

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

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Lidocaine is a local anaesthetic agent that is widely used in equine medicine. It is also an Association of Racing Commissioners International (ARCI) Class 2 foreign substance that may cause regulators to impose substantial penalties if residues are identified in post race urine samples. Therefore, an analytical/pharmacological database was developed for this drug. Using our abaxial sesamoid local anaesthetic model, the highest no-effect dose (HNED) for the local anaesthetic effect of lidocaine was determined to be 4 mg. Using enzyme-linked immunosorbent assay (ELISA) screening, administration of the HNED of lidocaine to eight horses yielded peak serum and urine concentrations of apparent lidocaine of 0.84 ng/mL at 30 min and 72.8 ng/mL at 60 min after injection, respectively. These concentrations of apparent lidocaine are readily detectable by routine ELISA screening tests (LIDOCAINE ELISA, Neogen, Lexington, KY).

ELISA screening does not specifically identify lidocaine or its metabolites, which include 3-hydroxylidocaine, dimethylaniline, 4-hydroxydimethylaniline, monoethylglycinexylidene, 3-hydroxymonoethylglycinexylidene, and glycinexylidene. As 3-hydroxylidocaine is the major metabolite recovered from equine urine, it was synthesized, purified and characterized, and a quantitative mass spectrometric method was developed for 3-hydroxylidocaine as recovered from horse urine. Following subcutaneous (s.c.) injection of the HNED of lidocaine, the concentration of 3-hydroxylidocaine recovered from urine reached a peak of about 315 ng/mL at 1 h after administration.

The mean pH of the 1 h post dosing urine samples was 7.7, and there was no apparent effect of pH on the amount of 3-hydroxylidocaine recovered. Within the context of these experiments, the data suggests that recovery of less than 315 ng/mL of 3-hydroxylidocaine from a post race urine sample is unlikely to be associated with a recent local anaesthetic effect of lidocaine. Therefore these data may be of assistance to industry professionals in evaluating the significance of small concentrations of lidocaine or its metabolites in postrace urine samples. It should be noted that the quantitative data are based on analytical methods developed specifically for this study, and that methods used by other laboratories may yield different recoveries of urine 3-hydroxylidocaine.

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INTRODUCTION

Lidocaine is a commonly used local anaesthetic (LA) agent in horses. In sensory neurons, it produces conduction blockade by retarding influx of Na⁺. Lidocaine is recognized by the American Association of Equine Practitioners (AAEP) (Norwood, 1995) as

a legitimate therapeutic drug to treat horses in training (Tobin, 1995). However, the Association of Racing Commissioners International (ARCI) also lists it as a Class 2 substance, 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 local anaesthesia produced by

this drug and the associated time periods for which lidocaine or its metabolites remain detectable in serum/urine samples.

Lidocaine was one of the first amide-type LA developed to replace procaine (Day & Skarda, 1991). The advantages over procaine include a faster onset of action, higher potency, and longer duration of action (Tobin *et al.*, 1977). Like procaine, lidocaine is detectable in urine long after its pharmacological effects have waned. Numerous identifications of lidocaine have been reported in post race samples; at least some of these identifications have resulted from use of 'over-the-counter' topical preparations that contain lidocaine (Lanabiotic^R, Combe; Myzitracin Plus^R, Upjohn; Gold Bond Ointment^R, Chatter; Camphophenique Ointment^R, Bayer). From 1989 to 1994, there were at least 75 identifications of lidocaine reported in racehorses (34 harness, 41 Thoroughbred) worldwide (Dr Robert Gowen, Racing Commissioners International, personal communication).

Although ARCI policy suggests that a veterinary pharmacologist review all chemical identifications, there is no well-defined analytical/pharmacological database for lidocaine on which pharmacologists can base such reviews. There is no information available on pharmacological/local anaesthetic responses to lidocaine and little quantitative information about the pharmacokinetics and pharmacodynamics of lidocaine in horses. Following subcutaneous (s.c.) injection of a 200 mg dose, lidocaine was detected in urine for 60 h, which is the longest detection time reported for this drug to date (Chalmers *et al.*, 1987). Following lidocaine (120 mg) infiltration as a nerve block in horses, very little parent lidocaine was detected in urine samples. Lidocaine was rapidly metabolized in the horse, with neither lidocaine nor any of its metabolites detectable 36 h after administration. The major identified metabolite in equine urine associated with lidocaine administration in that report was monoethylglycinexylidide (MEGX; Fig. 1) with smaller amounts of hydroxymonoethylglycinexylidide (OH-MEGX) and 3-hydroxylidocaine reported (Short *et al.*, 1988).

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

The ARCI has recently developed a policy requiring veterinary pharmacology review of all analytical identifications to evaluate the pharmacological and forensic significance of the findings (QAP Committee, 1995). The overall objective of this research was to provide regulators with an initial basis of technical information to allow interpretation of the pharmacological significance of chemical identifications of small concentrations

of lidocaine or its metabolites recovered from post race urine/serum samples.

MATERIALS AND METHODS

Horses

Nine mature Thoroughbred mares weighing 413–602 kg were used for this study. All horses were acclimatized to their stalls 24 h prior to 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 (Merck Agvet, Rahway, NJ). A routine clinical examination was performed prior to each experiment to insure that the animals were healthy. During experimentation, horses were provided water and hay *ad libitum*. Because of the critical role of superficial skin temperature in these experiments, no LA quantitation 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 Use Committee at the University of Kentucky, which also approved the experimental protocol.

Local anaesthetic response experiments

Horses were randomly allocated to treatment groups containing five horses. Each group was injected subcutaneously with a dose (0, 4, 10, or 40 mg) of 2% lidocaine HCl (Elkins-Sinn, Inc., Cherry Hill, NJ) 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 (2 mL) of normal saline was injected into the contralateral leg, which was tested in parallel with the lidocaine-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 lidocaine 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 lidocaine.

For the LAR experiments, dose and time response relationships for lidocaine 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 defined as the time between lamp illumination and withdrawal

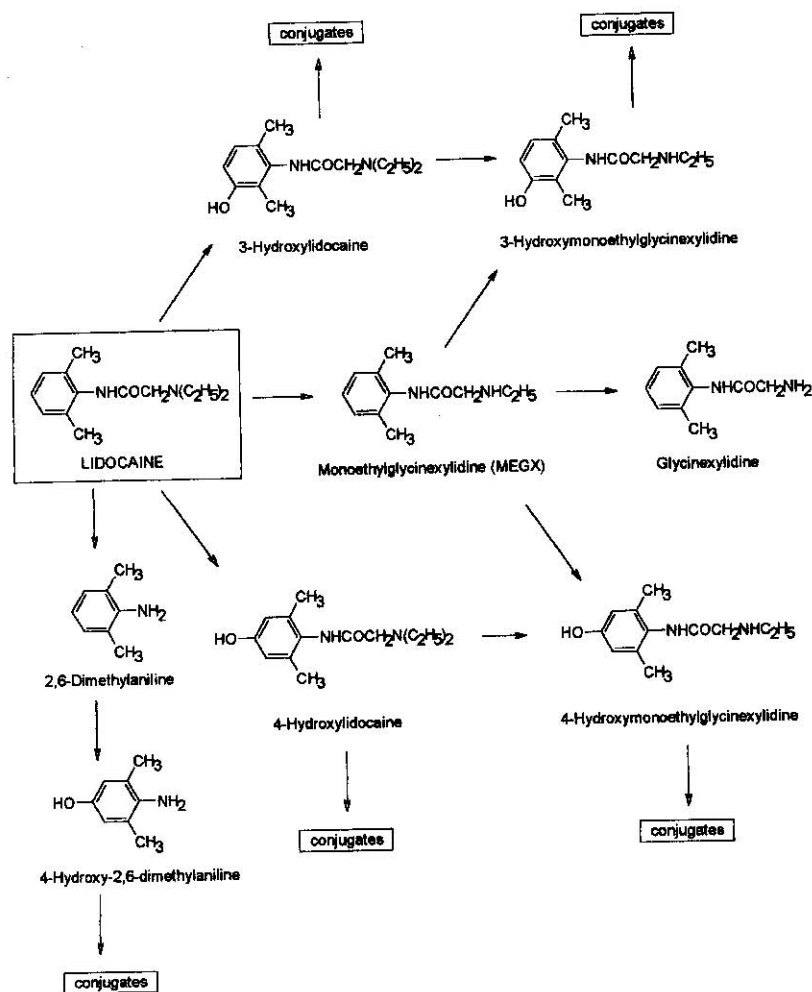


Fig. 1. Structures of lidocaine and its metabolites.

of the hoof. These times were adjusted by varying the intensity of the heat output with a rheostat. In general, the intensity of the light beam was adjusted so that the HWRL period was about 3–4 sec 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 due to visual rather than thermal perception of the focused light beam.

