Determination of a Bound Musk Xylene Metabolite in Carp Hemoglobin as a Biomarker of Exposure by Gas Chromatography–Mass Spectrometry Using Selected Ion Monitoring*

M.A. Mottaleb*, W.C. Brumley, S.M. Pyle, and G.W. Sovocool
U.S. Environmental Protection Agency, National Exposure Research Laboratory, Environmental Sciences Division, P.O. Box 93478, Las Vegas, Nevada 89193-3478

Abstract

Musk xylene (MX) is widely used as a fragrance ingredient in commercial toiletries. Identification and quantitation of a bound 4-amino-MX (AMX) metabolite was carried out by gas chromatography–mass spectrometry (GC–MS) with selected ion monitoring (SIM). Detection of AMX occurred after the cysteine adducts in carp hemoglobin (Hb), derived from the nitroso metabolite, were released by alkaline hydrolysis. The released AMX metabolite was extracted into n-hexane. The extract was preconcentrated by evaporation and analyzed by GC–SIM-MS. The concentration of AMX metabolite was found to range from 6.0 to 30.6 ng/g in the carp Hb, collected from the Las Vegas Wash and Lake Mead, NV areas. The presence of an AMX metabolite in the carp Hb was confirmed when similar mass spectral features and the same retention time of the AMX metabolite were obtained for both standard AMX and carp Hb extract solutions. In the nonhydrolyzed and reagent blank extracts, the AMX metabolite was not detected.

Introduction

Musk xylene (MX) and other synthetic musks are used as synthetic fragrances in formulations of cleaners, soaps, detergents, and body-care products. The commercial and domestic use and discharge of these compounds into municipal sewage systems has led to their ubiquitous occurrence in the aquatic environment and their presence at various concentrations in some organisms of certain aquatic ecosystems (1). Because of the low extent of biodegradation and high lipophilicity (2), MX and synthetic musks have high potentials as environmental contaminants and are capable of being bioconcentrated in aquatic and terrestrial organisms (3). In particular, the occurrence of MX in an aquatic organism was first reported in 1981 (4). Since then, MX has been detected in most environmental compartments: in the North Sea; rivers and freshwater (5–7); domestic and industrial sewage sludge (8); sewage treatment effluent (9); Norwegian air samples (10); human adipose tissue and breast milk (2,11); developing and adult rats (12); fish, mussels, and shrimp (1); and whole fish (13). Because of the ubiquitous distribution and bioaccumulation potential of nitro musks in the environment, MX was banned in Japan in the 1980s. In Europe, MX and musk ketone are under discussion (5) because of their environmental and toxicological impact.

Figure 1 shows the metabolic path of MX conversion to 4-amino-MX (AMX). MX and its metabolite have been identified and quantitated in samples of domestic and industrial sewage...
waters (14). However, despite widespread use and occurrence of MX, only limited information about its toxicology is available. The acute toxicity of MX is low, although a non-dose-dependent increase in the incidence of liver tumors was reported in mice after long-term administration of MX in the diet (15). A half-life for elimination for MX of less than a few days is found in the rat; however, this contrasts with a slow elimination and half-life of approximately 80 days in humans (16). MX is known as a co-mutagenic substance for a great number of polycyclic aromatic compounds and aromatic amines. MX was identified as an inducer of toxifying enzymes in rat liver, and it is a cytochrome P450 1A2 isoenzyme inducer (17).

The measurement of various biomarkers is used to indicate exposure to a variety of xenobiotics. Biomarkers have been found in urine, saliva, and hair, as well as serum proteins. One of the most promising biomarkers of exposure to xenobiotics involving nitroaromatics is the blood protein hemoglobin (Hb). Nitroaromatics and their metabolites form adducts with Hb. The binding of metabolites from nitroarenes to Hb may be used as a marker of exposure, which has resulted from the passage of the xenobiotic through one or more barriers of the organism (e.g., skin, intestinal mucosa, and lung mucosa). Hb-bound metabolites as biomarkers of exposure may be used to assess cumulative exposures over a longer time range (the life time of red blood cells) and, thus, may be better suited for risk assessment than quantitation of urinary metabolites (18,19). Nitroarenes are enzymatically reduced, and their metabolites, nitrosaeranes, react with the sulfhydryl group of cysteine in Hb to form an acid/base labile sulfanimide that hydrolyzes to aromatic amines in the presence of aqueous base.

