Development of a liquid chromatography–tandem mass spectrometry method for the determination of 23 endogenous steroids in small quantities of primate urine

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Abstract

A quantitative method using liquid chromatography–tandem mass spectrometry (LC–MS–MS) was developed for the simultaneous determination of 23 endogenous steroids in primate urine. The introduced method includes estrone, pregnandiol, cortisol, testosterone and several human urinary glucocorticoid and androgen metabolites. As the method is intended for the analysis of steroid hormones in behavioral studies on wild-living primates, it was adapted for a sample volume of 200 μL urine. The sample preparation consisted of an enzymatic hydrolysis of steroid glucuronides using β-glucuronidase from *E. coli* followed by a solvolytic cleavage of steroid sulfates employing sulfuric acid/ethyl acetate. The extraction of steroids from urine was optimized with respect to pH during extraction, type of ether and the amount of enzyme necessary for complete hydrolysis of glucuronides. The recovery of steroids spiked into urine before hydrolysis was 58.9–103.7% with an intra-day precision of 2.7–14.3% and an inter-day precision of 2.9–14.8%. Detection limits ranged from 0.1–0.5 ng/mL. The reproducibility of the whole sample preparation process was also demonstrated for unspiked urine (CV 1.2–16.5%). The proportion of steroid hormone excreted as sulfate was determined for 21 steroids in chimpanzee urine. The solvolysis proved to be essential for all investigated steroids except for pregnandiol, tetrahydrocortisol and tetrahydrocortisone, which were found to be less then 10% in the solvolysis fraction.
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1. Introduction

The analysis of steroid hormone concentrations in non-invasively collected samples has become increasingly important in a variety of behavioral ecology study contexts such as the evaluation of reproductive status, the impact of stress or the expression of aggression [1–4]. With respect to non-human primates, hormonal analyses have been carried out mostly with radioimmunoassays (RIA) or enzyme immunoassays (EIA). These methods have proven to be cost-effective and sensitive, but they have limitations with respect to specificity [5]. An important point is that the assays can show cross-reactivities of the specific antibody with other steroids that have different physiological functions than the hormone of interest [6,7]. For example,

a group-specific antibody raised against 11-oxoetiocholanolone has been suggested for monitoring adrenocortical activity in feces of several primate species [7]. However, this assay has 4.8% cross-reactivity towards etiocholanolone, which is a quantitatively important metabolite of testosterone. Given the great number of steroid metabolites present in urine, the determination of all relevant cross-reactivities is a difficult task. Therefore, the identity and the origin of the immunoreactive metabolites responsible for the response of the assay remain partially unclear. These uncertainties can lead to controversial results in behavioral endocrinological studies. For instance, the use of a cortisol-directed antibody has been described for monitoring stress reactions in fecal extracts of chimpanzees [8]. However, as known from a radio metabolism study, native cortisol is virtually absent in the feces of the macaque and chimpanzee [9]. Therefore, the response measured must be exclusively caused by cross-reacting metabolites, whose identity is not exactly known. Because different behavioral ecologists use different

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antibodies with different and often not thoroughly described cross reactivities, the hormonal data are difficult to compare and every author has to validate his assay for the species under study. Similarly, the analysis of testosterone concentrations in blood, urine or feces has to be evaluated carefully when using immunoassays. A comparison of ten commercially available immunoassays for testosterone revealed strongly divergent results in sera of women and children, when compared to gas chromatography–mass spectrometry as a reference method [5]. The metabolism of testosterone is characterized by a high number of metabolites, whose relative proportions can vary considerably among different species [10,11]. A radio metabolism study of testosterone [10] indicated the presence of at least five testosterone metabolites in chimpanzee urine, whereas native testosterone represented only 4% of administered radioactivity. Testosterone measurements are further complicated by the fact that C21-glucocorticoids are also metabolized down to androgen-like C19-structures, which could cross-react with testosterone-directed antibodies [6].

In order to overcome these difficulties, first, suitable marker compounds have to be identified for monitoring hormonal changes influencing behavior in a certain species. Then, these specific markers can be measured combining high pressure liquid chromatography with mass spectrometric detection, in order to be able to distinguish structurally similar metabolites. Within LC–MS/MS, a compound is characterized by its retention time on the chromatographic column, its parent mass deduced from its molecular weight, and two specific fragments, which have to be present in a certain ratio. Unless, there is no co eluting isobaric stereoisomer, the response is highly compound-specific. One main advantage of this approach is the possibility to measure very specifically many steroid hormones in parallel within one analysis, including different metabolites of the compound of interest. Furthermore, the dynamic range of the calibration spans four orders of magnitude instead of two for EIA or RIA, thus eliminating the need of measuring several dilutions of the same sample in order to meet the linear part of the calibration curve. Because of these characteristics, LC–MS/MS is a well-established technique for the analysis of steroid hormones in doping analysis of urine [12–16], the characterization of metabolic disorders in plasma or urine of humans [17–19] and the detection of illegal use of veterinary drugs in animal excrements [20,21]. A comprehensive review of profiling steroid hormones in plasma and urine by GC–MS and HPLC was published by Shackleton [22].

Thus far, studies on steroid hormone profiles of non-human primate urine employing mass spectrometric techniques are scarce. In one study, urine extracts were directly infused into the mass spectrometer without prior chromatographic separation of steroids [11], which did not enable exact quantification. Other authors obtained immunograms by chromatographic separation of urine extracts, collection of fractions and testing of these fractions in different immunoassays [9,10], but a mass spectrometric characterization, and therefore, clear identification of separated steroids, was not carried out. The aim of this study was to develop a multi-method for the quantitative analysis of glucocorticoids (cortisol and metabolites), androgens (testosterone and metabo-

lites), estrogens (E1, E2 and E3) and gestagens (progesterone and pregnandiol) in primate urine. Steroid hormone analysis with this method should be able to address questions about primate stress response to social or environmental factors, studies of dominance rank and aggression, as well as species comparisons with respect to androgen levels, and ovarian cycle profiles in relation to reproductive strategies.

Because samples have to be aspirated from vegetation or ground when collecting urine from wild-living primates, the volume that can be obtained is limited. Therefore, in contrast to already existing methods, we adapted our method to minimal quantities of urine.

2. Experimental

A number of quantitatively important human steroid hormones and several endocrinological markers known from behavioral ecology studies on great apes were chosen for implementation into the method.

In order to evaluate chromatographic interferences of isobaric androgen isomers, a great number of human testosterone metabolites were included in the method. We also implemented several human cortisol metabolites (tetrahydrocortisol, tetrahydrocortisone and α-cortol) in order to evaluate their relative abundances in primate urine and the potential to use them as additional markers for stress response. Furthermore, 11-oxoetiocholanolone was included, as it seems to be a major cortisol metabolite in urine and feces of the common marmoset, the macaque and the chimpanzee [9]. Because an EIA directed against 5β-androstane-3α, 11β-diol-17-one (11β-hydroxyetiocholanolone) has been described for monitoring adrenal activity in the African elephant [6], as well as in feces of ruminants [23] and chimpanzees [7], we examined this metabolite within our method. For characterizing ovarian function and estimating of the timing of ovulation the estrogen estrone, and the progesterone metabolite, pregnandiol, can be used in chimpanzees [24,25]. Additionally, the estrogens estradiol and estriol, were analyzed here.

The chromatography was optimized in order to achieve baseline separation of isobaric steroids. The best pair of precursor and fragment ion was chosen and mass spectrometric parameters were optimized, resulting in the highest possible response. Based on published procedures, the sample preparation was validated with respect to: (1) pH during extraction and use of *tert*-butyl methyl ether instead of diethyl ether for extraction of steroids; (2) amount of β-glucuronidase from *E. coli* used for hydrolysis of urine; (3) recovery and detection limits of steroid hormones spiked into urine before hydrolysis; (4) reproducibility of hydrolysis and solvolysis; (5) percentage of steroid hormone found after solvolysis (proportion of sulfates) in order to determine the necessity of this step for human and chimpanzee urine.

