POSTER NOTE

No. 90219

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

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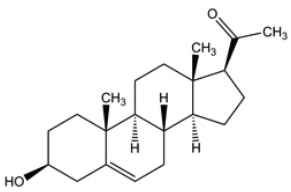
ABSTRACT

We developed an LC-MS/MS method for researchers to accurately measure pregnenolone in blood serum from 10 to 500 ng/dL with a throughput of at least 12 injections per hour. This was done by liquid-liquid extraction (LLE) of blood serum followed by derivatization with hydroxylamine and quantitative analysis using a 4-channel ultra high-performance liquid chromatography (UHPLC) system coupled to a tandem mass spectrometer (MS/MS). Oxime derivatives of pregnenolone and its internal standard were separated from sample matrix components by gradient elution through a UHPLC column packed with solid-core silica particles having an alkyl-aromatic bonded phase on its surfaces. Analytes were eluted to a heated electro-spray ionization (HESI) probe of a triple-quadrupole mass spectrometer where selected-reaction monitoring (SRM) detected the analytes. The desired analytical range of 10 to 500 ng/dL was achieved with inter- and intra-batch reproducibility of less than 8%, carryover less than 0.2% and acceptable correlation of specimen results with those from a reference laboratory. Throughputs of 13 to 52 injections/hour were achieved.

INTRODUCTION

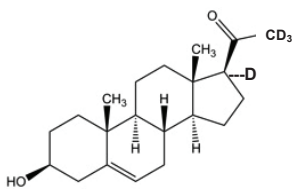
Pregnenolone is a biosynthetic precursor to other steroids such as corticosteroids, androgens and estrogens. It is converted to progesterone by 3-beta-hydroxysteroid dehydrogenase or to 17-OH-pregnenolone by 17-alpha-hydroxylase. Researchers investigating anomalies of these enzymes, which result in steroid hormone imbalances, need to measure pregnenolone within an analytical range of 10 to 500 ng/dL of blood serum. The chemical structures of pregnenolone and its deuterated internal standard (IS) are shown in Figure 1. Since pregnenolone does not ionize well in either atmospheric-pressure chemical-ionization (APCI) or electro-spray ionization (ES) sources, derivatization with hydroxyl amine was necessary to achieve the desired lower-limit of quantitation (LOQ). The resulting positive-ion oximes (Figure 2) were quantitated via UHPLC-MS/MS using pregnenolone-D4 as the IS.

Figure 1. Chemical structures of analyte and IS



Pregnenolone

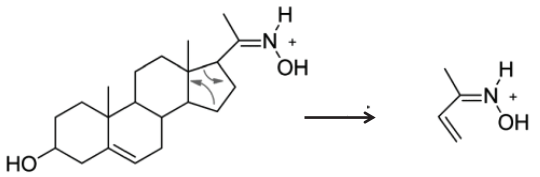
Formula: $C_{21}H_{32}O_2$
Mono-isotopic mass: 316.2397
Oxime derivative *m/z* = 332.3



Pregnenolone-17α,21,21,21-D_4
internal standard (**IS**)
Formula: $C_{21}H_{28}D_4O_2$
Mono-isotopic mass: 320.2648
Oxime derivative *m/z* = 336.3

Reactions with hydroxylamine form positive-ion oxime

Figure 2. Pregnenolone oxime fragmentation



$C_{21}H_{34}NO_2^+$
m/z 332.258

$C_4H_8NO^+$
m/z 86.060

Hydroxylamine derivative

MATERIALS AND METHODS

Sample Preparation
200 µL aliquots of fresh blood serum specimens, as well as calibrators and quality control specimens (QCs), were spiked with pregnenolone-D4 intern standard (IS) before being subjected to liquid-liquid extraction with 1 mL m butyl ether (MTBE). After drying the ether extracts by heated nitrogen flow residue of each was reacted with hydroxylamine to form positive-ion oxime derivatives. After drying the derivative preparations, the residue of each w reconstituted with water and methanol (1:1) to a total volume of 200 µL an µL injections were made into the LC-MS/MS system.

