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AROMATIC HYDROCARBON METABOLITES IN FISH: AUTOMATED EXTRACTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION INTO CONJUGATE AND NON-CONJUGATE FRACTIONS

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SUMMARY

An automated extractor–concentrator was used to extract metabolites of naphthalene, 2,6-dimethylnaphthalene, and benzo[*a*]pyrene from serum, bile and liver homogenate of rainbow trout (*Salmo gairdneri*). The extracts were analyzed by reversed-phase high-performance liquid chromatography (HPLC) with fluorescence detection. Recoveries of naphthalene and 2,6-dimethylnaphthalene metabolites from all matrices were generally greater than 90%; however, the recoveries of benzo[*a*]pyrene metabolites from serum ranged from 37–99%. In addition, conjugated metabolites of polycyclic aromatic hydrocarbons (PAHs) were separated from non-conjugated metabolites and parent PAHs by using two diol columns with normal-phase HPLC. The extraction and separation techniques were also applied to isolate metabolites in samples from fish fed 2,6-dimethylnaphthalene.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs), which are prominent xenobiotics in the marine environment, have been shown to be toxic to marine life[1–4]. PAHs are readily biotransformed and their metabolic products accumulate in tissues of exposed organisms[5–8]. An important requirement for assessing the effect of PAHs and their metabolites on marine organisms is the identification and quantitation of these compounds in the tissues and fluids of exposed organisms. Sometimes, analytes in biological fluids can be quantitated by injecting the fluid directly into a reversed-phase high-performance liquid chromatography (HPLC) system[9]. Usually, analytes must first be separated from their biological matrices before being determined by the conventional techniques of HPLC, gas chromatography (GC), or gas chromatography–mass spectrometry (GC–MS).

Solvent extraction[1,10–12] is used routinely to extract analytes from biological samples, but this method has its limitations. For example, some extraction techniques do not extract polar analytes efficiently[12]. Also, extracts often contain naturally occurring compounds (*e.g.*, lipids) that interfere with the analyses. Moreover, solvent

extraction can be the most laborious and least precise part of the assay[13]. The precision of HPLC assays with prior solvent extraction is generally poorer than that of assays with direct injection of samples (5–10% S.D. *vs.* 1–3%)[13]. However, direct injection is limited to biological fluids introduced into reversed-phase systems, because non-polar normal-phase solvents can precipitate sample components such as serum proteins and bile salts, which can then block the HPLC injector, tubing or column.

Two totally automated systems, column switching[14] and "FAST-LC"[15], allow for effective separation of analytes from biological fluids and rapid analysis by reversed-phase HPLC; however, tissue samples must be extracted and the extracts injected. Alternatively, Williams and Viola[16] developed a rapid and reproducible method for automated extraction of certain drugs from serum using an extractor–concentrator. Their method appeared promising for our work with PAH metabolites, so we adapted it for use with fish serum, bile and liver homogenate.

In addition, when determining PAH metabolites, separation of samples into conjugate and non-conjugate fractions is frequently desired. Non-conjugated metabolites can be determined directly, but the conjugates are often enzymatically hydrolyzed to aid in their analysis. Consequently, we devised a rapid normal-phase chromatographic method for separating conjugated PAH metabolites from non-conjugated metabolites and parent PAHs.

EXPERIMENTAL*

Chemicals

NPH**, DMN and BaP were obtained from Aldrich (Milwaukee, WI, U.S.A.). The NPH metabolite standards were purchased from Sigma (St. Louis, MO, U.S.A.), and the DMN metabolite standards were prepared in our laboratories[7]. The National Cancer Institute (Bethesda, MD, U.S.A.) provided the BaP metabolite standards. Citric acid monohydrate (U.S.P.) was obtained from Mallinckrodt (St. Louis, MO, U.S.A.). Disodium hydrogen phosphate heptahydrate and potassium chloride (both "analyzed-reagent" grade), Ultrex acetic acid, and the HPLC-grade solvents 2-propanol and water were purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). The "distilled-in-glass" solvents, acetone, hexane and methanol, were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Fish exposures

Rainbow trout (*Salmo gairdneri*) were maintained in flow-through freshwater

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* Reference to a company or product does not imply endorsement by the U.S. Department of Commerce to the exclusion of others that may be suitable.

