[TECHNOLOGY BRIEF]

Development of a Clinical Research Method for the Measurement of Testosterone and Dihydrotestosterone

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GOAL

To develop a method to measure Testosterone and Dihydrotestosterone (DHT) from plasma samples.

BACKGROUND

Testosterone is a steroid hormone from the androgen group and is the principal male sex hormone and an anabolic steroid (Figure 1).

In men, approximately 5% of testosterone undergoes 5α-reduction to form the more potent androgen, dihydrotestosterone (DHT) (Figure 1).

Measuring the male steroid hormones and related metabolites, particularly at the low levels found in pediatrics and females, has proven to be a major challenge. Immunoassays for steroids often suffer from poor sensitivity and specificity. While traditional LC-MS assays require derivatization procedures and/or long and complicated sample preparation protocols.

In the current study, a research method for the measurement of testosterone and DHT in plasma was developed. This method allows for the direct measurement of these two androgens at physiological levels in male and female samples by LC-MS without the need for derivitization. Moreover the method makes use of automated sample prep, minimizing sample pretreatment.

Direct measurement of androgens is made possible without derivatization using LC-MS with online SPE.

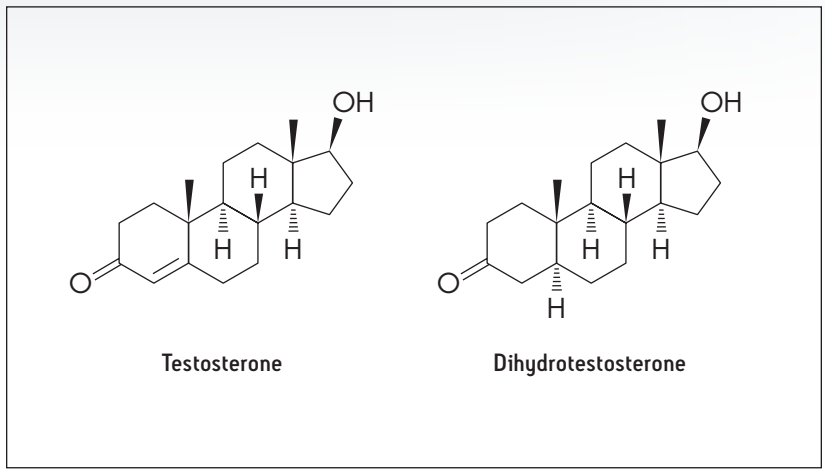

Figure 1: Structure of Androgen Hormones. Dihydrotestosterone (DHT) is the product of the reduction of the double bond in the a ring of testosterone by the enzyme 5-alpha reductase.

Method Details:

LC-MS System Configuration:	LC System: ACQUITY UPLC®
	Mass Spectrometer: Xevo® TQ-S
Column:	ACQUITY UPLC BEH C_{18}, 1.7 µm, 2.1 x 50 mm
Sample Preparation:	ACQUITY UPLC Online SPE Manager (OSM)
SPE:	MassTrak™ C_8 OSM Cartridge
Sample Pre-Treatment	200 µL Plasma
	20 µL Internal Standard (d3 Testosterone; 13C3 DHT)
	300 µL Zinc Sulfate:MeOH Solution; Centrifuge to pellet protein; remove supernatant
	After addition of zinc sulfate:MeOH we filled up to 1.0mL prior to centrifugation. 25 uL was then injected (no transfer of supernatant → needle adjusted so stays above pellet).
	Dilute to 1 ml with Water
	Inject 25 µL of final sample

Chromatography Conditions

Solvent A:	10% MeOH+0.05% Formic Acid
Solvent B:	MeOH+0.05% Formic Acid
Flow rate:	0.4 ml/min

