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Measurement of Glucocorticoid Metabolite Concentrations in Faeces of Domestic Livestock

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With 5 figures and 1 table

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Summary

After ^{14}C-labelled cortisol infusion in ponies and pigs, faecal samples were collected. Extraction of 0.5 g faeces with 5 ml 80–90 % methanol yielded the highest radioactivity in the supernatant. Most of the metabolites were ether soluble. After high performance liquid chromatography (HPLC), the presence of immunoreactive metabolites was demonstrated by measuring each HPLC fraction using enzyme immunoassays for cortisol, corticosterone and 11-oxoaetiocholanolone. Only the assay for 11-oxo-aetiocholanolone revealed peaks with co-eluting radioactivity. For biological validation of the test system, adrenocorticotrophic hormone (ACTH) and dexamethasone were injected intravenously successively in both species ($n = 6$). Cortisol concentration in blood and the 11-oxoaetiocholanolone immunoreactive substances in faeces were determined. In horse faeces, basal values of 2.3–35.2 nmol/kg were measured. After ACTH administration, an increase (more than 200 % above basal values) of these metabolites was seen about 1 day after ACTH administration. After dexamethasone injection the levels decreased, reaching minimum concentrations 2 days after administration. In pigs, an increase in these metabolites was measured in only three animals after ACTH; dexamethasone did not cause a decrease. The stability of the samples after defecation was tested by storing samples from cows, horses and pigs at room temperature. It was shown that there was a significant increase in the concentration of measured cortisol metabolites in bovine, equine and porcine faeces after storage for 1 h, 4 h and 24 h, respectively. In frozen samples this effect was diminished after thawing samples at 40°C; thawing the samples at 95°C prevented an increase in immunoreactive substances.

Introduction

There is no standard definition of stress and no single biochemical test system to evaluate stressful conditions (Hofer and East, 1998), but the response to stress causes an increased release of adrenocorticotrophic hormone (ACTH) which leads to elevated levels of glucocorticoids in the blood. These steroids (mainly cortisol and corticosterone) are secreted by the adrenal cortex. They have profound effects on glucose metabolism (Gower, 1975), act in a catabolic manner, induce lipolysis and cause an involution of the lymphatic tissue (Thun and Schwartz-Porsche, 1994).

Analysis of cortisol or corticosterone in blood has been proven as a parameter of stress in a variety of species (Broom and Johnson, 1993), but capturing and blood sampling themselves are known to cause disturbances in animals (Morton et al., 1995). Therefore, feedback-free methods are required to measure these hormones.

In farm animals, infused radioactive cortisol is mainly excreted in urine (Palme et al., 1996). Excretion via faeces is 28 % in sheep, 41 % in ponies and only 7 % in pigs. These authors

also described a species-specific lag-time between the infusion and the peak of radioactivity in the faeces (12 h in sheep, 24 h in ponies and about 48 h in pigs). The cortisol metabolites may be further metabolized during this period. For example, the side-chain of some C-21 steroids is cleaved by the faecal flora of humans and rats to C-19 compounds (Cerone-McLeron et al., 1981).

As steroids are excreted in faeces of domestic livestock, measurement of faecal glucocorticoid metabolites can potentially be used for monitoring adrenal activity. This non-invasive method offers the benefit that samples can be collected easily and perhaps provide an integrated measurement of those metabolites independent of short-term fluctuations. Despite these advantages, immunoassays of faecal glucocorticoid metabolites are still used predominantly in carnivores and primates (for review see Goymann et al., 1999).

In herbivorous mammals, there are few publications concerning the measurement of glucocorticoids or their metabolites in faeces. Miller et al. (1991) described that elevated levels of faecal cortisol can be measured during stress in the Rocky Mountain bighorn sheep. In domestic sheep, Palme and Möstl (1997) described that in faecal samples cortisol or corticosterone immunoreactive metabolites are present only in trace amounts. Some of the metabolites in the faeces of domestic ruminants have an 11,17-dioxoandrostane structure. Therefore, the authors established and validated an enzyme immunoassay for 11-oxoaetiocholanolone for measuring 11,17-dioxoandrostanes (11,17-DOA) as a parameter of cortisol concentrations in the blood (Palme and Möstl, 1997). Möstl and Palme (1998) reported that 11,17-DOA is also present in faecal samples of other species, such as roe deer, horses, pigs, okapis and rhinos. The biological relevance of this non-invasive method has been proven in ruminants (cattle, sheep) following stimulation (ACTH) or suppression (dexamethasone) of cortisol release by the adrenal cortex (Palme et al., 1999).

