References, 9, 14

#266

Further characterization of equine isoxsuprine metabolites by electrospray ionization tandem mass spectrometry

AF Lehner, A Hunsucker, JM Bosken, JD Harkins, WE Woods, W Karpiesiuk, P Almeida, L Dirikolu, WG Carter, J Boyles, C Holtz, M Fisher and Thomas Tobin

Abstract

Electrospray ionization tandem mass spectrometry (ESI-MS/MS) was applied to equine post-isoxsuprine administration urine to characterize the metabolite(s) present for this drug. In this report, we demonstrate that glucuronidase-hydrolyzed post-isoxsuprine urine samples evidenced a pattern of oxidative metabolites each on the order of 1% abundance or less including, among others, ~~a~~ phenolic ring hydroxylation, phenoxy ring hydroxylation and minor semiquininoid oxidation and paraquininoid oxidation products at the phenolic ring. Direct examination of urine by MS/MS showed a predominance of isoxsuprine-glucuronide versus free isoxsuprine. Unhydrolyzed samples examined by MS/MS applying collisionally induced dissociation under increasing levels of collision energy revealed potential information about the structure of the principal isoxsuprine glucuronides. The glucuronides present were identified by a unique combination of TMS derivatization and ESI-negative mode MS/MS. Several metabolites exist as glucuronides, including unmetabolized isoxsuprine and phenoxy and/or phenolic ring hydroxylated isoxsuprine. The site of glucuronidation of unmetabolized isoxsuprine was investigated preliminarily by model compound analysis and deconvolution of the relevant spectra, suggesting involvement of the benzylic alcohol function. Cone voltage optima assisted in interpretation and assignment of spectra and revealed that isoxsuprine-glucuronide occurred in complexes with urine components, including potassium ions and various metabolites of unknown structure nascent to urine.

Introduction

Isoxsuprine is a vasodilating agent routinely used in treatment of horses for navicular and other circulatory disorders. While it has often been thought to be a weak β-adrenergic agonist (Needleman, et al., 1985) and α-1 selective antagonist (Elliott and Soydan, 1995), its pharmacological effects are unaffected by the presence of propranalol, a known β-antagonist (Manley and Lawson, 1968). Oral treatment with isoxsuprine continues to occur in spite of evidence suggestive of its possible inefficacy for such disease states (Harkins, et al., 1998; Ingle-Fehr, et al., 1999). Presumably, it is the high susceptibility of isoxsuprine to "first pass" metabolism in the liver following oral administration that is responsible for relatively low serum levels and therefore poor oral effect (Dumasia, 1992).

We have recently demonstrated the presence in equine urine of an isoxsuprine-glucuronide post-administration for isoxsuprine (Bosken, et al., 1999). The glucuronide was most readily observed by application of negative mode electrospray ionization with introduction to a dual quadrupole tandem MS/MS. High pH conditions established as

Abstract, text 7/2/99.

(Isoxsuprine-HCl, Amide Pharmaceuticals, Inc., Little Falls, NJ 07424). Urine samples were

conducive to presentation of native isoxsuprine as an anion were successfully applied to the glucuronide of isoxsuprine, and it was primarily extensions of these methods which yielded further understanding of the glucuronide and other metabolites of isoxsuprine presented in this paper.

MATERIALS & METHODS

Sample preparation

Isoxsuprine·HCl was purchased from Sigma Chemical Co., St. Louis, MO.

Urine from horses treated 4, 6 or 8 hours previously with a 2000 mg oral dose of isoxsuprine was passed through a ~3000 m.w. cutoff Centrifree filter (Amicon, Inc., Beverly, MA, division of Millipore) to remove high molecular weight materials. Specifically, 900 ul urine was centrifuged 90 mins at 2500 rpm in a swinging bucket rotor (Type AH-4) in a Beckman AccuSpinFR centrifuge. The filtrate was diluted 1:10 with 50:50 acetonitrile:0.05% formic acid (aq) for positive mode, and 50:50 acetonitrile:0.5% (v/v) NH_4OH (aq, from conc) for negative mode. The mixture was infused at 0.6 ml/hr via a Harvard syringe pump equipped with a 500 ul Hamilton gas-tight syringe. Infusion was direct into the electrospray probe of the Quattro II MS/MS.

For glucuronide cleavage experiments, urine from horses dosed with isoxsuprine was treated for 16 hours at 37° C with *Helix pomatia* β-glucuronidase (1000 units of Sigma Type H-5 per ml of urine brought to 0.25 M sodium acetate, pH 5) or with *Patella vulgata* β-glucuronidase (1000 units of Sigma Type L-II per ml of urine brought to 0.25 M sodium acetate, pH 5) for 3 hours at 65° C. Resultant mixtures were centrifugally filtered and diluted as described above, or analyzed by solid phase extraction (SPE) and GC/MS.

Solid phase extraction and derivatization

Clean Screen SPE columns (Worldwide Monitoring, Philadelphia, PA) were conditioned by sequential addition of 3 ml methanol, 3 ml deionized water, 1 ml 100 mM sodium phosphate buffer, pH 6.0, after which samples were loaded. The column was then washed sequentially with 2 ml deionized water, 2 ml 1 M acetic acid and 4 ml methanol. The drug metabolites were eluted with 3 ml dichloromethane/isopropanol/NH_4OH, 78/20/2, v/v/v. The eluent was evaporated to dryness at ~ 40 °C. under a stream of nitrogen gas. For derivatization, each dried sample was dissolved in 40 ul BSTFA + 1% TMCS (Pierce Chemicals, Rockford, Ill.), vortexed briefly, and incubated at 75 °C. for 45 mins to produce the (trimethyl silyl) (TMS) derivatives of isoxsuprine compounds.

MS/MS (General)

Full scan electrospray ionization (ESI) mass spectra were obtained on analytical standards at 10 ug/ml in 50:50 acetonitrile:0.05% formic acid (aq), approximate pH 3, by infusion at 0.6 ml/hr 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), approximate pH 10. 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/ul isoxsuprine in 50:50 acetonitrile:0.05% formic acid (aq). The peak shape and intensity of the monoprotonated isoxsuprine 302 m/z ion were optimized by adjustment of capillary, HV lens, cone voltage, skimmer lens and RF lens settings. Skimmer lens offset was left at 5v. Negative ion mode was optimized similarly with focus placed on the isoxsuprine monodeprotonated 300 m/z ion and its dehydrated 282 m/z 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 MS2. Generally the collision gas was set to 1-3 x 10^{-3} mbar. Increasing the photomultiplier setting 100-150 above the regular 650 volts was found to increase our sensitivity sufficiently. In general, for positive mode the source cone voltage was set high at +70-80 volts, the collision energy was set between -25 and -70v, the capillary of the ESI probe was set at +3.0 kvolts, skimmer at 0.6 volts and the HV lens was set at +0.57 kvolts. For negative mode the source cone voltage was set at 23 volts, the collision energy was set between -25 and -70v, the capillary of the ESI probe was set at +2.6 kvolts, skimmer at 1.3 volts and the HV lens was set at +0.50 kvolts. Source temperature was set at 80 ^{o}C.

Gas chromatography/mass spectrometry

GC/MS analysis. Injection of TMS derivatives was made through the 250° C injector port of a Hewlett-Packard 6890/5972 GC/MSD, with sample deposition onto a 30 m long x 0.25 mm i.d. x 0.25 u film thickness HP-5MS 5% phenyl-95% methylsiloxane column with oven programming beginning at 70° C (held for 2 min), then ramping at 20° C/minute to 280° C (held for 12 min). Under these conditions isoxsuprine-bisTMS typically eluted with a retention time of 12.4 min and metabolites fall within the 11-15 min range. The mass spectrometer is set up to acquire from 50-550 m/z at 1.53 scans/sec with a threshold of 150.

Results

Effects of β-glucuronidase on visualization of isoxsuprine metabolites

Direct infusion negative mode electrospray MS/MS analysis of urine post-isoxsuprine with scans for the isoxsuprine m/z 300 and isoxsuprine glucuronide m/z 476 anions indicated that the samples overwhelmingly consisted of the glucuronide (see Fig. 1). Careful GC/MS measurements being carried out in our lab pre- and post-glucuronidase have confirmed this difference and will be reported separately (Bosken, et al., 1999, in preparation). Comparison of the two enzymatic treatments for glucuronide release (see

methods for *Patella vulgata* and *Helix pomatia* enzyme treatments) discerned a preference in our hands for the *Helix pomatia* method for the following reasons: 1) as shown in Table 1, the tendency was for greater proportion of released isoxsuprine with *Helix pomatia*; 2) the overall stability of isoxsuprine under mildly acidic conditions was relatively better at 37° C for 16 hours in contrast to 65° C for 3 hours (data not shown); 3) although the percentage of free isoxsuprine relative to glucuronide did not vary greatly between the two enzymatic treatments (Table 1), overall isoxsuprine values ranged 10-50% greater with the *Helix pomatia* results, perhaps owing in part to the greater stability of the released product at 37° C compared to 65° C. Our suggested method for enzymatic release of the conjugate matched that devised by Joujou-Sisic, et al. (1996).

Regardless of the method of enzyme treatment, 100% conversion to free isoxsuprine was not achieved. Figure 2 shows the ions visible in urine to ESI-negative MS/MS following treatment with β-glucuronidase, both for a zero and 4 hour sample post-administration for isoxsuprine. Many of the peaks evident in the zero hour sample matched those in the 4 hour sample, with the exceptions that the four hour sample disclosed a relatively high 300 m/z peak (released isoxsuprine) and lower 476 m/z peak (unhydrolyzed glucuronide of isoxsuprine). Figure 3 demonstrates that daughter ions of the released isoxsuprine 300 m/z anion matched a standard of isoxsuprine with nearly superimposable peaks, with the exception of an additional peak at m/z 187, presumably an artifact of m/z 300 ion native to urine.

