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Benzo-a-Pyrene Residues in Liver and in Bile of Fish Following Oral Exposure

M. U. Beg, P. G. Jacob, K. Al-Matrouk, K. R. Beg, M. Al-Bahloul, T. Saeed

Environmental Sciences Department, Kuwait Institute for Scientific Research, Post Office Box 24885, 13109-Safat, Kuwait

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A major challenge confronting environmental scientists is developing accurate and cost-effective analyses for contaminants in marine biota and their habitat. Polyaromatic hydrocarbons (PAHs) are ubiquitous in distribution (Neff 1979) and due to their hydrophobic nature have strong binding affinity to particulate matter and sediment in the aquatic environment, but are also known to be readily bioavailable to aquatic organisms (McCarthy and Jimenez 1985; Varanasi and Stein 1991). Determination of bile metabolites as fluorescent aromatic hydrocarbons has proven useful in estimating the exposure of fish to petroleum hydrocarbons (Varanasi et al. 1989; Tavendale et al. 1996). Krahn et al. (1986a,b), and Hellou and Payne (1987) identified a few individual aromatic hydrocarbon metabolites in the livers and bile of fish that had been exposed to fuel oil. Several individual metabolites were identified by gas chromatography/mass spectrometry (GC/MS) in the bile of fish captured in urban locations (Krahn et al. 1993). The products, resulting from the metabolism of Prudhoe Bay crude oil (from the Exxon Valdez) were identified in the enzymatically hydrolyzed bile of halibut (*Hippoglossus stenolepis*) and Dolly Varden trout (*Salvelinus malma*).

In addition, a good correlation between bile metabolites with liver lesions and the health status of fish has also been found. Aromatic hydrocarbons like naphthalene and phenanthrene are metabolized in the liver, and their metabolites appear in bile without causing liver damage. In contrast, fish with liver lesions contained higher amounts of BaP-like metabolites, which are known mammalian carcinogens (Steward et al. 1990). Experimental studies demonstrated a conversion of benzo-a-pyrene (BaP) into 7,8-dihydrodiol, a penultimate carcinogen in fish (Gmur and Varanasi 1982). The waterborne xenobiotics are concentrated in fish bile with a concentration factor of up to 10,000 as compared to a water environment (Statham et al. 1976). The metabolite of these aromatic hydrocarbons found in the fish bile would be useful tools for estimating the extent of exposure in the aquatic environment. Therefore, sheim (*Acanthopagrus latus*) a native fish of Arabian Gulf was orally exposed to varying concentrations of BaP mixed in food and its level in liver and elimination in bile was followed to assess the indicator value of the two parameters for exposure assessment (Lech et al. 1973; Collier and Varanasi 1991; Lin et al. 1994).

Correspondence to: M. U. Beg

MATERIALS AND METHODS

Adult sheim of more than one year of age weighing around 300 g were obtained from the hatchery and maintained in the laboratory for two weeks before treatment. BaP was dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution, which was then mixed at different concentrations with corn oil. The corn oil, containing the desired concentrations of BaP was mixed with fish food and given orally to each fish. After exposure, the fish were held in flow through aquaria and sacrificed at identified time intervals by blows to the head. Liver tissue and gall bladder were dissected carefully, avoiding rupturing the gall bladder. The liver was immediately transferred into a beaker on ice. Excess blood was removed from the tissue by blotting with filter paper and weighed. The liver samples were either processed fresh or stored in a freezer at -80°C.

The method used for BaP determination in liver tissue was according to Van der Weiden et al. (1994) with certain modifications. One gram of liver tissue was mixed with 15 g of sodium sulfate (anhydrous) and homogenized with 40 ml hexane:acetone (3:1 mixture) and ultrasonicated three times for 5 min each to separate the organic layer. The organic extract, containing BaP, was eluted through a silica column, containing 7 g SiO_2 (70 to 230 mesh) with 1 g SiO_2 (35 to 70 mesh) on top of the column. The sample was eluted with 40 mL of a 95:5 hexane:ether mixture. After reducing the volume in a rotavapour to 1 mL, the sample was passed through an alumina column (1 g aluminum oxide in a disposable Pasteur pipette) and eluted with 5 mL of methanol. The eluate was reduced to 1 mL under vacuum and quantified by high-pressure liquid chromatography (HPLC).

