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Faecal Metabolites of Infused ^{14}C-Progesterone in Domestic Livestock

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Contents

Non-invasive monitoring of the reproductive function in animals is possible by measuring steroid metabolites in the faeces. After infusion of ^{14}C-steroids, differences between species concerning both the percentage and the delay of faecal excretion can be observed. In domestic livestock almost all faecal ^{14}C-metabolites are found in an unconjugated form. The aim of this study was to characterize the main metabolites present in faecal samples of sheep, ponies and pigs after infusion of ^{14}C-progesterone and to evaluate different extraction procedures. This should help to standardize and improve faecal steroid analysis. Faeces containing peak radioactivity were suspended in methanol/water and Sep-Pak® C_{18} cartridges combined with Sephadex® LH-20 columns were applied to clean up the samples. Fractions were subjected to straight phase high pressure liquid chromatography (HPLC) and thin layer chromatography (TLC). Metabolites were classified by comparison with the elution patterns of steroid reference standards and by testing their immunoreactivity in different enzyme-immunoassays (EIAs). The results revealed considerable differences in the pattern of faecal progesterone metabolites between species. A great number of metabolites (mainly mono- and di-hydroxylated pregnanes as well as pregnanediones) was found and progesterone barely, if at all. In sheep, pregnanes containing a 20α-hydroxy (OH) group dominated (about 45%), whereas in ponies metabolites with a 20β-OH group (about 44%; mainly one 5α-pregnanediol), and in pigs progestagens with a 20-oxo group (46–58%) predominated. Suspending the faeces with a high percentage of methanol, ethanol or iso-propanol yielded the best recoveries and enabled a practicable extraction, without an evaporation step. As a wide range of metabolites was formed, group-specific immunoassays using antibodies with high cross-reactions with pregnanes sharing a similar C-20 group should be used for measuring faecal metabolites. To verify the data of the ^{14}C-experiments, various progesterone metabolites were measured in faecal samples of superovulated ewes. In accordance with the results of the characterized progesterone metabolites, an EIA measuring 20α-OH-pregnanes proved most suitable for non-invasive monitoring of the corpus luteum function in sheep.

Inhalt

Metaboliten von verabreichtem ^{14}C-Progesteron im Kot von Schafen, Ponys und Schweinen

Eine nicht invasive Überwachung der Fortpflanzung kann bei Tieren mittels Steroidbestimmungen im Kot erfolgen. Nach Verabreichung von ^{14}C-Steroiden können tierartliche Unterschiede bezüglich Ausscheidungsgrad und Verzögerung im Kot festgestellt werden, wobei bei Huftieren nahezu alle Metaboliten in nicht konjugiert Form vorliegen. Ziel unserer Arbeit war es, die Hauptmetaboliten von infundiertem ^{14}C-Progesteron im Kot von Schafen, Ponys und Schweinen zu charakterisieren und verschiedene Extraktionsverfahren zu vergleichen. Dies sollte zu einer Standardisierung bzw. Verbesserung der Steroidanalytik im Kot führen. Kotproben, die maximale Konzentrationen von ^{14}C-Steroiden enthielten, wurden in Methanol aufgeschlämmt. Zwecks Vorreinigung wurden Sep-Pak® C_{18} Kartuschen und Sephadex® LH-20 Säulen angewendet und entsprechende Fraktionen anschließend chromatographisch (HPLC und DC) aufgetrennt. Eine Zuordnung der Metaboliten erfolgte durch Vergleich des Laufverhaltens mit entsprechenden Standards und durch Bestimmung ihrer Reaktivität in verschiedenen Enzymimmunoassays (EIAs). Dabei wurden erhebliche tierartliche Unterschiede der Metabolitenmuster im Kot festgestellt. Es wurde eine große Anzahl von Metaboliten (hauptsächlich mono- und dihydroxylierte Pregnane, sowie Pregnandione) jedoch nahezu kein Progesteron ausgeschieden. Beim Schaf herrschten Pregnane mit einer 20α-Hydroxygruppe vor (cirka 45%), beim Pony hingegen solche mit einer 20β-Hydroxygruppe (ungefähr 44%; überwiegend ein 5α-Pregnandiol) und beim Schwein solche mit einer 20-Oxogruppe (46–58%). Eine Suspension der Kotproben in hochkonzentriertem Methanol bzw. Ethanol oder Isopropanol lieferte gute Ausbeuten und ermöglicht eine praktikable Extraktion ohne Eindampfschritt. Da eine weite Palette von Metaboliten gebildet wurde, sollten gruppenspezifische Immunoassays mit Antikörpern zur Erfassung von Pregnanen mit gleicher C-20 Gruppe zur Messung der Kotgestagene verwendet werden. Zur Bestätigung der Ergebnisse der ^{14}C-Experimente wurden Progesteronmetaboliten im Kot von superovulierten Schafen gemessen. Wie aufgrund obiger Ergebnisse zu erwarten, erwies sich ein EIA zur Bestimmung von 20α-Hydroxypregnanen als am Besten geeignet, um die Gelbkörperfunktion beim Schaf durch Messung der Progesteronmetaboliten im Kot zu untersuchen.

