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PHARMACODYNAMICS

Mepivacaine: its pharmacological effects and their relationship to analytical findings in the horse

J. D. HARKINS*
W. KARPIESIUK*
W. E. WOODS*
A. LEHNER*
G. D. MUNDY†
W. A. REES*
L. DIRIKOLU*
S. BASS‡
W. G. CARTER*
J. BOYLES* &
T. TOBIN*

Maxwell H. Gluck Equine Research Center and the Department of Veterinary Science, University of Kentucky, Lexington, KY 40506; †The Kentucky Racing Commission, Lexington, KY 40511; ‡Neogen Corp, Lexington, KY 40505, USA

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Mepivacaine is a local anaesthetic drug that is widely used in equine medicine and is classified by the Association of Racing Commissioners International (ARCI) as a Class 2 foreign substance that may cause regulators to impose significant penalties if residues are identified in post-race urine samples. Therefore, an analytical/pharmacological database was developed for this agent and its metabolites. Using an abaxial sesamoid local anaesthetic model, it was determined that the highest no-effect dose (HNED) for its local anaesthetic effect was 2 mg. Using enzyme-linked immunosorbent assay (ELISA) screening, it was determined that subcutaneous (s.c.) administration of the HNED of mepivacaine to eight horses yielded a peak urinary concentration of apparent mepivacaine of 63 ng/mL 2 h after injection.

The major identified metabolite recovered from equine urine after dosing with mepivacaine is 3-hydroxymepivacaine. Therefore, 3-hydroxymepivacaine was synthesised, purified and characterized, and a quantitative mass spectrometric method was developed for this metabolite as isolated from horse urine. Following subcutaneous injection of the HNED of mepivacaine, the concentration of 3-hydroxymepivacaine recovered from horse urine reached a peak of about 64.6 ng/mL at 4 h after administration as measured by GC/MS.

The concentration of mepivacaine or its metabolites after administration of a HNED dose are detectable by mass spectral techniques. Within the limits of this research, the study suggests that recovery of concentrations less than about 65 ng/mL of 3-hydroxymepivacaine from post-race urine samples may not be associated with a recent LA effect of mepivacaine.

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J. Daniel Harkins, 108 Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY 40506–0099, USA.

INTRODUCTION

Mepivacaine is a commonly used local anaesthetic (LA) agent in horses. It produces conduction blockade in sensory neurons by retarding the influx of Na^+ ions into the cells. Mepivacaine is recognised as a legitimate therapeutic medication by the American Association of Equine Practitioners (AAEP). However, it is also listed as a Class 2 substance by the Association of Racing Commissioners International (ARCI), and its identification in post-race urine samples can result in substantial penalties. Therefore, it is important to determine the dose and time response relationships for the local anaesthesia produced by this agent and the time periods for which mepivacaine or its metabolites remain detectable in plasma/urine samples after administration of therapeutic doses.

The potency of mepivacaine is reportedly twice that of procaine and similar to that of lidocaine. The duration of anaesthesia following mepivacaine administration is 2–3 times that of procaine (Booth, 1988) and similar to that of lidocaine (Ritchie & Greene, 1990). Further similarity to procaine and lidocaine is the possibility of its detection in urine long after the pharmacological effects have disappeared. During a 3.5-year period (January 1990 to June 1993), four identifications of mepivacaine were reported in North American racing (R. Gowen, personal comm.).

There is sparse information about the disposition or pharmacodynamics of mepivacaine in horses. The Agriculture Canada Equine Drug Evaluation Centre reports a 48-h detection time for mepivacaine following a dose of 300 mg administered subcutaneously or intra-articularly (Agriculture Canada, 1991).

and the Australian Equine Veterinary Association relates a detection time of 48 h after a subcutaneous or intramuscular dose of 400 mg (Australian Equine Veterinary Association, 1992). The European Horserace Scientific Liaison Committee reports a detection time of 4.5 days following a subcutaneous dose of 200 mg (European Horserace Scientific Liaison Committee, 1997). Detection time is defined as 'a known period of time after administration during which the agent or its metabolites have been detected in the plasma or urine of a horse' (Kellon & Tobin, 1995).

However, it should be noted that none of these detection time reports specified the analytical methods used, the sensitivity of the tests, or the nature of the analytes identified. Furthermore, the reports were not related to the possible pharmacological, therapeutic or performance effects of mepivacaine. These questions must be answered for veterinarians and pharmacologists to develop expert opinions about the possible performance-altering effects associated with a chemical identification of mepivacaine-related analytes in a post-race sample.

The objectives of this study were to: (1) determine the highest no-effect dose (HNED) of mepivacaine injected as an abaxial sesamoid nerve block; (2) synthesise, purify and characterise the major urinary metabolite of mepivacaine detected by forensic chemists, namely 3-hydroxymepivacaine; (3) validate a sensitive ELISA test for mepivacaine in equine serum or urine; (4) develop a sensitive and quantitative GC/MS method for mepivacaine or its major identifiable metabolite in equine serum/urine; and (5) determine the urine concentration of mepivacaine and its metabolites after administration of the HNED to establish analytical/pharmacological relationships for mepivacaine in the horse. As pharmacological effects and route of administration are linked, the HNED must be defined with regard to a specific route of administration.

MATERIALS AND METHODS

Horses

Nine mature Thoroughbred mares weighing 413–602 kg were used for this study. All horses were acclimatised to their stalls 24 h before experimentation. The animals were maintained on grass hay and feed (12% protein), which was a 50: 50 mixture of oats and an alfalfa-based protein pellet. Horses were fed twice a day. The animals were vaccinated annually for tetanus and were dewormed quarterly with ivermectin. A routine clinical examination was performed before each experiment to ensure that the animals were healthy and sound. During experimentation, horses were provided water and hay *ad libitum*. Because of the critical role of superficial skin temperature in these experiments, no LA quantification experiments were performed when the ambient temperature was less than 10°C. At least 7 days elapsed between individual LA dose–response curve experiments. Animals used in these experiments were managed according to the rules and regulations of the Institutional Animal Care and Use Committee at the University of Kentucky, the committee which also approved the experimental protocol.

Local anaesthetic response experiment

Horses were allocated to treatment groups containing five horses. Each group was injected subcutaneously with a dose (0, 2, 4, 7 and 10 mg) of 2% mepivacaine HCl (Steris Laboratories, Phoenix, AZ) in a randomized sequence with 7 days between doses. The site of injection was the lateral volar nerve where it passes lateral (abaxial) to the lateral sesamoid bone. In clinical practice, this block is known as an abaxial sesamoid block. To control for possible effects of pressure or volume, a similar volume of normal saline was injected into the contralateral leg, which was tested in parallel with the mepivacaine-treated leg. For a positive control, bupivacaine HCl (10 mg; Abbott Labs, Chicago, IL) was injected, and for a negative control, saline (2 mL) was injected in a similar manner.

Before each mepivacaine local anaesthetic response (LAR) experiment, the hair on the front and lateral sides of the fore leg pasterns was clipped, and the pastern was blackened with stamp pad ink (Dennison Manufacturing Co, Framingham, MA) to insure equal and consistent heat absorption independent of skin and hair colour. Contralateral legs were also clipped, blackened and tested to assess any systemic effect of mepivacaine.

For the LAR experiments, dose and time response relationships for mepivacaine were determined with a heat projection lamp described previously (Harkins *et al.*, 1996). Briefly, focused radiant light/heat was used as a noxious stimulus and was directed onto the pastern of the horse to elicit the classic flexion-withdrawal reflex. Hoof withdrawal reflex latency (HWRL) was defined as the time between lamp illumination and withdrawal of the hoof. These times were adjusted by varying the intensity of the heat output with a rheostat so that the HWRL period was about 3–4 s in the control legs, with the actual HWRL recorded on an electronic timer built into the lamp. In the anaesthetized leg, the duration of light exposure was limited to 10 s to prevent damage to the skin. A secondary unfocused light beam (sham light) was used to confound the horse, reducing the possibility that the flexion-withdrawal reflex was to visual rather than thermal perception of the focused light beam.

HWRL was measured at –30 and –15 min and immediately before injection of mepivacaine. These three HWRL times (–30, –15 and 0 min) were used to establish a baseline value for HWRL in each horse. The HWRL was also measured at 7.5, 15, 30, 45, 60, 75, 90 and 120 min after administration of mepivacaine HCl. The HWRL was expressed as a percentage of baseline values.

Development of a mepivacaine ELISA test

The one-step enzyme-linked immunosorbent assay (ELISA) tests were performed as described by Voller *et al.* (1976). Briefly, antimepivacaine antibodies were covalently linked to plastic, flat-bottom wells. Similarly, the mepivacaine hapten was linked to horse radish peroxidase (HRP) to create a covalently linked mepivacaine-HRP conjugate. This mepivacaine test is commercially available from Neogen Inc. of Lexington, KY.

When ELISA tests are used to screen horse urine, unidentified substances in the urine create variable levels of background or matrix effects that interfere with the assay (Stanley *et al.*, 1990).

To evaluate the endogenous background activity for the mepivacaine ELISA test, 20 mL aliquots of 40 post-race urine samples were assayed to measure the 'apparent drug' due to urinary background. The highest concentration of apparent drug was then compared with the I_{50} (the drug concentration with 50% less colour activity than the zero standard) of the standard curve for the drug.

