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RESEARCH ARTICLES

Urinary Steroid Evaluations to Monitor Ovarian Function in Exotic Ungulates: VII. Urinary Progesterone Metabolites in the Equidae Assessed by Immunoassay

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A direct enzyme immunoassay (EIA) for non-specific urinary progesterone (Po) metabolites, utilizing a non-specific monoclonal antibody against pregnanediol-3-glucuronide, was evaluated for the purpose of assessing luteal function in equids. Urinary pregnanediol-3-glucuronide (PdG) and immunoreactive PdG-like conjugate (iPdG) concentrations, indexed by creatinine, were compared to plasma Po concentrations in non-conceptive ovarian cycles through two ovulations in four mares. High-performance liquid chromatography (HPLC) of urine from luteal-phase mares and a pregnant zebra revealed an absence of significant concentrations of PdG and the presence of at least three immunoreactive compounds, all of which were more polar than PdG. The concentration of iPdG in the mare ranged from a nadir of approximately 3 ng/mg Cr at the time of ovulation to nearly 400 ng/mg Cr at the mid-luteal-phase peak and paralleled plasma Po concentrations. This non-radiometric assay for iPdG permits the assessment of ovulation, luteal formation and function, and luteolysis in unprocessed urine samples from domestic mares. Data from a single zebra indicate this approach also will permit simplified and non-invasive longitudinal studies of ovarian function among a wide range of Equidae.

Key words: Perissodactyla, ovulation, corpus luteum

INTRODUCTION

The assessment of luteal function in the cycling or pregnant mammal, reflected by circulating progesterone (Po), is necessary in order to understand ovarian dynam-

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ics and accurately manage female reproduction. Longitudinal studies of plasma Po require daily or frequent blood sampling, which is often a difficult or dangerous procedure in certain exotic species. An alternative approach is to monitor ovarian function through the measurement of urinary Po metabolite concentrations. Loskutoff et al. [1983] demonstrated the ability to measure luteal function in a wide variety of mammalian species through urinary pregnanediol-3-glucuronide (PdG) measurements. Whereas urinary progesterone metabolites in hominoid species [Lasley et al., 1982; Mitchell et al., 1982a,b] and some ungulate species [Loskutoff et al., 1986] were accurately assessed by this method, specific PdG measurements were insufficient to accurately monitor luteal function in the Perissodactyla [Loskutoff et al., 1983] and old world monkeys [Liskowski and Wolfe, 1972]. This problem was partially resolved by Shideler et al. [1985] when a variation of the PdG assay was developed. This assay employed an antiserum with significant cross-reactivity with 20∂-hydroxyprogesterone (hydroxypregn-4-ene-3-one, 20∂) (20-OHP) and was used successfully to characterize luteal function in the macaque [Shideler et al., 1985] and later the whale [Walker et al., 1988]. It seems clear that the failure of a single urinary progesterone metabolite assay to provide useful information in all species relates to the species-specific differences in progesterone metabolism, but these differences in steroid catabolism have not been systematically addressed.

The objective of this study was to demonstrate the utility of a modified iPdG assay to detect progesterone metabolites in the Perissodactyla, and specifically the Equidae. The horse was chosen for a model because of the requirement for matched plasma and urine samples. Furthermore, this report presents an assay which employs a monoclonal antibody in a non-radiometric format. These characteristics allow a broad application of the method both within and without laboratory facilities.

MATERIALS AND METHODS

Daily urine samples were collected from four domestic standard-bred mares during estrous cycles between June and August. Each of the four cycling mares was monitored and samples were collected through two ovulations, the first of which was induced by $PGF_{2\alpha}$ (10 mg, Lutalyse; Upjohn Co.). Urine samples were collected daily by means of a Mayo rubber catheter, between 0800 and 1000 hours, frozen without preservatives, and stored at −20°C until assay. Blood samples were collected by jugular venipuncture at the same time of day; plasma was separated and stored at −20°C until assay. The day of ovulation was determined by rectal palpation. All urine samples were diluted 1:100 in dH_2O and analyzed for creatinine (Cr) concentrations by the microcolorimetric method of Taussky [1954], in order to account for differences in urine concentration and to normalize urinary hormone values.

Plasma progesterone concentrations were measured by enzyme immunoassay (EIA) described previously by Munro and Stabenfeldt [1984]. The inter- and intraassay coefficients of variation were 10.5% and 4.9%, respectively. Urinary PdG immunoreactivity was determined by EIA as described by Munro et al. [1989]. The interassay (n = 17) and intraassay (n = 7) coefficients of variation were 17.77% and 10.6%, respectively.

