

RESEARCH ARTICLE

Fecal Steroid Research in the Field and Laboratory: Improved Methods for Storage, Transport, Processing, and Analysis

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Since the pioneering paper “Measurement of Excreted Steroids in *Macaca nemestrina*” [Risler et al., American Journal of Primatology 12:91–100, 1987] was first published, field primatologists have been using fecal extraction techniques to examine adrenal and gonadal hormones. These techniques have allowed investigators to determine reproductive conditions in wild primates without causing any disruption to the populations. Over the years, many techniques have been developed to improve the ease of analysis, transportation, and purification. More of the processing can now be done in the field. This paper describes the methodology developed or adapted at the Wisconsin National Primate Research Center, and the factors involved in preparing fecal samples for steroid analysis. We provide information on the steps involved in extracting and purifying steroids from feces for measurement. The latest methods include field processing of samples, such as drying collected material or separating steroids from the fecal material by solid phase extraction (SPE). How samples are processed in the field determines the requirements for international transportation and the methods used in the laboratory. The pros and cons of the different processing methods are discussed. We also report on recent advances in laboratory quantification, with implications for steroid isolation prior to analysis. The different processes involved in isolating and measuring fecal steroids discussed here will enable investigators to understand the components necessary to ensure accurate and reliable results. Am. J. Primatol. 67:159–174, 2005. © 2005 Wiley-Liss, Inc.

Key words: fecal steroids; reproductive function; sample processing

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INTRODUCTION

Primatologists who study nonhuman primates in the field know all too well the value of excrement for profiling the parasites, genetics, and internal physiology of an individual without having to handle the subject of interest. The use of animal excrement for a variety of reasons has been recorded throughout history. Perhaps some of the most unusual uses of animal feces occurred in ancient Egypt, where inhaling the fumes of charred crocodile dung was a treatment for female hysteria, and the excrement of lizards was used as a beauty product [Laporte, 2000]. Roman women would smear feces on their face to maintain their smooth complexion. During the 17th century “the purifying, healing and beautifying virtues of shit were sung by alchemy, medicine, chemistry, and the perfume industry” [Laporte, 2000]. Indeed, indole, a major fragrance in flowers such as orange blossom, jasmine, and lilac, is present in feces [Aftel, 2001]. Feces have been used for centuries in many civilizations as a fertilizer to enhance food growth, as is still practiced today.

The use of medical diagnostics on feces to determine an individual’s internal physical condition is not a new science. However, only recently has the connection been made that feces contain hormones that have been eliminated from the circulation, which can provide insights into the internal physiology of an individual. The first extraction of human feces to determine profiles of reproductive steroids was first reported by Adlercruetz et al. [1976]. However, another decade passed before steroids were extracted from nonhuman primate feces. Risler et al. [1987] first developed methods to noninvasively assess an animal’s reproductive condition. In their pioneering study, the authors described ways to measure fecal estradiol and progesterone to monitor the ovarian cycles of pigtailed macaques. The idea of using fecal steroids to determine the physiology of wild primates launched a new field: field-based studies of primate behavioral endocrinology.

One of the missions of the Wisconsin National Primate Research Center has been to advance our knowledge of nonhuman primates in their natural habitat. In Assay Services our expertise in hormone analysis has allowed us to work with investigators who are studying wild primates and need to assess their reproductive or adrenal function. We have measured steroid levels in over 20 nonhuman primate species, monitoring both reproductive and adrenal hormones (estradiol, estrone, progesterone, testosterone, DHT, and glucocorticoids (cortisol, cortisone, and corticosterone)). These analyses have allowed us to assess reproductive functions during seasonal changes in ovarian cycling, conception, pregnancy, and male fertility, and levels of glucocorticoids during reproductive functions and stress assessments. In many cases we have designed the analyses to fit the needs of the researcher. Reduced field processing of the material requires more laboratory processing.

In the late 1980s we began working on methods to determine reproductive function in wild muriquis, in collaboration with Karen Strier. For the initial collections, the fecal material was collected and stored in alcohol. These large fecal boluses required high quantities of alcohol and were bulky and heavy for transportation to Wisconsin. In the laboratory, the alcohol had to be evaporated from the feces before the sample was lyophilized, ground, and weighed for extraction. Over time, we began to have more of the processing performed in the field. Equipment for mixing, weighing, and initial extraction was set up in the field, which allowed the field workers to process the sample and leave the fecal material in the forest. Thus, only the fecal steroids in alcohol and water would be

transported to the United States. Recently, further field processing with solid phase extraction (SPE) has allowed transportation of only the steroids in small cartridges without the need for permits from the Centers for Disease Control (CDC) or bulky containers. This reduces the chance of bringing in infectious agents, and avoids expensive laboratory processing.

