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Fast and sensitive liquid chromatography–mass spectrometry assay for seven androgenic and progestagenic steroids in human serum

Pekka Keski-Rahkonen[a,*], Kaisa Huhtinen[b,c], Matti Poutanen[b,d], Seppo Auriola[a]

[a] Department of Pharmacy, University of Eastern Finland, Yliopistonranta 1 C, FI-70211 Kuopio, Finland
[b] Department of Physiology, Institute of Biomedicine, University of Turku, FI-20520 Turku, Finland
[c] Department of Obstetrics and Gynaecology, Turku University Hospital, FI-20520 Turku, Finland
[d] Turku Center for Disease Modeling, University of Turku, FI-20520 Turku, Finland

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ABSTRACT

A fast and sensitive LC–MS/MS method for the quantitative analysis of seven steroid hormones in 150 μl of human serum was developed and validated. The following compounds were included: 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, androstenedione, dehydroepiandrosterone, testosterone, pregnenolone, and progesterone. Individual stable isotope-labeled analogues were used as internal standards. Sample preparation was performed by liquid–liquid extraction, followed by oxime derivatization to improve the ionization efficiency of the analytes. In contrast to the common derivatization-based methods, the reaction was incorporated into the sample preparation process and the only additional step due to the derivatization was a short heating of the autosampler vials before the sample injection. Chromatographic separation was achieved on a reversed-phase column using a methanol–water gradient. For the analyte detection, a triple quadrupole instrument with electrospray ionization was used. Total run time was 7.0 min and the lower limits of quantification were in the range of 0.03–0.34 nM (0.01–0.10 ng/ml), depending on the analyte. The method was validated using human serum samples from both sexes and applied for the serum steroid profiling of endometriosis patients.

1. Introduction

Steroid hormones are endogenous compounds that are derived from cholesterol by a series of enzymatic reactions in the adrenal glands, gonads, and placenta. The steroids typically possess strong physiological effects at very low concentrations via binding to nuclear receptors at nano- or picomolar levels, and variations in the concentrations, e.g. of estradiol, progesterone, and testosterone are routinely used for diagnostic purposes. Although assaying a single steroid in serum or plasma can be a useful diagnostic parameter, in many cases the relative concentrations of several steroids and their metabolites are critical [1]. Thus, the simultaneous quantitative measurement of circulating steroids is expected to be an important part of the research, diagnosis, and treatment of the disorders of steroid hormone synthesis and metabolism in the future.

Classical methods for plasma and serum steroid analyses are based on immunoassays (IA) and related techniques. Although still in use, it is now known that many IAs suffer from specificity issues and often overestimate the concentrations, particularly at the lower end of the concentration range [2–5]. Also, the interassay precision of many IAs is poor [6] and multiple assays are needed for steroid profiling. For these reasons, the use of mass spectrometry-based methods for clinical steroid analysis is rapidly increasing [7,8].

While mass spectrometry has a long history in steroid analysis in the form of GC–MS, the current trend is towards LC–MS because of its potential for higher throughput and simpler sample preparation [8,9]. However, although steroids are well suited for liquid chromatography, their structural properties potentially lead to poor ionization efficiency, compromising the assay sensitivity. The weak gas-phase proton affinity of most steroids does not favor the use of positive-mode ESI, as different adducts are usually generated instead of $[M+H]^+$ ions [10,11]. The three major estrogens (estrone, estradiol, and estriol) have at least one phenolic hydroxyl group, which can be ionized with negative ESI [12], but the vast majority of steroids lack this property. To increase the assay sensitivity for these compounds, APCI or APPI have been used instead of ESI [13–15]. However, regardless of the ionization technique, improvements in the assay sensitivity have been realized after chemical derivatization of the analytes [10,11,16–19].

Although derivatization is shown to improve the ionization of many steroids, it can also complicate the sample preparation process. This can reduce the reliability of the method, usually due to the uncontrollable reaction recovery and the additional purification procedures needed for the reaction mixture [1].

* Corresponding author. Tel.: +358 40 3553682; fax: +358 17 162456.
E-mail address: pekka.keski-rahkonen@uef.fi (P. Keski-Rahkonen).

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However, some derivatization techniques, such as the recently introduced oxime derivatization of ketosteroids [10,20,21], enable the injection of the reaction mixture into the separation column as such [3], and the inclusion of stable isotope-labeled internal standards will effectively compensate any variability in the reaction recovery. The derivatization technique also significantly increases the ESI response of neutral keto- or oxosteroids [10,20].

The aim of the presented work was to develop a method for fast and reliable quantification of the major androgens and progestagens in the biosynthetic pathway from cholesterol to testosterone. Although various LC–MS assays for these compounds in serum have been published [22–31], methods for their simultaneous determination, with sufficient sensitivity for the analysis of both female and male samples have not been previously described. The presented assay employs oxime derivatization and positive-mode ESI, with a simplified sample preparation technique that incorporates the derivatization of the analytes into the sample extraction process.

2. Experimental

2.1. Materials, reagents, and equipment

Standard compounds 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, androstenedione, dehydroepiandrosterone (DHEA), testosterone, and pregnenolone were obtained from Sigma–Aldrich (St. Louis, MO). Progesterone was from Steraloids (Newport, RI). Internal standards d7-androstenedione and d9-progesterone were from Steraloids, d4-pregnenolone, d8-17α-hydroxyprogesterone, and d3-17α-hydroxypregnenolone from C/D/N Isotopes (Quebec, Canada), d3-testosterone and d6-dehydroepiandrosterone from Sigma–Aldrich. Additional compounds for the selectivity studies included aldosterone, androsterone, estradiol, estrone, etiocholanolone (all from Sigma–Aldrich), androstanedione, and dihydrotestosterone (Steraloids).

