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Simultaneous quantitation of seven endogenous C-21 adrenal steroids by liquid chromatography tandem mass spectrometry in human serum

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ABSTRACT

Quantitation of endogenous steroids is important in the diagnosis of several endocrine disorders. In this study we present a new method for simultaneous quantitation of cortisol, cortisone, 11-desoxycortisol, 21-desoxycortisol, corticosterone, 17-hydroxyprogesterone and 11-desoxycorticosterone in human serum by on-line extraction and LC–MS/MS. Analytes extraction was performed on-line using a 2-position and 6-port valve equipped with a monolithic silica cartridge. After chromatographic separation of all analytes, detection was performed in the multiple reaction monitoring mode using positive atmospheric pressure chemical ionization mode. Total imprecision of the assay ranged from 5.5 to 15.5%. Comparison with immunoassays yielded coefficients of 0.893 for cortisol, 0.848 for 11-desoxycortisol and 0.924 for 17-hydroxyprogesterone. The sensitivity of this method provides meaningful data for patients within normal and elevated range and it may be useful for the diagnosis of a variety of adrenal dysfunctions.

1. Introduction

Due to their low levels and their chemical similarity, the analysis of endogenous steroids is a bioanalytical challenge. Most methods for routine endogenous steroid quantitation are based on radioimmunoassay (RIA) that is simple and accessible to most of clinical laboratories and hospitals. However, this methodology is susceptible to interference from other structurally related endogenous and exogenous steroids [1]. It has been demonstrated that RIA, especially those based on direct assays often overestimate true steroid values [2,3]. Although RIA with previous extraction steps to eliminate interfering compounds improves specificity, especially when some chromatographic step such as HPLC is used [4,5], lengthy sample pretreatment procedures required limits their throughput and applicability in most of laboratories. Furthermore, even the use of analyte extraction techniques does not eliminate all interferences from some steroids [6].

Gas chromatography coupled to mass spectrometry (GC–MS) is considered the gold standard for steroids quantitation. In fact, it is highly specific and has been applied to quantify a large number of steroids [7,8]. However, this technique requires complicated and time consuming sample preparation procedures limiting its usage to few specialized laboratories.

Liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) is being rapidly introduced as a better alternative to quantify steroids in the clinical context. Methods have been described for the determination of cortisol and cortisone [9,10], 17-hydroxyprogesterone [3], 11-desoxycortisol [11,12], 21-desoxycortisol [13] and corticosterone [14]. Some of them still demand manual sample preparation procedures such as liquid–liquid extraction [13,14] or off-line solid-phase extraction [3,12]. Derivatization, which is often necessary to achieve the required sensitivity to quantify endogenous levels of these analytes requires several steps [12], reducing the throughput.

One of the most important features of LC–MS/MS for analysis of steroids is the possibility to measure several analytes simultaneously without all workup required by GC–MS [11,12,15,16]. This is especially interesting because it allows the discrimination among related disorders. For example, in congenital adrenal hyperplasia (CAH), the use of a steroid profile allows the acquisition of clinically more useful data than can be obtained through the measurement of a single steroid.

In this study, we report the development and clinical validation of a new method for simultaneous measurement of 7 related C-21 adrenal steroids: cortisol, cortisone, 11-desoxycortisol, 21-desoxycortisol, corticosterone, 17-hydroxyprogesterone and 11-

Abbreviations: RIA, radioimmunoassay; GC–MS, gas chromatography–mass spectrometry; LC–MS/MS, liquid chromatography–tandem mass spectrometry; CAH, congenital adrenal hyperplasia; APCI, atmospheric pressure chemical ionization; MRM, multiple reaction monitoring.

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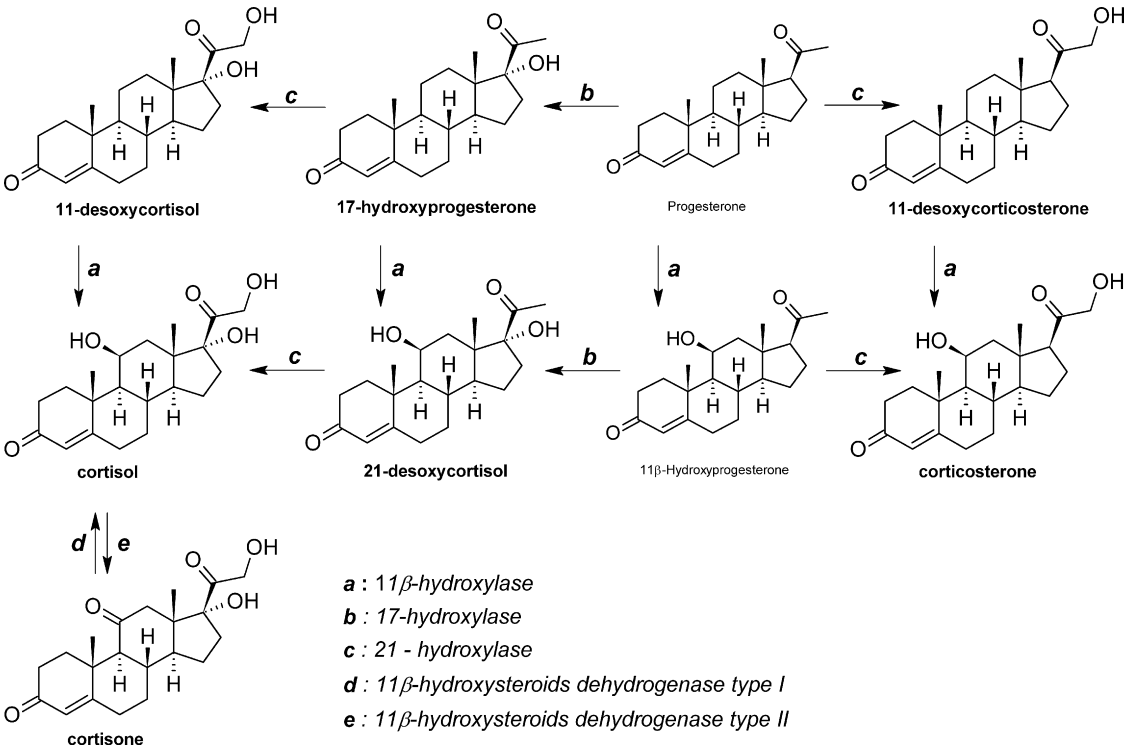

