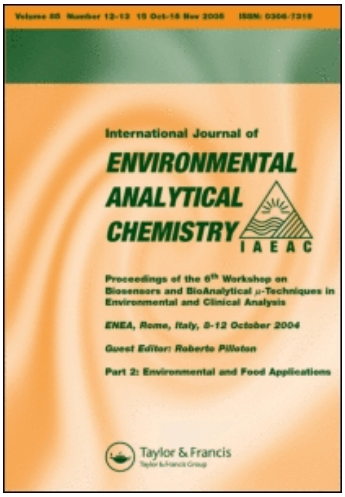

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

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To cite this Article Mottaleb, Mohammad A. , Moy, T. W. and Zimmerman, J. H.(2006) 'Biotransformation of musk xylene in trout haemoglobin: dose-response and toxicokinetics of musk xylene metabolites haemoglobin adducts by gas chromatography-mass spectrometry', International Journal of Environmental Analytical Chemistry, 86: 10, 743 — 756
To link to this Article: DOI: 10.1080/03067310500489528
URL: http://dx.doi.org/10.1080/03067310500489528

Intern. J. Environ. Anal. Chem.
Vol. 86, No. 10, 20 August 2006, 743–756

Biotransformation of musk xylene in trout haemoglobin: dose–response and toxicokinetics of musk xylene metabolites haemoglobin adducts by gas chromatography-mass spectrometry

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(*Received 29 August 2005; in final form 14 November 2005*)

Musk xylene (MX) is frequently used as a fragrance in commercial toiletries. Biotransformation of MX into 4-amino-MX (4-AMX) and 2-amino-MX (2-AMX) metabolites in rainbow trout haemoglobin (Hb) has been described. The dose–response relationship and toxicokinetics of the metabolites as adducts in the Hb were determined by gas chromatography (GC)–electron capture negative chemical ionization (NCI)–mass spectrometry (MS), and GC–electron ionization (EI)–MS/MS, using selected ion monitoring (SIM). The trout were subjected to a single exposure of 0.010, 0.030, 0.10, and/or 0.30 mg MX/g of fish. Hb samples were collected from exposed and control fish, and analysed subsequent to exposure at intervals of 24, 72, and 168 h. Alkaline hydrolysis released 4-AMX and 2-AMX metabolites from the Hb, and the solutes were extracted into *n*-hexane. The extracts were preconcentrated and analysed. The presence of the metabolites in the Hb extracts was confirmed based on agreement of similar mass spectral features from NCI/MS and EI-MS/MS spectra, and retention times of the metabolites with standards. The NCI/MS results were used for dose–response and toxicokinetics measurements. For dose–response, the concentrations of adducts of the metabolites increased with dosage, and a maximum adduct formation was observed at 0.10 mg g^{-1}, beyond which it decreased. The average concentrations of 4-AMX and 2-AMX at a dosage of 0.10 mg g^{-1} were 700 and 7.4 ng g^{-1}, respectively. For toxicokinetics, the concentration of the metabolites in the Hb reached a maximum in the 3 day sample after administration of MX. Further elimination of the metabolites exhibited kinetics with a half-life estimated to be 1–2 days, assuming first-order kinetics. Quantitations were made based on an internal standard and a calibration plot. In control samples, non-hydrolysed Hb, and reagent blank extracts, the metabolites were not detected. The limits of detection for 4-AMX and 2-AMX in the Hb were approximately 1.7 and 1.4 μg L^{-1}, respectively, based on a signal-to-noise ratio of 3 with NCI/MS.

Keywords: Biomarkers; Dose–response; Toxicokinetics; Hb adducts; Nitro musks; Environmental exposure

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ISSN 0306-7319 print/ISSN 1029-0397 online © 2006 Taylor & Francis
DOI: 10.1080/03067310500489528

1. Introduction

Pharmaceuticals and personal-care products (PPCPs) are 'emerging' environmental contaminants, because of their presence in different environmental matrices including air, sediments, waters, etc. and their possible adverse effect on ecological systems, aquatic organisms, and humans. The continued occurrence and fate of the PPCPs and their metabolites as contaminants and biomarkers have led many scientists to study this emerging issue. Musk xylene (1-*tert*-butyl-3,5-dimethyl-2,4,6-trinitrobezene, MX) is frequently used as a fragrance and additive material in the formulation of personal care products and perfumed household products, and its estimated annual production is about 1000 metric tons [1]. The commercial and domestic use and discharge of the compound into municipal sewage systems have contributed to its ubiquitous occurrence in the aquatic environment. Because of the persistence and high potential for bioaccumulation [2], MX has been detected as a contaminant in aquatic and terrestrial organisms [3, 4], human tissues [5–7], the North Sea and river water [1, 8, 9], sewage-treatment effluent [10], Norwegian air samples [11], human adipose tissue and breast milk [2, 12], developing and adult rats [13], and fish, mussels, and shrimp [14].

Metabolites of MX have been identified and quantified in samples of river waters, domestic and industrial sewage sludge [9, 15], and homogenized whole fish [16]. Some studies have been published on the ecotoxicity of MX and its metabolites [17, 18]. The acute toxicity of MX is low, though a non-dose-dependent increase in the incidence of liver tumours was observed in male and female mice after long-term administration of MX in the diet [19]. Several studies suggested that MX was not genotoxic [20–22]. 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 increase in liver tumours, analogous to phenobarbital. MX is also known as a co-mutagenic substance for polycyclic aromatics and aromatic amines, and was identified as an inducer of detoxifying enzymes in rat liver [23].