The aim of our study was to validate a method for measuring 11,17-DOA in the faeces of horses and pigs as a parameter for cortisol production. In addition, the stability of the cortisol metabolites in faecal samples of domestic livestock was investigated after defecation. This is important for the practicability of the method, especially in zoo animals and wildlife, where limited information on the time interval between defecation and sample collection is available.

Materials and Methods

Extraction, HPLC separation of radioactive and immunoreactive glucocorticoid metabolites in faeces of ponies and pigs

Radioactive faecal samples of the experiments described by Palme et al. (1996) were used for the present study. The authors infused ^{14}C-labelled cortisol in horses and pigs and collected urine and faecal samples. To find the optimum extraction conditions, these radioactive faecal samples were suspended with increasing percentages of methanol as described by Palme and Möstl (1997). Radioactivity was determined by liquid scintillation counting. Supernatants showing the highest radioactivity were considered to have the best extraction efficiency.

After extraction, straight-phase high performance liquid chromatography (HPLC) was performed for the separation of the metabolites. Radioactivity was measured in each fraction of the chromatogram. In addition, the presence of immunoreactive substances was determined using the 11-oxoaetiocholanolone, cortisol and corticosterone enzyme immunoassays (EIA). The chromatographic conditions and the assays were described by Palme and Möstl (1997). It was found that ^{3}H-cortisol eluted around fraction 82, the more apolar substance ^{3}H-20α-dihydroprogesterone eluted in fractions 20–22.

ACTH and dexamethasone administration in mares and pigs

A total of six (three of each sex) healthy, mature horses and pigs were used as experimental animals for an ACTH stimulation and a dexamethasone suppression test. All animals were sexually mature.

ACTH (Synacthen, Ciba-Geigy, Basel, Switzerland) and dexamethasone (Dexa-TAD, Fa. TAD, Cuxhaven, Germany) were administered (ACTH: in horses 1 mg, in pigs 0.75 mg; dexamethasone in horses 30 mg, in pigs 5 mg) by consecutive intravenous (i.v.) injections (minimum time interval between injections was 3 weeks).

Sampling

Permanent catheters were inserted into the vena jugularis (horses) or the vena auricularis (pigs) 3 days before the start of the experiments. In one boar catheterization was not possible. Blood samples were collected at 9 a.m. and 9 p.m. from day −2 till day +4 of the experiment. On the day of the ACTH infusion (day 0) blood samples were taken immediately before and 30, 60, 90, 120, 360 and 540 min after infusion. After extraction with diethylether, plasma cortisol concentrations were measured as described by Palme and Möstl (1997). Two or three faecal samples were collected on days −2 and −1 and on day +4. Faeces from all defecations were collected on days 0, 1, 2 and 3. Samples were frozen immediately and stored at −24°C until analysis.

As the concentrations of immunoreactive metabolites in the faeces of ponies and pigs were lower than in ruminants, an extraction step with diethylether was added after the suspension of the faeces (0.5 g) in 80 % methanol (5 ml). Following centrifugation, 1 ml of the methanolic supernatant was transferred to a new vial and 0.2 ml of 5 % sodium bicarbonate was added. The mixture was extracted using 5 ml diethylether. After freezing (−24°C) the supernatant was decanted into another vial and evaporated with a stream of nitrogen. The extracts were redissolved in assay buffer and the concentrations of 11,17-DOA were measured as described earlier (Palme and Möstl, 1997).

Stability of the immunoreactive metabolites

As many bacteria are present in faeces, we investigated the influence of the interval between defecation and freezing of the samples. Faecal samples (about 100 g) of cows, horses and pigs ($n = 8$) were collected directly from the rectum. Aliquots (0.5 g) were weighted in glass vials and frozen immediately. After 1, 4 and 24 h additional portions of samples were taken and frozen as previously described. Defrosting was done by adding 5 ml 80 % methanol to the samples. To test the influence of thawing conditions on the values of 11,17-DOA measured, an experiment using cattle faeces ($n = 5$) was performed. Half of the samples were defrosted at 40°C, the others at 95°C (20 min) in a water bath and incubated after that at room temperature for 0, 4 and 24 h. In all samples the concentration of 11,17-DOA was determined. Statistical differences in the median values of the groups were calculated using the Mann–Whitney rank sum test.