** Abbreviations: Naphthalene (NPH) metabolites: 1-naphthyl-β-D-glucuronic acid, naphthyl glucuronide; 1-naphthyl sulfate, naphthyl sulfate; 2-naphthyl-α-D-glucoside, naphthyl glucoside. 2,6-Dimethylnaphthalene (DMN) metabolites: 2,6-dimethyl-3-naphthol, 3-hydroxyDMN; 2,6-dimethyl-3,4-naphthoquinone, DMN 3,4-quinone; *trans*-3,4-dihydroxy-3,4-dihydro-2,6-dimethylnaphthalene, DMN 3,4-dihydrodiol; and 6-methyl-2-naphthalenemethanol, DMN 2-methanol. Benzo[*a*]pyrene (BaP) metabolites: 9,10-dihydro-9,10-dihydroxybenzo[*a*]pyrene, BaP 9,10-dihydrodiol; 7,8-dihydro-7,8-dihydroxybenzo[*a*]pyrene, BaP 7,8-dihydrodiol; 9-hydroxybenzo[*a*]pyrene, 9-hydroxyBaP; 1-hydroxybenzo[*a*]pyrene, 1-hydroxyBaP; 3-hydroxybenzo[*a*]pyrene, 3-hydroxyBaP; benzo[*a*]pyrene 3-sulfate, sodium salt, BaP 3-sulfate; 1-benzo[*a*]pyrenyl-β-D-glucopyranosiduronic acid, BaP 2-glucuronide.

aquaria. Two fish were each force fed 4 mg of DMN and 20 μCi of ^{14}C-labeled DMN (2,6-dimethylnaphthalene-4-^{14}C; 5.9 mCi/mmole, custom synthesized by Wizard Laboratories, Davis, CA, U.S.A.; at least 99% pure) as described by Collier *et al.*[17]. The DMN was dissolved in 100 μl of ethanol, and the ^{14}C-DMN was dissolved in 50 μl of salmon oil. Two control fish were similarly force fed only the carrier solvents. Twenty-four hours later, the fish were killed and liver, bile and blood samples were collected.

Extraction

Instrumentation. Biological samples were extracted with a Prep I automated extractor–concentrator (DuPont, Wilmington, DE, U.S.A.). Glass microbeads and Type W extraction cartridges packed with a styrene–divinylbenzene copolymer resin were also obtained from DuPont. The extractor–concentrator is a programmable centrifuge, capable of rotating in either direction. During clockwise rotation, the resin cartridge aligns with a waste cup. The prepared biological sample is placed in the cartridge reservoir, and centrifugal force from rotation drives the solution through the bed of resin. The analytes are sorbed by the resin, while water-soluble components elute into the waste cup. When the programmed rotor reverses direction, the outer ring rotates to align the resin cartridge with a sample-recovery cup. An organic solvent then elutes the analytes from the resin into this cup. Detailed operation of this instrument has been described by Williams and Viola[16].

Standard solutions. Solutions of NPH, DMN, BaP and their metabolites were prepared in water by diluting a concentrated methanol solution of each. These water standards were spiked into serum, bile and liver homogenate samples to determine recoveries. The aqueous NPH–DMN stock solution contained (ng/μl): naphthyl sulfate, 15.5; naphthyl glucuronide, 2.35; 1-naphthol, 87.0; NPH, 5.00; DMN dihydrodiol, 17.3; DMN 2-methanol, 3.95; 3-hydroxyDMN, 2.98, DMN quinone, 9.35, and DMN, 4.15. Two dilutions were prepared (40% and 8% of stock concentration). The 40% solution was used to determine recoveries in serum, bile and liver homogenate; the stock solution and the 8% solution were also used with serum. The BaP standard solution contained (ng/μl): BaP 9,10-dihydrodiol, 1.60; BaP 7,8-dihydrodiol, 1.19; 9-hydroxyBaP, 0.23; 1-hydroxyBaP, 0.18; 3-hydroxyBaP, 0.27; BaP 1-glucuronide, 0.36; BaP 3-sulfate, 0.20 and BaP, 0.205.

Buffers. A citrate–phosphate buffer (0.068 *M* citric acid, 0.064 *M* $Na_2HPO_4 \cdot 7H_2O$, pH 3.8) was prepared for extractions of NPH–DMN reference compounds from serum or liver homogenate. A second citrate–phosphate buffer (1.01 *M* citric acid, 0.2 *M* $Na_2HPO_4 \cdot 7H_2O$, pH 2.5) was prepared for extractions of NPH–DMN reference compounds from bile, for extractions of biologically produced metabolites from serum, bile and liver homogenate and for extractions of BaP reference compounds from serum. An internal standard, 2-naphthyl glucoside (5.75 ng/μl), was added to those portions of the buffer used in sample extraction.

