DETECTION AND QUANTITATION OF 3-HYDROXYMEPIVACAINE IN HORSE URINE: A PRELIMINARY REPORT

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ABSTRACT

The local anaesthetic mepivacaine is widely used in equine medicine but generally gives rise to penalties if detected in post race urine samples. A major metabolite 3-hydroxymepivacaine used by equine drug testing laboratories to confirm the presence of mepivacaine, has been synthesised. A gas chromatography/mass spectrometric (GC/MS) method was developed to detect and quantitate 3-hydroxymepivacaine recovered from urine samples collected from horses dosed with mepivacaine at the pharmacological threshold level.

Urine samples from a horse dosed with mepivacaine at the highest no-effect dose (HNED) were analysed by enzyme-linked immunosorbent assay (ELISA) and GC/MS. For GC/MS these samples were enzyme (β-glucuronidase) hydrolysed and extracted by a solid phase method. The extracts were reacted with N-methyl-N-(*tert*-butyldimethylsilyl) trifluoroacetamide derivatising agent and analysed by electron impact GC/MS with selected ion monitoring (SIM). Tetracaine was used as the internal standard, and standard curves for 3-hydroxymepivacaine were generated.

Peak urinary concentration of mepivacaine equivalents, as determined by ELISA, was about 46 ng/ml 2 h post subcutaneous injection of the HNED. By GC/MS, the concentration of 3-hydroxymepivacaine recovered from urine samples peaked at about 50 ng/ml at 4 h post dose. Therefore, this GC/MS-SIM method was able to detect and quantify 3-hydroxymepivacaine in urine samples from horses dosed at the HNED for mepivacaine.

INTRODUCTION

Mepivacaine (carbocaine) is an amide-type local anaesthetic used frequently in equine medicine, chiefly in minor surgery or as a diagnostic tool for the location of limb soreness. Although similar to lidocaine in structure and pharmacology, mepivacaine has a faster onset and a more prolonged duration (Gilman *et al.* 1990) and, thus, may be preferred in veterinary medicine. Mepivacaine, like other local anaesthetics, may be used as a nerve block when injected directly into a leg joint of a lame horse. This enables injured horses to perform normally when racing, but increases the risk of damage to the joint in question (Tobin 1981). Treatment of this nature, just prior to a race, may lead to permanent or catastrophic musculoskeletal injury with possible death to horse and jockey. For this reason the detection of mepivacaine or a mepivacaine metabolite in post race urine samples can lead to significant penalties being imposed by racing authorities.

The high sensitivity of modern methodology has lead to the detection of traces of therapeutic medications in equine post race urine samples. Many of these substances, when administered close to the time of racing, can affect the performance of the horse, yet residues of these compounds are often detected from medicinal administrations whose clinical effects have ended long ago (Tobin 1995). Highest no-effect doses (HNEDs), or thresholds, are being established for many therapeutic medications. Recently, using an abaxial sesamoid block model, our laboratory found the HNED of mepivacaine to be 2 mg, when given subcutaneously (Harkins *et al.* 1998a). Analytical procedures were then developed to ascertain the urine concentrations of a mepivacaine metabolite after administration of the established HNED. 3-hydroxymepivacaine is a mepivacaine metabolite used frequently by equine drug testing laboratories for mass spectral confirmation of mepivacaine administration. Recently, this compound has been synthesised and characterised (Harkins *et al.*

1998a). This report outlines the ELISA and GC/MS quantification of 3-hydroxymepivacaine using the synthesised compound as reference standard.

Materials and methods

Authentic mepivacaine reference standard was purchased from US Pharmacopeia (Maryland, USA). Local anaesthetic reference standards: bupivacaine, lidocaine, prilocaine, cocaine, dibucaine, procaine and tetracaine were all purchased from Sigma Chemicals (Missouri, USA). Etidocaine and ropivacaine were given by Astra Incorporation (Masachusetts, USA). 3-hydroxymepivacaine, 3-hydroxybupivacaine, 4-hydroxybupivacaine and 3-hydroxylidocaine were synthesised in the authors' laboratory (Harkins *et al.* 1998a). The mepivacaine ELISA kits were provided by Neogen Corporation, Kentucky, USA.