Fig. 1. Metabolic pathway for the adrenal corticosteroids.

desoxycorticosterone (Fig. 1). We aimed to achieve a simple, rapid, and sensitive assay using a reduced sample preparation procedure based on on-line extraction and LC–MS/MS.

2. Experimental

2.1. Materials

17-Hydroxyprogesterone-d_8 and 11-desoxycortisol-d_2 were from CDN Isotopes (Pointe-Claire, Canada). Cortisol-d_4, 21-desoxycortisol-d_8, corticosterone-d_8, and 11-desoxycorticosterone-d_8 were from Medical Isotopes (Pelham, NH). All other steroids were purchased from Steraloids (Newport, RI). Ultrapure water was prepared from MilliQ system (Millipore, Billerica, MA). HPLC grade solvents were from Tedia (Rio de Janeiro, Brazil). Fetal bovine serum was obtained from Invitrogen (São Paulo, Brazil). All other reagents were from Sigma–Aldrich (St. Louis, MO).

2.2. Preparation of standard solutions, calibrators and quality control samples

Stock solutions of all steroids standards and deuterated standards were prepared in DMSO at a concentration of 1 g/L. Working standard solutions of cortisol and cortisone were prepared in DMSO at concentration of 100 mg/L and at 10 mg/L for the other analytes. Steroid calibrators were prepared in bovine fetal serum at concentrations of 13.79, 27.59, 110.36, 344.87, 882.86 and 1379.46 nmol/L for cortisol; 6.94, 13.87, 27.74, 55.49, 88.78 and 138.72 nmol/L for cortisone, 0.43, 0.87, 2.60, 5.20, 10.39 and 25.98 nmol/L for 11-desoxycortisol; 1.15, 2.89, 8.66, 25.98, 51.95 and 129.88 nmol/L for 21-desoxycortisol; 1.44, 2.89, 8.66, 25.98, 51.95 and 129.88 nmol/L for corticosterone; 0.30, 0.76, 3.03, 7.57, 15.13 and 30.26 nmol/L for 17-hydroxyprogesterone; and 0.61, 1.51, 2.72, 5.45, 10.89 and 27.23 nmol/L for 11-desoxycorticosterone. Two levels of control samples were prepared by the addition of standard solutions from separate stock solutions to serum pools at target values of 270.37 and 899.41 nmol/L for cortisol; 58.26 and 108.20 nmol/L for cortisone; 1.44 and 27.42 nmol/L for 11-desoxycortisol, 5.20 and 72.16 nmol/L for 21-desoxycortisol; 8.37 and 95.25 nmol/L for corticosterone; 3.63 and 33.89 nmol/L for 17-hydroxyprogesterone; and 3.03 and 27.23 nmol/L for 11-desoxycorticosterone. Aliquots of calibrators and control samples were stored at −70 °C due to the low stability of cortisone in serum.

2.3. Specimens

The serum samples utilized in the study were collected from apparently healthy adult volunteers. For cortisol, cortisone and corticosterone, only samples collected in the morning were utilized. The samples were stored at −70 °C and thawed only once before the analysis. This study was approved by Institutional Research Ethics Committee.

2.4. Sample preparation

Sample preparation was performed as follows. An aliquot of serum (600 μL) sample (or blank, or calibrators or quality control samples) was combined with 20 μL of internal standards solution (cortisol-d_4 at 5.46 nmol/L, 21-desoxycortisol-d_8 and corticosterone-d_8 at 1.69 nmol/L, 11-desoxycortisol-d_8 at 0.57 nmol/L, 17-hydroxyprogesterone-d_8 and 11-desoxycorticostrone-d_8 at 0.59 nmol/L in DMSO) in 1.5 mL polypropylene tubes. Proteins were precipitated by the addition of 600 μL of 0.2 mol/L $ZnSO_4$/methanol (20/80) followed by vortexing for more 3 min and centrifugation for 10 min at 4000 × *g*. The supernatant was transferred to a polypropylene 96-well deep well plate and placed in a Waters 2777 sample manager equipped with a cooling stack set at 10 °C.