As cattle faeces containing radioactive cortisol metabolites were not available, we used sheep faecal samples containing the naturally occurring ^{14}C-cortisol metabolites (Palme et al., 1996) for stability tests.

In sheep, radioactive faecal samples from an earlier experiment (Palme et al., 1996) were used to demonstrate the conversion of cortisol metabolites. Immediately after defecation samples with high radioactivity had been divided. One half was frozen immediately, the other half was stored at room temperature for 1 week. The samples were extracted and the pattern of radioactivity was measured after reversed phase HPLC (Möstl and Palme, 1998). Radioactivity and the concentration of 11,17-DOA were measured in each fraction.

In order to test the chemical stability of the cortisol metabolites, faecal diethylether extracts were incubated with concentrated hydrochloric acid (10 µl dissolved in 5 ml ethylacetate) for 16 h at 80°C. After evaporation of the solvent, the extracts were separated by straight-phase HPLC as described by Palme and Möstl (1997) and were compared with extracts without this incubation.

Results

Extraction

The radioactivity of the supernatant increased by using increasing methanol concentrations. In the pony, best recoveries were obtained using 80 % methanol. In contrast to total radioactivity, the amount of non-ether-extractable metabolites (polar substances) decreased using methanol concentrations higher than 50 %. Maximum amounts of diethylether-extractable steroids were achieved by using 80–90 % methanol.

In pigs, about 70 % of the radioactivity obtainable by extraction with 80 % methanol was already in the supernatant after suspension in water, but only 20 % of these metabolites were ether soluble. By using 80 % methanol as solvent, the portion of ether-soluble radioactivity increased to 55 %.

HPLC separation of radioactive and immunoreactive metabolites in the faeces of ponies and pigs

After chromatography of diethylether extracts of faecal samples from ponies (Fig. 1) and pigs (Fig. 2) only trace amounts of radioactivity eluted in the same fractions as authentic cortisol

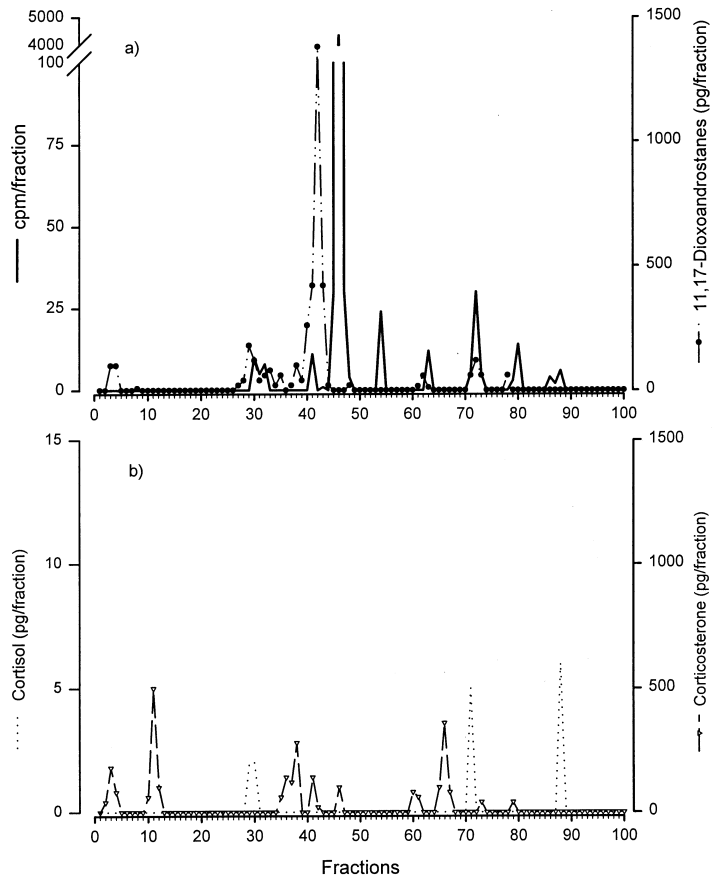

Fig. 1. High performance liquid chromatography (straight phase) of diethylether extracts of faecal ^{14}C-labelled cortisol metabolites of a pony. The sample was taken 1 day after infusion of ^{14}C-labelled cortisol. The fractions were analysed with enzyme immunoassays for 11,17-dioxoandrostanes, cortisol and corticosterone. Radioactivity was determined by liquid scintillation counting.

