Androgen and androgen metabolite levels in serum and urine of East African chimpanzees (*Pan troglodytes schweinfurthii*): Comparison of EIA and LC–MS analyses

Anna Preis a, Lawrence Mugisha b,c, Barbara Hauser a, Anja Weltring a, Tobias Deschner a,*

a Max Planck Institute for Evolutionary Anthropology, Department of Primatology, Deutscher Platz 6, D-04103 Leipzig, Germany
b Ngamba Island Chimpanzee Sanctuary, P.O.Box 884, Entebbe, Uganda
c Department of Wildlife and Animal Resources Management, Faculty of Veterinary Medicine, P.O. Box 7062, Kampala, Uganda

**ABSTRACT**

The primary male androgen testosterone (T) is often used as an endocrinological marker to investigate androgen-behaviour interactions in males. In chimpanzees and bonobos, studies investigating the relationship between T levels and dominance rank or aggressive behaviour have revealed contradictory results. The immunoassays used in these studies were originally developed for the measurement of steroids in serum. Their application to non-invasively collected samples, however, can lead to methodological problems due to cross-reacting metabolites, which might occur in urine or faeces but not in blood.

The overall aim of this study, therefore, is to clarify whether a T enzyme immunoassay (EIA) is an applicable method to monitor testicular function in adult male chimpanzees. To estimate the impact of cross-reacting androgens on the used T EIA, we compared the results of an EIA measurement with a set of androgen metabolite levels measured by LC–MS. In urine from male chimpanzees, cross-reactivities appear to exist mainly with T and its exclusive metabolites, 5α-dihydrotestosterone (5α-DHT) and 5α-androstanediol (androstanediol). Both urinary and serum T levels of male chimpanzees were significantly higher than female T levels when measured with the T EIA, indicating a reliable measurement of testicular androgens and their exclusive metabolites with the used EIA. In urine from female chimpanzees, the comparison between LC–MS and T EIA results indicated a higher impact of cross-reactions with adrenal androgen metabolites. Therefore, the investigation of urinary T levels in female chimpanzees with a T EIA seems to be problematic. Overall our results show that a T EIA can be a reliable method to monitor testicular function in male chimpanzee urine and that LC–MS is a valuable tool for the validation of immunoassays.

**1. Introduction**

Testosterone (T) is the primary biologically active male androgen. It has modulating effects on morphology, physiology, and behaviour in many vertebrate species, including primates. Through its organizational effects during early ontogeny and puberty, T mediates the differentiation of the primary and a number of secondary male sexual characteristics [48]. Activational effects of T during adulthood are related to traits that enhance male reproductive success, such as mating behaviour, male–male aggression, dominance relationships, and territory defence [5,12,22,67]. In a number of non-seasonal group-living primate species, dominance relationships and levels of aggression are positively correlated with T levels in unstable periods [4,55,58]. T is therefore used as an endocrinological marker to investigate androgen-behaviour interactions in males, which are related to reproductive effort and success.

For ethical and practical reasons, in most field studies on wild-living primates, endocrine profiles are monitored using non-invasively collected samples such as urine or faeces. Darting of free-living animals is risky, especially for arboreal species, and unpractical since the darting itself influences the steroid levels in the bloodstream [46,59]. Furthermore, measurements of steroids in serum or plasma are influenced by the pulsatile secretion of hormones [66]. Therefore, faeces and urine are easily collected sample mediums that represent an average of circulating hormone levels across days or hours, respectively. However, steroid measurements in these samples are subject to methodological problems since steroid hormones are highly metabolised before their excretion [49]. The resulting metabolites can differ enormously between species.
and even between sexes [41, 51]. Furthermore, due to the metabolism of steroids in the liver and kidneys prior to excretion, steroids in urine do not occur in the same ratios as they do in the circulation. For example, biologically active T occurs in serum of primate males in higher concentrations than other androgens and their metabolites while T as a urinary androgen is quantitatively of only minor importance [21, 23, 41, 60]. Despite this, urinary T concentrations in humans are known to parallel biologically active T in serum [10, 52]. Furthermore, although T is only a minor component of urinary androgen metabolites, it correlates best with serum T in adult male chimpanzees [24]. As in humans, two additional urinary androgen metabolites, 5α-dihydrotestosterone (5α-DHT) and 5α-androstenediol (androstanediol), seem to derive exclusively from circulating T in male chimpanzees [24, 64, 65].

