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A Review of Faecal progesterone metabolite analysis for non-invasive monitoring of reproductive function in mammals

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Abstract

The analysis of faecal progesterone metabolites for non-invasive reproductive monitoring is an established technique used in an increasing number of laboratories. All data confirm that faecal progesterone metabolites reflect luteal and/or placental function, and that faecal values show a similar pattern to that of plasma progesterone, but have a species-specific lag time of 12 h to >2 days. Although biological data are comparable within a single species, it is currently difficult to compare values of faecal progesterone metabolites among laboratories. This is due to the use of different extraction methods and antibodies with different cross-reactivities. The extraction efficiency in several studies was tested using [^{3}H]-progesterone added to faeces; the recovery rates varied from 10% to >90%, depending on the use of buffer or an increasing portion of methanol or ethanol in the extraction solvent. Progesterone is metabolised to several 5a- or 5β-reduced pregnanes (pregnanediones, mono- and dihydroxylated pregnanes) prior to its excretion into the faeces and unmetabolised progesterone is barely present, if at all. Faecal pregnanes either have a 20-oxo, a 20a-, or a 20β-hydroxyl group and several immunoreactive metabolites of each group are present in the faeces of all species as yet investigated. Hence, assay systems detecting different C20-groups can be employed for their determination, though antibodies against progesterone are most widely used. Recent studies have shown that antibodies raised against pregnane immunogens conjugated at the 3-position are superior to specific progesterone antibodies. These pregnane antibodies show considerable cross-reactivities and, therefore, concurrently detect several metabolites sharing a similar C20 group. For this reason the assays are termed group-specific. They can be generally applied to a wide range of species and their use, in combination with an extraction procedure using a solvent with a high percentage of alcohol, is recommended for the analysis of faecal progesterone metabolites.

Introduction

The importance of endocrinology in the conservation of wildlife has been emphasised in several recent, more general reviews (Lasley et al. 1991, 1994; Loskutoff et al. 1995, Heistermann et al. 1995; Wildt et al. 1995) and in a review specifically addressing faecal steroid analysis (Schwarzenberger et al. 1996 a). Techniques for non-invasive faecal monitoring of oestrogen in domestic and non-domestic animals have been available for over 10 years (for references see Schwarzenberger et al. 1996 a). Based on successful applications of these methods, efforts during the past 5–10 years focused on the development of techniques for monitoring faecal progesterone metabolites. These techniques are now available and routinely used in an increasing number of laboratories and animal species (for references see Schwarzenberger et al. 1996 a).

All available data confirm that faecal progesterone metabolites reflect plasma or milk profiles and therefore luteal and/or placental function. The analysis of faecal progesterone metabolites has been used for characterising ovarian cycles and diagnosing reproductive cycle abnormalities, for diagnosing pregnancy, for timing of placentation and

abortion, for monitoring treatment therapies, for correlation of hormone levels and social behaviours, and for seasonal hormonal profiling (for references see Schwarzenberger et al. 1996 a). Hence, non-invasive reproductive monitoring provides basic information on the reproductive physiology of many endangered species, and will also be fundamental for the development of assisted reproductive technologies such as artificial insemination, in vitro fertilisation and embryo transfer techniques.

Although biological data in a single species obtained in different laboratories are comparable, it is currently difficult to compare values of faecal progesterone metabolites among laboratories. This is a result of the use of different extraction procedures and antibodies with different cross-reactivities. Hence, in this paper we will focus on methodological considerations of faecal progesterone metabolite analysis and will summarise the current knowledge on the metabolism, extraction and analysis of progesterone metabolites from faecal samples.

Excretion of steroid hormones into faeces and urine

In general, steroid hormones are mainly metabolised by the liver and are excreted in the urine, or with the bile into the faeces; hormones also appear to a certain extent in milk and saliva. Steroids in the gut are subject to an enterohepatic circulation (Fig. 1). Faecal steroid metabolites show a similar pattern to plasma values, but, compared to the actual values in plasma have a lag time which, as was shown by studies on the metabolism of radioactively labelled steroids, largely depends on the time necessary for the intestinal passage of bile to the rectum. Thus the lag time is approximately 12–24 h in ruminants, 1–2 days in non-ruminants, and even more in herbivores, where the main site of fermentation is in the colon. The excretion time of steroids in non-ruminants roughly correlates to the passage time of ingesta, whereas it is appreciably shorter in ruminants, since in these species bile enters the intestine after the foregut (for references see Palme et al. 1996; Schwarzenberger et al. 1996 a).

