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Determination of steroid hormones in blood by GC–MS/MS

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Abstract This paper presents the development, optimization and validation of a methodology to determine nine key steroid hormones (viz. pregnenolone, progesterone, dehydroepiandrosterone, androstenedione, testosterone, dihydrotestosterone, estrone, 17α-estradiol and 17β-estradiol) expressed in the steroidogenesis in biological fluids. The analytical method allows for the determination of steroid hormones in blood plasma and serum down to 0.08–0.16 ng/mL for estrogens, 0.20–0.36 ng/mL for androgens and 0.36–0.43 ng/mL for progestagens. These limits of detection were obtainable using a two-step solid-phase clean-up for fractionation and elimination of interfering lipids (fatty acids, phospholipids, glycerides and sterols) from the steroid hormones. The accuracy of the method was 50–112% in the range 0.10 to 2.00 ng/mL.

Keywords Plasma · Serum · Solid-phase extraction · Gas chromatography · Lipid separation · Biological samples

Introduction

The vertebrate steroidogenesis is a highly conserved metabolic pathway responsible for producing a range of precursors and hormones fundamental to regulate important biological processes such as growth and reproduction [1]. Several chemicals, many of anthropogenic origin, are today known to disturb these finely tuned processes by interfering with the hormone homeostasis and the steroidogenesis [2–5] and are commonly referred to as endocrine disrupting chemicals (EDCs). In order to understand the effects of EDCs on endocrine systems such as the steroidogenesis, detailed studies on the interactions between steroid hormones are pivotal [4]. This requires a reliable analytical method for the simultaneous determination of several steroid hormones in biological matrices such as blood from endocrinologically active organs collected during experimentation. Key hormones that are essential to measure in this context include pregnenolone (PRE, synthesized from cholesterol and a prohormone to all other steroid hormones) and progesterone (PRO, involved in the female reproductive cycle and pregnancy), the male sex steroid hormones (androgens) dehydroepiandrosterone (DHEA), androstenedione (AN), testosterone (TS) and its active form dihydrotestosterone (DHT), along with the female sex steroid hormones (estrogens) estrone (E1), 17α-estradiol (αE2) and 17β-estradiol (βE2) as listed in Table 1. Conjugates and other types of metabolites, such as sulfate metabolites, have low occurrence in blood and are of less relevance in association with endocrine disruption [1]; therefore, they are not addressed in the present methodology.

Both biological methods, such as radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA), and modern GC-MS/MS techniques are often suggested methods to analyze steroid hormones, where the RIA and ELISA assays often are first technique of choice [6, 7]. Even though such biological techniques are sensitive, they also have several disadvantages such as being expensive and time consuming. Cross-reactivity may occur as these techniques rely on

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Table 1 Physicochemical properties and abbreviations of the investigated steroid hormones, surrogate interference compounds (SIC) and analytical control standards [26]

Compound	Abbrev.	CAS	Formula	MW (g/mol)	Log K_{ow}	S_w (mg/L)
Progestagens						
Pregnenolone	PRE	145-13-1	$C_{21}H_{32}O_2$	316.5	4.22	7.1[a]
Progesterone	PRO	57-83-0	$C_{21}H_{30}O_2$	314.5	3.87	8.8
Androgens						
Dehydroepiandrosterone	DHEA	53-43-0	$C_{19}H_{28}O_2$	288.4	3.23	63.5
Androstenedione	AN	63-05-8	$C_{19}H_{26}O_2$	286.4	2.75	57.8
Testosterone	TS	58-22-0	$C_{19}H_{28}O_2$	288.4	3.32	23.4
Dihydrotestosterone	DHT	521-18-6	$C_{19}H_{30}O_2$	290.4	3.55	NA
Estrogens						
Estrone	E1	53-16-7	$C_{18}H_{22}O_2$	270.4	3.13	30
Estradiol-17α	αE2	57-91-0	$C_{18}H_{24}O_2$	272.4	3.94	3.9
Estradiol-17β	βE2	50-28-2	$C_{18}H_{24}O_2$	272.4	4.01	3.6
Surrogate interference compounds (SIC)						
Cholesterol	CHOL	57-88-5	$C_{27}H_{46}O$	386.7	8.74	0.1
Coprostanol	COP	360-68-9	$C_{27}H_{48}O$	388.7	8.82	NA
Epi-coprostanol	eCOP	516-92-7	$C_{27}H_{48}O$	388.7	NA	NA
Sitosterol	SITO	5779-62-4	$C_{29}H_{50}O$	414.7	NA	NA
Control standards						
Estrone-3-methyl ether	ICS	1624-62-0	$C_{19}H_{24}O_2$	284.4	NA	NA
Estradiol-17-acetate	DCS	6045-67-6	$C_{20}H_{26}O_3$	314.4	NA	NA

