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# Metabolites of 2-chlorosyringaldehyde in fish bile: indicator of exposure to bleached hardwood effluent

V. S. HARITOS*, C. M. BRUMLEY, D. A. HOLDWAY and J. T. AHOKAS

Key Centre for Applied and Nutritional Toxicology, RMIT-University, GPO Box 2476V, Melbourne, Victoria 3001, Australia

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1. 2-Chlorosyringaldehyde (2-CSA) is the major chlorinated phenol produced by the 100% chlorine dioxide bleaching of eucalypt pulp and is found in other bleached hardwood effluents. Almost nothing is known of the environmental or metabolic fates of this chemical.

2. Sand flathead (*Platycephalus bassensis*) was given 2-CSA by intraperitoneal injection at 0·15, 1·5, 15 and 75 mg/kg doses and, 4 days later, bile was collected and solvent extracted before and after enzymatic cleavage of conjugates. The acetate derivatives of bile extracts were analysed by gas chromatography/mass spectrometry.

3. The major metabolite 4 days after administration was the glucuronide or sulphate conjugate of 2-chloro-4-hydroxy-3,5-dimethoxy-benzylalcohol (2-CB-alcohol). The identity of 2-CB-alcohol was confirmed by chemical synthesis.

4. The quantity of 2-CB-alcohol in the bile was linearly related to dose of 2-CSA and was detected at all dose levels. Minor metabolites identified were conjugated 2-CSA, unchanged 2-CSA and 2-chloro-4-hydroxy-3,5-dimethoxy-benzoic acid.

5. The amount of 2-CB-alcohol in bile has the potential to be a sensitive and specific indicator of fish exposure or bleached hardwood effluent.

## Introduction

The technology of kraft pulping and bleaching for modern mills is continually improving with the aim of reducing the organochlorine load discharged from bleaching plants (Crossland and Abel 1993). Consequently, the composition of chlorinated compounds in the effluents is changing. The bleached eucalypt kraft process using 100% chlorine dioxide replacement produces an effluent with no detectable 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or furans and adsorbable organohalogen (AOX) levels in the filtrate of well below 1 kg/oven-dried tonne of pulp (Nelson 1993).

Concomitant with the lower organochlorine content of the effluents is the expectation that they will lower the toxicological impact on the aquatic systems into which they are released. Nevertheless, concern regarding the impact of pulp mill effluents on the aquatic environment has led to the National Pulp Mills Research Program, which has included a program of biomarker development and validation using aquatic organisms (Commonwealth of Australia 1989). Environmental monitoring is expected to ensure that any significant, unknown impacts of effluent release are detected early, enabling remedial action to be taken before any irreversible degradation of the ecosystem occurs (Crossland and Abel 1993).

Sand flathead (*Platycephalus bassensis*) is a suitable species for the biomonitoring of Southern Australian marine waters receiving a modern bleached mill effluent as it is relatively abundant and widespread. Several biomarkers in sand flathead,

*Author for correspondence.

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including biliary metabolites, were recently investigated (and validated using Aroclor 1254) for use in marine pollution exposure biomonitoring (Brumley *et al.* 1994). Metabolites of pollutants extracted from the bile of fish have been used previously as specific indicators of exposure to softwood pulp mill effluent (Oikari and Ånäs 1985) and polycyclic aromatic hydrocarbon contaminated sediments (Krahn *et al.* 1993). Additionally, some pollutants found in fish bile are bioconcentrated by many orders of magnitude compared with levels found in the water and are therefore very sensitive indicators of exposure to contaminants of the aquatic environment (Stratham *et al.* 1976).

Pulping and bleaching of hardwood lignin produces a number of additional chlorinated phenolics such as syringaldehydes and syringols, which are not present in softwood pulps (Voss *et al.* 1980, Kringstad and Lindström 1984). 2-Chloro-syringaldehyde (2-CSA) is quantitatively the major chlorinated phenol produced by the 100% chlorine dioxide bleaching treatment of eucalypt pulp (Smith *et al.* 1994) and is formed after the chlorine and chlorine dioxide bleaching of other hardwood pulps (Kachi *et al.* 1980, Voss *et al.* 1981). Little is known of the environmental fate of chlorinated syringaldehydes in aquatic systems, however their toxicity toward marine invertebrates, alga and fish larvae have recently been investigated. 2-CSA was found to be much less acutely toxic than the more highly substituted chlorophenols found in effluents produced by elemental chlorine bleaching of softwood pulp (Stauber 1994). Chlorinated phenolics originating from bleached pulp mill effluent are generally metabolized in fish by conjugation with UDP-glucuronic acid or sulphate prior to excretion, with little evidence of phase I metabolism (Oikari and Ånäs 1985, Oikari *et al.* 1988).

