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USE OF ETHYL ETHERS, DEUTERIOMETHYL ETHERS AND CYCLIC *n*-BUTYLBORONATES OF HYDROXYCHLOROBIPHENYLS IN IDENTIFICATION OF METABOLITES OF POLYCHLORINATED BIPHENYLS

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SUMMARY

The mass spectra and gas chromatographic properties of a number of chlorobiphenylols, chlorobiphenyldiols, chlorophenols and naphthols, as well as their methyl, deuteriomethyl and ethyl ethers and some cyclic *n*-butylboronates have been investigated. Metabolism experiments with 4'-chloro-4-biphenylol showed that the selective use of ethylation and methylation is most effective in both detection and structure elucidation of metabolites partly methylated by metabolic processes. The usefulness of deuteriomethylation in such studies seems to be limited. The formation of cyclic *n*-butylboronates provides specific information on *o*-dihydroxy derivatives of chlorobiphenyls.

INTRODUCTION

Polychlorinated biphenyls (PCBs) are among the most ubiquitous and persistent chemical pollutants in the global ecosystem. In order to understand associated potential toxic effects, the metabolites should be fully characterised. PCB metabolism has been reviewed recently[1]. The main metabolites of PCBs in a variety of species are hydroxylated compounds. These phenolic substances are sensitive to oxidation and often show poor chromatographic properties. Therefore during sample purification and analysis in metabolism experiments a number of derivatization procedures, such as methylation, trimethylsilylation, acetylation and dansylation, have been used[1]. Methylation results in enhanced chemical stability and better chromatographic properties, and substantial structural information can be obtained by the specific mass fragmentation of the methylated compounds[2,3]. We have recently shown[4] that the characteristic mass fragmentation patterns of fluoro-, chloro- and bromobiphenylols allow unambiguous identification; the fragmentation patterns of the methyl ethers of chlorobiphenyldiols are specific, although not conclusive in all cases.

The reasons for studying different derivatization procedures are first, that in a number of documented cases[1,5–9] methoxy derivatives methylated by metabolic processes were found. If such metabolites are to be detected specifically, methylation

cannot be used during the sample purification. Secondly, in case of chlorobiphenylols, which are increasingly found as PCB metabolites[1,10], a specific derivatization procedure has advantages over the more elaborate synthesis of standards when mass fragmentation of the methyl ether does not provide unambiguous structural information[4].

MATERIALS AND METHODS

Gas chromatography–mass spectrometry (GC–MS) was performed on a Hewlett-Packard 5982A system. The all glass columns were 180 × 0.4 cm 0.2% Carbowax 20M on Chromosorb W 100–120 mesh[11] (Column A) and 200 × 0.4 cm 3% Apiezon L on Chromosorb W 100–120 mesh (Column B). Temperature programmes are in the table footnotes. The GC–MS operating temperatures were: injector 250°, jet separator 350°, transfer line 280° and ion source 200°. The electron energy was 70 eV. Mass spectra were scanned from 50 to 400 a.m.u. with a speed of 80 a.m.u./sec. For preparative thin-layer chromatography (TLC) silica gel 60 F_{254} plates (Merck, Darmstadt, G.F.R.), 20 × 20 cm, layer thickness 0.25 mm, were used. The developing solvent was hexane–ethyl acetate (9:1).

Derivatization procedures

Methylation was performed as previously described[10], using either methyl iodide or diazomethane. Deuteriomethylation was accomplished with deuteriodiazomethane (93% deuterium, synthesized with the Deutero Diazald Prep Set of Aldrich, Milwaukee, Wisc., U.S.A.). Ethylation with ethyl iodide was carried out as described for methylation. Demethylation was brought about with BBr_3 in dichloromethane[12]. Cyclic *n*-butylboronate formation was achieved by dissolving dried *n*-butylboronic acid in water-free dimethylformamide containing the substrate chlorobiphenyldiol and 2,2-dimethoxypropane[13,14].

Synthesis

The compounds investigated (Fig. 1) were synthesized as methyl ethers. Demethylation gave the free hydroxy compounds used as starting materials for the other derivatizations.

