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LC-MS/MS quantitative analysis of Endogenous Androgenic and Estrogenic Steroids in Serum for Research Use

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ABSTRACT

Purpose: An LC-MS/MS analytical method was developed and verified for the quantitation of endogenous Androgenic and Estrogenic steroids in serum for research use. Simple sample preparation techniques including protein crash and liquid-liquid extraction with and without derivatization were evaluated. A Thermo Scientific™ TSQ Quantiva triple quadrupole mass spectrometer in positive and negative Electrospray mode with a Thermo Scientific™ Dionex™ Vanquish™ Horizon HPLC system was used.

Methods: 200 µL (PPX) and 500 µL (LLE) of serum were used for the analysis of the androgenic and estrogenic steroids. Various columns were evaluated and a Thermo Scientific™ Accucore™ C18, 100 x 2.1 mm, 2.6 µm with 0.2mM Ammonium Fluoride in water and methanol mobile phases achieved baseline chromatographic separation in approximately 11 minutes run time. Quantitative analysis was performed using scheduled reactive monitoring (SRM) transition pairs for each steroid and internal standard in positive and negative mode and accuracy of the analytical method was verified using pooled reference samples.

Results: Good linearity and reproducibility were obtained across the dynamic range of the endogenous steroids with a coefficient of determination R^2>0.95 or better for all compounds in the various matrices. The limits of detection and quantitation were determined to the pg/ml levels with very good reproducibility observed for all compounds.

INTRODUCTION

Endogenous steroids are a class of diverse chemical compounds that are classified as androgenic or estrogenic depending on their chemical structure and physiological impact. There are many different kinds which are active parent and inactive forms that are then converted to their active parent and as well as active metabolites which can have a significant influence on the body.

Therefore in this case, we evaluated various columns and solvent combinations as well as simple and easy sample preparation techniques in order to develop an LC-MS/MS analytical method that can demonstrate the chromatographic separation, detection and quantification of the most common androgenic and estrogenic steroids and metabolites in serum for research. The steroids analyzed include Aldosterone, Allopregnanolone, Androstenedione, Androsterone, Corticiosterone, Cortisol, Cortisone, 11-Deoxycorticosterone, 11-Deoxycortisol, 21-Deoxycortisol, 18-Hydroxycorticosterone, Dehydroepiandrosterone, Dehydroepiandrosterone-Sulfate, Dihydrotestosterone, Etiocholanolone, Pregnenolone, 17-Hydroxy-Pregnenolone, Progesterone, 17-Hydroxy-Progesterone, Testosterone, Estradiol, Estriol and Estrone. The sample preparation choices were kept simple and included protein crash and a one step liquid-liquid extraction with and without derivatization. The methodologies were developed on a Quantiva triple quadrupole mass spectrometer in positive and negative Electrospray ionization modes with a Vanquish Horizon HPLC system with a 11 minute analytical gradient.

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MATERIALS AND METHODS

Standards

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

Aldosterone:	100 μg	Aldosterone-D7:	100 μg
Allopregnanolone:	100 μg	Allopregnanolone-D5:	100 μg
Androstenedione:	100 μg	Androstenedione-13C3:	100 μg
Androsterone:	100 μg		
Corticosterone:	100 μg	Corticosterone-D4:	100 μg
Cortisol:	100 μg	Cortisol-D4:	100 μg
Cortisone:	100 μg	Cortisone-D8:	100 μg
11-Deoxycorticosterone:	100 μg		
11-Deoxycortisol:	100 μg	11-Deoxycortisol-D5:	100 μg
21-Deoxycortisol:	100 μg	21-Deoxycortisol-D8-	100 μg
18-Hydroxycorticosterone:	100 μg		
Dehydroepiandrosterone:	100 μg	Dehydroepiandrosterone-D5:	100 μg
Dehydroepiandrosterone-Sulfate:	100 μg	Dehydroepiandrosterone-Sulfate-D5:	100 μg
Dihydrotestosterone:	100 μg	Dihydrotestosterone-D3:	100 μg
Etiocholanolone:	100 μg	Etiocholanolone-D5:	100 μg
Pregnenolone:	100 μg	Pregnenolone-13C2,14N2:	100 μg
17-Hydroxypregnenolone:	100 μg	17-Hydroxypregnenolone-13C2,14N2:	100 μg
Progesterone:	100 μg	Progesterone-D9:	100 μg
17-Hydroxyprogesterone:	100 μg	17-Hydroxyprogesterone-D8:	100 μg
Testosterone:	100 μg	Testosterone-13C3:	100 μg
Estradiol:	50 μg	Estradiol-13C3:	50 μg
Estriol:	50 μg		
Estrone:	50 μg	Estrone-13C3:	50 μg

