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Clinical determination of 17-hydroxyprogesterone in serum by LC–MS/MS: Comparison to Coat-A-Count™ RIA method

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Abstract

17α-Hydroxyprogesterone is a metabolic precursor of cortisol; elevated levels of 17α-hydroxyprogesterone are indicative of congenital adrenal hyperplasia. Traditional determination by immunoassay is plagued by poor antibody specificity, resulting in significant interferences. This study explores an LC–MS/MS method for the quantitation of 17OHP in serum. Deuterated 17α-hydroxyprogesterone was added as internal standard, followed by solid-phase extraction, HPLC separation with a C16-amide reverse-phase column with run time of 7 min, and quantification by MS/MS (positive electrospray ionisation) in the selected reaction monitoring mode (SRM). Transitions monitored were 331 > 109 for the analyte and 339 > 113 for the deuterated internal standard. Intra-assay precision (%R.S.D.) was 7.4% at 7 nmol/L, inter-assay precision (%R.S.D.) at 2, 7 and 27 nmol/L was 15.4, 10.0 and 7.9% and accuracy at 0.9 nmol/L was 100%. The method was linear from 0.156 to 80 nmol/L. Lower limit of quantitation was 0.2 nmol/L, providing meaningful data for patients within normal range as well as those with elevated levels.
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Keywords: LC–MS/MS; CAH; Congenital adrenal hyperplasia; 17-Hydroxy progesterone; RIA; SPE

1. Introduction

Congenital adrenal hyperplasia (CAH) is a family of inherited diseases affecting the adrenal gland, the most common being 21-hydroxylase deficiency, one of several enzymes responsible for steroid biosynthesis. Hormonal imbalances are reflected in decreased levels of mineralocorticoids (aldosterone) and glucocorticoids (cortisol) and increased 17α-hydroxyprogesterone (17OHP) (Fig. 1) and androgens. In classical CAH, these imbalances may result in salt-wasting with consequent failure to thrive and death, or incorrect gender assignment in virilized females [1]. Treatment is possible with early diagnosis. Non-classical CAH manifests in later childhood or young adult life and presents with symptoms of hirsutism and oligomenorrhea. Other conditions such as polycystic ovary syndrome mimic the symptoms of CAH, and differential diagnosis may be made using laboratory testing, including quantitation of 17OHP [2].

Quantitation of 17OHP in serum has traditionally employed immunoassay methods, which suffer from poor antibody specificity. These methods are subject to interferences by other similar steroids, which result in falsely elevated levels [3]. There has been increased interest in using LC–MS/MS for the profiling of steroid levels [4–6]; recent proficiency results published by the College of American Pathologists indicate for the first time various laboratories using mass spectrometry for steroid analysis.

Several chromatographic methods have been developed for analysis of 17OHP in serum, among others GC–MS [7] and LC–MS/MS [8–16]. However, in the former case 17OHP needs to be derivatized prior to analysis. Published methods that employ LC–MS/MS are either for screening purposes using blood-spots [8–12], or for serum matrix having detection limits which preclude quantitation of levels within normal ranges [13,14] or require derivatization prior to injection on the LC system in order to improve detection limits [15]. One method uses simple protein precipitation with acetonitrile [16] for sample pre-treatment followed by chromatographic clean-up. This method requires a mass spectrometer equipped

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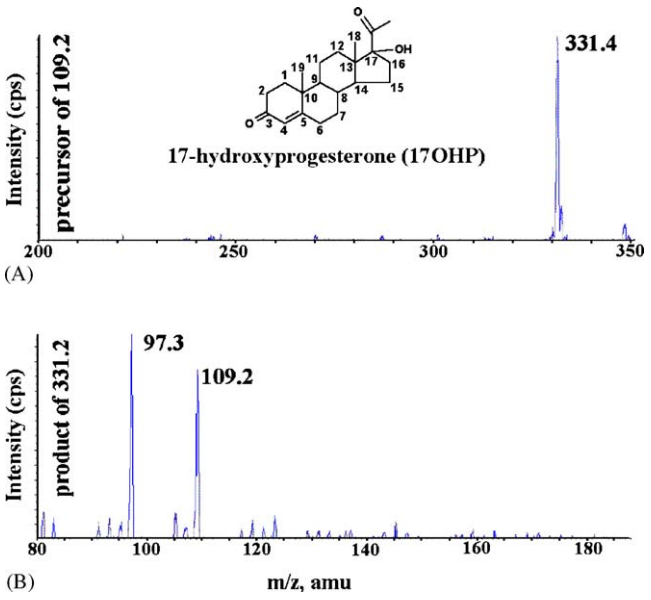

