

Ropivacaine in the horse: its pharmacological responses, urinary detection and mass spectral confirmation

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This report evaluates the pharmacological responses, urinary detection and mass spectral confirmation of ropivacaine in horses. Ropivacaine, a potent local anesthetic (LA) recently introduced in human medicine, has an estimated highest no-effect dose (HNED) of about 0.4 mg/site as determined in our abaxial sesamoid block model. Apparent ropivacaine equivalents were detectable by ELISA screening using a mepivacaine ELISA test after administration of clinically effective doses. Mass spectral examination of postadministration urine samples showed no detectable parent ropivacaine, but a compound indistinguishable from authentic 3-hydroxyropivacaine was recovered from these samples. The study shows that ropivacaine is a potent LA in the horse, that clinically effective doses can be detected in postadministration samples by ELISA-based screening, and that its major post administration urinary metabolite is 3-hydroxyropivacaine.

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INTRODUCTION

Ropivacaine is a long-lasting local anesthetic (LA) which acts by producing conduction blockade in sensory neurons and retarding the influx of Na⁺ ions. Ropivacaine is listed as a Class 2 substance by the Association of Racing Commissioners International (ARCI). Ropivacaine is not recognized as a therapeutic medication by the American Association of Equine Practitioners, and the possibility for abuse of the agent in racing horses is possible.

Ropivacaine has a potency and toxicity four times that of mepivacaine (Goehl *et al.*, 1973). The potency of ropivacaine is reported to be similar to that of bupivacaine (Hickey *et al.*, 1991; Vainionpää *et al.*, 1995); however, ropivacaine was 25% less toxic than bupivacaine when the acute central nervous and cardiovascular effects of the two LA were compared in humans (Scott *et al.*, 1989). There is little information available concerning the disposition or pharmacokinetics of ropivacaine in horses, and no researchers have published detection times for this agent. The structures of ropivacaine and its likely metabolites are shown in Fig. 1.

The objectives of this study were to (1) determine the dose and time-response curves for ropivacaine injected as an abaxial sesamoid block, (2) identify and validate a sensitive ELISA-based screening test for ropivacaine in equine urine and (3) develop and validate a sensitive mass spectral confirmation method for

the forensic detection of ropivacaine and/or its metabolites in postrace urine samples.

MATERIALS AND METHODS

Horses

Six mature Thoroughbred mares weighing 485–576 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 (MSD Agvet, Rahway, NJ, USA). 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 Institutional Animal Care Use Committee at the University of Kentucky, which committee also approved the experimental protocol.

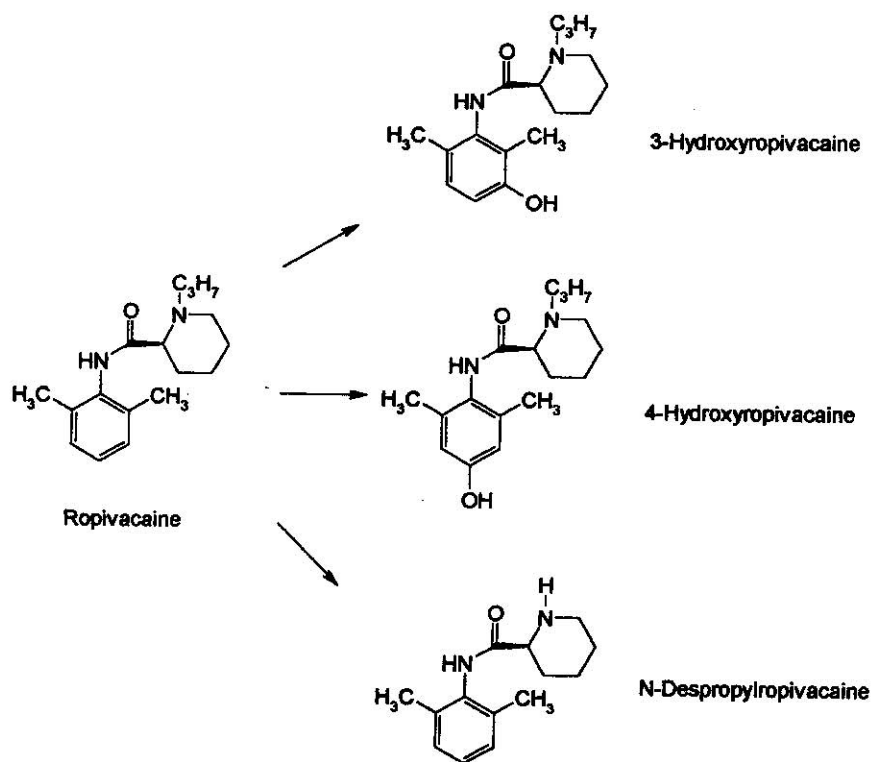


Fig. 1. Structures of ropivacaine and its metabolites.

Local anesthetic dose and time response experiments

Horses were randomly allocated to treatment groups containing five horses. Each group was injected subcutaneously with a dose (0.4, 1.0 or 4.0 mg) of 0.5% ropivacaine HCl (Astra USA, Inc., Westborough, MA, USA) in a randomized sequence with at least 7 days between doses. The site of injection was the lateral volar nerve where it passes lateral (abaxial) to the lateral sesamoid bone. In clinical practice, this block is known as an abaxial sesamoid block. To control for possible effects of pressure or volume, a similar volume of normal saline was injected into the contralateral leg, which was tested in parallel with the ropivacaine-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. There were at least 7 days between injections in the same horse.

Before each local anesthetic 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, USA) to insure equal and consistent heat absorption independent of skin and hair color.

