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Desorption Chemical Ionization and Fast Atom Bombardment Mass Spectrometric Studies of the Glucuronide Metabolites of Doxylamine

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Three glucuronide metabolites of doxylamine succinate were collected in a single fraction using high-performance liquid chromatography (HPLC) from the urine of dosed male Fischer 344 rats. The metabolites were then separated using an additional HPLC step into fractions containing predominantly a single glucuronide metabolite. Analysis of the metabolites by methane and ammonia desorption chemical ionization, with and without derivatization, revealed fragment ions suggestive of a hydroxylated doxylamine moiety. Identification of the metabolites as glucuronides of doxylamine, desmethyldoxylamine and didesmethyldoxylamine was accomplished, based on determination of the molecular weight and exact mass of each metabolite using fast atom bombardment (FAB) ionization. This assignment was confirmed by the fragmentation observed in FAB mass spectrometric and tandem mass spectrometric experiments. *Para*-substitution of the glucuronide on the phenyl moiety was observed by 500-MHz nuclear magnetic resonance (NMR) spectrometry. A fraction containing all three glucuronide metabolites, after a single stage of HPLC separation, was also analysed by FAB mass spectrometry, and the proton- and potassium-containing quasimolecular ions for all three metabolites were observed.

INTRODUCTION

The antihistamine doxylamine succinate, *N*, *N*-dimethyl-2-[1-phenyl-1-(2-pyridinyl)ethoxy]ethanamine succinate, is a component in a widely used drug, Bendectin.[1-5] Numerous studies have investigated the health effects and/or metabolic activity of this compound.[6-12] Early analytical studies of the urinary metabolites of doxylamine succinate and other related antihistamine drugs relied on colorimetric, thin-layer chromatographic and/or infrared measurements.[13-15] More recently chemical ionization mass spectrometry has been used to identify urinary metabolites of doxylamine and related compounds.[9-11,16] In this paper, we describe the characterization of three previously unidentified glucuronide metabolites of doxylamine succinate by both chemical ionization (CI) and fast atom bombardment (FAB) mass spectrometry,[17] as well as nuclear magnetic resonance (NMR) spectrometry.

Two stages of high-performance liquid chromatography (HPLC) were utilized to isolate and then separate three glucuronide metabolites from the urine of dosed rats. Initially, the purified fractions were analysed by desorption chemical ionization (DCI) mass spectrometry, before and after acetylation. The initial isolate and the three HPLC fractions were also analysed by FAB mass spectrometry. High resolution FAB mass spectrometry was used to determine the exact mass and the elemental compositions of the metabolites. Fragmentation in FAB mass spectrometry and tandem mass spectrometry provided data with which the glucuronide structures were elucidated. Finally, the NMR data, although largely obscured by unremoved components from the urine, were partially interpretable and allowed the most likely location of ring substitution to be deduced.

EXPERIMENTAL

Fischer 344 male rats were dosed by gavage with 50 mg of doxylamine succinate (Richard and Merril, 99+% pure) in 1 ml of aqueous solution. The glucuronide metabolites were isolated from rat urine using one or two stages of HPLC separation with a Waters Associates model 6000A high performance liquid chromatograph with a Supelco 5 μ CN analytical column 250×4.6 mm i.d.). Samples were injected into a Rheodyne 7125 injector fitted with a 20-μl sample loop and a Swagelok guard column filled with Waters 10 μ CN packing. A Waters Associates model 440 absorbance detector (254 nm) was used for detection of HPLC fractions. The first stage of HPLC separation served to isolate the three glucuronide metabolites from other metabolite components and the matrix. This separation employed an isocratic system using 70% methanol/30% 0·01 M KH_2PO_4-$N(CH_3)_3$ (0·02 M) buffer with an adjusted pH of 7·5. The flow rate was 1·0 ml min^{-1}. Under these conditions, the three glucuronide metabolites were collected in a single fraction from 2 to 5 min after injection. The three glucuronides were then separated in a second step, using an isocratic system of 5% methanol/95% 0.01 M KH_2PO_4-$N(CH_3)_3$ (0.02 M) buffer, with the same flow rate. The three separated glucuronide metabolites were collected in fractions having retention times of 4.5, 6.1

