

Metabolism of Hexachlorobenzene in Humans: Association between Serum Levels and Urinary Metabolites in a Highly Exposed Population

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Serum and urine from 100 subjects of a general population highly exposed to airborne hexachlorobenzene (HCB) were analyzed to obtain new insights into the metabolism of this ubiquitous compound. HCB was detected in all serum samples with concentrations ranging between 1.1 and 953 ng/ml. The major known metabolites of HCB were investigated in urine collected over 24 hr. Pentachlorophenol (PCP) was detected in all urines with values ranging between 0.58 and 13.9 μg excreted in 24 hr [mean \pm standard deviation (SD), 2.52 ± 2.05 ; geometric mean, 2.05]. A sulfur derivative that, after hydrolysis, yielded pentachlorobenzenethiol (PCBT) could also be identified and quantified in all the urines with values ranging between 0.18 and 84.0 μg of PCBT excreted in 24 hr (mean \pm SD, 3.47 ± 10.8 ; geometric mean, 1.39). The sulfur derivative assessed as PCBT appeared to be the main metabolite, with urinary concentrations surpassing those of PCP in the subjects with higher HCB accumulation (HCB in serum >32 ng/ml). PCBT concentration in urine collected over 24 hr showed a very strong association with HCB concentration in serum; the association was stronger in males than in females. An increase of 1 ng/ml of HCB in serum led to an increase of 2.12 μg of PCBT excreted in urine collected over 24 hr in males (95% CI, 1.82–2.44) and to an increase of 0.67 μg of PCBT in females (CI, 0.33–1.09). A weaker association was found between PCP in urine and HCB in serum, which was only statistically significant in males (an increase of 1 ng/ml of HCB in serum led to an increase of 0.63 μg of PCP excreted in urine collected over 24 hr; CI, 0.34–0.95). These results show that the formation of the cysteine conjugate is a quantitatively more important metabolic pathway in humans than the formation of PCP. Moreover, the association found suggests that PCBT is a good urinary marker of HCB internal dose and glutathione-mediated metabolism. *Key words:* exposed population, hexachlorobenzene, metabolism, pentachlorobenzenethiol, pentachlorophenol, urine. *Environ Health Perspect* 105:78–83 (1997)

Hexachlorobenzene (HCB) is a widespread environmental pollutant previously used as a fungicide for seed treatment (1). Currently, its main sources are impurities present in other chlorinated pesticides and by-products of several industrial emissions, notably in the chlorinated solvent industry through inappropriate manufacturing or waste disposal (2). HCB is a highly lipophilic chemical that is frequently found in food and accumulates in human tissues after ingestion (3,4). Porphyria is the major potential toxic manifestation of this chemical, both in experimental animals and in humans. A porphyria outbreak has been reported involving more than 3,000 people who had consumed food contaminated by HCB during the late 1950s in Turkey (5). HCB is also a potent carcinogen in rodents (6). No information on cancer incidence among the Turkish patients is currently available, but an excess risk for soft-tissue sarcoma and thyroid cancer has been found in a population exposed to airborne organochlorine mixtures with a high HCB content (7). Adverse reproductive effects induced by HCB have also been reported (8).

Little is known about the metabolism of HCB in humans despite several complete

rodent studies. Various phenolic derivatives, notably pentachlorophenol (PCP), tetrachloro-1,4-hydroquinone, and diverse tetra- and trichlorophenols have been identified in the excreta of rats dosed with HCB (9). The presence of PCP in human urine has been reported in several studies (10). Because HCB is ubiquitous in human tissues, some authors have hypothesized that most of the PCP found in urine may arise from the metabolism of the accumulated HCB (11). PCP, however, has been widely used as a wood preservative and is also common in some foods. Thus the potential direct ingestion of PCP through diet may hamper the possible correspondence between this polar compound and HCB.

