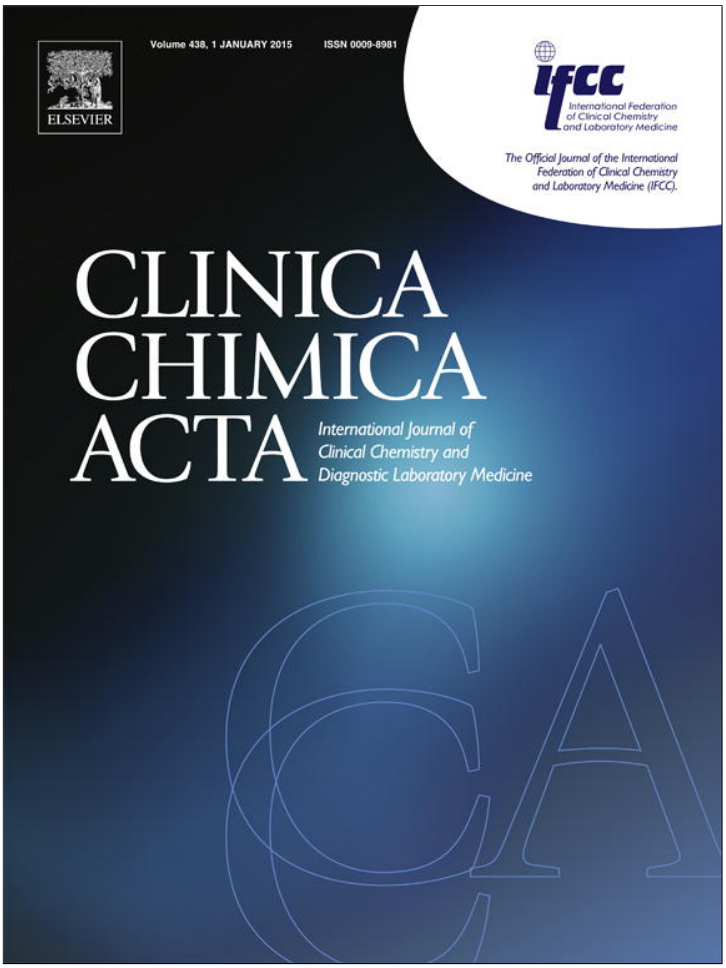

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Performance enhancement in the measurement of 5 endogenous steroids by LC–MS/MS combined with differential ion mobility spectrometry

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ABSTRACT

Background: Challenges for steroid analysis by LC–MS/MS include low ionization efficiency, endogenous isobars with similar fragmentation patterns and chromatographic retention. Differential ion mobility spectrometry (DMS) provides an additional degree of separation prior to MS/MS detection, and shows promise in improving specificity of analysis. We developed a sensitive and specific method for measurement of corticosterone, 11-deoxycortisol, 11-deoxycorticosterone, 17-hydroxyprogesterone and progesterone in human serum and plasma using an ABSciex 5500 mass spectrometer equipped with a differential ion mobility interface.
Methods: 250 μL aliquots of serum were spiked with deuterated internal standards and extracted with MTBE. The samples were analyzed using positive mode electrospray LC–DMS–MS/MS. The method was validated and compared with immunoassays and LC–MS/MS methods of reference laboratories.
Results: Inter and intra assay imprecision was <10%. Limits of quantification and detection in nmol/L were 0.18, 0.09 for corticosterone and 17-hydroxyprogesterone, 0.30, 0.16 for 11-deoxycortisol, 0.12, 0.06 for progesterone and 0.06, 0.03 for 11-deoxycorticosterone. Comparison for progesterone and 17-hydroxyprogesterone with immunoassay showed slopes of 0.97 and 1.0, intercepts of 0.16 and 0.10 and coefficients of determination (r^2) of 0.92 and 0.97, respectively. Progesterone by immunoassay showed positive bias in samples measuring <3.18 nmol/L. Reference intervals for progesterone and 11-deoxycorticosterone in post-menopausal women were found to be <2.88 and <0.28 nmol/L respectively.
Conclusions: We developed and validated an LC–DMS–MS/MS method for analysis of five endogenous steroids suitable for routine measurements in clinical diagnostic laboratories. Specificity gained with DMS allows reducing the complexity of sample preparation, decreasing LC run times and increasing speed of the analysis.

1. Introduction

Congenital adrenal hyperplasia (CAH) refers to a group of genetic disorders of steroid biosynthesis in the adrenal glands [1,2]. They are caused by the inability of the glands to produce enzymes required for biosynthesis of several steroid hormones, such as glucocorticoids, mineralocorticoids and sex hormones. Simultaneous measurement of corticosterone, 11-deoxycortisol (11-DOC-ol), 11-deoxycorticosterone (DOC) and 17-hydroxy progesterone (17-OHP) is useful in the diagnosis of these defects and monitoring of patients.

