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Measuring urinary testosterone levels of the great apes—Problems with enzymatic hydrolysis using *Helix pomatia* juice

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ABSTRACT

Helix pomatia (*Hp*) juice is a common enzymatic preparation for deconjugation of urinary steroids. It has been used in many published studies on urinary testosterone (T) in chimpanzees and bonobos, although the ability of *Hp* juice to convert other urinary steroids into T has been reported for human urine. We developed a protocol for determination of reliable T levels in primate urine using liquid chromatography–mass spectrometry. T levels were determined in a set of human, bonobo and chimpanzee urine samples (A) by measurement of intact testosterone glucuronide (TG) and testosterone sulfate (TS), (B) after hydrolysis/solvolysis with β-glucuronidase from *Hp* and (C) from *Escherichia coli*. When samples were hydrolyzed with *Hp* juice, results were not correlated with the direct assay of TG and TS, and determined T concentrations were considerably higher. By contrast, hydrolysis with *E. coli* β-glucuronidase yielded a good agreement of T concentrations. We demonstrated the ability of *Hp* juice to convert androst-5-ene-3β, 17β-diol (A^5diol) into T using commercial standards and within the urine of all three species. As A^5diol usually is present at higher levels in urine than T, this artifact leads to erroneous results for T concentrations in primate urine. The proportion of T excreted as sulfate (TS) is often neglected as TS can only be cleaved by additional solvolysis. In all three species, we found substantial amounts of TS in the urine of some subjects and a high variance of TS proportion between and within subjects. Therefore the inclusion of solvolysis into the sample preparation seems necessary.

1. Introduction

The measurement of testosterone (T) levels in body fluids is widely used in behavioral studies of reproductive function, dominance and aggression in primates (Wingfield et al., 1990). In order to minimize the influence of the observer on the animal, the use of non-invasively collected samples, either urine or feces, is necessary. It is assumed that urinary T concentrations parallel biologically active T circulating in blood as has been shown in humans (Deheninn and Matsumoto, 1993; Palonek et al., 1995). In non-human primates most analyses have been done using radioimmunoassays (RIA) or enzyme immunoassays (EIA). Although these methods are sensitive, cost-effective and relatively easy to establish, they suffer from cross-reactivities of the antibody towards structurally similar compounds, which may have different or no effects on the behavior under study.

Studies on the relationship of dominance rank, aggressive behavior and urinary T levels have produced inconsistent findings in chimpanzees. A study on wild-living chimpanzee males reported significant T increases and increased rates of male aggressions during periods when parous females had maximally tumescent swellings (Muller and Wrangham, 2004). In the same study a significant correlation between urinary T levels and dominance rank was found. A study on captive juvenile and adolescent male chimpanzees revealed the same association (Anestis, 2006). Contrary to these results, in a study on captive adult chimpanzee males there was no obvious relationship between urinary T levels and dominance rank (Klinkova et al., 2004). Testosterone concentrations were not related to rates of initiated aggression and copulatory behavior, but a significant negative relationship between male T level and the rates of strong aggression received was reported.

In a comparative study, significantly lower T metabolite levels were found in bonobo males than in chimpanzee males using a group specific 5α-androstane-17α-ol-3-one antibody (Sannen et al., 2003). The difference in mean T metabolite levels between males and females was significant but much smaller in bonobos than in chimpanzees. The author attributed these findings to the female-dominated social structure of bonobos. But, because the chosen antibody does not measure either T itself or several known quantitatively important T-metabolites, these conclusions seem questionable.

By contrast, in a study of free-living bonobos a pronounced sex difference in urinary T levels was found using an EIA against T. This difference was not seen with the above mentioned 5α-androstane-17α-ol-3-one assay (Dittami et al., 2007).

These discrepancies show the importance of knowing exactly what is being measured by an immunoassay. Otherwise

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metabolites that are not specific for T or structurally similar adrenal androgens will be wrongly assigned as T.

T is excreted in human urine as glucuronide and, to a much lesser extent, as sulfate (Borts and Bowers, 2000). Quantification of T is usually carried out after hydrolysis of conjugates followed by the analytical detection of the free hormone (Ayotte et al., 1996).

The digestive juice of *Helix pomatia* (*Hp*) is a very common enzymatic preparation for total deconjugation of steroids, as it contains both β-glucuronidase and arylsulfatase activities and is therefore able to cleave both steroid glucuronides and sulfates. It has been used in all studies on chimpanzees and bonobos mentioned above. However, several authors have reported on artifacts of *H. pomatia* juice, leading to steroid conversion. First, the conversion of 3β-hydroxy-5-ene steroids with *Hp* juice was described, such as the transformation of pregnenolone into progesterone (Vanluchene et al., 1982). The presence of 3β-hydroxysteroid oxidoreductase, specific for 3β isomers, and 3-oxosteroid-4,5-ene isomerase was identified within *Hp* juice (Messeri et al., 1984). During incubation of [^{3}H]DHEA with *Hp* DHEA was progressively transformed into androst-4-ene-3,17-dione (A^4dione). Likewise, Schmidt et al. (1985) reported a decreasing analytical recovery of DHEA from urine by the use of increasing amounts of *Hp* enzyme preparation. Another study compared two *Hp* preparations from different sources and β-glucuronidase from *E. coli* for their ability to transform DHEA, A^4dione and androst-5-ene-3β,17β-diol (A^5diol) (Massé et al., 1989). Only β-glucuronidase from *E. coli* did not transform any of the three compounds tested. The incubation of DHEA with *Hp* led to the formation of small amounts of A^4dione, while A^4dione was transformed to a minor extent into T. A^5diol, however, was rapidly and extensively converted into T. The transformation ability of the *Hp* preparation was dependent on the supplier. This was also confirmed by a study from Venturelli et al. (1995) in which the conversion of A^5diol into T occurred only with *Hp* juice of one of the two suppliers tested. In human urine, GC–MS analysis showed a significant increase in T and a decrease in urinary A^5diol using *Hp* juice. The natural level of epiT, however, remained unchanged. Houghton et al. (1992) demonstrated the conversion of A^5diol into T and of DHEA into A^4dione in horse urine after incubation with *Hp* preparation. By mass spectrometric characterization of byproducts they identified, in addition to 3β-hydroxysteroid oxidoreductase and 3-oxosteroid-4,5-ene isomerase, the presence of 6-hydroxylase, 6-dehydroxylase and 6-hydroysteroid oxidase activity within *Hp* juice. A recent study presents evidence that the transformation of 3β-hydroxy-5-ene steroids by *Hp* juice is, in fact, due to cholesterol oxidase (Christakoudi et al., 2008). The extent of transformation depended on the amount of *Hp* juice added, was temperature dependant with a maximum at 70 °C and diminishing thereafter, depended on the substrate concentration and was subject to competition by different substrates. The exclusion of oxygen as well as the addition of antioxidants such as sodium ascorbate diminished the formation of transformation products (Christakoudi et al., 2008).

