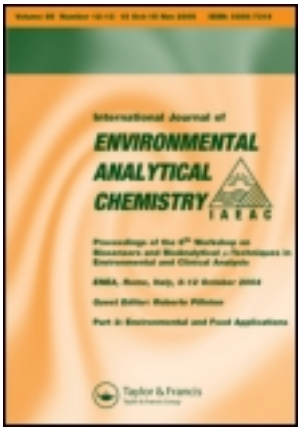

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Simultaneous determination of several natural steroids in blood plasma from perch (Perca fluviatilis) by GC-HRMS

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Simultaneous determination of several natural steroids in blood plasma from perch (*Perca fluviatilis*) by GC-HRMS

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A gas chromatography-high resolution mass spectrometry (GC-HRMS) procedure for the simultaneous determination of 18 endogenous steroid hormones in blood plasma from teleost fish has been developed. Proteins were removed by precipitation in methanol and lipids were removed by a liquid–liquid extraction. The protein and lipid free extract was further purified by using two successive solid phase extraction (SPE) methods (C18 and NH_2). The isolated steroid hormones were silylated with a mixture of N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA): iodotrimethylsilane (TMSI):dithioerythritol (DTE) prior to determination with GC-HRMS working in selective ion recording mode. A volume of 200 μL blood plasma was sufficient for accurate determination of the steroid hormone levels, which enabled determination in individual fish. The detection limits ranged from 0.0003 to 0.2 ng g^{-1} blood plasma from perch (*Perca fluviatilis*), approximately 10 to 100 times lower then previously reported in this field. The recoveries for the entire procedure were in the range 58 to 150% with a variation, expressed as standard deviation (SD), below 10% with some exceptions. Despite the multi-step clean-up procedure, the intra-assay coefficient of variation, i.e. the within-day variation, for most steroid hormones was well below 14%. Finally, the procedure has been successfully applied to the determination of steroid hormones in blood plasma from female perch caught in two Swedish lakes.

Keywords: endocrine disruption; testosterone; GC-HRMS; silylation

1. Introduction

Endocrine disrupting substances (EDSs) and their effects on reproductive success among aquatic organisms have gained increased interest in society and the research community during the last decades [1–5]. The endocrine and reproductive effects of EDSs are believed to be due to their ability to mimic or antagonise the effect of endogenous steroid hormones or by disrupting the synthesis and metabolism of the steroid hormones and important hormone receptors [6].

Endogenous steroid hormones are produced in different tissues and glands through a series of enzymatic conversions of the precursor steroid cholesterol as shown in Figure 1. The carbon numbering system for the steroid skeleton according to IUPAC is shown in Figure 2. Following synthesis, the steroid hormones are excreted into the blood and distributed in the body. Changes in the circulating levels of steroid hormones may reflect disrupted hormone synthesis and explain reproductive disturbances. Studies on fish

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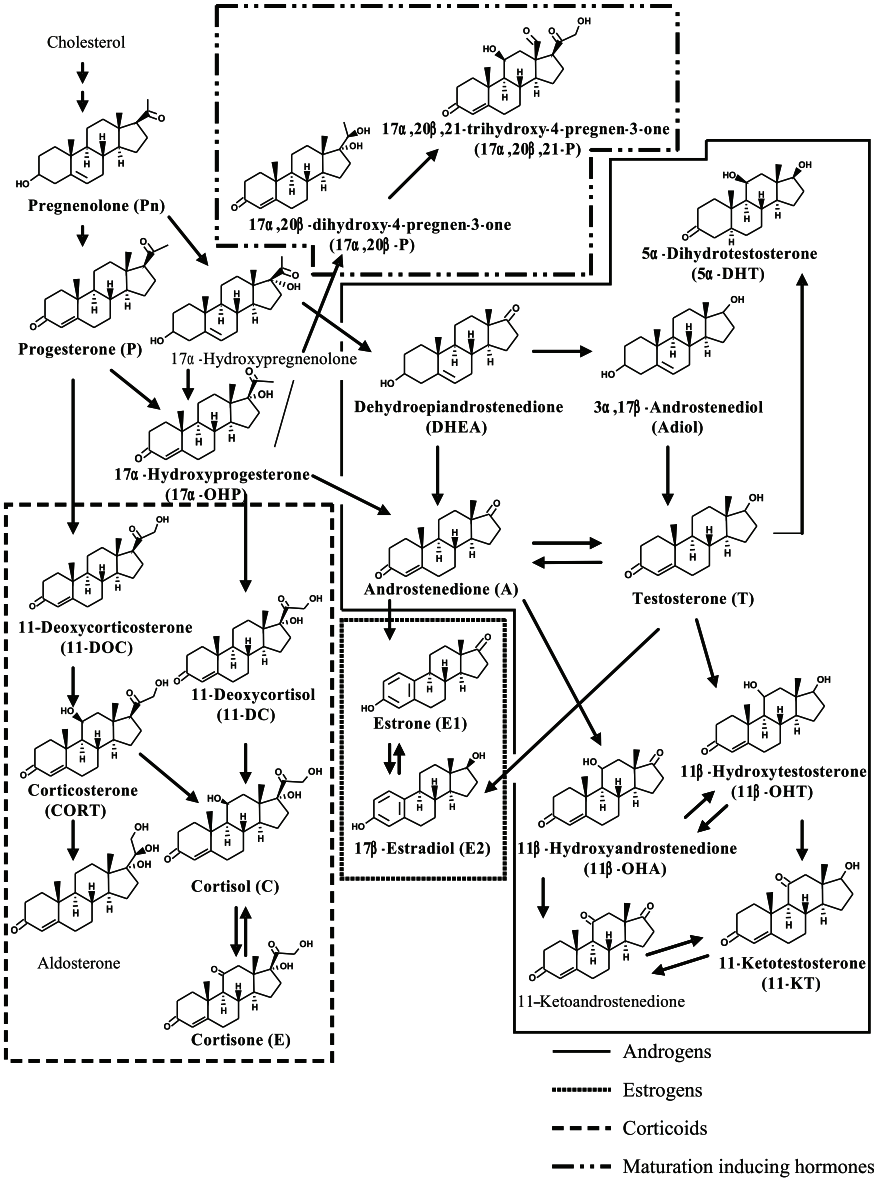