MW molecular weight, K_{ow} octanol–water coefficient, S_w water solubility at 25 °C, *NA* not available, *ICS* instrument control standard, *DCS* derivatization control standard

[a] 37 °C

antibody affinity between steroid hormones that are structurally similar. Cross-reactivity is not easily determined and may vary from sample to sample and from matrix to matrix. For example, during analysis of PRO, a substantial uncertainty is expected due to cross-reactivity with PRE amounting to more than 60% for some commercially available assays [3, 4]. In the human adrenocarcinoma H295R cell lines, PRE production may apparently be up to 75 times higher than that of PRO [8]. Measuring PRO in such cell lines, using an ELISA assay with 60% cross-reactivity, may thus lead to substantial errors. In a comment to an article by Taieb and coworkers [9], Herold and Fitzgerald [10] stated that "guessing seems to be nearly as good as most commercially available immunoassays and clearly superior to some".

This work for the first time presents a novel analytical procedure, which allows for the simultaneous determination of nine native key steroid hormones expressed in the steroidogenesis, relying on chemical analysis by solid-phase extraction (SPE) and clean-up followed by derivatization and gas chromatography tandem mass spectrometry (GC-MS/MS). The method is applicable for blood from several animal species with high variation in plasma lipids (e.g. rat, frog and polar bear). The steroid hormones can be determined in the low parts per billion range with high selectivity and in low concentrations. Therefore, the method is applicable to studies, such as in the steroidogenesis and other biochemical pathways affected by EDCs, wherein several steroid hormones are expected to interact at the same time and at low concentrations.

Experimental

Chemicals

Pregnenolone (PRE), progesterone (PRO), dehydroepiandrosterone (DHEA), androstenedione (AN), testosterone (TS), dihydrotestosterone (DHT), estrone (E1), 17β-estradiol (βE2) and 17α-estradiol (αE2) were achieved from Sigma-Aldrich (Glostrup, Denmark), all with a purity level above 96%. Deuterated analogues were applied as internal standards (IS); d_7-androstenedione (dAN), d_4-estrone (dE1) and d_5-17β-estradiol (dE2) were obtained from CDN isotopes (Pointe-Claire, QC, Canada), while d_9-progesterone (dPRO), d_3-testosterone (dTS) and d_3-dihydrotestosterone (dDHT) were purchased from Toronto Research Chemicals (North York, ON, Canada), all with a deuterated purity above 98%. The derivatization quality control standard (DCS), 17β-estradiol-17-acetate (>99% purity) and the instrument control standard (ICS), estrone-3-methyl ether (>98% purity) were both obtained from Sigma-Aldrich (Glostrup, Denmark). The derivatization reagents N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA), N-trimethylsilylimidazole (TMSI) and 1,4-dithioerythritol (DTE, 20.0 mg dissolved in 500 µL pyridine) were purchased from Sigma-Aldrich (Glostrup, Denmark).

In some experiments, lipid surrogate interference compounds (SIC) were applied with a structure similar to the steroid hormones to demonstrate the separation and clean-

up performance of the developed method. These compounds included cholesterol (CHOL), coprostanol (COP), epicoprostanol (eCOP) and sitosterol (SITO) all obtained from Sigma-Aldrich (Glostrup, Denmark). Two IS for the SIC were also implemented, these were d_6-cholesterol (dCHOL) and d_7-sitosterol (dSITO), both obtained from Toronto Research Chemicals (North York, ON, Canada) with a deuterated purity above 98%. Physicochemical properties of the steroid hormones and surrogate interferences are listed in Table 1. All utilized solvents were of analytical grade and obtained from Lab-scan analytical sciences (Fisher Scientific Biotech Line, Slangerup, Denmark). Chloroform (trichloromethane), diethyl ether and the utilized derivatization reagents should be handled with great care inside a fume hood and by qualified personnel only.
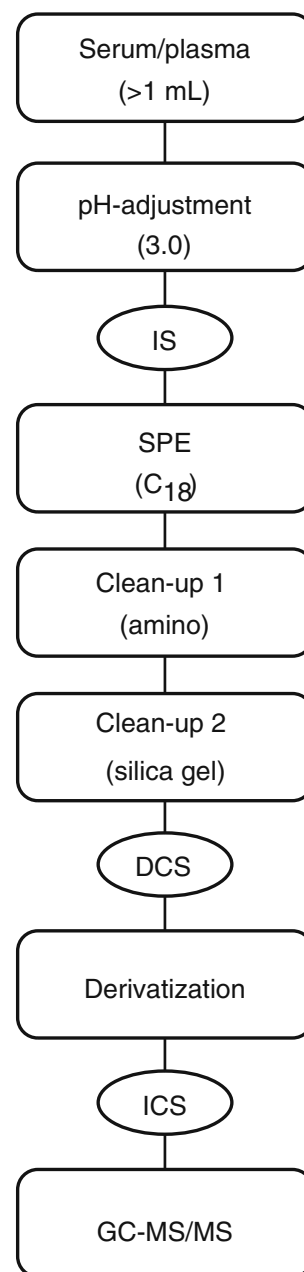
Standard solutions

