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Pharmaceutical Applications Compendium

- Counter-Ion analysis
- Impurity profiling and related substances
- MS and CAD detection
- Method development and transfer
- Improving method throughput

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Table of contents

Method development and transfer

- Method transfer of a USP-derived acetaminophen assay from an UltiMate 3000 SD system to a Vanquish Flex UHPLC system
- Method transfer of a USP derived acetaminophen assay from an Agilent 1260 Infinity system to an UltiMate 3000 SD system and a Vanquish Flex UHPLC system
- An instrument parameter guide for successful (U)HPLC method transfer
- Trifluoroacetic acid performance of the Vanquish Flex Binary UHPLC system
- Deoxycholic acid method transfer from the Corona ultra RS Charged Aerosol Detector to the Corona Veo (or Vanquish) Charged Aerosol Detector
- Guidelines for method transfer and optimization - from earlier model Corona detectors to Corona Veo and Vanquish charged aerosol detectors
- UHPLC method development for analyzing a once-daily tablet formulation for HIV-1 infection treatment
- A UHPLC method development system for efficient scouting of chromatographic elution parameters
- Metoprolol impurity testing by charged aerosol detection: method transfer and optimization of a USP method
- Validation of an improved ion chromatography method for the limit of choline test in the USP Succinylcholine Chloride monograph
- Comparison of suppressed to nonsuppressed conductivity detection for the determination of common inorganic cations
- Evaluating ion chromatography suppression options

Improving method throughput

- Simultaneous high-performance and ultra-high-performance liquid chromatographic analysis of acetaminophen impurities using a single instrument
- Doubling the throughput of long chromatographic methods by using a novel Dual LC workflow
- Fast methods for the determination of ibuprofen in drug products
- Simplified, high-throughput separation of glucocorticoids
- A rapid fenoprofen USP assay method
- A rapid ibuprofen USP assay method
- A rapid cephradine USP assay method
- Determination of gentamicin and related impurities in gentamicin sulfate using simple eluents

Counter-Ion analysis

Pharmaceutical substances are often manufactured in a specific salt form to promote solubility, stability, and bioavailability. Analysis of these counter-ions is an essential stage of drug development, QC and lot release processes to ensure patient safety and drug efficacy.

To determine the extent of salt formation and if the stoichiometry between the drug and counter-ion is correct, the concentration of the counter-ions must be known.

During early drug development, knowledge relating to the concentration of unknown ionic impurities is also critical.

Chapter highlights

Achieve best-in-class resolution, speed, and sensitivity with the **Thermo Scientific™ Dionex™ ICS-5000⁺ Capillary HPIC™ system**

Obtain high performance, convenience, and flexibility with the **Thermo Scientific™ Dionex™ ICS-6000 HPIC™ system**

Routine ion analysis through comprehensive application solutions and interactive wellness features with **Thermo Scientific™ Dionex™ Integrion™ HPIC™ system**

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Table of contents

Application Note 1144

Ion Chromatography Assay for Lithium in Lithium Hydroxide

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Key Words
Dionex IonPac CS16 Column, Suppressed Conductivity Detection, Pharmaceutical, USP Monograph

Goal

To develop an IC method for the determination of lithium in lithium hydroxide using an RFIC system with suppressed conductivity detection.

Introduction

Lithium is considered the primary therapeutic agent for acute and prophylactic treatment for biopolar disorder.[1] Practically, lithium is administered as salts such as lithium hydroxide. The United States Pharmacopoeia (USP) monograph for lithium hydroxide describes a titration-based assay.[2] This assay involves mixing lithium hydroxide with hydrochloric acid followed by observing the color change to calculate lithium content. This method is tedious and requires a hazardous/corrosive chemical.

The USP has initiated an effort to modernize existing monographs across all compendia.[3] In response to this effort, this application note describes an alternative method to assay the lithium content of lithium hydroxide that is automated, faster, and uses an aqueous mobile phase (eluent). This method uses ion chromatography (IC) and offers significant improvement over existing assays because it can simultaneously determine lithium, sodium, calcium, and other common cations.[4] Moreover, using a Thermo Scientific™ Reagent-Free™ Ion Chromatography (RFIC™) system with electrolytically generated methanesulfonic acid (MSA) eluent simplifies the method and enhances reproducibility. This approach was successfully used to design methods for lithium quantification in lithium carbonate[5] and lithium citrate[6].

The IC-based method described in this application note uses a Thermo Scientific™ Dionex™ IonPac™ CS16 cation-exchange column, an electrolytically generated MSA eluent, and suppressed conductivity detection to determine lithium in lithium hydroxide. The Dionex IonPac CS16 column is a high-capacity cation-exchange column packed with resin functionalized with carboxylic acid groups. This column is specifically designed for the separation of alkali metals, alkaline earth metals, and ammonium at diverse concentration ratios. Therefore, the Dionex IonPac CS16 column is suited for applications involving separation of lithium from low concentrations of other cationic contaminants, when the determination of the cationic contaminants is required as is true here, where we applied the same method to determine a low amount of calcium in lithium hydroxide samples. The eluent is generated using a Thermo Scientific Dionex EGC III MSA Eluent Generator Cartridge and purified online using a Thermo Scientific Dionex CR-CTC II Continuously Regenerated Cation Trap Column. The Thermo Scientific™ Dionex™ CERS™ 500 (2 mm) Cation Electrolytically Regenerated Suppressor produces the regenerant ions necessary for eluent suppression and allows continuous operation with minimum maintenance.

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ICS-5000+ RFIC

Table of contents

Because the RFIC system requires only deionized (DI) water as the carrier, it significantly simplifies system operation and improves analytical reproducibility. The method proposed in this application note was validated following the guidelines outlined in USP General Chapter <1225>, Validation of Compendial Procedures[7] to meet the requirements for lithium and calcium quantification prescribed in the lithium hydroxide USP monograph.

Equipment

- A Thermo Scientific™ Dionex™ ICS-5000⁺ RFIC system was used in this work. The Dionex ICS-5000⁺ is an integrated ion chromatograph that includes:
  - SP single pump module (P/N 061707) or DP Dual Pump (P/N 061712) with degas option
  - DC detector compartment (P/N 061767) with single-temperature zone
- Thermo Scientific Dionex AS-AP Autosampler with 10 µL injection loop
- Dionex EGC III MSA Cartridge (P/N 074535)
- Thermo Scientific™ Autoselect™ Polyvial™ 10 mL Autosampler Vials with caps and septa (P/N 055058)
- Dionex CERS 500 Suppressor, 2 mm (P/N 082543)
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) software version 7.2

Reagents and Standards

- DI Water, Type I reagent grade, 18 MΩ-cm resistance or better
- Lithium carbonate, 300 mg, USP Reference Standard (USP P/N 1369000, Lot Number GIJ227)
- Lithium hydroxide (monohydrate, $LiOH•H_2O$), Sigma-Aldrich®, P/N 450197-25G-F, Lot Number MKBD3360
- Thermo Scientific Dionex Six Cation-II Standard (P/N 046070, Lot Number 150216)

Conditions	
Columns:	Dionex IonPac CS16, Analytical, 3 x 250 mm (P/N 059596) Dionex IonPac CG16 Guard, 3 x 50 mm (P/N 059595)
Eluent:	10 mM MSA, 0–11 min; 65 mM MSA, 11–16 min; 10 mM MSA, 16–22 min
Eluent Source:	Dionex EGC III MSA cartridge (P/N 074535) with Dionex CR-CTC II trap column (P/N 066262)
Flow Rate:	0.45 mL/min
Background Conductance:	~0.3 µS
Detection:	Suppressed conductivity, Dionex CERS 500 suppressor, 2 mm (P/N 082543) recycle mode, 86 mA current
Noise:	~1–2 nS/min peak-to-valley
Run Time:	22 min
Injection Volume:	10 µL in Push-Full mode
Column Temperature:	40 °C

Preparation of Solutions and Reagents

Sample Preparation

Lithium Stock Solution 1000 mg/L, Prepared Using Lithium Carbonate, USP

Accurately weigh 0.5322 g of USP lithium carbonate and dissolve in DI water in a 125 mL polypropylene bottle and adjust the weight to 100 g with DI water.

Lithium Stock Solution 1000 mg/L, Prepared Using Lithium Hydroxide

Accurately weigh 0.6993 g of lithium hydroxide (hydrate) and dissolve in DI water in a 125 mL polypropylene bottle and adjust the weight to 100 g with DI water.

Working Lithium Carbonate Standard and Lithium Hydroxide Sample Solutions

To prepare working standard and sample solutions, the stock solutions were diluted appropriately with 10 mM acetic acid (final pH ~4).

Note – Use 10 mM acetic acid for sample preparation. This reduces sample pH and inhibits potential retention of divalent cations at weak cation exchange sites that are sometimes formed along the flow path. This can result in inaccurate reading for divalent cations. For more details refer to Product Manual for Dionex IonPac CS16 column, Document Number 031747-05.

Robustness Study

Following the guidelines of USP Physical Tests, <621> Chromatography,[8] evaluate the robustness of this method by examining the retention time (RT), peak asymmetry, and resolution after imposing small variations (±10%) in procedural parameters (e.g., flow rate, eluent gradient concentration, column temperature). Inject a standard mixture containing 10 mg/L lithium, 0.12 mg/L sodium, 0.06 mg/L magnesium, and 0.06 mg/L calcium in 10 mM acetic acid. Apply the same procedure to another column set from a different lot. Test the following variations:

- Flow rate: 0.405, 0.45, and 0.495 mL/min
- Column temperature: 36, 40, and 44 °C
- MSA eluent initial concentrations: 9, 10, and 11 mM
- MSA eluent final concentrations: 58.5, 65, and 71.5 mM

Results and Discussion

Separation

Separation of lithium was achieved with a Dionex IonPac CS16, 3 x 250 mm column using initial isocratic elution followed by a step change to a higher concentration that was used to elute the remaining cations. Figure 1 shows separation of a 10 mg/L lithium solution prepared using lithium hydroxide. Figure 2 shows separation of a commercially available six cation standard mix using the proposed method. In order to achieve good separation from the nearest cation, i.e. sodium, the initial eluent concentration was kept at 10 mM and then rapidly increased to 65 mM to elute the remaining cations quickly.

The other four common cations elute within next six minutes and the remaining time is used to re-equilibrate to starting conditions. This method can also be executed using manually prepared eluents, but the performance, especially for retention time reproducibility, will not be as good.

Columns:	Dionex IonPac CS16, 3 x 250 mm, IonPac CG16, 3 x 50 mm
Eluent:	10 mM MSA for 0 to 11 min, 65 mM MSA 11 to 16 min, 10 mM MSA for 16 to 22 min
Eluent Source:	Dionex ICS-5000⁺ EG with Dionex CR-CTC II trap column
Temperature:	40 °C
Flow Rate:	0.45 mL/min
Inj.Volume:	10 µL
Detection:	Dionex CERS 500 suppressor, 2 mm, recycle mode
Peak:	1. Lithium 10 mg/L

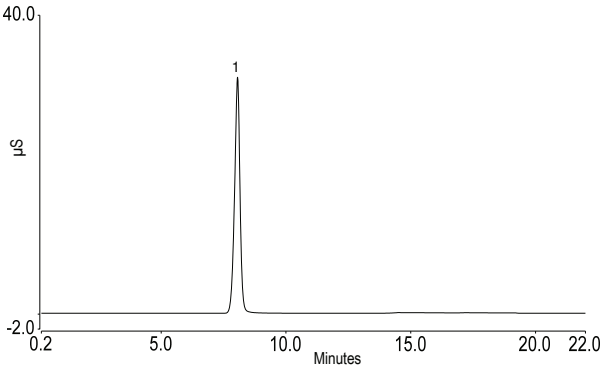

Figure 1A. Determination of 10 mg/L lithium in 10 mM acetic acid.

Columns:	Dionex IonPac CS16, 3 x 250 mm, IonPac CG16, 3 x 50 mm
Eluent:	10 mM MSA for 0 to 11 min, 65 mM MSA 11 to 16 min, 10 mM MSA for 16 to 22 min
Eluent Source:	Dionex ICS-5000⁺ EG with Dionex CR-CTC II trap column
Temperature:	40 °C
Flow Rate:	0.45 mL/min
Inj.Volume:	10 µL
Detection:	Dionex CERS 500 suppressor, 2 mm, recycle mode
Peaks:	1. Lithium 10 mg/L 2. Calcium 0.013 mg/L

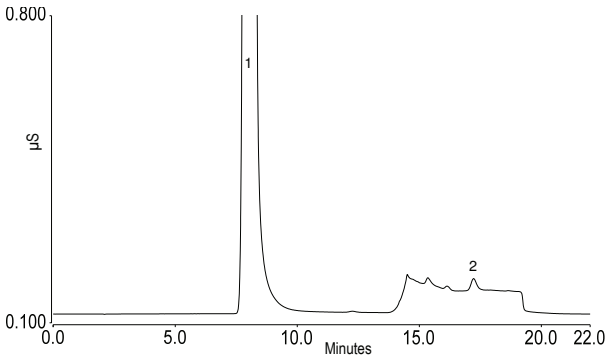

Figure 1B. Enlarged view of Figure 1A showing the calcium peak.

Columns:	Dionex IonPac CS16, 3 x 250 mm, IonPac CG16, 3 x 50 mm
Eluent:	10 mM MSA for 0 to 11 min, 65 mM MSA 11 to 16 min, 10 mM MSA for 16 to 22 min
Eluent Source:	Dionex ICS-5000⁺ EG with Dionex CR-CTC II trap column
Temperature:	40 °C
Flow Rate:	0.45 mL/min
Inj.Volume:	10 µL
Detection:	Dionex CERS 500 suppressor, 2 mm, recycle mode
Peaks:	1. Lithium 2. Sodium 3. Ammonium 4. Potassium 5. Magnesium 6. Calcium

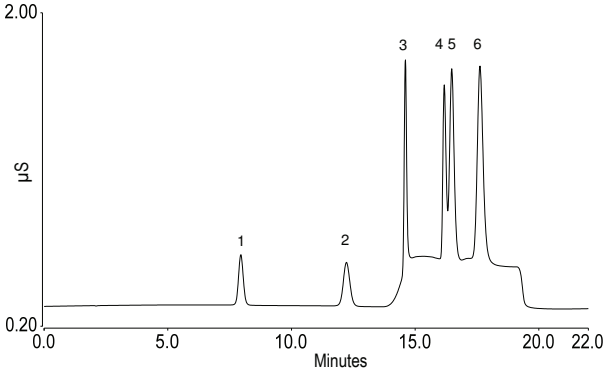

Figure 2. Separation of six common cations.

The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and the USP General Chapter <1225> guidelines recommend a minimum of five concentrations to establish linearity in an assay.[7,9] For a drug substance or finished product, the minimum specified range is from 80 to 120% of the test concentration. A minimum range from 50 to 120% is required for determination of an impurity. In this study, lithium was calibrated with eight concentration levels ranging from 0. 3 to 20 mg/L. The results yielded a linear relationship of peak area to concentration with a coefficient of determination (r^2) of 0.9999. Calcium was calibrated from 0.03 to 2 mg/L with an r^2 of 0.9999 (Table 1).

The limits of detection (LODs) and limits of quantitation (LOQs) were determined using a method described in ICH guidelines.[9] The method uses slope of the calibration curve and standard deviation of the lowest calibration standard response as described below.

$$LOD = 3.3\ \sigma / S$$

$$LOQ = 10\ \sigma / S$$

Where σ is the standard deviation of the response and S is the slope of the calibration curve.

The calculated LOD and LOQ for lithium were 2.0 and 6.1 µg/L, respectively. The LOD and LOQ values for calcium were 8.5 µg/L and 26 µg/L, respectively (Table 1).

Table 1. Method calibration, LOD, and LOQ data for lithium.

Parameter	Li	Ca
Linearity (r^2)	0.9999	0.999
LOD (µg/L)	2.0	8.5
LOQ (µg/L)	6.1	26.0

Sample Analysis

The USP monograph requires that lithium hydroxide contain not less than 98.0% and not more than 102% lithium calculated on the dried basis.[2] In this study, commercially available lithium hydroxide (≥ 99.995%) was used to prepare the test solution of 10 mg/L lithium. The calculated concentration of the test solution was 9.86 mg/L, equivalent to 98.6 % lithium content (Table 2), thus verifying the label claim. This indicates that the method is capable of determining lithium concentration within the USP specification. The USP requires that the lithium hydroxide be dried prior to assay for 1 h at 135 °C; this should result in weight loss of between 41.0 to 43.5% of its weight. In our hands, the 1 h drying process resulted in weight loss of 42.2%, as compared to a maximum 42.89% possible water loss, indicating residual water in the lithium hydroxide.

Table 2. Recovery data for lithium and calcium spiked in 10 mg/L lithium solution prepared using lithium hydroxide.

Cation (Concentration, mg/L)	Spike (mg/L)	Total Recovered (mg/L)	Recovery (%)	RT RSD (N=3)	Peak Area RSD (N=3)
Li (9.92)	1	0.99	99.6	0.01	0.03
	5	4.94	98.9	0.03	0.03
	10	9.86	98.6	0.03	0.06
Ca (0.013)	0.06	0.056	94.1	0.02	3.14
	0.12	0.11	94.7	0.01	4.19
	0.18	0.16	93.4	0.006	3.89

Note- values in parenthesis represent base concentrations determined in the 10 mg/L lithium solution before spiking.

Because we were uncertain that the drying process was successful, we used the water content provided by the manufacturer for making all lithium hydroxide solutions.

Sample Accuracy and Precision

To test sample accuracy, recovery studies were performed after spiking lithium samples prepared using lithium hydroxide with lithium from lithium carbonate. Three different spike levels of 1, 5, and 10 mg/L lithium were studied, and satisfactory recoveries were obtained for each spike level. The results of the lithium spike recovery experiment are 98.6 to 99.6% recovery of the spiked lithium amount. The USP monograph limits the amount of calcium in lithium hydroxide at 0.2%.[2] This corresponds to 1.2 mg/L calcium in 100 mg/L lithium. The 10 mg/L lithium solution was spiked with three calcium concentrations 0.06, 0.12, and 0.18 mg/L calcium, which correspond to 50, 100, and 150% of the prescribed limit. The calcium spike recoveries were 93.4 to 94.1%. The recovery results for lithium as well as calcium are summarized in Table 2. A chromatogram of the lithium hydroxide sample spiked with calcium at the concentration level prescribed in the USP monograph (0.2%) is shown in Figure 3.

Columns:	Dionex IonPac CS16, 3 x 250 mm, IonPac CG16, 3 x 50 mm
Eluent:	10 mM MSA for 0 to 11 min, 65 mM MSA 11 to 16 min, 10 mM MSA for 16 to 22 min
Eluent Source:	Dionex ICS-5000$^+$ EG with Dionex CR-CTC II trap column
Temperature:	40 °C
Flow Rate:	0.45 mL/min
Inj.Volume:	10 µL
Detection:	Dionex CERS 500 suppressor, 2 mm, recycle mode
Peaks:	1. Lithium 10 mg/L 2. Calcium 0.12 mg/L

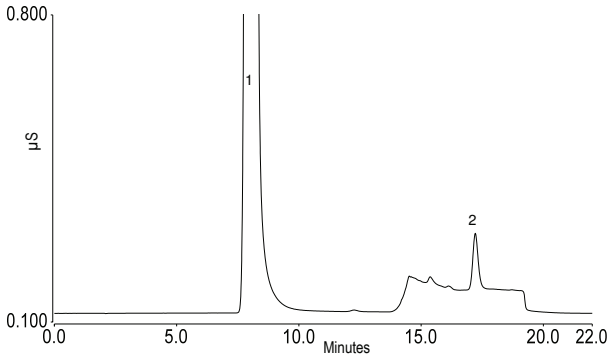

Figure 3. A 10 mg/L lithium sample spiked with calcium at the limit prescribed in the lithium hydroxide USP monograph.

Assay precision was evaluated by injecting seven replicates of 10 mg/L lithium spiked with 0.12 mg/L calcium and expressed as the RSDs of RT and peak area from the series of measurements. The RT RSDs were ≤0.03% and the peak area RSDs were ≤4.19% (Table 3).

Table 3. Retention time and peak area precision of 10 mg/L lithium sample spiked with 0.12 mg/L calcium.

Cation	Conc (mg/L)	RT RSD (N=7)	Peak Area RSD (N=7)
Li	9.99	0.03	0.04
Ca	0.11	0.01	4.19

Robustness

Assay robustness was evaluated by measuring the influence of small variations in procedural parameters (e.g., flow rate, eluent concentration during gradient, and column temperature) on the RT, peak asymmetry, and resolution of lithium from sodium, as well as calcium from magnesium on two columns from different lots. The peak asymmetry was measured using the USP formula.[8] A standard injection (10 mg/L lithium spiked with 0.12 mg/L sodium, 0.06 mg/L magnesium, and 0.12 mg/L calcium) was injected seven times (N=7) at each chromatographic condition. Tables 4 and 5 summarize the results for lithium and calcium robustness studies, respectively. These results indicate that the method is robust and suitable for lithium as well as calcium determination.

Table 4. Robustness of the IC-based assay for lithium determination performed using a 10 mg/L lithium sample spiked with 0.12 mg/L sodium, 0.06 mg/L magnesium, and 0.12 mg/L calcium.

Parameter	Value	Column 1						Column 2					
		Lithium RT (min)	Difference (%)	Asymmetry	Peak Asymmetry Difference (%)	Resolution (From Na)	Resolution (From Na) Difference (%)	Lithium RT (min)	Difference (%)	Asymmetry	Peak Asymmetry Difference (%)	Resolution (From Na)	Resolution (From Na) Difference (%)
Flow Rate (mL/min)	0.405	8.98	11.04	0.92	-0.36	9.58	3.19	9.81	11.12	0.84	0	15.51	50.70
	0.45	8.09	-	0.92	-	9.29	-	8.83	-	0.84	-	10.29	-
	0.495	7.36	-8.96	0.93	0.72	9.09	-2.21	8.08	-8.85	0.985	1.19	10.15	-1.33
Column Temp (°C)	36	8.09	0.04	0.93	0.72	9.50	2.30	8.84	0.11	0.85	1.19	11.49	11.63
	40	8.09	-	0.92	-	9.58	-	8.83	-	0.84	-	10.29	-
	44	8.08	-0.10	0.92	-0.72	9.01	-2.94	8.83	0.02	0.84	0.0	9.94	-3.43
MSA Eluent Initial Concentration (mM)	9	8.76	8.38	0.91	-1.44	9.76	5.10	9.57	8.43	0.82	-1.98	15.96	55.1
	10	8.09	-	0.92	-	9.29	-	8.83	-	0.84	-	10.29	-
	11	7.53	-6.85	0.93	1.08	9.04	-2.66	8.22	-6.85	0.85	1.19	9.96	-3.21
MSA Eluent Final Concentration (mM)	58.5	8.09	0.02	0.93	0.36	9.23	-0.57	8.83	0.08	0.84	0	10.31	0.29
	65	8.09	-	0.92	-	9.29	-	8.83	-	0.84	-	10.29	-
	71.5	8.08	-0.03	0.92	0.0	9.26	-0.32	8.84	0.06	0.84	0.4	10.33	0.39

Table 5. Robustness of the IC-based assay for calcium determination performed using a 10 mg/L lithium sample spiked with 0.12 mg/L sodium, 0.06 mg/L magnesium, and 0.12 mg/L calcium.

Parameter	Value	Column 1						Column 2					
		Calcium RT (min)	Difference (%)	Asymmetry	Peak Asymmetry Difference (%)	Resolution (From Mg)	Resolution (From Mg) Difference (%)	Calcium RT (min)	Difference (%)	Asymmetry	Peak Asymmetry Difference (%)	Resolution (From Mg)	Resolution (From Mg) Difference (%)
Flow Rate (mL/min)	0.405	17.92	3.99	1.16	-0.29	2.91	-0.11	17.97	1.82	1.08	-7.14	3.46	4.53
	0.45	17.23	-	1.16	-	2.91	-	17.65	-	1.17	-	3.31	-
	0.495	16.71	-3.02	1.11	-4.87	2.82	-2.98	17.11	-3.06	1.14	-2.57	3.26	-1.61
Column Temp (°C)	36	17.29	0.35	1.12	-3.72	2.91	-0.11	17.71	0.36	1.12	-4.29	3.32	0.10
	40	17.23	-	1.16	-	2.91	-	17.65	-	1.17	-	3.31	-
	44	17.18	-0.31	1.12	-3.72	2.89	-0.69	17.59	-0.30	1.13	-2.86	3.32	0.10
MSA Eluent Initial Concentration (mM)	9	17.28	0.27	1.14	-2.29	2.85	-2.18	17.69	0.27	1.18	0.86	3.31	-0.20
	10	17.23	-	1.16	-	2.91	-	17.65	-	1.17	-	3.31	-
	11	17.18	-0.29	1.13	-2.87	2.91	0.00	17.59	-0.30	1.20	2.86	3.34	0.70
MSA Eluent Final Concentration (mM)	58.5	17.96	4.22	1.22	4.58	3.09	6.07	18.42	4.38	1.09	-6.29	3.65	10.16
	65	17.23	-	1.16	-	2.91	-	17.65	-	1.17	-	3.31	-
	71.5	17.23	0.02	1.20	2.87	2.92	0.23	17.65	0.01	1.14	-2.29	3.30	-0.50

Conclusion

This study describes an IC-based assay for determination of lithium in lithium hydroxide. Lithium was separated on a cation-exchange column and detected by suppressed conductivity in 22 min. This method allows the concentration of lithium to be determined in an automated way and thus eliminates the need to perform the cumbersome titration-based assay. This assay for lithium was validated to meet the analytical performance characteristics outlined in USP General Chapter <1225>, Validation of Compendial Procedures, and was shown to measure accurately the lithium content of lithium hydroxide as per limits set in the USP monograph. Compared to the assay described in the USP lithium hydroxide monograph, this assay offers a simple, accurate, and robust measurement without handling hazardous reagents. Therefore, this method is a candidate to replace the existing assay for lithium hydroxide in the USP monograph, and thereby modernize the monograph.

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Application Note 1090

IC Assay for Lithium, Sodium, and Calcium in Lithium Carbonate

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Key Words
Dionex IonPac CS16 Column, Suppressed Conductivity Detection, Pharmaceutical, USP Monograph

Introduction

Lithium carbonate is used to treat a number of mental health problems associated with chemical imbalance in the brain, particularly bipolar disorder.[1,2] The U.S. Pharmacopeia (USP) monograph describes a lithium carbonate assay by titration with sulfuric acid, followed by sodium hydroxide, to titrate the excess acid using methyl orange as the indicator. Sodium and calcium are possible cationic impurities in lithium carbonate preparations. In the USP monograph, sodium is determined with a flame photometer at 589 nm and corrected for the background transmission at 580 nm. To determine calcium, the USP describes a chelometric titration assay that uses oxalate precipitation and permanganate titration and requires more than 4 h to complete.[3] These assays are time consuming and cumbersome procedures that use hazardous reagents.

The USP has embarked on a global initiative to modernize many of the existing monographs across all compendia.[4] In response to this initiative, an alternative analytical method was developed to determine these analytes in lithium carbonate.

Ion chromatography (IC) offers a significant improvement to the existing assays because it can simultaneously determine lithium, sodium, calcium, and other common cations in a single injection.[5] In addition, using electrolytically generated methanesulfonic acid (MSA) eluent significantly simplifies the method and enhances method reproducibility between laboratories.

This study describes a method that uses a Thermo Scientific™ Dionex™ IonPac™ CS16 cation-exchange column, an electrolytically generated MSA eluent, and suppressed conductivity detection to determine lithium, sodium, and calcium in lithium carbonate. The Dionex IonPac CS16 column is a high-capacity cation-exchange column packed with resin functionalized with carboxylic acid groups. This column is specifically designed for disparate concentration ratios of adjacent-eluting cations in diverse sample matrices. Therefore, the Dionex IonPac CS16 column is suitable for the separation of low concentrations of sodium and calcium in a sample with a high lithium concentration.

The required eluent is generated using a Thermo Scientific Dionex EGC III MSA Eluent Generator Cartridge and purified on line using a Thermo Scientific Dionex CR-CTC II Continuously Regenerated Cation Trap Column. The Thermo Scientific Dionex CERS 500 (2 mm) Cation Electrolytically Regenerated Suppressor produces the regenerant ions necessary for eluent suppression and allows continuous operation with minimum maintenance. Because the Reagent-Free™ IC (RFIC™) system requires only deionized (DI) water as the carrier, it significantly simplifies system operation and improves analytical reproducibility. This method was validated following the guidelines outlined in USP General Chapter <1225>, Validation of Compendial Procedures.[6]

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Goal

To develop an IC method for the determination of lithium, sodium, and calcium in lithium carbonate using an RFIC system with suppressed conductivity detection

Equipment

- Thermo Scientific™ Dionex™ ICS-5000+ HPIC™ system, capable of supporting high-pressure IC, including:
  - SP Single Pump
  - EG Eluent Generator
  - DC Detector/Chromatography Compartment
- Thermo Scientific Dionex AS-AP Autosampler with Sample Syringe, 250 µL (P/N 074306) and a Standard 1200 µL Buffer Line Assembly (P/N 074989)
- Dionex EGC III MSA Eluent Generator Cartridge (P/N 074535)
- Dionex CR-CTC II Continuously Regenerated Cation Trap Column (P/N 066262)
- Dionex CERS 500 (2 mm) Cation Electrolytically Regenerated Suppressor (P/N 082543)
- Vial Kit, 10 mL, Polystyrene with Caps and Blue Septa (P/N 074228)
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System software, version 7.2

Reagents and Standards

- DI water, Type I reagent grade, 18 M -cm resistance or better
- Lithium Carbonate, 300 mg, USP Reference Standard (USP P/N 1369000)
- Sodium Chloride, Certified ACS, ≥99% (Fisher Scientific P/N S271)
- Calcium Chloride Dihydrate, Certified ACS, 99 to 105% (Fisher Scientific P/N C79)
- Magnesium Chloride Hexahydrate, Certified ACS, 99 to 102% (Fisher Scientific P/N M33)

Conditions (Applicable to Figures 1–3)

Columns:	Dionex IonPac CG16 Guard, 3 × 50 mm (P/N 079931) Dionex IonPac CS16 Analytical, 3 × 250 mm (P/N 059596)
Eluent:	8 mM MSA from 0 to 15 min, 67 mM MSA from 15 to 20 min, 8 mM MSA from 20 to 25 min
Eluent Source:	Dionex EGC III MSA cartridge with Dionex CR-CTC II trap column
Flow Rate:	0.43 mL/min
Injection Volume:	10 µL
Temperature:	40 °C
Detection:	Suppressed conductivity, Dionex CERS 500 (2 mm) suppressor, recycle mode, 85 mA current
System Backpressure:	~2200–2300 psi
Background Conductance:	~0.3 µS
Noise:	~0.4 nS/min peak-to-peak
Run Time:	25 min

Preparation of Solutions and Reagents

Note: Do not use glassware to prepare the solutions. Polymeric containers made of high-density polyethylene (HDPE) are recommended.

Lithium Stock Solution, 1000 mg/L

Accurately weigh 0.5323 g of USP lithium carbonate and dissolve in 100 mL of DI water.

Sodium Stock Solution, 1000 mg/L

Accurately weigh 0.2542 g of sodium chloride and dissolve in 100 mL of DI water.

Calcium Stock Solution, 1000 mg/L

Accurately weigh 0.3668 g of calcium chloride dihydrate and dissolve in 100 mL of DI water.

Working Standard Solutions

To prepare working standard solutions, dilute the stock solutions to the appropriate concentrations with DI water. Prepare the working standard solutions for sodium and calcium on the day of analysis.

Sample Preparation

Prepare a 20-fold dilution of the 1000 mg/L lithium stock solution made from lithium carbonate to formulate a test solution of 50 mg/L lithium. The acceptance criteria of sodium and calcium in the USP monograph are 0.1% (0.26 mg/L) and 0.15% (0.40 mg/L), respectively. Dilute both the sodium and calcium stock solutions to prepare 100 mg/L each of the sodium and calcium solutions. Then spike 0.26 mL of 100 mg/L of sodium and 0.40 mL of 100 mg/L of calcium to 100 mL of 50 mg/L lithium to prepare the test solution of lithium carbonate fortified with sodium and calcium at the acceptance criterion levels.

Robustness Study

Following the guidelines of USP Physical Tests, <621> Chromatography, evaluate the robustness of this method by examining the retention time (RT), peak asymmetry, and resolution of the three analytes after imposing small variations (±10%) in procedural parameters (e.g., flow rate, eluent gradient concentration, column temperature).[7] Inject a standard mixture containing 50 mg/L lithium, 0.26 mg/L sodium, 0.20 mg/L magnesium (to determine the resolution of calcium), and 0.40 mg/L calcium. Apply the same procedure to two column sets from two different lots. Test the following variations:

- Flow rate at 0.43 mL/min, 0.39 mL/min, 0.47 mL/min
- Column temperature at 40, 36, and 44 °C
- MSA eluent initial/final concentrations at 8 mM/67 mM, 7.2 mM/67 mM, 8.8 mM/67 mM, 8 mM/61 mM, 8 mM/73 mM

Results and Discussion

Separation

The separation of lithium, sodium, and calcium was achieved on a Dionex IonPac CS16 column designed for disparate concentration ratios of close-eluting cations. This column enabled accurate determination of sodium in samples with a high concentration of lithium. The eluent program started with 8 mM MSA to separate sodium from lithium, followed by a step change to 67 mM MSA to quickly elute calcium without inferences from other common cations. The MSA concentration was then returned to 8 mM to equilibrate the column for the next injection. Figure 1 shows the separation of six common cations using a Dionex IonPac CS16 column set, indicating no interference from other common cations with lithium, sodium, and calcium. Figure 2 shows a lithium carbonate sample containing 50 mg/L lithium with an enlarged view (Figure 2, Chromatogram B) showing the separation of sodium and calcium.

Calibration, Limit of Detection (LOD), and Limit of Quantitation (LOQ)

The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and the USP General Chapter <1225> guidelines recommend a minimum of five concentrations to establish linearity in an assay.[6] For a drug substance or finished product, the minimum specified range is from 80 to 120% of the test concentration. A minimum range from 50 to 120% is required for determination of an impurity. In this study, lithium was calibrated at 11 concentration levels ranging from 2.5 to 150 mg/L. The results yielded a linear relationship of peak area to concentration with a coefficient of determination (r^2) of 1.000. Sodium and calcium were calibrated at seven concentration levels ranging from 0.05 to 5 mg/L with r^2 values of 0.9997 and 1.000, respectively (Table 1).

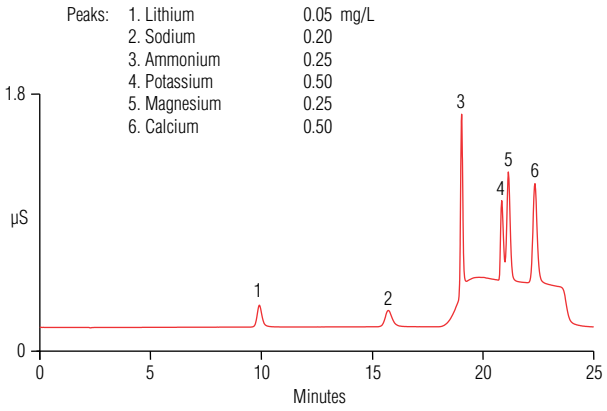

Figure 1. Separation of six common cations.

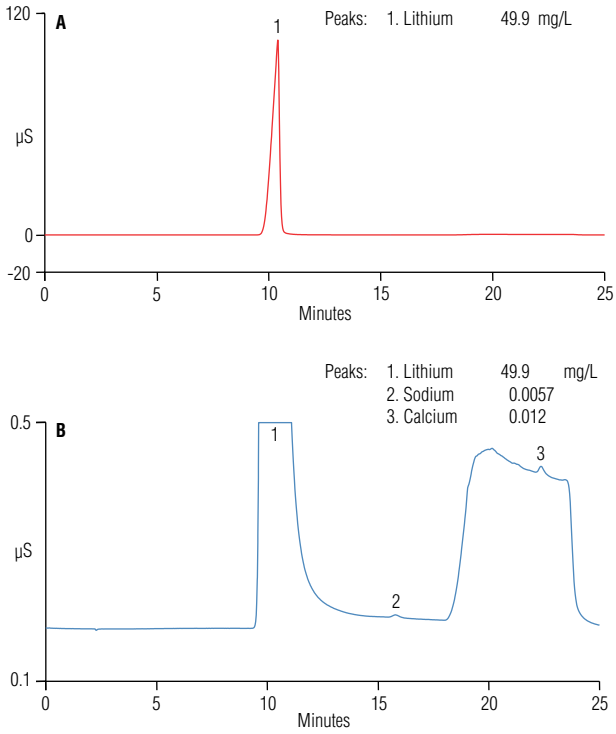

Figure 2. (A) A lithium carbonate sample (50 mg/L lithium) and (B) enlarged view of Chromatogram A showing sodium and calcium peaks.

Table 1. Calibration, LODs, and LOQs of lithium, sodium, and calcium.

Cation	Calibration Range (mg/L)	r^2	LOD (µg/L)	LOQ (µg/L)
Lithium	2.5–150	1.000	0.27	0.9
Sodium	0.05–5	0.9997	1.1	3.6
Calcium	0.05–5	1.000	7.4	25

Table 2. Recovery data for lithium, sodium, and calcium spiked in lithium carbonate samples containing 50 mg/L lithium.

Cation	Found (mg/L)	RT RSD (n = 3)	Peak Area RSD (n = 3)	Added (mg/L)	Total Found (mg/L)	RT RSD (n = 3)	Peak Area RSD (n = 3)	Recovery (%)
Lithium	49.9	0.01	0.12	25.1	75.5	0.03	0.04	102
				50	100	0.02	0.06	101
				75.5	125	0.01	0.03	100
Sodium	0.0057	0.03	4.39	0.130	0.120	<0.01	0.15	87.8
				0.260	0.240	0.01	0.07	90.0
				0.390	0.358	0.01	0.21	90.2
Calcium	0.012	0.02	4.66	0.200	0.207	<0.01	0.53	97.7
				0.400	0.423	0.01	0.26	103
				0.600	0.610	<0.01	0.33	99.6

To determine the LODs and LOQs, the baseline noise was first determined by measuring the peak-to-peak noise in a representative 1-min segment of the baseline where no peaks elute but close to the peaks of interest. The signal was determined from the average peak height of three injections of 1 µg/L lithium, 5 µg/L sodium, and 10 µg/L calcium. The LODs and LOQs were then determined by multiplying the signal-to-noise ratio 3× and 10×, respectively (Table 1). The LODs of lithium, sodium, and calcium were 0.27, 1.1, and 7.4 µg/L, respectively. The LOQs of those three analytes were 0.9, 3.6, and 25 µg/L, respectively, which correspond to 0.00034, 0.0014, and 0.0094% of lithium carbonate. The low LODs of sodium and calcium indicate that this IC method can easily assay these two cationic impurities well below the acceptance criteria specified in the USP monograph.

Sample Analysis

The USP monograph requires that lithium carbonate contain not less than 99.0% lithium carbonate calculated on the dried basis.[3] In this study, the USP lithium carbonate reference standard was used to prepare the test solution of 50 mg/L lithium. The concentration of the test solution was 49.9 mg/L, equivalent to 99.8% lithium carbonate, which is within the USP specification. The concentrations of sodium and calcium in the lithium carbonate sample at the test concentration were 0.0057 and 0.012 mg/L, corresponding to 0.004 and 0.008% of lithium carbonate, respectively.

Sample Accuracy and Precision

Method accuracy was validated by spiked recoveries of lithium, sodium, and calcium in lithium carbonate samples over three concentration levels (i.e., 50, 100, and 150%), with three replicates of each concentration. For a test solution concentration of 50 mg/L lithium, the recoveries of lithium at the three spike levels were in the range of 100–102%.

The USP monograph limits for sodium and calcium in lithium carbonate are 0.1 and 0.15%, respectively.[3] This correlates to 0.26 mg/L sodium and 0.4 mg/L calcium in 50 mg/L lithium. The lithium solution was spiked with 0.13, 0.26, and 0.39 mg/L of sodium and 0.2, 0.4, and 0.6 mg/L of calcium, respectively. The recoveries of the three spiked levels of sodium and calcium were in the range of 87.8–90.2% and 97.7–103%, respectively. These recoveries are summarized in Table 2. A chromatogram of a spiked lithium carbonate sample is shown in Figure 3.

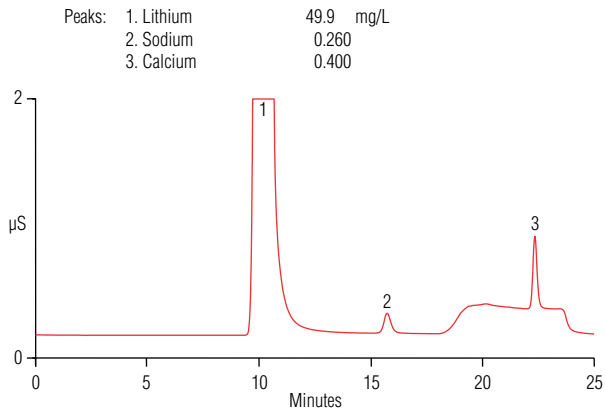

Figure 3. A lithium carbonate sample (50 mg/L lithium) spiked with sodium and calcium at the acceptance criterion levels.

Assay precision was evaluated by injecting seven replicates of the test concentrations (50 mg/L lithium, 0.26 mg/L sodium, and 0.40 mg/L calcium), and expressed as the RSDs of RT and peak area from the series of measurements for the three analytes. The RT RSDs were <0.02% and the peak area RSDs ranged from 0.36 to 1.04% (Table 3).

Table 3. Retention time and peak area precisions of 50 mg/L lithium spiked with 0.26 mg/L sodium and 0.40 mg/L calcium.

Cation	Concn (mg/L)	RT RSD (n = 7)	Peak Area RSD (n = 7)
Lithium	49.9	0.02	0.36
Sodium	0.24	0.01	0.44
Calcium	0.414	0.01	1.04

Robustness

Assay robustness was evaluated by measuring the influence of small variations (±10%) in procedural parameters (e.g., flow rate, eluent gradient concentration, column temperature) on the RT, peak asymmetry, and resolution of the three analytes on two column sets from two different lots. The peak asymmetry was evaluated following the USP formula. The resolution was determined using a USP formula relative to the previous peak in a chromatogram. A standard mix (50 mg/L lithium, 0.26 mg/L sodium, 0.20 mg/L magnesium, and 0.40 mg/L calcium) was injected three times at each chromatographic condition. The resolution of sodium to lithium ranged from 7.99 to 8.49 on Column 1 and from 7.13 to 7.86 on Column 2. The resolution of calcium to magnesium ranged from 3.56 to 4.18 on Column 1 and from 3.08 to 3.54 on Column 2. Tables 4–6 summarize the results for lithium, sodium, and calcium, respectively. These results indicate the method is robust for all three analytes.

Table 4. Robustness of the IC-based assay for lithium (injected sample: 50 mg/L lithium spiked with 0.26 mg/L sodium, 0.20 mg/L magnesium, and 0.40 mg/L calcium; average of three injections).

Parameter		Column 1				Column 2			
		Lithium RT (min)	Diff.* (%)	Asym.**	Diff.* (%)	Lithium RT (min)	Diff.* (%)	Asym.**	Diff.* (%)
Flow Rate (mL/min)	0.39	11.5	9.6	0.65	0.5	11.3	9.5	0.70	0.9
	0.43	10.4	—	0.66	—	10.2	—	0.70	—
	0.47	9.57	–8.9	0.66	–1.0	9.47	–8.6	0.71	–0.5
Column Temp (°C)	36	10.5	0.5	0.66	0	10.3	0.6	0.70	0
	40	10.4	—	0.66	—	10.2	—	0.70	—
	44	10.4	–0.4	0.66	0	10.2	–0.3	0.70	0
Eluent MSA Initial Concn (mM)	7.2	11.4	8.2	0.65	1.5	11.2	8.2	0.69	1.4
	8.0	10.4	—	0.66	—	10.2	—	0.70	—
	8.8	9.70	–7.5	0.67	–1.5	9.58	–7.4	0.71	–1.4
Eluent MSA Final Concn (mM)	61	10.5	0.7	0.66	0	10.4	0.8	0.70	0
	67	10.4	—	0.66	—	10.2	—	0.70	—
	73	10.4	–0.5	0.66	0	10.2	–0.4	0.70	0

* Difference
** Asymmetry

Table 5. Robustness of the IC-based assay for sodium (injected sample: 50 mg/L lithium spiked with 0.26 mg/L sodium, 0.20 mg/L magnesium, and 0.40 mg/L calcium; average of three injections).

Parameter		Column 1						Column 2					
		Sodium RT (min)	Diff.* (%)	Asym.**	Diff.* (%)	Resol.***	Diff.* (%)	Sodium RT (min)	Diff.* (%)	Asym.**	Diff.* (%)	Resol.***	Diff.* (%)
Flow Rate (mL/min)	0.39	17.3	9.5	1.19	0.8	8.44	0.8	16.9	9.4	1.18	0.8	7.56	0.9
	0.43	15.7	—	1.20	—	8.40	—	15.3	—	1.17	—	7.49	—
	0.47	14.4	–8.9	1.21	–0.8	8.29	–1.0	14.1	–8.6	1.19	–0.8	7.42	–0.9
Column Temp (°C)	36	16.1	2.6	1.19	0.3	8.78	4.6	15.7	2.5	1.18	0.3	7.86	4.9
	40	15.7	—	1.20	—	8.40	—	15.3	—	1.17	—	7.49	—
	44	15.4	–2.4	1.20	–0.6	7.99	–4.8	15.0	–2.4	1.18	–0.6	7.13	–4.8
Eluent MSA Initial Concn (mM)	7.2	17.2	8.8	1.16	2.2	8.49	1.2	16.8	8.7	1.17	0.6	7.60	1.4
	8.0	15.7	—	1.20	—	8.40	—	15.3	—	1.17	—	7.49	—
	8.8	14.5	–8.1	1.22	–2.8	8.28	–1.3	14.2	–8.0	1.18	–0.3	7.40	–1.3
Eluent MSA Final Concn (mM)	61	15.8	0.6	1.19	0.3	8.40	0.1	15.4	0.5	1.15	0.9	7.50	0.3
	67	15.7	—	1.20	—	8.40	—	15.3	—	1.17	—	7.49	—
	73	15.7	–0.4	1.20	–0.6	8.41	0.0	15.3	–0.4	1.16	–0.9	7.53	–0.2

* Difference
** Asymmetry
*** Resolution relative to lithium

Table 6. Robustness of the IC-based assay for calcium (injected sample: 50 mg/L lithium spiked with 0.26 mg/L sodium, 0.20 mg/L magnesium, and 0.40 mg/L calcium; average of three injections).

Parameter		Column 1						Column 2					
		Calcium RT (min)	Diff.* (%)	Asym.**	Diff.* (%)	Resol.***	Diff.* (%)	Calcium RT (min)	Diff.* (%)	Asym.**	Diff.* (%)	Resol.***	Diff.* (%)
Flow Rate (mL/min)	0.39	23.3	4.0	1.13	2.0	4.04	5.1	23.2	3.8	1.19	1.9	3.45	4.2
	0.43	22.3	—	1.16	—	3.82	—	22.2	—	1.22	—	3.28	—
	0.47	21.6	–3.7	1.18	–2.3	3.67	–4.5	21.5	–3.5	1.18	–1.4	3.20	–3.3
Column Temp (°C)	36	22.3	0.1	1.16	1.7	3.78	0.9	22.2	0.1	1.18	1.9	3.28	0.8
	40	22.3	—	1.16	—	3.82	—	22.2	—	1.22	—	3.28	—
	44	22.4	–0.1	1.19	–0.9	3.85	–1.0	22.2	0.0	1.19	–1.4	3.32	–0.4
Eluent MSA Initial Concn (mM)	7.2	22.4	0.2	1.16	1.1	3.80	0.7	22.3	0.2	1.19	1.7	3.27	1.3
	8.0	22.3	—	1.16	—	3.82	—	22.2	—	1.22	—	3.28	—
	8.8	22.3	–0.2	1.18	–0.6	3.85	–0.6	22.2	–0.2	1.19	–0.8	3.34	–0.8
Eluent MSA Final Concn (mM)	61	23.1	3.0	1.11	3.2	4.18	8.5	22.9	2.8	1.16	2.8	3.54	7.3
	67	22.3	—	1.16	—	3.82	—	22.2	—	1.22	—	3.28	—
	73	21.8	–2.7	1.19	–3.8	3.56	–7.6	21.7	–2.6	1.18	–2.2	3.08	–6.7

* Difference
** Asymmetry
*** Resolution relative to magnesium

Conclusion

This study describes an IC-based assay for simultaneous determination of lithium, sodium, and calcium in lithium carbonate. The three analytes were separated on a cation-exchange column and detected by suppressed conductivity within 25 min. The concentrations of lithium, sodium, and calcium in a lithium carbonate sample were determined in a single run. This assay for lithium, sodium, and calcium was validated to meet the analytical performance characteristics outlined in USP General Chapter <1225>, Validation of Compendial Procedures, and demonstrates detection limits well below the limits set in the USP monograph. Compared to the three time-consuming assays in the USP lithium carbonate monograph, this assay offers a simple, accurate, and robust measurement of the three analytes without handling hazardous reagents. Therefore, this method is a candidate to replace the existing assays for lithium carbonate, sodium, and calcium in the USP monograph, and thereby modernize the monograph.

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Fast Separations of Counter Ion in an Allergy Drug Tablet Using High-Pressure Capillary IC

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Technical Note 120

Key Words
Ion Chromatography, IonSwift MAX 200, HPIC, ICS-5000+, Pharmaceutical, Chloride

Goal
Demonstrating the rapid separation and quantification of a pharmaceutical counter ion.

Introduction

Many drugs are manufactured in a salt form to provide solubility, bioavailability, and stability. Counter ion determinations are important to confirm the correct stoichiometry and formula weight of the drug but also to test for drug impurities. High Pressure Capillary ion chromatography is the latest advancement in ion chromatography instrumentation. Typically with an Reagent-Free™ ion chromatography (RFIC™) system, the system pressure is limited to < 3000 psi because of the limitations of materials in the RFIC accessories. Now with the upgrade to high pressure, the Thermo Scientific™ Dionex™ ICS-5000+ HPIC™ capillary system can operate at system pressures < 5000 psi. This advance in technology allows increased capillary flow rates with all the same advantages as standard pressure capillary IC, resulting in low consumption of water (30 to 40 mL/d of water) and low waste generation. In capillary IC, the system can remain on without a loss in resources, i.e., capillary IC is always on and ready for analysis. These advantages result in:

- Greater ease-of-use
- Longer eluent generator cartridge life
- Lower cost of ownership

Additionally, with the increase in mass sensitivity, comparable results are achieved as with standard bore system using a sample injection of only 0.4 µL. In a previous study published in AB 136, counter ions were determined on a water extract of a pharmaceutical drug used to allergies using standard pressure capillary IC.[1]

In this study, inorganic anions from an allergy treatment pharmaceutical tablet dissolved in water are separated by anion-exchange chromatography on a capillary size Thermo Scientific™ Dionex™ IonSwift™ MAX 200 monolith IC column. The Dionex IonSwift MAX 200 column is designed with a monolith backbone and optimized with Thermo Scientific™ Dionex™ IonPac™ AS19 anion-exchange chemistry. This column was selected for these high pressure cap IC experiments because of the high efficiency and high flow rate characteristics. In these experiments, the chloride counteranion was separated at 10, 20, and 25 µL/min flow rates using an electrolytically-generated gradient. At 25 µL/min flow rate conditions, the system backpressure approaches 4500 psi which is well within the new high pressure tolerances while reducing the run time from 20 to 8 min. The analytes are detected by suppressed conductivity detection as they elute from the column, using the Thermo Scientific™ Dionex™ ACES™ Anion Capillary Electrolytic Suppressor, specifically optimized for capillary IC.

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Table of contents

Equipment

Dionex High Pressure ICS-5000+ HPIC Reagent-Free capillary IC system

- ICS-5000+ SP Single Pump or DP Dual Pump module
- ICS-5000+ EG Eluent Generator module
- ICS-5000+ DC Detector/Chromatography module with Thermo Scientific™ Dionex™ IC Cube™ and high pressure degas cartridge
- Thermo Scientific Dionex AS-AP Autosampler
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data system (CDS)

Reagents and Standards

- 18 M -cm degassed deionized water
- Thermo Scientific Dionex Combined Seven Anion II Standard (Dionex P/N 057590)

Samples

Pharmaceutical tablet prescribed for the treatment of seasonal allergies

Conditions	
Columns:	Dionex IonSwift MAX 200, 0.25 × 250 mm
Eluent Source:	Thermo Scientific Dionex EGC KOH capillary cartridge with Thermo Scientific Dionex capillary CR-ATC Continuously Regenerated Anion Trap Column
Gradient:	2 to 50 mM KOH
Flow Rate:	A: 10 µL/min
	B: 20 min
	C: 25 µL/min
Dionex IC Cube Temp.: *	30 °C
Compartment Temp.:	15 °C
Inj. Volume:	0.4 µL
Detection:	Suppressed conductivity, Dionex ACES 300, Thermo Scientific Dionex CRD 200 Carbonate Removal Device (Capillary), recycle mode, A: 8 mA; B: 15 mA; C: 18 mA
Background Conductance:	0.5–0.8 µS conductance
Noise:	< 0.3 nS
System Backpressure:	A: 1900 psi; B: 3700 psi, C: 4500 psi

* The Dionex IC Cube heater controls the separation temperature by controlling the column cartridge temperature. The original term of "column temperature" refers to the temperature in the bottom DC compartment which is not used for capillary IC. The part numbers of the consumables for this method are shown in Table 1.

Table 1. Consumables list.

Product Name	Type, Capillary	Dionex Part Number
Dionex EGC-KOH	Eluent Generator cartridge	072076
Dionex CR-ATC	Electrolytic trap column	072078
Dionex IonSwift MAX-200	Separation column	075889
Dionex CRD 200	Carbonate removal device	072054
Dionex ACES	Suppressor	072052
Dionex HP fittings (blue)	Bolts / Ferrules	074449 / 074373
EG Degas HP cartridge	High pressure degas cartridge, up to 5000 psi	074459
Dionex AS-AP autosampler vials	Package of 100, polystyrene vials, caps, blue septa	074228
Thermo Scientific™ Dionex™ OnGuard™ RP II cartridge	Sample preparation, pkg of 48	057084

Standard and Sample Preparation

The Dionex Seven Anion II Standard was diluted appropriately for calibration. The sample was a 60 mg tablet from a prescription allergy pharmaceutical drug, ground to a powder with a mortor and pestle and mixed thoroughly. Approximately 20 mg of the ground tablet was added to 20 mL of deionized water, and heated and stirred for 30 min at 80 °C. The sample mixtures were then cooled to room temperature, filtered with a 0.45 µm, IC syringe filter, Dionex OnGuard RP filter, and diluted 1:10 with deionized water prior to analysis.

Tip: It is important to use 18 M -cm resistivity, deionized water for standards, eluent, and autosampler flush solution. It is recommended to degas the deionized water intended for eluent. (An appropriate degassing method is vacuum filtration.) Using deionized water with resistivity less than 18 M -cm can reduce sensitivity, introduce contamination, and affect calibration, thereby resulting in inaccurate quantification. Results can vary and contamination introduced from samples can affect the chromatography.

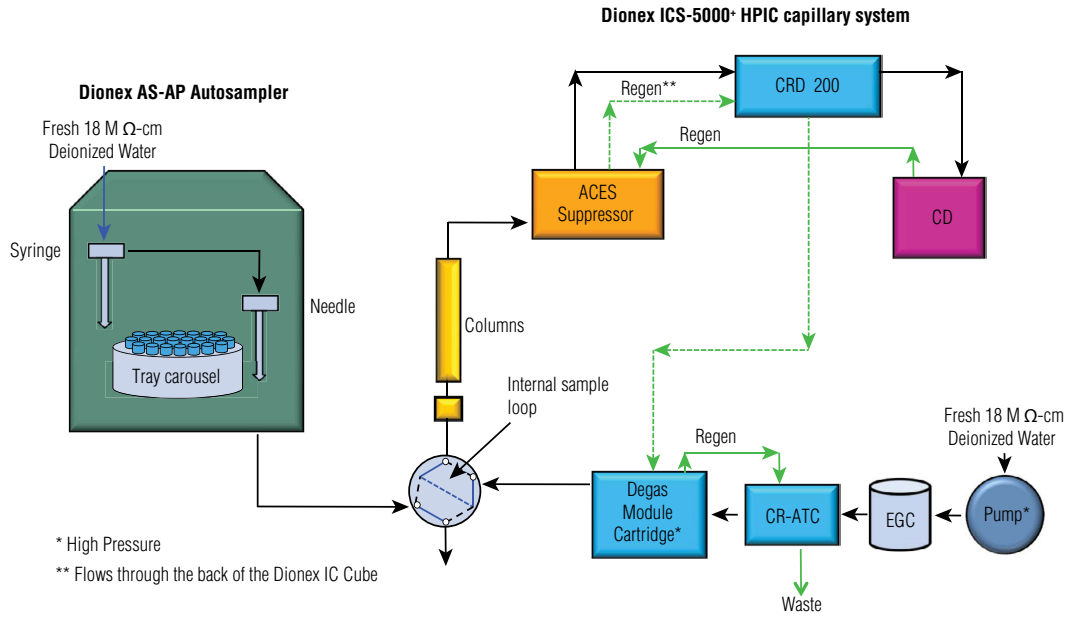

Figure 1. Flow diagram.

Instrument Setup and Installation

Tip: To achieve the best chromatography with capillary IC, it is important to minimize void volumes between connections by using precision cut tubing, high pressure connectors and fittings (colored blue), and by seating the ferrule > 2 mm above the end of the tubing. These tips are thorough discussed in "TN 113: Practical Guidance for Capillary IC".[2] Extra care should be used to prevent introducing air into any of the consumables or tubing by observing a steady liquid flow before installing the next device in line. The high pressure Dionex ICS-5000+ HPIC capillary IC system is designed to operate continuously up to 5000 psi which results in very low noise. To setup this application, plumb the consumables and modules of the Dionex ICS-5000+ HPIC system, according to Figure 1.

Install and hydrate the Dionex EGC-KOH capillary cartridge and Dionex CR-ATC trap column. Install the EG Degas cartridge, Dionex CRD 200 cartridge, and the Diones ACES Anion Capillary Electrolytic Suppressor capillary devices into the Dionex IC Cube (Figure 2). Hydrate the devices according to the product manuals and Section 3.18 of the Dionex ICS-5000 installation manual.[1–5] Install the columns and complete the configuration according to Figure 1. Detailed instructions are described in TN 131, the product manuals, and the instrument installation and operator's manuals.[1–5]

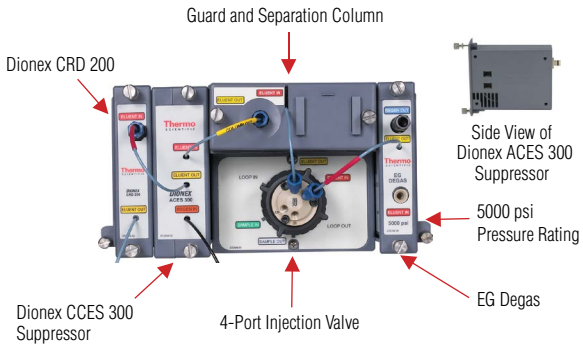

Figure 2. Dionex ICS-5000+ IC Cube .

Results and Discussion

Counter anions in drug formulations can include inorganic cations, inorganic anions, and organic acids. Therefore, to determine counteranions in this allergy pharmaceutical drug, the Dionex IonSwift MAX 200 was selected for its high resolution anion-exchange chemistry and fast separations of oxyhalides and inorganic anions. Although this sample contains only chloride, this column is suitable for separations of inorganic anions, oxyhalides, and some organic acids.

To determine the analyte concentrations, we determined the peak area response to concentration by calibrating with duplicate injections of the 50-, 100-, and 200-fold diluted Dionex Combined Seven Anion II Standard. A linear regression curve was used for the fluoride, chloride, nitrite, nitrate, bromide, sulfate, and phosphate peaks, resulting in linear coefficients of $r^2 > 0.999$.

Here we demonstrate the effects of flow rate on the 5-fold diluted extract from the allergy tablet (Figure 3). By just increasing the flow rate from 10 to 25 µL/min using the high pressure Dionex ICS-5000⁺ HPIC capillary IC capable of operating at 5000 psi, the elution time of single analyte - chloride is reduced from 7 min to 2.5 min.

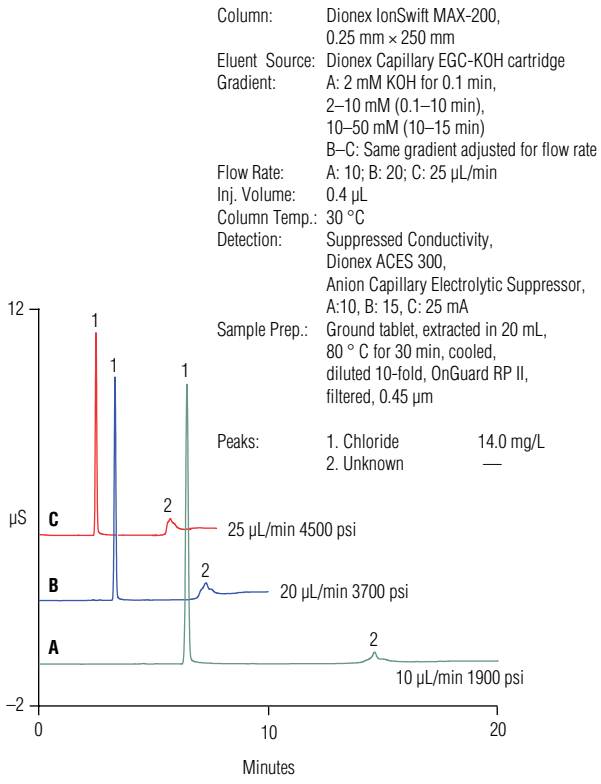

Figure 3. Fast separations of counter anions in an allergy pharmaceutical drug tablet using high pressure capillary IC.

Conclusion

This application demonstrates the advantages of high pressure capillary IC using the high efficiency Dionex IonSwift MAX 200 capillary size anion-exchange monolith column to provide high sample throughput by simply increasing the flow rate on a high pressure capable Dionex ICS-5000⁺ HPIC capillary IC, resulting in saving time and money.

For additional information on counter ion determinations in pharmaceutical drugs by high pressure and standard pressure capillary IC, please refer to AB 136, Thermo Fisher Scientific poster *High Pressure Capillary Ion Chromatograph for the Fast Separation of Pharmaceutical Relevant Inorganic Anions and Cations*, and Pharmaceutical Capillary IC Applications in Dionex Capillary IC Library website.[6–8] Counter ion determinations using standard bore and microbore IC are thoroughly discussed in AN 116, AN 164, AN 190, and AN 210 and De Borba etal poster.[9–13]

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Determination of Inorganic Counterions in Pharmaceutical Drugs Using Capillary IC

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Introduction

One of the most important applications of ion chromatography (IC) is to determine counterions in active pharmaceutical ingredients (API) and drug products in the pharmaceutical industry.[1,2] Approximately 50% of all drugs on the market are developed in salt forms.[3,4] Certain suboptimal physicochemical and biopharmaceutical properties of APIs can be overcome by pairing a basic or acidic drug molecule with a counterion to create a salt version of the drug with high solubility, stable crystalline form, and good bioavailability. Ion chromatography with suppressed conductivity detection plays an important role in the salt selection process to establish correct molecular mass of the entity in early stages of drug development. Ion chromatography can also be used in quality control to verify identity, strength, and purity of ionic APIs.

This study describes the determination of inorganic anions and cations in two different drugs using the capillary Thermo Scientific™ Dionex™ ICS-5000 system. Scaling down from standard bore to capillary scale brings many benefits to IC users. Capillary Reagent-Free™ IC (RFIC™) systems deliver fast results by reducing eluent preparation, system startup, and equilibration times. Perhaps most importantly, the system can be left on (i.e., running), always ready for analysis because of its low consumption of eluent (15 mL a day). Having the system always on and ready significantly streamlines the IC workflow. An always on system maintains stability and requires less frequent calibrations. The amount of waste generated is significantly decreased and the Thermo™ Scientific™ Dionex EGC Eluent Generator cartridge producing the eluent lasts 18 months under continuous operation mode, which translates into reduced overall cost of ownership.

Figure 1 shows the analysis of chloride in a drug used to treat type 2 diabetes using the Thermo Scientific™ Dionex™ IonPac™ AS19 capillary column designed for diverse sample matrices. This column is ideally suited for use with the RFIC system. The analysis time for this counteranion is less than 5 min.

Column:	Dionex IonPac AS19, Capillary, 0.4 × 250 mm		
Eluent Source:	Dionex EGC-KOH (Capillary)		
Eluent:	40 mM KOH		
Flow Rate:	10 µL/min		
Inj. Volume:	0.4 µL		
Column Temp.:	30 °C		
Detection:	Suppressed conductivity, Thermo Scientific™ Dionex™ ACES™ 300 Anion Capillary Electrolytic Suppressor		
Sample:	100 mg strength tablet		
Sample Prep.:	Thermo Scientific™ Dionex™ OnGuard™ RP cartridge, filtered, 1:10 dilution		
		mg/L	mg/tablet
Peak:	1. Chloride	16.6	0.453

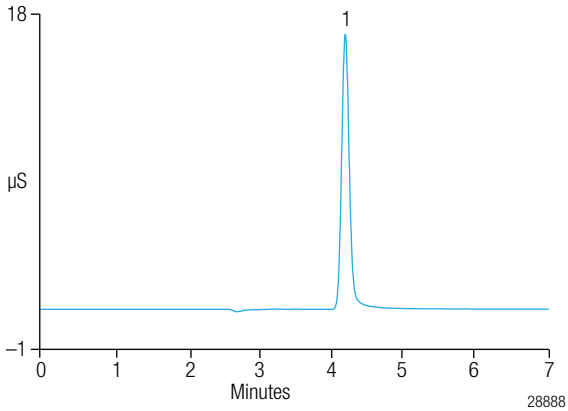

Figure 1. Determination of a counteranion in a drug used to treat type 2 diabetes.

Column:	Dionex IonPac CS12A, Capillary, 0.4 × 250 mm
Eluent Source:	Dionex EGC-MSA (Capillary)
Gradient:	20 mM MSA
Flow Rate:	10 µL/min
Inj. Volume:	0.4 µL
Column Temp.:	30 °C
Detection:	Suppressed conductivity, Thermo Scientific™ Dionex™ CCES™ 300 Cation Capillary Electrolytic Suppressor
Sample:	10 mg strength tablet
Sample Prep.:	Dionex OnGuard RP cartridge, filtered, 1:10 dilution

Peaks:		mg/L	µg/tablet
	1. Sodium	0.147	1.13
	2. Magnesium	0.705	5.47
	3. Calcium	3.59	27.8

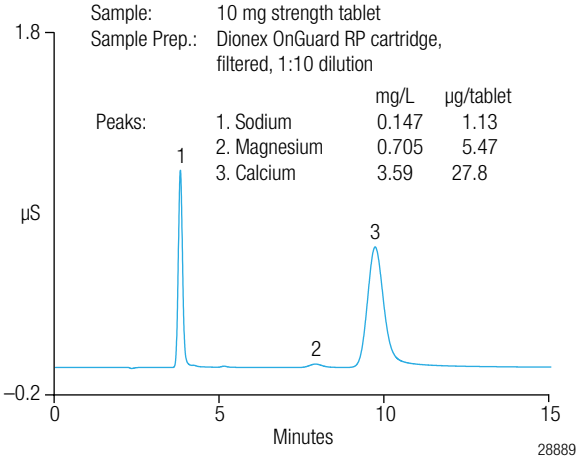

Figure 2. Determination of a countercation in a drug used to control cholesterol.

Analysis of the countercation calcium in a drug used to control cholesterol is illustrated in Figure 2. Using the Dionex IonPac CS12A column, calcium is well separated from sodium and magnesium present in the excipients.

Equipment and Conditions

The Dionex Capillary ICS-5000 system, Thermo Scientific™ Dionex™ AS-AP Autosampler, and Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System software are used in this experiment. All experimental parameters are listed in the figures above.

Sample Preparation

Extract the counterion analyte by dissolving the tablet in water after 50 °C. Treat the sample using the Dionex OnGuard RP cartridge, then filter through a 0.4 µm syringe filter, and dilute the sample solution 10-fold prior to analysis.

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Implementation of a Walk-Up High-Pressure Capillary Ion Chromatograph for the Fast Separation of Pharmaceutical Relevant Inorganic Anions and Cations

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Application Note 2967

Key Words

Pharmaceutical, high pressure, capillary IC, walk-up system, Dionex ICS-5000

Goal

The goal is to demonstrate the advantages of high pressure capillary IC for fast separations of pharmaceutical relevant anions and cations.

Overview

Purpose

This work reports the development and the advantages of a walk up capillary IC system using high pressure to provide ultrafast separations of inorganic anions and cations relevant to the pharmaceutical industry.

Methods

Counterions relevant to the pharmaceutical industry are separated by capillary ion-exchange chromatography and detected by suppressed conductivity on a capillary IC system using Thermo Scientific Dionex Capillary Electrolytic Suppressors (CES™).

Results

This work demonstrates the advantages of using a capillary IC system that is Always On, Always Ready for analysis.

Introduction

Ion chromatography (IC) with suppressed conductivity detection is a well-established technique for the determination of inorganic and organic ions in pharmaceuticals. The recent development of highpressure capillary IC brings additional advantages for the analysis of inorganic ions. Because the eluent consumption is very small, capillary IC systems can be operated continuously and therefore are always on and always ready for analysis, redefining the workflow for IC, and improving method performance. Capillary IC systems offer improved compatibility with applications where sample amount is limited. The operation of capillary IC at higher pressures and higher flow rates improves the separation efficiency and/or speed.

This work describes the development of a walk-up IC system using high pressure to provide ultrafast separations of inorganic anions and cations relevant to the pharmaceutical industry. Data will be presented on the identification, quantification, and control of inorganic impurities that are important during drug development, and the benefits an Always On, Always Ready system brings to IC analysis.

Methods

Sample Preparation

Samples

- Metformin HCl, Fexofenadine HCl tablets
- Naproxen sodium, Atorvastatin calcium tablets

The tablets were ground, dissolved in water at 45 °C , filtered using a Thermo Scientific Dionex OnGuard™ RP cartridge and 0.5 µm IC filter, and diluted 1:10 prior to analysis.

Liquid Chromatography Equipment and Data Analysis

Thermo Scientific Dionex ICS-5000 Reagent-Free™

Capillary IC system consisting of:

- DP Dual isocratic capillary pump
- DC Detector and Chromatography Module
- IC Cube™ capillary module compartment
- CD Capillary Conductivity Detector for Anions and Cations
- EG Eluent Generator
- AS-AP Autosampler with diverter valve
- Thermo Scientific Dionex Chromeleon™ Chromatography Data System

Conditions

All analytes were separated with ion-exchange chromatography and detected by suppressed conductivity using CES suppressors, as described in the figures.

Figure 1 shows the typical workflow for a continuously operated IC system. The system is equilibrated and ready to run samples. A check standard is run to verify system performance followed by the samples. This mode of operation lends itself ideally to environments where multiple users require the use of IC and method performance is of high importance.

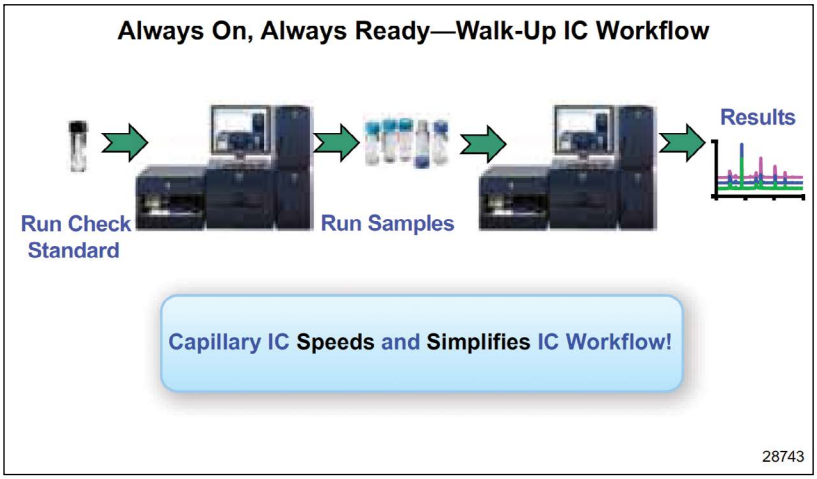

Figure 1. Always On, Always Ready – Walk-Up IC workflow

Results

Walk-Up IC Systems:

Operating an IC system in the Always On, Always Ready mode brings many advantages to IC:

- It simplifies and speeds up the analysis by eliminating time consuming and error prone steps such as manual eluent preparation, startup and equilibration time.
- The system is more stable in terms of noise and detector response.
- Enhanced stability saves time as fewer calibration sequences are required and the system can be quickly verified for system performance by just running a check standard.
- Lends itself to multiuser operation as it allows an operator to walk up to the system and obtain results with minimal training.
- Decreases preventive maintenance and down time.

Advantages:

The advantages of operating a capillary IC system in the Always On, Always Ready mode are:

1. Consumes only 15 mL/day of deionized water, equating to 5.2 L/year.
2. Reagent-Free IC (RFIC™) system technology plus capillary IC provides ease of use, high performance, and gradient eluent generation of potassium hydroxide.
3. More cost effective: The Thermo Scientific Dionex Eluent Generation Cartridge (EGC) lasts for 18 months of continuous operation under standard operating conditions, lowering the overall cost of ownership.
4. Higher eluent concentrations: The capillary eluent generator cartridge can generate up to 200 mM concentration, adding method flexibility and robustness.
5. Higher pressures and faster flow rates: The capillary format Dionex EGC is compatible with pressures up to 5000 psi, facilitating shorter run times, increased productivity, and faster turnaround of results.

System Performance

Peak-to-Peak Noise

Figure 2 illustrates the stability of a capillary IC system-running in continuous operation mode.

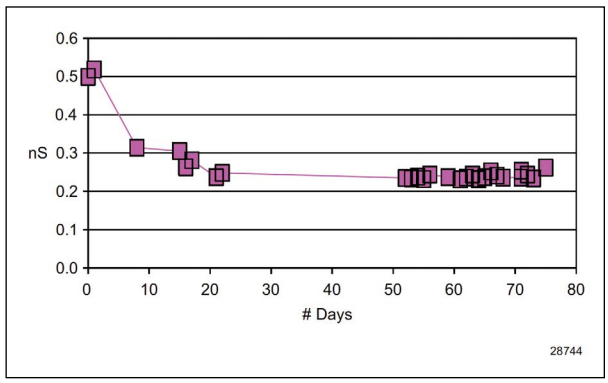

FIGURE 2. Peak-to-peak noise of capillary IC using Thermo Scientific Dionex IonPac™ CS16 cation columns over 80 days: Conditions—Eluent: 30 mM methanesulfonic acid (MSA); Flow 10 µL/min; Suppressor: Thermo Scientific Dionex CCES™ 300 Cation Electrolytic Suppressor

Retention Time and Peak Area Stability

Figure 3 illustrates the retention time stability of a capillary anion IC system running in continuous operation mode. The average retention time reproducibility for anions was well below 0.15%. This level of reproducibility provides accurate peak identification due to minimal shifts in analyte peaks.

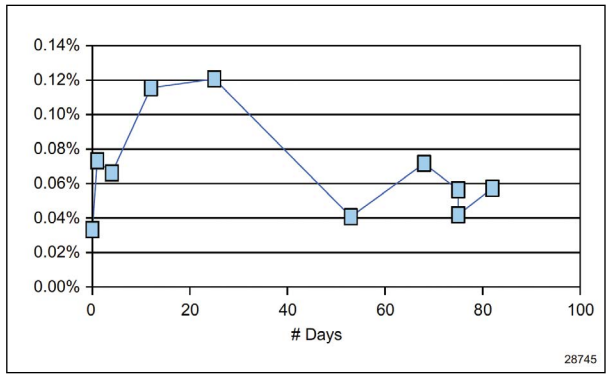

Figure 3. Average anion peak retention time reproducibility of Capillary IC using Dionex IonPac AS19 anion columns over 90 days: Conditions—Eluent: 20 mM KOH; Flow 10 µL/min; Suppressor: Thermo Scientific Dionex ACES™ 300 Anion Electrolytic Suppressor

Peak Area Stability and Linearity

Figures 4 and 5 illustrate the peak area stability of capillary anion and cation IC systems running under continuous operation mode. This level of enhanced stability saves time as fewer calibration sequences are required and the system can be quickly verified for system performance by just running a check standard. Tables 1 and 2 illustrate the linearity performance for the capillary anion and cation systems. The coefficient of linearity (r2) ranged from 0.9993 for magnesium to 1.000 for nitrite.

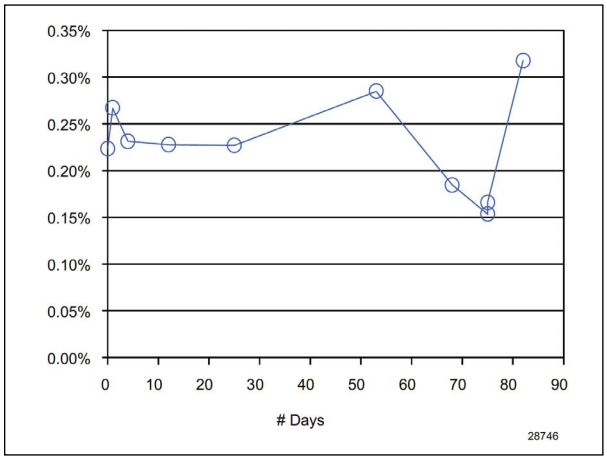

Figure 4. Average anion peak area reproducibility

Figure 5. Average cation peak area reproducibility

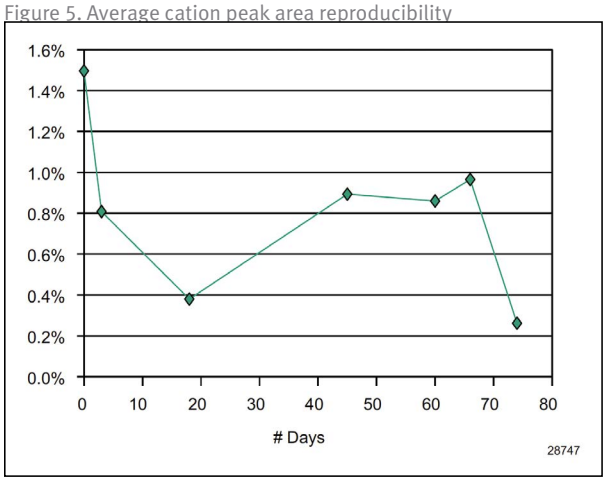

Table 1. Linearity for Anion Capillary System Using the Dionex IonPac AS19 Column

Table 2. Linearity for Cation Capillary System Using the Dionex

	Linear Range (µg/L)	Standard Deviation of % Residuals
Fluoride	1.5-150	0.068
Chlorite	5-500	0.97
Bromate	10-1000	1.24
Chloride	3-300	1.92
Nitrite	7.5-750	2.34
Chlorate	12.5-1250	2.41
Bromide	12.5-1250	2.62
Nitrate	12.5-1250	3.48
Sulfate	15-1500	14.0

	Linear Range (µg/L)	Standard Deviation of % Residuals
Lithium	0.5-50	0.043
Sodium	2-200	0.056
Ammonium+	2.5-250	0.093
Potassium	5-500	0.090
Magnesium	2.5-250	0.130
Calcium	5-500	0.161

High Pressure Capillary IC:

- With the introduction of high-pressure capillary IC, the system is now capable of continuous operation up to 5000 psi with RFIC system technology.
- These new capabilities allow it to take advantage of smaller particle size resin for high-resolution separations, and to accelerate the analysis by simply increasing the flow rate, resulting in higher throughput and sample turnaround.

Figures 6 and 7 illustrate the use of flow rate to decrease the run time for a multi-anion and cation mixture using the capillary platform. The separation of 19 anions relevant to the pharmaceutical industry can be cut in half by increasing the flow rate from 10 to 18 µL/min.

Similar acceleration can be achieved for the analysis of cations. Increasing the flow from 10 to 30 µL/min, the run time can be reduced from 18 min to under 6 min.

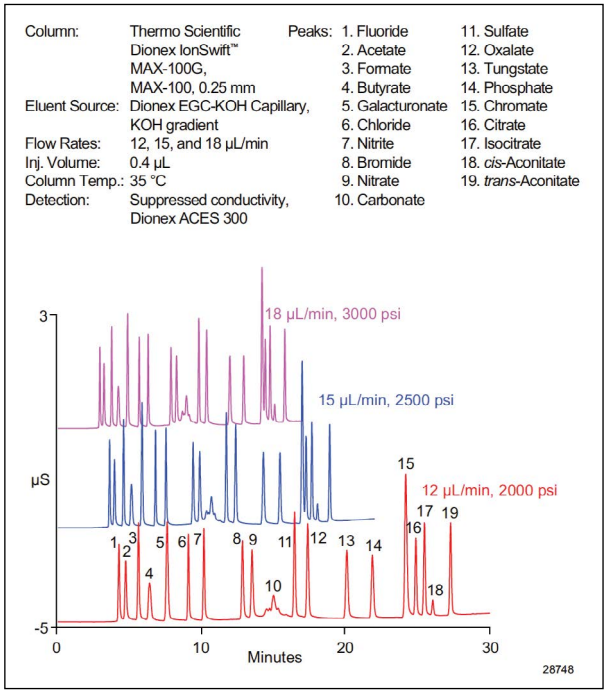

Figure 6. Fast anion determinations of 19 anions standard using high-pressure capillary IC

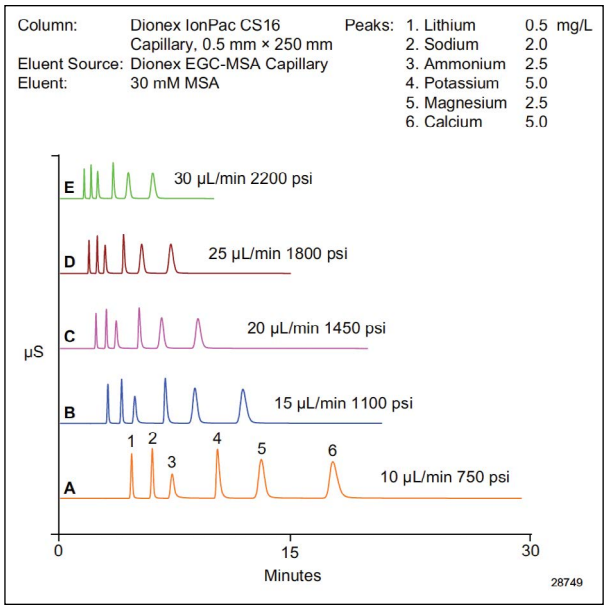

Figure 7. Fast cation determinations by high pressure capillary IC

Analysis of Counterions in Pharmaceutical Products

Pharmaceutical compounds are often charged compounds with a counterion. This counterion is an important part of the formula weight and impacts the effective concentration of the active pharmaceutical ingredient (API). Additionally, the regulatory agencies require that pharmaceutical companies determine the composition of all ingredients.

Figures 8–11 illustrate the potential of capillary IC for the sensitive and selective determination of counterions in pharmaceutical formulation using ion exchange with suppressed conductivity detection. High-pressure capabilities of the capillary format allow the use of higher flow rates to reduce run times and increase sample throughput and result turnaround.

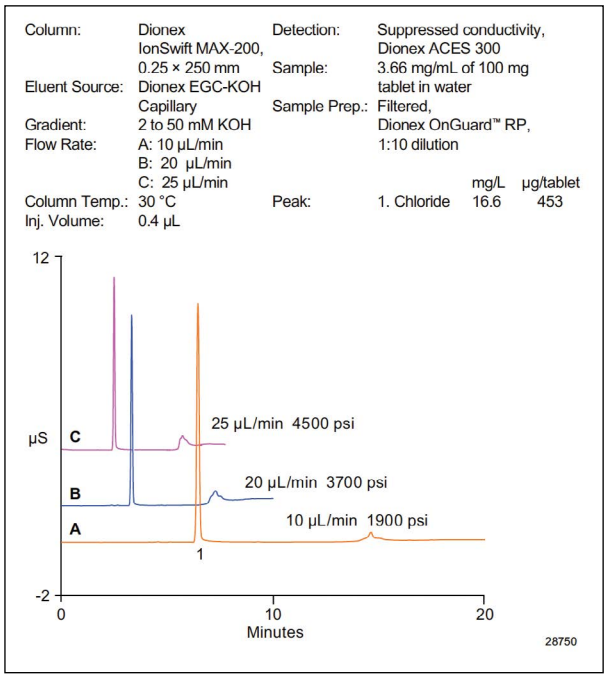

Figure 8. Fast IC: Counteranions in Allegra at different flow rates on Dionex IonSwift MAX-200 column.

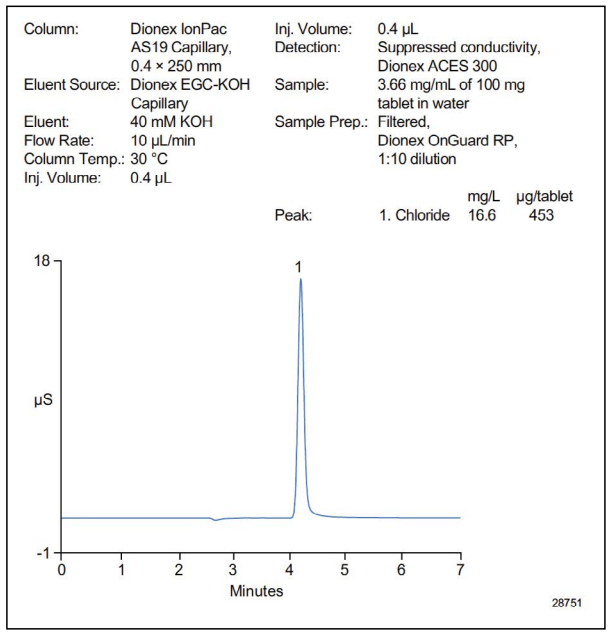

Figure 9. Counterion determinations in a Metformin HCl tablet by capillary IC

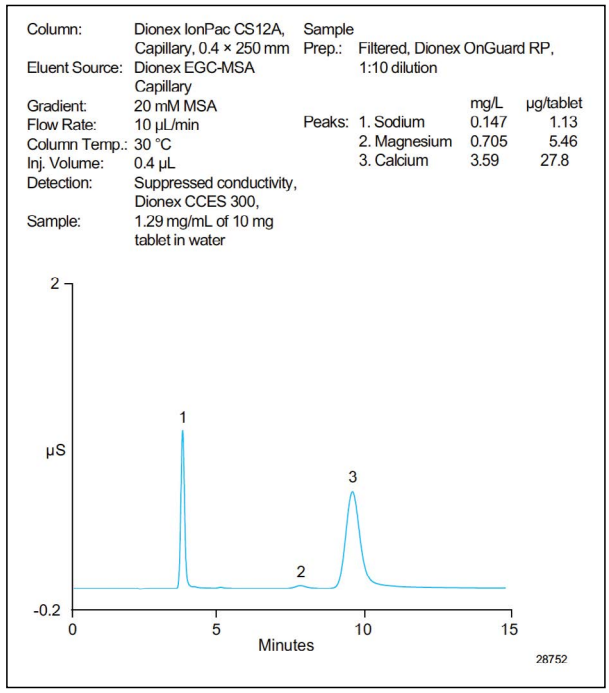

Figure 10. Counterion determinations in a atorvastatin calcium tablet by capillary IC

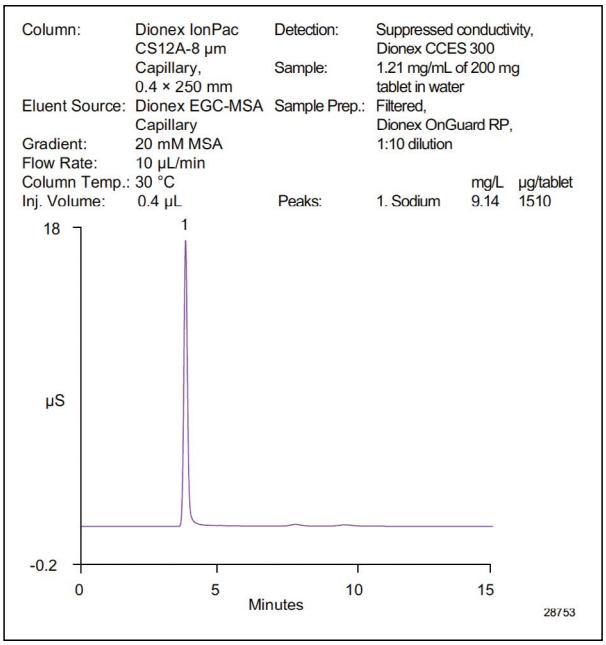

Figure 11. Counterion determinations in a naproxen sodium tablet by capillary IC

Conclusion

- Capillary IC with RFIC system technology redefines the workflow for ion chromatography by simplifying how IC is utilized in laboratories and speeding-up the overall process from sample to result, saving time and lowering the overall cost of ownership.
- The Always On, Always Ready mode of operation improves method performance of the ion chromatograph, providing high accuracy, reproducibility, sensitivity, and confidence in results.
- High pressure IC in the capillary format provides additional benefits of Fast IC and high-resolution ion analysis
- Ion exchange with suppressed conductivity detection provides a highly sensitive and selective detection mode for the analysis of counter ions in Pharmaceutical formulations.

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2. Thermo Fisher Scientific. *Dionex Application Note 164, Assay for Citrate and Phosphate in Pharmaceutical Formulations Using Ion Chromatography.*

Assay for Citrate and Phosphate in Pharmaceutical Formulations Using a High-Pressure Compact Ion Chromatography System

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Key Words
Small particle column, Microbore, Integrion, HPIC, Fast IC, USP method

Goal

To update Application Note 164 with fast separations using a Thermo Scientific™ Dionex™ Integrion™ HPIC™ system and a Thermo Scientific™ Dionex™ IonPac™ AS11-HC-4µm microbore column.

Introduction

Citric acid is an ingredient of many pharmaceutical formulations.[1] It provides an effervescent effect when combined with carbonates or bicarbonates in antacids and dentifrices. Citrate is added as a flavoring and stabilizing agent in some pharmaceutical products to mask the taste of medicinal flavors or as a buffering agent to maintain stability of the active pharmaceutical ingredient (API) and improve the effectiveness of an antioxidant. Additionally, citrate has been used in anticoagulants to preserve blood and to prevent excess bleeding during rectal enema treatments.

As the chromatographic technique of choice for citrate determinations, ion chromatography (IC) with suppressed conductivity detection has been validated in AN164[1,2] and featured in the United States Pharmacopeia (USP) General Chapter <345>, Assay for Citric Acid/Citrate and Phosphate.[3] The method for citric acid determination was first published in the official 2006 edition of the United States Pharmacopeia and National Formulary (USP 29–NF 24). In this method, citrate and phosphate are separated on a L61 column (a Dionex IonPac AS11 hydroxide-selective anion exchange column), using 20 mM NaOH or KOH at a 2 mL/min flow rate. Both phosphate and citrate are eluted from the column and determined within 10 minutes.

Ion chromatography technology has advanced greatly in the past 10 years. A recently introduced compact IC system, the Thermo Scientific™ Dionex™ Integrion™ HPIC™ System, includes many advances in IC instrument technology, such as high pressure capabilities for Reagent-Free™ IC (RFIC™) (up to 5000 psi), column heater control, and other new features designed to increase customer ease-of-use. These include:

- Simplified plumbing layout with easy-to-install Thermo Scientific™ Dionex™ IC PEEK Viper™ fittings connections in key positions to minimize void volume problems.
- Separate compartments for pump, column heater with injection valve, and detection-suppressor to provide separate temperature control and faster equilibration.
- Components tracked by consumable device tracking technologies for better GMP compliance tracking and to assure installation of compatible devices (i.e., installation of non-compatible devices is prevented).
- Detachable tablet for convenient IC control and continuous, full-screen monitoring. The tablet also provides the online instrument manual and trouble-shooting guides. All are available in local language.
- New Thermo Scientific™ Dionex™ Chromeleon 7 Chromatography Data System (CDS) software features, providing easy instrument configuration, monitoring of consumable devices, and online video instructions for conditioning columns, suppressors, and other electrolytic devices.

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Table of contents

Previously, it was shown that this analysis can be run successfully on the Dionex Integrion HPIC system.[4] Here, we update the method described in AN164 for citrate and phosphate determinations using the high-capacity Dionex IonPac AS11-HC-4µm column, which has similar selectivity to L61, on the Dionex Integrion HPIC system. This method demonstrates reduced run times from 10 to 5 min. Following the guidelines outlined in USP General Chapter <1225>, Validation of Compendial Methods[5], the improved IC method is evaluated in terms of linearity, precision, accuracy, robustness, and limit of quantitation (LOQ).

Equipment

Thermo Scientific™ Dionex™ Integrion™ HPIC™ system including:

- The Dionex Integrion HPIC system pump
- CD Conductivity Detector
- Detector compartment temperature control
- Column oven temperature control
- Tablet control
- Consumables device tracking capability
- Eluent generation

Thermo Scientific™ Dionex™ AS-AP Autosampler with 10 mL trays

Table 1 lists the consumable products needed for the Dionex Integrion HPIC system, configured for suppressed conductivity detection.

Software

Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data system (CDS) software CM 7.2 SR4.

Reagents and Standards

- Deionized water, 18 MΩ-cm resistivity or better
- Citric acid (USP, Catalog #1134368)
- Sodium dihydrogen phosphate monohydrate, $NaH_2PO_4 \bullet H_2O$ (EM Science)
- Sodium Hydroxide Solution, 50% (w/w), aqueous NaOH (Fisher Scientific)

It is important to use 18 MΩ-cm resistivity, deionized water (DI) for standards, eluent, and autosampler flush solutions to avoid system contamination, decreased sensitivity, and poor calibration. Degassing the DI water by vacuum filtration prior to use is a good practice.

Table 1. Consumables list for the Dionex Integrion HPIC system.

Product name	Product Description	Part Number
Thermo Scientific™ Dionex™ IC PEEK Viper™ fitting tubing assembly kits	Dionex IC Viper fitting assembly kit for the Integrion: Includes one each of P/Ns: 088805–088811	088798
Dionex IC PEEK Viper fitting tubing assemblies	Guard to separator column: 0.007 in i.d., 4.0 in long (102 mm)	088805
	Injection Valve, Port C (Port 2) to guard column: 0.007 in i.d., 5.5 in long (140 mm)	088806
	EGC Eluent Out to CR-TC Eluent In: 0.007 in i.d., 6.5 in long (165 mm)	088807
	Separator column to Suppressor Eluent In: 0.007 in i.d., 7.0 in (178 mm)	088808
	Suppressor Eluent Out to CD In: 0.007 in i.d., 9.0 in long (229 mm)	088810
	CR-TC Eluent Out to Degasser Eluent In: 0.007 in i.d., 9.5 in long (241 mm)	088811
Dionex AS-AP Autosampler vials	Package of 100, polystyrene vials, caps, blue septa,10 mL	074228
Thermo Scientific™ Dionex™ EGC 500 KOH Potassium Hydroxide Eluent Generator Cartridge	Eluent generator cartridge	075778
Thermo Scientific™ Dionex™ CR-ATC 600 Continuously Regenerated Anion Trap Column	Continuously regenerated trap column used with Dionex EGC 500 KOH cartridge	088662
HP EG Degasser Module	Degasser installed after Dionex CR-TC trap column and before the injection valve. Used with eluent generation.	075522
Thermo Scientific™ Dionex™ AERS™ 500 Anion Electrolytically Regenerated Suppressor	Suppressor for 2 mm columns, using recycle mode	082541
Dionex IonPac AG11-HC-4µm Guard Column	Anion guard column, 2 × 50 mm	078036
Dionex IonPac AS11-HC-4µm Analytical Column	Anion analytical column, 2 × 250 mm	078035
Thermo Scientific™ Nalgene™ syringe filter	Syringe filters, 25 mm, PES membrane, 0.2 µm. This type is compatible with IC analysis.	Thermo Scientific 725-2520*

* Fisher Scientific P/N 09-740-113

20 mM NaOH Matrix Solution

Pipet 1.05 mL of 50% (w/w) aqueous NaOH from the reagent bottle into a 1.00 L volumetric flask containing about 500 mL of degassed DI water. Bring to volume with degassed DI water. Prepare daily.

Stock Standard Solutions

To prepare a 1000 mg/L citrate stock standard, weigh 100 ± 0.1 mg of the citrate (as 101.6 mg of freshly opened official USP citric acid reference standard), add to a 100 mL volumetric flask. Add ~ 25 mL of DI water, swirl to dissolve the citrate reagent and dilute to volume with DI water. To prepare a 600 mg/L phosphate stock standard, weigh 60 ± 0.1 mg of phosphate (as 87.16 mg of $NaH_2PO_4 \bullet H_2O$), add to 100 mL volumetric flask. Dissolve with ~25 mL DI water, and dilute to volume with DI water. To prepare a 500 mg/L citrate and 300 mg/L phosphate mixed citrate/phosphate stock standard, add equal parts of the individual citrate and phosphate stock standards. Store all stock standards in polypropylene bottles at 4 °C.

Working Standard Solutions

To prepare working standard solutions in 1 mM NaOH, add an appropriate amount from the stock standard solutions, 5 mL of 20 mM NaOH and DI water. The 20 mM NaOH solution used for standard and sample preparation should be prepared fresh daily. The mixed standard containing 20 mg/L citrate and 12 mg/L phosphate is used to test the robustness of the assay for citrate and phosphate in samples.

Sample

Anticoagulant Citrate Phosphate Dextrose Solution (CPD) was purchased from Novateinbio (Cat# NIBB-410). According to Novateinbio, the CPD solution contains 90 mM trisodium citrate dihydrate, 17 mM citric acid, 18.5 mM monobasic sodium phosphate, and 142 mM dextrose.

The sample was diluted 100-fold for phosphate and 1000-fold for citrate determinations with 1 mM NaOH so that the concentration of citrate and phosphate were within the calibration ranges.

Chromatographic Conditions for the Assay

Columns	Dionex IonPac AG11-HC-4µm guard (2 × 50 mm) and Dionex IonPac AS11-HC-4µm analytical (2 × 250 mm)
Eluent	60 mM KOH
Eluent Source	Dionex EGC 500 KOH cartridge with Dionex CR-ATC 600 trap column and high pressure EG degasser
Flow Rate	0.35 mL/min
Column Temp	35 °C
Detector Compartment Temperature	20 °C
Injection Volume	2.5 µL, in Push Full mode
Detection	Suppressed conductivity, Dionex AERS 500 suppressor, 2 mm, recycle mode, 52 mA
Run time (min)	5
Background Conductance (µS)	< 1
Typical Noise (nS)	< 1
System backpressure (psi)	~ 4000

Instrument Setup and Installation

The Dionex Integrion HPIC system is a high-pressure-capable, integrated RFIC system. The Dionex Integrion HPIC system and the Dionex EGC 500 KOH cartridge and Dionex CR-ATC 600 trap column consumable products are designed for high pressure conditions up to 5000 psi.

To set up this application, connect the Dionex AS-AP autosampler and the Dionex Integrion HPIC system as shown in Figure 1.

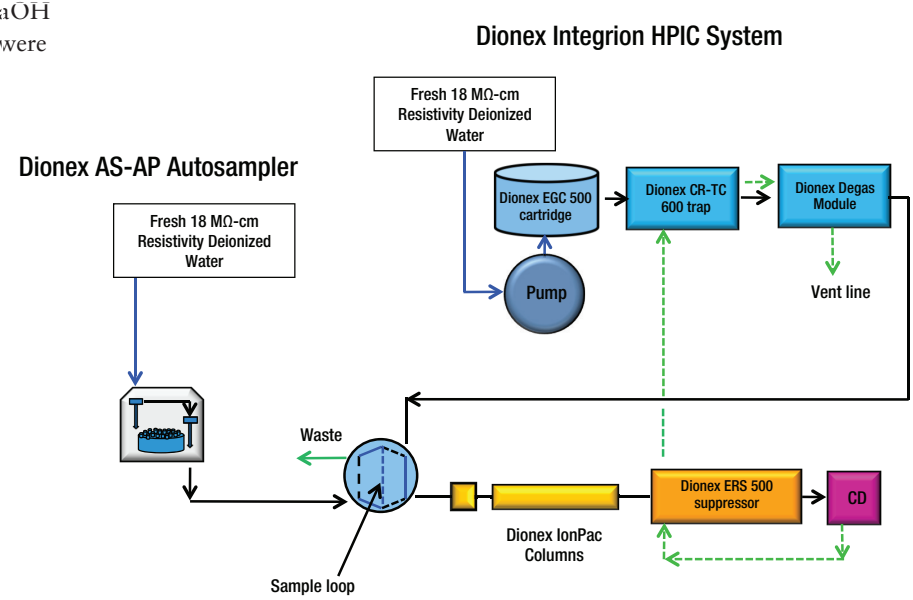


Figure 1. Flow diagram for the Dionex Integrion HPIC system.

Connect the USB cables from the Dionex Integrion HPIC system to the Dionex AS-AP autosampler and to the computer. Connect the power cables and turn on the IC instrument and the autosampler.

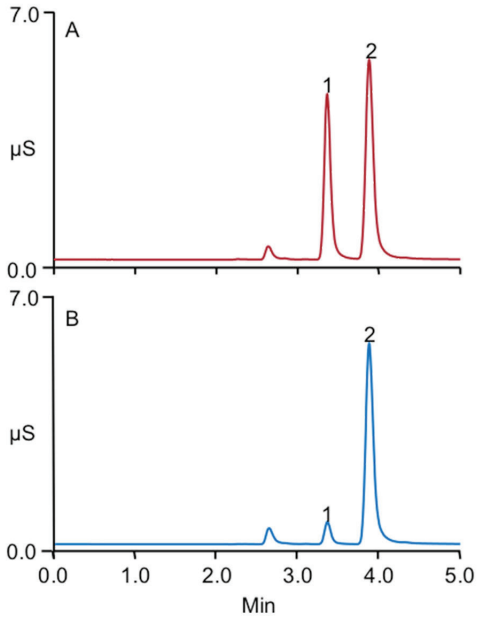
The following are important steps to prepare the system for the analysis. Details can be found in AU 200[6] or TN 175.[7]

- Configuring the modules in the Chromeleon CDS software
- Plumbing the high pressure Dionex Integrion HPIC system
- Conditioning electrolytic devices and columns
- Installing and optimizing the Dionex AS-AP autosampler
- Starting the Dionex Integrion HPIC system
- Creating an instrument method

Results

The Dionex Integrion HPIC system was designed to run analyses with eluent generation up to 5000 psi. An improved IC method for the assay for citric acid/citrate and phosphate was developed using a Dionex Integrion HPIC system and a 2 mm Dionex IonPac AS11-HC-4µm column set. The Dionex IonPac AS11-HC-4µm columns have smaller (4.0 vs. 13 µm) and higher porosity (pore size 2000 Å vs. <10 Å) resin particles with the same functional groups as the L61 column. As a result, the Dionex IonPac AS11-HC-4µm column has similar chromatographic selectivity as L61 combined with higher capacity and efficiency than an L61 column. The methods run on the L61, Dionex IonPac AS11 column can be easily transferred to the Dionex IonPac AS11-HC-4µm column with the benefit of increased peak resolution and faster analysis time. The microbore format provides reduced eluent consumption, which reduces operating costs.

Figure 2 shows the separation of phosphate and citrate on a 2 mm Dionex IonPac AS11-HC-4µm column in a (A) standard and (B) in an anticoagulant citrate, phosphate, dextrose, and adenine dosage form. Using an electrolytically generated 60 mM hydroxide eluent, phosphate and citrate were well separated in 5 min, saving 5 min compared to the separation time reported in AN164. Additionally, the lower flow rate (0.35 mL/min over the previous 2 mL/min) extends the lifetime of the Dionex EGC 500 KOH cartridge. Overall, each separation requires only 1.75 mL of eluent compared to 20 mL in the original application.



Columns:	Dionex IonPac AG11-HC-4µm Dionex IonPac AS11-HC-4µm, 2 mm
Eluent:	60 mM KOH
Eluent Source:	Dionex EGC-500 KOH cartridge, with Dionex CR-ATC 600 trap column, Dionex high pressure degasser
Flow Rate:	0.35 mL/min
Inj. Volume:	2.5 µL
Column Temp.:	35 °C
Detection:	Suppressed conductivity, Dionex AERS 500, 2 mm, 20 °C, 52 mA, recycle mode
Sample :	A: Mixed standard B: CPD Solution, 1000-fold diluted. (Anticoagulant Citrate Phosphate Dextrose)

Peaks:	A	B	
1. Phosphate	12	1.6	mg/L
2. Citrate	20	20	

Figure 2. Separation of phosphate and citrate on a 2mm Dionex IonPac AS11-HC-4µm column. (A) Standard. (B) Anticoagulant citrate, phosphate, dextrose, and adenine dosage form.

The LOQs were determined following the guidelines outlined in USP General Chapter <1225>, Validation of Compendial Methods.[3] The noise is calculated using eight injections of the lowest calibration standard 0.5 mg/L, and LOQ = 10 × δ (standard deviation of concentration by peak area). Table 2 summarizes the calibration and LOQs for citrate and phosphate. This method has lower LOQs with 25% the injection volume of the original method (phosphate 0.03 vs. 0.2 mg/L and citrate 0.06 vs. 0.2 mg/L). Over the calibration range of 0.5 to 200 mg/L for phosphate and 0.5 to 50 mg/L for citrate, the calibrations are linear. When the concentration range is extended above 50 mg/L, the peak area versus concentration relationship for citrate is best fit by a quadratic equation.

The method performance was measured by determining the precision of replicate sample injections and recovery of spiked samples. The relative standard deviations (RSDs) were calculated for samples prepared with the target analytes at a concentration of ~20 mg/L citrate and 12 mg/L phosphate. The intraday precisions for citrate and phosphate from independently prepared solutions analyzed on separate days were < 0.18–0.34% for phosphate and < 0.05–0.57% for citrate. The interday precisions over the four day period were 0.75% for phosphate and 1.25% for citrate. Recoveries were determined by adding known amounts of analyte to the sample solutions. The calculated recoveries were from 95.2–105.5% for all samples.

Table 3 summarizes the precision and recovery results for citrate and phosphate. The precision ranges are for each of the four days, using three independently prepared solutions each day. The spike recoveries are from spiking 2.0 mg/L of citrate or phosphate into the samples.

Table 2. Summary of calibration and limit of quantitation data for citrate and phosphate.

	Calibration Range (mg/L)	Calibration Type	Coefficient of Determination (r^2)	LOQ (mg/L)
Phosphate	0.5–200	Linear	1.0000	0.03
Citrate	0.5–200	Quadratic	0.9998	
Citrate	0.5–50	Linear	0.9997	0.06

Table 3. Accuracy and precision for citrate and phosphate in the pharmaceutical formulation.

	Intraday Precision Ranges (RSD)	Interday Precision (RSD)	Range of Recoveries (%)
Phosphate	0.18–0.34	0.75	95.6–105.5
Citrate	0.05–0.57	1.25	95.2–98.1

Table 4 compares the measured citrate and phosphate concentrations in the CPD solution to the amounts listed on the label and USP monographs.[8] The measured value was very close to the label amounts for citrate but about 6% different for phosphate. The label amount of phosphate appears incorrect. This hypothesis was supported when comparing the measured values to the USP specification. The label amount of phosphate is out of specification. The measured values were within the USP specification for both citrate and phosphate.

The USP defines robustness of an analytical method as a measure of its capacity to remain unaffected by small but deliberate variation in method parameters and provides an indication of its reliability during normal usage. Using the USP guidelines for chromatography, the robustness of this method was evaluated by examining the retention time (RT), peak asymmetry, and resolution of the two analytes in the mixed standard containing 20 mg/L of citrate and 12 mg/L of phosphate after imposing small variations (± 10%) in procedural parameters. (e.g., flow rate, eluent gradient concentration, and column temperature). Due to the maximum flow rate allowed for this column, a 0.380 mL/min flow rate was used instead of the 0.385 mL/min flow rate used for the faster flow test. Test results for different variations are summarized in Table 5. Peak asymmetry was calculated using the USP formula. The peak asymmetries were similar for all conditions. As expected, the reduced temperature (31.5 vs. 35.0 °C) and higher eluent concentration (66 vs. 60 mM KOH) decrease the retention time and resolution. In the worst case, at the highest eluent concentration and lowest

Table 4. Comparison of the citrate and phosphate concentrations obtained to the label amounts and USP Monograph.

	Label Amount (mg/mL)	USP Spec. (mg/mL)	Experimental Average ± standard deviation (mg/mL)
Phosphate	1.75	1.50–1.65	1.65 ± 0.01
Citrate	20.2	19.16–21.18	20.15 ± 0.04

Table 5. Robustness of the assay for citrate and phosphate. *

Parameter		Phosphate RT. (min)	Diff. (%)	Asym.	Diff. (%)	Resol.	Diff. (%)
Flow rate (mL/min)	0.315	3.72	10.68	1.31	1.35	3.28	0.54
	0.35	3.36		1.30		3.26	
	0.38	3.12	-7.20	1.27	-1.74	3.28	0.54
Column Temp. (°C)	31.5	3.19	-5.19	1.30	0.00	2.63	-19.34
	35.0	3.36		1.30		3.26	
	38.5	3.55	5.46	1.29	-0.19	3.96	21.49
Eluent Conc. (mM KOH)	54	3.85	14.47	1.27	-1.74	4.50	37.99
	60	3.36		1.30		3.26	
	66	3.05	-9.40	1.31	1.16	2.40	-26.25
Parameter		**Citrate RT. (min)**	**Diff. (%)**	**Asym.**	**Diff. (%)**	**Resol.**	**Diff. (%)**
Flow rate (mL/min)	0.315	4.29	10.65	1.36	-0.18		
	0.35	3.88		1.36			
	0.38	3.60	-7.10	1.38	1.65		
Column Temp. (°C)	31.5	3.59	-7.44	1.37	0.55		
	35.0	3.88		1.36			
	38.5	4.20	8.17	1.35	-0.74		
Eluent Conc. (mM KOH)	54	4.64	19.65	1.31	-3.49		
	60	3.88		1.36			
	66	3.40	-12.34	1.42	4.04		

*Average of three injections of the 12 mg/L phosphate and 20 mg/L citrate mixed standard

Table 6. Robustness of the assay for citrate and phosphate in a pharmaceutical formulation.*

Parameter		Phosphate (mg/L)	Diff. (%)	Citrate (mg/L)	Diff. (%)
Flow rate (mL/min)	0.315	16.55	0.30	20.23	0.39
	0.35	16.50		20.15	
	0.38	16.58	0.46	20.19	0.21
Column Temp. (°C)	31.5	16.44	-0.37	20.26	0.55
	35.0	16.50		20.15	
	38.5	16.57	0.42	20.20	0.24
Eluent Conc. (mM KOH)	54	16.58	0.47	20.17	0.13
	60	16.50		20.15	
	66	16.19	-1.88	20.19	0.21

* Average of three injections of phosphate in 100-fold diluted CPD solution and citrate in 1000-fold diluted CPD solution.

temperature, citrate and phosphate retained baseline resolution of > 1.5. In addition, a 10% shift in eluent concentration or temperature is unlikely to occur on a Dionex Integrion HPIC system as a result of its eluent generation capabilities and the temperature control in the column and detector compartments. The robustness of this method was also evaluated by comparing the amount of citrate and phosphate measured at these conditions for the samples. Table 6 summarizes the results from the assay of seven different conditions. The differences were minimal (< 2% and most < 1%) indicating that the method is robust.

Conclusion

This application demonstrates an improved IC method for the assay of citrate and phosphate using a Dionex IonPac AS11-HC-4µm microbore column on a Dionex Integrion HPIC system. The improved method has increased sensitivity with LOQs of 0.03 and 0.06 mg/L of phosphate and citrate, respectively, using 25% less sample than the previous method. The method was precise (< 2% RSDs over 4 days) and accurate (95–106% recoveries).

The robustness evaluations showed that the method was affected by variations in temperature and eluent concentration, but baseline separation of citrate and phosphate was maintained. These temperature and eluent variations are unlikely to occur on the Dionex Integrion HPIC system using eluent generation and temperature control in the column and detector compartments. Additionally, the new method doubles sample throughput by reducing the run time to 5 min.

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Impurity profiling and related substances

The aim during any drug synthesis is to produce a pure final product, however, the process often introduces unwanted components.

A variety of different impurities, including degradation products and ionic impurities, can be generated during manufacture, packaging and storage of pharmaceutical products. Some of these impurities may have toxic effects, reduce the efficacy of the final product, or even increase efficacy beyond acceptable levels.

Permitted impurity levels within drug formulations and active pharmaceutical ingredients (APIs) are controlled by regulatory authorities.

Chapter highlights

Acquire more results with better separations and easier interaction simultaneously, without compromise with the **Thermo Scientific™ Vanquish™ Horizon UHPLC system**

Obtain new benchmarks in accuracy, precision, and sensitivity with the biocompatible **Thermo Scientific™ Vanquish™ Flex Binary UHPLC System** or **Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC system**

Obtain high performance, convenience, and flexibility for IC separations with the **Thermo Scientific™ Dionex™ ICS-6000 HPIC™ System**

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Table of contents

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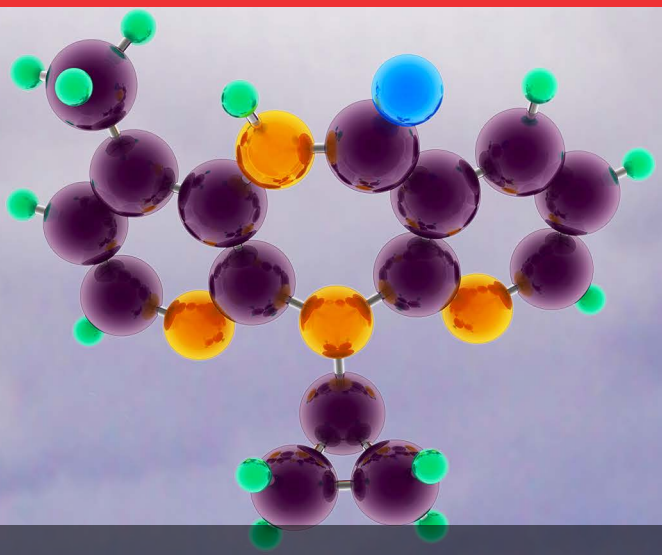

APPLICATION NOTE 72603

Simultaneous quantification of nevirapine and low-level impurities

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Keywords

Thermo Scientific Vanquish DAD FG, nevirapine, impurity profiling, linear range

Goal

To demonstrate the wide dynamic range of the new Thermo Scientific Vanquish DAD FG and how it facilitates the quantification of compounds of very different concentrations.

Application benefits

- The new Thermo Scientific™ Vanquish™ DAD FG provides an industry-leading linear range up to 3700 mAU.
- The detector linearity in combination with low baseline noise allows for the simultaneous quantification of nevirapine and its impurities within a single run.

Introduction

In the pharmaceutical industry, product safety and the quality of distributed drugs are of major importance to ensure proper and efficient therapy. However, the pharmacological-toxicological profile of a drug is not only affected by the active pharmaceutical ingredient (API) itself but also by its impurities originating from manufacturing processes or degradation during storage.[1] For that reason, strict regulations on impurity levels that have to be reported, identified, and/or qualified are defined by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH).[2,3] Thus analytical monitoring of substances and impurities is a crucial requirement in drug development and production. The challenge for the instrumentation is the absorption difference of high-concentrated API and low-level impurities that need to be reported down to a content of 0.03% of the API.[2]

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Vanquish Horizon UHPLC

Table of contents

Nevirapine is a non-nucleoside reverse transcriptase inhibitor drug that was approved for the antiretroviral therapy of HIV/AIDS patients by the regulatory authorities in the 1990s.[4] For impurity profiling, the United States Pharmacopoeia (USP)[5] provides an HPLC method with UV detection that was transferred into an optimized UHPLC method with ballistic gradient in a previous Thermo Fisher Scientific application brief.[6] This method was used in the current study to demonstrate the capabilities of the new Thermo Scientific Vanquish DAD FG to quantify both the API nevirapine and its impurities A, B, and C in a single run.

Experimental

Reagents and materials

- Deionized water, 18.2 MΩ·cm resistivity or higher
- Fisher Scientific™ Acetonitrile, Optima™ LC/MS grade (P/N 10001334)
- Fisher Scientific Ammonium acetate, Optima LC/MS (NH_4Ac) (P/N 11317490)
- Fisher Scientific Acetic acid, Optima LC/MS (P/N 10860701)

Certified standards of the following were purchased from reputable vendors:

- Nevirapine
- 11-Ethyl-4-methyl-5,11-dihydro-6H-dipyrido [3,2-b:2',3'-e][1,4]diazepin-6-one (Impurity A)
- 4-Methyl-5,11-dihydro-6H-dipyrido[3,2-b:2',3'-e][1,4] diazepin-6-one (Impurity B)
- 4-Methyl-11-propyl-5,11-dihydro-6H-dipyrido[3,2-b:2',3'-e][1,4]diazepin-6-one (Impurity C)

Sample preparation

Stock solutions of the API nevirapine (1 mg/mL) and the impurities A, B, and C (100–200 µg/mL each) were prepared by dissolving the solids in pure acetonitrile (ACN) and filling up the respective volumetric flasks with mobile phase A (see below). Calibration standards of nevirapine with concentrations of 0.1, 1, 5, 10, 50, 100, 250, 500, 750, and 1000 µg/mL were prepared by dilution of the stock solution with mobile phase A. These standards were injected with a volume of 1 µL. Further calibration levels were emulated by different injection volumes: 600 µg/mL by injection of 0.8 µL of 750 µg/mL standard; 850 µg/mL by injection of 0.85 µL of 1000 µg/mL standard; 1200 µg/mL by injection of 1.2 µL of 1000 µg/mL standard.

A sample that contained 900 µg/mL API and 0.45 µg/mL of each impurity (corresponding to 0.05% of the API) was prepared by mixing of stock solutions and filling up with mobile phase A.

Instrumentation

Thermo Scientific Vanquish Horizon UHPLC system consisting of:

- System Base Vanquish Horizon (P/N VH-S01-A-01)
- Binary Pump H (P/N VH-P10-A-01)
- Split Sampler HT (P/N VH-A10-A-01)
- Column Compartment H (P/N VH-C10-A-01)
- Diode Array Detector FG (P/N VF-D11-A-01)
- Flow Cell Semi-Micro 7 mm, 2.5 µL (P/N 6083.0530)

LC conditions

Column:	Thermo Scientific™ Syncronis™ C18, 2.1 × 100 mm, 1.7 µm, 100 Å (P/N 97102-102130)
Mobile phase:	A: 10 mM NH_4Ac, pH 5.0 with acetic acid/acetonitrile (85/15; v/v) B: Acetonitrile
Flow rate:	0.8 mL/min
Gradient:	0–0.73 min from 30 to 70% B 0.73–1.1 min 70% B 1.1–1.15 min from 70 to 30% B 1.15–2.8 min 30% B
Column temp.:	50 °C (still air mode)
Autosampler temp.:	8 °C
UV detection:	240 nm, 100 Hz, 0.05 s response time, 4 nm bandwidth, wide slit width
Inj. volume:	0.7–1.2 µL; usually 1 µL
Needle wash:	No wash

Data processing and software

Thermo Scientific™ Chromeleon™ 7.2 SR5 Chromatography Data System (CDS) software was used for data acquisition and analysis.

Results and discussion

In the current study, all standards and prepared samples were injected three times. Figure 1 shows a chromatogram of nevirapine and its impurities. Retention times with precision are summarized in Table 1.

In order to discover the linear detection range provided by the new Vanquish DAD FG, the dependence of detector response on the nevirapine amount was recorded over a concentration range from 0.1 µg/mL to 1200 µg/mL. Figure 2 shows the resulting curve. For a linear calibration with permitted offset and no weighting of data points, the regression line of peak heights exhibited a correlation coefficient of 99.965% and a relative standard deviation of less than 3% for standards from 0.1 µg/mL to 850 µg/mL. This corresponds to peak heights of 0.5 mAU to 3700 mAU. Further confirmation for linear behavior in the stated absorbance range was given by comparison of expected and measured detector responses for the following concentration standard. Each successive calibration point was successfully predicted to within an error of less than 5% from the respective preceding data. Thus, the detector provided an excellent linearity for the current application up to 3.7 AU.

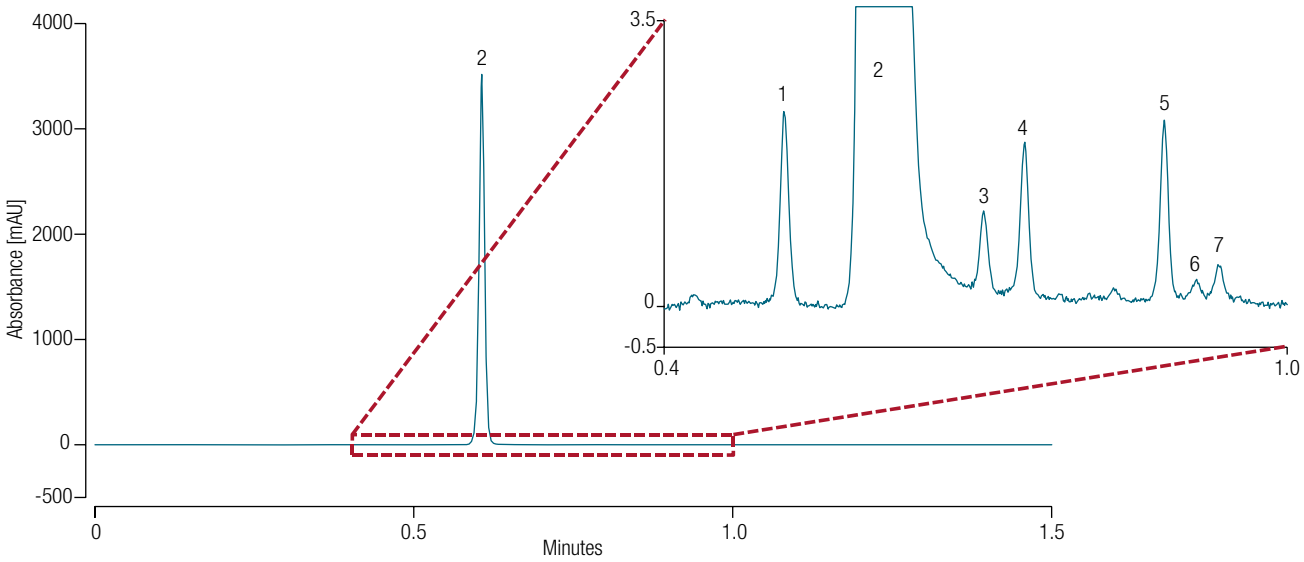

Figure 1. Impurity profile of nevirapine sample (injection volume 0.9 µL) with baseline zoom. For peak assignment see Table 1.

Table 1. Average retention times (t_R) and standard deviations (SD) of nevirapine impurity profiling; determined from all standard and sample injections where the respective peak could be integrated (18 ≤ n ≤ 51).

#Peak	Compound	t_R [min]	t_R SD [min]	t_R RSD [%]
1	Impurity B	0.5160	0.0007	0.14
2	Nevirapine	0.6067	0.0004	0.07
3	Unknown 1	0.7077	0.0005	0.07
4	Impurity A	0.7472	0.0005	0.06
5	Impurity C	0.8815	0.0005	0.05
6	Unknown 2	0.9116	0.0014	0.15
7	Unknown 3	0.9346	0.0008	0.09

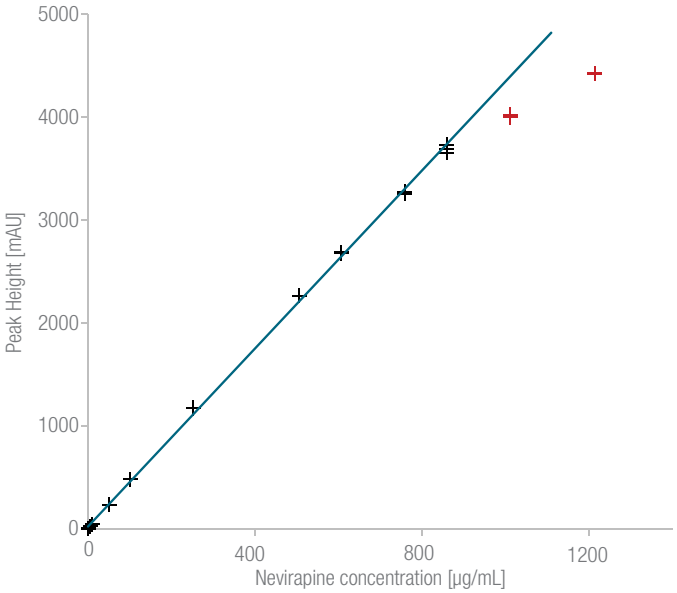

Figure 2. Concentration vs. peak height plot of nevirapine with data points that were considered for calibration (black) and data points that were eliminated from calibration due to curve decline (red). Linear calibration with permitted offset and no weighting.

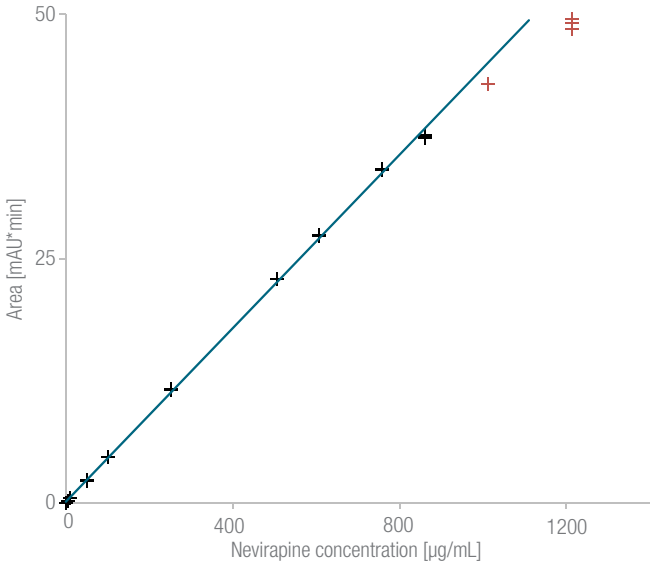

Figure 3. Concentration vs. peak area plot of nevirapine with data points that were considered for calibration (black) and data points that were eliminated from calibration due to curve decline (red). Linear calibration with permitted offset and weighting of calibration points by 1/amount.

This linearity supports the quantification of peaks of extremely different concentrations, eliminating the need for the preparation and injection of different dilutions. If responses are equivalent, this is also valid for the quantitative estimation of several compounds by calibrating with just one compound. This is common practice in pharmaceutical impurity profiling since impurities usually are structurally related to the API. Depending on the drug development stage, impurities may not be fully characterized and therefore are unavailable as reference material. For nevirapine profiling, we applied this procedure by quantifying API and impurities based on the nevirapine calibration curve. For this purpose, we did not utilize the calibration based on peak height that was shown in Figure 2. Instead, we calculated a calibration curve based on peak area with permitted offset and weighting of calibration points by 1/amount (Figure 3). This weighting is easily done in Chromeleon CDS software and cancels out the excessive influence of higher concentration points on the calibration curve. With this approach, low and high concentrations almost equally affect the curve and quantification of peaks over the whole range is improved. Over the same concentration range as before (0.1–850 µg/mL), a correlation coefficient of 99.984% was obtained and deviations of expected and measured response were less than 5% (procedure as described earlier).