The arylsulfatase present in *Hp* preparation is known to be inactive towards 17β-sulfates of androgens (Shackleton, 1986; Houghton et al., 1992), therefore TS, which has the sulfate group in this position, is not cleaved. The deconjugation of all steroid sulfates can be accomplished by solvolysis, a chemical hydrolysis of sulfates with sulfuric acid in ethyl acetate (Burstein and Liebermann, 1958; Vestergaard, 1978; Ayotte et al., 1996; von Kuk et al., 2003).

In doping analysis of T and structurally related androgens in human urine, standard methods use β-glucuronidase from *E. coli* for cleavage of TG but neglect T excreted as sulfate (Saugy et al., 2000; Mareck et al., 2004). The administration of exogenous T is detected by an elevated T/epiT ratio. To our knowledge, the proportion of T excreted as sulfate in urine has never been examined in bonobos or chimpanzees. Given the relatively small sample size available for most behavioral studies on wild-living primates, a high variance in the sulfate proportion of T could have a considerable impact on the findings when comparing T concentrations of different individuals.

The aim of this study was the comparison of different methods for measuring T excreted in urine of primates in order to determine the most suitable approach for obtaining reliable T concentrations. Quantification was done using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Within LC–MS/MS a compound is characterized by its retention time on the chromatographic column, its precursor mass depending on the molecular mass and two specific fragments, which have to be present in a certain ratio. This technique enables the specific quantification of T without interferences from other androgens. Three different approaches were compared. TG and TS were extracted from urine as intact conjugates using solid phase extraction (SPE) and quantified by LC–MS/MS. The direct measurement for the two possible conjugates of T was taken as reference for comparing two hydrolysis/solvolysis procedures. In the first procedure, β-glucuronidase from *H. pomatia* was used for hydrolysis of TG while the remaining TS was subsequently cleaved by solvolysis. The second approach used β-glucuronidase from *E. coli* for hydrolysis followed by the same solvolysis procedure. In both cases, T was quantified in its free form by LC–MS/MS. A set of 28 male urines from 10 humans, 8 bonobos and 10 chimpanzees was used to compare T concentrations determined with the three different methods. The proportion of TS was determined in order to evaluate whether the solvolytic step is necessary. Additionally, possible causes of artifact formation with the *Hp* enzyme were investigated in primate urine.

2. Materials and methods

2.1. Chemicals and reagents

Steroid reference materials were obtained from Steraloids (Newport, Rhode Island, USA (Table 1). The deuterated internal standard 16,16,17-*d3*-testosterone (*d3*-testosterone, *d3*-T) was supplied by Sigma Chemical Co. (St. Louis, MO, USA). 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 enzymes, β-glucuronidase type VII-A from *E. coli* and β-glucuronidase/arylsulfatase type H5 from *H. pomatia* were purchased from Sigma Chemical Co. (St. Louis, MO, USA, *Hp 1*). A solution of β-glucuronidase/arylsulfatase from *H. pomatia* containing 100,000 U/ml was obtained from Roche Diagnostics (Penzberg, Germany, *Hp 2*).

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 obtained at 30 °C on a Gemini C18 column (150 × 2 mm, 3 μm), protected by a guard column (Security Guard 4 × 2 mm, 5 μm) of the same stationary phase (Phenomenex, Torrance, CA, USA). The injection volume was 20 μl. For measurement of T, TG and TS in SPE extracted samples eluent A was composed of water/acetonitrile (95/5; v/v) and eluent B of acetonitrile/water (95/5; v/v), both containing 0.3% 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 (20–21 min), 100% B (21–30 min), stepwise back to 30%B (30–39 min) (method 1). The compounds A^4dione and androstenediol (A^5diol) were integrated in a multi-method (method 2, Hauser et al., 2008) using the same eluent composition for A and B, but containing only 0.1% formic acid. The gradient program was as follows: 30% B (0–2 min), linear increase to 70% B (2–20 min), 90% B (21–24 min), 30% B (24–34 min). Different LC methods had to be used because elution of TS required a lower pH and a higher acetonitrile content. The auto sampler 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 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 percent 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 2 if not already published (Hauser et al., 2008). Testosterone and both of its conjugates were measured with a separate MS method, when analyzing SPE extracted samples (method 1, Table 2). A^4dione, A^5diol and DHEA were integrated into an existing multi-method containing 23 steroid hormones (method 2, Table 2), (Hauser et al., 2008), which was used for measuring urine extracts after hydrolysis/solvolysis.

2.4. Solid phase extraction (SPE) of conjugates from urine

One milliliter of 0.5 M sodium acetate buffer and 20 μl of a methanolic internal standard mix containing 250 ng/ml each of *d4*-cortisol, *d4*-estrone, *d3*-T and *d9*-progesterone were added to 200 μl urine. Internal standards were used to compensate for differences in extraction yield as well as for matrix effects on ionization efficiency in the ESI source of the MS. Solid phase extraction cartridges (C18 ec, 200 mg, Chromabond, Macherey&Nagel, Düren, Germany) were conditioned with 2 ml methanol, 2 ml deionized water and 2 ml sodium acetate buffer. Then the sample was loaded and subsequently the cartridge was washed with 2 ml 10% potassium carbonate and 5 ml deionized water and dried for 10 min. Elution of steroids was carried out with 3 × 1 ml methanol. The eluate was evaporated and the residue dissolved in 100 μl 30% acetonitrile.

2.5. Enzymatic hydrolysis of urine

2.5.1. β-Glucuronidase from H. pomatia

Lyophilized β-glucuronidase type H5 from *H. pomatia* (100,000 U, Sigma, *Hp 1*) was dissolved in 5 ml 0.5 M sodium acetate buffer which had been adjusted to pH 4.7. The second *Hp 2* preparation (100,000 U/ml, Roche) was diluted 1:5 with deionized water. Each sample of 200 μl urine was diluted with 800 μl of the same sodium acetate buffer and 40 μl enzyme preparation and 20 μl of an internal standard mix, containing 250 ng/ml each of *d4*-cortisol, *d4*-estrone, *d3*-T and *d9*-progesterone, were added. This mixture was incubated at 37 °C for 22 h under gentle agitation. Then 750 μl 10% potassium carbonate was added in order to stop the enzymatic reaction and to adjust the sample to pH 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 a HPLC vial.

