Which LC-MS/MS Platform is Most Appropriate for the Quantitative Analysis of Steroids in Serum for Clinical Research? WP129

Rory M Doyle, Douglas McDowell, Thermo Fisher Scientific, 265 Davidson Avenue, Somerset, NJ, USA 08873

ABSTRACT

Purpose: Steroids control metabolism, inflammation, immune functions, salt and water balance, development of sexual characteristics, and help in the body's ability to withstand illness and injury. A robust, sensitive, accurate, and specific LC-MS/MS analytical method has been developed for the quantitation of steroids such as aldosterone, androstenedione, corticosterone, cortisol, cortisone, 11-deoxycortisol, 21-deoxycortisol, 11-deoxycorticosterone, 18-OH-corticosterone, DHEA-S, DHEA, DHT, 17-OH-pregnenolone, pregnenolone, 17-OH-progesterone, progesterone, testosterone in serum. A simple liquid-liquid extraction sample preparation achieves the required sensitivity and can be used for quantitating in all matrices. This research study was carried out to evaluate which LC-MS/MS instrument is best suited to characterize and quantitate the steroids.

Methods: Thermo Scientific™ TSQ Fortis™, Thermo Scientific™ TSQ Quantis™, and Thermo Scientific™ TSQ Altis™ tandem mass spectrometers in positive and negative electrospray modes and a Thermo Scientific™ Vanquish™ HPLC system were utilized. A 200 mL serum sample was used, various columns were evaluated, and initially a Thermo Scientific™ Accucore™ C18 100 x 2.1 mm, 2.6 mm column with 0.2 mM ammonium fluoride in water and methanol mobile phases achieved baseline chromatographic separation in approximately 11 minutes run time. Quantitative analysis was performed using selective reactive monitoring (SRM) transition pairs for each steroid and internal standard in positive and negative mode. Accuracy of the analytical method was verified using UTAK, NIST, and pooled reference samples.

Preliminary Data: Good linearity and reproducibility were obtained with the concentration range from 1 pg/mL to 1000 ng/mL for the steroids with a coefficient of determination > 0.995 for the sample preparation and both mass spectrometer platforms. The lower limit of detection (LLOD) and lower limit of quantitation (LLOQ) were determined range from 1 to 5 pg/mL. Excellent reproducibility was observed for both compounds (CV < 10%) for all steroids in the various matrices on both platforms with and without derivatization. It was observed that testosterone, cortisol, cortisone, etc., achieved results with the TSQ Fortis MS and TSQ Quantis MS that were comparable to those with the TSQ Altis MS, although DHT and the pregnenolones worked best on the TSQ Altis MS. A sensitive, simple, specific, and accurate liquid chromatography - triple quadrupole (QQQ) mass spectrometry analytical method was developed and verified for the simultaneous measurement of steroids in serum on multiple TSQ series mass spectrometer platforms. The sample preparation techniques are quick and easily applied for high-throughput analyses on any mass spectrometer platform.

INTRODUCTION

Endogenous steroids are diverse chemical compounds that are classified as androgenic or estrogenic depending on their chemical structure and physiological impact. These compoundsinclude active parent and inactive forms that may be converted to their active parent, as well as active metabolites.

In this research study, we evaluated various columns and solvent combinations as well as simple and easy sample preparation techniques to develop an LC-MS/MS analytical method that can demonstrate the chromatographic separation, detection, and quantification of various androgenic steroids on various TSQ series mass spectrometer platforms. The sample preparation choice was kept simple and included a one-step liquid-liquid extraction without derivatization. The methodologies were developed on TSQ Fortis, TSQ Quantis, and TSQ Altis tandem mass spectrometers in positive and negative electrospray ionization modes with a Vanquish HPLC system for a 10 minute analytical gradient.

MATERIALS AND METHODS

Standards

The following analytical reference standards and Internal standards were obtained from Isosciences, LLC (King of Prussia, PA).

