Determination of betamethasone and triamcinolone acetonide by GC-NCI-MS in excreta of treated animals and development of a fast oxidation procedure for derivatisation of corticosteroids†

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The use of corticosteroids in combination with other hormonal substances has long been known to result in increased mass gain with bovines. Practice has demonstrated, however, that even the single use of a glucocorticoid may result in growth promoting effects. In addition to the popular dexamethasone, more recently other corticosteroids have also been misused for fattening purposes. The first part of this study deals with the detection of two of them, namely betamethasone and triamcinolone acetonide. Betamethasone was administered orally to a cow at a dose of 50 mg d^{-1} for 5 d, then later the same cow was injected intramuscularly with a dose of 50 mg of betamethasone dipropionate. Excretion in urine and faeces was followed with both HPLC-enzyme immunoassay and a previously described method based on negative chemical ionization mass spectrometry (NCI-MS) after oxidation. For the triamcinolone acetonide study a cow was treated with 50 mg d^{-1} of the drug during a 7 d period. Excretion in faeces was followed with GC-NCI-MS. As triamcinolone acetonide is resistant to the previously described oxidation procedure, however, a hydrolysis step had to be introduced prior to oxidation. In addition to this specific modification necessary for triamcinolone acetonide, in a subsequent part of this study the original oxidation procedure with pyridinium chlorochromate was re-investigated especially to shorten the procedure. With the introduction of potassium dichromate the reaction time could be decreased from 3 h to 10 min.

Introduction

In recent years, corticosteroids have become one of the most important groups of illegal growth promoters in livestock production.[1,2] At first often combined with β-agonists and/or anabolic steroids,[3,4] more recently corticosteroids seem to be applied alone. Their use results in improved feed intake, increased live mass gain and a reduced feed conversion ratio.[5,6]

Belgium has very strict regulations concerning illegal growth promoters and severe sanctions are taken against farmers who administer non-registered corticosteroids (*e.g.*, betamethasone) or registered corticosteroids without a prescription from a veterinarian. Screening and confirmation procedures for these compounds are usually performed by immunoassays, followed by gas chromatography-mass spectrometry (GC-MS).

GC-MS in the negative chemical ionization (NCI) mode after oxidation remains one of the most powerful and attractive confirmation techniques for the determination of corticosteroids. Indeed, by a simple oxidation reaction these relatively large molecules with numerous polar groups are transformed into derivatives which are very well suited for GC and which can be detected extremely sensitively in the NCI mode. This transformation also permits the detection of most corticosteroids in a very specific way, as the matrix interferences are not sensitized. The high sensitivity is thought to be due to the strong response generated by the 1,4-dien-3-one system in ring A, combined with the 11-keto function.[7]

In the first part of the present study, the elimination of one of the most popular corticosteroids, betamethasone, was followed in the urine and faeces of a cow, first after oral treatment with betamethasone and second after intramuscular injection with the dipropionate ester. Both the HPLC-enzyme immunoassay (EIA) method and the GC-NCI-MS method (after oxidation of the corticosteroid) reported earlier were applied.[1]

In a subsequent elimination study, a cow was treated with triamcinolone acetonide. This compound, in contrast to most of the important representatives of the group of corticosteroids, is resistant to oxidation. Therefore, the earlier described oxidation procedure, preceding GC-NCI-MS detection, had to be adapted. A streamlined procedure was elaborated in which the cleavage of the acetonide function under acid conditions was followed by oxidation.

The growing number of samples to be analysed and especially those taken in slaughterhouses created a demand for a faster procedure. The original oxidation procedure with pyridinium chlorochromate was re-investigated and potassium dichromate was introduced. As outlined further in this study, by this means the oxidation reaction could be shortened from 3 h to only 10 min.