Reagents

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

HPLC grade Water	Methyl-Tert-Butyl Ether
Methanol	Acetonitrile
Ammonium Fluoride	Dansyl Chloride
Sodium Bicarbonate	

Sample Preparation- Protein Crash

- 200 μL of Serum/BSA mixture calibrators, controls and serum sample were added to 1.5 ml eppendorf tubes and 10 μL of Steroid ISTD at 500 ng/mL were added to each tube and vortexed briefly
- 400 μL of Acetonitrile was added to each tube and vortexed for 1 min prior to centrifugation for 10 minutes at 13000 rpm
- The supernatant was transferred to an MS vial and 200 μL of water was added to each vial and capped.
- All in-house calibrators were prepared in drug-free serum and bovine serum albumin mixture (Golden West Biological, Inc)

Sample Preparation- Liquid-Liquid Extraction Underivatized

- 500 μL of Serum/BSA mixture calibrators, controls and serum samples were added to a test tube and 25 μL of Steroid ISTD at 500 ng/mL were added to each and vortexed briefly
- 3 mL of Methyl-Tert-Butyl Ether was added to each tube and vortexed for 1 min prior to centrifugation for 10 minutes at 13000 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 water and methanol (100 μL of each)
- The supernatant was transferred to an MS vial and capped.

Sample Preparation- Liquid-Liquid Extraction Derivatized

- 500 μL of Serum/BSA mixture calibrators, controls and serum samples were added to a test tube and 25 μL of Steroid ISTD at 500 ng/mL were added to each and vortexed briefly
- 3 mL of Methyl-Tert-Butyl Ether was added to each tube and vortexed for 1 min prior to centrifugation for 10 minutes at 13000 rpm
- The upper organic layer was transferred to a new test tube and dried down under nitrogen at room temperature
- 50 μl of a 1g/L Dansyl Chloride in acetonitrile and 50 μl of 100 mM Sodium Bicarbonate at pH 10.5 was added to each tube, vortexed briefly and capped
- The tubes were then incubated at 70°C for 10 minutes then allowed to cool and then 100 μl of a 50%:50% Water:Acetonitrile mixture were added to each tube.
- 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 included for this method included the Thermo Scientific™ Xcalibur™ 3.1 SW, Thermo Scientific™ TSQ Endura Tune™ 2.1 SW, and Thermo Scientific™ Tracefinder™ 4.1 SW

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 °C
Injection Volume:	5 μL (PPX), 10 μL (LLE)
Sampler Temperature:	4 °C
Needle Wash:	Flush port (50%Methanol:50%Water) 10 seconds
Mobile Phase A:	0.2mM Ammonium Fluoride in Water
Mobile Phase B:	Methanol
Flow Rate:	0.5 ml/min
Gradient:	0 min- 50%A:50%B
	1.0 min- 50%A:50%B
	7.0 min- 40%A:60%B
	9.0 min- 5%A:95%B
	10.0 min-5%A:95%B
	10.1 min-50%A:50%B
Run time:	11.0 mins

MS and Ion Source Conditions-

Quantiva triple quadrupole mass spectrometer

Ion mode:	Positive and Negative Electrospray (H-ESI) Mode
Vaporizer Temperature:	300 °C
Ion Transfer Tube Temperature:	225 °C
Sheath Gas:	60
Aux Gas:	25
Sweep Gas:	0
Spray Voltage:	Positive Ion (V):3000 V/ Negative Ion (V): 3000V
Q1/Q2 Resolution:	0.7 (FWHM)
Cycle time (sec):	0.5
CID Gas (mTorr):	2
Chromatographic Peak Width:	6 secs