Sample preparation. Serum was obtained from whole blood by centrifugation; bile was used without preparation. For liver, the partially thawed sample was cut into small pieces, 1.5% (w/v) KCl soln, was added (1:4, w/v), and the mixture was homogenized for 1 min with a Tekmar Tissumizer (Tekmar, Cincinnati, OH, U.S.A.).

To determine recovery efficiencies, serum (0.50 ml), bile (0.10 ml) or liver homogenate (0.50 ml) was mixed with a PAH standard solution (0.50 ml), and the

mixture was added to a cleaned cartridge (see below). Buffer–internal standard (1.00 ml) was added. Samples from rainbow trout fed DMN were prepared as described above, except that the PAH standard solution was omitted.

Cartridge clean-up and extraction. Acid-rinsed glass microbeads (1 g) were added to the cartridge reservoir for extractions of matrices other than serum to prevent the cartridges from plugging. The cartridges were loaded into the instrument, 20 ml of acetone were added to reservoir 1, and the solvent was dispensed and collected. The instrument was reset, reservoir 1 was filled with 10 ml of buffer (without internal standard), and this solution was dispensed and collected. Cleaned cartridges containing the experimental samples (prepared as described above) were loaded into the instrument, and the wash and extraction solvents (see Table I) were added to reservoirs 1 and 2, respectively. The automated program was then executed up to the drying step, and the instrument was stopped and reset to dispense and collect the solvent (Table I) from reservoir 2. This extra extraction step was executed once for samples with spiked reference standards and three times for samples with metabolites produced *in vivo*. After the extraction steps were completed, the total extracts were evaporated at 30°C, under nitrogen, until *ca.* 0.7 ml remained (times of evaporation varied according to the total amounts and types of solvents used for extraction). The entire concentrated extract was transferred to a 1-ml volumetric flask, the volume was adjusted to 1.00 ml, and the solution was analyzed. Twelve samples could be extracted simultaneously, and the total instrument time for the entire procedure was less than 30 min.

TABLE I

WASH AND EXTRACTION SOLVENTS USED FOR AUTOMATED EXTRACTION OF PAH METABOLITES FROM RAINBOW-TROUT SERUM, BILE AND LIVER HOMOGENATE USING THE EXTRACTOR–CONCENTRATOR FOR PROCESSING 12 SAMPLES

Analyte	*Solvents*, ml*				
	Wash (reservoir 1)	*Extraction (reservoir 2)*			
		1	*2*	*3*	*4*
Spiked reference standards	10 W**	10 A***	10 M‡	—	—
DMN metabolites formed *in vivo*	10 W**	10 A/M	10 A/M	10 Q	10 Q

* W = water; A = acetone; M = methanol; A/M = acetone–methanol (1:1, v/v); Q = dichloromethane–2-propanol–water (75:25:2, v/v).

** No wash for bile.

*** 20 A for liver homogenate.

‡ 20 M for liver homogenate.

High-performance liquid chromatography

Instrumentation

Separations were performed with an 8000B high-performance liquid chromatograph (Spectra-Physics, Santa Clara, CA, U.S.A.). An MPF-44A fluorescence spec-

trometer (Perkin-Elmer, Norwalk, CT, U.S.A.) was used for detection and characterization. A UV detector, Spectra-Physics Model 8310, was connected in series with the fluorescence detector and was used to detect compounds with low fluorescence. Samples were injected by using a 710A variable-volume automatic sampler (Waters Assoc., Milford, MA, U.S.A.).