For nevirapine, a maximum daily dose of 400 mg translates into a reporting threshold of impurities in new drug substances of 0.05% given by the ICH.[2] In the pure nevirapine standard (850 µg/mL), all six aforementioned impurities were detectable but exhibited relative areas of 0.006% to 0.025%. This was far below the threshold of 0.05% and peaks were not quantifiable due to signal-to-noise ratios (S/N) of less than 10 except for Unknown 1 and Unknown 3. Because of this, a nevirapine sample spiked with 0.05% of impurities A, B, and C related to the API was prepared. The corresponding chromatogram is shown in Figure 1 and the quantitative results are listed in Table 2. The measured amount of the API deviates less than 2% from the expected amount. For the spiked impurities, this deviation is between 6% and 21%, reflecting excellent results under the consideration that equal responses of all compounds are just an approximated assumption and spiked impurity quantities are also affected by inherent concentrations below the quantification limit. Based on the highest and lowest standard that fit the calibration curve, a quantification down to 0.012% relative area is possible with the presented method.

Table 2. Average results of nevirapine and impurity quantification from calibration curve in Figure 3 and three injections of spiked nevirapine sample (see Figure 1). Numbers in red are not reliable as areas were smaller than the lowest calibration standard and/or S/N were less than 10.

#Peak	Compound	Area [mAU*min]	Relative Area [%]	S/N	Determined Amount [µg/mL]	Spiked Amount [µg/mL]
1	Impurity B	0.0225	0.063	70	0.49	0.405
2	Nevirapine	35.7293	99.791	137291	797.0	810
3	Unknown 1	0.0088	0.025	23	0.19	-
4	Impurity A	0.0175	0.049	43	0.38	0.405
5	Impurity C	0.0205	0.057	75	0.45	0.405
6	Unknown 2	0.0019	0.005	8.5	0.04	-
7	Unknown 3	0.0036	0.010	12	0.07	-

Conclusion

- The new Vanquish DAD FG combines a very wide linear range with the best noise performance, enabling for the simultaneous quantification of APIs and impurities within a single run.
- Excellent quantitative results were obtained for the API nevirapine and its impurities with an optimized UHPLC method with deviations from expected amounts of less than 2% for the API and 6–21% for impurities under the approximated assumption of equivalent responses. Impurity quantification was possible down to 0.012% relative area if the linear detection range is fully exploited.

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Fast Nevirapine Impurity Profiling Using UHPLC-DAD

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Key Words
Active Pharmaceutical Ingredient (API), Impurity profiling, Ballistic gradient, HPLC to UHPLC transfer tool, ICH requirements

Goal
To develop a fast ballistic gradient UHPLC method optimized for simultaneous analysis of an API and impurities in a nevirapine tablet

Introduction
Nevirapine is a non-nucleoside reverse transcriptase inhibitor with activity against human immunodeficiency virus type 1 (HIV-1), currently marketed for the treatment of HIV-1 infected adults.[1] The United States Pharmacopeia (USP) uses a reversed-phase high-performance liquid chromatography (HPLC) separation with UV detection to determine nevirapine and its impurities. The related column is a 4.6 × 150 mm column packed with L60 (spherical, porous silica gel, 5 µm in diameter).[2] Due to the strong retention of impurity C, the USP monograph method requires about 30 minutes to separate this API and known impurities. A previous Dionex, now part of Thermo Scientific, application note demonstrated that an HPLC-UV separation can meet or exceed the chromatographic requirements of the USP monograph method for nevirapine while requiring about half the analysis time per sample.[3]

Here we report further optimization of this approach, using a state of the art gradient UHPLC-UV method. Applying ballistic gradients with latest-generation UHPLC equipment achieves significantly shorter analysis time while maintaining compliance with ICH (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use) requirements.

Equipment
- Thermo Scientific™ Vanquish™ UHPLC System consisting of:
  - Binary Pump H (P/N VH-P10-A)
  - Split Sampler HT (P/N VH-A10-A)
  - Column Compartment H (P/N VH-C10-A)
  - Diode Array Detector HL (P/N VH-D10-A)
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System software 6.80 SR14

Table 1. Reagents and chemicals.

Compound	Supplier	P/N
Nevirapine Anhydrous	USP Standard	1460703
Related compound A	LGC Standards	MM1146.01
Related compound B	LGC Standards	MM1146.02
Impurity C	LGC Standards	MM1146.03
Ammonium acetate	Fisher Scientific	A114-50
Acetonitrile OPTIMA™ LC/MS	Fisher Scientific	A955-212
Ultra-pure lab water, 18.2 MΩ·cm at 25 °C	n.a.	n.a.

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Table of contents

Experimental Conditions	
Column:	Thermo Scientific™ Syncronis™ C18, 1.7 µm, 2.1 × 100 mm
Mobile Phase:	A) 10 mM NH_4OAc, pH 5.0 with acetic acid/ acetonitrile (85%/15% v/v) B) Acetonitrile
Gradient:	0–0.730 min: 30–70% B; 0.730–1.100 min: 70% B; 1.100–1.150 min: 70–30% B; 1.150–2.800 min: 30%
Flow Rate:	0.800 mL/min
Pressure:	950 bar (max.)
Temperature:	50 °C
Injection Volume:	1 µL
Detection:	240 nm, 100 Hz, 0.05 s response time, 4 nm slit width, 4 nm bandwidth
Flow cell:	LightPipe™, 10 mm

Results and Discussion

A number of rules should be followed for method transfer from HPLC to UHPLC, to adapt parameters such as flow rate, injection volume, or gradient profile (if applicable) to the new column characteristics. The Thermo Scientific Method Transfer Tool is a universal, multi-language tool that streamlines this process[4] and was used to transfer the USP method to a high-efficiency Syncronis UHPLC column. The application was accelerated by a factor of 1.3 by using the tool's boost factor functionality. The result was 130% of the initial linear mobile phase velocity through the column. With sub-2 µm particle columns this can easily be done while keeping the chromatographic efficiency almost constant.

The USP monograph uses 25 mM NH_4OAc, pH 5.0/ acetonitrile (80%/20% v/v) as mobile phase. In the method transfer, the buffer concentration was reduced to 10 mM and a gradient was applied with a maximum acetonitrile content of 80%. The buffer concentration was reduced to maintain compatibility of the buffer with the higher organic content. The detection wavelength was changed from 220 nm to 240 nm to eliminate baseline drift caused by varying absorption over the course of the gradient.

Figure 1 shows a calibration curve of the active pharmaceutical ingredient nevirapine with absorbances up to 3000 mAU. Even up to this high absorption, the calibration curve is almost perfectly linear with a correlation coefficient of $R^2 = 0.9998$.

Figure 2 shows a chromatogram of the 0.66 mg/mL standard. The zoom into the baseline reveals a number of impurities, including known compounds A, B, and C mentioned in the USP method as well as four additional impurities. Table 2 identifies the peaks of the chromatogram, the related signal-to-noise ratio and the relative area of the individual compound.

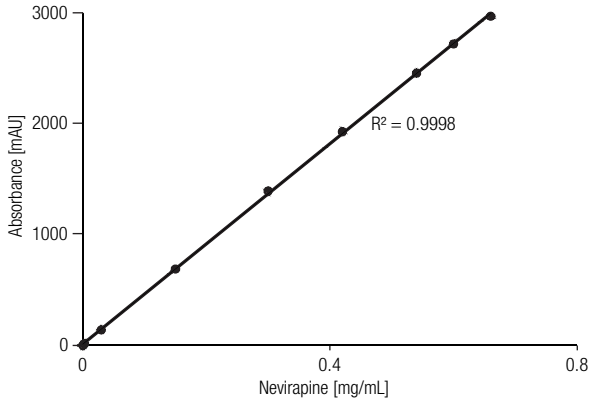

Figure 1. Calibration curve of nevirapine demonstrating excellent linearity up to 3000 mAU.

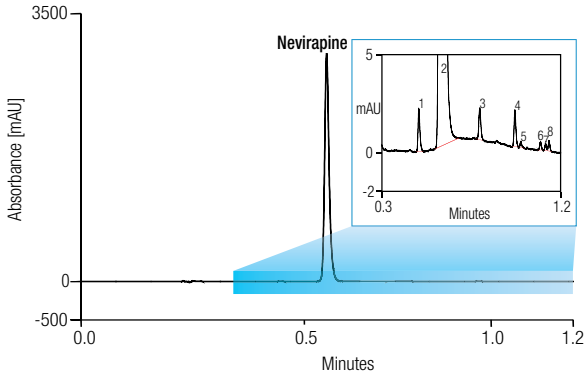

Figure 2. Impurity profiling of Nevirapine (0.66 mg/mL) with UHPLC gradient and zoom into the baseline to show related impurities.

Table 2. Peak identification for Figure 2 with related signal-to-noise ratio and area percentage of the individual compound.

Peak No.	Compound	Signal-to-Noise	Area [%]
1	Related compound B	28	0.065
2	Nevirapine	17602	99.790
3	Related compound A	16	0.050
4	Impurity C	53	0.053
5	Unknown 1	8	0.007
6	Unknown 2	17	0.011
7	Unknown 3	10	0.008
8	Unknown 4	11	0.013

The ICH defines the reporting threshold for impurities depending on the maximum daily dose. For nevirapine, a dosage of 400 mg/day translates into a reporting threshold of 0.05%.[5] According to the common definition of the Limit of Quantitation (LOQ)[6] defined as S/N ratio of at least 10, the nevirapine assay here described allows quantitation down to 0.008% relative area.

The compliance with the ICH guidelines was achieved despite challenging chromatographic conditions. We applied a ballistic 44 s linear gradient, achieving the elution of all relevant impurities within 1.2 min. The total run time was 2.8 min, using default detection parameters and 100 Hz data collection rate. This application is therefore a good example that the Vanquish system performance easily supports even ambitious analysis goals without the need for time-consuming instrument optimization.

Conclusion

This application describes an optimized method for the impurity profiling of nevirapine using a ballistic gradient method. The separation is completed in 2.8 min, compared to 80 min of the isocratic USP method. Even under challenging chromatographic conditions, the Vanquish UHPLC system easily enables the simultaneous detection of the API and related impurities while achieving compliance with the ICH guidelines on impurity monitoring.

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APPLICATION NOTE 72625

Fast UHPLC separation of budesonide diastereomers

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Keywords

Active Pharmaceutical Ingredient (API), USP, corticosteroid, Vanquish Flex, Accucore XL

Goal

Development of a fast assay for budesonide diastereomers based on isocratic UHPLC with a solid core reversed phase column

Introduction

Budesonide is a synthetic corticosteroid that is available as mixture of two diastereomers, 22R and 22S. The 22R form is two times as active as the 22S, and the ratio of the two diastereomers in medicinal products is therefore controlled by regulatory agencies. In contrast to enantiomers, which are chemically identical, diastereomers are chemically different and can be separated in achiral systems. Separation of the budesonide isomers is nonetheless difficult due to the very similar interaction of the almost identical molecules with the stationary phase. Because of this similar retention behavior, assays for active pharmaceutical ingredients (API) containing isomeric impurities are normally developed as isocratic methods rather than gradient methods. The USP monograph for the budesonide assay is an isocratic method that requires the fulfillment of three parameters: the plate count for R-budesonide must be at least 5500, the resolution between the two peaks must be at least 1.5 and the retention time of S-budesonide must be 1.1 times that of R-budesonide.[1]

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Vanquish Flex UHPLC

Table of contents

In this work, an assay for budesonide was developed using a Thermo Scientific™ Accucore™ XL C18, 4 µm column, operated with a Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC system. The solid core technology of the Accucore XL C18 column allowed fast and efficient separation of budesonide diastereomers. The Vanquish Flex Quaternary system provided the flexibility and reliability required to develop and optimize methods for the budesonide API analysis. This work describes the fine tuning of the challenging isocratic separation based on kinetic and thermodynamic analysis.

Experimental

Instrumentation

- Vanquish Flex Quaternary system:
  - Quaternary Pump, Vanquish Flex, P/N VF-P20-A, with 150 µL mixer
  - Split Sampler FT, P/N VF-A10-A
  - Column Compartment, P/N VH-C10-A
  - UV Detector, VWD F, P/N VF-D40-A, with 2.5 µL flow cell, 7 mm, P/N 6077.0360
- Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software version 7.2

Chemicals and consumables

- Acetonitrile, Optima™ LC-MS grade, Fisher Chemical (P/N A955-212)
- Deionized water, Thermo Scientific™ Barnstead™ GenPure™ xCAD Plus Ultrapure Water Purification System (P/N 50136149)
- Accucore XL C18, 4 µm, 3 × 150 mm (P/N 74104-153030)
- Budesonide, >99%, Sigma®, CAS 51333-22-3 (P/N B7777)

Separation conditions

The separation conditions are listed in Tables 1 and 2.

Table 1. Chromatographic conditions used in all experiments.

Column	Accucore XL C18, 4 µm, 3 × 150 mm
Mobile phase	60% water (18.2 MΩ at 25 °C), 40% acetonitrile
Injection volume	1 µL
Detection	244 nm, 2.5 µL flow cell, data collection rate 20 Hz, response time 0.20 s

Table 2. Optimized chromatographic conditions.

Column	Accucore XL C18, 4 µm, 3 x 150 mm
Mobile phase	60% water, 40% acetonitrile, pre-mixed in channel A
Flow rate	0.64 mL/min
Temperature	30 °C, forced air, Active pre-heater: 30 °C
Injection volume	1 µL
Detection	Variable wavelength detector 244 nm, data collection rate 20 Hz, response time 0.20 s 2.5 µL flow cell
Analytes	1) R-Budesonide, Diastereomer B 2) S-Budesonide, Diastereomer A
Run time	2.5 minutes

Results and discussion

Initial solvent screening showed that 40% acetonitrile in water provided values for resolution, plate number (N), and relative retention time (RRT) that fulfilled the compendial requirements (Figure 1). The initial flow rate was 0.52 mL/min and the run time was 5 minutes with separation of the diastereomers at 3 minutes.

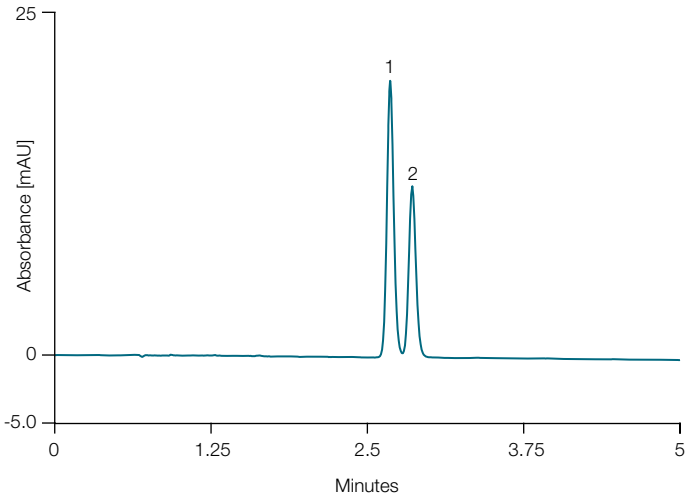

Figure 1. Initial method for the assay of budesonide. Flow rate 0.52 mL/min. Temperature 30 °C. Other conditions as in Table 1.

Flow rate optimization

Even though the method fulfilled the requirements and was sufficiently fast, further method optimization possibilities were explored. The first optimization step was to evaluate the dependence between flow rate and efficiency of the budesonide peak. Figure 2 shows the Van Deemter plot measured at 30 °C (blue trace). Observation of the plot indicates that the flow rate chosen to run the mobile phase optimization, 0.52 mL/min, is greater than the flow rate at the minimum of the Van Deemter curve. The flow rate for optimal efficiency is 0.106 mL/min. The separation of the budesonide diastereomers at a flow rate close to optimum would produce very efficient peaks, with more than 19,000 plates and resolution of 1.95. However, the run would take 14 minutes to separate the diastereomers at this decreased flow rate (Figure 3a). Because the minimum requirements of efficiency and resolution are easily met with a fast run, working close to the optimal flow rate is not recommended for this specific assay.

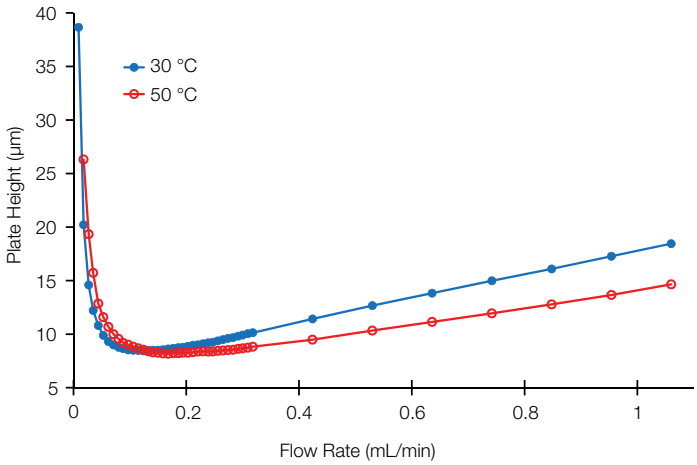

Figure 2. Van Deemter plot for the budesonide diastereomers. The plot shows plate height of the R-budesonide peak at different flow rates, at 30 °C (blue trace) and at 50 °C (red trace). Conditions listed in Table 1.

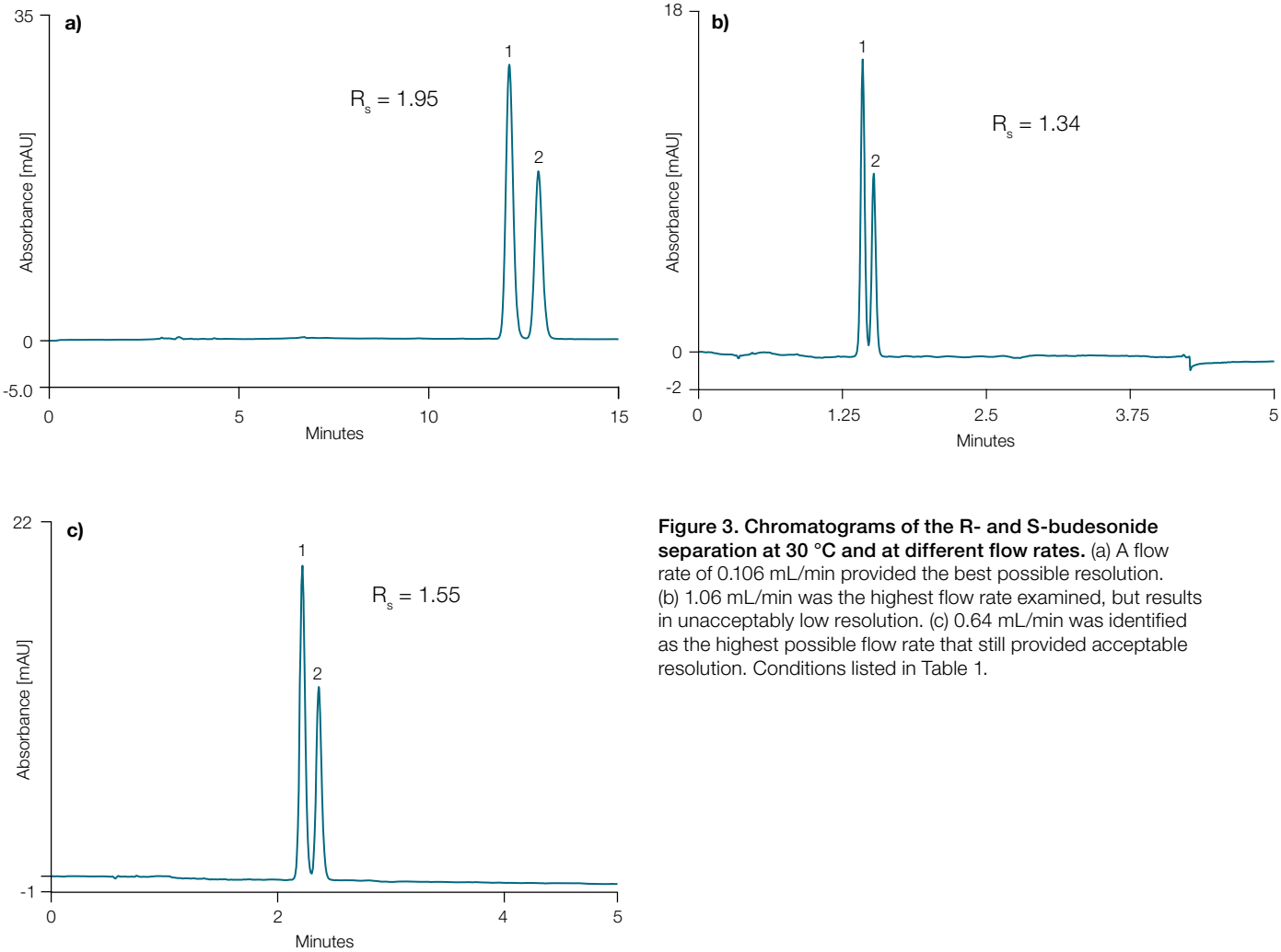

Figure 3. Chromatograms of the R- and S-budesonide separation at 30 °C and at different flow rates. (a) A flow rate of 0.106 mL/min provided the best possible resolution. (b) 1.06 mL/min was the highest flow rate examined, but results in unacceptably low resolution. (c) 0.64 mL/min was identified as the highest possible flow rate that still provided acceptable resolution. Conditions listed in Table 1.

In fact, because of the relatively flat behavior of the Van Deemter plot at high flow rate, caused by the small C term associated with the solid core particle column, the method run time can be decreased further. Equation 1 describes the relationship between plate height (H), plate number (N), and column length (L). The separation at 0.52 mL/min delivered more than 10,000 plates, which corresponds to H = 15 µm. This plate count of 10,000 is 1.8 times larger than the USP requirement of 5500 plates (Table 3). Applying this information, the Van Deemter equation can be used to estimate the fastest flow rate to fulfill the efficiency requirement of H = 27 µm. Although the Van Deemter curve does not extend this far, we know the flow rate would be above 1 mL/min, and the method at this flow rate would separate the diastereomers in less than 1.5 minutes (Figure 3b). However, resolution also changes with flow rate. The relationship between resolution (Rs), selectivity (α), and retention factor (k') is described in Equation 2.

$$\text{Equation 1: } H = \frac{L}{N}$$

$$\text{Equation 2: } Rs = \frac{\sqrt{N}}{4}\,\frac{(\alpha-1)}{\alpha}\,\frac{k'}{(k'+1)}$$

$$\text{Equation 3: } RRT = \frac{RT_2}{RT_1} = \frac{1+k_2'}{1+k_1'}$$

Of all the terms in Equation 1, only N depends on the flow rate. The Van Deemter equation can be used to predict the change of resolution with flow rate by easily combining Equation 1 and Equation 2. The flow rate of 0.64 mL/min is the fastest separation that could be achieved to fulfill the compendium requirements of both resolution and efficiency. This flow rate is within the USP's adjustment limits of +50% for isocratic methods, according to the <621> guidance. For this flow rate, the total analysis time, without the injection cycle, is 2.5 minutes.

Temperature optimization

Theory predicts that the method could be sped up by raising the temperature, which should improve efficiency at higher flow rates. The method, when run at a higher temperature and higher flow rate, would then be faster than and at least equally as efficient as the slower method. Figure 1 shows the Van Deemter measured at 50 °C (red empty circles). The comparison between the Van Deemter at 30 and 50 °C clearly points to the advantages of running chromatography at high temperature when fast methods are needed. In fact, the dependency of plate height, and therefore resolution, on flow rate is less steep at 50 °C. With this consideration we could conclude that further method optimization should be made at 50 °C. However, the monograph also dictates a minimum value for the RRT. As mentioned above, the RRT requirements were fulfilled at 30 °C. The RRT is described by Equation 3, where RT_1 is the retention time and k'_1 is the retention factor. Equation 3 shows that as long as the retention factors of the isomers do not change, the RRT remains constant. The influence of flow rate on retention factors is assumed constant in these experiments, therefore we can freely select the flow rate across the Van Deemter curve without significantly affecting the RRT. Temperature, on the other hand, influences the retention factor.

Because the diastereomers are chemically and structurally similar, it could be expected that the temperature dependence of their retention factors would also be similar. The Van't Hoff plot of Figure 4 shows that this is not true. The natural log of retention factor is linearly dependent on 1/T for both diastereomers, which is a common behavior in chromatography. However, the lines are not parallel, and the retention factors become closer as the temperature increases. The practical consequence is that the RRT also decreases with temperature. The minimum RRT of 1.1 is fulfilled only for temperature of 30 °C or lower for the mobile phase composition used in these experiments. In this case, the method cannot be accelerated further by increasing temperature, and the initial temperature of 30 °C is selected. Optimized conditions are listed in Table 2.

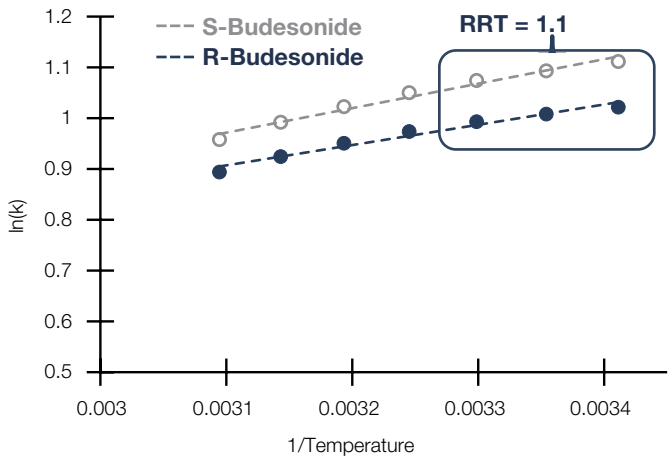

Figure 4. Van't Hoff plot for the budesonide diastereomers.

Table 3. USP requirements for the assay of budesonide drug substance by HPLC and performance of the optimized UHPLC method.

Parameter	USP requirements	Optimized method
Peak resolution	≥ 1.5	1.5
Theoretical Plates R-budesonide	≥ 5500	10,485
RRT	1.1	1.1

Conclusion

In this work, an assay for the quantification of budesonide diastereomers was developed with a Vanquish Flex Quaternary system fitted with an Accucore XL C18 column. It was shown how simple kinetic and thermodynamic tools, namely Van Deemter and Van't Hoff plots, can be used to speed-up a difficult isocratic separation. Although full Van Deemter plots were recorded in this work for educational purposes, such an extensive evaluation of flow rate influence is not needed for method optimization in practice. A few data points at flow rates above the minimum are usually sufficient to assess the separation performance at a high flow rate.

The findings also illustrate that separation speed optimization through elevated column temperature may sometimes fail, namely when selectivity of a critical pair is reduced with increasing temperature.

The selection of the Accucore XL C18 column allowed the use of a flow rate five times greater than the optimum due to the flatter Van Deemter plot associated with the low C-term value characteristic of solid core particles. This increased flow rate greatly increases the potential method throughput even at lower column temperatures. In general, Accucore columns allow excellent separation efficiency with limited back pressures. The final method back pressure was only 120 bar, which is easily attainable with standard HPLC instrumentation.

Reference

1. *United States Pharmacopeia, Revision Bulletin.* "Budesonide." June 1, 2011, 1-2 [Online] http://www.uspnf.com/official-text/accelerated-revision-process/accelerated-revision-history/budesonide (accessed March 20, 2017).

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Application Note 1129

Ternary Gradient for Tenofovir Disoproxil Fumarate Impurity Profiling

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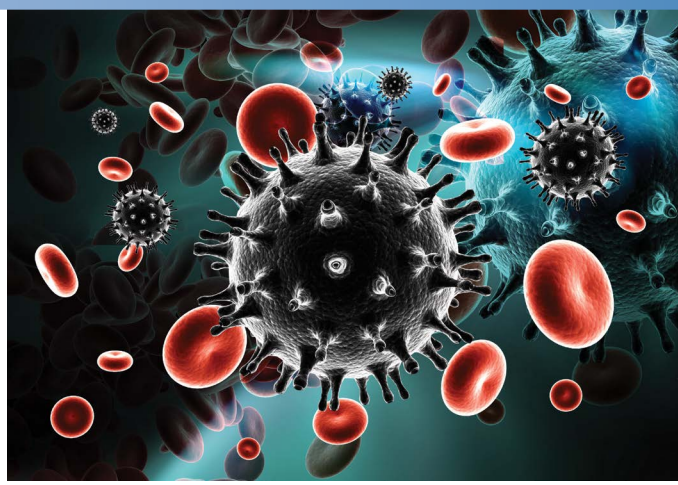
Key Words
USP, Aqueous Gradient, Active Pharmaceutical Ingredient, Equilibration, Accucore aQ Column

Goal
Demonstrate the robust performance of the Thermo Scientific™ Vanquish™ Flex UHPLC System in the challenging ternary gradient application starting at zero percent organic solvent.



Introduction
Tenofovir belongs to a class of antiretroviral drugs known as nucleotide analogue reverse transcriptase inhibitors (NRTIs), which block reverse transcriptase, an enzyme crucial to viral production. Tenofovir is in formulation given as the prodrug tenofovir disoproxil fumarate (TDF) in combination with the nucleoside reverse-transcriptase inhibitor emtricitabine. The combination drug is marketed under the tradename Truvada® by Gilead.

The organic impurities of TDF are analyzed following the instructions noted in a monograph posted on the USP website as USP Pending monograph.[1] The eluents of the original method of the procedure to analyze the organic impurities is modified to gain a mass spectrometry (MS) compatible method with easier eluent preparation and shortened run time. The system suitability testing of the original method requires the challenging separation of the polar compounds adenine and tenofovir. In this work, the simultaneous separation of early-eluting polar compounds and later-eluting nonpolar compounds is achieved by applying a ternary gradient with the Vanquish Flex UHPLC System[2] using a Thermo Scientific™ Accucore™ aQ column.[3]

Experimental
Equipment
Vanquish Flex UHPLC system consisting of:

- System Base (P/N VH-S01-A)
- Quaternary Pump F (P/N VF-P20-A)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Active Pre-heater (6732.0110)
- Diode Array Detector HL (P/N VH-D10-A)
- LightPipe Flow Cell, Standard (10 mm; P/N 6083.0100)

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Vanquish Flex UHPLC

Table of contents

Chromatographic Conditions	
Column:	Accucore aQ, 2.6 µm, 2.1 × 100 mm (P/N 17326-102130)
Mobile Phase:	A - 25 mM ammonium acetate buffer, pH 3.8 with acetic acid
	B - Methanol
	C - Acetonitrile
Gradient:	0–4.0 min 0% B–70% B, 0% C–15% C
	4.0–4.5 min 70% B, 15% C
	4.5–5 min 70% B–25% B, 15% C–70% C
	5–6 min 25% B, 70% C
	6.0–6.1 min 25% B–0% B, 70% C–0% C
	6.1–15 min 0% B, 0% C
Flow Rate:	0.6 mL/min
Temperature:	40 °C, Still air
	Active pre-heater: 40 °C
Injection Volume:	1 µL
Detection:	260 nm
	Data Collection Rate: 100 Hz
	Response time: 0.04 s
Analytes:	Test solution: Adenine (50 µg/mL), tenofovir (150 µg/mL), emtricitabine (100 µg/mL), tenofovir disoproxil fumarate (100 µg/mL) in mobile phase A Sample solution: Tenofovir disoproxil fumarate (100 µg/mL) in mobile phase A

Data Processing

Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System software, version 7.2, SR 3

Results and Discussion

The USP referenced mobile phases for *Organic Impurities, Procedure 1* requests dibasic sodium phosphate and tertiary butyl alcohol mixed with methanol. The reproducible premixing of eluents consisting of three different components is generally challenging. The consistent composition of the mobile phases from batch to batch might be questionable. In addition, these eluents are not MS-compatible, are prone to salt precipitation, and are inconvenient for the preparation. Specifically, the tertiary alcohol has a melting point of 25 °C, which makes it difficult to handle at room temperature. For these reasons, the described eluents might not be ideal for running routine methods with high eluent consumption. Here, the mobile phase was changed to an MS-compatible 25 mM ammonium acetate buffer, pH 3.8, as eluent, mixed with methanol and acetonitrile by the quaternary pump. The literature method uses a 250 mm long column with 5 µm fully porous particles and applies gradients between 60 and 70 minutes for organic impurity profiling. To decrease the method run time, a 100 mm, 2.6 µm fused core column is used.

The separation challenge of the here-described analytical problem is to achieve a resolution of at least 1.5 between the early eluting peaks adenine and tenofovir in the test solution. The Accucore aQ columns are compatible with 100% aqueous mobile phases and offer special selectivity for polar analytes. Starting at 100% aqueous mobile phase and the increase of acetonitrile content with a lower slope than methanol allowed the separation of adenine and tenofovir with a resolution of more than two (Figure 1). This is not achievable with a binary water/acetonitrile gradient and fast method. The increase of the acetonitrile content during the progression of the gradient allowed an earlier elution of the more hydrophobic emtricitabine, tenofovir disoproxil fumarate, and the impurities. An adequate equilibration time is beneficial for the retention time precision.

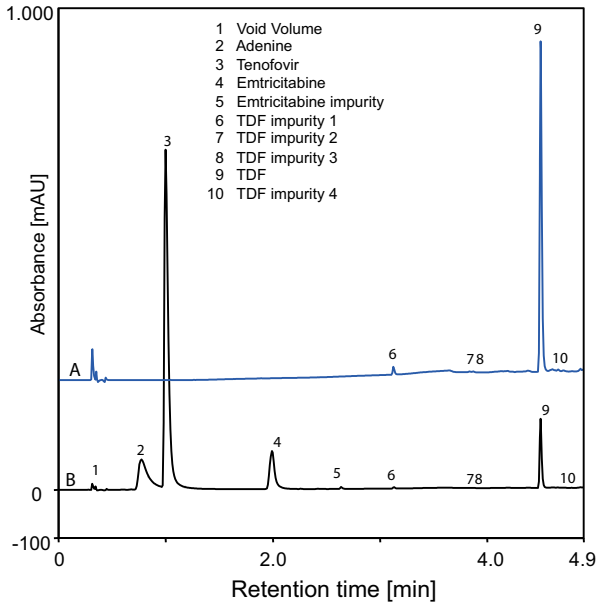

Figure 1. The sample solution (A) shows the active pharmaceutical ingredient and impurities. Test solution (B) shows sufficient resolution for the critical substances 2 and 3 running a ternary gradient starting with 100% aqueous conditions.

To investigate the long-term robustness of the ternary gradient method the sample solution was injected repeatedly over a 15 hour time period. For the tenofovir disoproxil fumarate, a retention time RSD of 0.03% and a peak area RSD of 0.3% was achieved. The robustness demonstrating results are visualized in the trendplot of Figure 2. These results are by far better than the requested limits in the pending monograph to be not more than 5–10% relative standard deviation.

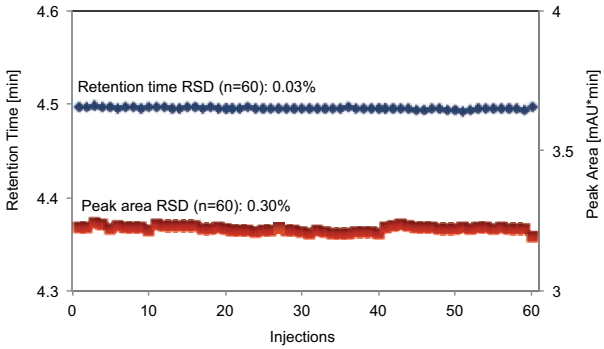

Figure 2. Trendplot showing the good retention time RSD of 0.03% and peak area RSD of 0.3% for the tenofovir disoproxil fumarate main peak analyzing 60 replicates over a run time period of more than 15 h.

Conclusion

This work combines an innovative column material with a versatile UHPLC instrument to solve a challenging separation problem. The capabilities of the Vanquish Flex System with quaternary pump allowed a selective increasing of the elution strength to create a fine-tuned gradient on the Accucore aQ column material. The modified method employs mass spectrometry compatible solvents. The column and instrument robustness allows the analysis of the active pharmaceutical ingredient with stable retention times and peak areas to give maximum confidence in the results.

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1. Authorized USP Pending Monograph for Tenofovir Disoproxil Fumarate, Version 1. [Online] http://www.usp.org/sites/default/files/usp_pdf/EN/USPNF/pendingStandards/m3429_authorized.pdf (accessed May 21, 2015)

2. Thermo Fisher Scientific. Vanquish UHPLC System. [Online] http://info1.thermoscientific.com/content/Vanquish-Overview/ (accessed May 21, 2015)

3. Thermo Fisher Scientific. Accucore aQ C18 Polar Endcapped LC Column. [Online] http://www.thermoscientific.com/en/product/accucore-aq-2-6-m-particle-size-hplc-columns.html (accessed May 21, 2015)

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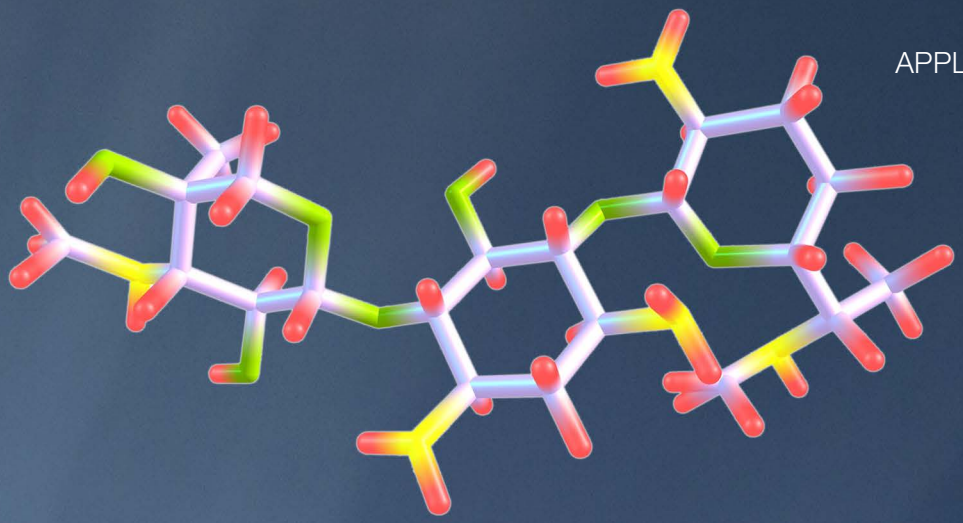

Determination of gentamicin and related impurities in gentamicin sulfate

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Keywords
Dionex IonPac AmG-3µm C18 column, aminoglycoside, Dionex ICS 5000+ HPIC system, PAD, electrochemical detection, drug substance, antibiotic, ion-pair reversed-phase HPLC, USP, EP, Dionex ICS-6000

Goal

To validate the gentamicin sulfate United States Pharmacopeia (USP) monograph method for gentamicin composition and impurities using a Thermo Scientific™ Dionex™ IonPac™ AmG-3µm C18 column

Introduction

Gentamicin is a broad spectrum water-soluble antibiotic belonging to the group of aminoglycoside antibiotics. It is valuable in the treatment of serious infections caused by gram-negative bacteria. Gentamicin is manufactured by a fermentation process and consists of a mixture of related gentamicin components. The main constituents are gentamicin C1, C1a, C2, C2a, and C2b. (Figure 1). Other related substances, such as sisomicin, garamine, gentamicin B1, and 2-deoxystreptamine are formed in small amounts during the manufacturing process.

All aminoglycosides have a narrow therapeutic range and their use is limited because of potential renal and otovestibular toxicity. The small difference between the effective and toxic concentrations call for monitoring of the given aminoglycoside levels to ensure optimal therapy and to minimize the risk of a toxic side effect, particularly in patients with renal failure.[1] It is essential to characterize a drug substance's purity by identifying and

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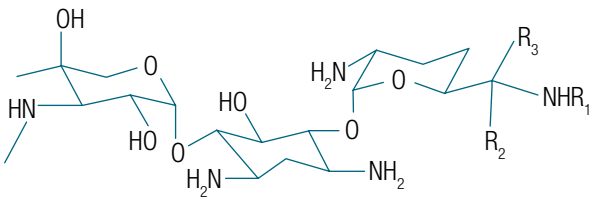

	R_1	R_2	R_3
C_{1a}	H	H	H
C_2	H	CH_3	H
C_{2b}	CH_3	H	H
C_{2a}	H	H	CH_3
C_1	CH_3	CH_3	H

Figure 1. Structure of gentamicin

quantifying the impurities, which ensures drug safety and efficacy. Gentamicin components differ in their antimicrobial potencies and toxicity in animals. It has also been reported that there is a wide variation in the major component ratio between different gentamicin products. Thus, this suggests the need to routinely investigate and control the ratio of major components of gentamicin C, as well as related substances in these commercial products.

The number of impurities and components makes the chromatographic analysis challenging. Detection of the different components of gentamicin is problematic because of the lack of a good UV-absorbing chromophore. Ion-pairing reversed-phase liquid chromatography is widely used to separate aminoglycosides by using volatile perfluorinated carboxylic acids, such as trifluoroacetic acid (TFA) and pentafluoropropionic acid, and this separation method has been paired with electrochemical detection. Pulsed amperometric detection (PAD), a powerful detection technique with a broad linear range and very low detection limits, is ideally suited for detecting aminoglycoside antibiotics and their impurities. Electrochemical detection has advantages relative to other techniques in that an oxidation potential can be selected for specific analytes while other compounds remain undetected, and derivatization is not required for detection, which simplifies the analysis. The analysis of gentamicin sulfate in pharmaceutical formulations based on ion-pairing HPLC-PAD is described in the U.S. and European Pharmacopoeias.[2,3]

The Dionex IonPac AmG-3µm C18 columns are specifically designed for ion-pairing reversed-phase separation of various aminoglycoside antibiotics. The stationary phase is prepared through the covalent bonding of C18 ligands onto a polymer-encapsulated silica media, which ensures ultra-stability when exposed to various mobile phase conditions such as low pH, high temperature, different organic solvents, and highly aqueous solutions.[4] The Dionex IonPac AmG-3µm column is packed in a PEEK column body rather than stainless steel. A stainless steel column can release significant levels of metal contamination, particularly when corrosive eluents are used. Metal ions can interfere with electrochemical detection.

Here we apply a 4-potential waveform to detect gentamicin components, rather than the 3-potential waveform reported in the USP Gentamicin Sulfate monograph for the Content of Gentamicins test.[2] Compared to the 3-potential waveform, the 4-potential waveform minimizes electrode wear and dramatically improves long-term peak area reproducibility.[5] The European Pharmacopeia (EP) Gentamicin Sulfate monograph describes organic impurity analysis and acceptance criteria in commercial samples. There is also an in-process revision for the USP Gentamicin Sulfate monograph that includes the addition of an organic impurities test that shares most of the conditions of the Content of Gentamicins test.

In this application note, the gentamicin sulfate analysis in the USP monograph was evaluated with a Dionex IonPac AmG-3µm C18 column using a 4-potential waveform for electrochemical detection of carbohydrates. Other than the waveform, the method and conditions were exactly as described in the USP Gentamicin Sulfate monograph. Key performance parameters were evaluated including system suitability separation, linearity, limits of detection, and precision. Two samples were analyzed. The percentage of gentamicin C major components results were compared with USP acceptance criteria. Impurity results were compared with EP Gentamicin Sulfate monograph and USP Gentamicin Sulfate in-process revision monograph's acceptance criteria.[6] We also compared results of the two analyses using the 4- and 3-potential waveforms.

Experimental

Equipment

- Thermo Scientific™ Dionex™ ICS-5000+ HPIC™ system including*:
  - Dionex ICS-5000+ DP Pump module
  - Dionex ICS-5000+ DC Detector/Chromatography module with ED Electrochemical Detector
  - Dionex AS-AP Autosampler with 250 µL sample syringe (P/N 074306) and 1200 µL buffer line (P/N 074989) and 1.5 mL vial trays (P/N 074936).
- Dionex™ ICS-5000+ ED Electrochemical Detector Cell (P/N 072044)
- ED conventional working electrode, gold, 3 mm (P/N 063723) with 5 mil gasket (P/N 063550)
- Reference electrode pH, Ag/AgCl (P/N 061879)
- Knitted reaction coil, 375 µL, unpotted (P/N 043700)
- Three-way manifold (P/N 48227)
- Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software, version 7.2.5

*This method can be run on a single Dionex ICS-5000+ or Thermo Scientific™ Dionex™ ICS-6000 system using a Thermo Scientific™ Dionex™ AXP pump to add the post-column reagent.

Consumables

- Glass autosampler vials 1.5 mL with slit septum (P/N 055427)
- Thermo Scientific™ Nalgene™ Rapid-Flow™ Sterile Disposable Filter Units with Nylon Membrane (1000 mL, 0.2 µm pore size, Fisher Scientific P/N 09-740-46)
- Nitrogen ultrahigh purity

Reagents and standards

- Deionized (DI) water, Type I reagent grade, 18 MΩ·cm resistivity or better
- Trifluoroacetic acid (Fisher Scientific P/N PI28901)
- Pentafluoropropanoic acid (Sigma-Aldrich® P/N 245917-50G)
- Sodium hydroxide 50% (w/w) (Fisher Scientific P/N SS254-500)
- Acetonitrile (Fisher Scientific P/N A955-4)
- USP Gentamicin Sulfate Reference standard (Sigma-Aldrich P/N 1289003-200MG)
- USP Sisomicin Sulfate Reference standard (Sigma-Aldrich P/N 1612801-500MG)

Samples

Two gentamicin samples were purchased from Sigma-Aldrich. Sample #1 claims to meet all USP specifications and sample #2 does not make that claim.

Chromatographic conditions

Columns:	Dionex IonPac AmG-3µm C18 Guard, 4 × 30 mm (P/N 302694) Dionex IonPac AmG-3µm C18 Separation, 4 × 150 mm (P/N 302693)
Eluent:	7 mL/L trifluoroacetic acid, 250 µL/L pentafluoropropanoic acid, adjust to pH 2.6 with NaOH, 30 mL/L acetonitrile
Flow Rate:	0.8 mL/min*
Column Temp.:	35 °C
Injection Volume:	20 µL (Full loop)
Autosampler Temperature:	5 °C
Reference Electrode:	Ag/AgCl
Working Electrode:	Conventional electrode gold, 3 mm diameter with a 5-mil gasket
Post-column Reagent	0.5 M NaOH
Post-column Reagent Flow Rate:	0.3 mL/min delivered by pump 2
Detection:	Pulsed Amperometric Detector (Electrochemical Detector)
Detection Compartment Temperature:	35 °C
Detection Waveform:	Gold, Carbohydrates, 4-Potential (Table 1)
System Backpressure:	~2600 psi
Run Time:	65 min

* The USP monograph describes the column as follows: Type – L1 (i.e. C18) size 250 mm, ID 4.6 mm; 5-µm packing L1. The diameter of the IonPac AmG-3µm C18 column is 4 mm. Therefore, the flow rate was adjusted from 1 mL/min (USP monograph condition) to 0.8 mL/min.

Table 1. Carbohydrates, 4-potential waveform

Time (s)	Voltage (V)	Integration
0	0.1	Off
0.20	0.1	On
0.40	0.1	Off
0.41	-2.0	Off
0.42	-2.0	Off
0.43	0.6	Off
0.44	-0.1	Off
0.50	-0.1	Off

Preparation of solutions and reagents

Eluent

To prepare 2 L, add 14 mL of trifluoroacetic acid, 500 µL of pentafluoropropanoic acid, and approximately 9.4 mL of 50% (w/w) NaOH into a glass 2 L volumetric flask containing approximately 1800 mL of degassed DI water. The pH of the solution should be around 2.6; if not, adjust the amount of 50% (w/w) NaOH to achieve 2.6. Add 30 mL of acetonitrile and bring the volume to 2 L with degassed DI water. Immediately transfer this solution to a glass eluent bottle and blanket it with nitrogen at 5 to 8 psi.

Post-column reagent (0.5 M NaOH)

To prepare 1 L of post-column reagent, weigh 40.0 g of 50% (w/w) NaOH into a plastic 1 L volumetric flask containing approximately 800 mL of degassed DI water. Briefly stir this solution (15–30 s) and then bring to volume. Immediately transfer this solution to the plastic eluent bottle on the HPAE-PAD system and blanket it with nitrogen at 5 to 8 psi. Gently swirl the bottle to complete mixing. Always maintain the eluents under 5 to 8 psi of nitrogen to reduce diffusion of atmospheric carbon dioxide. Prepare new NaOH eluent if left unblanketed for more than 30 min.

Stock standard solutions

Gentamicin sulfate stock, 1 mg/mL

Dissolve 25 mg of USP grade gentamicin sulfate in 25 mL of eluent.

Sisomicin sulfate stock, 1 mg/mL

Dissolve 25 mg of USP grade sisomicin sulfate in 25 mL of eluent.

Working standard solutions

Gentamicin sulfate standard, 0.2 mg/mL

Dilute 5 mL of gentamicin sulfate stock to 25 mL with eluent.

Sisomicin standard, 10 µg/mL

Dilute 1 mL of sisomicin sulfate standard stock to 100 mL with eluent.

System suitability solution, (100 µg/mL USP Gentamicin Sulfate RS and 20 µg/mL of USP Sisomicin Sulfate RS in eluent)

To 5 mL of gentamicin sulfate stock standard, add 1 mL of sisomicin sulfate stock standard, and dilute to 50 mL with eluent.

Sample preparation

Sample solution (a), 1 mg/mL

Dissolve 25 mg of sample in 25 mL of eluent. Use this sample preparation for impurity analysis.

Sample solution (b), 0.2 mg/mL

Dilute 5 mL of sample solution (a) to 25 mL with eluent. Use this sample preparation for the Content of Gentamicins analysis.

Notes: Store all standards and samples in a refrigerator after preparation.

System preparation and setup

A Dionex ICS 5000+ dual system has two pumps. Use the first pump to deliver eluent and the second pump to deliver post-column reagent. Connect extra tubing to the second pump outlet to achieve ~2000 psi pressure for lowering baseline noise.

The post-column addition of NaOH solution will require installation of a knitted reaction coil after the column but before the detector. Install a PEEK mixing tee (P/N 048227) after the column and use the second pump of the DP to deliver the post-column solution to the tee. Direct the third port on the tee to the reaction coil, followed by the electrochemical detector cell.

Rinse the cell body, working electrode, and gasket thoroughly with DI water and dry with a lab wipe. Caution: Do not touch the working electrode gold surface with any paper products as this can contaminate the working electrode. Assemble the cell following the Dionex ICS 5000+ operator manual[7] and Dionex ED User's Compendium for Electrochemical Detection[8] by first installing the working electrode gasket flat against cell body. Avoid any wrinkles in the gasket, as this will cause a poor fit and subsequent leaks and poor detection. Install the conventional working electrode with the metal face down over the gasket. Install the yoke block by squeezing the tabs and sliding it on the cell body. Align the yoke block parallel to the cell body and rotate the yoke block knob clockwise until you hear three "clicks". Install the cell into the ED module and connect the yellow cable to the yellow port.

To calibrate the pH-Ag/AgCl reference electrode, install the reference electrode blue cable into the black port. Immerse the reference electrode in pH 7 buffer to at least mid-level of the electrode. Select the "pH Calibration" button on the ED Panel and follow the instructions to calibrate the electrode including using pH 10 buffer. After calibration is complete, rinse the buffer solution off the electrode with DI water, and gently, but firmly, screw in or rotate the reference electrode clockwise into the reference electrode port of the electrochemical cell until the reference electrode is finger-tight. For best results, replace the reference electrode after six months of use.

While running the ED cell, bubbles may be trapped in the cell. Air bubbles in the cell can cause spikes in the baseline. To prevent air from becoming trapped in the cell, increase the backpressure on the cell by connecting backpressure tubing to the cell outlet. The backpressure limit for the ED cell is 690 kPa (100 psi). Do not exceed this limit. Six feet of black (0.01" i.d.) PEEK tubing at the cell outlet can generate 30–40 psi backpressure, which can prevent bubble formation.

Condition the column using the eluent at 0.8 mL/min for 20 min before connecting the column to detector.

After selecting the waveform, confirm flow is passing through the cell and turn the cell voltage to the ON position.

A layer of contamination may occasionally build up on the gold working electrode of the amperometry cell. When this occurs, the electrode must be polished to restore performance. Indications that the working electrode needs to be polished are visible electrode discoloration or a decrease in peak area response. The procedure for polishing the working electrode can be found in the product manual.[9]

When the system is idle for short periods (1–2 weeks), the pump should be left at a reduced flow rate of 0.05 mL/min to achieve rapid startup. When the system must be shut down for a period of several weeks, the pump and electrochemical cell may be simply turned off. For shutdown periods exceeding several weeks, all plumbing lines should be resealed, and the reference electrode should be removed from the electrochemical cell and stored in saturated KCl.

Results and discussion

System suitability

In the USP monograph for gentamicin sulfate, the system suitability requirements specify resolution between gentamicin C2 and gentamicin C2b as >1.5. The EP gentamicin sulfate monograph includes two additional requirements: Signal-to-noise ratio (S/N) > 20 for 10 µg/mL sisomicin and resolution > 1.2 between sisomicin and Gentamicin C1a.

The system suitability was evaluated using the chromatograms of a system suitability standard and 10 µg/mL sisomicin sulfate. Figure 2 shows the first chromatogram using a Dionex IonPac AmG-3µm C18 column set. The five congeners (C1, C1a, C2, C2a, and C2b) and sisomicin were well separated. Figure 3 shows the chromatogram of sisomicin sulfate. Sisomicin is sensitively detected.

Column:	Dionex IonPac AmG-3µm C18 Guard, 4 × 30 mm (P/N 302694) Dionex IonPac AmG-3µm C18 Separation, 4 × 150 mm (P/N 302693)
Eluent:	7 mL/L trifluoroacetic acid, 250 µL/L pentafluoropropanoic acid, adjust to pH 2.6 with NaOH, 30 mL/L acetonitrile
Inj. Volume:	20 µL
Column Temp.:	35 °C
Flow Rate:	0.8 mL/min
Post-Column Reagent:	0.5 M NaOH (0.3 mL/min)
Detection:	Pulsed Amperometric Detector (Waveform: Carbohydrates, 4-Potential)

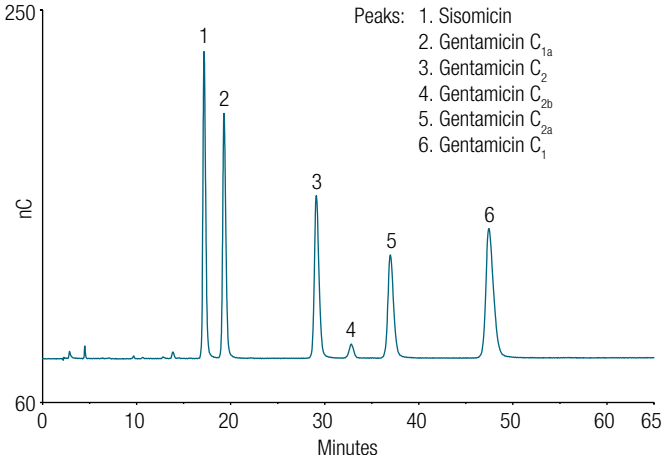

Figure 2. Separation of a system suitability standard (gentamicin 100 µg/mL + sisomicin 20 µg/mL) using a Dionex IonPac AmG C18 column

Column:	Dionex IonPac AmG-3µm C18 Guard, 4 × 30 mm (P/N 302694) Dionex IonPac AmG-3µm C18 Separation, 4 × 150 mm (P/N 302693)
Eluent:	7 mL/L trifluoroacetic acid, 250 µL/L pentafluoropropanoic acid, adjust to pH 2.6 with NaOH, 30 mL/L acetonitrile
Inj. Volume:	20 µL
Column Temp.:	35 °C
Flow Rate:	0.8 mL/min
Post-Column Reagent:	0.5 M NaOH (0.3 mL/min)
Detection:	Pulsed Amperometric Detector (Waveform: Carbohydrates, 4-Potential)
Peak:	1. Sisomicin

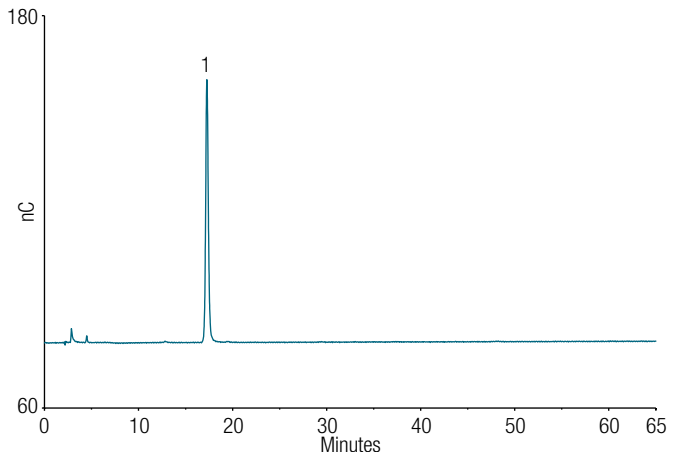

Figure 3. Sisomicin Sulfate USP standard (10 µg/mL)

The system suitability requirements are met for all parameters (Table 2). Peak resolution between C2 and C2b is 4.2, exceeding the USP and EP requirement of 1.5. Peak resolution between sisomicin and C1a is 3.75, exceeding the EP requirement of 1.2. The S/N of 10 µg/mL sisomicin sulfate is 233, easily exceeding the EP requirement of 20.

Table 2. System suitability using the 4-potential carbohydrate waveform

Test	EP Criteria	Measured
Resolution between Sisomicin and C1a	>1.2	3.75
Resolution between C2 and C2b	>1.5*	4.20
S/N (Sisomicin 10 µg/mL)	>20	233

*Also the USP criterion

Linearity

The linearity of gentamicin electrochemical response was investigated in the concentration range of 10 to 200 µg/mL (10, 25, 50, 100, 200 µg/mL). For all gentamicin derivatives, the coefficients of determination were better than 0.997. Figure 4 shows the calibration curve using C1 peak area; the coefficient of determination is 0.9991. This reveals that a sample concentration of 200 µg/mL is within the response linear range and can be used for analysis.

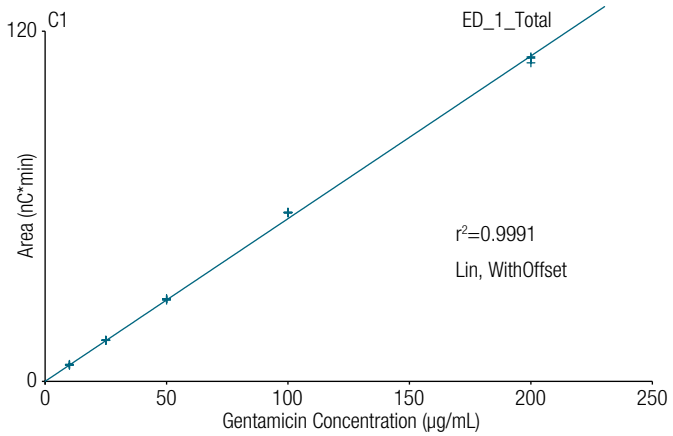

Figure 4. Calibration of gentamicin (C1 peak area)

Method limits of detection and quantification

The USP method for validation specifies a S/N of 3 for the determination of the limit of detection and a S/N of 10 for the determination of the limit of quantitation (LOQ).[10]

To determine the limit of detection (LOD) and limit of quantification (LOQ), the baseline noise was first determined by measuring the peak-to peak noise in a representative 1 min segment of the baseline where no peaks elute but close to the peak of interest. The LOD and LOQ were then calculated from the average peak height of three injections of sisomicin sulfate (0.2 µg/mL).

Table 3 summarizes the LOD and LOQ of sisomicin in sample solution and in gentamicin sulfate powder.

Table 3. LOD and LOQ

Analyte	LOD (µg/mL) in Sample Solution	LOQ (µg/mL) in Sample Solution	LOD in Gentamicin Sulfate Powder (µg/g)	LOQ in Gentamicin Sulfate Powder (µg/g)
Sisomicin	0.173	0.577	173	577

Method precision

Method precision performance was evaluated with five replicate injections of gentamicin sample #2 (0.2 mg/mL).

Figure 5 shows an overlay of the chromatograms from the precision analysis.

Column:	Dionex IonPac AmG-3µm C18 Guard, 4 × 30 mm (P/N 302694) Dionex IonPac AmG-3µm C18 Separation, 4 × 150 mm (P/N 302693)
Eluent:	7 mL/L trifluoroacetic acid, 250 µL/L pentafluoropropanoic acid, adjust to pH 2.6 with NaOH, 30 mL/L acetonitrile
Inj. Volume:	20 µL
Column Temp.:	35 °C
Flow Rate:	0.8 mL/min
Post-Column Reagent:	0.5 M NaOH (0.3 mL/min)
Detection:	Pulsed Amperometric Detector (Waveform: Carbohydrates, 4-Potential)

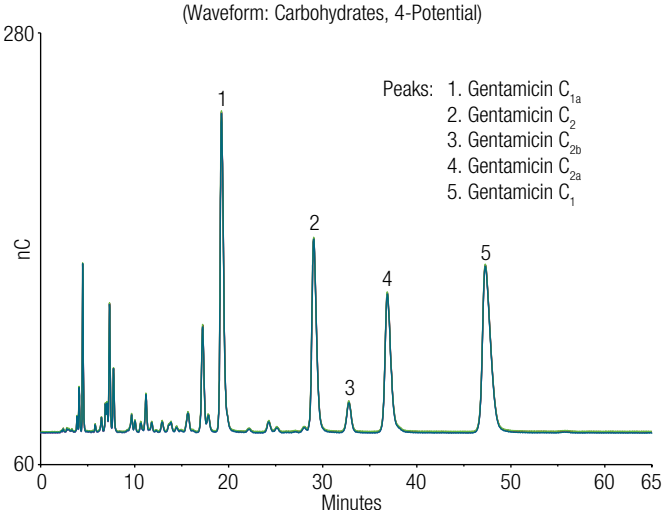

Figure 5. Overlay of five injections of sample #2 (0.2 mg/mL)

As shown in Table 4, the relative standard deviation (RSD) for 5 injections of sample #2 ranged between 0.1 and 0.5%.

Sample analysis

Content of gentamicins analysis

Standard and sample solution (b) were used for content of gentamicins analysis. Figure 6 shows the separation of a USP gentamicin standard. The five gentamicin constituents were well separated. Figure 7 shows the separation of gentamicin sample #1 (0.2 mg/mL); a few impurities were detected and they were separated from the five gentamicin constituents. Figure 8 shows the separation of gentamicin sample #2 (0.2 mg/mL); more than 20 impurities were observed and they were separated from the five gentamicin constituents.

Table 4. Peak area precision of five injections of sample #2, 0.2 mg/mL

Injection	C1a	C2	C2b	C2a	C1
1	69.1	57.8	8.96	53.2	86.5
2	69.3	58.2	9.07	53.4	86.2
3	69.4	58.1	9.04	53.5	86.5
4	69.3	58.3	9.02	53.0	86.4
5	69.3	58.3	9.01	53.5	86.2
RSD	0.17%	0.34%	0.45%	0.36%	0.15%

The relative percentage of each gentamicin constituent in the USP reference standard and the two samples was calculated using the peak areas obtained from the chromatograms shown in Figures 6, 7, and 8. The calculation method is shown below:

Result = (*rU/rT*) × 100

rU = Peak area response corresponding to the particular gentamicin from the sample solution

rT = Sum of all peak area response of gentamicin C1a, gentamicin C2, gentamicin C2a, gentamicin C2b, and gentamicin C1 from the sample solution.

Column:	Dionex IonPac AmG-3µm C18 Guard, 4 × 30 mm (P/N 302694) Dionex IonPac AmG-3µm C18 Separation, 4 × 150 mm (P/N 302693)
Eluent:	7 mL/L trifluoroacetic acid, 250 µL/L pentafluoropropanoic acid, adjust to pH 2.6 with NaOH, 30 mL/L acetonitrile
Inj. Volume:	20 µL
Column Temp.:	35 °C
Flow Rate:	0.8 mL/min
Post-Column Reagent:	0.5 M NaOH (0.3 mL/min)
Detection:	Pulsed Amperometric Detector (Waveform: Carbohydrates, 4-Potential)

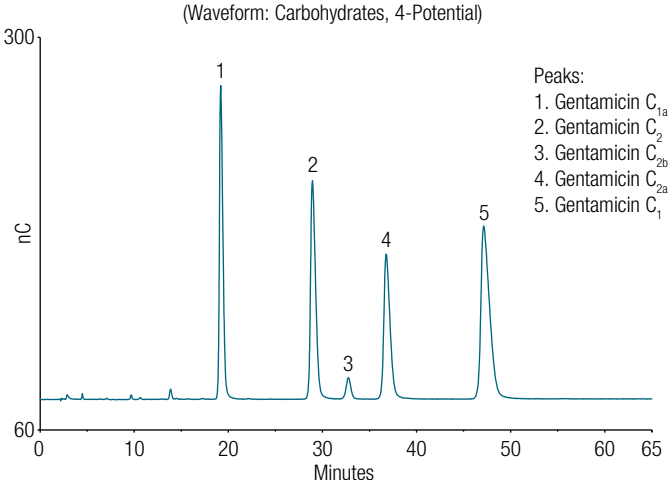

Figure 6. Separation of a gentamicin sulfate USP reference standard (0.2 mg/mL) using a Dionex IonPac AmG C18 column

Column:	Dionex IonPac AmG-3µm C18 Guard, 4 × 30 mm (P/N 302694) Dionex IonPac AmG-3µm C18 Separation, 4 × 150 mm (P/N 302693)
Eluent:	7 mL/L trifluoroacetic acid, 250 µL/L pentafluoropropanoic acid, adjust to pH 2.6 with NaOH, 30 mL/L acetonitrile
Inj. Volume:	20 µL
Column Temp.:	35 °C
Flow Rate:	0.8 mL/min
Post-Column Reagent:	0.5 M NaOH (0.3 mL/min)
Detection:	Pulsed Amperometric Detector (Waveform: Carbohydrates, 4-Potential)

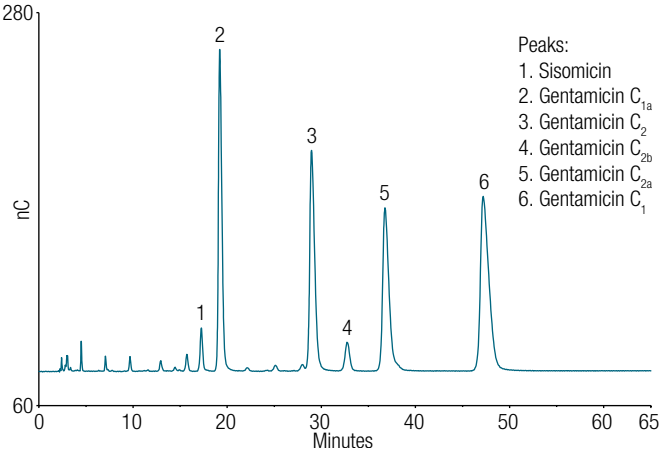

Figure 7. Separation of gentamicin sample #1 (0.2 mg/mL) using a Dionex IonPac AmG C18 column

Column:	Dionex IonPac AmG-3µm C18 Guard, 4 × 30 mm (P/N 302694) Dionex IonPac AmG-3µm C18 Separation, 4 × 150 mm (P/N 302693)
Eluent:	7 mL/L trifluoroacetic acid, 250 µL/L pentafluoropropanoic acid, adjust to pH 2.6 with NaOH, 30 mL/L acetonitrile
Inj. Volume:	20 µL
Column Temp.:	35 °C
Flow Rate:	0.8 mL/min
Post-Column Reagent:	0.5 M NaOH (0.3 mL/min)
Detection:	Pulsed Amperometric Detector (Waveform: Carbohydrates, 4-Potential)

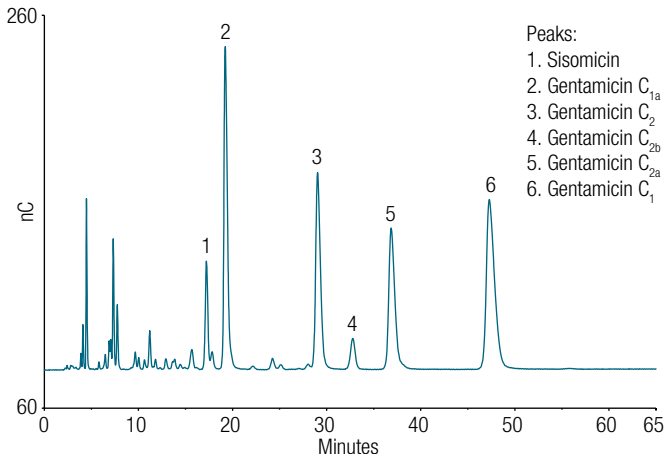

Figure 8. Separation of gentamicin sample #2 (0.2 mg/mL) using a Dionex IonPac AmG C18 column

As shown in Table 5, both samples met the USP acceptance criteria for the Content of Gentamicins test.

Table 5. Percentage of each gentamicin in gentamicin sulfate

Test	C1a	C2	C2b	C2a	C1	C2+C2a	C2b+C1
USP Standard	23.3	23.3	2.1	19.1	32.2	42.4	34.3
Sample #1	22.7	22.6	2.8	21.3	30.6	43.9	33.3
Sample #2	25.1	21.0	3.3	19.4	31.2	40.4	34.5
USP Acceptance Criteria	10–35					25–55	25–50

Percentage of impurities in gentamicin sulfate samples

Sample solutions (a) were used for impurities analysis. Figures 9 and 10 show the chromatograms of samples #1 and #2, respectively. The five times greater concentration of these samples compared to the samples used for the Content of Gentamicins analysis allows the impurity peaks to be more easily observed.

The EP Gentamicin Sulfate monograph and the USP in-process revision of the Gentamicin Sulfate monographs describe acceptance criteria for impurity levels in commercial samples. For that purpose, all impurities were calculated using the peak areas obtained from the chromatogram of the sample solutions (Figures 9 and 10) and compared to the response of the principle impurity sisomicin obtained from the chromatogram of sisomicin sulfate 10 µg/mL (Figure 3).

Column:	Dionex IonPac AmG-3µm C18 Guard, 4 × 30 mm (P/N 302694) Dionex IonPac AmG-3µm C18 Separation, 4 × 150 mm (P/N 302693)
Eluent:	7 mL/L trifluoroacetic acid, 250 µL/L pentafluoropropanoic acid, adjust to pH 2.6 with NaOH, 30 mL/L acetonitrile
Inj. Volume:	20 µL
Column Temp.:	35 °C
Flow Rate:	0.8 mL/min
Post-Column Reagent:	0.5 M NaOH (0.3 mL/min)
Detection:	Pulsed Amperometric Detector (Waveform: Carbohydrates, 4-Potential)
Peak:	1. Sisomicin

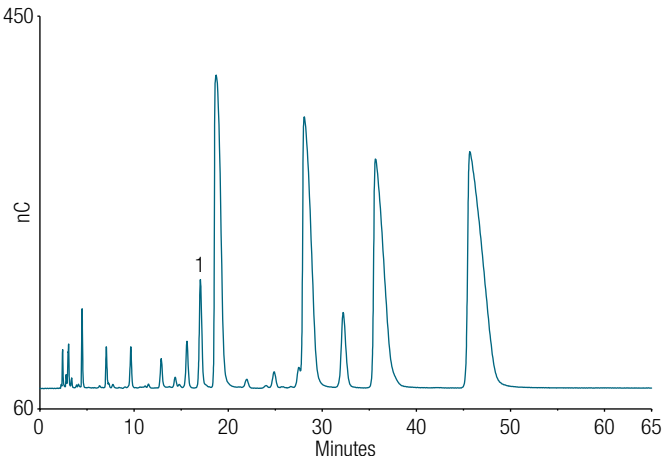

Figure 9. Separation of gentamicin sample #1 (1 mg/mL) using a Dionex IonPac AmG C18 column

Column:	Dionex IonPac AmG-3µm C18 Guard, 4 × 30 mm (P/N 302694) Dionex IonPac AmG-3µm C18 Separation, 4 × 150 mm (P/N 302693)
Eluent:	7 mL/L trifluoroacetic acid, 250 µL/L pentafluoropropanoic acid, adjust to pH 2.6 with NaOH, 30 mL/L acetonitrile
Inj. Volume:	20 µL
Column Temp.:	35 °C
Flow Rate:	0.8 mL/min
Post-Column Reagent:	0.5 M NaOH (0.3 mL/min)
Detection:	Pulsed Amperometric Detector (Waveform: Carbohydrates, 4-Potential)
Peak:	1. Sisomicin

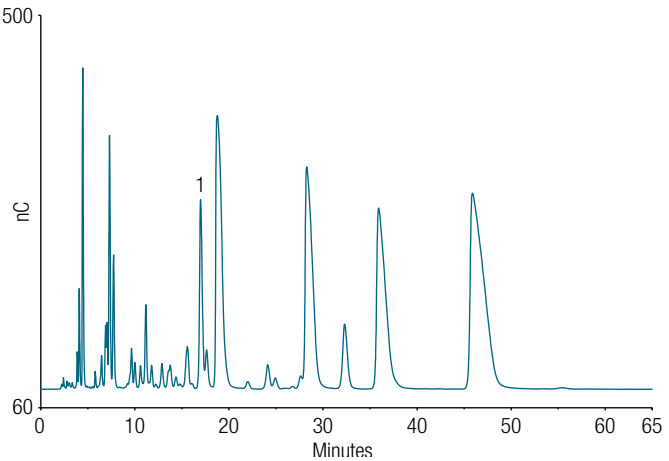

Figure 10. Separation of gentamicin sample #2 (1 mg/mL) using a Dionex IonPac AmG C18 column

Result = (*rU/rs*) × (*Cs/Cu*) × 100

rU = Peak response of each individually impurity from the 1 mg/mL sample solution

rs = Peak response of sisomicin from the 10 µg/mL standard solution

Cs = Concentration of USP Sisomicin Sulfate RS in the standard solution (mg/mL)

Cu = Concentration of Gentamicin Sulfate in the sample solution (mg/mL)

Table 6 shows the percentage of sisomicin and total impurities of samples #1 and #2 and compared with the USP acceptance criteria. Sample #1 met all USP impurity acceptance criteria as was claimed in its product description. Sample #2 did not pass the USP total impurities criteria.

Waveform comparison

The analysis of the gentamicin was evaluated using the 3-potential carbohydrate waveform that is in the USP and EP Gentamicin Sulfate monographs (Table 7). Figure 11 shows the separation of a system suitability standard using the 3-potential waveform. The five congeners (C1, C1a, C2, C2a, and C2b) and sisomicin were well separated.

Table 6. Percentage of impurity in gentamicin sulfate

	Sisomicin	Any Other Individual Impurity	Total Impurities
Sample #1	1.31	<1.31	4.1
Sample #2	2.64	<2.64	14.1
EP monograph/USP in process revision Acceptance Criteria	3.0	3.0	10

Table 7. Three-potential waveform (USP monograph method)

Time (s)	Voltage (V)	Integration
0	0.05	Off
0.1	0.05	On
0.4	0.05	Off
0.41	0.75	Off
0.55	0.75	Off
0.56	-0.15	Off
1.00	-0.15	Off

Column: Dionex IonPac AmG-3µm C18 Guard, 4 × 30 mm (P/N 302694)
Dionex IonPac AmG-3µm C18 Separation, 4 × 150 mm (P/N 302693)
Eluent: 7 mL/L trifluoroacetic acid, 250 µL/L pentafluoropropanoic acid, adjust to pH 2.6 with NaOH, 30 mL/L acetonitrile
Inj. Volume: 20 µL
Column Temp.: 35 °C
Flow Rate: 0.8 mL/min
Post-Column Reagent: 0.5 M NaOH (0.3 mL/min)
Detection: Pulsed Amperometric Detector (Waveform: 3-Potential)

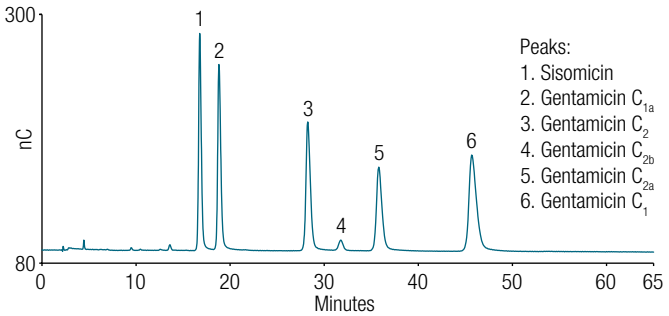

Figure 11. Separation of a system suitability standard (gentamicin 100 µg/mL + sisomicin 20 µg/mL) using a Dionex IonPac AmG C18 column with the 3-potential waveform

Figure 12 shows 10 µg/mL sisomicin with the 3-potential waveform. The system suitability requirements are met for all parameters (Table 8). Figure 13 shows the chromatogram of sample #1 (0.2 mg/mL) using the 3-potential waveform. The five congeners (C1, C1a, C2, C2a, and C2b) and sisomicin in the sample were well separated and the results using this waveform were equivalent with the results using the 4-potential waveform.

Column:	Dionex IonPac AmG-3µm C18 Guard, 4 × 30 mm (P/N 302694) Dionex IonPac AmG-3µm C18 Separation, 4 × 150 mm (P/N 302693)
Eluent:	7 mL/L trifluoroacetic acid, 250 µL/L pentafluoropropanoic acid, adjust to pH 2.6 with NaOH, 30 mL/L acetonitrile
Inj. Volume:	20 µL
Column Temp.:	35 °C
Flow Rate:	0.8 mL/min
Post-Column Reagent:	0.5 M NaOH (0.3 mL/min)
Detection:	Pulsed Amperometric Detector (Waveform: 3-Potential)
Peak:	1. Sisomicin,10 µg/mL

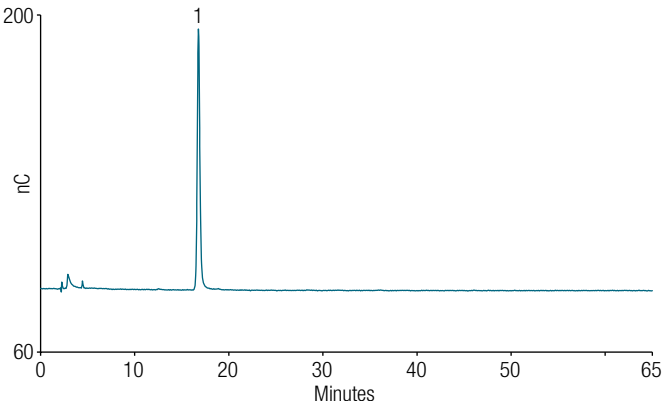

Figure 12. Sisomicin sulfate USP Reference standard (10 µg/mL) using a Dionex IonPac AmG C18 column with the 3-potential waveform

Table 8. System suitability using the 3-potential waveform (USP monograph) waveform

Test	EP Criteria	Measured
Resolution between Sisomicin and C1a	>1.2	3.72
Resolution between C2 and C2b	>1.5*	4.05
S/N (Sisomicin 10 µg/mL)	>20	304

*Also the USP criterion

Column:	Dionex IonPac AmG-3µm C18 Guard, 4 × 30 mm (P/N 302694) Dionex IonPac AmG-3µm C18 Separation, 4 × 150 mm (P/N 302693)
Eluent:	7 mL/L trifluoroacetic acid, 250 µL/L pentafluoropropanoic acid, adjust to pH 2.6 with NaOH, 30 mL/L acetonitrile
Inj. Volume:	20 µL
Column Temp.:	35 °C
Flow Rate:	0.8 mL/min
Post-Column Reagent:	0.5 M NaOH (0.3 mL/min)
Detection:	Pulsed Amperometric Detector (Waveform: 3-Potential)

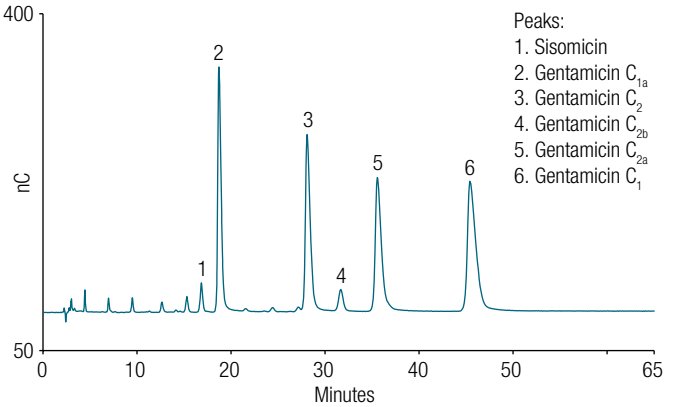

Figure 13. Separation of gentamicin sample #1 (0.2 mg/mL) using a IonPac AmG C18 column with the 3-potential waveform

All the gentamicin congeners evaluated had higher responses using the 3-potentential waveform than the 4-potential carbohydrate waveform. However, as discussed in Technical Note 21[5], the 4-potential waveform differs from the 3-potential waveform in that it uses a negative rather than positive potential for electrode cleaning. Therefore, electrode wear is greatly minimized and long-term reproducibility is improved. Overall, in our opinion, the 4-potential waveform is a better choice for this application.

Conclusions

This application note demonstrated that the USP Gentamicin Sulfate monograph Content of Gentamicins method and the USP in-process revision Gentamicin Sulfate monograph method for organic impurities method could be successfully executed with a Dionex IonPac AmG-3µm C18 column using either the 4-potential carbohydrate waveform or the 3-potential waveform described in the USP and EP monographs. The separation, linearity, reproducibility, and sensitivity were found to meet or exceed the current USP/EP Gentamicin Sulfate monograph performance requirements. This method is reliable and can be used for the routine monitoring of gentamicin.

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APPLICATION NOTE 72502

Assay of sodium thiosulfate and ionic impurities in sodium thiosulfate using ion chromatography

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Keywords
Dionex IonPac AS12A column, suppressed conductivity detection, pharmaceutical, USP Monograph, drug substance, drug product

Goal

To validate the ion chromatography (IC) methods for the assay of sodium thiosulfate and its ionic impurities in the proposed United States Pharmacopeia monographs

Introduction

Sodium thiosulfate is an active pharmaceutical ingredient (API) approved by the United States Food and Drug Administration. Dosing sequentially with sodium nitrite, Sodium Thiosulfate Injection solution is used for the treatment of acute cyanide poisoning that is judged to be life-threatening.[1,2] Sodium thiosulfate is being tested as an extravasation antidote for cancer treatment to lessen the side effects of cisplatin (a chemotherapy agent).[3,4]

The United States Pharmacopeia (USP) has embarked on a global initiative to modernize many of the existing monographs across all compendia. As part of the USP modernization effort, an ion chromatography (IC) method has been proposed to replace existing titration-based assays in the Sodium Thiosulfate and Sodium Thiosulfate Injection monographs. In addition, another IC method has also been proposed for determining chloride, sulfate, and sulfite impurities in Sodium Thiosulfate; and sulfate and sulfite impurities in Sodium Thiosulfate Injection.[5,6]

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This application note evaluates both methods with sodium thiosulfate following the guidelines outlined in USP General Chapter <1225>, Validation of Compendial Methods.[7–9] A Thermo Scientific™ Dionex™ ICS-5000+ ion chromatography system with a Thermo Scientific™ Dionex™ IonPac™ AS12A anion-exchange column and a Thermo Scientific™ Dionex™ AERS 500 (4 mm) Anion Electrolytically Regenerated Suppressor for suppressed conductivity detection were used for both method evaluations.

Experimental

Equipment

- A Thermo Scientific Dionex ICS-5000+ ion chromatography (RFIC) system*, which includes:
  - Pump
  - Column Heater
  - Degasser
  - Conductivity Detector
- Thermo Scientific™ Dionex™ AS-AP Autosampler, with 250 µL syringe (P/N 074306), 1.2 mL buffer line assembly (P/N 074989), 25 µL injection loop
- Thermo Scientific™ Chromeleon™ 7.2 Chromatography Workstation

*This method can be run on any system supporting an electrolytic suppressor or any Thermo Scientific Dionex ion chromatography system using a chemically regenerated suppressor. Please note that this method was not tested with a chemically regenerated suppressor.

Reagents and standards

- Deionized (DI) water, Type I reagent grade, 18 MΩ·cm resistance or better
- Sodium Thiosulfate anhydrous USP reference standard (USP, P/N 1615107)
- Sodium chloride (Sigma-Aldrich, 746398-500g)
- Sodium sulfite (Fisher Scientific, S430-500 98.1%)
- Sodium sulfate (EM, > 99%)
- D-mannitol (Acros Organic, 98+%)
- Sodium thiosulfate salt (J.T Baker, USP grade)

Conditions

Table 1. Chromatography conditions for the sodium thiosulfate assay.

Columns:	Dionex IonPac AS12A 4-mm Analytical, 4 × 250 mm (P/N 046034) Dionex IonPac AG12A 4-mm Guard, 4 × 50 mm (P/N 079801)
Eluent:	13.5 mM Na_2CO_3 /1.5 mM $NaHCO_3$
Flow Rate:	1.5 mL/min
Injection Volume	25 µL in Push-Full mode
Temperature:	30 °C
Detection:	Suppressed conductivity, Dionex AERS 500 (4 mm) Anion Electrolytically Regenerated Suppressor, recycle mode, 106 mA current
System Backpressure:	~2500 psi
Background Conductance:	~26 µS
Noise:	<5 nS/min
Run Time	10 min

Table 2. Chromatography conditions for the sodium thiosulfate ionic impurity method.

Columns:	Dionex IonPac AS12A 4-mm Analytical, 4 × 250 mm (P/N 046034) Dionex IonPac AG12A 4-mm Guard, 4 × 50 mm (P/N 079801)
Eluent Solution A:	2.7 mM Na_2CO_3/0.3 mM $NaHCO_3$
Eluent Solution B:	13.5 mM Na_2CO_3/1.5 mM $NaHCO_3$
Gradient:	

Time (min)	Solution A (%)	Solution B (%)
-5	100	0
0	100	0
14	100	0
16	0	100
21	0	100
23	100	0
30	100	0

Flow Rate:	1.5 mL/min
Injection Volume	25 µL in Push-Full mode
Temperature:	30 ˚C
Detection:	Suppressed conductivity, Dionex AERS 500 (4 mm) Anion Electrolytically Regenerated Suppressor, recycle mode, 106 mA current
System Backpressure:	~2500 psi
Background Conductance:	~13–26 µS
Noise:	<5 nS/min
Run Time	35 min (includes 5 min equilibrium time)

Preparations of solutions and reagents

Note: Do not use glassware to prepare the solutions. Polymeric containers made of high-density polyethylene (HDPE) are recommended.

Stock standard solution for sodium thiosulfate assay, 1.000 mg/mL in water

Accurately weigh 100.0 mg of USP Sodium Thiosulfate into a 125 mL polypropylene bottle and dissolve in 100 mL (100.00 g) of DI water. Keep at 4 ˚C for up to a month.

Standard solution for sodium thiosulfate assay, 100 µg/mL in water

Mix 1.0 mL (1.0 g) of 1.0 mg/mL of sodium thiosulfate stock standard solution and 9.0 mL (9.0 g of DI water to make the standard solution for assay. Prepare fresh for each sequence. This standard is also used as the system suitability solution for the assays.

Sodium thiosulfate calibration standards, 0.2, 20, 50, 75, 100, 125, 150, 200 µg/mL

To prepare calibration standard solutions, dilute the stock standard solution (1.0 mg/mL) to the appropriate concentrations with DI water.

Diluent: 2.0 g/L of D-mannitol in water

Accurately weigh 4.0 g of D-mannitol solid into a 2 L polypropylene bottle and dissolve in 2 L of DI water to make the diluent. The diluent is used to prepare the samples and standards in the sodium thiosulfate ionic impurity method.

Stock standard solutions for the ionic impurity method, in diluent

Accurately weigh a pure anhydrous salt (using USP reference standard if available) into a polypropylene bottle and dissolve in 100 mL (100.00 g) of diluent to make each stock standard solution. Mix 40.0 mg of sodium chloride to make 0.400 mg/mL sodium chloride stock, 100 mg of sodium sulfite to make 1.00 mg/mL sodium sulfite stock, and 200.0 mg of sodium sulfate to make 2.00 mg/ mL of sodium sulfate stock. Keep stock standard solutions at 4 ˚C.

Mixed standard stock solution for the ionic impurity method, in diluent

Mix the stock standard solutions (1.00 mL (1.0 g) each of sodium chloride and sodium sulfate stock, 2.00 mL of sodium sulfite stock) and 96.0 mL (96.0 g) of the diluent to make the mixed standard stock solution containing 4.0 µg/mL of sodium chloride, 20.0 µg/mL of sodium sulfite, and 20.0 µg/mL sodium sulfate.

Calibration standard solutions for the ionic impurity method, in diluent

Dilute the mixed standard stock solution to the appropriate concentrations with diluent to make the calibration standards (Table 3). The system suitability solution is the level 4 calibration standard solution.

Table 3. Concentration of standard solutions for the ionic impurity method (µg/mL of the salt (e.g., sodium chloride)).

Analyte	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6
Chloride	0.04	0.08	0.2	0.4	0.8	2
Sulfite	0.2	0.4	1	2	4	10
Sulfate	0.2	0.4	1	2	4	10

Sample preparation

Sodium thiosulfate stock sample solution, 1.000 mg/mL in water

Accurately weigh 100.0 mg of JT Baker sodium thiosulfate salt into a 125 mL polypropylene bottle and dissolve in 100 mL (100.0 g) DI water.

Sodium thiosulfate sample solution for assay, 0.100 mg/mL (100 µg/mL) in water

Mix 1.00 mL (1.00 g) of 1.00 mg/mL of the sodium thiosulfate sample stock and 9.00 mL (9.00 g) of DI water to make the sample solution for the sodium thiosulfate assay.

Spiked sodium thiosulfate sample solutions

Mix 1.00 mg/mL of sodium thiosulfate sample stock, 1.000 mg/mL of sodium thiosulfate standard stock solution, and DI water to make spiked samples (Table 4).

Sodium thiosulfate sample solution for ionic impurities, 2.0 mg/mL in diluent

Accurately weigh 40.0 mg of sodium thiosulfate solid sample into a 20 mL polypropylene bottle and dissolve in 20 mL (20.0 g by weight) diluent to make the sample solution for the ionic impurities determination.

Spiked sodium thiosulfate sample solutions for the ionic impurity recovery test

Accurately weigh 200.0 mg of sodium thiosulfate into a 100 mL polypropylene bottle and dissolve in 50 mL (50.0 g by weight) of diluent to make 4.0 mg/L sample stock solution. Mix 1.00 mL of 0.40 mg/mL sodium chloride stock, 2.00 mL of 1.0 mg/L of sodium sulfite stock, 1.00 mL of 2.0 mg/mL of sodium sulfate stock, and 96.0 mL (96.0 g) of the diluent to make the mixed spike stock solution containing 4 µg/mL sodium chloride, 20 µg/mL sodium sulfite, and 20 µg/mL sodium sulfate. Mix the sample stock and appropriate amount of the mixed spike stock with diluent to make the spiked samples (Table 5) for the recovery test.

Table 4. Preparation of spiked samples for assay recovery test.

Sodium Thiosulfate Spiked (µg/mL)	10	25	50*	50	75	100
Sample Stock (mL)	1.000	1.000	0.500	1.000	1.000	1.000
Standard Stock (mL)	0.100	0.250	0.500	0.500	0.750	1.000
DI water (g)	8.90	8.75	9.00	8.50	8.25	8.00

*50 µg/mL spiked in 50 µg/mL sample. All others are spiked in 100 µg/mL sample.

Table 5. Concentration of ionic impurities spiked in sodium thiosulfate samples.

	Sample	Spiked Sample 5	Spiked Sample 4	Spiked Sample 3	Spiked Sample 2	Spiked Sample 1
Sodium thiosulfate (mg/mL)	2.000	2.000	2.000	2.000	2.000	2.000
Sodium chloride (µg/mL)		2	1	0.4	0.2	0.02
Sodium sulfite (µg/mL)		10	5	2	1	0.1
Sodium sulfate (µg/mL)		10	5	2	1	0.1

Eluent preparation

Weigh 8.37 g of sodium carbonate monohydrate (mw = 124.0) and 6.3 g of sodium bicarbonate (mw = 84.0) in a 1 L polypropylene flask. Add DI water to the mark to make 50x concentrated eluent stock.

Dilute 20.0 mL of the 50x concentrated eluent stock to 1.00 L to make the eluent of 13.5 mM Na_2CO_3/1.5 mM $NaHCO_3$ for the sodium thiosulfate assay. This is also the eluent solution B used for the sodium thiosulfate ionic impurity method.

Dilute 22.0 mL of the 50× concentrated eluent stock to 1.00 L to make the +10% eluent (14.85 mM Na_2CO_3/ 1.65 mM $NaHCO_3$). Dilute 18.00 mL to 1.00 L to make -10% eluent (12.15 mM Na_2CO_3/1.35 mM $NaHCO_3$) for the robustness test.

Mix one part of the eluent solution B with four parts DI water to make eluent solution A for the sodium thiosulfate ionic impurity method, which is 2.7 mM Na_2CO_3/0.3 mM $NaHCO_3$.

Robustness study

Following the guidelines in USP General Chapter <1225>, Validation of Compendial Methods,[9] and USP General Chapter <621> Chromatography,[10] the robustness of this method was evaluated by examining the retention time (RT), peak asymmetry, and assay results of a 100 mg/L sodium thiosulfate sample after imposing small variations (±10%) in procedural parameters (e.g., flow rate, eluent gradient concentration, column temperature). A system suitability standard containing 100 mg/L of sodium thiosulfate was injected. The same procedure was applied to two column sets from two different lots. The following variations were tested:

- Flow rate at 1.5 mL/min, 1.35 mL/min, 1.65 mL/min
- Column temperature at 30 °C, 27 °C, 33 °C
- Eluent concentrations at, 13.5 mM Na_2CO_3/1.5 mM $NaHCO_3$, 12.15 mM Na_2CO_3/1.35 mM $NaHCO_3$, 14.85 mM Na_2CO_3/1.65 mM $NaHCO_3$

Results and discussion

Sodium thiosulfate assay

Separation

Figure 1 shows the chromatogram of sodium thiosulfate mixed with anions including fluoride, chloride, nitrite, bromide, sulfate, nitrate, and phosphate. Using a Dionex IonPac AS12A column set under the prescribed isocratic conditions, thiosulfate is well separated from the common anions. Figure 2 shows a chromatogram of 100 µg/mL of sodium thiosulfate. The retention time of thiosulfate is in agreement with the proposed USP method, which states about 7 min. For two lots of the Dionex IonPac AS12A column, retention time was 7.20 and 7.68 min. The data from both columns passed the proposed USP method suitability requirements. The asymmetry values for thiosulfate were 1.3 and 1.47 (USP requires these values be not more than (NMT) 2 and the relative standard deviations were 0.7% and 0.03% (USP NMT 2.0%), respectively.

Columns:	Dionex IonPac AG12A, 4 × 50 mm and Dionex IonPac AS12A, 4 × 200 mm
Eluent:	13.5 mM Na_2CO_3 / 1.5 mM $NaHCO_3$
Flow Rate:	1.5 mL/min
Inj. Volume:	25 µL
Column Temp.:	30 °C
Detection:	Suppressed conductivity, Dionex AERS 500 (4mm) Suppressor, 25 °C, 106 mA, recycle mode
Samples:	50 mg/L of Sodium Thiosulfate in DI-water spiked with anions (4 to 30 mg/L of Fluoride, Chloride, Nitrite, Bromide, Nitrate, Phosphate, and Sulfate)
Peak:	1. Thiosulfate

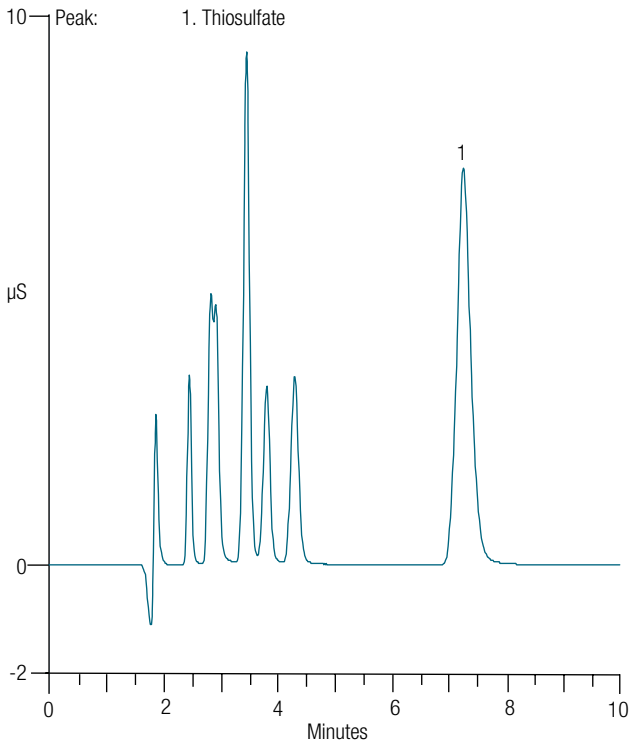

Figure 1. Separation of sodium thiosulfate from other anions.

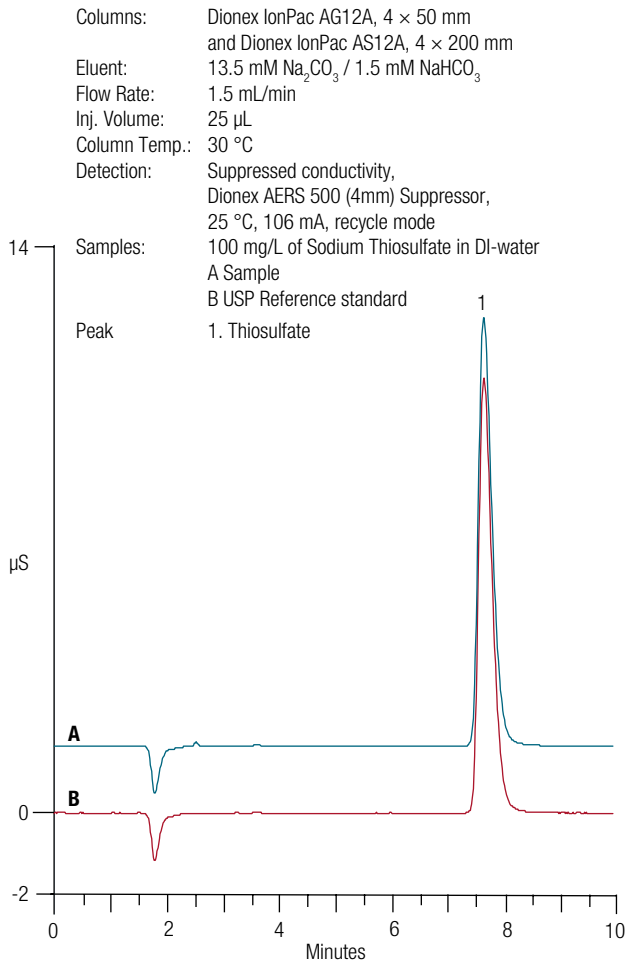

Figure 2. Chromatogram of 100 µg/mL of sodium thiosulfate.

Calibration, limit of detection (LOD), and limit of quantitation (LOQ)

The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and the USP General Chapter <1225> guidelines recommend a minimum of five concentrations to establish linearity in an assay. For a drug substance or finished product, the minimum specified range is from 80% to 120% of the test concentration.

In this study, sodium thiosulfate was calibrated at eight concentration levels ranging from 0.2 to 200 µg/mL. When the high concentration of 200 µg/mL is included, the measured coefficient of determination (r^2) was 0.998, therefore the data should be fit with using a quadratic function if including a concentration > 150 µg/mL. From 0.2 to 150 µg/mL, there was a linear relationship of peak area to concentration with a coefficient of determination (r^2) of 0.999. (Table 6 and Figure 3). As calibration is linear, the IC method for assay in the proposed USP Sodium Thiosulfate monograph using a one-point calibration at 100 µg/mL is an acceptable method for assay.

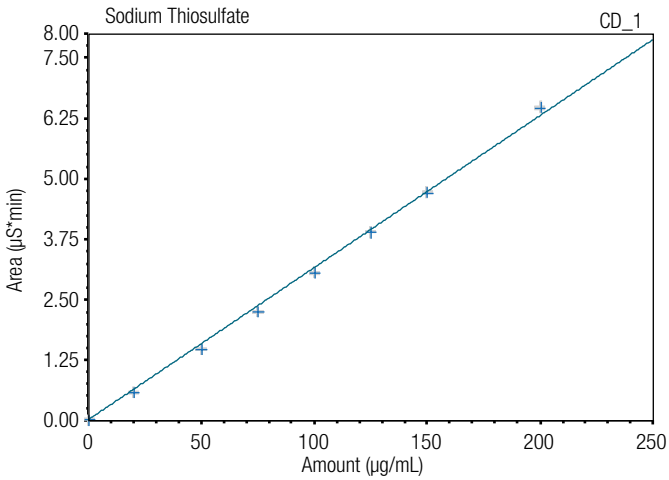

Figure 3. Calibration plot for sodium thiosulfate illustrating linearity.

Table 6. Comparison of calibration methods, LOD, and LOQ for sodium thiosulfate.

Method	Calibration Standards (µg/mL)	Calibration Type	r^2	Response Factor (µS*min/(µg/mL))	LOD (µg/mL)	LOQ (µg/mL)
A (USP method)	100	One level	n. a	0.031	0.05	0.17
B	0.2–200	Quadratic	1	n. a		
C	0.2–150	Linear, through origin	0.999	0.031		
D	0.2–200	Linear, through origin	0.998	0.032		

The LOD and LOQ were determined by seven injections of 0.20 µg/mL sodium thiosulfate. The baseline noise was determined by measuring the peak-to-peak noise in a representative 1 min segment of the baseline where no peaks elute but close to the peak of interest. The LOD and LOQ were determined for the concentration at the signal-to-noise ratio 3x and 10x (Table 6). The LOD is 0.05 µg/mL and the LOQ is 0.17 µg/mL.

Sample analysis

The proposed USP monograph requires that sodium thiosulfate contain 98.0–102.0% on the dried basis. In this study, the USP Sodium Thiosulfate Reference Standard was used to prepare the standard solutions. A purchased USP grade sodium thiosulfate salt was used to prepare the 100 µg/mL sample solution in DI water.

Two quantification methods were compared and evaluated to calculate the percentage of sodium thiosulfate ($Na_2S_2O_3$) in the portion of sodium thiosulfate taken. As shown in Table 7, the sodium thiosulfate % calculated from method A (proposed monograph method) is similar to that determined using a calibration curve (method B). The assay results from both methods show that this sodium thiosulfate sample (98.8% purity) passed the acceptance criteria of 98.0–102.0% in the proposed USP monograph. The assay result agrees with USP grade stated on the sample bottle.

Table 7. Percentage of sodium thiosulfate in a sample using two quantification methods.

	Method A* (%)	Method B**(%)
Average	98.80	98.85
RSD (n=3)	0.25	0.28

*Method A: Proposed USP IC method for assaying sodium thiosulfate using one-point calibration.
**Method B: Eight-point calibration using quadratic fitting.

Sample accuracy and precision

Assay precision was evaluated by injecting 0.1 mg/mL sodium thiosulfate sample solutions, and expressed as the RSD of the results (sodium thiosulfate % in sample by method A). The method is precise with intraday precision from 0.2% to 0.6% and interday precision of 0.8% (Table 8).

Table 8. Precision of the sodium thiosulfate assay.

Analyte	Injection Precision Range (%) *	Intraday Precision Range (%) **	Interday Precision (%) ***
Sodium Thiosulfate	0.04–0.3	0.2– 0.6	0.8

* Injection precisions calculated from n=3 injections/sample for each sample.
** Intraday precision range is from independently prepared 100 µg/mL sodium thiosulfate samples, n=3 injections/sample, 2-3 samples/day for four days.
*** Interday precision is from 10 independently prepared 100 mg/L sodium thiosulfate samples, n=3 injections/sample, the samples were analyzed on four separate days.

Method accuracy was validated by spiked recovery of USP Sodium Thiosulfate Reference Standard in sodium thiosulfate samples over five concentration levels from 10 to 100 µg/mL in both 50 and 100 µg/mL samples. Table 9 summarizes the recovery results. For the calibration range of 0.2–150 µg/mL (150% of assay concentration), the method is accurate with sodium thiosulfate recovery ranging from 99 to 108%. The results from two columns are similar.

Robustness

Assay robustness was evaluated by measuring the influence of small variations (±10%) in procedural parameters (e.g., flow rate, eluent concentration, and column temperature) on the RT, peak asymmetry, and sodium thiosulfate purity results. These tests were carried out on two column sets from two different lots. The peak asymmetry was measured following the USP standard. Table 10 summarizes the results for sodium thiosulfate. These results indicate the method was robust to both changes in chromatography parameters and column change.

Table 9. Recovery data for sodium thiosulfate spiked in 50 µg/mL and 100 µg/mL samples.

Sodium Thiosulfate Added (µg/mL)	Column A		Column A		Column B	
	50 µg/mL Sodium Thiosulfate		100 µg/mL Sodium Thiosulfate		100 µg/mL Sodium Thiosulfate	
	Total Found (µg/mL)	Recovery %	Total Found (µg/mL)	Recovery %	Total Found (µg/mL)	Recovery %
0			98.0–100.7*		98.6-99.0	
10	59.6	101	109.3–109.4**	103		
25			125.2–126.1**	101–105	125	104
50	99.2	99	152.6–154.1**	105–108	152.5	107
75			178.8–183.1**	105–111	180.8	109
100			202.7–210.4**	102–110	210.0	111

*n=7 independently prepared 100 µg/mL sodium thiosulfate samples over four days
**n=5 independently prepared spiked sodium thiosulfate samples over four days

Table 10. Robustness of the IC-based assay for sodium thiosulfate (injected sample: 100 µg/mL sodium thiosulfate).

Parameter	Value	Column A					
		Ret.Time (min)		Amount (µg/mL)		Asym.	
		Average	% Diff	Average	% Diff	Average	% Diff
Flow Rate (mL/min)	1.65	6.49	-9.8	98.04	0.0	1.27	-1.8
	1.5	7.20		98.08		1.30	
	1.35	7.88	9.5	98.09	0.0	1.30	0.3
Column Temp. (°C)	27	6.41	-11.1	98.07	0.0	1.26	-2.6
	30	7.20		98.08		1.30	
	33	6.25	-13.2	98.09	0.0	1.26	-3.1
Eluent Conc. (mM) Na_2CO_3 / $NaHCO_3$	12.15/1.35	7.71	7.1	98.20	0.1	1.29	-0.8
	13.5/1.5	7.20		98.08		1.30	
	14.85/1.65	6.81	-5.5	98.23	0.2	1.27	-1.8
Parameter	Value	Column B					
		Ret.Time (min)		Amount (µg/mL)		Asym.	
		Average	% Diff	Average	% Diff	Average	% Diff
Flow Rate (mL/min)	1.65	6.95	-9.5	98.59	-0.2	1.45	-1.1
	1.5	7.68		98.83		1.47	
	1.35	8.52	10.9	98.61	-0.2	1.47	0.0
Column Temp (°C)	27	7.01	-8.8	98.58	-0.3	1.44	-1.6
	30	7.68		98.83		1.47	
	33	6.91	-10.0	98.27	-0.6	1.44	-1.8
Eluent Conc. (mM) Na_2CO_3 / $NaHCO_3$	12.15/1.35	8.47	10.2	98.65	-0.2	1.49	1.6
	13.5/1.5	7.68		98.83		1.47	
	14.85/1.65	7.08	-7.9	98.82	0.0	1.46	-0.7

Ionic impurities in the sodium thiosulfate method

In the proposed Sodium Thiosulfate and Sodium Thiosulfate Injection monograph revisions, an IC method was also used to determine the ionic impurities (chloride, sulfite, and sulfate). The sample solution for the ionic impurities determination is 2.0 mg/mL of sodium thiosulfate in diluent. All sample and standards for impurity determination were dissolved in the diluent (2.0 g/L of D-mannitol) to prevent oxidation of sulfite.

Separation

Figure 4 shows a chromatogram of chloride, sulfite, and sulfate spiked in sodium thiosulfate with an enlarged view of the analytes of interest. Using a Dionex IonPac AS12A column set under the gradient conditions, chloride, sulfite, and sulfate are separated and also well resolved from thiosulfate in 30 min. The gradient is modified from the proposed USP revision method (eluent A from 0 to 14 min, instead of 12 min) to allow complete separation of sulfate from the rise in the baseline due to the eluent gradient. Resolution between sulfite and sulfate is 2.6 for column A and 3 for column B, both passing the proposed USP method suitability requirement NLT 2. Relative retention times for chloride, sulfite, and sulfate are 0.31, 0.84, and 1 for column A and 0.28, 0.84, and 1 for column B (Table 11). The proposed USP method suitability requirements are 0.22, 0.84, and 1. The relative retention of chloride varies from column to column and neither matched the proposed USP method value. However, it was observed that this had no impact on the determination of ionic impurities.

Columns: Dionex IonPac AG12A, 4 × 50 mm
and Dionex IonPac AS12A, 4 × 200 mm

Eluent: A: 2.7 mM Na_2CO_3/ 0.3 mM $NaHCO_3$
B: 13.5 mM Na_2CO_3/ 1.5 mM $NaHCO_3$

Time (min)	A (%)	B (%)
0	100	0
14	0	100
16	100	0
21	0	100
23	100	0
30	100	0

Flow Rate: 1.5 mL/min
Inj. Volume: 25 µL
Column Temp.: 30 °C
Detection: Suppressed conductivity, Dionex AERS 500 (4 mm) Suppressor, 25 °C, 106 mA, recycle mode

Peaks:			
	1. Chloride	2.0	µg/mL
	2. Sulfite	5.0	
	3. Sulfate	10.0	
	4. Thiosulfate	100.0	

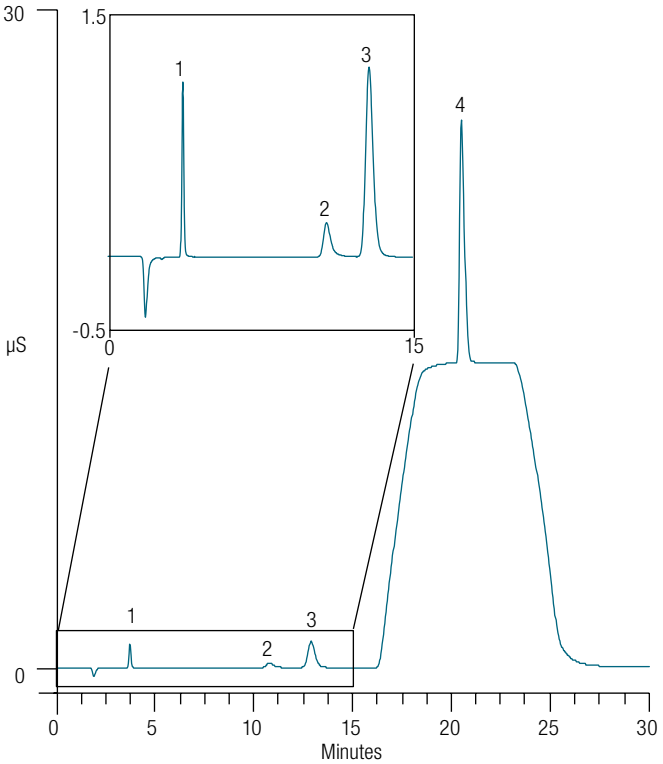

Figure 4. Chromatogram of chloride, sulfite, and sulfate in sodium thiosulfate.

Table 11. Retention time and resolution of impurity ions.

Analyte	Column A			Column B		
	Ret. Time (min)	Relative Ret. Time	Resolution	Ret. Time (min)	Relative Ret. Time	Resolution
Chloride	3.37	0.31	14	3.70	0.28	16
Sulfite	9.29	0.84	2.6	11.01	0.84	3
Sulfate	11.04	1.00		13.17	1.00	

Calibration, limit of detection (LOD), and limit of quantitation (LOQ)

According the ICH and the USP guidelines, a minimum calibration range of 50% to 120% is required for determination of an impurity with a minimum of five concentrations to establish its calibration curve.

In this study, ionic impurities (chloride, sulfite, and sulfate) were calibrated at six concentration levels following the proposed monograph revision method. The range of chloride is from 0.04 to 2 µg/mL, the range of sulfite is from 0.2 to 10 µg/mL, and the range of sulfate is from 0.2 to 10 µg/mL. The results yield a linear relationship of peak area to concentrations for all three impurities (Table 12 and Figure 5). The coefficients of determination (r^2), were 1 for chloride, 0.9995–0.9998 for sulfite, and 1 for sulfate, and all passed the suitability requirements (NLT 0.995).

Using similar methodology, LOD and LOQ were determined with repeat injection of low levels (approximately 3 times the LOQ or approximately 10 times the LOD) of chloride, sulfite, and sulfate standards. The impurity method is sensitive with LOD of chloride at 0.004 µg/mL, sulfite at 0.09 µg/mL, and sulfate at 0.02 µg/mL, and LOQ of chloride at 0.01 µg/mL, sulfite at 0.3 µg/mL, and sulfate at 0.08 µg/mL.

Sample accuracy and precision

The ionic impurities (chloride, sulfite, and sulfate) in the sodium thiosulfate sample ware determined using 2.0 mg/mL of sodium thiosulfate in the diluent. The limits of acceptance criteria are 0.02% for chloride, 0.1% for sulfite, and 0.5% for sulfate. Table 13 compares the results of chloride, sulfite, and sulfate in the sodium thiosulfate sample to the limit in the monographs revisions. This sodium thiosulfate sample did not pass the acceptance criteria limit stated in the proposed monographs revision because it exceeds the limit of 0.02% chloride. Recall that this sample was a purchased chemical and not an actual drug substance.

Table 12. Summary of calibration, limits of detection (LODs), and limits of quantitation data (LOQs) for ionic impurities.

Analyte	Calibration Standards (µg/mL)*	Coefficient of Determination Range (r^2)	LOD (µg/mL)	LOQ (µg/mL)
Chloride	0.04–2	1	0.004	0.01
Sulfite	0.2–10	0.9995–0.9998	0.09	0.3
Sulfate	0.2–10	1	0.02	0.08

*This is the concentration of its sodium salt

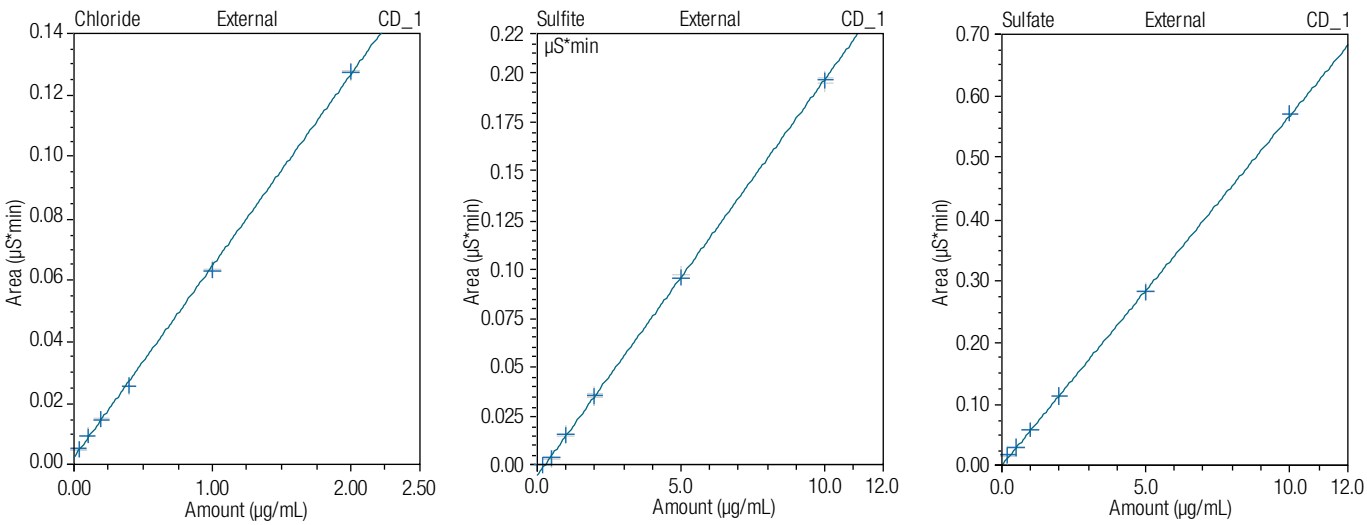

Figure 5. Calibration plots for ionic impurities illustrating linearity.

Table 13. Chloride, sulfite, and sulfate in sodium thiosulfate sample.

		Chloride	Sulfite	Sulfate
Acceptance Criteria (No more than)	In Sodium Thiosulfate (%)	0.02	0.1	0.5
	In 2.0 mg/mL Solution (µg/mL)	0.4	2	10
Sodium Thiosulfate Sample*	In Sodium Thiosulfate (%)	0.022	0.023	0.06
	In 2.0 mg/mL Solution (µg/mL)	0.44 ± 0.03	0.46 ± 0.06	1.19 ± 0.01

*Average result of n=3 each day over three days

Method accuracy was validated by spiked recovery of chloride, sulfite, and sulfate in 2.000 mg/mL of sodium thiosulfate in sample diluent solution at low concentration, with three replicates of each concentration and repeated twice on different days. Table 14 summarizes the recovery results. The method is accurate with chloride recovery ranges of 95–101%, sulfite of 86–100%, and sulfate of 107–109%.

Method precision was evaluated by injecting (n ≥ 3 per day) the system suitability solution containing 0.4 µg/mL of chloride, 1 µg/mL of sulfite, and 2 µg/mL of sulfate. The impurity method is precise with intraday precision range of chloride at 0.2–0.8%, sulfite at 1.0–3.3%, and sulfate at 0.04–0.9%. The interday precisions are chloride at 4.1%, and sulfite and sulfate at 1.9%. These precision results surpass the suitability requirement in the proposed USP method (<15%) (Table 15).

Table 14. Recovery data for mixed chloride, sulfite, and sulfate spiked in a sodium thiosulfate sample containing 2.0 mg/mL sodium thiosulfate.

	Chloride		Sulfite		Sulfate	
	Added (µg/mL)	Recovery (%)	Added (µg/mL)	Recovery (%)	Added (µg/mL)	Recovery (%)
Spiked in 2.0 mg/mL Sodium thiosulfate	0.4	95–99	2	86–88	2	107
	1	99–100	5	90–96	5	108
	2	101	10	98–100	10	109

*n=2 independently prepared spiked sample over 2 days

Table 15. Precisions for analysis of the system suitability solution.

Analyte	Chloride	Sulfite	Sulfate
Intraday Precision range* (%)	0.2–0.8	1.0–3.3	0.04–0.9
Interday** Precision (%)	4.1	1.9	1.9

*n=3 or > 3 for each day
**n=5 days. Two days with column set A and three days with column set B

Conclusion

This study evaluated two IC methods included in the proposed Sodium Thiosulfate and Sodium Thiosulfate Injection monograph revisions. Both IC methods use a Dionex IonPac AS12A anion-exchange column and suppressed conductivity detection. Following the guidelines outlined in USP General Chapter <1225> (Validation of Compendial Methods) and the monograph instructions for each method, both methods were validated. Deliberate variations in the IC method parameters (e.g., mobile phase concentration, column temperature, etc.) were also made to test robustness.

The sodium thiosulfate assay method, a 10 min isocratic method, is linear (r^2 = 0.999) over the established analytical range of 0.2 to 150 µg/mL. The method is sensitive (LOQ at 0.17 mg/L), accurate (recovery 99–108%), precise (intraday precision 0.2–0.6% and interday precision of 0.8%), and specific for sodium thiosulfate determination. The method is robust as IC method parameter changes had no impact on the purity determination. The sodium thiosulfate impurity method, a 35 min gradient method, is linear over the established analytical range for impurities, precise, and accurate.

In conclusion, both IC methods meet the guidelines outlined in USP General Chapter <1225> and can be used to replace existing titration-based assays in the Sodium Thiosulfate monograph and likely the Sodium Thiosulfate Injection monograph (we were unable to test the drug product).

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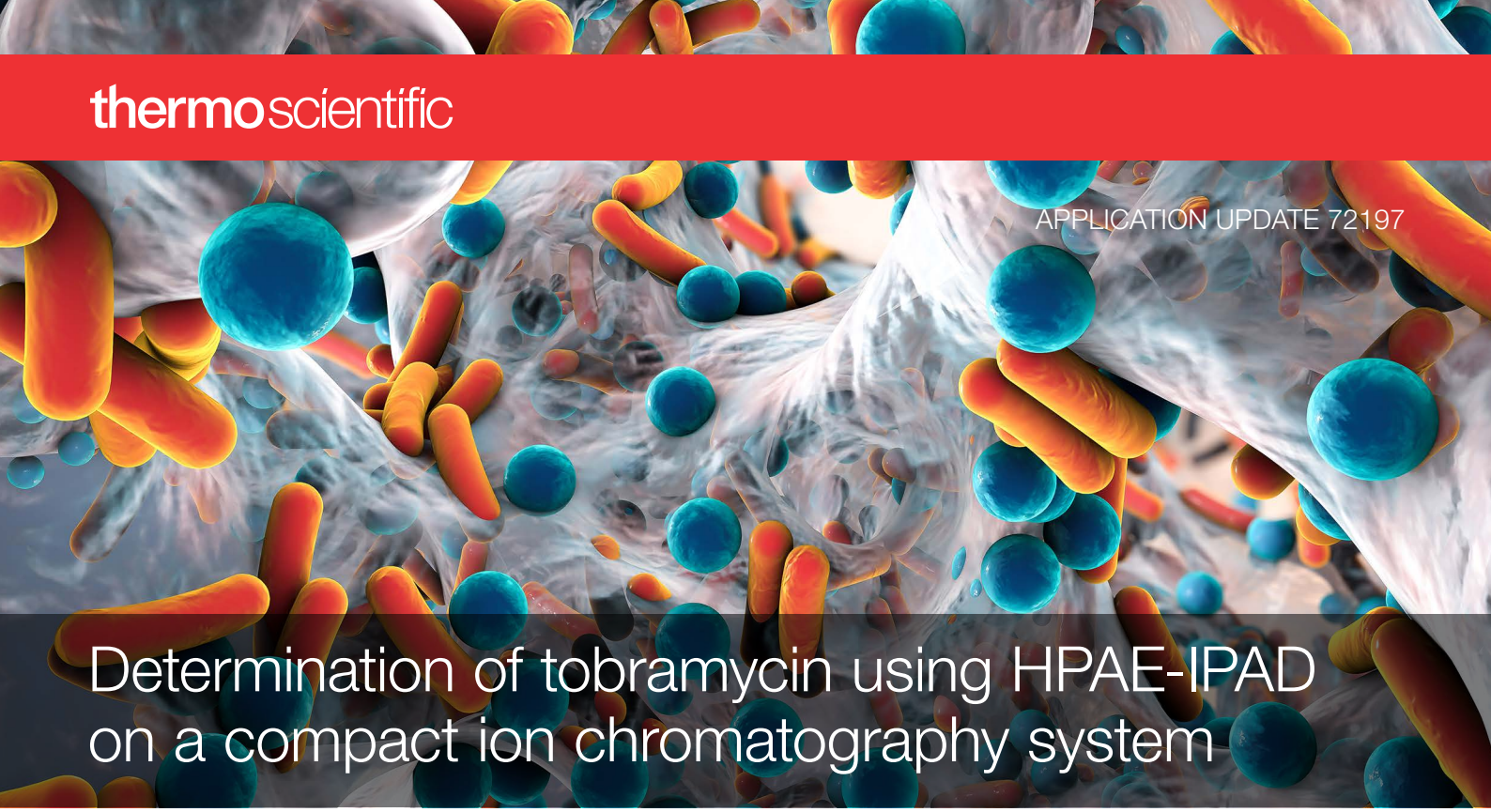

APPLICATION UPDATE 72197

Determination of tobramycin using HPAE-IPAD on a compact ion chromatography system

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Keywords
Integrion, CarboPac PA1, aminoglycoside, carbohydrate, antibiotic, drug substance

Goal

To develop an HPAE-IPAD method for the determination of kanamycin A, kanamycin B, and tobramycin using a high-pressure-capable Thermo Scientific™ Dionex™ Integrion™ HPIC™ system.

Introduction

Tobramycin is an important aminoglycoside antibiotic used in ophthalmic and intravenous treatments to treat bacterial infections by blocking protein synthesis.[1] Tobramycin is isolated from the fermentation of the actinomycete *Streptomyces tenebrarius* with kanamycin A and kanamycin B present as impurities from either incomplete isolation of the drug or from degradation of tobramycin. Therefore, it is important to assay the tobramycin content and quantify the related impurities of a tobramycin-based antibiotic. Determination of tobramycin, kanamycin, and other aminoglycoside antibiotics has been previously demonstrated by High Performance Anion Exchange with Integrated Pulsed Amperometric Detection (HPAE-IPAD).[2–6] These publications demonstrated the advantages of using eluent generation (EG) for this application.

This document updates Thermo Scientific™ Application Note 61 using a high-pressure-capable Dionex Integrion HPIC system equipped with updated EG technology and an electrochemical detector.

ThermoFisher SCIENTIFIC

Integrion HPIC

Table of contents

Experimental

Equipment

- Thermo Scientific Dionex Integrion HPIC System (P/N 22153-60305), including:
  - Temperature control in the detector and column oven chambers
  - EG capabilities
  - Eluent degas module
  - Device monitoring
  - Vacuum Degas Kit (P/N 00108-01-00046)
- Integrion ED detector (P/N 22153-62035) with Thermo Scientific™ Dionex™ Integrion electrochemical cell without electrodes (P/N 072044) (or the same P/N from the Thermo Scientific™ Dionex™ ICS-3000 IC, ICS-5000 IC or ICS-5000+ HPIC systems)
- Mobile Control Option (P/N 22153-62031) and Android tablet (P/N 22153-62100)
- Thermo Scientific™ Dionex™ AS-AP Autosampler temperature control option (P/N 074926) with 1.5 mL trays (P/N 074936) and 250 µL syringe (P/N 074306)

Reagents

- Degassed ASTM™ Type I deionized water,[7] vacuum degassed with ultrasonic agitation
- pH buffer solutions to calibrate pH – Ag/AgCl Reference electrode: Fisher Scientific™ P/N SB115-500 (pH 7) and P/N SB107-500 (pH 10)
- Tobramycin (Sigma-Aldrich® Chemical Co, P/N T40014)
- Kanamycin B (Sigma-Aldrich Chemical Co, P/N B5264)

Software

Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software, version 7.2 SR4

Consumables

Table 1 lists the consumable products needed for a Dionex Integrion HPIC Reagent-Free IC capabilities, configured for electrochemical detection.

Chromatographic Conditions

Columns:	Thermo Scientific™ Dionex™ CarboPac™ PA1 guard (4 × 50 mm) and separation (4 × 250 mm)		
Eluent:	2 mM KOH		
Eluent Source:	Thermo Scientific™ Dionex™ EGC 500™ KOH cartridge with Thermo Scientific™ Dionex™ CR-ATC™ 600 trap column and high pressure EG degas module (P/N 075522)		
Flow Rate:	0.5 mL/min		
Column Temperature:	30 °C		
Detector Compartment Temperature:	30 °C		
Injection Volume:	20 µL, in PushFull mode		
Waveform:	*Time (s)*	*Potential (V)*	*Integration*
	0.00	+0.13	
	0.04	+0.13	
	0.05	+0.33	
	0.21	+0.33	Begin
	0.22	+0.55	
	0.46	+0.55	
	0.47	+0.33	
	0.56	+0.33	End
	0.57	-1.67	
	0.58	-1.67	
	0.59	+0.93	
	0.60	+0.13	
Detection:	IPAD, AAA-Direct Waveform vs pH, 1.67 Hz		
Working Electrode:	AAA-Direct disposable gold working electrode, 0.002” thick Teflon® gasket		
Reference Electrode Mode:	pH/Ag/AgCl in pH mode		
Run Time:	16 min		
Typical Background:	50–90 nC		

Table 1. Consumables list.

