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Non-enzymatic Formation of a Novel Hydroxylated Sulfamethoxazole Derivative in Human Liver Microsomes: Implications for Bioanalysis of Sulfamethoxazole Metabolites

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Abbreviations: SMX, sulfamethoxazole; SMX-OH, hydroxy-sulfamethoxazole; SMX-NHOH, sulfamethoxazole hydroxylamine; liquid chromatography/mass spectrometry (LC/MS);

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Abstract

Sulfamethoxazole is metabolised by microsomal CYP2C9 to a hydroxylamine which is thought to be responsible for the relatively high incidence of hypersensitivity reactions associated with the drug. Accurate quantification of the hydroxylamine requires loss of metabolite through autoxidation to be blocked with ascorbate. In this study a partly non-enzymatically generated arylhydroxylated derivative of sulfamethoxazole was identified by liquid chromatography/mass spectrometry in incubations of human liver microsomes, and was found to co-elute with the isomeric hydroxylamine under the conditions of three published HPLC assays. Partial inhibition of the arylhydroxylation by 1-aminobenzotriazole suggested some involvement of P450. However, the formation of this compound was ascorbate-dependent, and was enhanced by the addition of Fe^{2+}/EDTA and inhibited by desferrioxamine but not by mannitol. These findings are consistent with the phenol being generated via an Fe^{2+}/ascorbate/O_2 oxygenating system not involving hydroxyl radicals. It was also produced by H_2O_2/ascorbate. Since the compound shares close chromatographic similarities with the hydroxylamine metabolite, it is possible that previous studies may have inaccurately characterized or quantified sulfamethoxazole metabolism.

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Introduction

The antimicrobial sulfamethoxazole (SMX; Fig. 1) is associated with a relatively high incidence of immune-mediated hypersensitivity reactions (Vilar et al., 2003). This is believed to be an idiosyncratic consequence of enzymatic generation of the hydroxylamine metabolite (SMX-NHOH) and subsequent autoxidation to a protein-reactive nitroso species (Cribb et al., 1991). The cellular distribution (Naisbitt et al., 1999), cytotoxicity (Vyas et al., 2005; Lavergne et al., 2006) and immunogenicity (Naisbitt et al., 2001) of SMX metabolites have been studied extensively.

SMX-NHOH formation has been found both in hepatic microsomes (Cribb and Spielberg, 1990a; Cribb et al., 1995) and in vivo (Cribb and Spielberg, 1992; Gill et al., 1996). Metabolism of SMX to SMX-NHOH is catalysed by human hepatic CYP2C9 (Cribb et al., 1995; Gill et al., 1999) and neutrophilic myeloperoxidase (Cribb et al., 1990). Although it was claimed that cyclooxygenase could also *N*-hydroxylate SMX, a recent study found the oxidation of SMX in cyclooxygenase incubations containing ascorbate was due to H_2O_2 in the reaction mixture (Vyas et al., 2006). Importantly, in the context of the present observations, accurate quantification of a hydroxylamine in vitro (Cribb and Spielberg, 1990a) and in vivo (Gill et al., 1996; Winter et al., 2004) requires loss of analyte through autoxidation to be blocked with a reducing agent; ascorbate being the agent of choice. Additionally, identification of the hydroxylamine has often depended upon co-chromatography with an authentic standard using relatively simple HPLC conditions.

In this study we identified a novel hydroxylated SMX derivative (SMX-OH) that is formed partly via a non-enzymatic, ascorbate-dependent pathway in microsomal incubations and is potentially a confounding factor in the quantification of SMX-NHOH. We have investigated the mechanism of its formation and determined the minimum bioanalytical requirements for accurate identification of SMX-NHOH, including a suitable chromatographic method for separation of SMX-NHOH from SMX-OH. We have also shown that SMX-OH rather than SMX-NHOH is the product of SMX's oxidation by H_2O_2 in the presence of ascorbate.

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Materials and Methods

Materials. SMX-NHOH was synthesised as described previously (Naisbitt et al., 1996), and its purity was confirmed by NMR and liquid chromatography/mass spectrometry (LC/MS) to be >99%. Unless specified otherwise, all reagents were purchased from Sigma-Aldrich Ltd. (Gillingham, Dorset, UK).