Methanol and formic acid (both of LC/MS grade), bovine serum albumin (BSA; fatty acid and globulin free, ≥99% purity), methyl *tert*-butyl ether (MTBE; ACS spectrophotometric grade), and hydroxylamine hydrochloride (ReagentPlus grade) were from Sigma–Aldrich. Sterofundin ISO was from B. Braun (Melsungen, Germany). BSA solution was made by dissolving 2 g of BSA in 50 ml of Sterofundin ISO. Hydroxylamine was used as a 100 mM solution in 50% (v/v) methanol.

Weighings were done with a calibrated analytical balance (Mettler Toledo AX205; Mettler-Toledo, Greifensee, Switzerland). Calibrated micropipettes were used throughout, with a dedicated 5–40 μl pipette for the spiking of standard and IS working solutions. Liquid–liquid extractions were performed in 2 ml clear glass screw top vials with PTFE lined silicone septa (Agilent Technologies, Palo Alto, CA), using a Multi Reax test tube shaker (Heidolph Instruments, Schwabach, Germany). All subsequent sample preparation steps were performed in 1.5 ml conical-bottomed clear glass vials with PTFE/red rubber crimp camps (Agilent Technologies). For the MTBE evaporation, a nitrogen evaporator N-EVAP 112 (Organomation Associates, Berlin, MA) was used. Sample heating was performed in a ULE500 laboratory oven (Memmert, Schwabach, Germany). Water was purified using a Milli-Q Gradient system (Millipore, Milford, MA).

2.2. Chromatographic equipment and conditions

The HPLC system (Agilent 1200 Series RRLC) consisted of a micro degasser, binary pump SL in low delay volume configuration (damper and mixer bypassed), high performance autosampler SL, and column thermostat SL (Agilent Technologies, Waldbronn, Germany). Zorbax SB-C18 column (50 mm × 2.1 mm, 1.8 μm) was used with a 0.2 μm in-line filter (Agilent Technologies). The column was connected to the mass spectrometer inlet using a 350 mm × 0.12 mm PEEK capillary. Column was maintained at 48 °C and autosampler tray at ambient temperature. Injection volume was 40 μl. The autosampler was set to perform vial bottom sensing and sampling from the bottom of the vials. Further autosampler functions in use were automatic delay volume reduction with default timing settings, and injection overlap at 5.0 min. Flow rate was 0.2 ml/min and gradient elution was used with water (eluent A) and methanol (eluent B), both containing 0.025% (v/v) of formic acid. The gradient was as follows: 0–0.7 min: 60% B, 0.7–5.0 min: 60 → 90% B, 5.0–5.4 min: 90% B, 5.4–5.41 min: 90 → 60% B; 5.41–7.0 min: 60% B. Total run time from injection to injection was 7.0 min.

2.3. Mass spectrometric equipment and conditions

Agilent 6410 Triple Quadrupole LC/MS (G6410A) with electrospray ion source was used (Agilent Technologies, Palo Alto, CA). Nitrogen was used as drying, nebulizer, and collision gas. The following ion source conditions were used: positive ion mode, drying gas temperature 300 °C, drying gas flow 5 L/min, nebulizer pressure 15 psi, and capillary voltage 3000 V. Instrument was tuned with the built-in autotune function using the associated tuning solution. The resulting voltage for the electron multiplier was then increased by 600 V. Multiple reaction monitoring (MRM) was used with both quadrupoles set at unit resolution. The MRM transitions, fragmentor voltages, and collision energies are shown in Table 1. Divert valve was used to allow eluent flow into the mass spectrometer from 2.0 to 5.5 min. Data was collected within this time window, which was divided into two segments at 4.3 min. Dwell times were 20 ms in the first and 100 ms in the second time segment (1.7 cycles/s in both segments). Data acquisition and quantification were performed using Agilent MassHunter Acquisition B.01.04 and Quantitative Analysis B.04.00, respectively.

2.4. Preparation of standard solutions

Stock solutions (5 mM) of all the steroid standards, except 17α-hydroxypregnenolone, were prepared by dissolving in 10 ml of methanol. A stock solution of the less soluble 17α-hydroxypregnenolone was made in 50 ml of methanol (1 mM). Stock solutions of the internal standards (IS) were also prepared individually by dissolving in methanol. The solutions were stored at room temperature, protected from light. For the working standard solutions, 1 ml of each stock solution was transferred into a 50 ml flask that was filled with methanol. This solution was further diluted with 50% (v/v) methanol to give a series of eleven standards that were stored in microcentrifuge tubes at +4 °C. The IS stock solutions were mixed and diluted with 50% (v/v) methanol to a working solution with following IS concentrations: d3-17α-hydroxypregnenolone: 20 nM, d6-dehydroepiandrosterone: 100 nM, d8-17α-hydroxyprogesterone: 25 nM, d7-androstenedione: 10 nM, d3-testosterone: 5 nM, d4-pregnenolone: 2.5 nM, and d9-progesterone: 10 nM. The IS working solution contained 55% (v/v) of methanol and was stored at room temperature.

2.5. Serum samples

After obtaining informed consent from six apparently healthy adult volunteers, separate male and female serum pools were prepared for the assay validation, by mixing equal volumes of sera from three donors and freezing the samples immediately. After the validation, the assay was used for the serum steroid profiling of 137

Table 1
Mass spectrometric conditions.

