

Bupivacaine in the horse: relationship of local anaesthetic responses and urinary concentrations of 3-hydroxybupivacaine

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Bupivacaine is a potent local anaesthetic used in equine medicine. It is also classified as a Class 2 foreign substance by the Association of Racing Commissioners International (ARCI). The identification of residues in postrace urine samples may cause regulators to impose significant penalties. Therefore, an analytical/pharmacological database was developed for this medication. The highest no-effect dose (HNED) for the local anaesthetic effect of bupivacaine was determined to be 0.25 mg by using an abaxial sesamoid local anaesthetic model. Administration of the HNED of bupivacaine to eight horses yielded a peak urine concentration of apparent bupivacaine of 23.3 ng/mL 2 h after injection as determined with enzyme-linked immunosorbent assay (ELISA) screening.

The major metabolite recovered from beta-glucuronidase-treated equine urine after dosing with bupivacaine is a hydroxybupivacaine, either 3-hydroxybupivacaine, 4-hydroxybupivacaine, or a mixture of the two. To determine which positional isomer occurs in the horse, 4-hydroxybupivacaine was obtained from Maxxam Analytics, Inc., and 3-hydroxybupivacaine was synthesized, purified, and characterized. Furthermore, a quantitative mass spectrometric method was developed for the metabolite as recovered from horse urine. Following subcutaneous injection of the HNED of bupivacaine, the concentration of the hydroxybupivacaine recovered from horse urine reached a peak of 27.4 ng/mL at 4 h after administration as measured by gas chromatography/mass spectrometry (GC/MS). It was also unequivocally demonstrated with ion chromatography that the hydroxybupivacaine metabolite found in horse urine is exclusively 3-hydroxybupivacaine and not 4-hydroxybupivacaine.

The mean pH of the 4-h urine samples was 7.21; the mean urine creatinine was 209.5 mg/dL; and the mean urine specific gravity was 1.028. There was no apparent effect of pH, urine creatinine concentration, or specific gravity on the concentration of 3-hydroxybupivacaine recovered. The concentration of bupivacaine or its metabolites after administration of a HNED dose are detectable by mass spectrometric techniques. This study also suggests that recovery of concentrations less than ≈ 30 ng/mL of 3-hydroxybupivacaine from postrace urine samples is unlikely to be associated with a recent local anaesthetic effect of bupivacaine.

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INTRODUCTION

Bupivacaine is a potent local anaesthetic (LA), has a potency and toxicity four times that of mepivacaine (Goehl *et al.*, 1973), and is able to provide significant anaesthesia with a dose of only 0.5 mg/site (Harkins *et al.*, 1996). The agent produces conduction

blockade in sensory neurons by retarding the influx of Na^+ ions and is listed as a Class 2 substance by the Association of Racing Commissioners International (ARCI). The identification of bupivacaine in postrace urine samples can result in substantial penalties. Therefore, it is important to determine the dose and time response relationship for the local anaesthesia produced by

this agent and the time periods for which bupivacaine or its metabolites remain detectable in plasma/urine samples after administration of therapeutic and subtherapeutic doses. During a 3.5-year period (January 1990 to June 1993), one 'positive' sample for bupivacaine was reported world-wide in Thoroughbred racing (Dr Robert Gowen, Racing Commissioners International, personal communication).

There is little information about the disposition or pharmacokinetics of bupivacaine in horses. The Agriculture Canada Equine Drug Evaluation Centre reports a 24-h detection time for bupivacaine following a 50-mg intramuscular dose (Agriculture Canada, 1991). However, this report did not specify the analytical methods used, the limits of detection of the tests, or the nature of the materials identified. Furthermore, the report did not relate the concentration of the detected material to the possible pharmacological, therapeutic, or performance effects of bupivacaine. These questions must be answered for veterinarians and pharmacologists to assess the possible performance-altering effects associated with a chemical identification of bupivacaine in postrace samples. No other racing agencies have reported detection times or recommended withdrawal times for bupivacaine. The structures of bupivacaine and its suggested metabolites are shown in Fig. 1.

The objectives of this study were to: (1) determine the highest no-effect dose (HNED; the highest dosage for which there was no effect) of bupivacaine injected as an abaxial sesamoid nerve block; (2) synthesize, purify and characterize the major urinary metabolite of bupivacaine detected by forensic chemists, namely 3-hydroxybupivacaine; (3) unequivocally distinguish between 3- and 4-hydroxybupivacaine as possible urinary metabolites of bupivacaine; (4) validate a sensitive enzyme-linked mepivacaine immunosorbent assay (ELISA) test for bupivacaine in equine serum or urine; (5) develop a sensitive and quantitative GC/MS method for bupivacaine or its major identifiable metabolite in equine serum/urine; and (6) determine the urine concentration of bupivacaine and its metabolites after administration of the

HNED to establish analytical/pharmacological relationships for bupivacaine 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

Ten mature Thoroughbred mares weighing 461–576 kg were used for this study. 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 assure 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 University of Kentucky Institutional Animal Care Use 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 (0.25, 0.5, 1.0 and 2.0 mg) of 0.5% bupivacaine (Abbott Laboratories, Chicago, IL) in a randomized sequence with at least 7 days between doses. The site of injection was the lateral volar nerve where it passes laterally (abaxially) 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 bupivacaine-treated legs. For a positive control, bupivacaine HCl (10 mg) was injected, and for a negative control, saline (2 mL) was injected in a similar manner.

Before each local anaesthetic response (LAR) experiment, the hair on the front and lateral side 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 bupivacaine.

For the LAR experiments, dose and time response relationships for bupivacaine 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 a horse to elicit the classic flexion-withdrawal reflex. Hoof withdrawal reflex latency (HWRL) was

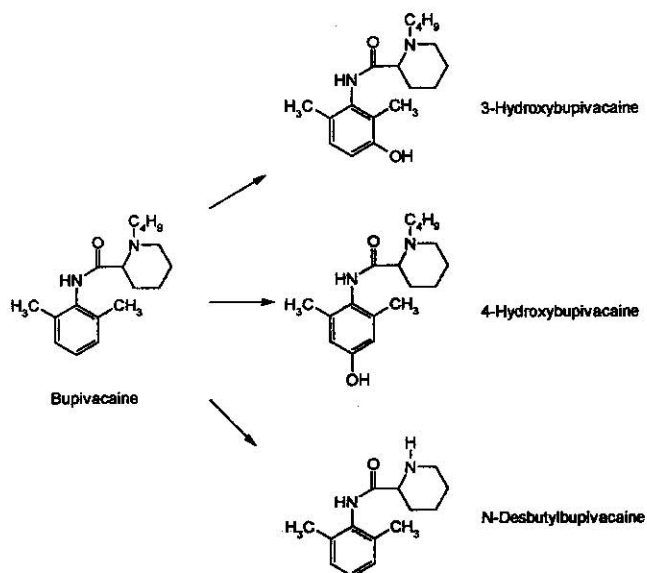


Fig. 1. Structures of bupivacaine and its metabolites.

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 each LA dose. 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, 120, 150 and 180 min after LA administration. The HWRL was expressed as a percentage of baseline values.

ELISA quantification of apparent bupivacaine in urine

The one-step ELISA used for this analysis has been described previously (Harkins *et al.*, 1998). All urine samples were first screened with an ELISA test to determine the concentrations of 'apparent bupivacaine' (i.e. the concentration of bupivacaine that would produce similar results) in the samples. The apparent bupivacaine is presumably composed of bupivacaine, the glucuronide of hydroxybupivacaine, and other metabolites. The assays were started by adding 20 µL of either the standard, test, or control samples to each well. During the test, the presence of bupivacaine or its metabolites in the sample competitively prevented the binding of the HRP (horse radish peroxidase) complex to the antibody. As the HRP enzyme was responsible for the colour-producing reaction in the ELISA, the log of the concentration of bupivacaine and its 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) \approx 30 min after addition of substrate. All assay reactions were run at room temperature (24 °C).

The ELISA method for the semiquantitation of the hydroxybupivacaine was validated by examining the precision and linearity of the assay. The between-run precision ($n = 6$) was determined for middle standard (10 ng/mL) of the standard curve to be 7.9%. The mean regression coefficient of the calibration curve (r) for the assays was 0.9949 ± 0.0027 SD. There was no extraction for the ELISA method, and therefore no recovery was calculated.

Analytical/pharmacological relationships after HNED administration

The HNED for bupivacaine (0.25 mg) was determined from the LA quantification experiment. Multiples of the HNED (2.5, 25 and 250 mg) were injected subcutaneously in four horses. During the first day, complete urine collection was accomplished with a Foley catheter (24 Fr, Rusch Inc, Duluth, GA) and attached plastic bag. Blank samples were collected during the

30-min period before administration. Samples were then collected during the following periods: 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 \times 152 cm; Seamless, Ocala, FL) was used to collect a maximum of 300 mL urine. Urine was placed in aliquots, and stored at –20 °C until assayed.

