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TECHNICAL REPORT

Measurement of Fecal Steroids in the Black Rhinoceros (*Diceros bicornis*) Using Group-Specific Enzyme Immunoassays for 20-Oxo-Pregnanes

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The metabolism and excretion of progesterone in different animal species results in several fecal 5-reduced progesterone metabolites (pregnanes), which in recent studies were quantified using progesterone antibodies. To increase the accuracy of fecal 20-oxo-pregnane evaluations in the black rhinoceros, enzyme immunoassays (EIA) using antibodies against 5α-pregnane-3β-ol-20-one 3HS:BSA (5α-20-one EIA) and 5β-pregnane-3α-ol-20-one 3HS:BSA (5β-20-one EIA) were developed. The assays showed high crossreactivities with pregnanes containing a 20-oxo group and are referred to as group-specific; results of these assays were compared with an EIA using an antibody against 6HS-progesterone (4-ene-20-one EIA). Fecal samples of both subspecies of the black rhinoceros (*Diceros bicornis michaeli*, n = 5, and *Diceros bicornis minor*, n = 1) during pregnancy were collected 1–3 times/week. HPLC separation showed three major immunoreactive fecal 20-oxo-pregnane peaks; their elution profiles and different crossreactivities in the three EIAs provided strong evidence that these peaks are 5α-pregnane-3,20-dione, 5α-pregnane-3α-ol-20-one, and 5α-pregnane-3β-ol-20-one. Pregnane values in the pregnant animals continuously increased between months 3–7 and were significantly ($P < 0.01$) elevated above the levels of nonpregnant animals (0.2 μg/g) by week 11. During months 6–13 concentrations in the 5α-20-one and in the 5β-20-one EIA (5–11 μg/g) were significantly ($P < 0.01$) higher than in the 4-ene-20-one EIA (1.5–3 μg/g). In conclusion, the immunoreactive fecal 20-oxo-pregnane metabolites in the black rhinoceros are determined more accurately with antibodies against pregnane-20-one-C3 conjugates, as compared with a progesterone antibody. © 1996 Wiley-Liss, Inc.

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INTRODUCTION

The two subspecies of African black rhinoceros, the eastern (*Diceros bicornis michaeli*) and the southern (*Diceros bicornis minor*), are critically endangered due to continued poaching. The African population was reduced to less than 2,000 animals by the end of 1992 [Klös and Frese, 1993]. To save the remaining black rhinoceros population the current conservation strategies consist of conservation breeding in zoos [Klös and Frese, 1993], strengthening of antipoaching measures, and translocation to sanctuaries in Africa [Kock et al., 1995].

Successful breeding management may benefit from reliable reproductive monitoring. Reproductive data of rhinoceroses in recent studies were gained from the analysis of steroids in blood [Kock et al., 1991; Berkeley, 1994]; saliva [Kuckelkorn and Dathe, 1990; Czekala et al., in press]; urine [Kassam and Lasley, 1981; Kasman et al., 1986; Wagner, 1986; Ramsay et al., 1987, 1994; Hodges and Green, 1989; Hindle et al., 1992]; and feces [Schwarzenberger et al., 1993a, 1994, 1995b; Berkeley, 1994]. Furthermore, the metabolism of radioactively labeled steroid hormones was studied in a white rhinoceros [Hindle and Hodges, 1990], and ultrasonographic imaging was used to investigate the reproductive tract of rhinoceroses [Adams et al., 1991; Schaffer et al., 1994].

The most appropriate techniques for reproductive monitoring in nondomestic animals are the analysis of noninvasively collected fecal and urine samples. Recent investigations of fecal immunoreactive progesterone metabolites in several animal species used progesterone antibodies [Desaulniers et al., 1989; Messier et al., 1990; Wasser et al., 1991, 1994; Graham et al., 1993; Kirkpatrick et al., 1993; Monfort et al., 1993; Möstl et al., 1993; Schwarzenberger et al., 1993a, 1995a; Berkeley, 1994; Brown et al., 1994; Czekala et al., 1994; Larter et al., 1994]. Resulting in useful biological data, most of these studies did not characterize the immunoreactive material by any method other than RIA. However, metabolism and fecal excretion of radioactively labeled progesterone in feline, primate, and farm animal species resulted in several progesterone metabolites [Shideler et al., 1993; Brown et al., 1994; Wasser et al., 1994; Palme, personal communication]. In addition, HPLC separation with subsequent immunoassay analysis displayed the presence of up to eight well defined immunoreactive fecal progesterone metabolites [Kirkpatrick et al., 1991; Schwarzenberger et al., 1991, 1992, 1993a,b, 1995a; Heistermann et al., 1993; Möstl et al., 1993; Shideler et al., 1993; Brown et al., 1994; Wasser et al., 1994]. These metabolites showed elution patterns similar to that of pregnanes, and "authentic" progesterone was either not detectable or detectable to a limited extent only.