Isoxsuprine metabolites seen by GC/MS after β-glucuronidase treatment

Fig. 4 displays full scan GC/MS analysis of a 6 hour sample post-administration and cleaved by β-glucuronidase treatment. The lower part of the figure shows the total ion chromatogram, whereas the upper part shows the relative contribution of the m/z 178 ion as an ion chromatogram. The m/z 178 ion represents the (1-methyl-2-phenoxyethyl) aminoethyl cleavage product from isoxsuprine, and is the base peak for the bis-TMS derivative of isoxsuprine. It is thus a potential indicator of isoxsuprine-related species. Table 2 summarizes the metabolites identified; those at 12.0 min and 12.2 min appear to be trace contaminants of isoxsuprine preparations carried unchanged through the animal, and visible in both the standard and in the pharmaceutical preparation. The others (12.7, 12.8, 13.0, 13.7, 14.2 and 14.4 min) are oxidation products of various types, at least two of which have been identified previously as early metabolites by Dumasia and Houghton (1991).

Fragmentation of isoxsuprine-glucuronide molecules is dependent upon collision energy and cone voltage settings

Figure 5 demonstrates the effects of increasing collision energy in the argon-saturated intermediate gas cell during MS/MS of the isoxsuprine glucuronide; there was a typical diminution of the parental 476 m/z anion with concomitant generation of diagnostic

fragments. As mentioned in our previous paper, most of the lower masses (≤175 m/z) corresponded to fragments generated from the glucuronic acid residue, as verified by comparison to authentic standards of morphine-3β- or 6β-glucuronides (Bosken, et al., 1999).

Another factor of importance for fragmentation effects concerned the cone voltage setting as measured in the initial quadrupole on MS scan. The sampling cone represents the initial transition from near atmospheric pressure to vacuum, and changes in voltage settings can accelerate ions in their transit towards high vacuum beyond the skimmer lens. This acceleration can result in additional collisions and therefore fragmentation, and many of these initial fragments can be diagnostic for organic functionalities (Johnstone editor, 1998; Willoughby, et al., 1998). Fig. 6 demonstrates that there was a distinct optimum setting for visualization of the dipotassium isoxsuprine-glucuronide 554 m/z cation by ESI^+ MS/MS at approximately 100 volts (cf. Bosken, et al., 1999, for more information on this complex), and that the optimum was the same when viewed by either m/z peak intensity value or by peak area on continuum data. The 476 m/z isoxsuprine-glucuronide anion had a much lower cone voltage dependence, but expanded plot shows that there is an optimum at around 25 volts.

Identification of isoxsuprine metabolite peaks visualized by TMS derivatization and ESI-neg MS/MS analysis

Extracts prepared for TMS derivatization and GC/MS analysis were also examined by direct infusion electrospray MS/MS in order to generate mass spectra of isoxsuprine metabolite glucuronides or other conjugates that might remain in the preparations. It was hoped that such a study might a) reveal conjugates not amenable to hydrolysis, and/or b) provide additional mass spectral information about glucuronide structure. This study demonstrated 1) that the glucuronides of isoxsuprine could in fact be extracted by the SPE protocol designed for free isoxsuprine, and 2) that a number of conjugates and their derivatives could be identified, the majority of which should have been candidates for β-glucuronidase hydrolysis; this suggests that the enzyme degrades prior to completion of hydrolysis and that additional enzyme must be added if complete hydrolysis is desired.

Fig. 7 shows representative spectra obtained following solid phase extraction of enzyme-hydrolyzed urine with TMS derivatization, both before and after isoxsuprine administration. A number of new peaks are visible in the top section of the figure, specifically post-isoxsuprine administration, implying relatedness to isoxsuprine metabolites. Table 3 lists identities for many of the peaks as derived by deconvolution of corresponding daughter ion spectra derived individually for each peak. Note that according to our identifications, some of the peaks, e.g. m/z 282, 432, 486, 602 and possibly others, may represent fragmentation products arising simultaneously from metabolites introduced into the instrument, whereas the remainder would be intact

unfragmented derivatized metabolites. For example, 602 m/z could represent a simple dehydration product arising from 620 m/z.

Cone voltage effects on fragmentation of isoxsuprine-specific metabolites and their derivatives

Table 3 lists an additional measurement of interest, specifically the optimal cone voltage for visualization of individual TMS-derivatized isoxsuprine metabolites. Such measurements provide information graphically similar to that in Fig. 6. We believe that these measurements relate to organic functionalities on the compounds of interest. In general, based on the listed structural assignments, there are distinct trends that may be summarized as follows: 1) decarboxylations (ions 432 and 486 m/z) are optimally visualized at low cone voltage settings, 10 volts; 2) intact TMS derivatives of non-carboxy functionalities such as hydroxyl and amino groups are most easily visualized at moderately high cone voltages, roughly 50-70 volts (ions 602, 620, 692,764, 780 and 852 m/z); 3) dehydration occurs with optimum visibility at higher cone voltages (ion 282 m/z), roughly 80 volts. Figure 8 demonstrates the range of cone voltage settings utilized, and the corresponding intensity measurements for select isoxsuprine- and isoxsuprine-glucuronide-related peaks. It appears that optimal cone voltages for visualization of certain peaks can assist in the further identification of unknowns. Thus, for example, the 468 m/z ion shown in Fig. 8 may involve a dehydration event such as secondary dehydration of the 486 m/z ion, whereas certain ions listed in Table 3, such as 545 and 877 m/z, may have arisen from decarboxylations.

Model compound analysis and cone voltages

The significance of electrospray cone voltage determinations are further demonstrated by the following model compound analysis. Salicylic acid is meaningful to isoxsuprine-glucuronide structure as a model in that it provides a phenolic ring with a carboxy function capable of anionic status in high pH media. When run by ESI-negative MS/MS similarly to isoxsuprine metabolites (Fig. 9), fragments are attainable with varying efficiencies depending upon the setting of the voltage at the initial transmittance of ions from high to low pressure, specifically the sampling cone. Figure 10 shows trends for some of the ions where they can be visualized.

It strikes us as significant that the anionic phenol of salicylic acid does not elicit tangible dehydration. This in turn would signify that the dehydration reaction seen with isoxsuprine arises predominantly from the benzylic hydroxyl group. Since the isoxsuprine-glucuronide elicits no dehydration, our reasoning suggests that the benzylic alcohol group is blocked, presumably by glucuronidation. Only after release of the glucuronide in the mass spectrometer, presumably in the source, does the freed isoxsuprine molecule undergo dehydration, revealing the 282 m/z ion as seen in Fig. 5. Although no anionic molecule containing a benzylic alcohol has been available to check the alternative aspect of this modeling, namely facile dehydration from benzylic

hydroxyls, results with the very weakly anionic epinephrine suggest that such dehydration does occur (Fig. 11).

Isoxsuprine metabolite complexation with urine constituents.

Electrospray set in positive ion mode uncovered a unique tendency for isoxsuprine-glucuronide to complex with potassium ions in urine, evidenced by a 554 m/z dipotassium isoxsuprine-glucuronide cation. This finding was supported by sodium titration, which sequentially yielded first an intermediate monosodium monopotassium and then a disodium isoxsuprine-glucuronide cation (Bosken, et al., 1999). We report now that this unique property apparently extends to other components in urine as well. Positive-mode parent ion spectrometry for the 554 m/z species revealed a large number of higher molecular weight peaks as shown in Fig. 12, bottom. The pattern (m/z values and intensities) and molecular weight differences were coincident with the values of normal unidentified urine components present in both blank urine and urine post-administration for isoxsuprine (cf. Fig. 12, top, for pattern of peaks evident in 4 hour post-isoxsuprine urine). Table 5 lists our interpretation of the complexes seen in the Fig. 12 (bottom) parent ion spectrum. Since the ions listed are all parental to a dipotassium complex, we can directly infer that unknown ions 256, 265, 270, 290 and 361 m/z (Fig. 12, top) must all contain at least one potassium.

Discussion

This exploration of isoxsuprine and its conjugates has revealed new chemical details about drug excretory products relevant to analytical techniques designed to detect them. Isoxsuprine undergoes oxidative metabolism to a variety of hydroxylated and oxidized products as listed in Table 2 (cf. also Dumasia & Houghton, 1991). However, the most prevalent metabolite is that which we have listed simply as isoxsuprine-glucuronide. This product can be seen early after administration (Fig. 1) with only low excretion of unmetabolized drug, suggestive of the effects of first pass hepatic metabolism. The glucuronide has rather stringent requirements for β-glucuronidase cleavage, which could possibly be a consequence of its rather extensive urinary complexation with potassium ions (Bosken, et al., 1999) and other urinary components (Fig. 12, Table 5), evident in electrospray-positive mode. Mass spectral evidence strongly suggests that the compound released by β-glucuronidase cleavage is indistinguishable from the original drug (Fig. 3 daughter ion analysis and similar GC/MS scans, not shown). The isoxsuprine-glucuronide as visualized by electrospray-negative mode has a discrete optimum cone voltage setting for maximum peak intensity. This setting is significantly lower than that utilized for positive mode visualization of the dipotassium complex of the glucuronide (Fig. 6).

Other experiments generated a solid phase extraction protocol for quantitation of glucuronide-liberated isoxsuprine (Bosken, et al., 1999; Bosken, ~~et al. in prep.~~). This in turn enabled us to apply our cone voltage optimization technique to species evident after trimethylsilyl derivatization. This unique approach allowed us to visualize derivatized species as they appear following SPE and derivatization and just prior to injection onto GC/MS. In addition to the isoxsuprine-bis(TMS) derivative visible on GC/MS at 12.35 min RT (Fig. 4), we were able to identify 1) several TMS derivatives of isoxsuprine-glucuronide differing in degree of derivatization; 2) several TMS derivatives of hydroxylated isoxsuprine-glucuronide also differing in degree of derivatization; and 3) several reaction products that are presumably specific to the instrument, including decarboxylations and dehydration. GC/MS results (Fig. 4 and Table 2) confirm the existence of substrates for formation of hydroxylated isoxsuprine-glucuronides, and indicate that they should occur in at least two forms indistinguishable in the absence of chromatography, one with hydroxylation of the isoxsuprine phenoxy ring (peak at 13.7 min in Table 2) and the other with hydroxylation of the isoxsuprine phenolic ring (peak at 12.8 min in Table 2). These are essentially the most important phase II metabolites other than the isoxsuprine-glucuronide itself, according to GC/MS TIC peak areas (Fig. 4).

