Direct MS-MS identification of isoxsuprine-glucuronide in post-administration equine urine

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Abstract

Isoxsuprine is routinely recovered from enzymatically-hydrolyzed, post-administration urine samples as parent isoxsuprine in equine forensic science. However, the specific identity of the material in horse urine from which isoxsuprine is recovered has never been established, although it has long been assumed to be a glucuronide conjugate (or conjugates) of isoxsuprine. Using ESI/MS/MS positive mode as an analytical tool, urine samples collected 4–8 h after isoxsuprine administration yielded a major peak at m/z 554 that was absent from control samples and resisted fragmentation to daughter ions. Titration of this material with increasing concentrations of sodium acetate yielded m/z peaks consistent with the presence of monosodium and disodium isoxsuprine-glucuronide complexes, suggesting that the starting material was a dipotassium-isoxsuprine-glucuronide complex. Electrospray ionization mass spectrometry negative mode disclosed the presence of a m/z 476 peak that declined following enzymatic hydrolysis and resulted in the concomitant appearance of peaks at m/z 300 and 175. The resulting peaks were consistent with the presence of isoxsuprine (m/z 300) and a glucuronic acid residue (m/z 175). Examination of the daughter ion spectrum of this putative isoxsuprine-glucuronide m/z 476 peak showed overlap of many peaks with those of similar spectra of authentic morphine-3- and morphine-6-glucuronides, suggesting they were derived from glucuronic acid conjugation. These data suggest that isoxsuprine occurs in post-administration urine samples as an isoxsuprine-glucuronide conjugate and also, under some circumstances, as an isoxsuprine-glucuronide-dipotassium complex.

Résumé

En expertise médico-légale équine, l'isoxsuprine détectée de routine à partir d'échantillons d'urine soumis à une hydrolyse enzymatique est considérée comme étant l'isoxsuprine initiale. L'identité spécifique du matériel à partir duquel l'isoxsuprine est détectée dans l'urine de cheval n'a jamais été clairement établie, même si on suspecte depuis un certain temps qu'il s'agisse d'un conjugé (ou de conjugés) d'isoxsuprine et de glucuronide. Des échantillons d'urine prélevés 4–8 h après administration d'isoxsuprine et analysés par ESI/MS/MS en mode positif ont permis de mettre en évidence un pic majeur (m/z de 554) qui était absent des échantillons témoins et qui était résistant à une fragmentation en ions de descendance. La titration de ce matériel avec des solutions de plus en plus concentrées d'acétate de sodium a démontré la présence de pics m/z concordant avec la présence de complexes glucuronide-isoxsuprine monosodique ou disodique, ce qui laisse à penser que le matériel initial serait un complexe de glucuronide et d'isoxsuprine dipotassique. En mode négatif, l'analyse par ESI/MS/MS a mis en évidence la présence d'un pic à 476 m/z. Toutefois, ce dernier a diminué suite à l'hydrolyse enzymatique, en même temps qu'apparaissaient deux nouveaux pics à 300 et 175 m/z qui sont conséquents avec la présence d'isoxsuprine (m/z de 300) et de résidus d'acide glucuronique (m/z de 175). L'examen du spectre des ions de descendance de ce supposé conjugé isoxsuprine-glucuronide a permis de constater que plusieurs pics chevauchaient ceux de spectres similaires obtenus de conjugés de morphine-3- et morphine-6-glucuronide, ce qui suggèrent qu'ils sont dérivés d'une conjugaison à l'acide glucuronique. Les résultats indiquent que suite à l'administration d'isoxsuprine, cette dernière se retrouve dans l'urine sous forme d'un conjugé isoxsuprine-glucuronide et que, dans certaines cirsonstances, sous forme d'un complexe glucuronide-isoxsuprine dipotassique.

