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Identification of betamethasone and a major metabolite in equine urine

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Abstract: Betamethasone and its major unconjugated metabolite, 6-β-hydroxybetamethasone, were detected in equine urine by thin-layer chromatography and characterized by micro-liquid chromatography/mass spectrometry (micro-LC/MS) [1, 2]. Their structures were confirmed by a combination of infrared spectroscopy and nuclear magnetic resonance spectroscopy.

Keywords: *Betamethasone; 6-β-hydroxybetamethasone; metabolites in equine urine; IR spectroscopy; NMR spectroscopy.*

Introduction

Betamethasone (9-fluoro-16-β-methyl-11β,17,21-trihydroxyl-1,4-pregnadiene-3,20-dione) is a powerful anti-inflammatory agent widely used in veterinary medicine for the treatment of various diseases including arthritis, rheumatic carditis and glomerulonephritis [3]. The anti-inflammatory action which makes corticosteroids one of the most useful groups of drugs at the same time causes them to be one of the most abused. At the racetrack, betamethasone and other corticosteroids enable unfit racehorses to remain in training without sufficient rest; if treated with these drugs for prolonged periods horses may suffer adverse reactions including joint damage, bone resorption, muscle wasting and immunoincompetency [3, 4]. To help prevent this flagrant disregard for animal well-being, the presence of betamethasone in horses must be determined and unknown metabolites unequivocally identified.

Several metabolic transformations in the corticosteroid skeleton have been reported to occur in the horse. Moss and Rylance [5] utilized thin-layer chromatography (TLC) retention data and characteristic colour reactions to tentatively report oxidation of the C-11 hydroxyl group as well as reduction of the C-1 double bond and the C-20 ketone. Dumasia *et al.* [6] employed gas chromatography together with TLC to provisionally report oxidation of the C-11 hydroxyl group of dexamethasone. Skrabalak and Maylin [7] used radiolabelling, mass spectrometry (MS), infrared spectroscopy (IR) and nuclear magnetic resonance spectroscopy (NMR) to confirm cleavage of the C-17 side-chain of

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dexamethasone with subsequent oxidation of C-17 and hydroxylation of both C-6 and C-16. The present work reports the identification of betamethasone and its major unconjugated metabolic product in the horse.

Experimental

Drug administration and sample collection

Mature Standardbred, Thoroughbred and Quarter Horse mares were housed in conventional box stalls and fed standard rations during this study. For isolation and identification of metabolites, 63 mg doses of betamethasone sodium phosphate (Chromalloy Pharmaceutical, Inc., Oakland, CA) were administered to horses either intravenously or intramuscularly. Urine samples were obtained by means of sterile Foley catheters (French size 30, 75-cm^3 balloon, C. R. Bard, Murray Hill, NJ) and were collected hourly for 48 h in disposable polypropylene bags. These samples were transferred to sterile 250-ml polypropylene beakers with polyethylene lids (Corning Glass Co., Corning, NY) and stored at 7°C until analysed.

Drugs and chemicals

Reference betamethasone was obtained from Sigma Chemical Company (St Louis, MO). Schering Corp. (Bloomfield, NJ) generously donated 6-β-hydroxybetamethasone (US Patent No. 4,201,778). Chemicals and solvents for extraction were of analytical reagent grade if not otherwise indicated. Acetone d_6 "100%" was obtained from Merck, Sharp, Dohme Canada Ltd (Montreal, Canada).

Isolation of betamethasone and its major urinary metabolite

Betamethasone and its major metabolite were extracted and isolated from horse urine by the method of Skrabalak and Maylin [7] but substituting diethyl ether–methylene chloride–isopropanol (2:1:1, v/v/v) for the solvent for extraction used in the original work.

Infrared spectroscopy

IR spectra were obtained with a Perkin–Elmer Model 457A Grating Infrared Spectrophotometer equipped with a 4× reflecting beam condenser. The spectrophotometer slit was set at the "N" (normal) position. Standards were scanned in the "fast" mode while urinary extracts were scanned in the "medium" scan mode. Potassium bromide (KBr) micro-pellets were formed in a Perkin–Elmer KBr Ultra Micro Die (Perkin–Elmer Corp., Norwalk, CT).

Sample preparation for micro-NMR

Following isolation, samples were placed in a water-bath at 70°C and carefully dried under a gentle stream of nitrogen. The residue was re-dissolved in 50 μl of glass-distilled ethyl acetate and transferred to a 4-in. capillary tube. Water was removed by freezing the sample in liquid nitrogen and immediately freeze-drying at −60°C and 40 millitorr in a Unitrap II freeze-drier (The Virtis Co., Gardiner, NY). The tube was capped immediately upon removal from the freeze-drier. The cap was removed only once for the injection of 25 μl of deuteroacetone into the capillary tube. The solvent was then frozen by dipping the tube in liquid nitrogen and while still frozen, the tube was sealed with the capillary tube sealer described by Skrabalak and Henion [8].

Nuclear magnetic resonance spectroscopy

NMR proton spectra were obtained with deuteroacetone on a Bruker (300 MHz) NMR spectrometer (USA Bruker Instruments, Billerica, MA). Acquisition parameters were: spectral width 3012.048 Hz; acquisition time 2.7197 s; pulse width 7 μs; relaxation delay 0.0 s; receiver gain 6400; temperature 300°C; and the number of scans varied between 275 and 668 according to sample concentration.