Figure 1. Biosynthetic pathways of steroids in female teleost fish. The steroid hormones are grouped as androgens, estrogens, corticoids and maturation inducing hormones (MIH) (marked with different lines) even though their function in fish does not fully correspond with the common definition of these groups. Structure, trivial or systematic name and abbreviation (in parentheses) are given for each steroid hormone. Analytes included in the procedure are indicated with bold text.

exposed to bleached kraft mill effluents [3,7], wastewater from refuse dumps [5,8–11], or tar sand process-contaminated water [12], have shown reduced circulating levels of testosterone (T) and 17β-estradiol (E2) in association with delayed sexual maturation.

An analytical procedure enabling the determination of a large number of natural steroid hormones in blood may thus be a useful tool for identifying possible effects of

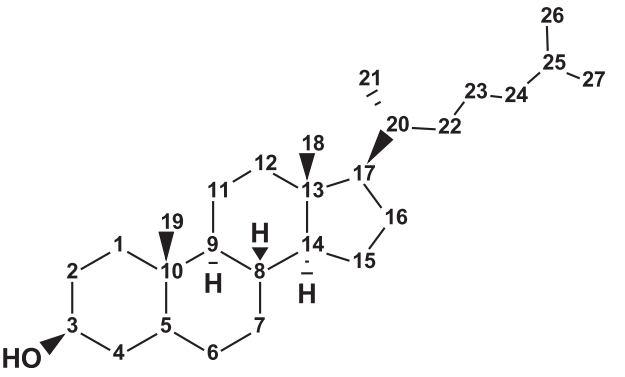

Figure 2. The carbon numbering scheme for the steroid skeleton according to IUPAC.

chemicals on steroid metabolism, as well as the mechanisms behind these disturbances. For instance, the build-up of one precursor steroid, in association with a decrease in a downstream steroid hormone, could indicate an inhibited enzyme activity.

Radio immunoassay (RIA) techniques have so far been the most frequently used methods for determining circulating levels of natural steroid hormones in fish [3,9,12]. Comparatively simple handling, relatively low cost and low limit of detection (LOD) are the most important reasons for the frequent use of RIA. Major drawbacks, however, are the limited number of steroids that may be determined in a single assay as well as the limited availability and specificity of the antibodies [13,14]. In combination with the often limited amount of available blood plasma, these drawbacks limit the number of assays that may be applied to a single fish, thereby limiting the number of steroid hormones that can be determined, often to less than five.

A couple of procedures for the determination of steroid hormones based on gas chromatography (GC) combined with mass spectrometry (MS) have recently been developed and applied in the field, e.g. in blood plasma from fish [15–18], in fish bile [18,19] and in fish muscle [20]. Chromatographic separation of the steroid hormones enables determination of a larger set of analytes (often >10) and improve the specificity compared to RIA. In most cases, however, the limit of detection reported for earlier GC-MS procedures is too high to handle the often small sample volumes available, which limit their usage for the determination of circulating steroid hormones in individual fish. Determinations on an individual basis provide information that in some cases is essential to distinguish between natural variations and disturbances induced by anthropogenic substances.

High resolution mass spectrometry (HRMS) is often used for determining low levels of analytes and could therefore be a useful tool in this field. GC-HRMS procedures have for years been used successfully for determining trace levels of anabolic steroids in, for example, human urine [21]. The high accuracy in measuring mass achieved with HRMS effectively separates compounds with nearly equivalent masses, resulting in lower limits of detection and more accurate determination [22].

The aim of this work was to develop a GC-HRMS procedure for the simultaneous determination of 20 natural endogenous steroid hormones (see Figure 1) in small volumes of blood plasma, to enable determination in individual female perch (*Perca fluviatilis*).

The procedure was applied to female perch caught in two Swedish lakes to investigate the steroid hormone levels.