Synthesis and characterization of 3-hydroxybupivacaine

Figure 2 presents a schema for the synthesis of 3-hydroxybupivacaine from bupivacaine, which was analogous to our previously reported synthesis of 3-hydroxylidocaine (Harkins *et al.*, 1998) and 3-hydroxymepivacaine (Harkins *et al.*, 1999).

Nitration of bupivacaine under classic conditions (mixture of concentrated sulfuric and nitric acid) resulted in the 3-nitro compound, which was reduced with zinc dust in 50% acetic acid to produce 3-aminobupivacaine in high yield. After diazotisation of this amine with sodium nitrite in sulfuric acid and following hydrolysis in acidic conditions at 70 °C, the desired 3-hydroxybupivacaine was obtained, which was purified upon column chromatography on silica gel and crystallized from acetone to yield fine white crystals.

General methods. ^1H -nmr (200 MHz) and ^{13}C -nmr (50 MHz) spectra were recorded on Gemini AC-200a (Varian, Palo Alto, CA). GC/MS analysis was performed on a Model 6890 gas chromatograph equipped with a Model 5972 A mass selective detector (Hewlett Packard, Bloomington, DE). Melting points are uncorrected.

Characterization data for 1-butyl-N-(3-hydroxy-2,6-dimethylphenyl)-2-piperidinecarboxamide (3-hydroxybupivacaine): Mp. 196–197 °C; ^1H -nmr (200 MHz, DMSO- d_6): δ (p.p.m.) 0.87 (t, 3 H, $\text{CH}_2\text{-CH}_3$), 1.18–1.86 (m, 11 H), 1.93 (s, 3 H, CH_3), 2.01 (s, 3 H, CH_3), 2.22 (m, 1 H), 2.50 (m, 1 H), 2.64 (m, 1 H), 2.84 (dd, 1 H, CH, J 6.4, J 2.2 Hz), 3.08 (dt, 1 H, J 7.6, J 2.6 Hz), 6.62 and 6.80 (AB, 2 H, J 5.6 Hz), 9.00 and 9.01 (2 s, 2 H, NH and OH); ^{13}C -nmr (50 MHz, DMSO- d_6): δ (p.p.m.) 10.94, 13.97, 17.64, 20.23, 23.13, 24.90, 28.26, 30.18 (8 C, piperidine ring, butyl chain, ring CH_3), 51.06, 55.86, 67.48 (3 C linked to piperidine N), 112.84, 121.94, 125.21, 126.80, 135.73 (5 C_{AR}), 153.54 ($\text{C}_{\text{AR-OH}}$), 171.75 (C = O). The authentic 4-hydroxybupivacaine standard was generously provided by Maxxam Analytics, Inc. of Mississauga, Ontario.

Determination of site for bupivacaine phenyl ring hydroxylation

A 25-mL urine sample taken from a horse 6 h after being dosed with bupivacaine (60 mg, subcutaneously) was mixed with 5 mL

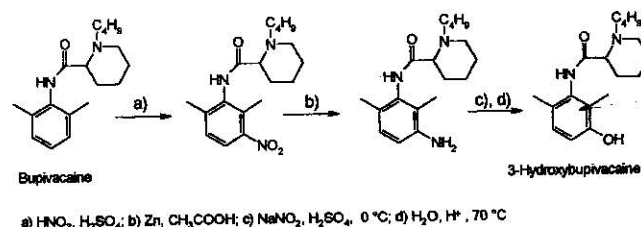


Fig. 2. Synthetic schema showing the sequential steps (a–d) in the conversion of bupivacaine to 3-hydroxybupivacaine.

of beta-glucuronidase (5000 units/mL; type L-II, Sigma, St. Louis, MO) and 10 mL of 1 M sodium acetate buffer (pH = 5.0). The samples were mixed by vortex and incubated at 65 °C for 3 h. After cooling overnight at 4 °C, the samples were sonicated on ice for 90 s, warmed to room temperature (24 °C), adjusted to a pH of 7.0, and mixed with 6 mL of dichloromethane. After vortexing the mixture for 5 min, the aqueous phase was removed and re-extracted with dichloromethane. The organic layers were combined and back extracted four times with 12 mL of deionized water and then centrifuged for 30 min (750 × g, 4 °C) in a swinging bucket rotor (Beckman Instruments, Palo Alto, CA). Aliquots (5 mL) of the organic layer were removed to each of two 5 mL Reacti-vials (Pierce, Rockford, IL) and evaporated under a stream of dry nitrogen gas. The dried extracts were reacted with either 100 µL of BSTFA + 1% TMCS (Pierce) or 100 µL MTBSTFA + 1% *t*-BDMSC (Regis Technologies, Inc., Morton Grove, IL). Following vortexing, the BSTFA reaction was performed at 70 °C for 60 min, while the MTBSTFA reaction was performed at 42 °C for 3 h.

MTBSTFA or BSTFA derivatized samples were run by GC/MS as described below (*Instrumentation*) with the following variations: initial oven temperature was 70 °C (held 2 min) then increased at a rate of 5 °C/min to 280 °C (held 12 min); the MSD was run in full scan mode, with acquisition in the 50–550 *m/z* range. The chromatographic method was found applicable in separating BSTFA derivatives only.

Development of a quantitative analytical method for 3-hydroxybupivacaine

The GC/MS derivatization method was designed according to the following considerations. It was previously determined that 3-hydroxylidocaine formed a single bis-*t*-butyldimethylsilyl (TBS) derivative with the silylating reagent *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA). This and its relative stability to beta-glucuronidase treatment led us to consider it as an internal standard for 3-hydroxybupivacaine quantitation. The following ions were chosen for each compound for selected ion monitoring (SIM), with *first ion* in each case chosen for quantitation purposes: 3-hydroxybupivacaine (retention time 14.6 min): 140.1, 73.0, 178.1, 192.1, 336.3, 403.4, 420.4, 433.4; and 3-hydroxylidocaine (retention time 12.8 min): 86.0, 73.0, 336.3, 421.4. 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 release of 3-hydroxybupivacaine from its glucuronide metabolite, urine samples, standards, and blanks (5 mL/sample) were first placed in culture tubes. To each sample were added 1 mL of beta-glucuronidase reagent (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 were the standard hydrolytic conditions routinely used in equine drug testing laboratories, specifically treatment at 65 °C for 3 h. The hydrolysis procedure was developed in our laboratory, was carefully optimized for the hydrolysis of morphine glucuronide (Combie *et al.*, 1982), and has been adapted to routine equine drug testing by Truesdail Laboratories (Tustin, CA). Researchers should be aware that, should conditions other than those used in this report be selected, glucuronidase hydrolysis of 3-hydroxybupivacaine conjugates could result in different threshold values.

Quantitative standard curve

Standard solutions of 3-hydroxybupivacaine and 3-hydroxylidocaine were prepared in methanol. Measured volumes of the 3-hydroxybupivacaine solutions were added to blank beta-glucuronidase-treated urine samples over a range of 1 ng/mL to 160 ng/mL to generate a standard curve. A constant volume of the 3-hydroxylidocaine standard (40 µL of a 40-µg/mL solution for a total of 1600 ng) was added to each sample, standard, and blank as an internal standard.

Extraction/derivatization procedure

Clean Screen[®] solid phase extraction (SPE) columns (Worldwide Monitoring, Bristow, PA, #ZSDAU020) 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 columns and allowed to pass through the columns at a rate of 1–2 mL/min. The columns were sequentially washed with 2 mL of water, 2 mL of 1 M acetic acid, and 4 mL of methanol. The columns were eluted with 3 mL of dichloromethane:isopropanol:ammonium hydroxide (78:20:2). The eluents were kept slightly warm (< 40 °C) and evaporated to dryness under a stream of N₂. For derivatization, each sample extract was dissolved in 40 µL MTBSTFA, 1% *tert*-butyldimethylchlorosilane (*t*-BDMCS), vortexed for 15 s, and heated at 40 °C for 45 min.

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-5MS, 30 m × 250 µm (i.d.) × 0.25 µm (film thickness) cross-linked 5% phenyl methyl polysiloxane column. The carrier gas was helium with flow of 1 mL/min. Each derivatized sample extract 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: 73.0, 86.0, 140.1, 178.1, 192.1, 336.3, 403.4, 420.4, 421.4, 433.4 m/z, with a 75 msec dwell time.

