Comparison of methylation methods for the determination of hydroxy-PCBs and preliminary results for polar bear blood plasma

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1. Introduction

The persistent nature of PCBs as well as the adverse health effects that may result from acute exposure is well known and characterized. Recently, interest in the possibility of deleterious health effects from low level exposure to PCBs has been investigated, especially in regards to endocrine disruption. It has been shown that the hydroxylated metabolites of PCBs may have an effect on the endocrine system by disrupting thyroid hormone transport. Brouwer *et al.* found that 4-OH-3,3',4',5-tetrachlorobiphenyl, a major metabolite of CB-77, selectively inhibits the binding of 3,3',5,5'-tetraiodo-L-thyronine (T_4) to transthyretin (TTR) in plasma of CB-77 exposed rats.[1] In the binding studies, it was found that 4-OH-3,3',4',5-tetrachlorobiphenyl binding strength is four times greater to TTR than T_4. This binding efficiency is due to the structural resemblance of the hydroxy ring of the metabolite and the diiodophenyl-ring of the thyroid hormone. This competitive binding to the TTR by the hydroxylated metabolites causes increased glucuronidation and biliary excretion of T_4 resulting in decreased plasma T_4 levels.[2]

PCB-77 may be of little importance to the study of these effects since it is only a very minor constituent in environmentally available PCBs. However, structurally similar metabolites of major PCBs have been found in the higher trophic levels, such as in Swedish grey seals and humans.[3] Some of these metabolites found in biota have also been shown to bind competitively with TTR. There is therefore reason to believe that the current exposure levels to PCBs may be affecting the plasma transport of thyroxine in higher trophic species.

With rising interest in hydroxylated metabolites of PCBs (HO-PCBs), it has become important to find a reliable method of quantitating the HO-PCBs found in tissues. Since gas chromatography (GC) is still the dominant method of separation and HO-PCBs give poor peak shapes when a GC is used, HO-PCBs must be derivatized in order to be efficiently quantitated. The standard method of derivatization is methylation using diazomethane. Ion-pair alkylation with methyl iodide was therefore compared to diazomethane. Preliminary application of the method to the identification of HO-PCBs in polar bear plasma is presented. Diazomethane is both toxic and explosive and must be used circumspectly, thus less toxic methylating reagents should be considered.

2. Procedure

Using a mixture of the following six synthetic HO-PCBs - 4-HO-2'5'-DiCB, 4-HO-2,5-DiCB, 3-HO-2',3',4',5'-TeCB, 4-HO-2',3',4',5'-TeCB, 2-HO-3,2',3',4',5'-PnCB and 4-HO-2,3,5,6,3',4'5'-HpCB, the two methods of methylation were tested.

Ion-Pair Alkylation (IPA)

A method developed by Hopper *et al.*[4], originally used for the methylation of pentachlorophenol and chlorophenoxy acid herbicides, was modified slightly for the methylation of HO-PCBs. The six congener mixture of HO-PCBs (8 nmol of each congener) was added to acetone (3 mL) followed by iodomethane (120 µL), and tetrabutylammonium hydroxide (60 µL). The samples were stoppered, vortexed and placed in a 40 °C water bath for 1.5 hours. The water level was kept just above the level of solvent in the reaction vessels. Each sample was then transferred to a round bottom flask and concentrated to a volume of approximately 1 mL, avoiding dryness. The samples were brought up to 4 mL with hexane and extracted with H_2SO_4 (2.0 mL, 0.5 M). The phases were allowed to separate and the organic layer removed. The aqueous phase was washed two more times with 3 mL hexane. The combined organic fractions were concentrated and applied to a neutral alumina column (3.0 g, 0.5% deactivated, prewashed with 30 mL hexane) using 1:1 dichloromethane:hexane as the mobile phase. The first 30 mL of eluent was collected and concentrated to the appropriate volume for determination. Final volumes differed depending on the method of detection used.