Stock solutions of 1,000 ng/μL in methanol were prepared for all compounds. Mixture dilution series of the nine steroid hormones were likewise prepared in methanol in the concentration range 0.01 to 50 ng/μL. A mixture of the six deuterated analogues (internal standards (IS)) was established at 0.40 ng/μL in methanol, and 50 μL of this mixture was applied to all samples, corresponding to 20 ng of all IS in each sample. A dilution of the derivatization control standard (DCS) was prepared at 0.20 ng/μL in methanol, and 100 μL (i.e. 20 ng) was added to the samples prior to the derivatization. All solutions were stored in darkness at −18 °C. The derivatization reagent mixture was prepared on a daily basis by mixing 1,000 μL MSTFA with 2 μL TMSI and 50 μL DTE mixture [11]. The instrument control standard (ICS) was diluted in heptane to a concentration of 0.10 ng/μL; 200 μL of this mixture was used as reconstitution solvent after derivatization equivalent to 20 ng ICS in every sample. A diluted mixture of the four SIC was prepared in methanol in the concentration range 0.1 to 50 ng/μL.

Sample preparation

An overview of the final sample preparation procedure is shown in Fig. 1 and is further described in details below. All utilized glassware was rinsed with soap and ethanol followed by heating to 300 °C for at least 8 h.

In order to stabilize the steroid hormones in the sample, pH was initially adjusted to pH 3.0±0.1 with diluted sulfuric acid, and 50 μL of the IS mixture was added to the solution. SPE was performed with C_{18} cartridges (500 mg, 10 mL reservoir, Varian Inc., Palo Alto, CA, USA) conditioned with 2×3 mL heptane, 3 mL acetone, 2× 3 mL methanol and lastly with 2×3 mL tap water adjusted to pH 3.0. Samples were transferred to the cartridge