Corticosterone is produced from DOC by the 11β-hydroxylase pathway. It is further converted to 18-hydroxy corticosterone and finally to the important mineralocorticoid, aldosterone. Measurement of corticosterone along with 11-DOC-ol and DOC is used in the diagnosis of CYP11B1 deficiency, hyperaldosteronism and 11β-hydroxylase deficiency [3,4]. DOC is also used in the diagnosis of suspected 11β-hydroxylase deficiency, differential diagnosis of 11β-hydroxylase 1 (CYP11B1) versus 11β-hydroxylase 2 (CYP11B2) deficiency and in the diagnosis of glucocorticoid responsive hyperaldosteronism [5,6]. Measurement of 17-OHP along with cortisol and androstenedione is useful for the diagnosis of 11- or 21-hydroxylase deficiencies [3,7]. Progesterone primarily produced in the placenta and corpus luteum, is used to monitor placental function during pregnancy and ovulation during the menstrual cycle. It is also used to evaluate patients with adrenal or testicular tumors [8–11].

The steroids measured in this assay have unique biological properties, while being closely structurally related (corticosterone: 11-DOC-ol and DOC: 17-OHP being isomeric pairs). Differentiation of these pairs by LC–MS/MS alone is difficult to achieve due to similar fragmentation

Abbreviations: 11-DOC-ol, 11-deoxycortisol; DOC, 11-deoxycorticosterone; 17-OHP, 17-hydroxy progesterone; DMS, Differential ion mobility spectrometry; IA, Immunoassay; CIA, Chemiluminiscent immunoassay; CAH, Congenital adrenal hyperplasia; LLE, liquid–liquid extraction; MTBE, Methyl-tert-butyl ether.

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patterns and chromatographic retentions. The last analyte on the list, namely progesterone is the most hydrophobic and elutes from the chromatographic column at very high percentage of organic modifier, along with strongly retained and unknown interferences from patient samples. Failing to resolve these co-eluents in samples with low concentrations of progesterone could lead to falsely elevated results, which is especially critical in post-menopausal women [12].

High specificity and sensitivity are essential for diagnosis of the above mentioned disorders. Immunoassays (IA) which are commonly used for steroid analysis are replete with inaccuracies for lack of sensitivity at low concentrations as well as poor selectivity due to cross reactivity with antibodies and matrix effects [13–18]. LC–tandem mass spectrometry is increasingly the choice of methodology due to better accuracies by virtue of improved sensitivity and specificity [19–23]. In addition to LC–MS/MS we evaluated the use of differential ion mobility spectrometry towards improving the performance of the assay. DMS (or field asymmetric ion mobility spectrometry, FAIMS) is based on gas-phase separations at atmospheric pressures and ambient temperature [24–35]. The Selexion equipped on an ABSciex 5500 mass spectrometer consists of a DMS cell comprised of 2 flat parallel plates (10 × 30 mm, separated by 1 mm). An asymmetric RF waveform (separation voltage, SV) is applied to the plates, causing the ions to oscillate, with different ionic species drifting with different velocities towards one plate or the other. A DC potential (compensation voltage, CoV) is also applied to compensate for the net drift of the analyte ions where the ions of the targeted analytes get resolved from interfering ionic species (Supplemental Fig. 1, courtesy Richard A. Yost, University of Florida).

The aim of this work was to develop a highly sensitive and robust method for quantification of the above 5 endogenous steroids in serum and plasma samples and to evaluate its performance. The novel method that we developed uses DMS in conjunction with MS/MS detection as a way of enhancing specificity of analysis and reducing the complexity of sample preparation [31,36–39]. The method has been fully validated and applied to the analysis of clinical samples.

2. Materials and methods

2.1. Reagents, standards and patient samples

Corticosterone, progesterone and d_9-progesterone were purchased from Cerilliant (Sigma), 11-DOC-ol, DOC, 17-OHP from Sigma, d_8-DOC and d_8-corticosterone from CDN Isotopes and d_2-11-DOC-ol and d_8-17-OHP from Cambridge Isotope Laboratories, Inc. Stock standards were prepared in methanol at concentrations of 1 g/l. A working calibration standard of the analytes was prepared at 144.5 nmol/l for corticosterone and 11-DOC-ol and 151.5, 159.0, 15.1 nmol/l for 17-OHP, progesterone and DOC respectively in 1:1 methanol: water. Combined working internal standards were prepared at 72.2 nmol/l for d_8-corticosterone and d_2-11-DOC-ol and 75.1, 79.5, 18.9 nmol/l for d_8-17-OHP, d_9-progesterone and d_8-DOC respectively in 1:1 methanol: water. Calibration standards were prepared in 0.05% BSA at nmol/l concentrations of 1.4, 2.8, 14.4, 28.9, 57.8, 86.7 for corticosterone and 11-DOC-ol, 1.5, 3.0, 15.1, 30.3, 60.6, 90.9 for 17-OHP, 1.5, 3.1, 15.9, 31.8, 63.6, 95.4 for progesterone and 0.1, 0.3, 1.5, 3.0, 6.0, 9.0 for DOC. Water, methanol, methyl t-butyl ether (MTBE), iso-propyl alcohol, acetone, acetonitrile and tri-fluoro acetic acid were purchased from VWR (Radnor, PA). Serum and plasma samples used in the assay were deidentified discard samples submitted to ARUP Laboratories for routine analysis. All studies with human serum and plasma samples were approved by IRB of the University of Utah.