Product	Description	Part Number
Thermo Scientific™ Dionex™ IC PEEK Viper™ fitting tubing assembly kits	Dionex IC PEEK Viper fitting assembly kit for the Dionex Integrion HPIC system: Includes one each of P/Ns: 088805–088807, 088809, 088811	088797
Dionex IC PEEK Viper fitting tubing assemblies	Guard to separator column: 0.007 × 4.0 in (102 mm)	088805
	Injection Valve, Port C (Port 2) to guard column: 0.007 × 5.5 in long (140 mm)	088806
	EGC Eluent Out to CR-TC Eluent In: 0.007 × 6.5 in (165 mm)	088807
	Separator to ED Cell In: 0.007 × 7.0 in (178 mm)	088809
	CR-TC Eluent Out to Degasser Eluent In: 0.007 × 9.5 in (241 mm)	088811
Dionex AS-AP Autosampler vials	Package of 100, polystyrene vials, caps, blue septa,10 mL	074228
Dionex AS-AP Autosampler vials	Package of 100, polypropylene vials, caps, 1.5 mL	079812
Thermo Scientific™ Dionex™ EGC™ 500 KOH Eluent Generator cartridge	Eluent generator cartridge	075778
EG Vacuum Degas Conversion Kit	Recommended for carbohydrate analysis methods using eluent generation. Included when ordering a Dionex Integrion HPIC system with ED detector	00108-01-00046
HP EG Degas module	Recommended for use with eluent generation. Included when ordering an Integrion with eluent generation	075522
Thermo Scientific™ Dionex™ CR-ATC™ 600 Electrolytic trap column	Continuously regenerated trap column used on Integrion systems with the Dionex EGC KOH 500 cartridge	088662
Thermo Scientific™ Dionex™ CarboPac™ PA1 Guard	Guard column, 4 × 50 mm	043096
Dionex CarboPac PA1 separation column	Separation column, 4 × 250 mm	035391
Thermo Scientific™ Dionex™ AAA-Direct disposable working electrodes	AAA-Direct carbohydrate gold working electrodes, preferred for this application	060082
Thermo Scientific™ Dionex™ Carbohydrate disposable working electrodes	Carbohydrate gold working electrodes, suggested alternative working electrodes for this application	060139
Thermo Scientific™ Dionex™ Ag/AgCl reference electrode	Reference electrode for this application	061879
Extra gaskets for working electrodes	Extra gaskets, PTFE, 0.002” thick	060141
Thermo Scientific™ Nalgene™ Rapid Flow™ sterile disposable filter units	1000 mL vacuum filtration flask, PES membrane, 0.2 µm,	5670020 (Fisher Scientific P/N 09-741-03)

Standard and eluent preparation

Use ASTM Type I deionized (DI) water for standards, eluent, and autosampler flush solution. It is important to degas the DI water to supply the eluent generator and to maintain 3–5 psi of inert gas headspace over the DI water eluent when running an HPAE-PAD method. Absorbed carbon dioxide gas can result in poor chromatography and variable retention times.

To prepare 2 L of the degassed ASTM Type I DI water to supply the eluent generator, degas 1 L of DI water by vacuum filtration (Nalgene, 1 L, PES, 0.2 µm) with applied ultrasonic agitation. Transfer the degassed water to the 2 L eluent bottle and cap the bottle. Prepare another 1 L in the same manner. Connect the 2 L eluent bottle containing degassed DI water to the Dionex Integrion HPIC system pump eluent line. Connect nitrogen or other inert gas to the eluent bottle to provide ~3–5 psi headspace pressure.

Weigh the tobramycin reagent and dissolve in DI water to prepare a 10 mg/mL stock standard. Use polypropylene volumetric flasks, storage containers, and autosampler vials to avoid sample losses. Tobramycin, and to a lesser extent kanamycin B, when dissolved in water adsorbs to glass surfaces. Significant losses due to adsorption occur at dilute concentrations.[2] The tobramycin working standards were prepared by diluting the stock standard with DI water to 0.5, 1.0, 10, 50, and 100 µM. Kanamycin A and kanamycin B solutions (0.1, 1, 5, and 10 µM) were prepared in the same manner. For recovery experiments, 10 µM and 26 µM tobramycin solutions were prepared from the stock standards. Method detection limit (MDL) standards were prepared by serial dilution from the 0.1 µM working standard. The samples were stored at -20 °C and thawed prior to analysis.

Instrument setup and installation

The Dionex Integrion HPIC System with RFIC capabilities is a high-pressure-capable, integrated system. The Dionex Integrion HPIC system, the Dionex EGC 500 KOH cartridge and Dionex CR-ATC 600 consumable products are designed for high pressure conditions up to 5000 psi.

To set up this application, connect the Dionex AS-AP autosampler and the Dionex Integrion HPIC system, equipped with an electrochemical detector, according to Figure 1. Note that the injection valve is plumbed through different ports than previous Dionex IC systems. See Thermo Scientific TN 176.[8]

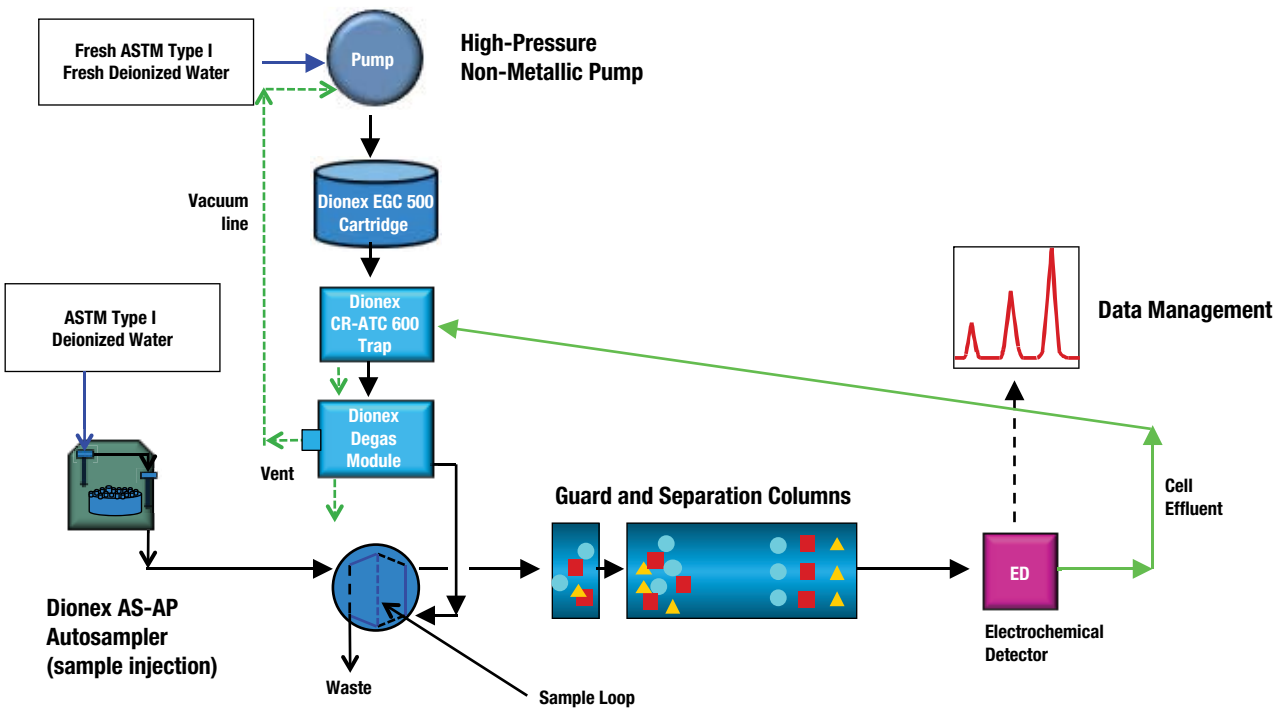

Figure 1. Flow diagram for the Dionex Integrion HPIC System configured for ED detection.

Connect the USB cables from the Dionex Integrion HPIC system to the Dionex AS-AP autosampler and to the computer. Connect the power cables and power on the IC instrument and the autosampler.

Configuring the modules in the Chromeleon CDS software

To configure the IC system:

1. Start the Chromeleon Instrument Controller program and select the link, *Configure Instruments* (opens Chromeleon Instrument Configuration Manager).
2. Right-click on computer name.
3. Select *Add an Instrument,* and enter an appropriate name (for example: Integrion_Tobramycin_1).
4. Select *Add a Module, IC: Dionex Integrated Modules,* and *Integrion HPIC System.*

In this application, only three modules are needed: the Dionex Integrion IC system, the autosampler, and the Integrion Pump Wellness module. The instructions to configure each module are summarized in Table 2.

To add the Dionex Integrion IC system in the configuration:

1. Right-click and select *Add a Module, IC: Dionex Integrated Modules, Integrion HPIC System* module.
2. Select USB address to link the module to the configuration.

The Chromeleon CDS software will automatically detect all Dionex Integrion IC system devices—the electrolytic devices, detectors, pump degasser, and seal wash—requiring minimal data entry during instrument configuration. The Chromeleon CDS software automates the system configuration process by automatically detecting these installed devices (Figure 2).

Table 2. Summary of system configuration for the Dionex Integrion HPIC system.

Tab	Action	Result
Dionex Integrion HPIC Module		
General	Link to USB address	
Pump		Flow Rate and pressure limitations are displayed
Detectors		Automatically detected
Electrolytics		Automatically detects Dionex eluent generator cartridges, and Dionex CR-TC trap columns (Figure 2)
Inject Device		Automatically detected
Thermal Controls		Automatically detects thermal control options for column, and detector
High-Pressure Valves		Automatically detected
Low-Pressure Valves		Not needed for this application, but automatically detected.
Options		Automatically detects Pump Degasser and Seal Wash pump
Pump Wellness Module		
Devices	Click pressure signal box	Activates pressure monitoring feature (Figure 3)
Dionex AS-AP Autosampler Module		
Add module	Link to USB address	
Sharing		Only if more than one instrument is detected. If this option is present, select Instrument
Segments / Pump Link		Select 10 mL polystyrene vials or 1.5 mL vials for “Red”, “Blue”, and “Green”
Options		Select Push, select syringe size, select 1.2 mL buffer line, enter the loop size

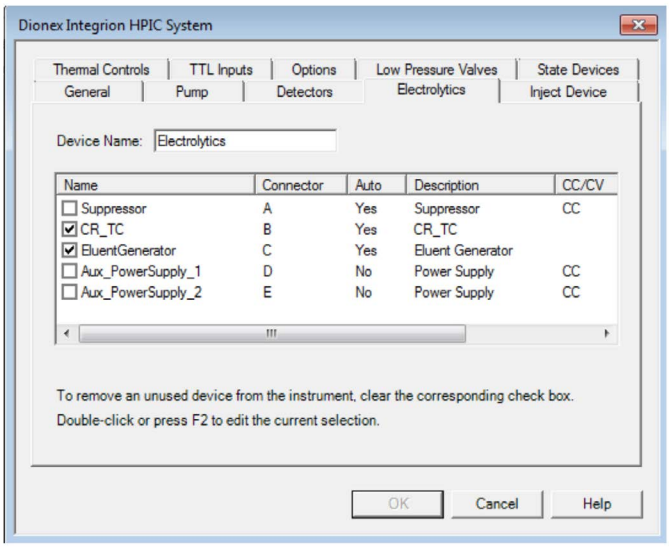

Figure 2. Automatic detection of electrolytic devices in the Dionex Integrion HPIC System module.

To add pressure monitoring capabilities:

1. Right-click and select *Add a Module, IC: Dionex Integrated Modules, Integrion HPIC Pump Wellness* module.
2. Select USB address to link the module to the configuration.
3. Select the Devices tab and click on the pressure signal box (Figure 3).

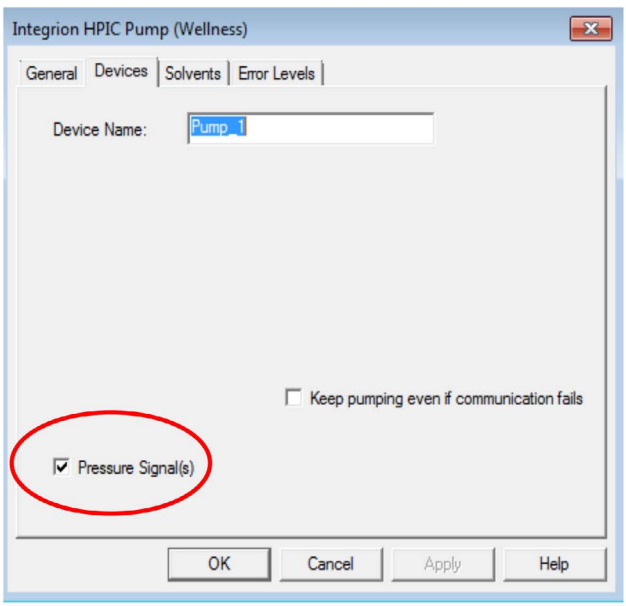

Figure 3. Adding the Dionex Integrion HPIC Pump Wellness module to instrument configuration.

Adding the Dionex AS-AP Autosampler to the configuration

1. Add the Dionex AS-AP Autosampler as a module, and select the USB address.
2. In the Segments/Pump Link tab, select the appropriate vial trays for each color zone.
3. In the Options tab, select Push, installed syringe size, 1.2 mL for buffer line, and enter the sample loop volume (20 µL).
4. Save the configuration.
5. Select *Check the Configuration.*
6. Close the Chromeleon Instrument Configuration program.

Plumbing the Dionex Integrion HPIC System

Decontaminate the IC system prior to installing the columns by puping a 500 mM NaOH solution at 0.5 mL/min from the pump to valves for 2–3 h, or overnight if the system was previously used for another application. Finish the decontamination process by pumping DI water at 0.5 mL/min for 30 min.

To achieve the best chromatography, use the Dionex IC PEEK Viper fitting assemblies (listed below) and tighten to finger-tight. It is important that they are not overtightened. These fittings are used in the following locations:

- Dionex EGC 500 KOH eluent generator cartridge - *Eluent Out to Eluent In* on Dionex CR-ATC 600 trap column
- Dionex CR-ATC 600 trap column - *Eluent Out to Eluent In* on the Dionex Degas Module
- Injection Valve - Port 2 (Column) to the guard column
- Between the guard and separation columns
- Separation column to *Eluent In* on the ED electrochemical cell

To install an HPAE-IPAD application on the Dionex Integrion HPIC system, follow the instructions in Technical Note 176.[8]

1. Loosen the waste lines, including the metal-wrapped waste line in the back of the instrument, and direct the free ends to a waste container.
2. To plumb the system, first connect the pump eluent line to the eluent bottle containing DI water previously degassed (vacuum filtration and ultrasonic agitation).
3. Apply 3 to 5 psi of nitrogen or other inert gas headspace to prevent carbon dioxide absorption.
4. Prime the pump by opening the priming knob ¼ turn and pressing the priming button.
5. Prime the pump until no bubbles are visible and water is flowing at a steady rate out of the pump waste line.
6. Turn off the pump priming and close the priming knob to finger-tight.

For more information, review the Dionex Integrion HPIC system Installation and Operator's manual.[9]

Install vacuum line to degasser module vent

Hydroxide eluents produced by eluent generation require inline vacuum degassing to achieve the optimum conditions for electrochemical detection. Vacuum degassing is accomplished by connecting the vent line of the degas module to the vacuum pump port on the back of the instrument (Figure 4). In the Dionex Integrion HPIC system configured with an electrochemical detector (ED), the vacuum pump connector is pre-installed, eliminating the manual installation previously required.

At the start of the application, to ensure that the degasser pump is working properly, reset the pump degasser to "Off" and then "On".

1. Press the "F8" key while on the instrument console page, which brings up the manual commands.
2. Place the cursor on the pump and set Degasser to "Off".
3. Close the dialogue window and repeat with "On".

The degasser pump should turn on for a short time and then shut off, indicating that the degasser pump is working and that the vacuum is acceptable.

To assist in degassing the eluent, install a ¼" i.d. air tubing from the EG Degas module (P/N 075522) vent port to the vacuum connection in the back of the Dionex Integrion HPIC system (Figure 4). The degasser pump will turn on again for a short time to re-establish the vacuum. If the degasser pump stays on for longer than 1 min, tighten the fitting (Figure 4) ¼ turn.

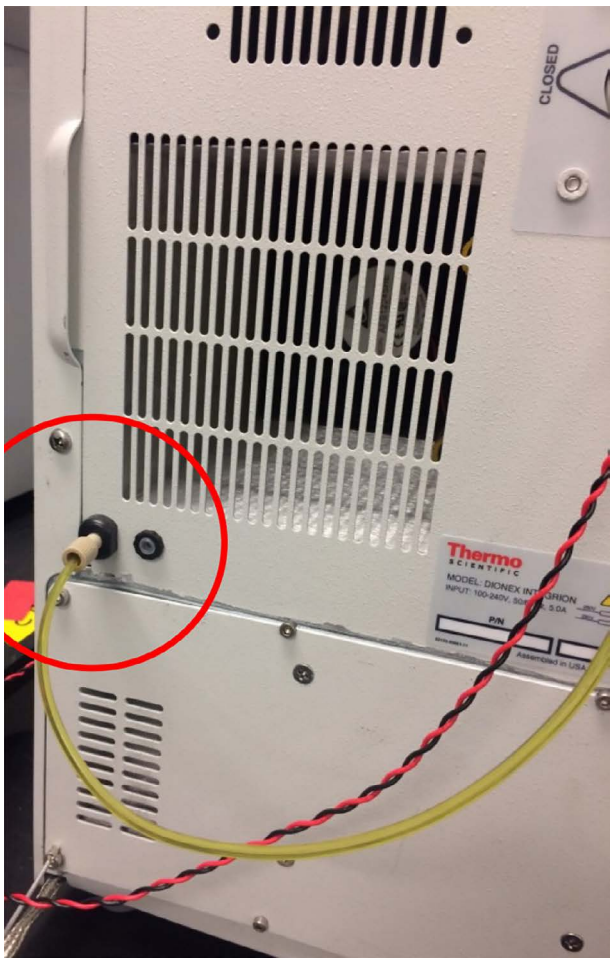

Figure 4. Installing the vacuum connection on the Dionex Integrion HPIC system.

Conditioning electrolytic devices and columns

Do not remove the tags on the columns and consumable devices. These tags are required for consumable device monitoring functionality.

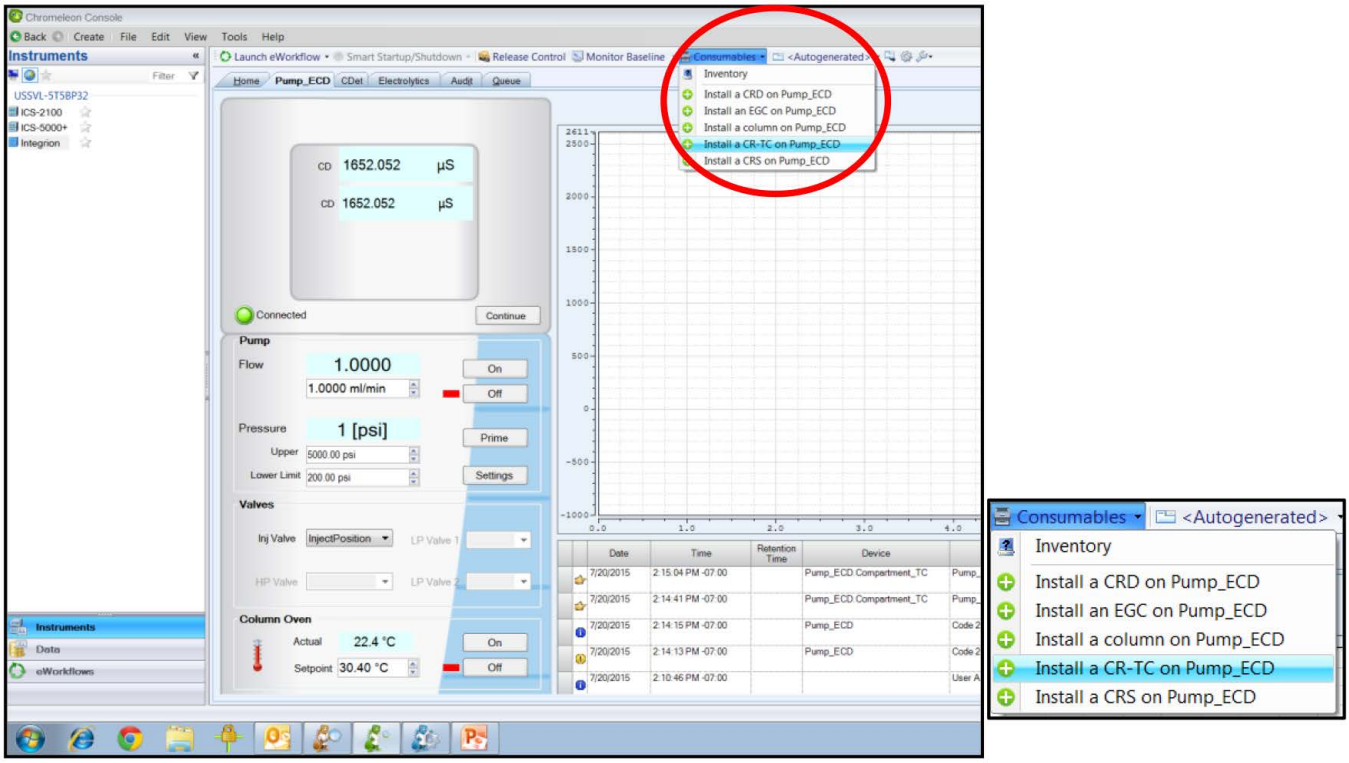

Figure 5. Consumables online installation instructions.

Install the Dionex EGC 500 KOH cartridge and Dionex CR-ATC 600 Continuously Regenerated Anion Trap Column in the Reservoir Tray compartment. Condition the devices according to instructions in the drop-down menu under Consumables, Install (Figure 5). (This information is also available in the product manuals and the system installation manual.[9-13])

Condition the columns for 30 min according to the instructions from the Consumables, Install Column section (Figure 5). The general practice is follow the eluent and flow rate conditions listed in the column's Quality Assurance Report (QAR) while directing the eluent exiting the column to a waste container. Complete the installation according to the flow diagram in Figure 1.

Installing and optimizing the Dionex AS-AP Autosampler

The Dionex AS-AP Autosampler needle must be aligned to the injection port. To align the autosampler needle:

1. Select the Sampler tab on the instrument panel, and press the Alignment button.
2. Follow the commands to align the autosampler needle to the Injection Port and Wash Port (Section B.12 in the Operator's Manual).[12]
3. Connect the autosampler syringe line to wash container containing degassed DI water to the syringe.
4. Prime the syringe to flush out any air in the Buffer Wash line and syringe.
5. Initially select a 5000 µL wash volume until a steady flow of water is observed at the Wash Port. Reset wash volume to 100 µL.
6. Calibrate the transfer line volume by following the prompts on the TLV Calibration button. This volume will be recorded automatically.

For more information review Section 5.9 in the Dionex IC Series AS-AP Autosampler Operator's Manual.[12]

Electrochemical cell

Always wear particle-free nitrile gloves (such as Fisher Scientific P/N 19-130-1597 series) when handling the electrochemical cell. If this is a new ED Cell, disassemble the cell and discard the shipping gasket. **Caution:** Do not touch the working electrode with any paper products, as this can contaminate the working electrode. Avoid wrinkles in the gasket.

The ED Cell is a three-electrode cell: the cell body as the counter electrode, a reference electrode (pH-Ag/AgCl), and a working electrode (conventional or disposable). The fully assembled cell also includes a Yoke Block Assembly (with a support block) to tighten the cell and gaskets for the working electrode. The installation procedures mentioned below describe an electrochemical cell with an AAA-Direct gold disposable working electrode.

Installing the disposable electrode

1. Rinse the cell body, the walls of the reference electrode, and the inlet tube thoroughly with DI water.
2. Shake off the excess water, and dry with a lab wipe.
3. Select the working electrode for the application and corresponding gasket and support block. Rinse them with DI water and shake off the excess water.
4. Dry the gasket (0.002” thick Teflon) and support block with an absorbent tissue.
5. Assemble the cell according to the Operator’s manual[7] and ED User’s Compendium for Electrochemical Detection[13] by first installing the working electrode gasket flat against cell body. Avoid any wrinkles in the gasket, as this will cause a poor fit and subsequent leaks and poor detection. To minimize the chance of using a worn gasket and developing a leak, the gasket should be replaced after three compressions (i.e. disposable electrode installations).
6. Install the AAA-Direct disposable working electrode with the metal face down over the gasket.
7. Install the support block firmly over the working electrode.
8. Install the Yoke Block by squeezing the tabs and sliding it on the cell body.
9. Align the Yoke Block parallel to the cell body and rotate the Yoke Block knob clockwise until you hear three “clicks”. (The cell with a conventional working electrode is assembled similarly with appropriate gasket, except the support block is not used.)

Calibrating and installing the pH-Ag/AgCl reference electrode in the electrochemical cell

To calibrate the reference electrode:

1. Condition the pH-Ag/AgCl reference electrode by removing the storage cap, rinsing the electrode with DI water to remove the potassium chloride storage solution, and then placing the electrode in a solution of pH 7 buffer.
2. Select pH buffer 7 and the corresponding buffer for the application, pH 10 for basic eluents and pH 4 for acidic eluents.
3. Install the cell into ED module and connect the yellow cable to the yellow port.
4. Install the reference electrode blue cable into the black port.
5. Immerse the reference electrode in pH 7 buffer to at least mid-level of the electrode.
6. Select the “pH Calibration” button on the ED Panel and follow the instructions to calibrate the electrode including using pH 10 buffer.
   If calibration fails, it will be reported in the audit trail.

To install the reference electrode into the cell:

1. After calibration is completed and to make the installation more convenient, first remove the reference electrode and cell body from the ED module.
2. Rinse the buffer solution off the electrode with DI water, and gently, but firmly, screw-in or rotate the reference electrode clockwise into the reference electrode port of the electrochemical cell until the reference electrode is finger tight.
3. Immediately complete the final plumbing as described below.

Completing the plumbing

For optimum cell performance, use 2–3 ft (60–90 cm) of black PEEK tubing or 1–2 in (5–10 cm) of red PEEK tubing at 0.5 mL/min for optimum cell backpressure of ~12 psi at 0.5 mL/min.

Caution: Excess tubing can cause band-broadening and thus reduce detection response.

The cell waste line should be removed prior to each reference electrode installation and installed after flow has started through the cell. (See discussion below.)

Complete the installation:

1. Install the cell in the ED detector module and connect the reference electrode cable (blue to black) and the counter/working electrode cable (yellow to yellow).
2. Remove the temporary waste line from the column and install the IC PEEK Viper fitting (P/N 088809, 0.007 × 7.0 in) to the column outlet.
3. Allow liquid to flow from the end of the column.
4. Connect the free end to the cell inlet tube.
5. Allow the liquid to flow from cell exit hole and then connect a 2 to 3 ft piece of black PEEK tubing (0.010 in, 0.254 mm i.d.).
6. Wait 60 s before tightening the connection. The tubing provides ~12 psi backpressure (at 0.5 mL/min) on the cell to prevent outgassing and detector noise. (The comparable length of red PEEK (0.005 in, 0.013 mm i.d.) tubing is 1–2 in.)
7. Connect the cell waste tubing to the CR-ATC Regen In line.

Starting the Dionex Integrion HPIC System

To start the system:

1. Turn on the pump.
2. Immediately turn on both the Dionex EGC 500 eluent generator cartridge and the Dionex CR-ATC 600 trap column when liquid is flowing through the device.

   The system backpressure is dependent on the flow rate and type of column but must be above 2000 psi to support the EGC cartridges.
3. Install yellow PEEK backpressure tubing (yellow PEEK, 0.076 mm i.d., 0.003 in i.d.) between the HP EG Degas module and the injection port (Port 1, Pump position).

   This application may require up to 1500 psi delivered by backpressure tubing.
4. Set the eluent concentration, column oven, compartment oven, and cell temperatures as shown in the Conditions section of the application.
5. Turn on the ED cell after the pH > 10.
6. Select Integrated Pulsed Amperometric for cell mode.
7. Select AAA-Direct waveform.
8. Enter the data collection frequency and the “pH” as reference electrode mode into the ED Panel.
9. Allow the system to equilibrate for 30 min.

For optimum chromatography equilibrate until the total background is stable, ~5 nC/min.

Creating an instrument sequence and instrument method

Use the Thermo Scientific™ AppsLab™ Library of Analytical Application database to download the Chromeleon sequence.[14]

To download the sequence:

1. Open AppsLab database.
2. Search for the title of this application note or search by using the key words, tobramycin and Integrion.
3. Select the AppsLab record.
4. Select the Downloads tab.
5. Select the eWorkflow to automatically generate the Chromeleon sequence.

Use Chromeleon Wizard to create a new method and a new sequence.

To create a new instrument method:

1. Select *Create, Instrument Method,* and select *Instrument* (such as Integrion_Tobramycin_1).
2. Enter the values from the Chromatographic Conditions section.
3. Save the instrument method.

To create a new sequence:

1. Select *Create, Sequence,* select the instrument, select ED or ED_Total as the preferred channel.
2. Save the sequence.

Device monitoring

A new feature of the Dionex Integrion HPIC system is the device monitoring and tracking, which automatically detects the electrolytic devices and the columns.

Review and approval of the devices is required to start the first sequence on the Dionex Integrion HPIC system and after installing new consumable devices. To access this approval:

1. Select Consumables and select Inventory (Figure 6). The device monitoring shows the device history, tracking: Part No., size, serial numbers, manufacture lot, installed location (On Device), and Best if Use by Date (Figure 6, top). Additionally, the device monitoring will provide warnings if there is incompatibility in the devices installed (Figure 6, bottom left).
2. To start the sequence, review the list of consumables listed as inventory.
3. Correct any errors and approve.
4. Close the page (Figure 6, bottom right).
5. Select the Instrument Queue tab.
6. Conduct a Ready Check on the sequence.
7. Press Start.

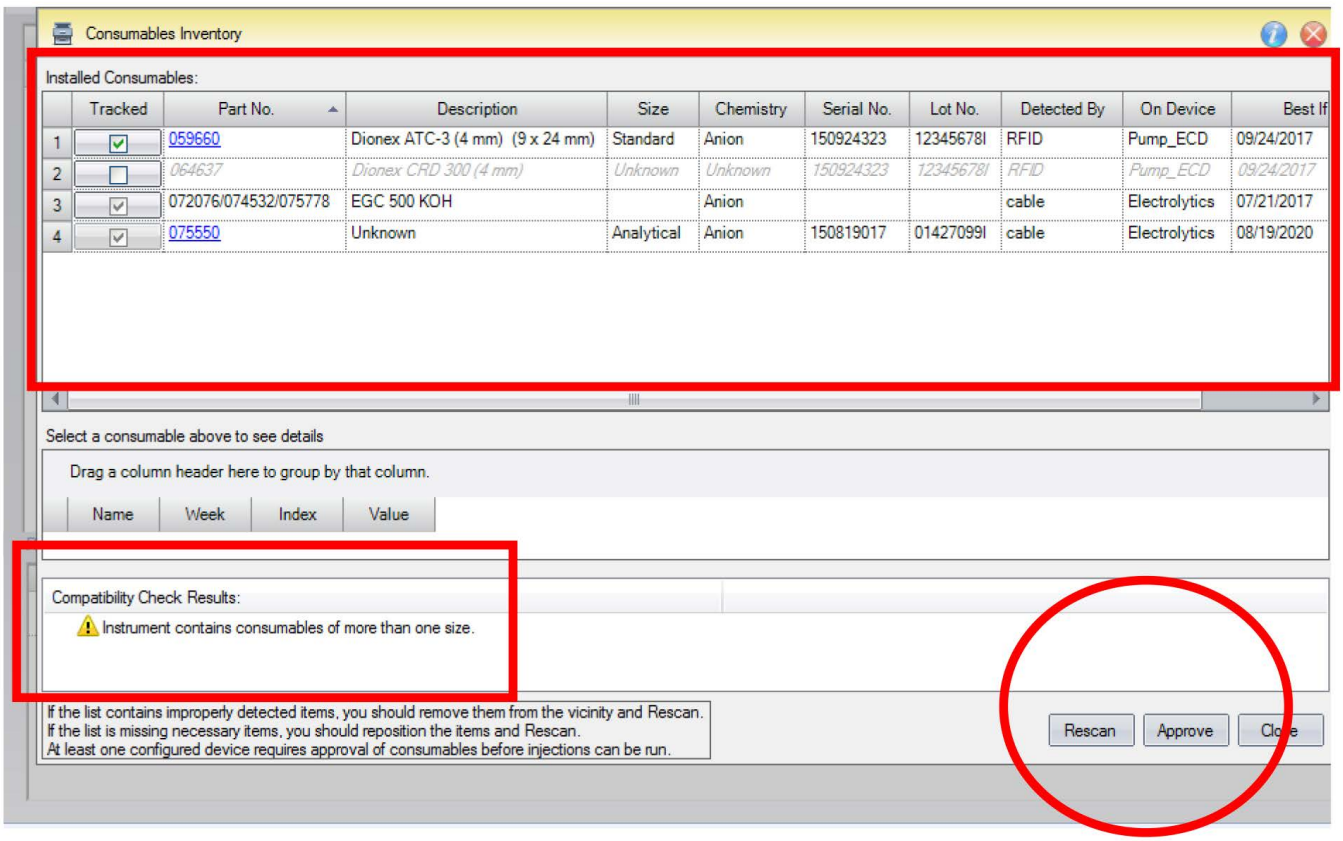

Figure 6. Consumables tracking.

Results and discussion

Tobramycin and other aminoglycoside determinations were previously demonstrated in AN61 using HPAE-PAD or IPAD with an IC system, autosampler, and EG device. Since AN61 was published, there have been many technology advances in IC instruments, consumables, and detectors. This application update demonstrates the same application executed on a Dionex Integrion HPIC system with an ED detector and ED cell, the latest technology in eluent generation consumables, and a Dionex AS-AP Autosampler.

Tobramycin and the aminoglycoside impurities were separated by an electrolytically generated eluent (2 mM KOH). Electrolytic eluent generation provides accurate eluent concentrations and precise retention times. The aminoglycosides were separated at 0.5 mL/min on the Dionex CarboPac PA1 anion-exchange column and detected by IPAD using the AAA-Direct waveform.

Figure 7 shows the chromatogram of tobramycin with kanamycin impurities. The run-time was extended two minutes from the AN61 conditions to 16 min to ensure that the oxygen dip (a dip in the baseline that is result of having less dissolved oxygen in the sample than in the eluent) did not appear near a peak of interest. The oxygen dip (~31-min retention time) is due to oxygen present in the samples and appears as a function of the gas permeation volume of the column. Like some organic impurities, eluting oxygen produces less background than the eluent, so there is a dip in the baseline. The retention times of the oxygen dip and other baseline dips vary from column to column, and depend on the flow rate, not the eluent strength. Eluting the baseline dips just prior to the end of run, or timing their elution to occur at the end of the following injection, prevents the baseline dips from interfering with the peaks of interest. The tobramycin peak exhibits some asymmetry and a small baseline slope after it elutes, but does not interfere with integration. At the low eluent conditions, carbonate can accumulate on the column resulting in a reduction of analyte retention time. A prudent practice is to run a column wash in the morning at 2 mM KOH for 16 min and 65 mM KOH for another 44 min. It may be necessary to add another column wash during the day, but it was not found to be necessary during these experiments.

Column:	Dionex CarboPac PA1 guard, 4 × 50 mm Dionex CarboPac PA1, 4 × 250 mm
Eluent:	2 mM KOH
Eluent Source:	Dionex EGC-500 KOH cartridge, with Dionex CR-ATC 600 trap column, Dionex high pressure degasser
Flow Rate:	0.5 mL/min
Column Temperature:	30 °C
Detector Compart.:	30 °C
Injection Volume:	20 µL
Detection:	IPAD, AAA-Direct Au disposable electrode, 0.002" thick gasket
Reference Electrode:	pH/Ag/AgCl, pH mode
Waveform:	AAA-Direct, versus pH , 1.67 Hz
Peaks:	1. Void volume 2. System peak 3. Kanamycin A 4. Kanamycin B 5. Tobramycin 6. Oxygen dip

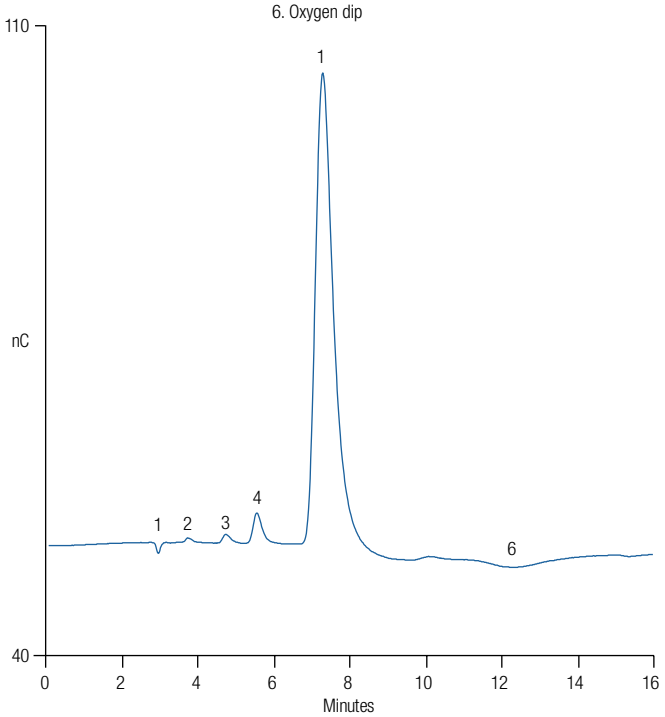

Figure 7. 20 µM tobramycin with trace amounts of kanamycin A and B.

The peak response of the tobramycin was also evaluated using the Four Potential Carbohydrate waveform. The response is significantly lower than with the AAA-Direct waveform, as previously reported in AN61, but if desired, the carbohydrate waveform can be used as demonstrated and discussed in AN61.

Method qualification

The tobramycin method was evaluated on the Dionex Integrion HPIC system using the previously described conditions. Additionally, general system suitability practices were followed as stated in USP® General Chapter <621> Chromatography.[15] The method was evaluated for reproducibility, linearity, accuracy, and sensitivity. The short-term stability was evaluated by determining the reproducibility of triplicate injections of 20 µM tobramycin and two of the impurities, 10 µM kanamycin A and 5 µM kanamycin B. The experiments showed good reproducibility with RSDs of 1.68, 0.46, 0.14%, respectively, for tobramycin, kanamycin A, and kanamycin B.

The peak response to concentration (linearity) was evaluated from 1–100 µM (0.5, 1.0, 10, 50, and 100 µM) for tobramycin and 0.1–10 µM (0.1, 1, 5, and 10 µM) for kanamycin A and kanamycin B. The responses were linear for both kanamycin analytes with coefficients of determination, $r^2 > 0.999$). These results were similar to those reported in AN61. In contrast to previous reports, the best correlation was obtained for tobramycin by applying a quadratic fit (Figures 8A, 8B, 8C).

Sensitivity was estimated by calculating the limit of detection (LOD) using nine replicate injections of a 0.010 µM tobramycin standard (S/N 24), diluted serially from 1 µM tobramycin working standard. The estimated LOD at 3 × S/N was 1.3 nM (0.026 pmol on column, 20 µL injection), which is comparable to previously reported values.

The LODs for kanamycin A and kanamycin B were determined similarly using 10-fold dilution of 0.1 µM working standards. The results were 14 (0.29 pmol on column) and 15 µM (0.30 pmol on column), respectively.

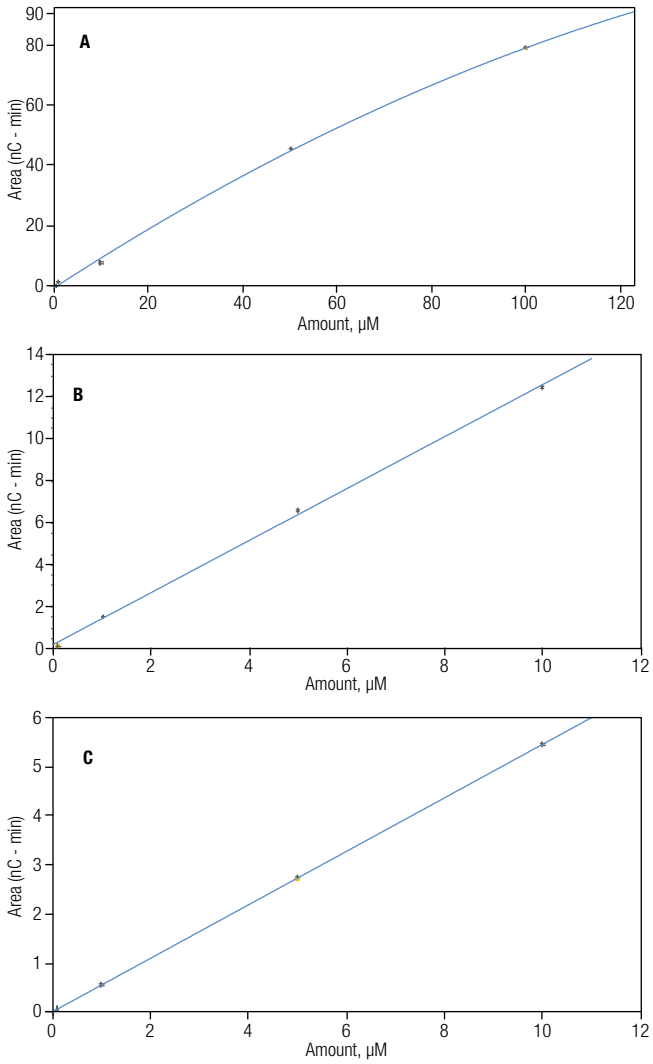

Figure 8. Calibration curves for (A) tobramycin, (B) kanamycin A, and (C) kanamycin B.

The method accuracy was determined by the average percent recovery of six replicate injections of 16.8 ± 0.24 µM tobramycin added to a 10.05 ± 0.10 µM standard. The experiments averaged 98.6% recovery, indicating that the method was accurate.

Conclusion

Determination of kanamycin A, kanamycin B, and tobramycin were demonstrated on a Dionex CarboPac PA1 anion exchange column by HPAE-IPAD. In this update, previously published AN61 is demonstrated on the Dionex Integrion HPIC system. Additionally, detailed instructions were added to support successful installations and operation.

The method was evaluated for linearity, reproducibility, and sensitivity which were found to be comparable to the values previously reported in AN61. All of the aminoglycoside antibiotic compounds evaluated had higher responses using the AAA-Direct waveform (Gold, AAA) than the Four Potential Carbohydrate waveform. However, as reported in AN61, the Four Potential Carbohydrate waveform (Gold, Carbo, Quad) is also suitable when pmol sensitivity is not needed.

More information on this application, including downloadable instrument methods, can be accessed through the Thermo Scientific AppsLab Library of Analytical Applications.[14]

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Application Note 1148

Assay of Nitrite and Determination of Nitrate Impurity in Sodium Nitrite Using a Reagent-Free Ion Chromatography System

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Key Words
Dionex IonPac AS12A Column, Suppressed Conductivity Detection, Pharmaceutical, USP Monograph

Goal

To confirm an ion chromatography (IC) method for the determination of nitrite and nitrate in sodium nitrite using a RFIC system with suppressed conductivity detection.

Introduction

Sodium nitrite is indicated for sequential use with sodium thiosulfate for the treatment of acute cyanide poisoning that is judged to be life-threatening. Sodium nitrate is the possible anionic impurity in sodium nitrite. The U.S. Pharmacopeia (USP) monograph describes a sodium nitrite assay by titration with potassium permanganate. That assay is time-consuming and uses hazardous reagents.

The USP has embarked on a global initiative to modernize many of the existing monographs across all compendia. In response to this initiative, an alternative analytical method to assay nitrite and determine nitrate impurity in sodium nitrite was developed and subsequently published in *Pharmacopeia Forum* (PF).[1] This method uses ion chromatography with a Thermo Scientific™ Dionex™ IonPac™ AS12A anion-exchange column and suppressed conductivity detection to assay the nitrite content of sodium nitrite. The same IC method is used to determine nitrate impurity in sodium nitrite and is also described in the proposed revision to the USP sodium nitrite injection monograph.[2]

Ion chromatography (IC) offers a significant improvement to the existing assay for nitrite because it can simultaneously determine nitrite and nitrate in a single injection. In addition, using a Reagent-Free™ Ion Chromatography (RFIC™) system with electrolytically generated potassium carbonate and bicarbonate eluent significantly simplifies the method and enhances method reproducibility between laboratories.

This application note reports our evaluation of the IC method for nitrite assay and nitrate determination in the proposed revision of the USP monograph for sodium nitrite using an electrolytically generated potassium carbonate/bicarbonate eluent to execute the method rather than the manually prepared sodium carbonate/sodium bicarbonate eluent described in the PF proposal. The required eluent is generated using a Thermo Scientific Dionex EGC 500 K_2CO_3 Eluent Generator and EPM 500 pH modifier. The Thermo Scientific Dionex AERS 500 (4 mm) Anion Electrolytically Regenerated Suppressor produces the regenerate ion necessary for eluent suppressor and allows continuous operation with minimum maintenance. Because the RFIC system requires only deionized (DI) water as the carrier, it significantly simplifies system operation and improves analytical reproducibility. This method was validated following the guidelines outlined in USP General Chapter <1225>, Validation of Compendial Methods.[3]

Thermo SCIENTIFIC

ICS-2100 RFIC

Table of contents

Equipment

- A Thermo Scientific™ Dionex™ ICS-2100 Reagent-Free Ion Chromatography (RFIC) system was used in this work. The Dionex ICS-2100 RFIC is an integrated ion chromatograph that includes the following:
  - Eluent Generator
  - Pump
  - Column Heater
  - Degasser
  - Conductivity Detector
- Thermo Scientific™ Dionex™ AS-AP Autosampler with 250 µL syringe (P/N 074306), 1.2 mL buffer line assembly (P/N 074989), and 25 µL injection loop
- Dionex EGC 500 K_2CO_3 Cartridge (P/N 088453)
- Dionex EPM 500 Electrolytic pH Modifier (P/N 088471)
- Dionex EGC Carbonate Mixer Kit, 4 mm (P/N 042126)
- Thermo Scientific Dionex AERS 500 Anion Electrolytically Regenerated Suppressor, 4 mm (P/N 082541)
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) software version 7.2

The column temperature of an ICS-2100 system can only be set at a minimum of 30 °C. Therefore, a Thermo Scientific™ Dionex™ ICS-5000 Reagent-Free Ion Chromatography (RFIC™) system was used in the method robustness test for column temperature (i.e. to set temperatures lower than 30 °C).

Reagents and Standards

- Deionized (DI) water, Type I reagent grade, 18 MΩ-cm resistance or better
- Sodium nitrite USP reference standard (Sigma-Aldrich® Cat # 1614454-1G)
- Sodium nitrite, extra pure (Sigma-Aldrich Cat # 13447-1KG-R)
- Sodium nitrate USP reference standard, 99.995% METALS BASIS (Sigma-Aldrich Cat # 229938-10G)
- Sodium and potassium salts, A.C.S. reagent grade for preparing the Quality Assurance Report (QAR) standard mix

Conditions	
Columns:	Dionex IonPac AS12A, 4 mm Analytical, 4 x 250 mm (P/N 046034) Dionex IonPac AG12A, 4 mm Guard, 4 x 50 mm (P/N 079801)
Eluent:	2.7 mM K_2CO_3 /0.3 mM $KHCO_3$
Eluent Source:	Dionex EGC 500 K_2CO_3 cartridge with EPM 500 electrolytic pH modifier
Flow Rate:	1.5 mL/min
Injection Volume:	25 µL in Push-Full mode
Temperature:	Ambient (~24 °C)
Detection:	Suppressed conductivity, Dionex AERS 500 (4 mm) Suppressor, recycle mode, 22 mA current
System Backpressure:	~2000–2100 psi
Background Conductance:	~12.5 µs
Noise:	<10 nS/min
Run Time:	18 min

Preparations of Solutions and Reagents

Notes: Do not use glassware to prepare the solutions. Polymeric containers made of high-density polyethylene (HDPE) are recommended.

Sodium Nitrite Stock Standard Solution, 1200 mg/L
Accurately weigh 12.0 mg of USP sodium nitrite into a 20 mL polypropylene bottle and dissolve in 10 g of DI water.

Sodium Nitrite Working Standard Solution, 120 mg/L
Transfer 1.0 mL of sodium nitrite stock standard solution (1200 mg/L) into a 20 mL polypropylene bottle and mix with 9.0 g of DI water.

Sodium Nitrite Calibration Standards, 30, 60, 90, 120, 150, 180 mg/L (Nitrite 20–120 mg/L)
To prepare calibration standard solutions, dilute the stock standard solution (1200 mg/L) to the appropriate concentrations with DI water (Table 1).

Table 1. Preparation of sodium nitrite calibration standard solutions.

Sodium Nitrite Concentration (mg/L)	30	60	90	120	150	180
Sodium Nitrite Stock Standard (1200 mg/L) (mL)	0.25	0.5	0.75	1	1.25	1.5
DI H_2O (g)	9.75	9.5	9.25	9	8.75	8.5

Sodium Nitrate Stock Standard Solution, 1200 mg/L

Accurately weigh 12.0 mg of USP sodium nitrate into a 20 mL polypropylene bottle and dissolve in 10 g of DI water.

Sodium Nitrate Working Standard Solution, 10 mg/L

Dilute the stock standard solution (1200 mg/L) to the appropriate concentration with DI water by pipetting 0.5 mL of Sodium Nitrate Stock Standard Solution to 59.5 g of DI H_2O.

Sodium Nitrate Calibration Standard, 0.5, 1, 1.5, 2, 5, 10 mg/L, (Nitrate 0.365–7.30 mg/L)

To prepare sodium nitrate calibration standard solutions, dilute the working standard solution to the appropriate concentrations with DI water (Table 2).

Table 2. Preparation of sodium nitrate calibration standard solutions.

Sodium Nitrate Concentration (mg/L)	0.5	1	1.5	2	5	10
Working Sodium Nitrate Standard (10 mg/L) (mL)	0.5	1	1.5	2	5	10
DI H_2O (g)	9.5	9	8.5	8	5	0

Robustness Test Standard, (Nitrite 10 mg/L, Nitrate 20 mg/L)

Sodium nitrite stock standard solution (1200 mg/L) contains 800.2 mg/L of nitrite. Sodium nitrate stock standard solution (1200 mg/L) contains 875.3 mg/L of nitrate. Dilute and mix the stock standard solutions to the appropriate concentration with DI water (Tables 3 and 4).

Table 3. Stock standard solutions.

Nitrite and Nitrate Stock Mixture	Nitrite (80 mg/L) + Nitrate (160 mg/L)
Nitrite Stock (800.2 mg/L) (mL)	2.00
Nitrate Stock (875.3 mg/L) (mL)	3.66
DI H_2O (g)	14.34

Table 4. Working mixtures.

Nitrite and Nitrate Working Mixture	Nitrite (10 mg/L) + Nitrate (20 mg/L)
Nitrite and Nitrate Stock Mixture (mL)	12.5
DI H_2O (g)	87.5

Sample Preparation

Sodium Nitrite Stock Sample Solution, 1200 mg/L, Prepared Using Sodium Nitrite, Extra Pure

Accurately weigh 12.0 mg of sodium nitrite into a 20 mL polypropylene bottle and dissolve in 10 g of DI water.

Sodium Nitrite Working Sample Solution, 120 mg/L

Transfer 1.0 mL of sodium nitrite stock sample solution (1200 mg/L) into a 20 mL polypropylene bottle and mix with 9.0 g of DI water.

Sodium Nitrite Recovery Test Sample Solution

To prepare 120 mg/L sodium nitrite sample solution spiked with 30, 60, 90, 120, 150, and 180 mg/L of USP grade sodium nitrite, dilute and mix sodium nitrite sample stock (1200 mg/L) with sodium nitrite USP standard stock (1200 mg/L) to the appropriate concentration with DI water (Table 5).

Table 5. Preparation of sodium nitrite sample solution.

Sodium Nitrite Spiked (mg/L)	30	60	90	120	150	180
Sodium Nitrite Stock Sample (1200 mg/L) (mL)	1	1	1	1	1	1
Sodium Nitrite Stock Standard (1200 mg/L) (mL)	0.25	0.5	0.75	1	1.25	1.5
DI H_2O (g)	8.75	8.5	8.25	8	7.75	7.5

Sodium Nitrate Recovery Test Sample Solution

To prepare 120 mg/L sodium nitrite sample solution spiked with 0.25, 0.5, 0.75, 1, 2.5, and 5 mg/L of sodium nitrate, dilute and mix sodium nitrite stock sample (1200 mg/L) with USP sodium nitrate working standard (10 mg/L) to the appropriate concentration with DI water (Table 6).

Table 6. Preparation of sodium nitrate sample solution.

Sodium Nitrate Spiked (mg/L)	0.25	0.5	0.75	1	2.5	5
Sodium Nitrite Stock Sample (1200 mg/L) (mL)	1	1	1	1	1	1
Sodium Nitrate Working Standard (10 mg/L) (mL)	0.25	0.5	0.75	1	2.5	5
DI H_2O (g)	8.75	8.5	8.25	8	6.5	4

Robustness Study

Following the guidelines of USP Physical Test, <621> Chromatography, evaluate the robustness of this method by examining the retention time (RT), peak asymmetry, and resolution of the two analytes in the robustness test standard after imposing small variations (±10%) in procedural parameters (e.g., flow rate, eluent gradient concentration, and column temperature). Apply the same procedure to two column sets from two different lots. Test the following variations:

- Flow rate at 1.5 mL/min, 1.35 mL/min, 1.65 mL/min
- Column temperature at 24 °C, 21 °C, 27 °C, and 30 °C (Using the ICS-5000 system)
- Eluent concentrations at 2.7 mM K_2CO_3/0.3 mM $KHCO_3$, 2.43 mM K_2CO_3/0.27 mM $KHCO_3$, and 2.97 mM K_2CO_3/0.33 mM $KHCO_3$

Results and Discussion

Separation

The separation of nitrite and nitrate was achieved using a Dionex IonPac AS12A column set with the specified isocratic conditions. Figure 1 shows the separation of ten anions using the proposed method. The other anions do not interfere with the determination of nitrite and nitrate.

Figure 2A shows the chromatogram of a sodium nitrite sample (120 mg/L sodium nitrite) with an enlarged view (Figure 2B) showing the separation of nitrate. The relative retention times are 1 for nitrite and 1.9 for nitrate, similar to the 1 and 1.85 reported in the proposed monograph revision. Peak resolution between nitrite and nitrate is >11, exceeding the USP requirement of 3. The asymmetry value for both nitrite and nitrate is <1.2 (USP specification is not more than (NMT) 2).

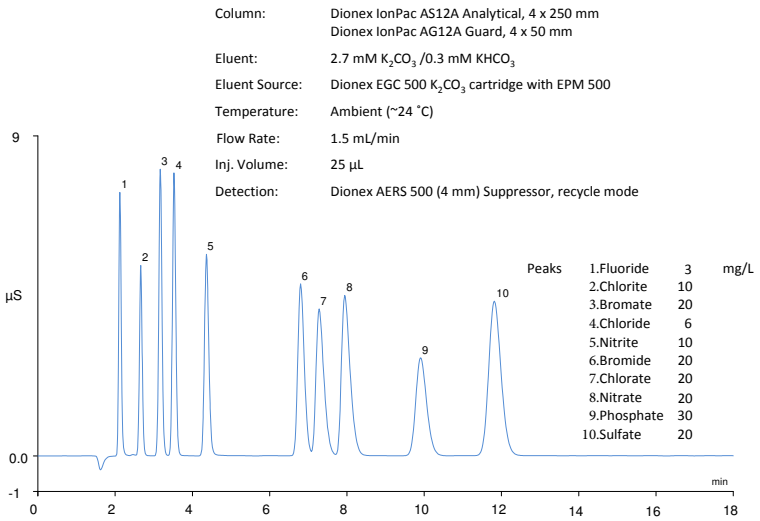

Figure 1. Separation of ten anions.

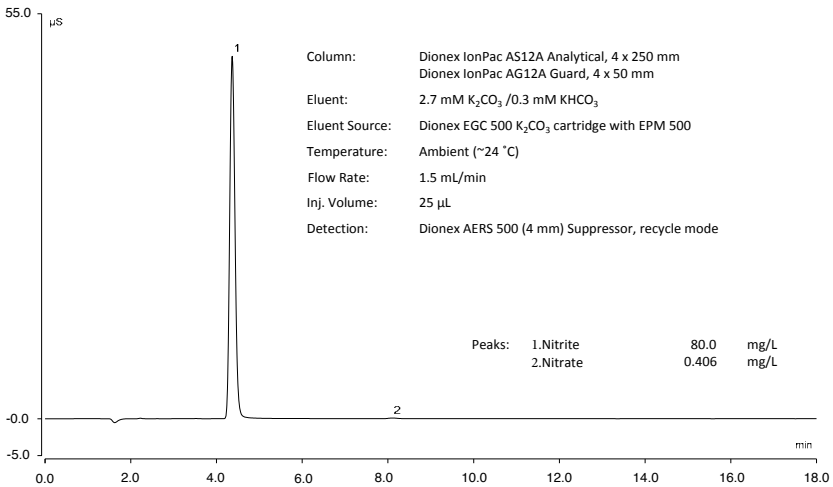

Figure 2A. Sodium nitrite sample (80 mg/L nitrite).

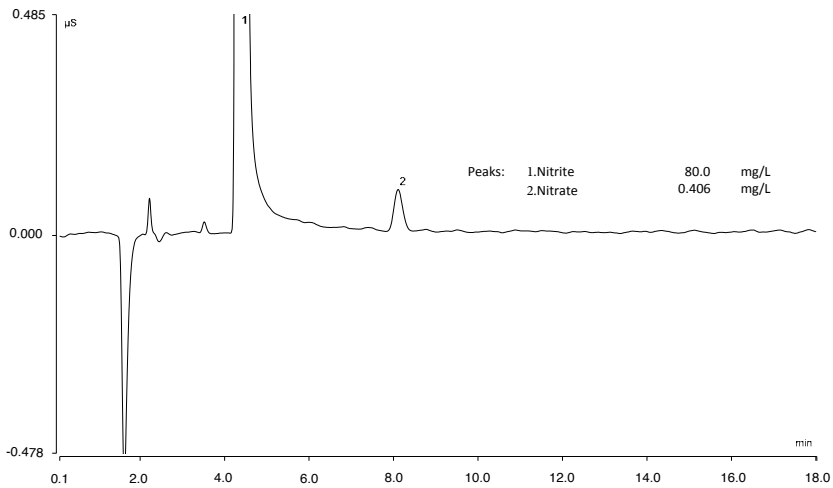

Figure 2B. Enlarged view of Chromatogram A showing the nitrate peak.

Calibration, Limit of Detection (LOD), and Limit of Quantitation (LOQ)

The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and the USP General Chapter <1225> guidelines recommend a minimum of five concentrations to establish linearity in an assay.[3-5] For a drug substance or finished product, the minimum specified range is from 80% to 120% of the test concentration. A minimum range from 50% to 120% is required for determination of an impurity. In this study, nitrite was calibrated at six concentration levels ranging from 20 to 120 mg/L. The results yield a linear relationship of peak area to concentration with a coefficient of determination (r^2) of 0.9999. Nitrate was calibrated at six concentration levels ranging from 0.365 to 7.20 mg/L with an r^2 of 0.9999 (Table 7).

Table 7. Calibration, LODs, and LOQs of nitrite and nitrate.

Anion	Calibration Range (mg/L)	r^2	LOD (µg/L)	LOQ (µg/L)
Nitrite	20–120	0.9999	43.6	145
Nitrate	0.365–7.30	0.9999	97.3	324

To determine the LODs and LOQs for nitrite and nitrate, the baseline noise was first determined by measuring the peak-to-peak noise in a representative 1-min segment of the baseline where no peaks elute but is close to the peaks of interest. The signal was determined from the average peak height of three injections of a sample of 200 µg/L nitrite and 365 µg/L nitrate. The LODs and LOQs were then determined by multiplying the signal-to-noise ratio 3x and 10x, respectively (Table 7). The LODs of nitrite and nitrate were 43.6 and 97.3 µg/L, respectively. The LOQs of those two analytes were 145 and 324 µg/L, respectively. In the sodium nitrite sample (120 mg/L), when nitrate is at the LOD level (97.3 µg/L), the sodium nitrate percentage is 0.11; when nitrate is at the LOQ level (324 µg /L), the sodium nitrate percentage is 0.37. Both are less than the USP acceptance criterion for nitrite (0.4%).

Sample Analysis

The USP monograph requires that sodium nitrite contain 98.0%–102.0% on the dried basis. In this study, the USP sodium nitrite reference standard (Sigma-Aldrich Cat # 1614454-1G) was used to prepare the standard solution of 120 mg/L sodium nitrite. Sodium nitrite (Sigma-Aldrich Cat # 13447-1KG-R) was used to prepare the sample solution of 120 mg/L.

Two quantification methods were compared and evaluated to calculate the percentage of sodium nitrite in the sample solution (Table 8).

Sodium Nitrite Percentage Method A: USP Sodium Nitrite Single Standard Point (120 mg/L) (Proposed monograph revision method)

Calculate the percentage of sodium nitrite ($NaNO_2$) in the portion of sodium nitrite taken:

$$\text{Result} = \left(\frac{ru}{rs}\right) \times \left(\frac{Cs}{Cu}\right) \times 100$$

ru = Peak area from the sample solution

rs = Peak area from the standard solution

Cs = Concentration of USP Sodium Nitrite RS in the standard solution (mg/L)

Cu = Concentration of sodium nitrite in the sample solution (mg/L)

Sodium Nitrite Percentage Method B: USP Sodium Nitrite Calibration Standard Curve

- Build a calibration curve with 30–180 mg/L of USP Sodium Nitrite Reference Standard.
- Prepare sodium nitrite sample solution of 120 mg/L.
- Calculate the true concentration of sample solution using the calibration curve.
- Calculate the percentage of sodium nitrite.

$$\text{Result} = \frac{\textit{Sodium Nitrite Concentration calculated from calibration curve (mg/L)}}{120\ \textit{(mg/L)}} \times 100$$

Table 8. Sodium nitrite percentage in sample using two quantification methods.

	Method A (%)	Method B (%)
Ave	99.1	99.5
RSD (n=3)	0.14	0.14

The USP monograph requires that sodium nitrite contain no more than 0.4% of sodium nitrate. Three quantification methods were compared and evaluated to calculate the percentage of sodium nitrate in the portion of sodium nitrite taken (Table 9).

Sodium Nitrate Percentage Method A: Nitrate Response Factor (Proposed monograph revision method)

Calculate the percentage of sodium nitrate ($NaNO_3$) in the portion of the sample taken:

$$\text{Result} = \left(\frac{ru}{rs}\right) \times \left(\frac{1}{F}\right) \times 100$$

ru = Peak response of nitrate from the sample solution

rs = Peak response of nitrite from the sample solution

F = Response factor for nitrate, 0.7

Sodium Nitrate Percentage Method B: USP Sodium Nitrate Single Standard Point (1 mg/L)

Calculate the percentage of sodium nitrate ($NaNO_3$) in the portion of sodium nitrite taken:

Calculate sodium nitrate concentration using single sodium nitrate standard as follows:

$$cu = cs \times \left(\frac{ru}{rs}\right)$$

ru = peak area from the sample solution

rs = peak area from the standard solution

Cs = concentration of USP Sodium Nitrate RS in the standard solution (mg/L)

Cu = concentration of sodium nitrate in the sample solution (mg/L)

$$\text{Result} = \frac{\textit{Sodium Nitrate Concentration calculated from single standard (mg/L)}}{\textit{Sodium Nitrite Concentration (mg/L)}} \times 100$$

Sodium Nitrate Percentage Method C: USP Sodium Nitrate Calibration Standard Curve

- Build a calibration curve with 0.5–3 mg/L of USP Sodium Nitrate Standard.
- Prepare sodium nitrite sample solution of 120 mg/L.
- Calculate true sodium nitrate concentration using calibration curve.
- Calculate % of sodium nitrate in the portion of sodium nitrite taken.

$$\text{Result} = \frac{\textit{Sodium Nitrate Concentration calculated from calibration curve (mg/L)}}{\textit{Sodium Nitrite Concentration (mg/L)}} \times 100$$

As shown in Table 8, the $NaNO_2$ percentage calculated from the method A (monograph revision method) gives a similar result to the standard curve calibration method. As shown in Table 9, the $NaNO_3$ percentage calculated from the method A (monograph revision method) also gives similar result as the calibration method.

Table 9. Sodium nitrate percentage in sample using three quantification methods.

Sample		Method A	Method B	Method C
120 mg/L $NaNO_2$	AVE	0.499	0.503	0.528
	RSD (n=3)	0.589	0.589	0.542
120 mg/L $NaNO_2$ spiked with 0.25 mg/L $NaNO_3$	AVE	0.694	0.699	0.721
	RSD (n=3)	0.442	0.442	0.459
120 mg/L $NaNO_2$ spiked with 0.5 mg/L $NaNO_3$	AVE	0.891	0.898	0.915
	RSD (n=3)	0.500	0.500	0.512
120 mg/L $NaNO_2$ spiked with 0.75 mg/L $NaNO_3$	AVE	1.08	1.09	1.10
	RSD (n=3)	0.707	0.707	0.624
120 mg/L $NaNO_2$ spiked with 1 mg/L $NaNO_3$	AVE	1.28	1.29	1.30
	RSD (n=3)	0.833	0.833	0.817
120 mg/L $NaNO_2$ spiked with 2.5 mg/L $NaNO_3$	AVE	2.53	2.55	2.53
	RSD (n=3)	0.492	0.492	0.506
120 mg/L $NaNO_2$ spiked with 5 mg/L $NaNO_3$	AVE	4.62	4.66	4.59
	RSD (n=3)	0.465	0.465	0.478

Sample Accuracy and Precision

Method accuracy was validated by spiked recovery of sodium nitrite and sodium nitrate in sodium nitrite sample over six concentration levels, with three replicates of each concentration. Tables 10 and 11 summarize recovery results for sodium nitrite and sodium nitrate. Sodium nitrite recovery ranges from 94 to 103% for the two quantification methods and sodium nitrate recovery ranges from 93% to 101% for all three quantification methods.

Table 10. Recovery data for sodium nitrite spiked in sodium nitrite sample containing 119 mg/L sodium nitrite.

Sodium Nitrite Added (mg/L)	Peak Area RSD	Method A		Method B	
		Total Found (mg/L)	Recovery %	Total Found (mg/L)	Recovery %
0	0.2	119	-	120	-
30	0.05	150	103	151	102
60	0.14	181	103	182	103
90	0.19	211	101	211	101
120	0.2	240	100	240	100
150	0.37	268	99.2	269	98.8
180	0.24	296	98.1	296	97.7

Table 11. Recovery data for sodium nitrate spiked in sodium nitrite sample containing 120 mg/L sodium nitrite.

Sodium Nitrate Added (mg/L)	Peak Area RSD	Method A		Method B		Method C	
		Total Found (mg/L)	Recovery %	Total Found (mg/L)	Recovery %	Total Found (mg/L)	Recovery %
0	0.19	0.59	-	0.599	-	0.63	-
0.25	0.5	0.83	94.4	0.837	95.1	0.86	93.5
0.5	0.31	1.07	95.5	1.08	96.2	1.10	94.4
0.75	0.49	1.30	94.6	1.31	95.3	1.33	93.4
1	0.79	1.54	95.1	1.55	95.3	1.56	93.5
2.5	0.24	3.06	98.5	3.08	99.3	3.06	97.2
5	0.21	5.60	100	5.64	101	5.57	98.8

Assay precision was evaluated by injecting seven replicates of the test sample 120 mg/L sodium nitrite (119 mg/L sodium nitrite + 0.568 mg/L sodium nitrate) spiked with 1 mg/L sodium nitrate and expressed as the RSDs of RT and peak area (Table 12). The assay exhibited good short-term precision.

Table 12. Retention time and peak area precisions of sodium nitrite 119 mg/L spiked with USP 0.5 mg/L sodium nitrate.

Compound	Conc (mg/L)	RT RSD (n=7)	Peak Area RSD (n=7)
Sodium Nitrite	119	0.050	0.070
Sodium Nitrate	1.56	0.060	1.66

Robustness

Assay robustness was evaluated by measuring the influence of small variations (±10%) in procedural parameters (e.g., flow rate, eluent concentration, column temperature on the RT, peak asymmetry, and resolution of the two analytes on two column sets from two different lots). The peak asymmetry was calculated using the USP formula. The resolution was determined relative to the previous peak in a chromatogram using the USP formula. A standard mix (10 mg/L nitrite, 20 mg/L nitrate) was injected three times at each chromatographic condition. The resolution of nitrate to nitrite ranged from 11.6 to 12.0 on column 1 and from 11.2 to 12.0 on column 2. Tables 13 and 14 summarize the results for nitrite and nitrate, respectively. These results indicate the method is robust for both analytes.

Table 13. Robustness of the IC-based assay for nitrite (injected sample: 10 mg/L nitrite and 20 mg/L nitrate; average of three injections).

Parameter		Column 1						Column 2					
		Nitrite RT (min)	Diff (%)	Asym.	Diff (%)	Resol.	Diff (%)	Nitrite RT (min)	Diff (%)	Asym.	Diff (%)	Resol.	Diff (%)
Eluent Conc (mM) $K_2CO_3/KHCO_3$	2.7/0.3	4.77	-	1.09	-	11.8	-	4.77	-	1.09	-	11.8	-
	2.43/0.27	4.94	3.72	1.07	-1.53	11.9	1.19	4.94	3.72	1.07	-1.53	11.9	1.19
	2.97/0.33	4.61	-3.22	1.10	0.92	11.7	-1.19	4.61	-3.22	1.10	0.92	11.7	-1.19
Flow Rate (mL/min)	1.5	4.77	-	1.09	-	11.8	-	4.77	-	1.09	-	11.8	-
	1.35	5.30	11.1	1.09	0.00	12.0	1.72	5.30	11.1	1.09	0.00	12.0	1.69
	1.65	4.31	-9.54	1.09	0.00	11.6	-2.06	4.31	-9.54	1.09	0.00	11.6	-2.09
Column Temp (˚C)	24	4.37	-	1.15	-	11.9	-	4.79	-	1.09	-	11.6	-
	21	4.44	1.58	1.16	0.87	12.0	0.93	4.86	1.52	1.10	0.92	11.7	0.60
	27	4.31	-2.93	1.17	0.86	11.8	-1.17	4.71	-3.00	1.10	0.00	11.5	-1.63
	30	4.25	-2.72	1.16	0.87	11.7	-1.43	4.65	-2.86	1.09	0.00	11.2	-3.45

Table 14. Robustness of the IC-based assay for nitrate (injected sample: 10 mg/L nitrite and 20 mg/L nitrate; average of three injections).

Parameter		Column 1				Column 2			
		Nitrate RT (min)	Diff (%)	Asym.	Diff (%)	Nitrate RT (min)	Diff (%)	Asym.	Diff (%)
Eluent Conc (mM) $K_2CO_3/KHCO_3$	2.7 /0.3	8.73	-	1.40	-	8.73	-	1.40	-
	2.43 /0.27	9.10	4.33	1.39	-0.24	9.10	4.33	1.39	-0.24
	2.97/0.33	8.41	-3.67	1.40	0.48	8.41	-3.67	1.40	0.48
Flow Rate (mL/min)	1.5	8.73	-	1.40	-	8.73	-	1.40	-
	1.35	9.71	11.3	1.41	0.95	9.71	11.3	1.41	0.95
	1.65	7.85	-10.0	1.39	-0.72	7.85	-10.0	1.39	-0.72
Column Temp (˚C)	24	7.90	-	1.53	-	8.73	-	1.38	-
	21	8.16	3.30	1.57	2.61	9.018	3.26	1.40	1.45
	27	7.67	-6.04	1.5	-4.46	8.47	-6.10	1.36	-2.86
	30	7.45	-5.76	1.48	-3.27	8.22	-5.87	1.33	-3.62

Conclusion

This study describes and evaluates an IC-based assay for simultaneous determination of nitrite and nitrate in sodium nitrite that was proposed to modernize two USP monographs.[1-2] The two analytes were separated on an anion-exchange column and detected by suppressed conductivity within 18 min. This assay for nitrite and nitrate was validated to meet the analytical performance characteristics outlined in USP General Chapter <1225>. Compared to the time-consuming assays in the USP sodium nitrite monograph, this IC-based assay executed with an RFIC system offers a simple, accurate, and robust measurement of the two analytes without handling hazardous regents.

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MS and CAD detection

Often analytes of interest are present in very low levels, or require additional information for identification, and not all contain suitable chromophores for UV detection. Alternative detectors can be used to improve analytical success.

The charged aerosol detector (CAD) allows quantification of analytes in the absence of a suitable, molecule-specific calibration standard, due to its near-linear detection response. With this detector, relative concentrations are proportional to analyte concentrations.

Mass spectrometry (MS) enables very low levels of detection, and quantification, with the added benefit of molecular characterization and compound identification.

Chapter highlights

Use the **Thermo Scientific™ Vanquish™ Duo UHPLC system** with the Inverse Gradient workflow to improve universal quantification capabilities with the **Thermo Scientific™ Vanquish™ Charged Aerosol Detector**

Achieve ultimate confidence in data quality with robust, sensitive, reproducible, and reliable targeted quantitation methods with a **Thermo Scientific™ TSQ™ LC-MS** workflow solution

Experience hassle-free, seamless integration with your IC or LC system for valuable MS data with the **Thermo Scientific™ ISQ™ EC Single Quadrupole Mass spectrometer**

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Table of contents

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APPLICATION NOTE 64977

Sensitive, robust quantitative analysis of a mixture of drug candidates in plasma using a TSQ Altis triple quadrupole mass spectrometer

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Keywords
Antidepressants, LC-MS/MS, TSQ Altis MS, pharmaceutical, small molecules, bioanalysis, bioequivalence

Goal

To develop a sensitive, robust, reproducible LC-MS/MS assay for determination and quantitation of a mixture of compounds of pharmaceutical interest in human plasma and plasma from preclinical animal species.

Introduction

Small molecules represent a significant proportion of drug discovery and development in the search for new chemical entities, in addition to the extensive work involved in the regulatory filings of generics. Targeted quantitation assays are a critical part of an optimal workflow, which is required to successfully develop a small molecule drug. These targeted quantitation analyses must be done in biological matrices, which can often create analytical challenges. In this study, we report the development of a sensitive, robust, reliable, and reproducible LC-MS/MS assay for multiple drug standards in rat plasma.

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Vanquish Horizon UHPLC

Table of contents

Experimental

Sample preparation

Crashed plasma stock solutions were prepared using an acetonitrile (ACN) crash at a ratio of 3:1, ACN to plasma. The resulting solution was centrifuged at 10,000 rpm for 10 minutes. The supernatant was removed and added to an equivalent volume of water to make the final crashed plasma stock solution. Stock solutions of each standard compound at 1 mg/mL were diluted in a pooled mix in the crashed plasma stock to concentration ranges of 1 pg/mL to 25,000 pg/mL and 10 pg/mL to 100,000 pg/mL. Isotopically labeled internal standards were added to each calibration level to produce a final internal standard concentration of 0.5 ng/mL. All reagents were obtained from Cerilliant Corporation, Round Rock, Texas, at 1 mg/mL in methanol.

Liquid chromatography

Chromatographic separation was performed using a Thermo Scientific™ Vanquish™ Horizon HPLC system. The column used was a Thermo Scientific™ Hypersil GOLD™ aQ C18 Polar Endcapped LC column (100 × 2.1 mm, 1.9 µm particle size). Mobile phases A and B consisted of 10 mM ammonium formate in Fisher Chemical™ Optima™ grade water and 0.1% formic acid in Fisher Chemical™ Optima™ grade acetonitrile, respectively. The column temperature was 50 °C. The total run time was 3.5 minutes (Table 1).

Table 1. Chromatography gradient for analysis.

Time (min)	Flow Rate (mL/min)	%A	%B
0	0.6	95	5
0.4	0.6	95	5
0.5	0.6	65	35
1.5	0.6	64	36
1.6	0.6	55	45
2.2	0.6	53	47
2.3	0.6	5	95
2.95	0.6	5	95
2.995	0.6	95	5
3.5	0.6	95	5

Mass spectrometry

Mass spectrometry analysis was carried out on a Thermo Scientific™ TSQ Altis™ triple quadrupole mass spectrometer equipped with the Thermo Scientific™ OptaMax™ NG source housing. Tables 2 and 3 show mass spectrometer source and SRM parameters used in the experimental setup.

Table 2. Mass spectrometer set-up.

Parameter	Setting
Run Time	3.5 min
Ion Source	HESI
Spray Voltage	3500 V
Sheath Gas	40 Arb
Auxiliary Gas	15 Arb
Sweep Gas	0 Arb
Ion Transfer Tube Temperature	350 °C
Vaporizer Temperature	325 °C
Experiment Type	SRM
Cycle Time	0.3 s
Chromatography Peak Width	6 s
Collision Gas Pressure	1.5 mTorr
Q1 Resolution	0.7 FWHM
Q3 Resolution	0.7 FWHM

Data analysis

Data was acquired and processed using Thermo Scientific™ TraceFinder™ software.

Table 3. SRM properties for experimental set-up.

Compound Name	Start Time (min)	End Time (min)	Polarity	Precursor *m/z*	Product *m/z*	Collision Energy (V)	RF Lens
Desomorphine	0.760	1.060	Positive	272.062	215.054	26	69
Desmethyldoxepin	1.230	1.530	Positive	266.062	107.000	23	56
Flecainide	1.310	1.610	Positive	415.050	398.054	24	84
Midazolam	1.410	1.710	Positive	326.012	291.054	28	87
Imipramine	1.660	1.960	Positive	281.462	86.054	17	48
Amitriptyline	1.800	2.100	Positive	278.075	233.111	18	53
Fluoxetine	1.890	2.190	Positive	310.362	43.889	11	39
Diazepam	2.230	2.530	Positive	285.012	193.071	33	78

Results and discussion

Table 4 shows the lower limits of quantitation (LLOQ) obtained with the TSQ Altis MS for each of the drug candidates, which were significantly lower than those obtained from previous generation MS systems. In addition, significantly lower %CV values for the IS also implies increased robustness and reproducibility for the TSQ Altis MS. The representative chromatogram of QC 2 at 300 pg/mL is show in Figure 1. Further details on linearity and reproducibility of the QCs are shown in Table 5.

Table 4. Limits of quantitation for the drug candidates in plasma and %CV (n=3) for the internal standards.

Compound	LOQ (pg/mL)	IS %CV
Desomorphine	5	3.5
Desmethyldoxepin	2.5	3.5
Flecainide	1	3.5
Midazolam	2.5	4.4
Imipramine	2.5	4.4
Amitriptyline	2.5	4.4
Fluoxetine	5	5.1
Diazepam	2.5	3.4

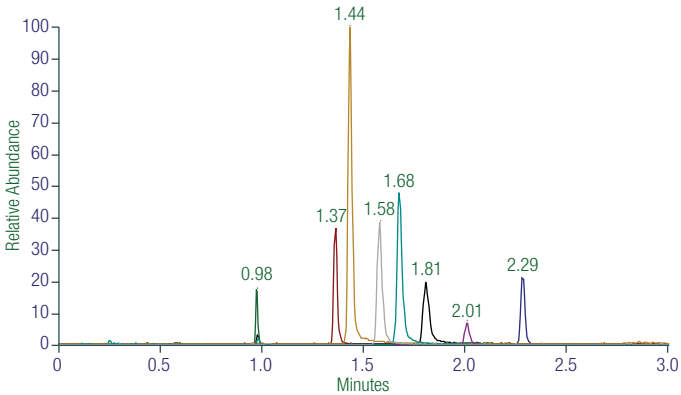

Figure 1. Representative chromatogram of QC 2 – 300 pg/mL.

Table 5. Linearity and reproducibility data for four QC points per calibration curve.

Compound	QC 1 %CV 30 pg/mL	QC 2 %CV 300 pg/mL	QC 3 %CV 3000 pg/mL	QC 4 %CV 15,000 pg/mL	R^2 Linear Fit
Desomorphine	7.93	4.72	3.47	1.15	0.9945
Desmethyldoxepin	5.28	1.55	0.67	1.01	0.9904
Flecainide	4.20	4.88	2.46	2.97	0.9924
Midazolam	2.96	1.52	1.71	2.77	0.9917
Imipramine	2.50	1.26	0.38	1.24	0.9913
Amitriptyline	7.04	3.16	0.68	0.83	0.9908
Fluoxetine	3.15	2.80	2.03	2.87	0.9901
Diazepam	5.77	3.15	0.53	2.69	0.9927

Conclusion

The method referenced in this application note shows excellent linearity and reproducibility over the dynamic range of the assay. This method demonstrates that the TSQ Altis MS provides the sensitivity and reproducibility required in the analysis of pharmaceutical compounds.

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APPLICATION NOTE 72594

Quantification of paclitaxel, its degradants, and related substances using UHPLC with charged aerosol detection

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Keywords

Vanquish Flex Duo UHPLC, charged aerosol detection, global calibration, impurity analysis, paclitaxel, taxane, forced degradation, stability analysis, inverse gradient

Application benefits

- Uniform response in gradient elution using charged aerosol detection with the Thermo Scientific™ Vanquish™ Flex Duo Inverse Gradient Workflow
- Quantification of multiple API- related species by a single calibrant

Goal

First, to demonstrate the ability to quantify multiple impurities with a single calibrant by using the inherent uniform response of charged aerosol detection (CAD). Second, to highlight the capabilities of the Thermo Scientific™ Vanquish™ Flex Duo UHPLC system to provide inverse gradient compensation, which is essential to achieve reliable single calibrant quantification with CAD.

Introduction

According to the World Health Organization, cancer is the second-leading cause of death globally, and was responsible for 8.8 million deaths in 2015. Worldwide, nearly 1 in 6 deaths is due to cancer.[1] There is considerable interest in the development of innovative drugs to support therapies (e.g., chemotherapy) or to treat patients individually with cutting-edge medication.

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Vanquish Flex Duo UHPLC

Table of contents

One example is paclitaxel (known under its tradename Taxol®), which belongs to the taxane family and can be either directly extracted from the bark of the Pacific yew (*Taxus brevifolia*) or be obtained by partial chemical synthesis from a precursor.

For any drug substance, unwanted impurities are a key concern during drug development and throughout a drug product's life cycle. Monitoring these impurities, which often are present in low abundance compared to the active pharmaceutical ingredient (API), is mandatory to ensure a safe and effective product. Even though some impurities may be closely related to the API, calibration standards are not likely to be available, particularly during early stages of development. Usually, (U)HPLC with UV/Vis detection is the preferred choice for analysis, but substances that lack a chromophore, or vary widely in their response factors, i.e. their extinction coefficients, are challenging to analyze. The issues with UV/Vis detection also apply to counter ion monitoring and especially to stability and degradation studies, which are necessary to support the product development. Consequently, it is often difficult to meet analytical requirements to demonstrate product quality. The uncertainties in the quantification make it challenging to accurately classify the impurities according to the ICH guidelines with respect to reporting, identification, or qualification threshold, which are in place to prevent severe side effects of the final drug formulation.[2]

CAD can be used to overcome these quantitation challenges (e.g., response or detectability issues). As CAD response is independent of the chemical structure of nonvolatile analytes, it is an ideal chromatographic approach when individual calibrants are unavailable.[3] Under isocratic conditions, the calibration curve obtained with CAD using an available standard, e.g. reference standard of an API, can be used to quantify all nonvolatile analytes in a sample, within a certain range of confidence. As CAD response is affected by mobile phase composition, it is necessary to ensure that the universal response is applicable in gradient elution. This can readily be achieved through a compensation gradient delivered by the Thermo Scientific™ Vanquish™ Flex Dual Pump.[4]

In this application note, a Thermo Scientific™ Accucore™ Pentafluorophenyl (PFP) column was used to separate paclitaxel from its related compounds and other impurities. Calibration using standards of paclitaxel and related compounds was used to estimate the quantities of unknown impurities present in the paclitaxel product. A thermal degradation study was also performed with the degradation products being analyzed and subsequently quantified using UHPLC-UV-CAD.

Experimental

Recommended lab consumables and equipment

- Accucore Pentafluorophenyl (PFP) column, 2.6 µm, 2.1 × 150 mm, L43 (P/N 17426-152130)
- Fisher Scientific™ LC-MS grade acetonitrile (P/N A955-212)
- Thermo Scientific™ Barnstead™ GenPure™ xCAD Plus Ultrapure Water Purification System (P/N 50136171)
- Thermo Scientific™ Digital Heating Shaking Drybath (P/N 88880028)

Sample preparation

Calibration

The API (paclitaxel) and the related impurities C (Impurity C) and A (cephalomannine) were bought from the European (Strasbourg, France) and United States Pharmacopeia (Rockville, MD, United States), respectively. Baccatin III (purity 97%) was obtained from Sigma-Aldrich® (Schnelldorf, Germany) and was only used for peak identification. All calibration standards were accurately weighed and transferred to volumetric flasks and were brought to volume with methanol to achieve a final concentration of 0.1 mg/mL. A 10 µg/mL stock solution containing all three substances was prepared by transferring each of them into a volumetric flask and bringing to volume with methanol. Accordingly, the 10, 5, 1, and 0.5 µg/mL calibration standards were produced by a dilution series with methanol and each was analyzed in three consecutive runs with blank injections in between the different concentrations. Due to the limited stability in solution, standards were prepared directly prior to analysis.

Forced degradation

For the thermal degradation study, a 100 µL volume of a 1 mg/mL paclitaxel solution was diluted with 350 µL methanol and 50 µL dimethyl sulfoxide (DMSO) in a 1.5 mL Eppendorf tube. The solution was exposed to 65 °C for 2 hours using a Digital Heating Shaking Drybath. The degraded sample was then analyzed immediately without further sample preparation.

Instrumentation

The separation was achieved using an Accucore PFP column, which is well suited for the separation of aromatic compounds. Chromatographic conditions are summarized in Table 1. Detection was performed using the Thermo Scientific™ Vanquish™ Flex Variable Wavelength Detector followed by the Thermo Scientific™ Vanquish™ Flex Charged Aerosol Detector.

The Vanquish Flex Duo UHPLC system for Inverse Gradient consisted of:

- System Base Vanquish Flex (P/N VF-S01-A-02)
- Dual Pump F (P/N VF-P32-A-01)
- Split Sampler FT (P/N VF-A10-A-02) with a 25 µL sample loop
- Column Compartment H (P/N VH-C10-A-02)
- Charged Aerosol Detector F (P/N VF-D20-A)
- Variable Wavelength Detector F (P/N VF-D40-A)
- Vanquish Duo for Inverse Gradient Kit (P/N 6036.2010)

Fluidic scheme

Figure 1 illustrates the Vanquish Duo Inverse Gradient Workflow setup. The right part of the Dual Gradient Pump delivers the eluent flow to the column (analytical gradient), and the left pump forms the second gradient (compensation gradient) directly to the T-piece immediately before the CAD.

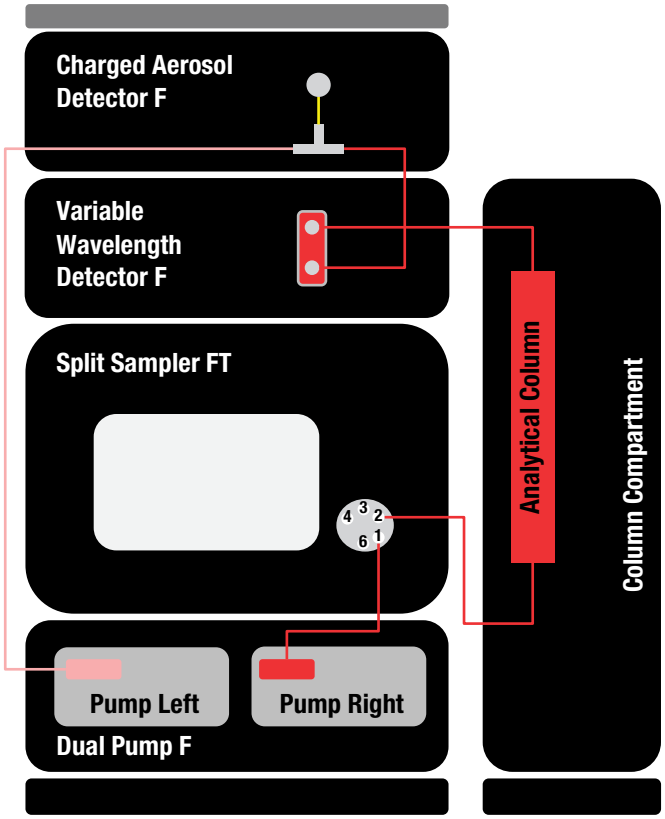

Figure 1. Fluidic scheme of the Vanquish Duo Inverse Gradient Workflow including a Charged Aerosol Detector F and Variable Wavelength Detector F.

Separation conditions

Table 1. Chromatographic conditions.

UHPLC Experimental Conditions	
Column:	Accucore PFP 2.1 × 150 mm, 2.6 µm
Mobile phase:	A: Water, Ultra-pure (18.2 MΩ·cm at 25 °C) B: LC-MS grade acetonitrile
Analytical gradient:	23–60 %B in 25 minutes; 0.3 mL/min
Compensation gradient:	23–60 %A in 25 minutes, 0.3 mL/min
Temperature:	35 °C Forced air; Active pre-heater 35 °C
Injection volume:	1 µL
UV detection:	227 nm, 5 Hz, Response time 1 s
CAD:	Evaporation Temp. 50 °C, 5 Hz, Filter 3.6

Data processing

The Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS), version 7.2.8 was used for data acquisition and evaluation.

Results and discussion

Global calibration

As mentioned above, the uniform response of the charged aerosol detector is routinely achievable under isocratic nebulization conditions. The nebulization is affected by solvent composition, so a change in organic solvent content during a gradient will lead to a change in detector response.[4] To compensate for this effect, an inverse gradient is applied post column using the Vanquish Flex Dual Pump to provide a uniform mobile phase composition. The inverse gradient bypasses the column and mirrors the analytical gradient composition to achieve isocratic flow to the nebulizer. The difference in response in UV and CAD using the Vanquish Duo Inverse Gradient Workflow is shown in Figure 2 (right). This shows that the UV response for 10 µg/mL of three different analytes differed by as much as 63%, while that of CAD differed by only 2%. This once again illustrates that the CAD response is independent of the chemical structure and response variation due to solvent gradients is minimal when using the Vanquish Flex Dual Pump to perform inverse gradient compensation.

The CAD calibration is linear for all three compounds in the concentration range from 0.5 to 10 ppm (Figure 2, left). The uniform response for the API and related components is demonstrated by the high similarity of the slopes of the calibration curves, and therefore the workflow is applicable to the measurement of related impurities. In addition, the slopes of the calibration curves show a relative deviation of average response of just 2%.

Analysis of API degradation products

Effect of Inverse Gradient compensation

The stressed API sample was analyzed using the Vanquish Duo Inverse Gradient with the Vanquish Flex Dual Pump and compared to the same system configuration without gradient compensation (Figure 3). Both setups can detect the same number of peaks, but a significant difference in peak response is noticeable.

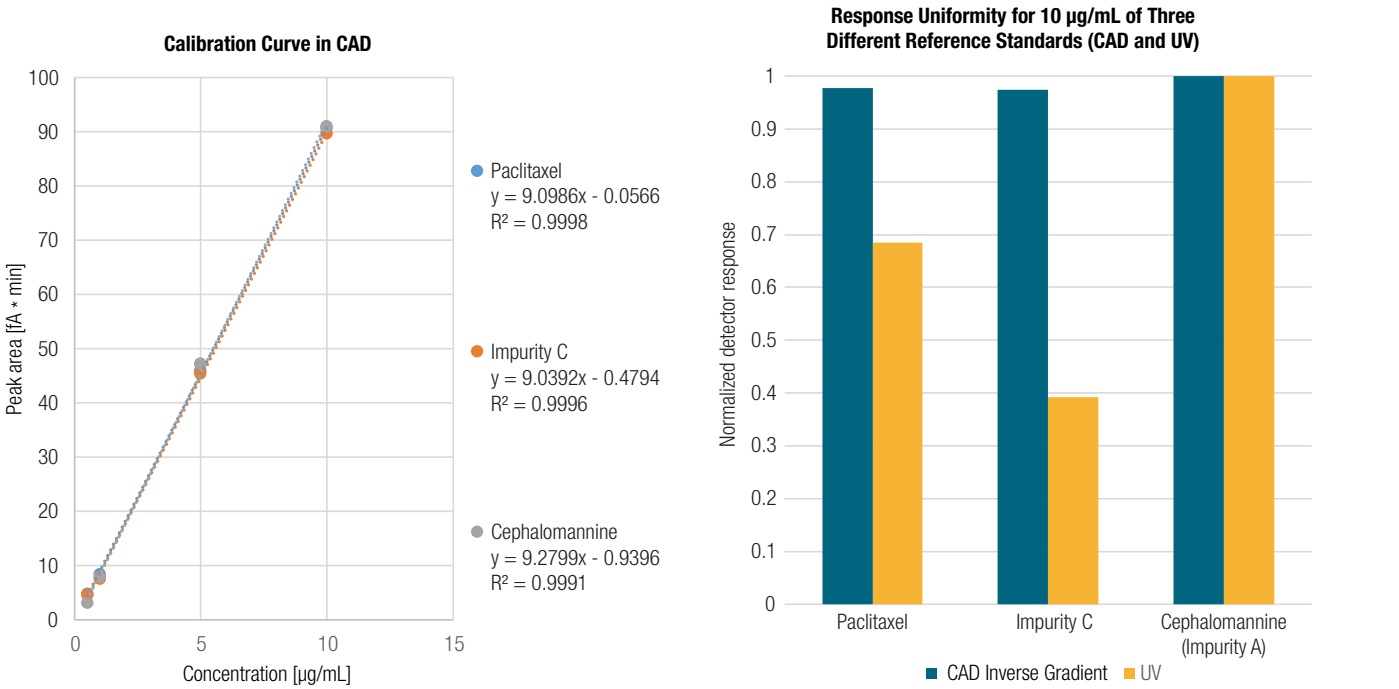

Figure 2. Uniform response was obtained using the Vanquish Duo Inverse Gradient with CAD as shown by the similar calibration curves obtained for the API and its related compounds (left side). By comparison, UV response was highly variable among analytes (right side). Response factors for both detectors were normalized to cephalomannine.

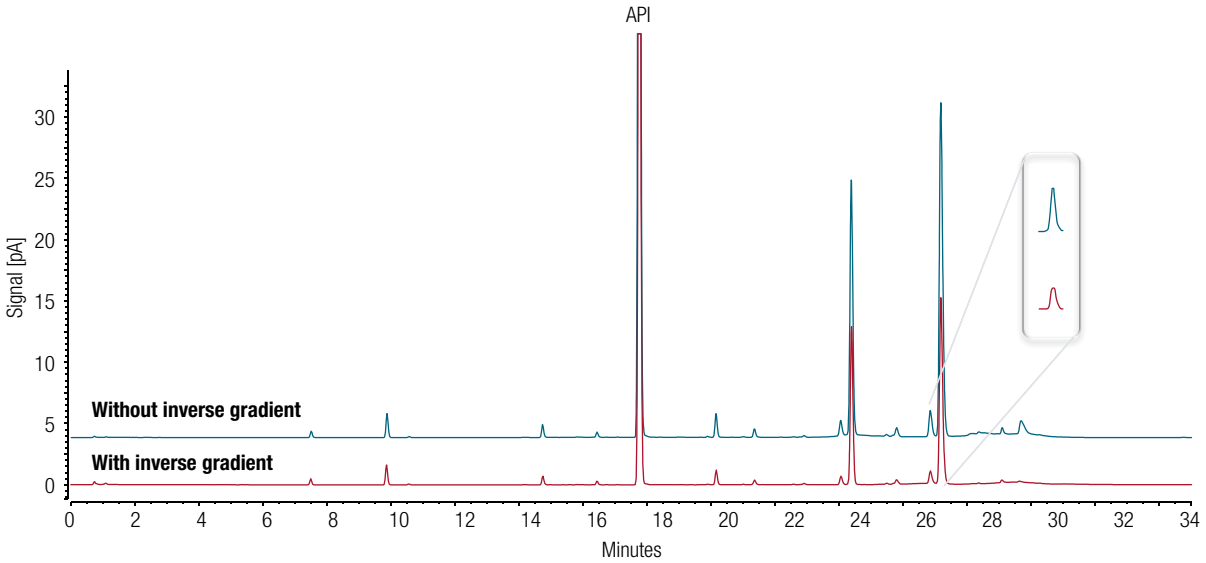

Figure 3. Comparison of CAD response with (red trace) and without (blue trace) applying inverse gradient compensation using the Vanquish Flex Duo system. Without inverse gradient, the quantity of analytes eluting before the API are underestimated while those eluting after the API are overestimated.

For the stressed sample shown in Figure 3, the combined peak areas for all impurities were found with CAD to be 63.9% of the API peak area when not using inverse gradient compensation (blue trace) and 53.8% of the API when using inverse gradient compensation (red trace). This large (>10%) difference in determined impurity content is attributed to the influence of solvent composition on CAD response. As expected in reversed-phase gradients, response factors for later eluting peaks are higher when not using inverse gradient compensation (see Figure 3 inset). These data highlight the capabilities of the Vanquish Duo Inverse Gradient Workflow to achieve uniform response with CAD and thus to minimize quantitation errors.

Comparison between UV and CAD

The combination of charged aerosol and ultraviolet absorbance detection can provide a more holistic approach when analyzing complex samples. As shown in Figure 4, some peaks lack UV activity and therefore cannot be measured using single wavelength UV at 227 nm. One of the degradation products eluting at 6.6 minutes is only visible using UV detection since it is too volatile to be detected by CAD. The two main impurities (24.3 and 27.1 min) are only detectable in CAD.

Comprehensive analysis of degradation products of new drugs is feasible using the multi-detector approach with UV and CAD. An example degradation product (peak at minute 9.8) with similar retention behavior as the precursor baccatin III can now be quantified using global calibration (Figure 2). Here, the example compound has a relative area with respect to the API of 1.1%. The relative standard deviation (n=5) of the peak area precision is 0.25% for the peak eluting at minute 9.8 and 0.76% for the API. The Vanquish Duo Inverse Gradient System, utilizing both CAD and UV, is an extremely powerful tool because of the complementary nature of the two detectors and is well suited for stability or degradation studies.

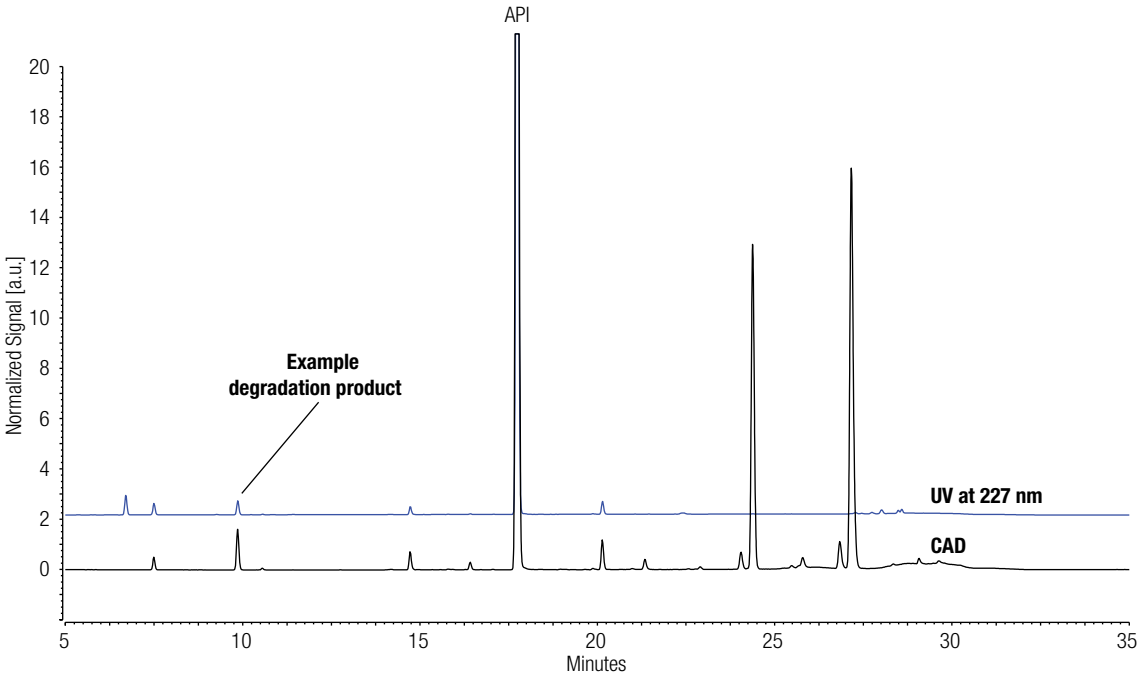

Figure 4. Comprehensive analysis of degradation products of paclitaxel using UV (blue) and CAD (black). UV signal normalized to the API.

Conclusion

Uniform response for degradation analysis with charged aerosol detection is easily achievable with a Vanquish Duo UHPLC Workflow and a post-analytical column inverse gradient. The combined analytical and inverse gradient flow yields a constant eluent composition in the CAD nebulizer, resulting in uniform response even during gradient runs. The proof of concept is presented here using the anti-cancer drug paclitaxel and two related impurities, which showed a variation in calibration slope of only 2%. Furthermore, the Inverse Gradient Workflow was successfully applied to impurity analysis of a paclitaxel standard resulting in a measurably more uniform analyte response throughout the gradient. Using the Vanquish Duo Inverse Gradient, the measured amounts of analytes were corrected by more than 10%, thus giving a more accurate and unbiased determination of critical impurities. When combined with UV detection, the resulting comprehensive profiling of a new drug candidate, its impurities, degradants, and related substances is straightforward, making this multi-detector setup a powerful tool for analytical laboratories.

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APPLICATION NOTE 65200

Tomorrow's quantitation with the TSQ Fortis mass spectrometer: quantitation of phenylephrine hydrochloride for QA/QC laboratories

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Keywords

Phenylephrine hydrochloride, QA, triple quadrupole MS, TSQ Fortis MS, quantitation workflow solution, TraceFinder software, Vanquish Flex UHPLC

Goal

Development of a robust, reliable, and reproducible quality control workflow for the analysis and quantitation of phenylephrine hydrochloride for QA/QC using a triple quadrupole mass spectrometer.

Application benefits

- Develop robust and easy quantitation workflows with reproducibility, reliability, and the required sensitivity for phenylephrine HCl in Milli-Q® water
- Implement easy-to-use and easy-to-implement quantitation workflows that allow quantitation of any molecule type in matrix regardless of user expertise and experience

Introduction

Phenylephrine HCl is a nasal decongestant that provides relief from discomfort caused by colds, allergies, and hay fever.[1] Quantitation of phenylephrine HCl in biological matrices (e.g., plasma) bears unique significance due to determining its optimal dosage as a nasal decongestant and also quantifying it as a drug of abuse if consumed. In addition, developing robust and accurate quantitation assays for quantifying phenylephrine HCl in water is critical for quality control (QC) and quality

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Table of contents

assurance (QA) processes. These QA/QC methods not only provide the concentration of phenylephrine HCl but also analyze and quantify any impurities that may have been added or formed during the manufacturing process. There have been several reports of quantitative assays of phenylephrine hydrochloride (phenylephrine HCl) that used a host of technologies.[2] These ranged from chromatographic to electrochemical and spectrophotometric techniques. Although liquid chromatography (LC) with ultraviolet or fluorescence detection has been well established, LC methods often face serious limitations due to poor sensitivity and longer analysis times.

Liquid chromatography coupled to triple quadrupole mass spectrometry (QqQ, also represented as MS/MS) offers some significant advantages, especially in offering higher sensitivity, selectivity, and productivity with remarkable reproducibility for quantitation assays. However, despite the outstanding quality of data and productivity gains offered by LC-MS/MS, for the typical QA/QC environment, minimizing the cost per sample continues to be a big challenge. The use of high-end QqQs often adds complexity to the challenge of successfully addressing the cost per sample issue, which in turn, can affect organizational profitability. Striking an optimal balance between choosing the right LC and MS platforms that can offer quality data while addressing other organizational challenges is extremely important for every QA/QC laboratory. In this report, we offer a robust, reliable, reproducible quantitation assay of phenylephrine HCl in Milli-Q water with a Thermo Scientific™ Vanquish™ Flex UHPLC system, Thermo Scientific™ TSQ Fortis™ triple quadrupole mass spectrometer, and Thermo Scientific™ TraceFinder™ 4.1 software.

Experimental

Sample preparation

A 100 mg/L phenylephrine HCl stock solution was prepared by dissolving 10 mg of phenylephrine HCl in a 100 mL volumetric flask using Milli-Q water. The stock solution was further diluted as shown in Table 1.

The QC analysis of phenylephrine HCl was carried out by preparing three different QC samples from the parent stock solution. The final concentrations of phenylephrine HCl in the QC A, QC B, and QC C solutions were 0.01, 0.002, and 0.001 ppm, respectively (Table 2).

Table 1. Dilution of phenylephrine HCl stock dilution using Milli-Q water

Calibration Level	Sample ID	Prep From	Stock (mL)	Mobile Phase A (mL)	Final Volume (mL)	Final Conc. (mg/L)
Cal 06	A	Stock	1.0	99.0	100.0	1.0
Cal 05	B	A	1.5	8.5	10.0	0.150
Cal 04	C	A	2.0	18.0	20.0	0.100
Cal 03	D	C	5.0	5.0	10.0	0.050
Cal 02	E	C	1.0	9.0	10.0	0.010
Cal 01	F	E	5.0	7.5	12.5	0.004

Table 2. Preparation of QC solutions from phenylephrine HCl stock solution

Sample ID	Prep From	Stock (mL)	Mobile Phase A (mL)	Final Volume (mL)	Final Conc. (mg/L)
QC A	A	1.0	99.0	100.0	0.01
QC B	QC A	2.0	8.0	10.0	0.002
QC C	QC A	1.0	9.0	10.0	0.001

Liquid chromatography

Liquid chromatographic analysis was done using the Vanquish Flex UHPLC system equipped with a Thermo Scientific™ Hypersil GOLD™ column (50 × 2.1 mm, 1.9 µm) that was conditioned and operated at 30 °C. A 2 µL injection volume was used for all analyses following the gradient indicated in Table 3 and Figure 1.

Mass spectrometry

The TSQ Fortis mass spectrometer was used for this analysis with positive heated electrospray ionization (HESI) mode. The experimental conditions were optimized with a static spray voltage of 3500 V, a cycle time of 0.3 s, and both Q1 and Q3 resolutions maintained at 0.7 Da FWHM. The ion transfer tube and vaporizer temperatures were maintained at 300 °C and 225 °C, respectively. The HESI probe position was optimized following the instrument control software guide and was locked. The SRM table and other critical MS features for all the target analytes are listed in Table 4.

Software

Data acquisition and processing were conducted using TraceFinder software version 4.1.

Table 3. LC gradient information

Time (min)	Flow (mL/min)	%B	Curve
Equilibration			
0.000	0.200	0.0	5
Run			
0.000	0.200	0.0	5
0.200	0.200	0.0	6
3.000	0.200	30.0	6
4.000	0.200	100.0	6
5.000	0.200	100.0	6
5.500	0.200	0.0	6
9.500	0.200	0.0	6

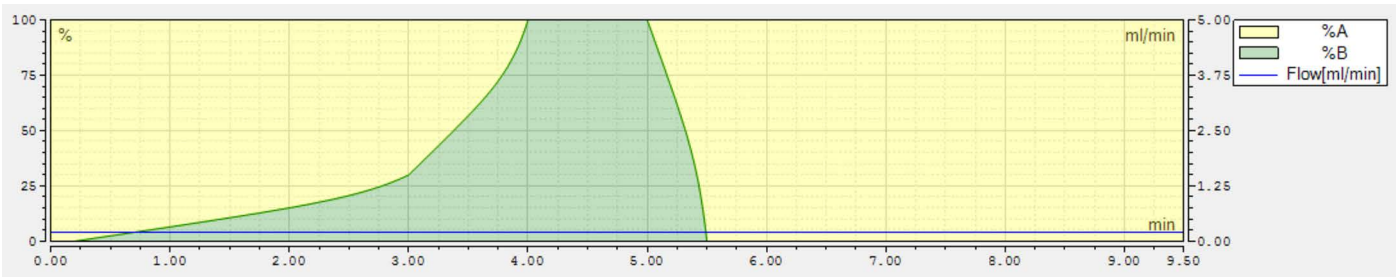

Figure 1. LC gradient profile for the analysis of phenylephrine HCl in water

Table 4. Optimized mass spectrometer transitions for phenylephrine HCl analyzed in this experiment

Compound	Precursor (*m/z*)	Product (*m/z*)	Collision Energy (V)	Min Dwell Time (ms)	Tube Lens (V)
Phenylephrine HCl	168.1	91.0	23	149	55
Phenylephrine HCl	168.1	150.0	10	149	55

Results and discussion

The superior performance and outstanding resolution offered by the Vanquish Flex UHPLC, combined with the robustness, selectivity, and sensitivity offered by the TSQ Fortis MS, enabled efficient quantitation workflows for phenylephrine HCl in water. The superior performance of the LC-MS/MS platform solution not only enabled identification but also routine quantitation of analytes without contamination of the ESI-MS/MS instrument source.

The calibration curve (Figure 2) highlights the linearity and range of sensitivity that were addressed by this quantitation workflow. A five-point linear calibration curve showed a minimum R^2 value of 0.9993 (Figure 2), when a linear curve fitting with weighting 1/x was applied to the curve. The lower limit of quantitation (LLOQ) calibration standard shows an acceptable CV value of 4.72%. Representative chromatograms of phenylephrine HCl at 4 µg/L and that of the blank sample are shown in Figure 3.

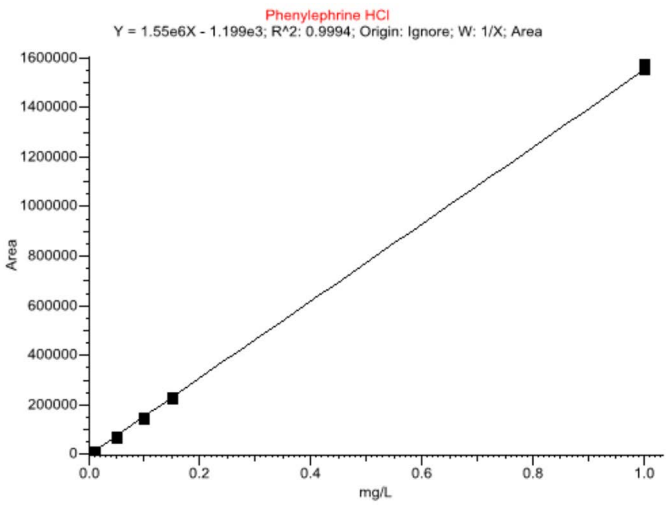

Figure 2. Calibration curve of phenylephrine HCl in Milli-Q water

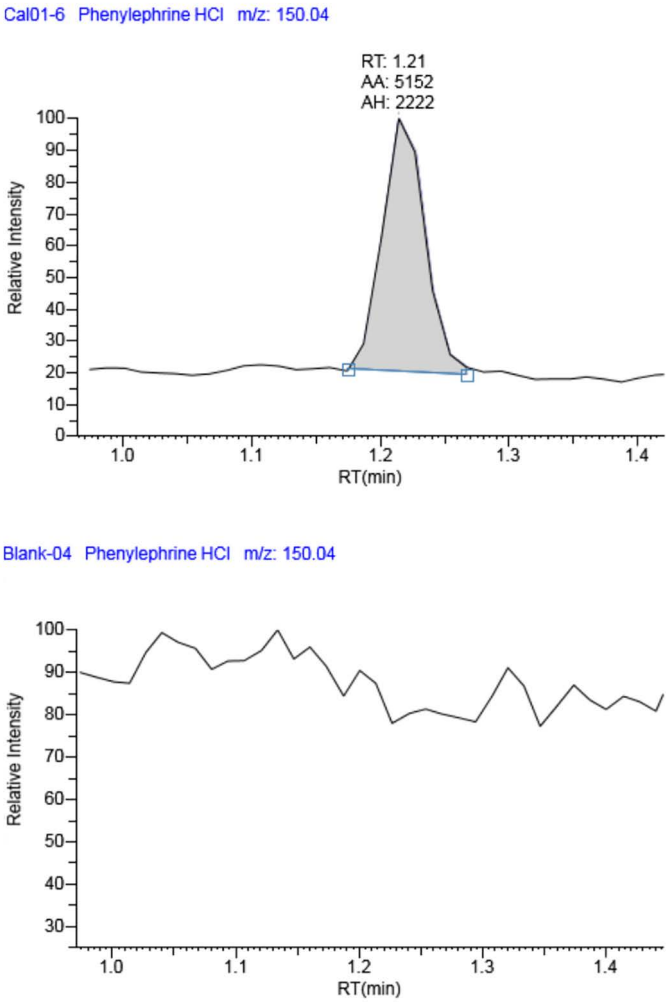

Figure 3. Chromatogram of phenylephrine HCl in LLOQ solution with a concentration of 4 µg/L (top) and that of the blank sample (bottom)

For the three analytical batch runs (Figure 4), the precision (% CV) ranged from 2.19% (QC C) to 4.76% (QC B) to 5.42% (QC A). The concentration ranges for the three shown QC samples are 10 µg/L (QC A) to 2 µg/L (QC B) to 1 µg/L ppm (QC C). The correlation coefficients (R^2) for these analytical batches of phenylephrine HCl were > 0.99. The results obtained for the analysis of the QC batches are well within the acceptable quantitation, precision, and accuracy limits.

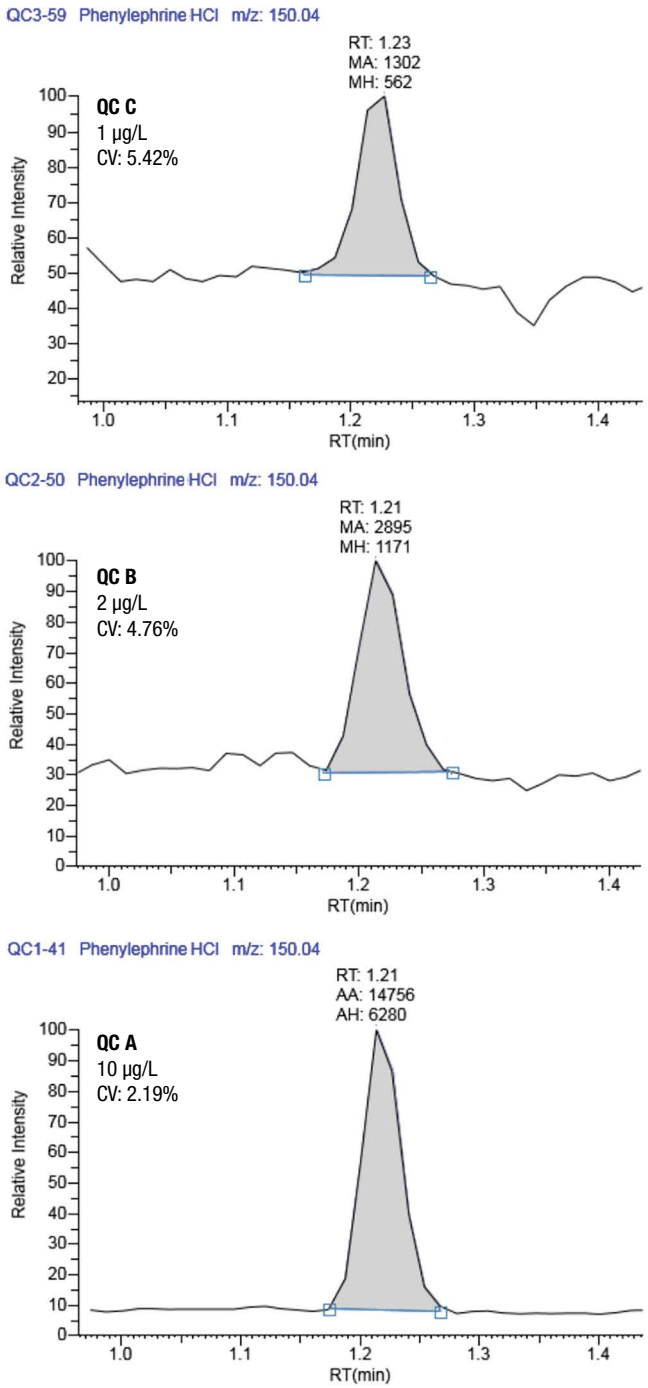

Figure 4. QC chromatograms of phenylephrine HCl at 10 µg/L (bottom), 2 µg/L (middle), and 1 µg/L (top)

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Conclusions

Robust, reproducible, sensitive, and affordable quantitation workflows for phenylephrine HCl are extremely important for determining the dosage amount, concentration in samples, and ensuring purity. An LC-MS/MS method for the quantitation of phenylephrine HCl in water was successfully developed using the Vanquish Flex UHPLC system and the TSQ Fortis mass spectrometer. The same quantitation workflow can also be transferred to quantify phenylephrine HCl in biological matrices. Five separate batches were analyzed for this study, with a linear fit R^2 value of 0.9993. The precision and accuracy results for the QC samples in all batches addressed the expectations.

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APPLICATION NOTE 72706

HPLC-CAD impurity profiling of carbocisteine using SCX-RP mixed-mode chromatography

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Keywords
Carbocisteine, amino acids, Vanquish CAD, mixed-mode chromatography, impurity profiling, LOQ

Goal

To analyze the non-proteinogenic amino-acid carbocisteine and its impurities by means of HPLC Charged Aerosol Detection (CAD) and to obtain improved sensitivity for the impurities when compared to the existing method.

Application benefits

- Modernized and translated UHPLC-CAD method
- LOQs reduced by average of 44% across all impurities

Introduction

Carbocisteine is a non-proteinogenic amino acid that is used in the treatment of acute and chronic respiratory diseases that require mucolytic agents.[1] As an amino acid, and thus with amino acids and similar structures in the impurity profile, it is challenging to establish a proper chromatographic procedure due to the substances being very similar in their physicochemical properties. The lack of a chromophore is often overcome with methods such as ninhydrin or ortho phthalaldehyde (OPA) derivatization, but this leaves the detection blind to other impurities such as organic acids or other substances that do not react with the derivatization agent such as cyclization products. The European Pharmacopoeia still sometimes uses simple TLC tests with ninhydrin-derivatization that only semi-quantitatively assess the impurity content relative to a reference spot.[2] Reliable and quantitative HPLC separation and detection methods are desirable. Therefore, charged aerosol detection (CAD) is a more convenient and direct approach for all non-volatile impurities making the Thermo Scientific™ Vanquish™ Charged Aerosol Detector a well-suited instrument.

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Table of contents

When used as a drug in humans, with a maximum daily dose of >2 g, the ICH guideline Q3A(R2) requires a reporting threshold for every impurity of 0.03% (m/m).[3] It is desirable to have reliable detection with a LOQ better than this value. The already published method lacked a sufficient LOQ for cystine, with only 0.09%, so an improvement of the sensitivity is the goal.

In this application, a method formerly published by Wahl and Holzgrabe[4] was slightly modified to result in a mobile phase of 18% acetonitrile and 10 mM trifluoroacetic acid (TFA). This method was run on a strong cation exchange reversed-phase (SCX-RP) mixed-mode column and the newest generation Vanquish Charged Aerosol Detector. The requirements according to the ICH guidelines of a reporting threshold of 0.03% for each impurity were met and LOQs of 0.02% or lower were obtained. These LOQs are far lower than LOQs of the original method on the Thermo Scientific™ Corona™ Charged Aerosol Detector (Corona CAD), which only reached 0.09% for cystine. Linear models of calibration curves for all impurities over a range of 0.05-0.25% of the assay's concentration yielded $R^2 > 0.995$. When analyzing the same batches as in the experiments from Wahl and Holzgrabe, batches that formerly could only be labeled with "not detected" for their cystine content could now be assigned with a low percentage value.

Experimental

Recommended consumables

- Deionized water, 18.2 MΩ·cm resistivity
- Fisher Scientific™ Optima™ UHPLC-MS grade acetonitrile (P/N A956-1)
- Fisher Scientific™ Optima™ LC-MS grade trifluoroacetic acid (TFA) (P/N A116-50)
- Fisher Scientific™ Analytical grade ammonia (P/N A/3295/PB05)
- Thermo Scientific™ Chromacol™ vial, clear 1.5 mL kit with septa and cap (P/N 2-SVWGKST-CPK)

Mobile phase preparation

Only high purity solvents are to be used with the Vanquish CAD because it will detect semi and non volatile contaminants present in the mobile phase and samples.

Prepare a 0.1 M TFA solution by adding 5.70 g of TFA to about 300 mL of 18.2 MΩ·cm resistivity deionized water, and then bring the volume up to 500 mL. Combine 180 mL of acetonitrile and 100 mL of the 0.1 M TFA solution in a 1000 mL volumetric flask and bring up to volume with water.

Sample preparation

Carbocisteine and its impurities (Figure 1) are polar and thus water-soluble with certain limitations as described below.

Stock solutions of the impurities were prepared at a concentration of 0.25 mg/mL by exactly weighing 2.5 mg and diluting with water to 10.0 mL. To overcome solubility issues, an addition of 3% of concentrated ammonia solution to the stock solution of cystine was made. For cystine the stock solution should be prepared at a concentration of 0.1 mg/mL for reasons of solubility.

All sample solutions must be freshly prepared by exactly weighing 50 mg of carbocisteine and diluting with water to 10.0 mL after the addition of 300 µL of concentrated ammonia solution.

The impurity stock solutions can be stored at 2 °C to 8 °C and diluted daily. Setting the autosampler temperature to 8 °C was found to be sufficient to use the vials in the rack on multiple days.

Method optimization summary

The initial method utilizes an acetonitrile content of 12% (v/v) with 0.1 mM TFA and detection with the Corona CAD with a filter setting of "high". Evaporation temperature could not be changed on that instrument.

The original method was used with a systematic variation of evaporation temperature settings of the CAD ranging from 25 °C to 60 °C. The signal-to-noise ratio for a cystine solution containing 0.0025 mg/mL was observed. It was calculated by the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software using the peak-to-peak noise in a fixed interval over the last two minutes of the run.

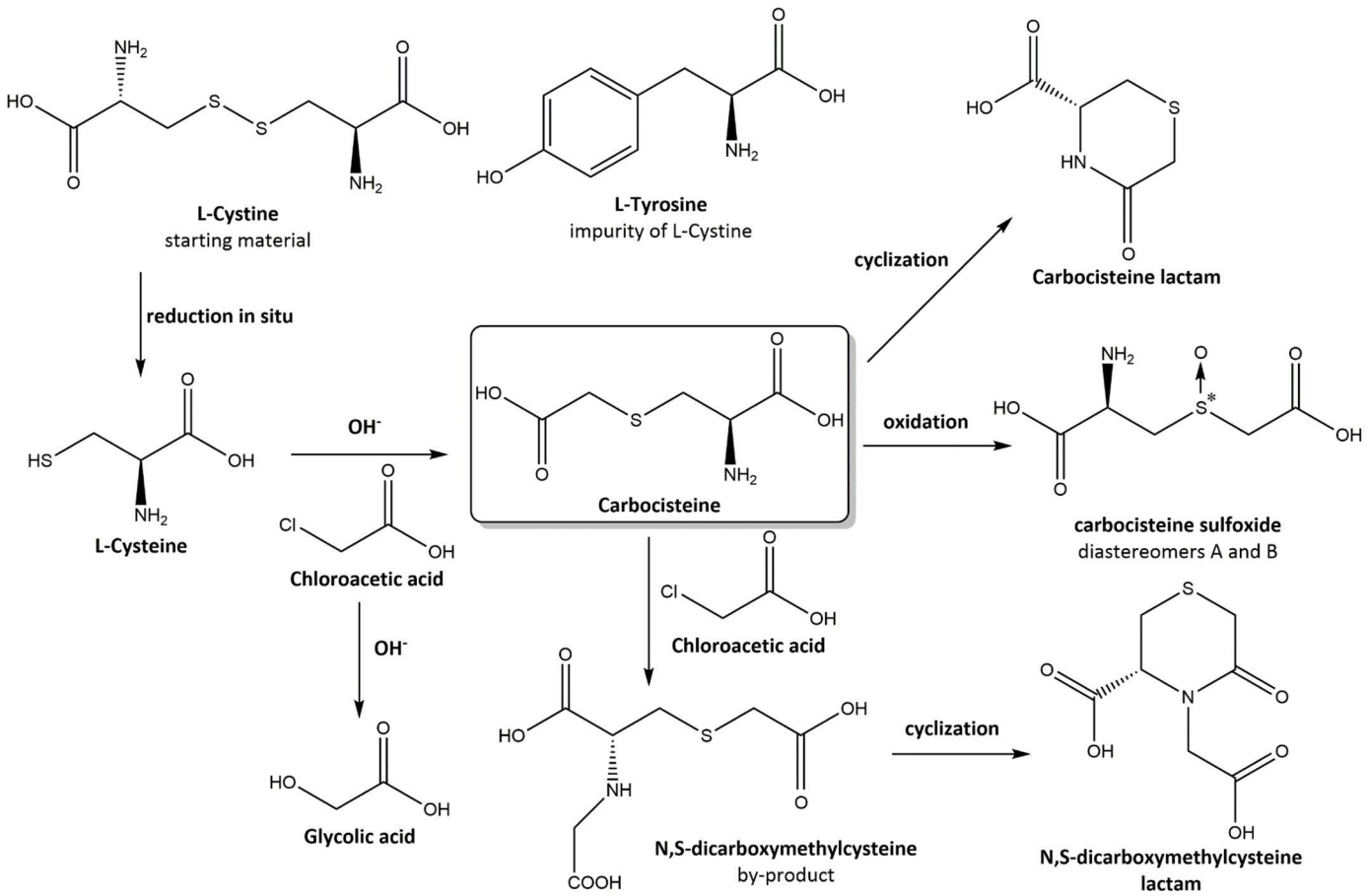

Figure 1. Structures and origin of carbocisteine and its impurities[1]

This concentration equaled 0.05% of the assay's concentration. The signal-to-noise-ratio of this 0.05% cystine solution obtained using the original method with the Vanquish CAD was >30 (see Figure 3) while it was below the LOQ with the older CAD. These preliminary experiments with the original mobile phase showed that the performance of the Vanquish CAD is superior to the earlier generation Corona CAD from Thermo Fisher Scientific.

The best result was achieved with an evaporation temperature of 50 °C and a filter setting of 10 s, which corresponds to the "high" setting on the Corona CAD.

When injecting an impurity mixture to check the separation of the method, an interesting observation was found with the late eluting analytes, tyrosine and cystine. On the one hand, they eluted too close to each other and on the other hand, they changed their elution order when injected with a carbocisteine sample instead of an impurity mixture or individually. The reason behind this was not further investigated because this phenomenon could be overcome by increasing the acetonitrile content.