Fast and reliable methods of analysis are important in environmental health and risk assessment to monitor the exposure of humans and other animals to nitro musks. Hb-adducts serve as suitable biomarkers for exposure to carcinogenic aromatic amines and nitroarenes. The metabolites of nitro musks or other related nitroarenes, bound to Hb as biomarkers of exposure, can be used potentially to assess continuous exposure over a longer time range, and thus may be better suited for risk assessment than quantitation of urinary metabolites [24, 25]. Nitroarenes are subject to enzymatic reduction, and their reactive intermediate, nitrosoarenes, react with sulphydryl (SH) functional group of cysteine in Hb to form an acid/base labile sulphinamide that hydrolyses to aromatic amines in the presence of aqueous base. The biological transformation routes of MX to its corresponding amines, involving Hb cysteine adduct formation, are shown in figure 1. It has been 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 [25].

The biotransformation of MX has been studied in rats [23]. A half-life for elimination for MX of less than a few days was found in the rat; however, this contrasts with a slow elimination half-life of about 80 days in humans [5]. The biotransformation and toxicokinetics of MX in human blood plasma were investigated, and an average half-life of 70 days for a 4-AMX metabolite was reported [26]. Recently, we detected a 4-AMX metabolite from carp Hb for the purpose of ecological assessment

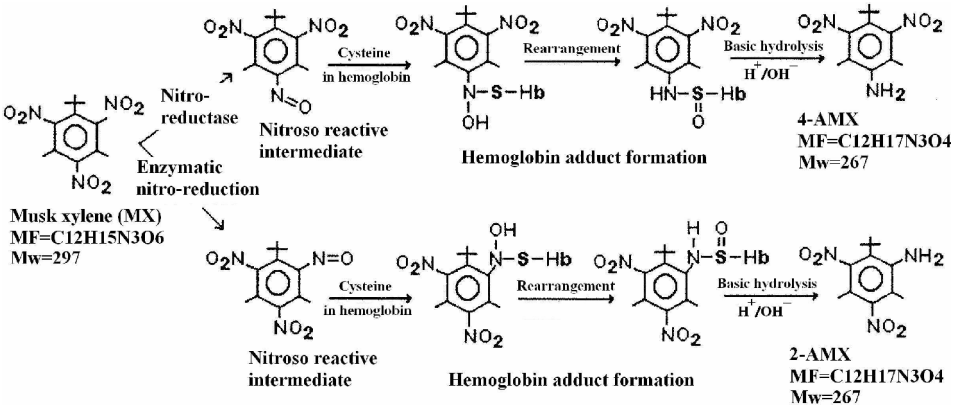

Figure 1. Metabolic pathway of cysteine Hb adduct formation with MX to 4-amino-MX and 2-amino-MX.

of MX exposures [27]. Riedel *et al*. demonstrated the nitro-reduction in MX that forms cysteine-Hb adducts in humans [28]. In earlier studies of trout exposed to MX, a trout Hb adduct of 4-AMX was found, suggesting that nitro-reduction in MX may occur in fish [29, 30]. The present investigation reports the dose–response and toxicokinetics of nitro musk Hb adducts from trout exposed to MX. In this work, the 4-AMX and 2-AMX metabolites bound to Hb, formed by enzymatic reduction in MX, are detected, characterized, and quantified by GC-NCI-MS and GC-EI-MS/MS using the SIM mode. Suitable controls are also employed.

2. Experimental

2.1 *Standards, chemicals, and solvents*

Sodium dodecyl sulphate (SDS), sodium hydroxide pellets, and *n*-hexane (HPLC grade) were purchased from Sigma-Aldrich (St Louis, MO), Fisher Scientific, and J.T. Baker, respectively. The internal standards (IS) 2,3,4,5,6-pentafluorobenzophenone (PFBP) (purity 99%), and naphthalene-d8 (NAP8) were obtained from Aldrich and Absolute Standard Inc., CT, respectively. The standard solutions of MX, 4-AMX and 2-AMX were provided by Dr L. I. Osemwengie, US Environmental Protection Agency (EPA), Las Vegas, Nevada [10]. Tricane methane sulphonate (MS 222) was obtained from Sigma-Aldrich. Known concentrations of the metabolite standards and IS solutions were used to prepare calibration plots. De-ionized water was used for all preparations.

2.2 *Exposure of trout to MX*

Trout exposure experiments were performed at the Department of Environmental & Molecular Toxicology, Oregon State University (OSU), Oregon, for the sampling periods 24 h (1 day), 72 h (3 days), and 168 h (7 days). A series of standard test solutions containing 10, 30, 100, and 300 mg mL^{-1} of MX were prepared in salmon oil as the vehicle (Pharmaceutical grade, Yukon Nutritional Company, Lake Wales, FL) for trout exposure to MX. At the highest intended concentration of 300 mg mL^{-1},

Table 1. *In vivo* rainbow trout exposure with musk xylene, vehicle (salmon oil) and observed concentration of 2-AMX and 4-AMX metabolites in trout Hb.

