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Development and Validation of a Fecal Testosterone Biomarker in *Mus musculus* and *Peromyscus maniculatus*[1]

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ABSTRACT

This is a report on the development and validation of an ELISA method to determine fecal testosterone levels, and on their evaluation as a biomarker for adverse effects of endocrine-disrupting chemicals on reproductive health using male rodents of the *Peromyscus maniculatus* and *Mus musculus* species as an animal model. The ELISA antibody had the highest specificity for testosterone (100%), followed by dihydrotestosterone (57.4%) and androstenediol (0.27%). Radiolabeled testosterone was injected i.p. into three mice. Fecal samples were collected, extracted, and analyzed by liquid scintillation counting. The ELISA was performed to characterize the excretion kinetics and metabolic fate of circulating testosterone. Solubilization of feces with 10% methanol overnight provided an extraction efficiency of 87% for all metabolites; an ethyl ether extraction was more selective for testosterone. The fecal excretion of the testosterone was a biphasic process with a majority of the radioactivity recovered in the first 24 hours. HPLC analysis revealed at least five testosterone metabolites in feces, with most metabolites being less polar than testosterone. This study forms the initial evaluation of what will become a field monitoring tool.

INTRODUCTION

When organisms are stressed by changes in the environment, their energies are utilized for immediate survival, and reproductive potential is reduced [1]. Thus, a reduction of fecundity, or reproductive potential, can be used as a measure of the adverse effects or cumulative negative impact of adverse environmental changes altering the fitness of an organism [2–4]. This paper describes a method of measuring changes in reproductive potential by looking at changes in steroid levels.

Conventional methods of assessing the reproductive health of males require invasive procedures and only infer endocrine function. These methods include determination of number of sperm in the testis, epididymis, or ejaculate; measures of sperm function; and histopathology [5, 6]. Serum steroid measurements require invasive procedures such as collection of blood samples and expensive assays. While it is well accepted that measurements of reproductive hormones, such as testosterone, are an adequate measure of reproductive potential, they have to be adapted to field studies. The objective of the present study was to develop a simple method for extraction and measurement of testosterone from feces of male rodents and to employ this parameter as a noninvasive biomarker of reproductive stress in *Peromyscus maniculatus*. This study was an examination of methods for extraction of fecal testosterone and its subsequent measurement using a competitive ELISA.

MATERIALS AND METHODS

Chemicals

Sodium bicarbonate, sodium carbonate, mono- and dibasic sodium phosphate, NaCl, BSA, Tween 20, 2,2′-azino-*bis*(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), hydrogen peroxide (H_2O_2), anhydrous citric acid, pentane, ethyl acetate, β-glucuronidase, horseradish peroxidase (HRP)-testosterone, testosterone, and all other steroids were purchased from Sigma Chemical Co. (St. Louis, MO). [^{14}C]Testosterone (specific activity 59.8 mCi/mmol) was purchased 97.9% pure in ethanol (NEN, Boston, MA). Rabbit polyclonal testosterone antibody was produced at UC Davis and directed to testosterone-6-CMO:BSA (VWR Scientific, San Francisco, CA). Anhydrous ethyl ether and methanol were purchased from Fisher Scientific (Fisher, Pittsburgh, PA).

Animals

Experiments were run on feces from both white laboratory (*Mus musculus*) and field (*Peromyscus maniculatus*) mice, subsequently referred to as Mus and Peromyscus, respectively. Peromyscus were housed individually, and Mus were housed in pairs, in 7.5 × 11.5 × 5-inch polypropylene cages with a 14L:10D cycle. Animals had access to Purina Mouse Chow (Ralston-Purina, St. Louis, MO) and water ad libitum. All procedures and animal care were approved by the UC Davis Animal Care Committee.

Solutions

Stock solutions used in the ELISA included coating buffer (0.05 M sodium bicarbonate, pH 9.6), assay buffer (0.1 sodium phosphate, pH 7.0, containing 0.87% NaCl and 0.1% BSA), wash solution (0.15 M NaCl containing 0.05% Tween 20), and substrate (40 mM 2,2′-azino-*bis*[3-ethylbenzthiazoline-6-sulfonic acid] and 0.5 M H_2O_2 in 0.05 M citrate, pH 4.0).