(Traduit par docteur Serge Messier)

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Introduction

Isoxsuprine (4-hydroxy-α-[1-[(1-methyl-2-phenoxy-ethyl)amino] ethyl]-benzenemethanol, hydrochloride) is a vasodilating agent. While it is speculated that isoxsuprine has some β receptor activity, it appears to behave in a papaverine-like manner, since propranolol, a β antagonist, does not appear to affect the efficacy of isoxsuprine (1). In humans, it has been used to treat cerebral vascular insufficiency, peripheral vascular disease, and to control premature labor (2), although it should be noted that it is now rarely prescribed in human medicine. However, in equine medicine, isoxsuprine has long been recommended for the treatment of navicular disease and other lower-limb problems (3).

Isoxsuprine is frequently identified in equine urine samples collected for post-performance drug screening (4,5). Among the reasons for such frequent discovery of this agent are that doses are relatively large, and the administration period may be for months or even years. Additionally, isoxsuprine apparently binds to melanin and may sequester in body fat (6).

Furthermore, isoxsuprine is efficiently converted to glucuronide metabolite(s), which are then excreted at high concentrations in equine urine. In this regard, Joujou-Sisic et al (7) have recently shown that orally administered isoxsuprine is 98% metabolized by the liver before it reaches the systemic circulation in the horse. This is a classic "first pass" effect, which greatly reduces the pharmacological effectiveness of orally administered isoxsuprine while at the same time increases its urinary excretion as the glucuronide metabolite.

The current methodology used in equine forensic science for detection of isoxsuprine consists of enzymatic hydrolysis of urine and recovery of parent isoxsuprine from the urine sample (8). While it is generally assumed that the material present in equine urine is a glucuronide-conjugated metabolite of isoxsuprine, these conjugated materials have never been directly identified in a post-administration urine sample. This communication reports the direct identification of a major glucuronide metabolite of isoxsuprine in post-administration urine samples, and describes at least one major form in which isoxsuprine-glucuronide may exist under some circumstances.

Materials and methods

Horses/drug administration/sample collection

Two mature Thoroughbred mares, weighing 513 and 602 kg, were acclimated to their stalls 24 h prior to experimentation. The animals were fed twice a day with grass hay and feed (12% protein), which was a 50:50 mixture of oats and an alfalfa-based protein pellet. The animals were vaccinated annually for tetanus and were dewormed quarterly with ivermectin (MSD Agvet, 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. Each mare served as its own control. 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. Isoxsuprine (2 g) was administered to horses, orally, in the form of isoxsuprine tablets (Isoxsuprine-HCl, Amide Pharmaceuticals, Inc., Little Falls, New Jersey, USA). Urine samples were collected immediately before and at 4, 6, and 8 h after administration using a Harris flush tube (24 Fr × 60 in; Seamless, Ocala, Florida, USA). Urine samples were divided into aliquots stored at −20°C until assayed.

Sample preparation collection

The isoxsuprine standard for mass spectral analysis was purchased as isoxsuprine-HCl (Sigma Chemical Co., St. Louis, Missouri, USA). Pre- and post-administration urine samples were filtered by centrifugation for 90 min 1200 × *g* in a swinging bucket rotor centrifuge (Type AH-4; Beckman AccuSpinFR) through a ~3000 molecular weight cutoff filter (Centrifree filter, Amicon Inc., Beverly, Massachusetts, USA) to remove high molecular weight materials. The filtrate was then diluted 1:10 with a mixture of 50:50 acetonitrile and 0.05% formic acid (aq) for positive mode mass spectrometry (MS), and with a mixture of 50:50 acetonitrile and 0.5% (v/v) NH_4OH (aq, from conc) for negative mode MS. The mixture was infused at 0.6 mL/h via 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 (Micromass, Beverly, Massachusetts, USA).