	MX exposure schedule				Concentration of 2-AMX and 4-AMX metabolites ($ng\,g^{-1}$)	
Exposure period (Day)	MX conc. ($mg\,mL^{-1}$)	Fish wet weight[a] (g)	MX dose per trout (mg)	Exposure level ($mg\,g^{-1}$)	2-AMX	4-AMX
1-day	10	202 (S1)	2.0	0.010	5.5	98
		256 (S2)	2.5	0.010	3.1	106
		165 (S3)	1.6	0.010	2.1	66
	30	180 (S4)	5.4	0.030	6.6	171
		256 (S5)	7.5	0.029	5.0	136
		280 (S6)[b]	8.4	0.030	31	1150
	100	236 (S7)	24	0.10	4.9	676
		264 (S8)	26	0.098	11	894
		204 (S9)	20	0.098	6.3	517
	300	250 (S10)	75	0.30	5.0	170
		310 (S11)	90	0.29	6.0	217
		227 (S12)	69	0.30	6.2	212
	Control	206 (C1)	0.20 mL, vehicle only		ND	
		304 (C2)	0.30 mL, vehicle only		ND	
		184 (C3)	0.18 mL, vehicle only		ND	
3-day	30	208 (S13)	6.3	0.030	17	555
		244 (S14)	7.2	0.030	13	529
		193 (S15)	6.0	0.031	18	596
	Control	253 (C4)	0.25 mL, vehicle only		ND	
		272 (C5)	0.27 mL, vehicle only		ND	
		233 (C6)	0.23 mL, vehicle only		ND	
7-day	30	212 (S16)[b]	6.3	0.030	28	865
		230 (S17)	6.9	0.030	2.9	39
		204 (S18)	6.0	0.029	1.5	31
	Control	273 (C7)	0.27 mL, vehicle only		ND	
		305 (C8)	0.30 mL, vehicle only		ND	
		250 (C9)	0.25 mL, vehicle only		ND	

[a] Indicates sample number in the parenthesis. [b] Trout was found sick (losing equilibrium) during collection of blood for the respective exposure periods, and the concentrations observed for 2-AMX and 4-AMX were not used in plotting the dose–response and the toxicokinetics curve. ND represents not detected.

the MX did not dissolve completely in the oil, but instead formed an emulsion. Well-shaken standard solutions were injected intraperitoneal into fish that were anaesthetized in an aqueous solution containing $75\,mg\,L^{-1}$ of MS 222. The anaesthetized trout were weighed prior to injecting the standard solutions.

In the dose–response study, three trout were exposed to one of four MX concentration for 1 day. For the toxicokinetic investigation, three trout were exposed to a single concentration of MX for a 3 day or 7 day period. As controls, nine fish were exposed to the vehicle (without MX) for the same sampling periods. After exposure, fish were returned to labelled tanks with circulating water at 13°C. Table 1 summarizes the *in vivo* fish exposure experiment parameters and data.

Following exposure, no food was given to the fish. In the dose–response study, one trout exposed to the $30\,mg\,mL^{-1}$ MX solution was found to be sick (losing equilibrium) in the tank. In the toxicokinetic study, one trout was also found to be sick in the 7 day exposure group.

2.3 *Collection of trout blood and isolation of Hb*

Trout were anaesthetized with MS 222 (250 mg L^{-1}) before collecting the blood. Blood samples were drawn from the anaesthetized trout into heparinized individual syringes from the caudal vein and placed into heparinized individual sterile interior Vacutainers (Becton Dickson VACUTAINER System 16939, NJ). All blood samples were placed on ice immediately after collection, and the fish were sacrificed.

Erythrocytes were separated from plasma by centrifuging at 3500 *g* for 10 min at 4°C, washed twice with equal volumes of 0.9% saline and re-centrifuged, and the cellular debris discarded. The erythrocytes were lysed by adding two volumes of distilled water. The Hb solutions were solidified in a freezer at −24°C. The water was eliminated from the solid Hb solutions by a freeze-drying procedure using a Sentry Microprocessor Control, Freezemobile and Benchtop Freeze-dryer (The VirTis Company, Inc., Gardiner, NY). The dried Hb was then placed in a freezer at −24°C for subsequent analysis of MX metabolites.

2.4 *Liberation of bound amine metabolites from the Hb*

Reference 27 describes the alkaline hydrolysis, extraction, and preconcentration procedures for liberation of the bound amino metabolites from carp Hb. The same procedures were used in this study. The volume of product from the aforementioned procedure (about 45 mL) was reduced under a stream of nitrogen at 45°C to about 50–65 μL, to which 10 μL of 100 pg μL^{-1} of IS (PFBP) was added for GC-NCI-MS analysis. The 4-AMX and 2-AMX metabolites detected by NCI-MS gave similar mass spectral signals, so to confirm the spectrum of each metabolite, GC-MS/MS analysis was performed. The PFBP was not a suitable IS for EI detection, so the preconcentrated extracts were spiked with IS (NAP8) using 10 μL of 20 ng μL^{-1}. The final volume was made up to 100 μL with *n*-hexane.

Two non-hydrolysed Hb control experiments were also performed to investigate the possible presence of unbound amino metabolites in the Hb samples. In the experiments, all chemicals and solvents except for the NaOH were added to the Hb (about 50 mg), and the same extraction and preconcentration procedures were followed as described in the alkaline hydrolysis work. A laboratory or reagent blank control experiment was carried out by using the same amounts of solvents, chemicals, and reagents used for the hydrolysis, except that no Hb was used.

