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Assessing the health risks following environmental exposure to hexachlorobutadiene

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Abstract

Hexachloro-1,3-butadiene (HCBD) has been reported to be toxic to the rat kidney in a 2 year study at doses higher than 0.2 mg/kg/day. The toxicity is known to be a consequence of the metabolism of HCBD by glutathione conjugation and the renal β-lyase pathway. Neither toxicity data, nor data on the metabolism of HCBD, are available in humans. In the current work, the potential of HCBD to cause kidney damage in humans environmentally exposed to this chemical has been assessed quantitatively by comparing the key metabolic steps in rats and humans. To that end, the hepatic conjugation of HCBD with glutathione, the metabolism of the cysteine conjugate by renal β-lyases and *N*-acetyltransferases, and the metabolism of the *N*-acetylcysteine conjugate by renal acylases has been compared in vitro in rat and human tissues. Rates for each metabolic step were lower in humans than in rats; 5-fold for glutathione conjugation, 3-fold for β-lyase and 3.5-fold for *N*-acetyltransferase. Acylase activity could not be detected in human kidney cytosol. Use of these data in a physiologically based toxicokinetic model to quantify metabolism by the β-lyase pathway demonstrated that metabolism in humans was an order of magnitude lower than that in rats. At the no effect level for kidney toxicity in the rat the concentration of β-lyase metabolites was calculated by the model to be 137.7 mg/l. In humans the same concentration would be achieved following exposure to 1.41 ppm HCBD. This is in contrast to the figure of 0.6 ppb which is obtained when it is assumed that the risk is associated with the internal dose of HCBD itself rather than β-lyase metabolites.

Keywords: Hexachlorobutadiene; Kidney; Modelling; Risk assessment

1. Introduction

Environmental contamination with hexachloro-1,3-butadiene (HCBD) has recently been discovered at an historic waste disposal site in the UK. The transfer of vapour from the site into the surrounding strata led to the detection of low concentrations of HCBD, typically less than 8 ppb, in the indoor air of a small number of houses immediately adjacent to the site. The consequences of this exposure on human health are uncertain, there being very little information on the toxico-

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logical effects of HCBD derived from studies on humans. Consequently, an assessment of the possible risks to human health has to be based on laboratory animal data.

The toxicity of HCBD has been studied extensively in the rat and to a lesser extent in a number of other laboratory species. The primary target organ in all species is the kidney. In the rat, the nephrotoxicity is well characterised with respect to dose, morphological changes and changes in blood and urine biochemistry. Following either a single oral or intraperitoneal dose, necrosis is seen in the pars recta of the proximal tubule (Ishmael et al., 1982) together with increases in blood urea nitrogen and marked increases in urinary protein, glucose and a number of enzymes normally found in the proximal tubule of the kidney (Lock and Ishmael, 1979; Nash et al., 1984; Ishmael and Lock, 1986). Following chronic dosing, a low incidence of kidney tumours was found in rats administered HCBD in the diet. The no observed effect level (NOEL) for kidney toxicity in this study was 0.2 mg/kg/day.

The mode of action of HCBD as a nephrotoxin is also understood. HCBD is metabolised in the liver to form a glutathione conjugate which is subsequently further metabolised to the equivalent cysteine conjugate (Nash et al., 1984; Wolf et al., 1984). The cysteine conjugate may be metabolised to its mercapturate, the *N*-acetylcysteine conjugate or undergo C–S bond cleavage by renal C–S or β-lyase enzymes (Jaffe et al., 1983; Green and Odum, 1985; Schrenk et al., 1988). It is the action of β-lyases that leads to the formation of a number of reactive intermediates and the observed kidney toxicity. The *N*-acetylcysteine conjugate may be excreted in urine or converted back to the cysteine conjugate by the action of acylases, the latter increasing the amount of cysteine conjugate available for cleavage by β-lyases. Nephrotoxicity is therefore directly linked to β-lyase activity and the amount of substrate, the cysteine conjugate, available to this enzyme.