Thoroughbred mares (450–520 kg) used for this study were kept in a 20 acre field where they were provided grass hay and feed (12% protein). The animals were maintained on pasture until approximately 24 h before dosing, when they were placed in box stalls and allowed free access to hay and water. A routine clinical examination of the mares was carried out to ensure they were healthy and sound. Throughout these experiments, animals were managed according to the rules and regulations of the University of Kentucky Institutional Animal Care Use Committee, which also approved the experimental protocol.

Horses were administered the HNED of mepivacaine (2 mg, Harkins *et al.* 1998a), and higher doses in the form of mepivacaine hydrochloride (Steris Labs, Arizona, USA) by subcutaneous injection in the fetlock area. Using a catheter, urine samples were collected after mepivacaine administration and stored frozen (-20°C) until assayed.

Measurement of mepivacaine by ELISA was performed as described by Woods *et al.* (1996). For the cross-reaction study, solutions of the local anaesthetics and their metabolites were dissolved in methanol (1 mg/ml) and diluted in Neogen assay buffer to concentrations appropriate for the standard curves. An aliquot (20 µl) of the standards was added to each well of a 96-well microtitre plate, together with 180 µl mepivacaine-horseradish peroxidase conjugate (mepivacaine-HRP), diluted in assay buffer. Wells were incubated at room temperature (RT) for 1 h then washed with Neogen wash buffer before Ky-Blue Substrate (Neogen, 150 µl) was added to each. After 30 min the optical density of the reaction mixture was measured using an automated microplate reader fitted with a 650 nm filter (Bio-Tek Inc., Vermont, USA).

Urine samples were assayed by ELISA for apparent mepivacaine concentration. The quantitative assay method was the same as that used for the cross-reactivity measurements, except that 20 µl of blank urine were added to each well containing standard to create a matrix comparable to the dosed-horse urine samples. In order to keep the total reaction mixture to 200 µl per matrix well the volume of mepivacaine-HRP solution added was reduced to 160 µl. All quantitative ELISA data were compared with a mepivacaine standard curve and reported as 'mepivacaine equivalents'.

Urine samples from a mepivacaine-dosed horse were assayed quantitatively for 3-hydroxymepivacaine by GC/MS as described previously (Harkins *et al.* 1998b). Standard solutions of 3-hydroxymepivacaine and tetracaine were prepared in methanol. Extraction standards were prepared by the addition of a known amount of 3-hydroxymepivacaine solution to blank urine samples at a range of 4–160 ng/ml. A known amount of tetracaine standard (18 µl of 100 µg/ml solution) was added to each sample, standard and blank, as an internal standard. Solutions of 3-hydroxymepivacaine and tetracaine were added after sample hydrolysis.

Urine samples (5 ml) were placed in culture tubes to each of which were added 1 ml ß-glucuronidase solution (Type L-II, 5,000 units/ml, Sigma Chemical Co) and 2 ml 1 M sodium acetate buffer, pH 5.0 were added. The samples were mixed briefly by vortex and incubated in a water bath at 65°C for 3 h. After cooling overnight to 4°C, 3-hydroxymepivacaine and tetracaine were added and the samples sonicated for 90 s before 2 ml 0.1 M sodium phosphate buffer, pH6 were added. Sample pH was adjusted to 6.0 ± 0.5 with 1 M sodium hydroxide or 1 M hydrochloric acid.

Clean Screen solid phase extraction columns (United Chemical Technologies, Pennsylvania, USA) were conditioned by adding sequentially 3 ml methanol, 3 ml water and 1 ml 0.1 M sodium phosphate buffer, pH 6.0. Samples were then loaded and the column washed sequentially with 2 ml water, 2 ml 1 M acetic acid and 4 ml methanol. The column was eluted with 3 ml dichoromethane/isopropanol/ammonium hydroxide (78/20/2) and the eluent evaporated to dryness under a stream of nitrogen (<40°C). For derivatisation, each sample was dissolved in 40 µl N-methyl-N-(*t*-butyldimethylsilyl) trifluoroacetamide (MTBSTFA), 1% *t*-butyldimethyl-chorosilane (Regis Technologies, Illinois, USA) and 1 µl triethylamine, mixed by vortex, sonicated by horn dismembrator and incubated at 40°C for 2 h, followed finally by overnight incubation at room temperature.

