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Simultaneous analysis by LC-MS/MS of 22 ketosteroids with hydroxylamine derivatization and underivatized estradiol from human plasma, serum and prostate tissue

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Highlights

- Validated LC-MS/MS method for quantifying 23 steroids
- 150 µl of plasma, serum or prostatic tissue homogenate
- LLOQs ranged from 0.9 to 91 pg/ml, and from 0.009 to 0.9 pg/mg tissue
- Androgens: A4, DHA4, DHEA, DHT, 11aOHA4, 11bOHA4, 11OHT, 11KA4, 11KDHT, 11KT, T

- Other steroids: A, B, E, E1, E2, F, S, 17OHP5, 17OHP4, 21OHP4, P4, P5

Abstract

This study describes a validated LC-MS/MS method for assaying 23 steroids within a single run from 150 µl of human plasma, serum or prostatic tissue homogenate. Isotope-labeled steroids were used as internal standards. Samples were extracted with toluene, and ketosteroids were derivatized with hydroxylamine prior to LC-MS/MS analysis. The steroids were separated on a C18 column and methanol was used as an organic solvent with the addition of 0.2 mM ammonium fluoride to improve underivatized estradiol (E2) ionization. Certified reference serums as well as plasma samples, and homogenates of prostate tissue were utilized in the method validation. The specificity of the method was inspected with a total of 27 steroids. The validation proved that the method was suitable for the quantitative analysis of a wide panel of androgens (testosterone, T (3.3 pM-13 nM); androstenedione, A4 (3.3 pM-13 nM); 5α-androstanedione, DHA4 (13 pM-13 nM); dehydroepiandrosterone, DHEA (67 pM-133 nM); dihydrotestosterone, DHT (33 pM-33 nM); 11-ketodihydrotestosterone, 11KDHT (13 pM-13nM); 11-ketotestosterone, 11KT (33 pM-6.7 nM); 11β-hydroxyandrostenedione, 11bOHA4 (33 pM-13 nM); 11β-hydroxytestosterone, 11OHT (13 pM-33 nM)), as well as estrogens (estrone, E1 (3.3 pM-13 nM)), progestagens (17α-hydroxypregnenolone, 17OHP5 (32 pM-127 nM); 17α-hydroxyprogesterone, 17OHP4 (67 pM-133 nM); progesterone, P4 (3.3 pM-13 nM); pregnenolone, P5 (6.6 pM-13 nM)), and glucocorticoids (cortisol, F (33 pM-134 nM); cortisone E (66 pM-131 nM); corticosterone, B (33 pM-67 nM); 11-deoxycortisol, S (33 pM-66 nM); 21-hydroxyprogesterone, 21OHP4 (32 pM-13 nM)). Furthermore, E2 (335 pM-134 nM) and 11α-hydroxyandrostenedione, 11aOHA4 (33 pM-33 nM) could be analyzed if the concentration in the sample was high enough. In addition, aldosterone, A (128 pM-64 nM) and 11-ketoandrostenedione, 11KA4 (33 pM-13 nM) could be analyzed semiquantitatively. The limits of quantification for all compounds ranged from 0.9 to 91 pg/ml, and from 0.009 to 0.9 pg/mg tissue. Compared to our previous method, this new method also permits the analysis of the more challenging steroids, like DHT, DHEA and P5, and a panel of 11-ketosteroids.

Abbreviations: A, aldosterone; A4, androstenedione; ACN, acetonitrile; B, corticosterone; CE, collision energy; CID, collision induced dissociation; E, cortisone; E1, estrone; E2, estradiol; ESI, electrospray ionization; F, cortisol; DHA4, 5α-androstanedione (or dihydroandrostenedione); DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; IS, internal standard; 11KA4, 11-ketoandrostenedione;

11KDHT, 11-ketodihydrotestosterone; 11KT, 11-ketotestosterone; LLE, liquid-liquid extraction; LLOQ, lower limit of quantification; ME, matrix effect; MRM, multiple reaction monitoring; 11aOHA4, 11α-hydroxyandrostenedione; 11bOHA4, 11β-hydroxyandrostenedione; 17OHP4, 17α-hydroxyprogesterone; 21OHP4, 21-hydroxyprogesterone; 17OHP5, 17α-hydroxypregnenolone; 11OHT, 11β-hydroxytestosterone; P4, progesterone; P5, pregnenolone; QC, quality control; QL, qualifier ion transition; QT, quantifier ion transition; RSD, relative standard deviation; S, 11-deoxycortisol; T, testosterone; tR, retention time; ULOQ, upper limit of quantification

Keywords: Steroids; Androgens; Tissue; Prostate; Serum; LC-MS;

1. Introduction

Steroids are important regulators of various central physiological processes and they are involved in the pathogenesis of many diseases and genetic disorders. In the context of prostate cancer, androgens are important, as they are required for both the initiation and progression of the disease. However, there does not seem to be any clear association between the serum level of the most common androgen, testosterone (T) and the prostate cancer risk [1]. It is currently assumed that very low androgen concentrations are thought to promote the development of prostate cancer [2]. Most of the currently available studies on this topic have examined the association between serum androgen concentrations and prostate cancer, while there are very few studies which have examined the role of androgen concentrations in the prostate tissue as a risk factor for prostate cancer. Prostatic cells express higher levels of androgen-metabolizing enzymes, such as 5α-reductase, as compared to other tissues with the expression being further upregulated in prostate hyperplasia [3]. Therefore, the local androgen milieu in the prostate differs from that in the serum. Methods for reliable measurement of androgens from both matrixes (serum and tissue) are needed to clarify the role of androgens in prostate hyperplasia and carcinogenesis.

Typically, steroid concentrations in body fluids and tissues are very low, from pM to nM quantities, and thus, they are analyzed by using sensitive methods like liquid chromatography (LC) or gas chromatography (GC) connected to mass spectrometry (MS), or by using immunoassays. Immunometric methods are rapid and simple but are susceptible to analytical interferences owing to the cross-reactivity with a variety of endogenous substances, and with other steroids with related structures [4]. Chromatography connected to mass spectrometry-based methods are more specific and enable the simultaneous analysis of several

analytes across a wide concentration range from a single run and with a small sample volume [5]. However, the majority of the published LC-MS methods have been developed for the analysis of steroids from serum samples. These include also very sensitive methods; some of these tend to have focused only on a few steroids [6,7], while others have examined up to dozens of steroids, but with somewhat greater lower limits of quantification (LLOQ) [8-11]. There are much fewer methods intended to analyze steroid profiles from different tissues, such as the prostate [12-16]. GC-MS may provide excellent sensitivity also for tissue samples [17], but the need for more laborious sample preparation and larger sample volumes limits its use.

