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Biotransformation and kinetics of 1,2- and 1,4-dichlorobenzene

A PB-PK approach in extrapolation from animal to man

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Biotransformatie en kinetiek van 1,2- en 1,4-dichloorbenzeen

Een PB-PK benadering in extrapolatie van dier naar mens

(met een samenvatting in het Nederlands)

Proefschrift ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. Dr. J.A. van Ginkel
ingevolge het besluit van het College van Dekanen
in het openbaar te verdedigen
op vrijdag 20 september 1996 des middags te 12.45

door

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geboren op 27 november 1965, te Vorden

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CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Hissink, Arendina Marieke

Biotransformation and kinetics of 1,2- and 1,4-dichlorobenzene.
A PB-PK approach in extrapolation from animal to man /
Arendina Marieke Hissink. Utrecht: Universiteit Utrecht,
Faculteit Diergeneeskunde.
Thesis Universiteit Utrecht. - With ref. - with summary in
Dutch.
ISBN 90-393-1326-1

Druk: Grafisch bedrijf Ponsen & Looijen BV, Wageningen.

The studies described in this thesis were carried out at the Toxicology Division, TNO Nutrition and Food Research Institute, Zeist, the Netherlands, and were financially supported by the Utrecht Toxicological Center (UTOX). Financial support for the publication of this thesis was provided by TNO Nutrition and Food Research Institute.

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Ter herinnering aan mijn broer Henk

Voor mijn familie

CHAPTER 1

GENERAL INTRODUCTION

1.1 Biotransformation.

All organisms are exposed constantly and unavoidably to foreign chemicals, or *xenobiotics*, which include both man-made and natural chemicals, such as drugs, industrial chemicals, pesticides, pyrolysis products in cooked food, alkaloids, and toxins produced by molds, plants and animals. Unfortunately, the physical property that enables many xenobiotics to be absorbed through the skin, lungs, or gastrointestinal tract, namely their lipophilicity, is an obstacle to their elimination. In the course of evolution, organisms have developed enzyme-systems that protect against toxic effects of xenobiotics. In general, these enzymes increase the hydrophilic properties of compounds, in such a way that the products (*metabolites*) are more easily transported through the body and excreted in the urine or bile. The enzymes catalyzing these processes of *biotransformation* are generally divided into two major classes, the phase I and phase II enzymes, as shown in Table 1.1 (Williams, 1971). Phase I reactions involve hydrolysis, reduction, and oxidation. These reactions expose or introduce a functional group (-OH, $-NH_2$, -SH or -COOH), and usually result in only a small increase in hydrophilicity. The cofactors for the phase II reactions react with functional groups that are either present on the xenobiotic or are introduced during phase I biotransformaton. Most phase II biotransformation reactions result in a large increase in xenobiotic hydrophilicity, hence they greatly promote the excretion of foreign chemicals. Although most xenobiotics are converted in the body to metabolites that are less toxic than the parent compound ('detoxication'), it has become increasingly clear that many toxicological effects of xenobiotics are mediated through the formation of reactive metabolites ('bioactivation'). Depending on both exogenous, e.g. induction by nutritional factors, as well as endogenous factors, e.g. genetic deficiencies, the relative concentration of activating and detoxifying enzyme systems may vary significantly between individuals, with a concomitant variation in the extent of toxicity. The balance

between these enzyme systems will ultimately determine whether a xenobiotic can elicit its hazardous effect.

Table 1.1. General pathways of xenobiotic biotransformation and their major subcellular location.

Reaction	Enzyme	Localization	Enzyme	Localization
	Phase I		Phase II	
Hydrolysis	Carboxylesterase	Microsomes, cytosol	Glucuronosyl-transferase	Microsomes
	Peptidase	Blood, lysosomes	Sulfotransferase	Cytosol
Reduction	Azo- and nitro-reduction	Microflora, cytosol microsomes	Glutathione S-transferase	Cytosol, microsomes
	Carbonyl reduction	Cytosol	Amino acid conjugation	Mitochondria, microsomes
	Disulfide reduction	Cytosol	Acetyl-transferase	Mitochondria, cytosol
	Sulfoxide reduction	Cytosol	Methyl-transferase	Cytosol
	Quinone reduction	Cytosol, microsomes	Epoxide hydrolase	Microsomes, cytosol
	Reductive dehalogenation	Microsomes		
Oxidation	Alcohol dehydrogenase	Cytosol		
	Aldehyde dehydrogenase	Mitochondria, cytosol		
	Aldehyde oxidase	Cytosol		
	Xanthine oxidase	Cytosol		
	Monoamine oxidase	Mitochondria		
	Diamine oxidase	Cytosol		
	Prostaglandin H synthase	Microsomes		
	Flavin-mono-oxygenase	Microsomes		
	Cytochrome P450	Microsomes		

1.1.1 Cytochrome-P450: general aspects.

Among the phase I biotransforming enzymes, the cytochrome P450 system ranks first in terms of catalytic versatility and the number of xenobiotics it detoxifies or activates to reactive intermediates (Guengerich, 1987; Waterman and Johnson, 1991). The highest concentration of P450 enzymes involved in xenobiotic biotransformation is found in liver endoplasmatic reticulum (microsomes), but P450 enzymes are present in virtually all tissues.

P450 enzymes are heme-containing proteins. The basic reaction catalyzed by cytochrome P450 is monooxygenation in which one atom of oxygen is incorporated into a substrate and the other is reduced to water with reducing equivalents derived from NADPH. The amino acid sequence of the numerous P450 isoenzymes has been determined, largely by recombinant DNA techniques, and such sequences now form the basis for classifying and naming P450 enzymes (Nebert *et al.*, 1991; Gonzalez, 1989; Nelson *et al.*, 1993). In general, P450 enzymes with less than 40 percent amino acid sequence identity are assigned to different gene families (1,2,3, etc.), with 40-55% identity are assigned to different subfamilies (2A, 2B, 2C, etc.), and with more than 55% identity are classified as members of the same subfamily (e.g. 2A1, 2A2). The number of P450 enzymes in each subfamily differs from one species to the next. Human liver microsomes can contain 15 or more different P450 enzymes that metabolize xenobiotics and/or endogenous substrates (Guengerich, 1994; Wrighton and Stevens, 1992). In Table 1.2, the concentrations of individual P450 enzymes in human liver microsomes are shown (data from Shimada *et al.*, 1994). The most abundant P450 enzymes in human liver microsomes belong to the CYP3A gene subfamily. The relative importance of these enzymes is smaller in the rat, a species which shows large sex differences in CYP3A activities, with a more than 10-fold

Table 1.2. Concentration of individual P450 enzymes in human liver microsomes.

	Concentration in liver microsomes (n = 60)	
P450 enzyme	Specific content (pmol/mg protein)	Percentage of total spectral P450
Total P450 (Determined spectrally)	344 ± 167	
Total P450 (Sum of individual enzymes determined immunochemically)	240 ± 100	72.0 ± 15.3
CYP1A2	42 ± 23	12.7 ± 6.2
CYP2A6	14 ± 13	4.0 ± 3.2
CYP2B6	1 ± 2	0.2 ± 0.3
CYP2C*	60 ± 27	18.2 ± 6.7
CYP2D6	5 ± 4	1.5 ± 1.3
CYP2E1	22 ± 12	6.6 ± 2.9
CYP3A†	96 ± 51	28.8 ± 10.4

* Sum of CYP2C8, 2C9, 2C18, and 2C19 and allelic variants (e.g., CYP2C10).
† Sum of CYP3A4, 3A5, and 3A7 and allelic variants (e.g., CYP3A3).
From Shimada *et al.* (1994).

higher expression in males compared to females. The second important class in human is P4502E1. In general 3A4 catalyzes the oxidation of large substrates, and 2E1 usually attacks small substrates. For extrapolation purposes, it is important to determine the (P450) enzymes which are responsible for detoxication or bioactivation of a compound, and to determine the activities of these enzymes in the test animal compared to man. Currently, such studies are being considered more and more in risk assessment. The interindividual differences between humans also may have consequences: the pharmacologic or toxic effects of certain drugs may be exaggerated in a significant percentage of the population due to a genetic deficiency in a P450 enzyme (Tucker, 1994; Meyer, 1994), which has been identified for CYP2D6 and CYP2C19. In addition, interindividual variability in levels of enzyme expression may be a consequence of inhibition or induction, e.g. by nutritional or environmental factors.

1.1.2 Conjugation reactons.

Phase II biotransformation reactions include glucuronidation, sulfation, acetylation, methylation (conjugation of *nucleophilic* substrates), and conjugation with glutathione (GSH) and amino acids (conjugation of *electrophilic* substrates) (Paulson *et al.*, 1986). In addition, metabolites which contain a (usually) highly reactive, electrophilic epoxide moiety may undergo hydrolytic cleavage (conjugation with water), which is catalyzed by epoxide hydrolase (EH).

