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Serum steroid profiling by isotopic dilution-liquid chromatography–mass spectrometry: Comparison with current immunoassays and reference intervals in healthy adults

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ABSTRACT

Background: The simultaneous, rapid and reliable measurement of a wide steroid panel is a powerful tool to unravel physiological and pathological hormone status. Clinical laboratories are currently dominated by high-throughput immunoassays, but these methods lack specificity due to cross-reactivity and matrix interferences. We developed and validated an isotopic dilution-liquid chromatography–tandem mass spectrometry (ID-LC–MS/MS) method for the simultaneous measurement of cortisol, corticosterone, 11deoxycortisol, androstenedione, deoxycorticosterone (DOC), testosterone, 17OHprogesterone, dehydroepiandrosterone (DHEA) and progesterone in serum, and compared it to routine immunoassays employed in our laboratory. We also established adult reference intervals in 416 healthy subjects.
Methods: 0.9 ml of serum were spiked with labelled internal standards (IS) and extracted on C18 cartridges. Eluate was injected into a two-dimensional LC-system, purified in a perfusion column and separated on a C8 column during a 21 min gradient run. Analytes were revealed by atmospheric pressure chemical ionization (APCI) followed by multiple reaction monitoring (MRM) analysis.
Results: Of the four immunoassays compared with the ID-LC–MS/MS method, only the results of ElecsysE170 for cortisol, testosterone in males and progesterone > 1 ng/ml were in agreement with ID-LC–MS/MS. ElecsysE170 for testosterone in females and progesterone < 1 ng/ml, Immulite2000 for androstenedione, DSL-9000 for DHEA and 17OHP Bridge for 17OHprogesterone, respectively, showed poor agreement. Reference intervals and steroid age and fertility related fluctuations were established.
Conclusion: Our ID-LC–MS/MS method proved to be reliable and sensitive in revealing steroid circulating concentrations in adults and in highlighting the limits of routine immunoassays at low concentrations.

1. Introduction

Steroid measurement remains a challenge for the endocrinological community. Since the RIA breakthrough 40 years ago [1,2], immunoassays have remained the most common tool to characterize the pathophysiological states of circulating steroids. RIA combined with extraction and chromatographic purification steps have been attributed acceptable specificity and good sensitivity. Nowadays, most methodologies are dominated by automated chemiluminescent or electro-chemiluminescent immunoassay platforms and by semi-automated RIAs. These assays offer simplicity and high throughput, key-factors for a large routine application, but due to cross-reactivity and matrix interferences they lack specificity [3]. Poor validation and standardization data are often provided for these methods, causing a huge variability among dif-

Abbreviations: ID-LC–MS/MS, isotopic dilution-liquid chromatography–tandem mass spectrometry; DOC, deoxycorticosterone; DHEA, dehydroepiandrosterone; IS, internal standard; APCI, atmospheric pressure chemical ionization; MRM, multiple reaction monitoring; GC–MS, gas chromatography–mass spectrometry; DHEA-S, DHEA-sulphate; BSA, bovine serum albumin; HPLC, high pressure liquid chromatography; SPE, solid phase extraction; QC, quality control; BMI, body mass index; CAD, collision activated dissociation; CUR, curtain gas; LLOQ, lower limit of quantification; S/N, signal to noise ratio; LOD, limit of detection; IR, ion ratio; IQR, interquartile range; CI, confidence interval; MW, molecular weight; RT, retention time; DP, declustering potential; CE, collision energy; CXP, cell exit potential; $S_{y/x}$, standard deviation of residuals; M, males; F, females; pre-M, pre-menopausal females; post-M, post-menopausal females; s.d., standard deviation.

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ferent kits and among different laboratories employing the same kits and leading to significant variations in reference intervals from one commercial assay to another [4,5]. These drawbacks limit diagnostic accuracy, appropriate treatment and follow-up in clinical praxis, and preclude epidemiological multicenter studies [6–9].

Gas chromatography–mass spectrometry (GC–MS) was first introduced in the 1960s and improved in the 1980s to represent the gold standard method for steroid analysis [10–12]. However, this technique requires complex time-consuming derivatization procedures for sample pre-treatment, limiting its application in routine clinical practice. Conversely, ID-LC–MS/MS is an innovative technology combining the high selectivity and sensitivity of mass spectrometry with the versatility of liquid chromatography. ID-LC–MS/MS allows reliable, simultaneous quantification of a wide panel of steroids in a broad concentration range with high throughput capabilities [13]. These advantages have yielded insights into biochemical changes and more useful clinical data, but a re-definition of age and sex specific reference intervals is urgently needed. Many ID-LC–MS/MS methods for steroid measurement have been proposed in recent years [14–22], often performing multi-hormone analysis in a short chromatographic run and requiring less-demanding sample preparation. However, a good pre-analytical treatment and a careful chromatographic separation of frequently occurring isobaric analytes are still needed for sensitive and specific steroid monitoring in complex matrices like serum or urine [23].

This study describes the development and validation of an ID-LC–MS/MS method for the simultaneous measurement of nine serum steroids of clinical relevance, comparing it with six routinely used immunoassays. In addition, we evaluated the steroid profile of 416 healthy normal weight drug-free subjects, aged 18–89 years, analyzing the influence of age and fertility status on steroid levels.

2. Materials and methods

2.1. Chemicals

The following compounds were used: cortisol, corticosterone, 11deoxycortisol, androstenedione, DOC, testosterone, 17OHprogesterone, DHEA, progesterone, 21deoxycortisol, epitestosterone, DHEA-sulphate (DHEA-S) and cortisone (Steraloids, Newport, RI); d4-cortisol, d8-corticosterone, d2-11deoxycortisol, d5-testosterone, d8-17OHprogesterone, d2-DHEA and d9-progesterone (CDN Isotopes, Pointe Claire, Canada); ^{13}C2-testosterone (Cambridge Isotope Laboratories, Andover, MA); bovine serum albumin (BSA), prednisone and prednisolone (Sigma–Aldrich, St. Louis, MO); betamethasone disodium phosphate (Defiante Farmaceutica, Madeira, Portugal); dexamethasone 21phosphate disodium salt (Visufarma, Rome, Italy); methylprednisolone acetate (Pfizer, New York City, NY); triamcinolone acetonide (Bristol-Myers Squibb, New York City, NY). Gradient grade methanol and zinc sulphate hepta-hydrated were from Merck (Darmstadt, Germany); ultrapure water was produced by MilliQ Gradient A10 system (Millipore, Volketswil, Switzerland). Steroid-free serum was from MP Biomedicals (Solon, OH). The solid phase extraction (SPE) cartridges were IST Isolute C18 100 mg, 1 cm^3 from Biotage (Uppsala, Sweden). Reference material was from the Reference Institute for Bioanalytics (Bonn, Germany).

