Quantitation of Endogenous Steroids in Serum Using Dried Blood Spot Serum Separator Card and Triple Quadrupole Mass Spectrometry

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1. Overview

This poster utilized LC-MS/MS for the quantitation of select endogenous steroids using dried blood spots (DBS) serum separator cards. Two different stationary phase columns and two DBS cards which result in different volumes of sample were evaluated for this study.

2. Introduction

Endogenous steroids are essential to the regulation of several metabolic pathways including energy metabolism, stress, and fertility. Dried blood spots (DBS) (Fig. 1) offer an alternative to conventional venipuncture blood collection by allowing less invasive sample collection at home. A challenge for the lab is the small sample size collected from the dried blood spot resulting in low concentrations of endogenous analytes. To assist with accurate quantitation of select steroids in this biological matrix, Dispersive Pipette XTRaction (DPX) technology was utilized for increased sensitivity and selectivity by reducing matrix interference. Triple quadrupole mass spectrometry with its sensitive quantitation capability was used to analyze endogenous steroids. Optimized source conditions and MRM transitions on the mass spectrometer were used to detect clinically relevant concentration limits in matrix.

Figure 1. Two types of DBS cards. Left card produces ~30µL serum; right produces 6µL of plasma

3. Experimental Methods

Calibrators and QCs were prepared from commercially available steroid standards (Cerilliant, TX) spiked in stripped human serum (Golden West, CA). Correlation samples were prepared by charging blank ADx cards with serum from venous drawn samples. The ADx sample cards were dried overnight and stored in the aluminum Ziploc sealed shipping bags provided with the cards.

Aliquots of each calibrator and QC were plated, while the ADx card samples were paper punched into culture tubes. All samples were spiked with internal standard and submerged in a water: methanol solution and incubated on a plate-rocker for 25min. The samples were then transferred to a well plate and the reconstituted protein was digested prior to dSPE.

Each sample was exposed to DPX XTR tips containing 20mg Supel™ Swift HLB (60 µm) (DPX170357) sorbent to isolate the target analytes from the matrix and further remove interferences using the Bind-Wash-Elute protocol (Fig. 2).

Figure 2. DPX sample preparation procedure

DPX's patented technology provides dispersive SPE in a pipette tip that improves recovery by allowing the loose sorbent to mix more efficiently with the sample solution and increases the available surface area of the sorbent for more adequate sample binding than other traditional SPE products.

4. Analytical Method

A triple quadrupole mass spectrometer (LCMS-8060NX, Shimadzu) coupled to an HPLC (Nexera XR, Shimadzu) equipped with a pump, autosampler, column oven, was used for analysis (Fig. 3). Two column stationary phases (biphenyl and C18) were tested for evaluation. The C18 column resulted in less matrix interferences with minimal gradient adjustments. The analytical conditions chosen for this evaluation are listed in Table1 and Table 2.

The IonFocus design of the Shimadzu LCMS-8060NX improves signal intensity with higher gas flows and higher effective temperatures. The probe position can be moved further from the MS inlet without losing signal intensity

Figure 3. Shimadzu LCMS-8060NX

Table 1. HPLC parameters

Column	:Shim-pack Velox C18 (100mm x 3.0mm I.D., 2.7µm
Mobile phases	: A) 0.1mM ammonium fluoride in water : B) Methanol
Gradient Program	: B 50% (0-0.1min); B 95% (0.1-4min); B 95% (4.0-5min); B 50% (5-7min)
Flow rate	: 0.6mL/min
Column Temperature	: 40°C
Injection volume	: 10µL

Table 2. LC-MS/MS parameters

Ionization	: ESI (+) and (-) mode
Mode	: MRM
Interface Voltage	: +3.0 kV/-3.0 kV
Focus Voltage	: 2kV
Nebulizing gas flow	: 3 L/min
Drying gas flow	: 15 L/min
Heating gas flow	: 25 L/min
DL Temp.	: 250°C
Heat Block Temp.	: 500°C
DL Temp.	: 250°C
Heat Block Temp.	: 500°C
Interface Temp.	: 400°C
Probe position	: 3mm

5. Results

A six-point calibration curve with three QC levels were prepared for all analytes. Figure 4 shows the representative calibration curve and chromatogram for calibrator 1 using 30µL serum aliquots. Correlation of coefficient (r^2) values were above 0.99 for all analytes. Percent accuracy for each calibrator and QC level were between 80-120% of the expected concentration. Intra-day (n=6) and Inter-day (n=21, over 4 days) precision was evaluated with replicate QC extractions. Table 3 summaries the precision for all analytes as less than 8% RSD. Correlation comparing immunoassay results to this newly developed method is shown in Table 4. Eight unique samples were charged on ADx serum separator cards and the percent difference for all analytes compared to immunoassay results were less than 30% for all analytes in all samples.