Introduction

Monitoring of the reproductive function in animals is possible by measuring steroid metabolites in the faeces. Faecal samples can be easily collected, an advantage which is especially useful in zoo and wildlife animals. Although faecal progestagens have been measured in a variety of species (for review see Schwarzenberger et al. 1996a), characterization of faecal metabolites after infusion of radio-labelled progesterone has not yet been reported in domestic livestock. After infusion of ^{14}C-steroids in a recent study, differences between species were observed concerning both the percentage and the delay of faecal radioactivity (Palme et al. 1996). Large amounts of administered progesterone were excreted via the faeces in ewes and mares (about 75%) but only 33% in sows (Palme et al. 1996). Although steroid metabolites are excreted into the bile as conjugates (Taylor 1971), in ungulates, faecal ^{14}C-metabolites were found mainly in an unconjugated form (Palme et al. 1996).

Currently it is difficult to compare the values of faecal progesterone metabolites among laboratories due to differences in the extraction procedures and in the

cross-reactivities of antibodies used in the immunoassays. Depending upon the solvents used for extraction, reported recoveries varied between 10% and more than 90% (Desaulniers et al. 1989; Hultén et al. 1995; Schwarzenberger et al. 1996a). However, as they were based on the extraction of radio-labels (mainly ^{3}H-progesterone) added to the sample just before processing, they probably do not reflect the real recovery of the mixture of progesterone metabolites present in the faeces. Besides, antibody and standard selection for the immunoassays is a serious concern as, in all the species investigated, several pregnanes (and barely progesterone) are present in the faeces (Schwarzenberger et al. 1996a). This explains why several immunoassays using different antibodies can be applied for measuring faecal gestagens. Only the equivalents of progesterone metabolites were estimated by measuring their immunoreactivity in different assays (Schwarzenberger et al. 1991, 1992, 1996b). A characterization and quantification of radio-labelled progesterone metabolites and the evaluation of different extraction methods, based on faecal samples containing the naturally metabolized, radio-labelled steroids, will be an important step towards a standardization of faecal steroid measurements and thus important for comparison of results obtained in different laboratories.

The aim of this study was to characterize the main metabolites of infused ^{14}C-progesterone in faeces of sheep, ponies and pigs, and to evaluate extraction procedures for measuring faecal progestagens. As the monitoring of corpus luteum activity by measuring faecal progesterone metabolites was reported in mares, sows and cows (Desaulniers et al. 1989; Schwarzenberger et al. 1992, 1996b; Hultén et al. 1995) but not in ewes, measurements of progesterone metabolites in faecal samples of superovulated ewes were carried out in addition.

Materials and Methods

Characterization of ^{14}C-progesterone metabolites

Faeces, excreted following intravenous administration of 0.1 mCi of ^{14}C-progesterone to ewes, pony mares and sows during the corpus luteum phase (n = 3 each; Palme et al. 1996), were used to identify the main metabolites in the samples that contained peak radioactivity. As many interfering substances were present in the faeces, a clean-up procedure, which was established by Palme et al. (1995) for identifying oestrogens in urine and faeces was used. Faeces (1 g) were suspended in methanol/water (4 + 1; 20 ml), the supernatant diluted with 0.2 M sodium acetate buffer (pH 4.8) and passed through a Sep-Pak® C_{18} cartridge (Waters, Milford, MA, USA). The cartridge was washed with 10 ml aqua bidest and dried with a stream of nitrogen. Elution was performed using 10 ml of dichloromethane, ethyl acetate/methanol (4 + 1) and methanol successively. Further analysis was limited to dichloromethane fractions, which contained almost all radioactivity. They were chromatographed on Sephadex® LH-20 columns (1 g; 6 × 0.6 cm; Merck, Germany) with the following solvent mixtures (10 ml): cyclohexane/iso-propanol (1 + 0; 6 + 1; 3 + 1; 2 + 1 and 1 + 1) and iso-propanol/methanol (9 + 1; 6 + 1 and 0 + 1).

In addition, faeces were directly extracted with methanol (3 ml) or diethylether (5 ml + 0.5 ml water). The extracts were evaporated, reconstituted in 1 ml of 20 mM $NaHCO_3$ and re-extracted with ether as described by Schwarzenberger et al. (1996c) and the remainder checked for possible loss of radioactivity.

Sephadex® fractions containing radioactivity or ether extracts were injected onto a Lichrosorb Si 60 (10 μm) column (25 × 0.4 cm; Forschungszentrum, Seibersdorf, Austria) and separated by high performance liquid chromatography (HPLC; gradient 1), as described by Schwarzenberger et al. (1996c), using a mixture of *n*-hexane/chloroform (75:25; v/v; linear methanol gradient from 0–6%; flow: 2 ml/min). Additionally, to enable a better separation of mono- and di-hydroxylated pregnanes, a modified methanol gradient (flow: 4 ml/min) was performed as follows (gradient 2): After 8 min of isocratic conditions a linear methanol gradient (0–2%) was run for another 16 min and fractions were collected every 15 s. After separation, the fractions were evaporated and the residues dissolved in 1 ml of methanol. The elution profiles of ^{14}C-steroids were determined by liquid scintillation counting (Packard Tricarb 4640, Warrenville, IL, USA). The immunoreactivity of the metabolites and the elution profiles of the reference standards were checked with different group-specific enzyme immunoassays (EIA). Two assays measuring 20-oxo-pregnanes (5α-20-one EIA; 5β-20-one EIA: Schwarzenberger et al. 1996c), two assays for 20α-OH-pregnanes (20α-dihydroprogesterone EIA; pregnanediol EIA: Schwarzenberger et al. 1991, 1993) and one assay for 20β-OH-pregnanes (Schwarzenberger et al. 1991) were used. In addition, suspected reference standards (Sigma, St. Louis, MO, USA or Steraloids, Wilton, NH, USA) were added to respective HPLC-fractions (of each peak, only the one containing maximal radioactivity) of the different metabolites, rechromatographed by the same HPLC system and checked for co-elution.