2.1. Chemicals and reagents

Steroid reference materials were obtained from Steraloids (Newport, Rhode Island, USA): 1,3,5(10)-estratrien-3,16α,17β-

triol (estriol, E3); 1,3,5(10)-estratrien-3ol-17-on (estrone, E1); 3, 5(10)-estratrien-3, 17β-diol (β-estradiol, E2); 5β-pregnan-3α, 11β, 17, 20α, 21-pentol (α-cortol); 4-pregnene-11β, 17, 21-triol-3, 20-dione (cortisol, C); 5β-Pregnan-3α, 11β, 17, 21-tetrol-20-one (tetrahydrocortisol); 5α-pregnan-3α, 11β, 17, 21-tetrol-20-one (allotetrahydrocortisol); 5β-pregnan-3α,17α, 21-triol-11,20-dione (tetrahydrocortisone); 5β-androstane-3α, 11β-diol-17-one (11β-hydroxyetiocholanolone); 5β-androsten-3α-ol-11, 17-dione (11-oxoetiocholanolone, 11-oxo); 4-androstene-17β-ol-3-one (testosterone, T); 4-androstene-17β-ol-3-one 17-glucuronide (testosterone glucuronide, TG); 4-androsten-17α -methyl-17β-ol-3-on (methyl testosterone); 5-androstene-3β-ol-17-one (dehydroepiandrosterone, DHEA); 4-androstene-17α-ol-3-one (epitestosterone, epiT); 5α-androstan-3β-ol-17-on (epiandrosterone, epiA); 5α-androstan-3α,17β-diol (androstandiol); 5α-androstan-17β-ol-3-on (DHT); 5β-Androstan-3β-ol-17-on (epietiocholanolone,epiE); 5β-androstan-3α-ol-17-on (etiocholanolone, E); 5α-androstan-3.17-dion (androstandione); 5α-androstan-3α-ol-17-on (androsterone, A); 5α-androstan-17α-ol-3-on (epiallodihydrotestosterone); 5β-pregnan-3α-20α-diol 3-glucoronide (pregnandiol glucuronide, PdG); 5β-pregnan-3α-20α-diol (pregnandiol, Preg); 4-pregnen-3,20-dion (progesterone). The deuterated internal standards 16,16,17-d3-testosterone and 2,4,16,16-d4-estrone were supplied by Sigma Chemical Co.(St. Louis, MO, USA), while 2,2,4,6,6,17,21,21,21-d9-progesterone (d9-progesterone) and 9,11,12,12-d4-cortisol (d4-cortisol) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Standard solutions were prepared at 1 mg/mL in methanol and stored at −20 °C. A mix standard was prepared at 10 μg/mL in methanol and diluted to give working solutions at 0.1–1000 ng/mL with 30% acetonitrile in water.

LC–MS grade methanol and acetonitrile, together with formic acid, were purchased from Fluka (Chromasolv, Riedel-de Haën/Fluka, Buchs, Switzerland). Water for chromatography was gradient grade (Mallinckrodt Baker, Phillipsburg, NJ, USA). For extraction of steroids, *tert*-butyl methyl ether (TBME) (VWR, Darmstadt, Germany) and ethyl acetate (Roth, Karlsruhe, Germany) were used. Deionized water for preparation of buffers came from a MilliQ® water purification system (Millipore, Bedford, MA, USA). The buffer salts potassium carbonate, sodium acetate, sodium dihydrogen phosphate, as well as disodium hydrogen phosphate, sodium hydroxide and sulfuric acid (98%) were supplied by VWR (Darmstadt, Germany). The lyophilized enzyme, β-glucuronidase type VII-A from *E. coli*, was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Liquid chromatography

A Waters Alliance 2695 separation module, equipped with a quaternary pump and a column oven, was used for chromatographic separation (Waters, Milford, MA, USA).

Separation was achieved at 30 °C on a Gemini C18 column (150 mm × 2 mm, 3 μm), protected by a guard column (Security Guard 4 mm × 2 mm, 5 μm) of the same stationary phase (Phenomenex, Torrance, CA, USA). The injection volume was 20 μL. Eluent A was composed of water/acetonitrile (95/5, v/v) and eluent B of acetonitrile/water (95/5, v/v), both containing 0.1% formic acid. A gradient elution was performed at a flow rate of 0.2 mL/min: 30% B (0–2 min), linear increase to 70% B (2–20 min), 90% B (21–24 min), 30% B (24–34 min). The autosampler tray was cooled to 5 °C. Due to the use of 150 μL inserts in the HPLC vials, the injection depth had to be adjusted to 2 mm.

2.3. Mass spectrometry

The analyses were carried out on a Quattro Premier XE tandem mass spectrometer (Micromass, Manchester, UK) equipped with a Z spray ESI interface. Nitrogen (NGM-11 nitrogen generator, CMC Instruments, Eschborn, Germany) was used as desolvation and cone gas, with flow rates of 900 L/h and 250 L/h, respectively. Source and desolvation temperature were 100 and 450 °C, respectively. The electric potential applied on the capillary was 3.8 V, and the sample cone voltage was set individually for each compound. LC–MS/MS experiments were performed using argon as collision gas at a pressure of 5.1×10^{-3} mbar and a collision energy setting adapted for each compound. Steroids were detected using multiple reaction monitoring (MRM) of the two most abundant product ions per analyte. Dwell time for each transition was 50 ms. For identification of steroids in urine extracts, the ion ratio of the two transitions was not allowed to exceed more than 30% compared to standards. The optimization of mass spectrometer parameters was carried out by infusing standards of 1 or 5 ng/μL at a flow rate of 10 μL/min into a carrier stream of 200 μL/min acetonitrile/water/formic acid (50/50/0.1, v/v/v) generated by the HPLC pump. Compound specific MRM parameters are listed in Table 1.

2.4. Enzymatic hydrolysis of urine with β-glucuronidase from E. coli

Lyophilized β-glucuronidase type VII-A from *E. coli* (25,000 U) was dissolved in 5 mL HPLC water. Each sample of 200 μL urine was diluted with 800 μL of 0.25 M potassium phosphate buffer (pH 6.9). Then 40 μL enzyme (200 U) and 20 μL of an internal standard mix, containing 250 ng/mL d4-cortisol, d4-estrone, d3-testosterone and d9-progesterone, were added. The phosphate buffer was prepared by mixing 35 mL of 0.5 M sodium dihydrogen phosphate, 65 mL 0.5 M disodium phosphate and 100 mL water. Samples were incubated at 37 °C for 22 h under gentle agitation. Then, 150 μL 10% potassium carbonate was added in order to stop the enzymatic reaction and to adjust the sample pH to 9.6. Steroids were extracted by adding 6 mL TBME and vortexing for 10 min. Afterwards phase separation was achieved by centrifugation at 1500 rpm for 5 min and freezing at −21 °C for at least 3 h. The ether phase was decanted and evaporated with pressurized air. The residue was reconstituted in 100 μL 30% acetonitrile in water and transferred to an HPLC vial.

