Veterinary Research Communications, 26 (2002) 127–139

Measurement of Cortisol Metabolites in Faeces of Ruminants

E. Möstl[1]*, J.L. Maggs[2], G. Schrötter[3], U. Besenfelder[4] and R. Palme[1]
[1]*Institut für Biochemie and Ludwig Boltzmann Institut für Veterinärmedizinische Endokrinologie, Vienna, Austria;* [2]*Department of Pharmacology and Therapeutics, University of Liverpool, UK;* [3]*II. Medizinische Universitätsklinik für Klauentiere, Vienna;* [4]*Institut für Tierzucht und Genetik, Vienna, Austria and IFA Tulln, Abt. Tierproduktion, Tulln, Austria*
**Correspondence: E-mail: Erich.moestl@vu-wien.ac.at*

Möstl, E., Maggs, J.L., Schrötter, G., Besenfelder, U. and Palme, R., 2002. Measurement of cortisol metabolites in faeces of ruminants. *Veterinary Research Communications*, **26(2)**, 127–139

ABSTRACT

Twenty-one metabolites were detected in faecal samples collected after infusion of (^{14}C)cortisol into the jugular vein of sheep, using high-performance liquid chromatography/radiometric analysis plus mass spectrometry. One group of metabolites had molecular weights of between 302 and 308, and another group of 350, which indicates that the substances have a $C_{19}O_3$ or a $C_{21}O_4$ structure. Therefore, an enzyme immunoassay against 5β-androstane-3α-o1-11,17-dione-17-CMO:BSA was established. Faecal samples were collected from 10 cows immediately after transport and then during a course in which non-invasive diagnostic procedures were being taught (course 1). For comparison, faeces were sampled from another 5 cows that were being used for teaching invasive procedures (course 2). Six cows from a university farm served as controls. In the animals used in course 1, the highest concentrations of cortisol metabolites were measured immediately after transport to the university (median value: 2.2 μmol/kg faeces). During the first 5 days at the university, the concentrations decreased to 0.52 μmol/kg (median) and remained at this level during the rest of the course. The median concentration in the samples that were taken during course 2 (collected about 2 months after transport) was 0.48 μmol/kg. There was no significant difference in the excretion of cortisol metabolites between these cows and the controls. We conclude from these data that, using the enzyme immunoassay against 5β-androstane-3α-o1-11,17-dione-17-CMO, we were able to detect transport/novel environment stress but not the potential disturbance that cows experience during diagnostic procedures.

Keywords: animal welfare, cortisol, cow, diagnosis, faeces, sheep, stress

Abbreviations: BSA, bovine serum albumin; CMO, *O*-carboxymethyloxime; EIA, enzyme immunoassay; 11,17-DOA, 11,17-dioxoandrostanes; HPLC, high-performance liquid chromatography; 3α,11oxo-A, 3α-hydroxy,11-oxoandrogens

INTRODUCTION

Numerous publications have shown that glucocorticoid production is increased during some adverse situations. The concentration of cortisol in blood has proved to be a useful indicator of stress (Broom and Johnson, 1993; Terlouw *et al.*, 1997), although caution is advised, since an increase in the concentration of glucocorticoids does not

occur with every type of stressor. In order not to confound the rating of stress levels, care has to be taken that the sample collection does not interfere and cause stress. As blood sampling may disturb the animals, some authors have investigated non-invasive sampling procedures such as cortisol determination in urine (Hultgren, 1988) or saliva (Cooper *et al.*, 1989). Measuring faecal steroid metabolites of hormones of placental or gonadal origin is an established procedure for monitoring reproductive function in farm and zoo animals (Schwarzenberger *et al.*, 1996), but there are only a few reports concerning the excretion of metabolites of glucorcorticoids in faeces.

Lindner (1972) stated that two-thirds of the radioactivity was found in the bile of sheep after infusion of (^{14}C)cortisol into the jugular vein. The predominant radio-metabolites in the bile were glucuronides of tetrahydrocortisol, tetrahydrocortisone and cortolones. $C_{19}O_3$ steroids were also formed. That author demonstrated an enterohepatic circulation of these substances and reported that cortisol metabolites are voided via the faeces.

The cortisol metabolites produced in the liver are further converted by bacterial enzymes in the gut. Winter and Brokkenheuser (1979) described a 21-dehydroxylation of glucocorticoid metabolites by some anaerobic strains of bacteria isolated from human faeces. In faeces from domestic livestock, Möstl and colleagues (1999) found an increase in immunoreactive 11,17-dioxoandrostanes (11,17-DOA) after incubating faecal samples at room temperature, which was most probably caused by bacteria.