Although MS-based measurements may be performed without derivatization, different derivatization strategies can be used to improve the detection properties of steroids, which are usually poorly ionized or provide only low-intensity fragment ions during collision-induced dissociation (CID) [18-21]. Thus, derivatization may be beneficial for the specificity and sensitivity of the method. However, the formation of several isomers is possible during the derivatization process, the rate of reaction may vary, and the presence of side products may hamper the analysis [18,19]. Hydroxylamine is one of the derivatization reagents utilized in steroid analysis; this compound can be used to form oximes from ketosteroids without the need to separate or purify the derivatized or underivatized steroids from the reaction mixture before the LC-MS analysis [4,22,23].

In this study, an LC-MS/MS method was developed for the simultaneous quantitative analysis of 23 steroids in human plasma, serum and prostatic tissue homogenate. The following compounds were included: Androgens (testosterone, T; androstenedione, A4; 5α-androstanedione, DHA4; dehydroepiandrosterone, DHEA; dihydrotestosterone, DHT; 11-ketoandrostenedione, 11KA4; 11-ketodihydrotestosterone, 11KDHT; 11-ketotestosterone, 11KT; 11α-hydroxyandrostenedione, 11aOHA4; 11β-hydroxyandrostenedione, 11bOHA4; 11β-hydroxytestosterone, 11OHT), estrogens (estradiol, E2; estrone, E1), progestagens (17α-hydroxypregnenolone, 17OHP5; 17α-hydroxyprogesterone, 17OHP4; progesterone, P4; pregnenolone, P5), glucocorticoids (cortisol, F; cortisone E; corticosterone, B; 11-deoxycortisol, S; 21-hydroxyprogesterone, 21OHP4), and a mineralocorticoid (aldosterone, A). Analytical conditions were selected to favor DHT measurements. We have recently reported a method allowing the simultaneous analysis of 14 steroids [24], where LC-MS parameters were tuned so that the E2 and E1 measurements were as sensitive and reliable as possible (LLOQ 6.7 pM for E2 and 1.3 pM for E1). However, that method was not suitable for DHT and P5 measurements, and the sensitivity for DHEA was low. DHT, P5 and other ketosteroids can be derivatized

using hydroxylamine, as it forms oximes in a selective reaction with aldehydes and ketones. Oxime formation increases the sensitivity of oxosteroids in electrospray ionization (ESI) [22,23]. In addition to the steroids measurable in our previous study, we were now able to detect also 11-oxygenated steroids [8,25,26], which are believed to be biologically active androgens in many clinical conditions, such as in prostate cancer [12], 21-hydroxylase deficiency [9] and polycystic ovary syndrome [27].

2. Material and methods

2.1. Reagents

Materials for chromatography and sample preparation were obtained as follows: Methanol (LC-MS Ultra chromasolv, tested for UHPLC-MS, ≥ 99.9 %) from Riedel-de Haën, acetonitrile (ACN, LC-MS grade, min 99.9 %) from BDH Prolabo Chemicals, VWR, ammonium fluoride (NH_4F, eluent additive for LC-MS, ≥98.0 %) from Fluka, toluene (Chromasolv plus for HPLC, ≥ 99.9 %) and hydroxylamine hydrochloride (NH_2OH, ReagentPlus, 99%) from Sigma-Aldrich and NaCl solution (9 mg/ml) from Braun Medical Oy, Finland. Sources for steroid standards were: A, E, E1, E2, DHEA, 11KA4, 17OHP4, 21OHP4, 17OHP5, and P5 from Sigma, A4 from Riedel-de Haën, B, F, DHA4, DHT, 11KDHT, 11KT, 11aOHA4, 11bOHA4, 11OHT and P4 from Steraloids, S from Toronto Research Chemicals, and T from Fluka. The specificity of the developed method was also tested with the following steroids: Androsterone, estriol, and etiocholanolone, from Sigma, and 5-androstenediol from Steraloids.

Isotope-labeled steroids were obtained from the following sources: A-d8, A4-13C3, DHEA-13C3, DHT-13C3, E-13C3, E1-13C3, E2-13C3, F-13C3, 21OHP4-13C3, 17OHP5-d2-13C2, P4-13C3, P5-d2-13C2, S-13C3, T-13C3 from IsoSciences, B-d8 from Toronto Research Chemicals and 11KT-d3, 11bOHA4-d4, 17OHP4-13C3 and 11OHT-d4 from Larodan AB, Sweden. The reference serum samples for F (ERM-DA192), E2 (BCR-577) and P4 (ERM-DA347) were from Sigma-Aldrich, and for T (ERM-DA346) from LGC standards. The reference serums for F, E2 and P4 were lyophilized material, which were diluted with water according to the manufacturer's instructions.

2.2. Samples for method validation

Human plasma was purchased from the Red Cross (FFP8), Helsinki, Finland. Tissue samples were collected from men with histologically verified prostate cancer and scheduled for radical prostatectomy as the primary management. The men were sampled from among the participants of a randomized clinical trial comparing whether intervention with atorvastatin would affect the proliferation activity of prostate cancer cells or the serum level of prostate-specific antigen (PSA) as compared to placebo [28]. Tissue samples were collected from a macroscopically non-cancerous area of the prostate immediately after its surgical removal from the body and were stored in liquid nitrogen. The frozen samples were ground in liquid nitrogen using a mortar and pestle. Then 9 mg/mL NaCl (100 mg ground tissue/1 mL saline) was added to the ground tissue sample. The samples were then carefully mixed and stored at -80 °C until used. Pooled samples of 14 subjects containing 9 placebo and 5 atorvastatin treated patients were used in all tissue containing studies.

2.3. LC-MS/MS conditions

The LC-MS/MS measurements were performed with an Agilent 1290 Rapid Resolution LC System connected to a model 6495 triple stage quadrupole MS (Agilent, San Jose, CA, USA). The data were acquired and analyzed using Agilent MassHunter Workstation software. LC was performed using Acquity UPLC CSH C18 column, 100 x 2.1 mm, 1.7 µm (Waters). Mobile phase solvents were 0.2 mM NH_4F in water (eluent A) and 0.2 mM NH_4F in methanol:water 95:5 (v/v) (eluent B) at a flow rate of 0.3 ml/min. The following gradient program was used: 0-3 min 57 % B; 3-7 min 57 → 63 % B; 7-12 min 63 → 95 % B; 12-12.5 min 95 → 100 % B; 12.5-14 min 100 % B; 14-14.1 min 100 → 57 % B; 14.1-16 min 57 % B. The column temperature was held at 40 °C, with the autosampler temperature held at 10 °C. The injection volume was 20 µl. The injection was performed using a 5 s needle wash with ACN in water 1:1 (v/v). Other tested columns were Kinetex biphenyl, 100 x 2.1 mm, 1.7µm (Phenomenex), and Acquity UPLC CSH Phenyl-Hexyl, 100 x 2.1 mm, 1.7µm (Waters).