2. Experimental

2.1 *Chemicals and material*

Unlabelled reference standards of 20 steroids were purchased from Sigma Chemical Company (St. Louis, MO, USA) [3α,17β-androstenediol (Adiol), dihydroepiandrosterone (DHEA), 5α-dihydrotestosterone (5α-DHT), estrone (E1), androstenedione (A), 17β-estradiol (E2), testosterone (T), 11-ketotestosterone (11-KT), 11β-hydroxyandrostendione (OHA), 11β-hydroxytestosterone (OHT), pregnenolone (Pn), progesterone (P), 17α-hydroxyprogesterone (17α-OHP), 11-deoxycortisol (11-DC), 11-deoxycorticosterone (11-DOC), cortisone (E), corticosterone (CORT), cortisol (C)]; Aldrich Chemical Company (Milwaukee, WI, USA) [17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-P)]; and Steraloids (Newport, RI, USA) [17α,20β,21-trihydroxy-4-pregnen-3-one (17α,20β,21-P)]. Deuterium labelled standards were purchased from Cambridge Isotope Laboratories (MA, USA) [testosterone 16,16,17-D_3, 17β-estradiol 2,4,16,16-D_4, cortisol 9,11,12,12-D_4 and pregnenolone 17,21,21,21-D_4] and Larodan Fine Chemicals AB (Malmö, Sweden) [Progesterone 2,2,6,6,17,21,21,21-D_8]. The ^{13}C-labelled polychlorinated biphenyl (PCB) standard, 2,2′,3,4,4′,5,5′-heptachlorobiphenyl (HpCB) (IUPAC #180) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). The silylation reagents N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) and dithioerythritol (DTE) were bought from Sigma Chemical Company (St. Louis, MO, USA) and iodotrimethylsilane (TMSI) from Fluka Chemie AG (Buchs, Switzerland). C-18 SPE cartridges (Supelclean^{TM} LC-18, 500 mg phase and tube volume 6 mL) and NH_2 SPE cartridges (Supelclean^{TM} LC-NH2, 500 mg phase and tube volume 3 mL) were purchased from Supelco (Bellafonte, PA, USA). Ethyl acetate and n-hexane were purchased from Scharlau (Barcelona, Spain). Acetonitrile and methanol was purchased from Merck (Darmstadt, Germany). All solvents were of analytical grade and the water used in the procedure was double-distilled. Stock solutions of individual steroids (20–50 mg mL^{-1}) and mixtures of steroids (0.5 mg mL^{-1}) were prepared in ethyl acetate. For the internal standard deuterium-labelled steroids were dissolved in methanol (100 ng mL^{-1}). All standard solutions were kept in the dark at 8°C and used within six months.

2.2 *Sample preparation*

The procedures for the sampling of perch and for plasma preparation have previously been described in detail by Noaksson *et al*. [10]. Briefly, perch were caught in gillnets and the blood was collected from the dorsal aorta by means of a heparinised syringe. The prepared blood plasma was instantly frozen in liquid nitrogen and stored at −140°C until analysis. Despite the addition of heparin during sampling, one in seven samples, randomly distributed, formed a gel during defrosting. The gel was insoluble in water and methanol, so to enable further processing the gel was manually homogenised with water in a Dounce homogeniser (Kontes Glass Co., Vineland, NJ, 7 mL). The samples were prepared according to Fritsche *et al*. [23] with some modifications. A schematic picture of the entire analytical procedure is presented in Figure 3.

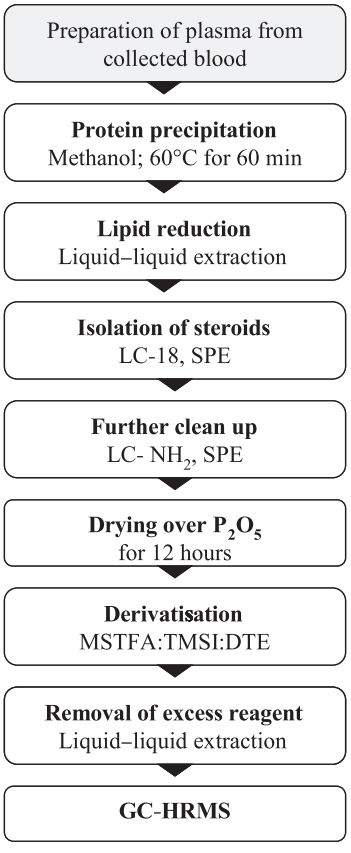

Figure 3. A schematic picture of the analytical procedure.

2.2.1 *Protein precipitation and removal of lipids*

Plasma collected from one individual (0.5–1 mL) was diluted with water to 2 mL. After addition of 7 mL methanol and 100 μL internal standard (100 ng mL^{-1}) the sample was placed in an oven at 60°C for 1 hour. The protein precipitate was removed by centrifugation at $2500g_{av}$ for 20 min and 2 mL of the protein-free supernatant was transferred to a new tube and diluted with 2 mL methanol : water (7 : 2). The methanol : water mixture was extracted twice with 200 μL n-hexane for removal of lipids.

2.2.2 *Isolation of steroids and clean-up*

The methanol : water mixture was evaporated in a vacuum centrifuge (RVC2-25 CD, CHRIST, Osterode am Harz, Germany) and the residual water, approximately 200 μL, was diluted with 6 mL water before application on the LC-18 cartridge. Prior to application of the sample, the cartridge was cleaned and preconditioned with 6 mL methanol followed by 6 mL water. After application the cartridge was washed with 2×2 mL water and 2×2 mL methanol : water (2 : 3). The cartridge was dried with air for 10 min before the steroids were eluted with 3 mL methanol, which then were evaporated

to dryness and the residue diluted to 4 mL with ethyl acetate : methanol (4 : 1). To further clean up the extract it was eluted through a LC-NH_2 cartridge. The cartridge was preconditioned with 4 mL water-saturated ethyl acetat and 4 mL ethyl acetat:methanol (4 : 1). Water-saturated ethyl acetat was prepared by adding 820 μL water to 100 mL ethyl acetat. After application of the extract, the cartridge was rinsed with 2 × 1 mL ethyl acetate : methanol (4 : 1). The total eluate was reduced to a suitable volume in the vaccum centrifuge and transferred to a 1.5 mL conical vial.