Fig. 1. (A) 17-hydroxyprogesterone (17OHP) precursor scan ($m/z = 331$ ion, M + 1) and structure. (B) 17OHP product ion scan: fragmentation of similar intensities for m/z 109.2 or 97.3.

with a photoionization source, not routinely available on all instruments.

This study employs extraction of 17OHP from serum by solid-phase extraction (SPE) using a deuterated analogue of the analyte as the internal standard (d8-17OHP, see Fig. 2) with subsequent analysis by LC–MS/MS. This provides a robust method for clinical determination of 17OHP in the normal range as well as elevated levels in patient samples. The developed technology is compared to a Coat-A-Count™ RIA method.

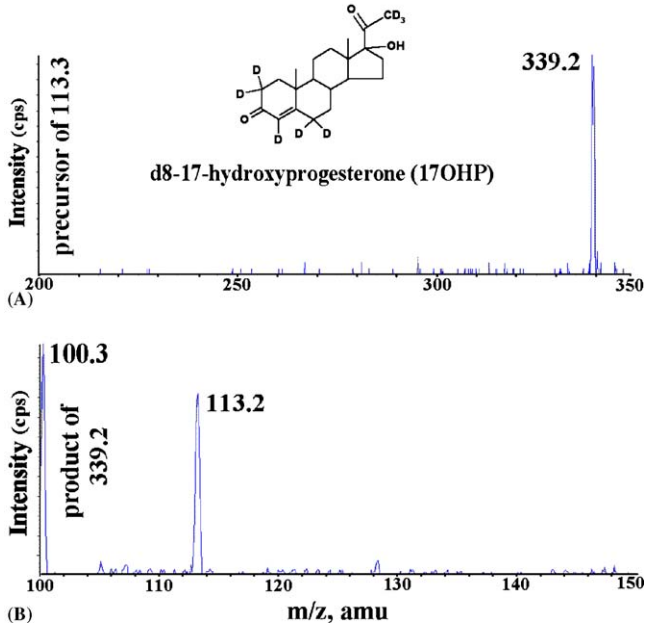

Fig. 2. (A) d8-17-hydroxyprogesterone (17OHP) precursor scan ($m/z = 339$ ion, M + 1) and structure. (B) d8-17OHP product ion scan: fragmentation of similar intensities for m/z 113.2 or 100.3.

2. Experimental

2.1. Reagents and chemicals

Stock standard 17OHP (Sigma Aldrich, Oakville, ON, Canada) was prepared by dissolving 13 mg, accurately weighed, in 10 mL of methanol (Fisher Scientific, Ottawa, ON, Canada), obtaining a final concentration of 4 mmol/L and stored at −20 °C. For analysis, the stock solution was mixed and diluted with methanol. Working standards were prepared daily and seven-point calibration curves were constructed for every 96-well plate using a linear regression based on concentration versus peak area ratio of analyte to internal standard. d8-17OHP was purchased in 10 mg quantities (C/D/N Isotopes, Pointe-Claire, QC, Canada), and was dissolved in methanol directly in the original vial to a volume of 5 mL (5.91 mmol/L) and also stored at −20°C. Serial dilutions were made with methanol to a final working internal standard solution of 59.1 nmol/L.