2.5.2. 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 and 20 μl of an internal standard mix, containing 250 ng/ml *d4*-cortisol, *d4*-estrone, *d3*-T and *d9*-progesterone, were added. The phosphate buffer was prepared by mixing 49 ml of 0.5 M sodium dihydrogen phosphate, 61 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 were 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 a HPLC vial.

2.6. Solvolysis of urine

In order to cleave steroid sulfates, the aqueous phase of the enzymatic hydrolysis (Section 2.5) was subjected to a solvolysis (Von Kuk et al., 2003) 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 MT were added. Solid phase extraction cartridges (C18 ec, 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 subsequently 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 1 h 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.

2.7. Creatinine assay

To compensate for variations in urine concentration, all steroid concentrations were indexed against creatinine and expressed as ng/mg creatinine (Bahr et al., 2000). Creatinine was measured by microtiter plate analysis based on the Jaffé reaction (Hauser et al., 2008).

2.8. Urine samples

Urine samples of 9 male volunteers (28–38 years) were collected at the Max Planck Institute for Evolutionary Anthropology in November and December 2006 using 50 ml polypropylene tubes. Samples were frozen within 2 h after collection. Bonobo urine samples were collected from April 2006 to June 2006 from 8 adult males (14–23 years) in the Zoos of Frankfurt am Main, Wuppertal (Germany), Planckendael (Belgium) and Milwaukee and San Diego (USA) and from 3 free-living adult males in Salonga National Park (DR Congo). Chimpanzee urine samples were collected in Taï National Park (Côte d'Ivoire) in January 2006 from 5 free-living males (15–42 years), from June to July in Budongo (Uganda) from 3 free-living adult males (15–26 years) and in January 2007 at the Leipzig Zoo from two adult males (13 and 31 years). Urine was aspirated from vegetation or the 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.

Table 1
Investigated steroids and internal standards: systematic name, trivial name and abbreviation used

Systematic name	Trivial name	Abbreviation
Androst-4-ene-17β-ol-3-one	Testosterone	T
Androst-4-ene-17β-ol-3-one 17-glucuronide	Testosterone glucuronide	TG
Androst-4-ene-17β-ol-3-one 17-sulfate	Testosterone sulfate	TS
Androst-4-ene-3,17-dione	Androstenedione	A^4dione
Androst-5-ene-3β,17β-diol	Androstenediol	A^5diol
Androst-5-ene-3β-ol-17-one	Dehydroepiandrosterone	DHEA
Androst-4-ene-17α-methyl-17-β-ol-3-one	Methyltestosterone	MT
16,16,17-*d3*-testosterone	*d3*-Testosterone	*d3*-T

Table 2
MRM parameters of investigated steroids not included in method 2 (Hauser et al., 2008)

Analyte	Retention time (min)	M_w (g/mol)	Precursor ion	Cone voltage (V)	Product ions (1/2)	Collision energy (eV)	Time segment/ polarity
Androst-5-en-3β,17β-diol[b]	13.9	290	273	18	255/159	12/21	1/+
Androst-4-ene-3,17-dione[b]	16.66	286	287	30	97/109	22/26	2/+
Testosterone-17β-sulfate[a]	21.75	368	369	38	97/109	26/26	1/+

[a] Method 1 for SPE extracted urine.
[b] Method 2 for urine after hydrolysis/solvolysis.

2.9. Method evaluation

Calibration 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*-T, *d4*-estrone, *d9*-progesterone and MT, 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 (*d3*-T) peak area ratios using linear regression with $1/x$ weighing.

For determination of recovery and reproducibility of solid phase extraction, TG and TS were spiked at 50 ng/ml into deionized water, which was then processed as described for urine under Section 2.4. The response of obtained eluates was compared with standards directly prepared in HPLC eluent.

For determination of hydrolysis rate TS and TG were spiked separately into 200 μl deionized water at 50 ng/ml. Solvolysis rate was investigated after spiking TS or TG separately into 1 ml methanol at 10 ng/ml. Spiked samples were processed as described in Sections 2.5 and 2.6 and the amount of T generated was compared with a standard directly prepared in HPLC eluent.

2.10. Transformation of androst-5-ene-3β, 17β-diol into T by Hp preparations

To investigate the transformation of androst-5-ene-3β, 17β-diol (A^5diol) into T, the former was spiked at 50 ng/ml into 200 μl of distilled water. Samples were incubated with *Hp* and *E. coli* β-glucuronidase as described under Sections 2.5.1 and 2.5.2 and the response of generated T was compared with a T standard directly prepared in HPLC eluent. Additionally two control experiments were performed: The A^5diol standard was incubated without addition of enzyme and the enzymes were incubated without adding A^5diol in order to obtain an enzyme blank.

Both *Hp* preparations (see Section 2.1) were applied at 800 U/sample, the *E. coli* enzyme was compared at 200 U/sample as described under Section 2.5.2.

2.11. Statistical analysis

Since in most of the data sets occasional outliers or skewed distributions were detected, assumptions of parametric tests were violated and therefore only non-parametric tests were used, since in these cases the corresponding non-parametric test is more reliable and may have more power (Hunter and May, 1993). In addition, the data sets fulfilling the assumptions of a parametric test were analyzed using such a procedure and throughout essentially the same results were obtained (with regard to significance or non-significance) compared to the corresponding non-parametric test. Occasionally, we tested a single null-hypothesis (e.g. no difference between *E. coli*-based measures and *Hp*-based measures) several times (i.e. separately for the three different species). In such a case we controlled for multiple testing using Fisher's omnibus test (Haccou and Meelis, 1994; Quinn and Keough, 2002). This procedure combines a number of *P*-values into a single χ^2-distributed variable with its degrees of freedom equaling twice the number of *P*-values. We calculated Fisher's omnibus test by hand, Spearman correlations using a software written by R. Mundry and all other tests using SPSS 11.0.1 or 15.0.0. We calculated exact tests when small samples required their use (Siegel and Castellan, 1988; Mundry and Fischer, 1998) and indicate two-tailed *P*-values throughout.

3. Results

3.1. Method evaluation

The efficiency and reproducibility of the different sample preparation methods for enrichment of testosterone (T) from urine was investigated using water spiked with known amounts of commercial standards.