Fig. 1 Overview of the sample preparation procedure for blood plasma and serum


```
Serum/plasma
(>1 mL)
      |
pH-adjustment
(3.0)
      |
     IS
      |
    SPE
   (C_18)
      |
 Clean-up 1
  (amino)
      |
 Clean-up 2
 (silica gel)
      |
     DCS
      |
 Derivatization
      |
     ICS
      |
  GC-MS/MS
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quantitatively (by flushing the sample test tube twice with 1 mL of pH-adjusted tap water) and applied at a rate of 1–2 mL/min. If necessary, an optional extra sample reservoir of 70 mL (PTFE container) was applied on top of the SPE. After enrichment, the SPE cartridges were air dried by suction for 30–60 min using the vacuum manifold (IST Vacmaster from Biotage, Uppsala, Sweden). Finally, analytes were eluted from the SPE into a test tube with 5 mL acetone and evaporated to dryness under a gentle stream of nitrogen, followed by reconstitution in 100 μL chloroform. These sample extracts were further cleaned by an optimized two-step procedure containing a 500-mg aminopropyl cartridge (Waters Sep-pak, Ireland) and a freshly prepared silica gel

(60 mesh, Merck, Darmstadt, Germany) packed in a glass cartridge (3 mL, LiChrolut, Merck, Darmstadt, Germany) containing a Chromabond filter in the bottom (Macherey-Nagel, Düren, Germany). The aminopropyl cartridge (clean-up 1) was washed with 2×2 mL heptane followed by sample application (reconstituted in chloroform). The test tube was rinsed with an additional portion of 100 μL chloroform:isopropanol (2:1) which was applied to the same cartridge. The steroid hormones were eluted from the cartridge with 5 ml chloroform:isopropanol (2:1) into a new test tube. Again, the eluate was evaporated to dryness and reconstituted in 50 μL chloroform followed by addition of 450 μL heptane. The samples were further cleaned on a silica gel (clean-up 2) prepared in heptane. The reconstituted sample from clean-up 1 was added on top of the silica gel, and the test tube was rinsed twice with an additional portion of 100 μL heptane which was applied to the same cartridge. The gel was flushed with 5 mL heptane followed by 10 mL heptane:acetone (90:10) solution. Finally, the steroid hormones were eluted from the column with a 5-mL portion of heptane:acetone (65:35) collected in a clean test tube. During this procedure, the silica gel may not run dry until the final elution.

DCS was added to the 5-mL clean-up 2 sample extract as 100 μL of the diluted standard followed by evaporation to dryness under nitrogen at 60 °C. The analytes were derivatized by adding 50 μL derivatization mixture and allowing it to react for 1 h at 60 °C. Hereafter, the sample was evaporated to dryness with nitrogen (60 °C) and dissolved in 200 μL ICS heptane solution and transferred to an autosampler vial with a 300-μL insert. Calibration standards were prepared in a similar way by transferring suitable volumes standard solutions (in methanol), followed by evaporation to dryness with nitrogen, and thereafter derivatization as described for the samples.

Gas chromatography-mass spectrometry

The analysis was done on a Varian CP-3800 gas chromatograph (Varian Inc., Palo Alto, CA, USA) with a large-volume programmable-temperature-vaporizer (PTV) injector coupled to a Varian 1200 triple-quadrupole mass spectrometry system (Varian Inc., Palo Alto, CA, USA) operated in the selective reaction monitoring (SRM) mode utilizing electron ionization (70 eV). Two SRM ion transitions per species were selected, optimized and verified by reasonable structural argumentation (Electronic Supplementary Material Table S1). The column was a Zebron-5HT Inferno (30 m×0.25 mm, 0.25 μm, Phenomenex Inc., Torrance, CA, USA) operated at a constant carrier gas flow of 1.0 mL/min. The column oven temperature program was

150 °C the first 2.0 min, ramped to 230 °C (25 °C/min) further on to 248 °C (1.0 °C/min) and finally to 325 °C (25 °C/min) with a 3.7-min hold. The total chromatographic time was 30.00 min. An autosampler (CTC Analytics AG, Switzerland) injected 10 μL of the prepared samples applying a solvent-split/splitless PTV-program starting at 120 °C (kept for 0.30 min after injection) followed by a ramp to 325 °C (200 °C/min) with an 11.50-min hold. The split ratio was 100 the first 0.30 min and then closed for 1.70 min followed by a split ratio of 30. The injector was installed with a glass split liner with a glass frit (Varian Inc., Palo Alto, CA, USA). Carbon dioxide was used as liquid injector coolant between injections. The GC-MS/MS system was software controlled with Varian MS Workstation 6.9 (Varian Inc., Palo Alto, CA, USA).

