Biological transformation, kinetics and dose-response assessments of bound musk ketone hemoglobin adducts in rainbow trout as biomarkers of environmental exposure

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Abstract
Low levels (ng/g) of musk ketone (MK), used as a fragrance additive in the formulation of personal care products, are frequently detected in the water and other environment. Thus, aquatic organisms can be continuously exposed to MK. In this study, kinetics and dose-response assessments of 2-amino-MK (AMK) metabolite, bound to cysteine-hemoglobin (Hb) in rainbow trout, formed by enzymatic nitro-reduction of MK have been demonstrated. Trout were exposed to a single exposure of 0.010, 0.030, 0.10, and 0.30 mg MK/g fish. Twenty-seven Hb samples were collected from exposed- and control fish subsequent to exposure intervals of 1 d (24 h), 3 d (72 h), and 7 d (168 h). Basic hydrolysis released bound AMK metabolite was extracted into n-hexane and then concentrated and analyzed by gas chromatography (GC) electron capture negative ion chemical ionization (NICI) mass spectrometry (MS) using selected ion monitoring (SIM). The presence of the AMK metabolite in Hb extracts was confirmed by agreement of similar mass spectral features and retention time with a standard. In the dose-response study, maximum adduct formation was obtained at the 0.10 mg/g dose with an average AMK metabolite concentration of 2.2 ng/g. For kinetics, the highest concentration of the AMK metabolite was found to be 32.0 ng/g at 0.030 mg/g dose in 3-d sample. Further elimination of the metabolite showed kinetics with a half-life estimated to be 2 d, assuming first-order kinetics. The metabolite was not detected in the control samples, non-hydrolyzed Hb, and reagent blank extracts. The detection limit for AMK in the Hb was approximately 0.30 ng/g, based on a signal to noise ratio of 3 (S/N = 3).

Key words: biotransformation; kinetics; hemoglobin adducts; dose-response; nitro musks; biomarker; fish

Introduction
Increasingly public interest in secondary effects of pharmaceuticals and personal care products (PPCPs) as emerging environmental pollutants has gained the attention of the global scientific community. Due to high domestic and commercial use and discharge of PPCPs, their ingredients enter into the environment and reach detectable and potentially harmful concentration levels because most are not regulated water contaminants (Bethesda, 2006). As such, ubiquitous occurrence and fate of PPCPs and their metabolites as pollutants and biomarkers in environments have driven many scientists to research this emerging issue (Kelly et al., 2007). Musk ketone (1-tert-butyl-3,5-imethyl-2,6-dinitro-4-acetylbenzene, MK) is an important member of synthetic nitro musks belonging to the class of PPCPs with an estimated annual production of about 8 × 105 tons (Rimkus, 1999). MK is widely used as a fragrance ingredient in formulation of commercial toiletries including soaps, detergents, cleaners, lotions, and other perfumed household products. The extensive use and discharge of the compound into municipal sewage systems make it available in the aquatic environment (Daughton and Ternes, 1999).

Musk ketone is not readily biodegradable and has high potential for bioaccumulation (Müller et al., 1996). It has been determined as an environmental pollutant in water (Gatermann et al., 1998), sewage treatment effluent (Osemwengie and Steinberg, 2001), air samples (Kallenborn et al., 1999), aquatic and terrestrial organisms (Oost et al., 2003), fish, mussels, and shrimp (Rimkus and Wolf, 1995) and human tissues (Liebl and Ehrenstofer, 1993), human adipose tissue and breast milk (Rimkus et al., 1994). A metabolite of MK has been quantified in samples of river waters, domestic and industrial sewage sludge (Berset et al., 2000), and homogenized whole fish (Osemwengie and Steinberg, 2003). Some studies have been documented on ecotoxicity of nitro musk and metabolites (Giddings et al., 2000). The acute toxicity of nitro musk such as...
musk xylene and MK is low, though a nondose-dependent increase in the incidence of liver tumors was observed in male and female mice after long-term administration of musk xylene in the diet (Maekawa et al., 1990). Emig et al. (1996) conducted a comparative study of five nitro musk compounds for genotoxicity and suggested that MK was not genotoxic. From a toxicological viewpoint, MK is known as a co-mutagenic substance for a great number of polycyclic aromatic compounds and aromatic amines. MK was identified as an inducer of detoxifying enzymes in rat liver and it is a cytochrome P450 1A1 and 1A2 isoenzyme inducer (Mersch-Sundermann et al., 1996).