Glucuronidation is a major pathway of xenobiotic biotransformation in mammalian species except for members of the cat family (Miners and Mackenzie, 1992; Burchell and Coughtrie, 1992). Glucuronidation requires the cofactor uridine diphosphate (UDP)-glucuronic acid, and the reaction is catalyzed by UDP-glucuronosyltransferases, which are located in the endoplasmatic reticulum of liver and other tissues, such as the kidney, intestines and brain. Glucuronide conjugates of xenobiotics and endogenous compounds are water-soluble conjugates that are eliminated from the body in urine or bile (molecular weight is larger than 350 for rat or larger than 500 for man).

Many of the xenobiotics and endogenous substrates that undergo *O*-glucuronidation also undergo sulfate conjugation (Mulder, 1981). Sulfate conjugation generally produces a highly water-soluble sulfuric acid ester. The reaction is catalyzed by sulfotransferases, a group of soluble enzymes found primarily in the liver, kidney, intestinal tract, lung, platelets, and brain. Sulfate conjugates of xenobiotics are excreted mainly in urine.

N-Acetylation is a major route of biotransformation for xenobiotics containing an aromatic amine ($R\text{-}NH_2$) or a hydrazine group ($R\text{-}NH\text{-}NH_2$), which are converted to aromatic amides ($R\text{-}NH\text{-}COCH_3$) and hydrazides ($R\text{-}NH\text{-}NH\text{-}COCH_3$), respectively (Evans, 1992). The *N*-acetylation of xenobiotics is catalyzed by *N*-acetyl-transferases and requires the cofactor acetyl-coenzyme A (acetyl-CoA). Genetic polymorphisms for *N*-acetylation have been documented in humans, hamsters, rabbits, and mice (Heim, 1988; Evans, 1992; Grant *et al.*, 1992; Vatsis *et al.*, 1995). A series of clinical observations in the 1950's established the existence of *slow* and *fast* acetylators of the antituberculosis drug isoniazid. The incidence of the slow acetylator phenotype is high (70%) in Middle eastern populations, intermediate (50%) in Caucasian populations, and low (<25%) in Asian populations. Whether slow or fast acetylators are at increased risk for xenobiotic-induced toxicity or carcinogenicity, depends on the nature of the xenobiotic and on other important risk factors, e.g. the presence of (de)toxifying enzymes.

Electrophilic centres in a xenobiotic may be conjugated with glutathione (GSH, a tripeptide comprised of glycine, cysteine, and glutamic acid) (Sies and Ketterer, 1988; Mantle *et al.*, 1987). Substrates for GSH conjugation include an enormous array of electrophilic xenobiotics, or xenobiotics that can be biotransformed to electrophiles, e.g. epoxidation. Glutathione conjugates are thioethers, which are formed by nucleophilic attack of GSH thiolate anion (GS^-) with an electrophilic site in the xenobiotic. The conjugation of xenobiotics with GSH is catalyzed by the multigene family of glutathione S-transferases (GST). These enzymes are present in most tissues, with high concentrations in the liver, intestine, kidney and lung, where they are localized in the cytoplasm (>95%) and endoplasmatic reticulum (<5%). The concentration of GSH in the liver is very high (5-10 mM); hence the nonenzymatic conjugation of certain xenobiotics with GSH can be significant.

Glutathione S-transferases are dimers composed of identical subunits, although some forms are heterodimers. Numerous subunits have been cloned and sequenced, which forms the basis of a nomenclature system the GST's, distinguishing four major classes: α, μ, π and θ (Mannervik *et al.*, 1992). GST is the major determinant of certain species differences in chemical-induced toxicity, e.g. low doses of aflatoxin B_1 cause liver toxicity and tumor formation in rats but not mice, due to the high expression levels of an alpha class GST in mice. Mice conjugate the highly reactive aflatoxin B_1 8,9-epoxide with GSH up to 50 times faster than rats or humans, which makes man also a susceptible species (Eaton and Gallagher, 1994). However, in some cases, conjugation with GSH enhances the toxicity of a xenobiotic (Monks

et al., 1990; Dekant and Vamvakas, 1993). For example, dibromoethane can be conjugated with GSH by the polymorphic GST theta, and the conjugate rearranges into the mutagenic episulfonium ion (Koga *et al.*, 1986). Another example involves the nephrotoxicity of bromobenzene, which is oxidized by cytochrome P450 to bromohydroquinone and subsequently conjugated with GSH, which ultimately results in the formation of reactive oxygen species (Monks *et al.*, 1990).

Epoxide hydrolase catalyzes the *trans*-addition of water to alkene epoxides and arene oxides, which can be formed during the cytochrome P450-dependent oxidation of aliphatic alkenes and aromatic hydrocarbons, respectively. Epoxide hydrolase plays an important role in detoxifying electrophilic epoxides that might otherwise bind to proteins and nucleic acids and cause cellular toxicity and genetic mutations. Within certain tissues, such as liver and lung, the distribution of epoxide hydrolase parallels that of cytochrome P450. There are three immunologically distinct forms of epoxide hydrolase in the liver; two in the endoplasmatic reticulum, and one in the cytosol (Oesch, 1972; Gill *et al.*, 1974). One of the microsomal enzymes hydrates cholesterol 5,6α-oxide but has virtually no capacity to detoxify xenobiotic oxides. The other microsomal epoxide hydrolase and the cytosolic epoxide hydrolase can hydrate a wide variety of alkene epoxides and arene oxides, as described above.

1.2 Dichlorobenzenes: Occurrence in the environment and human exposure.

Halogenated benzenes are widely dispersed in the environment, and are of concern regarding their possible hazardous effects to human health following (chronic) exposure, and their tendency for bioaccumulation in animal and human lipid-containing tissue (Lu and Metcalf, 1975). Virtually all chlorinated benzenes have been found in the various waterbodies (Oliver and Nicol, 1982), and reports on the presence of residues in human fat and breast-milk have been published (Mes *et al.*, 1986; Wagner *et al.*, 1991).

Of the halogenated benzenes, the chlorine-substituted compounds represent by far the most important group, with widespread economical use as chemical intermediates in industry, and as solvents, dye carriers, space deodorants and moth repellents. Distribution into the ecosystem results primarily from industrial and municipal waste water and from agricultural use (Deichmann, 1982). Natural sources of chlorinated benzenes in the environment have not been identified.

Of the dichlorobenzenes (DCB's, *ortho* (1,2-DCB), *meta* (1,3-DCB) and *para* (1,4-DCB)), 1,3-DCB has no documented use. Production of 1,2-DCB in 1981 was 4.99 million kg and of 1,4-DCB was 6.8 million kg (USEPA, 1987). For the latter, it is reported that the production in the United States in 1990 was 65 million kg and still increases yearly (Chem. Marketing Reporter, 1990). The general population is exposed to dichlorobenzenes principally in air (Pellizzari *et al.*, 1986; Meek *et al.*, 1994). Recently the exposure to 1,4-DCB among 1000 adults in the United States has been investigated (Hill *et al.*, 1995). It was found that 98% had detectable levels of 2,5-dichlorophenol (2,5-DCP), a metabolite of 1,4-DCB, in their urine and 96% had detectable amounts of 1,4-DCB in their blood. From a national survey of 210 Canadian women, it was found that 70% had detectable levels of 1,2-DCB in their breast milk, which results in exposure to 1,2-DCB for suckling infants (Mes *et al.*, 1986).

1.2.1 Metabolism of dichlorobenzenes.

The earliest *in vivo* biotransformation studies with all isomers of dichlorobenzene were performed in 1955 (Azouz *et al.*, 1955; Parke and Williams, 1955). The *in vivo* metabolism of 1,4-DCB has also been investigated in later studies (Kimura *et al.*, 1979; Hawkins *et al.*, 1980; Klos and Dekant, 1994) and one other metabolism study on 1,3-DCB has been reported (Kimura *et al.*, 1984). In addition, *in vitro* metabolism of 1,2- and 1,4-DCB has been investigated (Den Besten *et al.*, 1992). The general metabolic pathway of dichlorobenzenes is firstly an epoxidation step by cytochrome P450. The epoxide may be conjugated with glutathione (GSH), either non-enzymatically or catalyzed by GSH S-transferases. The epoxide can also be hydrolyzed by epoxide hydrolase to form a dihydrodiol. Thirdly, the epoxide can be rearranged to a phenol, which can be conjugated with sulphate or glucuronic acid *in vivo*. The phenol may also be further oxidized by cytochrome P450, to form a hydroquinone and subsequently a benzoquinone. The hydroquinone can be conjugated by sulphate or glucuronic acid, the benzoquinone can be conjugated by GSH. Thus, multiple pathways are involved in the generation of various metabolites. Of these metabolites, the epoxides, benzoquinones and GSH derivatives of hydroquinones (Monks and Lau, 1990) may be involved in eliciting toxicity. The various metabolic pathways for (di)chlorobenzenes are summarized in Figure 1.1.