The following MS conditions were used in the jet stream electrospray ion source: Sheath gas flow rate 12 l/min at 400 °C (nitrogen), drying gas flow rate 18 l/min at 230 °C (nitrogen), nebulizer gas pressure adjusted to 23 psi, capillary voltage 2500 V (ESI+) and 3000 V (ESI-), and nozzle voltage 0 V (ESI+) and 1500 V (ESI-). The ion funnel RF voltages were set as follows: 110 V (ESI+) and 210 V (ESI-) for the high-pressure ion funnel, and 100 V (ESI+) and 140 V (ESI-) for the low-pressure ion funnel. A divert valve was used to direct the LC flow to waste from start of the gradient to 1.7 min, and again after 12 min. Four time segments were used in the analyses with the turning points being at 5.6, 7.7 and 10.6 min. The measurement was

conducted with multiple reaction monitoring (MRM), and the resolution for the quadrupoles was set to 0.7 mass units, CID was made by nitrogen and the cell accelerator voltage was optimized to 2-7 V for each transition (Table 1). Stable isotope-labeled steroids were used as internal standards (IS). MS parameters including collision energies (CE), a list of precursor ions and fragments for the labeled and unlabeled steroids are shown in Table 1. The MRM calibration curves were created by plotting the peak area ratios of the analyte and IS against the concentration of the analytes. Development and validation of the method were based on the FDA guidelines [29], with further guidance for the measurement of endogenous analytes [30-32]. Calibration samples, including a blank and a zero sample, were run at the beginning and at the end of the analytical batch (two replicates for each level), and quality controls were run between the patient samples. System suitability was tested by injecting diluted IS before every batch to control the proper performance of the instrument. The LLOQ was determined as defined by the FDA guideline [29]. The accuracy had to be between 80-120 %, with the precision better than 20 %, and the area of the peak at LLOQ at least five times larger than that of the blank or zero (injection containing IS). Selectivity was controlled by following the (quantifier ion, QT)/(qualifier ion, QL) ratio in the standards and real samples.

2.4. Standard solutions

All steroids were dissolved in ACN to make stock solutions (0.2-5 mM) [24]. The stock solutions were mixed and diluted with 30 % ACN in water (v/v) to obtain a working solution, which contained all the analytes at the 5 µM level. The 5 µM working standard was used to make a set of working solutions in 30 % ACN at the following concentration levels; 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 50, 100, 250, 500 and 1000 nM. When making the IS solutions, the labeled steroid analogues were first dissolved in ACN, combined, and further diluted using 30 % ACN. The final concentration in the IS solution was 4 nM for A4-13C3 and T-13C3, 20 nM for 17OHP4-13C3 and 21OHP4-13C3, and 10 nM for the other labeled steroids.

2.5. Sample preparation for LC-MS/MS determination

Liquid-liquid extraction (LLE) was performed in 2 ml borosilicate glass autosampler vials as described previously [24], followed by derivatization with hydroxylamine. The IS working solution (20 µl) was pipetted to a 150 µl sample of plasma, serum or prostatic tissue homogenate followed by extraction with toluene (1 ml) for ten minutes. The upper layer was pipetted carefully into a conical autosampler vial, dried using stream of nitrogen, and redissolved in 50 µl of 30 % ACN containing 100 mM hydroxylamine hydrochloride. The vials were then heated at 60 °C for 30 min, and samples were then analyzed without further purification. The

calibration curve and quality control (QC) samples were prepared using 9 mg/ml NaCl as matrix. After addition of IS working solution (20 µl) and the standard working solution (20 µl), the standard and QC sample tubes were extracted and derivatized as described above.

2.6. Validation of the method

The precision of the measurement was tested by analyzing replicate samples (n = 4 - 6) of plasma or tissue homogenates on three separate runs. The accuracy of the T, P4, F and E2 measurements was studied by running reference serum samples. The following certified serum concentrations were applied: ERM-DA346 (890 ± 120 pM) for T, ERM-DA347 (10.13 ± 0.21 nM) for P4, ERM-DA192 (273 ± 6nM) for F and BCR-577 (690 ± 40 pM) for E2. The reference samples were analyzed also after dilution with NaCl solution (dilutions 1:3 and 1:10 for T, P4 and F, and 1:3 for E2). The number of replicates was four to five. In cases, when reference samples were not commercially available, the reliability of quantification was studied by spiking plasma, diluted plasma or tissue homogenate with known amounts of steroids before liquid-liquid extraction (LLE) with toluene. The plasma samples were enriched with steroids using four different concentrations, the diluted plasma (1:10) was spiked using one concentration, and the tissue homogenates were studied using three different steroid concentrations (5 - 6 replicates at each level). The spiked samples were measured, and for the calculation of the absolute recoveries, the following equation was used: (mean final concentration - mean initial concentration) / added concentration x 100 %. For the analytes with an endogenous concentration << LLOQ, the following equation was used to calculate absolute recovery (mean final concentration) / (added concentration) x 100 %.

To study the short term stability of steroids in tissue homogenates, thawed homogenates were kept for 60 min at 24 °C. Six replicate samples were analyzed for their steroid content and the mean concentrations were compared with the results obtained from the samples analyzed without delay. Post-preparative sample stability (autosampler stability) was determined by analyzing two sets of plasma samples and calibrators. One set was analyzed directly after the derivatization, while the other was stored for two days in the instrument autosampler. Post-preparative sample stability for tissue homogenate samples was determined by analyzing samples directly after derivatization and by reinjecting the same samples after storage for two days in the instrument autosampler.

The recovery was studied by adding IS either before or after extraction. The first set was prepared normally by adding IS into the samples before extraction with toluene, and the second set was prepared by adding 20 µl of 30 % ACN instead of the IS solution, followed by extraction with toluene, and the addition of the IS solution just before evaporation of the solvent (n=6). The following equation was used to calculate the extraction recovery: (the mean IS peak area of the pre-extraction IS spiked samples) / (mean IS peak area of the post-extraction spiked samples) x 100 %. The efficiency of tissue sample extraction was determined by increasing the extraction time from 10 min to 30 min, and also by two consecutive toluene extractions of the same homogenate, and analysis of samples from the two extraction rounds.

The matrix effect (ME) was evaluated by comparing the mean IS peak area in post-extraction spiked samples to the mean IS peak area in solutions prepared using pure standard steroids. The impact of carry-over on the accuracy was examined by first injecting the highest standard sample, and then injecting the three consecutive solvent samples (30 % ACN) in three runs. The steroid peak areas measured for the solvent injection were compared with those obtained from the highest calibrator standard. To study the selectivity of the method, solutions of common steroids with the potential for interference were added to the samples. The interference of other possible impurities was also constantly evaluated by following the QT/QL ion ratios for the steroids and IS in real samples, and they were compared with QT/QL ion ratios obtained from the calibrators and QC samples in the same batch. The results were considered acceptable if the difference for QT/QL ion ratios in samples and calibrants was below 30%. In addition, the purity of LC eluents, extraction solvents, steroids and other reagents, as well as the similarity of columns from different production batches was always verified when some parameter was changed, to ensure they were not introducing any artefacts.

