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Development and validation of a LC-MS/MS method for the establishment of reference intervals and biological variation for five plasma steroid hormones

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ABSTRACT

Background: With liquid chromatography–tandem mass spectrometry (LC-MS/MS) increasingly being used for the quantification of steroid hormones, there is a need for studies that re-establish reference intervals and biological variation in well-defined cohorts.

Methods: A plasma steroid hormone profiling method using LC-MS/MS for quantification of progesterone, 17-hydroxyprogesterone, androstenedione, testosterone and dihydrotestosterone was developed and validated. For reference interval assessment, 280 well-characterized healthy subjects from the LifeLines cohort were selected, including 40 women using oral contraceptive pills (OCP). The biological variation was examined in 30 healthy individuals. Samples were collected over a period of 4 months with 4 week intervals.

Results: The developed method proved to be robust and sensitive. The reference interval levels in men are higher, whereas in women the levels tend to decrease with increasing age. In addition, women using OCP had lower levels of 17-OH-progesterone and androstenedione. The biological variation is generally higher in women compared to men, especially with regard to the inter-individual variation.

Conclusions: The gender-specific determination of the reference intervals, together with the observation that the biological variation demonstrated a high degree of variation, allows interpretation of data on individual and group level for improved biochemical characterization of patients in clinical practice.

1. Introduction

Introduction of liquid chromatography-tandem mass spectrometry (LC-MS/MS) has resulted in increased specificity and accuracy of steroid hormone analysis. Additionally, LC-MS/MS enables profiling of steroid hormones, allowing combined steroid hormone status assessment, including determination of the ratio of specific precursors and products as proxy for enzyme activity [1].

For the interpretation of steroid hormone data we still rely mainly on data obtained with immunochemical assays, despite the proven discrepancies between MS-based and immunochemical methods, especially for low circulating steroid hormone levels [2–4]. This illustrates the need for reference intervals and data on individual variation, established with LC-MS/MS. Reference intervals are usually established in healthy individuals, representing the general population. Steroid hormones can vary considerably between males and females, can be age dependent and are influenced by medication such as oral contraceptive pills (OCP) in women [5,6]. Accordingly, these factors should be taken into account.

Data on individual variation can be found in the online biological variation database, hosted by the Westgard website [7,8]. However, the veracity of this database has been questioned, indicating the need for a harmonized approach [9]. In case of steroid hormones, when present in the database, the results are mainly based on immunochemical

Abbreviations: 17-OH-P, 17-hydroxyprogesterone; ACN, Acetonitrile; ADION, Androstenedione; CVa, Analytical variation; CVg, Between-subject variation; CVi, Within-subject variation; DHT, Dihydrotestosterone; FA, Formic Acid; II, Index of individuality; LC-MS/MS, Liquid chromatography-tandem mass spectrometry; LLOQ, Lower limit of quantitation; MeOH, Methanol; OCP, Oral contraceptive pills; P, Progesterone; PCOS, Polycystic ovary syndrome; QL, Qualifier; QN, Quantifier; RCV, Reference Change Value; RIA, Radioimmunoassay; SPE, Solid phase extraction; T, Testosterone

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methods, and accordingly less suitable to apply. Limited LC-MS/MS-based data is available on the biological variation, possibly because some of the steroids have been difficult to measure due to their presence in low concentrations, including testosterone (T) in women or dihydrotestosterone (DHT) in both men and women. Furthermore, it has been reported that data of the biological variation can be quite difficult to interpret [10].

The aim of our study was to develop and validate an LC-MS/MS method for simultaneous quantification of five steroid hormones in plasma, including progesterone (P), 17-hydroxyprogesterone (17-OH-P), androstenedione (ADION), T and DHT, with minimal sample preparation to allow high-throughput analysis. Reference intervals were established in 240 healthy subjects and in a sub-group of women using OCP. Additionally, the biological variation was established in 30 healthy individuals, generating valuable data for the assessment of serial measurements within a subject.

2. Materials and methods

2.1. Subjects

To established reference values, 240 healthy subjects from the Dutch LifeLines cohort study, a large population based cohort study, were selected [11]. The subjects consisted of 120 men and 120 women, stratified with 20 males and 20 females into 6 consecutive decade groups, ranging from 20 to 79 years. Inclusion criteria included normal blood pressure, normal blood count, no use of medication and BMI between 21 and 30 kg/m^2, the latter representing the general population visiting our medical center. Additionally, 40 women between 20 and 39 years using OCP were included. Women on OCP used combined contraceptives, mostly levonogestrel combined with ethinylestradiol ($n = 30$). Blood samples were collected between 8.00 and 10.00 a.m. after overnight fasting and were obtained by venepuncture using K_2-EDTA tubes (Becton Dickinson, Franklin Lakes, NJ, USA). The samples were centrifuged and the plasma was transferred to plastic tubes and stored at −80 °C until analysis. In women, no information was present on the menstrual cycle, and therefore reference intervals for P were not determined, except for women on OCP.

For the determination of the biological variation, 30 apparently healthy subjects were included at the University Medical Center Groningen (UMCG). All included subjects had no history of diseases related to steroid hormone metabolism and did not use medication that influence steroid hormone levels. In 15 males and 15 females, aged 21–63 years, blood samples were collected at a standardized time, at noon, with 4 week intervals from January to May. Plasma samples, obtained as mentioned above, were stored at −80 °C until analysis. Seven women were postmenopausal, 6 women were premenopausal and 2 women were on OCP. Due to these relative small groups and different phases of the menstrual cycle in the premenopausal women, the biological variation for P was not determined. Both studies were approved by the Medical Ethics Committee of the UMCG, according to the principals of the Declaration of Helsinki. All participants provided written informed consent.