The metabolic path of MX and Hb cysteine adduct formation is shown in Figure 1. It is reported that the Hb adduct of an aromatic amine is a good dosimeter for the target tissue dose of the ultimate carcinogenic metabolite of the amine (19). The biotransformation and toxicokinetics of MX metabolites in human Hb have been reported (20,21). Although there are studies conducted for the detection of an AMX metabolite in different environmental compartments (1–21), to our knowledge, no work has been performed on the determination of AMX metabolites from carp Hb for the purpose of ecological assessment of MX exposure. This paper describes the determination of a bound AMX metabolite, formed by enzymatic reduction of MX, followed by adduct formation with the carp Hb sample, collected from the Las Vegas Wash (LVW) and Lake Mead (LM), NV. The Hb adducts were detected and quantitated by gas chromatography mass spectrometry (GC–MS) with selected ion monitoring (SIM).

Materials and Methods

Reagents and chemicals
Sodium dodecyl sulfate (SDS), sodium hydroxide pellets, and n-hexane (high-performance liquid chromatography grade) were obtained from Sigma-Aldrich (St. Louis, MO), Fisher Scientific (Pittsburgh, PA), and J.T. Baker (Phillipsburg, NJ), respectively. The internal standard (IS), naphthalene-d₆, was purchased from Absolute Standard Inc. (Hamden, CT). The standard solution of AMX metabolite was obtained gratis from Dr. L.I. Osemwengie (U.S. Environmental Protection Agency, Las Vegas, NV). Solutions with known amounts of metabolite and IS were used to prepare calibration curves to quantitate the results. Deionized water was used for all preparations where necessary.

Collection of carp blood samples
Live carp were obtained from downstream (~ 100-m distance) of a publicly owned wash called LVW and from LM, NV. The domestic and sewage water treated by public sanitation treatment plants (STP) flowed down through a canal to LM. The distance between the LVW and LM sample-collection points was approximately 10 miles. Approximately 6.5–8.0-mL fresh blood samples were collected in whole blood tubes containing heparin solution (Daigger and Company Inc., Vernon Hills, IL). Blood was obtained from the carp by two methods: severing the caudal peduncle (SCP; used for smaller carp) and/or using cardiac puncture. After collection of blood, the tubes were shaken and placed on ice.

Isolation of Hb
Fresh, heparinized blood samples were centrifuged at 3000 x g for 10 min at 4°C, and the red blood cells (RBC) were separated from the plasma. The RBC were washed/twice with equal volumes of freshly prepared 0.9% NaCl solution. The cells were lysed by addition of two volumes of distilled water, and the solutions were centrifuged again at 3000 x g for 10 min at 4°C. The cellular debris was discarded after centrifugation. The Hb solutions were dialyzed for 72 h to remove small molecules and then placed in a freezer to freeze the Hb solution. A freeze-drying procedure was employed to eliminate water from the frozen Hb by a Sentry Microprocessor Control, Freezemobile, and benchtop freeze-dryer (The VirTis Company, Inc., Gardiner, NY). The dried Hb was then placed in a freezer for later analysis of the nitro musk metabolite.