Time	%A	%B
0	60	40
3.0	0	100
4.0	0	100
4.1	60	40

Online SPE Conditions

Conditioning	Step 1: 0.5 ml MeOH @ 4 ml/min
	Step 2: 2.0 ml Magic Mix* @ 3 ml/min
Equilibration	1.0 ml Water @ 4 ml/min
Sample loading	1.0 ml Water @ 2 ml/min
Cartridge wash	Step 1: 0.5 ml 80% 0.2% NH_4OH/20% MeOH @ 2 ml/min
	Step 2: 0.5 ml 70% 0.2% Formic Acid/30% MeOH @ 2 ml/min
Elution	Elution Time: 0.8 minutes
	Run Time: 0.8 minutes
Clamp flush	Step 1: 2.0 ml Magic Mix* @ 3 ml/min
	Step 2: 1.0 ml Water @ 3 ml/min

* Magic Mix= MeOH/ACN/IPA/Water+0.2% Formic Acid

RESULTS

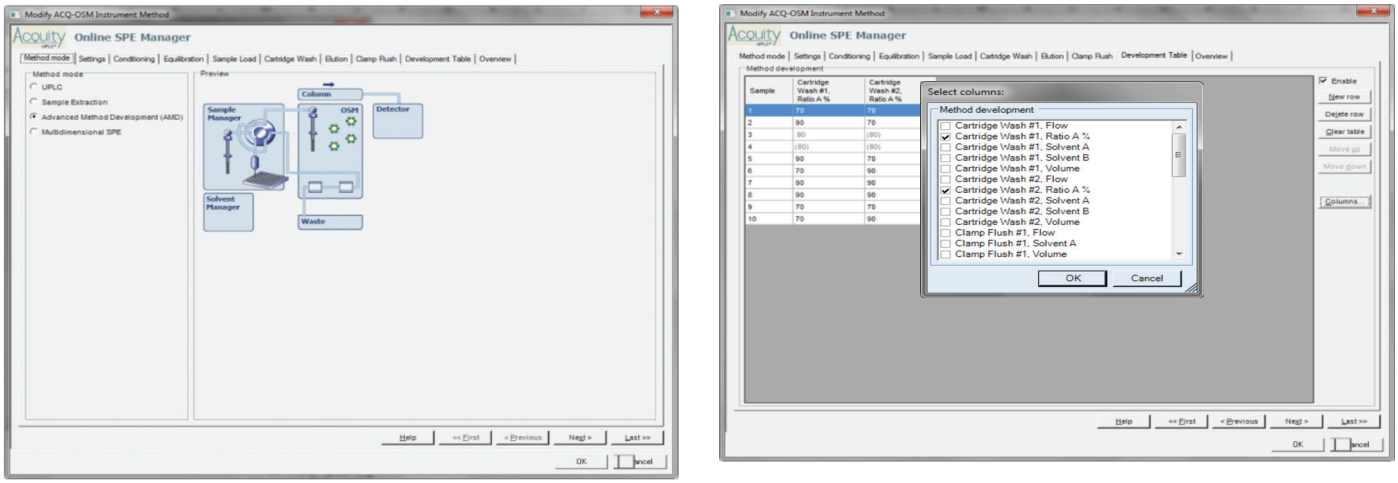

Figure 2: SPE Method Development. SPE sample preparation was facilitated by using an online SPE system and Design of Experiments (DoE) principles to manipulate multiple SPE variables simultaneously. This approach allowed for identification and optimization of significant sample preparation method development variables very quickly.

Compound 1: Testosteron-Quan

Name	Sample Text	ID	RT	Area
130529_033	70/70	0.4mL/min - steep	1.74	272098
130529_034	90/30	0.4mL/min - steep	1.75	1163025
130529_035	80/80	0.4mL/min - steep	1.75	1123519
130529_036	80/80	0.4mL/min - steep	1.75	1151666
130529_037	90/70	0.4mL/min - steep	1.75	1111988
130529_038	70/90	0.4mL/min - steep	1.75	1193007
130529_039	90/90	0.4mL/min - steep	1.76	1146458
130529_040	90/90	0.4mL/min - steep	1.76	1155421
130529_041	70/70	0.4mL/min - steep	1.74	103377
130529_042	70/90	0.4mL/min - steep	1.75	1114320