Quantitation

Standards were prepared, extracted and derivatized in duplicate, and ion areas were obtained by integration. The identity of a peak as 3-hydroxylidocaine or derivatized 3-hydroxybupivacaine

was confirmed by the coelution of ions specific to each compound at the retention times shown by authentic standards. 3-Hydroxybupivacaine areas were calculated as ratios relative to the area of the internal standard, and unknown concentrations were calculated by interpolation of the least squares-regression analysis line. Samples containing a higher concentration than the highest calibrator were diluted and rerun.

The GC/MS method for the quantitation of 3-hydroxybupivacaine was validated by examining the precision (% coefficient of variance, CV), linearity (coefficient of regression, r) and recovery of the assay. The within-run precision was determined

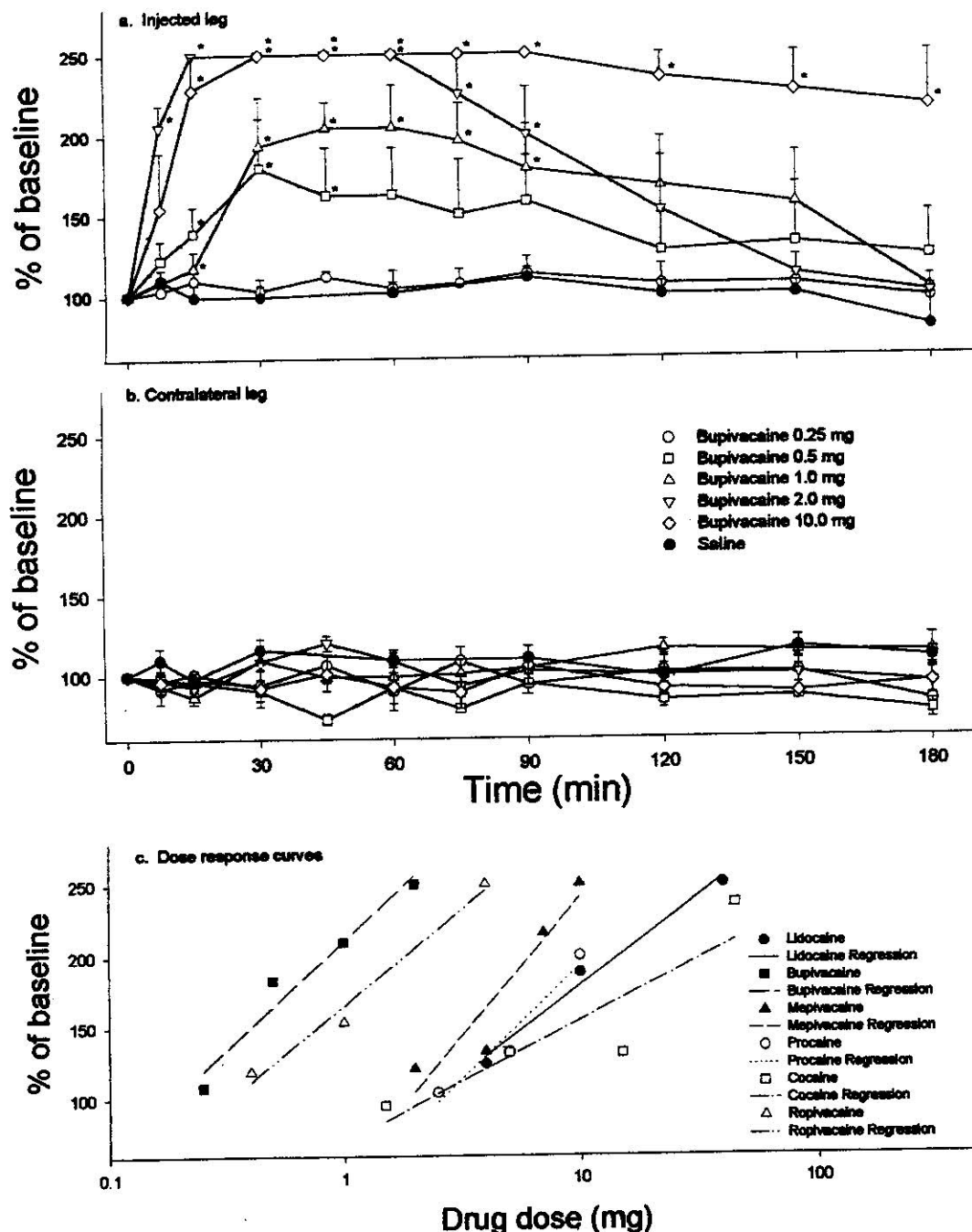


Fig. 3. (a) Mean \pm SE percentage increase in HWRL after injection of bupivacaine doses; (b) mean \pm SE percentage change in contralateral leg after saline injection; (c) dose response curves for bupivacaine, lidocaine, mepivacaine, procaine, cocaine and ropivacaine.

for the low (4 ng/mL: CV = 7.8%), middle (40 ng/mL: CV = 7.2%), and high (160 ng/mL: CV = 10.3%) concentrations of the 3-hydroxybupivacaine standard curve, with a mean CV of 8.4%. The between-run precision was determined for the low (4 ng/mL: CV = 9.3%), middle (40 ng/mL: CV = 6.8%), and high (160 ng/mL: CV = 1.2%) concentrations of the 3-hydroxybupivacaine standard curve, with a mean CV of 5.8%. The mean r for the assays was 0.9996 ± 0.0005 SD. The recovery was determined at two concentrations: 2 ng on column = 131.6% recovery, and 10 ng on column = 139.4% recovery. The mean recovery was $135.5\% \pm 5.5$ SD. We suggest that the high recovery values were due to relatively greater loss of 3-hydroxybupivacaine during the evaporation of the solvent. The 3-hydroxybupivacaine in the eluents of the extracted urine samples may have been protected during evaporation by extracted urine residue.

Stability of 3-hydroxybupivacaine

To assess the stability of the synthesized metabolite in horse urine under hydrolysis conditions, a stock solution of 3-hydroxybupivacaine in methanol (1 mg/mL) was prepared, and a suitable aliquot of stock solution was added to blank urine at a concentration of 80 ng/mL. Aliquots of urine to which the analyte was added were incubated at 65 °C under hydrolysis conditions for increasing durations (0–6 h). An internal standard (3-hydroxylidocaine) was added to each sample after incubation. The samples were then extracted, and the eluent was evaporated and derivatized according to methods described above. The derivatized samples were analysed by the GC/MS SIM method described previously. This provided quantitative information in the form of integrated peaks. The quantity of 3-hydroxybupivacaine in the incubated aliquots was reported as a percentage of the 3-hydroxybupivacaine solution in the pre-incubation (0 h) sample.

Statistical analysis

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

RESULTS

Dose and time response curves

The dose and time response curves of the LA effect of bupivacaine are presented in Fig. 3(a). There was a significant LA effect 7.5 min after administration of the 2 mg dose, and there was a significant LA effect beginning at 15 min after administration of the 0.5 and 1 mg doses. Significant local anaesthesia persisted for 45 min for the 0.5 mg dose and 90 min for the 1 and 2 mg doses. 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 0.25 mg bupivacaine HCl; based on review of the data, 0.25 mg was established as the HNED for bupivacaine in this model.

There was no LA effect in the contralateral leg (Fig. 3b). The dose response curve for the peak LA response to bupivacaine is presented in Fig. 3(c). Dose response curves for other local anaesthetic medications (lidocaine, procaine, ropivacaine, mepivacaine, cocaine) previously presented (Harkins *et al.*, 1996; Harkins *et al.* 1998, 1999) are included for reference.

Validation of the ELISA test

Standard curves for the ELISA test indicated that addition of 19.95 ng of bupivacaine/mL to the system produced 50% inhibition of the maximum ELISA response (I_{50} ; Fig. 4). Higher concentrations of bupivacaine increased the inhibition in a sigmoidal manner, with essentially complete inhibition of the ELISA test occurring at 100 ng of bupivacaine/mL. Additionally, the I_{50} s for mepivacaine, 3-hydroxybupivacaine, and 4-hydroxybupivacaine were 15.14, 138.0, and 467.7 ng/mL, respectively.