2.2. Standard solutions, calibrators and in-house quality control (QC) samples

Stock solutions were prepared in methanol for each standard and isotopically labelled IS at different concentrations in the mg/ml range. Working solutions were at 100 ug/ml for cortisol, progesterone and ISs, and at 10 ug/ml for other analytes. A stock calibrator was prepared by mixing each standard to obtain the following concentrations: cortisol, 500 ng/ml; DHEA and progesterone, 50 ng/ml; corticosterone, androstenedione and testosterone, 20 ng/ml; 11deoxycortisol, DOC and 17OHprogesterone, 10 ng/ml. An eight-point calibration curve was prepared by serial dilution of the stock calibrator in 4% BSA. The BSA solution represented the "zero" calibration point. The working IS solution was a mixture of d4-cortisol 50 ng/ml, d8-corticosterone and d2-11deoxycortisol 5 ng/ml, d2-DHEA 3 ng/ml, ^{13}C2-testosterone 2 ng/ml and d8-17OHprogesterone 1 ng/ml, d9-progesterone 10 ng/ml. Stock solutions, working solutions and calibrators were stored at −20 °C. Three in-house QCs were prepared by generating a serum pool used as the low level and by adding standard solutions to obtain the medium and high levels for each analyte. Calibrators for androstenedione determination at 0.5 and 5 ng/ml of Immulite2000 (Siemens Healthcare Diagnostics, Deerfield, IL), at 0.98, 2.5, 9.8 and 25.0 ng/ml of DSL-9000 RIA kit (Webster, TX) and at 0.1, 0.35, 2.0 and 10.0 ng/ml of 17OHP Bridge RIA kit (Adaltis, Guidonia, Italy) were measured by ID-LC–MS/MS as such for calibration assessment.

2.3. Specimens

De-identified samples for method comparison were collected among sera from S.Orsola-Malpighi Hospital routine laboratory. Healthy volunteers, males and females aged 19–89 years, were recruited for reference interval estimation, after having given their informed consent, at the local health service of the town of Massa Lombarda. The study was approved by the local Ethical Committee. Body mass index (BMI) ranged between 18.1 and 25.0 kg/m^2. The inclusion criteria were: body weight stability in the last 3 months, complete sexual development and menstrual cycle regularity in fertile women. Subjects taking drugs (except for antipyretic or anti-inflammatory compounds), or presenting endocrine, hepatic, renal, tumoral, autoimmune, cardiovascular, hematologic, neurologic or psychiatric diseases, sleep disorders, or allergies requiring treatment were excluded. Between 8 and 10 a.m., subjects were infused with saline for 10 min before blood collection in a Vacuette Z serum beads clot activator (Greiner Bio-One, Kremsmunster, Austria); samples were centrifuged at 2000 × *g* for 10 min at room temperature and sera were stored in 1.5 ml polypropylene tubes at −20 °C until analysis.

2.4. Sample preparation

Serum samples and in-house QCs were thawed and vortexed. Liophilic reference samples were reconstituted with 3 ml H_2O and gently mixed at room temperature for 60 min. For each sample, curve calibrator, immunoassay calibrators, and QCs 900 ul were transferred to 12 mm × 75 mm glass tubes before addition of 1 ml of MeOH:[$(ZnSO_4)(H_2O)_7$ aqueous solution 8.9%,w/v] = 80:20 containing IS. After 3 min vortex, tubes were centrifuged for 10 min at 2000 × *g* at room temperature. Supernatants were transferred on the SPE cartridge previously activated with 1 ml of MeOH and conditioned with 1 ml of H_2O. After washing with 3 ml of H_2O cartridges were eluted with 1 ml of MeOH. Eluates were dried under nitrogen stream, reconstituted with 0.3 ml of 50% MeOH and transferred into glass vials and placed into a Series 200 Autosampler thermostated at 10 °C (PerkinElmer, Waltham, MA).

2.5. On-line purification and LC separation

Two hundred microliters were injected into a two-dimensional LC-system consisting in a modular HPLC Series 200 by PerkinElmer, further purified on perfusion column POROS

