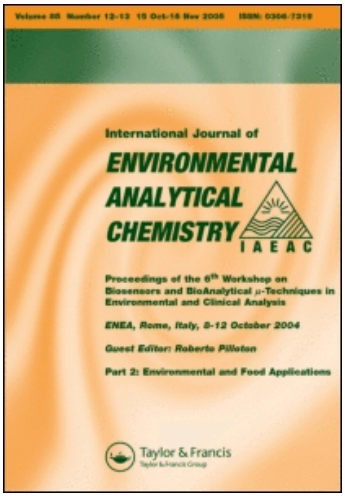

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The Determination of Polychlorinated Biphenyl in Small Samples of Monkey Milk and Tissues. I.

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The feasibility of analysing $\leqq 5$ ml blood and 1 ml monkey milk samples for polychlorinated biphenyls (PCBs) was tested by fortification of similar size human blood and milk samples with Aroclor 1260 at the 1, 5, 10 and 10 ng/g level, respectively. Recoveries were 71, 82 and 89% for blood and 95% for milk. Recoveries of >90% were obtained, when 100 mg samples of monkey liver, kidney and adipose tissue were fortified with Aroclor 1254 at the 0.2, 0.5 and 1 μg/g level. The methodology was then applied to blood, collected from monkeys receiving Aroclor 1254 at definite intervals of dosing. The initial PCB level rose from 2.2 to 4.5 ng/g after 120 days. Monkey milk analysed at different days of lactation showed little variation in the PCB content on a whole milk basis. The peak height ratios varied among the substrates and with those of standard Aroclor 1254.

KEY WORDS: Small, PCB, monkey, tissues, milk.

INTRODUCTION

Recently the monkey has been used as a test animal for toxicological studies of polychlorinated biphenyl (PCB).[1,2,3] The advantage being that the results of the monkey's PCB exposure should give a more accurate extrapolation to the human situation. A disadvantage, apart from higher cost of procurement and maintenance, is the relative difficulty in obtaining milk, blood and biopsy samples from monkeys. Therefore, sample sizes tend to be small and cannot be handled by established techniques, since 1 ml of monkey milk or 100 mg adipose tissue samples are not unusual.[4]

It is important that sound analytical methodology supports the generated data in order to evaluate with confidence the relationship between observed effects and tissue residue levels.

For this reason we report on the results of our preliminary work to develop methodology suitable for analysing PCBs in small samples of monkey fluids and tissues in view of future toxicity studies.

EXPERIMENTAL

Solvents and chemicals

All solvents were glass-distilled and free of interfering residues. Florisil, anhydrous Na_2SO_4 and glass wool were soxhlet extracted with methylene chloride until free of interfering residues (250:1 concentration). The Florisil was heated at 300°C overnight, while both Na_2SO_4 and glass-wool were heated at 100°C until free of CH_2Cl_2. Aroclors 1254 and 1260 were gifts from Monsanto Chemical Co. and the Environmental Protection Agency, Triangle Park, N.C., U.S.A. All glassware was acetone and hexane rinsed.

Fortification and blanks

Fortification solutions were made up in acetone or hexane and contained 0.5 and 5 ng/μl of Aroclor 1260 and 1254 respectively. At regular intervals solvent blanks were run through the entire analytical procedure.

Sampling

Glass syringes and vials were washed, heated at 300°C and rinsed with hexane. Blood samples were collected in glass syringes and transferred to vials with aluminum lined caps and containing 0.1 ml of a 10% EDTA solution as anticoagulant.

Human milk samples were collected as previously described,[5] while monkey milk was expressed into a vial by manually restraining the animal. Tissues were obtained at autopsy and weighed to the nearest milligram on hexane rinsed aluminum foil. Foil wrapped samples were transferred for storage to vials prepared as above.

Extraction

Extraction of the different substrates was carried out with the Silverson homogenizer as follows:

(a) *Blood.* Approximately 5 g blood, weighed in a widemouth 50 ml round

bottom centrifuge tube (Pyrex no. 8240), was extracted with 15 ml benzene for 2 min. The extraction was carefully started by slowly lowering the homogenizer shaft into the benzene in order to create a vortex of blood in benzene. This procedure prevented emulsions, which otherwise could develop by sudden mixing. Emulsions can be broken, however, by placing the centrifuge tube in an ethanol-dry ice bath ($< -70°C$).