2.2. Sample preparation

1.0 mL of sample or control, 2.0 mL phosphate buffer (100 mmol/L, pH 6) and 100 μL of working internal standard solution (59.1 nmol/L d8-17OHP in distilled water) were pipetted into disposable 16 × 100 glass culture tubes. Sample clean-up was achieved using "Clean-Screen DAU" copolymeric SPE columns (Diagnostix, Mississauga, ON, Canada) and a VacElut manifold (Varian, Mississauga, ON, Canada) as described in Table 1 [17]. This protocol is applicable to steroids having structural similarities to 17OHP. The final eluate was evaporated to dryness on a Labconco Centrivap Console (Fisher Scientific, Ottawa, ON, Canada), reconstituted in 50 μL of LC mobile phase (0.1% formic acid:methanol 50:50) and transferred to a v-bottom 96-well autosampler plate, which was covered with common aluminum foil to prevent evaporation, and placed on the autosampler (Agilent 1100).

2.3. Liquid chromatography

Chromatography was performed using an Agilent 1100 LC system including a vacuum degasser, binary pump, and well-plate autosampler. Chromatographic separation was achieved using a Supelco Discovery® RP-Amide C16 col-

Table 1
Conditions for SPE extraction of 17OHP from serum using Clean-Screen DAU copolymeric columns

Condition	1 × 3 mL CH_3OH 1 × 3 mL distilled H_2O 1 × 1 mL 100 mM phosphate buffer (pH 6.0)
Apply sample	Load at 1–2 mL/min
Wash column	1 × 3 mL 10% (v/v) CH_3OH in distilled H_2O Dry column (5 min at >10 in. Hg) 1 × 1 mL hexane
Elute analyte	1 × 3 mL elution solvent (CH_2Cl_2:*iso*-propanol:NH_4OH = 78:20:2)

umn (50 mm × 4.6 mm, 5 μm) with guard Discovery® RP-Amide C16 column (20 mm × 4.0 mm, 5 μm) (Sigma-Aldrich, Oakville, ON, Canada). Mobile phases were 0.1% aqueous formic acid and methanol, with a linear gradient starting at 50% methanol, increased to 70% at 5 min, held at 70% for 2 min, then re-equilibrated at 50%. Flow rate through the chromatographic system was 1.0 mL/min, with a post-column tee to divert 0.6 mL/min to waste. Injection volume was 10 μL; a 5 s flush-port needle rinse prior to injection was incorporated into the procedure to prevent cross-contamination.

2.4. Mass spectrometry

Detection was performed on an MDS Sciex API 4000 triple quadrupole mass spectrometer. Selected Reaction Monitoring (SRM) analysis was performed using the TurboIonSpray source at a temperature of 500 °C in positive ion mode. The mass spectrometer parameters were optimized using "Quantitative Optimization" in the Analyst software (v1.4, Applied Biosystems, Toronto, ON, Canada). The source parameter settings were as follows: curtain gas = 20 psi, source gas 1 = 5 psi, source gas 2 = 35 psi and ion spray voltage = 5500 V. Collision cell parameters were: CAD gas = 5 psi, declustering potential (DP) = 86 V, collision cell entrance potential (EP) = 10 V, collision energy (CE) = 37 V and collision cell exit potential (CXP) = 8 V. Q1 and Q3 were operated at unit mass resolution. The SRM mass transitions were 331.2 > 109.3 (17OHP) and 339.2 > 113.2 (d8-17OHP). The dwell time for all analytes was set at 500 ms. Data was acquired and quantitation performed using Analyst 1.4 Software.

2.5. Method validation

Linearity was evaluated by spiking serum at 80 nmol/L, and making serial dilutions to 0.156 nmol/L (80, 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0.156 nmol/L). For routine analysis, the calibration curve included 80, 40, 20, 5, 2.5 and 0.5 nmol/L and blank. Due to the use of a deuterated analogue of the analyte as internal standard, these standards were not extracted prior to analysis on the LC–MS/MS system.

Recoveries were obtained by analysing 10 replicates of a serum pool spiked at 5 nmol/L, as well as by analysing six separate serum samples, each spiked at 15 nmol/L. Percent recovery was calculated by dividing the measured concentration by the theoretical spiked concentration. Intra-assay precision was evaluated by determining 17OHP in serum at two levels (0.9 and 6.9 nmol/L) ($n = 10$). Commercial controls at three levels (1.8, 7.0 and 25.4 nmol/L) were analysed weekly ($n = 20$) to ascertain inter-assay precision.

