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Immunoaffinity chromatography combined with gas chromatography–negative ion chemical ionisation mass spectrometry for the confirmation of flumethasone abuse in the equine

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

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

H. Bertschinger

Department of Genesiology, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort 0110 (South Africa)

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ABSTRACT

Immunoaffinity chromatography using a synthesised immunosorbent was used to extract tritiated dexamethasone (with dexamethasone carrier) from equine urine at a recovery of 81.7 ± 8.4% (mean ± S.D.). A method utilising this procedure coupled to cool on-column injection gas chromatography negative ion chemical ionisation mass spectrometry is also described for the confirmation of low levels of flumethasone in equine urine samples.

INTRODUCTION

Corticosteroids increase blood glucose levels, cause stimulation and have a strong anti-inflammatory effect in the equine. These actions improve a horse's chance of winning a race [1]. Hence, these drugs have been reputed [1] to be used as a "pre-race shot" in the performance horse. This is illegal in the Southern African racing jurisdiction, and this laboratory routinely screens for the presence of these substances in post-race urine samples.

The synthetic corticosteroids (SC) are structural analogues of the endogenous hormone hydrocortisone which produce a stronger anti-inflammatory effect and, consequently, smaller amounts of SC are required to achieve the desired effect. For example, 40–50 μg/kg of the SC dexamethasone is required for anti-inflammatory therapy [2] and flumethasone, which is 700 times more potent than hydrocortisone [1], is recommended at dosages of less than 2.5 mg per 500 kg horse per 24 h [3]. Therefore the concentration of an SC found in urine is usually very low. For example, Seawright *et al.* [4] found that administration of 20 mg of a long-acting dexamethasone ester preparation gave urine concentrations of less than 30 ng/ml. Hence, methods of detecting SC abuse in the equine must be effective in the nanogram and sub-nanogram per millilitre of urine concentration range. Several techniques

* Corresponding author.

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have been investigated and the results reported. These include radioimmunoassay (RIA) [5–7], competitive protein binding (CPB) [8,9], high-performance liquid chromatography (HPLC) with ultraviolet (UV) [10–13] and mass spectrometric (MS) detection methods [14–16] and gas chromatography (GC) with various detection methods [17–19] including MS [20–22]. The drawback of many of these methods is that they require a separate confirmatory analysis to eliminate any false positive results.

We have found that RIA suits our requirements as a screening method for dexamethasone because it is sensitive and a large number of urine samples can be assayed in a short time. The antibody used in this assay lacks absolute specificity and this is beneficial for screening samples because it will interact with other structurally related SC, but it also gives a large number of false positive results. Thus samples suspected of containing an SC, as shown by the RIA results, must be examined by a confirmatory technique, and we have applied liquid–liquid extraction [23,24] and solid-phase extraction [25–27] methodologies followed by GC–negative ion chemical ionisation (NICI) MS analysis of the derivatised residues. These gave good recoveries, nevertheless we have found it difficult to confirm the presence of sub-nanogram per millilitre of urine concentrations of an SC because of the masking effect of the large amount of coextracted materials. A more selective method of isolating an analyte from a biological matrix is to use a highly specific immunosorbent prepared by coupling the specific antibody to a solid support [28]. This approach has proved to be effective in providing very clean extracts of picogram to sub-picogram concentrations of drugs from urine [29–34].

SC are usually derivatised [35] prior to GC–MS in order to stabilise the thermolabile C_{17} side-chain [36] which is lost at the high temperatures of conventional split and splitless injection ports. However, the derivatisation step, which is often time-consuming and not always stoichiometric, can be eliminated if a cool on-column injection port is used [37].

This paper describes the development and immobilisation of an anti-dexamethasone bovine serum albumin (BSA) antibody for extracting nanogram and sub-nanogram per millitre concentrations of dexamethasone and flumethasone from equine urine by immunoaffinity chromatography (IAC). Dexamethasone and [^{3}H]dexamethasone spiked into equine urine were isolated by this method. Urine samples from a horse that had been administered flumethasone pivalate were also subjected to IAC, and the presence of the flumethasone (cleaved from its ester in the horse) was confirmed by cool on-column injection GC–NICI-MS.