2.5 *Gas chromatography and mass spectrometry*

In several of the referenced studies EI-MS was employed, but as a result of a comparison study [31], we used NCI-MS. The analytical system consisted of an Agilent Technologies 6890 series GC equipped with an Agilent 5973 mass selective detector (MSD) and an Agilent 7683 auto sampler. The helium carrier gas was passed through a DB-5 (J&W Scientific, Agilent Technologies, CA) capillary column (40 m long, 0.18 mm i.d., 0.18 μm film thickness) at a constant flow rate of 0.50 mL min^{-1} (average linear velocity 22 cm s^{-1}) using the pulsed splitless mode. The auto sampler injected a 2 μL volume of sample extract or standard solution. See table 2 for analytical system parameters. By selecting the base peak and confirming ions of the

Table 2. Parameters for NCI/MS analytical instrumentation.

Parameter	Setting
Column	J&W Scientific DB-5 40 m × 0.18 mm × 0.1 μm
Column flow rate	Helium @ 0.5 mL min^{-1} (Average linear velocity 22 cm s^{-1})
Injector type	Pulsed splitless
Injector temperature	250°C
Injection volume	2 μL
Oven programme	60°C for 1 min 10°C min^{-1} to 150°C 8°C min^{-1} to 200°C 10°C min^{-1} to 300°C hold for 6 min
Transfer line temperature	280°C
Ion source temperature	150°C
Quadrupole temperature	106°C
Emission current	49.4 mA
Electron multiplier	1800 and 2000 V
Methane in carrier gas	38%
Dwell time	25 ms/ion

IS (PFBP) and the target compounds, the mass spectral acquisitions were performed with dwell times of 25 ms/ion using the GC/MSD Agilent ChemStation software, version B.02.05. Ions monitored included the IS (PFBP) *m/z* 272 molecular ion, *m/z* 267 (molecular ion) and *m/z* 268 for 4-AMX and 2-AMX.

In the GC-EI-MS/MS analysis, a Varian Saturn® 2200 GC/MS/MS consisting of a CP-3800 GC and 2000 Series Ion Trap MS/MS with a CTC Analytics Combi PAL auto sampler was used. See table 3 for analytical system parameters. Data acquisition, computation, and analysis were done using the Varian Saturn GC/MS Workstation software.

2.6 *Calibration curve for NCI/MS detection*

A regression analysis was carried out on the ratio of areas (analyte area divided by internal standard area) *versus* the ratio of 2-AMX and 4-AMX concentration to internal standard concentration resulting in a six-point calibration curve. The unweighted regression resulted in a coefficient of determination (R^2) of 0.996 for 4-AMX, and 0.997 for 2-AMX for forcing the equation through zero signal at zero concentration and also with no forcing through zero signal, and the resulting equation for the regression line was used to calculate the concentrations of the 4-AMX and 2-AMX in the samples and standards.

2.7 *Quality assurance/quality control (QA/QC)*

Each group of samples to be analysed was bracketed by a QC sample to verify that the instrument's response was within the established calibration QC parameter of ±10% difference. Results exceeding the QC parameters required corrective action before sample analysis could continue. Retention-time variations were generally less than 0.06% min for the analyte (metabolite) eluted from the GC capillary column, and peak widths at half-height were about 3 s. A laboratory reagent blank was included in each analytical batch. No amino metabolites were detected in these blanks.

Table 3. Parameters for EI/MS analytical instrumentation.

Parameter	Setting
Column	Varian FactorFour VF-5MS 30 m × 0.25 mm × 0.25 μm
Column flow	Helium @1.2 mL min^{-1} constant
Injector type	Split: ratio 50 : 1 from 0.75 to 5.00 min 10 : 1 after 5.00 min
Injector temperature	250°C
Injection volume	2 μL
Oven programme	60°C for 1 min 20°C min^{-1} to 180°C 6°C min^{-1} to 240°C 15°C min^{-1} to 260°C hold for 0.67 min
Transfer line temperature	250°C
Sim scan intervals	2-AMX: 15.01 to 15.20 min 4-AMX: 15.75 to 16.00 min
Ionization storage level	48.0 *m/z*
Low-edge offset	6 steps
High-edge offset	2 steps
High-edge amplitude	30.0 V
Isolation time	5 ms
Dissociation waveform type	Non-resident
Excitation storage level	117.7 *m/z*
Excitation amplitude	60.00 V
Excitation time	20 ms
Mass range scanned	40–350 *m/z* at 0.9 s/scan

3. Results and discussion

All extracts (Hb and control samples) were analysed by GC-NCI-MS and GC-EI-MS/MS. The GC-NCI-MS was used to determine the concentration of the 4-AMX and 2-AMX metabolites from all (18) samples. The control sample extracts did not show any characteristic mass signals of the metabolites. Thus, it was confirmed that the measured metabolites released from the alkaline hydrolysed extraction were originally bound to the trout Hb. In GC-NCI-MS analysis, both 4-AMX and 2-AMX metabolites gave similar mass spectral features, and eluted from the column with different retention times. GC-EI-MS/MS analysis was utilized to definitively identify the individual metabolites.