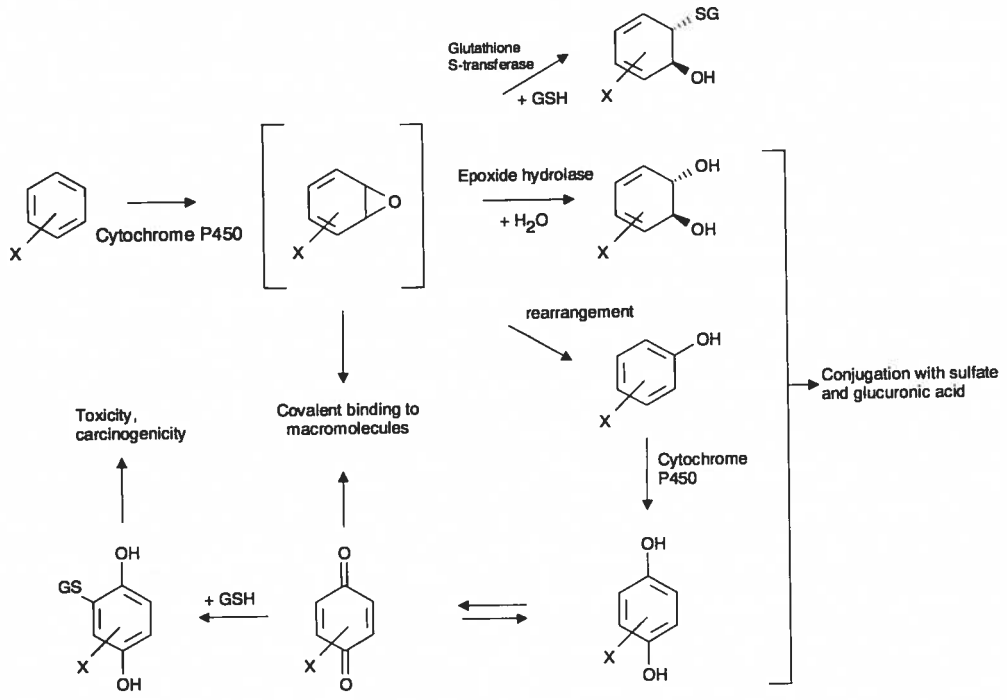

Fig.1.1. Possible metabolic pathways of halogenated benzenes.

Considerable species differences may exist in biotransformation of compounds, both in extent and the nature of metabolites formed (e.g. as is shown for 1,2,4-trichlorobenzene, in the rat and monkey; Lingg *et al.*, 1982). This interspecies variation in metabolism could very well reflect differences in the relative concentrations of the isoenzymes involved, as well as the type of enzyme responsible for biotransformation. Moreover, this variation could have toxicological implications. For example, studies on the metabolism of bromobenzene revealed the existence of a toxic route to *para*-bromophenol (which is catalyzed by P450 IIB1) and a non-toxic route to *ortho*-bromophenol (catalyzed by P450IA1) (Lau *et al.*, 1980). However, little data are available on the contribution of the various isoenzymes to the overall metabolism of dichlorobenzenes, and its toxicological implications. In order to make accurate risk estimates, knowledge of these data is of major importance, particularly with regard to the human cytochrome P450 isoenzymes involved.

1.2.2 Toxicity of dichlorobenzenes.

Various studies have been performed on the acute and subchronic toxicity and on the carcinogenicity of the three isomers of dichlorobenzene, with emphasis on 1,2- and 1,4-DCB (Hollingsworth *et al.*, 1956, 1958; Robinson *et al.*, 1991; Loeser and Litchfield, 1983; NTP, 1985, 1987). Only recently, a report has been published on the subacute and chronic toxicity of 1,3-DCB (McCauley *et al.*, 1995). Effects of 1,3-DCB were primarily found in the liver, kidneys and thyroid of both sexes of Sprague-Dawley rats. A NOAEL could not be firmly established, since various parameters were significantly altered in all dose groups of both sexes (9-735 mg/kg). The most pronounced effect following exposure to 1,2-DCB is acute hepatotoxicity, in mice as well as in rats (NTP, 1985). However, other toxic effects are reported, mainly for the kidneys, thymus, spleen and thyroid. The NTP bioassay using 1,2-DCB administered by gavage indicated that, under the conditions of the study, this isomer is not carcinogenic in Fischer 344 rats or B6C3F1 mice. No clear acute hepatotoxicity has been observed for 1,4-DCB. However, chronic exposure to 1,4-DCB resulted in histopathological changes in liver, kidneys, spleen and thymus, for Fischer 344 rats as well as B6C3F1 mice, of both sexes (NTP, 1987). Moreover, the *para*-isomer has been found to be a renal carcinogen in male, but not female Fischer 344 rats, and a liver carcinogen in male and female mice. Contradictory to these data, no increase in tumor incidence was found in (male) Wistar rats after exposure to 1,4-DCB (Riley *et al.*, 1980). Apparently, sex-, species-, and strain differences exist in the development of carcinogenicity following exposure to 1,4-DCB. Thus extrapolation to man is a complicated issue.

1.3 Extrapolation of toxicity data in risk assessment.

At the Chemical Abstracts Service (CAS) more than 1,000,000 different chemical compounds are registered. Of these compounds, 100,000 are applied commercially and therefore human and environmental exposure is widespread. Obviously, protection of man and environment against the possibly hazardous effects of these compounds is extremely important. One of the means which are available for the government to achieve this protection is determination of exposure limits, e.g. the maximum acceptable concentrations of compounds in air, food and (drinking) water. The determination of these exposure limits consists of several

steps, and the most crucial but also most uncertain step is extrapolation of toxicity data from testing animal to man.

1.3.1 Extrapolation in conventional risk analysis.

In the present conventional risk analysis, extrapolation of testing animal to man involves two steps: one real extrapolation step to account for the difference in body size between the testing animal and man, and the application of safety factors. These safety factors are needed to account for inaccurate observations, and possible intra- and interspecies differences in bioavailability and susceptibility to a certain chemical.

To account for differences in body size, two methods can be used, namely one based on body weight and one based on body surface. The most commonly used method in the case of oral exposure is extrapolation based on body weight, where the dose is expressed as mg compound per kg body weight per day (or week). The second method is applied in extrapolation of inhalation toxicity data based on assumed toxicological equivalence of a concentration of a chemical in the air for species with different body sizes. The first extrapolation step results in a 'no observable adverse effect level' (NOAEL) expressed as mg per kg body weight (oral exposure data), or mg per m^3 air (inhalation exposure data).

The second extrapolation step, namely the choice and application of safety factors serves to reduce the possibility that an exposure level is reached which may result in adverse effects for man. The safety factors serve firstly to obviate interspecies variations: differences may exist between testing animal and man regarding their susceptibility to a certain chemical, which will be larger after oral than after inhalation exposure due to the influence of the gastro-intestinal tract (e.g. differences in biotransformation). Secondly, considerable differences in susceptibility to a chemical may exist within an exposed population (intra-species or interindividual variation), especially compared with a genetically rather homogeneous group of testing animals. Also, these differences will be smaller within a population at the working place compared with the whole population. For the intra- as well as the interspecies variation, generally a safety factor of 10 is used, together resulting in a safety factor of 100.

1.3.2 The use of Physiologically Based Pharmacokinetic (PB-PK) modeling.

Pharmacokinetics is the study of the time-course for the absorption, distribution, metabolism, and excretion of a chemical substance in a biological system. Using pharmacokinetics, it is assumed that the response of a particular target tissue can be related to the concentration profile of the active form of the chemical in that tissue (Andersen, 1981; Monro, 1992). One of the first pharmacokinetic models was presented by Teorell (Teorell, 1937). The compartmental modeling which has originated from this first model, and which is currently still practiced, is largely an empirical exercise. In this empirical approach, data on the time course of the chemical of interest in blood or tissues are collected. Based on these data, a mathematical model is selected which possesses a sufficient number of compartments and parameters to describe the data. However, these compartments and parameters have no obvious physiological or biochemical meaning. The advantage of this modeling approach is that there is no limitation to fitting the model to the data. However, since these models do not possess a physiological structure, it is not possible to incorporate a description of e.g. non-linear biochemical processes.

Physiologically Based Pharmacokinetic (PB-PK) models differ from the conventional compartmental models in that they are based to a large extent on the actual physiology of the organism. Instead of compartments defined by the experimental data itself, actual organ and tissue groups are used with their weights and blood flows obtained from the literature (Bischoff and Brown, 1966). Instead of composite rate constants determined by fitting the data, actual physical-chemical and biochemical constants of the compound are used. This approach results in a model which can predict the behavior of an experimental time-course without being directly based on it. Refinement of the model yields a model which can be used for quantitative extrapolation well beyond the range of experimental conditions. In particular, a properly validated PB-PK model can be used to perform the high-to-low dose, dose-route, and interspecies extrapolations necessary for estimating human (cancer) risk from animal bioassays (Andersen *et al.*, 1987). The major advantage of a PB-PK model is its greater predictive power. Since fundamental metabolic parameters are used, dose extrapolation is possible over ranges where saturation of metabolism occurs. Since known physiological parameters are used, a different species can be modeled by simply replacing the appropriate constants with those for the species of interest (Dedrick, 1973). Figure 1.2 compares the interspecies scaling of doses using the body volume correction (see § 1.3.1) and the PB-PK approach. The behavior for a

different route of administration can be determined by properly describing the nature of the new input. The extrapolation from one exposure scenario to another (e.g. from a single 8 hours to 5 days a week) only requires correct equations for the dosing regimen in the model (Paustenbach *et al.*, 1988; Vinegar *et al.*, 1992).

