

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

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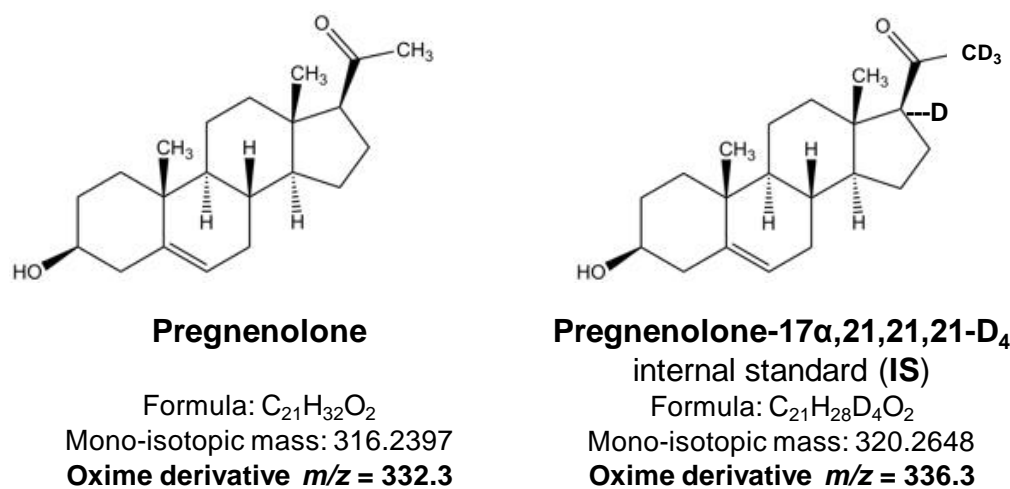
## ABSTRACT

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

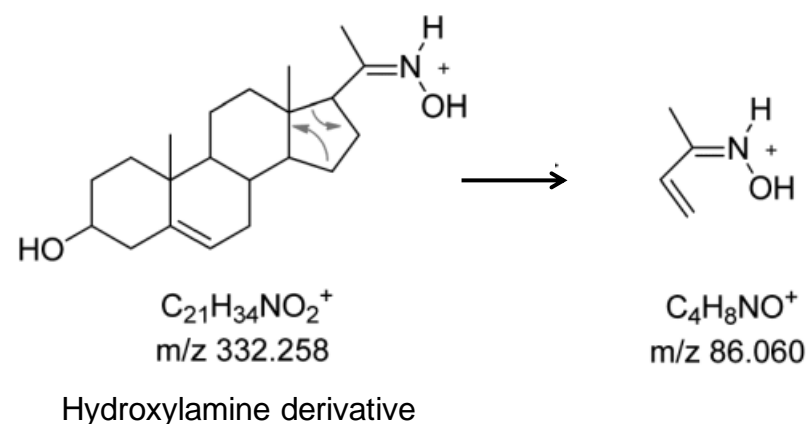
Pregnenolone is a biosynthetic precursor to other steroids such as corticosteroids, androgens and estrogens. It is converted to progesterone by 3-beta-hydroxysteroid dehydrogenase or to 17-OH-pregnenolone by 17-alpha-hydroxylase. Researchers investigating anomalies of these enzymes, which result in steroid 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 (ESI) sources, derivatization with hydroxylamine 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. Analyte & internal standard (IS)



Reactions with hydroxylamine form positive-ion oxime

Figure 2. Pregnenolone oxime fragmentation



## 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 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 reacted with hydroxylamine to form positive-ion oxime derivatives. After drying the derivative preparations, the residue of each was reconstituted with water and methanol (1:1) to a total volume of 200 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 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 40°C. Chromatographic conditions are described in Figure 3. The Thermo Scientific™ TSQ Endura™ triple-quadrupole mass spectrometer was used with a heated electro-spray ionization (HESI) probe. Ion source and MS/MS conditions are described in Figure 4.

