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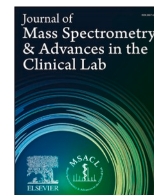

Research Article

A novel fully-automated method to measure steroids in serum by liquid chromatography-tandem mass spectrometry

François Fraissinet [a,*], Tony Pereira [a], Alizée Violin [b], Guillaume Feugray [a,c], Kalyane Bach-Ngohou [b,d], Valéry Brunel [a]

[a] Department of General Biochemistry, Rouen University Hospital, 76000 Rouen, France
[b] Nantes Université, CHU Nantes, Department of Biology, Laboratory of Biochemistry, F-44000 Nantes, France
[c] Normandie University, UNIROUEN, INSERM U1096 Rouen, France
[d] Nantes Université, CHU Nantes, INSERM, The Enteric Nervous System in Gut and Brain Disorders, IMAD, F-44000 Nantes, France

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ABSTRACT

Background: Steroids play a key role in numerous physiological processes. Steroid determination is a useful tool to explore various endocrine diseases. Because of its specificity, mass spectrometry is considered to be a reference method for the determination of steroids in serum compared to radioimmunoassay. This technology could progress towards more automation for the optimal organization of clinical laboratories and ultimately for the benefit of patients.
Methods: A fully automated ultra-high-performance liquid chromatography-tandem mass spectrometry method was developed and fully validated to determine five steroids in serum. Sample preparation was based on protein precipitation with filtration followed by online solid phase extraction. Chromatographic separation was performed using a biphenyl stationary phase.
Results: The method was successfully validated according to European Medicine Agency guidelines. Coefficients of variation did not exceed, respectively, 8.4% and 8.1% for intra- and inter-assay precision. Method comparison with radioimmunoassay showed a proportional bias for all compounds, except for testosterone in men. Comparison with another LC-MS/MS method demonstrated acceptable concordance for all steroids, although a small bias was observed for androstenedione.
Conclusion: The novelty of this method is that it has been fully automated. Automation provides benefits in traceability and allows significant savings in cost and time.

1. Introduction

Steroid hormones are synthetized in the adrenal gland from cholesterol and regulate processes such as growth, development, glucose homeostasis, stress response and maintaining sodium balance. These hormones are produced in three layers of the adrenal cortex: mineralocorticoids in the zona glomerulosa, glucocorticoids in the zona fasciculata and adrenal androgens in the zona reticularis. However, circulating sex steroid hormones are mainly derived from testicular steroidogenesis in men and from ovarian steroidogenesis in women.

The determination of steroids is particularly indicated in a wide array of endocrine diseases, such as Cushing's syndrome, polycystic ovary syndrome and congenital adrenal hyperplasia. Steroid determination in serum requires a great sensitivity for the determination of androgens in women and infants and estrogens in men and infants, because the circulating levels of these hormones are low. Specificity is

Abbreviations: 11DF, 11-deoxycortisol; 17OHP, 17-hydroxyprogesterone; DHEA, dehydroepiandrosterone; D4, delta4-androstenedione; EMA, European Medicine Agency; GC–MS/MS, Gas chromatography tandem mass spectrometry; LC-MS/MS, Liquid chromatography tandem mass spectrometry; LLE, Liquid-liquid extraction; LLOQ, Lower limit of quantification; MRM, Multiple reaction monitoring; QC, Quality control; PTFE, Polytetrafluoroethylene; RIA, Radioimmunoassay; SLE, Supported liquid extraction; SPE, Solid phase extraction; SRM, Standard reference material; T, Testosterone; UHPLC, Ultra-high performance liquid chromatography; 2D-UHPLC-MS/MS, Two dimensional ultra-high performance liquid chromatography-tandem mass spectrometry.

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* Corresponding author.
E-mail address: francois.fraissinet@chu-rouen.fr (F. Fraissinet).

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also essential because steroids have highly similar chemical structures. Steroids were first determined by radioimmunoassay (RIA), which was considered the reference method for many years [1]. However, RIA methods are manual, time-consuming, and require specific measures and approval for the handling of radioisotopes. Indeed, RIA methods sometimes require prior extraction of the serum by organic solvents to remove interferences by metabolites and may lack of specificity [2]. More recently, automated immunoassays have been used as an alternative to RIA. They are adapted to emergency situations and allow a rapid rendering of results with low volumes of serum, but sometimes lack specificity due to the cross-reactivity of antibodies with other endogenous or exogenous steroids [3]. Gas chromatography-tandem mass spectrometry (GC–MS/MS) requires a derivatization step to make steroids volatile and is no longer used in daily practice for steroid determination in serum, however it remains used for urinary steroid profiling [4].

In recent decades, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has become popular for steroid analysis due to its sensitivity, specificity and capacity for multiplexing. Due to greater specificity of the method, the reference values must be adapted to LC-MS/MS [5]. The determination of steroids by LC-MS/MS requires complex sample preparation. Protein precipitation is the simplest method, but may result in enhanced matrix effects. Different extraction methods are available for steroids after protein precipitation: liquid–liquid extraction (LLE) [6], solid phase extraction (SPE) [7] or online SPE. These preparation methods are time-consuming, but reduce matrix effects and remove interferences. Online solid-phase extraction (SPE) allows for the automation of extraction, however it offers less flexibility than classic SPE: the sample is loaded onto a cartridge, analytes are trapped and non-retained compounds are transferred to waste, then analytes are back-eluted to the analytical column [8]. Online SPE has already been used for the determination of steroid compounds in various matrices [9]. Automation improves the analytical traceability of different tasks and has gained ground in many clinical laboratories. If mass spectrometry is still reserved for specialized laboratories, it is now possible to automate some of the preparation steps as protein precipitation [10].

