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Excretion of infused ^{14}C-steroid hormones via faeces and urine in domestic livestock

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Abstract

The aim of this comparative study was to gain more information about the excretion of steroid hormones in farm animals. This should help to establish or improve non-invasive steroid monitoring procedures, especially in zoo and wildlife animals. Over a period of 4 h the ^{14}C-steroid hormones (3.7 MBq) progesterone (three females), testosterone (three males), cortisol and oestrone (two males, two females) were infused intravenously in sheep, ponies and pigs. Faeces were collected immediately after defecation. Urine was sampled via a permanent catheter in females and after spontaneous urination in males. A total of $88 \pm 10\%$ (mean $\pm$ SD) of the administered radioactivity was recovered. Considerable interspecies differences were measured both in the amounts of steroid metabolites excreted via faeces or urine and the time course of excretion. Progesterone and oestrone in ewes, and progesterone in mares were excreted mainly in the faeces (over 75%). The primary route of excretion of all other ^{14}C-steroids was via the urine but to a different extent. In general, sheep showed the highest degree of faecal excretion and pigs the least. The highest radioactivity in urine (per mmol creatinine) was observed during the infusion or in one of the next two samples thereafter, whereas in faeces it was measured about 12 h (sheep), 24 h (ponies) or 48 h (pigs) after the end of the infusion. Thereafter the radioactivity declined and reached background levels within 2–3 weeks. In faeces, steroid metabolites were present mainly in an unconjugated form, but in blood and urine as conjugates. Mean retention time of faecal radioactivity suggested that the passage rate of digesta (duodenum to rectum) played an important role in the time course of the excretion of steroids. The information derived from this investigation could improve the precision of sampling as well as the extraction of steroids from

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the faeces. Furthermore, the study demonstrates that it should be possible to establish methods for measuring faecal androgen and cortisol metabolites for assessing male reproductive endocrinology and stress in animals.

Keywords: Sheep—endocrinology; Pig—endocrinology; Pony; ^{14}C-steroids; Faeces; Urine; Blood

1. Introduction

The reproductive activity and stress of animals can be assessed by measuring various steroid hormones (McDonald, 1980; Edquist and Stabenfeldt, 1989). In recent years emphasis has been placed on faeces as sample material. Faecal samples can be collected easily and the animals are not subjected to stress. These advantages are especially important for zoo and wildlife animals. Pregnancy diagnosis based on faecal oestrogen alone or in combination with faecal progestagen was carried out on mares (Möstl et al., 1983; Palme et al., 1989; Lucas et al., 1991; Schwarzenberger et al., 1991), ruminants (Möstl et al., 1984; Busch and Bamberg, 1990) and sows (Choi et al., 1987). It was also applied successfully to some zoo animals (Safar-Hermann et al., 1987; Bamberg et al., 1991). Monitoring of corpus luteum function by measuring progestagens in faeces of non-pregnant mares (Schwarzenberger et al., 1992) and sows (Sanders et al., 1994) has been described. In the male, faecal oestrogen can be used for the diagnosis of equine cryptorchidism (Palme et al., 1994). The determination of androgens can be used to monitor sexual maturity and the annual rhythms of endocrine changes in males. Elevated plasma cortisol concentrations are an indicator of stress (McDonald, 1980). As stress during blood sample collection may increase the values of corticosteroids, faeces are of interest as sample material for studying the stress level of animals. So far determination of androgen and cortisol metabolites in faeces has not been described.

Steroid hormones are quickly eliminated from the blood (e.g. Challis et al., 1973; Ganjam et al., 1975). They are mainly metabolised by the liver and excreted in the urine and bile as conjugates (Taylor, 1971). In the gut most of them are deconjugated by bacteria and partially reabsorbed. Such an enterohepatic circulation of steroids was described in sheep (Lindner, 1972; Carroll and Cox, 1972) and pig (Ruoff and Dziuk, 1994; Symonds et al., 1994). Steroids, which are not reabsorbed, are eliminated via faeces. In addition, specific intestinal bacteria can metabolise steroids (Taylor, 1971; Lindner, 1972; MacDonald et al., 1983).

Only a few studies regarding the excretion of infused steroids in farm animals have been reported. In sheep, oestrogens were excreted mainly (about 90%) via the faeces (Terqui et al., 1968; Adams et al., 1994), but in sows (Terqui et al., 1968; Brown et al., 1970) and one pregnant mare (Heard et al., 1954) excretion was via the urine. In the urine of one sow, 49% of injected progesterone was found (Schomberg et al., 1965) compared with only 25% in ewes (Stupnicki et al., 1969). After infusion of ^{14}C-cortisol in one ewe, Lindner (1972) reported that 40% of the given dose was voided via faeces, whereas 79% or 97% was found in the urine of two sows (Brown et al., 1970). In gelded horses about 45% of injected ^{14}C-testosterone was recovered in the urine (Houghton and Dumasia, 1979).

These results indicate marked species differences. However, in most of the studies only one or two animals were used and different experimental settings make interspecies comparison difficult. In addition, excretion of some important steroid hormones in sheep, ponies and pigs has not been reported at all. Except for a few studies (mainly on sheep) the amount of faecal radioactivity was not determined. Little is known about the time course of the excreted steroid hormones in urine. Only one report (sheep: Adams et al., 1994) described the time course in faeces.

The aim of this comparative study was to gain more information about the excretion of steroids in domestic livestock. In addition to ruminants (sheep) and hindgut-fermenting herbivores (ponies), pigs were chosen, as they are also monogastric with simple alimentation. Domestic species were used as animal models for endangered zoo and wildlife species. To allow representative comparisons, studies in all species were performed in a similar way. Information derived from this study should help to establish or improve methods for monitoring the reproductive status or stress levels of animals by using non-invasive sampling techniques. Therefore, the proportion of excretion via faeces and urine, the time course of steroid excretion, the portion of conjugated versus free steroids in the excreta and the distribution within the faecal samples were studied after infusion of the steroid hormones progesterone, testosterone, cortisol and oestrone (all ^{14}C-labelled).

2. Materials and methods

2.1. Animals

A total of six (three of each sex) mature, healthy animals of each species from the research farm of the Veterinary University of Vienna were used. Austrian mountain sheep (56–70 kg) were 1–1.5 years old (one ewe was 4 years old). Shetland, bastard ponies (150–220 kg) were 2–2.5 years old. Pigs (German Edelschwein; 160–187 kg) were about 1 year old. During the experiment (4–7 days) the animals were kept in a metabolic cage (Fischer, 1993) or in a special box (Schildorfer, 1994) in the isotope laboratory of the Institute of Physiology (Veterinary University of Vienna). Afterwards the animals were transferred to a stall at the clinics.

Sheep and ponies were fed with the same, second cut batch of hay (approximately 1.5–2 kg and 5 kg day^{-1}, respectively). Pigs were fed twice daily with a total of about 2.5 kg day^{-1} of a commercially available complete diet based on cereals (supplemented with vitamins and minerals). Animals drank water ad libitum and had access to a lick block of mineral salts. Liver and kidney functions were determined before the experiment started by measuring activities of diagnostically relevant enzymes, total bilirubin, urea and creatinine in serum. The health status of the animals was checked daily by clinical examination of pulse, body temperature and respiratory rate. All animals remained healthy throughout the experiment.