The toxicity of HCBD in humans is not known, nor has the metabolism of HCBD been studied either in vivo or in vitro. All of the relevant enzymes needed to activate HCBD, including β-lyases, are known to exist in humans and hence HCBD has the potential to be nephrotoxic in humans. That potential can be assessed quantitatively by comparing the key metabolic steps in the activation of HCBD with those in the rat where the toxicity is known. To that end the hepatic conjugation of HCBD with glutathione, the metabolism of the cysteine conjugate by renal β-lyases and *N*-acetyltransferases, and the metabolism of the *N*-acetylcysteine conjugate by renal acylases have been compared in vitro in rat and human tissues. The data have then been used in a physiologically based toxicokinetic model (PB-TK model) to quantify the extent of metabolism by β-lyase in order to assess the risks to humans exposed to HCBD. This approach has been compared with that using default assumptions in the absence of metabolic data. A summary of the metabolism of HCBD and the key steps measured in vitro and used in the model is shown in Fig. 1.

2. Materials and methods

2.1. Chemicals

HCBD (99%) was supplied by BDH Chemicals Ltd, Poole Dorset. *S*-(1,2,3,4,4-pentachloro-1,3-butadienyl)glutathione (HCBD-GSH), *S*-(1,2,3,4,4-pentachloro-1,3-butadienyl)-L-cysteine (HCBD-CYS) and *N*-acetyl-*S*-(1,2,3,4,4-pentachloro-1,3-butadienyl)-L-cysteine (HCBD-NAC) were synthesised as described previously (Nash et al., 1984). [Acetyl-1-^{14}C]-coenzyme A (40–60 mCi/mmol) was obtained from Amersham-Pharmacia, Cardiff, UK.

2.2. Animals and tissues

Male Alderley Park (Alpk/APfSD) rats weighing 180–200 g were obtained from the Barriered Animal Breeding Unit, Alderley Park, Macclesfield, Cheshire, UK. The clinical condition of all animals was monitored prior to the start of the experiments and any animal showing adverse clinical signs was removed.

The animals received Rat and Mouse Number 1 pelleted diet from Special Diet Services, Witham, Essex, UK, and mains water ad libitum. The

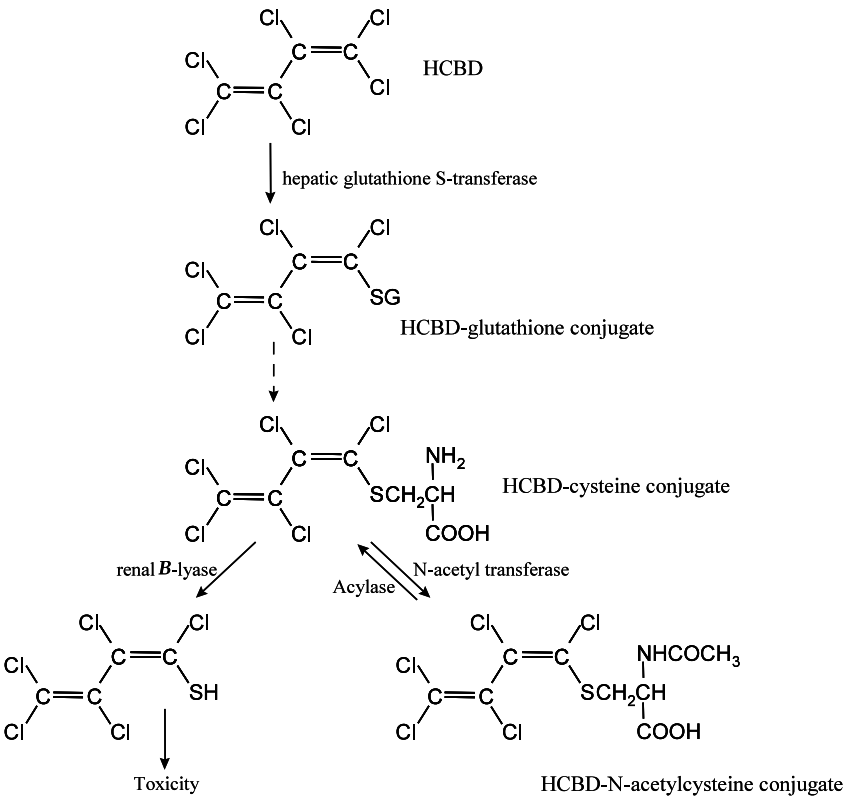

Fig. 1. The metabolic activation of HCBD. The enzymes shown are those compared between rats and humans and used in the PB-TK model.

environment of the animal room was controlled with the temperature being maintained within a target range of 19–23 °C, relative humidity within a target range of 40–70%, an artificial light cycle of 12 h light and 12 h darkness, and between 25 and 30 air changes per hour. All conditions were monitored by the Honeywell site monitoring system and any excursions outside these ranges were noted by means of an external alarm and recorded.

