A method for determination of one hundred endogenous steroids in human serum by gas chromatography-tandem mass spectrometry

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Abstract

Steroid profiling helps various pathologies to be rapidly diagnosed. Results from analyses investigating steroidogenic pathways may be used as a tool for uncovering pathology causations and proposals of new therapeutic approaches. The purpose of this study was to address still underutilized application of the advanced GC-MS/MS platform for the multicomponent quantification of endogenous steroids. We developed and validated a GC-MS/MS method for the quantification of 58 unconjugated steroids and 42 polar conjugates of steroids (after hydrolysis) in human blood. The present method was validated not only for blood of men and non-pregnant women but also for blood of pregnant women and for mixed umbilical cord blood. The spectrum of analytes includes common hormones operating via nuclear receptors as well as other bioactive substances like immunomodulatory and neuroactive steroids. Our present results are comparable with those from our previously published GC-MS method as well as the results of others. The present method was extended for corticoids and 17α-hydroxylated 5α/β-reduced pregnanes, which are useful for the investigation of alternative "backdoor" pathway. When comparing the analytical characteristics of the present and previous method, the first exhibit by far higher selectivity, and generally higher sensitivity and better precision particularly for 17α-hydroxysteroids.

Keywords: steroid metabolome; human blood; gas chromatography – tandem mass spectrometry; backdoor pathway; pregnancy; mixed umbilical cord blood

1. Introduction

For almost six decades, gas chromatography-mass spectrometry (GC-MS) served as an efficient tool for the routine quantification of endogenous steroids (Hill et al. 2010a, Hill et al. 2010b, Krone et al. 2010). At present, liquid chromatography-tandem mass spectrometry (LC-MS/MS) is also widely used and has become the gold standard for steroid quantification (Soldin and Soldin 2009). A number of LC-MS/MS based steroidomics studies was primarily focused on corticosteroids and their metabolites (Gomes et al. 2009, Haneef et al. 2013, Marcos et al. 2014). Other chromatographic strategies may involve a direct LC-MS/MS detection of unaltered glucuronoconjugated metabolites (Esquivel et al. 2017) or the use of supercritical fluids for extraction of steroidome (Kureckova et al. 2002). However, in steroid metabolomics (steroidomics), GC-MS remains the method of choice (Krone et al. 2010). A more advanced and therefore more sensitive, specific and precise GC-MS platform known as gas-chromatography tandem-mass spectrometry (GC-MS/MS) has lately been developed. The GC-MS/MS platform on the one hand retains the advantages of GC-MS in precisely distinguishing isomers with the same mass to charge ratio (m/z). However, the use of GC-MS/MS in the analysis of endogenous steroids has still been limited. Current studies using the GC-MS/MS platform have mostly focused on the quantification of anabolic steroids in the blood of athletes or farmyard animals (Gambelunghe et al. 2007, Impens et al. 2007, Marcos et al. 2002, Raro et al. 2016, Rossi et al. 1994, Shen et al. 2008, Van Vyncht et al. 1994, Wong et al. 2016, Yamada et al. 2008) or on steroid quantifications in wastewaters (Andrasi et al. 2013, Kelly 2000, Trinh et al. 2011, Zuehlke et al. 2005). Bloklanda *et al.* (Blokland et al. 2012) simultaneously quantified 47 steroids in the form of unconjugated steroids, glucuronides and sulfates in bovine urine. Regarding the number of steroids detected, the lead is still held by a series of studies from Christakoudi *et al.* who identified and quantified human urinary steroids. Their first study included 146 C21 steroids (Christakoudi et al. 2010), the second one 32 additional C21 steroids (Christakoudi et al. 2012a), the third 76 C19 steroids (Christakoudi et al. 2012b) and the fourth study additional 52 C21 steroids (Christakoudi et al. 2013). These studies have provided a complex qualitative picture of the urinary steroid metabolome in humans; however, the lack of validation of the methods used remains its weakness. The authors from research group headed by Man-Ho Choi (Molecular Recognition Research Center of Korea Institute of Science and Technology) published a series of extensive metabolomic studies on the GC-MS platform, which were focused on the role of urinary steroids in human physiology and pathophysiology (Ha et al. 2009, Choi and Chung 2014, Kim et al. 2013, Moon et al. 2016, Moon et al. 2009). There are few GC-MS/MS studies focused on circulating steroids in humans and other mammals, and all have quantified a limited number of steroids (Courant et al. 2010, Hansen et al. 2011, Matysik and Schmitz 2015, Nilsson et al. 2015, Styrishave et al. 2017).

The purpose of this study was to address the application of the GC-MS/MS platform for the simultaneous quantification of endogenous steroids. We developed and validated a GC-MS/MS method for the multicomponent quantification of unconjugated steroids and their polar conjugates (after hydrolysis). Of the original 120 steroids or their polar conjugates tested, only 100 of them met validation criteria for at least some physiological situations. Our method was validated not only for blood of men and non-pregnant women but also for blood of pregnant women and for umbilical cord blood. The spectrum of analytes in our method includes precursor steroids, active steroids and steroid metabolites, and covers the vast part of steroid metabolome in humans (see Figures 1 and 2). Steroid profiling helps various pathologies to be rapidly diagnosed. Moreover, the results from analyses investigating steroidogenic pathways may be used as a tool for uncovering pathology causations and proposals of new therapeutic approaches (Bicikova et al. 2013, Hill et al. 2010c, Kanceva et al. 2015, Parizek et al. 2016, Sosvorova et al. 2015, Sterzl et al. 2017, Vankova et al. 2016).

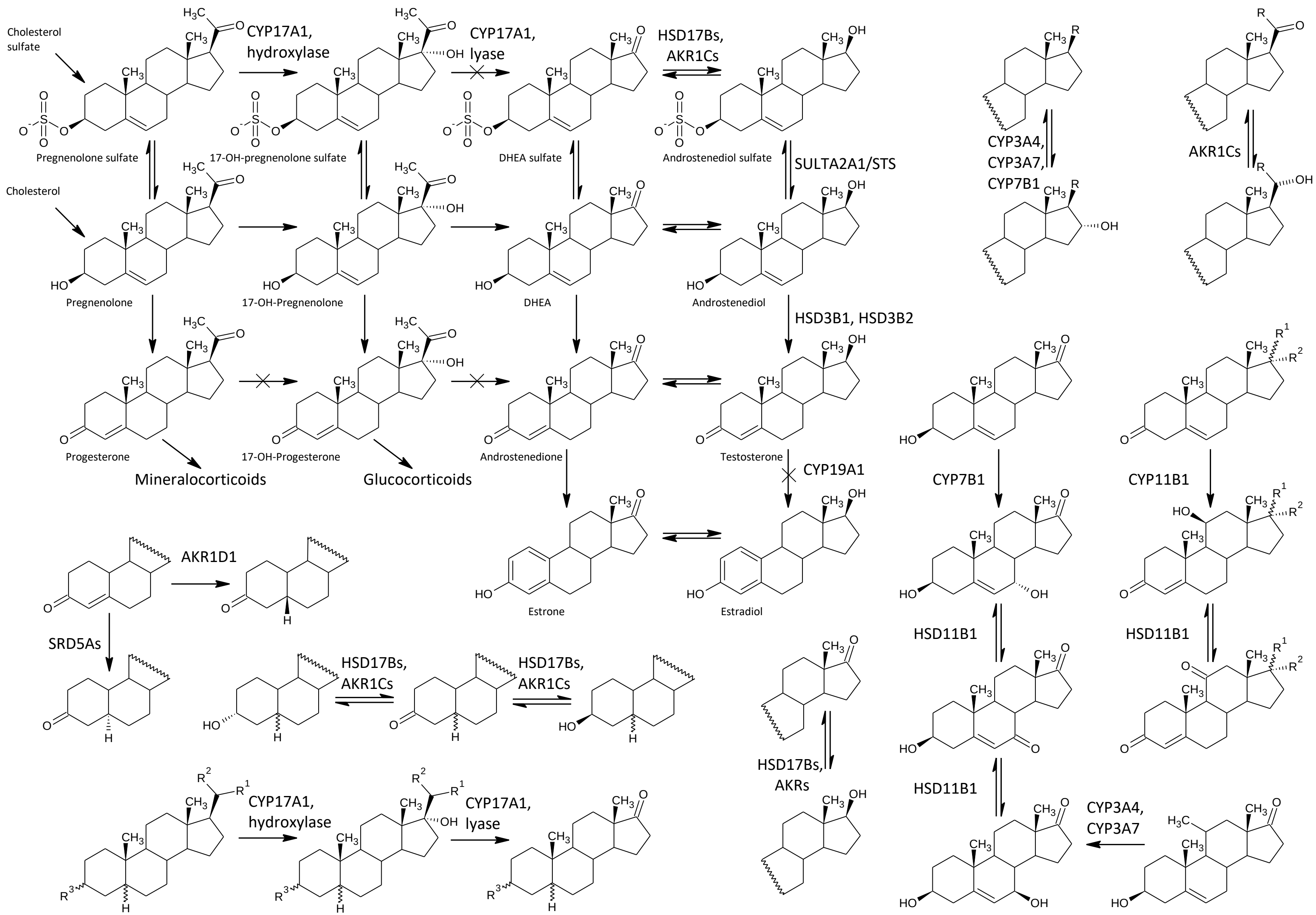


Figure 1. *Simplified scheme of human steroidogenesis. The symbol x signifies the minor or absent metabolic pathway in humans.*

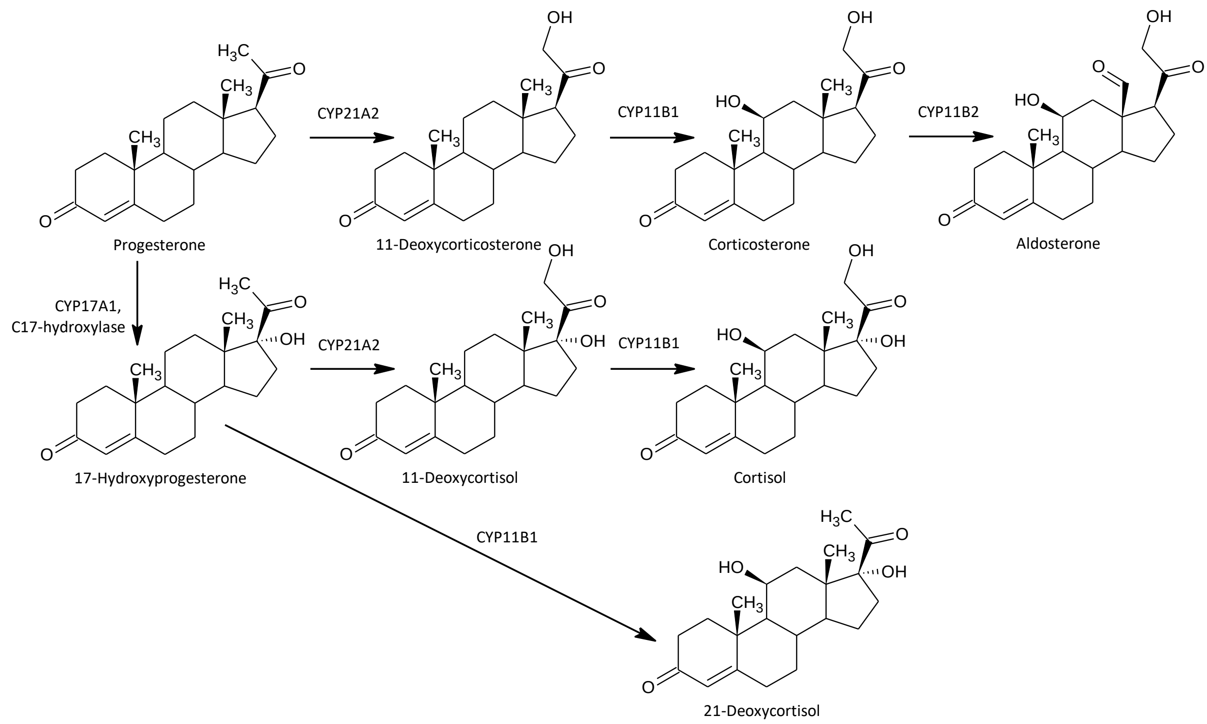


Figure 2. *Simplified scheme of corticosteroid pathways in human*

2. Experimental

2.1. Samples

Serum samples from non-pregnant subjects were collected from the employees of the Institute of Endocrinology, Prague, Czech Republic and their relatives, as well as from patients of the Institute of Endocrinology. Serum samples from pregnant women and umbilical cord serum at birth were obtained from patients of the Department of Gynecology and Obstetrics, General University Hospital and 1st Faculty of Medicine of Charles University in Prague. For all participants, the clinical protocol was approved by the Ethics Committee of the Institute of Endocrinology and by the Ethics Committee of the General University Hospital and 1st Faculty of Medicine of Charles University in Prague. Informed written consent was obtained from all participants. Serum from blood was obtained after centrifugation (5 min at 2000 × g at 2°C), and stored at −20°C until analyzed.

2.2. Chemicals

Most steroids and deuterated standards were purchased from Steraloids (Newport, RI, USA). The deuterated standard D7 cortisone [2,2,4,6,6,12,12-D7] and trimethylchlorosilane (TMCS) for hydrolysis of steroids conjugates were from Sigma-Aldrich (St. Louis, USA). Sylon BTZ, methoxyamine hydrochloride and all other solvents and chemicals were from Merck (Darmstadt, Germany). All solvents were of HPLC grade.

2.3. Stock solutions, calibration standards, and quality control samples

Stock solutions of external and internal standards (ISs) were prepared in methanol at the concentration of 1 mg/mL. The calibration curve samples (charcoal-stripped plasma with internal and external standards) were prepared in triplicate, blank samples (charcoal-stripped plasma without ISs) were made separately for unconjugated and conjugated steroids as well as zero samples (charcoal-stripped serum with ISs) were prepared. Charcoal-stripped serum was made using a multistep adsorption of steroids on charcoal. The absence of steroids in this matrix was checked by spiking of serum with [^{3}H]cortisol (10,000 dpm/mL) and measurement of the residual radioactivity close to zero. In brief, 100 g of Activated Charcoal Norit from Sigma-Aldrich (St. Louis, USA) was mixed with 1 liter of deionized water and let overnight. Then the water with fine particles of the charcoal was decanted, the charcoal was spread out on the filtration paper and let overnight. Then the charcoal was dried at 200°C in glass baking bowl for 2 hours. The dried charcoal was stored in wide mouth glass reagent bottle. Afterwards, 10^7 dpm of 3H cortisol from NEN® Life Science Products (Boston, MA, USA) was added to 1 liter of pooled human serum and 200 µL of the mixture was measured in triplicate in scintillation counter (1000 – 2000 dpm). Than the charcoal (50 g) was mixed with the pooled serum at 4°C for 3 hours. Then the centrifugation in cooled centrifuge followed at 4°C for 20 min (3500 rpm). Subsequently, the supernatant was decanted and filtered across the folded filter paper in refrigerator and the filtrate is then mixed with further 50 g of the charcoal overnight in the refrigerator and afterwards the further filtration followed. The filtrate was then treated (in parts) at 84000 g in ultracentrifuge at 4°C for 25 min and the centrifugation was repeated until the serum was free of charcoal particles. Finally, the 200 µL of the treated serum was measured (in triplicate) for 3H radioactivity together with the 200 µL of water (in triplicate) as negative control and the results were compared with initial activity of the 3H cortisol spiked serum.

Quality control (QC) samples were prepared using different serum pools from adult men, women in follicular menstrual phase and women in luteal menstrual phase, pregnant women (week 28-42 of pregnancy) and from mixed umbilical cord serum, which was collected at labor (week 28-42 of pregnancy). Using five pools differing according to gender, menstrual phase, pregnancy status and matrix (mixed umbilical serum) the QC control samples contained substantially different steroid levels covering gender differences and distinct physiological status in women. The number of samples in mixed pools in individual groups out of pregnancy was greater than 100 for each group, while the sample numbers for the groups of pregnant women and mixed umbilical serum were greater than 30 for each group.

From each stock solution of steroid (1 mg/mL), 10 µL was added into the glass tube. The mixture was dried in vacuum centrifuge (2 hours). Then the stock solution for calibration samples were prepared in concentrations 5000, 1000, 250, 62.5, 15.625, 3.906, 0.977, 0.244, 0.061 ng/mL in methanol. From these stock solutions 100 µL was administered to 10 mL extraction glass tubes vials and the mixtures were dried in the vacuum centrifuge at 45°C. Then 1 mL of charcoal-stripped serum and the solutions were mixed for 1 min. The next steps were identical for the calibration samples, zero samples, quality control samples and serum samples. The amount of 15 µL from the mixed stock solution containing ISs was added to the aforementioned samples. The mixed stock solution of

ISs for quantification of unconjugated steroids was prepared from the stock solutions of individual ISs as follows: 10 µL D6-dehydroepiandrosterone (D6-DHEA) ([2,2,3,4,4,6-D6]-DHEA, 1 mg/mL), 10 µL D8-Prog17 ([2,2,4,6,6,21,21,21-D8]-17α-hydroxyprogesterone 1 mg/mL), 10 µL D9-Prog ([2,2,4,6,6,17α,21,21,21-D9]-progesterone, 1 mg/mL), 100 µL D4-cortisol ([9,11,12,12-D4)-cortisol, 1 mg/mL), 50 µL D7-cortisone ([2,2,4,6,6,12,12-D7]-cortisone, 10 µg/mL) were mixed, the mixture was dried under the flow of nitrogen and the dry residue was dissolved in 1 mL of methanol. The internal standard of D6-DHEA sulfate ([2,2,3,4,4,6-D6]-DHEA sulfate, 1 mg/mL) for quantification of conjugated steroids was prepared similarly. The volume of 50 µL D6-DHEA sulfate, 1 mg/mL) was dried under the flow of nitrogen and the dry residue was dissolved in 1 mL of methanol.

2.4. Sample preparation

The sample preparation proceeded as follows: after addition of 15 µL of the mixed stock solution of ISs for quantification of unconjugated steroids to 1 mL of serum fluid and mixing (1 min), the unconjugated steroids were extracted from 1 mL of the mixture with diethyl-ether (3 mL). The diethyl-ether extract was dried in a block heater at 37°C. The lipids in the dry residue of the diethyl-ether extract were separated by partitioning between a mixture of methanol with water 4:1 (1 mL) and pentane (1 mL). The pentane phase was discarded and the polar phase was dried in a vacuum centrifuge at 60°C (2 h). The dry residue from the polar phase was firstly dissolved in 100 µL of acetonitrile. The solution was transferred into the 1 mL conical vial and dried in the flow of nitrogen. The dry residue was derivatized first with a methoxyamine hydrochloride solution in pyridine (2%) (60°C, 1 h) to convert the oxo-groups to methyloxime derivatives. After this first derivatization, the mixture was dried in a flow of nitrogen and the dry residue was treated with the reagent Sylon BTZ (90°C, 24 h. The Sylon BTZ is a mixture of N,O-bis(trimethylsilyl)acetamide (BSA) + trimethylchlorosilane (TMCS) + N-trimethylsilylimidazole (TMSI) (3:2:3). This sylilating agent forms trimethylsilyl derivatives on hydroxy-groups (TMS-MOX derivatives). After this second derivatization step, the mixture was dried in the nitrogen flow (2 min). After administration of approximately 1 mg of ammonium bicarbonate, the residue was partitioned between isooctane (100 µL) and N,N-dimethylformamide (50 µL). Then the volume of the vial was mixed (1 min) and centrifuged for 20 min at 3000 rpm. The lower, polar layer was aspirated with a Pasteur pipette and the upper non-polar layer remained in the vial for GC-MS/MS analysis. From the upper layer, 2 µL was injected into the GC-MS/MS system.

Steroid conjugates remaining in the polar residue after diethyl ether extraction were analyzed as follows: The volume of 15 µL D6-DHEA sulfate solution (50 µg/mL) was mixed with this residue (1 min mixing). Then 1 mL of methanol was added and mixed for additional 1 min. After the centrifugation of the mixture (20 min at 3000 rpm), the upper layer was transferred to the clean 10 mL extraction tube, dried in the vacuum centrifuge at 37°C (5 h), and the dry residues were chemically hydrolyzed according to Dehennin (Dehennin and Peres 1996). Briefly, 1mL of 1M TMCS was added to the dry residue of the upper layer and after 1 min mixing, the hydrolysis proceeded for 1 hour at 55°C. Then 100 mg of sodium bicarbonate was added and after short mixing, the hydrolyzed samples were again dried in the vacuum centrifuge at 37°C (5 h). The dried residues were reconstituted with 500 µL of chromatographic water and then further processed in the same way as the free steroids. The calibration samples for the conjugated steroids were prepared similarly as for their unconjugated analogues but the standards were mixed with the polar residues after diethyl ether extraction instead of the 1 mL of charcoal-stripped serum.

