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Toxicological and Kinetic Study of Musk Xylene in Rainbow Trout, *Oncorhynchus mykiss*

S. Boleas, C. Fernandez, J. V. Tarazona

Division of Environmental Toxicology, CISA-INIA, E-28130 Valdeolmos, Madrid, Spain

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Musk xylene, is a synthetic nitro musk that has been widely used as fragrance ingredient in soaps, detergents, lotions and even foods (Iwata et al. 1993a). These uses lead to the presence of the substance in the aquatic environment (Hahn 1993). The compound is moderately lipophilic, and a high bioaccumulation potential for aquatic organisms must be expected; in fact, the compound has been detected in freshwater and marine aquatic fish and molluscs in different areas of the world (Yamagishi et al. 1981; 1983; Hahn 1993; Rimkus and Wolf 1993a).

In rats, musk xylene has been detected as a cytochrome P450 inducer (Iwata et al. 1992). The compound is not mutagenic (Nair et al. 1986) but it has been reported as a hepatocarcinogen when chronically administered to B6C3F, mice (Maekawa et al. 1990). This paper studies the toxicity and accumulation of musk xylene in rainbow trout, *Oncorhynchus mykiss,* including the capability of the compound to induce the cytochrome P450 related activity EROD, and to affect hepatic levels of retinol. A CC/MS method for the analysis of musk xylene is also presented.

MATERIALS AND METHODS

Musk xylene, 2,4,6-trinitro-5-*ter*-butylxylene; CAS No 81-15-2, was obtained from EVSA (Spain), the purity of the chemical was confirmed by CC/MS.

Rainbow trout, *Oncorhynchus mykiss,* were obtained from a fish farm in central Spain and acclimated to laboratory conditions for at least one week. Exposures were performed in glass aquaria. Water quality conditions have been published elsewhere (Rodriguez-Moreno and Tarazona 1994). Basic parameters were, pH 7.47 ± 0.41, temperature 14.0 ± 0.5 C, dissolved oxygen 10.80 ± 0.50 mg/L, hardness 74.44 ± 0.40 mg CO_3Ca/L, and alkalinity 195.53 ± 21.88 mg CO_3Ca/L.

Correspondence to: J. V. Tarazona

The acute lethality of musk xylene was tested using a screening test on trout alevins (5-10 g weight) under standard general conditions (APHA, 1980), 96 hr of exposure and concentrations ranging in orders of magnitude up to 1,000 mg/L. The accumulation of musk xylene on fish and its effect on cytochrome P-450 related parameters were studied on 44.2±2.8 g trout exposed for 21 d to musk xylene concentrations of 1,10 and 100 µg/L, adding 5 mL of 0.01, 0.1 and 1 mg/mL musk xylene ethanolic solutions, respectively. Exposures were run under static conditions with daily water renewals. Trout samples (5 fish per aquaria) were collected weekly. Blood was sampled from the caudal vein and animals sacrificed by severing the spinal cord. In all cases, musk xylene was dissolved in ethanol and the same solvent and concentration, ethanol 0.1 ml/L, was use in the control group.

EROD (7-ethoxyresorufin-0-deethylase) activity was measured by fluorimetric assay (532/582 mn, Aminco Bowman, USA) according to Monod and Vindimian (1991). Activity was measured on hepatic S9 fractions instead of liver microsomes. Positive controls were performed on hepatic S9 fraction obtained from trout dosed i.p. with 50 mg/kg of β-Naphtoflavone. Retinol levels were analyzed in plasma samples, after microextraction with diethyl ether/n-hexane l:l, by HPLC on a Vydac C-18 column using isocratic methanol/water 90/10 conditions and UV detection at 325mn (Lim 1986). Retinol acetate was added before extraction and employed as internal standard.

Musk xylene was analyzed in the edible portion of trout by GC/MS (Hewlett-Packard 5890/5971, Germany) using splitless injection and SIM (Single Ion Monitoring) detection selected for the 282 m/e ion. Eviscerated fish were homogenized with distilled water (1:3 w/v). Aliquots, representing 20 g of fish tissue, were extracted with 80 mL of n-hexane and the n-hexane phase concentrated to 1 mL under vacuum (20 mm Hg, 45 C). Samples were injected into the GC/MS equiped with an HP-5 cross-linked 5% phenyhnethyl-silicone 30m x 0.25 mm i.d. x 0.25 mm film capillary column. The injection and detection temperatures were set at 250 C and 325 C respectively. Oven conditions started with 2 min at 60 C followed by: first temperature gradient at 15 C/min till 200 C, isothermic at 200 C for 9 min, second gradient at 30 C/min till 280 C, isothermic at 280 C for 5 min. Helium, 05ml/min, was used as carrier. Quantifications were done by the external standard method. Recoveries were higher than 95 %; nevertheless, to avoid recovery calculations, standard curves were done by adding fixed concentrations of musk xylene to preanalyzed musk xylene-free homogenates. The linear correlation coefficient between concentration and detector response was r =0. 9993. Variation coefficients were lower than 5 %. Quantification and detection limits were 2 and 1 µg/kg, respectively. The absolute limit of detection, 0.013 ng, allows detection of trace levels. No interferences have been observed using the SIM detection mode.

