Evaluation of Urinary Porphyrin Excretion in Neonates Born to Mothers Exposed to Airborne Hexachlorobenzene

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The existence of a link between hexachlorobenzene (HCB) and porphyria cutanea tarda has been known for a long time. However, the epidemiologic data on effects on health caused by prenatal exposure have not provided convincing evidence that HCB alters porphyrin metabolism. Our objectives were to analyze urinary porphyrin excretion and HCB in maternal serum and fetal cord blood in neonates born in a village (Flix) near a chlorinated solvent factory, to detect possible adverse effects in urinary porphyrin excretion caused by prenatal exposure, and to assess their relationship with HCB blood levels. We conducted a cross-sectional study in the Porphyria Unit at a tertiary care facility in Barcelona, Spain, and the Pediatric Unit of the Móra d'Ebre Hospital, the reference hospital of the study area. We included in the study all neonates (n = 68) born in Móra d'Ebre Hospital 1997–1999 and their mothers. We obtained 68 urine specimens of singleton neonates on the third day after birth to test for urinary porphyrin excretion. We obtained 52 fetal cord blood and 56 maternal serum samples for HCB analysis. Total urinary porphyrins were quantified using spectrofluorometry. Porphyrin profile was determined by HPLC. Serum HCB was analyzed by gas chromatography coupled with electron capture detection. In total population, median HCB levels were 1.08 ng/mL in cord blood and 3.31 ng/mL in maternal serum. Total urinary porphyrin concentration was 37.87 µmol/mol creatinine. Coproporphyrin I and coproporphyrin III were the major porphyrins excreted. We found no positive relationship between urinary porphyrin excretion and HCB levels. However, we observed an association between maternal smoking and coproporphyrin excretion. Although high environmental levels of HCB are reported in the town of Flix, we found no alteration in urinary porphyrin excretion. *Key words*: **coproporphyrin I, coproporphyrin III, hexachlorobenzene, neonates, porphyria, uroporphyrin.** *Environ Health Perspect* **110:205–209 (2002). [Online 18 January 2002]**
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Hexachlorobenzene (HCB) is a widespread, highly lipophylic environmental pollutant that accumulates in biologic systems. Nowadays, the major source of HCB is industrial emission as a by-product of the manufacture of organochlorinated products.

Porphyria cutanea tarda (PCT) is the most common of human porphyria. This disease is caused by a partial deficiency of the uroporphyrinogen decarboxylase (UROD) enzyme in the liver, and it is one of the major potential toxic manifestations of this chemical, as several studies in experimental animals have demonstrated (*1,2*). The disease is characterized biochemically by marked increases in uroporphyrin and heptaporphyrin in urine (*3*).

Although the existence of a link between HCB and porphyria has been known for a long time, the porphyrinogenic effect of this chemical on humans has not been widely studied. The first cases of PCT induced by HCB in humans were reported in southeastern Turkey in the late 1950s (*4*). The outbreak was related to the inadvertent ingestion of seed wheat contaminated with the fungicide HCB. It was estimated that 5,000 subjects developed acquired PCT. The syndrome commonly consisted of weight loss, weakness, thyromegaly, hepatomegaly, gross porphyrinuria, hypertricosis, and photosensitive dermopathy (*4*). Children were affected disproportionately more than adults; it was estimated that over 3,000 children under age 16 who had ingested contaminated bread developed the disease. However, the group most severely affected by this chemical were breast-fed babies. Over 1,000 babies born to mothers with clinical symptoms of PCT or who had ingested contaminated bread during gestation or lactation or both died before the age of 12 months with weakness, convulsions, and toxic erithema known as "pembe yara." Pembe yara was caused by HCB intake both transplacentally and via mother's milk (*5*). This was the first evidence that HCB is toxic to young children. Unfortunately, no dose–response data were recorded for the Turkish outbreak (*6*).

HCB and other organochlorine compounds are not readily metabolized or excreted. In pregnant mothers they persist in placenta tissue and are consequently transferred transplacentally from mother to fetus (*7–9*). However, little information is available about the relation between prenatal exposure to HCB and porphyrin metabolism in human populations. Several studies have reported the effects of other organochlorinated compounds such as polychlorinated biphenyls, dichlorodiphenyldichloroethylene, dichlorodiphenyltrichloroethane, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (*10–13*) on urinary porphyrin excretion in children and neonates, but the impact of HCB on these populations has not been analyzed to date.

