Online SPE/LC-MS Method for the Rapid Analysis of Thyroid Hormones in Plasma

Using Ascentis® Express Fused-Core® Columns for Trapping and Separation

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The power of bonded phase selectivity in both sample prep and separation is demonstrated here. The Ascentis Express RP-Amide was shown to be a better trapping phase than conventional C8, and the Ascentis Express Phenyl-Hexyl phase provided baseline resolution of the closely-related compounds. The technique uses standard hardware and is a viable approach to solve other clinical and bioanalytical challenges.

Introduction

Thyroid hormones play critical roles in the regulation of biological processes such as growth, metabolism, protein synthesis and brain development. Specifically, thyroid hormones 3, 3′, 5, 5′-tetraiodo-L-thyronine (thyroxine or T4) and 3, 3′, 5-triiodo-L-thyronine (triiodothyronine or 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. The physiological role and need for testing of 3, 3´, 5´-triiodo-L-thyronine (reverse triiodothyronine or rT3) is debated in clinical research circles.

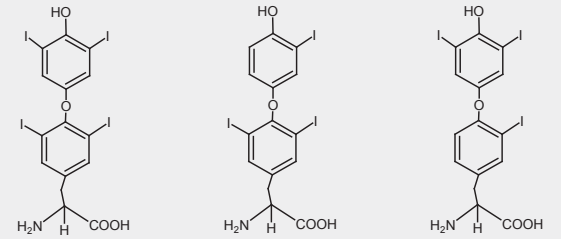
Analytical Challenges of Thyroid Hormone Measurement

Thyroid hormones are typically measured by an immunoassay technique that is subject to assay interference and easily perturbed by changes in protein levels that alter the free hormone availability.[1] Liquid chromatography with mass spectrometric detection (LC-MS) has been reported to offer superior specificity and speed over immunoassays for determination of thyroid hormones in biological matrices, such as serum, plasma and tissue. Nevertheless, the reported sample preparation procedures, typically by liquid-liquid extraction followed by solid phase extraction (SPE), involve multiple time-consuming steps, and are less compatible with automation.[2-3]

Study Goal and Overview of Approach

The present work exploited online SPE with LC-MS for rapid determination of T4, T3 and rT3 from plasma. Structures of these compounds appear in **Figure 1**. A 100 µL aliquot of rabbit plasma was spiked with T4, T3 and rT3. Protein was precipitated by addition of 25 µL $ZnCl_2$ and 200 µL methanol, vortex agitation for 1 minute and centrifugation at 9,000 *g* for 3 minutes. The resulting supernatant was collected and injected into the online SPE/LC-MS system described below. The LC-MS conditions are described in **Table 1**.

Figure 1. Chemical Structures of Thyroid Hormone 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 isomers.

Table 1. Online SPE LC-MS Method

instrument:	Shimadzu LCMS-8030
trapping column:	Ascentis Express RP-Amide (53514-U) or Ascentis Express C8 guard cartridges (53509-U), both 5 mm x 2.1 mm I.D., 2.7 µm particles
HPLC column:	Ascentis Express Phenyl, 5 cm x 2.1 mm I.D., 2.7 µm particles (53334-U)
mobile phase:	(A) water; (B) methanol, both with 0.1% acetic acid
column pressure:	1800 psi (88 bar)
injection vol:	5 µL
detection:	MS, ESI(+), MRM mode (777.7/731.8 for T4; 651.8/605.5 for T3 or rT3)

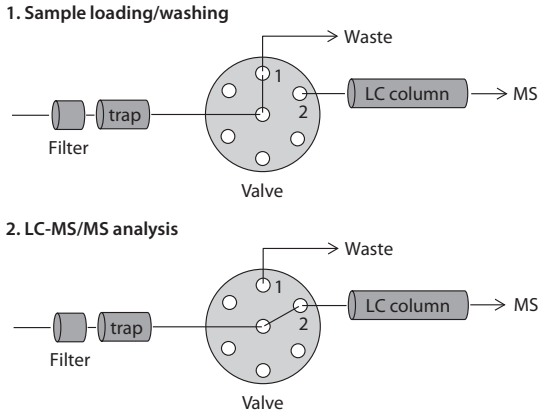
Online SPE/LC-MS Program

The online SPE/LC-MS system shown in **Figure 2** comprises a trapping column, a switching valve and an LC-MS instrument. The program consists of three distinct steps outlined in **Table 2**. The valve switches between position 1 for loading/washing (Step 1) and equilibration (Step 3), and position 2 for LC-MS analysis (Step 2). In Step 1 samples are loaded onto the trap and washed with mobile phase containing low percent organic and high flow rate to remove salts and other interferences which are directed to the waste. In Step 2 the analytes are eluted from the trap, separated and detected by the LC-MS at optimum flow for both chromatographic separation and MS signals. In Step 3, the system returns back to valve position 1 for re-equilibration under the sample loading/washing conditions.