For the LAR experiments, dose and time response relationships for ropivacaine 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 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 sec in the control legs, with the actual HWRL recorded on an electronic timer built into the lamp. In the anesthetized leg, the duration of light exposure was limited to 10 sec 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.

The HWRL was measured at –30 and –15 min and immediately before injection of each LA. The three pretreatment HWRL times (–30, –15 and 0 min) were used to establish a control value for HWRL in each horse. The HWRL was also measured at 7.5, 15, 30, 45, 60, 75, 90, 120, 150, 180 and 210 min after LA administration. Post-treatment HWRLs were expressed as a percent of control values.

Analytical/pharmacological relationships after HNED administration

The HNED for ropivacaine (0.4 mg) was determined from the LA quantification experiment. Multiples of the HNED (4.0, 40 and 400 mg) were injected subcutaneously in the area of the fetlock in four horses. During the first day, complete urine collection was accomplished with a Foley catheter and attached plastic bag. Blank samples were collected during the 30 min period before administration, then samples were collected during the following periods: 0–0.5, 1–2, 2–4, 4–6 and 6–8 h after administration. At 24, 48 and 72 h after administration, a Harris flush tube (24 Fr × 60 in; Seamless, Ocala, FL, USA) was used to collect a maximum of 300 mL urine. Urine samples were placed in aliquots and stored at –20 °C until assayed.

ELISA quantification of apparent ropivacaine in urine after incremental doses

All urine samples were first screened with a mepivacaine ELISA (Neogen, Lexington KY, USA) test to determine the concentrations of 'apparent ropivacaine' (i.e. the concentration of ropivacaine that would produce similar results) in the samples. The apparent ropivacaine is putatively composed of ropivacaine, 3-hydroxyropivacaine 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 mepivacaine-HRP solution to wells containing the test samples. A quantity of 160 µL of the mepivacaine-HRP solution was added to wells containing standard and control samples along with 20 µL of blank urine/serum to create a matrix comparable with the test samples. During the test, the presence of ropivacaine or its metabolites in the sample competitively prevented the binding of mepivacaine-HRP complex to the antibody. As the HRP enzyme was responsible for the color-producing reaction in the ELISA, the log of the concentration of ropivacaine and its metabolites in the sample was inversely related to the percent 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, USA) approximately 30 min after addition of substrate. All assay reactions were run at room temperature (20.5 °C).

The ELISA method for the semiquantitation of 3-hydroxyropivacaine was validated by examining the precision and linearity of the assay. The between-run precision was determined for middle standard (10 ng/mL) of the standard curve to be 4.1%. The mean *r* for the assays was 0.9907 ± 0.0004 SD.

Synthesis and characterization of 3-hydroxyropivacaine and 4-hydroxyropivacaine

The synthesis of 3-hydroxyropivacaine from ropivacaine was analogous to our previously reported syntheses of 3-hydroxyliclozine (Harkins *et al.*, 1998), 3-hydroxymepivacaine (Harkins *et al.*, 1999c) and 3-hydroxybupivacaine (Harkins *et al.*, 1999b). Nitration of ropivacaine under classic conditions (mixture of concentrated sulfuric acid and nitric acid) resulted in the formation of 3-nitroropivacaine, which was reduced with zinc dust in 50% acetic acid to produce 3-aminoropivacaine in high yield. After diazotization of this amine with sodium nitrite in sulfuric acid and hydrolysis in acidic conditions at 70 °C, the desired 3-hydroxyropivacaine was obtained, and this was purified upon column chromatography on silica gel and crystallized from acetone, giving fine, white crystals. The ¹H-nmr (200 MHz) spectra were recorded on VARIAN GEMINI AC-200. GC/MS analysis was performed on a Hewlett-Packard Model 6890 gas chromatograph (GC) equipped with a Model 5972 A mass selective detector (MSD). Melting points are uncorrected.

Characterization data for 1-propyl-N-(3-hydroxy-2,6-dimethylphenyl)-2-piperidinecarboxamide (3-hydroxyropivacaine):

Mp. 171–173 °C; ¹H-nmr (200 MHz, CDCl₃): δ (p.p.m.) 0.92 (3H, t, J 7.5 Hz), 1.26–1.84 (8H, m), 2.07 (3H, s, ring CH₃), 2.16 (3H, s, ring CH₃), 2.20–2.30 (1H, ddd), 2.80 (1H, ddd, J 6.3 Hz, J 10.5 Hz, 12.6 Hz), 2.9 (1 H, dd, J 3.9 Hz, J 10.5 Hz), 3.21 (1 H, dt, J 12.3 Hz, J 3.9 Hz), 5.64 (1H, s), 6.54 (1H, d, J 8.1 Hz), 6.85 (1H, d, 8.1 Hz), 8.20 (1H, s).

Molecular weight characteristics for the 3-hydroxyropivacaine mono-TMS derivative: *m/z* (relative intensity): 358 (9.5%), 359 (12%), 360 (51%), 361 (100%), 362 (83%), 363 (58%), 364 (14%), 365 (6%).

GC/MS Method

Glucuronide cleavage. Urine was treated with β-glucuronidase (1000 units of Siga Type L-II per mL of urine brought to 0.25 M sodium acetate, pH 5) for 3 h at 65 °C.

Sample extraction. Urine samples from dosed and undosed horses were treated with β-glucuronidase and then extracted as follows. Ten milliliter of sample were mixed with 10 mL saturated Na₂CO₃ bringing the pH to approximately 12. The resultant mixture was combined with 10 mL dichloromethane then set in a rotorack for approximately 20 min. The resultant emulsion was centrifuged at 730 × *g* at 4 °C for 1.5 h in a Beckman AccuSpinFR centrifuge equipped with a AH-4 rotor. The organic phase was transferred to a 5 mL Reacti-vial and concentrated with nitrogen gas at 40 °C. Controls in methanol were evaporated in Reacti-vials directly.