Analytical/pharmacological relationships after HNED administration

The HNED for mepivacaine (i.e. 2 mg; determined from the LA quantification experiment) was injected subcutaneously in the area of the fetlock in eight horses. During the first day, complete urine collection was accomplished with a Foley catheter and attached plastic bag. Negative control samples were collected during the 30-min period before administration; post administration samples were collected 0–0.5, 0.5–1, 1–2, 2–4, 4–6 and 6–8 h after administration. At 24, 48 and 72 h after administration, a Harris flush tube (24 Fr × 60 in; Sourcless, Ocala, FL) was used to collect a maximum of 300 mL urine. Urine was placed in aliquots, and stored at–20°C until assayed.

ELISA quantification of apparent mepivacaine in urine after incremental doses

All urine samples were first screened with an ELISA test to determine the concentrations of 'apparent mepivacaine' (i.e. the concentration of mepivacaine that would produce similar results) in the samples. The 'apparent mepivacaine' is composed of mepivacaine, 3-hydroxymepivacaine as its glucuronide, and possibly other metabolites. The assays were started by adding 20 mL of the standard, test, or control samples to each well. Volumes of 180 mL of the mepivacaine-HRP conjugate were added to wells containing the test samples, and a volume of 160 mL of the mepivacaine-HRP conjugate and 20 mL of negative control urine/serum (to create a matrix comparable to the test samples) were added to wells containing standard and control samples. During the test, mepivacaine or its metabolites competed with mepivacaine-HRP complex for binding to the antibody. As the HRP enzyme was responsible for the colour-producing reaction in the ELISA, the log of the concentration of mepivacaine and its cross-reacting metabolites in the sample was inversely related to the percentage of maximal optical density of the test well, which was determined at a wavelength of 650 nm with an automated microplate reader (Bio-Tek Instruments, Winooski, VT) ≈ 30 min after addition of substrate. All assay reactions were run at room temperature (20°C).

The ELISA method for the semiquantitation of 3-hydroxymepivacaine was validated by examining the precision and linearity of the assay. The between-run precision was determined for middle standard (10 ng/mL) of the standard curve to be 32.2%. The mean coefficient of regression (r) for the assays was 0.9857 ± 0.0190 SD. There was no extraction for the ELISA method; therefore, no recovery was calculated.

Synthesis and characterization of 3-hydroxymepivacaine

The structures of mepivacaine and its metabolites are shown in Fig. 1, and Fig. 2 presents a scheme for the synthesis of 3-hydroxymepivacaine from mepivacaine, which was adapted from a previously reported synthetic procedure (Thomas & Meffin, 1972). Nitration of mepivacaine under classic conditions resulted in the 3-nitro compound (R = NO_2), which was reduced with zinc dust in 50% acetic acid to produce 3-NH_2-mepivacaine in high yield. After diazotization of this amine with sodium nitrite in sulfuric acid and following hydrolysis in acidic conditions at 70°C, the desired 3-hydroxymepivacaine was obtained, which was purified upon sublimation in vacuum.

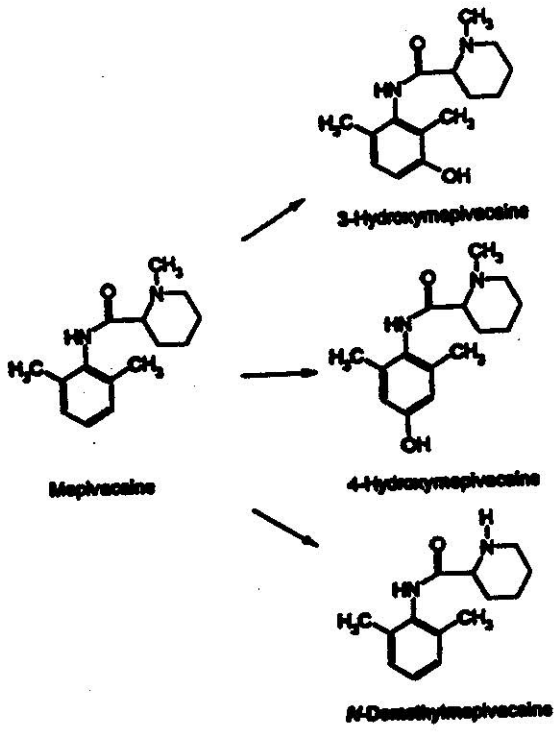

Fig. 1. Structures of mepivacaine and its metabolites

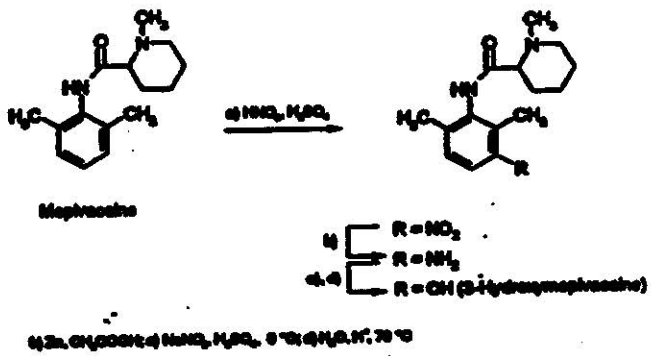

Fig. 2. Synthetic scheme showing the sequential steps (a-d) in the conversion of mepivacaine to 3-hydroxymepivacaine.

General methods

^{1}H-nmr (200 MHZ) and ^{13}C-nmr (50 MHZ) spectra were recorded on a GEMINI AC-200 (Varian, Palo Alto, CA) with tetramethylsilane as internal standard. GC/MS analysis was performed on a Model 6890 gas chromatograph equipped with a Model 5972A mass selective detector (Hewlett-Packard, Bloomington, DE). IR spectra were recorded on a 1640 FT-IR spectrometer (Perkin-Elmer Limited, Buckinghamshire, U.K.). Melting points are uncorrected.

Characterization data for N-(*3-hydroxy-2,6-dimethylphenyl*)-1-*methyl-2-piperidinecarboxamide* (3-hydroxymepivacaine): Mp. 194–197°C (sublimed 150°C, 0.1 mmHg): ^{1}H-nmr (200 MHZ, $CDCl_3$): δ (p.p.m.) 1.2–1.4 (m, 1 H), 1.5–1.9 (m, 4 H), 2.01 (s, 3 H, CH_3), 2.08–2.22 (m, 2 H) 2.14 (s, 3 H, CH_3), 2.43 (s, 3 H, CH_3), 2.68 (dd, 1 H, CH), 2.74–3.08 (m, 1 H), 6.45 and 6.78 (2 × ½ AB, 2 × 1H, 2 aromatic H, J 8.2 Hz), 8.08 (bs, 1 H, NH); ^{13}C-nmr (50 MHZ, $CDCl_3$): δ (p.p.m.) 11.25, 18.24 (2 × CH_3 $_{AR}$), 23.32, 25.31, 31.43 (3 × CH_2 piperidine), 45.41 (N-CH_3), 55.58, 70.02 (CH and CH_2 of piperidine), 114.73, 122.58, 126.21, 127.48, 133.26 (5 C_{AR}), 153.08 (C_{AR}-OH), 173.72 (C = O); FT-IR (KBr) 1660, 1500 cm^{-1}.

Stability of 3-hydroxymepivacaine

To assess the stability of the synthesized metabolite in frozen urine, a stock solution of 3-hydroxymepivacaine in methanol (1 mg/mL) was prepared, and a suitable aliquot of stock solution was added to negative control urine at a concentration of 4.0 mg/mL. Aliquots (6 mL) of both the supplemented urine and negative control urine were frozen and stored. We used the external standardization method, in which the recovered amount of metabolite from a given sample was expressed in terms of an identical sample prepared fresh on the day of analysis (McNair & Bonelli, 1968). Aliquots of urine to which the analyte was added and negative control urine were thawed at weekly intervals, and the stock solution was added to the negative control urine at a concentration of 4 mg/mL sample. Both samples were then extracted, and the eluent was evaporated according to methods described below. The residues were dissolved in 40 mL of ethyl acetate. One microlitre of this solution was injected into a GC/MS 6890/5972 in splitless mode at an injector temperature of 250°C. Initial oven temperature was 70°C (held 2 min) then increased at a rate of 20°C/min to 280°C (held 12 min). Total run time was 24.5 min. The GC to mass selective detector (MSD) interface temperature was kept at 280°C. Total ion chromatograms (TIC) were generated by scanning the 50–550 m/z range. This provided quantitative information in the form of integrated TIC peaks, mass spectral assurance of the metabolite's identity, and additional information as to the chemical nature of decomposition products. The quantity of 3-hydroxymepivacaine in the stored (supplemented) aliquots was reported as a percentage of the 3-hydroxymepivacaine solution prepared fresh each day (control). Similar experiments were also performed for the metabolite maintained in refrigerated and room temperature urine samples.

Development of a quantitative analytical method for 3-hydroxymepivacaine

The GC/MS derivatization method was designed according to the following considerations. Tetracaine was chosen as an internal standard owing to its relative stability to b-glucuronidase treatment and its lack of reactivity with the silylating reagent N-methyl-N-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA: Regis Technologies Inc., Morton Grove, IL).