### 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 3. UHPLC 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. MS/MS 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 shown in Figure 5. Ion ratios (Confirming/Quant 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 quantitative results

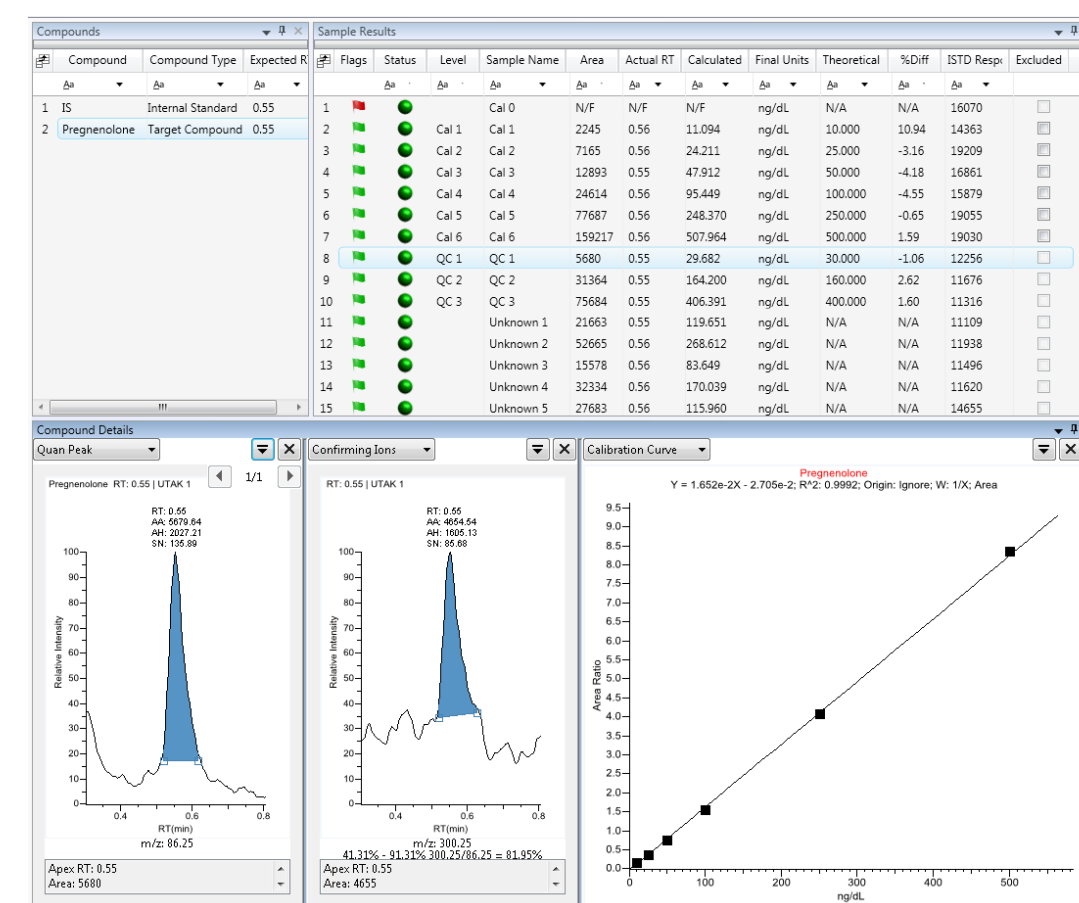


Table 1. Inter- and intra-batch precisions

Injection	Pregnenolone (ng/dL)	Date	Injection	Pregnenolone (ng/dL)
1	110	Day 1, 7/13/15	1	42
2	104		2	41
3	102		3	40
4	108		4	39
5	100		5	38
6	105	Day 2, 7/14/15	1	41
7	109		2	40
8	103		3	41
9	104		4	38
10	109		5	40
11	95	Day 3, 7/15/15	1	37
12	94		2	36
13	107		3	36
14	105		4	37
15	101		5	36
16	105	Day 4, 7/16/15	1	35
17	100		2	42
18	99		3	43
19	96		4	33
20	100		5	32
<b>Mean</b>	<b>102.80</b>	<b>Mean</b>	<b>38.35</b>	
<b>STD</b>	<b>4.69</b>	<b>SD</b>	<b>3.07</b>	
<b>CV %</b>	<b>4.6</b>	<b>CV %</b>	<b>7.99</b>	

a. Inter-batch precision

b. Intra-batch precision

### Throughput

Single-channel throughput was 13 injections per hour. When multi-channelled across 2, 3 or 4 channels, the throughput increased to 26, 39 and 52 injections per hour, respectively. Pregnenolone batches were also multi-channelled 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<sup>2</sup> = 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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