Therefore, together with T, these androgens are likely to be suitable urinary markers for testicular function in male chimpanzees [24].

Immunoassays, which function through a specific antibody–antigen reaction, are a commonly used method for steroid analyses. The majority of these assays were originally developed and validated for the analysis of steroids in blood, where gonadal and adrenal steroids occur in high concentrations in their biologically active forms. Due to these high concentrations, the impact of cross-reactivities with structurally similar antigens (e.g., metabolites) that also bind to the specific antibody of the assay are generally negligible. When these assays are adapted to the measurement of steroids in urine and faecal extracts, where metabolites usually occur in much higher concentrations than the biologically active steroid, formerly negligible levels of cross-reactivities can reach significant dimensions [2, 51, 63]. Still, existing cross-reactivities might not negatively influence the accuracy of the measurement of gonadal functioning as long as the cross-reacting metabolites indicate the same function as the steroid of interest, such as a shared testicular origin. However, cross-reactions with structurally similar but physiologically different hormones can have a major impact. For example, for the assessment of testicular function and activity in males, the co-measurement of adrenal androgens and their metabolites has to be avoided and vice versa. Such a co-measurement has been shown for a group-specific glucocorticoid assay where a major faecal androgen metabolite co-reacted with the group-specific antibody [17, 26].

Thus, before an immunoassay is used for the measurement of steroids in urine or faecal extracts, a careful validation is of paramount importance [25, 43, 49, 51, 63].

In male chimpanzees and bonobos, studies investigating the relationship between urinary T levels, aggression, and dominance relationships have revealed contradictory results. While T levels in wild-living East African male chimpanzees are positively correlated with rank and aggression, irrespective of periods of instability, this pattern was absent in a group of captive chimpanzees [32, 45]. Although the observed differences can be related to environmental and group size differences between the two study groups (11 males vs. 4 males; wild vs. zoo) [32], methodological problems in the T measurement could not be excluded. In a carefully conducted radio metabolism study, a 17α-HO EIA (T EIA) did not produce significant differences between the urinary androgen levels of male and female chimpanzees [41]. It was assumed that a high co-measurement of adrenal androgens (DHEA) with the T assay led to falsely elevated androgen levels in females [41].

Significant differences in urinary androgen levels between male and female chimpanzees and bonobos were found using a 17α-HO EIA [56]. However, another study using the same assay could not confirm these results for male and female bonobos [11]. Finally, while a positive correlation between urinary T concentrations and dominance rank of the long-term resident males was found in a group of wild-living bonobos [36], urinary T levels were not related to dominance rank in either of the two sexes in captive bonobos [57]. In the aforementioned studies, a number of different immunoassays for assessing testicular activity in males and T levels in females were used producing partly contradictory results.

In light of these opposing results, there is a need for further investigation to distinguish testicular and adrenal androgens and their metabolites in chimpanzee urine and to identify a reliable method to measure them. The overall aim of this study, therefore, is to investigate whether a T EIA reliably monitors testicular function in adult male chimpanzees. To estimate the impact of other androgens and androgen metabolites in serum and urine of male and female chimpanzees on the results of the T assay, we compared two different analytic methods. We used a T EIA system obtained from the Institute of Biochemistry and Ludwig Boltzmann Institute of Veterinary Endocrinology in Vienna and liquid chromatography–tandem mass spectrometry (LC–MS). The latter method is highly specific and allows one to analyse a number of metabolites in parallel [23].

To test if the measurement of chimpanzee urine samples with a T EIA can indeed reliably measure testicular activity, we aimed to answer the following questions:

1. How do T levels differ between the two methods?
2. How do serum and urinary T levels differ between males and females when measured with LC–MS and EIA?
3. How do urinary androgen levels measured with a T EIA correlate with testicular androgens and their metabolites measured with LC–MS?
4. How do urinary T levels correlate between EIA and LC–MS measurements in sexually mature female chimpanzees?