Normal-phase conjugate–non-conjugate separations

Column configuration. Two normal-phase diol columns (Brownlee, Santa Clara, CA, U.S.A.), a 0.46 × 4-cm OH-GU diol guard column and a 0.46 × 10-cm OH-MP diol analytical column, were connected to a six-port valve (see Fig. 1). With

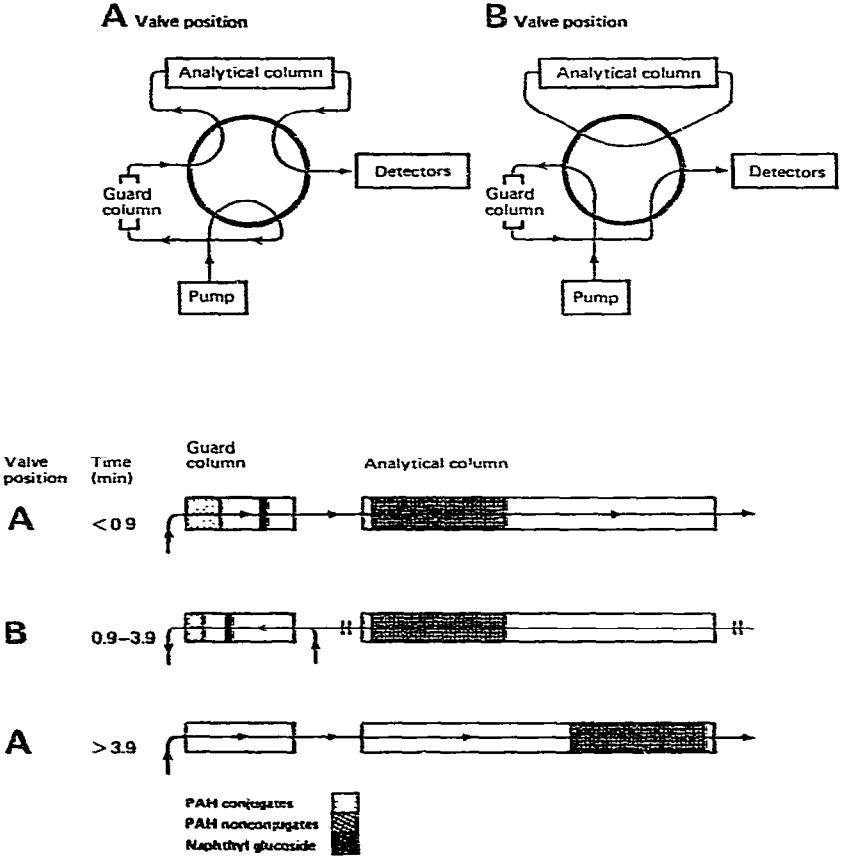

Fig. 1. Top: Connections of diol guard column, diol analytical column, pump and detectors to the six ports of the two-way valve; the flow-through system is shown for both valve positions A and B. Bottom: Flow through the two-colomn system as a function of valve position. At injection, with the valve in position A, eluent flowed through the guard column and the analytical column to the detectors. Conjugated PAH metabolites moved slowly through the guard column; non-conjugated metabolites and parent PAH moved quickly through the guard column and on to the analytical column. At 0.9 min, the valve was changed to position B, trapping the non-conjugated metabolites and parent PAHs on the analytical column and reversing the flow through the guard column so that the conjugated metabolites were eluted. At about 3.9 min, the valve was returned to position A to elute the non-conjugated metabolites and parent PAHs from the analytical column.

the valve in position A, the eluent traveled from the pump through the guard and analytical columns to the detectors. When the valve was moved to position B, the eluent traveled from the pump through the guard column in the reverse direction and then to the detectors. The analytical column was isolated with the valve in position B, and materials on this column remained there until the valve was shifted back to position A.

Standard solutions. The stock solutions of NPH–DMN and BaP prepared for the automated extractions were used to calibrate the normal-phase system for conjugate–non-conjugate separations.

Elution conditions. Conjugated and non-conjugated PAH metabolites were separated by using an eluent consisting of hexane–2-propanol (1:1, v/v). The flow-rate was 1 ml/min and the oven temperature was 40°C. The valve position remained at A until 0.9 min after injection, then it was shifted to B; at 3.9 min, the valve position was returned to A.

Fraction collection of serum extracts. Serum extracts from fish fed DMN were separated into two fractions; the first contained conjugated PAH metabolites and the second contained the non-conjugated metabolites and parent PAHs. The conjugated-metabolite fraction was collected while the six-port valve was in position B. The PAH parent compounds and non-conjugated metabolites were collected after the valve position was returned to A (see Fig. 2).

Reversed-phase analyses

Columns. The analytical column was a 0.26 × 25-cm reversed-phase HC-ODS column (Perkin-Elmer). A 0.21 × 5-cm stainless-steel guard column dry-packed with Vydac 37-μm reversed-phase packing (The Separations Group, Hesperia, CA, U.S.A.) was also used.