Human liver samples ($n = 3$) were obtained from the Queen Elizabeth Hospital, Birmingham, UK and were from fresh liver excess to transplantation requirements. The donors were male caucasians age 41–57 years, the causes of death were cardiac arrest (2) and intracranial bleed. Samples were provided in compliance with local ethical guidelines. Human kidney samples ($n = 6$) were obtained from the UK Human Tissue Bank, Leicester, UK. The donors were 4 males (age 54–73) and 2 females (ages, 41 and 51 years). Causes of death were cardiac arrest (3), cerebral oedema/haemorrhage (2) and cerebral tumour.

2.3. In vitro metabolism

2.3.1. Preparation of tissue fractions

Animals were sacrificed by asphyxiation with a rising concentration of CO_2 followed by cardiac puncture. The livers and kidneys were removed, pooled and thoroughly washed in ice cold 1.15% potassium chloride. The tissues were scissors minced, and homogenates, 30% (w/v) for liver and 10% (w/v) for kidney, prepared in 0.25 M sucrose, 20 mM tris, 5 mM EDTA pH 7.4 using a Potter Elvehjem homogenizer. The homogenates

were centrifuged at 16 900 × *g* for 15 min at 4 °C, the supernatants transferred to fresh tubes and centrifuged at 105 000 × *g* for 70 min at 4 °C. The supernatant (cytosol) was removed from the microsomal pellet and stored at −70 °C. The microsomal pellet was resuspended in 20 mM tris, 1.15% potassium chloride pH 7.4, centrifuged at 105 000 × *g* for 70 min at 4 °C, and the pellet resuspended in 0.1 M sodium phosphate pH 7.4 (2:1 w/v original tissue wet weight to buffer). The microsomes were stored at −70 °C until required.

For the preparation of kidney mitochondrial fractions, the tissues were scissors minced, and a 6.7% (w/v) homogenate was prepared in 0.25 M sucrose, 10 mM tris pH 7.4 using a Potter Elvehjem homogenizer. The homogenates were centrifuged at 600 × *g* for 10 min at 4 °C, the pellet discarded, and the supernatent centrifuged for a further 10 min at 600 × *g*. The supernatant was again removed and centrifuged at 10 000 × *g* for 20 min at 4 °C. The supernatant was now discarded, and the pellet washed in 0.25 M sucrose, 10 mM tris pH 7.4, before centrifuging at 10 000 × *g* for 20 min at 4 °C. The mitochondrial pellet was resuspended in 0.25 M sucrose, 10 mM tris pH 7.4 (1:6.67 w/v original tissue wet weight to buffer) and stored at −70 °C until required.

The human liver and kidney samples were processed as above, except that they were homogenised using an Ultra Turrux homogenizer, and the microsomal pellets were resuspended in 0.25 M sucrose, 20 mM tris, 5 mM EDTA pH 7.4 (1:1 w/v original tissue wet weight to buffer).

Protein concentrations in the tissue fractions was determined using the method of Lowry et al., 1951

2.3.2. Hepatic glutathione conjugation

The glutathione *S*-transferase catalysed metabolism of HCBD was determined by measuring the formation of HCBD-GSH.

The incubation mixtures contained 0.01–0.4 mM HCBD, 5 mM glutathione, and hepatic microsomes (1 mg), in a final volume of 1 ml 100 mM potassium phosphate buffer, pH 7.4 at 37 °C. The tubes were preincubated at 37 °C for 4 min before the reaction was initiated by the addition of the HCBD (in 5 μl DMSO). The reaction was terminated at 60 min by the addition of 1 ml 10% (w/v) trichloroacetic acid. The tubes were centrifuged at 3 000 × *g* to remove protein, and the sample neutralised by the addition of 0.5 ml 1 M sodium hydroxide.

The samples were analysed for HCBD-GSH using a 5 μm Hypersil ODS 4.6 × 200 mm analytical column with a 4.6 × 30 mm guard column (Hichrom, UK) on a Shimadzu LC-10 system, with UV monitoring at 220 nm. The glutathione conjugate was eluted in 10% acetonitrile (0.025% trifluoroacetic acid)/90% water (0.025% trifluoroacetic acid) at 1 ml/min using a gradient, which after rising to 75% acetonitrile over 20 min, rose to 100% acetonitrile over a further 2.5 min. Quantification was performed using HCBD-GSH standards. The HCBD-GSH standard eluted at 17.8 min.

