Quantitative Analysis of Steroids in Blood by Positive and Negative Ionization using QQQ LC-MS/MS

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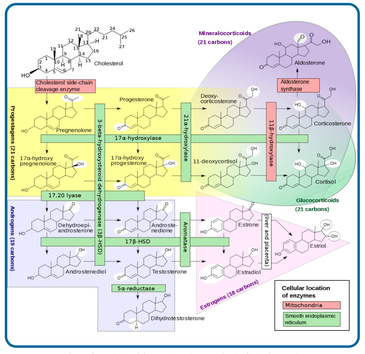
Introduction

Steroid hormones are derived from cholesterol and perform a number of important physiological functions. The steroids are synthesized mainly by endocrine glands – such as the gonads, adrenals and placental – and are then circulated through the blood stream. The main role of steroid hormones is to coordinate physiological and behavioral responses. They influence sexual differentiation, determine secondary sexual characteristics during development, trigger sexual maturation and control or modulate sexual behavior throughout adulthood.

Liquid chromatography triple quadrupole mass spectrometry (LC-MS/MS) has become an essential clinical research tool for analysis of endogenous steroids because of its ability to simultaneously analyze multiple analytes with high sensitivity, and excellent specificity and reproducibility.

In this study, thirteen major steroids were quantified. A robust, sensitive, reliable and fast method is presented for the quantitation of the major endogenous steroids in human serum using LC-MS/MS in both positive and negative ionization modes in a single run. This quantitative method demonstrates a wide dynamic range, excellent linearity, accuracy and reproducibility.

Figure 1. Human Steroidogenesis



Copy from http://en.wikipedia.org/wiki/File:Steroidogenesis.svg

Experimental

Sample Preparation:

Sample information: Thirteen steroid standards and four isotopic labeled internal standards are listed in Table 1.

Calibration curve: The calibration range of DHEA, estrone, estradiol, and estriol is from 0.2 to 2000 ng/mL. The calibration range of the other nine steroids is from 0.005 to 100 ng/mL. The dilution solvent is methanol:water 50:50.

Serum sample preparation: 250 µL human serum (obtained from UTAK Laboratories, Inc.) was crashed with 500 µL acetonitrile, votexed for 1 minute and centrifuged for 4 min at 10,000 rpm. 500 µL supernatant was transferred and diluted with 500 µL of water. 2 µL is injection onto LC-MS/MS.

LC Method:

Agilent 1290 Infinity UHPLC series binary pump, well plate sampler, thermostatted column compartment
Column: Extend C18, 2.1x50mm 1.8 um, 600 bar
Column temperature: 50 °C
Injection volume: 2 µL
Autosampler temp: 4 °C
Needle wash: flushport (MeOH:water 75:25), 10 sec
Mobile phase: A = 0.02 % ammonium hydroxide in water
B = methanol:isopropanol 75:25
Flow rate: 0.4 mL/min
Gradient: 20% B to 47% B in 7 minutes and up to 95% B in 1 min, hold at 95% B for 0.5 min, post run is 1.5 min

MS Method:

Agilent 6460 triple quadrupole mass spectrometer

Ion mode:	Agilent Jet Stream pos/neg
Gas temperature:	350 °C
Drying gas (nitrogen):	10 L/min
Nebulizer gas (nitrogen):	35 psi
Sheath gas (nitrogen):	350 °C
Sheath flow:	11 L/min
Capillary voltage:	+3000V/-3000V
Nozzle voltage:	+0V/-2000V
Q1/Q2 Resolution:	1.2/0.7 unit
Switching dwell time:	40 msec
Delta EMV:	+200V/-200V

Results and Discussion

Table 1. MRM acquisition table

Compound	Ion Mode	RT (min)	MRM	Dwell (msec)	Fragmentor (V)	CE (V)
DHEAS	ESI-	1.10	367.2>97.0	300	160	35
Estriol	ESI-	2.17	287.2>171.0, 145.0	100	140	37, 40
Aldosterone	ESI+	2.33	361.3>343.3, 315.2	100	100	15, 16
Cortisol	ESI+	3.23	363.2>327.2, 121.1	200	130	12, 20
11-Deoxycortisol	ESI+	4.45	347.3>329.3, 311.2	100	100	12, 12
Corticosterone	ESI+	4.81	347.3>109.1, 97.0	100	100	30, 30
Androsteronedione	ESI+	5.69	287.2>109.1, 97.0	50	100	25, 17
Estradiol-d5	ESI-	5.80	276.2>147.1	50	150	35
Estradiol	ESI-	5.87	271.2>183.1, 145.1	50	150	37, 35
Estrone	ESI-	5.97	269.2>183.1, 145.1	50	120	30, 37
Testosterone-d3	ESI+	6.27	292.2>97.0	50	100	25
Testosterone	ESI+	6.30	289.2>109.1, 97.0	50	100	25, 25
17-Hydroxyprogetserone	ESI+	6.71	331.3>109.1, 97.0	50	100	27, 27
DHEA-d5	ESI+	6.93	276.2>258.2	50	100	5
DHEA	ESI+	6.98	271.2>253.2, 197.2	50	100	5, 15
Progesterone-d9	ESI+	8.01	324.3>100.2	100	120	20
Progesterone	ESI+	8.03	315.3>109.2, 97.0	200	120	25, 20

