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Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood

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Abstract

Faecal samples excreted after infusion of ^{14}C-cortisol to sheep were used to characterise metabolites and evaluate extraction procedures in order to establish an enzyme immunoassay (EIA) for the quantification of some of the metabolites. Several (> 15) faecal metabolites were formed. Nearly all were unconjugated and showed a chromatographic mobility (straight phase HPLC, silica gel) between 20α-dihydroprogesterone and cortisol. "Authentic" cortisol and tetrahydrocortisol were at or below the limit of detection. An 11-oxoetiocholanolone-EIA (measuring 11,17-dioxoandrostanes) was established. Extraction with methanol (80%) yielded the highest recovery. The presence of immunoreactive ^{14}C-metabolites was confirmed by analysing the HPLC fractions with the established EIA. In addition, faecal samples were collected for four days from two rams infused with a large dose of cortisol (1 g). Only measures of the 11-oxoetiocholanolone-EIA but neither of the cortisol- nor the corticosterone-EIA showed the expected excretion patterns in the faeces. Therefore measuring 11,17-dioxoandrostanes should prove to be a valuable tool for monitoring stress in farm, zoo and wildlife ruminants, using the advantages of non-invasive sampling techniques.

Introduction

In mammals stress increases adrenal glucocorticoid production and cortisol concentrations in blood can be used as a parameter of stress (Morton et al. 1995). However, as blood sample collection itself causes stress and is dangerous or even impossible in some zoo and wildlife species, non-invasive methods for the determination of glucocorticoids (or their metabolites) seem desireable for assessing adrenal function in these animals. In this respect, faecal samples offer the advantage that they can be collected easily without any need to handle the animals. Glucocorticoids are extensively metabolised. Aside from the liver, intestinal bacteria in the gut can affect the structure of the metabolites (MacDonald et al. 1983, Brownie 1992). In sheep about 28% of infused ^{14}C-cortisol was excreted via the faeces, mainly (about 95%) as unconjugated metabolites (Palme et al. 1996). These authors found a delay time of faecal peak radioactivity of about 12 h in sheep, reflecting the time of passage of ingesta. The aim of this study was to establish an enzyme immunoassay (EIA) for the quantification of some of the faecal cortisol metabolites in this species. This should help to evaluate stress in farm and especially zoo and wildlife ruminants using the advantages of non-invasive methods of sample collection.

Material and Methods

An 11-oxoetiocholanolone-EIA was established. The characteristics of this newly developed EIA and the other two used (cortisol- and corticosterone-EIA) are shown in Tab. 1. All antibodies were raised in

rabbits and crossreactions (%) of relevant steroids in the three EIAs are given in Tab. 2. The biotinylated labels (DADOO-biotin = N-biotinyl-1,8-diamino-3,6-dioxaoctane) were synthesised and the EIAs performed as previously described (Palme and Möstl 1994, Schwarzenberger et al. 1996). Steroids were purchased from Steraloids (Wilton, NH, USA).

Faecal samples collected after intravenous administration of 0.1 mCi ^{14}C-cortisol to sheep (n = 4, Palme et al. 1996) were used to characterise metabolites. They contained peak radioactivity. Extraction was performed as described by Palme et al. (1997). Metabolites were separated by straight phase HPLC (n-hexane/chloroform: 70/30; flow: 2 ml/min) on a Lichrosorb Si 60 column (10 µm, 25×0.4 cm; Forschungszentrum Seibersdorf, Austria) using a linear methanol gradient from 0 to 6% in the first 30 min.,

Table 1. Characteristics of the three EIAs

EIA	11-oxoetiocholanolone	cortisol	corticosterone
Label (DADOO-biotin coupled with):	11-oxoetiocholanolone-3-gluc.	cortisol-3-CMO	cortisol-3-CMO
Antibody against: coupled with BSA)	11-oxoetiocholanolone-3-HS	cortisol-3-CMO	corticosterone-3-CMO
Standard	11-oxoetiocholanolone (= 5β-androstane-3a-ol-11,17-dione)	cortisol	corticosterone
Antibody (×10^3)	1:20	1:20	1:40
Label (×10^3)	1:250	1:100	1:200
Sensitivity (pg/well)	0.8	0.3	0.8
Intraassay-CV (n = 20)	9.5	8.9	10.0
Interassay-CV (n = 50)	11.3	11.1	13.4