Sample Preparation

Feces from several Peromyscus were collected and combined to obtain a single pooled sample for extraction comparisons. Samples were dried overnight under vacuum at 37°C and then ground using a household coffee grinder. Portions (100 mg each) were placed in 13 × 100-mm borosilicate tubes and used to determine extraction efficiency.

When measurements of individual mouse samples were

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required, the samples were collected, divided into 100-mg portions (3-6 fecal pellets), transferred to 16 × 100-mm borosilicate tubes, and then ground using a 10-ml Teflon (Dupont, Wilmington, DE) tissue grinder (Wheaton, Millville, NJ) and cordless drill at 600 rpm.

Assay

The testosterone was measured colorimetrically using a competitive heterogeneous ELISA with a column-purified polyclonal antibody as described by Munro and Lasley [7]. A 96-well microtiter plate (Immulon I; Dynatec, Chantilly, VA) was coated with antibody, incubated at 4°C for a minimum of 16 h, and washed; 50-μl buffer was then added. Next, 20 μl of sample was added, followed immediately by the HRP-testosterone conjugate. The competition reaction proceeded at room temperature for 2 h, or 4°C overnight, before the plate was washed to remove the unbound steroid and conjugate. To determine the amount of conjugate-bound antibody in the plate, a substrate solution of H_2O_2 and ABTS was added, and the absorbance was measured at 405 nm with a Bio-Tek EL 340 microplate reader (Bio-Tek Instruments Inc., Winooski, VT). A standard curve was run from 3 to 384 pg to determine the concentration in unknowns. Unknowns were run within the narrow range representing the most linear portion of the standard curve (typically between 40% and 70% displacement of HRP-testosterone) with $r^2 > 0.99$.

Assay Validation

Specificity of the assay was determined using three different methods. The cross-reactivities of steroids structurally related to testosterone (along with the bile acids found in feces that contain the same cholesterol backbone) were assessed for the antibody [8]. The percentage cross-reactivity was defined as 100X/Y where X is picograms of testosterone and Y is picograms of steroid required to produce 50% inhibition of HRP-testosterone hapten binding to antibody [9]. Parallel studies were done by taking feces and extracting them two times with 3.8 ml of ethyl ether as described below. The fecal extracts were serially diluted and compared to standards for parallelism over the range of 12–96 pg of testosterone (n = 4) [10, 11]. In a final determination, pooled fecal samples were extracted with ether, and the extract was separated on HPLC by methods described later. Fractions were analyzed using the ELISA.

Sensitivity was calculated from 95% confidence limits at the zero point of the standard curve [12]. The interassay coefficient of variation was determined using standards of testosterone at low, medium, and high concentrations (20 plates each with sample run in duplicate). The intraassay coefficient of variation was determined using pooled samples of fecal extract at low, medium, and high concentrations of testosterone (24 aliquots of each sample run on two separate plates) [13]. Student's *t*-tests were used to test the significance of the difference between slopes under the assumption that the standard errors of each of the populations were equal. Confidence limits were obtained using the computer spreadsheet Excel (Microsoft, Redmond, WA).

Extraction Efficiency

Two solvent extractions were compared to determine the optimum method for extracting testosterone from mouse feces. The first was 80% pentane/20% ethyl acetate, previously used by our laboratory for serum testosterone extraction. The second was ethyl ether, a solvent commonly used for steroid extraction [11]. Aliquots of 100 μl [^{3}H]testosterone (VWR Scientific) in ethanol were added to pooled fecal samples (n = 5) and empty borosilicate tubes (n = 3) as controls, then dried overnight. Each tube had 3.8 ml solvent added before vortexing for 60 min on a Thermolym Maxi-Mix III (Dubuque, IA) at medium speed (1100 rpm). After vortexing, 250 μl water was added and the tubes were placed in a dry ice and methanol bath. The organic layer was decanted from the frozen aqueous phase and placed in a second borosilicate tube. The extraction was then repeated for an additional 30 min. The solvent extract was evaporated in a bath at 37°C. After the second extraction, a 4-mm glass bead (Fisher) and 2 ml buffer were added to each extract tube and vortexed for 60 min at medium speed. An aliquot from each tube was transferred to a scintillation vial (Packard, Downers Grove, IL), and after addition of 4 ml triton-toluene scintillation fluid, radioactivity was counted for 10 min using a Packard Liquid Scintillation Counter (LSC) (Tri Carb 1600 CA). The counts were compared to dpm added to determine percentage recovery.

