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Abstract

Background: Urinary steroid profiling (USP) is a powerful diagnostic tool to asses disorders of steroidogenesis. Pre-analytical factors such as age, sex and use of oral contraceptive pills (OCP) may affect steroid hormone synthesis and metabolism. In general, USP reference intervals are not adjusted for these variables. In this study we aimed to establish such reference intervals using a newly-developed and validated gas chromatography with tandem mass spectrometry detection method (GC-MS/MS).

Methods: Two hundred and forty healthy subjects aged 20–79 years, stratified into six consecutive decade groups each containing 20 males and 20 females, were included. None of the subjects used medications. In addition, 40 women aged 20–39 years using OCP were selected. A GC-MS/MS assay, using hydrolysis, solid phase extraction and double derivatization, was extensively validated and applied for determining USP reference intervals.

Results: Androgen metabolite excretion declined with age in both men and women. Cortisol metabolite excretion remained constant during life in both sexes but increased in women 70–79 years of age. Progesterone metabolite excretion peaked in 30–39-year-old women and declined afterwards. Women using OCP had lower excretions of androgen metabolites, progesterone metabolites and cortisol metabolites. Method validation results met prerequisites and revealed the robustness of the GC-MS/MS method.

Conclusions: We developed a new GC-MS/MS method for USP which is applicable for high throughput analysis. Widely applicable age and sex specific reference intervals for 33 metabolites and their diagnostic ratios have been defined. In addition to age and gender, USP reference intervals should be adjusted for OCP use.

Keywords: GC-MS/MS; oral contraceptive pills; reference intervals; urinary steroid profile.

Introduction

Steroid biosynthesis is a complex process by which steroid hormones are produced from cholesterol through a series of unique enzymatic steps in steroidogenic tissue [1]. This tissue is mainly found in the adrenal cortex and gonads [2]. Steroid hormones can be classified according to their physiological function into progestins, androgens, estrogens, mineralocorticoids and glucocorticoids. As such, they regulate various biological processes including mineral balance, intermediate metabolism, sexual development, reproductive function, immune and stress responses [3]. Steroids are converted into a large number of metabolites by the liver and peripheral tissues before being excreted in the urine. The biochemistry of steroid biosynthesis and metabolism is largely known and specific steroid pathways are regulated by differential expression and activity of enzymes and cofactors involved in a developmental, sex, time and tissue specific fashion and might be perturbed in disease states [4].

Since the 1960s, urinary steroid profiling (USP) has been a powerful diagnostic tool to assess steroidogenesis. Nowadays, USP is usually being performed by application of gas chromatography-mass spectrometry (GC-MS) [4, 5]. This technique is able to measure a wide variety of urinary steroid hormone metabolites at the same time in one urinary sample, making it an efficient and patient friendly diagnostic tool. For the last 50 years almost all disorders

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of steroid hormone biosynthesis and metabolism have been characterized and first named following urinary steroid analysis [4].

USP has a broad range of applications. For example, it can be used for the diagnosis and follow-up of disorders resulting from steroid biosynthetic enzyme deficiencies, licorice-induced hypertension, hirsutism and other related diseases [6–8]. Furthermore, USP might be helpful in monitoring patients with an adrenocortical carcinoma (ACC) and could be useful in discriminating between malignant and benign adrenal tumors [9–12]. Reference intervals for USP using GC-MS have been described before [6, 11, 13–17]. Notably, those previous studies have several shortcomings, such as lack of adjustment for potential relevant preanalytical factors like age, sex or use of oral contraceptive pills (OCP) or limited external validity as a result of the examination of study subjects who may not accurately reflect the general population. Reliable reference intervals for urinary steroid metabolites are a prerequisite for correct interpretation of USP test results in clinical practice.

In this study we aim to establish age- and sex-specific USP reference intervals in a well-defined healthy adult population. In addition, USP reference intervals were determined in a subgroup of women using OCP. USP was performed by using a newly developed gas chromatography-tandem mass spectrometry (GC-MS/MS) assay. In comparison with other USP methods such as GC-MS, it has been suggested that GC-MS/MS demonstrates higher specificity, while being less laborious and more suitable for high-throughput analysis.

Materials and methods

Subjects

Two hundred and eighty healthy subjects with a body mass index between 21 and 30 kg/m^2 and age between 20 and 79 years were selected from the Life Lines Cohort Study, a large population-based cohort study in the Netherlands [18]. Of these, 240 subjects were stratified into six consecutive decade groups, each containing 20 males and 20 females. None of the subjects used any medication. In the subgroup of women between 20 and 39 years who were not using OCPs, any women using OCP were excluded.

In addition, 40 women aged 20–39 years (20 subjects per decade), using OCP were selected. Women on OCP used combined contraceptives with different progestogens, but mostly levonogestrel combined with ethinylestradiol. Urinary samples from 24 h collections had been stored at −80 °C until analysis. In women, urinary collections were not timed according to menstrual cycle or day of OCP use. The study was approved by the Medical Ethics Committee of the University of Groningen and all participants provided written informed consent.

Reagents and stock solutions

Methoxyamine HCl, trimethylsilylimidazole (TMSI) and sodium ascorbate were purchased from Sigma Aldrich Corp. (St. Louis, MO, USA). Pyridine was obtained from Merck (Kenilworth, NJ, USA), heptane and methanol from Biosolve BV (Valkenswaard, The Netherlands), and Suc d'Helix from Brunschwig Chemie (Amsterdam, The Netherlands).

Androsterone (A), etiocholanolone (E), dehydroepiandrosterone (DHEA), 11-keto-etiocholanolone (11-KE), 11-hydroxyandrosterone (11-HA), 11-hydroxyetiocholanolone (11-HE), epipregnanolone (polone), 16-α hydroxydehydroepiandrosterone (16-OH-DHEA), allo-pregnanediol (aP2), pregnanediol (P2), pregnanetriol (P3), 16-ketoandrostenediol (16-KA'2), androstenetriol (A'3), tetrahydrodeoxycortisol (THS), 11-deoxytetrahydrocorticosterone (TH-DOC), pregnanetriolone (PTL), 16-α hydroxypregnenolone (16-OH-P'OL), 17-hydroxypregnenetriol (17-P3), tetrahydrocortison (THE), 11-dehydrotetrahydrocorticosterone (THA), tetrahydrocorticosterone (THB), allo-tetrahydrocorticosterone (aTHB), tetrahydrocortisol (THF), allo-tetrahydrocortisol (aTHF), α cortolone (α-CTLN), β cortolone (β-CTLN), pregnanediolone (PDL) and allo-pregnanediolone (aPDL) were all obtained from Steraloids (Newport, RI, USA). Estriol and α-cortol (α-cortol) were from Sigma Aldrich Corp. (St. Louis, MO, USA). See Supplementary Table 1 for steroid nomenclature according to IUPAC, LOINC and Chemspider.