The analysis of the SIC analyte group during the method development experiments was done on the same system. The only differences between the two GC-MS/MS methods were a modified GC oven program as well as the SRM ion transitions, where the m/z values for the SIC were used instead of the steroid hormones (Electronic Supplementary Material Table S1). The GC oven program was 22.00 min in total: 150 °C the first 2.00 min, ramped to 270 °C (25 °C/min) and on to 282 °C (1.0 °C/min) and finally to 325 °C (25 °C/min) with a 1.50-min hold. The SIC were not derivatized and consequently analyzed as a separate sample. Good linear calibration curves were obtained for the surrogate interferences in the range 50 to 500 ng relative to one of the two internal standards (dCHOL and dSITO, Electronic Supplementary Material Table S1) each added as 100 ng and reconstituted in 200 μL heptane. The limits of quantification were approximately 50 ng for all SIC. A standard chromatogram of 50 ng of each SIC in 200 μL heptane is presented in Electronic Supplementary Material Figure S1.

Sample analysis

The method was developed and optimized on foetal bovine serum (charcoal-stripped, cat. no. 04-201-1A, Biological Industries, Israel) free from steroid hormones. Routine procedures for obtaining plasma and serum were used, e.g. blood was collected using heparin-flushed syringes (heparin 5,000 E/mL, LEO Pharma, Denmark) and transferred to 5-mL heparin-coated vacutainers (NH85 I.U., BD, Franklin Lakes, NJ, USA) and centrifuged at 4,000 rpm in 5 min at 20 °C. Depending on the sample type, different sample amounts are required; for serum and plasma, between 1 and 5 mL is a suitable volume.

Finally, the method was applied on plasma samples from male rats (*Rattus horuegicus*) to determine the native steroid hormone levels.

Results and discussion

GC-MS/MS development and optimization

Prior to the development and optimization of SPE and clean-up of samples, the GC-MS/MS procedure was established. The first step was to make the steroid hormones more volatile and thermal stabile, which was achieved using a derivatization procedure previously described for the estrogenic hormones by Ternes and colleagues [11]. It was revealed that this procedure was applicable also to the other steroid hormones investigated in this study. No literature could be identified demonstrating the separation of all nine steroid hormones; however, the GC separation was based on the aforementioned study [11]. The final separation of a standard mixture is shown in Fig. 2. In addition to the separation of the different steroid hormones, it was observed that the derivatization procedure combined with GC-MS/MS made it possible to differentiate between the two stereoisomers of estradiol (αE2 and βE2).

Once the separation and identification was set, the limit of detection (LOD), quantification (LOQ) and linear dynamic range were determined from the calibration curves based on steroid hormone and IS peak area ratio versus steroid hormone concentration. The calibration curves of all nine steroid hormones were established with a six-point calibration by derivatization of standards ranging from 0.10 to 20.0 ng with IS fixed at 20.0 ng at all calibration points. Finally, the derivatized calibration standards were dissolved in 200 μL heptane solution (containing the ICS), of which 10 μL was injected into the GC-MS/MS system. The LOD was calculated as 3.3 times the residual standard deviation of the linear regression divided by the slope. Likewise, the LOQ was calculated as ten times the same ratio. The results are presented in detail in Table 2. For the nine steroid hormones investigated, the LOD range was 0.08–0.43 ng/mL, and the LOQ range was 0.26–1.29 ng/mL based on 10-mL sample sizes, which also allows for calculating the absolute amount required to be injected into the chromatographic system in order to be detected by the MS corresponding to 0.04–0.21 ng for LOD_{instr} and 0.13–0.65 ng for LOQ_{instr}. Evidently, there is room for changes to the applied final chromatographic procedure by decreasing the final volume to below 200 μL and increasing the injection volume to above 10 μL, which may lead to improved LOD_{instr} and LOQ_{instr}. Yet, conditions were chosen based on previous experience of suitable volumes which were easy to handle. The calibration curves were linear in the entire investigated interval, covering the physiologically most important concentration ranges of most biological samples.
