CLINICAL & FORENSIC

Online Solid Phase Extraction and LC-MS Analysis of Thyroid Hormones in Human Serum

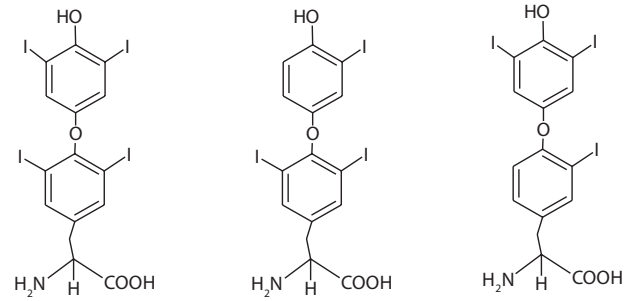
Hillel Brandes, Analytical Technology Specialist, **hillel.brandes@milliporesigma.com**

Introduction

Thyroid hormones play critical roles in the regulation of biological processes, such as: growth, metabolism, protein synthesis, and brain development. Specifically, both 3,3′,5,5′-tetraiodo-L-thyronine (thyroxine or T4) and 3,3′,5-triiodo-L-thyronine (T3), are essential for development and maintenance of normal physiological functions. For a clinical laboratory, measurements of total T4 and total T3, along with estimates of free T4 (FT4) and free T3 (FT3), are important for the diagnosis and monitoring of thyroid diseases. Most clinical laboratories measure thyroid hormones using immunoassays. The immunoassay-based methods offer a relatively rapid, high patient sample throughput that lends itself to automation, but are significantly compromised by problems with assay interference and are complicated by changes in protein levels that alter the free hormone availability.[1]

Liquid chromatography mass spectrometry (LC-MS) has been reported to offer superior specificity and speed over the immunoassays for determination of thyroid hormones in biological matrices such as serum and tissues. Nevertheless, the reported sample preparation procedures, typically liquid-liquid extraction followed by solid phase extraction (SPE), involve multiple time consuming steps, and are less compatible with automation.[2,3] The present work demonstrates successful online SPE with LC-MS for rapid determination of T4, T3, and 3,3',5'-triiodo-L-thyronine (rT3) from biological matrices.

Figure 1. Chemical structures of the thyroid analytes



Thyroxine (T4), $C_{15}H_{11}I_4NO_4$, Monoisotopic mass: 776.686 Da

3, 3', 5-Triiodothyronine (T3), $C_{15}H_{12}I_3NO_4$, Monoisotopic mass: 650.790 Da

3, 3', 5'-Triiodothyronine (Reverse T3), $C_{15}H_{12}I_3NO_4$, Monoisotopic mass: 650.790 Da

Note T3 and rT3 are isobaric

Experimental

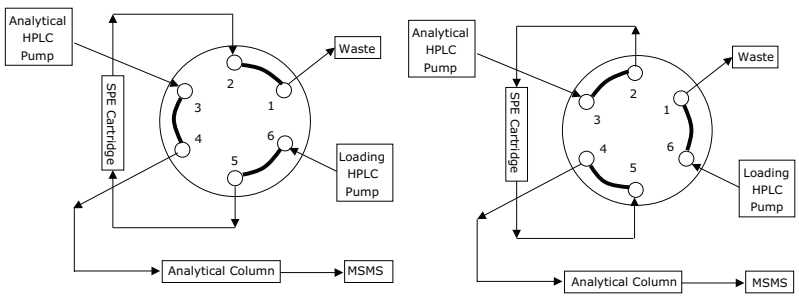
Materials: Supel™ Genie C8 and RP-Amide (RPA) online cartridges (2 cm × 4.0 mm I.D.), human serum (Cat. No. H1388), protein precipitation solvent: methanol with 1 % (w/v) ammonium formate.