Experimental

Reference compounds

Reference products were beclomethasone (9-chloro-11β,17,21-trihydroxy-16β-methylpregna-1,4-diene-3,20-dione), flume-

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thasone (6α,9-difluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione) and methylprednisolone (11β,17,21-trihydroxy-6α-methylpregna-1,4-diene-3,20-dione) from Sigma (St. Louis, MO, USA), betamethasone (9-fluoro-11β,17,21-trihydroxy-16β-methylpregna-1,4-diene-3,20-dione), dexamethasone (9-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione), prednisolone (11β,17,21-trihydroxypregna-1,4-diene-3,20-dione), prednisone (17,21-dihydroxypregna-1,4-diene-3,11,20-trione) and triamcinolone acetonide {9-fluoro-11,21-dihydroxy-16,17-[1-methylethylidenebis(oxy)]pregna-1,4-diene-3,20-dione} from Serva (Heidelberg, Germany) and betamethasone 17,21-dipropionate, fluorometholone (9-fluoro-11β,17-dihydroxy-6α-methylpregna-1,4-diene-3,20-dione) and isoflupredone (9-fluoro-11,17,21-trihydroxypregna-1,4-diene-3,20-dione) from Steraloids (Wilton, NY, USA).

Reagents

All solvents used were of analytical-reagent or HPLC grade and were obtained from Merck (Darmstadt, Germany). Sodium acetate, potassium dichromate and sodium hydroxide were of analytical-reagent grade from Merck and pyridinium chlorochromate was purchased from Sigma (St Louis, MO, USA). Trifluoroacetic acid was of Sequanal grade from Pierce (Rockford, IL, USA). EIA kits for corticosteroids were purchased from CER (Marloie, Belgium). Cross-reactivity for betamethasone was 50% (dexamethasone, 100%).

Instrumentation

The HPLC system for the follow-up of the oxidation reaction consisted of a SpectraSystem P1000XR pump equipped with a SpectraSystem UV6000LP diode array detector, both from Thermo Separation Products (San Jose, CA, USA), and a Merck-Hitachi AS-4000 automatic injector (Merck). The analytical column was an Alltima C_{18} (5 μm) column (15 cm × 3.2 mm id) supplied by Alltech (Deerfield, IL, USA).

Mass spectrometric determinations

Analysis was carried out as earlier described.[1] This included spiking with flumethasone, hydrolysis (for the urine samples only), extraction with diethyl ether, clean-up with HPLC fractionation and chemical oxidation as described below. For the HPLC fractionation the collection started at the retention time of flumethasone minus 0.5 min and ended at the retention time of betamethasone (or triamcinolone acetonide) plus 0.5 min. GC-MS determinations were performed with the equipment and conditions described earlier.[1] Quantitative measurements were made in the selected ion monitoring (SIM) mode at the following *m/z* values: betamethasone 330, 310, triamcinolone acetonide, 316, 296 and flumethasone 348, 328.

Enzyme immunoassay (EIA) procedure

The samples were analysed as for the GC-MS measurements, but without spiking. From the HPLC fractions, collected 0.35 min before until 0.35 min after the retention time of betamethasone, a portion of 50 μl was taken. After four-fold dilution with the dilution buffer delivered with the kit, an aliquot of 50 μl was taken for the assay. The kit applied was the CER corticosteroids EIA kit. Subsequently the test procedure was performed following the instructions provided with the kit.

Oxidation procedure

Original procedure used for the betamethasone excretion study. The residue obtained after evaporation of the HPLC fraction in a vacuum concentrator (45 °C) was dissolved in 50 μl of acetonitrile and 200 μl of an aqueous solution containing 50 mg ml^{-1} pyridinium chlorochromate and 25 mg ml^{-1} sodium acetate. The mixture was vortex mixed and heated at 92 °C for 3 h. After cooling, the oxidized compounds were extracted with 3 ml of *tert*-butyl methyl ether–dichloromethane (2 + 1 v/v) with vortex mixing and ultrasonication. Complete separation of the layers was achieved by centrifugation at 3500*g* for 5 min. After freezing, the organic phase was decanted and evaporated in a vacuum concentrator (45 °C). The residue was reconstituted with 25 μl of toluene.

Modified procedure for the oxidation of triamcinolone acetonide. The residue was dissolved in 50 μl of acetonitrile and 50 μl of 6 M HCl. The mixture was vortex mixed and heated at 85 °C for 30 min. After cooling, 50 μl of 5 M NaOH and 200 μl of an aqueous solution containing 50 mg ml^{-1} pyridinium chlorochromate and 25 mg ml^{-1} sodium acetate were added. The mixture was vortex mixed and heated at 92 °C for 3 h. Extraction was performed as above.

