Comparison of SPE Approaches for the Extraction of Thyroid Hormones: T3, rT3 and T4 prior to LC-MS/MS Analysis

Lee Williams[1], Helen Lodder[1], Rhys Jones[1], Adam Senior[1], Alan Edgington[1], Geoff Davies[1], Steve Jordan[1], Claire Desbrow[1], Victor Vandell[2], Frank Kero[2].

[1]Biotage GB Limited, Distribution Way, Dyffryn Business Park, Ystrad Mynach, Cardiff, CF82 7TS, UK.
[2]Biotage, 10430 Harris Oaks Blvd., Suite C, Charlotte North Carolina 28269, USA.

Introduction

Thyroid hormones are extremely important physiologically and involved in many biological processes such as growth and development. Reverse T3 is only produced at low levels compared to T3 and most notably to T4 so it is important to have a robust assay capable of detecting these low levels. Typically analysis uses RIA approaches which are generally expensive and can be problematic. LC/MS approaches also have their own issues with respect to assay robustness. This poster evaluates the performance of various polymer-based ion exchange SPE chemistries for the extraction of thyroid hormones prior to LC-MS/MS analysis. Method performance will be investigated from an analyte recovery perspective with particular emphasis on extract cleanliness.

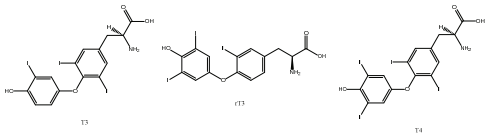

Figure 1. *Structure of Thyroid Hormones: T3, rT3, T4*

Experimental

Reagents

Formic acid, ammonium acetate and ammonium hydroxide were purchased from Sigma Chemical Co. (Poole, UK). Stripped and non-stripped serum was obtained from Sera Labs International (West Sussex, UK.) and discard patient plasma from the Welsh Blood Service (Pontyclun, UK). All solvents were HPLC grade from Fisher Scientific (Loughborough, UK) and MilliQ water used throughout.

Sample Preparation

All extractions were performed using polymer-based SPE. EVOLUTE EXPRESS® ion exchange chemistries (anion and cation) were evaluated in the 30 mg 96-well format.

Internal standard: 10 µL of the relative d6 ISTDs at 25 ng/mL were added to the samples and left to stand for one hour to replicate binding.

Matrix Pre-treatment:
EVOLUTE EXPRESS® AX: 500 µL of ISTD treated serum was pre-treated with 50/50 (v/v) 5% NH_4OH (aq) and acetonitrile (MeCN) and mixed thoroughly.
EVOLUTE EXPRESS® CX: 500 µL of ISTD treated serum was pre-treated with 80/20 (v/v) 4% formic acid (aq) and acetonitrile (MeCN) and mixed thoroughly.

Sample Application: 1 mL of pre-treated serum was applied to the 96-well plate.

Optimized Extraction Procedures

Table 1. *Optimized Extraction Procedures.*

Step	EVOLUTE EXPRESS® AX	EVOLUTE EXPRESS® CX
Conditioning	1 mL MeOH	1 mL MeOH
Equilibration	1 mL H_2O	1 mL 2% formic acid (aq)
Load	1 mL Pre-treated Serum	1 mL Pre-treated Serum
Wash 1	1 mL 50/50 (v/v) 5% NH_4OH (aq)/MeCN	1 mL 50mM NH_4OAc pH 6
Wash 2	1 mL 5% NH_4OH (aq)	1 mL 2% formic acid (aq)
Wash 3	1 mL MeOH	1 mL MeOH
Wash 4	1 mL 2% formic acid/DCM	-
Elution	500 µL 5% formic acid/MeOH	750 µl 5% NH_4OH/MeOH

Post Extraction: 2 µL of glycol was added to the collection plate prior to elution. The extracts were evaporated to dryness at 40 °C and reconstituted in 100 µL of 50:50 (v/v) mobile phase.

HPLC Conditions

Instrument: Waters Acquity UPLC (Waters Assoc., Milford, MA, USA).
Column: Kinetex C18 UHPLC column (1.7 µ, 100 x 2.1 mm id) (Phenomenex, Cheshire UK).
Mobile Phase: 2 mM NH_4OAc/0.1% formic acid (aq) and 2 mM NH_4OAc/0.1% formic acid/MeOH at a flow rate of 0.3 mL/min.
Gradient: 50/50 increasing to 10/90 over 2.5 minutes. Initial starting conditions resumed at 2.6 minutes.
Injection Volume: 15 µL *(partial loop with overfill)*
Column Temperature: 40 °C.