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and 7.8 min. Prior to mass spectrometric analysis, partial removal of the potassium salts from the HPLC extracts was accomplished via a Waters Associates C_{18} Sep – Pak column with a water-wash and methanol elution. Additional details of the dosing, collection and separation methodologies for various urine and fecal metabolites are described elsewhere.[18]

All DCI mass spectrometric experiments were performed with a Finnigan-MAT 4023 mass spectrometer operating in the CI mode. Because previous DCI mass spectrometric studies of doxylamine metabolites and related compounds[9] showed the 1-phenyl-1-(2-pyridinyl)ethyl carbonium ion (m/z 182) to be a marker ion for doxylamine metabolites resulting from modification of the ethanolamine moiety, the m/z 198 ion was carefully monitored with the expectation that either ring-hydroxylated or ring-conjugated doxylamine metabolites might be indicated by the presence of this ion. The intensity at m/z 198 was monitored in all DCI mass spectrometric experiments, and the scan before the signal for this ion maximized generally gave the best spectrum for all three glucuronide metabolites. The DCI mass spectra in Fig. 1 were obtained in this manner.

Samples were introduced via a Vacumetrics DCI probe incorporating a platinum filament and a heating range of 50 mA s^{-1} for 60 s. The reagent gas (methane or 10% ammonia in nitrogen) was set to an uncorrected nominal source pressure of 0.25 Torr. The source temperature was 270 °C for methane DCI mass spectrometric and 200 °C for ammonia DCI mass spectrometric experiments.

Low- and high-resolution FAB mass spectra were obtained with a Kratos MS-50 high-resolution mass spectrometer equipped with an M-Scan fast atom source and gun. FAB ionization employed 10-keV xenon atoms impinging on a copper target. Thioglycerol was used as a FAB liquid matrix. High-resolution FAB mass spectrometric measurements were obtained by peak matching one of the metabolites' quasimolecular ions against the prominent m/z 429 ion, $[C_{12}H_{29}O_8S_4]^+$, from the thioglycerol matrix. FAB tandem mass spectral data were acquired using an MS-50 triple analyser instrument (described previously in Ref. 19) equipped with an Ion Tech fast atom gun. Measurements were obtained by mass-selecting the $[M+H]^+$ ion for collisionally induced dissociation (CID) measurements.

Proton NMR measurements were made using a Bruker WM-500 spectrometer operated at 500 MHz, in methanol-d_4.