Microsomal oxidation of SMX. Human liver microsomes (1 mg/ml) were incubated in Tris buffer (50 mM; pH 7.4; final volume, 1 ml) at 37°C with SMX (500 µM), ascorbate (1 mM), $MgCl_2$ (3.3 mM) and NADPH (1 mM). Other reactions contained additionally either desferrioxamine (25 mM), mannitol (0.5 M) or 1-aminobenotriazole (1 mM). Control incubations contained SMX in the absence of either ascorbate or NADPH. Following 1-h incubations, protein was precipitated by addition of an equal volume of ice-cold acetonitrile, and supernatant analysed by LC/MS using one of the four LC systems listed below.

H_2O_2 oxidation of SMX. SMX (800 µM), ascorbate (1 mM), and H_2O_2 (1 mM) in Tris buffer (50 mM; pH 8.0; 1 ml) were incubated at 37°C. Other reactions contained additionally either desferrioxamine (25 mM), mannitol (0.5 M), or $FeSO_4$ (100 mM) and EDTA (100 mM). Control incubations contained SMX in the absence of either ascorbate or H_2O_2. Following 1-h incubations, aliquots of reaction mixtures were analysed by LC/MS using one of the four LC systems listed below, and the product was quantified by UV spectrophotometry.

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HPLC analyses. Three published chromatographic protocols were used to attempt to separate the novel hydroxylated species from an authentic SMX-NHOH standard, both to try to identify suitable conditions for analysis and to determine if any previous studies may have failed to adequately separate the two compounds. Samples were eluted from (1) a Prodigy 5-µm ODS-2 column (150 × 4.6 mm; Phenomenex, Macclesfield, Cheshire, UK) using an isocratic mobile phase of glacial acetic acid (1% v/v) and acetonitrile (20% v/v) in water at a flow rate of 1 ml/min (Gill et al., 1996), (2) an Ultrasphere 5-µm C-18 column (150 × 4.6 mm; Beckman Coulter, High Wycombe, Buckinghamshire, UK) using an isocratic mobile phase of acetonitrile (25% v/v), glacial acetic acid (1% v/v) and triethylamine (0.05% v/v) in water at a flow rate of 1 ml/min (Cribb and Spielberg, 1990a) and (3) a Nova-pak 4-µm C-18 column (150 × 3.9 mm; Waters, Elstree, Hertfordshire, UK) using a isocratic mobile phase of acetonitrile (18.95% v/v), glacial acetic acid (1% v/v), and triethylamine (0.05% v/v) in water at a flow rate of 1 ml/min (Coleman et al., 1989; Vyas et al., 2006). Complete resolution of the hydroxylated SMX derivatives was achieved with (4) an Ultrasphere 5-µm C18 column (250 × 4.6 mm; Beckman Coulter) using 10% acetonitrile in 50 mM formic acid for 5 min, 10-70% acetonitrile over 20 min and 70-100% acetonitrile over 2 min. The flow rate was 1 ml/min. The column was re-equilibrated with the starting eluent for 10 min prior to any further elutions.

Metabolite estimation. A BioTek 560 UV detector (Sci-Tek Instruments Ltd, Olney, Buckinghamshire, UK) connected to a reversed phase column was used to determine absorbances of eluted analytes at 254 nm. Metabolite formation in SMX/H_2O_2/ascorbate incubations was estimated by comparison of the compound's peak area with that of an internal standard of 10 µM sulfadimethoxine added immediately prior to analysis.

Mass spectrometric analysis. An LCQ Deca XP Plus ion-trap mass spectrometer (Thermo Finnigan, Hemel Hempstead, Hertfordshire, UK) interfaced to a reversed phase column was used to characterize SMX and its metabolites via negative-ion electrospray ionisation with data-dependant MS^2 fragmentation. It was controlled through Xcalibur software.