Results and Discussion

IR

Infrared spectroscopic data obtained from metabolic products and standards are shown in Table 1. As previously indicated, metabolic transformations of the corticosteroid skeleton reported in the horse include reduction of the C-20 ketone, cleavage of the C-17 side-chain and subsequent oxidation of C-17 to produce a five carbon ring ketone. With the metabolite as with the parent drug, absorptions corresponding to the C-20 carbonyl (1708 cm^{-1}) and the C-21 primary alcohol (1050 cm^{-1}) are present, indicating that the C-17 side-chain was neither cleaved nor altered. IR spectra of isolated parent drug and betamethasone are identical, as are the spectra of the isolated metabolite and authentic 6-β-hydroxybetamethasone. These data corroborate the proposed structures.

Table 1
Infrared spectral data* for betamethasone and related compounds

	Functional group				
Compounds	Saturated acyclic ketone	Unsaturated cyclic 6 member ring ketone	3°OH	2°OH	1°OH
Betamethasone	1708(s)	1658(s)	1180(m)	1138(m)	1044(m)
6β-Hydroxybetamethasone	1708(s)	1661(s)	1194(m)	1139(m)	1044(m)
"Parent drug"	1708(s)	1658(s)	1183(m)	1141(m)	1050(m)
"Metabolite"	1709(s)	1661(s)	1196(m)	1141(m)	1048(m)

* Absorbances are given in cm^{-1}. Abbreviations: s = strong; m = medium.

NMR

Nuclear magnetic resonance (NMR) spectral data of the isolated urinary products, reference betamethasone and authentic 6-β-hydroxy-betamethasone are shown in Table 2. The NMR spectrum of the parent drug matches that of authentic betamethasone and thus confirms its structure. When compared with the NMR spectrum of betamethasone, the NMR spectrum of the metabolite suggests three apparent changes indicative of its structural alteration. Downfield shift of the C-4 singlet by 0.07 ppm is consistent with a 6-β-hydroxyl addition [7]. This hydroxylation is further substantiated through downfield shifts of both the C-18 and C-19 angular methyl singlets by the expected amounts [9]. The structure of the metabolite is confirmed by its NMR spectral match to that of authentic 6-β-hydroxybetamethasone (Table 2). Structures of the two compounds are shown in Fig. 1.

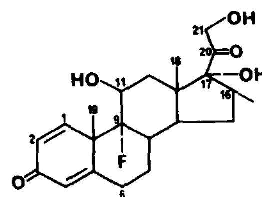
Conclusion

In conclusion, the administration of betamethasone to the horse generates at least two major unconjugated urinary components. These components have been isolated and

Table 2
Nuclear magnetic resonance spectral data* for betamethasone and related compounds

Compounds	Proton type C-1	C-2	C-4	C-16	C-18	C-19
Betamethasone	7.28, d J≃10.08 Hz	6.18, d of d J≃ 2.22 Hz J≃ 1.86 Hz J≃10.11 Hz	5.99, s	1.10, d J≃7.35 Hz	1.10, s	1.60, s
6β-Hydroxybetamethasone	7.28, d J≃10.17 Hz	6.17, d of d J≃ 1.92 Hz J≃ 1.83 Hz J≃10.23 Hz	6.06, s	1.11, d J≃6.42 Hz	1.12, s	1.79, s
"Parent drug"	7.28, d J≃10.26 Hz	6.18, d of d J≃ 1.86 Hz J≃ 1.86 Hz J≃10.29 Hz	5.99, s	1.10, d J≃7.35 Hz	1.09, s	1.60, s
"Metabolite"	7.29, d J≃10.09 Hz	6.17, d of d J≃ 1.86 Hz J≃ 1.47 Hz J≃10.08 Hz	6.06, s	1.11, d J≃6.93 Hz	1.12, s	1.78, s

*Chemical shifts are given in ppm downfield from tetramethylsilane. Coupling constants read directly from the spectra have an experimental error of 0.05 Hz. Abbreviations: s = singlet; d = doublet; d of d = doublet of doublets.



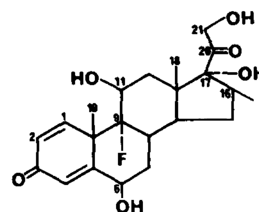


Figure 1
Structures of betamethasone and its metabolite (6-β-hydroxybetamethasone).

identified through spectroscopic analyses as betamethasone and its hydroxylated metabolite, 6-β-hydroxybetamethasone.

These results suggest that the major metabolism of betamethasone in the horse is rather simple, generating a metabolite very similar in structure to the parent drug. Nevertheless, this metabolite has been shown to produce quite different TLC, micro-high-performance liquid chromatographic (micro-LC) and micro-LC/MS data from those from the parent drug [1, 2]. Such discrepancies make screening for unknown metabolites difficult and thereby thwart determinations of drug usage. When the products of betamethasone metabolism are confirmed, their chromatographic properties can then be utilized in trackside drug screening. This screening coupled with mass spectral confirmation [1, 2] allows the forensic chemist to determine illicit betamethasone usage at the racetrack.

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