EXPERIMENTAL

Chemicals and reagents

Dexamethasone, flumethasone, hydrocortisone, BSA, Freund's complete adjuvant, murile dipeptide adjuvant, phosphatidylcholine dipalmitoyl, cellulose dialysis tubing, dextran and DEAE-Cibacron blue 3GA gel were purchased from Sigma (St. Louis, MO, USA). [1,2,4,6,7-^{3}H]Dexamethasone (9.25 MBq, 2.6–4.1 TBq/mmol) was obtained from Amersham International (Aylesbury, UK). Sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate, isobutyl chloroformate, pyridine, sodium sulphate, activated charcoal and succinic anhydride were from E. Merck (Darmstadt, Germany). Sodium azide was obtained from BDH (Poole, UK). Sodium chloride, 1,4-dioxane and sodium hydroxide were supplied by Saarchem (Krugersdorp, South Africa). Ethyl acetate, dichloromethane, methanol and 2-propanol, which were a minimum of 99.8% pure, and chloroform, which was 99% pure, came from Riedel-de Haën (Hannover, Germany). Bio-Rad Labs. (Hemel Hempstead, UK) supplied the Affi-gel immunoaffinity kit. Insta-gel liquid scintillation cocktail was supplied by Packard (Groningen, Netherlands). Fluvet injectable solution (flumethasone pivalate) was obtained from Hoechst (Johannesburg, South Africa). Water was purified with a Milli-Q reagent water system from Millipore.

Instrumentation

A Packard Minaxi Tricarb Series 4000 liquid scintillation spectrometer was used to measure β-radiation. A single-label efficiency correlation curve was generated for disintegrations per min (dpm) conversion. The spectrometer was normalised and time termination for sample measurement was set at 20 min, 2% terminator unchanged, with radionuclide preset region 1 selected. A Hewlett-Packard 5890 Series II/5989A gas chromatograph–mass spectrometer with a 7673 automatic liquid sample injector was used as listed below. The GC oven was fitted with a 30 m × 0.32 mm I.D. fused-silica capillary column from Quadrex (New Haven, CT, USA). The cross-linked bonded phase (007-OV17) was 50% methyl silicone and 50% phenyl methyl silicone at a film thickness of 0.5 μm. The helium carrier gas (99.996% purity) was at a head pressure of 83 kPa. The cool on-column inlet port, which had a 0.53-mm insert, was connected to the capillary column via a glass butt connector (GlasSeal, Supelco, Bellefonte, PA, USA) and a 15 cm × 0.53 mm I.D. deactivated retention gap (Quadrex) was programmed from 75°C (no hold) to 300°C at 150°C/min. The oven programme was 85°C (no hold) to 300°C (10 min hold) at 50°C/min. The capillary was directly interfaced into the ion source via a transfer tube at 250°C. The mass spectrometer was operated at an ion 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 and 100°C, respectively. The selected electron energy was 200 eV. The electron multiplier was set 200 above the value selected during a manual tune. The scan range was 250–450 a.m.u. Data were acquired and manipulated with a Hewlett-Packard HP-UX Series Chemstation and a HP9000 Series 300 computer.

Antibody production

Immunogen synthesis. Dexamethasone was conjugated to BSA via a 21-hemisuccinate bridge. This was achieved by the two-step method described by Erlanger *et al.* [38] and later modified by Cook and Beastall [39]. The product was characterised by UV spectroscopy [38].