3. Results and discussion

3.1. Sample preparation and LC-MS/MS method

In this method, we prioritized the quantification of DHT i.e. for optimal selectivity and sensitivity, and utilized the chromatographic and mass spectrometric parameters most favorable for this analyte. However, whenever possible, the instrumental conditions were optimized also for other steroids. Standards were prepared in NaCl solution, and toluene was used as an eluent for LLE, as this had been proven to work also in our previously described method [24]. Derivatization with hydroxylamine was used to improve the

ionization efficiency of DHT and other poorly ionizable ketosteroids, which do not have a conjugated double bond next to a carbonyl group, including DHEA and P5 [22,23]. The addition of ammonium fluoride (NH_4F) into the eluent has been shown to enhance the negative ion electrospray performance [33-35], and typically formic acid has been used as an eluent additive in positive mode measurements. However, when 0.025 % formic acid and 0.2 mM NH_4F were compared as eluent additives with optimized ionization conditions for both solvent systems, NH_4F provided a higher signal than formic acid. One possible explanation is that the higher amount of formic acid used compared to NH_4F contained more impurities, which may have suppressed the signal. As our previously described method [24] for the analysis of a different panel of steroids without derivatization utilized NH_4F as an eluent additive, we decided to use the same eluents in this current method. This also enabled the analysis of E2 together with the ketosteroids included in this method, if the sensitivity was not an issue. The reason for the lower sensitivity for E2 in this method (LLOQ 335 pM) compared to our previous method (LLOQ 6.7 pM) is unclear. The injection volume in the present study was half of that previous value. The sensitivity was not improved even if pure E2 was injected into the column (no added hydroxylamine in the sample or LLE with toluene). Thus, one reason for this phenomenon might be the presence of some impurity in the LC solvents, causing ion suppression for both E2 and E2-13C3.

LC-MS/MS analysis of steroids was performed by using MRM. The MRM transitions of 23 steroids analyzed with the method are presented in Fig. 1A-B. The C18 column achieved the best separation of DHT and isobaric compounds. The selection of the isomer used in the quantification was based on either achieving the optimal sensitivity or specificity. For example, for the E-13C3 derivative (m/z 379), the peak at the retention time 2.9 min overlapped with F (m/z 378), which gave a small response also in the MRM channel of the E-13C3 derivative. However, the peak for the second isomer of the E-13C3 derivative at retention time 3.33 min was separated from the second isomer of the F derivative at 3.56 min. Thus, the peak at a retention time of 3.33 min was used to quantify E.

Biphenyl and phenyl-hexyl columns were also tested during the method development. Examples of the chromatograms with different columns are shown in Fig. 2. The biphenyl column (Fig. 2A1-A7) resulted in a better separation of 11-keto and 11-hydroxy steroids from each other (Fig. 2A1-A3). The same was true for the separation of the isobaric compounds, androsterone and etiocholanolone (Fig. 2A6), and isobaric 21OHP4 and 17OHP4 (Fig. 2A4 and A5) from each other. Fig. 2A4 shows the bis-oxime derivatives of 21OHP4 and 17OHP4 (meaning that both carbonyls in the molecule have reacted with hydroxylamine). Fig.

2A5 illustrates the mono-oxime derivatives of 17OHP4 and 21OHP4 (only one of the carbonyls in the molecule has reacted with hydroxylamine). However, with the biphenyl column, DHT was not separated well from T or from androsterone, both of which produced a response also in the MRM channel of DHT (Fig. 2A6 and A7). The phenyl-hexyl column (Fig. 2B1-B7) was the best choice for separating isobaric androsterone and etiocholanolone from each other, as well as from DHT and T (Fig. 2B6 and B7). However, 11KDHT and 11OHT overlapped (Fig. 2B1 and B2). The C18 column (Fig. 2C1-C7) separated DHT well from T and from androsterone and etiocholanolone (Fig. 2C6 and C7), as well as differentiating between 11KDHT and 11OHT (Fig. 2C1 and C2). However, androsterone and etiocholanolone overlapped (Fig. 2C6), thus these two isobaric steroids could not be included in the method. In addition, 21OHP4 and 17OHP4 seemed to overlap with the C18 column, when following only the bis-oxime derivatives (Fig. 2C4). However, the reaction of the second carbonyl at position 20 was slower in comparison to the reaction in the carbonyl at the position 3 [22]. Unlike the bis-oxime derivatives, the mono-oxime derivatives of 21OHP4 and 17OHP4 could be separated from each other in the C18 column (Fig. 2C5). Thus, also these mono-oxime derivatives could be used in quantifying 17OHP4 and 21OHP4, instead of the bis-oxime derivatives. However, the progress of the reaction from mono-oxime forward to bis-oxime must be taken into account. This means that the derivatives are not stable in the sample (sensitivity of the method towards 17OHP4 and 21OHP4 decreases over time, which should be taken into account when defining the LLOQ) and also the used internal standard needs to react at a similar rate to compensate for the reaction in the sample. During the method development, it was noted that 17OHP4-d8, containing three deuteriums at position 21, next to the reaction center, was forming an oxime with hydroxylamine slightly faster than the corresponding non-labeled 17OHP4, and even faster than 21OHP4. Thus, 17OHP4-d8 was unable to compensate for the reaction process of 17OHP4 (or 21OHP4) in the sample. For this reason, 13C labeled internal standards were necessary for these two analytes. 17OHP5 contains a carbonyl group in a similar position. However, 17OHP5-d213C2 used as IS, had two deuteriums further from the reaction center (at position 16), and was able to compensate for the progressing reaction, as the reaction rates of IS and the non-labeled analyte were similar. The LC conditions were also tested with androsterone, etiocholanolone, estriol, and 5-androstenediol to ensure that they were not causing any interference.

3.2. Method validation

3.2.1 Calibration ranges and sensitivity

The accepted ranges for the validated calibration curves for all the analytes are presented in Table 1. The lowest and highest calibration points are used as the lower (LLOQ) and the upper limit of quantification (ULOQ), respectively. Calibration standards were injected into the system at the beginning and at the end of each analytical run. Quality controls were used to assess the performance of the assay, and they were run between the patient samples. Curve fitting with different functions and weightings was performed to obtain the most precise measurements. The sum of deviations of the measured concentrations from their nominal values was used to select the best curve fitting, and the degree of weighting was determined to obtain the lowest sum of deviations. The quadratic $1/x^2$ type calibration curve was the best for all analytes. The accepted intra- and inter-run precision error (RSD %) was < 15 % and the accepted accuracy was between 85 and 115 %. LLOQ was defined as the lowest calibration standard with a precision error of less than 20 %, and accuracy between 80-120 % of the nominal concentration.