2.2. Sample preparation

Aliquots of 250 μl of calibrators, controls and patient serum/plasma samples were transferred into 2 ml polypropylene microcentrifuge tubes. 20 μL of combined working internal standard and 1.5 ml MTBE were added to each tube, shaken and centrifuged. The organic layer was transferred to a 96-well plate and evaporated under nitrogen at 50 °C. The residues were reconstituted using 100 μl of 1:1 methanol: water. The plate was shaken, centrifuged for 5 min at 4000 ×g and the samples were analyzed by LC–MS/MS.

2.3. LC–MS/MS

Chromatographic separation was performed on a Kinetex C18, 50 × 3 mm, 2.6 μm particle HPLC column (Phenomenex) fitted with a Phenomenex Ultra security guard column (C18, 3 mm). The injection volume was 30 μl and oven temperature was set to 50 °C. The mobile phase consisted of 10 mmol/l formic acid in water and 10 mmol/l formic acid in acetonitrile and was delivered at 1 ml/min with a linear gradient 20% to 48% of organic in 3.5 min, followed by a gradient to 75% organic in 1.6 min. The column was conditioned with 98.5% organic for 1 min and then equilibrated to initial conditions. Total analysis time per sample was 6 min. The HTC PAL autosampler (LEAP Technologies,) injection syringe was washed twice with methanol: water (1:1) with 10 mmol/l formic acid, and 45% acetonitrile, 45% IPA, 9.4% acetone and 0.6% TFA.

Quadrupoles Q1 and Q3 were tuned to unit resolution and the MS parameters optimized for maximum signal intensity for each mass transition. The instrument was operated with electrospray ionization in positive mode; ion-spray voltage was 5500 V, gases 1, 2 and curtain gas were 60, 50 and 20, respectively; entrance potential (EP) of 10 V, ion source temperature of 500 °C and a separation voltage (SV) of the Selexion of 4000 V. The declustering potential (DP), collision energies (CE), exit potentials (CXP) and compensation voltages (CoV) of the 2 monitored MRMs for each analyte are shown in Table 1. The ratio of primary (1) to secondary mass transition (2) was used to evaluate specificity of the analysis. Quantitative calibration was performed with each batch of samples; data acquisition and processing was performed with Analyst™ 1.5.2.

2.4. Assay performance characteristics

Performance of the assay was assessed based on imprecision, limit of detection (LOD), limit of quantification (LOQ), upper limit of linearity (ULOL), method comparison, extraction recovery, carryover, interference and ion suppression studies. Imprecision of the assay was determined by analyzing three replicates of human serum sample pools (low, medium and high) with concentrations of the analytes ranging between 0.1 and 181.8 nmol/l in one run per day over a period of 20 days. These were also used as the quality control samples. LOQ was determined by analyzing 8 samples in triplicate over 6 days; the samples contained

Table 1
Mass transitions and corresponding optimized voltages used in the method.

Mass transitions	Q1(Da)	Q3(Da)	DP(V)	CE(V)	CXP(V)	CoV(V)
Corticosterone-1	347.3	121.1	90	30	15	5
Corticosterone-2	347.3	91.1	90	65	15	5
d8-Corticosterone-1	355.2	125.1	80	41	25	5
d8-Corticosterone-2	355.2	95.1	80	71	25	5
11-DOC-ol-1	347.2	109.1	90	35	10	5.8
11-DOC-ol-2	347.2	97.1	90	31	10	5.8
d2-11-DOC-ol-1	349.3	109.1	140	33	16	5.8
d2-11-DOC-ol-2	349.3	97.1	140	32	10	5.8
DOC-1	331.2	109.1	100	33	13	4.8
DOC-2	331.2	97.1	100	29	11	4.8
d8-DOC-1	339.2	113.1	100	32	25	4.8
d8-DOC-2	339.2	100.1	100	29	25	4.8
17-OHP-1	331.2	109	150	35	13	6
17-OHP-2	331.2	97.1	150	30	11	6
d8-17-OHP-1	339.2	113.1	150	37	13	6
d8-17-OHP-2	339.2	100.1	150	30	11	6
Progesterone-1	315.1	109	90	32	13	5.8
Progesterone-2	315.1	97.1	90	30	11	5.8
d9-progesterone-1	324.2	100.2	90	28	15	5.8
d9-progesterone-2	324.2	113.1	90	31	15	5.8

