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Determination of hemoglobin adduct of a musk xylene metabolite in trout as biomarker of exposure by gas chromatography mass spectrometry

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Abstract: Musk xylene(MX) is frequently used as fragrances in formulation of personal care products. Quantification of a bound 4-amino-MX(4-AMX) as cysteine adducts in trout hemoglobin(Hb) was made by gas chromatography-ion trap-mass spectrometry(GC/MS). The Hb samples were collected from trout after 24 h exposure to MX at 10 μg/g, and or menhaden oil(control). The formation of cysteine-Hb adduct was observed from nitroso derivative of MX, released by alkaline hydrolysis. The released 4-AMX metabolite was extracted in *n*-hexane. The extract was then reduced by evaporation, and analyzed by GC/MS. When similar agreement of mass spectral features and retention time of 4-AMX were obtained in both standard and sample solutions, the presence of 4-AMX metabolite in the Hb was confirmed. The concentration of 4-AMX was found to be 3.1×10^{-6}— 6.9×10^{-6} mg/g in the Hb solution. Quantitation was made based on an internal standard, a calibration plot, and response factor. In the non-hydrolyzed and laboratory blank extracts, the 4-AMX metabolite was not detected. Additionally, coeluting and interfering ions were observed in the biological samples.

Keywords: biomarkers; musk xylene metabolite; Hb adducts; fish exposure

Introduction

Nitro musks are widely used as synthetic fragrance ingredients in formulation of soaps, laundry detergents, lotions, and body-care products, and so on. The commercial and domestic use and discharge of these compounds from municipal sewage systems have led to their ubiquitous occurrence in the aquatic environment. Nitro musks enter city sewage systems and then the aquatic ecosystem, where they may potentially bioaccumulate in the tissues of aquatic organisms. Musk xylene (1-*tert*-butyl-3, 5-dimethyl-2, 4, 6-trinitrobezene(MX); Fig.1a) is the most important member of nitro musk group with an estimated annual production of up to 1000 metric tons (Rimkus, 1999). Due to its low biodegradability in the environment and high lipophilicity (Muller, 1996), MX is capable of being bioconcentrated in aquatic and terrestrial organisms (Daughton, 1999). It has been detected as contaminants in the river, North Sea, and freshwater (Yamagishi, 1981; Gatermann, 1995; 1998), sewage treatment effluent (Osemwengie, 2001), Norwegian air samples (Kallenborn, 1999), human tissue and milk (Muller, 1996; Rimkus, 1994), developing and adult rats (Suter-Eichenberger, 1998) and fish, mussels, shrimp (Rimkus, 1995). Metabolite of MX(4-amino-MX(4-AMX); Fig.1b) was identified in domestic and industrial sewage sludges (Berset, 2000; Herren, 2000), and whole fish tissues(Osemwengie, 2003).

Despite widespread occurrence of MX, some information about its ecotoxicity are available (Giddings, 2000). The acute toxicity of MX is low, though a nondose-dependent increase in the incidence of liver tumors was reported in mice after long-term administration of MX in the diet (Maekawa, 1990). Several studies suggested that MX was not genotoxic (Putman, 1993; Api, 1995). The fact that MX has been identified as an inducer of hepatic cytochrome P450 2B enzymes suggests that non-genotoxic mechanisms, such as increased cell proliferation, may be responsible for the increased liver tumors, analogous to phenobarbitual. MX is known as co-mutagenic substances for polycyclic aromatics and aromatic amines. It was identified as inducer of toxifying enzymes in the rat liver, and a cytochrome P450 1A2 isoenzyme (Minegishi, 1991).