3.2.2 Selectivity, carry-over and dilution linearity

Selectivity was studied by measuring plasma and prostatic tissue homogenate samples without added IS. No interfering peaks were observed for the IS. In addition, none of the 27 steroids tested disturbed the analytes. Isobaric steroids were separated by LC as shown in Fig. 2, with the steroid glucuronates and sulfates being left in the aqueous phase when samples are extracted with toluene. However, there may still be some co-eluting compounds, which produce interference with the MRM channels of the analytes. Thus, during the analysis, QT/QL ratios were constantly monitored. These MRM ratios were considered to be within the acceptable tolerance, when they differed by less than 30 % between the real samples and standards. Detection of carry-over % was based on the measurement of peaks in three consecutive solvent injections after injecting the highest concentration of the standard. Compounds P5 and P4 showed some carry-over, the mean values were 0.13 % (first injection) and 0.02 % (second injection) of the highest standard peak area for P5, and 0.10 % (first injection) and 0.02 % (second injection) for P4. The analysis of the other analytes did not display any signs of carry-over, as the value was below 0.01 %. Dilution integrity was studied to demonstrate that samples less than 150 µl could be measured reliably after the addition of the buffer, and to show that samples with values above the ULOQ could be safely diluted to a concentration within the working range. The dilution integrity was examined by measuring T reference standard serum, and the plasma samples after dilution with blank matrix (1:3 or 1:10, n = 5). The dilution integrity of the tissue samples was studied using 1:3 dilution (n = 6). The results are presented in Supplementary Table S1A. Dilution linearity was also tested with lyophilized reference serums for E2, P4 and F (Table S1B in

Supplementary material). The mean values corrected for the dilution were compared with the analyzed endogenous levels before dilution. The accuracy of the analysis was acceptable after dilution of the plasma or serum samples by 10-fold, and after dilution for prostatic tissue homogenates by 3-fold. Only 1:10 dilutions for T reference serum gave higher 11KA4 and DHA4 values, 149 % and 153 % respectively, as compared to the undiluted sample values. Furthermore, for lyophilized reference serums (E2, P4 and F reference serums, Table S1B in Supplementary material) 11KA4 gave higher values from diluted samples. In addition, F values were slightly higher in diluted samples, if the neat concentration was higher than ULOQ. Thus, the developed method also achieved reliable results, when the samples had to be diluted by 1:3 or 1:10, or when only 15 µl or 50 µl volume of sample was available.

3.2.3 Stability studies

The prostatic tissue homogenates were stored for 1h at room temperature prior to sample extraction in an evaluation of the stability of the steroids during sample processing. The results are presented in Table 2. The DHEA concentration increased by 149 %, the E1 concentration increased by 82 %, the P5 concentration increased by 65 %, the 17OHP5 concentration increased by 44 %, the 11KDHT concentration increased by 38 %, both DHT and A4 concentrations increased by 25 % and the DHA4 concentration increased by 20% in the prostatic tissue homogenate after 1 h storage at room temperature. This is most likely due to the endogenous steroid metabolizing enzyme activity remaining in the samples, when the homogenates are kept at room temperature. Thus, it is important that the samples are processed without delay after thawing, and homogenization should be carried out quickly and using as low a temperature as possible to maintain the original steroid profile. For the rest of the steroid compounds, only 2-10% variations were detected in the measured concentrations, when the homogenates were kept at room temperature. Analyte concentrations in the autosampler stability test samples changed by 0-19 %, thus the samples were considered to stable after the sample preparation.

3.2.4 Precision and Accuracy

The results of precision and accuracy tests for plasma and tissue homogenate extracts are shown in Table 3. The intra- and inter-day precisions were below 15 % for all steroids for the calibration range, except for A (variation between 11-21 %), 11KA4 (variation between 14-24 %) and 11KDHT (variation between 2-18 %). Furthermore, the 11bOHA4 concentration in the prostatic tissue homogenate used in this study was slightly lower than the LLOQ, with the variation ranging between 11-23 %. The precision results from the reference

serums, plasma and homogenized tissues, including samples diluted with blank matrix (1:3 or 1:10) are presented in Supplementary Table S1A-B, and were < 20 %, not including some of the 11KA4 and DHA4 levels. In the absolute recovery studies, the maximum RSD % was 10 % as shown in Table S2 in Supplementary material.

The determination of accuracy was based on the analysis of T, P4, F and E2 reference serums, presented in Table 4. The recovery %s from the reference values were between 91-118 % for all the reference serums highlighting the good accuracy of the method. The concentration of F in the undiluted sample was above the ULOQ. In addition, the trueness values for other analytes were verified by the measurement of absolute recovery after adding known concentrations of steroids to the samples. The spiked amount was close to that of the detected endogenous concentrations in the samples, and spiked levels of 32 pM or 320 pM were added in cases when the endogenous steroid levels were below the LLOQ. The results for the absolute recoveries are shown in Supplementary Table S2. For F, the endogenous concentration in the undiluted plasma sample was above the ULOQ, and the added standard concentration was less than adequate, below 15 % of the endogenous F amount. Thus, the absolute recovery value for F may not be reliable, as the accepted RSD % for the analysis was determined as 15 %. Moreover, in the undiluted plasma, the spiked concentration of T was > ULOQ. With tissue samples, the absolute recoveries of DHEA, P5 and DHT were only 168 %, 137 % and 134 %, and absolute recoveries for 11KT, E2, 11KDHT and B were 120 %, 119 %, 119 % and 81 %, respectively. With plasma samples, the absolute recoveries for A, E and 11KA4 were 79 %, 83 % and 76 %, respectively. For the rest of the compounds, the detected and expected levels were within the 15 % error margin. In case of the analytes, 11KA4, 11KDHT, DHA4 and 11aOHA4, the labeled steroids 11bOHA4-d4, 11KT-d3 and DHT-13C3 were used as the IS in the quantification. In these cases, the compensation for a matrix effect was presumably not as successful as in cases when labeled analogues were available. This may, at least partially, explain the poorer absolute recovery results for 11KA4, 11KDHT, DHA4 and 11aOHA4.

3.2.5 Extraction recovery and matrix effect

Extraction recoveries were determined using three standard concentrations and IS, one plasma sample pool and homogenized tissue, and the results are presented in Supplementary Table S3. The recovery of F from plasma samples was very low (7 %), but this did not have any deleterious impact on the accuracy of the method, based on the results obtained for the F reference serum in Table 4 and on the absolute recovery

presented in Supplementary Table S2. The results were similar for those steroids examined also in our previous method [24] For the homogenized tissues, the extraction efficiency using toluene was evaluated by calculating the ratio of the peak areas from the second and first toluene extract as shown in Table S4 in Supplementary material. It was considered that the sequential toluene extraction had not improved the analytical results, since the ratio of the analyte results was similar to the ratio of the corresponding IS results. The A4 and P5 analyte results were higher than the corresponding IS results, which indicates that the second extraction may have increased the yield of these two steroids. However, increasing the extraction time of tissue homogenate from 10 minutes to 30 minutes was not observed to enhance the yields from the solvent extraction, as shown in the results in Table S5 in Supplementary material.