Aldosterone:	Aldosterone-D7
Androstenedione	Androstenedione-13C3
Corticosterone	Corticosterone-D4
Cortisol	Cortisol-D4
Cortisone	Cortisone-D8
11-Deoxycorticosterone	
11-Deoxycortisol	11-Deoxycortisol-D5
21-Deoxycortisol	21-Deoxycortisol-D8
18-Hydroxycorticosterone	
Dehydroepiandrosterone	Dehydroepiandrosterone-D5
Dehydroepiandrosterone-Sulfate	Dehydroepiandrosterone-Sulfate-D5
Dihydrotestosterone	Dihydrotestosterone-D3
Pregnenolone	Pregnenolone-13C2,14N2
17-Hydroxypregnenolone	17-Hydroxypregnenolone-13C2,14N2
Progesterone	Progesterone-D9
17-Hydroxyprogesterone	17-Hydroxyprogesterone-D8
Testosterone:	Testosterone-13C3

Reagents

The following Fisher Scientific™ acids, reagents, and solvents were used:

HPLC Grade Water	Methyl-Tert-Butyl Ether
Methanol	Acetonitrile
Ammonium Fluoride	

The standards and internal standards were made up in methanol.

Sample Preparation- Liquid-Liquid Extraction

- 200 mL of serum/HSA mixture calibrators, controls, and serum samples were added to a test tube and
  10 mL of steroid ISTD at 500 ng/mL were added to each and vortexed briefly.
- 1.5 mL of methyl-tert-butyl ether was added to each tube and vortexed for 1 min prior to centrifugation for 10 minutes at 13,000 rpm.
- The upper organic layer was transferred to a new test tube and dried down under nitrogen at room temperature.
- The extract was reconstituted in 200 µL of 1:1 water:methanol.
- The supernatant was transferred to an MS vial and capped.

The calibration curves ranged from 0.1 pg/mL to 1000 ng/mL and various pooled samples were used as control material.

Data Analysis

The software used for this method included Thermo Scientific™ Xcalibur™ 3.1 software, Thermo Scientific™ TSQ Altis™ Tune™ 2.1 software, and Thermo Scientific™ TraceFinder™ 4.1 software.

METHOD

HPLC Conditions
Vanquish Horizon HPLC binary pump, well plate, thermostatted column compartment

Column:	Accucore C18, 100 x 2.1 mm, 2.6 µm	
Column Temperature:	50 ºC	
Injection Volume:	10 µL	
Sampler Temperature:	4 ºC	
Needle Wash:	Flush port (50% methanol:50% water) 10 s	
Mobile Phase A:	0.2 mM ammonium fluoride in water	
Mobile Phase B:	Methanol	
Flow Rate:	0.5 mL/min	
Gradient:	0.0 min	50% A:50% B
	1.0 min	50% A:50% B
	7.0 min	40% A:60% B
	9.0 min	2% A:98% B
	10.0 min	2% A:98% B
	10.1 min	50% A:50% B
Run Time:	11.0 min	

MS and Ion Source Conditions
TSQ series triple quadrupole mass spectrometer: TSQ Fortis MS, TSQ Quantis MS, TSQ Altis MS

Ion Mode:	Positive and negative electrospray (HESI) mode
Vaporizer Temperature:	300 'C
Ion Transfer Tube Temperature:	225 'C
Sheath Gas:	60
Aux Gas:	25
Sweep Gas:	0
Spray Voltage:	Positive Ion: 3000 V; Negative Ion: 3000 V
Q1/Q2 Resolution:	0.7/0.7 (FWHM)
Cycle Time:	0.8 s
CID Gas:	2 mTorr
Chromatographic Peak Width:	6 s

Table 1. Scan parameters - SRM table

Compound	Rt (min)	Polarity	Precursor (m/z)	Product (m/z)	Collision Energies (V)	Rf Lens (V)
DHEA-Sulfate	0.76	Negative	372.3	79.9/96.9	50/32	149
Aldosterone	0.86	Negative	359.2	189.1/331.2	19/16	67
18-OH-Corticosterone	1.00	Positive	363.3	121.1/269.1	28/16	73
Cortisone	1.1	Positive	361.3	121.1/163.1	31/24	73
Cortisol	1.18	Positive	363.3	121.1/327.2	26/16	66
21-Deoxycortisol	1.62	Positive	347.3	121.1/269.2	27/19	63
11-Deoxycortisol	1.95	Positive	347.3	96.9/109.1	24/28	67
Corticosterone	1.85	Positive	347.3	121.1/329.1	24/15	63
Androstenedione	2.69	Positive	287.3	97.1/109.1	22/25	62
11-Deoxycorticosterone	3.07	Positive	331.3	97.1/109.1	23/26	65
Testosterone	3.19	Positive	289.2	97.1/109.1	22/25	64
17-OH-Progesterone	3.75	Positive	331.3	96.9/109.1	24/28	64
DHEA	3.79	Positive	271.1	253.1/213.1	13/17	57
17-OH-Pregnenolone	3.81	Positive	315.1	297.1/279.2	10/15	58
DHT	4.9	Positive	291.2	159.1/255.2	23/16	57
Progesterone	6.25	Positive	315.3	97.1/109.1	23/25	65
Pregnenolone	7.93	Positive	299.5	281.2/159.1	12/21	61

