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Short Communication

Comparison of immunoaffinity chromatography combined with gas chromatography–negative ion chemical ionisation mass spectrometry and radioimmunoassay for screening dexamethasone in equine urine

S. M. R. Stanley*, B. S. Wilhelmi and J. P. Rodgers

Laboratory of the Jockey Club of South Africa, P.O. Box 74439, Turffontein, Johannesburg 2140 (South Africa)

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ABSTRACT

A comparison of the sensitive analytical methods of radioimmunoassay (RIA) and immunoaffinity chromatography (IAC) combined with gas chromatography–negative ion chemical ionisation mass spectrometry for the specific and reliable screening of dexamethasone in equine post-race urine is presented. Results from analyses of samples collected from a mare during 144 hours post administration of 26 mg of dexamethasone sodium phosphate are described.

INTRODUCTION

The time window for drug detection after administration may be short when the screening method is not very sensitive. Inconclusive or false negative results can even be obtained from samples taken where there was sufficient material present to exert a discernable pharmacological response in the horse. Low dosage (high potency) medications like synthetic corticosteroids (SCs) are particularly problematical in this respect. Where inadequate screening techniques are employed for SCs the time of administration can be tailored to gain an effect without the drug being detectable in the post-race urine. Thus sensitive and specific drug targeting procedures are required for these potent drugs.

A number of methods, including radioimmunoassay, have been published for the detection of SCs [1–13]. In general, when RIA is employed only urines that strongly depress binding [*i.e.* by >2 relative standard deviations (R.S.D.) from the mean of the "blank" urines] are considered potentially SCs containing [1]. This arbitrary criterion is used because urine contains other cross-reacting materials. Therefore, because of the inability of RIA to discriminate between "normal" urine and those with a low drug concentration, those that weakly depress binding are ignored. However many of the SCs exert a discernable pharmacological effect at these levels. This lack

* Corresponding author.

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of specificity and the need to use hazardous radio-labelled material are the major drawbacks of the method. We have recently reported [13] the use of IAC coupled to gas chromatography–negative ion chemical ionisation–mass spectrometry (GC–NICI-MS) as a method of confirming the abuse of the potent corticosteroid flumethasone in the equine. In the present study the technique was modified to include the chemical oxidation of the SCs to electrophilic compounds which are readily detectable by NICI-MS [13]. We have applied this technique and RIA to monitor the urinary excretion of 26 mg of dexamethasone sodium phosphate during 144 h after a single intramuscular administration to the horse. Our procedure is as sensitive as the radioimmunoassay, is more specific and therefore better suited to screen for dexamethasone abuse in post-race urine samples.

EXPERIMENTAL

Chemicals and reagents

Dexamethasone (9α-fluoro-11β,17α,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione), flumethasone (6α,9α-difluoro-11β,17α,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione), beclomethasone (9α-chloro-11β,17α,21-trihydroxy-16β-methylpregna-1,4-diene-3,20-dione), and pyridinium chlorochromate were purchased from Sigma (St. Louis, MO, USA). Celite® analytical filter aid was obtained from Manville Products (Denver, CO, USA). Anhydrous sodium acetate was supplied by Saarchem (Krugersdorp, South Africa). Acetone, toluene and dichloromethane (HPLC grade) high purity solvents were from Burdick and Jackson (Muskegon, MI, USA). Dexa 0.2 Phenix® injectable solution (dexamethasone sodium phosphate) was obtained from Phenix (Johannesburg, South Africa).

Instrumentation

A Hewlett-Packard (HP) 5890 Series II/5989A GC–MS with a HP 7673 automatic liquid sample injector was used as described below. The GC was fitted with either: (a) an HP-1 fused silica capillary column (12.5 m × 0.2 mm I.D.) from Hewlett-Packard (Palo Alto, CA, USA) with a crosslinked methyl silicone phase with a film thickness of 0.5 μm which was used for samples containing beclomethasone internal standard, or (b) a 30 m × 0.32 mm I.D. fused silica capillary column (007-OV17) from Quadrex (New Haven, CT, USA) for samples containing flumethasone internal standard. The helium carrier gas had a head pressure of 83 kPa. The splitless injection port was maintained at 250°C and the septum purge-flow was switched off until 0.75 min after injection. The oven temperature programme was 100°C (no hold) to 290°C (10 min hold) at 30°/min. The capillary was directly interfaced into the ion source via a transfer tube maintained at 250°C. The mass spectrometer was operated in the negative ion chemical ionisation mode with a source pressure of 0.266 kPa with methane (99.995% purity) as the chemical ionisation gas. The ion source and the quadrupole were operated at 150°C and 100°C, respectively. The selected electron energy was 200 eV. The electron multiplier was set 200 eV above the value selected during manual tuning. The scan range was 250 to 450 a.m.u. with a threshold of 20. Data were acquired and manipulated with a Hewlett-Packard HP-UX series Chemstation® and a HP9000 series 300 computer.

Immunoaffinity chromatography

Columns were prepared and used as described previously [13].

Radioimmunoassay

Radioimmunoassay was performed as described previously [13]. Dexamethasone was quantified by comparing the percentage bound with a calibration curve constructed with the data from known amounts of dexamethasone spiked into pre-administration urine and plotted in a logit–log graph.

Administration of dexamethasone sodium phosphate

The mare, which was kept in condition by daily exercise, was fed hay and grain three times a day and had water *ad libitum* during the trial. The

26 mg of dexamethasone sodium phosphate in sterile solution (Dexa 0.2 Phenix®) was injected intra-muscular and samples from the first 4 h were collected by means of an indwelling Foley catheter. Thereafter samples were collected if there was sufficient urine in the bladder by inserting an 18G polypropylene catheter. Urine was placed in either a 250-ml polypropylene bottle or a 20-ml glass bottle with a plastic lid and samples were frozen within 5 min after collection by immersion in an alcohol bath containing solid carbon dioxide and stored at < -15°C until analysis. Samples were thawed, extracted by IAC and the residue chemically oxidised before GC–NICI-MS analysis.