2.2.3 *Derivatisation*

The sample was then evaporated to dryness in the vacuum centrifuge and placed in a desiccator over P_2O_5 for 12 hour to remove remaining moisture. To the dry samples 20 μL derivatisation reagent mixture MSTFA : TMSI : DTE (1000 : 2 : 2; v : v : w) was added and the conical vials were sealed with a screw cap with a silicone-polytetrafluoroethylen (PTFE) septa and heated at 80°C for 120 min. The reagent mixture was prepared and stored in a dark bottle at 8°C and used within two weeks. Before injection on the gas chromatograph, excess silylation reagent was removed by a liquid–liquid extraction, as previously described by Vermeulen *et al*. [16]. The derivatised sample was transferred to a round-bottomed 3 mL test tube, and diluted with 2 mL n-hexane containing a volumetric standard, ^{13}C-labelled 2,2′,3,4,4′,5,5′-HpCB (IUPAC PCB #180, 0.5 μg mL^{-1}). The sample was extracted with 3 × 200 μL acetonitrile. The hexane phase was reduced in the vacuum centrifuge to 60 μL and transferred to a GC-vial with a 0.2 mL cone-shaped insert. A volume of 1 μL was injected on the gas chromatograph.

2.2.4 *Instrumentation*

The determination was performed on a HP6890 gas chromatograph (Hewlett Packard, Avondale, PA, USA) equipped with an HP6890 Series autosampler for on-column injections. It was coupled to an AutoSpec Ultima high resolution sector mass spectrometer (Micromass, Ultricham, UK). Four GC-capillary columns were tested: PTE-5 (30 m × 0.25 mm i.d., 0.25 μm film, 5% phenyl 95% dimethylarylenesiloxane), Equity-5 (30 m × 0.25 mm i.d., 0.25 μm film, 5% phenyl 95% dimethylarylenesiloxane) and SP2331 (30 m × 0.25 mm i.d., 0.20 μm film, 90% cyanopropyl 10% dimethylpolysiloxane) columns from Supelco, Bellafonte, PA, USA, and the OV-1 column (30 m × 0.25 mm i.d., 0.25 μm film, 100% dimethylpolysiloxane) from Ohio Valley Specialty Company (Marietta, OH, USA). The PTE-5 column with a 2 m × 0.53 mm i.d. retention gap (deactivated fused silica, Part. no 160-2535) from Agilent Technologies (Folsom, CA, USA), was finally chosen for the procedure. Helium (Alphagaz He, Air Liquide, Malmö, Sweden) was the carrier gas with a constant flow rate of 1 mL min^{-1}. The gas chromatograph oven was temperature programmed as follows: 70°C hold for 2 min; raised 20°C min^{-1} to 220°C and 7°C min^{-1} to 310°C; hold for 15 min. Electron ionisation (EI), 32 eV, was used and the resolution power was set to 10,000.

The m/z -values used for identification and quantification of each steroid hormone TMS-derivate are given in Table 1. The quantification ion (Q) (Table 1) was the molecular ion (M) or the M-15 fragment, representing the loss of a methyl group for most steroid hormone TMS-derivates. The only exceptions were for the derivates of corticoids with a dihydroxyacetone side chain, e.g. cortisol (Figure 1), which were quantified by their M-90 or M-105 fragment representing the loss of an additional methyl group as described in a previous study [20]. The volumetric standard HpCB #180 was identified

Table 1. Quantification (Q) and confirmation (C) ions for the studied steroid hormones and the deuterium-labelled steroid hormones used as internal standards.

Compound	Abbrev.	Ion Q (m/z)	Ion C (m/z)
Androgens			
3α, 17β-Androstenediol	Adiol	436.319	421.295
Dehydroepiandrosterone	DHEA	432.288	417.264
5α-Dihydrotestosterone	5α-DHT	434.303	419.280
Androstenedione	A	430.272	415.248
Testosterone	T	432.288	417.264
11-Ketotestosterone	11-KT	518.306	503.283
11β-Hydroxyandrostenedione	11β-OHA	518.306	503.283
11β-Hydroxytestosterone	11β-OHT	520.322	505.298
Oestrogens			
Estrone	E1	414.241	399.217
17β-Estradiol	E2	416.256	401.233
Corticoids			
11-Deoxycortisol	11-DC	634.372	619.349
11-Deoxycorticosterone	11-DOC	546.338	–
Cortisone	E	630.341	615.317
Corticosterone	CORT	634.372	619.349
Cortisol	C	632.356	617.333
Maturation inducing hormones (MIH)			
17α,20β-dihydroxy-4-pregnen-3-one	17α,20β-P	548.353	533.330
17α,20β,21-trihydroxy-4 pregnen-3-one	17α,20β,21-P	636.388	621.364
Pregnenolone	Pn	445.293	460.319
Progesterone	P	458.303	443.280
17α-Hydroxyprogesterone	17α-OHP	546.338	531.314
Deuterium-labelled standards			
17β-Estradiol 2,4,16,16-D_4	E2-D_4	420.281	405.843
Testosterone 16,16,17-D_3	T-D_3	435.306	420.281
Pregnenolone 17,21,21,21-D_4	Pn-D_4	448.315	463.338
Progesterone 2,2,6,6,17,21,21,21-D_8	P-D_8	464.341	449.318
Cortisol 9,11,12,12-D_4	C-D_4	636.381	621.364