The current study has investigated the metabolic fate of 2-CSA in sand flathead through the identification and characterization of the major biliary metabolites produced several days after treatment of the fist with 2-CSA at several doses.

## Materials and methods

*Materials*

Syringaldehyde and sodium borohydride were purchased from Aldrich (WIS, USA) and 2,4,6-tribromophenol was obtained from Sigma Chemical Co. (MO, USA). MS-222 (tricaine methanesulphonate) was purchased from Cresent Research Chemicals (USA). 2,3,7,8-Tetrachloronaphthalene (TCN) was obtained from ICN Biomedicals (OH, USA). All solvents used were of analytical grade. 2-CSA (m.p. 196–197°C) was a gift of Dr A. F. A. Wallis (CSIRO Division of Forest Products, Clayton, Australia).

*Synthesis of 2-chloro-4-hydroxy-3,5-dimethoxy-benzylalcohol*

2-CSA was synthesized from syringaldehyde by the method of Hyötyläinen and Knuutinen (1993) and recrystallized twice from 5:1 dichloromethane/methanol. Reduction of 2-CSA (2·08 mmol) was achieved with $NaBH_4$ (1 mmol) in alkaline ethanol. The product was recrystallized from dichloromethane/*n*-hexane to give 2-chloro-4-hydroxy-3,5-dimethoxy benzylalcohol (2-CB-alcohol) as white prisms (m.p. 95–97°C) in 65% overall yield from syringaldehyde. Microanalysis of the recrystallized product gave C 49·3%, H 5·2%, O 29·4%, Cl 16·2%; $C_9H_{11}ClO_4$ requires C 49·5%, H 5·1%, O 29·3%, Cl 16·0%. The mass spectrum of the diacetylated derivative of 2-CB-alcohol and the proposed fragmentation pathway obtained by GC/MS analysis are shown in figure 1. $^1$H-nmr chemical shift data: $\delta$ 1·80 (1H, s, benzyl-OH), $\delta$ 3·88 (3H, s, C3 or C5-$OCH_3$), $\delta$ 3·90 (3H, s, C3 or C5-$OCH_3$), $\delta$ 4·69 (2H, s, benzyl-H), $\delta$ 5·64 (1H, s, C4-OH), $\delta$ 6·79 (1H, s, C6-H). The $^{13}$C-nmr chemical shift data: $\delta$ 56·3 and 60·7 methoxy-C's, 62·8 benzyl-C, 106·7 C-6, 118·5 C-2, 129·3 C-4, 138·9 C-1, 143·3 C-5, 146·2 C-3. A distortionless enhancement by polarization transfer (DEPT) experiment confirmed the benzylic carbon assignment.

*Treatments and bile extraction*

Male and female sand flathead (166 ± 48 g, $n = 49$) were caught by reel and line from Port Phillip Bay, Melbourne, Australia, and acclimatized for at least 8 days in flow-through 30-litre seawater tanks. The