2.2. Administration of ^{14}C-steroids

Radiolabelled progesterone (and oestrone in sheep) was administered to three females, testosterone to three males, oestrone (ponies and pigs) and cortisol to two males

Table 1
Total recovered radioactivity (TRR), proportion of excretion via faeces, the delay (h) of the maximal concentration of radioactivity in urine ($mmol^{-1}$ creatinine) and faeces and the mean retention time (MRT) of faecal radioactivity after infusion of ^{14}C-steroid hormones in sheep

Hormone	Infusion No.	Animal No.	Sex [a]	TRR (%)	Faeces [b] (%)	Maximum (h) [c]		MRT (h)
						Urine	Faeces	
Progesterone	2	2	f	71.3	76.8	0.8	9.0	20.6
	5	5	f	78.8	83.8	0.8	9.3	19.4
	9	3	f	67.4	69.8	1.0	15.6	22.8
	12	2	f	86.4	76.5	0.9	11.0	18.9
Mean ± $SD_{(n-1)}$				76.0 ± 8.4	76.7 ± 5.7			
Testosterone	1	1	m	93.5	47.7	−1.4	11.3	16.1
	4	4	m	88.3	38.4	0.3	19.6	27.9
	11	6	m	88.6	46.3	−1.4	9.2	12.6
Mean ± $SD_{(n-1)}$				90.2 ± 2.9	44.1 ± 5.1			
Cortisol	3	3	f	99.8	26.5	0.1	11.8	19.9
	6	6	m	88.3	24.5	4.3	14.8	20.8
	8	1	m	92.2	27.1	−0.5	8.9	11.9
	13	5	f	99.2	35.1	−0.8	10.0	15.8
Mean ± $SD_{(n-1)}$				94.9 ± 5.6	28.3 ± 4.7			
Oestrone	7	2	f	86.1	91.6	0.3	10.9	18.9
	10	5	f	91.1	96.1	−0.6	9.0	14.1
	14	3	f	101.0	78.0	0.2	12.5	18.8
Mean ± $SD_{(n-1)}$				92.8 ± 7.6	88.6 ± 9.5			
Mean ± $SD_{(n-1)}$ of all infusions				88.0 ± 10.0			11.7 ± 3.1	18.5 ± 4.2

[a] f, female; m, male.
[b] Portion (%) of recovered radioactivity excreted via faeces.
[c] Delay time (h) of maximal concentration ($mmol^{-1}$ urinary creatinine or kg^{-1} faeces) of radioactivity after the completion of infusion (values with a minus sign represent hours before the end of infusion).

and two females (Tables 1–3). ^{14}C-steroids were infused at times when the endogenous hormones were present. In females, ^{14}C-progesterone was administered during the luteal phase and oestrone during the oestrus period. The interval between the infusions to the same animal was about 6 weeks. In sheep, oestrone was chosen as the oestrogen for ease of comparison with ponies and pigs, and because it is predominant during pregnancy. Conversion of oestradiol-17β to oestrone is documented in sheep (Kazama and Longcope, 1972) and neither oestrogen differed in its excretion pattern as reported by others (Terqui et al., 1968).

One infusion of progesterone (Infusion 2) contained only 1 MBq and was repeated later in the same animal with the usual dose (Infusion 12). In Infusion 15 (testosterone), which was repeated (Infusion 29) and Infusion 18 (cortisol), sampling was stopped too early. The two latter administrations were not included in the calculation of mean (± SD) values of total recovered radioactivity and the percentage of excretion via faeces and urine.

Table 2
Total recovered radioactivity (TRR), proportion of excretion via faeces, the delay (h) of the maximal concentration of radioactivity in urine ($mmol^{-1}$ creatinine) and faeces and the mean retention time (MRT) of faecal radioactivity after infusion of ^{14}C-steroid hormones in ponies

Hormone	Infusion No.	Animal No.	Sex [a]	TRR (%)	Faeces [b] (%)	Maximum (h) [c] Urine	MRT Faeces	(h)
Progesterone	40	17	f	70.0	79.8	0.2	26.8	34.8
	41	16	f	67.8	75.2	0.4	22.7	32.6
	43	18	f	71.2	68.8	0.3	21.9	37.3
Mean ± $SD_{(n-1)}$				69.7 ± 1.7	74.6 ± 5.5			
Testosterone	30	13	m	92.7	31.2	1.2	27.4	34.6
	32	15	m	95.1	33.4	−1.4	20.3	26.8
	34	14	m	92.2	18.5	1.0	18.8	21.5
Mean ± $SD_{(n-1)}$				93.3 ± 1.6	27.7 ± 8.0			
Cortisol	33	13	m	96.3	44.8	5.0	24.7	27.5
	36	15	m	98.3	47.9	3.4	29.1	34.7
	35	16	f	96.3	36.3	1.0	32.0	33.8
	38	17	f	95.8	36.2	1.0	18.1	37.5
Mean ± $SD_{(n-1)}$				96.7 ± 1.1	41.3 ± 6.0			
Oestrone	31	14	m	96.1	1.5	0.9	17.9	24.9
	37	13	m	92.6	1.3	3.2	26.7	28.0
	39	16	f	96.5	4.0	−0.9	18.1	22.0
	42	18	f	95.3	2.0	0.3	18.8	27.8
Mean ± $SD_{(n-1)}$				95.1 ± 1.8	2.2 ± 1.2			
Mean ± $SD_{(n-1)}$ of all infusions				89.7 ± 11.0			23.1 ± 4.7	30.3 ± 5.4

[a] f, female; m, male.
[b] Portion (%) of recovered radioactivity excreted via faeces.
[c] Delay time (h) of maximal concentration ($mmol^{-1}$ urinary creatinine or kg^{-1} faeces) of radioactivity after the completion of infusion (values with a minus sign represent hours before the end of infusion).

All ^{14}C-steroids were obtained from New England Nuclear (Dreieich, Germany). Immediately before the infusions the purity (over 98%) of ^{14}C-oestrone (NEC-512; specific activity: 1.9 GBq $mmol^{-1}$), ^{14}C-progesterone (NEC-081; 2.1 GBq $mmol^{-1}$), ^{14}C-testosterone (NEC-101; 2.0 GBq $mmol^{-1}$) and ^{14}C-cortisol (NEC-163; 2.2 GBq $mmol^{-1}$), was checked by thin layer chromatography (Silicagel 60, Merck, No. 5554; chloroform + acetone = 93 + 7 or 50 + 50 for cortisol only).