Biomarkers may serve to evaluate the completeness of exposure assessment. The use of biomarkers in assessing exposure for risk assessment increasingly may include consideration of susceptibility factors in conjunction with exposure factors, such as the presence of a specific genetic polymorphism for a metabolic enzyme (Bois, 1995; D'Erricco, 1996). One of the promising biomarkers of exposure to xenobiotics involving nitroarenes is blood protein Hb. The binding of metabolite from aromatic amines and nitroarenes to Hb may be used as a marker of internal exposure. The use of Hb bound metabolites as biomarkers of exposure may be used to assess cumulative exposure over a longer time range and thus may be better suited for risk assessment than quantitation of urinary metabolites (Farmer, 1987; Skipper, 1994). Nitrosoarenes are intermediates of the reaction(Fig.1) formed by reduction of nitro musk or other nitro compounds, or by oxidation of aromatic amines to nitroso derivatives and reaction of these with the sulfhydryl group(SH) of cysteine in Hb to form a base/acid labile sulfinamide that hydrolyzes to aromatic amines. The Hb adduct of aromatic amine may be a good molecular dosimeter for the target tissue dose of the ultimate carcinogenic metabolite of the amine (Skipper, 1994). The biotransformation and toxicokinetics of MX metabolites in human Hb have been reported(Riedel, 1999). Recently we reported the formation and recoveries of nitro musk adduct of trout Hb(Mottaleb, 2004) and the method was employed to detect a 4-amino-MX from carp Hb for the purposes of ecological assessment of MX exposure(Mottaleb, 2004). The present study describes identification and quantification of a bound 4-AMX metabolite, formed adduct with MX and cysteine-Hb of trout, by gas chromatography-ion trap-mass spectrometry(GC/MS). Trout were exposed to MX and suitable controls are also used.

1 Material and methods

1.1 Reagents and chemicals

The internal standard (IS), naphthalene-d8 was purchased from Absolute Standard Inc., CT. Sodium dodecyl

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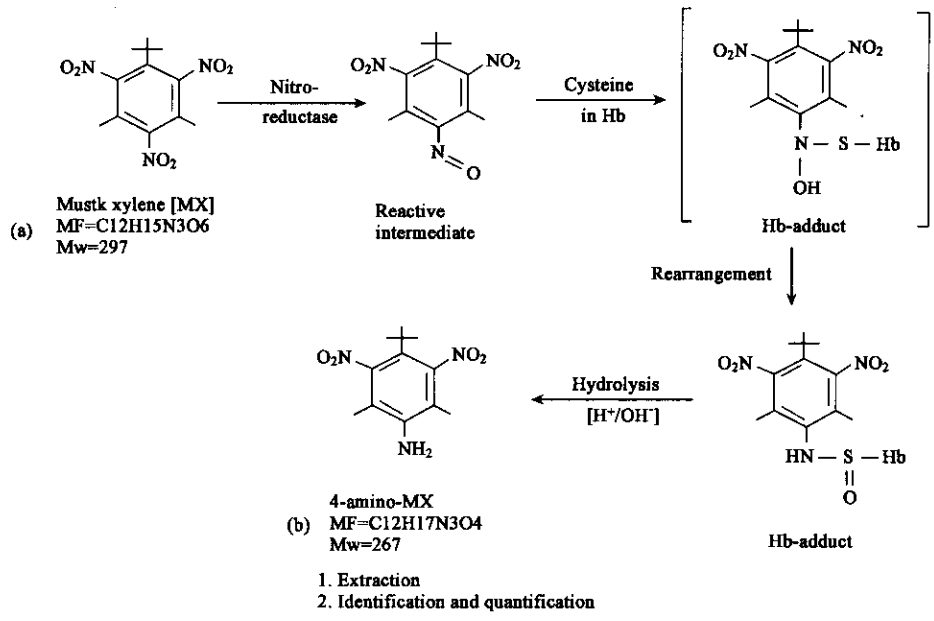


Fig.1 The metabolic path of hemoglobin cysteine adduct formation of 4-AMX metabolite
Musk xylene(a) to 4-amino-musk xylene(b); Hb represents hemoglobin

sulfate (SDS), sodium hydroxide pellets, and *n*-hexane (HPLC grade) were obtained from the Sigma-Aldrich, Fisher Scientific, and J. T. Baker, respectively. The standard solution of 4-amino-MX was obtained from Dr. L. I. Osemwengie, U. S. Environmental Protection Agency, Las Vegas, Nevada as gift. Tricane methane sulfonate(MS 222) was purchased from Sigma(St. Louis, MO). Solution with known amounts of metabolite and IS were used to prepare calibration. De-ionized water was used for all preparations where necessary.

1.2 Fish exposure to MX compound

To examine the Hb adducts with nitro musk, three trout were exposed to MX with a dose concentration of about 10 μg/g fish for 24 h. The test solution of MX was prepared in menhaden oil, and was intraperitoneally injected into the trout that were anaesthetized with 75 mg/L MS 222. For control experiment, three trout were exposed to menhaden oil without MX for the same time frame. Then control and MX exposed fish were placed in labeled two tanks with circulating water at 13℃. Table 1 details *in vivo* exposure of trout with MX.