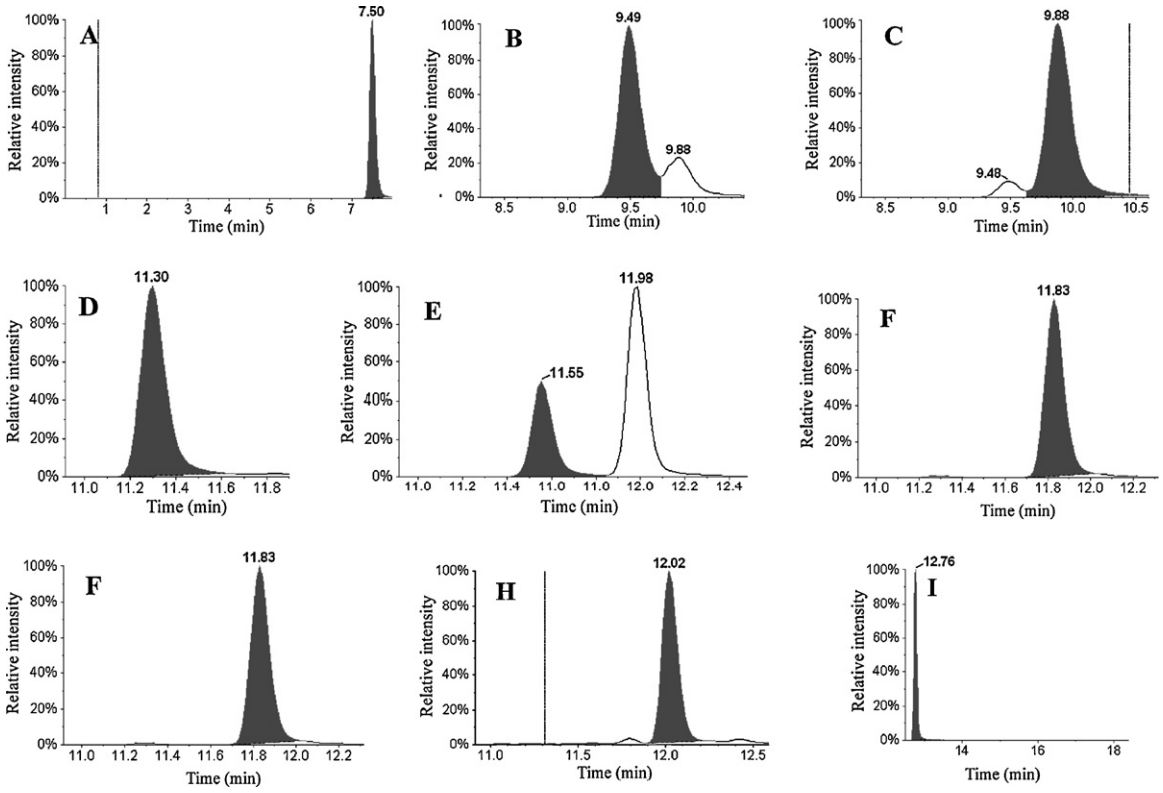

Fig. 1. Chromatographic peak with its retention time for cortisol (A), corticosterone (B), 11deoxycortisol (C), androstenedione (D), DOC (E), testosterone (F), 17OHprogesterone (G), DHEA (H) and progesterone (I).

R1/20 2.1 mm × 30 mm by Applied Biosystems (Foster City, CA). After washing with 10% MeOH at 3 ml/min for 1 min, the sample was back-flushed to the analytical column Luna RP-C8 100 mm × 4.6 mm, 5 um (Phenomenex, Torrance, CA) equipped with an RP-C8 4 mm × 2 mm, 5 um guard column, through a ten-port switching valve (VICI, Houston, TX) at 0.750 ml/min of an eluent made with 98% solvent A (20% MeOH) and 2% solvent B (100% MeOH). The 21 min gradient run program started with 45% solvent B and at min 8.5 a linear gradient to 100% in 2 min was activated with a subsequent 2 min washing step and a 6.5 min re-equilibration step to the initial conditions. Chromatographic peaks are shown in Fig. 1. Chromatographic resolution was achieved for the isobaric steroid pairs (corticosterone and 11deoxycortisol, DOC and 17OHprogesterone, testosterone and DHEA) and for the steroids, like testosterone and androstenedione, which differ in their molecular weight by only 2 amu and which could interfere through the specific +2 amu-isotopomer of one analyte over the mono-isotopic form of the other.

2.6. Mass spectrometry detection

Mass spectrometric measurements were performed by an API 4000-QTrap (AB-Sciex, Toronto, Canada) working in triple-quadrupole mode. Quantification was performed by the MRM mode, choosing for each analyte two specific transitions, one for the quantitative assessment (the "quantifier") and one for confirmation (the "qualifier"). The parameters pertaining to the MRM transitions were optimized by infusing standard solutions at concentrations ranging from 100 ng/ml to 10 ug/ml, into the Turbo-V source through an infusion pump set at 10 ul/min in addition to a makeup flow of 50% MeOH at 400 ul/min (Table 1). The APCI probe operated with a Corona discharge current of 3 uA in positive ion mode. Collision activated dissociation (CAD) gas was nitrogen set at a pressure of 10 mTorr and the other parameter settings were: probe temperature 400 °C, curtain gas (CUR) 30 psi, nebulising gas 30 psi. To maximize the dwell time for each targeted analyte, LC-run monitoring was divided into four periods: 0.0–8.3 min; 8.4–10.9 min; 11.0–12.5 min; 12.6–21.0 min. Unit mass resolution was set at both Q1 and Q3.

2.7. Quantitation

Data processing and quantitation were performed by Analyst 1.4.2 software package by AB-Sciex. Calibration was done through linear regression: concentrations for each analyte were back calculated by interpolation on the respective regression curve.

2.8. ID-LC–MS/MS method validation

Four per cent BSA was chosen as a suitable matrix for standard calibration points. The isotopic dilution quantitation method was assessed as follows: d4-cortisol was used as IS for cortisol, d8-corticosterone for corticosterone; d2-11deoxycortisol for 11deoxycortisol; ^{13}C2-testosterone for androstenedione, testosterone and DHEA; d8-17OHprogesterone for DOC, 17OHprogesterone and progesterone. D5-testosterone, d2-DHEA and d9-progesterone were discarded since their unsuitability for the remarkable signal instability with the APCI source (likely more than on the electrospray source), and for the cross-interference on the unlabelled standard steroid transitions; analogous phenomena were also reported by Vogeser and co-workers in a recent publication [24]. A $1/x$ weighting regression was chosen to ensure higher accuracy and precision at the low concentration end of the curve. Linearity was achieved for three-four orders of magnitude. The lower limit of quantification (LLOQ) was determined on five replicates as the lowest concentration exhibiting a signal to noise ratio (S/N) above ten, with an accuracy between 80 and 120% of the true value and with CV below 20%. The limit of detection (LOD) was determined as the lowest

Table 1
Experimental conditions for the ID-LC–APCI-MS/MS detection for each steroid and IS: molecular weight (MW), measurement period, retention time (RT, min), precursor ion (Q1 mass, *m*/*z*), fragment ion (Q3 mass, *m*/*z*), declustering potential (DP, V), collision energy (CE, eV), cell exit potential (CXP, V), and observed IR are reported for each targeted compound.

Analyte	MW	Period	RT	Transition	Q1 mass	Q3 mass	DP	CE	CXP	IR
Cortisol	362.46	1	7.4	Quantifier	363.2	121.2	60	45	3	4.3
				Qualifier	363.2	267.4	60	35	5	
D4-cortisol	366.46	1	7.4	IS	367.3	97.1	50	45	3	
Corticosterone	346.46	2	9.5	Quantifier	347.1	121.0	76	45	9	2.1
				Qualifier	347.1	97.1	76	45	5	
D8-corticosterone	354.46	2	9.4	IS	355.4	125.4	88	45	5	
11Deoxycortisol	346.46	2	9.9	Quantifier	347.2	109.1	82	45	5	1.1
				Qualifier	347.2	97.0	82	45	5	
D2-11deoxycortisol	348.46	2	9.9	IS	349.4	97.1	75	45	3	
Androstenedione	286.41	3	11.3	Quantifier	287.4	97.0	78	30	3	1.4
				Qualifier	287.4	109.0	78	40	5	
DOC	330.5	3	11.6	Quantifier	331.4	109.1	80	40	4	1.1
				Qualifier	331.4	97.0	80	30	4	
Testosterone	288.42	3	11.8	Quantifier	289.2	97.1	78	35	3	1.0
				Qualifier	289.2	109.1	78	35	5	
^{13}C2-testosterone	290.41	3	11.8	IS	291.4	111.1	74	35	5	
17OHProgesterone	330.46	3	12.0	Quantifier	331.1	97.0	70	40	3	0.9
				Qualifier	331.1	109.3	70	45	5	
D8-17OHProgesterone	338.46	3	11.9	IS	339.5	100.1	50	45	7	
DHEA	288.42	3	12.1	Quantifier	271.3	197.2	55	25	3	3.0
				Qualifier	271.3	213.3	55	25	4	
Progesterone	314.46	4	12.8	Quantifier	315.6	97.1	80	30	3	1.1
				Qualifier	315.6	109.1	80	40	4	

