



An isotope dilution LC–MS/MS-based candidate reference method for the quantification of androstenedione in human serum and plasma

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ABSTRACT

The accurate measurement of androstenedione in human serum and plasma is required for steroid profiling to assure the appropriate diagnosis and differential diagnosis of hyperandrogenism. In this work, we introduce an isotope dilution liquid chromatography–tandem mass spectrometry (LC–MS/MS) candidate reference measurement procedure for the quantification of androstenedione in human serum and plasma. The performance of the procedure enables its use in the evaluation and standardization of routine assays and for the evaluation of patient samples to ensure the traceability of individual patient results. As the primary standard, a certified reference material from NMIA (National Measurement Institute, Australia) was used. Additionally, a quantitative nuclear magnetic resonance (qNMR) method was developed for the value assignment of the primary reference material, which ensures the direct traceability to SI units, as well as the independence from the availability of reference materials. ¹³C₃-labeled androstenedione was used as the internal standard. The introduced method allows the measurement of androstenedione in the range of 0.05–12 ng/mL, and the assay imprecision was found to be <2% between 5 and 12 ng/mL, 3.5% at 1.5 ng/mL, and 5.2% at 0.05 ng/mL, with an accuracy of 95–105% for the serum and 91–103% for the plasma matrix. The transferability to a second laboratory was validated by method comparison based on 112 patient samples. The comparison of the results obtained from the presented method and an LC–MS/MS routine assay, using 150 native patient samples, showed a good correlation with a bias of the routine method of ≤4.0%.

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1. Introduction

Androstenedione is a sex hormone that belongs to the androgens, a class of steroid hormones, and it has a steroidal structure comprising 19 carbon atoms. It is primarily produced from dehydroepiandrosterone (DHEA) using the enzyme, 3 β -hydroxysteroid-dehydrogenase [1]. It is reduced to testosterone using testosterone-17 β -dehydrogenase and converted into estrone with aromatase [2]. In an adult male, androstenedione is formed primarily in the testicles, whereas in an adult female, it is produced in the adrenals and ovaries [3]. Since androstenedione is formed as an intermediate product in testosterone and estradiol biosyntheses, it is a hormone precursor and is, therefore, referred to as a pro-

hormone [4]. The reasons for increased androstenedione levels are hirsutism, polycyclic ovary syndrome, adrenal hyperplasia, and the Cushing syndrome [3,5]. Contrarily, adrenal insufficiency and ovarian failure may be the reasons for a lowered androstenedione level [3,5]. For many years, routine clinical methods for the detection of steroids have been based on immunoassays [6]. However, routine diagnostic assays may suffer from inappropriate calibration and are susceptible to interferences from related compounds, metabolites, or matrix effects (MEs), which lead to incorrect measurement results [7–9]. Thus, reference measurement procedures (RMPs), used for the standardization of clinical laboratory tests, are required [10,11]. To improve standardization, RMPs have been successfully applied within the Hormones Standardization Program of the CDC [11,12]. In addition to this established use of RMPs, there is a need to enable the evaluation of routine assays based on individual patient samples to identify

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patient-related interference effects and to offer the possibility of result traceability for individual samples.

There are several liquid chromatography–mass spectrometry (LC–MS)–based methods for the quantification of androstenedione published in the literature [13–15]; however, thus far, no reference measurement procedure is known to us.

In this paper, we report on an isotope dilution liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the quantification of androstenedione in human serum and plasma using a $^{13}\text{C}_3$ -labeled androstenedione as the internal standard. As the primary standard, a certified reference material from NMIA (National Measurement Institute, Australia) was used. Additionally, a quantitative nuclear magnetic resonance (qNMR)–based procedure was established that enables the determination of the absolute content of androstenedione in different raw materials. Thus, qNMR can be used for the value assignment of the primary reference material, being directly traceable to SI units and independent of the availability of the certified reference material.

The quality requirements for the reference methods were described by Thienpont et al. [16]; they differentiated between “genuine requirements,” including direct calibration with the primary reference materials and the absence of sample-related effects, and “performance specifications,” including the limits for random, systematic, and total error. Depending on the intended use (e.g., evaluation, validation or calibration of routine assays, method comparison studies, or analysis of complaint samples), the performance specifications of a RMP have to be aligned. Stöckl et al. described options to define goals for bias and imprecision for a reference measurement procedure [17], one of which is related to the biological variation. For androstenedione, the performance specifications could, thus, be defined as CV_{ref} half of CV_{rou} and B_{ref} one third of B_{rou} . Based on $\text{CV}_{\text{rou}} \leq 7.9\%$ and $\text{B}_{\text{rou}} \leq 10.47\%$ [18], the acceptance criteria for a RMP can be defined as $\text{CV}_{\text{ref}} \leq 4\%$ and $\text{B}_{\text{ref}} \leq 3.5\%$.

This development of this method was aimed at optimizing a candidate RMP for the quantification of androstenedione, which is (i) metrological traceable, (ii) allows the measurement of the “true value,” (iii) enables the evaluation and standardization of routine assays, and (iv) enables the evaluation of patient samples to ensure the traceability of individual patient results.

2. Materials and methods

2.1. Chemicals and reagents

Methanol, acetonitrile, and formic acid were of ULC/MS grade and purchased from Biosolve (Valkenswaard, The Netherlands). Deionized water was produced in-house with a Milli-Q IQ 7000 system from Merck (Burlington, Massachusetts, USA). CDCl_3 (865–49–6), 1,2,4,5-tetrachloro-3-nitrobenzene (CAS 117-18-0), progesterone (CAS 57-83-0), testosterone (CAS 58-22-0), 17-hydroxyprogesterone (CAS 68-96-2), and cortisol (CAS 50-23-7) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Zinc sulfate heptahydrate (CAS 7446-20-0), as the precipitating agent, and isopropanol were obtained from Honeywell (Muskegon, Michigan, USA). The standard for androstenedione (CAS 63-05-8) was purchased from the NMIA (NMIA M955, Batch: 04-S-02, West Lindfield, Australia) and as a USP reference standard (Lot F040W0) from U.S. Pharmacopeia (Rockville, MD, USA). A solution of the internal standard, androstene-3,17-dione-2,3,4- $^{13}\text{C}_3$ (CAS 327048-86-2), was purchased from Cerilliant (Round Rock, Texas, USA). The steroid-free human serum was charcoal-stripped (Roche Diagnostics GmbH, Mannheim, Germany). A multi-individual pooled human plasma was obtained from Roche Diagnostics GmbH (Mannheim, Germany) for use as the plasma matrix. The patient samples were anonymized leftover samples and were in accordance with the Helsinki Declaration.