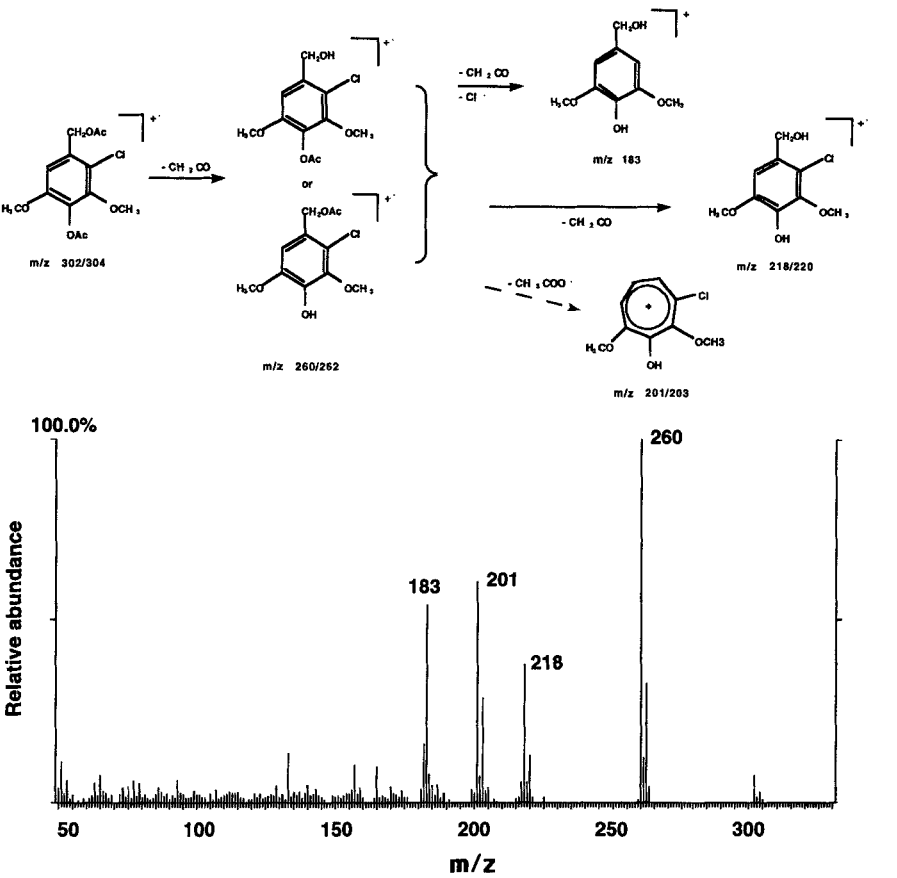
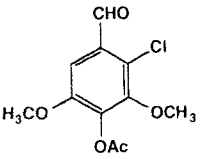
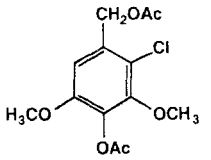
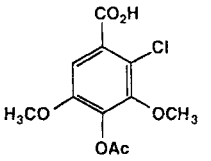



Figure 1. Mass spectrum of the diacetylated derivative of 2-chloro-4-hydroxy-3,5-dimethoxy-benzylalcohol and the proposed fragmentation pathways of the molecule. GC/MS conditons used are detailed in the text.

fish were anaesthetized with MS-222 (70 mg/l) prior to an injection of 2-CSA (0·15, 1·5, 15 or 75 mg/kg; 9–10 in each group) dissolved in dimethylsulphoxide (4% of total volume) and then diluted in corn oil, or were given vehicle alone. Four days after treatment the fish were anaesthetized with MS-222 and then killed by a blow to the head. The bile was aspirated from gall bladders by syringe and stored in liquid nitrogen until analysed. Bile was not available from all fish at time of sampling; one from the 0·15 mg/kg dose group and three from 1·5 mg/kg dose group were not obtained.

The methods for extraction and derivatization of chlorinated phenols from bile samples were based on those of Morales *et al.* (1992). Bile was removed from storage tubes and rinsed into glass test tubes with MiliQ water. 2,4,6-Tribromophenol (1 μg) was mixed into the bile sample prior to extraction, as an internal standard, from a 10-μg/ml stock solution in *iso*octane (100 μl). Each sample was treated with 1 M HCl (100 μl), extracted with 3:1 *tert*-butyl-methylether/dichloromethane (MTBE/DCM) (three times 2 ml) and vortexed for 2 min. The organic extracts were combined and evaporated to approximately 1 ml using a gentle stream of nitrogen. The remaining aqueous phase was incubated with a mixture of β-glucuronidase (4000 units) and aryl sulphatase (32 000 units) (Boehringer Mannheim, Germany) in 0·3 M sodium acetate buffer (2 ml), pH 5 for 20 h. The cleaved conjugate fractions were extracted with 3:1 MTBE/DCM and treated as described for the organic extracts. Two bile samples from the 75-mg/kg 2-CSA treated group were divided and extracted with ethyl acetate (three times 2 ml) or MTBE/DCM (three times 2 ml) to check the extraction efficiency of the latter solvent mixture toward polar metabolites. Acetate derivatives of the samples were formed by treatment with acetic anhydride (100 μl) and pyridine (50 μl) at 70°C in a tightly capped vial for 20 min. When cooled, the samples were washed with 0·5 M HCl (0·5 ml), dried with sodium sulphate and analysed after addition of instrument internal standard TCN (0·5 μg).