3.1 *GC-NCI-MS detection and identification of MX adduct metabolites in the Hb*

Figure 2 illustrates the typical selected ion chromatograms by GC-NCI-MS for (A) a mixture (100 pg μL^{-1} each) of a standard solution of 2-AMX and 4-AMX metabolites and (B) an extract from sample 4 (S4) in the 1-day exposure study (table 1). These chromatograms show an excellent agreement in the retention times of the metabolites. A variation of retention time (±0.01 min) was observed for 2-AMX (figure 2), and peak widths at half-height were about 3 s.

Fewer ions are available in the NCI detection for metabolite relative to the EI mode [27, 29]. The metabolites (2-AMX and 4-AMX) provided similar NCI mass spectra (figure 3). During the NCI analysis process, the 4-AMX and 2-AMX metabolites form a molecular anion and afford little or no fragmentation. An agreement (about ±10%)

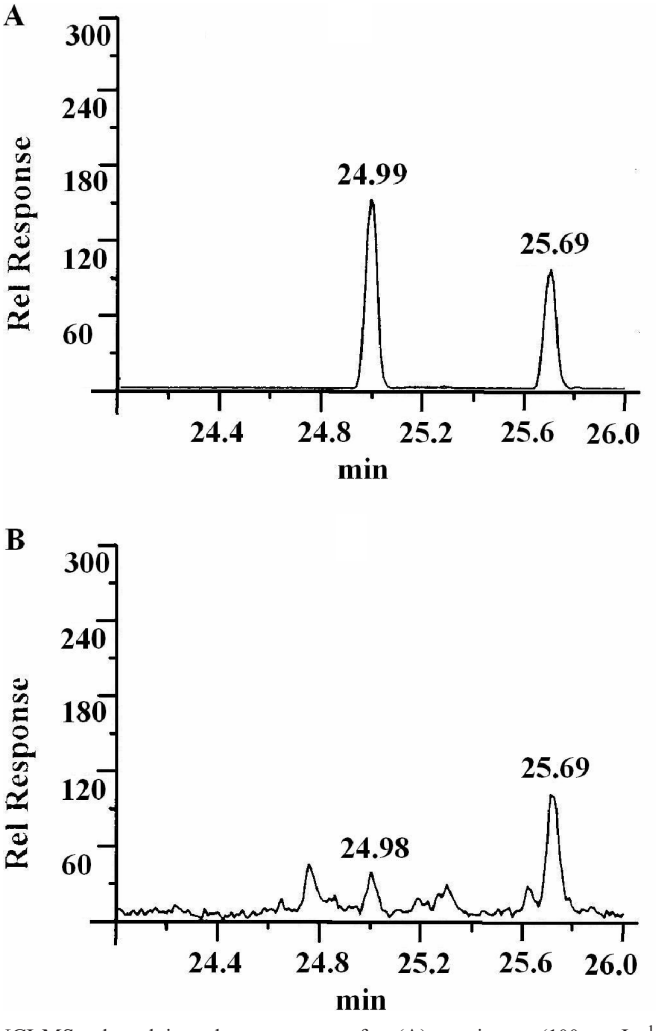

Figure 2. GC-NCI-MS selected ion chromatograms for (A) a mixture (100 pg μL^{-1} each) of standard solution of 2-AMX and 4-AMX metabolites, respectively, containing 1 ng of IS (PFBP) (IS not shown). Retention time 24.99 min for 2-AMX and 25.69 min for 4-AMX; and (B) extract from the sample after 1-day exposure to 5.4 mg MX (table 1, S4) containing 1 ng of IS (PFBP) (IS not shown). Retention times: 24.98 min for 2-AMX and 25.69 min for 4-AMX. The GC-MS conditions are given in table 2.

of the relative abundance of molecular and $(M+1)^-$ isotopic molecular ions was observed in both standard and sample extract solution spectra for 4-AMX and 2-AMX. Two signals at m/z 267 and 268 for 4-AMX and 2-AMX metabolites were observed. The signal m/z 267 is due to the molecular anion ($M^{-\bullet}$) of 4-AMX and 2-AMX, and signal at m/z 268 corresponds to the $(M+1)^-$ isotope. Since NCI gave similar mass spectra for both 2-AMX and 4-AMX, the retention times and GC-EI-MS/MS studies are also essential to the confirmation of the metabolites. The 2-AMX and 4-AMX eluted from the column at 24.98 and 25.69 min, respectively (figure 2), under conditions given in section 2.