2.2. Reagents and standard solutions

LC-MS and ultra-performance LC grade methanol (MeOH), acetonitrile (ACN) and formic acid (FA) were obtained from Biosolve BV (Valkenswaard, The Netherlands). P, 17-OH-P, ADION, T and DHT and their [$^{13}C_3$]-labeled analogues were purchased from IsoSciences (King of Prussia, PA, USA). For each steroid a stock solution was prepared in methanol, 1 g/L for the androgens and 100 µg/mL for the internal standard analogues. The stock solutions were serially diluted and mixed to generate a working solution of all steroid hormones.

2.3. Instrumentation

Online solid phase extraction (SPE) and LC were performed by an Acquity Online Sample Manager system from Waters (Milford, MA, USA). For online SPE, Waters XBridge™ C8-cartridges (10 × 1 mm) were used. Each cartridge was conditioned with 0.75 mL MeOH and 1.5 mL Magic Mix (MeOH/ACN/isopropanol/water (1:1:1:1), *v*/v + 0.2% FA) and equilibrated with 1 mL water in the right clamp position. Subsequently, 40 µL of the sample was loaded onto the cartridge with 1 mL water and was washed with 0.5 mL water, 0.5 mL 20% MeOH +0.2% ammonium hydroxide, and 0.5 mL 20% MeOH +0.1% FA. Next, the cartridge was transferred to the left clamp position and the steroid hormones were eluted with the gradient pumps towards the column for 1 min. After elution the cartridge was flushed with 1.5 mL Magic Mix and 1 mL water. A new cartridge was placed in the right clamp position, while chromatography was performed on the just eluted sample, allowing the next sample to undergo SPE, resulting in no loss of time for the SPE procedure during the entire cycle.

LC was performed using a Kinetex C18 column (2.6 µm, 100 × 2.1 mm) from Phenomenex (Torrance, CA, USA). The column temperature was kept at 40 °C. The mobile phase of the binary solvent system consisted of 10% methanol with 2 mM ammonium acetate and 0.05% formic acid (eluent A) and methanol with 2 mM ammonium acetate and 0.05% formic acid (eluent B). The flow rate was 0.4 mL/min, with the following gradient: 65%-A, 35%-B to 20%-A, 80%-B in 4 min; 20%-A, 80%-B from 4 to 5 min; and returning to 65%-A, 35%-B from 5 to 6 min. MS detection was performed by a Waters Xevo TQ-S tandem mass spectrometer in positive electrospray ionization (ESI+) and multiple reaction monitoring mode. Settings for the MS were as follows: capillary voltage was set at 0.5Kv, desolvation temperature at 600 °C, desolvation gas-flow at 1000 L/h, cone gas-flow at 150 L/h, and the collision gas-flow at 0.2 mL/min. Cone voltage and collision energies were optimized for all *m/z* transitions. For each androgen and corresponding internal standard the quantifier (QN) and qualifier (QL) m/z transitions were monitored (Supplemental Table 1). For P and T the collision energy was de-optimized due to saturation of the detector. All data were analyzed using Targetlynx™ software (Waters).

2.4. Sample preparation

For analysis, 200 µL of plasma or quality control sample was added to a 2 mL 96-well polypropylene plate (Greiner Bio-One, Kremsmünster, Austria). To each well 25 µL internal standard working solution was added together with 25 µL pepsin solution (Labor Diagnostika Nord, Nordhorn, Germany). The samples were vortex mixed for 1 min, and after incubation of 30 min at room temperature, ultrapure water was added to each well to a final volume of 1 mL. Subsequently, the plate was centrifuged (1500 *g*, 4 °C, 30 min). Following centrifugation, the plate was placed in the autosampler and 40 µL was injected on to the system. For construction of the calibration curve, dialyzed plasma was used, free of all steroid hormones.

2.5. Analytical validation

Method validation was performed according to the Dutch guidelines for validation of analytical methods in medical laboratories by the Dutch Society of Clinical Chemistry and Laboratory Medicine (NVKC) [12] and according to the ISO15189 regulations [13]. The intra- and interassay and reproducibility experiments were performed with plasma samples that were spiked for ADION, T, 17-OH-P, DHT and P in low, medium and high concentrations, the quality controls (QC). The concentrations of the QCs are chosen according to the concentration ranges that are generally found in the clinic, to assess the quality and validity of the method at the appropriate ranges each new day of analysis.

For the intra-assay the QCs were measured 10 times on one day and

Table 1
Steroids tested for interference.

Possible interfering substances (100 nM)	
11-Deoxycortisol	Cortisol
11-Dehydrocorticosterone	Cortisone
17-Hydroxypregnenolone (d5-Pregnen-3ß,17α-diol-20-one)	Dehydroepiandrosterone (DHEA)
21-Deoxycortisol (4-pregnene-11β-17α-diol,3,20-dione)	Dexamethasone
21-Deoxycortisone (pregnen-17α-ol-3,11,20-trione)	Etiocholanolone (5ß-Androstan-3α-ol-17-one)
5b-androstan-3α- 17β-diol	Epiandrosterone (5α-Androstan-3ß-ol-17-one)
Aldosterone	Estradiol
Androstandiol (5α-androstan-3α-17β-diol)	Estrone
Androstenediol (5-Androsten-3ß,17ß-diol)	Fluticasone propionate
Androsterone	Pregnenolone (d5-Pregnen-3β-ol-20-one)
Betamethasone	Triamcinolone
Corticosterone	