We have not considered isolating the isoxsuprine-glucuronide in sufficient quantities for ^{1}H- or ^{13}C-NMR or x-ray crystallography, nor have we considered synthesis of isoxsuprine-glucuronide candidates for chromatographic evaluation, although any or all of these remain possibilities. In lieu of such sophisticated approaches, we have opted to examine fragmentation patterns evident in mass spectra for clues regarding structure of the glucuronide. The most dramatic detail that can be seen on contrasting the isoxsuprine anion mass spectrum (Fig. 3) with the glucuronide (Fig. 5) is the ease with which unmetabolized isoxsuprine undergoes a dehydration reaction with loss of 18 mass units to 282, and the total lack of such a transition from the 476 m/z glucuronide anion. Analysis of model compounds including salicylic acid and epinephrine suggest that 1) the phenol functional group of salicylic acid is not capable of eliciting a loss of 18 amu, except to a very tiny extent, presumably the result of deformation of the carboxy group (McLafferty, 1973); 2) although difficult to detect as an anion under our conditions, epinephrine appears to disclose neutral loss of water as 18 amu, implying participation of its benzylic alcohol group. These considerations imply that the principal isoxsuprine-glucuronide has its attachment site at the benzylic alcohol functionality of isoxsuprine, and that only after fragmentary release of the glucuronic acid moiety can dehydration of the isoxsuprine-glucuronide occur (476-->300-->282 m/z). However, 1) facile glucuronidation is known to occur at the sterically less hindered phenolic groups, as has been demonstrated in our lab for the local anesthetic group including bupivacaine, ropivacaine, and lidocaine (Harkins, et al., 1998; Lehner, et al., 1998; Lehner, et al., unpublished); 2) it is difficult to predict without additional evidence what effects glucuronidation at the phenolic hydroxy group may have on dehydration at the benzylic alcohol. Therefore, although the chemical evidence currently suggests participation of the benzylic group, we must await the synthesis of glucuronide

candidates and the opportunity to pursue chromatographic evidence as to the structure of these metabolites.

If in fact the predominant isoxsuprine-glucuronide isomer eventually proves to involve benzylic alcohol conjugation, it does so in spite of thermodynamic, steric and possibly enzymatic preferences for phenolic conjugation. One hypothesis that may account for such an occurrence would involve cleavage *in vivo* of the thermodynamically favored phenolic conjugation product with gradual kinetically-favored accumulation of the benzylic alcohol conjugation product. This hypothesis would also preserve the pharmacological consequences of a high first pass hepatic metabolism and possibly account for the more stringent glucuronidase requirements we have observed relative to glucuronides of hydroxylated local anesthetics (Harkins, et al., 1999a and 1999b; Woods, et al., 1998).

Figure 12 discloses a peculiarity of isoxsuprine-glucuronide when visualized as a 554 m.w. dipotassium complex in positive mode-electrospray. Parent ion analysis for this 554 m/z reveal additional higher order forms that can collapse to yield this dipotassium complex, and whose molecular weights mathematically imply complexation with other components visible in urine (cf. Fig. 12, TOP and Table 5).

Figure 13 presents a summary of the data derived from the mass spectral observations specific to the structures of equine isoxsuprine metabolites. Note that the figure is much more a summary of reasonable functional group possibilities listing types of structures that are compatible with the molecular weights and other evidence; positional isomers are not excluded. This applies also to the isoxsuprine and phenolic- and phenoxy-specific hydroxyisoxsuprine glucuronides, where our initial evidence and reasoning supports the benzylic hydroxyl as the site of conjugation but does not conclusively exclude the phenolic hydroxyl.

References

1. Needleman, P., Corr, P.B., Johnson, E.M., Jr. Drugs used for the treatment of angina: organic nitrates, calcium channel blockers and β-adrenergic antagonists. In: Goodman and Gilman's The Pharmacological Basis of Therapeutics, 7th edition, A.G.Gilman, L.S. Goodman, T.W. Rall, F. Murad, eds. New York: Macmillan Publishing Co., 1985, p. 823.

2. Manley, E.S., Lawson, J.W. Effect of beta adrenergic receptor blockade on skeletal muscle vasodilation produced by isoxsuprine and nylidrin. *Arch. Int. Pharmacodyn. Ther.*, 175, 239-250, 1968.

3. Harkins, J.D.; Mundy, G.D.; Stanley, S.; Woods, W.E.; Sams, R.A.; Richardson, D.R.; Grambow, S.C.; Tobin, T. Absence of detectable pharmacological effects after oral administration of isoxsuprine. *Equine Veterinary Journal*, 30,(4),294-299, 1998.

4. Ingle-Fehr, J.E.; Baxter, G.M. The effect of oral isoxsuprine and pentoxifylline on digital and laminar blood flow in healthy horses. *Veterinary Surgery*, 28,(3), 154-160, 1999.

5. Bosken, J.M.; Lehner, A.F.; Hunsucker, A.; Harkins, J.D.; Woods, W.E.; Karpiesiuk, W.; Carter, W.G.; Boyles, J.; Fisher, M.; Tobin, T. Direct MS-MS Identification of Isoxsuprine-Glucuronide in Post-Administration Equine Urine. ~~Publication #260 from the Equine Pharmacology and Experimental Therapeutics Program at the Maxwell H. Gluck Equine Research Center and Department of Veterinary Science, University of Kentucky. Submitted to~~ *Veterinary Research*, ~~1999~~, In press, 1999.

6. Joujou-Sicic, K.; Andren, P.E.; Bondesson, U.; Fredriksson, E.; Lasson, C.I.; Kallings, P.; Persson, S.G.B. A Pharmacokinetic Study of Isoxsuprine in the Horse. *Proceedings of the 11th International Conference of Racing Analysts and Veterinarians*, Queensland, Australia, 453-458, 1996.

7. Dumasia, M.C.; Houghton, E.; Screening and confirmatory analysis of β-agonists, β-antagonists and their metabolites in horse urine by capillary gas chromatography-mass spectrometry. *Journal of Chromatography*, 564, 503-513, 1991.

8. Johnstone, R.A.W., editor. "Electrospray Ionization (and APCI)". In: *Back to Basics, the Mass Spectrometry Primer*. Beverley, MA (USA): Micromass, A Division of Waters Corporation, 1998.

Willoughby R.; Sheehan E.; Mitrovich S. "Electrospray Fundamentals". In: *A Global View of LC/MS*, A Global View Publishing, Pittsburgh, PA: 506-507, 1998.

10. McLafferty, F.W. "Postulation of Molecular Structures: Small Neutral Losses", in: *Interpretation of Mass Spectra*, W.A. Benjamin, Inc., Chap.5, 75-96, 1973.

11. Harkins, J.D.; Karpiesiuk, W.; Tobin, T.; Lehner, A.F. Direct identification of hydroxyropivacaine glucuronide by ESI$^+$/MS/MS in post-administration equine urine. ~~Publication #255 from the Equine Pharmacology and Experimental~~ Therapeutics Program at the ~~Maxwell H. Gluck Equine Research Center and~~ Department of Veterinary Science, ~~University of Kentucky. Submitted to~~ *Journal Vet. Pharmacol. Therap.* 1998.

12. Elliott, J.; Soydan J. Characterisation of beta-adrenoceptors in equine digital veins: implications of the models of vasodilatory action of isoxsuprine. *Equine Vet. J. Suppl.* 19,101-107, sep, 1995.

13. Dumasia, M.C. The Biotransformation and Detection of some β-Adrenergicmimectic Drugs in the Horse. *Proceedings of the 9th International Conference of Racing Analysts and Veterinarians*, New Orleans, 1992.

. Lehner, A.; Woods, W.E.; Karpesiuk, W.; Harkins, J.D.; Tobin, T. Detection and Quantitation of 3- Hydroxbupivacaine in Horse Urine: A preliminary report. In: *The 12th International conference of racing analysts and veterinarians*, Vancouver, Canada, ~~p-19, 1998~~.

15. Harkins, J.D.; Karpiesiuk, W.; Lehner, A.; Woods, W.E.; Dirikolu, L.; Carter, W.G.; Boyles, J.; Tobin, T. Ropivacaine in the horse: Its Pharmacological Responses, Urinary Detection and Mass Spectral Confirmation. ~~Publication #236 from the Equine Pharmacology and Experimental Therapeutics Program at the Maxwell H. Gluck Equine Research Center and Department of Veterinary Science, University of Kentucky~~. *J. Vet. Pharmacol. Therapeutics*, in press, 1999a.

16. Harkins, J.D.; Lehner, A.; Karpiesiuk, W.; Woods, W.E.; Dirikolu, L.; Boyles, J.; Carter, W.G.; Tobin, T. Bupivacaine in the horse: Relationship of local anaesthetic responses and urinary concentrations of 3-hydroxybupivacaine. *J. Vet. Pharmacol. Therap.* 22, in press, 1999b.

Final Program and Abstracts, #P-13, 1998.