Compound 3: DHT-Quan

Name	Sample Text	ID	RT	Area
130529_033	70/70	0.4mL/min - steep	2.01	235415
130529_034	90/30	0.4mL/min - steep	2.02	229790
130529_035	80/80	0.4mL/min - steep	2.02	226338
130529_036	80/80	0.4mL/min - steep	2.02	229653
130529_037	90/70	0.4mL/min - steep	2.02	231851
130529_038	70/90	0.4mL/min - steep	2.02	235417
130529_039	90/90	0.4mL/min - steep	2.02	232147
130529_040	90/90	0.4mL/min - steep	2.02	231634
130529_041	70/70	0.4mL/min - steep	2.02	227648
130529_042	70/90	0.4mL/min - steep	2.02	229368

Figure 3: SPE Method Development Results. Use of the Advanced Method Development (AMD) operation mode of the online SPE system to optimize sample preparation. AMD allowed a number of SPE conditions to be tested rapidly to identify the optimal SPE conditions for these two analytes. Also, the SPE method is also helping with separation of testosterone and DHT. Testosterone is breaking through, while DHT is retained under the conditions examined.

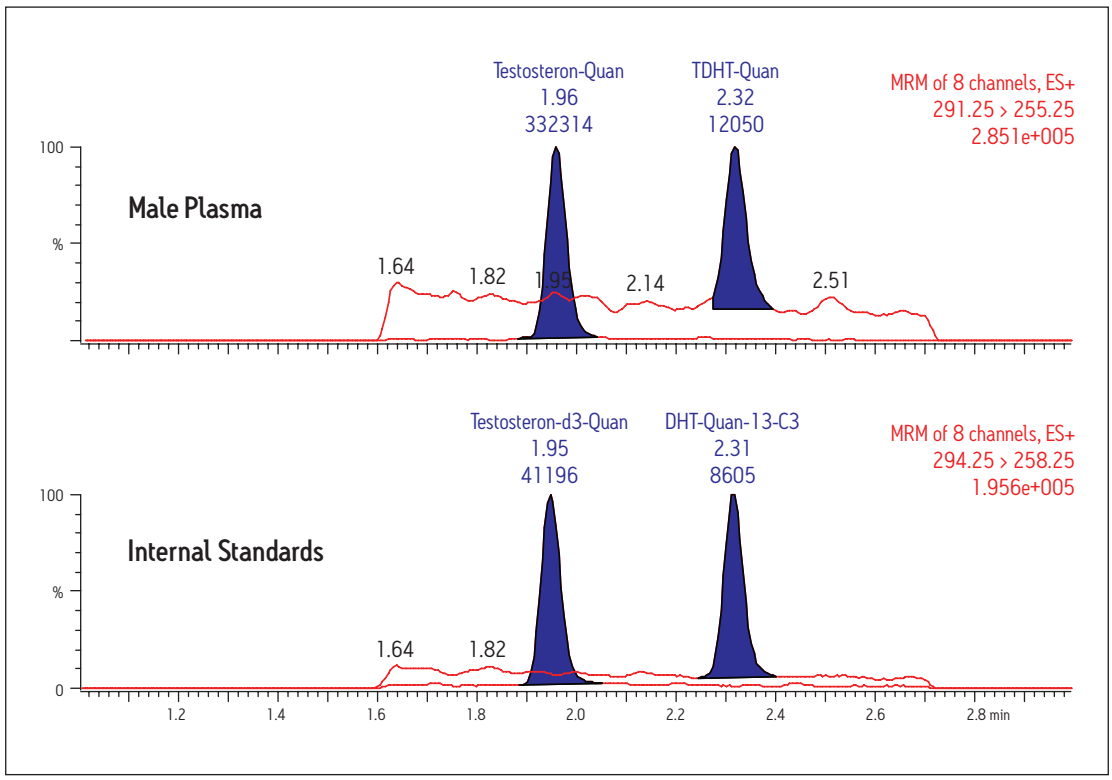

Figure 4: Separation and measurement of testosterone and DHT in male plasma samples (Sample from Male between 20 and 65 years of age). Testosterone and DHT are readily separated and quantitated.