2.3.3. Renal β-lyase

Cysteine conjugate β-lyase was measured in rat and human kidney cytosol and mitochondria using the method of Stevens and Jakoby (1983).

For the determination of cytosolic β-lyase activity, incubation mixtures contained 0.025–1.25 mM HCBD-CYS, 0.1 mM NADH, 0.1 units LDH, and kidney cytosol (1.3 mg/ml) in a final volume of 1 ml of 50 mM potassium phosphate, pH 7.4 at 37 °C. The mixture was pre-incubated for 4 min prior to the initiation of the enzyme reaction by the addition of HCBD-CYS. Pyruvate production over a 30 min period (60 min for the human samples), monitored spectrophotometrically at 340 nm, was used as a measure of enzyme activity.

For the determination of mitochondrial β-lyase activity, incubation mixtures contained 0.025–1.75 mM PCBD-CYS, mitochondria (1.0 mg/ml for rat and 1.75 mg/ml for humans) in a final volume of 1 ml of 50 mM potassium phosphate, pH 7.4 at 37 °C. The mixture was pre-incubated for 4 min prior to the initiation of the enzyme reaction by the addition of the mitochondria. The incubation was terminated after 5 min by the addition of 0.2 ml 10% trichloroacetic acid. The samples were spun at 1000 × *g*, for 10 min at 4 °C. 0.654 ml of the supernatant was neutralised by 0.196 ml 1 M

potassium hydrogen phosphate (K_2HPO_4). Enzyme activity was measured by determining the conversion of the pyruvate formed to lactate using 0.2 mM NADH, and 0.1 units LDH.

2.3.4. Renal *N*-acetyl transferase

Cysteine *N*-acetyl transferase activity was determined by measuring the amount of labelled acetyl coenzyme A conjugated to HCBD-CYS using the method of Duffel and Jakoby (1982).

Incubation mixtures contained 0.05–1.0 mM HCBD-CYS, 0.8 mM [acetyl-1-^{14}C]-coenzyme A (0.25 μCi/mmol), and kidney microsomes (0.11 mg for rat, and 0.09–0.15 mg for human), in a final volume of 0.25 ml 0.2 M potassium phosphate, pH 7.0 at 37 °C. The mixture was preincubated for 2 min at 37 °C, prior to the initiation of the reaction by the addition of the microsomes. The reaction was terminated at 5 min by the addition of 0.75 ml 1.33 M acetic acid and 2 ml cyclohexanone. The samples were vortexed and the layers separated by centrifugation at 500 × *g* for 3 min. 0.5 ml aliquots of the cyclohexanone layer were added to 10 ml Ultima-Gold (Canberra Packard), and the radioactivity determined by liquid scintillation counting.

2.3.5. Renal acylase

The de-acetylation of HCBD-NAC to HCBD-CYS was determined by measuring the amount of de-acetylated product formed using the method of Uttamsingh and Anders (1999).

Incubation mixtures contained 0.1–2.0 mM HCBD-NAC, 0.1 mM aminooxyacetic acid, and dialysed kidney cytosol (0.6–0.7 mg for rat, 1 mg for human) in a final volume of 1 ml of 50 mM potassium phosphate, pH 7.4 at 37 °C. The mixture was pre-incubated for 4 min prior to the initiation of the reaction by the addition of the HCBD-NAC. The reaction was terminated at 30 min (45 min for the human samples) by the addition of 200 μl ice cold 20% trichloroacetic acid. The samples were then spun at 1000 × *g* for 10 min at 4 °C. 75 μl of the supernatant was neutralised with 30 μl 1 M K_2HPO_4 and mixed with 100 μl 0.2 M borate buffer, pH 8.5. Fluorescamine (0.1 mg in 100 μl of acetone) was then added with mixing. After 10 min the samples were diluted to 2.5 ml with 50 mM potassium phosphate, pH 7.4, and the amount of product formed was measured fluorometrically using excitation at 390 nm, and emission at 475 nm with a band width of 4 nm. The amount of product formed was quantified against a HCBD-CYS standard.