Another major metabolic pathway observed in rodents is the conjugation of HCB with glutathione (12). The glutathione conjugate is further metabolized by cleavage of the glycine and glutamate residues to become pentachlorophenyl-*N*-acetyl-L-cysteine. Part of the mercapturate is eliminated unchanged via urine (13,14) and part may be further metabolized by cleavage of the C-S bond to produce pentachlorobenzenethiol (PCBT), pen-

tachlorothioanisole, tetrachloro-1,4-benzenedithiol, tetrachlorobenzenethiol, pentachlorobenzene, and other minor metabolites (15). A summary of the main HCB biotransformation pathways in rodents is shown in Figure 1.

In a previous study of human urine from a general population, a sulfur metabolite was identified that yielded PCBT after alkaline hydrolysis (16). However, the relative importance of this metabolic pathway for HCB kinetics and disposition, compared to the hydroxylation and formation of phenolic derivatives, remained unknown. Likewise, the significance of the different metabolites on the mechanism of action and toxicity of HCB also remains to be clarified.

A cross-sectional research project on the health effects of HCB is being carried out in Flix (Tarragona, Catalonia, Spain), a rural village of 5,000 inhabitants located near an organochlorine compound factory where high airborne HCB exposure has regularly occurred during approximately the last four decades (7). This project has also been used to obtain insight into human HCB metabolism. Thus, the HCB metabolites in urine samples collected over 24 hr (24-hr urine samples) of 100 individuals from this population have been identified, quantified, and correlated with HCB concentration in sera. The significant aspects of the resulting database for HCB metabolism in humans are described below.

Materials and Methods

Population of study. A total of 615 subjects of the general population (332 selected randomly and 283 volunteers) provided blood samples and 24-hr urine for the cross-sectional study. Previous written consent for inclusion in the study was obtained in all cases. A questionnaire that included inquiries about residence, occupation, lifestyle, and medical history was adminis-

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tered. Organochlorine levels in serum were analyzed in all the 615 participants. A subset of 92 subjects of both sexes was selected at random for analysis of HCB metabolites in urine. Eight additional 24-hr urine samples corresponding to the subjects with the four lowest and the four highest serum HCB concentrations were also included in the study. Urine samples were stored at -70°C until analysis.

Analysis of organochlorines in serum. Organochlorine compounds in sera were analyzed in the Department of Environmental Chemistry (CID-CSIC). Two-milliliter aliquots were spiked with an internal standard of 1,2,4,5-tetrabromobenzene (vortex stirring for 30/sec at 2,000 rpm). *n*-Hexane (3 ml) was added, followed by 2 ml concentrated sulfuric acid. After reaction, the mixture was stirred for 30 sec and the supernatant *n*-hexane phase was separated. The remaining sulfuric acid solution was reextracted two times with 2 ml *n*-hexane (stirring 30 sec). The combined *n*-hexane extracts (7 ml) were additionally cleaned with 2 ml sulfuric acid (stirring 30 sec). Then the *n*-hexane phase was separated and concentrated under a nitrogen stream to 500 μl .

The analyses were performed with a Hewlett-Packard model 5890 gas chromatograph (Hewlett Packard, Palo Alto, CA) equipped with an automatic injector and an electron capture detector. A column of 30 m \times 0.25 mm inner diameter coated with DB-5 was used (film thickness 0.25 μm). The temperature was held at 80°C for 1 min and then programmed to 300°C at $6^{\circ}\text{C}/\text{min}$, retaining the final temperature for 10 min. The injector and detector temperatures were 270 and 310°C , respectively. The injector was set in the splitless mode (solvent, iso-octane; hot needle technique), keeping the split valve closed for 35 sec. Helium was the carrier gas (50 cm/sec), and nitrogen was used as make-up gas (60 ml/min).