Table 2. Crossreactions (%) of relevant steroids in the various EIAs

Steroid	11-oxoetiocholanolone-	cortisol-	corticosterone-
4-Pregnene-			
11β,21-diol-3,20-dione	<0.01	6.2	100.0
11β,17a,21-triol-3,20-dione	<0.01	100.0	5.0
5a-Pregnane-			
11β,17a,21-triol 3,20-dione	<0.01	4.6	<0.01
3a,11β,17a,21-tetrol-20-one	<0.01	0.8	0.15
5β-Pregnane-			
3a,11β,17a,21-tetrol-20-one	<0.01	0.1	0.2
17a,21-diol-3,11,20-trione	<0.01	<0.01	<0.01
3a,11β,21-triol-20-one	<0.01	<0.01	0.25
3a,17a,21-triol-11,20-dione	<0.01	<0.01	<0.01
3a,17a,20a,21-tetrol-11-one	<0.01	<0.01	<0.01
3a,11β,17a,20a,21-pentol	<0.01	<0.01	<0.01
5a-Androstane-			
3a-ol-11,17-dione	5.7	<0.01	<0.01
3β-ol-11,17-dione	6.7	<0.01	<0.01
3,11,17-trione	14.7	<0.01	<0.01
5β-Androstane-			
3a,11β-diol-17-one	0.6	<0.01	<0.01
3a-ol-11,17-dione	100.0	<0.01	<0.01
3,11,17-trione	84.0	<0.01	<0.01

6% from the 30th to 35th min. and thereafter up to 10% until the 40th min. Fractions were collected every 30 sec for 8 min and every 15 sec afterwards until the end. They were evaporated and reconstituted in assay buffer. Radioactivity per fraction was determined by liquid scintillation counting and immunoreactivity was tested in the three assay systems (11-oxoetiocholanolone-, the cortisol- and the corticosterone-EIA, respectively).