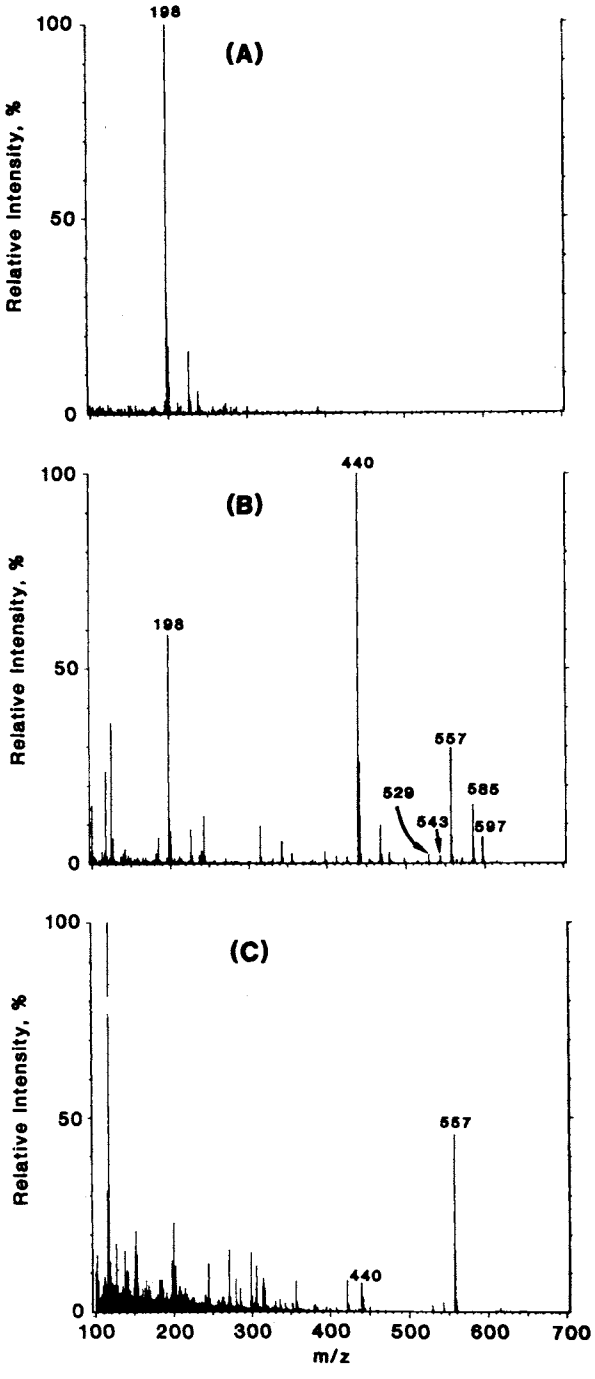


Figure 1. The methane DCI mass spectra of (a) metabolite II and (b) acetylated metabolite II. (c) Ammonia DCI mass spectrum of acetylated II.

RESULTS AND DISCUSSION

Initial analyses of the three metabolite-containing fractions (I–III) from the second stage of HPLC clean-up, by DCI mass spectrometry with either methane or ammonia reagent gas, showed a base peak at m/z 198 and essentially no other significant fragment ions. Figure 1(a) shows the methane DCI mass spectrum of metabolite II. Previous studies have shown significant differences in the behavior of doxylamine and related compounds via DCI mass spectrometry with different reagent gases, namely methane and ammonia.[16] With methane DCI mass spectrometry, the base peak at m/z 182 is attributed to the stable 1-phenyl-1-(2-pyridinyl)ethyl cation, presumably via simple cleavage of the protonated molecular ion. The base peak in the DCI mass spectra of metabolites I–III, observed 16 u higher in mass, is attributed to a ring-hydroxylated form of the 1-phenyl-1-(2-pyridinyl)ethyl cation. Reduction of metabolites I–III to give a fragment 2 u higher in mass was not observed with ammonia DCI mass spectrometry, as might be expected based on the base peak at m/z 184 observed with doxylamine and other related compounds using ammonia as the CI reagent.

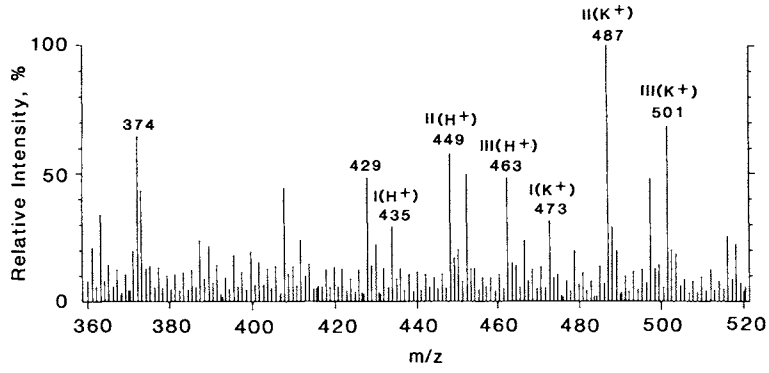


Figure 2. The partial FAB mass spectrum showing metabolites I–III after a single stage of HPLC separation for removal of the major portion of the urine matrix.

The three metabolite-containing fractions were also analysed after acetylation with acetic anhydride in pyridine. The methane DCI mass spectrum of II, after acetylation, is shown in Fig. 1(b). The large peak at m/z 557 is attributed to $[M+H-CH_3COOH]^+$ from a tetraacetylated monodesmethyldoxylamine glucuronide. Under methane CI mass spectrometric conditions, the loss of acetic acid from the protonated molecular ion is a well-known fragmentation pathway for acetylated sugar derivatives.[20] The ion at m/z 440 represents further loss of acetylated methyl-2-ethanolamine, consistent with a monodesmethyldoxylamine derivative. The ion at m/z 198 is also observed for the acetylated sample and is attributed to the ring-hydroxylated 1-phenyl-1-(2-pyridinyl)ethyl cation. Similar results were obtained for metabolite II after acetylation using ammonia as the reagent gas (see Fig. 1(c)). The major difference is the observation of the fragment ion at m/z 118 (base peak) corresponding to protonation and charge retention on the neutral fragment ($HOCH_2CH_2N(CH_3)COCH_3$), which was lost to give the m/z 440 ion under ammonia DCI mass spectrometric conditions.