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Results and Discussion

Identification of the novel SMX-OH derivative. Although standard microsomal incubations of SMX in the continuous presence of ascorbate, when they were analysed by HPLC method 1, were found to have produced a single mass chromatogram peak corresponding to a hydroxylated metabolite ($[M-H]^-$ *m/z* 268), this peak was also present, albeit at lower abundance, in control incubations without NADPH. Further investigations found that although the peak of *m/z* 268 present in these incubations coeluted with an SMX-NHOH authentic standard (Fig. 2A) under published HPLC conditions (method 1), it had a distinct MS/MS fragmentation pattern (Fig. 3) lacking the characteristic neutral loss of 17 amu seen with SMX-NHOH. In all other respects its fragmentation corresponded to that of SMX-NHOH. Unlike the previously identified plasma and urinary metabolite 5-hydroxysulfamethoxazole (Vree et al., 1994), the neutral loss of 96 amu (loss of the 5-methylisoxazole ring) was unchanged from the parent SMX molecule, demonstrating that the hydroxylation must be on the 4-aminophenyl ring, although the precise location was not determinable. Changes to the chromatography conditions (method 4) produced good separation of the novel compound (SMX-OH) from an SMX-NHOH standard (Fig. 2B), and also allowed an estimation of the relative abundance of the two hydroxylated metabolites by LC/MS. From the areas of their mass-chromatogram peaks, it was estimated that SMX-OH was produced at approximately one third of the amount of SMX-NHOH (Fig. 4). Neither HPLC method 2 nor method 3 resolved SMX-OH from SMX-NHOH (Fig. 2C and 2D). The presence of SMX-OH in microsomal incubations without NADPH and in incubations using boiled microsomes (data not shown) suggested that it is generated non-enzymatically.

However, the non-specific P450 inhibitor 1-aminobenzotriazole (Emoto et al., 2005) not only produced almost complete inhibition of the formation of SMX-NHOH in microsomes but also partially blocked the generation of SMX.OH (Fig. 4). Therefore it would appear that although the microsomal production of SMX.OH requires ascorbate, the formation of SMX.OH is partly dependent on a presently uncharacterized mechanism involving P450.

Investigation of the peroxidative mechanism of SMX-OH formation. An incubation of SMX with H_2O_2 and ascorbate in Tris buffer generated a single hydroxylated metabolite with an MS/MS fragmentation pattern identical to SMX-OH. This reaction was dependent on the presence of both H_2O_2 and ascorbate (Table 1). Contrary to the claim of a previous study (Vyas et al., 2006), SMX-NHOH was not detected when SMX was incubated with H_2O_2 and ascorbate. However, the HPLC conditions (method 3) used by Vyas et al. (2006) did not separate authentic SMX-NHOH from SMX-OH when they were tested by the present authors.

The kinetics of SMX-OH formation were investigated in an SMX/H_2O_2/ascorbate incubation. As appreciable quantities were detected within 0.1 h, it was not possible to determine the minimum required time for formation. Maximal quantities of SMX-OH were found after 1.5 h, which were stable for at least 4 h (Fig. 5). It is likely that the effective termination of the reaction after this point was due to depletion of one of the reactants, although this was not demonstrated experimentally.

Incubations of SMX with either H_2O_2 or human liver microsomes only generated SMX-OH in the presence of ascorbate. Furthermore, addition of the iron chelator desferrioxamine completely inhibited the generation of SMX-OH, whereas the hydroxyl

radical scavenger mannitol (Ingelman-Sundberg et al., 1991) had no effect, even at high concentrations. Addition of $FeSO_4$ and EDTA to the peroxide reaction increased the amount of SMX-OH formed over 1 h (Table 1). These data are consistent with the observed aromatic hydroxylation of SMX occurring via an Fe^{2+}/ascorbate/O_2 oxygenating complex (Hamilton, 1962), as first described by Udenfriend et al. (1954), in which the ascorbate acts as a complexing agent and the ultimate two-electron donor, rather than via hydroxyl radicals generated by Fenton (Mishin and Thomas, 2004) or Haber-Weiss (Johansson and Ingelman-Sundberg, 1983) reactions. Although there have been conflicting reports regarding the formation of hydroxyl radicals by Udenfriend's reagent (Hamilton, 1962; Ito et al., 1993; Li et al., 2003), the lack of inhibition by mannitol suggested they were not involved here. In summary, it is concluded that locally generated H_2O_2 was not involved significantly in the ascorbate-dependent formation of SMX-OH in these microsomal incubations. The non-enzymatic hydroxylation of SMX in microsomal incubations contrasts with aromatic hydroxylation of substrates such as salicylate (Ingelman-Sundberg et al., 1991; Halliwell and Kaur, 1997) and terephthalate (Mishin and Thomas, 2004) which has been attributed to hydroxyl radicals.

