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Research Article

Semi-automated serum steroid profiling with tandem mass spectrometry

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ABSTRACT

Objectives: Highly selective and sensitive multi-analyte methods for the analysis of steroids are attractive for the diagnosis of endocrine diseases. Commercially available kits are increasingly used for this purpose. These methods involve laborious solid phase extraction, and the respective panels of target analytes are incomplete. We wanted to investigate whether an improvement of kit solutions is possible by introducing automated on-line solid phase extraction (SPE) and combining originally separate analyte panels.

Methods: Sample preparation was performed using automated on-line SPE on a high-pressure stable extraction column. Chromatographic separation, including isobaric compounds, was achieved using a 0.25 mM ammonium fluoride-methanol gradient on a small particle size biphenyl column. Standard compounds and internal standard mixtures of two panels of a commercially available kit were combined to achieve an optimized and straightforward detection of 15 endogenous steroids. Validation was performed according to the European Medicines Agency (EMA) guidelines with slight modifications.

Results: Validation was successfully performed for all steroids over a clinically relevant calibration range. Deviations of intra- and inter-assay accuracy and precision results passed the criteria and no relevant matrix effects were detected due to highly effective sample preparation. External quality assessment samples showed the applicability as a routine diagnostic method, which was affirmed by the analyses of anonymized clinical samples.

Conclusions: It was found possible to complement a commercially available kit for quantitative serum steroid profiling based on isotope dilution LC-MS/MS by implementing automated on-line SPE, thereby improving the practicality and robustness of the measurement procedure.

1. Introduction

Endogenous steroids, which play a pivotal role as biomarkers in medical diagnosis, are interconverted into each other during metabolism within the body. This is enzymatically catalyzed by reactions, such as oxidation, hydroxylation and/or reduction [1]. As a result, the individual steroids within subclasses are structurally very similar. Besides isobaric structures, such as 17α-hydroxyprogesterone and 11-deoxycorticosterone, structurally similar steroids can be transformed into isobaric ones by, e.g., the loss of water during mass spectrometric analysis [2]. As a result, highly selective methods are required for the separation of steroids and especially in case of isobaric compounds. Due to low circulating concentrations and poor ionization efficiencies of some steroids, such as estradiol and dihydrotestosterone, methods have to also be very sensitive [3]. With regard to the diagnosis of certain diseases, such as adrenocortical adenoma or congenital adrenal hyperplasia (CAH) [4,5] a fingerprint of several steroids (steroid profiling) is helpful, for which multiplex steroid panels, covering a wide concentration range (pg/ml - μg/ml), are needed. Considering all above mentioned points, stable-isotope dilution (SID) LC-MS/MS is the current

Abbreviations: A4, Androstendione; ALDO, Aldosterone; APCI, Atmospheric pressure chemical ionization; CAH, Congenital adrenal hyperplasia; CE, Collision energy; CE-IVD, Certified in-vitro-diagnostic device; CV, Coefficient of variation; DHEA, Dehydro-epiandrosterone; DHEA-S, Dehydro-epiandrosterone sulfate; DHT, Dihydrotestosterone; DOC, 11-deoxycorticosterone; E, Cortisone; E2, Estradiol; EMA, European Medicines Agency; EQA, External quality assessment; ESI, Electrospray ionisation; F, Cortisol; IVD, In-vitro-diagnostic; IVDR, EU In vitro Diagnostic Regulation; LC, Liquid chromatography; LC–MS/MS, Liquid chromatography tandem mass spectrometry; LDT, Laboratory developed test; LLOQ, Lower limit of quantification; MRM, Multiple reaction monitoring; P4, Progesterone; QC, Quality control; S/N, Signal-to-noise ratio; SID, Stable-isotope dilution; SPE, Solid phase extraction; SST, System suitability test; UHPLC, Ultra high performance liquid chromatography; ULOQ, Upper limit of quantification.

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gold standard and preferable to the use of immunoassays, which critically lack specificity [6–8]. This is supported by the Endocrine Society, which appealed in 2014 for "high-quality, well-validated steroid assays to improve quality" [9]. Keevil et al. provided a review of published steroid LC-MS/MS methods that are applicable for routine analyses [10].

Nevertheless, inter-laboratory agreement is still not satisfactory due to methodological differences, e.g., the application of differently labelled internal standards or calibration [11]. Olesti et al. summarized these limitations in steroid analysis over the recent years, focusing on LC-MS/MS [12].

During recent years, commercial LC-MS/MS steroid kits have been introduced from several manufacturers, containing different and varying numbers of target analytes. These kits may be helpful to improve inter-laboratory agreement, as they include all steps of LC-MS/MS analysis, as well as calibration. However, time-consuming manual sample preparation techniques involving liquid–liquid extraction or off-line solid-phase extraction (SPE) are typically used. Notably, these methods are, in general, non-transparent in terms of the use of column, mobile phases or even internal standards.

Our aim was to investigate whether an improvement of two panels of a kit solution is possible by combining both panels and introducing automated on-line solid phase extraction instead of applying off-line extraction. In addition, the laboratory-developed test (LDT) is expected to bring practicality and high robustness, thus contributing to the standardization of multiplex steroid methods.

2. Materials and methods

2.1. Chemicals and reagents

Water and methanol (both UHPLC quality) were purchased from Biosolve (Valkenswaars, the Netherlands). A 0.1 mol/L zinc sulfate solution was obtained from Merck (Darmstadt, Germany). Ammonium fluoride used as a mobile phase additive was purchased from Sigma-Aldrich (Steinheim, Germany). Tuning mix solutions, system check solutions, an internal standard mix, calibrators and quality controls were individual obtained from the MassChrom® Steroid kit (Panel 1 and 2 including 15 steroids) by Chromsystems (Gräfelfing, Germany; subsequently referred to as manufacturer C). Additional quality control samples (ClinChek® controls for steroids) were purchased from Recipe (Munich, Germany; subsequently referred to as manufacturer R). All commercial calibrators and quality controls are based on human serum.

2.2. Calibrator samples, quality control samples and internal standards

Commercially available lyophilized serum-based calibrator and quality control material was used from a a certified in-vitro diagnostic (CE-IVD) kit kit from manufacturer C. Panel 1, including 11-deoxycortisol, 21-deoxycortisol, aldosterone, corticosterone, cortisol and cortisone, and panel 2, including 11-deoxycorticosterone, 17-OH-progesterone, androstenedione, dehydro-epiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEA-S), dihydrotestosterone (DHT), estradiol, progesterone and testosterone, were combined. This was achieved by dissolving the lyophilized material in only 1.5 ml water instead of 3 ml. After 20 min of shaking on a roller shaker, 1.2 ml of each panel were combined, whereby the desired concentration was obtained. Afterwards, this was again shaken for 15 min and vortexed before aliquoting. Six calibrators, one blank and three quality control samples, were prepared as described above. Additionally, quality control samples at three different levels were used from manufacturer R, including androstenedione, cortisol, DHEAS, 11-deoxycortisol, 21-deoxycortisol, 17-OH-progesteron, and testosterone. Individual concentrations of all 15 analytes can be found in Table 1. All reconstituted samples were stable at −20 °C for up to three months, according to the manufacturer.

