Chromatography Technical Note No AS104

Automating the analysis of androstenedione and testosterone in serum using a GERSTEL Multi Purpose Sampler coupled to liquid chromatography tandem mass spectrometry detection.

Paul H. Roberts & Bob Green, Anatune Ltd. Hardwick, Cambridgeshire, UK.

Introduction

The measurement of testosterone and androstenedione in females may be performed for a variety of reasons. Clinically, patients with androgen excess may present varying degrees of virilisation ranging from mild hirsuitism to deepening of the voice and male pattern baldness. Additional symptoms of androgen excess include acne, obesity, amenorrhea and infertility. There are many causes of androgen excess including polycystic ovarian syndrome, androgen secreting tumors, congenital adrenal hyperplasia and other endocrine and iatrogenic causes.

Historic methods used for the measurement of female testosterone and androstenedione were mostly immunoassay. These methods, particularly for testosterone, were prone to interference from related steroids and steroid like compounds which interfered with the assays. The method in use here is SPE-LC-MS/MS; this is a very sensitive and specific method and therefore produces detection limits which are considerably lower compared to the immunoassays.

Presented is methodology outlining fully automated sample preparation and analysis of serum samples including automated protein crash and centrifugation, coupled to tandem mass spectrometry analysis.

Instrumentation

GERSTEL MPS 2, fitted with 250 µl syringe and LC injection valve
Anatune CF-100 centrifuge
Instrument Top Sample Preparation (ITSP) Hardware Kit
Maestro Version 1.3.7.69
Agilent 6410 Triple Quadrupole Mass Spectrometer with HotBox and electrospray source.
Agilent 1200 Series HPLC
G1312B Binary Pump SL
G1316B Thermostatted Column Compartment SL
G1379B Degasser

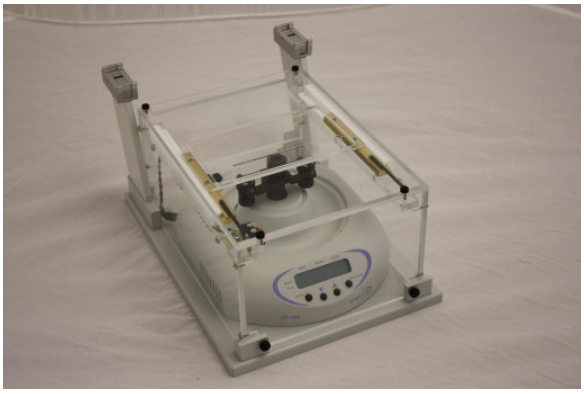

Figure 1 - Anatune CF-100 centrifuge.

Methodology

A set of working calibrators in charcoal stripped, mixed gender, human serum were prepared. See Table 1. These calibrators were prepared from a super stock solution (1 mg/ml) of the compounds in methanol, which was further diluted to an intermediate concentration (500 ng/ml) and then diluted further with serum to form the calibrants.
For sample preparation, 250 µl of the serum standard is placed in a 2 ml glass screw top autosampler vial and the vial capped using a magnetically transportable PolyMag™ caps (Gerstel, Germany). The sample is then placed on the vial tray of the multi purpose sampler (MPS). See Figure 2.
The following aspects of sample preparation are fully automated, conducted via the MPS and the CF-100.

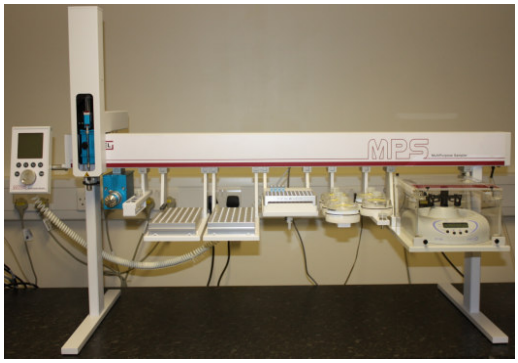

Figure 2 – Gerstel MPS for automated androstenedione and testosterone extraction.