Variations of TFA and acetonitrile content were examined. As already described in the original publication, the retention of the carbocisteine lactam and the elution order of cystine and tyrosine is governed by acetonitrile content. The TFA content affects peak shape and general retention for the two late eluting impurities. Increasing the acetonitrile content to 18% v/v instead of the initial 12% v/v showed the desired behavior of a good separation and tyrosine eluting before cystine. Additionally, sensitivity is increased with higher percentage of organic modifier.[5]

Instrumentation

Vanquish UHPLC system equipped with:

- System Base (P/N VH-S01-A)
- Vanquish Charged Aerosol Detector H (P/N VH-D20-A)
- Vanquish Binary Pump Flex (P/N VF-P10-A-01)
- Vanquish Split Sampler (P/N VF-A10-A)
- Vanquish Column Compartment H (P/N VH-C10-A)
- Vanquish Diode Array Detector F (P/N VF-D40-A)

Conditions

Column:	SIELC Primesep® 100; 250 × 4.6 mm, 5 µm
Mobile phase:	The mobile phase comprised 18/82 acetonitrile/ultrapure water (v/v) and 10 mM TFA.
Flow rate:	1.3 mL/min
Run time:	20 min
Column temp.:	20 °C
Injection volume:	20 µL
Vanquish CAD detector settings	
Evaporation temp.:	50 °C
Power function:	1.00
Data collection rate:	10 Hz
Signal filter:	10 s

Data processing

Chromeleon CDS Version 7.2.6 was used for data acquisition and analysis.

Results and discussion

Separation of carbocisteine and its impurities

Separation of carbocisteine and its possible impurities (Figure 1) was achieved using a mixed-mode column with both hydrophobic and strong cation exchange functionalities, SIELC Primesep 100 column. Due to the combined retention mechanisms, neutral impurities, e.g. carbocisteine lactam, and the polar amino acids tyrosine and cystine could be separated within 20 minutes isocratically (Figure 2). The two peaks due to the carbocisteine sulfoxide diastereomers A and B were analyzed by the sum of their respective areas.

The mobile phase comprised 18/82 acetonitrile/ultrapure water (v/v) + 10 mM TFA. The amount of acetonitrile in the mobile phase mainly affected the separation of the early eluting impurity carbocisteine lactam and the late eluting impurity tyrosine; whereas the TFA concentration was crucial for the retention of the late eluting impurities tyrosine and cystine. The acetonitrile content was increased compared to the previous method because more acetonitrile had a positive impact on signal height and resolution was still acceptable. Furthermore, a reliable resolution and elution order between tyrosine and cystine was obtained. The TFA content was not changed from the old method since it offered the best compromise in peak shape and retention times of the late eluting impurities.

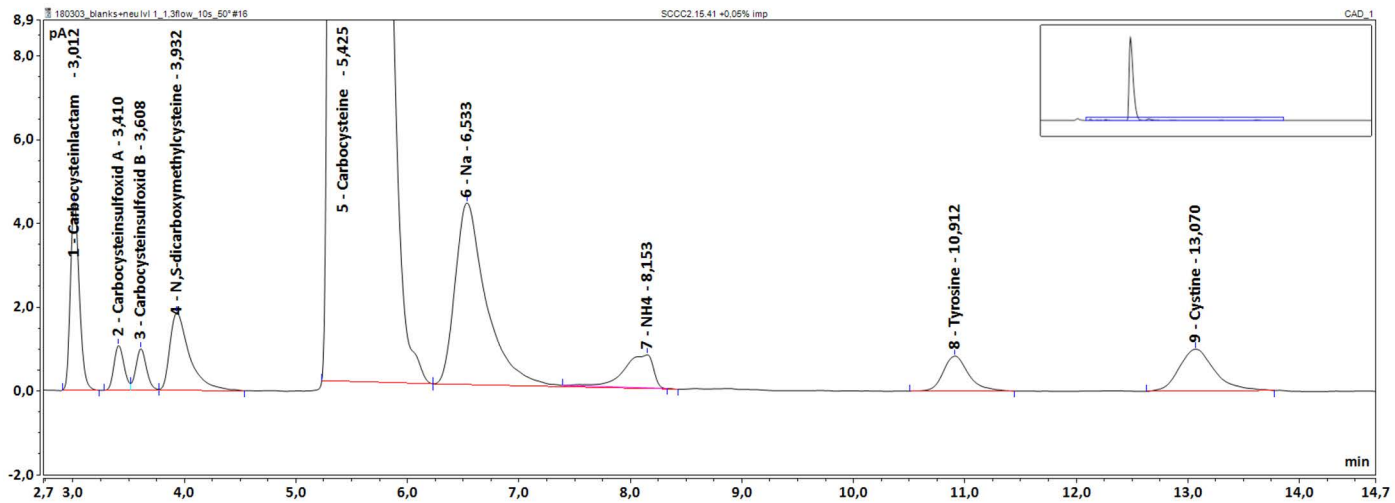

Figure 2. Batch sample of carbocisteine spiked with 0.05% of each impurity; transferred method

Linearity and limit of quantitation

Calibration curves for all detectable impurities were obtained by injecting five concentration levels covering the suspected range of impurity content in the samples (0.05%–0.25%). The coefficients of determination are shown in Table 1. Although the CAD generally is a non-linear detector, in the observed small concentration range an almost linear relation between analyte concentration and detector signal can be assumed.

The LOQ is crucial for this application because the relevant guidelines of the ICH and EDQM claim a reporting threshold of not more than 0.03% of each impurity. The analytical procedure's LOQ should not exceed that threshold, thus a LOQ of at least 0.03% of the test substance's concentration is highly desirable.

Compared to the old method using the Corona CAD as the detector, the Vanquish CAD showed improved sensitivity resulting in lower LOQs for all impurities except for carbocisteine sulfoxide (Table 1). The LOQ was obtained by the signal-to-noise (S/N) approach of ICH guideline Q2(R1) corresponding to an analyte's concentration that gives a S/N ratio of 10. For cystine, the S/N ratio was also determined at an acetonitrile content of 12% to enable comparison of the sensitivity of the new Vanquish CAD with the older model. A concentration of 0.05% cystine referred to the test substance carbocisteine at 5 mg/mL, thus corresponding to the qualification threshold of the ICH guidelines. This resulted in a S/N ratio of 30, demonstrating superior sensitivity of the Vanquish CAD model (Table 1 and Figure 3).

Table 1. Limits of quantitation and coefficients of determination

Analyte	R^2	LOQ [µg/mL]	LOQ [%]	LOQ Old Method [%]
Carbocisteine lactam	0.9983	0.70	0.01	0.02
Carbocisteinsulfoxide	0.9973	0.94	0.02	0.02
N,S-dicarboxymethyl cysteine	0.9990	0.97	0.02	0.04
Tyrosine	0.9990	1.14	0.02	0.03
Cystine	0.9995	0.69	0.01	0.09

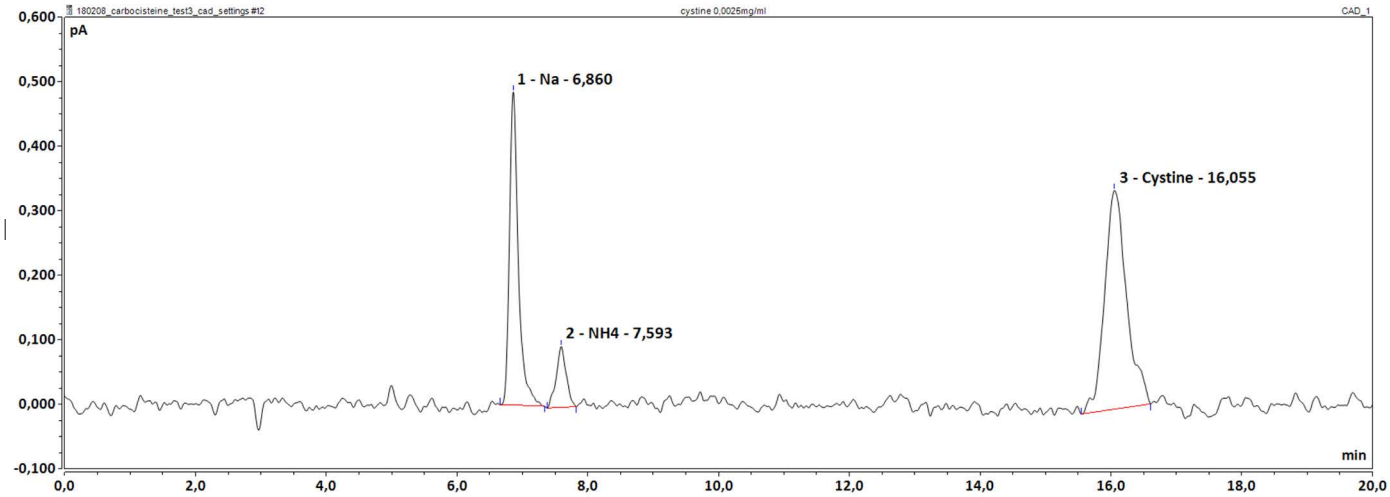

Figure 3. Cystine at 0.05% referred to the assay's concentration, run with 12% acetonitrile (v/v)

Accuracy and precision

Accuracy was assessed with spiked samples at three levels of impurity content (0.05%, 0.15%, and 0.25%). The spiked samples were injected repetitively (n=6) and the recovery of each impurity was calculated (Table 2). The average recovery rates were slightly increased at the 0.05% level. This can be accepted since a minor overestimation of impurities could be favorable around the reporting and qualification threshold with regard to drug safety.

Repeatability was investigated in terms of intra- and interday precision on a batch sample containing all specified impurities. The sample was injected repetitively (n=6) on day 1 and day 2 (Table 3). The RSD of each impurity was below 10%, indicating sufficient precision of the method.

Sample analysis

Eleven different batches of four manufacturers were analyzed using the described method. A specific impurity profile was observed for each manufacturer. Cystine, which was not always detectable by the old method, could be identified and estimated in every sample (Table 4). The transferred method on the Vanquish CAD shows superior sensitivity over the original method on the Corona CAD, leading to a more reliable assessment of low level impurities, especially for cystine. Due to the superior LOQs the regulatory requirements could be entirely met.

Table 2. Recovery rates at spiking level 0.05%, 0.15%, and 0.25%

Analyte	Recovery Rate (0.05%, n=6)	Recovery Rate (0.15%, n=6)	Recovery Rate (0.25%, n=6)
Carbocisteine lactam	111%	106%	105%
Carbocisteinsulfoxide	116%	99%	98%
N,S-dicarboxymethyl cysteine	112%	91%	95%
Tyrosine	119%	111%	108%
Cystine	111%	102%	101%

Table 3. Intra- and interday precision

Analyte	Repeatability Day 1 (RSD %, n=6)	Repeatability Day 2 (RSD %, n=6)	Interday Precision (RSD %, n=12)
Carbocisteine lactam	1.38	1.30	2.34
Carbocisteinsulfoxide	7.92	4.01	7.37
N,S-dicarboxymethyl cysteine	4.36	2.16	4.37
Tyrosine	9.16	7.08	8.19
Cystine	8.12	7.56	9.62

Table 4. Batch result comparison for cystine content, n.d. = not detectable

Batch	Cystine	Cystine Old Method
a1	0.333%	0.37%
a2	0.351%	0.41%
b1	0.039%	n.d.
b2	0.031%	n.d.
b3	0.040%	n.d.
c1	0.089%	0.09%

Conclusion

A method for the impurity profiling of the drug carbocisteine based on CAD detection was successfully transferred from the first-generation Corona CAD to the newest model, Vanquish CAD. The regulatory requirements that claim a reporting threshold of 0.03% impurity content could easily be met for most of the tested impurities, which was not the case before. The new approach offers superior sensitivity resulting in lower LOQs for most of the tested impurities.

Acknowledgements

We greatly appreciate Thermo Fisher Scientific for supplying the instrument for experimental research and the team involved in supporting us throughout the project. Special thanks are due to Paul Gamache, Frank Steiner, Tibor Muellner, Katherine Lovejoy, Jean-Phillippe Heedt, and Julian Görl.

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APPLICATION NOTE 72391

Achieve confident impurity detection with the Thermo Scientific ISQ EC single quadrupole mass spectrometer

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Keywords

Single quadrupole mass spectrometer, Impurity analysis, Peak purity, Quantitative LC MS, Tenofovir disoproxil fumarate

Goal

Demonstrate quantitative impurity analysis with the Thermo Scientific™ ISQ™ EC™ single quadrupole mass spectrometer and show its benefit for pharmaceutical development and quality control.

Introduction

Impurity analysis of produced chemicals is essential for small molecule pharmaceutical development or quality control. According to guidelines from the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), all side products above a certain threshold need to be first characterized and later monitored. Identification and qualification thresholds depend on the daily dose and range between 0.1% and 1.0%.[1] Analysis is often done by an array of different detection methods. In quality control, UV-based detection is still the standard, but MS detection is gaining acceptance because of its clear advantages. Besides its lower detection limit, it also allows immediate analyte identification based on its respective mass and straightforward peak purity analysis based on its mass spectrum. UV-based identification, on the other hand, is often ambiguous since analyte identities are inferred based on their retention time and UV absorption.

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Vanquish Flex UHPLC

Table of contents

Modern single quadrupole mass spectrometers, such as the ISQ EC single quadrupole mass spectrometer (ISQ EC MS), are reliable workhorses designed for routine applications. The ISQ EC MS can operate in Full Scan or Single Ion Monitoring (SIM) mode, to either scan a mass range for all detectable analytes or focus on a specific compound. It can run at scan rates suitable for fast UHPLC applications while delivering picogram detection limits. The new orthogonal source design provides high levels of instrument robustness, even with challenging matrices. Full integration into the Thermo Scientific™ Chromeleon™ 7.2 chromatography data system (CDS) and the Thermo Scientific™ AutoSpray™ smart method set-up make LC-MS operation and data analysis straightforward and intuitive.

In the current work, the advantages of ISQ EC MS based impurity profiling are exemplified using tenofovir disoproxil fumarate. This drug is used for HIV treatment, often in combination with other anti-retroviral drugs. In combination with emtricitabine it is marketed as Truvada® by Gilead. Several impurities are described by the United States Pharmacopeia (USP).[2] Two of them, adenine and tenofovir, were selected for showcasing an ISQ EC MS based impurity analysis workflow. Both of them are structurally related to tenofovir disoproxil (Figure 1). The upper impurity limit for each of them is 0.15% in relation to the amount of tenofovir disoproxil. The challenging chromatographic separation was developed in previously published work.[3]

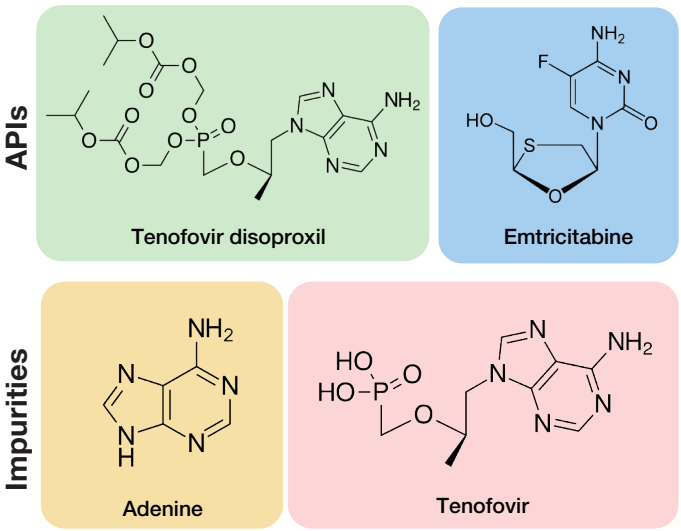

Figure 1. Chemical structures of tenofovir disoproxil, emtricitabine, adenine, and tenofovir. The first two are the active pharmacological ingredients (APIs) in Truvada while the latter two are structurally related impurities of tenofovir disoproxil.

Experimental

Fisher Scientific™ ACROS Organics™ adenine was used. Other sample reagents were purchased as USP reference standards.

Table 1. Overview of analytes. Tenofovir disoproxil is 1:1 complexed with fumarate in the formulation. During chromatographic analysis the complex separates and tenofovir disoproxil is detected. Therefore, only tenofovir disoproxil is mentioned here and in the following.

Analyte	CAS	Chemical Formula	Molecular Weight	Monoisotopic Mass [M]	$[M+H]^+$
Tenofovir disoproxil (fumarate)	201341-05-1	$C_{19}H_{30}N_5O_{10}P$	519.44	519.17	520.18
Emtricitabine	143491-57-0	$C_8H_{10}FN_3O_3S$	247.25	247.04	248.05
Adenine	73-24-5	$C_5H_5N_5$	135.13	135.05	136.06
Tenofovir	147127-20-6	$C_9H_{14}N_5O_4P$	287.21	287.08	288.09

Table 2. Solvents and additives.

Reagent	Grade	Supplier	Part number
Acetonitrile	Optima™ LC-MS	Fisher Chemical™	A955-212
Acetic acid	Optima LC-MS	Fisher Chemical	A113-50
Methanol	Optima LC-MS	Fisher Chemical	A456-212
Water	Ultra-Pure, 18.2 MΩ at 25 °C	Thermo Scientific™ Barnstead™ GenPure™ xCAD Plus Ultrapure Water Purification System	

Chromatographic separation was performed on a Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC system (Table 3). A 75 cm long MP35N capillary with 100 µm inner diameter (P/N 6042.2390) was used for connecting to the ISQ EC MS. LC and MS conditions are given in Tables 4 and 5, respectively.

Table 3. Vanquish Flex Quaternary UHPLC system modules.

Module	Part Number
Vanquish System Base F	VF-S01-A
Vanquish Quaternary Pump F (with 200 µL mixer)	VF-P20-A (6044.5110 and 6044.5026)
Vanquish Split Sampler FT	VF-A10-A
Vanquish Column Compartment H	VH-C10-A
Vanquish Variable Wavelength Detector F (2.5 µL SST flow cell)	VF-D40-A (6074.0360)

Table 4. HPLC conditions.

Parameter	Value
Column	Thermo Scientific™ Accucore™ aQ, 2.6 µm, 2.1 x 100 mm (P/N 17326-102130)
Mobile phase	A: Water with 0.1% acetic acid B: Methanol with 0.1% acetic acid C: Acetonitrile with 0.1% acetic acid
Gradient	0–4 min: 0–70% B, 0–15% C 4–4.5 min: 70% B, 15% C 4.5–5 min: 70–25% B, 15–70% C 5–6 min: 25% B, 70% C 6–6.1 min: 25–0% B, 70–0% C 6.1–15 min: 0% B, 0% C
Flow rate	0.6 mL/min
Column temperature	Still air, 40 °C Active pre-heater, 40 °C
Injection volume	1 µL or 10 µL
UV detection	260 nm, 100 Hz, easy mode

Table 5. MS conditions.

Parameter	Value
Vaporizer temperature	450 °C
Ion transfer tube temperature	350 °C
Source voltage	+750 V
SIM scan	
Compound Time Mass Source CID voltage	Adenine 0–1.5 min 136.1 *m/z* 20 V
Compound Time Mass Source CID voltage	Tenofovir 0–1.5 min 288.1 *m/z* 25 V
Compound Time Mass Source CID voltage	Emtricitabine 1.5–3.0 min 248.1 *m/z* 10 V
Compound Time Mass Source CID voltage	Tenofovir disoproxil 3.0–4.0 min 520.2 *m/z* 10 V
Full Scan	
Time Mass range Source CID voltage	0–15 min 120–600 *m/z* 10 V

The ISQ EC MS was fully integrated into the Chromeleon 7.2 CDS, which was used for system operation and subsequent data analysis.

Calibration standards (10 ppb–10 ppm) were prepared by serially diluting 10 ppm adenine and tenofovir in 5% methanol in water. Samples for measuring the impurity levels were prepared diluting 1000 ppm tenofovir disoproxil, 1000 ppm emtricitabine, and 10 ppm adenine/tenofovir solutions in 5% methanol in water. Prepared samples are listed in Table 6.

Table 6. Impurity samples and used sample concentrations (1 ppm = 1 ng/µL).

Impurity Level	Adenine (ppm)	Tenofovir (ppm)	Emtricitabine (ppm)	Tenofovir Disoproxil (ppm)
1%	1	1	66.7	100
0.2%	0.2	0.2	66.7	100
0.1%	0.1	0.1	66.7	100
0.02%	0.02	0.02	66.7	100
0.01%	0.01	0.01	66.7	100

Results and discussion

First, system suitability for the impurity analysis was assessed. The USP reference method was adapted in a previous publication to reduce cycle time and transfer from 4.6 to 2.1 mm columns.[3] The method in the presented work was further adapted. Solvent A was water with 0.1% acetic acid, solvent B methanol with 0.1% acetic acid, and solvent C acetonitrile with 0.1% acetic acid. Due to the modifications, the method is not equivalent to the USP method. Nevertheless, the developed method is expected to meet the chromatographic requirements stated by the USP, namely peak tailing of tenofovir disoproxil ≤ 2.0 with a relative standard deviation of ≤ 10%, and a resolution between adenine and tenofovir ≥ 1.5. The USP suitability requirements were determined using mass spectrometric detection doing quintuplicate injections of 10 ng adenine and tenofovir on column, and 100 ng tenofovir disoproxil and 66.7 ng emtricitabine on column (Figure 2).

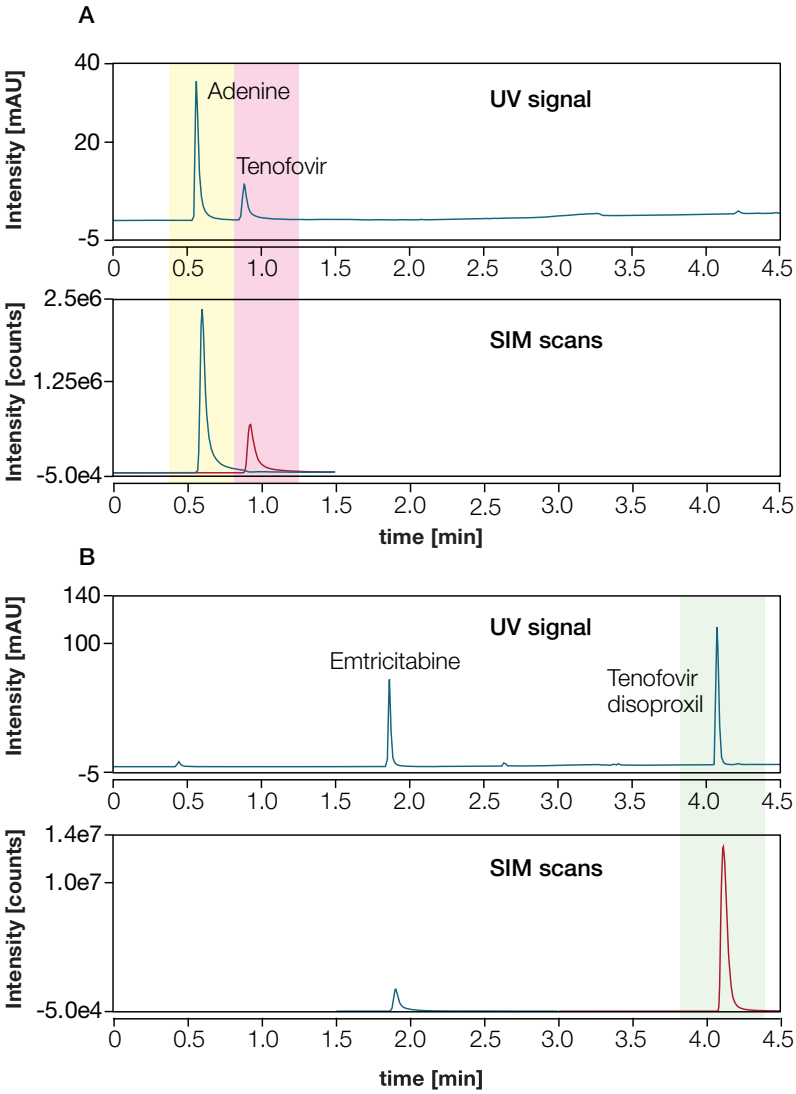

Figure 2. Chromatography of adenine, tenofovir, emtricitabine and tenofovir disoproxil. A) UV chromatogram (top) and SIM scans (bottom) of 10 ng adenine and tenofovir. B) UV chromatogram (top) and SIM scans (bottom) of 100 ng tenofovir disoproxil fumarate and 66.7 ng emtricitabine.

The tailing factor of tenofovir disoproxil was 1.7 with 4.0% RSD. The resolution of adenine and tenofovir was 4.0 (calculation based on Formula 1). Thus, the required suitability thresholds were met. Therefore, the method was considered suitable for impurity analysis.

Formula 1. Resolution according to US Pharmacopeia (USP) (t: time, W: peak width at base).

$$Resolution_{USP} = 2 * \frac{t_{Tenofovir} - t_{Adenine}}{W_{Tenofovir} + W_{Adenine}}$$

Next, the detection limits between UV detection and mass spectrometric detection were compared. Mass spectrometric detectors usually outperform UV detectors in terms of detection limits. Thus, the detection limits of the ISQ EC MS and the Vanquish Flex variable wavelength detector (VWD) were compared. Looking at the signal response of the ISQ EC MS and the VWD revealed differences in detection limits of up to three orders of magnitude (Figure 3). With the VWD, 1 ng of tenofovir on column was measured with a signal-to-noise ratio (S/N; peak to peak) of 10, and 100 pg adenine were detected with S/N 9. So, the limits of detection can be assumed to be 2 to 3 times lower (S/N 3). In single ion monitoring (SIM) mode, 1 pg adenine on column with S/N 10 and 10 pg tenofovir with S/N 7 were measured. Therefore, detection limits are probably 2 to 3 times lower S/N 3.

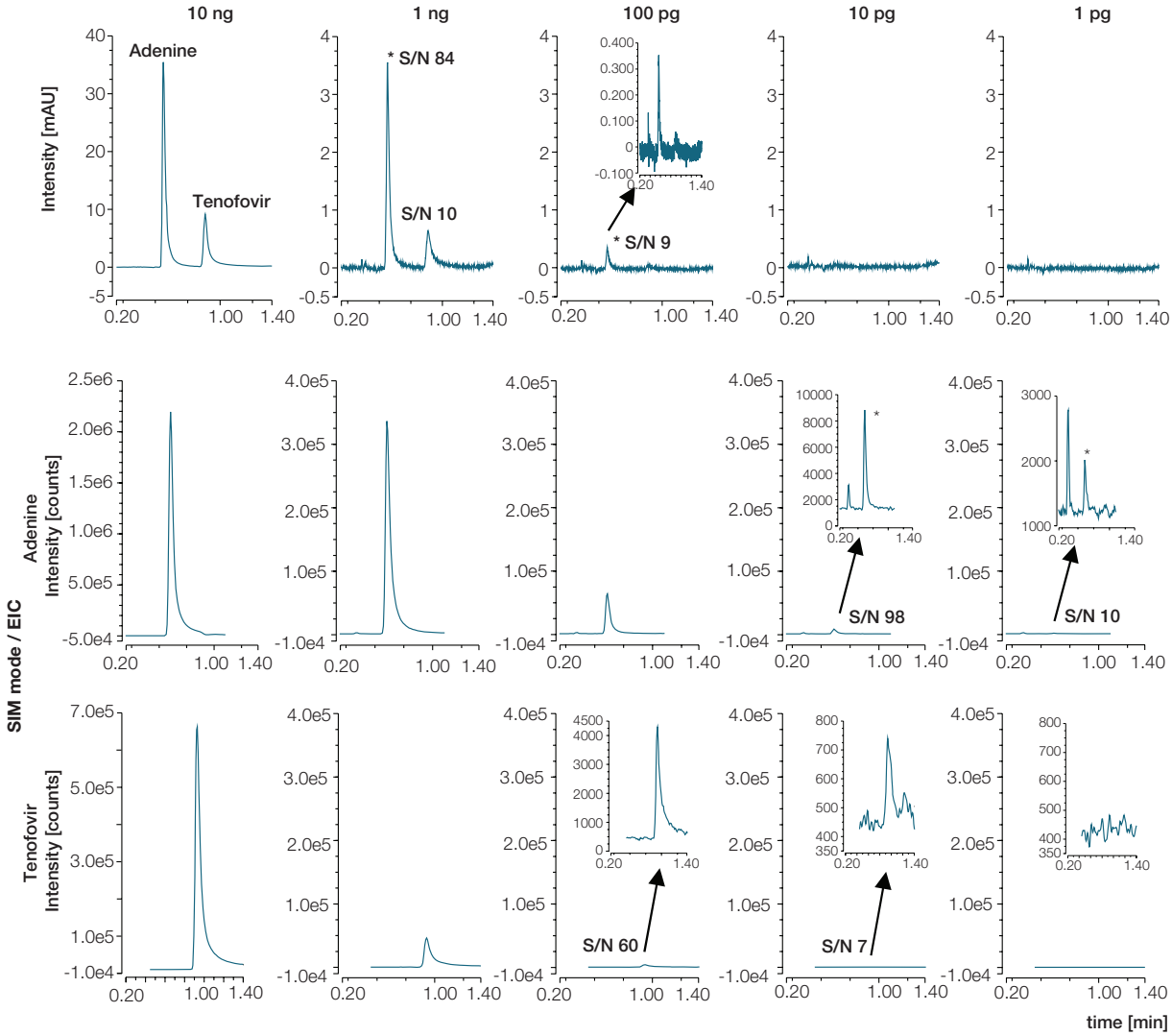

Figure 3. Comparison of signal response between the UV detector and the ISQ EC MS for adenine and tenofovir (EIC: Extracted ion chromatogram; S/N: signal-to-noise calculated by peak-to-peak method).

To prove that the ISQ EC MS can deliver accurate quantification of impurities at a low level, such as adenine and tenofovir levels between 0.01% and 0.2% of tenofovir disoproxil were analyzed. Timed-SIM mode was used for the targeted analysis of adenine, tenofovir, emtricitabine, and tenofovir disoproxil (Figure 4). SIM window (0.6 amu) and dwell time (0.2 s) parameters were selected to increase signal intensity and to assure at least 15 MS scans over the peak for good quantitation results. Full Scan (0.05 s dwell time) was used for determining peak purity and for untargeted background screening.

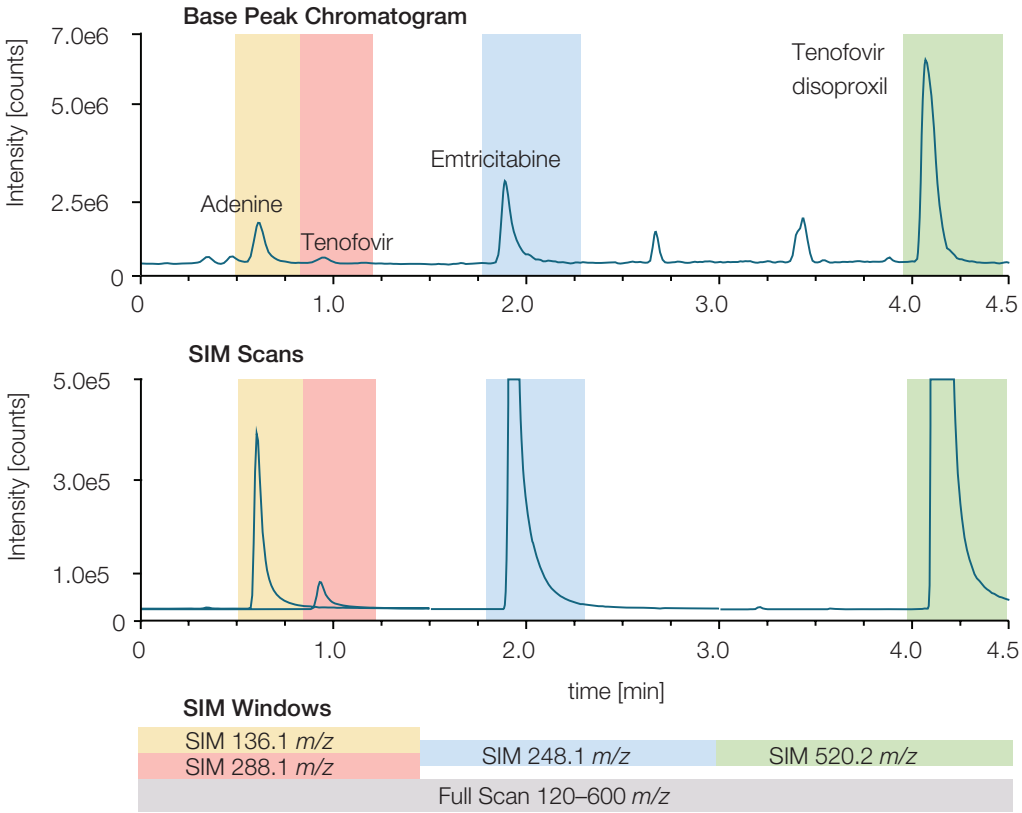

Figure 4. Chromatograms of 1% adenine and tenofovir (1 ppm each) in tenofovir disoproxil fumarate/emtricitabine (100 ppm / 67 ppm) analysis. Top: Base peak chromatogram. Middle: SIM scans. Bottom: SIM Windows - Acquisition windows for SIM scans.

Calibration curves for adenine and tenofovir spanning the relevant sample concentrations were generated (Figure 5). All injections were done in quintuplicate. Afterwards, reinjections of calibrants were done in triplicate to verify the accuracy of the calibration. Adenine and tenofovir showed good recovery rates, deviating by less than 10% at the lowest concentration and less than 5% at all other concentrations. The standard deviation between the reinjection replicates was below 10% indicating high precision (data not shown).

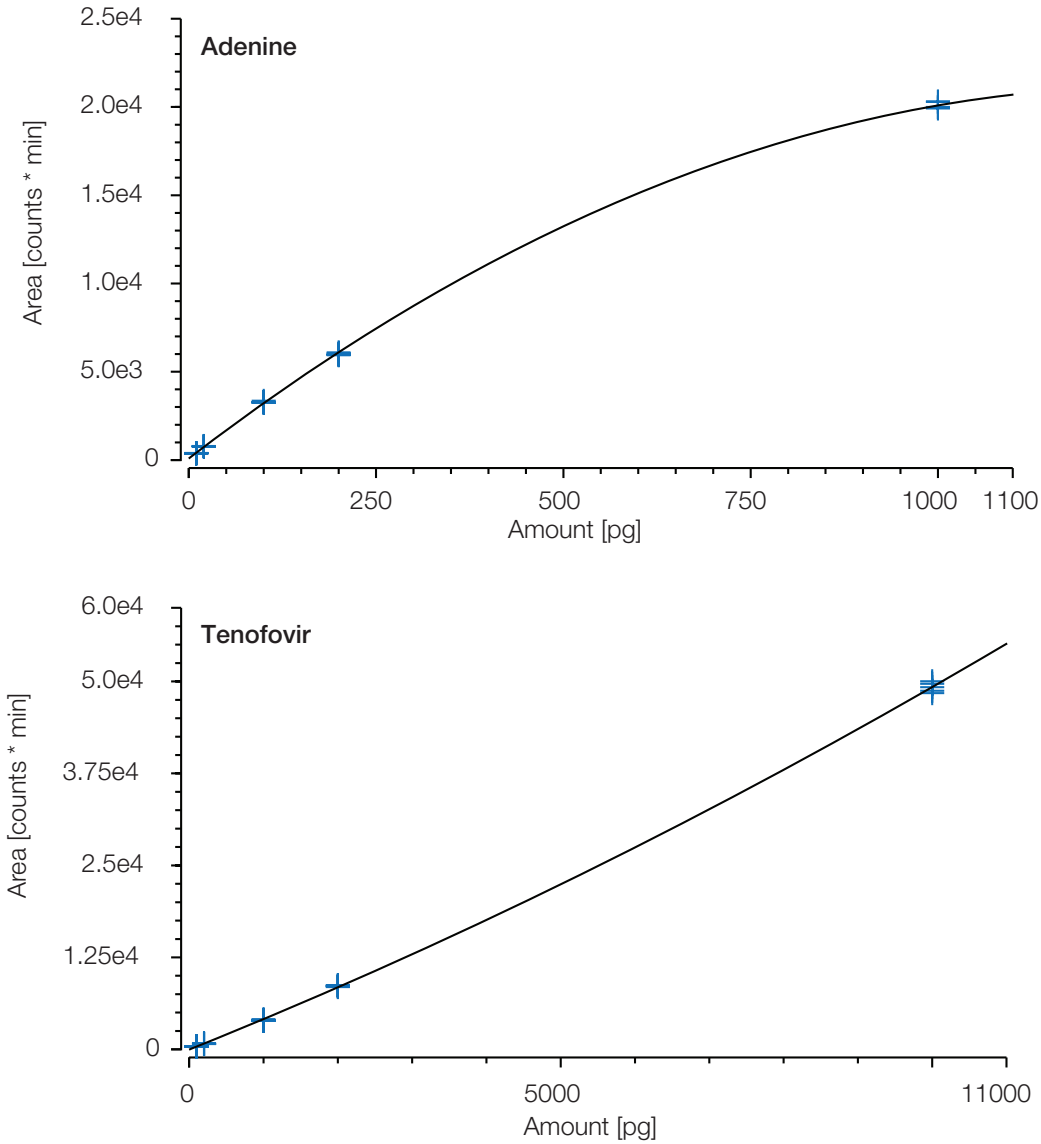

Figure 5. Calibration curves for adenine and tenofovir. Quadratic fit with 1/x weighting was applied. Adenine: 80.7655 + 32.6257x - 0.0126x^2, R^2 = 0.9994; tenofovir: -43.5406 + 4.0614x + 0.0001x^2, R^2 = 0.9996.

Quintuplicate analysis was done for impurity analysis. Adenine and tenofovir were confidently quantified down to an impurity level of 0.01% (Figure 6). Good accuracy was achieved for both compounds (Table 7). High precision was achieved with standard deviations smaller than 5% for most impurity levels. The lowest one showed a standard deviation smaller than 10%.

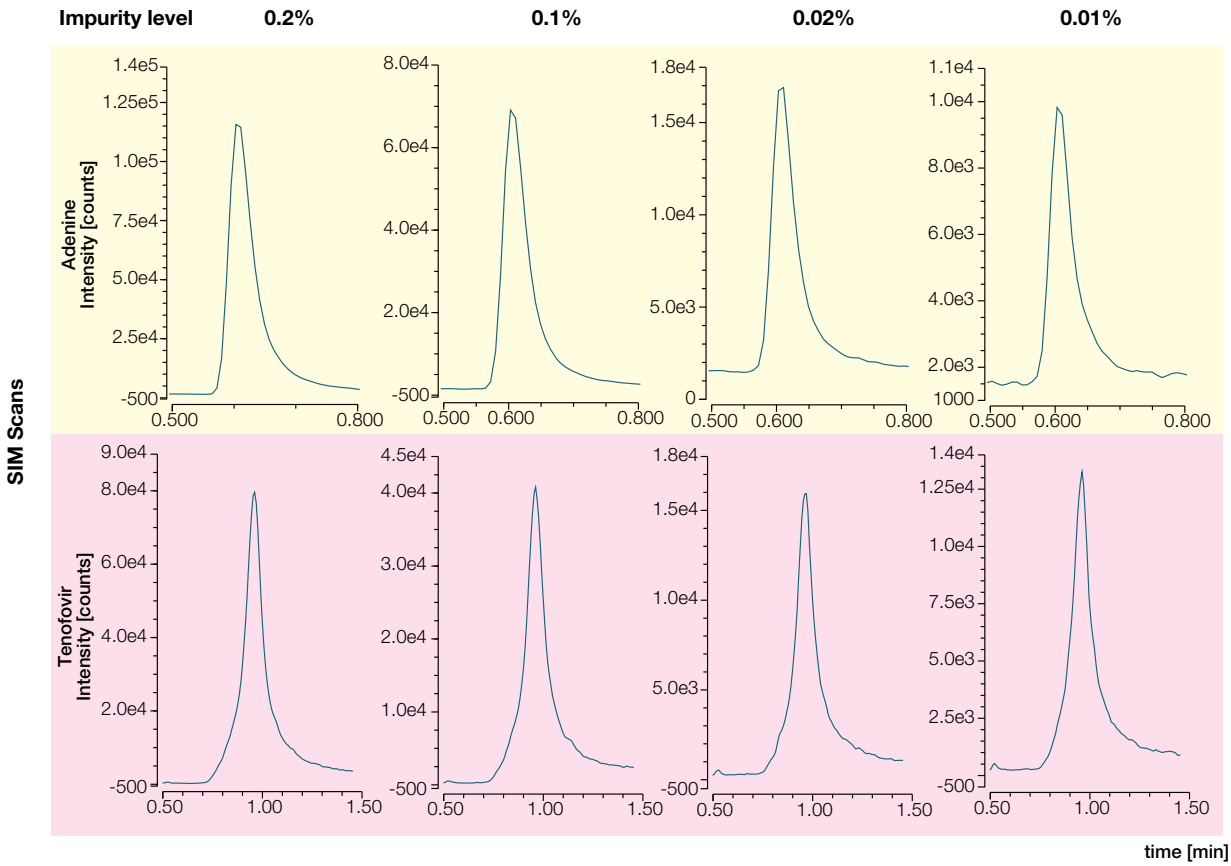

Figure 6 – Extracted ion chromatograms of adenine and tenofovir SIM scans for quantified impurity levels.

Table 7. Recovery rates and standard deviations (SD) for adenine and tenofovir at different impurity levels. Adenine was quantified using 1 µL injections, tenofovir using 10 µL injections to allow sufficient signal response for accurate quantification.

Impurity Level	Adenine Recovery ± SD	Tenofovir Recovery ± SD
0.2%	105.1% ± 0.8%	100.5% ± 2.0%
0.1%	111.3% ± 2.2%	93.7% ± 2.4%
0.02%	111.7% ± 3.7%	92.9% ± 4.4%
0.01%	103.7% ± 6.2%	100.0% ± 9.4%

The existence of additional components co-eluting with the API can be assessed using the Full Scan data which was acquired in parallel to the SIM data. The mass spectra of the front, apex, and tail of the tenofovir disoproxil peak were checked for the presence of additional masses (Figure 7). $[M+H]^+$ of tenofovir disoproxil was the dominant peak. Additionally, the sodium adduct $[M+Na]^+$ was detected (*m/z* 542.0). No other peaks were detected indicating peak purity.

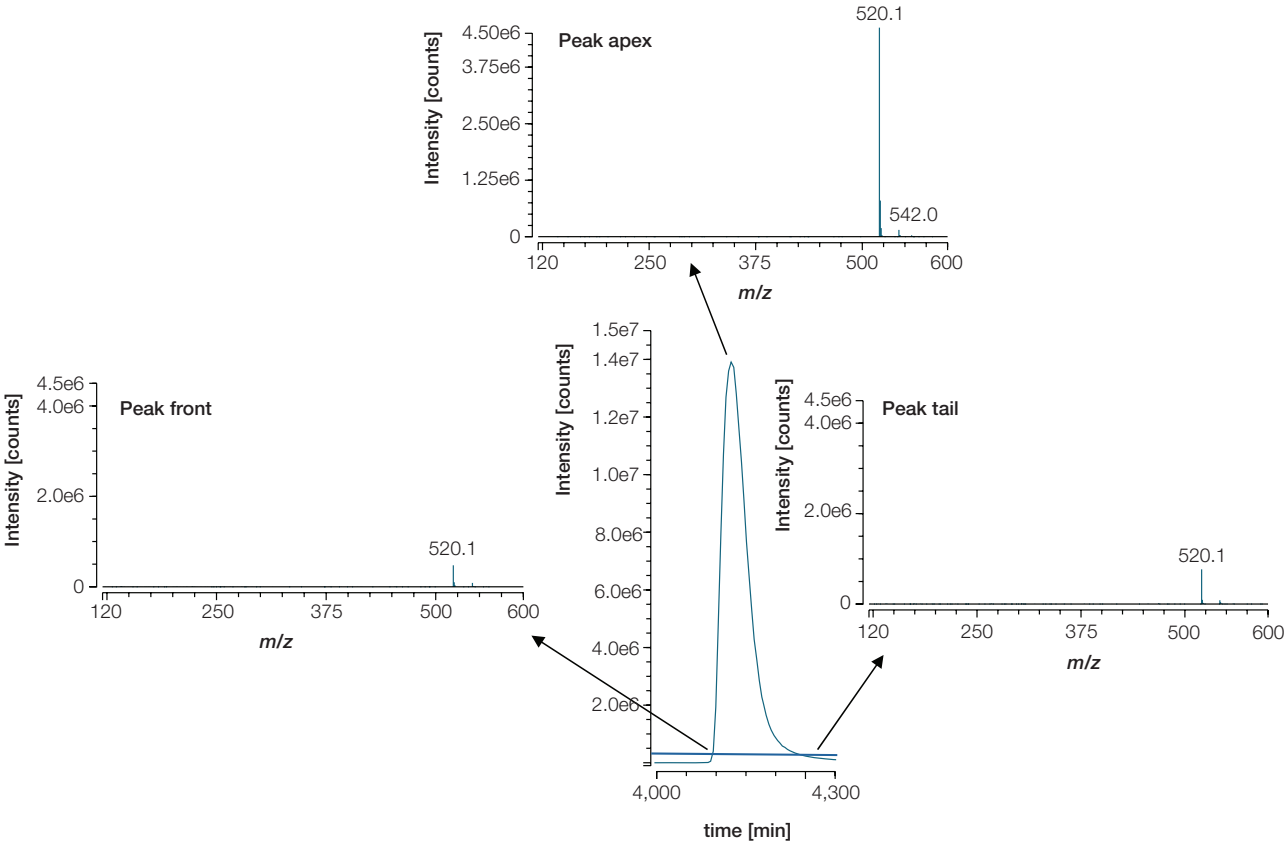

Figure 7. Peak purity analysis of tenofovir disoproxil. Mass spectra of the peak front, peak apex and peak tail of tenofovir disoproxil are shown. 15% peak height was used for the peak front and the peak tail. 1% of the main peak was used as detection threshold.

Another important aspect of impurity analysis is checking whether additional unexpected or unknown impurities are present in the sample. This is done by reviewing the Full Scan data. In the presented work, additional impurities eluting between emtricitabine and tenofovir disoproxil were detected (Figure 8). Combining the mass spectrometric information with impurity information provided by the USP allowed mass confirmation of two impurities: tenofovir isoproxil monoester and tenofovir isopropyl isoproxil. A third one could be identified as tenofovir methyl isoproxil, which is a degradation product formed by the replacement of one of the isoproxils with methanol. The observed masses for all three compound deviated 0.1 amu from the theoretical ones (Table 8).

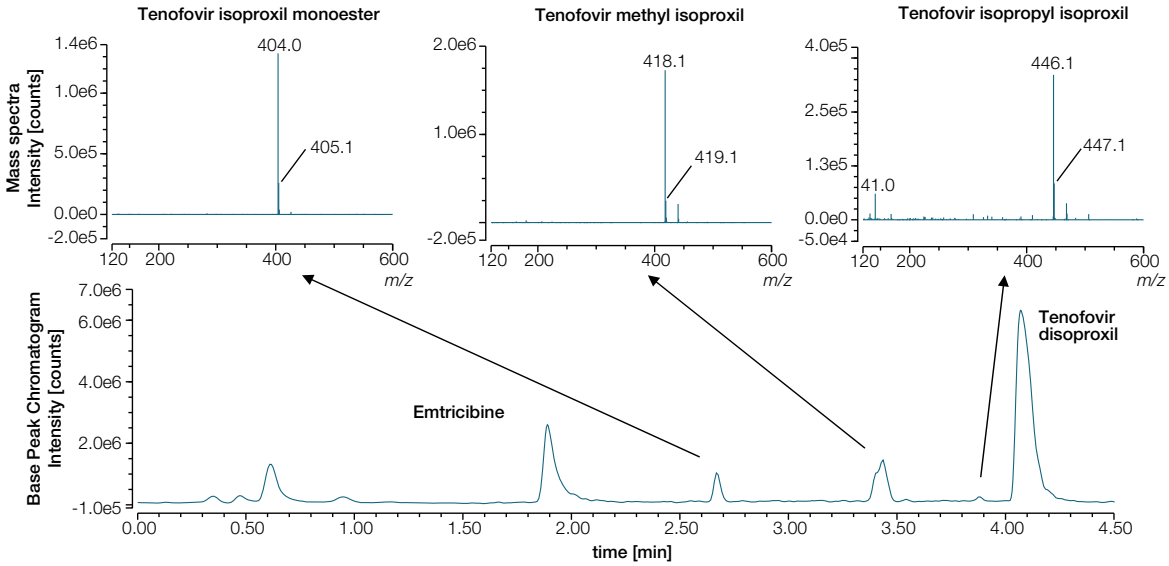

Figure 8. Identification of unknown impurities by mass spectrometric confirmation. Bottom: Base peak chromatogram of 1% adenine and tenofovir in tenofovir disoproxil/emtricitabine. Three additional peaks eluting between emtricitabine and tenofovir disoproxil were detected. Top: Mass spectra of these peaks identifying them as tenofovir isoproxil monoester, tenofovir methyl isoproxil, and tenofovir isopropyl isoproxil.

Table 8. Masses of discovered impurities.

Impurity	Chemical Formula	Theoretical Mass $[M+H]^+$	Observed Mass $[M+H]^+$	Mass Deviation (amu)
Tenofovir isoproxil monoester	$C_{14}H_{22}N_5O_7P$	404.1	404.0	0.1
Tenofovir methyl isoproxil	$C_{15}H_{24}N_5O_7P$	418.2	418.1	0.1
Tenofovir isopropyl isoproxil	$C_{17}H_{28}N_5O_7P$	446.2	446.1	0.1

Conclusion

- Quantitative impurity detection can be done with the ISQ EC single quadrupole mass spectrometer.
- SIM mode greatly increases sensitivity over UV detection and can be used for targeted quantification.
- Full Scan mode results in general detection of present analytes and provides their mass information. This facilitates determination of peak purity and detection of unknown impurities.

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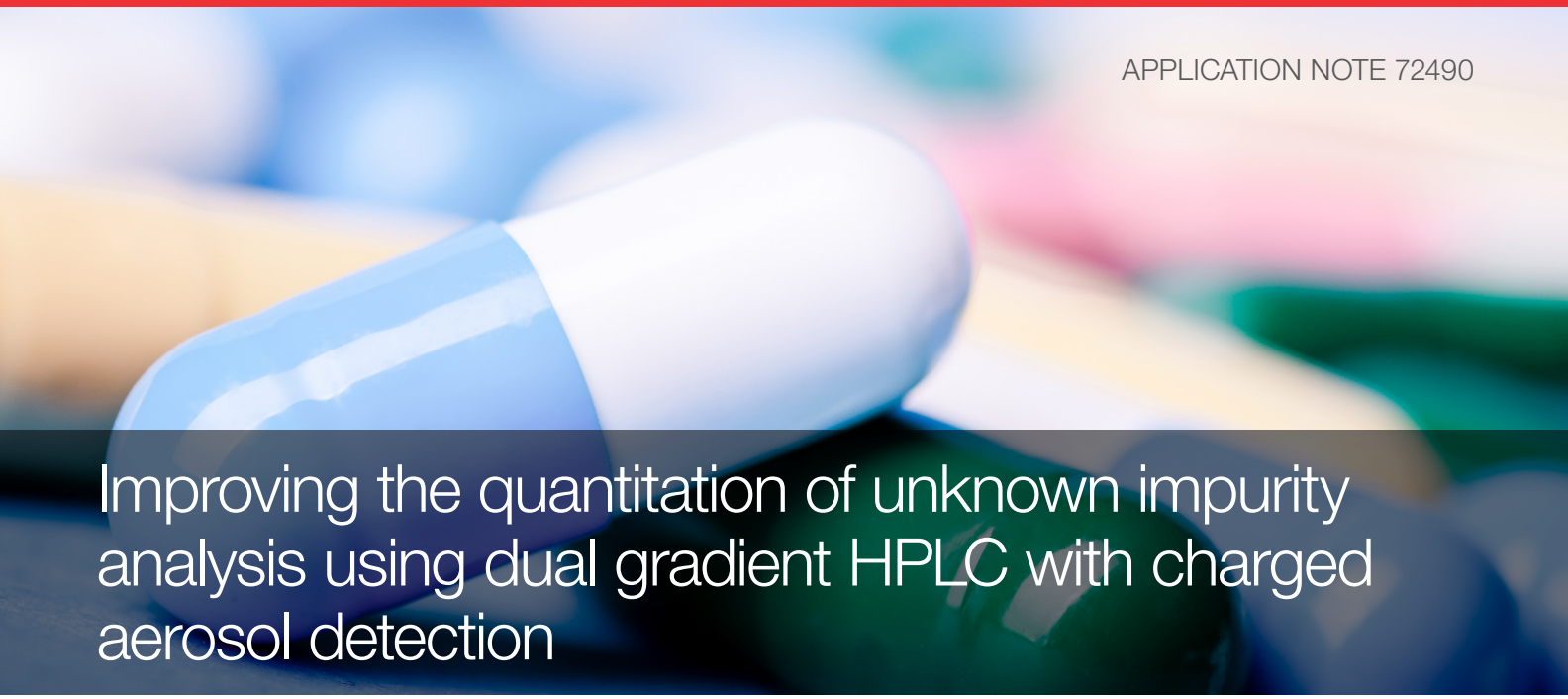
APPLICATION NOTE 72490



Improving the quantitation of unknown impurity analysis using dual gradient HPLC with charged aerosol detection

Author
Thermo Fisher Scientific

Keywords
HPLC, RSLC, charged aerosol detection, impurity analysis, pharmaceutical

Abstract

Quantification of substances, such as drug impurities or library compounds when pure standards are unavailable, is difficult yet often necessary. This is often accomplished by HPLC, based on relative response using low wavelength UV detection. The dependence of response on the optical properties of each component can lead to large errors in estimated quantity.

Charged aerosol detection is a mass sensitive detection technique with near uniform response for all nonvolatile analytes, provided that the eluent remains constant. However, response changes during gradient conditions are common with all nebulization-based detectors. The use of inverse gradient post-column addition can effectively normalize responses. Using a single platform capable of dual gradient HPLC or UHPLC combined with charged aerosol detection allowed a group of compounds ranging in chemical structure and properties, UV absorbance, HPLC retention and application in the pharmaceutical industry to be studied. The response deviation was significantly decreased across the compound set to ~13% compared to the 46% without the inverse gradient applied and >60% with UV-based detection.

The work demonstrated very good correlation in the linear response curves over the range tested. This allows for a single calibrant to be used to calculate the mass concentration of unknown impurities independent of their optical properties. This fully integrated system can be used to improve accuracy for

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Table of contents

mass balance calculations, analysis of impurities and degradants, monitoring compound synthesis and quality of library compounds, and cleaning validations while providing significant cost and time savings with identification and individual standard approaches.

Introduction

Interest in metabolite or trace impurity analysis in pharmaceutical industries is intensifying due to concerns with mass balance studies, regulatory commitments in reporting API impurities, metabolite in safety testing (MIST), and cleaning validation of manufacturing equipment. Most often an analytical requirement for accurately reporting the level of metabolites or impurities is to obtain reference standards. Since many of these standards remain unavailable, it makes exact quantification of impurities and metabolites difficult. The situation is further compounded since several types of HPLC detectors, such as UV or evaporative light scattering detection (ELSD) either lack the sensitivity to detect these compounds or do not provide uniform response across the target analytes.

The development of cleaning validation methods is an area facing similar challenges. The need for a fast turn-around-time of the cleaned equipment to help maintain production schedules does not allow for identification of every peak present. Therefore, quantitation of impurities by UV detection is often done on a peak area bases. The difficulty that can be encountered when using a specific technique like HPLC-UV is how to quantify unknown peaks. UV detectors suffer from varying extinction coefficients for different structures, and thus peak area percent calculations can result in significant errors in impurity calculations. Considering the major difference in UV response between an aromatic active ingredient and a non-aromatic surfactant, such as dodecylsulfate, can result in a potential source of significant underestimation of surfactant contamination. Another HPLC detection technique, evaporative light scattering detector (ELSD), often lacks sufficient sensitivity for trace analysis. Due to the need to optimize methods for different compounds, considerable response factor variation can occur even for compounds within a similar class structure.

The Thermo Scientific™ Corona™ Veo™ charged aerosol detector is mass sensitive and can be added to the traditional HPLC-UV platform. This detector provides the most consistent response across all nonvolatile and some semivolatile analytes of all HPLC detection techniques.[1] When running gradients from low organic to high organic content, all nebulizer-based detectors tend to show increased response as the organic solvent proportion increases due to improved nebulization efficiency. Aerosol-based detection techniques using CAD are also sensitive to this phenomen. Optimization of the detector response by delivering a second post-column solvent stream, which is inverted in composition relative to the elution gradient, enables a constant proportion of organic solvent to reach the detector and results in more uniform response factors for all compounds eluting from the column.[2-4]

This work illustrates the application of a Thermo Scientific™ UltiMate™ 3000 Dual Gradient HPLC system combined with the inverse gradient capillary kit for uniform responses that overcome gradient nebulization issues. To illustrate the power of this approach, its application for the low level quantification of a group of compounds ranging in diverse chemical structure and properties, UV absorbance, HPLC retention and application in the pharmaceutical industry is presented.

Equipment and software

The Thermo Scientific™ UltiMate™ 3000 Dual Gradient Rapid Separation (RS) LC system was used, which includes:

- Thermo Scientific™ UltiMate™ 3000 SRD-3600 Integrated Solvent and Degasser Rack (P/N 5035.9230)
- Thermo Scientific™ UltiMate™ DPG-3600RS Dual Gradient RS Pump (P/N 5040.0066)
- Thermo Scientific™ UltiMate™ 3000 WPS-3000TRS Thermostatted Split-Loop Autosampler (P/N 5840.0020)
- Thermo Scientific™ UltiMate™ TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.000)
- Thermo Scientific™ UltiMate™ DAD-3000RS Diode Array Detector (P/N 5082.0020), equipped with semi-micro flow cell, 2.5 µL, SST (P/N 6082.0300)
- Thermo Scientific™ Corona *ultra*™ Charged Aerosol Detector or equivalent Corona Veo RS Charged Aerosol Detector (P/N 5081.0020)*
- Thermo Scientific™ Viper™ Inverse Gradient Kit, RS System (P/N 6040.2820)
- Thermo Scientific™ Chromeleon™ Chromatography Data System

*Note: Corona Veo Detector was used in this application. For method transfer guidelines, see TN71290.[5]

Methods

Chromatographic conditions	
UHPLC System	UltiMate 3000 Dual Gradient Rapid Separation (RS) LC system (See Figure 1)
Column	Thermo Scientific™ Acclaim™ RSLC 120 C18, 3 µm 120 Å, 3.0 × 33 mm (P/N 066272)
Diode Array Detector	UV at 210 nm and 254 nm
Charged Aerosol Detector	Nitrogen 35 psi; filter, high
Mobile Phases	A) 10 mM ammonium acetate, pH = 4.5 B) Acetonitrile
Flow Rate	1 mL/min from both gradient pumps (2 mL/min to all detectors)

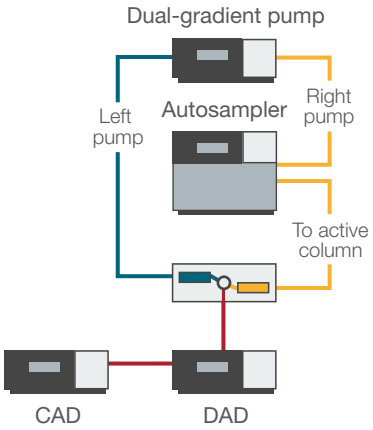

Figure 1. RSLC system flow path with primary column in orange and delay column in blue.

Table 1. Inverted analysis results

Time (min)	Inverse	Analytical
–2.0	98	2
0.102	98	2
3.1	2	98
4.1	2	98
4.6	98	2
5.0	98	2

Method development

The UltiMate 3000 Dual Gradient Rapid Separation (RS) LC system allows a single system to be used for analytical method development. The implementation of an inverse gradient can be achieved by different approaches:

1. Column/flow restriction approach: The delay times of both the primary gradient system with a column and second gradient system with an in-line filter (for pressure restriction) can be calculated. The delay time of the second gradient can then be used to determine the start of the inverse gradient so that it matches the primary gradient.

2. Two identical column approach: Using two identical columns with similar tubing lengths (Figure 1) may be preferable as this removes the need to calculate the delay volume. Both techniques were found to offer similar results (data not shown). The work described here was conducted using the second technique with two identical RSLC columns to test the feasibility of this approach for low level quantification.

Two sets of experiments were designed to test the effect of the inverse gradient first on response variability and then on low level quantification (Table 1):

1. The first experiment used a group of five test standards which were all common APIs with similar retentive properties. These compounds were prepared at approximately equal mass quantities. These samples were injected individually with and without the inverse gradient (Figure 2).

2. The second experiment used a group of nine standard materials selected for their range in chemical composition, molecular weight, industrial use and retention on a C18 column. These standards were then accurately weighed and individually dissolved in either 20% or 80% acetonitrile solutions (depending on solubility) at ~2 mg/mL. Aliquots of these solutions were then combined to give a mixture where each compound had a concentration of ~0.23 mg/mL. Five subsequent dilutions were then made creating six standard solutions from 7 to 230 µg/mL. The effect of the inverse gradient on nebulizer efficiency was measured by the comparison of multiple injections of the standard at 70 ng on column with and without the inverse gradient. The inverse gradient experiment was then used to analyze the standard mix at the six concentration levels.

Results and discussion

Improved response consistency with inverse gradient

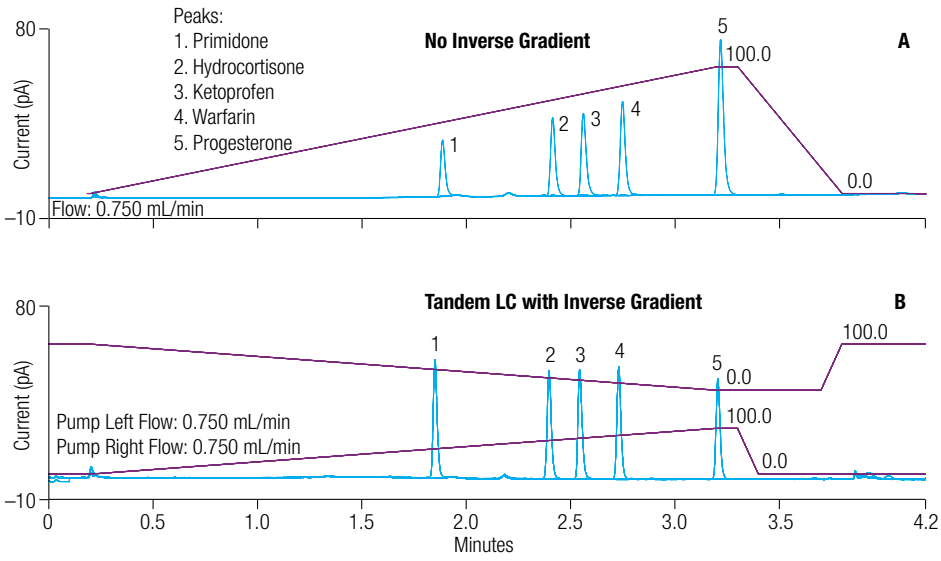

Figure 2. Data illustrating the effect of no inverse gradient vs. with inverse gradient on CAD response for five test compounds (gradients shown are slightly different from the final developed methods described in Figure 1).

An initial study using five test compounds evaluated the gradient effects (25 total injections) on response with and without using an inverse gradient (Figure 2). As expected, compound response improved significantly with the inverse gradient. Even though additional flow was going into the detector, no change in sensitivity for the test compounds was observed since the CAD is a mass sensitive detector and additional solvent does not influence response. Figure 3 illustrates that the CAD response deviation was reduced from 19% to 4.4% RSD by employing the inverse gradient. The response for early eluting compounds (primidone, hydrocortisone, and ketoprofen) was enhanced due to the addition of organic solvent during the inverse gradient. The responses for later eluting compounds (warfarin and progesterone) were decreased as the level of organic solvent going into the CAD was kept at a constant level during the inverse gradient.

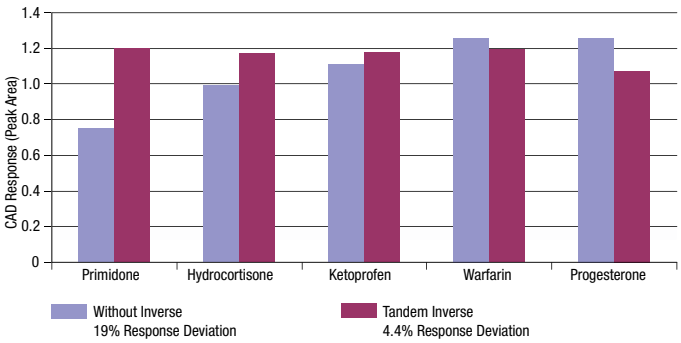

Figure 3. Comparison of the effect of inverse gradient on CAD response (peak area) deviation for five test compounds.

Improved quantification with inverse gradient

Additional experiments using nine different compounds were conducted to see if the charged aerosol detector, when operated with a post-column inverse gradient, could provide a sufficiently uniform response so that a single compound could be used as a calibrant. The method showed excellent resolution and reproducibility (Figure 4).

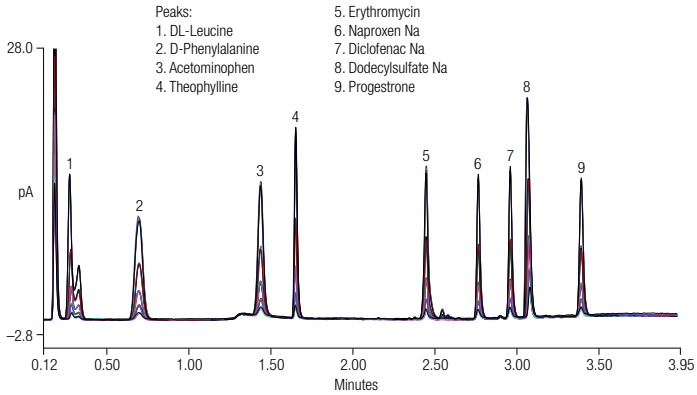

Figure 4. Overlay of five injections of standard mix at each of five concentration levels from 11 to 170 ng on column using charged aerosol detection with inverse gradient (25 total injections).

The response curves for each of the nine components are shown in Figure 5. The correlation coefficients for all nine linear fit curves were ≥0.999. Each curve was used to not only back-calculate the recovery of the standard at 20 ng on column but also to calculate the recovery for the other eight components. The results are shown in Table 2 and color coded according to the deviation from the expected value of 100%. Sixty-six percent of the results had recoveries within 25% of the expected values, and 87% were within 50%. The area result for sodium dodecylsulfate (peak 8) was higher than the rest of the values by ~50%. This peak was also observed in the solvent blank and indicates a potential carryover issue. When the results for sodium dodecylsulfate values were removed, the recoveries improved significantly.

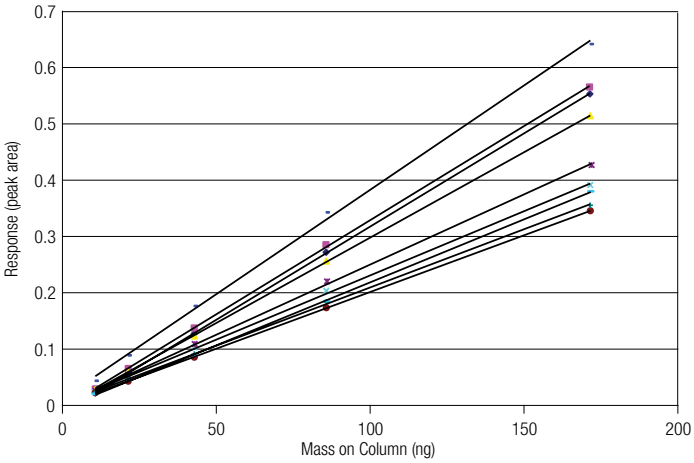

Figure 5. Response curves for data presented in Figure 4. Curve number correlates with the peak number (see Table 2). Identification from top to bottom: 8, 2, 1, 3, 5, 4, 9, 7, and 6.

The data collected at two common UV wavelengths (210 and 254 nm) are presented in Figure 6. No response was detected at either wavelength for components 1, 5, and 8 due to the lack of suitable chromophores. Those area results were assigned a value of zero, and the deviation in area calculations for the nine components was 101% and 125% for the UV at 210 and 254 nm, respectively. If only the compounds with UV chromophores are considered, the CAD results still show twice as much consistency as the UV at 210 nm and three times as much as 254 nm.

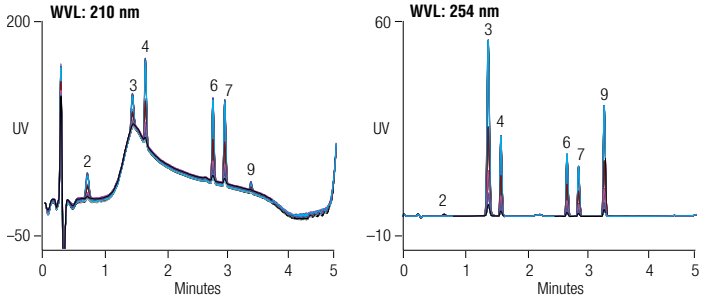

Figure 6. Data presented in Figure 2 for the UV at 210 nm (left) and the UV at 254 nm (right).

Table 2. Recovery of ~20 ng on column each, curves 1–9. Recovery calculated for each of the nine compounds using the nine different response curves. Results are colored according to deviation from expected value as follows: black <2%, purple <10%, blue <25%, green <50%, red >50%.

Compound	Peak #	Curve 1	Curve 2	Curve 3	Curve 4	Curve 5	Curve 6	Curve 7	Curve 8	Curve 9
DL-Leucine	1	100%	88%	97%	112%	106%	133%	124%	57%	131%
Phenylalanine	2	111%	99%	109%	128%	121%	152%	142%	67%	148%
Acetominophen	3	102%	89%	99%	115%	108%	136%	127%	59%	134%
Theophylline	4	89%	77%	85%	96%	91%	115%	106%	47%	115%
Erythromycin	5	93%	81%	89%	102%	97%	122%	113%	51%	121%
Naproxen Na	6	79%	67%	74%	82%	79%	99%	91%	39%	100%
Diclofenac Na	7	85%	73%	81%	90%	86%	109%	100%	44%	109%
Dodecylsulfate Na	8	144%	131%	145%	176%	164%	205%	194%	96%	196%
Progestrone	9	81%	69%	76%	84%	81%	102%	94%	40%	103%

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The UltiMate 3000 Dual Gradient Rapid Separation (RS) LC system equipped with a charged aerosol detector offers a new approach for the measurement of the active ingredients, potential degradants, byproducts and residual chemicals. Traditional approaches require several analytical techniques and often do not provide specific or quantifiable results. Consequently, long periods of time may be required for method development and validation. The approach discussed in this work uses a single HPLC platform and provides methods for quantification of known and unknown, nonvolatile residual materials overcoming many of the limitations found with common approaches. The use of the charged aerosol detector, with the inverse gradient, was shown to have very low response deviation across the mixture of nine compounds. When compared to the UV at either 210 or 254 nm with (101% and 125% RSD, respectively), the Corona CAD (23% RSD) offered a far superior approach. The estimation of unknown compounds by using response curves obtained from known compounds illustrates the power of this technique. By using one generic response curve of a non-volatile compound at known concentration (mass on column), the relative concentration of other material can be calculated.

Conclusion

The use of charged aerosol detection offers increased sensitivity in a more global mass sensitive approach. The LOD (S/N >3) of the compounds used in this study was estimated between 1 to 5 ng on column, while the LOQ (S/N >10) ranged from 6 to 11 ng on column for these test compounds. The application of the inverse gradient with the UltiMate 3000 system overcomes nebulization efficiency issues and allows for quantification of nonvolatile components at trace levels without the need for compound specific standards. The data using CAD clearly illustrates that improved quantitation can be achieved with inverse gradient over UV detection. A low response deviation of only 23% RSD can be obtained using CAD with the inverse gradient to control changes related to nebulization efficiency. The UV detector for the same mixture of nine compounds showed that the deviation in response was >100% RSD. The combination of the dual gradient HPLC and charged aerosol detection technologies presents the opportunity for manufacturers to implement significant cost savings over their current methods.

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APPLICATION NOTE 72398

A highly sensitive high-performance liquid chromatography-charged aerosol detection method for the quantitative analysis of polysorbate 80 in protein solution

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Keywords
Biopharmaceutical, polysorbate 80, Tween 80, protein, charged aerosol detection

Goal

To describe the development of a highly sensitive charged aerosol detection (CAD) method for the determination of polysorbate 80, also known as Tween™ 80, in biopharmaceutical products.

Introduction

Polysorbates, such as polysorbate 20 and polysorbate 80, are non-ionic surfactants. They are commonly used in biotherapeutic formulations to prevent surface adsorption and stabilize proteins against aggregation induced by stress, such as agitation and shear.[1] For quality control purposes, it is important to determine the concentration of polysorbate in the final products. However, the quantitative analysis of polysorbate is challenging—polysorbate is a complex mixture of many different species, which lack natural UV chromophores, and is therefore difficult to analyze by UV detection. Also, chromatographic separation often leads to peaks or peak groupings that consist of many unresolved components and poor peak shapes, thus making accurate and sensitive quantitation problematic.

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UltiMate 3000 RSLC

Table of contents

Chinese Pharmacopia 2015 introduced a derivatization method for the quantitative analysis of polysorbate 80,[2] which is used by manufacturers of protein-based therapies. Such a preparation method can improve the sensitivity of the detection of Tween 80 (Figure 1). However, reagents that are commonly used for the derivatization of Tween 80, such as cobaltous thiocyanate and dichloromethane, are toxic. In addition, the derivatization method is time-consuming, because the pretreatment of the polysorbate takes more than three hours. Further improvements are also needed in terms of sensitivity, accuracy, repeatability, and selectivity versus non-Tween 80 substances in a formulation.

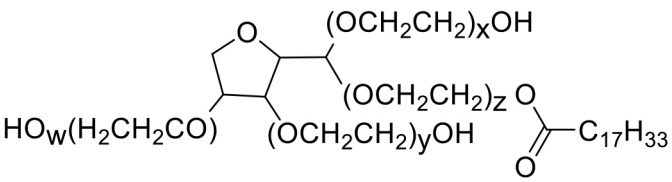

Figure 1. Structure of Tween 80 (x + y + z + w = 20).

CAD is a universal detection technique that can be used to detect non-volatile and some semi-volatile compounds with or without a strong UV chromophore.[3,4] Shi,[1] Fekete,[5] and Dixit[6] developed CAD methods for determining polysorbates in protein formulations. Compared to methods developed with evaporative light-scattering detection (ELSD),[7] CAD, as widely reported, is significantly more sensitive and its response its less dependent on analyte chemical structure.[8,9] The latter is particularly important to achieve accurate quantitation of Tween, since it consists of many different chemical species whose relative concentration can vary widely between manufacturer and lot. In this study, a previously reported method[10] was adapted for the quantitative analysis of Tween 80 using a new generation CAD, the Thermo Scientific™ Dionex™ Corona™ Veo™ detector. The quantitative parameters, including limit of detection (LOD), limit of quantification (LOQ), linearity, and precision, were systematically investigated, and then the method was used for the determination of Tween 80 in a protein formulation.

Experimental

Equipment and software

The Thermo Scientific™ UltiMate™ 3000 Dual Rapid Separation LC (RSLC) system was used, which includes:

- SRD-3600 Integrated Solvent and Degasser Rack (P/N 5035.9230)
- DGP-3600RS Dual Gradient Rapid Separation Pump (P/N 5040.0066)
- WPS-3000TRS Rapid Separation Wellplate Sampler, Thermostatted (P/N 5840.0020), equipped with a 100 µL sample loop and a 100 µL syringe
- TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.000)
- Thermo Scientific™ Dionex™ Corona™ Veo™ RS charged aerosol detector (P/N 5081.0020)
- Thermo Scientific™ Chromeleon™ Chromatography Data System software, version 7.2

Consumables

- Thermo Scientific™ Target2™ Polypropylene Syringe Filters (0.2 µm, 30 mm, P/N 03-376-219)
- Mixed-mode anion exchange column (2.1 × 20 mm, 30 µm)

Reagents and standards

- Deionized (DI) water, 18.2 MΩ·cm resistivity (generated from the Thermo Scientific™ Barnstead™ GenPure™ Pro UV-TOC Water Purification System, P/N 50131948)
- Isopropanol, HPLC Grade (Fisher Scientific™ P/N UN1219)
- Formic acid (FA), MS Grade (Fisher Scientific P/N TS-28905)
- Polysorbate 80, MP Biomedicals (Sigma®)

Preparation of standard solutions

Stock standard 1

Dissolve 10.0 mg of Tween 80 standard in 10 mL of DI water. The concentration of Tween 80 in stock standard 1 is 1000 mg/L.

Stock standard 2

Dissolve 1 mL of stock standard 1 in 10 mL of DI water. The concentration of Tween 80 in stock standard 2 is 100 mg/L.

Mixed standard solutions for calibration and sensitivity

For calibration, prepare 10, 20, 30, 40, and 50 mg/L of Tween 80 by diluting 100, 200, 300, 400, and 500 µL stock standard 2, respectively, with water to 1 mL. Prepare the standard solution for measuring the LOD by diluting 50 µL stock standard 2 with water to 1 mL.

Sample preparation

Dilute 1 mL chimeric anti-EGFR mAb solution (sample 1) to 5 mL with formic acid solution (formic acid/water, 2:100, v/v). All samples were provided by customers.

Sample solution for repeatability

Dilute two batches of protein samples (samples 2 and 3), which contain about 600–700 mg/L tenfold with formic acid solution (formic acid/water, 2:100, v/v), resulting in a Tween 80 concentration in the range of 60–70 mg/L.

Chromatographic conditions			
Column:	Mixed-mode anion exchange (2.1 × 20 mm, 30 µm)		
Mobile Phase:	A: Water (containing 2% (v/v) formic acid) B: Isopropanol (containing 2% (v/v) formic acid)		
Gradient:	*Time, min*	*A, %*	*B, %*
	0	90	10
	1	80	20
	3.4	80	20
	3.5	0	100
	4.5	0	100
	4.6	90	10
	10	90	10
Injection Volume:	30 µL		
Flow Rate:	1.0 mL/min		
Temperature:	30 °C		
Detection:	Evaporative temperature: 35 °C; collection frequency: 10 Hz; filter 5 s.; PFV 1.0		

Results and discussion

Chromatographic condition optimized

A chromatographic method reported previously for analyzing Tween 20 was used for analyzing Tween 80.[10] The resulting chromatogram for Tween 80 is shown in Figure 2. A step gradient was used for the elution of Tween 80 to achieve a sharper peak and higher response due to peak compression. However, a step gradient also contributes to a baseline artifact (Figure 2, red trace). To account for this artifact, a baseline subtraction was used (Figure 3). Except for Figure 2, all figures in this manuscript were obtained with baseline subtraction.

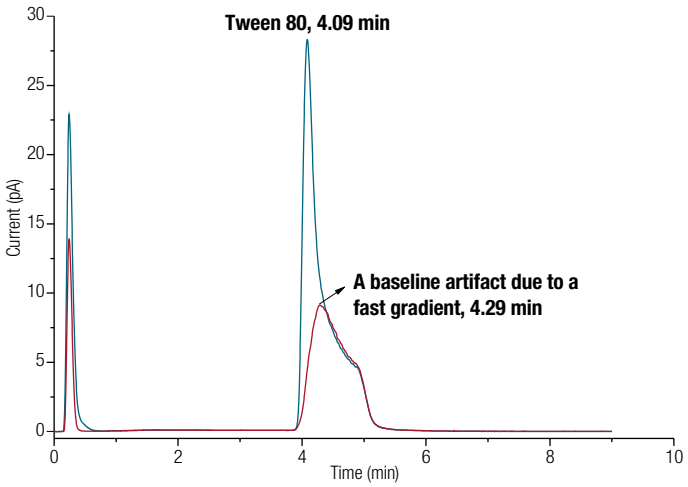

Figure 2. Chromatogram of 50 mg/L Tween 80 (blue trace) and blank (red trace).

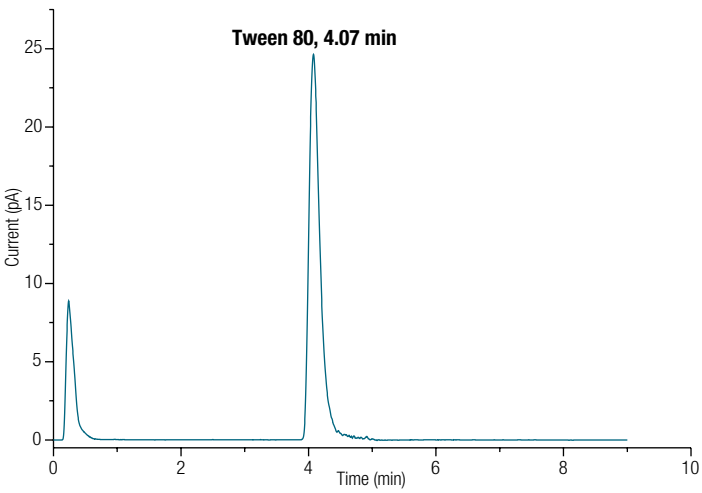

Figure 3. Chromatogram of 50 mg/L Tween 80 with chromatogram subtraction.

Sensitivity and linearity

For the detection of Tween 80 in a narrow concentration range (10–50 mg/L), a linear model can be used to fit to the calibration data. As shown in Figure 4, CAD can provide good linearity ($R^2 > 0.999$) for the detection of Tween 80 with a concentration range from 10–50 mg/L. The LOD and LOQ were taken as the minimum level at which the S/N ratio was above 3 and 10, respectively. The LOD and LOQ of the current method were 5 mg/L (S/N 5.6) and 10 mg/L (S/N 13.6), respectively.

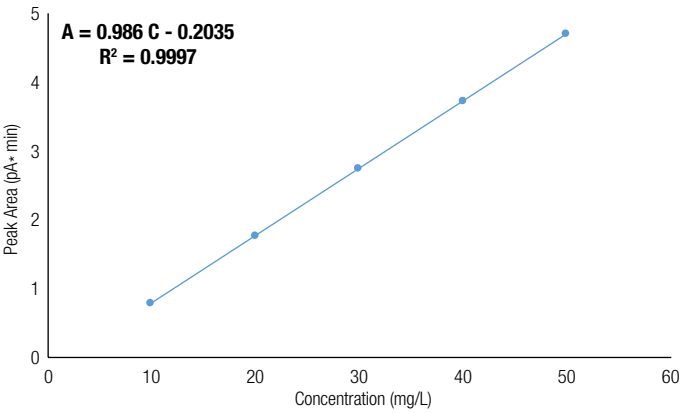

Figure 4. Calibration curve of Tween 80 (10–50 mg/L).

Repeatability

The repeatability of the current method was determined by evaluation of the RSD values of peak areas, which were obtained with five repetitive injections. Two concentrations of polysorbate in protein samples, 67.5 and 70.5 mg/L, were used for testing the repeatability. As shown in Table 1, the RSD values of these two concentrations were no more than 0.7%. This indicates that the current method can provide good repeatability for determining Tween 80 in protein samples.

Table 1. Repeatability (n = 5) of the current method.

Sample	Concentration (mg/L)	Peak Area Repeatability (%)
Sample 2	67.5	0.60%
Sample 3	70.5	0.63%

Sample analysis

Chimeric anti-EGFR mAb sample (Sample 1) was analyzed with the developed method. It can be seen from Figure 5 that Tween 80 can be well separated from the matrix of the protein samples. Almost the entire protein matrix can be eluted close to the dead time of the column due to ion exclusion interactions, since both protein and the column have a cationic group when 2% FA is used as a mobile phase additive. Small molecules such as sorbitol and phosphate, which are commonly used in protein samples, were also eluted close to the dead time due to the very weak hydrophobic retention and ionic repulsive interaction. Thus, many protein formulations can be analyzed by the presented method without any pretreatment. For high concentration samples (greater than 100 mg/L), only dilution was needed before HPLC analysis. The amount of Tween 80 was 105.1 ± 0.06 mg/L in sample 1, which was calculated by the calibration curve described previously. It should also be noted that several complementary approaches using CAD have been described, which provide additional specificity and profiling of polysorbate subspecies.[5,6] These are particularly useful for analysis of more complex formulations, for formulation development and in stability / forced degradation studies.

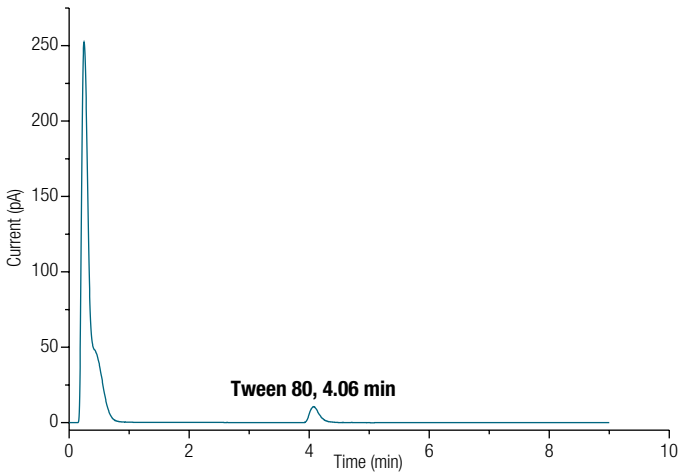

Figure 5. Chromatograms of Sample 1.

Conclusion

An HPLC-CAD method has been developed for the quantitative analysis of Tween 80 in protein formulations. Compared to the ChP 2015 method, the developed method was faster, less toxic, and of higher accuracy. No derivatization and pretreatment was needed and only nine minutes were used for the separation. Thus, the developed method had no pretreatment error. Furthermore, it was more accurate than the ChP 2015 method, since a column separation was used in the current method and there was less matrix disturbance.

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API and Counterions in Adderall® Using Multi-mode Liquid Chromatography with Charged Aerosol Detection

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Key Words
Acclaim Trinity P2, Corona Veo charged aerosol detector; pharmaceutical, Adderall, amphetamine, counterions

Abstract
This work demonstrates the determination of the API (amphetamine) and counterions (aspartate, saccharin and sulfate) in a medicine Adderall® within a single analysis using a Thermo Scientific™ Acclaim™ Trinity™ P2 column and Thermo Scientific™ Dionex™ Corona™ Veo™ Charged Aerosol Detector (CAD).

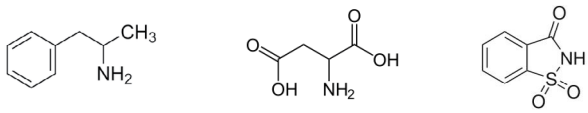
Introduction

Adderall is used to treat Attention Deficit and Hyperactivity Disorder (ADHD) and narcolepsy. It is a formulation of dextro-amphetamine sulfate, dextro-amphetamine saccharate, racemic amphetamine sulfate and racemic amphetamine aspartate monohydrate. The structures of amphetamine, aspartate, and saccharin are shown in Figure 1. The combination of dextro-amphetamine sulfate, dextro-amphetamine saccharate, racemic amphetamine sulfate and racemic amphetamine aspartate monohydrate optimizes the bioavailability profile over time. For quality purposes, it is necessary to measure the mass balance of the APIs versus the counterions. This set of analytes imposes analytical challenges which cannot be met by any reversed-phase columns, and mixed-mode HPLC permits the measurement of all the components in a single run.

The Acclaim Trinity P2 column is based on Nanopolymer Silica Hybrid (NSH™) technology, which consists of high-purity porous spherical silica particles coated with charged nanopolymer particles. The inner-pore area of the silica particles is modified with a covalently bonded hydrophilic layer that provides cation exchange retention, while the outer surface is modified with anion-exchange nano-polymer beads. This chemistry ensures spatial separation of the anion exchange and cation exchange regions.

The Corona Veo Charged Aerosol Detector (CAD) is the third generation of this technology. It converts the column effluent into a dry aerosol and applies an electrical charge to the particles; the amount of charge measured by the electrometer is proportional to the mass of analyte.

The Corona Veo and the diode-array UV detector are used for complementary detection. For the detection of UV-transparent analytes, CAD offers detection limits superior to evaporative light scattering detection, and unlike refractive index detection, gradient compatibility.



Amphetamine | Aspartate | Saccharin

Figure 1: Structures of API and counterions in Adderall®

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UltiMate 3000 HPLC

Table of contents

Experimental Details

Consumables	Part Number
Polypropylene vials, 1.5 mL	079812
Acetonitrile, Fisher Optima™ LC/MS grade	A955
Formic acid, >98%	
Ammonium formate, 99.995%	
(+/-)-Amphetamine, 1.00 mg/mL in methanol, Cerilliant	NC9909702
Deionized water	

Sample Preparation

A standard was prepared to mimic 200 µg/mL Adderall® XR in water. Stock solutions of amphetamine, aspartic acid, sodium saccharin, and ammonium sulfate (each 1000 µg/mL) were diluted in water to make the expected concentrations of 122 µg/mL amphetamine, 24 µg/mL aspartate, 24 µg/mL saccharate, and 26 µg/mL sulfate.)

Separation Conditions		Part Number
Instrumentation:	Thermo Scientific™ Dionex™ UltiMate™ 3000 System	
Column:	Acclaim Trinity P2, 3 µm, 50 × 3 mm	085433
Mobile phase A:	Acetonitrile	
Mobile phase B:	Water	
Mobile phase C:	100 mM ammonium formate, pH 3.65 (6.35 g/L NH_4HCO_2 + 4.5 g/L HCO_2H)	

Time (min)	A	B	C
-8.0	35	59	6
0.0	35	59	6
0.5	35	59	6
5.0	35	0	65
10.0	20	0	80
12.0	20	0	80

Table 1: LC gradient conditions

Flow rate:	0.60 mL/min
Column temperature:	30 °C
Injection volume:	5 µL
Detector 1:	Thermo Scientific™ Dionex™ DAD-3000RS Diode Array 200–400 nm; UV 254 nm shown (data rate 5 Hz, filter 0.5 sec)
Detector 2:	Corona Veo Charged Aerosol Detector (evaporator temperature 55 °C, gas pressure 60 psi, data rate 5 Hz, filter 2 s, power function 1.50)

Data processing

Software:	Thermo Scientific™ Dionex™ Chromeleon™ 6.8 SR13. Blank subtraction applied.

Results

Since Acclaim Trinity P2 columns provide both cation-exchange and anion-exchange retention mechanisms at the same time, they can adequately retain both amphetamine (cationic) and three counterions (anionic) under the same chromatographic conditions. The unique chemistry of the Acclaim Trinity P2 column, in which cation-exchange and anion-exchange regions are spatially separated, allows for great flexibility in method optimization by adjusting mobile phase buffer concentration, pH, and/or organic solvent content. For this particular application, aspartate, saccharin, sulfate and sodium were easily resolved, but amphetamine required certain amount of solvent to elute. Various buffer concentrations, solvent levels and gradient slopes were examined using three mobile phase bottles containing acetonitrile, 100 mM ammonium formate buffer and de-ionized water. The best result was achieved by the condition described in "Separation Conditions", according to the criteria of retention (k > 2), resolution (Rs > 2) and analysis time (<15 min), as shown in Figure 2.

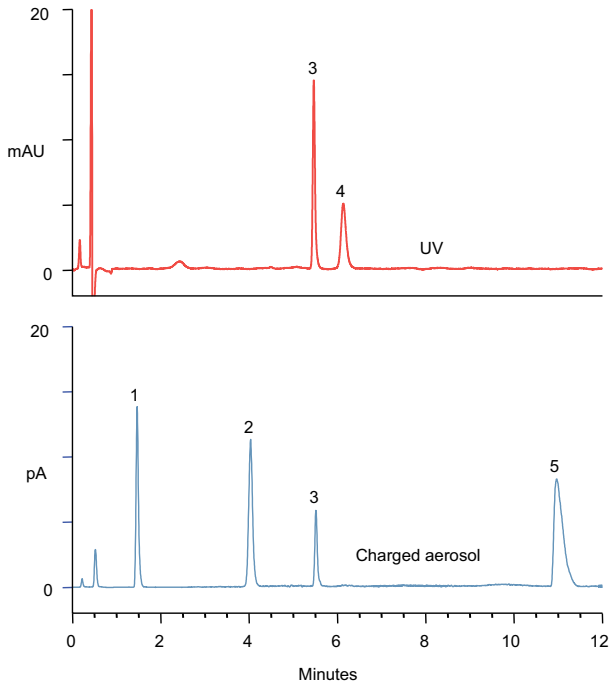

Figure 2: API and counterions of Adderall®.
Peaks: 1. aspartate (24 µg/mL), 2. sodium, 3. saccharin (24 µg/mL), 4. amphetamine (122 µg/mL), 5. sulfate (26 µg/mL)

Conclusion

- The Acclaim Trinity P2 column provides solutions for simultaneous determination of API (amphetamine) and counter-ions (aspartate, saccharin and sulfate).
- The separation is carried out using a simple mobile phase system of acetonitrile and ammonium formate buffer.
- The Corona Veo Charged Aerosol Detector and the UltiMate 3000 Diode Array Detector provide complementary detection for the API and its counterions.

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Application Note 1001

Quantitative Determination of Bisphosphonate Pharmaceuticals and Excipients by Capillary IC-MS

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Key Words

MSQ Plus, Chromeleon, Etidronate, Clodronate, Tiludronate, Excipients, ICS-5000

Goal

To develop and demonstrate a simple, fast, sensitive, and robust analytical method to quantify bisphosphonate pharmaceuticals and common excipients.

Introduction

Bisphosphonates are a group of compounds that are used as active pharmaceutical ingredients (APIs) to treat bone disorders including osteoporosis, Paget's disease, and hypercalcemia.[1,2] Typical methods for bisphosphonates analysis include liquid chromatography (LC) with derivatization and/or ion pairing,[3,4,5] ion chromatography (IC),[6,7] capillary electrophoresis (CE),[8,9] and gas chromatography (GC) with derivatization.[10,11] The reported analytical methodologies for bisphosphonates were summarized and compared in a review article published in 2008.[12] This article concluded that for pharmaceutical purpose quality control (QC), IC with conductivity is "an obvious solution," offering "simplicity, avoidance of derivatization steps, adequate sensitivity and simultaneous separation of ionic impurities." The authors also indicated that mass spectrometry (MS) would be a sensitive approach, but the application to bisphosphonate analysis is limited due to the obvious incompatibility of the ion-pairing agent used in the mobile phase.

Here we present a quantitative method for the direct analysis of bisphosphonates and excipients in pharmaceuticals using capillary IC with suppressed conductivity and mass spectrometric detection. A Thermo Scientific™ Dionex™ IonPac™ AS18-Fast Capillary anion-exchange column is used to achieve chromatographic retention and resolution for target analytes, and the elimination of derivatization steps simplifies the workflow and improves method throughput. The detection by suppressed conductivity provides sufficient sensitivity for QC analysis and MS offers additional selectivity and sensitivity for bisphosphonates in complex matrices, such as biological fluids. An isotope labeled internal standard (IS) citric acid-d_4 is used to ensure quantitation accuracy.

Equipment

- Thermo Scientific™ Dionex™ ICS-5000 Capillary IC* system with eluent generation
- Thermo Scientific™ MSQ™ Plus Mass Spectrometer (single quadrupole)
- Thermo Scientific™ Dionex™ AXP-MS Auxiliary pump (×2)
- Thermo Scientific™ Chromeleon™ Chromatography Data System software 6.8 SR11
- Thermo Scientific™ Xcalibur 2.0.7 with MSQ™ 2.0 SP1

*A Thermo Scientific™ Dionex™ ICS-6000 HPIC system or Thermo Scientific™ Dionex™ ICS-4000 HPIC system can be used for equivalent results

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Table of contents

Reagents and Chemicals

- All chemical standard chemicals were purchased from Sigma-Aldrich unless noted.
- Etidronate disodium hydrate (PN P5248)
- Clodronate disodium (PN D4434)
- Tiludronate disodium hydrate (PN T4580)
- Benzoic acid sodium salt (PN B3375)
- *p*-Hydroxybenzoic acid (PN H5376)
- Citric acid (PN 27788)
- Isotope labeled internal standard citric acid-d_4 (C/D/N Isotopes, Inc., PN D-3745)
- Deionized (DI) water with 18.2 MΩ-cm resistivity
- Acetonitrile (LC/MS grade, Fisher Scientific or equivalent)

Standard Preparation

Prepare individual stock solutions at 1000 µg/mL [parts per million (ppm)] by weighing each pure chemical to the nearest 0.1 mg, and dissolving in DI water. Prepare working standards containing six target analytes (etidronate, clodronate, tiludronate, benzoate, *p*-hydroxybenzoate, and citrate) from individual stock solutions at 10 ppm. Dilute working standard solutions to 1 ppm and 100 parts per billion (ppb) to prepare calibration standards.

Prepare IS stock solution at 1000 ppm in DI water and then dilute to 10 ppm to prepare calibration standards and spike unknown samples.

Prepare calibration standards at 6 levels with each of the target analytes (3 bisphosphonates, 3 excipients) at 5 ppb, 10 ppb, 50 ppb, 100 ppb, 200 ppb, and 500 ppb with IS spiked at 100 ppb in each level.

Sample Preparation

Etidronate disodium 200 mg tablets were supplied by a customer and analyzed in this laboratory. Weigh each tablet individually and calculate the average weight (0.346 g /tablet). Grind tablet samples into fine powder form and weigh three subsamples (10–15 mg each) to the nearest 0.01 mg. Dissolve each subsample in DI water to the concentration of 1.0 mg sample per mL DI water. Sonicate each solution in a water bath at room temperature for 30 min and filter through a 25 mm 0.2 µm PES syringe filter (PALL Life Science, PN 4583T). Dilute 5 µL of each filtrate to 10 mL with DI water and then inject for analysis and quantitation.

Conditions

Chromatographic Conditions

System:	Dionex ICS-5000 capillary IC system with eluent generation
Column:	Thermo Scientific™ Dionex™IonPac™ AS18-Fast Capillary Column (0.4 × 150 mm, PN 072062) Dionex IonPac AG18-Fast Capillary Guard Column (0.4 × 35 mm, PN 072063)
Eluent:	Hydroxide gradient

Time (min)	Concentration (mM)
-4.0	40
0.0	40
5.0	50
8.0	100
13.9	100
14.0	40

Eluent Source:	Dionex EGC-KOH (Capillary) Cartridge (PN 072076)
Flow Rate:	20 µL/min
Injection:	2 µL
Temperature:	40 °C
Detection:	1) Suppressed conductivity with Thermo Scientific Dionex ACES 300 Anion Capillary Electrolytic Suppressor (external water mode, 30 µL/min DI water delivered by AXP-MS pump) 2) MSQ Plus single quadrupole mass spectrometer

Mass Spectrometric Conditions

System:	MSQ Plus mass spectrometer, single quadrupole
Interface:	Capillary low-flow electrospray ionization (ESI) negative polarity
Probe:	MSQ Plus ESI probe with low-flow option (PN 078996)
Probe Temperature:	300 °C
Needle Voltage:	3500 V
Desolvation Solvent:	20 µL/min acetonitrile delivered by a Dionex AXP-MS pump
Nebulizer Gas:	Nitrogen at 65 psi
Acquisition:	Selected ion monitoring (SIM) with cone voltage set at 55 V for each SIM with 0.3 amu span See Table 1 for SIM events details

Table 1. Timed SIM scan events

Analyte	t_R (min)	SIM (*m/z*)	Timed Event (min)	Scan Time (s)
Benzoate	3.9	121	3.6–5.2	0.2
p-Hydroxybenzoate	4.4	137	3.6–5.2	0.2
Citrate	5.8	191	5.2–10.0	0.2
IS (citrate-d_4)	5.8	195	5.2–10.0	0.2
Etidronate	6.6	205	5.2–10.0	0.4
Clodronate	7.3	243	5.2–10.0	0.4
Tiludronate	12.1	317	10.0–14.0	1.0

Results and Discussion

Chromatography

Chromatographic methods have been used extensively for bisphosphonate analysis. Among the reported chromatographic methods included are reversed-phase LC, ion-paring LC, IC, CE, and GC. IC is an obvious method choice because of its ease of configuration, avoidance of derivatization, sensitive detection via suppressed conductivity for charged analytes, and also the capacity for simultaneous determination of impurities such as phosphate and other anionic species. Thus IC was selected as the chromatography method of choice in this study.

A Dionex ICS-5000 capillary IC system was used in this study because capillary IC offers improved sensitivity with injection of the same or less amount when compared to standard IC,[13] and better sensitivity when coupled with a capillary ESI interface to a mass spectrometric detector.[14] A Dionex IonPac AS18-Fast Capillary hydroxide selective anion-exchange column was selected for separation because it offers total resolution of three targeted bisphosphonates (clodronate, etidronate and tiludronate) and the three excipients (citrate, benzoate and *p*-hydroxybenzoate), as well as the seven commonly seen anions. The Dionex IonPac AS18-Fast Capillary column is also a shorter format (150 mm length) than regular 250 mm columns, thus improving method throughput while still offering sufficient chromatographic resolution. The optimized separation is shown in Figure 1: anionic impurities such as commonly seen anions were eluted as early peaks, with phosphate being the latest eluter. Phosphate may be a targeted impurity in a regulated environment, e.g., QC laboratories, and could be easily quantified since it was well separated from other anions. All bisphosphonates and excipient compounds were well separated from anionic species and from each other within a 14 min analytical run, thus allowing simultaneous accurate quantitation of each individual compound.

Mass Spectrometry

ESI is the most commonly used interface to couple IC-MS as it is more suitabile for polar and charged analytes than other atmospheric pressure ionization techniques, i.e., atmospheric pressure chemical ionization (APCI) and atmospheric pressure photo ionization (APPI). Standard ESI interfaces are generally optimized for analytical flow (100 µL to several mL/min) or nanoflow (<1µL/min) ranges. The capillary IC features a flow rate in the range from 10 to 50 µL/min, thus requiring modification and reoptimization of existing ESI interfaces.[15] Here, a standard MSQ Plus ESI probe with low flow option was used, showing significant improvement for low-flow applications,[15] and thus was used for the rest of the study.

The optimization of interface parameters such as probe temperature, nebulizer gas, needle voltage, type of desolvation solvent and the flow rate plays a critical role in establishing instrument sensitivity.[15] The observed optimum conditions are related to specific analytes and applications, thus optimization of interface parameters is highly recommended during method development. A general condition which serves as a starting point for optimization is recommended. When capillary IC is operating at 10 to 20 µL/min, set the probe temperature at 300 °C, needle voltage at 3 KV, nebulizer gas at 65 psi, and use acetonitrile as a desolvation solvent for anionic applications (isopropyl alcohol for cationic applications) at the same flow rate as the capillary IC. For this application, the source parameters are optimized for the best sensitivity of bisphosphonates, and are listed as follows: probe temperature at 300 °C, needle voltage at 3.5 KV, nitrogen gas at 65 psi, and acetonitrile at 20 µL/min.

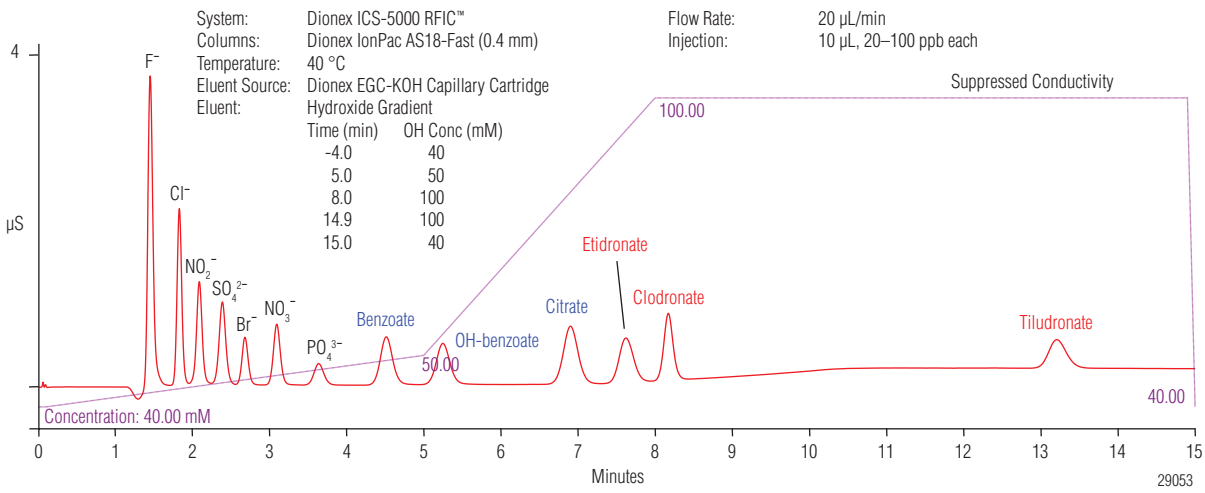


Figure 1. Total resolution of bisphosphonates, excipients and anions

All target analytes predominately show deprotonated molecular ions $[M-H]^-$ in negative polarity, and the respective deprotonated molecular ions were used in the SIM scans for quantitation. As shown in the full scan spectra in Figure 2, the observed pseudomolecular ions for etidronate, clodronate and tiludronate were 205, 243 and 317 *m/z*, respectively. Figure 2 also shows the observed isotopic peaks for clodronate and tiludronate. Matching the observed and theoretical isotope patterns can assist in compound identification or confirmation. The cone voltage of SIM scans was optimized and set at 55 volts, and each SIM scan had a span of 0.3 amu. The details of timed SIM scan events are shown in Table 1. Figure 3 shows the SIM chromatograms of target analytes under optimized conditions, each analyte selectively detected as seen by the single peak in each monitored SIM channel.

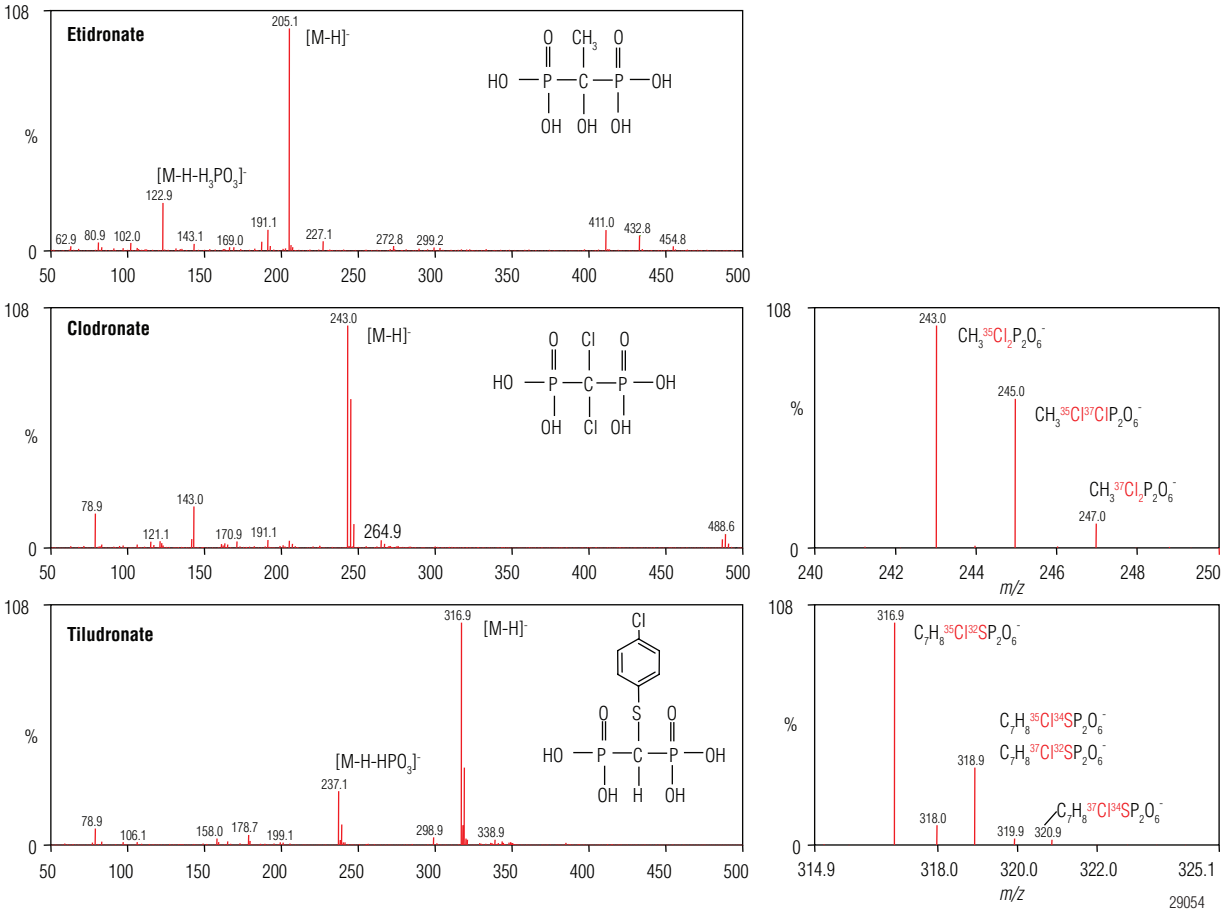
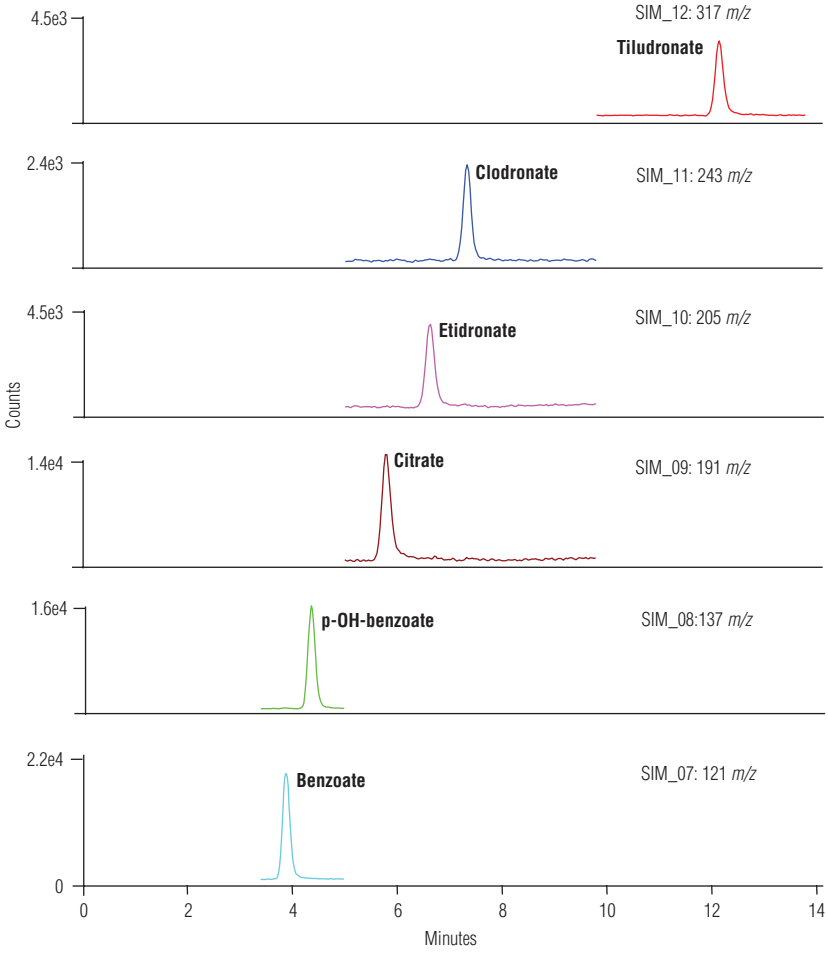


Figure 2. MS Spectra of three bisphosphonate pharmaceuticals



Chromatographic Conditions

System:	Dionex ICS-5000 RFIC
Columns:	Dionex IonPac AS18-Fast (0.4 mm)
Temperature:	40 °C
Mobile Phase:	Hydroxide from Dionex EGC-KOH Capillary Cartridge

Time (min)	OH Conc (mM)
-4.0	40
5.0	50
8.0	100
14.9	100
15.0	40

Flow Rate:	20 µL/min
Injection:	0.2 ng in 2 µL

MS Conditions

System:	Thermo Scientific MSQ Plus Mass Spectrometric Detector
Interface:	Electrospray ionization (ESI)
Probe Temp:	300 °C
Needle Voltage:	3500 V
Solvent:	20 µL/min acetonitrile
Nebulizer Gas:	Nitrogen at 65 psi
Detection Mode:	Selected ion monitoring (SIM)

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Figure 3. SIM chromatograms of bisphosphonates and excipients

Method Performance

Method performance was evaluated against quality parameters such as calibration range, correlation of determination, precision, accuracy, detection limits, and recovery. Additionally, this method was also used to quantify the target analytes in prescription tablets.

Calibration curves were generated from calibration standards with concentration from lower limit of quantification (LLOQ) to 500 ppb. The LLOQ was determined as the lowest concentration in prepared calibration standards that consistently demonstrated a signal-to-noise ratio (S/N) greater than 10 and within 20% bias of quantitation precision and accuracy. The LLOQ of the three excipients was observed at 5 ppb (10 pg injection) and at 50 ppb (100 pg injection) for the three bisphosphonates. The coefficient of determination (r^2) for each analyte was observed at greater than 0.99 with linear or quadratic fit and 1/x weighting factor. The method detection limit (MDL) was calculated by $MDL = S \times t_{99\%,\, n=5}$ where S is the standard deviation and t is the Student's t at 99% confidence interval. The standard deviation was obtained from five replicate injections of 10 ppb (excipients) or 50 ppb standard (bisphosphonates). The MDL was observed in the range from 1.20 ppb (*p*-hydroxybenzoate) to 15.5 ppb (clodronate). Results for above evaluations are listed in Table 2. The precision and accuracy were evaluated at 50 ppb and 500 ppb and the results are listed in Table 3. The precision was addressed by % RSD of three replicate assays and was observed in the range from 0.76 (citrate at 500 ppb) to 7.07 (*p*-hydroxybenzoate at 500 ppb). The accuracy was calculated by *Observed Amount/Specified Amount* × *100%* and was observed in the range from 83% (tiludronate at 50 ppb) to 108% (benzoate at 50 ppb).

This method was applied to the determination of etidronate in a prescription 200 mg etidronate disodium tablet. The sample preparation procedure was as described above. The tablets were quantified at 273 mg/tablet and the deviation was caused by the unknown number of water molecules in etidronate disodium hydrate standard used here, which was treated as anhydrous standard. This tablet sample was used to evaluate method recovery by spiking 100 ppb of each target analyte, and the result is listed in Table 3. The recovery was observed in the range from 89.5% (benzoate) to 134% (clodronate). The deviation of recovery from 100% can be explained by the different extent of matrix effect on the observed MS responses for IS and target analyte. This deviation could be corrected by using isotope-labeled analogues of each target analyte, as excellent recovery was observed for citrate due to the use of citrate-d_4 as internal standard.

Table 2. Calibration range, precision, and MDL

Analyte	Calibration Range	r^2	Fit	% RSD[a] (n = 5)	MDL[b]
Benzoate	5–500	0.9994	Quadratic	8.00	2.48
p-Hydroxybenzoate	5–500	0.9998	Quadratic	5.31	1.20
Citrate	5–500	0.9997	Linear	3.82	1.31
Etidronate	50–500	0.9978	Quadratic	5.33	9.36
Clodronate	50–500	0.9970	Quadratic	10.28	15.50
Tiludronate	50–500	0.9957	Quadratic	4.51	7.19

a: % RSD calculated based on 20 pg injection for benzoate, p-hydroxybenzoate and citrate; 100 pg injection for bisphosphonates

b: Calculated as MDL = S × $t_{99\%, n=5}$ where S is the standard deviation and t is the Student's *t* at 99% confidence interval

Table 3. Accuracy, precision and recovery

Analyte	50 ppb (n = 3)			200 ppb (n = 3)			Original	Observed	% Recovery*
	Mean	% RSD	% Accuracy	Mean	% RSD	% Accuracy			
Benzoate	54.2	2.75	108	499	4.71	99.9	ND	89.5	89.5
p-Hydroxybenzoate	52.4	1.53	105	499	7.07	99.9	ND	93.5	93.5
Citrate	49.4	2.58	98.7	497	0.76	99.5	ND	102	102
Etidronate	43.4	4.27	86.8	498	2.18	99.5	424	542	117
Clodronate	44.8	4.30	89.7	498	1.05	99.6	ND	134	134
Tiludronate	41.5	3.26	83.0	497	1.28	99.4	ND	121	121

Unit shown in ppb

* Recovery calculated based on [observed amount (original sample + 100 ppb spiked each analyte) – original amount]/100 × 100%

Conclusion

This study described a capillary IC-MS method for the simultaneous quantitation of three bisphosphonate pharmaceuticals (etidronate, clodronate and tiludronate) and three commonly used excipients (benzoate, hydroxybenzoate and citrate). Sensitive and selective quantitation can be achieved at as low a level as 5 ppb for excipients and 50 ppb for bisphosphonates using SIM acquisition within a 14 min run time. This configuration also provides confirmative information, such as molecular ions and isotope patterns for identity confirmation. This method was successfully applied for the analysis of etidronate disodium tablet samples.

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Method development and transfer

It is crucial that existing methods can be adapted to take advantage of the improvements in instrumentation and associated consumables. Laboratories need the ability to transfer from outdated legacy instruments to replacements offering enhanced analytical speed and detection sensitivity.

It is also common for laboratories to transfer methods between different instrument types, including those from different vendors.

Method transfer is complex and relies on robust method development. Adaptable instruments, able to accommodate for differences between systems, help to simplify the process.

Chapter highlights

Easily convert HPLC methods to UHPLC to speed up analysis and streamline chromatography, with simplified routine quantitative mass spectrometry workflows using **Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software**

For LC or LC-MS solutions, the flexibility of the **Thermo Scientific™ Vanquish™ Flex UHPLC Systems**, with binary or quaternary solvent blending, drives innovation without compromising quality

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Table of contents

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APPLICATION NOTE 72716

Method transfer of a USP-derived acetaminophen assay from an UltiMate 3000 SD system to a Vanquish Flex UHPLC system

Authors
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Keywords
HPLC method transfer, Vanquish Flex quaternary system, UltiMate 3000 Standard quaternary system, gradient delay volume, acetaminophen

Application benefits

- Flexible system volume adjustment in Thermo Scientific™ Vanquish™ UHPLC systems facilitates straightforward transfer of analytical HPLC methods from Thermo Scientific™ UltiMate™ 3000 HPLC systems
- Fine tuning of retention times can be achieved by adjustment of the idle volume of the autosampler metering device

Goal

To demonstrate the straightforward transfer of analytical HPLC methods between the UltiMate 3000 platform and the Vanquish platform.

Introduction

The transfer of analytical liquid chromatographic (LC) methods from one instrument to another is a frequent but challenging task in most industries and is of particular importance in regulated environments.[1,2] Transfers are performed between identical instruments as well as instruments of different configurations, vendors, or generations. The true complexity thus is highly dependent on the robustness of the method that needs to be transferred as well as on instrumentational differences of the systems in concern.[1,2] To obtain equivalent results with the sending and receiving systems, specific technical characteristics like gradient delay volume (GDV), hydrodynamic

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Table of contents

behavior, or thermostatting mode need to be accounted for, preferably without modification of method parameters to avoid elaborate revalidation.[3]

According to this, the current application note demonstrates the straightforward transfer of a USP-derived assay[4] of the active pharmaceutical ingredient (API) acetaminophen, a common pain killer, and its impurities from an UltiMate 3000 SD system to a Thermo Scientific™ Vanquish™ Flex UHPLC system by means of unique features of that platform.

Experimental

Reagents and materials

- Deionized water, 18.2 MΩ·cm resistivity or higher
- Fisher Scientific™ Optima™ Methanol, LC/MS grade (P/N 10767665)
- Fisher Scientific™ Sodium phosphate dibasic anhydrous (P/N 10182863)
- Fisher Scientific™ Potassium dihydrogen orthophosphate (P/N 10429570)
- Acetaminophen, 4-aminophenol, N-(4-hydroxyphenyl) propanamide (impurity B), 2-acetamidophenol (impurity C), acetanilide (impurity D), and 4'-chloracetanilide (impurity J) were purchased from reputable vendors.

Sample preparation

Stock solutions of acetaminophen (20 mg/mL), 4-aminophenol, and the impurities B, C, D, and J (1 mg/mL each) were prepared in methanol. By dilution with methanol and mixing of stock solutions, a sample was prepared that contained 1 mg/mL acetaminophen and 10 µg/mL of each of the other compounds (corresponding to 1% of the API).

Instrumentation

See Table 1 for the instruments used in this study.

LC conditions

Column:	Thermo Scientific™ Hypersil GOLD™ C8, 4.6 x 100 mm, 3 µm, 175 Å (P/N 25203-104630)
Mobile phase:	A: 1.7 g/L KH_2PO_4 and 1.8 g/L of Na_2HPO_4 in water B: Methanol
Flow rate:	1 mL/min
Gradient:	0 min 1% B 3 min 1% B 7 min 81% B 7.1 min 1% B 12 min 1% B
Column temp.:	35 °C (with or without eluent pre-heating)
Autosampler temp.:	8 °C
Detection:	230 nm, 10 Hz data collection rate, 0.5 s response time
Inj. volume :	1 µL
Needle wash:	Off

Table 1. Instruments used in this study

Standard configurations		
	UltiMate 3000 SD Quaternary	**Vanquish Flex Quaternary**
		System Base (P/N VH-S01-A-02)
Pump	Standard Quaternary Pump LPG-3400SD (P/N 5040.0031)	Quaternary Pump F (P/N VF-P20-A)
Sampler	Well Plate Autosampler WPS-3000TSL (P/N 5822.0020)	Split Sampler FT (P/N VF-A10-A)
Column Compartment	TCC-3000SD (P/N 5730.0010) with or without 7 µL eluent pre-heater (P/N 6722.0540)	Column Comp. H (P/N VH-C10-A)
Detector	Diode Array Detector DAD-3000 (P/N 5082.0010)	Diode Array Detector FG (P/N VF-D11-A)
Flow Cell	Analytical (10 mm, 13 µL, P/N 6082.0100)	Standard Bio (10 mm, 13 µL, P/N 6083.0540)
Modifications applied for method transfer		
		Modify idle volume from default 25 µL

Data processing and software

Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) 7.2.8 was used for data acquisition and analysis.

Results and discussion

All method transfer experiments were conducted with the same column and sample, with consistent method parameters and seven repeated injections. The chromatograms in Figure 1 display the starting situation for the transfer from the UltiMate 3000 SD system to the Vanquish Flex system (both quaternary). The corresponding retention times are summarized in Table 2. As was shown in a recent Thermo Scientific Application Note,[5] the effect of eluent pre-heating on retention times is not negligible, even not at moderate separation temperatures. Hence, a method transfer should be conducted under equivalent conditions. A method that is run on an UltiMate 3000 SD system in standard configuration (i.e. without eluent pre-heater) should be transferred to a Vanquish system with a disabled active pre-heater. If the UltiMate 3000 SD system was equipped with a 7 µL pre-heater, the active pre-heater, which is included in the Vanquish standard configuration, should be enabled for the Vanquish system. Both situations are covered in Figure 1. All gradient eluted peaks (2–6) exhibit earlier retention times with the Vanquish system. This is not surprising and can be attributed to the slightly smaller GDV of the Vanquish system. However, differences in the isocratic elution of the first peak (aminophenol) are not induced by gradient effects and might be the result of minor temperature differences or slight differences in the proportioning of the isocratic conditions with 1% of mobile phase B.

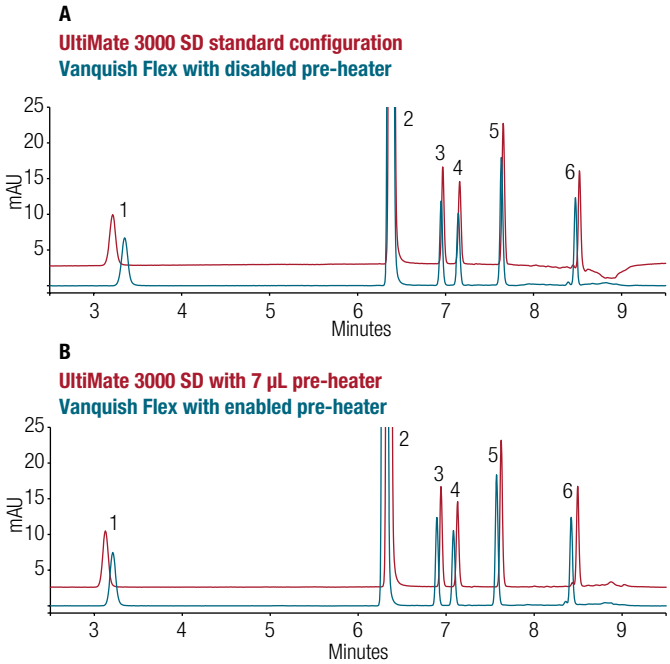

Figure 1. Starting situation of the method transfer. A) Comparison of UltiMate 3000 SD system and Vanquish Flex system without eluent pre-heating; B) Comparison of UltiMate 3000 SD system and Vanquish Flex system with enabled eluent pre-heating. For peak assignment see Table 2.

Three actions can be taken and combined to physically adapt the GDV of the Vanquish system to emulate the originating system:

1. The unique feature of adjustable idle volume of the autosampler metering device (the unit conducting sample aspiration) can be used to fine tune the GDV.
2. If the range of idle volume is not sufficient for the GDV difference compensation, the standard sample loop is replaceable by a larger volume one.
3. If GDV differences of major amount have to be emulated, a change of the static pump mixer should be considered.

Table 2. Averaged retention times in minutes over seven injections for the systems and configurations stated in Figure 1 and % deviation of Vanquish Flex target systems from originating UltiMate 3000 configurations

Peak No.	Compound	UltiMate 3000 SD w/o pre-heating	Vanquish Flex, pre-heater off	UltiMate 3000 SD w/ pre-heating	Vanquish Flex, pre-heater on
1	4-Aminophenol	3.21	3.35 (Δ -4.2%)	3.13	3.21 (Δ -2.6%)
2	Acetaminophen	6.39	6.37 (Δ 0.2%)	6.34	6.30 (Δ 0.7%)
3	Impurity B	6.97	6.95 (Δ 0.3%)	6.94	6.90 (Δ 0.7%)
4	Impurity C	7.16	7.14 (Δ 0.2%)	7.13	7.08 (Δ 0.6%)
5	Impurity D	7.66	7.63 (Δ 0.3%)	7.63	7.57 (Δ 0.7%)
6	Impurity J	8.52	8.47 (Δ 0.5%)	8.50	8.42 (Δ 0.9%)

An overview on respective volume ranges is given by Table 3. However, in the current case idle volume adaption was already sufficient. The very good retention time matches achieved are displayed in Figure 2. Without pre-heating idle volume adaption from default 25 µL to 53 µL was successful, with enabled pre-heating the idle volume was set to 79 µL. As expected, the peak that elutes under isocratic conditions was not affected by the adjustments, while gradient-eluted peaks were shifted accordingly, resulting in relative retention time differences of <0.3 % for these peaks with respect to the UltiMate 3000 SD chromatogram. The applied modulations are in full agreement with the allowed adjustments according to the USP General Chapter <621>, which states: "If adjustments are necessary, a change in […] the duration of an initial isocratic hold (when prescribed), and/or the dwell volume are allowed."[6]

All described systems (either with or without retention time adaption) easily pass the USP system suitability criteria, with a resolution of the critical peak pair of impurities B and C of 3.2 or larger, tailing factors from 0.99 to 1.1, and a relative standard deviation of peak heights of less than 0.5%. The relative areas of all impurity peaks were constant over all instruments, but signal-to-noise ratios improved during the transfer to the Vanquish Flex system (Figure 3).