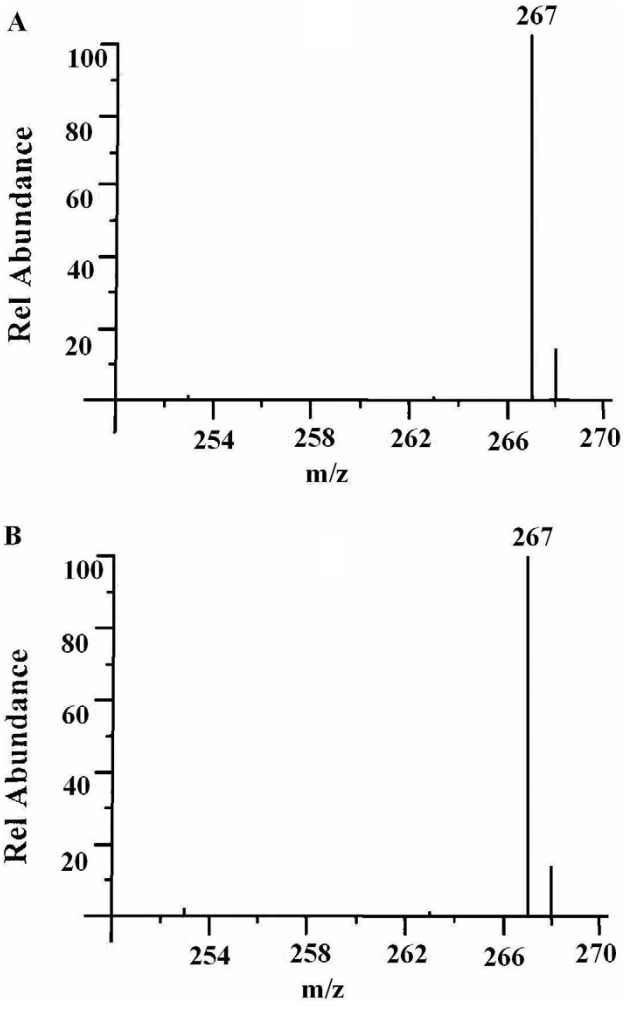

Figure 3. Typical NCI mass spectra for (A) standard 2-AMX (derived from figure 2A, peak retention time 24.99 min), and (B) sample extract (derived from figure 2B, peak retention time 24.98 min). Experimental conditions are the same as those in figure 2.

3.2 *GC-MS/MS characterization of MX adduct metabolites in the Hb*

In order to confirm the presence and characterize individual identities of the 2-AMX and 4-AMX metabolites in the Hb, all extracts were subjected to GC-EI-MS/MS analysis with SIM. Figure 4 shows typical spectra for the 2-AMX and 4-AMX metabolites obtained from the GC-MS/MS analysis of sample extract S4 (after 1 day's exposure to 5.4 mg MX, table 1) and standard solutions. It can be seen that the spectra show an excellent agreement with a variation of ±4% relative abundance of molecular and product ions for each metabolite observed from the sample and standard solutions. In the case of 2-AMX, the molecular ion (267 m/z) provided a lower abundance of product ion of 193 m/z. For the 4-AMX, the molecular ion (267 m/z) provided the highest abundance of product ion of 252 m/z. These suggest that the position

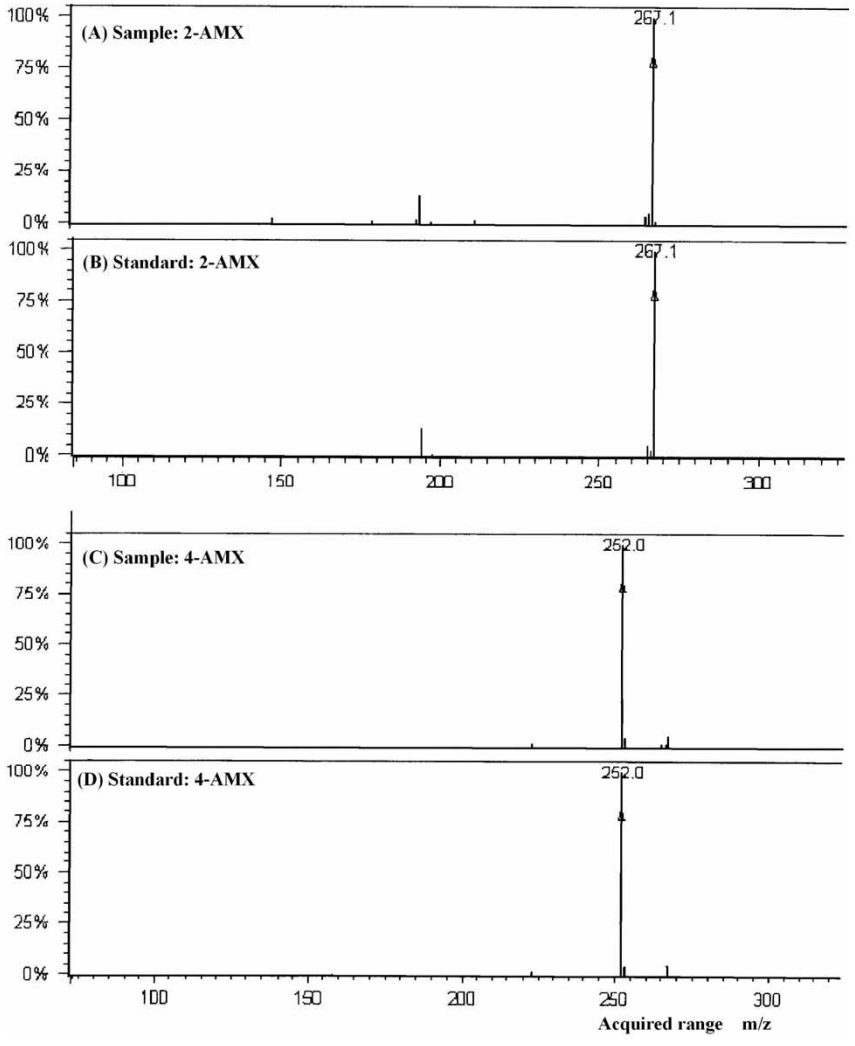

Figure 4. EI-MS/MS spectra for (A) sample [S4] extract, and (B) standard solution of 2-AMX (taken from GC-MS/MS chromatogram, peak retention time 15.08 min for both sample and standard, not shown); (C) sample [S4] extract and (D) standard solution of 4-AMX (taken from GC-MS/MS chromatogram, peak retention time 15.82 min for both sample and standard, not shown). The GC/MS conditions are given in table 3.

of amino groups in the metabolites (see figure 1) played a dominant role in fragmentation of molecular ion 267 m/z of the two isomers. Thus, the GC-EI-MS/MS spectra confirm the presence of 4-AMX and 2-AMX metabolites in the Hb.