The HWRL was measured at –30 and –15 min and immediately before injection of lidocaine. 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 lidocaine HCl. The HWRL is expressed as a percentage of baseline values.

Development of a lidocaine ELISA test

The one-step enzyme-linked immunosorbent assay (ELISA) tests were performed as previously described (Voller *et al.*, 1976). Briefly, antilidocaine antibodies were linked to flat bottom wells. Similarly, the lidocaine hapten was linked to horseradish peroxidase (HRP) to create a covalently linked lidocaine-HRP

complex. The lidocaine test is commercially available from Neogen Corporation of Lexington, KY.

When ELISA tests are used to screen horse urine, unknown substances in the urine create variable levels of background or matrix effect that interfere with the assay. To evaluate the endogenous background activity for the lidocaine ELISA test, 20 μ L aliquots of 40 post race urine samples were assayed to measure the 'apparent drug' due to matrix effect. The 40 post race urine samples used to establish the 'natural background' for lidocaine had been screened and declared 'lidocaine-free' by an equine testing laboratory using the best thin-layer chromatography screening technology available prior to the development of ELISA testing.

The highest concentration of apparent drug was then compared with the I_{50} (the drug concentration showing 50% less absorbance at 650 nm than the zero standard) of the standard curve for the drug, and the two values were approximately the same. Therefore, a 1:1 (v/v) dilution was considered necessary to reduce the natural background of horse urine.

Analytical/pharmacological relationships after HNED administration

The HNED for lidocaine (4 mg; determined from the LAR experiments) 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 at 0, 0.5, 1, 2, 4, 6 and 8 h after administration. At 24, 48 and 72 h after administration, a Harris flush tube (24 Fr \times 152.4 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. Blood samples were collected via venipuncture from the jugular vein at 0, 0.25, 0.5, 0.75, 1, 2, 4, 8 and 24 h after injection and centrifuged for 10 min at $1200 \times g$. The serum fractions of the samples were placed in aliquots and stored at -20°C until assayed.

ELISA quantitation of apparent lidocaine in urine/serum after HNED administration

All serum and urine samples were first screened with an ELISA test to determine the concentrations of 'apparent lidocaine' (i.e. the concentration of lidocaine that would produce similar results) in the samples. The 'apparent lidocaine' is presumably composed of lidocaine, 3-hydroxylidocaine as its glucuronide, and possibly other metabolites. The assays were started by adding 20 μL of the standard, test, or control samples to each well, along with 180 μL of the lidocaine-HRP solution to wells containing the test samples. To create a matrix comparable to the test samples, 160 μL of a lidocaine-HRP solution of greater concentration was added to wells containing standard and control samples along with 20 μL of blank urine/serum. During the test, the presence of lidocaine or its cross-reacting metabolites in the sample competitively inhibited the binding of lidocaine-HRP complex to the antibody. As the HRP enzyme was responsible for the colour-producing reaction in the ELISA, the log of the concentration of lidocaine and its metabolites in the sample was inversely related to the percentage of maximum 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 quantitation of apparent lidocaine was validated by examining the precision and linearity of the assay. The between-run precision for a Neogen-provided control sample was determined to be 29.3%. The mean r (regression coefficient) for the assays was 0.9808 ± 0.0307 SD. As there was no extraction recovery step in the ELISA analysis, recovery calculations were not appropriate.

Synthesis/characterization of 3-hydroxylidocaine and MEGX

Figure 2 presents a scheme for the synthesis of 3-hydroxylidocaine from lidocaine. Nitration of lidocaine under classic conditions resulted in the 3-nitro compound ($R = \text{NO}_2$), which

was reduced with zinc dust in 50% acetic acid to produce 3-NH₂-lidocaine in high yield. After diazotization of this amine with sodium nitrite in sulphuric acid and following hydrolysis in acidic conditions at 70°C , the desired 3-hydroxylidocaine (Thomas & Meffin, 1972) was obtained, which was purified upon crystallization from acetone. MEGX was obtained from Alltech Associates, Inc (Deerfield, IL) and checked for purity by gas chromatography/mass spectrometry (GC/MS).

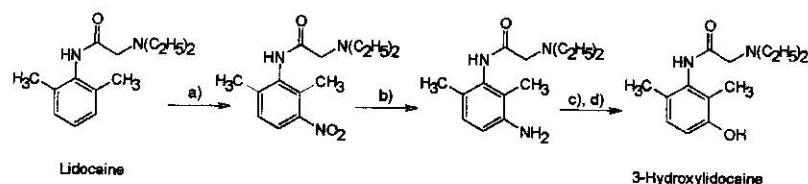
General chemical analytical methods

^1H -nmr and ^{13}C -nmr 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 5972 A mass selective detector (Hewlett-Packard, Bloomington, DE). Infrared spectra were recorded on a 1640 FT-IR spectrometer (Perkin-Elmer Limited, Buckinghamshire, UK). Melting points are uncorrected.

Characterization data for 2-diethylamino-N-(3-hydroxy-2, 6-dimethylphenyl)-acetamide (3-hydroxylidocaine)

Mp. $205\text{--}206^{\circ}\text{C}$; ^1H -nmr (200 MHz, CDCl_3): δ (p.p.m.) 1.13 (t, 6H, $2 \times \text{CH}_2\text{CH}_3$, J 7.1 Hz), 1.99 (s, 3H, Ph- CH_3), 2.11 (s, 3H, Ph- CH_3), 2.69 (q AB, 4H, $2 \times \text{CH}_2\text{CH}_3$, J 7.0 Hz), 3.24 (s, 2H, CH_2), 6.42 and 6.76 (2d AB, $2 \times 1\text{H}$, 2 aromatic H, J 8.1 Hz), 6.82 (bs, 1H, OH); 8.98 (bs, 1H, NH); ^{13}C -nmr (50 MHz, DMSO-d_6): δ (p.p.m.) 11.09, 12.15, 17.64 ($3 \times \text{CH}_3$), 48.00 ($2 \times \text{CH}_2\text{CH}_3$), 56.84 (CH_2), 112.79, 121.72, 124.93, 126.74, 135.77 (5C_{AR}), 153.54 ($\text{C}_{\text{AR}}\text{-OH}$), 169.37 ($\text{C}=\text{O}$); FT-IR (KBr) 1653, 1508 cm^{-1} .

To assess the stability of the synthesized metabolite in frozen urine, a stock solution of 3-hydroxylidocaine in methanol (1 mg/mL) was prepared, and a suitable aliquot of stock solution was added to blank urine at a concentration of 4.0 $\mu\text{g}/\text{mL}$. Aliquots (6 mL) of both the supplemented urine and negative control urine were frozen and stored. The external standardization method was used, in which the recovered concentration of metabolite from a given sample was expressed in terms of an identical sample prepared fresh on the day of analysis (Klee, 1990). Aliquots of urine to which the metabolite was added and blank urine were thawed at weekly intervals for the 8 weeks, and the stock solution was added to the blank urine at a concentration of 4 $\mu\text{g}/\text{mL}$ of sample. Both samples were then extracted, and the eluent was evaporated according to methods described below. The residues were dissolved in 40 μL of ethyl acetate. One microlitre of this solution was injected into a GC/MS 6890/5972 (Hewlett Packard Inc, Atlanta, GA) in splitless mode at an injector temperature of 250°C . Initial oven temperature



a) HNO_3 , H_2SO_4 ; b) Zn, CH_3COOH ; c) NaNO_2 , H_2SO_4 , 0°C ; d) H_2O , H^+ , 70°C

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

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-hydroxylicocaine in the stored aliquots was reported as a percentage of the 3-hydroxylicocaine solution prepared fresh each day (control). Similar experiments were also performed for the metabolite in horse urine samples stored at –20°, 4° and 24 °C.