Table 1. GC column retention times and mass spectral characteristics of the acetate derivatives of bile metabolites resulting from 2-CSA administration to sand flathead.

| Chemical* | RRT[a] | Mass spectral peaks (and relative abundance (%)) | | | |
|---|---|---|---|---|---|
| 2-CSA-acetate  | 0·897 | 258 (4)<br>215 (37) | 218 (35)<br>127 (10) | 217 (21) | 216 (100) |
| 2-CB-alcohol-diacetate  | 1·002 | 304 (2)<br>220 (13)<br>183 (54)<br>133 (13) | 302 (7)<br>218 (38)<br>182 (16) | 262 (33)<br>203 (29)<br>165 (10) | 260 (100)<br>201 (61)<br>157 (10) |
| 2-CB-acid-acetate  | 1·017 | 276 (7)<br>232 (100) | 274 (14)<br>219 (10) | 234 (32)<br>217 (25) | 233 (12)<br>215 (26) |

* 2-CSA, 2-chlorosyringaldehyde; 2-CB-alcohol, 2-chloro-4-hydroxy-3,5-dimethoxy-benzylalcohol; and 2-CB-acid; 2-chloro-4-hydroxy-3,5-dimethoxy-benzoic acid.

[a]RRT, relative retention time to tetrachloronaphthalene.

OAc, acetate derivative of phenol or alcohol.

*Analysis*

Extracted bile samples were analysed on a Shimadzu QP2000 gas chromatograph/mass spectrometer (GC/MS). Splitless injections of 1 $\mu$l were made onto a 30 m × 0·25 mm fused silica column coated with 0·25 $\mu$m DB-1 (J&W, USA). The initial column temperature was 70°C, held for 3 min increasing to 250°C at 12°C per min and held for 10 min. The injection port, mass spectrometer interface, and ion source were maintained at 250°C. Mass spectral acquisition was achieved at 70 eV at 1 scan/s between 650 and 50 amu. Selected ion monitoring was used for quantitation of metabolites in samples; mass spectral ions of the acetate derivatives monitored were 330/332 for 2,4,6-tribromophenol, 216/218 for 2-CSA, 266 for TCN and 260/201 for 2-CB-alcohol, and 232/234 for 2-chloro-4-hydroxy-3,5-dimethoxybenzoic acid (see table 1 for structures, mass spectra and relative abundances of selected chemicals). The underlined ions were used for quantitation as they were the most abundant ions in the mass spectra of the compounds and were of relatively high mass. The concentrations of 2-CSA and 2-CB-alcohol were calculated from five-point calibration curves constructed from prepared standards in the range 50 ng/ml to 3 $\mu$g/ml ($r^2 = 0{\cdot}99$ for both). Samples where the metabolite concentration was measured outside the quantitation range were diluted in *iso*octane and reanalysed.

Proton and carbon nmr spectra were obtained using a Varian Gemini 200 MHz (H) and 50 MHz (C-13) spectrometer in deuterated chloroform and given as ppm from tetramethylsilane.

*Statistical analysis*

Metabolite concentration data was log transformed due to heterogeneous variances between dosage groups. Differences between groups were assessed by one-way analysis of variance (ANOVA) using the software program SuperANOVA. Where significant differences were found, Fisher's protected LSD test was used to distinguish the differences between the means.

