Isolation of PCB Atropisomers for Toxicological Testing using Chiral High-Performance Liquid Chromatography

Peter Haglund

Institute of Environmental Chemistry, Umeå University, S-901 87 Umeå, Sweden.

1. Introduction

Stereoisomers that are chiral solely as a result of restricted rotation about a single bond are called atropisomers by Kuhn [1]. Seventy-eight out of the 209 PCB congeners are asymmetrically substituted and are thus displaying axial chirality in their non-planar conformations. Many of these will however rapidly interconvert at room temperature as their barriers of rotation about the pivot bond are low. An energy barrier of 16-20 kcal/mol is considered necessary for isolation at room temperatures [2]. Only the 19 asymmetrically substituted PCBs with three or four chlorines in the ortho positions will therefore exist as stable atropisomers in biota [3]. The structures, systematic numbers according to the IUPAC nomenclature [4,5], and estimated energy barriers of racemisation [3] for these stable atropisomers, are compiled in Table I. At room temperature there exist two enantiomeric forms of each PCB, see Fig. 1.

Three of the atropisomeric PCBs have been completely (PCB197) or partially (PCB88 and PCB139) separated by liquid chromatography on triacylcellulose [6,7]. These atropisomers have been subjected to various toxicological tests. Püttman studied the *in vivo* induction of total cytochrome P-450 content, aminopyrine N-demethylase, and aldrin epoxidase in immature male Sparque-Dawley rats. The racemic PCB139 was found to be a potent phenobarbital-type inducer, whereas (+)-PCB139 and (-)-PCB139 showed clearly differing potencies as inducers with (+)-PCB139 enhancing aminopyrine N-demethylase, aldrin epoxidase and total cytochrome P-450 content more potently than (-)-PCB139. In contrast, the racemic PCB197 and its individual enantiomers were only week phenobarbital-type inducers of cytochrome P-450, and the enantiomers were equally (weakly) potent [8].

IUPAC	Species	ΔE	IUPAC	Species	ΔE
45	22'36-Tetrachlorobiphenyl (TeCB)	25	**144**	22'345'6-HxCB	25
84	22'33'6-Pentachlorobiphenyl (PeCB)	29	**149**	22'34'5'6-HxCB	25
88	22'346-PeCB	25	**171**	22'33'44'6-Heptachlorobiphenyl (HpCB)	29
91	22'34'6-PeCB	25	**174**	22'33'456'-HpCB	29
95	22'35'6-PeCB	25	**175**	22'33'45'6-HpCB	29
131	22'33'46-Hexachlorobiphenyl (HxCB)	29	**176**	22'33'44'6-HpCB	58
132	22'33'46'-HxCB	29	**183**	22'344'5'6-HpCB	25
135	22'33'56'-HxCB	29	**196**	22'33'44'56'-Octachlorobiphenyl (OCB)	29
136	22'33'66'-HxCB	58	**197**	22'33'44'66'-OCB	58
139	22'344'6-HxCB	25			

Table 1: Structures, systematic numbers according to the IUPAC nomenclature 4,5, and estimated energy barriers of racemisation (kcal/mol) [3] for the stable tri- and tetra-*ortho* atropisomers.

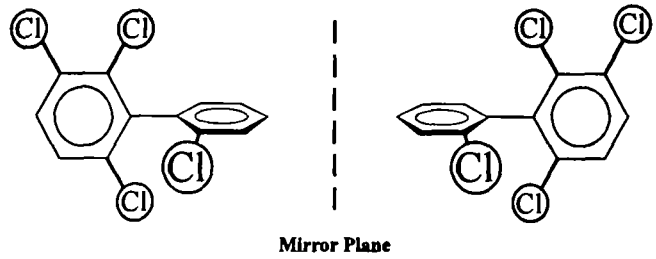

Figure 1: Optical isomers of an atropisomeric PCB, viz. IUPAC 45.