The other two metabolites gave DCI mass spectra that were nearly identical to II except that ions attributed to acetic acid loss from a protonated parent were also observed 14 u lower (metabolite I) and 28 u lower (metabolite III) in mass than the tetraacetylated desmethyldoxylamine glucuronide (II). Small amounts of acetylated metabolite I and III can be seen in the methane DCI mass spectrum of acetylated metabolite II, shown in Fig. 1(b) at m/z 543 and 529, respectively. These ions were also observed using ammonia DCI mass spectrometry (Fig. 1(c)). This observation was consistent with the assignment of acetylated metabolites I and III as the tetraacetylated didesmethyldoxylamine and triacetylated doxylamine glucuronides, respectively.

The partial FAB mass spectrum of metabolites I–III collected as a single fraction and isolated via HPLC separation from the urine matrix, but without further separation and without removal of the potassium salts from the HPLC mobile phase, is shown in Fig. 2. The peaks at m/z 435, 449 and 463 correspond to the protonated molecular ions for metabolites I, II and III, respectively. The corresponding ions at m/z 473, 487 and 501 represent the potassium adduct molecular ions. The constant difference of 14 u between the protonated or potassium adduct quasimolecular ion series confirms that the three metabolites differ only by the presence or absence of one or two methylene (or methyl) units. Moreover, the molecular weights of I–III were observed directly in the FAB mass spectrometric experiment, whereas with DCI mass spectrometry the molecular weights were inferred from the apparent $(M+H-60)$ ions (based on the assumption of a facile loss of acetic acid).

Other ions in Fig. 2 include a fragment ion at m/z 374, which is also observed in the FAB mass spectra of all three metabolites after further separation, and an ion at m/z 429 from the thioglycerol matrix. The remaining peaks are attributed to chemical noise from other components in the urine matrix as well as the addition of excess potassium from the HPLC mobile phase to the thioglycerol matrix.

In order to separate the three metabolites into three fractions, another stage of HPLC chromatography was utilized. Although the three fractions contained largely a single metabolite, the background level remained high, and the quality of the spectra for the separated glucuronide metabolites remained similar to that shown in Fig. 2. Removal of the bulk of potassium with a Sep-Pak column resulted in a significant improvement in the signal, with commensurate reduction in the background ions. For example, Fig. 3 shows the FAB mass spectrum of a single component, metabolite II, after two HPLC clean-up steps and a C_{18} Sep-Pak clean-up step. This fraction, showing a single component, was the cleanest fraction obtained. HPLC fractions containing I and III showed substantial FAB mass spectrometric signals for metabolite II, which eluted between metabolites I and III. The ions at m/z 449, 471 and 487 correspond to the proton, sodium and potassium adducts, respectively, of metabolite II. Assuming that the metabolites are in fact glucuronides, the ions at m/z 374 and 198 very likely correspond to structures *a* and *b* as depicted in Fig. 4. These same fragment ions are also observed with metabolites I and III (spectra not shown). This observation supports the assumption that the methyl groups that distinguish between metabolites I–III are indeed on the portion of the molecule lost as a neutral fragment. Ion *a* (Fig. 4) corresponds to loss of $HOCH_2CH_2NR_2$ ($R=H$ or CH_3) from the molecular ion, a loss also observed in the DCI spectra of the acetylated metabolites. Further loss of the sugar ring gives ion *b* at m/z 198, which was also observed via DCI mass spectrometry. This ion seems to be a characteristic fragment with these ring-substituted oxy-