Development of quantitative analytical methods for licocaine and 3-hydroxylicocaine

The GC/MS derivatization method was designed according to the following considerations. Tetracaine was chosen as an internal standard owing to its resistance to β -glucuronidase treatment, its lack of reactivity with the silylating reagent *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA), and its ability to elute between mono(*tert*-butyldimethylsilyl)MEGX and bis(*tert*-butyldimethylsilyl)-3-hydroxylicocaine with adequate resolution between all three compounds.

Generally, the internal standard is capable of derivatization in a manner similar to that of the analyte undergoing analysis. With 3-hydroxylicocaine, problems occurred in the creation of mixtures of mono- and di-TMS or *t*BuDMS derivatives, although much more so during TMS formation. 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 selected. MTBSTFA showed consistent reaction to form the bis(*t*BuDMS) derivative, and our ability to generate linear standard curves with tetracaine and MTBSTFA derivatization confirmed the selection of tetracaine as the internal standard.

The following ions were chosen for each compound for selected ion monitoring (SIM), with the *first ion* in each case chosen for quantitation purposes: MEGX (retention time 10.2 min): 58.0, 73.0, 206.2; tetracaine (retention time 11.9 min): 58.0, 176.1; 3-hydroxylicocaine (retention time 12.86 min): 86.0, 58.0, 73.0, 350.2. There was a 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. Although the sensitivity of the assay for MEGX was not established, this metabolite was not recovered at detectable concentrations; therefore, quantitation was not attempted.

Hydrolysis procedure

For enzymatic release of 3-hydroxylicocaine 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 β -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 sec, 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 not specifically optimized for 3-hydroxylicocaine glucuronide but 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 glucuronidase hydrolysis of 3-hydroxylicocaine conjugates could result in different threshold values should conditions other than those used in this report be selected.

Quantitative standard curve

Standard solutions of 3-hydroxylicocaine and tetracaine were prepared in methanol. Measured volumes of the 3-hydroxylicocaine solution were added to blank β -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 tetracaine standard (40 μ L of a 40 μ g/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 #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 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 μ L MTBSTFA, 1% *tert*-butyldimethylchlorosilane (*t*-BDMCS), vortexed for 15 sec, 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-5, 30 m \times 250 μ m (i.d.) \times 0.25 μ m (film thickness) cross-linked 5% phenyl methyl polysiloxane column. The carrier gas was helium with a 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: 350.0, 206.2, 176.1, 86.0, 73.0 and 58.0 m/z with a 60 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 MEGX, tetracaine, or derivatized 3-hydroxylidocaine was confirmed by the coelution of ions specific to each compound at the retention times shown by authentic standards. Lidocaine metabolite 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-hydroxylidocaine 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 for the low (4 ng/mL: CV = 17.5%), middle (40 ng/mL: CV = 10.1%), and high (160 ng/mL: CV = 9.1%) concentrations of the 3-hydroxylidocaine standard curve, with a mean CV of 12.2%. The between-run precision was determined for the low (4 ng/mL: CV = 53.6%), middle (40 ng/mL: CV = 5.8%), and high (160 ng/mL: CV = 2.8%) concentrations of the 3-hydroxylidocaine standard curve, with a mean CV of 20.7%. The mean *r* for the assays was 0.9966 ± 0.0034 SD. The recovery was determined at two concentrations: 2 ng on column = 90.6% recovery, and 10 ng on column = 81.3% recovery. The mean recovery was 85.9%. All data were based on a minimum of six determinations, and the variables were calculated according to the procedures of Shah *et al.* (1992) and Karnes *et al.* (1991).

Statistical analyses

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

RESULTS

Dose and time response curves

The dose and time response curves of the LA effect of lidocaine HCl after an abaxial sesamoid block are presented in Fig. 3(a). Following injection of 10 and 40 mg doses, there was a significant LA effect 15 min after administration. Significant local anaesthesia persisted for 30 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 4 mg lidocaine HCl, and this dose was defined as the HNED for lidocaine in this model.

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

Validation of the ELISA test

A standard curve for the lidocaine ELISA test indicated that an addition of lidocaine (2.0 ng/mL) to the system produced 50% inhibition of colour formation (Fig. 4). Higher concentrations of lidocaine increased the inhibition in a sigmoidal manner, with essentially complete inhibition of the ELISA test occurring at 100 ng/mL of lidocaine. The ELISA antibody also reacted well with 3-hydroxylidocaine and 3-hydroxymepivacaine, with 50% inhibition present following addition of 3.0 and 40 ng/mL of those compounds, respectively. There was minimal detectable cross-reactivity with MEGX at concentrations up to 1000 ng/mL. The glucuronide metabolite was not available for cross-reactivity studies.

Cross-reactivity was also evaluated for other local anaesthetic agents and other medications used in equine medicine. The ELISA test showed no significant cross-reactivity with ≈ 50 agents (Table 1) likely to be found in equine urine.

The results of ELISA tests are determined by the optical density of the test wells. Therefore, either lidocaine, its metabolites, and/or unknown materials found in horse urine can bind at the antibody binding site to influence the optical density. Accordingly, results are reported as 'apparent lidocaine'. The highest background reading (0.11 ng/mL) of the 40 post race samples (Fig. 5) was well below the apparent I_{50} (2.00 ng/mL) of the standard curve. The apparent lidocaine concentration of a typical positive control sample is shown at about 10.0 ng/mL, well above usual background interference. The 'positive' control was created by adding urine from a horse dosed with lidocaine to lidocaine-free (by ELISA) urine until the urine mixture was clearly 'positive' on the ELISA test.

ELISA quantitation of apparent lidocaine in serum/urine

The concentration of apparent lidocaine in serum reached a peak of 0.84 ng/mL 30 min after subcutaneous injection of the HNED (4 mg; Fig. 6). By 4 h after injection, there was no difference between optical density values of test and control horses. The concentration of apparent lidocaine in urine reached a peak of 72.8 ng/mL 60 min after administration. By 6 h after injection in six of the eight horses and by 24 h for all horses, there was no difference between optical density values of test and control horses.

Stability of 3-hydroxylidocaine

Figure 7(a) shows the relative stability of the 3-hydroxylidocaine metabolite in frozen urine. There was no substantial change in the concentration of 3-hydroxylidocaine detected after 8 weeks of storage at -20 °C, suggesting that the metabolite is stable in urine at this temperature. Furthermore, similar analyses of urine stocks kept refrigerated (4 °C) or at room temperature (24 °C)