In sheep, Palme and colleagues (1996) measured the percentage of cortisol excreted via urine and faeces by infusing radioactive cortisol. They found that 28% of the radioactivity was excreted via the faeces. Peak concentrations of faecal radioactivity were recorded about 12–13 h after the infusion. Palme and Möstl (1997) characterized radioactive faecal cortisol metabolites using HPLC and EIAs. They showed that the predominant faecal metabolites were unconjugated steroids but they could not detect immunoreactive cortisol. To quantify the excretion of cortisol metabolites, an enzyme immunoassay was developed for one group of these metabolites (11,17-DOA). After infusing 1 g cortisol into the jugular vein of sheep, the authors measured the increase of those metabolites in faecal samples. Palme and colleagues (1999) injected ACTH and dexamethasone into sheep and cattle. Changes in the faecal concentration of 11,17-DOA parallel those of cortisol in the blood, with a delay time of about 10–12 h. Adverse situations increase the concentration of faecal cortisol metabolites. In cows, Palme and colleagues (2000) demonstrated that road transport for 2 h is followed by an increase in 11,17-DOA concentrations in the faeces.

However, Palme and Möstl (1997) had shown that only some of the metabolites of (^{14}C)cortisol in ovine faecal samples can be measured using the 11,17-DOA EIA. Therefore, the aim of our investigations was to further characterize the faecal cortisol metabolites and to establish a new immunoassay that has cross-reactions with a broader spectrum of cortisol metabolites.

MATERIALS AND METHODS

Characterization of cortisol metabolites using liquid chromatography–mass spectrometry

To ensure a high concentration of metabolites in the faeces, samples were collected after infusing 1 g cortisol into the jugular vein of a ram (Palme and Möstl, 1997). Pooled faeces, voided about 13 h after infusion and therefore containing peak concentrations of cortisol metabolites were used, as Palme and colleagues (1996) stated that there is a delay of about 13 h between infusion and the peak of faecal excretion of cortisol. The sample (6 g) was divided into six equal portions. A methanolic extract containing radioactive faecal cortisol metabolites (Palme *et al.*, 1996) was added to each portion and straight-phase HPLC was performed as described by Palme and Möstl (1997). Using liquid scintillation counting of a small aliquot of each eluate, 10 radioactive peaks were found. The corresponding peaks of all the six chromatograms were pooled, so each of the 10 pools represented the material from 6 g faeces. The solvent was evaporated with a stream of nitrogen and the extracts were reconstituted in 100 µl acetonitrile–water (35:65).

Each solution of extract was injected onto an Ultratechsphere 5 µm C_{18} column (25 cm × 4.6 mm; HPLC Technology, Macclesfield, UK) via a Rheodyne 7125 loop injector. The column was protected with a cartridge guard column (1 cm × 3 mm ID; HPLC Technology). The mobile phase consisted of acetonitrile–water (35:65). After 10 min, the percentage of acetonitrile was linearly increased to 60% at 30 min and to 80% at 40 min, followed by a return to the starting conditions.

The mobile phase was delivered by two Jasco PU980 pumps (Jasco Corp., Tokyo, Japan) via an HG-980-30 mixing module (Jasco Corp., Tokyo, Japan) at a flow rate of 1.2 ml/min. The eluate passed through a Jasco UN-985 absorbance detector, a Valco tee-union stream splitter (Phase Separations, Deeside, Clwydd, UK), and a fused-silica capillary (75 µm ID, 1.5 m length, Phase Separations) to the electrospray probe (Micromass Ltd, Manchester, UK), and the interface of a Quattro II tandem quadrupole mass spectrometer (Micromass), as described by Maggs and colleagues (1995). The capillary flow rate was approximately 40 µl/min. For parallel radiometric analysis, the third tee-union outlet was connected via a capillary (180 µm ID, 80 cm length) to an A-200 radioactivity detector (Canberra-Packard, Pangbourne, UK). The flow rate of the eluate to the liquid chromatography–mass spectrometry interface was approximately 15 µl/min. Nebulizing and drying gas (N_2) was delivered at 10 L/h and 220 L/h, respectively. The interface temperature was 60°C; the capillary voltage was 3.75 kV and the cone voltage was 30 V. Selected ion recording was performed (dwell time: 1 s; inter-channel delay: 0.02 s; multiplier voltage: 650 V). The data were processed using MassLynx 2 software (Micromass).

Immunoassays

The existing 11,17-DOA EIA (Palme and Möstl, 1997) is group-specific so far as the oxo group in position 17 is concerned. A new EIA for cortisol metabolites was developed by using an immunogen linked to BSA at position C-17 of the molecule.

Antibodies were raised in a rabbit against 5β-androstane-3α-ol-11,17-dione (11-oxoetiocholanolone, Steraloids, Wilton, NH, USA) that had been converted to the CMO derivative and linked to BSA as described by Kohen and colleagues (1975). The biotinylated label was synthesized using a mixed anhydride reaction similar to that described by Palme and Möstl (1994). Some modifications had to be introduced because the biotin derivative used and the biotinylated steroid produced were more hydrophobic than the products described earlier. In short, the biotin derivative (10 mg of biotinyl-3,6,9-trioxaundecanediamine; EZ-Link Biotin-LC-PEO-Amine, Fa. Pierce, New York, USA) was dissolved in 900 µl dimethylformamide plus 100 µl distilled water (solvent A) and cooled to 4°C.