The solvent with the highest recovery, as determined in the experiments outlined above, was used for comparison with a 10% methanol solubilization method [14]. Three groups (n = 6) of pooled samples were weighed (25- to 30-mg portions) in 13 × 100-mm borosilicate tubes. For extraction of the first set, 1 ml of 10% methanol was added to feces to create a slurry. The samples were capped and shaken at room temperature (22°C to 25°C) for 16–24 h, then centrifuged for 30 min at 2000 × *g*. The supernatant was decanted and saved. For extraction of the second set, 50 μl 10% methanol was added to feces as a wetting agent. The samples were capped and set at room temperature for 16-24 h, then extracted two times with 4 ml ether and reconstituted in buffer (as described for the previous experiment). Samples in the last set were extracted two times with 4 ml ether and reconstituted in buffer. A 20-μl aliquot of each reconstituted extract was measured with the ELISA. An additional 60-min extraction was performed on all of the samples with 4 ml ether, and these extracts were reconstituted in buffer. A 20-μl aliquot of each of these reconstituted extracts was measured with the ELISA.

To determine the amount of enzyme-hydrolyzable testosterone conjugates, a subsample was buffered with 0.2 M acetate and then subjected to enzyme hydrolysis using β-glucuronidase (Sigma). A combination of 250 units glucuronidase containing 13 units sulfatase was added to 20 mg fecal samples, incubated at 37°C overnight, extracted with ethyl ether as above, and then measured with the ELISA.

Radiolabeled Testosterone Metabolism

Radiolabeled trace was used to determine how mice metabolized and excreted testosterone. Mus (n = 3) and Peromyscus (n = 3), ages 3–24 mo, were injected i.p. with [^{14}C]testosterone. Animals were caged in stainless steel metabolic chambers for separation of urine and feces; mice had access to food and water ad libitum.

To prepare for injection, [^{14}C]testosterone was transferred to a 5-ml serum vial (Fisher), evaporated under nitrogen, and reconstituted with 3 ml sterile 0.9% saline containing 3 mg cold testosterone. A 50-μl aliquot was removed with a 1/2-cc insulin syringe (Becton-Dickinson, Franklin Lakes, NJ), transferred to a scintillation vial, and counted on the LSC. The injectate was measured at 667

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000 dpm/50 μl and stored at room temperature to prevent precipitation. Urine and fecal samples were collected prior to injection of radiolabel to obtain baseline levels [15]. Peromyscus were injected i.p. with 50 μl of prepared injectate and placed in metabolic chambers for sample collection. In Mus the injection was increased to 70 μl. In both cases, identical injectates were placed into three scintillation vials, radioactivity was determined, and the average was taken to determine the dpm [^{14}C]testosterone injected into each mouse.

All urine and feces were collected in 13 × 100-mm borosilicate tubes separately at 4, 8, 12, 16, 24, 36, 60, 84, 108, 156, and 228 h for Peromyscus and at 4, 8, 12, 25, 35, 57, 98 h for Mus from the time of injection. Fecal samples were weighed, and urine volumes were estimated by comparison with known volumes. Urine (50 μl) from each time point was suspended in 4 ml triton-toluene scintillation fluid and counted for 10 min. Radioactivity was extracted from feces (25- to 30-mg samples), mixed with 1 ml 10% methanol to form a slurry, mixed for an additional 19 h on a shaker at medium speed, and centrifuged at 2000 × *g* for 20 min. A 500-μl aliquot of supernatant was removed from each tube, transferred to a scintillation vial, and counted on the LSC. Compartmental pharmacokinetic analysis was carried out on experimental data from [^{14}C]testosterone-treated mice. Data of the rates of excretion vs. time were fitted with WinNonlin (Scientific Consulting Inc., Apex, NC), a pharmacokinetics computer program, using the formulae for the pharmacokinetic parameters as described by Gibaldi and Perrier [16]. All rates were calculated from the radioactivity measured and did not take into account the metabolic state of the testosterone.