Previous studies made by our group, in adults of the same population, showed high atmospheric levels of HCB (mean 35 µg/m^3) in Flix (Tarragona, Catalonia, Spain), a rural village of 5,000 inhabitants located near a chlorinated solvent factory (*14*). We performed a cross-sectional epidemiologic study of the health effects of HCB on the population older than 14 years. We found high serum levels of HCB (mean 36.7 ng/mL), the highest ever recorded (*15,16*). The evaluation of the urinary pophyrin excretion showed one case of subclinical PCT and 5 subjects with coproporphyrinuria. No association between HCB serum concentrations and total urinary porphyrin excretion was found. The porphyrin profile of the highly exposed subjects was normal (*17*). Analysis of HCB metabolism and excretion in urine and feces revealed a strong correlation between HCB serum concentrations and pentachlorobenzenetiol (PCBT) in urine (*18*) and unmetabolized HCB (*19*).

Although PCT has been associated most frequently with excessive alcohol consumption in middle-aged men, it has also been

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reported in children (*20*). The availability of a population with high exposure to HCB over the last four decades prompted us to investigate the possible existence of subclinical changes in urinary porphyrin excretion in neonates exposed transplacentally to this chemical, so as to broaden our understanding of its toxic effects.

Materials and Methods

Study population. We performed a cross-sectional study of all 68 full-term singleton neonates born in the Department of Obstetrics at the Móra d'Ebre Hospital between 1997 and 1999. Thirty-eight were from the exposed population (Flix); the other 30 were from neighboring villages and were selected as a nonexposed group.

Information on maternal history, sex, and fetal exposure to alcohol and tobacco consumption was obtained through a questionnaire prepared ad hoc for this study.

Using urine collection bags, we collected fresh urine samples on the third day after birth from 68 full-term neonates. Specimens were immediately frozen and stored at –20°C until assayed. We obtained 52 fetal cord blood (23 from exposed vs. 29 nonexposed) and 56 maternal serum (24 exposed vs. 32 nonexposed) samples for HCB analysis. Informed consent was obtained from parents before collection.

Urinary porphyrin measurements. All urine specimens were analyzed without previous knowledge of HCB levels in fetal cord blood and maternal serum. We determined total urinary porphyrin concentrations by spectrofluorometry (model F-2000; Hitachi Ltd., Tokyo, Japan) (*21*). We analyzed urinary porphyrin excretion patterns by HPLC. Briefly, 1 mL of urine sample was acidified with 50 µL of concentrated HCl, and 200 µL of this solution was injected. For HPLC determination, we used Waters equipment (Waters Corp., Milford, MA, USA): two pumps (model 515), an autosampler injector (model 717 plus), and Millennium^{32} software. The porphyrins were detected using a fluorescence detector (model 474), under the following conditions: excitation 405 nm and emission 618 nm, both with bandwidths of 18 nm. Porphyrin separation was achieved with an analytic column BDS-Hypersil (250 × 4.6 mm, 5 µm particle size; Shandon HPLC, Cheshire, UK) and a gradient from 100% of solvent A (10:90 acetonitrile/ammonium acetate 1M pH 5.16) to 95% of solvent B (10:90 acetonitrile/methanol) in 25 min. The flow rate was 1.2 mL/min (*22*).

Urinary creatinine determinations. We measured urinary creatinine concentration (mmol/L) using the Jaffe method (*23*) on a Cobas Miras (Roche Diagnostics, F. Hoffmann-La Roche Ltd., Basel, Switzerland). Creatinine was analyzed in the department of biochemistry at the Hospital Clínic, Barcelona.

Analysis of organochlorine compounds. All HCB sera samples were extracted with *n*-hexane and the extracts blindly assayed with gas chromatography coupled to electron capture detection (GC-ECD) in the department of environmental chemistry at the Consejo Superior de Investigacion Cientifica in Barcelona.