Rodman et. al. used chick embryo hepatocyte cultures *in vitro* to determine if chirality plays a role in the recognition events associated with the induction of cytochrome P450 and the accumulation of uroporphyrine (URO) [9]. Concentration related induction of cytochrome P450 content, ethoxyresoryfin-*O*-deethylase (EROD) and benzphetamine N-demethylase (BPDM) activities was measured. The rank order of potency for total cytochrome P-450 induction was PCB139 > PCB197 ≥ PCB88. The (+)- and (-)- enantiomers of PCB88 and PCB197 were of equal potencies as inducers of cytochrome P-450, whereas the (+)- PCB139 was more potent than the (-)- PCB139. PCB139 was a much more potent inducer of EROD activity than either PCB88 or PCB139. EROD activity was induced to a much higher extent by the (+)-enantiomers of all compounds. BPDM activity was induced by all three compounds in the order PCB197 ≥ PCB139 > PCB88. The (-)-enantiomers were more potent than the (+)-enantiomers, except for PCB139, in which the (+)- was more potent than the (-)-enantiomer. Analysis of porphyrin accumulation revealed that (+)- PCB139 caused the greatest percentage URO accumulation.

PCBs are ubiquitous environmental pollutants that are biomagnifying in biota. Parts-per-million levels are found in species at the highest levels of the food webs, e.g. humans, marine mammals, and birds of prey. Much effort has been invested to assess the environmental impact of these compounds. Unfortunately, the existence of stable conformers among the axially asymmetric PCBs has not been considered in the toxicological testing that has been the basis for the risk evaluation of PCB. The discovery of enantioselective biological properties of the PCB atropisomers has therefore resulted in an increased concern among toxicologists. However, the limited availability of pure enantiomers currently restrains the efforts in the area of enantioselective toxicological testing. As far as we know, there are only the three atropisomeric PCBs discussed above that are available for toxicity studies.

The aim of the present study has therefore been to investigate if chiral high-performance liquid chromatography (HPLC) on permethylated cyclodextrin derivatized silica can be used to separate additional enantiomeric pairs of atropisomeric PCBs.

2. Experimental

2.1 Optimisation of the Chromatographic Conditions

The chromatographic conditions were optimised using a factorial design, specifically a three level face centred central composite design (CCF), cf. Fig. 2. Three additional centrepoints were included to estimate the experimental variance. In this type of design a quadratic polynomial model is fitted to the experimental results. The graphical meaning is that both planar and curved response surfaces can be accurately described. The experimental design was created, evaluated, and graphically illustrated using the MODDE software package (Umetri, Umeå, Sweden). The three

variables (and experimental domains) were flow rate (0.3 to 0.5 ml/min), buffer composition (0 to 1% triethylamine acetate buffer, pH 4, (TEAA) in 85% methanol), and column temperature (0 °C to 30 °C). The run order of the optimisation experiments was randomised to avoid systematic errors, cf. Table I.

A Hewlett-Packard 1050 liquid chromatographic system, consisting of a quaternary pump, an autosampler, and a variable UV detector, was used during the optimisation. A Hewlett-Packard Chemstation PC software was used for instrument control, data collection, and data analysis. Enantiomer separation was obtained by chromatography on two serially connected 4.6 x 250 mm Nucleodex β-PM columns (Macherey-Nagel, Düren, Germany). According to the manufactures documentation these columns are packed with 5 µm Nucleosil silica, which has been surface modified with a covalently bond chiral selector - permethylated β-cyclodextrin (PMCD). A column heater/chiller was used to regulate the temperature of the chromatographic columns.

Aliquots of a PCB174 standard solution were injected onto the PMCD columns, and the UV-absorption of the eluent was monitored at 210 nm. The resulting peaks were integrated and three performance measures, number of theoretical plates, selectivity, and resolution, were calculated by the performance algorithm of the Chemstation software.

2.2 Isolation and characterisation of atropisomeric PCBs

Ten to one hundred µl aliquots (5- 10 µg/µl) of nine racemic PCB solutions, viz. PCB84, PCB131, PCB132, PCB135, PCB136, PCB174, PCB175, PCB 176, and PCB196, were fractionated on the PMCD columns, and the enantiomers were collected in separate fractions. The fractionation process was repeated until ca. 1mg of pure enantiomers were obtained. The chromatographic equipment and settings were the same as above, and the separations were performed at the optimum chromatographic conditions, that is: pure 85% methanol at 0.4 ml/min, and a column temperature of 0 °C (see also Results and discussion). Finally, the solvent was evaporated and the residues were reconstituted in 1 ml of spectroscopic ethanol, and the specific optical rotation was determined using an electronic polarometer.