ELISA quantification of apparent bupivacaine in urine

For subcutaneous doses of 250 mg ($n = 2$), 60 mg ($n = 1$), 25 mg ($n = 2$), 20 mg ($n = 1$), 6.75 mg ($n = 1$), 2.5 mg ($n = 2$), 2.25 mg ($n = 1$), 0.75 mg ($n = 1$) and 0.25 mg ($n = 13$), the concentrations of apparent bupivacaine in urine reached

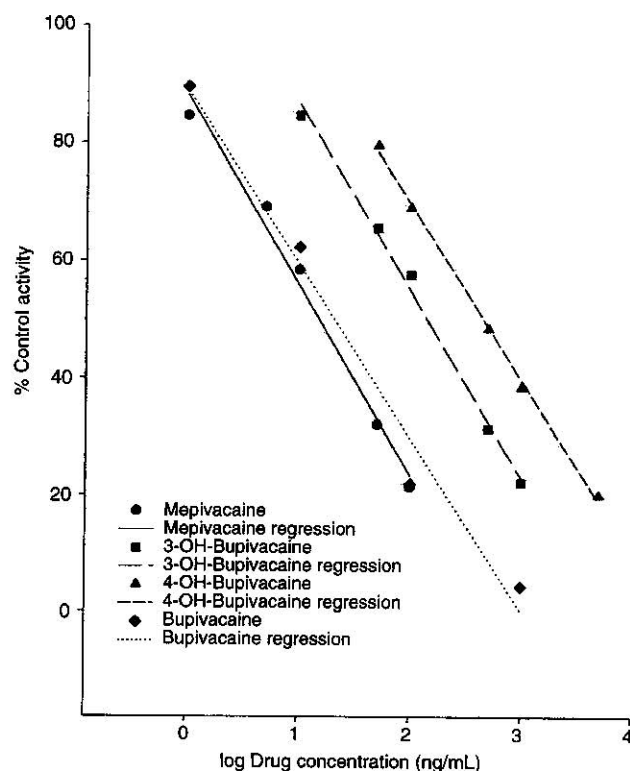


Fig. 4. Regression of ELISA standard curves for bupivacaine, 3-hydroxybupivacaine, 4-hydroxybupivacaine and mepivacaine in a mepivacaine ELISA test.

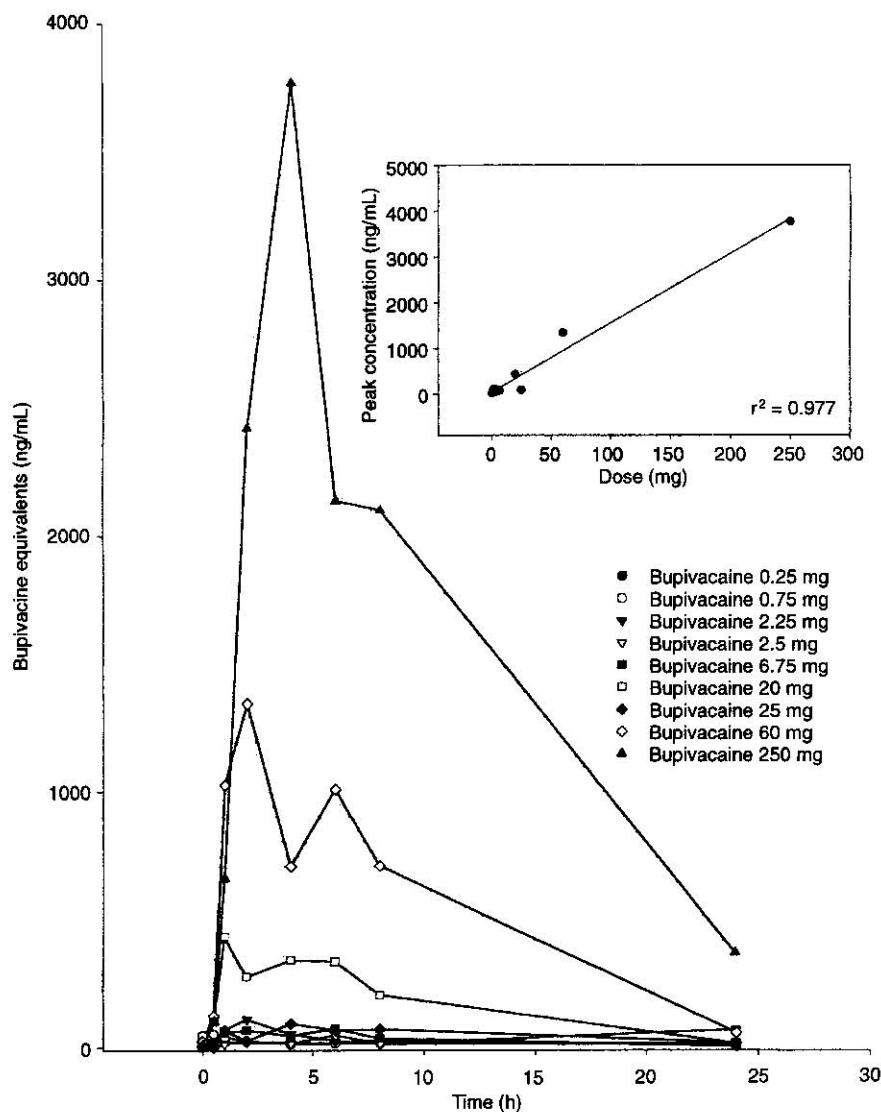


Fig. 5. ELISA measurement of apparent bupivacaine in urine of horses treated with incremental doses of bupivacaine. Inset graph shows the regression of bupivacaine dose and peak concentration.

peaks of 3768, 1343, 94, 432, 73, 48, 110, 51 and 24 ng/mL, respectively (Fig. 5). The peak 'apparent bupivacaine' concentrations were directly related to the dose administered (dose = $15.2 \times$ peak concentration + 41.1; $r^2 = 0.977$; Fig. 5, inset).

Figure 6 shows the percentage of maximum optical density of the ELISA tests following the incremental doses of bupivacaine. The dotted line represents 50% inhibition of the optical density. The inset graph shows the relationship between the bupivacaine dose on a log scale and the time of last detection (dose = $21.6 \times$ detection time - 26.3; $r^2 = 0.995$) when the criterion for detection was set at 50% inhibition of the bupivacaine ELISA test.

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

Figure 7(a) shows the total ion chromatogram for the TMS-derivatized dichloromethane extractable substances obtained from urine 4 h after treatment of a horse with 0.25 mg bupivacaine. The 38.4 min peak represents the peak for the

3-hydroxybupivacaine in urine collected 4 h after bupivacaine (0.25 mg) treatment. Figure 7(b) is an ion chromatogram for 140 m/z for TMS-derivatized dichloromethane extractable substances from the same urine sample shown in Fig. 7(a). Figure 7(c) shows the electron impact (EI)-mass spectrum of the 38.4 min peak derived from the chromatogram of the extract isolated from the urine sample analysed in Fig. 7(b).

Figure 8(a) shows the EI-mass spectrum of the mono-TMS derivative of the authentic 3-hydroxybupivacaine occurring at a retention time of 38.42 min (from Fig. 9a). The inset figure shows the structure of mono(TMS) derivative of 3-hydroxybupivacaine. Figure 8(b) shows the EI-mass spectrum of the mono-TMS derivative of the authentic 4-hydroxybupivacaine occurring at a retention time of 38.65 min (from Fig. 9a). The inset figure shows the structure of mono(TMS) derivative of 4-hydroxybupivacaine.

Figure 9(a) shows the total ion chromatogram (TIC) of one dosed-urine sample for the separation of 3- and 4-hydroxybupivacaine mono-TMS. Figure 9(b) is the ion chromatogram corresponding to the TIC in Fig. 9(a) that shows the differing