2.4. PB-TK modelling

A PB-TK model was constructed using Advanced Continuous Simulation Language (ACSL—Mitchell Gauthier Associates Inc, Concord, MA) software and was based on the inhalation model described by Ramsey and Andersen (1984), with the addition of a kidney compartment. Physiological parameters used in the model were taken from standard texts and metabolic rates were those determined experimentally as described above. Partition coefficients for blood:air and blood:fat were measured experimentally as described by Gargas et al., 1989. The model was used firstly to determine an inhalation dose in rats which gave the same body burden as that following dietary administration at the NOEL for kidney toxicity of 0.2 mg/kg/day. The concentration of β-lyase derived metabolites in the kidney was then calculated at this dose using the metabolic parameters. Subsequently, the model was used to predict a human exposure which gave the same concentration of β-lyase metabolites, in this case using the human metabolic parameters and physiological constants. Blood and fat concentrations of HCBD were also predicted to determine whether HCBD accumulated in humans upon continuous exposure to environmental concentrations.

2.4.1. Assumptions made in the model

Complete adsorption was assumed following dietary administration of HCBD and this route was not therefore described in the model. The model was used to calculate a continuous exposure concentration which gave a daily body burden of 0.2 mg/kg HCBD. The partition coefficients which were considered to have the greatest impact on the output of the model, air:blood and blood:fat, were measured experimentally. Blood:tissue compartment partition coefficients for small lipophilic molecules normally approximate to unity (Gargas

et al., 1989) and it was assumed that this was the case for HCBD. As such these values have little impact on the output of the model.

Glutathione conjugation of HCBD is catalysed by both microsomal and cytosolic glutathione *S*-transferases. The microsomal rate is 40-fold higher in human liver, and 3.4-fold higher in rat liver, than those found in the equivalent cytosolic fractions (Oesch and Wolf, 1989) and in the model, hepatic metabolism of HCBD is based on the rates measured in microsomal fractions. Metabolism of HCBD by glutathione conjugation is also known to occur in the kidney. However, the rates in the kidney are 15-fold lower than those in the liver on a mg protein basis and 90-fold lower on a whole organ basis (Morganstern et al., 1984). Consequently, in the model, glutathione conjugation only occurs in the liver.

In the rat, the glutathione conjugate of HCBD is known to be excreted in bile and to undergo enterohepatic recirculation (Nash et al., 1984; Reichert et al., 1985). This information is not available in humans, either quantitatively or qualitatively. In the absence of this information it is assumed, in both species, that all of the glutathione conjugate formed in the liver is available, as the cysteine conjugate, for further metabolism by the kidney. In the kidney, metabolism occurs by β-lyase, *N*-acetyltransferase and acylase. β-lyase metabolism is based on the rates in mitochondria since these were higher than cytosolic rates in the rat and a rate was not detectable in human cytosol. Furthermore, the mitochondrion is believed to be the target organelle for β-lyase activated nephrotoxins (Bruschi et al., 1998). The mercapturic acid (HCBD-NAC) is also eliminated in urine. Urinary excretion is based on that reported for the rat (Nash et al., 1984; Reichert et al., 1985).

3. Results

3.1. In vitro metabolism

The conjugation of HCBD with glutathione in rat liver microsomal fractions occurred at a rate (Vmax) which was approximately 5-fold higher than that found in human liver. The affinity constant (Km) for the reaction was approximately 1.3-fold higher in the rat than in the human samples. Less than a 2-fold variation was found in either the human Km or Vmax for this reaction (Table 1).

The metabolism of HCBD-CYS by renal β-lyases is shown in Table 2. A metabolic rate could not be detected in the human kidney cytosol samples. Based on the limit of detection of the assay, the rate is human kidney cytosol is at least 23-fold lower than that in the rat. Mitochondrial fractions were significantly more active than cytosolic fractions, the rate in rat kidney mitochondria being approximately 4-fold greater than that in the cytosol fraction. A significant rate, which was 3-fold lower than that in the rat, was also detected in human kidney mitochondria. The affinity constants Km for the mitochondrial enzyme were essentially similar between the two species. In humans, the variability between individuals was slight, between 2- and 3-fold.

Both rat and human kidney were highly active for the metabolism of HCBD-CYS to its *N*-acetylcysteine conjugate (Table 3). The rates (Vmax) were 27-fold greater in the rat and 21-fold greater in humans than the maximum rates for the competing β-lyase reactions in cytosolic and mitochondrial fractions combined.

A significant rate was detected for the metabolism of HCBD-NAC by acylases in rat, but not human kidney fractions (Table 3). Based on the limit of detection of the assay, the rate in humans was 74-fold lower than that in the rat.