Compounds I, II and III were synthesized using the Shu Huang and Cadogan modification of the Gomberg procedure[15,16] by treating 2,5-dichloroaniline with amyl nitrite in the presence of methoxybenzene. The syntheses of compounds IV, VI, VII and XI have been previously described[4,17]. Compound V was prepared from 4-methoxyaniline and 2,4,6-trichloromethoxybenzene. The hydroxy derivatives of compounds VIII, IX and X were purchased from commercial sources. Compound XII was synthesized from 4-methoxyaniline and 2-chloromethoxybenzene and compound XIII from 3,4-dimethoxyaniline and 2-chloromethoxybenzene. The latter two syntheses gave four isomers identified unambiguously by GC–MS, on the basis of the known relationship between structure (chlorine and methoxy substitution pattern) and retention time[2,4].

Metabolism of 4'-chloro-4-biphenylol

4'-Chloro-4-biphenylol of high purity (>99.9% by GC–MS) was dissolved in peanut oil (*Oleum arachidis*) and administered orally to male Wistar rats, weighing

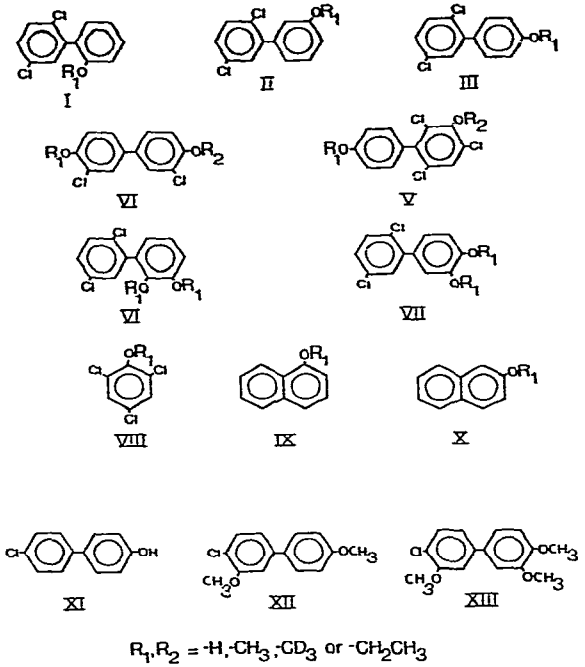

Fig. 1. Structures of compounds investigated.

250 g (TNO, The Netherlands) as a single dose of 100 mg/kg. The animals were housed in individual metabolic cages and freely supplied with water and food during the experimental period of 7 days. Urine was collected in 4 *N* sulphuric acid to prevent microbial metabolism after excretion.

Isolation of metabolites

The acidic urine was purified as depicted in Fig. 2. For experimental details see above and refs. 4 and 18.

RESULTS AND DISCUSSION

Ethylation

Nucleophilic substitution with ethyl iodide is a convenient way to obtain ethyl ethers of phenols, naphthols and biphenyl(di)ols in quantitative yield. Simultaneous reaction of these substrates with methyl and ethyl iodide results in both the methyl and ethyl ethers in an approximate ratio of 2:1.

In all cases investigated methyl and ethyl ethers of the same hydroxy compound gave excellent GC separation. Also, the ethyl ethers of structural isomers (*e.g.* the ethyl ethers of compounds I, II and III can be easily separated by GC. GC retention times of the ethylated compounds are given in Table I.

The ethylated compounds all gave molecular ions with intensities of 30–80% of the basepeak $M^+ - 28$, formed by loss of the ethyl group with concomitant hydrogen atom rearrangement to the phenolic analogue. The mass spectra of hydroxy com-