Isotope-labeled internal standards 11-KE-d5, pregnenolone-d4 and THE-d5 were purchased from CDN isotopes; DHEA-d6 from Sigma Aldrich Corp. (St. Louis, MO, USA). We used four deuterated internal standards divided over the 33 steroid metabolites representing polarity groups, because of availability and costs.

For 3α, 15β, 17α-trihydroxypregnanediolone (15-OH-PDL), 15-hydroxypregnenolone (15-OH-P'DL) and 16-β,18-dihydroxydehydroepiandrosterone (16,18-OH$_2$-DHEA) we have no standards available.

Stock solutions were prepared in methanol and serially diluted to form calibrators and quality control samples in urine by enrichment. The exact concentration range of calibrators varies with the analyte, for example, 0–27 μmol/L for THF and 0–36 μmol/L for E. Internal standards concentrations were 6 μmol/L.

Instrumentation

Solid phase extraction (SPE) was performed on Waters Oasis HLB (3 mL Vac cartridges, 60 mg sorbent per cartridge, 30 μm particle size (Waters Corporation, Milford, MA, USA). Steroids were chromatographically separated on a J&W CP-Sil 5 CB column (25 m × 250 μm × 0.12 μm; Agilent Technologies, Santa Clara, CA, USA). A 7890A GC with 7000 Triple Quadrupole Detector (Agilent Technologies, Santa Clara, CA, USA) was used for separation and detection using electron impact and selective reaction monitoring. Nitrogen was used as collision gas (flow 1.5 mL/min), helium as quench gas (flow 2.25 mL/min) and carrier gas (2 mL/min). The injection temperature was 65 °C, with the MS source at 270 °C and both quadrupoles at 150 °C. Chromatography was performed using a temperature program for optimal separation: 2 min 50 °C, ramp 40 °C/min until 160 °C, ramp 2.5 °C/min until 240 °C and finally ramp 4 °C/min until 270 °C. Electron impact was performed at 70 eV.

Data acquisition was performed with Masshunter Version B 06.01 (Agilent Technologies, Santa Clara, CA, USA) and data were processed with Masshunter Quantitative Analysis Version B07.00/ Build 7.0.457.0 (for QQQ).

Analytical principle

Glucuronide- and sulfate-conjugated steroid hormone metabolites were measured in samples from 24 h urine collections. First, conjugated hormones were converted to the free steroid form by enzymatic hydrolysis. Isotope-labeled internal standards 11-KE-d5, DHEA-d6, pregnenolone-d4 and THE-d5 were added and unconjugated steroids were extracted from urine by using SPE. Polar components were washed out of the extract. The extract was vaporized using an infrared vaporizer, after which the residue was derivatized at hydroxyl- and keto-residues, in a two-step reaction to decrease polarity. Keto-residues were derivatized using 2% methoxyamine in pyridine; hydroxyl-residues were silylated by N-trimethylsilyl imidazole.

Sample preparation

Before analysis, urinary samples were centrifuged at 1200 *g* before applying 1 mL to conditioned (methanol, water) HLB SPE columns. Cartridges were washed with water and eluted with methanol. The eluate was vaporized using an infrared vaporizer (Hettlab IR Dancer 300, Hettich AG, Switzerland) and rediluted in 2 mL acetate/sodium ascorbate (pH 4, 8) solution. One hundred microliters Suc d'Helix Pomatia was added and enzymatic hydrolysis of the conjugated groups took place during 2 h at 46 °C in a shaking temperature controlled bath. After cooling down, internal standards were added and a second SPE step took place on the HLB columns. Samples were washed with water, eluted with methanol and evaporated until dryness. The residue was derivatized with 150 µL methoxyamine in pyridine during 1 h at 80 °C. After evaporation until dryness a second derivatization step took place with 200 µL N-trimethylsilyl imidazole during 12 h (overnight) at 110 °C. In case of emergency diagnostics this last step can be reduced to 2 h at 140 °C.

Samples were washed with 4 mL heptane and 3 mL 0.1 M HCl by vortexing and centrifugation (1200 *g*). One milliliters of the upper heptane layer were transferred to a GC-MS/MS vial. Injection volume was 25 µL.

Analytical method validation

Prior to validation, claims were postulated for each validation parameter, according to the international ISO15189 regulation and the Dutch guideline for validation of analytical methods in medical laboratories by the Dutch Society of Clinical Chemistry and Laboratory Medicine (NVKC) [19]. Validation parameters applied were intra-assay (n = 20) and inter-assay (n = 16) imprecision, repeatability of the injection (n = 10), linearity (n = 6), recovery (n = 6 for three concentrations), lower limit of quantitation (LLOQ), minimal sample volume, carry-over, method comparison against the previous GC-MS method using liquid-liquid extraction and overnight hydrolysis at 37 °C using a buffer without sodium ascorbate [7] and stability of six different urinary samples (biological sample, freeze-thaw, autosampler).

Statistical analysis

GC-MS/MS and GC-MS methods were compared using Passing-Bablok regression analysis in Analyse-it (version 2.30 Excel 12+ Analyse-it Software). USP results from healthy volunteers were analyzed to obtain age, sex and OCP specific reference intervals for 33 steroid metabolites. Also diagnostic steroid ratio reference intervals were