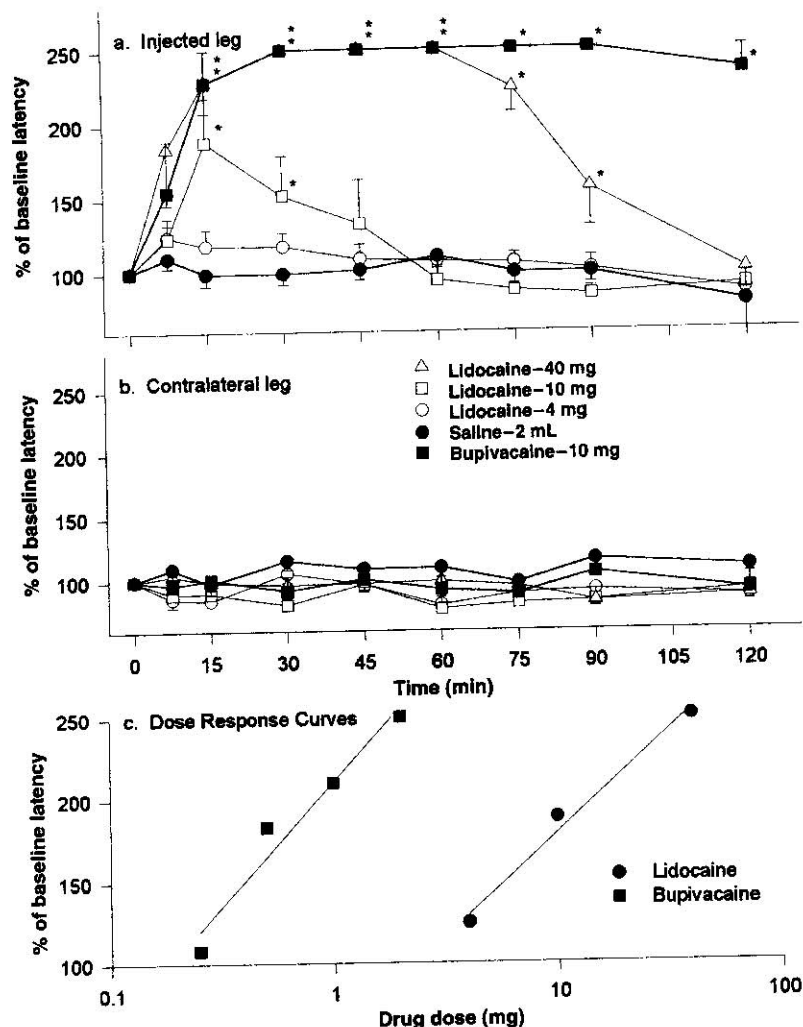


Fig. 3. (a) Mean \pm SE percentage increase in HWRL after injection of lidocaine doses; (b) mean \pm SE percentage change in contralateral leg after saline injection; (c) dose response curves for lidocaine and bupivacaine. *Significantly different from control values.

over a 5-day period also indicated substantial stability of the lidocaine metabolite in urine. Additionally, 3-hydroxylidocaine is stable for at least 6 h at 65 °C (Fig. 7b), the temperature at which the enzyme hydrolysis reaction is performed.

MS quantitation of 3-hydroxylidocaine: Ion chromatogram and electron impact (EI)-mass spectrum

Figure 8(a) shows the electron impact (EI)-mass spectrum of the tBuDMS derivative of the synthesized 3-hydroxylidocaine occurring at a retention time of 12.86 min. Figure 8(b) is the EI-mass spectrum of the 12.86 min peak derived from the chromatogram of the extract recovered from the urine sample in Fig. 9(a). Comparison of the spectra provides substantial evidence that the lidocaine metabolite chromatographing at 12.86 min is the 3-hydroxylidocaine derivative. In addition, the high relative abundance of ion 86 and the general lack of interferences with many of the ions of the spectra provide impetus for quantitation using these ions by SIM.

Figure 9(a) shows the total ion chromatogram of material recovered from an equine urine sample collected at the time of peak concentration (1 h) of 3-hydroxylidocaine following lidocaine HCl (4 mg) administration. The multiple peaks represent

normal urine components as well as derivatizing reagents. Each peak is the summation of all the ions detected when the particular compound is ionized. The peak associated with 3-hydroxylidocaine is marked with an arrow. Figure 9(b) shows the ion chromatograms for two diagnostic ions: ion 86, which is characteristic of lidocaine and certainly one of its metabolites, and ion 350, an ion specific for tBuDMS-derivatized 3-hydroxylidocaine. Although such ion chromatography reduces the scale of the abundances by an order of magnitude, it illustrates the substantial lack of interference by extraneous peaks resident in the matrix, at least for these two ions. Figure 9(c) shows similar ion chromatograms for six ions characteristic of derivatized 3-hydroxylidocaine. Note the simultaneous occurrence of ions at m/z 86, 350, 276, 392, 192 and 463 amu (atomic mass units) at 12.86 min. According to mass spectral data shown in Fig. 8, the appearance of these ions confirms that the compound recovered from equine urine by this methodology is 3-hydroxylidocaine.

GC/MS quantitation of 3-hydroxylidocaine recovered from urine

Single ion monitoring of the 350 m/z ion of derivatized 3-hydroxylidocaine provides excellent data for generation of a

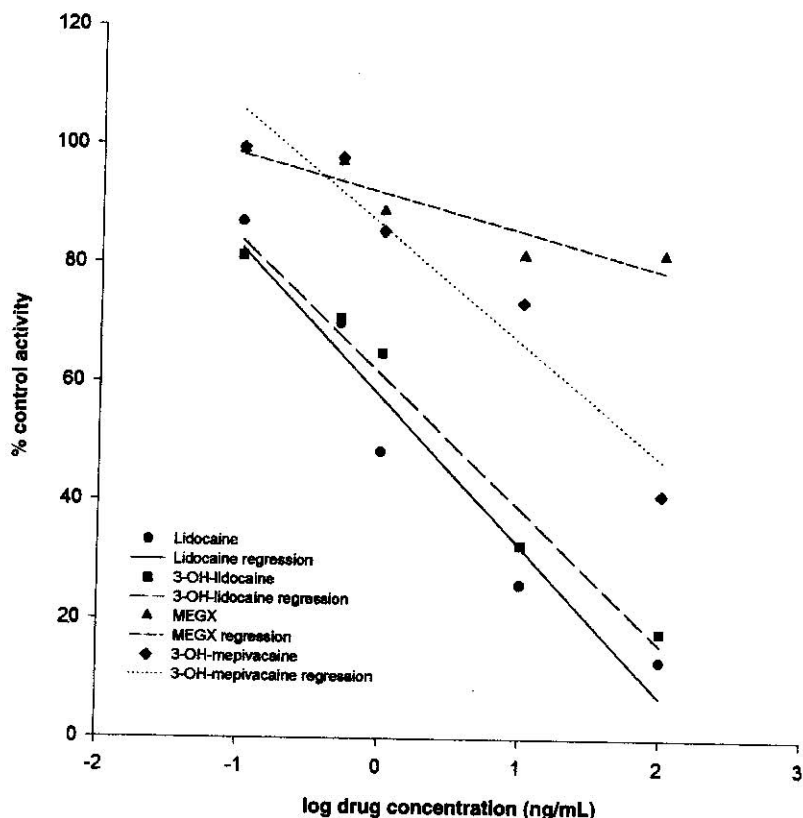


Fig. 4. Standard curves on ELISA for lidocaine, 3-hydroxylidocaine, MEGX and 3-hydroxylidocaine.

Table 1. Data for cross-reactivity of the anti-lidocaine antibody

Lidocaine	100%	Isoxsuprine	< 0.01%
3-Hydroxylidocaine	66%	Ketamine	< 0.01%
Mepivacaine	0.8%	Metaproterenol	< 0.01%
Bupivacaine	0.01%	Methocarbamol	< 0.01%
Dibucaine	0.01%	Methylene Blue	< 0.01%
Prilocaine	0.01%	6 α -Methylprednisolone	< 0.01%
Acepromazine	< 0.01%	Naproxen	< 0.01%
E-Amino-n-Caproic Acid	< 0.01%	Niacinamide	< 0.01%
Ascorbic Acid	< 0.01%	Orphenadrine	< 0.01%
Benzoylcegonine	< 0.01%	Oxyphenbutazone	< 0.01%
Butacaine	< 0.01%	Pentoxifylline	< 0.01%
Clenbuterol	< 0.01%	Phencyclidine	< 0.01%
Cocaine	< 0.01%	Phenothiazine	< 0.01%
Dexamethasone	< 0.01%	Phenylbutazone	< 0.01%
Diclofenac	< 0.01%	Polyethylene Glycol	< 0.01%
Dimethyl Sulfoxide	< 0.01%	Prednisolone	< 0.01%
Dipyron	< 0.01%	Procaine	< 0.01%
Ecgonine	< 0.01%	Promazine	< 0.01%
Ecgonine Methyl Ester	< 0.01%	Pyrantel	< 0.01%
Ethyl p-Amino-Benzoate	< 0.01%	Pyrilamine	< 0.01%
Fluxinin	< 0.01%	Salbutamol	< 0.01%
Furosemide	< 0.01%	Salicylamide	< 0.01%
Glycopyrrolate	< 0.01%	Salicylic Acid	< 0.01%
Hordenine	< 0.01%	Tetracaine	< 0.01%
Hydrocortisone	< 0.01%	Thiamine	< 0.01%
Ibuprofen	< 0.01%		

quantitative standard curve as shown in Fig. 10(a). However, owing to the lack of significant interference with the 3-hydroxylidocaine 86 m/z peak in derivatized equine urine at

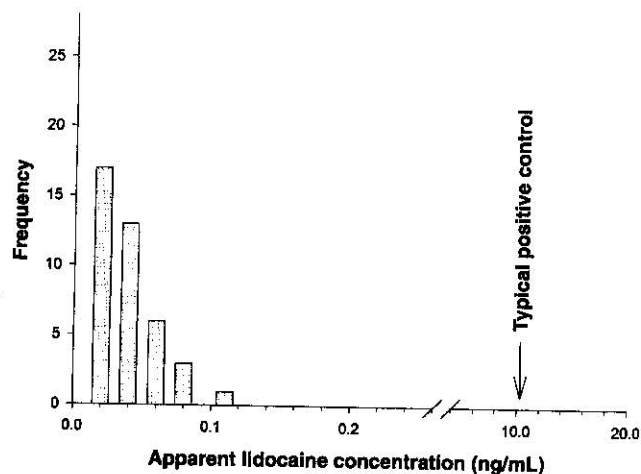


Fig. 5. Background interference in 40 post race equine urine samples. A dilution of 1:1 (i.e. 1 part sample to 1 part EIA buffer) is recommended to reduce natural background concentration. The apparent lidocaine concentration of a typical 'positive' control is shown at 10.0 ng/mL, well above background interference.