Cal Level	Analyte ng/dl	
	Testosterone	Androstenedione
Std_01	20	20
Std_02	50	50
Std_03	100	100
Std_04	200	200
Std_05	500	500
Std_06	1000	1000
Std_07	2000	2000

Table 1 – Levels of androstenedione and testosterone in the serum calibration standards.

25 µl of internal standard solution (testosterone-d5) is added to the serum sample to give a resulting concentration of 1000 ng/dl. Following this 250 µl of a 0.2 M zinc sulphate solution is added to the vial followed by 500 µl of methanol to precipitate proteins. The vial is then moved using magnetic transport to the CF-100 centrifuge whereby the contents are thoroughly vortexed for 1 minute to assist in the protein precipitation. The vial is then centrifuged at 3000 rpm for 1 minute to separate the proteins from the supernatant in preparation for solid phase extraction (SPE) clean-up.

Anatune Ltd, Broadway House, 149-151 St Neots Road, Hardwick, Cambridge, CB23 7QJ, UK
Tel: +44 (0) 1954 212909 Fax: +44 (0) 1954 212908 Email: info@anatune.co.uk Internet: www.anatune.co.uk

A 10 mg C8 ITSP SPE cartridge is solvated with 100 µl of methanol and then equilibrated with 100 µl of HPLC grade water. 500 µl of the supernatant is then loaded onto the SPE cartridge, before the cartridge is washed with 100 µl of 50 % methanol in water. The cartridge is then dried with 1 ml of air. Analytes are eluted with one 100 µl aliquot of methanol into a 300 µl high recovery vial. The polarity of the final solution is then adjusted by the addition of 100 µl of HPLC grade water

Sample analysis is fully automated by means of an external injection valve and loop fitted onto the MPS, 50 µl of extract is injected. Separation is achieved by means of an Agilent Eclipse Plus 2.1 x 50 mm; 1.8 µm particle size column. The chromatographic mobile phases consisted of 5 mM ammonium acetate plus 0.01 % formic acid in water (eluent A) and 5 mM ammonium acetate plus 0.01 % formic acid in methanol (eluent B). A gradient elution was performed from 45 % B held for 1.7 minutes then to 80 % B in 0.2 minute, with an isocratic hold at 80 % B until 3 minutes the column was then equilibrated to baseline conditions, giving an LC run time of 5 minutes. Column flow rate was 0.5 ml/min throughout the chromatographic run whilst the column temperature was maintained at 50 °C.

An Agilent 6410B tandem mass spectrometer with electrospray source was used in positive ionization mode. Instrument analysis time was 5 minutes per sample using the conditions listed in Table 2.

Parameter	Testosterone	Androstenedione	Testosetrone-d5
Precursor ion	289.3	287.4	294.4
Product ion (Q)	97.1	97.1	100.1
Product ion (q)	109.1	109.1	113.1
Dwell	50	50	50
Fragmentor (V)	140	140	140
Collision Energy (Q)	25	25	25

Gas Temp (°C):350 Gas Flow (l/min):13
Nebulizer (psi):45 Capillary (v):4000

Table 2 - Selected MS conditions for analysis

Results

Calibration curves were constructed for both androstenedione and testosterone. Linear calibrations were achieved from the seven point calibration standards. Correlation coefficients of 0.997 and 0.996 were achieved for androstenedione and testosterone respectively See Figure 3 & 4.

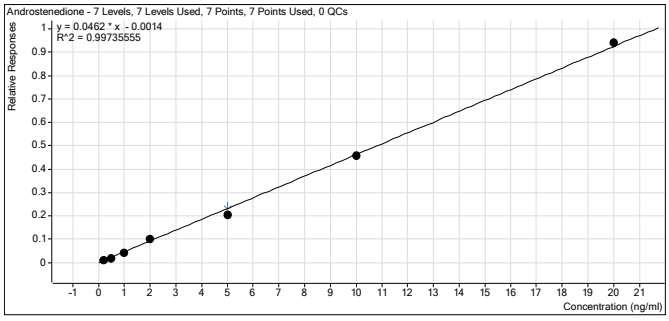

Figure 3 – Calibration curve for androstenedione.