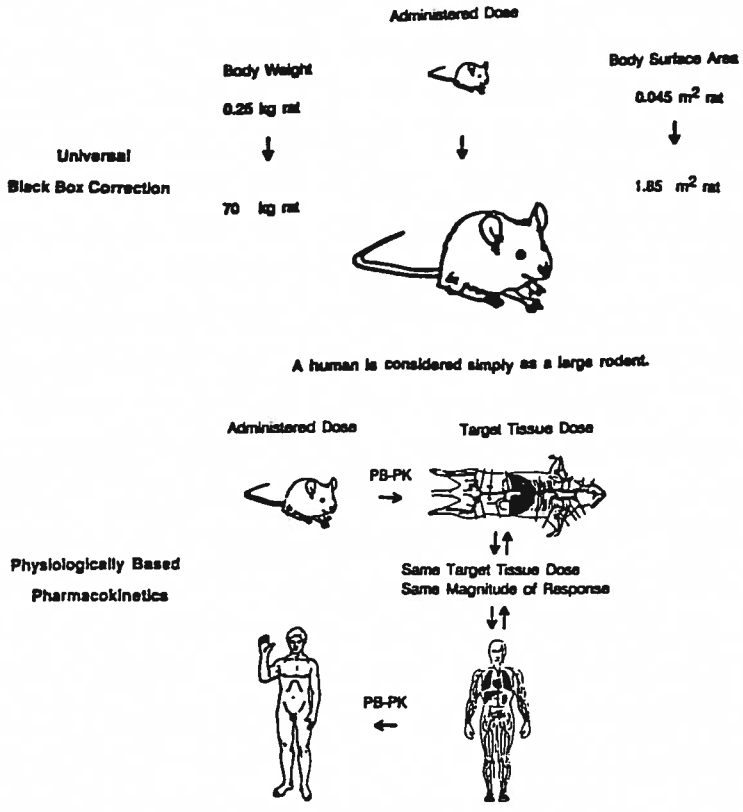

Fig.1.2. Comparison of approaches used in interspecies scaling. The universal black-box correction scales the external administered dose from animal to human on the basis of body size (body weight or body surface area). In the PB-PK approach, the equivalent human administered dose is estimated through a linkage of the internal tissue dose calculated by the respective animal and human PB-PK models.

The anatomical outline of the PB-PK model used by Ramsey and Andersen (1984) to study styrene inhalation by rats and humans (Figure 1.3) contains three multiple-tissue body compartments (fat, muscle/skin, viscera) and a separate liver compartment. In this formulation, all styrene metabolism in the body is assumed to occur in the liver. Each of the tissue groups is realistically defined with respect to their blood flow (Q), tissue volume, and their capacity to

store the chemical of interest. Blood flows and tissue volumes are available from the literature, whereas tissue solubility of the chemical (*partition coefficient*), a chemical-specific property, can be readily determined by simple experimentation *in vitro* (Sato and Nakajima, 1979; Gargas *et al.*, 1989). Several methods can be used to estimate metabolic constants, *in vivo* as well as *in vitro* (Andersen *et al.*, 1984; Reitz *et al.*, 1989; Gargas *et al.*, 1990; Kedderis *et al.*, 1993). When the physiological parameters, partition coefficients, biochemical constants, and the computer simulation model are available, pharmacokinetics can be predicted under a variety of conditions. Before accepting the model, these predictions should be tested by (limited) experimentation. This PB-PK approach requires less animals compared to conventional data-based modeling where time-course pharmacokinetic data are first obtained and then fitted with a particular mathematical model. To date, numerous PB-PK models for environmental toxicants as well as for drugs are available (Corley *et al.*, 1990; Medinsky *et al.*, 1989; Johanson, 1986; Menzel, 1988; Fisher *et al.*, 1989).

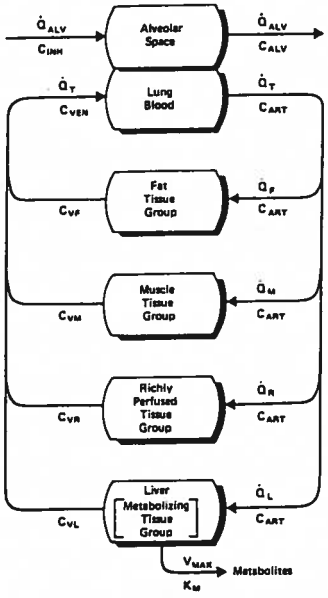

Fig.1.3. Diagram of a physiologically based pharmacokinetic model for volatile organic chemicals. In this description, groups of tissues are defined with respect to their volumes, blood flows (Q), and partition coefficients for the chemical. The uptake of vapor is determined by the alveolar ventilation (Q_{ALV}), cardiac output (Q_T), blood:air partition coefficient, and the concentration gradient between arterial and venous pulmonary blood (C_{ART} and C_{VEN}). Metabolism is described in the liver with a saturable pathway defined by a maximum velocity (V_{MAX}) and affinity (K_M). The mathematical description assumes equilibration between arterial blood and alveolar air as well as between each of the tissues and the venous blood exiting from that tissue (although diffusion limitations and saturable binding in the tissues can also be described when necessary).

1.4 Objectives of this thesis.

The primary interest of toxicology concerns the adverse effects of xenobiotics in humans. A major difficulty in this regard is that for ethical reasons almost no experiments can be performed in humans. Therefore, indirect approaches have been developed: testing in animals and extrapolation to the human situation. This extrapolation is far from straightforward, and thus large safety margins are applied in risk assessment (see also § 1.3.1). One way of overcoming some of the uncertainties in this process is to include the mechanism of the toxic action in the extrapolation process: are the same requirements for this action present in man and animal, and if not, which animal species shows the closest resemblance to the human situation? Since for many toxic compounds biotransformation plays a major role in the mode of action, much attention has been paid to this aspect.

As a model study for an exercise as described above we studied 1,2- and 1,4-dichlorobenzene, two closely related compounds inducing dissimilar effects in different species and organs. *In vivo* biotransformation of the rat is compared with *in vitro* biotransformation of the rat, and then *in vitro* biotransformation of rat and man is compared. Particular attention is paid to the cytochrome P450 enzymes involved, and the nature of the reactive metabolites (epoxides vs. quinones). For 1,4-DCB, the mouse is also included in the *in vitro* studies. The observed differences in effects in different species are compared to the metabolic profiles. Ultimately, these biotransformation data are, together with toxicity data, integrated in a PB-PK model. Such a model can be refined by incorporating interindividual differences, e.g. in enzyme activities, to determine the limits of a certain risk. For example, which concentration of a toxifying enzyme is necessary to produce a minimum amount of reactive metabolites eliciting toxicity? And is this minimum concentration of enzyme within the range observed for different individuals? Ultimately, when a PB-PK model is coupled to a pharmaco*dynamic* model, the effect of a certain concentration of reactive metabolites, e.g. cell death, can also be modeled.

Our first step was to investigate the *in vivo* metabolism of 1,2- and 1,4-dichlorobenzene (DCB) in the rat. These studies are described in chapter 2 and 3. To investigate *in vitro* biotransformation of DCB, different systems may be used, e.g. microsomes, hepatocytes and tissue slices. For our purpose, liver microsomes appeared to be the most suitable system to perform metabolism studies for rat, mouse, and for man. In addition to the metabolic pathways retrieved from these studies, an indication of toxic potential could be obtained, by measuring covalent binding of reactive metabolites to microsomal protein. Also, the nature of these

reactive metabolites was studied. In chapter 4, biotransformation of 1,2-DCB by rat and human liver microsomes is compared. In chapter 5, metabolism of 1,4-DCB by rat, mouse and human liver microsomes is compared. Also, different rat strains are used in this study, since the Fischer 344 rat has been shown to induce nephrocarcinogenicity, whereas the Wistar rat is insensitive to 1,4-DCB-induced adverse effects. The B6C3F1 mouse is included, since this species developed liver carcinogenicity following exposure to 1,4-DCB. In chapters 4 and 5, the relation between biotransformation and observed adverse effects *in vivo* is discussed, and an attempt is made to address this relation for the human situation.

Chapter 6 deals with the differential acute hepatotoxicity induced in male Wistar rats after administration of 1,2- or 1,4-DCB, and its relation with depletion of glutathione. Ultimately, data from the studies described in chapter 2, 4 and 6 (concerning 1,2-DCB) are incorporated into a Physiologically Based Pharmacokinetic (PB-PK) model. With this model, the concentration of reactive metabolites in the liver after exposure to a toxic dose level of 1,2-DCB was predicted, as well as the depletion of GSH, and lastly, this model was extended to the human situation.