with m/z 405.83. All chromatograms were recorded in selected ion recording (SIR) mode. Sector instruments show a considerable drop in sensitivity when scanning over a range of masses that is too wide; the largest mass recorded should not be more than 1.67 times larger than the smallest [24]. To limit the mass span in each scan cycle, the m/z values were divided into two windows according to the steroids' elution times from the GC column, window one (17–23.5 min) and window two (23.5–30 min). The scan time for each mass was 30 ms except for the lock mass that was scanned for 50 ms. The scan cycle time for window one was 1.3 s and for window two 1.14 s. Eight to ten data points were registered per chromatographic peak in both of the windows.

The final quantification of each steroid hormone was done with the isotope dilution methodology [25,26] and additional standard curves.

2.3 *Validation*

Blood plasma from 20 perch, both males and females caught in June 2001, was pooled and used for validation of the procedure. Portions of 1 mL pooled plasma were spiked

with 16 ng of each of the analysed steroid hormones, which were used for determining method recoveries and intra-assay coefficient of variations, i.e. within-day variations. Limit of detection (LOD) and limit of quantification (LOQ) were determined in natural blood plasma or plasma spiked at 0.5 ng. Plasma portions (1 mL), spiked at five different concentration levels in the range of 0.5–16 ng, were used for determining the linearity of the procedure.

3. Results and discussion

An analytical procedure for the simultaneous determination of 20 endogenous steroid hormones in blood plasma from teleost fish has been developed. The steroid hormones included (Table 1) are androgens (Adiol, DHEA, 5α-DHT, A, T, 11-KT, OHA, OHT), oestrogens (E1, E2), corticoids (11-DC, 11-DOC, E, C, CORT), Pn, P, 17α-OHP and two maturation inducing hormones (MIH) active in female teleost fish (17α,20β-P and 17α,20β,21-P).

Steroid hormones are found in blood plasma in both dissolved form and associated to transport proteins, mainly sex hormone binding globulin (SHBG). The strength of the steroid-protein association varies widely between different steroid hormones and fish species, but also throughout the annual reproductive cycle [27–29]. Studies of SHBG in human plasma have shown irreversible loss of the steroid-binding activity when heated at temperatures above 60°C [30,31]. Man *et al.* [32] suggested further that simple dilution with water (1 : 4) disrupt oestrogen-protein associations. Taking these observations into account the analytical procedure described in this paper, involving both dilution and heat treatment, was assumed to determine the total concentration of steroid hormones in blood plasma, both dissolved and protein-associated.

3.1 *Extraction and clean-up*

The formation of a gel, insoluble in methanol and water in some of the samples during thawing is a known problem when handling blood plasma from fish, especially perch, but it is yet not fully investigated and understood. Manual homogenisation of the gel in methanol : water (7 : 2) enabled further treatment according to the procedure. The concentrations and patterns of the steroid hormones in these samples agreed well with those found for non-coagulated samples, showing that the gel formation and additional homogenisation did not discriminate any analytes.

Prefabricated SPE cartridges with silica based LC-18 and LC-NH_2 phases were used for isolation of the steroid hormones and further clean-up mainly because of their well-defined and repetitive separation conditions. Simple handling and the opportunity for automatisation were also important factors. Elution volumes and eluate compositions were optimised with regard to good recovery of all relevant steroid hormones.

3.2 *Derivatisation*

A mixture of MSTFA : TMSI : DTE (1000 : 2 : 2; v : v : w), was used for silylation of the steroid hormones. The aliphatic hydroxyl functions reacted easily to tri-methyl-silyl (TMS)-ethers, while the keto-functions were converted to enols, before reaction with MSTFA to TMS-enol ethers as previously described [33]. The enolisation process is catalysed by TMSI.

All derivatisation conditions were optimised with regard to the highest and least variable GC-HRMS response for all steroid hormones in the standard. A set of reaction times (60–180 min) and temperatures (60–100°C), taken from the literature [23,33,34] were combined and tested. The silicon-PTFE septa on the derivatisation vials leaked at temperatures ≥100°C, which determined the upper temperature limit used in the test. The highest yield and lowest variation were achieved when the derivatisation reagent/sample mixture was heated at 80°C for 120 min.

The silylation procedure was efficient, but on days with high humidity (i.e. summer days with humidity >60%) the yield decreased remarkably, especially for the ketosteroids. This indicated that TMSI was negatively affected by moisture, i.e. it reacted with water before it was able to exert its catalytic effect. The problem was reduced, however, by drying the samples over P_2O_5 for 12 hours under argon prior to derivatisation.

To minimise the negative effects of unreacted silylation reagent on the background levels and on the performance of the mass spectrometer, the excess reagent was removed by a liquid–liquid extraction before introduction of the sample into the gas chromatograph. The loss was <2% for all steroid hormones in this step.