3.1.1. Solid phase extraction

As a first step the protocol for solid phase extraction of intact T conjugates was developed. The recovery of testosterone glucuronide (TG) and testosterone sulfate (TS) enriched by solid phase extraction (SPE) was 80.2% and 92.3%, respectively, with a relative standard deviation of 3.3% and 4.1% ($N = 6$). The elution had to be done with 3 ml methanol, as with 1 ml poor reproducibility was obtained (results not shown). In order to determine the limit of detection in SPE extracted samples, water samples spiked at 0.15, 0.25 and 0.5 ng/ml were extracted, and the signal to noise ratios (S/N) were compared. Based on the S/N criterion exceeding 3, the detection limit was 0.15 ng/ml for both TG and TS.

3.1.2. Hydrolysis and solvolysis

The efficiency of hydrolysis and solvolysis was tested by spiking TG and TS separately into distilled water and determining the amount of T generated. The cleavage rate of TG differed slightly between the two different β-glucuronidase preparations used (Table 3). About 65% of TG spiked was recovered as free T after hydrolysis using *Hp 1*, whereas 76% of TG was recovered as T after hydrolysis with the *E. coli* enzyme. A higher cleavage rate would be desirable. However, as the relative standard deviation of both reactions was below 4%, both methods seem appropriate for comparing T concentrations of different samples. TS is not cleaved by β-glucuronidase derived from *H. pomatia* (Shackleton, 1986; Houghton et al., 1992). Nevertheless, a small amount of T was found in TS spiked samples after hydrolysis with *Hp*. The reason is that the commercial TS standard always contains a small amount of free T.

In the solvolysis procedure 61.3% of TS is cleaved, whereas cleavage of TG is not observed.

3.2. Relative percentage of TG, TS and free T in urine

The relative percentage of TG, TS and free T was determined in three sets of human, bonobo and chimpanzee urine samples by SPE enrichment and subsequent quantification by LC–MS/MS. As known for human urine, TG represents the predominant conjugate in bonobo and chimpanzee urine (Fig. 1). About 20% of T is excreted as sulfate in all three species, while only minor amounts are present as free T. The relative proportions of TG and TS show a considerable variance between the different individuals of all three species (see also Section 3.5). The differences in TG and TS proportions were not significant when compared between

Table 3
Percentage of TG and TS cleaved after hydrolysis with β-glucuronidase from *Helix pomatia 1* and *E. coli* ($N = 8$) and percentage of TG and TS cleaved after solvolysis ($N = 8$) with relative standard deviation of cleavage rate (TG and TS were spiked separately at 50 ng/ml into deionized water for hydrolysis and at 10 ng/ml into 1 ml methanol for solvolysis)

Reaction		TG	TS
Hydrolysis *Helix pomatia* 1	Cleavage rate [%]	65.3	4.6
	CV [%]	3.2	12.0
Hydrolysis *E. coli*	Cleavage rate [%]	76.1	–
	CV [%]	1.9	–
Solvolysis	Cleavage rate [%]	0.3	61.3
	CV [%]	17.6	4.4

–, not determined.

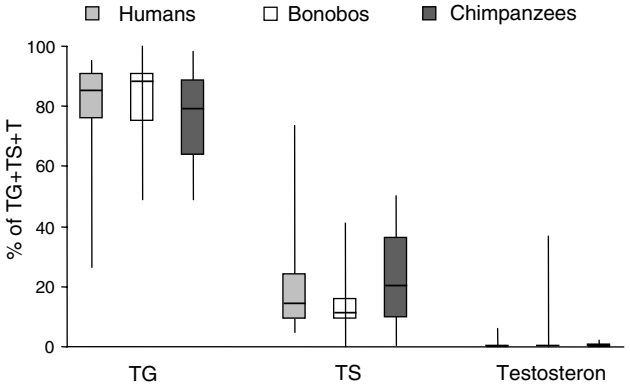

Fig. 1. Relative proportions of TG, TS and free T in male human ($N = 10$), bonobo ($N = 8$) and chimpanzee urine ($N = 10$) measured by method 1. The bold horizontal line represents the median, the top and bottom horizontal lines represent the 25th and 75th percentiles, the vertical line shows the range between minimum and maximum.

species (Kruskall–Wallis *H*-test, TG: $\chi^2 = 0.423$, df = 2, $P = 0.809$; TS: $\chi^2 = 1.014$, df = 2, $P = 0.602$).

3.3. Comparison of the three sample preparation methods for T extraction from urine

The enrichment of intact TG and TS by SPE involves only a single step, therefore the introduction of artifacts or the loss of analytes during sample preparation is minimized. Consequently, this technique was used as a reference method for comparing the results of urine hydrolysis/solvolysis procedures using *Hp 1* and *E. coli* β-glucuronidase. Fig. 2 depicts the correlation of total T concentrations (sum of TG, TS and free T) determined in three sets of human, bonobo and chimpanzee urine samples with the different methods. In human and bonobo urine no significant correlation was seen between results of SPE and *H. pomatia* hydrolysis/solvolysis (Spearman's rank correlation, humans: $r = 0.212$, $N = 10$, $P = 0.564$; bonobos: $r = 0.429$, $N = 8$, $P = 0.299$). For chimpanzee urine, a significant correlation existed between the results of both methods ($r = 0.786$, $N = 8$, $P = 0.028$; Fisher's omnibus test combining results of the three correlations: $\chi^2 = 10.72$, df = 6, $P = 0.098$). In general, the determined T concentrations were much higher after hydrolysis with *Hp 1* enzyme than after extraction of urine by SPE, the effect being most pronounced in human urine (up to 40-fold, Wilcoxon test: $T^+ = 55$, $N = 10$, $P = 0.001$), lesser in bonobo urine (up to 8-fold, $T^+ = 36$, $N = 8$, $P = 0.008$) and least in chimpanzee urine (up to 4-fold, $T^+ = 34$, $N = 8$, $P = 0.012$; Fisher's omnibus test: $\chi^2 = 32.46$, df = 6, $P < 0.001$).