12.86 min (Fig. 9b,c), as well as this ion's much greater intensity in the authentic standard and equine-derived metabolite (Fig. 8a,b), the 86 m/z ion was monitored for quantitative purposes to provide an increase in sensitivity over that obtained with 350 m/z. Figure 10(b) shows the curve obtained on single ion monitoring of 86 m/z ion in a standard quantitative series. Figure 11(a) shows the concentration of 3-hydroxylidocaine recovered

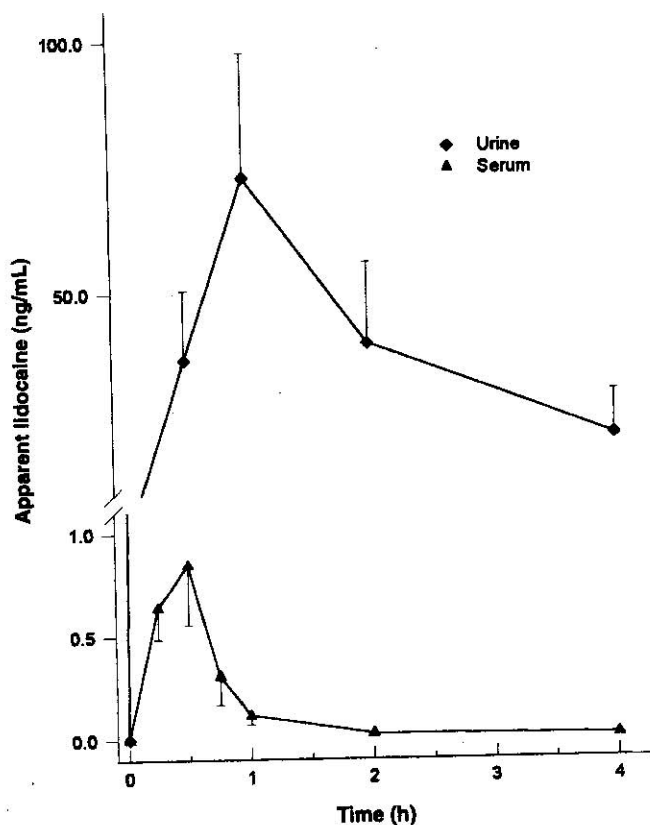


Fig. 6. ELISA quantitation of apparent lidocaine (\pm SE) in urine and serum of horses ($n = 8$) treated with the HNED (4 mg, subcutaneously) of lidocaine.

from horse urine, which was calculated by the interpolation of 3-hydroxylidocaine 86 m/z ion to tetracaine 58 m/z ion area ratios.

Figure 11(b) shows that the mean pH of the urine samples varied between 7.7 and 7.2, which is typical of urine from unexercised horses at pasture. Figure 11(c) shows that mean urine specific gravity varied between 1.028 and 1.031. Figure 11(d) shows that mean urine creatinine varied between 118 and 174 mg/dL. The inset graphs show there was no significant relationships between the peak concentration of recovered 3-hydroxylidocaine at 1 h and urine pH ($r^2 = 0.02$), urine specific gravity ($r^2 = 0.06$), or urine creatinine ($r^2 = 0.007$), respectively, over the measured range for those variables.

DISCUSSION

The goal of equine drug testing is to ensure fair competition without interfering with the proper veterinary care of horses. Reviewing this proposition, the European Horseracing Scientific Liaison Committee notes that the 'rules of horse racing are not intended to discourage the proper veterinary treatment of horses' if such treatment does not threaten 'fair competition' (European Horserace Scientific Liaison Committee, 1997).

However, veterinarians may be reluctant to treat horses with appropriate medications or may be forced to withdraw

them from racing for fear of incurring a medication violation. In this regard, appropriate limitations on the sensitivity of testing for approved therapeutic medications would be advantageous.

Specific limitations on the sensitivity of testing were first established by Canadian racing authorities (Agriculture Canada, 1991), followed by Australian (Australian Equine Veterinary Association, 1992) and European authorities (European Horserace Scientific Liaison Committee, 1997). However, the tests selected and the quantitative limits imposed by these tests are not described in the scientific literature, and the relationship between analytical findings and the disappearance of pharmacological effects, which is the critical relationship in equine medication control, is unknown. To establish this relationship, both of these factors must be accurately quantified. This paper describes development of a data base on this relationship for lidocaine in the horse.

The primary pharmacological effect of lidocaine in the horse is local anaesthesia. Figure 3 quantitates the local anaesthetic effects and shows that the HNED of lidocaine after an abaxial sesamoid block is about 4 mg/site. Therefore, 4 mg was the reference dose for analytical determinations of serum or urinary concentrations of lidocaine or its metabolites identified in these fluids.

Routine detection of potent drugs such as lidocaine is largely dependent on ELISA-based screening. Therefore, a previously developed ELISA test for lidocaine and its metabolites was evaluated by our group. As shown in Fig. 6, the ELISA test measured apparent lidocaine in serum and urine samples from horses dosed with the HNED of lidocaine. Peak serum concentrations of apparent lidocaine, which represent an unknown mixture of parent compound and metabolites, were less than 1 ng/mL at 30 min after administration. The concentration of apparent lidocaine in urine reached a peak at about 1 h after administration, and this activity presumably consisted largely of lidocaine metabolites, with variable quantities of parent drug. The concentration of parent lidocaine in urine was not determined as it was below the limit of detection by GC/MS, and also because 3-hydroxylidocaine was determined to be the critical forensic metabolite.

Lidocaine metabolites in urine of horses include 3-hydroxylidocaine, MEGX and dimethylaniline (Short *et al.*, 1988). One reason MEGX was not detected in these samples may be that the samples were not incubated with sulfatase. Also, MEGX may be unstable when stored at -20°C . However, it appears that the primary reason MEGX was not detected is because the recovery of MEGX from urine is relatively poor when solid phase extraction is used. For forensic reasons, this work was based on a standard solid-phase extraction SOP used in equine drug testing laboratories, and the selection of this method represents a significant design and interpretation constraint throughout the study.