Chemical oxidation

Chemical oxidation (COX) was performed using a modification of the method of Kayganich *et al.* [14]. Samples with 100 ng of flumethasone or beclomethasone internal standard were reconstituted in 1 ml of dichloromethane and transferred to a screwcap vial and 10 mg of anhydrous sodium acetate were added. Approximately 1 mg of pyridinium chlorochromate finely ground with Celite (1:3) was added and the reaction mixture was capped and heated at 80°C for 120 min. Products were purified by passage through a silicagel-60 column (3.5 cm bed height in a Pasteur pipette), elution was with 5 ml of 10% acetone in dichloromethane. The eluent was evaporated to dryness under a gentle stream of oxygen-free nitrogen at a temperature below 40°C and then 50 μl of toluene were added. With the exception of the 60-ml 144-h extract where 5 μl were used, a volume of 2 μl of sample was injected per GC–MS run.

RESULTS AND DISCsUSSION

Post administration urine samples were analysed by radioimmunoassay and the concentrations of dexamethasone determined from a calibration curve are listed in Table I. The results of this study and another RIA study [1] show that only those urine samples obtained during the first day (approx. 21–27 h) after a single i.m. injection (50 μg/kg) would be RIA "positives" on the basis of a >2 R.S.D. binding depression. When analysed by our method 15-ml urine aliquots of the samples up to and including the 96-h collection were conclusively shown (see Fig. 1) to contain dexamethasone. Identification was done on the basis of the peak retention times and a reverse search [15] of the averaged spectra with the library spectrum which gave probability matches (PBM) of >90%. Samples, with the exception of the 144-h urine sample, were quantitated (see Table I) with respect to a beclomethasone internal standard added prior to oxidation. When 60 ml of the 144-h sample (RIA result <10 pg/ml) was subjected to analysis (see Fig. 1) we were able to identify dexamethasone. We calculated the concentration of the dexamethasone in this sample to be 132 pg/ml of urine (see Table I) relative to flumethasone. This internal standard proved to be unsuitable and was not used any further. The peak to peak signal-to-noise ratio of the largest COX dexamethasone peak in the reconstructed *m/z* 295 ion chromatogram from the 144-h sample was 8.7.

IAC/COX/GC–NICI-MS can unambiguously detect dexamethasone in urine samples at lower levels than RIA. In addition to its use as a screen-

TABLE I

RADIO IMMUNOASSAY RESULTS FROM POSTADMINISTRATION SAMPLES

Time of collection (h)	Dexamethasone (pg/ml)	
	RIA[a]	IAC[b]
0.5	3300	7850
1	7790	16 100
2	17 500	48 544
4	8200	27 400
21	4300	10 400
48	110	1200
96	30	300
144	<10	130

[a] Determined from a calibration curve plotted on logit–log graph paper.

[b] Determined from the ratio of sample *m/z* 295 to *m/z* 313 of the I.S. using GC–MS.

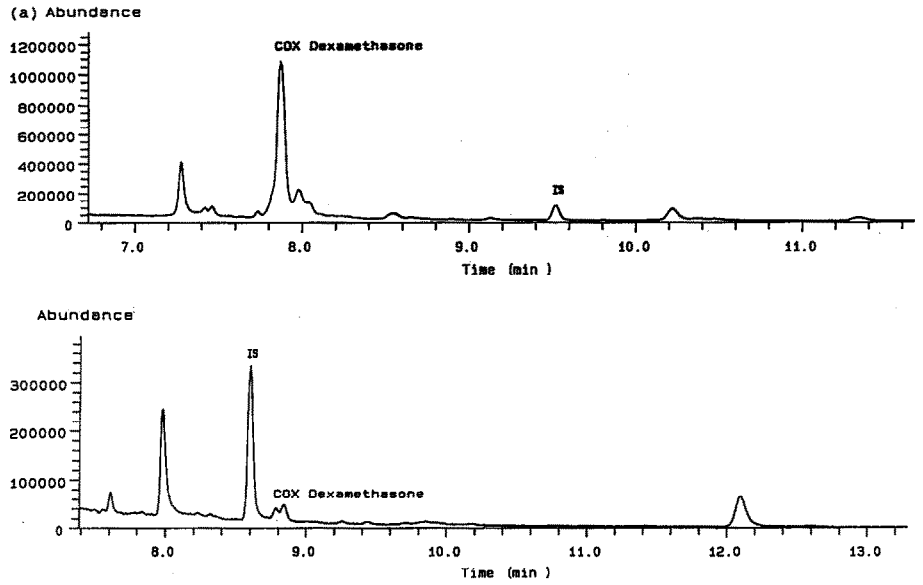

Fig. 1. (a) Total ion chromatogram of the 21-h post-administration (15 ml) sample separated on an HP-1 column, with beclomethasone added as the internal standard. (b) Total ion chromatogram of the 144-h post-administration (60 ml) sample separated on an OV-17 column, with flumethasone added as the internal standard.

ing procedure, it is also useful as an accurate and sensitive confirmation technique. For screening we have reduced the number of samples by combining four samples (4 × 15 ml) prior to analysis by this method. The ability of this procedure to confirm the presence of sub-nanogram concentrations in post race urines also eliminates the possibility that the time of administration before a race could be selected to gain an anti-inflammatory effect in a horse without the presence of the drug being detected and confirmed in the post-race sample.

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