The samples were analysed with a Varian 9010 solvent delivery system coupled with Shimadzu RF-551 spectrofluorometric detector. For the separation, the sample was eluted through a reversed-phase column (Vydac 201 PB-5, 25 x 4.6 mm) with a 80% methanol and 20% deionized water liquid phase for 20 min. The excitation and emission wavelengths were 380 and 418 nm. For the quantification, an external standard containing a known concentration of BaP was used. The recovery of BaP was checked by spiking with 10 µL of 1 ppm BaP to 1 g unexposed liver tissue . The recovery was about 80%.

Bile was drawn by rupturing gall bladders in a clean porcelain dish. A known volume of bile samples was taken and fluorescent aromatic compounds (FAC) were detected by direct dilution of the bile in 48% ethanol in water until the increase in fluorescence was proportional to the increase in the concentration (linear response) (Aas, 1994). Usually dilution of 1200 times was required. Excitation and emission wavelengths were set to 275 and 383 nm, respectively, for naphthalene and phenanthrene metabolites, 341 and 383 nm, respectively for pyrene metabolites, and 380 and 425 nm, respectively for BaP metabolites. Since in this study, exposure was given to BaP alone, the data for fluorescence measurement in bile at 380 and 425 nm alone are reported.

Table 1. BaP residues in hepatic tissue on the fourth day of oral exposure to varying doses of BaP

	Exposure Concentration mmole kg^{-1} body weight	BaP Residue in Liver nmole g^{-1} fresh tissue
1	19.84	0.018± 0.013
2	39.68	0.012± 0.002
3	59.52	0.024± 0.005
4	99.21	0.189± 0.103

Average ± Standard Deviation, n =3, ANOVA $p<0.008$

RESULTS AND DISCUSSION

The fish were fed with food containing varying concentration of BaP and sacrificed at identified intervals to determine its residue in hepatic tissue and elimination in bile in order to establish a relation between exposure and residue build-up. The analysis of tissue showed a non linear increase in the accumulation of BaP residues in liver with increase in exposure dose (Table 1). One way ANOVA showed the significant difference ($p<0.008$) in mean concentrations of BaP in liver in various treatments. Student-Newman-Keul multiple range test grouped treatment 1, 2, and 3 as one homogenous subset and the treatment 4 as the other.

In general, the data for BaP content in liver showed a low residue build-up in the tissue. The residue build-up was not high even at high exposure doses, which conforms to observations reported in the literature. Van der Weiden et al. (1994) detected the highest concentration of PAHs, equivalent to 2 to 25% of the dose, 1 or 2 d after treatment. In the current study, lower residues detected in the hepatic tissue of sheim were possible because of quick elimination of a great amount of residue in the bile. Collier and Varanasi (1991) showed efficient PAH metabolising capabilities in fish, especially when animals were fed during the study. The quantification of these biliary metabolites provide a sensitive biomarker of exposure to the parent chemical (Lech et al. 1973). In this study, a direct fluorescence detection technique was used for estimating relative concentrations of metabolites in the bile. Before conducting actual measurements in the bile of BaP-exposed fish, fluorescence was measured at different excitation and emission wavelengths by spiking bile taken from control fish with different aromatic compounds (Table 2). BaP had maximum fluorescence at excitation 380 nm and emission 425 nm spectra. At other excitation and emission wavelength pairings, i.e., 275/383 and 341/383 nm, the fluorescence value was minimal. At peak observation spectra, the fluorescence was linear to the concentration in the bile. Since BaP-ol, the main metabolite, was not available, the value in bile was calculated to be equivalent to that of the parent compound.

Naphthalene, phenanthrene and pyrene showed a maximum fluorescence value at 275/383 nm excitation/emission spectra. Among the three compounds, pyrene had