The structural identification of HCB was confirmed by analysis of selected samples by gas chromatography-mass spectrometry (GS-MS) in the chemical ionization-negative ion recording mode (NICI). A Varian Star 3400 (Walnut Creek, CA) coupled to a Finnigan Mat INCOS XL (San Jose, CA) was used for the analyses. The chromatographic conditions were the same as described above. A DB-5 column was used. The transfer line and ion source temperatures were 300 and 120°C , respectively. The reagent gas was methane. Data were acquired by scanning from 50 to 500 mass units as 1 sec/decade. Quantitation was performed by external standard. Standard curves for several linear concentration ranges of HCB (0.93–9.3,

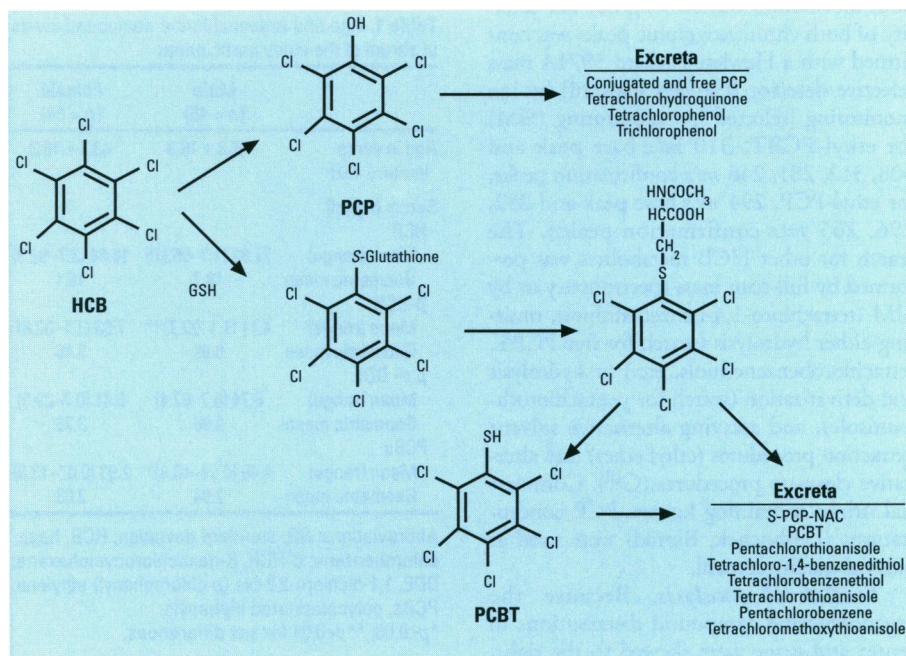


Figure 1. Main biotransformation pathways of hexachlorobenzene (HCB) in rodents and main metabolites found in urine and feces. Abbreviations: PCP, pentachlorophenol; GSH, reduced glutathione; PCBT, pentachlorobenzenethiol; S-PCP-NAC, pentachlorophenyl-*N*-acetyl-L-cysteine.

9.3–150, and 150–820 ng/ml), β -hexachlorocyclohexane (β -HCH; 1.75–15.5 and 15.5–1,550 ng/ml), 1,1-dichloro-2,2-bis (*p*-chlorophenyl)ethylene (*p,p'*-DDE; 1.5–13 and 13–130 ng/ml), and other organochlorine compounds were performed. The concentrations were calculated by interpolation on the linear curve corresponding to each compound. In the compounds eluting near HCB, these concentrations were corrected for recovery calculated from the internal standard. The samples were diluted in *n*-hexane in cases of concentrations higher than the linear range. Polychlorinated biphenyls (PCBs) were calculated by summation of the individual congeners 28, 52, 101, 118, 153, 138, and 180. Standard curves were determined for each congener. Detection limits were 0.2, 1.1, 0.7, and 0.05 ng/ml for HCB, β -HCH, *p,p'*-DDE, and PCBs, respectively.