Diazomethane

The solvent most often used for the collection and storage of diazomethane is diethyl ether. Using this solvent, a significant proportion of the less chlorinated HO-PCBs were ethylated. Therefore, hexane was the solvent of choice for the collection of diazomethane. All diazomethane:hexane solutions were prepared immediately prior to use, by adding as much N-nitrosomethylurea as needed to a 1:1 mixture of hexane:NaOH (50%) mixture until the organic phase reached a bright yellow colour.

The six congener mixture of HO-PCBs (8 nmol of each congener) was added to hexane (3 mL). Approximately 1 mL of the hexane:diazomethane solution was added to each centrifuge tube. The tubes were then stoppered, mixed and the reaction was allowed to proceed for 1.5 hours. The reaction mixture was then transferred to a 150 mL round bottom flask with hexane and concentrated to dryness to remove the excess diazomethane. The sample was immediately solvated with hexane and applied to a neutral alumina column, following the remaining steps as described for IPA.

The derivatized samples were analyzed by both GC-ECD and GC-MSD. The GC-ECD was performed on a Hewlett Packard 5890 equipped with a ^{63}Ni ECD detector and an HP 7673A automatic injector. The GC was fitted with a fused silica, Rtx-5 capillary column (30 m, 0.25 mm i.d., 0.25 µm film thickness). The injector and detector temperatures were 250 °C and 300 °C, respectively. Injections were 1 µL made in splitless mode. The GC-MSD (electron impact, EI) was performed on a Hewlett Packard 5890 equipped with a 5970 series mass selective detector and an HP 7673A automatic injector. Injections were 2 µL made in splitless mode. The GC was fitted with a DB-5 capillary column (30 m, 0.25 mm i.d., 0.25 µm film thickness). The injector and detector temperatures were 250 °C and 280 °C. The GC temperature program for both methods of detection was as follows: initial temperature held for 2 min at 80 °C, 10 °C/min to 150 °C and 8 °C/min until 280 °C.

The following list of standards were supplied by Prof. Åke Bergman (Environmental Chemistry, Wallenberg Laboratory, Stockholm University, Stockholm, Sweden) and were used for peak identification: 4-MeO-3,5,2',4',5'-PeCB, 4-MeO-3,5,2',3',4'-PeCB, 3-MeO-2,4,6,2',3',5',6'-HpCB, 3-MeO-2,4,5,2',4',5'-HxCB, 4-MeO-2,3,5,2',4',5'-HxCB, 3-MeO-2,4,5,2',3',4'-HxCB, 4-MeO-2,3,5,2',3',4'-HxCB, 3-MeO-2,4,5,2',3',5',6'-HpCB, 4-MeO-2,3,5,2',3',4',6'-HpCB, 4-

MeO-3,5,2',3',4',5'-HxCB, 3-MeO-2,4,5,2',3',4',5'-HpCB, 4-MeO-2,3,5,2',3',4',5'-HpCB, 4-MeO-2,3,5,6,3',4',5'-HpCB, and 4-MeO-2,3,5,6,2',4',5'-HpCB.

3. Results

IPA and diazomethane procedures both gave comparable rates of derivatization. Five replicates of each method were compared. The results from the GC-ECD analysis are given in Table 1. The 4-MeO-2,3,5,6,2',4',5'-HpCB was used as the reference standard and was added to each sample prior to final concentration.

Compound	Retention Time (min)	IPA Mean ARRF	% CV	Diazomethane Mean ARRF	% CV
4-HO-2'5'-DiCB	16.46	0.256	14	0.268	11
4-HO-2,5-DiCB	16.72	0.107	11	0.071	27
3-HO-2',3',4',5'-TeCB	22.66	0.334	8	0.229	27
2-HO-2'3,3',4',5'-PnCB	22.83	0.767	14	0.311	33
4-HO-2',3',4',5'-TeCB	23.44	0.123	12	0.089	25
4-HO-2,3,5,6,3',4',5'-HpCB	29.18	1.385	14	1.540	2

Table 1 - Mean apparent relative response factors (ARRF) (± %CV, n=5) for each compound compared to 4-MeO-2,3,5,6,2',4',5'-HpCB using GC-ECD mode of detection.