The aim of our study was to develop a fully automated method using LC-MS/MS (Shimadzu® LC-MS 8060 paired with the CLAM-2030 preparation module) to determine a panel of five steroids in serum (testosterone, delta4-androstenedione, 17-hydroxyprogesterone, 11-deoxycortisol, dehydroepiandrosterone). This panel allows the diagnosis of endocrine diseases such as congenital adrenal hyperplasia (21-hydroxylase and 11-hydroxylase deficiencies). The CLAM-2030 preparation module automatically performs extraction steps. We also compared the results obtained by this method with immunoassays and another LC-MS/MS method.

2. Material and methods

2.1. Chemicals and reagents

Ammonium fluoride, ammonium formate, formic acid, methanol, acetonitrile, propan-2-ol and water, all LC-MS/MS grade, were obtained from Carlo Erba (Val de Reuil, France).

2.2. Calibrators, quality control samples, internal standards

The calibration materials and quality controls used were, respectively, MassChrom Steroids 6PLUS1 Multilevel Serum Calibrator Set MassChrom® Steroid Panels 1 + 2 (lot 1419), and trilevel Chromsystems MassCheck® Steroid Panels 1 + 2 (level I and II: lot 2517, level III: lot 5016 from Chromsystems (Grafëlfing, Germany)). The concentrations of the lowest and highest calibrators were: testosterone (T) (0.048–11.5 ng/mL), delta4-androstenedione (D4) (0.168–12.7 ng/mL), 17-hydroxyprogesterone (17OHP) (0.094–13.8 ng/mL), 11-deoxycortisol (11-DF) (0.085–12.3 ng/mL) and dehydroepiandrosterone (DHEA) (0.918–57.7 ng/mL). The working internal standard solution was prepared by diluting 150 µL of MassChrom Steroids Internal Standard Mix from Chromsystems (Grafëlfing, Germany) (lot 1418) in 5 mL of acetonitrile.

2.3. Sample preparation

A simple deproteinization was carried out by an automated sample preparation system, the CLAM-2030 (Shimadzu Corporation, Marne-la-Vallée, France) paired with two-dimensional ultra-high performance LC-MS/MS (2D-UHPLC-MS/MS) system. Sample preparation was limited to 6 min and consisted of protein precipitation. Thirty microliters of serum were automatically pipetted in a pre-conditioned tube containing a filter, to which reagents were then added, mixed and filtered. Briefly, the PTFE (polytetrafluoroethylene) filter vial (0.45 µm pore size) was previously conditioned with 20 µL of methanol. Successively, 60 µL of working internal standard solution for 30 µL of serum were added. The mixture was agitated for 120 s (1900 rpm) then filtered by application of vacuum pressure (−60 to −65 kPa) for 120 s into a collection vial.

2.4. 2D-UHPLC-MS/MS **conditions**

Analysis was performed on a 2D-UHPLC–MS/MS consisting of the following Shimadzu® modules (Shimadzu Corporation, Marne-la-Vallée, France): a quaternary pump LC20AD SP, for the pre-treatment mode, a binary pump consisting of two isocratic pumps Nexera LC30AD for the analytical mode, an automated sampler SIL-30AC, a column oven CTO-20AC and a triple quadrupole mass spectrometer LCMS-8060.

The CLAM-2030 is a Clinical Laboratory Automated sample preparation Module to prepare biological samples (whole blood, plasma, serum, urine), to be analyzed on LCMS units. It consists of a filter storage module, a sample rack and a reagent rack. It also contains a mixing, filtration and heating unit. Using designated preparation sets of filtration and collection vials, the CLAM-2030 deproteinizes the biological samples through a filtration method, and automatically transfers the vials with the deproteinized and filtered samples to the LCMS autosampler for sample injection.

The extraction method was divided into two steps. During the first step, steroids were retained on hydrophobic cartridge and matrix compounds were eliminated, then, during the second step, the analytes were back-flushed from the cartridge to the analytical column with an elution gradient, which led to the distinction of steroids.

The deproteinized extract obtained by the CLAM-2030 was automatically transferred to the automated sampler, where 30 µL were directly injected into the chromatographic system.

The LC-integrated online sample clean-up was performed using a perfusion column MAYI-ODS (5 mm L × 2 mm I.D.) (Shimadzu Corporation®, Marne-la-Vallée, France). The first step consisted of loading the extract on the perfusion column with a mobile phase composed of 10 mM ammonium formate in water at a flow rate of 0.5 mL/min during 2 min. Then, the system switched to the analytical step to elute the analytes from the perfusion column to the analytical column to perform chromatographic separation. For this step, the loading line was washed with propan-2-ol during 3 min. Chromatographic separation was performed on a Raptor Biphenyl column (50 mm L × 3 mm I.D., 2.7 µm) (Restek®, Bellfonte, USA) maintained at 40 °C and a gradient of (A) ammonium fluoride 1 mM buffer in water and (B) methanol at a flow rate of 0.675 mL/min as follows: 0.0–2.10 min, 5 % (B); 2.1–3.0 min, 5 to 65 % (B); 3.0–4.75 min, 65 % (B); 4.75–5.0 min, 65 to 70 % (B); 5.0–6.6 min, 70 % (B); 6.6–8.0, 70 to 75 % (B); 8.0–8.5 min, 75 to 100 % (B); 8.5–9.5 min, 100 % (B); 9.5–9.6 min, 100 % to 5 % (B); 9.6–12.0 min, 5 % (B).