2.2. qNMR for the determination of the purity of the standard materials

The qNMR technique was utilized to estimate the absolute content of androstenedione within different raw materials (i.e., the standard from the NMIA M955 and the USP reference standard Lot F040W0). The quantification was performed with 1,2,4,5-tetrachloro-3-nitrobenzene, a qNMR internal standard available from Sigma-Aldrich, which is traceable to the SI units. Six individual sample weights were prepared using the USP reference standard to determine the reproducibility and standard deviation (SD). Therefore, androstenedione (in the range of 7.56–10.37 μmol) and the qNMR standard (between 5.98 and 9.58 μmol) were exactly weighed together in a glass vial on an ultra-microbalance (X6PRU) from Mettler Toledo. The molar ratios of the analyte and ISTD were normalized according to the number of protons, contributing to the considered signals of the analyte and ISTD (e.g., comparing a-CH₃ (3 protons) signal from A to a *tert*-butyl group (9 protons) signal from B should lead to an A:B molar ratio of around 3:1).

CDCl_3 (700 μL) was added to the glass vial, and after 15 s of vortexing, the solution was transferred to a 5 mm NMR tube (Bruker Biospin, Rheinstetten, Germany).

To prove the method as suitable for the determination of the absolute content, the certified NMIA material was determined by weighing androstenedione (7.89 μmol) and the internal standard (6.13 μmol) together in a glass vial on an ultra-microbalance (X6PRU). The NMR measurement was performed as described below.

The NMR measurements were performed on a JEOL 600 MHz NMR spectrometer equipped with an ultra-cool probe head, which provides 4- to 5- times sensitivity enhancement than the normal room temperature probe heads and enables the use of a lower amount of sample material. For the ^1H NMR measurement, a standard 1D 90° pulse was applied at 300 K, utilizing 64 K data points, and the olefinic proton signal ($\delta \approx 5.67$ ppm) was chosen for quantification. The number of scans was limited to 128 with automatic receiver gain conditions. The relaxation delay was set to 70 s, based on the T1 measurement of the analyte and qNMR standard signals in the given solution. Processing was performed with an exponential window function (line broadening = 0.3 Hz), followed by the manual phase and baseline corrections.

2.3. Calibrators, quality control samples (QC), and internal standard solution

For the preparation of the calibrators, two independent stock solutions (stock solutions I a and I b) were always freshly prepared using the certified reference material from NMIA M955. Therefore, 1 mg of androstenedione was weighed twice on an ultra-microbalance (XP6U/M, Mettler Toledo) and dissolved in 100 mL of acetonitrile using a volumetric flask. These solutions were further diluted by pipetting 1 mL into a 10 mL volumetric flask to achieve final androstenedione concentrations of 1 $\mu\text{g}/\text{mL}$ in 50% methanol in Milli-Q water (stock solutions II a and II b). Starting from these two stock solutions, eight calibrator spike solutions were generated by alternating dilution with 50% methanol in Milli-Q water, achieving concentrations of 2.50 ng/mL, 5.00 ng/mL, 12.5 ng/mL, 25.0 ng/mL, 75.0 ng/mL, 150 ng/mL, 300 ng/mL, and 600 ng/mL. The absolute concentrations of the spike levels were adjusted using the purity value of the NMIA certificate. The final matrix-matched calibration levels were prepared by a 1:50 dilution of each spike solution to a total volume of 1 mL steroid-free human serum, achieving a concentration range of 0.05–12.0 ng/mL. The samples were equilibrated for 30 min on

an overhead rotation mixer at 40 rpm. For the determination of the plasma samples, the serum calibration was used.

Three anonymized native patient samples with different concentrations (i.e., low, medium, and high) were used as QC samples to establish a control chart. Aliquots (100 μ L) of these samples were stored at -80°C until they were required.

The internal standard, $^{13}\text{C}_3$ -androstenedione, was delivered in acetonitrile with a concentration of 100 $\mu\text{g}/\text{mL}$. For the internal standard solution, the stock solution of $^{13}\text{C}_3$ -androstenedione was diluted (1:2000) with 50% methanol in Milli-Q water.

For the determination of linearity, three independent calibration curves (stock solutions I a and I b) were prepared, consisting of six individual sample weights. To show linearity over the entire calibration range (0.05–12 ng/mL), two additional spike solutions covering $\pm 20\%$ of the desired calibration range were prepared with final concentrations of 2.0 ng/mL and 750 ng/mL. The concentrations of the final spiked serum samples were 0.04 ng/mL and 15 ng/mL.

Accuracy and precision were assessed using the spiked samples with final concentrations of 0.05 ng/mL, 1.5 ng/mL, 5.0 ng/mL, 12 ng/mL, 20 ng/mL, and 30 ng/mL. Therefore, one of the existing stock solutions (from the linearity experiment) was used to prepare the spike solutions (see Table 1, Supplemental Material). The final samples were prepared by a 1:50 dilution of the spike solutions into 1 mL of the serum or plasma sample. Levels with final concentrations of 20 ng/mL and 30 ng/mL were only spiked in the serum and were diluted with acetonitrile (1:5) after solid-phase extraction (SPE) to test if the samples with concentrations above the calibration range could be properly measured.

2.4. Sample preparation

The internal standard solution (10 μ L) was added to aliquots of 100 μ L of serum/plasma/calibrators/controls and incubated on an overhead shaker for 10 min at room temperature; 0.5 mL polypropylene microtubes were used together with screw caps (Sarstedt, Nümbrecht, Germany). Subsequently, the samples were treated with 200 μ L of the precipitating agent (ZnSO_4 in water (89 g/L)/methanol 1/4 v/v), mixed on a thermomixer for 10 min at 1400 rpm (5382 thermomixer C, Eppendorf AG, Hamburg, Germany), and centrifuged for 10 min at 16,000 rcf and 10°C (centrifuge 5430 R, Eppendorf). For the final sample purification, 200 μ L aliquots of the supernatant were loaded onto Oasis prime HLB (30 mg) cartridges from Waters (Eschborn, Germany) by centrifuging the samples for 2 min at 50 rcf (centrifuge 5810 R, Eppendorf). The cartridges were washed with 500 μ L of water (centrifuging for 3 min at 100 rcf), after which the purified androstenedione was eluted by applying two times the volume of 100 μ L acetonitrile (centrifuging for 3 min at 50 rcf). Both phases were collected in a HPLC vial, and the samples were analyzed in duplicate. A schematic overview is given in Supplemental Fig. 1.

