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Method for Extraction, Isolation, and Detection of Free Polybrominated Biphenyls (PBBs) from Plasma, Feces, Milk, and Bile Using Disposable Glassware

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A rapid method was developed for the extraction, isolation, and detection of polybrominated biphenyls (PBBs) from plasma, feces, milk, and bile, using disposable glassware. Use of disposable equipment greatly reduced the amount of laboratory background and cross-contamination of samples. The procedure employed a multiple extraction with a mixture of diethyl and petroleum ethers, followed by cleanup on miniature Florisil, silica gel, and sodium sulfate columns. Detection was accomplished by gas chromatography. Recoveries were determined for the six major components of a commercial PBB mixture and were approximately 96% for plasma, 59% for feces, and 98% for milk. The background levels for plasma, feces, and milk were 0.0005, 0.0007, and 0.0007 ppm, respectively, bringing the minimum detectable limits of the major hexabromobiphenyl peak to 0.0010, 0.0014, and 0.0014 ppm on a whole tissue basis.

Polybrominated biphenyls (PBBs) were manufactured for use as a fire retardant from 1970 to 1973 by the Michigan Chemical Corporation (St. Louis, Mich.) and marketed both as fireMaster BP-6 and as fireMaster FF-1. In the summer of 1973, fireMaster FF-1 was accidentally mixed with dairy feed in place of the supplement magnesium oxide, or Nutrimaster, resulting in the contamination of about 30 Michigan dairy farms (Whitehead, 1975). Many animals which had ingested this contaminated feed attained PBB levels as high as 300 ppm on a fat basis (Jackson and Halbert, 1974). After the original contamination the PBB compound persisted in feed manufacturing, storage, and handling equipment. As other feed passed through the mills and was cross-contaminated, many more farms acquired relatively low levels of PBB contamination (Dunckel, 1975). Regulatory agencies have destroyed vast numbers of livestock and food products because of contamination, thus eliminating highly contaminated animals and products from the food chain. The present Food and Drug Administration guideline tolerance levels of PBB are 0.3 ppm in milk and meat on a fat basis and 0.05 ppm in whole eggs (Dunckel, 1975). These levels are largely based on the degree of sensitivity of available methods of analysis for the compound (Kolbye, 1975). PBB levels below tolerance have been detected in food products. Legislation in Michigan has been initiated to further reduce the tolerance to 0.02 ppm. Since these guideline levels are low, it will be necessary to have an extraction method that is rapid, reliable, sensitive, and relatively free of background contamination to accurately quantitate low levels of PBB.

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OBJECTIVE

The extraction, isolation, and detection method described here was developed during a controlled study of the toxicity in pregnant Holstein heifers which were fed fireMaster BP-6 at doses equivalent to 0 to 5000 ppm in their rations. Twenty-four animals were under observation with frequent sampling of blood, feces, and milk, resulting in a vast number of samples to be analyzed. A method for the analysis of these samples had to be developed that was both rapid and reliable throughout the wide range of concentrations.

Use of the standard method for the extraction and cleanup of halogenated hydrocarbons (USDHEW, 1971) was impractical and unusable because of the time and large amount of glassware necessary for each sample. Once samples containing high levels of PBB were passed through the glassware, it could no longer be used in the extraction and cleanup of low level samples. Control of background was impossible despite exhaustive cleaning and decontamination procedures. Methods which had proven to be effective for decontamination of PCBs in this laboratory were not effective for PBBs. It was apparent that all glassware needed to be disposable to prevent high background and cross-contamination.

METHOD

A method was developed for the extraction, isolation, and quantitation of PBBs from plasma, feces, milk and bile. All solvents were reagent grade and glass redistilled. Solvents concentrated 90% produced no gas chroma-

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Table I. Amounts of Sample and Reagent Appropriate to Each Procedure

	Plasma	Feces	Bile	Milk
Sample size, g	2.0	2.0	2.0	3.0
Ethanol, mL	1.0	0	1.0	3.0
Florisil column length, cm	6.0	1.5	1.5	6.0
Silica gel column length, cm	0	3.5	3.5	0
Na_2SO_4 column length, cm	1.0	1.0	1.0	1.0
Elution collected, mL	5	10	10	5

tography (GC) interference; reagents washed with petroleum ether (PE), concentrated 90%, produced no GC interference. All glassware was rinsed with acetone and PE prior to use.