Detection and quantification were performed for all analytes in positive ionization mode by scheduled-MRM (Multiple Reaction Monitoring) using 1 ms pause time and individual dwell times to achieve

sufficient points per peak. MRM transitions and dwell times are reported in Table 1.

The interface parameters and common settings were as follows: interface voltage: 1 kV; nebulizing gas flow: 3 L/min; heating gas flow: 10 L/min; drying gas flow: 10 L/min; interface temperature: 400 °C; DL (desolvation line) temperature: 150 °C; heat block temperature: 500 °C; collision gas pressure 300 kPa.

2.5. Method validation

The method was validated using the following criteria:

Intra-assay (n = 5) and inter-assay precision (n = 30), as well as the lower limit of quantification (LLOQ) were assessed with four levels of quality control (QC). The LLOQ corresponds to the lowest calibration standard according to European Medicine Agency (EMA) guidelines. The coefficient of variation (CV%) values had to be less than 20 % for the LLOQ and less than 15 % for the other levels as recommended by the EMA [11].

Calibration curves of the compounds-to-internal standard peak-area ratios of the quantification MRM transition were obtained using a linear regression analysis with $1/x^2$ weighting.

The dilution integrity was demonstrated using the highest calibration standard (T: 11.5 ng/mL, D4: 12.7 ng/mL, 17OHP: 13.8 ng/mL, 11-DF: 12.3 ng/mL, DHEA: 57.7 ng/mL). Five samples were diluted with sodium chloride (NaCl) 0.9 %, ratio 1:20. Recovery percentages have to be less than 15 % according to EMA guidelines.

Carryover was evaluated by successively analyzing three QC samples at the lowest QC level and three QC samples at the highest QC level. This sequence was repeated five times. The means of all the lowest QC levels of the first QC samples and the third QC samples were compared with a Student *t*-test. Recovery percentages must be less than 15 % according to EMA guidelines.

Matrix effects were evaluated in serum by spiking six samples of fully anonymized patient sera with the highest calibrator (2 %, v/v). Measured concentrations were compared to theoretical concentrations by calculating the percentage of recovery. Recovery percentages must be less than 15 % according to EMA guidelines.

The analyte stability in human sera was investigated under different storage conditions by analyzing QC samples at the lowest QC level and at the highest QC level. For short-term stability, samples were stored at 8 °C on the CLAM-2030 for 72 h. The freeze–thaw stability was evaluated after subjecting QC samples to 3 freeze–thaw cycles from the freezer (−20 °C) to room temperature. The mean concentration at each level had to be within ±15 % of the expected concentration.

Human sera from the French external quality program (Probioqual, Lyon, France) were assessed for the accuracy of this new LC-MS/MS method. The panel of these samples contained all analytes except DHEA. For DHEA, we joined an inter-laboratory comparison program (13 laboratories). For each sample, the difference between our LC-MS/MS result and other LC-MS/MS results, and the mean of bias assessed by the percentage of difference between results, were calculated.

2.6. Method comparison

Anonymized serum samples were used for comparison of steroid concentrations between RIA and LC-MS/MS. Comparison with RIA was performed for testosterone (n = 23 > 2 ng/mL and n = 29 < 2 ng/mL), androstenedione (n = 32) and 17-hydroxyprogesterone (n = 24). 11-deoxycortisol and DHEA were not determined in the laboratory before the use of mass spectrometry. All RIA assays were performed after liquid–liquid extraction (LLE) of serum with diethyl ether using Beckman-Coulter kits (Krefeld, Germany).

Sample results were also compared with another UHPLC-MS/MS method used in the biochemistry laboratory of Nantes University Hospital, France. Comparison was performed in anonymized serum samples for testosterone (n = 17), androstenedione (n = 20), 17-hydroxyprogesterone (n = 18), 11-deoxycortisol (n = 12). Institutional Review Board (IRB) approval was obtained for this study from CHU Rouen Normandie.

The calibrators and QCs used were exactly the same as our method (Chromsystems, lot 1419, Grafëlfing, Germany). This different method required manual sample preparation based on LLE. The sample, internal standard mix solution (prepared with testosterone-d3, androstenedione-^{13}C3, 17-hydroxyprogesterone-d8 and 11-deoxycortisol-d5 purchased from Merck, Darmstadt, Germany) and LLE solution reagent (*n*-Hexane:Ethyl acetate 80:20 (v/v)) were mixed together before centrifugation and evaporation of the supernatant. Then, methanol and water, of LC-MS quality, were added to the extract and mixed. After a second centrifugation, the supernatant was injected in a UHPLC-MS/MS system consisting of Acquity I-Class chromatographic technology with a binary pump and a Xevo TQ-XS mass spectrometer (Waters Corporation, Guyancourt, France).

2.7. Reference values

Reference values were retrieved from the literature, taking care to select studies carried out in a large number of healthy subjects [12,13]. We checked these values *a posteriori* by retrospectively analyzing the data from the assays performed with this method.

Table 1
MS/MS parameters of the steroids and their respective internal standards.