While our methods have been productive for assessing reproductive function, there are many factors to be considered in processing fecal steroids. Steroids are excreted into the feces at different lag times due to processing of the steroids in the liver and reabsorption in the gut. Some steroids, such as estradiol, are highly conjugated and may be delayed in their excretion into the feces [Ziegler et al., 1996]. While fecal analyses provide a means of obtaining information about the internal physiology of nonhuman primates without touching or interfering with them, the analyses can be costly because of the extensive techniques required to purify the samples. Here we describe the various aspects that need to be considered by investigators or field workers who want to use fecal steroid analyses to assess the physiological conditions of nonhuman primates in the field. We report on the trade-offs that have to be made between the cost of analysis and the precision of determining the most accurate methods for measuring individual steroids. While examinations of ovarian cycling may not require extensive methodologies to purify or isolate reproductive steroids, other steroids (such as testosterone and the glucocorticoids) will require more extensive processing. To determine the most appropriate method one must take into account the field conditions, transportation considerations, and laboratory analyses involved.

MATERIALS AND METHODS

Field Conditions

The amount of fecal processing that can be accomplished in the field where collection occurs influences both the transportation considerations and laboratory analyses. The more samples are processed in the field, the less they require laboratory processing. Traditional methods for storing fecal samples until analysis used alcohol as a preservative to cover the fecal sample for storage and shipping to the laboratory, without the need for subambient temperatures. However, this method can cause changes in some steroids [Khan et al., 2002; Lynch et al., 2003; Wasser et al., 1988], and results in restrictions on air transportation. Steroids left in the fecal material can be altered, and conjugation may change during storage. Furthermore, samples can only contain 20% alcohol by volume to be allowed on most air carriers, or the size of the container has to be decreased to require less alcohol. Other field methods for processing samples in the field are now considered to have advantages over the above methods, including freezing samples, drying fecal samples, field extraction, and SPE.

Freezing samples

Samples can be kept cold in a field pack during the day and transported back to the field camp in the evening. If a freezer is available, the samples can be frozen immediately and shipped on dry ice to the laboratory. Samples that remain frozen until they are thawed for analysis are considered to keep the steroids in excellent condition. Unfortunately, freezers are not common at field sites, and when available they will fill up fast with bulky fecal material. Alternatives for field workers who cannot freeze the feces and transport it this way are to either dry the feces in ovens or extract the feces in the field.

Drying samples

As a preservative, drying varies in its effectiveness depending on the drying temperature used. Drying temperatures in the literature range from 40–80°C and show considerable variation at the two extremes. Samples can be dried under field conditions with little equipment needed, and this method has been used on prosimian species in Madagascar [Brockman & Whitten, 1996; Whitten et al., 1998] (Gould et al., 2005, this issue) and New World howler monkeys [Zucker et al., 1995]. With this technique, the collected samples are brought to the field camp and heated in an oven until all the moisture is removed from the feces. Depending on the ambient humidity, this may take hours. The sample can then be stored in moisture-proof packages with desiccant and shipped back to the laboratory for analysis. This technique reduces the weight and size of the sample, making transportation easier. Water content in feces can range between 30–75% of fecal mass and vary from sample to sample within an individual or between individuals [Ziegler et al., 1996]. Steroids are reported in concentration per mass of the sample. Therefore, drying the sample will reduce variation in the fluid amount and result in more consistent steroid results [Wasser et al., 1993]. Unfortunately, steroid loss occurs with this technique, and there is also the disadvantage of bringing fecal material into the laboratory.

Field extraction

Extracting samples in the field requires setting up a small laboratory at the field station. To extract steroids from feces, a solvent is required. While methods for extracting feces in the field have been reported [e.g., Beener & Whitten, 2004; Shideler et al., 1995; Whitten et al., 1998], we choose to use what is most readily available in the primate source country. With distilled water and alcohol obtained at a pharmacy in a source country, steroids can be extracted from fecal material. Pharmacies will sell 85–95% ethanol, and this can be mixed 1:1 (v:v) with distilled water to extract all steroids, including water-soluble conjugated steroids. The samples are mixed thoroughly and weighed in 0.1–0.5 g amounts, depending on the anticipated amount of steroids in the feces [Strier et al., 1999]. New World primates have higher concentrations of steroids excreted into both urine and feces, and therefore only a small amount of fecal material is needed [Ziegler et al., 1997]. To 0.1 g of feces, 2.5 ml of distilled water and 2.5 ml of ethanol are added [Sousa & Ziegler, 1998; Strier & Ziegler, 1997]. The sample is mixed by using a vortex or hand-shaking for 5 min. The amount of steroids released from the feces will vary with the amount of shaking time, and therefore this time has to be consistent between samples. Five minutes of high-speed vortexing will extract 60–90% of the steroids from the fecal material and provide consistent results. The samples are then centrifuged at high speed for 10 min to bring the fecal material down to the bottom of the tube, leaving the supernatant or liquid layer to be poured off into another container. If a portable centrifuge cannot be obtained, field researchers have had great results with producing their own centrifuge by tying a string to the tube and whirling the sample over their head for a predetermined time (Anne Carlson and Leslie Seltzer, personal communication). Once the liquid is decanted, the fecal material can be left on site, thereby eliminating the problems of transporting possible infectious material. The 5-ml extracts can be stored in polypropylene containers in a cool dark place without fear of steroid alteration, or they can be purified and transported by SPE columns.