Progesterone is metabolized to pregnanes before its fecal excretion. Some of these pregnanes (namely 5α- and 5β-pregnane-3,20-dione) still posses two oxo groups in position C3 and C20 of the steroid molecule, and therefore these metabolites show crossreactivities with progesterone antibodies. However, depending on crossreactivities, specific progesterone antibodies quantify only a small proportion of the actual amount of fecal pregnane compounds. A possibility for determining these metabolites more accurately is the application of antibodies against pregnane-C3 conjugates. Since the functional group of the steroid in position C3 is masked, these

antibodies show considerable crossreactivities with several progesterone metabolites sharing a similar C20 configuration (a 20-oxo, a 20α-OH, or a 20β-OH group) and their measurement is combined in one assay, which we refer to as group-specific.

In our recent study on the black rhinoceros we (1) determined the estrous cycle length as 25 days; (2) diagnosed pregnancy by means of increasing fecal immunoreactive progesterone metabolites after the first quarter of gestation; and (3) demonstrated the presence of immunoreactive 20-oxo- and 20α-OH-pregnane compounds [Schwarzenberger et al., 1993a]. However, lacking specific enzyme immunoassays (EIA) for 20-oxo-pregnanes, we relied on the crossreactivities of an antibody against 6HS-progesterone, and assay specificity and accuracy were a serious concern. Therefore we raised antibodies against 5α- and 5β-pregnanes containing a 20-oxo group and developed EIAs.

The aims of the present study were: (1) to develop specific assays for pregnanes containing a 20-oxo group (20-oxo-pregnanes); (2) to compare EIA measurements of fecal 20-oxo-pregnanes in three EIAs with different degrees of specificity; (3) to characterize the possible structure of the fecal immunoreactive 20-oxo-pregnanes; and (4) to determine fecal 20-oxo-pregnane values in both subspecies of the black rhinoceros.

METHODS

Animals and Sample Collection

Female black rhinoceroses of the eastern (*Diceros bicornis michaeli*) and the southern (*Diceros bicornis minor*) subspecies were studied. Animals of the eastern subspecies (n = 5) were housed at Dvůr Kralové Zoo. At the times of parturitions reported, the animals were 10, 15, 16, and 23 years old and had (including deliveries reported in this study) 2, 3, 4, and 1 offspring, respectively. One 24-year-old animal had a late pregnancy abortion after 14 months of gestation; this was her fifth pregnancy and her previous pregnancies delivered viable calves. The rhino (n = 1) of the southern subspecies was housed at Frankfurt Zoo; she was seven years old and the report in this study was her first pregnancy. Each female was allowed access to a male during the daytime and housed alone at night. Estrous behavior and mating were recorded by the keeper staff. Animals were fed hay and grass, supplemented with leaves, branches, fruits, and vegetables, and had free access to water.

Morning fecal samples were collected off the ground and stored at −20°C until analysis. Samples were collected 2–3 times/week for the first 4 months of pregnancy and the month before parturition. Samples during midgestation were collected once a week or biweekly. Samples from nonpregnant animals were collected three times/week.

Fecal Extraction for Analysis With EIAs

Fecal samples (0.5 g) were extracted with methanol as described previously [Schwarzenberger et al., 1993a], except 1.0 g powdered aluminum oxide was added before extraction. Briefly, 0.5 g feces was mixed with 1.0 g powdered aluminium oxide, extracted with 4.5 ml aqueous methanol (80%), and defatted with petroleum benzene. The methanol was diluted and analyzed with the EIAs. Aluminium oxide was added to remove visible background pigment [Graham et al., 1993] and in an

TABLE 1. Enzyme immunoassays (EIA)