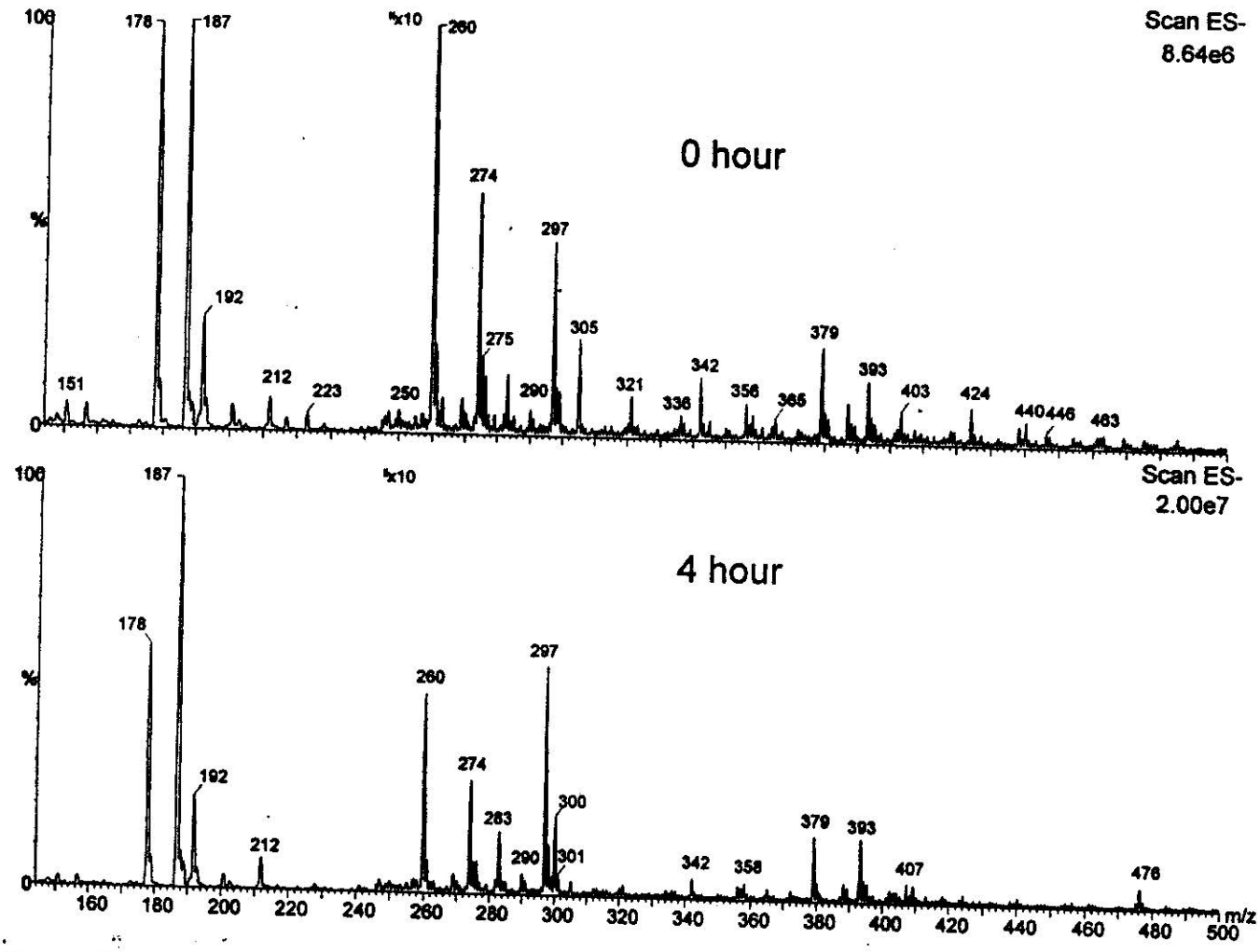


Figure 2. Equine urine before and after isoxsuprine administration, both with 37° C overnight β-glucuronidase treatment (*Helix pomatia*) and examined by ESI-negative mode MS/MS, full scan. Samples were diluted 1:10 in 0.5% NH_4OH, acetonitrile:H_2O, 50:50 prior to direct infusion analysis. The cone voltage was 25. Note 10-fold scale expansion above 245 m/z.

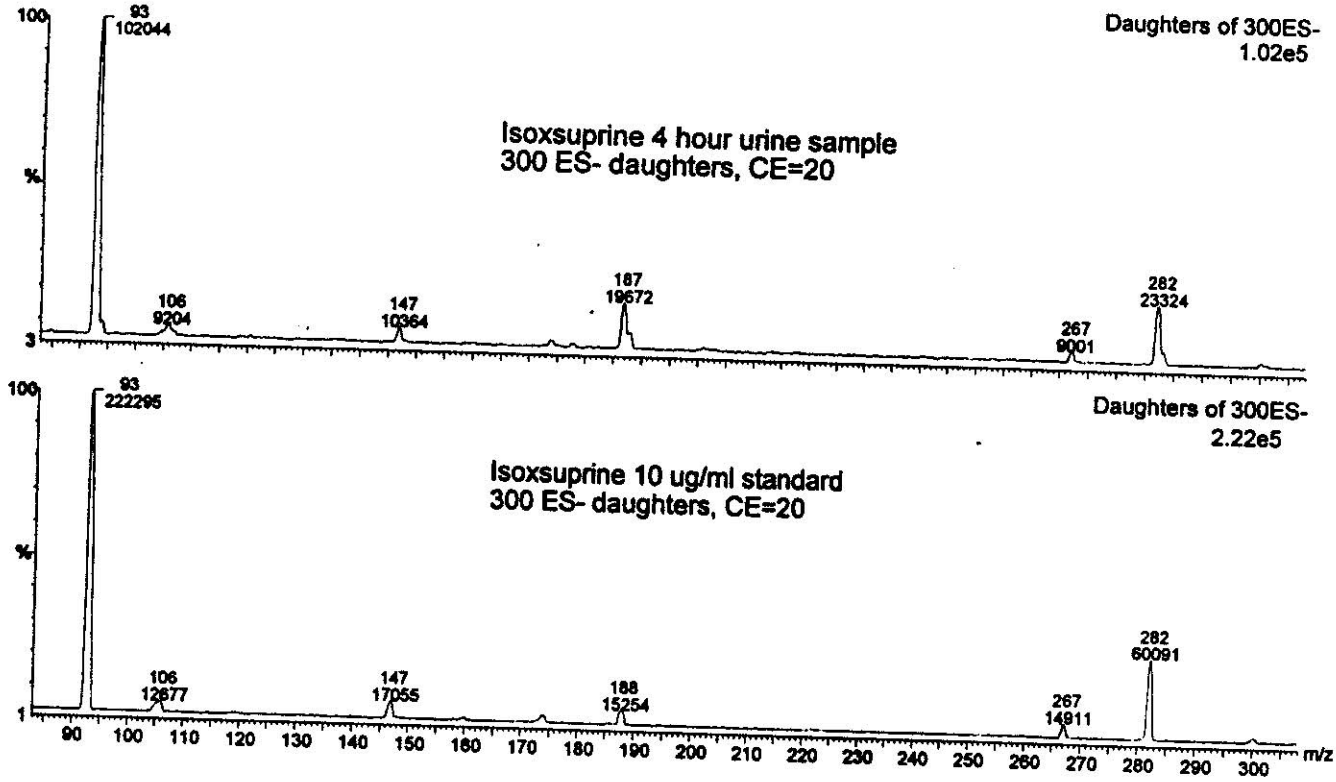


Fig. 3. Daughter ion spectrum of the released isoxsuprine molecule from isoxsuprine glucuronides in comparison to that from standard isoxsuprine. Continuum spectra provided by direct infusion ESI-MS/MS (negative mode), with daughters generated from m/z 300 anion. Samples were diluted 1:10 in 0.5% NH_4OH, acetonitrile:H_2O, 50:50 prior to direct infusion analysis. The cone voltage was 25, and the collision energy (CE) was 20 in both cases. Peak labels show m/z value (top), peak intensity (bottom).

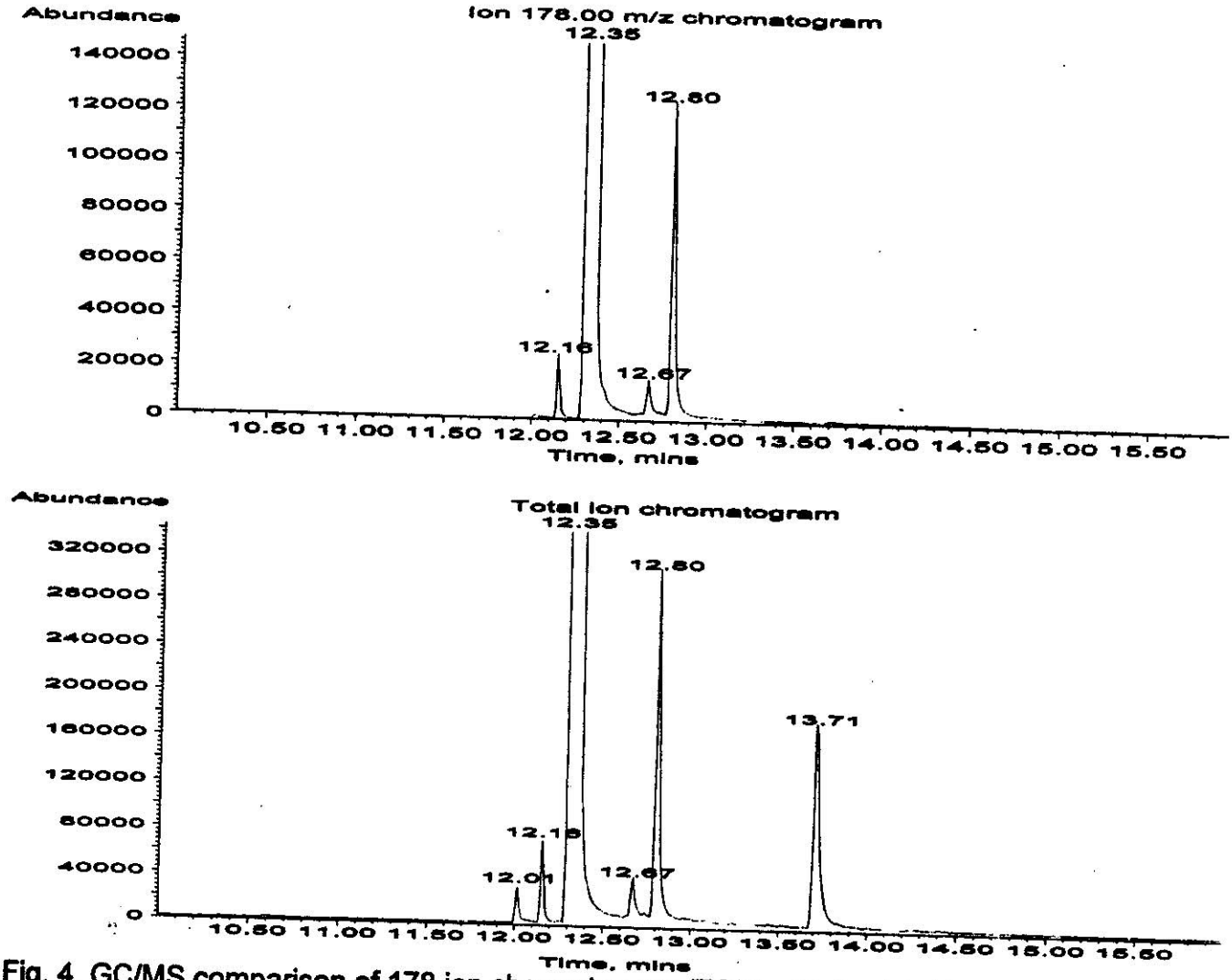


Fig. 4. GC/MS comparison of 178 ion chromatogram (TOP) with the total ion chromatogram (BOTTOM) of 6 hour post-administration isoxsuprine extract. The sample was treated sequentially with β-glucuronidase, SPE and derivatization, prior to GC/MS as described in Methods, with the exception that the sample was diluted 1:50 from the original BSTFA + 1% TMCS solution with dichloromethane. Peak labels indicate retention time in minutes.