Steroid	Precursor ion (*m/z*)	Fragment ion (*m/z*)	Q1 Pre Bias (V)	CE	Q3 Pre Bias (V)	Dwell time (ms)
11-deoxycortisol	347.2	97.1	−19	−28	−19	12
11-deoxycortisol confirmation	347.2	109.2	−19	−27	−12	12
11-deoxycortisol d5	352.4	100.2	−18	−26	−21	12
11-deoxycortisol d5 confirmation	352.3	113.2	−18	−30	–23	12
DHEA	271.2	213.2	−14	−17	−15	12
DHEA confirmation	271.2	253.2	−14	−13	−18	12
DHEA d5	276.1	218.3	−30	−17	–23	12
DHEA d5 confirmation	294.3	218.2	−11	−21	–25	12
Testosterone	289.1	109.2	−15	−31	–22	12
Testosterone confirmation	289.1	97.2	−16	−35	−19	12
Testosterone d3	292.1	109.2	−30	−26	−26	12
Testosterone d3 confirmation	292.1	97.1	−15	−29	−21	12
17-hydroxyprogesterone	331.2	109.1	−18	−26	−12	12
17-hydroxyprogesterone confirmation	331.2	97.2	−17	−24	−18	12
17-hydroxyprogesterone ^{13}C3	334.2	112.2	−17	−27	−12	12
17-hydroxyprogesterone ^{13}C3 confirmation	334.2	100.2	−17	−25	−11	12
Δ4 androstenedione	287.2	97.1	−15	−21	−19	16
Δ4 androstenedione confirmation	287.2	109.1	−15	−24	−21	16
Δ4 androstenedione ^{13}C3	290.2	100.2	−15	–22	−20	16
Δ4 androstenedione ^{13}C3 confirmation	290.2	112.1	−15	–23	−12	16

2.8. Statistical analysis

We used the Passing-Bablok and the Bland-Altman analysis to check concordance of the different assays. Results below the LLOQ were excluded. For the retrospective determination of reference values, we used the refineR package that is adapted to data not derived from healthy subjects [14]. Statistics were performed using MedCalc Statistical Software version 13 (Ostend, Belgium) and using R software (4.0.5).

3. Results

Chromatograms of the five steroids are presented in Fig. 1. Chromatographic separation of compounds was performed in 12 min with online SPE. The biphenyl stationary phase combined hydrophobic and π-π interactions (the latter due to methanol of mobile phase) [15]. Isobaric compounds of 11-deoxycortisol, such as 21-deoxycortisol or corticosterone, were distinguished from 11-deoxycortisol using their retention time, which faciliated efficient resolution of this compound.

The intra-assay and inter-assay coefficients of variation at three levels of commercial QC were within the acceptance interval of 15 % for all steroids. For the LLOQ, all coefficients of variation were within the limit of 20 % (Table 2). For all steroids, we established seven-point calibration curves (six calibrators and one blank) using commercial calibrators. Differences between calculated and theoretical results were within 15 % and 20 % for the LLOQ. We also studied the stability of steroids in serum on CLAM-2030 before automated preparation, the filtrate being discarded on waste. With an acceptance interval of 15 %, at low concentrations, the stability of steroids was less than 24 h in serum, except for 11-deoxycortisol which was stable for at least 24 h (Fig. 2). Stability was improved at high concentrations, for at least 24 h for all compounds, except for DHEA. Nevertheless, three freeze–thaw cycles did not affect stability for any of the compounds. Dilution in NaCl 0.9 % (ratio 1:20) was validated, recovery percentages were within the acceptance interval of 15 % for all compounds, with coefficients of variation of less than 8.5 %. (Table 3). For carryover testing, p values of *t* test between means of quality controls were: T ($p = 0.01$), D4 ($p = 0.11$), 17OHP ($p = 0.18$), 11DF ($p = 0.74$) and DHEA ($p = 0.78$). For testosterone, the percentage of carryover was 0.09 % and, therefore, considered negligible. Matrix effects correspond to a loss of response (ion suppression) or a gain of response (ion enhancement) during ionization. After spiking six different samples with the highest calibrator, mean recovery percentages did not exceed 15 % with coefficients of variation of less than 7.8 % (Table 3). Matrix effects were therefore considered insignificant. Thus, the stable isotope labeled internal standard for each analyte was able to compensate for matrix effects.

We compared our LC-MS/MS method to the RIA method previously used in our laboratory (Fig. 3). For testosterone >2 ng/mL, Passing-Bablok regression indicated that both methods provided similar results (slope 1.11, 95 % CI 0.98 – 1.22). In contrast, for steroids, a proportional

Table 2
Intra-assay and inter-assay precision of steroid hormone analysis.

Steroid	Intra-assay precision (n = 5)		Inter-assay precision (n = 30)	
	Mean (ng/mL)	Precision (%)[1]	Mean (ng/mL)	Precision (%)[1]
11-deoxycortisol	0.09 (LLOQ)	11.0	0.09	0.1
	0.32	4.2	0.26	5.5
	1.46	1.9	1.33	3.8
	9.98	0.8	8.20	5.5
DHEA	0.79 (LLOQ)	8.7	0.92	0.5
	1.92	8.4	1.85	8.1
	11.06	1.7	12.22	4.7
	37.57	3.6	39.47	5.3
Testosterone	0.04 (LLOQ)	9.3	0.048	2.8
	0.19	3.0	0.19	3.8
	1.44	0.9	1.47	2.3
	7.7	1.0	7.60	2.9
17-hydroxyprogesterone	0.10 (LLOQ)	3.5	0.09	0.9
	0.30	2.2	0.28	3.9
	1.51	2.3	1.46	2.0
	8.88	1.4	8.35	2.6
Delta4 androstenedione	0.18 (LLOQ)	3.7	0.17	0.8
	0.28	3.0	0.26	3.3
	1.24	1.2	1.09	1.7
	10.48	0.9	8.86	2.4

[1] coefficient of variation (%) = (SD/mean) × 100.