Solvent B was prepared by dissolving 10 mg 5β-androstane-3α-ol-11,17-dione, 17-CMO in 1 ml dimethylformamide, cooling it to 4°C, and adding 5 µl isobutyl-chloroformate plus 10 µl 4-methylmorpholine. Solvent B was then stirred for 3 min and added dropwise to solvent A. The mixture was then incubated overnight at room temperature.

For purification of the biotin label, the dimethylformamide–water mixture (2 ml) was diluted with 10 ml of water and extracted using a Sep-Pak C_{18} cartridge (Fa. Waters, Milford, MA, USA). After priming this minicolumn according to the instructions of the manufacturer, the diluted sample was passed through the cartridge, which was washed with 5 ml water, and the derivative was eluted using 4 ml methanol. The methanolic phase was evaporated and dissolved in 300 µl water–methanol (65:35 v/v) and the extract was purified by HPLC (Novapac C_{18} column 0.39×15 cm; Fa. Waters, Milford, MA, USA; solvent: water–methanol, linear gradient from 35% methanol to 75% within 30 min; flow 1 ml per min; 3 fractions per min were collected). The EIA was performed as described earlier (Palme and Möstl, 1997). Ten µl of each fraction (prediluted 1:1000) was transferred into a microtiter plate coated with affinity-purified anti-rabbit IgG. Anti-steroid antibody (100 µl, 1:1000) was added and the plate was incubated overnight at 4°C. After washing, the enzymatic reaction was performed. The peak of the immunoreactive biotinylated product eluted from the column in fractions 75 and 76. These fractions were pooled, evaporated and stored at –20°C. To determine the working dilutions of the antibody and label, a checkerboard titration (Meyer *et al.*, 1990) was performed. A dilution of the antibody of 1:60 000 and one of 1:2 000 000 for the label gave the best results and were used for the EIA. As standard, 11-oxoetiocholanolone was used. The standard curve ranged from 2 to 500 pg/well; the 50% intercept was about 20 pg. The cross-reactions were as follows: 5β-androstane-3α-ol-11,17-dione, 100%; 5β-pregnane-3α-ol-11,20-dione; 37%; 5β-androstane-3α,11β-diol-17-one, 3.3%; and 5β-androstane-3,11,17-trione, 1.2%. All other steroids tested (11-ketoandrosterone, etiocholanolone, pregnanediol, tetrahydrocortisol, 5β-dihydrocortisol, cortol, 5β-pregnane-3α,11β,21-triol-20-one, 5β-pregnane-3α,11β,17α,20α,21-pentol, 5β-pregnane-3β-ol-11,20-dione and 5β-pregnane-3α,11β-diol-20-one) had cross-reactions below 1%.

Immunoreactive substances in faeces

To assess whether androgens originating from the placenta cross-react with the new assay for 3α,11oxo-A, we collected faeces from cows that were not pregnant ($n = 3$) or were at the end of gestation ($n = 3$). Information on the androgens produced by the bovine placenta were given by Möstl and colleagues (1985). After extraction of the faecal samples, the metabolites were separated by HPLC as described by Palme and Möstl (1997). The immunoreactive substances in the HPLC fractions were measured using the EIAs for 11,17-DOA and 3α,11oxo-A. As Möstl and colleagues (1999) described further *in vitro* metabolism of cortisol metabolites after sampling, we also tested to ascertain whether the concentrations measured using the new assay remain constant during the storage of the samples. For this study, we used both fresh samples and frozen samples after thawing (thawing temperature 40 or 95°C). All the samples were stored at room temperature for 4 and 24 h.

Biological relevance

To investigate whether handling the cows during clinical courses for veterinary students caused an increase in the excretion of cortisol metabolites, faeces were collected twice a day during three 5-day periods during the winter term. Period 1 started immediately after the cows arrived at the university and one day before the start of the course that lasted throughout the experiment. Periods 2 and 3 started 2 and 16 weeks later, respectively. These cows were used to train students for non-invasive clinical investigations (course 1).

During the summer term, another 5 cows were sampled (course 2). These cows were brought to the university at the end of February and were used by students to practise sampling of blood and ruminal contents. A total of 100 faecal samples were collected during a 7-day period in April and a 4-day period in June. These cows were described by the veterinarians in the clinic as aggressive and difficult to handle. To investigate whether cortisol excretion by cows was in general higher at the university than at a farm, 6 cows from our university farm served as controls (64 faecal samples).

The samples (0.5 g) were extracted as described by Palme and Möstl (1997) and analysed using the 3α,11oxo-A EIA after dilution of the methanol extract with assay buffer. For comparison of the two assays, 35 samples were also measured by the 11,17-DOA EIA. Statistical differences between the time periods for the cows were calculated using the Friedman test. The Mann–Whitney rank sum test was applied to compare the basal values of the three groups.