Extraction. A sample of material was weighed or measured into a labeled 16 × 125 mm disposable glass test tube (Table I). Except in the case of feces samples, ethanol was mixed with the sample to denature proteins and decrease emulsion formation. Then 3 mL of a mixture of 1:1 petroleum ether–diethyl ether (PE:DE) was added to the test tube. Each sample was agitated vigorously on a Vortex mixer for 1 min and centrifuged in a Sorvall GLC-2 centrifuge at 2200 rpm (radius 6.47 cm) for 3 min. The ether extract was aspirated with a labeled disposable Pasteur pipet and placed in a labeled 18 × 150 mm disposable test tube. The transfer pipet was rinsed into the extract with 1:1 PE:DE. The extract was evaporated to dryness by shaking under vacuum in an evapo-mix (Buchler Inst., Fort Lee, N.J.) at room temperature. Three milliliters of 1:1 PE:DE was again added to each sample, and the extraction process was repeated two times so that a total of three extracts was evaporated. In the extraction of each bile and milk sample the large extract tube was preweighed to determine the amount of substance considered to be lipid that had been extracted from the sample. It was necessary to add several drops of acetone to the tube and heat (≃ 50 °C) during the final drying step in the evapo-mix, in order to remove water and solvent for accurate weighing of extract.

Isolation. Chromatography columns were packed in 23-cm disposable Pasteur pipets by first placing a small Pyrex wool plug in the bottom of the pipet and then tightly packing in the following order: Florisil (Magnesium silicate activated at 1250 °F, 60–100 PR, Sigma Chem. Co., St. Louis, Mo.), silica gel (SilicAR CC-7, Mallinckrodt, Inc., St. Louis, Mo.), and anhydrous sodium sulfate in appropriate amounts (Table I), all of which had been stored in a drying oven at 110 °C. In the event that pigment and lipid material passed through the column, it was sometimes necessary to adjust the amount of silica gel and Florisil in the column accordingly. Prior to loading of samples, all columns were washed with a minimum of 5 mL of PE and kept wet at all times. Following evaporation of solvents, the sample was dissolved in approximately 2 mL of 2% benzene in PE (BPE). The diluted extract was then loaded onto a column with the transfer pipet. The extract test tube and transfer pipet were rinsed onto the column with 2% BPE. The column was eluted with 2% BPE until the appropriate amount of solvent had been collected (Table I). The column eluate was collected in a clean, labeled, disposable screw-top glass vial with a metal cap liner. The solvent was evaporated under a stream of air that had been passed through a drying column of calcium sulfate. Slight changes were made in this procedure for extracting milk because of the large lipid content. The milk extract was diluted to a specific volume such that the lipid concentration was no greater than 15 mg/mL. Only 1 mL of this dilution was placed on a column for elution.

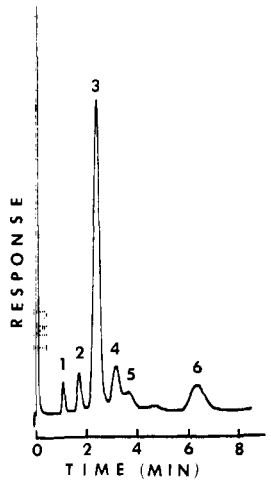

Figure 1. Retention times and relative sizes of peaks representing six components of the PBB mixture.

Detection. Sample extractions were analyzed for free PBB residue concentration on a Hewlett Packard 5736A gas chromatograph with two Ni^{63} electron-capture detectors. The injection ports and detectors were maintained at 300 °C, and the oven or column temperature was a constant 260 °C. The columns were 0.5 m long, 3 mm in diameter, packed with a 5% solution of OV-17 on 80/100 mesh Gas-Chrom Q, and silanized with Silyl-8 (Pierce Chem. Co., Rockford, Ill.). The carrier gas and purge was 5% methane:95% argon at a pressure of 30 lb/in.^{2} and a flow rate of approximately 120 mL/min. Equal volumes of standards and samples were injected, and both were altered when necessary to increase detection sensitivity.

Samples were quantitated by a Hewlett Packard 3380A integrator using an external standard program. By using a multiplication factor which adjusted for amount of sample extracted and dilution of the sample, the integrator output was reported in ppm of PBB in the sample on a whole tissue basis with four significant figures. Integration was carried out for six different peaks, each representing a different component of the PBB mixture. The retention times and relative sizes of each peak are illustrated in Figure 1. Samples were diluted in 2% BPE to volumes appropriate (0.5–10.0 mL) to maximize the GC response to the major hexa-peak, peak 3, and remain within the linear range of the detector. Sensitivity for the other five peaks was not optimized because peak 3 would have then been outside of the range of linearity. Periodically the detectors were thermally cleaned by raising the detector temperature to 330 °C for 24–48 h. This was successful in decreasing noise at low attenuations.