Test Methods
Using one or more channels of a Thermo Scientific™ Transcend™ LX-4 sy chromatographic segregation of the steroid oximes from unwanted sample components was achieved by gradient elution through a Thermo Scientific Accucore™ Phenyl-X column (2.6 µm, 50 x 2.1 mm), which was heated to Chromatographic conditions are described in Figure 3. The Thermo Scien TSQ Endura™ triple-quadrupole mass spectrometer was used with a heat electro-spray ionization (HESI) probe. Ion source and MS/MS conditions a described in Figure 4.

Instrument Control & Data Analysis
Thermo Scientific™ TraceFinder™ with Aria™ MX software was used to c the Transcend LX-4 and Endura MS/MS systems, submit batches to desire channels as well as for analyzing data and reporting results.

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Figure 3. Liquid chromatography parameters

Column:	Accucore Phenyl-X, 2.6 µ, 50 x 2.1 mm, @ 40°C
Solvent A:	Water + 0.1% Formic Acid
Solvent B:	Methanol

Step	Start	Sec	Flow	Gradient	%A	%B
1	0.00	10	0.5	Step	50	50
2	0.17	20	0.5	Ramp	20	80
3	0.50	120	0.5	Ramp	-	100
4	2.50	60	0.5	Step	-	100
5	3.50	60	0.5	Step	50	50

Start data: 1.0 min Duration: 1.0 min

Total run time: 4.5 min

Figure 4. Mass spectrometer HESI & SRM parameters

Ion Source:	HESI, +3500 V, vaporizer temp: 400°C
SRM Transitions:	Q1 & Q3 resolutions: 0.7

Analyte	Q1	Q2	CE	RF
Pregnenolone oxime (Quan)	332.25	86.25	27	95
Pregnenolone oxime (Confirm)	332.25	300.25	22	95
Pregnenolone-D4 oxime (Quan)	336.30	90.30	27	95
Pregnenolone-D4 oxime (Confirm)	336.30	304.25	22	95

RESULTS

Quantitation Reliability

Typical results from calibrators, QCs and specimens are show in Figure 5. Ion ratios (Confirming/Quan transitions) averaged 66% for pregnenolone and 50 % for its IS.

IS peak areas among calibrators & QC averaged 17,400 with RSD of 12%. IS peak areas in specimens ranged from 10,600 to 14,700 and showed an average recovery of 65%. Carryover was less than 0.2%. Inter- and intra-batch precisions were less than 5 and 8% coefficient of variation (CV), respectively (Tables 1a & 1b).

Figure 5. Typical pregnenolone quantitative results

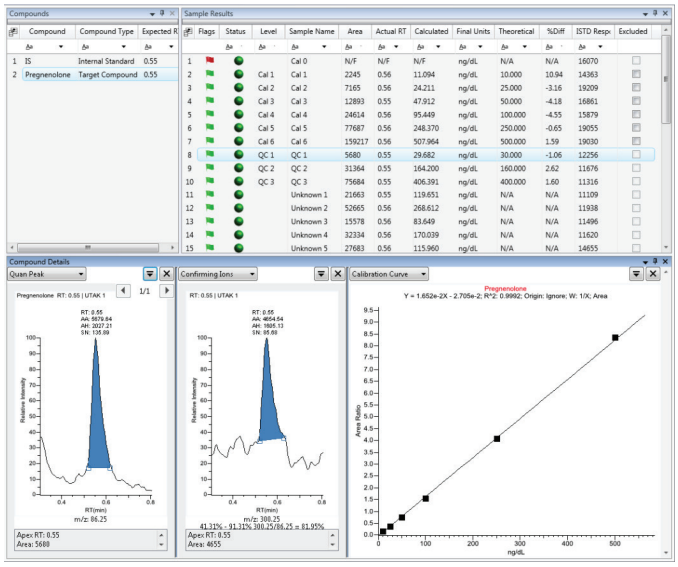

Table 1. Inter- and intra-batch precisions

Injection	Pregnenolone (ng/dL)
1	110
2	104
3	102
4	108
5	100
6	105
7	109
8	103
9	104
10	109
11	95
12	94
13	107
14	105
15	101
16	105
17	100
18	99
19	96
20	100
Mean	**102.80**
STD	**4.69**
CV %	**4.6**