In contrast, when comparing the results of SPE with hydrolysis/solvolysis employing *E. coli* β-glucuronidase, highly significant correlations were found for all three sample sets (humans: $r = 0.976$, $N = 10$, $P = 0.001$; bonobos: $r = 0.929$, $N = 8$, $P = 0.002$; chimpanzees: $r = 0.927$, $N = 10$, $P = 0.001$; Fisher's omnibus test: $\chi^2 = 39.84$, df = 6, $P < 0.001$). Testosterone concentrations determined after SPE tended to be slightly higher than after hydrolysis/solvolysis with *E. coli* enzyme in human urine (up to 1.4-fold, $T^+ = 48$, $N = 10$,

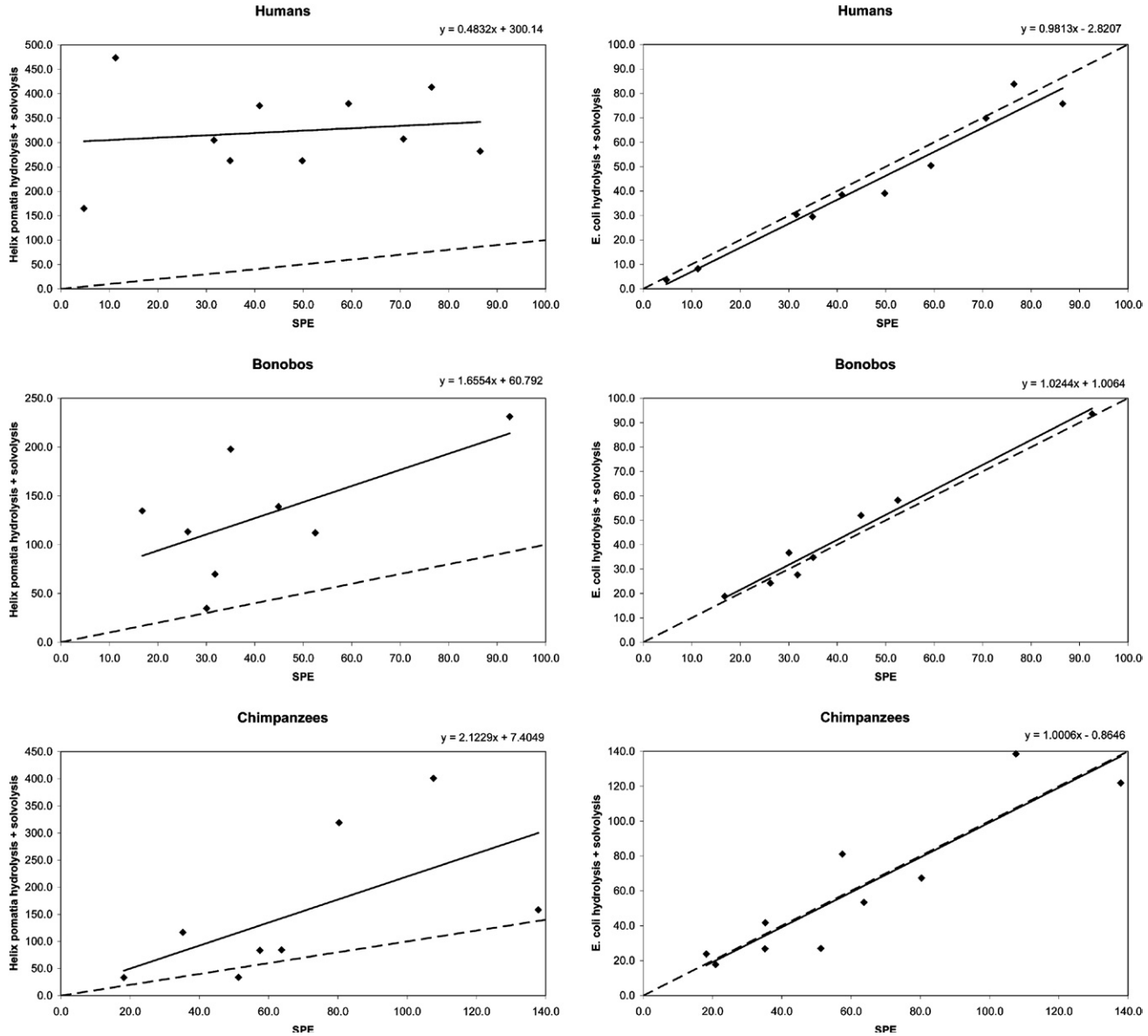

Fig. 2. Correlation of total T (sum of T+TG+TS) [ng/mg creatinine] determined after SPE of urine and LC–MS/MS analysis of T+TG+TS compared to hydrolysis of urine with *Helix pomatia* β-glucuronidase/solvolysis (*Hp 1*)and hydrolysis with *E. coli* β-glucuronidase/solvolysis followed by LC–MS/MS analysis of T. The dotted line represents the line of equality.

$P = 0.037$), whereas in bonobo und chimpanzee urine no systematic differences between both methods were seen (bonobos: $T^+ = 27$, $N = 8$, $P = 0.25$; chimpanzees: $T^+ = 32$, $N = 10$, $P = 0.695$; Fisher's omnibus test: $\chi^2 = 10.09$, df = 6, $P = 0.121$).

3.4. Enzymatic transformation of androstenediol (A^5diol) into T using *H. pomatia* juice

3.4.1. Transformation in spiked water

The incubation of A^5diol standard with both *Hp* enzymes led to the formation of T (Fig. 3). With *Hp* preparation 1 (our study, Sigma) 43% of spiked A^5diol were converted into T relative to an equally concentrated T standard. With *Hp* preparation 2 (Roche) 17% T were formed. However, below 5% of A^5diol were recovered after both experiments, indicating that beside the transformation into T other enzymatic reactions take place. This assumption is confirmed by the presence of some unidentified peaks in the chromatograms of A^5diol extracts after incubation with *Hp* enzyme 1 and 2. In both *Hp* enzyme blanks as well as in the *E. coli* blank virtually no T was found.

In contrast to *Hp* preparation, the β-glucuronidase from *E. coli* converted only 0.5% A^5diol into T, while 84.4% of A^5diol were recovered. The incubation of A^5diol without enzyme did not lead to the formation of T, which shows that the observed isomerization and oxidation is indeed caused by *Hp* enzymes.

3.4.2. Transformation in urine

After demonstrating the capability of the two *Hp* enzyme preparations to convert A^5diol into T, the extent of this reaction was examined within the three sets of urine samples by comparing the results of hydrolysis with *Hp* and *E. coli* β-glucuronidase followed by solvolysis. Additionally the transformation of DHEA into A^4dione was monitored. In all three urine sets treated with *Hp* enzyme the mean concentration of A^5diol is lower, while the mean T concentration is higher, when comparing to the same urine sets treated with the *E. coli* enzyme. Likewise, the mean concentration of DHEA is lower in samples hydrolyzed with *Hp* preparation, while the mean concentration of A^4dione is higher (exemplified by bonobo urine in Fig. 4). Therefore, the conversion of A^5diol into T and the conversion of DHEA into A^4dione take place in urine of all three species as a result of the additional enzymatic activities of *Hp* juice. The effect of this artifact on the determined T concentrations depends on the ratio of A^5diol/T present in urine. In all human urines examined, A^5diol was present in a much higher concentration than T. The ratio varied from 1:9 to 1:88, therefore even a conversion rate of only 17% A^5diol to T has a large impact. In bonobo urine, this influence is less pronounced. The ratio of A^5diol/T varied from 1:0.7 to 1:18, but only one individual out of 8 had a ratio <1. In chimpanzee urine the ratio varied from 1:0.4 to 1:16, and again only one individual out of 8 had a lower ratio than 1.