Sample derivatization

Nitrogen-evaporated samples were mixed with 50 µL of BSTFA + 1% TMCS (Pierce Chemical Co., Rockford, IL, USA), mixed thoroughly by brief vortexing and incubated at 75 °C for 30 min.

Instrumentation

Separations were carried out on an HP-5, 30 m × 250 µm (i.d.) × 0.25 µm (film thickness) cross-linked 5% phenyl methyl polysiloxane column. The carrier gas was helium with a flow of 1 mL/min. Derivatized samples were transferred to an autosampler vial. One microliter of derivatized extract was injected in splitless mode at an injector temperature of 250 °C. Initial oven temperature was 70 °C (held 2 min), with ramping at 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 scan mode over the 50–550 amu mass range with a 150 threshold.

Statistical analysis

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

RESULTS

Local anesthetic dose and time response experiments

The dose and time response curves of the LA effect of ropivacaine are presented in Fig. 2a. Following injection of the 1 and 4 mg doses, there was a significant LA effect 7.5 min after administration. Significant local anesthesia persisted for 30 and 150 min, respectively. Additionally, there was a significant difference between negative (saline) and positive (bupivacaine, 10 mg) control values at every time point after anesthetic injection. There was no significant LA effect following injection of 0.4 mg ropivacaine HCl; based on review of the data, 0.4 mg was established as the HNED for ropivacaine in this model.

There was no LA effect in the contralateral leg. The dose-response curve for the peak LA response to ropivacaine is presented in Fig. 2b. A dose-response curve for bupivacaine is included for reference.

Validation of the ELISA screening test

A standard curve for ropivacaine using the mepivacaine ELISA test indicated that an addition of ropivacaine (20 ng/mL) to

the system produced 50% inhibition of color formation (Fig. 3). Higher concentrations of ropivacaine increased the inhibition in a sigmoidal manner, with essentially complete inhibition of the ELISA test occurring at 100 ng/mL of ropivacaine. This ELISA test reacts well with mepivacaine ($I_{50} = 16$ ng/mL), the agent against which this antibody was raised. This ELISA test also reacts well with bupivacaine and with 3-hydroxybupivacaine, with 50% inhibition present following addition of 17 and 131 ng/mL of those compounds, respectively. There was minimal detectable cross-reactivity with 4-hydroxybupivacaine at concentrations up to 1000 ng/mL. The cross-reactivity of this ELISA test with other LA agents used in equine medicine has been reported previously (Harkins *et al.*, 1999c).

ELISA quantification of 'apparent ropivacaine' in urine

After subcutaneous doses of 400 mg ($n = 2$), 40 mg ($n = 2$) and 4 mg ($n = 2$), the concentrations of apparent ropivacaine in urine reached peaks of 476, 27 and 10 ng/mL at 4, 4 and 6 h after injection, respectively (Fig. 4). The peak 'apparent ropivacaine' concentrations were directly related to the dose administered ($\text{Dose} = 0.8 \times \text{Peak concentration} + 6.3$; $r^2 = 0.998$; Fig. 4, inset).

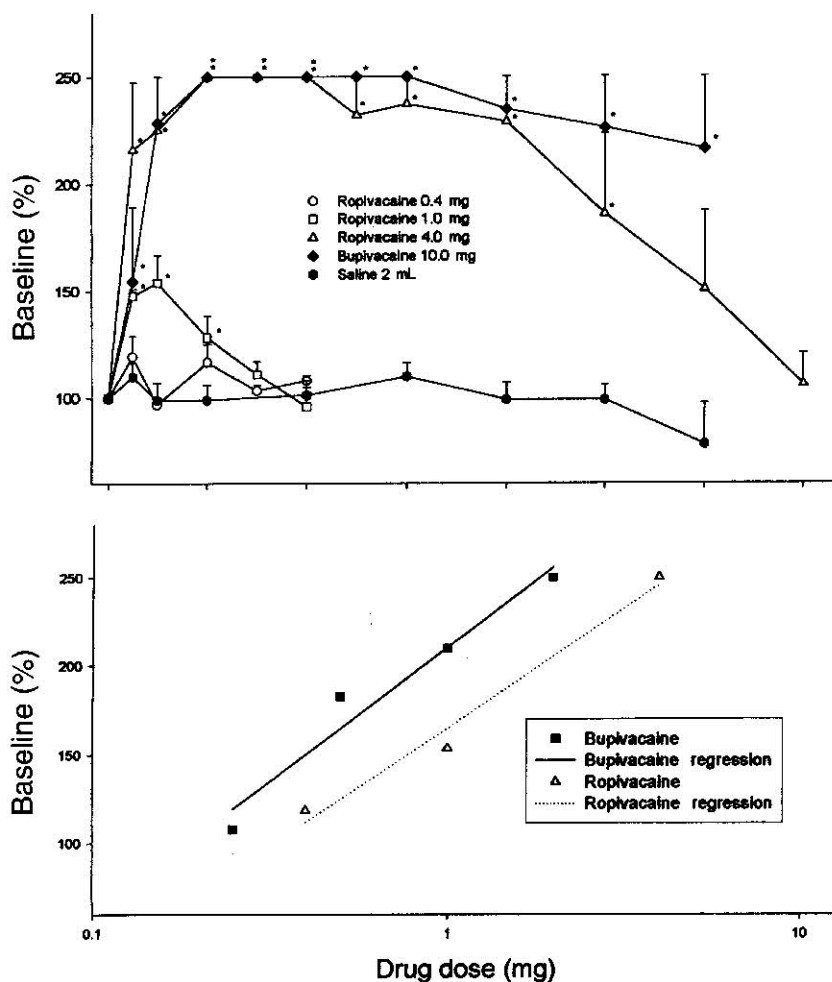


Fig. 2. (a) Mean $\pm$ SE% increase in HWRL after injection of ropivacaine doses; (b) dose-response curve for ropivacaine and bupivacaine (control). *Significantly different from control values.