SPE

This method can be used in the laboratory or the field, and is a precursor to any high-performance liquid chromatography (HPLC) separation methods. SPE

columns are readily available, which allows researchers to apply their samples, cap both ends, and store or ship them at ambient temperatures. We examined the recoveries of steroids in muriqui fecal extracts with SPE, and obtained the following results: cortisol 86%, progesterone 89%, estradiol 78%, and testosterone 70%. These recoveries were consistent between samples and indicate that the columns are reliable for recovering the steroids. SPE columns have a solid matrix consisting of sorbents, such as Octadecyl, which are similar to tiny porous beads. Bonded silica sorbents are commonly used to extract analytes from complex sample matrices, such as blood, urine, and feces. Under the proper conditions this matrix will adhere to the steroids as the liquid is pushed through the column and eliminated as waste. The steroids adhered to the matrix can be washed to eliminate contaminants and other undesirable components of the feces. Under the proper conditions, the steroids can be released and eluted for analyses by immunoassay or by further purification and separation by HPLC. The most common method uses 2 ml of methanol and then 2 ml of distilled water to condition the column. In the field a syringe can be used to push the substances through. One or 2 ml of fecal extract are pushed into a preconditioned column with a syringe, with the steroids adhering to the solid phase of the column, and the liquid is pushed through and eliminated. The columns can be washed with distilled water in the field to eliminate contaminants, and a preservative, such as sodium azide, can be added if the columns are to be stored for a long time. These columns are small (2 inches long) and lightweight, which allows many samples to be accumulated in a small space. Figure 1 shows the differences in the size of samples stored in alcohol, extracted steroids in ethanol/water, and SPE columns.

Sample Handling and Preparation

There are restrictions on the transportation of samples that originate from outside the United States. All nonhuman primate samples require either a CDC permit or a letter from the CDC indicating that the samples are not hazardous (the following website can be used to obtain an import permit application: <http://www.cdc.gov/od/ohs/biosfty/impptper.html>). Since frozen, dried, or alcohol-stored feces are considered to contain infectious agents, a permit is required to pass through customs, as is a sticker on the package indicating that it contains hazardous biological materials. Samples that contain more than 24% alcohol are also considered to be explosive, and are designated as a class III flammable substance. If samples fall under the diagnostic specimen category and contain more than 24% alcohol, they may be shipped as "Dangerous Goods in Excepted Quantities." However, it is difficult to find an airline carrier that will transport the samples. In the United States they will be shipped by ground after they pass through customs at the point of entry. Samples that are not accompanied by a field researcher usually require a broker to see them through customs, which increases the cost of transportation.

Laboratory Analyses: Sample Preparation

Once the samples are delivered to the laboratory, the type of analysis used depends on several factors. Samples that are brought back as frozen feces must be lyophilized (freeze dried) or prepared for weighing and extraction of the steroids. As mentioned above, eliminating the fluid variability allows for a more consistent measurement of fecal steroids. Each laboratory differs in terms of the percentage and type of solvent used for steroid extraction from fecal matter, but all



Fig. 1. Sizes of storage containers for storing and shipping muriqui fecal material to the United States. The container on the left is a 50-ml conical polypropylene tube for collecting fecal pellets and covering them in alcohol. The middle tube is a polypropylene container for storing fecal steroid extracts (5 ml of 50:50 ethanol : water). The column on the right is an SPE tube for storing steroids only, since the fecal material has been eliminated. This SPE tube has a top and bottom cap to keep the steroids moist.

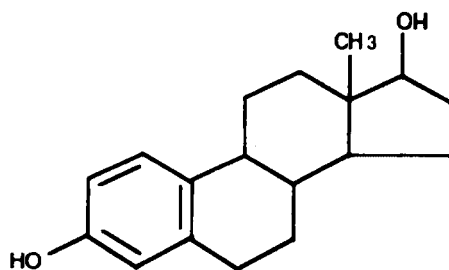
laboratories use at least a 30% solvent (ethanol and methanol). The advantage of using a mixture of solvent and aqueous solution is that both unconjugated steroids and conjugated steroids will be extracted from the fecal material.

Fecal steroids can be analyzed by immunoassay directly after extraction. However, further analyses are usually done, for the following reasons: 1) the majority of the steroid of interest is conjugated and has to be broken free before the antibody will recognize it, 2) samples are further purified by SPE to prevent interference in quantification, 3) samples must be separated by celite chromatography prior to immunoassay to prevent cross-reactivity of other steroids with the antibody, 4) samples are going to be separated by HPLC so that individual steroids can be collected from the same sample and analyzed by immunoassay, 5) samples are separated by HPLC and measured directly online through ultraviolet (UV) light detection, or 6) steroids are separated by HPLC and measured directly online through mass spectrometry (MS) to identify and quantify multiple steroids.