2.5. Instruments and chromatography conditions

2.5.1. Instrument settings

The instrument used was a GCMS-TQ8040 system from Shimadzu (Kyoto, Japan) consisting of a gas chromatograph equipped with an automatic flow control, an AOC-20s autosampler and a triple quadrupole detector with an adjustable electron voltage of 10–195 V. The analysis was conducted in multiple reaction monitoring (MRM) mode. A capillary column with a medium polarity RESTEK Rtx-50 column (diameter 0.25mm, length 15 m, film thickness 0.1 µm) was used for analyses. Electron-impact ionization with electron voltage fixed at 60 V and emission current set to 151 µA was used for the measurements. The temperatures of the injection port, ion source and interface were maintained at 220, 300, and 310°C, respectively. Analyses were carried out in the splitless mode with a constant linear velocity of the carrier gas (He), which was maintained at 60 cm/s. The septum purge flow was set to 3 mL/min. The samples were injected using a high-pressure mode, which was applied at 200 kPa and maintained for 1 min. The detector voltage was set to 2.2 kV. The temperature program was as follows: 1 min delay at 80 °C, increase to 190 °C (40 °C/min), increase to 210 °C (6 °C/min), increase to

300 °C (20 °C/min), increase to 320 °C (40 °C/min), 4 min delay at 320 °C, initial pressure 34 kPa, injector temperature 220 °C, analysis duration 16.08 min.

2.5.2. Optimization of method sensitivity

To optimize method sensitivity, the analysis was carried out using two separately injected aliquots (2 µL) for two different groups of steroids for each sample (see Table 1). The injection volume of samples was 2 µL. However, two steroid sulfates injected in the second aliquot exceeded the upper limit of linear dynamic range (LDR). To quantify these analytes, this measurement was repeated using the third aliquot with reduced injection volume (0.2 µL). The list of analytes with corresponding abbreviations, correlation coefficients (characterizing the linearity of the response) and the respective LDRs with indication of the abundant steroid conjugates quantified in the third aliquot are shown in Table 2.

For further improvement of sensitivity, the method used time-programmed MRM acquisition. The number of injection aliquot, number of time-programmed MRM acquisition window (AW), MRM transitions with corresponding optimum collision energies for individual steroids and ISs for the corresponding steroids are shown in Table 1. The optimization of collision energies for individual steroids was performed using the Microsoft Excel Macro-Enabled Worksheet named "MRM Optimization Tool" from Shimadzu (Kyoto, Japan).

The number of qualifiers ranged from no qualifier to three qualifiers with respect to the fragmentation patterns of individual steroid derivatives and sensitivity of the method, which is inversely related with the number of MRM transitions in the given AW (Table 1). For instance, in the case of 21-deoxycortisol (DOF) just a single MRM transition was selected 517>427 (12V) as the quantifier without a qualifier, because only this transition had a satisfactory response (Table 1). The case of PD3β5α20α was similar. In addition, the respective AW 7 included a relatively high number of transitions, which limited the sensitivity. On the other hand, in the AW 1, the androstanediols were measured using three confirmation MRM transitions as the total number of transitions in AW 1 was low (Table 1).

2.5.3. Selection of internal standards

To represent different chemical and physical properties of various steroid molecules we originally tried to use a maximum number of available ISs. However, we also respected the number of deuterium atoms in the steroid molecule, which is sufficient for separation of the signals from non-deuterated steroid and its deuterated counterpart and, at the same time, wide concentration range of steroids in serum samples, and isotopic purity of the ISs. In addition, we also considered an inverse relationship between the number of MRM-transitions in acquisition windows and sensitivity of the assay. Therefore, from the original number of 16 deuterated steroids we selected five deuterated standards with different polarity such as D6-DHEA sulfate (IS1) D6-DHEA (IS2), D8-Prog17 (IS3), D9-Prog (IS4), D4-cortisol (IS5), and D7-cortisone (IS6). For the conjugated steroids, only IS1 was applicable, because the remaining ISs were instable during the hydrolysis. Therefore, for the quantification of steroid conjugates, the IS1 was used instead of IS3 and IS4 (see Table 1).

2.6. Independent analytical methods used for accuracy testing

To compare some results of the present method, we measured 47 analytes using our previously published GC-MS method (Hill et al. 2010b), 6 analytes by our LC-MS/MS method (Vitku et al. 2016) and cortisol was also measured by radioimmunoassay from Immunotech (Marseilles, France).

Table 1. MRM acquisition windows (MRM-AW), retention times, transitions and optimum collision energies for individual steroids

Injection	MRM-AW	Steroid	IS[a]	Retention time [min] peak 1	peak 2	peak 3	peak 4	MRM transition (collision energy [V]) MRM transition 1	MRM transition 2	MRM transition 3	MRM transition 4
1	1	5β-Pregnane-3α,17α,20α-triol	3(1[a])	8.34				435>255 (12)	345>255 (9)		
1	1	5α-Pregnane-3α,17α,20α-triol	3(1[a])	8.37				435>255 (12)	345>255 (9)		
1	1	17α-Hydroxypregnanolone	3(1[a])	8.48				492>172 (24)	476>386 (12)	476>296 (15)	
1	1	17α-Hydroxyallopregnanolone	3(1[a])	8.59				492>172 (24)	476>386 (12)	476>296 (15)	
1	1	D6-DHEA sulfate (IS1, conjugates)		8.61				364>274 (9)			
1	1	D6-DHEA (IS2)		8.61				364>274 (9)			
1	1	11β-Hydroxyandrosterone	1	8.65				448>268 (12)	448>358 (9)		
1	1	11β-Hydroxyetiocholanolone	1	8.70				448>268 (12)	448>358 (9)		
1	2	5α-Pregnane-3β,17α,20α-triol	3(1[a])	9.00				435>255 (12)	345>255 (9)		
1	3	11β-Hydroxyepiandrosterone	1	9.19				448>268 (12)	448>147 (18)		
1	3	Estrone	1	9.37				371>340 (9)	340>231 (15)		
1	4	3α,5α-Tetrahydrocorticosterone	1	9.56				564>158 (18)	474>158 (18)		
1	4	3α,5β-Tetrahydrocorticosterone	1	9.60				564>158 (18)	474>158 (18)		
1	5	17α,20α-Dihydroxy-4-pregnen-3-one	3(1[a])	10.00	10.06			388>298 (9)	388>267 (12)	298>145 (15)	
1	6	21-Deoxycortisol	1	10.03	10.36			517>427 (12)			
1	6	D8-17α-Hydroxyprogesterone (IS3)		10.14	10.21			437>377 (18)			
1	6	11β-Hydroxyandrostenedione	2	10.20	10.32			401>279 (9)	401>311 (9)		
1	6	D9-Progesterone (IS4)		10.41	10.49			381>350 (9)			
1	7	D4-Cortisol (IS5)		10.69	10.76			609>519 (15)			
1	7	Cortisol	5	10.70	10.78			605>515 (12)	605>143 (21)	515>425 (15)	
1	7	11-Deoxycorticorticosterone	4(1[a])	10.76	10.84			460>286 (12)	429>298 (9)		
1	8	D7-Cortisone (IS6)		10.93	10.96			538>168 (18)			
1	8	Corticosterone	1	10.94	11.03	11.13	11.22	427>293 (15)	361>165 (12)		
1	8	Cortisone	6	10.96	10.99			531>168 (15)	441>160 (18)		
2	1	5β-Androstane-3β,17β-diol	1	6.76				421>255 (9)	346>256 (6)	346>241 (6)	331>241 (6)
2	1	5α-Androstane-3α,17β-diol	1	6.89				421>255 (9)	346>256 (6)	346>241 (6)	331>241 (6)
2	1	5β-Androstane-3α,17β-diol	1	6.97				421>255 (9)	346>256 (6)	346>241 (6)	331>241 (6)
2	2	5-Androstene-3β,7α,17β-triol	1	7.29				432>327 (12)	432>233 (24)	432>209 (15)	
2	3	5α-Androstane-3β,17β-diol	1	7.66				421>255 (12)	346>241 (15)	331>241 (6)	
2,3	3	Androstenediol	1	7.70				344>239 (15)	329>239 (9)	329>197 (18)	

[a]D6-DHEA sulfate (IS1) was used as internal standard for conjugated steroids

Table 1, continued. MRM acquisition windows (MRM-AW), retention times, transitions and optimum collision energies for individual steroids

				Retention time [min]				MRM transition (collision energy [V])			
Injection	**MRM-AW**	**Steroid**	**IS[a]**	peak 1	peak 2	peak 3	peak 4	MRM transition 1	MRM transition 2	MRM transition 3	MRM transition 4
2	4	Epietiocholanolone	1	7.95				360>270 (9)	270>213 (9)	270>157 (21)	
2,3	4	Androsterone	1	8.05				360>270 (9)	270>213 (9)	270>157 (21)	
2	4	Etiocholanolone	1	8.13				360>270 (9)	270>213 (9)	270>157 (21)	
2	4	5-Androstene-3β,7β,17β-triol	1	8.17				432>327 (15)	432>233 (21)	432>209 (18)	
2	5	5β-Pregnane-3β,20α-diol	4(1[a])	8.31				269>187 (12)	269>161 (12)	269>105 (30)	
2	5	7α-Hydroxy-DHEA	1	8.34				387>247 (15)	387>219 (30)		
2,3	5	5α-Pregnane-3α,20α-diol	4(1[a])	8.41				269>187 (12)	269>161 (12)	269>105 (30)	
2,3	5	5β-Pregnane-3α,20α-diol	4(1[a])	8.46				269>187 (12)	269>161 (12)	269>105 (30)	
1	1	D6-DHEA sulfate (IS1, conjugates)		8.61				364>274 (9)			
2,3	6	D6-DHEA (IS2)		8.61				364>274 (9)			
2	6	Estradiol	1	8.61				416>285 (15)	416>326 (6)	285>205 (15)	
2,3	6	Epiandrosterone	1	8.63				360>270 (9)	360>84 (18)	360>82 (21)	
2,3	6	Dehydroepindrosterone (DHEA)	1	8.64				358>84 (18)	268>82 (21)	260>213 (6)	
2,3	6	5-Androsten-3β,16α,17β-triol	1	8.65				432>327 (15)	432>239 (15)	329>239 (9)	
2	6,7	Epitestosterone	2	8.70	8.81			389>268 (9)	389>137 (12)		
2	7	5α-Dihydrotestosterone	2	8.78	8.79			391>360 (12)	391>286 (6)	286>254 (6)	
2,3	7	Epipregnanolone	4(1[a])	8.86				388>298 (15)	388>173 (18)	388>70 (18)	
2,3	7	5α-Pregnane-3β,20α-diol	4(1[a])	8.93				449>117 (12)			
2	7	20α-Dihydropregnenolone	3(1[a])	8.93				372>117 (18)	332>117 (12)		
2	7	7β-Hydroxy-DHEA	1	8.95				387>247 (15)	387>219 (30)		
2,3	7	Allopregnanolone	4(1[a])	8.96				388>298 (15)	388>173 (18)	388>70 (18)	
2	7	Testosterone	2	8.98	9.12			389>268 (9)	389>137 (12)	389>125 (9)	
2,3	7	Pregnanolone	4(1[a])	9.03				388>298 (15)	388>173 (18)	388>70 (18)	

[a]*D6-DHEA sulfate (IS1) was used as internal standard for conjugated steroids*

Table 1, continued. MRM acquisition windows (MRM-AW), retention times, transitions and optimum collision energies for individual steroids

Injection	MRM-AW	Steroid	IS[a]	Retention time [min] peak 1	peak 2	peak 3	peak 4	MRM transition (collision energy [V]) MRM transition 1	MRM transition 2	MRM transition 3	MRM transition 4
2	8	17α-Hydroxypregnenolone	3(1[a])	9.24				474>294 (9)	474>225 (12)	474>157 (21)	
2,3	9	Estriol	1	9.41				504>311 (18)	345>255 (12)		
2,3	9	Isopregnanolone	4(1[a])	9.42				388>173 (21)	388>107 (27)	388>70 (24)	
2	9	Pregnenolone	4(1[a])	9.43				402>239 (12)	312>239 (9)	239>157 (18)	
2,3	10	5β,20α-Tetrahydroprogesterone	4(1[a])	9.53	9.55			303>288 (9)	303>159 (27)		
2	10	5α-Androstane-3,17-dione	1	9.59	9.61			315>83 (27)	315>244 (21)		
2	10	16α-Hydroxypregnenolone	3(1[a])	9.61				474>156 (27)			
2	10	16α-Hydroxytestosterone	2	9.65	9.74			477>153 (18)			
2	10	Androstenedione	2	9.77	9.88			344>313 (9)	344>137 (24)	344>125 (15)	
2	10	5α,20α-Tetrahydroprogesterone	4(1[a])	9.80	9.82			303>288 (9)	303>159 (27)		
2	11	7-oxo-DHEA	1	9.99				401>148 (18)	386>235 (30)		
2	11	20α-Dihydroprogesterone	4(1[a])	9.99	10.10			417>117 (12)	301>286 (9)	301>138 (15)	
2	11	5β-Dihydroprogesterone	4(1[a])	10.01	10.03			343>259 (18)	343>244 (33)		
2	12	D8-17α-Hydroxyprogesterone (IS3)		10.14	10.21			437>377 (18)			
2	12	17α-Hydroxyprogesterone	3(1[a])	10.18	10.24			429>370 (18)	429>170 (12)		
2	12	5α-Dihydroprogesterone	4(1[a])	10.27	10.29			343>244 (24)	343>272 (18)	288>159 (18)	
2	13	D9-Progesterone (IS4)		10.41	10.49			381>350 (9)			
2	13	Progesterone	4(1[a])	10.45	10.55			372>341 (9)	341>269 (12)		
2	13	16α-Hydroxyprogesterone	3(1[a])	10.53	10.61			429>370 (15)	429>156 (18)	156>73 (15)	

[a]*D6-DHEA sulfate (IS1) was used as internal standard for conjugated steroids*

Table 2. List of abbreviations for endogenous steroids, linearity of the response and linear dynamic range

ID	Abbreviation	Steroid	Correlation coefficient *r*	Linear dynamic range [pg injected]
1	Preg	Pregnenolone	0.9995	0.077-2000
2	Preg17	17α-Hydroxypregnenolone	0.9996	0.12-2000
3	Preg16α	16α-Hydroxypregnenolone	0.9997	0.12-2000
4	DHPreg20α	20α-Dihydropregnenolone	0.9991	0.12-2000
5	DHEA	Dehydroepiandrosterone	0.9978	07.08.2000
6	DHEA7α	7α-Hydroxy-DHEA	0.9995	0.12-2000
7	DHEA7o	7-oxo-DHEA	0.9952	0.49-2000
8	DHEA7β	7β-Hydroxy-DHEA	0.9987	0.49-2000
9	5-Adiol	5-Androstene-3β, 17β-diol	0.9979	0.49-2000
10	AT7α	5-Androstene-3β,7α,17β-triol	0.9999	0.49-2000
11	AT7β	5-Androstene-3β,7β,17β-triol	0.9993	0.12-2000
12	AT16α	5-Androstene-3β,16α,17β-triol	0.9985	0.49-2000
13	P	Progesterone	0.9998	0.12-10000
14	P17	17α-Hydroxyprogesterone	0.9997	0.12-2000
15	DHP17α20α	17α,20α-Dihydroxy-4-pregnene-3-one	0.9957	0.12-10000
16	P16α	16α-Hydroxyprogesterone	0.9998	0.12-2000
17	DHP20α	20α-Dihydroprogesterone	0.9997	0.49-2000
18	A4	Androstenedione	0.9988	0.49-2000
19	T	Testosterone	0.9998	2.0-2000
20	T16α	16α-Hydroxytestosterone	0.9997	2.0-2000
21	DHT5α	5α-Dihydrotestosterone	0.9994	0.49-2000
22	E1	Estrone	0.9995	7.8-10000
23	E2	Estradiol	0.9996	0.12-2000
24	E3	Estriol	0.9999	7.8-10000
25	DHP5α	5α-Dihydroprogesterone	0.9995	0.12-10000
26	THP3α5α	Allopregnanolone	0.9996	0.12-2000
27	THP3β5α	Isopregnanolone	0.9995	0.49-2000
28	DHP5β	5β-Dihydroprogesterone	0.9986	7.8-10000
29	THP3α5β	Pregnanolone	0.9995	0.12-2000
30	THP3β5β	Epipregnanolone	0.9996	0.12-2000
31	THP5α20α	5α,20α-Tetrahydroprogesterone	0.9995	0.12-2000
32	PD3α5α20α	5α-Pregnane-3α,20α-diol	0.9995	0.12-10000
33	PD3β5α20α	5α-Pregnane-3β,20α-diol	0.9987	7.8-10000
34	THP5β20α	5β,20α-Tetrahydroprogesterone	0.9999	0.12-2000
35	PD3α5β20α	5β-Pregnane-3α,20α-diol	0.9995	0.12-2000
36	PD3β5β20α	5β-Pregnane-3β,20α-diol	0.9997	0.49-10000
37	PD3α5α17	17α-Hydroxyallopregnanolone	0.9994	0.49-2000
38	PD3α5β17	17α-Hydroxypregnanolone	0.9995	0.49-2000
39	PT3α5α17α20α	5α-Pregnane-3α,17α,20α-triol	0.9981	0.12-10000
40	PT3β5α17α20α	5α-Pregnane-3β,17α,20α-triol	0.9977	0.12-10000
41	PT3α5β17α20α	5β-Pregnane-3α,17α,20α-triol	0.9982	0.12-10000
42	DHA5α	5α-Androstane-3,17-dione	0.9993	0.12-10000
43	THA3α5α	Androsterone	0.9987	0.12-2000
44	THA3β5α	Epiandrosterone	0.9991	2.0-2000
45	THA3α5β	Etiocholanolone	0.9994	0.12-2000
46	AD3α5α17β	5α-Androstane-3α,17β-diol	0.9996	0.12-2000
47	AD3β5α17β	5α-Androstane-3β,17β-diol	0.9989	0.12-2000
48	AD3α5β17β	5α-Androstane-3α,17β-diol	0.9996	0.12-2000
49	F	Cortisol	0.9991	31-10000
50	E	Cortisone	0.9972	125-10000

[a]*Additional application of 0.2 μL sample (third injection aliquot) besides of the usual 2 μL injection volume (for unconjugated steroids and most steroid conjugates – first and second injection aliquots) to quantify two steroid conjugates above the upper limit of the linear dynamic range*

Table 2, continued. List of abbreviations for endogenous steroids, linearity of the response and linear dynamic range

ID	Abbreviation	Steroid	Correlation coefficient *r*	Linear dynamic range [pg injected]
51	B	Corticosterone	0.9987	7.8-10000
52	DOF	21-Deoxycortisol	0.9991	0.49-2000
53	DOC	11-Deoxycorticosterone	0.9999	2-10000
54	THB3α5α	3α,5α-Tetrahydrocorticosterone	0.9995	0.12-10000
55	THB3α5β	3α,5β-Tetrahydrocorticosterone	0.999	0.49-10000
56	11OHA4	11β-Hydroxyandrostenedione	0.9978	0.49-10000
57	THA3α5α11β	11β-Hydroxyandrosterone	0.9998	0.12-2000
58	THA3β5α11β	11β-Hydroxyepiandrosterone	0.9983	0.12-2000
59	THA3α5β11β	11β-Hydroxyetiocholanolone	0.9999	0.12-2000
60	PregC	Pregnenolone sulfate	0.9994	0.077-2000
61	Preg17C	17α-Hydroxypregnenolone sulfate	0.9996	0.12-2000
62	DHPreg20αC	20α-Dihydropregnenolone sulfate	0.9991	0.12-2000
63	DHEAC	DHEA sulfate	0.998	7.8-2000[a]
64	5-AdiolC	Androstenediol sulfate	0.9981	0.49-2000
65	AT16αC	5-Androstene-3β,16α,17β-triol sulfate	0.9986	0.49-2000
66	DHP17α20αC	Conjugated 17α,20α-dihydroxy-4-pregnen-3-one	0.9945	0.12-10000
67	DHP20αC	Conjugated 20α-dihydroprogesterone	0.9997	0.49-2000
68	TC	Conjugated testosterone	0.9993	2.0-2000
69	EpiTC	Conjugated epitestosterone	0.9997	0.49-2000
70	E1C	Estrone sulfate	0.9993	7.8-10000
71	E2C	Estradiol sulfate	0.9991	0.12-2000
72	E3C	Estriol sulfate	0.9994	7.8-10000
73	THP3α5αC	Allopregnanolone sulfate	0.9995	0.12-2000
74	THP3β5αC	Isopregnanolone sulfate	0.9997	0.49-2000
75	THP3α5βC	Conjugated pregnanolone	0.9994	0.12-2000
76	THP3β5βC	Conjugated epipregnanolone	0.9994	0.12-2000
77	THP5α20αC	Conjugated 5α,20α-tetrahydroprogesterone	0.9986	0.12-2000
78	PD3α5α20αC	Conjugated 5α-pregnane-3α,20α-diol	0.9994	0.12-10000
79	PD3β5α20αC	Conjugated 5α-pregnane-3β,20α-diol	0.9981	7.8-10000
80	THP5β20αC	Conjugated 5β,20α-tetrahydroprogesterone	0.9998	0.12-2000
81	PD3α5β20αC	Conjugated 5β-pregnane-3α,20α-diol	0.9995	0.12-2000
82	PD3β5β20αC	Conjugated 5β-pregnane-3β,20α-diol	0.9994	0.49-10000
83	PD3α5α17C	17α-Hydroxyallopregnanolone sulfate	0.9994	0.49-2000
84	PD3α5β17C	Conjugated 17α-hydroxypregnanolone	0.9996	0.49-2000
85	PT3α5α17α20α	5α-Pregnane-3α,17α,20α-triol	0.9981	0.12-10000
86	PT3β5α17α20α	5α-Pregnane-3β,17α,20α-triol	0.9977	0.12-10000
87	PT3α5β17α20α	5β-Pregnane-3α,17α,20α-triol	0.9982	0.12-10000
88	THA3α5αC	Androsterone sulfate	0.9987	0.12-2000[a]
89	THA3β5αC	Epiandrosterone sulfate	0.9993	2.0-2000[a]
90	THA3α5βC	Etiocholanolone sulfate	0.9995	0.12-2000
91	THA3β5βC	Epietiocholanolone sulfate	0.9992	0.49-2000
92	AD3α5α17βC	Conjugated 5α-androstane-3α,17β-diol	0.9994	0.12-2000
93	AD3β5α17βC	Conjugated 5α-androstane-3β,17β-diol	0.9996	0.12-2000
94	AD3α5β17βC	Conjugated 5β-androstane-3α,17β-diol	0.9992	0.12-10000
95	AD3β5β17βC	Conjugated 5β-androstane-3β,17β-diol	0.9992	0.12-10000
96	THB3α5αC	Conjugated 3α,5α-tetrahydrocorticosterone	0.9994	0.12-10000
97	THB3α5βC	Conjugated 3α,5β-tetrahydrocorticosterone	0.9994	0.12-10000
98	THA3α5α11βC	11β-Hydroxyandrosterone sulfate	0.998	0.12-2000
99	THA3β5α11βC	11β-Hydroxyepiandrosterone sulfate	0.9985	0.12-2000
100	THA3α5β11βC	11β-Hydroxyetiocholanolone sulfate	0.9982	0.12-2000