for the inter-assay the QCs were analyzed on 14 different days. The lower limit of quantitation (LLOQ) was determined by measuring serially diluted QC low samples on 6 different days. The LLOQ was set at the concentration were the coefficient of variation (CV) was ≤20%. Furthermore, the carry-over, by alternately injecting the high and low QC samples, was determined. Additionally, the linearity, by measuring 6 replicates of newly prepared calibration curves, consisting of 8 calibration points (P: 0.14–59.63 nmol/L, 17-OH-P: 0.15–61.47 nmol/L, ADION: 0.13–54.55 nmol/L, T: 0.13–54.14 nmol/L, DHT: 0.10–43.04 nmol/L), and the recovery were determined. The recovery was evaluated by spiking 3 samples at 3 different levels (the QCs) on 3 different days. The recovery should be between 85 and 115% to be accepted. The accuracy of the method for T and P was confirmed with National Institute of Standards and Technology - (NIST) standard reference material 971 (Gaithersburg, USA). For the other steroid hormones external quality assessment schemes were used to assess the accuracy. Also the stability of the stock-solutions and samples, ion suppression and interfering compounds were extensively analyzed, see Table 1 for the steroid hormones tested for interference. Furthermore, method comparison was performed (n = 40, n = 25 for DHT) with the newly developed method and with the former in-house developed radioimmunoassays (RIA) for P, 17-OH-P and ADION, with RIA combined with chromatographic purification method for DHT and an LC-MS/MS method for T [14,15].

2.6. Plasma reference intervals

Plasma reference intervals, the 2.5th and 97.5th percentiles (95% of the central population), were defined using SPSS version 23 (IBM, Chicago, IL, USA) and EP-evaluator (version 10, Data Innovations LLC, Burlington, VT, USA). Non-parametric data were log-transformed prior to analysis. The Mann-Whitney U test was used to assess the difference in steroid hormone levels between men and women, and women with and without OCP use. Differences in the steroid hormone levels between different age decades were compared with Kruskal-Wallis analysis followed by pairwise comparison when results were age-dependent.

2.7. Biological variation

The biological variation was established according to the method of Fraser and Harris, together with the recently published critical appraisal checklists for the biological variation [9,16,17]. The between-subject variation (CVg), the within-subject variation (CVi) and the analytical variation (CVa) were determined. To minimize variation, all samples of a subject were analyzed consecutive in the same run. The CVa was established by replicate analysis of each sample. The applied mathematical calculations for the biological variation have been published previously [18,19]. These include the calculations for the reference change value (RCV) and the index of individuality (II). The RCV can be used to quantify the significant difference between two consecutive measurements, expressed in percentages. In other words, the RCV allows the monitoring of serial results on individual level, to follow significant (disease) improvement or deterioration [20]. The II can be applied to find out whether the use of population based reference intervals are appropriate, based on cut-off values. With an II of > 1.4 the use of reference intervals are more appropriate than with a low II (< 0.6). In the latter case, consecutive results can best be interpreted with the biological variation data [21,22].

To assure homogeneity, outlier identification was performed. The homogeneity for the CVa was tested with either Bartlett or Levene's tests, depending on normality, and the homogeneity of the CVi was verified using the Cochran C-test [23]. Finally, the Dixon-Reed criterion was used to identify outliers between the mean values of the CVg [16]. When necessary, data were log transformed. All analyses were performed with Microsoft Excel 2010, XLSTAT (version 19.4) for Microsoft Excel and SPSS version 23 (IBM, Chicago, IL, USA).

3. Results

A representative patient sample chromatogram of both a male and female, obtained by the analysis of the five steroid hormones, with all peaks separated on baseline, is shown in Fig. 1. After addition of $^{13}C_3$-labeled standards to the samples and incubation with protein disruption buffer (pepsin), the samples were analyzed automatically with SPE followed by LC-MS/MS, according to the predefined conditions (Supplemental Table 1). The total cycle time, including automated sample extraction and chromatographic separation of all components, was 6 min.

3.1. Analytical validation

The results for the imprecision, recovery and LLOQ experiments of each steroid can be found in Table 2. Intra- and inter-assay CVs for all steroids were well below 10%. The recoveries ranged from 94 to 107% for all steroids at 3 different levels. The LLOQ for P was 0.03 nmol/L, for 17-OH-P 0.05 nmol/L, for ADION 0.04 nmol/L, for T 0.04 nmol/L and for DHT 0.12 nmol/L. The calibration curves for all five components were linear over the calibration range, with correlation coefficients (R^2) above 0.99, also when measured in reverse order or randomly throughout a set of samples.

Furthermore, for all steroid hormones no carry-over was observed. Of the 23 steroids tested for interference (Table 1), 11-deoxycortisol contributed 0.1% to the ADION concentration and androstenediol contributed 0.02% to the T concentration, in both the QN and QF m/z transitions. The other tested steroids did not show interference with the steroid hormones. No significant ion suppression was detected within the time-frame of the measured steroid hormones. The accuracy of the method for T and P, tested with the NIST 971 standard, was within the expected range (P: 6.2 ± 0.2 nmol/L; T (level 1): 0.96 ± 0.02 nmol/L;