Table 1 ***In vivo*** **exposure of trout to MX for 24 h, and concentration of 4-AMX metabolite in the trout Hb solution.**

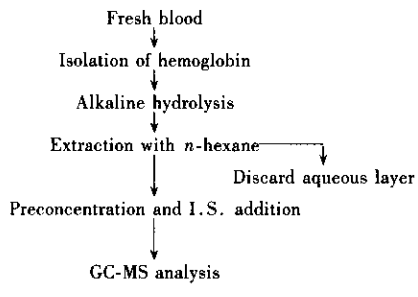
Fish exposed to compounds	Number of fish exposed	Fish wet weight, g	MX dose given to fish, mg	Average dose of MX, $\times 10^{-3}$ mg/g	Concentration of 4-AMX, $\times 10^{-6}$ mg/g
MX	3	153	1.5	9.97	3.1
		179	1.8		6.9
		168	1.7		4.5
Vehicle	3	164	None	None	Not detected
(menhaden		180			
oil)		134			

1.3 Collection of blood from fish and isolation of Hb

To draw the blood, trout were anaesthetized with 250 mg/L MS 222. This concentration was fatal for the trout. Blood samples were collected from the anaesthetized fish into heparinized individual syringes through the caudal vein, and placed into heparinized sterile Interior Vacutainers (VACUTAINER: BD Biosciences Blood Collection Sets, VWR International, Inc. Irving, TX). All blood samples were kept on ice immediately after collection. Erythrocytes or red blood cells were isolated from plasma by centrifuging at 3500 × g for 10 min at 4℃ and washed twice with 2 volumes of 0.9% saline. Adding 2 volumes of distilled water lysed the cells. The cellular debris was discarded. The Hb solutions were kept in a freezer at − 24℃ for further analysis.

1.4 Alkaline hydrolysis of Hb to release amine adduct and extraction of the adduct

The alkaline hydrolysis, and extraction procedures described by Mottaleb(Mottaleb, 2004) were followed in this study. Briefly, about 0.75 g Hb solution was taken into a dried and cleaned glass tube where 9 ml 0.5% of SDS followed by adding 1 ml of 10 mg/L NaOH solutions. The mixture was then stirred for 1 h at room temperature and then extracted. The extraction was performed 3 times with 10 ml of *n*-hexane. Pressure developed inside the tube was released by opening the cap. The tube was then placed in a refrigerator for about 30 min to freeze the sample. A clear *n*-hexane layer was obtained as an extract on the top of the aqueous layer in the tube. The extract was then drawn off, and passed through a drying column containing granular anhydrous Na_2SO_4 to remove the residual water. Prior to passing the extract, the drying column was activated with 5 ml of *n*-hexane. The dried extract was then concentrated under a stream of N_2 to 200—300 μl. The IS was added, the solution sealed in GC-Via, and the sample analysed by GC/MS. A schematic representation of experimental procedure as the follows:



To investigate the presence of unbound amino metabolite in the Hb sample, a non-hydrolysed experiment was performed. In the experiment, all chemicals and solvents except the NaOH were added to the Hb, and the same extraction and preconcentration procedures were followed as stated in the alkaline hydrolysis section. A laboratory or reagent blank control experiment was also carried out to check the status of laboratory supplies and consumables by using same amounts of solvents, chemicals, and reagents used for hydrolysis work, except the Hb.

1.5 Determination and quantification of Hb adduct

To determine the 4-AMX metabolite in the trout Hb solution, the preconcentrated *n*-hexane extract was spiked with IS at 4 ng/μl. A standard solution(50 pg/μl) of 4-AMX containing the same amount of IS was prepared. The blank, standard, sample and blank solutions were injected in this order into GC/MS, and at least three replicate runs were taken for each standard and sample solution. Quantification of 4-AMX metabolite was made using response factor(R_F), peak areas of IS, and sample solutions. The R_F was determined from a calibration curve by injecting five different levels of standard solutions, e.g. 0.01, 0.02, 0.05, 0.1, 1.0 ng/μl of 4-AMX containing 4 ng/μl of IS into GC/MS. At least four injections were made for each concentration by auto sampler with a volume of 1 μl. The peak areas of IS and 4-AMX metabolite were calculated using saturn software by selecting the 136 and 252 m/z ions, respectively