Internal standard mix was used from manufacturer C. This included internal standards for all target steroids that were stable-isotope labeled (11-deoxycortisol-d_5, 21-deoxycortisol-d_8, aldosterone-d_4, corticosterone-d_8, cortisol-d_4, cortisone-d_8, 11-deoxycorticosterone-d_8, 17-OH-progesterone-$^{13}C_3$, androstenedione-$^{13}C_3$, DHEA-d_5, DHEA-S-d6, DHT-d_3, estradiol-d_5, progestesteron-$^{13}C_3$ and testosterone-d_3).

2.3. Sample preparation

One hundred µL aliquots of each serum sample (calibrator, quality control or unknown) were mixed with 100 µL of a daily prepared precipitation solution including all stable-isotope labeled internal standards (97.75 % of a methanolic 15 mM zinc sulfate solution + 2.25 % of the internal standard mix). The blank sample was only mixed with 100 µL of the methanolic 15 mM zinc sulfate solution. After vortexing, the samples were shaken for 15 min at room temperature at 1400 rpm using a ThermoMixer C (Eppendorf, Germany). After centrifugation (15 min, 15 °C, 14,000×*g*), 120 µL of the clear supernatant were transferred to glass vials with micro-inserts and placed into the UHPLC autosampler (4 °C sample cooling).

2.4. UHPLC and MS/MS conditions

For sample analysis, a 1290 Infinity I LC system equipped with two binary pumps (Agilent, Santa Clara, California, USA), a thermostatted column compartment and a 10-port switching valve coupled to a TripleQuad 6500 + mass spectrometer (Sciex, Framingham, Massachusetts, USA) were used. Analyst 1.6.3 (Sciex) was available for instrument control and data acquisition. On-line solid phase extraction (SPE) was achieved on a recently established UHPLC applicable Strata C8 online extraction column (20x 2.1 mm) from Phenomenex (Aschaffenburg, Germany) using water and methanol as mobile phases A1 and B1. The extraction column was kept at 20 °C and 20 µL were injected. A Kinetex Biphenyl column (150 × 2.1 mm, 1.7 µm, Phenomenex) was used for chromatographic separation. The column oven was kept at 50 °C. As eluents, a 0.25 mM ammonium fluoride in water solution was used as mobile phase A2 and methanol as B2. Applied valve positions and gradients can be seen in Fig. 1.

All target analytes and their corresponding internal standards were tuned for MS/MS parameters. For this purpose, the Tuning Mix MassChrom® Steroid Panel 1 and 2 was diluted fivefold with methanol/water 1/1 (v/v). Most analytes were analyzed in positive ion mode (ESI +) with the exception of DHEAS, Estradiol and the qualifier of aldosterone, which were analyzed in the negative ion mode (ESI−). In addition, an advanced schedule multiple reaction monitoring (MRM) method from SCIEX was used to improve sensitivity of all analytes. Data processing was performed using MultiQuant 3.0.3 (Sciex). Peak integration was automatically performed using the retention times, and different smoothing was applied to each compound (Table 2). The following ion source settings were used: curtain gas 35 psi, collision gas 8 (medium), source temperature 600 °C, ion spray voltage 5000 V (respectively, −4500 V for the negative ion mode), ion source gas 1 55 psi (atomizing gas) and ion source gas 2 50 psi (heating gas), using nitrogen as gas. To verify the ideal collision energy in matrix, collision energy-breakdown curves were acquired for all mass transitions used for quantification in a matrix-based sample (calibrator 4 from manufacturer C). For this purpose, we applied a protocol previously published by our group [13]. All selected mass transitions, parameters and retention times can be found in Table 2.

2.5. Method validation

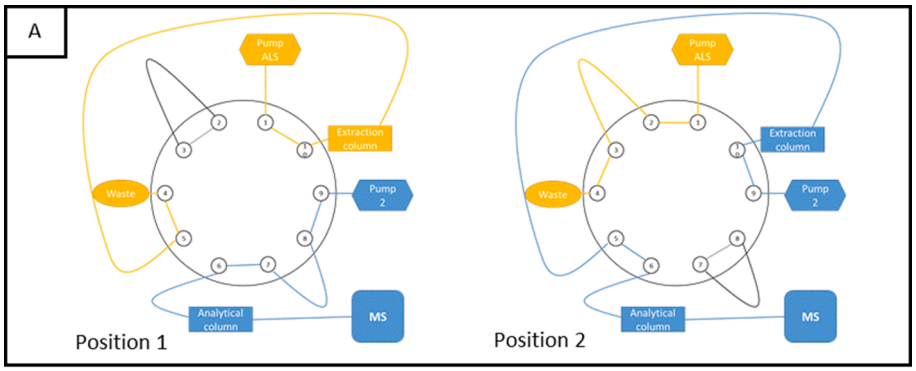
The characteristics of the presented bioanalytical method were validated essentially according to the European Medicines Agency (EMA) [14]. Each measurement series consisted of two system suitability solutions (SST, panel 1 and panel 2) for checking the retention times and performance of the LC-MS/MS, a blank sample, a zero sample (containing internal standards, but no analytes), calibrators measured at

Table 1
Concentrations [ng/mL] of calibrator and quality control samples from manufacturer C and R for all steroids.

Compound [ng/mL]	Manufacturer C									Manufacturer R		
	Cal 1	Cal 2	Cal 3	Cal 4	Cal 5	Cal 6	QC 1	QC 2	QC 3	QC 1	QC 2	QC 3
Cortisol	10.10	20.40	40.30	80.50	149.0	285.0	25.10	59.80	174.0	5.24	14.80	79.90
Cortisone	1.04	2.61	5.17	10.30	20.10	40.00	2.05	11.70	29.20			
21-Deoxycortisol	0.065	0.145	0.239	0.485	1.48	4.83	0.096	0.383	2.33	0.247	0.708	4.07
11-Deoxycortisol	0.108	0.530	1.06	2.12	5.16	15.10	0.304	1.49	9.71	0.229	0.719	3.94
Corticosterone	0.535	1.13	2.81	5.77	16.30	48.90	0.821	4.11	28.40			
Aldosterone	0.027	0.075	0.147	0.295	0.728	2.88	0.097	0.239	0.946			
DHEA	1.01	4.94	9.55	14.50	27.80	54.60	2.00	12.00	39.50			
DHT	0.053	0.102	0.232	0.458	0.890	1.35	0.078	0.365	1.10			
Testosterone	0.047	0.248	0.959	2.94	5.78	11.50	0.199	1.46	7.92	0.186	0.513	2.84
17-OH-Progesterone	0.106	0.499	0.957	1.98	3.83	22.10	0.286	1.42	8.61	0.238	0.658	3.53
11-Deoxycorticosterone	0.051	0.098	0.139	0.286	0.713	2.83	0.076	0.193	0.954			
Androstenedione	0.204	0.394	0.744	1.42	4.55	13.40	0.275	1.09	8.92	0.206	0.584	3.27
Progesterone	0.137	0.740	1.94	4.93	9.65	24.00	0.311	2.98	14.90			
DHEAS	119.0	580.0	1072	2072	3829	5675	268.0	1535	4857	97.0	256.0	1396
Estradiol	0.040	0.101	0.248	0.513	1.50	4.99	0.083	0.420	2.64			

Concentrations printed in grey are outside the calibration range. Calibrator, Cal; Quality control, QC; dehydroepiandrosterone; DHEA; dihydrotestosterone, DHT; dehydroepiandrosterone sulfate, DHEAS.