Gradient-elution separation of NPH–DMN metabolites. Acetic acid–water (0.5:100, v/v; solvent A) and methanol (solvent B) were used in a linear gradient going from 100% solvent A to 100% solvent B in 15 min, then 7 min at 100% B, 3 min to return to 100% A, and 8 min equilibration at 100% A. The flow-rate was 1 ml/min except during equilibration, when it was increased to 2 ml/min. The oven temperature was 50°C. BaP metabolites were separated as reported previously[18].

Quantitations. In chromatograms of analytes recovered from extractions of "spiked" serum, bile and liver-homogenate samples, peak areas were compared with those of chromatographed reference standards. A factor to correct for dilution was applied, and percentage recoveries were calculated. Peak areas of analytes in bile were corrected for contributions from interfering compounds. Responses of the standards were linear in the concentration ranges used.

RESULTS AND DISCUSSION

Automated extractions

Extractions of PAH metabolites from biological samples using the automated extractor–concentrator were rapid and reproducible. Previous automated extraction methods had been limited to biological fluids[14–16], but our adaptation of the extractor–concentrator method allowed extraction of both tissues and fluids. Analytes were separated from the biological matrices by chromatography on resin-packed

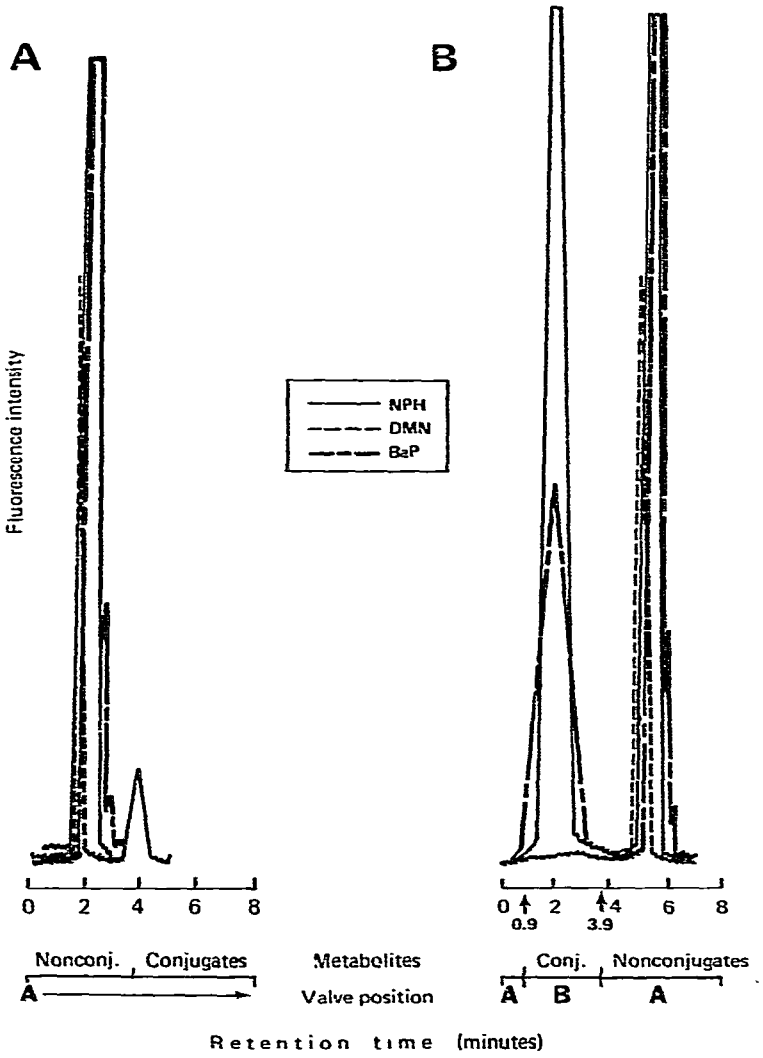

Fig. 2. Normal-phase HPLC chromatograms of NPH, DMN, BaP and their metabolites chromatographed on the configuration of diol columns shown in Fig. 1. The mobile phase was hexane-2-propanol (1:1). A: Valve in position A for the entire chromatogram. Among the conjugated metabolites, only naphthyl glucoside was eluted; the remainder were strongly sorbed to the guard column. B: Valve moved from A to B to A as shown in Fig. 1. Conjugated metabolites were eluted from 0.9–3.9 min and non-conjugates and parent PAHs after 3.9 min.

cartridges. This procedure allowed the processing of 12 samples in 30 min and replaced laborious conventional extraction schemes[1,11,12].