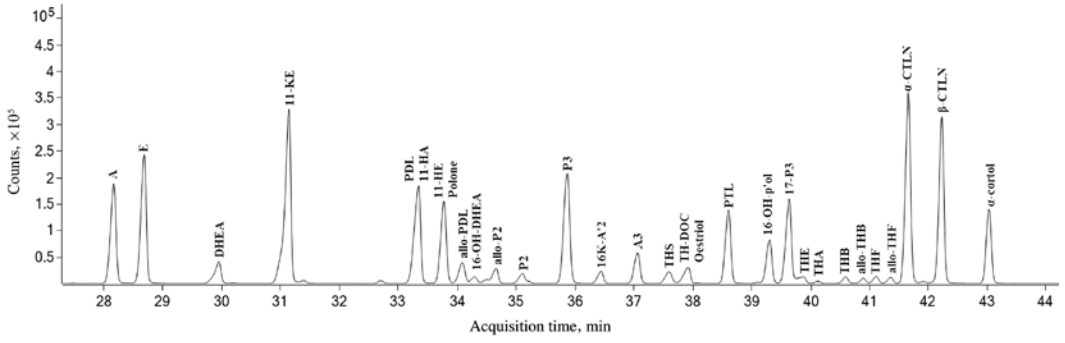

Figure 1: Total ion current chromatogram of a calibration standard mix of all the steroid metabolites.
A, androsterone; E, etiocholanolone; DHEA, dehydroepiandrosterone; 11-KE, 11-keto-etiocholanolone; 11-HA, 11-hydroxyandrosterone; 11-HE, 11-hydroxyetiocholanolone; polone, epipregnanolone; 16-OH-DHEA, 16-α hydroxydehydroepiandrosterone; aP2, allo-pregnanediol; P2, pregnanediol; P3, pregnanetriol; 16-KA'2, 16-ketoandrostenediol; A'3, androstenetriol; THS, tetrahydrodeoxycortisol; TH-DOC, 11-deoxytetrahydrocorticosterone; PTL, pregnanetriolone; 16-OH-P'OL, 16-α hydroxypregnenolone; 17-P3, 17-hydroxypregnenetriol; THE, tetrahydrocortison; THA, 11-dehydrotetrahydrocorticosterone; THB, tetrahydrocorticosterone; aTHB, allo-tetrahydrocorticosterone; THF, tetrahydrocortisol; aTHF, allo-tetrahydrocortisol; α-CTLN, α cortolone; β-CTLN, β cortolone; PDL, pregnanediolone; aPDL, allo-pregnanediolone; α-cortol, α-cortol. Standards for 3α, 15β, 17α-trihydroxypregnanediolone (15-OH-PDL), 15-hydroxypregnenolone (15-OH-P'DL) and 16-β,18-dihydroxy-dehydroepiandrosterone (16,18-OH_2-DHEA) were not available. See Supplementary Table 1 for steroid nomenclature according to IUPAC, LOINC and Chemspider.

Table 1: Mass spectrometric settings for steroid metabolites and internal standards.

Steroid metabolite	Precursor ion, *m/z*	Product ion, *m/z*	Collision energy, V	Dwell time, ms	Internal standard
[D5] 11-KE	305.1	258.2	10	50	
[D6] DHEA	364.2	274	3	50	
[D5] THE	583.3	403.2	12	50	
[D4] Pregnenolone	390.2	300.1	7	30	
11-HA	448.2	358.2	3	20	D6-DHEA
11-HE	448.2	268.2	10	40	D6-DHEA
11-KE	300.1	254.2	10	50	D5-11-KE
15-OH-PDL	258	168.1	8	40	D5-THE
15-OH-P'DL	562	472.3	8	30	D5-THE
16,18-OH_2-DHEA	534	444	10	30	D5-11-KE
16K-A'2	446.2	356.2	7	40	D6-DHEA
16-OH-DHEA	266	239.1	10	40	D5-11-KE
16-OH-p'ol	474.3	156	18	10	D5-11-KE
17-P3	433.2	253.3	7	10	D5-THE
A	360.2	270.2	5	50	D4-Pregnenolone
A'3	329	239.1	15	30	D5-THE
aP2	269.2	187	3	30	D5-THE
aPDL	476.3	386.3	10	40	D5-THE
aTHB	474	384.3	5	100	D5-THE
aTHF	472	382.1	15	80	D5-THE
α-cortol	343.3	199	5	30	D5-THE
α-CTLN	449.2	359.3	5	30	D5-THE
β-CTLN	449.2	359.3	3	30	D5-THE
DHEA	358.2	268	3	50	D6-DHEA
E	360.2	270.2	5	50	D4-Pregnenolone
Oestriol	504.3	414.1	3	40	D5-THE
P2	269.2	187	3	30	D5-THE
P3	435.3	255.2	5	20	D5-THE
PDL	476.3	386.3	10	40	D5-THE
Polone	388.2	298.2	10	40	D4-Pregnenolone
PTL	449.2	359.3	3	30	D5-THE
THA	400	241	5	30	D5-THE
THB	474	384.3	5	100	D5-THE
TH-DOC	476.3	241.2	10	30	D5-THE
THE	578.3	398.2	12	50	D5-THE
THF	472	382.1	15	80	D5-THE
THS	564.3	474.2	12	40	D5-THE

Abbreviations as in Figure 1 and Supplemental Table 1.

calculated from these data. Reference intervals were defined as the central 95% of the population (i.e. the 2.5th and 97.5th percentiles) and calculated using EP evaluator. Non-parametric data were logarithmically transformed before analysis.

Differences in the distributions of urinary steroid metabolitesin relation to OCP use were assessed using the Kolmogorov-Smirnov test. Metabolites were categorized in androgen (A, E, DHEA, 11-KE, 11-HA, 11-HE), cortisol (THE, THF, aTHF, α-CTLN, β-CTLN, α-cortol), progesterone (aP2, P2, P3, Polone), aldosterone (THA, THB, aTHB), intermediate (THS, PDL, PTL, aPDL, TH-DOC) and fetal (A'3, 16K-A'2, 16-OH-DHEA, 16,18-(OH)2-DHEA, 16-OH-p'ol, 15-OH-PDL, 17-P3, 15-OH-P'DL) metabolites. Sum scores were calculated per group and differences between groups were calculated using the Mann-Whitney test. Additional statistical analyses were performed using SPSS version 23.0 for Windows (IBM Corporation, Chicago, IL, USA). A two-sided $p < 0.05$ was considered to indicate statistical significance.

Results

Validation parameters

In one chromatographic run, we quantified 33 urinary steroid metabolites, as shown in the total ion current chromatogram (Figure 1) and mass spectrometric settings (Table 1). Calibration curves (weighed regression) and

validation samples were run with every batch of patient samples. Linearity was obtained over the 0–35 μmol/L range with corresponding correlation coefficients (R^2) consistently >0.99 for all steroids. Calibration curves were also reproducible between days (n = 6) with $R^2 > 0.95$. Coefficient of variation (CVs) of slopes between days were <3% (calibration data not shown).