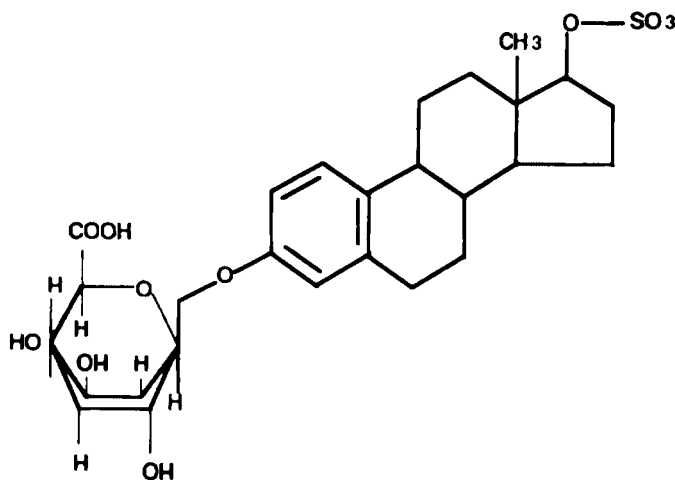
1. Steroid conjugation

Steroids are released into the circulatory system, where they can contact any tissue in the body. The liver acts as a filter for steroids and deactivates them

before they are eliminated from the body. Steroids such as estradiol and testosterone are highly bioactive and could be toxic in high amounts if left in the environment at high levels. Therefore, the body deactivates highly bioactive compounds by conjugating them to render them water-soluble and less active. Steroids are mainly conjugated as glucuronides or sulfates (Fig. 2). Conjugation can be simple, such as estrone-glucuronide and estradiol-sulfate, or conjugation can be complex, such as 17β estradiol-3-glucuronide-17 sulfate (the numbers refer to which carbons on the molecule have the attached conjugate). Substantial concentrations are excreted into the bile and then into the intestines. Steroids can be reabsorbed and recirculated before they are eliminated. Intestinal microbial action can hydrolyze steroids and break apart some of the conjugates, but many remain. Conjugated steroids can be more difficult to measure because the



17 β - Estradiol



Estradiol - 3 Glucuronide - 17 Sulfate

Fig. 2. The structure of 17β estradiol as a free steroid and a doubly conjugated steroid. In the lower diagram, estradiol is conjugated in the 3 and 17 positions. Antibodies made against 17β estradiol do not recognize this conjugated form.

conjugated part of the molecule may be hidden from an antibody raised against the individual steroid. Some antibodies, such as an estrone-glucuronide antibody, will recognize the conjugated steroid and hence deconjugation is not needed. However, steroids that are di- or triconjugated will not be recognized, and thus the conjugates must be broken off the steroid prior to quantification. Table I shows the level of conjugation of the major female reproductive steroids for four nonhuman primate species. Estradiol is highly conjugated in callitrichid species, but not in muriqui or patas monkeys [Strier & Ziegler, 1994; Ziegler et al., 1996]. The percentages of testosterone or glucuronides found as complex conjugates are presented in Table II for some nonhuman primate species. Failure to break the conjugates prior to analysis will result in measurement of only a portion of the steroid. If the conjugation does not remain stable under different physiological conditions, estimates of the steroid of interest will not be accurate.

Conjugated steroids are also problematic for HPLC separations. They are water soluble due to attached glucuronides and sulfides and this changes their polarity and retention times. Therefore, steroids are usually deconjugated prior to chromatography. Alternatively, a method can be developed that recognizes both the conjugated and free steroids because they elute at different retention times.

TABLE I. Percent of Female Reproductive Steroids Found in Free or Conjugated Form in the Feces of Four of Nonhuman Primate Species

Species	Conjugate	E2	E1	P	Pd
<i>Callithrix jacchus</i>	Free	16	88	64	95
	Simple	1	10	19	4
	Complex	83	2	17	1
<i>Saguinus oedipus</i>	Free	12	90	76	95
	Simple	3	9	12	2
	Complex	84	1	12	3
<i>Brachyteles arachnoides hypoxanthus</i>	Free	98	100	85.7	
	Simple	0.9	0	9.7	
	Complex	1.3	0	4.6	
<i>Erythrocebus patas</i>	Free	91	88	69	83
	Simple	6.4	12	20	8
	Complex	2.6	0.5	10.5	5

E2, estradiol; E1, estrone; P, progesterone; Pd, pregnanediol.

TABLE II. Percent of Testosterone (T) or Glucocorticoids (Gluc) Which Are Found as Complex Conjugates in the Feces

Species	T	Gluc
<i>Aloutta palliata</i>	74	80.5
<i>Brachyteles arachnoides (hypoxanthus)</i>	65	
<i>Cebus apella</i>	95	50
<i>Colobus badius</i>		61.5
<i>Laothrix lagoricha</i>		70
<i>Macaca mulatta</i>		86.5
<i>Pongo pygmaeus</i>		64

Liberating steroids from complex conjugates requires either solvolysis or acid hydrolysis. Our method uses solvolysis, as described by Eastman et al. [1984] and modified by Ziegler et al. [1996], which eliminates the use of both glucuronide and sulfide conjugates. To 500 μ l of fecal extract (consisting of water and ethanol), 100 μ l of saturated NaCl, 50 μ l of 2.5 M H₂SO₄, and 4 ml of ethyl acetate are added prior to incubation overnight at 40°C or at 60°C for 2 hr. To determine the level of conjugation of the steroid of interest, a procedure involving sequential hydrolysis and solvolysis can be performed as reported in Ziegler et al. [1996]. This method allows for the extraction of free steroids prior to hydrolysis to liberate simple conjugated steroids. The sample is extracted again before solvolysis is performed on the remaining sample. All three extractions provide the percentage of steroid that is found under the different conditions. When the majority of the sample is found in the complex conjugated portion, it is necessary to use solvolysis prior to analysis by any quantification method. The conjugation of a particular steroid in urine has been shown to change throughout the ovarian cycle; therefore, quantifying only the free steroids would misrepresent the steroid profile [Eastman et al., 1984].