RESULTS

Liquid chromatography–mass spectrometry of faecal cortisol metabolites in rams

The combination of straight-phase and reversed-phase HPLC plus MS revealed the presence of at least 21 metabolites of cortisol (Figure 1).

The mass spectra of the material in almost all of the radioactive fractions contained ions with masses of 302–308. The more polar peaks (peaks 4–10), which contained the predominant radioactive products, also yielded ions of mass 350. Metabolites of cortisol (e.g. cortisol, tetrahydrocortisol) with a higher molecular weight than 350 were not found.

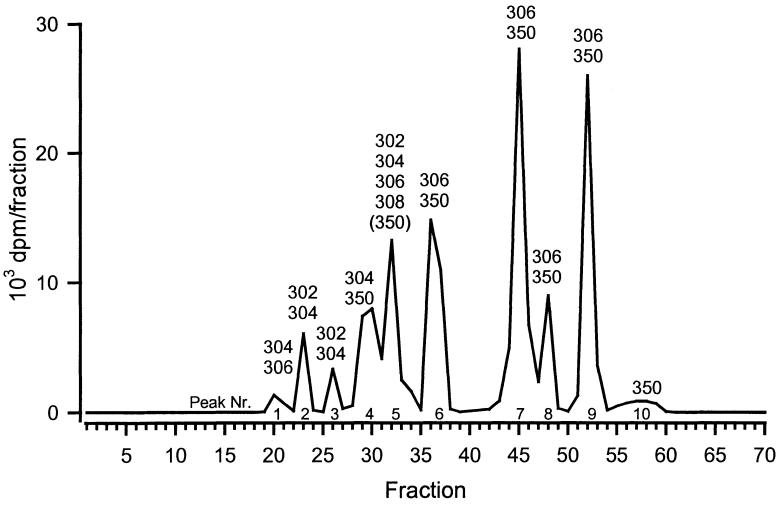

Figure 1. Radioactive metabolites of cortisol eluted from a straight-phase HPLC column. The numbers at the top of the peaks give the molecular masses of the molecules within the peak detected after reversed-phase HPLC-MS. The radioactivity of the sample originated from a ram after infusing (^{14}C)cortisol; the molecular masses are mainly from the experiment in which 1 g cortisol was infused

Immunoreactive substances in faeces of cows

In measuring the immunoreactive substances in the individual HPLC fractions of faecal samples from non-pregnant cows, one major peak was detected using 11,17-DOA EIA and two predominant peaks using the 3α,11oxo-A EIA. In addition to these peaks, less polar immunoreactive substances were found in extracts of faeces from pregnant cows. Examples of these chromatograms are shown in Figure 2.