1.6 Gas chromatography and mass spectrometry

A Varian gas chromatograph, Model 3400 equipped with 8200 auto sampler connected to a Varian Saturn II GC/MS was used. Separations were performed on a DB-5 MS(J&W Scientific, Agilent Technologies, CA) fused silica capillary column(30 m long, 0.25 mm i.d. and 0.25 μm film thickness) with a helium carrier gas at a constant flow rate of 1 ml/min setting at cylinder pressure, 10 psi. Manual and/or auto sampler injection was used to inject the sample into the GC at a temperature gradient starting at an oven temperature of 60℃ for 1 min, to 280℃ at 10℃/min and holding at 280℃ for 7 min. The injector and transfer line temperatures were kept constant at 300℃. The electron source temperature was adjusted to 250℃ in the electron ionization(EI) 70 eV mode. Mass spectra(50—500 m/z) were scanned from 6 to 30 min.

2 Results and discussion

2.1 Nitroarene adducts to Hb

Nitroarenes are enzymatically metabolized and their intermediates, nitrosoarenes react with DNA, proteins and glutathione. The resulting damage to the bimolecules is often responsible for toxic effects of aromatic amine. The Hb adduct formation involves the reaction of nitrosoarene with cysteine residues of Hb to form a sulfinamide that hydrolyses to aromatic amine. The metabolic path of cysteine-Hb adduct formation with nitro musk xylene is shown in Fig.1. Under strong alkaline condition(pH 12.5), the Hb was hydrolyzed and released the 4-AMX metabolite. The metabolite was then extracted into *n*-hexane and analyzed by GC-MS. In the non-hydrolyzed and reagent or laboratory blank extracts, the metabolite was not found. Thus, it was concluded that the metabolite observed from the hydrolyzed extraction was bound to the trout Hb.

2.2 Determination of 4-AMX metabolite in trout Hb

Fig. 2 compares a typical GC/MS selected ion chromatogram that was obtained by injecting for (1) standard and (2) sample solutions with a volume of 1 μl and 2.5 μl, respectively. The selected ion current profiles at 136 and 252 m/z corresponded to IS and 4-AMX peaks in the chromatograms, respectively. In the standard solution, the 4-AMX, giving peak(b), eluted from the capillary column with a retention time of 20.91 min. In the sample solution, the 4-AMX, giving peak(y), was isolated from other biological species at 20.92 min. The peaks (a) and (x) in the chromatograms represent the IS (naphthalene-d8). By selecting the peaks in the chromatograms, the mass spectra for 4-AMX in both standard and sample solutions were obtained and compared.

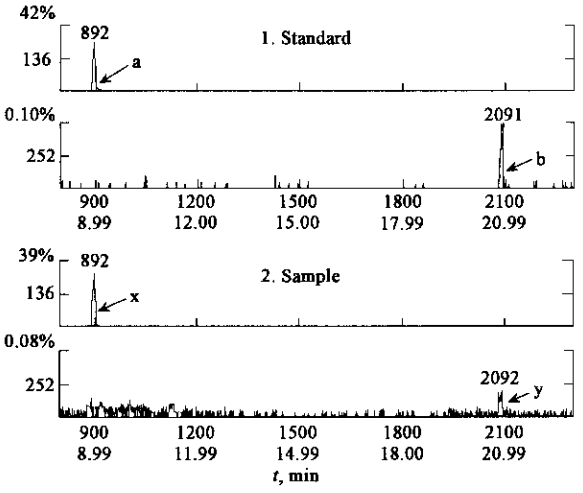


Fig.2 A typical GC/MS selected ion monitoring chromatograms for (1) standard solution containing 50 pg/μl 4-AMX and 4 ng/g IS and (2) sample solution containing 4 ng/g IS. Injection volume: 1 μl standard solution and 2.5 μl sample solution. GC/MS operation conditions are given in the experimental section