The extractor–concentrator techniques were adapted to various matrices and analytes by adjusting the operating parameters (*e.g.*, extraction time, centrifugation speed, drying time and drying temperature). In addition, other modifications to the original serum extraction technique[16] were made. For example, glass microbeads

were added to the cartridges to prevent plugging during liver and bile extractions. Also, extraction solvents were chosen to efficiently extract PAH metabolites from the resin. Originally, buffer had been added to the serum samples to aid retention of drugs by the resin in the cartridges[16]. However, in our applications, lowering the pH and increasing the ionic strength (creating a "salting-out" effect) of the buffer were necessary to optimize recoveries of NPH and DMN compounds from bile and of BaP compounds from serum.

Fish serum, bile and liver samples spiked with reference compounds were used to develop the automated extraction techniques, and metabolites of NPH and DMN were recovered efficiently and reproducibly (Table II). However, recoveries of some BaP metabolites from spiked serum were lower than from water (Table III) and were lower than recoveries of DMN and NPH metabolites from either serum or water (Table II). Some of the BaP and its metabolites remained in the water phase and were carried to waste, as confirmed by analysis of the waste-cup contents. By adjusting the pH and ionic strength of the buffer, the recoveries were improved, but the adjustments did not entirely prevent the losses to waste.

Aqueous solutions of the reference compounds were used to avoid quantities of organic solvents that could adversely affect sorption of the analytes by the resin bed, but the concentrations of some reference compounds were not stable in aqueous solution. After overnight storage at 5°C, the concentrations of DMN, BaP and the

TABLE II

RECOVERIES OF NPH, DMN AND THEIR METABOLITES FROM RAINBOW-TROUT SERUM, BILE AND LIVER HOMOGENATE USING AN AUTOMATED EXTRACTOR–CONCENTRATOR

Each recovery (± standard deviation) is the mean of three extractions each on two days ($n = 6$). See Experimental for concentrations. NR = not recovered; E = recovery erratic (see Results and Discussion).

Compound	*Percentage recovery of spiked compound*					
	Aqueous standard	*Serum*			*Bile*	*Liver homogenate*
Percentage of stock	*40%*	*8%*	*40%**	*100% (stock)*	*40%*	*40%*
Naphthyl glucuronide**	91 ± 3.2	83 ± 3.1	90 ± 3.4	90 ± 4.9	87 ± 3.7	90 ± 2.6
Naphthyl sulfate**	103 ± 2.9	96 ± 4.6	98 ± 4.7	97 ± 1.7	88 ± 3.9	82 ± 2.1
DMN dihydrodiol***	104 ± 2.1	99 ± 6.0	103 ± 2.3	101 ± 5.8	I‡	102 ± 3.1
1-Naphthol***	94 ± 3.6	100 ± 2.6	96 ± 6.5	97 ± 5.0	97 ± 4.3	99 ± 4.1
DMN quinone***	97 ± 0.6	NR	NR	NR	NR	NR
DMN 2-methanol**	101 ± 3.6	102 ± 2.6	103 ± 4.3	100 ± 2.6	102 ± 5.5‡‡	101 ± 3.1
3-Hydroxy DMN**	97 ± 1.0	102 ± 2.9	100 ± 3.2	95 ± 3.8	100 ± 1.8‡‡	96 ± 3.7
Naphthalene**	56 ± 3.5	106 ± 4.0	103 ± 3.1	90 ± 6.9	83 ± 4.0‡‡	E
DMN**	65 ± 8.9	E	E	E	91 ± 4.7‡‡	E
Internal standard (naphthyl glucoside)**	91 ± 6.6	98 ± 4.6	91 ± 3.7	94 ± 5.1	92 ± 5.8	89 ± 2.6

* ($n = 5$) because a cartridge failed.

** Quantitation by fluorescence at ex 290 nm, em 335 nm.

*** Quantitation by UV at 254 nm.

‡ Interfering compounds from bile matrix made quantitation impossible at the concentration used.

‡‡ Corrected for contributions to peak areas from bile matrix.

