Identification of hydroxyropivacaine glucuronide in equine urine by ESI$^+$/MS/MS

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Abstract

Ropivacaine is a local anesthetic that has a high potential for abuse in racing horses. It can be recovered from urine collected after administration as a hydroxylated metabolite following β-glucuronidase treatment of the urine. Based on these findings, it has been inferred that ropivacaine is present in equine urine as a glucuronide metabolite; however, these metabolites have never been directly identified. Using ESI$^+$/MS/MS, the presence of a $[M+H]^+$ molecular ion of m/z 467 was demonstrated in urine corresponding to the calculated mass of a hydroxyropivacaine glucuronide + 1. The abundance of this ion diminished after glucuronidase treatment with concomitant appearance of a m/z 291 peak, which is consistent with its hydrolysis to hydroxyropivacaine. In further work, the m/z 467 material was fragmented in the MS/MS system, yielding fragments interpretable as hydroxyropivacaine glucuronide. These data are consistent with the presence of a hydroxyropivacaine glucuronide in equine urine and constitute the first direct demonstration of a specific glucuronide metabolite in equine urine.

Résumé

La ropivacaïne est un anesthésique local ayant un potentiel élevé pour une utilisation abusive chez les chevaux de course. Ce médicament peut être retrouvé dans l'urine prélevée après administration de ropivacaïne sous forme d'un métabolite hydroxylé suite à un traitement de l'urine avec de la β-glucuronidase. Sur la base de ces résultats, il a été suggéré que la ropivacaïne est présente dans l'urine de cheval sous forme d'un métabolite conjugué à un glucuronide; toutefois, ces métabolites n'ont jamais pu être identifiés directement. À l'aide d'une technique ESI$^+$/MS/MS, la présence d'un ion moléculaire $[M+H]^+$ avec un m/z de 467 fut démontrée dans de l'urine et correspondait à la masse calculée d'un glucuronide d'hydroxyropivacaïne+1. L'abondance de cet ion diminuait après traitement avec de la glucuronidase en même temps qu'apparaissait un pic avec un m/z de 291, ce qui est cohérent avec l'hydrolyse de ce produit en hydroxyropivacaïne. Dans des études ultérieures, le matériel de m/z 467 fut fragmenté à l'aide du système MS/MS et donna des fragments correspondants au glucuronide d'hydroxyropivacaïne. Ces résultats sont conséquents avec la présence de glucuronide d'hydroxyropivacaïne dans l'urine de cheval et constitue la première démonstration directe d'un métabolite spécifique du glucuronide dans de l'urine de cheval.

(Traduit par docteur Serge Messier)

Introduction

Ropivacaine is a long-lasting local anesthetic with a potency similar to that of bupivacaine (1,2). Like most local anesthetics, it has a high potential for abuse in racing horses by interrupting pain transmission from injured tissue, and it also interrupts proprioception transmission from adjacent areas. Horses unaware of pain and joint/limb positions can have catastrophic accidents while racing, which can be life-threatening to the horse and its rider as well as trailing horses and riders. It is also devastating for the public perception of racing, especially during nationally-televised events. As such, most local anesthetics are listed as Class 2 substances by the Association of Racing Commissioners International (ARCI). Several veterinary local anesthetics are identified as therapeutic medications by the American Association of Equine Practitioners, and there are established forensic tests to control the abuse of those agents. However, ropivacaine is not an approved agent, and the identification of its metabolites in post-race urine samples may suggest that it was used to circumvent routine forensic testing for local anesthetics. Therefore, it is important to establish the mass spectrum for the metabolites of ropivacaine and other non-approved anesthetic agents.

It has been assumed that ropivacaine, like most local anesthetic agents, is cleared from the body through the urine as a glucuronide metabolite. In previous works by this laboratory (3), the concentration of a local anesthetic/metabolite in urine could be quantified only after the glucuronide moiety was removed from the hydroxy metabolite by β-glucuronidase hydrolysis (3–6). The hydroxylated metabolite is typically derivatized and injected into a GC/MS for characterization and quantitation. Under this system, the presence of a glucuronide metabolite in the urine is inferred from the

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increased yield of the hydroxylated metabolite after enzymatic cleavage of the glucuronide.