[a]Additional application of 0.2 µL sample (third injection aliquot) besides of the usual 2 µL injection volume (for unconjugated steroids and most steroid conjugates – first and second injection aliquots) to quantify two steroid conjugates above the upper limit of the linear dynamic range

2.7. Method performance characteristics

2.7.1. Calibration curve and linearity of the response

The calibration was performed in charcoal-stripped serum. The analytes were quantified using calibration curves based on known concentrations in the mixtures of analyzed standards with constant level of ISs. We used a 9-point logarithmic calibration curve. The values were corrected for procedural losses according to yields of ISs. The use of ISs for individual steroids is shown in Table 1. The amount of each steroid injected from the calibration samples into the GC-corresponded to amount of 10 ng, 2 ng, 500 pg, 125 pg, 31.2 pg, 7.81 pg, 1.95 pg, 488 fg and 122 fg. The calibration curves were constructed by plotting the logarithm of response factor (analyte area/internal standard area) against the logarithm of concentration of the calibration (external) standard to cover the large concentration differences for circulating steroids in different physiological and pathophysiological situations and even more explicit contrasts between unconjugated steroids and their conjugated counterparts at appropriate number of calibration points. This arrangement also provided equal weights for individual calibration points in the logarithmic calibration curve and therefore the use of weighted regression model was not necessary to apply. The assay acceptance criterion for each back-calculated standard concentration was set 15% deviation from the nominal value.

2.7.2. Precision

The method precision (intra-assay, within-day) and intermediate precision (inter-assay, between-day) was based on the concentrations of each analyte. Regarding gender differences in the levels of testosterone and its metabolites, elevated levels of progesterone and its metabolites in the luteal menstrual phase and excessive levels of numerous steroids in serum from pregnant women and in umbilical cord serum, the precision was evaluated separately in pooled sera for adult men, women in the follicular menstrual phase, luteal menstrual phase, pregnant women at labor and for mixed umbilical cord sera at labor. The method precision was calculated from steroid concentrations in six identical samples, which were prepared from the aforementioned pools within one batch prepared on the same day. Similarly, intermediate precision was estimated from the steroid concentrations in six identical samples but these were prepared in separate batches on different days. The precision was expressed as percent of relative standard deviation (RSD).

2.7.3. Recovery

The recovery indicates the extraction efficiency of an analytical process, reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method (2018). In the present method, the recovery was determined by spiking charcoal-stripped serum with three concentrations of the individual analytes taking into account steroid levels in the corresponding pools. The recovery experiments were performed by comparing the analytical results of extracted samples with corresponding extracts of blanks spiked with the analyte post-extraction (2018) in replicates from four independent runs.

2.7.4. Accuracy

Accuracy was expressed as relative error of the measured concentration of each steroid with respect to its true spiked concentration (% bias). The accuracy testing was performed for three different concentrations of analytes dissolved in charcoal-stripped plasma, which were close to their physiological levels. The bias was tested in both intra- and inter-day experiments. The corresponding samples for accuracy testing were processed in the same way as the calibration and unknown samples (see section 2.4 Sample preparation). The bias less then ±15% was met for all analytes in all tested concentrations in both intra- and inter-day experiments. The analytes, which did not meet these criteria were not included in this method.

Furthermore, we compared our present GC-MS/MS method with our previous GC-MS method for 45 steroids in samples covering all types of human sera (Supplementary Table S1) and also tested an agreement of six common steroids (pregnenolone, 17α-hydroxypregenolone, DHEA, androstenedione, testosterone and cortisol) measured by our present method with the LC-MS/MS method (Hill et al. 2010b) in samples mostly consisting of the women in follicular menstrual phase but there were also some women in the luteal phase, postmenopausal women and men (Supplementary Table S2). Besides the LC-MS/MS and GC-MS/MS, the cortisol was also evaluated using an RIA kit from Immunotech (Marseilles, France). The comparison was performed using Bland-Altman procedure (Bland and Altman 1986) and a robust Passing Bablok regression with the use of R library "mcr" (Manuilova and Schuetzenmeister 2014).

2.7.5. Limit of Detection and Limit of Quantification

Because the baseline noise was accessible for all analytes in all matrixes (pools), the limit of detection (LOD) and limit of quantification (LOQ) were estimated using charcoal stripped plasma spiked with steroids in three levels covering gender differences and distinct physiological status in women. The LOD was calculated as 3.3 times of the baseline noise using charcoal stripped plasma vs. charcoal stripped plasma spiked with steroid on the first level with lowest concentration of analyte.

The lowest nonzero standard on the calibration curve defined the LOQ. The satisfactory analyte response at the LOQ in the present method was at least five times the analyte response of the zero calibrator and the satisfactory bias at the LLOQ was at most ±20% of nominal concentration. Similarly, the satisfactory imprecision at the LLOQ was at most ±20% RSD. For this purpose, we tested the replicates prepared in six runs (2018). The determination of signal to noise ratios (S/N) for the calculation of LOD was completed using a functionality in the Shimadzu software GCMSsolution Version 4.20, which was a component of our GC-MS/MS system.

2.7.6. Efficiency of methanolysis and stability of non-deuterated and deuterated steroids

Unfortunately, the external standards for steroid sulfates and glucuronides are not available for the full spectrum of the quantified steroid conjugates. Therefore, we have tested the efficiency of methanolysis for only seven sulfated non-deuterated steroids (6 sulfates and one disulfate) and D6-dehydroepiandrosterone sulfate (D6-DHEA). The procedure was as follows. The 100 µL or 10 µL aliquots of the stock solution of unconjugated steroid and sulfated steroid were administered into the glass extraction tubes and dried under the flow of nitrogen. Then 20 µL of methanol was added and the solution was shortly mixed. The addition of 1 mL of charcoal-stripped mixed human plasma followed and the solution was then mixed for 1 min. The obtained samples for each steroid or steroid sulfate were processed in the same way as the calibration and unknown samples (see section 2.4 Sample preparation). The responses (areas under the peak) for polar and non-polar phases after diethyl ether extraction for individual unconjugated steroids, corresponding steroid conjugates and for internal standard (D6-DHEA) were used to calculate extraction efficiency for unconjugated steroids and sulfated steroids, as well as the efficiency of methanolysis in sulfated steroids.

The analysis of chemical stability during the methanolysis for unconjugated steroids was based on the comparison of calibration samples for unconjugated analytes, which were exposed to methanolysis procedure with the same samples, which did not undergo this route.

2.8. Terminology of steroid polar conjugates

Concerning the terminology of the steroid polar conjugates used here, the term steroid sulfate was used in the case of the dominance of 3α/β-monosulfate over other forms of steroid conjugates, while the term conjugated steroid was used in the case of comparable amounts of conjugate forms (sulfates, disulfates, and glucuronides). This terminology was based on the relevant literature, with appropriate citations for each steroid as follows: Preg sulfate (Brochu and Belanger 1987, Sanchez-Guijo et al. 2015), DHPreg20α sulfate, dehydroepiandrosterone (DHEA) sulfate (Brochu et al. 1987, Labrie et al. 1997, Sanchez-Guijo et al. 2015), 5-Adiol sulfate (Labrie et al. 1997, Sanchez-Guijo et al. 2015), THP3α5α sulfate , THP3β5α sulfate (Abu-Hayyeh et al. 2013), conjugated THP3α5β (sulfate + glucuronide) (Meng et al. 1997)), PD5α3β20α sulfate (3β,20α-disulfate + 3β-sulfate) (Meng et al. 1997), conjugated PD3α5β20α (3β,20α-disulfate + 3β-sulfate + glucuronide) (Meng et al. 1997), THA3α5α sulfate (Labrie et al. 1997, Sanchez-Guijo et al. 2015), THA3β5α sulfate (Labrie et al. 1997, Sanchez-Guijo et al. 2015), THA sulfate3α5β (Tokushige et al. 2013), THA sulfate 3β5β, conjugated (glucuronide + sulfate (Labrie et al. 1997)), and conjugated AD3β5α17β (sulfate + glucuronide (Labrie et al. 1997)).

3. Results and discussion

In total, the levels of 100 analytes (58 unconjugated steroids and 42 steroid conjugates) were quantified in samples of pooled sera from groups of adult men, women in the follicular menstrual phase, women in the luteal menstrual phase, pregnant women at labor and in umbilical cord serum at labor (see Tables 2 and 4). The steroid metabolome in the maternal circulation included the levels of C21 Δ^5 steroids, C19 Δ^5 steroids, C21 Δ^4 steroids, C19 Δ^4 steroids, estrogens, C21 and C19 5α/β-reduced steroids, 7α-hydroxy-, 16α-hydroxy-, 7β-hydroxy- and 7-oxo-derivatives of C19 Δ^5 steroids, and 20α-dihydro-metabolites of C21 steroids (20α-dihydro-pregnanes) (see Table 2). Figures 3-6 show a comparison of the chromatograms for calibration samples and samples prepared from five pools of human serum and recorded on quantification MRM transitions for unconjugated steroids, which are less abundant then their conjugated counterparts (Table 3).

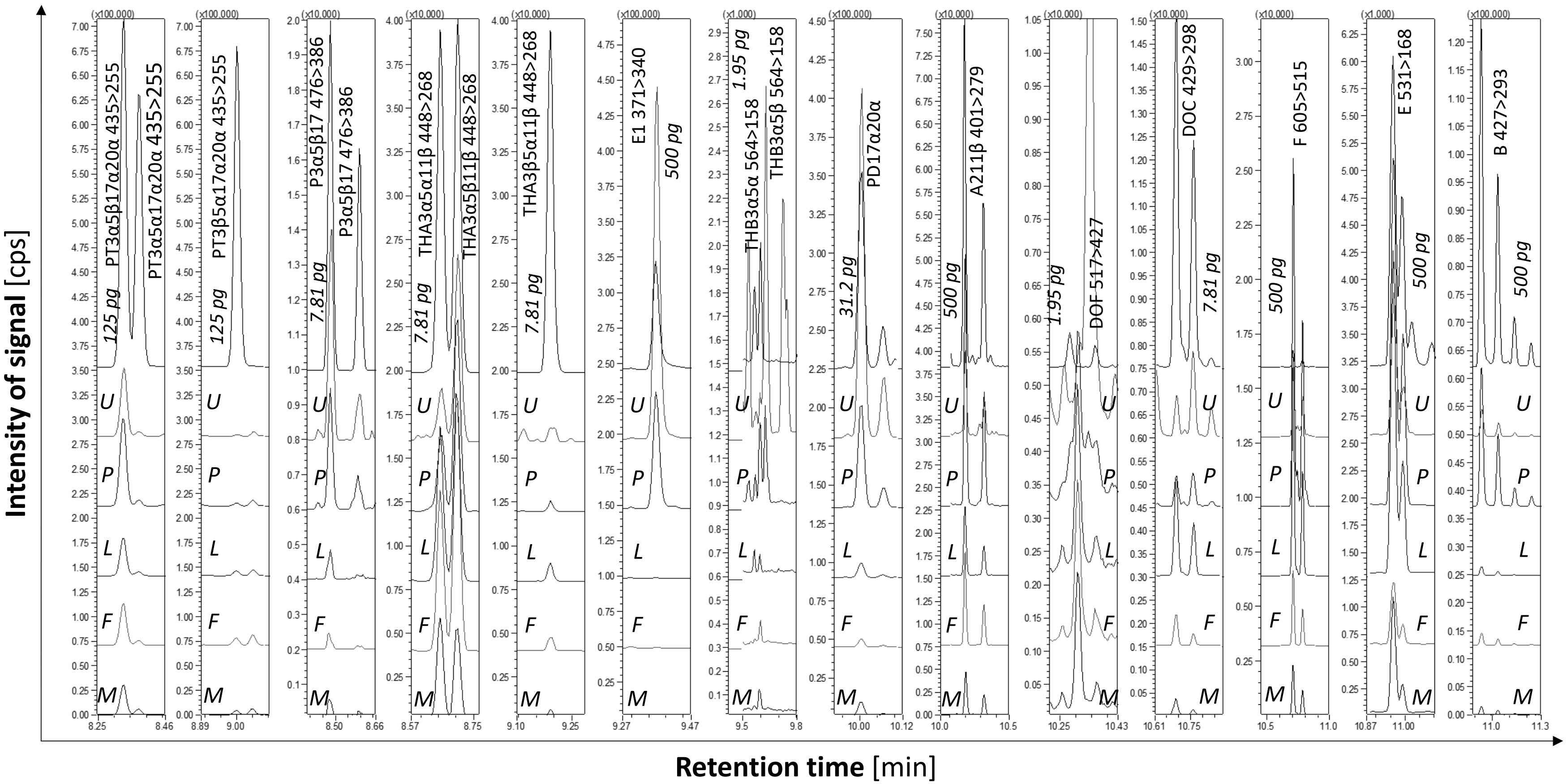


Figure 3. *Comparison of the chromatograms for calibration samples prepared from the charcoal stripped plasma and added steroids and samples of unconjugated steroids prepared from different pools of human serum and recorded on quantification MRM transitions. Numbers in embedded tables represent amounts of derivatized steroids in calibration samples (pg) injected to the GC-MS/MS system, M=males, F=follicular menstrual phase, L=luteal menstrual phase, P=pregnant women at labor, U=mixed umbilical serum at labor. Abbreviations of steroids are explained in Table 2.*

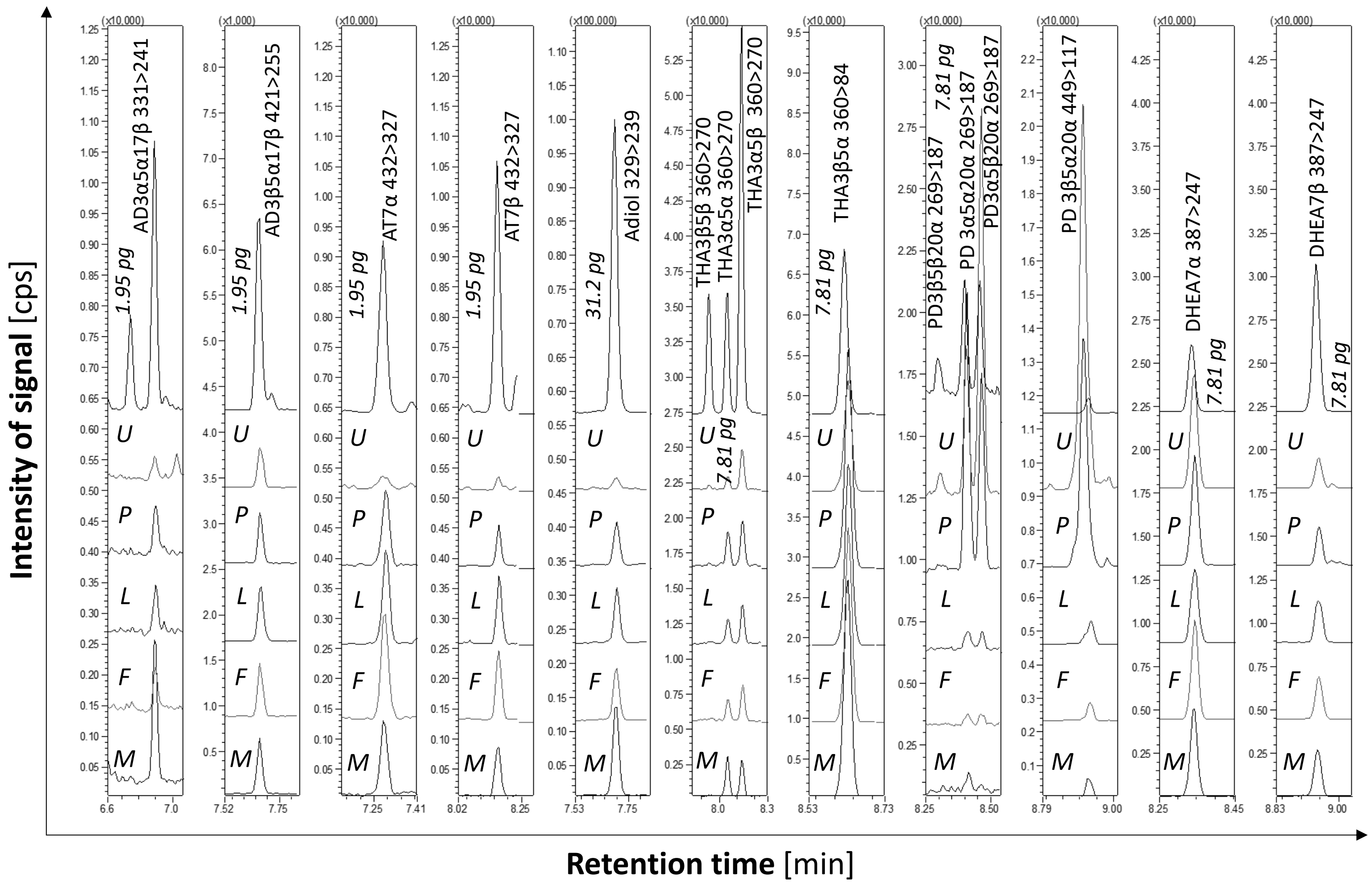


Figure 4. *Comparison of the chromatograms for calibration samples prepared from the charcoal stripped plasma and added steroids and samples of unconjugated steroids prepared from different pools of human serum and recorded on quantification MRM transitions. Numbers in embedded tables represent amounts of derivatized steroids in calibration samples (pg) injected to the GC-MS/MS system, M=males, F=follicular menstrual phase, L=luteal menstrual phase, P=pregnant women at labor, U=mixed umbilical serum at labor. Abbreviations of steroids are explained in Table 2.*