(fractions 80–85). In the horse, there was one dominant radioactive peak eluting in fractions 45–47, whereas most of the immunoreactive material eluted around fraction 41. A small radioactive peak co-chromatographed with this immunoreactive material. In the pig, the dominating cortisol metabolite was more polar and eluted in fraction 63–66. Some smaller radioactive peaks were seen in both species (Figs 1 and 2). Two immunoreactive peaks were detected. These fractions also contained radioactivity.

In all immunograms, higher amounts of immunoreactive material were found using the 11,17-DOA assay compared to the other two assays. The peaks measured by the other two assays were lower by a factor of three and some of them had no corresponding radioactivity. The dominant peak of radioactivity was not accompanied by a peak of immunoreactive substances in either the ponies or the pigs.

ACTH and dexamethasone administration in ponies

Before ACTH administration, morning cortisol values in blood were within the range of 7.6–29.7 nmol/l. Evening values were somewhat less (4.1–24.3 nmol/l). To show the change

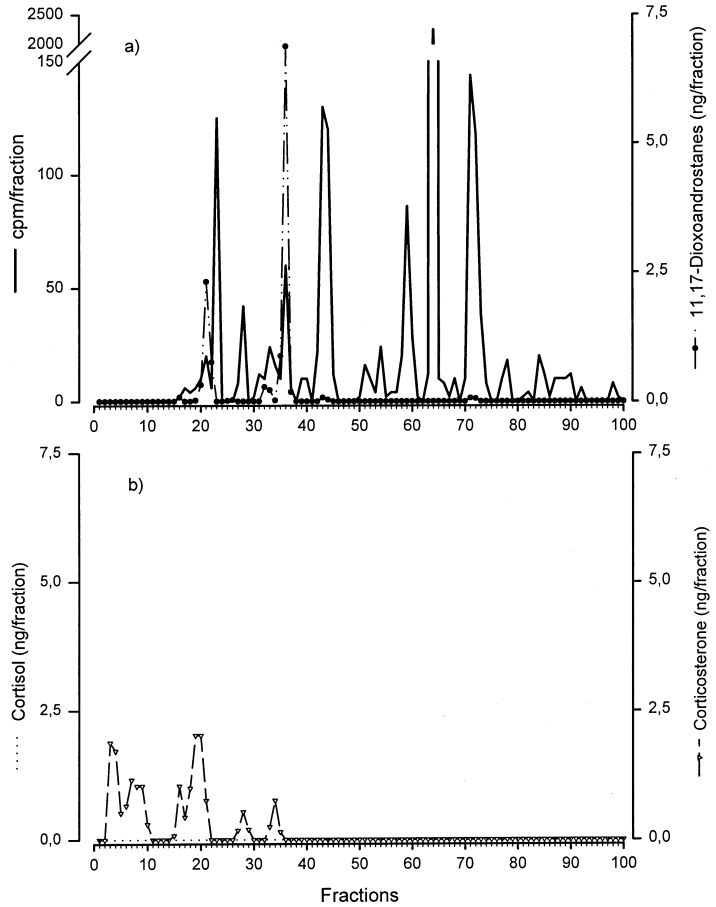

Fig. 2. High performance liquid chromatography (straight phase) of diethylether extracts of faecal ^{14}C-labelled cortisol metabolites of a pig. The sample was taken 1 day after infusion of ^{14}C-labelled cortisol. The fractions were analysed with enzyme immunoassays for 11,17-dioxoandrostanes, cortisol and corticosterone. Radioactivity was determined by liquid scintillation counting.

after drug administration independent from different individual starting values, the median cortisol concentration in samples before ACTH or dexamethasone applications was used as a reference point and the percentage increase was calculated.

Thirty minutes after ACTH administration a cortisol increase of 209–514 % was seen. Maximum values (85.8–107.7 nmol/l) were reached about 2 h after injection. After dexamethasone administration the lowest concentrations were seen 1 day after the injection (2.2–4.7 % of starting values).