progressively lower concentrations of the analytes, and were prepared by mixing serum pools containing high and low concentration of the analytes. A stripped serum which had negligible concentrations of all 5 analytes was used to dilute the high sample pool. The lowest concentration for which precision was within 15% and observed concentrations were within 20% of the expected value, was set as the lower limit of quantitation (LLOQ). Limit of detection (LOD) was determined as the lowest concentration at which the peaks of the analyte were present in both mass transitions at the expected retention time and signal to noise ratio for the quantitative mass transition was ≥5. Linearity was evaluated by analyzing seven samples in triplicate over a period of six days. The highest concentration at which precision was within 10% and accuracy was within 20% of the expected values was considered to be the upper limit of linearity (ULOL) of the method. Over 400 patient samples were analyzed during the method evaluation.

2.5. Method comparison

The method was compared with commercial IAs and LC–MS/MS methods of referral laboratories. Comparison with Radioimmunoassay (RIA) was performed for 17-OHP (n = 27); with chemiluminiscent immunoassay (CIA) for progesterone (n = 324) and with LC–MS/MS methods for 17-OHP (n = 32), 11-DOC-ol (n = 31), DOC (n = 20), corticosterone (n = 50) and progesterone (n = 20). The results for method comparison and bias estimation were evaluated using Deming regression.

2.6. Method recovery and carryover

Method recovery was evaluated by standard addition of the analytes at 2.89 nmol/l for corticosterone, 11-DOC-ol, 17-OHP and progesterone and 0.30 nmol/l for DOC in patient samples (n = 5) and analyzed in duplicate over a period of 2 days. Recovery was estimated from the difference between the observed and the expected concentrations of the analytes. Carryover potential for the method was evaluated by injecting negative controls after samples containing 3.0 to 909.0 nmol/l of the targeted analytes.

2.7. Interference and Ion suppression

Twenty-three steroids and steroid metabolites were analyzed using the method to evaluate possible interferences (Supplemental Table 1). Ion suppression was evaluated by analyzing extracted serum samples with standards of the analytes infused with a syringe pump at a flow rate 0.6 ml/h. Concentration of the 5 analytes infused into HPLC effluent was 50.0 nmol/l; concentration of the targeted analytes in the serum samples was less than 0.03 nmol/l. A drop in the baseline in the MRM transitions evidenced ion suppression [40].

2.8. Quality controls

Four controls (negative, low (LQC), medium (MQC) and high (HQC)) analyzed over a period of 20 days were prepared in patient serum pools by spiking with standards. Acceptability of runs was based on retention times of the analytes, concentrations of the analytes being within 20% of historical values and ratios of the primary to the secondary mass transitions >30% being interpreted as presence of interference. Negative controls were considered acceptable only when concentrations of the analytes were below LOQ of the method.

2.9. Sample stability and suitability

Stability of the analytes was evaluated over a period of 30 days at three storage conditions. A sample containing 14.4, 10.6, 12.8, 4.9 and 1.1 nmol/l of corticosterone, 11-DOC-ol, 17-OHP, progesterone and DOC, respectively, was stored at room temperature (RT), 4 °C and −20 °C. The tubes were placed in a −70 °C freezer after 1, 3, 7, 14, 21 and 30 days of storage and analyzed in a single batch.

Concentration of the analytes in different collection tubes was evaluated in duplicate by using samples from 5 individuals collected in six types of collection tubes: Li-Heparin, PST, SST, Na-Heparin, Serum and K_2EDTA. Concentrations in the samples were compared within same individuals.

2.10. Reference interval study for progesterone and DOC in post-menopausal women

Serum samples were collected from 125 post-menopausal women (ages 55–89 y; mean 61 y). Whole blood was collected in SST vacutainer tubes and allowed to clot at room temperature for 30 min. Tubes were centrifuged at 3000 rpm for 10 min, aliquoted, and frozen immediately at −80 °C. The samples were analyzed for progesterone and DOC. Statistical data analysis was performed using EP evaluator (v. 9.0; David G. Rhoads Associates, Inc.).

3. Results

A representative chromatogram of the primary MRM transition of the analytes in an extract from a patient sample is shown in Fig. 1; three periods are shown in separate traces, (A, 0–2.8 min; B, 2.8–3.9 min; and C, 3.9–6 min). The LLOQs (LODs) of the method were 0.18 (0.09), 0.33 (0.16), 0.18 (0.09), 0.12 (0.06), 0.06 (0.03) nmol/l for corticosterone, 11-DOC-ol, 17 OHP, progesterone and DOC, respectively. Linearity of the method was found to be 289, 116, 4545, 191, 91 nmol/l for corticosterone, 11-DOC-ol, 17 OHP progesterone and DOC, respectively. Values of the within-run, between-run and total imprecision are shown in Table 2. The results of method comparison are presented in Fig. 2A–F.

