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Journal of Chromatography B, 753 (2001) 217–223

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Accelerated solvent extraction and liquid chromatography–tandem mass spectrometry quantitation of corticosteroid residues in bovine liver

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Received 29 May 2000; received in revised form 7 September 2000; accepted 9 October 2000

Abstract

A new method for the rapid extraction and unequivocal confirmation of two highly potent fluorinated synthetic corticosteroids, dexamethasone and its β-epimer betamethasone, in bovine liver was developed. Flumethasone was used as internal standard. An extraction procedure using an accelerated solvent extraction system was employed for the isolation of the analytes in liver samples. The procedure was highly automated, including defatting and extraction steps, sequentially carried out under $1.0 \cdot 10^4$ kPa in about 35 min. The extracts were then directly analysed by tandem mass spectrometry with on-line liquid chromatography. The analytes were ionised in a heated nebulizer interface operating in the negative ion mode where the molecular related ions $[M\text{-}H\text{-}CH_2O]^-$ were generated for each analyte, at m/z 361 for betamethasone and dexamethasone and at m/z 379 for flumethasone. They served as precursor ions for collision-induced dissociation and three diagnostic product ions for the drugs were identified to carry out analyte confirmation by selected reaction monitoring. Assessment of recovery, specificity and precision for betamethasone, dexamethasone and flumethasone proved the method suitable for confirmatory purposes. The limit of quantification of betamethasone and dexamethasone in liver tissue was 1.0 μg/kg.

Keywords: Accelerated solvent extraction; Corticosteroids

1. Introduction

Corticosteroid drugs are synthetic analogues of hormones. They are widely used to combat inflammatory diseases in food-producing animals, but they are also frequently employed as growth promoters. The European Union banned their administration for fattening purposes, according to the 96/22/EC Directive [1]. At present, definitive maximum residue limits (MRLs) have been established only for dexamethasone in foodstuffs from bovine, equine and porcine species: 2.0 μg/kg in liver and 0.75 μg/kg in muscle and kidney. For bovines, an MRL of 0.3 μg/kg has been established for milk [2].

Residue depletion studies in bovines have revealed that dexamethasone tends to accumulate and persist in liver, which has therefore been identified as the target tissue for residue analysis [3].

A critical aspect of drug residue analyses is the

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0378-4347/01/$ – see front matter
PII: S0378-4347(00)00544-2

sample preparation required to isolate the analytes from complex biological matrices such as animal tissues.

Very few papers have been published on analysis of corticosteroids in liver tissue. Cumbersome sample preparation procedures, which are both time- and solvent-consuming and lack automation [4–7], are usually reported. On the other hand, although a more recent extraction technology, namely supercritical fluid extraction (SFE), has been evaluated for the isolation of dexamethasone from bovine liver, further improvement of extraction efficiency is necessary [8].

A new extraction technique, accelerated solvent extraction (ASE), has recently been reviewed for the extraction of a variety of compounds and a considerable number of applications are reported in environmental, food, polymer and pharmaceutical areas [9]. Conventional liquid solvents are used to perform extractions at elevated pressures ($1.0\cdot10^4$–$1.4\cdot10^4$ kPa) and temperatures (50–200°C) to extract solid or semi-solid samples quickly and with much less solvent than conventional techniques. With ASE, the sample is enclosed in a stainless steel vessel filled with an extraction solvent which is pressurised and heated. The sample is allowed to statically extract for 5–10 min, with the expanding solvent vented to a collection vial. Next, compressed nitrogen purges the remaining solvent into the same vial. The entire procedure typically requires less than 15 min and approximately 15 ml of solvent for a 10 g sample. The effectiveness of ASE is based on the increases in analyte solubilities that occur at temperatures above the boiling points of commonly used solvents. At higher temperatures, the kinetic processes for the desorption of the analytes from the matrix are accelerated compared with extractions using solvents at room temperature. Solvent usage is reduced as well, as a result of the higher analyte solubility in the heated solvent. Elevated pressures, used to maintain the solvents in a liquid state, facilitate extractions from samples in which the analytes are trapped in matrix pores [10].