Concentrations printed in grey are outside the calibration range. Calibrator, Cal; Quality control, QC; dehydroepiandrosterone; DHEA; dihydrotestosterone, DHT; dehydroepiandrosterone sulfate, DHEAS.



B

Gradient Pump ALS

Time [min]	B [%]	Flow [mL/min]
0.0	10	1.5
1.0	10	1.5
1.05	30	1.5
1.5	30	1.5
2.5	90	0.3
3.0	90	0.3
3.1	100	0.3
8.0	100	0.3
9.0	10	0.3
9.5	10	1.5
11.0	10	1.5

Gradient Pump 2

Time [min]	B [%]	Flow [mL/min]
0.0	100	0.3
1.0	30	0.3
1.5	30	0.3
1.6	70	0.3
2.6	70	0.3
5.8	85	0.3
6.8	85	0.3
7.8	100	0.3
11.0	100	0.3

Fig. 1. UHPLC settings for the 10-port switching valve and both binary pumps. A, on the left side, position 1 of the 10-port switching valve is shown. The extract is injected and loaded from the autosampler (ALS) to the extraction column, and afterwards washed (yellow line, time 0.0 – 1.5 min). On the right side, position 2 is visible. showing the elution of the analytes from the extraction column to the analytical column, where the chromatographic separation take place (blue line, time 1.5 – 11.0) and detected in the mass spectrometer (MS). B, UHPLC gradients for the autosampler (Pump ALS) and second pump (Pump 2), each with methanol as mobile Phase B, with the selected valve positions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the beginning and end of each sequence, and quality control samples. Besides using the quality controls from manufacturer C, those from manufacturer R were included for additional verification. Considering in particular that panel 1 and panel 2 from manufacturer C were combined (section 2.2.), an additional quality check is useful.

The calibration range for all steroids can be found in Table 1, with calibrator 1 indicating the lowest concentration limit (lower limit of quantification, LLOQ). A signal-to-noise ratio (S/N) of at least 10, combined with an inaccuracy and imprecision of $\leq$ 20 % was necessary for the LLOQ. The linearity of the method was tested by recalculating the concentrations of at least five calibration standards, which should be within $\pm$ 15 % (for the LLOQ $\pm$ 20 %). In addition, a 1/x (for DHEAS and corticosterone a $1/x^2$) weighting function was used and the correlation coefficient was determined.

Accuracy and imprecision (indicated by the coefficient of variation CV) was evaluated as intra- and inter-assay on three separate days covering quality controls from manufacturer C (n = 5 each day) and manufacturer R (n = 3 each day). All results should be within deviations of $\pm$ 15 % (for the LLOQ $\pm$ 20 %) according to the EMA guideline. In each series of measurements, MultiQuant automatically calculated the ion ratios of the mass transitions (qualifiers/quantifiers) of the calibration standards, the quality controls and the unknown samples. The mean value of the calibration standards was used as the expected ion ratio. The ion ratios of all other samples should not deviate from this by more than 20 %, as larger deviations would indicate interferences in the sample [15].

To investigate potential carry-over, a methanol/water (1/1, v/v) solution was analyzed after the injection of the highest calibrator (upper limit of quantification, ULOQ). Signals should be $\leq$ 20 % of the LLOQ and $\leq$ 5 % of the internal standard.

Due to the implementation of on-line solid phase extraction, matrix effects were only investigated by post-column infusion experiments [16] as a deviation from the EMA validation protocol [14]. This was realized by infusing each of the system check solutions (concentration unknown) from manufacturer C into the mass spectrometer while analyzing a blank serum sample, also from manufacturer C, or a solvent solution (neat). Ideally, both chromatograms are congruent.

Dilution experiments were performed for the highest quality control from manufacturer C. More specifically, samples were diluted three times for every dilution factor with water (1 + 1, 1 + 2, 1 + 4), processed, analyzed and concentrations back-calculated to undiluted concentration levels. Inaccuracy and imprecision should not exceed 15 % according to the EMA guideline.

With reference to the manufacturer's stability recommendations for calibrator and quality control samples, only 24 h autosampler stability of processed quality control samples (three concentrations and n = 5) was investigated.

2.7. Method applicability

As detailed standardized description, beyond validation, is receiving increasing attention in routine diagnostics [17], a measurement series was defined and acceptance criteria set, which were applied for the following measurements. To proof the method applicability for routine diagnostic, two samples of an external quality assessment (EQA) from the Reference Institute of Bioanalytics (Referenzinstitut für Bioanalytik, RfB) were measured (HM4/21), including aldosterone, cortisol, estradiol, progesterone, testosterone, DHEAS and 17OH-progesterone. In addition, randomized and fully anonymized leftover samples from 40 serum patient samples were analyzed. As only discarded, fully anonymized patient samples were used, neither informed consent nor IRB review were required.

3. Results

3.1. Method development

Method development was based on an existing SID UHPLC-MS/MS method in our laboratory [18]. Improved selectivity, especially for isobaric steroids, was achieved due to the application of a 150 mm Kinetex Biphenyl column from Phenomenex. Since all analytes present in this method are also part of the new method and three more steroids (estradiol, DHT and Androstenedione) were added, this column was selected as the analytical column as well. Chromatographic separation

Table 2
Multiple reaction monitoring transitions and parameters for the target steroids and their corresponding internal standards.