Table 3. Overview of options for GDV adjustments with the Vanquish platform

1) Autosampler metering device	Adjustable 0–100 µL (default 25 µL)
2) Sample loops	10 µL (V=23 µL, P/N 6850.1915) 25 µL (default, V=50 µL, P/N 6850.1911) 100 µL (V=130 µL, P/N 6850.1913)
3) Pump mixer kits	Available with total volume of: 35 µL (P/N 6044.3870) 100 µL (P/N 6044.5100) 200 µL (P/N 6044.5110) 400 µL (P/N 6044.5310, default in quaternary pump) 800 µL (P/N 6044.5750A) 1550 µL (P/N 6044.5450A)

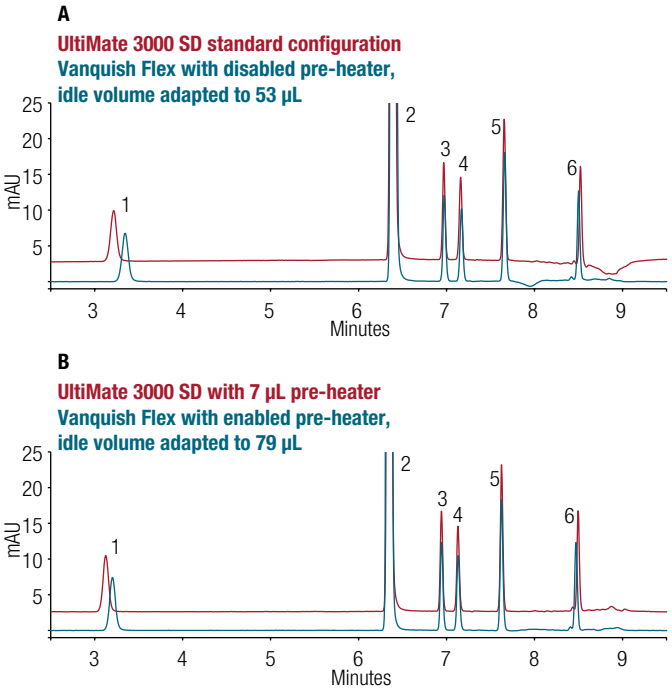

Figure 2. Method transfer from UltiMate 3000 SD system to Vanquish Flex system by idle volume adaption. A) Both systems without eluent pre-heating, idle volume adapted to 53 µL; B) both systems with enabled eluent preheating, idle volume adapted to 79 µL. For peak assignment see Table 2.

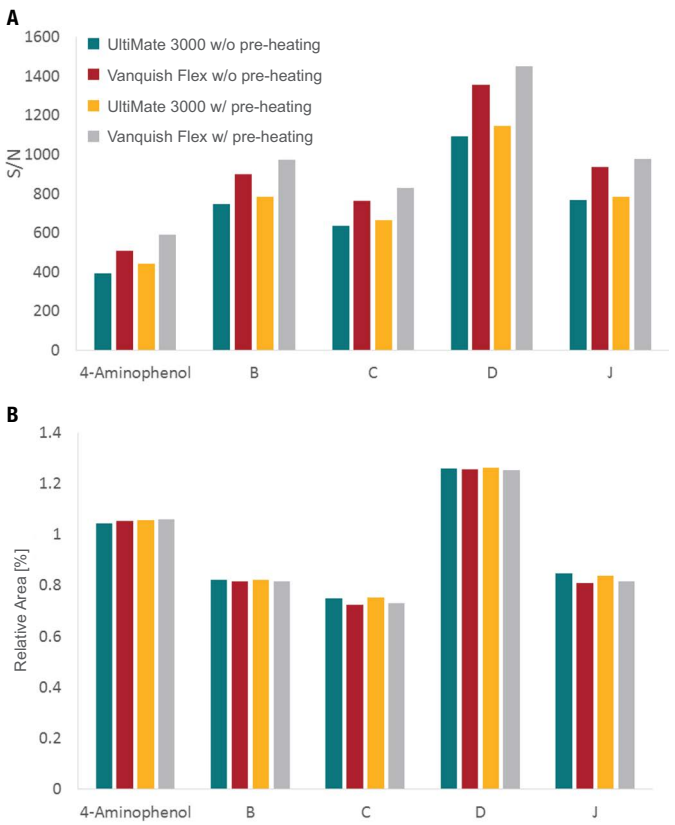

Figure 3. Averaged signal-to-noise ratios (A) and relative peak areas (B) for the originating UltiMate 3000 SD system with or without eluent pre-heater and the receiving system Vanquish Flex system with enabled or disabled eluent preheating. Noise calculated from the current chromatogram 4.1–4.6 min

In the context of method transfer, it is worthwhile to mention another unique feature of the Vanquish platform; that is the switchable thermostatting mode of the column compartment. The current experiments on the Vanquish system were conducted in forced air mode, as this reflects best the UltiMate 3000 column compartment. In a recent Application Note,[5] it was already shown that the type of thermostatting mode has a negligible effect on the acetaminophen assay. However, in applications where frictional heating of the column becomes significant due to high applied pressures, the thermostatting mode in the column compartment (still or forced air) can be important.[3]

Conclusions

- During method transfer of an acetaminophen assay from an UltiMate 3000 SD HPLC system to a Vanquish Flex UHPLC system (both quaternary), straightforward retention time matches were achieved by adjustments of the idle volume of the autosampler metering device.
- Critical chromatographic results like resolution of critical peak pair, peak asymmetries, peak height precision, and relative peak areas were easily maintained during transfer from one HPLC platform to another. Signal-to-noise ratios improved during the transfer.
- Unique features of the Vanquish platform such as the adjustable autosampler idle volume and switchable column thermostatting and eluent pre-heating modes provide helpful tools in the process of method transfer.

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APPLICATION NOTE 72717

Method transfer of a USP derived acetaminophen assay from an Agilent 1260 Infinity system to an UltiMate 3000 SD system and a Vanquish Flex UHPLC system

Authors
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Keywords
HPLC method transfer, Vanquish Flex quaternary system, UltiMate 3000 Standard quaternary system, Agilent 1260 Infinity quaternary system, gradient delay volume, acetaminophen

Application benefits

- Flexible system volume adjustment in Thermo Scientific™ UltiMate™ 3000 systems and Thermo Scientific™ Vanquish™ UHPLC systems facilitate straightforward transfer of analytical HPLC methods.
- Fine tuning of retention times can be achieved by shifting the gradient start relative to injection time.
- If detection sensitivity is a critical issue, Thermo Scientific™ LightPipe™ technology provides an excellent remedy.

Goal

To demonstrate the straightforward transfer of analytical HPLC methods from an Agilent™ 1260 Infinity system to the UltiMate 3000 platform and the Vanquish platform.

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UltiMate 3000 / Vanquish Flex UHPLC

Table of contents

Introduction

The transfer of analytical liquid chromatographic (LC) methods from one instrument to another is a frequent but challenging task in most industries and is of particular importance in regulated environments.[1,2] Reasons for the need to transfer methods are manyfold, and procedures comprise application switching between the same or different types of instruments within the same laboratory, as well as transfers from legacy instruments to new ones due to replacement. Also, the transfer from developing laboratories to implementing laboratories of diverse location and equipment is very common. Proper transfer is only achieved if equivalent results are obtained with the sending and the receiving LC system.[1,2] The true complexity of this task highly depends on the robustness of the method to be transferred as well as on instrumentational differences of both systems.[1,2] To succeed in the challenge of maintaining retention times, resolutions, and other critical factors, specific technical characteristics of the systems like gradient delay volume (GDV), hydrodynamic behavior, or thermostatting mode must be taken into account. Additionally, as revalidation is time-consuming and expensive, modification of method parameters must be avoided as much as possible. Thus, hardware solutions become attractive features in method transfer.[3]

The current application note demonstrates the use of helpful features provided by the Thermo Scientific UltiMate 3000 and Vanquish platforms, like tunable GDVs and switchable thermostatting modes for the method transfer from another vendor's instrument (here the Agilent 1260 Infinity system). The selected application is derived from a USP assay for the analysis of the active pharmaceutical ingredient (API) acetaminophen, a common pain killer, and its impurities.[4] Analysis is performed with a Thermo Scientific™ Hypersil GOLD™ C8 stationary phase that matches the required USP level L7 and is well suited for analytes of medium hydrophobicity.

Experimental

Reagents and materials

- Deionized water, 18.2 MΩ·cm resistivity or higher
- Fisher Scientific™ Optima™ Methanol, LC/MS grade (P/N 10767665)
- Fisher Scientific™ Sodium phosphate dibasic anhydrous (P/N 10182863)
- Fisher Scientific™ Potassium dihydrogen orthophosphate (P/N 10429570)
- Acetaminophen, 4-aminophenol, N-(4-hydroxyphenyl) propanamide (impurity B), 2-acetamidophenol (impurity C), acetanilide (impurity D), 4'-chloracetanilide (impurity J) were purchased from reputable vendors.

Sample preparation

Stock solutions of acetaminophen (20 mg/mL), 4-aminophenol, and the impurities B, C, D, and J (1 mg/mL each) were prepared in methanol. By dilution with methanol and mixing of stock solutions, a sample was prepared that contained 1 mg/mL acetaminophen and 10 µg/mL of each of the other compounds (corresponding to 1% of the API).

Instrumentation

See Table 1 for the instruments used in this study.

Table 1. Instruments used in this study

	Agilent 1260 Infinity Quaternary	UltiMate 3000 SD Quaternary	Vanquish Flex Quaternary
Standard configurations			
			System Base (P/N VH-S01-A-02)
Pump	Quaternary Pump (G1311B)	Standard Quaternary Pump LPG-3400SD (P/N 5040.0031)	Quaternary Pump F (P/N VF-P20-A)
Sampler	High Performance Autosampler (G1367E) with thermostat module (G1330B)	Well Plate Autosampler WPS-3000TSL (P/N 5822.0020)	Split Sampler FT (P/N VF-A10-A)
Column Compartment	TCC with 6 µL heat exchanger (G1316A)	TCC-3000SD (P/N 5730.0010)	Column Compartment H (P/N VH-C10-A)
Detector	Diode Array Detector DAD VL (G1315D)	Diode Array Detector DAD-3000 (P/N 5082.0010)	Diode Array Detector FG (P/N VF-D11-A)
Flow Cell	Standard: 10 mm, 13 µL (G1315-60022)	Analytical: 10 mm, 13 µL (P/N 6082.0100)	Standard bio: 10 mm, 13 µL (P/N 6083.0540)
Hardware modifications applied for method transfer			
		• Add 7 µL eluent pre-heater (P/N 6722.0540) • Replace default static mixer 350 µL (P/N 6040.5310) by static mixer 750 µL (P/N 6040.5750)	• Replace default loop 25 µL (V=50 µL, P/N 6850.1911) by loop 100 µL (V=130 µL, P/N 6850.1913) • Modify idle volume from default 25 µL
Modifications applied for additional sensitivity enhancement			
			• Replace DAD FG by DAD HL (P/N VH-D10-A) with LightPipe standard flow cell (10 mm, P/N 6083.0100B) or LightPipe high sensitivity flow cell (60 mm, P/N 6083.0200)

LC conditions

Column:	Hypersil GOLD C8, 4.6 × 100 mm, 3 µm, 175 Å (P/N 25203-104630)
Mobile Phase:	A: 1.7 g/L KH_2PO_4 and 1.8 g/L of Na_2HPO_4 in water B: Methanol
Flow Rate:	1 mL/min
Gradient:	0 min 1% B 3 min 1% B 7 min 81% B 7.1 min 1% B 12 min 1% B* (*when the UltiMate 3000 SD system was used with the 750 µL static mixer, equilibration was extended to 13 min)
Column Temp.:	35 °C (with eluent pre-heating)
Autosampler Temp.:	8 °C
Detection:	230 nm, 10 Hz data collection rate, 0.5 s response time
Inj. Volume:	1 µL
Needle Wash:	Off

Data processing and software

Thermo Scientific™ Chromeleon Software 7.2.8 Chromatography Data System was used for data acquisition and analysis.

Results and discussion

All method transfer experiments were conducted with the same column and sample, with consistent method parameters and seven repeated injections. The chromatograms in Figure 1 display the starting situation for the transfer from the Agilent 1260 Infinity system to the UltiMate 3000 SD system and to the Thermo Scientific™ Vanquish™ Flex system (all quaternary). The corresponding retention times are summarized in Table 2. In Figure 1a, Agilent 1260 Infinity system data are compared to data from the UltiMate 3000 Standard configuration system without an eluent pre-heater, and to data from the UltiMate 3000 system equipped with an optional 7 µL pre-heater. The distinct differences of both

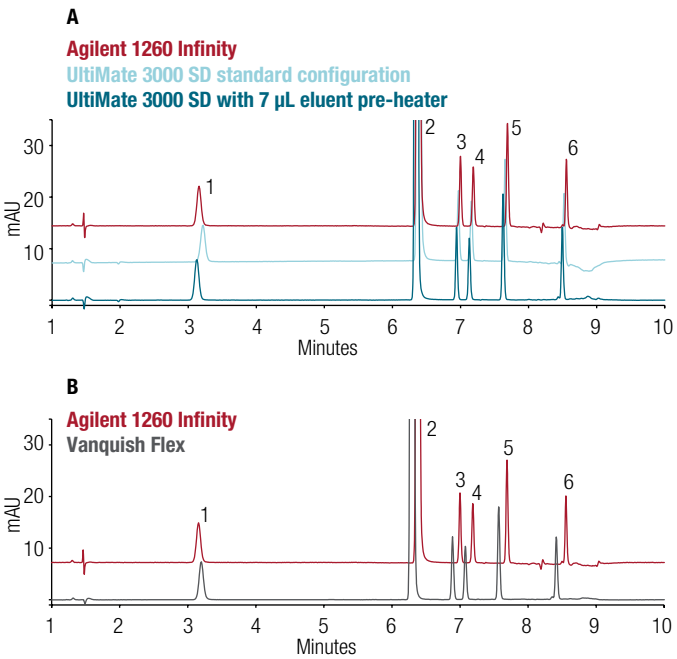

Figure 1. Starting situation of the method transfer. A) Chromatogram of Agilent 1260 Infinity system compared to UltiMate 3000 SD system in standard configuration and with optional eluent pre-heater; B) Chromatogram of Agilent 1260 Infinity system compared to Vanquish Flex system. For peak assignment see Table 2.

Table 2. Averaged retention times in minutes over seven injections for the systems and configurations stated in Figure 1 and % deviation for both pre-heated target systems from originating system

Peak No.	Compound	Agilent 1260 Infinity (originating system)	UltiMate 3000 SD system w/o pre-heating	UltiMate 3000 SD w/ pre-heating (target system)	Vanquish Flex (target system)
1	4-Aminophenol	3.16	3.21	3.13 (Δ 0.9%)	3.20 (Δ - 1.3%)
2	Acetaminophen (API)	6.38	6.39	6.34 (Δ 0.5%)	6.29 (Δ 1.3%)
3	Impurity B	7.00	6.97	6.94 (Δ 0.8%)	6.89 (Δ 1.6%)
4	Impurity C	7.19	7.16	7.13 (Δ 0.8%)	7.08 (Δ 1.5%)
5	Impurity D	7.69	7.66	7.63 (Δ 0.8%)	7.57 (Δ 1.6%)
6	Impurity J	8.56	8.52	8.50 (Δ 0.7%)	8.41 (Δ 1.7%)

UltiMate 3000 system chromatograms clearly illustrate the noticeable impact of eluent thermostatting even at moderate separation temperatures. Thus, a successful method transfer should be conducted with adjusted thermostatting conditions and an installed pre-heater on the UltiMate 3000 system. This is especially emphasized by the behavior of the first peak (4-aminophenol), which elutes under isocratic conditions and is not affected by gradient effects. Without eluent pre-heating, it elutes later than on the Agilent 1260 Infinity system and approximates when pre-heating is applied. For the Vanquish Flex system an active pre-heater is included in the standard configuration and was activated for this method transfer, yielding similar retention of aminophenol (Figure 1b). In contrast, all peaks that elute during the gradient elute earlier on both Thermo Scientific instruments than on the Agilent 1260 Infinity system with enabled eluent pre-heating for the three systems. This is mainly due to a larger (and furthermore back-pressure dependent) GDV of the Agilent 1260 instrument. For that reason, a physical GDV adjustment by several features provided by the UltiMate 3000 and Vanquish portfolio is a promising way to minimize system differences for a successful method transfer.

Tables 3 and 4 give an overview of UltiMate 3000 SD and Vanquish system accessories available to stepwise modify system GDVs. For the transfer of the acetaminophen assay from the Agilent 1260 Infinity system to the UltiMate 3000 SD system, the default static mixer (350 µL) was replaced by the larger 750 µL mixer. As this volume difference overcompensated the GDV difference between

Table 3. Available UltiMate 3000 SD system consumables that can be used to modify the system GDV

Description	P/N
Mixer kit for pump 35 µL (25 µL capillary mixer +10 µL inline filter)	6040.5000
Mixer kit for pump 100 µL (25 µL capillary mixer + 75 µL static mixer)	6040.5100
Mixer kit for pump 200 µL (50 µL capillary mixer + 150 µL static mixer)	6040.5110
Mixer kit for pump 400 µL **(default configuration quaternary pump)** (50 µL capillary mixer + 350 µL static mixer)	6040.5310
Mixer kit for pump 800 µL (50 µL capillary mixer + 750 µL static mixer)	6040.5750
Mixer kit for pump 1550 µL (50 µL capillary mixer + 1500 µL static mixer)	6040.5450
Sample loop 25 µL (V=40 µL)	6820.2452
Sample loop 100 µL (V=130 µL) **(default configuration)**	6820.2451
Sample loop 250 µL (V=344 µL)	6820.2453
Sample loop 500 µL (V=667 µL)	6820.2454

Table 4. Available Vanquish system consumables that can be used to modify the system GDV

Description	P/N
Mixing system for pump 35 µL (25 µL capillary mixer + 10 µL inline filter)	6044.3870
Mixing system for pump 100 µL (25 µL capillary mixer + 75 µL static mixer)	6044.5100
Mixing system for pump 200 µL (50 µL capillary mixer + 150 µL static mixer)	6044.5110
Mixing system for pump 400 µL **(default configuration quaternary pump)** (50 µL capillary mixer + 350 µL static mixer)	6044.5310
Mixing system for pump 800 µL (50 µL capillary mixer + 750 µL static mixer)	6044.5750A
Mixing system for pump 1550 µL (50 µL capillary mixer + 1500 µL static mixer)	6044.5450A
Sample loop 10 µL (V=23 µL)	6850.1915
Sample loop 25 µL (V=50 µL) **(default configuration)**	6850.1911
Sample loop 100 µL (V=130 µL)	6850.1913

sending and receiving instrument (see Figure 2a), a prestart of the gradient was then applied to match the retention times. With the prestart technique a smaller GDV can be emulated by shifting the point of injection relative to the method start. As the injection by definition is executed at 0.0 min, the method start is set to a negative time and all remaining steps of the method are shifted by the same value. Thus, no segment of the method is modified and the gradient table in total is not changed. For the current transfer, the extent of the time offset was -0.27 min and was derived from the average retention time difference of gradient-eluted peaks of the UltiMate 3000 system with the 750 µL static mixer and the Agilent 1260 Infinity system. Figure 2b illustrates the very good retention time match that was obtained by this technique, giving relative retention time deviations of <1% for aminophenol and <0.2% for peaks that elute in the gradient with respect to the originating method. While gradient-eluted peaks are shifted according to true or emulated GDV adjustments, peaks eluted under isocratic conditions are not affected. The minor difference in aminophenol retention thus might be the result of slightly different temperature conditions or proportioning of the isocratic conditions with 1 % of mobile phase B.

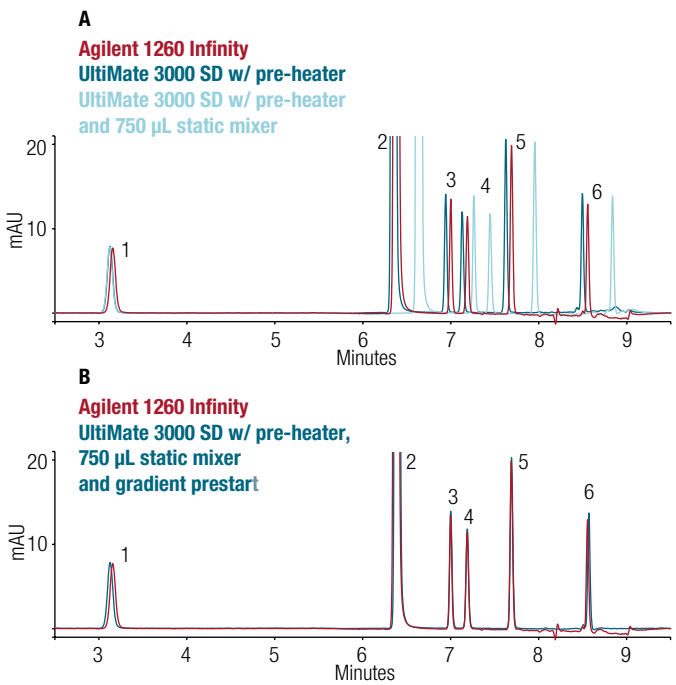

Figure 2. Method transfer from the Agilent 1260 Infinity system to the UltiMate 3000 SD system. A) Comparison of Agilent 1260 Infinity system and UltiMate 3000 SD system with eluent pre-heater and standard or larger static mixer; B) final transfer: comparison of Agilent 1260 Infinity system and UltiMate 3000 SD system with eluent pre-heater, larger static mixer, and gradient prestart. For peak assignment see Table 2.

In contrast, pre-starting the gradient was not necessary with the Vanquish Flex system to attain retention time congruence due to more flexible capabilities in GDV adjustment. At first the GDV difference of the Agilent 1260 Infinity and Vanquish Flex standard configured systems observed in Figure 1b was partially compensated by replacing the Vanquish standard sample loop by the 100 µL sample loop (actual GDV contribution 130 µL). The resulting retention times were closer to the originating instrument (see Figure 3a), and the remaining differences were in a range that could be offset by adjusting the idle volume of the autosampler metering device, the conducting unit of sample aspiration. This feature is unique to the Vanquish platform and can help in fine-tuning of the GDV as it is part of the sample loop flow path. The default idle volume setting of 25 µL was increased by 43 µL to a total of 68 µL, yielding the good alignment of retention times seen in Figure 3b with relative retention time deviations of 1.2 % for aminophenol and <0.4 % for peaks in the gradient.

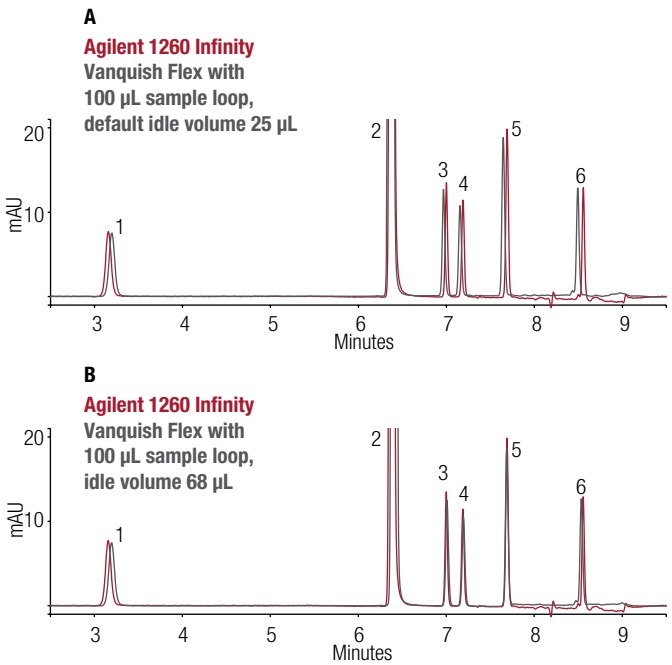

Figure 3. Method transfer from the Agilent 1260 Infinity system to the Vanquish Flex system. A) Comparison of Agilent 1260 Infinity system and Vanquish Flex system with 100 µL sample loop; B) final transfer: comparison of Agilent 1260 Infinity system and Vanquish Flex system with 100 µL sample loop and adapted idle volume to 68 µL. For peak assignment see Table 2.

Another unique feature of Vanquish instruments is the switchable thermostatting mode of the column compartment, giving the choice of still or forced air column heating. The previous chromatograms were recorded in still air mode, as this reflects the thermostatting mode of the Agilent column compartment best. Figure 4 shows that for the current application the thermostatting mode has minor influence on retention times and is negligible here. However, in applications of higher pressure ranges (ultra-high-performance LC, UHPLC, > 600 bar) where frictional heating of the column becomes relevant, the column compartment mode is of certain importance.[3]

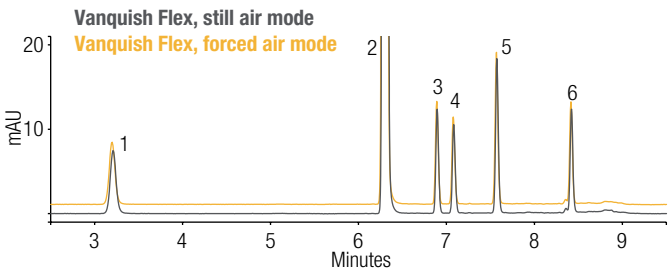

Figure 4. Negligible effect of column thermostatting mode for Vanquish Flex system in still and forced air mode. For peak assignment see Table 2.

In conclusion, retention times were successfully transferred from an Agilent 1260 Infinity instrument to an UltiMate 3000 SD instrument and a Vanquish Flex instrument by means of physical or simulated GDV adaption. This is in full agreement with the allowed adjustments according to the USP General Chapter <621>, which states: "If adjustments are necessary, a change in […] the duration of an initial isocratic hold (when prescribed), and/or the dwell volume are allowed."[5] Furthermore, critical chromatographic results were easily maintained during the transfer. The resolution of the critical pair of impurity B and C was 3.2 or better in all tested scenarios, and peak tailing factors ranged from 0.99 to 1.12. The relative standard deviation of peak heights was always far below 1% (Figure 5a). Thus, USP system suitability was accomplished by all three systems, both with and without GDV adaption. The relative areas of all impurity peaks were constant over the three instruments (Figure 5b).

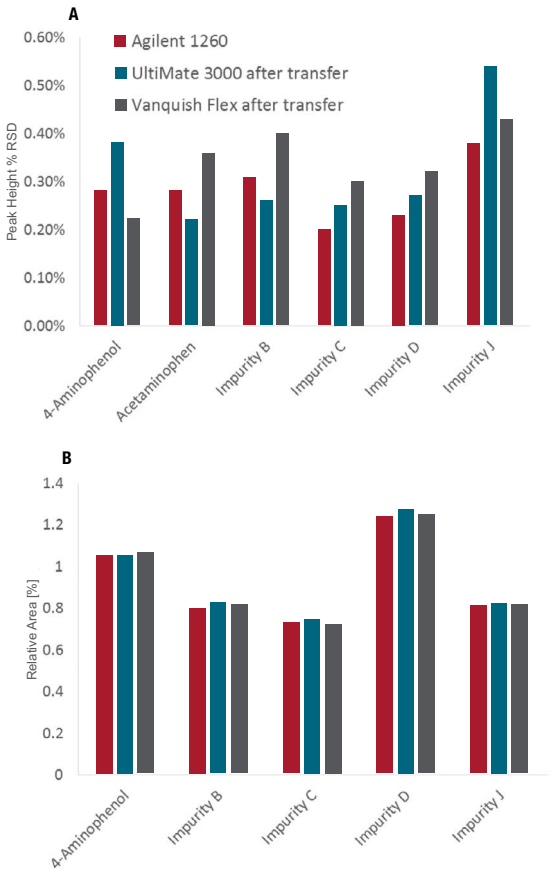

Figure 5. Averaged peak height precision (A) and relative areas of impurity peaks (B) for the originating system Agilent 1260 Infinity, UltiMate 3000 SD system after method transfer optimization (750 µL static mixer and gradient prestart), and Vanquish Flex system after method transfer optimization (100 µL sample loop and idle volume 68 µL)

In Figure 6, the signal-to-noise (S/N) ratios of the transferred method are summarized, illustrating a distinct improvement of S/N performance from the originating system to the Ultimate 3000 SD system and the Vanquish Flex system in the present configuration. As an alternative to the Thermo Scientific™ Vanquish™ DAD FG, the Thermo Scientific™ Vanquish™ DAD HL provides an outstanding S/N performance driven by Thermo Scientific™ LightPipe™ technology, which is demonstrated by the additional bars in that graph. These results were obtained with the same Vanquish system as before but with a swapped detector; with both the standard flow cell with equal light path length as the three previous systems of 10 mm and the high sensitivity flow cell with 60 mm light path. While the S/N enhancement by the standard LightPipe flow cell is mainly caused by further noise reduction, the enormous gain with the high sensitivity flow cell is particularly generated by the sensitivity gain due to the long light path. This cell is especially suited for analyses with columns of 4.6 mm inner diameter.[6] Thus, the DAD HL is very suitable for the analysis of low-abundant impurities, and if S/N performance or sensitivity are of critical concern, the utilization of that dedicated DAD technology is highly recommended.

Conclusions

- During method transfer of an acetaminophen assay from an Agilent 1260 Infinity system to an UltiMate 3000 SD system as well as to a Vanquish Flex system (all quaternary), straightforward retention time matches were achieved by true and emulated GDV adjustments by diverse tools provided by the Thermo Scientific platforms, like exchangeable eluent pre-heaters, pump mixers, sample loops, and adjustable autosampler idle volume.
- Further critical chromatographic results like resolution of critical peak pair, peak asymmetries, peak height precision, and relative peak areas were easily maintained during transfer. Signal-to-noise ratios improved distinctly during the transfer.
- If detection sensitivity of the method is of particular concern, the utilization of DAD LightPipe technology is recommended for LC-UV applications.

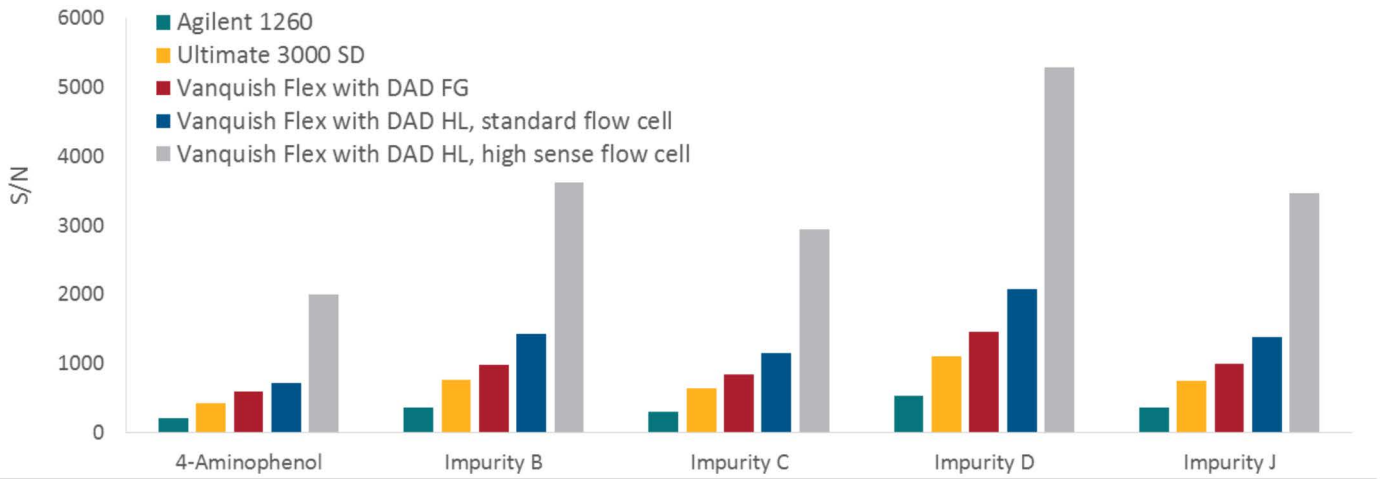

Figure 6. Signal-to-noise ratios (S/N) obtained with Agilent 1260 Infinity system, Ultimate 3000 SD system after method transfer optimization (750 µL static mixer and gradient prestart), Vanquish Flex system after method transfer optimization (100 µL sample loop and idle volume 68 µL) with DAD FG, DAD HL with 10 mm flow cell, and DAD HL with 60 mm high sense flow cell. Noise calculated from the current chromatogram 4.1–4.6 min.

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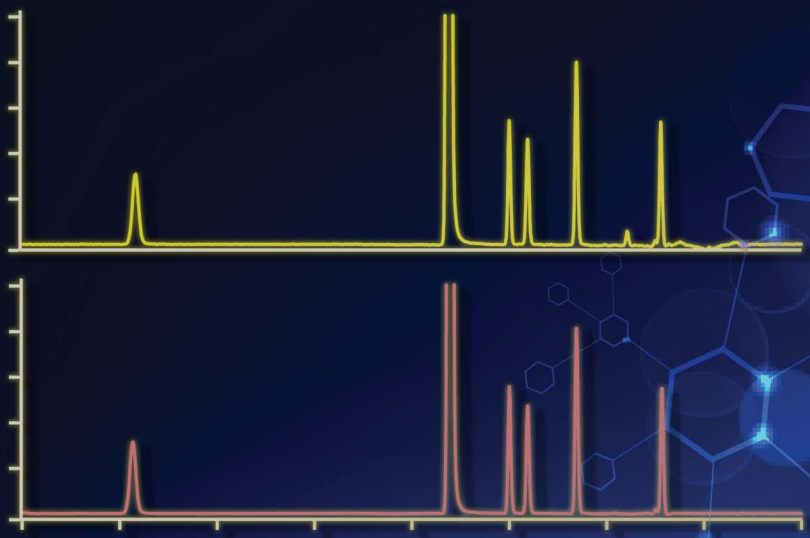

WHITE PAPER 72711

An instrument parameter guide for successful (U)HPLC method transfer

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Keywords
UHPLC, HPLC, USP <621>, gradient delay volume, column thermostatting, extra-column volume, detector settings

Goal

Explain in detail the instrumental parameters HPLC users need to consider during transfer of an analytical HPLC method between different instruments.

Introduction

The transfer of analytical procedures in liquid chromatography (LC) is a regular task in many laboratories. This challenge can be categorized into the following common scenarios:

A. Acceleration of methods, e.g. from HPLC to UHPLC methods
B. Method transfer to identical equipment, e.g. in another laboratory
C. Method transfer to a non-identical instrument, e.g. to a recently purchased system

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Table of contents

After the commercialization of ultra-high-performance liquid chromatography (UHPLC) instruments, and the simultaneous use of sub-2 µm column particles, scenario A became a common task in many laboratories. However, there are various publications available explaining the principles of method scaling.[1-3] Thus, scenario A is not further elaborated here, and the reader is referred to the existing literature.

For scenarios B and C, the aim of such a workflow is "simply" obtaining equivalent results between both systems to quickly have an operational method and to reduce revalidation efforts. For scenario B, the method robustness is the focus since the method is transferred between two identical systems. A discussion about criteria for method robustness/re-validation is not within the scope of this publication.

The challenge summarized under scenario C is often faced when transferring (validated) methods between different laboratories, e.g. from a developing laboratory to a QC laboratory or, similarly, from a sponsor laboratory to a contract laboratory. Here, the influence of instrument parameters on the chromatographic separation needs to be considered for successful transfer of an LC method from the originating to receiving laboratory.

This review explains instrumental parameters to be considered when transferring an LC method from one system to another. In addition, we will give recommendations on how to modify certain parameters to obtain equivalent results. These modifications are discussed with respect to USP General Chapter <621> Chromatography which describes the accepted limits of such modifications.[4] Finally, we give guidance on how to best characterize the root cause for common method transfer problems. This review focues solely on instrument parameters. Aspects such as correctly following an SOP, e.g. for buffer preparation, are not covered within this publication.

Categorization of (U)HPLC methods

The importance of instrument parameters for a successful method transfer became apparent over the last few years. The need to transfer methods gains importance due to the increasing involvement of external laboratories, such as contract research organizations, as well as the trend to transfer methods globally within a single company. In both cases, the chromatography instruments were often not identical, and difficulties occurred when reproducing results of the originating laboratory. In addition, the commercialization of UHPLC instruments with their significantly altered physical characteristics emphasized the influence of instrument parameters on a specific separation.

The extent to which a certain parameter influences the success rate of a method transfer process strongly depends on the actual application. Two important parameters are the column dimensions used (inner diameter and particle size) and the elution mode. Figure 1 shows the importance of the main instrument characteristics during the method transfer. For simplification purposes, the scenarios UHPLC (2.1 mm i.d. column, < 2 µm particles) versus HPLC (4.6 mm i.d. column, ≥ 3 µm particles) conditions, and isocratic versus gradient elution conditions are differentiated, as illustrated in Figure 1.

From these general considerations it becomes obvious that the gradient delay volume (GDV) is an important parameter for the transfer of a gradient elution method. Similarly, as the flow rates are generally lower for UHPLC separations, the importance of matching the GDV of the originating and receiving system is higher for UHPLC separations because small differences in GDV can affect retention times dramatically.

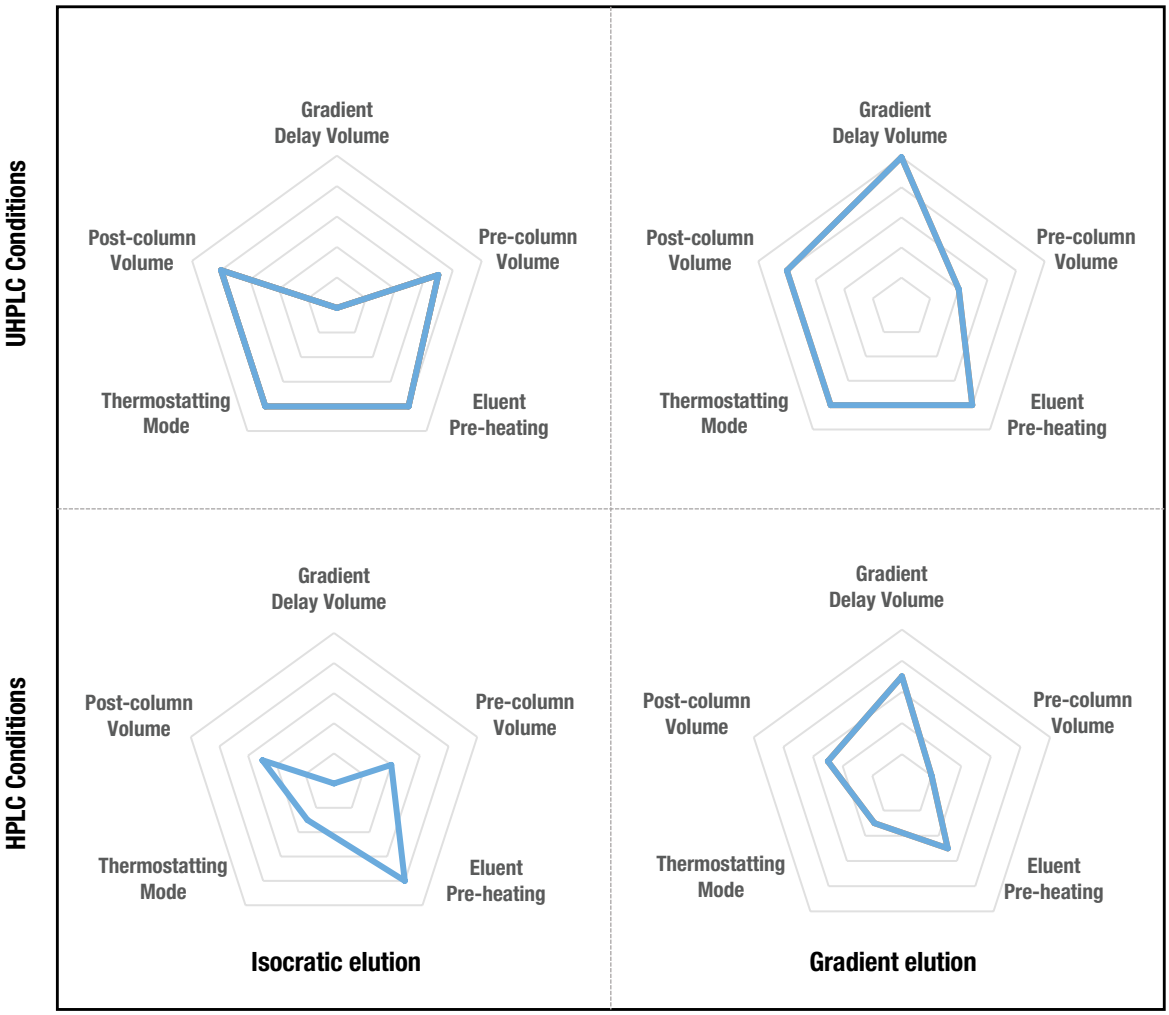


Figure 1. Instrument parameters and their importance for a successful method transfer. The further from the center of the graph, the more important the parameter. The importance value are estimates and dependent on additional method details such as separation temperature, flow rate, etc.

Furthermore, the thermostatting mode needs to be considered, which mainly describes how the instrument deals with frictional heating within the column. During standard HPLC separations, which regularly run below 400 bar (6000 psi), frictional heating is negligible. In contrast, under UHPLC conditions with pressures ranging up to 1500 bar (22,000 psi), significant frictional heating occurs. Thus, matching thermostatting modes is crucial when transferring UHPLC methods.

Gradient delay volume – What it is and how to measure it

The GDV is a physical characteristic of an HPLC system that describes the holding capacity of all interconnected components from the mixing point up to the entry of the column. Contributors to the GDV can include the pump, autosampler, and connecting capillaries. A consequence of the GDV is that a programmed elution gradient can enter the column with a delay, that can be calculated with the formula:

$$\textit{Delay time} = \frac{\textit{GDV}}{\textit{flow rate}}$$

As different HPLC instruments can have different GDVs, a particular solvent composition can arrive at different time points on the head of a column. Controlling the GDV can have a dramatic impact on reducing the amount of time required for a method transfer.

A common way to measure the GDV is to program a linear gradient from 0% to 100% B, with channel B containing a UV-absorbent compound. In this case, we used caffeine at a concentration of 12 mg/L (Figure 2).

The GDV is normally calculated by using the time when the UV trace reaches 50% of the maximal value (green arrow in Figure 2) according to the following formula:

$$GDV = (t_{50\%} - 0.5\ t_G) \times F$$

where $t_{50\%}$ is the time when the UV trace reaches 50% of the maximal value, t_G is the total gradient time, and F is the method flow rate.

An alternative approach is to use the time difference between the start of the gradient and the crossing of a linear extrapolation of the UV trace ramping up with the baseline (blue arrow in Figure 2). From our investigations, we found that using the method at 50% UV height (green arrow, Figure 2) is more reliable and thus we recommend this approach. In any case, care should be taken that no values are compared which originate from different evaluation methods.

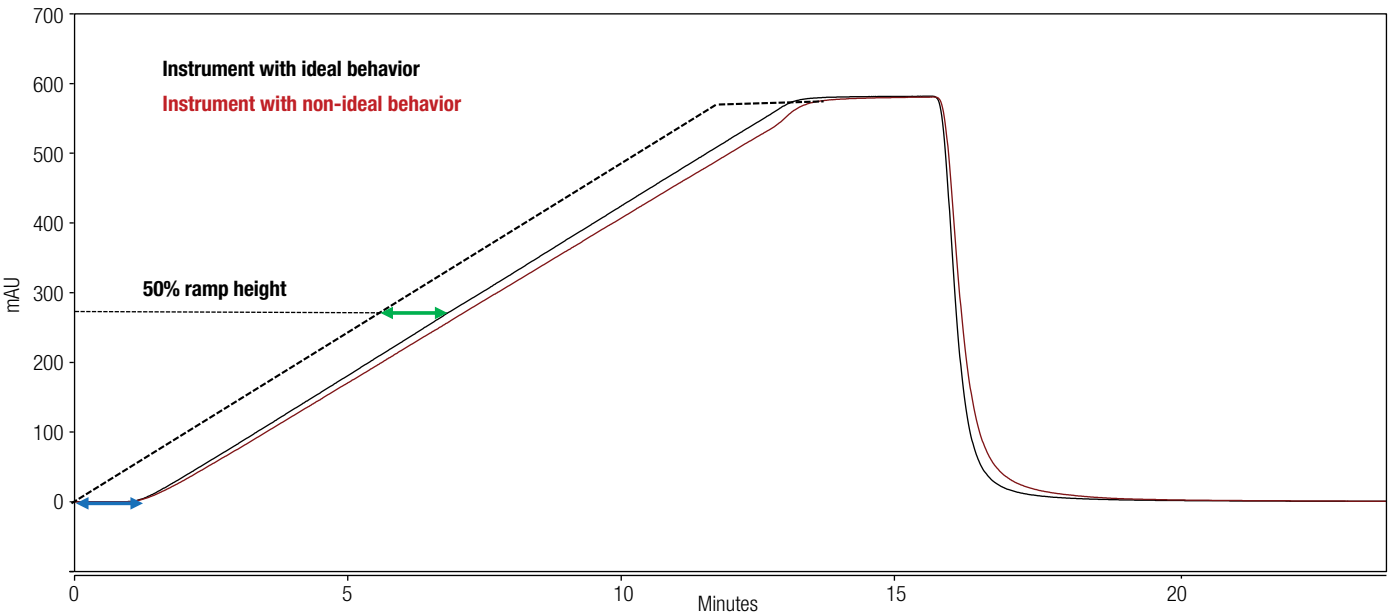


Figure 2. Method for determination of an instrument gradient delay volume. Two different instrument behaviors are shown as well as two commonly used data evaluation procedures (blue and green arrows).

In addition, the GDV is not a constant for specific HPLC or UHPLC instruments but depends on the flow rate and pressure applied. Figure 3 gives some examples for flow rate and pressure dependencies. Figure 3A shows the GDV of one system without a pulse damper and constant piston stroke volume at different flow rates while keeping the instrument backpressure constant. The differences between minimal and maximal GDV was up to 20%, with the lowest GDV observed at the highest tested flow rate of 3 mL/min. In contrast, Figure 3C shows the result of the same experiment using a system with a pulse damper and variable piston stroke volume. Here the GDV is more than 40% higher at the maximal flow rate of 3 mL/min compared to the lowest measured flow rate. This suggests that the GDV is not a fixed instrumental parameter but rather dependent on the applied method. For a successful method transfer, it will consequently be useful to determine the GDV under the original conditions.

Figure 3B shows the effect of the back pressure on the GDV. As expected, the GDV increases with increasing pressure by more than 40% when a pulse damper is used. However, in contrast to the flow rate, which is normally constant during one specific application, the pressure can change drastically during gradient elution. The result of this behavior is that retention times of compounds eluting during the gradient are affected by the dynamically changing GDVs and this needs to be considered for successful method transfer.

Table 1 gives an overview of commonly used HPLC systems equipped with a low pressure gradient type pump. As the measured GDV is flow rate dependent, a flow rate of 1 mL/min was used for all measurements to ensure best comparisons. Systems using a pulse damper have a high pressure dependency on their GDV. Even though it is not listed here, it should be noted that the GDV of high-pressure gradient type pumps is generally lower than for low-pressure gradient pumps, which makes the transfer between these instrument types more challenging.

Table 1. Summary of the GDV of several commonly used HPLC and UHPLC systems. Gradient tests were performed at a flow rate of 1 mL/min and a pressure of approximately 200 bar.

(U)HPLC System	GDV in µL
Thermo Scientific UltiMate 3000 SD Quaternary	1030
Thermo Scientific Vanquish Flex Quaternary	980
Agilent® 1100	1220
Agilent® 1260 Infinity® II Quaternary	1280
Waters® Alliance®	1150[5]
Shimadzu® LC-2010	1400
Shimadzu Nexera®-i	590 (40 µL mixer) 860 (300 µL mixer)

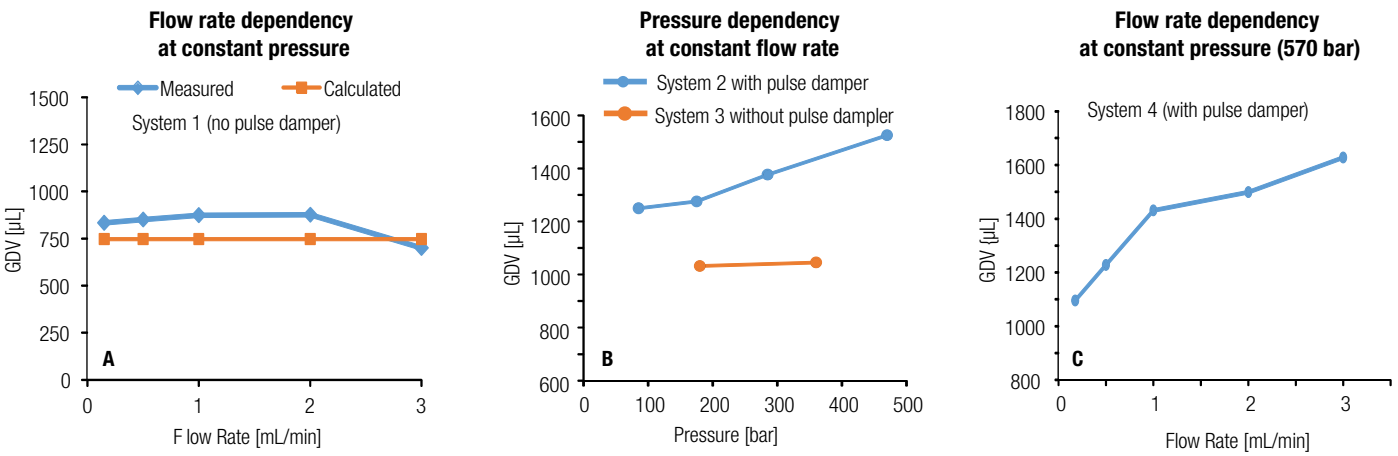


Figure 3. Dependency of gradient delay volume on flow rate and pressure for different types of instrumentation

Low-pressure vs. high-pressure mixing pumps

To form a gradient in liquid chromatography, two different gradient formation technologies exist—low-pressure gradient (LPG) and high-pressure gradient (HPG) proportioning. In the LPG, the convergence point of the solvents (normally up to 4) is before the pump head using a solenoid proportioning valve. LPG pumps generally have a higher GDV compared to HPG, since the pump heads contribute to the GDV.

Conversely, the HPG uses two independent pumps to deliver two solvents into the system. These two solvent streams converge after the pump on the high-pressure side of the HPLC. As the convergent point is after the pump heads on the high-pressure side, these pumps generally have a low GDV (Figure 4).

The difference in the gradient generation concept (e.g. solvent convergence either on the low- or on the high-pressure side of a pump) also has consequences on the flow and gradient accuracy as shown in Figure 5.

A simulated example is given for a programmed water/methanol gradient from 0% to 100% methanol at a flow rate of 1 mL/min (Figure 5A). For an HPG, both independent pumps deliver partial flow as determined by the desired gradient composition. For example, at a composition of 50% methanol, both pumps will deliver 500 µL/min. However, after converging both solvents on the high-pressure side of the pump, the resulting flow rate on the column will be less than 1 mL/min due to the volume contraction of both solvents. The contraction volume depends on the solvent and the mixture

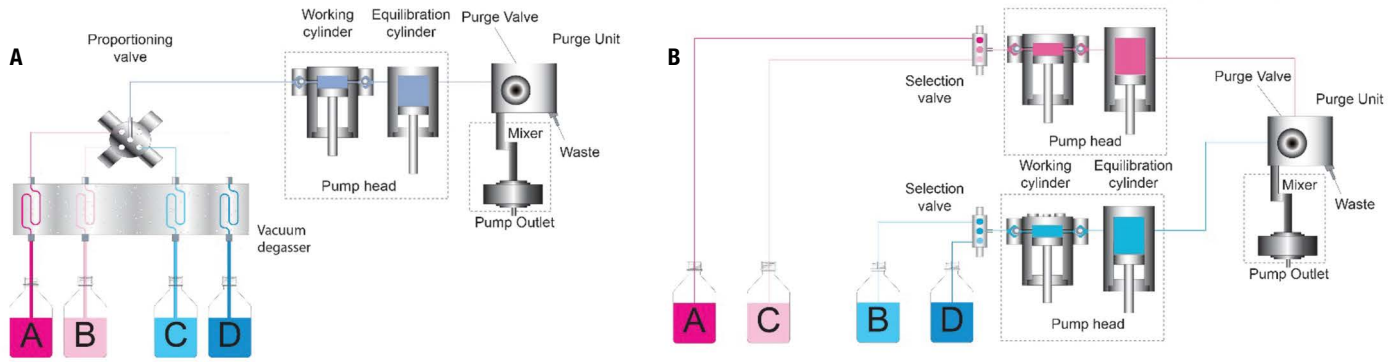


Figure 4. Schematic setup of a low-pressure gradient pump (A) and a high-pressure gradient pump (B). Note how the different solvent convergence points have effects on the gradient delay volume, which is defined as the volume between the convergent point of the solvents and the column head.

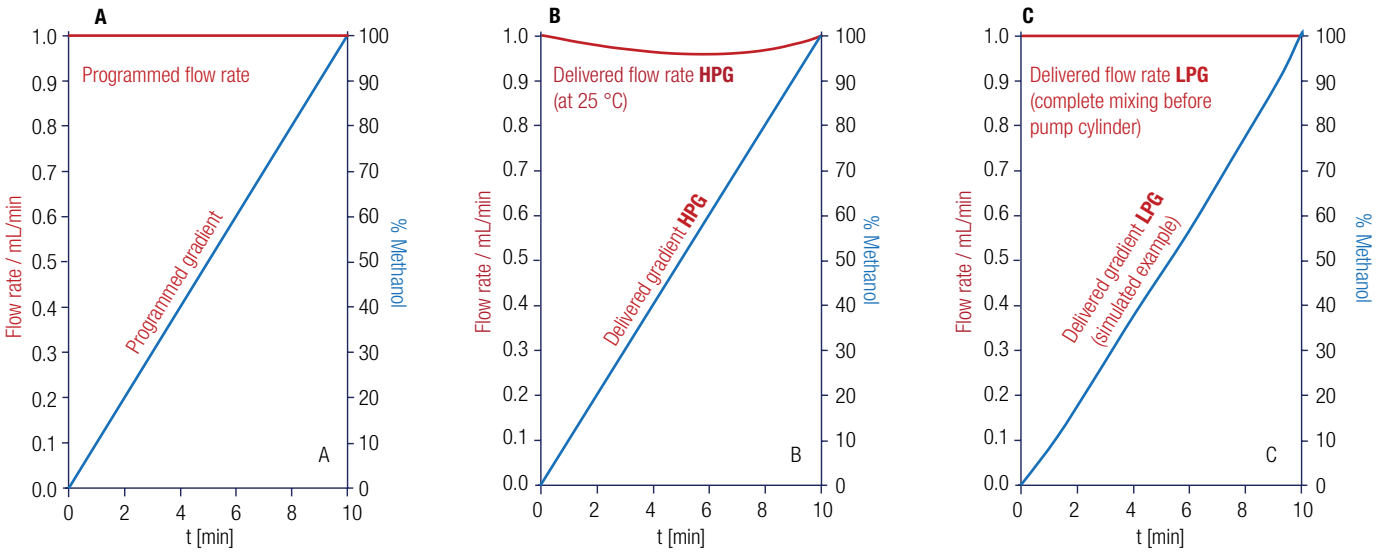


Figure 5. Flow rate and gradient accuracy of an HPG and LPG pump. Comparing A) programmed flow rate and gradient B) delivered flow rate and gradient of an HPG pump, and C) delivered flow rate and gradient of an LPG pump.

composition. For a methanol gradient, the error will be around 4% at a solvent composition of 55–60% methanol (Figure 5B). However, the gradient (solvent composition) delivered by an HPG pump is exactly as linear as the programmed gradient (Figure 5A). The LPG pump, in contrast, converges the solvents before the pump on the low-pressure side, and the delivered flow on the column will be 1 mL/min (Figure 5C). Furthermore, due to the volume contraction during the convergence of solvents at the proportioning valve, an LPG does not deliver the exact gradient composition as desired. Here the delivered gradient is not linear but rather bent.

As a consequence of this difference in the design of the pumps, it is generally recommended to consider the pump type (i.e., LPG or HPG) during a method transfer of the gradient. Preferably, methods should be transferred between the same pump type to avoid physical consequences of the design differences that may hamper method transfer results. Still, as described in the next chapter, care must be taken to reflect potential GDV differences that can appear even within one pump type.

Gradient delay volume adjustments

When a method is transferred, there are two general approaches used to adapt the different GDVs of the systems to facilitate the method transfer. Again, it should be considered that the transfer between HPG and LPG systems is normally accompanied with a significant difference in GDV and other differences that make method transfer more challenging. In addition to the two approaches explained in the next sections, the use of an isocratic hold at the beginning of a gradient program is a common practice in many HPLC laboratories. When such methods are transferred to a system with a larger GDV, the isocratic hold can simply be shortened. The change of the duration of the initial isocratic hold is allowed according to USP <621>.[4]

Adopting the GDV

An effective and straightforward way to compensate GDV differences between the originating and the receiving HPLC system is to physically change the GDV of the receiving system so that it matches the original system's GDV. An easy way to change the GDV is to adapt the mixer volume or sample loop volume of the instrument you are trying to transfer to. Such physical changes of the system are accepted and consistent with the USP guidelines.

Figure 6 gives an example of how compensation for the GDV differences was performed to transfer a method from an Agilent® 1260 Infinity® II system to a Thermo Scientific™ UltiMate™ 3000 Standard (SD) system. In this case, increasing the mixer volume from 400 µL to 800 µL on the UltiMate 3000 SD resulted in a good match of the gradient profile.

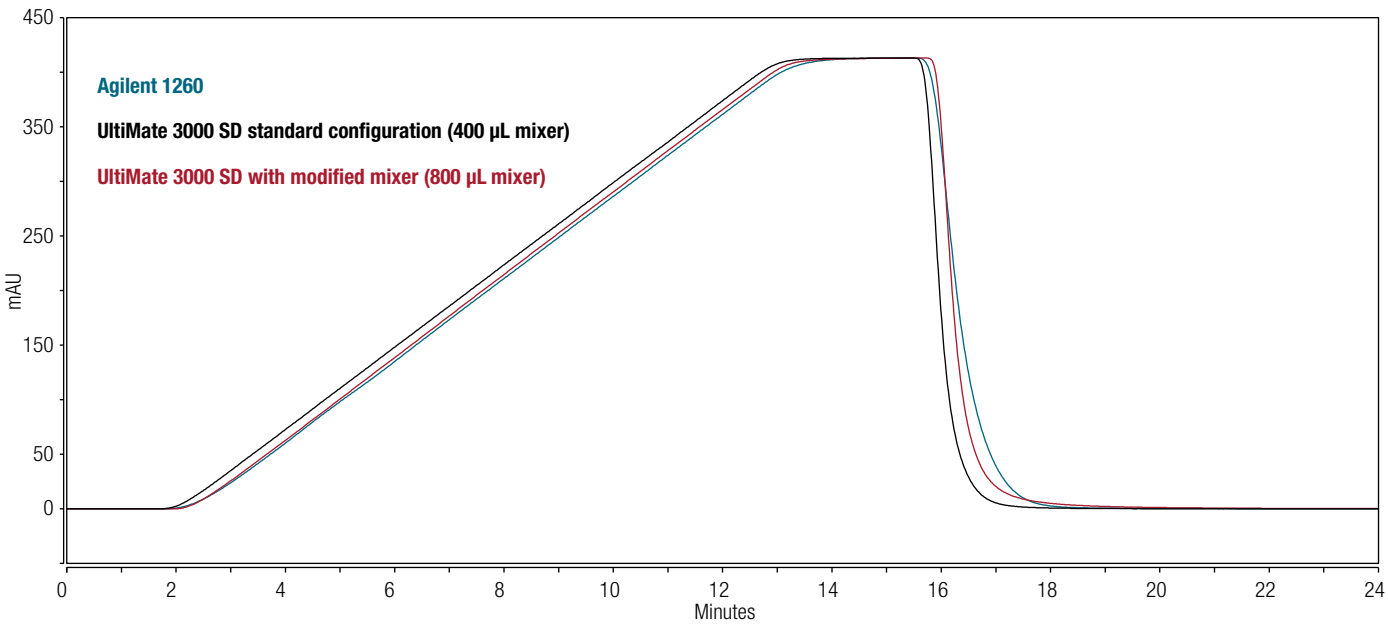


Figure 6. Overlaid gradient profiles of an Agilent 1260 LPG system, an UltiMate 3000 SD LPG system, and an UltiMate 3000 SD LPG system with increased mixer volume to compensate for GDV differences

Afterwards, the adopted instrumental setup was used to transfer the separation of 10 pesticides from the Agilent 1260 Infinity II system to the UltiMate 3000 SD system (Figure 7B). With this setup, the method could be transferred and a nearly identical separation was achieved. The same approach was also used to transfer a method for the separation of drugs used for the treatment of heart disease from an Agilent 1100 system to an UltiMate 3000 SD system. In this case, the installation of the 800 µL mixer kit also turned out to be successful (Figure 7A).

Besides changing the mixer of the pump (or the sample loop in the autosampler), the Thermo Scientific™ Vanquish™ UHPLC product line also allows the fine tuning of the GDV by adjusting the GDV via a metering device located in the autosampler which contributes to the system GDV. However, as this volume is adjustable with a simple software command, the user can gradually change the GDV for best method transfer. With this tool, it is possible to continuously vary the default GDV of any Vanquish system by a maximum of 100 µL. This feature is of help when already small differences in GDV hinder a successful method transfer (e.g. separation at flow rates around 400 µL/min or smaller or for the transfer between low GDV binary pumps of different vendors).

Changing the injection point relative to the gradient start

The second possibility to account for different GDVs between two HPLC systems is to move the injection time point relative to the gradient start. For instance, the originating system could have a GDV of 0.8 mL and the receiving system a GDV of 1.8 mL, resulting in a 1 mL difference. In this case, this difference can be compensated for by injecting the sample after the gradient start. For a flow rate of 1 mL/min, this would mean that the injection occurs one minute after the gradient program has started. In a practical sense, this would mean that the gradient starts at a time of -1 min relative to the injection, which always defines the zero point of a timetable. In this way, the slope and duration of the gradient would not be affected.

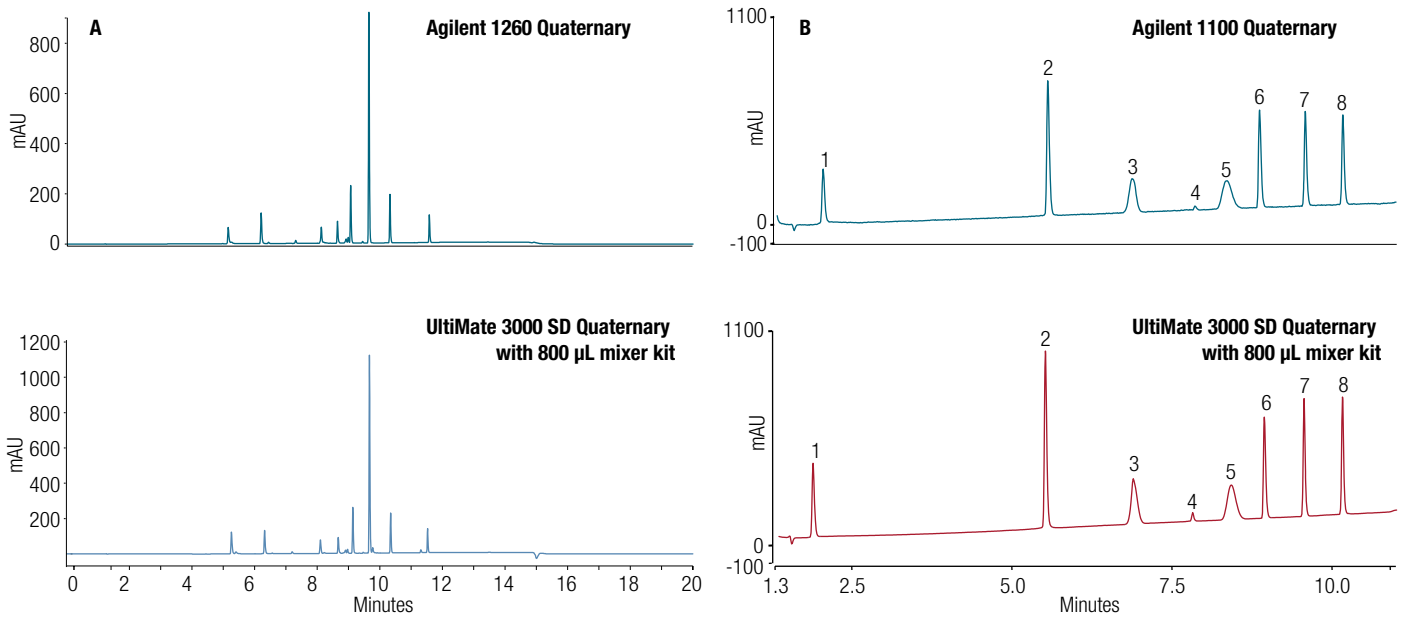


Figure 7. Transfer from an Agilent 1260 instrument to an UltiMate 3000 SD instrument (A) and transfer from an Agilent 1100 instrument to an UltiMate 3000 SD instrument (B). To match the gradient delay volume characteristics, the default mixer of an UltiMate 3000 SD system was exchanged to the 800 µL mixer kit.

In another example, Figure 8 shows the transfer of a method for acetaminophen and five impurities from an Agilent 1260 to an UltiMate 3000 SD instrument. The UltiMate 3000 SD system configuration has a lower default GDV. To compensate for this difference, an 800 µL mixer setup was installed. However, for this application that only runs at 120 bar, the additional mixer volume overcompensated the GDV difference (Figure 8, middle chromatogram). In such cases, a gradient pre-start can be programmed by the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software to start the gradient prior to the injection point. This resulted in a perfect overlay of both chromatograms (Figure 8, bottom) while smaller peak widths were observed for the UltiMate 3000 SD system.

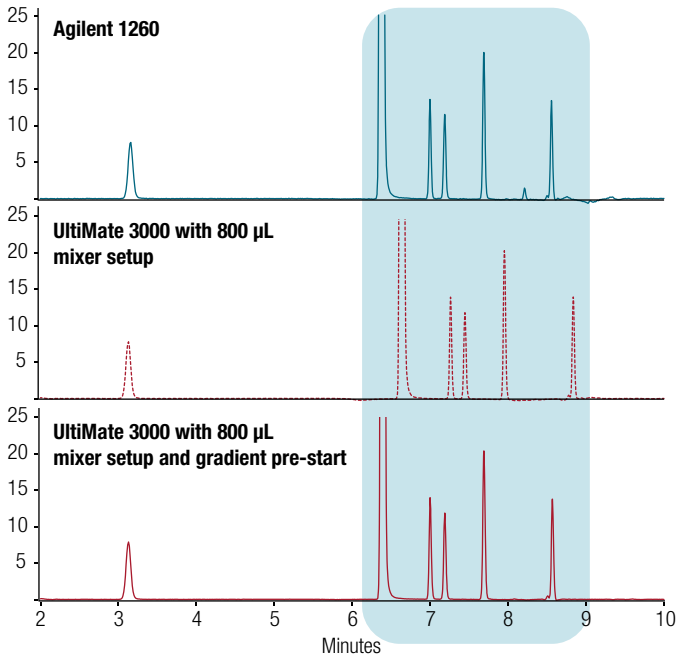


Figure 8. Transfer of a separation of acetaminophen and its impurities from an Agilent 1260 low pressure gradient to an UltiMate 3000 SD low pressure gradient system. For the UltiMate 3000 SD system the 800 µL mixer setup was used. To compensate for the higher gradient delay volume of the UltiMate 3000 SD system under these conditions, a gradient prestart was programmed.

Mobile phase pre-heating in front of the column

The temperature of a solvent entering a HPLC column may have an impact on both, resulting peak shapes and retention factors. Proper eluent temperature pre-conditioning is essential to achieve optimal column efficiencies, especially when working at column. temperatures above ambient. When the temperature of the incoming solvent is significantly lower than the column temperature, a radial temperature gradient between the center of the column and the column wall is formed, at least in the inlet part of the column. Such conditions are referred to as thermal mismatch effects and can have a strong impact on peak shape, resulting in peak broadening or peak distortion in the chromatogram. Thus, it is recommended to generally use the eluent pre-heating capability of an HPLC system.

For successful method transfer, care should be taken to also transfer the pre-heating capabilities of the originating system as accurately as possible. Beside the simple yes/no decision if a pre-heater needs to be included or not, the specific design, functional principle, and volume of the respective pre-heater must be considered.

Active and passive pre-heaters have two fundamentally different functional principles to distinguish. Passive pre-heaters (or temperature pre-conditioners) are more common and they work on the principle of a heat exchange device in mechanical contact to a temperature-controlled surface in the column compartment. From its surface, heat is transferred over the pre-heater into the incoming mobile phase along the temperature gradient. If this gradient has the opposite direction ($T_{Compartment} < T_{Eluent}$), heat flow occurs from the incoming eluent to the surface and the device acts as an eluent pre-cooler. This applies when the column compartment is cooled down below ambient conditions because the separation method requires low temperatures. Active pre-heaters are devices that are mostly independent from the temperature control of the column compartment. They use an internal heating element to regulate the temperature to actively control the resulting eluent temperature. The active eluent pre-heater of the Vanquish platform provides a unique opportunity to measure and control the temperature of the eluent streaming into the column, independent of the column compartment temperature. With this, it also allows the user to set the eluent temperature to a different value than the column compartment temperature, at least within certain limits. While column compartments mostly control the temperature by Peltier elements that can either heat or cool depending on the polarity of the applied voltage, the active eluent conditioners typically use a resistance heater, as this is a

much less bulky device to mount directly in front of the column. The consequence is that they can only heat and therefore cannot condition to sub-ambient temperatures. Table 2 provides an overview on the most important characteristics that distinguish active and passive pre-heaters.

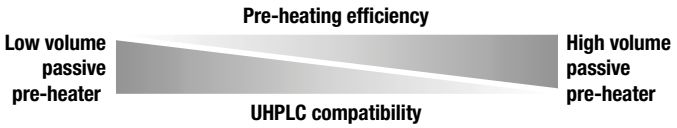


Figure 9. Passive pre-heater efficiency and UHPLC compatibility of different sized pre-heaters

Thanks to the flexible and independent temperature control of active pre-heaters, they provide clear advantages in method transfer scenarios. They can either mimic deviations from the expected outlet temperature of passive devices or compensate for deviations in the dissipation of frictional heat from the column. The advantage of these capabilities will be discussed in the section on column thermostatting.

In cases where a passive pre-heater is used, the volume should be considered, as this is normally the only readily available information. In general, a pre-heater with increased volume exhibits a more efficient pre-heating effect but also increases the extra column volume (Figure 9) and dispersion. That dispersion can be critical in method transfer especially for isocratic separations and UHPLC columns that generate very low peak volumes.

It is thus important to match the pre-heater volume to the specific method requirements, keeping in mind the impact of the column design and flow rate. Elaboration of the experimental setup is required to study the effects of pre-heating since the temperature cannot be directly controlled with passive pre-heaters. The effects of pre-heating were investigated with an UltiMate 3000 forced air column thermostat using different passive pre-heaters and passing ambient temperature water through a column under different elevated temperature settings in the column compartment. The outlet temperature was recorded with a PT-1000 sensor in close contact to the outer surface of the 1/32” stainless steel capillary with thorough insulation using carved Styrodur™ foam.

Table 2. Comparison of passive temperature conditioners and active pre-heaters for features and benefits

	Passive eluent temperature conditioners	Active eluent pre-heaters
Cost	• Not significantly higher than connection capillaries with advanced fitting technique	• Significantly higher than capillaries with advanced fitting techniques by integrated temperature control device and temperature sensor
Temperature control	• Linked to compartment temperature, therefore can also cool down eluents • Lower heating performance for high temperatures and elevated flow rates • No control of heat/cool efficiency	• Temperature control independent of column compartment • Provides highest heating performance at relatively low volume • Heating efficiency can be monitored • Can only heat eluents
Mounting flexibility	• Requires solid contact to temperature-controlled surface in column compartment • Requires fix mounting position and typical size complicates very short connections to column	• Requires electrical contact, otherwise position is independent • Relatively small devices can be directly connected to column inlet
Availability	• Very common type that all manufacturers provide (often) with wide flexibility in volumes, contact materials and internal diameters	• Small selection of manufacturers, different volumes for different flow rates not required, flexibility in contact materials

Figure 10 shows the results for column compartment temperatures of 50 °C, 85 °C, and 105 °C under flow rates between 0.25 mL/min and 5 mL/min and pre-heater volumes of 2 µL, 7 µL, and 11 µL. At the lowest temperature, the 2 µL and the 7 µL pre-heaters were not different, therefore the results of the largest pre-heater are not shown. At low flow rates, the plots of all temperatures indicate that the temperature of the outgoing eluent is above the set-point of the column compartment. This removes the common misconception that passive pre-heaters can never heat to temperatures higher than the column compartment. The reason is that the compartment temperature is measured in the air surrounding the column and not at the plate where the pre-heater is mounted. This plate can be at higher temperature than the air in the center of the column compartment because of heat loss during thermostatting. Another observation is that the increasing slope of eluent temperature decreases with higher flow rate. These curves also show differentiation between the individual pre-heaters. As the pre-heater volume increases and is run at very high flow rates, the heating effect is greater due to the longer (but still considerably short) time the solvent spends in the device. Interestingly, the 2 µL and 7 µL curves cross at all temperature settings. To understand this effect, several pre-heater properties should be considered (Table 3).

Table 3 shows that all devices used in this study had different internal capillary diameters, resulting in substantially different surface-to-volume ratios. Smaller volume pre-heaters have higher surface-to-volume ratios, which improved the pre-heating effect at low flow rates when the time the solvent spends in the heat-exchanger is sufficiently long. Table 3 also shows the total volume of (including the connection capillary volume, which is substantially larger than the heated volume) and the internal diameter of the pre-heaters; both of which have a pronounced effect on the pre-column dispersion. Dispersion, which is expressed as resulting peak volume, decreases with the square of the tubing diameter (right column, Table 3). The trade-off between heating and dispersion will be discussed below. From the data in Figure 10 it can be concluded that the 2 µL pre-heater is effective for flow rates up to 2 mL/min for pure water, which has a markedly higher heat conductivity (factor 3 at 25 °C) than methanol and acetonitrile.[6]

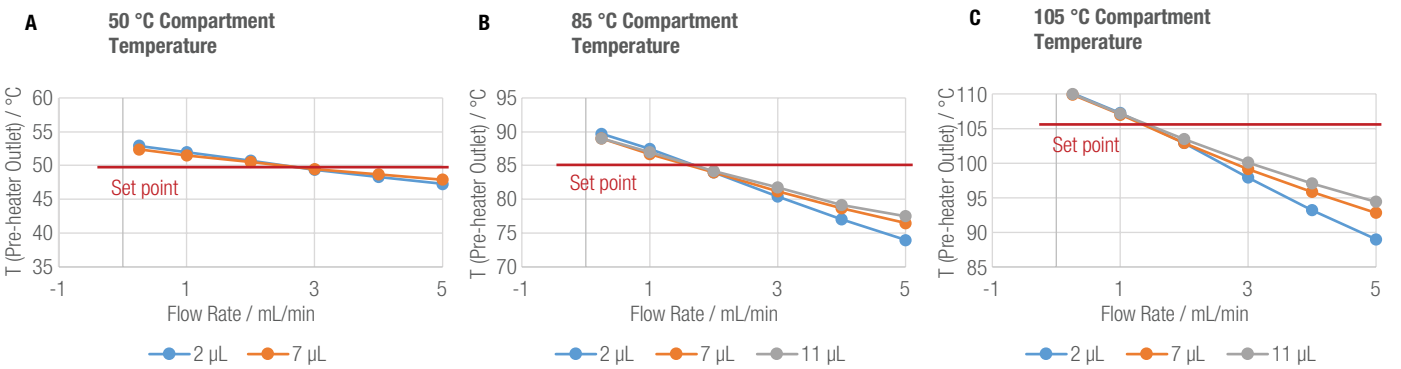


Figure 10. Passive eluent pre-heating effects on flow rate and pre-heater volume grouped by set compartment temperature

Table 3. Physical parameters of the different passive pre-conditioners studied

Nominal heated volume (µL)	Total volume with connectors (µL)	Internal capillary diameter (mm)	Surface to volume ratio (mm^2/mm^3)	Diameter induced dispersion effect (normalized to 1 µL pre-heater)
1	5	0.10	20	1.0
2	8	0.13	15	1.7
7	16	0.18	11	3.2
11	34	0.25	8	6.3

The combined effects of dispersion and eluent heating effectiveness of different passive pre-heaters can be seen from the chromatograms in Figure 11 and Figure 12. The black chromatograms on top show

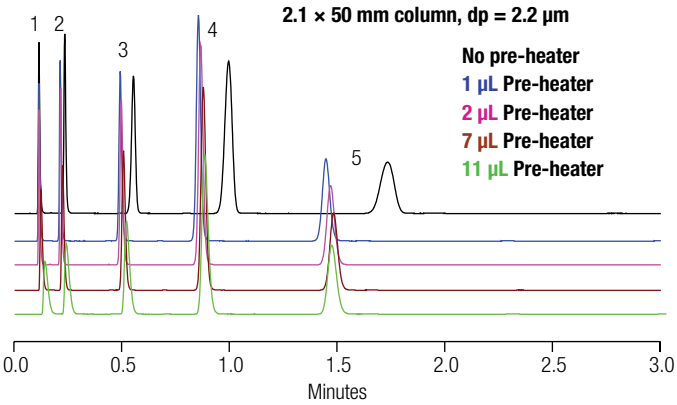


Figure 11. Standard isocratic column test on a column that produces small peak volumes show the effect of the pre-heater on peak shape and retention. Stationary phase: Thermo Scientific™ Acclaim™ RSLC 120 C18, eluent: water/acetonitrile 60/40 v/v, flow rate: 1.0 mL/min, column temperature: 70 °C. Peak assignment: 1: Uracil, 2: Nitroaniline, 3: Methylbenzoate, 4: Phenetole, 5: o-Xylene.

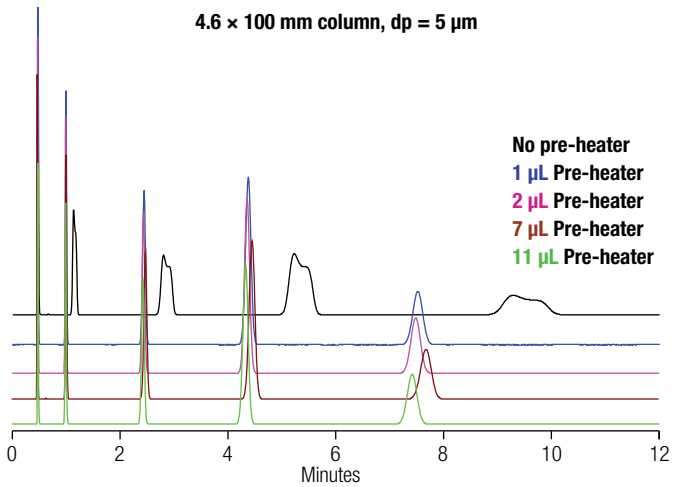


Figure 12. Standard isocratic column test on a wide bore column that produces relatively large peak volumes and is operated at elevated flow rate, show the effect of the pre-heater on peak shape and retention. Peak assignment, stationary phase, eluent, and column temperature as in Figure 11, flow rate: 2.0 mL/min.

the results without a pre-heater. The 2.1 mm column operated at 1 mL/min only shows broadened peaks (Figure 11), while the thermal mismatch in the 4.6 mm column leads to severe peak split or shoulder formation, which increases with the retention factor (Figure 12). This is caused by the less effective pre-heating in the connection capillary at high flow rates and the wider radial temperature gradient in a larger bore column.

As soon as a pre-heater is used, the peaks become much sharper and the retention factor is consistently reduced. These effects are more pronounced on the wide bore column and they result from the reduced thermal mismatch and higher average temperature inside the column when using a pre-heater. Also, the different pre-heater geometries have an effect on both retention and peak shapes that strongly varies with column dimension. While early eluting peaks become broad and asymmetric with the 2.1 mm column, there is no negative effect on peak shape with the conventional 4.6 mm column. It is also interesting to see how retention changes across the different pre-heaters. For both methods, the 7 µL pre-heater produces a lower internal temperature than the 2 µL pre-heater, which is in line with the data for 1 mL/min flow rate (Figure 9). When the 11 µL pre-heater is applied to the 4.6 mm column, it produces a separation with earlier elution of compounds than the 7 uL preheater. One might expect this with higher column temperatures, but it is due to the higher dwell time in a pre-heater with more similar surface-to-volume ratio. With the pre-heater outlet temperature measurement experiments applying pure water as mobile phase, this was at F = 2 mL/min only observed for T = 105 °C (Figure 9). Acetonitrile in the mobile phase of the chromatographic experiments conducts less heat, so the pre-heating conditions will be different relative to experiments with water.

The deepest insights into the effect of the pre-heater on peak shape can be obtained from plotting the determined plate number (N) of all peaks against their retention factor (k). Figure 13 compares the curves with and without pre-heaters in two different columns and methods. While the effect of thermal mismatch is expressed as a reduction in plate number with increasing retention, the effect of extra-column dispersion has the opposite characteristic. The N vs k plot can be used to characterize if the extent of extra-column dispersion of a system is appropriate for a certain column and method. Less extra-column dispersion can be tolerated with smaller peak volumes, in particular for early eluting peaks in isocratic methods. A basic rule of thumb demands 80% of the maximum efficiency that a column delivers in a given method should be achieved at a retention factor above 2. However, if plate numbers decrease in a method with increasing retention, a thermal mismatch effect is indicated. Although it is difficult to discriminate both effects occurring simultaneously, the N vs k plots can give valuable hints. The curves for small bore UHPLC columns are shown in Figure 13A. The operation without the pre-heater (blue) shows decreased efficiency with increasing retention, which clearly indicates thermal mismatch. The curve for the 1 µL pre-heater (orange) shows a normal characteristic of increasing plate number with the second peak at k=3.3 exhibiting 85% (6700) of the maximum plate number of 7900 which is acceptable. The curve for the 11 µL pre-heater (grey), starts with extremely low efficiency, while the second peak at k=2.7 only shows 37% (2200) of the maximum efficiency of 5900 plates, which is far below the 8000 plates that this column should provide in the respective method. Figure 13B shows the same scenarios for the conventional 4.6 mm column. The plate numbers without the pre-heater are included for completeness, but they are calculated from split peaks at high retention and are thus not meaningful. The curve for the 1 µL pre-heater (orange) shows a linear decrease in efficiency with increasing retention, thus pointing to a thermal mismatch effect. Looking at the curve from the 11 µL pre-heater (grey), one can see a normal behavior for an ideal column-to-system match. There is a slight effect of extra-column dispersion, which increases the plate number from 8400 to 9400 between the first and the second retained peak. After that, there is a slight decrease in plate number when going to very high retention. This effect is no thermal mismatch, but results from a stronger contribution of hindered mass transfer expressed as increasing C-term in the van Deemter or Knox equation with increased retention. This mass transfer effect is present in all scenarios and is more or less hidden by the thermal mismatch or extra-column dispersion effect. From the similarity of the orange and grey curve of the 4.6 mm column and from the generally good efficiencies with the 1 µL pre-heater, it can be deduced that the thermal mismatch with the small pre-heater and large column combination is not too severe, while the performance advantage of the 11 µL pre-heater is only minor. In other words, it would still be possible to use the 1 µL pre-heater for the conventional column, but the UHPLC column definitely requires a small volume pre-heater that keeps extra-column dispersion as low as possible.

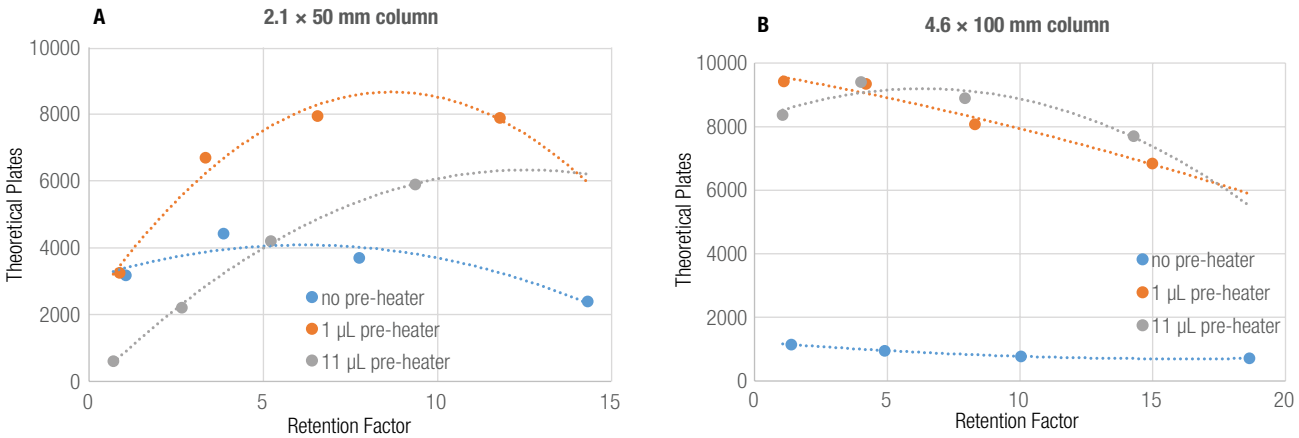


Figure 13. Plot of plate number against retention factor for both column type and experiment with no pre-heater, 1 µL pre-heater, and 11 µL pre-heater

The conclusion for the proper selection of a passive pre-heater in method transfer is not easy and straightforward. The simple rule to increase pre-heater volume with column volume could be demonstrated, but with highly heat-transfer effective small volume pre-heaters, the need for pre-heater volume increase is not always so strong, at least as long as flow rates do not exceed a certain limit. Predictions on the pre-heater volume that gives the best match to the behavior of the originating system will always be difficult, but it is advantageous to have a choice of devices to experimentally find the best one. In general, an appropriate pre-heater should always be used when the column temperature is 10 °C or more above ambient. If there is a choice, one should always start with the smallest available pre-heater. If the heating effect is not sufficient, this will be detected by poor efficiency of the peaks with higher retention and then the next larger pre-heater should be tested.

Column thermostatting and advantages of active pre-heaters

Effects of column thermostatting (even beyond the correct temperature control in the column compartment) are not typically considered in an HPLC or UHPLC method transfer scenario when it comes to root cause analysis of deviating chromatograms. For instance, if the retention times vary between the originating and the receiving system, differences in GDV or flush out behavior are often regarded as the only reason for the observed effect. Similarly, if differences in peak shapes are observed, an effect of the extra-column volume is regarded as the main problem. However, there are different column thermostatting modes applied for HPLC instruments that can have a significant effect on the chromatogram, especially when working at pressures above 400 bar (6000 psi).[7] For applications above 400 bar (6000 psi) the two thermostatting modes, forced and still air, will affect the produced frictional heating differently (Figure 14).

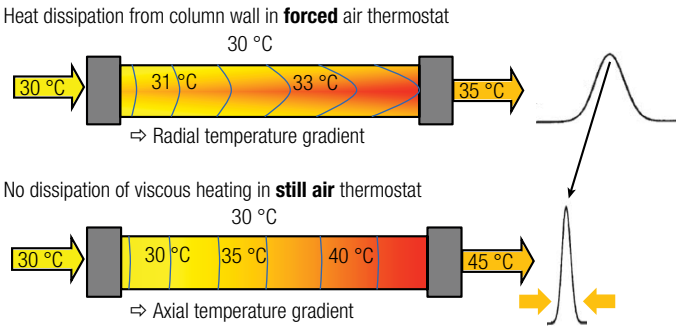


Figure 14. Schematic to show the differences in frictional heat dissipation for forced (top) and still air (bottom). For forced air, a radial temperature gradient occurs while for still air an axial temperature gradient occurs. The given temperatures are not real experimental data but simply serve to illustrate the effects.

In forced air, more frictional heat is removed, which causes a radial temperature gradient. Conversely, in still air thermostatting, the frictional heat is not removed, causing an overall higher separation temperature. The retention is dependent on the separation temperature as retention decreases with increasing temperature; the extent of this behavior is substance specific. In such a case, the effective column temperature also has an influence on the selectivity or distance of peaks.

This effect is illustrated with a separation of preservatives where the selectivity of the critical peak pair (dimethylphathalate/methylparabene) reacts strongly to the changes in column temperature. Moreover, the method produces relevant frictional heat at a pressure above 700 bar (10,000 psi), so a strong influence on the column thermostatting mode (or amount of heat dissipation) can be expected.

Figure 15 shows this effect in the context of transferring the respective isocratic method from an UltiMate 3000 BioRS system (top), which employs a forced air column thermostatting principle and passive eluent pre-heating, to a Vanquish Flex system operated in either forced air (bottom left) or still air thermostatting mode (bottom right) with an active pre-heater. In the forced air mode, the Vanquish Flex system allows method transfer with acceptable resolution of the critical peak pair. Still, the retention factors of peaks 2, 3, and 4 are somewhat reduced and so is the distance of peaks 2 and 3. These differences arise from the fact that the UltiMate 3000 TCC and the Vanquish TCC performance does not result in the exactly equivalent eluent pre-heating and temperature dissipation in their compartments. The still air mode, however, does not allow method transfer with sufficient separation of peaks 2 and 3 despite the overall better peak efficiency. The reason is that the overall higher temperature in the column, resulting from frictional heating, substantially reduces the selectivity between dimethylphathalate and ethylparabene. It would be desirable to take advantage of the still air thermostatting efficiency combined with the better selectivity from the lower column temperature with forced air thermostatting.

To influence the temperature in the column and thus the retention factors, one can take advantage of an independently controllable active pre-heater set at different temperatures. To test this, a series of separations starting from equal temperatures (40 °C) in the column compartment and active pre-heater was performed. The active pre-heater temperature was decreased gradually from 40 °C to 30 °C in 1 °C steps while keeping the column compartment temperature

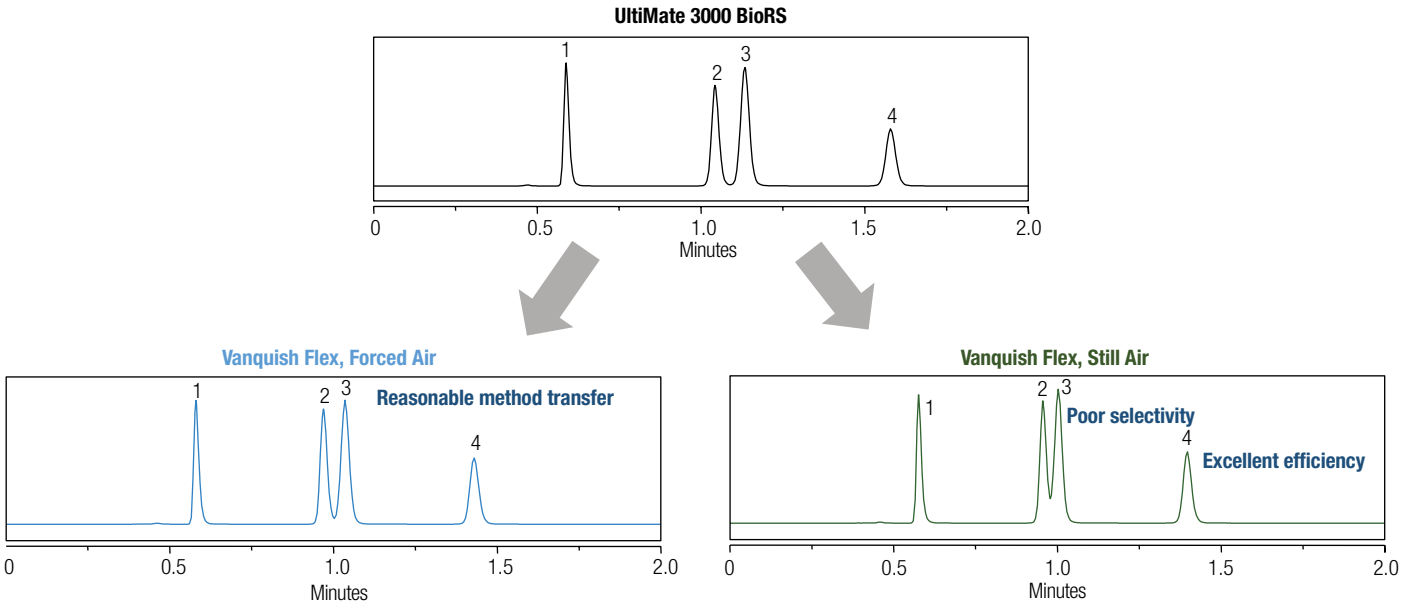


Figure 15. Influence of thermostatting mode on the transfer of a method

constant at 40 °C. To demonstrate the effect, the resulting retention factors were correlated with the temperature of the active pre-heater (Figure 16).

The retention factor of dimethylphthalate on the UltiMate 3000 BioRS system is shown as red dot in the chart at 40 °C with a value of 0.685 (Figure 16A). The retention factors on the Vanquish Flex system are represented as blue dots for the different active pre-heater temperatures. By plotting these two series in a chart, one can determine the intersection of the red and blue data on the y-axis to compare the retention factor on the Vanquish Flex system in still air mode with the retention factor on UltiMate 3000 BioRS system. The intersection can also indicate the corresponding temperature of the active pre-heater, on the x-axis, which in this case determines that an active pre-heater temperature of 30.5 °C leads to matching retention factors between the two systems for dimethylphthalate.

If applying this procedure to methylparaben and methylbenzoate accordingly (see other charts in Figure 16), one can find the active pre-heater temperature corresponding to matching retention factors for methylparabene at 34 °C and methylbenzoate at 32 °C. Since the compounds require three different incoming eluent temperatures to match the retention factor, one could take the average of 32 °C as a compromise to match all three retention factors as close as possible.

As stated above, one can benefit from the positive effects of still air mode under frictional heating at higher system pressures. Key criteria for this separation are the resolution of the critical pair and the overall peak efficiency translating into improved signal-to-noise ratio in the detector. To show the effects, the efficiency improvement of methyl benzoate in still air mode is plotted as a function of the set temperature in the active pre-heater. From Figure 17A, one can clearly see the

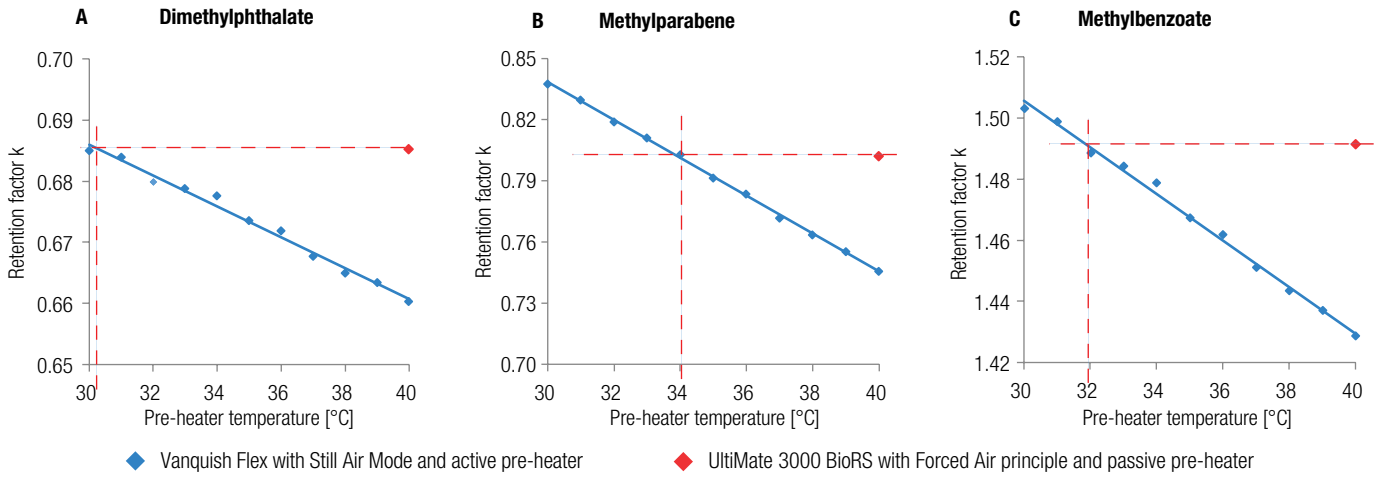


Figure 16. Influence of active pre-heater temperature on compound retention. Red for UltiMate 3000 RS system with passive pre-heater and blue for Vanquish Flex system with active pre-heater

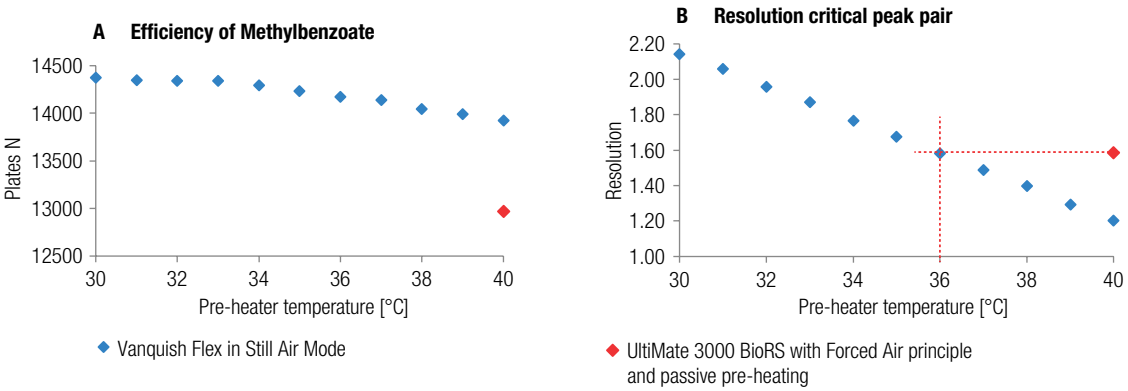


Figure 17. Influence of active pre-heater temperature on chromatographic efficiency and resolution

efficiency increase of 8% at 40 °C associated with still air thermostatting in the Vanquish Flex system. The red dot represents the result on the UltiMate 3000 BioRS system and the blue dots represent the result on the Vanquish Flex system in still air mode with varying pre-heater temperature. When reducing the active pre-heater temperature, it not only impacts the retention factors but also can increase the efficiency, in this case by 10%. The reason is a compensation of a minor radial temperature mismatch inside the column due to residual heat-flow (note that still air is not exactly adiabatic)—but this is only one part of the story. With this application, there is a critical peak pair that had a much worse resolution on the Vanquish Flex system in still air mode than on the UltiMate 3000 BioRS system. Because of influencing the retention factors by decreasing the active pre-heater temperature, the resolution of the critical peak pair changes. To demonstrate this, the resolution is plotted as a function of the active pre-heater temperature, and the intersection between the red dotted line and blue data points of the UltiMate 3000 BioRS system and the Vanquish Flex system, respectively, show the set point for the active pre-heater should be 36 °C. While the resolution is equivalent to the UltiMate 3000 BioRS system under these conditions, the retention factors do not match as shown before. When looking at the previously determined active pre-heater temperature of 32 °C (match of retention), the resolution of the critical peak pair on the Vanquish Flex system clearly exceeds the value observed on the UltiMate 3000 BioRS system.

Figure 18 compares the starting point on the UltiMate 3000 BioRS system at 40 °C and the optimized conditions for the run on the Vanquish Flex system, with the column compartment in still air mode at 40 °C and the active pre-heater set to 32 °C (setting values obtained from the previous evaluations).

By reducing the active pre-heater temperature to 32 °C while keeping the column compartment temperature at 40 °C, one can match the retention factors of the separated compounds of the UltiMate 3000 BioRS system with the Vanquish Flex system. These parameters on the Vanquish Flex system also exceed the resolution from the initial value of 1.58 to 1.93 and increased the efficiency by 11.5%. This example shows the positive effect of this unique property of active pre-heaters. Under frictional heating conditions, active pre-heaters can facilitate the transfer between different thermostatting modes, even without changing the controlled column compartment temperature, which is difficult in a regulated environment.

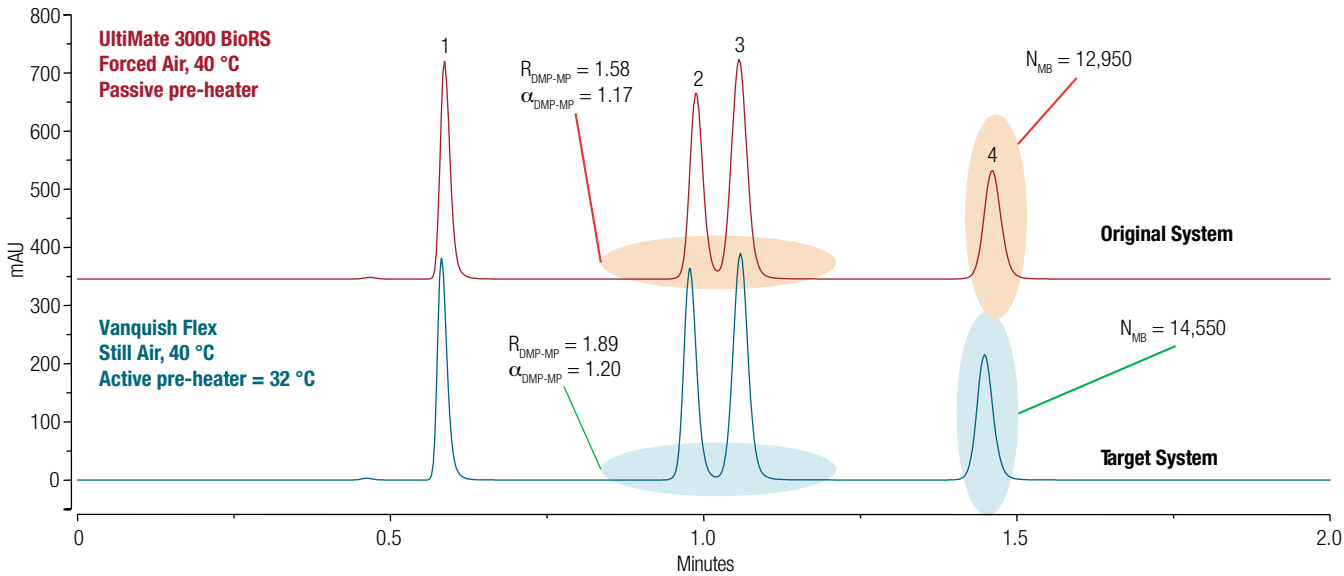


Figure 18. Match of retention times and improved peak shape and resolution with compensation of frictional heat by reduced inlet temperature of the column

Table 4 provides an overview on the column thermostatting modes of commonly used (U)HPLC systems.

Table 4. Thermostatting modes employed by various HPLC systems on the market

(U)HPLC System	Applied Thermostatting Mode
Thermo Scientific UltiMate 3000 Series	Forced Air
Thermo Scientific Vanquish Series	Still and Forced Air
Agilent 1100 and 1200 Series	Still Air
Waters Alliance Series	Forced Air
Waters® Acquity® Series	Still Air
Shimadzu series-i	Forced Air
Shimadzu LC-2010	Still Air

Effect of extra-column volume

The extra-column volume (ECV) is the volume from the injector to the detector excluding the volume in the column. The ECV can be further categorized into pre-column and post-column volume. The pre-column volume is determined mainly by instrument parts such as needle seat and connecting tubing, while the post-column volume also derives from the connecting tubing to the detector and capillaries within the detector, but mainly from the volume of the detector flow cell.

The impact of the ECV on the success rate of the method transfer strongly depends on the method itself. In general, the influence of the ECV becomes more prominent if the column volume decreases. This effect was reported for two column formats under isocratic elution conditions—adding an additional 15 µL ECV to a system with 4.6 × 150 mm column resulted in a small 1% loss in resolution for a low retaining compound (k=1) and no loss of resolution for a more retained compound (k=5). In contrast, for the more challenging column format of 2.1 × 150 mm, the loss in resolution was 19% and 3%, respectively, for the two compounds.[7] Thus, an instrument variation in ECV is of limited relevance when working with standard HPLC columns. If columns of 2.1 mm i.d. are used (UHPLC conditions) the effect of the ECV cannot be neglected.

Figure 19 shows the potential impact of additional ECV, generated by different tubing designs, on a chromatographic separation. Figure 19B gives a chromatographic example where, due to extended ECV, an impurity was not resolved from the main peak while with using Thermo Scientific™ Viper™ Fingertight capillaries and their minimized ECV, the impurity was distinguishable from the main compound. Such effects will be more pronounced for low diameter columns than for standard HPLC columns (4.6 mm i.d.). Thus, care should be taken on the fluidic connections when working with columns 2.1 mm i.d. or smaller.

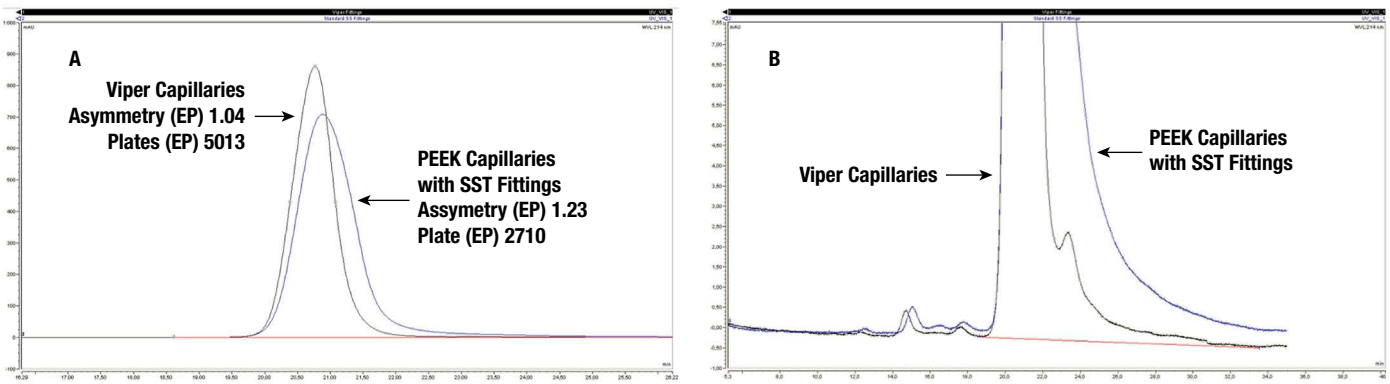


Figure 19. Comparison of Viper capillaries with ferrule-based fitting systems. (A) Asymmetry and plate counts of a single peak and (B) resolution of API and nearly eluting impurity

A significantly lower ECV in the receiving unit than in the originating unit has detrimental effects on the separation of early eluting substances when strong sample solvents are used.

To illustrate this behavior an isocratic separation was used under solvent mismatching conditions (sample in 100% methanol with 50:50 water/acetonitrile elution conditions). Figure 20A shows the plate counts for three different systems against the injection volume. The Vanquish Flex system clearly shows the highest chromatographic efficiency for the lowest injection volumes of 0.5 µL and 1 µL, whereas at 3 µL or higher no difference was observed. In addition, the sample mixing behavior was investigated by calculating a sample mixing factor (dividing the plate count at 3 µL injection volume by the plate count at 0.5 µL injection volume). In Figure 20B the mixing factor is plotted for the three instruments against the plate number at 0.5 µL injection volume and a correlation becomes obvious. Due to the lower general chromatographic efficiency, the Agilent 1260 system exhibits better pre-column sample mixing compared to the other systems. In this case it may make sense to artificially increase the pre-column volume, decrease the injection volume, or try to match the sample solvent with the eluent in order to transfer a method from a system with higher pre-column volume to a system with lower pre-column volume.

In Figure 21, the approach of reducing the injection volume to obtain a satisfactory peak shape is shown. The injection volume can be adjusted according to USP <621> if it fulfills the required precision and detection limits.[4]

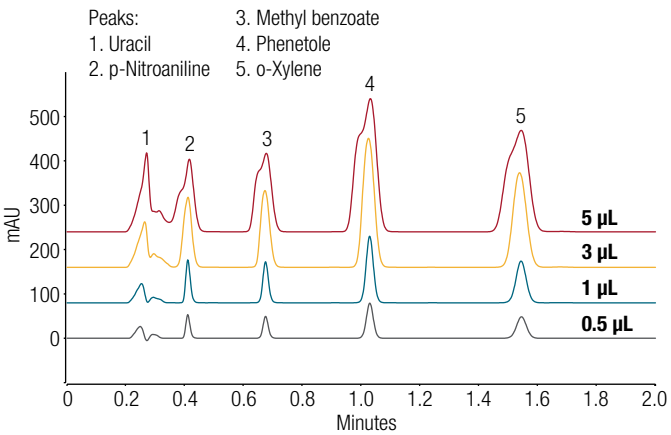


Figure 21. Effect of reducing the injection volume when the sample solvent (100% methanol) is stronger than the eluent (50:50 water/acetonitrile)

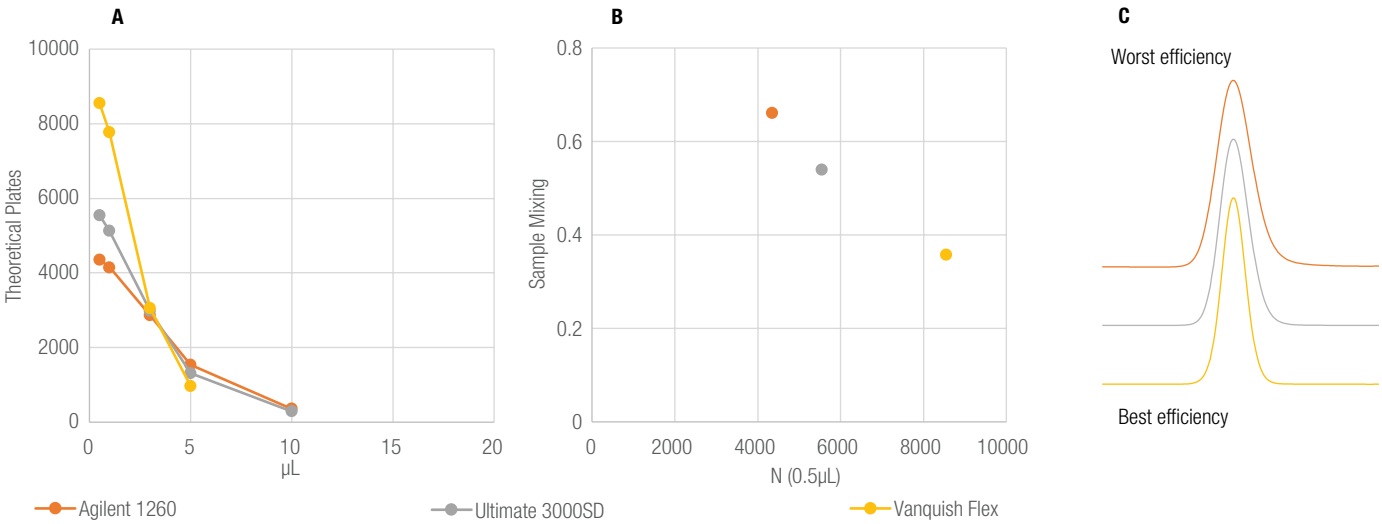


Figure 20. (A) Dependency of increasing injection volumes on system efficiency, (B) relationship between instrument sample mixing behavior and system efficiency with (C) respective peak shapes

For gradient separations the influence of the ECV is lower due to the peak re-focusing effect at the column head. Also, the post-column volume is more relevant than the pre-column volume, due to the on-column peak focusing in the gradient mode. Still, bad fluidic connections as well as inappropriate flow cell dimensions can result in different peak resolution between originating and receiving system when transferring a method (Figure 19).

Detector – flow cells and detector setting

The detector flow cell is critical to consider when transferring methods between different (U)HPLC systems. Care needs to be taken that the flow cell volume is in accordance with the peak volume and with the column diameter. As a rule of thumb, the flow cell volume should not be larger than 10% of the peak volume of the smallest peak. If the ratio between the peak volume and flow cell volume decreases, peak dispersion including a loss of efficiency and signal-to-noise will be the consequence.

The separations shown in Figure 22 were performed on a 1.0 × 100 mm, 2.1 × 100 mm, and 3.0 × 100 mm column, respectively.[8] For all separations a low dispersive UV monitor followed by a high sensitivity flow cell, with 13 µL illuminated flow cell volume and a light path of 60 mm, was used. In addition, the peak broadening factor was calculated by dividing the peak volume measured on the 13 µL flow cell by the peak volume measured with the UV monitor. From this data it becomes obvious that only marginal loss of resolution between the 45 nL and 13 µL flow cell is observed for the 3.0 × 100 mm column with peak volumes between 27 and 129 µL. For the last eluting peak in the 3.0 × 100 mm column, nearly no peak broadening is observed. Here the ratio of peak volume to flow cell volume is exactly 10. For the other column formats the high sensitivity 60 mm flow cell is not suitable. However, during a typical method transfer scenario it might be unrealistic that the column format is changed. Still, the same principle (flow cell volume 10% of peak volume) applies to method transfer scenarios where the column format is kept constant, but the flow cell volume is varied as different instruments are used.

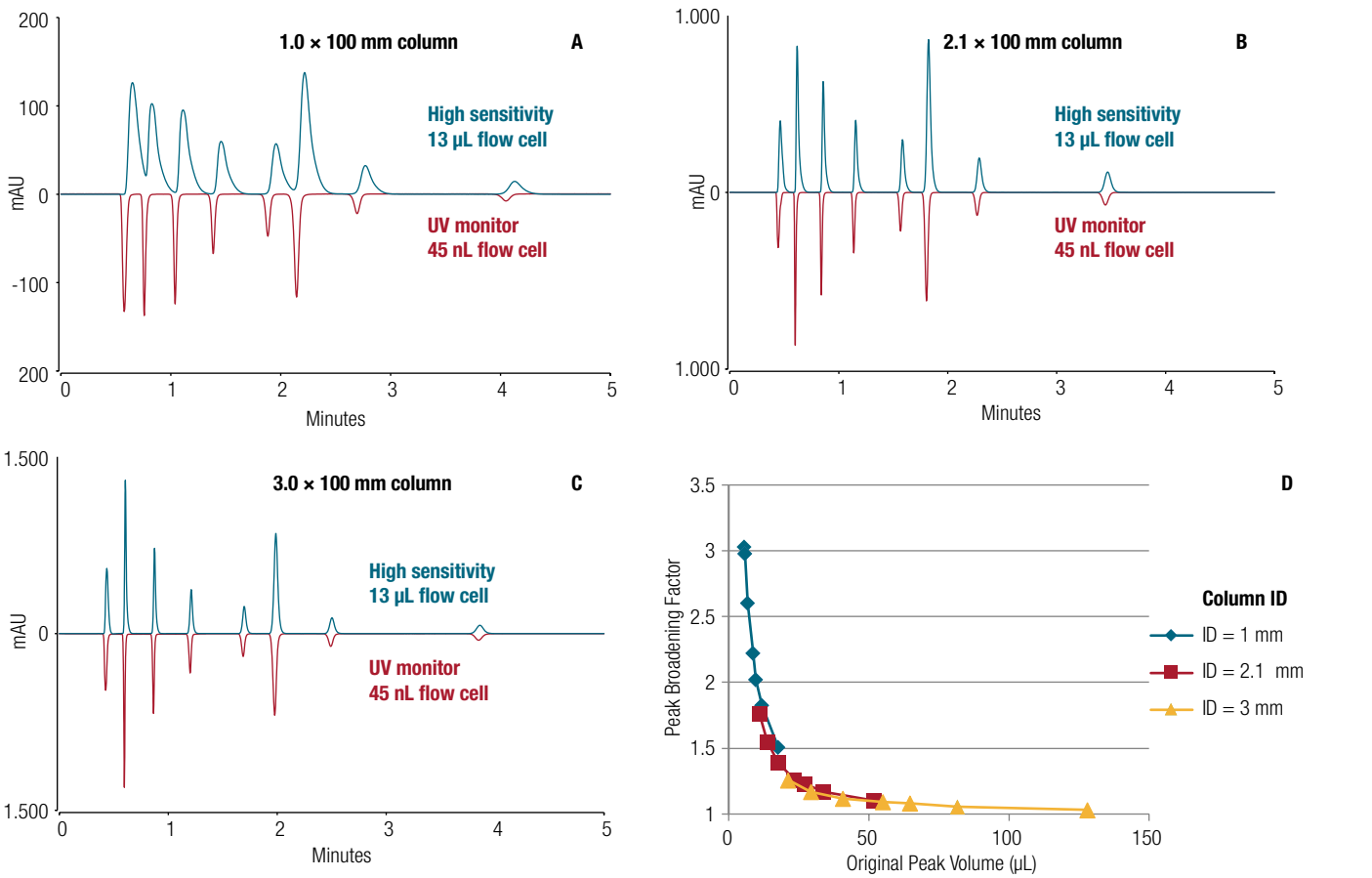


Figure 22. Isocratic separation of a standard mixture on a 1 mm column (A), 2.1 mm column (B), and 3.0 mm (C) using a high sensitivity 60 mm flow cell (blue trace) and a low dispersive flow cell (red trace). In addition, a peak broadening factor is given for all columns in dependence of the peak volume (D).

Besides the physical dimensions of the detector, or specifically the detector flow cell, the detector settings play a major role in obtaining similar results between different types of detectors or between different vendors. For successful method transfer, the setting for bandwidth, reference wavelength, and response time are of importance. The response time (also rise time or time constant) is in general a measure of how quickly the detector responds to a change in signal. An increasing response time reduces the signal noise but may simultaneously decrease the signal height and consequently influence the sensitivity. Furthermore, an increasing response time increases peak width and shifts the peak towards higher retention times.

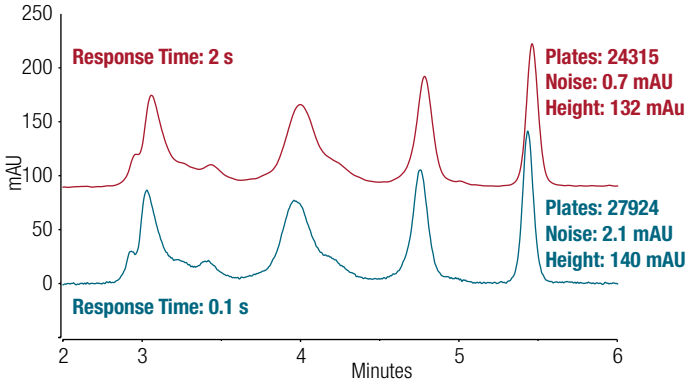


Figure 23. Comparison of isocratic size exclusion chromatography separations measured at different response times while all other parameters were kept constant

Figure 23 shows the effect on a practical example of a size exclusion chromatography (SEC) of a commercial standard. In this case, a decrease of the theoretical plates by nearly 13% was observed. This is especially critical for SEC as baseline separation between aggregates of biotherapeutics is often not easily achieved. In addition, the noise is dramatically decreased for the higher response time and improves overall signal-to-noise, so the user should find a compromise for best results. This compromise is normally provided by the CDS software, such as Chromeleon CDS software, which calculates optimal response times (and data collection rate) based on the obtained peak width.

A parameter influencing the relative quantitative results is the bandwidth of, for instance, a diode array detector. The bandwidth is the wavelength range that is used to record the chromatogram where the signal represents an averaged absorbance value for this wavelength range.

The effect of the bandwidth setting was investigated for an USP-based method analyzing acetaminophen with six different bandwidth settings. A first comparison of the spectra of acetaminophen and impurity B show very similar spectra for both compounds. Thus, the peak area ratio, which is often used for relative quantification purposes, is not affected (Figure 24, blue line). In contrast, the spectra of impurity C and 4-aminophenol have different spectra than the API, which is used for the calculation of the relative peak area. As a consequence, the relative quantification is affected by the bandwidth setting. For different analytes, this effect can even have different directions. While for aminophenol the relative response is decreasing with a broader bandwidth, the relative area of impurity C is increasing (Figure 24, green and purple line).

Thus, we recommend accurately considering corresponding detector settings during a method transfer. When the transfer is done on identical instruments this can be easily done. However, when instruments of different vendors are involved in the transfer, the standard instrument settings should be carefully evaluated.

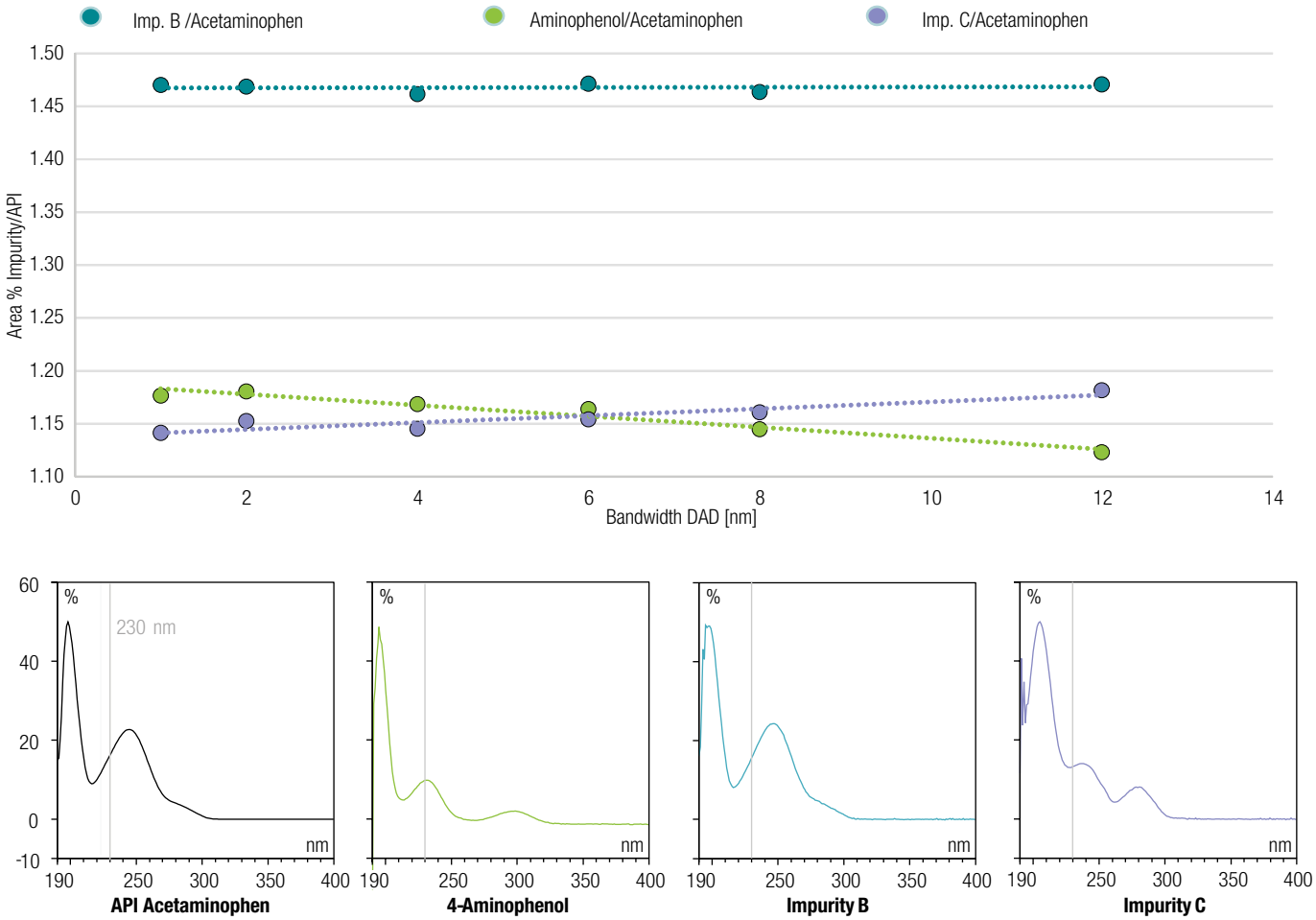


Figure 24. Relative peak areas of three impurities during the USP-based analysis of acetaminophen. Peak areas were recorded for six different bandwidth settings at 230 nm (indicated by gray vertical line) with the respective UV spectra of all involved compounds shown at the bottom.

Conclusions

Transferring HPLC methods depends on several different factors that often make this task very difficult for chromatographers. For instance, non-matching retention times can be caused by:

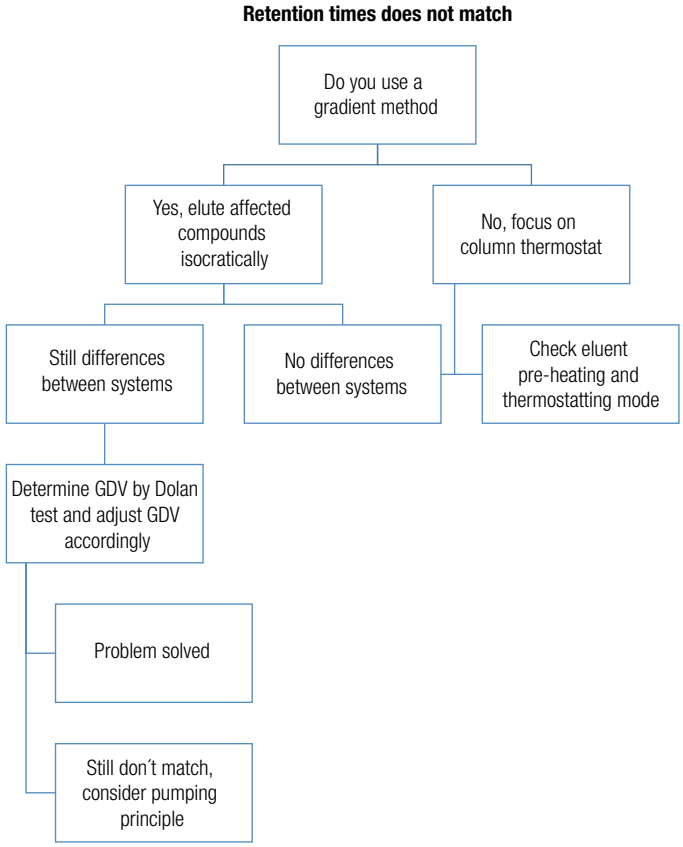
- Different pumping principles (LPG vs. HPG pumps)
- Different GDVs
- Different column thermostatting principles
- Different pre-heater usage

A loss of resolution also can be caused by multiple reasons such as:

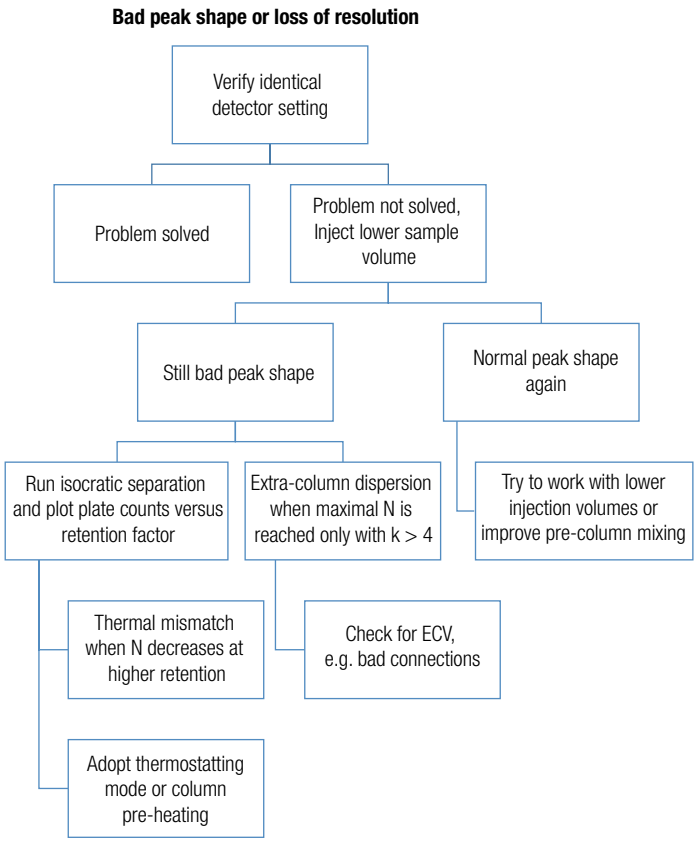
- Thermal mismatch due to pre-heating or column thermostatting
- Additional extra-column dispersion effects
- Sample solvent mismatch
- Detector settings

These two criteria illustrate how complex method transfer can be even when only the instrumental parameters are considered—aspects related to the column used, eluents, or other consumables are not even taken into account. The following flow schemes aim to provide guidance on how to transfer methods after certain observations. The guidance is primarily for the root cause analysis of deviation and not always the final fix of non-matching results, which was in depth discussed in all the sections above.

