

Application News

No. LCMS-152

Liquid Chromatography Mass Spectrometry

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

■ Introduction

Endogenous steroids are essential to the regulation of several metabolic pathways including energy metabolism, stress, and fertility. Dried blood spots (DBS) (**Figure 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. A Shimadzu LCMS-8060NX triple quadrupole mass spectrometry with its sensitive quantitation capability was used to analyze four endogenous steroids (**Figure 2**). Optimized source conditions and MRM transitions on the mass spectrometer were used to detect clinically relevant concentration limits in matrix. Correlation between traditional immunoassay assays and this newly developed LC-MS/MS method was determined using eight unique human serum samples.



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

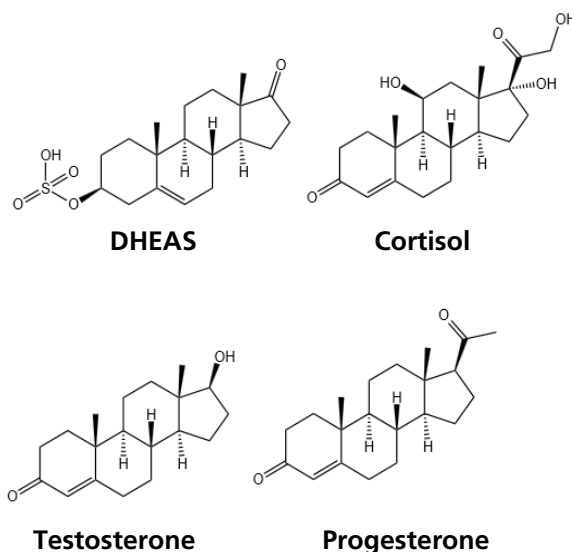


Figure 2: Four steroids quantitated in this LC-MS/MS method.

■ Experimental

Materials and Sample Preparation

A six-point calibration curve with three QC levels 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.

Thirty microliter 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 with 0.5% formic acid in 50% methanol in water 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 as shown in **Figure 3**. 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.

Bind - Wash - Elute



Figure 3: DPX Sample Preparation Procedure. Bind and wash solution: 50% methanol in water. Elution solution: acetonitrile.

Analytical Methods

Standards and samples were analyzed using the LCMS-8060NX triple quadrupole mass spectrometer coupled to Nexera HPLC equipped with a pump, autosampler, column oven as shown in **Figure 4**. Two column stationary phases (biphenyl and C18) were tested for evaluation. The biphenyl column was used for initial evaluation for correlation samples. The C18 column resulted in less matrix interferences with minimal gradient adjustments for spiked serum calibration and QC samples. The analytical conditions for both columns tested for this evaluation along with MS/MS conditions are listed in **Table 1**.



Figure 4: LCMS-8060NX configuration. The IonFocus design 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.

Table 1: HPLC and MS/MS Conditions

[LC Condition 1]		[LC Common Conditions]	
Column:	Shim-pack Velox C18 (100mm x 3.0mm I.D., 2.7µm	Mobile Phase A:	0.1mM ammonium fluoride in water
Gradient Program:	B 50% (0-0.1min); B 95% (0.1-4min); B 95% (4.0-5min); B 50% (5.01-7min)	Mobile Phase B:	Methanol
Flow Rate:	0.6mL/min	[MS/MS Conditions]	
Column Temperature:	40 °C	Ionization:	ESI (+) and (-) mode
Injection Volume:	10µL	Mode:	MRM
[LC Condition 2]		Interface Voltage:	+3.0 kV/-3.0 kV
Column:	Shim-pack Velox Biphenyl (100mm x 3.0mm I.D., 3.0 µm	Focus Voltage	2kV
Gradient Program:	B 30% (0min); B 55% (0.0-1min); B 80% (1.0-4.5min); B 80% (4.5min-6.3min); B 98% (6.30-6.31min); B 98% (6.31min-7.31min); B 30% (7.32min-8.7min)	Nebulizing gas flow:	3 L/min
Flow Rate:	0.8mL/min	Drying gas flow:	15 L/min
Column Temperature:	50 °C	Heating gas flow:	25 L/min
Injection Volume:	20uL	DL Temp:	250 °C
		Heat Block Temp:	500 °C

MRM Transitions

The quantitative and qualitative MRM transitions used for analysis are shown in **Table 2**.