Ideally, the internal standard should derivatize in a manner similar to that of the analyte undergoing analysis. However, 3-hydroxymepivacaine derivatized as either mono-or di-TMS or tBuDMS derivatives. To rule out as many factors as possible in the cause of this phenomenon, an internal standard that did not derivatize or show other reactivity toward the reagent was used. MTBSTFA showed consistent reaction to the bis(tBuDMS) derivative. The fact that we could generate linear standard curves with tetracaine and MTBSTFA derivatization encouraged us to continue using tetracaine as internal standard. On the other hand, the recent synthesis, characterization and development of a quantitative method for 3-hydroxylidocaine provides a possible superior internal standard for future work.

In a previous study (Harkins *et al.*, 1997), t-butyldimethylsilyl (TBS) derivatives were shown to be more stable than trimethylsilyl derivatives, a property critical for the development of a 3-hydroxylidocaine method. Specifically, the superior stability of t-butyldimethylsilyl (TBS) derivatives over trimethylsilyl (TMS) derivatives enabled complete derivatization of both phenolic and amido –H functions to give a bis(t-butyldimethylsilyl) derivative of 3-hydroxylidocaine. Similar reaction conditions were applied to 3-hydroxymepivacaine with the addition of a catalytic amount of triethylamine, which presumably displaces the phenolic and/or amido N-H protons to some extent prior to their substitution with TBS groups. However, for 3-hydroxymepivacaine, the reaction generally halted at mono-substitution, with only some bis(t-butyldimethylsilyl) 3-hydroxymepivacaine being formed.

However, when present, the bis(t-butyldimethylsilyl) derivative eluted as a shoulder on the mono-derivative under chromatographic conditions identical to those used for 3-hydroxylidocaine. Both mono-and bis-derivatives were found to produce the 98 m/z base peak fragment under electron impact ionization, similar to the 86 m/z base peak fragment ion of 3-hydroxylidocaine (Harkins *et al.*, 1997). Therefore, the areas for both species on a 98-m/z ion chromatogram were integrated together to represent the entire urine 3-hydroxymepivacaine. Most importantly, linear standard curves with *r*-values of 0.99 or greater were consistently generated by this method, verifying its applicability and dependability.

The results of this procedure are highly reproducible because the reaction can yield only mono-or bis-derivatives. More importantly, the base ion peak at 98 m/z of each individual derivative represents an identical molecular fragment of the 3-hydroxylidocaine, so that 'double counting' cannot occur.

The following ions were chosen for each compound for selected ion monitoring (SIM), with the *first ion* in each case chosen for quantitation purposes: tetracaine (retention time 11.9

min): *58.0*, 176.1: 3-hydroxymepivacaine (retention time 14.65 min): *98.0*, 178.1, 192.2, 250.2, 336.3. There was suitable lack of interferences with these ions from contaminants native to the matrix used. Lower molecular weight ions were chosen due to their high yields in the electron impact mass spectra and the conversely low yields of any other ions in the spectra including more diagnostic ones at higher molecular weights.

Hydrolysis procedure

For enzymatic hydrolysis of 3-hydroxymepivacaine glucuronide, urine samples, standards and negative control samples (5 mL/sample) were first placed in culture tubes. To each sample were added 1 mL b-glucuronidase solution (Sigma Type L-II, 5000 units/mL) and 2 mL of 1 M sodium acetate buffer (pH 5.0). The samples were mixed briefly by vortex and incubated (water bath) at 65°C for 3 h. After cooling overnight at 4°C, the samples were sonicated for 90 s, then 2 mL of 0.1 M sodium phosphate buffer (pH 6.0) was added, and the sample pH was adjusted to 6.0 ± 0.5 with 1 M NaOH or 1 M HCl.

Conditions for hydrolysis of glucuronide were the hydrolytic conditions used in equine drug testing laboratories, specifically treatment at 65°C for 3 h with *Patella vulgata* b-glucuronidase (Combie *et al.*, 1982). These conditions were optimised for recovery of morphine. Researchers should be aware that different glucuronidase hydrolysis conditions could result in quantitative yields of 3-hydroxymepivacaine different from those reported in this communication.

Quantitative standard curve

Standard solutions of 3-hydroxymepivacaine and tetracaine were prepared in methanol. Measured volumes of the 3-hydroxymepivacaine solution were added to negative control enzyme-treated urine samples over a range of 1 ng/mL to 160 ng/mL to generate a standard curve. A constant volume of the tetracaine standard (18 mL of a 100 ng/mL solution for a total of 1800 ng) was added to each sample, standard, and negative control as an internal standard.

Extraction/derivatization procedure

'Clean Screen' solid phase extraction (SPE) columns (Worldwide Monitoring #ZSDAU020, Bristol, PA) were conditioned by sequentially adding 3 mL of methanol, 3 mL of water and 1 mL of 0.1 M sodium phosphate buffer (pH 6.0). The samples were then loaded onto the column, and the column was sequentially washed with 2 mL of water, 2 mL of 1 M acetic acid and 4 mL of methanol. The analytes were eluted with 3 mL of freshly prepared dichloromethane:isopropanol:ammonium hydroxide (78: 20: 2). The eluent was kept slightly warm (< 40°C) and evaporated to dryness under a stream of N_2. For derivatization, each sample was dissolved in 40 mL of MTBSTFA, 1% *tert*-butyldimethylchlorosilane (*t*-BDMCS) and 1 mL of triethylamine, vortexed for 15 s, and incubated at 40°C for 2 h. Afterwards, the samples were incubated at room temperature overnight.

Instrumentation

The instrument used was a Hewlett-Packard (Model 6890) gas chromatograph equipped with a Model 5972 mass selective detector (MSD). Separations were performed on an HP-5, 30 m × 250 mm (i.d.) × 0.25 mm (film thickness) cross-linked 5% diphenyl 95% dimethyl polysiloxane column. The carrier gas was helium with flow of 1 mL/min. Each derivatized sample was transferred to an autosampler vial. One microlitre of derivatized extract was injected in the splitless mode at an injector temperature of 250°C. Initial oven temperature was 70°C (held 2 min) then increased at a rate of 20°C/min to 280°C (held 12 min). Total run time was 24.5 min. The GC to MSD interface temperature was kept at 280°C. The MSD was run in SIM mode with ions and their abundances measured during the 3–24.5 min interval as follows: 336.3, 250.2, 192.2, 178.1, 176.1, 98.0 and 58.0 m/z with a 60 ms dwell time.

Quantitation

Standards were prepared, extracted and derivatized in duplicate, and ion areas were obtained by integration. The identity of a peak as tetracaine or derivatized 3-hydroxymepivacaine was confirmed by the coelution of ions specific to each compound at the retention times shown by analyte standards. MTBSTFA derivatization of 3-hydroxymepivacaine under these conditions resulted in two products, mono (*t*-butyldimethylsilyl)-3-hydroxymepivacaine and di (*t*-butyldimethylsilyl)-3-hydroxymepivacaine, which were not resolved and peak area ratios varied from sample to sample. Therefore, the areas of the two products were combined by manual integration (MSD ChemStation User's Guide (1995) Hewlett-Packard Co., Palo Alto, CA). The combined-areas value was used as the area of the derivatized 3-hydroxymepivacaine. Derivatized mepivacaine metabolite areas were calculated as ratios relative to the area of the internal standard, and unknown concentrations were calculated by fitting data to a least squares-regression analysis line. Samples containing a higher concentration than the highest calibrator were diluted appropriately and reanalysed.

The GC/MS method for the quantitation of 3-hydroxymepivacaine was validated by examining the precision (% coefficient of variation, CV), linearity (coefficient of regression, *r*), and recovery of the assay. The within-run precision was determined for the low (4 ng/mL: CV = 3.9%), middle (40 ng/mL: CV = 8.8%), and high (160 ng/mL: CV = 6.3%) concentrations of the 3-hydroxymepivacaine standard curve. The between-run precision was determined for the low (4 ng/mL: CV = 22.6%), middle (40 ng/mL: CV = 7.0%), and high (160 ng/mL: CV = 0.9%) concentrations of the 3-hydroxymepivacaine standard curve. The mean *r* for the assays was 0.9995 ± 0.0004 SD. The recovery was determined at two concentrations: 2 ng on column = 132.7% recovery, and 10 ng on column = 125.2% recovery. The mean recovery was 128.9% ± 17.2 SD. The high recovery values were likely due to loss of 3-hydroxymepivacaine during evaporation of the solvent from samples not containing horse urine. The 3-hydroxymepivacaine in the eluents of the

extracted urine samples was presumably protected during evaporation by extracted urine residue.

Statistical analysis

Analysis of variance with repeated measures was used to compare control and treatment HWRL values for the different doses of mepivacaine. Significance was set at $P < 0.05$.

RESULTS

Dose and time response curves

The dose and time response curves of the LA effect of mepivacaine HCl are presented in Fig. 3(a). Following injection of 7 and 10 mg doses, there was a significant LA effect 15 min after administration which persisted for 45 and 90 min, respectively. Additionally, there was a significant difference between negative (saline) and positive (bupivacaine, 10 mg) control values at every time point after anaesthetic injection. There was no significant LA effect following injection of 2 or 4 mg mepivacaine HCl; based on review of the data, 2 mg was established as the HNED for mepivacaine in this model.

There was no LA effect in the contralateral leg (Fig. 3b). The dose–response curve for the peak LA response to mepivacaine is presented in Fig. 3(c). A dose–response curve for bupivacaine is included for reference.