EIA	4-ene-20-one[a]	5α-20-one	5β-20-one
Immunogens	4-pregnene-6α-ol-20-one-6HS:BSA	5α-pregnane-3β-ol-20-one-3HS:BSA	5β-pregnane-3α-ol-20-one-3HS:BSA
Labels	5α-pregnane-3β-ol-20-one-3HS:DADOO-B[b]	4-pregnene-3,20-dione-3CMO:DADOO-B[b]	5α-pregnane-3β-ol-20-one-3HS:DADOO-B[b]
Antibody titers	$1{:}40 \times 10^3$	$1{:}15 \times 10^3$	$1{:}15 \times 10^3$
Label titers	$1{:}250 \times 10^3$	$1{:}600 \times 10^3$	$1{:}600 \times 10^3$
Standard curve range	2–500 pg	2–500 pg	5–1,250 pg
Sensitivity (90% binding)	1.5 ± 0.12 pg	2.6 ± 0.33 pg	5.7 ± 0.37 pg
Intrassay coefficient of variation	10.4%	11.3%	9.6%
Interassay coefficient of variation	12.8%	16.2%	14.6%

[a]Assay described by Schwarzenberger et al. [1993].
[b]DADOO-B = biotin-DADOO = N-biotinyl-1,8-diamino-3,6-dioxaoctan.

initial study was found to increase differences between low and high 20-oxo-pregnane values in all three assays used.

Analysis With EIAs

Fecal extracts were analyzed with two newly developed EIAs (Tables 1,2) and the data compared with that using the 4-ene-20-one EIA described in our previous study [Schwarzenberger et al., 1993a]. The antibodies for the three EIAs were raised in rabbits. Progesterone was used as standard, and serial dilutions of fecal extracts gave displacement curves parallel to that of the standard curve in each assay.

The three assays followed the protocol described by Schwarzenberger et al. [1991, 1993a], except that steroid-DADOO-biotin conjugates (DADOO-biotin = N-biotinyl-1,8-diamino-3,6-dioxaoctane) were used as labels [Palme and Möstl, 1993; Pryce et al., 1994]. Briefly, the three EIAs used a double-antibody technique and were performed on anti-rabbit-IgG-coated microtiter plates. After overnight incubation of standard or samples, steroid antibody, and biotinylated label, the plates were emptied, washed, and blotted dry, before a streptavidin horseradish peroxidase conjugate was added. After a 45 min incubation, plates were emptied, washed, and blotted dry. The substrate (tetramethylbenzidine) was added and incubated for 45 min at 4°C before the enzymatic reaction was stopped with 2 mol/l sulfuric acid.

The synthesis of the steroid-DADOO-biotin conjugates followed the mixed anhydride reaction [Kellie et al., 1975]. Steroid:HS or steroid:CMO derivatives (5–10 mg) were dissolved in 1 ml dimethylformamide, and cooled to −18°C under constant stirring. Then 0.01 ml methylmorpholin and 0.01 ml isobutylchloroformiate were added and incubated at −18°C for 30 min. Thereafter, 1.5 ml of a mixture of 0.1 mol/l sodium phosphate buffer (pH 8.2) and dimethylformamide (1/2 v/v; −18°C), containing 5 mg biotinyl-DADOO, was slowly added under constant readjustment of the pH, using 1 mol/l KOH. The mixture was incubated at room temperature for 3 hr, under stirring and light protection. Then 7.5 ml of water was added and the solution was passed through a primed C18 cartridge. The cartridge was washed with 5 ml water, and the steroid and steroid conjugates were eluted with 4 ml methanol. The methanol was evaporated (40°C) under nitrogen and the residue dis-

TABLE 2. Crossreactivities (%) of steroids in the different enzyme immunoassays

Steroid	4-ene-20-one	5α-20-one	5β-20-one
4-pregnen-			
3,20 dione (progesterone)	100	100	100
3β-ol-20-one	26	14	68
3α-ol-20-one	8	390	20
20α-ol-3-one	<0.1	<0.1	<0.1
20β-ol-3-one	<0.1	<0.1	<0.1
5α-pregnane-			
3α-ol-20-one	5	89	8
3β-ol-20-one	18	56	102
3,20-dione	40	168	75
3α,20α-diol	<0.1	<0.1	<0.1
3α,20β-diol	<0.1	<0.1	<0.1
5β-pregnane-			
3α-ol-20-one	3	88	20
3β-ol-20-one	7	5	36
3,20-dione	71	26	151
3α,20α-diol	<0.1	<0.1	<0.1
3α,20β-diol	<0.1	<0.1	<0.1
5-pregnen-			
3β-ol-20-one	29	17	11
Cortisol	<0.1	<0.1	<0.1
Estrone	<0.1	<0.1	<0.1

solved in 2 ml of 20% methanol. The solution was injected onto a preparative RP18 chromatography system (310 × 25 mm, flow rate 1 ml/min). Separation of the steroid-biotin-DADOO conjugate from the free steroids was achieved using a methanol gradient, linearly increasing from 20 to 100% between fractions 0–70. Fraction size was 5 ml, and the steroid-biotin-DADOO conjugate eluted between fractions 65–70.