The ^{14}C-steroids (3.7 MBq = 0.1 mCi) were administered in 100 ml sterile 0.9% NaCl solution containing 10% (v/v) ethanol without sedating the animals. The infusions lasted for approximately 4 h using a canulation (Vasocan-Braunüle®, 0.8 mm × 25 mm, Melsungen, Germany) of the vena auricularis (sheep and pigs) or the vena jugularis (ponies) and a peristaltic pump (Model 101U/R, Co. Verder, Düsseldorf, Germany). A

Table 3
Total recovered radioactivity (TRR), proportion of excretion via faeces, the delay (h) of the maximal concentration of radioactivity in urine ($mmol^{-1}$ creatinine) and faeces and the mean retention time (MRT) of faecal radioactivity after infusion of ^{14}C-steroid hormones in pigs

Hormone	Infusion No.	Animal No.	Sex [a]	TRR (%)	Faeces [b] (%)	Maximum (h) [c]		MRT (h)
						Urine	Faeces	
Progesterone	16	8	f	72.5	33.7	1.1	113.3	116.5
	22	9	f	84.1	33.8	0.2	57.5	95.7
	23	10	f	79.2	34.1	0.7	91.8	125.5
Mean ± $SD_{(n-1)}$				78.6 ± 5.8	33.9 ± 0.2			
Testosterone	15	7	m			−0.1	43.0	45.5
	20	11	m	96.1	15.4	−0.5	49.4	60.2
	21	12	m	92.9	12.3	5.8	43.3	53.8
	29	7	m	95.6	15.5	19.8	24.4	37.3
Mean ± $SD_{(n-1)}$				94.9 ± 1.7	14.4 ± 1.7			
Cortisol	18	10	f			0.1	100.3	109.1
	24	7	m	84.3	11.0	−0.3	21.5	29.4
	26	9	f	96.5	5.1	1.0	48.2	59.8
	27	11	m	97.4	4.5	10.0	50.0	59.1
Mean ± $SD_{(n-1)}$				92.7 ± 7.3	6.9 ± 3.6			
Oestrone	17	9	f	89.8	2.5	−0.8	19.5	48.8
	19	7	m	83.7	3.9	−2.2	41.0	51.1
	25	12	m	75.7	4.2	19.1	43.7	51.4
	28	10	f	78.7	3.7	−1.9	80.1	85.5
Mean ± $SD_{(n-1)}$				82.0 ± 6.2	3.6 ± 0.7			
Mean ± $SD_{(n-1)}$ of all infusions				86.7 ± 8.6			48.2 [d]	59.1 [d]

[a] f, female; m, male.
[b] Portion (%) of recovered radioactivity excreted via faeces.
[c] Delay time (h) of maximal concentration ($mmol^{-1}$ urinary creatinine or kg^{-1} faeces) of radioactivity after the completion of infusion (values with a minus sign represent hours before the end of infusion).
[d] As the values are not normally distributed the median is given.

1.5 m teflon capillary tubing (No. 10-0041-01; Pharmacia, Uppsala, Sweden) was used, since compared with other tubing materials tested (silicone rubber, polyvinyl chloride) teflon showed minimal absorption of ^{14}C-steroids. Only the part in the pump consisted of silicone rubber.

2.3. Collection of samples

The animals were observed continuously for the first 48 h and then at intervals of 2–3 h (with longer intervals up to 10 h during the night) for the next 2–5 days. In females, urine was collected through inflatable catheters (Nos. 12, 30 and 18; Co. Ruesch, Vienna, Austria) to avoid contamination of faeces with urine and to minimise any loss of urine. Urine collections were made at 20–30 min intervals during infusion, at 60–90 min intervals until 8 h and then at every 2–3 h until 48 h after the start of

infusion, respectively. In males, urine was collected with a detachable collection apparatus comparable to that developed by Veenhuizen et al. (1984) for steers. During the first 2 days, urine samples of males and all faecal material of all animals were taken immediately after voidance. Afterwards, samples were collected in intervals as indicated above. In some infusions of sows, faecal sampling was continued until Day 14 after infusion. Only in cases of constipation in pigs was any attempt made to collect small samples from the rectum. Single urine and faecal samples were taken prior to infusion of ^{14}C-steroids to determine background levels of radioactivity and after 1, 2 and 3 weeks.

In addition, blood was sampled (only in sheep and ponies) in a similar pattern as the urine of females until 8 h after the start of infusion. Another two samples were collected during the next 2 days. Blood samples ($n = 310$) were taken from the vena jugularis (opposite side from infusion) by sterile disposable needle (1.5 mm × 5 mm) into EDTA-coated tubes and centrifuged at 2500 × g for 15 min. Plasma, urine ($n = 1465$) and faecal samples ($n = 1329$) were stored at −20°C. Aliquots of samples (see below) for measuring the radioactivity were taken immediately after collection.

2.4. Measurement of radioactivity and calculations

Duplicates of plasma samples (0.25 ml) or urine samples (0.5 ml) were mixed with 10 ml scintillation fluid (Quicksafe A®, No. 1008000, Zinsser Analytic, Maidenhead, UK) and measured in a liquid scintillation liquid-scintillation counter (Packard Tricarb 4640, Warrenville, IL, USA). Portions of 0.5 g per 10 g wet faeces (sheep) or 0.5 g per 50 g of homogenised, wet faeces (ponies and pigs) of each sample were used to determine radioactivity in the faeces. Samples ($n = 18\,400$) were extracted with 2 ml distilled water and 3 ml methanol. After shaking (30 min), the mixture was centrifuged at 2500 × g for 15 min. A 0.5 ml aliquot of the supernatant (in duplicate) was measured in the scintillation counter. To improve the recovery of apolar steroid metabolites a further extraction step was included, using a higher percentage of methanol. Therefore, 2.5 ml of the supernatant were discarded and another 3 ml methanol added to the remainder and extracted as before. Both steps were taken into account to determine total radioactivity of faecal samples.

As in ponies, only small amounts of radioactivity were present in faeces after infusion of oestrone, the concentration of ^{14}C in these samples was determined with a sample oxidiser 307 (Packard Instruments, Warrenville, IL, USA). Therefore, up to three portions (0.6 g) of homogenised faecal samples were combusted and the amount of radioactive CO_2 in filter papers determined with a beta-counter.

Quench correction was performed using ^{14}C-standards and spectral index of the sample (SIS) values, which were calculated using the beta-counter. Radioactivity was expressed in disintegrations per second (Bq) per kilogram faeces or per millimole of urinary creatinine. The concentration of creatinine in the urine samples was determined using a test-kit from Boehringer (No. 124192, Mannheim, Germany). In addition, the correlation between the radioactivity of urine samples related to creatinine or volume was calculated for the different infusions.

To evaluate the efficiency of quench correction and the recovery of the extraction procedure, ^{14}C-progesterone (dissolved in 0.5 ml methanol) was added at seven dilutions (1 + 1 with methanol) to 0.5 g non-radioactive faecal samples ($n = 8$ for each of the three species). In ponies, samples with added ^{14}C-testosterone and ^{14}C-cortisol were prepared in a similar way. Faeces were extracted as described above.

In ponies, the distribution of radioactivity within faecal balls was assessed by calculating the quotient between the amount of ^{14}C present in the outer and inner layers. Therefore, corresponding portions of 0.5 g from the outer parts (5 mm) and from the middle of faecal balls of nearly all defecations (over 15 Bq g^{-1}) after infusion of progesterone ($n = 184$), cortisol ($n = 202$) and testosterone ($n = 241$) were taken immediately after voidance and the radioactivity determined as described above. In addition the quotient of dry substances of paired samples from 70 faecal balls was calculated.

Amounts of ether soluble/insoluble steroids were determined too. A 0.5 ml aliquot of both urinary ($n = 10$ for each infusion) and plasma ($n = 6$ for each hormone and species; all taken after the end of the infusion) samples was extracted twice with 5 ml diethylether. After extraction of faeces ($n = 4$ for each infusion) the supernatants of the two extraction steps were combined, concentrated (to approximately 1.5 ml) and extracted with five times 5 ml diethylether. Radioactivity was measured in both the combined ether extracts and the remaining aqueous phases. In vitro ^{14}C-steroids were added to faecal samples taken before the infusions ($n = 5$ of each infused steroid). They were handled in the same way to test the efficiency of the extraction procedure for the unconjugated steroids.