	Precursor ion	Product ion	Fragmentor voltage (V)	Collision energy (V)
17α-Hydroxypregnenolone	348.2	330.1	80	6
d3-Internal standard	351.2	333.1	80	6
DHEA	304.2	253.1	140	17
d6-Internal standard	310.2	259.1	190	19
17α-Hydroxyprogesterone	361.2	112.0	190	35
d8-Internal standard	369.2	115.0	190	35
Androstenedione	317.2	112.0	150	30
d7-Internal standard	324.2	115.0	150	30
Testosterone	304.2	124.0	190	35
d3-Internal standard	307.2	124.0	190	35
Pregnenolone	332.2	86.0	130	30
d4-Internal standard	336.2	90.0	130	30
Progesterone	345.2	124.0	180	35
d9-Internal standard	354.2	128.0	180	35

patients with endometriosis and 66 healthy control subjects. All the serum samples were initially stored at −20 °C, transported between the participating centers in dry ice, and stored at −85 °C until analysis. The study protocol was approved by the Joint Ethics Committee of Turku University and Turku University Hospital, Turku, Finland. Details of the clinical study has been described earlier [32].

2.6. Sample preparation

Serum samples were thawed unassisted at room temperature, protected from light. An aliquot of 150 μl was transferred into 2 ml screw top vial, spiked with 20 μl of IS working solution and mixed shortly. Subsequently, 1 ml of MTBE was added, the vial was capped and shaken at 2000 rpm for 10 min. After the extraction, the organic layer was transferred into another vial, evaporated to dryness at 40 °C and reconstituted in 50 μl of hydroxylamine solution. The capped vial was then mixed shortly and heated at 60 °C for 30 min, before placing it into instrument autosampler for analysis.

Calibration samples were prepared similarly to the serum samples, only substituting the serum with 4% (w/v) BSA solution. In addition to the IS, 20 μl of working standard solutions were added into the vials before the extraction. The calibration curve consisted of eleven concentration levels: 0.033, 0.075, 0.10, 0.13, 0.33, 0.75, 1.0, 1.3, 6.7, 33, 80 nM, a zero sample (only IS added), and a blank (no standards added). For the quantification, accurate concentrations were calculated for each compound using the weighing results and certificates of analysis. Quality control (QC) samples were prepared similarly to the calibration samples at three concentration levels: 0.13, 1.3, and 33 nM, and incorporated in each assay run according to the FDA guideline [33].

2.7. Assay validation

Assay validation was carried out according to the FDA guideline for bioanalytical method validation [33], with emphasis on the requirements for the analysis of endogenous compounds [34].

Eleven calibration samples were analyzed before the serum samples within each run, in addition to the blank and zero samples. A calibration curve for each analyte was constructed from seven to eleven non-zero samples according to Table 2. The lower limit of quantification (LLOQ) for each analyte was determined by assaying five QC samples independent of the calibration curve, calculating the precision and accuracy, and comparing the analyte response to the zero sample response. Selection of the concentration range and the best curve fit for each analyte was based on the sum of deviations of standards from their nominal concentration: a calibration curve with the smallest sum of deviations was used. For quantification, peak area ratios of the analyte quantifier ion to the internal standard were calculated as a function of the concentration of the analyte.

Precision of the method was determined by analyzing six replicate samples from both male and female serum pools representing different steroid concentrations. This intra-day precision study was repeated on three days. In addition, samples ($n = 5$) from both serum pools were spiked before extraction with a working standard solution representing 6.7 nM sample concentrations and analyzed for precision. Furthermore, precision of the QC samples at three concentration levels was determined as a part of every assay run.

Absolute recovery was calculated by comparing the mean concentrations of the spiked serum samples described above to their nominal concentration using the following equation: $\text{Absolute recovery}(\%) = [(C_{\text{spiked}})/(C_{\text{unspiked}} + C_{\text{spike}})] \times 100$, where C_{spiked} = mean concentration measured for the spiked samples; C_{unspiked} = mean concentration measured for the unspiked samples; C_{spike} = nominal concentration of a spiked blank sample.

Extraction recovery was studied by comparing the described spiked serum samples to similar sets of serum samples that were spiked at the same concentration after the extraction. Peak areas of the pre- and post-extraction spiked samples were used for calculations, with spiking after extraction corresponding to 100% extraction recovery. The extraction efficiency of different solvents was determined by preparing three replicate samples with all the solvents and comparing the obtained analyte peak areas.

Table 2
Calibration curve characteristics and assay performance at the lower limits of quantification (LLOQ).

	Concentration range (nM)	Number of standards	Curve equation	R^2	LLOQ ($n = 5$)	
					RSD(%)	Accuracy(%)
17α-Hydroxypregnenolone	0.13–33	7	$0.0060x^2 + 0.82x + 0.0088$	0.987	16.9	103
DHEA	0.34–82	7	$0.0014x^2 + 0.22x + 0.0005$	0.992	17.4	103
17α-Hydroxyprogesterone	0.10–80	9	$0.0018x^2 + 0.43x - 0.0041$	0.992	10.8	90
Androstenedione	0.099–33	8	$0.0012x^2 + 0.56x - 0.0102$	0.989	19.6	95
Testosterone	0.076–81	10	$0.0060x^2 + 2.44x - 0.0132$	0.991	15.2	106
Pregnenolone	0.032–78	11	$0.0162x^2 + 3.60x + 0.1298$	0.987	19.4	105
Progesterone	0.034–82	11	$0.0044x^2 + 0.83x + 0.0134$	0.990	7.4	98