Immunisation of the sheep. An immunisation emulsion was prepared by adding 1000 μg of dexamethasone–BSA to 10 ml of normal saline solution. The mixture was sonicated with cooling until the solid had dissolved. Freund's complete adjuvant (10 ml) was added to this solution, and the mixture was vortex-mixed until a stable emulsion had formed. A second immunisation adjuvant was prepared by dissolving 1000 μg of the immunogen and 2 mg of murile dipeptide in 5 ml of water, 10 mg of phosphatidylcholine dipalmitoyl were added, and the mixture was sonicated to form micelles. This was followed by the addition of 16 ml of water. A sheep was immunised, and blood samples were collected from the jugular vein in plain plastic tubes. After clotting, the samples were centrifuged, and the small serum samples were transferred to cryo-tubes and the larger ones into 250-ml glass bottles for storage at < -15°C until required.

Titer determination

The antiserum to be tested was diluted with 0.275 *M* phosphate buffer pH 7.3 (PB) from 1:10 to 1:100 000. A 100-μl volume of the diluted antisera was vortex-mixed with 100 μl of equine urine (free of SC) diluted 1:10 with water and 500 μl of tritiated dexamethasone solution (giving approximately 25 000 dpm). This was incubated at 4°C for 120 min before 500 μl of dextran-coated charcoal solution were added. The samples were vortex-mixed, incubated for 90 min at 4°C, followed by centrifugation at a relative centrifugal force of 2000 *g* for 5 min. Other samples treated in a similar way included the total counts (no antibody added) and a blank (no antibody or radiolabel added). Supernatant (500 μl) was mixed with 500 μl of water and 4 ml of scintillation cocktail, vortex-mixed, and the dpm counted in a β-scintillation counter. The results were calculated as follows:

[(mean dpm of diluted antiserum − mean dpm of blank) × 100]/[(mean dpm of total counts − mean dpm of blank) × 1]

Radioimmunoassay procedure

A 100-μl volume of centrifuged (2000 *g* for 10 min) urine diluted 1:10 with water, 500 μl of tritiated dexamethasone (approximately 25 000 dpm) and 100 μl of antibody from day 261 diluted 1:40 000 in PB were vortex-mixed together in an assay tube. Samples were incubated at 4°C for 120 min, 200 μl of dextran-coated charcoal in PB were added per tube, and the mixture was vortex-mixed and incubated at 4°C for 90 min. Samples were centrifuged (2000 *g* for 10 min), and 500 μl of supernatant were mixed with 500 μl of water and 7 ml of scintillation cocktail in a scintillation vial; dpm were counted in the β-scintillation counter. The percentage bound is calculated as the dpm of the sample divided by the maximum binding dpm × 100, with allowance made for non-specific binding.

Specificity of sheep antiserum

The specificity of the antibody was determined on the 1:40 000 dilution of the day 261 serum in PB using [^{3}H]-dexamethasone as the radiolabel. The percentage cross-reactivity was calculated as follows:

(pg of dexamethasone that displaces 50% [^{3}H]dexamethasone × 100)/(pg of corticosteroid that displaces 50% [^{3}H]dexamethasone × 1)

Antibody purification

Aliquots of the freeze-dried serum (100 ml) obtained on day 162 were purified [40,41] by passing 500-mg samples in 10 ml of PB through a DEAE-Cibacron blue 3GA gel column (10 cm × 2 cm I.D.). The immunoglobulin G (IgG)- and transferrin-containing eluents were collected, pooled and freeze-dried.

Antibody immobilisation

A 49-mg mass of the purified antibody was treated as described in the manual supplied with the Affi-gel kit. At the final step 29 mg of periodate-modified material were mixed with 5 ml of the hydrazide gel. The coupling efficiency was estimated as the difference between the total protein in solution before and after coupling to the gel as measured by UV absorbance at 280 nm.

