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

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Application Note 650

Key Words
Steroid, pregnenolone, high-throughput, Transcend, TSQ Endura, multichannel, triple quadrupole MS, quantitation

Goal
To develop and evaluate a reliable, robust HPLC-MS/MS method for quantifying pregnenolone in human blood serum for clinical research purposes.

Application Benefits
- Simple, economical sample extraction/derivatization procedure
- Reliable quantitation from 10 to 500 ng/dL with ion-ratio confirmation
- Throughput capabilities of 13, 26, or 52 injections per hour from 1-, 2-, or 4-channel HPLC systems

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 how these enzymes function need to quantify pregnenolone within an analytical range of 10 to 500 ng/dL (0.3 to 1.5 nmol/L) in blood serum. Since pregnenolone does not ionize well by either atmospheric-pressure chemical-ionization (APCI) or electrospray ionization (ESI), derivatization with hydroxyl amine was necessary to reliably achieve the desired analytical range.[1] We pursued this strategy along with liquid-liquid extraction to develop a simple sample preparation that would provide robust and sensitive quantitation of pregnenolone using ESI.

Methods
Sample Preparation
Aliquots of fresh blood serum specimens (200 µL), as well as calibrators prepared in 1% BSA and quality control specimens (QCs), were spiked with pregnenolone-D_4 internal standard (IS) before being subjected to liquid-liquid extraction with 1 mL methyl *t*-butyl ether (MTBE). After drying the ether extracts with heated nitrogen, hydroxylamine was added to the residue of each extract to form positive-ion oxime derivatives. The preparations were dried and reconstituted with water and methanol (1:1) and then subjected to reversed-phase liquid chromatography coupled to tandem mass spectrometry with a heated electrospray ionization (HESI) probe.

Liquid Chromatography
Using one or more channels of a Thermo Scientific™ Transcend™ LX-4 multichannel HPLC, chromatographic separation 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, P/N 27926-052130), which was heated to 40 °C. The chromatographic conditions are described in Figure 1. This method was multichanneled with other methods that utilized the same MS ion source at the same temperatures and gas flows.

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 1. Chromatographic parameters.

Mass Spectrometry and Data Analysis
A Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer was used with a HESI probe. Pregnenolone oxime and its deuterated internal standard (IS) were measured by selected reaction monitoring (SRM), as described in Figure 2. All data were acquired and processed using Thermo Scientific™ TraceFinder™ software.

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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-D_4 oxime (Quan)	336.30	90.30	27	95
Pregnenolone-D_4 oxime (Confirm)	336.30	304.25	22	95

Figure 2. TSQ Endura parameters.

Method Evaluation

Method precision was assessed by calculating percent coefficient of variation (%CV) of peak areas from 20 replicate injections of two pools of test specimens. Carryover was measured in blanks immediately following injections of the highest calibrator. Matrix effects were evaluated by comparing IS peak areas in specimen samples to IS peak areas in calibrator and control samples. A method comparison experiment was performed by analyzing 40 donor samples (no informed consent was needed) following the procedures described in this study and comparing results with those obtained by a reference lab.

Results and Discussion

As shown in Figure 3, the desired analytical range from 10 to 500 ng/dL (0.3 to 1.5 nmol/L) was achieved and was consistently linear ($r^2 \geq 0.999$ with 1/X weighting). Carryover never exceeded 0.2%. Confirmation/quantitation of ion ratios among calibrators, QCs, and specimens were within 20% of averages calculated from the calibrators. The ion ratios for pregnenolone oxime and pregnenolone-D_4 oxime averaged 65% and 47%, respectively. Typical characteristics of chromatographic peaks from quantitation- and confirmation-ion transitions for pregnenolone oxime and its deuterated internal standard from a donor specimen are shown in Figure 4.