The infusion vial and tubing were rinsed with ethanol after the infusions and checked for residual radioactivity. This portion was subtracted from the whole dose to calculate the actual amount of infused ^{14}C-steroids. The portions of ^{14}C-steroid metabolites excreted via faeces and urine are expressed as a percentage of the radioactivity recovered. The mean retention time (MRT) of the excretion of ^{14}C-steroids in faeces was calculated according to that of markers as described by Blaxter et al. (1956) using the following equation: $MRT = \Sigma m_i t_i / \Sigma m_i$, where m_i is the amount of radioactivity excreted at the ith defecation at the time t_i after the end of infusion ($i = 1,2,...n$). The end of the infusion period was chosen as reference point as the maxima in blood were observed near the end of the infusion. In cases where mean ($\pm$SD) values of all infusions are given, the normal distribution was verified with the Kolmogorov–Smirnov test. The Mann–Whitney U-test or Student's t-test (where appropriate) were applied to check for significant differences between groups using SPSS© (SPSS Inc., Chicago, IL, USA). If not otherwise indicated, the order of presentation is sheep, ponies and pigs throughout.

3. Results

An average of 88 $\pm$ 10% (mean $\pm$ SD) of the administered radioactivity was recovered (see Tables 1–3 for individual and group values). This did not differ between species (86.7–89.7%) but was lower for progesterone (74.8%) than for the other steroids (90.0–94.7%) for all infusions. The times needed to excrete 90% of the recovered radioactivity of a certain steroid in a species are given in Table 4.

Table 4
The time (mean ± $SD_{(n-1)}$) needed to excrete more than 90% of the total recovered radioactivity and the percentage (mean ± SD) of ether extractable (unconjugated) steroids in faeces, urine and blood plasma after infusion of ^{14}C-steroids in domestic livestock

Steroid	Species	$t > 90\%$	ether extractable ^{14}C (%)			
		(days)	In vitro added	After infusions		
			Faeces	Faeces	Urine	Plasma
Progesterone	Sheep	1.59 ± 0.09	99.8 ± 0.04	98.9 ± 0.71	1.6 ± 0.93	1.0 ± 0.83
	Pony	2.45 ± 0.34	99.9 ± 0.02	96.6 ± 1.10	1.7 ± 0.20	21.7 ± 3.27
	Pig	5.79 ± 0.76	99.0 ± 0.20	95.5 ± 0.50	6.9 ± 5.40	ND
Testosterone	Sheep	1.13 ± 0.32	99.0 ± 0.02	98.1 ± 0.99	3.6 ± 2.74	3.6 ± 2.80
	Pony	1.52 ± 0.48	99.7 ± 0.03	81.60 ± 2.8	2.1 ± 1.10	14.0 ± 6.25
	Pig	2.04 ± 0.14	99.3 ± 0.20	97.6 ± 0.60	6.7 ± 3.56	ND
Cortisol	Sheep	1.16 ± 0.32	92.4 ± 0.37	94.6 ± 2.61	5.5 ± 3.33	10.1 ± 7.21
	Pony	2.02 ± 0.27	96.8 ± 0.30	77.2 ± 4.60	13.6 ± 3.00	48.7 ± 6.40
	Pig	1.88 ± 1.80	93.4 ± 2.30	94.0 ± 1.80	6.1 ± 1.05	ND
Oestrone	Sheep	1.26 ± 0.09	99.6 ± 0.15	99.5 ± 0.27	3.1 ± 1.86	7.1 ± 4.08
	Pony	0.45 ± 0.09	ND [a]	ND [a]	1.5 ± 1.40	0.7 ± 0.35
	Pig	1.41 ± 0.72	98.7 ± 0.30	96.7 ± 2.20	3.0 ± 3.86	ND

[a] Not determined owing to low concentrations of radioactivity in faecal samples of infused ponies.

Table 5
The coefficient of correlation (r) between Bq l^{-1} urine and Bq $mmol^{-1}$ creatinine (mean ± $SD_{(n-1)}$), the recovery of ^{14}C-steroids added to non-radioactive faeces and the portion of radioactivity in faecal samples extractable after the first extraction step alone (1st ESA; % of total) in the in vitro pilot study and in faecal samples after infusion of ^{14}C-steroids (mean ± SD)

Steroid	Species	r	In vitro pilot study		After infusions	
		(urine)	Recovery (%)	1st ESA (%)	1st ESA (%)	n
Progesterone	Sheep	0.87 ± 0.12	90.3 ± 3.0	91.2 ± 2.4	89.2 ± 4.5	529
	Pony	0.90 ± 0.04	94.4 ± 2.2	93.2 ± 3.0	61.3 ± 6.3	1462
	Pig	0.79 ± 0.19	97.2 ± 3.4	97.0 ± 2.0	70.6 ± 5.9	1421
Testosterone	Sheep	0.98 ± 0.02	ND [a]	ND [a]	95.7 ± 5.0	401
	Pony	0.98 ± 0.01	98.7 ± 3.7	86.2 ± 5.1	62.9 ± 8.5	1982
	Pig	0.91 ± 0.07	ND [a]	ND [a]	46.5 ± 7.9	784
Cortisol	Sheep	0.89 ± 0.10	ND [a]	ND [a]	97.3 ± 5.1	455
	Pony	0.95 ± 0.07	94.5 ± 4.5	100.4 ± 3.3	93.2 ± 4.7	1975
	Pig	0.95 ± 0.07	ND [a]	ND [a]	79.5 ± 8.7	766
Oestrone [b]	Sheep	0.91 ± 0.05	ND [a]	ND [a]	92.9 ± 3.8	682
	Pig	0.77 ± 0.10	ND [a]	ND [a]	84.4 ± 7.2	610

[a] Not determined.
[b] Pony: 0.90 ± 0.11.

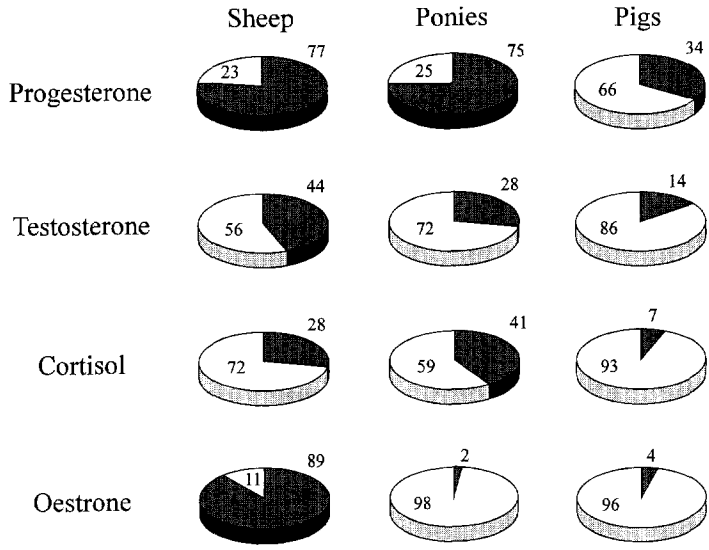

Fig. 1. Excretion of different ^{14}C-steroids via urine (○) and faeces (●) of sheep, ponies and pigs.

