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General and Comparative Endocrinology 129 (2002) 135–145

GENERAL AND COMPARATIVE ENDOCRINOLOGY

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Characterization of urinary and fecal metabolites of testosterone and their measurement for assessing gonadal endocrine function in male nonhuman primates

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Accepted 30 August 2002

Abstract

The aims of the present study were (i) to provide basic comparative data on the time course, route, and characteristics of excreted [^{14}C]testosterone (T) metabolites in three nonhuman primates: the common marmoset (*Callithrix jacchus*), the long-tailed macaque (*Macaca fascicularis*) and the chimpanzee (*Pan troglodytes*) and (ii) to use this information to help validate the measurement of urinary and fecal testosterone metabolites for assessing androgen status in Anthropoid primates. Radiolabeled ^{14}C-T (10–30 μCi) was injected intravenously into one adult male of each species and the excreta collected over the next 5 days. Peak radioactivity in urine was detected within 2 h and accounted for 67% (Mf), 80% (Cj) and 91% (Pt) of the total radioactivity recovered. The time course of excretion of radioactivity in feces showed a higher variation between species (4–26 h to peak values). In all three species, the majority (>90%) of urinary metabolites were excreted as conjugates whereas the proportion of conjugated metabolites in feces was substantially lower and more variable. High pressure liquid chromatography (HPLC) analysis of urinary and fecal extracts revealed multiple peaks of radioactivity in all three individuals, but each with a distinctive pattern. Native T was excreted in only small amounts into the urine, whereas it was virtually absent in the feces of all three individuals. Three C17 group-specific enzymeimmunoassays using antisera against testosterone, 5α-androstane-17α-ol-3-one and androsterone were evaluated for their ability to discriminate immunoreactive androgen levels between intact males, castrated males and females based on measurements in urine and feces. In the marmoset, all assays (except for T in feces) clearly discriminated between test groups; in the chimpanzee significantly higher levels of androgen immunoreactivity in intact versus castrated males were measured in urine, but not feces. In the macaque, only the 5α-androstanolone measurement in feces discriminated between groups. Data on the results of a radiometabolism study using ^{3}H-DHEA (a weak adrenal androgen) in a long-tailed macaque suggested that co-measurement of metabolites derived from T and DHEA in the assays tested might explain the difficulties in discriminating gonadal status in the two Old World primate species. Collectively, the data show that T metabolism in primates is highly complex and that no single method for noninvasive assessment of androgen status can be used for application across species. The importance of a proper validation of the methodology for each species is emphasised.

Keywords: Nonhuman primates; Testosterone metabolism; Urinary and fecal excretion; Long-tailed macaque; Chimpanzee; Common marmoset

1. Introduction

The degree to which competitive ability and reproductive (mating) success in male primates are mediated through variations in testicular endocrine function is a question that remains largely unresolved. Limited data exist for only a few species, and these mainly derive from studies of captive maintained animals in which testicular function has been assessed by measurements of circulating testosterone (e.g. Steklis et al., 1985; Wickings and Dixson, 1992). Given the negative and often unpredictable effects of capture and restraint procedures required for blood sampling on the adrenal–gonadal axis (e.g.

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PII: S0016-6480(02)00525-7

McGrady, 1984; Reinhardt et al., 1991) and of the practical difficulties in applying such methods to animals living in social groups, there are clear limitations to the use of invasive methodologies in this context. Furthermore, results obtained from animals in captive, laboratory conditions may not be of direct relevance to our understanding of proximate mechanisms underlying male mating success in naturally reproducing social groups. Thus, a noninvasive alternative, based on measurement of androgen metabolites in urine and feces would be useful, not only in avoiding the potential complications of capture stress, but also by facilitating studies of animals in more natural social settings in the wild (Whitten et al., 1998).

To date, there are few examples of studies involving noninvasive assessment of androgen status in primates. Most report on measurements of immunoreactive testosterone in urine (e.g. Maggioncalda et al., 1999; Robbins and Czekala, 1997; Ziegler et al., 2000), although it has been known for some time that native testosterone is quantitatively of only minor importance (e.g. Epple et al., 1991; Yamamoto et al., 1978a,b). Fecal androgen assays have been described only in lemurs (e.g. Brockman et al., 1998; Kraus et al., 1999; Von Engelhardt et al., 2000) and the muriqui (Strier et al., 1999) and were also restricted to testosterone immunoreactivity. Data for Old World monkeys and apes are not available. Among studies in nonprimate species, Brown et al. (1996) and Velloso et al. (1998) also report on the use of fecal immunoreactive testosterone measurements in carnivore species, but at the same time indicate that testosterone itself is either not detectable or present in only very small amounts. To date, there are no comparable data on the abundance of testosterone in relation to other androgen metabolites in feces for any primate species.

The overall aim of the present study was therefore to provide basic comparative data on testosterone metabolism and excretion in primates and to use this information to validate the measurement of urinary and fecal testosterone metabolites for reliably assessing male testicular endocrine function. Using radiometabolism studies in representative species of the three simian primate taxa, the chimpanzee (*Pan troglodytes*), the long-tailed macaque (*Macaca fascicularis*), and the common marmoset (*Callithrix jacchus*), our specific aims were firstly to describe (i) the time course and route of testosterone excretion, and (ii) the characteristics of the major urinary and fecal metabolites excreted. Based on this information, the second specific aim was to assess the suitability of different enzymeimmunoassays for the measurement of the major excreted urinary and fecal testosterone metabolites. Finally, we assessed the reliability of the different EIA systems in discriminating gonadal status by comparing androgen levels measured in urine and fecal samples collected from intact males, castrated males and females of each species.