Aside from the time course of excretion, the administration of radioactively labelled steroid hormones in several animals has been used to determine the route of excretion and the type of metabolic endproducts (for references see Schwarzenberger et al. 1996 a). Results of these studies demonstrate that the extent to which steroids are excreted in the urine or faeces varies considerably between species, but also between steroids within the same species. It is not possible to make a general statement on this topic, and extrapolation of results from one species to another could be misleading. For instance horses excrete 98% of oestrogens in the urine, but 75% of progesterone metabolites in the faeces

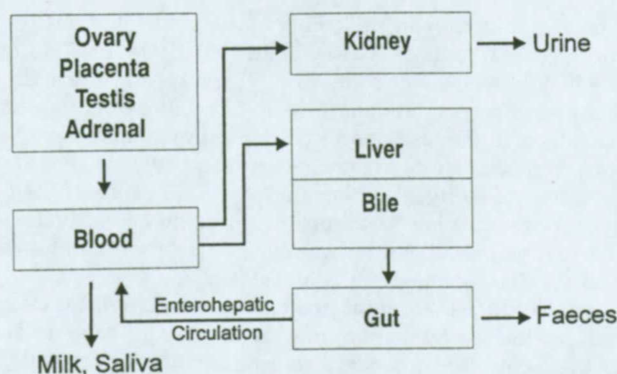

Fig. 1. Organs involved in the production, metabolism, and excretion of steroid hormones.

(Palme et al. 1996). Cats excrete almost all of progesterone, oestrogens and cortisol into the faeces (Brown et al. 1994; Goossens et al. 1995; Graham and Brown 1996). Nevertheless, despite low excretion rates, sensitive immunoassays can be used to measure oestrogen levels in the faeces of horses (for references see Schwarzenberger et al. 1996 a) or corticoid levels in the urine of cats (Goossens et al. 1995).

Although it would be advantageous to know the route of excretion, and thus use either urine or faeces as sample material, it is not feasible to study the metabolism of radioactively labelled steroid hormones in every species. Results from our laboratory demonstrate that these studies are not always necessary to successfully apply faecal steroid analysis. Oestrogen determination in faeces of mares was first published by Möstl et al. (1983). In view of these results it was surprising to learn from studies on the excretion of radioactively labelled oestrogens that only 2% is excreted in the faeces (Palme et al. 1996). In this case it was even an advantage that the route of excretion was not known before the practical application was established. In some animals it seems possible to learn the predominant route of excretion without conducting radioinfusion studies. Our experience with the determination of progesterone metabolites in okapis suggests that these metabolites are mainly excreted via the faeces. Concentrations of progesterone metabolites determined in a pregnanediol assay are about 100–200 fold higher in faeces than in urine. This observation explains why pregnancy diagnosis using urinary pregnanediol evaluations during the first half of gestation often produces invalid results (Schwarzenberger et al. 1997).

Extraction of faecal progesterone metabolites

In several studies [^{3}H]-progesterone added to faeces was used as a marker for the extraction efficiency. Results have shown that recovery rates vary from approximately 10% (Hulten et al. 1995) to more than 90% (Schwarzenberger et al. 1991, Brown et al. 1994), depending on the use of buffer or an increasing percentage of methanol or ethanol in the extraction solvent. In a recent report, using faeces of sheep, pigs and horses, Palme et al.

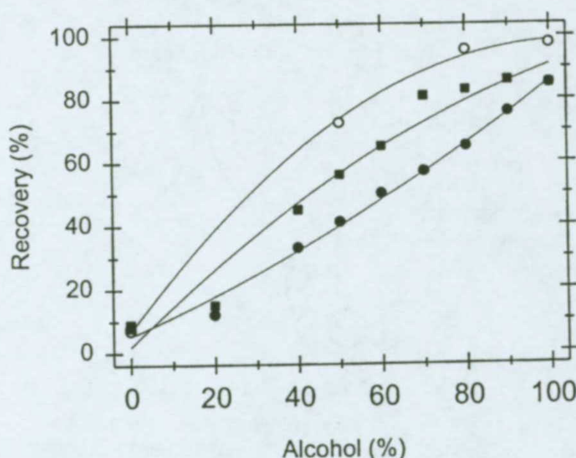

Fig. 2. Recovery rates (%) of radioactive [^{3}H]-progesterone and [^{14}C]-progesterone metabolites from faeces of mares. [^{14}C]-progesterone was infused into the animal and in vivo metabolised; the metabolites were extracted from the faeces with different percentages of methanol (l) and ethanol (n). In contrast, [^{3}H] -progesterone was added to faeces, and after soaking for 30 min, extracted with different percentages of methanol (¡). Adapted from Palme et al. 1997.