Intra-assay imprecision (n = 20), inter-assay imprecision (n = 16) and repeatability imprecision (n = 10) were <10% except for 16-KA'2, which showed an intra- and inter-assay imprecision of 14% and 16%, respectively (data not shown). Recoveries (n = 6) measured by spiking urine samples with three different concentrations of standard solution, ranged from 89% to 112%, as shown in Supplementary Table 2.

The LLOQ, or functional sensitivity, was at least 0.1 μmol/L for each analyte with a CV < 20% (data not shown). The minimal sample volume was established to be 500 μL.

The method did not suffer from carry-over (<0.1% for all analytes, data not shown).

Primary urine samples were stable at room temperature for at least 1 week, at 4 °C for at least 8 weeks and at –20 °C for at least 12 weeks. Samples were stable for at least 4 freeze-thaw cycles. Derivatized samples were stable for at least 2 weeks in the autosampler (room temperature). Stability data are shown in Table 2.

Method comparison

We compared the results obtained by the newly-developed GC-MS/MS and the former GC-MS method in a series of patients specimens routinely analyzed for USP at our laboratory. Passing-Bablok regression (n = 20) showed slightly lower concentrations for A, 11-KE, PDL, 11-HA, 11-HE, aPDL, aP2, P2, P3, A'3, PTL, 17-P'3, THA, THB, aTHB, THF, aTHF and α-cortol when quantified with the new GC-MS/

Table 2: Stability of steroid metabolites (n = 6).

Steroid metabolite	Room temperature, days	4 °C, days	–20 °C, days	Freeze/thaw, cycles	Autosampler, days
A	>90	>90	>90	4	>14
E	83	>90	>90	4	>14
DHEA	25	>90	>90	4	>14
11-KE	24	>90	>90	4	>14
PDL	41	>90	>90	4	>14
11-HA	>90	>90	>90	4	>14
11-HE	>90	>90	>90	4	>14
Polone	Not tested	Not tested	Not tested	Not tested	>14
aPDL	Not tested	Not tested	Not tested	Not tested	>14
16-OH-DHEA	6	55	>90	4	>14
aP2	>90	>90	>90	4	>14
P2	>90	>90	>90	4	>14
P3	46	>90	>90	4	>14
16-KA'2	Not tested	Not tested	Not tested	Not tested	>14
A3	11	>90	>90	4	>14
THS	19	>90	>90	4	>14
Oestriol	Not tested	Not tested	Not tested	Not tested	>14
TH-DOC	Not tested	Not tested	Not tested	Not tested	>14
PTL	>90	>90	>90	4	>14
16-OH-p'ol	Not tested	Not tested	Not tested	Not tested	>14
17-P3	12	>90	>90	4	>14
THE	23	>90	>90	4	>14
THA	33	>90	>90	4	>14
THB	>90	>90	>90	4	>14
aTHB	>90	>90	>90	4	>14
THF	>90	>90	>90	4	>14
aTHF	>90	>90	>90	4	>14
α-CTLN	29	65	>90	4	>14
β-CTLN	37	55	>90	4	>14
α-cortol	>90	>90	>90	4	>14

Abbreviations as in Figure 1 and Supplemental Table 1.

Table 3: Reference intervals of urinary steroid metabolites in men (μmol/24 h) per decade.