Many analytical techniques have been proposed for the detection of corticosteroids [11–20]. Immunochemical techniques play a predominant role in screening for the presence of corticosteroids. Gas chromatography–mass spectrometry (GC–MS) is currently applied for the identification and confirmatory analysis of these compounds although it requires cumbersome and time-consuming derivatisation steps. Recently, applications of LC using thermospray mass spectrometry (TSP-MS) [21] and atmospheric pressure chemical ionisation tandem mass spectrometry (APCI-MS–MS) [22] have been proposed for the determination of corticosteroids in urine. The latter technique provides structural information of the native compound and a high degree of sensitivity, although no separation of betamethasone and dexamethasone is achieved [22].

In this study the combination of two modern techniques, ASE and selected reaction monitoring (SRM) LC–MS–MS via a heated nebulizer (HN) interface, proved to be well suited for the determination of fluorinated synthetic corticosteroids such as betamethasone, dexamethasone and flumethasone in bovine liver.

2. Experimental

2.1. Chemical and reagents

Absolute ethanol, acetonitrile, ammonium acetate, ethyl acetate, hexane and methanol were HPLC grade and purchased from Farmitalia, Carlo Erba, (Milan, Italy). Diatomaceous earth–Hydromatrix was obtained from Varian (Harbour City, CA, USA). Water was purified in a Milli-Q system (Millipore, Bedford, MA, USA). Betamethasone, dexamethasone and flumethasone were provided by Sigma (St. Louis, MO, USA). Individual corticosteroid standard stock solutions of 0.5 mg/ml were prepared in methanol. Individual and composite working standard solutions were prepared daily by appropriate dilution of the standard stock solutions with methanol. All solutions were stored at 4°C and were stable for at least 1 month.

2.2. Accelerated solvent extraction

Extraction of liver samples was carried out by using an ASE 200 system (Dionex, Sunnyvale, CA, USA). The ASE system was equipped with an autosampler carousel and a collection tray that allowed up to 24 separate samples to be extracted

sequentially. Stainless steel extraction cells and glass collecting vials with 22 ml and 40 ml volumes, respectively, were used. An aliquot (5 g) of liver tissue was spiked with 15 ng of flumethasone (I.S.) and mixed with 7.5 g of diatomaceous earth–Hydromatrix. The mixture was loaded into the ASE cell and two extraction steps were sequentially performed under the ASE conditions described in Table 1. The ASE extract of about 35 ml was evaporated until dryness; the residue was dissolved in about 4 ml of absolute ethanol and centrifuged at 4000 rpm. The ethanol layer was evaporated to dryness and the residue dissolved in 500 μl of methanol.