Newly developed fast procedure for free corticosteroids. The residue was dissolved in 50 μl of acetonitrile and 50 μl of a solution of 1 g of $K_2Cr_2O_7$ dissolved in 20 ml of H_2O–H_2SO_4 (9 + 1 v/v). The mixture was vortex mixed and heated at 60 °C for 10 min. After cooling, 100 μl of 10% Na_2CO_3 and 800 μl of water were added, and the oxidized compounds were extracted with 3 ml of hexane–CH_2Cl_2 (2 + 1 v/v) with vortex mixing and ultrasonication. Separation and evaporation of the organic layer were performed as above.

Kinetics of the new oxidation procedure

For all of the corticosteroids listed in Table 4, six aliquots of 250 μl of their stock standard solutions containing 100 μg ml^{-1} were pipetted into glass vials. After evaporation under nitrogen the residues (25 μg of the corticosteroids) were subjected to the fast oxidation procedure described above. Only the time–temperature combination was different: vial 1 of each series was worked up immediately after mixing with the reagent, whereas vials 2–6 were held at 60 °C for 2.5, 5, 7.5, 10 and 15 min, respectively. After work-up and extraction, half of the organic layer was evaporated under nitrogen. The residues were dissolved in 50 μl of acetonitrile with vortex mixing and 10 μl were injected into the HPLC system. Chromatography was carried out under isocratic conditions with a mixture of acetonitrile and an aqueous solution of 5% v/v acetonitrile and 0.01% m/v trifluoroacetic acid. The volume ratios were 20 : 80 for betamethasone, fluorometholone, prednisolone, prednisone and methylprednisolone; 25 : 75 for dexamethasone and 30 : 70 for beclomethasone and flumethasone. The flow rate was held at 1.0 ml min^{-1}.

Betamethasone administration protocol

Two excretion studies were performed on a 5 year old white dairy-cow, first an oral administration of betamethasone and second an intramuscular administration of betamethasone dipropionate. Between both trials a rest period of 40 d was introduced in order to avoid interaction between the two treatments.

Oral administration and sample collection. After acclimatization, the cow (520 kg) received betamethasone for five

Table 1 Betamethasone concentrations observed in faeces and urine of a cow treated orally with 50 mg d^{-1} of the drug for 5 d. Times are given according to the the 24 h clock

Day	Treatment with 50 mg betamethasone at	Collection of faeces at	Betamethasone in faeces/ng g^{-1}		Collection of urine at	Betamethasone in urine/ng ml^{-1}	
			EIA	GC-MS		EIA	GC-MS
1	1000	1930	0.7	1.1			
2	0930	1600	29.5	35.2	1600	260	270
3	0830	1630	40.5	47.5	1630	434	346
4	0900						
5	0900	1600	68.5	55.3	1600	347	346
6		1000	81	57.9	1000	196	192
7		0930	20	23.4			
8		0900	6.5	7.3	0900	24	24
9		0900	1.7	3.4	0900	7.1	11
10		0900	< 2	< 2	0900	2.2	3.9
11		0900	< 2	< 2	1130	< 2	< 2
12		0815	< 2	< 2	0900	< 2	< 2
15		0900		< 2	0900		
16		0900			0900		
17		0900			0900		
18		0900			0900		
19		0900			0900		
22		0900			0900		
23		0900			0900	< 2	< 2

consecutive days at a dose of 50 mg d^{-1}. The 50 mg of betamethasone were dissolved in 20 ml of ethanol and further diluted with 200 ml of water. This solution was directly introduced into the paunch by means of a probe, which was rinsed with 400 ml of water.

Urine and faecal samples were collected from 8 h onwards after the first administration until 19 d after the last administration. A blank sample was taken 3 d before the start of the experiment. Samples were stored at −20 °C until analysis.

Intramuscular administration and sample collection. A 15 ml volume of an oily suspension containing 50 mg of betamethasone dipropionate was injected intramuscularly into the right cervical muscles of the cow (550 kg) at 9.30 am. Samples of urine and faeces were collected from the first until the 19th day after injection.

Triamcinolone acetonide administration protocol

A 5 year old black pied cow of about 600 kg received triamcinolone acetonide orally for 7 d, at a daily dose of 50 mg. Samples of faeces were collected from start until 19 d after the end of treatment.