To obtain further evidence, HPLC fractions and the corresponding reference standards were applied to thin layer chromatography (TLC) plates (Silica gel 60, no. 5554, Merck, Germany) using a Linomat III (Camag, Muttenz, Switzerland). TLC was carried out in glass chambers using a mixture of tetrahydrofuran/*n*-hexane (3 + 7) or chloroform/acetone (93 + 7) as solvents. All plates were scanned (Instant Manager, Packard Instruments, Warrenville, IL, USA) and/or exposed to conventional X-ray films to determine the location (and amounts) of radioactive metabolites. Steroid standards were located by spraying with sulphuric acid/methanol (1 + 1) and heating the plates at 120°C for 10 min.

Evaluation of extraction procedures

Thoroughly homogenized faecal samples of an ewe, a mare and a sow (infused with ^{14}C-progesterone), voided about 2 h, 3 h and 1 day, respectively, after the maximum of faecal radioactivity (= second defecation afterwards), were used to evaluate different organic solvents for extracting the faecal metabolites. Five ml of methanol, ethanol or iso-propanol (different percentages ranging

from 0 to 100) were added to 0.5 g of wet faeces (n = 5). The samples were vortexed (30 min) and centrifuged (2500 × *g*, 15 min). Thereafter, two 0.5 ml aliquots of the supernatant were measured in the scintillation counter to determine the radioactivity after quench correction (Palme et al. 1996). The total concentration of ^{14}C in the samples (n = 10 of each) was measured with a sample oxidizer 307 (Packard Instruments, Warrenville, IL, USA) as described by Palme et al. (1996). The amounts of extractable radioactivity for each condition is expressed as a percentage of the total radioactivity (both calculated for 1 g faeces). For comparison, unmetabolized ^{14}C-progesterone (30 000 dpm/0.5 g faeces) was added to non-radioactive faeces of an ewe, a mare and a sow and extracted with 50%, 80% and 100% of either alcohol, as described above.

Steroid measurements in superovulated ewes

Ewes were synchronized with progestagen vaginal sponges (40 mg Fluorogestone acetate, Cronogest®, Intervet, Boxmeer, Holland) and superovulation was induced by PMSG (Intergonan®, Vemin, Kempen, Germany) two days before sponge removal (Kühholzer et al. 1996). Blood and faeces of ewes (n = 10) were collected daily. Plasma progesterone concentrations were determined according to Möstl et al. (1985). In faecal samples concentrations of 20α-OH-pregnanes (pregnanediol EIA) and 20-oxo-pregnanes (5α-20-one EIA) were measured by EIA as described by Schwarzenberger et al. (1993, 1996b respectively).

Statistical analysis

The coefficient of correlation between plasma progesterone and faecal progesterone metabolites was determined. Data of different extraction procedures are presented as mean ± SD. Statistical significance of the differences was tested by the Student's *t*-test.

Results

Recoveries (n = 12) of Sep-Pak® C_{18} cartridges and Sephadex® LH-20 columns were 99% ± 1% and 93% ± 5%, respectively, for ^{14}C-progesterone metabolites. In faeces, extracted directly for HPLC without chromatographic clean-up procedures, the remaining aquous phase contained almost no radioactivity (< 1–4%). Therefore, the amounts of different metabolites are expressed as a percentage of the total radioactivity measured after HPLC separations.

Metabolites of infused ^{14}C-progesterone were classified according to similar chromatographic elution patterns both in HPLC and TLC (as compared to reference standards). Further evidence was obtained by checking their immunoreactivity in different EIAs. The proportion of metabolites in not separated HPLC-peaks was estimated by TLC and subsequent quantification.

Mainly reduced metabolites (pregnanes) were formed after administration of progesterone. Unmetabolized progesterone was barely (only in sows; about 1%; Fig. 3) present, if at all, in faeces. Almost all possible 5α/β-reduced progesterone metabolites (n = 18; containing either an oxo, α-OH or β-OH group at positions C-3 and C-20) were detected in the faeces of ewes, pony mares and sows, but with species-specific differences concerning their quantities (Table 1, Figs 1–3). In ponies and ewes about 58% of the metabolites were pregnanediols, whereas in sows less polar metabolites accounted for about 72% of the radioactivity. The elution order of the faecal ^{14}C-progesterone metabolites (a slash indicates the not clearly separated ones) in our HPLC system (gradient 2) is given in the legend of Figure 1. In pigs, pregnanediones (Fig. 3) accounted for about 15% of the metabolites. Only in ponies and pigs one less polar peak (< 6%, unidentified) was detected. One further unidentified peak (lacking immunoreactivity in all EIAs; about 20% in ponies) was present between fraction (fr.) 48 and 56 in all species. In pigs, the last group of pregnanolones (9/10) and in sheep the first group of pregnanediols (11–13) accounted for about 20% and 24%, respectively, of the radioactivity. The main metabolite in ponies (about 37%) was 5α-pregnane-3ξ,20β-diol. Unfortunately the OH-group at position C-3 could not be characterized due to the lack of standards and is represented with a ξ. The most polar pregnanediols (between fr. 80 and 90) were 5β-pregnane-3α,20β-diol (Figs 1–3; 17) and 5β-pregnane-3α,20α-diol (18). Only minor amounts of some (about 4–8; unidentified) even more polar metabolites were found (less than 9% in total; results from gradient 1, data not shown). In sheep, pregnanes containing a 20α-OH group dominated (about 45%), whereas in ponies metabolites with a 20β-OH group (about 44%; mainly one 5α-pregnanediol) and in pigs progestagens with a 20-oxo group (46–58%) predominated. Likewise, species differences were found in the amounts of faecal 5α- and 5β-pregnanes. Both were excreted in larger amounts in sheep, whereas in ponies mainly 5α- reduced progestagens and in pigs more 5β-pregnanes were found.