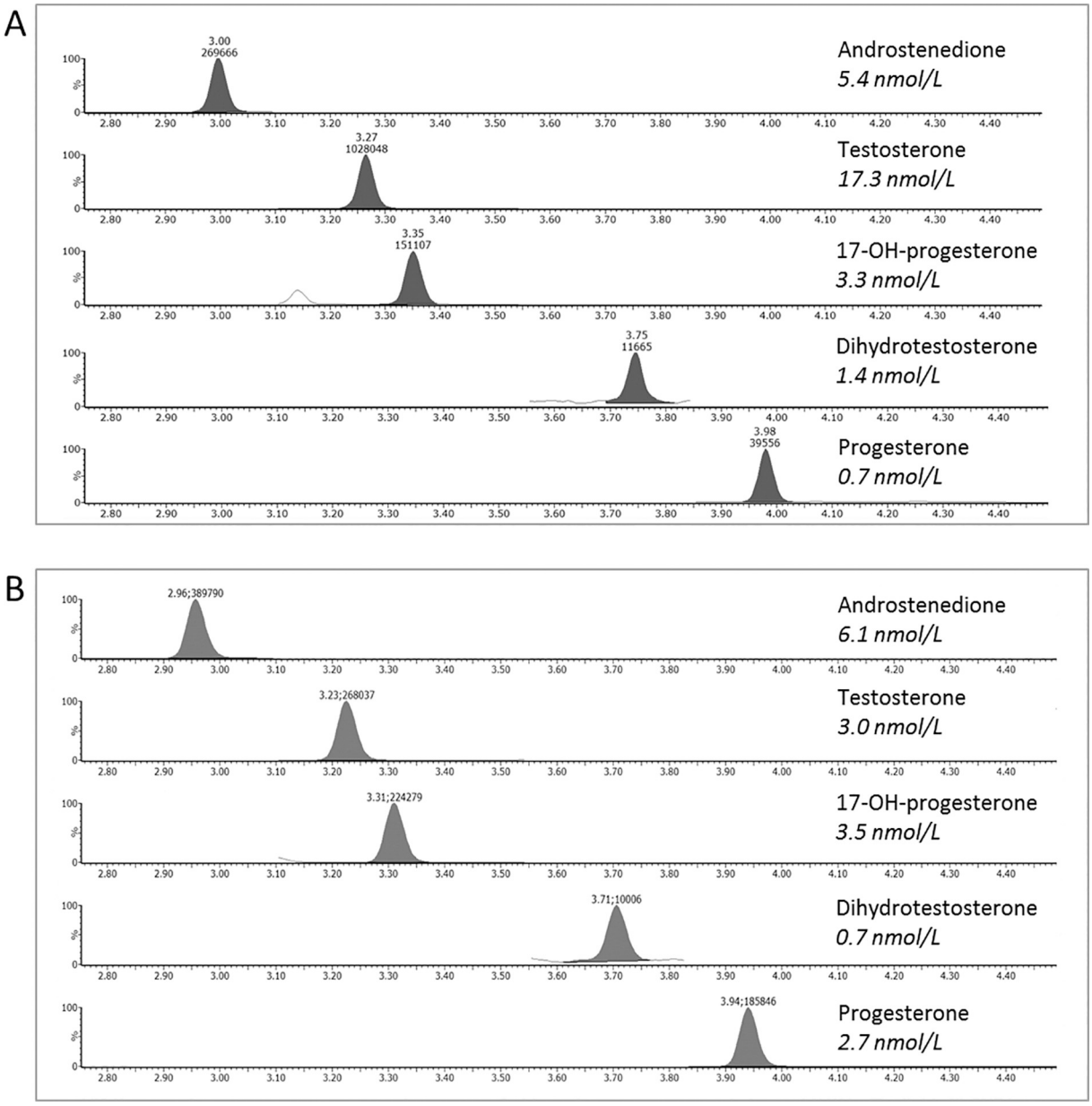

Fig. 1. Representative patient chromatograms of the steroid hormones in plasma.
A: male, 27 years and B: female, 48 years.

T (level 2): 22.3 ± 0.5 nmol/L), as were the steroid hormones compared with the external quality schemes. The stability experiments revealed that the stock solutions and samples are stable for at least one year at −20 °C. Samples already processed for analysis are stable for a week at 4 °C. The method was compared to the formerly used methods for the steroid hormones, RIA and LC-MS/MS. Passing-Blablok analysis revealed lower concentrations for ADION, 17-OH-P, DHT and P when measured with the new method. The previously used LC-MS/MS method for T showed good agreement with the new profiling LC-MS/MS method (Supplemental Table 2 and Fig. 1).

3.2. Reference intervals

Results from the reference interval study in a well-defined healthy population, separated by gender and for women additionally by OCP use, are shown in Table 3. Median overall concentrations of 17-OH-P, ADION, T and DHT were significantly higher in men (3.3 nmol/L, 3.4 nmol/L, 19.0 nmol/L and 1.7 nmol/L, respectively) compared to women (1.3 nmol/L, 3.1 nmol/L, 0.94 nmol/L and 0.24 nmol/L, respectively; $p < .001$ for all steroid hormones, except ADION: $p = .003$). In women using OCP, the median concentrations of 17-OH-P and ADION were significantly lower in comparison to women that were not using OCP in the same age-range from 20 to 39 years (17-OH-P: 0.97 [0.18–4.2] nmol/L vs. 1.5 [0.2–9.9] nmol/L respectively, $P = .002$ and ADION: 3.2 [1.2–7.6] nmol/L vs. 3.9 [1.3–8.6] nmol/L respectively, $P = .02$). There were no significant differences for T and DHT levels between women with and without OCP use.

Additionally, Kruskal-Wallis analyses was performed to investigate if the steroid hormones in men and women (not using OCP) showed age-dependency in this sample set of 120 men and 120 females. In men, the analysis showed that the concentration of P, 17-OH-P, T and DHT displayed no age dependency. Only ADION displayed lower levels with increasing age ($H(5) = 26.3$, $P < .001$). In women, concentrations of 17-OH-P, ADION, T and DHT were age dependent, the levels decrease with increasing age ($H(5) = 16.3$, $p = .006$; $H(5) = 38.7$, $p < .001$; $H(5) = 12.5$, $p = .03$; $H(5) = 55.1$, $p < .001$; respectively). Pairwise

Table 2
Intra- and inter assay variation, recovery and LLOQ of the LC-MS/MS method for the five plasma steroid hormones.