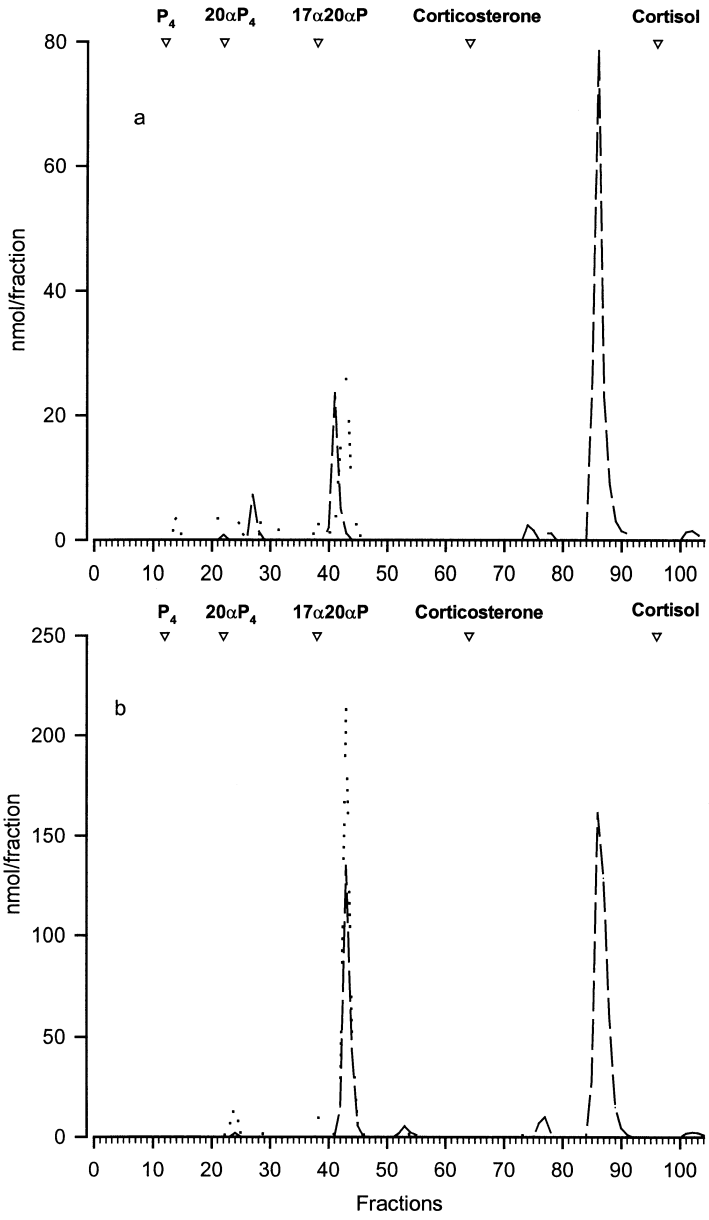

Figure 2. Elution pattern of immunoreactive substances, measured after straight-phase chromatography using the 11,17-DOA assay (dotted line) and the 3α,11oxo-A assay (dashed line) in a faecal sample of a cow near term (a) and from a non-pregnant cow (b). The elution of progesterone (P4), 20α-hydroxyprogesterone (20αP4), corticosterone and cortisol is marked with triangles

Storing fresh faecal samples of cows for 4 h at room temperature had no significant effect on the concentrations that were measured using the 3α,11oxo-A EIA.

After freezing of the samples, thawing at 40 or 95°C did not significantly influence the concentrations measured following the two thawing procedures if the methanol–water mixture for extraction was added immediately. If the organic solvent was added 4 h after thawing, there was a significant ($p = 0.014$) decrease in the concentrations of the immunoreactive 3α,11oxo-A following thawing at 40°C compared to samples in which the organic solvent was added immediately, but thawing at 95°C prevented this post-thawing decrease.

Biological relevance

In course 1, the maximum concentrations (median value 2.21 μmol/kg faeces) of 3α,11oxo-A were measured in the first samples, which were taken about 12 h after transport. The concentration decreased during the following 5 days (Figure 3) to a median level of 0.52 μmol/kg faeces. In the next sampling period (12 days later), the 3α,11oxo-A concentrations were in the range 0.11–0.80 μmol/kg faeces (median 0.41 μmol/kg). During the third sampling period, the concentrations were in a similar range (median 0.46 μmol/kg). During course 2, the median value of 3α,11oxo-A was 0.48

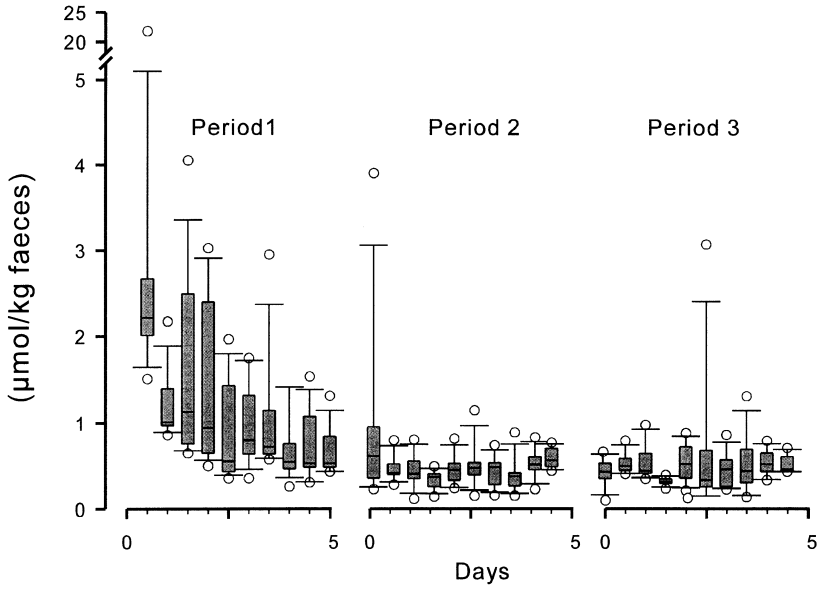

Figure 3. Boxplot of faecal 3α,11oxo-A concentrations (μmol/kg) during the first weeks after arrival at the university. Each box shows the median and the upper and lower quartile value; the whiskers show the 10th and 90th percentile of the 11,17-DOA values. The circles represent data points that are outside the centiles

μmol/kg, which was not statistically different from the concentrations in the samples from the university farm (median 0.49 μmol/kg).

The values for samples collected 12–48 h after arrival of the cows at the clinic were significantly higher (p = 0.045) than those for samples collected more than 120 h afterwards. The comparison of the results obtained with the two assays (35 samples collected 12–48 h after transport) showed that the immunoreactive substances measured using the 3α,11oxo-A assay were about 7 times higher compared to the results using the 11,17-DOA assay (Figure 4).