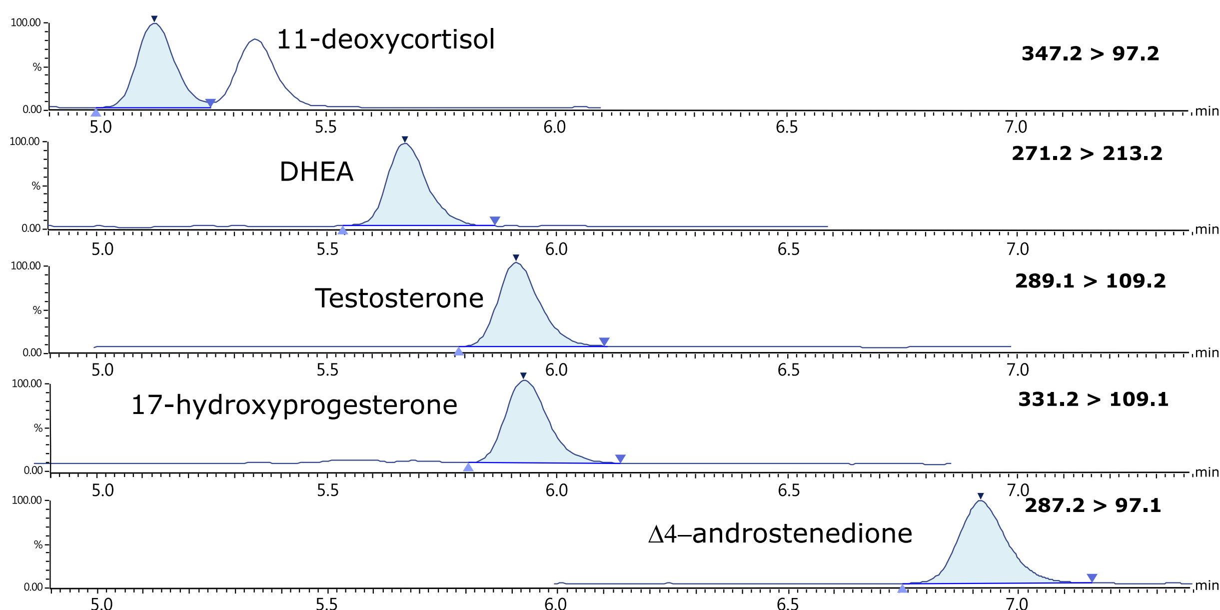

Fig. 1. Extracted ion chromatograms of five steroids measured by LC-MS/MS. Multiple Reaction Monitoring transitions are shown at the top right of each chromatogram. The peak on the right of the first chromatogram corresponds to corticosterone, an isobaric compound of 11-deoxycortisol.

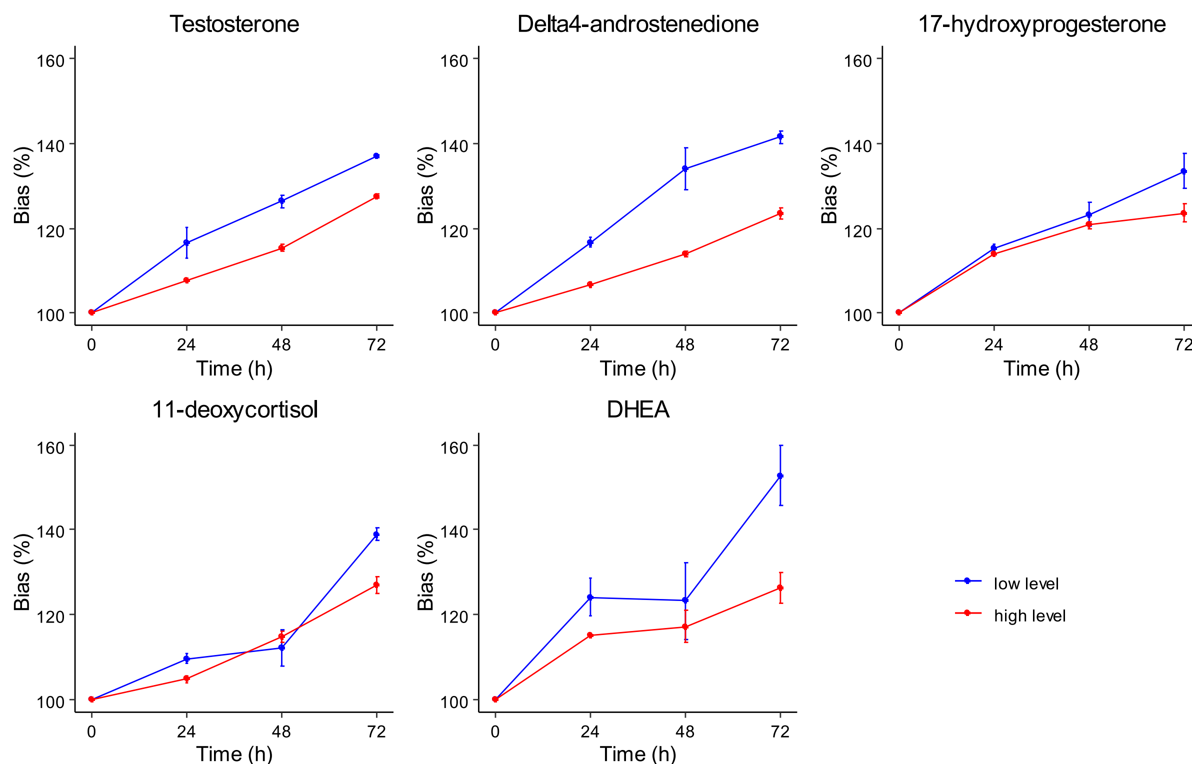

Fig. 2. Stability of steroids in serum on CLAM-2030 during 72 h. The recovery (Tx/T0*100) is expressed as mean ± SEM (n = 3 for each level).