RESULTS

Linearity/Sensitivity
The assays were linear over the calibration curve for the steroids in steroid-depleted serum/HSA mixture as shown in Table 2 with their mean of coefficient of determinations (R^2) from 10 pg/ml to 1000 ng/ml. The linearity of each extraction was determined in triplicate over 3 days and the results are shown with the LOQ being determined as 10:1 of signal to noise. The mean coefficient of determination (R^2) > 98 for each sample extraction technique and the %CV for each calibration point were all < 10% in order to be accepted. The analysis of the steroids by positive mode electrospray using the LC and source conditions shown on the three TSQ series mass spectrometer platforms are also shown.

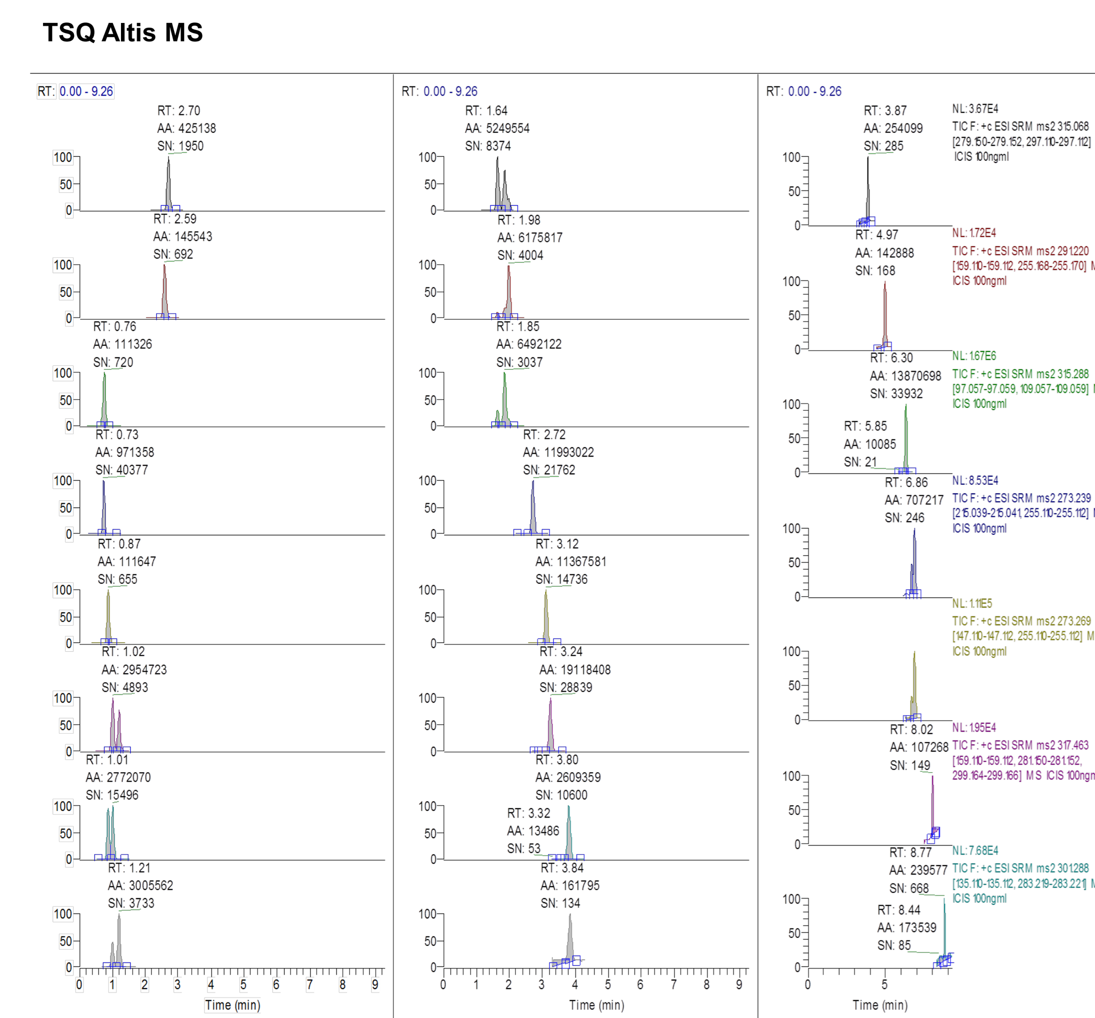
Precision/Specificity
The inter-assay precision for steroids was determined by extracting and quantifying three replicates of in-house control material resulting in %CV for the steroids of < 10% deviation from the targeted mean. The inter-assay precision was determined over three consecutive days and was found to have a %CV < 10% for each steroid within its respective linear range for the three levels of pooled serum sample control material respectively.