High Performance Liquid Chromatography (HPLC) of Fecal 20-oxo-pregnanes

The number, their relative proportions, and the polarity of the unconjugated fecal immunoreactive 20-oxo-pregnanes were determined by HPLC and subsequent analysis with the three respective EIAs [Schwarzenberger et al., 1991, 1993a]. Samples from both subspecies during different stages of pregnancy were used. Samples were mixed with ^{3}[H]-progesterone and ^{3}[H]-20α-dihydroprogesterone, and extracted with methanol. The methanol was evaporated, the residue reconstituted in 1 ml of 20 mmol $NaHCO_3$, and twice re-extracted with 5 ml of petroleum ether/diethylether (6/4 v/v). The ether was evaporated and the samples reconstituted in chloroform/n-hexane. Removal of polar substances was compatible with our straight phase HPLC system (Si 60, 10 μm; 250 × 4 mm; flow rate 2 ml/min); however, it is possible that conjugated steroids were removed.

The HPLC separation followed the principles described previously [Schwarzenberger et al., 1991, 1993a], but the elution time between ^{3}[H]-progesterone and ^{3}[H]-20α-dihydroprogesterone was increased by using chloroform/n-hexane (v/v 75/25) and a linear methanol gradient (0–6%). Retention times of the immunoreactive

fractions were compared with those of different crossreacting steroid standards (5α/β-pregnanes) and with ^{3}[H]-progesterone and ^{3}[H]-20α-dihydroprogesterone. Elution patterns of pregnane standards were determined by EIAs, and ^{3}[H]-steroids were determined by liquid scintillation counting.

Data Analysis

Data are presented as mean ± SEM. A total of 216 samples from nonpregnant animals (n = 5) during the estrous cycle was used for comparison with those of pregnant animals. The duration of pregnancy was calculated as the period between observed mating and parturition. Samples from the first 13 months and the last 2 months of pregnancy were standardized to the day of mating and to the day of parturition, respectively. Results were grouped in weekly intervals during gestational weeks 1–17 and 61–65, and in monthly intervals between these periods. Means calculated represent 5–18 samples per point. Statistical significance of the differences between pregnant and nonpregnant animals was tested by the Student's t test and the Mann–Whitney U test. Differences among the EIAs during pregnancy were analyzed by a paired t test and the Wilcoxon's signed-rank test.

RESULTS

Enzyme Immunoassays (EIA)

Characteristics of the EIAs are summarized in Tables 1 and 2. Antibodies for the 5α-20-one and 5β-20-one EIA were raised against steroid-C3-hemisuccinate protein conjugates; thus, group-specific antibodies directed against the C20 position of the steroid were obtained. The assays quantify closely related 5-reduced progesterone metabolites (pregnanes) containing an oxo group on the C20 atom, and the results are expressed as 20-oxo-pregnanes. Crossreactivities with pregnanes were considerably higher in the 5α-20-one and 5β-20-one EIA, as compared with the 4-ene-20-one EIA. However, the former two EIAs did not completely differentiate between the 5α- and the 5β-pregnane series. Either EIA could be used for fecal steroid monitoring, although HPLC separation provided strong evidence for the sole presence of 5α-pregnanes.

HPLC Separation of Fecal 20-oxo-pregnanes

HPLC separation and subsequent analysis of the fractions with the three EIAs were performed on fecal samples (n = 5) from both subspecies during different stages of pregnancy. A representative midpregnancy profile is shown in Figure 1. Results did not differ between the two subspecies; three major immunoreactive 20-oxo-pregnane peaks were detected in each sample. Their relative proportions remained unchanged throughout pregnancy. Immunoreactive material coeluting with ^{3}[H]-progesterone was not detectable.