Statistical analyses were performed using-ANOVA and the Student “t” test for $p<0.05$.

RESULTS AND DISCUSSION

The screening of the lethality tests showed a very low acute toxicity of musk xylene for rainbow trout. Mortality or clinical symptoms were not observed in

trout alevins even at the highest concentration, 1,000 mg/L. In addition, fish also showed normal behavior.

The EROD activity in control and exposed fish ranged between the detection level and 0.63 pmol/min.mg protein. No significant differences between control and exposed groups were observed. Additionally, dose/response or time/response relationships for the 21 d exposure were not observed. Similar results were observed for plasma retinol which ranged between the detection level and 296 ng/mL without significant differences between control and exposed animals.

Musk xylene has been reported as an inducer of EROD and other cytochrome P-450 activities in rats (Iwata et al. 199313); however, the induction is specific to P4501A2 isoforms, without change in the total cytochrome P450 content, and the level of induction is below 4-fold even at doses of 200 mg/kg (Iwata et al. 1992; 1993a; 1993b). The patterns of cytochrome P-450 induction in mammals and fish show large differences, and it is common that substances that are inducers in mammals do not have effect on fish (Stegeman and Kloepper-Sams 1987; Goksøyr and Förling 1992). Our data show that this is the case for musk xylene. Retinol levels were also not affected at any exposure level. Decreases in retinol have been considered as a physiological effect of the induction of cytochrome P-450 activities (Zile 1992); and suggested as markers for the exposure to typical inducers such as coplanar PCBs (Palace and Brown 1994). Thus, both EROD and retinol levels confirm a lack of induction under the exposure conditions used in this experiment.

Our GC/MS method for the analysis of musk xylene in fish avoided a cleanup procedure. Cleanup is the most critical and unpractical step of previous GC methods using EC detectors. The selectivity of the MS detector, with the improved sensitivity of the SIM mode, allows very rapid analysis and avoids the risk of losses during the cleanup and false positive errors of the nonselective EC detector. This possibility is illustrated in the chromatogram presented by Rimkus and Wolf (1993b) that shows an unidentified peak with almost the same retention time as musk xylene. In fact, the same authors recommend SIM mode GC/MS for the analysis of fish samples (Rimkus and Wolf 1993a). Figures 1 and 2 show a typical chromatogram and the mass spectra of musk xylene, respectively.

Musk xylene has not be considered a major problem for the aquatic environment, however, the exposure of human consumers via fish consumption is of concern in Germany. Thus, the accumulation study was designed to provide information on human exposure. The use of the edible portion and wet weight are ideal conditions for this assessment. Figure 3 shows the accumulation of musk xylene in the edible portion of rainbow trout after waterborne exposure. It can be observed that significant accumulation versus control fish appeared rapidly, being evident after 1 wk of exposure, with no further changes during the time span of the experiment. Only for the highest concentration, data show a tendency for further accumulation for the longest exposure time. No significant differences between the exposure to 1 and 10 µg/L were observed. As expected for a lipophilic substance, a rather high individual variability for data based on wet weight was observed, with variation coefficients ranging between 5 and 50 % . Bioconcentration factors, for the edible portion, between 10 and 60 can be estimated from the present data. A Pow of 4.38 was calculated for musk xylene