2. Purification by SPE

Liquid extraction by a solvent often carries impurities with it that can interfere with the accuracy of measuring steroids by radioimmunoassay (RIA) or enzyme-immunoassay (EIA). When interference precludes validity of a particular steroid from a particular species of nonhuman primate by accuracy or parallelism, we further purify the samples by SPE. Although SPE adds to the expense of quantifying fecal steroids, it is often a necessary process to obtain accurate concentrations in the sample. For samples that will be separated by HPLC and then quantified by UV or MS, it is essential for the sample to be pure. HPLC columns are very sensitive to impurities and clog quickly without SPE. Samples that are processed through SPE and stored for shipment to the lab are therefore ready for analysis (unless solvolysis is required). The procedure in the lab is equivalent to that described above for field SPE.

3. Celite chromatography

Another way to separate steroids without HPLC is to use celite chromatography. Celite columns also separate steroids by polarity, but instead of separating individual steroids they separate the steroids into groups [Abraham et al., 1972]. This method is particularly effective if the steroids of interest, such as testosterone and DHT, have different polarities. Steroids such as pregnanediol and estrone are found in the same group that is eluted by a particular percentage of ethyl acetate in iso-octane, but since these steroids are eluted for immunoassay, antibodies against pregnanediol do not cross-react with estrone, and vice versa. While celite chromatography is a well-established method that yields high recoveries of steroids [Ziegler et al., 1996], individual columns must be made and kept desiccated before each run of samples is performed. It can be time-consuming to make such columns. As with SPE, the samples are purified after they are run through the columns, and therefore are more readily validated in the assays.

4. HPLC separation for identification

HPLC is used for several reasons. We use HPLC to separate steroids from a particular primate species when we want to identify how many steroids

cross-react in the chosen immunoassay. For instance, to determine whether a testosterone antibody is specific enough to measure testosterone without measuring other steroids that provide a different profile from testosterone alone, we separate the sample by HPLC, collect the fractions (usually 1-min fractions), and measure each fraction by a testosterone immunoassay. If the assay indicates that the majority of reactivity to the antibody is due to the fraction that elutes testosterone, we can say the assay is specific for measuring testosterone in this particular species. However, most antibodies raised against testosterone also measure DHT and possibly other androgens. If these steroids are to be examined separately, they must be separated routinely by either HPLC or celite chromatography prior to assay. When we need to measure multiple steroids from a single sample, we use HPLC separation prior to the immunoassays.

Laboratory Analyses: Fecal Steroid Analyses

After the samples are prepared, the fecal steroids can be quantified by several methods: RIA, EIA, HPLC-UV, and HPLC-MS.

RIA

RIAs have been performed to quantify steroids since the 1960s. It is the most routine method available, but has the disadvantage of using radioactive material (though the doses are very low). RIAs are accurate and reproducible. However, the antibodies to specific steroids often cross-react with other steroids in the media, and thus chromatography is required to isolate the steroid of interest prior to analysis.

EIA

EIAs have an advantage over RIAs in that they do not involve the use of radioactive materials. These enzyme-linked assays are very reliable and are often more sensitive than RIAs for low concentrations. As with RIA, chromatography is often used to isolate the steroid of interest prior to quantification. EIAs offer the advantage of short incubation times, and results can be obtained quickly with a spectrophotometer plate reader.

HPLC and UV detection

Detectors are routinely attached to HPLC pumps, to allow for identification and quantification of the steroid content in a sample. Many types of detectors are used, the most common of which is the UV detector. These detectors measure the absorbance of UV light by the steroids. Steroids absorb light at different wavelengths and at different intensities depending on their structure, with double bonds and ketone groups providing the most absorption of light. If the UV max (wavelength of maximum absorbance) of a particular steroid is known, that wavelength is selected to measure the steroid. Steroid standards are calibrated at different concentrations and the method is used to quantify steroids as they are separated on the column. The advantage of this technique is that the samples do not have to be collected as fractions as they come off the column and then quantified by immunoassay. Multiple steroids are calibrated and measured in an individual sample during the time it takes to run the sample through the column. This is particularly advantageous when many steroids are of interest in a single sample. For instance, glucocorticoids and androgens can be measured simultaneously in males. Glucocorticoids, androgens, estrogens, and progestins in female