Statistical analysis. Because the data distribution on porphyrins was skewed, we performed a logarithmic transformation. The variable HCB was treated as a trichotomous variable with values HCB < 2.43, 2.43 < HCB < 4.07, and HCB > 4.07 ng/mL because the relationship between HCB and uroporphyrin isomer I (UPI), coproporphyrin isomer I (CPI), and coproporphyrin isomer III (CPIII) was not linear.

The concentration of porphyrins below the quantification limit was set at half the limit of detection. To evaluate the relationship among porphyrins (total porphyrin, UPI, CPI, CPIII) and possible confounding variables (sex, gestational age, maternal age, birth weight, alcohol, and tobacco) we used linear regression models.

We performed multiple linear regression analysis to examine the relationship between porphyrins (UP, CPI, CPIII) and HCB levels (fetal cord serum and maternal serum) adjusting for potential confounding variables such as sex, tobacco, alcohol, and maternal age. Statistical significance was defined as $p < 0.05$. All statistical analyses were performed using Stata (StataCorp., College Station, TX, USA).

Results

The anthropometric variables, gestational age, maternal age, and alcohol and tobacco habits in mothers of the population under study are shown in Table 1. Exposed and nonexposed neonates differed in terms of maternal alcohol consumption and smoking during pregnancy. The number of males born in the exposed group was higher than in the nonexposed group.

We found detectable levels of HCB (nanograms per milliliter) in all samples of fetal cord blood and maternal serum. The medians and interquartile ranges are shown in Table 2. In fetal cord blood 59% of cases showed HCB concentrations over 1 ng/mL, a figure that rose to 91% in maternal blood. HCB levels in both sets of samples were slightly higher in the exposed group than in the nonexposed group; the difference was statistically significant ($p < 0.05$).

We analyzed total porphyrin concentrations and individual porphyrins (HPLC) in all 68 urine specimens. The median and interquartile ranges for total porphyrin and the main individual porphyrins excreted are summarized in Table 2. CPI, CPIII, and UPI were the major porphyrins excreted in

Table 1. Characteristics of the study groups.

Variable	Total population ($n = 68$) Mean (SD)	Exposed ($n = 38$) Mean (SD)	Nonexposed ($n = 30$) Mean (SD)
Weight (g)	3,281 (457)	3,244 (503)	3,328 (396)
Height (cm)	49.5 (2.0)	49.4 (2.1)	49.6 (1.8)
Gestational age (weeks)	39.6 (1.5)	39.7 (1.5)	39.5 (1.5)
Maternal age (years)	30.8 (4.5)	30.3 (4.3)	31.5 (4.7)
Sex M/F	37/31	27/11	10/20
Maternal smoking during pregnancy (%)[a]	27.9	39.5	13.3
Maternal drinking during pregnancy (%)[b]	20.6	26.3	13.3

Abbreviations: F, females; M, males.
[a]At least a cigarette a day during gestation. [b]Moderate/high alcohol intake (as at least once a week) during gestation.

Table 2. Median (interquartile range) of HCB levels in fetal cord serum and maternal serum, and urinary prophyrin excretion in the study groups

Variable	Total population Median (IQR)	Exposed Median (IQR)	Nonexposed Median (IQR)
ng/mL			
HCB cord blood ($n = 52$)	1.08 (0.73–1.67)	1.41 (0.86–2.05)	0.92 (0.53–1.21)*
HCB maternal serum ($n = 56$)	3.35 (2.11–5.47)	3.83 (2.92–5.71)	2.44 (1.52–3.77)*
µmol/mol creatinine ($n = 68$)			
Total porphyrin	37.3 (27.9–50.5)	39.2 (27.4–56.6)	36.2 (27.9–48.3)
UPI	5.0 (1.9–6.6)	5.0 (2.2–6.7)	5.1 (1.6–6.8)
UPIII	0.05 (0.05–0.5)	0.05 (0.05–0.7)	0.05 (0.05–0.05)
UPI + UPIII	5.6 (1.9–7.3)	5.6 (2.2–7.5)	5.5 (1.6–6.8)
CPI	13.7 (6.3–18.7)	15.1 (6.7–21.5)	9.8 (5.3–17.1)
CPIII	10.2 (5.2–20.5)	11.6 (5.8–22.7)	7.4 (4.7–12.7)*
CPI + CPIII	23.0 (12.6–38.7)	27.8 (14.0–42.9)	19.2 (12.5–30.5)

IQR, interqualtile range (25th–75th percentile).
*$p < 0.05$ compared with exposed population using Kruskal-Wallis nonparametric test for equality of populations.

both groups. Neonates from the exposed group had higher levels of CPIII ($p < 0.05$) than did those of the nonexposed group. The uroporphyrin fraction was the third most excreted porphyrin; more UPI was excreted than UPIII (Figure 1).