After extraction the shaft was rinsed with benzene and the extract centrifuged for 15 min at 1600 rpm in an International centrifuge, size 2, model k. The benzene layer was carefully decanted and quantitatively filtered through anhydrous Na_2SO_4 on a 100 mm funnel with glasswool plug. The sides of the centrifuge tube were washed twice with ~ 5 ml benzene and filtered as above. The benzene filtrate was evaporated just to dryness in a 250 ml round bottom flask (RBF) on an all-glass rotatory-evaporator ($< 30°C$) and the residue redissolved in a minimum of hexane and transferred to a pre-weighed aluminum dish. The hexane was evaporated in the fumehood and the dish weighed to determine benzene soluble lipids. The residue was redissolved in hexane, transferred back to the same 250 ml RBF and concentrated to ~ 1 ml.

(b) *Milk.* Approximately 1 ml of milk was weighed in a 50 ml centrifuge tube (see above) and extracted with 15 ml benzene for 1.5 min at moderate speed. Centrifugation was carried out and oil content determined as above.

(c) *Tissue.* Approximately 100 mg of tissue (liver, kidney or adipose) was extracted for 1 min at high speed, filtered through anhydrous Na_2SO_4 and redissolved in hexane as above.

In the case of acetone : hexane (19 : 1 v/v) extractions, the acetone had to be removed and replaced with hexane before filtering over anhydrous Na_2SO_4.

Cleanup

Residues in ~ 1 ml of hexane were chromatographed on 4.5 g of 2% water deactivated Florisil (equilibrated by shaking for 2 to 3 hrs) in a 12 mm O.D. glass column with 50 ml reservoir and teflon stopcock.[6] The PCBs were eluted in 35 ml hexane. The latter was concentrated, transferred to a stoppered graduated 15 ml centrifuge tube, carefully brought to dryness and redissolved in 100–500 μl hexane, depending on PCB concentrations.

Identification and quantification

Samples were chromatographed on either a Varian Series 1200 gas chromatograph (GC) with electron capture detector (Tritium) under the following conditions:

Column: 0.6 × 183 cm glass, packed with 5% OV-210 (0.5 g OV-210 + 10 g solid support) on 80/100 Chromosorb W(AW).
Temperatures: Injection: 226°C; Column: 181°C; Detector: 226°C.
Flow rate: 50 ml N_2/min,
or alternatively on a Varian Series 3700 with a ^{63}Ni detector under similar conditions, but with a 4% SE-30 + 6% OV-210 (0.4 g SE-30 + 0.6 g OV-210 + 10 g solid support) on 100/120 Chromosorb W(AW) column and injector, column and detector temperature at 220, 208 and 299°C respectively.

PCBs were quantitated by summation of peak heights, using peaks no. 4–10 in Aroclor 1254 and 8 and 10–14 in Aroclor 1260, according to the numbering system of Reynolds[7] and Jensen and Widmark.[8]

Confirmation

The PCB fractions of 3 monkey milk samples were pooled and analysed by gas chromatography-mass spectrometry (GC-MS) on a Varian Mat 311A coupled to a Watson–Biemann Separator. The sample was monitored for a single ion at m/e = 359.8 and m/e = 325.9.

RESULTS AND DISCUSSION

All preliminary work on blood and milk was carried out with human blood and milk, since the quantities needed could not be obtained from the monkeys, especially the breast milk. Table I shows the effect on the fat and PCB content of a single blood sample, aliquots of which were extracted with 3 different solvents. Benzene appeared to be the most efficient solvent and its use eliminated one step in the procedure.

TABLE I

Effect of extraction solvent on recovery of PCB and lipids from human blood

Solvent	% Fat	ng PCB/g whole blood[a]
Hexane	0.13	1.0
Benzene	0.22	1.6
Acetone : Benzene (19 : 1)	0.21	1.2

[a]Corrected for background.

Table II shows PCB recoveries from fortified human blood samples extracted with benzene. Blood from Donors 1, 2, and 3 were all fortified at 1 ppb and gave recoveries of 65–82%. The volume of the fortification solution added to the blood did not seem to affect recovery. At the 5 and

TABLE II

Recovery of PCB from fortified human blood samples

Donor	Fortification		ng PCB/g whole blood[a]	% Recovery[b]
	ppb Aroclor 1260	Volume (μl)		
1	—	—	3.3	
	1.2	10	4.1	67
2	—	—	2.6	
	1	10	3.4	82
3	—	—	1.7	
	1	100	2.3	65
4	—	—	2.7	
	5	10	6.7	82
	10	10	11.4	89

[a]Average of 3 determinations, except for Donor 1.
[b]Corrected for background.
[-]Not fortified.