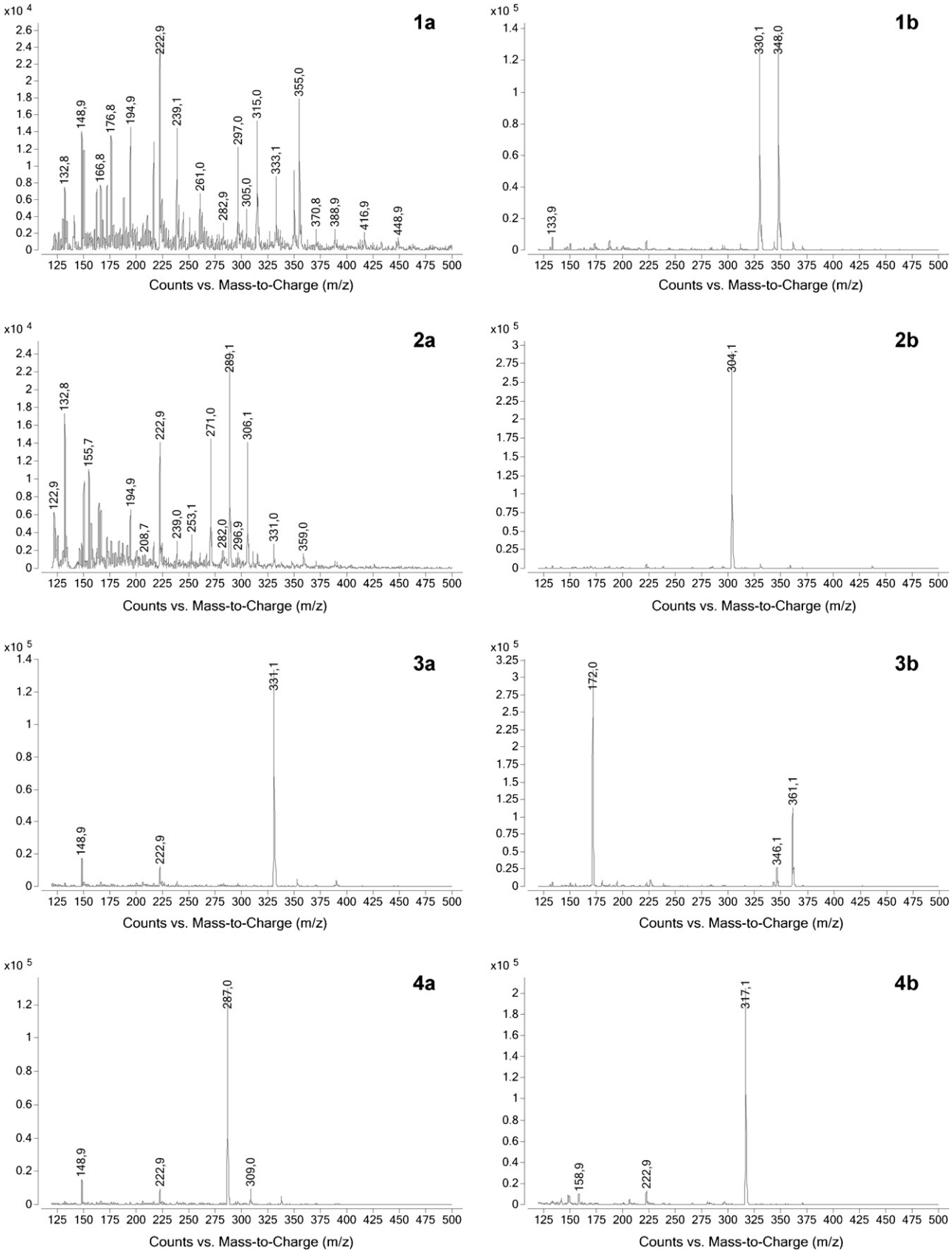

Fig. 1. Background-subtracted full scan spectra of the analytes before and after the oxime derivatization. Analyte spectra are marked (a) before and (b) after the derivatization. 1: 17α-hydroxypregnenolone, 2: DHEA, 3: 17α-hydroxyprogesterone, 4: androstenedione, 5: testosterone, 6: pregnenolone, 7: progesterone. Intensities between the spectra are not comparable.