The total amounts of ^{14}C and the percentage of recovered radioactivity (related to the total amount measured by combusting) after extraction with different

Table 1. Percentage of different progesterone metabolites present in faeces of sheep, ponies and pigs (median) classified according to the C-20 group or to their polarity

	Sheep	Ponies	Pigs
C-20 group			
20-oxo-pregnanes	24.1–29.8 (26.8)	10.0–14.5 (10.1)	46.0–58.1 (50.1)
20α-hydroxy-pregnanes	43.6–48.4 (44.5)	17.3–20.1 (18.5)	17.3–20.8 (18.9)
20β-hydroxy-pregnanes	24.8–28.1 (26.6)	43.7–44.6 (44.1)	22.6–25.8 (24.2)
Unidentified	1.0–1.4 (1.3)	23.7–27.4 (26.1)	0.5–8.5 (7.5)
Polarity			
Apolar	–	3.8–5.0 (4.2)	5.9–6.2 (6.0)
Pregnanediones	1.3–6.6 (3.6)	2.7–7.9 (2.8)	13.2–17.5 (15.0)
Pregnanolones	28.3–40.3 (37.0)	26.8–39.5 (33.3)	48.7–58.2 (52.3)
Pregnanediols	50.3–63.2 (58.1)	56.1–65.4 (58.2)	26.8–28.6 (27.7)

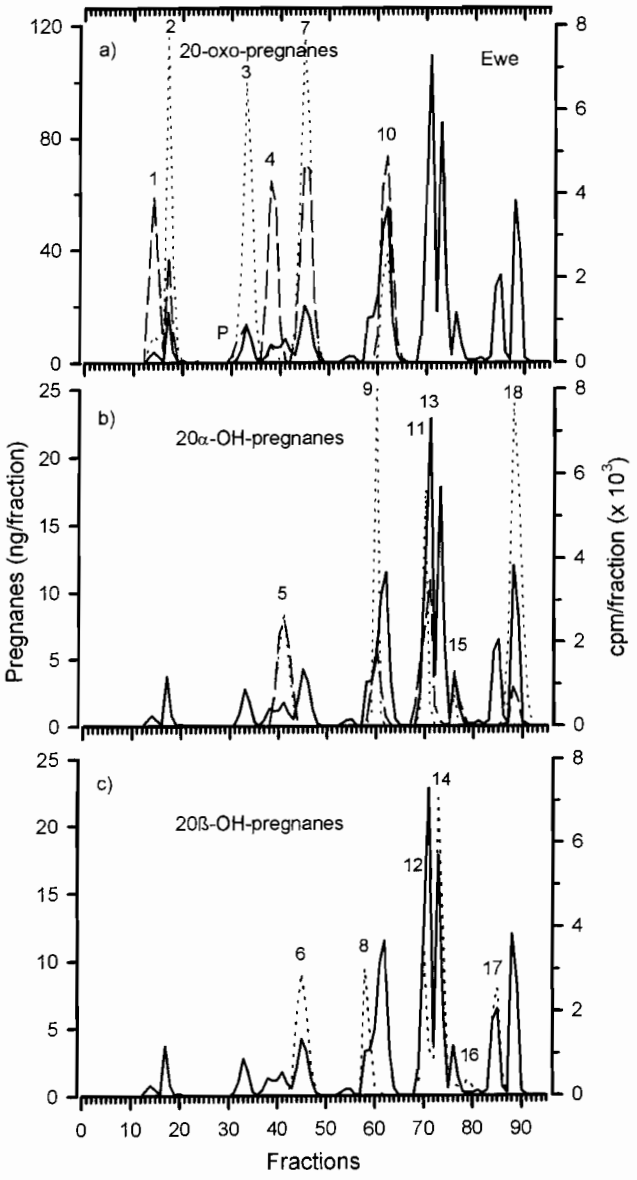

Fig. 1. High performance liquid chromatography (straight phase; gradient 2) separations of faecal ^{14}C-progesterone metabolites of an ewe. Fractions were analysed with EIAs for (a) 20-oxo-pregnanes (– – –5α-20-one EIA; ---- 5β-20-one EIA), (b) 20α-hydroxy-pregnanes (– – –20α-dihydroprogesterone EIA; ---- pregnanediol EIA × 10) and (c) 20β-hydroxy-pregnanes. ^{14}C-progesterone metabolites (——) in the same fractions were determined by liquid scintillation counting. 1/2 = 5α-/5β-pregnane-3,20-dione; P = progesterone; 3 = 5β-pregnane-3β-ol-20-one; 4/5 = 5α-pregnane-3α-ol-20-one/5α-pregnane-20α-ol-3-one, 6/7 = 5α-pregnane-20β-ol-3-one/5α-pregnane-3β-ol-20-one; 8 = 5β-pregnane-20β-ol-3-one; 9/10 = 5β-pregnane-20α-ol-3-one/5β-pregnane-3α-ol-20-one; 11/12/13 = 5β-pregnane-3β,20α-diol/5β-pregnane-3β,20β-diol/5α-pregnane-3β,20α-diol; 14 = 5α-pregnane-3ξ,20β-diol; 15 = 5α-pregnane-3α,20α-diol; 16 = 5α-pregnane-3ξ,20β-diol; 17 = 5β-pregnane-3α,20β-diol; 18 = 5β-pregnane-3α,20α-diol