2. Materials and methods

2.1. Animals

Urine and serum samples were obtained from chimpanzees living in the Ngamba Island chimpanzee sanctuary in Uganda. A total of 42 chimpanzees live in this sanctuary and all are orphans that were confiscated from poachers and bush meat dealers. At the time of sample collection, all individuals were kept in one social group except for two males, who were kept separate from the others. Altogether there were 16 adult and adolescent males (8–24 years of age), two of which were castrated, three juvenile males (3 years of age), and 23 adolescent and adult females (6–24 years of age). During the day, the chimpanzees were kept outside and were able to range freely in a forested fenced-in area. During the night they stayed in indoor enclosures. They were provisioned with fruit, vegetables, and water.

2.2. Sample collection

Serum samples were collected during a yearly health check in February and March 2008. For the health check the chimpanzees were anaesthetised with a combination of ketamine (3 mg/kg) and medetomidine (0.03 mg/kg). Sedation was carried out with a blowgun or by hand when animals were familiar with the keepers. One 7.5 ml blood sample was taken from each chimpanzee 10–15 min after the beginning of the anaesthetisation. The samples were centrifuged immediately and serum was stored at −80°C.

A total of 89 urine samples were collected from the anaesthetised chimpanzees 30–45 min after the onset of anaesthetisation and also at different times during the 2 month health check period. During anaesthesia, urine samples for some of the chimpanzees were obtained by catheterisation or abdominal manipulation. The other urine samples were collected in the indoor enclosures before the chimpanzees were released to the outdoor enclosures. Sampling was carried out with a collecting vessel and normally took...
place between 7:00 and 8:00 am. The collected urine was stored in 2 ml tubes at –80 °C. The samples were transported from Uganda to the MPI-EVA in Leipzig, Germany, on dry ice. At the MPI-EVA, the samples were stored at –20 °C until extraction.

2.3. Extraction of steroids

2.3.1. Serum

The extraction of steroids from serum followed the protocol of Hauser et al. (2011) [24]. We used the same extraction procedure for the enzyme immunoassay (EIA) as well as for the high-performance liquid chromatography–tandem mass spectrometry (LC–MS) measurements. To 200 µl of serum, we added 400 µl acetonitrile and 50 µl internal standard mixtures, which contained 10 pg/µl each of prednisolone, d4-estrone, d3-testosterone, and d9-progesterone (Dr. Ehrenstorfer, Augsburg, Germany). The internal standard mixture was only added to the serum extracts for LC–MS measurements to control for losses during extraction and purification, and matrix effects on ionisation of MS measurements. Samples were vortexed for 1 min and centrifuged for 10 min at 18000g. The supernatant of the precipitated protein pellet was aspirated and diluted with 3 ml deionised water. This was followed by a solid phase extraction with C18 cartridges (Chromabond C18, 200 mg, Macherey and Nagel, Dueren, Germany). Conditioning was carried out with 3 ml methanol and 3 ml deionised water (HPLC grade, Mallinckrodt Baker, Phillipsburg, NJ, USA). After the serum extract was applied, the cartridge was washed with 5 ml deionised water (HPLC grade) and 3 ml 20% methanol and dried for 30 min. Elution of steroids was carried out with 3 ml methanol. The eluate was evaporated to dryness and reconstituted with 300 µl acetonitrile and was then vortexed and centrifuged for 2 min. The extract was then transferred to a 650 µl Eppendorf tube and evaporated in a Speedvac. The residue was redissolved in 30 µl acetonitrile and transferred to a 150 µl insert of a HPLC vial. Extracts for the EIA measurement were stored in Eppendorf tubes until further use.

2.3.2. Urine

The steroids from the urine samples were extracted as described in detail by Hauser et al. [23,25]. We used 200 µl urine for steroid extraction and added 50 µl internal standard mixture only to extraction for the LC–MS measurement. The internal standard mixtures contained the same deuterated standards as described for serum above but with different concentrations (100 ng/µl). Steroid glucuronides were hydrolysed using β-glucuronidase from Escherichia coli (activity: 200 U/40 µl) (Sigma Chemical Co., St. Louis, MO, USA). β-Glucuronidase from E. coli was used for hydrolysis and a separate solvolysis was conducted in order to avoid enzymatic conversion of androgens as described for β-glucuronidase/sulphatase of Helix pomatia juice [25,37,38,64]. Extracts were purified by solid phase extractions (Chromabond C18, 200 mg, Macherey-Nagel, Dueren, Germany). Afterwards, steroid sulphates were cleaved by solvolysis with ethyl acetate/sulphuric acid (200 µg sulphuric acid, 98% in 250 ml ethyl acetate). Extraction of steroids was carried out with tert. butyl methyl ether, evaporated and reconstituted in 30% acetonitrile. Extraction efficiencies ranged from 60.4% to 103% [23].