Table 1
Investigated steroids in elution order and compound-specific MRM parameters

Analyte	Retention time (min)	M_w (g/mol)	Precursor ion	Cone voltage (V)	Product ions (1/2)	Collision energy (eV)	Segment/polarity
Estriol	4.35	288	271	25	133/157	26/20	1/+
α-Cortol	4.60	368	333	20	273/255	15/20	1/+
d4-Cortisol	5.60	366	367	30	121/97	26/30	1/+
Cortisol	5.64	362	363	30	121/91/97	26/54/30	1/+
Testosterone-17β-glucuronide	6.86	464	465	41	97/109	31/35	1/+
Allo-/Tetrahydrocortisol[a]	7.44	366	349	17	301/295	13/13	1/+
Tetrahydrocortisone	8.76	364	365	19	347/329	9/13	1/+
11-β-OH-Etiocholanolone	11.76	306	307	14	271/253	9/13	1/+
11-Oxoetiocholanolone	13.0	304	287	30	229/91	21/57	1/+
Pregnandiol glucuronide	14.71	496	495	58	75/85	38/38	2/-
17β-Estradiol	14.29	272	255	25	133/159	19/20	3/+
d3-Testosterone	14.34	291	292	33	97	26	3/+
Testosterone	14.44	288	289	33	97/109	26/26	3/+
17α-Methyltestosterone	16.02	302	303	33	97	28	3/+
Dehydroepiandrosterone	16.36	288	289	17	271/253	9/9	3/+
d4-Estrone	16.65	274	275	25	135	26	3/+
Estrone	16.72	270	271	25	159/157	20/20	3/+
Epitestosterone	16.74	288	289	33	109/97	26/26	3/+
Epiandrosterone	17.57	290	273	26	255/91	13/42	3/+
5α-Androstane-3α,17β-diol	17.82	292	275	24	257/95	11/25	3/+
5α-Dihydrotestosterone	18.20	290	291	32	255/105	15/41	3/+
Epietiocholanolone	18.34	290	273	26/30	255/147	13/20	3/+
Etiocholanolone	19.57	290	273	26	255/91	13/42	3/+
5α-Androstane-3,17-dione	20.18	288	289	30	271/253	13/17	3/+
Androsterone	20.43	290	273	26/30	255/147	13/20	3/+
d9-Progesterone	22.19	323	324	30	100	25	4/+
Progesterone	22.41	314	315	30	97/109	25	4/+
Pregnandiol	22.28	320	285	30	175/189	18/18	4/+

[a] Compounds coelute.

2.5. Solvolysis of urine

In order to cleave steroid sulfates, the aqueous phase of the enzymatic hydrolysis (Section 2.4) was subjected to a solvolysis [26] after the ether had been decanted. First, remaining TBME residues were evaporated. Then, 1 mL 0.5 M sodium acetate buffer (pH 4.7) and 20 μL methanolic internal standard containing 250 ng/mL methyl testosterone were added. Solid phase extraction cartridges (C18, 200 mg, Chromabond, Macherey & Nagel, Düren, Germany) were conditioned with 2 mL methanol, 2 mL deionized water and 2 mL sodium acetate buffer. Afterwards, the sample was loaded and the cartridge was washed with 5 mL deionized water and dried for 10 min. Elution of steroids was conducted with 3× 1 mL methanol. The eluate was evaporated to a volume of 1 mL, and 5 mL ethyl acetate/H_2SO_4 (250 mL ethyl acetate/200 mg sulfuric acid, 98%) were added. This solution was incubated for one hour at 55 °C under mild agitation. The reaction was stopped by adding 250 μL of 1 M sodium hydroxide. Samples were vortexed and centrifuged for 5 min at 1500 rpm. The ethyl acetate phase was evaporated and the residue dissolved in 2 mL deionized water. Steroid extraction was carried out by vortexing with 5 mL TBME for 10 min. Phase separation was achieved by centrifugation at 1500 rpm for 5 min and freezing at −21 °C over night. The next day the ether phase was decanted, evaporated and reconstituted in 100 μL 30% acetonitrile.

2.6. Creatinine assay

To compensate for variations in urine concentration, all steroid concentrations were indexed against creatinine and expressed as ng/mg creatinine [9]. Creatinine measurement was performed by micro titer plate analysis. Blanks (150 μL water), zero (50 μL water), 50 μL creatinine standards (range 0.075-10 μg/50 μL) and 50 μL of each sample dilution (bonobos 1:10, humans and chimpanzees 1:20 diluted with deionized water) were added to duplicate wells of a 96-well plate. Zero, standards and samples were incubated with 100 μL picrate reagent (1:1 mixture of 0.04 M picric acid and 0.75 M sodium hydroxide) in the dark on a mechanical shaker for 15 min. Following incubation, the absorbance was measured at 490 nm (MRX II, Dynex Technologies, Chantilly, VA, USA).

2.7. Urine samples

Urine samples of two male volunteers were collected at the Max Planck Institute for Evolutionary Anthropology in November 2006 using 50 mL polypropylene tubes. Samples were frozen, at the latest, 2 h after collection. Bonobo urine samples were collected in May 2006 from one adult male (23 years) and two adult females (8 and 19 years) in the Zoo of Frankfurt am Main (Germany) and one adult male (22 years) in the Zoo of Planckendael (Belgium). Chimpanzee urine samples were col-

lected in Taï National Park (Côte d'Ivoire) in January 2006 from five habituated adult males (15–42 years) and in January 2005 from eight habituated adult females (26–42 years); from June to July in Budongo Forest Reserve (Uganda) from three adult males (15–26 years); and in January 2007 in the Zoo of Leipzig from two adult males (13 and 31 years) and one adult female (26 years). Urine was aspirated from vegetation or ground using disposable plastic pipettes. Samples collected in the field were kept at 4 °C for ~1/2 day and then stored frozen. After shipment on dry ice to the lab, all samples were stored at –20 °C until analysis.

For method comparison eight samples of lyophilized human urine of four healthy individuals (two females, 27 and 55 years and two males, 49 and 54 years) provided by the Dutch Foundation for Quality Assessment in Medical Laboratories (SKML: www.skml.nl, Nijmegen, The Netherlands) were resuspended in 20 mL deionzed water.

2.8. Method evaluation

2.8.1. Linearity, LOD and QL

To evaluate linearity, standards were prepared at concentrations of 0.2, 0.6, 1, 2, 6, 10, 20, 60, 100, 200, 1000 and 2000 ng/mL of each steroid in 30% acetonitrile. These were then mixed 1:1 with an internal standard mix of each 100 ng/mL d4-cortisol, d3-testosterone, d4-estrone, d9-progesterone and methyl testosterone, resulting in final calibration concentrations of 0.1, 0.3, 0.5, 1, 3, 5, 10, 30, 50, 100, 500 and 1000 ng/mL and 50 ng/mL of the internal standards. Calibration curves were obtained from the analyte to internal standard peak area ratios using linear regression with $1/x$ weighing, except for pregnandiol glucuronide, which was calibrated externally in the negative ionization mode. The limit of detection (LOD) was calculated on the most intense transition, with the criterion of a signal to noise ratio exceeding 3. The quantitation limit (QL) was defined as the lowest calibration level, which fitted into the calibration curve with a residual of less than 20% and an ion ratio deviating less than 30% from that of a higher concentrated standard.

2.8.2. Selectivity

Two MRMs for each steroid were measured to check for interference. The intensity ratio of the quantifier and qualifier MRM was required to be ±30% of the standard ratio. Interferences were examined for each analyte within ±0.5 min of its retention time with all other analytes eluting in this time window. To check the selectivity of the method between the analytes, individual standard solutions at 100 ng/mL were separately injected and analyzed using the current MRM method. This should not produce a peak area >20% of the analyte under question injected at the quantitation limit.

2.8.3. Ether extraction

The performance of the ether extraction was first checked by spiking steroids at 25 ng/mL in 200 μL water—once by adjusting the solution to pH 4.7 by adding 800 μL of sodium acetate buffer, and once by adjusting the solution to pH 9.6 by adding 800 μL of sodium acetate buffer and 750 μL 10% potassium carbonate. The spiked samples were then extracted with 6 mL diethyl ether as described in Section 2.4. The extraction was then repeated with 6 mL TBME in order to evaluate if the latter can be used instead of diethyl ether. Every experiment was repeated six times. For determination of recovery, the response was compared with standards directly prepared in HPLC eluent without internal standardization.

2.8.4. Amount of β-glucuronidase used for hydrolysis

The completeness of the hydrolysis was examined by using 20, 40 and 80 μL β-glucuronidase (500, 1000 and 2000 U/mL urine) for hydrolysis of two male urines and looking for a potential increase of determined steroid concentrations.

In this study, two male human urines with relatively high creatinine contents (0.85 mg/mL and 2.118 mg/mL), and therefore assumed high steroid concentrations, were used. Each enzyme concentration was assayed in duplicate.