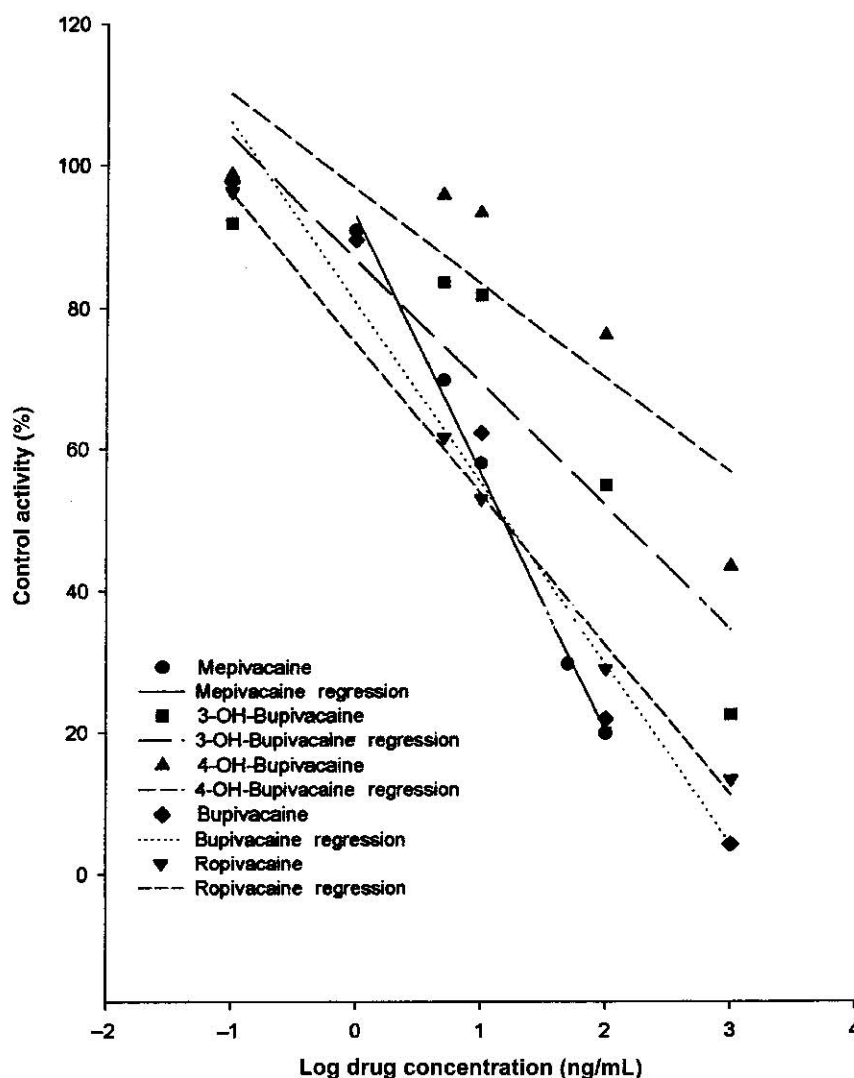


Fig. 3. Regression of ELISA standard curves for ropivacaine and its comparison to standard curves for mepivacaine, bupivacaine, 3-hydroxybupivacaine and 4-hydroxybupivacaine.

Mass spectral identification of 3-hydroxyropivacaine: ion chromatogram and EI-mass spectrum

Figure 5a shows the EI-mass spectrum of the mono TMS-derivative of the authentic standard 3-hydroxyropivacaine occurring at 12.9 min retention time. Figure 5b is the EI-mass spectrum of the 12.9 min peak from the chromatogram of a 4 h urine sample from a horse dosed with 400 mg of ropivacaine HCl. Note the similarity of masses and their abundances in the two spectra, supporting our contention that the major urinary metabolite of ropivacaine in equine urine is a hydroxyropivacaine, either 3- or 4-hydroxy.

Figure 6a is the total ion chromatogram of a urine sample taken 4 h after ropivacaine (400 mg) administration. As revealed by Fig. 6b, which is the ion chromatogram for ion 126, two major peaks related to ropivacaine can be seen. Ion 126 is specific to ropivacaine-related molecules with an intact N-(n-propyl) piperidine structure. The two peaks from the ropivacaine metabolite correspond in spectrum and retention time to the 3-hydroxyropivacaine bis-TMS derivative at

11.9 min and the 3-hydroxyropivacaine mono-TMS derivative at 12.9 min.

Figure 7a is the ion chromatogram for ions 126, 192, 178, 208, 347 and 361 of a urine sample collected 4 h after ropivacaine administration (400 mg). Figure 7b is the ion chromatogram for ions 126, 178, 192, 208, 347 and 361 of the authentic standard 3-hydroxyropivacaine, demonstrating the comparability of ion abundances between the equine sample and standard.

