[TECHNOLOGY BRIEF]

A Rapid and Analytically Sensitive LC-MS Method for the Simultaneous Analysis of a Panel of Steroid Hormones for Clinical Research

Laura Owen and Brian Keevil; University Hospital South Manchester, UK

GOAL

To demonstrate that the use of an online SPE system, ACQUITY UPLC® Online SPE Manager (OSM), enables simultaneous and analytically sensitive measurement of a panel of steroid hormones.

BACKGROUND

Many of the currently available measurement procedures for steroid hormones suffer from poor analytical sensitivity, selectivity, and a lack of harmonization, making it difficult to achieve an accurate assessment of their circulating levels. Additionally, tests for many of the steroid hormones or steroid precursors are not readily available or can require expensive and time consuming test regimes. Therefore, clinical research laboratories are increasingly turning to LC-MS as an alternative technique for measurement of steroid hormones. But, even this powerful analytical technique can struggle to measure steroid hormones with sufficient analytical sensitivity as many existing methods require extensive sample pre-treatment, derivatization, and lengthy chromatography.

Typically, measurements of serum testosterone (T) and other androgens such as dehydroepiandrosterone sulphate (DHEAS), androstenedione (A4), and the progestin, 17-hydroxyprogesterone (17-OHP) are performed in individual LC-MS methods.

Online SPE has a dramatic impact on the measurement of steroid hormones in serum.

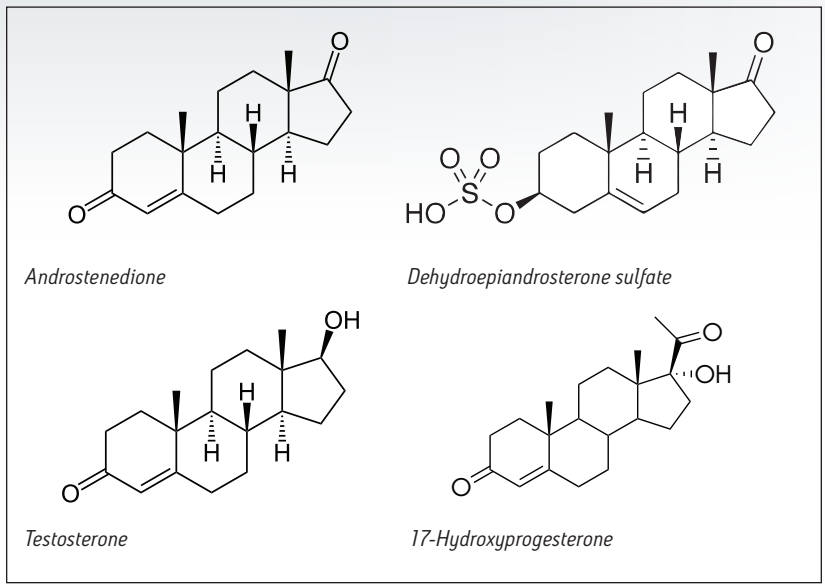

Figure 1. Structures of the steroid hormones analyzed.

Although it would be advantageous to measure these four steroids simultaneously using LC-MS, this is not straightforward because of the differing extraction conditions required to analyze these hormones.

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

THE SOLUTION

A clinical research method for simultaneously measuring four steroid hormones (T, DHEAS, A4, and 17-OHP) from serum has been developed. This method takes advantage of the unique capabilities of an online SPE system to enable analytically sensitive and reproducible measurements of these steroid hormones. The method enables direct measurement of these key steroid hormones and can provide valuable information to clinical researchers as they investigate the role these molecules play in normal biological function and disease. Simultaneous measurement of these key steroid hormones with this method can also provide valuable information to clinical researchers as they investigate novel models of disease.

ANALYTICAL METHOD DETAILS

LC System: ACQUITY UPLC®

MS: Xevo® TQ-S

Column: ACQUITY UPLC HSS SB C_{18}, 2.1 x 50 mm; 1.8 µm

Sample Preparation: ACQUITY UPLC Online SPE Manager (OSM)

SPE: MassTrak™ C_{18} OSM Cartridge

Sample Preparation and Online SPE

Calibrators were made by spiking methanolic standards into PBS/BSA. Initial sample pre-treatment involved the addition of 50 µL of serum to 150 µL of aqueous 0.4 M $ZnSO_4$ and 100 µL acetonitrile (including isotopically labelled internal standards). Following centrifugation, the supernatant was extracted using the C_{18} cartridges for the OSM. Online SPE cartridges and samples were prepared and extracted as follows:

Step	Solvent	Flow rate	Volume
Conditioning	MeOH	2 mL/min	0.5 mL
Equilibration	Water	2 mL/min	0.5 mL
Sample load	Water	1 mL/min	0.5 mL
Cartridge wash	30% MeOH	0.5 mL/min	0.25 mL
Clamp flush	Water	3 mL/min	0.5 mL