Group	ID	Q1 [*m/z*]	Q3 [*m/z*]	RT [min]	Window [*sec*]	P	TH	DW	DP [V]	EP [V]	CEV]	CXP [V]	ST
Cortisol	QN	363.2	121.1	4.70	40	1	2000	1	48	10	28	11	0
	QL	363.2	309.1	4.70		2			48	10	22	19	
	d_4	367.3	121.1	4.68		1			48	10	30	16	
(1) Cortisone	QN	361.2	163.1	4.91	40	1	3000	2	50	10	31	25	0
	QL	361.2	121.1	4.91		2			50	10	35	14	
	d_8	369.2	168.1	4.88		1			50	10	34	23	
(2) 21-Deoxycortisol	QN	347.2	311.3	5.06	40	1	6000	2	54	10	22	12	0.5
	QL	347.2	121.0	5.06		2			54	10	38	20	
	d_8	355.3	319.1	5.03		1			54	10	22	18	
(2) 11-Deoxycortisol	QN	347.2	97.0	5.79	40	1	2500	1	54	10	28	20	1.0
	QL	3472	109.0	5.79		2			54	10	34	21	
	d_5	352.3	100.0	5.75		1			54	10	29	21	
(2) Corticosterone	QN	347.2	121.1	6.09	40	1	5000	1	54	10	29	21	1.0
	QL	347.2	293.2	6.09		2			54	10	26	16	
	d_8	355.3	125.1	6.02		1			54	10	35	16	
(1) Aldosterone	QN	361.3	315.1	5.29	40	1	10,000	5	49	10	27	20	1.5
	QL	359.2	189.0	5.29		2			−40	10	−26	−14	
	d_4	365.2	319.3	5.29		1			49	10	27	20	
DHEA	QN	271.2	213.2	6.40	40	1	5000	1	70	10	23	19	1.0
	QL	289.3	253.1	6.40		2			43	10	12	22	
	d_5	276.2	218.1	6.36		1			70	10	23	19	
DHT	QN	291.3	255.2	7.02	40	1	5000	5	25	10	21	10	1.0
	QL	291.3	159.2	7.02		2			25	10	29	13	
	d_3	294.2	258.3	7.00		1			25	10	21	10	
Testosterone	QN	289.3	97.0	6.58	40	2	8000	1	66	10	27	14	1.0
	QL	289.3	109.1	6.58		1			66	10	31	14	
	d_3	292.3	97.0	6.56		1			56	10	27	12	
(3) 17-OH-Progesterone	QN	331.3	109.1	6.69	40	1	5000	1	10	10	32	16	1.0
	QL	331.3	97.0	6.69		2			10	10	29	14	
	$^{13}C_3$	334.4	112.0	6.69		1			10	10	32	16	
(3) 11-Deoxy-corticosterone	QN	331.3	97.1	7.32	40	1	1500	1	38	10	58	14	1.0
	QL	331.3	109.0	7.32		2			38	10	31	14	
	d_8	339.4	113.0	7.32		1			38	10	33	16	
Androstenedione	QN	287.3	109.0	7.44	40	1	5000	1	67	10	30	14	0
	QL	287.3	97.1	7.44		2			67	10	27	14	
	$^{13}C_3$	290.3	112.1	7.44		1			67	10	30	16	
Progesterone	QN	315.3	109.0	8.61	40	1	8000	1	10	10	30	18	0
	QL	315.3	97.0	8.61		2			10	10	27	14	
	$^{13}C_3$	318.2	112.0	8.61		1			10	10	30	14	
DHEAS	QN	367.1	97.0	3.77	40	1	3000	1	−55	−10	−12	−7	0
	QL	367.1	367.1	3.77		2			−55	−10	−50	−7	
	d_6	373.1	97.9	3.77		1			−55	−10	−20	−11	
Estradiol	QN	271.0	145.1	5.30	40	2	150	10	−182	−10	−51	−13	3.0
	QL	271.1	143.0	5.30		1			−182	−10	−68	−15	
	d_5	276.1	145.0	5.30		1			−182	−10	−67	−13	

(1), (2), (3) Respective isobaric compounds. Precursor ion, Q1; product ion, Q3: retention time, RT; priority, P; threshold, TH; dwell weight, DW; declustering potential, DP; entrance potential, EP; collision energy, CE; collision cell exit potential, CXP;
Quantifier, QN; qualifier, QL; dehydroepiandrosterone, DHEA; dihydrotestosterone, DHT; dehydroepiandrosterone sulfate, DHEAS.

was adapted through a slightly modified gradient. Different additives in the aqueous mobile phase, as well as different ionization techniques, ESI and atmospheric pressure chemical ionization (APCI), were tested to achieve the best sensitivity, especially for estradiol. As also stated in the previously mentioned study, ESI in combination with an aqueous 0.25 mM ammonium fluoride solution was selected. Since the column has a particle size of only 1.7 μm, it may rapidly clog with residual matrix components. To avoid sensitivity loss, further purification by an automated on-line SPE extraction was the method of choice. As chromatographic separation took place in the ultra high pressure range, a pressure-stable extraction column was essential. For this purpose, we could test a recently introduced extraction column from Phenomenex, the Strata C8 online extraction column (20 × 2.1 mm), which can withstand high backpressures. For the loading and washing of the extraction column, different water/methanol ratios were tested for the gradient, based on the logP values of the individual steroids. As mentioned previously, selected ratios are described in Fig. 1B.

Once the method was developed, CE-breakdown curves were generated in matrix to verify the method, especially all mass transitions used for quantification.

The CE with the highest relative response (100 %) was selected for most steroids to be used in the method. Due to an oversaturation at higher concentration levels for cortisol, 11-deocycorticosterone, and DHEAS, these were detuned along the CE curve. Another application of the CE-breakdown curves is the differentiation of isobars, especially if the same mass transitions are used [13]. This was not necessary for this method, since all structures and hence the isobaric ones were chromatographically separated (Fig. 2).

Along the retention time axis, a total ion current chromatogram for all 15 analytes is shown. A multiple reaction monitoring chromatogram of each analyte is depicted at the top of the figure. Analytes: Cortisol, F; cortisone, E; 21-deoxycortisol, 21-DF; aldosterone, ALDO; 11-deoxycortisol, S; corticosterone, B; dehydroepiandrosterone sulfate, DHEAS; estradiol, E2; dehydroepiandrosterone; DHEA; testosterone, T; 17-OH-progesterone, 17-OHP4; 11-deoxycorticosterone, DOC; androstenedione, A4; progesterone, *P*4.

3.2. Method validation

Linearity was attained over the entire calibration range with a correlation coefficient of $R^2 \geq 0.998$. The S/N of the LLOQ (calibrator 1) was at least 10 for all steroids. In addition, the mean accuracy and