Non-specific urinary Po metabolites were measured in 1:1 dilutions in dH_2O by means of an EIA based on a monoclonal antibody against pregnanediol-3-glucuronide (R. T. Chatterton, Northwestern University; 1:1,000) and a PdG standard.

This assay has been described previously by Shideler et al. (in press). Significant cross-reactivity with steroids includes 20-OHP (100%), PdG (164%), 20β-hydroxy-4-pregnen-3-one 3-oxime (41%), 20β-hydroxy-5β pregnan-3-one (10 %), 5β-pregnane-3β,20β-diol (4 %), and Po (2%). Cross-reactivity with all other steroids tested, including androsterone, 17β-estradiol, and cortisol, was less than 0.1% (R.T. Chatterton, personal communication). Interassay (n = 12) and intraassay (n = 7) coefficients of variation were 11.46% and 10.04% respectively. Urine was evaluated for parallelism to the standard curve as described by Shideler et al. [1983] by assaying serial halving dilutions ranging from neat to 1:16.

In order to validate the urinary iPdG assay for the mare, mid-luteal-phase urine samples from two different mares were subjected to high-performance liquid chromatography (HPLC) as described by Shideler et al. [1983]; 1 ml samples were extracted twice with 3.5 ml of diethyl ether in order to remove free steroids; 1 ml of MeOH:EtOH (1:1) was added to the urine; the mixture was vortexed, cooled to −5°C, and centrifuged at 2,500 rpm for 10 min in order to remove salts. Approximately 1,000 cpm of ^{3}H-PdG (20–40 Ci/mmole; Courtald Institute, London, UK) was added to the supernatant, which was reduced to approximately 1.0 ml volume under N_2; 25 µl was injected onto a 15 cm HPLC column (ALTEX, Ultrasphere-ODS, dp 5 µm). One milliliter fractions were collected during an MeOH/H_2O gradient (10 to 65 % MeOH over 40 min). From each fraction, 200 µl was mixed with 5.0 ml of scintillation cocktail (Ready Protein, Beckman) to monitor PdG recovery. The remaining 800 µl was evaporated and the residue dissolved in 100 µl of dH_2O and assayed for PdG and iPdG as described above. The inter- and intraassay coefficients of variation were 13% (N = 10) and 10% (N = 15) respectively. One milliliter urine samples from a North American bison (*Bison bison*) and a non-conceptive human, species with well-established and significant urinary PdG concentrations, and a Hartman's zebra (*Equus zebra hartmannae*) approximately 1 month pregnant, were subjected to HPLC as described, and compared to the progesterone metabolite HPLC profile of the mare.

Descriptive statistics and regression analysis were applied to the data by using Statworks© statistical program on a MacIntosh Plus computer.

RESULTS

The serial halving dilutions for four mares revealed parallelism to the standard curve ($r = 0.893$). High-performance liquid chromatography of urine for two luteal-phase mares confirmed the absence of significant concentrations of urinary PdG and the presence of three major peaks which demonstrated immunoreactivity with the iPdG antibody, all of which preceded eluates containing ^{3}H-PdG. Figure 1 illustrates the typical pattern of immunoreactive eluates for one of the two mares. Cochromatographic profiles obtained by assaying HPLC eluates of luteal-phase mare urine for immunoreactivity by specific PdG and iPdG assays were consistent with the immunoreactivity patterns seen in unprocessed urine, in which PdG accounted for only 8% of the iPdG during the times of high serum progesterone. In contrast to the HPLC profile of mare urine, bison and human urines (Figs. 2 and 3 respectively) revealed PdG peaks with both the PdG and iPdG assays. These data demonstrate the ability of both assays to detect PdG, and thereby substantiate the absence of significant PdG in the horse and the presence of relatively large concentrations of other Po metabolites