Therefore, the analytical method can achieve the required precision for the analysis of the androgenic and steroids in serum. Due to the similarity between the various steroids, there were interferences present as well as ion suppression from other steroids, which made obtaining consistent results difficult particularly for the 5-delta-steroids and their related steroids such as DHEA, DHEA-sulfate, pregnenolone, and 17-hydroxypregnenolone. The different TSQ series mass spectrometers also demonstrated differences; the TSQ Altis MS could see all the steroids analyzed while the TSQ Quantis MS and TSQ Fortis MS had varying results.

Accuracy
The accuracy was determined by the analysis of in-house control material as the percentage deviation from the targeted mean. The results were < 10% for all levels in each matrix. The serum in-house control material concentrations were 250 pg/mL, 25 ng/nL, and 250 ng/mL. Therefore, the analytical method can achieve research laboratory required accuracy for the analysis of the androgenic steroids in serum.

Figure 1. Chromatograms obtained on the TSQ Altis and TSQ Quantis platforms

TSQ Altis MS



TSQ Quantis MS

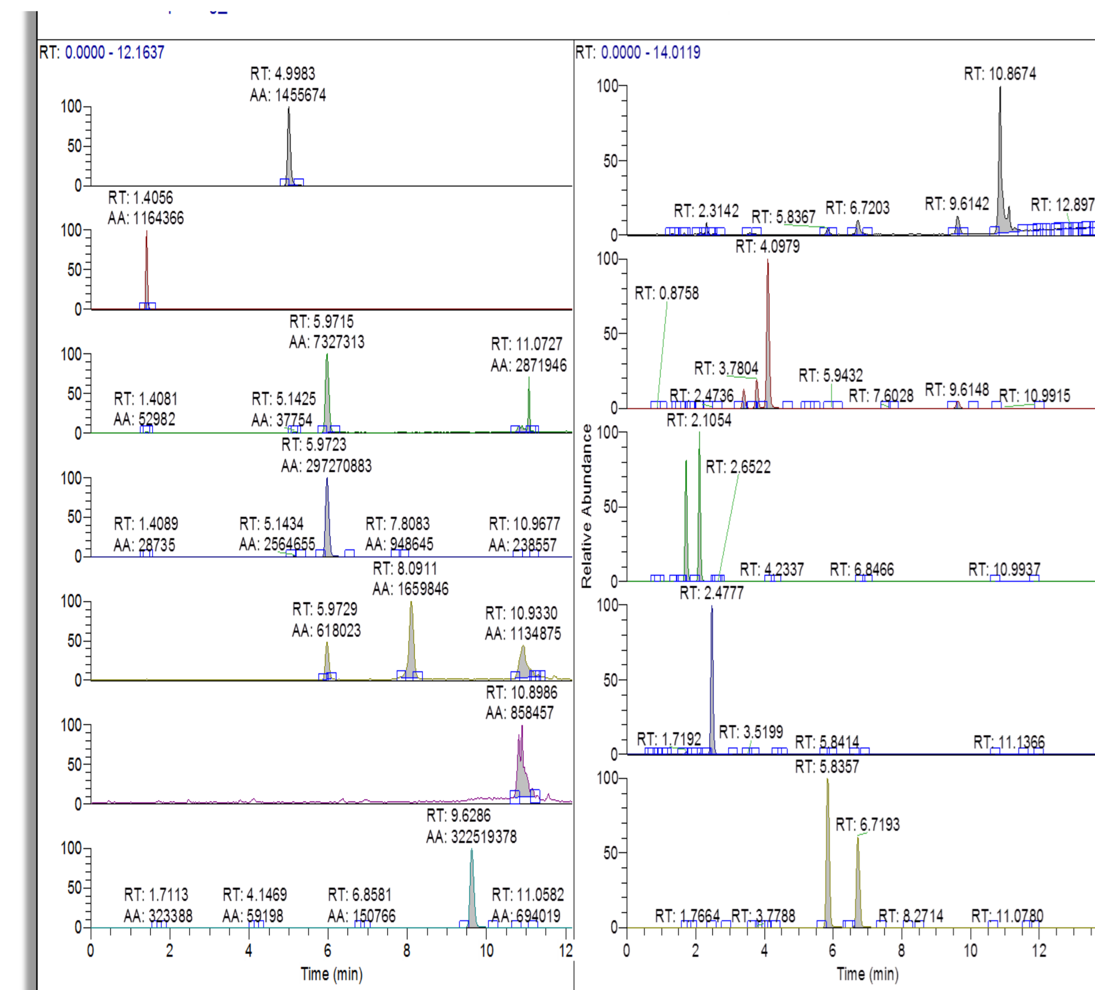


Table 2. Linearity and sensitivity for the extraction methodology of the TSQ series MS platforms

Compound	Linearity	TSQ Altis LOQ (pg/mL)	TSQ Quantis LOQ (pg/mL)	TSQ Fortis LOQ (pg/mL)
DHEA-Sulfate	50 pg/mL–1000 ng/mL	50	500	1000
Aldosterone	10 pg/mL–1000 ng/mL	10	25	50
18-OH-Corticosterone	10 pg/mL–1000 ng/mL	10	50	100
Cortisone	10 pg/mL–1000 ng/mL	10	25	25
Cortisol	5 pg/mL–1000 ng/mL	5	10	50
21-Deoxycortisol	10 pg/mL–1000 ng/mL	10	25	25
11-Deoxycortisol	5 pg/mL–1000 ng/mL	5	10	25
Corticosterone	10 pg/mL–1000 ng/mL	10	25	25
Androstenedione	1 pg/mL–1000 ng/mL	1	5	10
11-Deoxycorticosterone	5 pg/mL–1000 ng/mL	5	10	25
Testosterone	1 pg/mL–1000 ng/mL	1	5	5
17-OH-Progesterone	1 pg/mL–1000 ng/mL	1	10	10