Validation of the ELISA test

A standard curve for the mepivacaine ELISA test indicated that addition of 42 ng mepivacaine/mL to the system produced 50% inhibition (I_{50}; Fig. 4). Higher concentrations of mepivacaine increased the inhibition in a sigmoidal manner, with essentially complete inhibition of the ELISA test occurring at 100 ng/mL of mepivacaine. The ELISA antibody reacted poorly with 3-hydroxymepivacaine, with 50% inhibition of the test attained only after addition of > 1000 ng/mL of 3-hydroxymepivacaine. Cross reactivities were also evaluated for other common local anaesthetics used in equine medicine (Table 1).

The highest background reading (0.11 ng/mL) of the 40 post-race samples (Fig. 5) was well below the apparent I_{50} (42 ng/

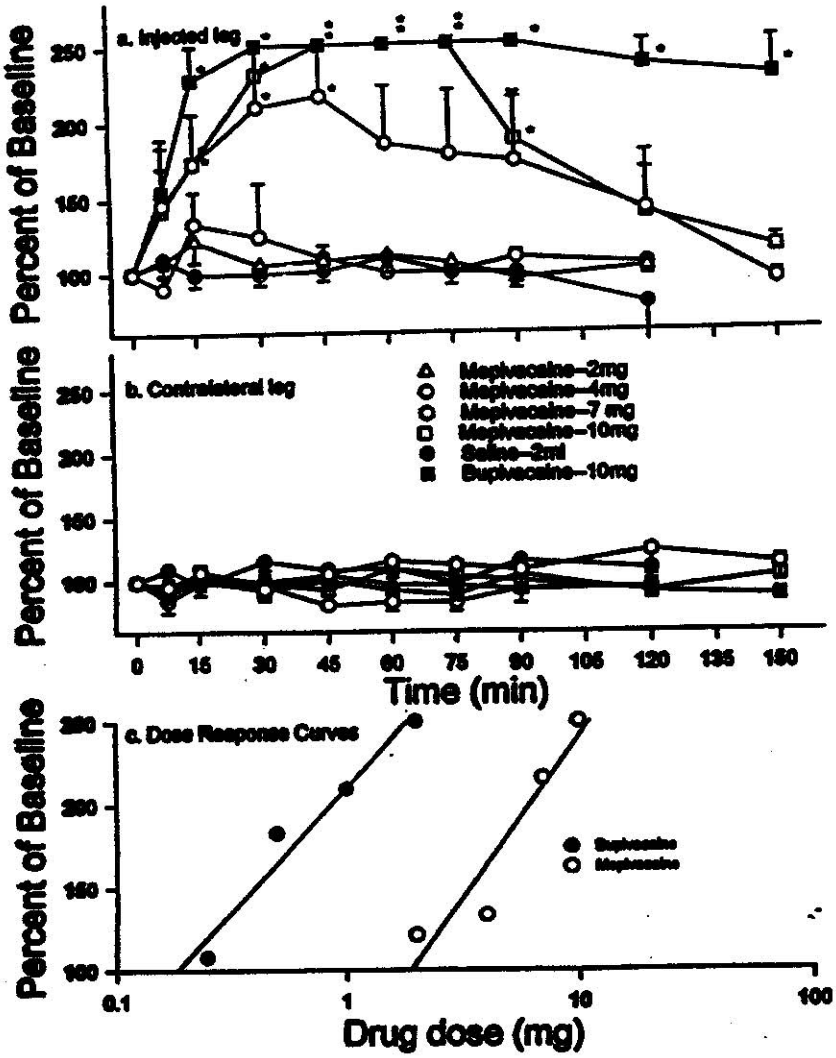

Fig. 3. (a) Mean ± SE percentage increase in HWRL after injection of mepivacaine dose; (b) mean ± SE percentage change in contralateral leg after saline injection; (c) dose–response curve for mepivacaine and bupivacaine. *Significantly different from control values.

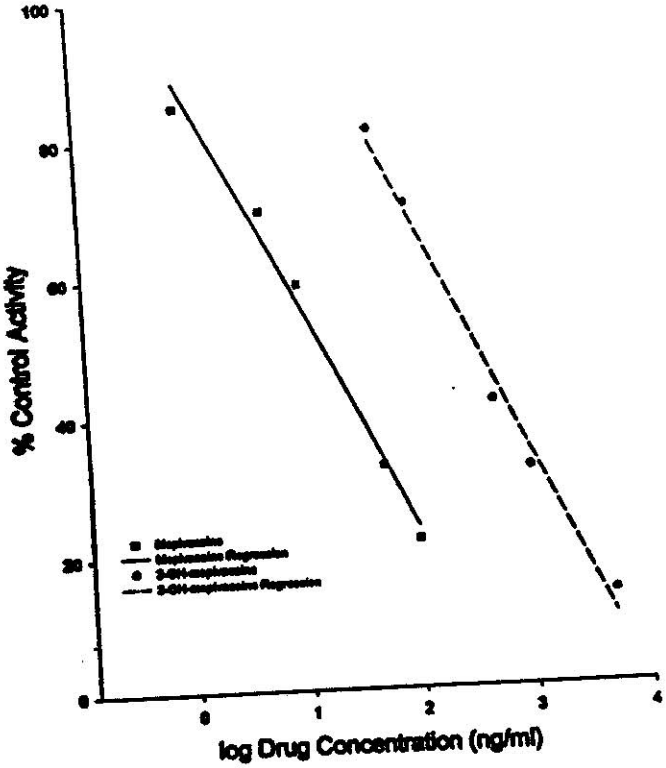

Fig. 4. Regression of ELISA standard curves for mepivacaine and 3-hydroxymepivacaine.

Table 1. Data for cross-reactivity of the antimepivacaine antibody

Mepivacaine	100%
Bupivacaine	94%
Lidocaine	13%
Etidocaine	5%
Prilocaine	5%
3-Hydroxymepivacaine	5%
Cocaine	< 0.1%
Dibucaine	< 0.1%
Procaine	< 0.1%
Tetracaine	< 0.1%

mL) of the standard curve. The apparent mepivacaine concentration of a typical positive control sample is shown at about 130.0 ng/mL, well above usual background interference. The typical positive control was created by adding urine from a horse dosed with mepivacaine (200 mg, subcutaneously) to mepivacaine-free (by ELISA) urine until the urine mixture was clearly 'positive' on the ELISA test.

ELISA quantification of apparent mepivacaine in urine

For subcutaneous doses of 486 ($n = 1$), 162 ($n = 1$), 54 ($n = 1$), 18 ($n = 1$), 6 ($n = 1$) and 2 ($n = 8$) mg, the concentrations of apparent mepivacaine in urine reached peaks of 2259, 1162, 414, 171, 65 and 63 ng/mL at 4, 4, 6, 4, 2 and 1 h after administration,

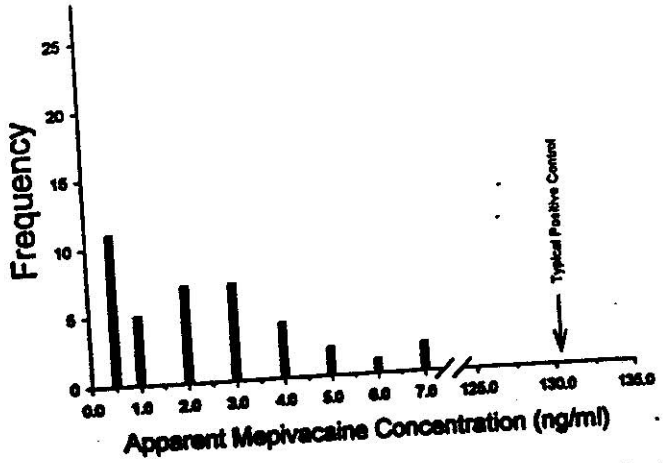

Fig. 5. Background interference in 39 post-race equine urine samples. A dilution of 1: 9 (i.e. 1 part urine to 9 parts EIA buffer) is recommended to reduce natural background concentration. The apparent mepivacaine concentration of a typical positive control is shown at about 130 ng/mL, well above usual background interference.

respectively (Fig. 6), and the apparent peak mepivacaine concentrations were directly related to the dose administered (Fig. 6a, inset). By 24 h after administration, the concentrations of apparent mepivacaine in urine for all doses were below the lowest concentration used in the standard curve (< 4.0 ng/mL).

Figure 7 shows the percentage of maximum optical density of the ELISA tests following the incremental doses of mepivacaine. The dotted line represents 50% inhibition of the optical density. The inset graph shows the relationship between the log of the mepivacaine dose and the time of last detection, when the criterion for detection was set at 50% inhibition of the mepivacaine ELISA test.

Stability of 3-hydroxymepivacaine

Figure 8(a) shows the relative stability of the 3-hydroxymepivacaine metabolite in frozen urine. There was no substantial change in the concentration of 3-hydroxymepivacaine detected after 8 weeks of storage, suggesting that the metabolite is stable in urine when stored frozen (–20°C). Furthermore, similar analyses of urine stocks kept refrigerated (4°C) or at room temperature (24°C) over a 5-day period also indicated substantial stability of the mepivacaine metabolite in urine. The slopes for these three concentrations were not significantly different from zero ($P < 0.05\%$).

Figure 8(b) illustrates the stability of 3-hydroxymepivacaine at 65 °C, the temperature at which b-glucuronidase treatment is performed, as described earlier. The slope of the concentration regression was –1.10 ($P < 0.05$) with a half-life of 49 h.