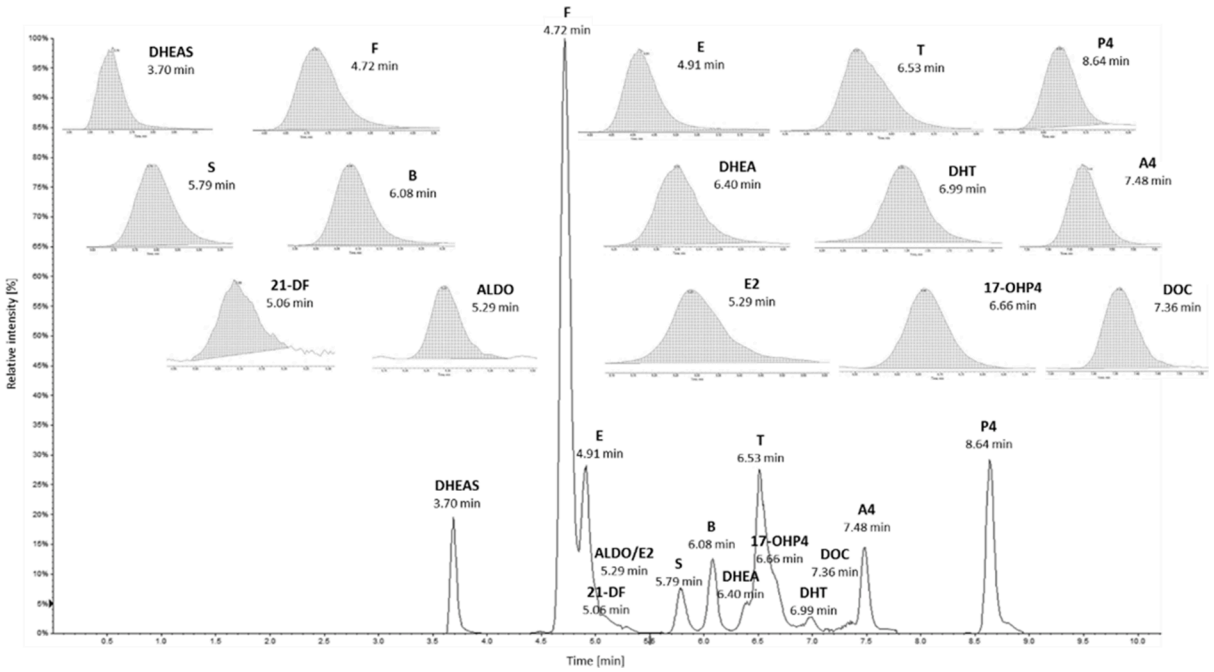


Fig. 2. Total ion current chromatogram for all 15 analytes of a UHPLC-MS/MS analysis of calibrator 4.

imprecision values were within ± 10 % for the three inter-assay days. Accuracy and imprecision (intra- and inter-assay) for all quality controls tested were within the limits of the EMA guideline (±15 %). In addition, 95.5 % of the accuracy values were even within ± 10 %. Maximum accuracy deviation was found for 11-deoxycortisol with 112.9 % (inter-assay, QC 1, manufacturer R) and imprecision for 21-deoxycortisol with 7.4 % (inter-assay, QC 1, manufacturer C). Table 3 shows the accuracy and imprecision values of all quality controls tested. Mean intensity values and retention times were calculated with the SST solutions for all analytes over the three inter-assay days, with deviations within ± 10 % (exception: progesterone with 12.6 %). Ion ratios were within the required 20 % for most of the compounds. For Aldosterone, 21-deoxycortisol and DHEA ion ratios at, and near, the LLOQ were higher. Highest carry-over signal was found for aldosterone with 6.2 % of the LLOQ. Dilution integrity could be demonstrated as deviations of all steroids for QC 3 were below the 15 % suggested by the EMA (between 0.1 % and 13.7 % across the three-dilution steps). All analytes were stable in the processed samples for at least 24 h stored in the autosampler, with a maximum accuracy deviation of 10.7 % for 17-OH-progesterone.

The result of the post-column infusion experiment indicated no relevant matrix effect (Fig. 3). An ion enhancement could be suspected for progesterone, showing a kind of peak in the blank serum line compared to the solvent. This can be disproved by the fact that the used blank serum from manufacturer C shows a recovery of progesterone of up to 70 % of the LLOQ defined herein.

On the left side, results of the post-column infusion experiment are shown for steroid panel 1, and on the right side for steroid panel 2 (both from manufacturer C). The infusion chromatogram of a solvent solution (methanol/water, 1/1) injection (blue line) and the one of a serum blank sample injection (red line) are lapped, with all analytes being post-column infused.

Analytes: Cortisol, F; cortisone, E; 21-deoxycortisol, 21-DF; aldosterone, ALDO; 11-deoxycortisol, S; corticosterone, B; dehydroepiandrosterone sulfate, DHEAS; estradiol, E2; dehydroepiandrosterone; DHEA; testosterone, T; 17-OH-progesterone, 17-OHP4; 11-deoxycorticosterone, DOC; androstenedione, A4; progesterone, P4.

3.3. Method applicability

The proposed and applied structured measurement series with defined acceptance criteria for additional standardization can be found in Table 4.

All acceptance criteria were met for the following measurement results. Accuracies for all included analytes of both EQA samples from the RfB were < 15 % and the values were within the validated calibration range. All geometric mean concentrations for the 40 measured anonymized and randomized leftover serum samples were within the literature reference ranges (Table 5). Concentrations below the LLOQ were not included in the calculations.

4. Discussion

In this article, we have demonstrated the improvement of two panels of a CE-IVD kit by introducing an automated on-line SPE. The improved, validated multiplex steroid SID UHPLC-MS/MS method resulted in one, short 11 min run for the detection of 15 endogenous steroids in human serum, instead of two separate runs (of 10 min and 11.7 min each) as part of the original method. Since sample preparation is an important and critical issue, we reduced both time and physical effort during sample preparation by upgrading from a manual off-line SPE to an automated on-line SPE. Protein precipitation in combination with on-line SPE is resource efficient, powerful and helps to extend the lifetime of the analytical column. Moreover, especially for routine tasks, rapid analysis is mandatory to achieve high-throughput. Even though the setup of on-line SPE may seem complex, once installed it can be used automatically over a long period of time. In our routine laboratory, we have already had decades of positive experience with this technique, e.g., in the high-volume analysis of immunosuppressive drugs. On-line SPE has been published frequently for steroids in combination with tandem mass spectrometry [19–21]. This shows the increasing importance of this technique in the field of laboratory medicine where it is already used for endocrinological diagnostics [22–24].

Within validation, all criteria proposed by the EMA were met for the described measurement procedure. Intra- and inter-assay accuracy deviations for all 15 steroids were ≤ 12.9 % for all tested quality control samples (manufacturer C and R). Imprecision values were ≤ 7.4 %. Ion

Table 3
Intra- and inter-assay accuracy and imprecision (CV) results for manufacturer C and R.