2.3. Liquid chromatography–mass spectrometry

Analyses were performed with a Phoenix 20 CU LC pump (Fisons, Milan, Italy) liquid chromatograph. A Valco injection valve (Valco, Houston, TX, USA) equipped with a 5-μl internal loop was used for injection by flow injection analysis (FIA)–MS, FIA–MS–MS and LC–MS–MS. Chromatographic separations were obtained under isocratic conditions using a reversed-phase Kingsorb C_{18} column (Phenomenex, Torrance, CA, USA) (250×2 mm, 5 μm), at room temperature, with a mobile phase of acetonitrile–5 m*M* ammonium acetate–methanol (35:60:5, v/v) and at a flow-rate of 100 μl/min. Mass spectral analyses were performed on a PE-Sciex API III plus triple-quadrupole (PE-Sciex, Thornill, Canada) equipped with an APCI source and a HN interface operating in the negative ion mode. Ultra-high-purity nitrogen was used as curtain gas (0.6 l/min), while air was used as nebulizer gas (400 kPa) and auxiliary gas (1.5 l/min). The HN temperature was set at 350°C and the discharge current at −4 μA. The orifice potential voltage (OR) was set at −80 V for betamethasone and dexamethasone and −70 V for flumethasone. Full-scan mass spectra were acquired in the negative ion mode from *m/z* 250 to *m/z* 500. In the MS–MS experiments, product ion mass spectra were acquired in the negative ion mode by colliding the precursor ion selected by quadrupole 1 (Q1), with argon (gas thickness $300 \cdot 10^{13}$ molecules/cm^2) in quadrupole 2 (Q2), operating in radio frequency (RF)-only mode, and by scanning (*m/z* 50 to *m/z* 400) the product ions by the third quadrupole mass spectrometer, Q3. A collision energy of −25 eV was chosen for the collision-induced dissociation (CID) experiments. The molecular related ions, $[M\text{-}H\text{-}CH_2O]^-$, at *m/z* 361 for betamethasone and dexamethasone and at *m/z* 379 for flumethasone, were the precursor ions for CID experiments. Precursor–product ion combinations of *m/z* 361→307, *m/z* 361→292, *m/z* 361→325 for betamethasone and dexamethasone and *m/z* 379→305, *m/z* 379→325 for flumethasone were used to carry out SRM-LC–MS–MS analyses. The dwell time for each monitored transition was 200 ms.

Table 1
ASE conditions

	Defatting	Extraction
Solvent	Hexane	Hexane–ethyl acetate (1:1, v/v)
Temperature	60°C	50°C
Pressure	$1.0 \cdot 10^4$ kPa	$1.0 \cdot 10^4$ kPa
Heat time	5 min	5 min
Static time	5 min	5 min
Flush volume	100%	60%
Purge time	100 s	100 s
Static cycles	3	1

2.4. Calibration, quantitation assessment of recovery and precision

Calibration and quantitation were computed using MacQuan version 1.3 software from PE-Sciex. Calibration curves were obtained from SRM-LC–MS–MS analyses of extracts of liver blank control samples spiked with mixtures of betamethasone and dexamethasone in the range 1–10 μg/kg and with 3 μg/kg of flumethasone, by plotting liver corticosteroids concentrations against measured peak area ratios of the analyte to the I.S., using a least-square regression model. Estimates of the amounts of analyte in fortified and real samples were interpolated from these calibration graphs.

To assess the recovery and precision of the SRM-LC–MS–MS method, three replicates of blank liver control samples, fortified with mixtures of the investigated analytes to obtain concentrations of 1, 2, 4 μg/kg were prepared and analysed on each of 3 days for each concentration. These extracted samples were used to estimate the recovery and precision of the method by comparing the amounts of each analyte

extracted from liver to that of known amounts of neat corticosteroid standard.

3. Results and discussion

3.1. FIA–MS, FIA–MS–MS, LC–MS–MS

The development of the SRM-LC–MS–MS method firstly required experiments carried out by FIA–MS on corticosteroid standard solutions to determine suitable HN parameters for absolute sensitivity and S/N ratio, as well as to select the molecular related ions. Representative HN full-scan (mass range m/z 250–500) mass spectra as obtained by FIA in the MS negative ion mode for the analytes under investigation are shown in Fig. 1. The molecular related ions $[M\text{-}H\text{-}CH_2O]^-$, at m/z 361, for betamethasone and dexamethasone and at m/z 379 for flumethasone were observed with no evidence of fragmentation. In order to obtain additional structural information, they were selected as precursor ions for