GC/MS procedures and data analysis were similar to those reported previously (Woods *et al.* 1996). The

TABLE 1: ELISA cross-reactivity of anti-mepivacaine antibody

Compound	I-50 concentration (ng/ml)	Cross-reactivity (%)
Mepivacaine	16	100
Bupivacaine	17	94
Ropivacaine	20	80
Lidocaine	120	13
3-hydroxybupivacaine	145	11
Etidocaine	310	5
Prilocaine	310	5
3-hydroxymepivacaine	330	5
4-hydroxybupivacaine	450	4
3-hydroxylidocaine	1,550	1
Cocaine	> 10,000	<0.1
Dibucaine	> 10,000	<0.1
Procaine	> 10,000	<0.1
Tetracaine	> 10,000	<0.1

% cross-reactivity relative to mepivacaine 50% maximum activity (I-50)

gas chromatograph (Hewlett-Packard Model 6890) employed was equipped with a mass selective detector (MSD Model 5972A) and the column was an HP-5MS, 30 m x 250 µm x 0.25 µm (Hewlett-Packard, Georgia, USA). The carrier gas used was helium, at a flow rate of 1 ml/min. Samples (1 µl) were injected in the splitless mode at an injector temperature of 250°C. Initial oven temperature was 70°C, held for 2 min, increasing at 20°C/min up to 280°C, at which it was held for 12 min. Total running time was 24.5 min. Detector temperature was 280°C. The MSD was operated in the selected ion monitoring (SIM) mode. Ions 98 m/z for the 3-hydroxymepivacaine *t*-butyldimethylsilyl derivatives (mono- and bis-*t*-butyldimethylsilyl forms) and 58 m/z for tetracaine were employed for quantification.

Results

Methanol solutions (1 ng/ml) of mepivacaine and several local anaesthetic analogues, together with the available synthesised equine metabolites, were diluted in ELISA assay buffer and analysed using the mepivacaine ELISA system (Table 1). Comparative ELISA reactivity with the anti-mepivacaine antibody was expressed as I-50, that is the concentration of compound required to reduce the maximum optical density at 650 nm by 50%. Mepivacaine (I-50 = 16 ng/ml), bupivacaine (17 ng/ml) and ropivacaine (20 ng/ml) were considered roughly equivalent. The major equine mepivacaine metabolite, 3-hydroxymepivacaine, was much less reactive (I-50 = 330 ng/ml) with only about 5% of the reactivity of mepivacaine.

A mepivacaine standard curve with added blank horse urine (urine matrix standard curve), was used for the quantification of mepivacaine equivalents in urine from dosed horses. The average I-50 for mepivacaine with the urine matrix standard curve was about 15 ng/ml.

TABLE 2: ELISA mepivacaine equivalents in dosed horse urine

Mepivacaine SC dose (mg)	Peak concentration (ng/ml)	% B_0	Time post administration (h)
2	46	66	2
6	65	66	1
18	172	51	4
54	414	36	6
162	1,162	22	4
486	2,259	15	4

% B_0 - percent maximum ELISA response

The mepivacaine ELISA system was employed to estimate the concentration of mepivacaine equivalents in urine samples collected from horses dosed subcutaneously with increasing levels of mepivacaine hydrochloride (Table 2). Peak concentrations of mepivacaine equivalents ranged from 46 ng/ml, measured after the lowest dose (2 mg) of mepivacaine hydrochloride, to 2,259 ng/ml, after the highest dose, at 486 mg. Results were also expressed as a percentage of the maximum optical density at 650 nm (%B_0). A %B_0 value considerably lower than 50% may indicate the presence of mepivacaine, or similar compound in a urine sample. This was true for the mepivacaine doses of 54 mg and higher.