MS quantification of 3-hydroxymepivacaine: Ion chromatogram and EI-mass spectrum

Figure 9(a) shows the electron impact (EI)-mass spectrum of the mono-TBS derivative of the authentic 3-hydroxymepivacaine

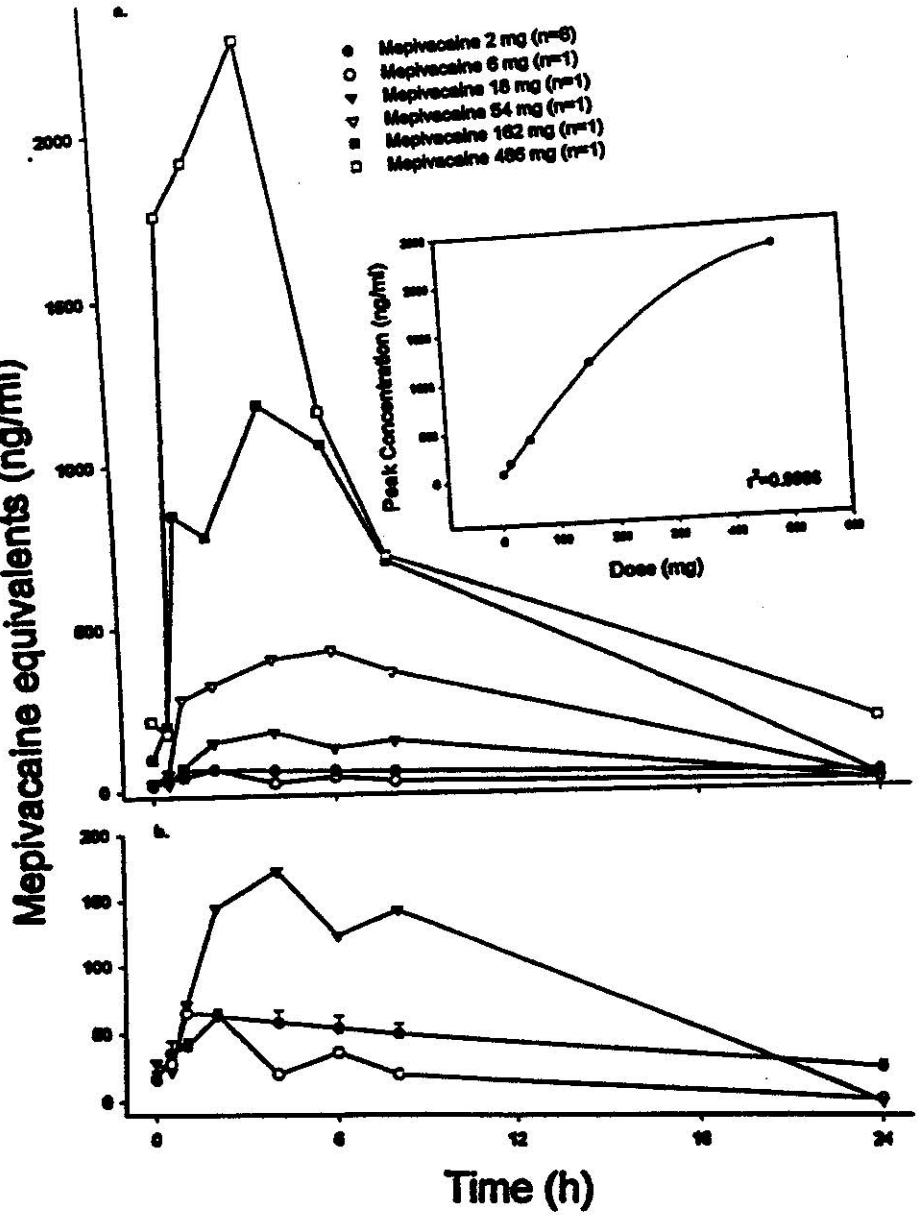

Fig. 6. (a) ELISA measurement of apparent mepivacaine in urine of horses treated with incremental doses of mepivacaine. Inset graph shows relationship of mepivacaine dose to the maximum concentration of mepivacaine equivalents detected at that dose; (b) expanded scale for lower doses.

occurring at a retention time of 14.7 min. Figure 9(b) is the EI-mass spectrum of the 14.7 min peak derived from the chromatogram of the extract isolated from the urine sample analysed in Fig. 10. Comparison of the two spectra (9a and 9b) provides substantial evidence that the horse mepivacaine metabolite eluting at 14.7 min (Fig. 9b) is 3-hydroxymepivacaine. Furthermore, the high relative abundance of ion 98 and the general lack of interferences with many of the ions of the spectrum provide impetus for quantitation using this ion by SIM.

Figure 10(a) shows the ion chromatograms for four diagnostic ions (98, 192, 178 and 361). As mentioned in the section on development of a quantitative method, ion 98 m/z is characteristic of mepivacaine and its TBS and TMS derivatives, owing to generation of the $[CH(CH_2)_4NCH_3]^+$ ion. Figure 10(a) and its enlargement in Fig. 10(b) illustrate the coincident elution of 98, 178, 192 and 361 ions at the retention time of 14.65 min. Ions 98, 178 and 192 are characteristic of both bis-and mono-TBS derivatives, while ion 361 is specific to the mono-derivative. Ions 177/178 and 192 are also present at the centre of small clusters in 3-hydroxymepivacaine TMS derivatives, as well as in 3-hydroxylidocaine TMS and TBS derivatives, and evolve from the phenyl ring portion of the respective molecules. Two fragmentation events are involved in the generation of these ions, with the two fragmentation schemes differing by the absence or presence of an additional N atom.

GC/MS quantitation of 3-hydroxymepivacaine recovered from urine

Because EI-mass spectrometry in general leads to an accumulation of lower molecular weight fragments, higher molecular weight ions such as 178, 192 and 361 m/z may be regarded as more specific to mono-TBS-3-hydroxymepivacaine, whereas one

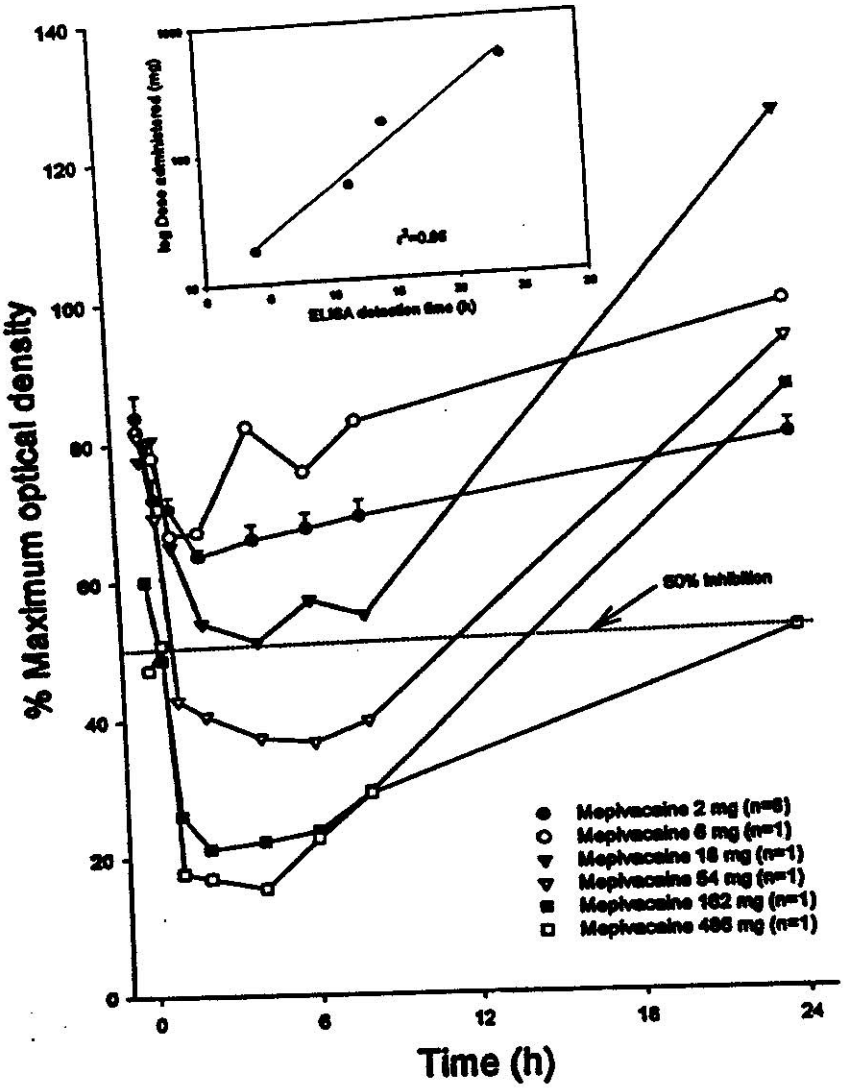

Fig. 7. Percent of maximum optical density of ELISA tests of urine from horses treated with incremental doses of mepivacaine. The dotted line is drawn at 50% of maximum optical density. Inset graph shows the regression of log drug dose against detection time.

might anticipate the base peak 98 m/z to be less so. As shown in Fig. 11(b), ions 178 and 192 integrated across mono-and bis-TBS derivatives and calculated relative to the internal standard provided standard curves suitable for interpolation of unknowns. Comparable data are shown for ion 98 m/z in Fig. 11(a). In light of the relative paucity of neighbouring 98 m/z peaks in ion chromatograms derived from equine urine (Fig. 10) and the much greater intensity of 98 m/z in the mass spectra of mono- and bis-TBS 3-hydroxymepivacaine (Fig. 9a), areas derived from this ion were chosen for quantitative purposes. This also avoided the somewhat lower intensity of 178 and 192 m/z peaks in the bis-TBS derivative (Fig. 10b) and any variance this might have eventually introduced. As indicated in Materials and Methods, this approach to quantitation of mepivacaine consistently yielded linear standard curves with r^2 values greater than 0.99.