Table 1. Scan Parameters- SRM Table

Compound	RT (Min)	Polarity	Precursor (m/z)	Product (m/z)	Collision Energies (V)	RF Lens (V)
Estriol	0.75	Negative	287.2	145/171.1	41/37	149
DHEA-Sulfate	0.76	Negative	372.3	79.9/96.9	50/32	98
Aldosterone	0.86	Negative	359.2	189.1/331.2	19/16	67
18-OH-Corticosterone	1	Positive	363.3	121.1/269.1	28/16	73
Cortisone	1.01	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
Estradiol	2.53	Negative	271.2	145.1/183.1	39/41	129
Estrone	2.65	Negative	269.2	145.1/183.1	38/38	103
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	3.79	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
Etiocholanolone	6.6	Positive	273.2	215.1/255.1	14/13	53
Androsterone	6.78	Positive	273.3	255.1/147.1	13/20	54
Pregnenolone	7.93	Positive	299.5	281.2/159.1	12/21	61
Allopregnanolone	8.76	Positive	301.288	283.2/135.1	12/18	58

RESULTS

Table 2. Sensitivity.

Compound	Protein Crash LOD/LOQ (pg/ml)	LLE-Underiatized LOD/LOQ (pg/ml)	LLE-Derivatized LOD/LOQ (pg/ml)
Estriol	1000/2500	100/250	DC-1/ 2.5
DHEA-Sulfate	25/50	1000/2500	Interferences
Aldosterone	1000/2500	100/250	500/1000
18-OH-Corticosterone	50/100	10/25	50/100
Cortisone	50/100	10/25	50/100
Cortisol	100/250	25/50	1000/2500
21-Deoxycortisol	50/100	10/25	25/50
11-Deoxycortisol	50/100	5/10	100/250*
Corticosterone	25/50	10/25	50/100
Estradiol	1000/2500	100/250	DC-1/ 2.5*
Estrone	250/500	50/100	DC-1/ 2.5
Androstenendione	25/50	1/ 2.5	2.5/5
11-Deoxycorticosterone	25/50	5/10	0.5/0.5
Testosterone	25/50	1/ 2.5	10/25
17-OH-Progesterone	25/50	1/ 2.5	2.5/5
DHEA	1000/2500	100/250	100/250
17-OH-Pregnenolone	2500/5000	100/250	500/1000
DHT	1000/2500	100/250	250/500
Progesterone	50/100	10/25	10/25
Etiocholanolone	2500/5000	250/500	250/500
Androsterone	1000/2500	100/250	100/250
Pregnenolone	1000/2500	100/250	500/1000
Alopregnanolone	1000/2500	1000/2500	1000/2500

*** Interferences present in matrix. LOD/LOQ achieved with 5%BSA/PBS only**

Linearity/Sensitivity

The linear range of the steroids in serum/BSA matrix was from 10 pg/ml to 100 ng/ml. The linearity of each matrix was determined in triplicate over 3 days and the results are shown with LOD and LOQ being determined as 3:1 and 10:1 of signal to noise respectively where possible and the mean coefficient of determination (R2) > 0.98 for each matrix and the %CV for each calibration point were all <15%.