## Results

Detection and quantitation of metabolites and standards were performed using GC/MS with selected ion monitoring however, total ion chromatograms (TICs) of

derivatized bile extracts effectively show the proportions of metabolites in the bile samples before and after enzymatic cleavage of conjugates. TICs of derivatized bile extracts from 2-CSA treated sand flathead (15 mg/kg) measured before and after enzymatic hydrolysis are shown in figure 2 (*a*) and (*b*). Extraction before hydrolysis gave up to three treatment-related peaks, which were identified as 2-CSA, 2-CB-alcohol and 2-chloro-4-hydroxy-3,5-dimethoxybenzoic acid (2-CB-acid) by the mass spectra of their acetate derivatives. The chemical structures, GC retention times and mass spectra of biliary metabolites are shown in table 1. The metabolite 2-CB-alcohol had an identical GC retention time and mass spectrum to that of synthesized 2-CB-alcohol. Enzymatic cleavage produced large quantities of 2-CB-alcohol and lesser amounts of 2-CSA in the extracts. Owing to the dominance of 2-CB-alcohol relative to 2-CSA concentration and normalization of the chromatogram, all peaks that were detected by selected ion monitoring are not visible in the TICs, as shown in figure 2 (*b*).

The quantity of both 2-CB-alcohol and 2-CSA in sand flathead bile were measured by GC/MS with selected ion monitoring. The major biliary metabolite 4 days after administration was conjugated 2-CB-alcohol, with lesser amounts of conjugated 2-CSA and unconjugated 2-CB-alcohol (table 2). The amount of the conjugated metabolite 2-CB-alcohol, in the bile of treated fish was significantly different ($p < 0{\cdot}05$) at each dose level as indicated in table 2. Some unconjugated 2-CSA (mean = 0·092 μg) was extracted from bile samples in most treated fish while the quantity of 2-CB-acid was not determined as there was no authentic standard available. The total quantity of metabolites measured in the bile 4 days after treatment was approximately 2–3% of the administered 2-CSA.

The amount of 2-CB-alcohol or conjugated 2-CSA in the bile were linearly related to the amount of 2-CSA administered to the fish with respective correlation coefficients ($r^2$) of 0·75 and 0·94 (figure 3). The individual amount of 2-CSA administered to the fish was calculated from the dose (mg/kg) and the weight of the fish.

The median recovery for the internal standard 2,4,6-tribromophenol from bile samples was 99·9% (range 83–117%). The lowest concentrations of 2-CSA and 2-CB-alcohol standards used in analysis (5 ng/ml each) were detected by the GC/MS selected ion monitoring technique with signal-to-noise ratios of about 40 and 25 respectively. The use of ethyl acetate as an extraction solvent for bile samples in place of MTBE/DCM did not significantly affect the quantity of 2-CB-acid recovered or extract any new metabolites from the bile of the 75-mg/kg 2-CSA dose group (data not shown).

## Discussion

As a substrate for metabolism, 2-CSA has several functional groups that can undergo phase I or II metabolism. Potential transformations are *O*-demethylation of the methoxy groups, oxidation or reduction of the carbonyl group and conjugate formation at the phenol group. Studies of the metabolism of phenol and pentachlorophenol in different fish species have reported the formation of glucuronide and sulphate conjugates as the sole detected products (Glickman *et al.* 1977, Layiwola and Linnecar 1981, Stehly and Hayton 1989). A related compound, pentachloroanisole, was reported to be metabolized via *O*-demethylation and conjugation by rainbow trout (Glickman *et al.* 1977). However, chlorinated guaiacols (*o*-methoxyphenols) were reported to be metabolized to glucuronide conjugates,

