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RESEARCH ARTICLE

Radioimmunoassay of Estrone Conjugates From Urine Dried on Filter Paper

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Hormonal analysis of urine from free-ranging primates has been limited due to the difficulty of preserving samples under field conditions. Drying urine on filter paper is an alternative for field preservation. This study describes a new laboratory method for eluting steroids from filter paper with methanol, along with a series of experiments used to develop and validate this method. The overall elution recovery of estrone sulfate (ES) from filter paper was 86.4%. Estrone conjugate (E1C) levels in humans and captive orangutans were analyzed by radioimmunoassay (RIA). Values from samples dried on filter paper were significantly correlated with values from matched frozen samples, with elution efficiencies ranging between 97.1% and 102.4%. Creatinine (Cr) measurements from frozen urine compared to urine dried on filter paper were also significantly correlated ($r=0.96$) with an elution efficiency of 101.7%. After the samples were stored for 2 years, the absolute values of E1C and Cr were significantly lower but were still significantly correlated with frozen urine values. These data demonstrate the effectiveness of filter paper as a medium for preserving urinary steroid samples, and the efficiency of methanol as a solvent for eluting E1C and Cr. This method thus provides a viable alternative to the traditional procedure of freezing urine for field studies, where freezers are not available. Am. J. Primatol. 67:121–135, 2005.

Key words: estrone conjugates; hormone; filter paper; orangutan; radioimmunoassay

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INTRODUCTION

Analysis of urinary steroids from wild primates has become increasingly more feasible within the past 10 years [e.g., Marshall & Hohmann, 2005; Muller & Lipson, 2003; Robbins & Czekala, 1997; Ziegler et al., 1997]. The results of such analyses have enriched and complemented what was known from captive studies [e.g., Czekala et al., 1988; Dahl, 1991; Faiman et al., 1981; French et al., 1983; Graham, 1981; Hearn, 1984; Kingsley, 1988; Maggioncalda et al., 2000; Masters & Markham, 1991; Nadler et al., 1985; Shideler & Lasley, 1982], and have greatly augmented our understanding of primate reproduction and the socioendocrinology of behavioral response.

The collection of urine and analysis of hormones from the wild provides investigators an opportunity to study the ecological context of reproduction by addressing questions about the effects of nutrition, energetics, and ultimately food availability on reproductive hormones. Nutrition, activity levels, and social behavior vary significantly between captive and wild populations [Knott, in press]. Thus, we should expect to find differences between these populations in the physiological correlates of energetics and behavior. Further, as demonstrated by Sapolsky [1982, 1986], van Schaik et al. [1991], and Muller and Wrangham [2004], evaluation of the hormonal correlates of social behavior in the wild can be effectively used to study how rank, aggression, and stress affect physiology and reproductive success. Analysis of urinary steroids also provides a way to investigate the effect of both male and female hormones on sexual behavior, mate choice, and the timing of mating in the wild.

Logistical considerations have hampered our ability to analyze the urinary steroids of wild primates in some settings. In the past, primate field studies that successfully analyzed urine samples collected from the wild had to rely on the use of a freezer, liquid nitrogen, and/or dry ice for transportation [Andelman et al., 1985; Czekala et al., 1994; Muller & Lipson, 2003; Muller & Wrangham, 2004; van Schaik et al., 1991]. However, this is not feasible at many remote field sites. Thus, an alternative method for urine preservation is needed. Analysis of steroids in feces has also been used in wild primates [e.g., Brockman et al., 1995; Starisky et al., 1995; Strier & Ziegler, 1997; Whitten et al., 1998; Ziegler et al., 1997] and can have some advantages in certain circumstances [Emery Thompson, in press; Whitten et al., 1998]. However, depending on the species involved, urine is often the medium of choice because many primates urinate more frequently than they defecate, urine has a shorter lag-time than feces [Whitten et al., 1998], and the analysis procedure requires less sample processing. Filter paper is easily transported and does not require the special packaging or permits that are often associated with exporting and airline transport of urinary or fecal samples stored in methanol or ethanol.