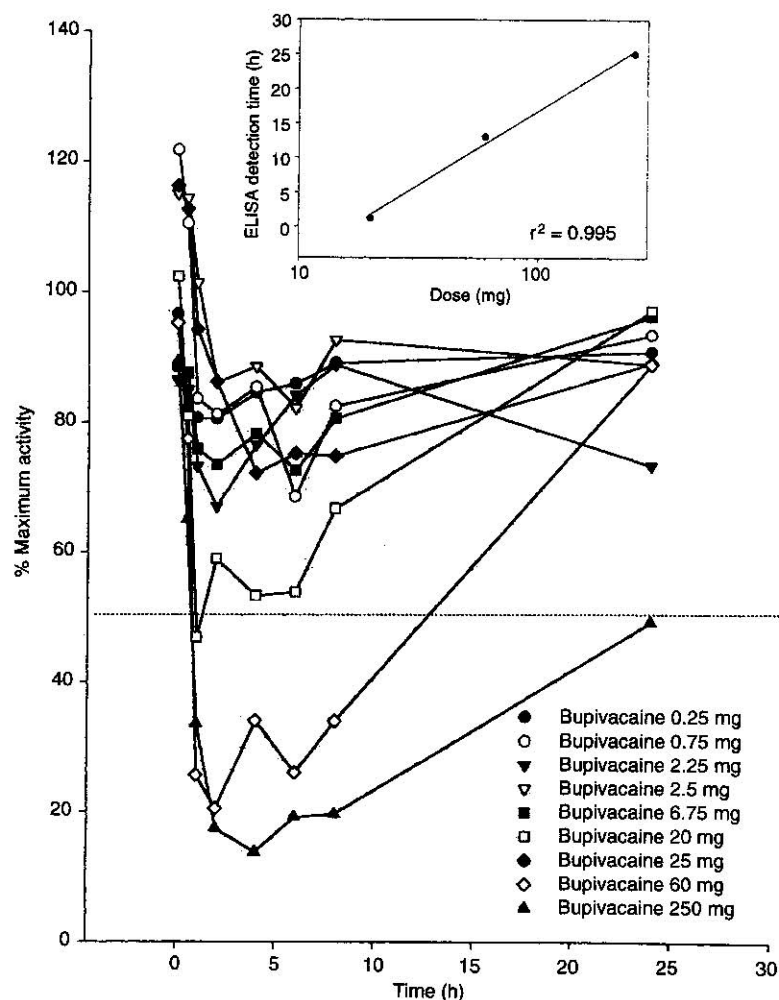


Fig. 6. Percent of maximum optical density of ELISA tests of urine from horses treated with incremental doses of bupivacaine. The dotted line is drawn at 50% of maximum optical density. Inset graph shows the regression of log drug against detection time.

ratios of ions 178, 192, 193 and 208 for 3- and 4-hydroxybupivacaine. Table 1 is a comparison of retention times for 3- and 4-hydroxybupivacaine mono-TMS derivatives from six horses dosed with 0.25 mg of bupivacaine.

Figure 10(a) is the ion chromatogram for ions 178, 192, 193 and 208 from urine of a horse treated with 0.25 mg of bupivacaine. Figure 10(b) is an enlarged segment of Fig. 10(a) showing the 38.4 min peak of these ions. This segment is placed alongside ion chromatograms for the 3-hydroxybupivacaine standard (Fig. 10c) and the 4-hydroxybupivacaine standard (Fig. 10d). Note the proportion similarity of the four ions in the urine from the treated horse (Fig. 10b) and the 3-hydroxybupivacaine standard (Fig. 10c). Note also that the ions for the 4-hydroxybupivacaine standard peak later (38.65 min; compare to Fig. 9a) and are not in the same proportion as in the urine sample from the treated horse (Fig. 10b). Therefore, it was concluded that 3-hydroxybupivacaine is the major metabolite of bupivacaine in the horse.

Stability of 3-hydroxybupivacaine

Figure 11 shows the stability of the 3-hydroxybupivacaine metabolite in horse urine at 65 °C as measured by GC/MS.

GC/MS quantitation of 3-hydroxybupivacaine recovered from urine

As shown in Fig. 12, ion 98 integrated across mono- and bis-TBS derivatives and calculated relative to the internal standard provided a standard curve suitable for interpolation of unknowns. As indicated in the Methods, this approach to quantitation of bupivacaine consistently yielded linear standard curves with r^2 values greater than 0.99.

Figure 13 shows the concentrations of 3-hydroxybupivacaine recovered from the urine of eight horses dosed with the HNED (0.25 mg/site) of bupivacaine. The concentration of recovered 3-hydroxybupivacaine rose rapidly after administration and reached a peak of 27.4 ng/mL 4 h after dosing. Thereafter, it declined to less than 4 ng/mL by 24 h after dosing. Figure 13(b)–(d) show that the pH of the urine varied between 7.1 and 8.3 (which is typical of unexercised horses at pasture), the urine creatinine varied between 116 and 274 mg/dL, and the urine specific gravity varied between 1.012 and 1.030 (normal values = 1.010–1.050). The inset graphs show there were no significant relationships between the concentration of recovered 3-hydroxybupivacaine at 4 h and urine pH, urine creatinine and urine specific gravity.

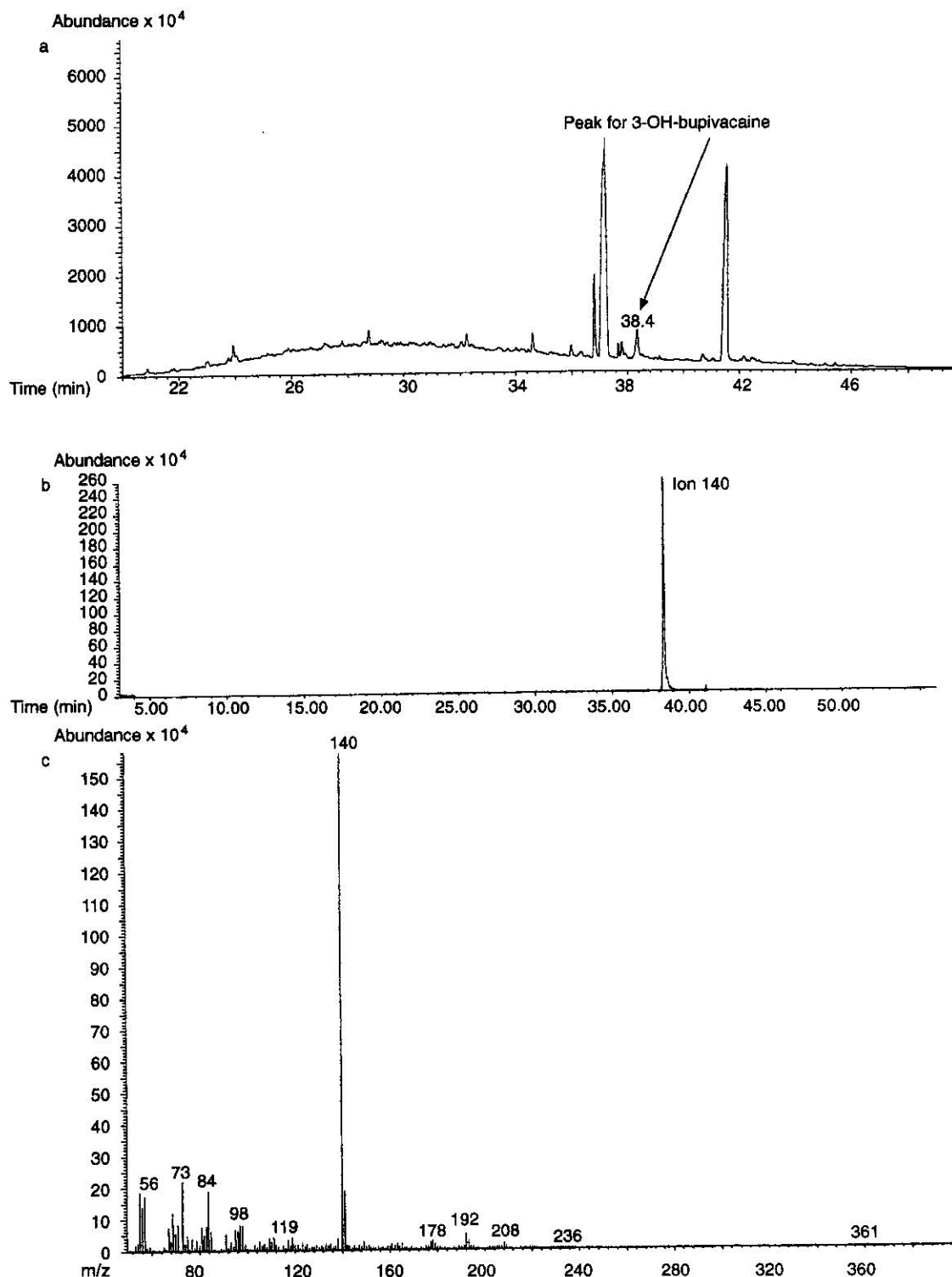


Fig. 7. (a) Total ion chromatograph for the TMS-derivatized dichloromethane extractable substances obtained from urine collected 4 h after treatment with the HNED for bupivacaine; (b) ion chromatogram for 140 m/z for TMS-derivatized dichloromethane extractable substances from urine collected 4 h after bupivacaine treatment; (c) EI-mass spectrum of the principal 140 m/z peak in Fig. 7(b), averaged across the peak (retention time at 38.4 min).