Results and discussion

Determination of betamethasone

As described earlier, betamethasone, differing from dexamethasone only in the β-position instead of the α-position of the 16-methyl group, on oxidation gives rise to the same two reaction products as dexamethasone: 9α-fluoro-16α-methyl-1,4-androstadiene-3,11,17-trione and its 16β-methyl epimer.[1] Both products can easily be separated by GC. The retention time of the α-epimer is lower than that of the β-epimer, and the spectra are also different. Distinction between dexamethasone and betamethasone is possible, however, by the large difference in the ratio of the two oxidation products obtained: the ratio of α/β, in the described procedure,[1] is about 5 for dexamethasone and about 0.2 for betamethasone. The ratios obtained are fairly constant, especially within one series. They are, however, influenced by several factors, of which temperature and reaction time seem to be important. This supports the thesis of Her and Watson[8] that the two isomeric products are the result of the enolization of the C-17 ketone under oxidation conditions. During the optimization of the oxidation conditions, we observed that an increase in temperature of 10 °C (from 87 to 97 °C) resulted in a decrease in the β/α ratio for betamethasone from 6.6 to 5.9 and of the α/β ratio for dexamethasone from 5.6 to 5.4. Concerning the influence of the reaction time, with an increase from 2 to 4 h the ratio for betamethasone dropped from 6.7 to 5.8 and that for dexamethasone from 5.4 to 5.3. Therefore, it is advisable to include in a series of samples at least one spiked sample of each of the two corticosteroids. In case of doubt, one can always resort to standard additions. Another possibility to obtain complete evidence for the presence of either dexamethasone or betamethasone is the combined application of a generic EIA kit with reaction towards dexamethasone and betamethasone and a specific kit for dexamethasone. The 'enhanced dexamethasone kit' from Biognost (Wevelgem, Belgium) has a cross-reactivity for betamethasone of only 1.5%. For dexamethasone a reaction is obtained with both kits, whereas betamethasone only reacts in the first kit.

Excretion profile of betamethasone. The results from the excretion study, for betamethasone, obtained by EIA and GC-MS, for a cow after oral administration of the drug and intramuscular injection of the dipropionate ester are given in Tables 1 and 2, respectively. The excretion profiles in urine and faeces after oral administration, based on GC-MS measurements, are shown in Fig. 1. The elimination of betamethasone seems to proceed essentially *via* urine and only to a lesser extent *via* the faeces. This is different to dexamethasone, for which an earlier study[1] indicated that elimination *via* urine and faeces was comparable. Steady state conditions for betamethasone in urine are reached very fast: the highest concentration was obtained already the third day of the 5 d treatment. The concentrations measured in the faeces gradually increased and reached a maximum at the first day after the end of the treatment, which may indicate an enterohepatic circulation. Resorption of the drug after oral administration seems to be complete, whereas elimination is fast.

Also after intramuscular injection of betamethasone dipropionate, the elimination proceeds primarily *via* the urine (Fig.

2). Here the elimination is slower than after oral administration, which also may originate from the application of the ester.

Determination of triamcinolone acetonide

The sensitive determination *via* GC-NCI-MS after oxidation is applicable to most of the important representatives of the group of corticosteroids, such as dexamethasone, betamethasone, flumethasone, isoflupredone, prednisolone, prednisone and methylprednisolone. Triamcinolone acetonide, however, which has an acetonide function between C-16 and C-17, is resistant to oxidation with pyridinium chlorochromate.

LC-MS has been reported for the confirmation of triamcinolone acetonide.[9] We tried to apply GC-NCI-MS after cleavage of the acetonide and subsequent oxidation. Although it has been reported that ethers may be cleaved by heating with concentrated hydrogen iodide and hydrogen bromide, and that hydrochloric acid is only seldom successful,[10] we obtained the best results with the latter. After a number of preliminary experiments, the reaction mixture was chosen as 50 μl of acetonitrile, in order to dissolve the residue, and 50 μl of 6 M hydrochloric acid. Temperature–time studies gave the optimum conditions as 85 °C for a reaction time of 30 min. Fig. 3 shows the response found after 30 min at different temperatures. The use of hydrochloric acid allowed us to perform the oxidation step after a simple neutralisation without any further manipulations.