2. Materials and methods

2.1. Animals

2.1.1. Radiometabolism study

One adult male chimpanzee (*P. troglodytes* Pt, 10 years, 57 kg), long-tailed macaque (*M. fascicularis* Mf, 9 years, 7 kg), and common marmoset (*C. jacchus*, Cj, 4 years, 0.38 kg) were used in this study. During the experimental phase, the subjects were housed individually in metabolic cages at either the Biomedical Primate Research Center (BPRC), Rijswijk, The Netherlands (chimpanzee) or the German Primate Center in Göttingen, Germany (long-tailed macaque, marmoset). All animals were fed twice daily a diet consisting of commercial monkey pellets supplemented with bread, fresh fruits, and vegetables. Diet remained constant during the experimental period and water was available ad libitum.

During the experimental phase, each animal was housed in a metabolic cage fitted with a metal waste pan. To facilitate the collection of fecal samples and avoid cross-contamination between urine and feces, a wire mesh was placed between the metal pan and the foot end of the cage (cf. Bahr et al., 2000). The total volume/mass of each urine/fecal sample was immediately collected after voiding using a syringe (urine) and spatula (feces). The pan and the wire mesh were cleaned with ethanol every evening to prevent a possible contamination from residual urine and feces on samples collected the next day.

2.1.2. Biological validation test

Urine and fecal samples were collected from each of five adult intact males, castrated males, and females of each species for testing the biological validation of three androgen assays in discriminating gonadal status (see below). Animals were kept in different locations (Pt: Burger Zoo Arnhem; BPRC Rijkswijk, The Netherlands; Zoo Karlsruhe, Germany; Mf: German Primate Center, Göttingen, Covance, Münster; University of Münster, all Germany; Cj: German Primate Center, Göttingen). While chimpanzees and marmosets were kept in social groups or pairs, all macaques were housed individually. Diets of the animals were the same as for the animals used for the radiometabolism studies (see above) and remained constant throughout the study period. Ages in chimpanzees ranged between 8–21 years (intact males), 10–25 years (castrates), and 17–35 years (females). Ages in the macaques ranged from 6–12 years (intact males), 10–18 years (castrates), and 4.5–6 years (females). Marmosets were 2.5–7 years (intact males), 3–7 years (castrates), and 2–7 years (females) old.

2.2. Administration of [^{14}C]testosterone

Following anesthesia with ketamine (Ketavet, Pharmacia Upjohn GmbH, Erlangen, Germany), radiola-

beled testosterone ([4(C)-^{14}C]testosterone; NEN Life Science Products; sp. act. 45–60 Ci/mmol) was administered intravenously to the chimpanzee (30 μCi), long-tailed macaque (20 μCi), and the common marmoset (10 μCi). The ^{14}C-labeled testosterone (^{14}C-T) was diluted in either 5 ml (chimpanzee, macaque) or 200 μl (common marmoset) of 0.9% sterile NaCl containing (v/v) 10% ethanol. For the chimpanzee and macaque, the solution additionally contained 1 mg of unlabeled testosterone (No. T 1500, Sigma Chemie, Deisenhofen, Germany) as carrier. Syringes were sonicated for 30 s immediately before isotope injection and 10 μl were removed and counted with scintillation fluid (Lumasafe, Groningen, The Netherlands) to determine the total pre-injection radioactivity. After isotope administration, the syringe and the tube containing the radiolabeled hormones were rinsed twice with scintillation fluid, and the residual radioactivity counted and subtracted from the pre-injection total to determine the amount of radioactivity administered.

Following isotope injection, animals were observed continuously throughout the light phase (12 h) for the following 5 days and all urine and fecal samples collected (see above) and stored at −20 °C until analysis.

2.3. Time course and route of testosterone excretion

In all three species, urine samples collected 0–24 h after radiolabel administration were measured individually, whereas samples collected after 24 h post-injection were pooled in 4-h intervals. In the macaque, all fecal samples were analyzed individually, whereas fecal samples of the marmoset were pooled in 4-h intervals prior to analysis. In the chimpanzee, fecal samples were analyzed individually until 70 h post-injection, while later samples were also pooled in 4-h intervals.

To determine the radioactivity in urine, samples (marmoset: 10–20 μl, macaque and chimpanzee: 500 μl) were counted directly in 10 ml scintillation fluid as described by Bahr et al. (2000). To adjust for variations in water content, urine samples were measured for creatinine (Cr) concentrations as described by Bahr et al. (2000) and urinary radioactivity was indexed against creatinine and expressed as dpm/mg Cr. For determination of radioactivity in feces, samples were thawed, homogenized, and duplicate aliquots of 0.2–0.5 g wet weight were analyzed through catalytic oxygen combustion (Peterson et al., 1969) as described by Bahr et al. (2000). Average recovered radioactivity per fecal sample ($n = 2$) was expressed as dpm/g wet weight. The percentage of total recovered radioactivity was determined by summarizing the radioactivity excreted into the urine and feces and relating this to the total amount of radioactivity administered. The portions of ^{14}C-steroid metabolites excreted via urine and feces were determined as percentages of the total radioactivity recovered. For all subsequent analyses, urine and fecal samples containing peak radioactivity were used.

2.4. Separation of unconjugated and conjugated steroids

The relative proportion of conjugated and unconjugated ^{14}C-T metabolites in urine and feces was assessed by ether–water extraction. A total of 250 μl PBS buffer (pH 6.8) was added to 50 μl- (Pt), 100 μl- (Mf), and 25 μl- (Cj) aliquots ($n = 5$) of urine which was then extracted with 7 ml diethyl ether by vortexing for 15 min. The aqueous phase was frozen, the ether was decanted, evaporated to dryness, and finally reconstituted in 150 μl absolute ethanol. Radioactivity was determined by counting 50 μl aliquots (unconjugated fraction). The aqueous phase was subjected to sequential enzymatic hydrolysis by incubation with specific β-glucuronidase (No. G-7396, Sigma Chemie, Deisenhofen, Germany) followed by β-glucuronidase/sulfatase No. G-1512, Sigma Chemie, Deisenhofen, Germany) as described in detail by Bahr et al. (2000). Combined hydrolysis and extraction efficiency was checked by subjecting duplicate aliquots of the same amounts of nonlabeled urine (25, 50, 100 μl for Cj, Mf, and Pt) with the addition of 20 μl of [^{3}H]estradiol, [^{3}H]estrone-glucuronide, or [^{3}H]estrone-sulfate to the above procedure. The extraction recovery of [^{3}H]estradiol was 95.8 ± 11.6% (mean ± SD, $n = 3$) and the combined hydrolysis and extraction efficiency of [^{3}H]estrone-glucuronide and [^{3}H]estrone- sulfate was 76.2 ± 5.2% ($n = 3$) and 83.2 ± 4.6 ($n = 3$), respectively.