Assessment of previously used analytical techniques. With very few exceptions (Cribb and Spielberg, 1990a; Cribb and Spielberg, 1990b), all previous studies to have quantified SMX-NHOH formation in microsomal or peroxidase systems, or in clinical samples, have used HPLC separation with either UV or MS detection, and identified SMX-NHOH by co-chromatography with an authentic standard (Gill et al., 1996, 1999; Winter et al., 2004). We tested three published HPLC methods, designated 1, 2 and 3, for their ability to separate SMX-OH from SMX-NHOH, but none of the systems achieved this separation (Fig. 2). Furthermore, the use of either UV or simple mass detection is clearly insufficient for

accurate metabolite verification, given the chromatographic similarity of SMX-NHOH to SMX-OH. The minimum requirements for accurate identification and quantification should be confirmed separation from SMX-OH, and either MS-MS fragmentation analysis or conversion to nitro-SMX by addition of excess base (Rieder et al., 1988).

While there is no question that the major oxidative metabolite of SMX in microsomal incubations containing NADPH is SMX-NHOH, previous studies are likely to have insufficiently separated this from the non-enzymatically generated SMX-OH, and as a result may not have accurately identified or quantified the SMX-NHOH. These data have major implications for designing future analyses of SMX metabolite formation.

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Footnotes

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Figure legends.

Figure 1. Sulfamethoxazole (SMX)

Figure 2. LC-UV chromatograms (254 nm) of incubations of SMX with human liver microsomes. (A) SMX-OH generated in a microsomal incubation with ascorbate (grey trace) co-eluted with an SMX-NHOH authentic standard (black trace) under HPLC conditions used previously (method 1); (B) SMX-OH (grey trace) separated effectively from a SMX-NHOH standard (black trace) by novel chromatographic conditions (method 4); (C,D) SMX-OH (grey trace) and SMX-NHOH (black trace) display chromatographic similarities under two previously used HPLC conditions (methods 2 and 3). All the UV traces are representative of three independent experiments.

Figure 3. HPLC-MS/MS spectra of SMX, SMX-NHOH and SMX-OH with diagnostic fragment ions.

Figure 4. LC/MS mass chromatograms (negative-ion detection) of incubations of SMX with human liver microsomes when the incubations were analysed by HPLC method 4. (1) Incubations containing SMX, NADPH and ascorbate. (2) Incubations containing SMX, NADPH, ascorbate and 1-aminobenzotriazole. SMX was monitored in channel *m/z* 251.5-252.5 ($[M-1]^-$); SMX-OH and SMX-NHOH were monitored in channel *m/z* 267.5-268.5 ($[M-1]^-$).

Figure 5. Time course of SMX-OH formation in an SMX/H_2O_2/ascorbate incubation.

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TABLE 1

Ascorbate-dependent oxidation of SMX to SMX-OH by H_2O_2

Incubation	Incubation Components[a]	SMX-OH generation[b]
1	SMX (800 µM)	0
2	1 + H_2O_2 (1 mM)	0
3	2 + ascorbate (1 mM)	0.262
4	3 + Fe^{2+} (100 mM) + EDTA (100 mM)	1.258
5	3 + mannitol (0.5 M)	0.283
6	3 + desferrioxamine (25 mM)	0.053

[a]SMX was incubated in Tris buffer (50 mM; pH 8.0) at 37ºC for 1 h.

[b]Formation of SMX-OH was assessed from the chromatographic peak-area ratio SMX-OH:sulfadimethoxine at 254 nm.