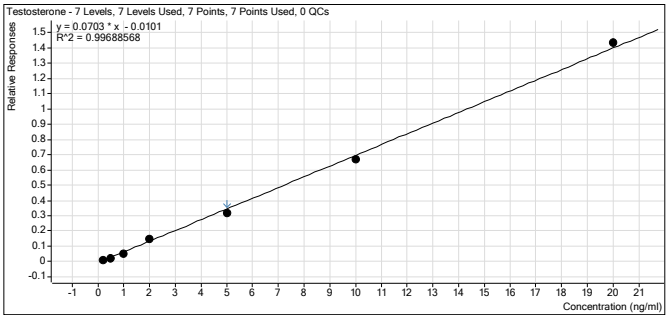

Figure 4 – Calibration curves for testosterone.

In order to fully validate the method, quality control materials obtained from SERO were reconstituted as per the manufacturer's instructions, extracted and analysed using the automated procedure. Data for testosterone is presented in Table 3 and 4 and the units are in ng/dl.

Sample	Testosterone
Sample_01	312
Sample_02	264
Sample_03	314
Sample_04	269
Sample_05	259
Sample_06	251
Sample_07	267
Sample_08	260
Target Value	310
Range	250 – 370
Pass/Fail	Pass
Average	275
SD	24.4
% RSD	8.9

Table 3 – Validation data from the Seronorm Low QC material

Sample	Testosterone
Sample_01	515
Sample_02	518
Sample_03	567
Sample_04	449
Sample_05	680
Sample_06	558
Sample_07	424
Sample_08	683
Target Value	570
Range	330 – 810
Pass/Fail	Pass
Average	549
SD	95.1
% RSD	17.3

Table 4 – Validation data from the Seronorm High QC material

Anatune Ltd, Broadway House, 149-151 St Neots Road, Hardwick, Cambridge, CB23 7QJ, UK
Tel: +44 (0) 1954 212909 Fax: +44 (0) 1954 212908 Email: info@anatune.co.uk Internet: www.anatune.co.uk