Table 2. Fluorescence analysis of spiked bile samples

Spiked Compound	Fluorescence Value in Bile			
	Dilution μM (x)	Ex 275/Em 383 (y1)	Ex 341/Em 383 (y2)	Ex 380/Em 425 (y3)
Bile alone*		14.83	4.63	1.57
Benzo(a)pyrene	0.329	28.92	11.55	93.34
	0.663	41.44	19.06	406.31
	0.992	53.52	26.10	593.62
Regression Equation		y1=37.10x +16.8	y2=21.95x + 4.4	y3=755x - 135
Naphthalene	0.648	20.73	5.54	1.87
	1.305	30.21	7.35	2.17
	1.953	46.15	10.77	3.18
Regression Equation		y1=19.47x + 7.0	y2=4.0x + 2.7	y3=1.0x + 1.1
Phenanthrene	0.466	42.99	6.45	1.95
	0.938	67.61	8.39	2.31
	1.404	84.13	9.77	2.68
Regression Equation		y1=43.88x + 23.8	y2=3.54x + 4.9	y3=0.78x + 1.6
Pyrene	0.411	108.52	34.34	1.89
	0.827	154.53	47.38	2.30
	1.237	207.10	66.91	2.75
Regression Equation		y1=119.3x + 58.3	y2=39.4x + 17.0	y3=1.04x + 1.4

*Bile was diluted to 1200 times
Ex = excitation wavelength, Em = emission wavelength

maximum fluorescence, followed by phenanthrene and naphthalene. These compounds showed minimal fluorescence at 380/425 nm excitation/emission spectra, at which BaP showed maximum fluorescence. The metabolites which fluoresced at the BaP wavelength pair includes 1-hyroxy BaP, 3-hyroxy BaP and hydroxy and dihyrodiol metabolites of pyrene and fluoranthene (Krahn et al. 1986). Therefore, the chances of interference with other compounds are minimal at BaP wavelength pair at which measurements in bile were made in this study.

The current data showed a concentration-dependent elimination of BaP in bile. As the exposure concentration increased, the level of fluorescence in the bile also increased. However, above 2.5-mg kg^{-1} treatment dose, this linearity was not observed. The fluorescence in the bile increased marginally at the 5-mg kg^{-1} dose compared to that at the 2.5-mg kg^{-1} dose. Further increases in the treatment dose brought decreases in elimination (Table 3). This observation suggests interference in the elimination process at higher treatment concentrations. This is possible if phase-2 metabolic reactions are also influenced, like inhibition of the conjugation mechanism, a necessary step in the clearance of aromatic hydrocarbons.

The data from the current study also suggest that clearance was faster in the early days of treatment. Subsequently, it decreased even though a substantial amount of a given compound might still be present in the body. At all the treatment doses, the fluorescence value obtained in bile after 48 h was more than at 72h.

Table 3. Elimination in bile after oral exposure to BaP at varying doses

BaP Exposure mmoles kg^{-1} body weight (x)	Fluorescence in Bile, Equivalent to µmoles BaP ml^{-1}			
	48 h (y1)	Fold Increase	96 h (y2)	Fold Increase
0	0.009±0.003 [a]	1.00	0.009±0.001 [b]	1.00
0.50	0.015±0.008 [a]	1.67	0.010±0.002 [b]	1.11
0.99	0.024±0.008 [a]	2.78	0.013±0.001 [b]	1.33
1.98	0.044±0.025 [a]	5.11	0.019±0.002 [b]	2.11
3.97	0.301±0.052 [b]	33.11	0.194±0.005 [b]	21.56
9.92	2.226±0.012 [b]	246.76	1.125±0.134 [b]	124.89
19.84	2.279±0.073 [b]	253.33	1.251±0.138 [b]	138.89
39.68	ND	ND	1.087±0.276 [c]	119.78
59.52	ND	ND	0.847±0.483 [c]	94.00
99.21	*1.019±0.097 [b]	113.22	0.657±0.373 [c]	73.00
Regression Equation	y1=0.0086x + 0.5929		y2=0.0073x + 0.3475	

Mean values ± SD, a (n = 4), b (n=3), c (n=5); ND=not done.
*Value substituted from another experiment.

This phenomenon was further explored by administering a single dose and following elimination from immediately after exposure until the background level was attained (Fig. 1). There was a substantial increase in the fluorescence in the bile of treated fish even at one day of treatment. The value was 20.2-times higher than the value obtained in control bile. The elimination in the bile increased further to 92.4-times than that in the control bile on the day two of treatment. At that time, the fluorescence in the bile of treated animals was the highest . On the third day, the increase in the fluorescence value over controls in the bile of treated fish dropped to 87-times and on day four to 65.3-times. Further increases in time resulted in low levels of fluorescence in the bile, and on day 16 the fluorescence was only 3-times greater than that in the control bile. On day 32, the mean value in the treated bile was 1.7-times greater than that in the controls. Taking into consideration the standard deviation which overlapped at some points, this may beviewed as the time when fish could eliminate a body burden of as high as the 25-mg kg^{-1} dose level.