Comparison with corresponding reference steroids together with the different crossreactivities in the three respective EIAs provided good evidence that the fecal immunoreactive peaks are (1) 5α-pregnane-3,20-dione, (2) 5α-pregnane-3α-ol-20-one, and (3) 5α-pregnane-3β-ol-20-one. Further evidence for the identity of peaks 2 and 3 was gained from the chromatography of fecal samples spiked with the authentic steroid standards, and after ether extraction and rechromatography of the fractions containing peaks 2 and 3. These separations resulted in chromatograms similar to that

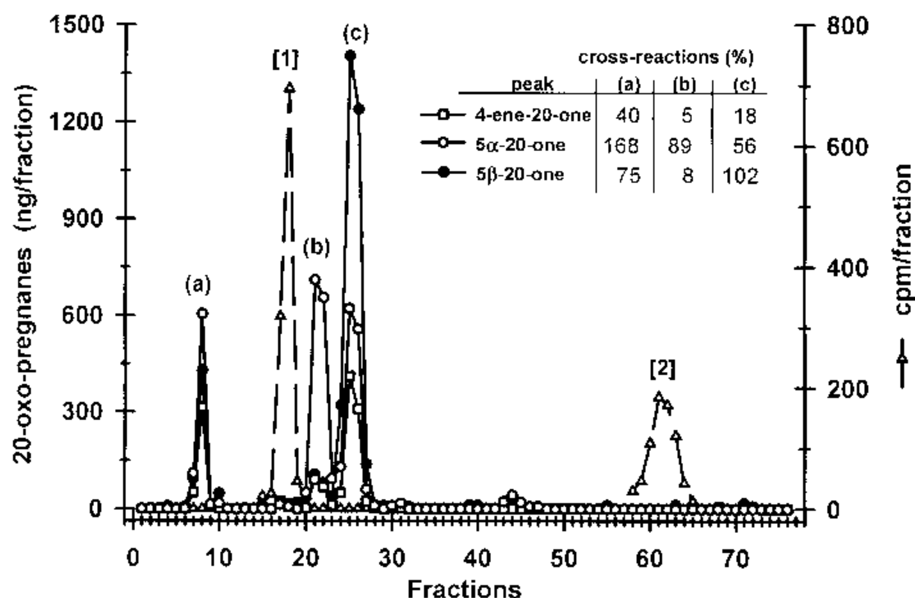

Fig. 1. HPLC separation of immunoreactive 20-oxo-pregnanes in a fecal sample of a 7-months-pregnant black rhinoceros (*Diceros bicornis michaeli*). Elution patterns of (1) ^{3}[H]-progesterone, (2) ^{3}[H]-20α-dihydroprogesterone were determined by liquid scintillation counting (△ cpm/fraction). Fractions were analyzed with EIAs for 20-oxo-pregnanes (○ 5α-20-one EIA; ● 5β-20-one EIA; □ 4-ene-20-one EIA). Concentrations in ng/fraction were calculated for 1 g of feces without correction for methodological losses. Crossreactivities of immunoreactive peaks (a–c) in the three EIAs are given in the inset. Retention times of peaks a–c were comparable to: (a) 5α-pregnane-3,20-dione, (b) 5α-pregnane-3α-ol-20-one, and (c) 5α-pregnane-3β-ol-20-one.

obtained after fecal sample separation. The elution patterns of crossreacting 5β-pregnanes were different from the fecal immunoreactive metabolites; the steroid standards 5β-pregnane-3,20-dione, 5β-pregnane-3α-ol-20-one, and 5β-pregnane-3β-ol-20-one eluted in fractions 10, 39–42, and 42–46, respectively (Fig. 1).

Fecal 20-Oxo-Pregnane Profiles

Estrous cycle profiles were comparable to those described previously [Schwarzenberger et al., 1993a]. Distinction between follicular and luteal phases was possible in the three EIAs used, and mating corresponded with low fecal 20-oxo-pregnane concentrations. Therefore, mean values were calculated from samples collected during follicular and luteal phases, and used for comparison only with the results obtained during pregnancy. Values of the nonpregnant animals were 117 ± 8, 136 ± 8, and 140 ± 9 ng/g feces in the 4-ene-20-one, 5α-20-one, and 5β-20-one EIA, respectively.

Gestation lengths for the animals of the *Diceros bicornis michaeli* subspecies were 442, 455, 467, and 469 days, respectively; one animal had a late pregnancy abortion on day 427 (Fig. 2). Values of this animal were used for mean ± SEM calculation during the first 9 months of gestation only. Duration of pregnancy was 464 days for the animal of the *Diceros bicornis minor* subspecies.