The intra- and inter-batch precisions were better than 5% and 8%, respectively. IS peak areas among calibrators and QCs averaged 17,400 cps with an RSD of 12%. Specimen IS peak areas ranged from 10,600 to 14,700 cps with an average recovery of 65%, relative to the averaged IS peak areas in calibrators and QCs. However, the IS in each sample successfully compensated for matrix effects as proved by method comparison results. In the method comparison experiment, pregnenolone values in analyzed samples ranged from 13 to 130 ng/dL and the percent difference between two analytical methods for 92.5% of analyzed samples was 20% or less (Table1).

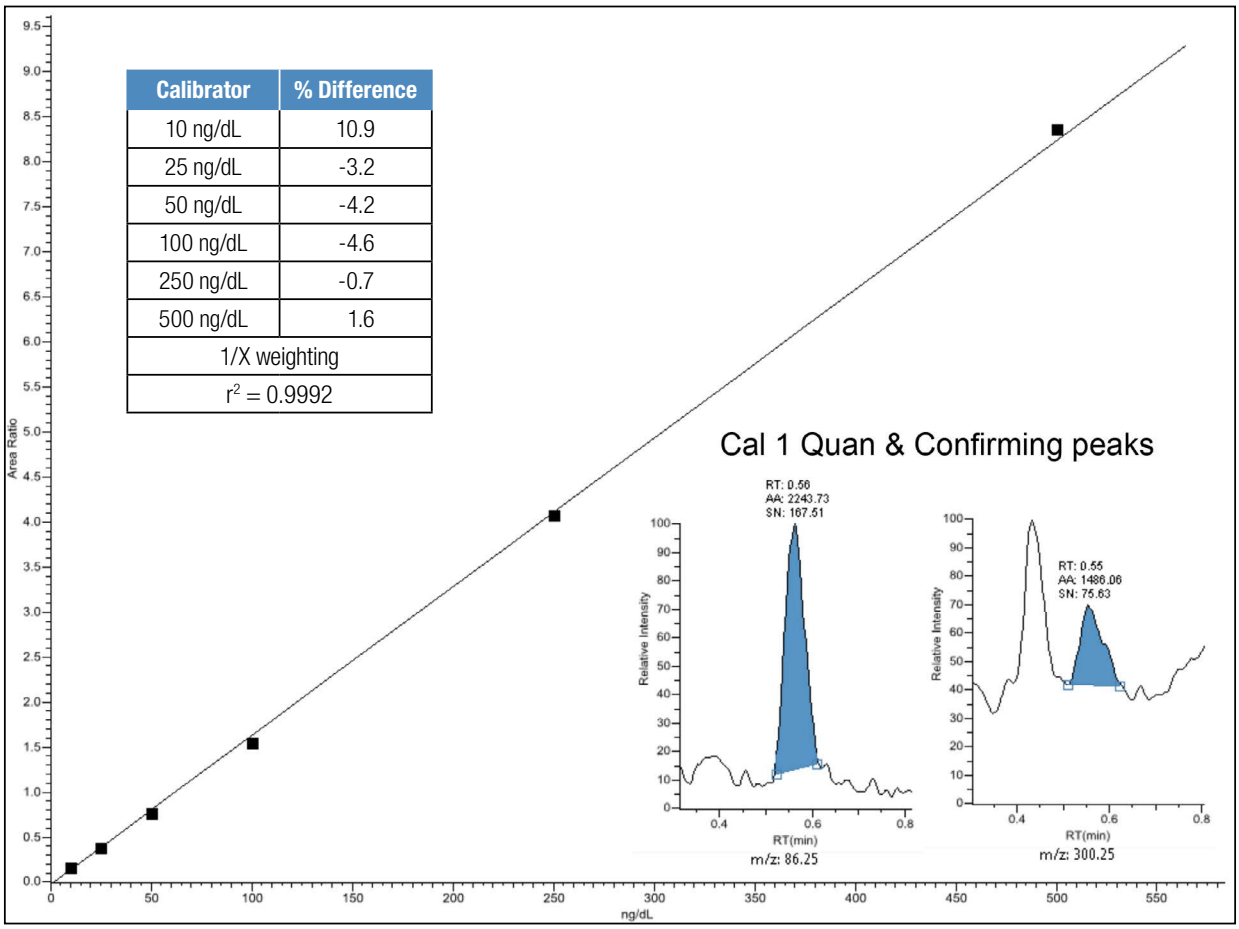

Figure 3. Typical pregnenolone quantitation results.