Immunoaffinity extraction

Aliquots (1 ml) of the immunoaffinity gel were poured into glass columns (10 cm × 1 cm I.D.) fitted with Nylon membranes to retain the beds. These were stored at 4°C with PB containing 0.1% sodium azide until required for use. Immunoaffinity columns were conditioned with 5 ml of 2-propanol–water (9:1, v/v) and 5 ml of PB prior to sample application. A 15-ml volume of supernatant from a centrifuged (2000 *g* for 15 min) urine sample was diluted 1:1 with distilled water and passed down a column at a flow-rate of approximately 1 ml/min. Unbound material was eluted with sequential 5-ml washes of 0.5 *M* NaCl and purified water. The analytes were eluted with 10 ml of 60% 2-propanol in water. Eluent was removed under a stream of oxygen-free nitrogen at temperatures <40°C until approximately half the volume remained. SC were then extracted with 2 × 5 ml of diethyl ether. These extracts were dried at temperatures less than 40°C under oxygen-free nitrogen. Samples for GC–MS were dissolved in 25 μl of ethyl acetate and transferred to an autosampler microvial. A 1-μl sample was injected per GC–MS run.

Determination of the percentage recovery of corticosteroids from equine urine

Horse urine, free from SC, was centrifuged (2000 *g* for 15 min) and 15-ml aliquots were spiked with dexamethasone at 0, 1.0, 5.0, 10.0, 25.0, 50.0 and 100.0 ng/ml of urine. Dexamethasone radiolabel was added in direct proportion to the amount of dexamethasone present (see Table I). The samples were extracted as described previously and the residue was made up in 1 ml of water plus 7 ml of scintillation cocktail. Radioactivity was measured and the percentage recovery calculated from a calibration curve constructed with values obtained using known amounts of the [^{3}H]dexamethasone solution.

Influence of hydrocortisone on recovery

Samples, spiked as described above, had hydrocortisone added at a ratio of 10:1 to the amount of dexamethasone in the urine. These were extracted by IAC, and the percentage recovery was determined as described previously.

Fluvet equine administration trial

A 10-ml volume of a 0.25 mg/ml flumethasone pivalate solution (Fluvet) was administered intravenously to a thoroughbred mare of approximately 500 kg mass. Urine was collected by means of an indwelling Foley catheter at hourly intervals starting at the time of administration. Samples placed in either 250-ml polypropylene bottles or 20-ml glass bottles with plastic lids were frozen within 5 min of collection by immersion in a solid CO_2 and alcohol bath followed by storage at < -15°C until required. Samples were thawed, extracted by IAC, and the residues analysed by GC–NICI-MS.

RESULTS AND DISCUSSION

The 1:10 000 dilution of the antiserum collected on day 162 after the start of the immunisation programme gave a 50% binding with [^{3}H]dexamethasone. The percentage cross-reactivity of the 1:40 000 dilution of the antiserum from day 261 with dexamethasone and several other corticosteroids is listed in Table I. These results show that although the antibody is most reactive with dexamethasone and desoxymethasone, it also interacts with other structurally similar SC, including flumethasone. The cross-reactivity with the endogenous corticosteroid hydrocortisone is very low.

TABLE I

CROSS-REACTIVITY BETWEEN THE ANTI-DEXAMETHASONE–BSA ANTIBODY AND SOME CORTICOSTEROIDS

Corticosteroid	Cross-reactivity (%)
Betamethasone	28.20
Betamethasone disodium phosphate	8.30
Carbenoxolone sodium	<0.01
Desoxymethasone	100.00
Dexamethasone	100.00
Flumethasone	80.10
Hydrocortisone	0.16
Methylprednisolone	0.04
Prednisone	0.83
Prednisolone	1.03
Triamcinolone	2.87
Triamcinolone acetonide	0.57