concentration exhibiting a S/N above three. Data are summarized in Table 2.

Interference with other endogenous and exogenous steroids was investigated. Amounts of 21deoxycortisol, epitestosterone, DHEA-S, cortisone, prednisone and prednisolone were injected into the LC–MS/MS system: traces shown in Supplemental Fig. 1 highlight the good immunity of the monitored steroids from the massive presence of the others. Triamcinolone acetonide, methylprednisolone, dexamethasone and betamethasone, were spiked at 500 ng/ml into the QC samples and processed as unknown samples: no interference over calculated concentrations of monitored steroids was found. No interference by serum matrix non-steroid compounds was shown by injecting steroid-free serum samples. Intensity ratios between quantifier and qualifier transitions (ion ratio, IR) were monitored in each sample to check for any unexpected interference. Sample IR was accepted within 20% of the calibrator IR.

Method imprecision was assessed on six replicates per day of in-house QC samples at low, medium and high concentrations (intra-assay), and on six different days (inter-assay). CV was accepted below 15% in the intra-assay and below 20% in inter-assay.

Trueness, expressed as the percentage of found concentration over nominal concentration, was assessed in the low, medium and high range by reference certified sera for cortisol, testosterone, 17OHprogesterone and progesterone, and by in-house validation for corticosterone, 11deoxycortisol, androstenedione, DOC and DHEA, by spiking gravimetrically determined quantities of pure standards in steroid-free serum, previously checked for absence of steroids above the LOD. Data are summarized in Table 3.

Sensitivity was also evaluated in serum matrix, as reported for LLOQ, by spiking minimal amounts of analyte standards in steroid-free serum in three replicates (Table 2).

Ion suppression was investigated by spiking equal amounts of standard analytes either on pre-extracted BSA solutions or steroid-free serum, to exclude any procedural losses from the yield calculation. Analyte peak areas were compared in BSA and in steroid-free serum, and both were compared to peak areas of pure standards (Supplemental Table 1). Negligible deviation from 100%, denoting absence of suppression, was observed. A post-column infusion of a mixture containing the nine steroids at concentrations suitable for generating measurable steady-state signals was performed during injections of blank and steroid-free serum

Table 2
ID-LC–MS/MS method calibration curve and sensitivity ($S_{y/x}$: standard deviation of residuals).

	Linear range (ng/ml)	Slope	Intercept	$S_{y/x}$	r^2	LLOQ				LOD (pg on column)	Sensitivity in serum matrix (ng/ml)
						ng/ml	S/N	CV %	Accuracy %		
Cortisol	0.244–500.0	0.0551 ± 0.0022	0.0050 ± 0.0022	1.673	0.9997	0.2440	49	9.0	99.3	4.8	0.244
Corticosterone	0.039–20.0	0.2652 ± 0.0213	−0.0003 ± 0.0019	0.100	0.9995	0.0391	13	3.4	95.9	4.0	0.313
11Deoxycortisol	0.019–10.0	0.1748 ± 0.0106	0.0025 ± 0.0014	0.075	0.9993	0.0195	14	15.3	94.1	2.0	0.078
Androstenedione	0.019–20.0	0.4577 ± 0.0139	0.0032 ± 0.0013	0.047	0.9998	0.0195	14	13.2	100.7	2.3	0.039
DOC	0.019–10.0	0.8857 ± 0.0451	−0.0006 ± 0.0018	0.032	0.9994	0.0195	11	9.6	107.8	2.5	0.078
Testosterone	0.019–20.0	0.4922 ± 0.0145	0.0158 ± 0.0027	0.110	0.9993	0.0195	11	6.2	94.2	2.8	0.019
17OHProgesterone	0.010–10.0	0.9012 ± 0.0649	0.0011 ± 0.0023	0.033	0.9996	0.0098	11	8.9	107.5	1.7	0.078
DHEA	0.195–50.0	0.0164 ± 0.0036	0.0002 ± 0.0001	0.348	0.9995	0.1953	10	11.2	97.4	29.2	0.781
Progesterone	0.024–50.0	0.4527 ± 0.0273	0.0035 ± 0.0024	0.117	0.9999	0.0244	29	9.3	103.4	1.2	0.049

Table 3
ID-LC–MS/MS method trueness and imprecision at low, medium and high range.

	Low range					Medium range				High range				
	ng/ml	Trueness %	ng/ml	Intra-assay CV %	Inter-assay CV %	ng/ml	Trueness %	ng/ml	Intra-assay CV %	Inter-assay CV %	ng/ml	Trueness %	ng/ml	Intra-assay CV %	Inter-assay CV %
Cortisol	63.7	94.9	25.4	3	8	129.0	103.7	144.8	3	7	446.2	93.6	415.1	2	5
Corticosterone	1.00	92.5	1.12	2	10	5.00	98.3	5.41	6	5	15.00	98.5	16.25	3	7
11Deoxycortisol	0.400	106.2	0.260	8	8	2.000	99.1	0.782	4	5	8.000	102.3	4.407	3	2
Androstenedione	0.400	86.3	0.291	10	11	5.000	97.9	4.239	10	10	15.000	100.6	17.810	7	11
DOC	0.080	100.7	0.114	5	6	1.000	100.0	0.702	6	9	8.000	104.1	7.783	6	9
Testosterone	0.300	99.9	0.299	4	7	2.300	97.4	2.218	4	7	8.800	99.6	13.126	3	4
17OHProgesterone	0.529	104.4	0.696	5	9	2.430	102.1	4.562	4	5	7.534	101.4	9.810	4	5
DHEA	1.00	94.6	2.05	8	8	15.00	101.8	14.00	7	9	40.00	98.2	43.64	8	10
Progesterone	0.842	83.7	0.129	8	11	4.214	92.4	5.369	5	6	29.340	92.0	23.123	5	6

extracts. Negligible increase or decrease in MRM scan intensities were observed, denoting a negligible ion suppression effect.