3.5. Variability of TS proportion

The average percentage of TS is low compared to TG (Fig. 1). For cleavage of TS a solvolysis has to be carried out, which introduces an additional and time-consuming step into the extraction of urine samples. Therefore, we investigated if this step is necessary for the comparison of T levels of different individuals by analyzing the size and the variance of TS proportion between different subjects and within subjects of one species. Samples were hydrolyzed using *E. coli* β-glucuronidase and submitted to solvolysis. For comparing between subjects, only one sample per individual was analyzed. The samples for comparison within one subject were collected on different days and times of the day. On average between 10% and 20% of T are excreted as sulfate (Table 4). But there are single subjects with a much higher TS ratio. In humans we found 73% as TS in one male, in bonobos the highest TS ratio was 36% and in chimpanzees one individual excreted 79% of T as sulfate. When testing for individual differences in TS proportions we found no significant effect in bonobos (Kruskall–Wallis *H*-test, $\chi^2 = 0.596$, df = 2, $P = 0.742$) but clear differences in chimpanzees ($\chi^2 = 42.239$, df = 3, $P < 0.001$). This means there were individual chimpanzees with a consistently higher ratio of TS.

4. Discussion

4.1. Validation of methods used for measurement of TG, TS and T

We demonstrated the suitability of the measurement of intact TS and TG by LC–MS/MS as a reference method for the evaluation of different approaches for hydrolysis/solvolysis. The enrichment of TG and TS from urine by solid phase extraction (SPE) could be conducted with high recovery and reproducibility. The limit of detection was sufficiently low to quantify TS in male urine. The positive ionization mode enabled the specific and sensitive quantification of T, TG and TS on their intense fragment ions at *m/z* 97 and 109 fragments, which are typical for steroids with a 4-ene-3-one structure (Williams et al., 1999). With respect to TG and TS, the frag-

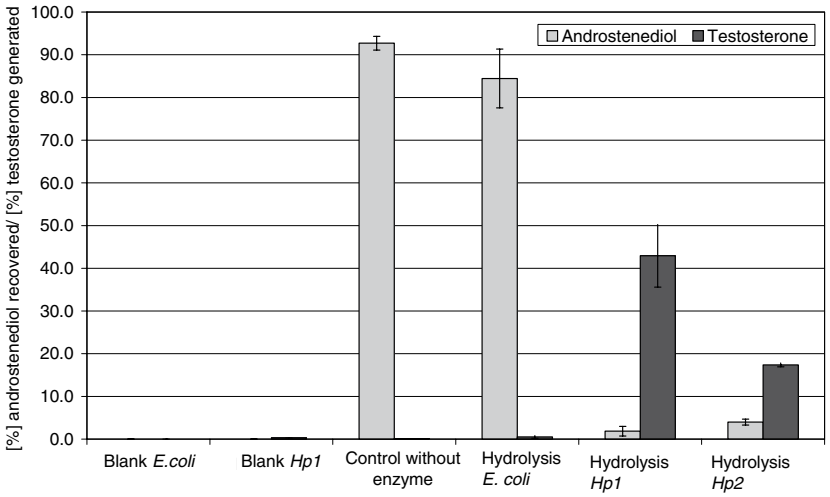

Fig. 3. Conversion of androst-5-ene-3β,17β-diol into T (A^5diol was spiked at 50 ng/ml into deionized water and incubated with *Helix pomatia* as described under Section 2.5.1.

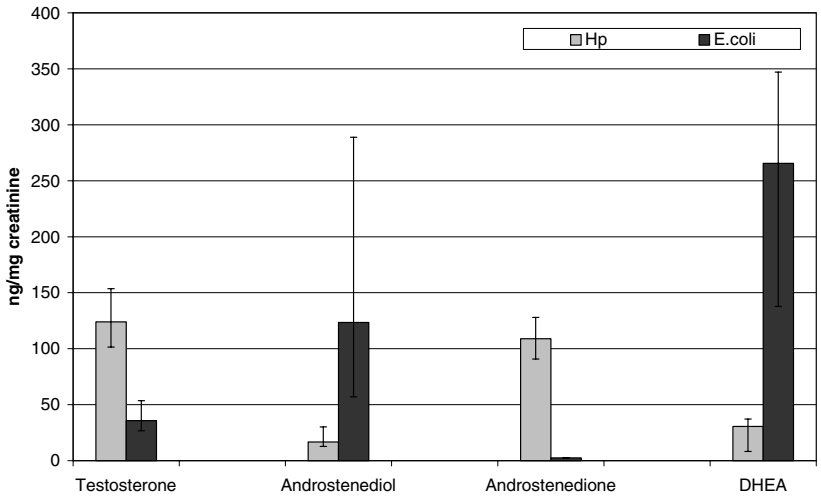

Fig. 4. Mean concentration of T, 5-A[5]diol, A[4]dione and DHEA in male bonobo ($N=8$) urine after hydrolysis with *Helix pomatia* and *E. coli* β-glucuronidase and solvolysis. The bars represent the median, the errors bars show the 25th and 75th percentiles.

mentation of the $[M+H]^+$ ions in the positive ionization mode has been shown to provide better structural information on the steroid backbone than the analysis of fragments derived from the $[M-H]^-$ ions (Bowers and Sanaullah, 1996). The direct measurement of intact steroid conjugates by LC–MS/MS has been used by several authors (Antignac et al., 2005; Borts and Bowers, 2000; Buiarelli et al., 2004; Saudan et al., 2006). All of these studies describe the enrichment of glucuronides and sulfates from urine by SPE.