	Intra-assay (n = 10)		Inter-assay (n = 14)		Recovery (n = 3)	LLOQ (n = 6)
	Mean (nmol/L)	CV (%)	Mean (nmol/L)	CV (%)	Mean (%)	(nmol/L)
P						0.03
Low	0.27	7.0	0.27	3.7	104	
Medium	7.56	1.2	7.54	2.7	97	
High	46.2	2.1	45.0	2.3	94	
17-OH-P						0.05
Low	0.49	2.8	0.50	2.6	105	
Medium	7.41	0.6	7.40	2.6	99	
High	36.4	2.2	36.1	2.4	99	
ADION						0.04
Low	0.36	3.6	0.35	2.4	102	
Medium	2.64	1.6	2.63	2.7	100	
High	23.8	1.9	23.4	2.2	99	
T						0.04
Low	0.37	3.1	0.37	2.8	101	
Medium	3.89	1.5	3.84	2.7	99	
High	51.3	1.2	50.3	2.9	99	
DHT						0.12
Low	0.13	5.3	0.12	9.6	107	
Medium	1.63	2.4	1.62	3.9	97	
High	5.28	4.3	5.26	3.6	96	

Abbreviations: 17-OH-P - 17-hydroxyprogesterone; ADION - androstenedione; CV – coefficient of variation; DHT- dihydrotestosterone; P- progesterone; T-Testosterone.

comparison showed that this age dependency can possibly be related to post-menopausal age, since from the age of 50 the decline becomes more evident. This decline after post-menopausal age is underlined by performing Kruskal-Wallis analyses on only the women younger than 50 years, where for 17-OH-P, ADION, T and DHT no age-dependency is present ($H(2) = 3.67$, $p = .16$; $H(2) = 1.09$, $p = .58$; $H(2) = 1.99$, $p = .37$; $H(2) = 0.67$, $p = .72$ respectively).

3.3. Biological variation

Characteristics of the subjects have been previously published [18]. Sample exclusion based on the Cochran and Dixon-Reed tests resulted in generally one to two outliers per steroid hormone for both males and females (Fig. 2). Furthermore, for 5 of the 15 women the DHT results were below the LLOQ. Therefore, these data were not included in the calculations.

In line with the results from the analytical validation, the CVa results were ≤ 3% except for DHT in women, as shown in Table 4. The CVi is relatively comparable in men and women, i.e. 12–24% for men and 11–34% for women, and with exclusion of 17-OH-P the CVi for women is between 11 and 20%. The CVg is higher in women compared to men (36–61% vs 15–40%, respectively). In men, the CVg of T is low in comparison to the CVg of the other steroid hormones. To assess the relative change of serial measurements that represents a true statistical change, the RCV was determined. RCVs are relatively comparable in men and women for most of the steroid hormones, ranging from 35 to 68% in men and 36–94% (36–55% without 17-OH-P) in women. Furthermore, nearly all steroid hormones in both men and women demonstrate an II of < 0.6, except the II for T and 17-OH-P in men, 0.92 and 0.89 respectively.

4. Discussion

In this study, we describe an automated and sensitive LC-MS/MS method for high-throughput analysis of total plasma P, 17-OH-P, ADION, T and DHT. The LC-MS/MS method was extensively validated and reference intervals have been established in a well-defined, healthy sample-set. Furthermore, the biological variation has been determined in 30 apparently healthy volunteers.

The method proved to be robust, sensitive and displays excellent performance, as confirmed with the results of the complete method validation. The use of online SPE resulted in an automated, short and simple pre-analytical phase, improving sample throughput. The efficient sample preparation is partly explained by the addition of pepsin. Pepsin effectively releases the steroid hormones from their binding proteins, by proteolyzing the binding proteins, with no need of centrifugation. The pepsin step resulted in improved signal and reduced ion-suppression for especially the more apolar steroid hormones, in comparison to a precipitation step with zinc sulfate in methanol (data not shown). As a result of optimal sample preparation and a sensitive mass-spectrometer, there was no need to increase sensitivity by derivatization [24]. On the contrary, the signal for T and P had to be de-optimized in order to accommodate simultaneous quantification of the steroid hormones present at lower concentrations. Elution of the steroid hormones via focusing, i.e. a concentration step of the hormones at the beginning of the analytical column after SPE, resulted in excellent chromatography.

Even though LC-MS/MS assays are sensitive, i.e. low LLOQ, lower concentrated steroid hormones, including DHT, posted a challenge to measure. DHT is present in very low concentrations, is bound to carrier proteins and ionizes poorly in comparison to other steroid hormones [25]. With the method described in this paper we are able to quantify plasma DHT routinely, without derivatization, in combination with other relevant steroids. This hormone-profiling can enable quick insight in potential disorders and the underlying mechanisms, for example in the diagnosis of polycystic ovary syndrome (PCOS) and the determination of the origin of adrenocortical carcinomas [26,27].

Method comparison with in-house developed immunoassays revealed disagreement for most of the steroid hormones. Deviations in the

Table 3
Reference intervals of the plasma steroid hormones in men and women (in nmol/L).