Retention time problem



Peak shape problem



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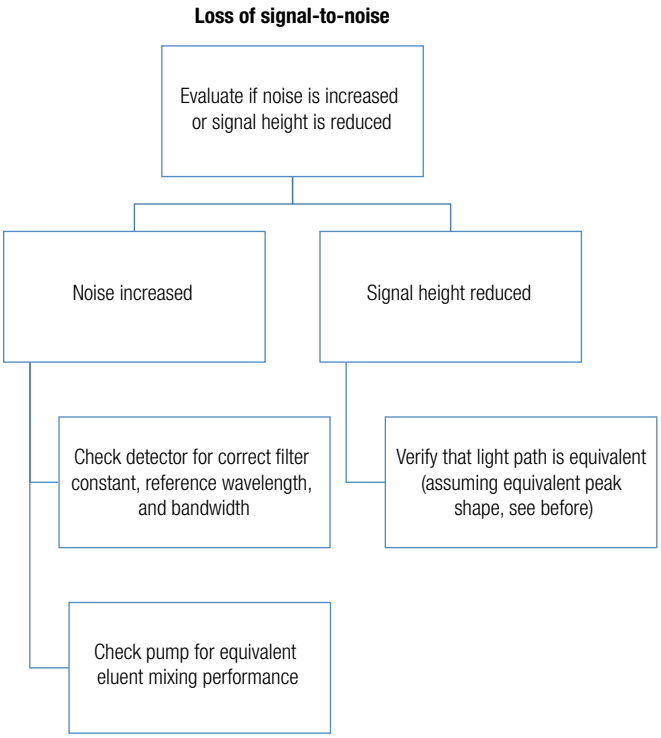
Signal-to-noise problem



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TECHNICAL NOTE 72505

Trifluoroacetic acid performance of the Vanquish Flex Binary UHPLC system

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Keywords
Trifluoroacetic acid, baseline ripples, mixer volume, stroke volume, flow consistency

Goal

Mixing ripple free TFA applications using the Vanquish Flex Binary UHPLC system

Introduction

Trifluoroacetic acid (TFA) is the most common ion-pairing agent used in reversed-phase (RP-) UHPLC for peptide and protein separations. It lowers the pH and modifies the interaction of the molecules with the stationary phase to control selectivity and thus enhance separations. Common conditions for peptide and protein separations include linear and shallow, low organic to high organic, LC gradients where the mobile phase is composed of water and acetonitrile containing approximately 0.1% TFA. Typically, the analytes are detected with a UV detector at 210–220 nm for peptide bonds, as well as at 280 nm for aromatic amino acid residues.

However, under these analytical LC conditions TFA shows some undesirable effects. TFA strongly absorbs UV light below 250 nm, depending on the water/acetonitrile ratio,[1] resulting in a strong shift in baseline during gradient elution. In addition, TFA is retained on RP columns causing the TFA concentration of the mobile phase within the column to fluctuate with varying organic solvent concentration. In the case of incomplete mixing or fluctuating mobile phase content, the dynamics of TFA equilibrium in the column are disturbed causing a strong amplification of mixing noise. Because TFA absorbs 50–100 times stronger than water or acetonitrile in the UV range, significant baseline ripples are observed.[1]

As a consequence, the TFA associated baseline ripples can significantly increase the limit of detection (LOD) for analytes. The LOD is defined as the lowest analyte concentration that can be detected over baseline noise and

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Table of contents

is usually expressed as the concentration at a signal-to-noise ratio of at least 3:1. Those baseline ripples can mask the detection of low concentrated and harmful impurities.

A solution to reduce baseline ripples is to use larger mixer volumes,[2] which also increase the gradient delay volume (GDV) of a LC system. However, by increasing the mixer volume, the separation is delayed, which translates into longer LC run times and therefore limits sample throughput per day. When throughput is a concern, UHPLC systems with small GDVs, and therefore with small mixer configurations, are the preferred option.[3]

When faced with a challenging TFA application that requires high throughput ("small mixer volume required") and low LOD ("large mixer volume required"), one fundamental requirement is that the pump flow must be extremely consistent to avoid fluctuations of TFA concentration.[4]

In this context, the new Thermo Scientific™ Vanquish™ Flex Binary system was compared to the Thermo Scientific™ UltiMate™ 3000 Binary RS system in TFA mixing ripples using the standard configuration of the mixer volume of 200 µL (Figure 1). The standard setup of the Vanquish Flex Binary UHPLC system already showed a significant improvement in TFA baseline ripples due to new technologies used in the Vanquish UHPLC platform. However, more adjustments can be made to reduce the TFA mixing ripples further.

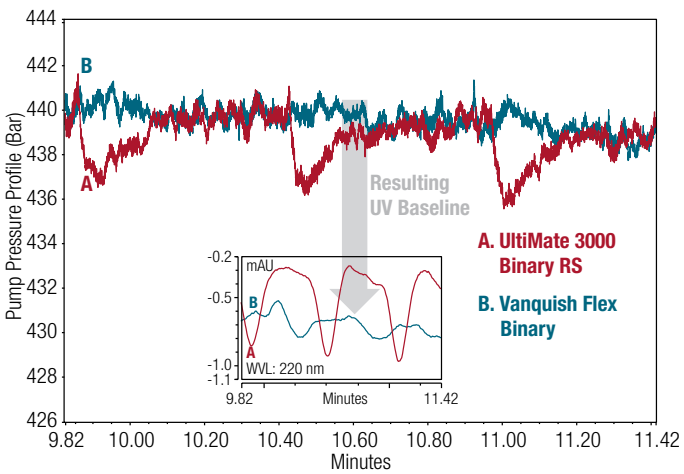

Figure 1. Pump pressure profiles and UV baseline absorbance of the Vanquish Flex Binary system and the UltiMate 3000 Binary RS system. The standard mixer volume of 200 µL was used for both systems.

To improve chromatographic separations for TFA related applications, this technical note will focus on three specific aspects of the pump that contribute to baseline ripples and what can be done to minimize their effects:

1. Mixer volume

2. Stroke volume

3. Flow consistency

Experimental

System equipment

- Vanquish Flex Binary system consisting of the following:
  - System Base (P/N VF-S01-A)
  - Binary Pump F (P/N VF-P10-A)
  - Split Sampler FT (P/N VF-A10-A)
  - Column Compartment H (P/N VH-C10-A)
  - Diode Array Detector HL (P/N VH-D10-A)
  - Flow Cell, 10 mm Thermo Scientific™ LightPipe™ (P/N 6083.0100)
- UltiMate 3000 Binary RS system consisting of the following:
  - Solvent Rack with Degasser (SRD-3600; P/N 5035.9230)
  - Binary High-Pressure Gradient Pump (HPG-3400RS; P/N 5040.0046)
  - Thermostatted Autosampler (WPS-3000TRS; P/N 5730.0000)
  - Diode Array Detector (DAD-3000RS; P/N 5082.0020)
  - Flow Cell, semi-micro, 7 mm, 2.5 µL, SST (P/N 6082.0300)
- Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software, version 7.2 SR4

Consumables

Reagents and chemicals

- Ultra-pure lab water, 18.2 MΩ·cm at 25 °C
- Acetonitrile Optima™ LC/MS grade (Fisher Scientific P/N A955-212)
- Trifluoroacetic acid, LC/MS grade (Thermo Scientific P/N 85183)

LC conditions	
Column:	Thermo Scientific™ Accucore™ C18, 2.6 µm, 2.1 × 100 mm (P/N 17326-102130)
Eluents:	A. Water containing 0.1% TFA
	B. Acetonitrile containing 0.085% TFA
Gradient:	0–0.1 min: 5% B, 0.1–30 min: 5–55% B, 30–30.02 min 55–100% B, 30.02–32 min 100% B, 32–32.02 min 100–5% B, 32.02–37.1 min 5% B
Flow Rate:	0.6 mL/min
Pressure:	From 300 to 450 bar
Temperature:	25 °C
Injection:	1.0 µL

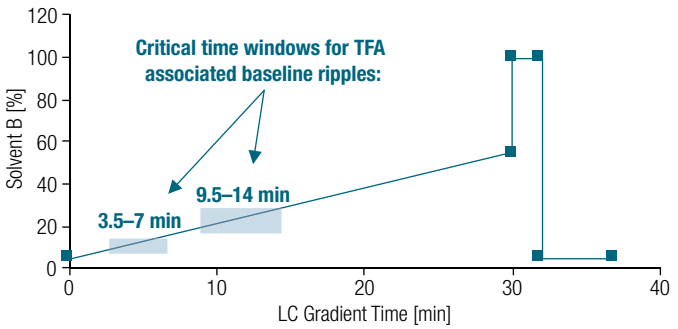

Figure 2. LC gradient with critical time windows for TFA associated baseline ripples highlighted.

Results and discussion

Effect of mixer volume

The pump mixer receives the volume period of solvent A and B delivered by the pump. As a theoretical experiment for better illustration, let us think of the two pump blocks as conveyor belts that run at different velocities, *v*, representing different flow deliveries (Figure 3A), for example:

Conveyor belt A: 4 *v*, e.g. 80% water

Conveyor belt B: 1 *v*, e.g. 20% acetonitrile

This reflects starting conditions for the LC run of 20% solvent B. The parcels (red: from pump A; yellow: from pump B) represent the “disturbances” within the consistent pump flow. The distance between the parcels (here: 1 *m*) is the volume period, which is 80 µL for the Vanquish Flex Binary system (Figure 3A). The parcels on conveyor belts A and B are transferred to a larger conveyor belt, corresponding to the mixing of solvent A and B at the mixing point of a high pressure gradient pump. The speed of the large conveyor belt is the sum of both velocities (here: 5*v*), which corresponds to the volume period that is the sum of all compressed solvent volumes A and B at the mixing point (here: 100%). Subsequently, each parcel (representing the disturbances from the pump) that is delivered from its conveyor belt will accelerate:

Red parcels from pump A:

$$v_A = v \cdot \frac{100\%}{80\%} = 1.25v$$

Yellow parcels from pump B:

$$v_B = v \cdot \frac{100\%}{20\%} = 5v$$

The increase of each velocity results in larger distances between parcels on the larger conveyor belt, meaning larger volume periods (Figure 3A):

Red parcels from pump A:

$$distance_A = \frac{5v}{4v} \cdot 1m = 1.25m$$

Yellow parcels from pump B:

$$distance_B = \frac{5v}{1v} \cdot 1m = 5m$$

The assumption that the distance of 1 *m* on the conveyor belt, corresponding to the volume period of 80 µL in the pump, results in the following single volume period for each solvent after the mixing point:

(1) v_A = 1.25 · 80 µL =100 µL
(2) v_B = 5.00 · 80 µL = 400 µL

V_A: Volume period A (after mixing point)

V_B: Volume period B (after mixing point)

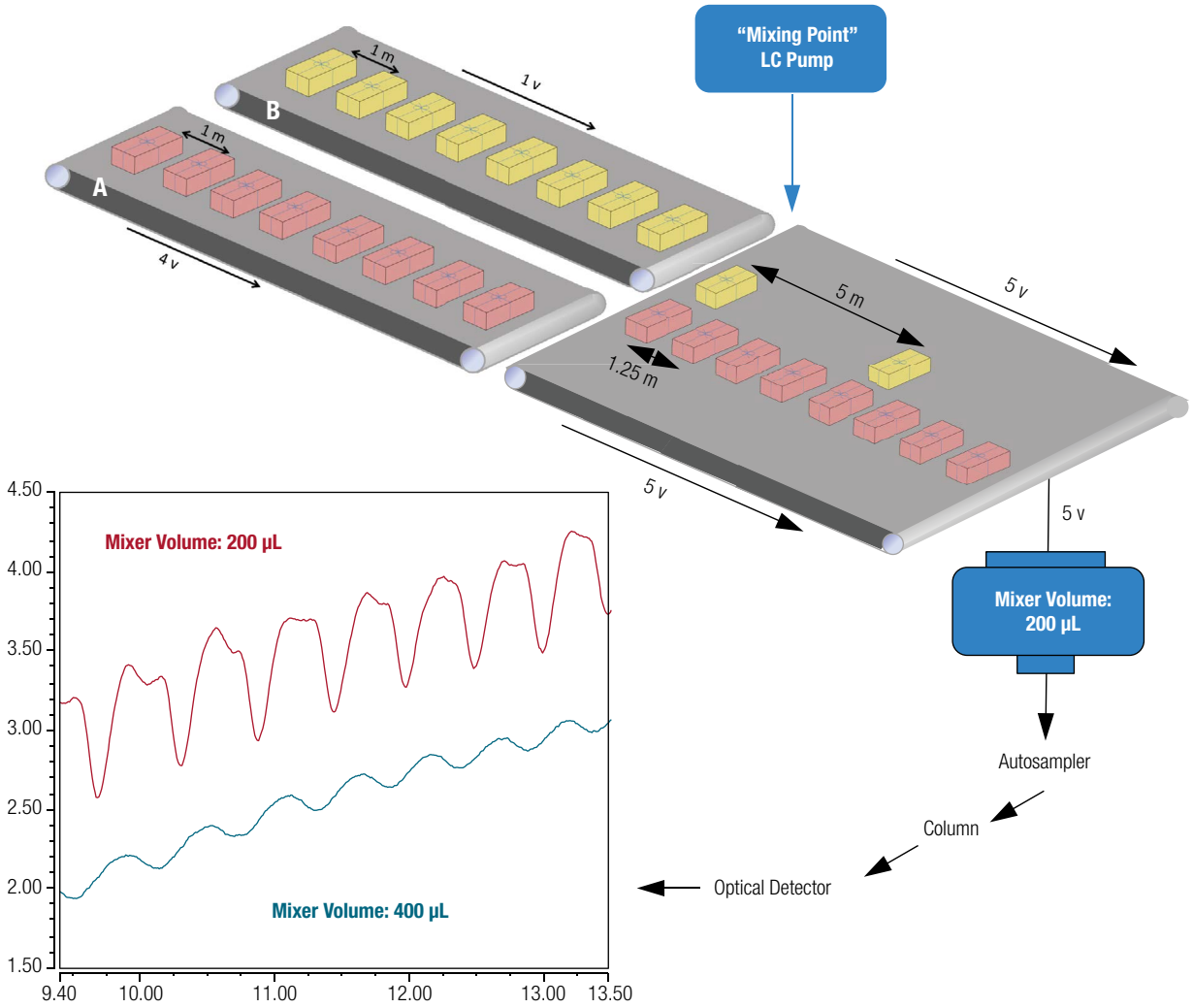

Figure 3. The volume period of the LC pump. (Top) Illustration of solvent flows A and B (represented as conveyor belts A and B) converging at the mixing point and a third conveyor belt to the mixer. The volume period of the LC pump is depicted as the space between parcels (e.g., 1 m) on the conveyor belts. (Bottom) Influence of the mixer size on baseline ripples at constant volume period using the UltiMate 3000 Binary RS system.

To achieve good mixing, the volume period of the disturbance needs to be smaller than the mixer volume. In the example, this is the case for A (100 µL < 200 µL) but not for B (400 µL > 200 µL). As a consequence, incomplete mixing of solvent B within the total flow occurs resulting in fluctuations of mixing concentrations. When using solvents with TFA and UV detection, the fluctuations generated by the pump are detectable and are amplified by the retention of TFA on the column (Figure 3B). Shallow LC gradients, typical for TFA applications, have very large volume periods for solvent B since the initial level of solvent B is very low and increases very slowly during gradient formation (typically < 2% B per min). This slow increase results in long time segments in the LC run where baseline ripples can appear (Figure 2). Therefore, even large pump mixer volumes cannot completely mix this large volume period. As a result, TFA associated baseline ripples can be significantly reduced, but not resolved, by using a larger mixer volume of e.g. 400 µL (Figure 3B) at the cost of increased system GDV.

Effect of stroke volume

The volume of each compressed solvent volume A and B depends on the stroke volume of the pump pistons. In the conveyor belt illustration, the distance between two parcels (the volume period) was assumed to be 1 m, or 80 µL (Figure 3A). To experimentally achieve a small stroke volume, the fixed mixing point of the pump (Figure 3A) can be replaced by a network of different arranged Thermo Scientific™ Viper™ capillaries via mixing T's (Figure 4). The volume period A flows through capillaries with an inner diameter wider than that of the volume period B. At the same time, the split volumes of the volume period B flow into the volume period A, at four different positions. Subsequently this assembly of capillaries causes the splitting of flow B (acetonitrile) to simulate a reduced volume period B of 16 µL, whereas the volume period A remains at 80 µL.

This would correspond to a reduction of the parcel distance from 1 m to 0.2 m in this theoretical experiment. As a result, the volume period B behind the mixing point decreases significantly from 400 µL (see equation on page 3) to 80 µL. Now complete mixing of each solvent within the total flow can be achieved at comparable GDVs using the UltiMate 3000 Binary RS system with the 200 µL mixer (Figure 5A). Residual baseline ripples are reduced significantly, but not resolved, with this flow splitting approach (Figure 5B). In TFA based applications, the extent of volume period A and B divergence is so pronounced that the successful use of small stroke volumes has its limitation. In the example, using a 16 µL stroke volume and 5% or less solvent B as the LC starting condition will translate into a volume period B of 480 µL or higher, which cannot be completely mixed using a 200 µL mixer.

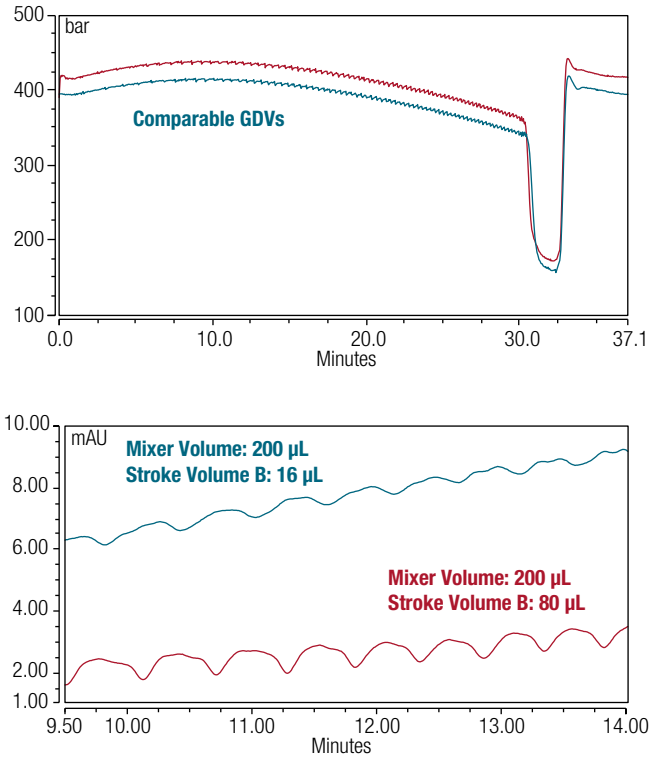

Figure 5. Effect of small stroke volume. (Top) Comparable GDVs between the UltiMate 3000 Binary RS system with 200 µL mixer (red color) and with mimicked mixing point. (Bottom) Influence of small stroke volumes on TFA baseline ripples at fixed mixer volume (blue color).

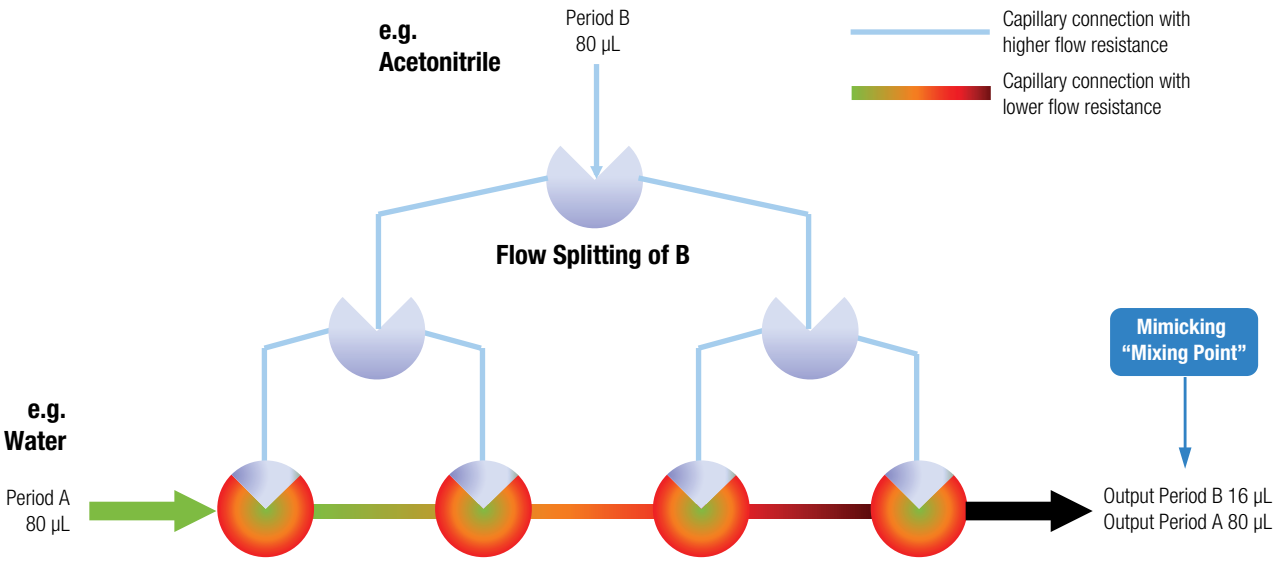

Figure 4. Simulation of a small stroke volume of the pump.

Flow consistency

So far it was described that reoccurring disturbances within the pump flow could not be resolved by using larger mixer volumes. Smaller stroke volumes may improve, but not resolve, the TFA baseline behavior; moreover, smaller stroke volume cannot be set for pump designs that have fixed stroke volumes like the pump of the Vanquish Flex Binary system.

It is important to understand that disturbances of the pump flow can be caused by the thermal constants of the solvents. These effects are less pronounced using water, as it has a high thermal capacity and a low coefficient of expansion, but are generally more significant when using organic solvents, e.g. acetonitrile. These physical properties affect how the solvent volume changes during the pressurization phase in a pump cycle. For water, less compression work must be done compared to that for acetonitrile. From a thermodynamic perspective, water warms up negligibly, whereas acetonitrile heats up notably. After the compression phase of the pump cycle, a time-dependent cool-down to ambient temperature of the compressed solvent takes place in the piston chamber corresponding to a volume reduction, resulting in a flow delivery lower than intended.

Subsequently, the fluctuation of the flow causes the disturbance that is responsible for the variation of the TFA concentration and is amplified by the column. To achieve a low-noise UV trace over the entirety of an applied LC gradient run in TFA applications, disturbances of the flow must be eliminated appropriately (Figure 6).

Smart pump control algorithms are able to compensate this negative flow portion and thus provide a more consistent and accurate flow. This results in a much more even UV baseline with only minimal residual ripples and without the need of major mixer volume adjustments that negatively impact the analysis time. Figure 6 impressively illustrates how substantial the impact of enhanced flow control is on the UV baseline behavior. The left graph displays a TFA baseline for an established UltiMate 3000 Binary RS system with a high level of baseline ripples, which may interfere with analyte peaks, thus reducing reproducible peak integration. The right graph illustrates the superior pump control technology of the Vanquish Flex Binary system, revealing a massive improvement in the TFA baseline noise (Figure 6).

Conclusion

The new pump control algorithm enables the Vanquish Flex Binary system to offer both ultra-low baseline ripple and low GDV at the same time. The system does not require a pulse damper, which would increase the system GDV (and pressure-dependent), thus limiting throughput. A variety of mixers can be used to tailor the system to the LOD needs of various applications. Nonetheless mixer volume adjustment can be minimized due to the new pump control algorithm of the Vanquish Flex Binary system.

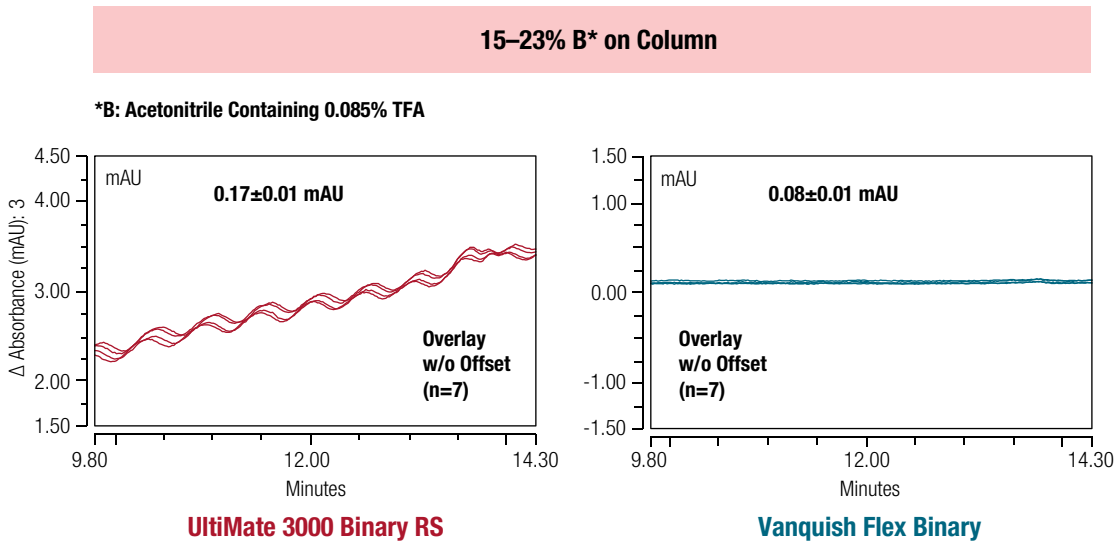

Figure 6. System comparison of TFA mixing performance. The UltiMate 3000 Binary RS system and the Vanquish Flex Binary system tested for TFA triggered mixing ripples with the same pump mixer volume (400 µL) in a challenging TFA application (for details, see Figure 2).

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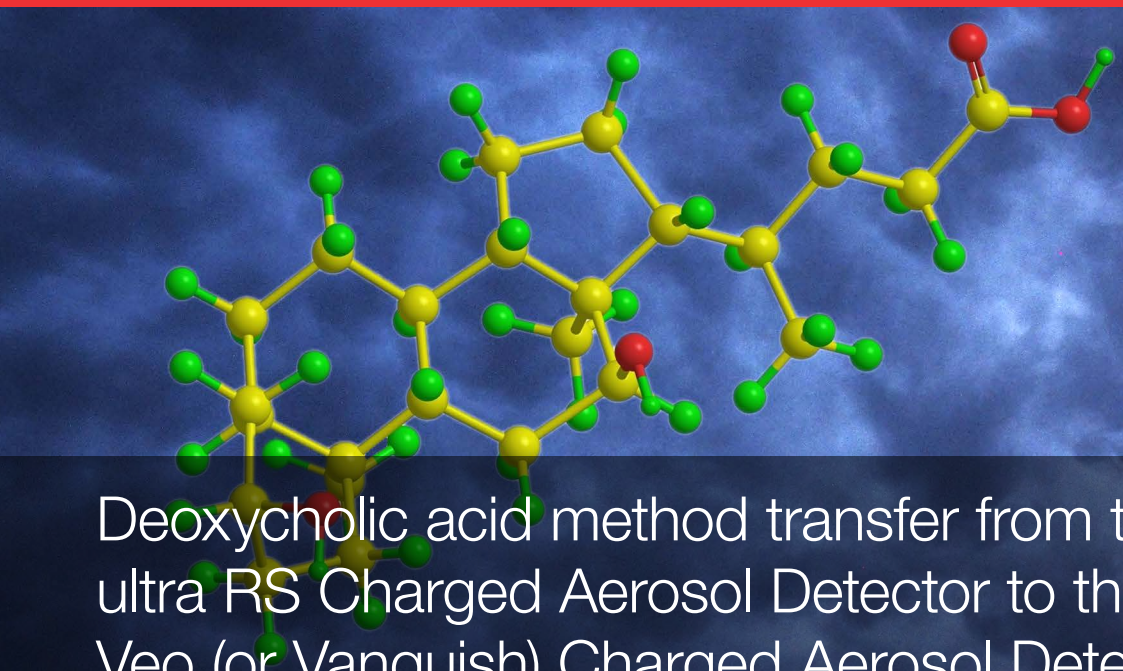
APPLICATION NOTE 72600



Deoxycholic acid method transfer from the Corona ultra RS Charged Aerosol Detector to the Corona Veo (or Vanquish) Charged Aerosol Detector

Authors

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Keywords

United States Pharmacopoeia, desoxycholic acid, method transfer

Goal

To provide guidance for transferring the United States Pharmacopeia (USP) Monograph method for deoxycholic acid, also known as desoxycholic acid, from the Thermo Scientific™ Corona™ ultra RS™ Charged Aerosol Detector (CAD) to the Thermo Scientific™ Corona™ Veo™ Charged Aerosol Detector or Thermo Scientific™ Vanquish™ Charged Aerosol Detector (VCAD).

Introduction

The United States Pharmacopoeia (USP) monograph USP 40-NF 35 describes the use of an HPLC-CAD method for the measurement of both deoxycholic acid, its primary impurity, cholic acid (Figure 1), and several minor impurities. This application note replicates the original USP method, which used a Corona ultra RS CAD, and provides guidance for transfer of the method to the new generation Vanquish Flex CAD (VCAD), which is identical to the Corona Veo CAD.

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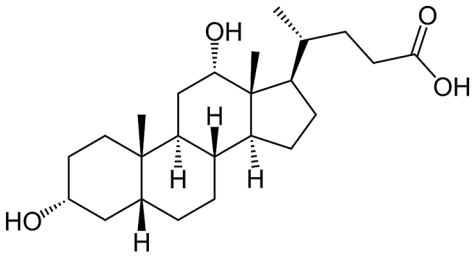
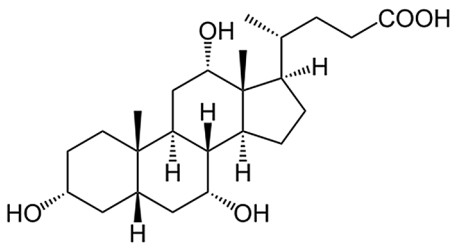
Vanquish Flex UHPLC

Table of contents



Deoxycholic acid



Cholic acid

Figure 1. The chemical structures of deoxycholic acid and cholic acid.

Experimental

Equipment

Chromatographic separation was performed on a Thermo Scientific Vanquish Flex Quaternary UHPLC system including:

- System Base Vanquish Flex (P/N VF-S01-A)
- Quaternary Pump F (P/N VF-P20-A)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment (P/N VH-C10-A)
- Thermo Scientific™ Chromeleon™ Chromatography Data System Software 7.2 SR 5

and either

- Vanquish Charged Aerosol Detector F with concentric flow nebulizer (P/N VF-D20-A, identical to Corona Veo Charged Aerosol Detector, P/N 5081.0010)

or

- Corona ultra RS Charged Aerosol Detector (P/N 70-9406), no longer sold

Reagents and standards

- Acetonitrile, Fisher Scientific™ LC-MS grade (P/N A/0638/17)
- Formic acid, Acros Organics™, 99% for analysis grade (P/N 270480010)
- Water, Ultra-pure (18.2 MΩ·cm at 25 °C) from a Thermo Scientific™ Barnstead™ GenPure™ xCAD Plus Ultrapure (P/N 50136171) Water Purification System
- Cholic acid, Sigma-Aldrich®, USP Reference Standard grade (P/N 1133503)
- Deoxycholic acid, Sigma-Aldrich, USP Reference Standard grade (P/N 1171273)

Conditions

Column:	Thermo Scientific™ Acclaim™ 120 C18*, 4.6 × 150 mm, 3 µm (P/N 059133)		
Mobile Phase A:	0.1% (v/v) Formic acid in water		
Mobile Phase B:	0.1% (v/v) Formic acid in acetonitrile		
Gradient Profile:	*Time (min)*	*% A*	*% B*
	0.0	75.0	25.0
	2.0	55.0	45.0
	14.0	42.0	58.0
	24.0	0.0	100.0
	35.0	0.0	100.0
	35.0	75.0	25.0
	38.0	75.0	25.0
Flow Rate:	1.0 mL/min		
Column Temp.:	30 °C, forced air mode, 30 °C active pre-heater		
Inj. Volume:	25 µL		
Corona ultra RS CAD:	PFV = 1.00; Filter = 3 s; Neb. Temp. = On, 25 °C		
Corona Veo CAD/VCAD:	PFV = 1.20; Filter = 5 s; Evap T = 50 °C		

The USP column requirement is for a 4.6 × 150 mm column with 3 µm particle size of type L1, which is fulfilled by the Acclaim 120 C18 4.6 × 150 mm column with 3 µm particle size.

Preparation of solutions and reagents

Mobile phase preparation

- Mobile phase A: 1 L of 0.1% aqueous formic acid was prepared by adding 1 mL of formic acid to 1 L ultrapure water in a 1 L graduated cylinder.
- Mobile phase B: 1 L of 0.1% formic acid in acetonitrile was prepared by adding 1 mL of formic acid to 1 L acetonitrile in a 1 L graduated cylinder.

Stock standard solutions

Samples were prepared as 1 mg/mL stock solutions in the diluent, 80/20 methanol/water, by adding 10 mg of the sample or reference standard to a 10 mL volumetric flask and filling to the line with diluent. The diluent was prepared by adding 800 mL methanol to 200 mL water.

Working standard solutions

The working standard solutions were prepared as 0.01 mg/mL by adding 1 mL of the stock standard solution to a 100 mL volumetric flask and filling to the line with diluent. The 0.01 mg/mL concentration is required by the compendial method. Calibration solutions of 0.01, 0.005, 0.002, 0.001, and 0.0005 mg/mL were prepared by serial dilution in 100 mL volumetric flasks starting from a 1 mg/mL stock solution.

Results and discussion

System suitability

Figure 2 shows the separation of deoxycholic acid and cholic acid standards using the Acclaim 120 C18 column. Both peaks were well separated and easily quantified. Deoxycholic acid elutes at 15.8 min, which is slightly later than the retention time stated in the USP monograph of "about 13.0 min"; the relative retention time of cholic acid of 0.54 min closely matches the USP-given value of 0.56 min. Neither of these values are required for system suitability, however. The system suitability test requires a %RSD for the signal area of not more than 3.0% for a 0.01 mg/mL solution and a signal-to-noise ratio (SNR) of not less than 10 for a 0.0005 mg/mL solution. As shown in Table 1, both the Corona ultra RS CAD and the Corona Veo CAD/VCAD are suitable for assaying deoxycholic acid and its organic impurities using USP 40-NF 35.

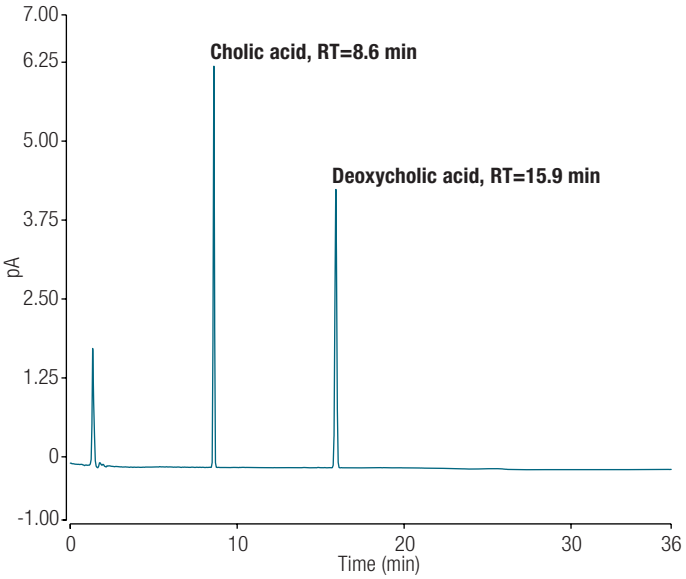


Figure 2. Chromatogram of 0.01 mg/mL cholic acid and 0.01 mg/mL deoxycholic acid.

Method transfer (from Corona ultra RS CAD to Corona Veo CAD/VCAD)

Technical Note 157[1] and Chapter 3 of Charged Aerosol Detection for Liquid Chromatography and Related Separation Techniques[2] were used to provide guidance for method transfer from the Corona ultra RS CAD to the Corona Veo CAD/VCAD. Data acquisition parameters were optimized in the following sequence.

Power Function Value

The first data acquisition parameter that should be optimized is the Power Function Value (PFV). The PFV is used to help linearize the signal output of the CAD over the desired range of quantitation so that SNR is a more accurate measure of sensitivity limits and peak shape is a more accurate measure of chromatographic performance.[4] When evaluating changes in PFV, it is very important to study its effects on response for low levels of analyte and to choose the best curve fit model. Several different PFVs were evaluated including 1.0, 1.10, 1.15, 1.20, and 1.30. The PFV of 1.2 produced the best calibration curve based on a robust evaluation of goodness of fit.

Table 1. Results of system suitability testing.

	Corona ultra RS CAD	Corona Veo CAD/VCAD	USP Requirement
%RSD of area	0.28% (mean, N = 6)	0.63% (mean, N = 6)	< 3.0%
S/N ratio	32 (lowest value of three injections)	42 (lowest value of three injections)	> 10

Evaporation Temperature

There is little or no relationship between the nebulizer temperature (Nebulizer T) setting on the Corona ultra RS detector and the evaporation temperature (Evap T) setting on the Corona Veo CAD/VCAD detector. The Nebulizer T setting is used to prevent freezing of the nebulizer due to evaporative cooling that occurs with highly volatile solvents. It has limited use as a method control variable. The Evap T setting on the Corona Veo CAD/VCAD is an important method parameter enabling greater analytical flexibility. However, the correct choice of Evap T is essential. A low Evap T has the advantage of producing more uniform response between analytes, and the accompanying reduction in selectivity enables the measurement of a broader range of analytes. However, it can be associated with increased noise due to greater contribution from semivolatile impurities. A higher Evap T, on the other hand, is associated with decreased noise, but as more analytes behave as semivolatiles, there may be a loss of signal, especially when measuring low levels. As part of the method transfer, three different Evap Ts were evaluated – 35, 50 and 70 °C. Although an Evap T of 70 °C produced the highest SNR for both deoxycholic acid (SNR = 14 for 0.25 µg/mL) and cholic acid (SNR = 20), due to the concern that it could have an adverse effect on sensitivity for other impurities, an Evap T of 50 °C was chosen as a compromise. At 50 °C the SNR for deoxycholic acid and cholic acid at 0.25 µg/mL, a lower level than the USP-required LOD of 0.5 µg/mL, were 8 and 15, respectively. The background noise was 0.012 pA for all three Evap Ts evaluated.

Signal filter

Several different digital filter settings were evaluated (2, 3.6, 5, and 10 s). The 5 s filter was chosen because it showed a slightly better SNR of 14 for deoxycholic acid at a concentration of 0.25 µg/mL. The SNR was about 10 for the other filter settings.

Method Performance

Using PFV = 1.20, Evap T = 50 °C, and a filter of 5 s, the Corona Veo CAD/VCAD met USP criteria for precision (for 10 µg/mL deoxycholic acid, N = 6, %RSD = 0.63%); and LOD (SNR = 42 for 0.5 µg/mL deoxycholic acid).

Linearity

For all experiments, a linear plot weighted by 1/area^2 was used.[2] Because larger concentrations show larger deviations and therefore have a greater influence on the linear regression line, weighting is necessary to ensure that every concentration is equally well represented by the calibration curve. This model was chosen by following the FDA's guidelines for validation of bioanalytical methods, which require "applying the simplest model that adequately describes the concentration-response relationship using appropriate weighting and statistical tests [such as the residual plot shown in Figure 3] for goodness of fit."[3]

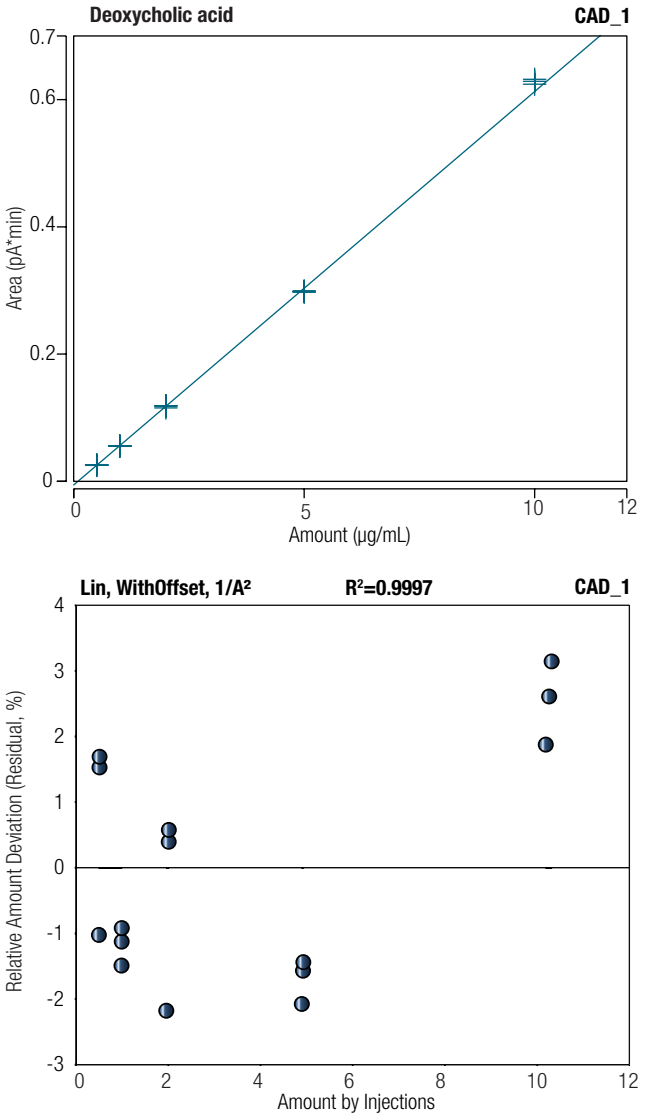


Figure 3. Calibration curve and residual plot for deoxycholic acid using a power function value of 1.20.

Robustness

No adverse effects were found (e.g., on retention time, peak shape, or quantitative accuracy) when doubling the injection volume of a 0.001 mg/mL sample of deoxycholic acid.

Quantification of deoxycholic acid

The percentage of deoxycholic acid in the portion of deoxycholic acid taken was calculated according to the following equation from the USP monograph:

$$\text{Result} = (r_U / r_S) \times (C_S / C_U) \times P$$

r_U = peak area of deoxycholic acid from a 10 µg/mL sample solution

r_S = peak area of deoxycholic acid from a 10 µg/mL standard solution

C_S = concentration of the USP deoxycholic acid RS in the standard solution

C_U = concentration of deoxycholic acid in the sample solution

P= labeled purity of USP deoxycholic acid RS in %

As shown in Table 2, sample 1 met the acceptance criteria on the Corona Veo CAD/VCAD and on the Corona ultra RS CAD. Sample 2, a mixture of bile salts, did not meet the acceptance criteria on the Corona Veo CAD/VCAD or on the Corona ultra RS CAD. Sample 2 was found to contain approximately 50% deoxycholic acid by both the Corona Veo CAD/VCAD and Corona ultra RS CAD. These results are as expected because this was labeled as a mixture of equal parts cholic acid and deoxycholic acid. Subsequent quantification of impurities of this sample verified that the sample was 50% cholic acid.

Quantification of impurities

The percentage of each impurity in a commercial sample of deoxycholic acid, advertised as 98% pure, was calculated according to the following equation from the USP monograph and is shown in Table 3:

$$\text{Result} = [r_U / (r_S \times 100 + r_T)] \times 100$$

r_U = peak area of individual impurity from a 1 mg/mL sample stock solution of deoxycholic acid

r_S = peak area of deoxycholic acid from a 10 µg/mL sample solution

r_T = sum of peak areas of all impurities from the 1 mg/mL sample stock solution of deoxycholic acid

Table 2. Percentage of deoxycholic acid in 10 µg/mL samples.

Sample	Percentage Found, Corona Veo CAD/VCAD	Percentage Found, Corona ultra RS CAD	Acceptance Criteria
1	98.4%	98.7%	97.0–103.0%
2	50.3%	50.1%	97.0–103.0%

A sample with a stated purity level of 98% was analyzed for individual and total impurities using both a Corona Veo/VCAD (Table 3) and a Corona ultra RS CAD (Table 4). Both detectors produced nearly identical results for known impurities cholic acid, 3α,12β-dihydroxy-5β-cholan-24-oic acid, and 3α,12α-dihydroxy-5β-chol-9(11)-en-24-oic acid while a higher level (0.25%) of ethyl 3α,12α-dihydroxy-5β-cholan-24-oate was found with the Corona Veo/VCAD than with the Corona ultra RS CAD (0.14%). A possible unknown impurity was found only when using the Corona ultra RS CAD (relative retention time of 2.01) and contributed to a higher total impurity determination of 1.82% compared to 1.37% with the Corona Veo/VCAD. Further study is required to determine whether these differences are related to detection or other factors. Peaks of less than 0.10% total area summed to 0.40% of total area on the Corona ultra RS CAD and to 0.39% of total area on the Corona Veo CAD/VCAD. With both detectors, the total impurity level met the acceptance criteria of not more than 2% and confirms this sample's stated purity of 98%.

Table 3. Impurities found by the Corona Veo CAD/VCAD in a commercial sample of deoxycholic acid, advertised and verified to be 98% pure.

Impurity Name	Relative Retention Time (Actual)	Relative Retention Time (Compendial)	Acceptance Criteria NMT (%)	% Found	Pass / Fail
Cholic acid	0.52	0.56	1.0	0.10	Pass
3α,12β-Dihydroxy-5β-cholan-24-oic acid	0.72	0.69	0.15	0.13	Pass
3α,12α-Dihydroxy-5β-chol-9(11)-en-24-oic acid	0.88	0.87	0.15	0.26	Fail
Ethyl 3α,12α-dihydroxy-5β-cholan-24-oate	1.62	1.61	0.15	0.25	Fail
Impurity at 33.6 min	2.15	-	0.10	0.25	Fail
Total impurities	-	-	2.0	1.37	Pass

Table 4. Impurities found by the Corona ultra RS CAD in a commercial sample of deoxycholic acid, advertised and verified to be 98% pure.

Impurity Name	Relative Retention Time (Actual)	Relative Retention Time (Compendial)	Acceptance Criteria NMT (%)	% Found	Pass / Fail
Cholic acid	0.52	0.56	1.0	0.10	Pass
3α,12β-Dihydroxy-5β-cholan-24-oic acid	0.72	0.69	0.15	0.13	Pass
3α,12α-Dihydroxy-5β-chol-9(11)-en-24-oic acid	0.88	0.87	0.15	0.26	Fail
Ethyl 3α,12α-dihydroxy-5β-cholan-24-oate	1.62	1.61	0.15	0.14	Pass
Impurity at 31.4 min	2.01	-	0.10	0.41	Fail
Impurity at 33.6 min	2.15	-	0.10	0.39	Fail
Total impurities	-	-	2.0	1.82	Pass

Conclusion

As charged aerosol detection achieves increasing prominence in compendial methods, it gets increasingly important to provide guidelines for method transfer between detectors.

The USP Monograph (USP 40-NF 35) method for deoxycholic acid, originally developed with a Corona ultra RS detector, was easily transferred from the Corona ultra RS CAD to the Corona Veo CAD/VCAD charged aerosol detector. A standard method transfer procedure was followed, resulting in final Corona Veo CAD/VCAD parameters of PFV = 1.20, Evap T = 50 °C, and a filter of 5 s.

The performance of the Vanquish CAD (Corona Veo CAD) readily met the standard set by the Corona ultra RS CAD. The signal-to-noise ratio for the low-level standards was generally better on the Vanquish CAD (Corona Veo CAD) than on the Corona ultra RS CAD and peak area reproducibility was about the same. Both detectors easily satisfied the SNR and peak area reproducibility tests for system suitability specified in the USP compendial method.

Either instrument can be used to perform the USP compendial procedures for both content and impurity levels of deoxycholic acid.

References

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TECHNICAL NOTE 71290

Guidelines for method transfer and optimization—from earlier model Corona detectors to Corona Veo and Vanquish charged aerosol detectors

Authors

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Keywords

Corona Veo CAD, CAD, Charged Aerosol Detection, CAD Method Transfer, CAD Method Optimization

Summary

This technical note provides guidelines for method transfer and optimization of Thermo Scientific™ Corona™ Veo™ charged aerosol detectors and Thermo Scientific™ Vanquish™ charged aerosol detectors VH-D20-A and VF-D20-A.

Initial settings

It is highly recommended that the respective default settings of Corona Veo and Vanquish charged aerosol detectors (CAD) are used as a starting point for all method development.

- Evap T = 35 °C
- Power Function Value = 1.0
- Filter Time Constant = 5.0 sec

Note: When comparing chromatographic data between a Corona Veo or Vanquish CAD and an earlier model Corona detector, using the default conditions, it is fairly common to see a somewhat higher baseline level, noise and drift with Corona Veo and Vanquish detectors. This is typically due to a non-linear 'drop-off' in sensitivity of the earlier Corona models at the extreme low end of the dynamic range. This 'drop off' in sensitivity can mislead the user to think that the achievable lower limits of detection or quantitation with the Corona Veo or Vanquish detectors are poorer than that of earlier models. However, this is more likely due to the better sensitivity (relative absence of signal drop-off) of Corona Veo and Vanquish CAD to very low levels of non-volatile residue.

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Table of contents

– It is important to check the limits of sensitivity by analyzing standards at levels approaching the noise or near the desired limits of detection and quantitation before choosing to adjust instrumental settings or method conditions

– Do not be misled by signal to noise ratios obtained solely from higher level standards since the detector response may be non-linear

Selecting Evaporation Temperature (Evap T)

- Evap T is a variable available on Corona Veo and Vanquish detectors that can be adjusted to optimize performance for a given application

Note: There is little to no relationship between the Nebulizer T setting on the Thermo Scientific™ Corona™ ultra RS™ detector and the Evap T setting on Corona Veo or Vanquish charged aerosol detectors.

  – The Nebulizer T setting on the earlier Corona ultra RS detector is provided mainly to prevent freezing due to rapid evaporative cooling that occurs with highly volatile eluents. However, the usefulness of Nebulizer T as a method control variable is very limited due to a very short residence time within the nebulizer.

- In general, use the lowest Evap T that provides acceptable limits of sensitivity
- As a starting point, we recommend the use of an Evap T of 35 °C
- Higher Evap T settings can be used when analytes of interest have low volatility, relative to the background. Since the volatility of background residue is typically unknown, experimentation is required for optimizing this parameter for a given method.
- With a Corona Veo RS or VH-D20-A detector, it is recommended to perform screening runs at a minimum of two Evap T settings, typically 35 and 40 °C, or additional tests in a maximum of 5 °C increments if needed
- Consider that higher Evap T settings may inadvertently reduce response for low analyte levels—even if considered as a non-volatile
- Remember to check limits of sensitivity by actual analysis of low level standards. Also, check analyte signal reproducibility at each Evap T setting.
- For detection of semivolatiles, lower Evap T settings may be used, however this may lead to higher background levels and noise

Calibration and Power Function Value (PFV) settings

- Recommend using a PFV of 1.0 as a starting point or the same setting as used with an existing method on the Corona ultra RS detector
- Use the simplest curve fitting model that adequately describes the response-amount relationship over the required range of interest
- As with earlier Corona detector models, a linear fit can often be used over a small range, but response is inherently non-linear over a wide dynamic range
- For calibration when a linear fit is inadequate, consider using a:

  – Linear fit of log response vs log amount

  – Quadratic fit (second order polynomial)

- PFV settings other than the default of 1.0 can be used to extend the linear range of the detector output

  – PFV settings of greater than 1.0 are much more common than those less than 1.0

  – PFV settings of <1.0 may, in limited cases, help ‘linearize’ response for semivolatile analytes, albeit over a relatively narrow range

  – Avoid using too high a PFV as it could create or exaggerate a ‘drop-off’ in response near the lower limit of the dynamic range

- Always evaluate goodness of fit over the entire range with special consideration to the upper and lower limits

  – Consider that correlation coefficient (r) and coefficient of determination (r^2) are only aggregate measures of goodness of fit for least squares regression and may not adequately reflect goodness of fit near the sensitivity limits

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Filter settings

- Corona Veo and Vanquish CAD filter settings are chosen based on a time constant
- The digital filtering algorithm used the with Corona Veo and Vanquish CAD is the same as earlier model Corona detectors
- The time constant in the Thermo Scientific™ Corona™ CAD detector (and Thermo Scientific™ Corona™ CAD Plus detector), however, is further modified by the slower electronic circuitry in these models. Table 1 provides a matrix to choose a Corona Veo filter setting that corresponds to a given setting used with an earlier model Corona detector.

Table 1. Matrix for method transfer.

Corona CAD/CAD Plus	Corona ultra	Corona ultra RS	Rate for Corona CAD/CAD Plus/ ultra/ultra RS	Corona Veo	Vanquish CAD
n/a	none	0	0.1 sec	0.1 sec	0.1 sec
n/a	Low	1	0.2 sec	0.2 sec	0.2 sec
n/a	Medium	2	0.4 sec	0.5 sec	0.5 sec
n/a	High	3	1.0 sec	1.0 sec	1.0 sec
None	Corona (Default)	4 (Default)	3.6 sec	2.0 sec	2.0 sec
Low	n/a	5	5.9 sec	3.6 sec (Corona)	3.6 sec (Corona)
Medium	n/a	6	10.1 sec	5.0 sec (Default)	5.0 sec (Default)
High	n/a	7	18.87 sec	10.0 sec	10.0 sec

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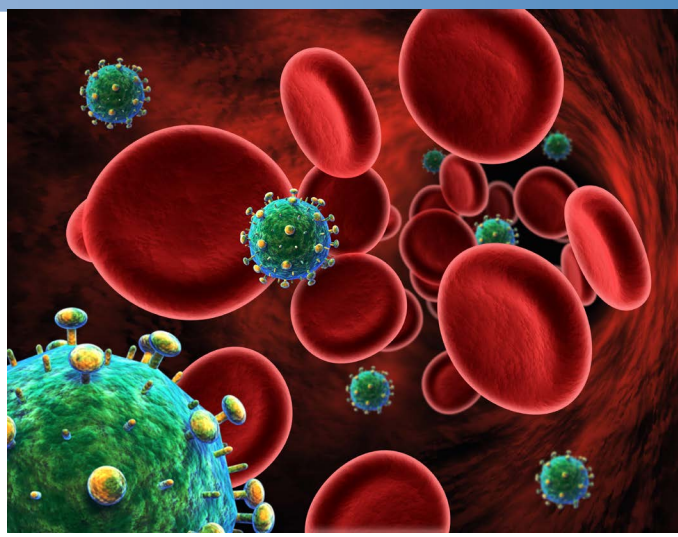
UHPLC Method Development for Analyzing a Once-Daily Tablet Formulation for HIV-1 Infection Treatment

Susanne Fabel
Thermo Fisher Scientific, Germering, Germany

Application Note 1115

Key Words
UHPLC Instrumentation, Stribild® Drug, Autosampler Linearity, Pharmaceuticals, HIV, Generic Method

Goal
To develop an easy to use method for the fast separation of four main compounds of the Stribild pill utilizing the latest UHPLC equipment and technology. The method was further optimized to consider the simultaneous analysis of a variety of possible impurities in this rather complex drug formulation.



Introduction
The HIV-1 specific medication has progressed from monotherapy in the early 1990s towards combination therapies of different antiretroviral agents (ARVs), resulting in numerous pills that a patient must consume.[1] A new formulation for the therapy of HIV-1 is marketed by Gilead Science under the trade name Stribild.[2] This novel "once-daily" tablet was approved by the Food and Drug Administration (FDA) in 2012.[3] Instead of consuming several pills a day, a patient only has to take a single tablet. This tablet formulation is comprised of different types and varying amounts of active pharmaceutical ingredients (API). Emtricitabine (200 mg) is a nucleoside reverse-transcriptase inhibitor (NRTI), tenofovir disoproxil fumarate (300 mg) a nucleotide reverse-transcriptase inhibitor (NtRTI), elvitegravir (150 mg) an integrase strand transfer inhibitor (INSTI), and cobicistat (150 mg) a pharmacokinetic boosting agent.[4]

Several High Performance Liquid Chromatography (HPLC) methods that are used to separate some of these APIs have already been published.[5–7] However, an HPLC method that enables the separation of all those four APIs in a single run has not yet been published within the literature. In this application note, we describe the development of an Ultra High Performance Liquid Chromatography (UHPLC) method for the simultaneous determination of the four components of a simulated Stribald formulation by applying the latest-generation of UHPLC-instrumentation.[8]

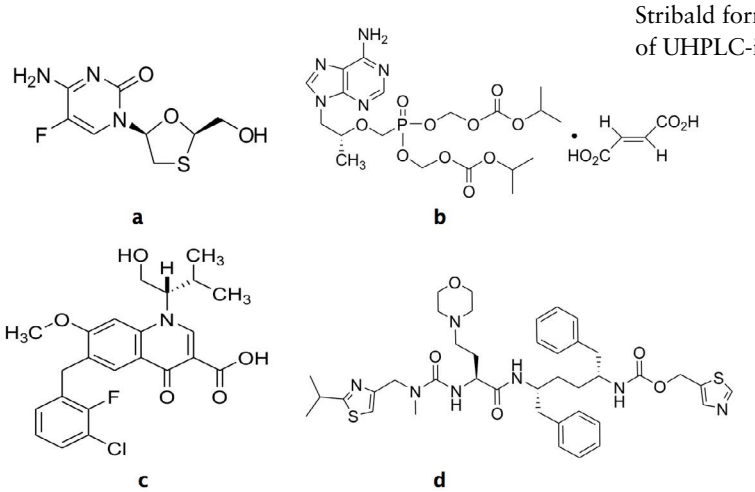


Figure 1. a–d: APIs in Stribild tablets are emtricitabine (a), tenofovir disoproxil fumarate (b), elvitegravir (c), and cobicistat (d).

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Table of contents

Reagents and Chemicals

Compound	Supplier	P/N
Cobicistat	Selleckchem	S2900
Elvitegravir	Selleckchem	S2001
Emtricitabine	USP Standard	1235106
Tenofovir Disoproxil Fumarate	Selleckchem	S1400
Formic Acid	Fisher Scientific™	F/1900/PB15
Methanol Optima™ LC/MS	Fisher Scientific	A456-212
Acetonitrile Optima LC/MS	Fisher Scientific	A955-212
Ultra-Pure Lab Water, 18.2 MΩ·cm at 25 °C	NA	NA

Equipment

- Thermo Scientific™ Vanquish™ System consisting of:
  - Binary Pump H (P/N VH-P10-A)
  - Split Sampler HT (P/N VH-A10-A)
  - Column Compartment H (P/N VH-C10-A)
  - Diode Array Detector HL (P/N VH-D10-A)
- Thermo Scientific™ Dionex™ Chromeleon™ 7.2 Chromatography Data System (CDS)

Experimental Conditions

Column:	Thermo Scientific™ Accucore™ Vanquish™ C18+, 1.5 µm, 2.1 × 100 mm (P/N 27101-102130)
Mobile Phase:	A – water with 0.1% formic acid B – methanol with 0.1% formic acid
Gradient:	0–6.0 min 5–90% B, 6.0–9.0 min 90% B, 6.9–7.0 min 90–5% B, 7.0–10.0 min 5%B
Flow Rate:	0.500 mL/min
Pressure:	1320 bar (max.)
Temperature:	50 °C, still air, easy mode
Injection Volume:	0.5 µL (for calibration: 0.01 µL, 0.05 µL, 0.1 µL, 0.3 µL 0.5 µL, 0.8 µL, 1.0 µL)
Autosampler wash mode: after draw, wash solvent: 40% acetonitrile	
Detection:	214 nm, 260 nm, 50 Hz, 0.1 s response time, 4 nm slit width, 4 nm bandwidth
Flow Cell:	LightPipe™, 10 mm

Standard Preparation

Single stock solutions of 1 mg/mL were prepared for all APIs by dissolving the appropriate amount in methanol, except for emtricitabine which was dissolved in water. The single standard solutions were mixed and diluted with water to yield a standard mixture containing 100 µg/mL emtricitabine, 150 µg/mL tenofovir disoproxil fumarate, 75 µg/mL elvitegravir and 75 µg/mL cobicistat. The concentration ratios of the APIs reflect their dosage ratios in the Stribild formulation.

Results and Discussion

When developing a method, it is a well-tried procedure to begin this approach with a generic elution gradient. As rule of thumb, such a mobile phase gradient starts from 5% and goes up to 90% organic content within a run time that reflects 20 column volumes divided by an appropriate flow rate. Assuming the porosity of 0.6 for an Accucore Vanquish column, the generic mobile phase gradient was performed at a flow rate of 0.4 mL/min within a 10.35 minute gradient time. This generic method already allowed a separation of all APIs and their impurities (data not shown). Nevertheless, the mobile phase gradient was optimized by decreasing the gradient rise-time and increasing the flow rate for faster separation. The limiting factor for this method speed-up was the resolution of a critical peak pair at the detection wavelength of 260 nm (see Figure 2, peak 9 and 10). After final method optimization, a resolution factor of 2.1 was achieved between cobicistat and the elvitegravir impurity. With a flow rate of 0.5 mL/min and a generated system back pressure of 1320 bar, the method worked well within the operational optimum of the Vanquish UHPLC system and Accucore Vanquish column.

Simultaneous recording of a wide range of detection wavelengths (3D-field) with the Vanquish diode array detector revealed that the cobicistat peak is not interfered by the elvitegravir impurity at a detection wavelength of 214 nm (see Figure 2, blue line in zoom). The further analysis of cobicistat was, therefore, made at 214 nm detection wavelength.

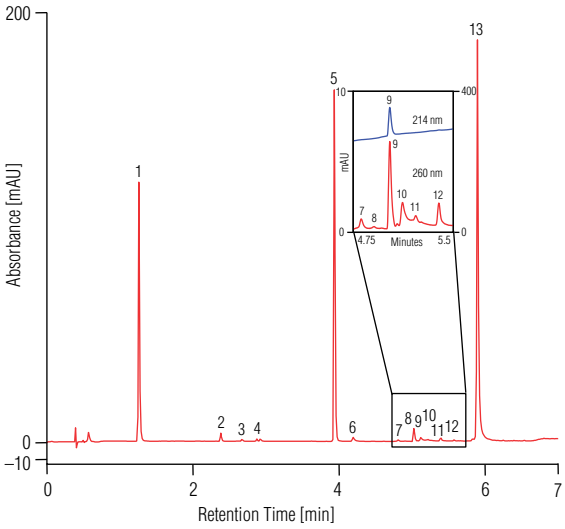

Figure 2. Separation of the four APIs and their impurities (for peak details refer to Table 1) detected at 260 nm. The zoom shows the separation of compound Cobicistat (peak 9) and impurities being compared at 214 nm and 260 nm.

The sources of impurities were identified by injections of single API standard solutions. This approach in method development gives a hint to the possible occurrence of secondary components in the formulation. The occurence of impurities in the commercial formulation has not been evaluated here.

The excellent retention time reproducibility allows for reliable peak assignments. Relative standard deviations of the retention times were <0.008% as shown in Table 1.

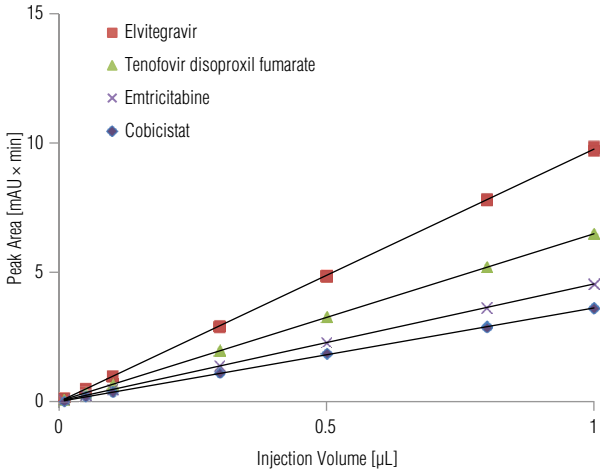

Figure 3. Calibration curves generated for the four APIs of the Stribild formulation by applying injection volumes from 0.01 µL up to 1 µL.

Table 1. Peak identification for Figure 2 with chromatographic performance indicators for three replicates.

Peak No.	Compound	Retention Factor k	Peak Width (min)	Asymmetry (EP)	Resolution (EP)	Retention Time (min)	Retention Time RSD (%)
1	Emtricitabine	2.23	0.02	1.5	41.5	1.26	0.008
2	Tenofovir disoproxil fumarate impurity	5.11	0.02	1.5	10.6	2.38	0.004
3	Elvitegravir impurity 1	5.86	0.02	1.3	9.2	2.67	0.003
4	Unknown 1	6.50	0.02	1.4	35.8	2.92	0.001
5	Tenofovir disoproxil fumarate	9.09	0.02	1.4	6.6	3.94	0.000
6	Elvitegravir impurity 2	9.75	0.03	2.1	13.5	4.19	0.000
7	Elvitegravir impurity 3	11.33	0.03	1	5.3	4.81	0.000
8	Elvitegravir impurity 4	11.58	NA	NA	NA	4.9	0.000
9	Cobicistat	11.88	0.02	1.5	2.1 (24.0 at 214 nm)	5.02	0.000
10	Elvitegravir impurity 5	12.13	0.03	5.3	6.1	5.12	0.000
11	Unknown 2	12.38	NA	NA	NA	5.22	0.000
12	Unknown 3	12.83	0.02	1.9	13.8	5.39	0.000
13	Elvitegravir	14.12	0.02	1.7	NA	5.9	0.000

A calibration curve was recorded for each API by utilizing the wide injection volume range of the Vanquish autosampler. Injection volumes from 0.01 µL up to 1 µL were injected with three replicates. The excellent injection linearity resulted in superior calibration coefficients (see Table 2).

The wide range of the sampler injection volumes allowed the determination of the limit of detection (LOD) and limit of quantitation (LOQ) without any standard dilution. The recorded 3D-field helped to discover the best detection wavelength for every single API to ensure best signal-to-noise ratios. The calculated values for the four APIs are listed in Table 2.

Table 2. Calibration coefficient and LOD/LOQ determination for the four APIs.

Compound	Detection Wavelength	Calibration Coefficent	Calibration Points	S/N (Injection Volume)	LOD (ng on Column)	LOQ (ng on Column)
Emtricitabine	260 nm	0.99998	21	159 (0.01 µL)	0.02	0.06
Tenofovir Disoproxil Fumarate	260 nm	0.99998	21	124 (0.01 µL)	0.04	0.12
Cobicistat	214 nm	0.99980	18	11 (0.05 µL)	1.02	3.41
Elvitegravir	260 nm	0.99987	21	23 (0.01 µL)	0.10	0.33

Conclusion

This application note demonstrates fast and easy method development with the latest generation of UHPLC equipment. The highly efficient Accucore Vanquish C18+ column with 1.5 µm particles provides a fast separation of the four main compounds and several impurities of a simulated Stribild pill. Excellent retention time reproducibility from run to run is utilized for reliable peak identification. Superior calibration curves were generated by applying the wide injection volume range of the Vanquish autosampler. The optimum detection wavelength was determined by acquiring a 3D-field and identifying the best signal-to-noise ratio for LOD and LOQ calculations.

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TECHNICAL NOTE

A UHPLC method development system for efficient scouting of chromatographic elution parameters

Carsten Paul, Susanne Fabel, Anthony Squibb; Thermo Fisher Scientific, Germering, Germany

Key words

Column switching, eluent screening, column screening, solvent selection valve, optimization, method development

Goal

Show the straightforward method development capabilities of the Thermo Scientific™ Vanquish™ UHPLC platform in combination with Thermo Scientific™ Chromeleon™ 7.2 Chromatography Data System (CDS) software

Introduction

The speed of analysis in HPLC has dramatically improved over the last decade due to the development of columns with sub-2 µm particles and the respective UHPLC instrumentation. (U)HPLC method development is, however, still a bottleneck in laboratory workflows and can take from weeks to months, especially if extensive column and eluent scouting is required to complete the development. This often limits the laboratory productivity and increases operational costs. A high degree of automation in column and eluent scouting is required to fully exploit the speed potential of UHPLC.

In reversed-phase HPLC, several parameters are subject to optimization, such as mobile phase pH, column chemistry, and separation temperature, in addition to the gradient profile. The pH value, for instance, is of high importance when analyzing ionizable compounds. In general, the eluent pH should be adjusted according to the pKa value of the compound, which can be a challenge for mixtures of acidic and basic compounds and requires a screening of various pH values. For polar compounds the selectivity might vary between different C18 chemistries, depending on endcapping and additional polar selectivities, and it can be helpful to investigate the separation on a range of C18 columns. Finally, the separation temperature can change the selectivity dramatically and is worth exploring during method development workflows. In order to avoid thermal

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Table of contents

mismatch between column temperature and incoming eluent, best chromatographic practice should include the use of an eluent pre-heater.[1] If all these parameters are used for the method development, a large number of chromatographic runs is required. An instrument and software package enabling an automated sequence and instrument method setup facilitates the task of method development significantly.

In this technical note we present an Automated Method Scouting solution for all Thermo Scientific Vanquish UHPLC systems. This solution combines the leading Vanquish technology with the intelligence of Chromeleon CDS software. It provides quaternary or binary (two out of six solvents) solvent blending and column scouting capabilities of up to six columns using 6-position 7-port switching valves. Other features include advanced thermostatting scouting options, and extensive solvent screening possibilities using a low-pressure solvent selection valve of a dedicated extension kit. With this extension kit, for example, up to 12 aqueous buffers can be screened in an automated manner (Figure 1). These large data sets are automatically evaluated by Chromeleon CDS, and methods providing the best results according to pre-defined criteria, for example best resolution between critical peak pairs, are reported.

This development approach was used for the separation of two isomeric forms of budesonide, a steroid used in the long-term treatment of asthma. Four different columns, spanning a broad selectivity range, were scouted with six different aqueous buffers from pH 3 to pH 8. The method results were evaluated by the best resolution between the critical peak pair.

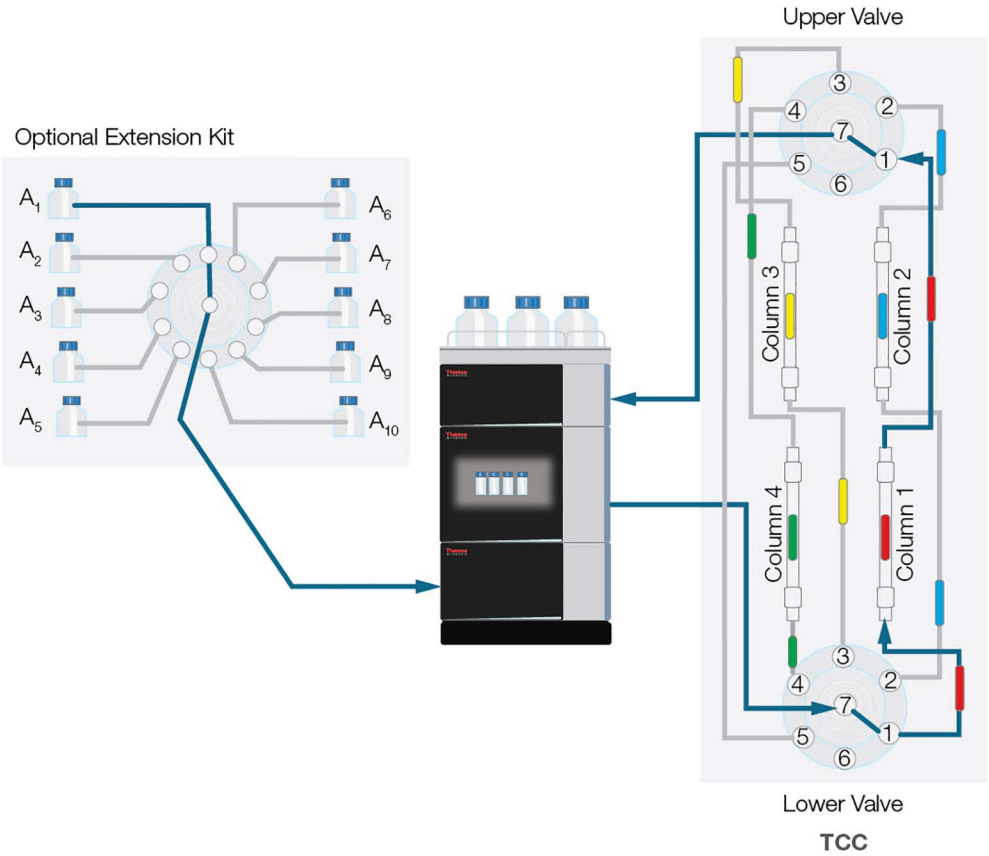

Figure 1. Vanquish flow scheme.

Experimental Conditions	
Columns	Thermo Scientific™ Hypersil GOLD™ VANQUISH™ (2.1 × 100 mm, 1.9 µm), P/N 25002-102130-V Thermo Scientific™ Hypersil GOLD™ VANQUISH™ aQ (2.1 × 100 mm, 1.9 µm), P/N 25302-102130-V Thermo Scientific™ Accucore™ Vanquish™ C18+, (2.1 × 100 mm, 1.9 µm), P/N 27101-102130 Thermo Scientific™ Hypersil GOLD™ C4 (2.1 × 100 mm, 1.9 µm), P/N 25502-102130
Mobile Phase	A1: 20 mM Ammonium formate in water, pH 3, (P/N Ammonium formate A114-50) A2: 20 mM Ammonium formate in water, pH 4 A3: 20 mM Ammonium acetate in water, pH 5, (P/N Ammonium acetate A115-50) A4: 20 mM Ammonium acetate in water, pH 5.6 A5: 20 mM Sodium phosphate in water, pH 7, (P/N NaH_2PO_4 BP329-500, P/N Na_2HPO_4 BP332-500) A6: 20 mM Sodium phosphate in water, pH 8 B: Acetonitrile (v/v), P/N TS-51101
Gradient	0–6.5 min: 5–80% B, 6.5–7.5 min: 80% B 7.5–7.6 min: 80–5% B 7.6–10.5 min: 5% B
Flow Rate	0.5 mL/min
Temperature	30 °C Still Air
Injection Volume	1 µL
Detection	254 nm
Data Collection Rate	20 Hz
Response Time	0.2 s

Experimental

Equipment

Thermo Scientific™ Vanquish™ Horizon UHPLC system consisting of:

- System Base (P/N VH-S01-A)
- Binary Pump H (P/N VH-A10-A)
- Split Sampler HT (P/N VH-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Diode Array Detector HL (P/N VH-D10-A)
- Flow Cell, 10 mm Thermo Scientific™ LightPipe™ (P/N 6083.0100)
- Viper Automated Method Scouting Kit, Vanquish Systems (P/N 6036.2807)
- Column Switching Valve 6-pos 7-port 150 MPa, (P/N 6036.1570)
- Extension Kit for Automated Method Scouting, Vanquish Systems (P/N 6036.0100)

Chromeleon 7.2 CDS software was used.

Sample preparation

Commercially available budesonide powder (Sigma-Aldrich®) was dissolved in acetonitrile (P/N A955-212) to a concentration of 1 mg/mL. This stock solution was diluted 1:1 with water to give a working sample of a concentration of 0.5 mg/mL.

Method scouting workflow

eWorkflow download and sequence setup

For a fast and efficient sequence setup and experimental design, an eWorkflow can be downloaded from the Thermo Scientific™ AppsLab Library of Analytical Applications (name: *Method Scouting on Vanquish Horizon*).[2] After launching the eWorkflow, a sequence with instrument methods for scouting and column switching will appear. This sequence also includes Custom Variables. Custom Variables are associated with a sequence and can be used to set parameters for instrument methods in an elegant way. In this eWorkflow, the active column position, aqueous buffer, flow rate, column temperature, and solvent selector can be set with customer variables (Figure 2). All important parameters of a method development process can be changed with one universal instrument method, making the sequence setup very easy. Details on the use of Custom Variables in Method Scouting experiments were also published in a previous Technical Note.[3]

#	UV_VIS_1	Name	Type	Level	*PercentageASele	*PercentageBSele	*ColumnSele	*ColumnNam	*SSV_Pos	*BufferName	*FlowRate	*ColumnOvenTem
1	None	Column Switching	Blank		%A1	%B1	1	<enter nam...	1	<enter name of...	0.600	30
2	None	Equilibration	Unknown		%A1	%B1			1	<enter name of...	0.600	30
3	None	Blank injection	Unknown		%A1	%B1			1	<enter name of...	0.600	30
4	None	Standard, injectio...	Calibrat...		%A1	%B1			1	<enter name of...	0.600	30
5	None	Standard, injectio...	Calibrat...		%A1	%B1			1	<enter name of...	0.600	30
6	None	Equilibration	Blank		%A1	%B1			2	<enter name of...	0.600	30
7	None	Blank injection	Unknown		%A1	%B1			2	<enter name of...	0.600	30
8	None	Standard, injection...	Calibrat...		%A1	%B1			2	<enter name of...	0.600	30
9	None	Standard, injection...	Calibrat...		%A1	%B1			2	<enter name of...	0.600	30
10	None	Equilibration	Blank		%A1	%B1			3	<enter name of...	0.600	30
11	None	Blank injection	Unknown		%A1	%B1			3	<enter name of...	0.600	30
12	None	Standard, injection...	Calibrat...		%A1	%B1			3	<enter name of...	0.600	30
13	None	Standard, injection...	Calibrat...		%A1	%B1			3	<enter name of...	0.600	30
14	None	Equilibration	Blank		%A1	%B1			4	<enter name of...	0.600	30
15	None	Blank injection	Unknown		%A1	%B1			4	<enter name of...	0.600	30
16	None	Standard, injectio...	Calibrat...		%A1	%B1			4	<enter name of...	0.600	30
17	None	Standard, injectio...	Calibrat...		%A1	%B1			4	<enter name of...	0.600	30

Figure 2. Sequence setup as created by the Automated Method Scouting for Vanquish Horizon eWorkflow. The red boxes highlight the used Custom Variable (please see Reference 3 for a detailed explanation).

For the development of a separation of two epimeric forms of budesonide, four different reversed-phase columns were utilized, one of which was the Accucore Vanquish C18+ column, employing 1.5 µm solid core particles. In addition, the effect of six aqueous buffers ranging from pH=3 to pH=8 was investigated. The separation temperature was kept at 30 °C including passive pre-heaters for all four columns. In general, up to three different organic solvents can be used in the standard configuration of the Vanquish Horizon system, which allows additional screening of different organic eluent types like methanol or solvents blended with modifiers. However, in this example, only acetonitrile was used as organic eluent.

Efficient data evaluation

Chromeleon 7 CDS features the *Intelligent Run Control* to check which injections pass certain criteria. Common test cases are the number of detected peaks, minimal resolution between critical peak pairs, or the peak asymmetry. The most promising conditions are easily found with the powerful Query tool of Chromeleon CDS, which is also part of the downloadable eWorkflow. This tool condenses the most promising injections into a virtual sequence allowing comfortable access to the raw data of the best results. Details on the use of the Intelligent Run Control and the Query functionality were published previously[3] and will not be discussed in more detail here. Figure 3 gives the retention time of all detected critical peak pairs, while the resolution is represented by the bubble size, with larger bubbles meaning better resolution. In total, 24 different chromatographic conditions were tested in less than 20 hours and needed no manual interaction. The data processing was facilitated by Chromeleon 7.2 CDS software, which shortened the time effort for the data analysis to less than 1 hour.

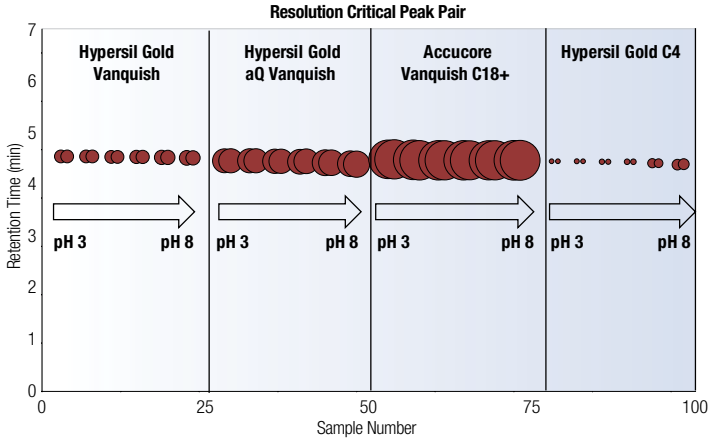

Figure 3. Retention time and resolution of the method scouting of budesonide epimers using the Vanquish Horizon system. The resolution correlates with the bubble size. Two injections were performed for each of the six buffers, resulting in 12 injections on each column. The bubble chart is part of the default report template within the eWorkflow.

As Figure 3 illustrates, the retention time is very similar among all columns and is not affected by the pH of the eluent. The Accucore Vanquish C18+ column with the 1.5 µm solid core particles clearly gave the best resolution and was the only column that delivered a resolution of more than 1.5 as required by the USP method[4] (Figure 4). The Accucore Vanquish C18+ column not only offers an improved separation but also improves the signal-to-noise ratio by 20 percent compared to the second-best resolution method due to the decreased peak width achieved with that column.

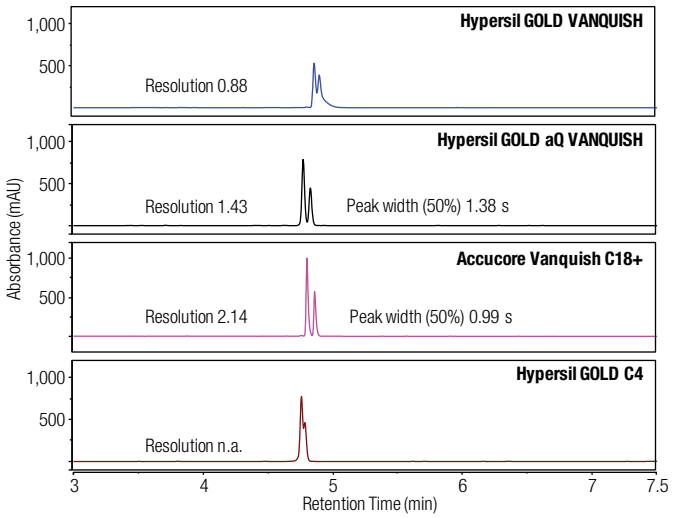

Figure 4. Comparison of the separation of budesonide epimers with four different columns during the method development.

Clearly this method could be further optimized especially regarding the mobile phase gradient and separation temperature to increase the speed of the analysis. This intuitive and fast method development approach would recommend performing the subsequent optimization with the Accucore Vanquish C18+ column. This optimization was done for the separation temperature. For an improved separation of the two epimers, the effect of a sub-ambient separation temperature was investigated using the passive pre-heating capability of the Vanquish UHPLC system.

Figure 5 illustrates the effect of the separation temperature on the resolution of the two epimers of budesonide. While the resolution is 2.04 and lower for a separation temperature of 40 °C and above, the resolution can be increased when working at sub-ambient temperatures. In this case the difference in resolution between 10 °C and 20 °C is marginal. Still, the highest resolution of 2.2 was obtained at a temperature of 10 °C. With the Vanquish systems these temperature settings can be scouted while simultaneously using a passive eluent pre-heater for all four columns in order to avoid any thermal mismatch. This can be very helpful for separations of labile substances or enantiomers.

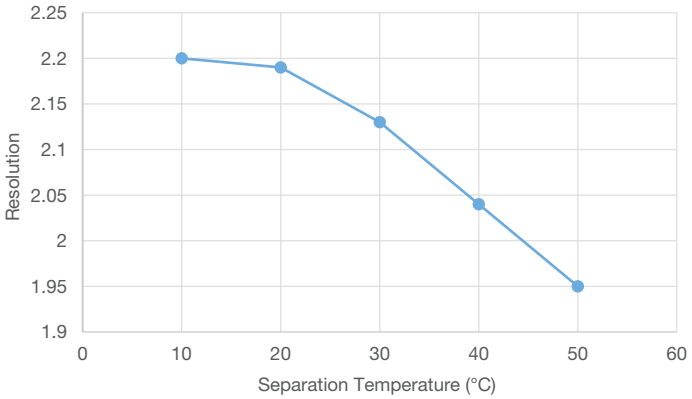

Figure 5. Effect of separation temperature on resolution for the separation of budesonide epimers.

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Conclusion

The Automated Method Scouting solution for the Vanquish UHPLC platform, in combination with Chromeleon 7.2 CDS, enables chromatographers to develop (U)HPLC methods easily and efficiently because of the automation. The instrument enables the screening of up to six columns and an extensive range of solvents. Chromeleon CDS facilitates this workflow by automated sequence setup using pre-defined eWorkflows and Custom Variables, post-processing tools, and graphical user interfaces for method evaluation. This way the complete method development process can be accelerated significantly.

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APPLICATION NOTE 72763

Metoprolol impurity testing by charged aerosol detection: method transfer and optimization of a USP method

Authors

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Keywords

United States Pharmacopoeia, USP modernization, beta blocker, Lopressor™, Toprol™, metoprolol succinate, metoprolol tartrate, anti-doping, impurity testing

Goal

Simple step-by-step optimization for the Corona Veo/Vanquish CAD of a USP method for impurity analysis of the small molecule pharmaceutical metoprolol.

Introduction

As part of the United States Pharmacopoeia (USP) monograph modernization effort, USP 41(3) In-Process Revision includes a proposed change to the official USP Metoprolol Succinate monograph (USP 38, page 4370) for the determination of organic impurities (USP related compounds H and I) that lack UV chromophores (Figure 1). The older TLC method is replaced by one that uses a hydrophilic interaction chromatography (HILIC) method coupled with a charged aerosol detector (CAD).

This application note replicates the updated USP method and related publication,[2] both of which used older models of CAD (e.g., Thermo Scientific™ Dionex™ Corona™ ultra RS™ Charged Aerosol Detector), and provides guidance for transfer of the method to the new generation Thermo Scientific™ Vanquish™ Flex CAD (VCAD), which is equivalent to the Thermo Scientific™ Dionex™ Corona™ Veo™ CAD. This method also works flawlessly and without modification on a Thermo Scientific™ Vanquish™ Horizon CAD, which is equivalent to the Thermo Scientific™ Dionex™ Corona™ Veo™ RS CAD. The following Corona Veo/VCAD data acquisition parameter settings are recommended for optimal performance: power function value (PFV) = 1.30;

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Vanquish Flex UHPLC

Table of contents

evaporation temperature (Evap T) = 35 °C; Filter = 5 s. A doubling of the injection volume is also recommended for any model CAD. Using these values, the Corona Veo/VCAD easily meets all USP requirements. This work also optimizes CAD digital filter settings to ensure resolution of metoprolol from other substances, besides impurities H and I, likely to be present in real samples (USP related compounds A, B, C, D, and succinate). Metoprolol (Figure 1) is an active pharmaceutical agent present in the commercial products Lopressor™ as the tartrate salt and Toprol™ as the succinate salt.

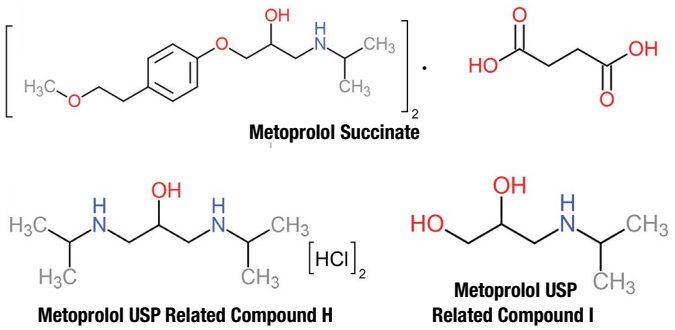

Figure 1. Metroprolol compounds

Experimental

Equipment

Chromatographic separation was performed on a Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC system including:

- System Base Vanquish Flex (P/N VF-S01-A)
- Vanquish Flex Quaternary Pump (P/N VF-P20-A)
- Vanquish Flex Split Sampler (P/N VF-A10-A)
- Vanquish Column Compartment (P/N VH-C10-A)
- Thermo Scientific™ Chromeleon™ Chromatography Data System Software 7.2.8

and either

- Vanquish Flex Charged Aerosol Detector with concentric flow nebulizer (P/N VF-D20-A, identical to Corona Veo Charged Aerosol Detector, P/N 5081.0010)

or

- Corona ultra RS Charged Aerosol Detector (P/N 70-9406)

Reagents and standards

- Acetonitrile, Fisher Scientific™ LC-MS grade (P/N A/0638/17)
- Formic acid, Acros Organics™, 99% for analysis grade (P/N 270480010)
- Water, Ultra-pure (18.2 MΩ·cm at 25 °C) from a Thermo Scientific™ Barnstead™ GenPure™ xCAD Plus Ultrapure Water Purification System
- Metoprolol succinate, USP Reference Standard (P/N 1441298)
- Metoprolol Related Compound H: purchased as the dichloride form of European Pharmacopoeia Metoprolol Impurity M (CAS 73313-36-7). LGC Standards (P/N MM0027.28)
- Metoprolol Related Compound I: purchased as European Pharmacopoeia Metoprolol Impurity N (CAS 6452-57-9). Enamine Store, Monmouth Junction, New Jersey, USA (P/N EN300-138953)
- Metoprolol Related Compound A (CAS 109632-08-8): Enamine Store (P/N EN300-223895)
- Metoprolol Related Compound B (CAS 56718-76-4): LGC Standards (P/N MM0027.17)
- Metoprolol Related Compound C (CAS 29122-74-5): Enamine Store (P/N EN300-649742)
- Metoprolol Related Compound D (CAS 1486464-40-7): USP Reference Standard (P/N 1441265). Note: This standard is sold containing about 25% of an impurity that coelutes with metoprolol related compound H and is UV-transparent.

Conditions

Column:	HALO™ Penta HILIC 4.6 x 150 mm, 5 µm, polyol column with 1,2,3,4,5-pentahydroxypentyl derivatization. Advanced Materials Technology, Wilmington, Delaware, USA (P/N 95814-705).
Mobile Phase:	85% acetonitrile, 15% 0.1 M ammonium formate in water, pH 3.2
Flow Rate:	1.0 mL/min
Column Temp.:	25 °C, forced air mode
Inj. Volume:	10 µL
Corona ultra RS:	PFV = 1.00; Filter = 3 s; Neb. Temp. = on, 25 °C
Corona Veo/VCAD:	PFV = 1.30; Filter = 5 s; Evap T = 35 °C. These parameters were individually optimized (see below)

Preparation of solutions and reagents

Mobile phase preparation

A 200 mL solution of 100 mM ammonium formate was prepared, adjusted to pH 3.20 with formic acid, and subsequently filtered through a 0.45 µm cellulose acetate filter. A 150 mL portion of the filtered ammonium formate buffer was added to 850 mL acetonitrile.

Diluent

The diluent was prepared by adding 850 mL acetonitrile to 150 mL water.

Stock solutions

- A 1.0 mg/mL stock solution of metoprolol succinate was prepared by adding 6.116 mg of the 99.8% pure substance (2:1 ratio of metoprolol to succinate) to a 5 mL volumetric flask and filling to the line with diluent.
- A 1.0 mg/mL stock solution of Metoprolol Related Compound H (impurity H) was prepared by adding 7.16 mg of the 99% pure dichloride salt to a 5 mL volumetric flask and filling to the line with diluent.
- A 1.0 mg/mL stock solution of Metoprolol Related Compound I (impurity I) was prepared by adding 5.26 mg of the 95% pure substance to a 5 mL volumetric flask and filling to the line with diluent.
- A 1.0 mg/mL stock solution of Metoprolol Related Compound A (impurity A) was prepared by adding 5.26 mg of the 95% pure substance to a 5 mL volumetric flask and filling to the line with diluent.
- A 1.0 mg/mL stock solution of Metoprolol Related Compound B (impurity B) was prepared by adding 5.06 mg of the 98.8% pure substance to a 5 mL volumetric flask and filling to the line with diluent.
- A 1.0 mg/mL stock solution of Metoprolol Related Compound C (impurity C) was prepared by adding 5.26 mg of the 95% pure substance to a 5 mL volumetric flask and filling to the line with diluent.
- A 1.0 mg/mL stock solution of Metoprolol Related Compound D (impurity D) was prepared by adding 5.79 mg of the 93% pure HCl salt to a 5 mL volumetric flask and filling to the line with diluent.

Standard solution, system suitability solution, calibration solution, LOD/LOQ solution

- The standard solution was prepared as 2 µg/mL each of impurities H and I by adding 20 µL of each of the stock standard solutions of H and I to a 10 mL volumetric flask and filling to the line with diluent.
- The system suitability solution was prepared as 100 µg/mL of metoprolol succinate and 10 µg/mL each of H and I, by adding 100 µL of the stock standard solutions of H and I and 500 µL of a 2 mg/mL solution of metoprolol succinate to a 10 mL volumetric flask and filling to the line with diluent.
- Calibration solutions of 100, 50, 20, 10, 5, 2, 1, and 0.5 µg/mL were prepared by serial dilution in the diluent in 10 mL volumetric flasks starting from 10 mL of a 1 mg/mL stock solution.
- Solutions for determining LOQ and LOD were prepared at 0.2 and 0.1 µg/mL by further serial dilution in 10 mL volumetric flasks.

Sample solutions

The sample solutions of 2 mg/mL metoprolol succinate used to produce the data for “E8-H” and “E8-I” were prepared by weighing 20.00 mg of the metoprolol succinate USP reference standard, adding to a 10 mL volumetric flask, and filling to the line with diluent. The samples C7, C8, C9, and E9 were prepared in the same manner and spiked with varying amounts of compounds H and I.

Results and discussion

Figure 2 shows the separation of metoprolol, succinate, and impurities H and I in the system suitability solution. Both related compounds were well separated and easily quantified.

Method transfer (from Corona ultra RS CAD to Corona Veo/VCAD)

Technical Note 157[3] and Chapter 3 of *Charged Aerosol Detection for Liquid Chromatography and Related Separation Techniques*[4] were used to provide guidance for method transfer from the Corona Ultra RS CAD to the Corona Veo/VCAD. Data acquisition parameters were optimized in the following sequence:

a) PFV

The first data acquisition parameter that should be optimized is the PFV. The PFV is used to help linearize the signal output of the CAD over the desired range of quantitation so that SNR is a more accurate measure of sensitivity limits and peak shape is a more accurate measure of chromatographic performance.[3] When evaluating changes in PFV it is very important to study its effects on response for low levels of analyte and to choose the best curve fit model using residual plots. Several different PFVs were evaluated including 1.10, 1.20, 1.30, and 1.40. The PFV of 1.3 produced the best calibration curve based on a robust evaluation of goodness of fit.

b) Evap T

There is little to no relationship between the Nebulizer T setting on the Corona ultra RS detector and the Evap T setting on the Corona Veo/VCAD detector. The Nebulizer T setting is used to prevent freezing of the nebulizer due to evaporative cooling that occurs with highly volatile solvents. It has limited use as a method control variable. The Evap T setting on the Corona Veo/VCAD is an important method parameter enabling greater analytical flexibility. However, the correct choice of Evap T is essential. A low Evap T has the advantage of producing more uniform response between analytes, and the accompanying reduction in selectivity enables the measurement of a broader range of analytes; however, it can be associated with increased noise due to greater contribution from semivolatile impurities. A higher Evap T, on the other hand, is associated with decreased noise, but as more analytes behave as semivolatiles, there may be a loss of signal, especially when measuring low levels. As part of the method transfer, three different Evap Ts were evaluated: 35, 50, and 70 °C. Four concentrations of related compound I around its limit of detection were evaluated: 0.5, 1, 2, and 5 µg/mL.

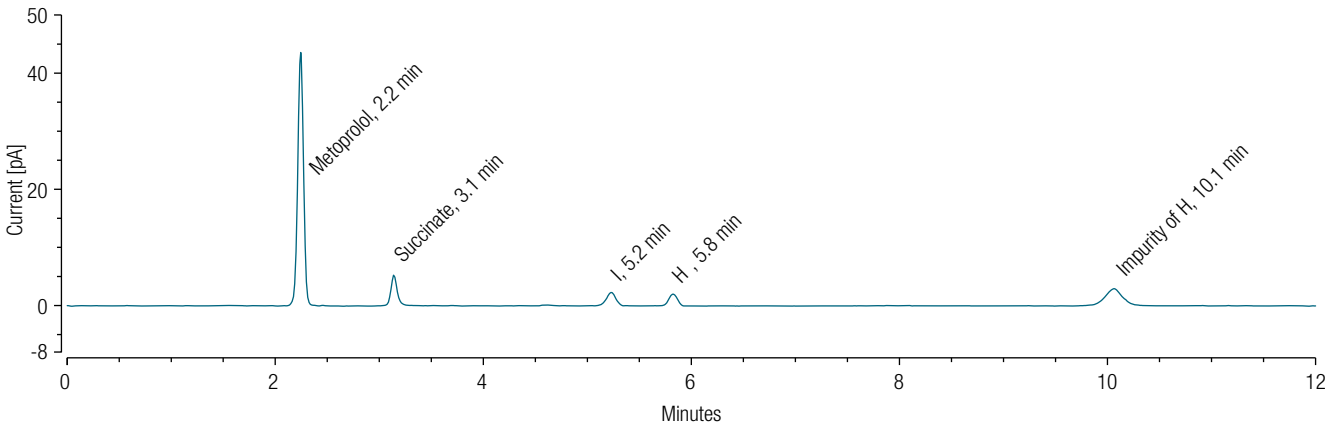

Figure 2. HPLC-Corona Veo CAD chromatogram of a 10 µL injection of the system suitability solution (0.1 mg/mL metoprolol succinate, 0.01 mg/mL H, and 0.01 mg/mL I)

The signal decreased with increasing temperature, as shown in Table 1. Similarly, the background noise decreased with increasing temperature. As described above, the mobile phase buffer was prepared by adjusting a 100 mM ammonium formate (pKa 3.77) solution to pH 3.2 with formic acid. This results in an aqueous buffer concentration of >200 mM and a final mobile phase buffer concentration of >30 mM. This rather high additive concentration (≤15 mM is typically recommended for CAD and MS) may result in a relatively high background signal and baseline noise for this application.

c) Signal filter

Four different digital filter settings were evaluated (2, 3.6, 5, and 10 s) for a 1 µg/mL concentration of related compound I. The SNR increased with higher filter settings and the noise decreased (see Table 1). The 5 s filter was chosen because it offered a balance between good SNR and acceptable separation between metoprolol and other impurities that are not quantified in this monograph, but which may be present in real metoprolol samples (metoprolol related compounds A, B, C, and D). Figure 3 shows that metoprolol is resolved from its major impurities with a filter value of 5. The 10 s filter resulted in broadened peaks that were poorly resolved.

Method performance

Using a PFV = 1.30, Evap T = 35 °C, and a filter of 5 s, the Corona Veo/VCAD met USP system suitability criteria for precision (for 2 µg/mL H and 2 µg/mL I, N = 6, %RSD = 2.03% for I and 1.90% for H using a doubled injection volume); and resolution (for a solution of 100 µg/mL metoprolol succinate, 10 µg/mL H and 10 µg/mL I, resolution of 5.41 between H and I using a doubled injection volume). These requirements comprise the system suitability test defined in the monograph. Doubling the injection volume from 10 µL to 20 µL, a modification allowed under USP guidelines, improves the precision and is a recommended modification (see *Robustness,* below). See Table 2 for more details.

Table 1. Noise and signal-to-noise ratio (SNR) for 5 µg/mL related compound I (Evap T) or for 1 µg/mL related compound I (filter at 35 °C)

	Evap T (°C), 5 µg/mL I			Filter (at 35 °C), 1 µg/mL I			
	35	50	70	2	3.6	5	10
SNR	12.2	4.0	1.7	5.1	5.7	7.6	9.0
Noise	0.214	0.170	0.155	0.145	0.072	0.066	0.058

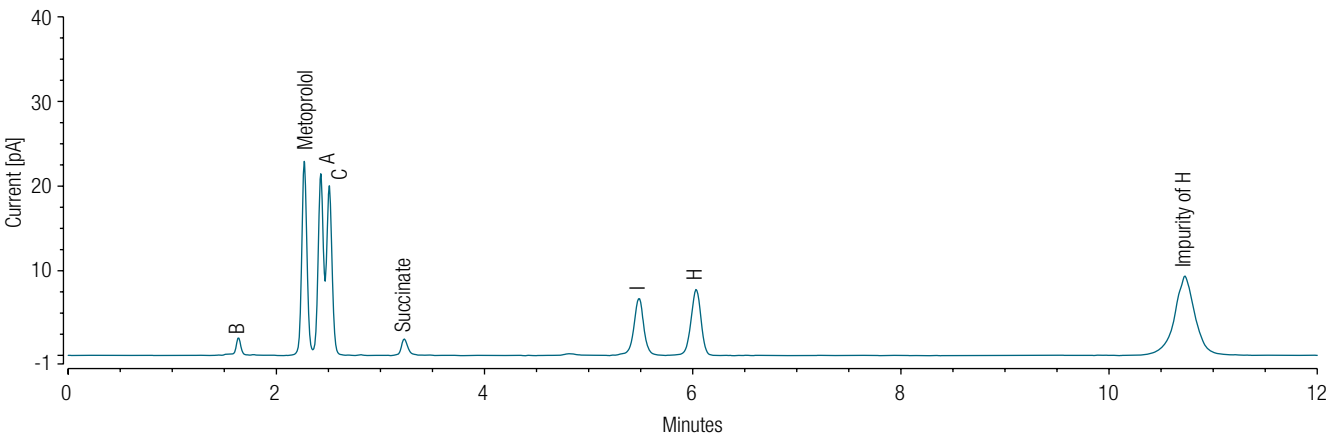

Figure 3. HPLC-Corona Veo CAD chromatogram of a 20 µL injection of 20 µg/mL of each of metoprolol succinate and related compounds A, B, C, H, and I, showing that metoprolol is resolved from its major impurities with a filter value of 5. The peak at around 11 minutes is an impurity of compound H. Related compound D was not injected because it has an impurity that interferes with quantification of related compound H.

Table 2. Results of system suitability testing

	Corona ultra RS CAD	Corona Veo/VCAD	Doubled Injection Volume, Corona Veo/VCAD	USP Requirement
%RSD, peak area, 2 µg/mL H and I	2.56% for H 1.92% for I (mean, N = 6)	2.82% for H 2.71% for I (mean, N = 6)	1.90% for H 2.03% for I (mean, N = 6)	≤ 3.0%
Resolution between H and I	3.42	3.81	5.41	≥ 2.0

Response curves

Response curves for compounds H and I using the 10 µL injection volume are shown in Figure 4. A linear fit is applied in both cases. The correlation coefficient, R^2, for compound H is 0.9997 and for I is 0.9995. Note: An R^2 near 1, by itself, does not necessarily prove linearity as this metric is based on the assumption that the data show equal absolute error throughout the concentration range. Since most HPLC analyses show somewhat higher absolute error at higher amounts, it is generally recommended to closely examine goodness of fit especially at the extremes of the required quantitation range.

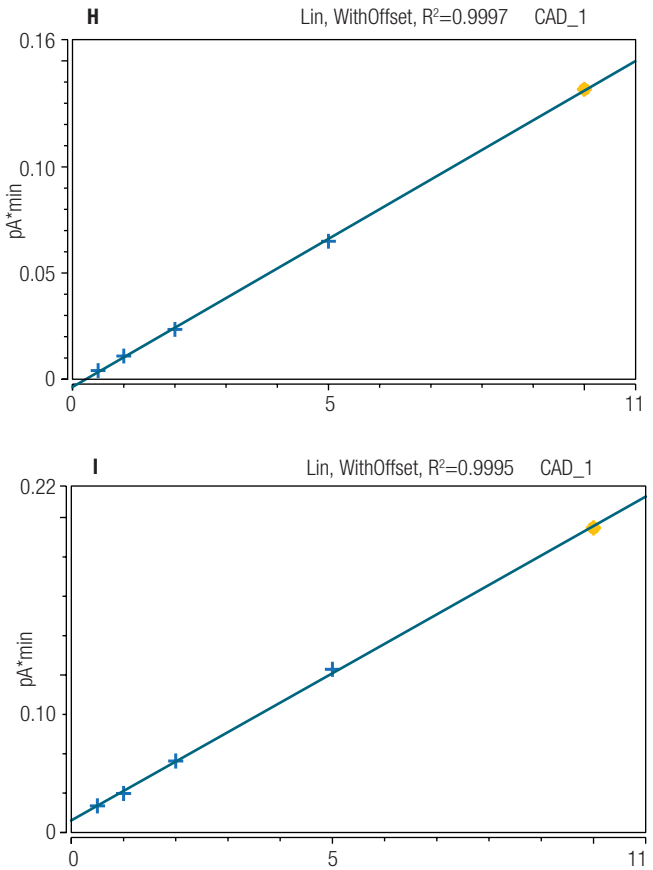

Figure 4. Five-point calibration curve (0.5, 1, 2, 5, and 10 µg/mL) for compounds H and I using a linear fit and 10 µL injection volume

Robustness

No adverse effects were found (e.g., on retention time, resolution, peak shape, or quantitative accuracy) when doubling the injection volume for the Corona Veo/VCAD or the Corona ultra RS CAD. Based on these results, we recommend that the user double the injection volume given in the monograph from 10 µL to 20 µL. Such a change is explicitly allowed by USP. The method is robust with respect to injection volume and not adversely affected by the change. The %RSD for the area of repeated injections improves (see Table 2) and the limit of quantification is 0.2 µg/mL or better.

Quantification of impurities

The standard solution was used to calculate the amount of H and I in the metoprolol succinate sample solution using the 10 µL injection volume (Tables 3 and 4).

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

Where r_U is the peak area of H or I in the sample solution,
r_S is the peak area of H or I in the standard solution
C_S is the concentration of H or I in the standard solution (mg/mL)
C_U is the concentration of metoprolol succinate in the sample solution

The results show acceptable reproducibility for the same samples measured by the Corona Veo/VCAD and the Corona Ultra RS CAD. Use of the 20 µL injection volume improved reproducibility for both instruments (Table 4).

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Table 3. Results for quantification of impurities with 10 µL injection volume

Sample ID	Corona Veo CAD (% H or I)	Corona ultra RS CAD (% H or I)
C7-I	0.13	0.20
C8-I	0.14	0.22
C9-I	0.12	0.19
E9-I	0.04	0.05
E8-I	0.00	0.00
C7-H	0.19	0.25
C8-H	0.21	0.27
C9-H	0.20	0.26
E9-H	0.06	0.06
E8-H	0.01	0.03

Table 4. Results for quantification of impurities with 20 µL injection volume

Sample ID	Corona Veo CAD (% H or I)	Corona ultra RS CAD (% H or I)
F1-I	0.15	0.17
F2-I	0.18	0.18
F3-I	0.17	0.16
F1-H	0.19	0.21
F2-H	0.24	0.21
F3-H	0.22	0.20

Conclusion

As charged aerosol detection achieves increasing prominence in USP compendial methods, it becomes increasingly important to ensure all models of charged aerosol detectors are suitable for use, as well as provide guidelines for method transfer between detectors.

The USP 41(3) In-Process Revision to USP 38 (page 4370) for determination of impurities in Metoprolol Succinate originally developed with a Corona ultra RS detector, was easily transferred from the Corona ultra RS detector to the Corona Veo/VCAD charged aerosol detector. A standard method transfer procedure was followed, resulting in final Veo/VCAD parameters of PFV = 1.30, Evap T = 35 °C, and a filter of 5 s.

Performance of the Corona Veo/VCAD readily met the standard set by the Corona ultra RS detector. Peak resolution between H and I was better with the Corona Veo/VCAD detector than with the Corona ultra RS detector, and peak area reproducibility was about the same. Both detectors easily satisfied the resolution and peak area reproducibility tests for system suitability specified in the USP compendial method. Both resolution and peak area reproducibility improve with a doubled injection volume, a change that is explicitly allowed by USP. Based on the data presented here, we recommend use of the doubled injection volume for both instruments. Either instrument can be used to perform the USP compendial assay for impurities in metoprolol succinate.

References

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2. Xu, Q.; Tan, S.; Petrova, K. Development and validation of a hydrophilic interaction chromatography method coupled with a charged aerosol detector for quantitative analysis of nonchromophoric α-hydroxyamines, organic impurities of metoprolol. *J. Pharm. Biomed. Analysis* **2016,** *118,* 242–250.
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APPLICATION NOTE 72649

Validation of an improved ion chromatography method for the limit of choline test in the USP Succinylcholine Chloride monograph

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Keywords

Dionex IonPac CS19 column, suppressed conductivity detection, pharmaceutical, USP monograph, drug substance, drug product, impurity, United States Pharmacopeia, ICS-5000+, ICS-6000

Goal

To validate an improved ion chromatography method developed for the limit of choline test in the United States Pharmacopeia (USP) Succinylcholine Chloride monograph

Introduction

Succinylcholine chloride, also known as suxamethonium or suxamethonium chloride, is a United States Food and Drug Administration (FDA) approved intravenous (IV) medication used as a skeletal muscle relaxant during procedures of short duration (e.g., endotracheal intubation, endoscopic examinations, electrically or pharmacologically induced convulsive therapy) after general anesthesia has been induced.[1,2]

Pharmaceutical companies have tried using the ion chromatography (IC) method to test the limit of choline in succinylcholine chloride according to the USP Succinylcholine Chloride monograph,[3] but they observed a problem. These companies reported that after about 6–7 h of analysis a large peak elutes. This interferes with subsequent analyses. They also reported that during the 6–7 h there is a loss of choline retention time. We confirmed these observations and developed a new method to solve the problem. The modified IC method has been proposed for the limit of choline test in the USP Succinylcholine Chloride monograph.[4]

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ICS-5000+ HPIC

Table of contents

This application note reports the method development and then the evaluation of the improved IC method for the limit of choline test. The evaluation follows the guidelines given by the International Conference on Harmonization (ICH) and the USP, which are outlined in the ICH Guideline Q2A and Q2B Validation of Analytical Procedures,[5,6] the USP General Chapter <1225> Validation of Compendial Methods,[7] and USP General Chapter <621> Chromatography.[8] A Thermo Scientific™ Dionex™ ICS-5000+ HPIC™ system with a Thermo Scientific™ Dionex™ IonPac™ CS19 anion-exchange column (USP L97) and a Thermo Scientific™ Dionex™ CERS™ 500 Cation Electrolytically Regenerated Suppressor for suppressed conductivity detection were used to execute the method.

Experimental

Equipment

- A Dionex ICS-5000+ HPIC system* was used in this work. It includes:
  - Eluent Generator
  - Pump
  - Column Heater
  - Degasser
  - Conductivity Detector with cell
- Thermo Scientific™ Dionex™ AS-AP Autosampler, with 250 µL syringe (P/N 074306), 1.2 mL buffer line assembly (P/N 074989), 5 µL injection loop
- Thermo Scientific™ Dionex™ EGC 500 MSA Methanesulfonic Acid Eluent Generator Cartridge (P/N 075779)
- Dionex CERS 500 Cation Electrolytically Regenerated Suppressor (2 mm) (P/N 082543)
- Thermo Scientific™ Dionex™ CR-CTC 500 Continuously Regenerated Cation Trap Column (P/N 075551)
- Thermo Scientific™ Chromeleon™ 7.2 Chromatography Workstation

*This method can be run on any system supporting an electrolytic suppressor or any Thermo Scientific™ Dionex™ ion chromatography system using a chemically regenerated suppressor. Please note that this method was not tested with a chemically regenerated suppressor. This method can also be run with manually prepared MSA but was tested using electrolytically generated MSA.

Reagents and standards

- Deionized (DI) water, Type I reagent grade, 18 MΩ·cm resistance or better
- Choline chloride USP reference standard (Sigma-Aldrich® Cat# 1133547-500MG, Lot R060C0)
- Succinylcholine chloride (Acros Organics™ 96%, Cat# AC460110050)
- Potassium chloride (Mallinckrodt®, 99.7%, Cat# 6858)

Conditions

Table 1. Chromatography conditions of the original USP monograph method[3] to test the limit of choline in succinylcholine chloride

Columns:	Thermo Scientific™ Dionex™ IonPac™ CS19 2-mm Analytical, 2 × 250 mm (P/N 076028) Thermo Scientific™ Dionex™ IonPac™ CG19 2-mm Guard, 2 × 50 mm (P/N 076029)
Eluent:	6.4 mM (0.62 g/L) Methanesulfonic acid (MSA)
Eluent Source:	Dionex EGC 500 MSA cartridge with Dionex CR-CTC 500 continuously regenerated cation trap column (may not have been used by the method contributor)
Flow Rate:	0.25 mL/min
Injection Volume:	5 µL (full loop)
Column Temperature:	35 °C
Detection:	Suppressed conductivity, Dionex CERS 500 (2 mm) Suppressor, recycle mode, 5 mA current
Detection/ Suppressor Compartment:	30 °C
Cell Temperature:	35 °C
System Backpressure:	~2700 psi
Noise:	< 3 nS/min
Run Time:	18 min

Table 2. Chromatography conditions of the improved IC method to test[4] the limit of choline in succinylcholine chloride

Columns:	Dionex IonPac CS19 2-mm Analytical, 2 × 250 mm (P/N 076028) Dionex IonPac CG19 2-mm Guard, 2 × 50 mm (P/N 076029)		
Eluent:	Methanesulfonic acid (MSA)		
	Time (min)	*MSA (mM)*	*Curve*
	-3	6	5
	0	6	5
	14	6	5
	15	50	5
	33	50	5
	34	6	5
	40	6	5
Eluent Source:	Dionex EGC 500 MSA cartridge with CR-CTC 500 continuously regenerated cation trap column		
Flow Rate:	0.25 mL/min		
Injection Volume:	5 µL (full loop)		
Column Temperature:	30 °C		
Detection:	Suppressed conductivity, Dionex CERS 500 (2 mm) Suppressor, recycle mode, 37 mA current		
Detection/ Suppressor Compartment:	30 °C		
Cell Temperature:	30 °C		
System Backpressure:	~2900 psi		
Noise:	< 2 nS/min		
Run Time:	43 min		

Preparation of solutions and reagents

Note: Do not use glassware to prepare the solutions. Polymeric containers made of high-density polyethylene (HDPE) are recommended.

Stock standard solutions 1000 µg/mL

Accurately weigh 100.0 mg of pure anhydrous salts (choline chloride using USP reference standard, potassium chloride using 99.7 % salt) into 125 mL polypropylene bottles, and dissolve in 100 mL (100.00 g) of DI water to make 1000 µg/mL stock solutions. Keep stock standard solutions at 4 ˚C.

Choline chloride calibration standard, 0.2, 2, 4, 8, 16, 25, 50 µg/mL

To prepare calibration standard solutions, dilute the choline chloride stock standard solution (1000 µg/mL) to the appropriate concentrations with DI water.

System suitability solution

Mix the stock standard solutions 1.00 mL (1.00 g) of choline chloride and 0.50 mL (0.50 g) of potassium chloride stock) and 98.5 mL (98.5 g) of DI water to make the system suitability solution containing 10.0 µg/mL of choline chloride and 5.0 µg/mL of potassium chloride. Keep the solution at 4 °C.

Sample preparation

Succinylcholine chloride sample solution, 2.000 mg/mL

Accurately weigh 200.0 mg of succinylcholine chloride into a 125 mL polypropylene bottle and dissolve in 100 mL (100.0 g) DI water. Store at 4 °C immediately following preparation.

Spiked succinylcholine chloride sample solution

Accurately weigh 400.0 mg of succinylcholine chloride into a 125 mL polypropylene bottle and dissolve in 100 mL (100.0 g) DI water to make 4.000 mg/mL succinylcholine chloride sample stock. Mix the 4.000 mg/mL succinylcholine chloride sample stock, choline chloride standard, and DI water to make the 1, 2, 4, and 8 µg/mL of choline chloride spiked in 2.000 mg/mL sample solution. (For example, to make 2 µg/mL of choline chloride spiked in 2.000 mg/mL sample, mix 4 mL of the 4.000 mg/mL sample stock, 1 mL of 16 µg/mL choline chloride standard, and 3 mL of DI water.) These are 0.04% to 0.3% of choline chloride in succinylcholine chloride.

Robustness study

Following the guidelines of USP General Chapter <1225>, Validation of Compendial Methods[6] and USP General Chapter <621>, Chromatography[7], the robustness of this method was evaluated by examining the results of the suitability standard (concentration, retention time (RT), peak asymmetry of choline, and resolution between potassium and choline) after imposing a small variation (±10%) in procedural parameters (e.g., flow rate, eluent gradient concentration, column temperature).

The following variations were tested:

- Flow rate at 0.275 mL/min, 0.25 mL/min, 0.225 mL/min
- Column temperature at 27 °C, 30 °C, 33 °C
- Eluent: Methanesulfonic acid (MSA) concentrations: ±10% as shown in Table 3.

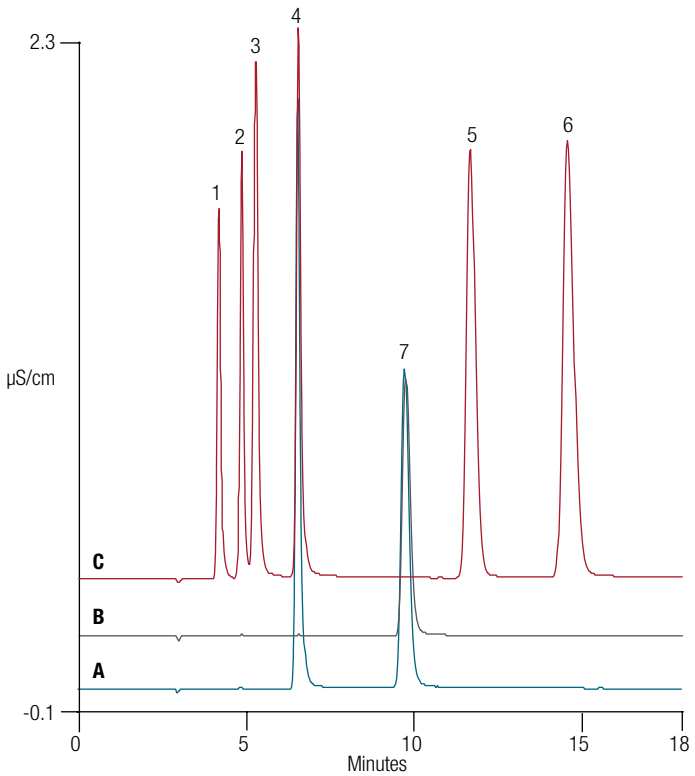
Table 3. Eluent concentrations for robustness

Time (min)	MSA (mM)	+10% MSA (mM)	-10% MSA (mM)
-3	6	6.6	5.4
0	6	6.6	5.4
14	6	6.6	5.4
15	50	55	45
33	50	55	45
34	6	6.6	5.4
40	6	6.6	5.4

Results and discussion

Separation of choline from cations

Figure 1 shows the separation of choline (retention time about 10 min) from common cations using the chromatography conditions of the original USP monograph method (Table 1); choline is well separated from the common cations (lithium, sodium, ammonium, potassium, magnesium, and calcium).



Columns:	Dionex IonPac CG19, 2 × 50 mm Dionex IonPac CS19, 2 × 250 mm
Eluent:	6 mM Methanesulfonic acid (MSA)
Flow Rate:	0.25 mL/min
Inj. Volume:	5 µL (Full loop)
Column Temp.:	35 °C
Sampler Temp.:	4 °C
Detection:	Suppressed conductivity, Dionex CERS 500 (2 mm) Suppressor, 30 °C, 37 mA, recycle mode
Samples:	A: System suitability solution B: 8 µg/mL USP Choline Chloride standard C: Mixed common cations

Peaks:	A	B	C (µg/mL)
1. Lithium	0	0	0.25
2. Sodium	0	0	1.00
3. Ammonium	0	0	1.25
4. Potassium	0	50	2.50
5. Magnesium	0	0	1.25
6. Calcium	0	0	2.50
7. Choline	8.0	10.0	0

Figure 1. Separation of choline from common cations using the isocratic IC method described in the USP Succinylcholine Chloride monograph

It was confirmed that choline retention was less after each injection of the sample containing succinylcholine chloride. It was also confirmed that the baseline shifted upward ~ 6.5 h after the injection of the first succinylcholine chloride sample (data not shown). This interferes with subsequent sample injections. An improved IC method (Table 2) was developed based on the assumption that the baseline rise and retention time problem of the original USP method are caused by the retention of succinylcholine on the column

Figure 2 shows separation of choline from succinylcholine and common cations with the improved IC method for choline analysis. Similar to the original method, choline is well separated from the common cations. With the addition of a 50 mM MSA wash for 18 min to each injection (Table 2), succinylcholine is eluted at about 26 min. The total run time of this method is 43 min, which includes an additional 3 min of column re-equilibrium at the starting conditions. By eluting succinylcholine, choline retention time is stable during choline analysis and no baseline upset (rise) is observed after 6 to 7 h of sample analysis. The remainder of this application note will report data from the evaluation of this method and discuss this evaluation.

Calibration, limit of detection (LOD) and limit of quantitation (LOQ)

First, a calibration curve for choline chloride was established at seven levels from 0.2 to 50 µg/mL. Figure 3 shows the calibration plot for choline. A linear relationship was observed for peak area to concentration with a coefficient of determination (r^2) of 0.9998 (Figure 3 and Table 4).

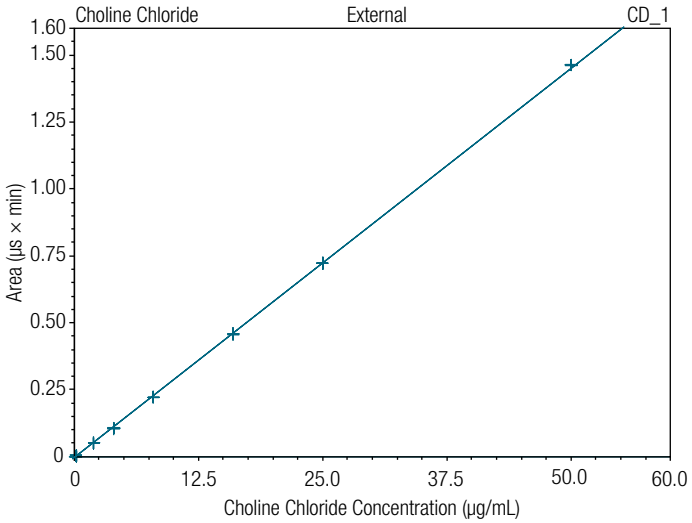
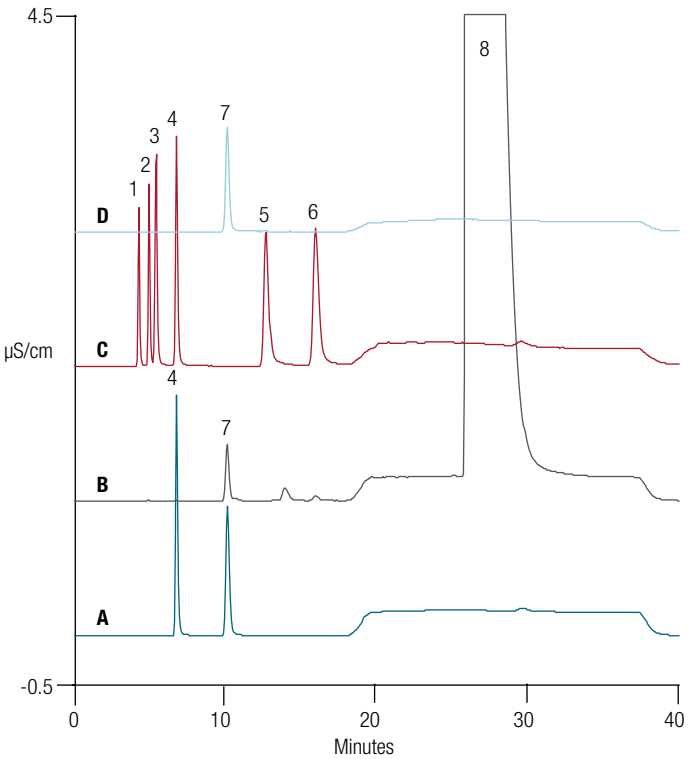


Figure 3. Calibration plot for choline chloride analysis (n=3)



Columns:	Dionex IonPac CG19, 2 × 50 mm Dionex IonPac CS19, 2 × 250 mm
Eluent:	Methanesulfonic acid (MSA): 6 mM 0–14 min; 6 to 50 mM, 14–15 min; 50 mM, 15–33 min; 50 to 6 mM, 33–34 min; 6 mM 34–40 min.
Flow Rate:	0.25 mL/min
Inj. Volume:	5 µL (Full loop)
Column Temp.:	35 °C
Sampler Temp.:	4 °C
Detection:	Suppressed conductivity, Dionex CERS 500 (2 mm) Suppressor, 30 °C, 37 mA, recycle mode
Samples:	A: System suitability solution B: 2 mg/mL of succinylcholine chloride sample C: Mixed common cations D: 8 µg/mL USP Choline Chloride standard

Peaks:

	A	B	C	D (µg/mL)
1. Lithium	0	0	0.25	0
2. Sodium	0	0	1.00	0
3. Ammonium	0	0	1.25	0
4. Potassium	5.0	0	2.50	0
5. Magnesium	0	0	1.25	0
6. Calcium	0	0	2.50	0
7. Choline	10.0	3.0	0	8.0
8. Succinylcholine	0	2000	0	0

Figure 2. Separation of choline from succinylcholine and common cations using an improved IC method

Table 4. Calibration, LOD, and LOQ for choline

Calibration Standards (µg/mL)	Calibration Type	r^2	Response Factor (µS × min)/(µg/mL)	LOD (µg/mL)	LOQ (µg/mL)
0.2–50	Linear, through origin	0.9998	0.0291	0.06	0.2

The LOD and LOQ were determined by seven injections of the 0.20 mg/L choline standard. The baseline noise was determined by measuring the peak-to-peak noise in a representative 1 min segment of the baseline where no peaks elute but close to the peak of interest. The LOD and LOQ were determined for the concentration at the signal-to-noise ratio 3× and 10× (Table 4). Using this method, the LOD was determined to be 0.06 mg/L and the LOQ was 0.2 mg/L.

System suitability

The system suitability solution contains 10.0 µg/mL of choline chloride and 5.0 µg/mL of potassium chloride. Table 5 shows the system suitability results from three different sequences over three days. The method has relative standard deviation (RSD) of 0–0.1% for retention time and 0.2–1% for peak area. The resolution between choline and potassium is 10. These results surpass the suitability requirements of RSD, not more than (NMT) 3% for choline, and resolution, not less than (NLT) 5.0, between choline and potassium in the current Succinylcholine Chloride USP monograph and the proposed revision.[3,4]

Sample analysis and precision

As a linear relationship of peak area to concentration was established for choline chloride from 0.2 to 50 µg/mL, the proposed monograph revision IC method uses a single standard point of 8 µg/mL choline chloride standard to determine the percentage of choline in the portion of succinylcholine chloride taken.

The percentage of choline in the portion of succinylcholine chloride taken was calculated as follows:

$$\text{Result (\%)} = \left(\frac{ru}{rs}\right) \times \left(\frac{Csd}{Cu}\right) \times \left(\frac{Mr1}{Mr2}\right) 100$$

ru= Peak area of choline from the succinylcholine chloride *Sample* solution

rs= Peak area of choline from the choline chloride *Standard* solution

Csd= Concentration of USP Choline Chloride RS in the *Standard* solution (8 µg/mL)

Cu= Concentration of succinylcholine chloride in the *Sample* solution (1000 × 2.000 mg/mL)

Mr1= Molecular weight of choline, 104.17

Mr2= Molecular weight of choline chloride, 139.62

Table 5. System suitability data of the improved IC method over three days

Day	Retention Time				Peak Area				Resolution	
	Potassium		Choline		Potassium		Choline			
	Average (min)	RSD	Average (min)	RSD	Average (µS × min)	RSD	Average (µS × min)	RSD	Average	RSD
1	6.7	0.1	10.1	0	0.31	1	0.28	0.4	10	0.1
2	6.7	0	10.0	0	0.31	0.2	0.28	0.2	10	0.2
3	6.7	0	10.1	0	0.31	0.2	0.28	0.3	10	0.2

The USP monograph requires that succinylcholine chloride contain no more than 0.3% of choline, which is equal to 8 µg/mL choline chloride in 2.00 mg/mL of succinylcholine chloride.