A 49-mg amount of the purified material was prepared for coupling as described in the instruction manual supplied with the Affi-gel kit until, at the final step, 29 mg remained which were coupled to 5 ml of hydrazide gel at a coupling efficiency of 47%. The immunosorbent was divided into five (1-ml gel) columns. Chromatographic conditions for isolating corticosteroids were developed by extracting tritiated dexamethasone spiked into equine urine. The eluents including progressively stronger 2-propanol in water solutions were collected, and the radioactivity was measured. It was found that the 60% 2-propanol elution was the most appropriate to elute the [^{3}H]dexamethasone quantitatively. The eluted material was extracted with diethyl ether to eliminate the large volume of water and traces of salts that were present in the corticosteroid-containing effluent. IAC of various amounts (see Table II) of [^{3}H]dexamethasone, plus a proportionately larger amount of unlabelled carrier, spiked into equine urine gave a mean recovery of 81.7% for the radiolabel with a standard deviation of ± 8.4% (n = 60) and a coefficient of variation of 10.3%. It was assumed that the inter-column variation was small because the same batch of gel was used to prepare all the columns. The mean percentage recovery of the largest [^{3}H]dexamethasone and dexamethasone spiking (1.0 ml + 100.0 ng/ml) was lower than that of the other samples on both the inter- and intra-day assays (around 70%), and this may be due to the maximum column loading being exceeded. We also found that IAC extractions of solutions containing [^{3}H]dexamethasone plus dexamethasone at amounts greater than 1.0 ml [^{3}H]dexamethasone and 100 ng/ml dexamethasone all gave approximately the same dpm readings (see Table III). Hence it could be assumed that the amount of tritiated dexamethasone and dexamethasone in these samples may have saturated the immunosorbent. If the figures of the samples suspected of exceeding the column capacity are excluded the mean recovery is >85%. The individual columns were used 25 or more times each without a no-

TABLE II

VARIABILITY IN THE PERCENTAGE RECOVERY OF [^{3}H]DEXAMETHASONE EXTRACTED FROM URINE BY IAC

Volume of [^{3}H]dexamethasone solution added per sample[a] (ml)	Precision		
	Mean ± S.D.	n	C.V. (%)
Intra-day assay			
0.01 (1.0 ng/ml)	78.9 ± 10.5	6	13.4
0.05 (5.0 ng/ml)	81.3 ± 1.8	6	2.2
0.10 (10.0 ng/ml)	82.1 ± 4.7	6	5.7
0.25 (25.0 ng/ml)	79.7 ± 3.5	6	4.4
0.50 (50.0 ng/ml)	83.5 ± 2.5	6	3.0
1.00 (100.0 ng/ml)	74.7 ± 5.5	6	7.4
Inter-day assay			
0.01 (1.0 ng/ml)	85.7 ± 9.9	4	11.6
0.10 (10.0 ng/ml)	85.7 ± 5.6	5	6.5
0.25 (25.0 ng/ml)	83.4 ± 4.6	5	5.5
0.50 (50.0 ng/ml)	87.6 ± 6.2	5	7.1
1.00 (100.0 ng/ml)	69.9 ± 5.7	5	8.2
Cumulative results	81.7 ± 8.4	60	10.3

[a] 1 ml of [^{3}H]dexamethasone solution gave 105 396 dpm (activity 2.6–4.1 TBq/mmol). Figures in brackets represent the concentration of dexamethasone spiked into the urine sample.

ticcable decrease in performance and this is in agreement with the results produced elsewhere [32].

TABLE III

MAXIMUM LOADING OF [^{3}H]DEXAMETHASONE AND DEXAMETHASONE PER MILLILITRE OF GEL

Volume of [^{3}H]dexamethasone solution added to sample[a] (ml)	dpm
0.60 (60.0 ng/ml)	67 000
0.70 (70.0 ng/ml)	93 000
0.80 (80.0 ng/ml)	103 000
0.90 (90.0 ng/ml)	111 000
1.00 (100.0 ng/ml)	128 000
1.20 (120.0 ng/ml)	132 000
1.40 (140.0 ng/ml)	127 000
1.60 (160.0 ng/ml)	128 000

[a] 1 ml of [^{3}H]dexamethasone solution gave 168 933 dpm (activity 2.6–4.1 TBq/mmol). Figures in brackets indicate the amount of dexamethasone added per sample.