2.9. Immunoassays

Cortisol, testosterone and progesterone were measured by electro-chemiluminescence immunoassay on the Modular Analytics ElecsysE170 by Roche Diagnostics (Mannheim, Germany); androstenedione by the solid-phase, competitive chemiluminescent enzyme immunoassay Immulite2000; DHEA by DSL-9000 RIA and 17OHprogesterone by 17OHP Bridge RIA.

2.10. Data analysis and statistics

2.10.1. Method comparison

ID-LC–MS/MS method was compared with the immunoassay measurements for cortisol ($n = 159$), testosterone ($n = 162$) progesterone ($n = 85$), androstenedione ($n = 137$), DHEA ($n = 143$) and 17OHprogesterone ($n = 99$). To avoid bias attributable to different sensitivity limits, results below the sensitivity limit were excluded. Calibration agreement between Immulite2000, DSL-9000 and 17OHP Bridge and ID-LC–MS/MS was assessed by measuring kit calibrators by ID-LC–MS/MS. No comparison was performed for corticosterone, 11deoxycortisol and DOC since no routine methods were available in the reference laboratory.

Non-normally distributed variables were compared by the Mann–Whitney test and all data are expressed as median and interquartile range (IQR). The Deming regression was applied to account for the imprecision of both methods [25]. Bland and Altman plots were drawn for agreement estimation, representing the percentage difference between the methods against the mean [26].

2.10.2. Estimation of reference intervals

Median values and non-parametric 2.5th and 97.5th centiles [27] were estimated in males ($n = 217$) and females ($n = 199$). The female group was subdivided according to fertility status into premenopausal ($n = 134$ of whom 51 women in the follicular phase [days 1–10]), and post-menopausal ($n = 65$) subgroups. Reference intervals in luteal phase were not evaluated because of the small number of samples available. The effect of age on steroid level was evaluated by the Spearman regression analysis.

Data analysis was performed on MedCalc v9.3.7.0 (Mariakerke, Belgium).

3. Results

3.1. Method comparison study

By the Mann–Whitney comparison, ElecsysE170 and ID-LC–MS/MS methods provided non-different results for testosterone in males and for progesterone above 1 ng/ml. Lower median values were obtained by ID-LC–MS/MS compared with ElecsysE170 for determination of cortisol (−16%, $p = 0.0052$), testosterone in females (−26%, $p = 0.0080$) and progesterone below 1 ng/ml (−84%, $p < 0.0001$). ID-LC–MS/MS, compared to Immulite2000, DSL-9000 and 17OHP Bridge for measurement of androstenedione, DHEA and 17OHprogesterone, respectively, provided significantly lower results ($p < 0.0001$), immunoassay medians being 2–3-fold higher than ID-LC–MS/MS medians (Supplemental Table 2).

Deming regression graphs, slope and intercept coefficients with respective standard errors and correlation coefficient between immunoassays and ID-LC–MS/MS are reported in Fig. 2. In males, the slope and the intercept obtained from the regression between testosterone results by ElecsysE170 and ID-LC–MS/MS were not different from one and zero, parameters of the curve of best