Campbell [1994] and Shideler et al. [1995] pioneered the analysis of steroids stored on filter paper as an alternative to freezing for studies of humans and captive primates. In this study I present a new technique for eluting steroids from filter paper, and describe its application to radioimmunoassay (RIA) and the experiments conducted to validate the procedure. Shideler et al.'s [1995] procedure used hole-punched samples from urine-soaked filter paper that were directly introduced into enzyme assays. Hole-punched samples cannot be used with RIA. Thus, I tested the ability of several solvents to elute estrone conjugates (E1C) directly from filter paper. This technique allows the eluted sample to be used in multiple assays, and because the entire sample is eluted, the problem of uneven absorption of urine across the filter paper is avoided.

MATERIALS AND METHODS

Wild Orangutan Urine Collection

Samples were collected from wild orangutans at the Cabang Panti research site in Gunung Palung National Park, West Kalimantan, Indonesia, on the island of Borneo (1°13′S, 110°7′E). This was done in the context of a large study on the reproduction and socioecology of wild orangutans [Knott, 1996, 1998, 1999, 2001]. The methods used for urine collection and handling followed those previously described by Knott [1996, 1999]. Urine was collected from a large (1.5×1.5 m) clean plastic tarp that was placed on the ground beneath the night nest before the animal awakened, or from a clean plastic bag that was positioned on a forked stick beneath the urinating animal. A disposable pipette was used to collect urine from the plastic, which was then stored in 15-ml screwtop polystyrene centrifuge tubes for large samples or 1.5-ml snap tubes for small samples. Collection tubes of polyethylene should not be used, because they have been shown to adsorb substantial amounts of steroid [Banjeree & Levitz, 1985]. Plastic disposable gloves were worn for protection and to avoid contamination of the sample. Care was taken to avoid collecting urine that had intermixed with feces on the plastic sheet.

Urine Preservation

The urine samples were dried on pieces of filter paper with the use of a method modified from a similar procedure employed for human and captive primate urine samples [Campbell, 1994; Shideler et al., 1995]. Wearing gloves, I cut highly absorbent filter paper (Schleicher and Schluell #16110) into 2.5×2.5 cm pieces. Because of the high humidity of the rain forest, the cut pieces of filter paper were stored in an air-tight container with silica gel desiccant to prevent moisture absorption. The filter papers were placed on a nonabsorbent surface (usually aluminum foil or parafilm) for sample application. On each piece of filter paper the animal's name, collection date, sample letter, and aliquot amount were written in pencil. Samples of undiluted urine (200 μl) were aliquoted onto pieces of filter paper with a micropipette. After the urine was transferred to filter paper, the aluminum foil with the samples on it was placed in an airtight container of approximately 5 L in volume, containing approximately 0.5 L of silica gel. The drying time depended on the number of samples in the container (i.e., the fewer number of samples the quicker the drying process). The samples normally dried within 12 hr. Urine samples dried in this manner exhibited no mold growth. After the samples were dried they were stored in transparent plastic slide sheets, as recommended by Shideler et al. [1995]. Slide sheets containing samples were also kept in a plastic container with silica gel to avoid the moisture absorption and mold growth that can occur under tropical rain forest conditions. The silica gel used for these procedures had a color indicator and was ''cooked'' once a week to maintain its effectiveness. The samples were also kept away from light and heat, as suggested by Campbell [1994]. Samples prepared in the laboratory were processed using the identical methodology.

Captive Orangutan Urine Collection

Samples from captive orangutans were obtained from two cycling females at the San Diego Zoo, and four cycling females from the Seattle-Woodland Park Zoo. The zoos recorded the name of the animal, date of sample collection, signs of menstruation, and other behavioral notes. The samples were stored frozen at the

respective zoos and mailed on dry ice to the Reproductive Ecology Laboratory in the Department of Anthropology, Harvard University, where they were placed in a freezer upon arrival. Filter paper samples from captive orangutans were preserved by a procedure identical to that described above for wild samples.