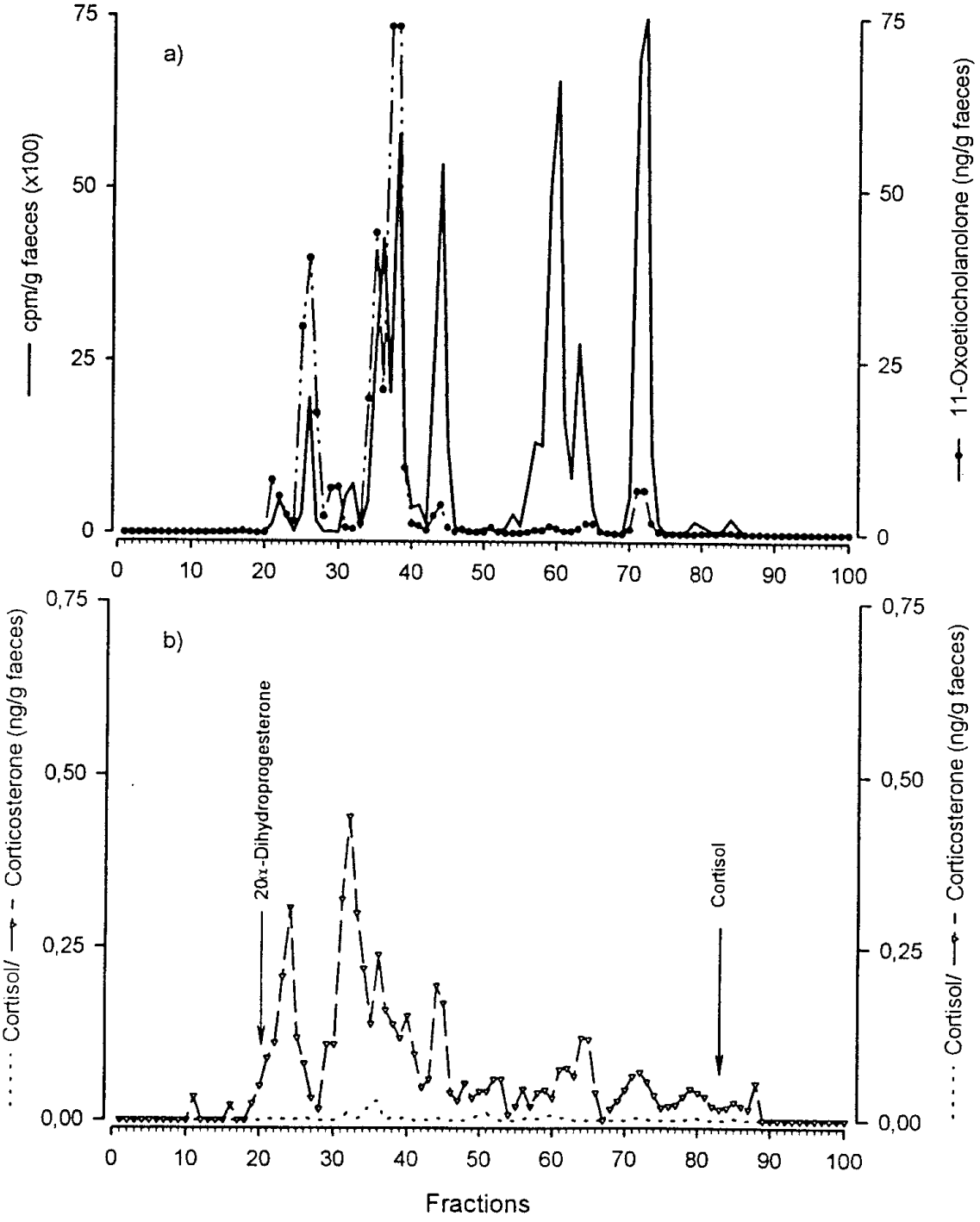

Fig. 1. High performance liquid chromatography (straight phase) separations of faecal ^{14}C-cortisol metabolites of a sheep. Fractions were analysed with the 11-oxoetiocholanolone- (a), a cortisol- and a corticosterone-EIA (b; note different scale of the y-axis!). ^{14}C metabolites were determined by liquid scintillation counting.

Efficiency of extraction was tested as described by Palme et al. (1997) for progesterone metabolites. Briefly, 0.5 g of homogenised faeces, containing the mixture of naturally excreted metabolites of infused ^{14}C-cortisol, (voided about 1 h after faecal peak radioactivity) were extracted with different percentages of methanol (ranging from 0% to 100%).

In addition, all voided faecal samples were collected for four days from two rams infused (for 2 h) with a high dose of cortisol (1 g). After extraction with methanol (80%), cortisol metabolites were measured by the 3 EIAs.

Results

Several (> 15) faecal ^{14}C-metabolites were formed and could be separated by our straight phase HPLC system (Fig. 1 a). They showed a chromatographic mobility between 20 a-dihydroprogesterone and cortisol. "Authentic" cortisol and tetrahydrocortisol were at or below the limit of detection. The presence of immunoreactive ^{14}C-metabolites was confirmed by analysing the HPLC fractions with the newly established 11-oxoetiocholanolone-EIA (Fig. 1 a). They showed similar chromatographic elution patterns as 11,17-dioxoandrostanes which were tested as standards. Little or almost no immunoreactive substances were detected by testing the HPLC-fractions with the corticosterone- and cortisol-EIA, respectively (Fig. 1 b; note the different scale of the axis).