2.5. On-line extraction and LC separation

On-line extraction of the serum supernatants were performed using Onyx monolithic C_{18} 10 mm × 4.6 mm cartridge (Phenomenex, Torrance, CA) connected to a 2-position, 6-port Phenomenex Synergi fluid processor. One milliliter aliquots were injected into the LC system. Samples were loaded and washed with 5% acetonitrile at 4 mL/min pumped by a Waters 510 pump (Millford, MA) for 2 min. The analytes were eluted for 1.5 min to a Synergi Fusion 4 μ 250 mm × 4.6 mm analytical column (Phenomenex) kept at 50 °C in a Thermasphere TS-130 column oven. The analytical column was eluted with a multistep binary gradient pumped by a Waters 1525 μ pump. The elution mobile phase consisted initially of a mixture of 55% (v/v) methanol in 0.5 mmol/L pH 3.0 ammonium formate at a flow rate of 1 mL/min. The methanol content was increased to 63% in 9 min using the binary gradient curve 6 and then to 79% in 3 min using the gradient curve 2.

2.6. Mass spectrometry detection

Detection was performed on a Quattro Premier tandem mass spectrometer (Waters/Micromass, Manchester, UK) equipped with an IonSabre atmospheric pressure chemical ionization (APCI) probe operating at positive mode. The mass spectrometer operating conditions were as follows: desolvation temperature 550 °C, desolvation gas (nitrogen) flow 600 L/h, source temperature 60 °C, cone gas flow (nitrogen) 70 L/h, with corona current set at 2 μA. Collision-induced dissociation was performed using argon as the collision gas at 4×10^{-3} mbar. For product ion spectra and multiple reaction monitoring (MRM) analyses, unit resolution was maintained for both parent and product ions. Instrument optimization for the analytes were conducted by infusing standard solution (1 μg/mL) of the analytes by the built-in syringe pump at a flow rate of 10 μL/min combined with a makeup-flow of 55% (v/v) methanol in 0.5 mmol/L pH 3.0 ammonium formate at a flow rate of 1 mL/min. System control and data acquisition were achieved with the MassLynx 4.0.

2.7. Quantitation

Data processing and quantitation were performed by the QuanLynx Application Manager. For cortisone, cortisol-d_4 was used as internal standard. Calibration was performed using a 6 points curve through linear regression with fit weighting to $1/x^2$ to give higher priority to calibration points with a low concentration. The accepted range for ion ratios was within 20% of the calibration standards.

2.8. Method validation

Within-run and total assay imprecision was assessed using the EP5 guideline from the Clinical and Laboratory Standards Institute (CLSI) [17]. Lower limits of detection and quantitation were determined following the CLSI EP17-A guideline [18]. Linearity and recovery were determined altogether through a spiked curve with 11 concentrations analyzed in triplicate. Percent recovery was calculated by dividing the measured concentration by the theoretical spiked concentration. Linearity was evaluated according to the CLSI EP6-A guideline [19].

Evaluation of matrix effect was performed using the post-column infusion method [20]. A solution containing the seven steroids at 1 μg/mL in methanol was infused post-column at a constant rate into the LC stream through a T-connection. Ion suppression or enhancement was evaluated comparing the MS response between mobile phase injections and 10 specimens with undetectable concentration of the studied steroids.

Potential carryover effects were investigated by assaying 2 specimens with low and high concentration of steroids. The low-concentration pool was obtained combining serum samples from 30 healthy donors. This pool was quantified to determine the concentration of each analyte (cortisol: 220.71 nmol/L, cortisone: 27.74 nmol/L, 21-desoxycortisol and corticosterone: 5.77 nmol/L, 11-desoxycortisol: 2.31 nmol/L, 11-desoxycorticosterone and 17-hydroxyprogesterone: 2.42 nmol/L). The high-concentration specimen was prepared spiking the low-concentration pool with standard solutions of the seven analytes resulting in the following concentrations: 2207.14 nmol/L of cortisol, 277.44 nmol/L of cortisone, 216.47 nmol/L of 21-desoxycortisol and corticosterone, 57.73 nmol/L of 11-desoxycortisol and, 54.47 nmol/L of 11-desoxycorticosterone and 17-hydroxyprogesterone. The samples were run in the following order: 3 low specimens, 2 high specimens, 1 low, 2 high, 4 low, 2 high, 1 low, 2 high, 1 low, 2 high, and 1 low specimen. Results were analyzed using EP Evaluator On-line (David G. Rhoads Associates, Inc. Kennett Square, PA).