The method reproducibility and precision were evaluated by running the test for choline in the succinylcholine chloride sample. Table 6 lists the results over three separate days. Three sample solutions, 2.000 mg/mL of succinylcholine chloride in water, were independently prepared on each day. Each sample solution was tested with multiple (n=3) injections. The succinylcholine chloride sample contained 0.11% choline, which passes the acceptance criteria of NMT 0.3%. Table 6 also shows that the method is precise with intraday precision from 0.5% to 1.2% and interday precision of 3.4%

Method accuracy

Method accuracy was validated by spiked recovery of choline in a succinylcholine chloride sample over four concentration levels and over three days, with three replicates of each concentration (Table 7). The method was shown to be accurate with good recovery (average from 104% to 118%) for a low level of choline (0.04% to 0.3%) spiked in succinylcholine chloride.

Robustness

Using the system suitability solution containing 10.0 µg/mL of choline chloride and 5.0 µg/mL of potassium chloride, robustness of the improved IC method was evaluated by measuring the influence of small variations (±10%) in chromatography parameters (e.g., flow rate, eluent concentration, and column temperature) on the measured choline concentration, RT, peak asymmetry, and resolution between potassium and choline. The peak asymmetry was measured using the USP formula. The resolution was determined relative to the previous peak in the chromatogram using the USP formula. The system suitability solution was injected three times at each chromatographic condition. These experiments were run on two columns from different lots. Table 8 summarizes the robustness test results. Although choline RT changed when the chromatography condition changed, the choline peak asymmetry and resolution between choline and potassium only change a small amount (<4%), and the measured choline concentrations were about the same (<1.1% variation) for all chromatography conditions. These results indicate the method was robust to changes in chromatography conditions.

Table 6. The percentage of choline in the succinylcholine chloride sample

	Day 1		Day 2		Day 3	
	Average (%)	RSD	Average (%)	RSD	Average (%)	RSD
Sample 1	0.11	0.6	0.11	0.4	0.12	3.3
Sample 2	0.11	0.5	0.11	0.2	0.12	0.2
Sample 3	0.11	0.5	0.11	0.1	0.12	0.1
Average	0.11	0.5	0.11	0.3	0.12	1.2
Overall average = 0.11% RSD = 3.4%						

Table 7. Recovery of choline spiked in succinylcholine chloride

Choline Spiked in 2.000 mg/mL Succinylcholine Chloride Sample	Day 1		Day 2		Day 3		Average Recovery (%)
	Recovery (%)	RSD	Average (%)	RSD	Average (%)	RSD	
0.04%	127	1	98	0.8	130	1.1	118
0.07%	104	0.5	99	0.4	114	0.3	106
0.15%	103	0.2	101	0.4	108	0.3	104
0.3%	104	0.1	103	0.3	107	0.3	105

Table 8A. Robustness of the improved IC method for choline in succinylcholine chloride for Column A*

Parameter		Retention Time		Asymmetry		Resolution	
		Min	Diff. (%)		Diff. (%)		Diff. (%)
Flow Rate (mL/min)	0.275	9.15	-9	1.28	1.3	9.7	-2.6
	0.25	10.06		1.27		9.9	
	0.225	11.17	11	1.29	2.1	10.2	2.4
Eluent Conc. (mM)	5.445	10.84	8	1.26	-0.5	10.3	4.0
	650	10.06		1.27		9.9	
	6.655	9.43	-6	1.28	0.8	9.5	-3.8
Column Temp. (°C)	27	10.39	3	1.28	0.8	9.8	-0.8
	30	10.06		1.27		9.9	
	33	9.77	-3	1.27	0.3	9.9	0.3

*Injected sample: 10.0 µg/mL of choline chloride and 5.0 µg/mL of potassium chloride in water; average of three injections for each condition

Table 8B. Robustness of the improved IC method for choline in succinylcholine chloride for Column B*

Parameter		Retention Time		Asymmetry		Resolution	
		Min	Diff. (%)		Diff. (%)		Diff. (%)
Flow Rate (mL/min)	0.275	9.76	-9	1.23	-2	10.1	-2
	0.25	10.71		1.26		10.2	
	0.225	11.88	11	1.27	1	10.5	0
Eluent Conc. (mM)	5.445	11.55	8	1.23	-2	10.0	-3
	650	10.71		1.26		10.2	
	6.655	9.99	-7	1.26	0	10.6	3
Column Temp. (°C)	27	11.05	3	1.25	-1	10.2	0
	30	10.71		1.26		10.2	
	33	10.34	-3	1.25	-1	10.3	1

*Injected sample: 10.0 µg/mL of choline chloride and 5.0 µg/mL of potassium chloride in water; average of three injections for each condition

Conclusion

This study described a modified IC method for the Limit of Choline Test in the USP Succinylcholine Chloride monograph. The IC method, with the addition of a 50 mM MSA wash for 18 min to each injection to elute succinylcholine, meets the parameters specified in the USP Succinylcholine Chloride monograph and was validated following USP and ICH guidelines. The study showed that the IC method is reproducible, has a linear calibration, and is sensitive for choline determination in succinylcholine chloride. The method is precise, accurate, and robust. Therefore, it is suitable to replace the current limit of choline method in the Succinylcholine Chloride USP monograph, which was found to be problematic.

Acknowledgement

The authors acknowledge Chetan Chavan, Chanakya Thaker, Vishnuvardhanrao T. of the Thermo Scientific team in India for confirming the problem with the limit of choline test and determining that the probable cause was the strong retention of succinylcholine on the column.

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APPLICATION NOTE 157

Comparison of suppressed to nonsuppressed conductivity detection for the determination of common inorganic cations

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Keywords
Ion chromatography, suppressor, suppression, Dionex IonPac CS16, Dionex IonPac SCS 1, lithium, sodium, ammonium, potassium, calcium, magnesium

Goal

To demonstrate the capabilities of suppressed conductivity detection using the high-capacity Thermo Scientific™ Dionex™ IonPac™ CS16 column and nonsuppressed conductivity detection using a lower-capacity Thermo Scientific™ Dionex™ IonPac™ SCS 1 column for the determination of common inorganic cations

Introduction

In 1975, Hamish Small and coworkers at The Dow Chemical Company first introduced the concept of ion chromatography (IC) that allowed the sensitive detection of ions using suppressed conductivity detection.[1] A significant portion of this work was dedicated to cation analysis. The original components described by Small et al. for the separation of cations included a low-capacity, sulfonated polystyrene/divinylbenzene (PS/DVB) column followed by a packed-bed suppressor in the hydroxide form and a conductivity detector. The primary purpose of the suppressor was to achieve sensitive detection of the ionic species by chemically modifying the eluent.[2] This detection is accomplished by converting the mineral acid eluent to water and thereby achieving a very low background signal and low noise, while converting the

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ICS-2500 RFIC

Table of contents

analyte to its base form. Although mineral acid eluents are sufficient to elute alkali metals and ammonium, the low affinity of hydronium ions for sulfonated resins required a stronger eluting component, *m*-phenylenediamine, to elute the more retained alkaline earth metals. However, the concentrations of *m*-phenylenediamine required to separate the alkaline earth metals resulted in the alkali metals coeluting in the void volume. In addition to requiring two eluent systems for this analysis, the difficulty in converting the column from the *m*-phenylenediamine to the hydronium form essentially required a separate column dedicated for the analysis of alkaline earth metals. Another major drawback of this system was the requirement for periodic regeneration of the suppressor column.[3] Today, suppressor technology has improved considerably and the chemical regeneration requirement is a distant memory. Figure 1 shows a historical timeline of suppressor development.

In 1979, a conductometric method for the determination of inorganic anions without a suppressor was first reported.[4,5] This method was later commercialized and is known by various names, such as single-column IC, direct conductivity, and nonsuppressed conductivity detection.[6] To achieve a lower background signal and therefore lower noise, nonsuppressed conductivity methods required low-capacity resins with dilute eluents. At higher conductivity levels, the influence of temperature changes become more significant, resulting in an increase in the baseline noise.[7] Therefore, the low background requirement precludes the use of high-capacity columns that require high acid concentrations to elute the cationic species within a reasonable time.

As with suppressed conductivity applications, sulfonated resins were also commonly used for nonsuppressed cation analysis, and a stronger eluting component—such as ethylenediamine—was required to separate the highly retained alkaline earth metals.[6]

Improved separation performance using latex agglomerated anion-exchange columns suggested that similar performance could be achieved for cation-exchange columns. This development resulted in the first latex cation column, the Dionex™ IonPac™ CS3, which was introduced in 1985. A layer of anion-exchange latex, functionalized with a tertiary amine, was attached to a surface-sulfonated PS/DVB substrate bead. A layer of sulfonated cation-exchange latex particles was then electrostatically attached to the positively charged surface.[8] Due to the high mass transfer between the analytes and the latex material, a significant improvement in peak efficiencies for cations was observed. This column allowed the use of 2,3-diaminopropionic acid monohydrochloride (DAP·HCl) in combination with a mineral acid eluent for the separation of alkali and alkaline earth metals. DAP is effective for eluting alkaline earth metal ions because it can be protonated to form a divalent ion and therefore has a significantly higher selectivity for the cation-exchange resin than a monovalent eluent component. This higher selectivity allows lower eluent concentrations to be used, resulting in lower background conductivity during a gradient elution. Another advantage of using DAP with suppressed conductivity systems is that it makes only a minor contribution to the total background conductivity.[9]

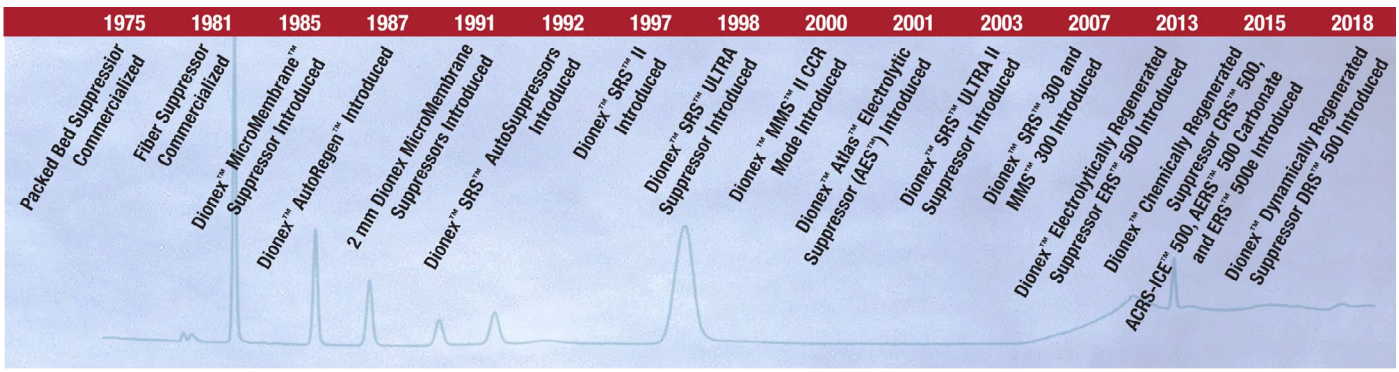

Figure 1. Thermo Scientific™ suppressor history

In 1987, Schomburg et al. introduced a silica-based, polymer-coated, cation-exchange column.[10] The poly(butadiene-maleic acid) copolymer silica column was functionalized with carboxylic acid groups. The high selectivity for hydronium ions of these weak acid functional groups, in comparison to previous sulfonated resins, allowed the separation of alkali and alkaline earth metals and ammonium within a reasonable time (<20 min) using only tartaric acid, a mildly acidic complexing agent, as the eluent. This system was designed exclusively for detection with nonsuppressed conductivity. Additional eluents that are appropriate for use with these columns include dilute mineral acids, pyridine-2,6-dicarboxylic acid (PDCA), oxalic acid, and citric acid. The retention mechanism uses the unique selectivity of the carboxylate functional groups with the complexing agent in the eluent that forms complexes with divalent cations, reducing their effective positive charge. Thus, the retention times of the divalent cations are significantly reduced. However, the silica substrate allows only a relatively narrow sample and eluent pH range of 2–8. In a highly acidic environment (pH < 2), the covalent bonds linking the functional groups become unstable, while basic conditions (pH > 8) may dissolve the silica material.[11]

In 1992, Dionex Corporation (now part of Thermo Fisher Scientific) introduced the Dionex IonPac CS12 column, a polymer-based cation-exchange column with grafted carboxylate functional groups for IC with suppressed conductivity detection. This column separated the six common cations in less than 10 min using a simple isocratic acidic eluent. DAP·HCl was no longer required to separate divalent cations, which allowed the use of the Thermo Scientific™ Dionex™ CSRS™ Cation Self-Regenerating Suppressor in the recycle mode.[9] The recycle mode requires no external base for regeneration. The Dionex CSRS suppressor improved the ease of use of the IC system, provided low baseline noise, and therefore enhanced detection sensitivity for cations. These columns are also compatible with the Thermo Scientific™ Dionex™ EG50 Eluent Generator because only a single component eluent, such as methanesulfonic acid (MSA), is required. The Dionex EG50 Eluent Generator electrolytically generates the MSA online, requiring only deionized water to operate the system and therefore significantly enhancing the flexibility and convenience of operation.[12] Unlike previous latex columns, the grafted Dionex IonPac CS12 resin used a macroporous high-surface-area polymeric substrate to increase the exchange capacity. Following the introduction of the Dionex IonPac CS12 column, additional hydronium-selective carboxylate-functionalized resins that use MSA as the eluent were developed to resolve common cations and amines. Table 1 summarizes the cation-exchange columns commercially available from Thermo Fisher Scientific for suppressed conductivity eluent systems.

This application note compares suppressed to nonsuppressed conductivity detection for the determination of inorganic cations. The Dionex IonPac CS16 column was used to demonstrate the capabilities of a suppressed cation system, in terms of capacity, linearity, detection limits, and typical baseline noise using a self-regenerating suppressor. A silica-based cation-exchange column, the Dionex IonPac SCS 1, was evaluated for nonsuppressed cations and the results were compared to the suppressed system. Please note that since the completion of this work, the Thermo Scientific™ Dionex™ IonPac™ CS18 column has been introduced and that column can be used with suppressed or nonsuppressed conductivity detection.

Table 1. Properties of Dionex IonPac cation-exchange columns used for suppressed IC

Cation-Exchange Column	Particle Diameter (µm)	Substrate X-Linking[a] (%)	Latex Diameter (nm)	Latex X-Linking[b] (%)	Column Capacity[c] (µequiv)	Functional Group
CS3	10	2	300	5	100	Sulfonic acid
CS5A[d]	9	55[e]	140	10	20	Sulfonic acid and
			76	2	40	Alkyl quaternary amine
CS10	8.5	55[e]	200	5	80	Sulfonic acid
CS11	8.5	55[e]	200	5	35[f]	Sulfonic acid
CS12	8	55[e]	N/A[g]	N/A[g]	2800	Carboxylic acid
CS12A	8	55[e]	N/A[g]	N/A[g]	2800	Carboxylic acid and phosphonic acid
CS14	8	55[e]	N/A[g]	N/A[g]	1300	Carboxylic acid
CS15	8.5	55[e]	N/A[g]	N/A[g]	2800	Carboxylic acid/Phosphonic acid/Crown ether
CS16	5.5	55[e]	N/A[g]	N/A[g]	8400[h]	Carboxylic acid
CS17	6.5	55[e]	N/A[g]	N/A[g]	1450	Carboxylic acid
CS18	6	55	N/A	N/A[g]	290[f]	Carboxylic Acid
CS19	4	55	N/A	N/A[g]	2410	Carboxylic Acid
CS20	5	55	N/A	N/A[g]	3000	Carboxylic Acid/Sulfonic Acid/Phosphonic Acid

[a]Substrate is PS/DVB, unless otherwise noted

[b]Cation-exchange latex is PS/DVB

[c]Capacity is given for 4 × 250 mm i.d. column, unless otherwise noted

[d]Column designed for transition metal determination with Vis detection

[e]Substrate is EVB/DVB and is solvent compatible with 100% acetonitrile, 100% acetone, and 20% tetrahydrofuran, but not alcohols (exception: Dionex IonPac CS14 and Dionex IonPac CS17 columns are compatible with the above solvents, including alcohols)

[f]Capacity is for a 2 × 250 mm i.d. column

[g]Grafted resin

[h]Capacity is for a 5 × 250 mm i.d. column

[i]Coated with anionic and cationic latex materials; contains both anion- and cation-exchange capacity

Experimental

Equipment

Suppressed cation system

- Thermo Scientific™ Dionex™ ICS-2500 Reagent-Free™ Ion Chromatography (RFIC™)* System consisting of:
  - Thermo Scientific™ Dionex™ GP50 Gradient Pump with vacuum degas option
  - Dionex EG50 Eluent Generator
  - Thermo Scientific™ Dionex™ EluGen™ EGC II MSA cartridge (P/N 058902)
  - Thermo Scientific™ Dionex™ ED50A Electrochemical Detector with conductivity cell and DS3 Detector Stabilizer
- Thermo Scientific™ Dionex™ AS50 Autosampler with thermal compartment (or any other Thermo Scientific Dionex autosampler)
- Thermo Scientific™ Chromeleon™ Chromatography Workstation

*Any Thermo Scientific Dionex RFIC system may be used, including the Thermo Scientific™ Dionex™ Integrion™ or Thermo Scientific™ Dionex™ ICS-6000 IC systems.

Nonsuppressed cation system*

- Thermo Scientific™ Dionex™ ICS-1000, ICS-1500, or ICS-2000 Ion Chromatography System consisting of:
  - Dual-piston pump
  - Column heater
  - Digital conductivity detector
- Dionex AS50 Autosampler
- Chromeleon Chromatography Workstation

*Any current Thermo Scientific Dionex IC system can be used for this work including the Thermo Scientific™ Dionex™ Aquion™, Dionex Integrion, or Dionex ICS-6000 IC systems. Any current Thermo Scientific Dionex autosampler could be used including a Dionex AS-DV or Dionex AS-AP.

Reagents and standards

- Deionized water, Type I reagent-grade, 18 MΩ-cm resistivity or better
- Lithium standard, 1000 mg/L (Ultra Scientific; VWR P/N ULICC 104)
- Sodium standard, 1000 mg/L (Ultra Scientific; VWR P/N ULICC 107)
- Ammonium standard, 1000 mg/L (Ultra Scientific; VWR P/N ULICC 101)
- Potassium standard, 1000 mg/L (Ultra Scientific; VWR P/N ULICC 106)
- Magnesium standard, 1000 mg/L (Ultra Scientific; VWR P/N ULICC 105)
- Calcium standard, 1000 mg/L (Ultra Scientific; VWR P/N ULICC 103)
- Lithium chloride (LiCl; Fisher L-121-100)
- Sodium chloride (NaCl; Fisher S-271)
- Ammonium chloride (NH_4Cl; Fisher A-5666)
- Potassium chloride (KCl; Sigma P-3911)
- Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$; Aldrich 24,696-4)
- Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$; Fisher C79-500)
- Combined Six Cation Standard-II (P/N 046070)

Suppressed cation conditions[13,14]

Columns:	Dionex IonPac CS16 Analytical, 5 × 250 mm (P/N 079805) Dionex IonPac CG16 Guard, 5 × 50 mm (P/N 057574)
Eluent:	26 mM MSA
Eluent Source:	Dionex EG50
Flow Rate:	1.5 mL/min
Temperature:	30 °C
Injection:	10 µL
Detection:	Suppressed conductivity, Dionex CSRS ULTRA (4 mm) suppressor, AutoSuppression recycle mode, current setting 100 mA
Background:	<1 µS
Noise:	~0.2 nS peak-to-peak
Backpressure:	~2300 psi
Run Time:	30 min

Nonsuppressed cation conditions

Columns:	Dionex IonPac SCS 1 Analytical, 4 × 250 mm (P/N 079809) Dionex IonPac SCG 1 Guard, 4 × 50 mm (P/N 079933)
Eluent:	3 mM MSA
Flow Rate:	1 mL/min
Temperature:	30 °C
Injection:	10 µL
Detection:	Nonsuppressed conductivity
Background:	~1100 µS
Noise:	~5–10 nS peak-to-peak
Backpressure:	~2100 psi
Run Time:	35 min

Preparation of solutions and reagents

Eluent solution for suppressed cation system

Generate 26 mM MSA by pumping deionized (DI) water through the Dionex EGC II MSA cartridge. Alternatively, prepare 1.0 N MSA stock solution by adding 96.10 g of methanesulfonic acid (MSA, >99%, P/N 033478) to a 1 L volumetric flask containing about 500 mL of DI water. Dilute to the mark and mix thoroughly. Prepare 26 mM MSA by diluting 26 mL of the 1.0 N MSA stock solution to 1 L with DI water. Degas the eluent by sonicating under vacuum for 10 min or by sparging with helium. Store the eluent in a plastic eluent bottle.

Eluent solution for nonsuppressed cation system

Prepare 3 mM MSA by diluting 3 mL of the 1.0 N MSA stock solution to 1 L with DI water. Degas the eluent by sonicating under vacuum for 10 min or by sparging with helium. Store the eluent in a plastic eluent bottle. The eluent generator is not recommended for use with the nonsuppressed cation system, because a significant increase in the baseline noise will be observed.

Stock standard solutions

Certified stock solutions may be purchased or 1000 mg/L standards may be prepared for the cations of interest. Dissolve the appropriate amounts of the required analytes in DI water in a 100 mL plastic volumetric flask according to the amounts in Table 2. Dilute to volume with DI water. Store in plastic containers at 4 °C. Stock standards are stable for at least three months.

Table 2. Mass of compound required to prepare 100 mL of 1000 mg/L solution of cation

Cation	Compound	Mass (g)
Li^+	Lithium (LiCl)	0.6108
Na^+	Sodium (NaCl)	0.2542
NH_4^+	Ammonium (NH_4Cl)	0.2965
K^+	Potassium (KCl)	0.1907
Mg^{2+}	Magnesium ($MgCl_2 \cdot 6\ H_2O$)	0.8365
Ca^{2+}	Calcium ($CaCl_2 \cdot 2H_2O$)	1.433

Working standard solutions

Composite working standard solutions at lower analyte concentrations are prepared by diluting the appropriate volumes of the 1000 mg/L stock standard solutions with DI water. Prepare working standards daily if they contain less than 100 mg/L of the cations.

System preparation and setup

Suppressed cation system

Prepare the Dionex CSRS ULTRA suppressor for use by hydrating the eluent chamber. Use a disposable syringe to push approximately 3 mL of 200 mN NaOH through the “Eluent Out” port and 5 mL of 200 mN NaOH through the “Regen In” port. Allow the suppressor to sit for approximately 20 min to fully hydrate the suppressor screens and membranes. Note: The Dionex CSRS ULTRA suppressor has been replaced. Please follow the start up instructions of the current cation suppressor.

Install the Dionex EG50 Eluent Generator, connect it to the system, and configure it with the Chromeleon chromatography workstation. Condition the Dionex EluGen MSA cartridge as directed in the Dionex EG50 Eluent Generator manual by setting the MSA concentration to 50 mM at a flow of 1.0 mL/min for 30 min. Note: The Dionex EluGen EGC II has been replaced. Please follow the start up instructions of the current cation eluent generation cartridge used with your Dionex IC system.

Remove the backpressure tubing temporarily installed during conditioning of the Dionex EluGen MSA cartridge. Install a 5 × 50 mm Dionex IonPac CG16 column and a 5 × 250 mm Dionex IonPac CS16 column. Make sure the system pressure is at least 2000 psi when 26 mM MSA is delivered at 1.5 mL/min. If necessary, install backpressure coils supplied with the Dionex EG50 Eluent Generator ship kit to bring the system pressure between 2000 and 2800 psi. Do not exceed 3000 psi.

The Dionex IonPac CS16 column storage solution is 30 mM MSA; before use, equilibrate the column with 26 mM MSA eluent for 60 min. An equilibrated system has a background signal of <1 µS, and peak-to-peak noise should be between 0.2 and 0.5 nS. There should be no peaks eluting at the same time as the cations of interest.

Prepare a 500× dilution of the Six Cation Standard-II (P/N 046070) and make a 10 µL full-loop injection. The column is equilibrated when two consecutive injections of standard produce the same retention times. Confirm that the resulting chromatogram resembles the chromatogram in Figure 2.

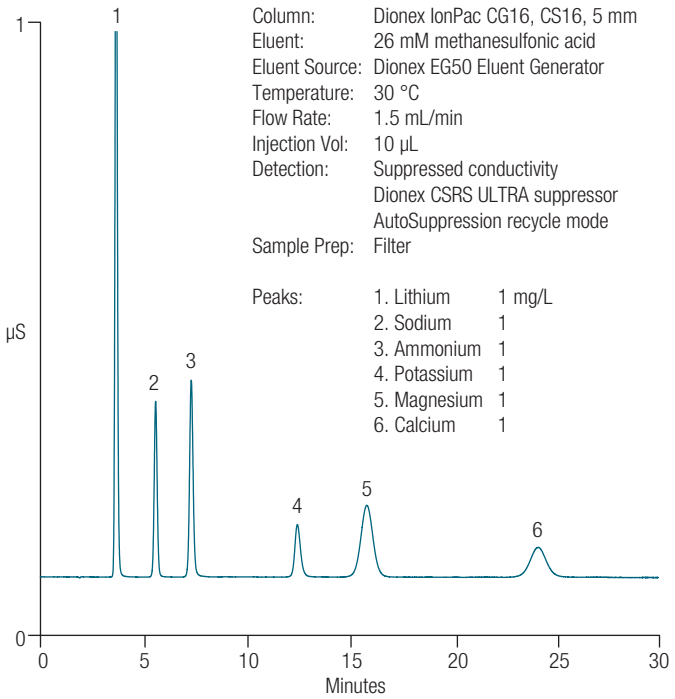

Figure 2. Separation of inorganic cations and ammonium on the Dionex IonPac CS16 column

Nonsuppressed cation system

The Dionex ICS-1000, ICS-1500, or ICS-2000 integrated IC systems may be used for nonsuppressed cations. This application note describes the proper setup and system preparation for a Dionex ICS-2000 system. Install the 4 × 50 mm Dionex IonPac SCG 1 column and 4 × 250 mm Dionex IonPac SCS 1 column in the column oven. Set the signal polarity by navigating to the dropdown menu on the LCD screen and press "DETECTOR". In the conductivity polarity option, set the polarity to "Inverted". For the Dionex ICS-1000 system, the polarity must be changed using Chromeleon CDS software.

Because the Dionex ICS-2000 system contains an eluent generator cartridge, this portion of the system should be bypassed by placing a 10-32 in. union in place of the inlet and outlet fittings for the Dionex EluGen cartridge. A separate union should also be placed between the inlet and outlet fittings for the continuously regenerated trap column. Because a suppressor is not used for this system, the outlet of the conductivity detector may be connected to the tubing labeled "Regen Out" to direct the column effluent to waste. The Chromeleon CDS program (*.pgm file) should be set for "0 mM" MSA and the suppressor should be set to "None".

Equilibrate the columns with 3 mM MSA at 1 mL/min for at least 60 min. Prior to sample analysis, analyze a system blank of reagent water. An equilibrated system has a background signal of < 1100 µS, and peak-to-peak noise should be < 10 nS. There should be no peaks eluting at the same retention time as the cations of interest.

Prepare a 100× dilution of the Six Cation Standard-II and make a 10 µL full-loop injection. The column is equilibrated when two consecutive injections of standard produce the same retention times. Confirm that the resulting chromatogram is similar to the chromatogram shown in Figure 3.

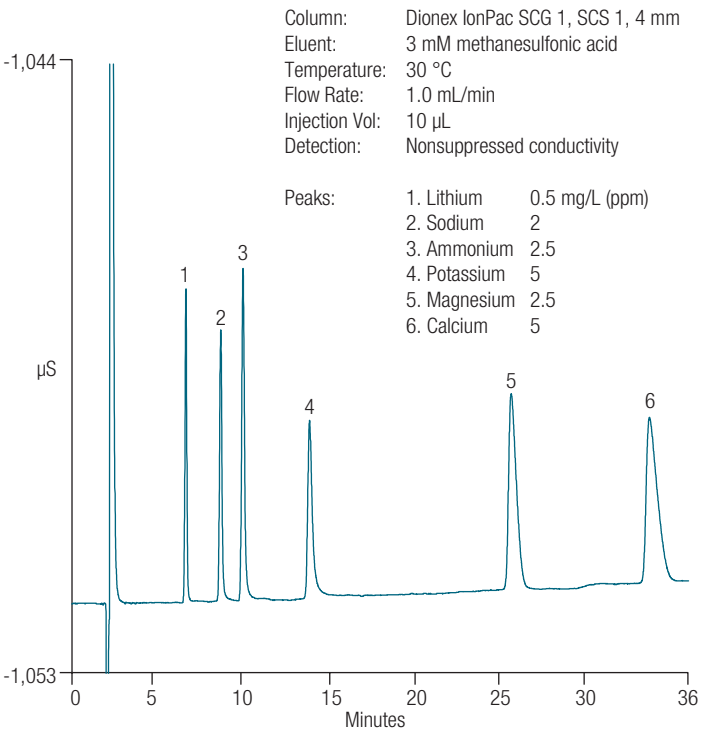

Figure 3. Separation of common inorganic cations on the Dionex IonPac SCS 1 column

Results and discussion

Conductometric detection is the major detection technique used to determine ionic species by IC. However, the measurement of conductance had some serious faults in the early attempts to apply it to IC. A major limitation was attempting to determine relatively low concentrations of an analyte in the presence of a highly conductive eluent species. This limitation was overcome when Small and coworkers introduced the concept of *suppressed* IC. The suppressor eliminated the highly conductive background and therefore enhanced the sensitivity of the measured analytes.[1] In this system, an eluent species of HX (X being the anion associated with the eluent) passes through the suppressor that exchanges X^- for OH^- to produce a background of H_2O. Noise is proportional to the background signal and therefore elimination of the background electrolyte lowers the noise and improves analyte sensitivity.

In 1979, a method was reported that used IC directly coupled to a conductometric detector. A low-capacity analytical column, using dilute eluent concentrations, was required to achieve a low background signal. In this case, the background is directly proportional to the equivalent conductances of the eluent species, HX, as shown in the following equation:

$$G = C_E(\lambda_H + \lambda_X) \qquad (1)$$

where G is the conductance ($S \cdot cm^2/equiv$), C_E is the concentration of the eluent, and λ_H and λ_X are the limiting equivalent conductances of H_3O^+ and X^-, respectively. The equivalent conductances ($S \cdot cm^2/equiv$) for common ions of interest in the context of this application note are[15]: Li^+, 38.7; Na^+, 50.1; Mg^{2+}, 53.1, Ca^{2+}, 59.5; NH_4^+, 73.5; K^+, 73.5; H^+, 350; OH^-, 198. Because the conductance of hydronium is significantly greater than any other cation, analytes appear as negative peaks. Therefore, it is common to reverse the polarity of the output signal when performing cation analyses by nonsuppressed conductivity.

Suppressed and nonsuppressed conductometric methods may be differentiated in terms of sensitivity, linear range, column capacity, and the ability to perform gradient separations. Consider two identical systems with the primary difference being that the effluent first passes through a suppressor before entering the conductivity cell in the first system, whereas in the second system the effluent flows directly through the conductivity cell.

In the second nonsuppressed system, the analyte signal is measured as the difference between the limiting equivalent conductance of the analyte (e.g., sodium) and the eluent cation (e.g., hydronium):

$$\Delta G = C_{Na}(\lambda_{Na} - \lambda_H) \qquad (2)$$

where ΔG is the change in conductance, C_{Na} is the concentration of sodium injected on the column, and λ_{Na} and λ_H are the limiting equivalent conductances for sodium and hydronium, respectively. If C_{Na} is neglected for this discussion, then the change in conductance for equation 2 is –300, resulting in a negative peak. Positive peaks can be obtained by reversing the signal polarity of the detector.

In the suppressed system, the sodium analyte first passes through the suppressor, converting it to sodium hydroxide, while the acidic eluent is converted to water. Therefore, the analyte is essentially determined in a background of pure water, resulting in a positive analyte response. The response can be calculated from the following equation:

$$\Delta G = C_{Na}(\lambda_{Na} + \lambda_{OH}) \qquad (3)$$

This results in a change in conductance of +248 using suppressed conductivity detection.

In comparing the change in conductance between these two systems, the analyte response is –300 compared to +248 for the nonsuppressed and suppressed systems, repectively. It would be erroneous at this point to say the nonsuppressed system is more sensitive than the suppressed without factoring the difference in baseline noise. In this application note, the typical background conductance of a suppressed system is <1 µS compared to ~1100 µS for the nonsuppressed system. An increase in the background signal generally results in a proportional increase in baseline noise. Therefore, in a nonsuppressed system, it is critical to use relatively dilute concentrations of acid to produce the lowest possible background signal and separate the common cations within a reasonable time. To meet this requirement, a low-capacity cation-exchange column must be used. However, column choice is not critical for suppressed systems, because high eluent concentrations may be used without any significant change in the background conductance, as long as the suppressor capacity is not exceeded. In this context, suppressed conductivity

detection may easily deliver baseline noise of <0.5 nS compared to ~5–10 nS for a nonsuppressed system. Using the signals calculated from equations 2 and 3 and baseline noise of 0.4 nS for a suppressed system and 7 nS for a nonsuppressed systems, a theoretical S/N may be calculated as follows:

Suppressed: S/N = 248/0.4 = 620 (4)

Nonsuppressed: S/N = 300/7 = 43 (5)

Dividing equation 4 by 5 results in a S/N difference of ~14. This exercise demonstrates that the lower noise and drift generated with a suppressor results in *superior* sensitivity of at least an order of magnitude (i.e., factor of 10) compared with nonsuppressed detection.[7,16] In addition, the calculated values agree with the experimental results determined in this application note.

The requirement of a low-capacity column for nonsuppressed detection restricts its ability to analyze high-ionic-strength matrices and lowers the dynamic range to avoid overloading the column. In addition, gradient elution is impossible because an increase in eluent strength will significantly increase the background signal and therefore preclude the detection of analytes. In contrast, columns used with suppressed systems may calibrate over four orders of magnitude in concentration due to the higher column capacity and can easily accommodate a change in eluent strength during a sample run without any significant change in the background signal. This feature allows a suppressed system to determine cations in a wide range of sample matrices. However, for analytes that form weak bases from the suppressor reaction, such as NH_4^+ or other amines, a nonlinear calibration curve is observed. Thus, a quadratic curve fit is typically required for acceptable correlation of the calibration curve. A linear calibration curve is observed using nonsuppressed conductivity detection.

In this application note, the Dionex IonPac CS16 and Dionex IonPac SCS 1 columns were used to demonstrate the capabilities of suppressed and nonsuppressed conductivity detection, respectively. The Dionex IonPac CS16 column is a high-capacity cation-exchange column with 100% solvent compatibility and medium hydrophobicity. The high capacity of 8400 µeq/column is achieved by using a higher density of grafted carboxylic acid groups and a larger column format (5 × 250 mm). The higher capacity is particularly advantageous for analyzing high-ionic-strength matrices and resolving analytes at disparate concentration ratios, such as sodium and ammonium in wastewater samples.

The nonsuppressed Dionex IonPac SCS 1 column is a 4.5 µm silica-based poly(butadiene-maleic acid) copolymer column functionalized with carboxylic acids. To achieve a separation of the six common cations within a reasonable time using a dilute acidic eluent, the capacity of the 4 × 250 mm Dionex IonPac SCS 1 column (318 µeq/column) needs to be considerably less than that of the Dionex IonPac CS16 column. The Dionex IonPac SCS 1 column is also 100% solvent-compatible with acetone or acetonitrile that may be used to change the selectivity or alter retention times. Figures 2 and 3 show separations of common cations using the Dionex IonPac CS16 and Dionex IonPac SCS 1 columns, respectively. The higher-capacity Dionex IonPac CS16 column required nearly ten times the eluent strength of the Dionex IonPac SCS 1 column to achieve the separation in less than 30 min. The higher eluent strength required by the Dionex IonPac CS16 column, due to its higher capacity, precludes its use for nonsuppressed conductivity detection.

Because retention times vary with temperature, maintaining constant temperature is critical. Although both systems can be operated at ambient temperatures, the temperature should be controlled at 30 °C for good retention time reproducibility. However, the high stability of the polymeric Dionex IonPac CS16 column allows temperatures up to 60 °C to be used. *Temperatures above 35 °C may result in irreversible damage to the silica-based Dionex IonPac SCS 1 resin and therefore should not be used.*

Retention time and background signal may also vary slightly between eluent preparations for the nonsuppressed Dionex IonPac SCS 1 column. In contrast, the suppressed system can generate very reproducible retention time and peak area data by electrolytically generating the MSA online. This online eluent generation also significantly increases the flexibility of the suppressed cation system in comparison to manually preparing the eluents.

Tables 3 and 4 summarize the calibration data and method detection limits (MDLs) obtained for the six cations using the Dionex IonPac CS16 and Dionex IonPac SCS 1 columns, respectively. The higher capacity of the Dionex IonPac CS16 column results in a calibration curve *over three* orders of magnitude for most cations, except for ammonium. The nonlinear dependence of peak area (or height) on concentration is common for weak bases such as ammonia that are not completely protonated at high concentrations in the suppressor.[6] A quadratic curve fitting function extends the calibration curve for ammonium to 40 mg/L. For the nonsuppressed Dionex IonPac SCS 1 column, the calibration curve extends *up to three* orders of magnitude for all cations. Unlike the suppressed system, nonsuppressed detection results in a linear curve for ammonium, using a least squares fit, with a coefficient of determination (r^2) of 0.9999. However, sodium was calibrated up to four orders of magnitude for the suppressed system, compared to three orders of magnitude for the nonsuppressed system.

High concentrations of sodium and other cations will overload the Dionex IonPac SCS 1 column due to its significantly lower capacity compared to the Dionex IonPac CS16 column. Overloading can cause peak splitting, especially for weakly retained analytes. This peak splitting is illustrated in Figure 4A, showing a standard injection containing 1000 ppm sodium, 40 ppm ammonium, and 100 ppm of the other common cations using the Dionex IonPac SCS 1 column. The Li^+ peak is split and the divalent cation peaks severely tail. Figure 4B shows a chromatogram of the same standard injected on the high-capacity Dionex IonPac CS16 column with suppressed conductivity detection. Due to the significantly higher capacity of the Dionex IonPac CS16 column, the sample does not cause column overloading. Figure 4C shows the same standard diluted by a factor of two analyzed with the Dionex IonPac SCS 1 column. Although, the lower concentration has removed the splitting of the lithium peak, tailing is still observed for the

Table 3. Linearity and MDLs using suppressed conductivity detection[a]

Analyte	Range (mg/L)	Linearity (r^2)	Calculated MDL[b] (µg/L)	MDL Standard (µg/L)
Lithium	0.05–80	0.9999	0.19	1
Sodium	0.1–1000	0.9999	1.81	4
Ammonium[c]	0.05–40	0.9993	1.23	5
Potassium	0.05–80	0.9999	2.64	10
Magnesium	0.05–80	0.9999	1.00	5
Calcium	0.05–80	0.9998	1.09	5

[a] Dionex ICS-2500 IC system with a 10 µL injection
[b] Dionex IonPac CS16 column can tolerate a higher upper concentration than shown
[c] Quadratic fit
[d] MDL = $\sigma t_{S,99}$ where $t_{S,99}$ = 3.14 for n = 7

Table 4. Linearity and MDLs using nonsuppressed conductivity detection[a]

Analyte	Range (mg/L)	Linearity (r^2)	Calculated MDL[b] (µg/L)	MDL Standard (µg/L)
Lithium	0.05–50	0.9999	2.0	10
Sodium	0.25–250	0.9999	5.8	20
Ammonium	0.05–50	0.9999	10.9	25
Potassium	0.2–50	0.9999	30.0	100
Magnesium	0.2–50	0.9999	19.6	100
Calcium	0.2–100	0.9999	36.6	150

[a] Dionex ICS-2000 IC system with a 10 µL injection
[d] MDL = $\sigma t_{S,99}$ where $t_{S,99}$ = 3.14 for n = 7

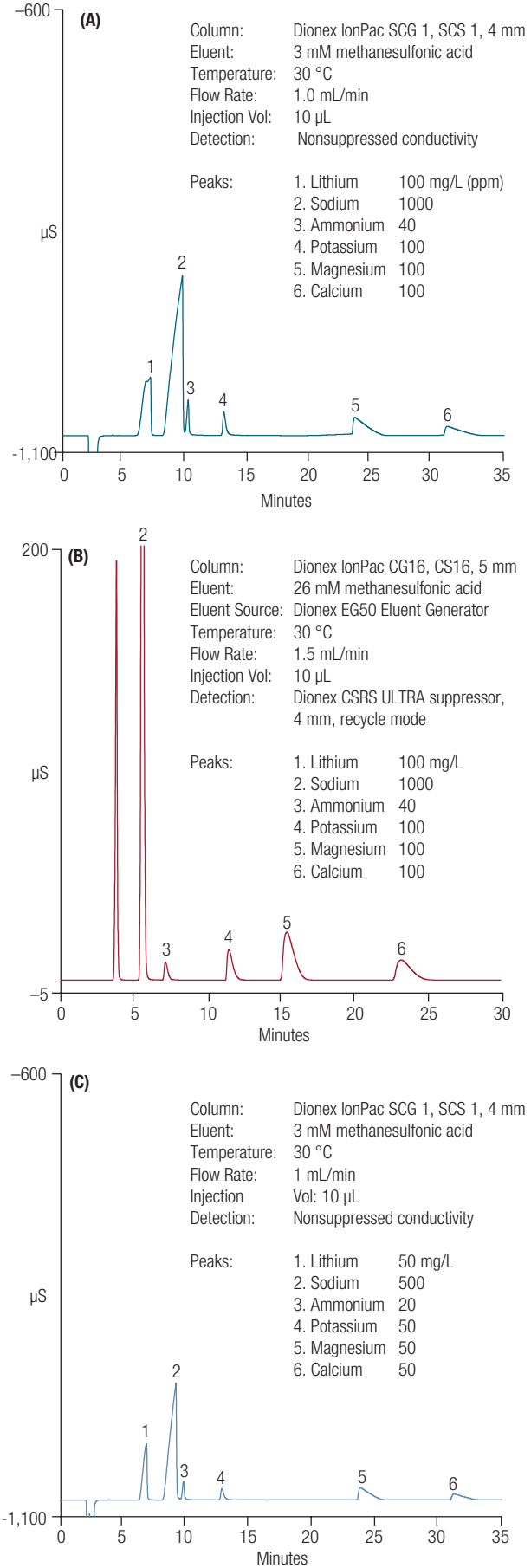

Figure 4 A–C. Separation of inorganic cations in a high-ionic strength matrix

divalent cation peaks. Therefore, analysis of high-ionic-strength matrices on the Dionex IonPac SCS 1 column requires an appropriate dilution or lower injection volume to avoid column overloading.

As previously discussed, the sensitivity for suppressed cations is significantly better than the nonsuppressed system (Tables 3 and 4). The suppressed system MDLs were lower by at least an order of magnitude for most cations compared to the nonsuppressed system. Lower detection limits may be achieved for either system by injecting a larger sample volume. The amount of sample injected onto either column depends on its ionic strength. Higher capacity columns, such as the Dionex IonPac CS16 column, will tolerate larger injection volumes than lower capacity columns. Although the MDLs for the suppressed system were better than the nonsuppressed system, in a truly fair comparison the column dimensions should be considered. A further improvement in detection limits than shown in Table 3 would be expected for a smaller i.d. Dionex IonPac CS16 column format, such as a 4 × 250 mm column.

An important application, particularly for environmental samples, is the ability to determine trace concentrations of ammonium in the presence of high concentrations of sodium. The high-capacity Dionex IonPac CS16 column is ideal for this analysis by providing an improved resolution of sodium from ammonium, even in high-ionic-strength samples. Figure 5 illustrates the determination of trace-level ammonium in the presence of high sodium. The sodium to ammonium ratio shown in this chromatogram is ~6700:1. However, the Dionex IonPac CS16 column is capable of tolerating ratios of up to 10,000:1. The Dionex IonPac SCS 1 column is not ideal for analyzing these types of matrices due to its lower capacity. The maximum ratio determined for this column was 1000:1 sodium to ammonium (Figure 6).

Column:	Dionex IonPac CG16, CS16, 5 mm
Eluent:	26 mM methanesulfonic acid
Eluent Source:	Dionex EG50 Eluent Generator
Temperature:	30 °C
Flow Rate:	1.5 mL/min
Injection Vol:	10 µL
Detection:	Suppressed conductivity Dionex CSRS ULTRA suppressor AutoSuppression recycle mode
Sample Prep:	Filter

Peaks:	1. Lithium	<0.2 mg/L
	2. Sodium	200
	3. Ammonium	0.03
	4. Potassium	0.5
	5. Magnesium	8.0
	6. Calcium	20

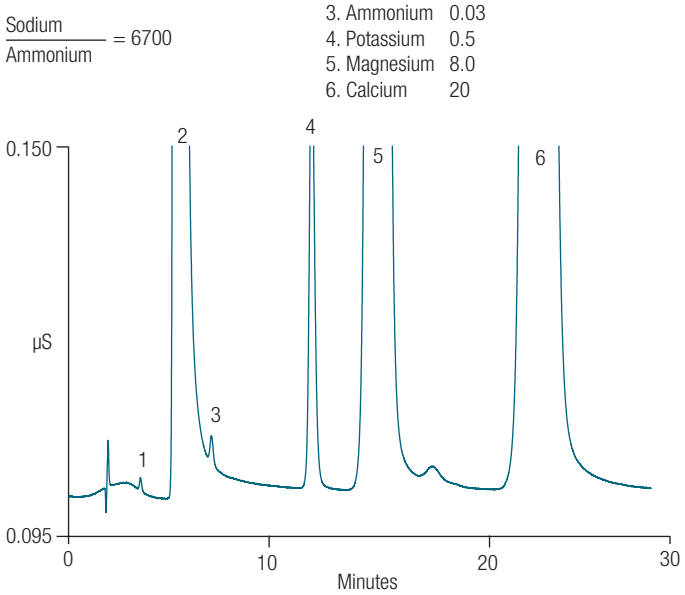

Figure 5. Resolution of trace ammonium from high sodium with the Dionex IonPac CS16 column

Column:	Dionex IonPac SCG 1, SCS 1, 4 mm
Eluent:	3 mM methanesulfonic acid
Temperature:	30 °C
Flow Rate:	1.0 mL/min
Injection Vol:	25 µL
Detection:	Nonsuppressed conductivity

Peaks:	1. Sodium	100 mg/L (ppm)
	2. Ammonium	0.1

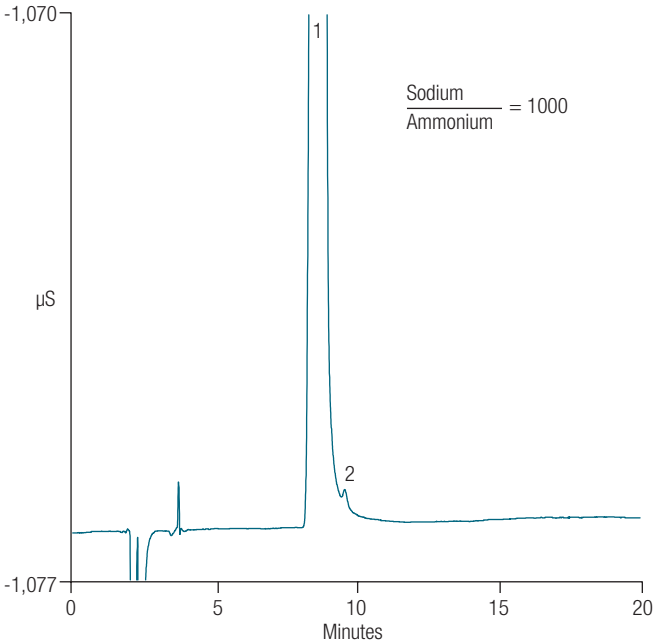

Figure 6. Determination of low concentrations of ammonium in high concentrations of sodium on the Dionex IonPac SCS 1 column

The high capacity of the Dionex IonPac CS16 cation-exchange column is an advantage when injecting low pH samples, such as acidic digests, acid-preserved samples, and acidic soil extracts. These samples can contain up to 100 mM hydronium ion (pH 1) and can be injected (25 µL) without pH adjustment. However, because the functional groups are weakly acidic carboxylic acids, a sample pH <1 will impact the separation of cations on the column. The significantly lower cation-exchange capacity of the Dionex IonPac SCS 1 column prevents the analysis of these types of samples without sample preparation to remove the excess hydronium ions. *Therefore, samples with a pH less than 2 (10 mM hydronium ion) should not be injected on the Dionex IonPac SCS 1 column.*

Alternative cation eluents for nonsuppressed conductivity detection are weakly acidic complexing agents, such as tartaric acid and PDCA. The high affinity of PDCA for divalent metal ions, such as calcium and magnesium, causes a significant decrease in their retention. Calcium forms a particularly strong complex with PDCA, reducing its effective positive charge, and therefore causing it to elute before magnesium. Alkali metals are not affected by a change in the concentration of PDCA due to their low complexing ability. Figure 7 shows a separation of common cations using 4 mM tartaric acid and 0.75 mM PDCA. The significant increase in run times, compared to other commercially available nonsuppressed cation-exchange columns, results from the higher capacity of the Dionex IonPac SCS 1 column. Therefore, the optimum eluent for the Dionex IonPac SCS 1 column is 3 mM MSA, as specified under the method conditions in this application note.

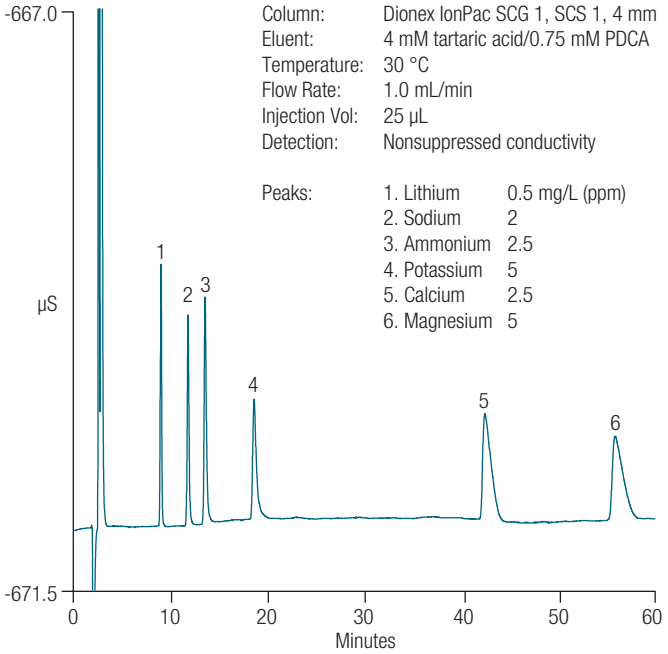

Figure 7. Separation of common inorganic cations using a weak acid eluent on the Dionex IonPac SCS 1 column

Conclusion

This application note demonstrates the capabilities of suppressed conductivity detection using the high-capacity Dionex IonPac CS16 column and nonsuppressed conductivity detection using a lower-capacity Dionex IonPac SCS 1 column for the determination of common inorganic cations. The lower noise generated with suppressed systems results in an improved S/N ratio of at least one order of magnitude compared to a nonsuppressed cation system. This improved ratio enables the determination of trace levels of cations that may otherwise prove difficult using a nonsuppressed system. The use of nonsuppressed conductivity as a detection mode requires a low capacity column using dilute acidic eluents to achieve a low background signal. This requirement limits the linear range of common cations, prevents the use of eluent gradients limits sample pH, and prevents the possibility of analyzing high-ionic-strength matrices without overloading the column. However, nonsuppressed conductivity detection does produce linear calibration curves for ammonium and weakly basic amines.

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Evaluating Ion Chromatography Suppression Options

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Keywords
Eluent suppressor, ICS-6000, ICS-4000, Integrion, water, carbohydrates, environmental, food safety, pharma

Introduction

In the years leading up to 1971, Hamish Small and the other ion chromatography (IC) pioneers at the Dow Chemical Company abandoned ion-exchange chromatography as a practical technique. They had found that the highly concentrated solutions needed to displace some analytes of interest created such a high level of noise that it masked the analyte signal.

Fortunately, 1971 saw the invention of a “stripper” (later termed a suppressor) to convert the highly-conducting molecules in the eluent to a low-conducting form against which the analyte signal could be clearly seen.

Since that time, suppressors have evolved and several types are now commercially available, and it can be difficult to compare the many options. In this white paper, we will look at the benefits and drawbacks of each.

ThermoFisher SCIENTIFIC

ICS-6000 / ICS-4000 / Integrion

Table of contents

Non-suppressed

All IC systems can be run without suppression simply by bypassing whatever suppression devices are present. In order to lower background noise as much as possible, non-suppressed IC should utilize very low-capacity ion-exchange columns (such as the Thermo Scientific™ Dionex™ IonPac™ SCS 1 Silica Cation Separator Column) to ensure that only weak eluents are necessary (Figure 1). This requirement means that selectivity options are limited.

Chemically Regenerated Suppressors

Chemically regenerated suppressors, including packed bed, automatically-switching packed bed, and continuously regenerated suppressors, use chemical solutions to regenerate the suppressor.

Packed Bed Suppressor

The first suppressor type developed was the "packed-bed suppressor", and is the only suppressor type available from some vendors. This type of suppressor contains a large volume of high-capacity ion-exchange resin (cation-exchange resin for anion analysis, and anion-exchange resin for cation analysis), often packed into a column body.

The high capacity of packed bed suppressors extends their suppression ability, typically to eight hours, before they are exhausted. Once they are exhausted, they must be taken offline and regenerated for an extended period, typically overnight, before they can be used again. The drawbacks of the extended downtime for each regeneration meant that hydroxide eluents were not used in the early years of IC, despite their clear advantages.

Unfortunately, the large internal volume of these suppressors also led to peak dispersion and poor chromatographic performance.

Automatically-Switching Packed Bed Suppressor

One way to reduce the volume of packed bed suppressors is to take the suppressor offline and regenerate it after every analysis, but that is an inconvenient process. To avoid this, it is possible to attach multiple suppressor cartridges to a rotor and change cartridges after every run.

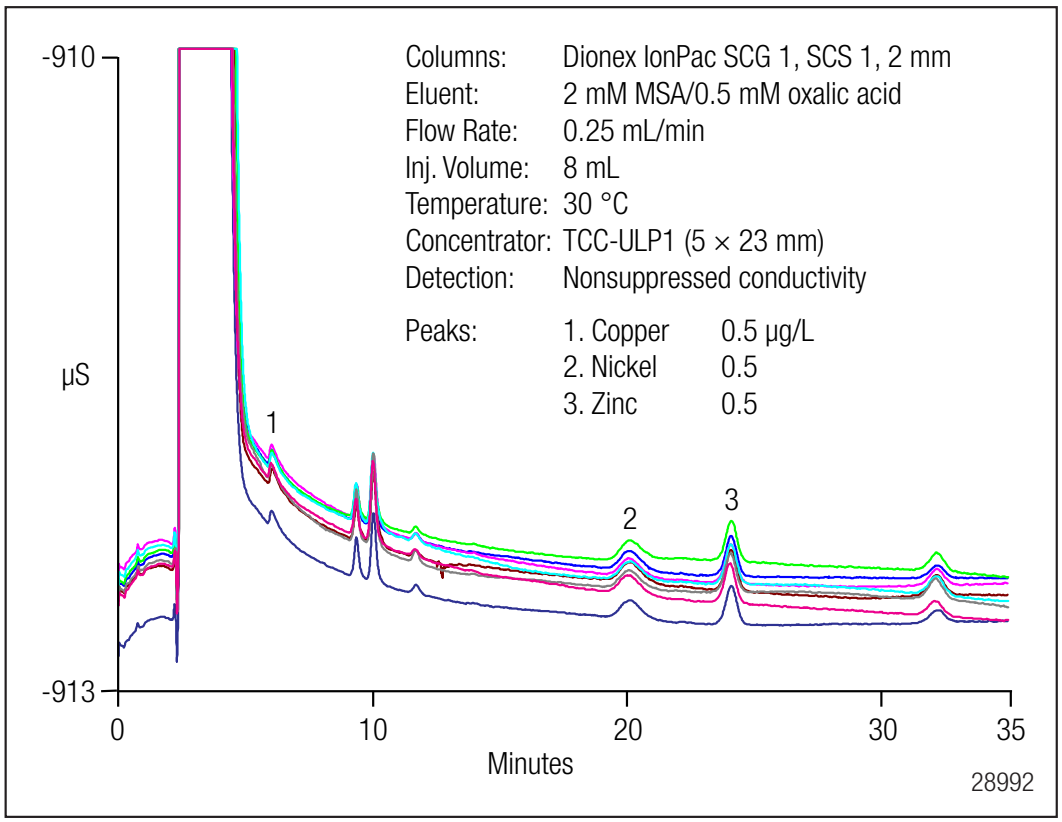


Figure 1. Non-suppressed conductivity detection of cations using a Dionex IonPac SCS 1 column separation.

Automating the switching, regenerating, and rinsing of the suppressor cartridges minimizes labor and downtime. However, to maintain chromatographic performance, the capacity of the suppressor cartridges has to be reduced significantly. As a result, these types of suppressors may run out of capacity during an analysis.

Additionally, automated switching makes it difficult to track which suppressor cartridge has been used for a given analysis, leading to validation issues. These include:

- When a cartridge becomes contaminated, it can be impossible to prove that certain injections were unaffected.
- Samples are likely to be run through a different cartridge than the standards used for calibration (Figure 2).

This approach is equivalent to running separate injections on different analytical columns during a sequence or calibration curve. It is impossible to verify true compliance and regulatory accuracy when switching packed bed suppressor columns between injections.

Continuously Regenerated Suppressor

Continuous regeneration addresses the issue of limited capacity in packed bed suppressors. When using continuous regeneration, regenerant solution is continuously passed through the suppressor via a parallel channel. This enables analyses of any duration without the prospect of running out of suppression capacity.

Regenerant solution must still be prepared. To mitigate the time taken for regenerant preparation, concentrated regenerant solutions can be purchased for dilution. The expense of an additional mechanism for moving the regenerant through the suppressor can be alleviated using techniques such as displacement chemical regeneration (DCR), where the conductivity cell effluent displaces the regenerant back through the suppressor.

Electrolytically Regenerated Suppressors

Electrolytically regenerated suppressors, including constant current and dynamically regenerated suppressors, use water and a potential applied across two electrodes to regenerate the suppressor, eliminating the need to prepare regenerant solution.

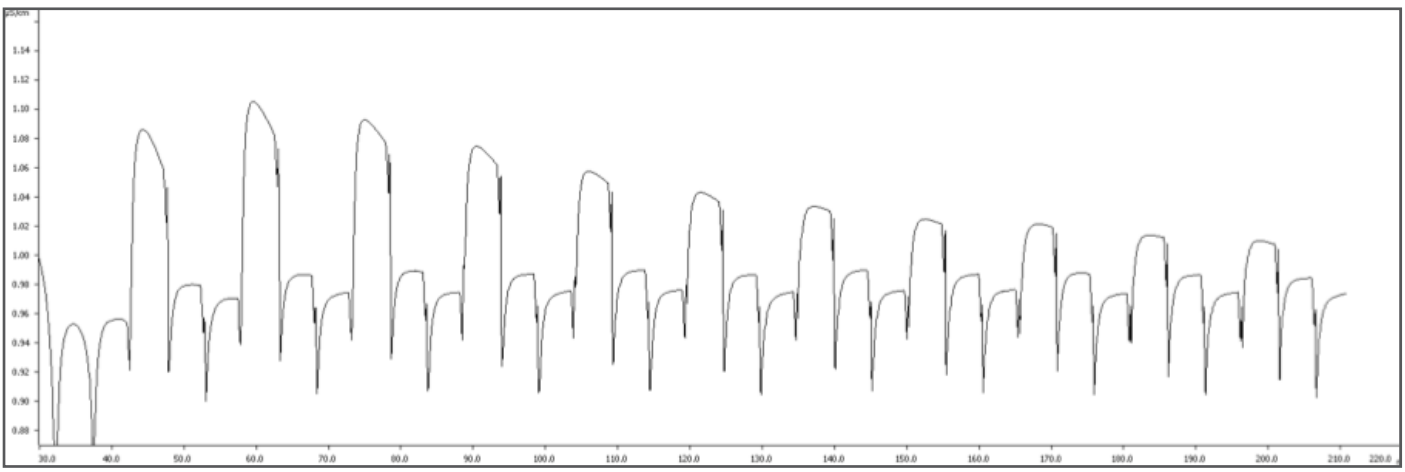


Figure 2. Example of baseline disruptions resulting from automatically switching packed bed suppressors. If standards are run on different suppressor cartridges from samples, spurious results may be obtained.

Constant Current Suppressor

The water required for regeneration can simply be the cell effluent (so-called “recycle” mode), or it can be delivered externally if the cell effluent is unsuitable for re-use (“external water” mode). Recycle mode removes the need for additional pumps or chemicals, and is therefore cost-effective, low-labor, and reagent-free. External water mode is used when the cell effluent is unsuitable, or unavailable, for re-use.

Early electrolytic suppressors generated 3–5 times the noise of the best performing chemical suppressors, so trace ion analysis was still best performed with chemical suppression. However, noise is considerably reduced in modern electrolytic suppressors, so they can be used successfully for trace-level work (Figure 3).

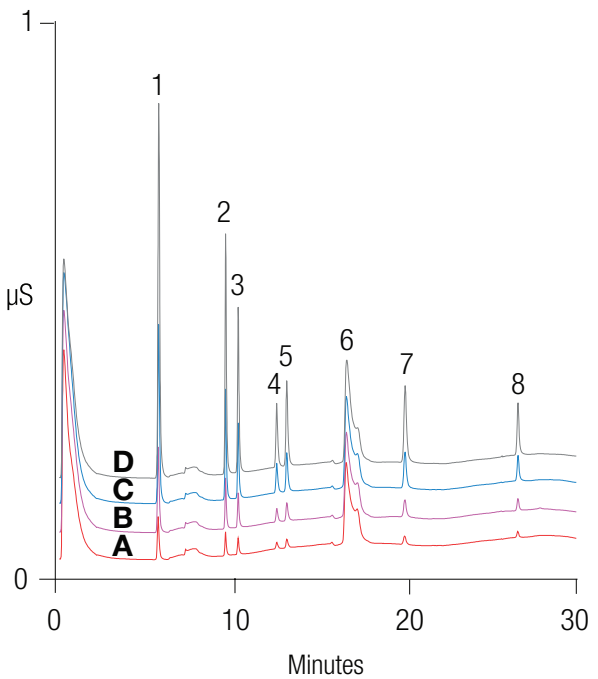
Until recently, all electrolytic suppressors operated by applying a fixed current across the electrodes. One drawback of this approach is that the operator must determine what current needs to be applied (usually by reference to the application material). More significantly, when running an eluent concentration gradient, the current applied needs to be high enough to regenerate the highest eluent concentration used. current applied needs to be high enough to regenerate the highest eluent concentration used. However, during the times when the eluent concentration is low, the excess current applied may increase noise and decrease the lifetime of the suppressor.

Finally, electrolytic suppressors are not suitable for eluents or samples containing high levels of organic solvents. These applications require a chemical suppressor.

Dynamically Regenerated Suppressor

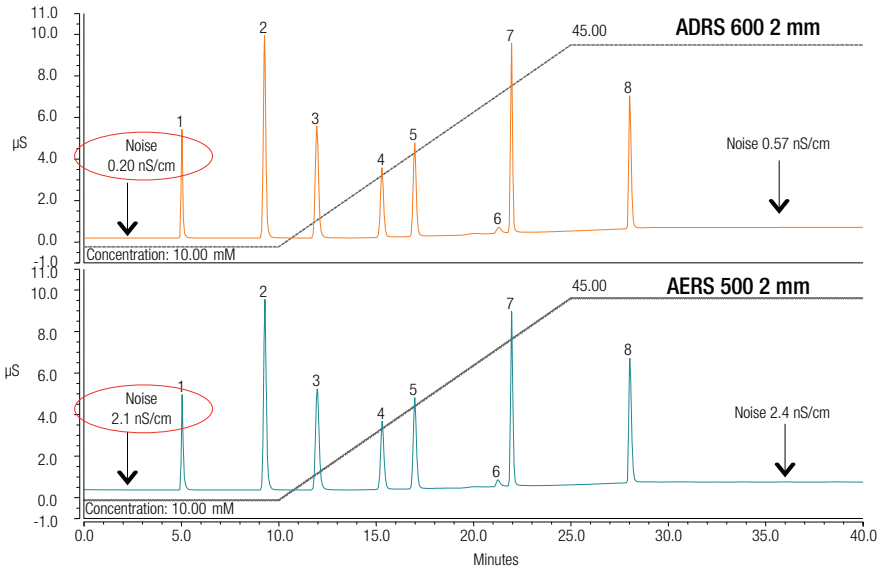
Dynamically regenerated electrolytic suppression overcomes some of the issues of constant current suppressors by maintaining a constant potential across the electrode throughout the analysis (Figure 4). As a result, only the current necessary to regenerate the suppressor is used. In this way, a dynamically regenerated suppressor:

- Minimizes training and set-up time
- Eliminates the potential for errors in the manual calculation of electrical currents
- Improves signal-to-noise ratio with concentration gradients by dynamically adapting to the changing eluent composition



Columns:	Dionex IonPac AG17-C, 4 × 50 mm Dionex IonPac AS17-C, 4 × 250 mm
KOH Eluent:	50 mM wash (4.5 min); 1 mM (-7 to 4 min), 1–12.5 mM (4–10 min); 12.5–20 mM (10–20 min); 20–35 mM (20–30 min)
Eluent Source:	Dionex EGC-500 KOH cartridge, with Dionex CR-ATC 600 trap column, Dionex high pressure degasser
Flow Rate:	1 mL/min
Inj. Volume:	Sample (10 mL), Standard (10–80 µL)
Column Temp.:	30 °C
Detection:	Suppressed conductivity, Dionex AERS 500 suppressor,
Sample Prep.:	Dionex CRD 300 inline degassing, Dionex AutoPrep, EWP, 30 mA
Concentrator:	Dionex UTAC-LP2, 4 mm
Sample Vol.:	Sample (10 mL), Standard (10–80 µL)
Samples:	A: 50 ng/L C: 200 ng/L B: 100 ng/L D: 400 ng/L
Peaks:	1. Fluoride 5. Nitrate 2. Chloride 6. Carbonate 3. Nitrite 7. Sulfate 4. Bromide 8. Phosphate

Figure 3. Calibration standards of 50 to 400 ppt on a system utilizing the Thermo Scientific™ Dionex™ AERS™ 500 Anion Electrolytically Regenerated Suppressor in recycled water mode.



Column:	Thermo Scientific™ Dionex™ IonPac™ AS19-4µm 2 mm
Eluent (EG gradient):	10 mM from 0–10 min; 10 mM to 45 mM from 10–25 min; 45 mM from 25 min to 40 min
Flow rate:	0.25 mL/min
Inj. volume:	2.5 µL
Detection:	Suppressed conductivity
ADRS 600	4.5 V
AERS 500	28 mA
Oven Temp:	30 °C

Peaks:
1. Fluoride
2. Chloride
3. Nitrite
4. Bromide
5. Nitrate
6. Carbonate
7. Sulfate
8. Phosphate

Figure 4. Comparison of baseline noise on a system running a potassium hydroxide gradient using constant current (Dionex AERS 500 Anion Electrolytically Regenerated Suppressor) and dynamically regenerated (Thermo Scientific™ Dionex™ ADRS 600 Anion Dynamically Regenerated Suppressor) suppressors.

Summary

Thermo Fisher Scientific offers the widest choice of suppression capabilities, ensuring that you can find the most suitable solution for your application.

Suppression Technique		Summary	# of vendors offering	
	Non-suppressed	Simple, but low sensitivity and selectivity	7+	ThermoFisher SCIENTIFIC
Chemically Regenerated	Packed-bed	High sensitivity, but large dead volume and downtime	3	
	Automatically switching packed-bed	Reduces downtime, but limited capacity and no traceability	3	
	Continuously regenerated	"Infinite" capacity, but still requires regenerant preparation	2	ThermoFisher SCIENTIFIC
Electrolytically Regenerated	Constant Current	Reagent and labor-free, not suitable for exotic applications	2*	ThermoFisher SCIENTIFIC
	Dynamically Regenerated	Always optimized, not solvent compatible	1	ThermoFisher SCIENTIFIC

*** Only Thermo Fisher Scientific offers *continuously regenerated* electrolytic suppression**

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Improving method throughput

Many current pharmaceutical analytical methods were developed on instrumentation and column geometry that result in long run-times.

There is always a drive to increase analytical throughput, and reduce analysis time per sample, within accepted limits of the relevant Pharmacopeial methods. The USP Chapter <621> defines acceptable modifications to column dimensions, as well as allowable flow rate alternations for transferring methods from HPLC to UHPLC.

Many laboratories are also limited by spatial constraints and cannot increase throughput by increasing the number of individual systems available for use.

Chapter highlights

Thermo Scientific™ Vanquish™ Duo UHPLC systems combine two flow paths in one integrated UHPLC solution. The supported workflows save time, reduce cost per sample and increase capacity without requiring added bench space.

The **Thermo Scientific™ Vanquish™ Horizon UHPLC System** features high sample capacity for high-throughput workflows.

The **Thermo Scientific™ Vanquish™ Flex UHPLC Systems** increase productivity without compromising quality.

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Table of contents

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APPLICATION NOTE 72593

Simultaneous high-performance and ultra-high-performance liquid chromatographic analysis of acetaminophen impurities using a single instrument

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Keywords
Vanquish Flex Duo UHPLC system for Dual LC, Vanquish Flex Dual Pump UHPLC, Vanquish Flex Dual Split Sampler, acetaminophen

Goal

To demonstrate the capabilities of the Thermo Scientific™ Vanquish™ Flex Duo UHPLC system for Dual LC to run independent HPLC and UHPLC methods simultaneously using one instrument.

Application benefits

- Dual LC technology provides two independent LC channels with the footprint of only one instrument.
- Established HPLC methods and their UHPLC counterparts can be implemented in parallel on the same instrument.

Introduction

In current analytical laboratories, vast numbers of analytical methods are typically established and used for the analysis of hundreds of samples. To increase throughput and generate more results, there is a growing need for faster methods as well as for additional analytical instrumentation. Thus, UHPLC-compatible instruments and spatial constraints play an increasing role in equipping these labs. In this respect, LC systems that house two independent LC channels with two separate, individually configurable, flow paths in the footprint of a single instrument are beneficial in multiple ways. For example, the newly developed Vanquish Flex Duo system for Dual LC, allows for optimization of each flow path to specific requirements, e.g. regarding extra column or gradient delay volumes, giving the opportunity to have one HPLC and one UHPLC instrument in the same stack.

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Vanquish Flex Duo UHPLC

Table of contents

Such a setup can be utilized for parallel implementation of completely independent HPLC and UHPLC methods but also for speed-up of legacy HPLC methods at the same workstation. This application demonstrates the latter case.

Here, the left chromatographic channel of the novel Vanquish Flex Duo system for Dual LC was configured with HPLC common system volumes (see instrumentation section) and was run with a 4.6 mm i.d. column with 3 µm particles for the analysis of acetaminophen as an active pharmaceutical ingredient (API) and its impurities derived from an USP assay.[1] System volumes were reduced at the right channel and the respective UHPLC counterpart method, which was created by the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) UHPLC speed-up tool[2], was run in parallel with a 2.1 mm i.d. column with 1.9 µm particles.

Both analyses were performed with Thermo Scientific™ Hypersil GOLD™ C8 stationary phase of different column dimensions. Hypersil GOLD C8 matches the required USP level L7 and is well suited for analytes of medium hydrophobicity.

Experimental

Reagents and materials

- Deionized water, 18.2 MΩ·cm resistivity or higher
- Fisher Scientific™ Optima™ Methanol, LC/MS grade (P/N 10767665)
- Fisher Scientific Sodium phosphate dibasic anhydrous (P/N 10182863)
- Fisher Scientific Potassium dihydrogen orthophosphate (P/N 10429570)
- Acetaminophen, 4-aminophenol, N-(4-hydroxyphenyl) propanamide (Impurity B), 2-acetamidophenol (Impurity C), acetanilide (Impurity D), and 4'-chloracetanilide (Impurity J) were purchased from reputable vendors.

Table 1. LC conditions.

	Left Flow Path: HPLC	Right Flow Path: UHPLC
Column	Hypersil GOLD C8, 4.6 x 100 mm, 3 µm, 175 Å (P/N 25203-104630)	Hypersil GOLD C8, 2.1 × 100 mm, 1.9 µm, 175 Å (P/N 25202-102130)
Mobile phase	A: 1.7 g/L KH_2PO_4 and 1.8 g/L of Na_2HPO_4 in water B: Methanol	
Flow rate	1 mL/min	0.5 mL/min
Gradient	0-3 min 1% B, 3-7.2 min from 1 to 85% B, 7.2-7.3 min from 85 to 1% B, 7.3-12.2 min 1% B	0-1.25 min 1% B, 1.25-3.001 min from 1 to 85% B, 3.001-3.043 min from 85 to 1% B, 3.043-6 min 1% B or alternative 0-1.751 min from 1 to 85% B, 1.751-1.792 min from 85 to 1% B, 1.792-4.8 min 1% B
Mixer volume (static + capillary mixer)	(350+50) µL	(150+50) µL
Column temperature	35 °C (Still air mode) with active pre-heater	
Autosampler temperature	8 °C	
UV wavelength	230 nm	
UV data collection rate	10 Hz	20 Hz
UV response time	0.5 s	0.2 s
Injection volume	1 µL	0.17 µL or alternative 0.5 µL
Needle wash	Off	

Sample preparation

Stock solutions of acetaminophen (20 mg/mL), 4-aminophenol and the impurities B, C, D, and J (1 mg/mL each) were prepared in methanol. By dilution with methanol and mixing of stock solutions, a sample was prepared that contained 10 mg/mL acetaminophen and 10 µg/mL of each of the other compounds (corresponding to 0.1% of the API).

Instrumentation

Vanquish Flex Duo system for Dual LC consisting of:

- System Base Vanquish Dual (P/N VF-S02-A-02)
- Dual Pump F (P/N VF-P32-A-010)
- Left pump with Static mixer, volume 350 µL (P/N 6044.5310)
- Right pump with Static mixer, volume 150 µL (P/N 6044.5110)
- Dual Split Sampler FT (P/N VF-A40-A-020)
- Column Compartment H (P/N VH-C10-A-020)
- Variable Wavelength Detector at left flow path (P/N VH-D40-A0)
  - With Standard flow cell, 10 mm, 11 µL (P/N 6077.0250)
- Variable Wavelength Detector at right flow path (P/N VH-D40-A0)
  - With Semi-micro flow cell, 7 mm, 2.5 µL (P/N 6077.0360)

Data processing and software

Chromeleon CDS software version 7.2.8 was used for data acquisition and analysis.

Results and discussion

Figure 1 illustrates the schematic fluidic setup of the Dual LC system used in this study.

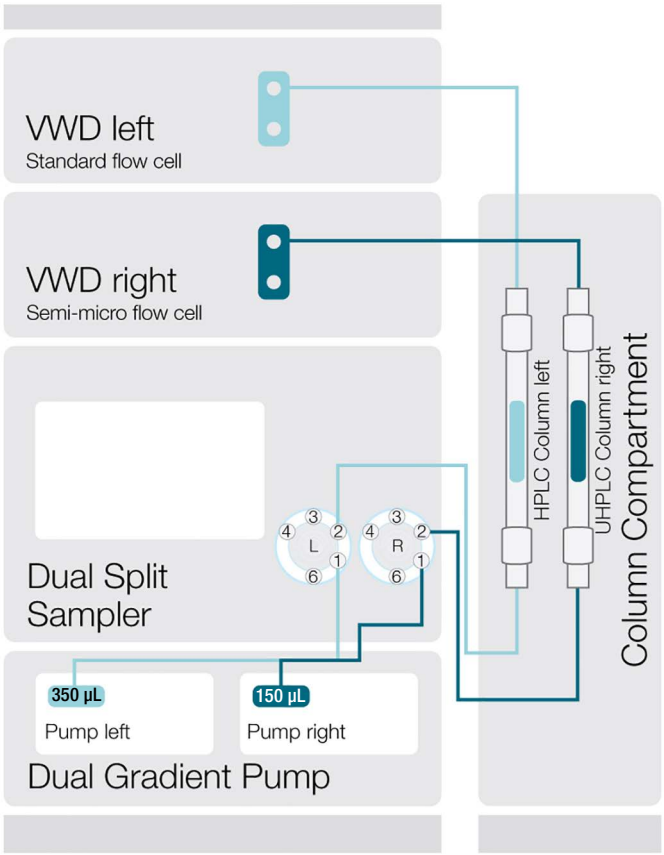

Figure 1. Fluidic setup of Vanquish Flex Duo system for Dual LC with one HPLC (light blue) and one UHPLC (dark blue) flow path.

The method parameters of the UHPLC channel of this experiment were derived from the original HPLC method by the Chromeleon CDS UHPLC speed-up tool with a boost factor of 1.52 for a flow rate of 0.5 mL/min and additional flush time to ensure sufficient equilibration. Both chromatographic channels were run with 10 repeated injections of the prepared sample. Figure 2 shows two example chromatograms with average resolutions (R_S) that easily meet the USP requirements.[1] Table 2 summarizes the retention times (t_R) and their precision. The absolute and relative standard deviations (SD and %RSD) of retention times are comparable for both methods.

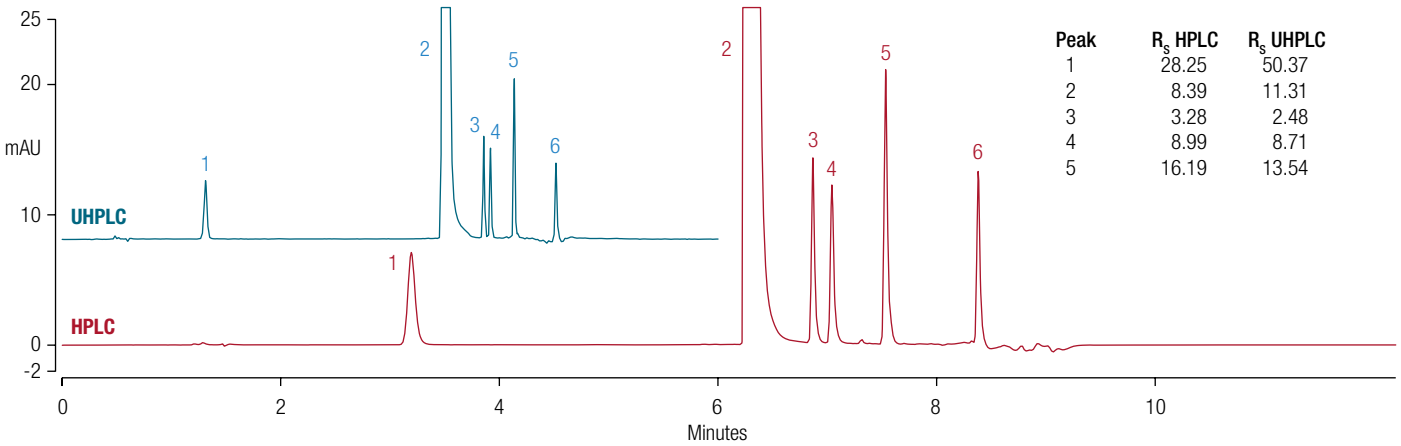

Figure 2. Chromatograms of HPLC (bottom, red) and UHPLC (top, blue) run at same time and signal scale and peak resolutions. For peak assignment see Table 2.

Table 2. Retention times (t_R) and standard deviation (SD) and relative standard deviations (%RSD) for HPLC and UHPLC analysis.

Peak #	Compound	HPLC Method			UHPLC Method		
		t_R [min]	t_R SD [min]	t_R %RSD	t_R [min]	t_R SD [min]	t_R %RSD
1	4-Aminophenol	3.195	0.002	0.058	1.313	0.001	0.034
2	Acetaminophen	6.261	0.001	0.018	3.498	0.001	0.046
3	Impurity B	6.868	0.001	0.017	3.858	0.001	0.015
4	Impurity C	7.042	0.001	0.018	3.919	0.001	0.014
5	Impurity D	7.534	0.001	0.020	4.136	0.001	0.015
6	Impurity J	8.382	0.002	0.019	4.519	0.001	0.017

Regarding peak area precision and signal-to-noise values (S/N), the UHPLC method was inferior to the HPLC method due to two impacts (see Figure 3, blue and red bars). For one, UV sensitivity is affected by the length of the light path provided by the flow cell, which is 30% shorter for the UHPLC setup. Furthermore, injection precision (and thus area %RSD) is negatively affected by the very low injection volume of 0.17 µL in the UHPLC method as it comes closer to the autosampler's specification limit. Due to downscaling to the smaller UHPLC column volume, this small injection volume results from automatic parameter calculation by the Chromeleon CDS speed-up calculator originating from an already small injection volume of just 1 µL that had to be applied in the original HPLC method. In HPLC mode, analysis volumes greater than 1 µL caused distorted peak shapes for the early eluting 4-aminophenol because of the high elution strength of the sample solvent methanol and insufficient pre-column mixing in the low system volumes. In contrast to a fronting peak shape in HPLC for injection of 3 µL, an equivalent triplication of the downscaled injection volume did not cause any peak disturbance for the UHPLC method as the sample volume of 0.5 µL is small enough to be adequately mixed with the surrounding mobile phase before entering the column. Both cases are illustrated in Figure 4 and clearly demonstrate the advantage of the UHPLC method for sample volume loading capacity. Thus, a simple improvement of the UHPLC method by increasing the injection volume from 0.17 µL to 0.5 µL is recommended to improve S/N and yield area %RSDs in a similar range as the HPLC method, which is also depicted in Figure 3, yellow bars. However, all three methods resulted in well integrable peaks with S/N values all greater than 50.

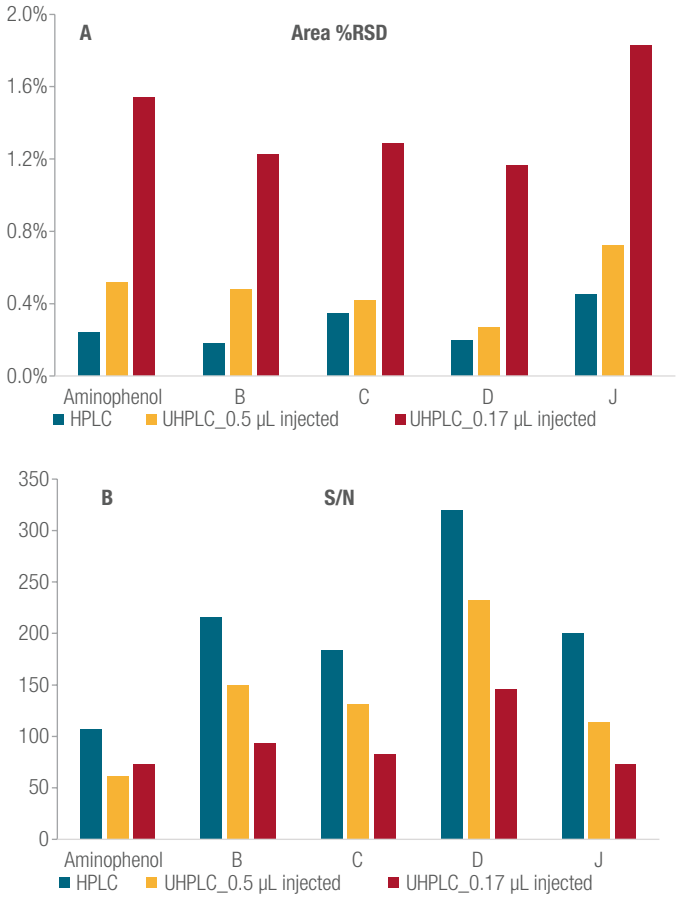

Figure 3. Area precision (A) and signal-to-noise values (B) for HPLC with 1 µL and UHPLC with 0.17 µL and 0.5 µL injection volume for acetaminophen impurities.

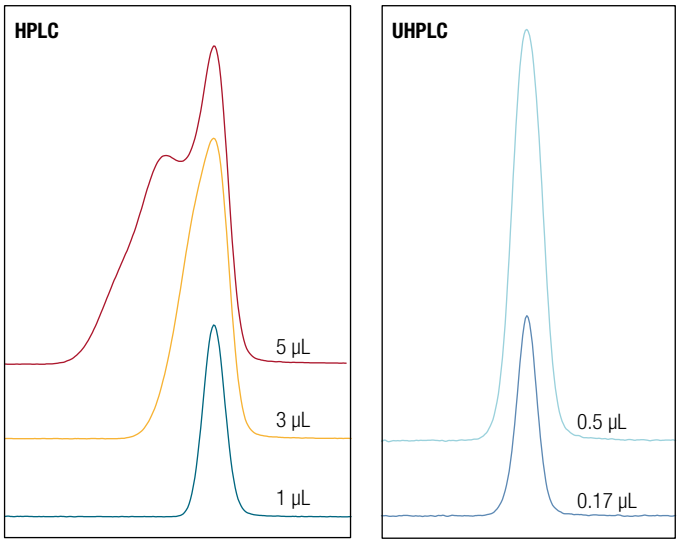

Figure 4. 4-Aminophenol peak depending on injection volume in both assays. Injecting the threefold volume in HPLC already causes peak fronting. In UHPLC, the peak shape is not affected.

Considerable benefits of the UHPLC method are substantial savings in sample volume, solvent consumption, and cycle time (t_C), with additional optimization capabilities if the gradient delay volume and thus equilibration time were further reduced, for example by configuring the Dual LC system with a high-pressure mixing pump (HPG) for the UHPLC path. Another option to increase throughput and save costs and time is the elimination of the first isocratic step from the gradient table, as the column experiences a sufficiently long isocratic step due to gradient delay. The respective UHPLC chromatograms are depicted in Figure 5, and it can be deduced that the resolution of the critical pair (peak 3 and 4) is improved (R_S=3). With this method the run time could be shortened by another 1.2 min without compromising area %RSDs or S/N (see Figure 6).

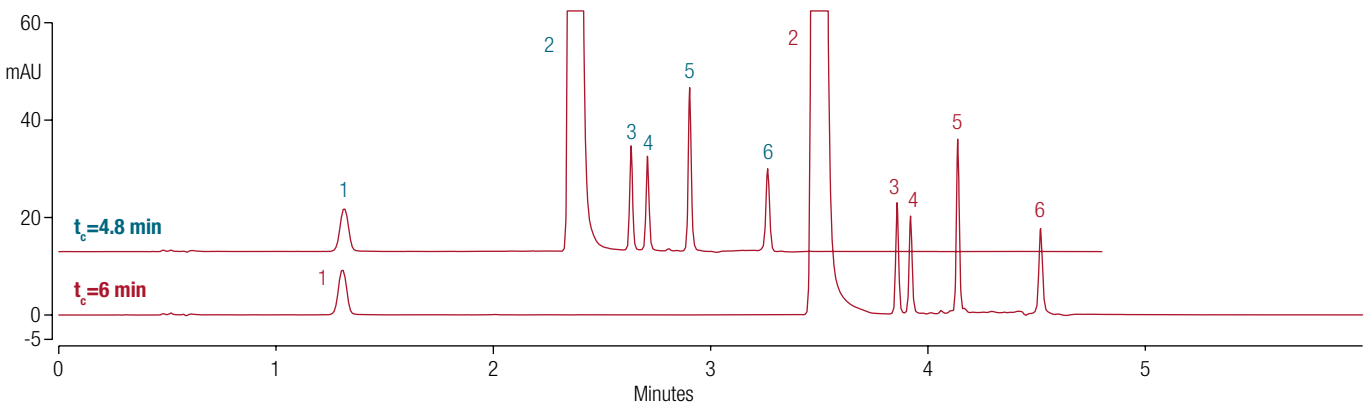

Figure 5. Chromatograms of UHPLC runs with (bottom) and without (top) programmed isocratic start at same time and signal scale. Injection volume was 0.5 µL. For peak assignment see Table 2.

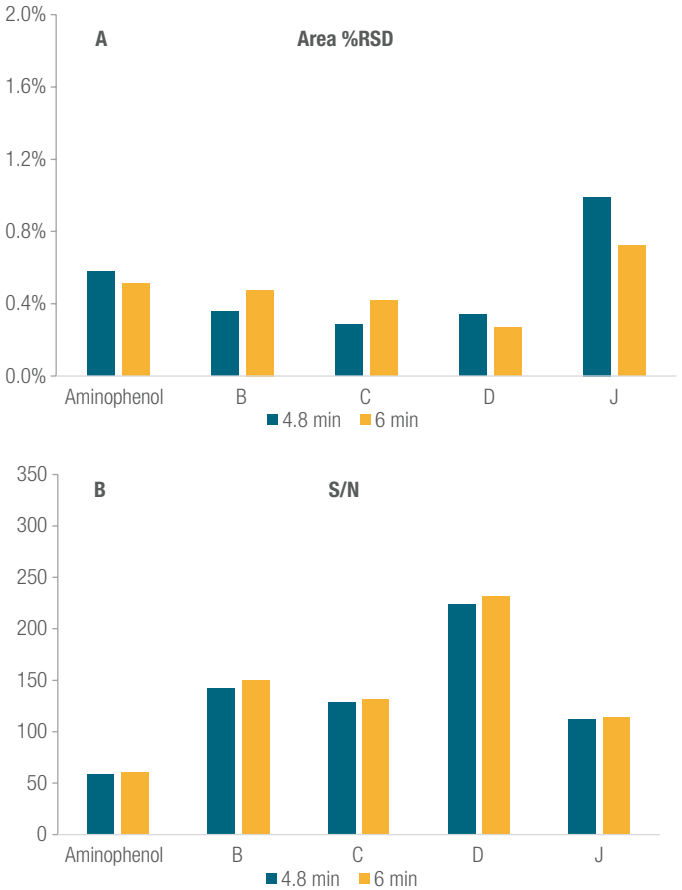

Figure 6. Area precision (A) and signal-to-noise values (B) for UHPLC analysis with 0.5 µL injection volume and 4.8 min cycle time without isocratic step or 6 min cycle time with isocratic step.

Compared to the HPLC analysis, the optimized UHPLC method (without isocratic step, injection volume 0.5 µL) resulted in 50% sample, 80% solvent, and 60% time savings and a 2.5-fold throughput improvement (Figure 7). One hundred samples could be analyzed during an 8 h working day by UHPLC instead of more than 20 h. Assuming costs of $25 per liter of solvent plus 10% for disposal, switching to UHPLC implies cost savings of around $27 per 100 samples or $5400 per year (with an estimation of 20,000 samples per year).

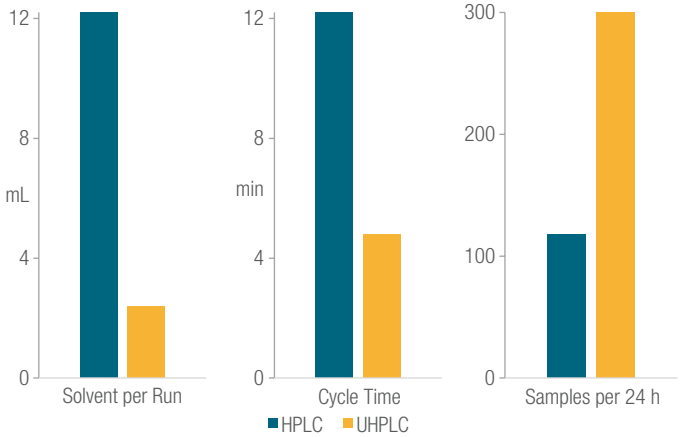

Figure 7. Comparison of the HPLC method and the most-optimized UHPLC method (without an isocratic step and injection volume of 0.5 µL) regarding throughput, solvent, and time expenses.

Conclusion

- The Vanquish Flex Duo system for Dual LC provides the opportunity to have one HPLC and one UHPLC channel in a single system stack, both working independently from each other.
- Speed-up of legacy HPLC methods to fast UHPLC methods can be easily conducted at the same workstation. Both channels can also be used independently for separate analyses.
- In the current study, a 2.5-fold throughput increase and savings of up to 80% mobile phase and 60% cycle time were achieved by speeding up a HPLC method to UHPLC conditions.

References

1. United States Pharmacopeia USP40-NF35 S1, Acetaminophen method, The United States Pharmacopeial Convention, 2017.
2. Franz, H.; Fabel, S.: Thermo Fisher Scientific Technical Note 75: A Universal Tool for Method Transfer From HPLC to UHPLC, 2016, https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/TN-75-HPLC-UHPLC-Universal-Tool-Method-Transfer-TN70828-EN.pdf (accessed December 5, 2017).

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APPLICATION NOTE 72601

Doubling the throughput of long chromatographic methods by using a novel Dual LC workflow

Authors

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Keywords

Vanquish Duo UHPLC system for Dual LC, ezetimibe, simvastatin, pharmaceutical, related impurity analysis

Application benefits

- Dual LC technology enables the simultaneous analysis of two samples, doubling the throughput of a stability-indicating method.
- The Thermo Scientific™ Vanquish™ Flex Duo UHPLC system for Dual LC duplicates the analysis capacity per bench space in the lab.

Goal

The Vanquish Flex Duo system for Dual LC was used for the analysis of a stressed drug mixture of ezetimibe and simvastatin. It enabled the simultaneous analysis of two samples, doubling the throughput of the stability-indicating method.

Introduction

Purity analyses of drugs are routinely run in the pharmaceutical industry for purposes such as batch releases and stability studies. In most cases, reversed phase HPLC is used.

The purity analysis of drug products is frequently performed by isocratic elution. Compared to gradient methods, isocratic elution provides the required selectivity to separate related impurities with high structure similarity. Additionally, isocratic methods have better instrument portability compared to

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Table of contents

gradient methods. For instance, typical method transfer difficulties, such as gradient delay volume discrepancies, do not affect the transfer of isocratic methods. Still, the method must be able to retain and separate components with wide hydrophobicity range, and this results in long run times, particularly when columns packed with 5 µm particles are used. The method total run time is further increased by the column washing steps required to remove possible hydrophobic contaminants. When many samples must be processed, for instance during stability studies, long isocratic methods will decrease the number of samples that can be processed per day, extending the length of studies with obvious cost consequences and blocking of lab resources.

In this work, we introduce a novel Dual LC workflow, which provides a unique concept by using two separated flow paths in one system. The Dual LC workflow enables the simultaneous analysis of two samples by the same instrument, in practice doubling the laboratory throughput within the footprint of one instrument. The Vanquish Flex Duo system for Dual LC consists of a Dual Pump F with two individual pumping units, a Dual Split Autosampler FT with two separate injection valves and sample loops, one—or optionally two—Column Compartments H, and two detectors.

The value of the Dual workflow is here demonstrated for an isocratic stability-indicating method to profile the combined impurities of simvastatin (SMV, Figure 1) and ezetimibe (EZE, Figure 2). SMV and EZE are drugs used to reduce the total cholesterol value and triglycerides in blood. In a combinatorial therapy they are used for the

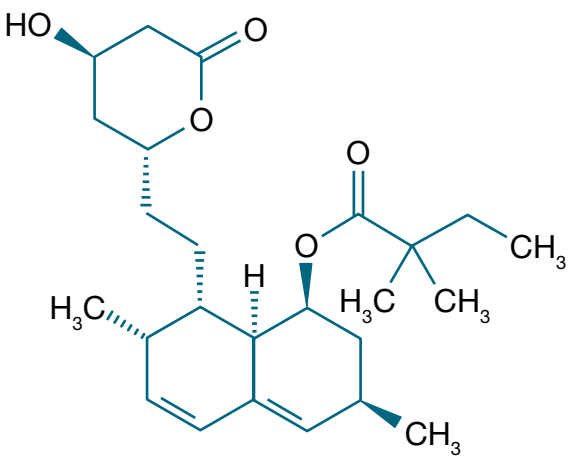

Figure 1. Chemical structure of simvastatin (SMV).

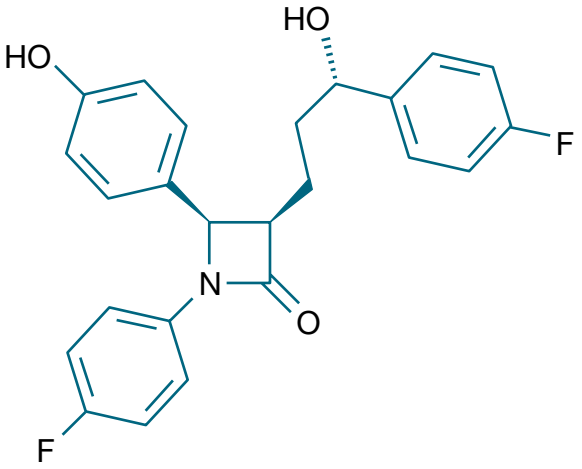

Figure 2. Chemical structure of ezetimibe (EZE).

treatment of hyperlipidemia.[1] EZE and SMV reduce the 'bad' LDL-cholesterol, while increasing the 'good' HDL-cholesterol. The LDL-cholesterol can produce serious issues to the arteria walls by building up plaques. These plaques could cause arterial occlusion, which could finally result in heart attack or stroke.

The column selected for the stability indicating method is a Thermo Scientific™ Hypersil GOLD™ PFP column. The fluorinated hydrocarbon groups provide enhanced selectivity for positional isomers of halogenated compounds, like EZE, and at the same time provide good retention for SMV and non-halogenated impurities.

Experimental

Recommended consumables

- Deionized water, 18.2 MΩ·cm resistivity
- Fisher Scientific™ Optima™ LC-MS grade acetonitrile (P/N 100001334)
- Fisher Scientific Ortho-phosphoric acid, HPLC grade (P/N 10644732)
- Fisher Scientific Sodium dihydrogen phosphate, anhydrous (P/N 12615157)
- Fisher Scientific Sodium hydroxide (P/N 10528240)
- Hypersil GOLD PFP column (250 × 4.6 mm, 5 µm) (P/N 25405-254630)
- Vials (amber, 2 mL) (P/N 15508760)
- Septa (silicone/PTFE) (P/N 11548180)

Sample handling equipment

- Fisher Scientific Ultrasonic bath
- Thermo Scientific™ Orion™ 3 Star pH meter
- Fisher Scientific Magnetic stirrer with heating option
- Fisher Scientific Conical tubes (15 mL) (P/N 11307211)
- Syringe filter, Minisart® cellulose acetate (CA) (Ø 26 mm; 0.45 µm pore size) (purchased from a reputable vendor)

Sample preparation

The respective drugs containing the active ingredients EZE and SMV were bought from a local pharmacy. One tablet of each drug was ground with a mortar and pestle. The powder was weighed out and transferred to a 15 mL conical tube. Then, 10 mL acetonitrile were added and the solution sonicated for 10 min at room temperature. One aliquot of each sample was filtered through a 0.45 µm CA membrane and used as a reference during the analysis.

To generate a wide spectrum of possible impurities of both drug substances, the extracts of EZE and SMV were combined and treated by a hydrolytic degradation described in Reference 1. The mixture was treated by adding 0.1 N NaOH and stirred for 30 min at 60 °C. Afterwards, the solution was filtered through a CA syringe filter with 0.45 µm pore size. This solution was used to detect the impurities.

Instrumentation

Vanquish Flex Duo system for Dual LC equipped with:

- System Base Vanquish Dual (P/N VF-S02-A-02)
- Dual Pump F (P/N VF-P32-A-01)
- Dual Split Sampler FT (P/N VF-A40-A-02)
- Column Compartment H (P/N VH-C10-A-02) with 2 active pre-heaters, (P/N 6732.0110)
- 2 Variable Wavelength Detectors (P/N VH-D40-A) each equipped with a 7 mm semi-micro PEEK™ flow cell, 2.5 µL (P/N 6074.0300)

Separation conditions

Column:	Hypersil GOLD PFP (250 x 4.6 mm, 5 µm) (P/N 25405-254630)
Mobile phase A:	62% 20 mM Sodium phosphate buffer, pH 3.5 / 38% acetonitrile
Mobile phase B:	Acetonitrile

Gradient (isocratic separation with column wash):

Time (min)	*% A*	*% B*
0	100	0
55	100	0
56	20	80
66	20	80
67	100	0
75	100	0

Flow rate:	0.9 mL/min
Column temperature:	40 °C
Active pre-heater temperature:	40 °C
Injection volume:	15 µL
Autosampler temperature:	4 °C
Detector wavelength:	238 nm
Data collection rate:	2 Hz
Response time:	2 s

Data processing

The Thermo Scientific™ Chromeleon™ 7.2.8 Chromatography Data System was used for data acquisition and analysis.

Results and discussion

The Vanquish Flex Duo system for Dual LC provides two separated fluidic pathways in one instrument, as can be seen in Figure 3. Each flow path consists of a sample loop and column, and each is connected to a variable wavelength detector. The same chromatographic method described above was applied to both flow paths.

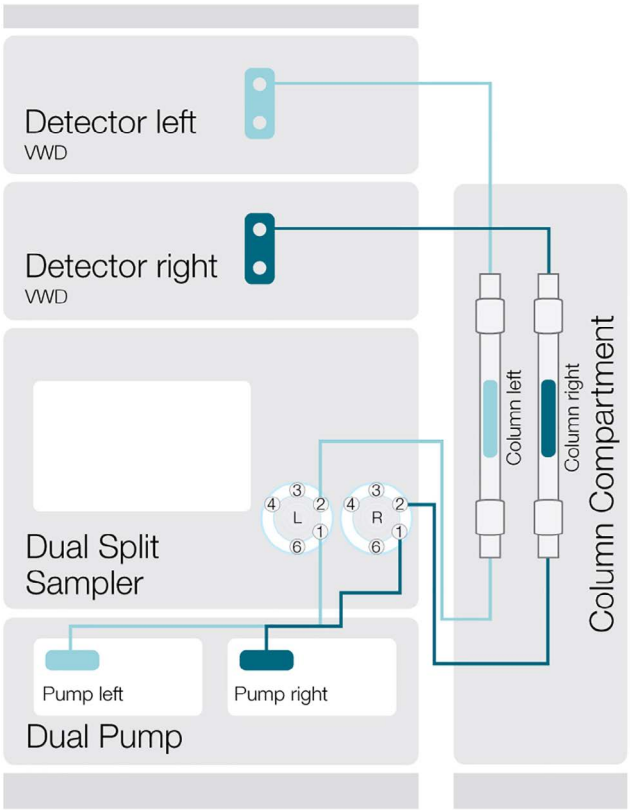

Figure 3. Schematic instrument configuration of the Vanquish Flex Duo system for Dual LC.

First, the reference samples for the EZE and SMV were run simultaneously by the Vanquish Flex Duo system for Dual LC to generate the reference chromatograms. Afterwards, the stressed sample was analyzed using the same method on both flow paths. The stress conditions generated a substantial number of impurities. In Figure 4, the chromatograms of the reference EZE sample, SMV reference sample, and the stressed EZE and SMV drug mixture are overlaid. SMV could not be detected in the stressed sample, indicating a complete degradation of this molecule under the stress conditions.

Figure 5 shows mirrored chromatograms of the stressed drug mixture analyzed with the Vanquish Flex Duo system for Dual LC using both flow paths simultaneously, whereby the same vial was used for injections into both flow paths. The chromatograms' profiles match almost perfectly.

Chromatographic consistency between the two flow paths is demonstrated by calculating the average of relative retention times of each peak for five consecutive injections. EZE is used as the reference peak for the calculation. Table 1 illustrates an almost perfect match of relative retention times obtained in the simultaneous analysis.

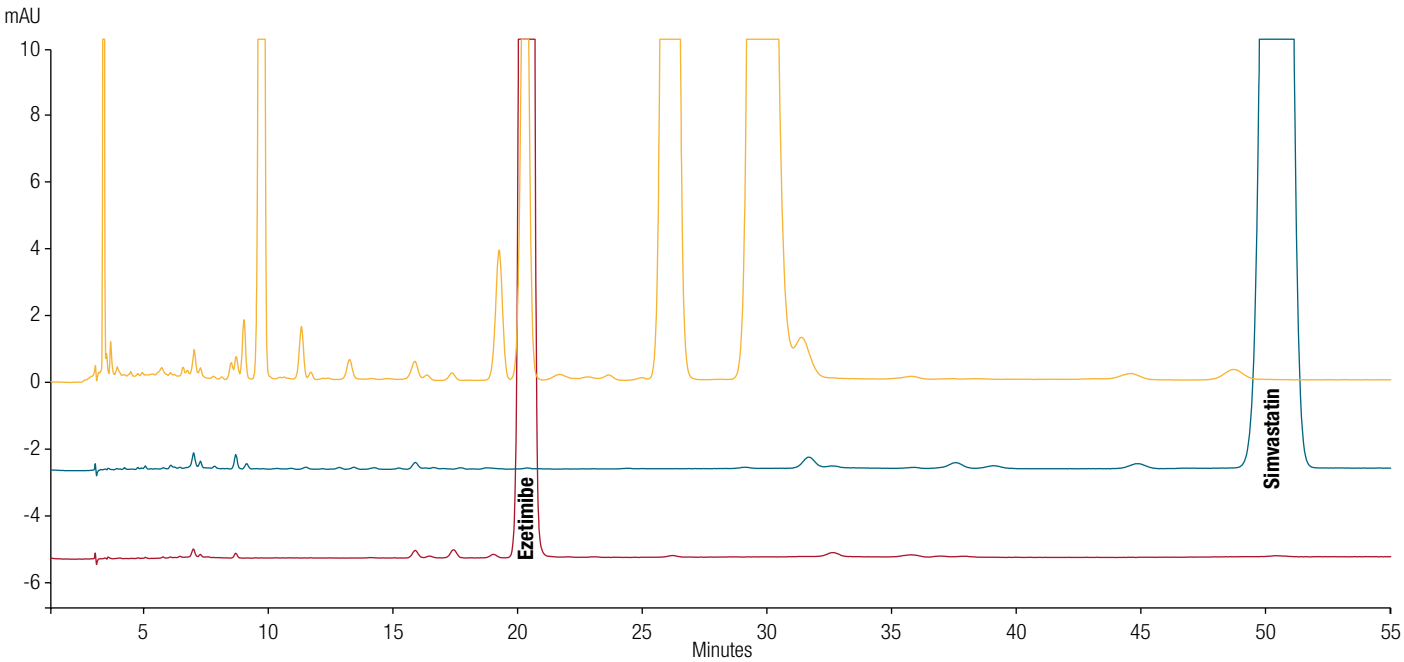

Figure 4. Chromatogram overlays of stressed mixture of EZE and SMV (orange); untreated SMV (blue), and untreated EZE (red).

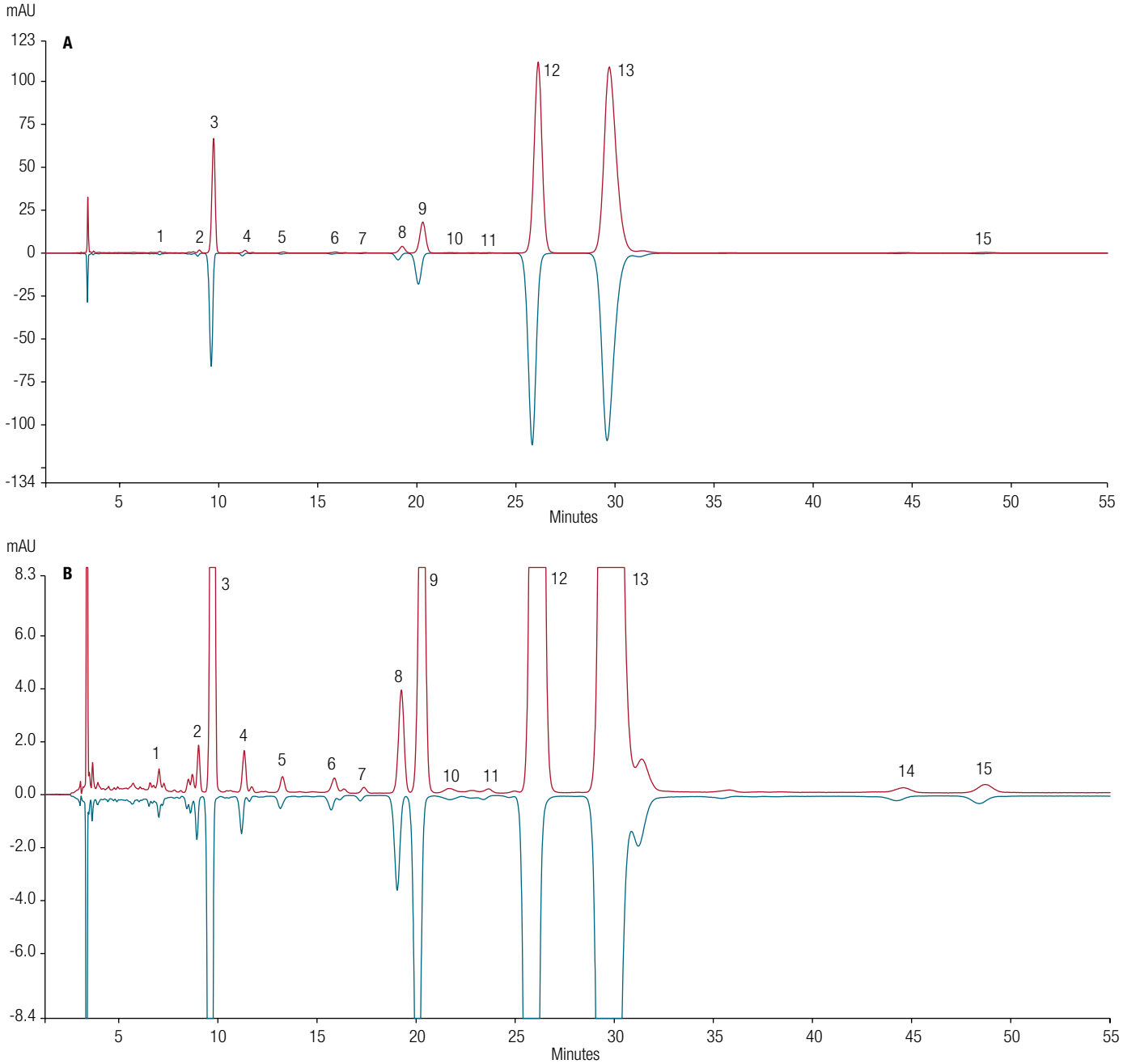

Figure 5. Mirrored chromatograms of the stressed mixture of EZE and SMV tablets (red: left flow path, blue: right flow path); Peak number assigned to all components with relative peak area > 0.05 %. A) un-zoomed view; B) zoomed view to spot related impurity peaks with lower intensity.

Table 1. Average of relative retention time with standard deviation of each peak (n=5 for each flow path). The EZE peak (peak 9) was used as the reference.

Peak	Average Relative Retention Time ± S.D. Left Flow Path	Average Relative Retention Time ± S.D. Right Flow Path
1	0.35 ± 0.001	0.35 ± 0.001
2	0.45 ± 0.002	0.45 ± 0.002
3	0.48 ± 0.002	0.48 ± 0.002
4	0.56 ± 0.002	0.56 ± 0.002
5	0.66 ± 0.002	0.66 ± 0.002
6	0.79 ± 0.003	0.78 ± 0.003
7	0.86 ± 0.005	0.85 ± 0.005
8	0.95 ± 0.005	0.95 ± 0.006
9	1.00 ± 0.006	1.00 ± 0.007
10	1.07 ± 0.009	1.07 ± 0.010
11	1.17 ± 0.008	1.16 ± 0.008
12	1.29 ± 0.008	1.28 ± 0.009
13	1.46 ± 0.012	1.47 ± 0.013
14	2.19 ± 0.015	2.20 ± 0.016
15	2.39 ± 0.020	2.40 ± 0.023

A decrease of the active ingredient EZE peak area can be observed over the five consecutive injections. On the other hand, several impurities show area increase with time (Figure 6). This indicates that the degradation process was still ongoing even when the sample was placed in the autosampler tray at 4 °C. Based on these observations, an assessment of the run-to-run peak area precision was pointless. However, the comparison of the relative peak area of two simultaneous injections is provided to evaluate the consistency of quantitative results delivered by the two channels. The data are visible in Table 2, and indicate a good agreement between the relative peak areas.

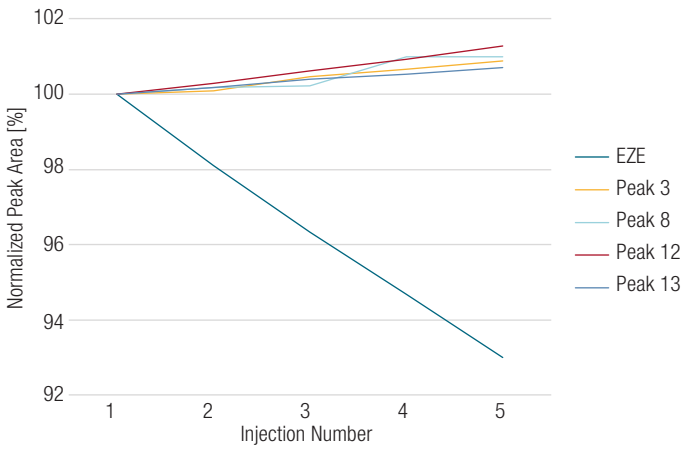

Figure 6. Normalized peak area plot over five injections, where the areas of injection 1 are normalized to 100%.

Table 2. Relative peak areas of the peaks shown in Figure 7 of the first and last injection in each flow path.

Peak	Relative Area [%] Left Flow Path	Relative Area [%] Right Flow Path	Relative Area [%] Left Flow Path	Relative Area [%] Right Flow Path
	First Injection		Last Injection	
3	6.64	6.56	6.67	6.55
8	0.67	0.67	0.67	0.67
12	27.08	26.95	27.34	26.97
13	40.67	40.67	40.83	40.45

Conclusion

- The Dual LC capabilities increased the number of analyses run on one instrument from 19 to 38 per day for this method with 75 minutes total run time.
- The Vanquish Flex Duo system for Dual LC duplicates the analysis capacity per bench space in the lab.
- Chromatographic results of both flow paths of the Vanquish Flex Duo system for Dual LC exhibit very good consistency both in relative retention time and relative peak area.

Reference

1. Dixit, R.P. et al., Stability Indicating RP-HPLC Method for Simultaneous Determination of Simvastatin and Ezetimibe from Tablet Dosage Form, *Indian J Pharm Sci,* **2010**, *72* (2), 204–210

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APPLICATION NOTE 72709

Fast methods for the determination of ibuprofen in drug products

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Keywords

Pharmaceutical, drug, USP 40 monograph, ibuprofen, valerophenone, Chromeleon CDS, DII for Empower software

Goal

To demonstrate straightforward approaches to improve the throughput compared with the USP 40 method by using a 50 × 2.1 mm column, with a 2.6 µm particle size, operated in a UHPLC instrument (Thermo Scientific™ Vanquish™ Horizon UHPLC system), while maintaining USP acceptance criteria on relative retention time, resolution, and tailing factors.

Application benefits

- Thermo Scientific™ Vanquish™ UHPLC instruments can be controlled by Waters™ Empower™ 3 software
- Rapid chromatographic method determining ibuprofen in drug products
- Fast method with 94% reduced analysis time, along with 97% solvent reduction and 69% cost per sample savings compared to the USP method

Introduction

In the pharmaceutical industry, United States Pharmacopeia (USP) guidelines and methods are used to standardize analytical processes and give the capability to compare results between laboratories. Those methods are often developed based on columns packed with 5 µm particles. The run times are typically long compared with modern UHPLC standards. USP General Chapter <621> describes permitted modifications in terms of mobile phase composition and pH, column length, inner diameter and particle size, as well as flow rate settings;[1] however, the possibilities of increasing the method throughput by adhering to the permitted modifications remain limited. An example is the method for ibuprofen.[2] Much faster methods can be used that fulfill the quality requirements set by the USP methods.[3–5] However, the conditions were changed beyond those permitted by the USP monograph, and the methods would need full qualification prior to implementation.

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Vanquish Horizon UHPLC

Table of contents

This application explores several options to improve existing USP methods[2] for the determination of ibuprofen in a reference standard and a tablet by using modern instrumentation, in combination with smaller column dimensions and smaller particle size. Furthermore, the influence of the organic content in the mobile phase composition will be discussed. For these studies a Vanquish Horizon UHPLC system was used. The Vanquish Horizon system was controlled by the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) version 7.2, or by Waters Empower 3 software.

Experimental

Chemicals	Part number
Deionized water, 18.2 MΩ·cm resistivity or higher	N/A
Fisher Scientific™ Optima™ Acetonitrile LC/MS grade	10001334
Fisher Scientific™ Optima™ Methanol LC/MS grade	10767665
Fisher Scientific™ Chloroacetic acid (99+%)	10216660
Fisher Scientific™ Ammonium hydroxide solution, for LC/MS, ≥25% in H_2O	15655540
Ortho-phosphoric acid, HPLC grade	10644732
Ibuprofen (purchased from a reputable vendor)	
Valerophenone (purchased from a reputable vendor)	
Equipment	**Part number**
Fisherbrand™ Isotemp™ Stirring Hotplate, Fisher Scientific	15353518
Fisherbrand™ Mini Centrifuge	10243043
Vials (amber, 2 mL), Fisher Scientific	11545884
Snap Cap with Septum (Silicone/PTFE), Fisher Scientific	10547445

Preparation of standards

Three stock solutions with 50 and 60 mg/mL of ibuprofen and 7 mg/mL of valerophenone were prepared in acetonitrile. The 50 mg/mL ibuprofen solution was used for spiking the recovery sample. Mixed working solutions and individual working standards were prepared in water at a concentration of 250 µg/mL each, and in 60% acetonitrile + 0.4% chloroacetic acid at a concentration of 12 mg/mL for ibuprofen and 0.35 mg/mL for valerophenone by diluting the 60 mg/mL ibuprofen and 7 mg/mL valerophenone stock solutions to the appropriate volume.

Five calibration standards of ibuprofen were prepared by diluting the 60 mg/mL stock solution with 60% acetonitrile + 0.4% chloroacetic acid to obtain concentrations of 1, 2.5, 5, 7.5, and 10 mg/mL.

Preparation of samples

The drug containing 400 mg ibuprofen per tablet was purchased in a local pharmacy. A placebo tablet (used for the determination of recovery) was provided by a local pharmacy for the study.

One ibuprofen tablet was weighed and ground with a mortar and pestle. The powder was transferred to a 100 mL volumetric flask and filled up to approximately 50% with solvent (60% acetonitrile + 0.4% chloroacetic acid) and subsequently stirred for 1 h. Afterwards it was filled up to volume with solvent (60% acetonitrile + 0.4% chloroacetic acid) and an aliquot centrifuged for 10 min. The supernatant was transferred into a HPLC vial for injection.

The placebo tablet was ground with a mortar and pestle. The powder was transferred to a 10 mL volumetric flask and 1 mL of 50 mg/mL ibuprofen solution added. Following the procedure of the ibuprofen tablet, it was filled up to approximately 50% with solvent (60% acetonitrile + 0.4% chloroacetic acid) and stirred for 1 h before filling up to volume. Subsequently, an aliquot was centrifuged for 10 min and the supernatant was transferred into a HPLC vial for injection.

Instrumentation	Part number
Vanquish Horizon UHPLC system consisting of:	
System Base Vanquish Horizon	VH-S01-A-02
Binary Pump H	VH-P10-A-01
Sampler HT	VH-A10-A-02
Column Compartment H	VH-C10-A-02
Diode Array Detector with Lightpipe™ Standard flow cell, 10 mm	VH-D10-A-01 6083.0100

Column and instrument settings used in the mobile phase screening and the USP compliant methods are shown in Tables 1 and 2, respectively.

Data processing and software

Chromeleon CDS version 7.2 SR5 was used for data acquisition and processing for the isocratic mobile phase screening.

For data acquisition and processing of method 1 and 2, Thermo Scientific™ Dionex™ Instrument Integration (DII) 1.15 for Empower software and Waters Empower 3 software (Build 3471) were used.

Table 1. Column and instrument settings used in the mobile phase screening

Column	Thermo Scientific™ Accucore™ XL C18, 100 × 3 mm, 4 µm (P/N 74104-103030)
Mobile phase	A: Water + 0.1% H_3PO_4 B: Acetonitrile + 0.1% H_3PO_4
Flow rate	1.125 mL/min
Isocratic mobile phase condition	Variable, 25% B to 50% B
Isocratic run time	Variable, 2–36 min
Mixer volume	10 + 25 µL
Column temperature	30 °C (forced air mode, fan speed 5)
Autosampler temperature	10 °C
UV wavelength	215 nm
UV data collection rate	20 Hz
UV response time	0.2 s
Injection volume	5 µL
Needle wash	10:90 water/methanol (v/v)

Table 2. Columns and instrument settings for USP compliant method (method 1) and fast method (method 2)

	Method 1	Method 2
Column	Thermo Scientific™ Acclaim™ C18, 250 × 4.6 mm, 5 µm (P/N 59149)	Thermo Scientific™ Accucore™ C18, 50 × 2.1 mm, 2.6 µm (P/N 17126-052130)
Mobile phase	40:60 water/ACN (v/v) + 0.4% chloroacetic acid, pH 3.0 ± 0.2	
Flow rate	2 mL/min	1.1 mL/min
Isocratic run time	8 min	0.5 min
Mixer volume	350 + 50 µL	
Column temperature	30 °C (forced air mode, fan speed 5)	
Autosampler temperature	10 °C	
UV wavelength	254 nm	
UV data collection rate	10 Hz	50 Hz
UV response time	0.5 s	0.1 s
Injection volume	10 µL	1 µL
Needle wash	10:90 water/methanol (v/v)	

Results and discussion

Several methods are described in the USP 40 monograph for the identification and quantification of ibuprofen and ibuprofen-related impurities either in reference standards, tablets, or oral suspensions.

1) Mobile phase screening in isocratic mode by varying the organic content

Within the USP 40 monograph for ibuprofen, under the section Chromatographic Purity, an L1 column with dimensions of 150 × 4 mm and 5 µm particles is reported. A complete list of columns belonging into the L1 category can be found in the USP40-NF35 S2 section under chromatographic columns and packings.[6] The method runs under isocratic conditions with a mobile phase composition of 66% water, adjusted to pH 2.5 with phosphoric acid, and 34% of acetonitrile at a flow rate of 2 mL/min. To study the effect of the organic content in the mobile phase with respect to selectivity, single standards and a mixture of ibuprofen and valerophenone at a concentration of 250 µg/mL of each were injected into the mobile phase. From run to run, the acetonitrile content was changed in steps of 5% starting at 25% acetonitrile and going up to 50%. As can be seen in Figure 1, valerophenone eluted before ibuprofen when mobile phases with acetonitrile content of 35% or lower were used. At 35% acetonitrile, the resolution was 2.56, which is above, albeit close to, the requirements of the USP method (Rs > 2). With 40% acetonitrile, the selectivity changed and ibuprofen eluted before valerophenone with a resolution of 1.29, hence below the acceptance criteria. When the acetonitrile content was further increased to 50%, the resolution increased again to 4.87. At the same time, the run-time strongly decreased at higher organic content, from over 35 minutes at 25% organic to less than 2 minutes at 50%. Based on these results, it can be shown that varying the organic content in the mobile phase significantly reduces analysis time. The obtained backpressure measured at 50% acetonitrile was 230 bar, which would allow the use of conventional HPLC instruments with a pressure specification limited to 400 bar.

2) Use of a fast method with reduced column dimensions and particle size

The USP 40 assay method for ibuprofen reports a column with dimensions of 250 × 4.6 mm and 5 µm particles, running with a mobile phase composition of 40% water and 60% acetonitrile containing 0.4% of chloroacetic acid, adjusted with ammonium hydroxide

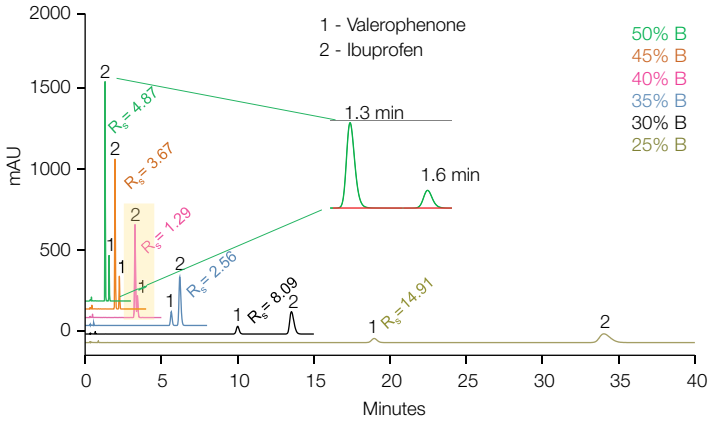

Figure 1. Overlaid chromatograms (1) valerophenone and (2) ibuprofen, obtained by the method shown in Table 1

to pH 3.0 (Table 2, method 1). Under these conditions, ibuprofen elutes before valerophenone. Figure 2 shows the comparison between the USP method and one developed with a 50 × 2.1 mm column packed with 2.6 µm particles (Table 2, method 2). The Plate Height (H) of the column packed with the 2.6 µm solid core particles is expected to be substantially lower than the one with the 5 µm particles. Consequently, a shorter column will be capable of delivering the required efficiency to resolve the ibuprofen and valerophenone peaks, with shorter run times. Moreover, the 2.6 µm solid core particles allow the use of a higher linear flow rate compared to the fully porous 5 µm particles without compromising in efficiency, thereby enabling even faster methods to be developed. The ibuprofen peak in the USP method (method 1) eluted at RT 5.69 min and the valerophenone peak at 7.40 min. By using a 50 mm column with an inner diameter of 2.1 mm packed with 2.6 µm particles, the retention times could be reduced to 0.26 min and 0.32 min, respectively. Even though the linear velocity in the short column was much higher than the one used for the original method, the combination of shorter run time and smaller diameter, resulted in a savings of organic solvent of 97%. The original method required more than 16 mL mobile phase per run, versus the 0.55 mL required for the fast method, as can be seen in Figure 3. One reference[5] describes more in depth the separation speed up of valerophenone and ibuprofene by several particle size and column length combinations, resulting in backpressures up to 1320 bar, while achieving the USP requirements of resolution and tailing factors. They concluded that for this application 50 mm columns packed with 2.6 µm particles delivered the fastest method.