The binding of metabolite from aromatic amines and nitroarenes to Hb may be used as an indicator of internal exposure. The Hb bound metabolite serves as suitable biomarkers for exposure to chemical carcinogens. The metabolites of nitro musks or other related nitroarenes, bound to Hb as biomarkers of exposure, can potentially be used to assess continuous exposure over a longer time range, and thus, may be better suited for risk assessment than quantitation of urinary metabolites (Farmer et al., 1987). Nitroarenes are subject to enzymatic reduction, and their reactive intermediate. Nitrosoarenes, react with sulphydryl (SH) group of cysteine in Hb to form an acid/base labile sulfonamide that hydrolyzes to aromatic amines in the presence of aqueous base (Sabbioni, 1994). The biological transformation process of MK to an anime metabolite compound, with cysteine-Hb adduct formation, is shown in Fig.1. As such DNA adducts may also be formed in the organisms or mammals. Hb adduct of an aromatic amine is a good dosimeter for the target tissue dose of the ultimate carcinogenic metabolite of the amine (Skipper and Tannenbaum, 1994).

The biotransformation of nitro musk xylene was studied in rats and a half-life for elimination of musk xylene and its metabolite of less than a few days was reported (Minegishi et al., 1991). The toxicokinetics of nitro musk xylene in human blood plasma was estimated about 70 d (half-life) for a 4-amino musk xylene metabolite (Riedel and Dekant, 1999). In our earlier studies, the biotransformation and toxicokinetics (half-life) of the musk xylene metabolites were estimated about < 2 d (Mottaleb et al., 2006). The present contribution describes the kinetics and dose-response assessments of musk ketone cysteine-Hb (hemoglobin) adducts in rainbow trout. The fish are exposed to MK and the bound Hb adduct of AMK metabolite in trout, formed through enzymatic digestion of MK, is identified and quantified with gas chromatography (GC) electron capture negative ion chemical ionization (NICI) mass spectrometry (MS) using selected ion monitoring (SIM) mode. Suitable controls are also used.

1 Experiment
1.1 Reagents and chemicals

Sodium dodecyl sulfate (SDS), sodium hydroxide pellets, n-hexane (HPLC grade), internal standard 2,3,4,5,6-pentafluorobenzophenone (IS) and methane sulfonate (MS 222) were purchased from the Sigma-Aldrich Inc., USA. The standard solutions of MK and AMK were obtained from Dr. L. I. Osemwengie, U.S. Environmental Protection Agency (EPA), Las Vegas, Nevada, USA, as gratis. Known concentrations of the metabolite standard and IS solutions

![Fig. 1](https://example.com/image1.png)

**Fig. 1** Biological transformation process—formation of AMK (2-amino-MK) metabolite, a product of an adduct between cysteine Hb (hemoglobin) and MK (musk ketone) through enzymatic metabolism.
were used to prepare calibration plots. De-ionized water was used for all preparations.

1.2 Test solution and fish exposure

A series of standard test solutions containing 10, 30, 100, and 300 mg MK/ml of salmon oil as the vehicle (Pharmaceutical grade, Yukon Nutritional Company, USA) were prepared for trout exposure. At the highest concentration of 300 mg/ml, the MK did not dissolve completely in the oil, but instead, formed an emulsion. Exposure experiments were conducted at the Department of Environmental and Molecular Toxicology, Oregon State University (OSU), Corvallis, Oregon, USA. Well-shaken standard solutions were injected intraperitoneally into fish that were anaesthetized in an aqueous solution containing 75 mg/L of MS 222. The anaesthetized trout were weighed prior to injecting the standard solutions. To perform dose-response investigation, twelve fishes were exposed to MK solutions, three trout for each concentration level for 1 d exposure period. For the kinetics study, three fishes were exposed to a single concentration of MK (30 mg/ml) for 3 or 7 d. As controls nine fish were exposed to the vehicle (with no MK) for the same sampling periods. After exposure, fish were returned to labeled tanks with circulating water at 13°C. The details of fish exposure experiments are given in Table 1. After exposure, no food was supplied to the fish and one fish exposed to 10 mg/ml MK was found to be dead in 1-d exposure water tank.