Figure 1

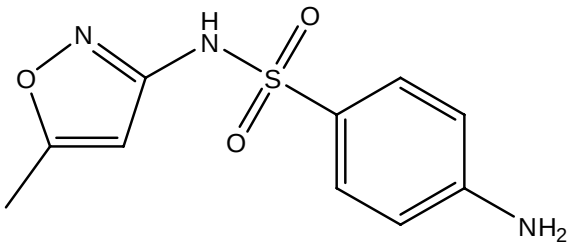

Figure 2

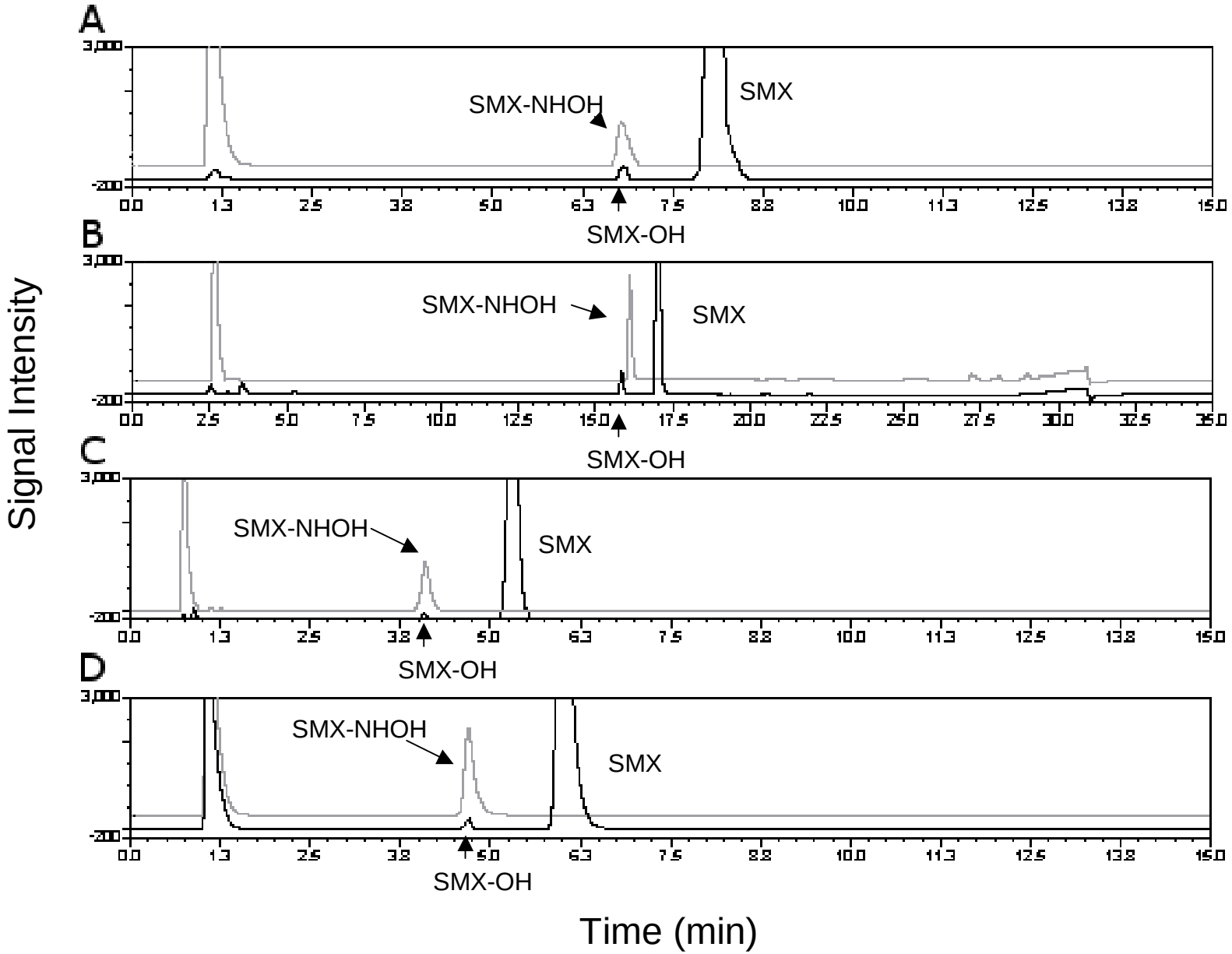

Figure 3

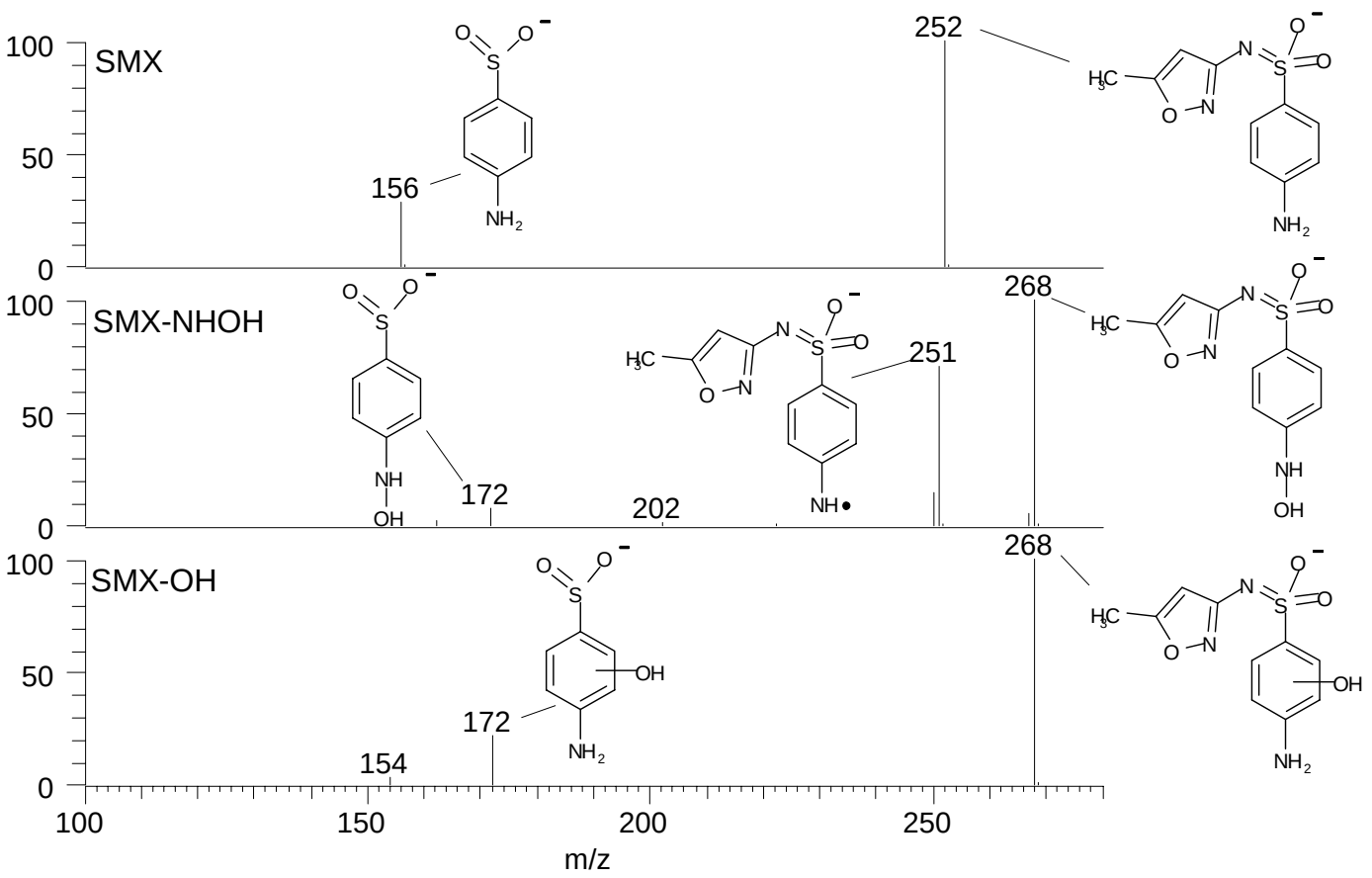