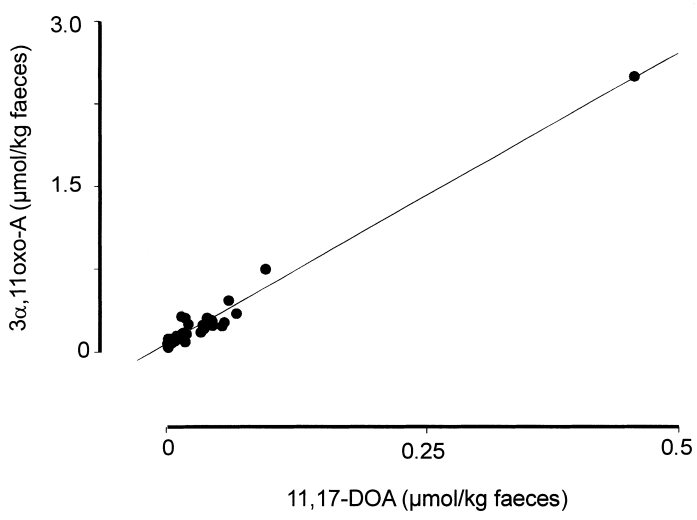

Figure 4. Comparison of the results obtained with the two immunoassays

DISCUSSION

Liquid chromatography–mass spectrometry

The mass spectrometry data indicate that the metabolites of cortisol in sheep are mainly $C_{21}O_4$- and $C_{19}O_3$-steroids, as the mass spectra contained masses of 350 ($C_{21}O_4$) and 302–308 ($C_{19}O_3$). At least 21 different cortisol metabolites were detected. As the molecular weights of the metabolites co-chromatographing with the radioactive cortisol metabolites were measured after straight-phase plus reversed-phase HPLC, we may assume that the substances measured by mass spectrometry originate from cortisol.

Lindner (1972) found that the dominant steroids excreted via the bile in sheep are glucuronidated $C_{21}O_5$-steroids such as tetrahydrocortisol, tetrahydrocortisone or cortolones. Our data showed only unconjugated cortisol metabolites in faeces. The hydrolysis of the conjugated cortisol metabolites during passage through the gut may be explained by bacterial activity. Also, the conversion of $C_{21}O_5$ steroids, such as tetrahydrocortisol (in conjugated or unconjugated form) to $C_{21}O_4$-steroids is most probably carried out by bacteria. Winter and Brokkenheuser (1979) described certain strains of anaerobic bacteria that convert cortisol metabolites into the corresponding 21-dehydroxylated products.

For the LC-MS-analysis, only prepurified fractions (straight-phase HPLC) were used. The reversed-phase LC-MS-analysis of these individual fractions showed metabolites of molecular weight 350, which suggests that (like the main peak of metabolites of (^{14}C)cortisol) the predominant faecal metabolites of cortisol, which have the same chromatographic pattern as the (^{14}C)cortisol metabolites in two chromatographic systems, have configurations such as 5α/β-pregnane-3α/β,11β,17α-triol-20-one or 5α/β-pregnane-3α/β,17α,20α/β-triol-11-one, with a CH_3 structure at the C-21 position ($C_{21}O_4$-steroids). Metabolites like tetrahydrocortisol (MW = 366), cortol (MW = 368), cortolone (MW = 366) or tetrahydrocortisone (MW = 364) were not found in the samples, which can be explained by bacterial metabolism of these substances.

Our mass spectrometry data confirm the previous observation (Palme and Möstl, 1997) that there is no cortisol, corticosterone or tetrahydrocortisol present in the faecal samples in substantial amounts, the cortisol being converted into the various $C_{21}O_4$ and $C_{19}O_3$ metabolites.

Immunoassay and immunoreactive substances

The 3α,11oxo-A EIA cross-reacted mainly with 5β,3α,11oxo-structures and, to a lesser extent, with C_{19} and C_{21} steroids with a 11β-hydroxy or a 11-oxo group.

The cross-reactions of the new enzyme immunoassay with other C_{19} and C_{21} steroids are plausible, as some cross-reactions occur when the differences in structure between cross-reacting molecules are located close to the position of linking (Kohen *et al.*, 1975; Niswender and Migley, 1970). The antibodies used for our investigations also have some cross-reactions with gonadal steroids of placental origin, as seen by the occurrence of apolar immunoreactive metabolites in the faeces of animals near term. As the bovine placenta produces large amounts of androgens (Möstl *et al.*, 1985), even minor cross-reactions may cause elevated levels of immunoreactive products in faeces near term. Therefore, both EIAs (11,17-DOA and 3α,11oxo-A) have to be used with caution in cows at the end of gestation. Prolonged storage at room temperature caused a decrease in the immunoreactive substances measured by the 3α,11oxo-A assay, whereas with the 11,17-DOA EIA Möstl and colleagues (1999) found that the values increased. This may be explained by the effect of bacterial side-chain cleavage, forming increased amounts of $C_{19}O_3$ steroids. The 3α,11oxo-A EIA cross-reacts with some $C_{21}O_4$ steroids, whereas the 11,17-DOA EIA does not. Hence, an explanation for the

decrease in immunoreactive substances using the 3α,11oxo-A EIA may be that the assay has more cross-reactions with some $C_{21}O_4$ steroids than with the $C_{19}O_3$ metabolites formed from the precursors during incubation.