2.5. HPLC and MS/MS conditions

An ABSciex QTrap 6500 instrument equipped with an electrospray ionization source (ESI) (Framingham, MA, USA) coupled to an Agilent Infinity 1290 LC system (Waldbronn, Germany), which includes two binary pumps, a column temperature control device, and an autosampler, was used for the measurements. The ABSciex Analyst 1.6.2 software (Framingham, Massachusetts, USA) provided data collection and instrument control.

Chromatographic separation was performed on a Raptor biphenyl column (100 \times 2.1 mm, 2.7 μm , Restek, Bellefonte, Pennsylvania, USA) equipped with a Raptor C18 EXP guard column (5 \times 2.1 mm, 2.7 μm , Restek). The column oven was maintained at 30°C , and the injection volume was 5 μ L. The mobile phases consisted of water (A) and acetonitrile (B), each containing 0.1% formic acid. The chromatographic separation was performed at a flow rate of 500 $\mu\text{L}/\text{min}$ with a linear gradient starting from 20% to 45% of mobile phase B within 2.5 min. Subsequently, mobile phase B was increased to 95% from 2.5 to 5.0 min and held for 1.0 min. Subsequently, mobile phase B was ramped to 20% and a 2 min equilibration process was performed to give a total run time of 8.5 min.

The parameters of the mass spectrometer were optimized as follows: curtain gas pressure, 30 psi; collision gas, high; spray voltage, 5000 V; temperature, 550°C ; ion-source gas pressure 1 60 psi; ion-source gas pressure 2 50 psi; and entrance potential, 10 V. To avoid charging, the MS was switched between positive and negative modes. The measurements were started in the negative mode, and after 0.5 min, the MS was switched to the positive mode. After a further 0.5 min, the MS was switched again to the negative mode. From 1.5 to 8.5 min, the MS was used in the positive mode. For all the analytes and internal standards, multiple reaction monitoring was performed. The acquired mass transitions are shown in Table 1.

2.6. Long-term traceability and data acquisition

To assure the long-term traceability of the method, a system suitability test (SST) was established to examine the sensitivity and chromatographic resolution before every sequence.

Therefore, two samples were analyzed: sample 1 was a mixture of testosterone and androstenedione, both at a concentration of 10 ng/mL in 50% methanol; for sample 2, a processed sample of level 2 of the calibrator (0.13 ng/mL) was used. The resolution was calculated as follows:

$$R_s = (1.18 \times (tr_2 - tr_1)) / (w_{0.5(1)} + w_{0.5(2)}) \geq 3.00$$

(tr_1 = retention time of testosterone, tr_2 = retention time of androstenedione, $w_{0.5(1)}$ = full width half maximum of testosterone, $w_{0.5(2)}$ = full width half maximum of androstenedione).

The sensitivity of the system was tested using sample 2. The signal to noise ratio (S/N) was calculated using the Analyst software, calculating it as the peak height divided by the noise. To calculate

Table 1
MS/MS parameters for the target steroids and their internal standards.

Analyte	Parent	Daughter	DP* (V)	CE* (V)	CXP* (V)	Time (ms)
Androstenedione quantifier	287.1	97.1	76	27	16	100
Androstenedione qualifier	287.1	109.0	76	29	14	100
$^{13}\text{C}_3$ -Androstenedione quantifier	290.1	99.9	56	27	10	100
Testosterone	289.2	96.9	101	27	10	100
Progesterone	315.2	97.1	26	25	12	100
17-Hydroxyprogesterone	331.2	109.0	1	31	14	100
Cortisol	363.2	120.9	81	31	14	100
11-Deoxycortisol	347.1	96.9	71	29	10	100

*DP = declustering potential, CE = collision energy, CXP = collision exit potential.

the noise, the software uses the SD (using a mean of zero) of all the chromatographic data points between the specified background. The S/N ratio of sample 2 must be ≥ 10 to fulfill the acceptance criteria. To calculate the S/N ratio, the background from 3.3 to 3.6 min is used. A chromatogram of the SST is shown in Fig. 1.

Additionally, three levels of anonymized native patient samples (QCs) covering the measuring range were analyzed within every sequence, and the results were monitored using a control chart (chromatograms of QC 2 and 3 are shown in Fig. 2(a and b)).

If the SST passed, data were acquired as follows: calibrator block, acetonitrile blank, native patient samples (QCs), acetonitrile blank, unknown patient samples (first injection), acetonitrile blank, unknown patient samples (second injection), acetonitrile blank, calibrator block, and patient samples. (QCs)

2.7. Data processing

For the raw file processing, the Analyst software, Version 1.6.2, was used with Analyst Classic as the Quantitation Integration Algorithm. For the peak integration, we adjusted the Bunching Factor to one and the Number of Smooths to three. The calibration curve was linear with a $1/x$ weighting; the origin was ignored, and for the response, the area was obtained. The mean value of two injections was reported as the result in ng/mL. The calibration functions (using 8 levels) were obtained by linear regression of the area ratios of the analyte and internal standard (y) against the analyte concentration (c_A) resulting in the function, $y = ac_A + b$.

2.8. Requirements for general lab equipment

The ultra-microbalance (XP6U/M, Mettler Toledo) used was calibrated and certified. The minimum sample weight was determined according to USP guidelines (USP Chapters 41 and 1251). Each weighing process had to be in accordance with the determined minimum sample weight. The pipettes used were from Eppendorf (Eppendorf AG, Hamburg, Germany); each pipette was calibrated and certified by the manufacturer. The requalification

intervals were compliant with the requirements communicated by the manufacturers.

All the solvents were used at room temperature.

3. Method validation

The intended use of this method is the quantification of androstenedione in human serum and plasma. Assay validation was performed according to existing guidelines [19].

3.1. Selectivity, specificity, and ME

The selectivity of the method was verified by analyzing analyte-free samples of stripped human serum, which was used for the preparation of the calibrators. The analyte-free stripped serum should show no interfering signals for the Selected Reaction Monitoring (SRM) transition at the expected retention time of androstenedione. The steroids, progesterone, testosterone, 17-hydroxyprogesterone, and cortisol, were added to the stripped serum to show the separation of these steroids from androstenedione.

Specificity was determined by performing a post-column infusion experiment, as well as an experiment based on Matuszewski et al. [20,21]. A neat androstenedione solution with a concentration of 10 ng/mL in 50% methanol was infused post-column, and processed serum and plasma samples were injected (for sample preparation see Section 2.4). Any change (i.e., decrease or increase) of the analyte signal indicates that the matrix components are interfering with the analyte [22].