DISCUSSION

Analysis. The PBB compound used for standards and for dosing animals was fireMaster BP-6 (lot 6244A). The composition of fireMaster BP-6 as reported by the manufacturer is 2% tetrabromo, 11–12% pentabromo, 62% hexabromo, 13–14% heptabromo, 1% octabromo, and 1% other brominated biphenyls. Six major peaks account for 98% of the product, and although the quantity of each component varies somewhat with each batch, the third peak is largest and the sixth is second largest (Jacobs et al., 1976). By comparing lot 6244A to semipurified standards of individual brominated biphenyls (isomers not identified) by GC analysis, Willett and Irving (1976) identified the peaks in Figure 1 as follows: peak 1, tetrabromo; peak 2, pentabromo; peaks 3, 4, and 5, hexabromo; and peak 6, heptabromobiphenyl. More recent

Table II. Background Levels (in ppm) and Coefficient of Variation (C.V.) for Each Material Analyzed

	Peak (Figure 1)											
	1		2		3		4		5		6	
Tissue	ppm	C.V.	ppm	C.V.	ppm	C.V.	ppm	C.V.	ppm	C.V.	ppm	C.V.
Plasma	0.0051	104	0.0020	103	0.0005	75	0.0008	130	0.0039	164	0.0015	81
Feces	0.0047	41	0.0015	121	0.0007	53	0.0001	149	0.0007	91	0.0002	95
Milk	0.0092	72	0.0054	143	0.0007	51	0.0015	67	0.0062	136	0.0018	68
Bile[a]	0.0017		0.0004		0.0004		0.0002		0.0004		0.0006	

[a] Information limited, C.V. not meaningful.

Table III. Percent Recoveries (%) and Coefficients of Variation (C.V.) for Plasma

	Peak (Figure 1)												
	1		2		3		4		5		6		
Concentration, ppm	%	C.V.	%	C.V.	%	C.V.	%	C.V.	%	C.V.	%	C.V.	Mean % of six peaks
0.05	73	53	86	14	110	8	99	15	97	16	104	10	95
0.1	86	12	87	8	109	2	98	4	97	5	111	3	98
0.2	84	7	84	5	108	5	95	6	101	8	110	6	97
0.5	79	13	86	6	106	5	95	3	100	4	107	2	95
5.0[a]	76		106		95		93		101		98		95
10.0[a]	80		107		91		94		98		95		94
50.0[a]	69		114		95		100		113		105		99
Overall mean recovery 0.05-50.0	78		96		102		96		101		105		96

[a] Information limited, C.V. not meaningful.

Table IV. Percent Recoveries (%) and Coefficients of Variation (C.V.) for Feces

	Peak (Figure 1)												
	1		2		3		4		5		6		
Concentration, ppm	%	C.V.	%	C.V.	%	C.V.	%	C.V.	%	C.V.	%	C.V.	Mean % of six peaks
0.05	74	39	56	16	68	15	70	15	65	16	70	27	67
0.1	55	13	62	19	63	25	59	25	54	25	48	29	57
0.2	45	7	47	9	54	6	52	10	48	16	48	17	49
0.5	49	15	55	11	60	14	58	14	56	21	58	15	56
1.0	59	16	62	12	67	12	64	9	65	11	69	12	64
5.0	57	11	68	22	59	11	61	15	60	12	58	8	60
10.0	60	22	71	26	63	26	64	27	68	34	65	25	65
50.0	49	15	61	26	51	15	52	20	51	15	49	6	52
Overall mean recovery 0.05-50.0	56		60		61		60		58		58		59

research using the more sophisticated procedures of gas chromatography combined with mass spectrometry has shown peaks 1 and 2 to be pentabromo, peaks 3, 4, and 5 hexabromo, and peak 6 heptabromobiphenyl (Jacobs, et al., 1976). Most routine analyses of PBB levels in animal tissues or products have been reported based on analysis of only the major hexa-peak, peak 3 (Willett and Irving, 1976; Fries and Marrow, 1975; Fehringer, 1975; Gutenmann and Lisk, 1975). Recovery values for the method described here were calculated for all of the six major peaks.

Samples spiked with known concentrations of PBB, samples known to have no PBB, and glassware blanks were frequently run through the procedure to ascertain what the background and percent recovery levels were for each type of material analyzed. Samples with 0.0 ppm added PBB were used to calculate the background levels. Glassware blanks served to monitor any possible contamination. Spiked samples were used to calculate a standard curve of recovery over a predetermined range of concentrations.