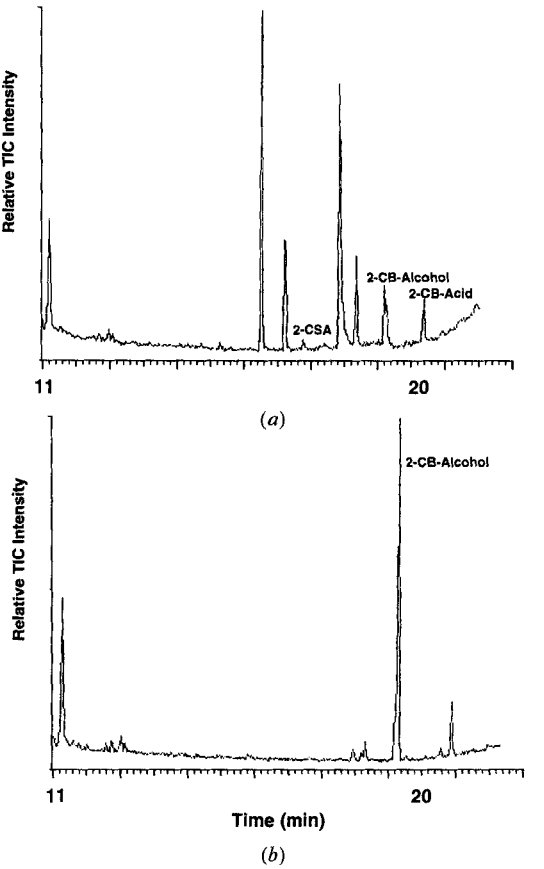



Figure 2. Total ion chromatograms (TICs) of a solvent-extracted, acetate-derivatized bile sample from sand flathead administered 2-chlorosyringaldehyde (15 mg/kg). (*a*) 'Free' metabolites and (*b*) extracted metabolites after cleavage of the glucuronide and sulphate conjugates. Analytes were detected by the mass spectrometer scanning between 650 and 50 amu. (Not all analytes detected by selected ion monitoring are visible in the TICs and unlabelled peaks are not treatment related.) 2-CSA, 2-chlorosyringaldehyde; 2-CB-alcohol, 2-chloro-4-hydroxy-3,5-dimethoxy-benzylalcohol; and 2-CB-acid, 2-chloro-4-hydroxy-3,5-dimethoxy benzoic acid.

Table 2. Mean quantity of metabolites (± SE) extracted from the bile of sand flathead 4 days after treatment with 2-chlorosyringaldehyde by intraperitoneal injection.

| Dose (mg/kg) | Number | 'Free' 2-CB-alcohol (μg) | Conjugated 2-CB-alcohol (μg) | Conjugated 2-CSA (μg) |
|---|---|---|---|---|
| 0 | 9 | 0·00[a] | 0·00[a] | 0·00[a] |
| 0·15 | 9 | 0·01 (0·01)[a] | 0·94 (0·13)[b] | 0·09 (0·01)[a, b] |
| 1·5 | 7 | 0·04 (0·01)[a] | 3·97 (0·80)[c] | 0·14 (0·02)[b] |
| 15 | 10 | 0·17 (0·02)[a] | 69·17 (11·95)[d] | 1·42 (0·11)[c] |
| 75 | 8 | 0·81 (0·29)[b] | 267·18 (41·51)[e] | 5·99 (0·43)[d] |

2-CB-alcohol, 2-chloro-4-hydroxy-3,5-dimethoxy-benzylalcohol; and 2-CSA, 2-chlorosyringaldehyde. Data is presented as mean (± SE) of raw data; statistical analyses were performed on log-transformed data. Values in columns without a superscript letter in common are significantly different ($p < 0·05$) based on Fisher's LSD test.

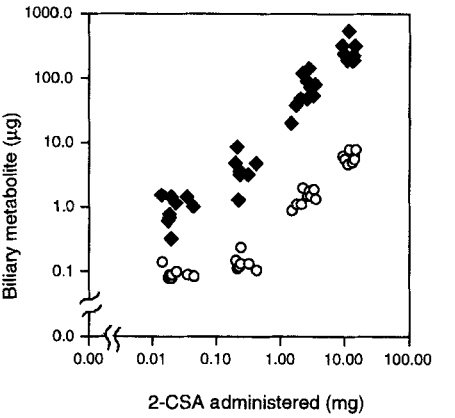



Figure 3. Relationship between the amount of metabolite measured by GC/MS with selected ion monitoring in the whole bile sample 4 days after intraperitoneal injection versus quantity of 2-chlorosyringaldehyde (2-CSA) given to sand flathead. 2-CSA in corn oil was administered to weighed fish in dose groups 0, 0·15, 1·5, 15 and 75 mg/kg. The equation for the total amount (conjugated and non-conjugated) of major metabolite 2-chloro-4-hydroxy-3,5-dimethoxy-benzylalcohol (2-CB-alcohol, solid diamonds) is $y = 20{\cdot}80x + 6{\cdot}12$, $r^2 = 0{\cdot}75$, $n = 43$, $p < 0{\cdot}001$ and 2-CSA (cleaved of phase II conjugates, open circles) has the equation $y = 0{\cdot}47x + 0{\cdot}11$, $r^2 = 0{\cdot}94$, $n = 43$, $p < 0{\cdot}001$.

with no evidence of *O*-demethylated products, by two Lake Baikal fish species (Oikari *et al.* 1988).