As 3-hydroxylidocaine, the metabolite commonly recovered by analytical chemists from post race urine samples, was not available, it was necessary to synthesize, purify and characterize it. As 3-hydroxylidocaine is designated the critical metabolite,

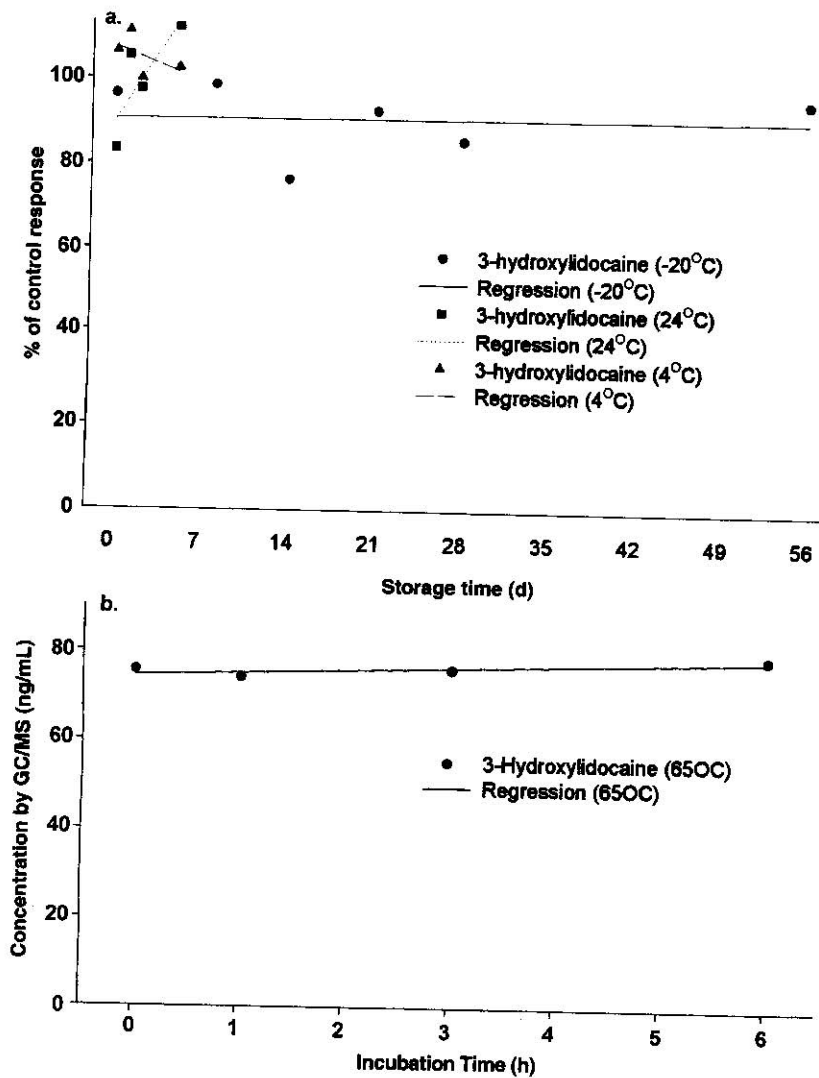


Fig. 7. (a) Stability of 3-hydroxylidocaine in urine at room temperature (24 °C), refrigerated (4 °C), and frozen (-20 °C); (b) stability of 3-hydroxylidocaine in horse urine at 65 °C as measured by GC/MS.

synthesizing the authentic compound has advanced equine forensic chemistry in the following ways:

- 1 It has enabled the development of authentic high quality mass spectral data on 3-hydroxylidocaine and its various derivatives for use as forensic reference standards. Without an authentic standard, mass spectral data must be derived from equine administration samples, a source that cannot be claimed to yield authentic mass spectral data.
- 2 It has enabled accurate quantification of 3-hydroxylidocaine recovered from biological samples. The standard is a prerequisite for quantitative analytical work and for development of the central theme of this paper, which is the creation of an analytical/pharmacological database for lidocaine in the horse.
- 3 It has simplified the creation of quality assurance samples used to monitor the efficacy of equine drug testing. Because the metabolite is stable in frozen urine, samples can be created by direct addition of specific amounts of authentic 3-hydroxylidocaine to samples, which are then stored and shipped frozen, a significant advantage in quality assurance work. This approach is much simpler and more accurate than the previous standard method, which required the dosing of

horses, collection of urine samples and laborious preparation, characterization and approximate quantification of the sample prior to shipment.

With regard to the recovery and quantification of 3-hydroxylidocaine from equine urine samples, preliminary work with trimethylsilyl (TMS) derivatization showed that the ratios of the mono- and di-TMS products varied and appeared to be a function of different factors. These factors include injector and oven temperatures, relative injector and column cleanliness, glassware active sites, presence of oxygen or other reactants, and active sites in different individual GC injector configurations. We have also observed inter-instrumental variation in mono-/di-TMS derivative ratios between HP 5890 and 6890 GCs, although other analysts have reported > 99% conversion to the di-TMS product (Dr Richard Sams, The Ohio State College of Veterinary Medicine, personal communication). The same problem extends to trimethylsilyl derivatization at the amide functionalities of MEGX and lidocaine itself.

Because of this inherent variability, both the phenol and amide sites were derivatized with MTBSTFA containing 1% *tert*-butyldimethylsilylchloride (TBDMSCI) as catalyst. The *tert*-

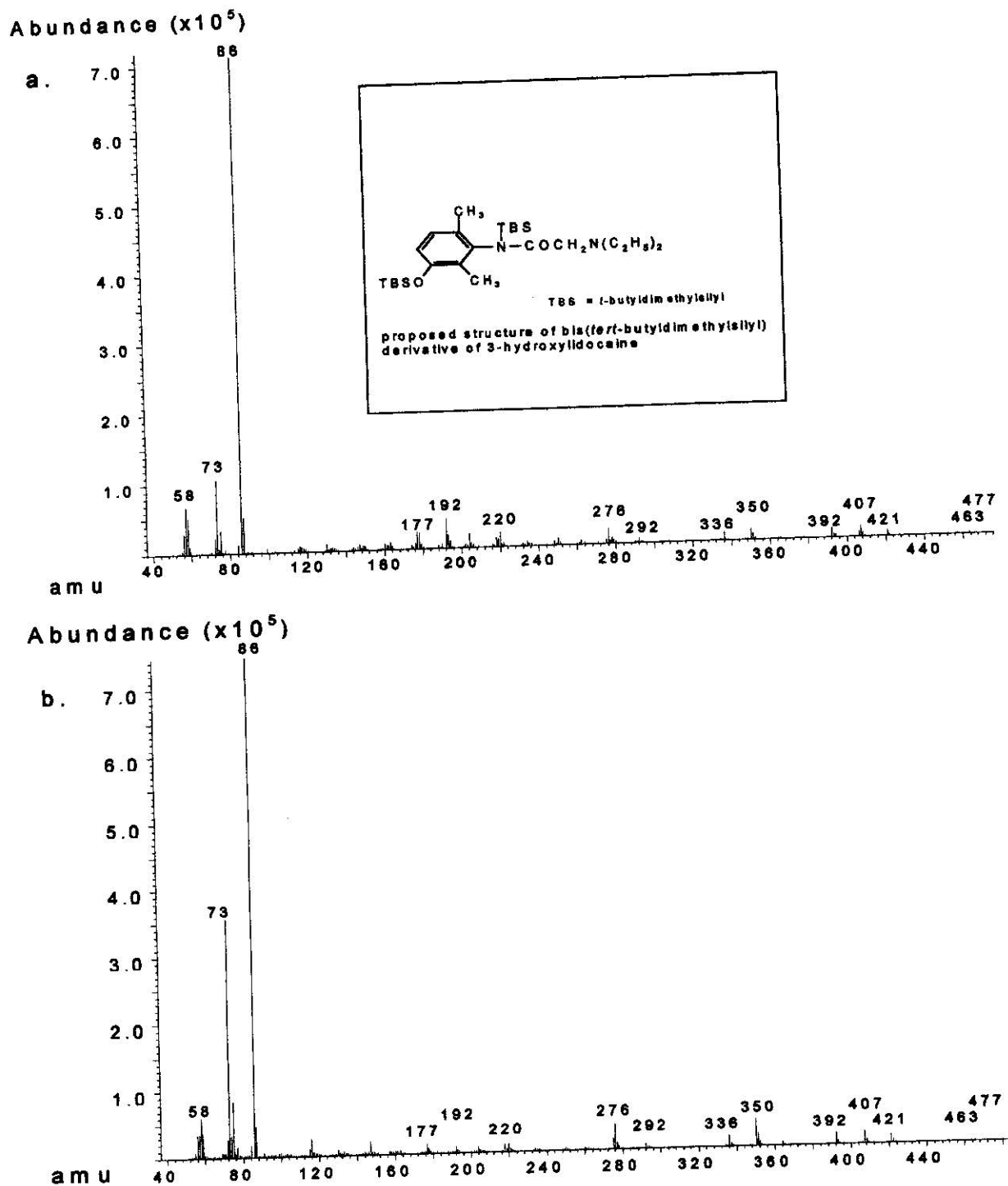


Fig. 8. (a) EI-mass spectrum of the derivative of authentic 3-hydroxylicocaine (bis[*t*-butyldimethylsilyl]) occurring at 12.86 min retention time. Inset figure shows structure of the 3-hydroxylicocaine derivative; (b) EI-mass spectrum of the 12.86 min peak from the chromatogram of a urine sample from a lidocaine-dosed horse shown in Fig. 9.

butyldimethylsilyl ethers derived from such reactions are reportedly up to 10 000 times more stable than TMS-ethers (Early *et al.*, 1987).