Table 3
Matrix effects and effect of dilution in NaCl (n = 5) in all compounds.

Steroid	Effect of dilution in NaCl 1:20 (n = 5)		Matrix effects (n = 5)	
	Mean of percentage recovery (%)	Precision (%)[1]	Mean of percentage recovery (%)	Precision (%)[1]
11-deoxycortisol	90.1	3.5	101.3	2.6
DHEA	82.5	8.4	105.0	7.8
Testosterone	90.8	3.1	105.4	4.4
17-hydroxyprogesterone	87.4	2.5	103.1	3.5
Delta4 androstenedione	92.4	2.4	102.2	2.1

[1] coefficient of variation (%) = (SD/mean) × 100.

bias between LC-MS/MS and RIA was observed, the RIA method gave higher concentrations than LC-MS/MS (D4: slope 0.62, 95 % CI 0.49–0.73; testosterone <2 ng/mL: slope 0.89, 95 % CI 0.75–1.00). Surprisingly, RIA underestimated 17-hydroxyprogesterone concentrations (slope 1.28, 95 % CI 1.15–1.36). We also compared our LC-MS/MS method with the LC-MS/MS method of another laboratory: results matched for testosterone (slope 1.00 95 % CI 0.96–1.13), 17-hydroxyprogesterone (slope 0.93, 95 % CI 0.81–1.00) and 11-deoxycortisol (slope 0.82, 95 % CI 0.54–1.00). For androstenedione, a proportional bias (slope 1.14, 95 % CI 1.03–1.24) was observed between the two LC-MS/MS methods (Fig. 4).

To assess the accuracy of this new LC-MS/MS method, we analyzed different samples from the French external quality evaluation program. The mean bias relative to the other laboratory's LC-MS/MS method was: testosterone (+2.7 % [−4.2 % to 8.9 %], n = 16), delta4-androstenedione (−1.8 % [-11.9 % to 4.7 %], n = 10), 17-hydroxyprogesterone (+2.1 % [−7.4 % to 15.4 %], n = 18), 11-deoxycortisol (−5.2 % [−20.9 % to 13.0 %], n = 13). For DHEA, the estimated bias with the inter-laboratory comparison program was −13.3 % [−32.6 % to 1.0 %] (n = 9).

We, retrospectively, evaluated the reference values that we selected from the literature. Reference intervals of four steroids were: testosterone men (0.44–7.26 ng/mL), testosterone women (0.05–0.64 ng/mL), delta4-androstenedione men (0.09–1.38 ng/mL), delta4-androstenedione women (0.10–3.22 ng/mL), 17-hydroxyprogesterone men (0.38–1.17 ng/mL), 17-hydroxyprogesterone women (0.01–1.37 ng/mL) (Supplemental Table 1). The most important difference concerned testosterone in men. The lower reference interval (0.63 ng/mL) seemed weak compared to the literature (2.82 ng/mL) [13].

4. Discussion

In this study, we describe for the first time a fully automated analytical method to determine five steroids in serum. Our panel of steroids is limited to five molecules, which correspond to the majority of the orders that we receive at our university hospital's clinical laboratory. Nonetheless, the panel allows the diagnosis of congenital adrenal hyperplasia due to 21-hydroxylase or 11-hydroxylase deficiencies. The panel could be extended to other steroids, such as mineralocorticoids (e.g., corticosterone, 11-deoxycorticosterone) or precursors (e.g., pregnenolone, 17-hydroxypregnenolone). Isobaric compounds of 11-deoxycortisol, such as corticosterone and 21-deoxycortisol, were correctly separated in the biphenyl stationary phase; poor chromatographic separation of these steroids could result in incorrect diagnosis [16]. This column type has already been described for steroids with the same elution order for isobaric compounds of 11-deoxycortisol [17]. The elution mobile phase contains ammonium fluoride, which enhances the ionization of steroids, including in positive mode [9].