Figure 12(a) shows the concentration of 3-hydroxymepivacaine recovered from the urine of eight horses dosed with the HNED (2 mg/site) of mepivacaine. The concentration of recovered 3-hydroxymepivacaine rose rapidly after administration and reached a peak of 64.6 ng/mL 4 h after dosing. Thereafter, it declined to less than 2 ng/mL by 24 h after dosing.

DISCUSSION

The goal of this research was to create a data base to assist veterinarians, pharmacologists and toxicologists in assessing the possible performance-altering effects of mepivacaine when chemical residues of that agent are identified in a post-race urine sample. As mepivacaine is a potent local anaesthetic, the identification of mepivacaine residues in a post-race sample creates a presumption that the agent may have been administered as a clinically effective dose close to race time, creating a local anaesthetic effect which could influence the horse's performance (Tobin, 1981).

The primary pharmacological effect of mepivacaine in the horse is local anaesthesia and is essentially the only pharmacological effect likely to influence performance. Therefore, local anaesthesia is the pharmacological effect of interest to racing regulators.

Figure 3 quantifies the local anaesthetic effect of mepivacaine using the heat lamp/abaxial sesamoid block model and shows that the HNED of mepivacaine is about 2 mg/site. Therefore, 2 mg was the reference dose for determination of urinary concentrations of mepivacaine and its metabolites.

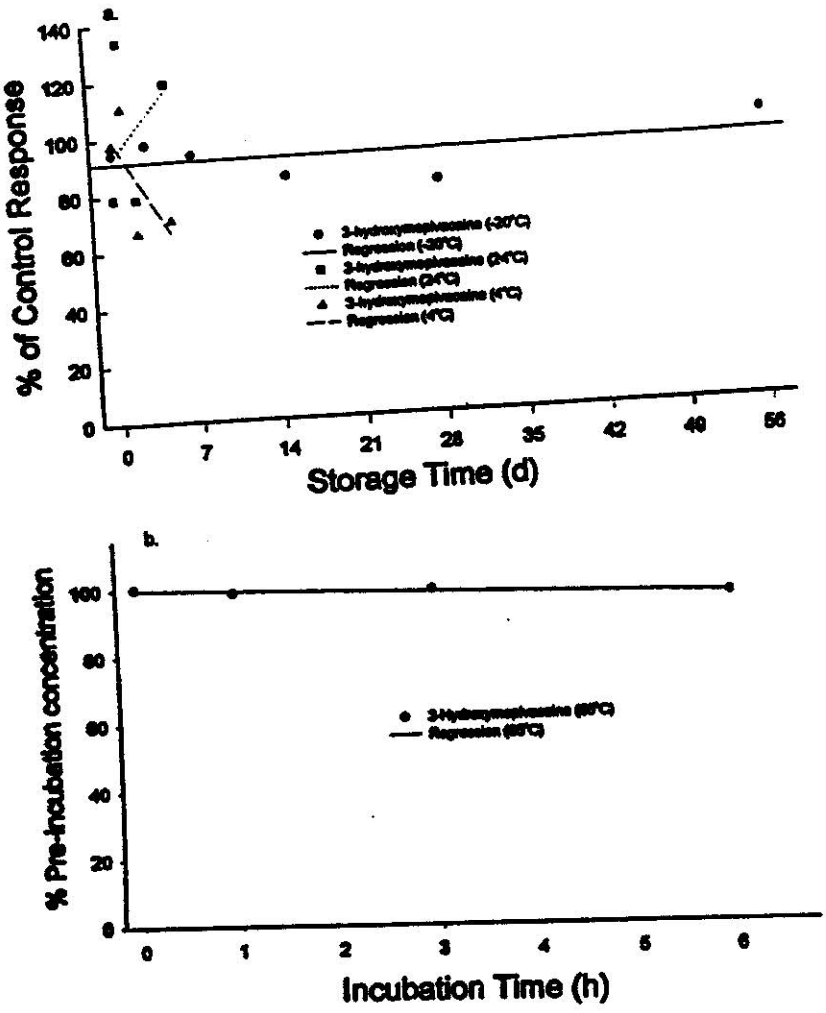

Fig. 8. (a) Regression lines showing the stability of 3-hydroxymepivacaine in urine at room temperature (24°C), refrigerated (4°C), and frozen (–20°C); (b) stability of 3-hydroxymepivacaine in horse urine at 65°C as measured by GC/MS.

Screening of urine from performance horses for potent agents like mepivacaine is largely dependent on ELISA testing. An ELISA test had been developed previously for mepivacaine, and Fig. 4 shows the reactivity of the ELISA test with parent mepivacaine and its principal urinary metabolite in the horse, 3-hydroxymepivacaine. This ELISA test is much more sensitive for mepivacaine (I_{50} = 42 ng/mL) than for 3-hydroxymepivacaine (I_{50} = > 1000 ng/mL). This indicates that mepivacaine is detectable in urine at much lower concentrations than is the metabolite. With appropriate dilution of post-race samples to reduce endogenous background, this screening test has the capability of detecting apparent mepivacaine concentrations of 10–100 ng/mL in post-race urine samples (Fig. 5). Figure 6 shows that the incremental doses of mepivacaine became increasingly detectable by the ELISA test. Additionally, the inset graph of Fig. 6 shows there is an approximately linear relationship between the dose of mepivacaine administered and the peak concentration of apparent mepivacaine found in urine.

It is also apparent from these data that doses of mepivacaine capable of producing a significant LA effect may not be readily detectable by ELISA, depending on the optical density threshold in a given laboratory. Possible approaches to this problem include the addition of a hydrolysis/extraction step prior to ELISA screening of the urine sample, which is known as 'enhanced ELISA'. An alternative and more challenging approach would be the development of an ELISA test with greater sensitivity for mepivacaine or its major urinary metabolite.

The relationship between dose and peak concentration of apparent mepivacaine in post-race urine samples raised the question as to whether there was a similar relationship between dose and last time of detection. If a cut-off for detection of 50% inhibition of the ELISA test is assumed, there appears to be a linear relationship between logarithm of the mepivacaine dose and the time for which the residue of the agent can be detected (Fig. 7).

The apparent mepivacaine detected by the ELISA test is presumably composed of mepivacaine and structurally related metabolites of mepivacaine excreted in equine urine. Mepivacaine itself has not been found in significant concentrations in these samples and is not commonly found in post-race urine samples. Therefore, 3-hydroxymepivacaine was selected as the important metabolite detected in post-race samples. Because authentic 3-hydroxymepivacaine was not available commercially, the metabolite was synthesized and chemically characterized. However, for 3-hydroxymepivacaine to be useful in forensic