Separation of Free Steroid

Samples initially extracted with 10% methanol were reextracted using ethyl ether to obtain primarily unconjugated testosterone and nonpolar metabolites of testosterone. Extracts were concentrated into 2-ml vials, dried under nitrogen, and reconstituted in ethanol. HPLC separations were obtained using a Waters 5 × 100-mm Nova-Pac C18 column contained in an RCM Radial Compression Cartridge Holder (Waters, Milford, MA). A linear gradient of 20–75% acetonitrile (Fisher) in water at a 1 ml/min flow rate over 30 min, followed by 75% acetonitrile for 5 min, was used to separate testosterone metabolites [14]. After separation on the C18 column, samples were passed through a UV detector to determine retention time of cold steroid samples or spiked ^{14}C samples. HPLC elution fractions containing ^{3}H-labeled and nonlabeled steroid standards were run to determine separation efficiency and retention times of the metabolite peaks.

Samples were collected using a fraction collector. Half of each sample was measured on the LSC and the other half with the testosterone ELISA. Some samples were also measured using a Packard Flo-One Beta Series A-500 radio-chromography in-line detector. The percentage unconjugated testosterone excreted in feces and the number of major metabolites were determined.

Animal Validation

To validate the use of the biomarker for measuring changes in male reproductive condition, a fecal sample was obtained from three male Peromyscus to determine a basal testosterone level. The animals were then weighed and injected s.c. with 2.5 IU/g hCG in 0.9% sterile saline [17]. Fecal samples were collected at 20, 24, and 28 h after injection to obtain the peak testosterone excreted. Four days after hCG dosing, the animals were castrated. Fecal samples were collected from all mice at 6 and 8 days after castration. Animals were weighed and injected again with hCG before a final fecal sample collection the following day.

A preliminary field validation was performed to show the use of the testosterone biomarker on control populations of free-ranging male *Microtus californicus* through collaboration with Michael Johnson (Department of Civil and Environmental Engineering at UC Davis). Two live-trap grids of 10 × 10 stations had been previously established in the pickleweed marsh at Mare Island Naval Shipyard (Vallejo, CA). A Sherman trap was used twice monthly at each station, and animal number, location of capture, sex, reproductive condition, and body mass were recorded when an animal was trapped. The reproductive condition for males was recorded as abdominal (reproductively inactive) or scrotal testes (reproductively active). Fecal samples were collected during handling, and brought to UC Davis for analysis.

RESULTS

Assay Validation

The cross-reactivity of several steroids with the polyclonal antibody shows very high specificity for testosterone. The 5-reduction product of testosterone, 5-alpha-dihydrotestosterone (5α-DHT), resulted in 57% cross-reactivity; the next highest cross-reactivity, 0.30%, was shown by androstenediol. Androsterone, dehydroepiandrosterone, estradiol, progesterone, and pregnenolone all had cross-reactivities of less than 0.04%. The bile acids had reactivities of less than 0.02%. The slopes generated from the serially diluted samples to determine parallelism were not different from the slope of the standard curve ($p > 0.1$). In the final cross-reactivity experiment in which a nonradioactive fecal sample was separated on HPLC and fractions were analyzed using the ELISA, the only immunoreactive peak occurred in fractions coinciding with the UV peak for testosterone.

The detection limit of the ELISA was less than 3 pg/well. The average interassay coefficients of variation for the three concentrations of testosterone were 10.5%, 12.4%, and 11.0% at, respectively, 66%, 51%, and 21% displacement of labeled testosterone. The intraassay coefficients of variation for the fecal extract were 8.04%, 8.37%, and 4.52% at, respectively, 40%, 50%, and 75% displacement.

Extraction Efficiency

The extraction of tritiated testosterone metabolites from pooled fecal samples using pentane:ethyl acetate (80:20) and ethyl ether resulted in 82% and 86% recovery, respectively. Decreasing the sample size to less than 30 mg and increasing the solvent volume to 4 ml increased the efficiency to 90%.