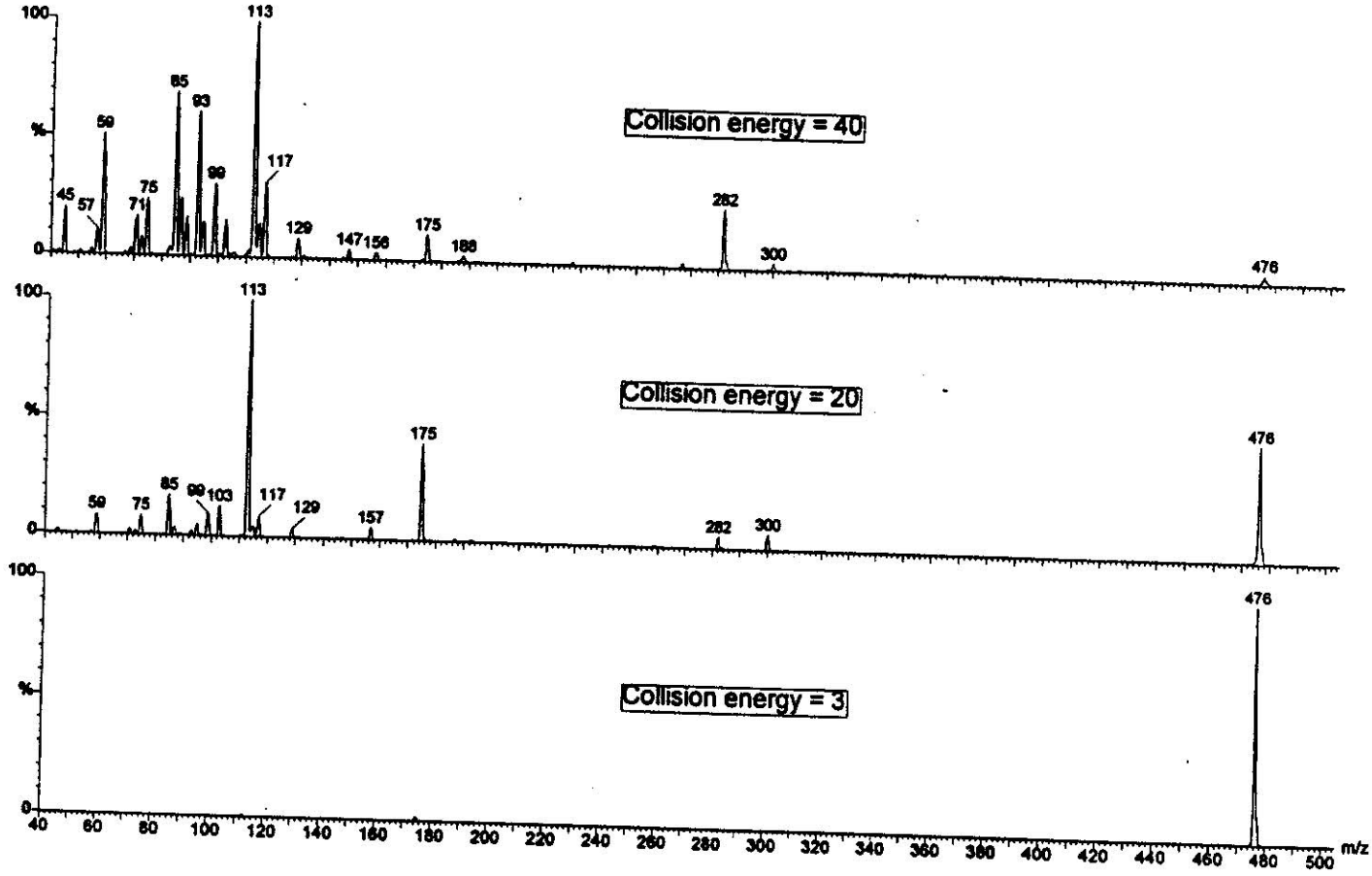
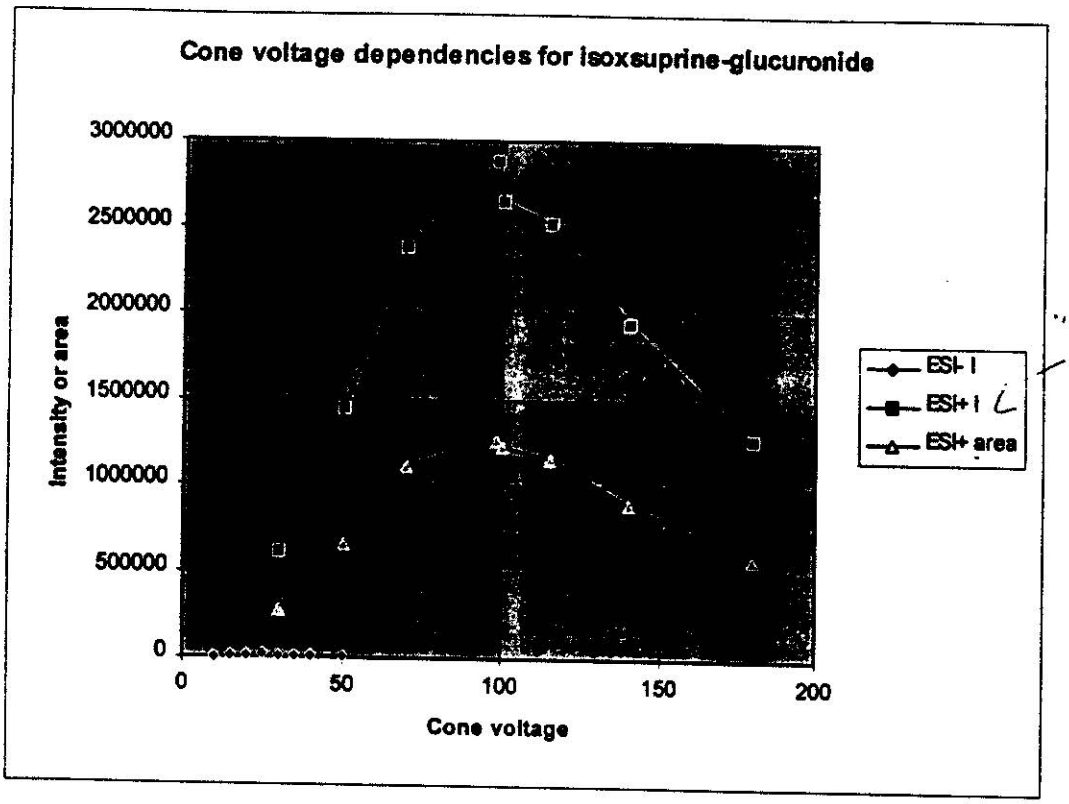


Fig. 5. Isoxsuprine glucuronide as seen by ESI⁻ MS/MS: 476 m/z daughter ions at different collision energies for the four hour post-isoxsuprine administration sample. Note that daughter ions can be generated with much greater ease by using negative ion mode than positive mode (Bosken, et al., 1999). Identical results were obtained with six and eight hour urine samples post-administration.



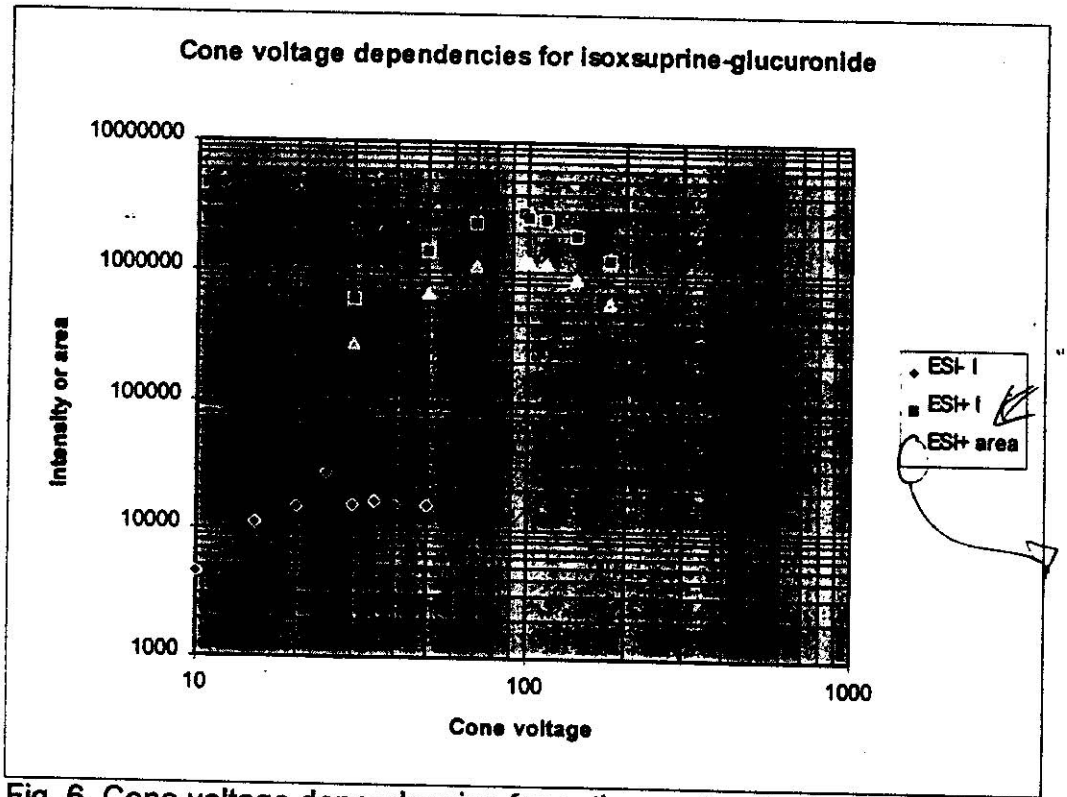


Fig. 6. Cone voltage dependencies for optimum visualization of isoxsuprine glucuronide, with comparison of ESI^+ intensities and areas, and also with ESI^- mode intensity. TOP, normal Cartesian plot; BOTTOM, log-log plot to emphasize contrast with ESI^- data.