Steroid metabolite	Age decade, years											
	20–29		30–39		40–49		50–59		60–69		70–79	
	Reference interval	Mean[a]	Reference interval	Mean[a]	Reference interval	Mean[a]	Reference interval	Mean[a]	Reference interval	Mean[a]	Reference interval	Mean[a]
Androgen												
A	6.8–36.4	21.6	6.4–29.0	13.7(NP)	5.5–22.7	14.0	3.2–17.6	10.4	3.5–14.7	6.8(NP)	2.6–11.0	6.8
E	5.0–39.9	13.5(NP)	0.3–16.9	8.6	3.1–16.1	9.6	1.2–16.1	8.6	1.5–13.0	7.3	2.1–12.1	7.0
DHEA	0.5–41.2	6.6(NP)	0.2–17.3	1.5(NP)	0.6–12.9	2.6(NP)	0.2–7.0	1.1(NP)	0.2–3.2	0.6(NP)	0.1–7.8	0.9(NP)
11-KE	0.1–3.6	1.8	0.1–3.5	1.6	0.1–3.4	1.7	0.4–5.2	1.8(NP)	0.5–4.8	1.9(NP)	0.4–4.6	1.7(NP)
11-HA	1.1–9.5	5.3	1.1–6.9	4.0	2.3–7.2	4.8	2.2–8.1	4.2(NP)	0.9–8.2	4.5	1.6–7.3	4.4
11-HE	0.1–4.0	1.9	0.0–4.7	1.6(NP)	0.1–4.1	1.9	0.1–4.6	2.0	0.2–6.3	1.4(NP)	0.5–7.6	1.9(NP)
Estriol	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Cortisol												
THE	5.0–25.3	15.2	6.5–24.7	12.9(NP)	7.1–20.9	14.0	5.1–24.2	14.7	5.4–23.0	14.2	5.6–22.6	14.1
THF	3.5–13.5	6.9(NP)	3.1–10.0	6.5	3.8–10.6	7.2	2.5–13.4	7.9	3.9–16.0	8.2(NP)	4.0–15.3	9.7
aTHF	2.5–21.6	7.4(NP)	1.8–15.3	8.5	3.9–12.8	8.3	3.3–14.7	7.1(NP)	3.0–13.2	5.9(NP)	1.6–12.4	7.0
α-CTLN	2.2–8.5	5.4	2.7–6.7	4.3(NP)	2.8–6.7	4.8	1.9–7.3	4.6	1.5–8.6	5.0	2.1–8.3	5.2
β-CTLN	1.4–6.1	2.7(NP)	0.9–4.5	2.7	1.0–4.5	2.7	0.9–4.5	2.7	1.3–4.8	2.5(NP)	0.9–4.1	2.5
α-cortol	0.5–1.7	0.8(NP)	0.5–1.2	0.7(NP)	0.5–1.2	0.9	0.4–1.6	0.8(NP)	0.2–1.8	1.0	0.3–2.1	1.2
Progesterone												
aP2	0.2–1.3	0.4(NP)	0.1–0.9	0.4(NP)	0.1–0.7	0.4	0.0–0.6	0.3	0.0–0.6	0.2(NP)	0.0–0.5	0.2(NP)
P2	0.6–3.7	1.3(NP)	0.3–2.4	1.0(NP)	0.5–2.2	1.0(NP)	0.3–2.6	0.8(NP)	0.2–2.4	0.8(NP)	0.1–2.0	1.1
P3	2.2–8.4	4.3(NP)	0.6–6.0	3.3	1.2–5.3	3.3	1.4–6.4	2.8(NP)	1.0–6.5	2.2(NP)	1.3–5.2	2.6(NP)
Polone	0.0–0.7	0.2(NP)	0.0–0.3	0.2(NP)	0.0–0.3	0.2(NP)	0.0–0.3	0.1(NP)	0.0–0.3	0.1(NP)	0.0–0.3	0.1(NP)
Aldosterone												
THA	0.1–1.1	0.6	0.1–1.1	0.4(NP)	0.1–0.8	0.4	0.1–0.9	0.5	0.0–1.1	0.4(NP)	0.1–0.9	0.5
THB	0.3–1.4	0.6(NP)	0.1–1.1	0.5	0.2–1.1	0.5(NP)	0.2–1.3	0.5(NP)	0.1–1.7	0.56(NP)	0.2–1.3	0.7
aTHB	0.3–3.3	1.8	0.1–3.4	1.7	0.3–2.7	1.5	0.6–3.2	1.3(NP)	0.3–3.4	1.0(NP)	0.1–3.0	1.6
Intermediate												
THS	0.0–0.6	0.2(NP)	0.0–0.4	0.2(NP)	0.0–0.3	0.2	0.0–0.5	0.2	0.1–0.7	0.3(NP)	0.1–0.7	0.3(NP)
PDL	0.4–2.4	0.9(NP)	0.3–2.0	0.8(NP)	0.4–1.8	0.8(NP)	0.2–2.0	0.9(NP)	0.3–1.9	0.7(NP)	0.2–1.6	0.9
PTL	<0.1	<0.1	<0.1	<0.1	0.0–0.2	0.05(NP)	0.0–0.2	0.06(NP)	0.0–0.4	0.06(NP)	0.0–0.2	0.07(NP)
aPDL	0.0–0.4	0.2(NP)	0.0–0.3	0.1(NP)	0.0–0.3	0.1(NP)	0.0–0.3	0.1(NP)	0.0–0.2	0.1(NP)	0.0–0.2	0.1(NP)
TH-DOC	≤0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	≤0.1	<0.1	<0.1	<0.1
Fetal												
A'3	1.0–9.6	2.5(NP)	0.5–9.9	2.2(NP)	0.5–5.1	2.1(NP)	0.5–4.4	1.4(NP)	0.1–3.0	1.3	0.5–3.4	1.2(NP)
16K-A'2	0.2–1.5	0.5(NP)	0.1–1.5	0.4(NP)	0.0–0.7	0.3	0.0–0.8	0.3(NP)	0.0–0.8	0.2(NP)	0.0–0.8	0.3(NP)
16-OH-DHEA	1.2–11.3	4.2(NP)	0.2–12.6	2.0(NP)	0.4–3.4	1.9	0.1–4.2	1.2(NP)	0.1–3.2	0.7(NP)	0.2–4.6	1.0(NP)
16,18-$(OH)_2$-DHEA	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
16-OH-p'ol	0.0–0.4	0.2	0.0–0.4	0.1(NP)	0.0–0.2	0.1(NP)	0.0–0.2	0.1(NP)	<0.1	<0.1	0.0–0.2	<0.1
15-OH-PDL	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
17-P3	0.1–4.0	2.0	0.2–3.9	1.1(NP)	0.2–2.3	1.2	0.1–1.7	0.8	0.1–1.3	0.5(NP)	0.1–1.5	0.8
15-OH-P'DL	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

[a]Data are presented as mean for parametric data or median for non-parametric (NP) data. Abbreviations as in Figure 1 and Supplemental Table 1.

MS method compared to the GC-MS method, as shown in Supplementary Table 3.

Reference intervals and influence of OCP

As shown in Tables 3 and 4, almost all steroid excretions were different between the various age and sex groups. Androgen metabolite excretion declined with age in men and women, with absolute concentrations much higher in men compared to women (Figures 2 and 3). Cortisol metabolite excretion remained constant during life in both sexes but increased in women 70–79 years of age. Progesterone metabolite excretion peaked in 30–39 year old women and declined afterwards. Fetal metabolites declined in both sexes during life. In addition to absolute

Table 4: Reference intervals of urinary steroid metabolites in women (μmol/24 h) per decade.