In an in vitro pilot study, evaluating the recovery of the extraction procedure, more than 90% of ^{14}C-steroids, added to non-radioactive faecal samples of sheep, ponies and pigs, could be recovered using the described extraction method (Table 5; $r = 1.00$ for

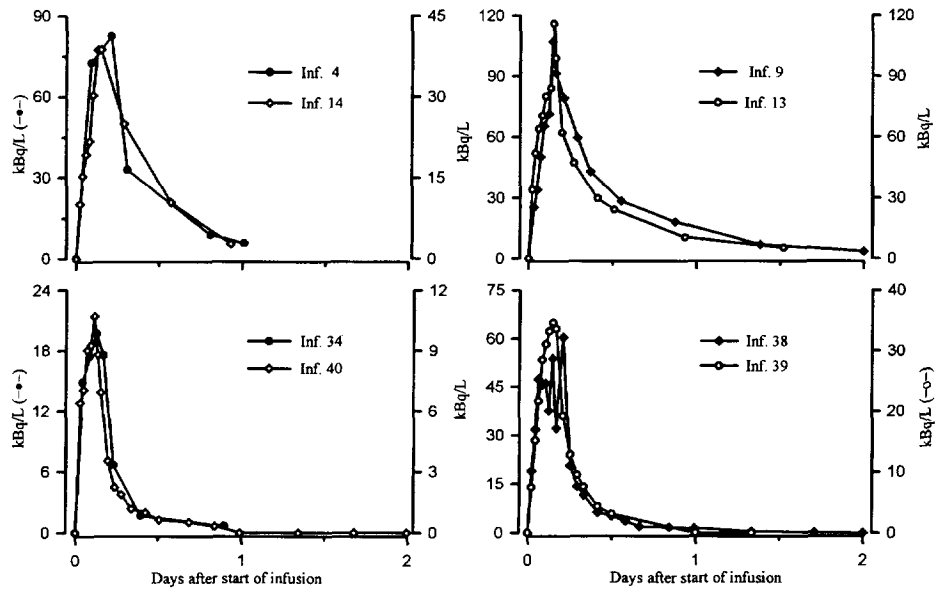

Fig. 2. Radioactivity in the blood of sheep (upper panels) and ponies (lower panels) after infusion of ^{14}C-progesterone (Infusions 9 and 40), ^{14}C-testosterone (Infusions 4 and 34), ^{14}C-cortisol (Infusions 13 and 38) and ^{14}C-oestrone (Infusions 14 and 39).

all). The coefficient of variation (*CV*) was between 1.07 and 6.43%. The second extraction step improved recovery and minimised *CV*. This improvement of the extraction was even greater in faecal samples after infusions of ^{14}C-steroids (Table 5).

The percentages of excretion of different steroids via faeces and urine are presented in Fig. 1. Progesterone and oestrone in ewes and progesterone in mares were excreted mainly in the faeces (over 75%). All other administered steroid hormones were eliminated preferentially via the urine but to different extents (Tables 1–3). In faeces, steroid metabolites were present mainly in an unconjugated form, but in blood and urine as conjugates (Table 4).

In blood, radioactivity peaked around the end of the infusion period (Fig. 2) and quickly disappeared afterwards. In ponies maximal concentrations were lower after infusions of progesterone (5–11 kBq l^{-1}) and testosterone (11–20 kBq l^{-1}) than after infusions of cortisol (57–87 kBq l^{-1}) and oestrone (25–73 kBq l^{-1}).

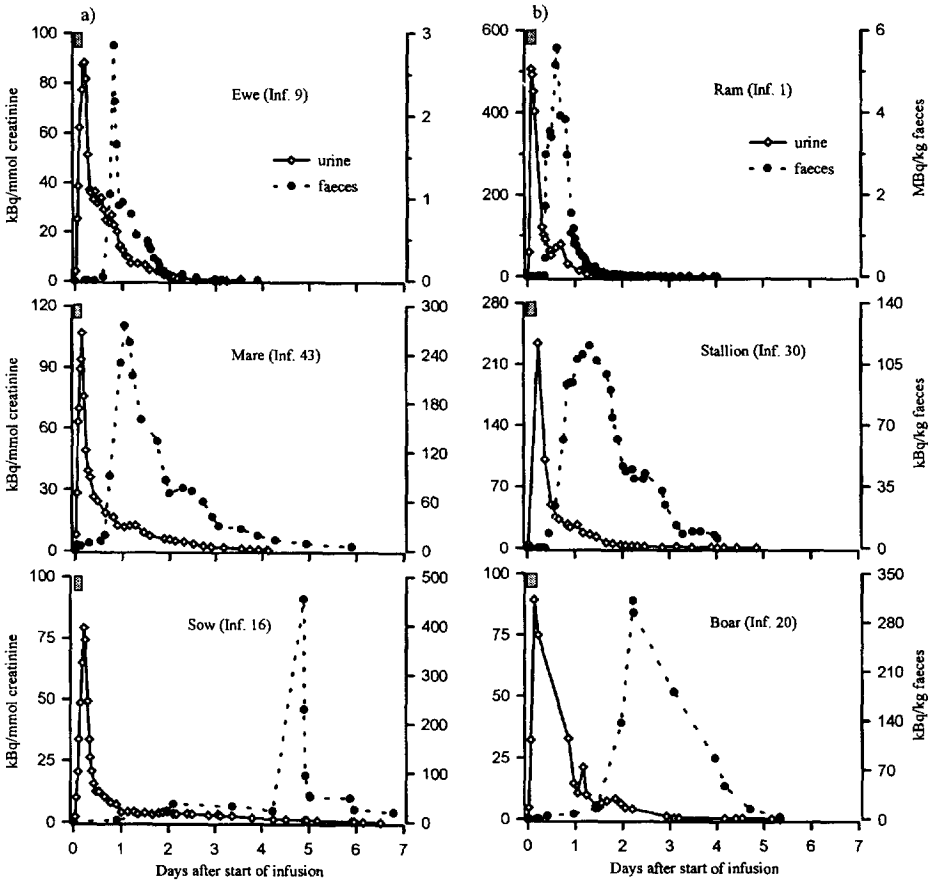

Fig. 3. Radioactivity after infusion of ^{14}C-progesterone (a) and ^{14}C-testosterone (b) in urine and faeces of one sheep (Infusions 9 and 1), pony (Infusions 43 and 30) and pig (Infusions 16 and 20). The hatched box indicates the time of infusion.