TABLE IV

INFLUENCE OF HYDROCORTISONE ON THE RECOVERY OF [^{3}H]DEXAMETHASONE FROM URINE

Volume of [^{3}H]dexamethasone solution added to sample[a] (ml)	Hydrocortisone (ng/ml)	Recovery (%)
0.10 (10.0 ng/ml)	–	85
0.10 (10.0 ng/ml)	100	80
0.25 (25.0 ng/ml)	–	84
0.25 (25.0 ng/ml)	250	81
0.50 (50.0 ng/ml)	–	78
0.50 (50.0 ng/ml)	500	84
1.00 (100.0 ng/ml)	–	73
1.00 (100.0 ng/ml)	1000	68

[a] 1 ml of [^{3}H]dexamethasone solution gave 100 010 dpm (activity 2.6–4.1 TBq/mmol). Figures in brackets represent the concentration of dexamethasone spiked into the urine sample.

Hydrocortisone present in urine was shown not to have a significant effect upon the percentage recovery of [^{3}H]dexamethasone (see Table IV). Thus, even in the unlikely event of this endogenous material being present in a urine sample at the same time as a SC (which suppress glucocorticoid secretion), there will be no significant interference with the recovery.

The results of RIA on urine samples from a horse administered 2.5 mg of flumethasone pivalate ester (intravenously) are shown in Table V. These urines were extracted by IAC and the residues analysed by cool on-column GC–NICI-MS in the full-scan mode. Flumethasone could be positively identified (probability matches of >95% against the standard using the McLafferty search algorithm [42]) in all the post-administration samples including the last collection at 6 h. This had the highest percentage bound of all the RIA results, with the obvious exception of the pre-administration trial urine, and therefore was the weakest flumethasone-containing urine sample equating to approximately 740 pg/ml. A peak corresponding to almost 0.5 ng of flumethasone on-column (as compared to an external calibration curve) which translates to a recovery of 105% can be clearly seen at 13.273 min in the chromatogram of this sample (Fig. 1a) and the spectrum average across the peak, shown in Fig.

TABLE V

RADIOIMMUNOASSAY RESULTS OF URINE SAMPLES COLLECTED FROM A HORSE DOSED WITH FLUMETHASONE PIVALATE

Time of collection	Average % bound
Pre-administration	100
Post-administration	
1 h	0
2 h	7
3 h	10
4 h	17
5 h	17
6 h	24

2a, matches that obtained from an authentic flumethasone standard (Fig. 2b). The other peaks are, as yet, unidentified. Fig. 1a and b also show that the extracts exhibit GC–MS chromatograms with low backgrounds because of the limited amount of extraneous material that is coextracted by IAC. This demonstrates that flumethasone abuse in the equine can be confirmed by IAC followed by cool on-column GC–MS even when the concentration of the analyte in urine is at subnanogram per millilitre levels. This is in direct contrast to the results obtained from the analysis of the dichloromethane extract of 15 ml of the 6-h sample where the high background masked