Analysis of HCB metabolites in urine. Urinary metabolites of HCB were analyzed at the Toxicology Unit (Hospital Clínic, Facultat de Medicina). After hydrolysis of the urine, conjugated pentachlorophenol and pentachlorophenyl-*N*-acetyl-L-cysteine were assessed as PCP and PCBT, respectively (9). After addition of ascorbic acid (20 mg), the urine samples (4 ml) were spiked with an internal standard (aldrin) and digested under N_2 with 2 ml of 2N NaOH for 3 hr at 70°C . This alkaline hydrolysis yielded free PCP and PCBT. After cooling and acidification with HCl (pH = 1), the derivatives were extracted twice with toluene

(5 ml) and the solvent extracts were concentrated to 0.5 ml. Diazoethane in diethyl ether (0.25 ml) was added for derivatization (30 min in the dark). The excess diazoethane was removed under a nitrogen stream, the solvent extracts were concentrated to approximately 0.1 ml, and 2 ml *n*-hexane was added. The resulting mixtures were cleaned up with H_2SO_4 (17). The organic phase was separated and concentrated to approximately 50 μl . The quantification of the ethyl derivatives of PCP and PCBT was performed in a Hewlett Packard 5890 II gas chromatograph (Hewlett Packard) equipped with a 5% diphenyl-95% dimethylpolysiloxane (HP-ultra 2, 25 m \times 0.2 mm inner diameter) capillary column and a ^{63}Ni electron capture detector. The temperature was held at 60°C for 1 min and then programmed to 260°C at $5^{\circ}\text{C}/\text{min}$, retaining the final temperature for 45 min. Injector and detector temperatures were 280 and 300°C , respectively. Injection was in the splitless mode. Helium was the carrier gas and nitrogen was used as make-up gas. Quantitation of both derivatives was performed by internal standard. Peak area integration was used (Hewlett Packard 3396A integrator, Hewlett Packard), and linearity was checked with standard calibration curves of PCP and PCBT. The samples with concentrations higher than the linear range were diluted with *n*-hexane and quantified after addition of aldrin. Recovery (88–109%) was assayed with spiked urines. The detection limit for

both metabolites was 0.15 ng/ml. The identity of both chromatographic peaks was confirmed with a Hewlett-Packard 5971A mass selective detector (Hewlett Packard) by ion monitoring [selected ion monitoring (SIM) for ethyl-PCBT, 310 *m/z* base peak and 308, 312, 281, 246 *m/z* confirmation peaks; for ethyl-PCP, 294 *m/z* base peak and 292, 296, 265 *m/z* confirmation peaks]. The search for other HCB metabolites was performed by full-scan mass spectrometry or by SIM (tetrachloro-1,4-benzenedithiol), omitting either hydrolysis (search for free PCBT, tetrachlorobenzenethiols, etc.) or hydrolysis and derivatization (search for pentachlorothioanisole), and assaying alternative solvent extraction procedures (ethyl ether) and alternative clean-up procedures (C¹⁸). Commercial urines containing known PCP concentrations (Lyphochek, Biorad) were used as internal quality control.

Statistical analysis. Because the organochlorine compound distributions in serum and urine were skewed to the right, the natural logarithmic transformation (ln) was used in the analysis. To perform all the statistical analyses, the concentrations of organochlorine compounds below the detection limit were set at half the limit of detection. Multiple linear regression models were fitted to assess the association between HCB levels in serum and its urine metabolites while adjusting for other possibly confounding variables. The potential confounders examined included age (in years), body mass index (kg/m²), and current alcohol consumption. The possible confounding effects of other measured organochlorine compounds was also assessed. The regression models were stratified by sex including an interaction term. All analyses were performed in SPSS.PC (SPSS Inc., Chicago, IL).

Results

The study included 100 subjects (46 males and 54 females) with a mean age of 49.1 years. HCB was detected in all serum samples with values ranging between 1.1 and 953.0 ng/ml. HCB levels were higher in males than in females, but the differences were not statistically significant. Among the other major organochlorines analyzed, PCB levels were detected in all samples, ranging from 0.07 to 48.9 ng/ml. Levels of β -HCH were detected in all except nine cases (8 males, 1 female), ranging from 1.1 to 32.6 ng/ml and levels of *p,p'*-DDE were detected in all except three cases (2 males, and 1 female), ranging from 0.7 to 67.4 ng/ml (Table 1).