A comparison of the calibrations in the native (pool of 15 samples) and steroid-free serum samplers, as well as in the neat solution, was conducted to evaluate the specificity of the method during the method development. The calibration with the native human serum and that with the analyte-free serum were conducted as described in Section 2.4. For the calibration in the neat sample, the spike solutions (see Sections 2.3 and 2.4) were diluted to obtain the same final concentrations as in the two calibrations in

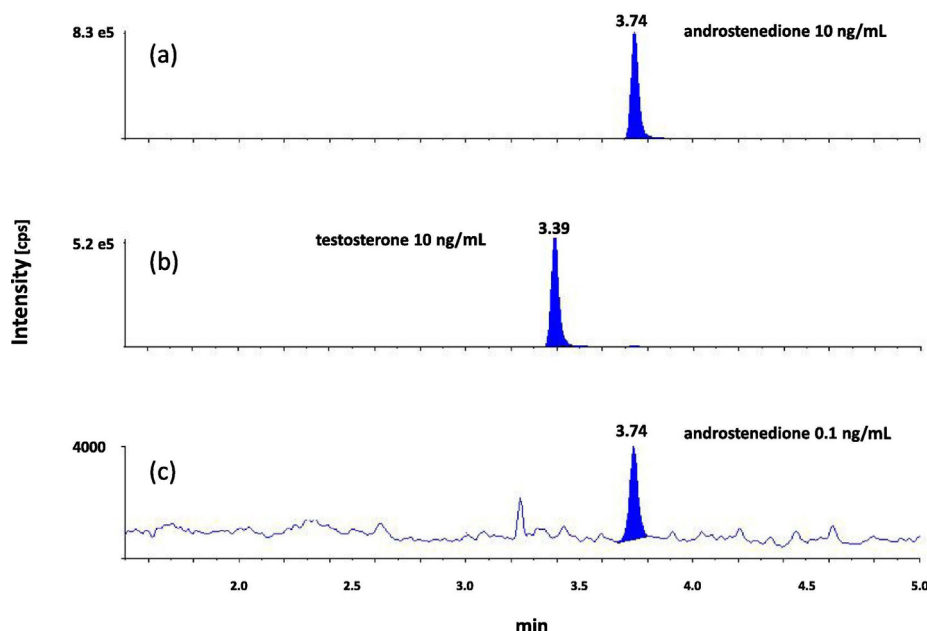


Fig. 1. Chromatograms of the SST. (a) The androstenedione peak of the SST sample in 50% methanol with a concentration of 10 ng/mL; (b) shows the testosterone peak of the SST sample with a concentration of 10 ng/mL; (c) shows the androstenedione peak of calibrator 2 in the stripped serum with a concentration of 0.1 ng/mL.

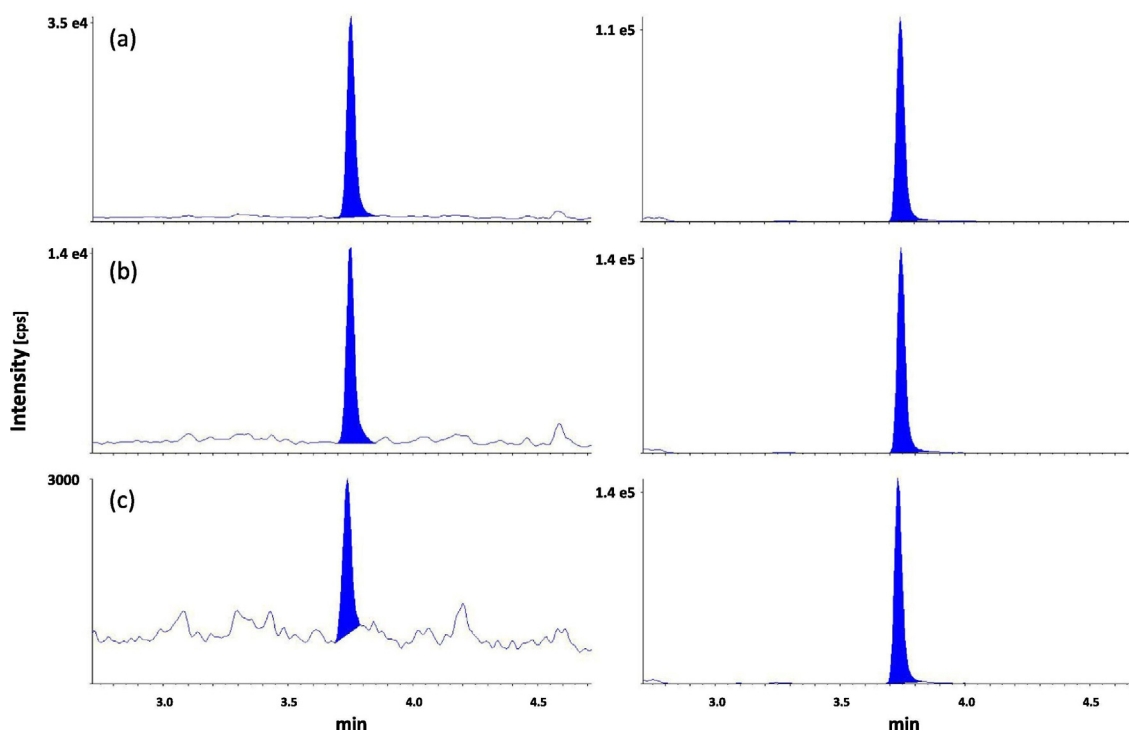


Fig. 2. (a) Chromatogram of a female native patient sample (QC 2) with a concentration of 1.2 ng/mL, analyte (left) and internal standard (right); (b) Chromatogram of a male native patient sample (QC 3) with a concentration of 0.45 ng/mL, analyte (left) and internal standard (right); (c) Chromatogram of a spiked stripped serum sample with a concentration of 0.05 ng/mL.

the matrix. No difference in the slope between the three calibrations should be observed if the ME is excluded [23].

In addition, to examine possible MEs, the ion-suppression experiment according to Matuszewski et al. [20,21] was carried out.

For this purpose, three sets of samples were generated in either acetonitrile (set 1) or in the native human serum (set 2 and set 3), at three different concentrations, each in five replicates. Since there was no analyte-free native serum available, a pool consisting of 15 individual donors was prepared to use the most diverse matrix. The androstenedione concentration of this pool was estimated using the above-described method. The measured androstenedione level was 0.21 ng/mL. For the preparation of set 1, a solution of acetonitrile with the same analyte concentration (0.21 ng/mL) was used.

Set 1 and set 3 were prepared by spiking different analyte solutions (1:50) into the androstenedione-enriched acetonitrile solution (set 1) or the human serum (set 3) before sample extraction. The second set consisting of the human serum (set 2) and analyte was spiked after the extraction procedure to give the same nominal concentration level as in sets 1 and 3. The final concentration levels were 0.25, 1.50, and 12.00 ng/mL.