Alkaline hydrolysis
To release the bound amino metabolite from the carp Hb, alkaline hydrolysis was performed. The detailed descriptions of basic hydrolysis, extraction, and preconcentration procedures were described in our earlier work (22). Briefly, approximately 13–76 mg dried Hb was placed in cleaned and dried tubes, along with 9 mL 0.5% SDS solution and 1 mL 10N NaOH solution. The pH of the solution was determined to be 12. The mixture was then stirred for 1 h at room temperature and extracted 3 times with 10 mL n-hexane. The tube was placed in a refrigerator for approximately 45 min to freeze the aqueous sample. A clear hexane layer was obtained as an extract on the top of the aqueous layer in the tube. The residual water from the extract was removed by passing the extract through a drying column containing granular anhydrous Na₂SO₄. The dried extract was then concentrated by evaporation under a stream of nitrogen to a volume of approximately 0.5 mL. The IS was added, the solution sealed in GC vials, and the sample analyzed by GC–SIM–MS. A schematic representation of the experimental procedure is shown in Figure 2.
Nonhydrolyzed and reagent blank experiments

To investigate whether any unbound AMX metabolite was present in the carp Hb, a control experiment without basic hydrolysis was carried out. In this experiment, except for the NaOH, all chemicals and solvents were added to the Hb, and the same extraction and preconcentration procedures were followed as described in the hydrolysis work. A laboratory or reagent blank control experiment was also performed by taking the same amounts of solvents, chemicals, and reagents used for the hydrolysis experiment, except the Hb.

GC–MS

An Agilent Technologies HP 6890 series GC system equipped with a HP 5973 mass selective detector (MSD) connected to an Agilent 7890 autosampler and Agilent 6890 GC were used (Palo Alto, CA). The helium carrier gas was passed through a DB-5 (J&W Scientific, Agilent Technologies) capillary column (40 m long, 0.180-mm i.d., and 0.18-µm film thickness) at a constant flow rate of 0.5 mL/min (average linear velocity, 22 cm/s) using the pulsed splitless mode. The autosampler injected a 2-µL volume of sample or standard solution into the GC with gradient oven temperature starting at 60°C for 1 min, 150°C at 10°C/min, 250°C at 8°C/min, 300°C at 10°C/min, and holding the final temperature for 6 min. The injector and transfer line temperatures were 250 and 280°C, respectively. The ion source temperature was 230°C and operated in the 70 eV electron ionization (EI) mode. By selecting base peak and confirming ions of the IS and the target compounds, the mass spectral acquisitions were performed with dwell times of 25 ms/ion using the GC–MSD Agilent ChemStation software (v. B.02.05). In the case of the IS (naphthalene-d3), the base peak ion m/z 136 was also the molecular ion, and for the target compound (AMX), the base peak ion was m/z 252 (M-15) with five confirming ions at m/z 268, 267 (molecular ion), 253, 218, and 215 selected for monitoring.

Calibration curve

A regression analysis was carried out on the ratio of areas (analyte area divided by internal standard area) versus the ratio of AMX concentration to internal standard concentration resulting in a six-point calibration curve. Unweighted regression was considered and resulted in an $R^2$ of 0.998 for forcing the equation through zero signal at zero concentration and also with no forcing through zero signal. The calibration curve resulting from forcing the equation through the point 0,0 was employed to determine the AMX in Hb samples because the concentration of AMX was expected to be near zero. The resulting equation of the line was used to calculate the concentrations of AMX in the samples and check standards run during the course of analysis. The concentration of AMX was calculable based on the known volume and concentration of IS added to the sample and the known mass of the sample being analyzed.

Quality assurance/quality control (QA/QC)

Each group of samples to be analyzed was bracketed before and after by a representative standard/IS QC sample to establish adherence to the calibration curve equation and agreement with the retention time of the standard. Deviations from the calibration curve greater than ±10% would cause rerunning of standards, construction of a new calibration curve, or replacement of the capillary GC injector as a result of poor peak shape (tailing), which affected quantitation. Retention time variations were generally less than 0.08%, and peak widths at half-height were approximately 3 s.

A laboratory reagent blank was analyzed using the same procedure as was used for the Hb samples, except no Hb was extracted. No AMX was detected as a background contaminant. Standards run on the day of analysis were followed by a solvent blank run, followed by the extract. No carryover of AMX was observed.

Confirmation of identity was based on the presence of six ions whose relative abundances agreed to within ±20% of the relative abundances of the standard and whose retention time was within 2 s of that of the standard.