2.4. Analytical methods

2.4.1. LC–MS

LC measurements were carried out using a Waters Alliance 2695 separation module equipped with a quaternary pump and a column oven (Waters, Milford, MA, USA) and separation was performed on a reverse phase C18 material (Gemini; 150 × 2 mm, 3 μm) with gradient elution (acetonitrile/water and 0.1% formic acid) [23]. MS analyses were carried out on a Quattro Premier XE tandem mass spectrometer (Micromass, Manchester, UK) with an electro spray interface (ESI) in positive mode [23].

The quantitative analysis of 11 urinary and five serum androgens by LC–MS was realised in the range of 0.3 – 1000 ng/µl [23,25] (Table 1). We excluded samples that had an internal standard recovery of more than ±50% from the expected values from our analysis. We examined LC–MS data with MassLynx (Version 4.1; QuanLynx-Software).

2.4.2. EIA

For the measurement of urinary and serum androgens with EIA, we used a T EIA, which detects 17β-OH-androgens and their metabolites. Antisera for this T assay were raised in rabbits against testosterone-3-CMO [41,50]. As label we used 5α-androstane-3β, 17β-diol-3-Hemisuccinat-DADDO-Biotin, and T (0.31–40 pg/well) as standard [40,41,50]. Incubation of streptavidin and substrate solution took place for 45 min at 4 °C.

Assay procedures and cross-reactivities have been described in detail [40,41,50] (Table 2). In addition, we tested the cross-reactivity of three more androgen metabolites, DHEA (androst-5-ene-3β,17β-diol-17-one), androstenedione (androst-4-ene-3,17-dione; A4dione), and androstenediol (androst-5-ene-3β,17β-diol; A4diol). Cross-reactivity was determined by detecting the concentration of the tested substance at 50% binding in the T EIA through a duplicate dilution series. This concentration was then compared with the corresponding concentration of the T standard at 50% binding and expressed in percentages [40]. Cross-reactivities of the T antibody were 0.01% for DHEA, 1.6% for A4dione, and 1.5% for A4diol (Table 2).

The inter-assay coefficients of variation (CV) of high and low concentration quality controls were 2.7% and 11.9% for serum extracts and 12.4% and 13.3% for urine extracts, while intra-assay CVs were 9.5% and 12.3%, respectively.

Urinary androgens and their metabolites were indexed against creatinine to compensate for variations in urine concentration [2,14]. Therefore, urinary steroids are expressed as ng/mg creatinine (ng/mg crea). Creatinine was measured by microtiter plate analysis based on the Jaffé reaction [23,62].

2.5. Statistical analysis

For the comparison of serum T levels between the two methods and sexes, 16 serum samples from 16 females between 7 and 24 years of age and 12 serum samples from 12 males between 8 and 22 years of age were used. For urine analysis, we used a total of 24 samples from 12 males and 22 samples from 15 females. We only included sexually mature individuals in our statistical analyses. Captive chimpanzees reach sexual maturity at around 7 years of age [7,42].

We used only urine samples that were collected between 6:00 and 8:00 a.m. in order to avoid diurnal effects of hormone concentrations in urine [44,45]. To compare T levels between the two methods and sexes, we used mean values per individual. All statistical analyses were carried out with non-parametric tests. When sample sizes were small, we used exact tests [47].

To investigate the comparability of T levels measured with EIA and with LC–MS in urine of male and female chimpanzees and to detect cross-reactivities of the T EIA, we calculated Spearman rank correlations [61].

Since we had different numbers of urine samples from each of the study individuals (ranging from 1 to 5), we had to avoid pseudo-replication [30]. Therefore, we used a program written in R (Version 2.8.1) by Mundry to choose one urine sample per individual by chance. With these random selections, a Spearman rank correlation between different measures of T levels was calculated.