In faecal samples the median of 11,17-DOA values collected on days −2 and −1 (2.3–35.2 nmol/kg faeces) was used as an individual reference value and the other concentrations were expressed as per cent deviation from this. As the time and frequency of defecation differed, all values were allocated into time frames of 0.5 days (Fig. 3). In individual mares the increase in 11,17-DOA concentrations started after 5.3–13.2 h. Individual maxima of 11,17-DOA concentrations were reached between 16.3 and 34 h after ACTH stimulation. These peak concentrations were more than twice as high (209–662 %) as the reference range in all animals. Afterwards the values decreased to basal concentrations within half a day (Fig. 3a).

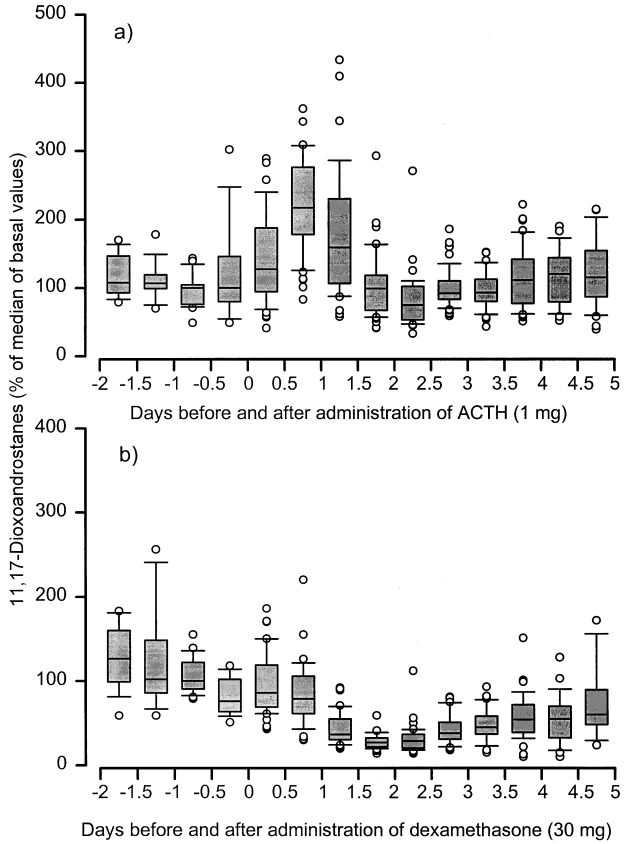

Fig. 3. Time–course of faecal immunoreactive cortisol metabolites using the immunoassay for 11,17-dioxoandrostanes after ACTH (a) or dexamethasone (b) administration in ponies. Levels are shown as boxplots.

Dexamethasone administration was followed by a decrease in 11,17-DOA concentrations (Fig. 3b). Lowest values were measured 22–29 h after injection, declining to between 31 and 61 % of the reference range. Afterwards the concentrations increased again, reaching the range of starting values by the end of the collection period. The plasma cortisol and faecal 11,17-DOA concentrations after stimulation and suppression tests of one individual animal each are shown in Fig. 4. In general, concentrations of cortisol in the blood were reflected by the 11,17-DOA concentrations in the faeces with a delay time of about 1 day.

ACTH and dexamethasone administration in pigs

Before ACTH administration median cortisol concentrations in the plasma of individual pigs varied between 4.1 and 26.4 nmol/l. Maximum concentrations (81.2–181.3 nmol/l) were measured about 80 min after ACTH administration (354–1489 %). As in ponies, we calculated individual median values from all samples before injection (6.9–19.1 nmol/kg).

In contrast to the results in horses, a distinct period of higher 11,17-DOA concentrations was only measurable in faecal samples from three out of the six pigs. Dexamethasone injection caused a decrease in plasma cortisol values, but the suppression was not as complete as in horses. In faecal samples, no distinct suppression of 11,17-DOA excretion could be measured (Fig. 4).