2.6. Comparison to RIA

Serum samples were determined using a "DPC Coat-A-Count® 17α-OH Progesterone" RIA kit, including reagents and serum controls purchased from Inter Medico (Markham, ON, Canada) according to manufacturer's protocol [18]. Standards and samples were counted on a LKB Wallac Multigamma 1261 Gamma Counter (Perkin Elmer, Woodbridge, ON, Canada).

The LC–MS/MS method was compared to results from this RIA method using EP Evaluator™ (Release 6, D.G. Rhoads Associates, Pennsylvania, PA, USA). The comparison of the two methods involved analysing a total of 25 patient samples on 4 separate days with analysis dates at least 1 week apart. Serum samples were frozen after collection and were stored frozen until analysis. Comparison with other laboratories was available through an external quality assurance program (External Quality Assurance Services, Bio-Rad Laboratories, Irvine, CA, USA) and data accumulated from this program ($n = 20$) have also been compared using EP Evaluator™.

3. Results and discussion

3.1. Infusion experiments

Infusion at a flow rate of 20 μL/min of pure solutions (40 nmol/L in 0.1% formic acid: methanol) of both 17OHP and d8-17OHP showed similar collisionally activated dissociation products as previously reported [9,10]. Product ion scans of the corresponding molecular ions of 17OHP (*m/z* 331.2) and d8-17OHP (*m/z* 339.2) indicated fragmentation of similar intensity for *m/z* 109.2 or 97.3 (17OHP) and for *m/z* 113.2 or 100.3 (d8-17OHP) (Figs. 1 and 2). Quantitative optimisation was performed using the Analyst 1.4 software for the SRM's *m/z* 331.2 to 109.2 for 17OHP and *m/z* 339.2 to 113.2 for d8-17OHP. Suggested fragmentation mechanisms for d8-17OHP are presented in Fig. 3.

3.2. Liquid chromatography

Fig. 4 shows a typical chromatogram for an extracted serum control at a stated level of 1.8 nmol/L [19]. The use of a deuterated analogue of the analyte compensates for any ion suppression effects, as both internal standard and analyte will be affected to the same extent.

The method described and validated here used a post-column split to restrict flow to the mass spectrometer's source. This was done in order to remain cautious and ensure that no overspray would pass through the orifice and contaminate the Q0 region. However, the orthogonal geometry of the API 4000 source significantly decreases this risk over earlier Sciex source designs. The post-column splitter has subsequently been removed, and the entire 1 mL/min flow is directed to the source. This has resulted in improved chromatography without the turbulence imparted by the splitter. Increased flow into the source has also produced gains in sensitivity of approximately three times.

3.3. Method validation

The limit of detection (LOD) was 0.1 nmol/L (signal/noise = 10), and the lower limit of quantitation (LLOQ) was 0.2 nmol/L when extracting a sample volume of 1 mL. At the LLOQ, precision (%R.S.D.) was equal to 10%. CAH is