The direct measurement of conjugates is often used to determine the relative proportions of several possible conjugates in order to discriminate endogenous production from exogenous administration. In addition, the influence of chemical or enzymatic hydrolysis on steroid recovery can be avoided. The cleavage of steroid conjugates with subsequent quantification of the free hormone is necessary if several steroid hormones are to be analyzed in parallel. Many hormones have multiple hydroxyl-groups that can be conjugated either with sulfuric or glucuronic acid resulting in numerous possible conjugates. As it is often not known exactly which conjugates of a specific steroid are excreted in urine and not every possible compound is commercially available, the cleavage of all glucuronides and sulfates is the most common approach (Ayotte et al., 1996; Mareck et al., 2004; Shackleton, 1986). The enzymatic hydrolysis with all three β-glucuronidase preparations tested in this study only cleaved TG, whereas TS was not affected (confirmed by Shackleton, 1986 and Houghton et al., 1992). Likewise, the solvolysis only cleaved TS and left TG intact (confirmed by Von Kuk et al., 2003). Therefore, both steps must be carried out in order to measure total T excreted in urine. The cleavage rates of hydrolysis and solvolysis were not complete but ranged between 61% and 78%. However, the relative standard deviations of cleavage rates were below 5%. Consequently, T levels can be determined and compared precisely between individuals, but due to lower recovery from urine, concentrations can be expected to be lower than after SPE. The high variance of TG/TS proportions between different individuals of humans, bonobos and humans underlines the necessity of either summing up TG, TS and free T or cleaving the different conjugates before quantifying the free hormone in order to compare T levels of different individuals.

Table 4
Range and variance of TS proportion from total testosterone (TG+TS+T) between and within subjects measured by method 2

Species	Range [%]	Average ± SD [%]
Between subjects		
Humans ($N=9$)	6–73	23 ± 21
Bonobos ($N=8$)	2–22	11 ± 7
Chimps ($N=9$)	4–31	14 ± 8
Within subjects		
Bonobo 1 ($N=8$)	4–36	11 ± 11
Bonobo 2 ($N=11$)	2–34	11 ± 12
Bonobo 3 ($N=7$)	4–12	8 ± 3
Chimpanzee 1 ($N=21$)	6–62	27 ± 14
Chimpanzee 2 ($N=19$)	14–79	55 ± 18
Chimpanzee 3 ($N=24$)	1–57	21 ± 12
Chimpanzee 4 ($N=20$)	2–22	11 ± 6

4.2. Hydrolysis with Hp juice falsifies determination of T in urine

When comparing the results of the different sample preparation methods no correlation was found between the results of SPE and hydrolysis with *Hp* preparation in human and bonobo urine, while a weak correlation existed for chimpanzee urine. Especially for human urine, hydrolysis with *Hp* enzyme resulted in much higher T concentrations than SPE, and concentrations exceeded the reference range reported for humans 12- to 14-fold (Borts and Bowers, 2000; Von Kuk et al., 2003). For bonobo and chimpanzee urine, determined T levels were also significantly higher after hydrolysis with *Hp* enzyme, the effect being smaller in chimpanzee urine. By contrast, when using *E. coli* β-glucuronidase for hydrolysis, highly significant correlations were found between the results of SPE and hydrolysis/solvolysis for all three species. Furthermore, the relative magnitude of T concentrations was similar in both methods. Because of the good accordance to the results of SPE, the use of *E. coli* β-glucuronidase is a better alternative for determination of reliable T levels in primate urine. The incubation with *Hp* preparation apparently leads to transformation of other steroids present in urine into T. These artifacts have been reported for human urine (Massé et al., 1989; Venturelli et al., 1995; Houghton et al., 1992, Christakoudi et al., 2008). *Hp* juice contains not only a β-glucuronidase/arylsulfatase, but also a cholesterol oxidase, which is capable of transforming androst-5-ene-3β,17β-diol into T.

4.3. Hp juice transforms urinary A[5]diol into T

The ability of the *Hp* preparations used in this study to convert A[5]diol into T could be demonstrated with commercial standards. However, this conversion was not complete—only 43% of spiked A[5]diol was transformed into T with *Hp* 1 (Sigma) and 17% with *Hp*

2(Roche). This finding is confirmed by other authors. Massé et al. found a conversion rate of 70% for a 3 h incubation with *Hp* at 55 °C and a 50% conversion for a 16 h incubation at 37 °C. Venturelli et al. reported a 29.2% conversion rate with one *Hp* preparation and only 9.8% with another batch from the same supplier. The majority of transformation products of A^5diol have been identified as testosterone, 6-dehydro-testosterone, 6-oxo-testosterone and 6α- and 6β-hydroxy-testosterone (Houghton et al., 1992).

The *E. coli* preparation used in this study does not transform A^5diol into T. This result is in accordance with other studies (Massé et al., 1989; Houghton et al., 1992), and it is the reason laboratories involved in doping control of urine exclusively use β-glucuronidase from *E. coli* (Spyridaki et al., 2006; Mareck et al., 2004; Gotzmann et al., 2004; Saugy et al., 2000).

Hp preparation induced the transformation of A^5diol into T and DHEA into A^4dione in urine of humans, bonobos and chimpanzees and thus has a considerable influence on the T levels determined. The size of this effect depends on the ratio of A^5diol/T present in urine. In most samples investigated for this study, A^5diol occurred in higher concentrations than T, thus also a conversion rate of 17% A^5diol into T would affect the result of T quantification. For samples with an A^5diol/T ratio <1, the elevated T concentrations measured after hydrolysis with *Hp* enzyme can not be explained by A^5diol conversion alone. Another possible source of T is the transformation of A^4dione (Massé et al., 1989). Furthermore, the presence of at least five other enzymatic conversions within *Hp* juice (Houghton et al., 1992) can lead to a lot of unpredictable reactions in urine, which could potentially result in the formation of T or have an effect on other steroids of interest. Therefore, the use of *Hp* juice for cleavage of urinary steroid glucuronides should be avoided if T is the main steroid of interest. Otherwise, cholesterol oxidase activity within *Hp* juice can be inhibited by concomitant use of ascorbic acid (Christakoudi et al., 2008). If this procedure suppresses any unwanted transformation should be tested for the specific *Hp* preparation used. The amount of *Hp* juice required for optimal hydrolysis of steroids can be minimized by removing inhibiting glucuronic acid, galacturonic acid, saccharolactones and phosphates by SPE prior to hydrolysis (Vestergaard, 1978; Shackleton, 1986).