3.3 *GC-HRMS*

To achieve the best possible separation of all steroids, which represent a wide span of properties, four capillary columns with different polarities were tested SP2331, OV-1, Equity-5 and PTE-5. Of these the PTE-5 column gave the best result, separating 18 of the 20 steroid TMS-derivates satisfactorily and providing the best separation of A, T and E2, which continue to be the most commonly determined steroids.

To retain the good separation the pre-column was changed once a month (i.e. after approximately 100 injections of real samples) or as soon as the peak shapes were visibly affected. The PTE-5 column was changed after approximately a year.

A typical HRMS total ion chromatogram (TIC) for a standard solution (100ng mL^{-1}, 1 μL injected) monitored in SIR mode following separation on a PTE-5 column, is shown in Figure 4a and 4b. T and E2 are not baseline separated, but having different diagnostic masses they could still be determined separately with good accuracy (Figure 4a). During the silylation procedure P and Pn each reacted to two chromatographically separable TMS-derivates. The registered peak profile was reproducible and could be used to identify the peaks as previously reported [34]. Both of the TMS derivates showed good linearity with $R^2 > 0.997$ over the investigated concentration range (0.01–300 ng mL^{-1}). Only the most abundant derivate, the first peak eluted, was used for quantification. Silylation of 11-KT resulted in two non-separable TMS derivates, in this case the sum of the peak areas was used for quantification.

C and CORT are not baseline separated (Figure 4b) and their fragmentation patterns overlap, i.e. they give fragments with identical masses, which obstruct individual determination. In spite of this, it is possible to determine them separately as long as they occur in about the same concentrations. In blood plasma from perch, however, the concentration of C was normally much higher, overwhelming the CORT peak. Separate determination of C and CORT in blood plasma requires a different chromatographic system than the one tested here.

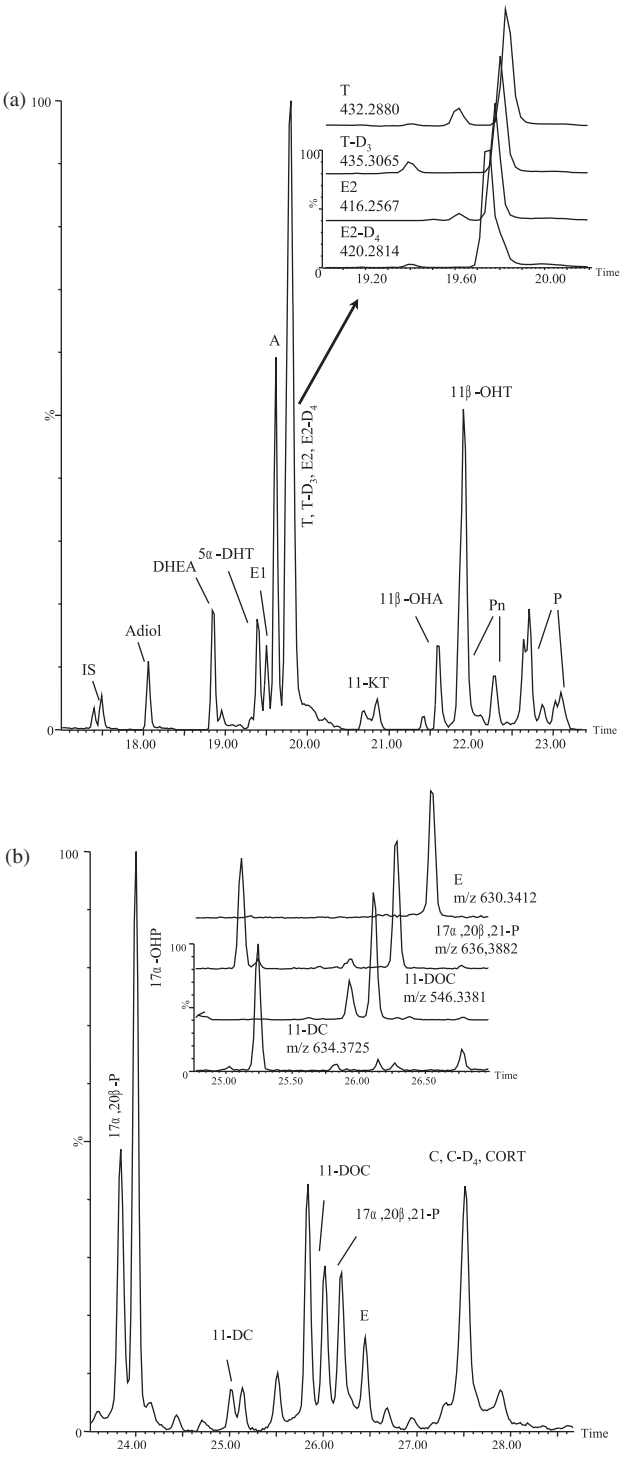

Figure 4. A typical GC-HRMS total ion chromatogram (TIC) for a steroid hormone mixture (100 ng/mL, 1 μL injected) after silylation with MSTFA:TMSI:DTE (100:2:2, v:v:w) recorded in selected ion recording (SIR) mode.

