UTILISING MIXED MODE µELUTION SPE FOR THE LC-MS/MS ANALYSIS OF STEROID HORMONES IN SERUM FOR CLINICAL RESEARCH

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OVERVIEW

Here we evaluate an LC-MS/MS method used to measure serum testosterone and androstenedione enabling investigation of metabolic dysfunction for clinical research purposes. An analytically selective method was developed using a mixed-mode Solid Phase Extraction (SPE) sorbent in 96-well plate µElution format. The use of this µElution chemistry provides precise and accurate measurement of testosterone and androstenedione to high analytical sensitivity.

INTRODUCTION

Testosterone and its precursor, androstenedione, are androgenic steroid hormones that play a central role in the regulation of sexual characteristics.

As seen in Figure 1, the steroid pathways consist of many structurally similar steroid hormones. Therefore, care must be taken when developing steroid panel analytical methods, to limit interferences which affect accuracy and precision. MS/MS provides a way to differentiate between these hormones, while the LC dimension provides separation of isobaric species. However, sample preparation is the key in providing optimal LC-MS/MS analytical sensitivity. The use of Oasis MAX SPE reduces background noise and removes interferences, which allows accurate and precise quantification of testosterone and androstenedione in serum at low concentrations using the Xevo TQD mass spectrometer (Figure 2). To demonstrate the effectiveness of this methodology, we employed CDC Hormone Standardization (HoSt) testosterone samples to evaluate the accuracy and therefore suitability of this method for analysing testosterone for clinical research.

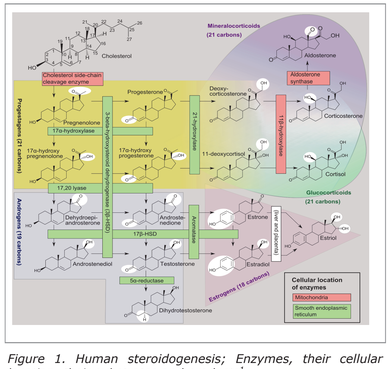

Figure 1. Human steroidogenesis; Enzymes, their cellular location, their substrates and products[1].

METHODS

Materials

- Certified testosterone and androstenedione reference material purchased from Cerilliant (Round Rock, TX) was used to prepare calibrators (0.05—15 ng/mL) and QC material in MSG4000 pooled human serum obtained from Golden West Biologicals (Temecula, CA).
- Total precision was determined by extracting and quantifying three replicates of tri-level QC material on two occasions per day over five consecutive days (n=30). Repeatability was determined by analyzing three replicates at each QC level.
- A comparison was performed using anonymized testosterone and androstenedione samples previously analyzed using an independent LC-MS/MS method (n=35).
- Analytical method bias for testosterone was determined using Hormone Standardization (HoSt) samples obtained from the CDC (Atlanta, GA) (n=40).

Methods

- All samples were pre-treated with internal standard, ammonia, zinc sulphate, methanol and water.
- Sample supernatant was transferred to a Waters® Oasis® MAX µElution plate, washed with 0.1% ammonia in 20% methanol and eluted with methanol and then water.
- Automation was performed using the Tecan Freedom Evo 100 Liquid Handler.
- Using a Waters ACQUITY UPLC® I-Class System, samples were injected onto a 2.1x50mm Waters ACQUITY UPLC HSS C18 SB column using a water/methanol/ammonium acetate gradient and analyzed with a Waters Xevo® TQD Detector, using MRM parameters in Table 1.
- The analysis time per sample was approximately 4.0 minutes injection to injection.

Figure 2. ACQUITY UPLC I-Class with Xevo TQD Detector

Compound	MRM Transition (m/z)	Cone (V)	Collision (eV)
Testosterone	289.2 > 97.0(109)	38	25 (28)
Testosterone $-^{13}C_3$	292.2 > 100.0	38	25
Androstenedione	287.2 > 97.0 (109)	38	25 (28)
Androstenedione $-^{13}C_3$	290.2 > 100.0	38	25

Table 1. MRM parameters for the analysis of testosterone and androstenedione. (Qualifier ion parameters)

RESULTS

Chromatography

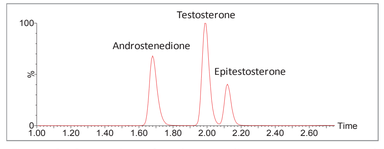

Figure 3. Separation of androstenedione, testosterone and it's epimer; epitestosterone

Extraction Selectivity

- The use of Oasis MAX SPE reduces background noise in both testosterone and androstenedione (Figure 4) MRM transitions in comparison to protein precipitation, liquid-liquid and Oasis HLB extraction techniques.
- For each extraction, a serum sample was extracted in triplicate and the mean S/N is shown. S/N was calculated on the raw data using PtP at ±2 SD.

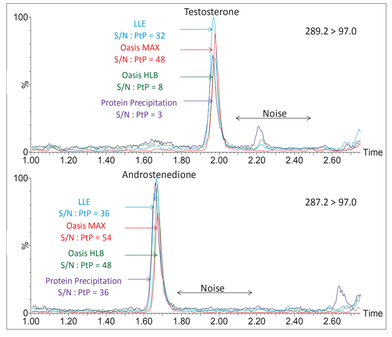

Figure 4. Comparison of testosterone and androstenedione MS sensitivity using four different extraction techniques

- Oasis MAX SPE has been shown to remove interferences that could affect integration reproducibility (Figure 5).