Steroid metabolite	Age decade, years															
	20–29				30–39				40–49		50–59		60–69		70–79	
	OCP−		OCP+		OCP−		OCP+									
	Reference interval	Mean[a]	Reference interval	Mean[a]	Reference interval	Mean[a]	Reference interval	Mean[a]	Reference interval	Mean[a]	Reference interval	Mean[a]	Reference interval	Mean[a]	Reference interval	Mean[a]
Androgen																
A	2.8–20.8	6.7$^{(NP)}$	0.9–12.2	6.5	0.1–17.3	8.0	1.9–10.9^{b}	6.4	2.1–15.4	5.2$^{(NP)}$	1.1–7.0	2.7$^{(NP)}$	0.5–8.8	2.5$^{(NP)}$	0.3–6.0	1.9$^{(NP)}$
E	1.8–14.4	8.1	1.1–10.4	5.8	1.4–13.2	7.3	1.9–17.8	6.9$^{(NP)}$	2.6–17.7	6.2$^{(NP)}$	0.9–7.4	4.2	1.2–9.7	3.3$^{(NP)}$	0.8–7.6	2.5$^{(NP)}$
DHEA	0.2–11.8	1.2$^{(NP)}$	0.1–2.5^{b}	0.7$^{(NP)}$	0.1–12.2	0.8$^{(NP)}$	0.2–3.4	0.9$^{(NP)}$	0.1–5.7	0.7$^{(NP)}$	0.2–1.0	0.3$^{(NP)}$	0.1–0.8	0.3$^{(NP)}$	0.0–0.6	0.2$^{(NP)}$
11-KE	0.4–3.1	1.0$^{(NP)}$	0.1–2.3^{c}	0.5$^{(NP)}$	0.5–2.8	1.1$^{(NP)}$	0.2–3.1^{c}	0.8$^{(NP)}$	0.1–3.1	1.6	0.6–3.9	1.4$^{(NP)}$	0.4–3.6	1.5$^{(NP)}$	0.5–3.8	1.9
11-HA	0.6–6.3	1.7$^{(NP)}$	0.5–3.1	1.0$^{(NP)}$	0.6–6.0	2.2$^{(NP)}$	0.5–3.7^{c}	1.5$^{(NP)}$	0.6–5.2	2.9	1.0–5.3	2.3$^{(NP)}$	0.3–4.8	2.5	0.3–5.0	2.8
11-HE	0.1–2.3	1.2	0.0–2.2^{b}	0.6$^{(NP)}$	0.3–3.8	1.3$^{(NP)}$	0.1–3.3	0.8$^{(NP)}$	0.2–3.1	1.6	0.2–3.0	1.6	0.1–2.1	1.0	0.4–4.7	1.4$^{(NP)}$
Estriol	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.0–0.3	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Cortisol																
THE	3.1–23.4	7.8$^{(NP)}$	2.3–11.2^{c}	4.7$^{(NP)}$	2.6–14.4	8.5	1.3–15.1^{c}	5.3$^{(NP)}$	4.7–18.2	9.2$^{(NP)}$	4.5–18.4	8.3$^{(NP)}$	4.8–19.4	9.1$^{(NP)}$	3.4–18.8	11.1
THF	1.0–7.1	4.1	1.6–5.6	3.0$^{(NP)}$	2.2–8.7	4.2$^{(NP)}$	0.4–7.4	3.9	2.8–8.0	4.5$^{(NP)}$	1.8–7.7	4.7	2.6–12.7	5.4$^{(NP)}$	2.2–10.0	6.1
aTHF	0.4–10.4	3.2$^{(NP)}$	0.4–5.0^{c}	1.3$^{(NP)}$	0.1–7.6	3.6	0.7–4.2^{c}	1.8$^{(NP)}$	0.9–5.4	3.1	1.1–6.3	2.4$^{(NP)}$	0.7–4.8	2.8	0.5–9.0	2.3$^{(NP)}$
α-CTLN	0.9–7.4	4.1	1.1–5.6	3.4	1.5–7.4	3.3$^{(NP)}$	1.3–5.4	3.3	1.3–6.6	3.9	0.9–6.4	3.7	2.1–7.1	3.5$^{(NP)}$	1.8–7.5	4.7
β-CTLN	0.1–3.3	1.7	0.4–2.4^{b}	0.9$^{(NP)}$	0.4–2.8	1.6	0.4–2.4^{c}	1.2	0.7–4.0	1.6$^{(NP)}$	0.8–3.2	1.5$^{(NP)}$	0.8–3.1	1.6$^{(NP)}$	0.7–3.5	2.1
α-cortol	0.1–1.1	0.6	0.3–1.0	0.6	0.3–1.3	0.5	0.3–1.5	0.7$^{(NP)}$	0.3–1.0	0.6	0.3–1.2	0.5$^{(NP)}$	0.3–1.4	0.7$^{(NP)}$	0.3–1.4	0.9
Progesterone																
aP2	0.0–3.5	0.3$^{(NP)}$	0.0–0.4	0.2$^{(NP)}$	0.0–3.6	0.5$^{(NP)}$	0.0–0.4^{c}	0.1$^{(NP)}$	0.1–5.6	0.3$^{(NP)}$	0.0–0.3	0.2$^{(NP)}$	0.0–0.4	0.1$^{(NP)}$	0.0–0.2	0.1$^{(NP)}$
P2	0.3–14.2	1.5$^{(NP)}$	0.2–2.1^{b}	0.7$^{(NP)}$	0.4–21.9	2.2$^{(NP)}$	0.2–4.4^{c}	1.0$^{(NP)}$	0.2–25.6	1.6$^{(NP)}$	0.2–1.4	0.6$^{(NP)}$	0.2–1.7	0.7$^{(NP)}$	0.1–1.2	0.6
P3	0.6–5.7	2.1$^{(NP)}$	0.3–2.9^{b}	0.8$^{(NP)}$	0.7–6.5	2.7$^{(NP)}$	0.3–5.6^{c}	1.4$^{(NP)}$	0.1–5.3	2.6	0.5–2.6	1.1$^{(NP)}$	0.5–2.7	1.0$^{(NP)}$	0.2–1.8	1.0
Polone	0.0–1.3	0.2$^{(NP)}$	0.0–0.3^{c}	0.1$^{(NP)}$	0.0–2.2	0.2$^{(NP)}$	0.0–0.4^{b}	0.1$^{(NP)}$	0.0–2.5	0.2$^{(NP)}$	0.0–0.2	0.1$^{(NP)}$	0.0–0.2	0.1$^{(NP)}$	<0.1	<0.1
Aldosterone																
THA	0.1–1.3	0.3$^{(NP)}$	0.1–0.6	0.3$^{(NP)}$	0.0–0.5	0.3	0.0–0.8	0.2$^{(NP)}$	0.2–0.6	0.3$^{(NP)}$	0.1–0.8	0.3$^{(NP)}$	0.1–0.9	0.3$^{(NP)}$	0.0–0.8	0.3$^{(NP)}$
THB	0.1–1.3	0.4$^{(NP)}$	0.1–0.7	0.3$^{(NP)}$	0.1–0.8	0.4$^{(NP)}$	0.1–1.0	0.3$^{(NP)}$	0.2–0.7	0.4	0.2–0.9	0.4$^{(NP)}$	0.1–1.3	0.4$^{(NP)}$	0.2–1.0	0.4$^{(NP)}$
aTHB	0.2–3.5	0.8$^{(NP)}$	0.2–1.5^{b}	0.4$^{(NP)}$	0.1–1.5	0.8	0.2–1.6^{b}	0.5$^{(NP)}$	0.2–1.3	0.8	0.2–1.5	0.5$^{(NP)}$	0.2–1.0	0.6	0.2–1.7	0.6$^{(NP)}$
Intermediate																
THS	0.0–0.3	0.1$^{(NP)}$	0.0–0.3	0.1$^{(NP)}$	0.0–0.3	0.2$^{(NP)}$	0.0–0.3	0.1$^{(NP)}$	0.1–0.4	0.2$^{(NP)}$	0.0–0.6	0.2$^{(NP)}$	0.1–0.7	0.2$^{(NP)}$	0.0–0.6	0.2$^{(NP)}$
PDL	0.1–1.9	0.4$^{(NP)}$	0.0–0.6^{b}	0.2$^{(NP)}$	0.1–2.1	0.9	0.0–1.0^{c}	0.2$^{(NP)}$	0.1–2.6	0.6$^{(NP)}$	0.0–0.7	0.2$^{(NP)}$	0.0–0.6	0.2$^{(NP)}$	0.0–0.4	0.2$^{(NP)}$
PTL	0.0–0.2	0.04$^{(NP)}$	<0.1^{c}	<0.1	0.0–0.2	0.03$^{(NP)}$	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.0–0.4	0.06$^{(NP)}$	0.0–0.3	0.05$^{(NP)}$
aPDL	0.0–0.2	0.1$^{(NP)}$	<0.1	<0.1	0.0–0.2	<0.1	<0.1	<0.1	0.0–0.2	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
TH-DOC	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Fetal																
A'3	0.3–5.8	1.1$^{(NP)}$	0.1–2.4	1.2	0.2–2.8	1.1$^{(NP)}$	0.3–4.0	1.0$^{(NP)}$	0.3–2.4	0.9$^{(NP)}$	0.1–1.1	0.6	0.1–1.9	0.5$^{(NP)}$	0.0–0.8	0.4
16K-A'2	0.0–1.2	0.3$^{(NP)}$	0.0–0.4	0.1$^{(NP)}$	0.0–0.6	0.2$^{(NP)}$	0.0–0.7	0.2$^{(NP)}$	0.0–0.5	0.2$^{(NP)}$	0.0–0.3	0.1$^{(NP)}$	0.0–0.2	0.1$^{(NP)}$	0.0–0.2	0.1$^{(NP)}$
16-OH-DHEA	0.1–3.4	1.7	0.1–2.2	1.0	0.1–5.0	1.0$^{(NP)}$	0.2–4.4	0.9$^{(NP)}$	0.2–3.3	0.6$^{(NP)}$	0.0–1.5	0.3$^{(NP)}$	0.0–1.1	0.2$^{(NP)}$	0.0–0.5	0.2$^{(NP)}$
16,18-$(OH)_2$-DHEA	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
16-OH-p'ol	0.0–0.2	<0.1	0.0–0.2	<0.1	<0.1	<0.1	0.0–0.2	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
15-OH-PDL	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
17-P3	0.1–2.2	0.8$^{(NP)}$	0.0–1.1^{c}	0.3$^{(NP)}$	0.0–2.8	0.5$^{(NP)}$	0.0–2.1	0.3$^{(NP)}$	0.0–1.1	0.3$^{(NP)}$	0.0–0.7	0.2$^{(NP)}$	0.0–0.4	0.2$^{(NP)}$	0.0–0.4	0.1$^{(NP)}$
15-OH-P'DL	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