LC-MS Method

After extraction, the samples were eluted from the Mass Trak C_{18} OSM Cartridge onto the analytical column using the following gradient of water (A) and methanol (B) both containing 2 mM ammonium acetate and 0.1 % formic acid:

Time	%A	%B	Curve
Initial	50	50	Initial
3.0	30	70	6
3.01	0	100	11
4.0	50	50	11

Eluent was directed (without stream splitting) into the ion source of a Waters® Xevo TQ-S tandem quadrupole MS, operated in the negative ion mode for DHEAS and the positive ion mode for the other steroids. The following quantifier and (qualifier) transitions were utilized:

Steroid	MRM Transition
Testosterone	289.1>97 (109)
Androstenedione	287.1>97 (109)
17-OH Progesterone	331.1>97 (109)
DHEAS	367.0>96.9

Steroid	MRM Transition
d2-Testosterone	291.1>111
d7-Androstenedione	294.1>113
d8-17-OH Progesterone	339.2>100
d2-DHEAS	369>96.9

RESULTS

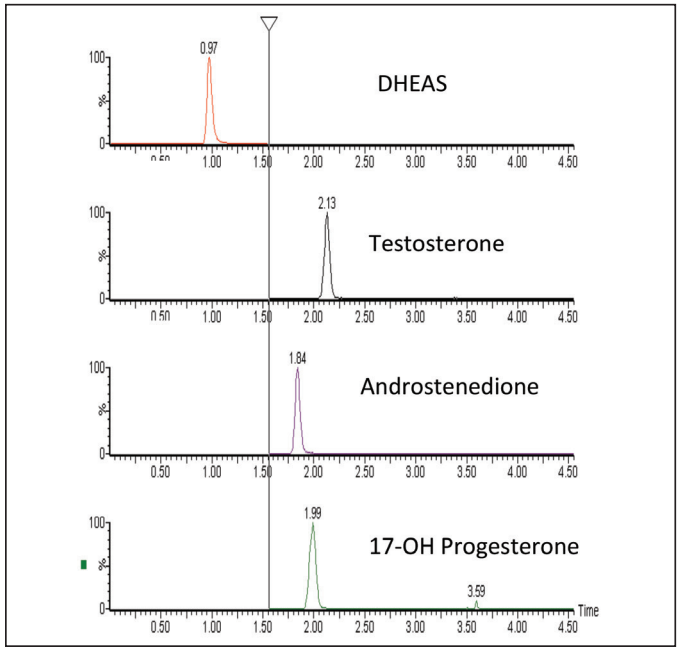

Figure 2. Separation of steroids and LC-MS analysis after online SPE. Separation of all four steroids was achieved within a run time of 5.5 minutes per sample. All four steroids were base line separated with DHEAS measured in negative ion mode and the polarity changed to positive ion mode to measure the other three steroids as indicated by the solid line on the chromatogram.

Further studies demonstrated that the lower limits of quantitation (LLOQ) for these steroids were as follows:

Steroid	LLOQ	LLOQ Giving <8% Imprecision
Testosterone	0.12 nmol/L (34.6 pg/ml)	0.4 nmol/L (115.4 pg/mL)
Androstenedione	0.26 nmol/L (74.5 pg/ml)	1.7 nmol/L (487.1 pg/mL)
17-OH Progesterone	0.27 nmol/L (89.2 pg/ml)	0.6 nmol/L (198.2 pg/mL)
DHEAS	0.17 µmol/L (49.0 ng/ml)	0.2 µmol/L (57.9 ng/mL)

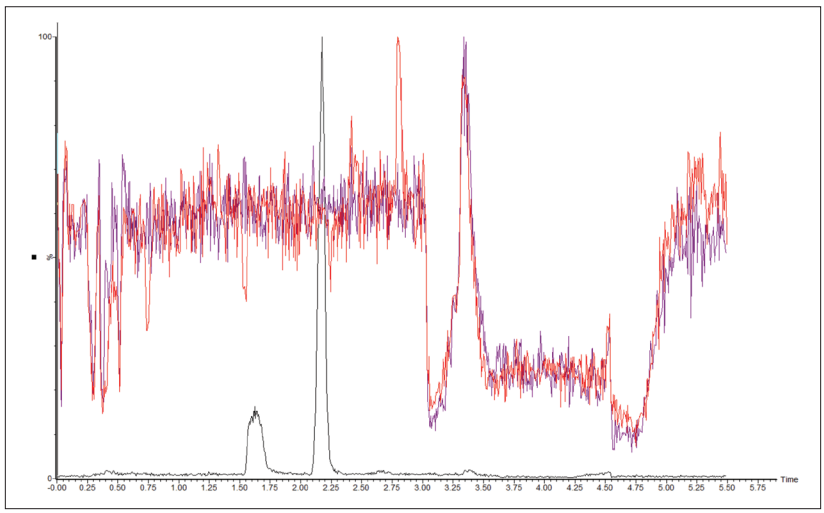

Figure 3. Ion suppression determination. Signal from solvent background (purple) is compared with a blank serum sample treated with the online SPE system (red). The online SPE serum blank shows no ion suppression in the area where testosterone elutes (green).