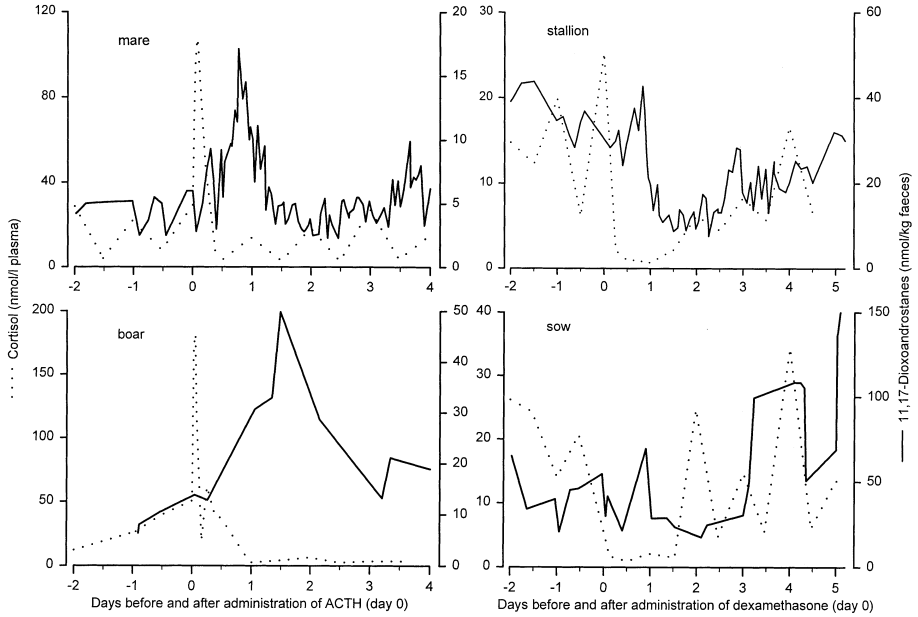

Fig. 4. Concentration of cortisol in plasma (nmol/l) and 11,17-dioxoandrostanes in faecal samples (nmol/kg) before and after ACTH or dexamethasone administration in single horses and pigs.

Stability of the immunoreactive metabolites

To demonstrate changes in the 11,17-DOA concentrations during storage at room temperature the values measured immediately after defecation were considered to be 100 % and the variations were calculated as per cent increase or decrease (Table 1).

In cattle, there was a significant ($P < 0.01$) increase in 11,17-DOA concentrations within 1 h (Table 1). After 4 h of incubation, the concentrations were significantly elevated also in the faeces of horses compared to starting values. Because of the high variations measured in faeces from pigs, a significant ($P < 0.01$) increase was seen only after 24 h.

Also in samples from cows thawed at 40°C the 11,17-DOA concentrations increased after storing the samples at room temperature. After 4 h the concentrations were higher ($P = 0.014$) compared to samples without prolonged storage. Thawing at 95°C prevented this effect and even after storing the samples for 1 day the values remained in the same range as without incubation.

HPLC chromatograms of extracted samples, which were stored overnight in the presence

Table 1. Increase (%) in 11,17-DOA concentrations after storing the samples at room temperature for 1–24 h

	Hours of incubation			
Species	0	1	4	24
Cattle	100	136 ± 47	213 ± 76	835 ± 534
Horse	100	108 ± 16	145 ± 27	962 ± 862
Pig	100	77 ± 71	175 ± 116	375 ± 337

of hydrochloric acid at 80°C, showed the same concentrations and elution patterns of mass and radioactivity as samples without incubation.

Storing radioactive faecal samples from sheep at room temperature for 8 days caused a completely different elution pattern of radioactivity compared to samples frozen immediately after defecation. There was a shift to more polar metabolites eluting at about fraction 18 from the column. New immunoreactive substances were also found in these fractions reacting with the 11,17-DOA and the corticosterone assay (Fig. 5). The total amount of immunoreactive material was higher, whereas the total radioactivity measured was the same in both chromatograms.

Discussion

The suspension of radioactive cortisol metabolites in the faeces of ponies and pigs in water dissolved a larger proportion of radioactivity compared to the values found for sheep