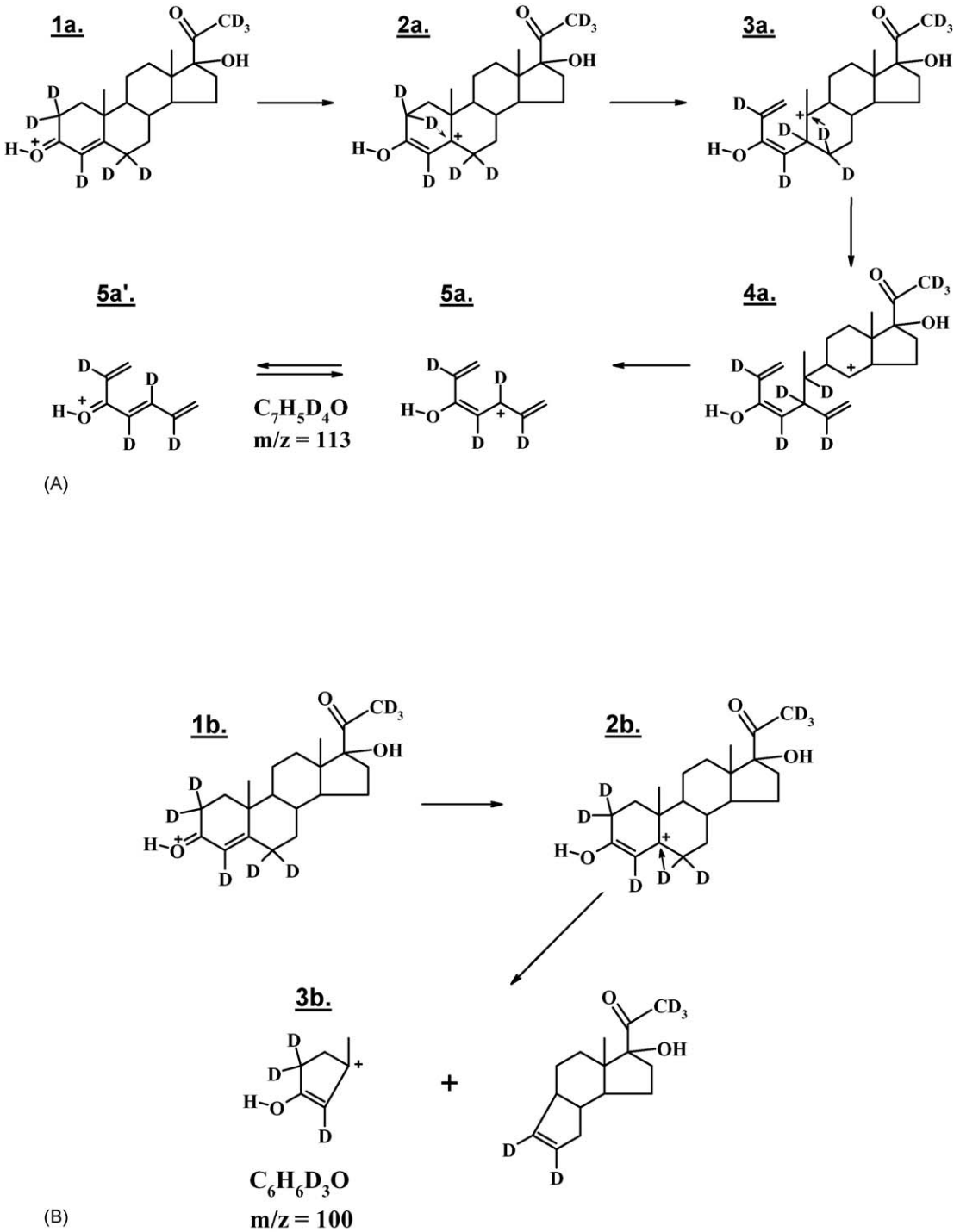

Fig. 3. Suggested fragmentation mechanisms for d8-17OHP. (A) $m/z = 339–113$ and (B) $m/z = 339–100$.

determined by an increase in 17OHP, and ranges for normal populations (Table 2) [20] are higher than the LLOQ; the method thus provides acceptable LOD and LLOQ for clinical interpretation. Extraction of smaller volumes of serum as would be available from neonates may not provide LLOQ sufficient to quantify levels within normal ranges, but elevated levels would be quantified allowing diagnosis of CAH.

Recovery of spiked 17OHP to a pooled serum sample at 5 nmol/L ($n = 10$) was 115% (%RSD = 8.8%), and average recovery in six separate serum samples each spiked at 15 nmol/L was 102% (%R.S.D. = 6.2%). Analysis of a certified control with stated value of 0.9 nmol/L ($n = 10$) gave a measured value of 0.90 nmol/L (100% accuracy) with %R.S.D. = 3.5%. Intra- and inter-assay precision results are summarized in Table 3.