3.4 *Method validation*

Studies of blood plasma from perch collected once a month during a 14-month period showed that plasma collected in June, right after spawning, was the most suitable for method validation. In June the circulating levels of T and A, the dominant steroid hormones except for C, decreased to a minimum, which resulted in a narrower concentration span between the different steroid hormones [10].

3.4.1 *Linearity and repeatability*

The linearity for the whole procedure obtained with plasma samples spiked at different levels, 0.5–16 ng steroids added, was good, with R^2-values between 0.980 and 0.995.

The intra-assay coefficient of variation (intra-assay CV; within-day variation) for most steroid hormones was lower than 14%, 3/5 were well below 10% (Table 2). E1, OHA and 17α, 20β-P showed a slightly larger variation, 20%, which reflected the somewhat lower sensitivity for those compounds in the procedure.

Table 2. Limit of detection (LOD), limit of quantification (LOQ), intra-assay coefficient of variation (within-day variation) and recoveries obtained from spiked blood plasma samples.

Compound	LOD (S/N > 3) ng/g plasma	LOQ (S/N > 10) ng/g plasma	Intra-assay COV %[a]	Recovery % Average ± SD[b]
Androgens				
Adiol	0.007	0.02	6	85 ± 6
DHEA	0.003	0.01	4	93 ± 4
5α-DHT	0.003	0.01	4	125 ± 5
A	0.0003	0.001	2	104 ± 1
T	0.002	0.01	2	101 ± 2
11-KT	0.01	0.03	10	90 ± 4
11β-OHA	0.002	0.01	21	121 ± 14
11β-OHT	0.004	0.01	12	102 ± 3
Oestrogens				
E1	0.004	0.01	20	150 ± 28
E2	0.002	0.01	2	96 ± 18
Corticoids				
11-DC	0.2	0.8	8	67 ± 3
11-DOC	0.01	0.04	14	80 ± 10
E	0.04	0.1	14	61 ± 21
C/CORT	0.01	0.03	4	148 ± 23
MIH				
17α,20β-P	0.007	0.02	20	89 ± 7
17α,20β,21-P	0.02	0.06	10	73 ± 9
Pn	0.003	0.01	11	58 ± 8
P	0.03	0.1	7	103 ± 3
17α-OHP	0.005	0.02	16	84 ± 9
Samples (n)	(2)	(2)	(5)	(4)

[a]Coefficient of variation (COV) = (standard deviation/average)*100 (%) based on calculations of obtained concentrations.
[b]Standard deviation (SD).

Cholesterol, the precursor to endogenous steroid hormones, had an intra-assay CV >70% and was excluded from the procedure. Cholesterol is slightly more lipophilic because of the larger side chain (Figure 2) compared to the steroid hormones and may be lost during the hexane-methanol : water extraction or in the LC-18 SPE.

3.4.2 *Limits of detection, limits of quantification, blanks and sample volume*

LODs, defined as S/N > 3, were calculated for 1g blood plasma yielding 60 μL extract of which 1 μL was injected into the gas chromatograph. The LODs ranged from 0.0003 to 0.2 ng g^{-1} blood plasma (Table 2), of which a majority of the values were between 0.002 and 0.04 ng g^{-1} blood plasma, for example T 0.002 ng g^{-1} and P 0.03 ng g^{-1}. These values are 50 to 100 times lower than previously reported for fish blood plasma [15,18] and for fish muscle [20]. LODs obtained by Budzinski *et al.* [18] in blood plasma from trout ranged from 0.1 ng g^{-1} for T, A, E1 and E2 to 0.4 ng g^{-1} for OHP.

The LOD for 11-DC was 100 times, and for E 20 times higher than the LOD for T. Corticoids contain from three to six oxygen-containing functions, all of which are silylated [23]. This results in TMS derivates that are heavy, have numerous isomers and give EI fragmentation patterns with a large number of equally intensive fragments. The latter two issues make peak identification more difficult and cause increased LODs because of decreased S/N ratios.

LOQs, defined as S/N >10, ranged from 0.001 ng g^{-1} for A to 0.8 ng g^{-1} for 11-DC (Table 2).

No relevant signal, i.e. above LOD, was registered for any of the steroid hormones in the method blanks.

By processing three different volumes of unspiked pooled plasma, 0.01, 0.1 and 0.8 mL, and comparing signal to noise ratios (S/N) for all steroid hormones above the limit of quantification (LOQ, S/N >10), it was shown that 0.1 mL sample gave the same steroid profile as 0.8 mL, except for 11-DC. This led to the conclusion that 0.1 mL is sufficient for the determination of steroid concentrations. For the determination of the four most dominant steroid hormones T, A, C/CORT, and 11β-OHA with S/N >10, 0.01 mL blood plasma from perch was sufficient (Table 3).

3.4.3 *Recovery*

Recoveries for the entire procedure for all steroid hormones are shown in Table 2. They ranged from 58 to 150% with a variation, expressed as standard deviation (SD), below 10% except for E1, E2, OHA, E and C/CORT (28%, 18%, 14%, 21% and 23%, respectively). The recovery of Pn was 58% but due to the low variation (8%) the procedure could still be used to gain valuable information about its presence and variation in blood plasma. The high recovery (150%) and slightly larger variation (28%) obtained for E1 are probably caused by insufficient separation. Blood plasma contains, in addition to steroids not identified in this procedure, other compounds with steroid-like structures and properties, e.g. vitamins [35], which may interfere with the steroids of interest.