For the preparation of the samples from set 1, a 100 μ L aliquot of the sample was transferred into HPLC vials and mixed with 10 μ L of the internal standard solution, followed by 200 μ L of acetonitrile instead of the precipitation agent. For the preparation of the samples from the second and third sets, the described sample preparation procedure was used. Since the samples from the second set were spiked after the sample preparation, the respective volumes for the spiked and internal standard solutions were substituted using a solution of 50% methanol in water. After SPE, the samples were evaporated to dryness and reconstituted in 200 μ L of acetonitrile containing the analyte and the internal standard. For this purpose, 980 μ L of acetonitrile was mixed with 20 μ L of the spiked solution. Subsequently, 100 μ L of the internal standard solution and 2000 μ L of acetonitrile were added.

All the samples were prepared in five replicates and injected twice. With the average value of the analyte and internal standard peak area of every set and concentration, the ME, recovery (RE), and the process efficiency (PE) were calculated with the following equations: $ME (\%) = \text{Set 2}/\text{Set 1} \times 100$; $RE (\%) = \text{Set 3}/\text{Set 2} \times 100$; $PE (\%) = \text{Set 3} / \text{Set 1} \times 100$ [17,18].

3.2. Accuracy and imprecision

For the determination of accuracy and precision, analyte-free serum was spiked in four different concentrations (0.05, 1.50, 5.00 and 12.00 ng/mL). All the levels were prepared in six replicates and injected twice. The whole experiment was repeated independently on two different days including columns from two different batches. To prove the suitability of the method for the plasma matrix, four concentration levels were prepared in triplicates. Additionally, samples with high concentrations of 20.00 ng/mL and 30.00 ng/mL were spiked and diluted (1:5) with acetonitrile after the sample preparation. Accuracy was reported as the percentage of recovery of the measured concentration in relation to the spiked concentration. The inter-day precision data were estimated from this experimental setup with a variance component analysis (VCA) approach and are expressed as CV. The inter-day precision was calculated over two days, considering the calibration effect, sample preparation, and repeated measurements.

3.3. Linearity and lower limit of measuring interval

Linearity was determined by measuring three independently prepared calibration curves (each calibration curve consists of two sample weights), each with two injections, by the analysis of eight calibrator levels and two additional spiked samples to extend the working range by $\pm 20\%$ (0.04, 0.05, 0.10, 0.25, 0.50, 1.50, 3.00, 6.00, 12.00, and 15.00 ng/mL). The ratio of the analyte peak area to the internal standard peak area was plotted against the respective

androstenedione concentration (ng/mL). A $1/x$ weighting was used for the linear regression; the correlation coefficient and residuals for each curve were determined.

Based on the intended use of the assay, the lower limit of the measuring interval (LLMI) was set to the lowest calibration level with a concentration of 0.05 ng/mL. The acceptance criterion for precision at the LLMI was defined as $CV \leq 10\%$.

3.4. Sample stability

The stability of the processed samples on the autosampler was investigated at 4 °C, and the samples were protected from light for 96, 120, 168, and 192 h. For this purpose, three patient samples at different concentration levels were used. The recoveries were calculated by comparing the measured value with the original value ($t = 0$ h).

The stability of the spiked serum samples was investigated at a storage temperature of -20 °C for 1, 3, 6, 9, 12, 14, and 15 days. For this purpose, three spiked serum samples with concentrations of 0.10 ng/mL, 1.50 ng/mL, and 12.00 ng/mL were prepared and measured using freshly prepared serum calibrators. Using these concentrations, 3-point-calibrations were generated over the 15 days and the slopes were compared.

3.5. Uncertainty of measurements

The uncertainty of measurements was determined according to the “Evaluation of measurement data – Guide to the expression of uncertainty in measurement” GUM [24]. For the calculation of uncertainty, the following steps were considered: preparation of calibrators, preparation of internal standard and precipitating agents, preparation of samples, measurement of calibrators, generation of the calibration curve, and measurement and evaluation of the sample results. The estimation of the uncertainty for the preparation of calibrators was performed as type B evaluation. All other aspects such as calibration, sample preparation, and measurement and evaluation of the sample result were evaluated in the above-described precision experiment. The total uncertainty for the whole measurement procedure was calculated as a combination of the uncertainty of the calibrator preparation (unc_{cal}) and the uncertainty estimated from the precision experiment (unc_{prec}). The two uncertainties, unc_{cal} and unc_{prec} , were combined as

$$unc_{total} = \sqrt{unc_{cal}^2 + unc_{prec}^2}$$

The derived total uncertainty was multiplied by a coverage factor of $k = 2.23$ to obtain an expanded uncertainty. The coverage factor of $k = 2.23$ corresponds to a confidence level of 95%, considering that 10 degrees of freedom was available for the estimations in the precision experiment [25].

The uncertainty of the purity of the material determined via qNMR was calculated according to GUM. The sample preparation steps, including weighing of the primary reference material, weighing of the internal standard, and all the steps associated with the measurement, were evaluated as type A by repeated weighing and measurement. The purity of the internal standard was considered as type B evaluation.

For details about the uncertainty calculations, see [Supplemental Description 1](#).

4. Method comparison

To assess the agreement of the reference method between two independent laboratories (Laboratory 1: Roche Penzberg, and Laboratory 2: Universitätsklinikum Erlangen), a method comparison study including 112 native anonymized leftover patient samples

was performed. Therefore, the described reference method was established at Laboratory 2. The calibrator levels were prepared independently in each laboratory as described above. Both sites used the certified reference material (NMIA M955) as the primary standard.

To demonstrate and validate the use of the RMP for the evaluation of the bias and sample-related effects of a routine assay, the RMP described in this paper was compared with a routine assay based on an online SPE-HPLC-MS/MS method developed by Rauh et al. [26]. All the routine measurements were performed at Laboratory 2. For a more detailed description of the SPE-HPLC-MS/MS assay, see [Supplemental Description 2](#).

For method comparison, 149 native anonymized patient samples were measured at both sites. The concentrations of the native patient samples for both comparisons were distributed over the entire measurement range, and the measurements were randomized to simulate the conditions in a routine clinical laboratory. All the patient samples were anonymized leftover samples. No information about gender or origin was available.

5. Results

5.1. Traceability to SI units

Regarding the aim to develop a RMP, a higher-order reference material is essential to establish traceability to SI units. In this method, we used the NMIA certified reference material, androstenedione (M955), which was characterized by a combination of traditional analytical techniques, such as gas chromatography with flame ionization detection, thermogravimetric analysis, Karl Fischer analysis, and 1H NMR.