Figure 1 shows the putative structure of hydroxyropivacaine glucuronide with a non-isotopic molecular weight of 466. Hydrolysis of the glucuronic acid residue would be expected to yield hydroxyropivacaine with a molecular weight of 291, as the molecular weight of ropivacaine itself is 274. Additionally, the typical fragmentation pattern of ropivacaine under electrospray ionization (ESI) conditions includes the generation of a prominent m/z 126 ion, which is characteristic of ropivacaine related species, as shown in Figure 1.

Direct introduction of small quantities of urine into an MS/MS enables identification of a mass spectral peak at m/z 467 present only in urine from horses treated with ropivacaine, which corresponds to the molecular weight of hydroxyropivacaine glucuronide, plus 1 for addition of a proton (H^+), to give the molecular ion, $[M+H]^+$. Furthermore, this $[M+H]^+$ ion can be diagnostic for the effects of enzymatic treatments. Energetic challenges to the m/z 467 ion during collisionally-induced dissociation (CID) experiments results in a fragmentation pattern which, along with the results of enzymatic treatments, is consistent with this material being the glucuronide metabolite of phase I-metabolized ropivacaine. To the authors' knowledge, this is the first reported direct identification of the glucuronide metabolite of ropivacaine or any other drug in an equine urine sample.

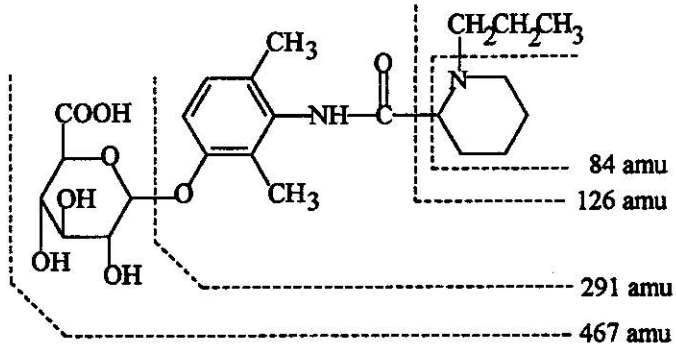

Figure 1. Proposed structure of hydroxyropivacaine glucuronide, which is tentatively assigned as arising from conjugation to a 3-hydroxyropivacaine ($C_{23}H_{34}N_2O_8$). The 466 amu structure becomes 467 amu after ionization with a H^+. Dashed lines show cleavages that produce the principal fragments seen in Figure 2. Some cleavages require the concurrent shift and/or addition of a proton to account for the full fragment size indicated. For example, 291 requires the presence of the phenolic hydrogen as well as ionization with H^+.

Materials and methods

Horses

Two mature Thoroughbred mares, weighing 503 and 527 kg, respectively, 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 daily. The animals were vaccinated annually for tetanus and were dewormed quarterly with ivermectin (Merck Agvet Division, Rahway, New Jersey, 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. Animals used in these experiments were managed according to the rules and regulations of the University of Kentucky's Institutional Animal Care and Use Committee, which also approved the experimental protocol.

MS/MS (General)

Full scan electrospray ionization (ESI) mass spectra were obtained on analytical standards at 10 μg/mL in 50:50 (v/v) methanol:1% formic acid (aq), by infusion at 0.9 mL/h with a Harvard syringe pump into the electrospray probe of a mass spectrometer (Micromass Quattro II MS/MS; Micromass, Beverly, Massachusetts, USA) set in positive ion ($^+$) mode. All spectra were optimized by combination of 1–2 min of uniformly acquired data, background subtraction, and peak smoothing. Complex spectra were further simplified by conversion from continuum data to centroid data. Interpretation of mass spectra was assisted by specialized software (Mass Spec Calculator Pro, v. 4.03; ChemSW, Fairfield, California, USA).

MS/MS Calibration

After tuning the ESI^+ source with 1:1 (v/v) acetonitrile:2 mM ammonium acetate and 0.1% formic acid solution, the instrument was calibrated with a mixture of polypropylene glycols (PPGs) of average molecular weights 425, 725, 1000, and 2000. These were dissolved in 1:1 acetonitrile:aqueous 2 mM ammonium acetate at 20–50 ng/μL, depending on the PPG. Calibration selected the following monoisotopic ions: 59.04969, 291.2172, 541.3952, 964.7148, 1312.9660 and 1835.3427 amu. Note that 59-233 are fragments of the form $(C_3H_6O)_n$, 291-541 are (PPG + H^+), and above 616 are (PPG + NH_4^+). Calibration enabled more accurate assignment of masses within the ± 1–2 amu window factory adjustment for each mass value.