Analysis of the bile of 2-CSA treated fish indicates that the primary fate of the chemical in sand flathead is reductive metabolism to the corresponding alcohol (2-CB-alcohol), which is then conjugated with UDP-glucuronic acid or sulphate and excreted. An enzyme family capable of this transformation is the aldehyde reductases, cytoplasmic enzymes concentrated in the liver and kidney of vertebrates of which aromatic aldehydes are the best model substrates (Von Wartburg and Wermuth 1980). These low molecular weight enzymes have broad substrate specificity, both exogenous and endogenous, and depend on NADPH as a cofactor (Von Wartburg and Wermuth 1980), which occurs in excess of $NADP^+$ and may allow reduction of aldehydes under oxidative conditions in the cell.

The second most prevalent metabolite in bile was the glucuronide or sulphate conjugate of 2-CSA. As the major metabolite is also excreted after it is acted on by the phase II enzymes, UDP-glucuronosyl transferases and sulphur transferases, conjugation is clearly an important detoxication pathway for this chemical. The process of glucuronide and sulphate conjugation of xenobiotics enhances excretion of the chemical by increasing the hydrophilicity of the molecule. Chlorinated phenolic compounds from bleached mill effluent are predominantly excreted in the bile as glucuronides and sulphate conjugates by fish. Oikari and Ånäs (1985) reported that almost all of the chlorinated phenolics investigated were excreted by trout as conjugated forms and 9–25% of the total were sulphate conjugates with the remainder being glucuronides.

The detection of 2-CB-acid as a minor metabolite of 2-CSA in sand flathead bile is of interest as it has been reported that aromatic aldehydes are predominantly metabolized *in vivo*, in vertebrates, to oxidized products at the expense of reduced products (McMahon 1982). The opposite situation has been found in 2-CSA administration to sand flathead. We are currently investigating 2-CSA metabolism

in fish and mammalian liver preparations to determine whether there are interspecies differences.

The relationship between the amount of 2-CSA administered and the main metabolite produced in sand flathead bile 4 days after treatment was linear and the variability was relative to amount of 2-CSA given. The variation from a direct relationship may be due to differing physiology and metabolic capability of individual fish. As this study has investigated the production of metabolites of 2-CSA at one time point only, allowances cannot be made for these variables between fish.

Reduction of the carbonyl group of 2-CSA combined with glucuronide or sulphate formation is a major pathway of 2-CSA metabolism in sand flathead, regardless of the dose of 2-CSA. The consistency of the metabolic route and other metabolic characteristics strengthen the use of biliary 2-CB-alcohol measurement as a sensitive and specific exposure marker for bleached hardwood and mixed effluents. At the lowest dose of 2-CSA given to sand flathead (mean 25 $\mu$g per fish), the metabolite 2-CB-alcohol was readily detected in bile 4 days later. Also, the concentration of 2-CB-alcohol in the fish bile is proportional to the level of 2-CSA exposure. We measured 2-CB-alcohol in the bile of sand flathead exposed for 4 days to highly diluted, untreated 100% chlorine dioxide bleached kraft pulp effluent in a series of flow-through tanks containing approximately 0·1 $\mu$g/l 2-CSA (Brumley 1994, unpublished data). This indicates that 2-CSA is readily taken up by the fish from diluted effluent, metabolized and excreted in the bile. In addition, chlorosyringaldehydes are found in high levels in Canadian rivers and ponds receiving effluent from hardwood and mixed pulp mills (Lee *et al.* 1989, Morales *et al.* 1992).

In summary, hardwood and mixed effluents account for much of the effluent from pulp mills, chlorosyringaldehydes are found in the final effluents and specific biliary metabolites are relatively easy to detect. The analysis of fish bile for effluent exposure monitoring could also be carried out using a routine gas chromatographic-electron capture detection procedure.



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