Interference studies were performed by spiking a pooled serum with 10 μmol/L of the following compounds: prednisolone, 6α-methyl-prednisolone, prednisone, dexamethasone, aldosterone, cortisol-21-glucuronide, cortisol-21-sulfate, 5α-dihydrocortisol, 20α-dihydrocortisol, 6β-hydroxycortisol, 5α-tetrahydrocortisol, 5α-tetrahydro-11-desoxycortisol, betamethasone, triamcinolone, dihydrotestosterone, progesterone, fludrocortisone, 5β-tetrahydrocortisone, pregnenolone and androstenediol. The average of three measurements for each compound tested was compared with the average for the pool of serum before additions.

The present method was compared with three different immunoassays. For 17-hydroxyprogesterone, 86 samples were compared with an *in-house* developed method based on liquid–liquid extraction followed by HPLC purification and detection by RIA [21]. For 11-desoxycortisol, 51 samples were analyzed by the present method and by an *in-house* RIA method with previous liquid–liquid extraction and Celite® chromatography [22]. One hundred and ten samples were compared for cortisol with an Auto DELFIA commercial kit (Wallac Oy, Turku, Finland). Data were evaluated through Deming Regression using the EP Evaluator software.

2.9. Estimation of reference intervals

Reference intervals for 11-desoxycortisol, 21-desoxycortisol and 11-desoxycorticosterone were determined analyzing serum samples from 138 healthy donors with ages ranging from 18 to 65. 17-Hydroxyprogesterone reference interval was calculated using only males samples ($n = 56$) since we had no information about phase within the menstrual cycle and menopausal status. Reference intervals for cortisol, cortisone and corticosterone were verified measuring serum samples obtained from 58 healthy adult donors (ages ranging from 18 to 67). As these steroids present a circadian variation of their serum concentrations, all the samples were collected between 7 and 9 a.m. The central 95% interval was calculated by a non-parametric method in accordance with the IFCC recommendations, using the EP Evaluator software.

3. Results

3.1. Mass spectrometry detection

Positive mode electrospray, APCI and atmospheric pressure photoionization (using toluene as dopant) were evaluated on the Quattro Premier tandem mass spectrometry in order to detect the seven steroids (data not shown). We found higher signal to

ratio intensities detecting protonated forms $[M+H]^+$ in positive mode APCI. Suitable precursor and product ions for each steroid and parameters optimization were performed by direct infusion of single-analyte solutions at 10 μmol/L into the mass spectrometer.

Among the steroids tested there are two groups of isomers: 11-desoxycortisol, 21-desoxycortisol and corticosterone and 17-hydroxyprogesterone and 11-desoxycorticosterone. Therefore, they present same precursor ions and very similar fragmentation patterns (Fig. 2). 11-Desoxycortisol dissociation spectrum presented two dominant product ions at *m/z* 97 and 109. 21-Desoxycortisol and corticosterone presented extensive fragmentation with *m/z* 121 and 97 as the most abundant product ions. The pair of isomers 17-hydroxyprogesterone and 11-desoxycorticosterone presented almost identical dissociation spectra with dominance of *m/z* 97