DISCUSSION

The goal of this research was to establish the LA efficacy of ropivacaine in the horse and to develop, evaluate, and validate sensitive screening and confirmation methods for ropivacaine or its major urinary metabolite in equine urine samples. The overall goal of this research is to assist regulatory personnel in detecting and controlling the use of this LA in racing horses. As ropivacaine is a potent LA, highly sensitive and specific

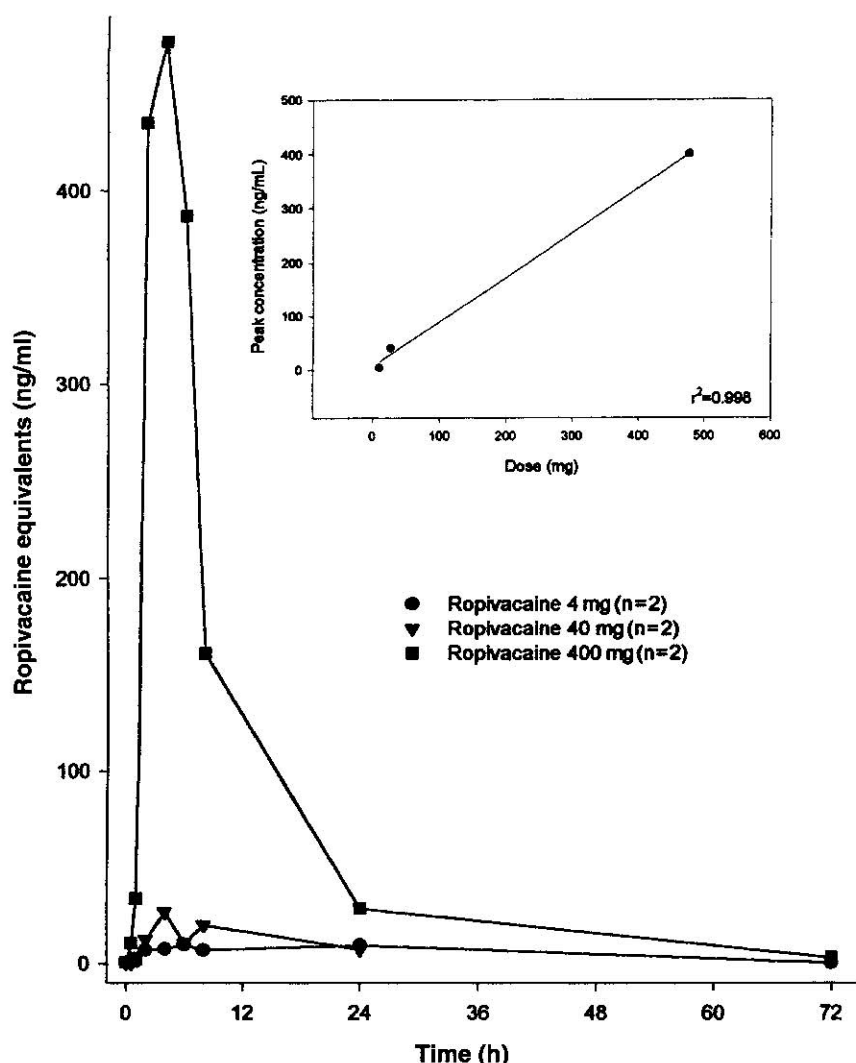


Fig. 4. ELISA measurement of apparent ropivacaine in urine horses treated with incremental doses of ropivacaine. Inset graph shows relationship of ropivacaine dose to the maximum concentration of ropivacaine equivalents detected at that dose.

screening and confirmation methods are required to control its abuse in racing horses (Tobin, 1981).

The primary pharmacological effect of ropivacaine is local anesthesia and is essentially the only pharmacological effect likely to influence racing performance. Therefore, the LA efficacy of ropivacaine is the pharmacological effect of interest to racing regulators. Figure 2 describes the dose and time response relationships for the LA effects of ropivacaine using the heat lamp/abaxial sesamoid block model and shows that the HNED of ropivacaine is about 0.4 mg/site.

Having established the clinical efficacy of this agent as a local anesthetic, the next step was to evaluate and/or develop methods to screen for the presence of ropivacaine in posttrace urine samples. A third and equally important step was to develop sensitive, specific, and scientifically defensible methods to confirm the presence of ropivacaine or its metabolites in posttrace urine samples after administration of clinically significant doses.

Postrace screening of urine samples from performance horses for potent medications like ropivacaine is largely dependent on ELISA testing. Based on previous experience with the mepiva-

caine ELISA test (Harkins *et al.*, 1998), it was evaluated as a screening test for ropivacaine. Figure 3 shows the reactivity of this ELISA test with parent ropivacaine, mepivacaine, bupivacaine, 3-hydroxybupivacaine and 4-hydroxybupivacaine. The test was less sensitive for ropivacaine ($I_{50} = 20$ ng/mL) than for bupivacaine ($I_{50} = 17$ ng/mL) and mepivacaine ($I_{50} = 16$ ng/mL) but more sensitive than for 3-hydroxybupivacaine ($I_{50} = 131$ ng/mL) and 4-hydroxybupivacaine ($I_{50} > 1000$ ng/mL). With appropriate dilution of postrace samples to reduce endogenous background, this screening test is able to detect the concentration of apparent ropivacaine in postrace urine samples at concentrations of 10–100 ng/mL. As shown in Fig. 4, the incremental doses of ropivacaine became increasingly detectable by the ELISA test. Additionally, the inset graph of Fig. 4 shows there is a largely linear relationship between the dose of ropivacaine administered and the peak concentration of apparent ropivacaine recovered in urine.