The qNMR technique is becoming increasingly prominent for evaluate the absolute content of reference standards, as the amplitude of the measured response for a particular signal in a highly optimized qNMR experiment is directly proportional to the number of the nuclei (nuclear spins). For this purpose, a method was developed with the current USP lot of androstenedione (Lot F040W0). The determination of the absolute content entails the unambiguous realization of the chemical identity and purity; therefore, a suite of 1D and 2D NMR pulse sequences (1H NMR, ^{13}C NMR, COSY, TOCSY, HSQC, and HMBC) was resorted to reaffirm the structure of the androgen steroid. The six different measurements with USP androstenedione yielded an average purity of 98.89% with a CV of 0.13%. With such slight variance and consistent content results available, the afore-mentioned method was evaluated by determining the chemical purity (content g/g) of the NMIA androstenedione reference standard. The structure (identity) of the NMIA standard was confirmed by comparison with the 1H NMR spectra of the USP material, since the USP lot was already characterized by the relevant 1D and 2D NMR experiments. When the afore-mentioned procedure was applied to the NMIA androstenedione, a content value of $99.12 \pm 0.41\%$ ($k = 2.57$) was obtained, which is in excellent conformity with the certificate value of $99.5 \pm 1.4\%$ ($k = 2$) (see [Supplemental Description 3](#)). For future applications and long-term traceability, qNMR will provide the opportunity for independence from the availability of a certified reference material.

5.2. Specificity/selectivity

The goal was to optimize an assay with the highest possible selectivity and to minimize the occurrence of possible interferences coming from the matrix or structurally related compounds. For this reason, the focus was on a baseline separation to other common steroids and on the optimization of the MS parameters to be as selective as possible. Therefore, various HPLC columns

and solvent compositions were tested. The use of a Raptor biphenyl column with water and acetonitrile, each containing 0.1% formic acid, as eluents allowed the baseline separation of androstenedione, 11-deoxycortisol, testosterone, progesterone, 17-hydroxyprogesterone, and cortisol within a total runtime of 8.5 min (see Fig. 3). The addition of formic acid was important, since the carryover of the analyte could be resolved.

In addition, the calibrators were prepared in the neat solution, and the slope was compared to those of the serum-based calibrators. No difference between the slope of the calibrators with the different equilibration times (10, 30, and 60 min) and those in the neat solution was observed.

Sample preparation was done by protein precipitation, followed by SPE using an Oasis HLB material, which allowed the removal of common matrix interferences, such as salts, proteins, and phospholipids. Since the used sorbent is water-wettable, the conditioning and equilibration of the sorbent were not required, and the samples could be applied directly. Afterward, the purified and enriched analyte was eluted using acetonitrile.

5.3. MEs

Validation was performed using spiked matrix samples in human serum and plasma. The selectivity and specificity were determined by analyzing the analyte-free and spiked human serums. No interfering signals were detected at the respective retention time of androstenedione. The possible ion-suppression or enhancement effects from the serum or plasma were evaluated by performing a post-column infusion experiment, which showed no interfering signals at the retention time of androstenedione independent of the

evaluated matrix. Additionally, the MEs were determined as described by Matuszweski et al. [20,21]. Therefore, ME, RE, and PE were calculated by comparing the three sets described in the method validation section, and the results were reported as percent recovery. The values for ME > 100% indicate ionization enhancement, and a value < 100% suggests ionization suppression [20,21]. The ME values ranged from 95 to 100% for androstenedione and from 94 to 96% for the internal standard, $^{13}\text{C}_3$ -androstenedione, proving the method to be matrix independent.

The overall RE and PE varied from 90 to 101% for the analyte and from 89 to 95% for the internal standard, with relatively low RE and PE obtained at high concentrations (see Table 2). However, the comparison of the area ratios for the different sets showed comparable values, which confirms the compensating effect of the presence of the labeled internal standard. Thus, the reliable quantification of androstenedione is possible.

The representative calibration for each matrix (neat, pooled, and stripped serums) was evaluated to confirm the method as being matrix independent. The slope and *r* values for the different calibration curves in the neat, native, and stripped serums were determined for the evaluation of the ME. The *r* values were 1.00 for all the three calibration models. The slope (95% CI) was 0.061 (0.059–0.064) for the calibration curve in the pooled native serum, 0.062 (0.059–0.064) for the calibration curve in the stripped serum, and 0.062 (0.060–0.064) for the calibration curve in acetonitrile, wherefore the absence of the ME can be confirmed.

The comparison of the different calibration curves in different matrices and the neat sample show a similar extractability for androstenedione from the calibrators and the native samples. Thus, for this particular RMP, a matrix-matched calibration is preferred to have a calibrator material that is as similar as possible to the patient samples.

5.4. Accuracy

Within the validation, the bias was determined at four different concentration levels in the serum and plasma. Additionally, to show the bias for highly concentrated samples, two levels were spiked (20 and 30 ng/mL), and the samples were prepared as described above and diluted with acetonitrile (1:5) before measurement. The bias for the individual measurements ranged from –5% to 5% (median: –2%) for the serum and between –9% and 3% (median: –6%) for the plasma matrix (see Table 3).

5.5. Imprecision

For the determination of the assay imprecision, the serum samples were spiked in four different concentrations, each prepared six times. The CVs are shown in Table 3 and range from 1.8% to 5.2% for the levels close to the LLMI.

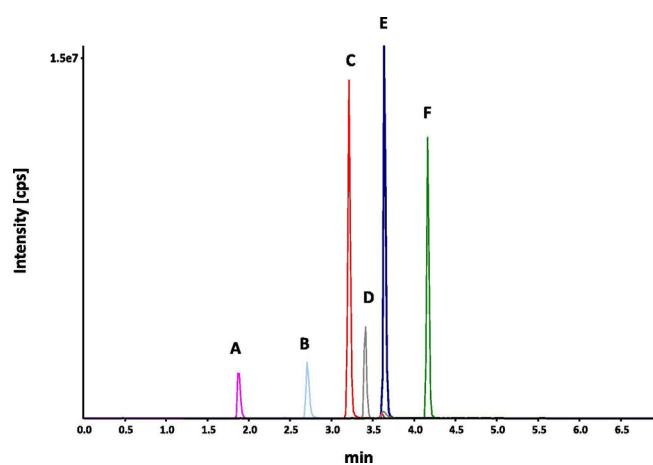


Fig. 3. Representative LC-ESI-MS/MS ion-chromatogram of the quantifier mass transitions in acetonitrile for (A) cortisol, (B) 11-deoxycortisol, (C) testosterone, (D) 17-hydroxyprogesterone, (E) androstenedione, and (F) progesterone.

Table 2

Matrix effect (ME), recovery (RE), and process efficiency (PE) data.