After thawing at 95°C, the concentration of the metabolites was stable, probably because bacteria and bacterial enzymes were destroyed by the heat.

The presence of more than 21 cortisol metabolites in faeces from sheep will complicate the standardization of faecal cortisol metabolite measurements because, as with progesterone metabolites (Schwarzenberger *et al.*, 1996), different laboratories will use different immunoassays and extraction procedures.

Relating the amounts of the metabolites of cortisol to other substances, such as the water content of the faeces, will not give better results with ruminants, as the steroids are not water-soluble and the diet of the cows is more or less constant.

Biological relevance

Similar metabolites to those in sheep can also be expected in cows. Palme and colleagues (1999) found the concentration of 11,17-dioxoandrostanes in faeces from sheep and cattle to be a good measure of cortisol production.

The corticosterone concentration in the faeces of carnivores, as measured by immunoassays (Graham and Brown, 1996; Jurke *et al.*, 1997; Monfort *et al.*, 1998; Goymann *et al.*, 1999), reflects glucocorticoid production. Our data indicate that the broad spectrum of cortisol metabolites present in ruminants can be used to monitor the production of cortisol by the adrenal cortex.

The values we obtained with the 11,17-DOA EIA are in agreement with those described by Palme and colleagues (1999). The higher concentrations detected by the new assay can be explained by the differences in the cross-reactions, as shown by HPLC analysis.

The passage of the cortisol metabolites through the gut causes a delay between cortisol infusion into a vein and the excretion via faeces for a species-specific time (Palme *et al.*, 1996). In ruminants, the concentration of the metabolites of cortisol in a faecal sample reflects the cortisol production about 12 h earlier.

The stress arising from transport and the novel housing conditions caused an increase in the concentration of faecal metabolites of cortisol. Tarrant (1990) reported that one of the most stressful aspects of transportation is the confinement in a moving vehicle. In our experiment, the highest value for the metabolites of cortisol were measured in the first samples obtained about 12 h after transportation. These elevated concentrations are in agreement with the data of Palme and colleagues (2000). These authors showed that the maximum excretion of 11,17-DOA occurs about 12 h after transport and that the concentrations decreased to baseline within one day after the cows were in their home environment.

In the present experiment, the elevated excretion of the metabolites of cortisol lasted for about 5 days, probably because the cows were brought to a new environment. Clark and colleagues (1997) emphasized that mental and biological well-being is more likely to exist in animals if they are familiar with their environment. As also seen in our

experiment, not all kinds of disturbances cause an increase in cortisol production. The cows used for teaching invasive diagnostic techniques were described as aggressive, but no elevated excretion of metabolites of cortisol was measured compared to cows at the university farm. Hopster and colleagues (1999) investigated the effects of repeated jugular puncture on the concentration of cortisol in the plasma of dairy cows. They concluded that an increase in the concentrations of cortisol during successive blood sampling depends on the handling experience of the cows and on individual differences.

Even better immunoassays could be developed for the measurement of faecal metabolites of cortisol, especially for the group of $C_{21}O_4$ metabolites. However, the results of our study should assist in improving assays for faecal metabolites of cortisol in ruminants and may help to establish assay systems that can be used in other species.

ACKNOWLEDGEMENTS

The authors thank Mrs A. Kuchar-Schulz and Mrs M. Stark for laboratory assistance. The financial support of the Austrian Science Fund (FWF; project no. P12376-Med) is gratefully acknowledged. The LC-MS system in the University of Liverpool was purchased and maintained by means of grants to Professor B.K. Park from The Wellcome Trust.

REFERENCES

Broom, D.M. and Johnson, K.G., 1993. *Stress and Animal Welfare*, (Chapman and Hall, London), 87–144

Clark, J.D., Rager, D.R. and Calpin, J.P., 1997. Animal well-being: II. Stress and distress. *Laboratory Animal Science*, **47**, 571–579

Cooper, T.R., Trunkfield, H.R., Zanella, A.J. and Booth, W.D., 1989. An enzyme-linked immunosorbent assay for cortisol in the saliva of man and domestic farm animals. *Journal of Endocrinology*, **123**, R13–R16

Goymann, W., Möstl, E., Van't Hof, T., East, M.L. and Hofer, H., 1999. Noninvasive fecal monitoring of glucocorticoids in spotted hyenas (*Crocuta crocuta*). *General Comparative Endocrinology*, **114**, 340–348

Graham, L.H. and Brown, J.L., 1996. Cortisol metabolism in the domestic cat and implications for non-invasive monitoring of adrenocortical function in endangered felids. *Zoo Biology*, **15**, 71–82