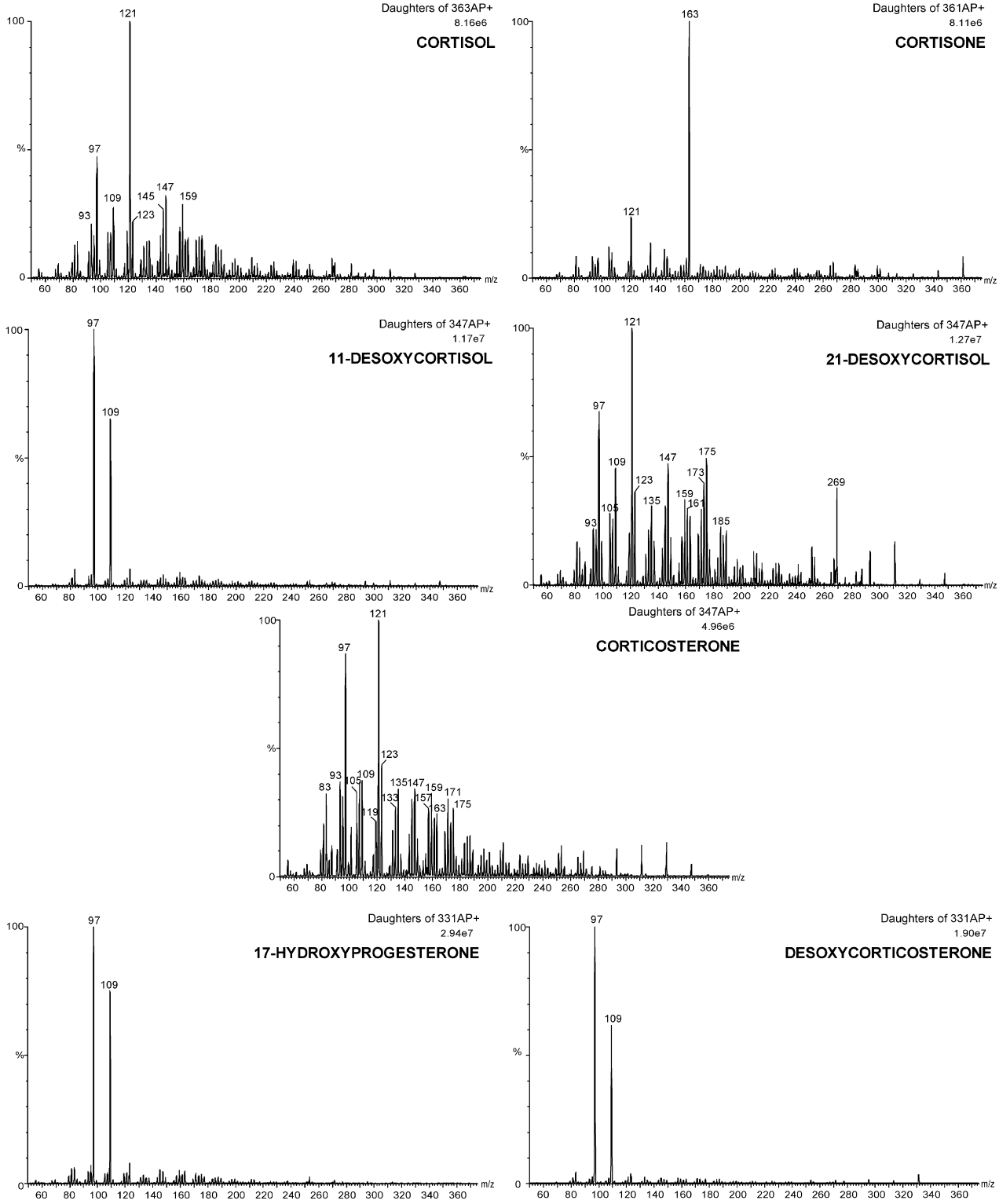

Fig. 2. Dissociation spectra of $[M+H]^+$ ions of cortisol, cortisone, 11-desoxycortisol, 21-desoxycortisol, corticosterone, 17-hydroxyprogesterone and 11-desoxycorticosterone.

Table 1
Tandem mass spectrometry parameters for positive APCI–MS/MS detection of the seven C-21 steroids and respective deuterated internal standards: retention times (RT, min), cone voltages (CV, V), SRM transitions, collision energy (CE, eV) and ion ratios

Compound	Group	RT	Precursor ion	Quantifier transition			Qualifier transition			Ion ratio
				Product ion	CV	CE	Product ion	CV	CE	
Cortisone	1	7.1	361.2	163.1	38	24	121.1	38	24	4.8
Cortisol		7.7	363.2	121.1	32	20	267.1	30	28	3.2
Cortisol-d_4		7.7	367.2	121.1	32	20	271.1	30	28	4.3
21-Desoxycortisol	2	9.0	347.2	121.1	32	25	147.1	36	25	1.9
Corticosterone		9.7	347.21	121.11	36	28	97.11	32	24	1.4
11-Desoxycortisol		10.1	347.2	97.1	38	24	109.1	38	24	1.3
21-Desoxycortisol-d_8		9.0	355.2	125.1	36	25	180.1	36	25	1.1
Corticosterone-d_8		9.7	355.21	125.11	32	28	100.1	32	28	1.0
11-Desoxycortisol-d_2		10.1	349.2	97.1	38	25	109.1	38	24	1.3
11-Desoxycorticosterone	3	12.9	331.21	97.11	38	22	109.11	38	22	1.5
17-Hydroxyprogesterone		13.5	331.2	109.1	40	28	97.1	40	28	1.2
11-Desoxycorticosterone-d_8		12.9	339.21	100.11	38	22	113.11	38	22	2.2
17-Hydroxyprogesterone-d_8		13.5	339.2	100.1	40	28	113.1	40	28	1.6

and 109. Two mass transitions were optimized for each analyte and correspondent deuterated internal standards resulting in 26 mass transitions. The main optimized acquisition parameters are depicted in Table 1. Mass transitions were divided into three segments to ensure maximum sensitivity. Although corticosterone and 11-desoxycorticosterone share identical MRM channels with, respectively, 21-desoxycortisol and 17-hydroxyprogesterone, we applied a 0.01 mass difference in parent and product ions for corticosterone (347.21 > 121.11) and 11-desoxycorticosterone (331.21 > 97.11) to allow the acquisition of separate detection channel for each analyte.

3.2. Chromatography and on-line extraction

On-line extraction of the deproteinated serum was achieved using a C_{18} silica monolithic pre-column as an extraction column. Due to its low backpressure it was possible to use high flow rates for efficient extraction of unwanted components in only 2 min. We found that a pre-column can be reused up to 300 injections and no carryover was observed for all analytes studied.

Separation of the seven steroids was achieved in 14 min (including on-line extraction) using a Synergi Fusion column eluted with a binary gradient optimized to provide the complete chromatographic separation in a minimum time period. As shown in Fig. 3, 21-desoxycortisol and corticosterone, and 17-hydroxyprogesterone and 11-desoxycorticosterone present very similar MRM profiles. The most intense MRM transition for 11-desoxycortisol (347.2 > 97.1) also revealed correspondent peaks for 21-desoxycortisol and corticosterone. Deuterated internal standards presented slightly differences in retention time in comparison with their correspondent non-labeled steroids due to isotopic effects. The chromatographic reproducibility was assessed from 50 injections. The RSD, calculated from retention times from 50 injections proved to be less than 0.5% for all analytes.