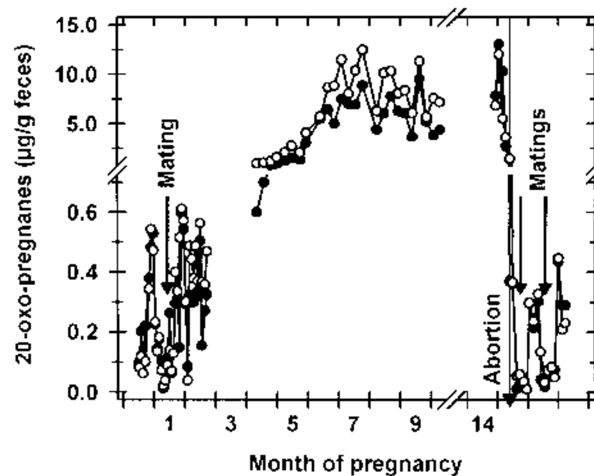

Fig. 2. Concentrations of immunoreactive 20-oxo-pregnanes in fecal samples of a black rhinoceros (*Diceors bicornis michaeli*) during an estrous cycle, subsequent pregnancy, and after late pregnancy abortion. Samples were analyzed with EIAs for 20-oxo-pregnanes (○ 5α-20-one EIA; ● 5β-20-one EIA). Note breaks of horizontal and vertical axes.

Pregnane concentrations during pregnancy were analyzed with the three EIAs and results of the *Diceros bicornis michaeli* (mean ± SEM) and the *Diceros bicornis minor* subspecies are shown in Figures 3 and 4, respectively; endocrine profiles of the two subspecies were comparable. Mean 20-oxo-pregnane values increased to luteal phase values within 2 weeks after mating and remained in that range during the first 2 months of pregnancy. Pregnane concentrations continuously increased during months 3–7, and values were significantly ($P < 0.01$) elevated above mean values of non-pregnant animals from week 11 onwards. The increase in the 4-ene-20-one EIA values was moderate as compared with the pronounced increase in the 5α-20-one and 5β-20-one EIA. During months 6–13 mean values in the 5α-20-one and the 5β-20-one EIA (5–11 µg/g) were significantly higher ($P < 0.01$) than those in the 4-ene-20-one EIA (1.5–3 µg/g feces). The 20-oxo-pregnane values decreased during the last 3 months of gestation and returned to basal levels within 2–3 days after parturition.

DISCUSSION

The principal objective of this study was to compare measurements of fecal 20-oxo-pregnane values in EIAs with different degrees of specificity. Results of our recent study on fecal immunoreactive progesterone metabolites in the black rhinoceros (*Diceros bicornis*) demonstrated the presence of 20-oxo- and 20α-OH-pregnanes; progesterone was not present, or present to a limited extent only [Schwarzenberger et al., 1993a]. However, lacking specific assays for 20-oxo-pregnanes, we relied on the crossreactivities of an antibody against 6HS-progesterone (the 4-ene-20-one EIA used in this study). Thus, assay specificity and accuracy of fecal 20-oxo-prenane determinations were a reasonable concern. Therefore, two antibodies against 5α- and 5β-pregnane-20-one 3HS:BSA derivatives (5α-20-one EIA and 5β-

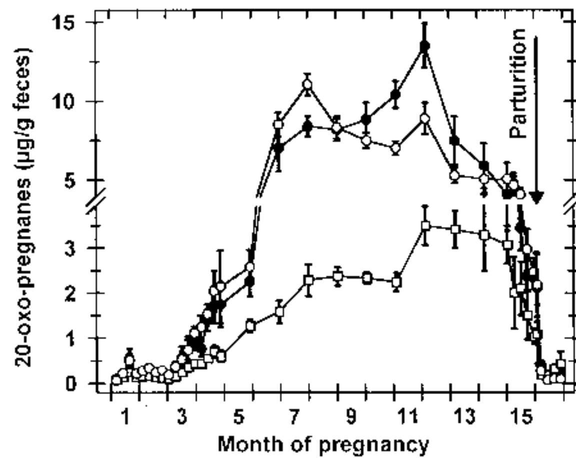

Fig. 3. Concentrations (mean ± SEM; n = 5–18 per point) of immunoreactive 20-oxo-pregnanes in fecal samples of black rhinoceroses (*Diceors bicornis michaeli*; n = 5) during pregnancy. Samples were analyzed with EIAs for 20-oxo-pregnanes (○ 5α-20-one EIA; ● 5β-20-one EIA; □ 4-ene-20-one EIA). Note break of vertical axis.