We detected the heptacarboxylporphyrin isomer I (hepta I) only in four cases in the exposed group (10.26%), at very low concentrations (median 0.21). We detected the heptaporphyrin isomer III (hepta III) in 14 cases (35.9%) in the exposed group and three cases (9.4%) of the nonexposed group ($p < 0.012$). All values were within the normal range (median 0.94). We found no difference in HCB concentrations between the subjects with and without hepta III. The hexa and pentacarboxylporphyrin fractions were not detected in any group.

Table 3 shows the relationship among total porphyrin, UPI, CPI, and CPIII concentrations, HCB levels, and other characteristics of the cohort studied. We observed a decrease of urinary porphyrin excretion with HCB levels. Moreover, the neonates in the highest tertile of HCB had lower levels of CPI and CPIII ($p < 0.05$).

In neonates born to cigarette-smoking mothers, the excretion of total porphyrin and CPIII fraction were higher than in nonsmokers ($p < 0.05$).

The association between CPIII and the exposed group disappeared after adjusting for smoking (Table 4), because mothers from the exposed group smoked more during pregnancy. However, the negative association of CPI and CPIII with the highest tertile of HCB did not disappear after adjusting for smoking and alcohol, and remain statistically significant for CPI ($p < 0.05$).

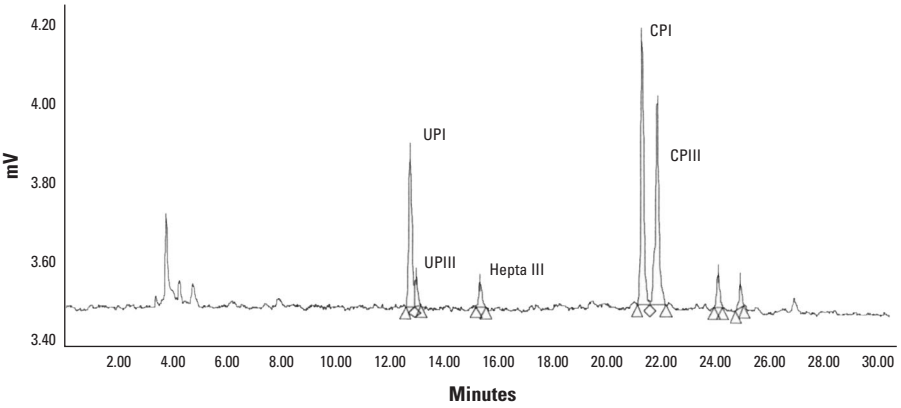

Figure 1. Porphyrin excretion pattern in a 3-day-old neonate.

Table 3. Median (IQR) of variables UPI, CPI, and CPIII, by birth weight, gestational age, maternal age, sex, smoking, alcohol, HCB maternal serum, and HCB fetal cord blood.