β-glucuronidase hydrolysis

For β-glucuronidase hydrolysis, the urine samples were treated for either 3 h at 65°C with *Patella vulgata* β-glucuronidase (1000 units of Sigma Type L-II/mL urine brought to 0.25 M sodium acetate, pH 5) or for 16 h at 37°C with *Helix pomatia* β-glucuronidase (1000 units of Sigma Type H-5/mL urine brought to 0.25 M sodium acetate, pH 5) (9). The resultant hydrolysates were centrifuged, filtered, and diluted in acid or base as described above.

MS/MS analysis

Full scan electrospray ionization (ESI) mass spectra were obtained on analytical standards at 10 µg/mL in 50:50 acetonitrile:0.05% formic acid (aq), pH 4, by infusion at 0.6 mL/h via a Harvard syringe pump into the electrospray probe of a Micromass Quattro II MS/MS set in positive ion mode. Negative mode spectra were obtained similarly but with dissolution in 50:50 acetonitrile:0.5% (v/v) NH_4OH (aq, from conc), pH 9. 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.

MS/MS tuning

The mass spectrometer was tuned for positive ion spectra by direct infusion of 10 ng/µL isoxsuprine in 50:50 acetonitrile:0.05% formic acid (aq). The peak shape and intensity of the monoprotonated isoxsuprine m/z 302 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. Negative ion mode was optimized similarly with focus placed on the isoxsuprine monodeprotonated m/z 300 ion and its dehydrated m/z 282 product as

visualized in 50:50 acetonitrile:0.5% (v/v) NH_4OH (aq, from conc). Collision gas (argon) and collision energy were adjusted for collisionally-induced dissociation (CID) in the central hexapole by optimizing settings as needed for the second quadrupole. Generally, the collision gas was set to 1–3 × 10^{-3} mbar. Increasing the photomultiplier setting 100–150 V above the regular 650 V sufficiently increased sensitivity. In general, for positive mode, the source cone voltage was set high at +70 to 80 V, the collision energy was set between −25 and −70 V, the capillary of the ESI probe was set at +3.0 kV, the skimmer at 0.6 V, and the HV lens was set at +0.57 kV. For negative mode, the source cone voltage was set at 23 V, the collision energy was set between −25 and −70 V, the capillary of the ESI probe was set at +2.6 kV, the skimmer at 1.3 V, and the HV lens was set at +0.50 kV. Source temperature was set at 80°C.

Results

Under ESI positive conditions (ESI^+) in pH 4 acidic solution, isoxsuprine presented as a m/z 302 cation by ESI^+-MS/MS (Figure 1), presumably due to protonation at the pair of nonbonding electrons of the secondary amine. The daughter ions of this protonated form of isoxsuprine include an ion at m/z 284 (presumably due to the loss of a H_2O molecule [-18 amu] from the m/z 302), an ion at m/z 190 (due to loss of a phenyloxy +H [-94 amu] from m/z 284), and a major peak at m/z 150 (due to loss of 1-oxophenyl-2-methyl-2-aminoethyl fragment). Other major daughter ions include the phenyloxypropyl fragment (m/z 135), and the phenyloxymethylene fragment (107 m/z; Table 1).

Under ESI negative conditions (ESI^-) in pH 9 basic solution, isoxsuprine (final concentration: 10 µg/mL) presented as an anion due to deprotonation at the relatively acidic phenolic group. Anionic isoxsuprine generated a family of daughter ions from the m/z 300 molecular ion, as shown in Figure 2. This scan was performed with ESI^--MS/MS to serve as a baseline for future biological sample scans.

Anionic isoxsuprine provided its most definitive daughter ion peak at m/z 282, which represents isoxsuprine minus a water molecule, and is most likely from the benzylic hydroxyl group. The intensity of the m/z 282 peak is 25% that of the m/z 93 base peak; the small peak at m/z 300 represents the isoxsuprine anion and is only 2% the size of the base peak under the conditions of the experiment. A similar loss of a water molecule occurs from cationic isoxsuprine seen under ESI^+ conditions, except that the base peak is the m/z 302 molecular ion.