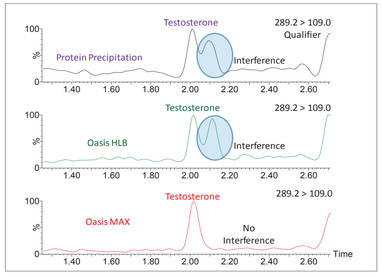

Figure 5. Comparison of testosterone qualifier traces using protein precipitation, Oasis HLB and Oasis MAX extractions

Matrix Effect

- Matrix Factor (MF) assessments demonstrate a mean 33% ion suppression for testosterone (MF=67%, RSD = 10.4%) and 23% ion suppression for androstenedione (MF=77%, RSD= 8.0%) using this method based on the analysis of 6 extracted individual serum samples.
- The normalized Matrix Factor based on analyte:internal standard response ratio showed no significant ion suppression or enhancement for testosterone and androstenedione, indicating that the internal standard compensates for the matrix effect (MF=100%).
- A qualitative matrix effect profile of an extracted serum sample is shown in Figure 6 and demonstrates that both testosterone and androstenedione elute in a region free from major ion suppression.

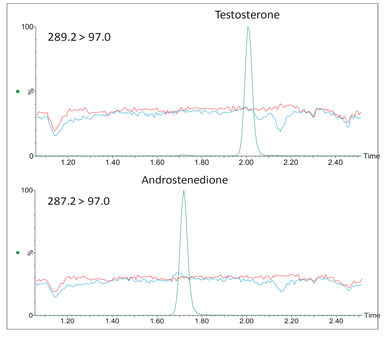

Figure 6. Ion suppression trace following injection of an extracted sample (blue) compared to a solent sample (red)

Linearity and Analytical Sensitivity

- The method was shown to have a linear fit over the range of 0.05—15ng/mL (n=4) for both analytes.
- Calibration lines were linear with $r^2 > 0.994$ (n=10) for both analytes.
- The S/N ratios for the lowest calibrator (Figure 7) for spiked serum were >15:1 over 10 separate occasions, with imprecision of <7% RSD (n=30) at the lowest calibrator (0.05ng/mL) for both analytes.
- Analytical sensitivity investigations reveal that the sensitivity of this method would allow precise quantification (<20% RSD, n=30) at 0.025 ng/mL for both testosterone and androstenedione.

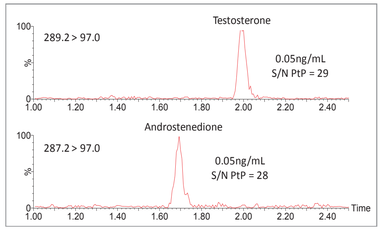

Figure 7. Chromatogram (unsmoothed) of an extracted calibrator sample in stripped serum at a concentration of 0.05ng/mL. S/N was calculated on the raw data using PtP at ±2 SD

Precision

- Low, mid and high QC concentrations were 0.15, 1.0 and 10.0 ng/mL for both testosterone and androstenedione.
- Total precision and repeatability using the Tecan Freedom Evo 100 Liquid Handler were ≤ 4.7% for both analytes (Table 2).

	Total QC Precision			QC Repeatability		
Compound	Low	Mid	High	Low	Mid	High
Testosterone	4.0%	2.8%	4.0%	3.1%	2.6%	4.0%
Androstenedione	4.0%	2.7%	4.7%	3.5%	2.7%	4.7%

Table 2. Total precision and repeatability for the analysis of testosterone and androstenedione

Comparison

- Comparison with samples previously analyzed by an independent LC-MS/MS method (n= 35) was described by the Deming equation y = 1.07x + 0.01 for testosterone and y= 0.96x + 0.02 for androstenedione.
- Proportional bias was observed for testosterone ($p<0.05$) but there was no constant bias ($p>0.05$).
- There was no proportional or constant bias for androstenedione ($p>0.05$).

Accuracy

- The HoSt program is a voluntary program run by the CDC to assist labs and manufacturers to produce accurate and precise hormone measurements
- Phase I samples comprising of 40 individual, single donor serum samples having reference values assigned were analyzed to assess analytical method bias
- Comparison with HoSt samples was described by a Coefficient of Determination (r^2) of 0.9992 (Figure 8).
- The mean method bias was determined to be 3.3%.

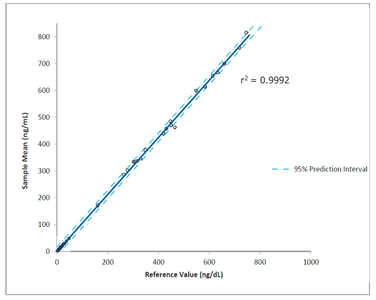

Figure 8. A simple linear regression of the reported mean values of the CDC HoSt samples against the reference values.

CONCLUSION

- **We have successfully quantified testosterone and androstenedione for clinical research purposes**
- **Through the use of Oasis MAX SPE sample preparation, a highly analytical selective and sensitive method for the analysis of testosterone and androstenedione can be performed, with detection using the Xevo TQD**
- **The method provides excellent precision and accuracy at low physiological concentrations**
- **Further studies include the use of this sample preparation technique in the LC-MS/MS analysis of other steroid panels**

References

1. Häggström M, Richfield D *(2014). "Diagram of the pathways of human steroidogenesis". Wikiversity Journal of Medicine* ***1*** *(1).*

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