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.

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 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 was reconstituted with water and methanol (1:1) to a total volume of 200 µL and µL injections were made into the LC-MS/MS system.

Test Methods
Using one or more channels of a Thermo Scientific™ Transcend™ LX-4 or 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), which was heated to Chromatographic conditions are described in Figure 3. The Thermo Scien TSQ Endura™ triple-quadrupole mass spectrometer was used with a heated electro-spray ionization (HESI) probe. Ion source and MS/MS conditions as described in Figure 4.

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

**Quantitation Reliability**

Typical results from calibrators, QC samples, and specimens show that for pregnenolone, 66% of the IS area is within 10% of the expected area. IS peak areas for calibrators and QC samples averaged 17,400 with RSD of 12%. Recovery of IS was less than 0.2%. The intra- and inter-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**

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<th>Injection</th>
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| Mean      | 102.80              | Mean      | 38.35     |
| SD        | 4.69                | CV        | 7.99      |

**Througput**

Single-channel throughput was 13 injections per hour. When multi-channeled across 2, 3, and 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 the test 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**

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<th>Reference Lab</th>
<th>Difference %</th>
<th>Test Sample</th>
<th>Current Method</th>
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CONCLUSIONS

Pregnenolone can be accurately measured in blood serum by this method which achieved:
- Analytical range from 10 to 500 ng/dL
- Throughput 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


ACKNOWLEDGEMENTS

The authors thank Dr. Lori Vermeulen, Dean of the College of Arts & Sciences of West Chester University of Pennsylvania and Dr. Fred Monson, Director of the Center for Microanalysis & Imaging Research & Training, for the use of space and resources needed for some of this work. We also thank Dr. Stephen Zimniaki, Director, Pharmaceutical Product Development Program, for providing student internship opportunities for this project.

TRADEMARKS/LICENSING

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channels as well as for analyzing data and reporting results. Chromatographic conditions are described in Figure 3. The Thermo Scientific™ m, 50 x 2.1 mm), which was heated to 40ºC.

Components was achieved by gradient elution through a Thermo Scientific™ residue of each was reacted with hydroxylamine to form positive-ion oxime butyl ether (MTBE). After drying the ether extracts by heated nitrogen flow, the aliquots of fresh blood serum specimens, as well as calibrators and quality control specimens (QCs), were spiked with pregnenolone-D4 internal IS quantitated via UHPLC-MS/MS using pregnenolone-D4 as the IS. The resulting positive-ion oximes (Figure 2) were since pregnenolone does not ionize well in either atmospheric-pressure hydroxylase. Researchers investigating anomalies of these enzymes, which result in steroid hormone imbalances, need to measure pregnenolone within an

Table 1. Inter- and intra-batch precisions

<table>
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Results

We developed an LC-MS/MS method for researchers to accurately measure pregnenolone. The throughput increased to 26, 39 and 52 injections per hour from a 1-, 2- or 4-channel multi-channeling. Deming regression analysis showed R² = 0.9706, slope = 1.080, intercept -20.0 to 24.7% (Table 2). Only 5 of 40 test samples (7.5%), deviated 20% or more. Deming regression analysis showed R² = 0.9706, slope = 1.080, intercept -20.0 to 24.7% (Table 2). Only 5 of 40 test samples (7.5%), deviated 20% or more. Deming regression analysis showed R² = 0.9706, slope = 1.080, intercept -20.0 to 24.7% (Table 2). Only 5 of 40 test samples (7.5%), deviated 20% or more. Deming regression analysis showed R² = 0.9706, slope = 1.080, intercept -20.0 to 24.7% (Table 2). Only 5 of 40 test samples (7.5%), deviated 20% or more. Deming regression analysis showed R² = 0.9706, slope = 1.080, intercept -20.0 to 24.7% (Table 2). Only 5 of 40 test samples (7.5%), deviated 20% or more.

**ICES**


**EDGEMENTS**

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