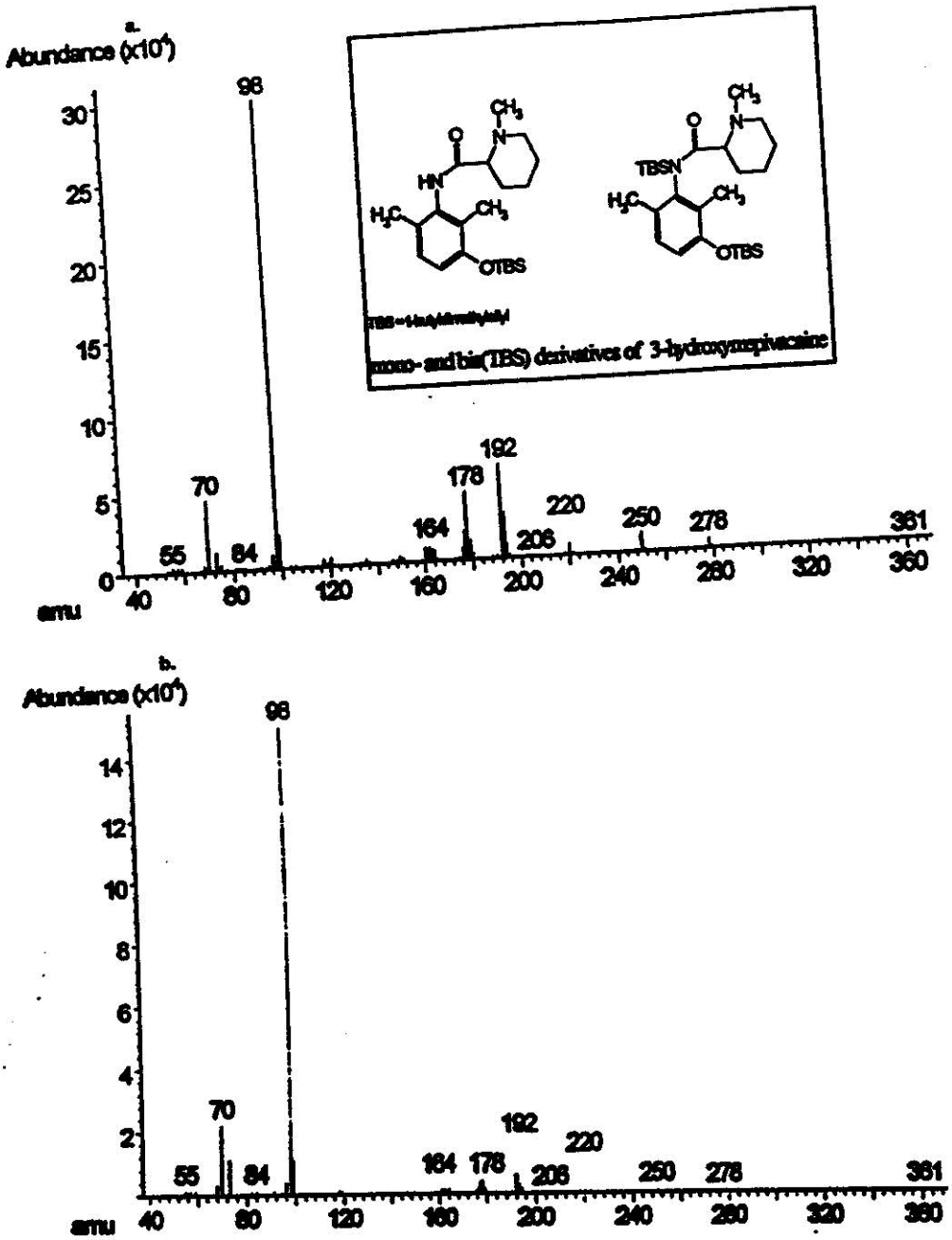

Fig. 9. (a) EI-mass spectrum of the mono-t-butyldimethylsilyl derivative of authentic 3-hydroxymepivacaine occurring at 14.7 min retention time. Inset figure shows structures of the 3-hydroxymepivacaine derivatives: (b) EI-mass spectrum of the 14.7 min peak from the chromatogram of a urine sample from a mepivacaine-dosed horse shown in Fig. 10. Both spectra were derived by ion chromatography for mass 98, derivation of the spectrum at the top of the appropriate peak, and subtraction of the spectrum associated with the background immediately before the peak.

analyses, the metabolite must be stable. Figure 8 shows that the synthesized metabolite is stable under most laboratory conditions and therefore has the potential to be a useful authentic standard in equine forensic work.

The synthesis of authentic 3-hydroxymepivacaine allows for improvement in the qualitative chemistry of 3-hydroxymepivacaine by making an authentic standard available to analytical chemists. Prior to the synthesis of this substance, the only mass spectra available were those of 3-hydroxymepivacaine isolated from horse urine. Figure 9 shows the first authentic mass spectra for 3-hydroxymepivacaine and its derivatives from the analytically pure substance.

The synthetic metabolite can also be used to create quality control samples. Prior to the synthesis of 3-hydroxymepivacaine, the generation of quality control samples required administration of the parent compound to horses followed by urine collection. With synthesized 3-hydroxymepivacaine, samples can be created by direct addition of specific amounts of the metabolite to equine urine samples, which is more accurate and economical and less cumbersome than dosing horses. Finally, this approach allows for the generation of blind proficiency samples, as 3-hydroxymepivacaine can be added to urine samples previously tested and found to be free of detectable drug residue, which are difficult to distinguish from regular track samples. In contrast, urine samples from dosed experimental horses at pasture can be recognized immediately by experienced analysts because of their physical characteristics, thereby defeating the purpose of 'blind' and proficiency testing.

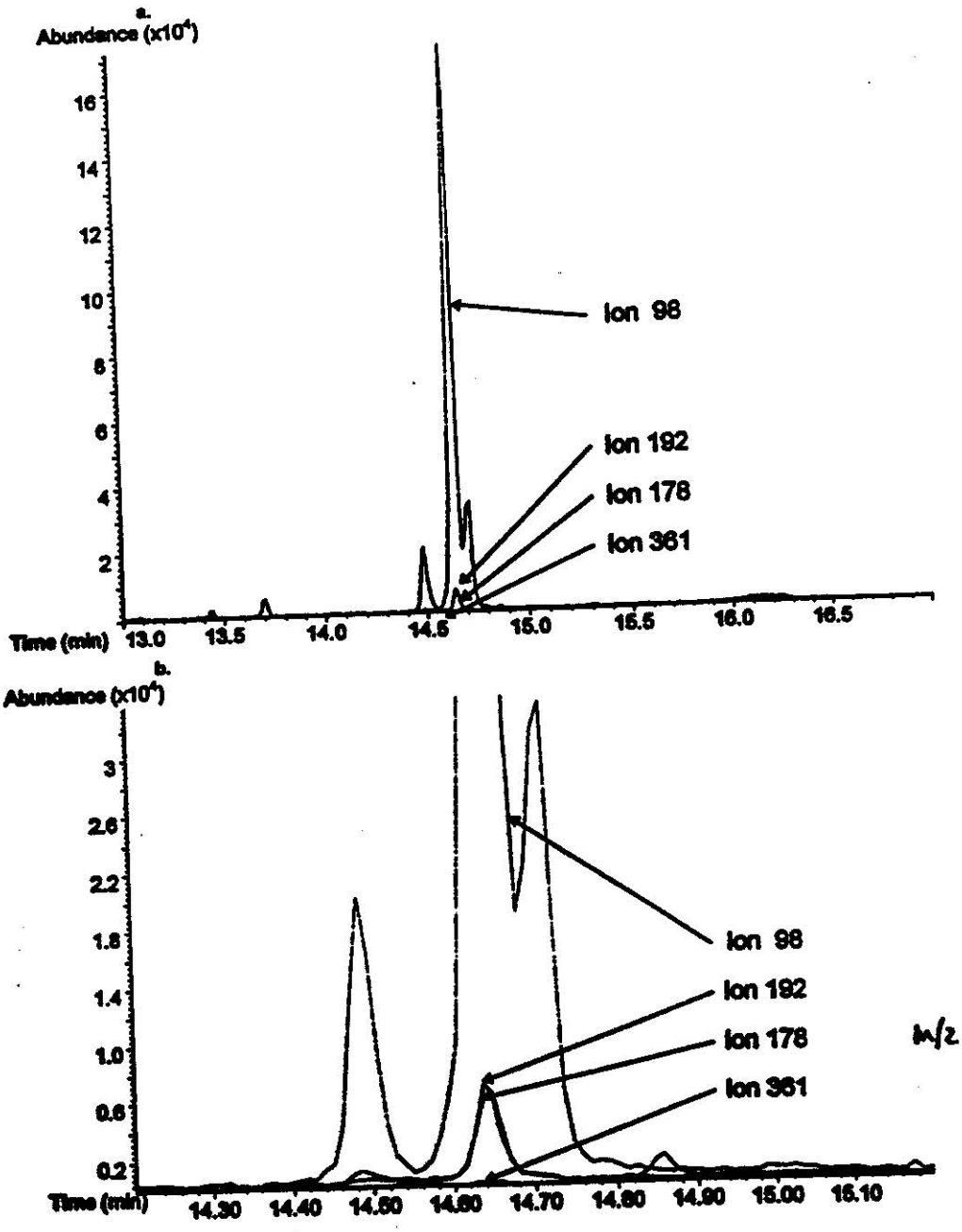

Fig. 10. (a) Ion chromatogram of the same urine sample in Fig. 9(b) for ions 98, 192, 178 and 361 amu at the retention time appropriate for the mono(*t*-butyldimethylsilyl) derivative of authentic 3-hydroxymepivacaine: (b) enlargement of ion chromatogram to show the smaller peaks.

With regard to the quantitation of 3-hydroxymepivacaine, MTBSTFA + 1% TBDMCS derivatises 3-hydroxymepivacaine to yield mono-and bis-derivatives, with the mono-form generally predominating. This contrasts with 3-hydroxylidocaine (Harkins *et al.*, 1997) where the bis-derivative predominates or is the exclusive product. The contrast is best explained by inductive or steric effects introduced by the carbonyl R-group adjacent to the internal amide linkage. The second derivatisation occurs at this amide, as amides are generally much more difficult to silylate than phenolic groups with reaction taking place at either the N or the O atom. O-silyl derivatives are thermodynamically favoured over N-silyl derivatives owing to the greater thermal stability of the Si-O bond in comparison with the Si-N bond (Pierce, 1982). However, N-derivatives may be kinetically favoured, specifically because the iminol form necessary for O-reaction is extremely unfavoured during amido-iminol tautomerism.

Despite the propensity of the amide group to silylate during the derivatisation reaction, this approach is remarkably reliable and reproducible, consistently yielding linear standard curves with *r*-values of greater than 0.99. For this assay, the mean *r* was 0.9995 ± 0.0004 SD, with appropriate between-run and within-run precision.