Sample Processing Procedure: the human serum spiked with analytes was protein precipitated by vortex mixing with the precipitation solvent at a 1:3 ratio. Then the mixture was centrifuged at 10,000 x g for 3 min and the resulting supernatant was collected and directly injected for LC-MS analysis.

Online SPE-LC-MS Setup: As shown in **Figure 2.**, the setup consists of a 6-port switching valve and two

Figure 2. Configuration of the online SPE-LC-MS system

column:	Ascentis® Express Biphenyl, 10 cm × 2.1 mm I.D., 2.7 µm (64065-U)
mobile phase:	(A) water; (B) methanol, each with 0.1 % acetic acid
isocratic:	70 % B for 10 min
flow:	0.3 mL/min
column temp:	35 °C
SPE online cartridge:	Supel™ Genie C8 and RP-Amide, 2 cm x 4 mm I.D.
sample loading/ washing:	Equilibrate in Pos-1: 2.5 min (10 % MeOH). Load sample by injecting 2 µl in Pos-1. Leave to wash for 2 min. Switch to Pos-2 for online analytical separation (70 % MeOH, reverse flow)
sample loading solvent:	10 % methanol
injection vol:	2 µL injection
detection:	MS, ESI(+), MRM mode
instrument:	Shimadzu® LCMS-8030 with 2DLC setup



Position-1: Sample loading/washing

Position-2: Online SPE → LC-MS analysis

pumps; one for sample loading and washing, the other for sample elution. To minimize the potential peak broadening from the cartridges, the flow of sample loading/washing and the subsequent elution are in reversed directions.

Results and Discussion

The conventional (off-line) sample preparation by SPE typically involves multiple labor-intensive and time-consuming steps, including: conditioning, sample loading, washing, elution, and finally evaporation and reconstitution of the sample in mobile phase. The Supel™ Genie C8 and RPA online cartridges have been developed to automate the sample preparation process, minimize hands-on time and human error, and reduce overall sample processing time. The present work utilized the C8 and RPA online cartridges with LC-MS for the detection of thyroid hormones from human serum. **Figures 3** and **4** show the representative LC-MS chromatograms of T3, rT3, and T4 spiked in human serum with C8 and RPA online cartridges, respectively. The human serum samples were simply protein precipitated with methanol containing ammonium formate and then directly injected for online SPE and LC-MS analysis. The sample loading/washing were carried out entirely by the instrument, without any hands-on effort. Additionally, the time-consuming solvent evaporation and reconstitution steps were eliminated.

As can been seen from **Figures 3** and **4**, both C8 and RPA were capable of capturing a trace amount (100 ng/mL x 2 µL in this case) of thyroid hormones from complicated human serum. All three analytes are resolved from each other, with a peak width at half height <6s and tailing factor from 1.4-1.8. The total run time is within 6 min.

Tables 1 and **2** show the ruggedness of the online SPE-LC-MS with C8 and RPA cartridges, respectively, from 120 consecutive injections of human serum samples. As can be seen, the retention times of the analytes with C8 or RPA are very reproducible, with RSD's of 0.1 % - 0.2 %. Reproducibility (%RSD) of the peak areas for both C18 and RPA were very good, with %RSDs of 6.2 % - 7.0 % and 5.1 % - 7.7 %, respectively.

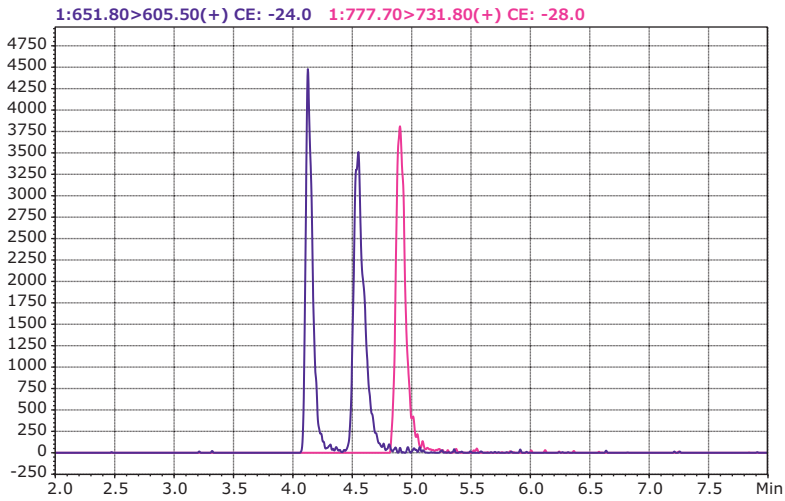
Table 1. Ruggedness of the System with C8 Cartridge