Results. Background levels for each of the four materials analyzed are presented in Table II. The maximum background level for the major hexa peak, peak 3, was 0.0007 ppm, bringing the minimum detectable limit of the procedure, defined as twice the background level, to 0.0014 ppm for that peak on a whole tissue basis. The minimum detectable limits of the other five peaks can be similarly calculated. It was noted that background levels were not significantly altered by varying the amounts of Florisil or silica gel in the columns used for cleanup.

Mean percent recovery values and coefficients of variation for each of the six peaks of fireMaster at each concentration tested are presented for plasma (Table III), feces (Table IV), milk (Table V), and bile (Table VI). Also presented in these tables are the mean percent recoveries of all concentrations for each peak and for each concentration as an average of all six peaks.

The coefficients of variation of these recoveries were low. Generally, the coefficients of variation decreased as the concentration of PBBs increased. It is likely that the error represented by this variability is in part a summation of the human error involved in the mixing of standard solutions, the spiking of samples with known quantities of PBBs, the dilution of extracts for analysis, and the injection of samples on the GC. The coefficients of variation for individual peaks was smallest for peak 3 in the analysis of all samples. Gas chromatography conditions were optimized for peak 3, making the error of the GC procedure less for peak 3 than the other five peaks. For each material extracted, the percent recoveries at each con-

Table V. Percent Recoveries (%) and Coefficients of Variation (C.V.) for Milk

Concentration, ppm	Peak (Figure 1) 1 %	1 C.V.	2 %	2 C.V.	3 %	3 C.V.	4 %	4 C.V.	5 %	5 C.V.	6 %	6 C.V.	Mean % of six peaks
0.05	76	56	79	20	101	12	84	12	78	19	101	12	87
0.1	81	62	105	51	107	24	98	32	88	43	91	36	95
0.2	77	39	93	22	110	8	99	16	102	15	112	10	99
0.5	90	22	96	14	111	7	101	14	108	18	116	9	104
1.0	92	9	100	7	114	7	109	3	104	7	106	14	104
5.0	81	3	99	8	106	4	102	4	98	9	101	11	98
Overall mean recovery 0.05-5.0	83		95		108		99		96		105		98

Table VI. Percent Recoveries for Bile[a]

Concentration, ppm	Peak (Figure 1) 1	2	3	4	5	6	Mean % of six peaks
0.05	72	80	94	87	77	93	84
0.5	84	95	94	94	94	97	93
5.0	83	93	87	84	92	94	89
Overall mean recovery 0.5-5.0	80	89	92	89	88	95	89

[a] Information limited, coefficients of variation not meaningful.

centration were not very different from each other. The range of standard errors of recoveries included the mean recovery at each concentration. Therefore, the overall mean recovery was a good single representative value for the recovery of each peak (Tables III–VI).

The recovery of peaks 3 and 6 was consistently over 100% in the analysis of plasma and milk. However, the mean recovery of all six peaks for each concentration was less than 100%. Peaks 3 and 6 represented the majority of fireMaster BP-6, totalling approximately 71% of the analyzed mixture. This percentage was consistent in GC standards of spiking material and extracts of spiked samples. It was concluded that no differential extraction or elution of the biphenyls had occurred. The information also suggested that no degradation or transformation of the biphenyls had taken place.

Evaluation. The procedure described for the extraction, isolation, and cleanup of PBBs from various samples is useful for a rapid screening of PBBs in suspect samples and for the comparison of the levels of PBBs in samples from animals with different exposure to PBBs under experimental conditions. The main advantage is the very minimal background level achieved by using disposable glassware. It is also useful in that many samples can be handled at one time. Recovery is both linear and repeatable. The experimental work for which this procedure was designed did not require that a routine method for verification of the identity of the residue as PBB be developed. All samples were collected from animals known to have received a given amount of fireMaster BP-6 over a given period of time and known to have been kept under environmental conditions whereby no other halogenated hydrocarbons were likely to have been ingested. Several of the samples of this study were verified by the use of alternate GC columns. Under nonexperimental conditions where the amount of PBB in a sample is being analyzed, and where the probability of unknown contaminants is likely, it is necessary to have a method for identification of the residue as well (Burke, 1976). Appropriate selection from several procedures may confirm the identity of the residue as PBB. Some of these procedures are photolytic degradation (Ruzo et al., 1976; Erney, 1975), alternate gas chromatographic column, halogen selective gas chromatographic detectors, partitioning between a series of solvent pairs (p values), and mass spectrometry both alone and combined with gas chromatography (Burke, 1976). It would be possible to use the same extract for all of these verification procedures.

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