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CHAPTER 2

DOSE DEPENDENT KINETICS AND METABOLISM OF 1,2-DICHLOROBENZENE IN RATS: EFFECT OF PRETREATMENT WITH PHENOBARBITAL

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Xenobiotica 26, 89-105, 1996

ABSTRACT

Toxicity of halobenzenes has been ascribed mainly to their epoxides, but recent studies with bromobenzene have shown that secondary quinone metabolites are also involved in the alkylation of hepatic proteins. However, the relative contribution of the quinones and the epoxides to the toxicity of halobenzenes is still unclear. In order to investigate the relation between metabolism and toxicity of 1,2-dichlorobenzene (1,2-DCB), the biotransformation, tissue distribution, blood kinetics and excretion at three different oral dose levels (5, 50 and 250 mg/kg) of the radiolabelled compound were investigated in male Wistar rats. A toxic dose level (250 mg/kg, as demonstrated by Allis *et al.*, 1992) was included.

The major route of elimination (75-85%) was renal excretion. Excretion via the faeces ranged from 19% for the low dose to 7% for the high dose level. Excretion was nearly complete within 24 hours for the low and mid dose level, and within 48 hours for the high dose level. Pretreatment with phenobarbital resulted in a more rapid excretion for the high dose level and an overall higher urinary excretion. Biliary excretion was 50-60%, indicating a considerable enterohepatic circulation. Highest concentrations of radioactivity after a low dose were found in fat, liver and kidneys at 6 hours after administration and then declined rapidly.

The maximum concentration of radioactivity in blood was reached at 6-8 hours for the low and mid dose level, and at 24 hours for the high dose level. The concentration of parent chemical was essentially constant during 3 and 6 hours for the mid and high dose level,

respectively and then declined. 1,2-DCB could only be detected in blood in the first two hours after administration of the 5 mg/kg dose.

The major route of biotransformation was via the glutathione pathway and 60% of the urinary metabolites were mercapturic acids. In addition, the major metabolites in bile were conjugates of glutathione. Other major metabolites in urine were the sulfate conjugates of 2,3- and 3,4-dichlorophenol (DCP). No significant differences in metabolic profiles were observed between the different doses. Induction with phenobarbital resulted in the increased excretion of sulfate conjugates (30% in induced rats, 20% in control rats), mainly the conjugate of 3,4-DCP. The mercapturic acids in urine and glutathione conjugates in bile were epoxide-derived, whereas no quinone- or hydroquinone-derived metabolites were observed. Therefore the hepatotoxicity of 1,2-dichlorobenzene is assumed to be related, at least partly, to the presence of the intermediate arene oxide. A high dose of 1,2-DCB will result in depletion of GSH, followed by oxidative stress and possible binding to macromolecules.

INTRODUCTION

For many years 1,2-dichlorobenzene (1,2-DCB) has been used extensively in the industrial synthesis of various organic compounds and therefore represents a significant environmental pollutant (Oliver and Nicol, 1982; Zoeteman *et al.*, 1980). 1,2-DCB is a potent hepatotoxicant (Cameron *et al.*, 1937; Brondeau *et al.*, 1986; Robinson *et al.*, 1991; Stine *et al.*, 1991). The hepatotoxicity of halogenated benzenes is thought to be mediated by the generation of reactive metabolites as a result of their oxidation by cytochrome P450 (Brodie *et al.*, 1971; Reid *et al.*, 1973; Reid and Krishna, 1973). Enhanced toxicity by induction of the cytochrome-P450 enzymes, mostly by phenobarbital, has been reported (Reid *et al.*, 1973; Stine *et al.*, 1991; Gunawardhana *et al.*, 1993; Valentovic *et al.*, 1993). Toxicity and covalent binding to macromolecules have been ascribed mainly to the epoxides of the halobenzenes (Jollow *et al.*, 1974). However, more recent studies have shown that secondary quinone metabolites are also involved in the alkylation of hepatic proteins (Narasimhan *et al.*, 1988; Slaughter and Hanzlik, 1991). The relative contribution of the quinones and the epoxides to the toxicity of halobenzenes is still unclear.

Many studies have been conducted on the toxicity of 1,2-DCB and also several *in vitro* studies have been performed (Fisher *et al.*, 1990). Den Besten *et al.*, (1992) suggested a key

role for quinones in the protein binding of reactive metabolites of 1,2-DCB in a microsomal system. Few reports have been published on the *in vivo* metabolism of 1,2-DCB. In order to correlate *in vitro* data to *in vivo* data, toxicity data to biotransformation and ultimately animal data to human risk, *in vivo* metabolism studies are necessary. Azouz and co-workers (1955) have reported the metabolism of 1,2-DCB in rabbits after an oral dose of 500 mg/kg. They showed that mainly conjugated phenols (sulfates and glucuronides) were excreted and that only 5% of the dose was excreted as a mercapturic acid. However, the ability of 1,2-DCB to deplete glutathione (GSH) and the enhanced toxicity after GSH depletion has been observed by several investigators (Reid, 1973; Stine *et al.*, 1991; Den Besten *et al.*, 1991).

In the current work, we investigated the kinetics and metabolism of 1,2-DCB in the rat at three different oral dose levels, including a toxic dose level, in order to correlate the existing toxicity data to the kinetics and biotransformation. Also the effect of induction of the oxidation by cytochrome P450 on the metabolic profile was examined.

MATERIALS AND METHODS

Chemicals

1,2-Dichloro[U-^{14}C]benzene (1,2-DCB) with a radiochemical purity of >98% and a specific activity of 326 MBq/mmol was obtained from Sigma Chemical Co., St. Louis, MO, USA. 1,2-Dichlorobenzene (purity at least 99%) was from Merck, Darmstadt, Germany, 2,3- and 3,4-dichlorophenol were obtained from Aldrich Chemical Company Ltd, Wembley, UK. Phenobarbital (PB) was obtained from CAV, Utrecht, the Netherlands. β-Glucuronidase (from E.coli, sulfatase-free) and β-Glucuronidase/Arylsulfatase from Helix pomatia (100 μl containing 10,000 Fishman units b-glucuronidase and 80,000 Roy units arylsulfatase) were obtained from Boehringer Mannheim GmbH, Germany. All other chemicals were of reagent grade.

Animals

Adult male Wistar rats (strain Crl:(WI)WUBR) weighing 250-300g and 9-11 weeks old, were obtained from Charles River Wiga GmbH, Sulzfeld, Germany. The rats were provided *ad libitum* with the Institute's cereal-based rodent diet (SDS Special Diets Services,

Witham, England) and tap water. The phenobarbital pretreated rats received drinking water with 0.1% (w/v) PB during four days before dosing with 1,2-dichlorobenzene.

Blood kinetics

Three groups of three rats were orally dosed by gavage with 5, 50 or 250 mg/kg bw 1,2-dichlorobenzene (0.74 MBq/kg bw), dissolved in corn oil (4 ml/kg bw). After administration, blood samples of approximately 150 μl were taken via the tail vein at the following time points: 1, 2, 3, 4, 6, 8, 12, 24, 48, 72 and 96 hours. The samples were mixed with an equal amount of heparinized saline and stored at -20 °C until further processing.

Tissue distribution

Four groups of three rats were orally dosed by gavage with a non-toxic dose (10 mg/kg bw) of 1,2-DCB (1.48 MBq/kg bw) dissolved in corn oil (4 ml/kg bw). The animals were placed in metabolism cages and sacrificed after 6, 15, 30 and 75 hours. Urine and faeces were collected until sacrifice. The rats were killed by aortal bleeding under ether anaesthesia. The following organs and tissues were removed and weighed: liver, kidneys, spleen, pancreas, lungs, heart, brains, skin, femur bone, skeletal muscle, perirenal fat, testis, urinary bladder, stomach, small intestine, cecum and colon. The contents of the gastro-intestinal tract were collected separately. The stomach, small intestine, cecum and colon were rinsed thoroughly to remove residues of the contents. Blood was centrifuged and separated into plasma and red blood cells. The residual carcass was dissolved in 1.5 M KOH in 20% (v/v) ethanol and heated during 24 hours at 60 °C. The metabolism cage was washed with 70 ml 0.5% (v/v) triton X100. Aliquots of the tissues and excreta were analyzed for radioactivity.

Excretion in urine and faeces

Two groups of nine rats were orally dosed by gavage with 1,2-dichlorobenzene (0.74 MBq/kg), dissolved in corn oil (4 ml/kg). One group was pretreated with phenobarbital. Each group was divided in three groups of three rats, which received 5, 50 and 250 mg/kg 1,2-DCB, respectively. After dosing, the rats were placed in metabolism cages. Urine and faeces were collected separately after 8 hours, from 8 to 24 hours and then every 24 hours up to 6 days. Urine and faeces were stored at -20 °C until further processing.

Excretion in bile

The bile ducts of two rats were cannulated under halothane anaesthesia. Up to xenobiotic administration, the bile was carried back into the duodenum by a re-entry cannula. The rats were allowed to recover for several days and then dosed with 10 mg/kg 1,2-DCB (2.78 MBq/kg). Bile was collected immediately after dosing every 15 minutes up to 2 hours, then every 30 minutes up to 3 hours, every hour up to 3 hours, from 8 to 24 hours and from 24 to 48 hours. Urine and faeces were collected from 0 to 8 hours and from 8 to 48 hours. The samples were analyzed for total radioactivity. Bile- and urine samples from different time intervals were treated with β-glucuronidase and arylsulfatase and treated and untreated samples were analyzed on HPLC.