Determination of Solvent

I conducted several experiments to determine the best solvent for eluting steroids from urine dried onto filter paper. In tests 1 and 2 (see Table I), the recovery abilities of methanol, ether, ethanol, and acetylnitrile were investigated. In these tests, 100 μl of titrated estrone sulfate (ES) (6,500 cpm) were aliquoted onto 2.5×2.5 cm pieces of filter paper. Control samples of tritiated ES were aliquoted into scintillation vials at the same time. The filter paper samples were dried in a container with silica gel to mimic field conditions. After the samples were dried, each filter paper sample was folded in half and placed with forceps into a 6×130 mm glass test tube. Each tube received 5 ml of solvent, which was enough to fully immerse the filter paper. The filter paper was allowed to elute in the solution for approximately 1 hr. After elution, I removed the filter paper squares from the test tubes by grasping the top edge of each square with forceps. I squeezed out as much of the solvent as possible from the filter paper by pressing the forceps against the side of the test tube. The forceps were cleaned with ethanol in between samples. The samples were placed in a heated dry bath, set at 37°C, and the solvent was evaporated with nitrogen. After the solvent was completely dried off, 2 ml of Tris buffer (0.1 M Tris, 0.9% NaCl, 0.1% NaN_3, 0.1% gelatin; pH 8.4) were added to each sample tube. Each tube was then vortexed for 2 min, sealed with a rubber stopper, and refrigerated overnight before counting. The sample aliquots were counted and compared with the control aliquots of tritiated ES solution.

In test 3 (Table I) I investigated the ability of Tris buffer (pH 8.4) to elute ES, in comparison with methanol. The methanol samples were eluted as described above in 5 ml of methanol. The buffer-eluted samples did not require the evaporation and reconstitution step. Thus, the samples could be eluted in buffer, and the aliquots could be taken directly from the buffer for counting. However, this required a smaller elution volume to prevent overdilution of the sample. Consequently, additional folds of the filter paper were necessary for complete immersion of the sample. I increased the elution time in the test to 2 hr. The sample aliquots were counted and compared with the control aliquots of tritiated ES solution.

In solvent test 4 (Table I), I evaluated the ability of water, Tris buffer, and methanol to elute E1C from urine as measured by RIA. Urine samples were obtained from three noncontracepting human females. I used human urine for the initial tests to ensure judicial use of the valuable samples of orangutan urine. Each 2.5 cm $\times$ 2.5 cm piece of filter paper was aliquoted with 200 μl of urine. The samples were eluted as described above, with water following the procedure for buffer. However, after the solvent was added, the tubes were sealed with rubber stoppers, placed in a refrigerator, and eluted overnight to ensure maximum elution of the steroid. The samples were then assayed by RIA following the procedure described below.

After I determined that methanol was the most effective solvent for elution (see Results), I tested the ability of methanol to elute tritiated ES added to human (test 5) and orangutan (test 6) urine, to assess the complete methodology using a large number of samples (Table I). In these tests 200 μl of urine were aliquoted

TABLE I. Description of Tests Conducted in Developing Elution Methodology

Test no.	n	Description of test	Sample	Measurement	Solvents	Test conditions
1	12	Solvent for elution	Tritiated estrone sulfate	Tritiated ES	Methanol, ether	1 hour elution
2	6	Solvent for elution	Tritiated estrone sulfate	Tritiated ES	Methanol, ethanol, ether, acetylnitrile	1 hour elution
3	4/2	Solvent for elution	Tritiated estrone sulfate	Tritiated ES	Buffer, methanol	2 hour elution
4	9	Solvent for elution	Human urine	Estrone conjugates	Water, buffer, methanol	Overnight elution
5	36	Solvent for elution with human urine	Human urine+ tritiated estrone sulfate	Tritiated ES	Methanol	Overnight elution
6	59	Solvent for elution with orangutan urine	Orangutan urine+ tritiated estrone sulfate	Tritiated ES	Methanol	Overnight elution
7	12	Procedure for filter paper removal	Human urine	Tritiated ES	Methanol	Paper removed after drying; paper removed before drying: methanol decanted before drying
8	10	Procedure for filter paper removal	Human urine	Tritiated ES	Methanol	Paper removed after drying; paper removed before drying

onto filter paper along with recovery counts of tritiated ES. The samples were eluted overnight, reconstituted, and compared with control aliquots of tritiated ES solution as described above.