Analyte	MRM Quantifier	Retention Time (Min) (Avg. n = 120)	Retention Time Reproducibility (%RSD, n=120)	Peak Area (Avg. n = 120)	Peak Area Reproducibility (%RSD, n = 120)
3,3',5-triiodo-L-thyronine (T3)	651.8 / 605.5	4.13	0.1	17711	6.9
3,3',5-triiodo-L-thyronine (rT3)	651.8 / 605.5	4.53	0.2	22081	7
3,3',5,5'-tetraiodo-L-thyronine (T4)	777.7 / 731.8	4.89	0.1	22233	6.2

Table 2. Ruggedness of the System with RPA Cartridge

Analyte	MRM Quantifier	Retention Time (Min) (Avg. n = 120)	Retention Time Reproducibility (%RSD, n=120)	Peak Area (Avg. n = 120)	Peak Area Reproducibility (%RSD, n = 120)
3,3',5-triiodo-L-thyronine (T3)	651.8 / 605.5	4.03	0.2	27046	5.1
3,3',5-triiodo-L-thyronine (rT3)	651.8 / 605.5	4.43	0.2	33723	6.2
3,3',5,5'-tetraiodo-L-thyronine (T4)	777.7 / 731.8	4.79	0.2	23766	7.7

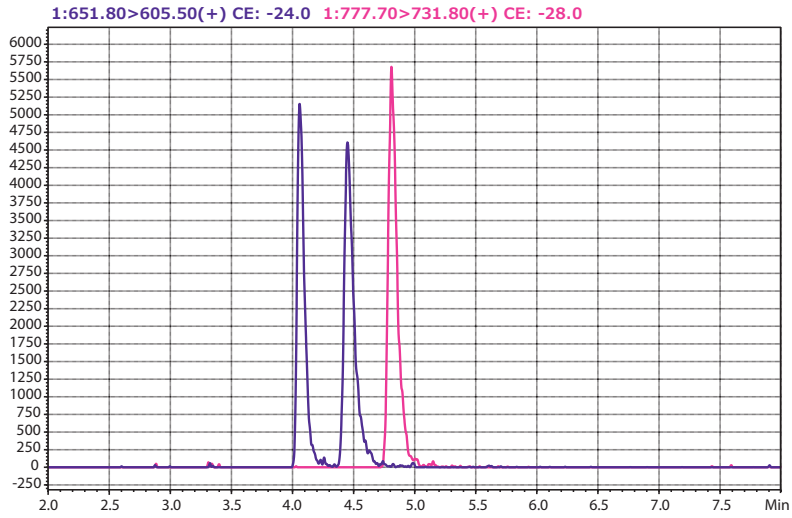
Figure 3. Representative LC-MS chromatogram of thyroid hormones in human serum with C8 online cartridge



Peak	Analyte	Peak Width at 50 % Height (s)	Tailing Factor
1	T3	3.7	1.6
2	rT3	5.2	1.5
3	T4	4.9	1.4

(continued on next page)

Figure 4. Representative LC-MS chromatogram of thyroid hormones in human serum with RPA online cartridge



Peak	Analyte	Peak Width at 50% Height (s)	Tailing Factor
1	T3	3.7	1.6
2	rT3	5.2	1.5
3	T4	4.9	1.4

Comparing the two online cartridges, RPA appears to deliver better signals (peak height and area) for all three thyroid analytes compared with the C8 cartridge. The mechanism behind this is not clear, however, the RPA is known to offer better retention for analytes with polar moieties which form hydrogen bonds. Otherwise C8 and RPA provide similar results in terms of peak shape and reproduciblity of peak area.