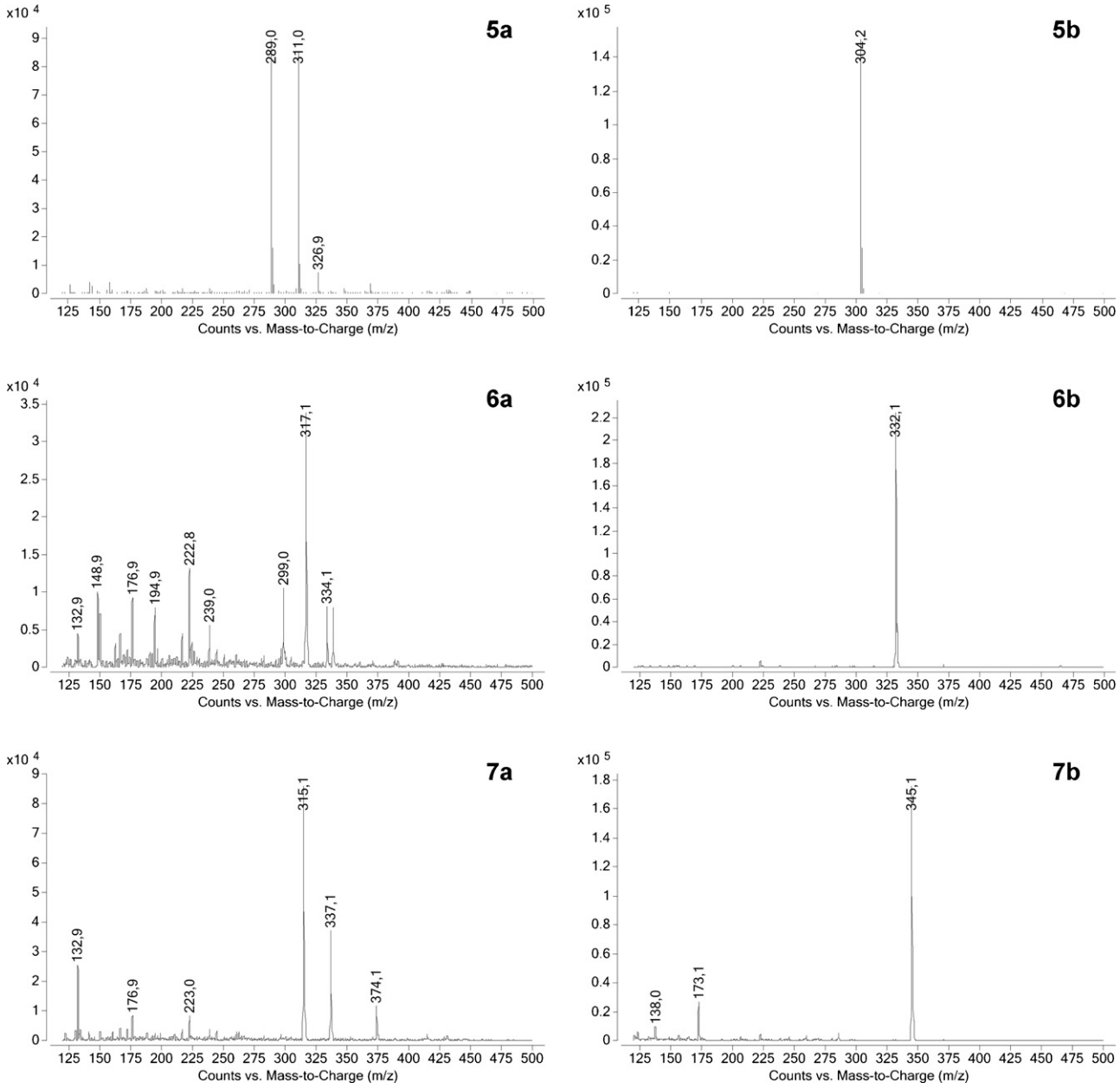

Fig. 1. (*Continued*).

Matrix effect was investigated by performing post-column infusions. The infusion setup consisted of a post-column T-piece and syringe pump as reported elsewhere [35]. Constant flow of derivatized 30 nM solution containing the standard compounds was delivered via T-piece to the mobile phase at a flow rate of 15 μl/min. Changes in the intensity of the baseline of each MRM transition were monitored after the injection of blank and serum samples.

The stability of the analytes was determined by comparing the analyte peak areas from freshly prepared stock and working standard solutions against stored solutions. Storage conditions were as described in the Section 2.4. Stock solutions were diluted, derivatized, and analyzed as six replicate injections. Working standard solutions at four concentration levels were spiked with internal standard working solution, derivatized, and analyzed against fresh calibration standards. Post-preparative sample stability was determined by analyzing two sets of samples from both female and male sera: one set was analyzed directly after the derivatization, while the other was stored overnight at the instrument autosampler. Isotopic stability of the internal standards was determined by storing the IS working solution, diluting to the sample concentration, derivatizing, and monitoring the MRM chromatograms for the corresponding unlabeled analytes. Long-term stability was studied by storing two sets of female serum samples in different conditions. One set of samples was kept in −85 °C while the other was thawed once and placed in a −20 °C freezer. Samples from both storage conditions were analyzed in three replicates after 11-month storage.

Selectivity of the method was examined by analyzing a set of structurally similar steroid metabolites (aldosterone, androstenedione, androsterone, dihydrotestosterone, estradiol, estrone, and etiocholanolone). In addition, to ensure that the possible incomplete derivatization of the analyzed steroids would not affect the method selectivity, they were analyzed in their underivatized forms.

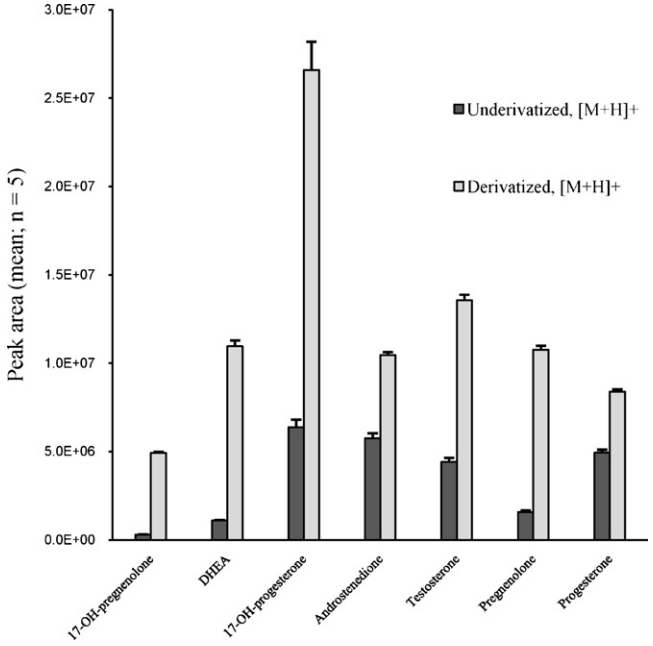

Fig. 2. Intensities of the $[M+H]^+$ ions of the studied compounds before and after oxime derivatization. Flow injection analysis, mean peak areas of five injections with standard deviations. Mobile phase: 70% methanol with 0.025% formic acid, flow rate: 0.2 ml/min, injected amount: 25 pmol (5 μM × 5 μl).