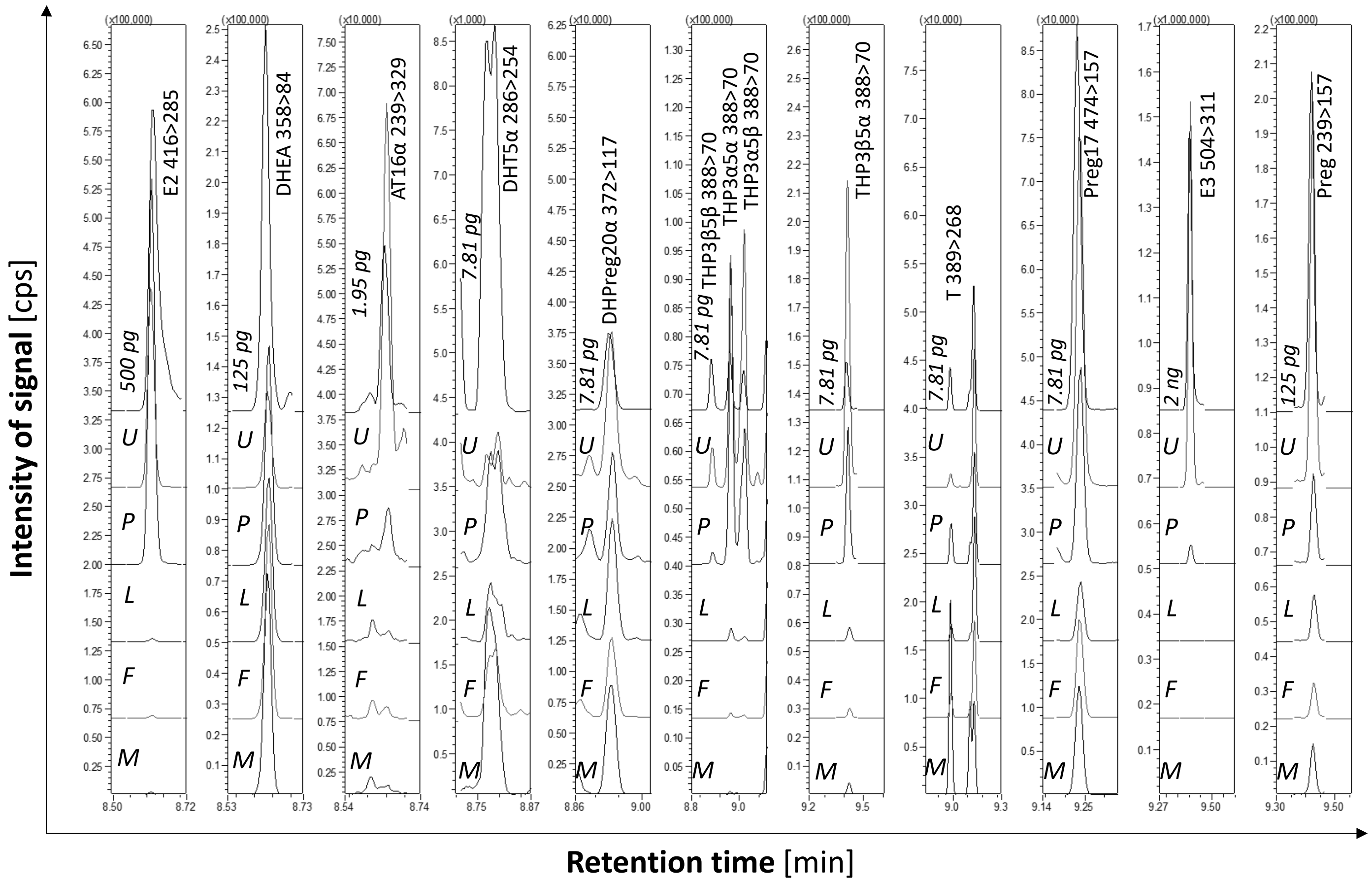


Figure 5. *Comparison of the chromatograms for calibration samples prepared from the charcoal stripped plasma and added steroids and samples of unconjugated steroids prepared from different pools of human serum and recorded on quantification MRM transitions. Numbers in embedded tables represent amounts of derivatized steroids in calibration samples (pg) injected to the GC-MS/MS system, M=males, F=follicular menstrual phase, L=luteal menstrual phase, P=pregnant women at labor, U=mixed umbilical serum at labor. Abbreviations of steroids are explained in Table 2.*

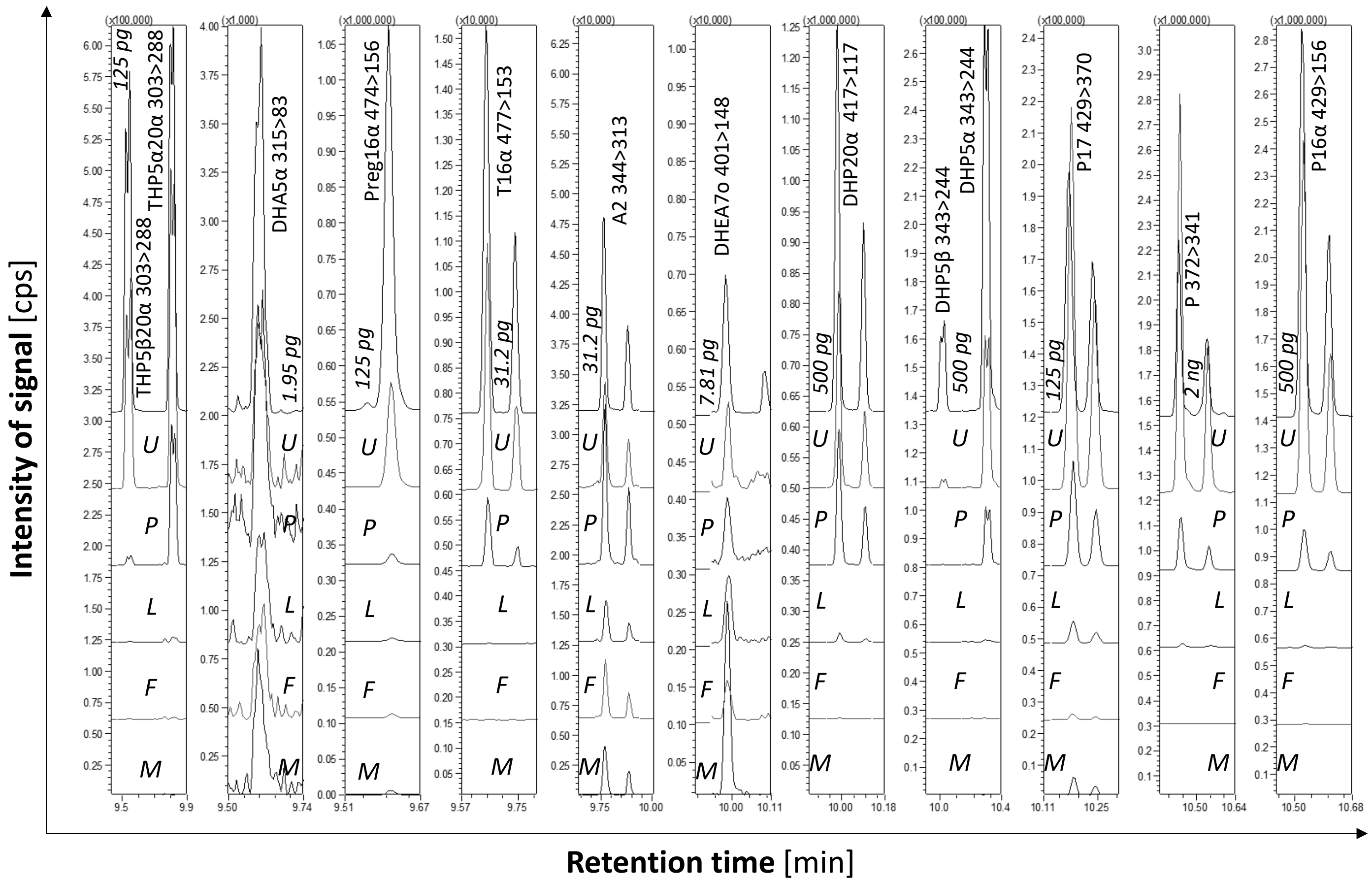


Figure 6. *Comparison of the chromatograms for calibration samples prepared from the charcoal stripped plasma and added steroids and samples of unconjugated steroids prepared from different pools of human serum and recorded on quantification MRM transitions. Numbers in embedded tables represent amounts of derivatized steroids in calibration samples (pg) injected to the GC-MS/MS system, M=males, F=follicular menstrual phase, L=luteal menstrual phase, P=pregnant women at labor, U=mixed umbilical serum at labor. Abbreviations of steroids are explained in Table 2.*

3.1. Validation parameters

3.1.1. Linearity of the response

Sufficient linearity was found for broad range of concentrations (Table 2). The 15% deviation from the nominal value for each back-calculated standard concentration as the criterion of assay acceptance was not exceeded in any case.

3.1.2. Precision

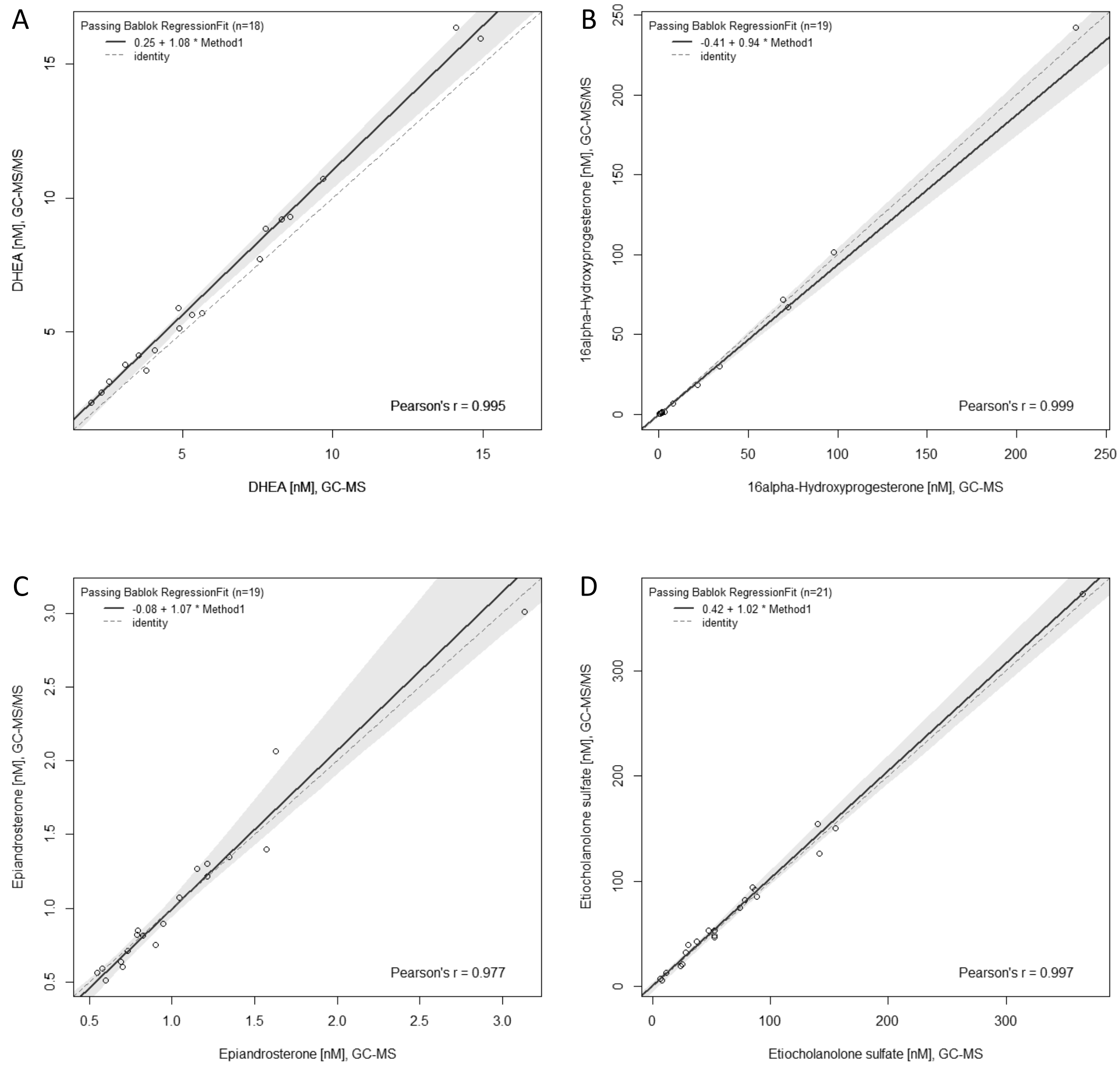
As expected, the higher precision was typically obtained for more abundant steroids. For instance, better results were obtained for C19 steroids in non-pregnant subjects but for C21 steroids in pregnant women and in mixed umbilical serum. Higher precision was achieved for more abundant steroid conjugates when compared with their less abundant unconjugated counterparts. The results for T, DHT5α and 5-Adiol were generally better in pooled serum from adult men when compared with other groups. As concerns the accessibility of hydroxy-group for derivatization, the 11β-hydroxy-steroids showed lower precision when compared with their 11-deoxy-counterparts due to difficult accessibility of 11β-hydroxy-group for the sylilating agent.
If the intra- and/or inter assay exceeded the 15% RSD in some of the tested pooled samples, the validation in this biological material was considered as unsatisfactory. For instance, the levels of several reduced 5β-reduced C21 steroids are insufficient to quantify these analytes out of pregnancy. However, in a nutshell, most analytes may be quantified in all investigated matrixes (Table 3).

3.1.3. Recovery

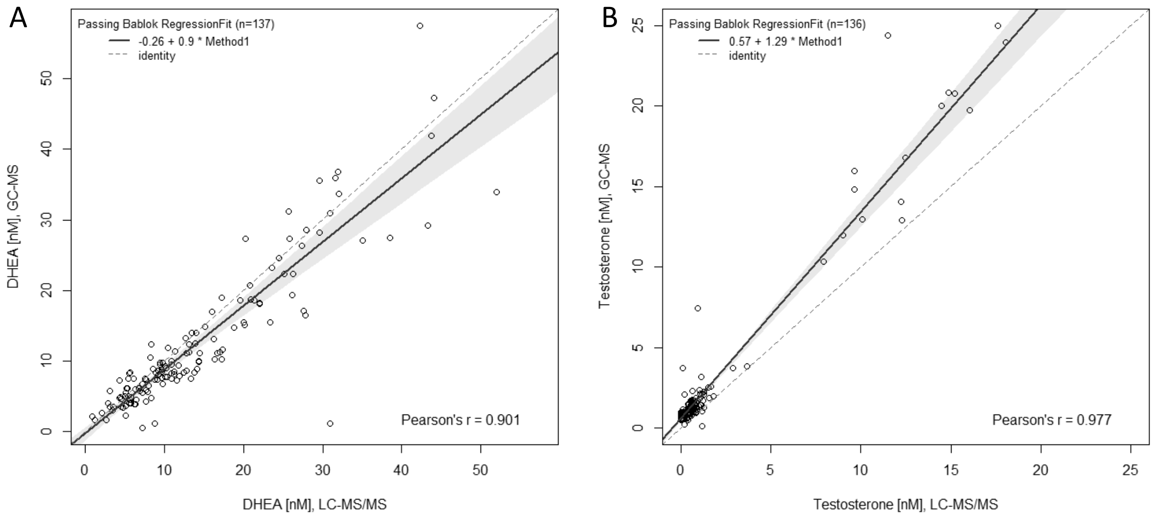
In general, the additions of steroids for the computation of recovery were derived from steroid levels in the pooled sample. In two steroid sulfates such as DHEA sulfate and THA3α5αC, the samples for recovery were diluted to be within the LDR (Supplementary Table S3). As expected, the recovery rates differed according to the steroid polarity. On the one hand, the diethyl-ether extraction step should be more favorable for the less polar steroids but on the other hand, partitioning between the methanol-water mixture and pentane should be less efficient for the steroids with low polarity. When testing the recovery, we found lower values for less polar steroids such as 5α/β reduced C21 steroids but high values for the polar ones such as cortisol. The number of hydroxy-groups positively correlates with the recovery rate (for instance allopregnanolone vs. 5α-pregnane-3α,20α-diol or allopregnanolone vs. 17-hydroxyallopregnanolone). The 5α/β-reduced steroids showed lower recovery rates in comparison with their unsaturated counterparts (for instance 5α-dihydroprogesterone vs. progesterone or 5α-dihydrotestosterone vs. testosterone). The C19 steroids generally exhibit higher recovery rates in comparison with their C21 analogues (for instance androsterone vs. allopregnanolone).

3.1.4. Accuracy

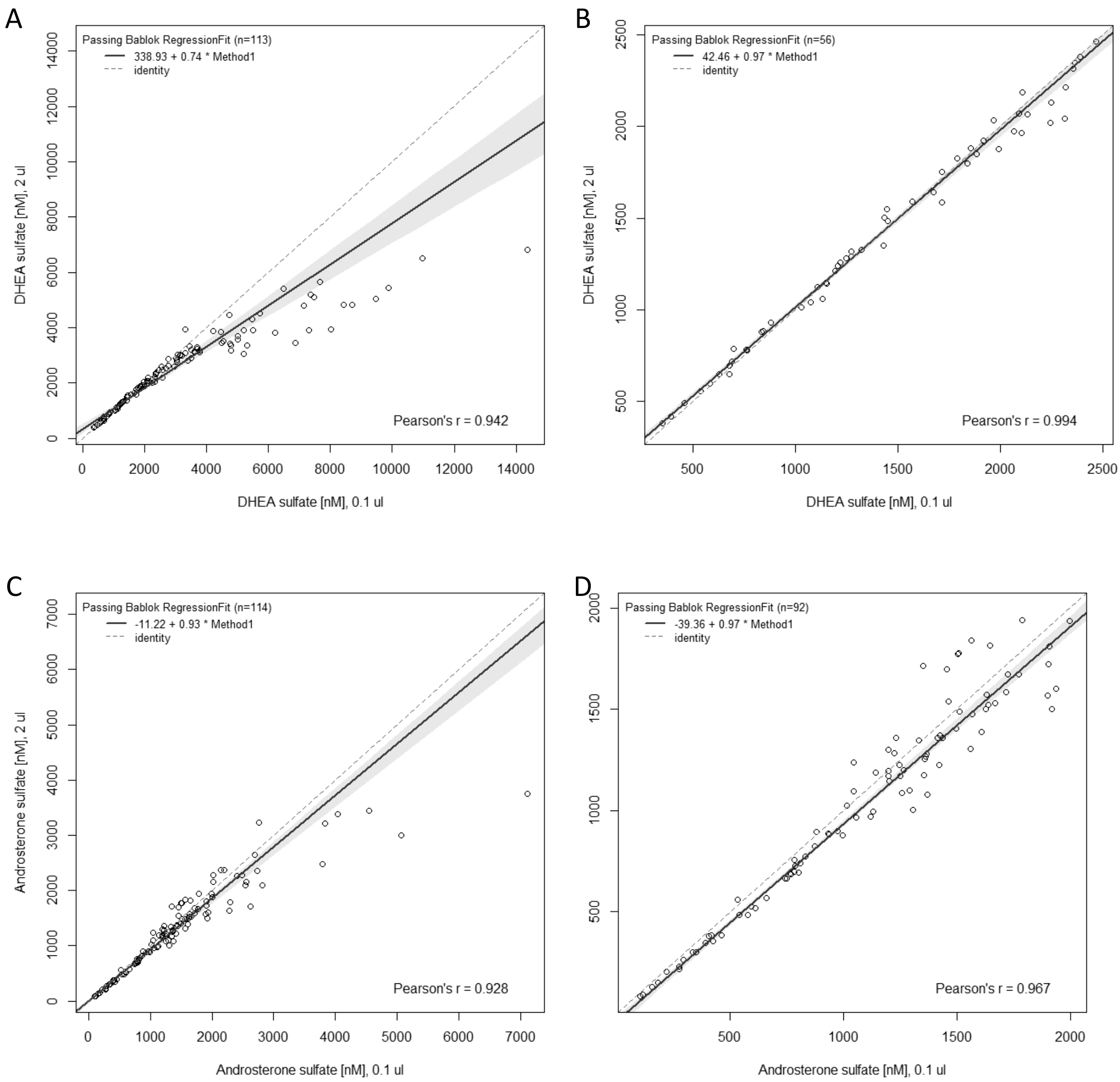
The accuracy test was not carried out if the If the intra- and/or inter assay for precision exceeded the 15% RSD (see Table 3). When the precision testing was acceptable, the bias less then ±15% was met for all analytes in all tested concentrations in both intra- and inter-day experiments (Supplementary Table S4).



Supplementary Figure S1. *Comparison of GC-MS and GC-MS/MS data (1 pooled serum sample, 4 men, 4 women in the follicular menstrual phase 4 women in luteal menstrual phase women, 4 women at labor, 4 samples of mixed umbilical cord serum), unconjugated steroids for DHEA (Panel A), 16α-hydroxyprogesterone (Panel B), epiandrosterone (Panel C) and etiocholanolone sulfate (Panel D).*



Supplementary Figure S2. *Comparison of GC-MS/MS and LC-MS/MS data (adult men, premenopausal and postmenopausal women in follicular and luteal menstrual phases).*



Supplementary Figure S3. *Comparison of concentrations calculated from the same samples injected at volume of 2 µL and 0.2 µL (adult men, premenopausal and postmenopausal women in follicular and luteal menstrual phases) as evaluated by Passing Bablok robust regression. Panel A and represent the relationship for the full concentration range of DHEA sulfate and panel B shows only the samples, which were within the linear dynamic range. Panels C and D show the similar situation for androsterone sulfate.*

3.1.5. Stability tests

A stability test after three freeze and thaw cycles did not show statistically significant differences. There were also no significant differences found for a temperature stability test after leaving the sample for one day at room temperature, a 3-day post-preparative stability test for steroids after derivatization at room temperature, or for one-month stability test for the stock solutions of analytes.

3.1.6. Limit of Detection and Limit of Quantification

The lowest nonzero standard on the calibration curve defined the sensitivity. The analyte response at the LOQ was at least five times the analyte response of the zero calibrator and the bias at the LOQ was at most ±20% of nominal concentration (as found using replicates prepared in six different runs). Similarly, the imprecision was at most ±20% RSD as found using six replicates in four runs (2018) (see Table 3).

The LOD was sufficient in all cases where the intra- and/or inter assay for precision did not exceeded the 15% RSD (see Table 3) but the LOQ was borderline for the levels of AD3α5β17β and AD3α5β17β, E1 and PD3α5β17 levels in subjects out of pregnancy, and E2 and THP3α5α levels in men.