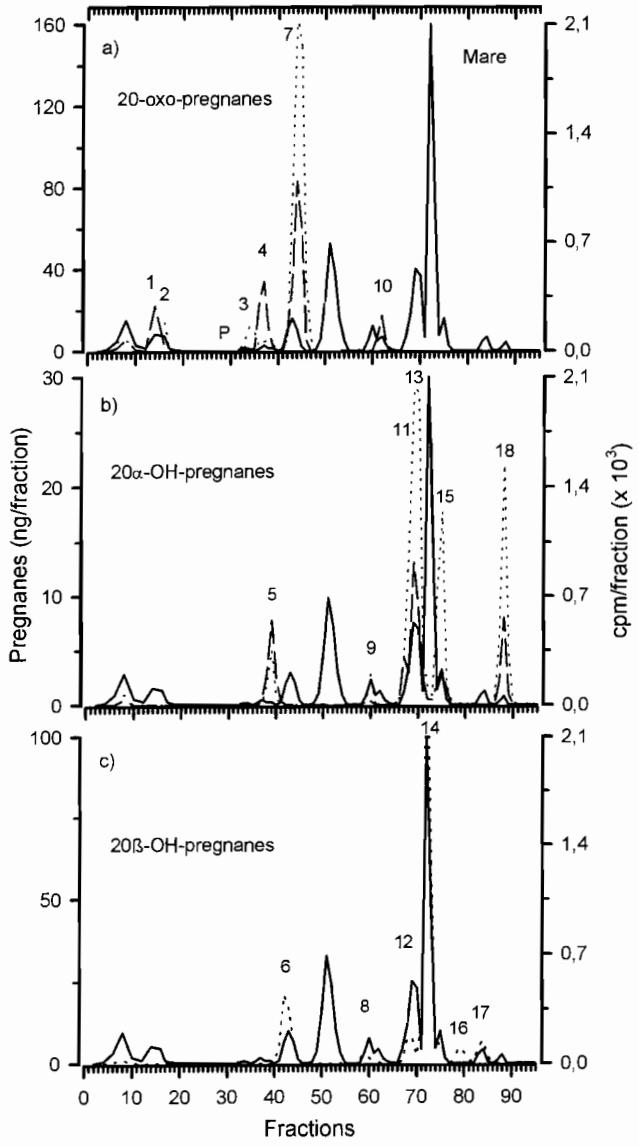

Fig. 2. High performance liquid chromatography (straight phase; gradient 2) separations of faecal ^{14}C-progesterone metabolites of a pony mare. Fractions were analysed with EIAs for (a) 20-oxo-pregnanes (– – –5α-20-one EIA; ---- 5β-20-one EIA), (b) 20α-hydroxy-pregnanes (– – –20α-dihydroprogesterone EIA; ---- pregnanediol EIA × 10) and (c) 20β-hydroxy-pregnanes. ^{14}C-progesterone metabolites (——) were determined by liquid scintillation counting. P; 1–18: see legend of Fig. 1 for explanation

concentrations of alcohols are shown in Figure 4. Extracting the faeces of ewes with 80% methanol yielded the highest proportion (88% ± 2%) of progesterone metabolites (but differences between 70 and 90% of methanol were not significant, $p > 0.01$). When methanol was applied to extract the faeces of ponies and pigs, the best results were obtained at 100%. However, utilizing ethanol or propanol (70–100% or 60–100%, respectively, in ponies and 70–90% or 60–80%, respectively, in pigs) gave equal or even better results ($p < 0.01$ only for 95% ethanol in ponies). Extractions of unmetabolized ^{14}C-progesterone added to non-radioactive faeces resulted in recoveries between 90.5 and 99.6% (ewe), 91.1 and 100.7% (mare) and 95.2 and 102.9% (sow), for either alcohol, with the exception of the 50% methanol, which provided lower recoveries (67.1%, 72.5% and 83.5%, respectively).