Steroid		QC 1 (C)		QC 2 (C)		QC 3 (C)		QC 1 (R)		QC 2 (R)		QC 3 (R)	
		Intra-Assay	Inter-Assay	Intra-Assay	Inter-Assay	Intra-Assay	Inter-Assay	Intra-Assay	Inter-Assay	Intra-Assay	Inter-Assay	Intra-Assay	Inter-Assay
Cortisol	c [ng/mL]	25.10		59.80		174		5.24		14.80		79.90	
	Accuracy [%]	100.2	99.5	101.2	100.3	101.8	100.4	99.1	100.9	102.1	102.3	104.9	104.1
	CV [%]	0.9	1.5	2.1	1.6	2.3	1.8	1.1	1.4	0.3	0.7	0.7	1.2
Cortisone	c [ng/mL]	2.05		11.70		29.20							
	Accuracy [%]	98.3	98.0	100.6	99.9	100.3	99.8						
	CV [%]	1.3	1.6	1.6	1.4	1.2	1.2						
21-Deoxycortisol	c [ng/mL]	0.096		0.383		2.33		0.247		0.708		4.07	
	Accuracy [%]	101.2	98.6	97.4	96.9	95.7	97.0	105.0	105.1	99.3	100.5	103.2	101.5
	CV [%]	6.6	7.4	3.0	4.6	3.4	5.2	5.3	5.3	4.1	5.2	1.1	5.9
11-Deoxycortisol	c [ng/mL]	0.304		1.49		9.71		0.229		0.719		3.94	
	Accuracy [%]	102.6	102.0	100.1	100.3	100.7	100.5	111.0	112.9	102.4	103.2	104.0	103.9
	CV [%]	3.2	2.4	3.5	2.2	2.5	1.6	2.4	1.9	0.8	1.0	2.2	1.4
Corticosterone	c [ng/mL]	0.821		4.11		28.4							
	Accuracy [%]	102.9	101.0	97.9	97.9	105.5	105.5						
	CV [%]	2.3	2.1	3.1	2.1	1.4	1.4						
Aldosterone	c [ng/mL]	0.097		0.239		0.946							
	Accuracy [%]	99.1	99.3	101.8	102.3	105.9	103.6						
	CV [%]	3.9	5.5	4.2	3.3	1.5	3.1						
DHEA	c [ng/mL]	2		12		39.5							
	Accuracy [%]	103.1	106.0	93.6	94.3	95.1	95.0						
	CV [%]	3.1	3.5	2.3	2.0	1.7	1.8						
DHT	c [ng/mL]	0.078		0.365		1.1							
	Accuracy [%]	101.6	101.4	98.0	98.4	96.1	98.0						
	CV [%]	4.0	4.5	1.1	1.6	1.9	2.8						
Testosterone	c [ng/mL]	0.199		1.46		7.92		0.186		0.513		2.84	
	Accuracy [%]	101.2	101.1	99.9	99.1	96.4	96.3	109.3	110.4	107.6	107.8	106.7	105.6
	CV [%]	2.3	1.8	1.1	1.2	1.7	1.4	0.9	1.1	0.8	1.2	0.6	1.0
17-OH-Progesterone	c [ng/mL]	0.286		1.42		8.61		0.238		0.658		3.53	
	Accuracy [%]	108.5	108.4	102.8	103.3	99.2	99.2	107.9	108.6	108.5	110.6	109.5	109.5
	CV [%]	3.0	3.2	2.1	2.0	1.5	1.3	2.9	2.6	1.2	2.0	0.4	1.1
11-Deoxy-corticosterone	c [ng/mL]	0.076		0.193		0.954							
	Accuracy [%]	105.8	107.1	104.1	104.3	104.2	104.2						
	CV [%]	2.6	2.7	1.9	1.6	1.8	1.3						
Androstenedione	c [ng/mL]	0.275		1.09		8.92		0.206		0.584		3.27	
	Accuracy [%]	103.9	103.2	97.4	97.8	97.7	98.2	104.5	105.7	100.9	100.9	98.4	98.2
	CV [%]	1.4	1.5	1.2	1.1	0.4	0.9	0.6	1.3	1.0	1.1	0.5	0.5
Progesterone	c [ng/mL]	0.311		2.98		14.9							
	Accuracy [%]	97.6	97.8	95.7	96.8	93.1	94.7						
	CV [%]	1.6	2.4	1.1	1.9	0.6	1.5						
DHEAS	c [ng/mL]	268		1535		4857		97		256		1396	
	Accuracy [%]	96.9	96.6	91.8	90.3	89.3	89.8	109.4	108.8	107.0	107.3	97.2	96.9
	CV [%]	2.4	1.8	4.3	3.1	1.8	1.7	0.9	1.4	1.1	1.0	0.9	1.1
Estradiol	c [ng/mL]	0.083		0.42		2.64							
	Accuracy [%]	102.6	95.7	96.0	94.8	92.3	92.9						
	CV [%]	4.0	7.5	5.3	4.5	3.6	3.3						

Concentrations and results printed in grey are outside the calibration range.

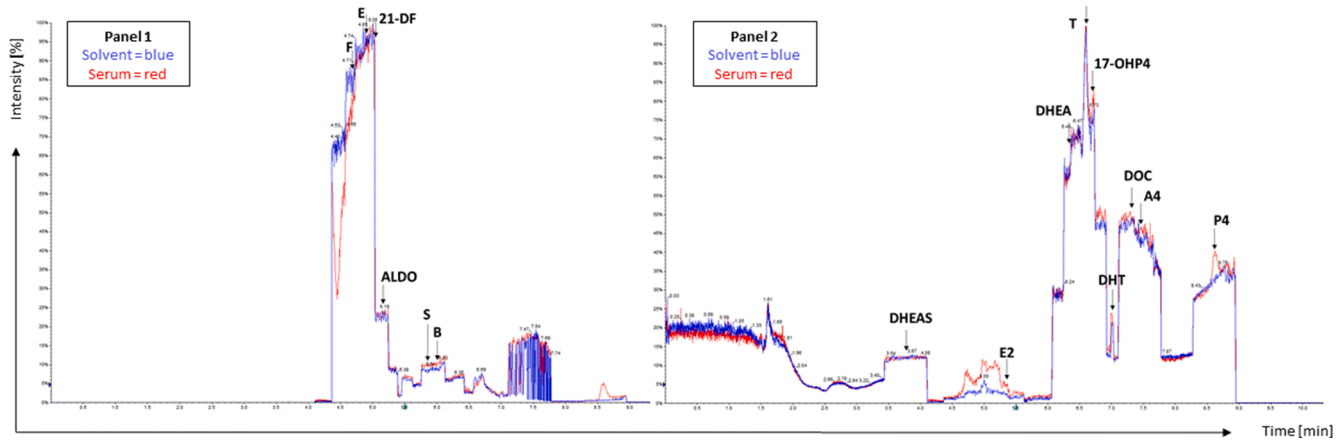


Fig. 3. Chromatograms of the post-column infusion experiment.

Table 4
Overview of the defined acceptance criteria for routine analyses.

Sample	Acceptance criteria
SST	Retention time ± 10 %; intensity ± 20 %
Solvent	
Blank	Area < 20 % of Calibrator 1
Zero	No analyte signal > Blank
Calibrator 1–6	R^2 > 0.995, minimum of 5 with ± 15 % accuracy
Solvent	Carry over < 20 % of Calibrator 1
Quality control 1–3	±15 % accuracy
Solvent	
Unknown samples (max. 15)	If more than 4 series of unknown samples are measured, another series of quality controls should be measured in between; QL/QN < 20 %
Solvent	
Unknown samples (max. 15)	
Solvent	
Solvent	
Quality control 1–3	±15 % accuracy
Solvent	
Calibrator 1–6	
Solvent	

ratios for aldosterone, 21-deoxycortisol and DHEA at and near the LLOQ were partially higher than the maximum 20 % expected by the EMA. For the first two steroids, this is related to insufficient sensitivity of the qualifier in the LLOQ region. For aldosterone, in particular, it was difficult to select a second mass transition for the qualifier because no second mass transition was sensitive enough in the positive ion mode. As a result, a negative ion mode had to be selected, which also did not match the transition for the internal standard. For DHEA, different adducts were used for quantification ($[M-H_2O]^+$) and qualification ($[M+H]^+$), and only one internal standard transition was used, which fits to the quantifier. This can lead to different physicochemical properties of qualifier and internal standard and imperfectly compensated matrix effects, especially in the low concentration range. However, this would only be an issue for unknown concentrations near the LLOQ and would then need to be reviewed on a case-by-case basis. Carry over was far below the maximum tolerance of 20 % of the LLOQ for all analytes studied and was, therefore, negligible.