10 ppb level recoveries were >80%. Donors 1, 2, and 4 were laboratory workers and their PCB blood levels were very similar.

The methodology for PCB analysis in human blood was applied to blood samples of a cynomolgus monkey fed 5 μg Aroclor 1254/kg/day. The results of the monkey blood analysis are shown in Table III. The PCB concentration doubled during the 120 day treatment period. Blood volumes of dosed monkeys as low as 1 ml can be handled by our method.

The results of studies on the effects of the solvent and volume used for fortification, and extraction solvent, on the recovery of Aroclor 1260 from a human milk sample are reported in Table IV. Samples 4 and 5 gave the highest recoveries. Benzene was preferred over acetone:benzene, because it eliminated one step in the procedure as mentioned earlier.

TABLE III

PCB levels in monkey blood at various time intervals, during dosing (5 μg Aroclor 1254/kg/day)

Days after initial dosing	Weight of blood (g)	ng PCB/g whole blood
1	4.45	2.2
20	3.13	3.2
36	5.23	3.9
75	4.23	4.9
120	4.00	4.5

TABLE IV

Effect of various parameters on the recovery of PCB from human milk fortified at the 10 ppb level

Sample no.	Fortification		Extraction solvent	ng PCB/g whole milk[a] (as Aroclor 1260)	% Recovery[b]
	Solvent	Volume (μl)			
1	—	—	Benzene	3.1	—
2	Acetone	20	Benzene	7.4	44
3	Acetone	100	Benzene	7.7	47
4	Hexane	100	Benzene	12.3	95
5	Acetone	100	Acetone : Benzene	13.4	106

[a]Average of 3 determinations.
[b]Corrected for background.
—Not fortified.

The human milk method was now applied to milk from a monkey mother, which had not been deliberately exposed to PCB. Results in Table V indicate that PCB levels in the monkey milk increased only slightly during the first 4 days of lactation. However, on a fat basis, the PCB level would be considerably higher on day 2 than days 3 and 4 of lactation. PCB levels in milk from the P.M. feedings were slightly higher than the A.M. feedings, but the difference may not be significant at these levels.

The sample size of monkey tissues obtainable by biopsy is understandably small and therefore, for fortification and recovery experiments 10–150 mg tissue samples were obtained at autopsy.

Because of the high background levels, relative to blood and milk, fortification of tissues was carried out at the ppm level. Recovery of PCB

TABLE V

PCB levels in 1 ml monkey milk samples from control monkeys at different days after delivery

Sample no.	Days after delivery	Time of sampling	% Fat	ng PCB/g whole milk (as Aroclor 1260)
1	2	[a]	1.2	3.5
2	3	A.M.	[b]	3.6
3	3	P.M.	3.0	3.9
4	4	A.M.	[b]	3.9
5	4	P.M.	3.1	4.1

[a]A.M. and P.M. samples were pooled, to give sufficient sample size.
[b]Not determined due to limited sample size.

was >90% in all cases (Table VI). Figure 1 illustrates the gas chromatographic elution pattern of PCBs found in the various monkey substrates. These chromatograms were taken from controls other than

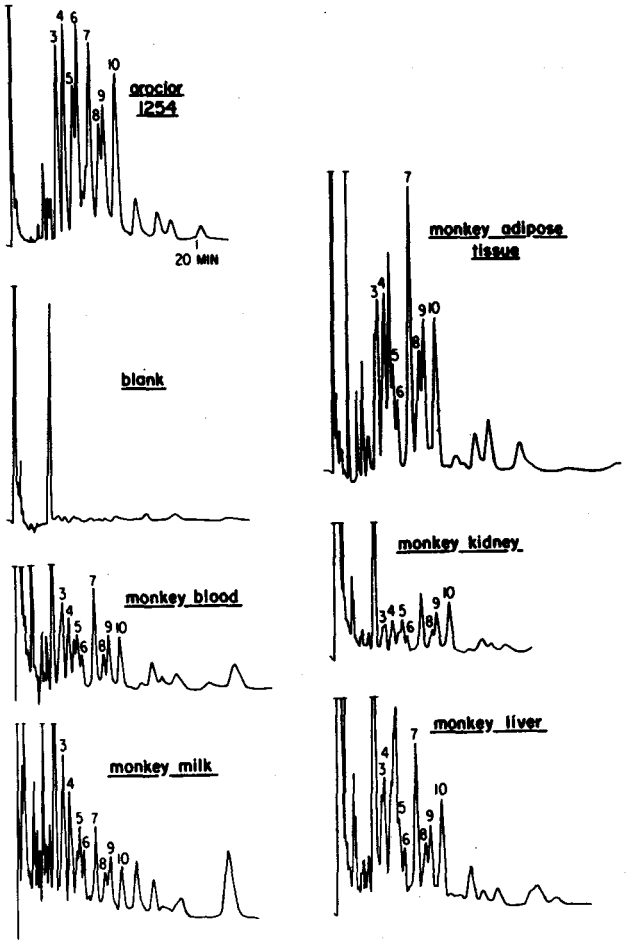

FIGURE 1. GC elution patterns of PCBs in Aroclor 1254, solvent blank and monkey blood, milk, adipose, kidney and liver.