Table 1
The conjugation of HCBD with glutathione in rat and human liver microsomes

Species	Km (mM)	Vmax (nmol/min/mg)
Rat	0.21	1.23
Human 90	0.16	0.22
Human 94	0.16	0.19
Human 98	0.16	0.34
Human–mean	0.16	0.25

Table 2
The metabolism of HCBD-CYS by β-lyases in rat and human kidney fractions

Species	Cytosol		Mitochondria	
	Km (mM)	Vmax (nmol/min/mg)	Km (mM)	Vmax (nmol/min/mg)
Rat	0.30	1.13	0.12	4.17
Human 003	–	<0.05[a]	0.12	0.90
Human 004	–	<0.05[a]	0.16	1.43
Human 005	–	<0.05[a]	0.67	1.78
Human 006	–	<0.05[a]	0.28	1.56
Human 007	–	<0.05[a]	0.15	0.73
Human 008[b]	–	<0.05[a]	ND	1.74[b]
Human–mean	–	<0.05[a]	0.25	1.76

[a] No measurable rate, limit of detection shown.
[b] Measured at a single substrate concentration of 1.25 mM.

3.2. PB-TK model parameters

The blood:air partition coefficients for rat and human blood were 115 and 75 respectively. A fat:air partition coefficient of 2717 was obtained for rat fat and used in the model for both rat and human fat. This gave fat:blood partition coefficients of 24 for the rat and 36 for human. The metabolic constants used in the model were those shown in Tables 1–3 expressed in model units (mg/h/kg tissue). Where metabolic rates could not be detected (human acylase Table 3), the rate used in the model was the limit of detection of the assay. The values of physiological parameters used in the model are shown in Table 4.

3.3. PB-TK model predictions

For the purposes of modelling the metabolism of HCBD in rats and humans, it has been assumed that exposures occur at concentrations of less than 1 ppm. At these exposure levels the concentrations of HCBD found in tissues increase linearly with increasing dose (Table 5). Fig. 2 illustrates the blood and fat concentrations of HCBD in humans exposed to 0.01 ppm, continuously for 10 days. Steady state has been reached in both blood and fat compartments by the end of exposure and post-exposure the concentrations of HCBD decline with half-lives of 6 h in blood and 26 h in fat. Because steady state has been reached, blood and fat levels

Table 3
N-Acetyl transferase and acylase activities in rat and human kidney fractions

Species	*N*-Acetyl transferase[a]		Acylase[b]	
	Km (mM)	Vmax (nmol/min/mg)	Km (mM)	Vmax (nmol/min/mg)
Rat	0.39	144.9	1.43	7.36
Human 003	0.21	40.8	–	<0.10[a]
Human 004	0.20	8.8	–	<0.10[a]
Human 005	0.10	25.6	–	<0.10[a]
Human 006	0.05	27.5	–	<0.10[a]
Human 007	0.24	60.6	–	<0.10[a]
Human 008	0.13	62.5	–	<0.10[a]
Human–mean	0.19	37.6	–	<0.10[a]

[a] Measured in microsomal fractions with HCBD-CYS as the substrate.
[b] Measured in cytosol fractions with HCBD-NAC as the substrate.

Table 4
Partition coefficients and physiological parameters used in the HCBD PB-TK model

Parameter	Rat	Human
Partition coefficients[a]		
Blood/air	115	75
Fat/blood	24	36
Tissue compartment—% body weight		
Liver	4	2.6
Kidney	1.1	0.44
Fat	7	21
Richly perfused	5	4.5
Slowly perfused	75	62
Blood flow—% cardiac output		
Liver	25	24
Kidney	17	18
Fat	5	5
Richly perfused	34	34
Slowly perfused	19	19
Alveolar ventilation rate (l/h)	5.4	348
Cardiac output (l/h)	5.4	348
Body weight (kg)	0.25	70

[a] Liver/blood, kidney/blood, slow/blood, rapid/blood coefficients were set to unity.

would not be expected to increase with continued exposure beyond 10 days.

In order to assess the likelihood that kidney toxicity would occur in humans following exposure to HCBD, the model was first used to predict metabolism by the β-lyase pathway in rats at the NOEL of 0.2 mg/kg/day for kidney toxicity. In the rat, a body burden of 0.2 mg/kg HCBD/day was calculated by the model to be equivalent to