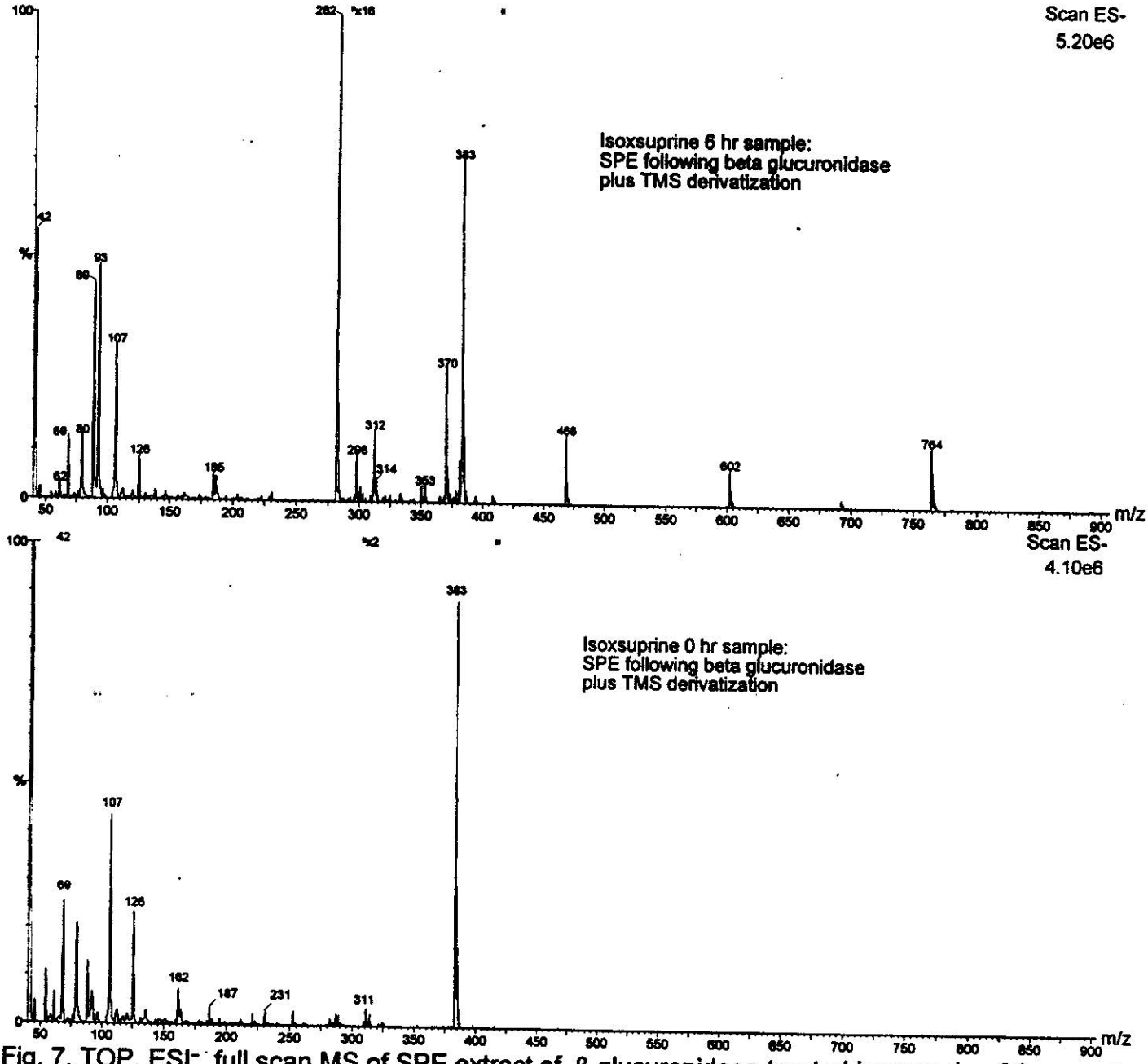


Fig. 7. TOP, ESI⁻ full scan MS of SPE extract of β-glucuronidase-treated isoxsuprine 6 hr sample derivatized with TMS groups. The scan included the 40-900 m/z range. Samples were infused by dilution of BSTFA + 1% TMCS solutions (see methods) 1:20 with acetonitrile containing 1% triethylamine. BOTTOM, ESI⁻ scan of SPE extract of glucuronidase-treated isoxsuprine 0 hr sample (blank) derivatized with TMS groups and infused as above. Note the prominent 383 peak evident also in the 6 hr sample, as well as the absence of peaks above 383 relative to the 6 hr sample. Note also relative scale expansion between roughly 300 and 420 m/z in both spectra.

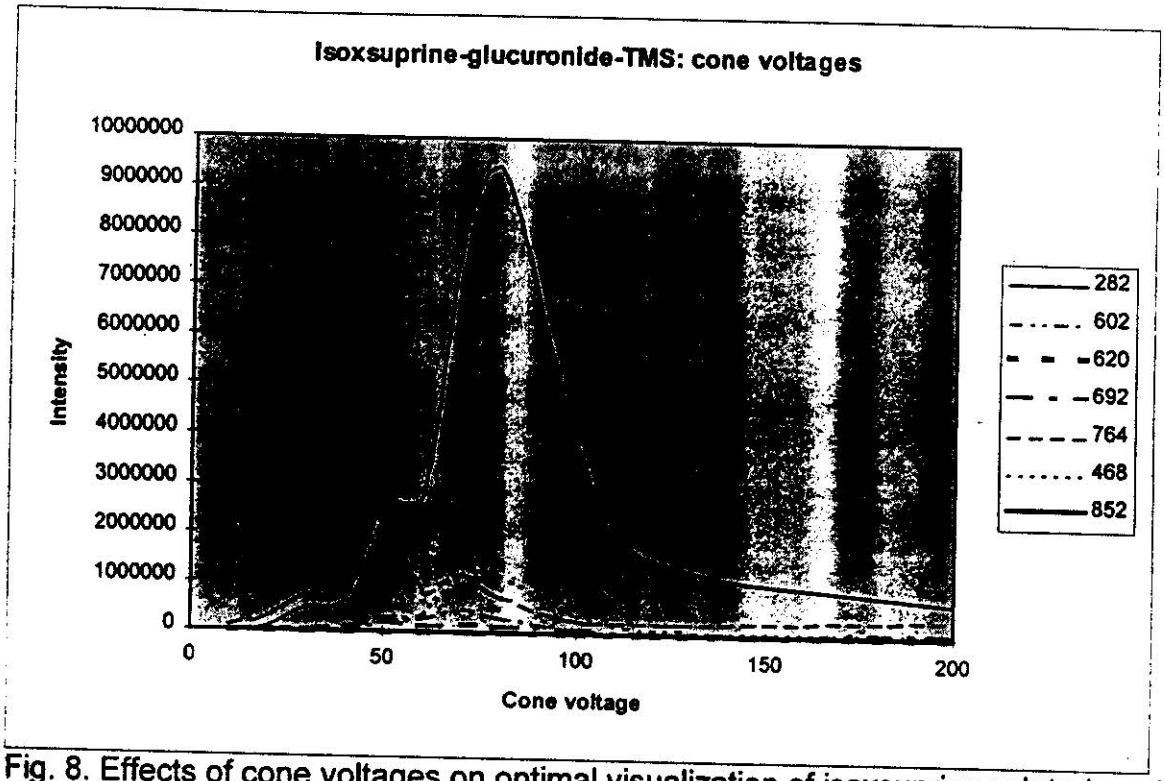


Fig. 8. Effects of cone voltages on optimal visualization of isoxsuprine-related peaks derivatized with TMS.

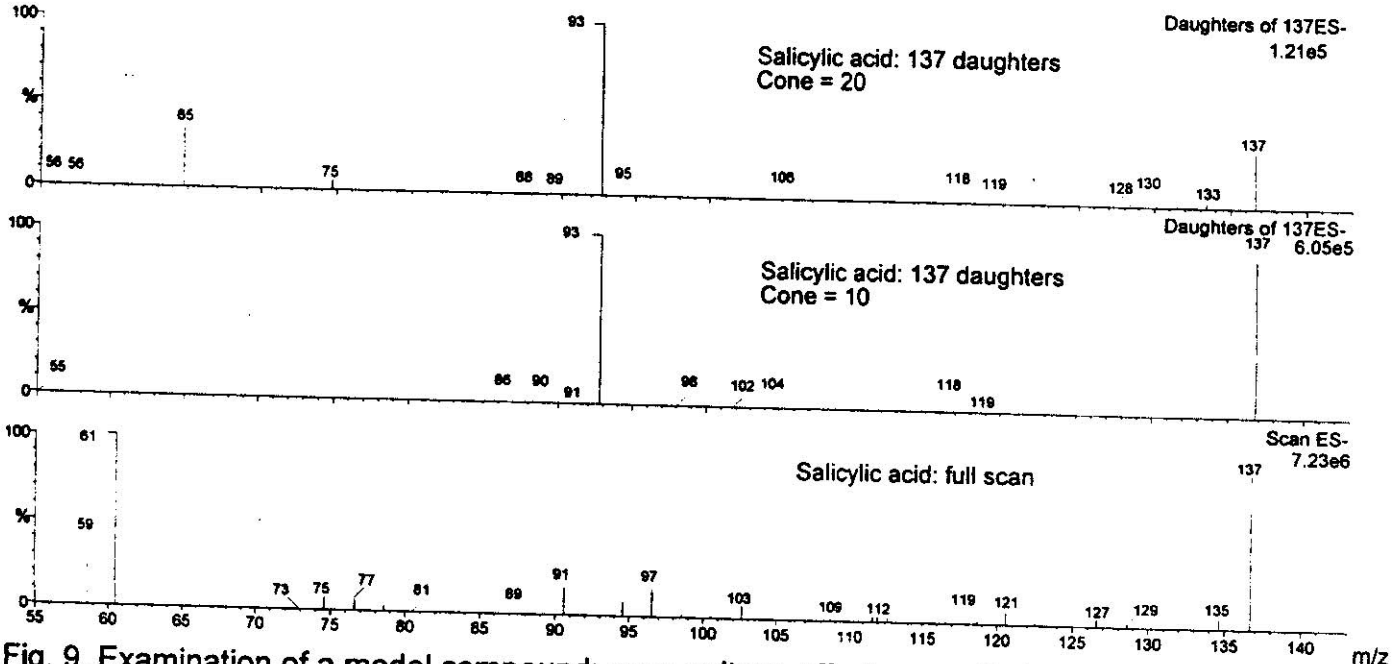


Fig. 9. Examination of a model compound: cone voltage effects on optimization of various fragment ions of salicylic acid as visualized by ESI in negative mode. Note the lack of evidence for facile dehydration of the phenolic hydroxyl (as loss of 18 amu).