1.3 Collection of fish blood and separation of Hb

Before collection of blood, fish were anaesthetized with the MS 222 solution and blood samples were drawn from the anaesthetized fish into heparinized individual syringes from the caudal vein and placed into heparinized individual sterile interior Vacutainers (Becton Dickson VACUTAINER System 16939, USA). All blood samples were placed on ice immediately after collection, and the fish were sacrificed.

Red blood cells/erythrocytes were isolated from plasma by centrifuging at 3,500×g for 10 min at 4°C, washed twice with equal volumes of 0.9% saline and re-centrifuged, and the cellular debris was discarded. The erythrocytes were lysed by adding two volumes of distilled water. The Hb solutions were then solidified in a freezer at −24°C, and 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., USA). The dried Hb was then placed in a freezer at −24°C until further analysis of the MK metabolite.

1.4 Bound amine metabolite from the Hb

Previously we described the alkaline hydrolysis, extraction, and preconcentration procedures for liberation of the bound amino metabolites from carp Hb (Mottaleb et al., 2004a). The same procedures were employed in this study. The volume of extract in n-hexane was about 45 ml,

| Table 1 In vivo fish exposure schedule showing exposure time, dosage and exposure level of test material and vehicle (salmon oil), and obtained metabolite concentration in Hb (hemoglobin) |
|---|---|---|---|---|
| Fish exposure time (d) | MK conc. (mg/ml) | Fish wet weight a (g) | MK dose per trout (mg) | Exposure level (mg/g) | Concentration of AMK metabolite (ng/g) |
| | | | | | Individual fish | Mean ± SD |
| 1 | 10 | 257 (S1) | 2.6 | 0.010 | 0.61 | 0.55±0.07 c |
| | | 237 (S2) | 2.4 | 0.010 | 0.52 |
| | | 222 (S3) b | 2.2 | 0.010 | – |
| | 30 | 199 (S4) | 6.0 | 0.030 | 1.9 | 2.0±0.10 |
| | | 230 (S5) | 6.9 | 0.030 | 2.1 |
| | | 212 (S6) | 6.3 | 0.030 | 2.0 |
| | | 272 (S7) | 27 | 0.099 | 1.9 | 2.2±0.26 |
| | | 271 (S8) | 27 | 0.100 | 2.3 |
| | | 197 (S9) | 20 | 0.100 | 2.4 |
| | 100 | 190 (S10) | 57 | 0.300 | 1.1 | 1.2±0.21 |
| | | 270 (S11) | 81 | 0.300 | 1.4 |
| | | 250 (S12) | 75 | 0.300 | 1.0 |
| Control | 206 (C1) | 0.20 ml, vehicle only | ND | ND |
| | 304 (C2) | 0.30 ml, vehicle only | ND | ND |
| | 184 (C3) | 0.18 ml, vehicle only | ND | ND |
| 3 | 30 | 278 (S13) | 8.4 | 0.030 | 33 | 32.0±1.7 |
| | | 156 (S14) | 4.5 | 0.029 | 30 |
| | | 196 (S15) | 6.0 | 0.031 | 33 |
| Control | 253 (C4) | 0.25 ml, vehicle only | ND | ND |
| | 272 (C5) | 0.27 ml, vehicle only | ND | ND |
| | 233 (C6) | 0.23 ml, vehicle only | ND | ND |
| 7 | 30 | 121 (S16) | 3.6 | 0.030 | 6.9 | 5.7±1.1 |
| | | 241 (S17) | 7.2 | 0.030 | 4.7 |
| | | 167 (S18) | 5.1 | 0.031 | 5.6 |
| Control | 273 (C7) | 0.27 ml, vehicle only | ND | ND |
| | 305 (C8) | 0.30 ml, vehicle only | ND | ND |
| | 250 (C9) | 0.25 ml, vehicle only | ND | ND |