Urine samples collected from a horse dosed subcutaneously with 2 mg mepivacaine HCl were analysed by ELISA using a urine matrix mepivacaine standard curve (Table 3). The results are expressed as ng mepivacaine equivalents/ml urine. ELISA analysis of the pre-dose urine sample (0 h) resulted in an apparent mepivacaine equivalents concentration of 20 ng/ml. The apparent mepivacaine equivalents concentration reached a maximum value of approximately 46 ng/ml 2 h post administration and dropped to a value of about 6 ng/ml 48 h post administration.

The same urine samples, subjected to ELISA analysis, were analysed quantitatively by GC/MS for 3-hydroxymepivacaine concentration (Table 3). The results are expressed as ng 3-hydroxymepivacaine/ml urine. The pre-dose sample was negative for 3-hydroxymepivacaine although, by 4 h post mepivacaine HCl (2 mg) administration, the concentration of 3-hydroxymepivacaine increased to a maximum of about 50 ng/ml and returned to 0 ng/ml by 48 h post administration.

TABLE 3: Mepivacaine concentrations measured in urine samples from one horse post subcutaneous mepivacaine HCl administration (2 mg) using GC/MS and ELISA methods

Time post administration (h)	Concentration (ng/ml)	
	GC/MS*	ELISA†
0	0.0	20.1
0.5	0.0	35.7
1	0.8	31.8
2	30.6	45.8
4	49.7	30.8
6	38.8	31.6
8	40.9	32.6
24	1.7	44.3
48	0.0	6.0

* ng 3-hydroxymepivacaine/ml urine
† ng mepivacaine equivalents/ml urine

Discussion

Under the GC/MS conditions employed in this report, the MTBSTFA derivatisation of 3-hydroxymepivacaine resulted in the formation of 2 compounds, mono-*t*-butyldimethylsilyl-3-hydroxymepivacaine (MW = 376) and bis-*t*-butyldimethylsilyl-3-hydroxymepivacaine (MW = 490), which have very close retention times, with a separation of about 0.08 min. The relative areas of the MSD peaks of these substances appear to vary with individual urine samples, the 3-hydroxymepivacaine concentration and the degree of GC column and injector contamination. In general, however, mono-*t*-butyldimethylsilyl-3-hydroxymepivacaine was the dominant form while the bis-derivative appeared as a much smaller, but variable, shoulder peak.

Both mono- and bis- species of hydroxymepivacaine produced the 98 m/z base peak fragment under electron bombardment in the MSD. The areas for both derivatives on a 98 m/z ion chromatograph were integrated to represent the total concentration of 3-hydroxymepivacaine. Using the standard curves, with tetracaine (underivatised) as the internal standard, linear responses with regression coefficients of 0.9990 or greater were generated consistently by this method, verifying its applicability and dependability.

Several observations justify the integration of the areas under the MSD peaks for mono-*t*-butyldimethylsilyl-3-hydroxymepivacaine and bis-*t*-butyldimethylsilyl-3-hydroxymepivacaine of ion 98 m/z.

First, the areas of the 2 derivatives appeared to be proportional to one another; in the standard curves for a given concentration of 3-hydroxymepivacaine, as one species of derivative increased the other decreased.

Secondly, in both species, the ion 98 m/z represented the same molecular fragment, that is, the N-methyl piperidinyl ion derived from mepivacaine and 3-hydroxymepivacaine. The chemical similarities of the derivatives allowed almost identical patterns of ionisation and associated spectra.

Lastly, the consistent linearity of the standard curves derived from several assays and the considerable inter- and intra-assay variability of the mono-*t*-butyldimethylsilyl-3-hydroxymepivacaine to bis-*t*-butyldimethylsilyl-3-hydroxymepivacaine ratio, provides substantial empirical support for the validity of the data treatment.