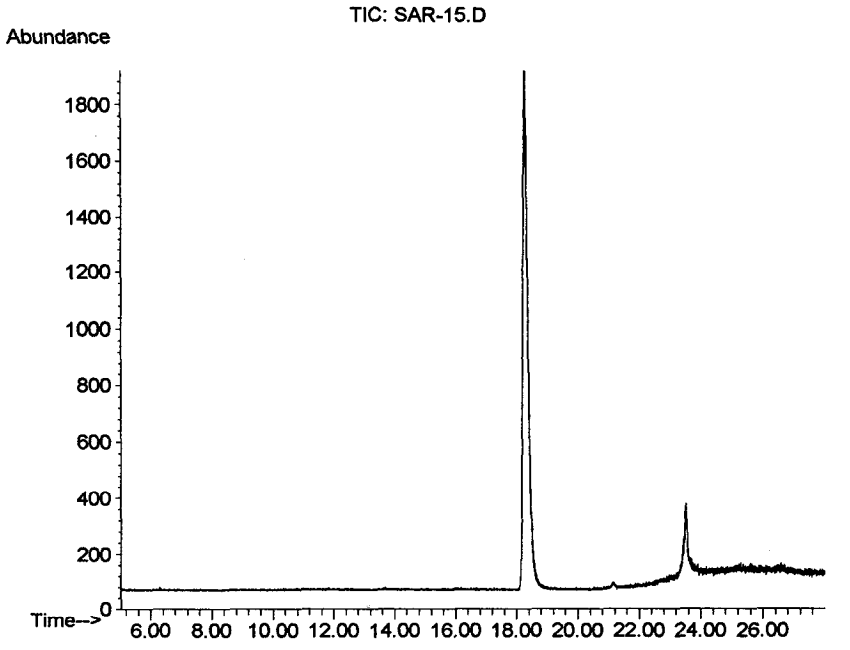

Figure 1 Typical chromatogram of musk xylene

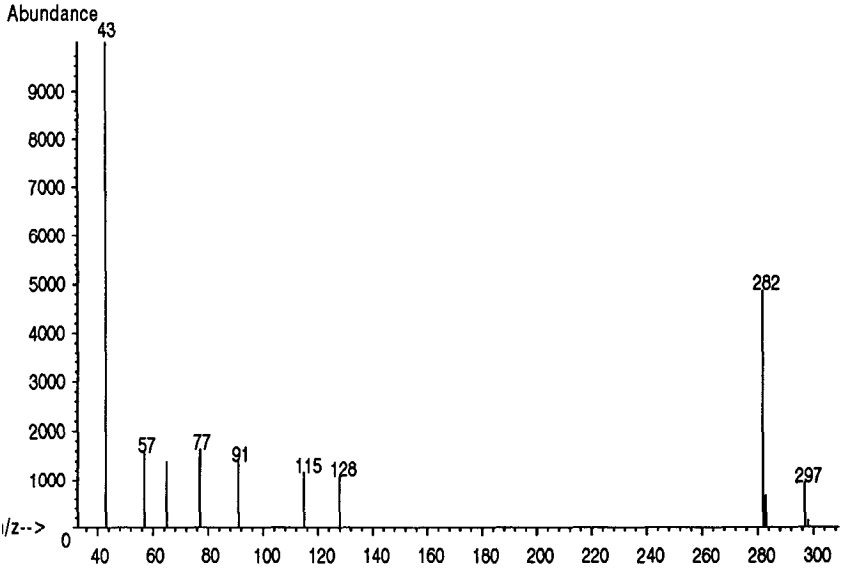

Figure 2. EI mass spectrum of musk xylene

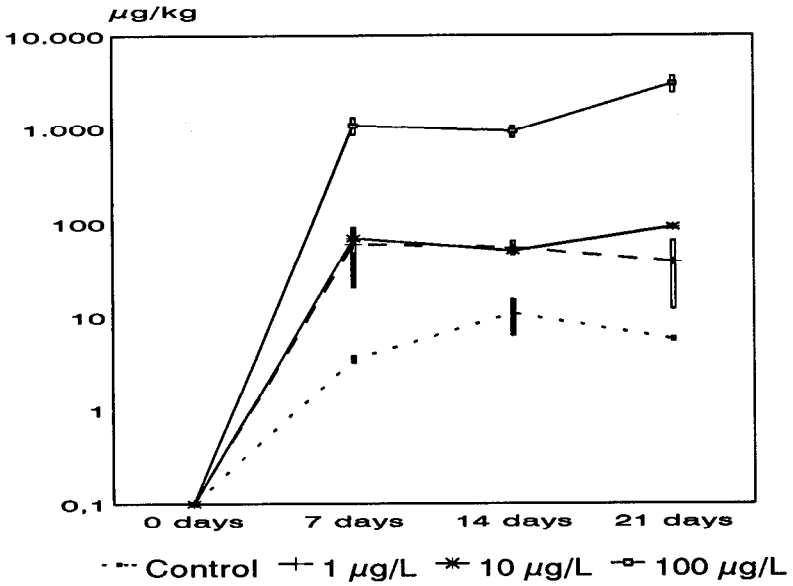

Figure 3. Accumulation of musk xylene in the edible portion of rainbow trout

using the Hansch and Leo (1979) procedure; suggesting a higher potential for bioaccumulation than that observed. Obviously, the use of the edible portion instead of whole body produces lower BCFs for lipophilic chemicals. However, even for the edible portion, the observed bioaccumulation was lower than expected. The low potential for bioaccumulation observed in this study agrees with reported field data. Field BCFs lower than 100 can be estimated from published data, which showed concentrations of musk xylene between 11 and 82 µg/kg wet weight in fish collected downstream a musk xylene point-pollution-source in a German river with musk xylene concentrations ranging between 1 and 3 µg/L in the river and up to 39 µg/L in the mixing zone (Hahn 1993). Considering the purpose of this study, these results indicates a lower concern for the exposure of human consumers to musk xylene than that expected according to its lipophilicity.

Nitro musks have been extensively used as fragrance ingredients in food and cosmetic products, but nowadays are cause of concern due to some indications of exposure in humans (Liebl and Ehrenstorfer 1993a), although the toxicological relevance of the present findings for humans is still unclear (Liebl and Ehrenstorfer 1993b). This paper shows the lack of acute toxicity and MFO induction of musk xylene in trout, and its accumulation patterns characterized by a very rapid accumulation but with bioconcentration factors relatively low compared to those observed for lipophilic substances.

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