3.3 *Limit of detection by NCI/MS*

The limit of detection (LOD) for the metabolites was calculated based on a signal-to-noise ratio of 3 : 1 (signal-to-noise ratio = 3). The LODs for 4-AMX and 2-AMX were approximately 3.4 and 2.8 pg, respectively, at the lowest level of calibration used.

These correspond to the detection limits in the Hb sample of approximately 1.7 and 1.4 ng g^{-1} (based on 50 mg of Hb per 50 μL final volume of extract), respectively.

3.4 *Concentration of MX adduct metabolites in the Hb by NCI/MS*

Table 1 depicts the concentration of 4-AMX and 2-AMX metabolites formed in the individual trout Hb for the exposure period of 1 day, 3 days, and 7 days. A considerable variation of metabolite concentrations was found among the individual trout. This is expected because different trout have their own individual metabolic activity, so variation of the adduct formation in individual trout is normal. Two trout (noted by asterisk marks, sample S6 and S16 in table 1) exposed to the 30 mg mL^{-1} MX solution were found to be sick when the blood samples were collected from them 1 day or 7 days after exposure. For the sick fish sampled 1 day after exposure, the concentrations of 2-AMX and 4-AMX were 31 and 1150 ng g^{-1}, respectively. These values were five- to ninefold higher than other fish exposed during the same period. In the case of the sick fish sampled 7 days after exposure, the concentrations of 2-AMX and 4-AMX were 28 and 865 ng g^{-1}, respectively, and 10–28 times higher than that observed values for other fish over the same time frame. These results suggest that some additional metabolic activity of the sick fish promotes increased formation of the Hb adducts. Alternatively, the removal process for Hb adducts may be impaired in the sick fish.

3.5 *Dose–response of MX adduct metabolites in the Hb by NCI/MS*

The dose–response relationship curves for 4-AMX and 2-AMX metabolites formation in the trout Hb at 24 h (1 day) after exposure are presented in figure 5. The data show that the binding of MX as adducts to the Hb increases in a dose-dependent fashion with maximum formation at dose 0.10 mg g^{-1}. The average concentrations of 4-AMX and

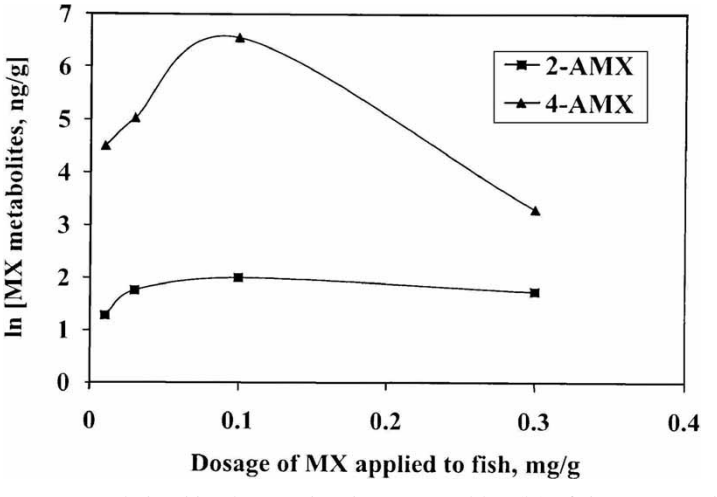

Figure 5. Dose–response relationship plots produced as a natural log (ln) of the concentration of 4-AMX and 2-AMX metabolites observed in Hb at 1 day exposure *vs.* dose of MX given to trout.

2-AMX metabolites at a dose level of 0.10 mg g^{-1} were found to be 700 and 7.4 ng g^{-1}, respectively. We note that the rate of formation of 4-AMX adduct is almost 100-fold greater than the rate of the 2-AMX. A drop in the adduct formation was seen at a dose of 0.30 mg g^{-1}, which may be a result of this standard not being a true solution; rather, it was a thick emulsion that did not dissolve all the solutes. The rates of formation of adducts may have experienced a drop because of the solid states of the MX as suspensions, and therefore, they were not readily available for intracellular transport. The difference in the formation of 4-AMX and 2-AMX may reflect differences in absorption, distribution, metabolism, or excretion of the different compounds.