In the chromatograms, two peaks with almost the same spectra are obtained, the ratio of which was found to be strongly dependent on the reaction conditions. Under the given condi-

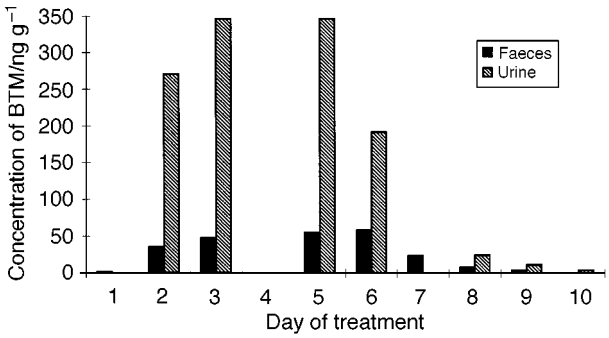

Fig. 1 Betamethasone excretion profiles in faeces and urine of a cow treated orally with 50 mg d^{-1} of the drug for 5 d.

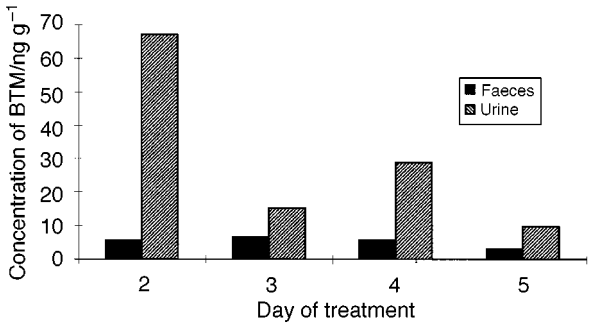

Fig. 2 Betamethasone excretion profiles in faeces and urine of a cow injected intramuscularly with 50 mg of betamethasone dipropionate on day 1.

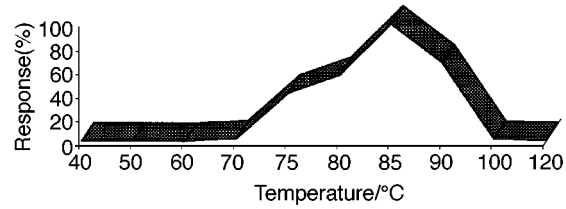

Fig. 3 Cleavage of the acetonide function of triamcinolone acetonide with 6 M HCl for 30 min at different temperatures. Response expressed as percentage relative to the optimum.

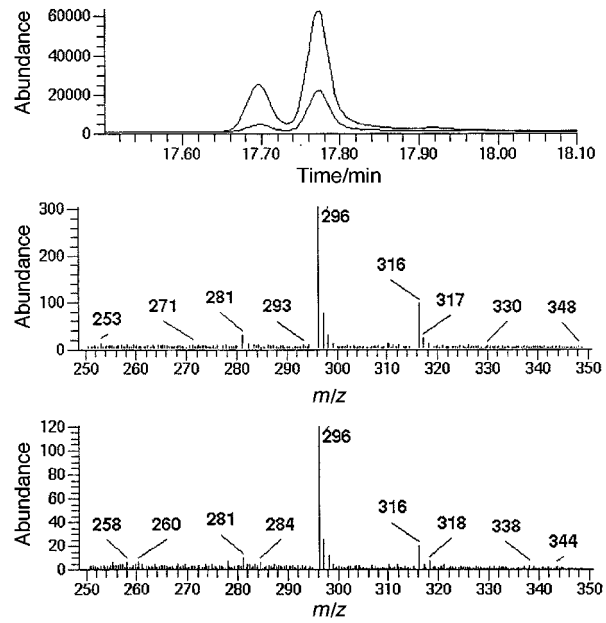

Fig. 4 Selected ion chromatograms of ions of *m/z* 296 and 316 under NCI conditions for oxidized triamcinolone acetonide, with full scan spectra of the two observed peaks.

Table 2 Betamethasone concentrations observed in faeces and urine of a cow treated intramuscularly with betamethasone dipropionate on day 1 at 9.30 am. Times are given according to the 24 h clock

Day	Collection of faeces at	Betamethasone in faeces/ng g^{-1} EIA	GC-MS	Collection of urine at	Betamethasone in urine/ng ml^{-1} EIA	GC-MS
2	1100	3.9	5.5	0900	103	67
3	0900	4.3	6.6	0900	19.8	15.2
4	0900	3.1	5.7	0900	32.6	28.8
5	0900	1.7	< 2	0900	5.6	9.7
8	0900	< 2	< 2	0900	< 2	< 2
9	0900	< 2		0900	< 2	
10	0900	< 2		0900	< 2	
11	0900	< 2		0900	< 2	
12	1100	< 2		1430	< 2	
16	1100	< 2		1100	< 2	< 2
19	0830	< 2	< 2	0830	< 2	

tions the first peak is about 40% of the second. Fig. 4 shows the selected ion chromatogram of the ions of *m/z* 316 and 296 and the spectra of both observed peaks. The response of the oxidized triamcinolone acetonide obtained in this way is about 10 times lower than that for the same amount of dexamethasone.

