Low Level LC-MS/MS Analysis of Steroid Hormones in Human Serum and Plasma

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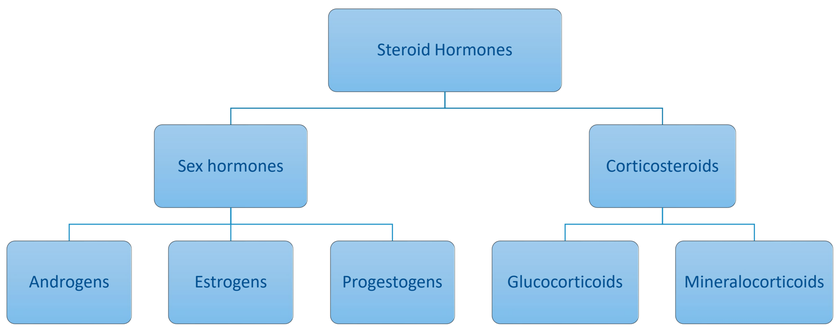
Introduction

Analysis of steroid hormones provides clinical insight into many biological processes within the human body allowing clinicians to diagnose and monitor numerous diseases/conditions. LC-MS/MS is the gold standard for this type of testing, offering superior detection capabilities over other techniques. However, analysis of steroid hormones by LC-MS/MS is very complex due to extremely low detection limit requirements, presence of isomers and isobaric matrix interferences, and poor ionization.

Figure 1. Steroid hormones subclasses.



To combat these challenges, method developers will often employ ammonium fluoride as a mobile phase additive, which has shown to significantly increase sensitivity for steroid hormone compounds. However, ammonium fluoride may form hazardous byproducts such as hydrofluoric acid gas (HF) and is also known to damage the silica of HPLC columns, so alternative solutions may be preferable. In this work, we explore the development of an LC-MS/MS workflow for the analysis of steroid hormones in serum/plasma that avoids the use of ammonium fluoride while still achieving low-level detection limits.

Method Development

A list of analytes was selected to encompass multiple classes of steroid hormones. The analytes and their classifications are shown below.

Analyte List

Androgens
- Androstenedione
- Dehydroepiandrosterone (DHEA)
- Dihydrotestosterone (DHT)
- Dehydroepiandrosterone sulfate (DHEAS)
- Testosterone

Estrogens
- Estrone (E1)
- Estradiol (E2)

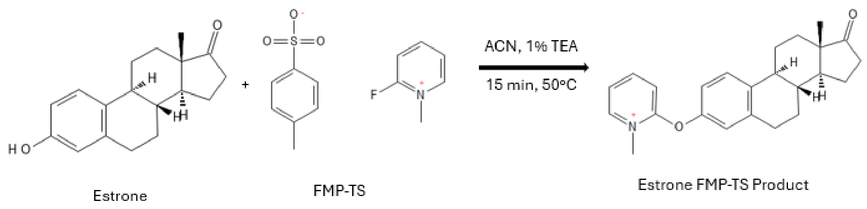
Progestogens
- Progesterone
- 17α-hydroxyprogesterone

Corticosteroids
- Cortisol
- Cortisone
- Aldosterone
- Corticosterone
- 11-Deoxycorticosterone
- 11-Deoxycortisol
- 21-Deoxycortisol

Derivatization

Multiple derivatization agents were evaluated for use in this workflow including dansyl chloride, methoxyamine, and methyl piperazine. These agents were not chosen for the final workflow for various reasons, including as unstable derivatization products, formation of side isomers, and long reaction times. 2-fluoro-1-methylpyridinium p-toluenesulfonate (FMP-TS), which reacts with primary and secondary hydroxyl (–OH) groups, was selected for the final workflow. An example of the derivatization reaction for the compound estrone (E1) is shown in Figure 2 below.

Figure 2. Derivatization of estrone with FMP-TS.