3.6 *Toxicokinetics of the MX adduct metabolites in the Hb by NCI/MS*

Figure 6 illustrates the curves for sampling times *versus* the natural logarithm of average concentration of the metabolites. The concentrations of 2-AMX and 4-AMX metabolites obtained (table 1) from the sick trout were not considered in the kinetics study. The time variable of the adduct formation for each of the metabolites shows a maximum at about 3 days after exposure, although the frequency of the sampling precludes definitive analysis of the data. The kinetics of the removal of the Hb-adduct metabolites is non-linear and occurs significantly faster than would be expected based on human erythrocyte elimination kinetics. The lifetime of trout erythrocytes is unknown. We estimate a half-life of 1–2 days for the metabolites in the trout Hb based by assuming a first-order pharmacokinetics single-compartment model [32, 33], and the lack of more sampling points precludes a more definitive analysis. This is comparable with the reported half-life for MX of less than a few days in the rat [23]. This does not seem to correlate with the Hb erythrocyte life span of 60 days reported

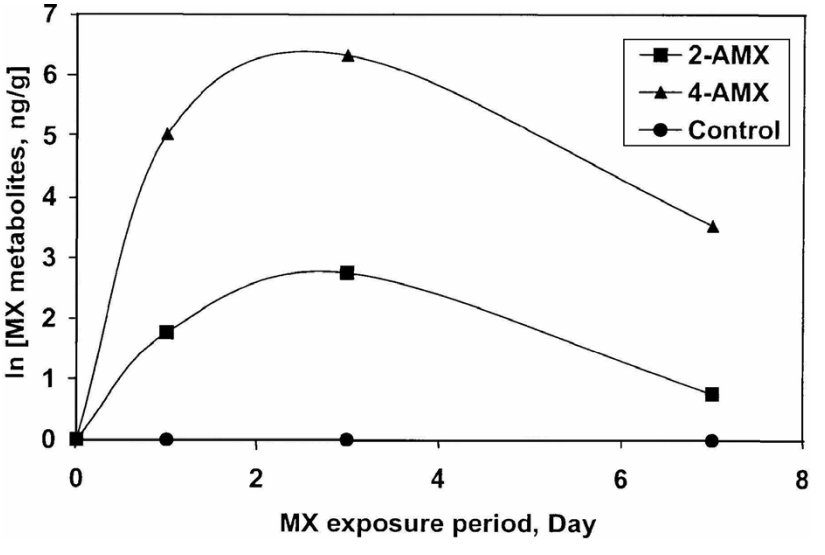

Figure 6. Toxicokinetics plot made as natural log (ln) of the concentration of 4-AMX and 2-AMX metabolites observed in Hb *vs.* MX exposure periods. The elimination rate constant and half-life of the metabolites in Hb were calculated from this plot.

Table 4. Elimination rate constant (k) and half-life ($t_{1/2}$) of the bound metabolites in trout Hb based on first order kinetics.

Metabolites found in the trout Hb	Natural log (ln) of average concentration of the metabolites over 3-day and 7-day exposure period ($ng\,g^{-1}$)		Elimination rate constant (k) (Day^{-1})	Half-life ($t_{1/2}$) (Day)
	3-day	7-day		
4-AMX	6.3	3.6	0.68	1.0
2-AMX	2.8	0.79	0.50	1.4

for the rat [34]. Table 4 depicts the individual values of the elimination rate constants and half-lives of 4-AMX and 2-AMX metabolites found in the trout Hb. These data represent the first study of this kind for fish exposure and, as such, can be used as a guide to design more definitive experiments.

4. Conclusion

The bioavailability of metabolites in trout Hb is investigated after a single administration of MX and salmon oil (dosage vehicle) into 27 trout for exposure periods of 1, 3, and 7 days. One nitro-group of the MX compound is enzymatically reduced to an intermediate nitroso compound, capable of forming an adduct with Hb, that subsequently yields an amine. That amine could be suitable as a biochemical end-point in monitoring and assessing exposure to MX. The 4-AMX and 2-AMX were identified and quantified as metabolites bound to Hb, released by alkaline hydrolysis of the extracts of 18 trout blood samples, and measured by GC-NCI-MS and GC-EI-MS/MS with SIM. No metabolites were observed in the control samples, non-hydrolysed Hb samples, or reagent blank extracts. The formation of Hb adducts as metabolites of MX in trout increases with the dose up to $0.10\,mg\,g^{-1}$, beyond which it decreases. We estimate 1–2 days for the half-life of the metabolites, assuming first-order kinetics. To our knowledge, this is first report of toxicokinetics and dose–response studies of trout Hb adducts with nitro musks. The data suggest that more sampling points are required for definitive treatment of data.

Acknowledgements

This work was performed while the author (MAM) held a National Research Council Research Associateship Award at the National Exposure Research Laboratory, US Environmental Protection Agency (EPA), Las Vegas, Nevada. The authors would like to thank Dr L. I. Osemwengie for providing the 4-AMX and 2-AMX metabolite standards. The authors would like to thank Professor L. R. Curtis, Department of Environmental and Molecular Toxicology, 1007 Agricultural and Life Sciences, Oregon State University, Corvallis, OR 97331-7301, USA for providing all sorts of fish exposure facilities. The US Environmental Protection Agency, through its Office of Research and Development, collaborated in the research described in this article. It has been subjected to the EPA's peer review and has been approved

for publication. Mention of trade names or commercial products does not constitute an endorsement or recommendation for use. This article has been presented as poster in Society for Environmental Toxicology and Chemistry (SETAC) – South Central Regional Conference, 12–14 May 2005, Marble Falls, TX.

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