

High-Sensitivity Analysis of a Steroid Panel Samples using Micro-Flow LC-MS/MS for Clinical Research

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1. Overview

Development of a high-sensitivity method to assay a steroid panel in serum samples. Thanks to micro-flow LC/MS/MS, lower limit of quantification can be reached than usual methods.

2. Introduction

As they are involved in many physiological processes, steroids are important biomarkers to assess health status and to investigate environmental effects on exposed populations. In pediatric or post-menopausal samples, circulating levels of reproductive hormones are very low and therefore challenging to measure. In addition, sample volume availability can be limited adding difficulties for high-sensitivity analysis. Here we present a method to simultaneously measure seventeen steroids in serum samples using micro-flow LC/MS/MS at high sensitivity.

3. Methods

3-1. Reagents

Standard of aldosterone, androstenedione, corticosterone, cortisol, cortisone, dehydroepiandrosterone, 5-a-dehydrotestosterone, 11-deoxycorticosterone, 11-deoxycortisol, estradiol, estriol, estrone, 17a-hydroxypregnenolone, 17a-hydroxypregesterone, pregnenolone, progesterone and testosterone were obtained from Sigma-Aldrich (St Louis, USA). D4-aldosterone (IsoSciences, Ambler, USA) , d3-estradiol (Kanto Chemicals, Japan) and d3-Testosterone (Sigma-Aldrich) were used as internal standards. All solvents were of LCMS grade or analytical grade from Wako Chemicals (Osaka, Japan). Double charcoal-stripped serum (pool of healthy donors) from BioIVT (Westbury, USA) was used to prepare calibration curves and quality controls.

3-2. Sample Preparation

300 µL of serum sample were spiked with 30 µL of ISTD solution at 500 pg/mL in water/methanol (1/1) and submitted to supported liquid-liquid extraction (SLE+400, Biotage, Sweden). After elution with 2x500µL of ethyl acetate/hexane (75/25 v/v) and evaporation to dryness, samples were reconstituted with 50 µL of methanol and transferred to a vial with glass-integrated insert prior to injection in the system.

3-3. Analytical Conditions

A Nexera™ Mikros LC-MS/MS system with trap-and-elute configuration was used for analysis (Shimadzu Corp., Japan). Micro-LC system composed of a binary gradient microflow pump (LC-Mikros), a binary gradient with two analytical pumps for trapping process (LC-30AD), an autosampler (SIL-30AC), a 6-ports/2-positions switching valve (FCV-32AH) and a column oven (CTO-Mikros).

This system was coupled to high-sensitivity triple quad mass spectrometer (LCMS™-8060) with micro-ESI ionization source .

Other analytical parameters are described in Tables 1 and 2.

Table 1: LC Conditions

Trapping Column	: CERI C8 5µm 5*0.3mm
Trapping Flow Rate	: 200 µL/min
Trapping Mobile Phases	: A: Water/Methanol 95/5 v/v B: Acetonitrile/Isopropanol 1/1 v/v
Trapping Gradient	: 100% A (2min) → 100 %B (2-15min) → 100 %A (15-19min)
Injection Volume	: 10 µL
Microbore Column	: Shimadzu PLONAS Biphenyl 2.7µm 100*0.2mm
Temperature	: 50°C
Mobile Phases	: A: Water + NH ₄ F 0.15mM B: Methanol + NH ₄ F 0.15mM
Flow Rate	: 4 µL/min
Gradient	: 40% B (0-2.5 min) → 40 to 50%B (2.5-2.7 min) → 50 to 95%B (2.7-11 min) → 95%B (11-13 min) → 40%B (13.5-19 min)

Table 2: MS/MS conditions

System	: LCMS-8060
Ionization	: Micro-ESI
Probe Voltage	: +1.8 kV / -2.8 kV
Temperature	: Interface: 150° C Desolvation Line: 250° C Heater Block: 300° C
Gas Flow	: Nebulizing Gas: 1.7 L/min Heating Gas: 3 L/Min Drying Gas: Off
Dwell Time	: 5 ms
Pause time	: 1 ms

MRM	Compound	MRM Quant	MRM Qual
	Aldosterone (-)	359.20 > 189.30	359.2 > 331.35
	11-Deoxycorticosterone (+)	331.05 > 109.20	331.05 > 97.25
	11-Deoxycortisol (+)	347.05 > 109.20	347.05 > 97.30
	17-hydroxypregnenolone (-)	331.05 > 287.25	331.05 > 303.30
	17-hydroxypregesterone (+)	331.10 > 109.20	331.10 > 97.20
	Androstenedione (+)	287.05 > 97.20	287.05 > 109.1
	Corticosterone (+)	347.05 > 121.20	347.05 > 91.10
	Cortisol (+)	363.05 > 121.10	363.05 > 267.20
	Cortisone (+)	361.00 > 163.25	361.00 > 121.25
	DHEA (+)	289.05 > 253.20	289.05 > 271.25
	Dihydrotestosterone (+)	291.10 > 255.15	291.10 > 91.20
	Estradiol (-)	271.15 > 145.10	271.15 > 143.10
	Estriol (-)	287.10 > 143.10	287.10 > 145.10
	Estrone (-)	269.00 > 145.10	269.00 > 143.00
	Pregnenolone (+)	317.10 > 281.30	317.10 > 159.35
	Progesterone (+)	315.15 > 109.20	315.15 > 97.30
	Testosterone (+)	289.05 > 109.10	289.05 > 97.15

4. Results

4-1. Calibration

Calibration curve was calculated by linear regression with 1/x² weighting using internal standardization. Acceptance criteria for calibration levels was an accuracy comprised between 85-115% (80-120% at LOQ).