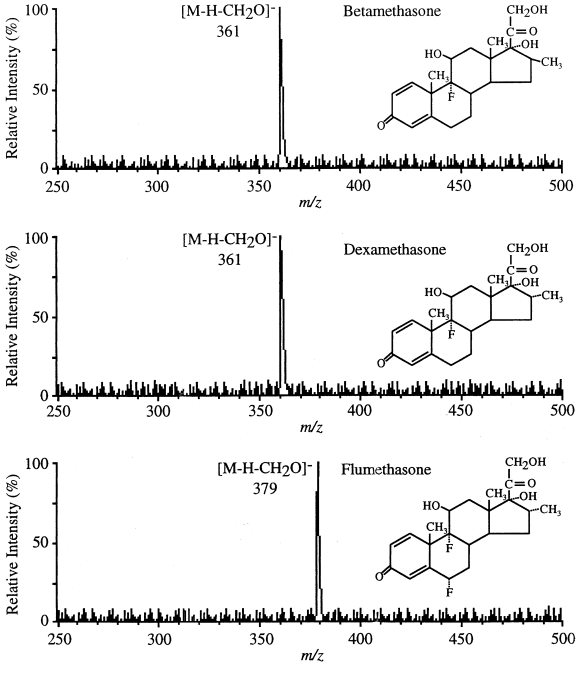

Fig. 1. Negative ion mass spectra of betamethasone, dexamethasone and flumethasone. Conditions as described in the Experimental section.

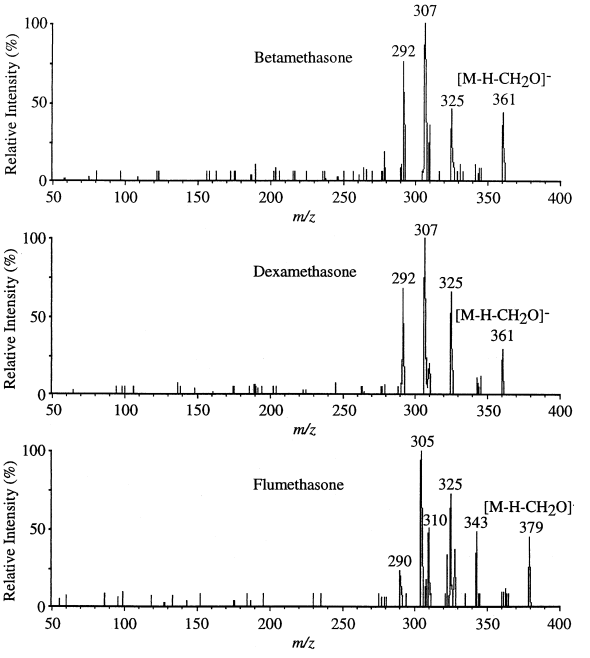

Fig. 2. Negative product ion mass spectra of betamethasone, dexamethasone and flumethasone. Conditions as described in the Experimental section.

CID in the MS–MS experiments carried out by FIA–MS–MS on the individual corticosteroid standard solutions. Fig. 2 shows the negative product ion mass spectra (mass range m/z 50–400) of the molecular related ions $[M\text{-}H\text{-}CH_2O]^-$, of betamethasone, dexamethasone and flumethasone. Consistent with previous investigations [22], the MS–MS spectra revealed the production of the most abundant product ions at m/z 292, 307 and 325 for betamethasone and dexamethasone and m/z 305 and 325 for flumethasone. Precursor–product ion combinations of m/z 361→307, m/z 361→292, m/z 361→325 for betamethasone and dexamethasone and m/z 379→305, m/z 379→325 for flumethasone were used to carry out SRM-LC–MS–MS analyses.

3.2. ASE

Experiments were performed on blank and fortified control samples, in order to optimise the relevant extraction parameters of the ASE for each analyte. The extraction of corticosteroids was therefore investigated considering various ASE parame-