(1997) compared recovery rates of [^{3}H]-progesterone added to faeces shortly before extraction (in vitro), to those of radioactive progesterone metabolites appearing in faeces after infusion of [^{14}C]-progesterone (in vivo). The authors tested the extraction efficiency of different concentrations of ethanol, methanol and iso-propanol, and found that recovery rates of [^{3}H]-progesterone added in vitro seemed to be overestimated (Fig. 2). Regardless of this limitation, recovery testing with radioactively labelled steroids gives a rough estimate of extraction efficiency and is, therefore, an invaluable tool for establishing extraction methods since in vivo metabolised radioactively labelled steroids are not available in every species. However, a general recommendation for the extraction of faecal progesterone metabolites is that the concentration of the alcohol used should be more than 80%.

Progesterone metabolism and assay methodology

Studies using chromatographic techniques in combination with immuno-assays and radiometabolism studies indicated that almost all progesterone is metabolised to 5a- or 5*β*-reduced pregnanes (pregnanediones, mono- and dihydroxylated pregnanes) prior to its excretion in the faeces. Nonmetabolised progesterone is barely present, if at all, in the faeces. In all species studied so far, there is no single major metabolite but rather several metabolites (for references see Schwarzenberger et al. 1996 a). Progesterone is metabolised by reduction of its double bond located between the C4 and C5 atom of the steroid molecule, leading to 5a- or 5*β*-reduced pregnanes. Further reductions affect the oxo-groups at the C3 and/or the C20 atoms. Considering these possibilities 18 different pregnane metabolites (2 pregnanediones, 8 monohydroxylated and 8 dihydroxylated pregnanes) are possible (Fig 3). Not included in our considerations are 8 possible pregnene metabolites, since to our current understanding these are not, or to a limited extent only, present in the faeces.

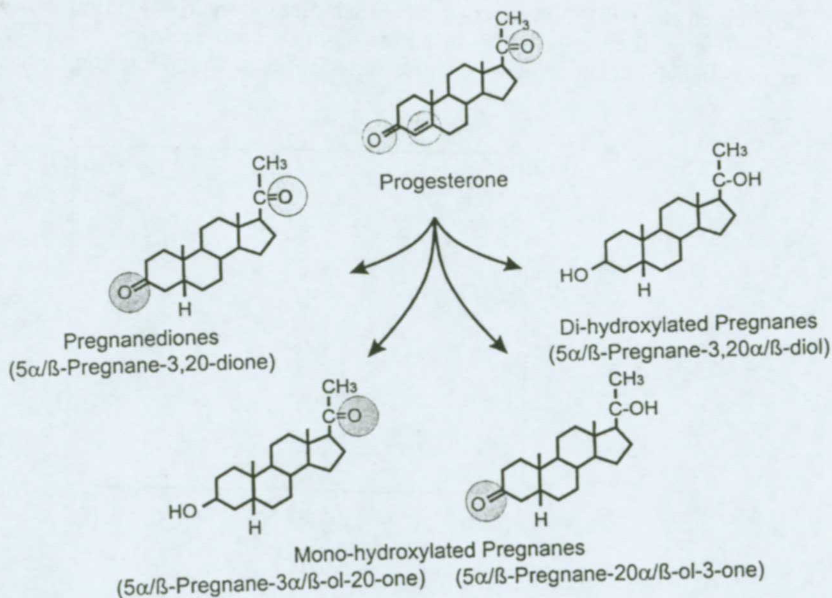

Fig. 3. Faecal progesterone metabolites. The circles mark the oxo groups located at the C3- and the C20-atom, and the double bond between C4–C5 of progesterone. Reduction of these double bonds leads to different pregnane metabolites.