In order to determine the proportion of conjugated and unconjugated metabolites in feces, aliquots ($n = 4$) of 0.02–0.03 g (Pt, Mf) and 0.01 g (Cj) of lyophylized and pulverized fecal powder were homogenized in 0.5 ml H_2O and unconjugated metabolites were extracted with 5 ml diethyl ether (Heistermann et al., 1995). Following centrifugation (1800*g*, 1 min), the ether phase of each extraction step was decanted, pooled, and evaporated to dryness in Lexan combustion capsules. The capsules were subjected to catalytic oxygen combustion as described above and radioactivity was counted to determine the fraction of unconjugated steroids.

2.5. High pressure liquid chromatography (HPLC)

Urinary and fecal T metabolites were separated by reverse phase high pressure liquid chromatography (HPLC) using a Nova-Pak C18 column (3.9 × 300 mm, Millipore, Milford, MA, USA) and an isocratic solvent system of acetonitrile:water (ACN:H_2O, 40:60, v:v) at a flow rate of 0.3 ml/min. Prior to HPLC, urine samples (chimpanzee: 1 ml, long-tailed macaque: 3 ml, marmoset: 100 μl) were ether-extracted to remove unconjugated metabolites and the water phase was hydrolyzed with β-glucuronidase/sulfatase (1250 IU) and extracted with

diethyl ether as described by Bahr et al. (2000). Following evaporation, steroids were reconstituted in 300 μl ACN:H_2O (40:60), 100 μl were injected onto the HPLC column and 130 fractions of 0.3 ml were collected.

Fecal samples were lyophylized and pulverized and 0.05–0.5 g (dependant on the species) were extracted three times with 5 ml 80% MeOH in water. Extracts were pooled, mixed with 45 ml sodium acetate buffer (0.2 M, pH 4.8) and passed through a primed SepPak C18 cartridge (Millipore, Milford, Massachusetts, USA) at a flow rate of 2–4 ml/min for purification (Palme et al., 1995). The cartridge was washed with 10 ml of water, dried under a stream of nitrogen and unconjugated metabolites were eluted with 10 ml dichloromethane. Following evaporation to dryness, steroids were reconstituted in 300 μl ACN:H_2O (40:60, v:v). An aliquot of 100 μl was injected onto the HPLC column and 130 fraction of 0.3 ml were collected.

From each of the HPLC fractions collected, aliquots of 100 μl were counted in 3 ml scintillation fluid to determine the profile of radioactivity. In addition, elution positions of different androgen standards were determined separately in the HPLC system.

2.6. Antibody screening and determination of immunoreactivity

For determination of immunoreactivity in HPLC fractions, 200 μl of each fraction was evaporated to dryness, reconstituted in 300 μl assay buffer and measured in three C17 group-specific androgen enzymeimmunoassay (EIA) systems, which were expected to detect the vast majority of testosterone metabolites excreted. A testosterone assay was used to measure the group of 17β-OH-androgens, whereas a 5α-androstanolone and epiandrosterone assay were used to detect 17α-OH- and 17oxo-androgens, respectively. All antisera were raised in rabbits against testosterone-3-CMO, 5α-androstane-17α-ol-3-one-CMO and 5α-androstane-3α-ol-17-one-HS, each coupled with bovine serum albumin. The cross-reactivities of the T and epiandrosterone antibodies are described by Palme and Möstl (1994). Cross-reactivities of the 5α-androstane-17α-ol-3-one antibody (100%) were 1.3% for 5α-androstanedione and <1% for 5β-androstanedione, androsterone, epiandrosterone, androstenedione, dehydroepiandrosterone, testosterone, 5α-dihydrotestosterone, progesterone, 11-oxoetiocholanolone, and estradiol. The labels used in the three EIA systems were 5α-androstane-3β,17β-diol-3-HS, 5α-androstane-17α-ol-3-on-CMO- biotinamin, and 5α-androstane-3,17-dione-thioether-DADOO-biotin, respectively. The standards were testosterone (0.16–20 pg/well), 5α-androstane-17α-ol-3one (0.78–200 pg/well), and epiandrosterone (1.9–500 pg/well).

In brief, duplicate 50 μl aliquots of sample or standard were combined with label (50 μl) and antiserum (50 μl) and incubated overnight at 4 °C. Following incubation, the plates were washed four times with PBS washing solution (containing 0.05% Tween 20), blotted dry, 150 μl streptavidin horseradish peroxidase was added to each well and plates were left in the dark on stirring tables for 30 min. Plates were then washed again before 150 μl of substrate solution (including 0.025% tetramethylbenzidine and 0.05% H_2O_2) was added to each well and plates incubated in the dark for further 0.5–3 h (depending on the assay). The enzyme reaction was then stopped with 50 μl/well 4 mol/L sulfuric acid and absorbance measured at a wavelength of 450 nm (reference filter 630 nm) on an automatic plate reader (MRX, Dynatech Technologies, Denkendorf, Germany).

Assay sensitivity at 90% binding was 0.2 pg for the testosterone assay and 1.2 and 2.5 pg for the 5α-androstanolone and epiandrosterone assay, respectively. Intra-assay coefficients of variation of high and low concentrated quality controls ranged between 3.8% and 11.1% ($n = 16$–18), while inter-assay coefficients of variation ranged between 10.4% and 21.0% ($n = 64$–82).