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This procedure was repeated 1000 times in order to avoid results being dependent on a single random selection. If there was only one sample for an individual, this was used for each correlation. At the end, the program calculated mean statistical parameters such as r and p values derived from all random selections, which were then used as final results.

Testing the same null-hypothesis (e.g., no correlation between T (EIA) and any of the androgens and their metabolites (LCMS)) several times required some correction for multiple testing. We achieved this using Fischer’s omnibus test. This procedure combines a number of P-values into a single chi-square distributed variable with degrees of freedom equaling twice the number of P-values [20,54]. If this is significant, it means that the collective of correlations is significant. However, since several correlations between variables obtained from the same set of individuals are not independent, an essential assumption of this procedure was not met. We hence established significance based on a permutation procedure [1,35]. For this we randomised the variable to be correlated with all others, derived all its correlations with the other variables and combined the obtained P-values into a single chi-square distributed variable with degrees of freedom equaling twice the number of P-values [20,54]. If this is significant, it means that the collective of correlations is significant. However, since several correlations between variables obtained from the same set of individuals are not independent, an essential assumption of this procedure was not met. We hence established significance based on a permutation procedure [1,35].

<table>
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<tr>
<th>Systematic name</th>
<th>Trivial name</th>
<th>Percentage (%)</th>
<th>Tested by</th>
</tr>
</thead>
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<td>Testosterone</td>
<td>100</td>
<td>Palme and Möstl [50]</td>
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<tr>
<td>7β-Hydroxy-5α-androstane-3-one</td>
<td>5β-Dihydrotestosterone</td>
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<td>Palme and Möstl [50]</td>
</tr>
<tr>
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<td>androstenediol</td>
<td>12.3</td>
<td>Palme and Möstl [50]</td>
</tr>
<tr>
<td>Androst-4-ene-3β,17β-diol</td>
<td>Androstenediol</td>
<td>7.6</td>
<td>Palme and Möstl [50]</td>
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3. Results

3.1. T levels in serum

T levels measured with EIA in male serum were significantly higher than measured with LC–MS (Wilcoxon signed-rank test: T = 77.0, N = 12, p < 0.001). The EIA produced 1.4-fold higher T levels than the LC–MS (Table 3). In female serum, the mean T level was 0.89 ± 0.33 ng/ml measured with EIA and 0.11 ± 0.06 ng/ml measured with LC–MS. In female serum, T levels of the EIA measurement were significantly higher than T levels measured with LC–MS (Wilcoxon signed-rank test: T = 136.0, N = 15, p < 0.001); in our study, the EIA produced 8.9-fold higher T levels than the LC–MS measurement.

With both methods, T levels of males were significantly higher than those of females (Mann–Whitney U test, males N = 12, females N = 16; EIA: U = 0.0, p < 0.001; LC–MS: U = 0.0, p < 0.001).

Of all androgens and androgen metabolites measured with LC–MS in male serum, T, together with DHEA, had the highest proportions of 36% each (Table 3). In female serum, DHEA had the highest proportions of 36% each (Table 3).
correlations = 22.10, permutation test:
lites or metabolites that could derive from both the testis and adre-
proportion of all androgens and their metabolites measured with
Table 3
Table 4
Table 5

3.2. T levels in urine

3.3. Correlations between male urinary T levels measured with EIA and

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connections between T and other androgens and androgen metabolites, correlations between T and androgen metabolites within the LC–MS measurement were used as a control (Table 5). Only one of these correlations between T and androstaneol was significant (r = 0.65, N = 12, p = 0.04). Androstaneol also showed a nearly significant correlation with T measured with EIA (r = 0.59, N = 12, p = 0.06). There was no significant correlation between T LC–MS and 5α-DHT (N = 12, r = 0.26, p = 0.46). Furthermore, T LC–MS did not correlate significantly with any of the adrenal androgens and their metabolites measured with LC–MS (Table 5). Correction for multiple testing revealed that the collectives of both sets of correlations within the LC–MS measurement were not significant. However, there was a trend toward significance in the collective of correlations between testicular androgens and their metabolites (chi-square combining P-values of spearman correlations = 9.15, permutation test: p = 0.085), as opposed to the collection of correlations between T and adrenal androgen metabolites of both steroid sources (chi-square combining P-values of spearman correlations = 12.29, permutation test: p = 0.61).