Table 2: MRM transitions

Analyte	Ret. Time, (min), biphenyl	Ret. Time, (min), C18	Quant. Transition	Collision Energy (V)	Qual. Transition	Collision Energy (V)	Polarity
DHEAS-d5	2.023*	2.323*	372.10>98.0	13.0	---	---	-
DHEAS	2.029*	2.401*	367.05>97.05	13.0	---	---	-
Cortisol-d5	3.021	2.136	367.20>121.10	-23.0	---	---	+
Cortisol	3.034	2.142	363.20>105.10	-21.0	363.20	-23.0	+
Testosterone-d3	4.824	3.120	292.20>109.10	-23.0	---	---	+
Testosterone	4.834	3.125	289.20>109.10	-23.0	289.20>97.10	-22.0	+
Progesterone-d9	6.608	3.787	324.30>113.15	-11.0	---	---	+
Progesterone	6.690	3.812	315.20>109.05	-11.0	315.20>97.10	-17.0	+

*adequate column conditioning should be completed to avoid retention time shifting for DHEAS

■ Results

Calibrators and QCs

Representative calibration curves and chromatograms for calibrator 1 using 30µL serum aliquots for biphenyl and C18 column phases are show in **Figure 5** and **Figure 6**. Correlation of coefficient (r^2) values were above 0.99 for all analytes and percent accuracy was between 80-120% for all calibration and QC levels.

Intra-day (n=6) and Inter-day (n=21, 4 days) for replicate QC injection were less than 8% RSD as shown in **Table 3**.

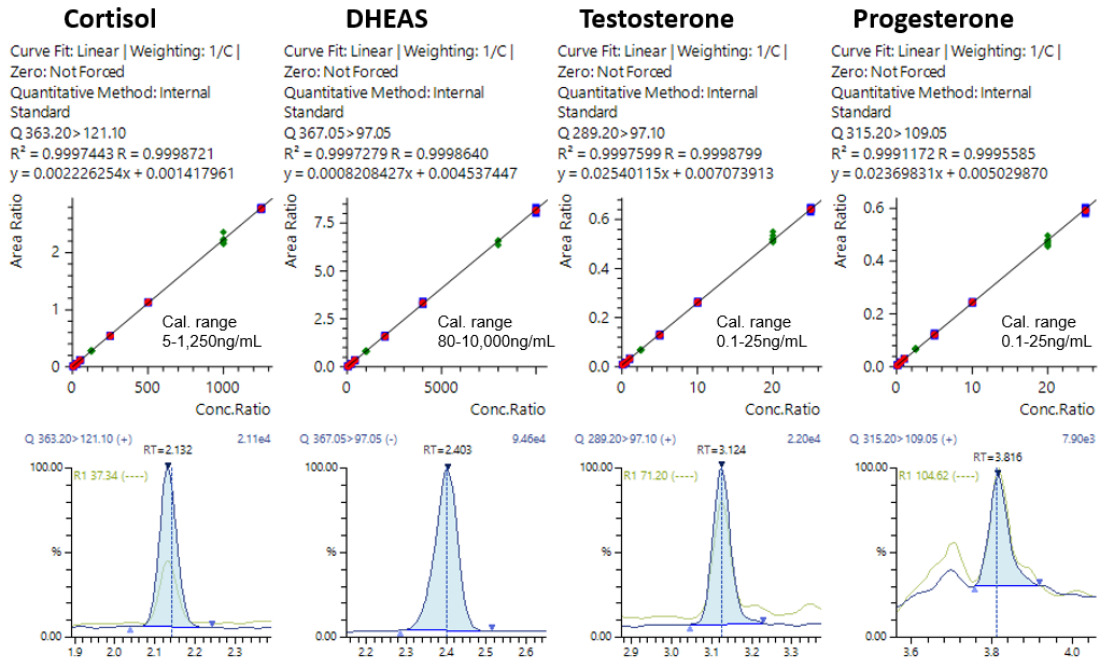


Figure 5: Calibration curves and chromatogram for calibrator 1 on C18 column.