2.7. Biological validation

To determine the suitability of the different enzymeimmunoassays for discriminating gonadal status through urine and fecal analysis, early morning urine ($n = 5$) and fecal samples ($n = 5$) were collected from five individuals per group from each species. Urine samples were hydrolysed with β-glucuronidase/sulfatase and ether extracted as described above, while fecal samples were lyophilized and pulverized (cf. Heistermann et al., 1995) prior to extraction with 80% methanol in water (see above). After appropriate dilution in assay buffer, urinary, and fecal extracts were finally measured in all three androgen EIA's as described above. Urinary immunoreactive androgen concentrations were indexed by creatinine and expressed as mass/mg Cr, while fecal hormone concentrations were expressed as mass/g dry weight.

2.8. Radiometabolism study of DHEA

In order to test the extent to which metabolites of testosterone and dehydroepiandrosterone (DHEA, a weak, but abundant androgen from the adrenal cortex) might be similar and thus result in a co-measurement in the three androgen assays used, a second radiometabolism study was performed on an individual male long-tailed macaque. For this purpose, the male was injected with radiolabeled DHEA (100 μCi [1,2,6,7-^{3}H (N)]dehydroepiandrosterone, NEN Life Science Products,; spec. act. 60–100 Ci/mmol) and testosterone (25 μCi [4(C)-^{14}C]testosterone; NEN Life Science Products,; spec. act. 45–60 mCi/mmol) as described above and all

urine and fecal samples collected for the following 4 days. Peak samples of radioactivity from urine and feces were then analyzed by HPLC using the same methods and HPLC system as described above in order to determine the presence of radioactivity peaks of both androgens.

2.9. Statistical analysis

For comparison of concentrations of immunoreactive androgens in the three validation groups, the individual hormone values were first averaged for each animal and from these mean values an overall group mean (±SD) was calculated and presented in Fig. 4. Statistical differences between the group means were tested using one-way analysis of variance (ANOVA) with post-hoc comparisons using Tukey test. The level of statistical significance was set at $p < 0.05$.

3. Results

3.1. Time course and route of testosterone excretion

The total radioactivity recovered in urine and feces after 5 days of sample collection was 82.5% (Cj), 83.4% (Mf), and 95.3% (Pt), respectively. The time courses of radiolabeled ^{14}C-T excretion for the three species are shown in Fig. 1A–C. In all animals, maximum levels of urinary radioactivity were already detected within 2 h post-injection (first sample after ^{14}C-T administration). In contrast, the time course of excretion of radioactivity in feces was more variable, with maximum levels being detected after 4 h in the marmoset and 22 and 26 h in the chimpanzee and long-tailed macaque, respectively. There were also considerable differences in the dynamics of T excretion. Whereas radioactivity decreased to baseline levels in urine within 24 h (>90%

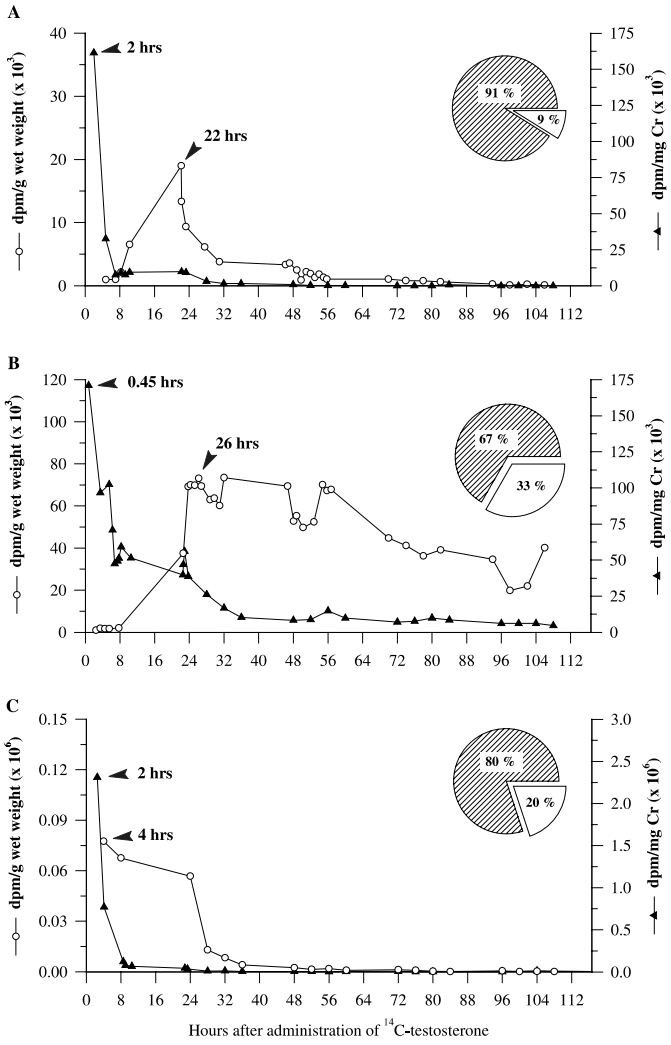

Fig. 1. Time course and route of excretion (pie charts) of [^{14}C]testosterone in urine (▲) and feces (○) after i.v. administration at hour 0 in an adult male chimpanzee (A), long-tailed macaque (B), and common marmoset (C). Pie charts show the proportion of T metabolites recovered in urine (▨) and feces (□). The values are presented as percentages and are based on the total radioactivity excreted during the first five days post-injection.

of total urinary radioactivity excreted) and in feces within 48 h (81–96% of total fecal radioactivity excreted) post-injection in the chimpanzee and the common marmoset, elimination of radioactivity in the long-tailed macaque was much slower with only 54% of urinary and 40% of fecal radioactivity excreted after 24 and 48 h, respectively.