MS/MS Tuning

The mass spectrometer was tuned by direct injection of 10 ng/μl ropivacaine in 50:50 (v/v) methanol:1% formic acid (aq). The peak shape and intensity of the monoprotonated ropivacaine 275 ion were optimized by adjustment of capillary, HV lens, cone voltage, skimmer lens, and RF lens settings. Skimmer lens offset was left at 5 V. Collision gas (argon) and collision energy were adjusted for CID in the central hexapole by optimizing settings for the m/z 126 daughter ion from 275 in MS2. Generally, the collision gas was set to 2.5×10^{-3} mbar. Increasing the photomultiplier setting 100–150 above the regular 650 V sufficiently increased the sensitivity. In general, the source cone voltage was set at +27 V, the collision energy was set between −25 and −70 V, the capillary of the ESI probe was set at +3.70 kV and the HV lens was set at +0.87 kV. Source temperature was set between 120–140°C.

Sample preparation

Ropivacaine·HCl·H_2O was utilized (Astra Pharmaceutical Products, Westboro, Massachusetts, USA). The 3-hydroxyropivacaine was prepared as described previously (4). Urine was collected from horses 2 h after subcutaneous administration of ropivacaine (400 mg) and stored at −20°C until analysis. The urine was passed through a filter (~30 000 mw cutoff Centrifree filter; Amicon Inc.,

Table 1. Summary of mass spectral fragments derived from ropivacaine and 3-hydroxyropivacaine by ESI-MS and ESI-MS/MS

Compound	Spectrum type	Ions measured, m/z (relative intensity)
Ropivacaine standard	Full scan	275 (M+H 70%), 126 (100%)
Ropivacaine standard	275 m/z daughter	275 (M+H, 100%), 242 (10%), 186 (5%), 126 (30%)
3-hydroxyropivacaine standard	Full scan	291 (M+H, 80%), 126 (30%), 114 (10%), 61 (100%), 44 (5%)
3-hydroxyropivacaine standard	291 m/z daughter	291 (M+H, 100%), 126 (80%)

Division of Millipore, Beverly, Massachusetts, USA) to remove high molecular weight materials. Specifically, 900 μL of urine was centrifuged 60–90 min at 2000 rpm in a swinging bucket rotor (Type AH-4 rotor, Beckman AccuSpinFR centrifuge; Beckman, Palo Alto, California, USA). The filtrate was diluted 1:10 with 50:50 (v/v) methanol:1% formic acid (aq). The mixture was infused at 0.9 mL/h with a Harvard syringe pump equipped with a 500 μL Hamilton gas-tight syringe. Infusion was direct into the electrospray probe of the Quattro II MS/MS set in positive mode.

For glucuronide cleavage experiments, urine from horses treated 2 h previously with a 400 mg subcutaneous dose of ropivacaine was treated for 3 h at 65°C with β-glucuronidase (Type L-II; Sigma Chemical Company; 1000 units added per mL of urine, previously brought to pH 5 with 0.25 M sodium acetate, final concentration). Resultant mixtures were centrifugally filtered and diluted.

Results

MS/MS identification of ropivacaine and its characteristic fragmentation pattern

Figure 1 is the proposed structure for the glucuronide of 3-hydroxyropivacaine, which has a molecular weight of 466, which becomes 467 after protonation with H^+. Dashed lines show the cleavages that occur to produce the more common fragments (m/z 126, 84, and 291). The m/z 126 fragment is characteristic of ropivacaine-related species, with an intact N-propyl piperidinyl ring, and can be seen both in ESI and electron impact (EI) spectra.

Table I lists the ropivacaine mass spectra obtained by direct infusion electrospray ionization in positive mode for the full-scan spectrum and for the daughter ion spectrum of the m/z 275 ion after CID using argon gas and measured in the second quadrupole. Both spectra were obtained with ropivacaine at 10 μg/mL in a 50:50 (v/v) mixture of 0.4% formic acid:methanol solution. Peaks m/z 242 and 186 are related to each other according to daughter ion analysis and represent an unidentified contaminant in the ropivacaine standard. Following the full-scan electrospray ionization, the m/z 275 ion had an abundance of 1.32×10^8, and the abundance of the m/z 126 ion was 30% of the parent ion. After CID, the abundance of the m/z 126 ion was 6.5×10^7, and the m/z 275 ion was 65% of the daughter ion. Also included in Table I is the full-scan mass spectrum and the daughter ion spectrum of the m/z 291 ion from the 3-hydroxyropivacaine standard. The abundance of the m/z 126 ion is roughly 30% of the m/z 291 ion in full scan, whereas the abundances of daughter ions are roughly the same as for the m/z 291 parent. The spectrum for the m/z 291 ion from β-glucuronidase-treated horse urine matched that of 3-hydroxyropivacaine.