There is no direct comparison between the results of mepivacaine quantification using GC/MS and ELISA analyses (Table 3), due to the nature of their detection methods. Analysis by ELISA is employed generally as a screening method for drug detection and its sensitivity depends on the reaction of anti-mepivacaine antibody with substances in the unextracted horse urine. The cross-reactivity of the ELISA antibody (Table 1) suggests a much greater sensitivity for the parent compound mepivacaine, than for its metabolite. Because the concentrations are measured in mepivacaine (parent compound) equivalents, a very high concentration of the metabolite would be assumed incorrectly if that were the only mepivacaine ELISA reactive substance present. However, studies of mepivacaine metabolism in man and other animals indicate a possible mixture of metabolites and the parent compound (Reynolds 1971). Furthermore, 3-hydroxymepivacaine occurs in urine as the glucuronide conjugate and thus there may be little, if any, unconjugated 3-hydroxymepivacaine in horse urine prior to hydrolysis. The ELISA reactivity of 3-hydroxymepivacaine-glucuronide is unpredictable so may be entirely different to that of 3-hydroxymepivacaine. However, the 3-hydroxymepivacaine-glucuronide standard is not available for ELISA reactivity studies. It is impossible to determine the proportion of mepivacaine reactive substances in the urine samples analysed, as any one of these substances may have a high affinity for the anti-mepivacaine antibody.

The relatively high concentration of mepivacaine equivalents in the pre-race urine samples (20 ng/ml) suggests that compounds in equine urine interfere with the ELISA test. These 'background' values in urine vary greatly from sample to sample and the use of urine matrix standard curves did not fully compensate for the

presence of these interfering substances. Therefore, the concentrations of mepivacaine equivalents reported here are probably higher than the true values for mepivacaine-related substances present in the samples.

The GC/MS method was specific for the 3-hydroxymepivacaine metabolite. It is characteristic of MSD methodology to identify a detected chemical and confirm its structure. In the present study, urine samples were enzyme hydrolysed under conditions known not to compromise the stability of 3-hydroxymepivacaine (Harkins *et al.* 1998a). Therefore, concentrations determined by GC/MS are a measure of 3-hydroxymepivacaine released from the glucuronide conjugate in the urine samples.

This research demonstrates that, although the ELISA and GC/MS procedures may measure entirely or partially different compounds derived from mepivacaine in the horse, the ELISA method is useful as a screening device for mepivacaine administration and the GC/MS method is useful for the establishment of quantitative data for the concentration of 3-hydroxymepivacaine recovered from post race urine samples.

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References

Gilman, A.G., Rall, T.W., Nies, A.S. and Taylor, P. (eds) (1990) *The Pharmacological Basis of Therapeutics.* 8th edn., Pergamon Press, New York, pp 311-331.

Harkins, J.D., Karpiesiuk, W., Woods, W.E., Lehner, A., Rees, W.A., Dirikolu, L., Bass S., Carter, W.G., Boyles, J. and Tobin, T. (1998a) Mepivacaine: Its pharmacological effects and their relationship to analytical findings in the horse. *J. vet. Pharmac. Ther.* **22,** 107-121.

Harkins, J.D., Mundy, G.D., Woods, W.E., Lehner, A., Karpiesiuk, W., Rees, W.A., Dirikolu, L., Bass, S., Carter, W.G., Boyles, J. and Tobin, T. (1998b) Lidocaine: Its pharmacological effects and their relationship to analytical findings in the horse. *J. vet. Pharmac. Ther.* **21,** 462-476.

Reynolds, F. (1971) Metabolism and excretion of bupivacaine in man: a comparison with mepivacaine. *Brit. J. Anaesth.* **43,** 33-37.

Tobin, T. (1981) *Drugs and the Performance Horse.* Charles C. Thomas, Springfield, IL, pp 261-275.

Tobin, T. (1995) Fifties rules, nineties testing: The need for thresholds for therapeutic medications. *Racing Update* **18,** 89-92.

Woods, W.E., Mundy, G.D., Stanley, D.S., Sams, R.A., Harkins, J.D., Carter, W., Boyles, J., Rees, W.A., Bass, S. and Tobin, T. (1996) Detection and quantification of cocaine and its metabolites in equine body fluids after administration by different routes. *Proc. 11th int. Conf. racing Anal. Vet.* Eds: D.E. Auer and E. Houghton. R&W Publications, Newmarket, pp 503-507.