The 'apparent ropivacaine' detected by the mepivacaine ELISA test is presumably composed of ropivacaine and structurally related metabolites of ropivacaine, including ropivacaine

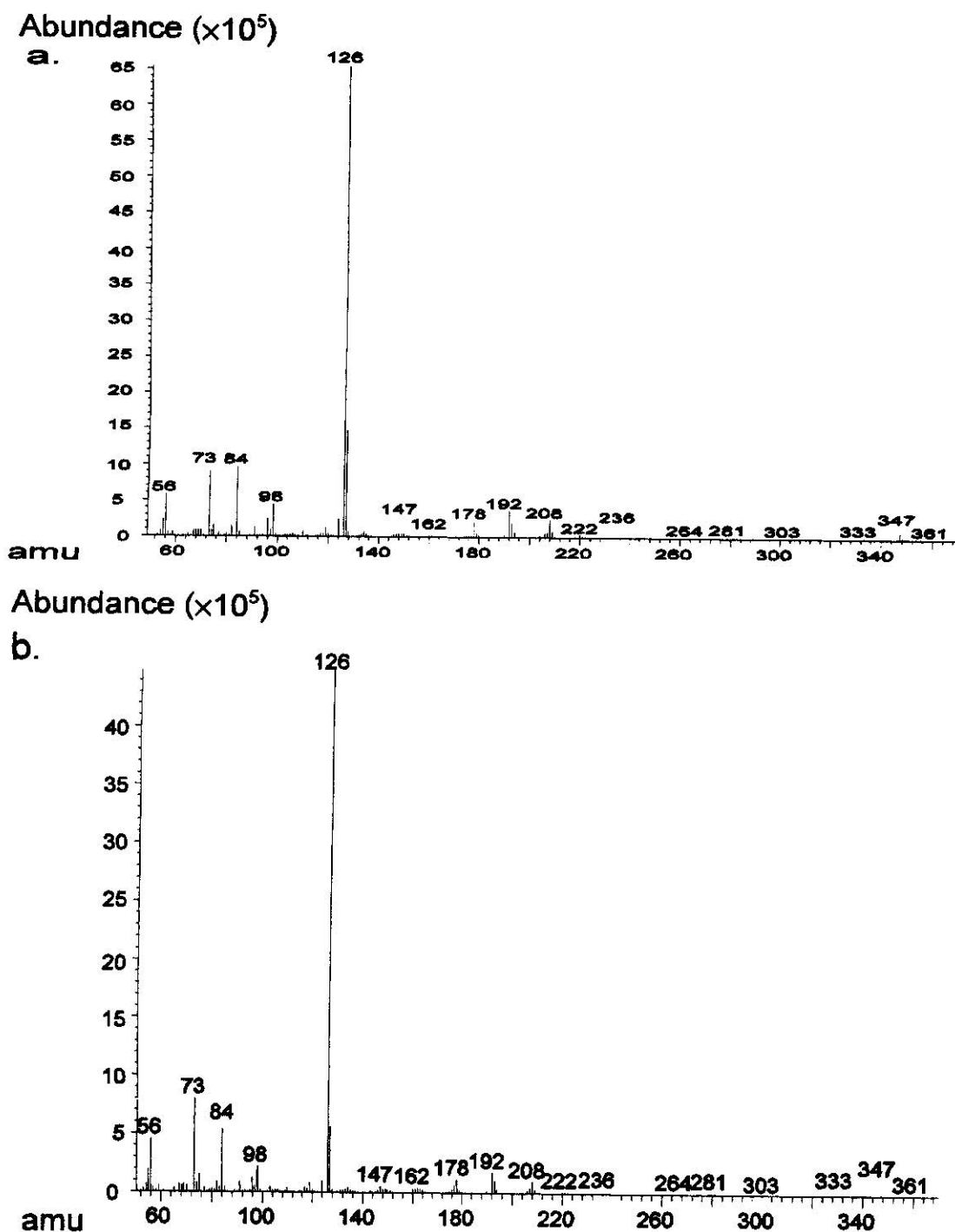


Fig. 5. (a) EI-Mass spectrum of the TMS-derivative of authentic 3-hydroxyropivacaine occurring at 12.9 min retention time; (b) EI-Mass spectrum of the 12.9 min peak from the chromatogram of a urine sample from a ropivacaine-dosed horse shown in Fig. 7a. Note the similarity of ion peaks between the two spectra.

glucuronide (Harkins *et al.*, 1999a), excreted in equine urine. However, parent ropivacaine has not been detected in significant concentrations in urine samples after administration of the doses of ropivacaine reported in these experiments. Based on previous experience with detection of the urinary metabolites of other local anesthetics in the horse, 3-hydroxyropivacaine was

proposed to be the critical metabolite most likely to be detected in postrace urine samples and was synthesized in our laboratory.

The spectrum of the authentic (synthesized) 3-hydroxyropivacaine was compared with the spectrum of the material recovered from horse urine in Fig. 5. There is an excellent