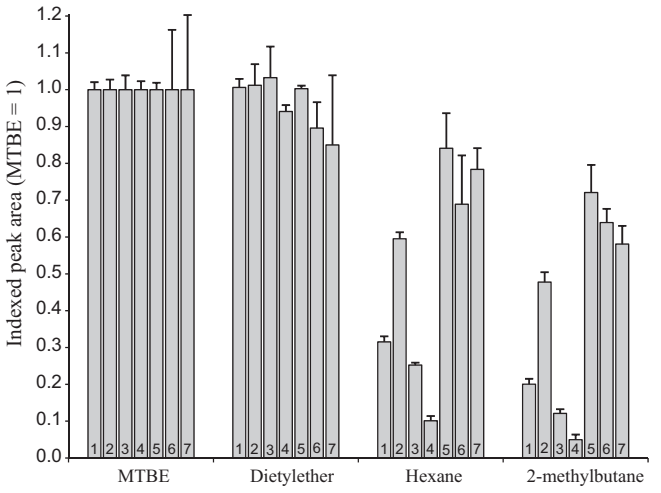

Fig. 3. Extraction recovery of the analytes with different organic solvents. 1: testosterone, 2: DHEA, 3: 17α-OH-pregnenolone, 4: 17α-OH-progesterone, 5: androstenedione, 6: pregnenolone, 7: progesterone. Indexed mean peak areas of three replicate samples with standards deviations.

3. Results and discussion

3.1. Chromatography

During oxime derivatization of ketosteroids, two geometric stereoisomers may be formed, with the hydroxyl group in either *syn*- or *anti*-position. These isomers can be separated chromatographically [36], which is usually undesirable as it decreases the overall response achievable from a single derivatized compound. On the other hand, adequate chromatographic separation of structurally similar, isobaric steroids must be achieved. For these reasons, the method development was focused on achieving the best compromise between isomer resolution, compound separation, and run time.

Several different reversed phase columns were tested, with the best overall result achieved using a short, non-endcapped C18 column with 1.8 μm silica particles. With fully endcapped phases, slightly less tailing was seen, but the unwanted isomer resolution was evident. Selection of the organic solvent and the flow rate were based on the MS response.

Column back-pressure was monitored during the routine use of the method, and varied between 99 and 146 bar (1435 and 2117 psi) during the gradient run. A typical set of around 100 serum samples with calibrators and QC samples did not increase the pressure. The chromatographic separation was tested with three similar columns from different manufacturing lots to ensure that column replacement would not affect the separation. No difference was seen in resolution, retention times, or peak shapes.

3.2. Mass spectrometry

Using the described mobile phase, the ESI mass spectra of the underivatized analytes showed abundant sodium and potassium adducts (Fig. 1). After oxime derivatization, however, the spectra of the analytes were completely devoid of adducts, showing only protonated molecule ions with considerable increase in the MS response when compared to underivatized compounds (Fig. 2).

Loss of water was evident in the spectra of non-derivatized 17α-hydroxypregnenolone, DHEA, and pregnenolone. However, only 17α-hydroxypregnenolone showed loss of water after derivatization (*m/z* 348.2 and 330.1). This reaction could not be avoided, regardless of instrument conditions, and as the MRM transition of 17α-hydroxypregnenolone was based on these ions, the inclusion of isotope-labeled IS was of paramount importance.

The derivatization reactions were monitored by flow injection analyses from the sample vials during their heating at 60 °C. For the compounds having more than one ketone group, complex mass spectra were observed before the derivatization was complete. With the exception of 17α-hydroxypregnenolone, the compounds with one ketone group showed a single $[M+H]^+$ peak after complete derivatization. All compounds capable of forming two oxime groups were seen as doubly or singly charged species. The collisional fragmentation of all the discussed oxime derivatives has been described in detail previously [10].

MRM conditions were optimized for the most intense, selective transitions for each analyte (Table 1). Highest overall signal for all the analytes was observed when 0.025% (v/v) formic acid was added to both mobile phase constituents. Data acquisition rate was set to be uniform in both time segments, resulting in around 30 data points across a typical peak width.

3.3. Sample preparation

Liquid–liquid extraction was experimented using 2-methylbutane, diethyl ether, hexane, and MTBE. MTBE was found to produce the best extraction recovery for most of the compounds, even with single extraction, and with sample-to-solvent volume ratios less than 1:10 (Fig. 3). Extraction was initially performed in polypropylene microcentrifuge tubes [3,20], but it was found that late-eluting, unidentified material was extracted from the plastic. This was seen as a broad, high-intensity chromatographic peak at the MRM transition of pregnenolone, after the injection of two blank extractions. It was possible to adjust the chromatography to retain selectivity, but later experiments with post-column infusions revealed a negative, ion-suppressing peak at the retention time of 17α-hydroxyprogesterone. When the extraction was performed in glass tubes, both issues were avoided.

Due to the small sample volumes, extraction in 2 ml autosampler vials instead of the glass tubes was experimented. The use of vials

Table 3
Precision of the method.