Results of the two EQA samples showed the applicability of the method for routine diagnostic use.

With well-defined acceptance criteria of each measurement series, such as intensity and retention time of the SST or carryover, important method characteristics can be routinely checked (Table 4). This will contribute to improved standardization in addition to the applied commercial material. The analysis of the 40 anonymized samples showed the suitability of the method for a wide concentration range described in the literature and, hence, the routine use in endocrionological diagnostics. This was demonstrated by the fact that all samples were evaluable, no overlap of analyte and internal standard occurred and no additional peaks were visible.

The procedure described herein represents the modification of IVD-

Table 5
Results for anonymized test samples. Geometric mean results of anonymized test samples of 40 patients and reference ranges obtained from adults based on the literature (1) [29–34], (2) [35] and (3) [36]. Samples below the lower limit of quantification (LLOQ) were not included in the calculation of the geometric mean.

Compound	Geometric mean [ng/mL]	Minimal c [ng/mL]	Maximum c [ng/mL]	Reference range [ng/mL]
Cortisol	142.1	10.2	970.5	7.20–216 (1)
Cortisone	13.0	1.12	84.48	10.1–32.5 (2)
21-Deoxycortisol	0.193	0.073	0.559	< 0.103 (2)
11-Deoxycortisol	0.427	0.105	8.66	0.082–1.35 (3)
Corticosterone	1.76	0.068	44.14	0.45–12.60 (3)
Aldosterone	0.095	0.038	0.255	0.029–0.31 (1)
DHEA	4.15	1.50	11.58	0.80–27.00 (3)
DHT	0.130	0.050	0.548	0.05–1.10 (1)
Testosterone	0.293	0.040	5.14	0.077–8.18 (3)
17-OH-Progesterone	0.805	0.112	4.69	0.152–2.54 (3)
11-Deoxycorticosterone	0.101	0.050	1.74	< 0.16 (2)
Androstenedione	0.948	0.205	2.57	0.20–2.60 (1)
Progesterone	0.529	0.140	2.30	0.078–17.82 (3)
DHEAS	718.6	131.6	2598	441–3749 (1)
Estradiol	1.01	0.172	2.93	0.01–4.00 (1)

(1) Lexikon der Medizinischen Laboratoriumsdiagnostik (2019)
(2) Eisenhofer et al. (2017)
(3) Fanelli et al. (2011)

CE certified products beyond their intended purpose / intended use – which, thereby, represents in vitro diagnostics from in-house manufacturing. In the European Union this has to comply with the requirements of the EU In Vitro Diagnostic Regulation (IVDR) [25–27].

Fanelli et al showed, in a recent study (HarmoSter) involving nine European centers, that the use of commercially available calibrators can improve interlaboratory agreement, supporting the use of these calibrators to harmonize steroid analyses. However, the authors also noted that LDTs can be as powerful as kits and that methods with high HPLC resolution are required for specificity [28]. The method presented here addresses both of these important considerations by using commercial calibration and high-resolution chromatography.

All analytes covered by the method have well-established diagnostic significance. The availability of a multi-analyte measurement method, as described, is attractive to diagnostic laboratories and much more convenient from a practical point of view compared to separate methods addressing single analytes.

In conclusion, we describe an innovative validated stable-isotope dilution UHPLC-MS/MS measurement procedure for the simultaneous quantification of 15 steroids in serum with good sensitivity and selectivity. The use of commercially available calibrator and quality control samples and an internal standard solution in combination with the automation by on-line SPE improved practicability and robustness substantially.

Ethical approval

As only discarded, fully anonymized patient samples were used, neither informed consent nor IRB review were required.

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.

References

[1] A.H. Payne, D.B. Hales, Overview of Steroidogenic Enzymes in the Pathway from Cholesterol to Active Steroid Hormones, Endocr. Rev. 25 (6) (2004) 947–970.

[2] M. Vogeser, C. Seger, Pitfalls Associated with the Use of Liquid Chromatography-Tandem Mass Spectrometry in the Clinical Laboratory, Clin. Chem. 56 (8) (2010) 1234–1244.

[3] J. Marcos, O.J. Pozo, Current LC–MS methods and procedures applied to the identification of new steroid metabolites, J. Steroid Biochem. Mol. Biol. 162 (2016) 41–56.

[4] G. Di Dalmazi, F. Fanelli, M. Mezzullo, E. Casadio, E. Rinaldi, S. Garelli, E. Giampalma, C. Mosconi, R. Golfieri, V. Vicennati, U. Pagotto, R. Pasquali, Steroid Profiling by LC-MS/MS in Nonsecreting and Subclinical Cortisol-Secreting Adrenocortical Adenomas, J. Clin. Endocrinol. Metab. 100 (9) (2015) 3529–3538.

[5] N. Janzen, M. Peter, S. Sander, U. Steuerwald, M. Terhardt, U. Holtkamp, et al., Newborn Screening for Congenital Adrenal Hyperplasia: Additional Steroid Profile using Liquid Chromatography-Tandem Mass Spectrometry, J. Clin. Endocrinol. Metab. 92 (7) (2007) 2581–2589.

[6] M. Rauh, Steroid measurement with LC–MS/MS. Application examples in pediatrics, J. Steroid Biochem. Mol. Biol. 121 (3) (2010) 520–527.

[7] M. Vogeser, K.G. Parhofer, Liquid Chromatography Tandem-mass Spectrometry (LC-MS/MS) - Technique and Applications in Endocrinology, Exp. Clin. Endocrinol. Diabetes 115 (09) (2007) 559–570.

[8] T. Jl, C. Benattar, A.S. Birr, A. Lindenbaum, Limitations of Steroid Determination by Direct Immunoassay, Clin. Chem. 48 (3) (2002) 583–585.

[9] M.E. Wierman, R.J. Auchus, D.J. Haisenleder, J.E. Hall, D. Handelsman, S. Hankinson, W. Rosner, R.J. Singh, P.M. Sluss, F.Z. Stanczyk, Editorial: The new instructions to authors for the reporting of steroid hormone measurements, J. Clin. Endocrinol. Metab. 99 (12) (2014) 4375.

[10] B.G. Keevil, LC–MS/MS analysis of steroids in the clinical laboratory, Clin. Biochem. 49 (13) (2016) 989–997.

[11] R.M. Büttler, F. Martens, M.T. Ackermans, A.S. Davison, A.E. van Herwaarden, L. Kortz, J.G. Krabbe, E.G.W. Lentjes, C. Syme, R. Webster, M.A. Blankenstein, A. C. Heijboer, Comparison of eight routine unpublished LC–MS/MS methods for the simultaneous measurement of testosterone and androstenedione in serum, Clin. Chim. Acta 454 (2016) 112–118.