Mass spectra obtained in the ESI^+ mode (pH 4) from post-administration urine samples displayed a peak at m/z 554. Figure 3 shows the decreasing concentration of the m/z 554 ion and the increasing concentration of the m/z 522 ion as the urine samples were titrated with increasing concentrations of Na^+.

Figure 4 shows the full MS scan for urine samples collected at 0, 4, 6, and 8 h after isoxsuprine administration analyzed in ESI^- ion mode. For the 4, 6, and 8 h samples, the analysis yielded a molecular ion at m/z 476, the expected molecular ion for isoxsuprine-glucuronide. This ion was absent in samples taken prior to administration. Analysis of the daughter ions of this peak for the 4 h sample shows the presence of ions characteristic of isoxsuprine (Figure 5).

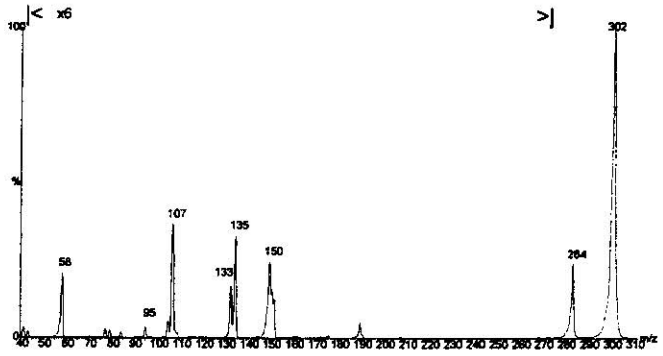

Figure 1. ESI^+ continuum spectrum of isoxsuprine in acidic solution (pH 4), as derived by daughter ion analysis of m/z 302 isoxsuprine cation. Argon was the collision gas. Note scale expansion for 40–270 m/z

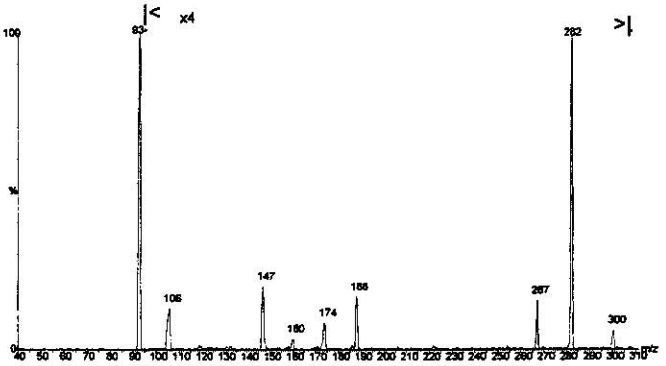

Figure 2. ESI^- continuum spectrum of isoxsuprine in basic solution (pH 9), as derived by daughter ion analysis of m/z 300 isoxsuprine anion. Note scale expansion for m/z 95–310 range.

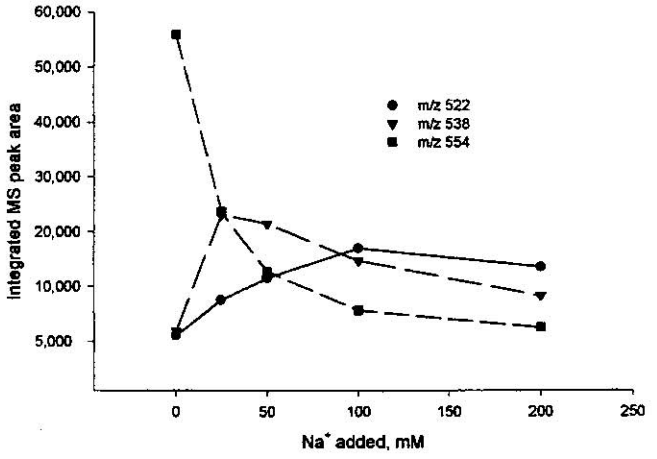

Figure 3. Integrated mass spectral peak areas for isoxsuprine-glucuronide with Na^+ acetate titration as measured by ESI^+.