	Men	20–79 years	Women	20–79 years	Women	20–39 years
	n = 120		n = 120		n = 40	OCP
	Interval	Median	Interval	Median	Interval	Median
P	0.10–0.66	0.3	–	–	0.06–0.57	0.19
17-OH-P*	1.4–7.7	3.3	0.27–6.7	1.3	0.18–4.2	0.97
ADION*	1.6–8.4	3.4	0.93–7.6	3.1	1.2–7.6	3.2
T	10.8–35.4	19.0	0.34–2.0	0.94	0.33–2.0	0.82
DHT	0.91–3.4	1.7	< 0.73	0.24	< 0.84	0.34

Data presented as median for non-parametric data with central 95% interval (2.5 and 97.5 percentiles) separated for men, women and women using OCP. Abbreviations: 17-OH-P - 17-hydroxyprogesterone; ADION - androstenedione; DHT- dihydrotestosterone; OCP – oral contraceptive pills; P- progesterone; T-Testosterone. *For women: results of 17-OH-P and to a lesser extent ADION should be interpreted with care since these hormones are known to be influenced to some extent by the menstrual cycle. For women not using OCP data of P is not shown as a result of the large fluctuations due to the menstrual cycle and menopause.

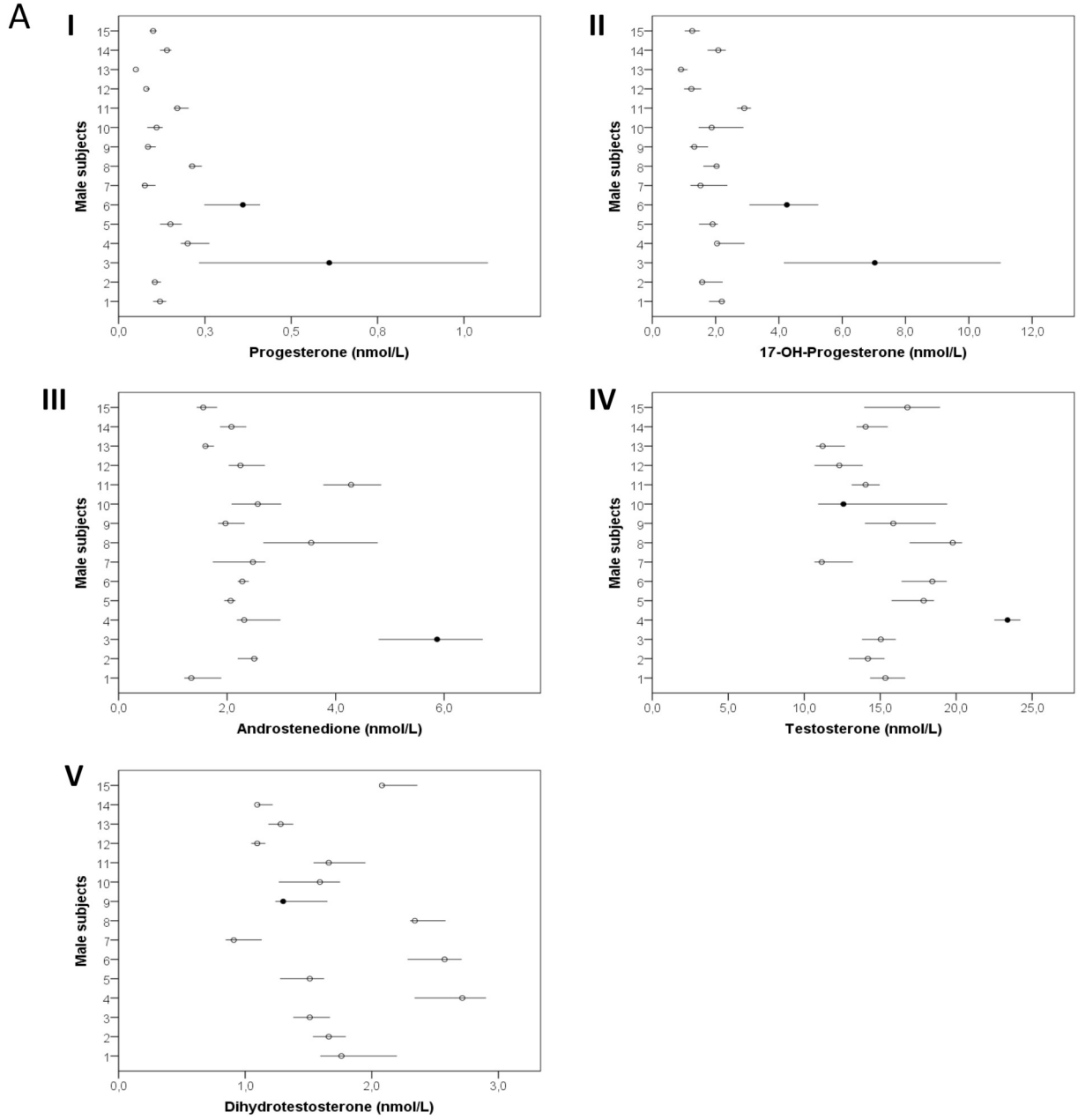

Fig. 2. Biological variation of the steroid hormones in men (A) and women (B).
Median (transparent dot) and interquartile range (horizontal line) for each individual. Solid dots represent outliers determined by either Cochran (CVi) or Dixon-Reed (CVg) tests. Solid squares represent data below the lower limit of quantitation of the assay (DHT in Women) (I): Progesterone, (II): 17-OH-Progesterone, (III): Androstenedione, (IV): Testosterone and (V): Dihydrotestosterone.