3. Results and discussion

3.1. Optimisation of the Chromatographic Conditions

A response surface model was fitted, using the Partial Least Square (PLS) algorithm, to each of the response variables: number of theoretical plates (N), selectivity (α), and resolution (R). Three principal components were found to be statistically significant, as determined by cross-validation. All experimental data were nicely fit to the model, and the residual error sum of squares (R2) and prediction error sum of squares (Q2) values were satisfactory: 0.992 and 0.860 for N, 0.984 and 0.736 for α, and 0.964 and 0.781 for R.

The statistical data treatment revealed that the column temperature was by far the most important variable for all of the studied response variables. The flow rate ranked as the second most important variable. The buffer composition was not very crucial, and it was even so, that the best chromatographic performances were obtained without any buffer addition at all. Optimum performance was achieved under the following conditions: pure 85% methanol at 0.4 ml/min, and a column temperature of 0°C.

3.2 Isolation of PCB atropisomers

The capacity factors (k'), selectivity factors (α), and resolution (R) of the nine atropisomeric PCBs are compiled in Table II. All congeners can be completely resolved by using PMCD columns. However, in the present study the columns were severely overloaded and the resolution was therefore deteriorated, c.f. Figure 2. This problem was overcomed by using a shaving technique. The first enantiomer was collected until 0.5 min before the elution time of the second enantiomer, and the collection of the second enantiomer was started 1 min later. The interjacent fraction was directed to waste. In this way a purity of > 98% was achieved.

PCB#	Substitution	k' 1	k' 2	α	R
84	23 6-23	2.43	3.15	1.30	3.05
131	234 6-23	3.37	4.12	1.22	1.57
132	234 -23 6	3.87	5.05	1.30	3.15
135	23 5 -23 6	3.45	4.39	1.28	2.80
136	23 6-23 6	2.31	3.48	1.51	3.15
174	2345 -23 6	4.63	5.84	1.26	3.03
175	234 6-23 5	4.49	5.52	1.23	2.51
176	234 6-23 6	3.27	4.03	1.23	2.12
196	2345 -234 6	5.50	6.63	1.21	2.65

Table II: Capacity factors (k'), selectivity factors (α), and resolution (R) of nine atropisomeric PCBs for the HPLC separation on PMCD. The HPLC instrumentation and experimental conditions are described in detail in the Experimental section.

The results of the polarometry measurements are shown in Table III. Interestingly, the (-)-enantiomer always elute from the PMCD columns prior to the (+) enantiomer. The magnitude of the specific optical rotation is similar to the values reported earlier for PCB88, PCB139, and PCB197 [7].

λ	84 E1	84 E2	131 E1	131 E2	132 E1	132 E2	135 E1	135 E2	136 E1	136 E2	174 E1	174 E2	175 E1	175 E2	176 E1	176 E2	196 E1	196 E2
578 nm	-115	125	-52	60	-51	54	-21	17	-35	37	-15	14	-17	16	-33	31	-22	21
546 nm	-135	138	-63	63	-62	61	-22	18	-41	43	-21	20	-20	20	-39	35	-24	26
436 nm	-242	241	-110	111	-111	110	-42	31	-70	73	-30	31	-34	34	-66	63	-29	30

Table III: Specific optical rotation (degr. x ml x dm-1 x g-1) of the pure PCB atropisomers. E1 and E2 means first and second eluting enantiomer, respectively, in the HPLC separation.

3.3 Summary

The atropisomers of PCB have been successfully separated using a permethyl-β-cyclodextrin derivatized HPLC column. Milligram quantities of pure enantiomers have been isolated and their optical rotation characteristics have been determined. In the near future the PCB atropisomers will be subjected to various toxicity tests to screen for enantioselective toxicological properties.