Typical calibration curves with their respective linear range are shown in Figure 1.



Figure 1 – Calibration Curves

4-2. Lower Limit of Quantitation

The lower limit of quantitation (LOQ) was established was injecting 5 independent replicates of a Quality Control spiked at each target compound respective LOQ. Acceptance criteria were an accuracy comprised between 80-120% and a %RSD< 20%.

The Figure 3 shows LOQ QC chromatograms and Table 3 the results obtained.

Table 3: LOQ results

	11-Deoxycorticosterone	11-Deoxycortisol	17-Hydroxypregesterone	Androstenedione	Corticosterone	Cortisol	DHEA	Dihydrotestosterone	
LOQ (pg/mL)	0.2	0.1	0.4	0.2	0.2	0.4	10	1	
Mean Accuracy (%)	107	98.3	100.5	98.2	93.6	85.2	117	105	
RSD (%)	5.2	13	9.0	15	8.1	11	4.5	5.0	
	Pregnenolone	Progesterone	Testosterone	Cortisone	17-Hydroxypregnenolone	Aldosterone	Estradiol	Estriol	Estrone
LOQ (pg/mL)	10	0.2	0.1	0.4	2	0.4	0.8	10	0.2
Mean Accuracy (%)	108	102	101	98.6	108	96.0	105	106	89.6
RSD (%)	7.1	4.2	6.6	10	14	20	14	4.0	3.5

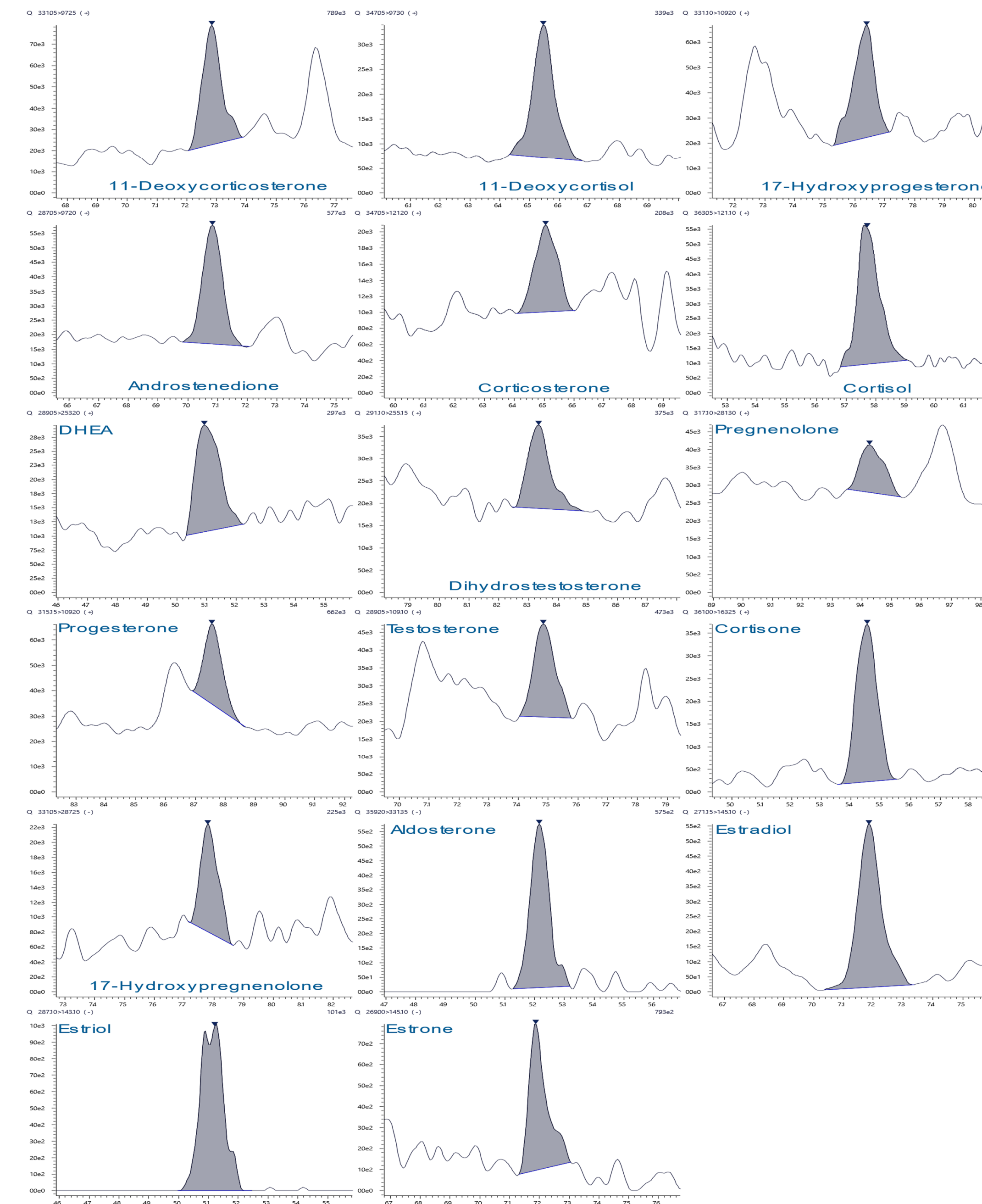


Figure 2– LOQ Chromatograms

5. Conclusions

A high-sensitivity assay was developed for 17 steroids in human serum samples to support clinical research and reached LOQ as low as 0.1 pg/mL (6 fg on column). Separation of isobaric compounds was ensured to prevent quantitative bias.