a Indicates sample number in the parenthesis; b trout was found dead and blood sample was not collected; c depicts that SD was calculated from two measurements. SD represents standard deviation; ND represents not detected. S1 to S18 represent fish sample exposed to MK and C1 to C9 indicate control fish sample exposed to salmon oil vehicle.
and was then evaporated under gentle flow of nitrogen at 45°C to about 50 µl, to which 10 µl of 100 pg/µl of IS was added. The solution was sealed in GC-vials and analyzed with GC-NICI-MS using SIM. A schematic diagram is shown in Fig. 2.

1.5 Test of unbound amine metabolite

The presence of unbound amino metabolites in the Hb sample was examined with non-hydrolyzed Hb control experiments. In the experiment, all chemicals and solvents (except NaOH) were added to the Hb (about 50 mg), and the same extraction and preconcentration procedures were followed as described above. Laboratory/reagent blank control experiments were also carried out by using the same amounts of solvents, chemicals, and reagents used for the hydrolysis, except no Hb was used.

1.6 Gas chromatography and mass spectrometry

An Agilent Technologies 6890 series GC equipped with an Agilent 5973 mass selective detector (MSD) and an Agilent 7683 auto sampler was used in this investigation. The helium carrier gas was passed through a DB-5 (J&W Scientific, Agilent Technologies, USA) capillary column (40 m × 0.18 mm × 0.18 µm) at a constant flow rate of 0.50 ml/min (average linear velocity 22 cm/s) using the pulsed splitless mode. Table 2 illustrates detailed GC-MS operating conditions. By selecting the base peak and confirming ions of the IS and the target compounds, the mass spectral acquisitions were performed with dwell time of 25 ms/ion using the GC/MSD Agilent ChemStation software, version B.02.05. Ions monitored included the IS m/z 272 molecular ion; m/z 264 (molecular ion) and m/z 265 (isotopic molecular ion) for AMK.

1.7 Calibration curve

A regression analysis was carried out on the ratio of areas (analyte area divided by internal standard area) versus the ratio of AMK concentration to IS concentration resulting in an 8-point calibration curve. Unweighted regression resulted in a coefficient of determination ($R^2 = 0.997$) 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 concentration of the AMK and standards.

1.8 Quality assurance/quality control (QA/QC)

Each group of samples to be analyzed was checked 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. Deviation from the calibration curve greater than ±15% would cause of the rerunning of standards, construction of new calibration curve or replacement of the GC capillary injector tubing. Contaminated tubing resulted in poor peak shape (tailing) which affected quantitation. Retention time variations were generally less than 5 s, and peak widths at half height were approximately 3 s. On the day of analysis, solvent blank, standard, solvent blank, extract solutions, and solvent blank were injected in this order into the GC-MS instrument, and no carry over of the metabolite was detected. In control specimens, the metabolite was not determined. Moreover laboratory reagent blank, non-hydrolyzed extracts were included in each analytical batch and the metabolite was not observed in these blanks.

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from the alkaline hydrolyzed extraction were bound to cysteine-Hb in trout blood (Fig. 1).

2.1 Detection and characterization of the bound AMK metabolite

Figure 3 compares a typical selected ion chromatogram detected using GC-NICI-MS. Fig. 3a shows a mixture containing 100 pg/µl of AMK metabolite, and Fig. 3b exhibits an extract from sample S6 in the 1-day exposure study (Table 1). The standard solution and extracts were spiked with 1.0 ng IS, before being injected into the instrument. These show an excellent agreement in the retention time of the metabolite which eluted from the column at 25.17 min. The IS was eluted at 15.45 min from the column (data are not shown). Fewer ions are available in the NICI detection for metabolite relative to the EI mode (Mottaleb et al., 2004b). The AMK metabolite form negative ion spectra by an electron capture process of collisionally moderated, electrons, and afford little or no fragmentation. An agreement (about ±8%) of the relative abundance of molecular and (M+1)− isotopic molecular ions was observed in both standard and sample extract solution spectra of the AMK metabolite (Fig. 4). The mass signal of m/z 264 corresponds to molecular anion (M−) and m/z 265 represents to the (M+1)− isotope.