2.8.5. Matrix effect/ion suppression

Two pools of equal volumes of four bonobo and four chimpanzee urines (two males, two females) were prepared. These pools were submitted to hydrolysis and solvolysis (Sections 2.4 and 2.5) and evaporated. Dry extracts were dissolved using internal standard solutions at three concentration levels (2, 20 and 200 ng/mL). Matrix effect was determined by comparing the analytical response of these samples with that of standard solutions. Each experiment was performed in duplicate.

2.8.6. Precision and recovery

As no commercial quality control samples containing all analytes implemented into our method were available, quality controls were prepared by spiking a low concentrated urine pool of a female bonobo (9 years) at three levels with appropriately diluted methanolic composite standards. First, the basal level of the urine pool was determined. The concentration of hormones spiked was adjusted to the physiological relevant range of each hormone. Hormones were spiked in their free from, except for testosterone and pregnandiol, which were spiked as glucuronides and DHEA, which was spiked as sulfate. Each QC level was submitted to hydrolysis and solvolysis six times on the same day (intra-day precision) and five times on different days (inter-day precision). The response was compared with a set of calibration standards. The recoveries were calculated by linear regression. The precision acceptance criterion for each quality control sample concentration was ≤15%. Additionally, the reproducibility of the whole method including hydrolysis and solvolysis was determined by submitting two samples of unspiked male human urine to the hydrolysis and solvolysis procedure (Sections 2.4 and 2.5) eight times.

2.8.7. Method comparison

Eight human urine samples from an international survey (SKML, Nijmegen) were analyzed by the presented LC–MS/MS method in duplicate. Results for 9 compounds were compared to the median of the survey. The predominant method used by the other laboratories taking part in the survey was GC–MS. Regression analysis was performed by Spearman's rank correlation, in
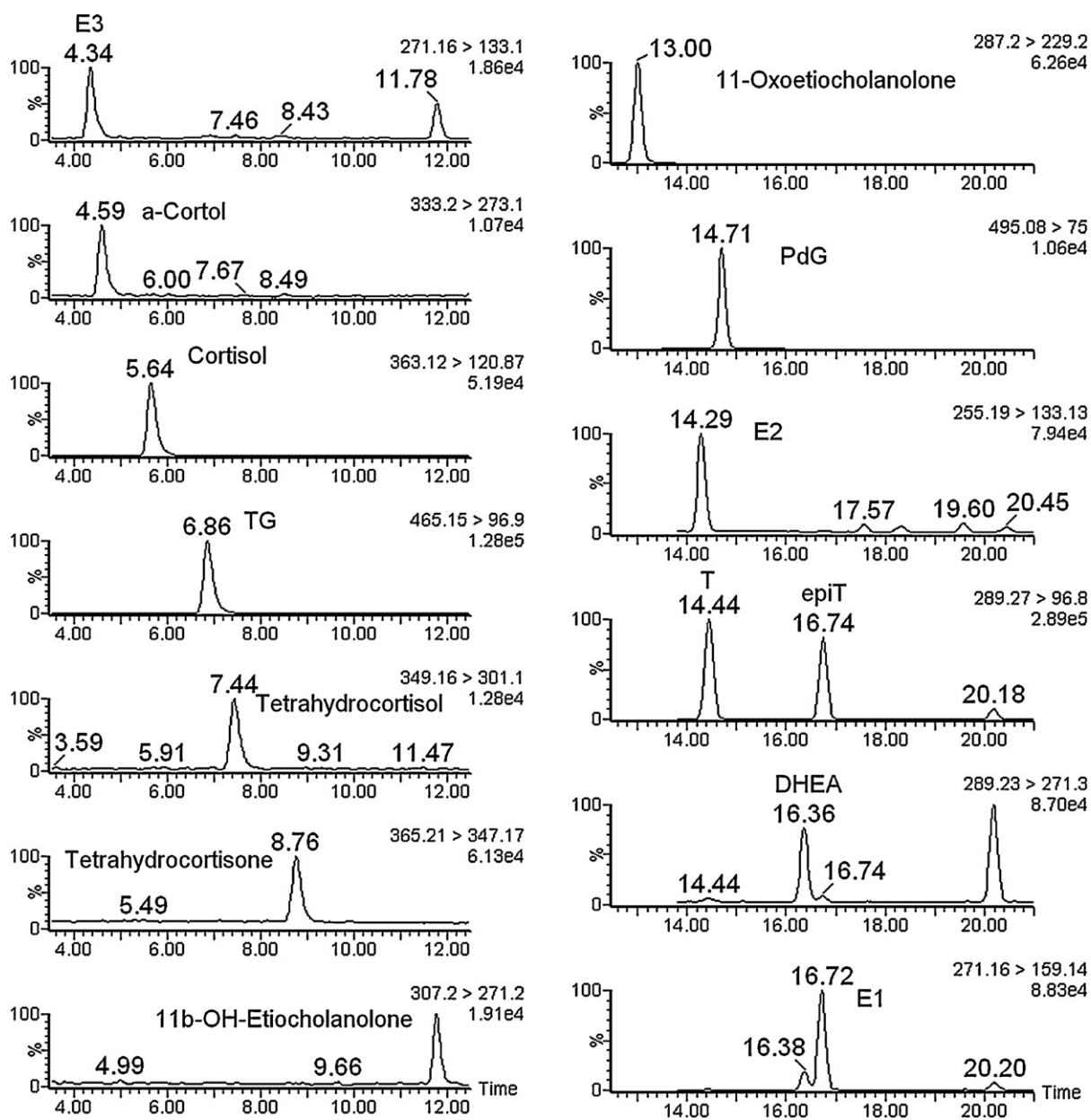
Fig. 1. Chromatographic separation of 23 steroids in 30 ng/mL standard (x-axis: retention time (min); y-axis: relative abundance (%); E3, estriol; TG, testosterone glucuronide; PdG, pregnandiol glucuronide; E2, β-estradiol; T, testosterone; epiT, epitestosterone; DHEA, dehydroepiandrosterone; E1, estrone; epiA, epiandrosterone; epiE, epietiocholanolone; E, etiocholanolone; A, androsterone; Androstanediol, 5α-Androstane-3α, 17β-diol; DHT, 5α-dihydrotestosterone; Androstanedione, 5α-Androstane-3,17-dione.

order to test whether the relative magnitude of measures was comparable between the two methods. Systematic differences between both methods were examined with a Wilcoxon test.

3. Results and discussion

3.1. Mass spectrometry

For quantification in MRM mode, usually the two most intense transitions of a compound are chosen. The daughter scan spectra of six steroids are presented in Fig. S1 (supplement). The MS^2-spectra of most steroids show abundant water losses. The first water loss $[M+H-18]^+$ is often more intense