Matrix effect results are shown in Table S6 in Supplementary material. The data indicated ion suppression for P5 (ME 68-73 %), P4 (ME 72-75 %), A4 (ME 13-84 %), DHEA (ME 68-81 %), 17OHP5 (ME 83 %), and B (ME 82-84 %). In addition, ion suppression was observed in the plasma sample for F (ME 69 %), E (ME 54 %), both 11KT and 21OHP4 (ME 74 %), 11bOHA4 (ME 64 %), both E1 and S (ME 75 %), T (ME 76 %), DHT (ME 73 %) and for 17OHP4 (ME 77 %).

4. Conclusions

An analytical method based on liquid extraction and LC-MS/MS was successfully developed and validated for the quantification of 21 steroids (T, A4, DHA4, DHEA, DHT, 11KDHT, 11KT, 11bOHA4, 11OHT, E1, 17OHP5, 17OHP4, P4, P5, F, E, B, S, 21OHP4, E2, and 11aOHA4) from serum, plasma and prostatic tissue homogenate samples. The method allows also the quantification of steroids in low volume samples after the addition of blank matrix or after dilution of the samples if the concentration in samples is higher than the ULOQ. In addition, two compounds (A and 11KA4) can be measured semiquantitatively in cases when approximate values will suffice. The analysis of all of the 23 steroids can be achieved within a single run from 150 µl sample using toluene for LLE, oxime derivatization with hydroxylamine in the sample preparation, and isotope-labeled internal standards in the MS detection. The method enables simultaneous analysis of the most important adrenal and gonadal steroids and their metabolites from a small sample volume in the pmol/l or pg/g range, as required for several steroids, including DHT, in tissue, plasma and serum samples. The method can be utilized to examine steroid hormone profiles in serum and prostate tissue homogenates in different experimental studies and clinical trials.

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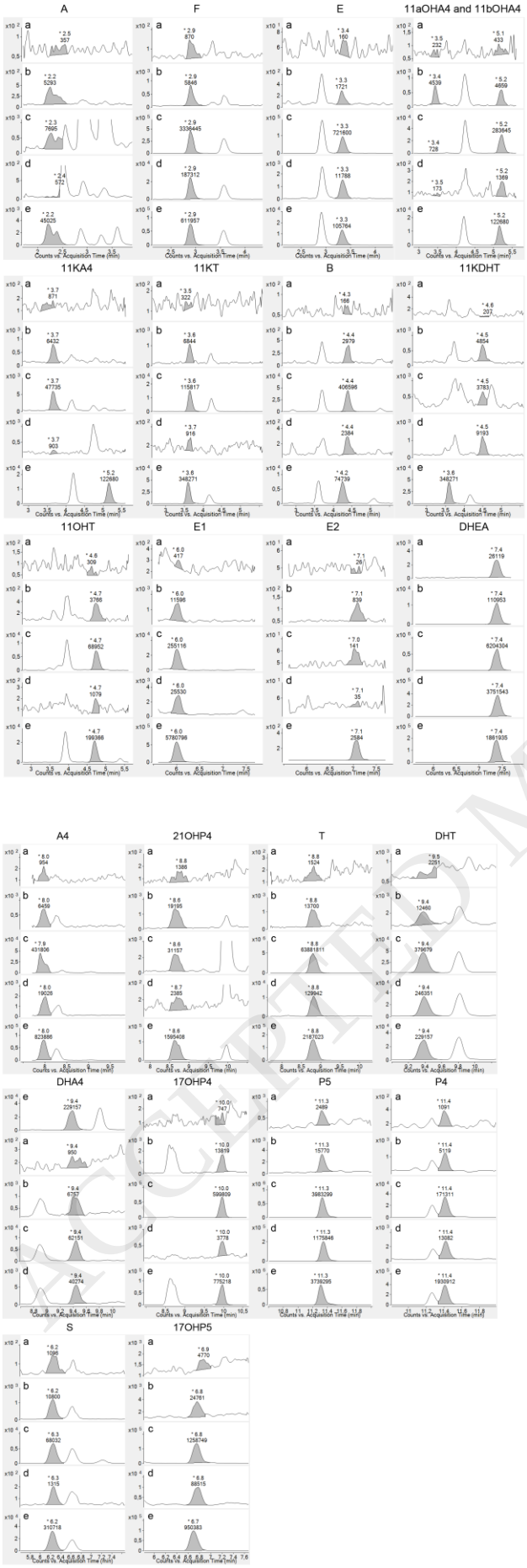

Figure captions

Figure 1. Chromatograms of the 23 steroids included in the method. a) zero sample, b) LLOQ sample, c) plasma sample, d) prostatic tissue homogenate sample, and e) internal standard (IS). 11bOHA4-d4 was used as IS for 11aOHA4 and for 11KA4. 11KT-d3 was used as IS for 11KDHT. DHT-13C3 was used as IS for DHA4. The peak for the isomer used in the quantification is integrated. Some of the analytes were below the LLOQ in these samples (11aOHA4 and E2 in plasma, and A, 11aOHA4, 11bOHA4, 11KA4, 11KT, B, 11OHT, E2, 21OHP4, 17OHP4 and S in prostatic tissue homogenate).