Results and Discussion

The alkaline hydrolysis, nonhydrolyzed, and reagent blank experiments were performed to obtain and support the identification and quantitation of the AMX metabolite as truly coming from the carp Hb. In the alkaline hydrolysis, the MX bound to carp Hb was released as the AMX metabolite that was extracted into n-hexane. It was considered that the unbound AMX metabolite might also be present in the carp Hb. To investigate the possible presence of unbound AMX metabolite, the nonhydrolyzed and reagent blank experiments were also performed. The preconcentrated extract obtained from the nonhydrolyzed and reagent blank experiments were spiked with a known concentration of IS solution. The AMX metabolite standard solution (50 pg/µL) containing 200 pg/µL IS was prepared. The standard, solvent blank, nonhydrolyzed Hb extract, solvent blank, and reagent blank extract were injected into the GC–MS, in this order. A 2-µL volume of each solution was injected into the GC by the autosampler. In the case of the standard solution, it was observed that the IS and AMX metabolite eluted from the
GC capillary column at 11.19 and 24.43 min, respectively, and the appropriate base peak and confirming ions, in the correct relative intensity ratios, were also observed at these times. The mass chromatograms and spectra of the standard AMX metabolite will be shown in the detection of a bound AMX metabolite in the carp Hb section. When nonhydrolyzed and reagent blank extracts were considered, the GC–MS chromatograms did not show any peak for AMX at 24.43 min, and no mass signals of the ions for AMX metabolite were observed. This indicated that no unbound AMX metabolite was present in the carp Hb investigated in this study. This further supports the inference that AMX results from liberation by hydrolysis from the Hb-bound metabolite (21).

Detection of a bound AMX metabolite in the carp Hb
To determine the AMX metabolite in the carp Hb, the preconcentrated extracts obtained from the hydrolyzed experiments were spiked with a known concentration of IS solution. A standard solution (50 pg/μL) of AMX metabolite containing 200 pg/μL IS was also prepared. A 2-μL volume each of standard, solvent blank, and hydrolyzed Hb extract was injected in this order into the GC–MS by the autosampler. The same base peak and confirming ions were monitored for identification of the IS and AMX metabolite as were monitored for the nonhydrolyzed extract. The AMX metabolite was found in the hydrolyzed extract solution. Figure 3 shows an overlay of mass chromatograms of selected m/z 267, 252, and 218 ions obtained from (A) standard solution of AMX metabolite and for (B) hydrolyzed carp Hb extract for detection of AMX metabolite. These show a similarity within ± 20% of the selected ion relative abundances for the AMX metabolite eluted from the capillary column at a retention time of 24.43 min.

By selecting the peaks in the SIM chromatograms of the standard and sample extract solutions and then subtracting the background level by ChemStation software, the mass spectra were produced. Figure 4 shows the mass spectra for (A) standard AMX solution and (B) sample extract solution. The mass spectra are only shown between the ranges of m/z 210 and 270 ions because the base peak and confirming ions for the identification of the AMX metabolite were selected in this range. In general, the mass spectral features compared very well to each other and indicate the presence of bound AMX metabolite in the carp Hb. A small difference of relative abundances could be observed in some confirming ions relative to the standard. This could be a matrix effect on the ion levels detected.

Quantitation of the AMX metabolite in the carp Hb samples
The AMX metabolite previously bound to the carp Hb cysteines was detected. A GC–MS response calibration curve for AMX quantitation in the carp Hb was prepared by injecting a series of standard solutions (1.0, 10.0, 25.0, 50.0, 100.0, and 200 pg/μL) of AMX containing IS (200 pg/μL) into the...
was found in the carp Hb in the range 6.0-30.6 ng/g. The use of
populations appears to be worthy of further study.