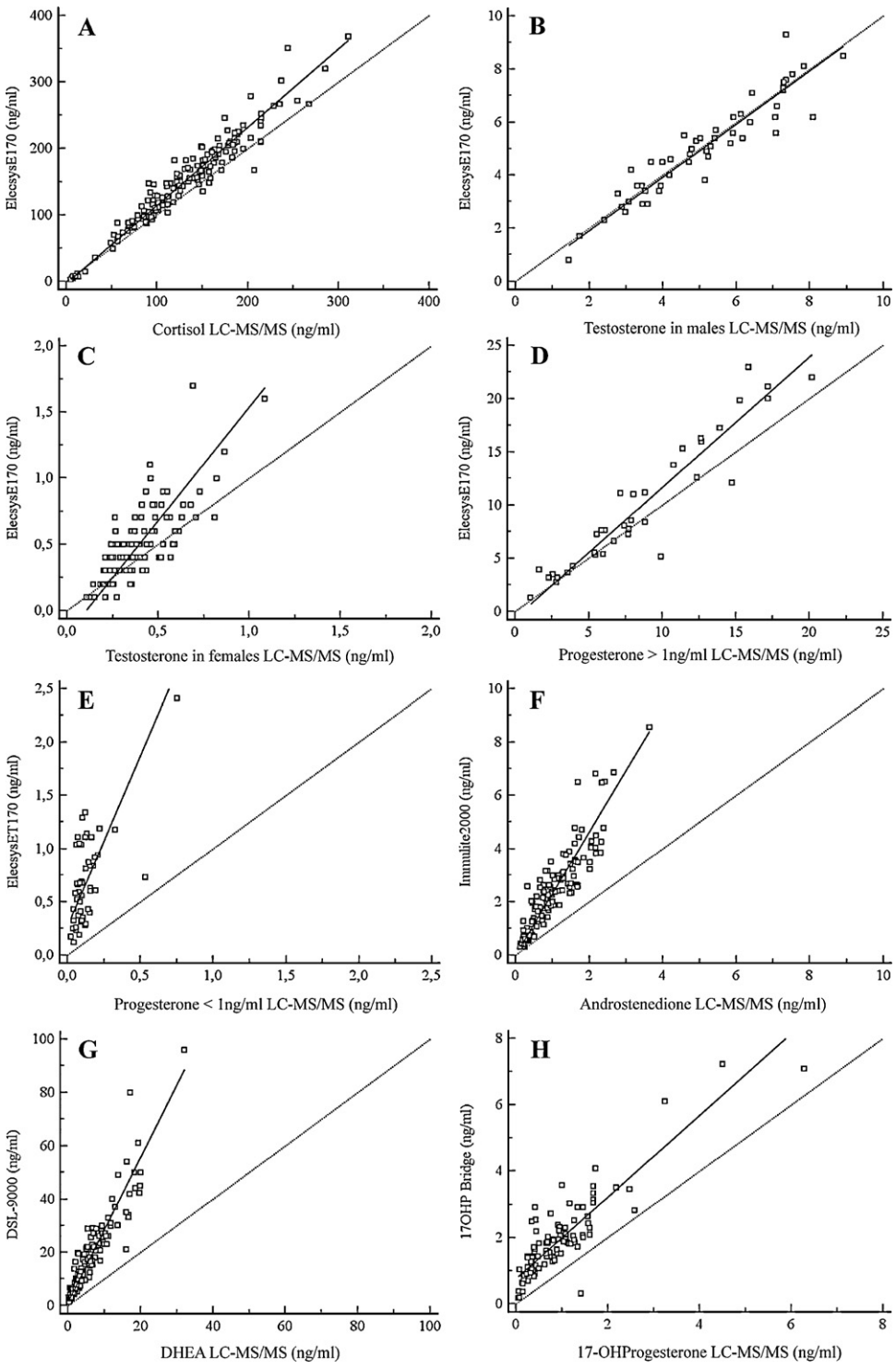

Fig. 2. Deming regression line (black) and line of best fit (dotted) for cortisol (A: $m^* = 1.176 \pm 0.029$; $q^{\#} = -3.093 \pm 3.147$; $r = 0.968$), testosterone in males (B: $m^* = 1.004 \pm 0.062$; $q^{\#} = -0.097 \pm 0.283$; $r = 0.938$), testosterone in females (C: $m^* = 1.724 \pm 0.208$; $q^{\#} = -0.181 \pm 0.073$; $r = 0.773$), progesterone > 1 ng/ml (D: $m^* = 1.227 \pm 0.074$; $q^{\#} = -0.587 \pm 0.573$; $r = 0.946$), progesterone < 1 ng/ml (E: $m^* = 3.201 \pm 1.466$; $q^{\#} = 0.270 \pm 0.166$; $r = 0.637$), androstenedione (F: $m^* = 2.299 \pm 0.117$; $q^{\#} = 0.042 \pm 0.093$; $r = 0.906$), DHEA (G: $m^* = 2.708 \pm 0.182$; $q^{\#} = 1.478 \pm 0.800$; $r = 0.929$) and 17OHprogesteorne (H: $m^* = 1.230 \pm 0.181$; $q^{\#} = 0.769 \pm 0.152$; $r = 0.874$); m^* = slope; $q^{\#}$ = intercept.

fit, respectively. A slight but statistically elevated slope and an intercept not different from zero were obtained for cortisol and progesterone above 1 ng/ml [95% confidence interval (CI): 1.119–1.233 and 1.077–1.378, respectively].

Testosterone in females and progesterone below 1 ng/ml exhibited elevated slope coefficients (95%CI: 1.311–2.137 and 0.253–6.149, respectively) and testosterone in females had a negative intercept coefficient (95%CI: −0.326 to −0.036). The regression between ID-LC–MS/MS and Immulite2000, DSL-9000 and 17OHP Bridge, for the determination of androstenedione, DHEA and 17OHprogesterone provided significantly elevated slopes (95%CI: 2.068–2.530; 2.348–3.068; 0.870–1.589, respectively), but only 17OHprogesterone regression provided a non-negligible positive intercept coefficient (95%CI: 0.468–1.071).