Method recovery was found to be greater than 95% for all analytes. No carryover was detected in the blanks and solvents injected after samples containing very high concentrations of the analytes. None of the steroids and steroid metabolites evaluated for potential interference produced peaks at the retention times of the analytes of interest. There was no ion suppression at the retention times of the analytes (Supplemental Fig. 2).

The 3 controls analyzed with every batch of samples showed ≤20% imprecision and the ratios of the primary and secondary mass transitions for the analytes in the controls were within the expected limits. Supplemental Fig. 3 shows results for the three controls per analyte analyzed over a period of one month. The % CV for LQC, MQC and HQC were 13, 11 and 11 for corticosterone, 11, 11 and 11 for DOC, 11, 8 and 8 for 11-DOC-ol, 10, 9 and 9 for 17-OHP and 7, 10 and 8 for progesterone evidencing ruggedness of the assay.

The 5 analytes, corticosterone, 11-DOC-ol, DOC, 17-OHP and progesterone were found to undergo 12, 21, 41, 15 and 32% degradation, respectively, over a period of 4 weeks when stored at room temperature. Except DOC which degraded by 20% at the end of 4 weeks at 4 °C, other analytes were stable under refrigeration for a month. No degradation was observed in samples stored at −20 °C and −70 °C, and after 3 freeze thaw cycles. Specimen type evaluation showed that both serum and plasma samples were acceptable for the test. Concentrations observed in the patient samples collected in various collection tubes was found to be <10%.

Reference intervals of progesterone and DOC in post-menopausal women (n = 125) were established using nonparametric method as the central 95% of the distribution and determined to be <2.88 and <0.28 nmol/l respectively (Fig. 3).

4. Discussion

In evaluating the ion mobility technology towards improving the efficiency of the method, we approached the development of the assay from 2 directions: simplification of sample preparation and

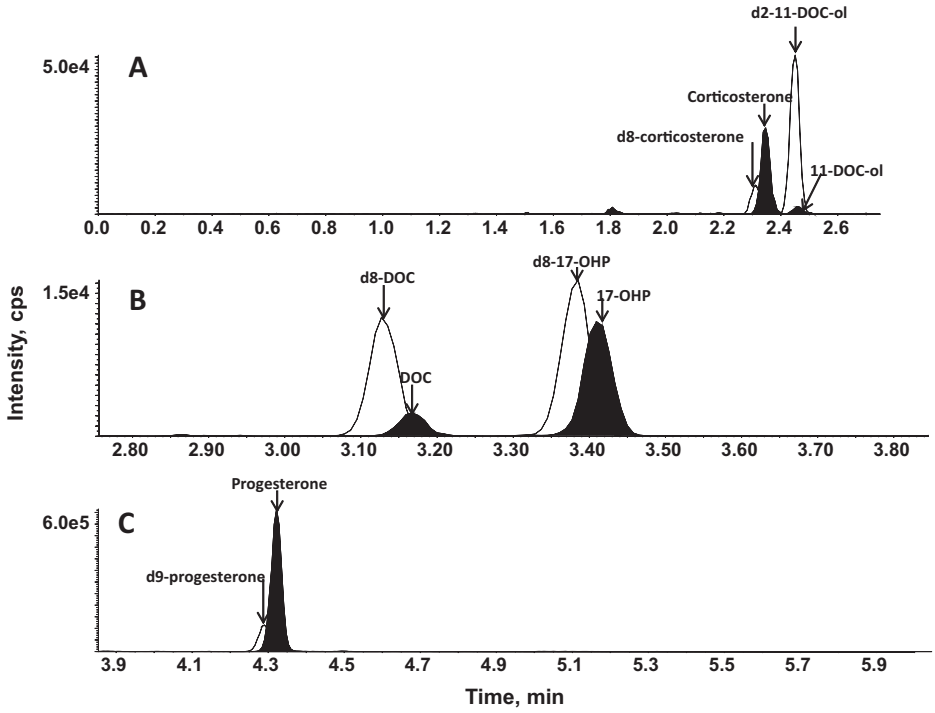

Fig. 1. Representative chromatogram (separated into three time periods) of patient serum sample containing 6.3, 0.3, 2.8, 34.3 and 1.2 nmol/L of corticosterone, 11-DOC-ol, 17-OHP progesterone and DOC, respectively. The black and white peaks correspond to the quantitative mass transitions of the analytes and the internal standards, respectively. Mass transitions of corticosterone and 11-DOC-ol were collected during period 1 (A); DOC and 17-OHP during period 2 (B) and progesterone during period 3 (C).

enhancement of specificity. Earlier published method for 11-DOC-ol, 17-OHP, pregnenolone and 17-hydroxypregnenolone used 2 extractions (SPE and LLE) in conjunction with derivatization, for enhancing sensitivity and specificity of the assay [19].