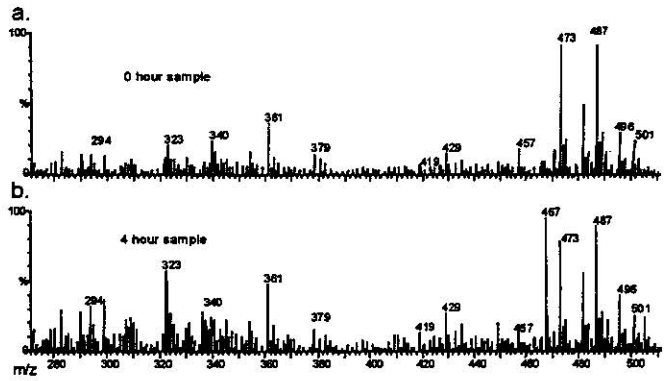

Figure 2. Full-scan electrospray of a) urine samples from pretreatment (control) horse, and b) urine sample taken 4 h after ropivacaine treatment. Note the appearance of the m/z 467 ion peak in the sample collected after ropivacaine administration.

MS/MS identification of the m/z 467 peak

Figure 2a shows the full-scan ESI^+ mass spectrum of a control urine sample collected before administration of ropivacaine. Figure 2b is the full-scan mass spectrum of a urine sample collected 4 h after ropivacaine adminstration. Note the appearance of the m/z 467 peak in the ropivacaine-treated urine, suggesting the presence of hydroxyropivacaine glucuronide (Figure 1) in this sample. Based on intensities alone, the m/z 291 ion is attributed to no more than ~10% that of the m/z 467 ion, although subsequent experiments demonstrated that almost none of this 291 ion is derived from free hydroxyropivacaine, but rather from background ions (data not shown).

CID of m/z 467 peak to yield m/z 126

Daughter ion spectra were recorded for the m/z 467 peak following CID of urine samples from horses treated with ropivacaine. Measurements were made at 2 different collision energies for induction of different levels of fragmentation (data not shown). Under relatively low collision energy of −25 V, the putative hydroxyropivacaine glucuronide at m/z 467 yielded a daughter ion of mass 126, the major fragmentation product of ropivacaine (Table I). The abundance of the m/z 126 daughter ion is only about one-eighth that of the m/z 467 parent ion. A higher collision energy of −70 V resulted in a dramatic decrease in the abundance of the parent ion and an increase in the abundance of the m/z 126 daughter ion. Table II lists the principal ions derived under these conditions from the 467 m/z daughter ion spectrum. Since the m/z 126 fragment is a characteristic fragmentation product of ropivacaine, these findings support the possibility that the m/z 467 material represents hydroxyropivacaine glucuronide. A control daughter ion

Table 2. Interpretation of the principal ions derived from daughter ion analysis of the putative hydroxyropivacaine glucuronide with a collision energy of −70 V

Ion (m/z)	Relative intensity (%)	Interpretation	Supported by
43	4.1	n-propyl group	
55	2.5	n-buty fragment of piperidine ring with a shift of a proton	56 m/z in EI-MS of ropivacaine
84	8.4	N-Dealkylated piperidine ring + 1, deriving from 126 m/z minus propyl group with proton shift	84 m/z in EI-MS of ropivacaine
97	3.1	N-(n-propyl)-piperidine fragment (126 m/z) minus ethyl group with shift of proton	98 m/z in EI-MS of ropivacaine
126	100	N-(n-propyl)-piperidine fragment	base peak in ropivacaine and 3-hydroxyropivacaine-TMS ether
165	2.0	291 m/z minus N-(n-propyl)-piperidine fragment (126 amu)	
248	2.4	291 m/z minus n-propyl group (43 amu)	
291	6.5	Loss of glucuronic acid moiety by hydrolysis	
467	23.5	Parent molecule + 1	