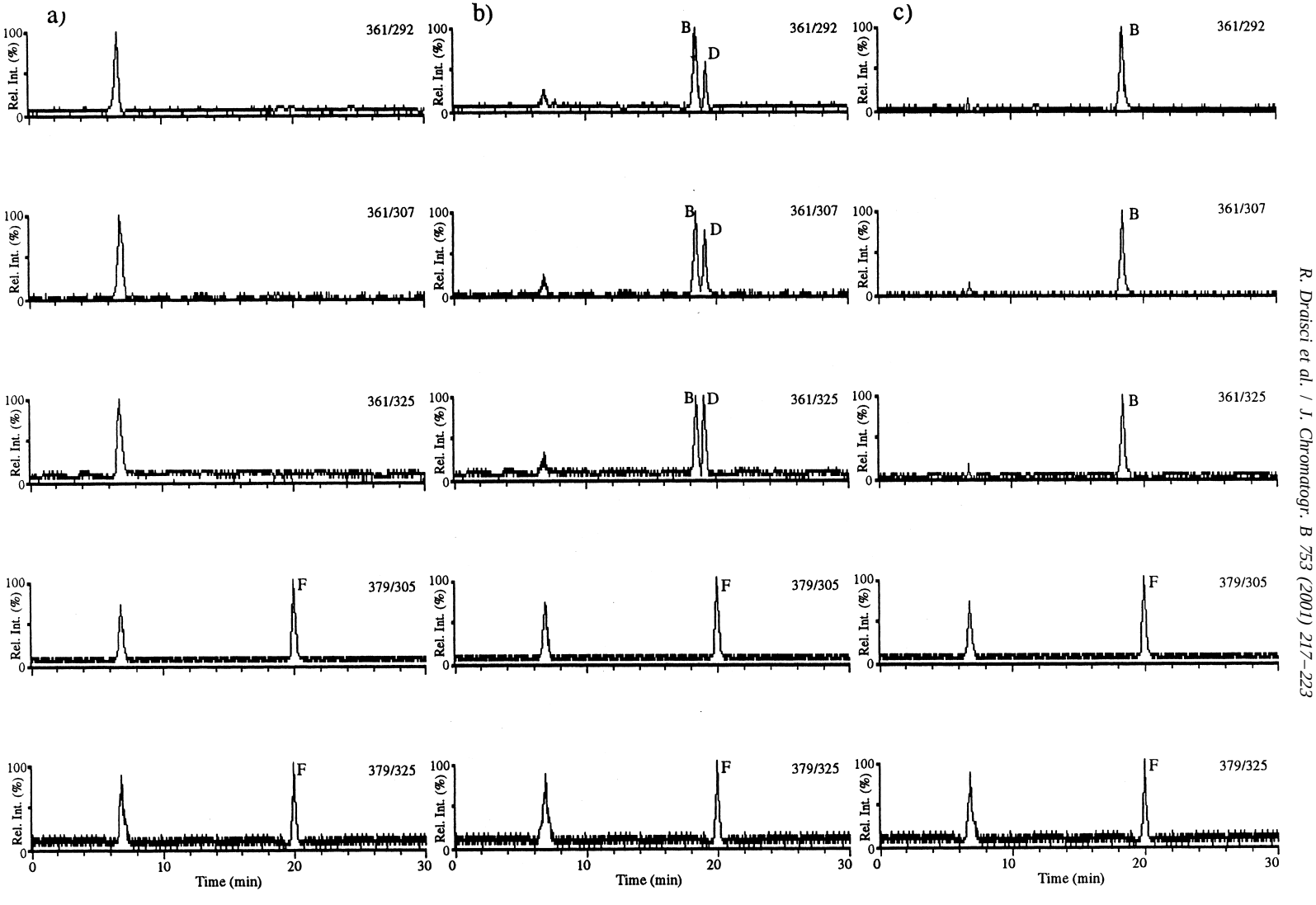

Fig. 3. SRM-LC–MS–MS chromatograms of: (a) extract of blank control liver spiked with 3 ng/g of flumethasone (F) as I.S.; (b) extract of blank control liver spiked with 2.0 ng/g of betamethasone (B), dexamethasone (D) and 3 ng/g of flumethasone (F); (c) extract of positive liver sample containing betamethasone (B) (3.8 ng/g). Precursor–product ion combinations used in SRM detection are shown. Conditions as described in the Experimental section.

ters, among which temperature, solvent composition and number of static cycles appear to be the most critical ones affecting the extraction behaviour of the analytes from the liver matrix. An efficient fat removal was obtained using hexane through three static cycles of 5 min at 60°C. The exhaustive extraction of the target drugs was then obtained with a mixture of hexane–ethyl acetate (1:1, v/v) by one static cycle at 50°C (Table 1). The entire procedure was completely automated and required about 35 min.