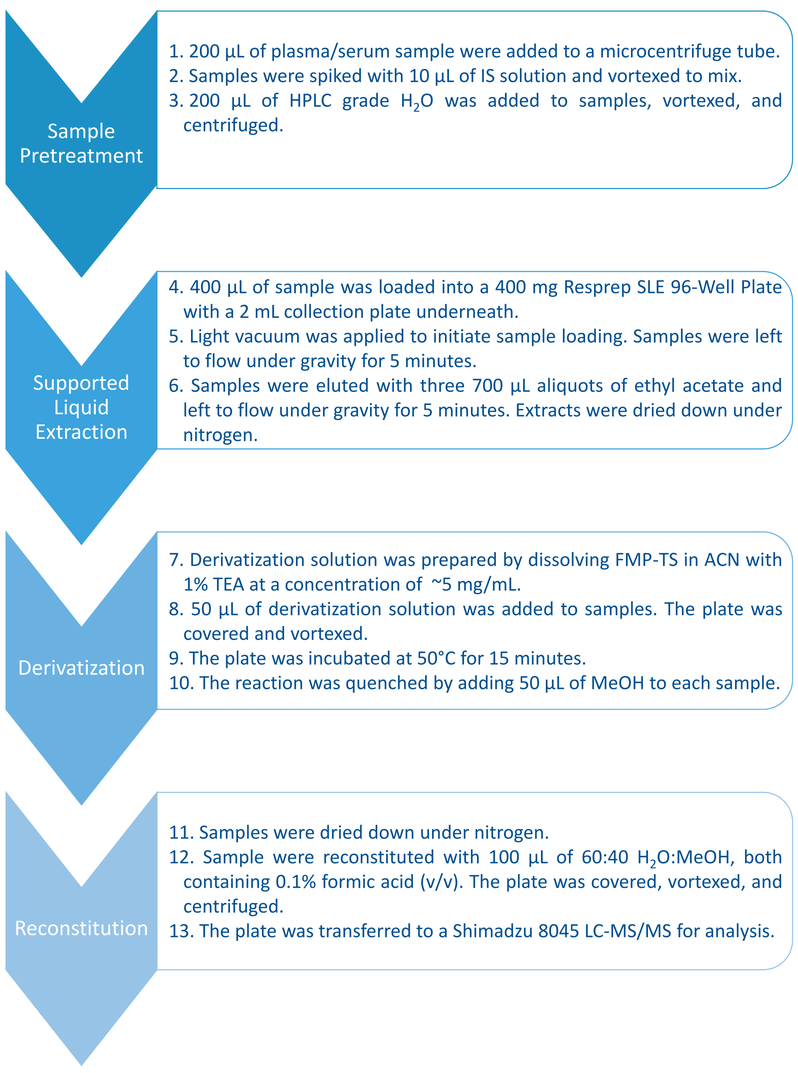


Method Development cont.

Sample Preparation

A supported liquid extraction (SLE) procedure was optimized for the recovery of steroid hormones in plasma/serum samples. While SLE materials are available in both 96-well plate or cartridge format, the 96-well plate is preferred for this workflow as it reduced the number of times sample transfers required, thus reducing analyte loss due to non-specific adsorption. The complete sample preparation workflow is described in Figure 3.

Figure 3. Steroid hormones sample preparation workflow.



Compound Optimization

The precursor/product ions for the 16 steroid hormone compounds and deuterated internal standards were optimized by infusing neat standards on the mass spectrometer in ESI mode. Compounds that reacted with FMP-TS formed a precursor ion of $[M+92]^+$ and compounds that did not react formed a precursor ion of $[M+1]^+$. The optimized MRMs are shown in Table 1 below.

Table 1. Optimized MRM transitions.

Analyte	Precursor Ion	Product Ion 1	Product Ion 2	Polarity
Androstenedione	287.1	97.1	109.2	+
Testosterone	289.1	97.1	109.1	+
Testosterone-D3	292.3	97.1	108.9	+
Progesterone	315.1	97.1	109.2	+
Progesterone-D9	324.2	100.2	113.1	+
17α-hydroxyprogesterone	331.1	97.2	109.2	+
21-Deoxycortisol	347.1	311.3	121.1	+
Estrone*	362.1	252.2	238.2	+
Estradiol*	364.1	110.1	128.0	+
DHEAS	367.0	97.0	79.7	-
Estradiol-D3*	367.1	110.1	128.1	+
DHEAS-D5	372.1	97.9	-	-
DHEA*	380.2	110.1	271.2	+
DHT*	382.2	110.1	255.2	+
11-Deoxycorticosterone*	422.2	110.1	187.5	+
Corticosterone*	438.2	110.1	120.3	+
11-Deoxycortisol*	438.2	110.1	311.3	+
Cortisone*	452.1	109.9	-	+
Aldosterone*	452.1	109.9	325.1	+
Cortisol*	454.1	110.1	142.9	+
Cortisol-D4*	458.5	110.1	-	+

*Analyte is analyzed as an FMP-TS derivative $[M+92]^+$.

Method Development cont.

Instrument Method

The following chromatographic conditions were selected for the final method.