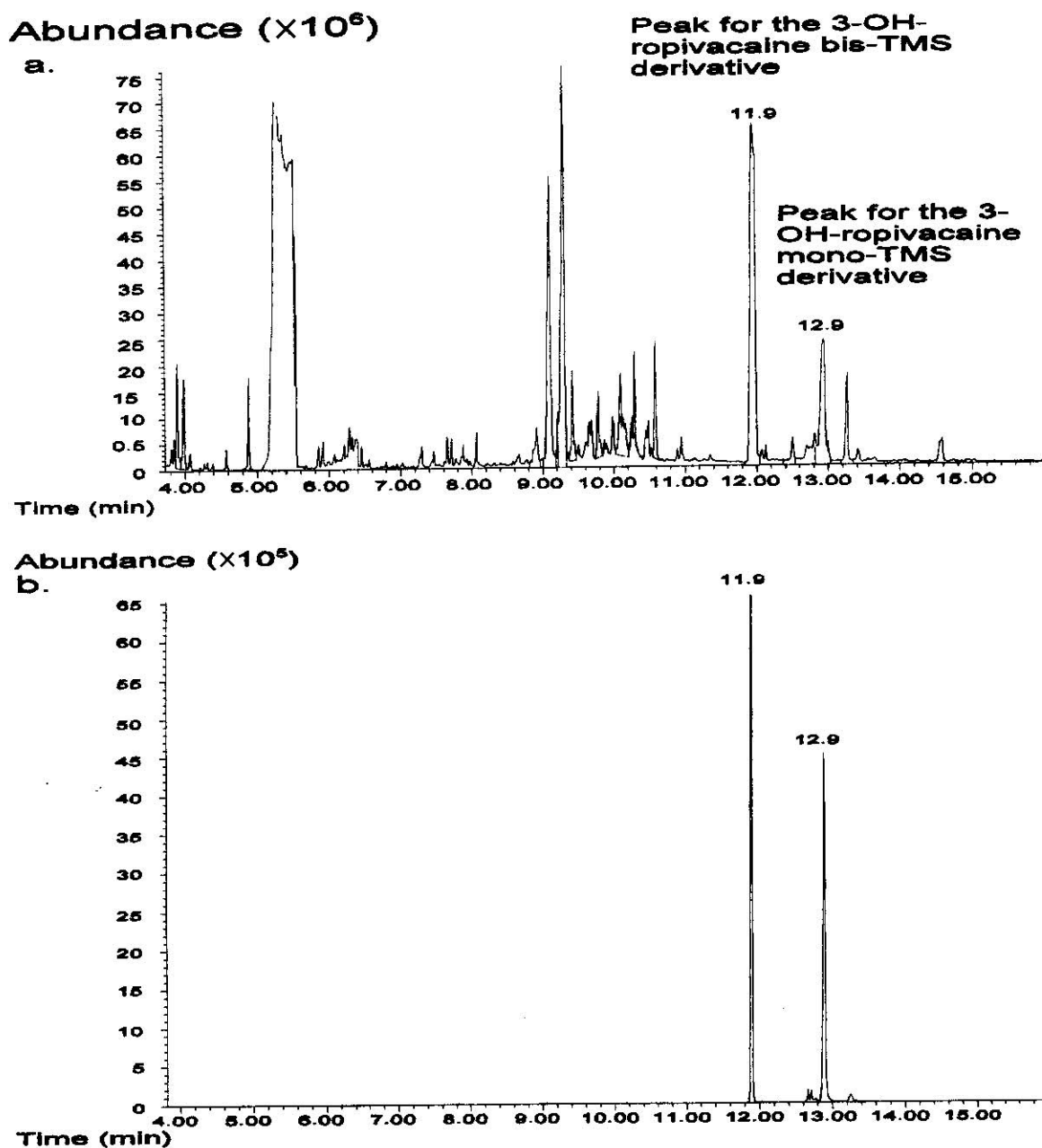


Fig. 6. (a) Total ion chromatogram of urine sample taken 4 h after administration of ropivacaine HCl (400 mg); (b) ion chromatogram derived from the sample depicted in Fig. 7a for the 126 m/z ion, which is characteristic of ropivacaine-related species.

match of the spectra from Fig. 5a (authentic standard) and Fig. 5b, which is glucuronidase-treated urine from a horse injected with 400 mg of ropivacaine. These data support our contention that the glucuronidase-released metabolite of ropivacaine from postadministration samples is a hydroxyropivacaine, possibly the 3-hydroxy metabolite by analogy to work carried out with bupivacaine (Lehner *et al.*, 1998). Molecular weight analysis indicates that one TMS unit must be taken up for a 362 molecular weight complex (274 [ropivacaine] + 16 [oxygen to give hydroxy] + 72 [TMS] = 362). However, instead of a principal upfield peak at 362, there was a range of ions with varying intensities (listed previously under Characterization

data for 1-propyl-N-(3-hydroxy-2,6-dimethylphenyl)-2-piperidinecarboxamide). The consistently higher 361 amu peak relative to 362 amu suggests the presence of a labile proton in the mono-TMS 3-hydroxyropivacaine, possibly at the internal amide linkage.

As discussed in our recent paper on mepivacaine (Harkins *et al.*, 1999c), derivatization of hydroxy metabolites of the structurally related local anesthetics (lidocaine, mepivacaine, ropivacaine and bupivacaine) by silylating agents is possible both at the easily derivatized hydroxyl group or at the internal amido linkage. This accounts for the consistency with which this group of compounds gives both bis- and mono-derivatives.