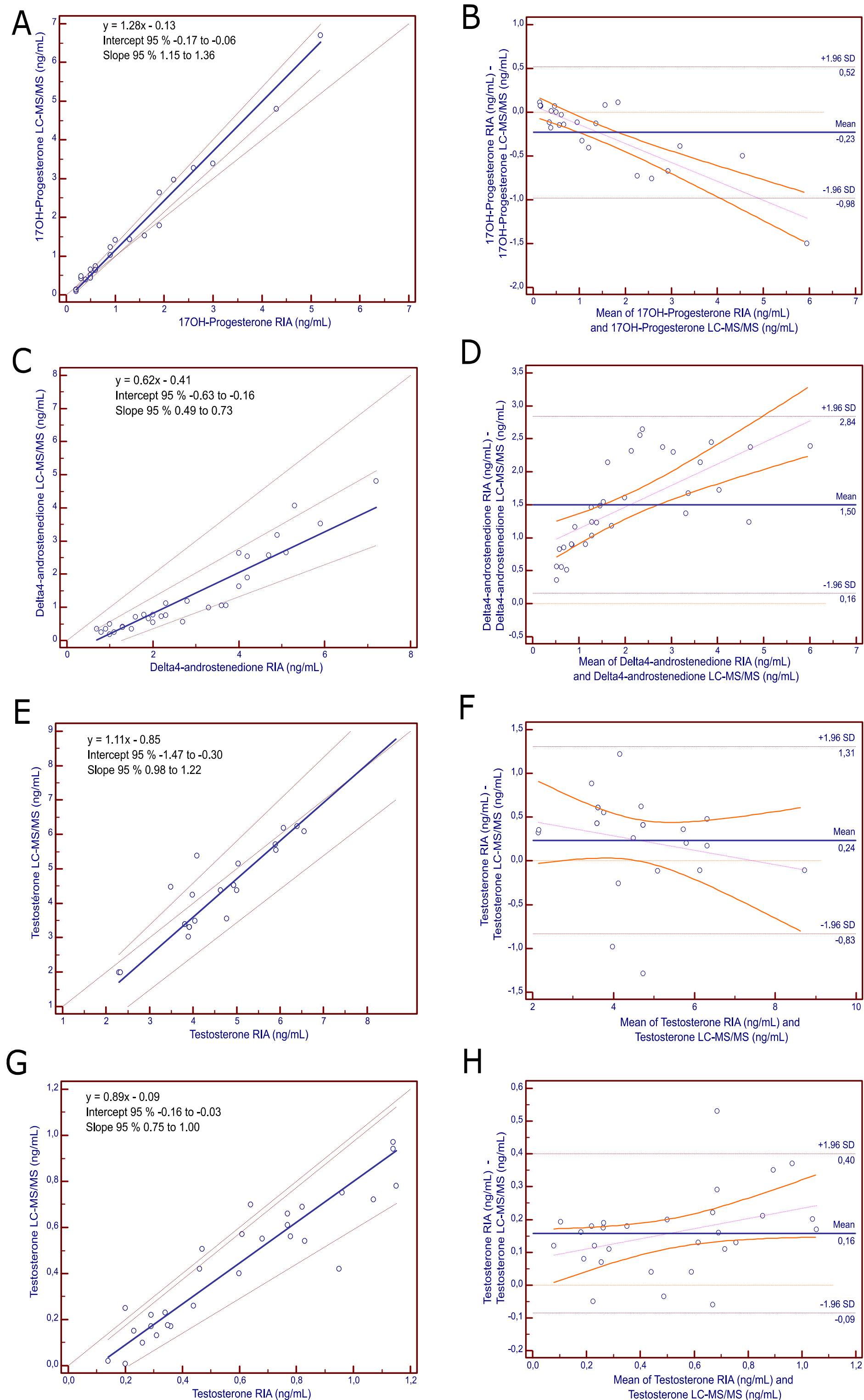

Fig. 3. Passing-Bablok regression and Bland-Altman diagrams for method comparison between our LC-MS/MS method and the RIA method (A-B, 17-hydroxyprogesterone, C-D, delta4-androstenedione, E-F, testosterone <2 ng/mL, G-H, testosterone >2 ng/mL).

In this study, we used a Mayi-ODS cartridge for online SPE extraction, which contains polymeric support with pores coated by C18 phase. The separation of nine steroids with online SPE was performed using a similar polymeric cartridge (i.e., Poros R1, Applied Biosystems) [9].

The novelty of our method is the full automation of sample preparation on the CLAM-2030 automatic pretreatment device paired with HPLC system material. This type of sample preparation has already been used with the same material for uracil and dihydrouracil determination [18], for drug quantification [19] or for targeted toxicological screening [20]. However, these methods did not use online SPE following protein precipitation. Total laboratory automation of preanalytical and analytical systems reduces the risk of laboratory errors and improves the quality of results [21].

This method has been validated according to EMA guidelines.