DISCUSSION

The goal of this research was to create a database to assist veterinarians, pharmacologists, and toxicologists in assessing

the forensic significance of chemical residues of bupivacaine in postrace urine samples. As it is a potent local anaesthetic, the identification of bupivacaine residues in a postrace sample opens the possibility that the medication may have been

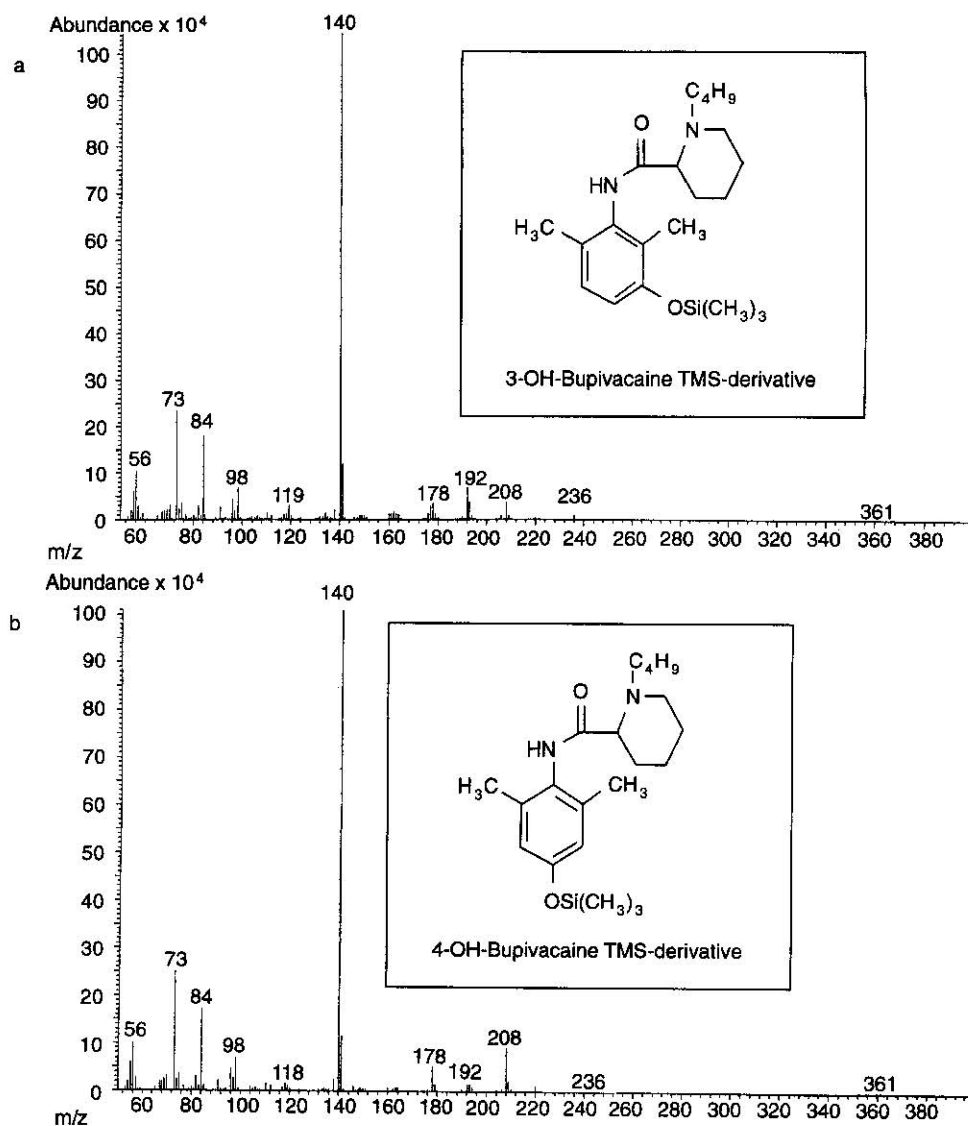


Fig. 8. (a) EI-mass spectrum of 3-hydroxybupivacaine mono-TMS derivative, averaged across the TIC peak. Inset figure shows structure of mono(TMS) derivative of 3-hydroxybupivacaine; (b) EI-mass spectrum of 4-hydroxybupivacaine mono-TMS derivative, averaged across the TIC peak (run separately from 3-hydroxybupivacaine). Inset figure shows structure of mono(TMS) derivative of 4-hydroxybupivacaine.

administered close to race time, creating a local anaesthetic effect which could influence performance of a horse during a race (Tobin, 1981).

Local anaesthetics cause CNS stimulation at relatively high doses (Tobin *et al.*, 1976), and the no-effect concentration of bupivacaine for this effect has not been studied. However, the primary pharmacological effect of bupivacaine when administered at the smaller doses used in this paper is local anaesthesia and is essentially the only effect likely to affect performance. Therefore, local anaesthesia is the pharmacological effect of interest to racing regulators.

Figure 3 quantifies the local anaesthetic effect of bupivacaine using the heat lamp/abaxial sesamoid block model and shows that the HNED of bupivacaine is about 0.25 mg. Therefore, the next step was to determine the urinary concentration of bupivacaine or its metabolites after administration of this dose. It should be noted that a stimulatory source other than heat or a different injection site may lead to a different no-effect dose.

Screening of urine from performance horses for potent medications like bupivacaine is largely dependent on ELISA testing. A previously described mepivacaine ELISA test (Harkins *et al.*, 1998, 1999) was used, and Fig. 4 shows the reactivity of the ELISA test with mepivacaine, parent bupivacaine, 4-hydroxybupivacaine, and 3-hydroxybupivacaine, the principal urinary metabolite of bupivacaine in the horse. This ELISA test is much more sensitive for bupivacaine ($I_{50} = 11.38$ ng/mL), mepivacaine ($I_{50} = 13.29$ ng/mL), and 3-hydroxybupivacaine ($I_{50} = 131.5$ ng/mL) than for 4-hydroxybupivacaine ($I_{50} > 1000$ ng/mL), which is not found in equine urine. With appropriate dilution of postrace samples to reduce endogenous background, this screening test was able to detect the concentration of apparent bupivacaine in postrace urine samples of 10–100 ng/mL. Figure 5 shows that the incremental doses of bupivacaine became increasingly detectable by the ELISA test. Additionally, the inset graph of Fig. 5 shows there is an approximately linear relationship between the dose of bupivacaine administered and the peak concentration of apparent bupivacaine recovered in urine.