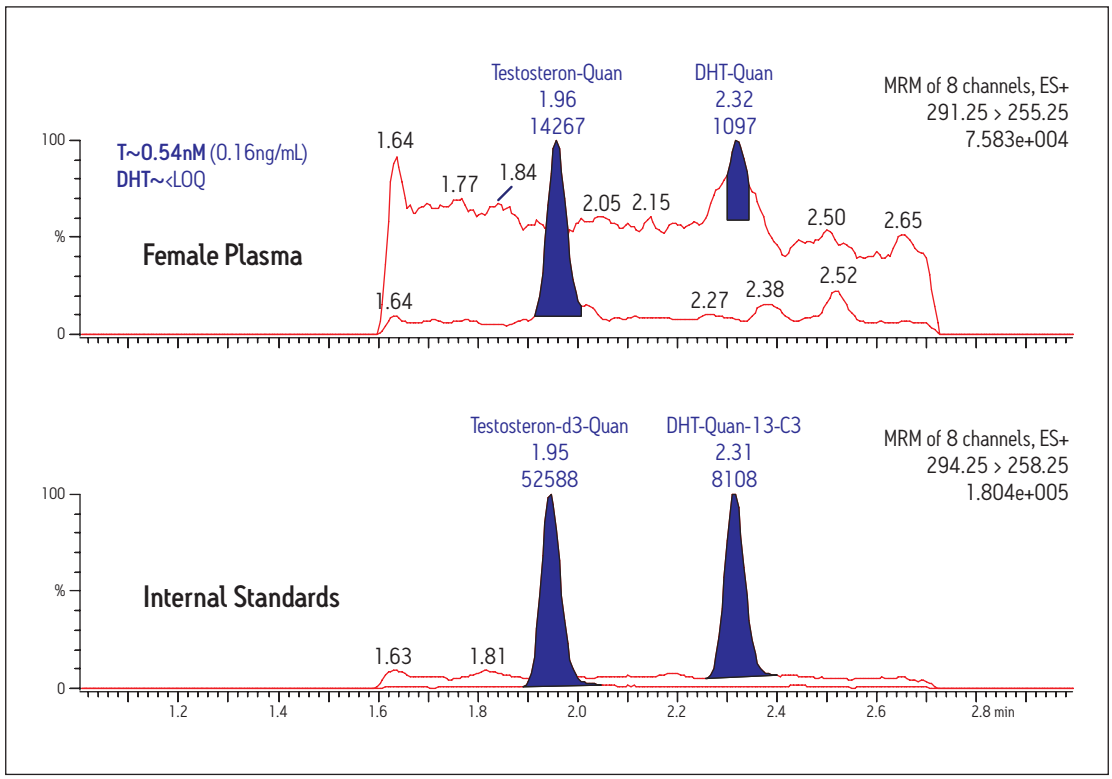

Figure 5: Separation and measurement of testosterone and DHT from Female plasma sample from pre-menopausal females. Testosterone can be measured at pM range in these samples, but DHT is below the LOQ in female samples.

SUMMARY

The wide variation in testosterone and DHT concentrations as well as the inherent difficulties in measuring these molecules, has made it difficult to develop test methods for them.

To measure these molecules successfully by LC-MS requires an effective sample preparation method. Using an online SPE system greatly accelerated the development of an LC-MS method by quickly identifying and utilizing optimized SPE conditions for isolating these androgens from plasma samples.

The focus of the clinical research method described herein was on quickly and efficiently developing an integrated SPE sample preparation method with high sensitivity mass spectrometry to simultaneously measure testosterone and DHT. The method developed uses online SPE to provide a high quality sample that is relatively free from sample matrix effects. By using LC-MS augmented with online SPE, the direct measurement of androgens, even at low levels, is possible.

The clinical research method developed here provides:

- Simultaneous analysis of testosterone and DHT in a single assay
- Simple sample preparation and androgen detection without derivatization
- Fast analysis time of < 5 min/sample
- Measurement of testosterone and DHT in male and female samples; where applicable
- Highly efficient SPE sample preparation integrated with LC-MS

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