comparison between RIA and LC-MS/MS were anticipated, due to the well-known disadvantages of steroid analysis with immunoassays, such as cross-reactivity and a relatively poor analytical performance [28]. Therefore, new reference values were established in well-defined healthy individuals and have been in use for the last three years in our clinical laboratory. For T and 17-OH-P, reference intervals have previously been established with both immunoassay and LC-MS/MS methods [5,6,29,30]. However, the other steroid hormones are less well described and reference intervals of DHT over a wide age-distribution, in a well-characterized healthy population, are even more scarce. Comparison of the overall results of the reference intervals for P, 17-OH-P, ADION and T with the study of Eisenhofer et al. and Fanelli et al. shows relatively good agreement [6,31]. Differences were found in the 17-OH-P and ADION levels, respectively. These comparisons show that with LC-MS/MS, using different (pre-) analytical methods, comparable results are obtained. However, also differences are observed, indicating the need for harmonization [32]. This can be achieved by standardization and the use of predefined calibrators and certified reference materials, for which LC-MS/MS is perfectly applicable [4]. For this study the accuracy for T and P was demonstrated with NIST reference material, indicating the validity of the method for these compounds. However, it must be noted that for women we were not able to include the reference values for P, due to the lack of information on the menstrual cycle during blood sampling. P, and to some extent 17-OH-P and ADION, are known to be influenced by the menstrual cycle, but we were not able to control for this in the present study, and the results should be interpreted as such.

Interestingly, in men aged from 20 to 79 only ADION displayed age dependency. Whereas in women, 17-OH-P, ADION, T and DHT were age dependent. This age dependency in women is most likely associated

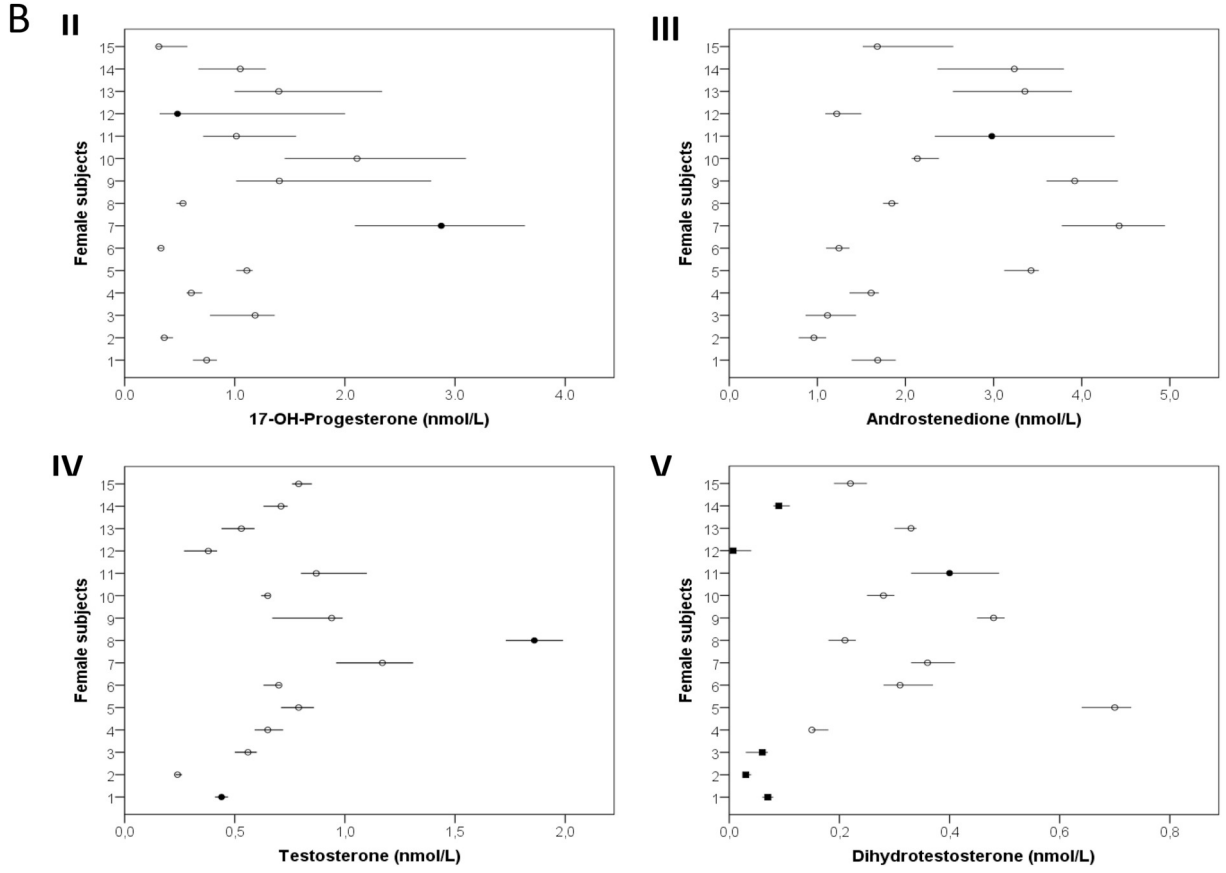

Fig. 2. (*continued*)

with menopause, usually occurring between the age of 45 and 55 [33]. In this study, we did not find the generally known age-related decline of T in men. T concentrations may be affected by health status, such as obesity, diabetes, metabolic syndrome, acute illness and medication use, not present in de men included in this study [34]. This underlines that prescription of T therapy to older men is not necessarily required and that more assessment is needed to find the cause of the possible underlying hypogonadism in men with reduced T levels, in line with the 'Testosterone therapy in men guideline' [34]. Reference values were also determined in a subgroup of women using OCP. The use of OCP resulted in reduced plasma levels of 17-OH-P and ADION, possibly as a result of inhibition of the hypothalamic-pituitary-gonadal axis, in line with other studies [6,35]. These data emphasize the importance of considering OCP use in women when interpreting steroid hormone data.