Measurement of radioactivity

Samples of urine (0.5 ml), bile (20 μl), plasma (0.5 ml) and cage wash (0.7 ml) were mixed with 4.5 ml Ultima Gold scintillation cocktail (Packard Instrument Company, Reading, UK), residual carcass (1 ml) was mixed with 15 ml Hionic Fluor (Packard). Radioactivity was measured in a Liquid Scintillation Analyzer (Packard Instrument Company) using automatic external standard quench correction, and was counted at efficiencies >85%. Faeces were homogenized with 3 parts of water. Samples of ± 0.5 g were burned in oxygen using an Automatic Sample Oxidizer (Model 307 Mk.2, Packard Instrument Company). The combustion products were absorbed in CarboSorb and mixed with Permafluor V scintillator system (Packard) for measurement of radioactivity. This method of analysis of the faeces cannot exclude partial evaporation of non-absorbed parent 1,2-DCB in the period between excretion and combustion of the faeces. Therefore it is possible that the amount of radioactivity in the faeces was underestimated. Samples of blood, organs and tissues were weighed in combustible cups, burned in oxygen and analyzed for radioactivity.

Enzymatic hydrolysis of metabolites in urine and bile

For determination of glucuronide conjugates, 200 μl urine or 100 μl bile was added to an equal volume of a 200 mM sodium acetate buffer, pH = 7, and 60 μl of a β-glucuronidase solution (12000 Fishman units at 37 °C). The mixtures were then incubated at 37 °C for 20 hours. After incubation, the mixtures were stored at -20 °C until analysis. The same procedure was followed for control incubations without enzyme. To hydrolyze the sulfate conjugates, 200 μl urine or 100 μl bile were added to equal volumes of a 200 mM sodium acetate buffer, pH =

5, and 60 μl Helix pomatia which contained 6000 Fishman units β-glucuronidase and 48000 Roy units arylsulphatase. The mixtures were then incubated at 37 °C for 20 hours and stored at -20 °C.

High Pressure Liquid Chromatography

HPLC was carried out with a Pharmacia LKB HPLC Pump 2248 (Pharmacia, Sweden), fitted with a Pharmacia LKB-VWM 2141 variable wavelength u.v. detector operating at 254 nm. Radioactivity was measured on line with a Flo-one Beta Radio-Chromatography Detector, series A-515 (Packard Instrument Co, USA). A liquid flow cell of 1 ml was used and radioactivity was measured using Flo Scint A (Packard) with a flow of 2 ml/min. Separations were carried out using a reversed phase column, 250 x 4.6 mm, C18 hypersil ODS 5 μm (Chrompack). Eluting solvents were methanol and 20 mM ammonium acetate, pH = 6.7, used with the following gradient: 0-5 min.: 15% methanol; 5-10 min.: 15->30% methanol; 10-30 min.: 30->50% methanol; 30-38 min.: 50->95% methanol; 38-45 min.: 95% methanol. The flow rate was 1 ml/min. Injections of 100-200 μl were made automatically using a Pharmacia LKB-Autosampler 2157 or manually with a Rheodyne injector.

Gas Chromatography

The concentration of the parent 1,2-dichlorobenzene in blood after oral dosing was determined using gas chromatography. Aliquots of blood were extracted with 3 parts of cyclohexane, containing chlorobenzene as internal standard. Extraction efficiency was determined by adding a known amount of 1,2-DCB to blood and to determine the recovery after extraction, which was at least 95 %. One microliter of the extracts was injected onto a Perkin-Elmer 8500 Gas Chromatograph. Starting temperature of the oven was 80 °C, and after 11 minutes increased by 5 °C/min to 105 °C. Temperature of the injection port was 175 °C and the carrier gas was helium. Separations were accomplished on a 30 m DB-5 column, 0.25 x 0.25 μm. Detection was accomplished with an electron capture (ECD) detector.

Mass Spectrometry

Metabolites of 1,2-DCB excreted in the urine and bile were collected separately using HPLC and the fractions dried under a flow of nitrogen. The urinary residues were mixed with glycerol as matrix for FAB-MS (fast atom bombardment mass spectrometry). A Finnigan-MAT 900 instrument was used with a magnetic scan range of 50-1000 amu. The primary

energy of the cesium ionizing beam was approximately 70 eV. Accelerating voltage was 5 kV, dynode voltage was 16 kV and the electron multiplier voltage was 1.9 kV. All measurements were performed at a resolution of 1100 (10%) and the source temperature was 50 °C. Biliary metabolites were measured using LC-MS (liquid chromatography mass spectrometry). A spherisorb S5 ODS-2 (200*0.32 mm) column was used. The mobile phase A was 0.05% trifluoracetic acid in bidest, and mobile phase B was 60 ml bidest in 200 ml methanol. The solvent programme started with 100% A, followed by a linear gradient to 100% B in 30 min, and then the programme remained at 100% B for 10 min. The flow was 0.2 ml/min. Mass spectrometric analyses were performed using electrospray ionization (Analytica) on a Finnigan MAT TSQ 700.

RESULTS

Blood kinetics

The concentration of 1,2-DCB as well as the total radioactivity in blood were determined at different time points at the three oral dose levels (5, 50 and 250 mg/kg body weight, Fig. 1A and B). For the low dose, the highest concentration of radioactivity (Cmax[ra]) was reached after 6 hours; the parent compound could only be detected in the first two hours after administration (Detection limit was 0.05 μmol/l). At 50 mg/kg body weight, the highest concentration of 1,2-DCB (Cmax[DCB]) was reached 3 hours after administration. Cmax[ra] was reached at 8 hours after administration. For the high dose level, the concentration of parent chemical was essentially constant during the first 6 hours, and then decreased. Cmax[ra] was reached after 24 hours.

Tissue distribution

The distribution of radioactivity between the different organs and tissues at four different time points is summarized in Table 1. Highest concentrations were reached after 6 hours for all tissues except for cecum and colon. Highest concentrations were found in kidneys, liver, urinary bladder, small intestine, abdominal skin (including subcutaneous fat) and perirenal fat.

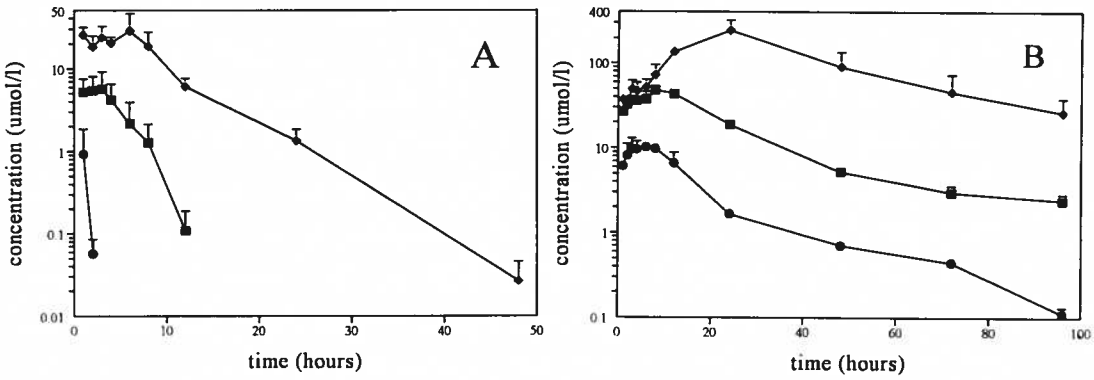

Fig.1. A. Blood concentrations of 1,2-dichlorobenzene after an oral dose of 5 (●), 50 (■) or 250 (◆) mg/kg body weight. Mean values and standard deviations are given for each timepoint and dose (n = 3 animals). B. Blood concentration of total radioactivity after an oral dose of 5 (●), 50 (■) or 250 (◆) mg 1,2-DCB/kg body weight. Mean values and standard deviations are given for each timepoint and dose (n = 3 animals).

Excretion in urine and faeces

In Fig. 2A the cumulative excretion in the urine and faeces is presented as percentage of the dose at three different dose levels. Excretion was predominantly in the urine (75-84% of the dose). Faeces contained 16, 12 and 7% of the dose, for the low, mid and high dose level,

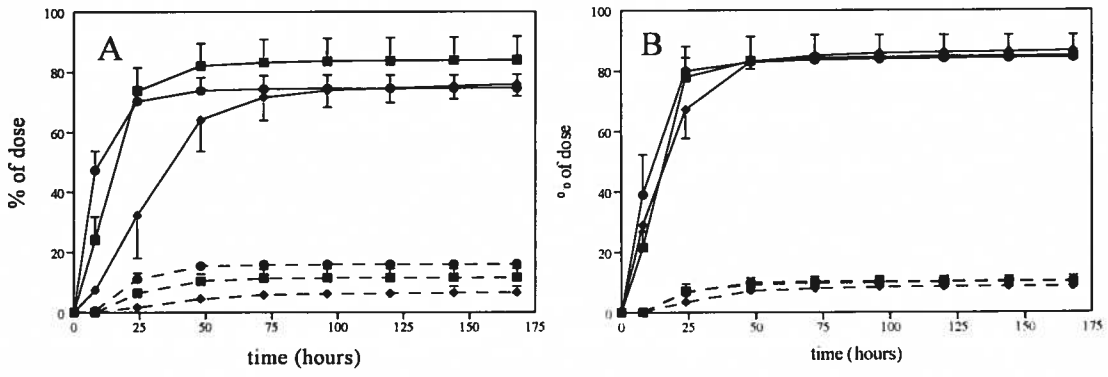

Fig.2. A. Cumulative excretion of total radioactivity in urine (solid lines) and faeces (dotted lines) as percentage of the dose after an oral dose of 5 (●), 50 (■) or 250 (◆) mg 1,2-DCB/kg body weight. Mean values and standard deviations are given for each timepoint and dose (n = 3 animals). B. Cumulative excretion of total radioactivity in urine (solid lines) and faeces (dotted lines) as percentage of the dose after an oral dose of 5 (●), 50 (■) or 250 (◆) mg 1,2-DCB/kg body weight in phenobarbital pre-treated rats. Mean values and standard deviations are given for each timepoint and dose (n = 3 animals).