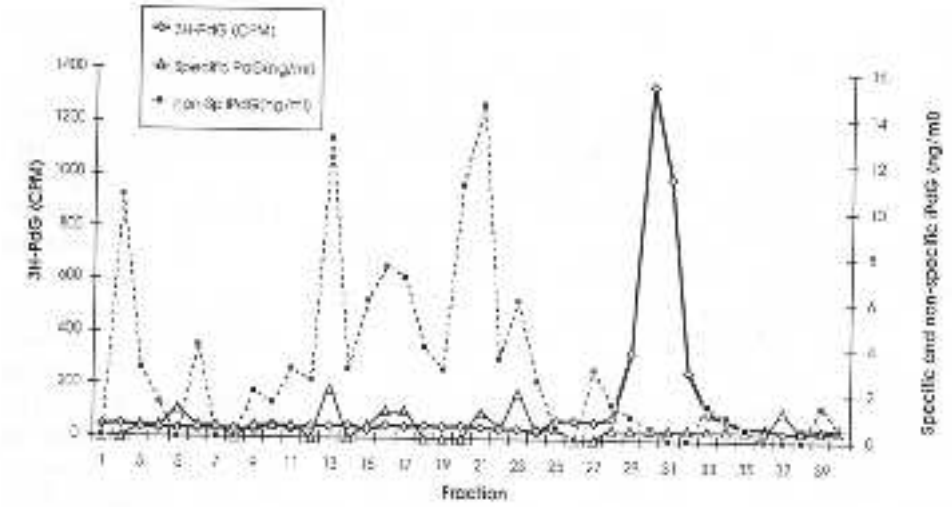

Fig. 1. HPLC elution profile of radiolabelled standard (^{3}H-PdG) and urinary-specific PdG and non-specific iPdG for a luteal-phase mare.

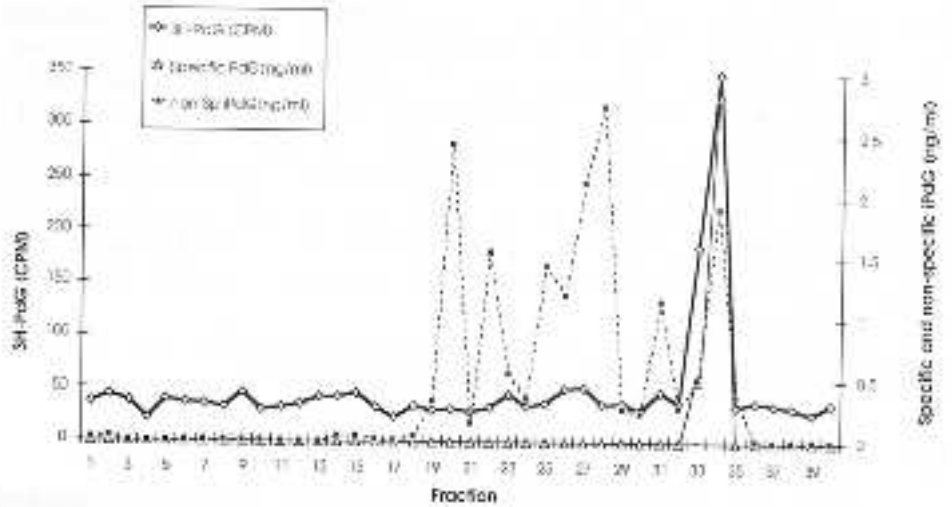

Fig. 2. HPLC elution profile of radiolabelled standard (^{3}H-PdG) and urinary-specific and non-specific iPdG in a pregnant bison (*Bison bison*).

which are detected collectively by the less specific assay used in this study. Similarly, the zebra revealed no measurable PdG and at least one major immunoreactive peak preceding ^{3}H-PdG.

Plasma progesterone, urinary PdG, and iPdG metabolites are shown for representative ovarian cycles of two of the mares in Figure 4a,b. Urinary PdG concentrations were minimal and constant throughout the cycles, averaging 7.04 ng/mg Cr. ± 0.686 (SEM) for four mares not shown and did not reflect luteal regression, whereas plasma Po decreased following $PGF_{2\alpha}$ administration. In contrast, the less specific antibody (iPdG) revealed parallelism between plasma Po and urinary Po metabolites

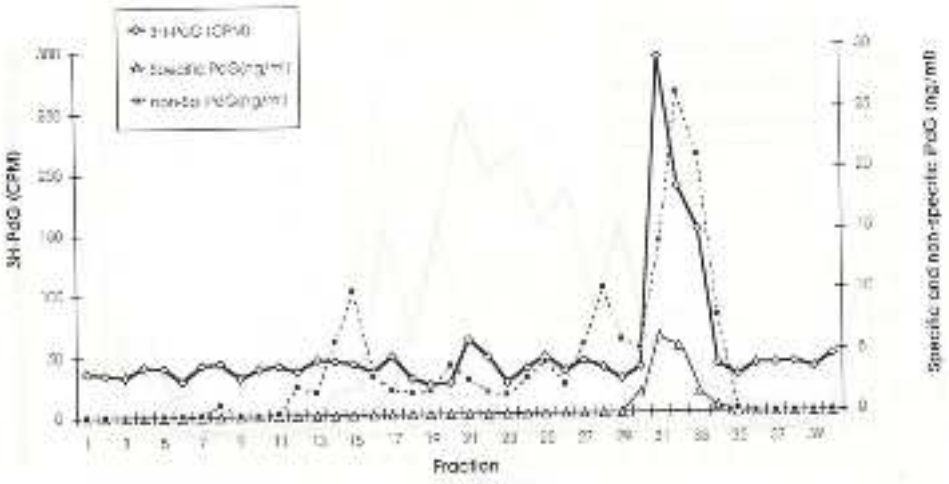

Fig. 3. HPLC elution profile of radiolabelled standard (^{3}H-PdG) and urinary specific and non-specific iPdG in the luteal-phase human.