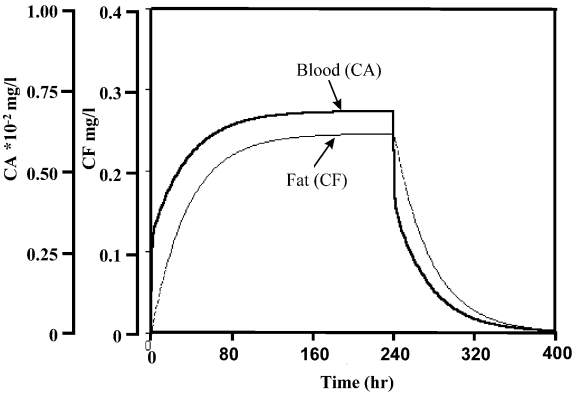

Fig. 2. Predicted concentrations of HCBD in human blood and fat during and post-exposure to 10 ppb HCBD 24 h/day for 10 days. CA is the concentration of HCBD in arterial blood and CF the concentration in fat.

continuous exposure to 0.07 ppm/day. Exposure at this concentration gave a renal concentration of β-lyase metabolites of 137.7 mg/l. The NOEL for kidney toxicity in the rat equates, therefore, to a target organ dose of β-lyase derived metabolites of 137.7 mg/l. To produce the same concentration of β-lyase metabolites in the human kidney, the model calculates that humans would have to be continuously exposed to 1.41 ppm HCBD.

As indicated above, a rate was not detectable for the metabolism of HCBD-NAC by human kidney acylase. Consequently, the limit of detection of the assay was used as the metabolic rate for this pathway in the model. If the rate for human acylase is set to zero, the difference in β-lyase metabolism between rats and humans increases from approximately 20- to almost 50-fold.

Table 5
PB-TK model predictions

Species	Exposure ppm	Blood level mg/l	Fat concentration mg/l
Rat	1	0.62	14.9
	0.01	0.0062	0.148
Half-life (t 1/2-h)	0.01	4.16	5.80
Human	1	0.68	24.6
	0.01	0.0068	0.245
Half-life (t 1/2-h)	0.01	6.0	26.0

Blood and fat levels in rats and humans continuously exposed to HCBD.

4. Discussion

Assessment of the risk following continuous exposure to very low levels of a chemical in the environment is always problematical and can normally only be estimated from laboratory animal data with the application of appropriate safety margins to allow for the uncertainties involved. Frequently, the animal data will have been obtained at considerably higher dose levels with different exposure scenarios and possibly even by a different route of administration. The data available to assess the risks to humans from environmental exposure to HCBD is typical. The primary data source is a 2 year rat chronic toxicity and carcinogenicity study which was conducted at a maximum tolerated dose with the test material administered in the diet (Kociba et al., 1977). Thus, assessment of human risks requires extrapolation across doses which differ by orders of magnitude and extrapolation between dietary and inhalation exposure. Currently, the only tool available to make this type of extrapolation is PB-TK modelling.

Although nothing is known about the toxicity of HCBD in humans it is known that the enzymes involved in its metabolic activation in the rat are present in humans. Consequently, it must be assumed that HCBD has the potential to be toxic in the human kidney, as it is in the rat. HCBD itself is not nephrotoxic, the effects in the kidney are in fact induced by a metabolite produced at the end of a metabolic cascade involving a number of enzymes in more than one organ. Thus, in addition to comparing uptake between the two routes of exposure, a comparison of the metabolic cascade that leads to a nephrotoxic metabolite is also required in rats and humans.

Although PB-TK modelling is an ideal means of incorporating these types of data into risk assessments, there were a number of constraints during the construction of the model used here. There are no human metabolic or kinetic data of any type available to validate the model, nor under the circumstances is there any opportunity to generate such data. Similarly, there are no data in animals following inhalation exposure. Attempts to exposed rats to low concentrations (≈ 1 ppm) of HCBD failed because of difficulties in maintaining a stable atmosphere of this high boiling chemical. This meant that the model was constructed using a limited set of existing data and the in vitro studies described here. Nevertheless, the human exposures to HCBD were a real issue, they were a matter of immediate concern and required a rapid resolution of the associated risks. Thus, incorporation of the data describing the complex metabolic pathway into the risk assessment process was considered to be a worthwhile improvement over the default which is based on inhaled dose alone.