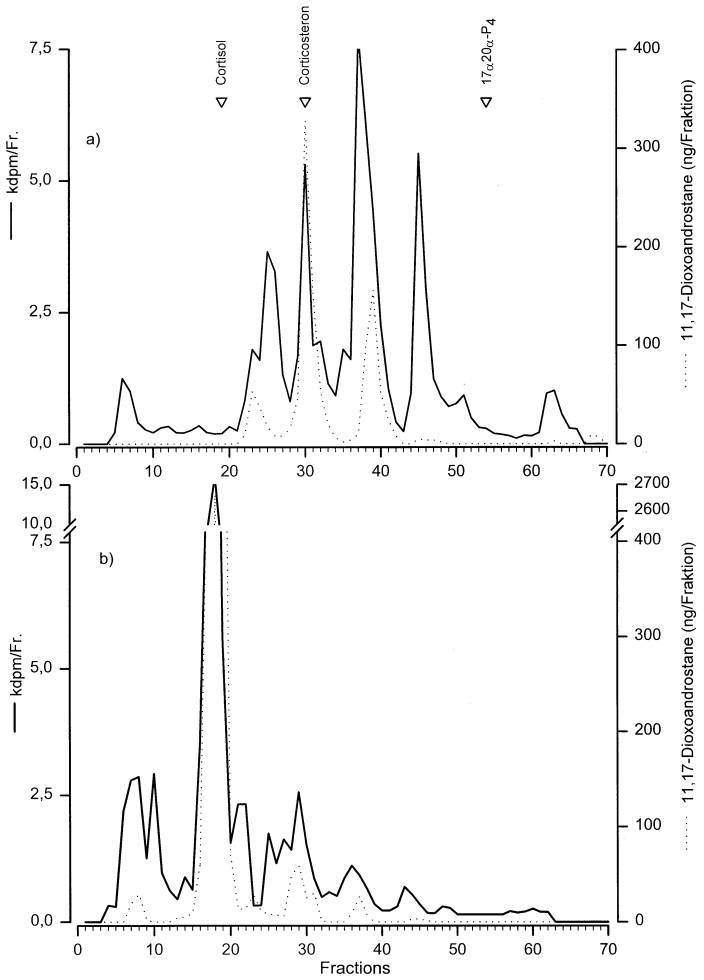

Fig. 5. Elution pattern of radioactive and immunoreactive substances in the faeces of sheep after infusion of ^{14}C-labelled cortisol using faeces immediately after defecation (a) or after storing the sample for 8 days at room temperature (b).

faeces (about 65 % compared to 35 %; Palme and Möstl, 1997). This is an indication that the cortisol metabolites excreted via faeces of ponies and pigs are more polar than in sheep. The highest recovery was obtained by using 80 % methanol. As expected, the percentage of ether-extractable radioactivity increased by extraction with higher methanol concentrations, as more apolar substances can be extracted using an apolar solvent. Like the gonadal steroid metabolites (Palme et al., 1996), cortisol metabolites are excreted in the faeces of ponies and pigs predominantly in an unconjugated (ether-extractable) form. The-non-ether-soluble radioactive substances can be either conjugated steroids, which were not hydrolysed by the bacterial flora, tetra- or pentahydroxylated compounds, or addition products formed by steroid metabolites and amino acids (Glass et al., 1982).

Chromatographic separation of the various radioactive metabolites showed that the dominating metabolites elute earlier from the column than cortisol and have a chromatographic mobility between ^{3}H-labelled 20α dihydroxyprogesterone and cortisol. Therefore, mono- and dihydroxylated metabolites are the dominating ether-extractable metabolites. In ponies, more apolar metabolites were excreted compared to pigs. All these metabolites of both species showed almost no cross-reaction with the cortisol and corticosterone assay used. Therefore, the use of these assays for measuring cortisol metabolites in the faeces of horses and pigs was not possible. This is in agreement with the situation in sheep, where cortisol immunoreactive material is excreted only in trace amounts via faeces (Palme and Möstl, 1997).

Ruminants, horses and pigs differ in the proportion of cortisol metabolites excreted via faeces and the interval between infusion of ^{14}C-cortisol and the maximum excretion via faeces (Palme et al., 1996). As shown in this study, there are considerable differences in cortisol metabolites excreted among species. In ponies and pigs the 11,17-DOA are not the dominating excretory products in faeces, as the dominating peaks of radioactivity did not show immunoreactivity. Therefore, the potential of the measurement of cortisol metabolites in faeces has to be improved by developing assays which react with the dominating metabolites excreted in faeces.