PCP was detected in all urines analyzed with values ranging between 0.58 and 13.9 μ g for 24 hr excretion [mean \pm standard deviation, (SD) 2.51 \pm 2.05; geometric

Table 1. Age and organochlorine compound levels in serum of the study participants

	Male (n = 46)	Female (n = 54)
Age in years (mean \pm SD)	48.8 \pm 18.9	49.2 \pm 18.3
Serum (ng/ml)		
HCB		
Mean (range)	71.82 (1.1–953.0)	18.84 (3.7–92.3)
Geometric mean	18.7	15.1
β -HCH		
Mean (range)	4.11 (1.1–22.7)**	7.08 (1.1–32.6)
Geometric mean	0.95	3.46
<i>p,p'</i> -DDE		
Mean (range)	6.74 (0.7–67.4)	6.41 (0.7–28.0)
Geometric mean	2.98	3.76
PCBs		
Mean (range)	4.49 (0.49–48.9)*	2.93 (0.07–13.8)
Geometric mean	2.94	2.03

Abbreviations: SD, standard deviation; HCB, hexachlorobenzene; β -HCH, β -hexachlorocyclohexane; DDE, 1,1-dichloro-2,2-bis (*p*-chlorophenyl) ethylene; PCBs, polychlorinated biphenyls.

* $p < 0.05$; ** $p < 0.01$ for sex differences.

mean, 2.05; Table 2, Fig. 2]. Only one sample showed a concentration higher than 9 ng/ml, which may be considered a reference value (upper normal limit) for PCP in urine according to Wrbitzky et al. (20). PCBT (the sum of all metabolites that yield PCBT after hydrolysis plus free unconjugated PCBT) was also detected in all urines with values ranging between 0.17 and 84 μ g eliminated for 24 hr (mean \pm SD, 3.47 \pm 10.8; geometric mean, 1.39; Table 2, Fig. 2). Because no reference limits exist for this derivative, the values previously published for the general population of Barcelona (16) were used as baseline normal levels, and the highest PCBT concentration found in that survey (4.5 ng/ml) was tentatively used as upper normal limit. Eight urine samples of the population under study exceeded the upper limit according to this criterion.

When hydrolysis was omitted, traces of free PCBT could also be detected in the samples with total PCBT >5 ng/ml. The values ranged between 3 and 9% with respect to the total PCBT. Other major HCB metabolites identified in rodent studies, such as tetrachlorobenzene, tetrachloro-1,4 benzenedithiol, tetrachloro-1,4 hydroquinone, pentachlorothioanisole, and pentachlorobenzene, could not be detected in any urine at a concentration above 0.2 ng/ml.

The relationship between urinary metabolites and HCB in serum is depicted in Figure 3. The shape of the relationship was not completely linear. At low HCB levels, the values of PCP were higher than those of PCBT, whereas at high HCB levels, PCBT was higher than PCP. Hence, the PCBT/PCP ratio in urine could be related to HCB in serum. Thus, ln(PCBT/PCP) and

Table 2. Urinary levels (μ g eliminated in 24 hr) of pentachlorophenol (PCP) and pentachlorobenzenethiol (PCBT) by hexachlorobenzene (HCB) serum levels and sex

	Male	Female
PCP (mean \pm SD)		
All cases (n = 100)	2.52 \pm 2.22	2.53 \pm 2.00
HCB, <32 ng/ml (n = 82)	1.89 \pm 1.07	2.59 \pm 2.03
HCB, >32 ng/ml (n = 18)	4.08 \pm 3.35	1.70 \pm 0.62
PCBT (mean \pm SD)		
All cases (n = 100)	6.38 \pm 16.16*	1.29 \pm 0.79
HCB, <32 ng/ml (n = 82)	1.35 \pm 1.01	1.20 \pm 0.65
HCB, >32 ng/ml (n = 18)	18.78 \pm 26.93	2.39 \pm 1.62

SD, standard deviation.

* $p < 0.01$ for sex differences.