3.1.7. Specificity/selectivity of the method

In the co-eluting steroids, the selectivity was tested by injecting large amounts of the individual steroids and checking the potential contribution to other steroids respecting circulating levels of the potential interferents. For instance, for the transition 360>84 between DHEA and epiandrosterone we found some interference. On the other hand, the interference of DHEA for transition 360>270 in epiandrosterone was absent. Therefore, we choose the transition for quantitation of epiandrosterone 360>270 instead of the 360>84 transition. We also tested partly co-eluting pregnenolone and isopregnanolone and found some interference on 388>70 but no interference on 388>173 transition, which was then chosen for quantitation of isopregnanolone. The interferences were also tested for 388>70 transition between partly co-eluting 7β-OH-DHEA and allopregnanolone but there was no perceptible interference. Some interference was found for 421>255 transition between partly co-eluting 5-androstene-3β,17β-diol and 5α-androstane-3β,17β-diol but the corresponding peaks only marginally coincided and the quantitation was possible. Besides the cases mentioned above and DOF, Preg16α, T16α, in which only a single MRM transition was recorded, no further perceptible interferences were found and the remaining ion ratios were within the tolerance according to WADA Technical Document – TD2010IDCR "Identification Criteria for Qualitative Assays Incorporating Column Chromatography and Mass Spectrometry".

The levels of DOF were higher in male serum pool when compared with our previously published data from RIA assays (Hill et al. 1995), possibly due to the unintentional inclusion of patients with Cushing syndrome or congenital adrenal hyperplasia in some pooled samples. However, the recording of a single MRM transition for DOF did not rule out the possibility of some endogenous co-eluting interference being responsible of the apparent larger concentrations.

3.1.8. Efficiency of methanolysis and stability of non-deuterated and deuterated steroids

The deconjugating step in the present method was performed using the methanolysis according to Dehennin (Dehennin et al. 1996). This harsh acid hydrolysis is an adopted method of deconjugation that efficiently and rapidly cleaves both sulfates and glucuronides simultaneously. However, the formation of artefactual by-products is a known weakness of this method (Dehennin et al. 1996, Shackleton et al. 2004, Viljanto et al. 2018).

The results characterizing the efficiency of methanolysis for seven steroids sulfates/disulfates are summarized in in Supplementary Table S5. The efficiency of the methanolysis step for individual steroid sulfates was high, ranging from 85% to 116% (98 ± 11%, shown as mean ± SD) (see Supplementary Table S5). Furthermore, we have tested the methanolysis efficiency for the DHEA sulfate using the same protocol but sulfated D6-DHEA as the internal standard. The efficiency of methanolysis step was close to absolute and almost the same when using the unconjugated or sulfated D6-DHEA as the internal standard (102.6 ± 0.9%, shown as mean ± SD).

Considering the high efficiency of the methanolysis step, there is probably lessened necessity to use sulfated internal standards instead of the unconjugated ones as the deconjugation step does not represent a critical point in methanolysis. Thus, the more available unconjugated deuterated steroids may be used as satisfactory surrogates instead of their more appropriate conjugated equivalents. However, all internal standards (regardless their conjugation status) should possess isotopic stability in strongly acidic environment, which occurs during the methanolysis.

Some steroids, have also limited chemical stability during the methanolysis (Dehennin et al. 1996, Viljanto et al. 2018). Dehennin *et al.* (Dehennin et al. 1996) report, that while the sulfates of androsterone, epitestosterone, testosterone, 5-androstene-3β,17β-diol (5-Adiol) and DHEA and glucuronides of androsterone and testosterone are almost totally recovered using the methanolysis, steroids with tertiary alcohol in the steroid 17 position and secondary alcohol in the steroid 11β-position may dehydrate in strongly acidic conditions. This environment stimulates a protonation of the oxygen attached to steroid C-17 position and the nucleophilic attack by methanol, which consequently induces a cleavage of sulfate and glucuronide moieties on steroid molecules. However, there is also a risk of partial dehydration and formation of double bond (Viljanto et al. 2018).

The analysis of steroid chemical stability of unconjugated steroids (see section 2.7.6.) showed that most of them were relatively stable during the methanolysis, however in a part of them the stability was low, particularly in 7α/β-hydroxy-metabolites of 5-Adiol. Similarly, estrogens, 16α -hydroxy-metabolite of 5-Adiol and 11β-hydroxy- and 3-oxo- steroids showed a limited stability (Supplementary Table S6). Nevertheless, even in these cases, one can expect a similar degree of conversion to artefacts in standard and unknown samples on condition that they are processed in the same way in one run. So, the obtained results may be still acceptable as apparent in tables presenting analytical criteria for conjugated forms of these analytes (see section 3.1.). We are aware that the use of chemically and isotopically stable deuterated external standards with sufficient isotopic purity in conjugated forms would be a by far better approach.

The accessibility of appropriate deuterated conjugated internal standards is even more critical. Moreover, the deuterated internal standards are often isotopically unstable. The strongly acidic environment during the methanolysis promote deuterium-hydrogen exchange, which considerably limits the number of applicable deuterated standards. For instance, a complete deuterium-hydrogen exchange was observed in a deuterium-labelled, D9-progesterone during methanolysis but no change was observed when the samples spiked with D9-progesterone were incubated with methanol in the neutral environment. The deuterium-hydrogen exchange is induced by acid-catalyzed enol tautomer formation when the double bond rapidly moves between the keto and enol forms. Although the equilibrium usually favors the keto-tautomer, it can be shifted to the enol-one by acidic or alkaline environment. Steroids labelled on an α-carbon adjacent to a ketone functional group(s) exhibit the hydrogen exchange, whereas other labelled analytes are unlikely to cause any problems. In extreme situations, such as in the case of D9-progesterone, the deuterium-hydrogen exchange via keto-enol tautomerism may lead to the formation of unlabeled product (Viljanto et al. 2018). We observed this effect during the methanolysis when using D9-progesterone and D8-17α-hydroxyprogesterone as internal standards. Besides the problems with the isotopic stability, the relatively frequent drawback of deuterated internal standards may be also their insufficient isotopic purity, which is specifically critical in analytes showing wide biological variability such as pregnane steroids exhibiting extreme changes during the menstrual cycle and pregnancy.

In contrast to some authors discriminating between glucuronide, monosulfate and disulfate moieties on steroid molecules, we did not test their levels separately (Mareck et al. 2008, Meng et al. 1997) but measured only the total polar conjugates. On one hand, the concurrent deconjugation of sulfates and glucuronides is a weakness of our method but on the other hand, the methanolysis is more robust and less laborious then the enzymatic hydrolysis or microcolumn pre-separation of sulfate, disulfate or glucuronide moieties from each other. Nevertheless, the discrimination between these moieties may be desirable in the diagnostics of some disorders such as the intrahepatic cholestasis of pregnancy. In this pathology, from a variety of pregnanediols, only the of 5α-pregnane-3α,20α-diol disulfate is considered as toxic for fetus (Abu-Hayyeh et al. 2013, Meng et al. 1997).

Supplementary Table S1. Comparison of results from GC-MS and GC-MS/MS using Passing-Bablok regression and Bland-Altman procedure (1 pooled serum sample from non-pregnant subjects, and sera from 4 adult men, 4 women in follicular phase, 4 women in luteal phase, 4 women at labor, and 4 samples from mixed umbilical blood at labor were analyzed).

Steroid abbreviation	**Matrix**	***r***[a]	**Intercept (95% CI)**	**Slope (95% CI)**	**Difference (95% CI)**	**Range** GC-MS	GC-MS/MS
Preg	MFLPU	0.994	-0.16 (-0.93, 0.29)	0.93 (0.84, 1.1)	-0.71 (-1.2, -0.25) *	2.1 - 27	2 - 24
PregC	MFLPU	0.976	-120 (-190, 1.7)	0.96 (0.74, 1.2)	-45 (-220, 130)	35 - 4900	29 - 5600
DHPreg20α	MFLPU	0.760	1 (-0.4, 1.6)	0.83 (0.58, 1.3)	0.42 (0.069, 0.78) *	1.2 - 5.2	1.9 - 5.7
DHPreg20αC	MFLPU	0.965	140 (-46, 220)	0.9 (0.76, 1.1)	-6.1 (-110, 93)	260 - 3000	230 - 2600
Preg16α	MFLPU	0.974	0.018 (-0.065, 0.067)	1 (0.8, 1.3)	-0.017 (-0.32, 0.29)	0.11 - 9.3	0.11 - 7.6
DHEA	MFLPU	0.995	0.25 (-0.41, 0.51)	1.1 (0.98, 1.2)	0.66 (0.38, 0.93) *	1.9 - 15	2.3 - 16
DHEAC	MFLPU	0.987	-92 (-260, 230)	1.2 (1, 1.3)	250 (150, 360) *	620 - 6300	530 - 6600
DHEA7α	MFLPU	0.951	0.24 (0.074, 0.5) *	1.1 (0.81, 1.4)	0.33 (0.18, 0.47) *	0.35 - 3.9	0.6 - 3.7
DHEA7β	MFLPU	0.993	-0.13 (-0.39, -0.052) *	0.91 (0.8, 1.4)	-0.23 (-0.34, -0.11) *	0.25 - 6.2	0.15 - 5.2
5-Adiol	MFLPU	0.964	0.13 (-0.17, 0.46)	1.4 (1.1, 1.9) *	0.61 (0.4, 0.81) *	0.18 - 5.3	0.25 - 6.3
5-AdiolC	MFLPU	0.988	120 (-7.7, 220)	1.1 (0.98, 1.2)	290 (130, 440) *	230 - 5800	310 - 6000
AT7α	MFLPU	0.969	-0.05 (-0.1, -0.022) *	1 (0.89, 1.2)	-0.05 (0.077, -0.023)	0.046 - 0.81	0.013 - 0.82
AT7β	MFLPU	0.958	-0.072 (-0.095, -0.029)*	1.1 (0.79, 1.2)	-0.07 (-0.091, -0.049) *	0.064 - 0.49	0.0031 - 0.45
P	MFLPU	0.999	-1.2 (-2.6, 0.24)	0.95 (0.91, 0.98)*	-31 (-63, -0.12) *	0.22 - 2300	0.072 - 2100
P16α	MFLPU	0.999	-0.41 (-0.64, -0.31) *	0.94 (0.88, 1)	-0.25 (-1.7, 1.2)	0.49 - 230	0.13 - 240
A4	MFLPU	0.974	-0.27 (-0.81, 0.041) *	1.3 (1.1, 1.5) *	0.71 (0.12, 1.3) *	1.2 - 10	1.2 - 12
T	MFLPU	0.954	-1.1 (-1.5, -0.46) *	1.3 (0.84, 1.6)	0.39 (-0.99, 1.8) *	0.61 - 21	0.21 - 30
DHT5α	MFLPU	0.820	-0.032 (-0.13, 0.047)	1.1 (0.59, 1.4)	-0.011 (-0.13, 0.11)	0.018 - 1.6	0.035 - 1.4
E2	LPU	0.979	-0.27 (-3.4, 0.27)	1.5 (1.1, 1.7) *	7.6 (-1, 16)	0.33 - 60	0.2 - 96
E2C	LPU	0.937	0.27 (-0.29, 5.4)	0.99 (0.73, 1.6)	1.4 (-2.2, 5)	0.33 - 46	0.2 - 45
DHP5α	PU	0.969	-8.7 (-68, 43)	1.5 (0.99, 2.3)	38 (7.2, 70) *	24 - 200	27 - 260
THP3α5α	PU	0.789	5.3 (-250, 18)	0.81 (0.23, 13)	0.6 (-2.4, 3.6)	18 - 30	19 - 30
THP3α5αC	MFLPU	0.971	-8.8 (-14, -3) *	1.2 (0.91, 1.3)	11 (-30, 53)	7.9 - 1200	1.2 - 1200
THP3β5αC	MFLPU	0.948	-4.7 (-8.2, -0.66) *	1.2 (0.94, 1.3)	56 (-28, 140)	3.3 - 920	2.9 - 1600

[*]Intercept and difference between method significantly different from zero and slope significantly different from 1. Results are shown in nmol/L. [a]correlation coefficient between results from GC-MS and GC-MS/MS. M, F, L, P, and U represent inclusion of samples from men, women in luteal phase, women in follicular phase, pregnant women and umbilical cord blood, respectively.

Supplementary Table S1, continued. Comparison of results from GC-MS and GC-MS/MS using Passing-Bablok regression and Bland-Altman procedure (1 pooled serum sample from non-pregnant subjects, and sera from 4 adult men, 4 women in follicular phase, 4 women in luteal phase, 4 women at labor, and 4 samples from mixed umbilical blood at labor were analyzed).

Steroid abbreviation	**Matrix**	r^a	**Intercept (95% CI)**	**Slope (95% CI)**	**Difference (95% CI)**	**Range** GC-MS	GC-MS/MS
THP3β5β	MFLPU	0.940	-0.28 (-0.45, -0.056) *	1.2 (0.46, 1.8)	0.048 (-0.3, 0.4)	0.14 - 2.5	0.0091 - 3.9
THP3β5βC	MFLPU	0.977	-6 (-8.5, -3.5) *	1.1 (0.97, 1.3)	5 (-7.3, 17)	1.4 - 260	0.21 - 330
THP5α20α	MFLPU	0.945	0.96 (-0.68, 1.1)	1.4 (1, 1.9)	6.6 (1.6, 12) *	0.057 - 78	0.7 - 91
THP5β20α	MFLPU	0.984	-0.16 (-0.37, -0.09) *	1.3 (1.1, 1.8)*	2.1 (-0.08, 4.2)	0.028 - 51	0.024 - 58
PD3α5α20α	PU	0.962	-4.4 (-13, 1.7)	1.1 (0.65, 1.5)	-3.5 (-6, -1.1) *	7.8 - 29	3.6 - 28
PD3α5α20αC	MFLPU	0.964	1.7 (-12, 4.7)	1.2 (1.1, 1.6) *	680 (-20, 1400)	11 - 6400	10 - 10000
PD3β5α20αC	MFLPU	0.951	56 (-460, 270)	0.98 (0.84, 1.3)	3300 (-6800, 13000)	180 - 170000	220 - 220000
PD3α5β20αC	MFLPU	0.974	-3.8 (-11, 2.9)	1.2 (1, 1.3)	200 (-39, 430)	1.5 - 3600	4.4 - 4300
PD3β5β20αC	MFLPU	0.962	-13 (-38, 1)	1.1 (0.9, 1.6)	93 (-14, 200)	5.4 - 2100	7.9 - 2300
THA3α5α	MFLPU	0.923	0.062 (0.0067, 0.14) *	0.87 (0.58, 1.1)	-0.0078 (-0.078, 0.062)	0.15 - 1.4	0.19 - 1.1
THA3α5αC	MFLPU	0.987	53 (0.67, 190) *	1 (0.88, 1.1)	47 (-55, 150)	23 - 4500	30 - 3700
THA3β5αC	MFLPU	0.990	56 (28, 86) *	1.1 (0.96, 1.2)	93 (59, 130) *	52 - 1600	90 - 1900
THA3α5β	MFLPU	0.960	0.025 (0.07, 0.082) *	0.96 (0.73, 1.3)	-0.0049 (-0.037, 0.027)	0.12 - 1.1	0.1 - 1.1
THA3α5βC	MFLPU	0.997	0.42 (4.7, 4.3) *	1 (0.96, 1.1)	1.3 (-1.8, 4.3)	6.7 - 360	5.6 - 370
THA3β5βC	MFLPU	0.996	0.05 (-0.42, 2)	0.92 (0.79, 0.98)*	-1.7 (-4.1, 0.67)	0.39 - 210	0.39 - 200
AD3α5α17β	MFLPU	0.990	-0.026 (-0.045, -0.002) *	1.3 (1, 1.4) *	0.019 (-0.012, 0.051)	0.044 - 0.52	0.029 - 0.64
AD3α5α17βC	FLPU	0.774	6.3 (-5.9, 16)	0.79 (0.37, 1.3)	1.6 (-4.4, 7.5)	2.2 - 64	9.2 - 63
AD3β5α17β	MFLPU	0.754	-0.043 (-0.17, -0.00026)*	1.5 (0.92, 3.1)	0.005 (-0.016, 0.026)	0.044 - 0.2	0.043 - 0.27
AD3β5α17βC	MFLPU	0.995	2 (-0.46, 6.2)	1 (0.97, 1.1)	5.9 (1.5, 10) *	3.9 - 360	4.6 - 340

[*]Intercept and difference between method significantly different from zero and slope significantly different from 1. Results are shown in nmol/L. [a]correlation coefficient between results from GC-MS and GC-MS/MS. M, F, L, P, and U represent inclusion of samples from men, women in luteal phase, women in follicular phase, pregnant women and umbilical cord blood, respectively.

Supplementary Table S2. Comparison of results from LC-MS/MS and RIA (cortisol) and GC-MS/MS using Passing-Bablok regression and Bland-Altman procedure (sera from women in follicular phase, women in luteal phase, and from adult men).

						Median (quartiles)	
Steroid	**n**	***r***	**Intercept (95% CI)**	**Slope (95% CI)**	**Difference (95% CI)**	LC-MS/MS(RIA)	GC-MS/MS
Pregnenolone	135	0.751	0.53 (0.22, 0.85) *	1.0 (0.81, 1.2)	0.65 (0.44, 0.87)*	1.6 (0.9, 2.9)	2.2 (1.6, 3.5)
17α-Hydroxypregnenolone	134	0.949	0.56 (0.36, 0.66) *	1.1 (1.0, 1.2)	0.74 (0.48, 1.0)*	3.8 (1.4, 6.2)	4.0 (2.1, 7.1)
Cortisol (vs. LC-MS/MS)	127	0.791	-38 (-120, 17)	1.2 (1.0, 1.4)	14 (-3.3, 31)	400 (310, 470)	430 (300, 510)
Cortisol (vs. RIA)	119	0.864	-63 (-140, -11) *	0.93 (0.83, 1.1)	-99 (-110, -85)*	520 (430, 650)	
Testosterone	129	0.993	0.57 (0.48, 0.67) *	1.2 (1.1, 1.3)	1.0 (0.81, 1.3)*	0.57 (0.27, 0.98)	1.2 (0.89, 1.7)
Androstenedione	127	0.737	0.091 (-0.57, 0.26)	0.99 (0.77, 1.2)	-0.039 (-0.065, -0.012)*	2.4 (1.7, 3.6)	2.1 (1.6, 3.3)
DHEA	131	0.928	-0.15 (-1, 0.75)	0.90 (0.80, 0.99)*	-0.055 (-0.073, -0.037)*	11 (7.1, 20)	9.0 (6.3, 16)

**Intercept and difference between method significantly different from zero and slope significantly different from 1. Results are shown in nmol/L.. Symbol r represent a correlation coefficient between results from LC-MS/MS (RIA) and GC-MS/MS.*

Table 3. Sensitivity, Intra-assay and Inter-assay relative standard deviations (RSDs) for GC-MS/MS analysis of endogenous unconjugated steroids in human serum