At least partially, 11,17-DOA are formed within the gut. Cerone-McLeron et al. (1981) demonstrated that the faecal flora of humans and rats contains a desmolase, an enzyme which removes the side-chain from some C-21 steroids. This may cause the increase in the concentration of this group of metabolites during incubation at room temperature. This effect was most pronounced in the cow, where an increase to 136 % was seen within a time interval of 1 h between defecation and freezing. The increase of up to 375–962 % in the three species implies that there are a lot of precursors for 11,17-DOA available in the faeces. As the time interval between defecation and freezing of the sample is critical, this is a limiting factor for the use of the assay in measuring faecal samples from wild animals.

Some of the 11,17-DOA-producing enzymes are still active after freezing whereas enzyme activity is lost after heating the sample to 95°C. Without enzymes from the bacterial flora, the metabolites are quite stable, as demonstrated by incubation with HCl at 80°C.

From our data, we conclude that measurement of faecal 11,17-DOA is an indicator of adrenocortical activity in horses. This technique offers the advantage of easy sampling, which does not interfere with the results of the experiment.

As in all species investigated in this experiment the time interval between defecation and freezing of the sample was critical, this non-invasive technique for measuring adrenal activity requires more careful sampling as it is necessary for measuring gonadal steroids. In our experiment we found only small amounts of metabolites cross-reacting with the cortisol or corticosterone assay. This was similar in ruminants (Palme and Möstl, 1997). In carnivores, corticosterone immunoassays can be used for monitoring adrenal activity. The difference between species may be caused by the length of the gut. In herbivores and omnivores a more intensive metabolism may take place, probably caused by bacteria.

There is a considerable variation in the formation of immunoreactive substances in the faeces during storage. This is best shown in pig faeces and may be in part explained by formation or decomposition of metabolites by bacteria. Winter et al. (1982) showed that a variety of substances are formed by the faecal flora, including 20β-hydroxylated products. Bokkenheuser et al. (1975) described differences in ability to metabolize 11-deoxycorticosterone in pure

cultures of faecal origin as different metabolites were formed. After side-chain cleavage, these substances may form products which react with the 11,17-DOA-assay.

In sheep we observed an increase in polarity of the ^{3}H-cortisol metabolites in faeces, which may be caused by additional hydroxylations of the molecule.

In ruminants, basal levels of 11,17-DOA ranged from 34 to 445 nmol/kg (median 93 nmol/kg) of cattle faeces and from 93 to 1031 nmol/kg (median 312 nmol/kg) of sheep faeces, respectively (Palme et al., 1999). The immunoreactive substances in the faeces of horses and pigs were much lower. In faecal samples of ponies 11,17-DOA basal levels were in the range of 2.3–35.3 nmol/kg faeces, in pigs between 6.9 and 19.1 nmol/kg. The factors contributing to these findings may be different percentages of excretion via faeces, different mass of faeces or cross-reactions of the metabolites. Additionally, the amount of steroids excreted via faeces reflects the production not the concentration in the blood.

In the horses the highest 11,17-DOA concentrations were measured about 1 day after ACTH administration. This is in agreement with the findings of Palme et al. (1996) that the time interval between infusion and peak excretion via faeces is about 24 h.

In the pig, the biological validity of the assay could not be proved, as there was no distinct period of elevated 11,17-DOA values after ACTH injection in any of the animals and no decrease after dexamethasone application. This may be caused by the relatively low percentage of cortisol excretion via faeces (7 %) in this species and because only a small part of these metabolites was immunoreactive. Additionally, the time interval between infusion of radioactive steroids and excretion via faeces shows considerable variation (Palme et al., 1996) and was longer in pigs than in ruminants and ponies. This may cause a higher dampening of the signal in faeces, as the metabolites are excreted over a longer period of time. Low percentage of excretion does not limit the measurement itself. In the horse, the percentage of oestrogens in faeces is only 2 % compared to that in urine, but a pregnancy test can be performed based on the measurement of faecal oestrogens (Choi et al., 1985). As there was also no continuous suppression of cortisol in plasma, the absence of a period of depressed 11,17-DOA excretion may have been caused by too low a dose of dexamethasone. In the pig there is a lag time of about 2 days between infusion of ^{14}C-labelled cortisol and the maximum level of radioactivity in faeces (Palme et al., 1996). Therefore, in this species a longer period of suppression may be necessary to see the effect in faeces.

It remains unknown whether severe disturbances (stress) in pigs can be measured in faeces. The data of the immunogram show that the use of another assay reacting with the more polar metabolites present in faecal samples may be a successful approach in measuring elevated glucocorticoid values in pigs.

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