The data generated on oral exposure of fish to BaP and subsequent determination of its metabolites were concentration-dependent. Therefore, bile metabolites could be a good indicator of exposure concentrations. The method can be applied in field monitoring as biomarker of pollution due to its simplicity and requirement for less manipulation of samples. The gallbalder bile is easy to collect and store, and unlike other tissues such as liver, bile does not need to be homogenized. It is relatively free of lipids and can be analyzed directly without extraction or cleanup procedures (Hellou and Payne 1987).

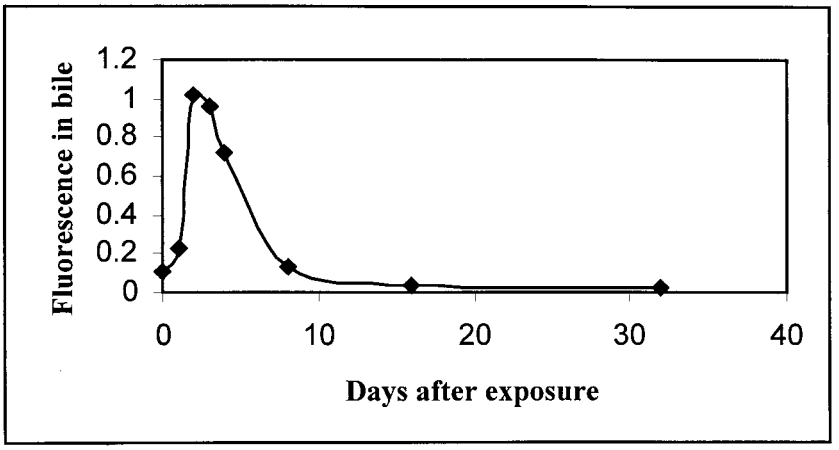

Figure 1. Elimination in bile after a single BaP dose (Fluorescence equivalent to μmole BaP ml^{-1} bile)

The direct fluorescence measurement at fixed excitation/emission wavelength pairs usually does not distinguish PAH metabolites from other compounds because the excitation and emission spectra of different PAHs and other compounds often overlap and may be quite similar. Therefore, direct fluorescence is less PAH-specific than the HPLC with fluorescence detection. However, environmental sampling often requires screening a large number of samples. It would be desirable to use a simpler, less expensive method of assessing biliary PAH concentration than the exhaustive HPLC-fluorescence detection as advocated by Lin et al. (1994).

Krahn et al. (1984) attempted further to simplify the HPLC procedure with fluorescence detection at a fixed wavelength for estimating the relative amount of biliary PAH metabolites in fish without complete separation or identification of individual metabolites. After direct injection of bile to HPLC, the sum of all fluorescent peak areas for a fixed excitation/emission wavelength at 380/430 nm was used to estimate the exposure of fish to PAHs (Krahn et al. 1986a,b). Similar to the sum of peaks determined by HPLC fluorescence detection, direct spectrofluorometry provides total fluorescence by PAH-type metabolites in the current determination, which for all practical purposes indicate the level of exposure to PAHs since at 380/430 nm other compounds like napthalene, phenanthrene and pyrene produce no fluorescence interference. In field samples, it is advisable to measure the fluorescence also at other fixed wavelength pairing in order to detect and differentiate other types of metabolites and determine the type of pollution.

In this study, two end points were monitored in experimentally exposed fish. The first end point examined was the level of BaP in liver tissue. The data suggest a low residue build-up in liver tissue, even at considerably high exposure

concentrations. Also, BaP residue monitoring is complex; therefore, this parameter is not recommended for routine monitoring of environmental status. The second end point examined was the level of PAH-like metabolites in the bile of exposed fish determined spectrofluorometrically by direct dilution of bile at a fixed excitation/emission wavelength (i.e., 380/425 nm). The metabolites of other petroleum-related compounds are detected at different wavelength pairings. The detection of BaP like metabolites in fish bile can, therefore, be used as simple marker to infer exposure to this class of environmentally important chemicals.

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