3.3. Analytical data

Specificity of the SRM-LC–MS–MS method was proved by processing and analysing liver blank control samples (Fig. 3a). No interference was noticed around the retention times of the analytes.

Under the adopted chromatographic conditions, the separation of betamethasone (t_R=18.6 min), dexamethasone (t_R=19.2 min) and flumethasone (t_R=19.9 min), observed in SRM profiles of liver spiked control samples (Fig. 3b), was acceptable.

The calibration curves using fortified samples provided correlation coefficients (r^2) higher than 0.995 for both the analytes in the whole range of tested concentrations.

The overall repeatability, as determined by calculating the relative standard deviation (RSD) for the repeated measurements, ranged from 2.57 to 7.40% (Table 2). The recovery of the analytes was calculated by comparing the peak areas of fortified samples with those of the corresponding standards. Recovery values ranged from 75.1 to 77.3% (Table 2). The accuracy and precision data obtained in our study can be defined acceptable according to the ranges established by the Commission Decision 93/256/EEC [23].

Table 2
Inter-day precision and recovery for corticosteroids in bovine liver samples

Analyte	Spike (ng/g)	Found[a] (ng/g)	Recovery (%)	RSD (%)	*n*
Betamethasone	1.00	0.75±0.05	75.4	6.89	9
	2.00	1.53±0.08	76.6	5.46	9
	4.00	3.10±0.09	77.4	2.79	9
Dexamethasone	1.00	0.75±0.06	75.1	7.40	9
	2.00	1.54±0.08	76.8	5.32	9
	4.00	3.09±0.08	77.3	2.57	9
Flumethasone	1.00	0.74±0.05	74.4	7.05	9
	2.00	1.52±0.08	76.2	5.00	9
	4.00	3.09±0.08	77.3	2.63	9

[a] Mean±SD.

The limit of quantification (LOQ) of each corticosteroid residue in bovine liver (i.e., 1 μg/kg; signal-to-noise ratio of at least 10:1) was the lowest concentration for which acceptable accuracy and precision were obtained. The LOQ was lower than the MRL (2 μg/kg dexamethasone), thus proving the SRM-LC–MS–MS method suitable for confirmatory purposes.

SRM-LC–MS–MS was then used to analyse real samples collected from animals of both sexes, as part of the national program for hormone control in Italy. Representative SRM-LC–MS–MS chromatograms from the analysis of a violative bovine liver sample, containing 3.8 μg/kg of betamethasone, are shown in Fig. 3c.

4. Conclusion

The major goal of this research was to investigate for the first time the suitability of ASE for the extraction of some corticosteroids from bovine liver samples. The results demonstrated that this technique offers many advantages over extraction methods currently used in the isolation of corticosteroids. The extraction procedure is rapid, simple and highly automated, requiring mixing the samples with a drying agent and transferring the mixture to an extraction cell. One sample can be extracted in about 35 min and up to 24 separate samples can be extracted sequentially by the ASE system. No chlorinated solvents are employed.

The LC–APCI-MS–MS method was used for the determination of the analytes in liver extracts with a high degree of specificity. The developed LC–MS–MS method, providing unequivocal identification and accurate quantification of betamethasone and dexamethasone, complies with the criteria laid down by the Commission Decision 93/256/EEC [23] for confirmatory methods.

In conclusion the combination of the two investi-

gated modern techniques, ASE and SRM-LC–MS–MS, was shown valuable for the determination of fluorinated synthetic corticosteroids such as betamethasone, dexamethasone and flumethasone in bovine liver. The proposed approach seems particularly useful for routine control of the illegal use of corticosteroids in food-producing animals.

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