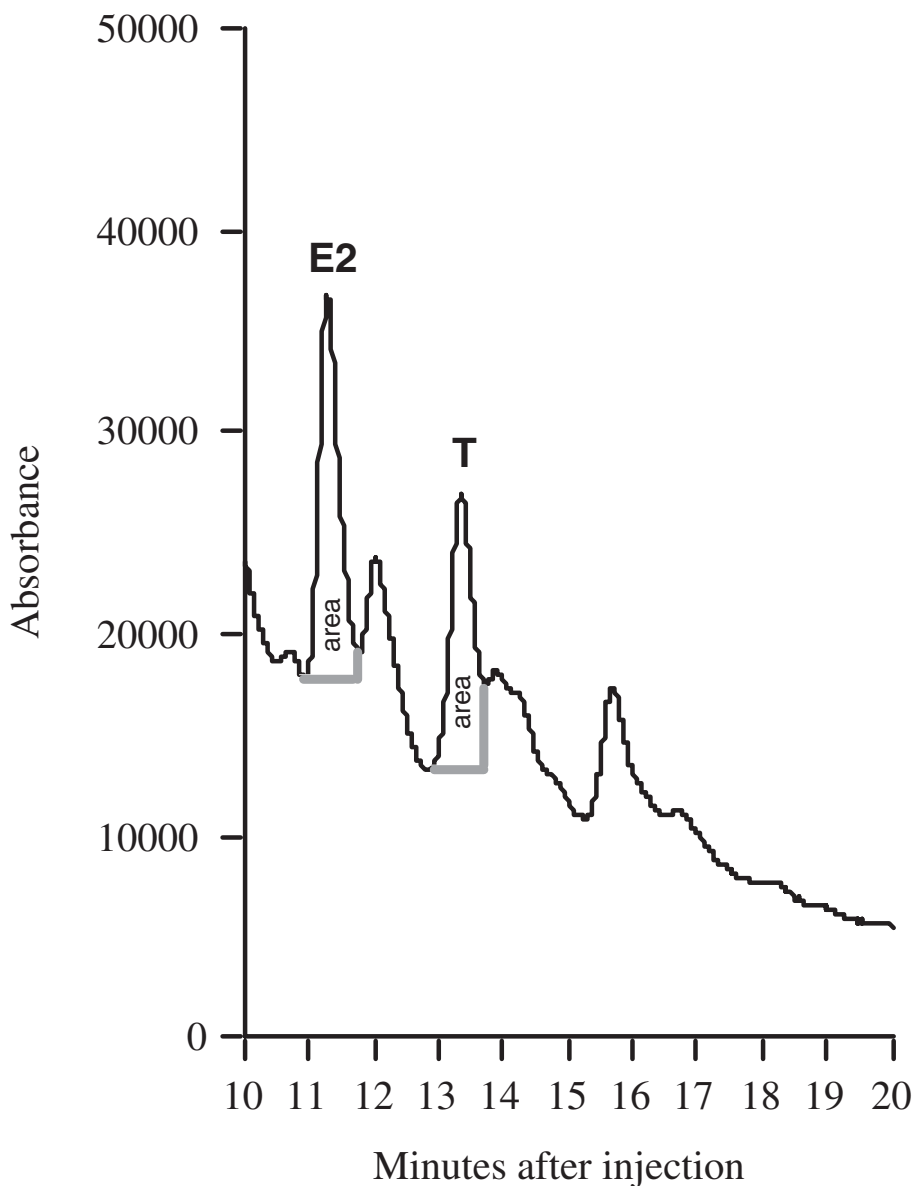


Fig. 3. HPLC chromatograph of an extracted and purified fecal sample from a female murrelet, with UV detection at 238 nm. The chromatograph is limited to 10–20 min to illustrate peaks of estradiol (E2) and testosterone (T) in this female. Concentrations of the steroids are determined by area under the curve for known standards.

samples can be measured during a 40-min run through the HPLC column. These steroids will be seen as peaks as they separate, and thus can be identified on the computer screen (Fig. 3). The disadvantages of this technique include the need to perfect the chromatography to ensure that no other steroid elutes at the same time. Additionally, not all steroids absorb light well. Many of the 17-ketosteroids, including DHT, do not absorb light and must be manipulated before they can be

measured by UV (i.e., measured at an ultralow wavelength or derivatized). UV detection is not as sensitive as immunoassay, and for most steroids the sensitivity does not go below 100 ng. Most fecal steroids are above this level and can be measured; however, if multiple steroids are examined at one time, some may be detected while others of interest are not. We have used this technique to measure urinary steroids in the cotton-top tamarin, with good success [Ziegler et al., 2004]. Highly light-absorbant compounds (e.g., estradiol and the glucocorticoids cortisol, cortisone, and corticosterone) are reliably measured, whereas other steroids (e.g., DHT and estrone) are not as reliable and must be collected and analyzed by immunoassay.