In superovulated ewes, the concentration of faecal

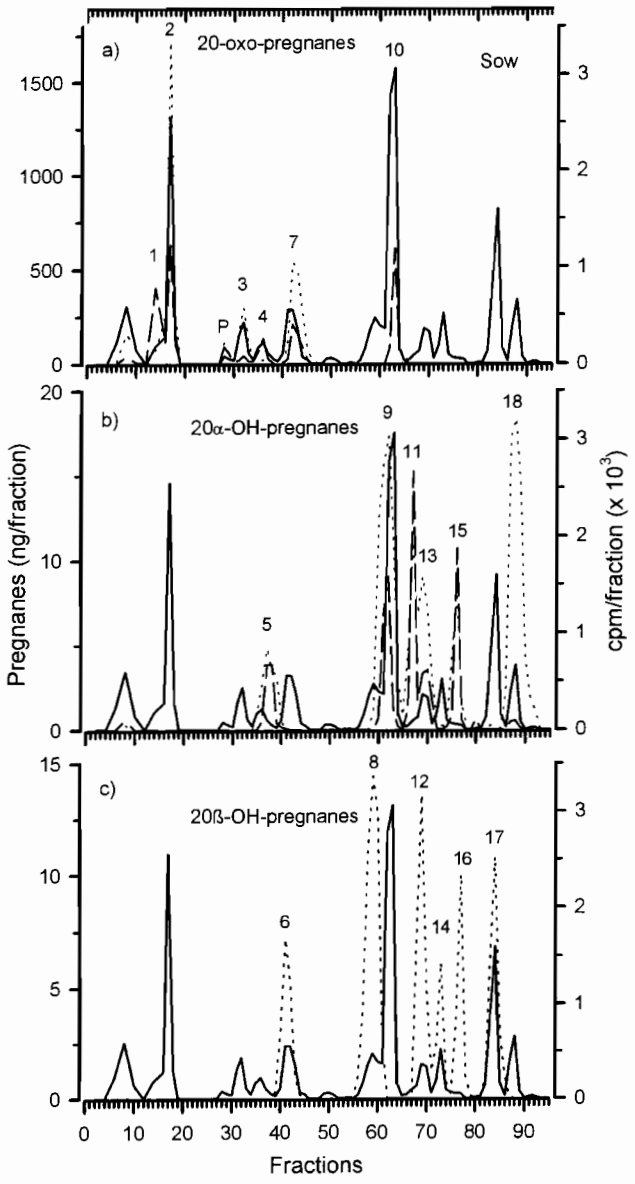

Fig. 3. High performance liquid chromatography (straight phase; gradient 2) separations of faecal ^{14}C-progesterone metabolites of a sow. Fractions were analysed with EIAs for (a) 20-oxo- pregnanes (– – –5α-20-one EIA; ---- 5β-20-one EIA), (b) 20α-hydroxy-pregnanes (– – –20α-dihydroprogesterone EIA; ---- pregnanediol EIA × 10) and (c) 20β-hydroxy-pregnanes. ^{14}C-progesterone metabolites (——) were determined by liquid scintillation counting. P; 1–18: see legend of Fig. 1 for explanation

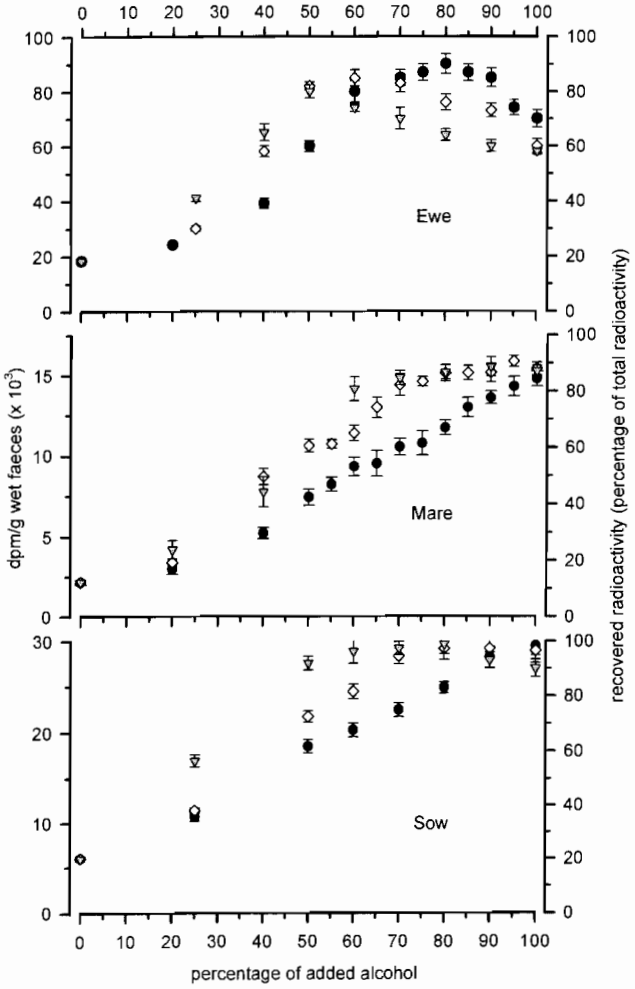

Fig. 4. Recovered amounts of metabolites (mean ± SD in faeces of an ewe, a mare and a sow, infused with ^{14}C-progesterone after extraction with different percentages of methanol (●), ethanol (◇) and isopropanol (▽)

progesterone metabolites reflected that of blood progesterone (Fig. 5). The coefficients of correlation ($p < 0.001$) between plasma progesterone and faecal 20-oxo- or 20α-OH-pregnanes were 0.77 and 0.85 ($y = 0.6x - 0.3$ and $y = 2.0x + 5.0$), respectively.

Discussion

The main objective of this study was to characterize the faecal metabolites of infused ^{14}C-progesterone in domestic livestock and to evaluate extraction procedures for faecal progestagens in order to standardize and advance faecal steroid analysis. The applied clean-up and extraction procedures of faeces proved suitable for subsequent chromatographic separations of the ^{14}C-steroids. With the modified HPLC gradient, a better separation of pregnanolones and pregnanediol was achieved. Besides interspecies differences concerning the percentage of excretion and the delay of faecal peak radioactivity (Palme et al. 1996), differences regarding the formed faecal metabolites of infused ^{14}C-progesterone were found in domestic livestock.