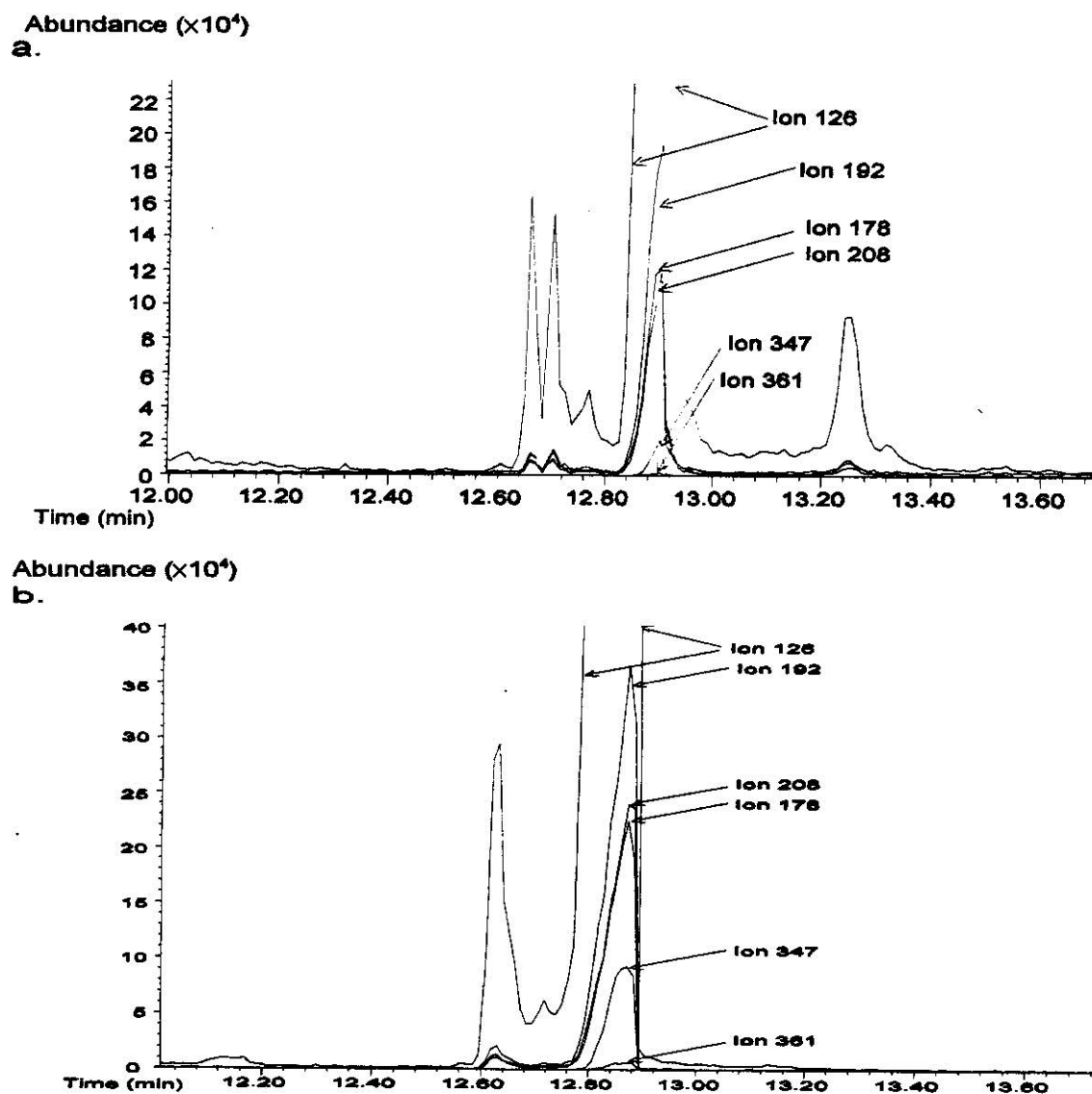


Fig. 7. Ion chromatogram for ions 126, 178, 192, 208, 347 and 361 of (a) postropivacaine administration urine sample shown in Fig. 6a; (b) the authentic standard 3-hydroxyropivacaine. Note the qualitative and proportional similarity of the peaks.

At the excess concentrations of derivatizing agents used, the resultant mixture of mono- and bis-products is possibly reflective of an equilibrium between the two species or between these species and the BSTFA reagent, rather than the results of incomplete derivatization. The two peaks revealed by ion chromatography at 11.9 and 12.9 mins (Fig. 6b) in any case reveal the great majority of glucuronidase-released metabolites of ropivacaine with intact N-(n-propyl)-piperidinyl rings. When compared with an authentic standard of 3-hydroxyropivacaine-mono-TMS derivative, relative ion abundances for significant mass spectral peaks in the equine substance at 12.9 min match very closely, offering support for our contention that released metabolite is a hydroxyropivacaine. The GC peaks in Fig. 6 have been labeled as arising from 3-hydroxyropivacaine, based on the excellent match of ionic abundances to authentic 3-hydroxy standard in Fig. 7 and in light of analogous work

done with bupivacaine (Lehner *et al.*, 1998) in which the hydroxylated metabolite was chromatographically established as being in the 3-position. However, we caution that additional work must be completed in order to ascertain conclusively that hydroxylation exclusive to the 3-position is also the case with ropivacaine.

An additional group of minor peaks can be seen in the vicinity of the 12.9 min mono-TMS hydroxyropivacaine peak in Fig. 6. These peaks are expanded in Fig. 7a in the 12.6–12.8 min range and appear to share most diagnostic ions with those of the large peak at 12.9 min, which is labeled 3-hydroxyropivacaine-mono-TMS. There is also at least one analogous peak in the authentic 3-hydroxy standard in Fig. 7b at ~12.65 min. We suggest that one or more of these peaks represent alternative sites for mono-TMS formation, such as the less easily derivatized N or O atoms of the internal amide functionality.

In summary, ropivacaine is a potent local anesthetic, with an estimated HNED of about 0.4 mg/site in our abaxial sesamoid block model. Apparent ropivacaine equivalents were detectable by ELISA screening using a mepivacaine ELISA test for up to 5 h after administration of a clinically effective dose. However, because of the relatively high potency of the agent, an enhanced ELISA test may be needed to screen for smaller doses of ropivacaine (< 40 mg/site). Mass spectral examination of post-administration urine samples showed no detectable parent ropivacaine, but a material indistinguishable from authentic 3-hydroxyropivacaine was recovered from these samples. The identification of the 3-hydroxyropivacaine metabolite in post-administration urine samples, the availability of an authentic standard, and the availability of sensitive screening and mass spectral identification techniques will enable effective regulatory control of this agent in racing horses.

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