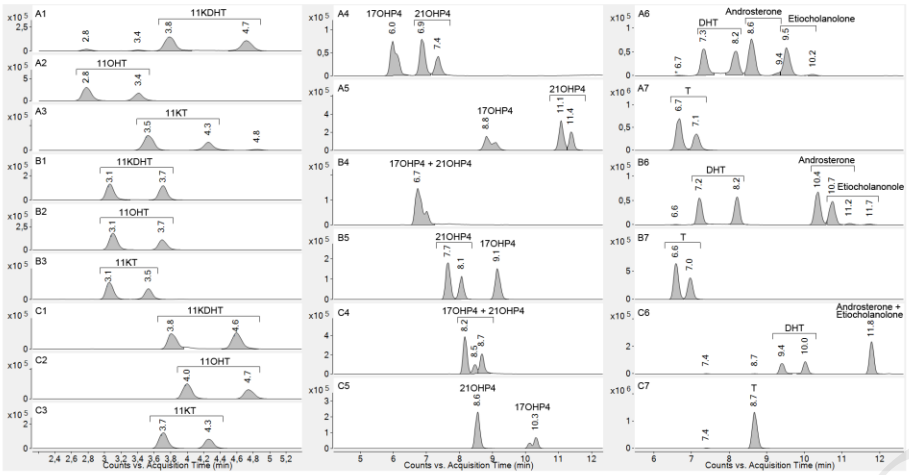

Figure 2. Examples of the chromatograms with a biphenyl column (A1-A7), phenyl-hexyl column (B1-B7) and C18 column (C1-C7). 11-Keto and 11-hydroxy steroids were separated better from each other with the biphenyl column (A1-A3), than with the C18 column (C1-C3). With the phenyl-hexyl-column, 11KDHT and 11OHT were overlapping (B1 and B2). The phenyl-hexyl column was the best choice for separating isobaric androsterone and etiocholanolone from each other, and from DHT (B6) compared to the biphenyl column (A6). With the C18 column, androsterone and etiocholanolone overlapped (C6). The C18 column (C1-C7) separated DHT more efficiently from T and from androsterone and etiocholanolone (C6 and C7), when compared to the biphenyl (A6 and A7) and phenyl-hexyl columns (B6 and B7). All three columns separated the isobaric mono-oxime derivatives of 17OHP4 and 21OHP4 (A5, B5, C5). The bis-oxime derivatives were separated only with the biphenyl column (A4), but overlapped with the phenyl-hexyl (B4) and C18 columns (C4). The double peaks for hydroxylamine derivatives were due to *anti* and *syn* isomers of the formed hydroxylamine derivative, which could either be separated (e.g. T in A7 and B7) or not separated (e.g. T in C7) during chromatography.

Table 1. Retention time, MS parameters and calibration ranges for all analytes. 11bOHA4-d4 was used as IS for 11aOHA4 and 11KA4. 11KT-d3 was used as IS for 11KDHT. DHT-13C3 was used as IS for DHA4. For the rest of the analytes, a corresponding labeled standard was used as IS. ESI- was used for the E2, and ESI+ for the rest of the analytes.

Compound	Derivative	t_R (min)	QT	CE	QL	CE	CAV	Calib. range (pM)
A	1xNH_2OH	2.24	376 > 112	36	376 > 91	67	2	128-63924
A-d8	1xNH_2OH	2.21	384 > 115	46	384 > 128	45	2	
F	1xNH_2OH	2.89	378 > 136	28	378 > 112	36	5	33-133900
F-13C3	1xNH_2OH	2.89	381 > 139	29	381 > 115	36	5	
E	1xNH_2OH	3.33	376 > 178	37	376 > 121	43	4	66-131326
E-13C3	1xNH_2OH	3.33	379 > 181	39	379 > 123	43	4	
11aOHA4	2xNH_2OH	3.44	333 > 138	29	333 > 79	65	2	33-32990
11KA4	2xNH_2OH	3.66	331 > 121	33	331 > 178	37	7	33-13236
11KT	1xNH_2OH	3.64	318 > 121	35	318 > 120	35	5	33-6665
11KT-d3	1xNH_2OH	3.61	321 > 121	36	321 > 120	37	5	
B	2xNH_2OH	4.38	377 > 138	31	377 > 91	63	3	33-66740
B-d8	2xNH_2OH	4.26	385 > 143	35	385 > 115	41	3	
11KDHT	1xNH_2OH	4.50	320 > 108	41	320 > 110	35	5	13-13148
11OHT	1xNH_2OH	4.74	320 > 138	29	320 > 288 320 > 105 320 > 147	39 45 29	5	13-32832
11OHT-d4	1xNH_2OH	4.70	324 > 139	32	324 > 151	27	5	
11bOHA4	2xNH_2OH	5.21	333 > 138	29	333 > 79	63	2	33-13412
11bOHA4-d4	2xNH_2OH	5.17	337 > 139	30	337 > 79	76	2	
E1	1xNH_2OH	6.01	286 > 253	13	286 > 213	21	5	3.3-13177
E1-13C3	1xNH_2OH	6.01	289 > 256	14	289 > 216	21	5	
S	1xNH_2OH	6.25	362 > 124	38	362 > 112	33	4	33-65918
S-13C3	1xNH_2OH	6.25	365 > 127	39	365 > 115	35	4	
17OHP5	1xNH_2OH-H_2O	6.75	330 > 312	15	330 > 105	46	2	32-127202
17OHP5-d213C3	1xNH_2OH-H_2O	6.71	334 > 316	18	334 > 105	50	2	
E2	-	7.08	271 > 145	47	271 > 183	49	2	335-133960
E2-13C3	-	7.08	274 > 148	47	274 > 186	48	2	
DHEA	1xNH_2OH	7.38	304 > 213	21	304 > 253	17	6	67-133278
DHEA-13C3	1xNH_2OH	7.38	307 > 216	22	307 > 256	18	6	
A4	2xNH_2OH	7.96	317 > 124	34	317 > 112	31	4	3.3-13277
A4-13C3	2xNH_2OH	7.96	320 > 127	35	320 > 115	32	4	
21OHP4	1xNH_2OH	8.64	346 > 112	32	346 > 124	35	5	32-13293
21OHP4-13C3	1xNH_2OH	8.64	349 > 115	34	349 > 127	35	5	
T	1xNH_2OH	8.80	304 > 124	33	304 > 112	30	5	3.3-13293
T-13C3	1xNH_2OH	8.80	307 > 127	33	307 > 115	31	5	
DHT	1xNH_2OH	9.37	306 > 81	47	306 > 93	42	2	33-33299
DHT-13C3	1xNH_2OH	9.37	309 > 81	47	309 > 93	47	2	
DHA4	2xNH_2OH	9.44	319 > 286	16	319 > 161	25	3	13-12984
17OHP4	1xNH_2OH	9.97	346 > 112	32	346 > 124	35	5	67-133329
17OHP4-13C3	1xNH_2OH	9.97	349 > 115	35	349 > 127	37	5	
P5	1xNH_2OH	11.33	332 > 86	29	332 > 300	23	2	6.6-13176
P5-d213C2	1xNH_2OH	11.31	336 > 90	30	336 > 304	24	2	
P4	2xNH_2OH	11.41	345 > 124	34	345 > 112	31	5	3.3-13259
P4-13C3	2xNH_2OH	11.41	348 > 127	34	348 > 115	34	5	

t_R, retention time; QT, quantifier ion transition; QL, qualifier ion transition; CE, collision energy; CAV Cell accelerator voltage. Calibration curve type Quadratic $1/x^2$ for all.

Table 2. Short-term and autosampler stabilities of the samples. Short-term stability: Thawed homogenates were stored for one hour at room temperature, and the mean measured concentrations were compared with samples that were prepared immediately after thawing (n = 6). Autosampler stability: Samples were stored in the autosampler (10ºC, 2d) and the mean measured concentrations were compared with samples that were measured at the beginning of the batch.