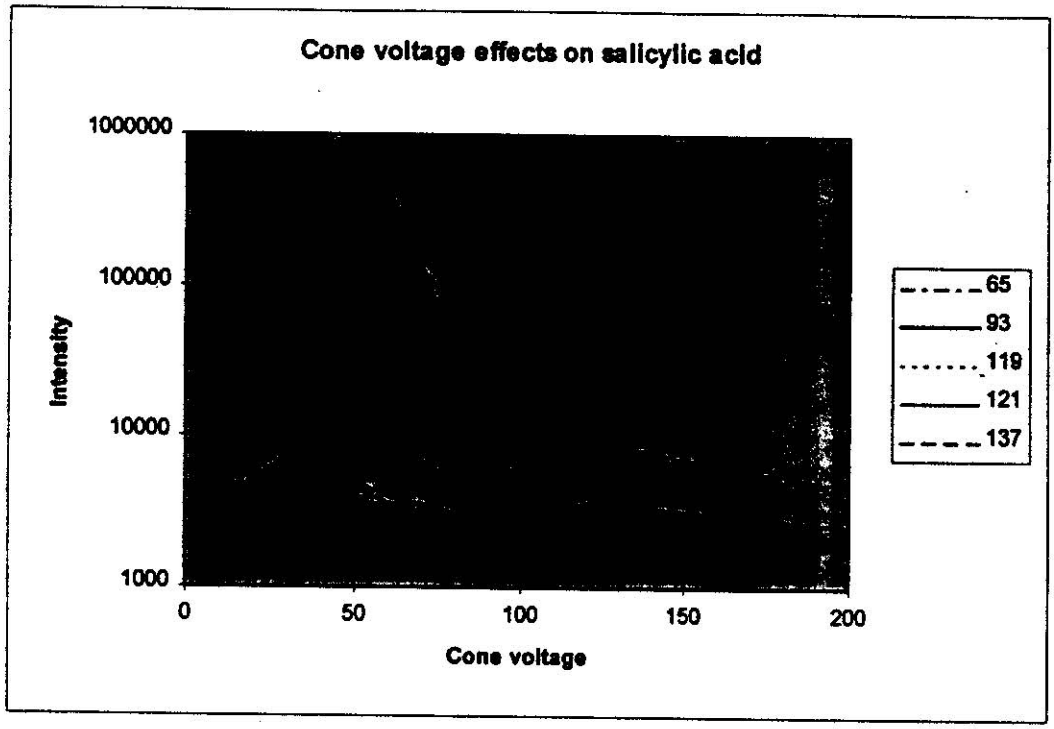


Fig. 10. Optimization of cone voltage settings for visualization of principal ionic fragments of salicylic acid, as seen with ESI-negative mode MS/MS. Mass spectral intensity is plotted as a function of cone voltage.

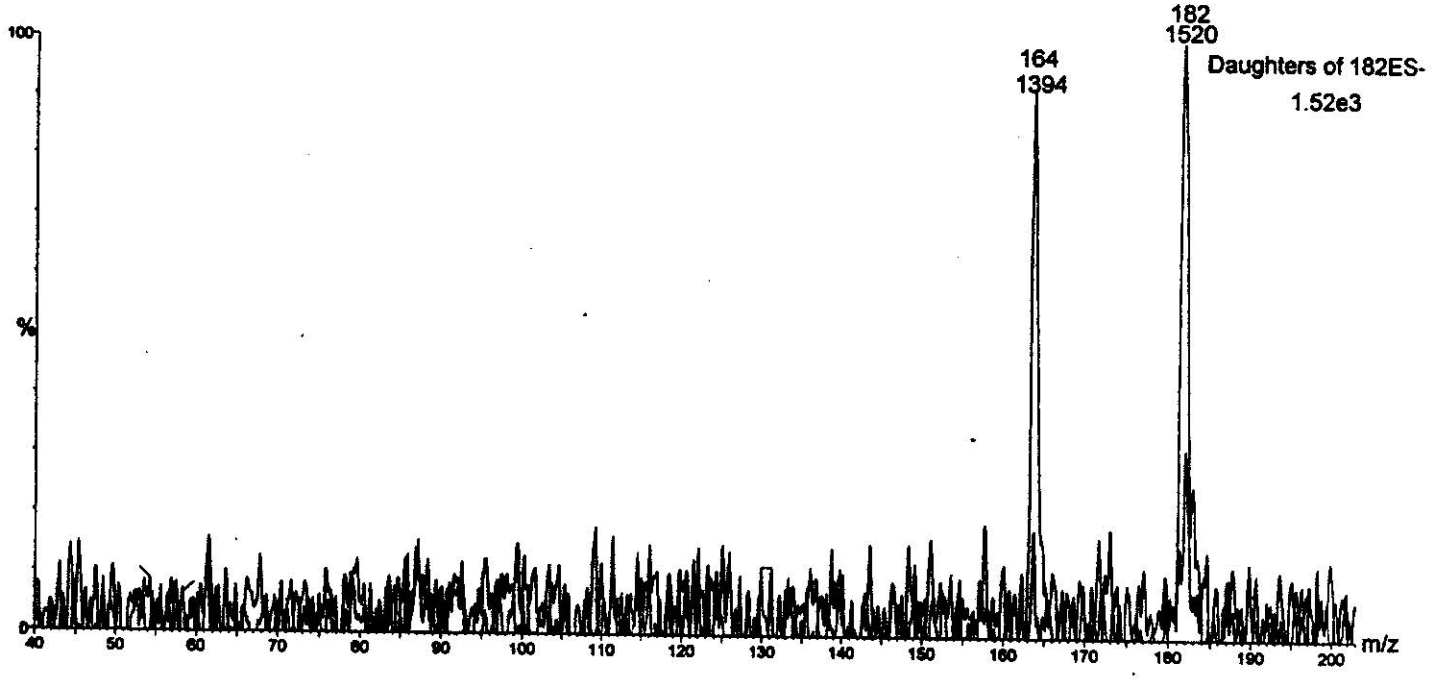


Fig. 11. Negative mode ESI daughter ion analysis for m/z 182 of 7.5 ug/ml epinephrine in 0.05% NH_4OH, 50:50 acetonitrile:water, infused at 0.6 ml/hr. Visualization of the loss of water from a benzylic alcohol (overlay of two experiments: one at collision energy (CE) = 3 identifies parent 182; CE=10 discloses loss of 18 to 164). Peak labels: upper = m/z value; lower = intensity.

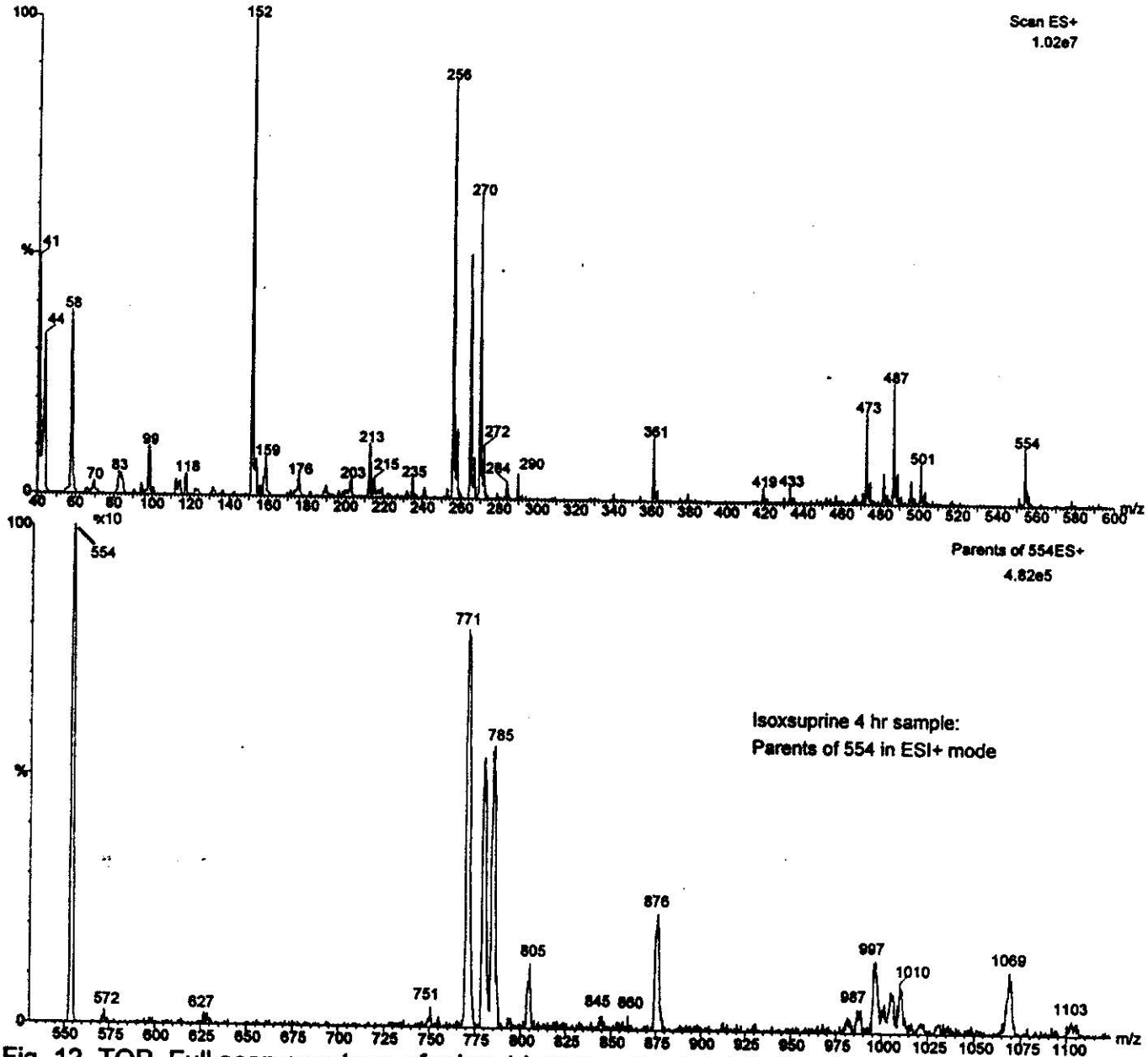


Fig. 12. TOP, Full scan spectrum of urine 4 hours post-administration for isoxsuprine as visualized by ESI^+ mode. Note that, with the exception of the 554 m/z dipotassium isoxsuprine-glucuronide anion, almost all of the ions are identical to those seen in a 0 hour blank. BOTTOM, Parent ion spectrum for the 554 m/z species disclosing additional complexes with isoxsuprine glucuronide and visible in ESI^+ mode.