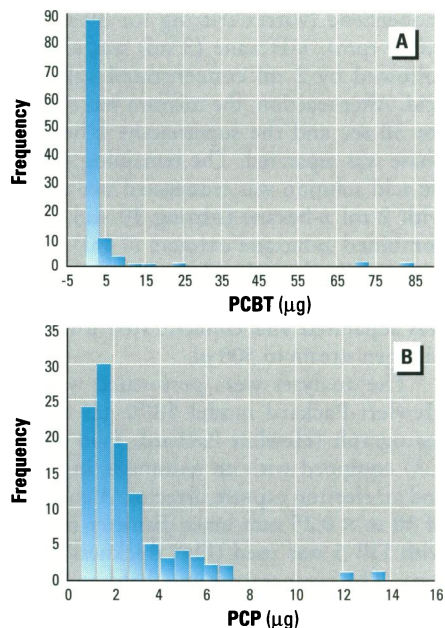


Figure 2. Frequency distribution of pentachlorophenol (PCP) and pentachlorobenzenethiol (PCBT) eliminated in urine collected over 24 hr.

ln(HCB) were linearly correlated. The PCBT/PCP ratios were lower and higher than unit for HCB concentrations below and above 32 ng/ml, respectively. Table 2 shows the average levels of metabolites stratified by sex and HCB levels.

The results of multiple linear regression analysis are shown in Table 3. An increase of 1 ng/ml HCB in serum led to statistically significant increases of PCBT urine in both males and females. The association was stronger in males than in females ($p < 0.05$ for interaction) when considering all the individuals, as well as when only assessing individuals with low levels of HCB ($p < 0.1$ for interaction). We could not assess sex-related differences in the association at high levels of HCB given the small number of women in that group. The association with PCBT in males did not substantially change at low and high HCB levels.