A six-point calibration curve with three QC levels were also prepared using 12µL serum aliquots. Correlation of coefficient (r^2) values were above 0.99 for all analytes. Percent accuracy for each calibrator and QC level were between 80-120% of the expected concentration. The representative chromatogram for calibrator 1 is shown in Fig. 5. These initial results indicate Telimmune plasma serum separator cards may be a viable option as these cards produce smaller volumes of plasma.

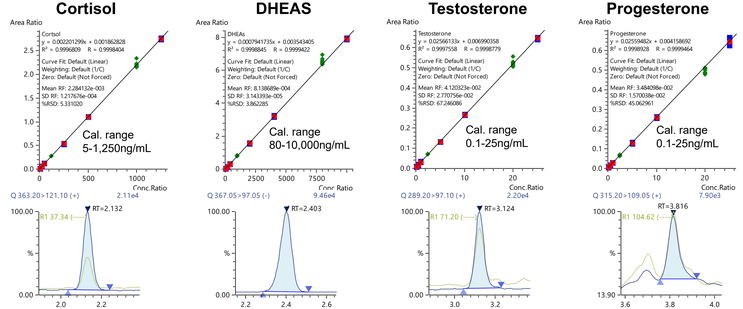

Figure 4. Calibration curves and chromatogram for calibrator 1 (30µL aliquot). Injection volume 10µL

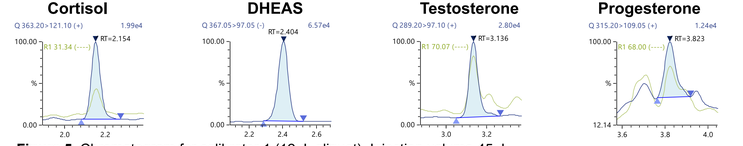

Figure 5. Chromatogram for calibrator 1 (12µL aliquot). Injection volume 45µL

Table 3. Intra- and inter-day precision for each QC level

Analyte	Sample ID	Intra-day %RSD (n=6)	Inter-day %RSD (n=21, 4 days)
Cortisol	LowQC	2.46	2.26
	MidQC	1.15	1.31
	HighQC	3.04	2.49
DHEAS	LowQC	2.52	2.3
	MidQC	2.64	2.38
	HighQC	3.4	3.85
Testosterone	LowQC	4.84	5.39
	MidQC	1.81	1.94
	HighQC	3.65	2.97
Progesterone	LowQC	5.46	7.2
	MidQC	2.98	2.22
	HighQC	2.61	3.28

Table 4. Correlation comparing LCMS results from charged ADx DBS cards vs. immunoassay

Sample Name	Analyte Name	Conc. (ng/mL)		
		LCMS Avg. Results	Immunoassay Results	%Diff
Sample A	DHEAs	778.50	763	-2.03
	Cortsol	65.95	66	0.07
	Testosterone	0.13	0.15	14.92
	Progesterone	4.32	5.29	18.26
Sample B	DHEAs	4133.22	4099	-0.83
	Cortsol	114.98	126	8.75
	Testosterone	0.24	0.32	26.45
	Progesterone	3.72	3.89	4.36
Sample C	DHEAs	1257.13	1234	-1.87
	Cortsol	87.24	102	14.47
	Testosterone	10.63	15	29.12
	Progesterone	0.35	0.43	18.29
Sample D	DHEAs	2056.68	1825	-12.69
	Cortsol	101.74	105	3.11
	Testosterone	1.65	2	17.26
	Progesterone	0.36	0.36	1.48
Sample E	DHEAs	3109.40	2585	-20.29
	Cortsol	88.97	92	3.30
	Testosterone	1.80	2	9.89
	Progesterone	0.37	0.32	-14.22
Sample F	DHEAs	3769.05	3373	-11.74
	Cortsol	202.04	241	16.17
	Testosterone	1.47	1.66	11.28
	Progesterone	0.37	0.34	-10.59
Sample G	DHEAs	525.91	513	-2.52
	Cortsol	146.87	150	2.09
	Testosterone	0.11	0.15	24.07
	Progesterone	0.63	0.55	-14.23
Sample H	DHEAs	766.75	909	15.65
	Cortsol	62.98	70	10.02
	Testosterone	0.10	0.13	19.97
	Progesterone	0.69	0.77	9.65

5. Conclusion and Future Studies

A highly sensitive and accurate LC-MS/MS method was developed for the quantification of four steroids on DBS cards. Further studies are currently underway to include five additional steroids (estrone, estradiol, estriol, DHT and DHEA) into the current method. Correlation studies are under evaluation using Telimmune DBS plasma separator cards which produce smaller volume of plasma. Telimmune cards provide 3µL of plasma per collection disk, which eliminates the requirement for manual punch. Quicker turnaround times are possible as the card is dried and ready to ship in 15min.