Since 'native' progesterone is not detectable in the faeces, the term faecal progesterone is misleading and, therefore, should not be used to report values of faecal progesterone metabolites determined by progesterone assays. These type of assays do not report progesterone but values of cross-reacting 5a- and 5β-reduced pregnane metabolites containing a 20-oxo-group. Since it is unclear to what extent faecal progesterone metabolites could have progestational activity several researchers have used the expression progestagens instead of progestogens; progestogens are defined as naturally occurring progesterone metabolites which have progestational activity. Other researchers have used the term progestin, but, referring to textbooks of pharmacology, this term should preferably be used for synthetic progestational agents. Therefore, it is suggested to use the term progestagens or progesterone metabolites to characterise faecal metabolites of progesterone.

For analytical purposes faecal progesterone metabolites can be grouped into pregnanes having either a 20-oxo, a 20a-, or a 20β-hydroxyl group. In general, antibodies for the analysis of progesterone metabolites identify the C20 position of the steroid molecule and the large numbers of progesterone metabolites present in the faeces explain why several assay systems can be used for their measurement. However, due to their widespread availability, antibodies against progesterone are most widely used. Since most progesterone antibodies are prepared against immunogens conjugated at the position C11, these antibodies have high affinity to the C4–C5 double bond and the 3-oxo- and the 20-oxo-group of progesterone. Therefore these antibodies are rather specific for progesterone (Fig. 4) and have low cross-reactivities with pregnanes. Thus they considerably underestimate the actual amount of faecal 20-oxo-pregnanes and, therefore, are less suitable for faecal progesterone metabolite analysis.

Schwarzenberger et al. (1996 b, 1996 c) compared 3 enzyme-immunoassays with different degrees of specificity for several 20-oxo-pregnanes using faecal samples of domestic cows and black rhinoceroses. The authors suggested that antibodies for faecal steroid analysis should be raised against 5a- or 5β-pregnanes conjugated at the position 3 of the steroid molecule. These group-specific antibodies mainly recognise the C20-position, whereas the functional group on C3 and the hydrogen atom on C5 of the steroid molecule are not recognised as well (Fig. 4). Thus these antibodies show considerable cross-reactivities with several pregnanes sharing a similar C20 group and concurrently detect

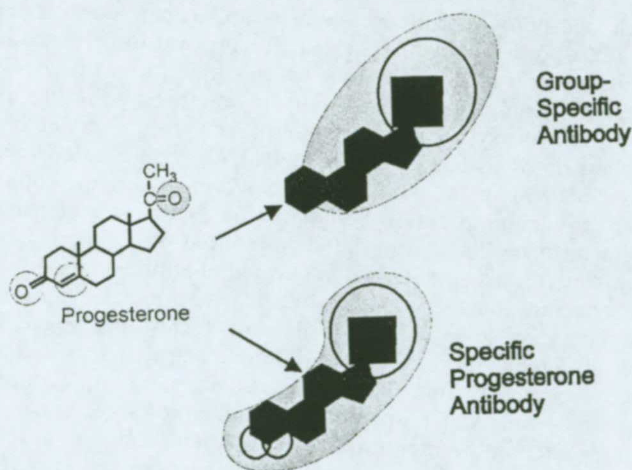

Fig. 4. Group-specific and progesterone antibodies reacting with steroid molecules. The arrows direct to the C3 atom of the group-specific and to the C11 atom of the specific antibody, which are the conjugation sides for the preparation of the respective immunogens.

these; for this reason they are termed group-specific. The concept of these assays is similar to that used for the total oestrogen assays, which recognise the phenolic A-ring common to several oestrogens. Consequently, results obtained with group-specific assays are considered as measurement of total immunoreactive progesterone metabolites. Schwarzenberger et al. (1996 b, 1996 c) concluded that the combined measurement of several pregnanes containing a similar C20 group is superior to assays detecting only a limited number of progesterone metabolites. Due to the improved specificity, group-specific assays show better sensitivity, accuracy, reliability and practicability for faecal pregnane analysis than specific progesterone assays.