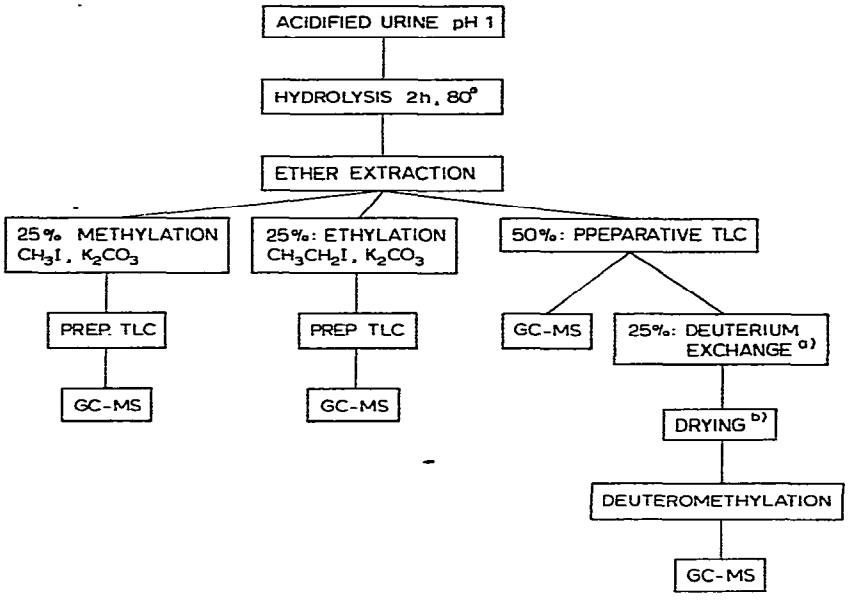

Fig. 2. Isolation of metabolites. a) Residue dissolved in dried[b] ether, shaken with a 30% solution of NaOD in D_2O and acidified with DCl. b) Molecular Sieve 4A.

TABLE I

GC RETENTION TIMES OF ETHYLATED AND METHYLATED COMPOUNDS

For structures of compounds I–X see Fig. 1: Et = ethyl, Me = methyl.

Compound	R_1	R_2	*Retention time (min)* *	*Compound*	R_1	R_2	*Retention time (min)* *
I	Et	—	2.0 (170°)	V	Me	Me	6.4 (170°)
II	Et	—	3.2 (170°)	V***	Me	Et	6.8 (170°)
III	Et	—	3.6 (170°)	VIII	Me	—	2.8 (100°)
I	Me	—	1.8 (170°)	VIII	Et	—	3.6 (100°)
II	Me	—	3.1 (170°)	IX	Me	—	2.9 (120°)
III	Me	—	3.3 (170°)	IX	Et	—	3.7 (120°)
IV**	Me	Me	8.7 (170°)	X	Me	—	3.1 (120°)
IV	Me	Et	8.9 (170°)	X	Et	—	3.9 (120°)
IV	Et	Et	9.1 (170°)				

* Column A (see Materials and methods). Temperature in parentheses is the starting temperatures that was maintained for 2 min and elevated to 240° at a rate of 8°/min.

** The dihydroxy derivative of IV ($R_1 = R_2 = H$) was partially methylated with diazomethane to give $R_1 = R_2 = H$, $R_1 = H$, $R_2 = Me$ and $R_1 = R_2 = Me$. This mixture then was ethylated to yield $R_1 = R_2 = Me$, $R_1 = Me$, $R_2 = Et$ and $R_1 = R_2 = Et$.

*** Prepared by treating 4-methoxyaniline with 2,4,6-trichloroethoxybenzene (see *Synthesis*).

pounds and their ethyl ethers are virtually identical, except for the additional M^+ in the latter (*e.g.* Fig. 3). Thus, in contrast to the mass fragmentation of methyl ethers of chlorobiphenylols, the mass fragmentation of their ethyl ethers does not allow further structural assignments. This is important in the interpretation of the mass spectra of biphenyldiols with one hydroxy group methylated and one ethylated. As

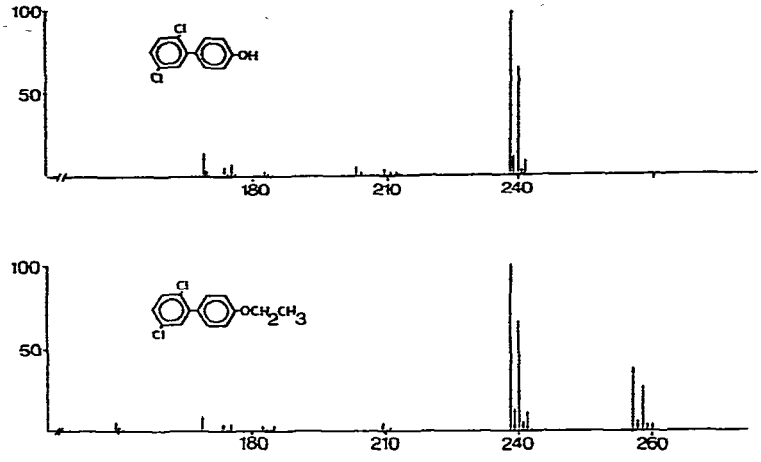

Fig. 3. Partial mass spectra of 2′,5′-dichloro-4-biphenylol and its ethyl ether.

shown in Fig. 4 the specific fragmentation pattern of the methoxy group is unchanged. Apart from M^+ of the ethyl ether the spectra are almost superimposable.