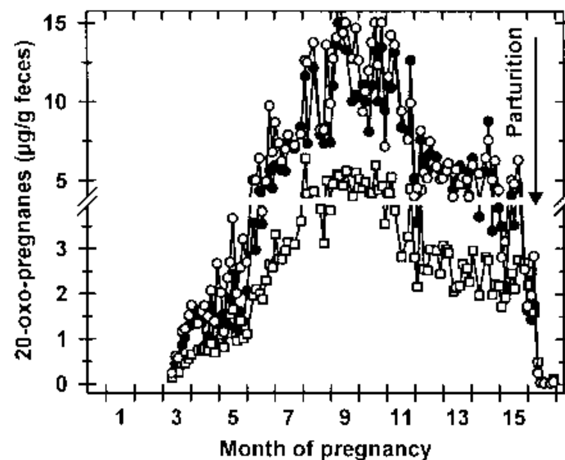

Fig. 4. Concentrations of immunoreactive 20-oxo-pregnanes in fecal samples of a black rhinoceros (*Diceors bicornis minor*) during pregnancy. Samples were analyzed with EIAs for 20-oxo-pregnanes (○ 5α-20-one EIA; ● 5β-20-one EIA; □ 4-ene-20-one EIA). Note break of vertical axis.

20-one EIA) were produced. In both assays considerably higher crossreactivities with 20-oxo-pregnanes were obtained, as compared to the 4-ene-20-one EIA. However, the newly developed EIAs had crossreactions with 5α- and 5β-pregnanes containing a 20-oxo-group and thus were not specific for the 5α- and 5β-pregnane series, respectively. Therefore, values obtained in these EIAs were comparable, but they were considerably higher than those in the 4-ene-20-one EIA.

In fecal samples of the black rhinoceros "authentic" progesterone, which would have coeluted with ^{3}[H]-progesterone and which had crossreactivities of 100%

in all three EIAs used, was not detectable. HPLC separation showed three major immunoreactive peaks; their elution patterns and the different crossreactivities in the three respective EIAs provided strong evidence that these peaks are 5α-pregnane-3,20-dione, 5α-pregnane-3α-ol-20-one, and 5α-pregnane-3β-ol-20-one. Although rigorous identification was not achieved (GC/MS was not available), our findings of several 20-oxo-pregnanes are in accordance with other studies in different species. Using progesterone antibodies, the presence of 20-oxo-progesterone metabolites in the feces of primates [Wasser et al., 1991, 1994], felids [Graham et al., 1993; Möstl et al., 1993; Brown et al., 1994; Czekala et al., 1994], ruminants [Desaulniers et al., 1989; Messier et al., 1990; Monfort et al., 1993; Larter et al., 1994], white rhinoceroses [Schwarzenberger et al., 1994, 1995b], and vicunas [Schwarzenberger et al., 1995a] was demonstrated. In addition, radioinfusion studies [Shideler et al., 1993; Brown et al., 1994; Wasser et al., 1994; Palme, personal communication] and studies using HPLC separation with immunoassay analysis [Kirkpatrick et al., 1991; Schwarzenberger et al., 1991, 1992, 1993a,b, 1995a; Heistermann et al., 1993; Shideler et al., 1993; Brown et al., 1994; Wasser et al., 1994] showed that progesterone metabolism and excretion results in several fecal steroid metabolites showing elution patterns similar to that of pregnanes.

The comparisons made in this manuscript deal with progesterone assays and not assays recognizing either a 20α-OH or a 20β-OH group. Pregnanediol (5β-pregnane-3α,20α-diol) has a 20α-OH group in position C20 of the steroid molecule, and its crossreactivities in our 20-oxo-assays are $<0.1\%$ (see Table 2). However, most pregnanediol antibodies are position-3 conjugates against a 20α-ol-5β-pregnane and therefore correspond with our definition of group-specific. Pregnanediol antibodies were used for fecal steroid analysis in mares [Kirkpatrick et al., 1991], primates [Heistermann et al., 1993; Shideler et al., 1993], okapis [Schwarzenberger et al., 1993b], and black rhinoceroses [Schwarzenberger et al., 1993a; Berkely, 1994].