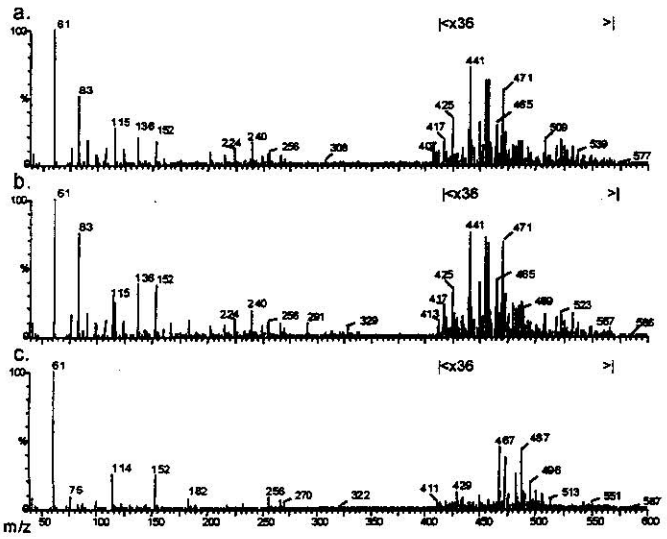

Figure 3. Full-scan electrospray of a) pretreatment urine sample with β-glucuronidase added, b) urine sample collected 4 h after treatment, with β-glucuronidase added, and c) urine sample collected 4 h after treatment without β-glucuronidase added. Note the presence of the m/z 467 ion in 3c and the appearance of the m/z 291 ion in 3b. Note also the lack of m/z 291 in 3a and 3c and the lack of m/z 467 in 3a and 3b. Peaks have been magnified 36-fold for clarity between m/z 410–570.

spectrum obtained for m/z 467 in pretreatment urine (data not shown) evidenced none of the peaks listed in Table II, supporting the contention that they are unique to ropivacaine treatment.

β-Glucuronidase hydrolysis of the m/z 467 species (putative hydroxyropivacaine glucuronide) to yield m/z 291 (putative hydroxyropivacaine)

Figure 3a is a full-scan electrospray of a pretreatment urine sample following β-glucuronidase hydrolysis. There is no m/z 467 peak, which is consistent with the absence of hydroxyropivacaine glucuronide, nor is there a peak at m/z 291, consistent with the absence of hydroxyropivacaine. Similarly, Figure 3b is a full-scan electrospray of urine collected after ropivacaine administration treated with β-glucuronidase. Again, there is no peak at m/z 467. However, the appearance of the m/z 291 ion illustrates the presence of the hydroxyropivacaine ion after the glucuronide moiety has been hydrolyzed with the enzyme. Figure 3c is an electrospray of a urine sample collected after ropivacaine administration to which β-glucuronidase has not been added. Note the abundance of the characteristic m/z 467 fragment, consistent with the presence of hydroxyropivacaine glucuronide and the absence of a m/z 291 peak.

Daughter ion spectra were measured after CID for the m/z 291 ion in β-glucuronidase treated samples. The m/z 291 daughter ion spectrum for a 0 h sample treated with β-glucuronidase yielded only meager, low-intensity fragments at m/z 207, 123, and 80 and was no match for the hydroxyropivacaine standard described in Table I. In contrast, the 4 h urine sample treated with β-glucuronidase provided a m/z 291 daughter ion spectrum that was an excellent match for the 3-hydroxyropivacaine standard described in Table I, with the exception of minor traces of m/z 123 and 207 ion that are presumably matches to background ions seen in the 0 h sample. The abundances of the m/z 291 ion in the 0 and 4 h samples were 4.01×10^4 and 2.84×10^5, respectively. The relatively small amount of the m/z 291 ion in the 0 h sample is background and is, therefore, independent of the m/z 291 ion resulting from β-glucuronidase treatment of urine collected after ropivacaine dosing. Furthermore, the daughter ion spectrum for the pretreatment sample did not contain the m/z 126 ion, which is the ionized N-(n-propyl)-piperidine fragment.