Variable	No.	Total porphyrin	UPI[a]	CPI[a]	CPIII[a]
Birth weight (g)					
< 2,500	3	41 (29–57)	5.3 (0.1–10)	17 (15–22)	25 (6.4–32)
≥ 2,500	65	37 (27–50)	5.0 (2.0–6.6)	13 (6.2–18)	10 (5.0–19)
Gestational age (weeks)					
< 37	2	37 (33–41)	5.1 (0.1–10)	24 (23–25)	15 (7.4–23)
≥ 37	66	37 (27–52)	5.0 (2.2–6.6)	13 (6.2–17)	10 (5.0–20)
Maternal age (years)					
≤ 30	28	39 (33–57)	5.5 (2.7–7.3)	15 (7.3–23)	11 (6.8–22)
> 30	40	37 (27–49)	4.8 (1.6–6.4)	12 (5.1–17)	7.6 (3.5–21)
Sex					
Male	37	38 (29–49)	5.8 (2.7–7.5)	15 (6.8–21)	11 (3.8–20)
Female	31	37 (25–57)	4.0 (0.7–6.3)	10 (5.2–17)	9.8 (5.8–21)
Smoker					
No	49	35 (26–43)	4.9 (2.4–6.6)	12 (5.3–17)	7.4 (4.1–18)
Yes	19	47 (40–60)*	5.8 (0.1–6.9)	15 (9.0–22)	16 (10–23)*
Alcohol					
No	54	36 (26–48)	4.8 (1.4–6.6)	14 (6–18)	10 (4.7–21)
Yes	14	42 (37–59)	6.0 (4.1–8.0)	15 (6.3–23)	10 (6.4–20)
HCB fetal cord (ng/mL)					
<0.8	16	48 (28–70)	6.5 (2.6–8.7)	13 (7.3–23)	13 (5.2–23)
0.8–1.48	20	39 (35–53)	5.9 (3.8–6.6)	15 (8.6–19)	13 (7.4–23)
> 1.48	16	26 (19–39)*	3.1 (0.9–6.2)	6.2 (2.9–13)*	5.1 (2.1–11)*
HCB mother serum (ng/mL)					
< 2.43	19	48 (34–68)	6.1 (3.4–8.8)	15 (9.7–23)	7.4 (5.1–21)
2.43–4.07	17	40 (30–45)	5.8 (3.3–6.7)	17 (8.3–22)	12 (7.7–22)
> 4.07	20	29 (25–38)*	4.5 (1.6–6.2)	6.8 (5.0–14)*	7.1 (2.1–18)

IQR, interquartile range.
[a]μmol/mol creatinine. *$p < 0.05$ using Kruskal-Wallis nonparametric test for equality of populations.

Discussion

Because long-term exposure to HCB can cause its accumulation in humans, adverse effects on health are expected in exposed populations. Public attention has been drawn to HCB when high concentrations are found in the environment, as is the case in Flix (*14,15*). Detecting subtle alterations in urinary porphyrin excretion may be one of the most useful methods for identifying the biologic response to environmental chemicals.

In the Turkish outbreak, high HCB levels were found in maternal milk (*6*), but urinary porphyrin excretion was not studied in the breast-fed babies. Porphyria acquired transplacentally in response to HCB exposure has been demonstrated in experimental animals (*24*) but little is known about the effects in infants (*25*). The present study is the first to analyze the urinary porphyrin excretion in human neonates in relation to HCB levels in cord blood and maternal serum. The neonate's HCB burden depends on the mother's level of contamination. In this cohort, the mothers had been living for a long time in a population with the highest environmental (mean 35 ng/m^3) (*14*) and serum (36.7 ng/mL) levels of this chemical ever reported (*15*), although in recent years HCB levels in the area around Flix have decreased (*16*) because of protective measures implemented in the factory. However, the HCB concentrations found in cord blood of exposed neonates and in maternal serum were still higher than in the nonexposed group (Table 2). The values in both exposed and nonexposed groups are higher than those found in neonates born in Germany between 1994 and 1995 (median 0.61 ng/mL), but lower than neonates born in Germany between 1984 and 1985 (median 2.03 ng/mL) (*26*). These values in Europe are much higher than those found in Canada (median 0.04 ng/mL) (*27*) during 1993–1995 and the United States (median 0.03 ng/g) during 1993–1998 (*28*).

The results of this study showed no alteration in total urinary porphyrin excretion. The quantitative analysis did not reveal any differences between groups concerning total porphyrin excretion (Table 2). The total urinary porphyrin concentrations detected here are within the ranges observed in other studies of neonates and pediatric groups (*29–32*). Unfortunately, in the Turkish

Table 4. Coefficient (SE) obtained with multivariate linear regression between log-transformed porphyrins variables and exposed group, maternal HCB and cord HCB in separate models, adjusting for mother's age, smoking, alcohol, and sex of children.