Summary

An online SPE-LC-MS method has been developed for the rapid detection of thyroid hormones in human serum with minimal hands-on effort and time-consuming steps. Both C8 and RP-Amide online cartridges were shown to be capable of capturing a trace amount of analyte from protein precipitated human serum samples. All three analytes, T3, rT3 and T4 were resolved on a Biphenyl column, with sharp and symmetric peak shapes. In addition, reproducibility (%RSD) of the retention times of the thyroid hormones from 120 consecutive injections is between 0.1% and 0.2%, with either C8 or RPA online cartridges, while the peak area reproducibility (%RSD) is between 5.1% and 7.7%. These RSD's indicate great ruggedness of the online SPE-LC-MS system.

References

1. Kahric-Janicic N, Soldin SJ, Soldin OP, West T, Gu J, Jonklaas J, Tandem mass spectrometry improves the accuracy of free thyroxine measurements during pregnancy. *Thyroid*. 2007;*17(4)*: 303-11.
2. Susan S-C. Taia, Lorna T. Sniegoski and Michael J. Welch, Candidate Reference Method for Total Thyroxine in Human Serum Use of Isotope-Dilution Liquid Chromatography-Mass Spectrometry with Electrospray Ionization. *Clinical Chemistry*, 2002; *48(4)*: 637-642.
3. Dongli Wang and Heather M. Stapleton, Analysis of thyroid hormones in serum by liquid chromatography-tandem mass spectrometry. *Anal Bioanal Chem*. 2010; *397(5)*: 1831–1839.

Featured Products

Description	Cat. No.
Ascentis® Express Biphenyl HPLC Column, 10 cm × 2.1 mm I.D., 2.7 µm	**64065-U**
Supel™ Genie RP-Amide Online Starter Kit*	**55516-U**
Supel™ Genie RP-Amide Online SPE Cartridge, pk. of 2	**55519-U**
Supel™ Genie RP-Amide Online SPE Cartridge, pk. of 6	**55522-U**
Supel™ Genie C8 Online Starter Kit*	**55274-U**
Supel™ Genie C8 Online SPE Cartridge, pk. of 2	**55512-U**
Supel™ Genie C8 Online SPE Cartridge, pk. of 6	**55515-U**

Related Products

Description	Cat. No.
Supel™ Genie HybridSPE® Online Starter Kit*	**55324-U**
Supel™ Genie HybridSPE® Online SPE Cartridge, pk. of 2	**55326-U**
Supel™ Genie HybridSPE® Online SPE Cartridge, pk. of 6	**55327-U**
Thyroid Hormones	
L-Thyroxine (T4), 100 µg/mL in 0.1 N NH_3 in methanol, 1 mL	**T-073**
3,3',5-Triiodo-L-thyronine (T3), 100 µg/mL in 0.1 N NH_3 in methanol, 1 mL	**T-074**
3,3',5'-Triiodo-L-thyronine (reverse T3) 100 µg/mL in 0.1 N NH_3 in methanol), 1 mL	**T-075**
3,3',5-Triiodo-L-thyronine-$^{13}C_6$ (T3-$^{13}C_6$), 100 µg/mL in 0.1 N NH_3 in methanol, 1 mL	**T-077**
3,3´,5'-Triiodo-L-thyronine-$^{13}C_6$ (reverse T3-$^{13}C_6$), 100 µg/mL in 0.1 N NH_3 in methanol, 1 mL	**T-078**

* 1 Holder, 1 cartridge

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