Table 1. Tissue distribution of radioactivity at 4 time points after an oral dose of 1,2-dichlorobenzene (10 mg/kg). Results are expressed as nmol compound per gram tissue or percentage of the dose (residual carcass, contents of gastro-intestinal tract (GIT)). The half life ($t_{½}$) is calculated assuming first order depletion kinetics.

Tissue	6 hours	15 hours	30 hours	75 hours	$t_{½}$ (hr)
Liver	32.7 ± 3.4	9.4 ± 1.9	3.1 ± 1.1	1.4 ± 0.4	17.0
Kidneys	132.5 ± 107	15.7 ± 4.8	3.8 ± 0.7	1.5 ± 0.4	13.1
Spleen	8.0 ± 5.3	2.0 ± 0.9	0.59 ± 0.14	0.20 ± 0.07	15.2
Pancreas	9.5 ± 5.6	2.6 ± 0.9	1.11 ± 0.4	0.23 ± 0.08	14.5
Lungs	6.6 ± 0.6	3.4 ± 0.9	1.02 ± 0.12	0.29 ± 0.11	16.0
Heart	4.7 ± 0.8	2.6 ± 0.8	0.7 ± 0.08	0.18 ± 0.03	15.1
Brains	1.1 ± 0.1	0.7 ± 0.08	0.3 ± 0.08	0.09 ± 0.04	19.3
Skin	18.9 ± 10.9	2.9 ± 1.1	1.11 ± 0.46	0.41 ± 0.12	15.1
Femur bone	5.2 ± 2.6	1.3 ± 0.4	0.55 ± 0.18	0.14 ± 0.0	15.1
Skel. muscle	4.7 ± 3.1	1.3 ± 0.6	0.45 ± 0.2	0.09 ± 0.04	13.5
Perir. fat	33.4 ± 12.1	14.0 ± 2.6	2.18 ± 0.3	0.18 ± 0.03	9.4
Testis	3.6 ± 0.8	1.9 ± 0.4	1.13 ± 0.9	0.20 ± 0.07	17.2
Urin. blad.	183 ± 121	17.3 ± 13.6	6.6 ± 6.4	0.32 ± 0.04	8.7
Stomach	6.5 ± 1.7	1.7 ± 0.2	0.98 ± 0.46	0.16 ± 0.03	14.3
Sm. intest.	29.1 ± 9.3	10.7 ± 0.6	3.5 ± 2.4	0.43 ± 0.28	11.6
Cecum	16.4 ± 4.8	16.7 ± 1.1	2.8 ± 2.2	0.27 ± 0.07	11.1
Colon	7.5 ± 2.2	12.0 ± 2.4	1.4 ± 0.9	0.20 ± 0.07	12.0
Plasma	22.3 ± 2.0	8.8 ± 3.0	1.8 ± 0.1	0.41 ± 0.14	12.5
Reb.bl.cells	9.2 ± 1.0	3.4 ± 0.6	1.6 ± 0.4	0.57 ± 0.22	18.8
Resid. carc.	13% ± 3%	4% ± 2%	1% ± 0.2%	0.3%±0.07%	--
GIT cont.	13% ± 4%	15% ± 4%	2% ± 1%	0.1%±0.04%	--

respectively. The total excretion was almost complete within 24 hours after administration of the low and mid dose. For the high dose, the faecal excretion was maximal between 24 and 48 hours. The residual carcass contained less than 1% of the dose for all dose levels. After induction with phenobarbital, the contribution of the urinary excretion increased (84-87% of the dose). Also, differences in the rate of excretion between the three doses were smaller than without induction (Fig. 2B).

Excretion in bile

Fig. 3 presents the cumulative excretion in bile after an oral dose of 10 mg/kg. The concentration of radioactivity in the bile was maximal at 6 hours after administration. 60% of the dose was excreted in bile and 25% in urine. Nearly all radioactivity was excreted within the first 24 hours. The amount excreted in the faeces was very low (<4%).

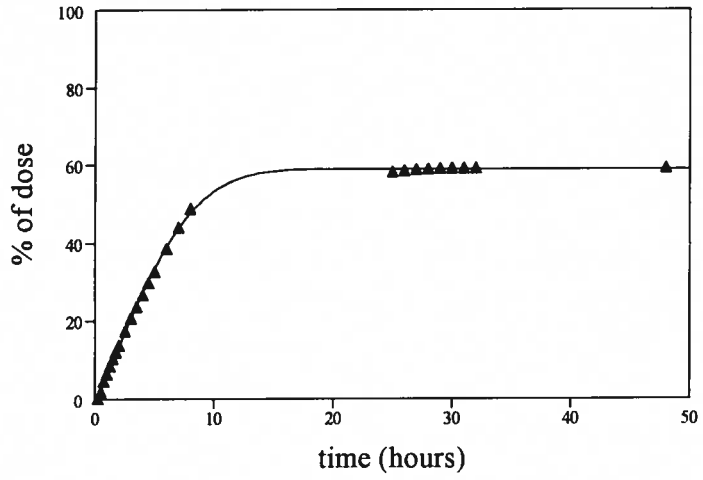

Fig.3. Cumulative excretion of total radioactivity in bile as percentage of the dose after an oral dose of 1,2-DCB (10 mg/kg, n = 2 animals).

Identification of urinary metabolites

Since only minor differences in urine HPLC profiles between the different doses were observed, the profiles of one dose level (250 mg/kg) are presented. Fig. 4A and B show chromatograms of a urine sample before and after hydrolysis of conjugates by Helix pomatia. The two peaks in the chromatogram which increased in intensity after hydrolysis (peaks 6 and 7) were identified as 2,3- and 3,4-dichlorophenol (DCP) respectively by co-elution with non-radiolabeled reference compounds (data not shown). Since incubation of urine with β-glucuronidase alone did not change the profile, peaks 3 and 4 were assigned to the sulfate esters of the two isomers of dichlorophenol. This was confirmed by FAB-MS (molecular ion at m/z = 242 and a characteristic fragment without SO , m/z = 162 and without chlorine, m/z = 207), as shown in Fig. 5A. Fig. 4C shows a HPLC-chromatogram of urine from a rat dosed with 1,2-DCB after induction with phenobarbital. The major difference from urine of non-treated rats is the increase in amount of a sulfate conjugate (peak 4). This was observed for all doses (data not shown). Hydrolysis of this urine sample resulted in the formation of mainly 3,4-

dichlorophenol (peak 7, data not shown). Therefore peaks 3 and 4 were assigned to the sulfates of 2,3- and 3,4-dichlorophenol respectively. HPLC-analysis of urine from a low dose rat, containing much less material with a high specific activity, resulted in a better separation and revealed that peak 1 consisted of two different metabolites (results not shown); the three compounds represented by peak 1 and 2 were also analyzed by FAB-MS. These metabolites were identified as phenylmercapturic acids of 1,2-DCB since the ions, displaying a two-