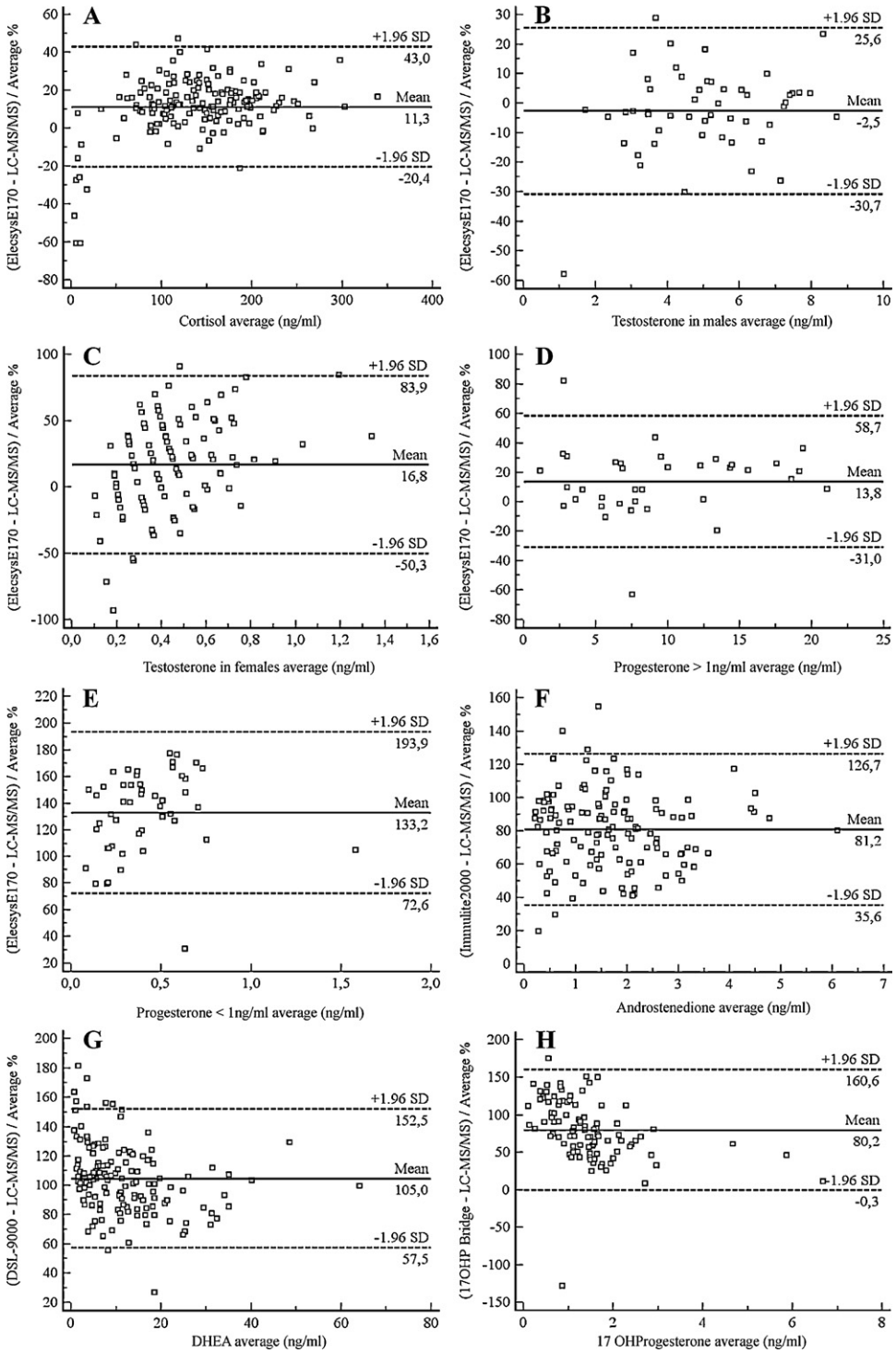

Fig. 3. Bland and Altman plot of cortisol (A), testosterone in males (B), testosterone in females (C), progesterone > 1 ng/ml (D), progesterone < 1 ng/ml (E), androstenedione (F), DHEA (G), 17OHprogesterone (H); *y*-axis: percentage difference between results by immunoassay and LC–MS/MS on average; *x*-axis: average results of the two methods.

By the Bland and Altman agreement estimation we found that among the four immunoassays compared to ID-LC–MS/MS method, only ElecsysE170 method for all analytes, except progesterone below 1 ng/ml, provided an acceptable mean difference. However, only for testosterone in males the mean difference was not different from 0% (95%CI: −6.6% to 1.5%). For all other analytes worse agreement was obtained, with mean differences ranging from 80.2% of 17OHprogesterone to 133.2% of progesterone below 1 ng/ml. Furthermore, despite the notable width, agreement intervals for androstenedione, DHEA, 17OHprogesterone and progesterone below 1 ng/ml were entirely located on the positive side of the graph, with only one case in which measurement of 17OHprogesterone was higher for immunoassay compared to ID-LC–MS/MS (Fig. 3). These data indicated a good accuracy and calibration of ElecsysE170 for the determination of cortisol, testosterone and progesterone, analytes for which trueness of the ID-LC–MS/MS method was confirmed against GC–MS certified sera. Nevertheless, Deming regression, correlation of coefficients and Bland and Altman analysis showed that ElecsysE170 is affected by cross-reactivity at low ranges of testosterone and progesterone.

Table 4
Steroid median concentrations and 2.5–97.5th percentiles (ng/ml) in adult males (M) and pre-menopausal (pre-M), follicular and post-menopausal (post-M) females (F) (s.d.: standard deviation). Blood samples were taken from 8:00 to 10:00 a.m.

	M	F pre-M	F follicular	F post-M
Age	18–89	18–54	18–54	45–86
BMI (mean ± s.d.)	23.2 ± 1.7	21.7 ± 2.0	21.8 ± 2.0	23.2 ± 1.7
N	217	134	51	65
Hormone	Median (2.5–97.5P)	Median (2.5–9 7.5P)	Median (2.5–97.5P)	Median (2.5–97.5P)
Cortisol	119.9 (45.7–199.4)	101.5 (47.4–199.7)	113.1 (40.5–199.8)	114.7 (56.9–180.4)
Corticosterone	3.23 (0.46–12.60)	2.62 (0.62–11.85)	2.89 (0.45–11.94)	2.74 (0.68–8.54)
11Deoxycortisol	0.325 (0.086–1.094)	0.239 (<1.081)	0.249 (<1.345)	0.284 (0.082–0.838)
Androstenedione	0.571 (0.262–1.263)	0.748 (0.277–1.638)	0.727 (0.308–1.602)	0.299 (0.095–0.773)
Testosterone	5.34 (2.82–8.18)	0.248 (0.104–0.454)	0.248 (0.116–0.431)	0.147 (0.077–0.392)
DHEA	4.97 (1.40–14.28)	5.09 (1.19–18.93)	5.68 (2.03–27.04)	2.45 (0.80–6.60)
17OHProgesterone	1.095 (0.415–2.542)	0.578 (0.152–2.266)	0.411 (0.161–0.947)	0.209 (<0.527)
Progesterone	0.078 (<0.189)	0.203 (<17.816)	0.093 (<1.673)	<0.049 (<0.080)

The results given by the measurement of 17OHP Bridge calibrators by ID-LC–MS/MS assessed the good calibration of this kit, trueness of the ID-LC–MS/MS method being confirmed against GC–MS certified sera: accuracy at points 0.1, 0.35, 2.0 and 10.0 ng/ml was 92.8%, 101.3%, 96.7% and 101.6%, respectively. The data above, together with the poor correlation coefficient (Fig. 2), the discrepancies observed in the Mann–Whitney comparison (Supplemental Table 2) and the results obtained by the Bland and Altman analysis (Figs. 2 and 3), clearly explain that the overestimation by 17OHP Bridge RIA is due to the cross-reactivity and not to the miscalibration. By measuring DHEA in DSL-9000 calibrators by ID-LC–MS/MS, we found an accuracy of 53.9%, 48.5%, 46.2% and 55.5% at points 0.98, 2.5, 9.8 and 25 ng/ml, respectively. Such a miscalibration can only in part explain the huge overestimation shown by the Mann–Whitney comparison and by the regression analysis. The wide agreement range (57.5–152.5%) obtained in the Bland and Altman plots suggested the presence of a non-proportional bias, probably due to the cross-reactivity of the antibody employed in the assay. The accuracy obtained by the measurement of androstenedione in Immulite2000 calibrators was 133.1% and 82.5% at 0.5 and at 5.0 ng/ml, respectively. Similarly to DSL-9000, this miscalibration may explain only part of the overestimation exhibited by Immulite2000 (Fig. 1, Supplemental Table 2), but both the 2.5-fold increase observed in the Mann–Whitney comparison and the wide range of agreement observed in the Bland and Altman analysis (35.6–126.7%) suggest a further contribute due to cross-reactivity (Fig. 3).

3.2. Reference intervals

Reference intervals and median values are listed in Table 4. The reference interval for DOC was not set because, despite a LLOQ of 19.5 pg/ml, the sensitivity in serum matrix (78 pg/ml) proved unsatisfactory for the measurement of this hormone. A single measurement of corticosterone, testosterone and 17OHprogesterone, two of DHEA, four of 11deoxycortisol and 28 of progesterone were discarded because of the questionable IRs.

In females, a single measurement of corticosterone, androstenedione and DHEA, 13 of 11deoxycortisol (8% of pre-menopausal and 3% of post-menopausal), three of 17OHprogesterone and 65 of progesterone, 46 of them in the post-menopausal subgroup (71%), whereas in males four measurements of 11deoxycortisol, and 39 of progesterone were below the sensitivity limit.