4.1. Extraction, ionization, HPLC separation

During evaluation of the method we observed an overall reduction in the signal of all the analytes by approximately 5 times by the use of DMS. However an efficient extraction method such a LLE which provided higher signal of the analytes as well as higher background noise of the chromatograms was a trade-off for the loss of the signal intensity due to the use of DMS.

Comparison of the ionization efficiencies between ESI and APCI modes showed approximately 2-fold greater sensitivity for DOC, 11-DOC-ol, 17-OHP and progesterone using ESI while corticosterone was more efficiently ionized using APCI. Aside from the differences in ionization efficiencies among the analytes, HPLC separation also presented a problem. Progesterone eluted late in the gradient along with several co-eluting peaks which posed a problem of poor specificity especially in samples with low concentration.

LLE in conjunction with ESI boosted the signal of the analytes and the use of DMS overcame the challenges of selectivity and high background noise, resulting in an assay with simplified sample preparation. It also permitted combining all the analytes into a single assay.

Table 2
Inter and intraassay imprecision.

Sample	Mean, nmol/L	Within run, CV%	Between- run/day, CV%	Total Imprecision %
Corticosterone				
Level 1	1.5	4.6	7.1	8.5
Level 2	30.0	5.0	6.9	8.5
Level 3	115.1	5.4	4.3	6.9
11-DOC-ol				
Level 1	1.6	5.5	4.1	6.9
Level 2	60.7	3.1	1.1	3.3
Level 3	138.7	2.4	3.5	4.3
17-OHP				
Level 1	1.4	3.7	3.9	5.4
Level 2	15.3	2.7	2.3	3.6
Level 3	59.3	2.6	3.2	4.1
Progesterone				
Level 1	1.6	3.0	1.8	3.5
Level 2	101.1	2.0	2.4	3.1
Level 3	126.4	2.5	3.2	4.1
DOC				
Level 1	0.1	9.3	5.0	9.9
Level 2	1.5	5.4	2.3	5.9
Level 3	15.9	2.8	2.7	3.9

CV, coefficient of variation.

4.2. Separation of isomers using DMS

Among the 5 steroids, there are 2 pairs of isomers: corticosterone and 11-DOC-ol (sharing mass transitions m/z 347/96 and 347/91); and DOC and 17-OHP (sharing mass transitions m/z 331/109 and 331/97). As a way of enhancing specificity we evaluated the use of DMS for resolving peaks of the analytes from coeluting interfering substances. Optimal SV for all analytes was 4000 V; optimal CoVs were determined by ramping the DC voltage over a range of −100 to +100 V. While DOC and 17-OHP were well separated by DMS (CoVs of 4.8 V and 6.0 V) and could be resolved chromatographically, baseline separation of 11-DOC-ol and corticosterone by LC alone was somewhat inadequate. To enhance the selectivity for these 2 analytes, we employed CoV of 5 V for corticosterone, and 5.75 V for 11-DOC-ol (Supplemental Fig. 4). Combination of the partial DMS separation and partial chromatographic separation allowed better resolution of the isomers than could be achieved by either technique alone.