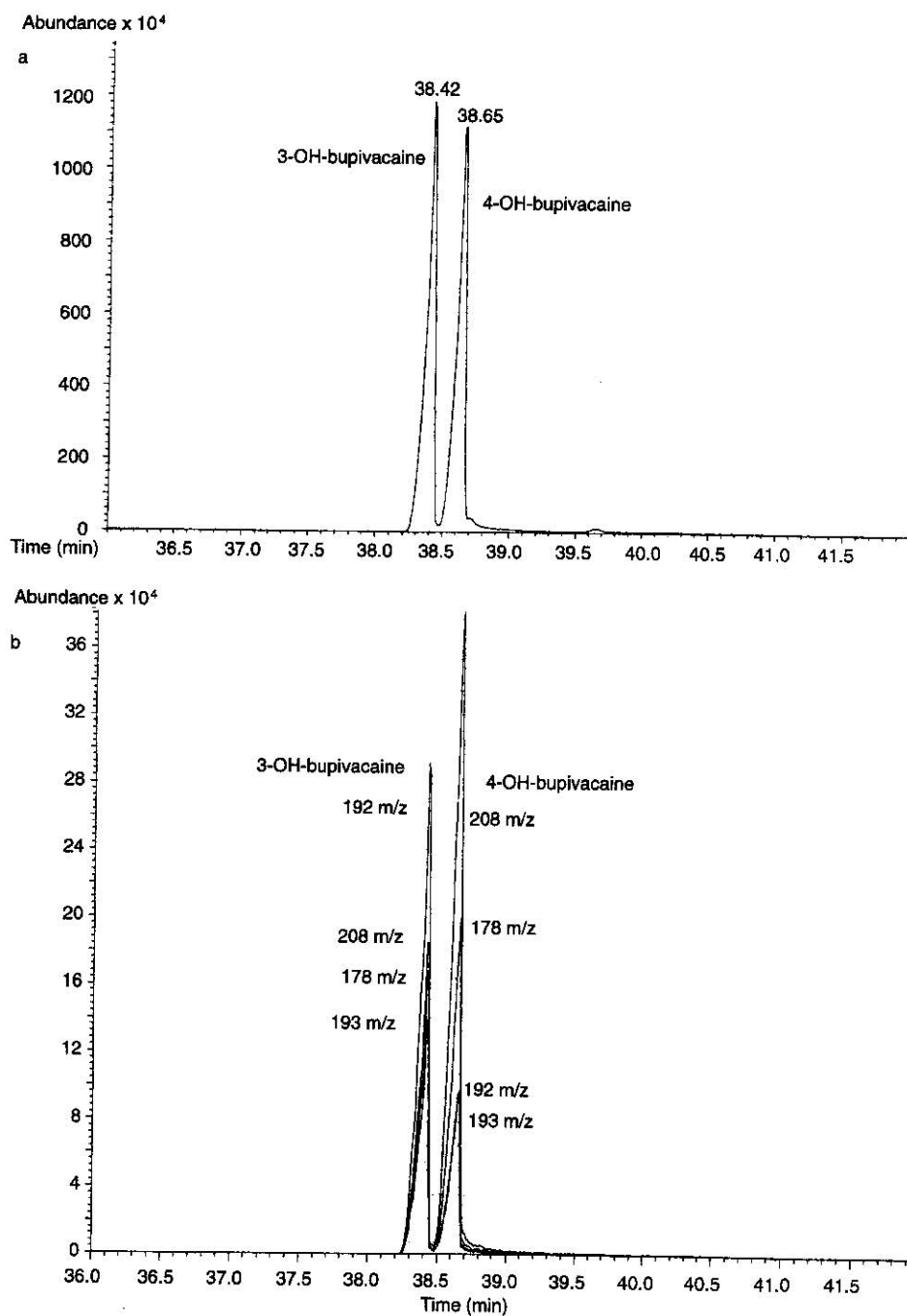


Fig. 9. (a) Total ion chromatograph for the separation of 3- and 4-hydroxybupivacaine mono-TMS derivatives by slow increase in the GC oven temperature; (b) ion chromatogram showing the differing ratios of 178, 192, 193, and 208 *m/z* molecular species for 3- and 4-hydroxybupivacaine (also seen later in Fig. 10).

Table 1. Comparison of retention times for 3- and 4-hydroxybupivacaine mono-TMS derivatives with urine from horses dosed with 0.25 mg bupivacaine

Compound	Mean retention time (min)	Standard Deviation
Bupivacaine-dosed urine samples (<i>n</i> = 6)	38.36 ^a	0.055
3-Hydroxybupivacaine (<i>n</i> = 6)	38.37 ^a	0.024
4-Hydroxybupivacaine (<i>n</i> = 6)	38.60 ^b	0.028

Mean retention times with different superscripts are significantly different (*P* < 0.05).

The relationship between dose and peak concentration of apparent bupivacaine in postrace 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 the logarithm of the bupivacaine dose and the time for which the residue of the medication can be detected (Fig. 6, inset).

The apparent bupivacaine detected by the ELISA test is presumably composed of bupivacaine and structurally related metabolites of bupivacaine excreted in urine. Bupivacaine itself has not been found in significant concentrations in these samples

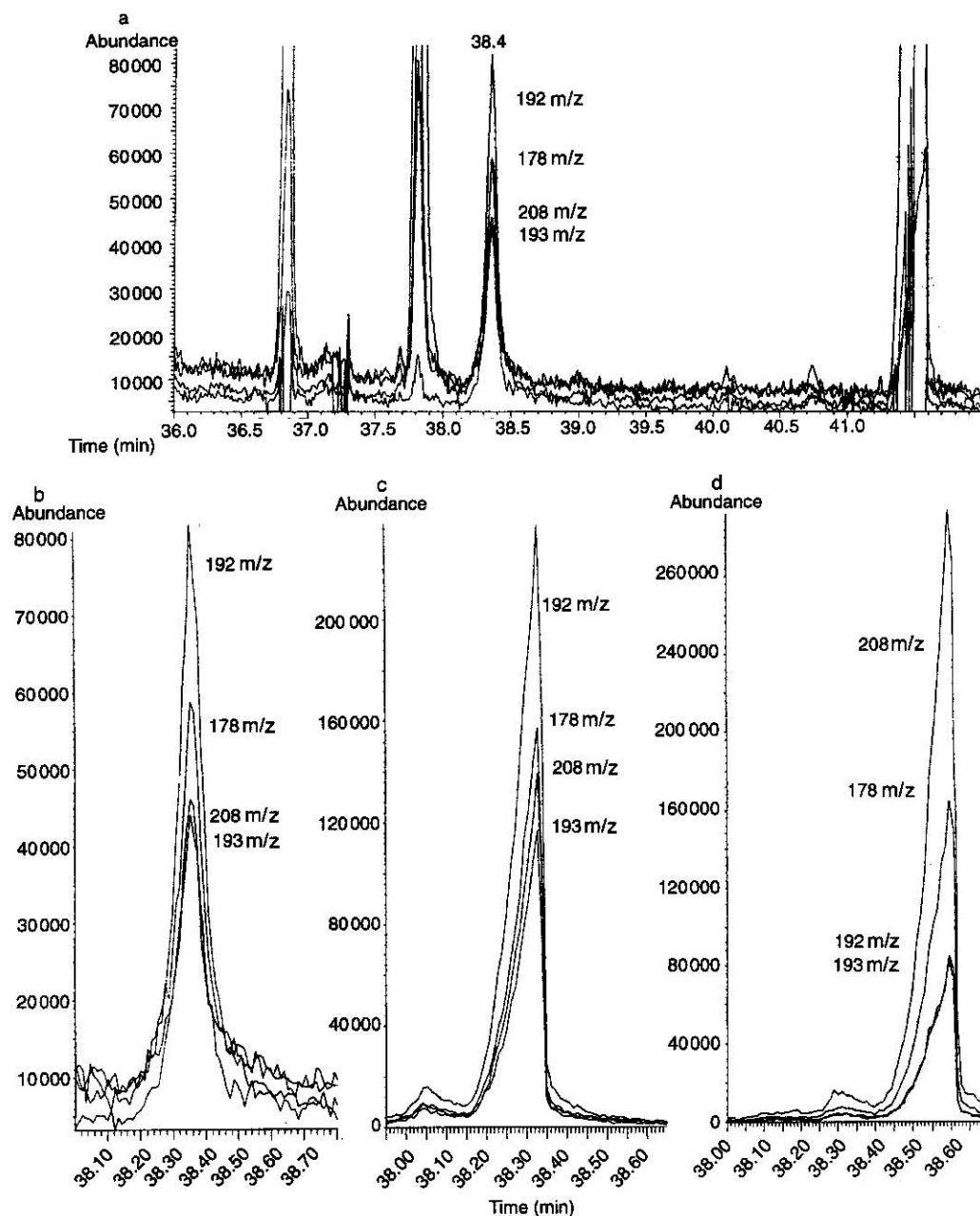


Fig. 10. GC/MS of TMS-derivatized samples: (a) Ion chromatogram for 178, 192, 193, and 208 m/z molecular species from urine of horse treated with bupivacaine (0.25 mg); (b-d) Side-by-side comparison of 178, 192, 193, and 208 m/z ion chromatograms for: (b) urine from horse treated with bupivacaine [enlarged segment of Fig. 10(a)]; (c) 3-hydroxybupivacaine standard; and (d) 4-hydroxybupivacaine standard.

and is not commonly found in postrace urine samples. Therefore, 3-hydroxybupivacaine was selected as the important metabolite detected in glucuronidase-treated postrace samples. Because authentic 3-hydroxybupivacaine was not available commercially, the metabolite was synthesized and chemically characterized. However, for 3-hydroxybupivacaine to be useful in forensic analyses, the metabolite must be stable. Figure 11 shows that the synthesized metabolite is stable at 65 °C, the temperature at which beta-glucuronidase hydrolysis occurs and also the maximum temperature to which the synthesized compound is

exposed. Therefore, it has the potential to be a useful authentic standard in equine forensic work.

The synthesis of authentic 3-hydroxybupivacaine allows for improvement in the qualitative chemistry of 3-hydroxybupivacaine 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-hydroxybupivacaine isolated from horse urine. Figure 8(a) shows the first authentic mass spectra for 3-hydroxybupivacaine and its derivatives from the analytically pure substance.

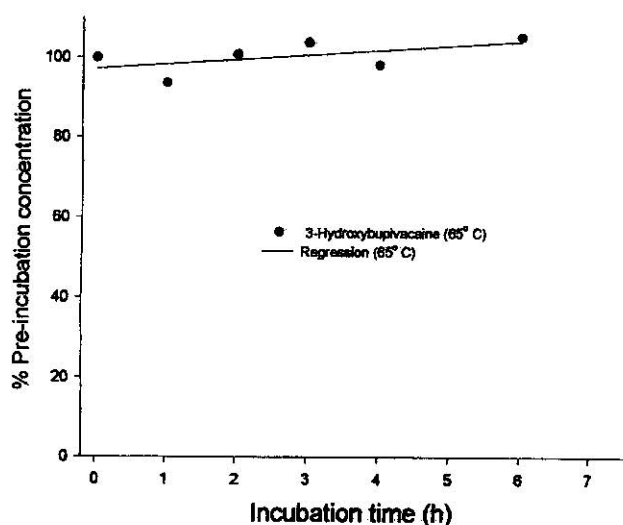


Fig. 11. Regression line showing the stability of 3-hydroxybupivacaine in horse urine at 65 °C as measured by GC/MS.