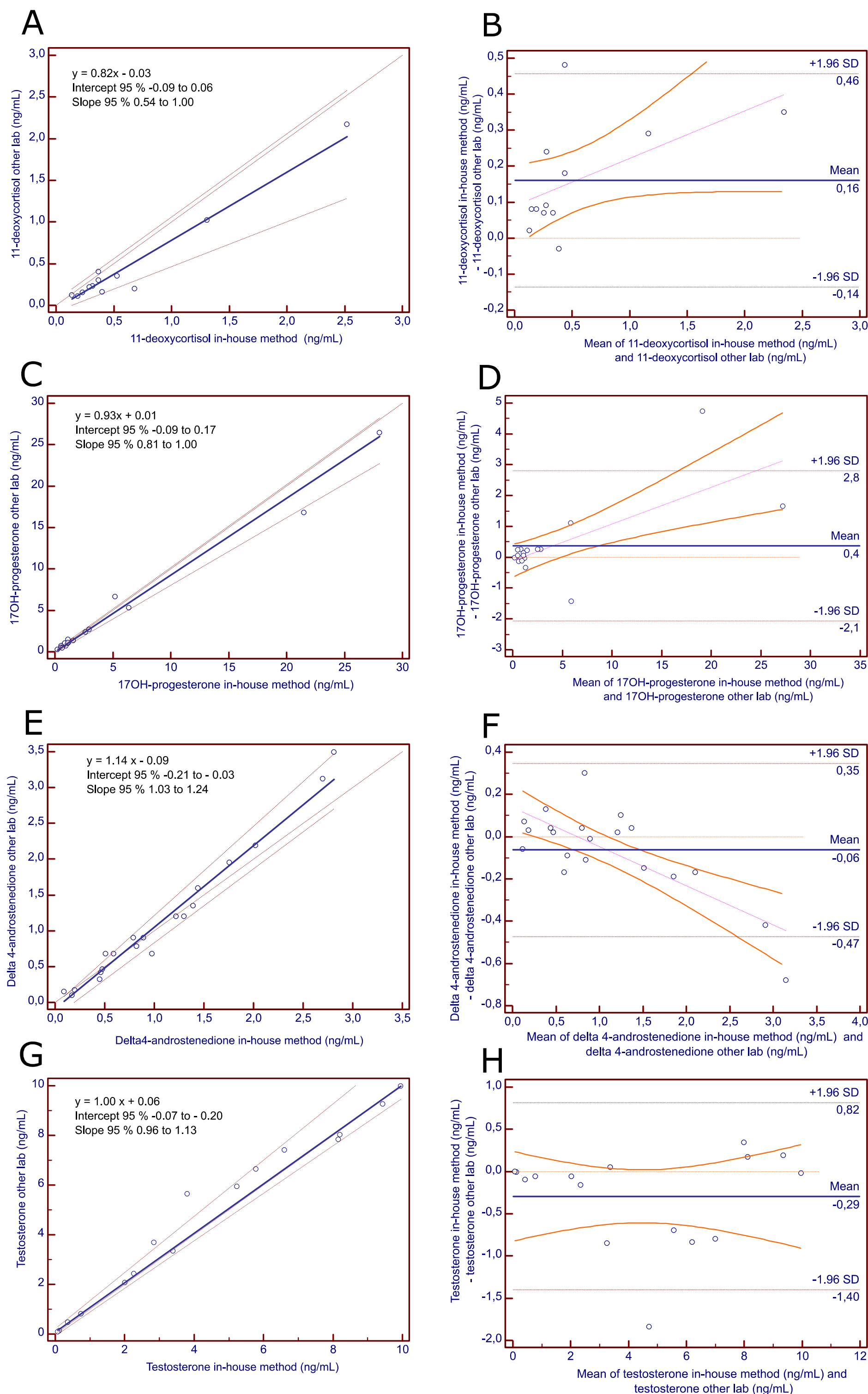

Fig. 4. Passing-Bablok regression and Bland-Altman diagrams for method comparison between our LC-MS/MS method and another laboratory's LC-MS/MS method. (A-B, 11-deoxycortisol, C-D, 17-hydroxyprogesterone, E-F, delta4-androstenedione, G-H, testosterone).

Clinical and Laboratory Standards Institute (CLSI) C62-A and C57-Ed1 propose more extensive validation criteria for mass spectrometric assays, in particular for accuracy: comparison with reference procedure methods and analysis of certified reference materials [22]. Nevertheless, EMA guidelines have already been used for method validation of exogenous substances, but also for determination of steroids [23]. We show here that the stability of steroids on the CLAM-2030 does not exceed 24 h at 8 °C. Other studies have shown that androgen levels increase moderately over 1–5 days after storage at 2–8 °C [24], or are stable in serum up to 48 h at ambient temperature [25].

Our study illustrates the lack of specificity of the RIA method for all steroids, except for testosterone in men for whom the results were comparable between the two methods. The same results were reported for androgens in two previous studies using LC-MS/MS and RIA assays [26,27]. In general, steroid concentrations are higher with RIA assays than with LC-MS/MS, depicting the lack of antibody specificity. This is

especially relevant for low testosterone concentrations in women and infants. If the standardization of immunoassays improves the accuracy of measurement, differences within LC-MS/MS methods persist [28]. Surprisingly, according to Fiet et al [26], 17-hydroxyprogesterone concentrations were 20 % to 30 % higher with the LC-MS/MS method. Results of the French external QC confirm this hypothesis. Prior to RIA analysis, the LLE of serum allows one to avoid interference of similarly structured compounds, such as 17-hydroxypregnenolone sulfate, which has a high neonatal concentration [2].

We also compared two LC-MS/MS methods for four steroids. Results were comparable for all four steroids, except androstenedione. Our results were different to those of the other laboratory (mean bias + 14 %). This is in accordance with a previous study that compared eight LC-MS/MS methods for testosterone and androstenedione and showed the existence of biases between different methods already published for androstenedione [29]. To avoid such biases, standard reference material (SRM), NIST SRM 971, and a reference measurement procedure are available for testosterone. The lack of comparison with a reference method constitutes a limitation of this work [30]. These standardization efforts have improved the analytical accuracy of testosterone immunoassays and LC-MS/MS methods. For androstenedione, the two LC-MS/MS methods used Chromsystems calibrators, which cannot explain observed differences; however, extraction procedures (LLE and online-SPE), and many calibrators and quality controls were different. Recently, a reference method has been published [31]. Standardization efforts could improve analytical performance for androstenedione. The existence of a bias between the RIA method and the LC-MS/MS method requires the use of reference values specific to the mass spectrometry method. We checked retrospectively selected reference values from the literature using a statistical method suitable for a global population not exclusively composed of healthy volunteers. The number of results was insufficient for DHEA and 11-deoxycortisol. It should be noted that, for testosterone in men, the lower limit was abnormally low compared to previously published values. This is probably related to our population, which included non-healthy subjects and, thus, does not fully reflect a population of healthy volunteers.

In European countries, the In Vitro Diagnostic Medical Devices Regulation (IVDR) 2017/746 requires the use of certified reagents and materials and limits the use of in-house methods called laboratory developed tests (LDT). A high volume of serum, 500 μL, is required to perform the SPE proposed by the Chromsystems kit. This high volume of serum is not suitable for pediatric assays. With our method, the sample volume is low, 30 μL, which is a significant advantage compared to the Chromsystems kit. Our method also has the advantage of being fully automated, which reduces the risk of error in sample preparation. It is also easier to troubleshoot an in-house method than a commercial kit because the exact composition of kits is not provided by the manufacturer.

5. Conclusion

In this study, we present original data in support of a novel fully automated analytical method allowing the measurement of five steroids in serum. Automation saves time in sample preparation, improves the traceability of different preparation steps and helps to reduce the cost of reagents. This novel automated LC-MS/MS method is an advantageous alternative to automated immunoassays for the determination of steroids.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmsacl.2022.12.004.

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