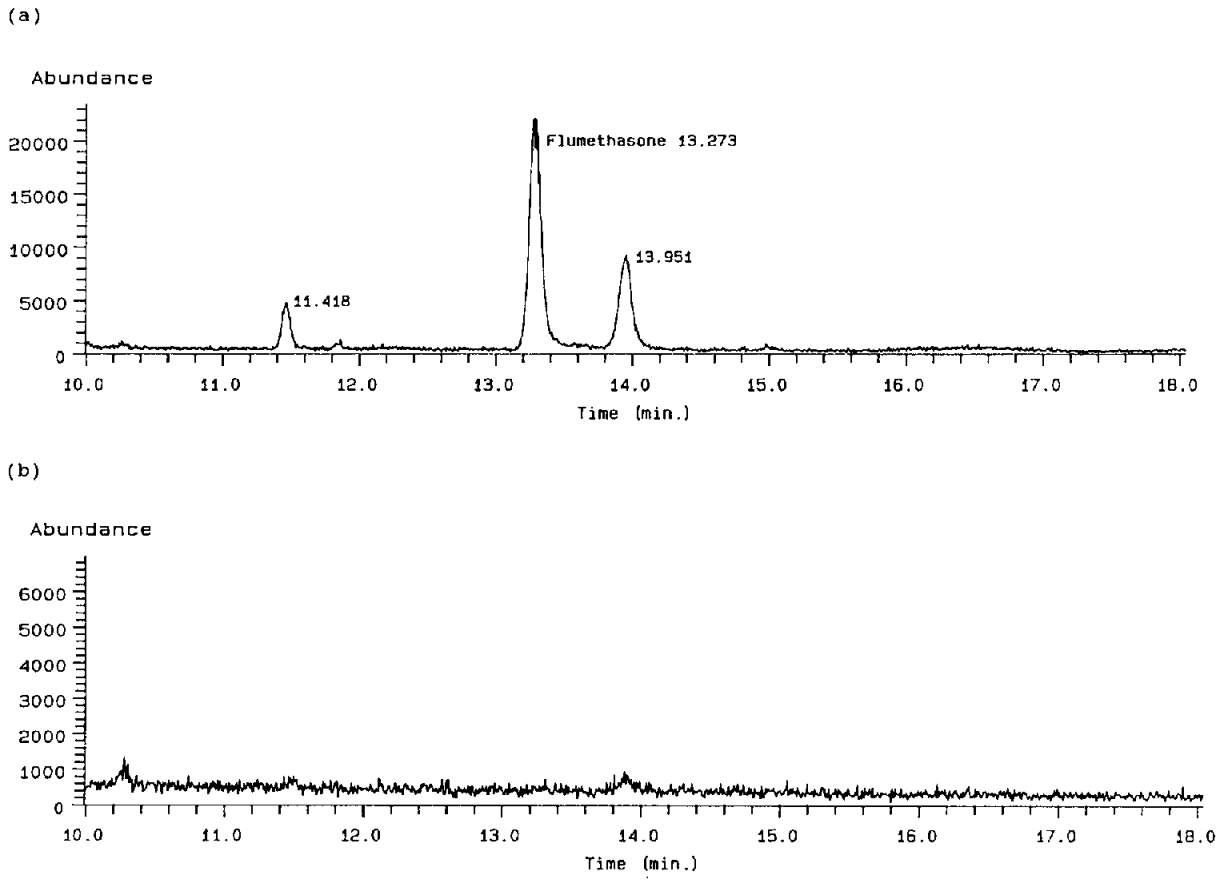

Fig. 1. Total ion chromatograms (GC–NICI-MS) of samples extracted by IAC. (a) Urine collected 6 h post-administration of flumethasone pivalate. (b) Pre-administration urine.