Patient follow-up can be important to assess clinical improvement or disease progression. Besides the reference intervals, the biological variation was determined for the steroid hormones, to gain insight in the individual variability and the significance of serial measurements within a patient. Assessment of the biological variation in 30 healthy individuals for the five plasma steroid hormones shows that T and DHT display the lowest variation in men, with T having a relatively low CVg

Table 4
Biological variation of the steroid hormones in men and women.

Men	CVa	CVi[a]	CVg	II	RCV (%)	RCV Log Normal (%)		
						+	–	Mean
P	3.0	18.5 (12.6–24.4)	39.7	0.48	55.2	75.0	−38.9	57.0
17-OH-P	1.8	24.4 (18.5–30.4)	27.6	0.89	68.1	102.3	−46.8	74.6
ADION	1.9	18.1 (13.5–22.8)	32.1	0.57	50.8	69.4	−37.9	53.7
T	2.0	13.5 (11.4–15.6)	15.1	0.92	38.0	46.6	−31.2	38.9
DHT	2.7	12.2 (9.2–15.2)	31.6	0.40	35.1	43.1	−28.8	36.0
Women								
17-OH-P*	2.2	33.8 (23.7–43.8)	60.7	0.56	94.4	171.4	−54.3	112.9
ADION*	2.7	19.5 (13.3–25.8)	49.4	0.40	54.9	80.5	−39.0	59.8
T	2.1	14.9 (11.5–18.3)	35.7	0.42	42.0	52.9	−32.7	42.8
DHT	7.1	10.8 (8.5–13.2)	48.1	0.27	36.4	44.3	−30.1	37.2

[a] CVi with 95% Confidence interval. Abbreviations: CVa – analytical variation; CVi – within-subject variation; CVg – between-subject variation; II – index of individuality; RCV – Reference Change Value; 17-OH-P - 17-hydroxyprogesterone; ADION - androstenedione; DHT- dihydrotestosterone; P- progesterone; T-Testosterone. *Results of 17-OH-P and to a lesser extent ADION should be interpreted with care since these hormones are known to be influenced to some extent by the menstrual cycle. For women data of P is not shown as a result of the large fluctuations due to the menstrual cycle and menopause.

(15.1%). These data indicate a relatively constant T level between men, and therefore appears to be more controlled in comparison to the other steroid hormones. Similarly in women, T displays the lowest CVg (35.7%) of the measured steroid hormones, although the variation is more than twice as high compared to men. In women, 17-OH-P, formed from the precursor P, displays the highest variation, likely influenced by the menstrual cycle. Of the 15 samples analyzed for DHT in women, 5 were below the LLOQ. This indicates that in some cases DHT is too low in women to measure accurately with the described analytical configuration. However, it should be noted that to our knowledge this study is the first to describe the determination of the reference intervals and biological variation of DHT in women using an automated LC-MS/MS method, and in this context we were still able to analyse DHT quantitatively in 10 of the 15 women.

With data collected from the biological variation analysis, the II and the RCV were calculated. The II results, for most steroid hormones below the cut-off of 0.6, indicate that the biological variation is a valuable addition in the interpretation of data in patient follow-up, instead of solely using reference intervals. When interpreting the results of consecutive measurements, RCV calculations reveal that on average a 50% change represents a significant change on individual level. The calculations of the RCV with the log-normal approach reveal similar results. The log-normal approach however allows, in addition, the assessment of the RCV separate for increasing or decreasing values in consecutive analysis.

Comparison of several studies shows that the CVg of T in men varies between 16.5 and 40.8% and the CVi between 8.3 and 12.6%, illustrating a degree of variability between studies [36–39]. This might be explained by the use of different methods, since most of these studies used immunoassays for measuring T. Additionally, time of sampling and time-interval between blood sampling can be of influence on the variation. However, in line with this, Bui et al. described the biological variation of T in 25 healthy women both measured with LC-MS/MS and immunoassay [29]. No clear differences in the CVi and CVg were found when both methods were compared. It must be noted that the T measurements were performed with a 2nd generation immunoassay, a more specific and accurate assay than the 1st generation immunoassay. For the other steroid hormones much less data on the biological variation has been published. One study determined the CVg and CVi of 17-OH-P and ADION, besides T [39]. Since different assays are used, together with a different study set-up makes it hard to compare these results to ours.

For the biological variation, quality specification or measurement uncertainty can be estimated by using the following formulae: $CVa \leq 0.5CVi$ (desired performance), $CVa \leq 0.25CVi$ (optimum performance) and $CVa \leq 0.75CVi$ (minimum performance) [16,40]. Examining our data on the biological variation we see that for all steroid hormones, except DHT in women, the optimum performance is achieved. For DHT women the minimum performance was achieved as quality specification. From these results we can assume that the data on the biological variation is reproducible enough to be used.

In general, these novel data on biological variation show a high degree of individual variation, illustrating their importance and the need for careful interpretation of single measurements of steroid hormones. These data can be useful to monitor disease progression or improvement in patients. Moreover, these data will contribute to the further understanding of the importance of the biological variation of steroid hormones, especially for DHT as this study is one of the firsts to report the biological variation of DHT measured with LC-MS/MS. As example, the T/DHT-ratio has been proposed as new biomarker for an adverse metabolic phenotype in women with PCOS [41]. In obese women with PCOS, lifestyle intervention has shown to be associated with the resumption of ovulation [42]. During intervention, monitoring is essential and the biological variation, in particular the RCV, can be greatly informative in individual patient follow-up.

5. Conclusion

We developed a specific and sensitive method for high-throughput analysis of P, 17-OH-P, ADION, T and DHT. The establishment of reference intervals and the biological variation in healthy subjects will allow improved biochemical characterization of patients in clinical practice.

Declaration of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clinbiochem.2019.03.013.

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