Figure 4

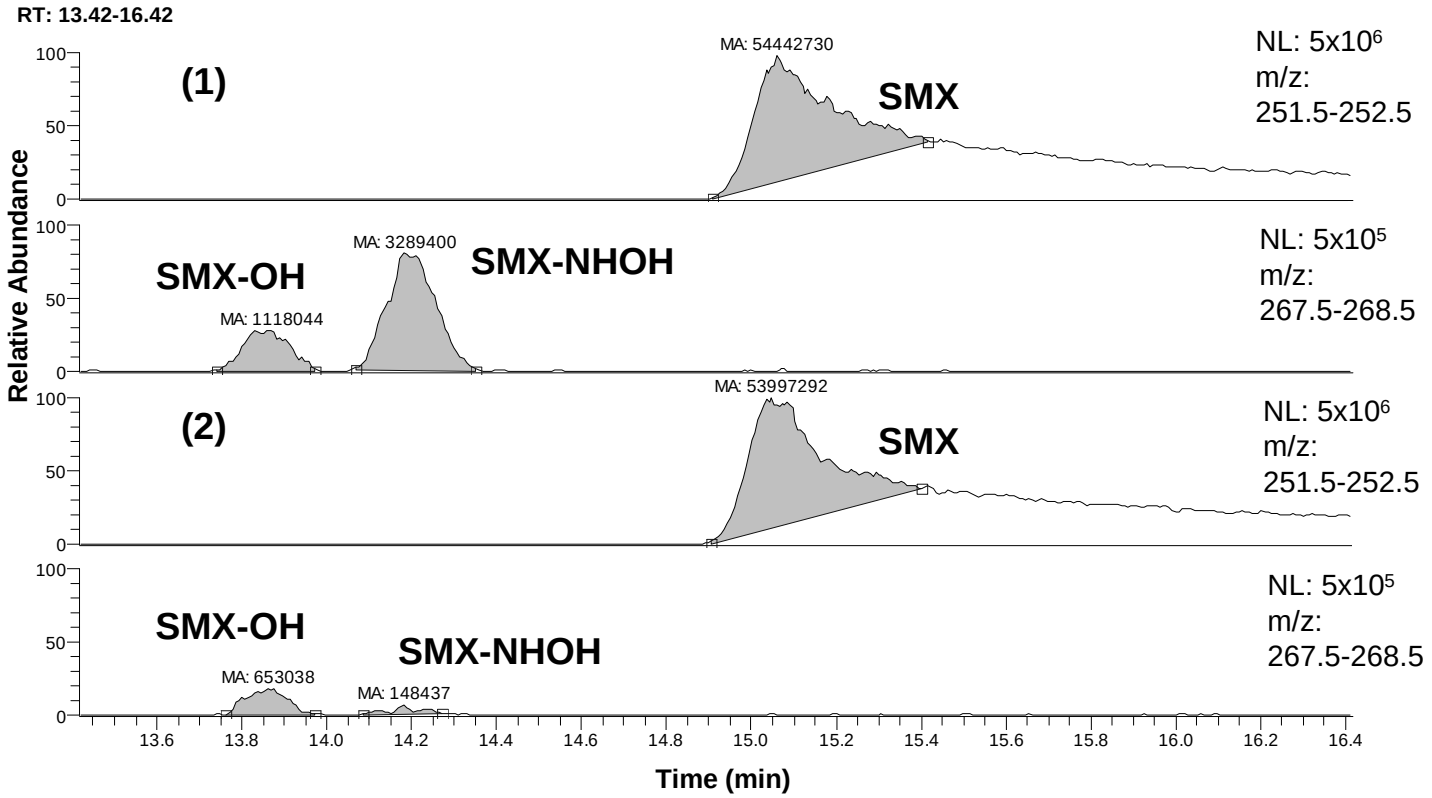

Figure 5