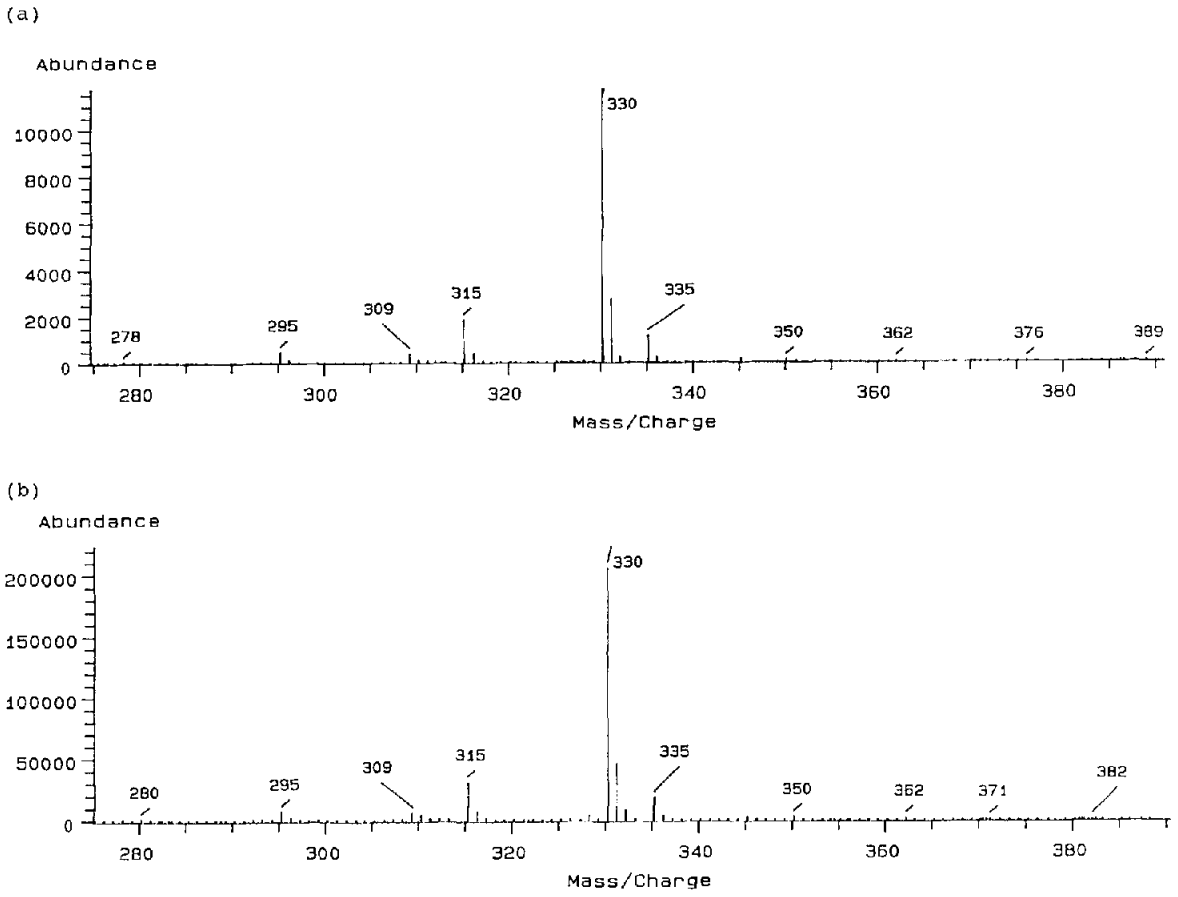

Fig. 2. Mass spectra (a) obtained from the peak at 13.237 min in the TIC of the 6-h sample and (b) of an authentic flumethasone standard.

the flumethasone peak in the full-scan mode and the spectra were of very poor quality. Flumethasone in this extract could only be tentatively identified by selective-ion monitoring.

We were unable to establish the lowest concentration at which flumethasone could be positively identified in a sample using the IAC method. This is because, in theory, the selectivity of the isolation procedure allows the complete sample to be extracted without producing samples with large amounts of unwanted material which could mask the analyte peak. Hence the total amount of SC (*i.e.* concentration × sample volume) is the important factor and, provided that this is greater than the lower limit of detection of the mass spectrometer, flumethasone can be positively identified. However, we suggest that given the sensitivity of our mass spectrometer and if the sample was limited to 30 ml, for example, we would require a flumethasone concentration of at least 50 pg/ml of urine to obtain a better than 10:1 peak-to-peak signal-to-noise ratio in the full-scan mode. This cut-off point could possibly be lower if the residue was reconstituted in a volume of ethyl acetate less than 25 μl.

Affinity chromatography using an anti-dexamethasone–BSA antibody bound to a support is a simple and effective way of isolating dexamethasone and flumethasone and, on the basis of the cross-reactivity studies, probably several other SC. IAC has the advantage over the conventional liquid–liquid and solid-phase extractions because

it produces cleaner samples that provide chromatograms with a low background making it easier to identify even picogram amounts of the compound of interest. The procedure could quite easily be automated. Moreover, our results indicate that the immunosorbent can be regenerated without loss of performance and we have reused a column more than 25 times and still found it to be reliable. This factor makes the time-consuming development of an IAC procedure a practical proposition when looking for clean extraction methodology to isolate weak analytes on a routine basis.

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