ID	Steroid	**LOD** [pg]	**LOQ** [pg] (bias + precision at LOQ)	**Men** Level [pg inj.]/[nM]	**Men** Intra-/Inter-assay [%]	**Women, follicular phase** Level [pg inj.]/[nM]	**Women, follicular phase** Intra-/Inter-assay [%]	**Women, luteal phase** Level [pg inj.]/[nM]	**Women, luteal phase** Intra-/Inter-assay [%]	**Women, pregnancy** Level [pg inj.]/[nM]	**Women, pregnancy** Intra-/Inter-assay [%]	**Mixed umbilical blood** Level [pg inj.]/[nM]	**Mixed umbilical blood** Intra-/Inter-assay [%]
1	Preg	0.02	2(5.8%,18%)	32/5.1	1.3/12	53/8.4	2.5/9.4	58/9.2	2/13	110/18	0.91/7.9	470/74	0.91/7.9
2	Preg17	0.05	0.5(-5.8%,10%)	80/12	1.5/11	86/13	0.98/9.5	56/8.4	1.2/7.4	160/24	1.7/6.4	220/33	0.87/6.6
3	Preg16α	0.009	0.5(13%,3.3%)	2.9/0.43	4.8/8.5	2.9/0.43	2.8/4.5	2.2/0.33	8.6/8.5	5.4/0.81	3.5/5.9	47/7.1	1.1/7.1
4	DHPreg20α	0.03	0.5(-6%,11%)	15/2.3	4.3/9.8	22/3.4	6.1/9.4	27/4.2	1.2/10	25/4	2.9/5.4	32/5.1	2.9/5.9
5	DHEA	0.008	2(9.5%,6%)	58/10	1.4/6.8	86/15	1.4/4.7	69/12	1.6/3.8	100/18	1.8/4.7	44/7.7	2.6/5.1
6	DHEA7α	0.02	0.5(-1.7%,11%)	7.9/1.3	1.6/8.3	9.1/1.5	2.8/4	5.8/0.96	4.3/6.3	5.5/0.91	1.3/7.4	12/2	1.8/6.2
7	DHEA7o	0.09	0.5(7.7%,11%)	6.6/1.1	6.7/11	2.5/0.41	13/9.9	2.4/0.39	7.7/12	3.2/0.53	8.4/15	4.8/0.79	7.2/7.9
8	DHEA7β	0.03	0.5(3.2%,13%)	2.9/0.48	7.1/14	1.5/0.25	4/14	2.4/0.4	8.5/13	1/0.17	5.2/7.3	2/0.33	7.5/9.7
9	5-Adiol	0.1	2(0.61%,10%)	15/2.5	1.4/6.7	13/2.3	2/8	11/1.9	2.9/10	8.7/1.5	2.6/7	2.6/0.44	6.5/6.4
10	AT7α	0.02	0.5(15%,4.9%)	2.3/0.37	2.7/10	2.7/0.44	2.4/6.8	1.7/0.28	5.2/8.9	0.6/0.098	3.4/10	---	15/11
11	AT7β	0.02	0.5(13%,8.4%)	1.9/0.31	8.6/12	2.1/0.35	5.3/5.7	1.5/0.25	12/7.3	0.42/0.068	6.9/11	---	7.7/13
12	AT16α	0.04	0.5(-2.6%,20%)	3.1/0.51	13/12	2.8/0.45	9.9/13	3.1/0.51	12/13	4.9/0.8	13/12	19/3.1	5.5/11
13	P	30	0.5(-2.1%,12%)	1.5/0.24	6.3/11	1.6/0.25	13/11	75/12	2.2/14	2000/320	0.61/8	14000/2300	0.53/7.5
14	P17	0.1	0.5(5.1%,11%)	18/2.8	4.3/9.1	7.3/1.1	4.4/5.4	21/3.2	3.7/14	120/18	1.3/8.9	650/99	0.79/8.4
15	DHP17α20α	1	0.5(6.3%,7.6%)	10/1.5	1.3/8.6	4.5/0.68	1.7/11	6.6/1	1.7/4.4	56/8.4	1.3/15	170/26	0.74/10
16	P16α	0.02	0.5(7.2%,3.6%)	5/0.76	3.3/11	3.2/0.48	1.5/7.6	6.3/0.96	3/6.2	130/19	0.64/5.9	920/140	0.58/8.4
17	DHP20α	0.02	0.5(13%,8.6%)	1.3/0.21	6.9/8.9	1.8/0.29	3.1/8.5	31/4.9	0.81/11	580/92	0.6/6	630/99	0.69/6.7
18	A4	0.09	2(-4.3%,9.8%)	15/2.6	0.88/11	15/2.7	3.2/7.9	13/2.3	4.9/14	49/8.6	2.5/8.2	86/15	4.4/7.7
19	T	0.02	2(18%,1.6%)	86/15	2.2/8	8.1/1.4	10/10	5.8/1	6.2/11	19/3.3	5.8/6.9	4.3/0.74	12/6.8
20	T16α	0.3	2(-5.8%,15%)	---	---	---	---	---	---	26/4.2	2.9/13	67/11	4.6/8.2
21	DHT5α	0.04	0.5(9.5%,5.8%)	8.7/1.5	6.4/9.1	3/0.51	9/8.6	2.9/0.5	15/8.5	3.4/0.58	4.3/9.8	0.81/0.14	14/15
22	E1	0.07	0.5(-1.3%,8.6%)	0.86/0.16	4.8/14	1.3/0.24	7.8/10	1.4/0.26	4.8/11	260/48	6.6/9	650/120	0.4/7.5
23	E2	0.02	0.5(-2.6%,11%)	0.54/0.1	7.7/15	2.1/0.38	5.3/13	2.3/0.42	8.9/6.5	370/68	0.41/9	180/33	0.83/8.1
24	E3	0.05	2(-5.8%,8.2%)	---	---	---	---	---	---	110/18	0.91/7.9	470/74	0.91/7.9
25	DHP5α	0.2	2(9%,17%)	---	---	---	---	---	---	390/61	1.3/10	1100/170	0.8/8.2

Table 3, continued. Sensitivity, Intra-assay and Inter-assay relative standard deviations (RSDs) for GC-MS/MS analysis of endogenous unconjugated steroids in human serum

Steroid				Men		Women, follicular phase		Women, luteal phase		Women, pregnancy		Mixed umbilical blood	
ID		**LOD** [pg]	**LOQ** [pg] (bias + precision at LOQ)	Level [pg inj.]/ [nM]	Intra- /Inter- assay [%]	Level [pg inj.]/ [nM]	Intra- /Inter- assay [%]	Level [pg inj.]/ [nM]	Intra- /Inter- assay [%]	Level [pg inj.]/ [nM]	Intra- /Inter- assay [%]	Level [pg inj.]/ [nM]	Intra- /Inter- assay [%]
26	THP3α5α	0.02	0.5(1.9%,11%)	0.43/0.068	13/15	1.5/0.24	11/12	5.5/0.87	4.5/13	200/32	1.6/8.2	150/24	1/8.1
27	THP3β5α	0.02	0.5(4%,15%)	1.6/0.25	3.5/9.8	3.8/0.59	5.1/8.1	5.3/0.83	2.3/11	110/18	1.6/7.6	240/38	3.8/7.8
28	DHP5β	0.7	8(-12%,4.8%)	---	---	---	---	---	---	20/3.1	14/14	280/45	4.1/6.8
29	THP3α5β	0.04	0.5(-9.9%,10%)	---	---	---	---	---	---	130/20	1.1/7.1	180/29	1.4/7.4
30	THP3β5β	0.03	0.1(14%,6.9%)	---	---	---	---	---	---	8.9/1.4	1.6/8.6	22/3.4	1/6.7
31	THP5α20α	0.2	0.5(-4.3%,11%)	1.5/0.24	11/7.1	3.8/0.6	5.2/12	8.9/1.4	5.9/13	220/34	0.65/7.1	390/62	1.1/5.7
32	PD3α5α20α	0.6	0.5(16%,6.1%)	1.7/0.26	5.3/5	2.6/0.41	4.9/12	7/1.1	8.5/12	160/25	2.2/7.5	63/9.8	4.7/7.2
33	PD3β5α20α	2	0.5(17%,3.5%)	9/1.4	12/12	15/2.4	14/8.8	23/3.6	8.7/10	470/73	4.1/7.7	580/90	2.4/7.2
34	THP5β20α	0.2	0.5(1.9%,10%)	---	---	---	---	---	---	15/2.3	2.1/10	250/40	1.3/4.6
35	PD3α5β20α	0.2	0.5(-2.2%,16%)	1.8/0.28	14/11	1.5/0.23	9.2/15	2.2/0.35	14/9.3	52/8.2	2/8.1	70/11	1.4/8.7
36	PD3β5β20α	0.5	0.5(6.4%,13%)	---	---	---	---	---	---	5.4/0.85	13/14	15/2.3	9.8/8.4
37	PD3α5α17	0.2	0.1(-1.8%,13%)	0.51/0.077	19/14	0.44/0.066	11/11	0.42/0.063	12/13	4.6/0.69	6.7/10	6/0.9	4.9/11
38	PD3α5β17	0.1	0.5(-3.5%,13%)	0.8/0.12	13/9.1	0.48/0.072	8.6/14	1.2/0.18	9.7/11	9.4/1.4	3.2/8.2	11/1.7	1.8/5.8
39	PT3α5α17α20α	0.07	0.5(0.56%,11%)	1.9/0.28	3/14	1.3/0.2	3.9/7.9	1.5/0.22	5.6/5.1	1.5/0.23	4.2/15	0.87/0.13	5.1/15
40	PT3β5α17α20α	0.1	0.5(6%,9.4%)	1.6/0.24	3/8.5	2.1/0.31	1.9/8.8	2.2/0.33	2.1/5.5	1.6/0.24	2.5/14	0.81/0.12	5.3/15
41	PT3α5β17α20α	0.06	0.5(0.33%,12%)	10/1.5	1.9/9.1	10/1.5	2.5/8.5	11/1.7	1.2/3.4	49/7.3	1/12	20/3	0.65/11
42	DHA5α	0.3	0.5(3.6%,12%)	1.6/0.27	5.7/8.8	1.8/0.32	13/9	1.4/0.24	11/13	2.8/0.49	8.1/4.9	3.5/0.6	8.6/9.2
43	THA3α5α	0.1	0.5(5.7%,7.5%)	2.7/0.46	2.6/12	1.8/0.31	1.7/11	1.7/0.29	4.8/12	4.1/0.7	4.6/8.6	2.3/0.4	6.7/6.9
44	THA3β5α	0.03	0.5(9.9%,6.6%)	1.8/0.31	1.9/12	2.3/0.4	1.1/8.9	1.9/0.33	3/8	3.3/0.57	3.3/8.8	1.8/0.31	2.1/13
45	THA3α5β	0.01	0.5(5.6%,6.2%)	1.5/0.25	2.8/6.2	1.8/0.31	4.2/6.9	1.4/0.24	3.2/5.6	3.1/0.54	4.7/14	3.7/0.63	6.5/6.5
46	AD3α5α17β	0.2	0.5(5.3%,11%)	1.7/0.29	4.1/9.7	0.49/0.084	8.5/9.2	0.46/0.078	7.1/6.7	0.76/0.13	9.2/13	---	6.7/11
47	AD3β5α17β	0.02	0.5(16%,7.8%)	0.81/0.14	9.4/11	0.7/0.12	12/10	0.58/0.1	10/7.9	0.64/0.11	8.8/11	---	6.4/12
48	AD3α5β17β	0.09	0.5(0.74%,6.5%)	0.93/0.16	8.4/11	0.64/0.11	8.4/9.4	0.93/0.16	13/5.5	0.55/0.095	5.1/15	---	13/15
49	F	30	100(-4.7%,8.8%)	2200/310	2.9/11	2200/300	5.9/5.8	2200/310	3.9/5.6	4900/680	2.9/6.2	1900/260	2.8/4.7
50	E	30	100(-0.35%,6.6%)	370/51	4.6/8.2	350/49	9/10	360/50	7.4/9.8	1000/140	4/6.1	2200/310	5.4/7.1

Table 3, continued. Sensitivity, Intra-assay and Inter-assay relative standard deviations (RSDs) for GC-MS/MS analysis of endogenous unconjugated steroids in human serum

	Steroid			Men		Women, follicular phase		Women, luteal phase		Women, pregnancy		Mixed umbilical blood	
ID		**LOD** [pg]	**LOQ** [pg] (bias + precision at LOQ)	Level [pg inj.]/ [nM]	Intra- /Inter- assay [%]	Level [pg inj.]/ [nM]	Intra- /Inter- assay [%]	Level [pg inj.]/ [nM]	Intra- /Inter- assay [%]	Level [pg inj.]/ [nM]	Intra- /Inter- assay [%]	Level [pg inj.]/ [nM]	Intra- /Inter- assay [%]
51	B	1	2(3.2%,10%)	90/13	4.7/9.8	97/14	2.2/7.1	76/11	4.1/7.7	510/74	1.3/6.3	120/18	2.9/8.9
52	DOF	0.8	0.5(1.3%,12%)	1.3/0.19	11/13	1.5/0.22	11/7.4	1.7/0.24	7.6/8.5	5.2/0.75	13/10	5.3/0.76	11/8.4
53	DOC	2	8(-4.7%,11%)	---	---	---	---	---	---	23/3.3	7.3/10	49/7.1	12/6.7
54	THB3α5α	0.5	0.5(-4.9%,3.4%)	1.9/0.27	9.9/11	2.6/0.37	14/9.2	1.9/0.27	11/14	2.6/0.37	12/10	---	---
55	THB3α5β	0.4	0.5(6.6%,17%)	3.5/0.5	15/12	3.6/0.51	14/9	3.3/0.47	13/12	3.4/0.48	12/11	1.1/0.16	2.9/15
56	11OHA4	0.9	8(-3.8%,11%)	470/77	3.4/6.5	450/75	1.9/14	280/47	3.5/15	1600/270	1.9/9.1	600/100	1.4/9.9
57	THA3α5α11β	0.04	0.5(3.3%,10%)	50/8.2	2.4/11	23/3.7	2/6.8	23/3.7	3.6/13	7.3/1.2	4.3/13	8/1.3	5.1/8.2
58	THA3β5α11β	0.04	0.5(6.5%,6.7%)	3/0.49	8.2/11	1.6/0.26	9.3/12	1.6/0.26	9.8/13	0.61/0.1	15/14	1/0.17	9.3/14
59	THA3α5β11β	0.05	0.5(7.7%,6%)	32/5.3	2.9/12	30/4.9	2/13	23/3.8	2.6/13	18/3	2.7/15	32/5.2	2.4/9.3
60	PregC	5	30(10%,2.5%)	1600/250	1.3/7.2	1600/250	1.3/12	1900/300	0.75/9.5	3300/530	1.7/11	28000/4400	0.86/13
61	Preg17C	1	8(1.5%,6.2%)	270/41	1.3/10	280/42	1.6/11	390/59	2.9/9.8	730/110	1.3/7.3	39000/5800	0.93/8.6
62	DHPreg20αC	4	30(3.2%,8.2%)	8900/1400	0.76/11	6300/990	0.66/14	9500/1500	1.1/4.9	5000/790	1.3/9.1	15000/2300	0.91/7.8
63	DHEAC	0.2	30(-0.17%,4.3%)	27000/4700	1.3/7.3	26000/4600	1.2/6	25000/4400	1.3/4.1	12000/2100	0.59/5.5	29000/5000	0.51/6.6
64	5-AdiolC	4	30(5.9%,9.2%)	20000/3400	1.2/13	17000/2900	0.78/12	13000/2300	0.54/13	2100/360	1.2/11	26000/4500	1.3/7.7
65	AT16αC	0.7	8(-0.063%,9.4%)	310/50	2.8/12	410/67	1.2/11	370/61	1.2/13	1000/170	1.3/12	14000/2300	0.37/11
66	DHP17α20αC	5	8(5.2%,7.2%)	86/13	1.5/14	130/20	7.2/11	140/21	4.8/13	---	---	---	---
67	DHP20αC	2	8(1.9%,12%)	18/2.9	2.8/9	23/3.6	4/10	52/8.2	4.9/12	190/30	1.5/13	880/140	1.5/8.2
68	TC	2	8(8.2%,8%)	---	---	---	---	---	---	110/19	5.3/12	290/51	3.7/10
69	EpiTC	5	8(6.4%,10%)	---	---	---	---	---	---	92/16	5.8/9.3	2400/410	0.76/4.2
70	E1C	10	8(3.2%,7.9%)	---	---	---	---	---	---	3700/680	9.4/9	200/37	0.42/3.4
71	E2C	0.2	2(3.5%,8.7%)	---	---	---	---	---	---	160/29	1.1/14	29/5.3	7.2/10
72	E3C	0.6	8(3.9%,9.4%)	---	---	---	---	---	---	3300/530	1.7/11	28000/4400	0.86/13
73	THP3α5αC	0.3	2(-2.8%,4.1%)	46/7.3	3/14	89/14	3.1/14	430/68	1.6/5.8	9500/1500	0.97/7.2	2600/410	0.75/8.2
74	THP3β5αC	0.3	8(4%,9%)	110/17	3.2/9.6	160/25	1.8/12	290/45	1.8/11	6400/1000	1/9.6	3100/490	0.89/13

Table 3, continued. Sensitivity, Intra-assay and Inter-assay relative standard deviations (RSDs) for GC-MS/MS analysis of endogenous unconjugated steroids in human serum

	Steroid			Men		Women, follicular phase		Women, luteal phase		Women, pregnancy		Mixed umbilical blood	
ID		**LOD** [pg]	**LOQ** [pg] (bias + precision at LOQ)	Level [pg inj.]/ [nM]	Intra- /Inter- assay [%]	Level [pg inj.]/ [nM]	Intra- /Inter- assay [%]	Level [pg inj.]/ [nM]	Intra- /Inter- assay [%]	Level [pg inj.]/ [nM]	Intra- /Inter- assay [%]	Level [pg inj.]/ [nM]	Intra- /Inter- assay [%]
75	THP3α5βC	0.7	2(0.29%,5.5%)	200/31	1.9/9.9	260/41	2.7/12	450/70	0.9/10	5600/880	0.99/7.7	2900/450	1/7
76	THP3β5βC	0.5	2(-5.5%,5.4%)	25/3.9	2.9/11	76/12	3/8.5	89/14	1.5/15	1500/240	0.94/7.3	1000/160	0.73/4.7
77	THP5α20αC	5	2(2.3%,8.7%)	4/0.63	8.3/6.9	8.9/1.4	6.6/15	89/14	5.5/11	830/130	1.7/13	1100/180	1.1/15
78	PD3α5α20αC	10	8(1.4%,8.8%)	210/33	2.1/14	400/62	3.1/7.2	1700/270	0.58/12	33000/5100	1/10	19000/3000	0.35/11
79	PD3β5α20αC	5	30(-7.3%,7.2%)	3200/500	5.2/8.9	17000/2700	12/9.4	50000/7800	12/12	420000/65000	0.96/8.9	220000/35000	0.47/8.8
80	THP5β20αC	6	30(-9.8%,18%)	---	---	---	---	---	---	220/35	1.1/13	620/98	0.47/12
81	PD3α5β20αC	2	8(7.4%,6.2%)	130/20	2.5/11	330/52	1.4/9.6	1400/220	1.2/13	11000/1700	0.73/11	13000/2000	0.83/8.6
82	PD3β5β20αC	9	8(4.9%,7.7%)	150/24	5.6/12	770/120	2.9/11	1200/190	1.4/14	7700/1200	0.98/9.7	6300/990	0.51/13
83	PD3α5α17C	0.5	2(-1.6%,7.6%)	29/4.4	4.8/7.5	15/2.2	5.7/6.9	41/6.1	3.5/6.7	150/22	0.88/7.6	63/9.5	2.6/7.8
84	PD3α5β17C	0.3	8(-3.2%,10%)	120/18	1.5/6.7	80/12	3.4/7.8	170/26	0.89/9.1	670/100	0.32/6.9	460/69	1.4/6.8
85	PT3α5α17α20αC	0.2	8(0.31%,6%)	---	---	---	---	---	---	260/39	4.4/14	550/82	14/11
86	PT3β5α17α20αC	0.2	8(-5.8%,17%)	---	---	---	---	---	---	52/7.7	4.9/14	35/5.2	2.9/12
87	PT3α5β17α20αC	0.2	8(1.3%,13%)	---	---	---	---	---	---	2400/360	1.8/13	2600/390	4.1/15
88	THA3α5αC	1	8(2%,6.8%)	11000/1900	0.67/8.6	16000/2700	2.1/11	19000/3200	0.77/13	3700/630	0.68/7.9	580/100	0.91/14
89	THA3β5αC	0.9	8(-0.81%,8.5%)	3000/510	1.2/7.6	3000/510	1.4/11	2900/500	1.4/5.7	870/150	1.2/5.9	320/55	0.73/6.9
90	THA3α5βC	3	8(-2.5%,20%)	580/100	0.63/5	750/130	0.7/5.9	640/110	0.67/8.2	360/62	0.81/7	120/20	1.2/7.6
91	THA3β5βC	0.9	8(-1.6%,8.2%)	310/54	0.97/8.7	420/73	2.3/12	570/99	0.94/14	58/10	2.2/10	15/2.5	3.8/10
92	AD3α5α17βC	1	8(-0.64%,5.8%)	990/170	0.98/10	460/78	1.9/6.5	440/76	0.94/13	180/31	0.82/8.6	190/33	0.99/11
93	AD3β5α17βC	0.1	8(-1.6%,6%)	1500/250	1.2/8.6	870/150	1.8/11	1200/200	5.9/12	170/29	1.8/8.4	87/15	3.7/8.1
94	AD3α5β17βC	2	8(2.2%,11%)	140/24	2.5/11	130/22	1.9/12	140/24	3.1/12	33/5.6	2.8/12	52/8.9	4.7/12
95	AD3β5β17βC	2	2(11%,13%)	7/1.2	4.4/11	7/1.2	12/13	12/2.1	4.6/12	4/0.69	12/14	9.3/1.6	9.2/11
96	THB3α5αC	20	30(3.1%,12%)	---	---	---	---	690/98	9.4/10	280/40	7/14	140/20	5.3/14
97	THB3α5βC	10	30(3.5%,11%)	---	---	---	---	---	---	330/47	7.9/14	---	---
98	THA3α5α11βC	0.2	2(8.6%,9%)	270/44	0.98/5.1	230/38	0.91/7.8	230/37	1.3/8.2	120/19	1.7/7.7	86/14	2.4/5.1
99	THA3β5α11βC	0.2	2(-8.1%,8.6%)	13/2.2	6.3/6.6	14/2.3	7.5/7.6	12/2	7.2/9.9	6.7/1.1	2.8/14	61/10	2.9/6.8
100	THA3α5β11βC	0.3	2(6.2%,7.7%)	56/9.2	1.2/11	92/15	2/13	92/15	1.8/13	20/3.2	3.8/5.5	6.7/1.1	3.8/12

Supplementary Table S3. Recovery for endogenous steroids in human serum as measured by GC-MS/MS