Figure 2. Chromatography

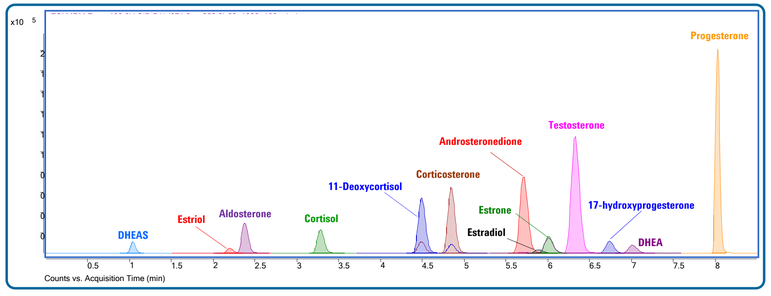
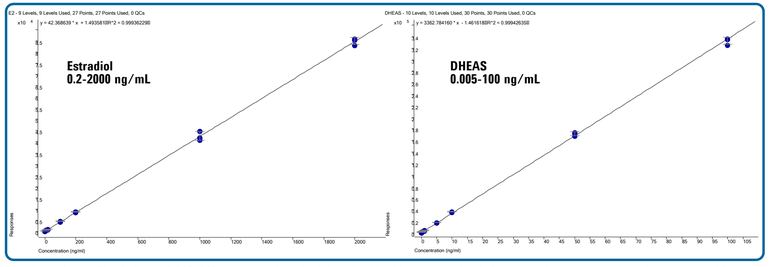


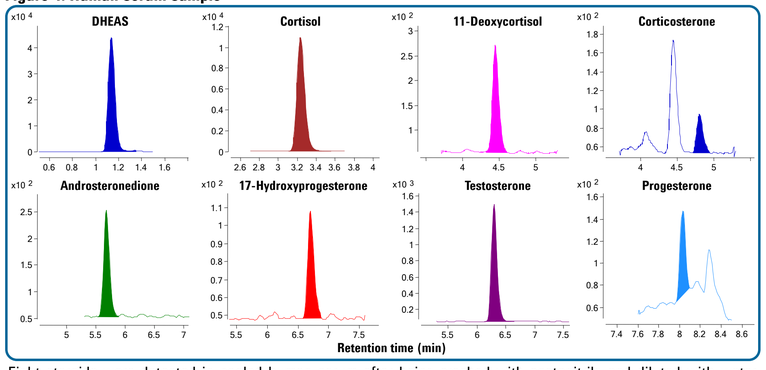
Figure 3. Calibration curves



Please Note : The Agilent LC-MS/MS system and method described in this presentation is for research purposes only and not approved for diagnostic use

Results and Discussion

Figure 4. Human serum sample



Eight steroids were detected in pooled human serum after being crashed with acetonitrile and diluted with water (Figure 4). The detected human serum levels are listed in Table 2. The simple sample preparation procedure results in a 6 factor dilution from the original serum. Estradiol, estrone, estriol and DHEA, which have lower normal levels, are not detected at very low levels. Increasing injection volume and using an enrichment column or drying down the extracts are alternatives that would improve detection limits. In a future study, a double charcoal stripped serum will be used.

Table 2. Summary

Compound	LOQ (ng/mL)	Range (ng/mL)	R^2	Accuracy (%)	Reproducibility (%)	Serum (ng/mL)
DHEAS	0.005	0.005-100	0.9994	91.8-104.8	0.87-5.30	1116
Estriol	0.2	0.2-2000	0.9981	92.8-113.2	0.09-5.02	n.d.
Aldosterone	0.05	0.05-100	0.9975	85.6-109.1	0.32-6.81	n.d.
Cortisol	0.05	0.05-100	0.9993	87.6-100.7	0.47-6.28	141
11-Deoxycortisol	0.01	0.01-100	0.9989	85.6-110.6	1.23-7.64	2.82
Corticosterone	0.01	0.01-100	0.9989	82.1-113.6	0.98-8.20	0.18
Androsteronedione	0.01	0.01-100	0.9987	89.0-108.7	1.01-7.70	0.84
Estradiol	0.2	0.2-2000	0.9993	85.6-103.9	0.99-9.26	n.d.
Estrone	0.2	0.2-2000	0.9990	91.4-101.8	1.39-4.07	n.d.
Testosterone	0.005	0.005-100	0.9988	93.4-115.7	0.76-4.93	3.42
17-Hydroxyprogesterone	0.01	0.01-100	0.9990	84.6-110.9	1.56-8.51	1.38
DHEA	0.2	0.2-2000	0.9954	87.2-110.2	0.79-8.73	n.d.
Progesterone	0.005	0.005-100	0.9982	87.5-107.4	0.91-7.59	0.042

Conclusion

- Baseline separation of thirteen steroids with the exception of estradiol is achieved under 8.5 minutes. However, estradiol is not isobaric to androsteronedione or estrone, so the quantitation calculation is not effected.
- The calibration curves show excellent linearity (> 0.995) with greater than three orders of dynamic range.
- Great accuracy, precision, reproducibility, and signal stability of LC-MS/MS (QQQ) analyses were observed for the 13 steroids.
- This fast and simple LC-MS/MS method is suitable for analyzing several endogenous steroids in biological matrices in a single run.