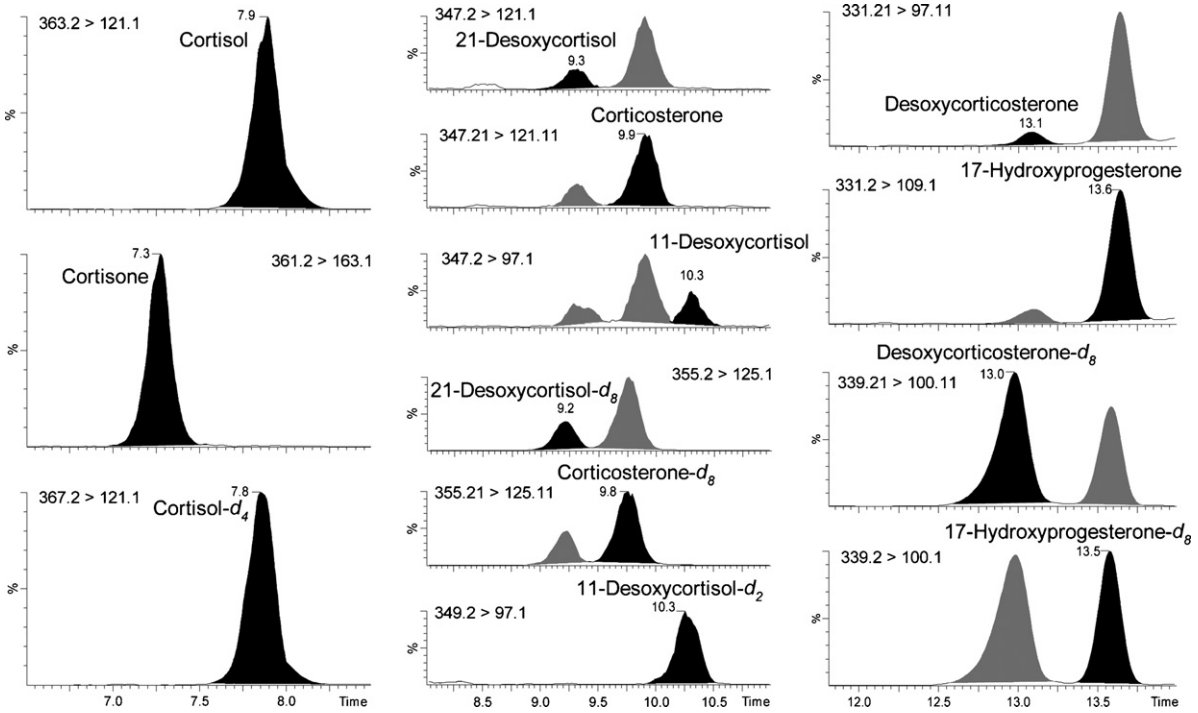

Fig. 3. Chromatograms of patient serum sample containing depicting the quantifying transitions for cortisol, cortisone, cortisol-d_4, 21-desoxycortisol, corticosterone, 11-desoxycortisol, 21-desoxycortisol-d_8, corticosterone-d_8, 11-desoxycortisol-d_2, 17-hydroxyprogesterone, 11-desoxycorticosterone, 17-hydroxyprogesterone-d_8 and 11-desoxycorticosterone-d_8.

Table 2
Imprecision, lower limits of detection (LD) and quantitation (LQ) of the LC–MS/MS method for analysis of serum cortisol, cortisone, 11-desoxycortisol, 21-desoxycortisol, corticosterone, 17-hydroxyprogesterone and 11-desoxycorticosterone

Steroid	Imprecision				LD (nmol/L)	LQ (nmol/L)
	Level	Mean (nmol/L)	Within-day (%CV)	Total (%CV)		
Cortisone	1	59.65	6.1	15.5	0.85	2.80
	2	440.85	5.1	10.4		
Cortisol	1	340.45	3.0	8.7	2.75	12.40
	2	830.16	2.9	6.1		
21-Desoxycortisol	1	3.55	10.0	13.3	0.15	0.70
	2	12.82	6.9	8.6		
Corticosterone	1	12.32	7.8	7.8	0.60	2.75
	2	56.66	5.0	6.1		
11-Desoxycortisol	1	1.44	10.4	12.1	0.10	0.45
	2	9.93	4.5	6.1		
11-Desoxycorticosterone	1	2.09	8.4	11.5	0.10	0.30
	2	10.38	3.8	5.5		
17-Hydroxyprogesterone	1	4.54	7.5	7.9	0.15	0.45
	2	12.29	5.2	6.6		

3.3. Method performance

The intra-assay and total coefficient of variation are summarized in Table 2. The intra-assay CV ranged from 2.9% (cortisol) to 10.4% (11-desoxycortisol). Total imprecision results yielded CVs of 7.8% (cortisol) to 15.5% (cortisone) at low-concentration level and 5.5% (11-desoxycorticosterone) to 10.4% (cortisone) at high concentration.