This analytical method was used to identify 3-hydroxylicocaine recovered from post administration samples from eight horses. Urinary concentrations of recoverable 3-hydroxylicocaine rose

rapidly following lidocaine administration and peaked at 1 h after treatment. The peak concentration (310 ng/mL) of recovered 3-hydroxylicocaine was considerably higher than the apparent peak (73 ng/mL) from the ELISA screening data, which is a semi quantitative estimate based on the lidocaine ELISA. However, the ELISA test is less reactive with 3-hydroxylicocaine than with

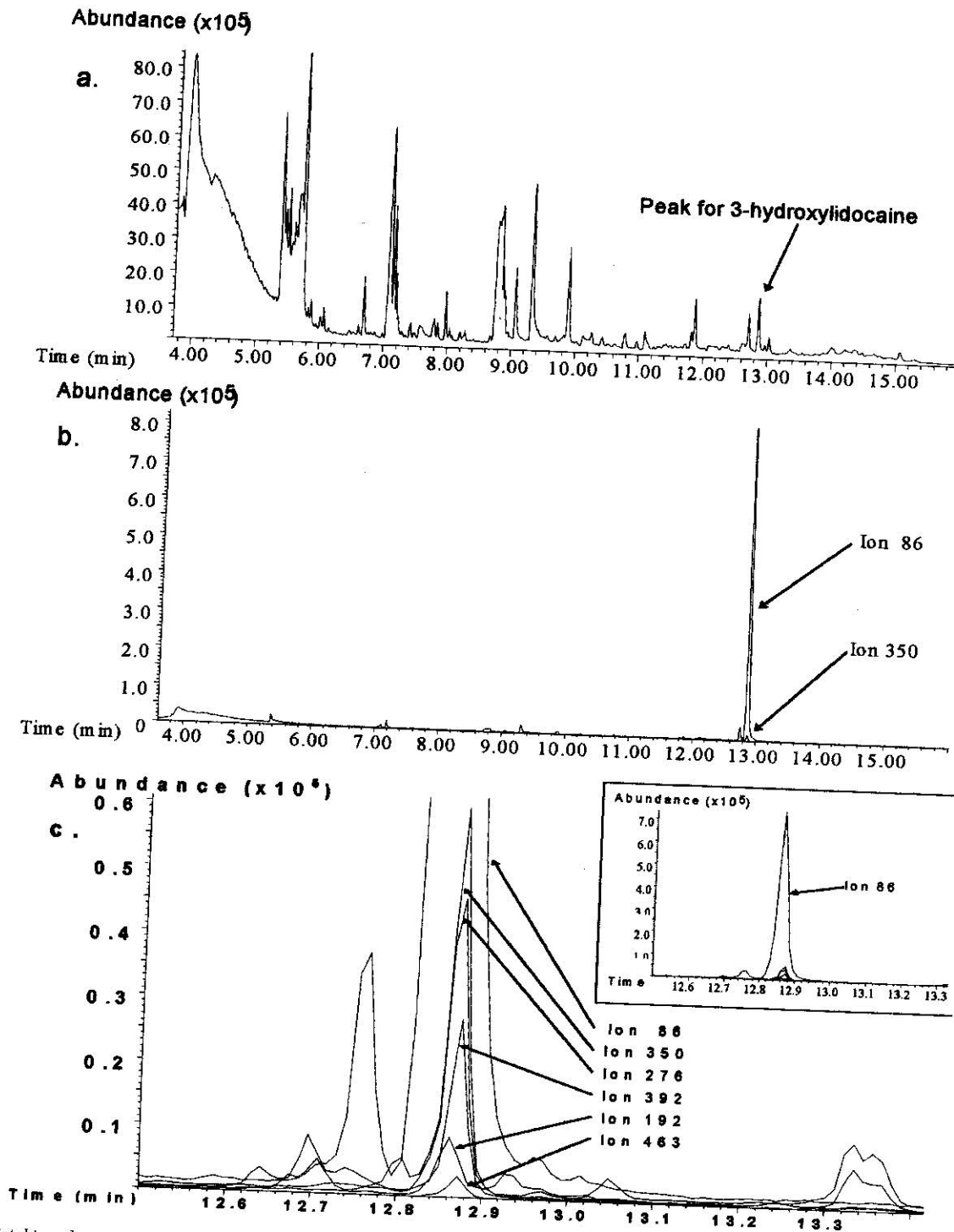


Fig. 9. (a) Total ion chromatogram of a urine sample taken 1 h after administration of 4 mg lidocaine HCl; (b) ion chromatograms for ions 86 and 350 only of the urine sample shown in Fig. 8 (a); (c) ion chromatograms of the same urine sample in Fig. 8 (a) for ions 86, 350, 276, 392, 192 and 463 amu at the retention time appropriate for the bis(*t*-butyldimethylsilyl) derivative of authentic 3-hydroxylidocaine. Inset graph shows full range for abundances of these ions.

lidocaine, and presentation of the data as apparent 3-hydroxylidocaine identifies this difference as an apparent discrepancy.

When comparing the concentrations of apparent 3-hydroxylidocaine determined by ELISA and GC/MS, the actual concentration of 3-hydroxylidocaine recovered from urine by GC/MS was higher than the concentrations of apparent lidocaine

estimated by ELISA. The relationship between apparent concentrations of lidocaine or its metabolites in equine urine by ELISA and GC/MS are not inconsistent, although they clearly suggest that the ELISA test tends to underestimate the total amount of lidocaine-related material actually present in the urine sample.

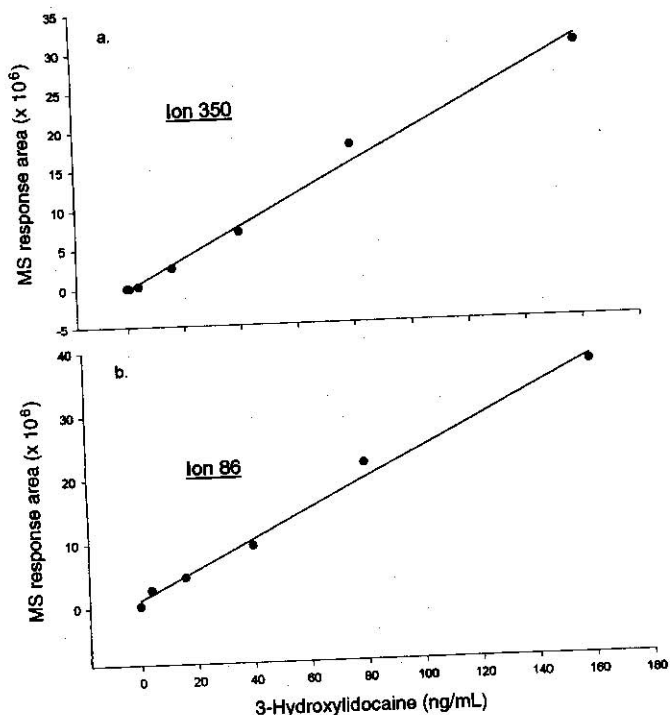


Fig. 10. Typical standard curves for derivatized 3-hydroxylidocaine extracted from urine and generated by least squares fit of integrated GC/MS areas for (a) ion 350 ($r = 0.9957$) and (b) ion 86 ($r = 0.9997$).

Therefore, the results show that administration of the HNED of lidocaine for an abaxial sesamoid block produces concentrations of apparent lidocaine in urine samples that are readily detectable by ELISA screening and are also detectable/recoverable as 3-hydroxylidocaine by GC/MS. As these concentrations of 3-hydroxylidocaine (310 ng/mL) in urine are unlikely to be associated with a pharmacological effect, lesser concentrations of this metabolite recovered from post race urine samples are also unlikely to be associated with local anaesthesia in horses at the time of racing. The database reported above applies primarily within the context of these experiments or closely related circumstances.