Filter Paper Removal

I also investigated alternative procedures for removing the filter paper from the eluting solvent (tests 7 and 8). The alternative procedures were to remove the filter paper after the solvent was dried off, to remove the filter paper when the top of the paper was partially exposed (avoiding putting the forceps into the solvent), to decant the solvent into a separate test tube before drying, and to remove the paper using forceps before drying (the forceps were cleaned with ethanol in between samples). For each treatment, 2.5×2.5 cm pieces of filter paper received 100 μl aliquots of tritiated ES. The filter paper samples were dried and eluted with methanol as described above. After the solvent had been dried off, 1 ml of Tris assay buffer was added to each tube and vortexed for 2 min. Sample aliquots were counted and compared with control aliquots of tritiated ES solution.

Tests of Complete Elution Methodology and RIA of E1C

After the filter paper elution method was developed, I tested the effectiveness of the complete procedure for recovering E1C from human and orangutan urine. The human urine came from three noncontracepting females. I used samples from two of the captive orangutan females after I eliminated those that had incomplete cycles or were pregnant or lactating. The urine samples from both humans and orangutans were collected daily, with occasional missed days. Using data provided on menstrual bleeding, I divided the samples into menstrual cycles. For all samples, frozen urine was thawed, and 200 μl were aliquoted onto 2.5×2.5 cm pieces of filter paper and stored in a container with silica gel. The samples were stored dry for 1 week to 1 month before they were assayed. Matched samples of urine stored by freezing, and urine dried on filter paper were analyzed as described above.

Briefly, the finalized procedure was performed as follows: Using sterilized forceps, I folded dried pieces of filter paper in half and placed them into 16×100 mm test tubes. I then added 5 ml of methanol to each test tube. The tubes were capped and refrigerated overnight to elute the sample from the filter paper. Using forceps sterilized with methanol between each sample, I squeezed the filter paper against the side of the test tube and then removed it before drying down the solvent under nitrogen. The samples were reconstituted with 2 ml of Tris assay buffer, vortexed for 2 min, and capped. The samples were then analyzed by RIA, as described below. In addition, for each assay I ran three pool samples from frozen urine and three matched pool samples from urine eluted off of filter paper. Pool samples came from human females. The mean interassay coefficient of variation (CV) was 9% for the frozen urine pools, and 15% for the filter paper pools. The mean intra-assay CV was 9.7%. The mean assay sensitivity was 2002 pg E1C/ml.

RIA Methodology

I analyzed samples for urinary E1C samples using RIA, following Shideler et al. [1983], Czekala et al. [1987, 1991], and Ellison [1988]. For samples that had been eluted in methanol, ethanol, ether, and acetylnitrile, and dried down, aliquots of 0.1 ml of sample reconstituted in 2 ml of Tris buffer (thus equivalent

to 0.01 ml of undiluted urine) were combined with 0.3 ml of Tris assay buffer. For samples eluted in water and buffer, aliquots of 0.25 ml of eluted sample (equivalent to 0.01 ml of undiluted urine) were combined with 0.15 ml of Tris assay buffer. Urine stored by freezing was thawed, and sample aliquots of 0.01 ml of urine were combined with 0.39 ml of Tris assay buffer. Antiserum that cross-reacts equally with ES and estrone glucuronide (antiestrone glucuronide, 0.1 ml, 1:4500; D. Collins, Emory University) and tritiated ES (0.1 ml, 6,500 cpm, sp. act. 55 Ci/mmol; New England Nuclear, Boston, MA) was added to the urine and standard samples (50–8,000 pg ES). The assay was incubated at 4°C overnight. Charcoal-dextran (0.3 ml, 0.625% charcoal Norite A, 0.0625% in 0.1 M phosphate buffer, pH 7.0) was used to separate bound and free steroids during a 10-min incubation at 4°C. After centrifugation for 10 min the supernatant was decanted and mixed with scintillation fluid (Packard, Meriden, CT). Each vial was counted on a beta counter for 5 min each.

Creatinine (Cr) Validation

Variation in fluid intake can have a significant effect on the concentration of steroids in urine. Thus, urinary steroid values must be indexed by an appropriate indicator of concentration. The concentration of Cr, which is a product of muscle metabolism that is released in urine at a constant rate, is the standard measure used to index urinary steroid values and measured by means of the Jaffe reaction [Taussky, 1954].