Excretion profile of triamcinolone acetonide. The administration protocol and GC-MS results for the excretion study of triamcinolone acetonide, given orally to a cow, are given in Table 3. In Fig. 5 the elimination profile of triamcinolone acetonide after oral administration for 7 d is compared with that of dexamethasone under identical conditions.[1] Although the concentrations for both drugs during the first few days are more or less comparable, the concentrations of triamcinolone acetonide are generally lower. After treatment, the concentration of triamcinolone acetonide decreases very rapidly and can be hardly followed until 1 week after the end of treatment. Elimination in another form, such as free triamcinolone, may be a possible explanation.

Fast oxidation procedure

The original oxidation procedure with pyridinium chlorochromate described earlier has been widely used for various matrices.[11] One of the more important applications nowadays is the determination of corticosteroids in liver. One of the drawbacks to the application of the procedure to urgent samples from slaughterhouses is the long oxidation time of 3 h. As the oxidation temperature already was high, namely 92°C, we investigated the use of stronger oxidizing agents. One of these, potassium dichromate in acidic conditions, seemed very suitable, and we started to optimize the reaction conditions. Of important help here was that we were able to follow the reaction kinetics by HPLC (Table 4). Therefore, we first optimized the HPLC conditions so that we could follow both the corticosteroid and the oxidation products, which was impossible with GC-MS.

For the 11-hydroxycorticosteroids the oxidation reaction includes two steps: oxidation of the 17-hydroxy function with cleavage of the C-17–C-20 bond and oxidation of the 11-hydroxy function. These two steps could be followed during reaction: the HPLC traces from the reaction mixtures showed in addition to the corticosteroid and the 3,11,17-trione, also an intermediate compound. This compound was readily obtained by mixing the standard with the reagent at room temperature, while the second step with formation of the 3,11,17-trione proceeded much more slowly. As Her and Watson[8] reported that the 17-OH function can be selectively oxidized, without affecting the 11-OH function, with sodium bismuthate under acidic conditions, we first thought that this intermediate was the 17-keto derivative. However, the oxidation of prednisone, the 11-keto analogue of prednisolone, was completely comparable to that of the intermediate of the reaction of prednisolone, proving that not the 17-OH function, but the 11-OH function, was oxidized first. Another indication for this was that the retention times for the glucocorticosteroid and the intermediate 11-keto analogue were very close, which was not expected for the 17-keto analogue, which should be much more apolar owing to the loss of the side chain. The final proof was obtained in an experiment in which the intermediate compound of the oxidation of prednisolone, obtained at room temperature within 1 min, was identified as prednisone by LC-MS.

After some preliminary oxidation experiments, the reaction temperature was chosen as 60 °C and sampling was performed immediately after addition of the oxidation reagent and subsequently at 2.5, 5, 7.5, 10 and 15 min. The oxidation was followed for the nine corticosteroids listed in Table 4. The areas, given as a percentage of the maximum area measured of the 3,11,17-trione, of both the intermediate 3,11-dione and the 3,11,17-trione, are given in Table 4. In general, the intermediate was completely oxidized after 10 min, and the maximum

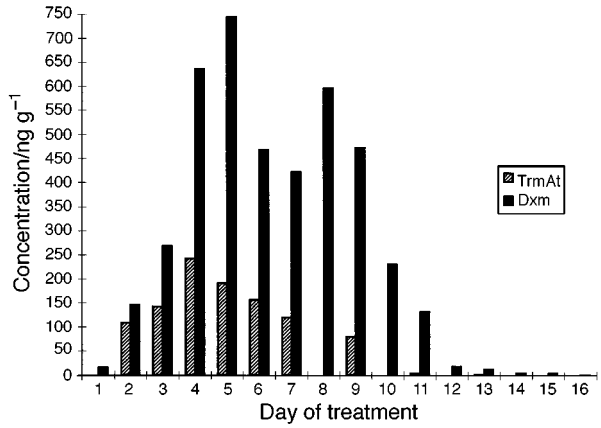

Fig. 5 Triamcinolone acetonide (TrmAt) excretion profile in the faeces of a cow treated orally with 50 mg d^{-1} of the drug for 7 d, in comparison with the excretion profile of dexamethasone (Dxm).