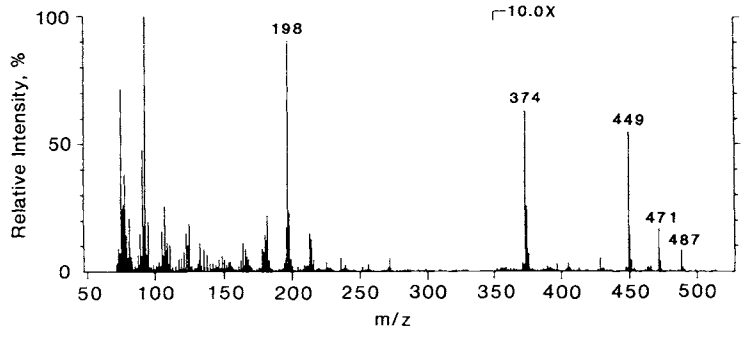
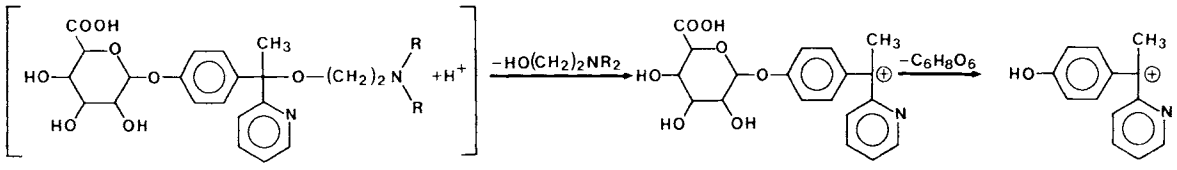


Figure 3. The FAB mass spectrum of metabolite II after additional HPLC separation from I and III.



I: R = H
II: 1R = H, 1R = CH_3
III: R = CH_3

a: m/z = 374

b: m/z = 198

Figure 4. Proposed scheme for the fragmentation observed in the FAB mass spectrum of I–III.

gen-linked doxylamine metabolites. Indeed, loss of a sugar moiety (176 u) has been observed previously in the FAB mass spectra of glucuronides of alcohols, phenols, acids and quaternary amines, although generally from the $[M+H]^+$ ion rather than from a fragment ion.[21]

The elemental compositions of the metabolites were checked by high-resolution peak matching of the FAB mass spectrometric quasimolecular ions against the prominent *m/z* 429 matrix ion. The exact masses obtained (Table 1) indicated that the metabolites had the correct exact masses for assignments as glucuronides, with elemental compositions given by $[C_{21}H_{24}N_2O_8R_2X]^+$, where R = H and/or CH_3 and X = H or Na.

To confirm the assumption that the ions at *m/z* 374 and 198 observed with all three metabolites were indeed fragment ions from the proposed molecular ions, rather than impurities due to degradation or background from the HPLC mobile phase, the $[MH]^+$ ion from each metabolite was mass-selected and subjected to high-energy CID.[22] This experiment (FAB tandem mass spectrometry) produced fragment ions whose association with the parent ion is essentially unequivocal. The FAB tandem mass spectrum of metabolite II is shown in Fig. 5. The two most prominent ions are the *m/z* 374 and 198 ions observed previously and depicted as fragment ions *a* and *b* in Fig. 4. An additional fragment is also observed at *m/z* 182, corresponding to loss of an additional oxygen with the sugar ring, presumably the atom linking the glucuronide sugar to the doxylamine moiety. The FAB tandem mass spectra of the other metabolites (I and III) give identical fragment ions to those of metabolite II and differ only in the mass of the parent protonated quasimolecular ion. These data confirm the conclusions reached, based on the previous DCI and FAB mass spectrometric experiments, and further suggest that the three metabolites are O-linked glucuronides of doxylamine, desmethyldoxylamine and didesmethyldoxylamine. However, the actual location of the glucuronide–doxylamine bond could not be determined by mass spectrometry. For instance, either a benzyl or a pyridinyl glucuronide, with *ortho*, *meta* or *para* substitution, could be explained by the mass spectral data alone.

Table 1. Exact masses for selected quasimolecular ions from metabolites I–III

Metabolite	Cation	Mass	Elemental composition	Error
I	H	435.174	$C_{21}H_{27}N_2O_8$	0.003
II	Na	471.175	$C_{22}H_{28}N_2O_8Na$	0.001
III	H	463.208	$C_{23}H_{31}N_2O_8$	0.000

The 500-MHz NMR data for metabolite fractions containing I and II showed *para* substitution on the phenyl ring with no indication of any pyridinyl ring substitution. The aromatic portion of the NMR spectrum of metabolites I and II indicated a *para*-substituted phenyl moiety based on the observation of a set of transitions from an AA'XX' spin system centred at 7.2 ppm, which is characteristic of this type of *para*-substituted aromatic ring system. The protons from the pyridine ring remained unaffected by the glucuronide substituent, based on a comparison with the parent antihistamine. Protons observed elsewhere in the NMR spectrum, where they could be unambiguously identified, were similarly unaffected. However, all of the proton NMR spectrum of III and much of the spectrum of I and II were