Ether extraction of pooled fecal samples wetted with 10% methanol gave the highest recovery of free (ether soluble) testosterone, 94.9% (Table 1). Ether extraction without wetting recovered 74.1%. Solubilization with 10% methanol recovered only 35.7%. All fecal samples were wetted for the second extraction with the 250 μl H_2O that had been added during the first extraction.

The addition of β-glucuronidase to the fecal sample with subsequent ether extraction did not increase the amount of

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TABLE 1. Amount and percentage of uncogugated testosterone extracted.

Parameter	10% Methanol solubilization	Ether extraction	Ether extraction with wetting
Primary extraction*	64 ± 4	114 ± 8	150 ± 6
Additional ether extraction*	115 ± 11	40 ± 5	8 ± 1
Total testosterone*	179	154	158
% Extraction of ether-soluble testosterone of primary extraction**	36	74	95

* Values for the amount of unconjugated testosterone extracted represent nanograms testosterone per gram of feces.
** Percentage recovery is based on the maximum recovery of a second ether extraction.

testosterone measured with the ELISA in comparison to that in nonhydrolyzed controls. The extraction efficiency of the polar and nonpolar radiolabeled testosterone metabolites excreted into feces was measured using the 10% methanol solubilization method and recovered 87 ± 2% (n = 3, mean ± SD).

Radiolabeled Testosterone Metabolism

Averages of 5.61 ± 0.62 g and 10.43 ± 1.74 g feces total weight and of 10.74 ± 5.38 ml and 9.78 ± 0.73 ml total volume were collected per animal for Mus and Peromyscus, respectively (n = 3, mean ± SD). The proportion of the injected testosterone excreted in feces was 62 ± 14% for Mus and 58 ± 13% for Peromyscus.

In Peromyscus, an average of 72.8 ± 12.8% of the total radiolabeled steroid was recovered from each animal. The testosterone excreted into urine and feces was very consistent between the three animals injected. Of the 73% recovered, 91.8 ± 1.5% was excreted in the first 24 h (Fig. 1). The rate vs. time profile showed two elimination phases. The first phase (α phase) occurred between 10 and 30 h after dosing and had a first-order apparent $t_{1/2}$ of 2.8 h. The second phase (β) occurred between 30 and 192 h after dosing and had a first-order apparent $t_{1/2}$ of 89.4 h. Urinary excretion kinetics showed three elimination phases. The alpha, beta, and gamma elimination phases occurred between 2 and 30 h, 30 and 72 h, and 72 and 192 h with $t_{1/2}$ of 2.3 h, 10.6 h, and 334 h, respectively.

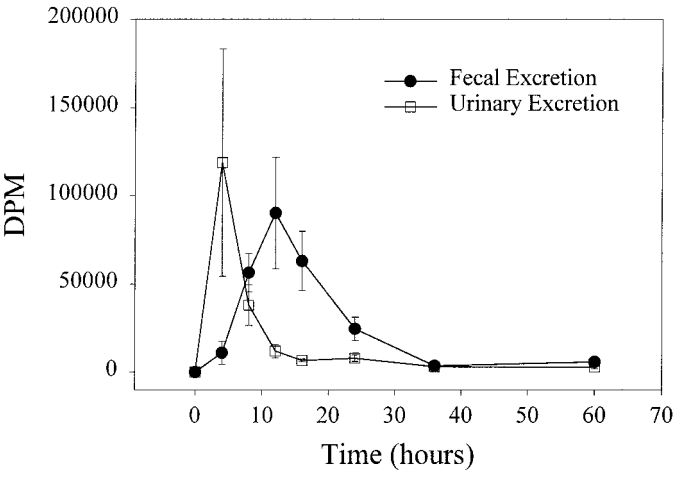

FIG. 1. Measurement of total dpm in excrement over time from Peromyscus after s.c. injection of [^{14}C]testosterone. Radioactivity was measured in both urine and feces to determine the excretion profile shown (n = 3, mean ± SEM).