HPLC and MS

Comparisons among RIA, EIA, and LC/MS for a range of linearity are shown in Fig. 4 for testosterone. Serial dilutions of testosterone are linear for a longer range on the LC/MS than the RIA or EIA. The testosterone was accurately measured from 0.1 pg to 1 ng, whereas both EIA and RIA were much less sensitive for testosterone. The LC/MS technique has been around for many years, but only

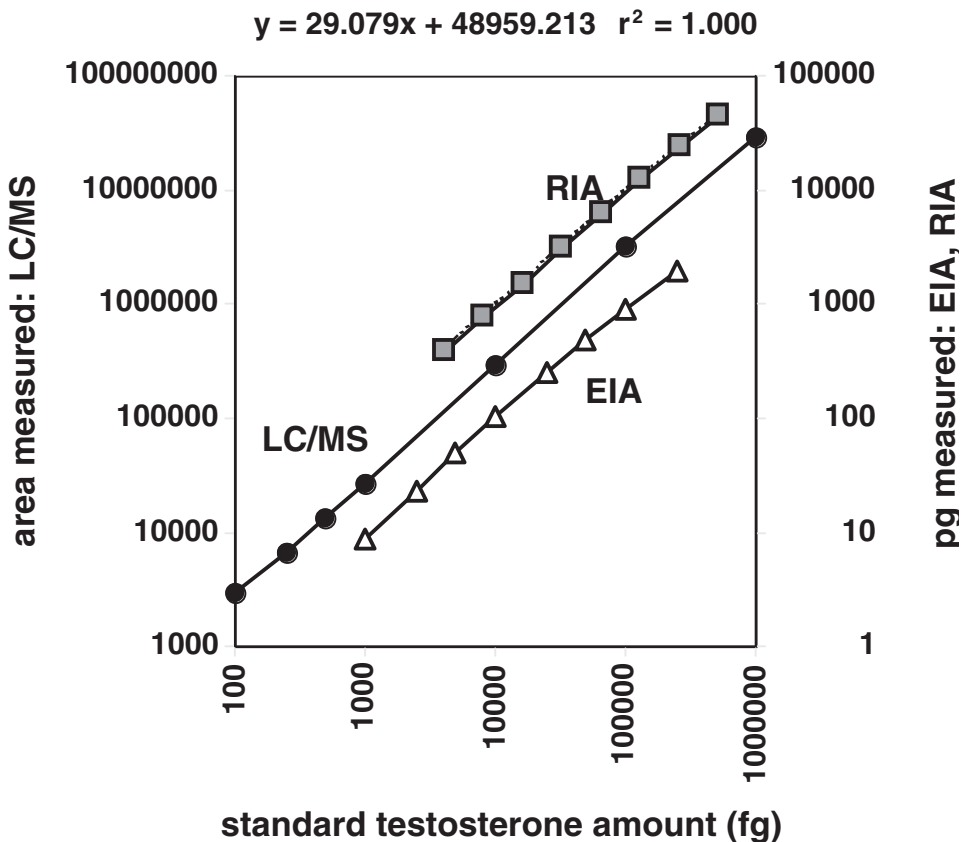


Fig. 4. Comparison of linear ranges for measurement of testosterone using three methods: RIA, EIA, and LC/MS. Serial dilutions of testosterone from 100 femtogram (0.1 pg) to 1,000,000 femtogram (1 ng). The serial dilutions for the RIA and EIA were for a shorter range than that for the LC/MS.

recently has it become an easily used bench-top technique. Samples are separated by HPLC, usually with the use of capillary columns that separate steroids and render them measurable at extremely low levels. Mass spectrometers often have UV detectors as well to help identify steroids at higher levels until the separation techniques are perfected well enough to go to the low concentrations used for MS analyses. This type of analysis can identify and quantify the steroids found in a sample collected from any bodily fluid. The steroid levels can be as low as a subpicogram (femtogram) and can be measured in a volume as low as 1 μ l. MS measures the mass-to-charge ratio of an individual steroid (Fig. 5). For instance, testosterone has a mass of 288, and depending on the mobile phase (a mixture of buffers and solvents) will take on electrons, such as hydrogen or sodium, and thus will be identified at a mass of 289 or 311. Once the mass and its charge are known, one can readily find a steroid and quantify it by calibrating the steroid as a standard. This technique is highly reliable and sensitive, and potentially offers a means of measuring multiple steroids in a single sample. All steroids can be measured this way as long as the chromatography method for separating the steroids is well developed. We are currently developing this technique for fecal steroids because it will allow us to examine all steroids regardless of their absorption or concentration. Since the methodology is highly sophisticated, users of the equipment must undergo intensive training. Additionally, the equipment is very expensive. However, it should prove to be the most reliable method for identifying and quantifying steroids at any concentration.

DISCUSSION

Field researchers make extensive efforts to habituate nonhuman primates and develop methods to obtain fecal material from individuals. These samples must be treated with the utmost care to ensure accurate assessment of the steroids found within them. As indicated in this study, measuring steroids in fecal material is not a simple, straightforward procedure. One must determine what equipment is available for field sites, how motivated the technical staff on site is to do the necessary lab work, and what techniques should be used to isolate and quantify the steroids.