Steroid	**Added [pg]**			**Recovery (RSD)** [%]		
	level 1	level 2	level 3	level 1	level 2	level 3
Pregnenolone	10000	500	31.3	74 (15)	67 (12)	69 (13)
17α-Hydroxypregnenolone	2000	500	31.3	65 (11)	59 (6.2)	55 (7.1)
16α-Hydroxypregnenolone	500	31.3	7.81	120 (16)	110 (6.7)	120 (10)
20α-Dihydropregnenolone	2000	500	31.3	76 (4)	79 (10)	77 (6.8)
Dehydroepiandrosterone (DHEA)	10000	500	31.3	120 (7.7)	120 (2.7)	110 (15)
7α-Hydroxy-DHEA	31.3	7.81	0.488	110 (5.1)	120 (4.3)	100 (2.4)
7-oxo-DHEA	31.3	7.81	1.95	120 (12)	120 (14)	120 (14)
7β-Hydroxy-DHEA	31.3	7.81	1.95	100 (5.8)	88 (8.5)	96 (6.6)
Androstenediol	10000	2000	7.81	110 (4.7)	82 (3.9)	110 (5.7)
5-Androstene-3β,7α,17β-triol	7.81	1.95	0.488	90 (12)	78 (13)	85 (11)
5-Androstene-3β,7β,17β-triol	7.81	1.95	0.488	99 (12)	88 (5)	98 (11)
5-Androstene-3β,16α,17β-triol	2000	500	125	82 (3.3)	84 (17)	83 (7.5)
Progesterone	10000	500	1.95	87 (5.2)	76 (12)	69 (12)
17α-Hydroxyprogesterone	500	125	7.81	82 (4.2)	92 (1)	77 (16)
17α,20α-Dihydroxy-4-pregnene-3-one	500	125	7.81	88 (7.4)	47 (5.6)	60 (9)
16α-Hydroxyprogesterone	2000	31.3	0.488	100 (18)	92 (12)	100 (14)
20α-Dihydroprogesterone	500	31.3	1.95	89 (3.6)	90 (6.4)	99 (11)
Androstenedione	125	31.3	7.81	120 (13)	120 (7.2)	110 (8.9)
Testosterone	125	31.3	7.81	110 (10)	110 (7.6)	120 (2.9)
Epitestosterone	2000	500	125	85 (6.3)	99 (7.6)	100 (5)
16α-Hydroxytestosterone	500	125	31.3	74 (14)	66 (13)	58 (2.6)
5α-Dihydrotestosterone	31.3	7.81	1.95	83 (6.1)	79 (8.1)	82 (12)
Estrone	10000	2000	31.3	95 (11)	110 (6.5)	95 (16)
Estradiol	2000	125	31.3	79 (18)	70 (17)	60 (5.9)
Estriol	2000	500	125	54 (0.43)	76 (14)	74 (12)
5α-Dihydroprogesterone	2000	500	125	50 (4)	46 (4.7)	43 (1.9)
Allopregnanolone	2000	125	7.81	56 (3.8)	49 (1.9)	50 (2.1)
Isopregnanolone	2000	125	7.81	67 (4.2)	50 (5)	58 (12)
5β-Dihydroprogesterone	500	125	31.3	50 (0.93)	46 (4.4)	46 (0.61)
Pregnanolone	2000	500	125	68 (6.3)	63 (7.4)	56 (3.8)
Epipregnanolone	2000	125	31.3	53 (0.52)	48 (4.1)	48 (6.8)

Supplementary Table S3, continued. Recovery for endogenous steroids in human serum as measured by GC-MS/MS

Steroid	**Added [pg]**			**Recovery (RSD)** [%]		
	level 1	level 2	level 3	level 1	level 2	level 3
5α,20α-Tetrahydroprogesterone	2000	125	7.81	61 (2.4)	61 (15)	56 (8.3)
5α-Pregnane-3α,20α-diol	2000	125	31.3	66 (2.7)	84 (13)	80 (4.8)
5α-Pregnane-3β,20α-diol	2000	125	7.81	74 (4.9)	66 (9.1)	71 (4.5)
5β,20α-Tetrahydroprogesterone	500	125	31.3	63 (5.3)	55 (5)	61 (8.4)
5β-Pregnane-3α,20α-diol	2000	125	7.81	75 (5.9)	68 (5.1)	74 (12)
5β-Pregnane-3β,20α-diol	2000	500	31.3	65 (5.4)	66 (4.1)	69 (7.9)
17α-Hydroxyallopregnanolone	125	7.81	1.95	65 (8.3)	62 (9)	65 (4.2)
17α-Hydroxypregnanolone	125	7.81	1.95	68 (9.9)	66 (8.7)	71 (1.6)
5α-Pregnane-3α,17α,20α-triol	500	125	1.95	57 (15)	81 (8.1)	74 (4.7)
5α-Pregnane-3β,17α,20α-triol	125	7.81	1.95	55 (5.6)	62 (1.2)	64 (11)
5β-Pregnane-3α,17α,20α-triol	500	125	1.95	54 (15)	59 (11)	55 (2.2)
5α-Androstane-3,17-dione	125	7.81	1.95	100 (8.3)	94 (11)	97 (6.4)
Androsterone	10000	125	1.95	99 (11)	110 (6.7)	100 (1.6)
Epiandrosterone	500	7.81	1.95	110 (8.4)	110 (7.6)	120 (2.1)
Etiocholanolone	500	125	1.95	85 (4.5)	63 (5.9)	75 (6.4)
Epietiocholanolone	500	125	31.3	110 (14)	93 (6)	110 (8.7)
5α-Androstane-3α,17β-diol	2000	125	7.81	88 (5.6)	91 (8.5)	89 (14)
5α-Androstane-3β,17β-diol	2000	1.95	0.488	88 (6.8)	77 (13)	70 (6.2)
5β-Androstane-3α,17β-diol	125	31.3	0.488	110 (7.6)	110 (8.7)	100 (13)
5β-Androstane-3β,17β-diol	125	31.3	0.488	95 (8.1)	99 (12)	86 (7.6)
Cortisol	10000	2000	500	98 (2.1)	94 (7.3)	95 (4.1)
Cortisone	10000	2000	500	95 (8.5)	100 (7.3)	100 (5.6)
Corticosterone	500	125	7.81	87 (8.1)	81 (13)	86 (15)
21-Deoxycortisol	31.3	7.81	1.95	84 (11)	78 (5.7)	81 (10)
11-Deoxycorticosterone	500	125	31.3	110 (7.2)	90 (7.9)	95 (0.37)
3α,5α-Tetrahydrocorticosterone	500	125	1.95	92 (15)	85 (6.4)	95 (10)
3α,5β-Tetrahydrocorticosterone	500	1.95	0.488	89 (3.6)	110 (10)	100 (9.1)
11β-Hydroxyandrostenedione	2000	500	7.81	120 (9.3)	120 (4.6)	120 (7.1)
11β-Hydroxyandrosterone	500	125	31.3	110 (11)	99 (3.4)	96 (3.5)
11β-Hydroxyepiandrosterone	125	31.3	1.95	110 (9.3)	110 (6.7)	91 (8.4)
11β-Hydroxyetiocholanolone	125	31.3	1.95	110 (6.4)	110 (11)	110 (12)

Supplementary Table S4. Accuracy testing

No	Steroid	Concentration in blood [nM]					Level 1 Added [pg inj. /nM]	Bias (SD) [%] Intraday/interday (n=6)	Level 2 Added [pg inj. /nM]	Bias (SD) [%] Intraday/interday (n=6)	Level 3 Added [pg inj. /nM]	Bias (SD) [%] Intraday/interday (n=6)
		M	F	L	P	U						
1	Preg	5.1	8.4	9.2	18	74	500/79	1.5(0.62) / 2.7(5.8)	125/20	-7.9(1.2) / 2.3(7.1)	31.25/4.9	4.3(0.55) / -1(8.6)
2	Preg17	12	13	8.4	24	33	500/75	7.3(0.023) / 3.5(3.4)	125/19	-14(0.2) / -0.84(11)	31.25/4.7	1.8(0.28) / -0.37(4.4)
3	Preg16α	0.43	0.43	0.33	0.81	7.1	31.3/4.7	5.8(0.2) / 3.8(11)	7.81/1.2	1.3(1.6) / 2.7(9.5)	1.95/0.29	-3.1(2) / -1.8(6.1)
4	DHPreg20α	2.3	3.4	4.2	4	5.1	125/20	-12(2.2) / 3.5(8.5)	31.3/4.9	-0.015(1.9) / 2.5(6.9)	7.81/1.2	-3.5(1.5) / -0.12(7.5)
5	DHEA	10	15	12	18	7.7	500/87	4.5(0.21) / -0.79(6.9)	125/22	-3.9(0.27) / -0.7(3.2)	31.25/5.4	9.9(1.5) / -1.1(8.6)
6	DHEA7α	1.3	1.5	0.96	0.91	2	31.3/5.1	-4.3(0.25) / 1.2(8.9)	7.81/1.3	1.7(1.2) / 1.2(8.3)	1.95/0.32	0.12(2.7) / 1.2(8.4)
7	DHEA7o	1.1	0.41	0.39	0.53	0.79	31.3/5.2	-6.7(3.3) / -2.3(6.1)	7.81/1.3	-2.1(0.95) / -0.35(9.9)	1.95/0.32	8.7(9.7) / 4.1(12)
8	DHEA7β	0.48	0.25	0.4	0.17	0.33	31.3/5.1	-13(0.06) / -0.82(9.2)	7.81/1.3	0.013(1.2) / 2.1(2.5)	1.95/0.32	7.7(3.9) / 2(13)
9	5-Adiol	2.5	2.3	1.9	1.5	0.44	31.3/5.4	-2.4(0.47) / 2.9(13)	7.81/1.3	6.9(1.2) / 2.4(10)	1.95/0.34	0.65(1.1) / -0.43(5)
10	AT7α	0.37	0.44	0.28	0.098	0.016	31.3/5.1	-3.5(1.2) / 1(6.8)	7.81/1.3	4.2(0.8) / 6.4(2.9)	1.95/0.32	-1.9(3.9) / -2.8(6.3)
11	AT7β	0.31	0.35	0.25	0.068	0.019	31.3/5.1	-3.4(0.62) / 2.2(7)	7.81/1.3	4.7(0.89) / 4.2(5.6)	1.95/0.32	-2.2(1.3) / -0.49(3.8)
12	AT16α	0.51	0.45	0.51	0.8	3.1	31.3/5.1	2.2(0.21) / -2.9(0.74)	7.81/1.3	-5.6(4.1) / 1.8(3.9)	1.95/0.32	-1.5(10) / 2.8(1.3)
13	P	0.24	0.25	12	320	2300	2000/320	0.64(0.83) / 2.1(5)	125/20	-5(0.13) / -3.3(7.1)	1.95/0.31	-1.8(0.35) / -0.024(7)
14	P17	2.8	1.1	3.2	18	99	500/76	4.6(0.55) / 1.1(5)	125/19	-14(1.2) / -7.5(5.9)	7.81/1.2	5.1(11) / 2.5(6.4)
15	DHP17α20α	1.5	0.68	1	8.4	26	500/75	8.7(0.64) / 1.7(5.7)	31.3/4.7	-13(0.2) / -6.9(12)	7.81/1.2	6.1(1.7) / 1.3(3.6)
16	P16α	0.76	0.48	0.96	19	140	500/76	7.8(0.13) / 1.2(11)	125/19	-11(0.38) / -10(2.8)	7.81/1.2	-0.54(0.83) / -4.2(9.2)
17	DHP20α	0.21	0.29	4.9	92	99	500/79	2.6(0.42) / 6.6(5.4)	31.3/5	3.3(0.63) / 1.5(10)	7.81/1.2	-4.7(0.67) / -2.9(8.6)
18	A4	2.6	2.7	2.3	8.6	15	125/22	-2.9(0.55) / -4.9(4.9)	31.3/5.5	6.9(0.19) / -0.39(13)	7.81/1.4	-4.8(6.4) / -4.3(8.9)
19	T	2.6	2.7	2.3	8.6	15	125/22	-12(0.12) / -0.66(6.2)	31.3/5.4	2.5(2.6) / 5.3(9.1)	7.81/1.4	1.7(0.82) / -13(15)
20	T16α	---	---	---	4.2	11	125/21	1.6(1.3) / -3.2(7.7)	31.3/5.1	-1.8(0.64) / 4.9(4.9)	7.81/1.3	-0.81(14) / 1.2(14)
21	DHT5α	1.5	0.51	0.5	0.58	0.14	31.3/5.4	4.3(1.4) / -0.51(6.8)	7.81/1.3	2(1.6) / -0.54(8.6)	1.95/0.34	-2.3(2.9) / 3(4.9)
22	E1	0.16	0.24	0.26	48	120	500/93	3.1(0.41) / 2.9(9.9)	7.81/1.4	4.4(2) / -0.012(7.5)	1.95/0.36	-7.9(1.7) / 3.7(8.7)
23	E2	0.1	0.38	0.42	68	33	500/92	-8.7(1.7) / -4.9(9.2)	31.3/5.8	-0.52(1.5) / -4.2(13)	1.95/0.36	12(8.3) / -9.8(9.5)
24	E3	---	---	---	22	415	2000/350	-2.2(0.039) / -1.1(8)	500/87	4.6(1.3) / -8.7(6.3)	125/22	-2.5(0.31) / -6.4(13)
25	DHP5α	---	---	---	61	170	2000/320	3.6(0.51) / 0.56(6.2)	500/79	-5.3(1) / 2.5(5.9)	125/20	-7.4(0.42) / 2.3(11)

Supplementary Table S4, continued. Accuracy testing

No	Steroid	Concentration in blood [nM]					Level 1		Level 2		Level 3	
							Added [pg inj. /nM]	Bias (SD) [%] Intraday/interday (n=6)	Added [pg inj. /nM]	Bias (SD) [%] Intraday/interday (n=6)	Added [pg inj. /nM]	Bias (SD) [%] Intraday/interday (n=6)
		M	F	L	P	U						
26	THP3α5α	0.068	0.24	0.87	32	24	500/79	1.4(1.1) / 5.9(5.5)	31.3/4.9	-0.017(0.5) / -0.48(12)	1.95/0.31	12(8.3) / 4(9.5)
27	THP3β5α	0.25	0.59	0.83	18	38	500/79	-0.52(0.98) / 0.36(9.1)	31.3/4.9	3.3(2.7) / -0.32(10)	7.81/1.2	3.9(3.1) / -2.7(5.8)
28	DHP5β	---	---	---	3.1	45	500/79	6.9(2.1) / 3.6(9.3)	125/20	-13(2.6) / -6.3(3.9)	31.25/4.9	5.3(0.18) / 1.1(6.2)
29	THP3α5β	---	---	---	20	29	500/79	2.5(0.12) / 0.25(8.9)	125/20	-6.5(0.69) / 1.3(13)	31.25/4.9	5.3(2.2) / 0.51(9.4)
30	THP3β5β	---	---	---	1.4	3.4	31.3/4.9	2.9(1.6) / 1.9(8.2)	7.81/1.2	-13(3.9) / -3.2(10)	1.95/0.31	12(0.97) / -3.2(8.5)
31	THP5α20α	0.24	0.6	1.4	34	62	500/79	1.4(0.93) / 1.6(8.3)	7.81/1.2	2.9(1.8) / 0.46(6.9)	1.95/0.31	-5.2(5.2) / -2.6(5.3)
32	PD3α5α20α	0.26	0.41	1.1	25	9.8	500/78	4.4(0.95) / 8.2(5.7)	31.3/4.9	5.3(3.1) / -1.7(8.5)	7.81/1.2	-0.48(5.9) / 0.57(13)
33	PD3β5α20α	1.4	2.4	3.6	73	90	2000/310	2.2(1.4) / 2.9(2.2)	500/78	3.4(0.83) / -0.028(5.6)	31.3/4.9	-13(1.7) / -11(11)
34	THP5β20α	---	---	---	2.3	40	500/79	4.8(0.84) / 1.5(4.7)	125/20	-11(0.94) / -0.14(5.3)	31.25/4.9	10(0.76) / 1.5(10)
35	PD3α5β20α	0.28	0.23	0.35	8.2	11	125/20	-8.5(0.75) / 3.2(8.1)	31.3/4.9	11(0.68) / 5.5(7.2)	7.81/1.2	-7.3(8.7) / -1.4(9.9)
36	PD3β5β20α	---	---	---	0.85	2.3	125/20	-2.3(1.7) / 4.3(12)	31.3/4.9	4.8(0.92) / -3(9.4)	7.81/1.2	-2.8(5) / 1.9(6.3)
37	PD3α5α17	0.077	0.066	0.063	0.69	0.9	7.81/1.2	1.3(0.26) / -1.8(6.4)	1.95/0.29	11(0.58) / 0.038(15)	0.488/0.073	-11(5) / -5.2(24)
38	PD3α5β17	0.12	0.072	0.18	1.4	1.7	31.3/4.7	3(1.4) / -4.5(5.5)	7.81/1.2	2.7(1.1) / -3.6(3.9)	1.95/0.29	-1.8(4.8) / 0.92(8.8)
39	PT3α5α17α20α	0.28	0.2	0.22	0.23	0.13	31.3/4.7	-4.3(0.19) / 1(5.1)	7.81/1.2	-1.1(1.6) / -6.1(4.1)	1.95/0.29	3(0.1) / -2.9(8.2)
40	PT3β5α17α20α	0.24	0.31	0.33	0.24	0.12	31.3/4.7	-2.7(0.94) / -0.96(7.5)	7.81/1.2	4(0.25) / -4.2(7.1)	1.95/0.29	-2.3(0.46) / -6.3(11)
41	PT3α5β17α20α	1.5	1.5	1.7	7.3	3	31.3/4.7	-2.4(0.4) / 0.55(3.8)	7.81/1.2	1.2(2.1) / -8(8.7)	1.95/0.29	0.21(1.7) / -1.6(11)
42	DHA5α	0.27	0.32	0.24	0.49	0.6	31.3/5.4	12(0.47) / 4.5(12)	7.81/1.4	-3.6(3.2) / -2.8(7.7)	1.95/0.34	-2(1.3) / 3.1(4.6)
43	THA3α5α	0.46	0.31	0.29	0.7	0.4	31.3/5.4	7.8(0.043) / 0.72(10)	7.81/1.3	4.8(0.54) / 2.7(6.5)	1.95/0.34	-6.4(1.2) / -2.8(3.8)
44	THA3β5α	0.31	0.4	0.33	0.57	0.31	31.3/5.4	5.4(0.51) / 2.3(2.5)	7.81/1.3	11(2.7) / 2.7(10)	1.95/0.34	-10(4) / -3.9(7.1)
45	THA3α5β	0.25	0.31	0.24	0.54	0.63	7.81/1.3	4.3(1.1) / 3.3(6.8)	1.95/0.34	2.8(0.34) / -6.6(12)	0.488/0.084	-8.8(4.9) / -11(8.2)
46	AD3α5α17β	0.29	0.084	0.078	0.13	0.038	7.81/1.3	11(0.59) / 5.6(8)	1.95/0.33	4.3(1.2) / -6.1(8.4)	0.488/0.084	-10(9) / -0.081(6.7)
47	AD3β5α17β	5.1	8.4	9.2	18	74	7.81/1.3	6.1(1.7) / 3.5(4.2)	1.95/0.33	-1.3(1.9) / 5.6(8.2)	0.488/0.084	1.7(7.9) / -21(29)
48	AD3α5β17β	5.1	8.4	9.2	18	74	7.81/1.3	6.1(1.7) / 8(5.9)	1.95/0.33	-1.3(1.9) / -4(2.9)	0.488/0.084	1.7(7.9) / 1.8(2.5)
49	F	310	300	310	680	260	10000/1400	3.1(1.7) / 3.3(2.8)	2000/280	-6.5(4.9) / -3(4.7)	500/69	3(1.8) / 5.5(5.8)
50	E	51	49	50	140	310	10000/1400	4.1(0.25) / 1.7(5.2)	2000/280	-8.5(1.4) / 1.4(6.5)	500/69	3.8(4.6) / -2.6(7.4)