Table 3 Triamcinolone acetonide concentrations measured by GC-NCI–MS in faeces of a cow treated orally with 50 mg d^{-1} of the drug for 7 d. Times are given according to the 24 h clock

Day	Treatment with 50 mg triamcinolone acetonide at	Collection of faeces at	Triamcinolone acetonide in faeces/ng g^{-1}	Corresponding dexamethasone levels/ng g^{-1}
1	0800	2000		15.1
2	0800	0800	108	146
3	0800	2000	142	269
4	0800	0800	242	636
5	0800	2000	191	744
6	0800	0800	156	468
7	0800	2000	120	422
8		0800		596
9		0800	80	473
10		0800		231
11		0800	3.93	132
12		0800		18.3
13		0800	1.48	12.5
14		0800		4.89
15		0800	0.43	3.41
16		0800		1.05
17		0800		0.81
18		0800		0.65
19		0800		0.48

Table 4 Kinetics of the oxidation reactions of some corticosteroids with $K_2Cr_2O_7$ at 60 °C, expressed as a percentage relative to the maximum response of the 3,11,17-trione

		Reaction time/min					
Oxidation of	Reaction product	0	2.5	5	7.5	10	15
Beclomethasone	3,11-Dione	236	115	19	4[a]	8[a]	9[a]
	3,11,17-Trione	0	85	93	90	100	88
Betamethasone	3,11-Dione	42	60	9	5	0	0
	3,11,17-Trione	0	76	99	100	99	98
Dexamethasone	3,11-Dione	26	38	69	37	16	9
	3,11,17-Trione	3	25	42	70	93	100
Flumethasone	3,11-Dione	13	79	58	43	32	15
	3,11,17-Trione	0	34	45	72	89	100
Fluorometholone	3,11-Dione	35	199	187	170	160	133
	3,11,17-Trione	0	23	37	49	67	100
Isoflupredone	3,11-Dione	9	36	22	19	11	4
	3,11,17-Trione	0	36	64	76	86	100
Methylprednisolone	3,11-Dione	76	37	33	16	15	4
	3,11,17-Trione	13	51	79	84	99	100
Prednisolone	3,11-Dione	29	24	18	10	8	3
	3,11,17-Trione	2	40	61	80	100	96
Prednisone	3,11-Dione	40	21	23	10	8	3
	3,11,17-Trione	2	41	77	85	100	90

[a] Disturbed by side products.

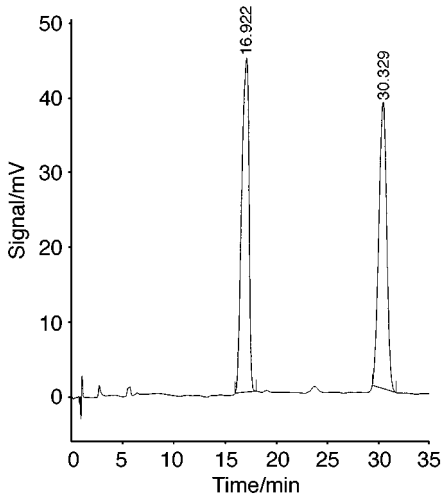

Fig. 6 HPLC trace of the reaction mixture, with the intermediate 3,11-dione and the 3,11,17-trione of prednisolone, after oxidation with $K_2Cr_2O_7$ at 60 °C for 2.5 min.

response of the trione was also reached. The reaction conditions adopted with potassium dichromate under acidic conditions were therefore 60 °C for 10 min. Fig. 6 shows the intermediate 3,11-dione and the 3,11,17-trione from oxidation of prednisolone after an oxidation time of 2.5 min at 60 °C.

In this oxidation procedure also, the earlier used *tert*-butyl methyl ether–dichloromethane (2 + 1 v/v) for extraction of the triones prior to GC-MS analysis was replaced with hexane–dichloromethane (2 + 1 v/v), as the latter was found to be more selective in not dissolving the oxidation reagents. Changing the original oxidation procedure to the fast procedure did not influence the visual aspect of the chromatograms.

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Paper 8/04921A