As shown in the inserts of Figs. 1A–C (pie charts) in all species investigated, the majority (67–91%) of radioactivity recovered was excreted into the urine. Diethylether extraction revealed that in each species almost all of the excreted metabolites were conjugated (90–99%) and enzyme-hydrolysable (80–90%), with 76% (Cj) to 98% (Pt) being accounted for by glucuronides and 1.5% (Pt), 7.5% (Mf), and 24.3% (Cj) by sulfates. The remaining 9–33% of the radioactivity was excreted via the feces. Ether:water extraction revealed that fecal metabolites were mainly unconjugated in Pt and Mf (74% and 76%, respectively), but not in Cj (42%).

3.2. HPLC analysis

HPLC co-chromatography of extracts of urine and feces revealed multiple peaks of radioactivity in all three species (see Figs. 2A–C). Comparison of radioactivity profiles, however, indicated differences both between urine and feces within a species and between the three species investigated.

The urinary HPLC profile of the chimpanzee (Fig. 2A) revealed 5 distinct radioactive peaks with the majority of radioactivity being found in fractions 80–86 and co-eluting with 5β-androstane-3α-ol-17-on and 5α-androstan-3,17-dione standards. In contrast, the chromatographic separation of the urine samples of both the long-tailed macaque (Fig. 2B) and common marmoset (Fig. 2C) revealed (i) a higher number of different T metabolites and (ii) excretion of larger quantities of more polar T metabolites. In the macaque, the majority (65%) of radioactivity was associated with T metabolites eluting in fraction 10–28, whereas in the marmoset the

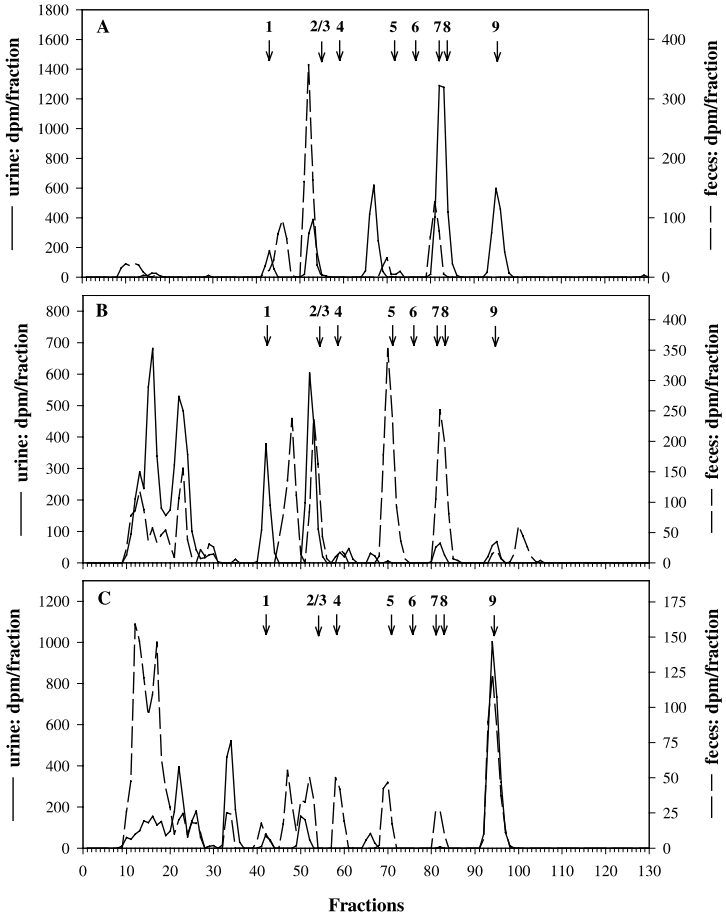

Fig. 2. HPLC profiles of radioactivity excreted into urine (—) and feces (- - -) following i.v. injection of [^{14}C]testosterone in an adult male chimpanzee (A), long-tailed macaque (B), and common marmoset (C). Arrows indicate the retention times of androgen reference standards: 1, 4-androsten-17β-ol-3-one (testosterone, T); 2, 4-androsten-3,17-dione (androstendione); 3, 5-androsten-3β-ol-17-one (dehydroepiandrosterone); 4, 5-androsten-3α-ol-17-one (dehydroandrosteron); 5, 5-androstan-3β-ol-17-one (epiandrosterone); 6, 5α-androstan-17β-ol-3-one (5α-dihydrotestosterone); 7, 5β-androstane-3α-ol-17-on; 8, 5α-androstan-3,17-dione; 9, 5α-androstan-3α-ol-17-one (androsterone).

figure was 33%. None of the androgen reference tracers eluted at these positions. The major peak of radioactivity in the common marmoset was more apolar, eluting in fractions 92–98, similar to the elution position of the androsterone standard. A major radioactive peak in the chimpanzee was also found at this position. In all three species, a small radioactive peak co-eluted with the testosterone reference tracer, indicating that native T was present in extracts of urine but not in substantial amounts (Pt: 4%, Mf: 9.5%, and Cj: 1.6% of total radioactivity).

Feces: Three substantial peaks of radioactivity were obtained after HPLC of the fecal extract in the chimpanzee (Fig. 2A), with the major peak eluting in fraction 51–54. More radioactive peaks were found in the other two species (Figs. 2B and C). As in urine, both the macaque and the marmoset excreted a higher proportion of more polar T metabolites into the feces compared to the chimpanzee. In the macaque, the major peak of radioactivity was found in fraction 68–74 (co-eluting with epiandrosterone standard) with three additional peaks in fractions 45–50, 52–56 (co-eluting with androstendione and dehydroepiandrosterone standard), and 81–86 (co-eluting with 5β-androstane-3α-ol-17-on and 5α-androstan-3,17-dione standard). In contrast, the majority of radioactivity in the marmoset was found in fractions 10–27 (52.5% of total radioactivity eluted), with a second prominent peak (fractions 92–98), co-eluting with the androsterone standard. No radioactivity was detected at the elution position of authentic T in any species.