2.2 Limit of detection and concentration of the metabolite

The limit of detection (LOD) of the metabolite was estimated based on a signal-to-noise ratio of 3:1 (S/N = 3). The LOD of the metabolite was found to be about 0.60 pg at the lowest level of calibration. This corresponds to the LOD in the Hb sample of about 0.30 ng/g based on 50 mg of Hb per 50 µl final volume of extract.

The concentrations of AMK metabolite formed in the individual trout Hb for the exposure period of 1, 3, and 7 d are illustrated in Table 1. A small variation of the metabolite concentration was observed in individual trout. Although different trout have their own individual metabolic process, variation of the adduct formation in individual trout is common. The average concentration of AMK metabolite formed with cysteine-Hb adduct for each exposure level was calculated (Table 1) and maximum formation of adduct was observed as 32.0 ng/g from 3 d sample exposed with 0.030 mg/g MK. One trout died during exposure period (1 d) (noted by markb in Table 1), and no blood sample was received from this fish.

2.3 Dose-response and kinetics assessments of the metabolite

Figure 5 shows the dose-response assessment of bound AMK metabolite formed in the trout Hb at 1 d after exposure. It can be seen that binding of MK as adduct to the cysteine-Hb increases in a dose-dependent fashion and maximum formation was obtained from 0.030 to 0.10 mg/g dosage. Average concentration was found to be 2.2 ng/g at a dose level of 0.10 mg/g. A drop in adduct formation was
observed at a dose of 0.30 mg/g. We attempt to prepare a concentration of 300 mg/ml of MK for a dose level of 0.30 mg/g fish. This dose level was not a true solution; rather, it was a thick emulsion that did not dissolve all the solutes. The rate of formation of adduct may have experienced a drop due to solid state of the MK as suspensions and, thus active ingredient of MK was not readily available or transported into intracellular system.

The kinetics assessment of the AMK metabolite is depicted in Fig.6. This plot shows the average concentration of the metabolite as natural logarithm (ln) versus sampling times. A maximum adduct formation could be seen at approximately 3 d after exposure, although the frequency of the sampling precludes definitive treatment of data. The kinetics of the elimination of the Hb adduct metabolite is nonlinear, and occurs significantly faster than human erythrocytes elimination process. The lifetime of trout erythrocytes is unknown. The average concentration, derived from the Fig.6, of the metabolite for 3 and 7 d exposure were found to be 3.5 and 1.8 ng/g, respectively. The elimination rate constant was calculated as 0.43 d$^{-1}$. We estimate 2 d half-life of the metabolite in the trout Hb based on assuming first-order kinetics. This value is comparable to the reported half-life for musk xylene metabolite of less than a few days in rats (Minegishi et al., 1991) and fish (Mottaleb et al., 2006).

3 Conclusions

The bioavailability of nitro musk metabolite in trout Hb is investigated after a single administration of MK and salmon oil (dosage vehicle) into rainbow trout for 1, 3, and 7 d exposure periods. One nitro-group (m-position) of MK is enzymatically reduced to reactive intermediate nitroso compound, capable of binding with sulfahydryl group of cysteine-Hb that subsequently yields an amine. That amine could be suitable as a biochemical endpoint in monitoring and assessing exposure to MK hazards. The AMK was identified and quantified as metabolite bound to cysteine-Hb of trout exposed to MK by GC-NICI-MS with SIM. The half-life of the AMK metabolite was calculated as 2 d based on first-order kinetics. This study suggests that more sampling data points are required for definitive examination of toxicokinetics assessment of AMK metabolite.

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References


Bethesda M, 2006. Environmental impacts of emerging con-