Since "authentic" progesterone is not (or to a limited extent only) present in fecal samples, antiserum selection for the analysis of progesterone metabolites is a serious concern. Some of the currently available antibodies for progesterone (namely 11HS- or 6HS-progesterone antisera) are rather specific and show little crossreactivity to other metabolites. However, fecal progesterone metabolites derive from one parent molecule and it is desirable to quantify several metabolites in one assay. In this respect, group-specific assays using steroid C3-conjugate antibodies are superior; they show high crossreactivities with several progesterone metabolites sharing a similar C20 configuration and their measurement is combined in one assay. This concept was also the basis for the "total estrogen assays," which employ 17-hemisuccinate derivatives of estradiol and which recognize the phenolic A ring, common to several naturally occurring estrogens [Niswender et al., 1975].

Instead of using a 5-reduced 20-oxo-pregnane as standard, we used progesterone. Progesterone is easily available, and application-orientated laboratories can use group-specific 20-oxo-pregnane assays for fecal steroid analysis in several animal species without laborious identification of the major species-specific metabolite(s). Since fecal 20-oxo-pregnanes are present in different species, group-specific assays can be used confidently across species. However, assay results will depend upon the summarized crossreactivities of the fecal steroid metabolites. Therefore, absolute values obtained in different assays may vary considerably, so for diagnostic purposes laboratories using different assays might use their own threshold values. Crossreac-

tivities with 20-oxo-pregnanes in the present study were considerably higher than those of a progesterone antibody used in a comparable study [Berkeley, 1994]. Our results were 3–5 times higher, although our calculations were based on wet feces and those of Berkeley [1994] on dry feces. Similarly, a study on feline species using a monoclonal progesterone antibody with high crossreactivities to 5α-reduced pregnanes reported 10–100-fold higher progesterone metabolite concentrations as compared to other studies [Brown et al., 1994].

The increase in assay specificity enabled us to determine fecal 20-oxo-pregnane values more accurately as compared with the 4-ene-20-one EIA [Schwarzenberger et al., 1993a]. Thus, differences in the fecal pregnane values between nonpregnant and >2 months pregnant animals increased. The increase in accuracy causes an increase in reliability of pregnancy diagnosis. Although this is not of the utmost importance for animals kept in zoos, where several samples can be collected over prolonged time periods, the high assay reliability is important for the study of free-ranging animals. Confirmation of pregnancy from one sample is possible by monitoring the high fecal 20-oxo-pregnane values 3–4 months after mating. These high pregnane values most probably reflect placental steroid production. The increase in fecal steroid excretion in the black rhinoceros after the third month of pregnancy was also described by Berkeley [1994]. Serum hormone levels of >1,000 ng/ml were obtained in a progesterone RIA during the last third of pregnancy [Kock et al., 1991].

During the last quarter of gestation fecal 20-oxo-pregnane values declined. This is in contrast to the pattern of fecal progesterone metabolites containing a 20α-OH group which, in black rhinoceroses and mares, increased throughout gestation [Schwarzenberger et al., 1991, 1993a]. HPLC separation of fecal immunoreactive progesterone metabolites in black rhinoceroses [Schwarzenberger et al., 1993a] suggested that metabolites with higher polarity became more prominent as pregnancy progressed. A shift in steroid production/metabolism and/or excretion to more hydroxylated and thus more polar progesterone metabolites might explain this phenomenon. The patterns of 5α-pregnanes containing a 20α-OH group using group-specific assays need to be elucidated in future investigations. These assays might improve estrous cycle monitoring, since the conversion of radioactively labeled progesterone into 20α-dihydroprogesterone in a rhinoceros was demonstrated [Hindle and Hodges, 1990], and since urinary analysis of 20α-dihydroprogesterone was successfully used for estrous cycle monitoring [Hindle et al., 1992].

CONCLUSIONS

1. Fecal 20-oxo-pregnanes in the black rhinoceros consist of closely related immunoreactive metabolites. Their elution profiles on HPLC and different crossreactivities in the three EIAs used provided strong evidence that these metabolites are 5α-pregnane-3,20-dione, 5α-pregnane-3α-ol-20-one, and 5α-pregnane-3β-ol-20-one.

2. Values of fecal 20-oxo-pregnanes are determined more accurately using group-specific assays against 5-pregnane-20-one C3 conjugates, as compared to a progesterone antibody.

3. Using the newly developed assays, differences between values of nonpregnant and pregnant animals increased and thus reliability of pregnancy diagnosis was improved.

4. Application of group-specific assays for 20-oxo-pregnanes reaffirms that they might be of general interest for future analysis of fecal progesterone metabolites in several animal species.

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