In males, androstenedione, testosterone, DHEA, 17OHprogesterone and progesterone exhibited a significant negative correlation with age (Supplemental Fig. 2) [rho = −0.236 ($p = 0.0005$); −0.224 ($p = 0.0010$); −0.576 ($p < 0.0001$); −0.185 ($p = 0.0066$) and −0.268 ($p = 0.0002$), respectively]. In the pre-menopausal subgroup an age-dependent decrease of androstenedione, testosterone and DHEA was also observed (Supplemental Fig. 3) [rho = −0.334 ($p = 0.0001$); −0.232 ($p = 0.0076$) and −0.466 ($p < 0.0001$), respectively] and it continued for DHEA, but not for androstenedione and testosterone, in the post-menopausal subgroup (rho = −0.282, $p = 0.024$), whereas an age-dependent increase was observed for cortisol and 11deoxycortisol: rho = 0.369 ($p = 0.0031$) and 0.401 ($p = 0.0015$), respectively. In the post-menopausal subgroup, levels of androstenedione, testosterone and DHEA were significantly lower than pre-menopausal levels, and levels of 17OHprogesterone and progesterone lower than follicular phase levels ($p < 0.0001$ for all). No significant changes were observed for cortisol and 11deoxycortisol.

4. Discussion

We developed a sensitive ID-LC–MS/MS method for the simultaneous measurement of nine serum steroids. This is a powerful tool in clinical praxis to depict various pathophysiological alterations in steroid secretion in a single run. By using a sample volume higher than those reported in other published multi-analyte ID-LC–MS/MS methods (15–20; 22), we were able to provide a general better sensitivity for the 9 hormones, determined not only as LLOQ, but also in a complex matrix, more similar to the real samples, as steroid free serum.

Such high sensitivity is very much needed for the careful definition of the lower reference limits, although DOC detection in the healthy population is still unsatisfying. However, in the routine application of our method, less sample volume may be used if the determination of steroids whose circulating levels are close to method sensitivity, like 11deoxycortisol, corticosterone, DOC, DHEA and progesterone, are not required. By increasing sample volume, noise and interferences may also be of relevance, and a second purification step on the perfusion column after SPE concentration was therefore adopted. The double purification guaranteed ruggedness across the analysis of many samples per run in several runs per week, helping to keep the system stable and clean and minimizing matrix interference. The chromatographic conditions guaranteed an adequate resolving power for isobaric compounds like corticosterone and 11deoxycortisol, DOC and 17OHprogesterone, respectively, and for the isotopic pattern cross-interferences between androstenedione and testosterone. The method also exhibited a good immunity from matrix components, as proved by European certified sera analyses and by the in-house validation.

The comparison of our ID-LC–MS/MS method with routine immunoassays employed in the clinical laboratory revealed a good agreement for ElecsysE170 in determining elevated levels of cortisol, testosterone in males and progesterone above 1 ng/ml. However, the ElecsysE170 performance was not reliable for low

levels of testosterone and progesterone both for lack of specificity and general overestimation, proving severely inadequate in depicting females' health status, especially for testosterone, thereby confirming other reports [5,8,28]. The huge overestimation obtained by Immulite2000 and DSL-9000 in measuring androstenedione and DHEA, respectively, is not only due to miscalibration between ID-LC–MS/MS and these immunoassays, but also to a sub-optimal specificity that may complicate evaluations in clinical borderline situations. In agreement with previous reports [16,29–31], we also demonstrated that the overestimation exhibited by 17OHP Bridge should be attributed to cross-reactivity, and finally to a severe lack of specificity in 17OHprogesterone measurement, being the ID-LC–MS/MS accuracy verified against reference material. Our present study took into account only one immunoassay per analyte; however we cannot exclude that other immunometric platforms or RIAs may perform better and give more consistent results.

Few steroid ID-LC–MS/MS reference intervals on adult populations have been published to date, and the topic remains a matter of debate. We therefore provided preliminary reference intervals for a wide pattern of steroids in an adult male and female population by analyzing 416 healthy drug-free normal-weight subjects. Our data showed a strong age-dependent decrease of androgens both in males and, to a greater extent, in pre-menopausal females. DHEA showed the highest rate of decrease in both sexes including post-menopausal females, whereas testosterone and androstenedione remained stable in post-menopausal women. The post-menopausal group showed an age-dependent increase in cortisol and 11deoxycortisol. In the male group, 17OHprogesterone and progesterone declined with age, but the sensitivity limit of progesterone probably masks an even higher extent of this tendency. In females, reference intervals were examined in the follicular phase, but more subjects distributed through the menstrual cycle are needed for the complete reference intervals of these hormones during physiological fluctuations.

Some discrepancies between our values and literature data could be due to sample collection: our subjects were saline infused for 10 min before blood withdrawal to avoid stress-related alterations in the glucocorticoid cascade. In particular, our cortisol and corticosterone intervals are lower than those reported by others, whereas 11deoxycortisol and 17OHprogesterone are generally higher [14,15,19,21]. Our data on 17OHprogesterone intervals in females cannot be compared with a previous work as no information on fertility was provided [15]. The intervals we obtained for androstenedione are in agreement with those reported by Kushnir in 2010 [22]. Male testosterone values are similar to those previously published [22,32–34], whereas the upper limit found in pre-menopausal females is generally lower than those reported [22,32–34]. Since circulating androgen levels are strongly age-related, comparison with cohorts of different ages is difficult. Multicenter studies involving a larger number of subjects grouped for decades are needed to establish age-related intervals and steroid trends. Not only the sampling procedures, but also ethnicity or statistical calculation of reference limits may account for differences among reports. A further confounding factor is the inclusion of subjects with BMI above 25. We took particular care to exclude overweight or obese subjects to define as normal values that are modified by subtle changes in body weight. However, the main cause of poor agreement among literature reports lies in the miscalibration and generally poor standardization of ID-LC–MS/MS methods, since reference procedures and matrix-based calibrators for most steroids are not available, and in-house validation alone is not sufficient to achieve a consensus [12,35]. To date, few studies have compared data from different ID-LC–MS/MS laboratories, and most focused on testosterone measurement [36,37]. Further comparative studies of steroid measurement are urgently needed to gain advances in biomedical research and enhance clinical care.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.steroids.2010.11.005.

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