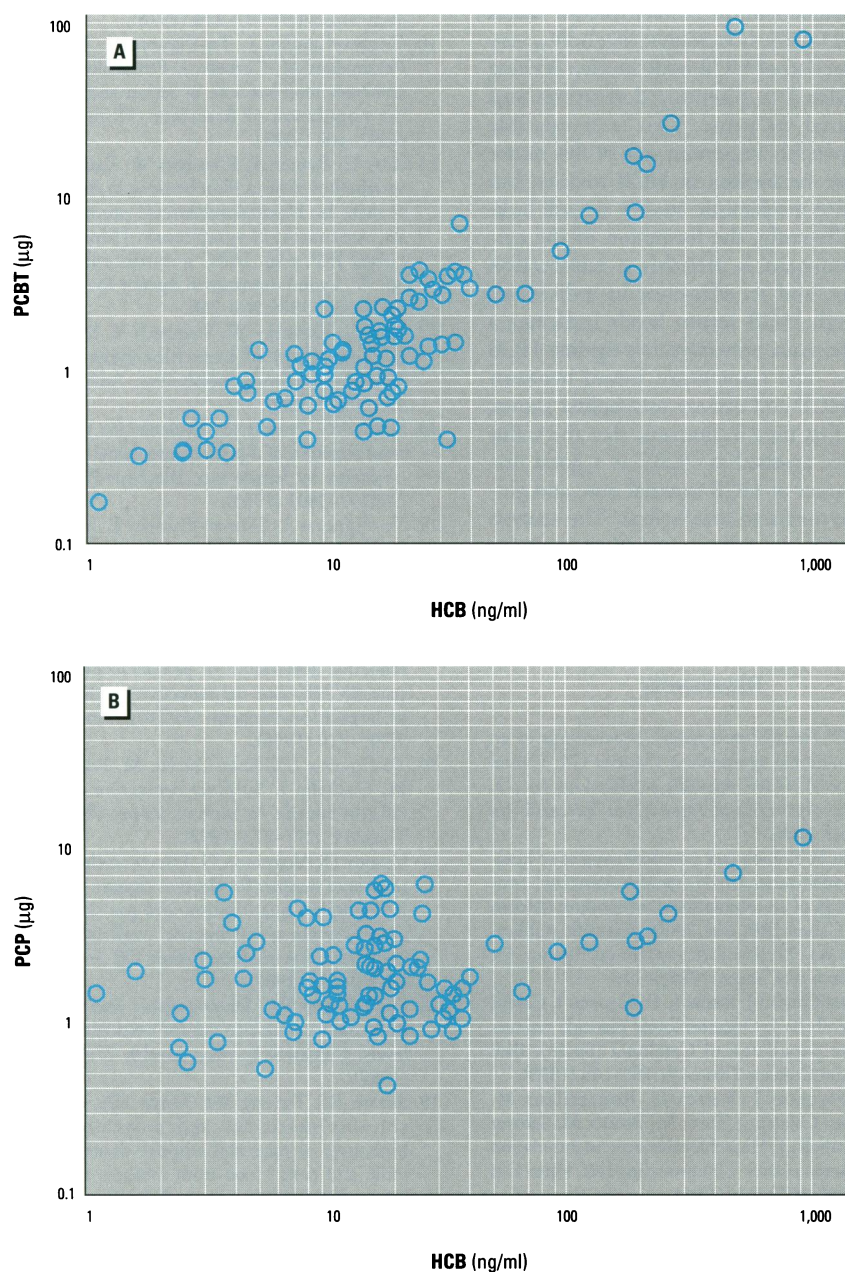


Figure 3. Correlation plots of pentachlorophenol (PCP) and pentachlorobenzenethiol (PCBT) eliminated in urine collected over 24 hr versus hexachlorobenzene (HCB) concentration in serum.

The association between HCB and PCP was less consistent (R^2 was 0.78 in the regression model with PCBT, and it was reduced to 0.19 in the regression model with PCP). The association only occurred in males and at high levels of HCB. The inclusion of age, body mass index, and alcohol consumption in the models did not confound the above results, and neither did the inclusion of other organochlorines (HCH, *p,p'*-DDE, PCBs).

Discussion

The serum HCB values found in the subjects from Flix are significantly higher than

those found in other European and U.S. studies (18,19), including those found in a recent survey of the population of Barcelona (4). Other ubiquitous organochlorines such as *p,p'*-DDE and β -HCH were not particularly high compared to these other reports. This indicates that in Flix there is a particular exposure to HCB but not a general exposure to organochlorines.

These elevated serum HCB concentrations were not reflected, however, in notable increases in PCP excretion via urine. Concentrations above 9 ng/ml were found only in one sample. These observations con-

Table 3. Change ($\mu\text{g}/\text{urine}$ collected in 24 hr) and 95% CI of pentachlorobenzenethiol (PCBT) and pentachlorophenol (PCP) for each increase of 1 ng/ml of hexachlorobenzene (HCB) in sera for all individuals and stratified by HCB levels

	Male	Female
All individuals		
PCBT	2.12 (1.82–2.44)*	0.67 (0.33–1.09)*
PCP	0.63 (0.34–0.95)*	-0.08 (-0.51–1.23)
HCB <32 ng/ml		
PCBT	1.95 (1.41–2.58)*	0.79 (0.33–1.39)*
PCP	0.54 (-0.15–2.27)	0.20 (-0.18–2.80)
HCB >32 ng/ml		
PCBT	2.09 (1.15–3.33)*	— ^a
PCP	0.77 (0.45–1.16)*	— ^a

^a $n < 5$.

* $p < 0.01$

tradict previous statements (11) and show that HCB hydroxylation leading to the formation of PCP in humans is very small in comparison with HCB accumulation. Moreover, some PCP in urine may arise from PCP present in food that is absorbed and eliminated unchanged or after conjugation with glucuronic acid. The PCBT/PCP ratio in the subjects with low HCB levels and the weak association between PCP and HCB reinforces this possibility.