3.3. Antibody screening

Profiles of immunoreactivity were determined after HPLC using three different C-17 group-specific assay systems. The testosterone assay was used to measure 17β-OH-androgens, whereas the 5α-androstanolone assay and the epiandrosterone assay were used to determine 17α-OH- and 17oxo-androgens, respectively. Information on the specificity of the measurements and the relative levels of immunoreactivity as determined by each of these assay systems is given in Table 1. All antibodies recognized at least one or more of the radioactive peaks in both urine and feces (data not shown). As indicated in Table 1, the highest levels of immunoreactivity were consistently obtained with the epiandrosterone assay, whereas, with a single exception (chimpanzee feces), the lowest titers were measured by the 5α-androstanolone assay.

Using the testosterone assay, a single immunoreactive peak co-eluting with the T standard was detected in the urine in all three individuals, indicating that T is present and that its measurement using this antibody is highly specific. In contrast, the antibody detected several peaks of radioactivity in feces, none of which co-eluted with the T standard. Multiple peaks of immunoreactivity were usually obtained when using the 5α-androstanolone assay, although there were two exceptions. Only one prominent immunoreactive peak was found in the urine of the long-tailed macaque (corresponding to one of the main peaks of radioactivity in fraction 51–55) and a single immunoreactive peak (fraction 53–58) was also measured in chimpanzee feces. Using the epiandrosterone assay, multiple immunoreactive peaks were obtained in the majority of cases, although single peaks indicated a high degree of specificity for marmoset urine and macaque feces. Major peaks of immunoreactivity (fractions 93–98 in urine, fractions 67–73 in feces) corresponding to radioactive peaks at the elution positions of androsterone and epiandrosterone, respectively, were detected in all three individuals.

3.4. Biological validation

Fig. 3 shows the data on urinary and fecal immunoreactive androgen concentrations in the different

Table 1
Qualitative information on specificity and relative abundance of immunoreactivity measured with the three different enzyme immunoassays using three different C17 group-specific antibodies

Assay	Species	Urine		Feces	
Testosterone [17β-OH-androgens]	Pt	S	++	NS	+
	Mf	S	++	NS	++
	Cj	S	++	NS	++
5α-androstanolone [17-α-OH-androgens]	Pt	NS	+	S	++
	Mf	S	+	NS	+
	Cj	NS	+	NS	+
Epiandrosterone [17oxo-androgens]	Pt	NS	+++	NS	+++
	Mf	NS	+++	S	+++
	Cj	S	+++	NS	+++

Abbreviations used: Pt = chimpanzee, Mf = long-tailed macaque, Cj = common marmoset; S = specific measurement (one peak of immunoreactivity), NS = nonspecific measurement (>one peak of immunoreactivity); + = low titer (⩽600 pg/fraction), ++ = medium titer (600–4000 pg/fraction), +++ = high titer (4000–30,000 pg/fraction) of immunoreactivity.

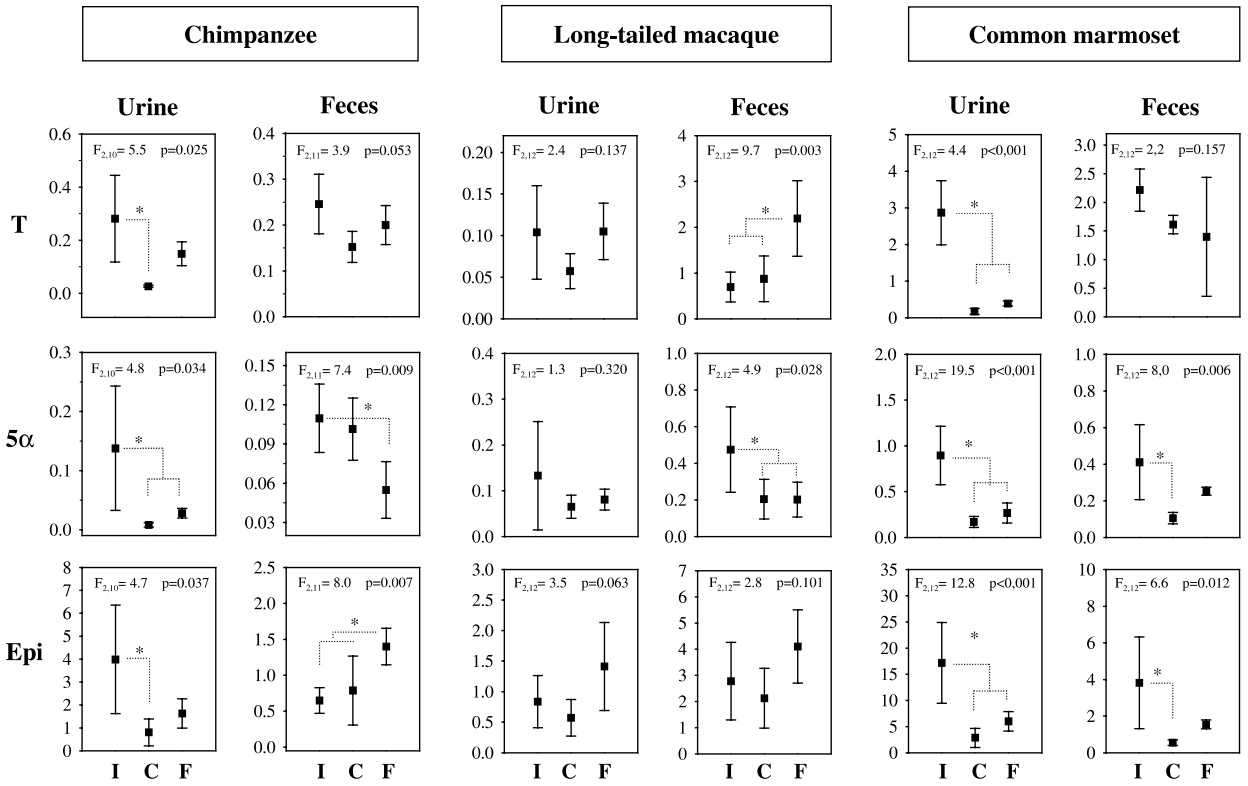

Fig. 3. Mean (±SD) concentrations of immunoreactive urinary and fecal testosterone (T), 5α-androstanolone (5α), and epiandrosterone (Epi) in the chimpanzee, long-tailed macaque and common marmoset. I, C, and F indicate groups of intact, adult males (I), castrated males (C), and females (F) ($n = 5$ individuals per group). Statistics: ANOVA, * post-hoc analysis Tukey test, $p < 0.05$. Values are given in μg/mg Cr for urine and μg/g dry weight for feces.