Fig. 3 shows the mass spectra of 4-AMX metabolite derived from GC/MS chromatograms(Fig.2) for(1) standard and (2) sample solutions. In the electron-impact mass spectrum, the ions m/z = 267(molecular ion), m/z = 252 (M-15), m/z = 235, m/z = 218 are typical characteristic molecular ion and mass fragments for the 4-AMX metabolite (Herren, 2000). The standard 4-AMX spectrum (a) in Fig. 3, derived from peak(b) at 20.91 min(Fig.2), showed a good agreement of mass signals with Herren(Herren, 2000). The molecular ion and mass fragments are accountable for identification of the 4-AMX, these ions/mass signals agreed well with the sample spectrum(b) with a variation of relative abundance ± 4%. Thus, the compound, giving mass spectrum(b) in Fig.3, obtained from the peak(y), eluted from the capillary column at 20.92 min (Fig. 2), was assigned for 4-AMX metabolite.

Additionally, a few lower masses, coeluting, and interfering ions were also present in the biological samples. Some interfering ions are also observed in the spectrum(b) above the molecular ions and the isotopically substituted molecular ions.

2.3 Quantification of 4-AMX metabolite

The R_F was calculated using the EPA Method 625(U.

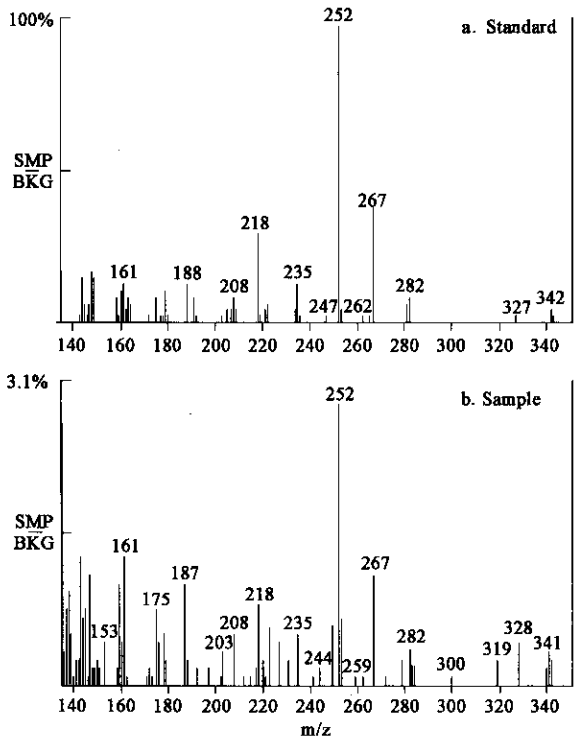


Fig.3 GC/MS derived mass spectra of 4-AMX metabolite: (a) standard solution spectrum(taken from Fig.2, peak, retention time 20.91 min), and (b) sample solution spectrum(taken from Fig.2, peak, retention time 20.92 min). Conditions are the same as in Fig.2

S. EPA, 1982), and was found to be 0.219 with a relative standard deviation of $< 8\%$. The concentration of 4-AMX in the Hb solution was calculated employing the peak areas of IS and 4-AMX of sample solution, and the R_F value. Table 1 includes the concentration of 4-AMX observed in the Hb solution, and it was found to be 3.1×10^{-6}—6.9×10^{-6} mg/g. The difference of 4-AMX concentration in trout Hb solution suggested that various enzymatic activities and or metabolism process occurred during the exposure of the individual fish. Observed concentrations of the metabolite were low compared to exposure level at about 10×10^{-3} mg/g. This indicates that the MX given to the trout was distributed, absorbed, and or metabolized in the whole body, or excreted from the fish. In the control, non-hydrolysed and laboratory blank extracts, the 4-AMX metabolite was never be detected.

3 Conclusions

The bound 4-AMX metabolite with cysteine was detected in trout Hb by GC-ion trap-MS. Enzymatically reduced one nitro group of MX to nitroso compounds formed cysteine-Hb adducts and subsequently yielded an amine. That amine could be suitable as a biochemical endpoint useful for monitoring and assessing of MX hazard exposure. The enzymatic metabolic processes in individual trout may influence the formation of nitroso-derivatives of MX that yielded various concentration of the 4-AMX metabolite formed as trout Hb adducts. The use of Hb adducts as biomarkers of exposure of other nitro musks including polycyclic musks in the general fish populations appears to be worthy of further investigation.

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