Lowest limits of detection and quantitation for the analytes are presented in Table 2. For 11-desoxycorticosterone and 11-desoxycortisol, for example, it is possible to detect 60 fmol of injected steroid. Among 20 related endogenous or synthetic steroids evaluated for interference, none interfered up to 10 μmol/L. No ion suppression or enhancement was observed in the elution interval of the seven steroids.

The present method was compared with immunoassays for 17-hydroxyprogesterone, 11-desoxycortisol and cortisol (Fig. 4). The correlation found for cortisol comparing the present method and a commercial Auto DELFIA method ($n = 103$) was 0.8927. The comparison for 11-desoxycortisol with an in-house RIA method with previous sample treatment by liquid–liquid extraction followed by SPE ($n = 50$) showed a correlation of 0.8477. The correlation for 17-hydroxyprogesterone with an in-house method based on liquid–liquid extraction/HPLC/RIA ($n = 67$) showed a correlation of 0.9242.

The reference ranges from the seven analytes determined by analysis of the samples for healthy adult volunteers are shown in Table 3.

Table 3
Reference ranges for cortisol, cortisone, 11-desoxycortisol, 21-desoxycortisol, corticosterone, 11-desoxycortisol and 17-hydroxyprogesterone in adults[a]

Steroid	*N*	nmol/L
Cortisol[b]	58	176.6–714.6
Cortisone[b]	58	22.1–97.1
11-Desoxycortisol	138	≤2.3
21-Desoxycortisol	138	≤1.1
Corticosterone[b]	58	≤67.4
17-Hydroxyprogesterone[c]	53	≤5.1
11-Desoxycorticosterone	138	≤0.7

[a] Results are the non-parametric estimates of the central 95% interval.
[b] Samples collected between 7 and 9 a.m.
[c] Results obtained from samples from males.

4. Discussion

LC–MS/MS is becoming the method of choice for steroid quantitation in clinical samples. Despite the high selectivity and sensitivity achieved by LC–MS/MS, sample preparation procedures are still necessary and they often bottleneck the throughput of this technique. Here we present a simple strategy for on-line extraction of seven endogenous steroids from serum samples. A single silica monolithic guard cartridge connected to a 6-port 2-position diverter valve is used as an extraction column. Due to the porous nature of monolithic silica, it is possible to apply high flow rates resulting in efficient sample clean-up and analyte concentration [23]. This strategy provides a low cost alternative to dedicated systems for on-line extraction and reduces off-line sample preparation to two steps.

The presence of 5 positional isomeric compounds among the 7 steroids analyzed posed an additional challenge to the method development. The related isomers presented a very similar fragmentation pattern (Fig. 2) which is characteristic for the group of 3-oxo-4-ene steroids (Δ^4-structure). Therefore, chromatographic separation plays a key role in this method as complete resolution is necessary. This method well illustrated the complexity involving the determination of endogenous steroids which present physiological concentrations of isobaric compounds with identical product ions. Consequently, in order to assure the absence of interferences, besides the confirmatory ion ratios, it is also important to observe chromatographic parameters such as retention times, peak shapes and correspondence with isotopic internal standards.

An extensive method validation was performed and confirmed its applicability to clinical routine. The combination of on-line extraction and the use of six deuterated internal standards reduced variability and contributed to the good precision achieved for all compounds. The sensitivity reached by this method allowed the detection of all analytes in non-disease ranges. Due to different efficiencies in ionization and fragmentation, higher sensitivities were achieved for 11-desoxycortisol, 11-desoxycorticosterone and 17-hydroxyprogesterone. Since cortisol and cortisone are presented in about 100× higher levels in serum compared to the other 3 compounds, the method was adjusted to satisfy the clinical needs for these two steroids. This is another advantage of LC–MS/MS methods over RIA since, due to their extended dynamic range, it is possible to determine compounds with large differences in their relative concentrations in the same analysis.