	Measured concentration (nM)[a]			Precision (RSD%)	
	Day 1	Day 2	Day 3	Intra-day	Inter-day[b]
Female serum					
17α-Hydroxypregnenolone	4.78 ± 0.36	4.47 ± 0.26	4.65 ± 0.06	1.3	6.6
DHEA	17.37 ± 0.86	17.43 ± 0.95	18.10 ± 1.07	5.5	5.2
17α-Hydroxyprogesterone	0.89 ± 0.05	0.87 ± 0.05	0.85 ± 0.06	6.5	5.7
Androstenedione	3.16 ± 0.18	3.12 ± 0.09	3.22 ± 0.07	1.9	3.6
Testosterone	0.68 ± 0.06	0.71 ± 0.06	0.66 ± 0.03	4.5	7.4
Pregnenolone	3.09 ± 0.18	2.95 ± 0.11	2.89 ± 0.10	3.1	4.9
Progesterone	2.39 ± 0.09	2.30 ± 0.08	2.35 ± 0.06	2.3	3.4
Male serum					
17α-Hydroxypregnenolone	6.69 ± 0.34	6.21 ± 0.24	6.70 ± 0.28	3.9	6.0
DHEA	21.17 ± 0.76	19.97 ± 1.00	21.75 ± 0.58	2.5	5.0
17α-Hydroxyprogesterone	2.41 ± 0.12	2.28 ± 0.12	2.34 ± 0.04	1.8	4.5
Androstenedione	2.63 ± 0.11	2.54 ± 0.15	2.72 ± 0.11	3.7	5.0
Testosterone	21.96 ± 1.10	21.95 ± 1.12	22.75 ± 0.72	3.0	4.3
Pregnenolone	4.06 ± 0.12	4.14 ± 0.13	4.08 ± 0.10	2.3	2.6
Progesterone	0.15 ± 0.01	0.15 ± 0.01	0.16 ± 0.01	2.4	5.0

[a] Mean values ±95% confidence intervals ($n = 6$).
[b] Values obtained from all runs on three separate days ($n = 18$).

resulted in efficient extraction and convenient handling of the samples using vial trays. Centrifugation of the vials and snap freezing of the aqueous layer were experimented, but proved unnecessary, as the phase separation was clear and the organic layer was easy to remove.

To minimize the amount of sample preparation steps, the organic layer was transferred from the extraction vial into another, cone bottomed glass vial that could be used for all subsequent sample preparation steps. After the evaporation residue was redissolved in the derivatization solution, pressure-tight crimp caps enabled the use of heat for derivatization without drying the sample. After the derivatization, samples could be injected directly from the vials without opening the caps.

3.4. Calibration curve and sensitivity

The calibration curve characteristics are shown in Table 2. The best curve fit was achieved for all compounds using a weighted ($1/x^2$) regression. Deviation of the calibration standards from their nominal concentrations at the LLOQ was always less than 20% and less than 15% at all other concentration levels. The LLOQ was defined as the lowest concentration analyzed with a precision less than 20% and accuracy of 80–120%. In addition, the response from a zero sample had to be less than 20% of the LLOQ sample response.

3.5. Precision

The method had good precision for serum sample analysis as shown in Table 3. Spiked serum samples (as in Section 2.7) showed precision values below 6.5% (RSD). The QC samples at three concentration levels, analyzed within every assay run, where within the acceptance limits of the FDA guideline [33].

3.6. Matrix effect

Post-column infusion experiments showed no clear ion suppression zones at the retention times of the analytes after the polypropylene tubes were changed to glass vials. The issues encountered during the method development suggest that if LLE is performed in test tubes or well plates made of plastic, matrix effects from the possible extractables have to be taken into consideration.

3.7. Recovery

Absolute recovery and extraction recovery are shown in Table 4. Absolute recovery was determined to evaluate the measurement accuracy of the analytes added to the female and male serum in known quantities. Although not as accurate as the traditional method of spiking true blank samples with the compounds of interest [33], it was used as an estimate of the method accuracy for the endogenous steroids, as true blank matrix is not available [34].

3.8. Stability

Stock solutions of all compounds were stable for at least six months. Stored solutions had concentration-to-area ratios within 96–102% of the new stock solutions. Working standard solutions were stable for at least four months, with mean concentration of the stored solutions within 90–110% of the freshly prepared ones. Derivatized serum samples were stable in the instrument autosampler for at least 18 h, having analyte concentrations within 90–105% of the samples that were analyzed directly after their derivatization. The IS working solution was stable for at least one month, showing no response at the MRM transitions of the assayed steroids. The samples that were thawed and stored at −20 °C had 92–100% of the analyte concentrations of the samples that were stored uninterrupted at −85 °C, with a precision (RSD) below 6.7%.

3.9. Selectivity

All co-eluting analytes (Fig. 4) had fully selective MRM transitions. It is remarkable that in their underivatized forms,

Table 4
Absolute recovery and extraction recovery.

	Absolute recovery[a] (%)		Extraction recovery[a] (%)	
	Female	Male	Female	Male
17α-hydroxypregnenolone	113	107	84	79
DHEA	105	103	89	84
17α-hydroxyprogesterone	107	103	84	82
Androstenedione	110	104	85	82
Testosterone	114	106	83	88
Pregnenolone	110	106	85	90
Progesterone	110	107	89	82

[a] Mean values, $n = 5$.