those reported in the tables to enable presentation of tissues and fluids from the same monkey under identical GC conditions. All solvent blanks contained a single, but not interfering large peak as shown in Figure 1. Although such a blank would have a negligible effect on the data under dosing conditions, in control samples it would have to be taken into

TABLE VI

PCB recovery from fortified monkey tissues

Substrate	Sample weight[a] (mg)	Fortification Level (ppm)	Fortification Volume (μl)	μg PCB/g wet tissue (as Aroclor 1254)	% Recovery[b]
Adipose	79	—	—	0.380	
	80	0.2	50		89
	90	0.5	50		107
	96	1.0	20		99
Liver	104	—	—	0.093	
	96	0.2	50		106
	112	0.5	50		102
	108	1.0	50		98
Kidney	98	—	—	0.027	
	99[c]	0.2	50		100
	104[c]	0.5	50		92
	102	1.0	50		93

[a]Average of 3 determinations.
[b]Corrected for background.
[c]Average of 5 determinations.
⁻Not fortified.

account. All samples showed the same peak pattern, but peak ratios varied compared to those of standard Aroclor 1254 (Table VII). Peak ratios in monkey milk differed most from those in standard Aroclor 1254.

To separate PCBs from DDT and its metabolites, a combined Florisil-silicic acid column could be used.[6] However, the PCB dosage in most toxicity studies would be such that *p,p'*-DDT and *p,p'*-DDE interference would become negligible, justifying sample cleanup on a Florisil column only. Peak 5 in Figure 1 was not used for peak height ratios in Table VII, because of possible *p,p'*-DDE interference. The control samples were not corrected for *p,p'*-DDT background, since interference was considered minimal as shown in Table VIII.

PCBs were only confirmed in monkey milk, because of limited access to a mass-spectrometer (MS).

The methods outlined prove suitable for routine PCB analysis in toxicity studies, where often only small samples of monkey fluids and tissues are available.

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TABLE VII

Peak height ratios from PCB chromatograms of various monkey substrates

Ratio of peaks	Aroclor 1254[a]	Blood	Milk	Adipose	Liver	Kidney
3/4	0.96	1.21	1.32	0.96	1.01	0.88
3/6	0.96	1.56	2.58	2.43	2.33	0.88
3/7	1.04	0.84	1.80	0.60	0.77	0.48
3/8	1.73	2.42	3.97	1.45	2.09	1.36
3/9	1.51	1.56	2.77	1.13	1.51	0.71
3/10	1.27	1.61	3.37	1.12	1.11	0.57
4/6	1.01	1.29	1.95	2.53	2.31	1.00
4/7	1.09	0.70	1.36	0.63	0.75	0.55
4/8	1.90	2.00	3.00	1.50	2.07	1.55
4/9	1.58	1.29	2.09	1.17	1.50	0.81
4/10	1.32	1.33	2.54	1.16	1.10	0.64
6/7	1.08	0.54	0.70	0.24	0.33	0.55
6/8	1.89	1.55	1.54	0.60	0.90	1.55
6/9	1.57	1.00	1.07	0.46	0.65	0.81
6/10	1.32	1.04	1.30	0.46	0.48	0.64
7/8	1.74	2.87	2.21	2.40	2.76	2.82
7/9	1.45	1.75	1.54	1.87	2.00	1.47
7/10	1.21	1.91	1.87	1.86	1.47	1.17
8/9	0.83	0.64	0.70	0.78	0.73	0.52
8/10	0.70	0.67	0.85	0.76	0.53	0.42
9/10	0.84	1.04	1.22	0.99	0.73	0.79

[a]Average of 3 determinations.

TABLE VIII

Effect of DDT removal on the PCB level in monkey adipose tissue

Adipose tissue sample no.	μg PCB/g wet weight	
	Florisil column	Florisil-silicic acid column
1	0.608	0.587
2	0.353	0.354
3	0.277	0.266

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