The base peak M^+-56 in the mass spectrum of IV ($R_1 = R_2 = Et$) (see Table I), is formed by loss of both ethyl groups and rearrangement to the diol analogue. From M^+-56, at 254 a.m.u., the spectrum of this compound is identical with that of 3,3′-dichloro-4,4′-biphenyldiol. This implies that a polyhydroxy-chlorobiphenyl with one of the hydroxy groups methylated by metabolic processes can be successfully identified after ethylation. The mass spectrum of such a compound will unambiguously reveal the position of the methoxy group.

Deuteriomethylation

Deuteriomethylation with deuteriodiazomethane proceeds swiftly and quantitatively with phenols, biphenylols, biphenyldiols and naphthols. However, this reac-

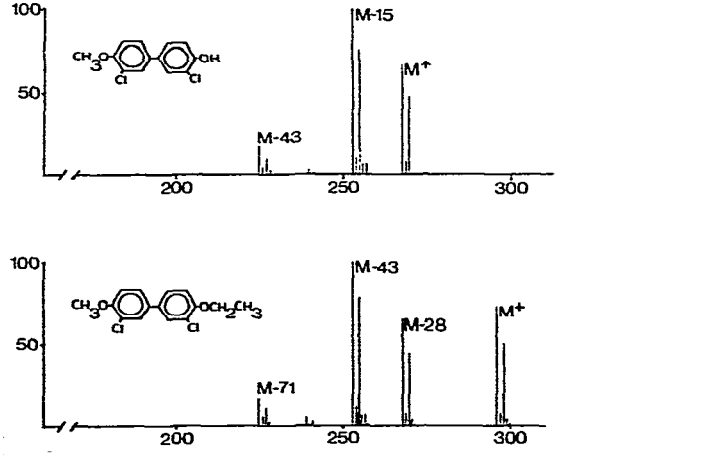

Fig. 4. Partial mass spectra of 3,3′-dichloro-4′-methoxy-4-biphenylol and its ethyl ether.

tion gives a mixture of three derivatives: $-OCD_3$, $-OCD_2H$ and $-OCDH_2$. These three, and the corresponding $-OCH_3$ derivative, all have identical retention times. Because of the interference of the specific isotope clusters of each isomer, it becomes impossible to calculate relative intensities, since there is no unequivocal molecular ion. This difficulty is illustrated by Fig. 5.

The mass spectra of chlorobiphenylols treated with deuteriodiazomethane also give the characteristic fragmentation pattern that is observed with the methyl ethers[2,3]. Deuteriomethylated samples show quite rapid deuterium exchange. Upon standing or after partial evaporation of solvent, spectra with a changed M^+-cluster are obtained.

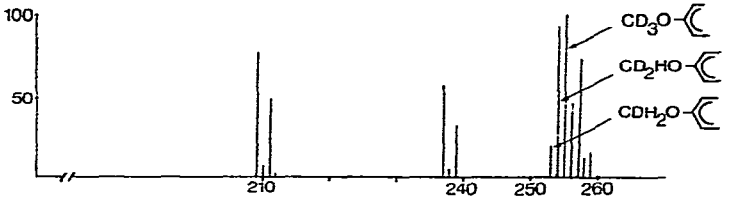

Fig. 5. Partial mass spectrum of 2′,5′-dichloro-4-biphenylol treated with deuteriodiazomethane.

Cyclic n-butylboronate formation

The two isomers of chlorobiphenyldiols having two hydroxy groups in the *ortho*-position relative to each other are structures A and B (Fig. 6). The methyl ethers of compounds with structures A and C and those of B and D give identical mass fragmentation patterns[4]. In metabolism studies with PCB isomers having two adjacent unsubstituted positions, chlorobiphenylols with structures as shown in Fig. 6 are possible metabolites, and to distinguish between A and C or B and D, synthesis of either one isomer is necessary for structural proof.