	Spiked Concentration (ng/mL)	Androstenedione (%)	$^{13}\text{C}_3$ -Androstenedione (%)	Area Ratio (Analyte/Internal Std.) (%)
ME ^a (%)	0.25	100	96	105
	1.50	97	96	102
	12.00	95	94	101
RE ^b (%)	0.25	101	94	107
	1.50	93	93	101
	12.00	95	95	100
PE ^c (%)	0.25	101	90	113
	1.50	91	89	102
	12.00	90	89	101

^a ME expressed as a percentage of the ratio of the mean peak area of set 2 to the mean peak area of set 1.

^b RE expressed as a percentage of the ratio of the mean peak area of set 3 to the mean peak area of set 2.

^c PE expressed as a percentage of the ratio of the mean peak area of set 3 to the mean peak area of set 1.

Table 3
Accuracy and imprecision data of the method for serum and plasma samples.

Concentration (ng/mL)	Accuracy (%)		Precision (CV%)
	Serum	Plasma	
0.05	97	97	5.2
	100	99	
	103	103	
1.50	105	94	3.5
	95	94	
	100	95	
5.00	97	94	2.0
	97	94	
	97	94	
12.00	99	95	1.8
	99	91	
	97	92	
20.00	98	–	
	97		
	98		
30.00	102	–	
	102		
	102		

For the determination of accuracy and precision, analyte-free serum was spiked in different concentrations in the serum and plasma matrix. All levels were prepared in six replicates and injected twice.

5.6. Linearity and lower limit of measuring interval

The linearity was proven by the analysis of ten spiked serum calibrators covering $\pm 20\%$ of the anticipated working range (preparation $n = 3$, six individual sample weights). For determining the linearity, residuals were calculated that represent the vertical distance between the observation point and the estimated regression line. The residuals were determined for linear and quadratic regressions. The visual evaluation of the residuals showed that a linear regression was well suited (see [Supplemental Fig. 2](#)).

The ratios of the analyte peak and internal standard peak area were plotted against the androstenedione concentration in ng/mL. The correlation coefficients were used to judge the quality of the calibration curve, calculated by a weighted linear regression. If the correlation coefficient was better than 0.99, the calibration curve was used for the calculation of the results. The correlation coefficients were $r = 0.999$ for all three preparations. Based on these working range investigations, the calibration range was set to 0.05–12.00 ng/mL.

The imprecision data at the concentration level of 0.05 ng/mL showed a CV of 5.2%, which is slightly higher than the proposed bias criterion of 4%. Considering the acceptance criterion for precision at the LLMI ($CV \leq 10\%$), the LLMI was set to this concentration. A chromatogram of a spiked sample in the stripped serum with a concentration of 0.05 ng/mL is shown in [Fig. 2\(c\)](#).

5.7. Stability

The sample stability of the processed samples was determined by comparing the original value ($t = 0$ h) with the values of 4, 5, 7, and 8 days later. The samples were found to be stable for 8 days protected from light and stored at a temperature of 4 °C with a bias

of less than $\pm 5\%$ compared to the original value. The stability of the spiked serum samples was determined by measuring the stored serum samples with freshly spiked calibrators over a storage time of 15 days. The comparison of the slopes of the generated 3-point calibrations showed a CV of 1.7%. Thus, the sample stability was shown for at least 14 days at a storage temperature of -20 °C.

5.8. Uncertainty of results

The expanded uncertainties ($k = 2.23$) for the individual measurements were found to be within 12.9% and 6.6%, covering the measuring range of androstenedione (see [Table 4](#)).

To further reduce uncertainty, e.g., for different intended uses, repeated measurements can be performed. Depending on the requirements of both parts, calibrator preparation, and/or calibration, the sample preparation and measurement can be performed in multiple repetitions. The uncertainty estimates for the repeated measurements can be calculated from the reported values.

5.9. Method transferability to a second laboratory

The evaluation of the transferability of the candidate RMP is an essential part of the validation of the procedure; therefore, a method comparison study containing 112 native patient samples was performed at two independent laboratory sites (Roche Penzberg and Universitätsklinikum Erlangen). The regression analysis involving performing a Passing–Bablok procedure resulted in a regression equation of $y = 1.042 \times -0.0183$ with a 95% confidence interval from 1.030 to 1.053 for the slope and from -0.0263 to -0.0109 for the intercept (see [Table 5](#) and [Fig. 4](#)). The Spearman rank correlation value was 0.997. The comparison of the results by Bland–Altman analysis showed a very good agreement between the two laboratories with a mean bias of 0.9%, fulfilling performance specifications (see [Fig. 5](#)). The slightly observed concentration dependency is within the defined error range, particularly if the low number of samples in the higher concentration range is considered.

5.10. Method comparison study to a routine LC–MS/MS method

A method comparison study involving 149 anonymized native leftover patient samples was performed with the LC–MS/MS routine assay at the Universitätsklinikum in Erlangen. The results showed a very good correlation between the reference method and the routine assay with a Passing–Bablok regression equation of $y = 1.004 \times +0.0258$. The 95% confidence intervals for the slope and intercept ranged from 0.9647 to 1.046 and from 0.0045 to 0.0534, respectively (see [Supplemental Fig. 3](#)). The Spearman rank correlation value was 0.987. Bland–Altman analysis showed a mean bias of 4.0% between the two methods (see [Supplemental Fig. 4](#)). The routine assay is calibrated using a commercially available kit with no information about the primary material used; consequently, this bias is in accordance with the desired specifications for routine assays. Furthermore, on a sample basis, all the differences were within the maximum allowed difference of 31.3%.

Table 4
Total uncertainties of the method.

Level (ng/mL)	Total Uncertainty SD (ng/mL)	Total Uncertainty CV (%)	Exp. Uncertainty SD (ng/mL) ($k = 2.23$)	Exp. Uncertainty CV (%) ($k = 2.23$)
0.05	0.003	5.8	0.006	12.9
1.50	0.062	4.3	0.138	9.7
5.00	0.167	3.4	0.372	7.6
12.00	0.338	3.0	0.754	6.6

Table 5
Method comparison studies.

	Comparison to routine LC–MS/MS method ^a	Inter-laboratory comparison RMP ^b
Intercept A	0.0258	–0.0183
Slope B	1.004	1.042
Correlation coefficient	0.987	0.997
Bias	4.0%	0.9%
+1.96 SD	22.1%	11.8%
–1.96 SD	–14.2%	–10.1%

^a Method comparison study between the reference method (laboratory 1, ROCHE) and the routine LC–MS/MS method (laboratory 2, Universitätsklinikum Erlangen).

^b Method comparison study between the reference method performed at laboratory 1 (ROCHE) and laboratory 2 (Universitätsklinikum Erlangen).