categories of animals used for assessing the biological validity of the three group-specific assays used. ANOVA indicated that for the marmoset and chimpanzee there were significant differences in urinary androgen concentrations between groups for all assays. Post-hoc analysis showed, that in both species, intact males had significantly higher levels than castrates. Levels of all three immunoreactive androgens in intact male marmosets were also significantly elevated compared with those in females, whereas although mean levels in male chimpanzees were also clearly higher than those in females (particularly in the 5α-androstanolone assay), differences were not significant. Urinary androgen levels in the long-tailed macaque were not significantly different between groups, regardless of the assay used (ANOVA, $p > 0.05$).

Fecal androgen levels in the marmoset were higher in intact males than in castrates and females for all three assays, but the differences were statistically significant only for the 5α-androstanolone and epiandrosterone assay. Although ANOVA indicated significant between-group differences in all fecal androgen measurements in the chimpanzee, post-hoc analysis showed that none of the assays discriminated significantly between intact and castrated males. In the long-tailed macaque, significant differences in hormone levels between groups were obtained using the T and 5α-androstanolone assays, but only in the latter were levels in intact males significantly higher than those in castrated males and females.

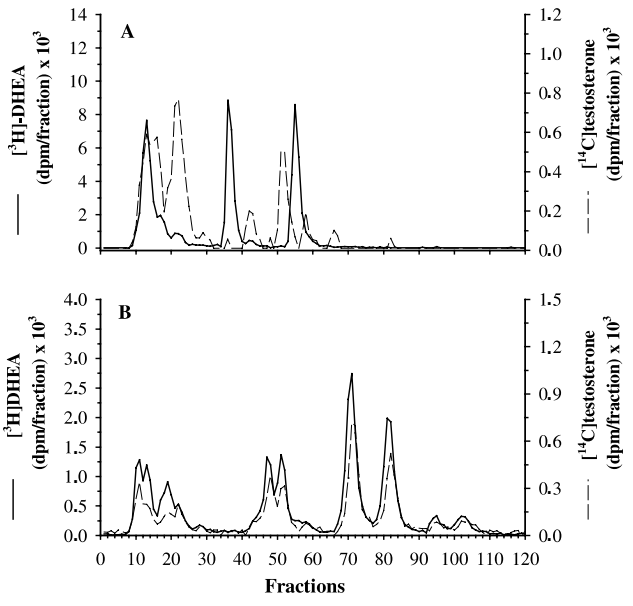

Fig. 4. HPLC profiles of excreted radioactivity into urine (A) and feces (B) following administration of [^{3}H]DHEA (—) and [^{14}C]testosterone (- -) in a male long-tailed macaque.

The finding that in the two Old World primates the measurement of fecal and, to some extent, urinary androgens did not consistently discriminate between validation groups was unexpected. HPLC of peak radioactive samples collected after the administration of ^{14}C-T and ^{3}H-DHEA to a second long-tailed macaque indicated close similarities in the metabolic products of the two isotopes, particularly in feces where elution profiles were almost identical (Fig. 4). This suggests that a substantial proportion of our measurements might not be confined to androgens of testicular origin but that in both urine and feces a co-measurement of metabolites derived from DHEA is very likely.

4. Discussion

The present study provides the first detailed comparative information on androgen metabolism and excretion in nonhuman primates. Specifically, we describe the metabolic fate of ^{14}C-T administered to representatives of the three major Anthropoid primate taxa, the common marmoset, long-tailed macaque, and chimpanzee, and evaluate a number of different enzyme immunoassays for use in assessing testicular endocrine function based on the measurement of urinary and fecal androgens.

In all three individuals studied, T metabolites were excreted predominantly into the urine, a finding consistent with reports on the route of excretion of testosterone in other mammals (Monfort et al., 1995; Palme et al., 1996) including the baboon (Kulkarni et al., 1979) and human (Sandberg and Slaunwhite, 1956), but different from results obtained for carnivores (Brown et al., 1996; Velloso et al., 1998). Urinary androgen excretion occurred relatively rapidly and in terms of time lag was consistent between the three individuals. On the other hand, excretion of T metabolites into the feces was much slower and more variable. The interval to peak excretion of T metabolites of 24–36 h obtained for the macaque and chimpanzee is similar to that reported for fecal steroid excretion in many other species (see Schwarzenberger et al., 1996, for review). The more rapid excretion of T metabolites into the feces of the marmoset is consistent with the short gut passage time of 4–5 h described for this (Krombach et al., 1984) and several other callitrichid species (Price, 1993). Although time to peak excretion was comparable between the two Old World primate species, elimination of radioactivity thereafter occurred much more slowly in the long-tailed macaque (both animals). Both macaques also showed secondary peaks in feces at about 30 and 50 h, suggesting pronounced enterohepatic re-circulation of steroids in this species as previously described for domestic animals (Lindner, 1972; Symonds et al., 1994). HPLC separation of sample extracts indicated the presence of multiple peaks of radioactivity in all three individuals, although each had a distinctive pattern. In urine, androsterone (standard 9, see Fig. 2) appears to be the predominant T metabolite in the marmoset, whereas in the chimpanzee, the major peak of radioactivity coeluted with 5β-androstane-3α-ol-17-one/5α-androstan-3,17-dione (standards 7 and 8, see Fig. 2); neither of these was abundant in the urine of the long-tailed macaque. Androsterone, previously described as an abundant metabolite of T in the chimpanzee (Layne et al., 1963), rhesus macaque, and baboon (Yamamoto et al., 1978a,b), was identified in all three individuals. A further common finding was that T itself was excreted in only small amounts, confirming findings for other primate (e.g. Epple et al., 1991; Yamamoto et al., 1978a) and nonprimate species (e.g. Brown et al., 1996) that T is quantitatively of minor importance as a urinary androgen. Despite difficulties in characterization of many of the radioactivity peaks in feces, androsterone appears to be the major T metabolite in marmoset feces, whereas epiandrosterone (standard 5, see Fig. 2) and 5β-androstane-3α-ol-17-one/5α-androstan-3,17-dione probably represent major fecal androgens in the macaque. The absence of radioactivity at the elution position of authentic T in any of the animals studied is conspicuous and significant. The virtual absence of T from feces has also been demonstrated in a variety of nonprimate species (Brown et al., 1996; Velloso et al., 1998), suggesting that this may be a common finding among mammalian taxa.