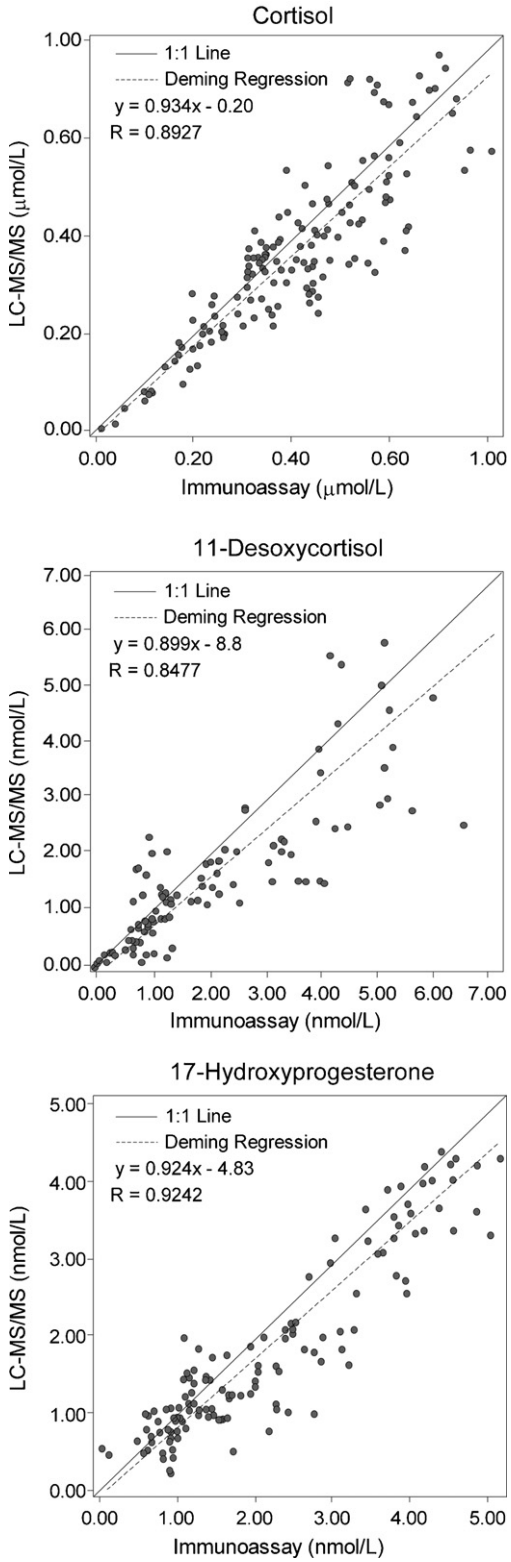

Fig. 4. Comparison of measurements of cortisol, 11-desoxycortisol and 17-hydroxyprogesterone by immunoassays and LC–MS/MS. Regression lines obtained by Deming Regression are represented in dashed line. The continuous line represents the line of equality.

As has been previously reported, compared to LC–MS/MS, immunoassays yield higher values for cortisol [24,25], 11-deoxycortisol [12] and 17-hydroxyprogesterone [2,26]. This may occur due to cross-reactivity with endogenous steroids in the immunoassay methods. As expected, the correlation was better for 17-hydroxyprogesterone where an extensive analyte extraction including HPLC purification was performed before quantitation by RIA.

One of the main advantages of LC–MS/MS over immunoassays is the possibility to analyze several compounds simultaneously. This feature is valuable in the diagnostic of adrenal disorders, since the use of more than one marker allows a better assessment of the genetic errors implicated in a certain condition. Steroid profiles in CAH not only allow the acquisition of more clinically useful data than can be obtained through the measurement of a single steroid alone, but also allows differential identification of the CAH subtypes. Although 17-hydroxyprogesterone and 11-desoxycortisol are the most used markers to diagnose CAH defects, the use of other steroids from C21-steroid metabolic pathway is relatively unexplored, mainly due to the lack of practical methodologies. 21-Desoxycortisol has been described as a more sensitive marker than 17-hydroxyprogesterone for 21-hydroxylase deficiency detection and its determination after ACTH stimulation is becoming the new strategy for the identification of heterozygote carriers of an impaired 21-hydroxylase gene [27–29]. Increased 11-desoxycorticosterone levels may signal a mineralocorticoid excess in situations when aldosterone levels remain within the normal limits and it is a specific marker of the mineralocorticoid pathway [5,30]. It has been described that the ratio between 17-hydroxyprogesterone/11-desoxycorticosterone after ACTH stimulation can be used to detect CAH heterozygotes [31]. The ratio between cortisol and cortisone is a relatively well established indicator of the activities of the 11β-hydroxysteroid hydroxylases types I and II (Fig. 1) which is important in the diagnosis of apparent mineralocorticoid excess syndrome [32].

Another important application of simultaneous quantitation of several steroids is the diagnosis of adrenal incidentalomas. Measurement of multiple steroid precursors before and after ACTH stimulation showed significantly increased levels of 17-hydroxyprogesterone, 11-deoxycortisol, and 11-deoxycorticosterone in certain conditions [33,34] and corticosterone in others [35]. Precursors of the glucocorticoid and mineralocorticoid pathways have been pointed not only as biochemical markers to confirm imaging procedures, but also as markers of malignancy in adrenal incidentalomas [35].

In summary, we present a sensitive and specific method for simultaneous quantitation of seven related endogenous C-21 adrenal steroids. The use of on-line extraction coupled to LC–MS/MS allowed automated analyte extraction and simultaneous measurement of endogenous concentrations of cortisol, cortisone, 11-desoxycortisol, 21-desoxycortisol, corticosterone, 17-hydroxyprogesterone and 11-desoxycorticosterone in less than 15 min. The limit of quantitation and linear range achieved makes it possible to analyze and quantify all seven adrenal steroids in both healthy and diseased subjects. This strategy is a simple alternative to the labour intensive RIA and GC/MS methods and it has been used to explore the clinical value of steroids profiling.

Acknowledgments

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