Progesterone is mainly metabolized by the liver and partly excreted via the bile into the intestine (Taylor 1971). During its sojourn in the gut microbial transformations, too, can alter the molecule (MacDonald et al. 1983). Besides conjugation in the liver and deconjugation in the gut (Taylor 1971), certain reductions at the steroid molecule take place. In agreement with others (for literature see Schwarzenberger et al. 1996a) 'authentic' ^{14}C-progesterone was almost absent in the faeces. Reduction of the double bond at C-5 took place yielding 5α- and/or 5β-derivatives. In addition reduction of the C-3 and/or C-20 keto group can form a 3α- or 3β- and/or a 20α- or 20α-hydroxy- (OH) moiety. Taking this into account, a total of 18 different pregnanes can be formed from progesterone. Our results demonstrate the presence

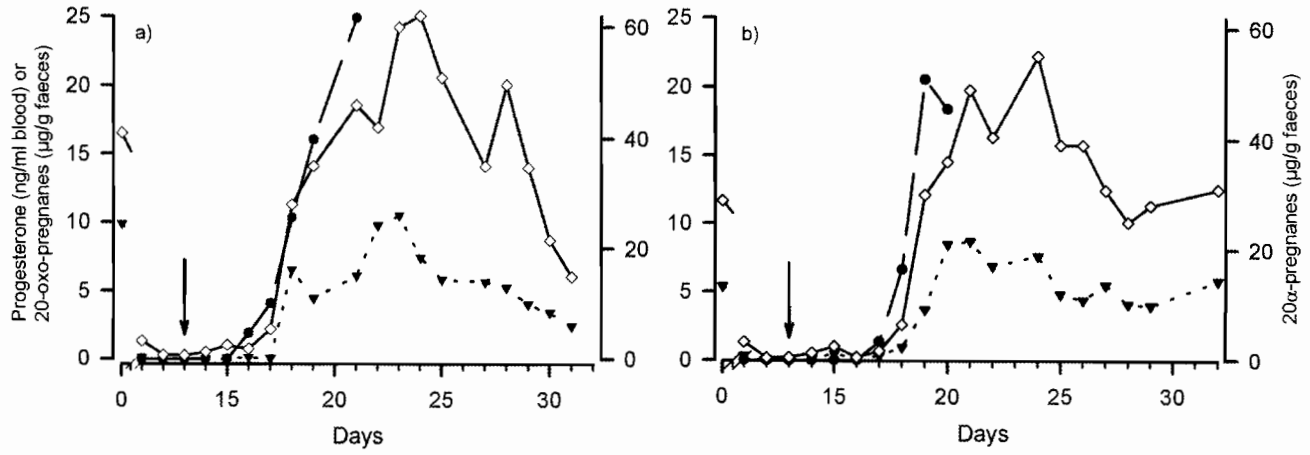

Fig. 5. Concentration of progesterone in the blood (– –●– –) and concentrations of 20-oxo-(· · · ▼ · · ·) and 20α-hydroxy-(—◇—) pregnanes in the faeces of two superovulated ewes (a, b; arrow indicates day of sponge removal)

of almost all of these ^{14}C-labelled pregnanes in the faeces after infusion of progesterone into ewes, mares and sows, but with significant differences regarding their quantities. Pregnanediones and several mono- and di-hydroxylated pregnanes were identified in the faeces of different animal species (Schwarzenberger et al. 1996a). However, the relative portions of these metabolites were only determined on the basis of their immunoreactivity in different EIAs. Consequently, values (even for the same group of pregnanes) obtained in different assays vary considerably, thus making comparison between different laboratories difficult. In contrast, the proportions of faecal metabolites of infused progesterone in this study are calculated on the basis of the amounts of radioactivity of each metabolite. Differences between faecal metabolites in sheep, ponies and pigs concerned the reduction at C-5 (mainly 5α- and 5β-pregnanes or 5α- or 5β-pregnanes, respectively) and (more significant for practical applications) at position C-20 (mainly 20α-OH or 20β-OH or 20-oxo, respectively). This information will be helpful for predicting the usefulness of different antibodies for measuring faecal gestagens in domestic livestock and probably in related zoo and wildlife animals.

Besides blood, urine and milk, faeces was also used as sample material for monitoring corpus luteum activity in cows, mares and pigs (Desaulniers et al. 1989; Schwarzenberger et al. 1992, 1996b; Hultén et al. 1995). Such measurements in the faeces of ewes during the estrous cycle have not been reported. Our results showed that an EIA for 20α-OH-pregnanes was most suited for measuring corpus luteum activity in superovulated sheep; a fact, which is in agreement with the dominance of this sort of metabolite after infusion of ^{14}C-progesterone. Schwarzenberger et al. (1996b) tested different EIA measuring 20-oxo- but not 20α-OH-pregnanes in faeces of cows. By measuring the later group of metabolites it might be possible to achieve better results in other ruminants as well.