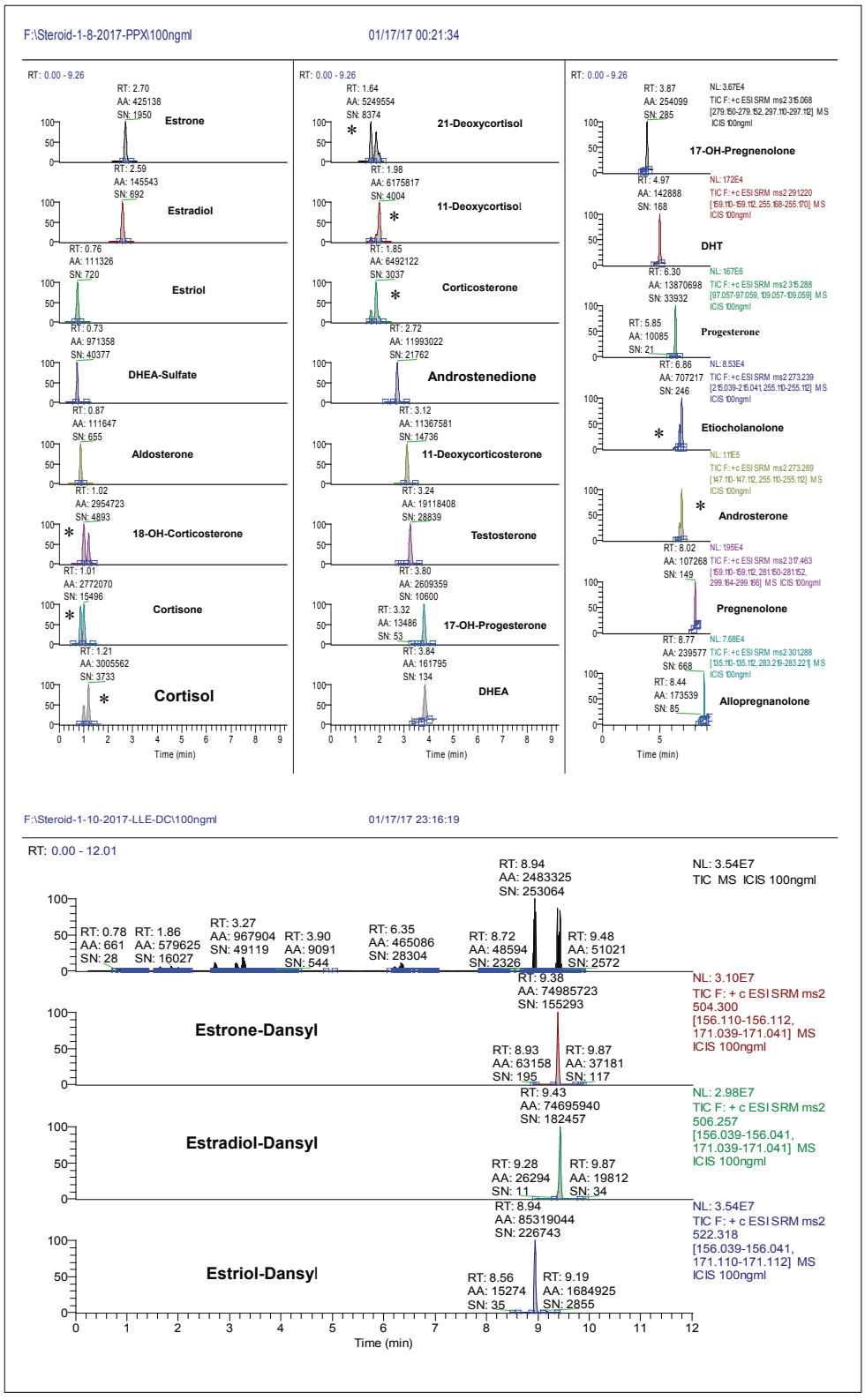
Accuracy

The accuracy was determined by the analysis of pooled sample control material as the percentage deviation from the targeted mean and the results were <15% for all levels in each matrix. The serum pooled 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 and estrogenic steroids in serum.

Precision/Specificity

The intra–assay precision (%CV) of the steroids in serum were determined by extracting and quantifying three replicates of the pooled sample control material. The inter-assay precision was determined over 3 consecutive days and was found to have a %CV <15% for each steroid within their 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 estrogenic 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, 17-Hydroxypregnenolone, Allopregnanolone, Etiocholanolone and Androsterone. Interferences also caused issues obtaining consistent results for Dansylated Estradiol. Separation of Etiocholanolone and Androsterone proved difficult and may require a larger diameter LC column.

Figure 1: Chromatograms



CONCLUSIONS

- Baseline separation of the androgenic and estrogenic steroids with good LOD/LOQ was achieved in serum
- Simple sample preparation achieved desirable LOD/LOQ to the relevant levels with further work to be carried out to fine tune these techniques to obtain more sensitive results and to remove the interferences and achieve better separation particularly for Etiocholanolone and Androsterone while maintaining ease of use and low cost
- Good linearity of calibration curves with acceptable accuracy, precision and reproducibility in positive and negative mode was achieved <15% for %CV for each steroid within their linear range and the sample preparation techniques and analytical methodologies will be further verified and optimized

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