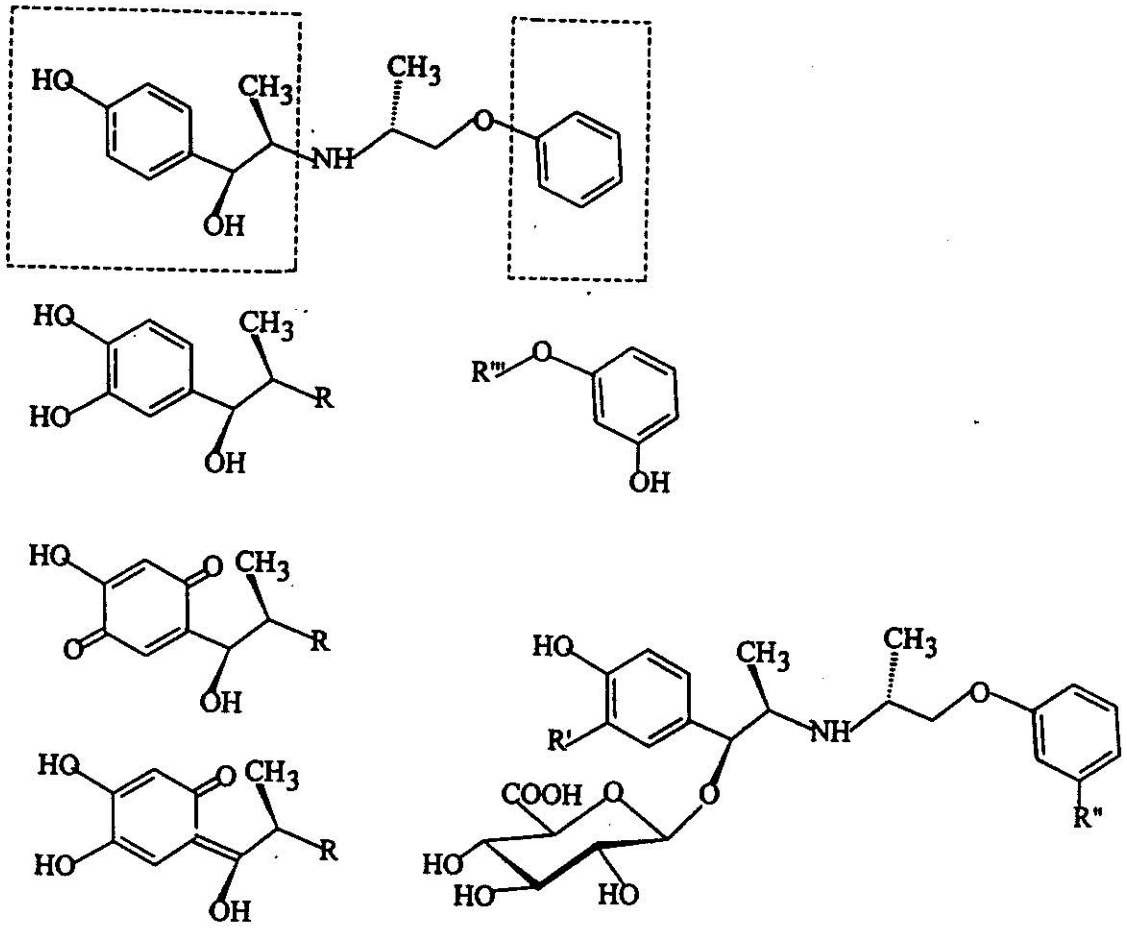


Fig. 13. Summary of isoxsuprine metabolites revealed by GC- and ESI-MS. GC/MS scans revealed 6-7 metabolites of isoxsuprine, whereas isoxsuprine-glucuronides were seen by ESI^+. Strucures on the left depict oxidation events specific to the phenol ring of isoxsuprine, whereas that on the right indicates oxidation of the phenoxy ring. The structure at lower right represents our proposed structure for isoxsuprine- and phenoxy- and/or phenolic-ring hydroxyisoxsuprine-glucuronides, based on model compound analysis which at present suggests the benzylic alcohol as the site for glucuronidation. In regards to the latter, the R' and R" functional groups can be either H or OH.

Table 1. Percentages of free isoxsuprine present relative to isoxsuprine-glucuronide as measured by direct infusion ESI-MS/MS under conditions of various enzymatic treatments.

Hours post-administration	No enzyme	*Helix pomatia* β-glucuronidase	*Patella vulgata* β-glucuronidase
0	0.0	0.0	0.0
4	5.8	84.1	73.0
6	6.9	87.3	84.0
8	6.2	91.4	88.0

Table 2. List of isoxsuprine metabolites released by β-glucuronidase treatment of equine urine and derivatized with TMS groups: possible identities following interpretation of GC/MS-derived spectra.

Peak RT, min	**Poss. M.W.**	**Evidence for m.w. (highest m/z peak)**	**% based on TIC area**	**base peak, m/z**	**other significant peaks, m/z**	**possible identity**
12.0	451	436 m/z ($-CH_3$)	0.2	184	267,251,220,178, 102,73,55	possible contaminant in original isoxsuprine prepn.
12.2	445	430 m/z ($-CH_3$)	0.3	178	340,267,193,135, 107,73	isomer of 12.3'; possible contaminant in original isoxsuprine prepn.
12.3	445	430 m/z ($-CH_3$)	96	178	340,267,193,135, 107,73	isoxsuprine-bisTMS
12.7	475	460 m/z ($-CH_3$)	0.3	178	370,297,267,223, 193,163,135,107, 73	paraquininoid oxidation product at phenolic ring ($+O_2 - H_2$) + bis-TMS
12.8	533	518 m/z ($-CH_3$)	1.7	178	355,281,251,135, 107,73	second phenolic ring OH + tris TMS
13.0	547	532 m/z ($-CH_3$)	<0.1	178	468,306,272,217, 147,107,73	hydroquininoid oxidation product at phenolic ring ($+O_2 - H_2$) + tris-TMS
13.7	533	518 m/z ($-CH_3$)	1.4	266	375,248,223,207, 193,181,167,151, 135,84,73	phenoxy ring OH + tris TMS
14.2	ca. 457	442 m/z ($-CH_3$)	<0.1	266	340,297,248,223, 205,180,84,73	unidentified phenoxy ring OH species + bis TMS
14.4	ca. 370	355 m/z ($-CH_3$)	<0.1	266	336,320,233,207, 181,167,84,73	unidentified phenoxy ring OH species (loss of phenolic ring?) + bis TMS

Table 3. Optimal cone voltages for various compounds that are relevant to isoxsuprine metabolism and which could be visualized by ESI^- MS/MS with direct infusion of SPE-extracted TMS-derivatized sample.

Ion, m/z	Optimal cone voltage	Tentative Identification	Principal daughter ions, m/z
282	80	Isoxsuprine minus H_2O OR Isoxsuprine glucuronide minus (glucuronide & H_2O)	267,188,174,147,105,93
372	20	MonoTMS of isoxsuprine	
408	20	Unidentified	
432	10	Decarboxylated underivatized isoxsuprine-glucuronide	
468	80	Unidentified	378,374,330,282,147,112,93
486	10	Decarboxylated mono TMS of isoxsuprine-glucuronide minus H_2O	
499	70	Unidentified	
545	10	Unidentified	
560	80	Unidentified	
602	70	Bis TMS of isoxsuprine-glucuronide minus H_2O	282,185,139,131,93
620	50	Bis TMS of isoxsuprine-glucuronide	282, 468, 486
692	50	Tris TMS of isoxsuprine-glucuronide	468,282,185,93
764	50	Tetrakis TMS of isoxsuprine-glucuronide	602,468,282,93
780	50	Tetrakis TMS of hydroxyisoxsuprine-glucuronide	370, 408, 298
794	70	Unidentified	
800	50	Unidentified	
852	50	Pentakis TMS of hydroxyisoxsuprine-glucuronide	
877	10	Unidentified	
891	30	Unidentified	
892	30	Unidentified	

Table 4. Summary of salicylic acid ESI^- mass spectral fragments, their respective optimal cone voltages, and interpretation of each peak's origin. Data summarizes values derived from Fig. 8.

Mass spectral peak, m/z	Optimal cone voltage	Possible origin of peak
65	60	possible ring contraction to C_5H_5
93	50	base peak: loss of CO_2 (-44)
119	120	minor water loss peak (-18)
121	40	minor/negligible O loss peak (-16)
137	20	salicylic acid anion

Table 5. Interpretation of isoxsuprine-glucuronide complexes evidenced in Fig. 12, Bottom.

m/z	interpretation
554	Isoxsuprine-glucuronide + K_2 (301 + 176 - 1 [proton] + 39 + 39)
572	Isoxsuprine-glucuronide + K_2 + H_2O (554 + 18)
771	Isoxsuprine-glucuronide + K + unknown 256 (476 + 39 + 256)
780	Isoxsuprine-glucuronide + K + unknown 265 (476 + 39 + 265)
785	Isoxsuprine-glucuronide + K + unknown 270 (476 + 39 + 270)
805	Isoxsuprine-glucuronide + K + unknown 290 (476 + 39 + 290)
876	Isoxsuprine-glucuronide + K + unknown 361 (476 + 39 + 361)
1069	Isoxsuprine-glucuronide dimer + K_3 (476 + 476 + 39 + 39 + 39)

Not listed: 981, 987, 997, 1005, 1010, 1103 m/z (currently unidentified).