DHEA	100 pg/mL–1000 ng/mL	100	250	1000
17-OH-Pregnenolone	100 pg/mL–1000 ng/mL	100	250	250
DHT	10 pg/mL–1000 ng/mL	10	100	500
Progesterone	10 pg/mL–1000 ng/mL	10	25	50
Pregnenolone	100 pg/mL–1000 ng/mL	100	500	1000

CONCLUSIONS

- Baseline separation of steroids in serum in 11 minutes with good LOD/LOQ in positive and negative mode was achieved on multiple TSQ series mass spectrometer platforms.
- A clean serum matrix is required to achieve the desired calibration curve and LOQ as the thyroid hormones bind to proteins and albumin within serum that can result in interfering responses.
- Good linearity of calibration curves with acceptable accuracy, precision, and reproducibility in positive mode was achieved with < 10% for %CV for each steroid within their linear range on each TSQ series platform.
- The TSQ Altis MS could obtain the most sensitive results down to the pg/mL levels required for all the steroids analyzed. The TSQ Quantis MS and TSQ Fortis MS could easily analyze testosterone, androstenedione, progesterone, and the corticosteroids down to their pg/mL levels but the TSQ Fortis MS had difficulty getting consistent results for DHT, DHEA, and the pregnenolones.

For Research Use Only. Not for use in diagnostic procedures.

REFERENCES

1. Therese Koal et al., Standardized LC–MS/MS based steroid hormone profile-analysis, *Journal of Steroid Biochemistry & Molecular Biology* **2012**, *129*, 129–138.
2. Mikael Levi, et al., Fast, Sensitive, and Simultaneous Analysis of Multiple Steroids in Human Plasma by UHPLC–MS–MS, *LCGC*, Mar 1, **2015**, 186.

TRADEMARKS/LICENSING

© 2018 Thermo Fisher Scientific Inc. All rights reserved. All trademarks are the property of Thermo Fisher Scientific and its subsidiaries. This information is not intended to encourage use of these products in any manner that might infringe the intellectual property rights of others.

ThermoFisher SCIENTIFIC

PO65277-EN 0518S