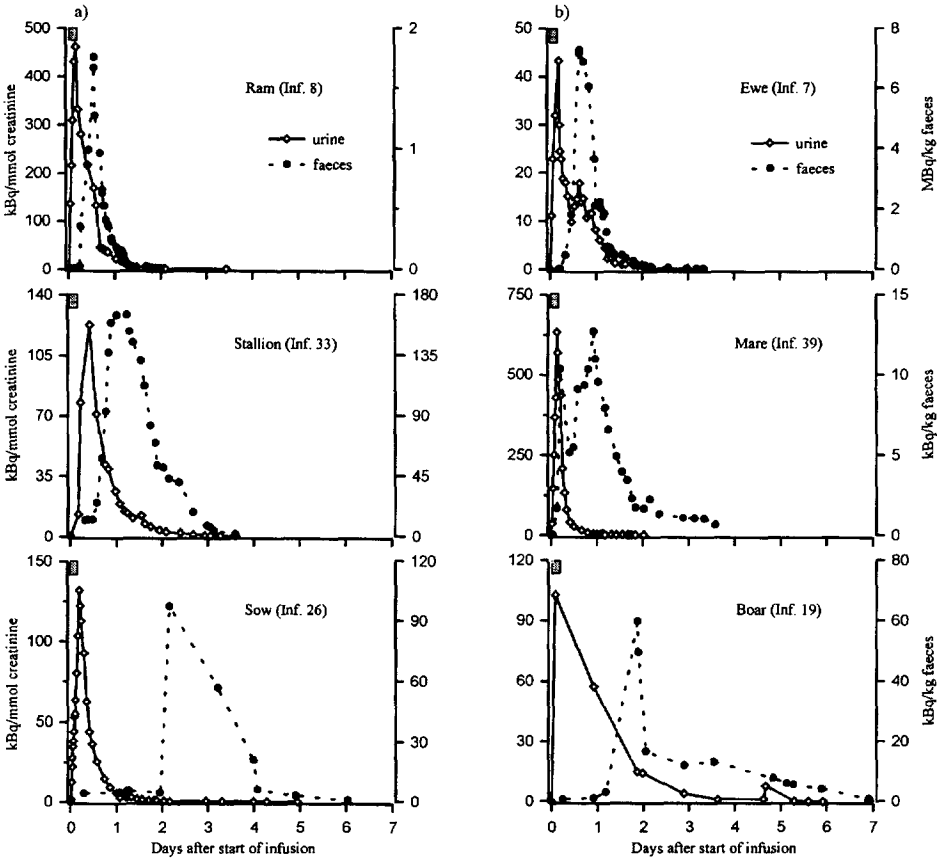

Fig. 4. Radioactivity after infusion of ^{14}C-cortisol (a) or ^{14}C-oestrone (b) in urine and faeces of one sheep (Infusions 8 and 7), pony (Infusions 33 and 39) and pig (Infusions 26 and 19). The hatched box indicates the time of infusion.

The different time courses of the excreted radioactivity in urine and faeces are shown individually. Graphs of representative infusions of each hormone are given in Figs. 3 and 4 (note different scales of Y axes). An increase in radioactivity was detected in the first urinary sample after the infusion started. In urine, maximum radioactivity ($mmol^{-1}$ creatinine) was observed during the infusion or in the first sample afterwards. Only with infusion of progesterone (in ewes and sows) or cortisol (ponies) were the maxima observed in the first or second sample after the infusions were completed. Maximal faecal concentrations were measured about 12 h, 24 h or 48 h, respectively, after the end of the infusion (Tables 1–3; Fig. 5). From then on, the radioactivity declined and reached background levels within 2–3 weeks. In contrast to the increase of radioactivity, the decline was protracted in blood, urine and faeces. During this phase additional minor peaks of faecal or urinary radioactivity were observed in some infusions (Figs. 3 and 4).

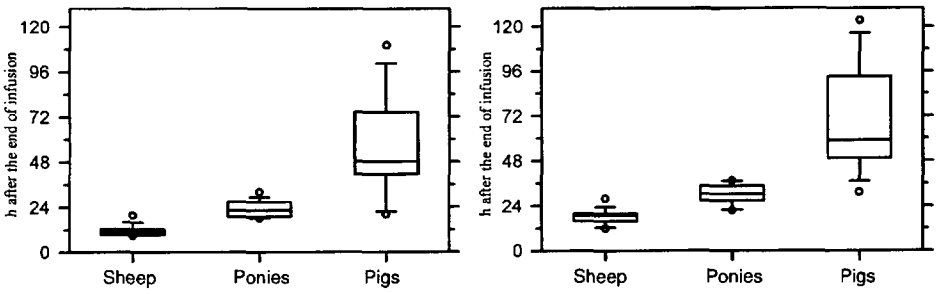

Fig. 5. Boxplots of delay times (h) of maximal concentration (left) and mean retention times (MRT; right panel) of faecal radioactivity in sheep, ponies and pigs.

In ponies, the skewed nature of this elimination curve was more pronounced in blood and urine than in faeces. In some infusions (Infusions 32, 36, 38–43), small peaks of radioactivity around the end of the infusion period were found in the faeces. Boxplot diagrams of both, delay times of maximal faecal radioactivity and MRT in all investigated species are given in Fig. 5. In general, compared with boars, sows showed longer intervals until peak concentrations of faecal radioactivity were reached and higher MRT.

Coefficients of correlation between the radioactivity of urine samples related to creatinine or volume are given in Table 5. Females (especially mare 16, which produced large amounts of urine 4–8 h after start of the infusions) showed lower coefficients of correlation. Higher concentrations of radioactivity were found in the outer layers of the faecal balls from ponies, than in the inner ones (Fig. 6).

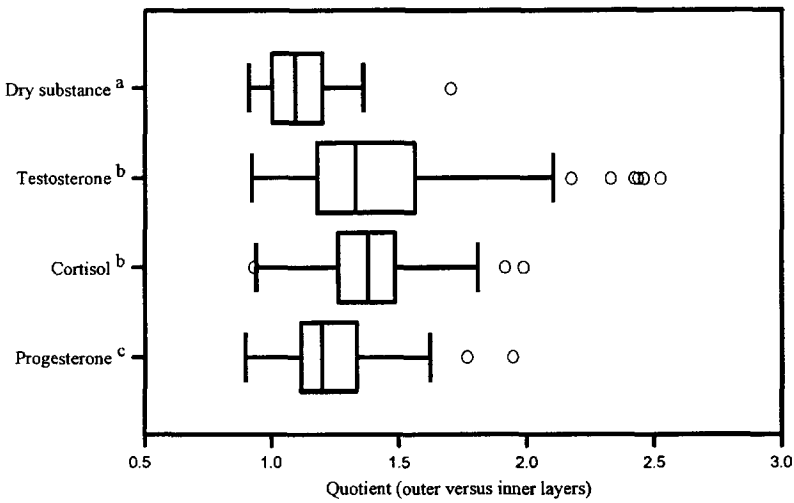

Fig. 6. Boxplots of quotients (outer vs. inner layers) of dry substance and radioactivity in faecal balls after infusion of ^{14}C-steroids (outliers: o). Groups with different superscripts differ ($P < 0.01$).

4. Discussion

^{14}C-steroids were used to minimise possible loss of radioactivity of ^{3}H-steroids during hydroxylation or aromatisation. In addition, quench correction of ^{3}H-marked material in faecal samples containing low amounts of radioactivity is harder to achieve. The steroids were infused over a period of 4 h to mimic physiological conditions and at times when the corresponding endogenous hormones were present (except Infusions 7 and 14, in which an induction of oestrus was not successful). In this study, emphasis was placed on the time course of the excreted steroids in urine and faeces and the amount of metabolites in the excreta. Only a small number of plasma samples was collected, as many authors have already reported metabolic clearance rates (e.g. Challis et al., 1973) and the half life (e.g. Paterson and Harrison, 1968; Ganjam et al., 1975) of different steroid hormones in blood of farm animals. In agreement with their findings, measurement of radioactivity revealed a quick elimination of steroids from the blood. In addition, high amounts of conjugated steroids in the blood (in mares even during infusion) indicate effective metabolisation. Lack of progesterone-specific binding proteins in the blood of mares (Ganjam et al., 1975) probably accounts for the low radioactivity after infusion of ^{14}C-progesterone. On the other hand, high concentrations of corticosteroid-binding globulins in horses (Seal and Doe, 1966) might explain the high amounts of radioactivity in the blood after the administration of ^{14}C-cortisol, delayed excretion in the urine and also the higher portion of unconjugated metabolites of the polar cortisol in blood and urine of ponies.