than the pseudo-molecular ion $[M+H]^+$. If the $[M+H]^+$ could not be detected at all or if a more abundant mass spectrum could be generated from the first water loss, the latter was chosen as the precursor ion. This was the case for nine of the 23 steroids examined here (see also Table 1). For the compounds, α-cortol and pregnandiol, the second water loss had to be chosen as precursor. As is widely known, higher cone voltages promote the in-source collision induced dissociations (CID) leading to the loss of water. Therefore, the cone voltage was optimized carefully depending on the precursor chosen to achieve maximum sensitivity. After loss of all hydroxyl- and keto-groups, the fragments obtained from the steroid backbone are very similar for the non-aromatic steroids. This can be seen
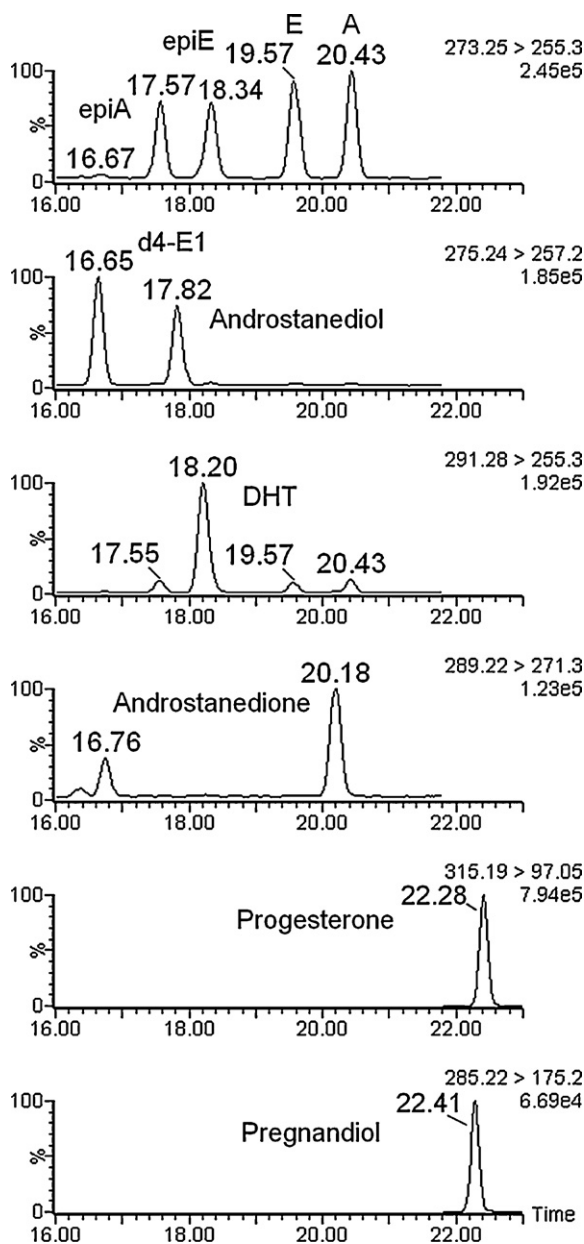
Fig. 1. (*Continued*).

when comparing the high-energy spectra of etiocholanolone and cortisol in Fig. S1. Common fragments in these line-rich spectra are 91 and 105. As these are not more compound-specific than water-losses, and furthermore were less abundant, water-losses were preferred as daughter ions for MRM transitions [27,28]. An exception is the compound pregnandiol, which produces numerous intense fragments, even when applying low collision energies. The aromatic steroids E1, E2 and E3 all show relatively abundant fragments at $m/z = 133$ and 159. For E1 and E3, 157 also represents an intense fragment ion. Estrogens can also be measured sensitively as $[M − H]^-$ in the negative mode, which usually exhibits a lower background than the positive mode [29,30]. As this would require a different eluent with a basic pH, this was not possible within the multi method presented here. The compounds DHEA and E1 elute close to each

other after 16.36 and 16.72 min (see also Fig. 1). Both show the water losses at $m/z = 271$ and 253. In order not to influence the detection of DHEA by overlapping E1 ($M_r = 270$), DHEA was quantified as $[M + H]^+ = 289 > 271$ and $289 > 253$. However, detection of small quantities of E1 can be negatively influenced by a large DHEA peak. Best sensitivity was obtained for testosterone, epitestosterone and progesterone, all forming intense ions at m/z 97 and 109, typical for steroids having the 4-ene-3-one structure [31]. In order to examine the completeness of enzymatic cleavage in SPE-extracted urine samples (results not shown), two glucuronides were implemented into the method. Testosterone glucuronide was detected more sensitively in the positive ionization mode resulting in the typical fragment ions of m/z 97 and 109. Pregnandiol glucuronide (PdG) was acquired in the negative ionization mode. The daughter ions monitored for PdG are characteristic of the glucuronide moiety and correspond to the loss of CO, CO_2 and H_2O: $[Glu-2CO-CO_2]^-$ ($m/z = 75$) and $[Glu-CO-CO_2-H_2O]^-$ ($m/z = 85$).

3.2. Chromatography

All 23 steroids could be separated within 23 min using a water–acetonitrile gradient, as specified in Section 2.2. A chromatogram of a 30 ng/mL standard mix is shown in Fig. 1. A chromatogram of hydrolyzed urine of a female chimpanzee can be found in the supplement (Fig. S2).

All compounds are baseline separated when using their optimized MRM transitions. However, we observed two co-elutions, which resulted in exclusion of these compounds from our method. Under the chromatographic conditions used, tetrahydrocortisol cannot be separated from allotetrahydrocortisol. As we concluded from the ion ratio of both transitions in bonobo and chimpanzee urine, tetrahydrocortisol is the predominant isomer in these two species. Therefore we calibrated only tetrahydrocortisol. When quantifying it in human urine, a higher than 30% deviation of the ion ratio has to be accepted, since both isomers are present. If necessary, it is possible to separate both isomers with the HPLC phase used here by modifying the gradient program and starting at 25% B and keeping this constant for 6 min (results not shown).

The second co-elution was observed between androsterone and 5α-androstane-17α-ol-3-one. As androsterone is present in much higher concentrations in urine of all three species, we excluded 5α-androstane-17α-ol-3-one from our calibration mix. The separation of the two isomeric pairs etiocholanolone/androsterone and epietiocholanolone/epiandrosterone is remarkable, as these only differ in the in the 5β/5α-position of one H-atom.

3.3. Method validation

3.3.1. Linearity, LOD and QL

Table 2 summarizes the method validation parameters for the calibration in HPLC eluent.

The limit of detection varied between 0.1 and 1 ng/mL depending on the compound. This corresponds to 2–20 pg on column (20 μL injected). By increasing the injection volume, the

Table 2
Detection limits (LOD) and quantitation limits (QL) of investigated steroids in HPLC eluent and statistics of calibration curves, range <QL − 1000 ng/mL

Compound	LOD (ng/mL)	QL (ng/mL)	Internal standard	Slope[b] (mean ± S.D.)	Intercept[b] (mean ± S.D.)	Correlation coefficient r
Estriol	1	3	d4-Estrone	0.3356 ± 0.0258	−0.0416 ± 0.1463	0.9993
α-Cortol	1	3	d4-Cortisol	0.2358 ± 0.0124	−0.0403 ± 0.0710	0.9996
Cortisol	0.1	0.3	d4-Cortisol	1.7612 ± 0.0189	−0.0848 ± 0.0749	0.9998
Testosterone-17β-glucuronide	0.1	0.3	d3-Testosterone	0.4539 ± 0.0104	0.0054 ± 0.0129	0.9998
Allo-/Tetrahydrocortisol[a]	1	3	d4-Cortisol	0.2976 ± 0.0463	−0.1297 ± 0.1194	0.9995
Tetrahydrocortison	0.5	1	d4-Cortisol	1.3208 ± 0.1079	−0.0143 ± 0.2482	0.9997
11-β-OH-Etiocholanolone	0.5	1	d4-Cortisol	0.3909 ± 0.1132	0.0933 ± 0.1111	0.9993
11-Oxoetiocholanolone	0.3	0.5	d4-Cortisol	1.0730 ± 0.0360	0.1220 ± 0.0709	0.9996
Pregnandiol glucuronide	0.3	0.5	none	58.8507 ± 2.6680	25.6400 ± 11.6612	0.9975
17β-Estradiol	0.1	0.3	d4-Estrone	4.1164 ± 0.1277	−0.0021 ± 0.1130	0.9997
Testosterone	0.1	0.3	d3-Testosterone	0.8593 ± 0.0286	0.0115 ± 0.0114	0.9998
Dehydroepiandrosterone	0.5	1	d3-Testosterone	0.1809 ± 0.0061	−0.0112 ± 0.0194	0.9997
Estrone	0.3	0.5	d4-Estrone	1.2794 ± 0.0245	0.1701 ± 0.0763	0.9994
Epitestosterone	0.1	0.3	d3-Testosterone	0.6825 ± 0.0230	0.0273 ± 0.0237	0.9998
Epiandrosterone	0.5	1	d3-Testosterone	0.4466 ± 0.0173	0.0363 ± 0.0512	0.9998
5α-Androstane-3α,17β-diol	0.3	0.5	d3-Testosterone	0.3409 ± 0.0104	0.0198 ± 0.0392	0.9997
5α-Dihydrotestosterone	0.3	0.5	d3-Testosterone	0.5447 ± 0.0435	0.0032 ± 0.0480	0.9999
Epietiocholanolone	0.5	1	d3-Testosterone	0.4753 ± 0.0173	0.1184 ± 0.0627	0.9998
Etiocholanolone	0.5	1	d3-Testosterone	0.5322 ± 0.0260	0.1137 ± 0.0496	0.9997
5α-Androstane-3,17-dion	0.5	1	d3-Testosterone	0.3168 ± 0.0274	−0.0073 ± 0.0344	0.9998
Androsterone	0.5	1	d3-Testosterone	0.6137 ± 0.0217	0.1165 ± 0.0692	0.9998
Progesterone	0.1	0.3	d9-Progesterone	1.1725 ± 0.0263	0.0460 ± 0.0153	0.9998
Pregnandiol	0.3	0.5	d9-Progesterone	0.0970 ± 0.0079	0.0083 ± 0.0048	0.9997