These results indicated that the IPA method of methylation gave as good or better yields of MeO-PCBs than diazomethane. The reproducibility of the IPA method was also superior. Although the IPA method may be superior for derivatization, more residual interferences were observed in the GC/ECD chromatogram of the final fraction relative to diazomethane. The use of diazomethane for derivatization resulted in very clean chromatograms using both ECD and MSD methods of detection. While IPA did produce a variety of extraneous peaks, perhaps interferences could be avoided by using a better clean up procedure or simply using MSD detection in the SIM mode. Under these conditions, IPA could replace diazomethane as a derivatization approach. The reagents for IPA are somewhat less toxic and much easier to handle, but derivatization must still be completed in a well ventilated fume hood.

4. Application

Three pools of polar bear plasma (mothers, cubs and juveniles) were prepared from the available samples. The polar bear plasma was analyzed for HO-PCBs using the method of extraction described previously by Bergman *et al.*[3]. The HO-PCBs were extracted and subsequently derivatized using diazomethane in hexane and analyzed by both GC-ECD and GC-MSD in the SIM mode using the same conditions as described above. The ions monitored were 320, 322, 356, 358, 390, 392, 424, 426, 458, 460, 492, and 494. The SIM chromatograms for each of the pools is given along with the identified derivatized metabolites in Figure 1.

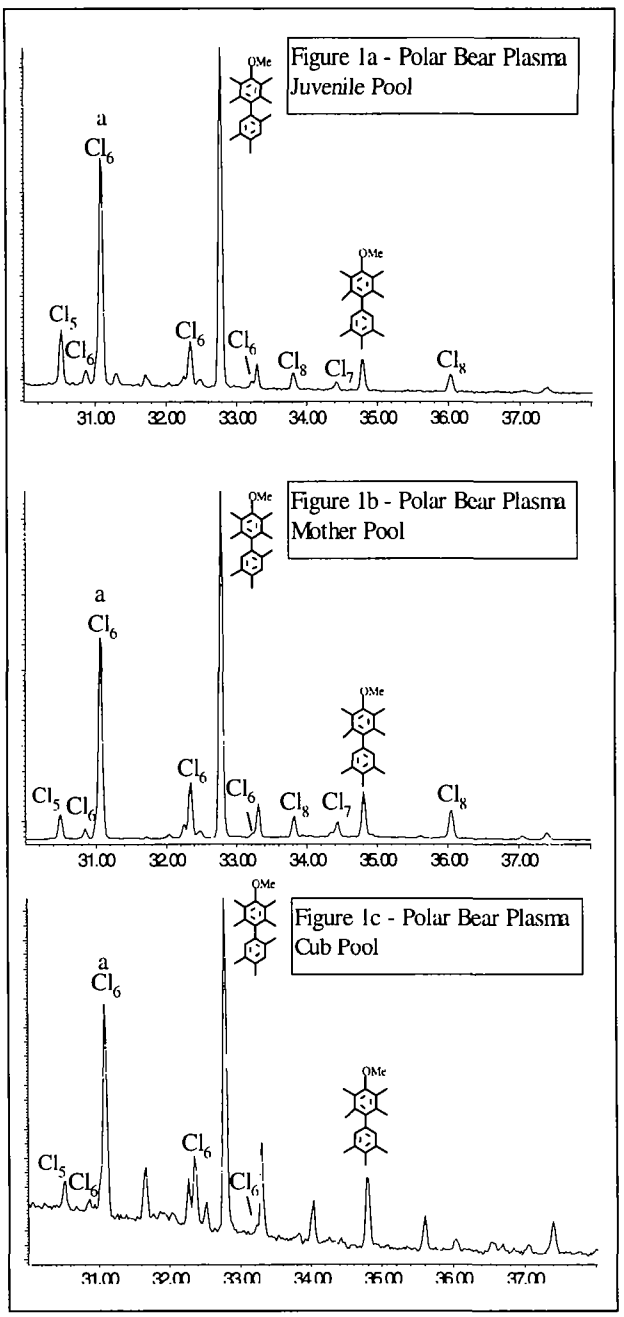

Figure 1 - Gas chromatograms, using SIM detection, of the methylated hydroxy metabolites of PCBs of three different pools of polar bear plasma. The "Cl_x" numbers above peaks indicate the degree of chlorination. (a) pool of juvenile polar bear plasma. (b) pool of mother polar bear plasma. (c) pool of cub polar bear plasma. Structures indicated are tentative assignments.