Mass Spectrometry

Instrument: Quattro Premier XE triple quadrupole mass spectrometer (Waters Assoc., Manchester, UK) equipped with an electrospray interface for mass analysis. Positive ions were acquired in the MRM mode.
Desolvation Temperature: 450 °C
Ion Source Temperature: 150 °C
Collision Gas Pressure: 3.5 x 10^{-3} mbar

Table 2. *Waters Quattro Premier XE parameters.*

Analyte	MRM Transition	Cone Voltage (V)	Collision Energy (eV)
T3	651.8 >605.8 (651.8 > 479.0)	35 (35)	21 (35)
rT3	651.8 >605.8 (651.8 > 508.0)	35 (35)	21 (20)
T3/rT3 d6 ISTD	657.9 > 611.9	35	21
T4	777.8 > 731.8 (777.8 > 351.2)	40 (40)	25 (45)

Results

Initial experiments investigated non-specific binding during the evaporation process. Under acidic evaporation conditions > 25% analyte losses can be observed. The addition of 2 µL of ethylene glycol to the collection plate eliminated this issue, as demonstrated in ***Figure 2.***

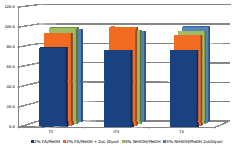

Figure 2. *Investigation of non-specific binding during evaporation*

Due to the amphoteric nature of the thyroid hormones we evaluated extraction using both anion and cation exchange SPE sorbents. Depending on sorbent selection it was necessary to suppress the ionization of one of the functional group on the hormones. Initial experiments extracting 100 µL of serum ruled out the use of either of the weak anion or cation exchange sorbents, as demonstrated in ***Figure 3***.

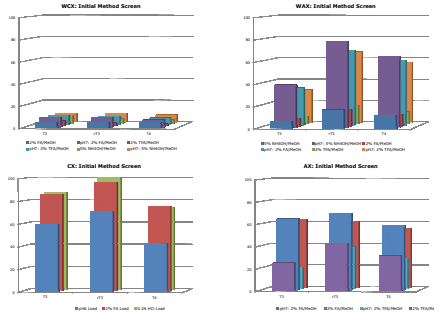

Figure 3. *Initial sorbent screen using various extraction conditions.*

Serum volumes were increased to 500 µL in order to meet the LOQs required. Protein binding was observed to be more of an issue with larger matrix volumes. Optimization of the CX and AX procedures involved the use of high organic proportions in the pre-treatment in order to help with analyte release from protein binding. Patient samples for the AX extracts showed considerable pigmentation. The final wash incorporating 2% formic acid in DCM eliminated this pigmentation. Final recoveries for each procedure are detailed in ***Figure 4***.

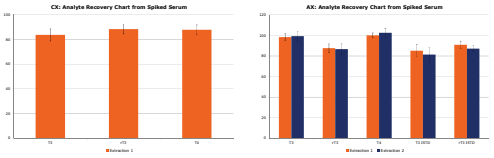

Figure 4. *Optimized CX and AX recovery profile from serum.*

Calibration curves were generated using stripped serum spiked at concentrations from 50-5000 pg/mL. Both CX (data not shown) and AX (***Figure 5***) demonstrated good linearity and coefficients of determination for T3 and rT3 (r^2 > 0.99). Endogenous levels of T4 contributing towards a substantial intercept affected calibration curve performance, demonstrating r^2 values of only 0.97.

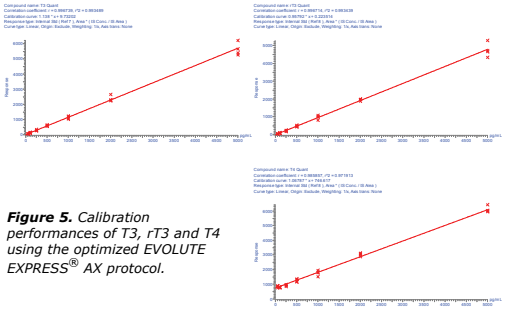

Figure 5. *Calibration performances of T3, rT3 and T4 using the optimized EVOLUTE EXPRESS® AX protocol.*

Phospholipid analysis (non stripped serum) of both protocols demonstrated relatively high levels of lyso PLs remaining in the CX extracts but full PL removal using AX, as shown in ***Figure 6***. Optimization of the CX elution eliminates the lyso phospholipids at the expense of analyte recoveries (data not shown). Due to the impact of these lyso PLs the AX provides a more robust mechanism for extraction.

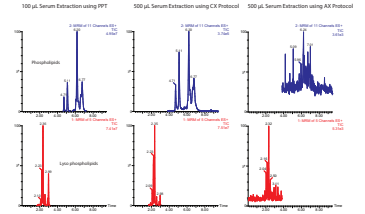

Figure 6. *Phospholipid TICs comparing PPT with CX and AX extraction protocols.*

The final AX protocol was tested using patient plasma samples. No chromatographic deterioration or method performance was observed for injection of > 400 samples overnight.

Conclusion

- Both EVOLUTE EXPRESS® CX and AX provided good extraction of the various thyroid hormones, demonstrating acceptable calibration parameters.
- Further investigation into matrix interferences demonstrated that the optimized cation exchange protocol didn't effectively remove phospholipids, whereas excellent removal was afforded by the optimized anion exchange protocol.
- EVOLUTE EXPRESS® AX provides a robust and reliable method for the analysis of thyroid hormones, providing high analyte recoveries and low matrix interference.

© 2014 Biotage. All rights reserved. All trademarks are the property of their respective companies.
www.biotage.com