The collection of urine and faeces was extremely rigorous and perhaps accounted for the high recovery of infused ^{14}C-steroids. The reason for the lower amount of total recovered radioactivity after infusion of progesterone could be an overestimation of the dose of infused progesterone, if significant amounts of this apolar steroid were absorbed in the tubing (especially the silicone rubber part) as described by Bedford et al. (1972).

The different time courses of excreted steroid metabolites are shown individually (Figs. 3 and 4). In each species, mean ($\pm$SD) values of radioactivity in samples of all infusions of one hormone for a certain time period were not calculated, since the maximal concentrations and the time courses of excretion depended upon the intervals between infusions, and the amount of voided samples. Concentrations of faecal ^{14}C-steroid metabolites were highest in sheep, as smaller amounts of faeces were voided and in general a higher percentage of steroids was excreted via faeces.

In males, urine was sampled after spontaneous voidance and consequently the time of maximal radioactivity in urine could not be defined as exactly as in females. The urinary samples did not reflect the radioactivity of the urine actually produced but only the pooled concentration of ^{14}C for the whole period since the last urination (in boars often around 24 h) related to the time of voidance. This resulted in a delayed excretion of urinary radioactivity compared with females. This phenomenon was especially pronounced in one boar (Infusion 27), perhaps due to an incomplete emptying of the bladder. As this boar was used only once, this assumption could not be verified. On the other hand, differences in the concentration of produced urine, which could be observed in urine sampled via the catheter, would not be seen in males. This could account (at least in part) for the generally better correlation between radioactivity expressed per litre

of urine and per millimole of creatinine in these infusions. However, as the correlation was high in all infusions, relating the amount of urinary steroids to creatinine concentrations in longitudinal studies of one animal should be necessary only if urine is collected via catheter or in special cases, when for example urine-soaked snow or soil was used (Kirkpatrick et al., 1992).

It was possible to extract faecal samples of all infused steroids with good recovery using the procedures described in this paper. In faecal samples of ponies and pigs, only 61% and 71% respectively of the total extractable radioactivity of the progesterone metabolites (vs. 93% and 97% of in vitro added ^{14}C-progesterone), and an even smaller amount of ^{14}C (63% and 47%) after infusion of testosterone was recovered after the first extraction step. The second extraction step with a higher percentage of methanol significantly improved the recovery and minimised coefficients of variation. From this we conclude that a great portion of the faecal metabolites in these species was more apolar than the infused steroids (in pigs even after administration of cortisol). This is in accordance with the great portion of ether extractable radioactivity present in faecal samples. In ponies only, greater amounts (about one-fifth) of the faecal metabolites of cortisol and testosterone could not be extracted with diethylether and therefore probably consisted of polar unconjugated and/or conjugated steroids. The advantage of recovery testing with faecal samples after infusions of labelled steroid is that they contain the naturally occurring metabolites in physiological distribution. Therefore, the efficiency of extraction procedures for the particular steroid metabolites can be better evaluated and consequently the extraction procedures can be effectively improved. As labelled steroids added to non-radioactive faecal samples have been commonly used to study the efficiency of extraction procedures (e.g. ^{3}H-progesterone: Schwarzenberger et al., 1991), reported recoveries might have been overestimated. As a consequence the solvent system (80% methanol) described by Schwarzenberger et al. (1991) or that (distilled water) described by Sanders et al. (1994) is too polar. A higher proportion of methanol (or a more apolar alcohol) for extracting faecal samples for measuring progestagens (and perhaps androgens) will yield a better recovery of steroid metabolites.

Radioactivity was detected even in the first urine sample after the start of the infusion. Other authors (Schomberg et al., 1965; Brown et al., 1970; Taylor, 1971; Lindner, 1972; Carroll and Cox, 1972) also observed such an immediate appearance of radioactivity in the urine. Delayed urinary excretion of ^{14}C-progesterone (especially in sheep and pigs) might be due to the fact that progesterone is apolar (compared with the other steroids tested) and therefore was stored in adipose tissue for some time. In general, a greater urinary excretion of a steroid results in its quicker elimination. The occurrence of additional peaks of radioactivity after the maximum in urine and faeces in some infusions could be caused by an enterohepatic circulation, which was demonstrated for steroids in sheep (Carroll and Cox, 1972; Lindner, 1972) and pigs (Ruoff and Dziuk, 1994; Symonds et al., 1994). Coombe and Kay (1965) suggested that the fluctuation in the concentration of markers of the digesta (stained straw) during this phase could be caused by the fact that digesta were stored and mixed to some extent in the large intestine (caecum). This could also account for the observed peaks of faecal radioactivity in our study and for the fact that in ponies, the usually skewed nature of the excretion curve was not so pronounced in faeces.

In some infusions in ponies small peaks of faecal radioactivity around the end of the infusion were observed. As they appeared too soon to result from biliary secretion, it is possible they originate from the mucosa of the distal portion of the intestine. The higher concentrations of radioactivity in the outer layers of the faecal balls compared with the inner ones underlines this postulate. Mere differences in dry matter content can be excluded, as the quotients of radioactivity were higher than those of dry substances ($P < 0.01$). In addition, great variations of radioactivity within a faecal sample from pigs were found. In some infusions, portions containing low levels of radioactivity and some showing peak concentrations of metabolites were present in the same defecation. As a consequence, faeces of a defecation were divided into subsamples (in sequence of voidance). All subsamples were thoroughly homogenised prior to the determination of radioactivity. It seems advisable to routinely homogenise faecal samples of pigs or ponies before measuring steroid metabolites, or to take the portions from defined sites within the faecal balls (e.g. from the centre). This is not necessary in sheep, because variations within one sample were small.

Warner (1981) reviewed the literature on the rate of passage of digesta through the gut of mammals and birds and favoured measuring the mean retention time (MRT). Retention times are affected by a number of factors and mean retention time is quite variable in animals (Warner, 1981). As steroid metabolites are excreted via the bile into the gut and are transported with the digesta we calculated MRT in addition to the maximum of radioactivity in faeces. The observed values for MRT lay within the range of reported retention times (from the small intestine to the rectum) in sheep, ponies and pigs (Warner, 1981). We postulate that the rate of passage (duodenum to rectum) can give a rough estimate of the delay of steroid metabolites in faeces of animals. This is underlined by the fact that the observed faecal excretion patterns of the steroid metabolites paralleled those of markers used to determine retention times of the digesta (Coombe and Kay, 1965). No differences between infused steroid hormones in the time course of excretion could be found. This is in accordance with results of Wasser et al. (1994) in baboons and supports the stated postulate. As much information about that rate of passage has accumulated in domesticated, zoo and wildlife species (see review by Warner, 1981) this can be used for estimating delay times of faecal steroid excretion in various species.