Figure 6 shows the effect of β-glucuronidase hydrolysis on the intensity of the peaks at m/z 476 and at m/z 300 following analysis of the 4 h samples in the ESI- mode. Exposure of the sample to β-glucuronidase under standard hydrolysis conditions resulted in a 6-fold decrease of the m/z 476 peak, while the m/z 300 peak increased 15 fold in intensity. This data is further support of the hypothesis that urine samples from horses treated with isoxsuprine contain an isoxsuprine-glucuronide species.

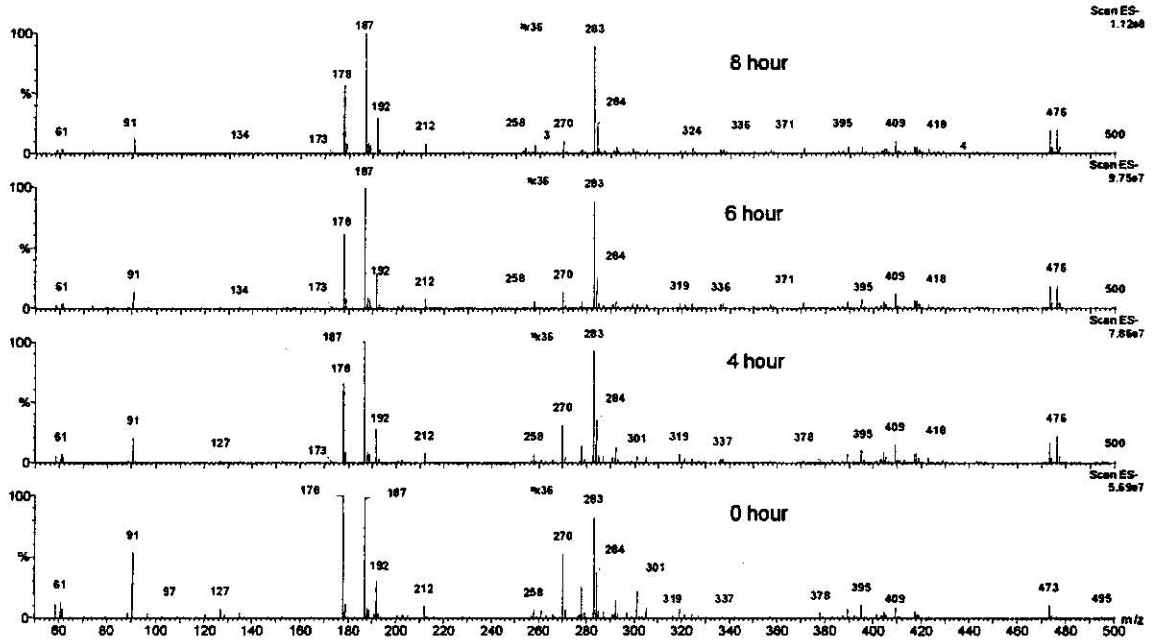

Figure 4. Full MS centroid scans by ESI⁻ mode for urine samples taken 0, 4, 6, and 8 h after isoxsuprine administration. Note that values above m/z 250 have been enlarged to show detail.