As was found in the blood of pregnant mares (Holtan et al. 1991) and in the faeces of mares (Schwarzenberger et al. 1991, 1992), almost all of the ^{14}C-metabolites in ponies were also 5α-pregnanes. Most of them had a 20β-OH configuration. The small amounts of 5β-pregnanes could be formed by microbial metabolism. The direction of the 3-OH-group of the 5α-pregnane-3ξ,20β-diol, the main metabolite (about 40%) in mare faeces, could not be characterized due to the lack of standards. However, as antibodies raised against 5- reduced 20β-OH-pregnanes coupled at position C-3 can detect a 3α- as well as a 3β-OH group, this is, for practical purposes, unimportant. One unidentified metabolite, which eluted between fr. 48–56, was present in faeces of all species, but showed significant amounts only in ponies (20%). It could be a 6-hydroxylated pregnane. This would be in agreement with the lack of immunoreactivity in any EIA and the fact that such metabolites were found in sow urine (Jones and Erb 1968). In earlier investigations specific 20α- or 20β-dihydroprogesterone assays were applied in order to measure faecal progestagens to monitor estrous cycle and pregnancy in mares (Bamberg and Schwarzenberger 1990; Schwarzenberger et al. 1991, 1992). However, measuring 20β-OH-pregnanes might produce better results.

Monitoring of ovarian activity in sows was reported by measuring faecal progestagens with specific progesterone assays (Sanders et al. 1994; Hultén et al. 1995). As approximately one half of the metabolites of infused ^{14}C-progesterone in sow faeces were 20-oxo-pregnanes, these studies underestimated the actual amounts of faecal metabolites. In accordance with results obtained in cows (Schwarzenberger et al. 1996b) an assay for 20-oxo-pregnanes should yield higher values during the luteal phase and thus enhance the accuracy of this method in sows. This effect could be additionally increased by using a high percentage of alcohol (Fig. 4.) instead of buffer (Hultén et al. 1995) for extracting these apolar metabolites.

As species differences of faecal progesterone metabolites seemed to be more quantitative than qualitative, every group-specific immunoassay (for 20-oxo- or 20α-OH- or 20β-OH-pregnanes) can be used across species. However, only one (e.g. for 20-oxo-pregnanes in sows) will give the best results in a given species and thus the highest accuracy for non-invasive monitoring of the reproductive function. Schwarzenberger et al. (1996b,

1996c) emphasized the importance of the selection of antibodies for measuring 20-oxo-progestagens in cows and rhinoceroses. EIAs measuring several 20-oxo-pregnanes were superior to a progesterone-EIA. As almost all metabolites were 5- reduced pregnanes, this concept also applies for the measurement of 20α-OH- or 20β-OH-pregnanes.

Selection of extraction procedures is a serious concern, too, as faecal progesterone metabolites are a mixture of several pregnanes of different polarity. In most studies reported (for review see Schwarzenberger et al. 1996a), recoveries were based on the extraction of radio-labels (mainly ^{3}H-progesterone) added to the sample just before processing. As the results of this study have shown, in general a higher proportion of radioactivity could be extracted from faeces after *in vitro* added ^{14}C-progesterone compared with faecal samples containing the natural ^{14}C-progesterone metabolites. The reasons are that the metabolites are of different polarity and complex interactions between sample matrix and steroids, which affect extraction, are less pronounced with *in vitro* added steroids. Therefore, the recoveries reported in the literature probably do not reflect the real recovery of the progesterone metabolites present in the faeces, but are over- (or under-) estimates, depending upon the steroid added, the metabolites measured by the immunoassay and the species under investigation. The advantage of the recovery testing used in this study was that it was based on naturally metabolized, radio-labelled steroids infused into the animals. The results should be considered as estimates of the summarized recoveries of all progesterone metabolites. If a factor for the correction of the methodological losses for an extraction in combination with a certain EIA needs to be evaluated, a mixture of all immunoreactive metabolites or at least a group of representative markers of the relevant polarities has to be used. However, this is not practicable and not necessary as the extraction procedures which recover almost all natural ^{14}C-progesterone metabolites are suited for all assays.

A possible method for the extraction of unconjugated steroids is the use of diethylether. This yields high recoveries (Palme et al. 1996) but has the disadvantage of including an additional evaporation step. On the other hand, extraction procedures of faeces with buffer, especially in combination with immunoassays measuring apolar steroids (e.g. 20-oxo-pregnanes in sows; Hultén et al. 1995), seem to be less suited, due to low recoveries (about 20%). The use of a less polar solvent increases recovery and thus the accuracy of the applied EIA. In addition, blank values and the differences between measures during estrous and luteal phases have to be considered. For measuring faecal gestagens, the authors recommend (depending upon the species) extraction by simply suspending the faeces in 80–100% methanol or higher concentrated ethanol or iso-propanol. This has the advantage of being practicable (no evaporation step is needed) and yields high recoveries.

On the basis of the results obtained in this study, it is concluded that for measuring faecal progestagens the selection of both the antibody used in the immunoassay and the extraction procedure is of great importance. It is essential to use immunoassays with antibodies showing high cross-reactions with 5α- and 5β-pregnanes. In addition, depending upon the species (sheep, ponies or pigs), an antibody which detects metabolites of a certain C-20 group (20α-OH, 20β-OH or 20-oxo, respectively) should provide best results. Extraction procedures with 80% methanol (sheep), 100% methanol or 80–100% ethanol or iso-propanol (in mares and sows) yielded the highest recoveries. These results will help to standardize and improve faecal steroid analysis. In accordance with the results of the characterized ^{14}C-progesterone metabolites, non-invasive monitoring of corpus lutem function in ewes by measuring faecal pregnanes possessing a 20α-OH group proved most suited and the best results can be expected by measuring 20-oxo-pregnanes in sows and 20β-OH-pregnanes in mares.

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