After administration of ^{3}H-oestradiol-17β, Adams et al. (1994) found that excretion was slower in food-restricted ewes compared with animals given supplementary food, although the final amount of radioactivity excreted in the faeces was similar. The maximal concentration of faecal radioactivity in our study was reached even sooner, probably because of the larger volume of food consumed, which results in an accelerated passage through the gut. In addition, great individual differences were found (e.g. ewe 3 showed a slower faecal excretion of all three steroids than ewes 5 and 2). These differences were also observed by others (Adams et al., 1994) and account for the high variation in the results.

Constipation in some pigs (mainly sows) during the experiments and generally long intervals between defecations in all animals and urinations in boars (only once or twice a day) resulted in great variations in the delay time of maximal radioactivity in the excreta

and faecal MRT. Sows showed significantly ($P < 0.05$) higher levels of delay time of faecal peak radioactivity and mean retention time (MRT). However, these differences did not seem to reflect different metabolism and excretion of the steroids but mere differences in the rate of gut passage. This is underlined by the fact that all sows showing long delay times of faecal radioactivity had problems with constipation, reflected in low amounts of faecal bulk produced during the first few days after infusion. The sow (No. 15) with the greatest daily output of faeces (especially Infusion 17) showed the shortest delay times. The reason for the constipation in sows could have been the fact that the sows were irritated by the presence of the permanent catheters and therefore tried to delay defecation. As a consequence the time course of faecal radioactivity in boars probably reflected the physiological situation better than that in sows. A delayed excretion of progesterone metabolites via the bile into the gut probably contributed only to a minor extent to the delay in sows in these infusions.

In pigs, only minor individual variations of the percentage of excreted metabolites via faeces or urine and maximal concentrations of faecal radioactivity of a certain steroid were found. This is remarkable, as great differences in the time course of faecal radioactivity were observed. Therefore, concentrations of faecal steroid metabolites seemed quite stable in pigs. This is in accordance with Naletoski (1993), who did not find significant differences in faecal progestagen and oestrogen concentrations during early pregnancy in sows, regardless of whether animals were fed different diets, antibiotics or charcoal.

The infusion of ^{14}C-progesterone in animal 2 (Infusion 2), which was repeated (Infusion 12), resulted in much the same percentage of excretion via faeces and urine and a similar time course of radioactivity in the excreta. Excretion patterns of one hormone seemed constant for one animal (even when different amounts of steroid were used). The reason for the high amount of radioactivity excreted via urine after infusion of oestrone in one animal (Infusion 14) compared with the other two (Infusions 7 and 10) could be the fact that this ewe (No. 3) showed an individual tendency to excrete more metabolites via urine.

Our comparative study of the excretion of different steroid hormones in domestic livestock underlines the observation (Taylor, 1971; Wasser et al., 1994) that differences in the amount of radioactivity excreted via faeces and urine depends upon the hormone administered and the species. Although sex differences in the excretion of steroids (oestrone in ponies and pigs and cortisol in ponies) were detectable, further investigations with a larger number of animals are necessary to evaluate their significance. The percentage of radioactivity, which was accumulated in the excreta in our study was comparable to that found by others (so far as data is available), if it is taken into account that the percentages in some of these studies were calculated from the given doses (Schomberg et al., 1965; Terqui et al., 1968; Brown et al., 1970; Lindner, 1972; Houghton and Dumasia, 1979; Adams et al., 1994). The predominance of unconjugated (ether extractable) faecal ^{14}C-steroids proved their deconjugation (probably by intestinal bacteria), as they are secreted with the bile as conjugates (Taylor, 1971). Urinary radioactivity consisted mainly of steroid conjugates, which is in accordance with results of others (Taylor, 1971; Lindner, 1972).

Only about 2% and 4% of the recovered radioactivity following administration of

^{14}C-oestrone could be found in the faeces of ponies and pigs, respectively. The development of sensitive immunoassays in recent years has made it possible to determine small amounts of steroids in faeces. Determination of faecal oestrogens has proved useful for pregnancy diagnosis in mares and sows (Möstl et al., 1983; Choi et al., 1987; Lucas et al., 1991; Schwarzenberger et al., 1991) and diagnosis of equine cryptorchidism (Palme et al., 1994). Large amounts of faecal metabolites and the relatively short delay of excretion have allowed determination of progestagens to be used for monitoring corpus luteum activity (Schwarzenberger et al., 1992). In ewes, ^{14}C-oestrone is excreted mainly via faeces (89%) and it is possible to diagnose pregnancy in ruminants (Möstl et al., 1984; Safar-Hermann et al., 1987; Busch and Bamberg, 1990) based on the measurement of faecal oestrogens. Because of the short delay in excretion and the preferential elimination of infused ^{14}C-progesterone via faeces, faecal samples would seem suitable to monitor the oestrous cycle of ewes. In pigs short-lasting variations in the concentration of steroids in the blood probably cannot be related reliably to faecal concentrations, as the maximum of faecal excretion was observed a long time after the infusion and with great variations. This fact limits the usefulness of determinations of faecal progestagens for oestrous cycle control in sows. Faecal sampling will be suitable only for events which are connected with higher steroid concentrations for longer times (e.g. pregnancy). This disadvantage may turn into an advantage in the case of faecal androgen and cortisol metabolites, as fluctuations might be merged.

Even though adrenal steroids can be measured in the blood, the levels may not reflect the actual stress level of the animal because sample collection may increase cortisol levels. As urine samples can be difficult and/or time-consuming to obtain, faeces are recommended as sample material. Because radioactive metabolites of cortisol and testosterone were excreted to an even greater extent than oestrone (in ponies and pigs) in the faeces, the amount of radioactivity detected in faecal samples indicated that the determination of faecal metabolites (mainly unconjugated) with sensitive immunoassays should be possible. The results of our study encourage investigations to characterise faecal metabolites and to establish immunoassays for their measurement. They would be a helpful tool for studying seasonality, puberty or stress (especially in stress-sensitive animals), as they can be collected easily without causing stress.

5. Conclusion

The results of this comparative study in sheep, ponies and pigs investigating the excretion of different steroid hormones via faeces and urine can be summarised as follows.

1. Significant differences concerning the amount of excretion of different steroid hormones via faeces and urine were found in each species. Progesterone was excreted mainly via faeces and oestrone via urine in ponies and pigs.
2. Remarkable differences in the excretion of specific steroid hormones were detected among these three species. In general, sheep showed the highest degree of faecal excretion and pigs the least. In all three species, the amounts of faecal metabolites of

the four infused steroid hormones are, or seem to be, sufficient for quantitative measurement.

3. Our results underline the importance of the rate of passage of digesta (duodenum to rectum) for the excretion of steroids in the faeces. Rate of digesta passage gives an approximate estimate of delay times of faecal steroid excretion for a species. Peak concentrations of faecal radioactivity were reached most rapidly in sheep, followed by ponies and pigs (about 0.5 day, 1 day and 2 days, respectively).
4. Relating the amounts of urinary steroids to creatinine concentrations in longitudinal studies should only be necessary in special cases.
5. Taking portions of faecal samples for steroid analysis from defined sites or after homogenisation seems advisable in horses and pigs.
6. Extraction procedures utilising a higher percentage of methanol or an even more apolar alcohol will yield better recoveries of steroids from faecal samples of horses and pigs.
7. Plasma and urinary radioactivity consisted mainly of steroid conjugates, thus proving a quick metabolisation of steroids. The predominance of unconjugated faecal ^{14}C-steroids demonstrated their deconjugation in the gut.

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