Hopster, H., van der Werf, J.T.N., Erkens, J.H.F. and Blokhuis, H.J., 1999. Effects of repeated jugular puncture on plasma cortisol concentrations in loose-housed dairy cows. *Journal of Animal Science*, **77**, 708–714

Hultgren, J., 1988. *The Function of Electric Cow-trainers and Effects of Them on Behaviour and Physiology. A Review and Methodological Study*, (Rapport Institutionen vor Husdjurshygien, Sveriges Lantbruksuniversitet, No. 24)

Jurke, M.H., Czekala, N.M., Lindburg, D.G. and Millard, S.E., 1997. Fecal corticoid metabolite measurement in cheetah (*Acinonyx jubatus*). *Zoo Biology*, **16**, 133–147

Kohen, F., Bauminger, S. and Lindner, H.R., 1975. Preparation of antigenic steroid-protein conjugates. In: E.H.D. Cameron, S.G. Hillier and K. Griffiths (eds), *Steroid Immunoassay*, (Omega Publishing, Cardiff), pp. 11–32

Lindner, H.R., 1972. Enterohepatic circulation and patterns of urinary excretion of cortisol metabolites in the ewe. *Journal of Endocrinology*, **52**, XIX–XX

Maggs, J.L., Williams, D., Pirmohamed, M. and Park, B.K., 1995. The metabolic formation of reactive intermediates from clozapine, a drug associated with agranulocytosis in man. *Journal of Pharmacology and Experimental Therapeutics*, **275**, 1463–1475

Meyer, H.H.D., Sauerwein, H. and Mutayoba, B.M., 1990. Immunoaffinity chromatography and a biotin-streptavidin amplified enzyme-immunoassay for sensitive and specific estimation of estradiol-17β. *Journal of Steroid Biochemistry*, **35**, 263–270

Monfort, S.L., Mashburn, K.L., Brewer, B.A. and Creel, S.R., 1998. Evaluating adrenal activity in African wild dogs (*Lycaon pictus*) by fecal corticosteroid analysis. *Journal of Zoo and Wildlife Medicine*, **29**, 129–133

Möstl, E., Choi, H.S. and Bamberg, E., 1985. Stimulation of androgen and oestrogen concentrations in plasma of cows after administration of a synthetic glucocorticoid (flumethasone) at the end of gestation. *Journal of Endocrinology*, **105**, 121–126

Möstl, E., Messmann, S., Bagu, E., Robia, C. and Palme, R., 1999. Measurement of glucocorticoid metabolite concentrations in faeces of domestic livestock. *Journal of Veterinary Medicine A*, **46**, 621–632

Niswender, G.D. and Midgley, A.R. Jr, 1970. In: F.G. Peron and B.V. Caldwell (eds), *Immunologic Methods in Steroid Determination*, (Appleton-Century-Crofts, New York), 149–173

Palme, R. and Möstl, E., 1994. Biotin-streptavidin enzyme immunoassay for the determination of oestrogens and androgens in boar faeces. In: S. Görög (ed.), *Advances of Steroid Analysis 93*, (Akadémiai Kiadó, Budapest), 111–117

Palme, R. and Möstl, E., 1997. Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood. *International Journal of Mammalian Biology*, **62** (Supplement 2), 192–197

Palme, R., Fischer, P., Schilddorfer, H. and Ismail, M.N., 1996. Excretion of infused ^{14}C-steroid hormones via faeces and urine in domestic livestock. *Animal Reproduction Science*, **43**, 43–63

Palme, R., Robia, C., Messmann, S., Hofer, J. and Möstl, E., 1999. Measurement of faecal cortisol metabolites in ruminants: a non-invasive parameter of adrenal function. *Wiener Tierärztliche Monatsschrift*, **86**, 237–241

Palme, R., Robia, C., Baumgartner, W. and Möstl, E., 2000. Transport stress in cattle as reflected by an increase in faecal cortisol metabolites. *The Veterinary Record*, **146**, 108–109

Schwarzenberg, F., Möstl, E., Palme, R. and Bamberg, E., 1996. Faecal steroid analysis for non-invasive monitoring of reproductive status in farm, wild and zoo animals. *Animal Reproduction Science*, **42**, 515–526

Tarrant, P.V., 1990. Transport of cattle by road. *Applied Animal Behaviour Science*, **28**, 153–170

Terlouw, E.M., Schouten, W.G.P. and Ladewig, J., 1997. Physiology. In: M.C. Appleby, and B.O. Hughes (eds), *Animal Welfare*, (CAB International, University Press, Cambridge), 143–158

Winter, J. and Brokkenheuser, V.D., 1979. Bacterial metabolism of corticoids with particular reference to the 21-dehydroxylation. *Journal of Biological Chemistry*, **254**, 2626–2629

(Accepted: 24 July 2001)