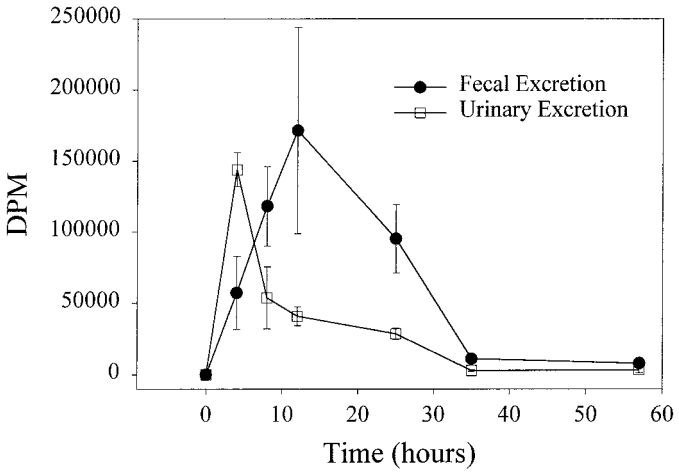

FIG. 2. Measurement of total dpm in excrement over time from Mus after s.c. injection of [^{14}C]testosterone. Radioactivity was measured in both urine and feces to determine the excretion profile shown (n = 3, mean ± SEM).

In Mus, an average of 83 ± 15% of the total radiolabeled steroid was recovered from each animal. Of this recoverable fraction, testosterone excretion in urine and feces proved similar to values for Peromyscus, with 95.5 ± 2.0% being excreted in the first 25 h (Fig. 2). There were two elimination phases in feces. The alpha and beta elimination phases occurred between 10 and 30 h and between 30 and 77.5 h with $t_{1/2}$ of 3.01 h and 11.5 h, respectively. There were also two urinary elimination phases. The alpha and beta elimination phases occurred between 2 and 30 h and between 30 and 77.5 h with $t_{1/2}$ of 4.0 h and 32.0 h, respectively.

HPLC Separation

HPLC separation of ether extract from feces of a [^{14}C]testosterone-injected Peromyscus produced two major and three minor nonpolar peaks. A polar peak comprising < 20% of the total dpm injected was detected in the first few minutes of the HPLC run. The [^{3}H]testosterone tracer coeluted with another compound with a similar elution time in the HPLC run. The area of the peak composed of testosterone indicated that a small percentage of unconjugated testosterone had been excreted in the feces. Though peaks were not positively identified, some coeluted (± 1 fraction) with metabolites identified by Brooks: estriol at fractions 19-22 and androsterone at fractions 50-54 [18]. Analysis of the aqueous component remaining after ether extraction yielded a single large polar peak (the presumably conjugated metabolites were not enzyme hydrolyzable and were not identified). Further HPLC analyses using an in-line radiomatic detector on samples from Mus indicated that testosterone made up less than 8% of nonpolar steroids from feces.

Animal Validation

Fecal testosterone concentrations increased to 8 times the basal level when animals were given hCG, decreased below basal after surgical castration, and did not change with hCG treatment of castrated mice (Table 2). No gross abnormalities of the testes, such as atrophy or discoloration, were observed.

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TABLE 2. Testosterone levels in feces after hCG challenge to obtain maximum levels and castration to obtain a minimum level.

Reproductive state	n	Testosterone*	SD
Basal	3	173	39
hCG stimulated	3	1352	60
Castrated	3	52	23

* Values for the amount of unconjugated testosterone extracted represent nanograms testosterone per gram of feces.

The preliminary data resulting from field trials demonstrate the utility of this biomarker as an accurate indicator of reproductive condition in a control population. The mean fecal testosterone content of reproductively active males (testes in scrotal position) was 510 ± 32 ng/g feces (n = 86, mean ± SEM), while the fecal testosterone of inactive males (testes in abdominal position) was 380 ± 18 ng/g feces (n = 261, mean ± SEM). Analysis using a one-way ANOVA showed that the difference between the two groups was statistically significant ($p < 0.05$).

DISCUSSION

The use of feces for steroid analysis enables laboratory and field data to be collected in situations in which blood and urine collection may prove either detrimental to the animal or impossible to obtain. Blood collection is an invasive technique through which only limited number of samples can be obtained from a single animal. Furthermore, fecal measurements of testosterone correlate well with measurements of plasma testosterone [19]. The method described and evaluated in this paper combines a noninvasive and simple collection procedure with an analytical technique that has been shown to accurately measure testosterone concentration. The methods were designed for eventual use of the technique as a biomarker to be used on field populations.