Table 1 reinforces the message from Figs 10 and 11, namely that the BSTFA-derivatized bupivacaine metabolite seen in horse urine is 3-hydroxybupivacaine rather than 4-hydroxybupivacaine. The table shows that the mean retention time for 3-hydroxybupivacaine was not significantly different from the mean retention time for bupivacaine-dosed urine. MTBSTFA provided no advantage in distinguishing 3- and 4-hydroxybupivacaine chromatographically.

With regard to the quantitation of 3-hydroxybupivacaine, MTBSTFA + 1% TBDMCS derivatizes 3-hydroxymepivacaine to yield mono- and bis-derivatives, with the mono-form generally predominating. This contrasts with 3-hydroxylidocaine (Harkins *et al.*, 1998) 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 derivatization occurs at this amide, as amides are generally much more difficult to silylate than phenolic groups (Pierce, 1982) 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 derivatization 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.

After review of the analytical data and their relationship to the pharmacological data, the results of this study show that the HNED of bupivacaine yielded concentrations of apparent bupivacaine in urine samples that were detectable by ELISA screening and were also detectable/recoverable as 3-hydroxy-

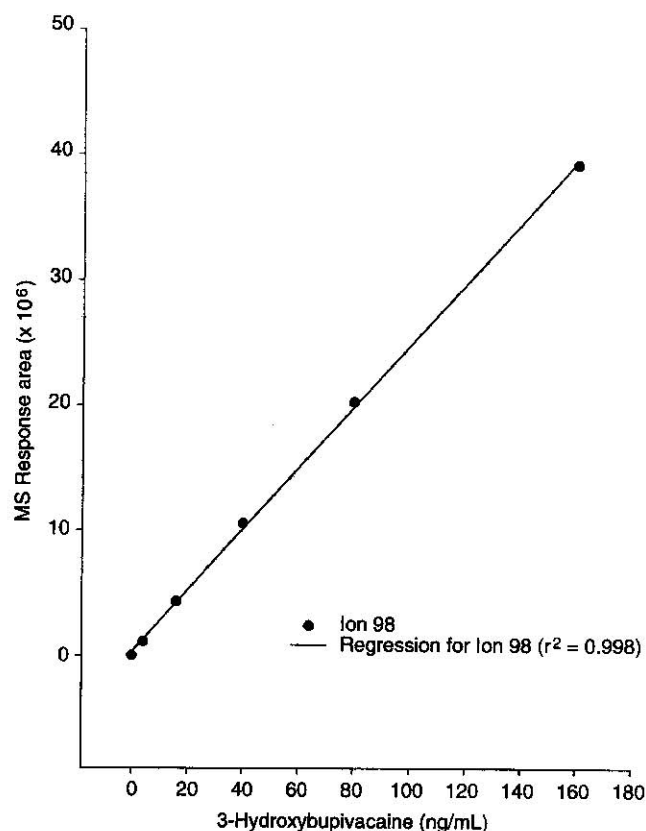


Fig. 12. Standard curve for derivatized 3-hydroxybupivacaine extracted from urine and generated by least squares fit of integrated GC/MS area of ion 140.

bupivacaine by GC/MS. The simplest interpretation of the data is that the detection of a urinary concentration of 3-hydroxybupivacaine of less than ≈ 30 ng/mL is unlikely to be associated with a pharmacological effect.

No parent bupivacaine was detected in the urine of any horses treated with the HNED of bupivacaine. Presumably, parent bupivacaine was present in the urine in trace quantities but at concentrations undetectable by this methodology. A critical factor which determines the detectability of parent bupivacaine is the pH of the urine. For example, the concentration of parent lidocaine in horse urine increases dramatically if pH is reduced (Sams, 1997). Therefore, it would not be unexpected to find parent bupivacaine at detectable concentrations in equine urine with an acidic pH, which is often the case in postrace 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 parent bupivacaine in urine can presumably increase dramatically (Sams, 1997). In contrast, the concentration of the glucuronidated metabolite is likely to be independent of pH. As the recovered 3-hydroxybupivacaine metabolite likely entered the urine by glomerular filtration and exists in the urine as a glucuronide conjugate, it should not move across 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

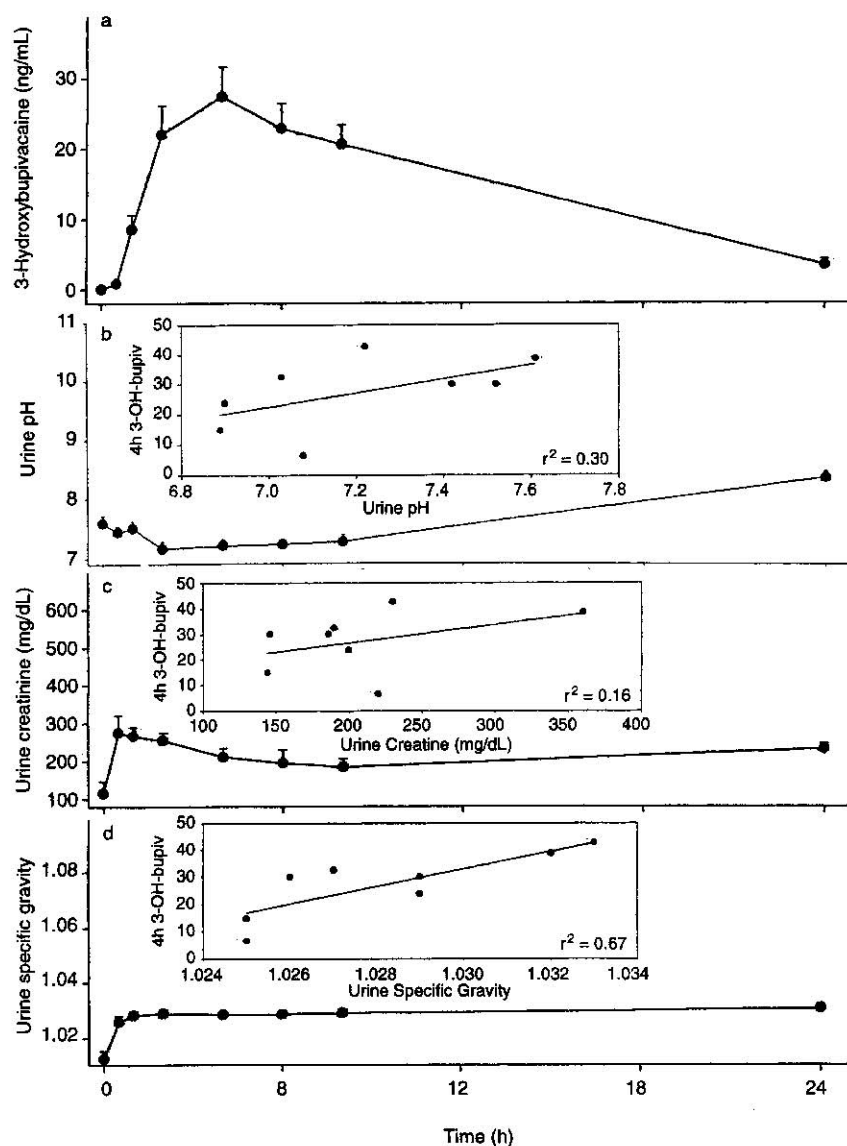


Fig. 13. Mean concentrations \pm SE of 3-hydroxybupivacaine recovered from urine of 8 horses after administration of the HNED (0.25 mg, subQ) of bupivacaine as measured by GC/MS in selected ion monitoring mode; (b) mean pH of urine; (c) mean creatinine; (d) mean urine specific gravity. The inset graphs show the nonsignificant relationships between peak 3-hydroxybupivacaine recovered at 4 h (4 h 3-OH-bupiv) and urine pH, urine creatinine, and urine specific gravity, respectively.

of the experiment. Figure 13(d) shows that the urine became more concentrated after a few hours in the stalls, probably because of a decrease in water intake. The alkaline urine (pH 7.2–8.4) is consistent with our previous experience. Because bupivacaine is a basic drug, parent or unchanged bupivacaine would tend to concentrate in acidic urine. As the concentrations of bupivacaine would be expected to be greater in acidic urine, any urinary thresholds based on this data are likely to be very conservative.

Finally, furosemide is commonly administered as a prophylactic medication 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-hydroxybupivacaine in dilute urine samples is to correct the concentration value for the specific gravity or creatinine content of the urine sample.

ACKNOWLEDGMENTS

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