The renal toxicity of HCBD is known to be a consequence of the metabolism of the cysteine conjugate of HCBD by renal β-lyases. The amount of this conjugate available for metabolism by these enzymes is determined by a number of factors. These are principally the uptake and distribution of HCBD itself, and the activities of those enzymes involved in the formation and further metabolism of the cysteine conjugate. In the PB-TK model the uptake and distribution of HCBD is determined using measured partition coefficients and standard values for physiological parameters such as ventilation rates and blood flows. Metabolism is described by four key enzymes which control the formation, activation and further metabolism of the cysteine conjugate of HCBD (Fig. 1). The glutathione *S*-transferases catalyse the conjugation of HCBD with glutathione and determine the amount of cysteine conjugate available for further metabolism in the kidney. The cysteine conjugate is a substrate for both renal β-lyases and *N*-acetyltransferases. The β-lyases which activate the cysteine conjugate, are pyridoxal phosphate dependent enzymes and are only active with the cysteine conjugate. Consequently, metabolism of the cysteine conjugate by *N*-acetyltransferases to form the mercapturic acid is a true detoxification reaction. The fourth enzyme system, the renal acylases convert the *N*-acetylcysteine conjugate back to the cysteine conjugate thus making more substrate available for the β-lyases. Each of these processes has been included in the model.

The β-lyases comprise a number of enzymes which are able to cleave the C–S bond of haloalkene cysteine conjugates to give a reactive thiol, pyruvate and ammonia (Abraham et al.,

1995; Stevens and Jakoby, 1983; Stevens et al., 1988). The lyases are found in both the soluble (cytosolic) and mitochondrial fractions of kidney homogenates, the relative activities of the enzymes vary according to the structure of the cysteine conjugate. The HCBD conjugate is metabolised mainly by the mitochondrial enzymes which is consistent with the known toxicity of cysteine conjugates of this type towards the mitochondrial respiratory chain (Bruschi et al., 1998).

Two major forms of the *N*-acetyltransferases are known, one of which (NAT2) is known to be polymorphic in the human population. Which of the two isoforms is responsible for the acetylation of cysteine conjugates of this type is not known. Both forms are found in the microsomal fractions and hence total activity has been measured in the current work. The variability between individuals was relatively low, 25.6–62.5 nmol/min/mg, with a single sample having an activity of 8.8 nmol/min/mg protein. Whether the single low value reflects a slow acetylator and polymorphism, or the lower end of the normal range, is not known.

The metabolism of the cysteine conjugate of HCBD by *N*-acetyltransferases occurred at significantly faster rates than that by β-lyases in both rat and human kidney thus minimising the amount metabolised to toxic intermediates. In human kidney, the rate of metabolism of the *N*-acetylcysteine conjugate back to the cysteine conjugate by acylases is low, below the limit of detection, further minimising the amount of cysteine conjugate available for metabolism by β-lyases. In contrast, in the rat acylase activity was high thus increasing the amount of cysteine conjugate available. Using these experimentally measured metabolic rate constants in a PB-TK model has enabled the uptake and metabolism of HCBD to be compared in rats and humans and the potential toxicity of HCBD to humans to be predicted from the known toxicity in the rat. Following exposure of rats and humans to atmospheric concentrations of HCBD, metabolism by the β-lyase pathway is approximately 20-fold less in humans than in the rats at the same dose level. This prediction by the model is consistent with the lower uptake, lower glutathione *S*-transferase and β-lyase activities, and the lack of acylase activity in human kidney.

As noted, the only available toxicity data on which to make a human health assessment is the lifetime study in rats in which HCBD was administered in the diet for 2 years (Kociba et al., 1977). In that study, the NOEL for kidney pathological change was 0.2 mg/kg/day. In the absence of the metabolic data described in this paper data, the UK Committee on Toxicity (COT, 2000) used the toxicity data and default assumptions to calculate safety margins. The uptake of HCBD from inhaled air was assumed to be complete, and humans were considered to be as sensitive as the rat to the toxic or carcinogenic effects of HCBD. This approach calculates that a gavage dose of 0.2 mg/kg/day is equivalent to an inhaled dose of 60 ppb. Their application of a safety margin of 100 to account for cross-species and intra-species variability for non-carcinogenic and reproductive effects resulted in an exposure of 0.6 ppb which they regarded as being without appreciable adverse health effect (COT, 2000). When the metabolic data described above is used in a PB-TK model, the concentration of β-lyase metabolites in the rat kidney at the NOEL of 0.2 mg HCBD/kg/day is calculated to be 137.7 mg/l. To achieve the same concentration in humans would require continuous exposure to 1.41 ppm HCBD which is equivalent to an uptake of 2.0 mg/kg/day. Applying the same 100-fold safety margin (COT, 2000) results in an exposure of 14.1 ppb which can be regarded as being without appreciable adverse health effect. This is in contrast to the figure of 0.6 ppb obtained using default assumption without the species specific metabolic data.

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