Reviewing the analytical data and how they relate to the pharmacological data, the results of this study show that the HNED of mepivacaine yielded concentrations of apparent mepivacaine in urine samples that were detectable by ELISA screening and were also detectable/recoverable as 3-hydroxymepivacaine by GC/MS. The simplest interpretation of the data is

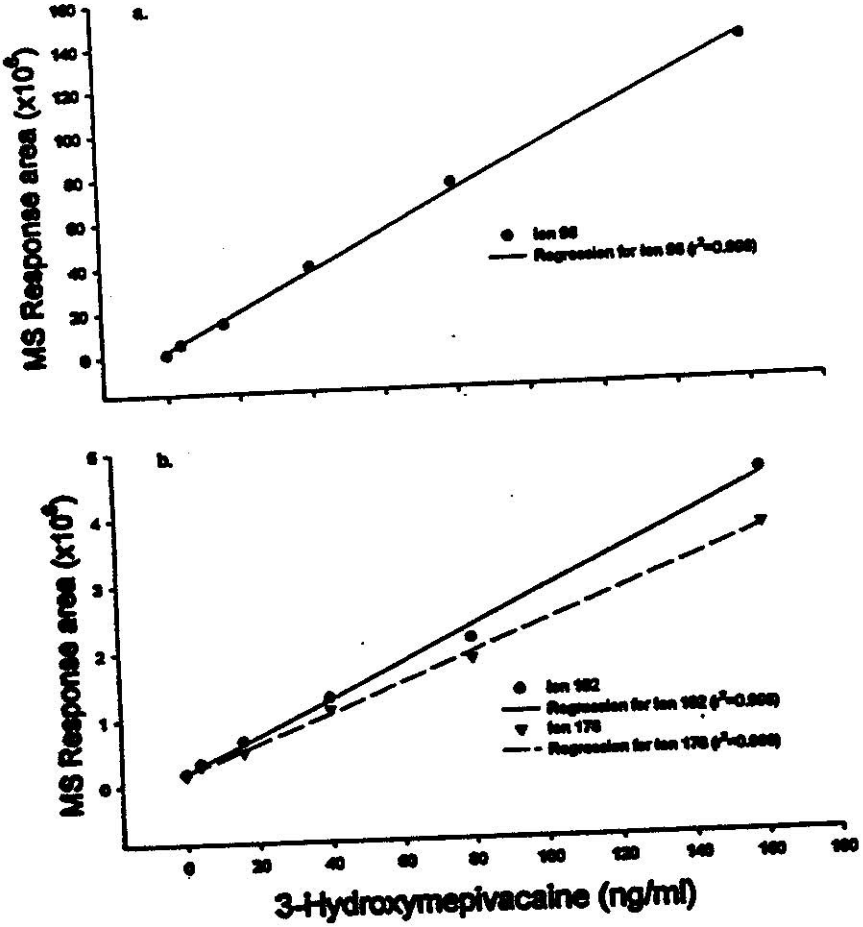

Fig. 11. Standard curves for derivatised 3-hydroxymepivacaine extracted from urine and generated by least squares fit of integrated GC/MS areas for (a) ion 98 and (b) ions 178 and 192

that the detection of a urinary concentration of 3-hydroxymepivacaine of less than ≈ 65 ng/mL is unlikely to be associated with a pharmacological effect.

No parent mepivacaine was detected by mass spectral testing in the urine of any horses treated with the HNED of mepivacaine. Presumably, parent mepivacaine was present in the urine but at concentrations undetectable by this methodology. A critical factor which determines the detectability of parent mepivacaine is the pH of the urine. For example, the concentration of lidocaine in horse urine increases dramatically if pH is reduced (Sams, 1997). Therefore, it would not be unexpected to find mepivacaine at detectable concentrations in equine urine with an acidic pH, which is often the case in post-race urine samples.

For these reasons, the pH of a urine sample is needed to accurately interpret the analytical results. If urinary pH is lowered in association with exercise, then the concentration of mepivacaine in urine can increase dramatically (Sams, 1997). In contrast, the concentration of the glucuronidated metabolite should remain constant as urine pH changes. As the recovered 3-hydroxymepivacaine metabolite is likely to have entered the urine by glomerular filtration and active tubular secretion of the glucuronide conjugate, it should not be reabsorbed from the renal tubule in response to pH changes.

These experiments were performed in untrained horses routinely kept at pasture and stalled in a barn for the duration of the experiment. Figure 12(b) -(d) show that the urine became more concentrated after a few hours in the stalls, probably due to a decrease in water intake. The alkaline urine (pH 8.1–8.4) is consistent with previous experiments. Because mepivacaine is a basic drug and will concentrate in acidic urine, the concentrations of mepivacaine would be expected to be greater in acidic urine. Therefore, any pharmacological conclusions based on these data are likely to be very conservative.

This is because the effect of decreased urinary pH is most likely to be an increase in the urinary concentration of mepivacaine and, possibly, but less likely, an increased concentration of the glucuronide metabolite. As the concentrations of parent mepivacaine are probably higher in acidic urine, urinary values reported in this communication are likely to be the lowest associated with administration of a HNED of mepivacaine.

Finally, furosemide is commonly permitted in American racing. Previous work from our group has shown that furosemide-induced diuresis reduces urinary concentration of water-soluble glucuronide metabolites in parallel with its effect on urine specific gravity (Tobin *et al.*, 1977; Roberts *et al.*, 1978; Tobin *et al.*, 1978). Therefore, one approach to interpreting the recovery of 3-hydroxymepivacaine in dilute urine samples is to correct the concentration value for the specific gravity or creatinine content of the urine sample. Therefore, if a

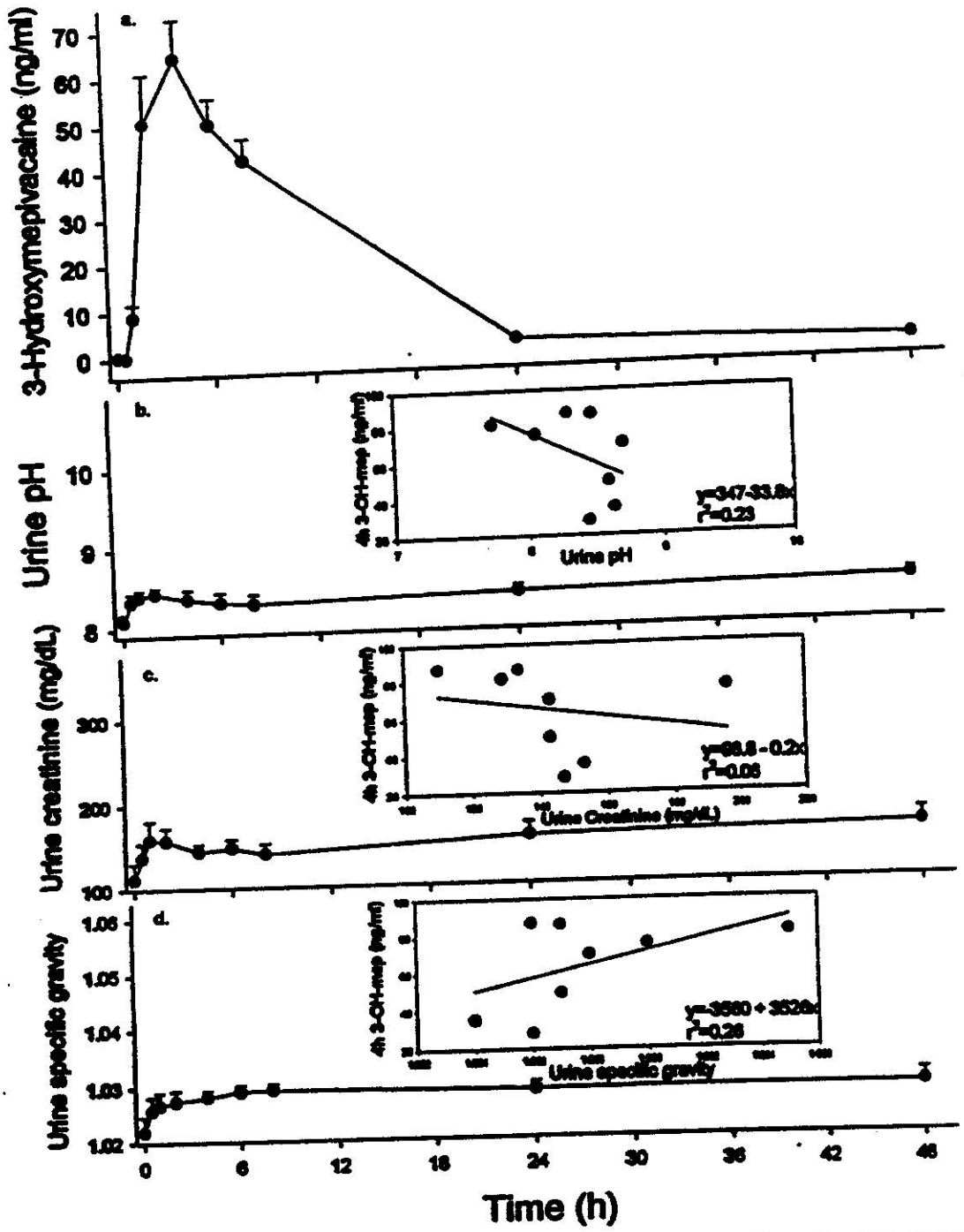

Fig. 12. (a) Mean concentrations ± SE of 3-hydroxymepivacaine recovered from urine of horses ($n = 8$) following administration of a 2-mg dose as measured by GC/MS in selected ion monitoring mode; b-d) mean pH, creatinine and specific gravity of urine samples. Inset graphs show nonsignificant relationships between those variables and peak 3-hydroxymepivacaine (3-OH-mep) recovered at 4 h. The slopes of these regressions were not significantly different from zero ($P < 0.05$).

subthreshold concentration of 3-hydroxymepivacaine is recovered from a dilute urine, particularly one associated with furosemide administration, one approach would be to recalculate the apparent concentration based on urinary dilution, as estimated from the actual creatinine content of the urine sample in question.

ACKNOWLEDGMENTS

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