Table 2. Online SPE/LC-MS Program

Final online SPE/LC-MS Program:	min	Flow (mL/min)	Valve	Pump B%
1. Sample loading/washing	0.00	0.4	1	5
	2.79	0.4	1	5
	2.80	0.25	1	5
2. LC-MS/MS	3.00	0.25	2	5
	5.00	0.25	2	70
	8.00	0.25	2	70
3. Equilibration	8.01	0.25	1	70
	8.09	0.25	1	70
	8.10	0.4	1	5
	12.0	0.4	1	5

Figure 2. Configuration of the LC-MS System and Valve Positions for Online SPE/LC-MS



Choosing the Trapping Column

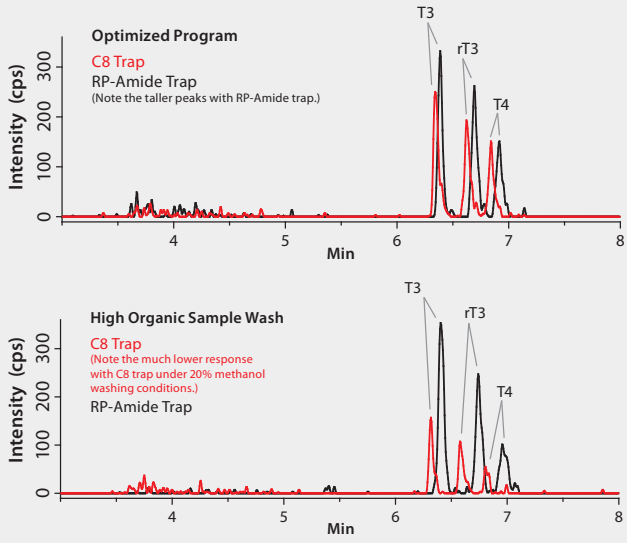
The choice of the right trapping column is critical for successful online SPE/LC-MS method development as it affects both trapping efficiency (e.g., the recovery of the analytes) and the downstream LC separation. As a rule of thumb, the trapping column should be less retentive than the analytical column to avoid possible peak broadening. Two guard cartridges (traps), Ascentis Express Fused-Core RP-Amide and C8, of the same dimensions were evaluated. As can be seen in **Figure 3**, both trapping columns led to comparable sharp peak shape (high separation efficiency). However, the RP-Amide delivered higher peak responses than the C8 trap under the trapping conditions with 5% methanol (top panel) as the washing solvent. Further increase of the methanol content to 20% in the washing solvent (**Table 3**) resulted in a 50% signal decrease on the C8 traps, but almost no change in signals with the RP-Amide traps (**Figure 3**). These results indicate better washing can be achieved with minimal sample loss with RP-Amide traps.

Table 3. Online SPE/LC-MS Conditions (high organic sample wash)

LC Program:	min	Flow (mL/min)	Valve	Pump B%
1. Sample loading/washing	0	0.4	1	5
	0.5	0.4	1	20
	2.8	0.25	1	20
2. LC-MS/MS	3.0	0.25	2	20
	5.0	0.25	2	70
	8.0	0.25	2	70
3. Equilibration	8.0	0.25	1	70
	8.1	0.4	1	5
	12.0	0.4	1	5

Figure 3. Comparison of the LC-MS Chromatograms with Online Trapping with C8 and RP-Amide Guard Cartridges

Online SPE/LC-MS conditions as in Tables 1 and 2.



Summary

An online SPE/LC-MS method was developed and described here for the determination of the thyroid hormones T4, T3 and rT3 from plasma. The method uses an Ascentis Express RP-Amide cartridge to trap the analytes, and an Ascentis Express Phenyl-Hexyl column to resolve them. Compared to the commonly used C8 trap, the RP-Amide trap gave higher analyte signals and was compatible with 20% methanol washing. Another advantage of the RP-Amide traps is their compatibility with 100% aqueous mobile phases and samples. This is due to the hydrophilic nature of the amide-embedded phase, which makes it very useful for polar analytes.

References

1. Kahric-Janicic, N.; Soldin, S.J.; Soldin, O.P., 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. Taia, S.S.; Sniegoski, L.T.; Welch, M.J.; 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. Wang, D. and Stapleton, H.M.; Analysis of thyroid hormones in serum by liquid chromatography-tandem mass spectrometry. *Anal Bioanal Chem.* **2010**, *397(5)*, 1831-1839.

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Description	Cat. No.
HPLC Column	
Ascentis Express Phenyl-Hexyl, 5 cm x 2.1 mm I.D., 2.7 µm particles	**53334-U**
Trap Columns	
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Ascentis Express C8 Guard Cartridge, 5 mm x 2.1 mm I.D., 2.7 µm particles	**53509-U**
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Methanol LC-MS Ultra CHROMASOLV, tested for UHPLC-MS	**14262**
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3, 3', 5-Triiodo-L-thyronine (T3); 100 µg/mL (Cerilliant Certified Reference Material)	**T-074**
3, 3', 5'-Triiodo-L-thyronine (Reverse T3); 100 µg/mL (Cerilliant Certified Reference Material)	**T-075**

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