However, only A and B will form cyclic *n*-butylboronates, thus providing a simple unambiguous method to distinguish between the two types of isomer. We prepared cyclic *n*-butylboronates of 2,5-dichloro-2′,3′-biphenyldiol and 2,5-dichloro-3′,4′-biphenyldiol. GC–MS was performed with the reaction mixture. No further purifica-

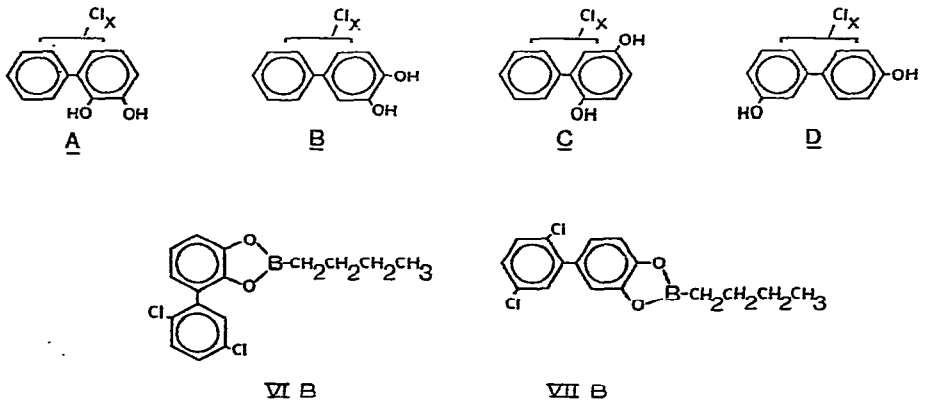

Fig. 6. Cyclic *n*-butylboronates and (potential) substrates.

tion was necessary. In the total ion chromatogram both compounds gave a sharp peak with *m/e* 320 a.m.u., in accordance with the structures VI B and VII B (Fig. 6); retention times were 8.3 and 8.4 min, respectively, using column B (temperature programme: 170° for 2 min, 8°/min to 240°).

In both cases the base peak was M—56 (loss of C_4H_8, known to occur with cyclic *n*-butylboronates[13,14] whilst the molecular ions were 61 and 63% of the base peaks, respectively.

Metabolism of 4'-chloro-4-biphenylol

4'-Chloro-4-biphenylol has been shown to give partly methylated metabolites in rats[6] and rabbits[7]. As described above, the metabolites in the urine were purified in four different ways after isolation, resulting in methylated, ethylated, deuteriomethylated and untreated samples. GC retention times and MS data of the metabolites are in Table II. The mass spectra of the methylated metabolites showed that M-1 (see Table II) was the unchanged methylated starting material. M-2 had either the structure of the methyl ether of m-2 (see Fig. 7) or that of compound XII[4]. A synthetic sample of compound XII showed a different retention time and, in the mass spectrum, different relative intensities than M-2. Metabolite M-4 was a trimethoxychlorobi-

TABLE II

GC RETENTION TIMES AND MASS SPECTRAL DATA OF METABOLITES

*Compound**	*Molecular formula*	*Retention time (min)***		*Mass spectrum****							
		A	*B*	*m/e*	*M⁺*	*M–15*	*M–28*	*M–43*	*M–56*	*M–71*	*M–99*
M-1	$C_{13}H_{11}OCl$	7.0	3.4	218	100	55	—	41	—	—	—
M-2	$C_{14}H_{13}O_2Cl$	11.0	7.2	248	100	31	—	24	—	—	—
XII	$C_{14}H_{13}O_2Cl$	—	8.5	248	100	44	—	22	—	—	—
M-4	$C_{15}H_{15}O_3Cl$	12.8	9.7	278	100	78	—	14	—	—	—
XIII	$C_{15}H_{15}O_3Cl$	—	12.9	278	100	21	—	28	—	—	—
E-1	$C_{14}H_{13}OCl$	7.9	4.0	232	55	—	100	—	—	—	—
E-2	$C_{15}H_{15}O_2Cl$	10.9	7.4	262	100	—	40	20	—	36	—
E-3	$C_{16}H_{17}O_2Cl$	11.2	7.6	276	95	—	19	—	100	—	—
E-4	$C_{17}H_{19}O_3Cl$	13.0	10.2	306	100	16	—	22	8	89	31
DM-1	§	6.8	—	221§	+§	+	—	+	—	—	—
DM-2	§	10.8	—	251	+	+	—	+	—	—	—
DM-3	§	10.8	—	254	+	+	—	+	—	—	—
DM-4	§	11.7	—	284	+	+	—	+	—	—	—
m-1	$C_{12}H_9OCl$	12.6	§§	204	100	—	—	—	—	—	—
m-3	$C_{13}H_{11}O_2Cl$	10.1	§§	238	100	39	—	37	—	—	—

