POSTER NOTE

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High-Throughput LC-MS/MS Measurement of 17-Hydroxyprogesterone in Human Blood Serum for Research Purposes

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OVERVIEW

Purpose: To develop an LC-MS/MS method for researchers to accurately measure 17-hydroxy-pregnenolone (17-OHP) in blood serum from 10 to 1,000 ng/dL with a throughput of at least 12 injections per hour and can be multi-channeled with other LC-MS methods utilizing the same ion source.

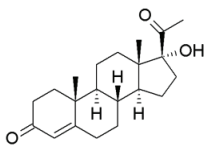
Methods: Liquid-liquid extraction (LLE) of blood serum followed by quantitative analysis using a 4-channel ultra high-performance liquid chromatography (UHPLC) system coupled to a tandem mass spectrometer (MS/MS). 17-OHP and its internal standard were separated from sample matrix components by water-to-methanol gradient elution through a UHPLC column packed with solid-core silica particles having an alkyl-bonded phase on its surfaces. Analytes were eluted to an atmospheric-pressure chemical ionization (APCI) probe of a triple-quadrupole mass spectrometer where selected-reaction monitoring (SRM) detected the analytes.

Results: An analytical range of 10 to 1,000 ng/dL was achieved with inter- and intra-batch reproducibility less than 6%, carryover less than 0.1% and acceptable correlation of specimen results with those from a reference laboratory. Throughputs of 14 to 56 injections/hour were achieved. Method accuracy and reproducibility were maintained while other channels ran other methods, such as testosterone.

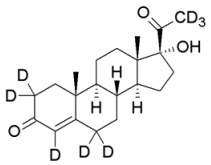
INTRODUCTION

17-OHP is a biosynthetic precursor to other steroids such as cortisol, androgens and estrogens. It is converted to 11-deoxycortisol by 21α-hydroxylase or to androstenedione by 17, 20 lyase. Researchers investigating anomalies of these enzymes, which can respectively be manifested as congenital adrenal hyperplasia and pseudohermaphroditism, need to measure 17-OHP within an analytical range of 10 to 1,000 ng/dL of blood serum. The chemical structures of 17-OHP and its deuterated internal standard (IS) are shown in Figure 1.

Figure 1. Chemical structures of analyte and IS



17α-Hydroxyprogesterone
(**17-OHP**) internal standard
Formula: $C_{21}H_{30}O_3$
Mono-isotopic mass: 330.2189



17α-Hydroxyprogesterone-D_8
(**17-OHP-D_8**) internal standard
Formula: $C_{21}H_{22}D_8O_3$
Mono-isotopic mass: 338.2692

MATERIALS AND METHODS

Consumables

HPLC-grade solvents, reagents, and most other consumables were from Thermo Fisher Scientific. 17α-Hydroxyprogesterone and 17α-Hydroxyprogesterone -D8 were from Cerilliant (Round Rock, TX). Calibrators were made by mixing stock standard with a diluent of 1% bovine serum albumin in phosphate-buffered saline. 17-OHP controls were from UTAK (Valencia, CA).

Sample Preparation

200 µL aliquots of fresh blood serum specimens, as well as calibrators and quality control specimens (QCs), were spiked with 17-OHP-D8 internal standard (IS) before being subjected to liquid-liquid extraction with 1 mL methyl t-butyl ether (MTBE). After drying the ether extracts by heated nitrogen flow, the residue of each was reconstituted with water and methanol (1:1) to a total volume of 150 µL and 50 µL injections were made into the LC-MS/MS system.

Test Methods

Using one or more channels of a Thermo Scientific™ Transcend™ LX-4 system, chromatographic segregation of the steroids from unwanted sample components was accomplished by gradient elution through a Thermo Scientific™ Accucore™ RP-MS column (2.6 µm, 50 x 2.1 mm), which was heated to 50°C. Chromatographic conditions are described in Figure 2. The Thermo Scientific™ TSQ Endura™ triple-quadrupole mass spectrometer was used with an APCI probe. Ion source and MS/MS conditions are described in Figure 3.

Instrument Control & Data Analysis

Thermo Scientific™ TraceFinder™ with Aria™ MX software was used to control the Transcend LX-4 and Endura MS/MS systems, submit batches to desired channels as well as for analyzing data and reporting results.

Figure 2. Liquid chromatography parameters

Column:	Accucore RP-MS, 2.6 µ, 50 x 2.1 mm
Solvent A:	Water + 0.1% Formic Acid
Solvent B:	Methanol

Step	Start	Sec	Flow	Gradient	%A	%B
1	0.00	10	0.5	Step	90	10
2	0.17	20	0.5	Step	50	50
3	0.50	60	0.5	Ramp	30	70
4	1.50	30	0.5	Ramp	-	100
5	2.00	30	0.5	Step	-	100
6	2.50	30	0.5	Step	90	10
7	3.00	60	0.7	Step	90	10

Start data: 2.0 min Duration: 1.0 min

Total run time: 4.0 min

Figure 3. Mass spectrometer APCI & SRM parameters

Ion Source:	APCI, + Ion Current 3 µA, vaporizer temp: 400°C
SRM Transitions:	Q1 & Q3 resolutions: 0.7

Analyte	Q1	Q2	CE	RF
17-OHP (Confirm)	331.20	97.20	22	150
17-OHP (Quan)	331.20	109.20	26	150
17-OHP-D_8 (Confirm)	339.25	100.22	24	160
17-OHP-D_8 (Quan)	339.25	113.22	28	160

RESULTS

Quantitative Analysis Reliability

Typical results from calibrators, QCs and specimens are show in Figure 4. Ion ratios (Confirming/Quan transitions) averaged 110% for 17-OHP and 140% for 17-OHP-D8.