and provided a clear picture of ovarian activity through two ovulations, substantiating the source of the non-specific urinary Po metabolites as luteal Po. Nadirs obtained by iPdG measurements occurred within 24 hours following ovulation in the two mares represented in Figure 4a,b and in all four mares monitored. Fluctuations of plasma Po during the luteal phase were also accurately reflected by these same metabolite measurements. The values for the immunoreactive non-specific Po metabolites for all four mares ranged from a low of 3.6 ng/mg Cr at the time of ovulation to a high of 390.3 ng/mg Cr at the mid-luteal Po peak. Without accounting for a 24-48 hour delay in the excretion of the Po metabolites, the correlation coefficient for plasma Po and urinary non-specific Po metabolites for the four mares collectively was $r = 0.91$.

DISCUSSION

This study provides the first detailed description of luteal-phase urinary progesterone metabolites in equids and the direct measurement of urinary iPdG which reflects changing concentrations of plasma progesterone throughout the ovarian cycle of the mare. Although urinary PdG has been used successfully to evaluate luteal and placental activity in a variety of other species, the concentrations in the Equidae are too low to be useful for these purposes. Consequently, the description of luteal function in the horse and other equids has been entirely reliant upon plasma Po measurements. Conversely, the relatively high concentrations of progesterone metabolites in the Equidae which react with the less specific iPdG antibody point to significant differences in the metabolic end-products of progesterone from species to species. Loskutoff et al. [1983] showed examples of these species differences by demonstrating high concentrations of urinary PdG in the domestic cow and gorilla (*Gorilla gorilla*), intermediate concentrations in the bongo (*Taurotragus eurycerus*) and oryx (*Oryx tao*), and very low concentrations in the Hartman's zebra. The lion-tailed macaque (*Macaca silenus*) is a species with very low concentrations of