3.4. Correlations between female urinary T levels measured with EIA and urinary androgens and androgen metabolites measured with LC–MS

Overall, the collective of correlations between T EIA and LC–MS androgens and androgen metabolites was significant (chi-square combining P-values of spearman correlations = 49.13, permutation test: p = 0.016).

In female urine, there were several significantly positive correlations between T EIA and adrenal gland androgens such as DHEA (r = 0.61, N = 15, p = 0.02), A4dione (r = 0.63, N = 15, p = 0.02) and A4diol (r = 0.63, N = 15, p = 0.02) measured with LC–MS (Table 6). We found no significant correlation between T levels measured with EIA and T levels of the LC–MS analysis (r = 0.45, N = 15, p = 0.11). Within the LC–MS measurements, there were also many significant or nearly significant correlations between T and other androgens and androgen metabolites, for which a significant correlation was also found for T levels measured with EIA. There was only one exception where a significant correlation between T EIA and A4dione (LC–MS) did not show a significant correlation with the T concentrations measured with LC–MS. The collective of correlations within the LC–MS measurement was not significant, but revealed a trend towards significance (chi-square combining P-values of spearman correlations = 55.53, permutation test: p = 0.087).

4. Discussion

The data presented in this study provide a detailed analysis of the T EIA measurement in urine and serum of male and female chimpanzees validated and compared with LC–MS analyses. T levels were significantly higher in both matrices and sexes when measured with the T EIA than with the LC–MS. Males had significantly higher T levels than females in serum and urine in both measurement types. When comparing urinary T measured with EIA and LC–MS androgens, we found that only androgens of the LC–MS measurement, which are known to exclusively derive from T in humans, correlated significantly with the T measured with EIA and no indications of co-measurements of adrenal androgens were found [24,64,65]. Taken together, these results indicate that the tested T EIA is a reliable method for monitoring testicular function in male chimpanzees. However, T levels measured with the T EIA in female urine showed indications of considerable co-measurements of adrenal androgens and their metabolites, which is probably due to the much lower T concentrations in relation to these cross-reacting metabolites than in male urine. No significant correlation between the T levels of the EIA and the LC–MS was found, indicating that the tested T EIA is not a reliable method to measure urinary T in female chimpanzees.

4.1. T levels in serum and urine

T levels measured with the EIA were generally higher than measured with LC–MS. This was true for both matrices as well as for both males and females. These results are in concordance with other comparative studies in which immunoassays generally revealed higher steroid levels than mass spectrometry measurements [3,8,15,29] but see [13]. Most of the former studies related the higher steroid levels measured with immunoassays to the co-measurement of additional steroids by the used antibody. This is consistent with our findings in male chimpanzees. Differences between the two T measurements were higher in urine than in serum. Since, in contrast to in urine, steroids in serum primarily occur in their natural form and are not highly metabolised, the higher proportion of metabolites in male urine cross-reacting with the T antibody led to more elevated T readings. However, for female chimpanzees, the differences between T levels within urine and serum measurements of the two methods were more similar. This could be due to the already very low concentration of T and the relatively higher influence of cross-reacting metabolites in female serum. Alternatively, the low T concentrations in female serum might have been insufficient for a reliable measurement. Both explanations seem to be possible since T levels in female serum were extremely low (detection limits of the LC–MS for T was reached) and cross-reacting steroids, such as A4dione and DHEA, had much higher concentrations in relation to T than in male serum.