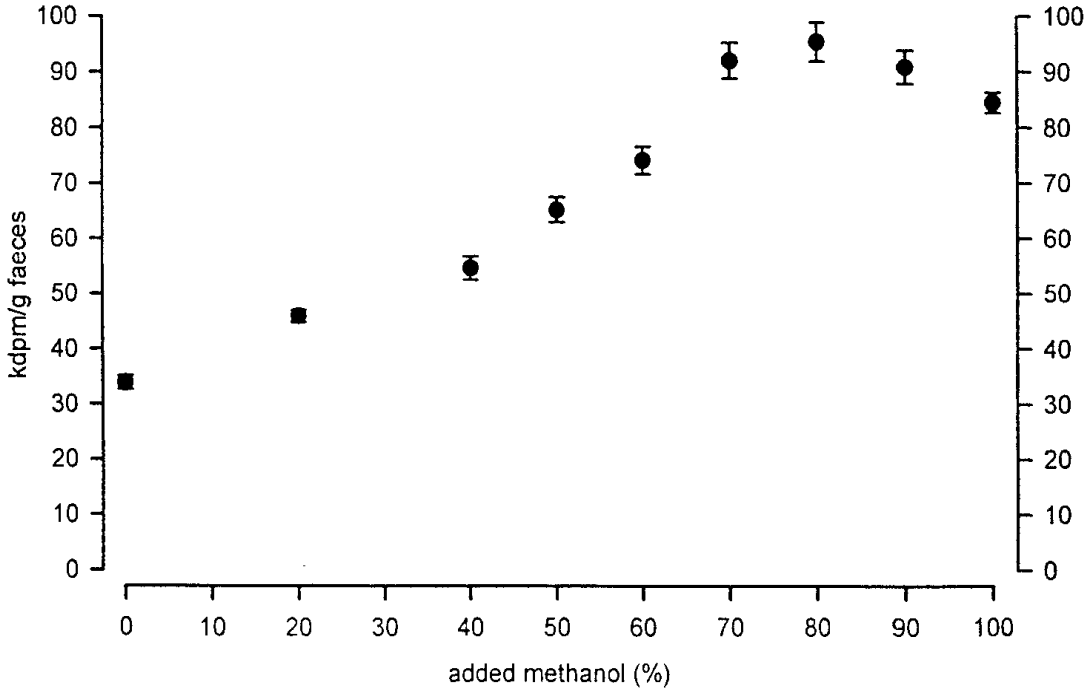

Fig. 2. Amounts (mean ± SD) of recovered metabolites (kdpm/g faeces) in faeces of a sheep, infused with ^{14}C-cortisol, after extraction with different percentages of methanol.

Extracting the faeces (0.5 g) with 1 ml water and 4 ml methanol (= 80% methanol) yielded the highest recovery, although the differences were not significant ($p > 0.05$) when compared to 70% or 90% methanol (Fig. 2). The time course of the concentrations of faecal metabolites, following an infusion of 1 g of cortisol, as determined by the three EIAs, is shown in Fig. 3.