TABLE III

PERCENTAGE RECOVERIES OF BENZO[*a*]PYRENE AND METABOLITES FROM RAINBOW-TROUT SERUM USING AN AUTOMATED EXTRACTOR–CONCENTRATOR

The compounds were determined by fluorimetry at 430 nm, with excitation at 380 nm. Each recovery (± standard deviation) is the mean of three extractions ($n = 3$). See Experimental for concentrations.

Compound	*Aqueous standard**	*Serum***
BaP 1-glucuronide	83 ± 1.5	66 ± 4.9
BaP 3-sulfate	94 ± 2.3	66 ± 9.5
BaP 9,10-dihydrodiol	94 ± 3.2	98 ± 5.1
BaP 7,8-dihydrodiol	100 ± 5.6	99 ± 6.8
9-HydroxyBaP	73 ± 16.5	49 ± 9.5
1-HydroxyBaP	61 ± 15.3	37 ± 2.6
3-HydroxyBaP	59 ± 7.1	41 ± 2.6
BaP	80 ± 3.6	62 ± 5.9

* Concentrations of some BaP reference compounds are unstable in aqueous solution (see Results and discussion).

** BaP and some of the metabolites appear in the waste cup (see Results and discussion).

hydroxyBaPs decreased 30, 20 and 70–80%, respectively. Consequently, even when freshly prepared solutions were used, recoveries calculated for these compounds were erratic.

To determine if metabolites formed *in vivo* could be extracted as efficiently as spiked metabolites by our techniques, fish were fed radiolabeled DMN. Recoveries of total radioactivity from liver and serum were low when the extraction conditions were identical to those used to extract the spiked samples (Table I, extraction with acetone and with methanol). However, recoveries were excellent (Table IV) when the method was modified to use four extractions with solvents similar to those used for conventional tissue extractions[11] (Table I). When this modified technique was used to extract spiked biological samples, the recoveries were equal to or greater than those reported for two-solvent extractions in Table II.

TABLE IV

RECOVERIES OF TOTAL RADIOACTIVITY FROM [^{14}C]DMN AND METABOLITES EXTRACTED FROM RAINBOW-TROUT SERUM, BILE AND LIVER USING THE EXTRACTOR–CONCENTRATOR: ANALYSIS OF THE ORGANIC EXTRACTS, WASH SOLUTION, AND CARTRIDGE CONTENTS BY LIQUID SCINTILLATION COUNTING

Each recovery (± standard deviation) is the mean of three extractions ($n = 3$).

Matrix	*Percentage of radioactivity recovered in:*			*Total recovery*
	Organic extract	*Wash*	*Cartridge**	
Serum	102.9 ± 4.7	1.1 ± 0.2	4.6 ± 1.6	108.6 ± 3.2
Bile	102.8 ± 1.5	1.7 ± 1.1	0.1 ± 0	104.6 ± 2.1
Liver homogenate	94.8 ± 1.7	1.6 ± 0.1	3.6 ± 0.2	100.0 ± 2.0

* Cartridge resin, glass microbeads and liver residue.

Separations of conjugated from non-conjugated PAH metabolites

Conjugated PAH metabolites were readily separated from non-conjugates by normal-phase HPLC on diol columns (Fig. 2). The two-column system allowed for a more rapid analysis than a previous separation on alumina[19]. and the analytes were recovered in a small volume of solvent. Naphthyl glucoside, the only conjugated metabolite among the reference standards not retained by the guard column, was