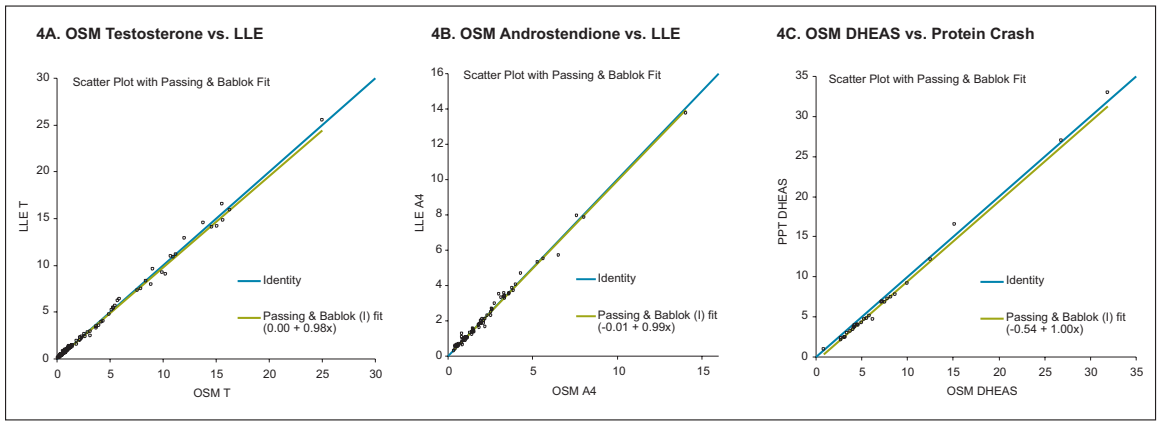

Figure 4. Comparison of online SPE with existing LC-MS/MS methods using liquid/liquid extraction (LLE) and protein precipitation for sample clean up. Figures 4A and 4B show the Passing-Bablok regression plots comparing the OSM with LLE methods for testosterone and androstenedione. Figure 4C shows this same comparison for the OSM and a protein precipitation method for DHEAS.

SUMMARY

In many clinical research laboratories, two or more separate analytical runs for measuring T, A4, 17-OHP and then DHEAS have been required. Using the OSM method in conjunction with LC-MS will reduce both the direct staff and instrument time required to perform these analyses while allowing for simultaneous analysis of all four steroids. The Waters ACQUITY UPLC HSS SB C_{18} Column used in this study showed excellent performance in terms of separation of these steroids. While the ion suppression experiment showed negligible matrix effects (Figure 3) from the serum sample matrix after treatment with online SPE. The clinical research method developed here demonstrated excellent performance when compared to existing single analyte LC-MS/MS methods incorporating various sample prep techniques such as LLE.

Sample preparation using LLE as used in many laboratories can produce clean sample extracts with minimal matrix interference. But, the use of highly flammable solvents is precluded in some laboratories because of health and safety concerns. In addition, LLE does not lend itself easily to automation and high sample throughput. Using a simple protein precipitation method, samples can be quickly and easily prepared for online sample cleanup.

This clinical research method is suitable for multiple steroid analysis and can also accommodate ionic compounds such as DHEAS which do not extract into organic solvents.

In this study, a rapid clinical research method for the LC-MS/MS measurement of T, DHEAS, A4 and 17OHP has been developed. The method is suitable for research use and the small volume of serum used (50 µL) is desirable for clinical research laboratories that are sample limited.

The method developed here provides:

- Simultaneous analysis of testosterone, androstenedione, 17-hydroxyprogesterone and dehydroepiandrosterone sulfate from serum.
- Effective separation of all four steroids.
- Efficient SPE sample preparation integrated with LC-MS in ~5 mins/sample.
- Very good analytical sensitivity (LLOQs for all analytes in the low nanomolar ranges).
- No significant ion suppression effects with online SPE.
- Excellent agreement with single analyte LC-MS methods but requiring less time and resources.

For Research Use Only. Not for Use in Diagnostic Procedures.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters, ACQUITY, ACQUITY UPLC, and Xevo area registered trademarks of Waters Corporation. MassTrak and The Science of What's Possible is a registered trademark of Waters Corporation. All other trademarks are the property of their respective owners.

©2014 Waters Corporation. Produced in the U.S.A. August 2014 720005037EN KP-PDF

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com