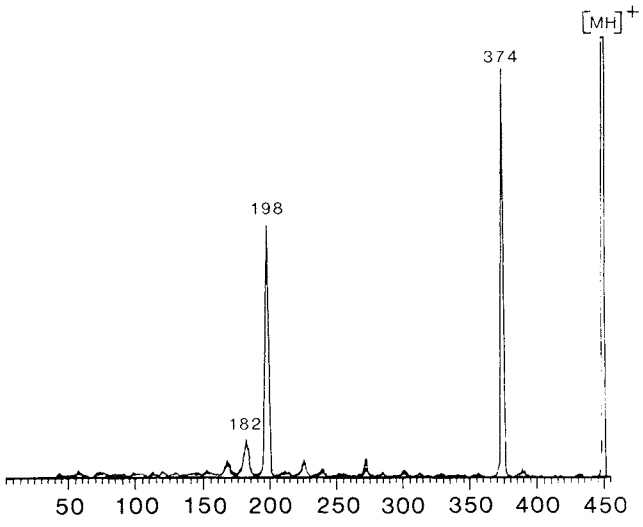


Figure 5. The FAB tandem mass spectrum from the $[MH]^+$ ion of metabolite II.

obscured by other components in these HPLC-separated fractions. The impurities in the 1.0–5.5 ppm region were of a level such that they obscured the aliphatic and glucuronide moieties of the antihistamine metabolites I and II. The aromatic region of metabolite III was also obscured. These unknown components, attributed to the urine, were unseparated from the glucuronides even after two HPLC steps and one Sep-Pak clean-up step. Complete separation of the glucuronide metabolites from all other components in the urine matrix was particularly difficult because some of the compounds are UV transparent, and hence undetected during the HPLC separations. These data suggest that the glucuronides I and II are substituted on the *para* position of the phenol moiety and we have assumed, though not proven, that the position of substitution for III is identical.

CONCLUSION

Three new rat urine metabolites of doxylamine succinate were identified after HPLC separation as *p*-benzyl glucuronides of doxylamine, and the mono- and didesmethyl derivatives. Simple FAB mass spectrometry allowed identification of all three quasimolecular ions, even with minimal HPLC clean-up. Fragment ions from the FAB mass spectra of individual components provided the evidence for the assignment of glucuronide structures to the metabolites. This assignment could also be made, based on the DCI mass spectral data from the acetylated samples, provided that a facile acetic acid loss is assumed. High-resolution measurements of FAB-produced quasimolecular ions allowed the assignment of elemental compositions that were consistent with the proposed glucuronide structures. FAB tandem mass spectrometry allowed the fragment ions to be unequivocally associated with each parent quasimolecular ion, even though the HPLC separation was incomplete. The ability of tandem mass spectrometry to sort out problems of this type with complex (or even fairly simple) mixtures cannot be overemphasized. This can be particularly useful with incompletely separated metabolites, where each additional separation step introduces the concurrent problems of lower sample recovery and possible metabolite alteration. Indeed, the prominent quasimolecular ions associated with FAB ionization and the easily recognized molecular ion patterns from H-, Na- and K-containing quasimolecular ions might recommend that some metabolite studies would benefit from FAB mass spectrometric (and, if possible, FAB tandem mass spectrometric) examination after minimal separation.

The NMR data, although insufficient for complete structural characterization, provided a key piece of evidence unavailable from mass spectral studies, namely the exact location and position of the glucuronide substituent on the *para* position of the phenyl moiety in metabolites I and II. Complete assignments of the glucuronide and aliphatic resonances by proton NMR were not possible because of the large amount of urine-related impurities remaining in the samples. Interestingly, these components were not observed by either DCI or FAB mass spectrometry. In this instance, mass spectrometry acted to clean-up the samples, based on an apparent discrimination between the glucuronide metabolites and any other components. That the smaller masses in the FAB and DCI mass spectra are fragments from the metabolites, and not due to the other components giving rise to the unwanted NMR signals, is established by the tandem mass spectrometric experiments.

These experiments allowed characterization of three previously unidentified metabolites from doxylamine succinate. Metabolites I and II can be considered to be unambiguously identified, while the structure for III is tentatively assigned based on the assumption that all three metabolites have the same site for the glucuronide substituent.

Acknowledgement

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