IS peak areas among calibrators & QC averaged 8,050 with RSD < 6%. IS peak areas in specimens ranged from 6,670 to 10,290 and showed an average recovery of 102%. Carryover was less than 0.1%. Inter- and intra-batch precisions were less than 5 and 6% coefficient of variation (CV), respectively (Tables 1a & 1b).

Throughput

Single-channel throughput was 14 injections per hour. When multi-channeled across 2, 3 or 4 channels, the throughput increased to 28, 42 and 56 injections per hour, respectively. 17-OHP batches were also multi-channeled with MMA (1) batches which utilized the same MS source conditions.

Figure 4. Typical 17-OHP quantitative results

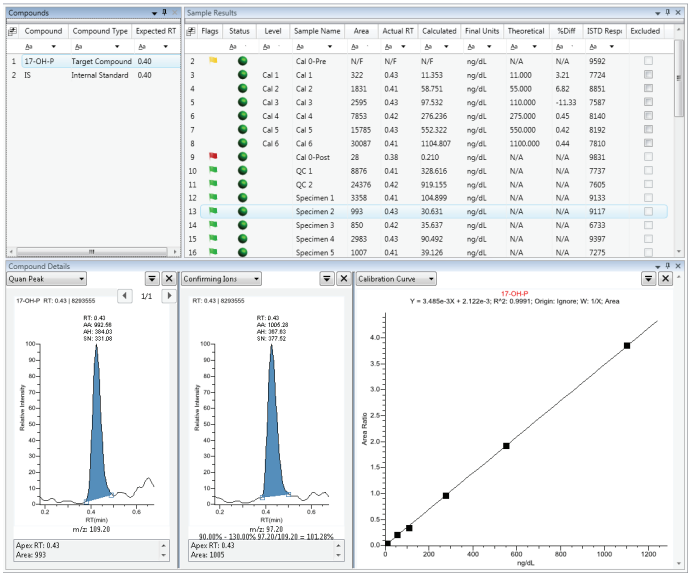


Figure 5. Inter- and Intra-batch Precision

Injection	17-OHP (ng/dL)
1	86
2	74
3	80
4	85
5	84
6	84
7	84
8	79
9	83
10	76
11	75
12	86
13	81
14	78
15	83
16	83
17	87
18	83
19	84
20	86
Mean	**82.05**
STD	**3.85**
CV %	**4.7**

a. Inter-batch

Date	Injection	17-OHP (ng/dL)
Day 1, 10/7/14	1	86
	2	74
	3	80
	4	85
	5	84
Day 2, 10/8/14	1	80
	2	77
	3	78
	4	82
	5	89
Day 3, 10/9/14	1	85
	2	85
	3	76
	4	77
	5	87
Day 4, 10/10/14	1	77
	2	82
	3	74
	4	86
	5	77
	Mean	**81.05**
	SD	**4.63**
	CV %	**5.71**

b. Intra-batch

Accuracy Assessment

Comparison of 40 specimen results ranging from 11 to 828 ng/dL with reference-lab results showed differences that averaged 3.0% and ranged from -29.6 to 15.4 (Table 2). Only 3 of 40 test samples (7.5%), deviated 20% or more. Deming regression analysis showed $R^2 = 0.9961$, Slope = 0.979, Intercept = 2.8, Standard Error of Estimate = 10.6%

Table 2. Reference lab results comparison

Test Sample	Current Method	Reference Lab	Difference %	Test Sample	Current Method	Reference Lab	Difference %
1	35	27	-29.6	21	81	71	-14.1
2	37	32	-15.6	22	54	58	6.9
3	28	27	-3.7	23	108	97	-11.3
4	34	27	-25.9	24	112	127	11.8
5	48	48	0.0	25	177	161	-9.9
6	23	23	0.0	26	34	35	2.9
7	22	26	15.4	27	34	31	-9.7
8	18	18	0.0	28	164	168	2.4
9	31	32	3.1	29	37	35	-5.7
10	71	72	1.4	30	91	82	-11.0
11	67	77	13.0	31	94	88	-6.8
12	82	85	3.5	32	207	181	-14.4
13	53	55	3.6	33	53	49	-8.2
14	93	98	5.1	34	63	61	-3.3
15	41	41	0.0	35	84	70	-20.0
16	44	43	-2.3	36	13	11	-18.2
17	46	48	4.2	37	80	85	5.9
18	121	114	-6.1	38	246	213	-15.5
19	42	39	-7.7	39	185	199	7.0
20	25	24	-4.2	40	12	11	-9.1

CONCLUSIONS

17-OHP can be accurately measured in blood serum by this method which achieved:

- Analytical range from 10 to 1,000 ng/dL
- Throughputs of 14, 28 or 56 injections per hour from a 1-, 2- or 4-channel system
- Inter- & intra-batch precisions less than 6% and carryover less than 0.1%
- Multi-channeling with other methods utilizing the same APCI source

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