[12] E. Olesti, J. Boccard, G. Visconti, V. González-Ruiz, S. Rudaz, From a single steroid to the steroidome: Trends and analytical challenges, J. Steroid Biochem. Mol. Biol. 206 (2021) 105797.

[13] S. Mörlein, C. Schuster, M. Paal, M. Vogeser, Collision energy-breakdown curves – An additional tool to characterize MS/MS methods, Clinical Mass Spectrom. 18 (2020) 48–53.

[14] European Medicines Agency, Guideline on bioanalytical method validation, Committee for Medicinal Product for Human Use (CHMP). London, UK2011.

[15] Clinical and Laboratory Standards Institute (CLSI): C62-A - Liquid Chromatography-Mass Spectrometry Methods; Approved Guideline.October 2014.

[16] R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle, The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds, Rapid Commun. Mass Spectrom. 13 (12) (1999) 1175–1185.

[17] M. Vogeser, C. Schuster, A.L. Rockwood, A proposal to standardize the description of LC-MS-based measurement methods in laboratory medicine, Clin. Mass Spectrom. 13 (2019) 36–38.

[18] J.M. Lindner, M. Vogeser, S.H. Grimm, Biphenyl based stationary phases for improved selectivity in complex steroid assays, J. Pharm. Biomed. Anal. 142 (2017) 66–73.

[19] C. Seger, K. Tentschert, W. Stöggl, A. Griesmacher, S.L. Ramsay, A rapid HPLC-MS/MS method for the simultaneous quantification of cyclosporine A, tacrolimus, sirolimus and everolimus in human blood samples, Nat. Protoc. 4 (4) (2009) 526–534.

[20] M. Vogeser, C. Fleischer, B. Meiser, J. Groetzner, U. Spöhrer, D. Seidel. Quantification of Sirolimus by Liquid Chromatography-Tandem Mass Spectrometry Using On-Line Solid-Phase Extraction. 2002;40(1):40-5.

[21] T. Koal, M. Deters, B. Casetta, V. Kaever, Simultaneous determination of four immunosuppressants by means of high speed and robust on-line solid phase extraction–high performance liquid chromatography–tandem mass spectrometry, J. Chromatogr. B 805 (2) (2004) 215–222.

[22] M. Vogeser, J. Briegel, K. Jacob. Determination of Serum Cortisol by Isotope-Dilution Liquid-Chromatography Electrospray Ionization Tandem Mass Spectrometry with On-line Extraction. Clinical chemistry and laboratory medicine 2001. p. 944.

[23] G. Karvaly, K. Kovács, K. Mészáros, I. Kocsis, A. Patócs, B. Vásárhelyi, The comprehensive characterization of adrenocortical steroidogenesis using two-dimensional ultra-performance liquid chromatography – electrospray ionization tandem mass spectrometry, J. Pharm. Biomed. Anal. 153 (2018) 274–283.

[24] D. Luque-Córdoba, F. Priego-Capote, Fully automated method for quantitative determination of steroids in serum: An approach to evaluate steroidogenesis, Talanta 224 (2021) 121923.

[25] M. Vogeser, IVDR and diagnostic application of mass spectrometry in the European Union, J. Mass Spectrom. Adv. Clin. Lab. 19 (2021) 32–33.

[26] M. Vogeser, M. Brüggemann, Complex analytical procedures in diagnostic laboratories and the IVDR, Clin. Chem. Lab. Med. 59 (3) (2020) 457–458.

[27] M. Vogeser, M. Brüggemann, J. Lennerz, A. Stenzinger, U.M. Gassner, Laboratory-Developed Tests in the New European Union 2017/746 Regulation: Opportunities and Risks, Clin. Chem. 68 (1) (2021) 40–42.

[28] F. Fanelli, M. Cantù, A. Temchenko, M. Mezzullo, J.M. Lindner, M. Peitzsch, J. M. Hawley, S. Bruce, P.-A. Binz, M.T. Ackermans, A.C. Heijboer, J. Van den Ouweland, D. Koeppl, E. Nardi, F. MacKenzie, M. Rauh, G. Eisenhofer, B.G. Keevil, M. Vogeser, U. Pagotto, Report from the HarmoSter study: impact of calibration on comparability of LC-MS/MS measurement of circulating cortisol, 17OH-progesterone and aldosterone, Clin. Chem. Lab. Med. 60 (5) (2022) 726–739.

[29] M. Bidlingmaier. Estradiol. In: Gressner AM, Arndt T, editors. Lexikon der Medizinischen Laboratoriumsdiagnostik. Berlin, Heidelberg: Springer Berlin Heidelberg; 2019. p. 811-3.

[30] M. Bidlingmaier. Dihydrotestosteron. In: Gressner AM, Arndt T, editors. Lexikon der Medizinischen Laboratoriumsdiagnostik. Berlin, Heidelberg: Springer Berlin Heidelberg; 2019. p. 703-5.

[31] M. Bidlingmaier. Androstendion. In: Gressner AM, Arndt T, editors. Lexikon der Medizinischen Laboratoriumsdiagnostik. Berlin, Heidelberg: Springer Berlin Heidelberg; 2019. p. 128-9.

[32] W. Hubl. Kortisol. In: Gressner AM, Arndt T, editors. Lexikon der Medizinischen Laboratoriumsdiagnostik. Berlin, Heidelberg: Springer Berlin Heidelberg; 2019. p. 1392-3.

[33] W. Hubl. Aldosteron. In: Gressner AM, Arndt T, editors. Lexikon der Medizinischen Laboratoriumsdiagnostik. Berlin, Heidelberg: Springer Berlin Heidelberg; 2019. p. 62-5.

[34] W. Hubl. Dehydroepiandrosteronsulfat. In: Gressner AM, Arndt T, editors. Lexikon der Medizinischen Laboratoriumsdiagnostik. Berlin, Heidelberg: Springer Berlin Heidelberg; 2019. p. 665-6.

[35] G. Eisenhofer, M. Peitzsch, D. Kaden, K. Langton, C. Pamporaki, J. Masjkur, G. Tsatsaronis, A. Mangelis, T.A. Williams, M. Reincke, J.W.M. Lenders, S. R. Bornstein, Reference intervals for plasma concentrations of adrenal steroids measured by LC-MS/MS: Impact of gender, age, oral contraceptives, body mass index and blood pressure status, Clin. Chim. Acta 470 (2017) 115–124.

[36] F. Fanelli, I. Belluomo, V.D. Di Lallo, G. Cuomo, R. De Iasio, M. Baccini, E. Casadio, B. Casetta, V. Vicennati, A. Gambineri, G. Grossi, R. Pasquali, U. Pagotto, Serum steroid profiling by isotopic dilution-liquid chromatography-mass spectrometry: comparison with current immunoassays and reference intervals in healthy adults, Steroids 76 (3) (2011) 244–253.