[a] Compounds coelute.
[b] Results are expressed as mean values ±S.D. of five independent calibrations.

detection limits could be improved. Best sensitivity was obtained for steroids with a 3-oxo-4-ene structure, resulting in the typical fragments of 97 and 109. Quantitation limits were between 0.3 and 3 ng/mL. The response was linear up to 1000 ng/mL, except for pregnandiol glucuronide, which was acquired in the negative ionization mode and could only be calibrated up to 500 ng/mL.

3.3.2. Selectivity

The selectivity criteria were not met for the pairs estrone/epitestosterone and DHT/epietiocholanolone. The interference is 1.1% if estrone and epitestosterone are both injected at 100 ng/mL and 240% if epitestosterone is injected at 100 ng/mL and estrone at its QL of 0.5 ng/mL. Given the physiological ranges of both hormones in urine this interference is relevant. However, we were able to obtain biological meaningful estrone profiles in cycling females. The interference of DHT/epietiocholanolone is 9% if both are injected at 100 ng/mL and 1550% if epietiocholanolone is injected at 100 ng/mL and DHT at its QL of 0.5 ng/mL. Consequently, both hormones cannot be analyzed independently from each other.

3.3.3. Ether extraction

The extraction of steroid hormones from urine is usually carried out at pH 9–10, in order to minimize the co-extraction of acidic matrix compounds [26,37,38]. In order to evaluate, if the recovery of any of the steroids examined here is affected by the higher pH, water was spiked with steroids and adjusted once to pH 4.7 and once to pH 9.6, and extracted with diethyl ether (Section 2.8.3). The results are shown exemplarily for four compounds in Fig. S3 (supplement).

The differences in the extraction recovery at different pH were below 10% for all compounds, which is within the standard deviation of the extraction at pH 4.7. Consequently, samples can be extracted at pH 9.6 without loss in sensitivity.

Due to its high toxicity and narcotic effect [39,40] diethyl ether should be replaced by other solvents if possible. Several authors describe the use of *tert*-butyl methyl ether (TBME) instead of diethyl ether [26,41–43]. When comparing diethyl ether and TBME as extraction solvent at pH 9.6, TBME resulted in 1–6% higher recoveries with a standard deviation of 5% (Fig. S3), whereas for α-cortol the average recovery was 24% higher using TBME. Therefore, the ether extraction can be carried out at pH 9.6 with TBME replacing diethyl ether.

3.3.4. Amount of β-glucuronidase used for hydrolysis of urine

In order to cleave steroid glucuronides in urine and convert them to their ether-extractable free form, a β-glucuronidase from *E. coli* was used. This enzyme does not have a sulphatase activity and is therefore unable to cleave steroid sulfates. Consequently, a subsequent solvolytic step is necessary to evaluate whether a significant proportion of the steroid of interest is excreted as sulfate. In solvolysis, sulfates are hydrolyzed with sulfuric acid in ethyl acetate. To avoid the necessity of this additional step, several authors describe the use of β-glucuronidase from *H. pomatia*. Because this enzymatic preparation also contains a sulphatase activity, most steroid sulfates are cleaved simultaneously. However, several authors have reported on artefacts using *H. pomatia* juice for hydrolysis of urine, resulting from a

Table 3
Validation results

Analyte	Level 1				Level 2				Level 3			
	Mean (ng/mL)	Intra-day CV (%)	Inter-day CV (%)	Recovery (%)	Mean (ng/mL)	Intra-day CV (%)	Inter-day CV (%)	Recovery (%)	Mean (ng/mL)	Intra-day CV (%)	Inter-day CV (%)	Recovery (%)
Estriol	11.5	6.3	4.2	65.9	37.8	6.3	2.9	79.7	90.6	7.2	7.3	84.3
α-Cortol	22.1	6.9	6.6	101.2	62.8	2.7	5.0	95.1	97.1	6.6	6.7	89.3
Cortisol	58.4	5.4	13.0	87.3	94.7	4.6	8.8	88.7	185.7	4.1	10.0	81.9
Allo-/THF[a]	135.1	6.5	10.8	81.4	218.6	5.9	11.2	65.2	364.3	4.2	11.2	58.9
Tetrahydrocortisone	234.7	8.7	11.4	74.0	368.2	7.1	12.8	71.2	512.3	5.8	14.1	71.4
11-OH-Etiocholanolone	34.7	14.3	11.9	77.0	70.0	10.3	12.5	82.3	158.3	9.5	8.5	77.2
11-Oxoetiocholanolone	166.5	6.9	5.3	100.6	275.5	6.0	14.8	103.7	465.0	6.7	6.5	99.9
17β-Estradiol	10.1	7.4	9.0	69.3	34.2	6.5	8.0	76.8	99.3	5.4	10.8	95.0
Testosterone[b]	71.5	8.0	9.9	84.6	135.6	6.5	8.0	82.4	313.1	5.9	10.7	77.4
Dehydroepiandrosterone[c]	134.3	7.5	7.7	60.4	253.4	7.4	7.6	60.0	390.3	5.9	8.5	62.7
Estrone	11.2	7.8	12.3	77.2	36.9	6.7	9.5	83.1	104.4	6.7	11.5	99.9
Epitestosterone	10.8	8.7	11.5	103.0	35.8	6.0	8.6	88.6	101.2	5.6	10.8	100.7
Epiandrosterone	11.0	10.7	8.2	93.2	33.5	6.0	5.5	80.2	94.7	5.7	10.9	93.0
5α-Androstane-3α,17β-diol	12.0	9.9	4.3	89.4	34.3	4.9	7.8	78.9	92.8	6.7	9.7	89.7
5α-Dihydrotestosterone	11.6	10.5	8.7	92.4	33.7	6.2	8.4	79.2	93.9	5.7	10.3	91.5
Epietiocholanolone	14.1	6.7	7.6	97.6	37.6	7.5	8.2	84.5	99.9	6.2	8.3	95.6
Etiocholanolone	142.4	8.5	8.9	89.8	237.2	6.7	7.0	91.7	418.7	6.0	10.9	91.3
5α-Androstane-3,17-dione	11.1	5.9	6.6	93.7	32.8	6.8	6.7	78.5	92.4	6.6	9.6	90.8
Androsterone	56.4	9.4	8.2	96.5	96.5	7.4	5.8	98.0	204.8	7.4	10.1	93.7
Progesterone	9.2	10.0	9.8	92.2	31.3	8.1	5.5	78.2	90.9	6.8	10.9	90.9
Pregnandiol[b]	40.6	11.0	9.0	77.4	66.0	9.3	8.1	71.5	142.2	7.4	12.9	67.0

[a] Compounds coelute; THF, tetrahydrocortisol.
[b] Spiked as glucuronide.
[c] Spiked as sulfate.