All three pools of plasma contained the same peaks. It was possible to get full EI spectra of the two main peaks found in the polar bear plasma. Both of these peaks had the characteristic *para* methoxy fragmentation pattern determined by Safe *et al.*[5] and Bergman *et al.*[3] Using the available MeO-PCB standards and the distinctive hepta-chlorination pattern, the main metabolite in each of the samples was tentatively identified to be 4-MeO-2,3,5,6,2',4',5'-HpCB, based on retention time on a DB-5 column. Retention times on at least two other columns of differing properties will be necessary to confirm this identity. This is the major metabolite found in human plasma and was suggested to result from the hydroxylation of CB-187 or CB-183.[3] If CB-187 is the precursor, metabolism of the PCB would have to have proceeded via *para* oxygen insertion. None of the other standards in our possession exactly matched the retention time of any of the MeO-PCBs in polar bear plasma except for 4-MeO-2,3,5,6,3',4',5'-HpCB. If this indeed is the identity of the peak, it possesses the same 2,3,5,6-chloro, 4-MeO structure as the major peak. CB-193 is a minor peak in commercial PCBs, but may be present at low levels in polar bears.

The second largest peak, labeled peak (a), is a hexachlorinated compound that was very close in retention time to both the 3-MeO-2,4,5,2',4',5'-HxCB and 4-MeO-2,3,5,2',4',5'-hexachlorobiphenyl standards. These are derivatives of metabolites formed from CB-153 through direct oxygen insertion and NIH shift of chlorine, respectively. Both of these compounds were identified in human plasma by Bergman *et al.*[3] Since the unknown hexachlorinated compound is found in both females and cubs, the compound or its precursor PCB must be present and persistent enough to be transferred via lactation. It is unlikely that the HO-PCB metabolites would be transferred to the cub through mother's milk and absorbed efficiently to produce the same patterns in plasma as the females. Therefore, the precursor PCB is most likely carried in the milk to the cub where it is then biotransformed to the hydroxy-metabolite. CB-163 co-elutes with CB-138 on DB-5. Using an SP2330 column, we have found that the combined DB-5 peak actual consists of approximately 30% CB-163. CB-163 possesses the 2,3,5,6-substitution of the other two tentatively identified peaks, and may be the precursor of peak (a) via direct insertion of oxygen, by analogy to the two other tentatively identified peaks.

5. Conclusion

Ion pair alkylation was more quantitative and reproducible than diazomethane at derivatizing the six HO-PCBs used in this experiment, but produced more interferences. Tentatively, polar bear plasma had the same major metabolite found in human plasma, 4-MeO-2,3,5,6,2',4',5'-HpCB. We propose to test the hypothesis that *para* oxygen insertion into 2,3,5,6- chlorine substituted PCBs is the mechanism of formation of the major HO-PCBs in polar bears. Both polar bear females and cubs had similar congener patterns. Age and feeding status therefore had an insignificant effect on the congener pattern.

6. References

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7. Acknowledgments

We thank Åke Bergman and Eva Klasson-Wehler (Environmental Chemistry, Wallenberg Laboratory, Stockholm University, Stockholm, Sweden) for supplying us with the MeO-PCB standards. The polar bear plasma was supplied by Malcolm Ramsay (Department of Biology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada). Special thanks to Robert Letcher for his assistance and guidance as well as his preliminary work with this project. This study was made possible through funding given by the Canadian Chlorine Coordinating Committee (C4).