Despite this, we found highly significant differences between the T levels of males and females with both methods and in both serum and in urine. These results are in contrast to an earlier validation study that did not find significant differences in urine with the same T immunoassay [41]. Mean urinary T levels of males and females measured with the T EIA were not in the same range as measured in the study from Möhle and colleagues [41]. Indeed they found much higher T levels in male and especially female chimpanzees than we found in our study. Since the aforementioned study used the same T antibody, higher levels of cross-reacting metabolites could not explain the opposing results. We assume that the reason for the elevated urinary T levels and the non-significant differences between the T levels of males and females in the study by Möhle and colleagues [41] was the use of a special enzyme from H. pomatia (H. p. juice), β-glucuronidase/sulphatase. H. p. juice has the additional ability to artificially increase the original T levels in samples by converting A4diol to T through a cholesterol oxidase which is also present in the juice [25,28,37,64]. It has been shown that even a slow conversion rate of the H. p. juice enzymes can have a strong impact due to significantly increasing the origin-

![Image]

Table 6: Spearman rank correlations between urinary T concentrations measured with EIA and urinary androgen concentrations of the LC–MS analysis of 15 females between 7 and 24 years of age. Significant correlations are highlighted with bold script [58].

<table>
<thead>
<tr>
<th>Androgens (LC–MS)</th>
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<th>T (EIA)</th>
<th>T (LC–MS)</th>
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</thead>
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<td></td>
<td></td>
<td>r</td>
<td>p</td>
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<tr>
<td>T</td>
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<td>Epandrostosterone</td>
<td>15</td>
<td>0.66</td>
<td>0.01</td>
</tr>
<tr>
<td>Epitriocanolone</td>
<td>13</td>
<td>0.56</td>
<td>0.06</td>
</tr>
<tr>
<td>Etiochlorolone</td>
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<td>0.21</td>
<td>0.45</td>
</tr>
<tr>
<td>Androsterone</td>
<td>15</td>
<td>0.61</td>
<td>0.02</td>
</tr>
<tr>
<td>A4diol</td>
<td>15</td>
<td>0.63</td>
<td>0.02</td>
</tr>
<tr>
<td>Androstaneol</td>
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<td>0.35</td>
<td>0.26</td>
</tr>
<tr>
<td>5α-DHT</td>
<td>15</td>
<td>0.73</td>
<td>0.04</td>
</tr>
</tbody>
</table>

4.2. Correlations between male urinary T levels measured with EIA and urinary androgens and androgen metabolites measured with LC–MS

Correlations were calculated to investigate the comparability of T levels measured with EIA and with LC–MS in urine of male chimpanzees, and to detect possible cross-reactivities of androgen metabolites with the T EIA. Correlations between T and other androgens and androgen metabolites within the LC–MS measurement were used to control for physiological coherences between T and other androgen metabolites due to metabolic origin. There was a strong correlation between T concentrations measured with EIA and LC–MS. The correlation was even stronger when all main urinary testicular androgens known for humans, which could be measured with the LC–MS [24], were summed up and correlated with the T EIA measurement. No significant correlation between adrenal androgens of the LC–MS measurement and T levels measured with EIA was found. There was an additional significant correlation between T measured with EIA and 5α-DHT of the LC–MS measurement, which was probably due to the known cross-reactivity of 5α-DHT with the T antibody [50]. Among the correlations within the LC–MS measurement, there was only one significant correlation between T and androstenediol. Androstenediol is known to be produced from T through 5α-DHT, and therefore, a known metabolic association between these two androgens [24,64]. The measurement of 5α-DHT within our LC–MS method can be confounded by epiandrosterone, as both compounds coelute and have the same retention time [23]. This could explain the non-significant correlation between T and 5α-DHT within the LC–MS measurements as well as the absence of an overall significance in the collective of correlations of the testicular androgens (T-androstenediol and 5α-DHT) within the LC–MS measurement.

But besides that, our results indicate that significant cross-reactivities of the tested EIA mainly occur with testicular androgens and their metabolites and that existing cross-reactivities of the T EIA with adrenal androgens seem to have a negligible impact on the measurement of testicular function in male chimpanzees. Therefore, contrary to results from earlier studies [21,41], T immunoassays can be a reliable method to investigate male gonadal activity and to monitor male reproductive state in urine of chimpanzees.