[a]Data are presented as mean for parametric data or median for non-parametric (NP) data. [b]$p < 0.1$ compared to women of the same age not taking OCP. [c]$p < 0.05$ compared to women of the same age not taking OCP. Abbreviations as in Figure 1 and Supplemental Table 1.

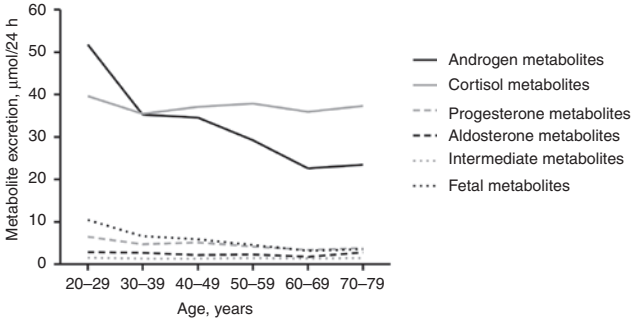

Figure 2: Median sum scores of urinary steroid metabolite excretion in men per decade (n = 120).

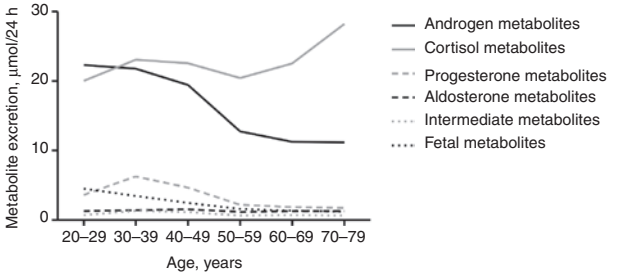

Figure 3: Median sum scores of urinary steroid metabolite excretion in women not using OCP per decade.

reference intervals we also calculated age and sex specific reference intervals for diagnostic ratios, as presented in Supplementary Table 4.

There were significant differences between distributions of metabolite excretions for women with or without the use of OCP (Table 4 and Figure 4). Women using OCP had lower excretions of androgen metabolites in the 20–29 year old age group (16.3 [10.6–19.8] vs. 21.8 [13.9–23.9] µmol/24 h, p = 0.042). Progesterone metabolite excretion was decreased in both age groups using OCP compared to corresponding age groups not using OCP (20–29 years: 1.9 [1.5–3.3] vs. 3.6 [2.5–7.6] µmol/24 h, p = 0.006 and 30–39 years: 2.6 [1.5–4.0] vs. 6.3 [3.4–13.2] µmol/24 h, p = 0.001). Multiple cortisol metabolites were excreted in significantly lower amounts in the women using OCP (Table 4). Total cortisol metabolite excretion showed a trend toward lower excretion in women using OCP in both age groups (20–29 years: 13.1 [10.6–21.4] vs. 20.0 [12.4–31.2] µmol/24 h, p = 0.058 and 30–39 years: 15.8 [11.1–22.5] µmol/24 h, p = 0.055, Figure 4).

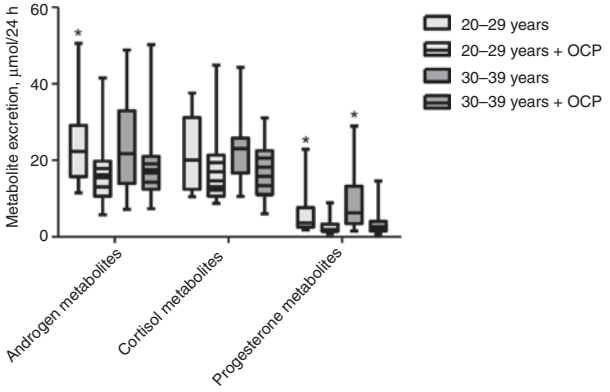

Figure 4: Urinary metabolite excretion in women with versus without use of OCP.
Boxes represent median with interquartile range. Whiskers represent minimum and maximum values. OCP, oral contraceptive pills.
*p < 0.05 compared to the same age group using OCP.