Studies using HPLC separation of faecal extracts and immunoreactivity testing of the fractions in different assays determined the relative proportions of pregnane metabolites on the basis of their immunoreactivity (for references see Schwarzenberger et al. 1996 a). Based on these studies it was somehow questionable, whether there might not be a single major metabolite not adequately detected by the antibodies used, and whether it, therefore, might have been preferential to produce a specific antibody for an, at that time, unknown progesterone metabolite. However, in a recent study on the metabolism of [^{14}C]-progesterone and immunoreactivity testing using different antibodies, Palme et al. (1997) found that more than 75% of in vivo metabolised faecal [^{14}C]-progesterone metabolites co-elute with immunoreactive metabolites cross-reacting in assays which detect either a 20-oxo, a 20a-, or a 20β-hydroxyl group. The authors found that almost all of the 18 possible pregnanes appear in the faeces, though species specific differences concerning the type of pregnanes and the C-20 group occur. Metabolites in sheep were 5a- and 5β-pregnanes, whereas in pigs mainly 5β-pregnanes, and in horses 5a-pregnanes were found. Several immunoreactive metabolites of each group were present and the predominant pregnanes in the faeces of sheep were those containing a 20a-hydroxyl-group (45%), in horses a 20β-hydroxyl-group (44%), and in pigs a 20-oxo-group (50%). The authors, thus, confirmed that immunoreactivity testing after HPLC separation is a valid method to determine the number of cross-reacting faecal metabolites, and also confirmed that the use of group-specific antibodies is an appropriate strategy for the determination of faecal progesterone metabolites.

Comparability of faecal progesterone analysis between laboratories

Although faecal progesterone metabolites can be analysed with assays recognising either a 20-oxo, a 20a-, or a 20β-hydroxyl group, it needs yet to be tested in each and every single case, which assay is most suitable for which species. In many species assay systems detecting different C20-groups give quantitatively rather similar results. In mares assays against a 20a- or a 20β-hydroxyl group can be used (Schwarzenberger et al. 1991, 1992), whereas in black rhinos those against 20-oxo or a 20a-hydroxyl group are suitable (Schwarzenberger et al. 1993 a, 1996 b). In some species, however, a certain group clearly dominates and, thus, only one immunoassay is best suited for faecal progesterone metabolite analysis. In okapis for instance, 20a-hydroxylated 5β-reduced pregnane metabolites are quantitatively dominant and, thus, assays using a pregnanediol-antibody provide higher differences of the steroid concentrations between the follicular and luteal phase of the oestrous cycle, and are also most suitable for pregnancy diagnosis (Schwarzenberger et al. 1993 b).

Since different laboratories use different extraction procedures and antibodies with different cross-reactivities, values of various laboratories are difficult to compare. Although recoveries of [^{3}H]-progesterone added to faeces as an indicator of extraction efficiency reported in the literature varies between 10 and 90%, the major drawback in the comparison of faecal values is that many publications report only very limited data about the cross-reactivities of their assays. Cross-reactivities with pregnanes could at least give indications about comparability of results between laboratories, and should, therefore, be

Table 1. Cross-reactivities (%) of steroids in immunoassays published by Brown et al. (1994), and by Schwarzenberger et al. (1996 b).

Source	Brown et al. (1994)	Schwarzenberger et al. (1996 b)	
		(5a-20-one)	(5β-20-one)
Steroid			
4-pregnene-			
3,20 dione (progesterone)	100	100	100
3β-ol-20-one	–	14	68
3a-ol-20-one	–	390	20
5a-pregnane-			
3a-ol-20-one	36	89	8
3β-ol-20-one	96	56	102
3,20-dione	–	168	75
5β-pregnane-			
3a-ol-20-one	7	88	20
3β-ol-20-one	15	5	36
3,20-dione	–	26	151
5-pregnene-			
3β-ol-20-one	13	17	11

(– not tested)

published in any study on faecal progesterone metabolite analysis. Depending on the type of assay, relevant cross-reactivities in the description of a faecal steroid assay include those against pregnanes containing either a 20-oxo, a 20a-, or a 20β-hydroxyl group. This, in conjunction with immunoreactivity testing after HPLC separation of faecal extracts, would be an important step towards the comparability and possibly the standardisation of faecal progesterone metabolite values.

For instance, based on their cross-reactivities, the assays published by Brown et al. (1994) and Schwarzenberger et al. (1996 b) seem to be rather comparable (Table 1), although different antibodies were used. The immunogen for the monoclonal antibody used by Brown et al. (1994) was described as 11-OH-conjugated progesterone. However, recent results of our laboratory (Möstl and Schwarzenberger 1997, unpublished observations) indicate that this antibody was most likely produced against a position 3-conjugated progesterone, thus giving it the characteristics of a group-specific antibody and explaining its high crossreactivities with 20-oxo-pregnanes.

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