The elimination of HCB as a sulfur metabolite was higher than the elimination as PCP in those individuals with higher internal dose of HCB (HCB in serum >32 ng/ml). These observations show that the conjugation of HCB with glutathione, leading to the formation of hydrosoluble sulfur metabolites, is a quantitatively more important biotransformation pathway than the hydroxylation and formation of phenolic metabolites. This is evident in the individuals with higher HCB levels for which the ratio PCBT/PCP (>1) is not as confounded by exogenous PCP as it may be in those with low HCB levels. The urinary metabolite that yields PCBT after alkaline hydrolysis is most probably pentachlorophenyl-*N*-acetyl-L-cysteine, although the presence of other closely related conjugates cannot be totally excluded. These results confirm an earlier report that showed the presence of a sulfur derivative in human urine assessed as PCBT (16). Urinary PCBT could also arise from ingestion and metabolism of the fungicide pentachloronitrobenzene (quintozone), but no exposure to that compound has been reported for the population under study. Moreover, the association between PCBT and HCB clearly suggests that most of the PCBT in urine arises from the metabolism of HCB.

When hydrolysis was omitted, free PCBT could be detected in some urines. This suggests that a minor part of the mercapturate may undergo *in vivo* cleavage of the C-S bond to yield free PCBT. This

pathway has been clearly documented in rodents (12,15), but the possibility of some degree of bacterial mediated hydrolysis during urine collection or spontaneous hydrolysis during acidification cannot be ruled out.

The very strong association between PCBT in urine and HCB in serum is striking and suggests a biotransformation pathway with small interindividual variations and scarcely affected by modifiers of hepatic enzymatic activity. These results are enhanced by the fact that the two parameters (HCB in serum, PCBT in urine) were analyzed and quantified by two independent laboratories. The addition of eight subjects with extreme values to the analysis of the randomly selected individuals did not modify any of the findings.

The excretion of the sulfur conjugate in urine showed a sex-related difference: males eliminated more PCBT than females. This could reflect some sex-related differences including perhaps a more efficient HCB metabolism in men. The confirmation of this finding requires further investigation, which is difficult to carry out in humans, but the results are in accordance with some studies made on populations exposed to background HCB, which have found higher HCB body burdens in women (22,23). Several studies in rats have shown sex-related differences in HCB metabolism together with higher susceptibility of females to HCB-induced porphyria (24,25), but a mechanistic link between both has not been elucidated. Another sex-related difference has been shown in rats, with females eliminating pentachlorophenyl-*N*-acetyl-L-cysteine in urine with a higher efficiency than males (13,14). The difference appears after rats are treated with either HCB or pentachloronitrobenzene, but it also appears after dosing the animals directly with pentachlorophenyl-*N*-acetyl-L-cysteine. As a consequence, the differences do not originate in a different rate of formation of the final cysteine derivative, but in a sex-dependent renal secretion mechanism particularly developed in female rats (13). The existence of a similar mechanism in species other than rats, however, has been not reported.

The relationship between HCB metabolism and toxicity is still not clear. Cytochrome P450-mediated oxidative metabolism has been implicated in several toxic effects of HCB, notably hypothyroidism (26) and porphyria (27,28) in rodent chronic studies.

These results show a low PCP urinary output, confirming the poor efficiency of HCB oxidative metabolism in humans. Taking into account the accumulation kinetics of HCB in adipose tissue and the

high adipose: blood concentration ratios reported (4,21), it is evident that only a very small fraction of HCB body burden is being metabolized to phenolic derivatives and eliminated via urine. This is compatible with a possibly important role of this minor oxidative metabolism in HCB toxicity, but conclusive mechanistic studies are still lacking. Differently, the formation of the cysteine conjugate is probably a detoxication pathway, and its efficiency in individuals with high HCB serum levels indicates a probable main protective role against HCB toxicity. The association found between serum HCB and urinary metabolites shows, for the first time in humans, that the sulfur derivative assessed as PCBT is a good urinary marker of HCB internal dose and glutathione-mediated metabolism. The analysis of feces in the same population is under study and will eventually provide a more complete approach to the kinetics of HCB in humans.

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