Hb adducts as biomarkers for nitro musk exposure in fish

hazards exposure. The concentration of bound AMX metabolite
chemical endpoint, useful for monitoring and assessment of MX
detected in carp by GC-SIM-MS. The reduction of a nitro group

Conclusions

Lib that could yield an amine that would be suitable as a bio-

masses of AMX metabolite that were near the GC-MS detec-
or 19.8 mg, taken for hydrolyzed extractions were performed.
The extraction performed with the Hb (13.3-76.1 mg) gave
concentration levels of AMX metabolite ranging from 6.0
to 30.6 ng/g. In the case of LVW samples, the 19.8 or 13.3-mg
HB samples that released the AMX metabolite by hydrolyzed
extraction were approximately 4 or 5 times lower than the
amount obtained from the 76.1-mg Hb sample. This may be
explained by the fact that the smaller sample weights of 13.3
or 19.8 mg, taken for hydrolyzed extraction, liberated small
amounts of AMX metabolite that were near the GC-MS detec-
tion level. Another possibility is sample inhomogeneity because
the carp Hb was extracted from approximately 6.5 to 8 mL carp
blood, and after freeze-drying, the dried Hb samples were not
homogenized. The AMX was quantitated for a signal-to-noise
ratio of 3:1, with a limit of detection of 2.5 to 3.5 ng/g, based on
the matrices.

Table I. Concentration of AMX Metabolite in the Hb Obtained from the
LVW and LM Carp Blood Samples

<table>
<thead>
<tr>
<th>Live Carp Sample Collection Points</th>
<th>Experiments Performed for Extraction of AMX</th>
<th>Amount of Hb Extracted (mg)</th>
<th>Quantitated Ions</th>
<th>Conc. of AMX Metabolite (ng/g)</th>
<th>Conc. of AMX Metabolite in Downstream (ng/L)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVW Hydrolyzed</td>
<td>76.1</td>
<td>252</td>
<td>30.6</td>
<td>1.4-31.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>19.8</td>
<td>252</td>
<td>8.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>13.3</td>
<td>252</td>
<td>6.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonhydrolyzed</td>
<td>43.6</td>
<td>252</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LM Hydrolyzed</td>
<td>30.1</td>
<td>252</td>
<td>14.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonhydrolyzed</td>
<td>30.6</td>
<td>252</td>
<td>16.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reagent or laboratory blank*</td>
<td></td>
<td></td>
<td>ND†</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Concentration of AMX metabolite found downstream of the LVW sewage treatment plant was taken from reference 9.
† ND represents not detected.
‡ Reagent blank experiment was performed by taking the same amount of reagents used for the hydrolysis experiment, except Hb.

GC-MS instrument. The linear calibration curve fitted the experimental data with an $R^2$ of 0.998. Using the ChemStation software, the concentration of the AMX metabolite liberated from the carp Hb was calculated. Table I summarizes the concentrations of AMX metabolite that were obtained from the alkaline hydrolysis, nonhydrolyzed, and reagent blank experiments. Because of the limited amount of the carp Hb samples, only a few hydrolyzed extractions were performed. The extraction performed with the Hb (13.3-76.1 mg) gave concentration levels of AMX metabolite ranging from 6.0 to 30.6 ng/g. In the case of LVW samples, the 19.8 or 13.3-mg HB samples that released the AMX metabolite by hydrolyzed extraction were approximately 4 or 5 times lower than the amount obtained from the 76.1-mg Hb sample. This may be explained by the fact that the smaller sample weights of 13.3 or 19.8 mg, taken for hydrolyzed extraction, liberated small amounts of AMX metabolite that were near the GC-MS detection level. Another possibility is sample inhomogeneity because the carp Hb was extracted from approximately 6.5 to 8 mL carp blood, and after freeze-drying, the dried Hb samples were not homogenized. The AMX was quantitated for a signal-to-noise ratio of 3:1, with a limit of detection of 2.5 to 3.5 ng/g, based on the matrices.

Conclusions

The binding of AMX metabolite and cysteine in Hb has been detected in carp by GC–SIM–MS. The reduction of a nitro group in MX yielded an intermediate capable of forming an adduct of Hb that could yield an amine that would be suitable as a biochemical endpoint, useful for monitoring and assessment of MX hazards exposure. The concentration of bound AMX metabolite was found in the carp Hb in the range 6.0-30.6 ng/g. The use of Hb adducts as biomarkers for nitro musk exposure in fish populations appears to be worthy of further study.

Acknowledgments

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References


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