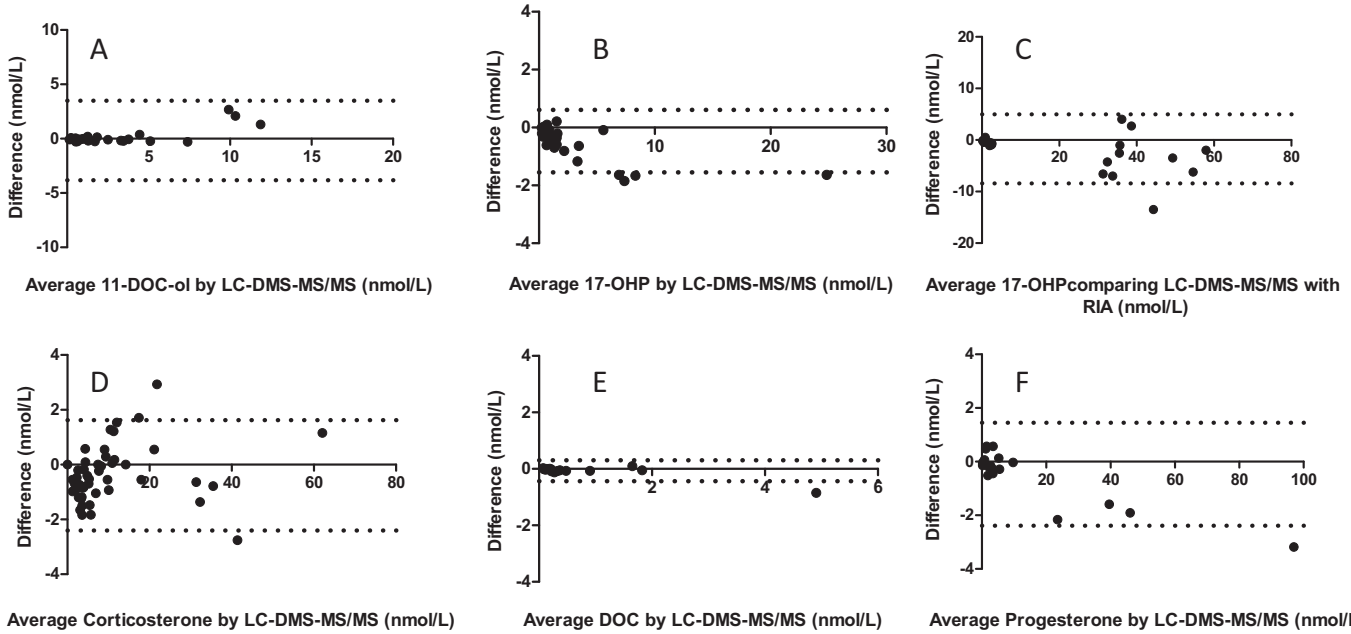

Fig. 2. Bland Altman plots for method comparisons. A) Comparing 11-DOC-ol measured by current LC–DMS–MS/MS method with reference laboratory LC–MS/MS method. Deming regression equation: Ref LC–MS/MS = 1.12 * LC–DMS–MS/MS − 0.46, n = 31, r = 0.99, Sy/x = 0.72. B) Comparing 17-OHP measured by current LC–DMS–MS/MS method with reference laboratory LC–MS/MS method. Deming regression equation: Ref LC–MS/MS = 1.08 * LC–DMS–MS/MS + 0.26, n = 32, r = 0.99, Sy/x = 0.28. C) Comparing 17-OHP measured by current LC–DMS–MS/MS method with reference laboratory RIA method. Deming regression equation: Ref RIA = 1.08 * LC–DMS–MS/MS + 0.32, n = 27, r = 0.97, Sy/x = 2.15. D) Comparing corticosterone measured by current LC–DMS–MS/MS method with reference laboratory LC–MS/MS method. Deming regression equation: Ref LC–MS/MS Corticosterone = 0.98 * LC–DMS–MS/MS + 0.55, n = 50, r = 0.99, Sy/x = 0.72. E) Comparing DOC measured by current LC–DMS–MS/MS method with reference laboratory LC–MS/MS method. Deming regression equation: Ref LC–MS/MS = 1.10 * LC–DMS–MS/MS − 0.022, n = 20, r = 0.99, Sy/x = 0.07. F) Comparing progesterone measured by current LC–DMS–MS/MS method with reference laboratory LC–MS/MS method. Deming regression equations: Ref LC–MS/MS = 1.03 * LC–DMS–MS/MS + 0.00, n = 20, r = 0.99, Sy/x = 0.31 Divide by 0.029 to convert nmol/L to ng/dL for corticosterone and 11-DOC-ol, by 0.030 for 17-OHP and DOC and by 3.18 for converting nmol/L to ng/mL for progesterone.

4.3. Removal of unknown interferences using DMS

The role of DMS in improving selectivity could be more clearly assessed by evaluating the performance of the method with and without the DMS. As shown in Fig. 4, for samples containing low concentrations of progesterone or DOC, the DMS effectively removed peaks of interfering substances from the peaks of interest. Fig. 4B and D demonstrate the elimination of coeluting peaks with progesterone and DOC at 4.28 and 3.32 min, respectively (a 0.03 min difference in retention time of progesterone between Fig. 1 and Fig. 4 is attributed to typical variation in LC retention times between runs). In the experiments without DMS, the apparent concentrations of progesterone (due to coeluting interfering peaks) were up to 3 times higher than concentrations in the method employing DMS (Supplemental Fig. 5). This clearly establishes the utility of DMS as a tool for resolving peaks of interest from co-eluents, which becomes especially noticeable in samples containing low concentrations of the analyte.