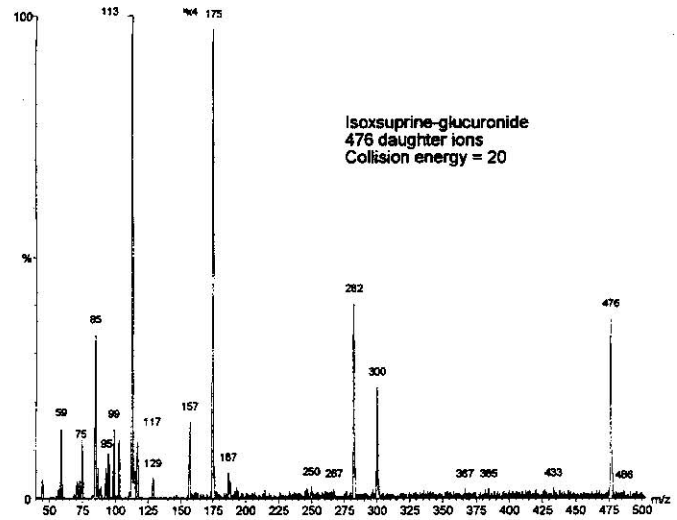

Figure 5. Daughter ion continuum scan in ESI⁻ mode of the m/z 476 material from a urine sample collected 4 h after isoxsuprine administration. No enzymatic hydrolysis was attempted. Note that the data above m/z 150 were magnified (× 4) to show detail.

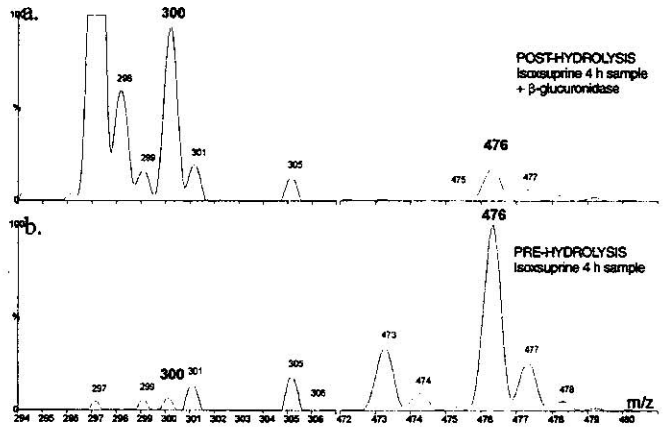

Figure 6. Comparison of 4 h urine samples (a) with and (b) without standard β-glucuronidase treatment by ESI⁻ mode continuum scanning. Note the nearly 6-fold reduction in intensity of the m/z 476 ion and the 15-fold increase of the m/z 300 ion following enzyme treatment.

Discussion

When isoxsuprine is reported as a forensic identification (a "positive") in a post-administration sample, the urine sample has usually been subjected to glucuronidase hydrolysis, and free isoxsuprine has been recovered from the urine. In this regard, the wording of the forensic report often implies or explicitly states that the urine sample contains isoxsuprine. However, the identified isoxsuprine is almost certainly not parent isoxsuprine, but rather a poorly extractable glucuronide metabolite (or metabolites) of isoxsuprine that yields recoverable isoxsuprine only after enzymatic hydrolysis (11).

Current practices in equine forensic chemistry suggest that isoxsuprine exists in equine urine as glucuronide conjugates, based on the need to perform a β-glucuronidase hydrolysis step to recover significant concentrations of isoxsuprine from urine samples. The calculated mass of isoxsuprine-glucuronide ($C_{24}H_{31}NO_9$) is 477. Therefore, in post-administration urine samples, the complex should present at m/z 476 as a singly charged anion, or as a monoprotonated cation at m/z 478, depending on urine pH.

This report directly identifies specific evidence for such glucuronide metabolite(s) of isoxsuprine in post-administration urine samples. Parent and daughter ions of authentic isoxsuprine were first characterized by MS-MS identification. Authentic isoxsuprine

fragmented in characteristic patterns following both negative and positive ESI analysis. Based on the characteristic fragmentation patterns of isoxsuprine, urine samples from horses treated with the agent were examined for the presence of isoxsuprine-glucuronide conjugates.