I tested the ability of filter paper to retain Cr after drying, and the ability of methanol to serve as an effective solvent, using procedures modified from those described by Czekala et al. [1987, 1991] and Shideler et al. [1983]. Shideler et al. [1995] used hole punches to sample pieces of urine-soaked filter paper disks. They found, however, that urine retention varied across the filter paper disks, which confounded their ability to measure Cr. They suggested that urine collected in a standardized way and kept dry after collection could be successfully analyzed for Cr. Thus, I aliquoted a standard amount of urine (200 μl) on pieces of filter paper and eluted the Cr from the entire piece of filter paper using the exact methanol extraction procedure described above. The same eluted sample, reconstituted in 2 ml of Tris buffer, could be used for steroid analysis and Cr analysis.

I aliquoted 0.1 μl of distilled water (blanks), Cr standards (0.01 and 0.3 mg/ml; Sigma Co.), and eluted urine samples (equivalent to 1:10 dilutions of original urine sample) onto microtiter plates. I also tested three matched pairs of urine pools in their frozen (diluted 1:10 in water) and eluted forms from filter paper. Sample and standard wells were aliquoted with 50 μl of 0.75 N NaOH and 50 μl of 0.02 N picric acid. All wells were read on a microtiter plate reader (Dynatec MR5000) after a development time of approximately 60 min. Standards and blanks were run in quadruplicate, and urine samples were run in triplicate. If one replicate value was less than half of the average optical density of the other values, it was rejected.

Storage Time

In many field studies, urine samples may have to be stored on filter paper for 1 year or more before they are brought back to the laboratory. Therefore, I tested the stability of urine stored for longer than 1 month in several experiments. E1C values obtained from human and orangutan frozen urine were compared with those obtained from matched filter paper samples that had been stored for periods

ranging between 3 months to 2 years. All filter paper samples were stored in a plastic container with silica gel, in the dark, at room temperature. Elution, RIA, and Cr measurement procedures were performed as described above.

Sample Contamination

Urine collected under field conditions may sometimes come in contact with feces from the animal, and dirt, leaves, and bark from the canopy or the ground. Therefore, I conducted an experiment to test the effect of contact between the urine and these substances in the field. A large-volume urine sample was collected from three wild orangutans and separated into five separate bottles. One bottle was left as uncontaminated urine. One bottle was intermixed with a small amount of feces from the animal, as might happen if urine were collected adjacent to where the feces dropped. The remaining bottles were intermixed with small amounts of dirt, leaves, and bark, respectively, as might happen during routine sample collection. Sample aliquots (200 μl) were put onto filter paper and dried in a container with silica gel. The samples were assayed by RIA following the procedure described above.

Statistical Analyses

In tests comparing samples dried on filter paper with matched samples of frozen urine, I calculated the measured value of E1C or Cr from filter paper as a percentage of the value determined from frozen urine. I also determined the Pearson correlation coefficients between frozen and filter paper samples, and tested for significant differences between the two types of samples using paired t-tests.

RESULTS

Determination of Solvent

The results from tests 1–3 to determine the most effective solvent are shown in Table II. These revealed that in all tests methanol was the most effective solvent for recovering the steroid from filter paper. Buffer and ethanol recovered less than half of the steroid. Ether and acetylnitrile were particularly poor at eluting the sample. Methanol elution also recovered the most conjugated steroid and produced the highest correlation between E1C values obtained through RIA of frozen urine compared to urine dried on filter paper (test 4). Tests 5 and 6, which were performed with the finalized methanol elution methodology, showed an overall ES recovery rate of $86.4\% \pm 8.4$.