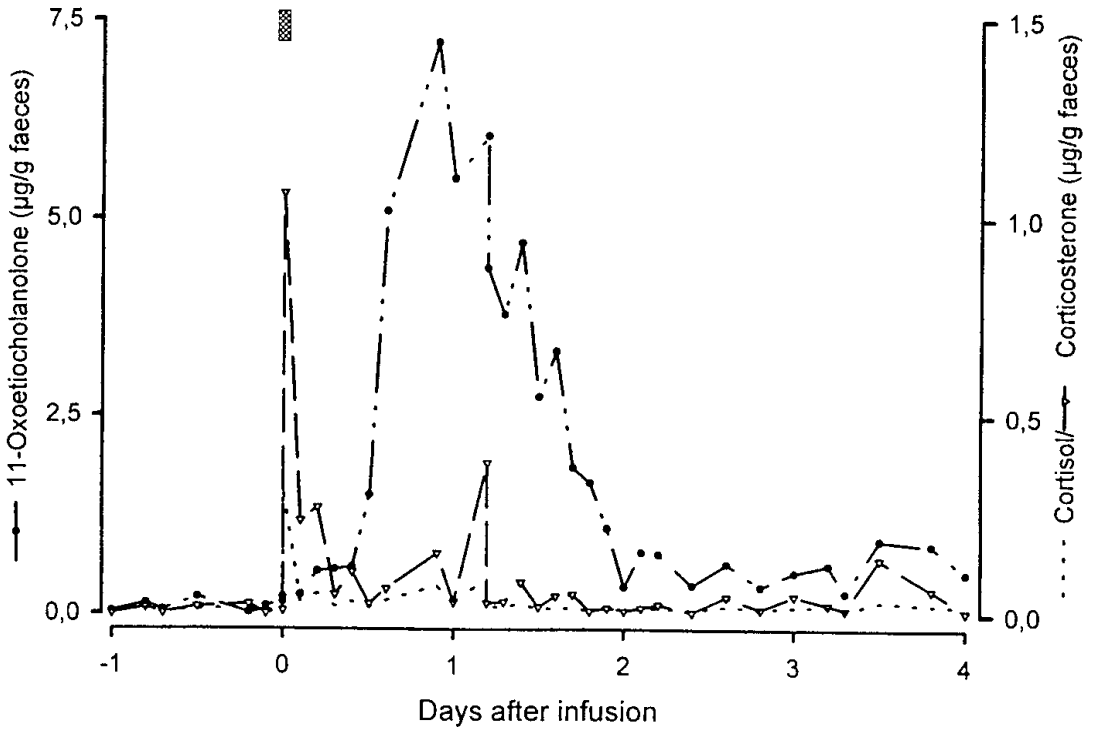

Fig. 3. Time course of faecal metabolites following infusion of a high dose (1 g) of cortisol measured by the 11-oxoetiocholanolone- (left axis), a cortisol- and a corticosterone-EIA (both right axis; note different scale of the axis!). The hatched box indicates the time of infusion.

Discussion

As was found after administration of ^{14}C-progesterone (Palme et al. 1997), the administered steroid (^{14}C-cortisol) itself was almost absent in the faeces of the sheep. According to the crossreactions the established 11-oxoetiocholanolone-EIA can be regarded as a group-specific EIA measuring 11,17-dioxoandrostanes. Although a definite identification has not yet been achieved, there is good evidence (immunoreactivity of ^{14}C-metabolites; comparison with the elution pattern of standards) that 11,17-dioxoandrostanes, derived from cortisol by side chain cleavage, are excreted in sheep faeces. In accordance with results obtained in studies measuring faecal progesterone metabolites (Palme et al. 1997, Schwarzenberger et al. 1996) it also seems advisable to apply the concept of group specific EIAs for measuring faecal cortisol metabolites. More specific immunoassays as generally applied for the determination of cortisol- or corticosterone must be considered inadequate since they will not show significant crossreactions with the faecal cortisol metabolites (Fig. 1 b and 3).

Thus after infusion of 1 g of cortisol the expected excretion pattern (Palme et al. 1996) in the faeces was only seen by application of the 11-oxo-etiocholanolone-EIA but not after application of the cortisol- and the corticosterone-EIA. Only during the infusion small peaks were determined in the cortisol- and corticosterone-EIA. This is in accordance with Palme et al. (1996), who found such peaks of radioactivity around the end of the infusion of ^{14}C-steroids in the faeces of ponies. They may result from a secretion of the mucosa of the distal portion of the intestine. However, such a direct elimination from the blood would only be important in the case of very high steroid concentrations in the blood and probably represents more or less non-metabolised steroids.

With the above described 11-oxoetiocholanolone-EIA it was possible to measure fae-

cal cortisol metabolites in domestic livestock for the first time. Therefore measuring 11,17-dioxoandrostanes appears to be a valuable tool for monitoring adrenal function in farm, zoo and wildlife ruminants, due to the non-invasive sampling techniques. However, further studies are necessary to evaluate the biological relevance of this EIA.

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