a. Inter-batch

Date	Injection	Pregnenolone (ng/dL)
Day 1, 7/13/15	1	42
	2	41
	3	40
	4	39
	5	38
Day 2, 7/14/15	1	41
	2	40
	3	41
	4	38
	5	40
Day 3, 7/15/15	1	37
	2	36
	3	36
	4	37
	5	36
Day 4, 7/16/15	1	35
	2	42
	3	43
	4	33
	5	32
	Mean	**38.35**
	SD	**3.07**
	CV %	**7.99**

b. Intra-batch

Throughput

Single-channel throughput was 13 injections per hour. When multi-channeled across 2, 3 or 4 channels, the throughput increased to 26, 39 and 52 injections per hour, respectively. Pregnenolone batches were also multi-channeled with estrogen batches which utilized the same MS source conditions.

Accuracy Assessment

Comparison of 40 specimen results ranging from 13 to 130 ng/dL with reference-lab results showed differences that averaged 3.8% and ranged from -20.0 to 24.7% (Table 2). Only 5 of 40 test samples (7.5%), deviated 20% or more. Deming regression analysis showed R^2 = 0.9706, slope = 1.080, intercept = -1.8 and standard error of estimate = 7.3%.

Table 2. Reference lab results comparison

Test Sample	Current Method	Reference Lab	Difference %	Test Sample	Current Method	Reference Lab	Difference %
1	116	93	24.73	21	27	30	-10.00
2	85	78	8.97	22	49	52	-5.77
3	86	73	17.81	23	66	73	-9.59
4	13	14	-7.14	24	22	24	-8.33
5	74	68	8.82	25	55	60	-8.33
6	32	32	0.00	26	110	121	-9.09
7	43	42	2.38	27	20	25	-20.00
8	17	16	6.25	28	35	30	16.67
9	85	69	23.19	29	55	64	-14.06
10	16	16	0.00	30	77	62	24.19
11	51	46	10.87	31	36	31	16.13
12	20	19	5.26	32	90	90	0.00
13	131	118	11.02	33	38	33	15.15
14	52	48	8.33	34	33	30	10.00
15	52	65	-20.00	35	53	45	17.78
16	28	30	-6.67	36	64	56	14.29
17	52	48	8.33	37	18	17	5.88
18	83	86	-3.49	38	37	36	2.78
19	13	13	0.00	39	50	50	0.00
20	28	27	3.70	40	42	37	13.51

CONCLUSIONS

Pregnenolone can be accurately measured in blood serum by this method which achieved:

- Analytical range from 10 to 500 ng/dL
- Throughputs of 13, 26 or 52 injections per hour from a 1-, 2- or 4-channel system
- Inter- & intra-batch precisions less than 8% and carryover less than 0.2%
- Multi-channeling with other methods utilizing the same HESI source

REFERENCES

1. Kushnir MM, et al. Development and Performance Evaluation of a Tandem Mass Spectrometry Assay for 4 Adrenal Steroids. Clin Chem 2006; 52:1559–1567.

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TRADEMARKS/LICENSING

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1. Nelson, RE, et al. Liquid chromatography–tandem mass spectrometry assay for simultaneous measurement of estradiol and estrone in human plasma. Clin Chem, 2004, 50:373-384.
2. Kushnir, MM, et al. High-sensitivity tandem mass spectrometry assay for serum estrone and estradiol. Am J Clin Pathol, 2008, 129:530-539.

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Reference Lab	Difference %		Test Sample	Current Method	Reference Lab	Difference %
93	24.73		**21**	27	30	-10.00
78	8.97		**22**	49	52	-5.77
73	17.81		**23**	66	73	-9.59
14	-7.14		**24**	22	24	-8.33
68	8.82		**25**	55	60	-8.33
32	0.00		**26**	110	121	-9.09
42	2.38		**27**	20	25	-20.00
16	6.25		**28**	35	30	16.67
69	23.19		**29**	55	64	-14.06
16	0.00		**30**	77	62	24.19
46	10.87		**31**	36	31	16.13
19	5.26		**32**	90	90	0.00
118	11.02		**33**	38	33	15.15
48	8.33		**34**	33	30	10.00
65	-20.00		**35**	53	45	17.78
30	-6.67		**36**	64	56	14.29
48	8.33		**37**	18	17	5.88
86	-3.49		**38**	37	36	2.78
27	3.70		**40**	42	37	13.51

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