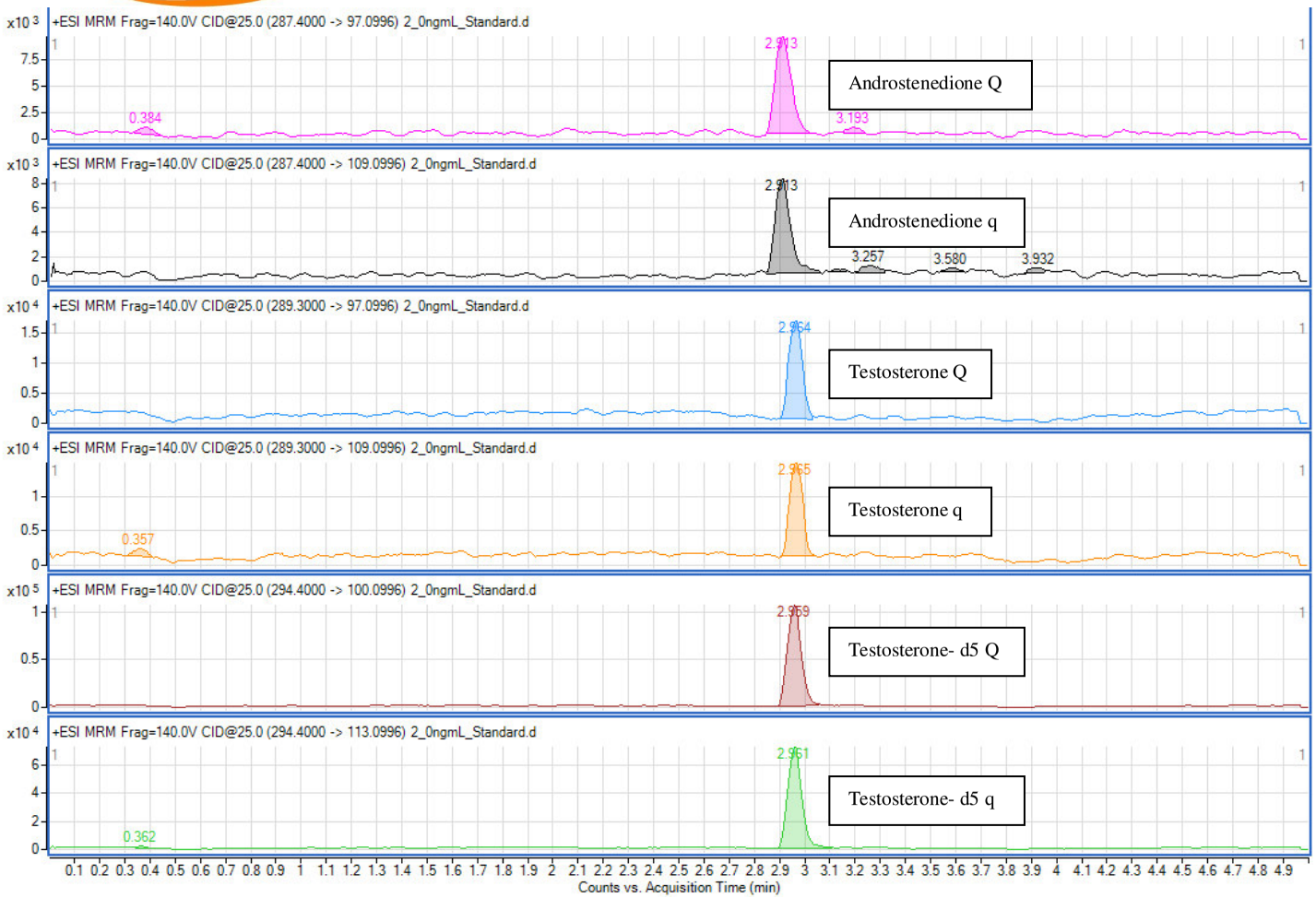

Figure 4 – Example chromatogram for a 200ng/dl extracted serum standard.

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Conclusions

Presented is a fully automated method for androstenedione and testosterone analysis featuring automated, internal standard and reagent addition, protein precipitation and centrifugation. Sample preparation is coupled directly to LC-MS/MS and is fully integrated within the Masshunter software. Alternatively it is possible to configure a standalone workstation. The system is capable of handling 98 samples in 13 hours and 15 minutes.

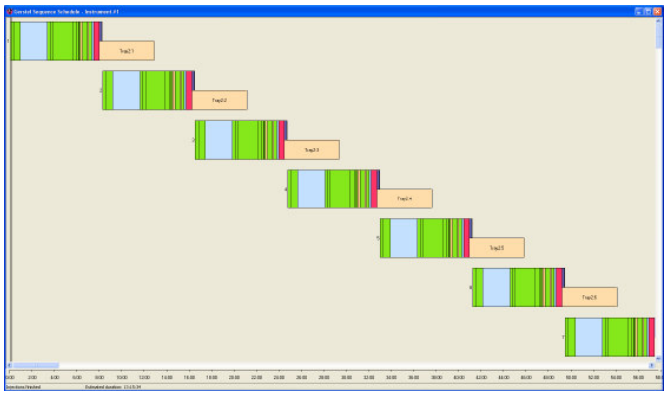

Figure 5 – Illustrating the Prepahead functionality of the automated system.

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