Standard curves showed excellent linearity for serial dilutions of spiked serum (ranging from 80 to 0.156 nmol/L) [$y = 0.067x - 0.023$; $r = 0.9996$] during method validation, and for aqueous standards (ranging from blank to 80 nmol/L)

Table 2
Ranges of 17OHP in normal patient population [20]

Classification	Birth weight (g)	Normal 17OHP level (nmol/L)
Neonate		
Normal	All	<120
Possible abnormal	<2200	120–270
Definite abnormal	<2200	≥270
	≥2200	≥120
Male		2.2–10.9
Female		0.3–12.1

Table 3
Method validation results for intra- and interassay precision

N	Concentration (nmol/L)	Precision (%R.S.D.)
Intra-assay precision		
10	0.9	3.5
10	6.9	7.4
Inter-assay precision		
20	1.9	15.4
20	7.1	10.0
20	27.0	7.9

[$y = 0.081x - 0.023$] with correlation coefficients consistently greater than 0.996 [$n = 25$ weeks, average $r = 0.999$] for routine weekly analysis. Calibration curves for routine analysis are not made up in serum, rather are simple dilutions in distilled water. These standards are also not subjected to the SPE extraction, but are injected straight onto the HPLC system. The results of recovery experiments and the analysis of certified control materials confirm that this technique is valid and provides accurate results.

3.4. Comparison to RIA

As has been previously reported [8,9,13], immunoassay methods are subject to significant interferences and yield higher values for 17OHP than LC–MS/MS methods. Comparison of results obtained by LC–MS/MS with the DPC Coat-A-Count™ RIA method are consistent with previous reports; statistical analysis using EP Evaluator indicates a mean bias of −1.2 nmol/L. Aside from this bias in the results, there is good correlation ($y = 0.945x - 1.06$; y = LC–MS/MS; x = RIA; $r = 0.87$) between both methods (Fig. 5).

Minutti et al. [8] also report that an opposite trend is observed in patients with CAH, with immunoassay methods underestimating the 17OHP levels. The inter-laboratory proficiency testing provided by the EQAS program yielded comparison of higher 17OHP levels, and supports these findings. All results from the LC–MS/MS method were within two S.D. (average = 0.95 S.D., range: −0.57 to 1.88) of the comparator mean. At levels indicative of CAH, there is a positive bias for the LC–MS/MS method (Fig. 6), which increases linearly with concentration [$y = 0.337x - 3.38$; $r = 0.965$; x = LC–MS/MS result; y = bias]. Correlation between the LC–MS/MS method and the "All Lab" mean showed a correlation coefficient of 0.99 ($y = 1.50x - 4.789$; y = LC–MS/MS; x = "All Lab" mean).

Table 4 compares the analytical characteristics of the two techniques. LC–MS/MS compares favourably with RIA, being more sensitive (0.1 versus 0.2 nmol/L), with greater linear range (0.16–80 nmol/L versus 0.3–37.8 nmol/L).

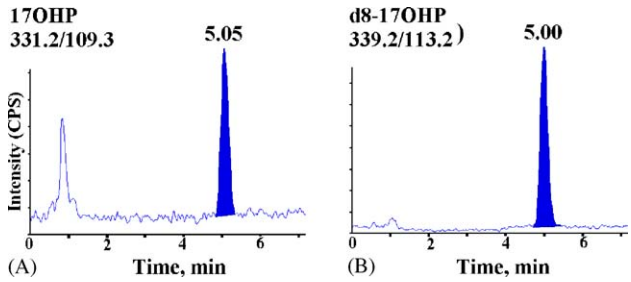

Fig. 4. Chromatogram for extracted serum control (1.8 nmol/L). (A) Retention time of 17OHP = 5.05 min (m/z = 331–109). (B) Retention time of d8-17OHP = 5.00 min (m/z = 339–113).