Variable	Total porphyrin	UPI[a]	CPI[a]	CPIII[a]
Model with exposed group[b]				
Exposed	–0.04 (0.16)	–0.21 (0.48)	0.09 (0.22)	0.31 (0.26)
Smoker	0.32 (0.17)	–0.89 (0.51)	0.16 (0.24)	0.56 (0.27)*
Alcohol	0.13 (0.18)	1.02 (0.55)	0.03 (0.25)	–0.03 (0.29)
Model with HCB fetal cord[b]				
HCB fetal cord (ng/mL)				
0.8–1.48	0.05 (0.20)	0.11 (0.61)	0.26 (0.24)	0.31 (0.29)
> 1.48	–0.49 (0.22)*	–0.56 (0.65)	–0.69 (0.26)*	–0.58 (0.32)
Smoker	0.16 (0.20)	–1.04 (0.61)	0.32 (0.24)	0.67 (0.29)*
Alcohol	0.28 (0.24)	1.18 (0.72)	–0.14 (0.29)	–0.46 (0.35)
Model with HCB maternal serum[b]				
HCB maternal serum (ng/mL)				
2.43–4.07	–0.23 (0.18)	–0.10 (0.55)	–0.09 (0.27)	0.46 (0.35)
> 4.07	–0.47 (0.19)*	–0.48 (0.56)	–0.79 (0.27)*	–0.06 (0.34)
Smoker	0.08 (0.19)	–0.97 (0.57)	0.11 (0.28)	0.72 (0.35)*
Alcohol	0.29 (0.19)	1.29 (0.56)*	0.19 (0.27)	0.02 (0.35)

[a]μmol/mol creatinine. [b]Coefficient for maternal age and sex not shown. *$p < 0.05$.

outbreak, urinary porphyrin concentrations were not recorded. Although levels of porphyrins were within the normal range (even subclinical normality), we observed a decrease of CPI and CPIII with the highest tertile of HCB (only statistically significant for CPI). This negative association between CP fractions and HCB levels does not agree with the results on experimental porphyria where moderate increases of CP are observed at an early stage (*2*). However, the small number of subjects and the known variations on the profile of porphyrin excretion during the first days of life preclude a firm conclusion.

The increase of the uroporphyrin and heptaporphyrin fractions is a specific indicator of UROD deficiency. In our cohort, no neonates showed subclinical alterations in the urinary porphyrin profile. CPI was the major porphyrin excreted, in accordance with the normal pattern observed in neonates < 7 days of age, followed by CPIII fraction (*32*). We found no difference in CPI excretion between both groups, but there was a statistically significant difference in CPIII excretion between exposed and nonexposed groups (Table 2). However, the increase in CPIII excretion cannot be explained by higher HCB concentrations in blood because no positive association was found between the two variables. The UP fraction, the third most excreted porphyrin, was also within normal ranges (*30,31*). Although the heptaporphyrin fraction is present most frequently in exposed populations, we found no evidence of a relationship between the presence of this porphyrin and HCB concentrations in blood. This lack of association between urinary porphyrin excretion and HCB levels has been observed previously in adults of the same population (*17*).

The information obtained through the questionnaire reflected differences in response between populations studied regarding tobacco and alcohol habits. In the exposed group the proportion of mothers who smoked and consumed alcohol was higher than in the nonexposed (Table 1), and a relationship between CP excretion and tobacco smoking was found (Table 3). Therefore, the greater CPIII excretion detected in the exposed population may be explained by the deleterious effect of tobacco smoking. Because some cytochrome P450 isoenzymes, such as P4501A2, are involved in disturbances of porphyrin metabolism a possible indirect effect of smoke through a P450 induction cannot be ruled out (*33*).

We conclude that although high environmental levels of HCB have been reported in Flix, no major alteration in urinary porphyrin excretion is present in neonates. The placental transfer of HCB to the fetus may not reach the threshold for subclinical alteration in porphyrin excretion pattern, considering that HCB levels in mothers are not high at the time of the study. Our findings show only a greater urinary CPIII excretion in the exposed population. However, we found no evidence for an association between HCB levels and the amount of CP. Probably, this increases of CP III is caused by a higher tobacco consumption by the mothers in this group, but further research must be done to elucidate the tobacco effect on porphyrin metabolism. The HCB levels described here could be considered a guideline for evaluating further research in other populations.

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