Filter Paper Removal

Removing the filter paper before drying, and removing the filter paper after the top was exposed resulted in the highest recovery of tritiated steroid from the filter paper (Table III). Allowing the paper to remain in the test tube during the drying process and then removing it resulted in a recovery of less than 50%. In this case the steroid adhered to the filter paper during drying, which I determined by placing the eluted paper itself in a scintillation vial and counting it. Decanting the methanol into a separate tube and then drying the sample resulted in the lowest percentage recovery. The steroid may have remained adhered to the original test tube in this case. Based on these results, I decided to remove the paper before the drying process began. This allowed for more consistent

TABLE II. Solvent Tests*

Test no.	n	Sample	Test conditions	Water	Buffer	Methanol	Ethanol	Ether	Acetylnitrile
1	12	Tritiated ES	1 hour elution	–	–	86.6% ± 2.5	–	0.8% ± 0.1	–
2	6	Tritiated ES	1 hour elution	–	–	79.7% ± 2.6	47.5% ± 1.8	0.6% ± 0.1	14.5% ± 1
3	4/2	Tritiated ES	2 hour elution	–	41.9% ± 3.2 (n=4)	80.1% ± 5.4 (n=2)	–	–	–
4	9	Human urine	Overnight elution	43.1% ± 24.1 r=.514	28.5% ± 13.5 r=.787[a]	75.5% ± 10.6 r=.965[b]	–	–	–
5	36	Human urine + tritiated ES	Overnight elution	–	–	81.4% ± 8.5	–	–	–
6	59	Orangutan urine + tritiated ES	Overnight elution	–	–	89.5% ± 6.7	–	–	–

*Tests 1–3 show the recovery efficiency of various solvents in eluting tritiated estrone sulfate (ES) from filter paper. Values (followed by the standard deviation) are expressed as the percentage of tritiated ES applied to filter paper recovered after the procedure compared to control samples. Test 4 shows the percentage recovery of estrone conjugates measured from samples dried on filter paper compared to matched samples of frozen urine. Percent efficiencies are followed by the standard deviation with the Pearson correlation coefficients expressed below. Test 5–6 show the recovery efficiency of methanol in eluting tritiated ES from filter paper using the finalized elution methodology.

[a]$P < 0.001$.

[b]$P < 0.0001$.

treatment of samples than waiting until the top of the paper was exposed, since not all of the samples could be removed with precisely the same degree of paper exposure.

Tests of Complete Methodology for E1C and Cr

For human and orangutan urine samples dried for 1 week to 1 month, recovery of E1C eluted from filter paper ranged between 97.1–102.4% (Table IV), with a mean overall recovery of 101.3 ± 23.3 (n=86). Values from the matched frozen and filter paper samples were moderately to highly correlated. The matched samples were not significantly different ($P > 0.05$).

All Cr assays showed a highly significant correlation between Cr values from frozen urine and Cr values from filter paper (r=.959, $P < 0.0001$). The mean recovery from filter paper compared to frozen samples was 101.3%. This indicates that Cr was retained on the filter paper after the sample was dried, and that methanol was an effective solvent.

Additionally, I calculated the ng E1C/mg Cr for each sample. Figure 1 presents a cycle from one captive female, showing the matched samples and the high degree of correspondence (r=.904, $P < 0.0001$). The cycle was 28 days long, as determined by days in between menstrual bleeding. The cycle shows a smaller

TABLE III. Filter Paper Removal*

Test no.	n	Paper removed after drying	Paper removed after top exposed	Methanol decanted before drying	Paper removed before drying
8	12	42.7% ± 21.6	85.6% ± 2.5	0.4% ± 0.1	–
9	10	29.4% ± 3.0	–	–	91.9% ± 3.2

*Comparison of recovery efficiency using various procedures for removal of filter paper after elution. Results (followed by the standard deviation) are expressed as the percentage of tritiated estrone sulfate recovered after methanol evaporation and reconstitution in buffer. Methanol was used to elute all samples.

TABLE IV. Tests of Recovery Efficiency and Storage Time Using Complete Extraction and RIA Procedure With Human and Orangutan Urine Samples*