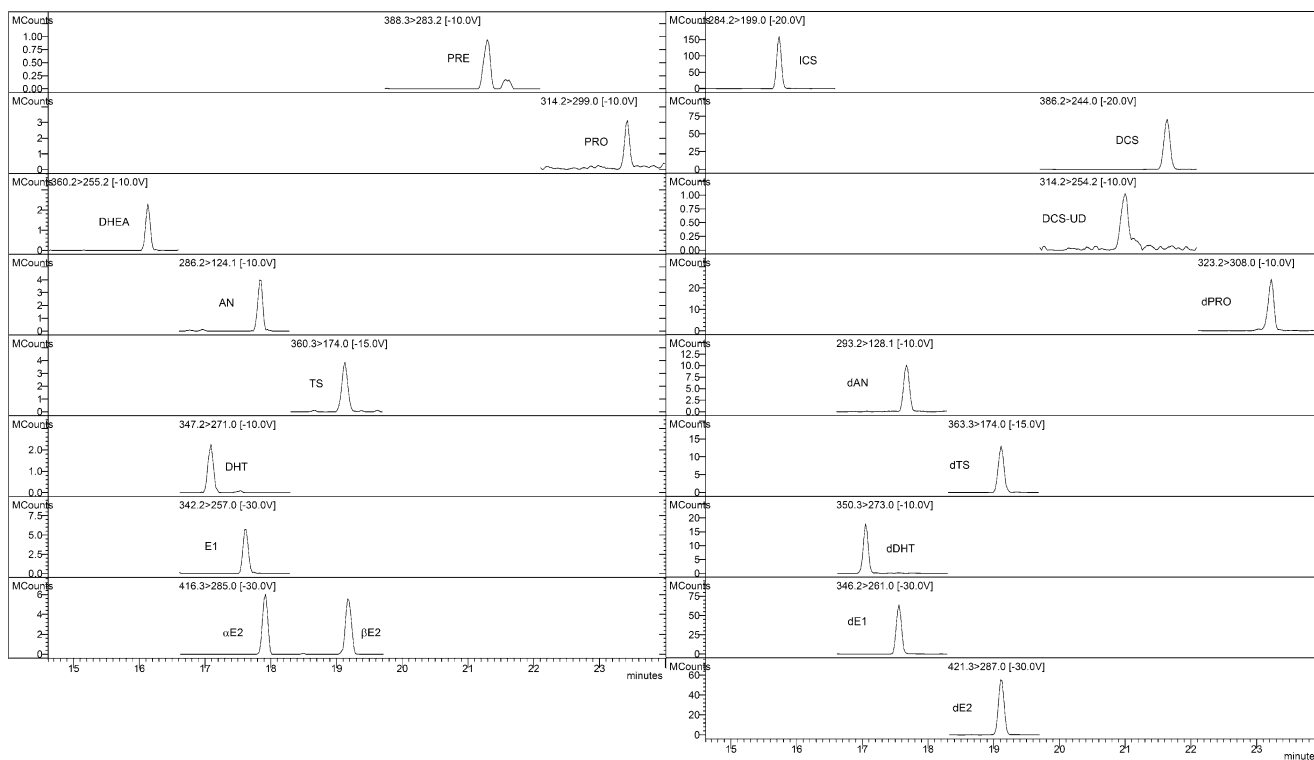

Fig. 2 Standard chromatogram of 3.0 ng derivatized steroid hormones present in 200 μL heptane with 10 μL injection. The ICS, DCS and IS are all spiked at 20 ng. The SRM quantifier ion transitions (product ion 1 in Electronic Supplementary Material Table S1) for each analyte are depicted. DCS-UD is the underivatized form of DCS

Table 2 Limit of detection (LOD) and quantification (LOQ) when using 10 mL plasma determined from individual calibration curves as 3.3 and ten times the residual standard deviation of the regression relative to the slope, respectively. All steroid hormones present in derivatized form in 200 μL heptane, injecting 10 μL into the chromatographic system

	LOD[a] (ng/mL)	LOQ[a] (ng/mL)	LOD$_{instr}$[b] (ng)	LOQ$_{instr}$[b] (ng)
Progestagens				
PRE	0.43	1.29	0.21	0.65
PRO	0.36	1.08	0.18	0.54
Androgens				
DHEA	0.35	1.05	0.17	0.52
AN	0.26	0.79	0.13	0.40
TS	0.36	1.10	0.18	0.55
DHT	0.20	0.60	0.10	0.30
Estrogens				
E1	0.09	0.28	0.05	0.14
αE2	0.08	0.26	0.04	0.13
βE2	0.16	0.48	0.08	0.24

[a] Based on 10 mL sample sizes
[b] Absolute amount of steroid hormone injected into GC-MS/MS

Clean-up development and optimization

During the method development, it became evident that a purification of the steroid hormones from matrix interferences, such as fatty acids, phospholipids, sterol esters and glycerides, was required. Therefore, the well-described lipid classes fractionation methodology by Kaluzny and co-workers [12] applying two aminopropyl SPEs was tested to see if the steroid hormones and the SIC could be separated. Furthermore, this methodology was tested with the goal to simplify and adapt the procedure for biological samples and tissues. Results are outlined in Electronic Supplementary Material Figure S2 and Table S2. The steroid hormones and the SIC were eluted from the first aminopropyl cartridge (A) using chloroform:isopropanol (2:1), as in accordance with previous findings by Kaluzny and co-workers [12]. In this initial fractionation step, the fatty acids and phospholipids should be removed from the steroid hormones [12]. Two additional elution steps from the same cartridge (A), applying first ethanol:acetic acid (98:2) and then pure methanol (eluate 2 and 3, respectively), did not cause elution of substantial amounts of the analytes verifying that this initial aminopropyl cartridge is a suitable clean-up step removing a number of interfering components (fatty acids and phospholipids) as described by Kaluzny and co-workers [12]. The eluted analytes (eluate 1) were added to a second aminopropyl cartridge (B), where no steroid hormones were eluted with hexane (eluate 4) and hexane:dichloromethane:diethyl ether (89:10:1, eluate 5), while some of the SIC (CHOL and COPs) were partly eluted. Eluate 6 comprise of hexane:ethyl acetate (95:5), eluted the chief part of the SIC. In elution steps 7 and 8 (hexane:ethyl acetate (85:15) and chloroform:methanol (2:1), respectively) the steroid hormones are (partly) eluted along with small amounts of SIC.

Due to the observed co-elution of steroid hormones and residues of SIC, the use of a silica gel was investigated for a potential replacement of the second aminopropyl cartridge to obtain improved fractionation (B cartridge in Electronic Supplementary Material Figure S2). Consequently, fractionation experiments were made by applying standards (100 ng) of the steroid hormones and SIC reconstituted in 500 μL chloroform:heptane (10:90) on top of freshly prepared silica gels (1.0 g) eluted with different compositions of heptane and acetone (Electronic Supplementary Material Table S3). These experiments show that the SIC were removed from the silica gel with 5 mL heptane:acetone (90:10), while the steroid hormones were partly eluted with 5 mL heptane:acetone (80:20) and quantitatively eluted with 5 mL heptane:acetone (65:35). In biological samples, cholesterols may be found in the microgrammes per gramme to milligrammes per gramme range [13, 14], which are much higher concentrations than the concentration range for steroid hormones (nanogrammes per gramme). Therefore, fractionating these SIC from the steroid hormones is necessary in order to avoid derivatization problems and overloading of the GC-MS/MS system.