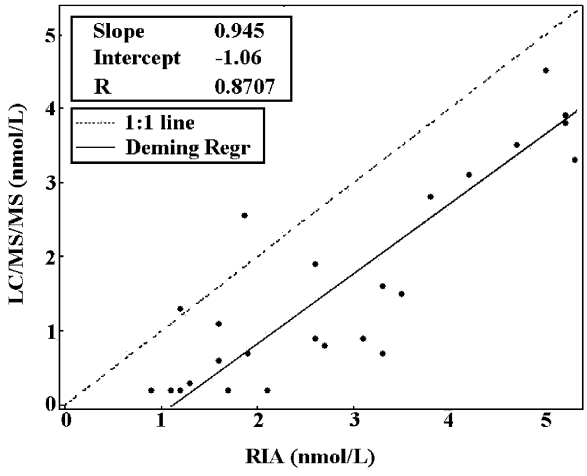

Fig. 5. 17OHP scatter plot: LC–MS/MS vs. DPC Coat-A-Count® RIA ($n = 25$).

Table 4
Analytical characteristics: LC–MS/MS vs. DPC Coat-A-Count® RIA

	DPC Coat-A-Count® [18] 17OHP RIA	LC–MS/MS
Intra-assay precision	%RSD = 7.1 @ 1.4 nmol/L	%RSD = 3.5 @ 0.9 nmol/L
Inter-assay precision	%RSD = 7.3 @ 1.3 nmol/L	%RSD = 15.4 @ 1.9 nmol/L
Detection limit	0.2 nmol/L	0.1 nmoL/L
Calibration range	0.3–37.8 nmol/L	0.16–80 nmol/L
Linearity (correlation coefficient)	$r = 0.999$	$r = 0.9996$

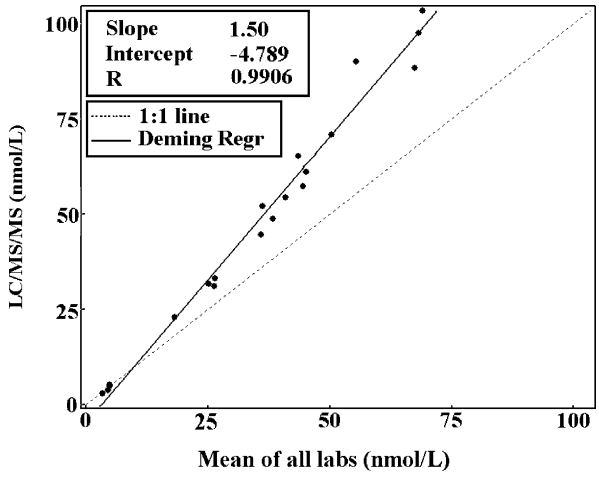

Fig. 6. 17OHP bias plot: LC–MS/MS vs. EQAS program ($n = 20$).

4. Conclusions

SPE extraction combined with the use of an isotopically labelled analogue of the analyte has provided relatively quick, simple and robust sample preparation. This method rivals the Coat-A-Count™ RIA method in terms of sample preparation time, as the immunoassay required a 3 h incubation prior to reading on the gamma counter [18]. Moreover, the major benefits of LC–MS/MS are sensitivity and specificity; providing more accurate results than RIA and making possible analysis of serum samples where interferences are likely to cause false high results.

Turpeinen et al. [13] describe a method for the quantitation of 17OHP in serum by LC–MS/MS, also without the need for derivatization. However, their extraction of analyte from serum employs two liquid–liquid extractions, using a greater volume of organic solvent than is required for the SPE method. This SPE-LC-MS/MS method achieved a LLOQ 10 times lower than the LLE method; providing meaningful data for all patients within normal range (see Table 2) as well as accurate measurement of elevated levels.

External interlaboratory proficiency testing of serum specimens has validated the accuracy of this SPE-LC–MS/MS method for a range of analytical results. Comparison to the results obtained by other laboratories using immunoassay techniques shows that for levels indicative of CAH, the immunoassay results are lower than those from the SPE-LC–MS/MS method, allowing the possibility that late-onset CAH may not be recognized.

Traditionally, 17OHP levels have been the sole indicator of CAH; however, Marsden [4], Speiser [6] and Lacey [9] discuss the importance of using steroid profiles including the measurement of 17OHP, androstenedione and cortisol for the diagnosis of CAH. This SPE protocol and quantitation by LC–MS/MS may prove to have applicability to the quantitation of other steroids, imparting value on this method for the determination of steroid profiles. Future work involves further development and validation of this chromatographic method for the separation and quantitation of other significant steroids.

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