The specificity of the assay gives good evidence of a highly specific assay. The only compound that was found to cross-react with the antibody at > 1% was 5α-DHT. This does not present a problem because 5α-DHT is the more potent form of testosterone, is found in much lower concentrations, and is also of biological interest because of the equivalent cellular targets of both compounds.

Comparison of an ether extraction, with and without wetting, to a 10% methanol solubilization extraction showed that the former was the better method of extraction for unconjugated testosterone. An additional extraction with ether on the wetted sample recovered only an additional 5%, indicating this additional step was not necessary because it provided only a minor increase in extraction efficiency. Though the exact percentage of unconjugated testosterone extracted has not been definitively shown, the experiments indicate that a consistent extraction with greater than 90% recovery can be achieved. The high volatility and purity of ethyl ether make it convenient for use in the extraction. Additionally, it does not interfere with the ELISA, and the extracts can be easily concentrated if they fall below the sensitivity of the assay.

The kinetic studies done with radiolabeled testosterone provided data regarding the time course of steroid excretion in order to provide a better understanding of the significance of a measurement relative to the time of an event (chemical or environmental stress). In Peromyscus, the kinetic profile for testosterone excreted in feces followed a first-order two-compartment model. The first phase (α) corresponded to the rapidly available portion of the [^{14}C]testosterone that went directly to highly perfused tissues such as liver and kidney. The second phase (β) corresponded to a slower component in which the testosterone is sequestered in slowly perfused tissues such as adipose tissues. Intraperitoneal dosing may favor the excretion of testosterone in one phase over another. Fecal excretion of testosterone for Mus followed the same pharmacokinetic trends as that of the Peromyscus, but fewer time points were used in determination of the kinetic parameters. Knowledge of the lag times for excretion is important in determination of the relationship between physiological events and the detection of changes in the feces [20].

Radioactivity in feces was associated with both the polar and the nonpolar phases of extraction. These results are consistent with studies of other steroids, which have been shown to exist in the conjugated [21] and the unconjugated form [22] in the feces. Extraction using solubilization of the sample with 10% methanol effectively recovered 87% of the radiolabel, but HPLC separation showed large polar peaks with negligible nonpolar peaks. Reextraction with ethyl ether and HPLC separation resulted in an increase in the size of the nonpolar peaks. When one is measuring total radiolabel excretion, the feces should be extracted using both solubilization and solvent extraction. For measurement of unconjugated testosterone with the ELISA, only an ether extraction is necessary.

The radioactive tracer experiment indicated substantial metabolism of the [^{14}C]testosterone resulting in only a small percentage of injected radiolabeled testosterone being excreted in the feces. Though measurable, androsterone and etiocholanone were not good indicators of testes function, probably because these steroids are also formed as metabolites of adrenal steroid secretion [18]. Despite the extensive metabolism of testosterone, there is sufficient amount excreted to quantify using the ELISA. It should be noted that the effect that a single large dose of testosterone may have had on the metabolic profile was unknown.

The production and release of endocrine-disrupting chemicals has been occurring for decades and is a concern in environmental research [23, 24]. These chemicals can suppress reproductive development in wild male mice, and this suppression is due in part to decreased production and release of testosterone [17]. Because reproduction is not required for the immediate survival of the individual, fecundity may be reduced, and despite survival of the individual, important reduction in the quality and numbers of the succeeding generations could result.

In conclusion, the present study employed a technique for extracting and measuring fecal steroids that can measure androgen status of animals in the laboratory and field. Mus were studied because of their availability, the abundance of baseline data, and their prevalence in the fields of California. Peromyscus were chosen because they have many characteristics of good sentinel species for field studies [25]. Feces can be easily collected in the field and frozen. With fecal analysis, samples can be collected frequently without the stress associated with venipuncture. This fecal testosterone biomarker should prove to be a valuable marker in the study of endocrine-disrupting chemicals on free-ranging wild mice.

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mice feces and for providing information on the reproductive condition of the mice trapped.

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