* M = methylated, E = ethylated, DM = deuteriomethylated, m = untreated = true metabolite. For structures m-1–m-4 see Fig. 7, for compounds XII and XIII see Fig. 1.

** A = Column A, B = column B (see Materials and methods). Temperature programmes: A, 150° 2 min, 8°/min to 240°; B, 170° 2 min, 4°/min to 240°.

*** Calculations based on fragments containing ^{35}Cl only.

§ Mixtures of the $-CD_3$, $-CD_2H$ and $-CDH_2$ ethers. "Molecular ions" at *m/e* corresponding to each isomer. Intensities could not be calculated (see *Deuteriomethylation*).

§§ Could not be eluted.

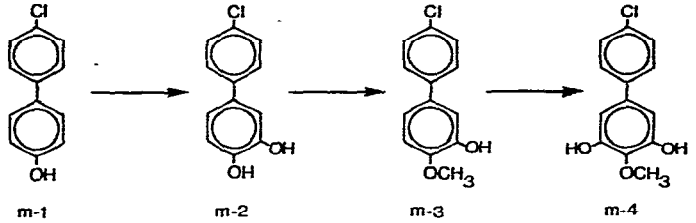

Fig. 7. Rat metabolism of 4'-chloro-4-biphenylol.

phenyl. A number of these compounds were synthesized and their mass spectra investigated[19]. It appeared that all isomers with a methoxy group *ortho* to the biphenyl bond showed a fragment M—50 (—CH_3—Cl). However, M-4 did not show this fragment which meant that two structures were possible: the methyl ether of m-4 or compound XIII (see Fig. 1). MS of a synthetic sample of XIII showed that this compound has different relative intensities and a different retention time than M-4. The structures deduced for M-1, M-2 and M-4 agree with earlier findings[6,7].

GC–MS investigation of the ethylated sample showed two partially methylated metabolites. E-2 has both a methoxy and an ethoxy group and the mass spectrum revealed both fragments M—43 and M—71[—(28 + 15) and —(28 + 43)], indicating that the methoxy group is in the 4-position. E-4 has two ethoxy groups and one methoxy group, and the mass spectrum showed that the methoxy group is in the 4-position, since both the fragments M–71 and M—99 [—(56 + 15) and —(56 + 43)] are present. E-1 is the unchanged ethylated starting material, E-3 is the ethyl ether of m-2. Compounds with *m/e* identical with M-1, M-2 and M-4 were not present in the ethylated sample, thus indicating that the methyl ethers of m-1, m-2 and m-3 are no metabolites. No triethoxychlorobiphenyl was found either, which implies that m-4 is formed from m-3, since an unmethylated analogue of m-4, the triol, would have resulted in a triethyl derivative.

The same conclusions were drawn from the GC–MS investigation of the deuteriomethylated sample. DM-2 with *m/e* 251 has one methoxy and one deuteriomethoxy group, whilst DM-4 has one methoxy and two deuteriomethoxy groups. M-1, M-2 and M-4 were not found in the deuteriomethylated sample, and no trideuteriomethoxy compound (*m/e* 287) was detected either.

Investigation of the untreated sample showed the necessity of derivatization: none of the metabolites with one or more free hydroxy groups could be eluted from column B, and from column A only m-1 and m-3 could be detected. The mass spectrum of m-3 confirmed the methoxy group to be at the 4-position.

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