3β-hydroxy-steroid-dehydrogenase and a $\Delta^{5\text{-}4}$-isomerase activity present in this preparation [32,33]. As this can result in altered hormone profiles for several androgens, especially testosterone, the β-glucuronidase from *E. coli* was chosen for this study.

The amount of β-glucuronidase from *E. coli* used for hydrolysis of steroid glucuronides in human urine varies in the literature from 1.6 U/mL [34], to 4 U/mL [35], to 1000 U/mL [36]. In this study, two male human urines with relatively high creatinine contents (0.85 mg/mL and 2.118 mg/mL), and therefore assumed high steroid concentrations, were hydrolyzed in duplicate with 20, 40 and 80 μL enzyme, prepared as described in Section 2.4, corresponding to 500, 1000 and 2000 U/mL urine, respectively. Hydrolyzing urine with 40 μL enzyme, instead of 20 μL, results in only minor differences in steroid concentrations for the majority of steroids, ranging from −6 to 7% (average deviation 1.9%). The same was true when further increasing the amount of enzyme to 80 μL—the average deviation was only 0.6%, ranging from −5 to 7%. Only the glucuronides of tetrahydrocortisol, α-cortol and 5β-androstane-3α, 11β-diol-17-one (11β-hydroxyetiocholanolone) were hydrolyzed to a higher extent by a higher amount of enzyme. When using 40 μL enzyme, instead of 20 μL, the concentrations of the latter rose about 16, 26 and 49%, respectively. By adding 80 μL enzyme, an additional increase of only 4% for tetrahydrocortisol, 18% for α-cortol and 10% for 5β-andro was observed. As the β-glucuronidase precipitates after adding potassium carbonate and ether and can impede phase separation, we decided to use as little enzyme as necessary and performed the hydrolysis with 40 μL β-glucuronidase from *E. coli*.

3.3.5. Matrix effect/ion suppression

The possibility of matrix effects on ionization was explored by comparing the response of the four deuterated internal standards spiked into extracted and evaporated urine pools of bonobos and chimpanzees with the response of these standards spiked into HPLC eluent. There was no significant decrease of peak areas of reference solutions and standards spiked into extracted urine (Table S1, supplement). The ESI source used has a Z-spray configuration, which results in a twofold redirection of the ion-beam and makes it relatively stable against matrix effects. However, at the low level of 2 ng/mL a significant positive matrix effect was seen for d4-cortisol in both bonobo and chimpanzee urine pools. This effect is due to a coeluting compound having the same transition of 367 > 121 and can impede the use of d4-cortisol as internal standard in bonobo urine. In these cases glucocorticoids are at present referred to d3-testosterone, but a replacement of d4-cortisol is planned when analyzing bonobo urine.

3.3.6. Precision and recovery

Precision and recovery were calculated at three different concentration levels of low, medium and high QC samples analyzed on 6 days. The results are presented in Table 3.

Both intra-day ($N = 6$) and inter-day ($N = 5$) precision was ≤15% at all levels examined and ≤20% at basal level of the unspiked bonobo urine pool. Therefore the precision acceptance criterion is fulfilled. Recoveries were between 60 and 103%. For most analytes no concentration dependence of recovery was seen. However, the recovery of tetrahydrocortisol decreases with increasing concentration. This could be an ion suppression

Table 4
Reproducibility of sample preparation and analysis for two male human urine samples ($N = 8$)

Compound	Concentration (ng/mL)		CV Hydrolysis (%)		CV Solvolysis (%)		Solvolysis in (%) of total	
	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2
Estriol	17	53	8.7	4.2	<QL	<QL	–	–
α-Cortol	135	531	4.8	4.8	<QL	<QL	–	–
Cortisol	154.2	237.8	1.9	3.6	6.2	16.5	26.7	13.1
Allo-/THF[a]	942	2853	2.0	4.6	13.5	18.1	4.9	7.7
Tetrahydrocortison	4562	11012	1.9	4.2	15.5	24.5	1.3	2.7
11-OH-Etiocholanolone	481	875	3.4	1.2	4.1	4.2	8.5	6.0
17β-Estradiol	<QL	<QL	<QL	<QL	<QL	<QL	–	–
Testosterone	132.2	144.5	1.5	2.6	2.2	3.9	19.3	11.6
Dehydroepiandrosterone	8585	15226	1.3	3.2	1.9	2.9	98.3	98.2
Estrone	7.1	27.4	5.1	4.6	<QL	<QL	-	-
Epitestosterone	105.7	309.3	1.7	2.6	1.9	2.4	37.4	19.0
Epiandrosterone	856	1049	2.3	1.5	1.6	2.6	93.5	86.2
5α-Androstane-3α,17β-diol	154.5	535.0	2.2	2.3	3.1	5.5	12.8	11.9
5α-Dihydrotestosterone	20.5	28.5	2.9	3.8	3.7	5.8	36.9	10.9
Epietiocholanolone	81	42	2.9	5.9	2.5	7.2	70.7	56.3
Etiocholanolone	3657	5776	1.4	3.3	2.3	14.3	10.8	3.9
5α-Androstane-3,17-dione	NQ	NQ	NQ	NQ	NQ	NQ	–	–
Androsterone	6671	11484	1.4	3.3	0.8	2.1	35.1	22.8
Progesterone	<QL	<QL	<QL	<QL	<QL	<QL	–	–
Pregnandiol	676.0	984.0	1.7	2.3	14.5	20.3	1.5	1.7

Percentage of steroid concentration found after solvolysis.
CV, coefficient of variation; <QL, below quantitation limit; NQ, not quantifiable due to incorrect ion ratio; –, not determinable.
[a] Compounds coelute; THF, tetrahydrocortisol.

effect caused by coeluting allo-tetrahydrocortisol or the result of an incomplete hydrolysis (see also Section 3.3.4) The lowest recovery was obtained for DHEA, which was spiked as sulfate.

Finally the repeatability of the whole sample preparation including solvolysis and hydrolysis was checked by eightfold extraction of two unspiked male urines. The results are presented in Table 4.

The reproducibility of the hydrolysis and solvolysis is good. For hydrolysis the relative standard deviation ranged from 1.4 to 8.7%. In general, the variance of extracted steroid concentrations after solvolysis is bigger than that of the hydrolysis. This is especially true for compounds that are found in small concentrations in the solvolysis fraction. As the recovery of steroids from hydrolyzed urine employing one single ether extraction is not 100% (Table 3), about 5–10% of steroids found in solvolyzed urine can be attributed to the additional extraction step with ethyl acetate. This was determined by comparing the peak areas of the deuterated internal standards spiked before hydrolysis and remaining in solvolyzed urine. Therefore, only steroids with a solvolysis fraction exceeding 10% can be considered as sulfates which were cleaved and extracted by the solvolysis procedure. For those, the relative standard deviation was between 1.6% and 16.5%.

3.3.7. Method comparison

The comparison of survey results with our own results could only be carried out for 9 analytes, as only those were included in both schemes. The range of survey results was very wide, values differed up to 6 times between the different labs. The number of labs, which analyzed a particular compound ranged from 3 to 23. Concentrations were given in μmol/L, the lowest value being 0.1 μmol/L. The sensitivity of the presented LC–MS/MS method was higher (Section 3.3.1). All concentrations determined by our method were within the range of the survey results. The results of the eight samples determined by LC–MS/MS correlated well with the median of the survey, correlation coefficients were all ≥0.807 with all P-values ≤0.02 (Spearman's rank correlation). The only exception was cortisol, for which no significant correlation was found. However, cortisol was only analyzed by three labs, the result being either 0.1 or 0.2 μmol/L, therefore no significant correlation could be expected.

Systematic differences between LC–MS/MS results and the median of the survey were examined with the Wilcoxon test. Only for cortisol no significant difference was found. For 11-hydroxyetiocholanolone and 11-oxoetiocholanolone the survey median was higher, whereas for the other compounds the LC–MS/MS results were higher. The differences were significant ($P < 0.05$) for all compounds except for pregnandiol and 11-oxoetiocholanolone. Detailed statistical results can be found in the supplement (Tables S2 and S3).