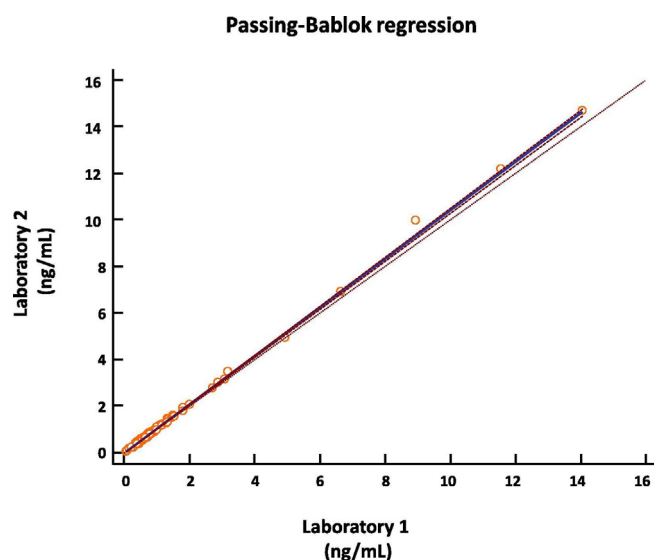


Fig. 4. Passing–Bablok regression plot for the method comparison study of the reference method ($n = 112$ patients) between two independent laboratories (Laboratory 1: Roche Penzberg, and Laboratory 2: Universitätsklinikum Erlangen). The regression analysis resulted in a regression equation of $y = 1.042 \times -0.0183$ with a 95% confidence interval from 1.030 to 1.053 for the slope and from -0.0263 to -0.0109 for the intercept. The Spearman rank correlation value was 0.997.

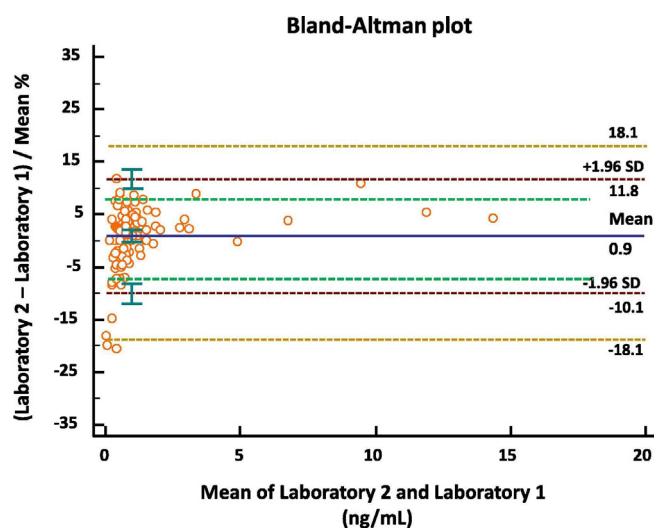


Fig. 5. Relative Bland–Altman plot for the method comparison study of the reference method ($n = 112$ patients) between two independent laboratories (Laboratory 1: Roche Penzberg, and Laboratory 2: Universitätsklinikum Erlangen). The performance specifications for an androstenedione RMP were defined based on the biological variation as $CV_{ref} \leq 4\%$ and $B_{ref} \leq 3.5\%$. The bias limit for the shown difference between the two labs was defined as $2 B_{ref} = 7\%$ (green dotted line). The maximum allowed difference was defined following the total error idea, as $2B_{ref} + 1.96\sqrt{CV_{ref}^2 + CV_{ref}^2} = 18.1\%$ (yellow dotted line). Method comparison showed a very good agreement with a mean bias of 0.9% (blue line).

6. Discussion

This paper describes a LC–MS/MS-based candidate RMP for the quantification of androstenedione in human serum and plasma.

The calibration of the method is directly performed with the primary reference material. The purity assignment of this material is based on certificates or by qNMR; thus, there is no dependence on the future availability of certified materials. The qNMR technique directly ensures the traceability to SI units and has the potential to be a generic approach for other reference materials used in RMPs. The performance of the method proved to be appropriate for the intended use. The genuine requirements described by [16] are fulfilled, including the direct calibration with a certificated primary reference material and, additionally, by the optional use of a qNMR-characterized primary material. The absence of sample-related effects was shown by extensive evaluation of the MEs. The performance specifications including the limits for random, systematic, and total error met the requirements derived from the biological variation [17,18] for androstenedione ($CV_{ref} \leq 4\%$ and $B_{ref} \leq 3.5\%$). The measured imprecision was found to be $<2\%$ between 5 and 12 ng/mL, 3.5% at 1.5 ng/mL, and 5.2% at the LLMI of 0.05 ng/mL, with a median accuracy of 98% for the serum matrix. This method is comparable to the methods described in the literatures in terms of performance [13–15].

The transferability of the RMP to a second independent laboratory was shown based on 112 native patient samples with a bias of 0.9% and a 1.96 SD interval of -10.1 to 11.8% , which is within the maximum allowed difference of 18.1%. This method shows good comparability to a routine LC–MS/MS method with a bias of 4% (based on 149 patient samples). The 1.96 SD interval of -14.2 to 22.1% is within the maximum allowed difference between the reference method and the routine method of 31.3%.

The method can serve as a higher-order standard for the measurement of traceability and can be used for the evaluation, validation, and calibration of new and existing routine methods. The method is configured to enable the measurement of large sample sets of patient samples within a reasonable time, and the measurement of critical individual patient samples for the evaluation of routine assays.

7. Conclusion

In this paper, an analytical protocol based on the isotope dilution LC–MS/MS method for the quantification of androstenedione is reported. The use of highly characterized primary reference standards combined with a highly selective LC–MS/MS method provides a traceable and reliable platform for the standardization of routine assays and for the assessment of clinical samples.

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.

Acknowledgment

Human subjects/informed consent statement

All procedures were in accordance with the Helsinki Declaration. All samples used were exclusively anonymized leftover samples.

Conflict of interest

Katrin Gradl, Judith Taibon, Neeraj Singh, Eva Albrecht, Andrea Geistanger, Stephan Pongratz, Stefan Hutzler, Magdalena Mayer, Christine Kleinschmidt, Verena Hofmann, and Uwe Kobold are all employees of Roche Diagnostics GmbH. Manfred Rauh and Daniel Köppl are employees at the Universitätsklinikum Erlangen. All authors have no conflict of interest to declare.

Honoraria

Manfred Rauh receives a consultant honorarium from Roche Diagnostics GmbH.

Shareholdership, Patent situation, Research funding, Role of sponsor

None declared.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.clinms.2020.01.003>.

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