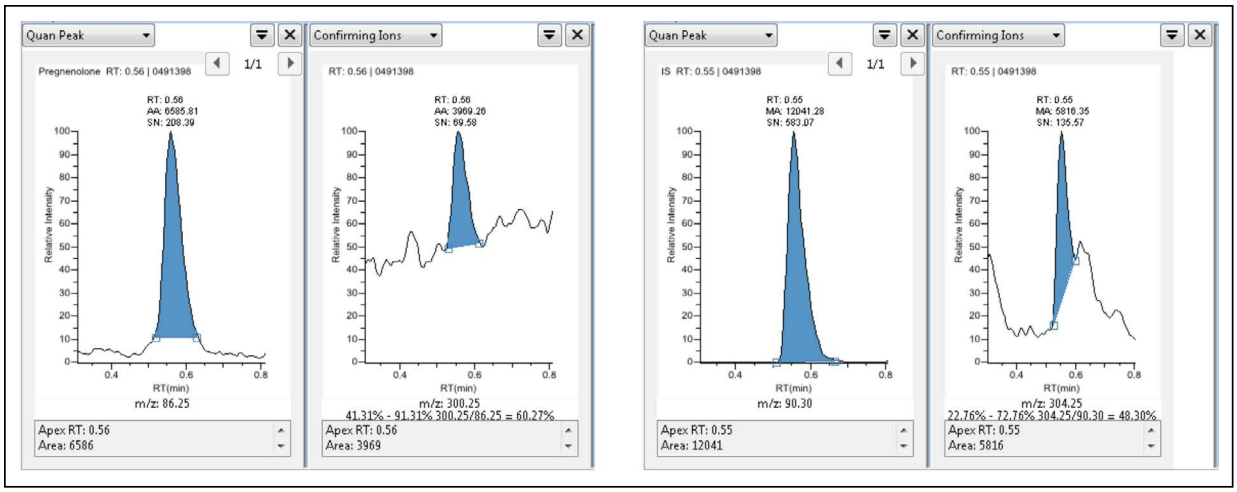

Figure 4. Analyte & IS chromatograms from donor specimen containing 35 ng/dL pregnenolone.

Table 1. Pregnenolone results comparison with reference lab.

Test Sample	Current Method	Reference Lab	Difference %
1	116	93	24.73
2	85	78	8.97
3	86	73	17.81
4	13	14	-7.14
5	74	68	8.82
6	32	32	0.00
7	43	42	2.38
8	17	16	6.25
9	85	69	23.19
10	16	16	0.00
11	51	46	10.87
12	20	19	5.26
13	131	118	11.02
14	52	48	8.33
15	52	65	-20.00
16	28	30	-6.67
17	52	48	8.33
18	83	86	-3.49
19	13	13	0.00
20	28	27	3.70

Test Sample	Current Method	Reference Lab	Difference %
21	27	30	-10.00
22	49	52	-5.77
23	66	73	-9.59
24	22	24	-8.33
25	55	60	-8.33
26	110	121	-9.09
27	20	25	-20.00
28	35	30	16.67
29	55	64	-14.06
30	77	62	24.19
31	36	31	16.13
32	90	90	0.00
33	38	33	15.15
34	33	30	10.00
35	53	45	17.78
36	64	56	14.29
37	18	17	5.88
38	37	36	2.78
39	50	50	0.00
40	42	37	13.51

Conclusion

A robust, high-throughput, sensitive quantitation assay of pregnonelone in blood serum is described in this application note. Some of the key features of this research method are:

- Sensitive quantitation from 10 to 500 ng/dL (0.3 to 1.5 nmol/L)
- Excellent reliability with inter- and intra-batch precisions less than 8% and carryover less than 0.2%
- High-throughput capabilities and multichanneling with other HESI methods

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References

1. Kushnir M.M. 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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