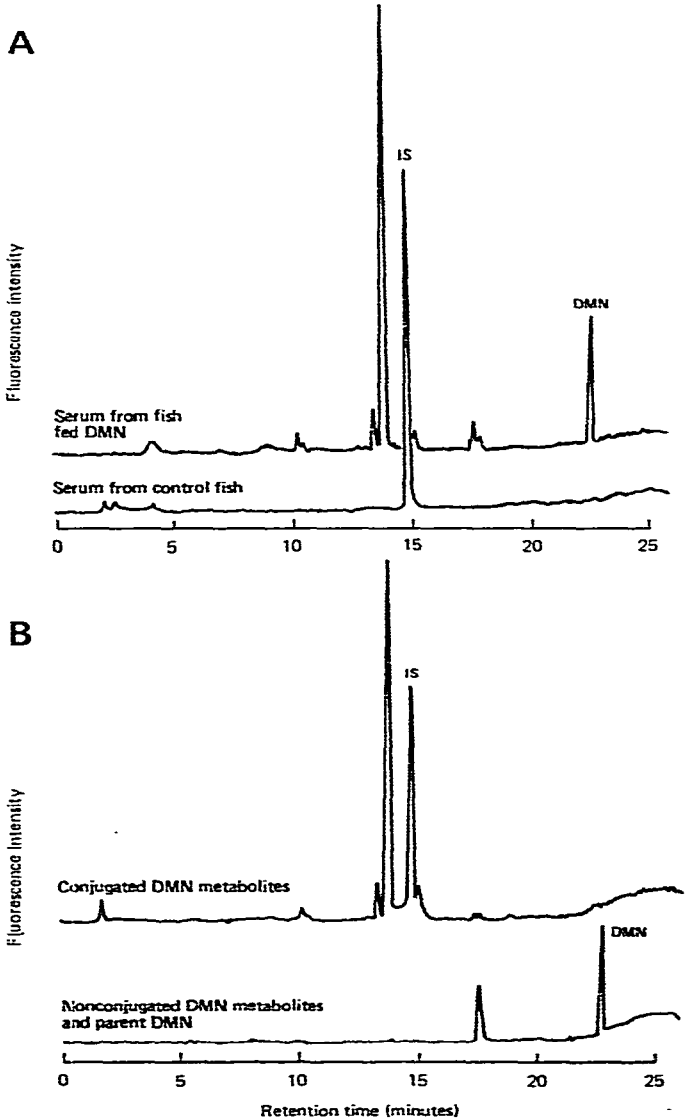

Fig. 3. A: Reversed-phase HPLC–fluorescence chromatograms (excitation at 290 nm; measurement at 335 nm) of serum from fish fed DMN and from control fish. Extracts were prepared with an automated extractor–concentrator. Injection represented extracts from *ca.* 14 mg serum (wet weight). B: Reversed-phase HPLC chromatograms of conjugated and non-conjugated metabolite fractions from extracts of serum of fish fed DMN. The extracts were separated into fractions using the normal-phase HPLC techniques shown in Fig. 1. See Experimental for HPLC conditions.

prevented from eluting with the non-conjugated metabolites by reversing the direction of eluent flow through the guard column after 0.9 min (Fig. 1). The reversal of flow did not appear to degrade the guard column, but, should this occur, it is inexpensive to replace.

This separation technique is an important step in preparing the experimental sample for HPLC analysis of the non-conjugate fraction or for additional procedures to determine the conjugates. For example, enzymatic hydrolyses could be carried out on the conjugate fraction, and the resulting liberated aromatic compounds analyzed by GC or HPLC to identify and quantitate the resulting conjugated metabolites.

Applications to samples from fish fed non-radiolabeled DMN

Serum, bile and liver homogenate from fish fed non-radiolabeled DMN were extracted using the extractor–concentrator, and the extracts were analyzed by reversed-phase HPLC. Extracts of serum (Fig. 3A), bile and liver gave similar HPLC profiles, but the concentrations of metabolites varied; bile had the highest concentration and liver the lowest. From HPLC retention times, most of the metabolites appeared to be conjugates. Similar findings were reported by Gruger *et al.*[7] for metabolites of DMN in bile of starry flounder (*Platichthys stellatus*) fed radiolabeled DMN.

Separation of the conjugated DMN metabolites in serum extracts from DMN and its non-conjugated metabolites was carried out by the normal-phase HPLC technique. Fractions were collected and concentrated, and the resulting solutions were analyzed by reversed-phase HPLC (Fig. 3B). As predicted from the HPLC retention times, most of the DMN metabolites were found in the conjugate fraction. In future research, we plan to hydrolyze this fraction enzymatically and then identify the liberated aromatic compounds by HPLC or GC–MS.

CONCLUSIONS

NPH and DMN metabolites were extracted efficiently and reproducibly from serum, bile and liver homogenate using the commercially available extractor–concentrator. A simple modification of the extraction procedure was necessary to recover DMN metabolites produced *in vivo* as efficiently as those spiked into biological samples. However, extraction efficiencies for BaP metabolites varied widely, even after modifications in the method were made. Extracts were suitable for reversed-phase HPLC analysis of individual components and for normal-phase HPLC separation into conjugate and non-conjugate fractions. Ultimately, these methods are intended for determination of xenobiotics and their metabolites in aquatic organisms exposed via their natural environment.

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