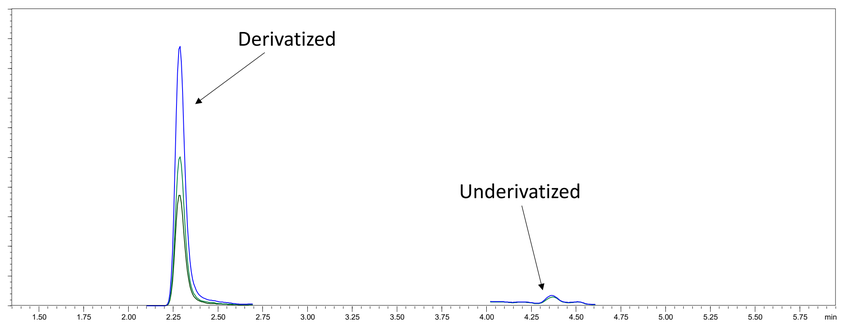
Table 2. Instrument conditions.

Column:	Restek Force Inert C18, 1.8 µm, 100 x 2.1 mm (cat# 9634212-T)		
Injection Volume:	12 µL		
Column Temperature:	60 °C		
Mobile Phase A:	Water, 2 mM ammonium formate, 0.1% formic acid		
Mobile Phase B:	Methanol, 2 mM ammonium formate, 0.1% formic acid		
Flow Rate:	0.4 mL/min		
Gradient:	**Time (min)**	**%A**	**%B**
	0.00	60	40
	6.00	10	90
	6.01	0	100
	7.00	0	100
	7.01	60	40
	8.00	60	40

Results & Discussion

The derivatization procedure was highly effective in enhancing the ionization of many analytes, particularly the estrogens. In Figure 4, estrone was analyzed both derivatized and underivatized at a concentration of 125 ng/dL. Derivatization with FMP-TS resulted in an increase in signal of more than 4000% for this analyte.

Figure 4. Estrone analyzed derivatized vs. underivatized.



An example chromatogram for the 16 steroid hormones in plasma/serum analyzed using the described method is shown below in Figure 5. An inert column was chosen for the final method to mitigate metal interactions for compounds that are prone to chelation, specifically DHEAS. Acceptable accuracy, precision, and linearity were demonstrated for each analyte over the calibration range listed in Table 3. LLOQs for individual analytes were determined based on these experiments. The calibration range encompasses expected clinical levels of each analyte in serum/plasma specimens.

Figure 5. 16 steroid hormones in plasma/serum analyzed using described method.

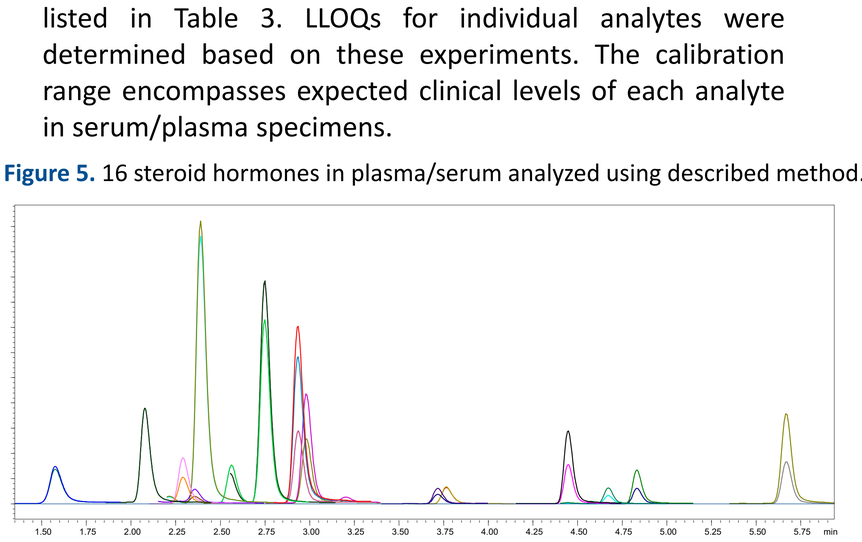


Table 3. Elution order, retention time, LLOQ, and calibration ranges.

Peak #	Analyte	RT (min)	LLOQ (ng/dL)	Calibration Range (ng/dL)
1	Aldosterone*	1.57	10	10-5000
2	Cortisone*	2.08	40	40-40000
3	Estrone*	2.29	0.5	0.5-500
4	Estradiol*	2.36	1	1-500
5	Cortisol*	2.39	40	40-40000
6	Corticosterone*	2.56	5	5-5000
7	11-Deoxycortisol*	2.75	5	5-5000
8	DHT*	2.92	10	10-5000
9	11-Deoxycorticosterone*	2.94	1	1-1000
10	DHEA*	2.98	10	10-5000
11	21-Deoxycortisol	3.71	5	5-5000
12	DHEAS	3.76	1000	1000-1000000
13	Androstenedione	4.49	5	5-5000
14	Testosterone	4.67	1	1-1000
15	17α-hydroxyprogesterone	4.83	5	5-5000
16	Progesterone	5.66	5	5-5000

*Analyte is analyzed as an FMP-TS derivative $[M+92]^+$.