It should also be understood that this database applies primarily within the context of these experiments or closely related circumstances. If the drug is administered by a different route, as repeated doses, as a different formulation, or with another therapeutic rationale, the analytical-pharmacological database reported here may not be applicable to the specific regulatory circumstances.

Furthermore, the database is method-sensitive as no validated qualitative or quantitative analytical methods were (or have become) available since we commenced this work. The methodologies on which this database was developed are specific to this work, and any variation, adaptation, or substitution of these methods may affect interpretation of the database. However, the methodologies used are direct and in some cases unchanged adaptations from those frequently used in racing chemistry; for example, the enzymatic hydrolysis method was adapted from the work of Combie *et al.* (1982) and is used by

Truesdall Laboratories, which performs post race testing for Kentucky, California, and other states.

In this regard, development of a standardized, validated enzyme hydrolysis method for conjugated metabolites would significantly improve the reproducibility of quantitative data involving a glucuronide hydrolysis step. Similarly, no validated quantitative analytical methods were available for the unique hydroxylated metabolites that form the basis of this research. Progress in this field would be facilitated by the development of validated quantitative methods for these drugs and metabolites.

Finally, it must be remembered that the data were developed in a research setting. Only experienced regulators who can evaluate a research database and determine its applicability to field situations should apply these data to a regulatory setting.

In these experiments, no parent lidocaine was detected in the urine of any horses treated with the HNED dose of lidocaine. Presumably, parent lidocaine was present in the urine, but the concentrations were less than those detectable by this methodology. A critical factor that determines the detectability of parent lidocaine is the pH of the urine. These experiments were performed in untrained horses at pasture, horses that routinely produce alkaline urine. Review of Fig. 11(b) shows a mean pH value of about 7.5, which is consistent with our previous experiences. Because lidocaine is a basic drug, parent or unchanged lidocaine would tend to concentrate in acidic urine (Tobin, 1981). As the concentrations of parent lidocaine might be expected to be greater in acidic urine (Gerken *et al.*, 1991), any urinary 'cut-offs' based on the data presented in this paper are likely to be very conservative.

Information concerning the pH of a urine sample is important to accurately interpret the analytical results. If urinary pH is lowered in association with exercise, then the concentrations of parent lidocaine and its detectability in urine may be expected to increase dramatically (Sams, 1997).

In contrast, the concentration of the glucuronidated metabolite should remain constant as urine pH decreases, as the concentration of 3-hydroxylidocaine recovered from urine is likely to be independent of pH. As the 3-hydroxylidocaine metabolite recovered is likely to have entered the urine and to exist in the urine as a glucuronide conjugate, it will have entered the urine primarily by glomerular filtration and tubular secretion and should not move across the renal tubule in response to pH gradients. Therefore, the expectation is that concentrations of 3-hydroxylidocaine will not be affected by urinary pH changes, while the concentration of parent lidocaine will increase in response to reductions in urinary pH.

In summary, after administration of a sub-HNED dose for an abaxial sesamoid block, relatively high concentrations of free lidocaine in an acidic post race urine sample would be expected, due to the influence of urinary pH. However, relatively modest recovery of 3-hydroxylidocaine would be expected, which would relate more closely to the actual dose of lidocaine administered and the actual concentrations of parent lidocaine in the system of the horse, consistent with the serum ELISA data of Fig. 6.

On the other hand, the 3-hydroxylidocaine glucuronide metabolite excreted in urine is likely to be susceptible to the

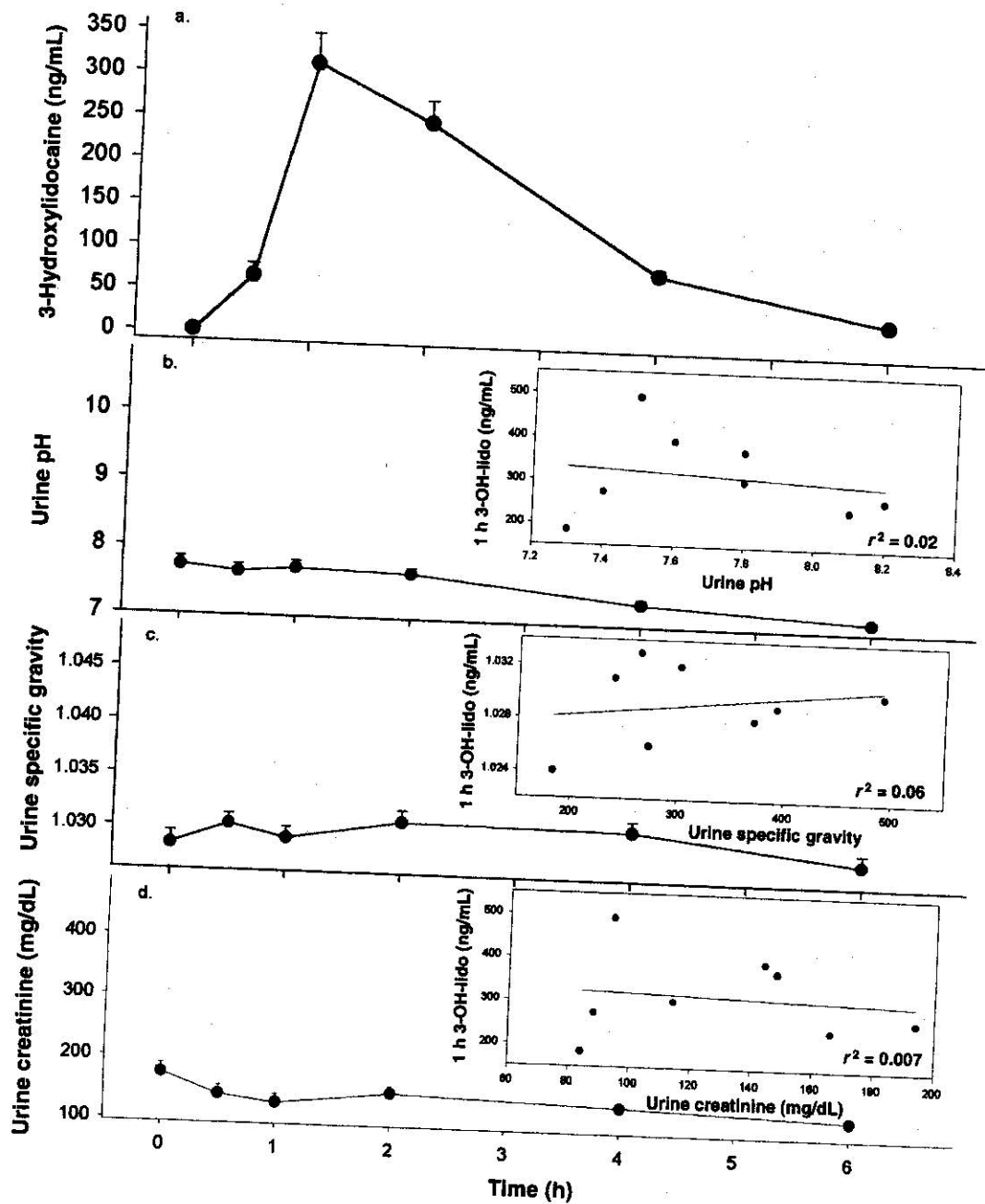


Fig. 11. (a) Mean concentrations \pm SE of 3-hydroxylidocaine recovered from urine of 8 horses after administration of HNED (4 mg, s. c.) of lidocaine as measured by GC/MS in selected ion monitoring mode; (b) mean pH of urine; (c) mean urine specific gravity; (d) mean urine creatinine. The inset graphs show the nonsignificant relationships between peak 3-hydroxylidocaine recovered at 1 h and urine pH, urine specific gravity, and urine creatinine, respectively.

effects of drugs that produce urinary dilution, such as furosemide. Therefore, the specific gravity and creatinine concentration of urine samples should also be reviewed, and appropriate concentration adjustments should be made if it appears that the urine samples are dilute.

Finally, the ELISA detection of what is most likely largely parent lidocaine in serum samples raises the possibility of establishing a database of analytical/pharmacological relationships for serum concentrations of lidocaine in the horse, an approach currently being pursued in our laboratory.

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