In the present study, three C-17 group-specific enzymeimmunoassay systems using antisera raised against testosterone (17β-OH androgen), 5α-androstane-17α-ol-3-one (5α-reduced 17α-OH androgen), and androsterone (5α-reduced 17-oxo-androgen) were assessed for their ability to discriminate between intact and castrated males and females. All assays detected substantial amounts of immunoreactivity, although relative levels and specificity of measurement differed between assays and according to substrate being measured. Since, irrespective of assay specificity, 17-oxo androgen immunoreactivity predominated in individuals of all three species, 17-oxo metabolites are likely to be generally more abundant than 17-hydroxylated compounds. This conclusion is supported by our HPLC data showing the excretion of mainly 17oxo-metabolites (see above) and is also in line with findings from earlier studies on urinary androgens in other primate species (Yamamoto et al., 1978a; Layne et al., 1963).

The attempted biological validation of the three assay systems for assessing testicular endocrine function revealed interesting species differences. In the marmoset, 5α-androstanolone, and epiandrosterone appeared equally reliable in their ability to discriminate androgen status when applied to feces, whereas in urine, clearest differences between validation groups were obtained

with the testosterone assay. The situation in the two Old World species was less clear. In the chimpanzee, intact and castrate males could be distinguished by measurements in urine, but not in feces. Since the 5α-androstanolone assay produced the largest differences between groups, the lowest concentrations in castrates and was the only assay that also significantly discriminated between males and females, it appears to be most suitable for measuring testicular androgen status in chimpanzees. In contrast, there were no differences between groups in terms of fecal measurements, suggesting that all assays were also detecting androgens and/or other substances of nontesticular origin. Similarly in the long-tailed macaque, gonadal status could not be discriminated by most of the measurements attempted and only fecal 5α-androstanolone levels showed statistical differences between groups. However the degree of overlap of values obtained suggests that even this assay is of little practical use as a means of noninvasively assessing male gonadal status in this species.

Although the reason for these difficulties in the two OW species is not fully clear, the similar nature of the metabolic products of testosterone and DHEA as shown in Fig. 4, suggests that co-measurement of DHEA metabolites by the assays employed provides at least a partial explanation. DHEA is known to be present in the circulation of both men and women and some nonhuman primates in concentrations exceeding those of T by 100–500 fold (Labrie et al., 1998) and, due to its adrenal origin, is also present in high amounts in castrated animals. Thus, the high levels of androgen immunoreactivity measured in females and castrated males of both species is not surprising (Callies et al., 2000). In contrast, Callitrichid species in general produce much lower amounts of DHEA, probably due to the absence of the adrenal reticular zone in adults (Levine et al., 1982). Co-measurement of substantial amounts of DHEA would therefore not be expected and this is verified by the higher degree of success in discriminating gonadal status in the marmoset.

Collectively, the data indicate that T metabolism in primates is complex and likely to be highly variable between species. Testosterone itself is of minor importance in urine and virtually completely absent from feces. Despite the limited data-set presented, we suggest that these findings will apply to most Anthropoid primates. In those cases where clear differences between intact and castrated males were obtained, we can be reasonably confident that the androgens being measured are predominantly of testicular origin. Clearly, part of the problem in discriminating gonadal status in males by the methods described is that there exists marked individual variation in androgen levels in intact animals. The source of this variation is unlikely to be the assays themselves, but is most likely to reflect the inherent biological variation in androgen levels found in males. Many factors are known to potentially affect testosterone output (pulsatile secretion, rank, age, season, health status, environmental/social stress) and it is the need for suitable methods to investigate how these operate under natural settings that prompted this study in the first place. Nevertheless, given that other factors can also potentially affect steroid concentrations in excreta (e.g. diet, health, diurnal variations), it is clearly important that this be taken into account when designing studies to quantitate androgen excretion in male primates using noninvasive methodologies. Further, this study clearly illustrates the potential problems associated with co-measurement of androgens of nontesticular (adrenal) origin, particularly in OW primates and this should also be taken into consideration when selecting assays and interpreting results of fecal androgen analysis.

Animal experiments: The sedation and administration of ^{3}H-labeled testosterone to the common marmoset and ^{3}H-labeled testosterone and DHEA to the long-tailed macaques, as described in the following study, was approved by the Bezirksregierung Braunschweig (Aktenzeichen 604.42502/08-05.97), Germany. The sedation and administration of ^{3}H-labeled testosterone to the chimpanzee was approved by the relevant animal welfare committee in The Netherlands.

Acknowledgments

We are indebted to S. Rensing, N. Stolte, and H. van der Eerd for performing the veterinary procedures and the keepers at the German Primate Center for care of the animals. We are also grateful to Ton Kos, Biomedical Primate Research Center, Rijswik, The Netherlands, for access to the male chimpanzee, and to M. Horstmann, Zentrales Isotopen Laboratorium, Universität Göttingen, for helping with radioactive counting and performing catalytic oxygen combustion on fecal samples. A. Heistermann and J. Hagedorn provided valuable support in all laboratory techniques. This research was supported by a grant from the German Research Council (Grant No. GRK 289/1-97).

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