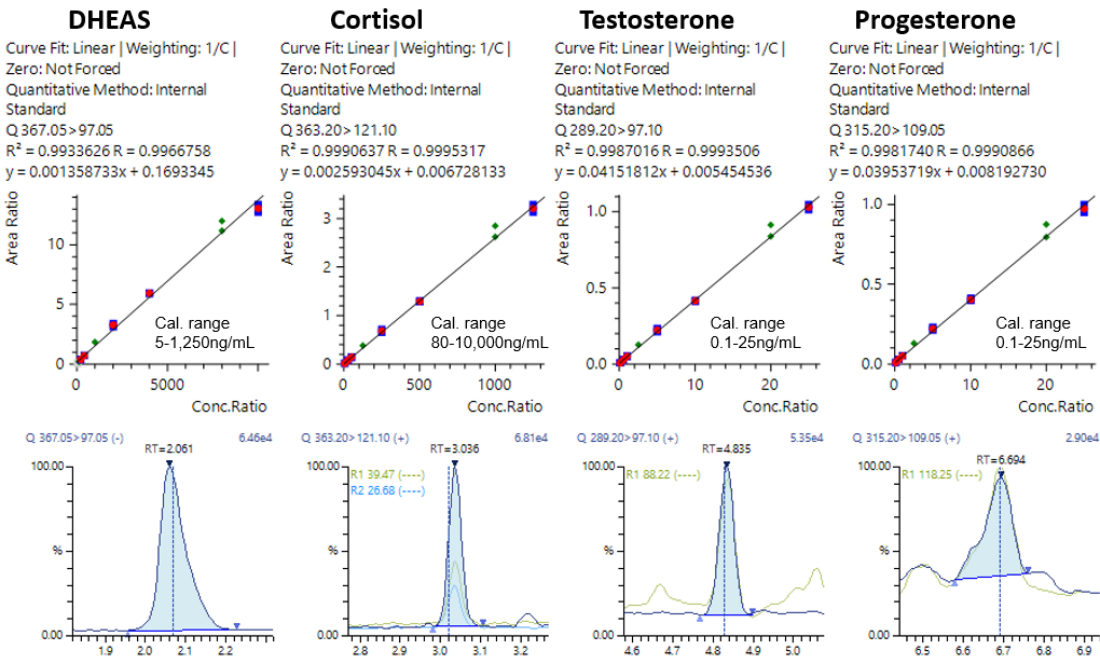


Figure 6: Calibration curves and chromatogram for calibrator 1 on biphenyl column.

Table 3: Intra- and inter-day precision for each QC level on C18 column.

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
	HighQC	2.98	2.22

Correlation

Correlation comparing immunoassay results for eight human venous drawn samples to this newly developed quantitative method is shown in **Table 4**. Each sample was charged with 30µL of serum on ADx cards. The percent difference for all analytes compared to immunoassay results were less than 30% for all analytes in all samples. A representative sample chromatogram is shown in **Figure 7**.

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

Sample Name	DHEAs			Cortisol			Testosterone			Progesterone		
	Conc. (ng/mL)		%Diff	Conc. (ng/mL)		%Diff	Conc. (ng/mL)		%Diff	Conc. (ng/mL)		%Diff
	LCMS	Immunoassay		LCMS	Immunoassay		LCMS	Immunoassay		LCMS	Immunoassay	
Sample A	779	763	-2.0	66	66	0.1	0.13	0.15	14.9	4.32	5.29	18.3
Sample B	4133	4099	-0.8	115	126	8.8	0.24	0.32	26.5	3.72	3.89	4.4
Sample C	1257	1234	-1.9	87	102	14.5	10.63	15	29.1	0.35	0.43	18.3
Sample D	2057	1825	-12.7	102	105	3.1	1.65	2	17.3	0.36	0.36	1.5
Sample E	3109	2585	-20.3	89	92	3.3	1.8	2	9.9	0.37	0.32	-14.2
Sample F	3769	3373	-11.7	202	241	16.2	1.47	1.66	11.3	0.37	0.34	-10.6
Sample G	526	513	-2.5	147	150	2.1	0.11	0.15	24.1	0.63	0.55	-14.2
Sample H	767	909	15.7	63	70	10.0	0.1	0.13	20.0	0.69	0.77	9.7

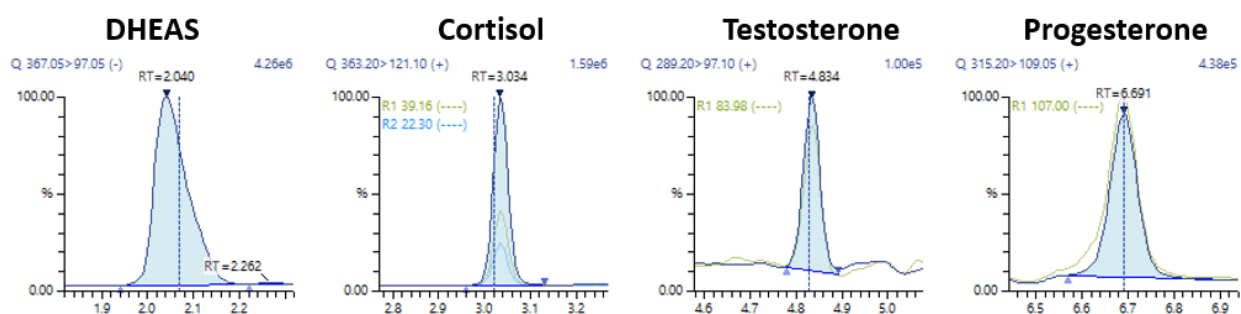


Figure 7: Chromatogram for Sample B on biphenyl column

■ **Conclusion**

A highly sensitive and accurate LC-MS/MS method was developed for the quantification of four steroids on ADx DBS cards, with calibration ranges for all compounds within published reference ranges. The sensitivity of LC-MS/MS analysis using the small sample volume from DBS has been comparable to classical immunoassay for all analytes.

This method has shown that fingerstick collection on DBS cards could potentially be a substitute to conventional venipuncture blood collection.

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