The urine samples were first analyzed by ESI^+ MS/MS. Surprisingly, the samples showed the presence of a m/z 554 ion, with no evidence of the expected m/z 478 ion. Furthermore, the 554 material could not be fragmented by altering the analysis conditions; therefore, its composition could not be simply interpreted by analysis of its daughter ions. Review of these results suggests that m/z data on this material could be explained by an isoxsuprine-glucuronide-dipotassium complex.

To test this hypothesis, increasing concentrations of sodium acetate were added to the samples. The added Na^+ ions displaced the loosely bound K^+ by mass action from the m/z 554 isoxsuprine-glucuronide-dipotassium complex and generated the appearance of complexes of m/z 538 (554 minus 16) and m/z 522 (554 minus 32). Furthermore, as the sodium concentration increased from 0 to 100 mM, the peak areas of m/z 554, 538, and 522 complexes shifted appropriately. Under the highly acidic conditions of the ESI^+ MS/MS experiments, it appears that the isoxsuprine-glucuronide complex binds native potassium ions in the urine to yield a relatively stable isoxsuprine-glucuronide-dipotassium complex of m/z 554.

The samples were next analyzed by ESI^- MS/MS, and an apparent isoxsuprine-glucuronide conjugate of the predicted m/z 476 was identified. To establish that certain of the daughter ions of the m/z 476 material represented fragments of the glucuronide portion of isoxsuprine-glucuronide, they were compared with the fragmentation patterns of morphine-3- and morphine-6-β-glucuronides, 2 commercially available drug-glucuronide conjugates. The appropriate daughter ion masses of the isoxsuprine-glucuronide and the morphine-3- and -6-β-glucuronides show excellent correspondence. These findings are consistent with the concept that the m/z 476 material identified in post-administration urine samples is indeed an isoxsuprine-glucuronide conjugate. In further support of this interpretation, when the urine samples were subjected to β-glucuronidase hydrolysis, the m/z 476 material representing the putative isoxsuprine-glucuronide declined in intensity, and there was a concomitant appearance of a peak at m/z 300, representing anionic isoxsuprine.

In summary, this study shows that urine samples from horses treated with isoxsuprine and examined by ESI^- mode contained a material of m/z 476 that was consistent with an isoxsuprine-glucuronide complex. Under acidic conditions, the material bound 2 potassium ions and presented in MS-MS spectra as a relatively stable m/z 554 material, which appeared to be a dipotassium-isoxsuprine-glucuronide complex. Furthermore, the m/z 554 material could not be fragmented to yield daughter ions. Under alkaline conditions the urine samples yielded an expected m/z 476 peak, consistent with the presence of an isoxsuprine-glucuronide conjugate. When the urine samples were subjected to β-glucuronidase hydrolysis, the m/z 476 peak declined, and m/z 300 and 175 peaks appeared, consistent with hydrolysis of the isoxsuprine-glucuronide conjugate to yield free isoxsuprine (m/z 300) and free glucuronic acid residue (m/z 175). Therefore, the data establishes that at least one source of the material in equine urine from which isoxsuprine is recovered is an isoxsuprine-glucuronide conjugate.

What these experiments do not address is the actual chemical structure of the isoxsuprine-glucuronide conjugate. There are two potential sites for substitution by the glucuronic acid moiety on the parent isoxsuprine molecule. Both sites are hydroxyl groups, one is the terminal phenoxy group and the other is the internal benzylic alcohol group. At this time, it is reasonable to assume that the substitution site utilized by the glucuronic acid moiety is on the phenoxy-hydroxyl group, but the benzylic site cannot be excluded by current data.

However, it should be noted that suggested preference of phenolic conjugation is tentative and requires confirmation with definitive data. Continued work utilizing gas chromatography/mass spectrometry GC/MS and organic synthesis of authentic standards is warranted to determine the exact site of substitution.

A further point of interest in these findings is the identity of the form of the glucuronide metabolite normally occurring in equine urine. At this point, the physiological and pharmacological relevance of the dipotassium isoxsuprine-glucuronide complex identified in acidified urine samples remains unclear.

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