Supplementary Table S4. Accuracy testing

No	Steroid	Concentration in blood [nM] M	F	L	P	U	Level 1 Added [pg inj. /nM]	Level 1 Bias (SD) [%] Intraday/interday (n=6)	Level 2 Added [pg inj. /nM]	Level 2 Bias (SD) [%] Intraday/interday (n=6)	Level 3 Added [pg inj. /nM]	Level 3 Bias (SD) [%] Intraday/interday (n=6)
51	B	13	14	11	74	18	500/72	2.8(1.6) / 5.6(5.7)	125/18	-12(0.21) / -2.5(7.5)	31.3/4.5	-1.5(0.45) / 9.6(8.3)
52	DOF	0.19	0.22	0.24	0.75	0.76	31.3/4.5	-10(2.1) / -6.6(9.5)	7.81/1.1	5.2(0.43) / 0.94(9.3)	1.95/0.28	3.8(0.3) / -4.4(9.5)
53	DOC	---	---	---	3.3	7.1	125/19	-13(1.5) / -2(10)	31.3/4.7	-7.4(1.6) / -6(7.6)	7.81/1.2	7.5(0.66) / 2(10)
54	THB3α5α	0.27	0.37	0.27	0.37	---	31.3/4.5	-10(5.2) / -1.8(4.4)	7.81/1.1	3(0.89) / 3.9(11)	1.95/0.28	6.5(8.5) / 2.7(12)
55	THB3α5β	0.5	0.51	0.47	0.48	0.16	31.3/4.5	1.9(2.5) / 3.4(9.5)	7.81/1.1	3.2(0.49) / 2.2(7.6)	1.95/0.28	1(1.5) / -9.8(12)
56	11OHA4	77	75	47	270	100	2000/330	-4.4(0.82) / 3.3(5.2)	500/83	6.8(0.95) / -2.1(7.7)	125/21	-4.9(0.78) / -1.4(8.6)
57	THA3α5α11β	8.2	3.7	3.7	1.2	1.3	125/20	-1.5(0.043) / 1.2(3)	31.3/5.1	0.13(0.21) / 0.54(8.6)	7.81/1.3	4.5(3.8) / -0.94(3.8)
58	THA3β5α11β	0.49	0.26	0.26	0.1	0.17	125/20	-1.3(0.2) / -8.9(15)	31.3/5.1	-0.5(0.5) / 0.68(9.6)	7.81/1.3	5(0.44) / 2.2(11)
59	THA3α5β11β	1.5	0.68	1	8.4	26	7.81/1.3	8.5(2.2) / 2.5(3.1)	1.95/0.32	13(0.76) / -7.8(21)	0.488/0.08	-14(11) / -28(37)
60	PregC	250	250	300	530	4400	10000/1600	-1.2(0.93) / -7.7(2.3)	2000/320	-0.034(1.1) / 5.1(6.1)	500/79	3.8(1.3) / -2.5(5.8)
61	Preg17C	41	42	59	110	5800	10000/1500	-2.4(0.49) / -5.6(3.5)	500/75	4.8(0.71) / -2.4(9.3)	125/19	-4.4(6.3) / 0.45(5.7)
62	DHPreg20αC	1400	990	1500	790	2300	10000/1500	0.39(0.47) / -7.4(2.9)	2000/300	-0.87(0.5) / 3.5(7)	500/75	0.5(1.6) / 2.1(8.1)
63	DHEAC	4700	4600	4400	2100	5000	10000/1700	2.5(0.99) / -7.2(3.2)	2000/350	-5.7(1.1) / 7.8(5.3)	500/87	3.4(2) / -3.6(4.8)
64	5-AdiolC	3400	2900	2300	360	4500	10000/1700	0.53(1.2) / -5.4(2.6)	2000/340	0.57(0.78) / 8(4.8)	500/86	5.9(1.4) / -3.8(3.4)
65	AT16αC	50	67	61	170	2300	2000/330	-6.9(0.97) / -3(6)	500/82	3.8(0.78) / 0.81(0.78)	125/20	0.5(1.2) / 1.3(7.9)
66	DHP17α20αC	13	20	21	---	---	500/75	5.4(0.58) / -2.6(3.7)	125/19	-7.1(1.9) / -11(5.5)	31.3/4.7	-14(3.9) / 5.3(7.2)
67	DHP20αC	2.9	3.6	8.2	30	140	2000/320	2.6(1.3) / 4.9(2.1)	125/20	0.76(4.5) / -9.3(6.6)	7.81/1.2	3.8(3.2) / -1.5(6.1)
68	TC	---	---	---	19	51	500/87	-2.9(4.7) / 5.7(2.5)	125/22	2.2(5.8) / -0.54(7.3)	31.3/5.4	0.3(5.5) / -1.3(1.7)
69	EpiTC	---	---	---	16	410	2000/350	-2.9(4.7) / -4.5(3.6)	500/87	2.2(5.8) / 3.2(4)	125/22	0.3(5.5) / -1.3(3.8)
70	E1C	---	---	---	680	37	2000/370	6.9(1.2) / 3.5(6.2)	500/93	-2.3(1.5) / 1.1(5.1)	125/23	-13(3.8) / -0.6(10)
71	E2C	---	---	---	29	5.3	500/92	9.4(1.1) / 1.3(9.8)	125/23	-4.1(1.2) / 2(7.7)	31.3/5.8	9.3(2.6) / -3.7(5.5)
72	E3C	---	---	---	16	410	10000/1700	-8.1(0.6) / 1.6(2.4)	2000/350	-3.7(0.68) / -5.8(5.5)	500/87	8(0.86) / 7.4(8.1)
73	THP3α5αC	7.3	14	68	1500	410	10000/1600	1.9(1.1) / -9.8(19)	2000/310	-3.4(0.58) / -2.3(5.5)	125/20	-13(2.8) / 1(6.3)
74	THP3β5αC	17	25	45	1000	490	2000/310	-6(0.46) / -3.7(6.3)	500/79	3.6(0.83) / 7(3.2)	125/20	-6.2(1.4) / -5.1(4.7)

Supplementary Table S4. Accuracy testing

No	Steroid	Concentration in blood [nM]					Level 1		Level 2		Level 3	
							Added [pg inj. /nM]	Bias (SD) [%] Intraday/interday (n=6)	Added [pg inj. /nM]	Bias (SD) [%] Intraday/interday (n=6)	Added [pg inj. /nM]	Bias (SD) [%] Intraday/interday (n=6)
		M	F	L	P	U						
75	THP3α5βC	31	41	70	880	450	10000/1600	-5.9(0.9) / -7.9(3.9)	2000/310	2.5(0.78) / -0.45(9.6)	125/20	-3.9(2.9) / 1.4(5.8)
76	THP3β5βC	3.9	12	14	240	160	2000/310	-3.9(1.1) / -3.2(8.3)	125/20	-8.8(3.2) / 0.7(4.5)	31.3/4.9	-9.3(2.9) / -3.4(4.1)
77	THP5α20αC	0.63	1.4	14	130	180	2000/310	-1.9(0.51) / 3.4(6.2)	125/20	-9(2) / -2.1(11)	7.81/1.2	10(2.6) / -4.3(7.4)
78	PD3α5α20αC	33	62	270	5100	3000	10000/1600	-4.4(0.98) / -7.2(1.1)	500/78	-3.3(0.41) / -3.1(6.8)	125/20	-14(4.2) / -3.5(6.4)
79	PD3β5α20αC	500	2700	7800	65000	35000	10000/1600	0.28(2.1) / -8.9(2.1)	2000/310	-0.72(1.5) / 9.8(8.1)	500/78	0.57(4.2) / -2.8(8.1)
80	THP5β20αC	---	---	---	35	98	2000/310	-11(0.48) / 2.6(5.4)	500/79	14(2) / 0.22(8.5)	125/20	-4.2(2.8) / 1.6(9.4)
81	PD3α5β20αC	20	52	220	1700	2000	10000/1600	-9.2(2.1) / -11(1.7)	2000/310	4(1.4) / 4(10)	125/20	-12(4) / -1.8(6.3)
82	PD3β5β20αC	24	120	190	1200	990	10000/1600	-7.1(0.67) / -8.4(7)	2000/310	-2.3(0.97) / -2.7(7.2)	125/20	-12(3.1) / 1.2(0.99)
83	PD3α5α17C	4.4	2.2	6.1	22	9.5	500/75	5.6(0.8) / 7.2(4.7)	125/19	-9.8(1.6) / 2.4(7.1)	31.3/4.7	3.4(2.4) / 0.99(8.2)
84	PD3α5β17C	18	12	26	100	69	2000/300	-11(0.64) / 1.1(7.3)	500/75	15(1.6) / 4.7(3.8)	125/19	-2.3(1.9) / 0.52(5.9)
85	PT3α5α17α20αC	---	---	---	39	82	2000/300	0.93(0.83) / 3(8.8)	500/74	-2.9(1.4) / -0.6(9.9)	125/19	2.1(0.001) / -2.2(4.5)
86	PT3β5α17α20αC	---	---	---	7.7	5.2	125/19	-4.3(1.5) / -5.2(5.6)	31.3/4.7	-11(0.58) / 6.1(8.1)	7.81/1.2	11(4.5) / 4.1(1.1)
87	PT3α5β17α20αC	---	---	---	360	390	10000/1500	-11(1.6) / 1.2(4.7)	2000/300	15(2.7) / -3.9(10)	125/19	1.9(1.1) / 2.6(6.7)
88	THA3α5αC	1900	2700	3200	630	100	10000/1700	5.1(1.4) / -4.8(1.3)	2000/340	-12(0.8) / 9.8(2.3)	500/86	7.9(1.7) / -4.6(3.8)
89	THA3β5αC	510	510	500	150	55	10000/1700	-0.57(0.74) / -9.2(3.8)	2000/340	0.34(0.55) / 5.2(7.1)	125/22	-0.82(1.7) / -1.7(5.2)
90	THA3α5βC	100	130	110	62	20	2000/340	-2(0.75) / 5.3(7.4)	500/86	-1.4(0.95) / 0.74(7)	125/22	-7.3(1) / -3.4(8.7)
91	THA3β5βC	54	73	99	10	2.5	500/86	14(0.7) / 3.4(7.3)	125/22	-5.1(1.7) / -2.7(8.2)	7.81/1.3	10(5) / 2.4(7.3)
92	AD3α5α17βC	170	78	76	31	33	2000/340	-1.7(0.95) / 2.1(7.6)	125/22	-13(1.4) / -1.3(7.5)	31.3/5.4	-8.4(1.6) / -3.1(3.8)
93	AD3β5α17βC	250	150	200	29	15	2000/340	4.4(1.2) / 2.7(5.9)	125/22	-9.8(1.6) / 3.7(9.2)	31.3/5.4	-7.5(2) / 0.84(5.1)
94	AD3α5β17βC	24	22	24	5.6	8.9	500/86	14(0.45) / 1.6(8.2)	125/22	-11(2.7) / 0.22(5.1)	31.3/5.4	-8.7(2.9) / -0.89(10)
95	AD3β5β17βC	1.2	1.2	2.1	0.69	1.6	125/22	-13(1.2) / 0.72(9.9)	31.3/5.4	-9(1.6) / -1.3(9.2)	7.81/1.3	9.6(4.4) / -4(5.7)
96	THB3α5αC	---	---	98	40	20	10000/1400	-4.2(1.7) / -3.4(5.9)	2000/290	9.9(13) / 6.5(1.2)	500/71	5.3(12) / 2.2(7.3)
97	THB3α5βC	---	---	---	47	---	10000/1400	-2.8(2.2) / -2.9(9.2)	125/18	6.8(8.5) / 2.8(11)	31.3/4.5	-2.4(13) / -6.4(4.8)
98	THA3α5α11βC	44	38	37	19	14	500/82	9.6(0.82) / 4.5(5.5)	125/20	4.8(0.95) / 0.46(5.5)	31.3/5.1	3.2(1.3) / -1.9(1.6)
99	THA3β5α11βC	2.2	2.3	2	1.1	10	125/20	4.5(1.3) / 2.4(6.3)	31.3/5.1	-2.5(2.9) / -1.6(5)	7.81/1.3	4.4(4.8) / -6.3(2.2)
100	THA3α5β11βC	9.2	15	15	3.2	1.1	125/20	4.3(2.2) / 0.8(4.8)	31.3/5.1	0.61(2.6) / -2.3(3.5)	7.81/1.3	0.82(4) / -3.7(6.8)

Supplementary Table S5. Testing of extraction efficiency and efficiency of the methanolysis step using available steroid sulfates

				Unconjugated steroid				Sulfated steroid						Extraction efficiency		
				Diethylether layer			Polar layer	Diethylether layer		Polar layer						
Steroid/sulfate	Injected steroid [ng]	Injected IS (D6-DHEA) [µL]	MW sulfated steroid/MW steroid	Peak area, steroid [AUC]	Peak area, IS [AUC]	Peak area ratio (steroid/IS)	Peak area, steroid [AUC]	Peak area, steroid [AUC]	Peak area corrected to MWs [AUC]	Peak area, steroid [AUC]	Peak area corrected to MWs [AUC]	Peak area, IS [AUC]	Peak area ratio corrected to MWs (steroid/IS)	Unconjugated steroid [%]	Sulfated steroid [%]	**Efficiency of methanolysis step, corrected to IS [%]**
Androsterone	20	20	1.41	14944357	8801862	1.70	2140798	55249	77730	5441067	7655018	3880859	1.97	87	99	116
Epiandrosterone	20	20	1.41	13952600	9379142	1.49	1129634	2235	3144	2818461	3965283	3130178	1.27	93	100	85
Etiocholanolone	20	20	1.41	17330257	9379142	1.85	1806954	3542	4983	4508911	6343571	3130178	2.03	91	100	110
Pregnanolone	20	20	1.37	4802104	5681583	0.85	1161327	4494	6162	1829402	2508237	3021745	0.83	81	100	98
Epipregnanolone	20	20	1.37	6941351	6715295	1.03	2179828	9243	12673	1981282	2716475	2951234	0.92	76	100	89
DHEA	20	20	1.41	12402619	9334823	1.33	845542	1319	1859	3298765	4650342	3610621	1.29	94	100	97
Estradiol (disulfate)	2	20	1.87	3168922	6309477	0.50	47475	28	52	881558	1646439	3623213	0.45	99	100	90
													Minimum	76	99	85
													Maximum	99	100	116
													Mean	88	100	98
													SD	7.2	0.3	10.5

Supplementary Table S6. Stability of unconjugated steroids during the methanolysis procedure

No	Steroid	Ratio of peak areas **+hydrolysis/-hydrolysis** (mean ± SD)	No	Steroid	Ratio of peak areas **+hydrolysis/-hydrolysis** (mean ± SD)
1	Preg	111 ± 16 [%]	20	PD3α5α20α	106 ± 15 [%]
2	Preg17	87 ± 18 [%]	21	PD3β5α20α	117 ± 11 [%]
3	DHPreg20α	115 ± 17 [%]	22	THP5β20α	73 ± 4 [%]
4	DHEA	83 ± 12 [%]	23	PD3α5β20α	110 ± 12 [%]
5	Adiol	116 ± 9 [%]	24	PD3β5β20α	112 ± 15 [%]
6	AT16α	58 ± 5 [%]	25	P3α5α17	83 ± 6 [%]
7	DHP20α	41 ± 4 [%]	26	P3α5β17	70 ± 10 [%]
8	DHP17α20α	46 ± 5 [%]	27	THA3α5α	90 ± 5 [%]
9	T	34 ± 6 [%]	28	THA3β5α	64 ± 3 [%]
10	EpiT	22 ± 3 [%]	29	THA3α5β	70 ± 5 [%]
11	DHT5α	47 ± 5 [%]	30	THA3β5β	79 ± 5 [%]
12	E1	53 ± 9 [%]	31	AD3α5α17β	92 ± 6 [%]
13	E2	31 ± 2 [%]	32	AD3β5α17β	79 ± 6 [%]
14	E3	43 ± 3 [%]	33	AD3α5β17β	79 ± 8 [%]
15	THP3α5α	102 ± 14 [%]	34	AD3β5β17β	85 ± 6 [%]
16	THP3β5α	100 ± 13 [%]	35	THB3α5α	14 ± 5 [%]
17	THP3α5β	102 ± 15 [%]	36	THA3α5α11β	51 ± 5 [%]
18	THP3β5β	103 ± 14 [%]	37	THA3β5α11β	62 ± 14 [%]
19	THP5α20α	65 ± 5 [%]	38	THA3α5β11β	59 ± 15 [%]

3.2. Comparison of the present GC-MS/MS method with our previous GC-MS method

Due to the high number of analytes and variety of steroids measured in human circulation, a comparison of all steroids with results from other methods was unachievable. Nevertheless, a number of our present results are comparable with those data from our previously published GC-MS method (Bicikova et al. 2013, Duskova et al. 2012, Hill et al. 2010b, Hill et al. 2011a, Hill et al. 2014, Hill et al. 2011b, Hill et al. 2010c, Kancheva et al. 2011, Majewska et al. 2014, Parizek et al. 2016, Paskova et al. 2014, Pospisilova et al. 2012, Vankova et al. 2016) as well as with the results of other authors (see citations in (Hill et al. 2010b)).

The agreement between GC-MS, LC-MS/MS, RIA (for cortisol) and our present method for individual analytes mostly ranged from satisfactory to excellent results (Supplementary Table S1 and Supplementary Table S2, Supplementary Figure S1 and Supplementary Figure S2,) even if there were little deviations from identity line and problems with LDR in two analytes. We compared responses to samples injected in high- (2 µL) and low injection volumes (0.2 µL) for analytes with high circulating levels (some conjugated steroids) and found two of them, in which a considerable number of responses was not proportional to the injected volume (DHEA sulfate and androsterone sulfate). In these analytes, the samples from subjects with lower analyte circulating levels showed tight correlations between concentrations calculated from low- and high injection volume and slopes (using the same calibration curve) of the corresponding regression lines did not significantly differ from 1. However, in the samples from subjects with higher analyte concentrations, the divergence between concentrations calculated for samples injected at high and low injection volume began to grow. Here the samples injected in low volume showed higher concentrations when compared with the same ones injected in the high volume (see Supplementary Figure S3). It is evident that samples from subjects with higher analyte circulating levels underwent the same treatment as those from subject with the lower analyte circulating levels. Thus, the only cause of the differences in the former group should be the different injection volumes. As expected, the only change at lower injection volume was the shift of the analyte response to LDR in the samples from subjects with higher analyte circulating levels without significant influence on results in the samples from subject with the lower analyte circulating levels. These levels evidently remained sufficiently high for analysis at lower injection volumes. Based on these data, the sulfates of DHEA and androsterone were measured at low injection volumes of samples in the present method and the method validation for these steroid conjugated was also completed at low injection volumes.

In addition to the steroids quantified in the previous method (Hill et al. 2010b), the present one was extended for corticoids, 11β-hydroxy-androstanes and 17α-hydroxylated 5α/β-reduced pregnanes. The last-mentioned substances may be useful for the investigation of the alternative "backdoor" pathway. When comparing the analytical characteristics of the present and previous methods, the first exhibited by far higher selectivity, generally higher sensitivity and better precision particularly for 17α-hydroxysteroids. However, in the case of estrogens the precision was worse and even unsatisfactory for estrone in non-pregnant subjects, which may be associated with the use of different derivatization agent in the silylation step and worse repeatability (during the drying of derivatized mixture under nitrogen because to its higher heterogeneity in comparison with our previous method). On the other hand, the more intense and lengthier derivatization together with the use of more advanced GC-MS/MS platform resulted in substantially improved sensitivity and precision in 17α-hydroxy-steroids and enabled the quantification of corticoids and 11β-hydroxy-androgens, which were undetectable by our previous method.

3.3. Limitations of our method

We acknowledge that our proposed method has some limitations. The first is the absence of conjugated external and deuterated internal standards in most conjugated steroids and absence of deuterated internal standards even for most unconjugated steroids. The first reason was a limited accessibility of these substances. The further serious problem especially in quantification of conjugated steroids was chemical and isotopic instability as well as isotopic impurity of various deuterated standards (as discussed above). Therefore, we excluded the analysis of four steroid conjugates, which were well detectable but extremely instable during the hydrolysis such as conjugated 7α/β-hydroxy-metabolites of DHEA and 5-Adiol. The difficult accessibility, isotopic and chemical instability were also the reasons for which we used only a single (but pure and stable) deuterated steroid conjugate (D6-DHEA sulfate) as the internal standard for the quantification of conjugated steroids.

Furthermore, in spite of wide spectrum of the measured steroids some diagnostically important steroids remained, which were not included. Partly due to unfavorable fragmentation pattern of the steroid even after derivatization resulting in low sensitivity as in the case of 11-deoxycortisol. In addition, 11-deoxycorticosterone was below the LOQ for non-pregnant subjects and 21-deoxycortisol was above the LOQ for all groups but the sensitivity was also relatively low. Also, the sensitivity for estrogens in non-pregnant subjects was low. The quantification of interesting steroids such as 11β-hydroxy-testosterone, 11-oxo-testosterone and 11-oxo-androstenedione was not tested as well as the measurement of steroid 6α/β-hydroxy-catabolites.

4. Conclusions

To the best of our knowledge, in spite of the limitations described above, this is the first GC-MS/MS method for multicomponent quantitation of circulating steroids validated for different physiological conditions in humans including gender differences and pregnancy status. In addition, this method currently includes the largest spectrum of human circulating steroids and steroid polar conjugates, at least for the GC-MS/MS platform. As have been demonstrated in our previous papers, steroid profiling enables various pathologies to be rapidly diagnosed (Bicikova et al. 2013, Hill et al. 2010c, Kanceva et al. 2015, Parizek et al. 2016, Sosvorova et al. 2015, Vankova et al. 2016). The present GC-MS/MS method includes a wide range of analytes, which reflect activities of most steroidogenic enzymes. Thus, it could be used for the estimation of changes in steroidogenesis for various physiological and pathophysiological situations and subsequently the data obtained can be utilized for uncovering the mechanisms of some steroid-related human pathologies (Parizek et al. 2016, Sterzl et al. 2017, Vankova et al. 2016).

Nevertheless, the hydrolysis step is laborious and may carry problems with stability of some steroid conjugates. Furthermore, some positions of sulfate or glucuronide groups in steroid molecule may be resistant to hydrolysis although the deconjugation step used in the present method appears to be quite efficient. Moreover, the physiological and pathophysiological importance of steroid sulfates and glucuronides may be different. Therefore, the future work in steroid assay development should strive to measure the entire conjugated molecule without hydrolysis.

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