4.3. Correlations between female urinary T levels measured with EIA and urinary androgens and androgen metabolites measured with LC–MS

To investigate whether the T EIA is a reliable method to measure T levels in urine of female chimpanzees, we conducted correlations between T measured with EIA and T and other androgens and androgen metabolites measured with LC–MS, as described for males above. We did not find a significant correlation between the T levels of the two methods. Instead there were significant correlations between T measured with EIA and adrenal androgens measured with LC–MS, such as A4dione, A5diol, and DHEA. In addition, T measured with EIA correlated with two other androgen metabolites, androsterone and epiandrosterone, from the LC–MS measurement, which are not directly produced by the adrenal gland or gonads but are known to be converted from both types of androgens through the adrenal androgen A4dione in humans [16,39]. These metabolites were also significantly or nearly significantly correlated to T within the LC–MS measurement, indicating an association with T through metabolic pathways rather than detecting cross-reacting metabolites. There was one exception where T levels of the EIA measurement did significantly correlate with A4dione measured with LC–MS, but no significant or nearly significant correlation was observed within the LC–MS measurement. Since no cross-reactivity with the tested T antibody for A4dione had been described we conducted a cross-reactivity test which revealed a cross-reactivity of 1.6% (unpublished data). This finding could explain the significant correlation between T measured with EIA and A4dione of the LC–MS measurement. Furthermore, we tested A4dione and DHEA for cross-reactivities with the T antibody and found cross-reactivities of 1.9% and 0.01%, respectively. The significant correlations between T measured with EIA and DHEA and A4dione of the LC–MS measurement can thus partly be explained by cross-reactions with the T antibody. However, the cross-reactivity of DHEA with the antibody is on the low side and although DHEA concentrations are high in female urine, this cannot fully explain the strong correlation between T measured with EIA and DHEA (LC–MS), nor can it explain the nearly significant correlation between T and DHEA within the LC–MS measurement.

In contrast to the production of T in males, T in females is not produced from the gonads in high proportions [34,64]. In human females, the production of T occurs equally in the gonads and adrenal glands, each contributing around 20%, and the majority of the T originates from the peripheral conversion of adrenal androgens such as A4dione (60%) and DHEA (1–8%) [6,27,31,34]. Since the main precursor of T in human females is A4dione, we expected to find a significant correlation between urinary T and A4dione levels in female chimpanzees within the LC–MS measurement as well. The absence of a significant correlation between these two androgens within the LC–MS measurement could indicate a difference between human and chimpanzee females in terms of sources of T production, however, this issue needs further investigation. In addition the non-significance of the collective of correlations within the LC–MS measurement makes the interpretation of these results even more difficult. Nevertheless, there was a trend towards significance within the collective of correlations that cannot be ignored. Further investigation would be important to clarify these results.

Furthermore, the correlations between the T EIA and the LC–MS measurement of male and female chimpanzees were highly diverse, which indicates that T production and metabolism differs between the two sexes, which would agree with results from studies on humans [33,34,64]. In summary, due to the numerous correlations between T (EIA) and various androgens and androgen metabolites from the LC–MS measurement, including adrenal androgens, which are not all known to be converted into T in peripheral tissue in humans and the low concentration of T in female chimpanzee urine in relation to other cross-reacting androgens and androgen metabolites, the T EIA does not seem to be a reliable method to investigate female urinary T levels.

Our results showed that the tested T EIA is a reliable method to assess testicular activity and function in male chimpanzees. This was verified with the strong correlation between urinary T levels measured with EIA and LC–MS and with the lack of indications for influential cross-reactivities of adrenal androgens with the T antibody. Furthermore, we found highly significant differences between T levels measured with EIA and LC–MS of male and female chimpanzees, which indicates that the T EIA can discriminate between T levels measured with EIA of male and female chimpanzees in relation to behavioral patterns and could also be explained as artifacts of the used H. p. juie.

In contrast to males, the performance of the T EIA in measuring urinary T levels in females was not reliable. This was due to the low concentration of T in female chimpanzee urine and due to the high proportion of cross-reacting adrenal androgens. Considering all possible validation procedures and methods, the LC–MS seems to be a valuable option for a detailed validation of immunnoassays.

Acknowledgments

We thank all involved veterinarians and keepers from Ngamba Island Sanctuary (Uganda), run by the Chimpanzee Sanctuary and Wildlife Conservation Trust, for the collection of urine and blood samples. We thank Roger Mundry for help with the statistical analysis and Carolyn Roewney for comments on earlier versions of the manuscript. This research was supported by the Max Planck Society.

References