4.4. Method comparison

The method showed good agreement with LC–MS/MS assays of other reference laboratories with slopes of Deming regression line of 1.12, 1.08, 0.98, 1.10 and 1.03 and for 11-DOC-ol, 17-OHP, corticosterone, DOC and progesterone respectively (Fig. 2). 17-OHP measured by RIA showed good agreement with the current method (slope of regression line being 1.08). Comparison with CIA method for progesterone

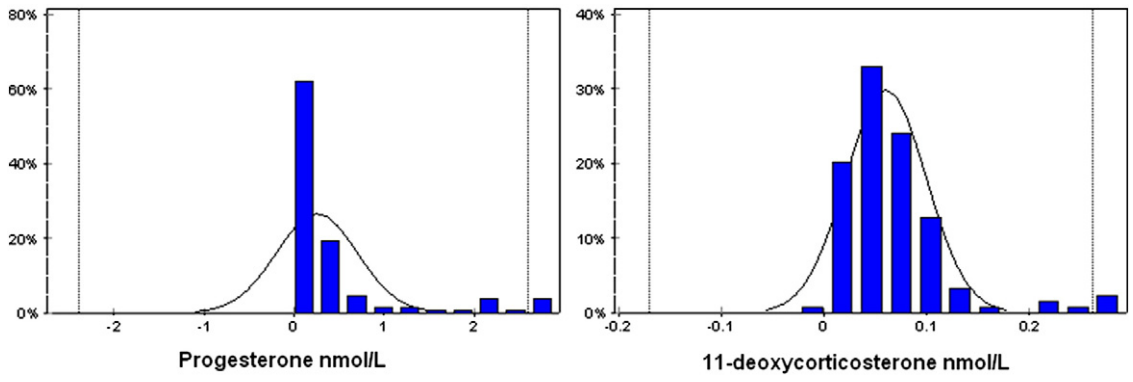

Fig. 3. Histograms with distributions of concentrations of progesterone and 11-deoxycorticosterone in samples from post-menopausal women (n = 125).

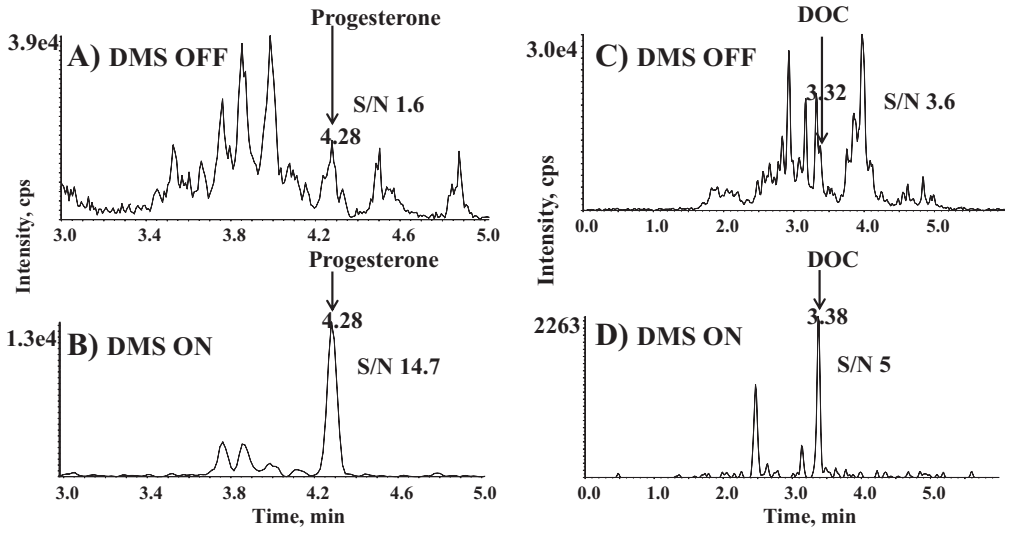

Fig. 4. Patient serum sample containing 2.2 nmol/L progesterone in DMS OFF (A) and DMS ON (B) mode and 0.30 nmol/L DOC in DMS OFF (C) and DMS ON (D) mode.

(n = 324) showed a regression line slope of 0.97 and correlation coefficient of 0.92. However, CIA was overestimating concentration of progesterone by an average of 2.3 times in samples measuring <3.18 nmol/l (by the current LC–MS/MS method) (Supplemental Fig. 6). Considering these findings, the reported analytical sensitivity of the CIA method (0.31 nmol/l) could be misleading, especially when measuring concentrations in samples of post-menopausal women.

Performance of the method validated according to CLSI guidelines (Supplemental Table 2) was found to be consistent throughout the evaluation of the assay, suggesting sufficient robustness of the technique for use in routine analysis and the practical utility of the hyphenated technique LC–DMS–MS/MS.

5. Conclusion

We developed an LC–DMS–MS/MS method for analyzing corticosterone, 11-deoxycortisol, 11-deoxycorticosterone, 17-hydroxy progesterone and progesterone in human serum and plasma that has acceptable performance characteristics for diagnostic applications. We were able to reduce background noise, improve performance and separate the isomeric pairs of analytes by the effective combination of LC and ion mobility spectrometry. Reference intervals for progesterone and DOC in post-menopausal women were established. Use of DMS allowed us to simplify sample preparation, while achieving high specificity and sensitivity of analysis, especially in measuring low concentrations of progesterone in samples from post-menopausal women.

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