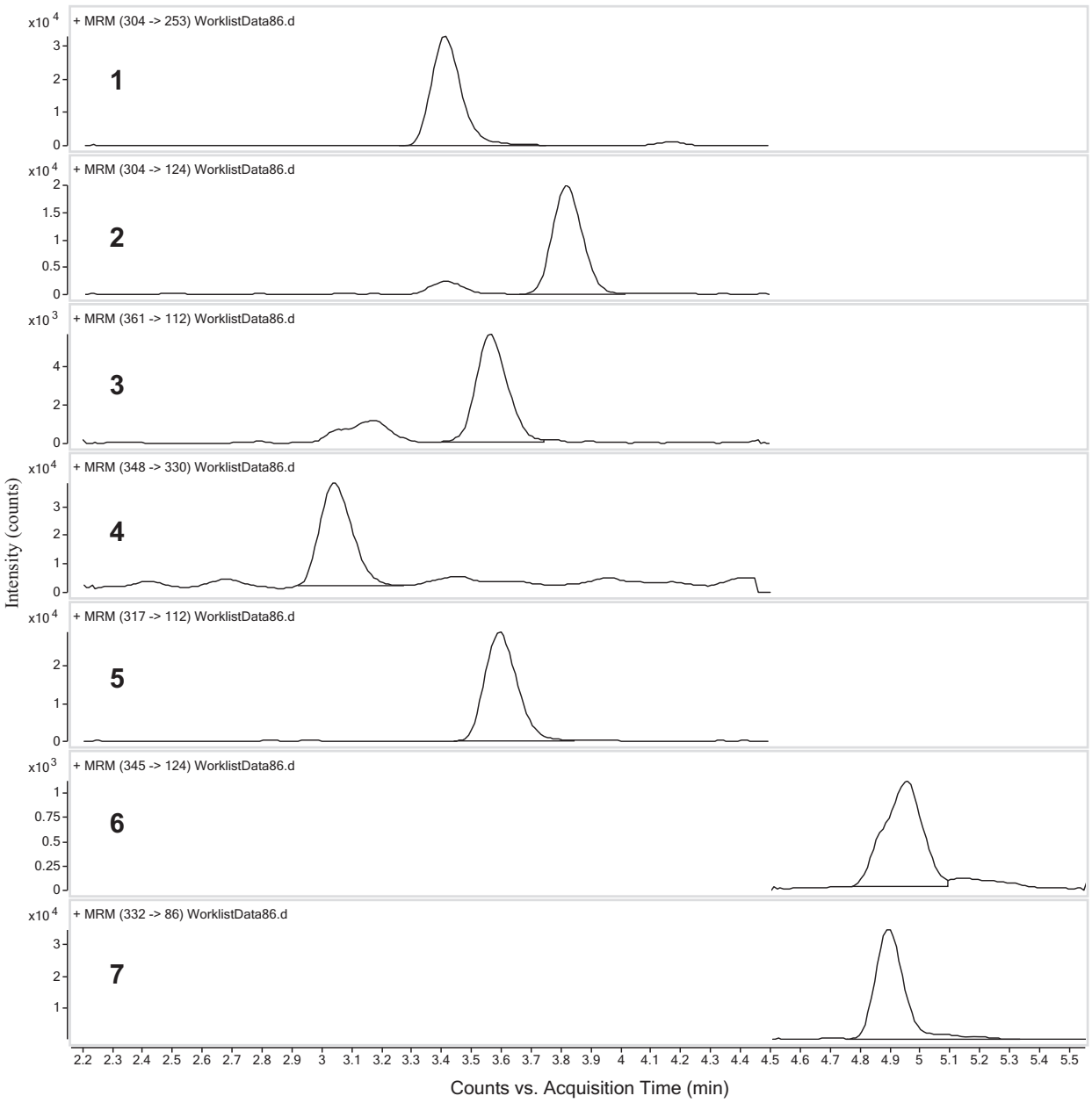

Fig. 4. Representative MRM chromatograms of an endometriosis patient serum sample. Measured concentrations in parentheses. 1: DHEA (20 nM), 2: testosterone (1.0 nM), 3: 17α-hydroxyprogesterone (1.1 nM), 4: 17α-hydroxypregnenolone (1.9 nM), 5: androstenedione (2.9 nM), 6: progesterone (0.15 nM), 7: pregnenolone (4.0 nM).

mass spectrometric selectivity could not be achieved for 17α-hydroxyprogesterone, DHEA, and testosterone, nor could they be separated by the described chromatography. When derivatized, however, the peaks were separated. No effect on method selectivity was found to arise from the presence of underivatized analytes or structurally close steroid metabolites. There was no detectable signal at the MRM transitions of the assayed compounds when a derivatized zero sample was analyzed. Analysis of more than three hundred clinical samples has showed no selectivity-affecting chromatographic artifacts.

3.10. Application

The presented method was used for serum ketosteroid profiling of endometriosis patients and healthy controls. The analyte concentrations were within the range of the calibration curves, as shown in Table 5. Chromatograms representing an endometriosis patient serum sample are shown in Fig. 4. The quality control samples that were analyzed together with the patient samples were within the limits according set in the FDA guideline [33]. No change in the column selectivity or separation efficiency were seen. The assay throughput was approximately 90–100 serum samples per day.

Table 5
Serum sample concentrations of the endometriosis study subjects ($n = 217$).

	Mean (nM)	SD	Range (nM)
17α-Hydroxypregnenolone	3.58	3.87	0.10–17.32
DHEA	20.98	12.77	2.70–85.11
17α-Hydroxyprogesterone	2.32	2.18	0.12–11.21
Androstenedione	4.23	2.12	0.81–10.56
Testosterone	1.04	0.59	0.24–5.76
Pregnenolone	5.88	3.85	0.70–33.13
Progesterone	7.29	13.76	0.04–72.38

4. Conclusions

The presented method describes a simple, sensitive, and fast assay for seven steroid metabolites in human serum. Unlike methods employing column-switching techniques [24,27,30], the chromatographic separation was achieved using a robust setup of single column and HPLC pump and the ionization was carried out with a conventional ESI source. Contrary to the common practice involving several sample preparation steps, the entire process of extraction, derivatization, and injection was accomplished by using only two widely available autosampler vials. By employing oxime derivatization of the analytes, significant increase in sensitivity and selectivity were obtained. Even if derivatization is usually avoided with LC–MS, in the presented work it does not add unnecessary complexity to the method, as heating of the autosampler vials shortly before the sample injection was the only additional step needed. The straightforward sample preparation, together with a 7-min analysis time enabled the measurement of large amounts of serum samples in a single assay run. To the authors' knowledge, this is the first published method to include all the main ketosteroid metabolites of the progestagen and androgen groups in a single, validated, sub 8-min quantitative assay that has adequate sensitivity for the analysis of both female and male samples.

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