This lipid fractionation procedure can also be used for studying the relationships between the steroid hormone levels and the specific lipid class concentration (with a proper lipid quantification method), as the lipid recoveries presented here and by Kaluzny are quantitative.

Finally, the aminopropyl and silica gel cartridge were validated in a combined procedure applying 100 μL chloroform containing 100 ng of each analyte and SIC. The results demonstrate that steroid hormones were retained in the silica gel (clean-up 2) when applying 5 mL heptane for washing, followed by 10 mL heptane:acetone (90:10), while the SIC were completely eluted when applying these solvents (Table 3). Lastly, the steroid hormones were quantitatively eluted with a single volume of 5 mL heptane:acetone (65:35), as demonstrated by a second 5-mL elution with the same solvent showing only traces of steroid hormones. Unexplainable problems with PRO were encountered at this stage with 87% recovered in the 10-mL heptane:acetone (90:10) eluate. Yet, this was ignored at this stage and considered as an artefact

Table 3 Absolute recoveries (% ±SD, *n*=3) of steroid hormones and surrogate interference compounds (SIC) from the optimized clean-up procedure combining aminopropyl cartridge (clean-up 1) with silica gel (clean-up 2) according to Fig. 1. A sequential elution procedure was utilized, applying 5 mL chloroform: isopropanol (2:1) for the aminopropyl cartridge and washing the silica gel with 5 mL heptane prior to the final elution of analytes with different mixtures of heptane: acetone according to the table. One hundred nanogrammes (in 100 μL chloroform) of each analyte was spiked on top of the aminopropyl cartridge

Eluate (hep:ace)[a]	10 mL (90:10)	5 mL (65:35)[b]	5 mL (65:35)[c]
Progestagens			
PRE	23±5	42±20	0
PRO	87±2	5±2	0
Androgens			
DHEA	NA	NA	NA
AN	6±2	67±3	4±0.4
TS	2	68±22	0
DHT	14±4	56±13	0
Estrogens			
E1	14±4	75±30	5
αE2	1±0.2	94±14	5±1
βE2	0	96±29	6±2
SIC			
SITO	88±19	0	0
CHOL	149±28	0	10
COPs	78±12	1	3

NA not applied in the current study

[a] Mixture of heptane:acetone by volume given in parenthesis

[b] First 5 mL aliquot

[c] Second 5 mL aliquot from the same silica gel

and was confirmed in the later successful entire method validation (discussed later).

Solid-phase extraction evaluation

Prior to clean-up steps 1 and 2, a sample enrichment and initial clean-up were performed using C_{18} SPE cartridges as previously used to enrich steroid estrogens from various aqueous matrices and published in several papers [15–18]. This procedure was verified, and absolute recoveries of the steroid hormones were obtained to be between 78% and 120% at 10 ng spike level (Electronic Supplementary Material Table S4), demonstrating that the C_{18} SPE successfully can be applied for extracting not only estrogens but also progestagens and androgens from samples.

Validation and quality assurance of the final procedure

In order to assure the total quantitative transfer of analytes from matrix to GC vials along with assessment of potential (adverse) influence of matrix components, the absolute and relative recoveries were determined using spiked charcoal stripped bovine serum. The charcoal stripped bovine serum free of steroid hormones made it suitable for spike recovery experiments. The samples were spiked with analytes and IS prior to sample preparation, and other identical samples were spiked after sample preparation prior to derivatization. Thereby, the absolute recoveries could be obtained along with the relative recoveries for the analytes relative to the IS [11, 19–21]. Results for the absolute and the relative recoveries for the steroid hormones in question for carbon stripped bovine serum are shown in Table 4. Three different concentration ranges were investigated by spiking 10 mL stripped bovine serum with 1, 10 and 20 ng. Absolute recoveries for the steroid hormones varied from 50% to 112%, and the relative recoveries were all in the range 81–127% with the exception of AN spiked at 0.10 ng/mL (201±113%) and 2.00 ng/mL (191±29%) and PRO spiked at 2.00 ng/mL (157±54%). The deuterated internal standards provided good relative recoveries and thereby ensured a high analytical reliability. The repeatability was investigated at 0.10 and 2.00 ng/mL levels by injecting the same standard six times, giving relative standard deviations of 5–22% and 1–8%, respectively.