	Prostate tissue homogenate 1h at room temperature	Prostate tissue sample autosampler stability	Plasma sample autosampler stability
A	< LLOQ	< LLOQ	94 %
F	110 %	96 %	101 %
E	108 %	97 %	104 %
11aOHA4	< LLOQ	< LLOQ	< LLOQ
11KA4	< LLOQ	< LLOQ	112 %
11KT	< LLOQ	< LLOQ	101 %
B	91 %	92 %	100 %
11KDHT	138 %	114 %	119 %
11OHT	< LLOQ	< LLOQ	95 %
11bOHA4	97 %	99 %	101 %
E1	182 %	100 %	92 %
S	< LLOQ	< LLOQ	96 %
17OHP5	144 %	102 %	98 %
E2	< LLOQ	< LLOQ	< LLOQ
DHEA	249 %	101 %	96 %
A4	125 %	102 %	99 %
21OHP4	< LLOQ	< LLOQ	104 %
T	104 %	100 %	97 %
DHT	125 %	99 %	101 %
DHA4	120 %	101 %	102 %
17OHP4	< LLOQ	< LLOQ	99 %
P5	168 %	102 %	96 %
P4	98 %	102 %	101 %

Table 3. Endogenous steroid concentrations (pM) and intra-day and inter-day precisions (RSD %) for plasma and tissue homogenates.

	Plasma					Prostate				
	Day 1 (n=6)	**Day 2 (n=6)**	**Day 3 (n=5)**	**Intra-day precision**	**Inter-day precision**	**Day 1 (n=6)**	**Day 2 (n=4)**	**Day 3 (n=6)**	**Intra-day precision**	**Inter-day precision**
A*	208 ± 22	136 ± 18	192±24	11 / 14 / 13	21	< LLOQ	< LLOQ	< LLOQ		
F**	242118 ± 6194	254465 ± 15645	243744±19702	3 / 6 / 8	6	1889 ± 154	1892 ± 165	2026 ± 138	8 / 9 / 7	8
E	64550 ± 4265	62327 ± 3078	59106 ± 4874	7 / 5 / 8	7	357 ± 26	346 ± 37	321 ± 21	7 / 11 / 7	9
11aOHA4	< LLOQ	< LLOQ	< LLOQ			< LLOQ	< LLOQ	< LLOQ		
11KA4	519 ± 89	558 ± 84	351 ± 49	17 / 15 / 14	24	< LLOQ	< LLOQ	< LLOQ		
11KT	809 ± 59	822 ± 81	889 ± 41	7 / 10 / 5	8	< LLOQ	< LLOQ	< LLOQ		
B**	8618 ± 632	9333 ± 901	8820 ± 327	7 / 10 / 4	8	27 ± 2	32 ± 1	31 ± 2	6 / 4 / 7	10
11KDHT	15 ± 3	17 ± 2	13 ± 0	18 / 10 / 2	15	30 ± 5	30 ± 3	23 ± 1	15 / 10 / 5	17
11OHT	370 ± 15	389 ± 24	323 ± 19	4 / 6 / 6	9	< LLOQ	< LLOQ	< LLOQ		
11bOHA4**	2791 ± 105	2778 ± 207	2631 ± 118	4 / 7 / 4	6	15 ± 2	9 ± 2	15 ± 2	11 / 19 / 14	23
E1	82 ± 4	83 ± 6	92 ± 3	5 / 7 / 4	7	13 ± 2	12 ± 1	11 ± 2	15 / 4 / 15	15
S	346 ± 13	344 ± 13	327 ± 8	4 / 4 / 2	4	< LLOQ	< LLOQ	< LLOQ		
17OHP5	2268 ± 119	2190 ± 122	2170 ± 36	5 / 6 / 2	5	136 ± 16	124 ± 9	123 ± 9	12 / 7 / 7	10
E2	< LLOQ	< LLOQ	< LLOQ			< LLOQ	< LLOQ	< LLOQ		
DHEA	5752 ± 178	5497 ± 358	5409 ± 95	3 / 6 / 2	5	4277 ± 483	3925 ± 205	3840 ± 313	11 / 5 / 8	10
A4	1715 ± 77	1726 ± 114	1720 ± 79	4 / 7 / 5	5	17 ± 3	16 ± 0	15 ± 1	16 / 2 / 5	12
21OHP4	55 ± 3	58 ± 3	53 ± 2	5 / 5 / 3	6	< LLOQ	< LLOQ	< LLOQ		
T**	13966 ± 450	13823 ± 404	13158 ± 223	3 / 3 / 2	4	34 ± 2	31 ± 1	31 ± 2	6 / 4 / 7	7
DHT	1273 ± 35	1301 ± 54	1263 ± 21	3 / 4 / 2	3	1004 ± 63	882 ± 17	839 ± 22	6 / 2 / 3	9
DHA4	147 ± 12	155 ± 15	158 ± 3	8 / 10 / 2	8	100 ± 7	94 ± 3	90 ± 5	7 / 3 / 6	7
17OHP4	2144 ± 64	2167 ± 120	1979 ± 60	3 / 6 / 3	6	< LLOQ	< LLOQ	< LLOQ		
P5	1908 ± 76	1609 ± 91	1780 ± 16	4 / 6 / 1	8	548 ± 64	472 ± 24	489 ± 36	12 / 5 / 7	11
P4	150 ± 2	152 ± 11	146 ± 3	2 / 7 / 2	4	10 ± 1	11 ± 0	11 ± 1	6 / 4 / 14	10

*A: Matrix disturbs the analysis, no better than a semiquantitative result.

**F: ULOQ = 133900, B: LLOQ = 33.5pM, 11bOHA4: LLOQ = 33.5, T: ULOQ = 13293. Plasma F was >> ULOQ. Prostate B and 11bOHA4 were below LLOQ.

Table 4. Recovery % of T, P4, F, and E2 from reference serum samples.

	Dilution Factor	Measured ± sd (pM)	RSD %	n	Recovery % from reference value
T Ref. serum (ERM-DA346) 890 ± 120 pM	neat	966 ± 60	6 %	4	109 %
	1:3	293 ± 6	2 %	5	99 %
	1:10	91 ± 2	3 %	5	102 %
P4 Ref. serum (ERM-DA347) 10.13 ± 0.21 nM	neat	10473 ± 449	4 %	4	103 %
	1:3	3727 ± 99	3 %	5	110 %
	1:10	1141 ± 52	5 %	5	113 %
F Ref. serum (ERM-DA192) 273 ± 6 nM	neat	249648 ± 17418	7 %	5	91 %
	1:3	97182 ± 9082	9 %	5	107 %
	1:10	31705 ± 1799	6 %	5	116 %
E2 Ref. serum (BCR-577) 690 ± 40 pM	neat	812 ± 46	6 %	5	118 %
	1:3	243 ± 15	6 %	4	106 %