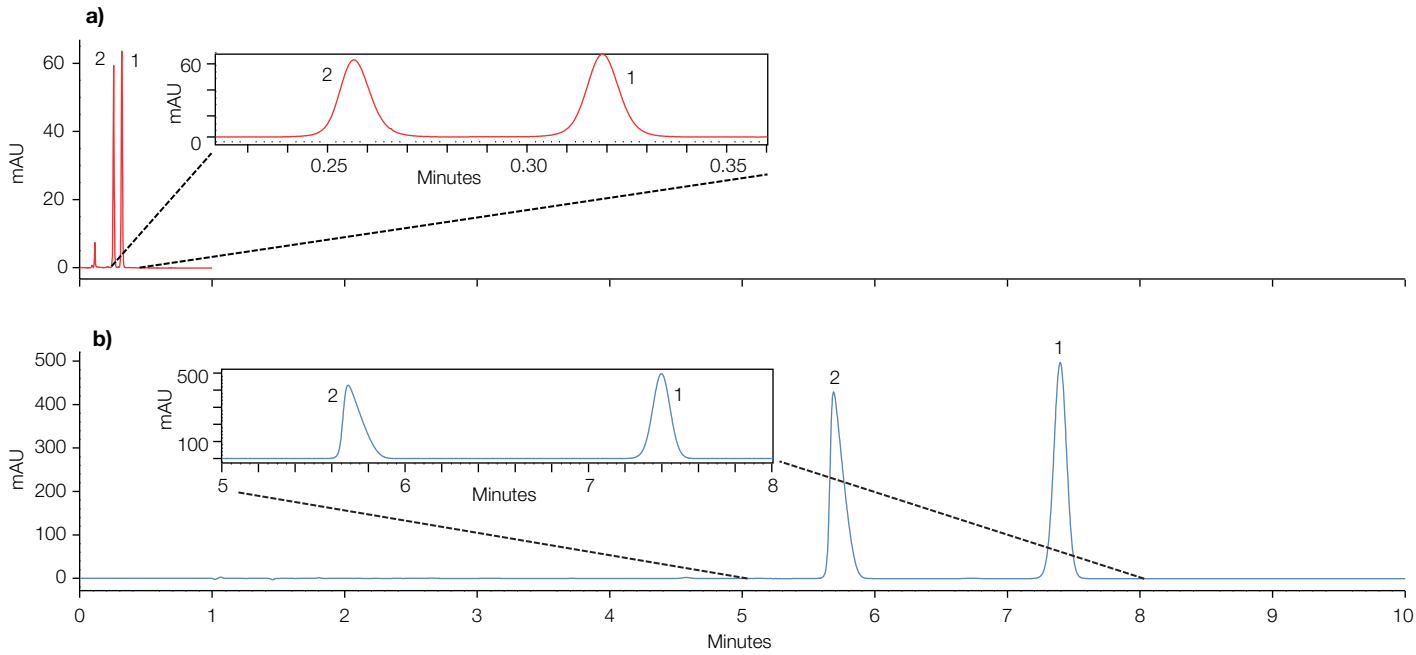

Figure 2. a) Chromatogram of the fast method (method 2) with zoomed view on (2) valerophenone and (1) ibuprofen; b) chromatogram of USP method (method 1) with zoomed view on (2) valerophenone and (1) ibuprofen

Reducing the particle size from 5 µm to 2.6 µm does not entail a large increase in pressure if the column length is reduced from 250 mm to 50 mm. The backpressure of the fast method (method 2) increased just to 330 bar compared to 305 bar for the USP method (method 1).

The acceptance criteria described in the USP 40 monograph regarding relative retention times (RRT) and resolution (Rs) of ibuprofen and valerophenone is 1.4 for valerophenone and a minimum resolution of 2.5. As can be seen in Table 3, a RRT of 1.3 for valerophenone was observed when the USP method was applied, and a RRT of 1.2 with the fast method indicating excellent chromatographic consistency. The resolution decreased in the fast method to 4.2 compared to 9.1 in the USP method. This is more than adequate to meet the requirements of the USP monograph. Additionally, Figure 3 shows that the analysis time could be reduced by a factor of 16 and the associated acetonitrile consumption is clearly decreased, which also contributes significantly to the reduction of cost per sample (considering prices of organic solvent and columns, assuming 1000 injections per column).

Table 3. Comparison of RRT and Rs between the original USP method (method 1) and the fast method (method 2)

Analyte	RRT		R_s	
	USP method	Fast method	USP method	Fast method
Ibuprofen	1.0	1.0	—	—
Valerophenone	1.3	1.2	9.1	4.2

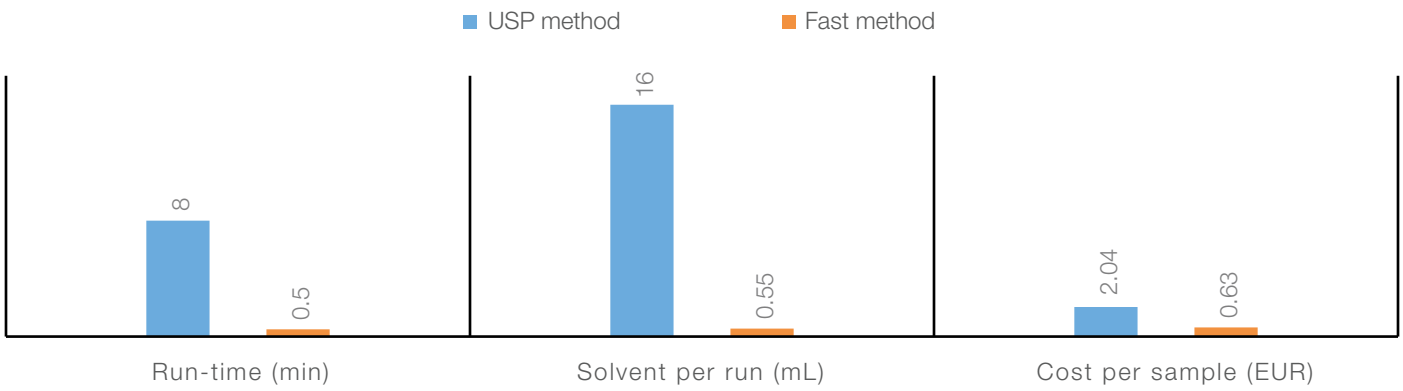

Figure 3. Comparison between USP method and fast method with respect to run time, organic solvent consumption, and cost per sample

3) Quantitation of ibuprofen in tablets

Quantitation was performed using the methods in Table 2. Both calibration standards and recovery samples were injected once, while the tablet sample was injected three times to obtain method-reproducibility data.

The USP monograph reports acceptance criteria with a tailing factor < 2.5 and the relative standard deviation (% RSD) of the area not more than 2.0%. The tailing factor was determined to be 1.2 for the USP method (method 1) and 1.1 for the fast method (method 2).

As can be seen in Table 4, the % RSD Area is slightly higher (0.25%) for the fast method compared with the USP method (0.04%) but is still below the given acceptance criterion of a maximum of 2.0%. For both the USP method and the fast method, the % RSD of retention time (RT) is excellent.

Both methods were run using the combination of Empower 3 software and DII 1.15 for Empower software. Figure 4 shows an overview of the data analysis part with the sample set and instrument controller on the left side and the data processing part on the right side within the software. The sample set contains the calibration standards, recovery sample, and ibuprofen tablet sample. The calibration curve and calculation of sample amounts were done within the software by using an appropriate processing method.

Table 4. Comparison of % RSD RT and Area of ibuprofen peak between USP method and fast method for three consecutive injections of sample

USP method		Fast method	
% RSD		% RSD	
RT	Area	RT	Area
0.01	0.04	0.02	0.25

Calibration standards of ibuprofen were prepared in the concentration range of 1 mg/mL to 10 mg/L. Linearity was found to be 0.9999 for the USP method and 0.9986 for the fast method.

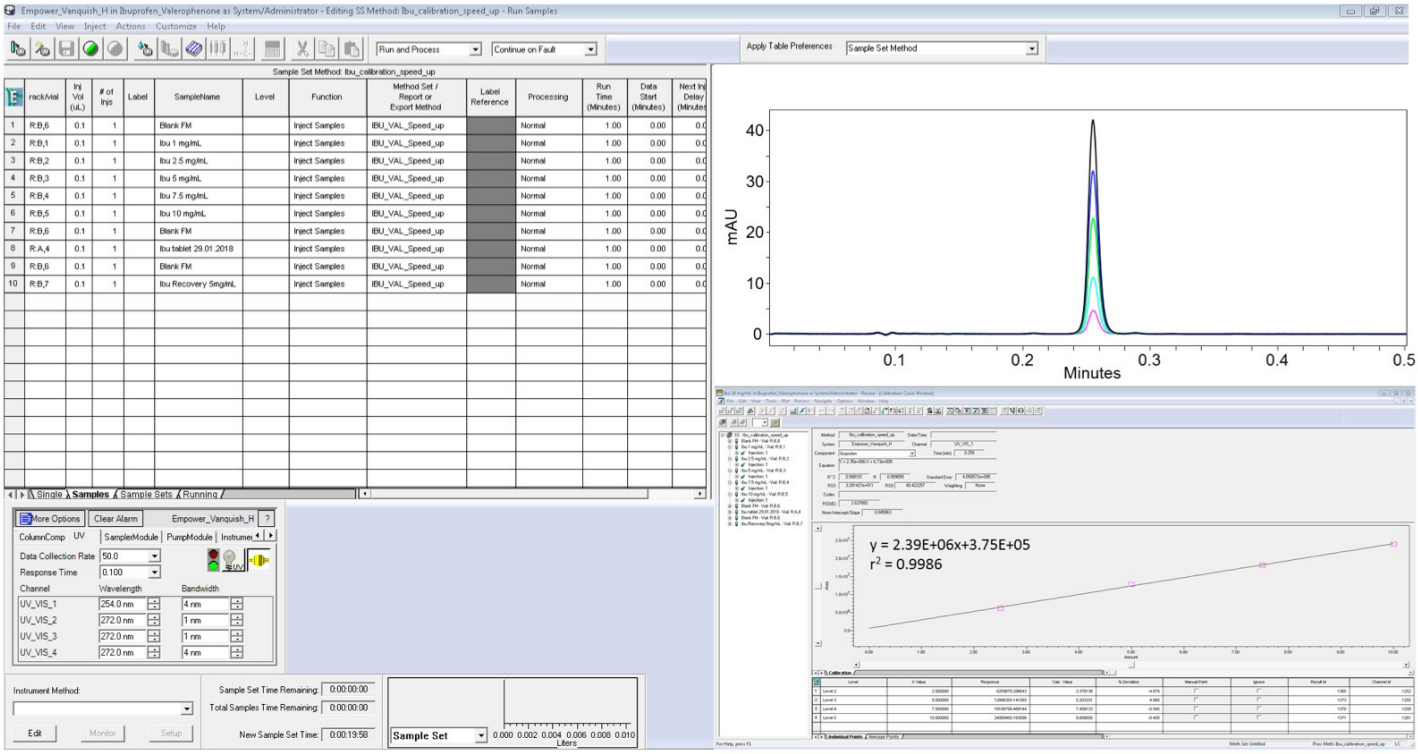

Figure 4. Insight to the Empower 3 software with an example sample set and the instrument controlling status on the left (section run samples) and an example of overlaid chromatograms and a calibration curve on the right (section data review)

The quantitative results are summarized in Table 5. Good recovery could be achieved with 103% for ibuprofen with the USP method and 108% for the fast method, respectively. The content of ibuprofen in the tablet was calculated to be 397 mg/tablet in the USP method and 391 mg/mL with method 2, already corrected by recovery rate. This corresponds to an ibuprofen content of 99% and 98%, respectively. Both methods therefore meet easily the USP requirements of 90–110% labeled amount of ibuprofen in the tablet.

Table 5. Quantitative results of ibuprofen in tablet with each method. The measured amount was corrected by the recovery rate.

	Stated amount on label [mg/tablet]	Measured amount [mg/tablet]	Recovery [%]	% Amount ibuprofen in tablet
USP method	400	397	103	99
Fast method	400	391	108	98

The fast method shows comparable results with the USP method, demonstrating clear advantages in analysis throughput and solvent consumption. Instrumentation and software from multiple vendors are commonly employed within pharmaceutical laboratories, and this workflow improvement enables greater flexibility.

Conclusion

- The analysis of ibuprofen in a tablet with the fast method and the USP method provides comparable results in terms of RRT, resolution, and tailing factors.
- The measured amount of ibuprofen in the tablet with the fast method was 98%, which is in line with the USP requirements of 90–110%.
- Analysis time could be reduced by 94%, along with solvent reduction of 97% and a 69% cost per sample savings.
- The Vanquish UHPLC systems can be controlled by the combination of DII for Empower software and Empower 3 software.

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6. United States Pharmacopeia, chromatographic columns and packings USP40-NF35 Section S2

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APPLICATION NOTE 21686

Simplified, high-throughput separation of glucocorticoids

Author
Derek Hillbeck, Thermo Fisher Scientific, Runcorn, UK

Keywords
Pharmaceutical, Drug development, QA/QC, USP, Modernization, USP-NF Chapter <621>, Small molecule, Glucocorticoids, Prednisone, Cortisone, Prednisolone, Hydrocortisone, Corticosterone, Betamethasone, Dexamethasone, Solid core, Accucore C30, Vanquish Flex, UHPLC, USP monograph modernization

Application benefits

- Improved separation of closely related glucocorticoids with reduced method complexity
- High-throughput analysis possible through a reduced complexity, rapid, two-minute isocratic method
- Associated reduction in cost per sample through reduced mobile phase consumption and waste generation

Goal

To demonstrate how the use of alternate UHPLC stationary phase selectivities can simplify the separation of structurally similar analytes and facilitate method speed up

Introduction

Glucocorticoids are a group of hormones, both naturally occurring and synthetic. Structurally similar (Figure 1), they can be challenging to separate.

Previous application notes[1] have shown that a rapid separation, in under 4 minutes, can be achieved using a ternary mobile phase and a C18 column chemistry. This application note extends that work to a solid core C30 column.

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Vanquish Flex UHPLC

Table of contents

One of the key goals for the chromatographer is to achieve a consistent, reproducible separation. The selection of a highly reproducible HPLC column is essential if this goal is to be attained. Based on solid core technology, Accucore HPLC columns allow users of conventional HPLC methods to enjoy performance beyond that of columns packed with 5 µm or even 3 µm fully porous particles. High separation efficiencies provide increased peak resolution. An ultra-stable packed bed results in exceptionally robust columns that demonstrate excellent retention and response reproducibility. Accucore columns are available in a wide range of chemistries and particle sizes making them an ideal choice for this type of work.[2]

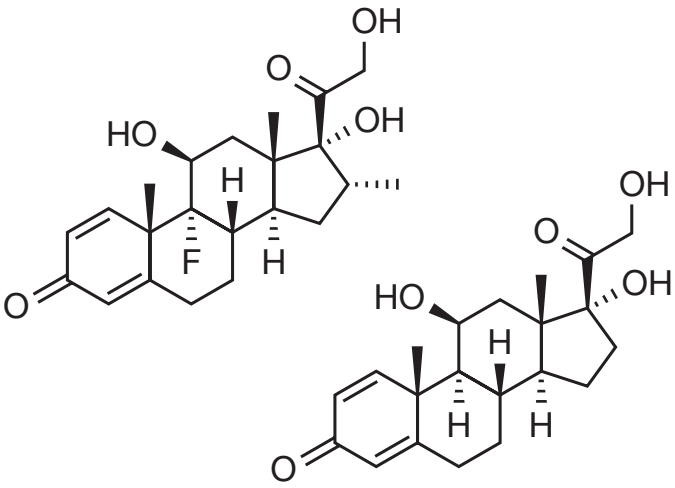

Figure 1. Structure of dexamethasone and prednisone.

The Vanquish Flex Quaternary UHPLC system has the benefit of SmartInject technology, and improvements in injection system hardware synchronization. This results in excellent retention time precision providing the user with greater data confidence during method development. The Vanquish Flex Quaternary system also utilizes Thermo Scientific™ LightPipe™ flow cell technology designed for the diode array detector (DAD), which provides the user with superior sensitivity and low peak dispersion due to small internal volume.

Experimental

Consumables and apparatus

- Accucore C30, 150 × 2.1 mm, 2.6 µm column (P/N 27826-152130)
- LC-MS grade 18 MΩ water from Thermo Scientific™ Smart2Pure™ system (P/N 50129845)
- Fisher Scientific™ LC-MS grade methanol (P/N A456-212)
- Fisher Scientific LC-MS grade tetrahydrofuran (P/N 268290025)
- Thermo Scientific™ Virtuoso™ 9 mm wide opening, 2 mL screw thread vial and cap kit (P/N 60180-VT400)

Standards

The compounds used were representative of this class and were purchased from a reputable supplier: prednisone (1), cortisone (2), prednisolone (3), hydrocortisone (4), corticosterone (5), betamethasone (6), and dexamethasone (7). The number relates to their elution order and peak labelling in the subsequent chromatograms.

Instrumentation

Analyses were performed using a Vanquish Flex Quaternary UHPLC System consisting of:

- Quaternary Pump F (P/N VF-P20-A)
- System Base Vanquish Flex (P/N VF-S01-A)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Active Pre-heater (P/N 6732.0110)
- Diode Array Detector HL (P/N VH-D10-A)
- LightPipe Flow Cell, 10 mm (P/N 6083.0100)
- Thermo Scientific Virtuoso Vial Identification System (P/N 60180-VT-100)

Software

Thermo Scientific™ Chromeleon™ 7.2 SR4

Sample preparation

Solutions of the compounds were prepared by dissolving a known amount in water/acetonitrile (20:80, v/v) to produce 1 mg/mL primary solutions. A mixed working standard solution and individual working standards were used to assess method development and were prepared in water/acetonitrile (80:20, v/v) at a concentration of 0.1 mg/mL.

Sample handling

Vial labeling was supported by the Virtuoso Vial Identification System.

UHPLC conditions (final method)

UHPLC column:	Accucore C30, 2.6 µm, 150 mm × 2.1 mm
Mobile phase A:	Water
Mobile phase B:	Methanol
Mobile phase C:	Tetrahydrofuran
On-pump mixing:	73% A/8% B/19% C
Flow rate:	0.6 mL/min
Column temperature:	60 °C, still air with eluent pre heating
Injection volume:	1 µL
Mixer:	50 µL capillary + 350 µL static in combination
UV detection:	240 nm

Note that prolonged use of significant levels of THF as a mobile phase component may require replacement of UHMW polyethylene piston seals with those more tolerant of this solvent, please refer to pump technical manual for further guidance.

Results and discussion

Using the method outlined in the previous application note[1], the C30 column was configured and the mixed standard analyzed with a flow rate of 0.4 mL/min and a column temperature of 50 °C (Figure 2a). This resulted in all the standards eluting within four minutes and presented a good starting point for further development. This experiment was also repeated with a column oven temperature of 60 °C (Figure 2b).

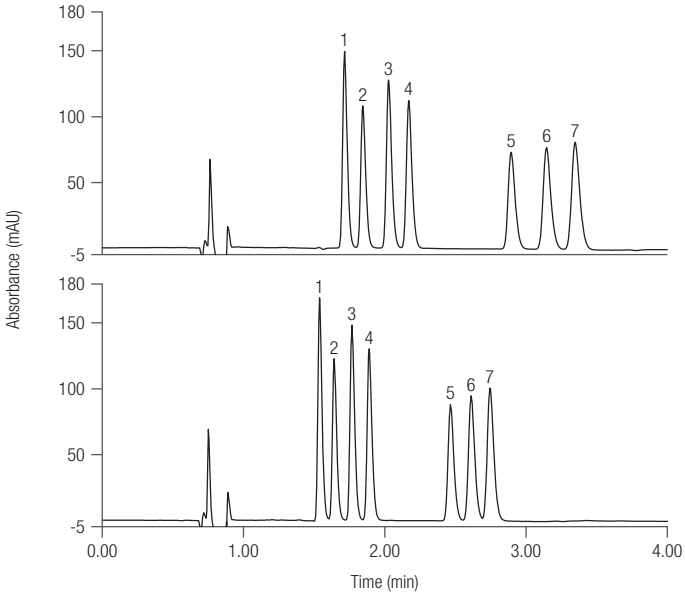

Figure 2. Chromatogram showing mixed standards analyzed at two different column temperatures a) 50 °C and b) 60 °C. (1) prednisone, (2) cortisone, (3) prednisolone, (4) hydrocortisone, (5) corticosterone, (6) betamethasone and (7) dexamethasone.

The elevated temperature shows the expected reduction in retention time and peak width. There were no overt selectivity changes; however, the peak resolution dropped below the usual USP guidance of greater than 2 but was still greater than the 1.5 value usually considered as baseline resolution, and well within the consistent integration capabilities of the Chromeleon software (Figure 3).

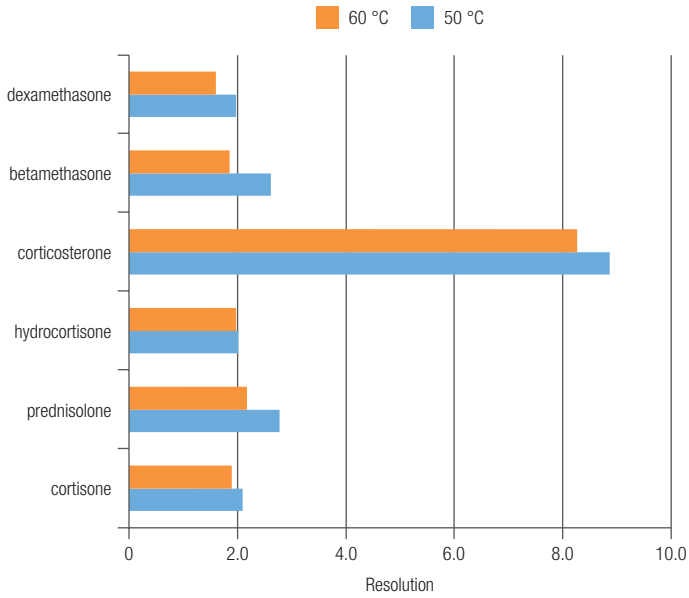

Figure 3. Comparison of resolution values at two column temperatures. Compounds are listed in reverse elution order.

Solid core HPLC columns are capable of equivalent efficiencies to much smaller fully porous particles. This allows high efficiency separations at a fraction of the cost in pressure. This, coupled with the solid-core columns ability to run at optimum (highest) efficiency over a much larger linear velocity range[1], enables chromatographers to significantly speed up their methods.

Maintaining the column temperature at 60 °C, the effect of flow rate was investigated by injecting the standards mixture at flow rates from 0.4 to 0.7 mL/min.

Figure 4 shows the efficiency of the separation normalized to that achieved with a 0.4 mL/min flow rate. There is a gradual reduction in efficiency as the flow rate departs from the optimal flow, but even at 0.6 mL/min it is still within 80% of the lower flow efficiency. Table 1 shows the effect of flow rate on resolution.

A flow rate of 0.6 mL/min allows the separation to maintain a minimum resolution value, between the last two peaks, of 1.5 with the column temperature at 60 °C. Twenty-four replicate injections were made under these conditions (Figure 5 and Table 2).

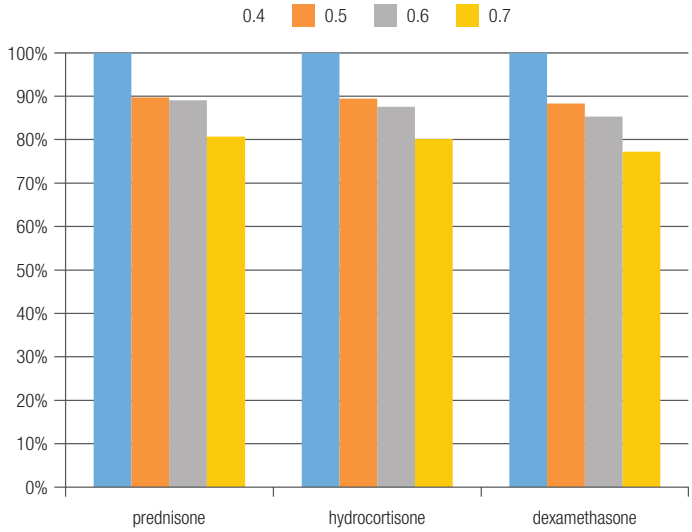

Figure 4. Relative efficiencies for three compounds at four different flow rates normalized to their values at 0.4 mL/min.

Table 1. Resolution values between peaks at different flow rates using USP criteria.

Flow rate (mL/min)	Cortisone	Prednisolone	Hydro-cortisone	Corti-costerone	Beta-methasone	Dexa-methasone
0.4	1.9	2.2	2.0	8.3	1.9	1.6
0.5	1.8	2.1	1.9	7.8	1.7	1.5
0.6	1.8	2.0	1.9	7.8	1.6	1.5
0.7	1.7	1.9	1.8	7.3	1.5	1.4

These results show that the method is very stable with differences between maximum and minimum retention times of less than 0.5 seconds across the replicate injections. This is much better than the 2% RSD criteria usually associated with legacy USP-type methods. The separation of all seven components is achieved within 2 minutes.

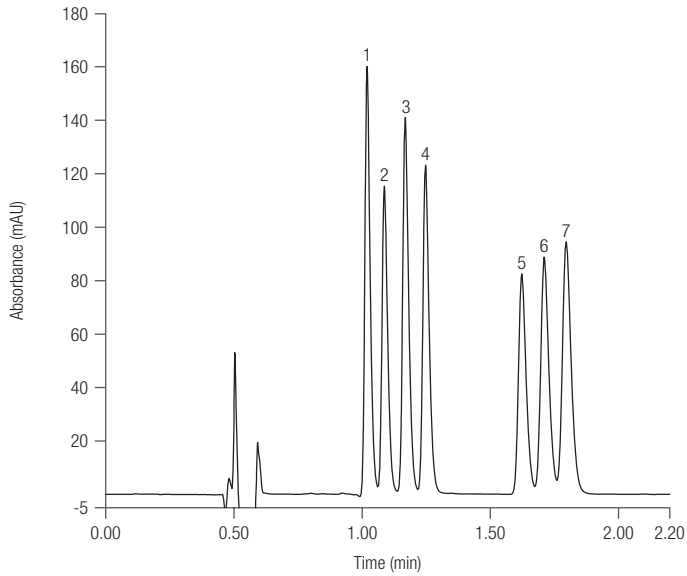

Figure 5. Overlay of 24 replicate injections of standards mixture at 60 °C and a flow rate of 0.6 mL/min.

Table 2. Peak summary data for 24 replicate injections of standards mixture at 60 °C and a flow rate of 0.6 mL/min.

	Parameter	Pred-nisone	Cort-isone	Pred-nisolone	Hydro-cortisone	Corti-costerone	Beta-methasone	Dexa-methasone
Minutes	Maximum RT	1.020	1.087	1.170	1.249	1.626	1.714	1.800
	Average RT	1.018	1.085	1.167	1.247	1.623	1.711	1.797
	Minimum RT	1.016	1.083	1.164	1.243	1.619	1.706	1.792
	Standard Deviation	0.001	0.001	0.002	0.002	0.002	0.002	0.002
	RSD%	0.10%	0.11%	0.13%	0.14%	0.11%	0.11%	0.10%

When compared to typical legacy methods (20 min, 1.2 mL/min), this method development has provided a 10-fold increase in sample throughput and a 20-fold reduction in mobile phase consumption / waste generation (Figure 6).

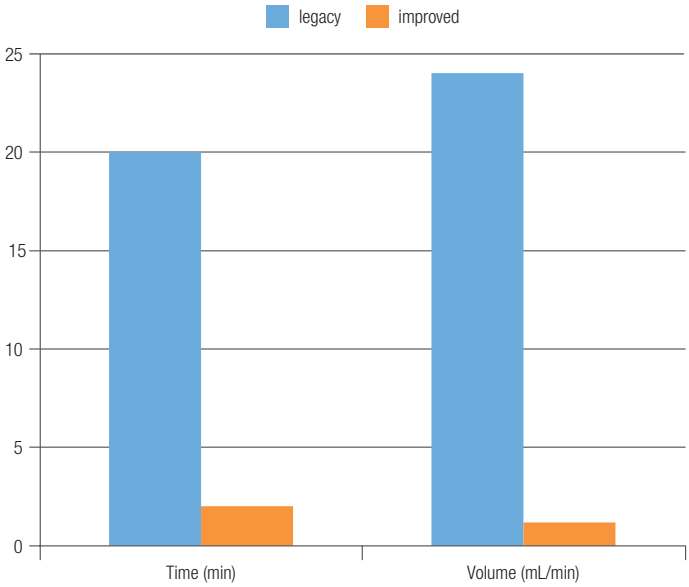

Figure 6. Relative differences in method time and mobile phase consumption between typical legacy methods and this improved method.

Conclusions

A high-throughput application has been demonstrated showing the separation of seven closely related glucocorticoids in under two minutes with a simplified UHPLC method that demonstrates the following:

- Critical pair resolution maintained in under two minutes with a simple isocratic method
- Ten-fold increase in method throughput when compared to typical legacy methods on 250 × 4.6 mm columns
- Associated reduction in cost per sample through reduced mobile phase consumption and waste generation

References

1. Rapid analysis of nine corticosteroids using a Thermo Scientific Acclaim RSLC C18 column https://appslab.thermofisher.com/App/205/rapid-analysis-nine-corticosteroids-using-a-thermo-scientific-acclaim-rslc-c18-column
2. Accucore HPLC Columns Technical Guide https://tools.thermofisher.com/content/sfs/brochures/TG-20666-Accucore-HPLC-Columns-TG20666-EN.pdf

Find out more at **thermofisher.com/Accucore**

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APPLICATION NOTE 21687

A rapid fenoprofen USP assay method

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Keywords
Pharmaceutical, Drug development, QA/QC, USP, Modernization, USP-NF Chapter <621>, Small molecule, Fenoprofen, Gemfibrozil, Solid core, Accucore C18, Vanquish Flex, UHPLC, USP monograph modernization

Application benefits

- Five-fold increase in method throughput compared to original method (fifty samples/hour)
- Associated 10-fold reduction in cost per sample through reduced mobile phase consumption and waste generation
- Additional reduced method complexity from easy-to-prepare mobile phase

Goal

To demonstrate practical approaches that can be used to significantly improve throughput of the fenoprofen USP assay monograph keeping to the spirit of USP-NF Chapter <621> guidelines while maintaining USP quality acceptance criteria. To then take this optimized assay monograph and reduce analysis time even further.

Introduction

Most existing pharmacopeial methods were established prior to the turn of the century and are configured for large particle size (≥5 µm) and long columns (>200 mm). As a consequence, the method times are long and the mobile phase consumption is high compared to modern equivalents.

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Vanquish Flex UHPLC

Table of contents

Since 2014 the USP-NF Chapter <621> has allowed adjustments to these methods, within certain criteria, to benefit from the increased performance of smaller particle size products. For isocratic methods, the main changes relate to particle size, column length, and flow rate.

- Particle size and column length can be changed but must maintain a constant length to particle size ratio or in a -25% to +50% range.

- Flow rate can be adjusted using a defined formula to take into account changes to particle size and column diameter, or ±50%.

One of the key goals for the chromatographer is to achieve a consistent, reproducible separation. The selection of a highly reproducible HPLC column is essential if this goal is to be attained. Based on solid core technology, Accucore HPLC columns allow users of conventional HPLC methods to enjoy performance beyond that of columns packed with 5 µm or even 3 µm fully porous particles. High separation efficiencies provide increased peak resolution. An ultra-stable packed bed results in exceptionally robust columns that demonstrate excellent retention and response reproducibility. Accucore columns are available in a wide range of chemistries and particle sizes making them an ideal choice for this type of work.[1]

The Vanquish Flex Quaternary UHPLC system has the benefit of SmartInject technology and improvements in injection system hardware synchronization. This results in excellent retention time precision providing the user with greater data confidence during method development.

The Vanquish Flex Quaternary system also utilizes Thermo Scientific™ LightPipe™ flow cell technology designed for the diode array detector (DAD), which provides the user with increased sensitivity for analytes due to fiber optics and total internal UV light reflection, and minimum peak dispersion due to small internal volume.

The fenoprofen method was selected due to the widespread use by generic pharmaceutical manufacturing and the potential for significant improvement. This will be demonstrated by direct comparison of legacy and modern column formats firstly within the USP guidelines for equivalence and then beyond those guidelines to demonstrate the savings that can be applied to reduce operating costs through mobile phase and waste reduction.

Experimental

Consumables and apparatus

- Thermo Scientific™ Hypersil GOLD™ C8, 150 × 4.6 mm, 5 µm column (P/N 25205-154630)
- Accucore C8 XL, 150 × 4.6 mm, 4 µm column (P/N 74204-154630)
- Accucore C8, 100 × 4.6 mm, 2.6 µm column (P/N 17226-104630)
- Accucore C8, 100 × 2.1 mm, 2.6 µm column (P/N 17226-104630)
- Accucore C8, 50 x 2.1 mm, 2.6 µm column (P/N 17226-054630)
- LC-MS grade 18 MΩ water from Thermo Scientific™ Smart2Pure™ system (P/N 50129845)
- Fisher Scientific™ LC-MS grade acetonitrile (P/N A955-212)
- Fisher Scientific™ Optima™ LC-MS grade formic acid (P/N A117-50)
- Fisher Scientific certified AR, orthophosphoric acid (P/N O/0500/PB08)
- Thermo Scientific™ Virtuoso™ 9 mm wide opening, 2 mL screw thread vial and cap kit (P/N 60180-VT400)

Standards

The two compounds specified in the USP chromatographic purity method were fenoprofen (1) and gemfibrozil (2). The number relates to their elution order and peak labelling on subsequent chromatograms. These were purchased from a reputable supplier.

Instrumentation

Analyses were performed using a Vanquish Flex Quaternary UHPLC system consisting of:

- Quaternary Pump F (P/N VF-P20-A)
- System Base Vanquish Flex (P/N VF-S01-A)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Active Pre-heater (P/N 6732.0110)
- Diode Array Detector HL (P/N VH-D10-A)
- LightPipe Flow Cell, 10 mm (P/N 6083.0100)

- Thermo Scientific™ Virtuoso™ Vial Identification System (P/N 60180-VT-100)

Software

Thermo Scientific™ Chromeleon™ 7.2 SR4

Sample preparation

Solutions of the compounds were prepared by dissolving a known weighed amount in methanol/water (70:30 v/v) to produce 1 mg/mL primary solutions. A mixed standard solution and individual working standards were used to assess method development and were prepared in methanol/water (70:30 v/v) at a concentration of 0.1 mg/mL.

Sample handling

Vial labeling was supported by the Virtuoso Vial Identification System.

HPLC conditions

Various columns and conditions were explored as part of the method development described below. These values represent the initial and final method.

Initial USP HPLC method

HPLC column:	Hypersil GOLD, 5 µm HPLC column, 150 mm × 4.0 mm
Mobile phase A:	Water/phosphoric acid (92:8 v/v)
Mobile phase B:	Acetonitrile
Flow rate:	2.0 mL/min
Column temperature:	30 °C, still air, no eluent pre-heating
Injection volume:	5 µL
On-pump mixing:	50% A : 50% B
Mixer:	50 µL capillary + 350 µL static in combination UV detection at 272 nm

Final UHPLC method

UHPLC column:	Accucore, 2.6 µm HPLC column, 50 mm × 2.1 mm
Mobile phase A:	Water/phosphoric acid (92:8 v/v)
Mobile phase B:	Acetonitrile
Flow rate:	1.0 mL/min
Column temperature:	45 °C, still air with eluent pre-heating
Injection volume:	1 µL
On-pump mixing:	66% A : 34% B
Mixer:	50 µL capillary + 350 µL static in combination UV detection at 214 nm

Results and discussion

A Hypersil GOLD column was configured on the Vanquish Flex Quaternary system and data obtained, using the existing USP method, to provide a starting point for further method development.

Initial development focused on the column length and particle size. The initial analysis was repeated with an Accucore XL C8, 150 × 4.6 mm, 4 µm column as the direct equivalent and also on an Accucore C8, 100 × 4.6 mm, 2.6 µm column, selected to maintain the length to particle size ratio within the +50% / -25% limits as stated in the USP <621> guidance.

Figure 1 shows the chromatograms obtained with these three columns. There is a slight change in selectivity and hydrophobicity moving from the Hypersil GOLD column to the Accucore column families due to the differences in stationary phase bonding (surface area and carbon load). However, the USP criteria of peak resolution exceeding 2 is still attained and all the columns have the USP L1 designation. The core-shell particles provide narrow peaks with increased peak height due to their narrow particle size distribution and efficient packing. A reduced column length allows the method run time to be decreased from 6 to 4.5 minutes.

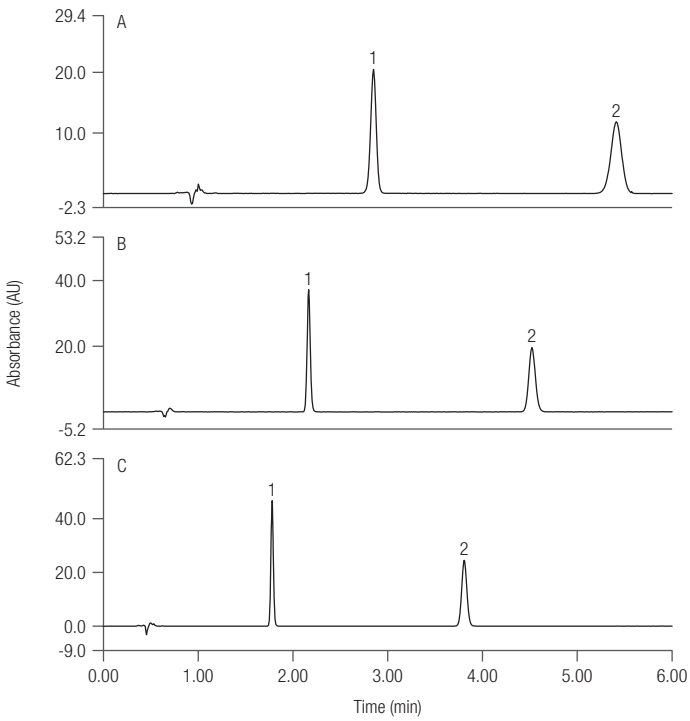

Figure 1. Separation of fenoprofen and gemfibrozil on three different columns A) Hypersil GOLD, 150 mm × 4.0 mm, 5 µm, B) Accucore XL, C8 150 × 4.6 mm, 4 µm, C) Accucore C8, 100 × 4.6 mm, 2.6 µm.

For isocratic methods, USP guidance allows changes in column internal diameter, providing that the flow rate is scaled. Recent updates also take particle size into consideration.

$$F_2 = F_1 \times (dc_2^2 / dc_1^2) \times (dp_1 / dp_2)$$

Where F is the flow rate; dc relates to the diameter of the column and dp relates to the diameter of the particle. Subscripts 1 and 2 relate to the original and modified methods, respectively.

Converting from a 4.6 mm diameter column at 2 mL/min to a 2.1 mm diameter column provides a scaled flow rate of 0.417 mL/min. The resulting chromatogram is shown in Figure 2.

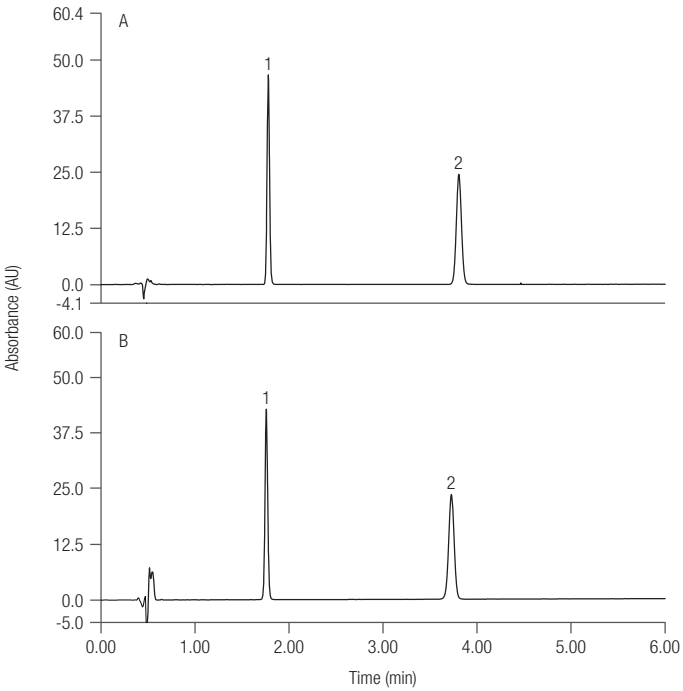

Figure 2. Separation achieved on Accucore 100 mm columns of two different diameters, flow rate scaled.
A) Accucore C8, 100 × 4.6 mm, 2.6 µm, 2.0 mL/min
B) Accucore C8, 100 × 2.1 mm, 2.6 µm, 0.417 mL/min

The separation is achieved within the same time frame but with a seven-fold reduction in mobile phase consumption when compared to the original method on the Hypersil GOLD column.

The final aspect of adjustment that lies within the USP guidance is to increase the flow rate. The guidance allows for an increase of ± 50% or until a 20% drop in column efficiency.

The 100 × 2.1 mm column was tested at flow rates from 400 to 1000 µL/min. Representative chromatograms can be seen in Figure 3 and the column plate values in Figure 4.

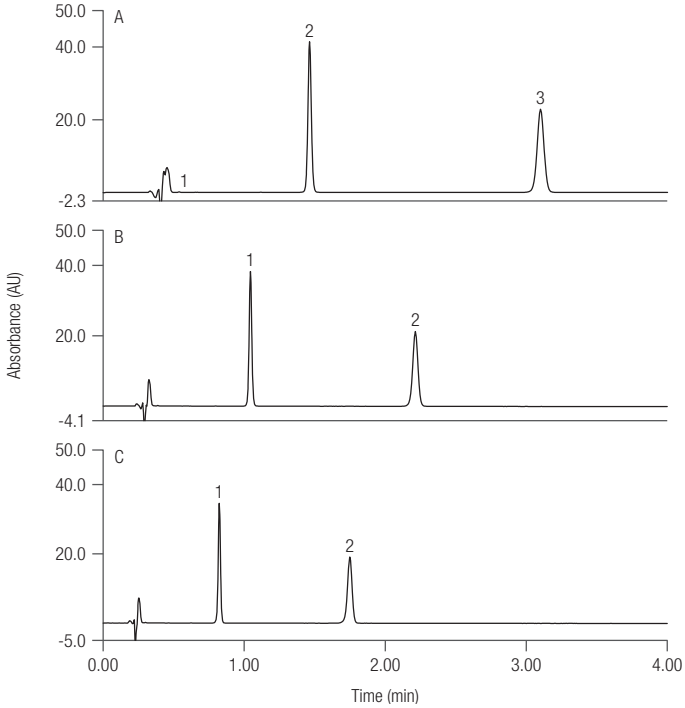

Figure 3. Representative chromatograms observed when increasing the method flow rate on an Accucore C8, 100 × 2.1 mm, 2.6 µm column.
A) 0.5 mL/min B) 0.7 mL/min C) 0.9 mL/min

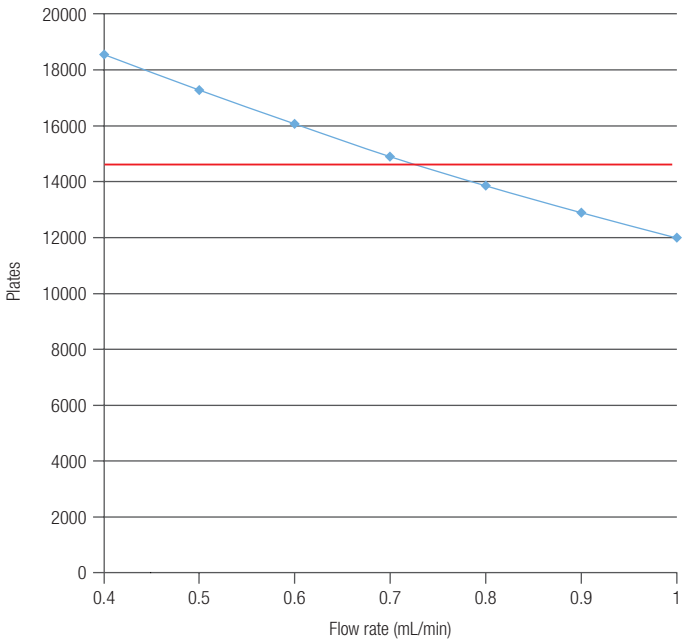

Figure 4. Plate count values when increasing the method flow rate on an Accucore C8, 100 × 2.1 mm, 2.6 µm column. The red line represents the 80% value of the plate count at 0.4 mL/min and the limit for USP equivalence.

Using the Accucore C8, 100 × 2.1 mm, 2.6 µm column at 0.7 mL/min represents the limit to which the USP approved adjustments can be applied. The speed of the assay has been reduced from 6 minutes to 2.5 minutes and the solvent consumption per assay reduced from 12 mL to 1.75 mL.

It is possible to extend the method beyond the conservative USP equivalence guidance and still produce an assay that meets the resolution requirements, and this was explored further using the flow rate of 1.0 mL/min and investigating the effect of column temperature. The mixed standard was analyzed at column temperatures of 25, 30, 35, 40, and 45 °C. The expected shift to earlier retention time with narrowing of the peak width was observed. The resolution value remained greater than 20 across all the experiments, again still above the USP limit (Figure 5).

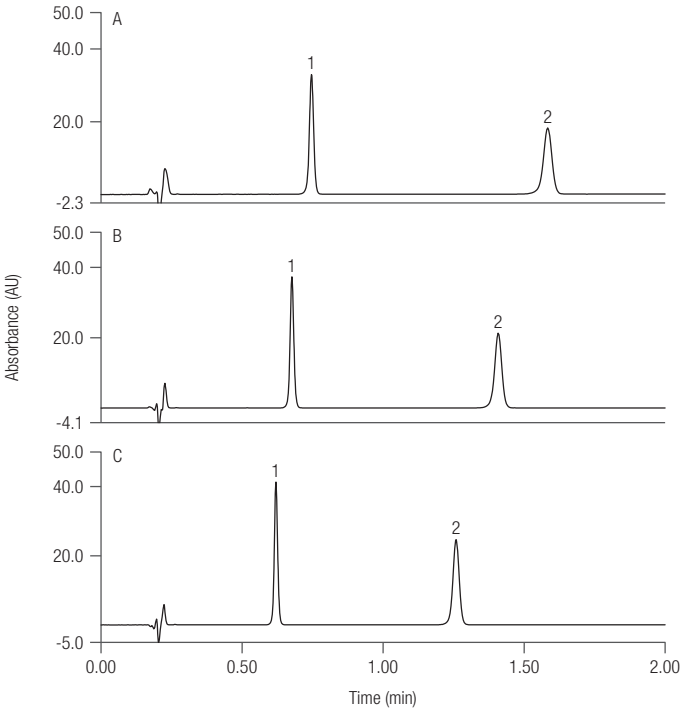

Figure 5. Chromatogram showing standards mixture analyzed on an Accucore C8, 50 × 2.1 mm column at three different temperatures A) 25 °C, B) 35 °C, and C) 45 °C.

By applying the developed method, the method time has been decreased further from 6 minutes to 1.5 minutes and the consumption of mobile phase (and waste) per assay has also been further reduced, from 12 mL to 1.5 mL, thus contributing a savings in both assay cost and an increase in throughput (Figure 6).

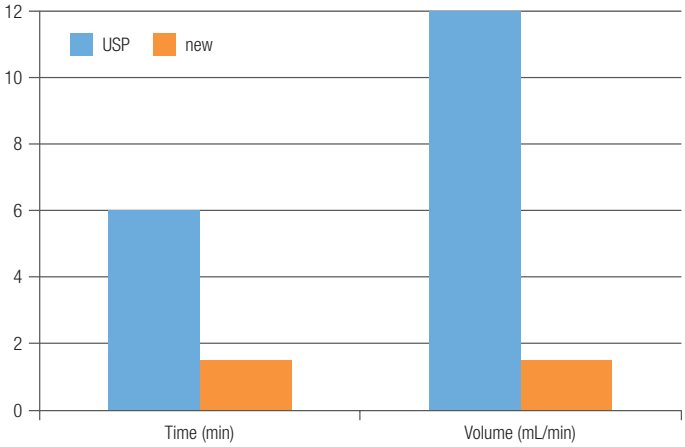

Figure 6. Indicative savings in time and mobile phase volume/waste between the original USP and the improved method.

A comparison of the key stages in this method development is shown in Figure 7.

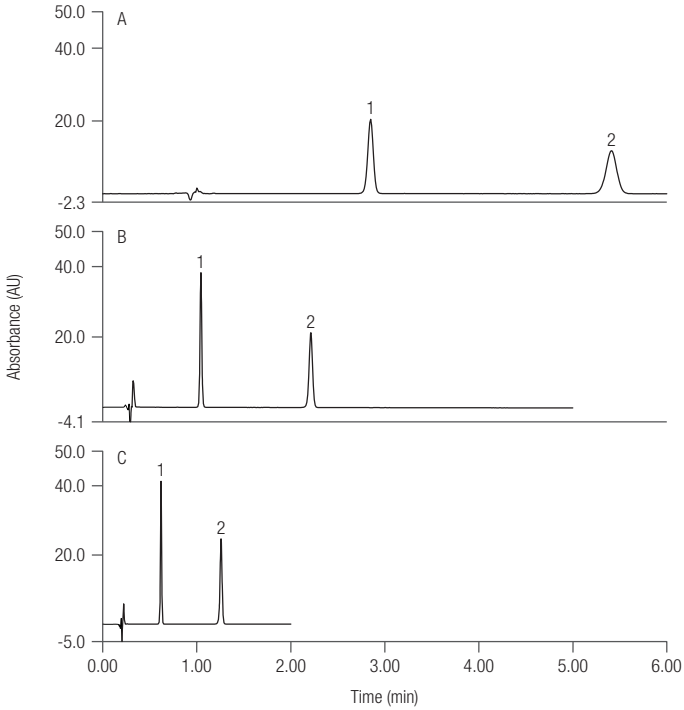

Figure 7. A comparison of the key stages of the method development. A) Original USP method, Hypersil GOLD, 150 mm × 4.6 mm, 5 µm column, 2 mL/min, 25 °C, B) Scaled USP method, Accucore C8, 100 × 2.1 mm, 2.6 µm column, 0.7 mL/min, 25 °C, C) Beyond USP method, Accucore C8, 100 × 2.1 mm, 2.6 µm column, 1.0 mL/min, 45 °C

To conclude this work, method repeatability was investigated by making 24 replicate injections using the final developed method. The results are shown in Figure 9 and Table 1.

Table 1. Data from 24 replicate injections of standards mixture using the final developed method on an Accucore C8, 100 × 2.1 mm, 2.6 µm column, 1.0 mL/min, 45 °C.

Compound	% RSD			
	RT	Area	PW (50%)	As
Fenoprofen	0.07	0.16	0.20	1.56
Gemfibrozil	0.06	0.17	0.25	1.00

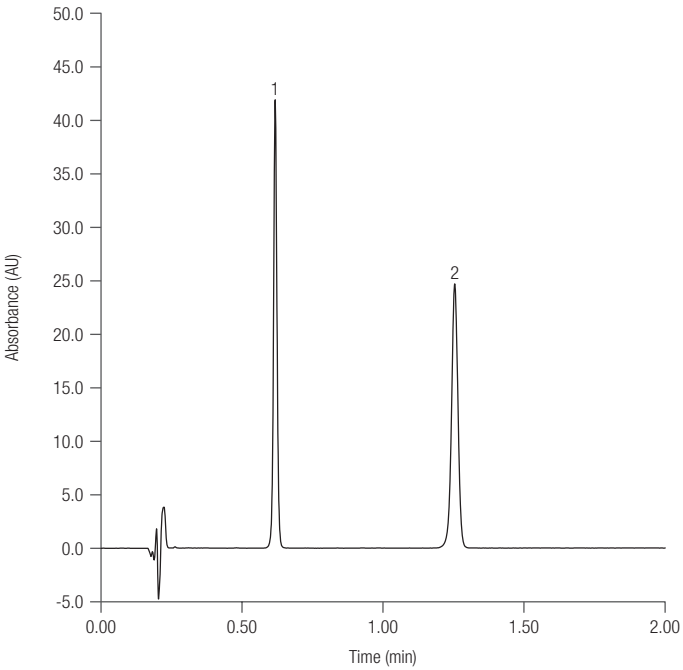

Figure 8. Overlay of 24 replicate injections of the standards mixture using the final developed method on an Accucore C8, 100 × 2.1 mm, 2.6 µm column, 1.0 mL/min, 45 °C.

Conclusions

A high-throughput assay for fenoprofen was developed keeping to the spirit of USP-NF Chapter <621> guidelines for method modernization that doubled throughput and maintained USP quality acceptance criteria. The method, when further optimized, demonstrated the following when compared to the original USP method:

- Four-fold increase in method throughput, allowing more samples to be analyzed in a given time
- Eight-fold reduction in cost per sample through reduced mobile phase consumption and waste generation

References

1. Accucore HPLC Columns Technical Guide https://tools.thermofisher.com/content/sfs/brochures/TG-20666-Accucore-HPLC-Columns-TG20666-EN.pdf

Find out more at **thermofisher.com/Accucore**

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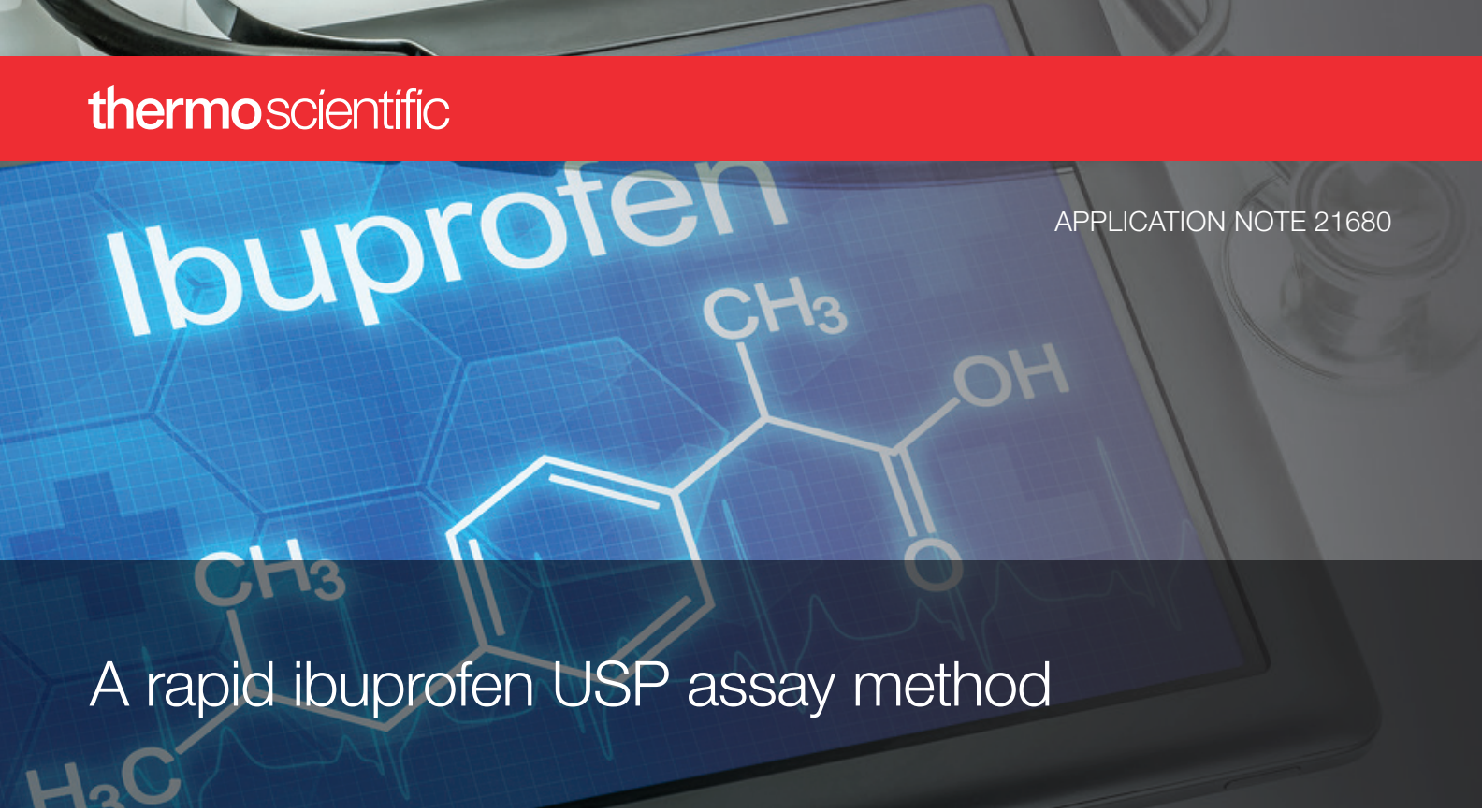
APPLICATION NOTE 21680



A rapid ibuprofen USP assay method

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Runcorn, UK

Keywords
Pharmaceutical, Drug development, QA/QC, USP, Modernization, USP-NF Chapter <621>, Small molecule, Ibuprofen, Valerophenone, Solid core, Accucore C18, Vanquish Flex, USP monograph modernization

Application benefits

- Five-fold increase in method throughput compared to original method (fifty samples/hour)
- Associated 10-fold reduction in cost per sample through reduced mobile phase consumption and waste generation
- Additional reduced method complexity from easy-to-prepare mobile phase

Goal

To demonstrate practical approaches that can be used to significantly improve throughput of the ibuprofen USP assay monograph keeping to the spirit of USP-NF Chapter <621> guidelines while maintaining USP quality acceptance criteria. To then take this optimized assay monograph and reduce analysis time even further.

Introduction

Most existing pharmacopeial methods were established prior to the turn of the century and are configured for large particle size (≥5 µm) and long columns (>200 mm). As a consequence, the method times are usually >20 minutes and the mobile phase consumption is high compared to modern equivalents.

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Vanquish Flex UHPLC

Table of contents

Since 2014, the USP-NF Chapter <621> has allowed adjustments to these methods, within certain criteria, in order to benefit from the increased performance of smaller particle size products. For isocratic methods, the main changes relate to particle size, column length, and flow rate.

- Particle size and column length can be changed, but must maintain a constant length to particle size ratio or in a -25% to +50% range.
- Flow rate can be adjusted using a defined formula to take into account changes to particle size and column diameter, or ±50%.

One of the key goals for the chromatographer is to achieve a consistent, reproducible separation. The selection of a highly reproducible HPLC column is essential if this goal is to be attained. Using solid core particles, the Accucore HPLC columns allow users of conventional HPLC methods to enjoy performance beyond that of columns packed with 5 µm or even 3 µm fully porous particles. High separation efficiencies provide increased peak resolution. An ultra-stable packed bed results in exceptionally robust columns that demonstrate excellent retention and response reproducibility. The Accucore columns are available in a wide range of chemistries and particle sizes making them an ideal choice for this type of work.[1]

The Vanquish Flex UHPLC system offers intelligent SmartInject Technology to mitigate injection pressure drops and improvements in injection system hardware synchronization. This results in excellent retention time precision, providing the user with greater data confidence during method development.

The Vanquish Flex system also utilizes Thermo Scientific™ LightPipe™ flow cell technology, designed for the diode array detector (DAD). It provides the user with increased sensitivity for analytes due to fiber optics and total internal UV light reflection and minimum peak dispersion due to small internal volume.

The ibuprofen method was selected due to the widespread use by generic pharmaceutical manufacturing and the potential for method improvement. This will be demonstrated by direct comparison of legacy and modern column formats within the USP guidelines for equivalence and then beyond those guidelines to demonstrate the savings that can be applied to reduce operating costs through mobile phase and waste reduction.

Experimental

Consumables and apparatus

- Thermo Scientific™ Hypersil GOLD™, 150 × 4.0 mm, 5 µm column (P/N 25005-154030)
- Accucore C18 XL, 150 × 4.6 mm, 4 µm column (P/N 74104-154630)
- Accucore C18, 100 × 4.6 mm, 2.6 µm column (P/N 17126-104630)
- Accucore C18, 100 × 2.1 mm, 2.6 µm column (P/N 17126-102130)
- Accucore C18, 50 x 2.1 mm, 2.6 µm column (P/N 17126-052130)
- LC-MS grade 18 MΩ water from Thermo Scientific™ Smart2Pure™ system (P/N 50129845)
- Fisher Scientific™ LC-MS grade acetonitrile (P/N A955-212)
- Fisher Scientific™ Optima™ LC-MS grade formic acid (P/N A117-50º)
- Fisher Scientific certified AR, orthophosphoric acid (P/N O/0500/PB08)
- Thermo Scientific™ Virtuoso™ 9 mm wide opening, 2 mL screw thread vial and cap kit (P/N 60180-VT400)

Standards

The two compounds specified in the USP chromatographic purity method were ibuprofen and valerophenone. These were purchased from a reputable supplier.

Instrumentation

Analyses were performed using a Vanquish Flex Quaternary UHPLC System consisting of:

- Quaternary Pump F (P/N VF-P20-A)
- System Base Vanquish Flex (P/N VF-S01-A)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Active Pre-heater (P/N 6732.0110)
- Diode Array Detector HL (P/N VH-D10-A)

• LightPipe Flow Cell, 10 mm (P/N 6083.0100)

Thermo Scientific™ Virtuoso™ vial identification system (P/N 60180-VT-100)

Software

Thermo Scientific™ Chromeleon™ software 7.2 SR4

Sample preparation

Solutions of the compounds were prepared by dissolving a known amount in acetonitrile to produce 1 mg/mL primary solutions. A mixed standard solution and individual working standards were used to assess method development and were prepared in water/acetonitrile (2:1, v/v) at a concentration of 0.2 mg/mL

Sample handling

Vial labeling was supported by the Virtuoso vial identification system

HPLC conditions

Various columns and conditions were explored as part of the method development described below. These values represent the initial and final method.

Initial USP HPLC method

HPLC column:	Hypersil GOLD, 5 µm HPLC column, 150 mm × 4.0 mm
Mobile phase A:	Water adjusted to pH 2.5 with orthophosphoric acid
Mobile phase B:	Acetonitrile
Flow rate:	2.0 mL/min
Column temperature:	30 °C, still air, no eluent pre-heating
Injection volume:	5 µL
On-pump mixing:	66% A : 34% B
Mixer:	50 µL capillary + 350 µL static
UV detection:	214 nm

Final UHPLC method

UHPLC column:	Accucore, 2.6 µm HPLC column, 50 mm × 2.1 mm
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile
Flow rate:	1.0 mL/min
Column temperature:	50 °C, still air with eluent pre-heating
Injection volume:	1 µL
On-pump mixing:	66% A : 34% B
Mixer:	50 µL capillary + 350 µL static
UV detection:	214 nm

Results and discussion

A Hypersil GOLD column was configured on the Vanquish Flex system and data obtained, using the existing USP method, to provide a starting point for further method development.

Initial development focused on the column length and particle size. The initial analysis was repeated with an Accucore XL C18, 150 × 4.6 mm, 4 µm column as the direct equivalent and also on an Accucore C18, 100 × 4.6 mm, 2.6 µm column, selected to maintain the length to particle size ratio within the +50% / -25% limits as stated in the USP <621> guidance.

Figure 1 shows the chromatograms obtained with these three columns. There is a slight change in selectivity and hydrophobicity moving from the Hypersil GOLD columns to the Accucore column range due to the differences in stationary phase bonding (surface area and carbon load). However, the USP criteria of peak resolution R exceeding 2 is still attained and all the columns have the USP L1 designation. The solid core particles provide narrow peaks with increased peak height, due to their narrow particle size distribution and efficient packing.
A reduced column length allows the method run time to be decreased from 20 to 15 minutes.

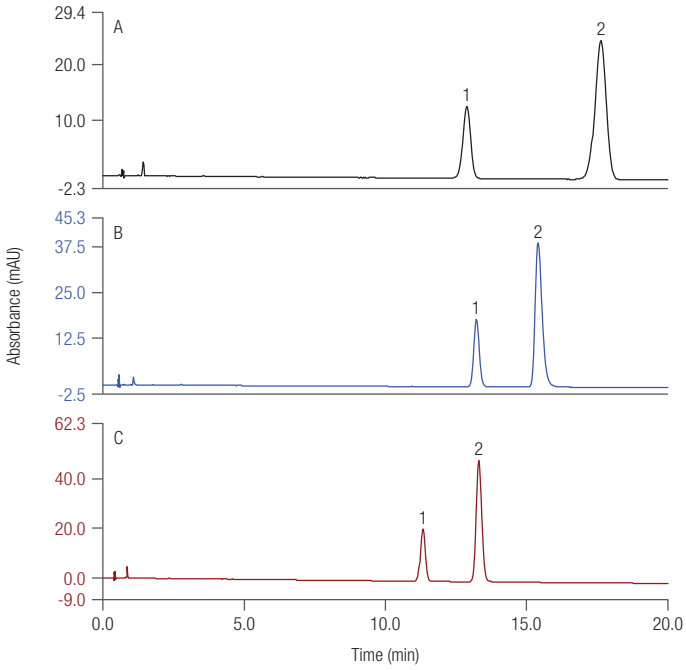

Figure 1. Separation of valerophenone (1) and ibuprofen (2) on three different columns.
(A) Hypersil GOLD, 150 mm × 4.0 mm, 5 µm
(B) Accucore XL, C18 150 × 4.6 mm, 4 µm
(C) Accucore C18, 100 × 4.6 mm, 2.6 µm

For isocratic methods, USP guidance allows changes in column internal diameter, providing that the flow rate is scaled. Recent updates also take particle size into consideration.

$$F_2 = F_1 \times (dc_2^2 / dc_1^2) \times (dp_1 / dp_2)$$

Where F is the flow rate; dc relates to the diameter of the column and dp relates to the diameter of the particle. Subscripts 1 and 2 relate to the original and modified methods, respectively.

Converting from a 4.6 mm diameter column at 2 mL/min to a 2.1 mm diameter column provides a scaled flow rate of 0.417 mL/min. The resulting chromatogram is shown in Figure 2.

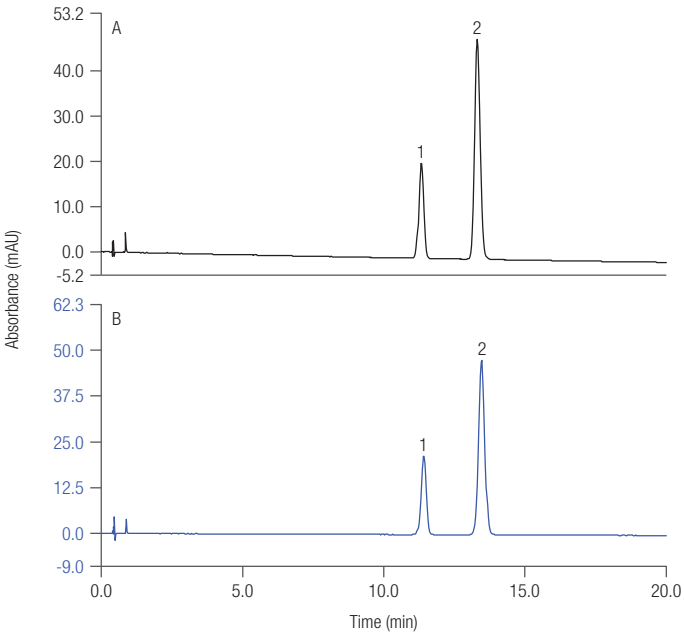

Figure 2. Separation achieved on Accucore 100 mm columns of two different diameters, flow rate scaled.
(A) Accucore C18, 100 × 4.6 mm, 2.6 µm, 2.0 mL/min
(B) Accucore C18, 100 × 2.1 mm, 2.6 µm, 0.417 mL/min

The separation is achieved within the same time frame, but with an eight-fold reduction in mobile phase consumption when compared to the original method on the Hypersil GOLD column.

The final aspect of adjustment that lies within the USP guidance is to increase the flow rate. Guidance allows for an increase of ±50% or until a 20% drop in column efficiency. The 100 × 2.1 mm column was tested at flow rates from 400 to 1000 µL/min. Representative chromatograms can be seen in Figure 3 and the column plate values in Figure 4.

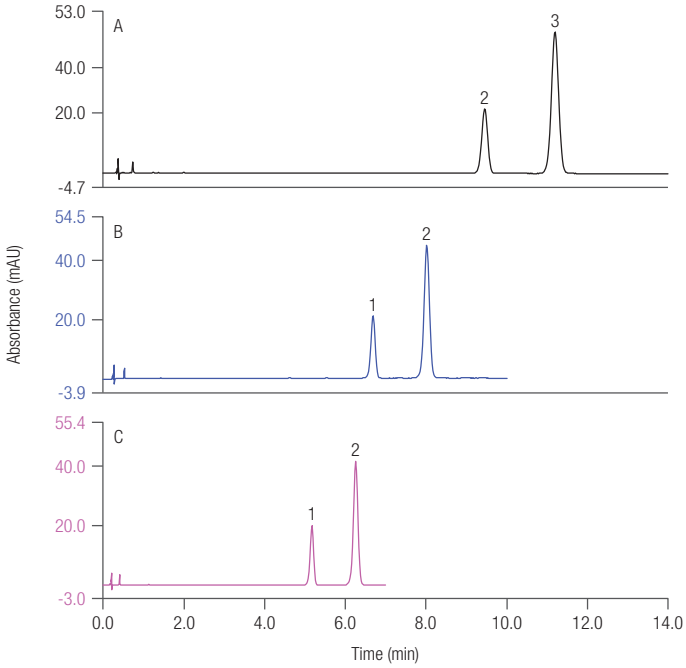

Figure 3. Representative chromatograms observed when increasing the method flow rate on an Accucore C18, 100 x 2.1 mm, 2.6 µm column.
(A) 0.5 mL/min (B) 0.7 mL/min (C) 0.9 mL/min

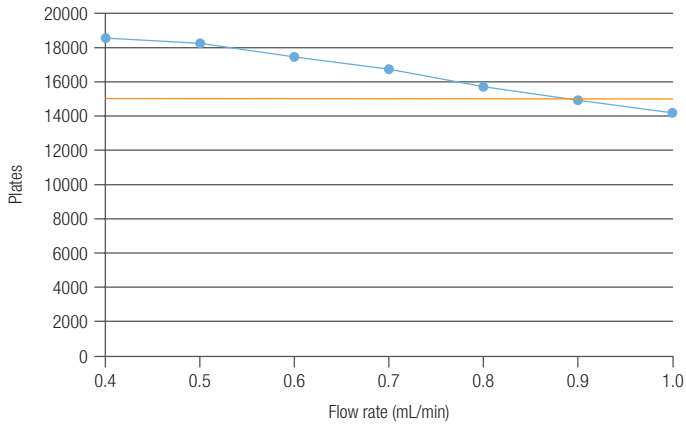

Figure 4. Plate count values when increasing the method flow rate on an Accucore C18, 100 x 2.1 mm, 2.6 µm column. Red line represents 80% value of the plate count at 0.4 mL/min.

Using the Accucore C18, 100 × 2.1 mm, 2.6 µm column at 0.9 mL/min represents the limit to which the USP approved adjustments can be applied. The speed of the assay has been reduced from 20 minutes to 7 minutes and the solvent consumption per assay reduced from 40 mL to 6.37 mL.

There is still opportunity for further assay improvement beyond the USP guidance, yet still meet the USP method guidance on resolution. The column length can be decreased further, column temperature can be increased, and mobile phase composition can be simplified.

Column length was reduced from 100 mm to 50 mm resulting in a decrease of both retention time and peak resolution. The latter shifted from 6.0 to 3.4 and was still above the usual USP limit of 2.0. This can be seen in Figure 5.

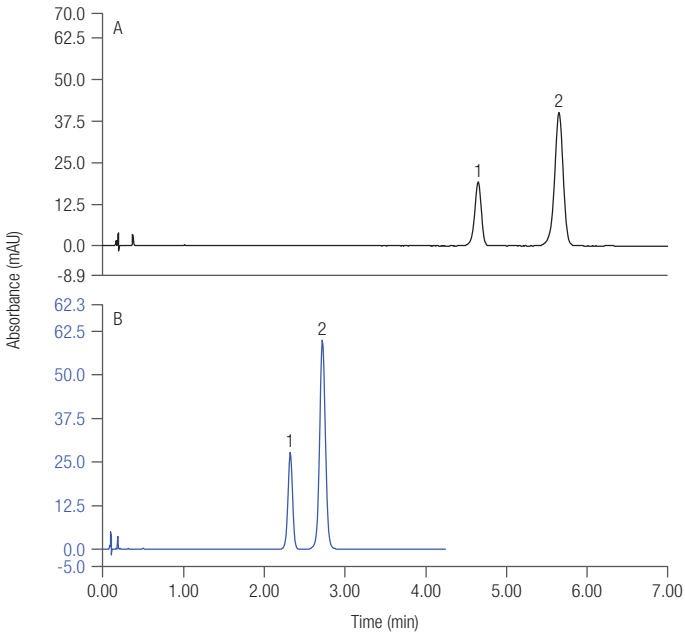

Figure 5. Standards mixture (A) on 100 mm × 2.1 mm 2.6 µm Accucore column, (B) standards mixture on 50 mm × 2.1 mm 2.6 µm Accucore column.

The mobile phase was simplified by substituting the original mobile phase with water and acetonitrile, both containing 0.1% formic acid. The 50 mm column was conditioned with the new mobile phase and the standards analyzed. There was no significant difference in the peak shape and retention.

Finally, the analysis was carried out at with column oven temperatures of 30, 40, and 50 °C.

The expected shift to earlier retention time with narrowing of the peak width was observed. There was a further slight decrease in resolution but again still above the USP limit (Figure 6.) The method with a temperature of 50 °C was selected for further development.

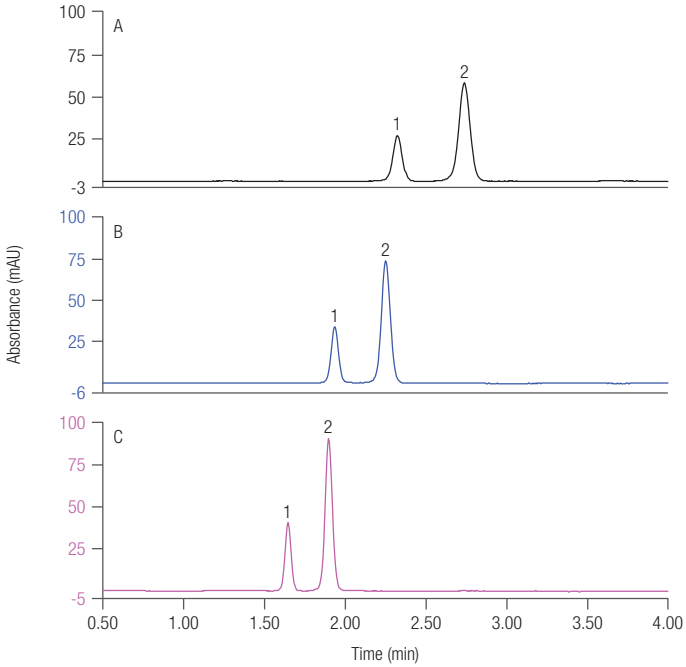

Figure 6. Chromatogram showing standards mixture analyzed on an Accucore 50 mm column at three different temperatures. (A) 30 °C, (B) 40 °C, and (C) 50 °C.

By applying the developed method, the method time has been decreased from 20 minutes to 4 minutes and the consumption of mobile phase (and waste) per assay has also been reduced from 40 mL to 4 mL, thus contributing a saving in both assay cost and an increase in throughput (Figure 7).

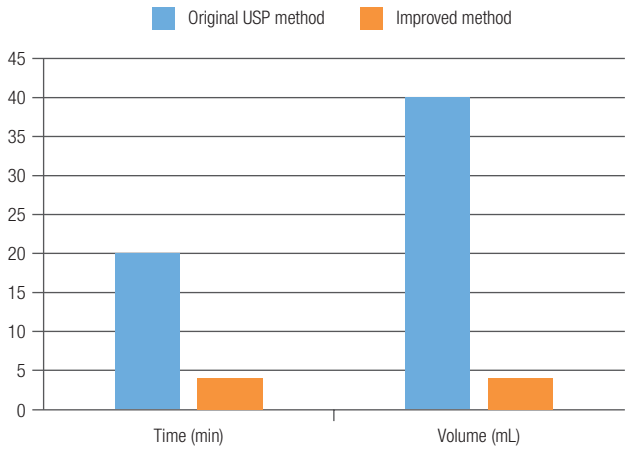

Figure 7. Indicative savings in time and mobile phase volume/waste between the original USP and the improved method.

A comparison of the key stages in this method development is shown in Figure 8.

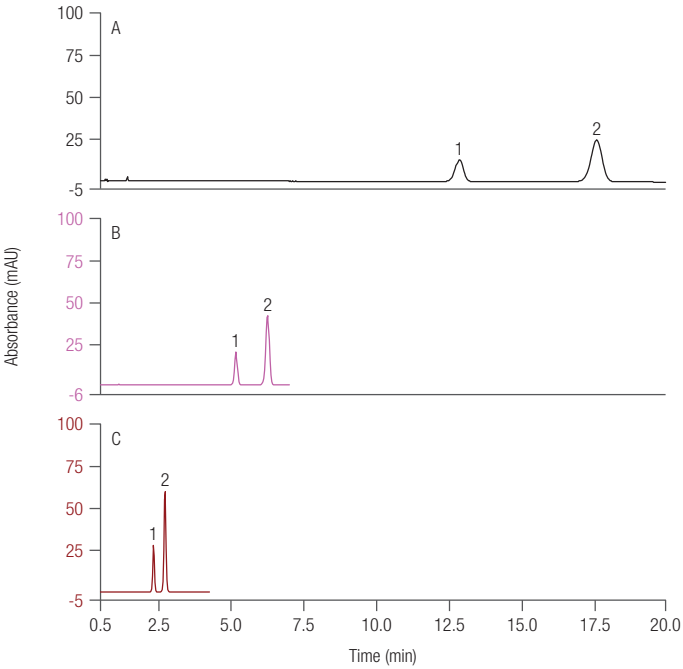

Figure 8. (A) Original USP method, Hypersil GOLD, 150 mm × 4.0 mm, 5 µm, 2 mL/min, 30 °C, (B) Scaled USP method, Accucore C18, 100 × 2.1 mm, 2.6 µm, 0.9 mL/min, 30 °C, (C) Beyond USP method, Accucore C18, 50 × 2.1 mm, 2.6 µm, 1.0 mL/min, 50 °C.

To conclude this work, method repeatability was investigated by making 24 replicate injections using the final developed method. Results are shown in Figure 9.

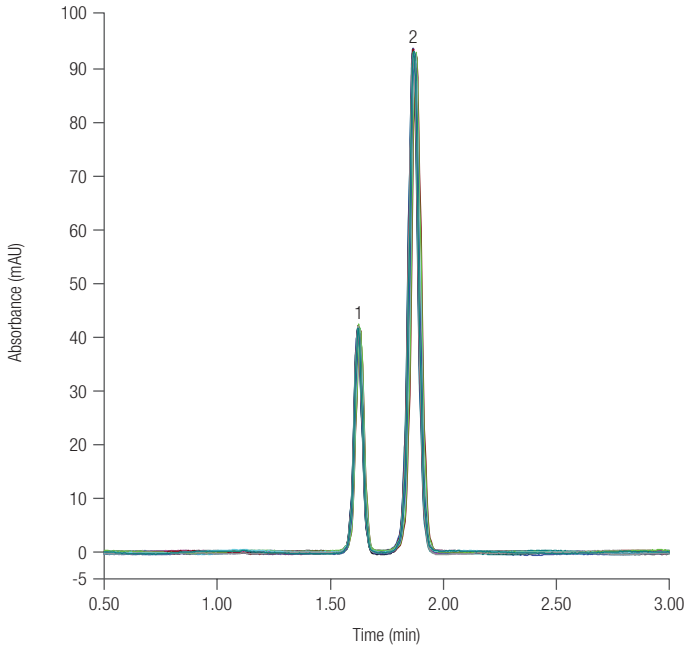

Figure 9. Overlay of 24 replicate injections of the standards mixture using the final developed method on an Accucore C18, 50 × 2.1 mm, 2.6 µm column, 1.0 mL/min, 50 °C.

Conclusions

A high-throughput assay for ibuprofen was developed keeping to the spirit of USP-NF Chapter <621> guidelines for method modernization that significantly increased throughput and maintained USP quality acceptance criteria. When compared to the original USP method, the updated method demonstrates the following:

- Significant increase in assay throughput (five-fold)
- Substantial associated cost reduction, through reduced mobile phase consumption and waste generation
- Associated reduced method complexity from simplified mobile phase preparation

References

1. Accucore HPLC columns technical guide https://tools.thermofisher.com/content/sfs/brochures/TG-20666-Accucore-HPLC-Columns-TG20666-EN.pdf

Find out more at **www.thermofisher.com/LC-columns**

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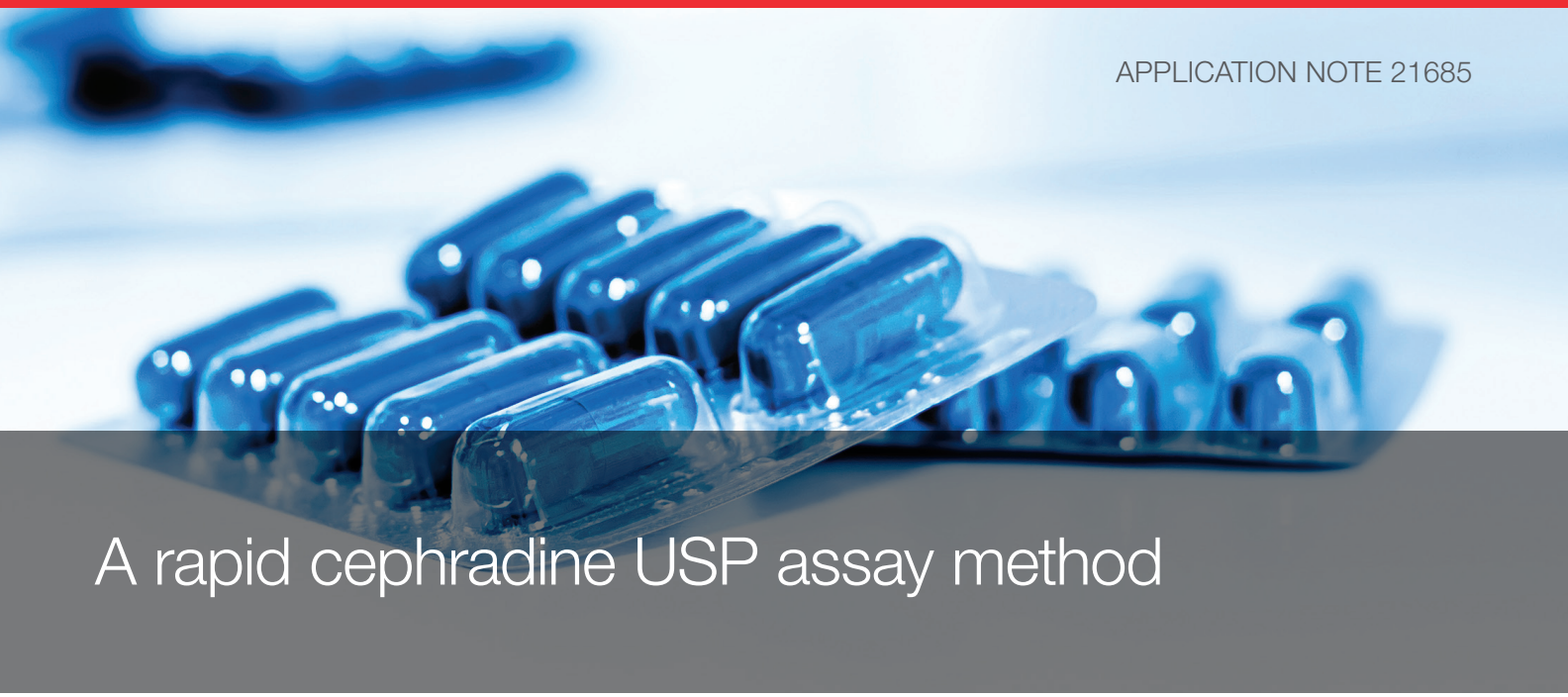

A rapid cephradine USP assay method

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Keywords

Pharmaceutical, Drug development, QA/QC, USP, Modernization, USP-NF Chapter <621>, Small molecule, Cephradine, Cephalexin, Solid core, Accucore aQ, Vanquish Flex, UHPLC, USP monograph modernization

Application benefits

- Seventeen-fold increase in method throughput compared to original method (fifty samples/hour)
- Associated 30-fold reduction in cost per sample through reduced mobile phase consumption and waste generation
- Additional reduced method complexity from easy to prepare mobile phase

Goal

To demonstrate practical approaches that can be used to significantly improve throughput of the cephradine USP assay monograph keeping to the spirit of USP-NF Chapter <621> guidelines while maintaining USP quality acceptance criteria

Introduction

Most existing pharmacopeial methods were established prior to the turn of the century and are configured for large particle size (≥5 µm) and long columns (>200 mm). As a consequence, the method times are long and the mobile phase often contains additives that are not compatible with modern aerosol-based HPLC detectors such as MS, ELS, and CAD. The volume of mobile phase consumption and subsequent waste disposal is high compared to modern equivalents, contributing to a significant cost when using these types of assay.

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Table of contents

Since 2014 the USP-NF Chapter <621> has allowed adjustments to these methods, within certain criteria, to benefit from the increased performance of smaller particle size products. However, there are still restrictions on changing the mobile phase composition and chemistry and this can limit the benefit compared to establishing a new method without the legacy restrictions.

One of the key goals for the chromatographer is to achieve a consistent, reproducible separation. The selection of a highly reproducible HPLC column is essential if this goal is to be attained. Based on solid core technology, Accucore HPLC columns allow users of conventional HPLC methods to enjoy performance beyond that of columns packed with 5 µm or even 3 µm fully porous particles. High separation efficiencies provide increased peak resolution. An ultra-stable packed bed results in exceptionally robust columns that demonstrate excellent retention and response reproducibility. Accucore columns are available in a wide range of chemistries and particle sizes making them an ideal choice for this type of work.[1]

The Vanquish Flex Quaternary UHPLC system has the benefit of SmartInject technology, and improvements in injection system hardware synchronization. This results in excellent retention time precision providing the user with greater data confidence during method development. The Vanquish Flex Quaternary system also utilizes Thermo Scientific™ LightPipe™ flow cell technology designed for the diode array detector (DAD), which provides the user with low peak dispersion due to small internal volume.

The cephalexin method was selected as a good example of a legacy method with a complex mobile phase using a large column dimension (250 × 4.6 mm) and potential for method improvement. This will be demonstrated by developing a new method with simple mobile phase, compatible with MS and CAD, and a short 50 mm column providing a method time capable of fifty samples per hour and excellent retention time reproducibility. This process can be applied to other legacy methods to improve the productivity of the laboratory and reduce operating costs through mobile phase and waste reduction.

Experimental

Consumables and apparatus

- Accucore aQ, 50 × 2.1 mm, 2.6 µm column (P/N 17326-052130)
- LC-MS grade 18 MΩ water from Thermo Scientific™ Smart2Pure™ system (P/N 50129845)
- Fisher Scientific™ LC-MS grade acetonitrile (P/N A955-212)
- Fisher Scientific™ Optima™ LC-MS grade ammonium acetate (P/N A114-50)
- Fisher Scientific Optima LC-MS grade acetic acid (P/N A113-50)
- Thermo Scientific™ Virtuoso™ 9 mm wide opening, 2 mL screw thread vial and cap kit (P/N 60180-VT400)

Standards

The two compounds specified in the USP chromatographic assay method were cephalexin (1) and cephradine (2). These were purchased from a reputable supplier. The numbers relate to their elution order and peak labelling in the subsequent chromatograms.

Instrumentation

Analyses were performed using a Vanquish Flex Quaternary UHPLC System consisting of:

- Quaternary Pump F (P/N VF-P20-A)
- System Base Vanquish Flex (P/N VF-S01-A)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Active Pre-heater (P/N 6732.0110)
- Diode Array Detector HL (P/N VH-D10-A)
- LightPipe Flow Cell, 10 mm (P/N 6083.0100)
- Thermo Scientific™ Virtuoso™ Vial Identification System (P/N 60180-VT-100)

Software

Thermo Scientific™ Chromeleon™ 7.2 SR4

Sample preparation

Solutions of the compounds were prepared by dissolving a known amount in water/acetonitrile (80:20, v/v) to produce 1 mg/mL primary solutions. A mixed working standard solution and individual working standards were used to assess method development and were prepared in water/acetonitrile (80:20, v/v) at a concentration of 0.1 mg/mL.

Preparation of mobile phase

The 25 mM ammonium acetate solution was prepared by dissolving 1.575 g of ammonium acetate in 1 L of 18 MΩ water and adjusting the pH to 5.0 with Optima grade acetic acid.

Vial labeling was supported by the Virtuoso Vial Identification System.

USP criteria

Relative retention times are approximately 0.8 for cephalexin and 1.0 for cephradine, with resolution between the peaks not less than 2.0 and RSD not more than 2%.

HPLC conditions (USP method)

HPLC column:	L1, C18, 10 µm, 250 mm × 4.6 mm
Mobile phase:	Water/methanol/0.5M sodium acetate/0.7 N acetic acid
Off-pump mixing:	782:200:15:3
Flow rate:	Approximately 1 mL/min
Column temperature:	Not specified
Injection volume:	Approximately 10 µL
UV detection:	254 nm

UHPLC conditions (final method)

UHPLC column:	Accucore aQ, 2.6 µm, 50 mm × 2.1 mm
Mobile phase A:	25 mM Ammonium acetate pH 5.0
Mobile phase B:	Acetonitrile
On-pump mixing:	95% A: 5% B
Flow rate:	0.6 mL/min
Column temperature:	50 °C, still air with eluent pre-heating
Injection volume:	1 µL
Mixer:	50 µL capillary + 350 µL static in combination
UV detection:	254 nm

Results and discussion

The Accucore aQ UHPLC column was configured on the Vanquish Flex Quaternary system and an initial flow of 0.4 mL/min established with a column temperature of 40 °C. The mobile phase proportioning was adjusted to provide a retention time correlating to a capacity factor of at least two and resolution between the cephradine and cefalexin peaks of at least two. Figure 1 shows the UV chromatogram with a mobile phase proportion of 95:5 (buffer/acetonitrile).

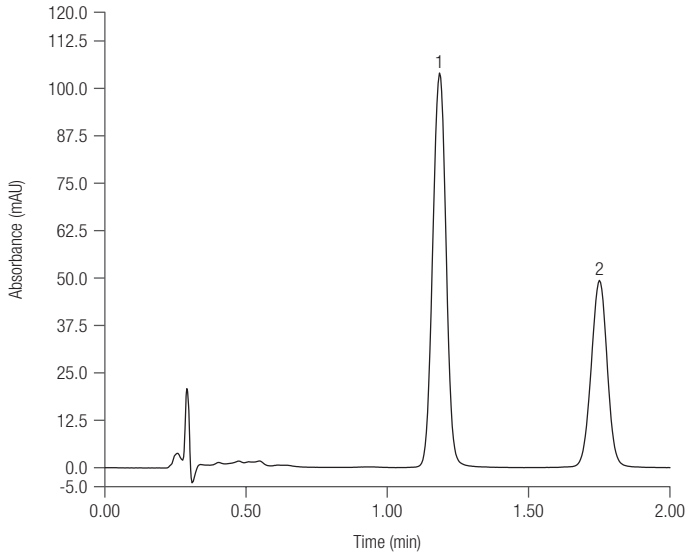

Figure 1. UV chromatogram of standard mixture with a flow rate of 0.4 mL/min of buffer: acetonitrile (95:5) at a column temperature of 40 °C.

The typical retention time for cephradine using the standard USP method[2] is nearer 20 minutes, so already a 10-fold reduction in method time is feasible.

Modern UHPLC systems are equipped with accurate column ovens, and for robust method transfer between different laboratories it is essential to maintain the column at a consistent temperature.

The effect of temperature on this separation was investigated by running the same standard mixture at column temperatures of 40, 50, and 60 °C (Figure 2). The mobile phase proportion and flow rate remain unchanged. The results are shown in Figure 2 and Table 1.

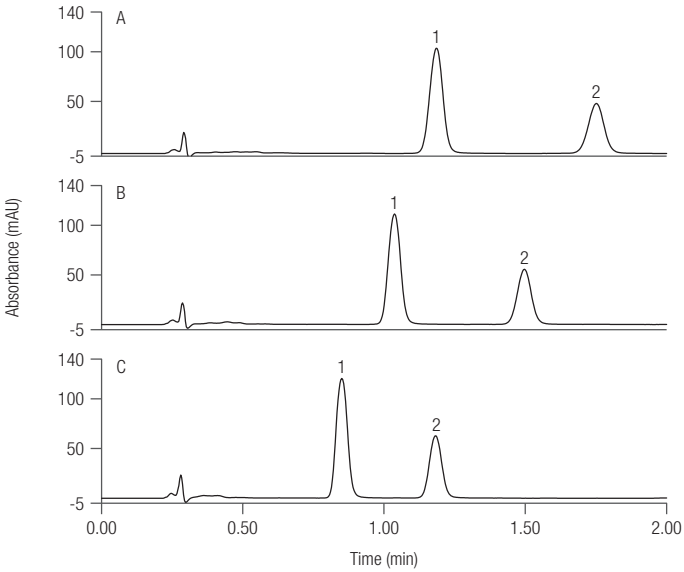

Figure 2. Chromatogram showing standards mixture analyzed at three different temperatures a) 30 °C, b) 40 °C, and c) 50 °C.

Table 1. Peak parameters for cephradine and resolution against cephalexin, under different column temperatures.

Temp. (°C)	Retention Time (min)	Peak Height (mAU)	Peak Width @50% (min)	Resolution
40	1.751	49	0.103	6.0
50	1.497	56	0.091	5.4
60	1.182	63	0.080	4.4

As expected, the retention time decreases with the increase in temperature. Peaks become narrower and taller, thus improving signal to noise, and there is a slight reduction in resolution though still well above the USP criteria of at least two.

Solid core HPLC columns are capable of equivalent efficiencies to much smaller fully porous particles. This allows high efficiency separations at a fraction of the cost in pressure. This, coupled with the solid-core columns ability to run at optimum (highest) efficiency over a much larger linear velocity range[1] allows the chromatographer to significantly speed up their methods.

The effect of flow rate was investigated at temperatures of 50 and 60 °C with flows of 0.4, 0.5, and 0.6 mL/min. Figure 3 and Table 2 show key data obtained from these experiments.

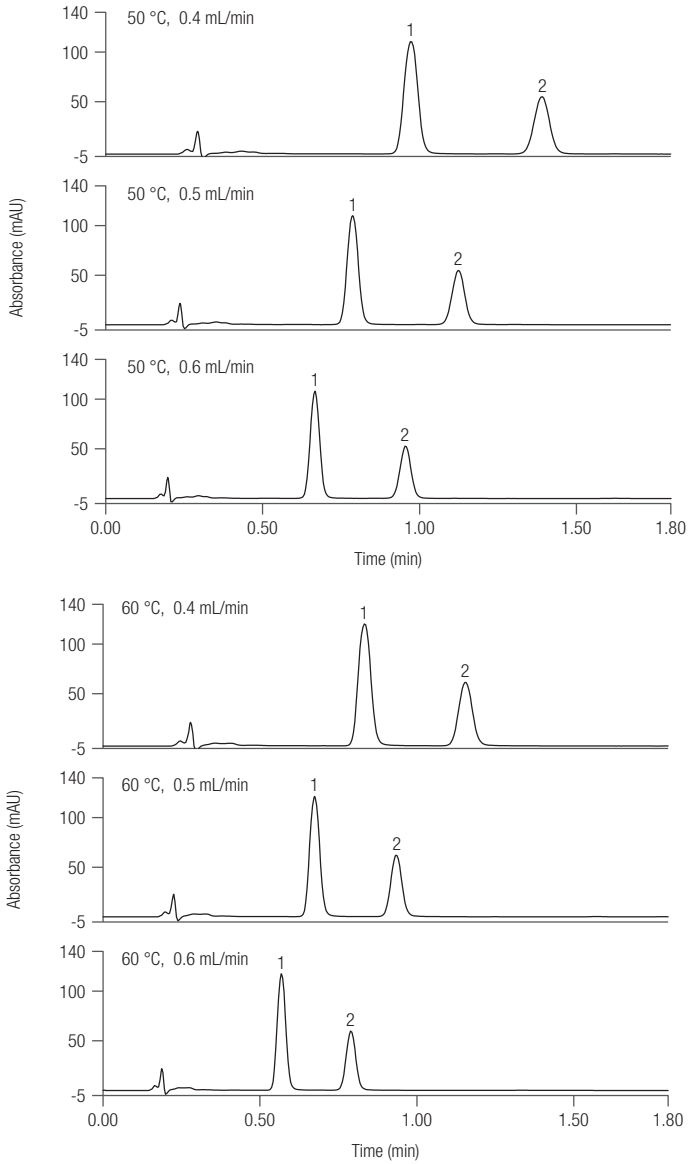

Figure 3. Chromatograms showing separation of cephalexin and cephradine under different temperature and flow rate.

Table 2. Retention time and resolution data from experiments at two temperatures and three flow rates.

	Temp. (°C)	Flow Rate (mL/min)		
		0.4	0.5	0.6
RT cephradine (min)	50	1.390	1.127	0.966
Rs		4.9	4.9	4.8
RT cephradine (min)	60	1.155	0.935	0.790
Rs		4.3	4.3	4.2

Some customers prefer working with lower column temperatures so this was set to 50 °C to collect method reproducibility data using a flow rate of 0.6 mL/min. Twenty-four replicate injections were made under these conditions (Figure 4).

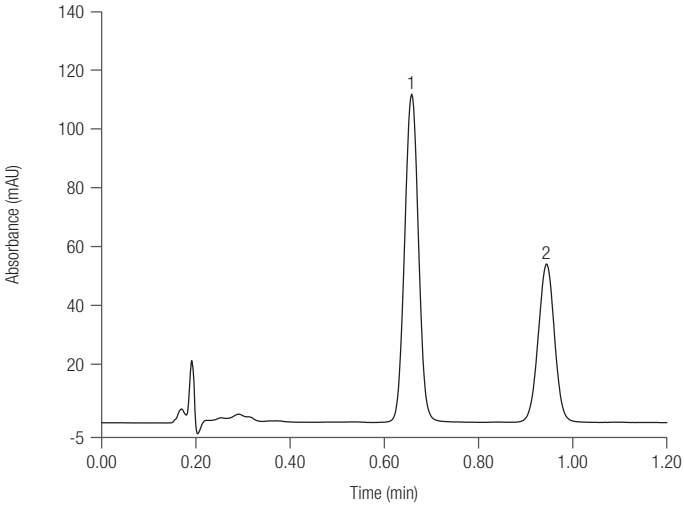

Figure 4. Overlay of 24 replicate injections of standards mixture at 50 °C and a flow rate of 0.6 mL/min. RT RSD for cephradine was 0.14%.

By applying the developed method, the method time has been decreased from ~20 minutes to 1.2 minutes. The consumption of mobile phase (and generation of waste) per assay has also been reduced, from 20 mL to 0.72 mL, thus contributing a saving in both assay cost and an increase in throughput (Figure 5).

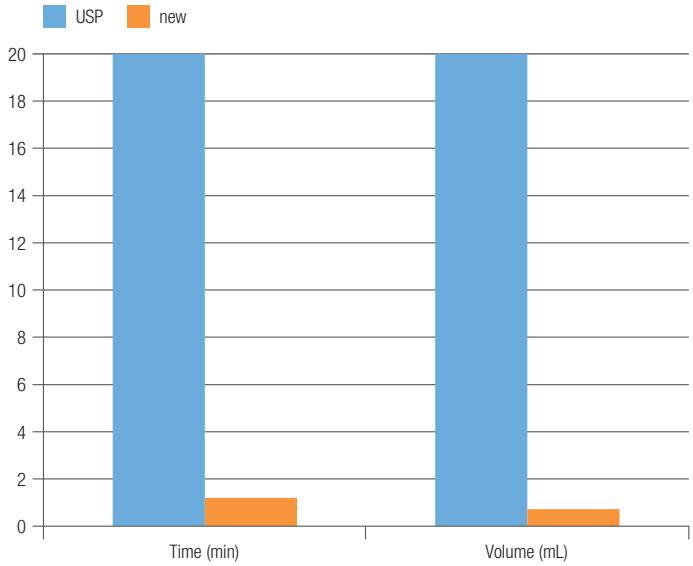

Figure 5. Indicative savings in time and mobile phase volume between the original USP and the improved method.

Conclusions

A high-throughput assay for cephradine was developed keeping to the spirit of USP-NF Chapter <621> guidelines for method modernization that significantly increased throughput and maintains USP quality acceptance criteria. When compared to the original USP method, the updated method demonstrates the following:

- Significant increase in assay throughput (seventeen fold) allowing fifty samples per hour to be assessed
- Substantial associated cost reduction, through reduced mobile phase consumption and waste generation
- Associated reduced method complexity from simplified mobile phase preparation

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APPLICATION UPDATE 72648

Determination of gentamicin and related impurities in gentamicin sulfate using simple eluents

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Keywords

Dionex IonPac AmG-3µm C18 column, aminoglycoside, Dionex ICS 5000+ system, PAD, electrochemical detection, drug substance, antibiotic, USP, EP, Dionex ICS-6000 system

Goal

To simplify and speed up the determination of gentamicin composition and impurities using a Thermo Scientific™ Dionex™ IonPac™ AmG-3µm C18 column

Introduction

The analysis of gentamicin sulfate in pharmaceutical formulations based on an ion-pairing HPLC-PAD method using a C18 silica-based column is described in United States Pharmacopeia (USP) and The European Pharmacopeia (EP) monographs.[1-3] Application Note 72647[4] has demonstrated that the USP Gentamicin Sulfate monograph Content of Gentamicins method and the USP in-process revision Gentamicin Sulfate monograph method for organic impurities could be successfully executed with a Thermo Scientific™ Dionex™ IonPac™ AmG-3µm C18 column using either the 4- or 3-potential carbohydrate waveform. The separation, linearity, reproducibility, and sensitivity were found to meet or exceed the current USP performance requirements.

The eluent of the USP and EP monograph methods contains trifluoroacetic acid, pentafluoropropionic acid, and acetonitrile. Eluent (mobile phase) pH is adjusted to 2.6 with sodium hydroxide to avoid silica-bonded phase hydrolysis when exposed to lower pH conditions. The Dionex IonPac AmG-3µm C18 columns are specifically designed for ion-pairing reversed-phase analysis of various aminoglycoside antibiotics with superior resistance towards acidic conditions.[5] Therefore, an aqueous TFA solution can be used as the eluent without adjusting its pH to a higher value.

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Table of contents

In this application update, the eluent in USP/EP monograph is modified in two ways, with each modification used to make a new method. Method A uses 100 mM TFA as the eluent. Method B is similar to Method A, but it includes 2% acetonitrile to accelerate the analysis. Because sodium hydroxide was not added into the eluents, the pH is lower than in the USP/EP monograph method. Therefore, both methods use 0.76 M NaOH as the post-column agent instead of 0.5 M NaOH used in the USP/EP monograph to achieve a similar pH for detection. The system suitability of each method was evaluated and compared with the monograph performance requirements. Two samples were analyzed. The percentage of gentamicin C major components results were compared with USP acceptance criteria. Impurity results were compared with the acceptance criteria of the EP Gentamicin Sulfate monograph and USP Gentamicin Sulfate in-process revision monograph.

Experimental

Equipment

- Thermo Scientific™ Dionex™ ICS-5000⁺ HPIC™ system including*:
  - Dionex ICS-5000⁺ DP Pump module
  - Dionex ICS-5000⁺ DC Detector/Chromatography module with ED Electrochemical Detector
  - Dionex AS-AP Autosampler with 250 µL sample syringe (P/N 074306) and 1200 µL buffer line (P/N 074989) and 1.5 mL vial trays (P/N 074936)
- Dionex ICS-5000⁺ ED Electrochemical Detector Cell (P/N 072044)
- ED conventional working electrode, gold, 3 mm (P/N 063723) with 5 mil gasket (P/N 063550)
- Reference electrode pH, Ag/AgCl (P/N 061879)
- Knitted reaction coil, 375 µL, unpotted (P/N 043700)
- Three-way manifold (P/N 48227)
- Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software, version 7.2.5

*This method can be run on a single Dionex ICS-5000⁺ system or the Thermo Scientific™ Dionex™ ICS-6000 system using a Thermo Scientific™ Dionex™ AXP pump to add the post-column reagent.

The procedure for system preparation and setup can be found in Thermo Scientific Application Note 72647[4] with support from specific product manuals.[5-8]

Consumables

- Glass autosampler vials 1.5 mL with slit septum (P/N 055427)
- Thermo Scientific™ Nalgene™ Rapid-Flow™ sterile disposable filter units with nylon membrane (1000 mL, 0.2 µm pore size, Fisher Scientific P/N 09-740-46)
- Nitrogen, ultrahigh purity

Reagents and standards

- Deionized (DI) water, Type I reagent grade, 18 MΩ·cm resistivity or better
- Trifluoroacetic acid (Fisher Scientific P/N PI28901)
- Sodium hydroxide (w/w) 50% (Fisher Scientific P/N SS254-500)
- Acetonitrile (Fisher Scientific P/N A955-4)
- USP Gentamicin Sulfate Reference standard, (Sigma-Aldrich® P/N 1289003-200MG)
- USP Sisomicin Sulfate Reference standard, (Sigma-Aldrich P/N 1612801-500MG)

Samples

Two gentamicin samples were purchased from Sigma-Aldrich. Sample #1 claims to meet all USP specifications and sample #2 does not make that claim.

Chromatographic conditions

Columns:	Dionex IonPac AmG-3µm C18 Guard, 4 × 30 mm (P/N 302694) Dionex IonPac AmG-3µm C18 Separation, 4 × 150 mm (P/N 302693)
Eluent:	Method A: 100 mM TFA Method B: 100 mM TFA (98%) + acetonitrile (2%)
Flow Rate:	0.8 mL/min*
Column Temperature:	35 °C
Injection Volume:	20 µL (Full loop)
Auto Sampler Temperature:	5 °C
Reference Electrode:	Ag/AgCl
Working Electrode:	Conventional electrode gold, 3 mm diameter with a 5-mil gasket
Post-column Reagent:	0.76 M NaOH
Post-column Reagent Flow Rate:	0.3 mL/min delivered by pump 2
Detection:	Pulsed amperometric detector (electrochemical detector)
Detection Compartment Temperature:	35 °C
Detection Waveform:	Gold, Carbohydrates, 4-potential (Table 1)
System Backpressure:	~ 2600 psi
Run Time:	Method A: 65 min Method B: 25 min

*The USP monograph describes the column as follows: Type – L1 (i.e. C18) size 250 mm, ID 4.6 mm; 5 µm packing L1. The diameter of the Dionex IonPac AmG-3µm C18 column is 4 mm. Therefore, the flow rate was adjusted from 1 mL/min (USP monograph condition) to 0.8 mL/min.

Table 1. Carbohydrates, 4-potential waveform

Time (s)	Voltage (V)	Integration
0	0.1	Off
0.20	0.1	On
0.40	0.1	Off
0.41	-2.0	Off
0.42	-2.0	Off
0.43	0.6	Off
0.44	-0.1	Off
0.50	-0.1	Off

Preparation of solutions and reagents

Eluent

Method A, 100 mM TFA

To prepare 2 L of eluent, add 15.3 mL of trifluoroacetic acid into a glass 2 L volumetric flask containing approximately 1800 mL of degassed DI water. Immediately transfer this solution to a glass eluent bottle and blanket it with nitrogen at 5 to 8 psi.

Method B, 100 mM TFA (98%) + acetonitrile (2%)

Mix 100 mM TFA and acetonitrile at a ratio of 98:2 using IC pump channels A and B. Alternatively, the eluent could be premixed and one channel used (not tested).

Post-column reagent (0.76 M NaOH)

To prepare 1 L of post-column reagent, add 40.0 mL of 50% (w/w) NaOH into a plastic 1 L volumetric flask containing approximately 800 mL of degassed DI water. Briefly stir this solution (15–30 s) and then bring to volume. Immediately transfer this solution to the plastic eluent bottle on the HPAE-PAD system and blanket it with nitrogen at 5 to 8 psi. Gently swirl the bottle to complete mixing. Always maintain the eluents under 5 to 8 psi of nitrogen to reduce diffusion of atmospheric carbon dioxide. Prepare new NaOH eluent if left unblanketed for more than 30 min.

Stock standard solutions

Gentamicin sulfate stock, 1 mg/mL

Dissolve 25 mg of USP grade gentamicin sulfate in 25 mL of eluent.

Sisomicin sulfate stock, 1 mg/mL

Dissolve 25 mg of USP grade sisomicin sulfate in 25 mL of eluent.

Working standard solutions

Gentamicin sulfate standard, 0.2 mg/mL

Dilute 5 mL of gentamicin sulfate stock to 25 mL with eluent.

Sisomicin standard, 10 µg/mL

Dilute 1 mL of sisomicin standard stock to 100 mL with eluent.

System suitability solution, (100 µg/mL USP Gentamicin Sulfate RS and 20 µg/mL of USP Sisomicin Sulfate RS in eluent)

To 5 mL of gentamicin sulfate stock standard, add 1 ml of sisomicin sulfate stock standard, and dilute to 50 mL with eluent.

Sample preparation

Sample solution (a), 1 mg/mL

Dissolve 25 mg of sample in 25 mL of eluent. Use this sample preparation for impurity analysis.

Sample solution (b), 0.2 mg/mL

Dilute 5 mL of sample solution (a) to 25 mL with eluent. Use this sample preparation for the Content of Gentamicins analysis.

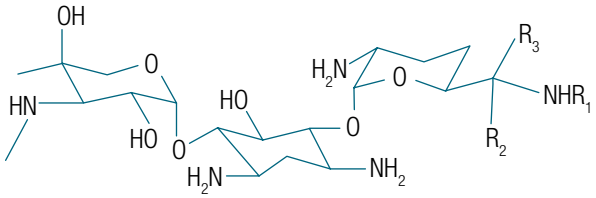
Note: Store all standards and samples in a refrigerator after preparation.

Results and discussion

System suitability

In the USP monograph for gentamicin sulfate (Figure 1), the system suitability requirements specify resolution between gentamicin C2 and gentamicin C2b as >1.5. The EP gentamicin sulfate monograph includes two additional requirements: Signal-to-noise ratio (S/N) >20 for 10 µg/mL sisomicin and resolution >1.2 between sisomicin and gentamicin C1a.

The system suitability was evaluated using the chromatograms of the system suitability standard and 10 µg/mL sisomicin sulfate. Figure 2 shows this separation with a Dionex IonPac AmG-3µm C18 column set using the two methods. The five congeners (C1, C1a, C2, C2a, and C2b) and sisomicin were well separated using both methods. TFA acts as the ion-pairing



	R_1	R_2	R_3
C_{1a}	H	H	H
C_2	H	CH_3	H
C_{2b}	CH_3	H	H
C_{2a}	H	H	CH_3
C_1	CH_3	CH_3	H

Figure 1. Structure of gentamicin

agent and plays an important role in the gentamicin separation. Gentamicin separation is normally completed in 60 min when using 100 mM TFA as the eluent (Figure 2A). To accelerate the separation, 2% acetonitrile was added to the 100 mM TFA eluent, and this resulted in a separation that was less than 25 min (Figure 2B).

Column:	Dionex IonPac AmG-3µm C18 Guard, 4 × 30 mm (P/N 302694) Dionex IonPac AmG-3µm C18 Separation, 4 × 150 mm (P/N 302693)
Eluent:	A) 100 mM Trifluoroacetic acid, B) 100 mM Trifluoroacetic acid (98%) + Acetonitrile (2%)
Inj. Volume:	20 µL
Column Temp.:	35 °C
Flow Rate:	0.8 mL/min
Postcolumn Reagent:	0.76 M NaOH (0.3 mL/min)
Detection:	Pulsed Amperometric Detector (Waveform: Carbohydrates, 4-Potential)

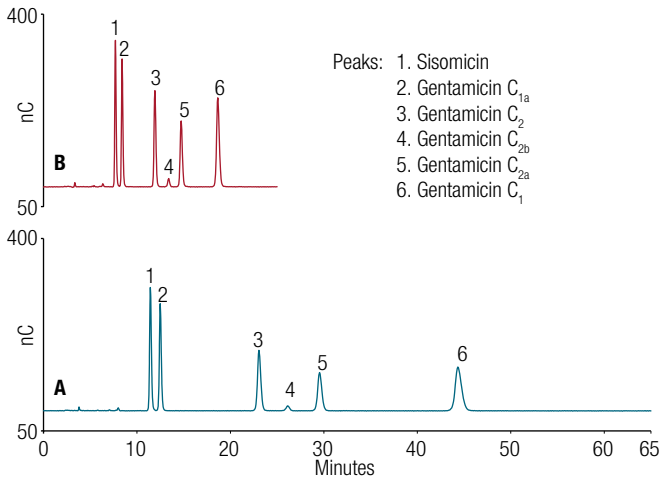


Figure 2. Separation of system suitability standard

Figure 3 shows chromatograms of sisomicin sulfate using both methods. Sisomicin is detected with good sensitivity using either method.

Column:	Dionex IonPac AmG-3µm C18 Guard, 4 × 30 mm (P/N 302694) Dionex IonPac AmG-3µm C18 Separation, 4 × 150 mm (P/N 302693)
Eluent:	A) 100 mM Trifluoroacetic acid, B) 100 mM Trifluoroacetic acid (98%) + Acetonitrile (2%)
Inj. Volume:	20 µL
Column Temp.:	35 °C
Flow Rate:	0.8 mL/min
Postcolumn Reagent:	0.76 M NaOH (0.3 mL/min)
Detection:	Pulsed Amperometric Detector (Waveform: Carbohydrates, 4-Potential)
Peak:	1. Sisomicin 10 µg/mL

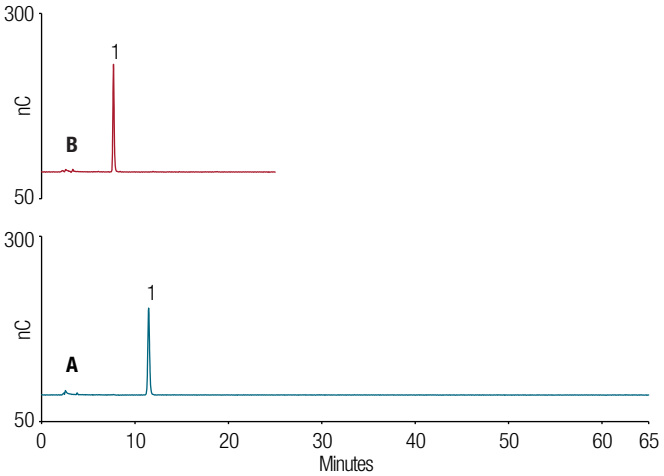


Figure 3. Sisomicin (10 µg/mL)

Using either method the system suitability requirements are met for all parameters (Table 2). Peak resolution between C2 and C2b is 4.53 and 3.97 for Methods A and B, respectively, exceeding the USP and EP requirement of 1.5. Peak resolution between sisomicin and C1a is 2.85 and 2.63 for Method A and Method B, respectively, exceeding the EP requirement of 1.2. The S/N of 10 µg/mL sisomicin sulfate is 248 and 242 for Methods A and B, respectively, easily exceeding the EP requirement of 20.

Sample analysis

Content of gentamicins analysis

Standard and sample solution (b) were used for content of gentamicins analysis. Figure 4 shows the separation of a USP gentamicin standard using both methods. The five gentamicin constituents were well separated from each other. Figure 5 shows the separation of gentamicin sample #1 (0.2 mg/mL) using both methods. A few impurities were detected and they were separated from the five gentamicin constituents. Figure 6 shows the separation of gentamicin sample #2 (0.2 mg/mL) using both methods, more than 20 impurities were observed and they were separated from the five gentamicin constituents.

Column:	Dionex IonPac AmG-3µm C18 Guard, 4 × 30 mm (P/N 302694) Dionex IonPac AmG-3µm C18 Separation, 4 × 150 mm (P/N 302693)
Eluent:	A) 100 mM Trifluoroacetic acid, B) 100 mM Trifluoroacetic acid (98%) + Acetonitrile (2%)
Inj. Volume:	20 µL
Column Temp.:	35 °C
Flow Rate:	0.8 mL/min
Postcolumn Reagent:	0.76 M NaOH (0.3 mL/min)
Detection:	Pulsed Amperometric Detector (Waveform: Carbohydrates, 4-Potential)

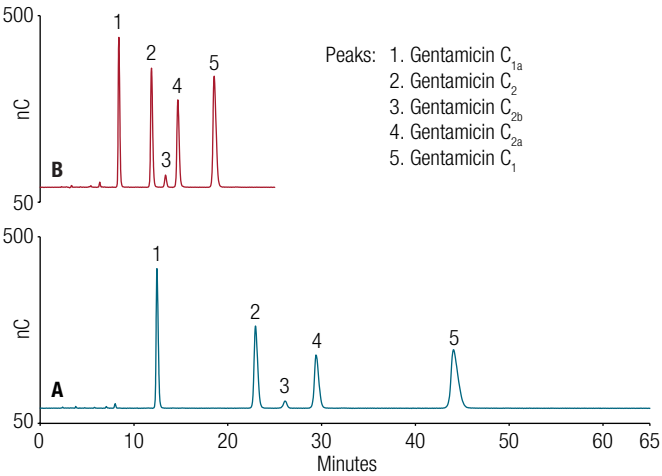


Figure 4. Separation of a gentamicin USP reference standard (0.2 mg/mL) using Methods A and B

Table 2. System suitability

Test	EP Criteria	Measured (Method A)	Measured (Method B)
Resolution between Sisomicin and C1a	>1.2	2.85	2.63
Resolution between C2 and C2b	>1.5*	4.53	3.97
S/N (Sisomicin 10 µg/mL)	>20	248	242

*Also the USP criterion

Column:	Dionex IonPac AmG-3µm C18 Guard, 4 × 30 mm (P/N 302694) Dionex IonPac AmG-3µm C18 Separation, 4 × 150 mm (P/N 302693)
Eluent:	A) 100 mM Trifluoroacetic acid, B) 100 mM Trifluoroacetic acid (98%) + Acetonitrile (2%)
Inj. Volume:	20 µL
Column Temp.:	35 °C
Flow Rate:	0.8 mL/min
Postcolumn Reagent:	0.76 M NaOH (0.3 mL/min)
Detection:	Pulsed Amperometric Detector (Waveform: Carbohydrates, 4-Potential)

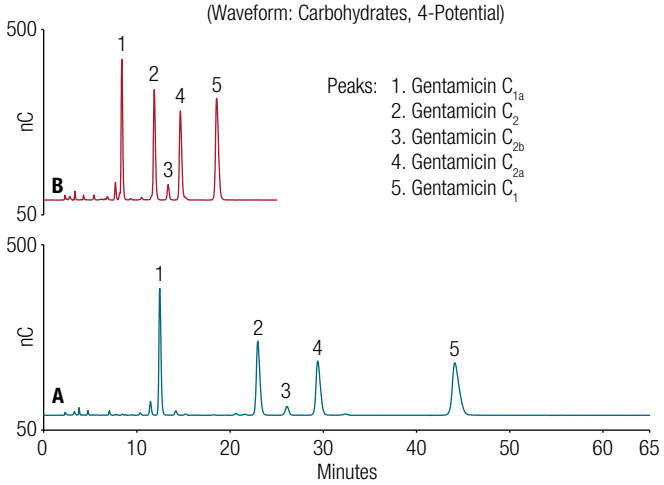


Figure 5. Separation of gentamicin sample #1 (0.2 mg/mL) using Methods A and B

Column:	Dionex IonPac AmG-3µm C18 Guard, 4 × 30 mm (P/N 302694) Dionex IonPac AmG-3µm C18 Separation, 4 × 150 mm (P/N 302693)
Eluent:	A) 100 mM Trifluoroacetic acid, B) 100 mM Trifluoroacetic acid (98%) + Acetonitrile (2%)
Inj. Volume:	20 µL
Column Temp.:	35 °C
Flow Rate:	0.8 mL/min
Postcolumn Reagent:	0.76 M NaOH (0.3 mL/min)
Detection:	Pulsed Amperometric Detector (Waveform: Carbohydrates, 4-Potential)

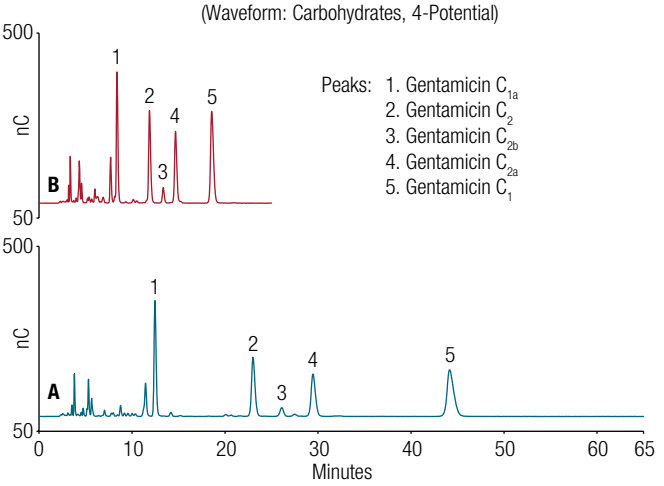


Figure 6. Separation of gentamicin sample #2 (0.2 mg/mL) using Methods A and B

The relative percentage of each gentamicin derivative in the USP reference standard and the two samples were calculated using the peak areas obtained from the chromatograms shown in Figures 4, 5, and 6. The calculation method is shown below:

Result = (*rU*/*rT*) × 100

rU = Peak area response corresponding to the particular gentamicin from the sample solution

rT = Sum of all peak area response of gentamicin C1a, gentamicin C2, gentamicin C2a, gentamicin C2b, and gentamicin C1 from the sample solution

As shown in Tables 3 and 4, both samples met the USP acceptance criteria. The results agree with the results from the same samples reported in Application Note 72647.

Percentage of impurities in gentamicin sulfate samples

Sample solutions (a) were used for impurities analysis. Figures 7 and 8 show the chromatograms of samples #1 and #2, respectively. The five times greater concentration of these samples compared to the samples used for the Content of Gentamicins analysis allows the impurity peaks to be more easily observed.

Table 3. Percentage of each gentamicin in gentamicin sulfate (Method A)

Test	C1a	C2	C2b	C2a	C1	C2+C2a	C2b+C1
USP Standard	23.3	23.2	2.1	18.6	32.7	41.8	34.9
Sample #1	22.6	22.7	2.9	20.7	31.2	43.3	34.1
Sample #2	24.3	21.0	3.4	18.8	32.5	39.8	35.9
USP Acceptance Criteria	10–35					25–55	25–50

Table 4. Percentage of each gentamicin in gentamicin sulfate (Method B)

Test	C1a	C2	C2b	C2a	C1	C2+C2a	C2b+C1
USP Standard	22.8	22.9	2.3	19.5	32.5	42.4	34.8
Sample #1	22.4	22.4	3.0	21.0	31.2	43.4	34.2
Sample #2	23.8	21.5	3.5	19.3	31.9	40.8	35.4
USP Acceptance Criteria	10–35					25–55	25–50

Column: Dionex IonPac AmG-3µm C18 Guard, 4 × 30 mm (P/N 302694)
Dionex IonPac AmG-3µm C18 Separation, 4 × 150 mm (P/N 302693)
Eluent: A) 100 mM Trifluoroacetic acid,
B) 100 mM Trifluoroacetic acid (98%) + Acetonitrile (2%)
Inj. Volume: 20 µL
Column Temp.: 35 °C
Flow Rate: 0.8 mL/min
Postcolumn Reagent: 0.76 M NaOH (0.3 mL/min)
Detection: Pulsed Amperometric Detector (Waveform: Carbohydrates, 4-Potential)
Peak: 1. Sisomicin

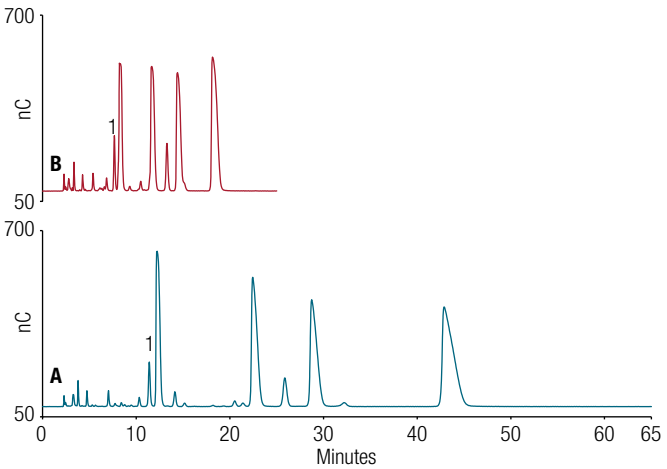


Figure 7. Separation of Gentamicin sample #1 (1 mg/mL) using Methods A and B

Column: Dionex IonPac AmG-3µm C18 Guard, 4 × 30 mm (P/N 302694)
Dionex IonPac AmG-3µm C18 Separation, 4 × 150 mm (P/N 302693)
Eluent: A) 100 mM Trifluoroacetic acid,
B) 100 mM Trifluoroacetic acid (98%) + Acetonitrile (2%)
Inj. Volume: 20 µL
Column Temp.: 35 °C
Flow Rate: 0.8 mL/min
Postcolumn Reagent: 0.76 M NaOH (0.3 mL/min)
Detection: Pulsed Amperometric Detector (Waveform: Carbohydrates, 4-Potential)
Peak: 1. Sisomicin

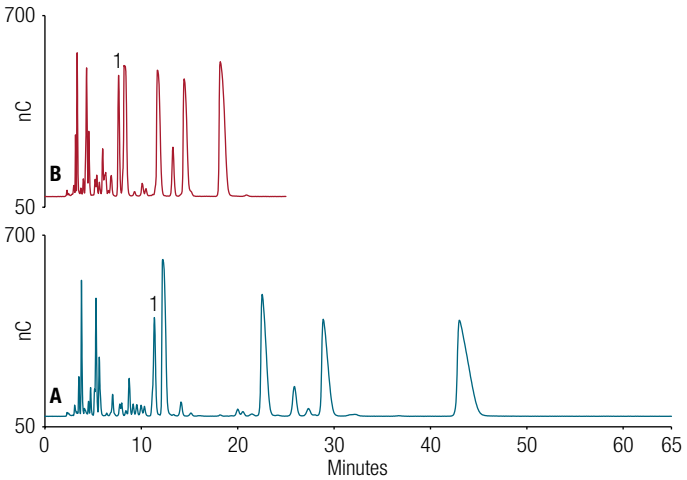


Figure 8.Separation of gentamicin sample #2 (1 mg/mL) using Methods A and B

EP Gentamicin Sulfate monograph and the USP in-process revision of the Gentamicin Sulfate monograph describe acceptance criteria for impurity levels in commercial samples. For that purpose, all impurities were calculated using the peak areas obtained from the chromatogram of the sample solutions (Figures 7 and 8) and compared to the response of the principal impurity sisomicin obtained from the chromatogram of 10 µg/mL sisomicin sulfate (Figure 3).

Result = (*rU/rs*) × (*Cs/Cu*) × 100

rU = Peak response of each individual impurity from the 1 mg/mL sample solution

rs = Peak response of sisomicin from the 10 µg/mL standard solution

Cs = Concentration of USP Sisomicin Sulfate RS in the standard solution (mg/mL)

Cu = Concentration of Gentamicin Sulfate in the sample solution (mg/mL)

Tables 5 and 6 show the percentage of sisomicin and total impurities of samples #1 and #2 using Methods A and B, respectively, and compare this with the USP acceptance criteria. Sample #1 met all USP impurity acceptance criteria as was claimed in its product description. Sample #2 did not pass the USP sisomicin and total impurities criteria. The results agree with the results for the same samples reported in Application Note 72647.

Conclusion

This application update demonstrated that gentamicin sulfate and related impurities can be separated with a Dionex IonPac AmG-3µm C18 column using two modified methods. Method A is a simple eluent method (100 mM TFA). Method B is a fast method that involves the addition of 2% acetonitrile to the eluent to accelerate the separation 2.5 times without compromising resolution and column performance. The separation and sensitivity of both methods were found to meet or exceed the current USP/EP Gentamicin Sulfate monograph performance requirements.

Table 5. Percentage of impurities in gentamicin sulfate (Method A)

	Sisomicin	Any Other Individual Impurity	Total Impurities
Sample #1	1.29	<1.29	4.7
Sample #2	3.05	<2.73	15.5
EP Monograph/ USP In-process Revision Acceptance Criteria	3.0	3.0	10

Table 6. Percentage of impurities in gentamicin sulfate (Method B)

	Sisomicin	Any Other Individual Impurity	Total Impurities
Sample #1	1.29	<1.29	4.7
Sample #2	3.05	<2.73	15.5
EP Monograph/ USP In-process Revision Acceptance Criteria	3.0	3.0	10

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