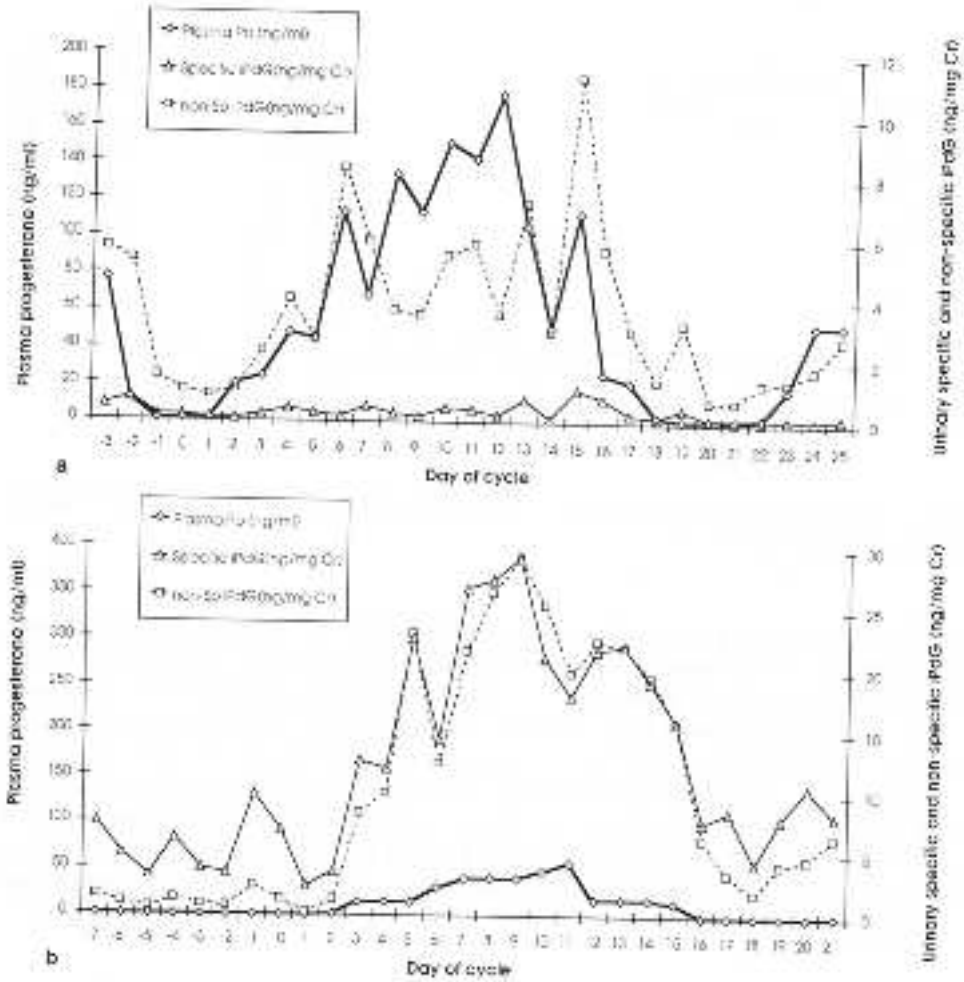

Fig. 4. Plasma progesterone and urinary-specific and non-specific PdG concentrations in two representative cycling mares. Day 0 represents the day of ovulation, verified by rectal palpation.

urinary PdG, but Shideler et al. [1985] used a modified PdG RIA which reacted with 20∂-hydroxyprogesterone (20-OHP) to demonstrate luteal function in this species. In the case of the killer whale (*Orcinus orca*) both specific (PdG) and non-specific (20-OH) PdG assays were capable of evaluating luteal function or placental activity; however, the less specific assay provided more resolution [Walker et al., 1988]. Despite the fact that progesterone is a highly conserved steroid among animals, including the reptiles and birds, it is the metabolic fate of this steroid which reveals species diversity.

Care must be exercised in evaluating non-specific progesterone metabolites because of differences in antiserum cross-reactivity. Thus far, two different antibodies have been used for non-specific urinary Po metabolite evaluation. The antibody utilized in this study was not the same as the one used in either the macaque study

[Shideler et al., 1985] or the whale study [Walker et al., 1988]. HPLC analysis revealed significant immunoreactivity of the iPdG antibody with at least three compounds more polar than PdG. The precise characterization of these molecules was not attempted in this study, but, because they collectively correspond with plasma concentrations of Po during the ovulatory cycle of the mare, their origin as ovarian Po is not in doubt. Nevertheless, the assessment of urinary Po metabolites in any species must be carried out systematically in order to match the appropriate assay with the species-specific metabolite.

Urinary estrone conjugates (E_1C) have previously been measured in a wide variety of species [Czekala et al., 1986; Monfort et al., 1986, 1987; Walker et al., 1988], including the Indian rhinoceros (*Rhinoceros unicornis*) [Kassam and Lasley, 1981] and the domestic horse [Ammon et al., submitted], in order to assess follicular and placental activity. Coupled with the use of these urinary E_1C measurements, the application of the iPdG assay to equids makes it possible to evaluate ovarian dynamics non-invasively across the entire ovulatory cycle. Testing the application of this assay to other species of the Perissodactyla is a next step in the study of reproductive function in captive exotic ungulates. Beyond the potential logical application of this assay to zoo animals, the ability to extract urine from the soil and to measure steroid conjugates without interference [Kirkpatrick et al., 1988] permits a more precise evaluation of ovarian dynamics in free-ranging wildlife as well. Finally, the non-isotopic nature and stability of reagents used in this study lend themselves to the development of non-laboratory, semi-quantitative field kits for urinary steroid metabolite assessment.

CONCLUSIONS

1. Species-specific differences in the metabolism of Po produce an undetermined number of urinary Po metabolites among mammals.
2. The horse and the zebra do not produce significant quantities of urinary PdG during the ovulatory cycle or pregnancy, but both species do excrete several other immunoreactive Po metabolites in the urine.
3. These equine urinary Po metabolites can be measured and quantified by means of an EIA utilizing the non-specific Chatterton monoclonal 1284-1 anti-pregnanediol glucuronide antibody (iPdG).
4. Equine non-specific urinary Po metabolites correlate significantly with, and accurately reflect, luteal-phase plasma progesterone concentrations.
5. Equine urinary non-specific Po metabolites range in concentration from approximately 3.0 ng/mg Cr at the time of ovulation to 400 ng/mg Cr during the mid-luteal-phase Po peak, and can be used presumptively or retrospectively to confirm ovulation.
6. Coupled with the measurement of urinary estrone conjugates, the assay of urinary non-specific Po metabolites permits a broad assessment of equine ovarian dynamics, including folliculogenesis, ovulation, luteal formation, and luteolysis.

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