4.4. Interconversion and physiological functions of adrenal androgens

A^5diol and DHEA are both androgens' of primarily adrenal origin (Arlt, 2004; Felig et al., 1995). DHEA is converted into A^4dione in the adrenal cortex, which is a precursor of T (Fig. 5). The adrenal cortex produces only about 5% of T in males as a result of very low concentrations of the relevant enzymes for its production. However, in the Leydig cells of the testis a high amount of A^4dione is converted into T. Androgen biosynthesis in the human testis preferentially proceeds via the Δ^5 pathway from pregnenolone to DHEA and A^5diol before entering the Δ^4 pathway as A^4dione and T (Felig et al., 1995, see Fig. 5). Though being precursors of T, different physiological functions have been reported for DHEA, A^4dione and A^5diol. Both, DHEA and A^4dione do not bind to the androgen receptor (Arlt, 2004). DHEA sulfate (DHEAS) levels show a pronounced age-dependency, therefore DHEA has been discussed as anti-ageing hormone. DHEA interacts with different neurotransmitters, thereby suggesting a putative anti-depressant action (Arlt, 2004). A^5diol binds to the androgen receptor, but also to the estrogen receptor. It has been suggested to play a role in the genesis of prostate cancer in males and may be involved in the genesis of estrogen-sensitive carcinomas, such as breast cancer (Miyamoto et al., 1998). Other authors report an influence of A^5diol on the regulation of immune response (Loria, 1997). Given the complex interactions and different and not completely understood physiological functions of these adrenal steroids, it does not seem appropriate

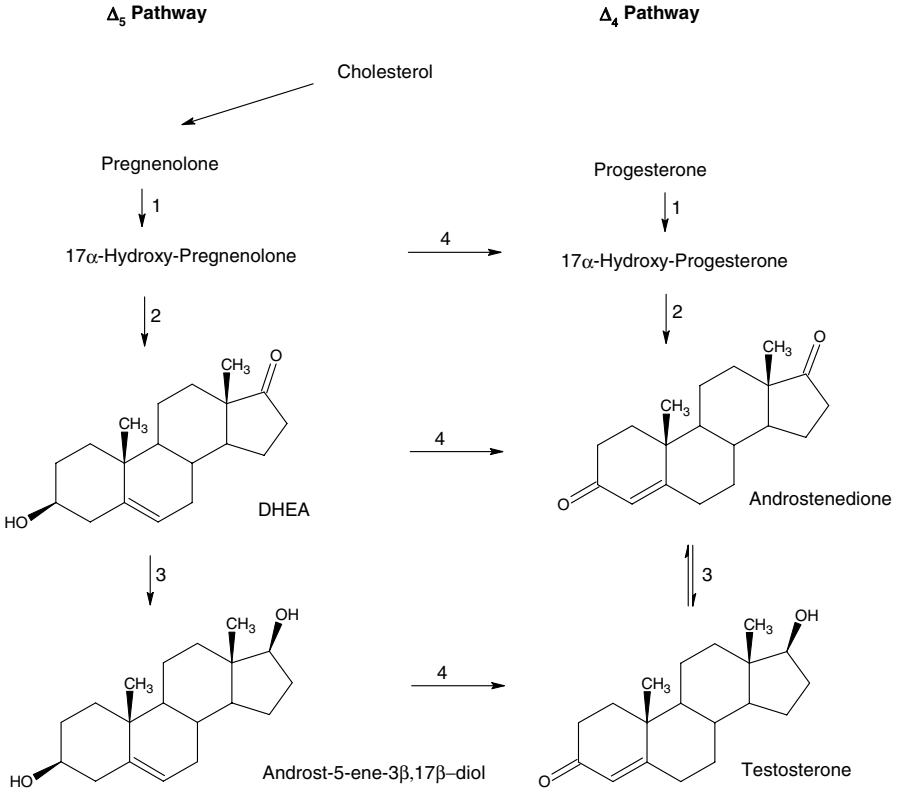

Fig. 5. Androgen synthetic pathway. Enzymes are represented by numbers: 1 = 17α-hydroxylase, 2 = 17,20-lyase (desmolase), 3 = 17β-hydroxydehydrogenase, 4 = 3β-hydroxydehydrogenase/$\Delta^{4,5}$-isomerase.

to convert them into T and measure them together with T, without being able to differentiate between them.

4.5. Size and variability of TS proportion in primate urine

The analysis of the three sets of 8–10 urines from male humans, bonobos and chimpanzees by SPE of intact conjugates revealed a high inter-individual variance of both total T levels and the relative proportions of TG and TS. However, the average TG/TS ratio was very similar in all three species. On average 20% of T is excreted as sulfate in humans, bonobos and chimpanzees, while the excretion of unconjugated T usually is below 1%. These data are in accordance with the excretion pattern reported for humans (Deheninn and Matsumoto, 1993).

Although, in general, the proportion of TS was around 20%, there were individuals with a much higher ratio. In humans one individual excreted 73% of T as sulfate, in bonobos the highest TS ratio was 36% and in chimpanzees one individual had a ratio of 79% TS. As the variance of TS proportion was high within all three species, no significant difference between the species was found. However, when analyzing TS proportion repeatedly within one individual, among chimpanzees one male with a consistently high rate of TS excretion could be identified. The T level of this individual would be underestimated if a solvolysis with subsequent analysis of TS were not carried out. Given the small sample size typically available in studies on wild-living primates, the inclusion of TS in the estimate of total T therefore seems necessary.

The high variance observed within subjects shows that even if studies on behavioral correlates to T excretion are carried out within the same subject, it is not appropriate to neglect TS.

In humans, a study on six quantitative important androgen sulfates in 141 male athlete's urines showed a median TG concentration of 32 ng/ml (0.025 quantile 0.7 ng/ml; 0.975 quantile 187 ng/ml) and a median TS concentration of 3 ng/ml (0.025 quantile 0.3 ng/ml; 0.975 quantile 19 ng/ml) (Von Kuk et al., 2003). Another study on exogenous T administration reported a mean TS ratio of 25% in the control and recovery phase. The ratio of TG/TS varied between 1.2 and 212.38 in a reference group of 45 adult human males with a mean TG/TS ratio of 27.65 and a standard deviation of 49.2 (Borts and Bowers, 2000). These data demonstrate a high variance of individual TG and TS excretion rates for human urine. We now provide reference data that confirm this high variance for chimpanzees and bonobos.

Existing studies on behavioral correlates to urinary T levels in bonobos or chimpanzees (Sannen et al., 2003; Muller and Wrangham, 2004; Klinkova et al., 2004), as well as comparative studies on testosterone metabolism in different apes (Möhle et al., 2002; Hagey and Czekala, 2003; Dittami et al., 2007) all used *Hp* enzyme for hydrolysis of urine. This study shows that this procedure not only falsifies T quantification by conversion of A^5diol into T, but also neglects T excreted as sulfate. As a better alternative, we propose hydrolysis with β-glucuronidase from *E. coli* with a subsequent cleavage of steroid sulfates by solvolysis.

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