Recoveries of the internal standards (E2-D_4, T-D_3, Pn-D_4, P-D_8 and C-D_4) were 98–108% for the entire procedure in the absence of plasma, with variations below 10% ($n = 6$). When plasma spiked with the internal standards was processed, the recoveries of Pn-D_4 (51 ± 31%) and P-D_8 (187 ± 43%) were more strongly affected by the presence

Table 3. Signal to noise ratios (S/N) for all studied steroid hormones obtained by processing different amounts of blood plasma from female perch (*Perca fluviatilis*) caught in June.

	Signal to noise ratio (S/N)		
	mL plasma processed		
Compound	0.01[a]	0.1	0.8
Adiol	–[b]	–	–
DHEA	–	3	7
5α-DHT	–	3	6
A	30	40	200
T	200	300	700
11-KT	13	10	20
11β-OHA	50	80	400
11β-OHT	–	30	40
E1	–	–	–
E2	3	5	5
11-DC	–	–	3
11-DOC	–	3	6
E	10	10	80
C/CORT	40	100	2500
17α,20β-P	–	4	8
17α,20β,21-P	–	–	–
Pn	–	10	30
P	–	–	–
17α-OHP	–	–	–

[a]The sample volume was reduced to 20 μL instead of 60 μL prior to determination; 20 μL is the minimum volume required for sample introduction by a HP6890 Series autosampler for on-column injections.
[b]No matching peak found or S/N < 3.

of the plasma, than the recoveries of E2-D_4, T-D_3 and C-D_4 ($88 \pm 15\%$; $109 \pm 12\%$; $143 \pm 27\%$; $n = 4$).

Insufficient separation and variations in the stability of the deuterium labelling during the derivatisation and ionisation procedure may explain the results for Pn-D_4 and P-D_8. The observations agree with previous results obtained for a P-D_9-standard [21]. Because of the unsatisfactory recovery, Pn-D_4 and P-D_8 were excluded from future work and both P and Pn were satisfactory determined with reference to T-D_3.

4. Environmental application

This procedure has successfully been used in a study of the annual variations in circulating levels of the natural steroid hormones T, A, E2, E1, 11-KT, 17α-OHP, P, 11-DC, C/CORT and 17α,20β-P, in blood plasma from female perch caught in two Swedish lakes: Lake Molnbyggen, which was for many years exposed to leachate from a local municipal refuse dump, and Djursjön, located in the same area but connected to another water system.

Table 4. Circulating levels of steroid hormones obtained in blood plasma from individual female perch collected in April 2001. The levels are shown as ng steroid hormone/g blood plasma.

Compound	DHEA	5α-DHT	E1	A	E2	T	11-KT	11β-OHA	Pn	P	17α,20β-P	17α-OHP	11-DC	17α,20β,21-P	E	C/CORT
Djursjön																
1	trace[a]	trace	1.9	6.4	trace	29	trace	4.1	0.43	trace	0.30	trace	trace	trace	16	[b]
2	trace	trace	3.5	4.7	3.4	45	trace	2.5	0.44	trace	0.47	trace	trace	trace	31	187
3	trace	trace	1.2	5.5	1.0	49	trace	3.2	0.24	trace	0.15	trace	trace	trace	7.6	211
Mean			2.2	5.6	2.2	41		3.2	0.37		0.31				18	199
Molnbyggen																
1	trace	trace	1.4	4.4	trace	15	trace	5.3	0.26	trace	0.37	trace	trace	0.60	17	[b]
2	trace	1.0	2.7	3.0	trace	16	trace	4.3	0.48	trace	0.26	trace	trace	0.70	10	348
3	trace	0.1	2.0	1.7	trace	9	trace	3.6	0.27	trace	0.28	trace	trace	0.51	19	260
Mean		0.59	2.0	3.0		13		4.4	0.34		0.30			0.60	15	304

[a]Detected but showed signal to noise ratios below 10.
[b]The intensity of the signal was out of range.

A detailed descripton of the study and the results thereof have been published by Noaksson *et al.* [10]. A volume of 200 μL blood plasma from six individual female perch, three from each lake sampled in April, were re-analysed looking at all 18 steroid hormones included in the analytical protocol. The determined circulating levels, ng steroid hormone/g blood plasma, are given with two or three significant figures in Table 4. In April, 11 endogenous steroid hormones could be quantified ($S/N > 10$) and an additional five (DHEA, 11-KT, P, 17α-OHP and 11-DC) could be detected ($S/N > 3$), Table 4, out of the 18 included in the analysis. The steroid hormone levels in April are except for T and C, very similar irrespective of where the perch were caught.

5. Conclusions

The proposed procedure has proved to be suitable for the separate determination of the circulating levels of 18 endogenous steroid hormones in blood plasma from female perch. Because of co-eluted peaks and overlapping fragmentation patterns it was not possible to determine C and CORT separately in the chromatographic system tested here. Satisfactory recoveries were obtained for all recorded analytes, despite the large number of clean-up steps. The efficient clean-up, in combination with HRMS, showed LODs 10 to 100 times lower than previously reported in this field. The low LODs enable determination of hormone levels in small volumes of sample, which makes it possible to investigate hormone levels on an individual level in smaller organisms than were previously studied.

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