Sample	Storage time	Measurement	% Recovery	r	Paired t-test	n
New samples						
Human urine	1 Week	E1C	102.4% ± 16.6	.879[a]	$P > 0.05$	8
Human urine	1 Week	E1C	97.1% ± 27.2	.774[b]	$P > 0.05$	12
Human urine	2 Weeks	E1C	101.8% ± 17.8	.923[c]	$P > 0.05$	16
Orangutan urine	1 Month	E1C	102.0% ± 25.1	.905[c]	$P > 0.05$	50
Orangutan urine	1 Month	Creatinine	101.7% ± 21.5	.959[c]	$P > 0.05$	50
Stored samples						
Human urine	3 Months	E1C	103.1% ± 14.4	.981[c]	$P > 0.05$	8
Human urine	6 Months	E1C	101.2% ± 20.7	.802[c]	$P > 0.05$	16
Orangutan urine	2 Years	E1C	76.3% ± 31.7	.957[c]	$P < 0.05$	21
Orangutan urine	2 Years	Creatinine	86.3% ± 31.2	.520[c]	$P < 0.05$	21

*Percent recovery is the value of estrone conjugate (E1C) or creatinine determined from samples stored on filter paper compared to matched samples of frozen urine.
[a]$P < 0.005$.
[b]$P < 0.001$.
[c]$P < 0.0001$.

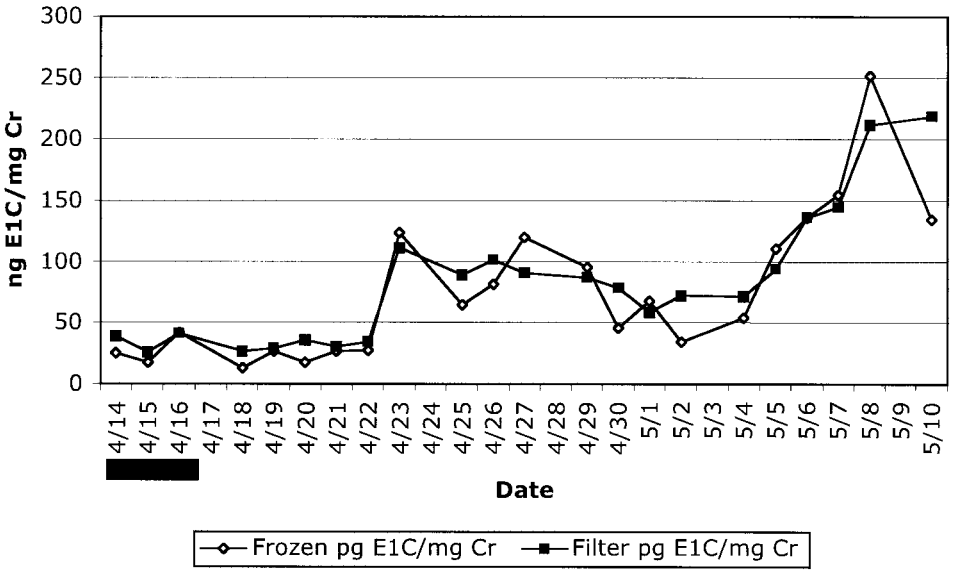

Fig. 1. Comparison of ng E1C/mg Cr values from one orangutan menstrual cycle using frozen urine and matched samples of urine dried on filter paper and eluted with methanol. Black bar indicates menstruation. The values obtained with the two methods were highly correlated (r=.904, $P<0.0001$).

rise in E1C at mid-cycle and a larger peak in E1C during the luteal phase, as is typical for orangutans [Masters & Markham, 1991].

Storage Time

Samples preserved on filter paper and stored for 3–6 months showed a highly significant correspondence with frozen urine, and no degradation in absolute recovery for both E1C and Cr (Table IV). However, samples that were stored for 2 years showed a significant loss of approximately 24% for E1C and 14% for Cr. The measured values were still significantly correlated after 2 years.

Sample Contamination

Uncontaminated urine and urine intermixed with contaminants were highly correlated (feces: r=0.983; dirt: r=0.987; leaves: r=0.999; bark: r=1.000, all $P<0.0001$). A comparison between matched samples of contaminated and uncontaminated urine showed no significant differences between samples (paired t-test, $P>0.05$, n=12).