We have outlined the methods used at the Wisconsin National Primate Center to process feces from nonhuman primates. Our newest techniques allow for more thorough extraction of steroids in the field, with less processing in the laboratory. While this does require more skill and equipment in the field, it also reduces time and expense in the laboratory. Steroids that are purified in the field are cleaner and less likely to have altered levels of conjugation or degrade over time. The overall cost decreases because less laboratory work is necessary, and the steroids are kept intact and purified before they are shipped to the laboratory. Additionally, processing samples in the field eliminates the need to travel with primate fecal material, as well as requirements for international transport of infectious material (such as a permit from the CDC for entry into the United States). Recovery rates from solid phase-extracted steroids are high, and provide a reliable measurement of the steroids of interest.

New methods for quantifying fecal steroids allow multiple steroids to be measured simultaneously, and therefore one can better assess the internal physiology of the primate of interest. When online assays are performed after HPLC, multiple steroids can be measured by instant calculations, and there is no need to resort to RIA or EIA for each separate steroid. This reduces laboratory time and eliminates expenses. For example, the time it takes to

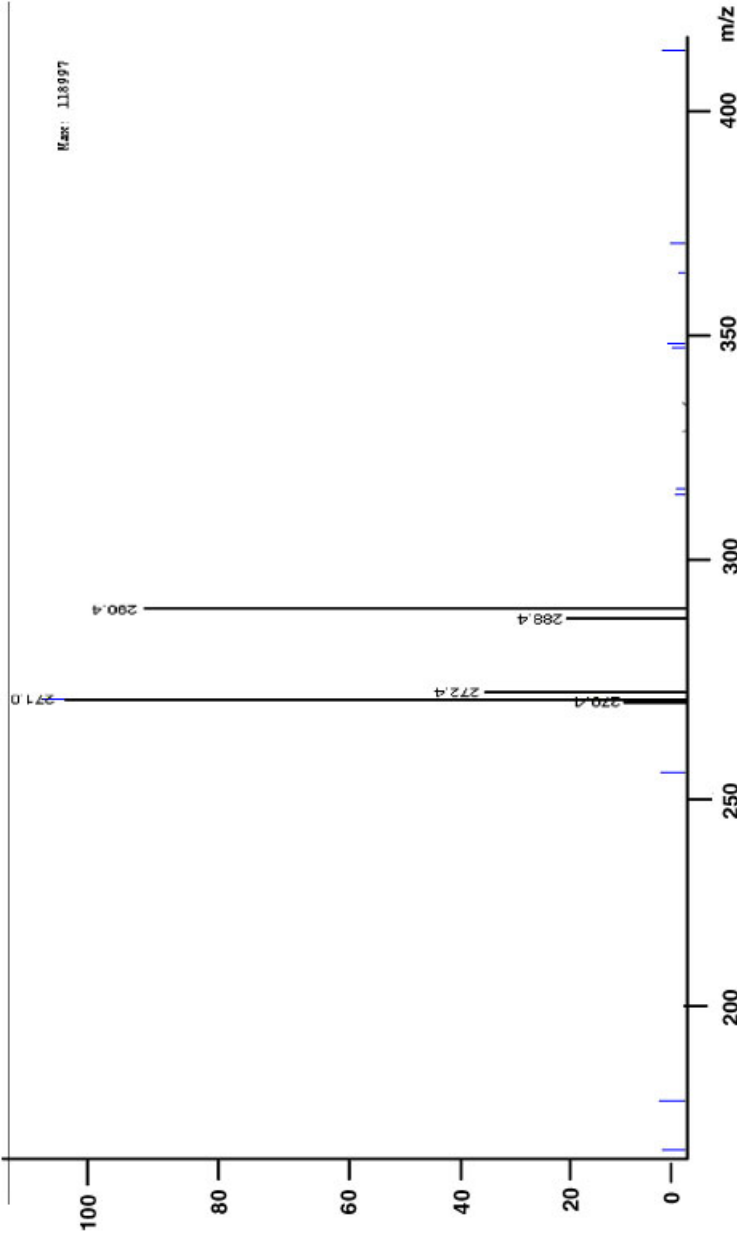


Fig. 5. An MS analysis of two steroids (estrone and testosterone) is shown as an on-screen analysis. The two steroids have similar retention times with this particular method (estrone = 5.75, testosterone = 5.66), but different masses. Estrone, with a molecular weight of 270, is mainly ionized by hydrogen to yield a mass of 271. Testosterone, with a molecular weight of 288, is shown to be ionized mainly by two hydrogen ions to yield 290.4. These patterns of ionization provide the fingerprint of the individual steroids for identification.

measure four steroids (estradiol, testosterone, androstenedione, and dihydrotestosterone) by LC/MS is less than 5 min from the injection time, while it takes 240 min from the start of an assay to the end for each steroid by either RIA or EIA. LC/MS may be more expensive for quantifying an individual steroid, but is less expensive for measuring several steroids in each sample. Since there are no antibodies, no cross-reactivities occur, and the standards are linear for a greater range.

In summary, there are many methodologies to extract steroids from the fecal matrix, and many quantification methods to obtain the final concentration. We hope this paper will elucidate many of the choices field primatologists must make to obtain the most accurate analyses, and offer alternatives to methods that are more laborious or require international transportation of the samples.

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