A quality assurance (QA) system was established to ensure reliable analytical results. This QA system is a compilation of the integrated and extracted data from the GC-MS/MS system, i.e. retention time, peak area of qualifier and quantifier transition ions of all analytes. Samples with deviations higher than 50% on the ICS and DCS peak areas were discarded from the current result scheme, and if required, samples were re-analyzed, or if applicable, the sample preparation was performed again from the beginning. Furthermore, the following identification criteria were set for each analyte in the samples compared to a 1.00-ng/mL reference standard: (a) the retention time were never allowed to deviate more than 2% and (b) the deviation on the peak area ratio between the qualifier and the quantifier ion transitions should be less than 50%. These criteria were rarely triggered by the GC-MS/MS system. Most often, any problems could be tracked back to standard GC maintenance procedures (inlet liner exchange and column cut) or MS ion volume cleaning for every 100 injections.

Applications

The developed method was applied on male rats to determine natural occurring levels of steroid hormones in blood plasma. These determinations were compared to other studies available in the literature using RIA and are outlined in Table 5. Overall, the steroid

Table 4 Recoveries (% ±SD) from 10 mL bovine serum (*n*=3), following the entire optimized methodology, spiked with 1, 10 and 20 ng of individual steroid hormones. The deuterated internal standards (IS) are as well spiked at the same three levels (i.e. 1, 10 and 20 ng). The IS used for PRE is dTS; for DHEA, dDHT is used and for αE2, dE2 is used

	Absolute			Relative		
	0.10 ng/mL	1.00 ng/mL	2.00 ng/mL	0.10 ng/mL	1.00 ng/mL	2.00 ng/mL
Progestagens						
PRE	91±27	74±14	93±16	81±45	95±16	103±27
PRO	68±43	50±13	60±46	110±46	91±22	157±54
Androgens						
DHEA	80±20	77±10	78±6	127±30	106±15	107±20
AN	70±46	67±12	90±18	201±113	101±21	191±29
TS	112±26	90±10	107±16	100±45	115±13	118±27
DHT	53±38	64±8	79±22	84±44	88±14	109±29
Estrogens						
E1	89±18	65±3	55±4	97±27	89±6	114±9
αE2	83±16	62±4	62±9	104±21	84±6	98±13
βE2	84±11	65±4	66±6	105±18	89±6	104±11
Internal standards (IS)						
dPRO	61±17	55±18	38±28			
dAN	35±103	66±17	47±23			
dTS	112±36	78±8	91±21			
dDHT	63±22	73±11	73±19			
dE1	92±21	73±5	48±8			
dE2	80±14	73±5	63±9			

hormone concentrations in natural samples demonstrated that the levels were somewhat in the same range as those obtained with the RIA methodologies [22–25]. However, plausible cross-reactivities for the progestagens (PRE and PRO) can be drawn from Table 5. It was not possible to find immunoassays in the literature used for determination of DHEA and DHT in rat plasma. In addition to rats, the developed method has been used, successfully, for a number of samples from other vertebrate taxonomic groups (data not shown), such as Japanese quail (*Coturnix coturnix japonica*) and African clawed frog (*Xenopus laevis*).

Conclusions

The developed method allows for the detection of nine key steroid hormones expressed in the steroidogenesis (two progestagens, four androgens and three estrogens) in blood, plasma and serum samples down to 0.09 to 0.43 ng/mL (depending on the steroid hormone). These low limits of detection are possible due to an efficient clean-up procedure separating interfering lipid classes from the steroid hormones obtained with quantitative absolute recoveries in the range 53–112% at 0.10 ng/mL. Furthermore, the combination with tandem mass spectrometry (SRM) detection yielded extremely low background noise and therefore good signal-to-noise ratios of

the steroid hormones. This methodology has successfully been applied on blood plasma samples from different vertebrate taxonomic groups, i.e. bird, canine, pigs, rats and frog studies.

Table 5 Natural concentration levels of steroid hormones present in blood plasma (nanogrammes per millilitre) from male rats (*n*=3) applying the optimized method and compared to other studies utilizing RIA methods

	Present study	Other studies
Progestagens		
PRE	1.58±0.17	3.4±0.1 [24]
PRO	ND	5.35±0.32 [22]
		3.2±0.2 [24]
Androgens		
DHEA	11.3±3.4	
AN	0.38±0.14	0.13±0.08 [23]
		0.43±0.09 [25]
TS	6.38±0.38	1.67±0.12 [23]
		3.75±0.49 [22]
		2.64±0.37 [25]
DHT	0.16±0.03	
Estrogens		
E1	1.86±0.43	0.24±0.01 [22]
αE2	ND	
βE2	0.85±0.23	0.14±0.06 [22]

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