Discussion

To the best of our knowledge, we here describe for the first time age- and sex-specific reference intervals for urinary steroid metabolite excretion in a well-defined and large healthy adult population with a broad age range. These urinary steroid profiles were determined with a newly-developed and validated GC-MS/MS assay.

Well-defined reference intervals for the adult population are essential, as USP in adult subjects has proven to be a valuable diagnostic tool in several clinical situations. For example, congenital adrenal hyperplasia (CAH), in particular the milder variants, might be diagnosed for the first time in adulthood [6, 7] by noticing moderately elevated P3, PTL and PDL excretions and/or elevated diagnostic ratios for these enzyme deficiencies (Supplementary Table 4). In addition, USP can be helpful to evaluate the efficacy of glucocorticoid treatment in adult patients with CAH or for the diagnosis of licorice induced hypertension, which is reflected by an impaired hydroxysteroiddehydrogenase activity [8]. Patients with ACC often demonstrate several disorders in steroid biosynthesis. Retrospective data demonstrate the diagnostic potential of USP to differentiate between a benign adenoma and ACC [10, 11]. These abnormalities in steroid biosynthesis are clinically useful for both diagnosis as well as for the follow-up of patients with ACC [9].

Our data show that excretions of almost all 33 analyzed steroid metabolites are affected by age and gender, underscoring the need for age and sex specific reference intervals (Tables 3 and 4). In addition, it was demonstrated that use of OCP resulted in a significantly lower urinary excretion of progesterone and androgen metabolites. The decrease in progesterone and androgen excretion can be explained by an OCP induced inhibition of the hypothalamic-pituitary-gonadal axis. In addition, there was a trend for a lower urinary cortisol metabolite

excretion in women on OCP, which is likely to result from an OCP induced elevation of the plasma cortisol-binding globulin (CBG) concentration [20, 21].

Our data are in agreement with previous reports showing a decline in androgen production with age, while cortisol production remains relatively stable throughout adult life (Figures 2 and 3) [16, 22, 23]. The observed increase in cortisol metabolite excretion among women aged 70–79 years might be due to a decline of CBG levels or differences in body composition [24]. In addition, a survivor effect cannot be excluded. The decrease in progesterone metabolite excretion in women older than 50 years of age most likely reflects their postmenopausal status. Importantly, reference intervals for the diagnostic ratios of disorders in steroid biosynthesis are also significantly affected by age and sex (Supplementary Table 4). Calculation of these diagnostic ratios is very helpful in clinical practice for the detection of various inborn errors of steroid biosynthesis and the follow-up of these conditions throughout adulthood [4, 6, 14]. Reference intervals for urinary steroid metabolites have been reported in several previous studies [4, 6, 11, 13–17]. We believe our study design is the most optimal one currently available. Previous published reference intervals did not take the age dependency of metabolite excretion into account as demonstrated in the current study. It is therefore reasonable that our reference intervals differ slightly compared to studies in which the population was analyzed as a whole irrespective of age. Obviously, the latter approach results in regression to the mean. Also, analytically we optimized the setting by applying isotope labeled internal standards as much as possible, using the most specific technique currently available. Moreover, the strength of our study is the selection of a large healthy population. Consequently, we believe that our reference intervals are useful in an adult population for anyone using a similar GC-MS/MS (or GC-MS) method to ours.

The here described newly-developed GC-MS/MS assay has several advantages over our previous GC-MS method. First of all, GC-MS/MS methods have higher specificity than GC-MS methods. Tandem mass spectrometry has the ability to select one mass-to-charge ratio (*m/z*) ion and create specific fragmented ions out of this precursor ion in the second mass spectrometer. Consequently, interference by other analytes with the same precursor *m/z*, but different product ions, is avoided. In addition, the GC-MS/MS equipment provides an improved baseline separation, which enables automatic peak integration and reduces time of analysis [25]. Pre-set requirements (based on ISO15189 and Dutch guidelines for validation of analytical methods) for precision, linearity, recovery, LLOQ, carry-over and stability were met for all steroid metabolites and results were improved compared to the former GC-MS method. Only for 16-KA'2 the inter- and intra-assay CVs were noted to be relatively high. This particular steroid metabolite, however, is of limited diagnostic value. Another advantage of the GC-MS/MS is the reduction of analysis time with 1 day. In addition, the integrated software provides automatic chromatographic data integration, whereby obtained results are transferred to the laboratory information system, which in turn generates user-friendly graphical data reports.

For more than a decade we and 27 other laboratories from all over the world participate in an external steroid profile quality assessment scheme organized by the University College London Hospitals (London, UK) and the Stichting Kwaliteitsbewaking Medische Laboratoriumdiagnostiek (SKML, The Netherlands). Since a standard reference method is lacking, participation guarantees quality and is the best way of demonstrating accuracy. In this quality scheme our GC-MS/MS method performs better than average and scores in the top 7 (range 1–7) of 28 laboratories in the year 2016 with a constant MOM score of 2 or higher.

Although liquid chromatography-tandem mass spectrometry (LC/MS-MS) is expected to become the high-throughput method of choice for targeted limited steroid determinations, we agree with other authors [4] that USP by GC-MS will remain the most powerful discovery tool for defining disorders of steroid biosynthesis for years to come. Advantages of applying USP instead of targeted blood measurements of adrenal steroids are the elimination of diurnal variation by using 24 h urine collections and the generation of a so-called metabolome including characterization of unknown metabolites. LC-MS/MS is not capable of profiling >20–30 metabolites simultaneously, although a method for glucocorticoid metabolites has been described [26]. For such profiling chromatography with high plate numbers remains very important and therefore GC-MS is still the superior technique. A future development could be the use of a multidimensional statistical approach of data interpretation (metabolomics). Data from the current study might be very useful for the development of such a tool.

In conclusion, we developed a new GC-MS/MS method for USP which is suitable for high-throughput analysis. Widely applicable age and sex specific reference intervals for 33 metabolites and their diagnostic ratios have been defined for male and female individuals aged 20–79 years. In addition, we have shown that the use of OCP influences USP in women 20–39 years of age, which should be taken into account when interpreting these results.

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