DISCUSSION

On the basis of these experiments, I concluded that methanol is the best solvent to use for filter paper elution. Methanol is a strong solvent that is also effective for extracting fecal steroids [Brockman & Whitten, 1996; Brockman et al., 1995; Heistermann et al., 1995; Shideler et al., 1993; Staviksy, et al. 1995]. Methanol elution is also advantageous because it is possible to dry off the solvent and reconstitute the steroid at the desired concentration for the assay. Water and

buffer were not highly correlated with the matched frozen samples in this study, possibly because I had to fold the filter paper multiple times to achieve complete immersion in the smaller amounts of solvent that were required. Buffer has been found to be an effective solvent in enzyme assays that use hole-punched samples (Lipson, personal communication). Thus, researchers developing other assays could also investigate buffer and/or water as potential solvents. Further, a solution combining buffer or water and methanol could also be tested.

Shideler et al. [1995] found that samples that had been soaked, dried, and stored on filter paper for 1 year produced hormone concentrations that were quantitatively and qualitatively similar to results obtained from samples that were newly placed on filter paper. After 5 years of storage, the hormonal results were qualitatively similar, but quantitatively they were 10–50% lower than those originally obtained. In the current study I found that both E1C and Cr remained significantly correlated after 2 years of storage on filter paper. However, because of the drop in absolute recovery that occurs after 2 years, samples should be frozen after they are brought back from the field, and assays should be run as soon as it is convenient. Samples assayed after a period of 2 years are still highly correlated with frozen urine, and thus allow for *relative* comparisons to be made within a given time period.

Shideler et al. [1995] were not able to validate the Cr assay because of variation in absorption of urine across the filter paper when samples were soaked in urine. They recommended aliquoting a known amount of urine instead. This was done in the current study, and the results demonstrate that Cr can consistently be recovered from filter paper. Thus, I recommend the allocation of a standard amount of urine for future studies using this method. After 2 years I found a drop in the correlation between Cr samples on filter paper compared to frozen urine, although the correlations were still highly significant. I thus recommend assaying samples first for Cr after they are returned from the field. If identical filter paper aliquots are made, as in this study, these Cr values can be used for subsequent steroid assays of replicate samples. Additional storage time tests of Cr on filter paper are also warranted.

Because of differences in methodologies and extraction techniques, the absolute values of samples eluted from filter paper should not be directly compared with those obtained from samples preserved in other ways, such as freezing. Instead, relative comparisons of samples preserved using the same methodology are indicated. The percentage recovery did vary among samples, and thus I would caution against overinterpretation of individual samples.

The sample orangutan cycle shown in Fig. 1 shows that analysis of steroids from filter paper is sufficiently robust to allow for the identification of biologically meaningful phenomena. The two peaks in E1C, and the relatively higher luteal phase peak are revealed by both freezing and the current method. This pattern is similar to that reported for orangutans by Masters and Markham [1991], and provides another orangutan cycle to add to the small number of published cycles.

Since I found that filter paper could be used successfully as a preservative, I have employed this method for over 10 years in my study of wild orangutans at Gunung Palung National Park. One piece of filter paper is sufficient for analyzing Cr and at least one steroid, depending on the concentration needed for the assay. I recommend making five to 10 sample replicates, if the sample is sufficient, to allow for multiple assays. I have also successfully used this method for RIA of testosterone, and enzyme immunoassay of E1C and PDG (Knott, unpublished data). The method should also work well with cortisol. Alternatives to preserving

steroids and Cr on filter paper could also be explored. For example, Brown et al. [1995] stored urine in liquid form by adding sodium azide and ethanol as preservatives. However, Kesner et al. [1995] found that sodium azide did not prevent a significant decrease in the activity of estrone 3-glucuronide or Cr when samples were stored at 25°C or 37°C for 2 weeks. I also stored replicate samples under these conditions in a subsequent study (to be reported elsewhere). If it is feasible, freezing urine is still the preferred methodology; however, as shown here, filter paper storage is an acceptable alternative.

These sets of experiments demonstrate that the desiccation of urine onto filter paper and elution using methanol is an effective method for preserving primate urine samples for later hormonal analysis. This method can be applied to any species in which urine can be readily obtained from individual animals. The collection and assay protocols may have to be modified depending on local conditions and the species involved. This method for noninvasively collecting urine from free-ranging orangutans and preserving urine without freezing provides an effective way to monitor primate reproductive physiology in the wild. It is particularly valuable under field conditions where storage and transport of frozen urine is not feasible.

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