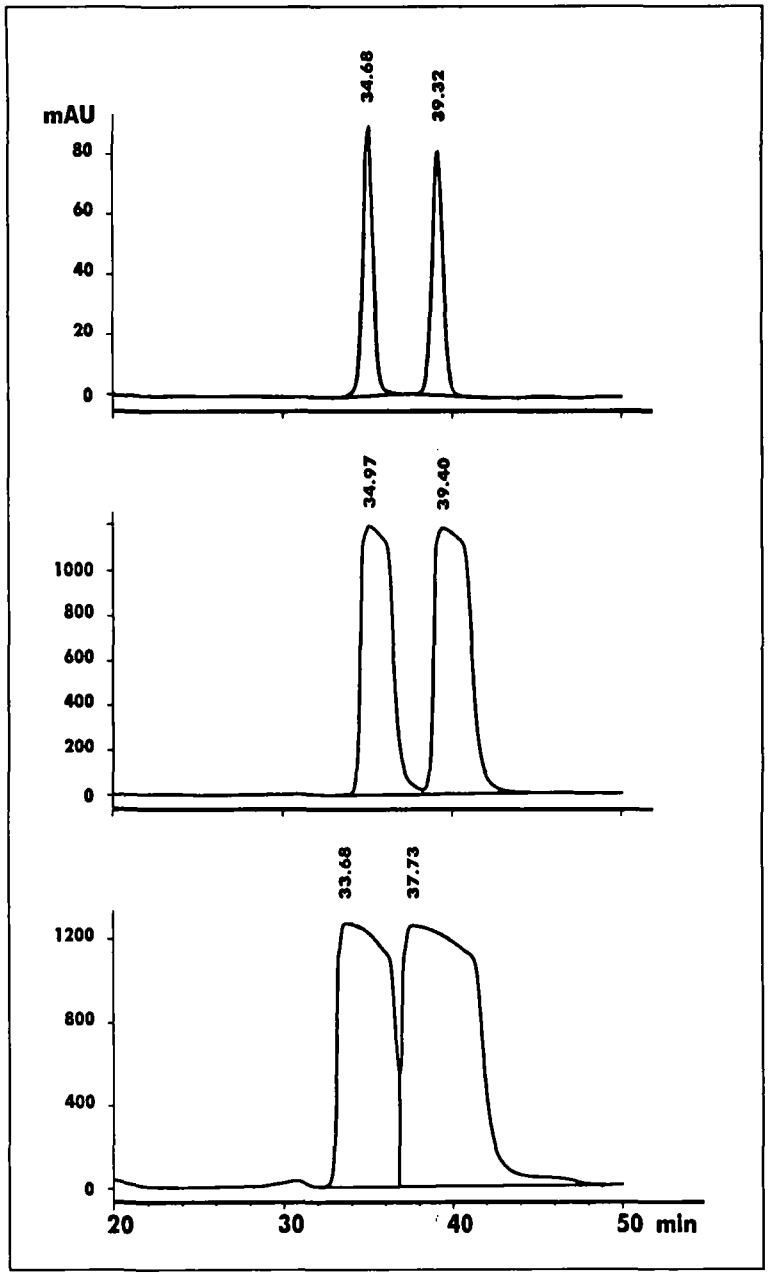

Figure 2: Chromatograms illustrating the effect of column load on the separation of PCB atropisomers. The injected amounts are (from top to bottom): 5 μg, 100 μg and 500 μg. The instrumentation and chromatographic conditions are described in detail in the Experimental section.

4. References

1 Kuhn R. (1933): *Moleculare Assymetrie* in *Stereochemie*, edited by H. Freudenberg, Franz Deutike: Leipzig-Wien, pp. 803-824.

2 König W.A., Gehrcke B., Runge T., and Wolf C. (1993): *J. High Res. Chromatogr.*, 16, 376-378.

3 Kaiser K.L.E. (1974): *Environ. Polut.*, 7, 93-101.

4 Ballschmitter K. and Zell M., (1980) *Frezenius Z. Anal. Chem.*, 302, 20-31.

5 Schulte E. and Malisch R. (1983): *Frezenius Z. Anal. Chem.*, 314, 545-551.

6 Mannschreck A., Pustet N., Robertson L.W., Oesch F. and Püttmann M. (1985): *Liebigs Ann. Chem.*, 2101-2103.

7 Püttmann M., Oesch F. and Robertson L.W. (1986): *Chemosphere*, 15, 2061-2064.

8 Püttmann M., Mannschreck A., Oesch F. and Robertson L.W. (1989): *Biochem. Pharmacol.*, 38, 1345-1352.

9 Rodman L.R., Shedlofsky S.I., Mannschreck A., Püttmann M., Swim A.T. and Robertson L.W. (1991): *Biochem. Pharmacol.*, 41, 915-922.