Discussion

The goal of this research was to identify the glucuronide metabolite of metabolized ropivacaine in urine. The data demonstrate the presence of a 467 amu molecule in urine after ropivacaine administration, which is consistent with the molecular weight of hydroxyropivacaine glucuronide. The molecule was not found in a pre-adminstration urine sample, suggesting that its presence was due to ropivacaine administration, nor was it present in a sample taken after ropivacaine administration to which β-glucuronidase had been added. Considering that the addition of β-glucuronidase eliminated the m/z 467 species, the hypothesis that the m/z 467 molecule is the glucuronide of hydroxyropivacaine is further supported

by the appearance of a m/z 291 fragment, which corresponds to the calculated molecular weight of hydroxyropivacaine + 1. Similarly, when the m/z 467 ion was subjected to CID, the fragments of this molecule were consistent with it being a hydroxyropivacaine glucuronide. Taken together, the data strongly suggest that the m/z 467 molecule identified in urine collected after ropivacaine administration is hydroxyropivacaine glucuronide.

The principal ions derived by fragmentation of the putative hydroxyropivacaine glucuronide and observed via m/z 467 daughter ion analysis are listed in Table II. As predicted for a ropivacaine derivative, the base peak by electrospray ionization (ESI/MS/MS) at higher collision energies is m/z 126, which is obtained by the cleavage shown in Figure 1 and is identical in value and, presumably, in structure, to the base peak obtained by electron impact (EI-MS) fragmentation from ropivacaine and 3-hydroxyropivacaine-trimethyl silyl ether (data not shown), implying a similar cleavage mechanism.

The m/z 55 ion remains unassigned, but like ions 84 and 97, there may be a corresponding ion in the parent ropivacaine EI mass spectrum, which would have been obtained with a more fragment-inducing technique. It is reassuring that peaks requiring 2 fragmentation events (m/z 248 and 165) are of the lowest intensity (ie, of decreasing likelihood). In summary, the assignments, while not proven, are nonetheless internally consistent and may be considered as additional support for our conclusions.

The tentative assignment of the structure for the glucuronide of the principal ropivacaine metabolite has 2 facets for which additional support is merited. One is the implication that the glucuronide is attached through an O-ether linkage, the other is the possibility of positional isomerism for the initial hydroxylation on the aromatic ring.

At the present time, the internal consistency of the fragmentation analysis is offered as support that the linkage is to a phenolic oxygen. Other sites for oxidation are generally excluded; for example, by the retention of the intact N-(n-propyl)-piperidine ring, as signified by the m/z 126 base peak. The possibility of aromatic hydroxylation followed by glucuronic acid conjugation at a remote site such as at the amide linkage is a formal possibility, albeit one with little precedent.

The other problem is more serious, as, admittedly, the m/z 467 ion peak can easily represent the glucuronide conjugated at either the 3 or 4 position, or some mixture of the two. For the moment, bupivacaine, a structurally related local anesthetic with an N-(n-butyl) substitution on the piperidine ring, is offered for comparison. De novo synthesis of both 3- and 4-positional isomers enabled chromatographic and EI mass spectral verification that bupivacaine is hydroxylated at the 3-position, with no 4-position variant present in measurable quantities (7). The hydroxyropivacaine glucuronide is, thus, hypothesized to be the 3-hydroxyropivacaine-O-glucuronide. Chromatographic isolation of the naturally-occurring glucuronide and its de novo organic synthesis will provide ideal proof of that contention.

The physiological role of glucuronide metabolites is well understood. By increasing the polarity of the drug-glucuronide complex, these agents are rapidly cleared by glomerular filtration and are found in relatively high concentrations in equine urine. As such, these agents may be more concentrated in equine urine than are the parent drug or other metabolites and are likely to appear in forensic screening. It is interesting to note that the critical forensic metabolites of lidocaine, mepivacaine, bupivacaine and ropivacaine are all glucuronide metabolites and that these conjugates are the metabolites of the agents most commonly identified in forensic samples. Therefore, these agents are of considerable forensic importance, and their proper identification and the correct description of their role in the urinary elimination of drugs is highly significant.

To our knowledge, this is the first direct identification in equine urine of a drug metabolite glucuronide. Previous work with glucuronides has always required hydrolytic cleavage of the metabolite glucuronide bond, resulting in release of the free drug or a hydroxylated metabolite with subsequent identification of the actual material released from the glucuronide complex. Thus, the evidence heretofore available for the identification of glucuronide metabolites in equine urine has almost always been indirect. In this regard, many chemical identification reports state that the material identified is the compound actually present in the urine sample, when, in fact, the material identified is a fragment hydrolyzed from the actual conjugated material in the urine sample. If the analytical identification has been made in the context of a forensic finding, such reporting may be significantly in error.

Acknowledgments

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