3.4. Percentage of sulfate—necessity of solvolysis

The percentage of sulphoconjugate versus glucuronide varies very much depending on the steroid. Table 5 presents the concentration ranges of 21 steroids in male ($N = 10$) and female ($N = 8$)

Table 5

Mean and concentration range of urinary steroids in chimpanzee urine (ng/mg creatinine) determinined after hydrolysis and solvolysis, including mean percentage found after solvolysis ± standard deviation

Compound (ng/mg creatinine)	Male ($N = 10$)	Female ($N = 8$)
Estriol		
Mean	<QL	20.6
Range	–	8.5–41.4
Solvolysis (%)	–	62.3 ± 20.8
α-Cortol		
Mean	65.5	56.4
Range	21.8–165.0	25.3–77.1
Solvolysis (%)	17.0 ± 23.0	40.0 ± 13.0
Cortisol		
Mean	179.2	86.4
Range	57.4–347.8	33.6–228.5
Solvolysis (%)	46.2 ± 14.7	39.5 ± 14.4
Tetrahydrocortisol/allotetrahydrocortisol		
Mean	854.9	538.8
Range	356.4–1812.9	99.8–1282.3
Solvolysis (%)	6.3 ± 2.4	6.0 ± 3.8
Tetrahydrocortisone		
Mean	1200.7	831.1
Range	352.5–2086.9	67.7–1522.1
Solvolysis (%)	4.2 ± 2.2	4.8 ± 3.2
5β-Androstane-3α, 11β-diol-17-one		
Mean	170.7	156.1
Range	28.4–501.8	36.9–412.2
Solvolysis (%)	11.0 ± 5.1	14.6 ± 7.3
11-Oxoetiocholanolone		
Mean	304.9	286.6
Range	110.9–665.2	73.6–536.1
Solvolysis (%)	NQ	NQ
17β-Estradiol		
Mean	<QL	0.8
Range	–	0.1–1.4
Solvolysis (%)	–	5.7 ± 16.2
Testosterone		
Mean	69.4	11.8
Range	17.8–138.5	1.7–42.4
Solvolysis (%)	14.0 ± 8.9	19.6 ± 10.2
Dehydroepiandrosterone		
Mean	2548.4	377.7
Range	99.5–6473.2	118.1–927.2
Solvolysis (%)	77.9 ± 25.2	69.3 ± 31.6
Estrone		
Mean	10.0	9.4
Range	2.6–15.6	2.2–30.3
Solvolysis (%)	24.4 ± 23.6	7.2 ± 9.4
Epitestosterone		
Mean	39.8	11.6
Range	8.6–94.3	2.4–50.3
Solvolysis (%)	60.8 ± 26.2	78.2 ± 11.4
Epiandrosterone		
Mean	43.3	35.9
Range	10.7–107.4	8.1–67.7
Solvolysis (%)	86.1 ± 28.3	96.3 ± 8.5
5α-Androstane-3α,17β-diol		
Mean	32.4	35.9
Range	7.5–44.6	0–22.2

Table 5 (*Continued*)

Compound (ng/mg creatinine)	Male (*N* = 10)	Female (*N* = 8)
Solvolysis (%)	19.7 ± 32.8	15.6 ± 31.3
5α-Dihydrotestosterone		
Mean	14.2	7.9
Range	6.2–42.6	0.8–18.4
Solvolysis (%)	11.9 ± 11.1	19.9 ± 14.2
Epietiocholanolone		
Mean	12.2	34.2
Range	0–84.3	3.8–187.4
Solvolysis (%)	59.0 ± 41.5	76.5 ± 36.8
Etiocholanolone		
Mean	950.4	641.9
Range	59.8–2360.7	165.5–1070.3
Solvolysis (%)	24.7 ± 11.5	30.5 ± 17.2
5α-Androstane-3,17-dione		
Mean	105.4	88.3
Range	0–830.3	0–692.9
Solvolysis (%)	NQ	NQ
Androsterone		
Mean	434.2	323.7
Range	137.2–1006.4	86.8–447.9
Solvolysis (%)	44.7 ± 28.5	48.1 ± 26.1
Progesterone		
Mean	0.1	0.2
Range	0–0.4	0–0.6
Solvolysis (%)	0	10 ± 18.7
Pregnandiol		
Mean	113.7	36.0
Range	3.7–403.9	14.0–101.3
Solvolysis (%)	4.0 ± 5.2	3.4 ± 2.7

<QL, below quantitation limit; NQ, not quantifiable due to incorrect ion ratio; –, not determinable.

chimpanzee urine together with their mean and the percentage of total steroid concentration found in the solvolysis fraction representing the proportion excreted as sulfate.

For dehydroepiandrosterone, we found 98.2% as sulfate in humans (see Table 4) and 73.6% in chimpanzees (72.9% according to [44], data for humans). Androsterone occurred to 28.9% in the solvolysis fraction of human urine and to 46.4% in chimpanzee urine (17.2% [44]), and etiocholanolone to 7.4% in humans and 27.6% in chimpanzees (10.9% [44]). For chimpanzees, 91.2% and 67.8% of epiandrosterone and epietiocholanolone, respectively, were found in the sulfate form. These values were 89.8% and 63.5% for humans. As dehydroepiandrosterone, both are 3β-hydroxy-steroids, which are expected to be excreted predominantly as sulphoconjugates [44]. A detailed statistical comparison of hormone profiles and conjugation patterns of chimpanzees, humans and bonobos is beyond the scope of this article. Reference values for several androgens and a number of glucocorticoids have been established for humans [45,46], but data for chimpanzees or bonobos are scarce. Except for the two glucocorticoids, tetrahydrocortisol and tetrahydrocortisone, and the progesterone metabolite pregnandiol, the solvolysis proved to be necessary for all steroids investigated here, as their sulfate proportion exceeds 10%, with standard deviations up to 41%. Therefore, especially for the

comparison of hormone concentrations of several individuals or species, the solvolytic cleavage of sulfates is essential.

The data presented in Table 5 show that all investigated steroids can be measured with sufficient sensitivity in chimpanzee urine, except for progesterone, estriol and β-estradiol, which occur at lower concentrations in male urine. The three most abundant steroids are dehydroepiandrosterone, tetrahydrocortisone and tetrahydrocortisol—for a correct determination of these sample extracts have to be diluted 10-fold. For all other steroids, the dynamic range of the calibration is suitable if samples are concentrated twofold as described in Sections 2.4 and 2.5.

As a next step, we will investigate which of the tested urinary metabolites allow for the most reliable monitoring of gonadal and adrenal activity in different primate species.

4. Conclusions

The developed positive ion LC/ESI–MS/MS method enabled the measurement of 23 endogenous steroids in primate urine at low nanogram per milliliter level. The method comprises four classes of steroid hormones that are relevant to primate behavioral ecology—namely glucocorticoids, androgens, estrogens and gestagens. Given the small sample volume typically available from wild-living primates, the achieved detection limits of 0.1–0.5 ng/mL have been shown to be sufficient for analyzing hormone concentrations in only 200 μL of urine. However, estrogens often occur at lower levels in urine, and at present require a higher sample volume. The optimization of sample preparation parameters led to the following final conditions: the hydrolysis was performed with 5000 U β-glucuronidase/mL urine with a subsequent extraction of steroids at pH 9.6 using *tert*-butyl methyl ether.

The sample preparation, including hydrolysis and subsequent solvolysis, can be conducted with a high reproducibility. Since within the hydrolysis with β-glucuronidase from *E. coli*, only steroid glucuronides are cleaved, the solvolysis step has proven to be necessary for the correct determination of all investigated steroids except of tetrahydrocortisol, tetrahydrocortisone and pregnandiol.

The described method represents an attractive alternative to commonly used enzyme immunoassays, whenever a greater number of steroids have to be analyzed within the same urine sample, especially when cross reactivities of the antibody might negatively influence the results.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2007.11.009.

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