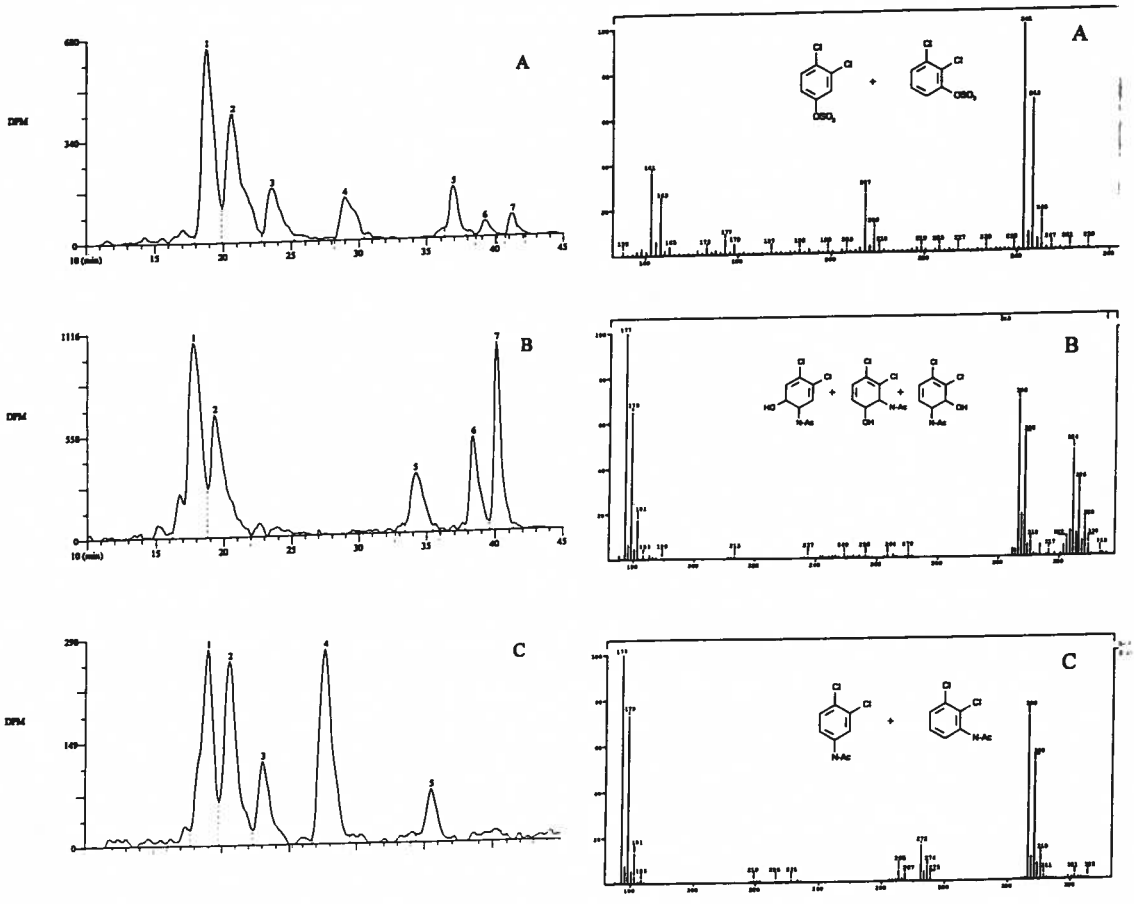

Fig.4. A. HPLC-chromatogram of urine from a rat dosed with 1,2-DCB (250 mg/kg body weight). Peaks 1 and 2: phenylmercapturic acids; peaks 3 and 4: sulfates of 2,3- and 3,4-DCP respectively; peak 5: mercapturic acid; peaks 6 and 7: 2,3- and 3,4-DCP respectively. B. HPLC-chromatogram of the same urine sample as in A above, but after enzymatic hydrolysis of the conjugates with Helix pomatia. C. HPLC-chromatogram of urine from a rat dosed with 1,2-DCB (250 mg/kg body weight) after induction with phenobarbital.

Fig.5. Mass-spectra of the major urinary metabolites of 1,2-DCB. A) Peaks 3 and 4. B) Peaks 1 and 2. C) Peak 5. Peak numbers refer to those described in Figure 4. N-Ac = N-acetylcysteinyl.

chlorine isotope pattern with a molecular ion at *m/z* = 325 and fragments at *m/z* = 307 and 178, were attributed to loss of water and fragmentation of the N-acetylcysteinyl side-chain; the fragment at *m/z* = 272 is formed due to loss of chlorine (Fig. 5B). Because no differences between the mass-spectra of the three compounds were observed, these are assigned as the three possible isomers. The mass-spectrum of component 5 showed a molecular ion at *m/z* = 307. The structure of this metabolite can also be assigned to a mercapturic acid; the fragment at *m/z* = 178 can be attributed to the thio-ether. The difference with the other mercapturic

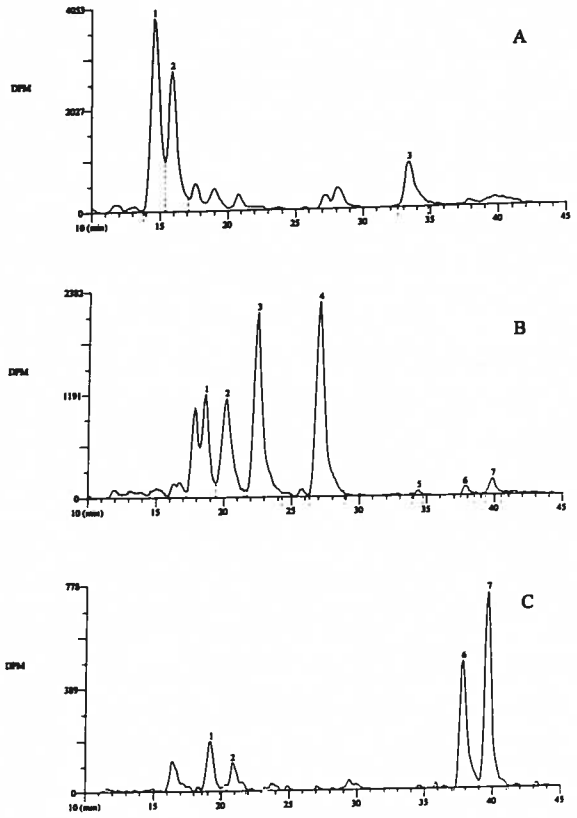

Fig.6. A. HPLC-chromatogram of bile from a rat, dosed with 1,2-DCB (10 mg/kg). Peaks 1 and 2: glutathione conjugates; peak 3: mercapturi acid B. HPLC-chromatogram of urine from the same rat as decribed in A above, collected during the first 8 hours. Peak numbers correspond to the numbers in Figure 4. C. HPLC-chromatogram of the same urine sample as in B above, but after enzymatic hydrolysis of the conjugates with Helix pomatia.

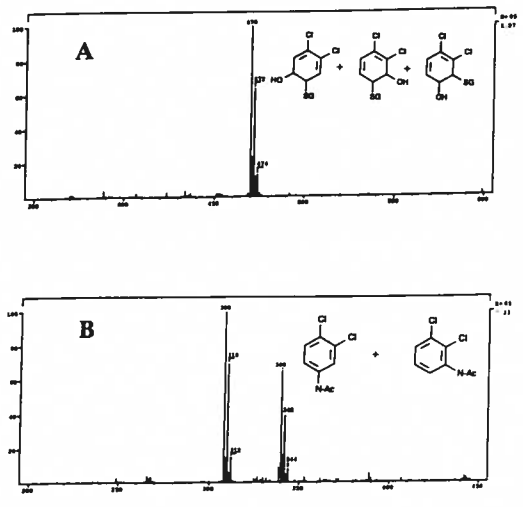

Fig.7. Mass-spectra of the major biliary metabolites of 1,2-DCB. A) Peaks 1 and 2. B) Peak 3. SG = glutathione, N-Ac = N-acetylcysteinyl.

acids is due to loss of water, resulting in regaining the aromatic structure (Fig. 5C). The radioactivity in faeces, which accounted for only minor amounts, was not identified by structural analysis. However, since the method of analysis of radioactivity allowed for evaporation of the volatile 1,2-DCB, the radioactivity was tentatively assigned to (non-volatile) metabolites.

Identification of biliary metabolites

The bile of rats dosed with 10 mg/kg 1,2-DCB contained two major metabolites (1 and 2), which were not affected by enzymatic hydrolysis (Fig. 6A). These metabolites, and the metabolite eluting at 34 minutes (peak 3), were analyzed by LC-MS: metabolites 1 and 2 both had a mass of 470 and were assigned to the precursors of the phenylmercapturic acids, i.e. the glutathione conjugates of the epoxides of 1,2-DCB (Fig. 7A). Metabolite 3 had a mass of 308 (340 = + MeOH), and was assigned to the same mercapturic acid as was found in the urine (Fig. 7B). The urine of these rats predominantly contained sulfate conjugates and minor amounts of dichlorophenols and mercapturic acids (Fig. 6B). Enzymatic hydrolysis of this urine fraction resulted in disappearance of the sulfates and an increase in the amount of phenols (Fig. 6C).

DISCUSSION

1,2-Dichlorobenzene is predominantly metabolized to phenylmercapturic acids and phenolic sulfates, 60% and 20% respectively of the urinary metabolites (Fig. 8). Azouz and co-workers (1955) reported that only 5% of the dose was excreted as mercapturic acids by the rabbit after a 500 mg/kg oral dose. The extensive metabolism to mercapturic acids by the rat has not been reported before and explains the depletion of glutathione (GSH) demonstrated in other studies (Stine *et al.*, 1991; Reid, 1973). The conjugation by GSH, which has been extensively studied for bromobenzene, is assumed to occur via the intermediate arene oxide (Brodie *et al.*, 1971; Reid and Krishna, 1973; Jollow *et al.*, 1974). The reactivity towards glutathione also indicates the ability to react with biological macromolecules (proteins and DNA), a possible explanation for the hepatotoxic effects found for 1,2-DCB (Hollingsworth *et al.*, 1958; Robinson *et al.*, 1991; Stine *et al.*, 1991; Allis *et al.*, 1992). Depletion of glutathione enhances the toxicity of 1,2-DCB (Stine *et al.*, 1991). Also induction of the first

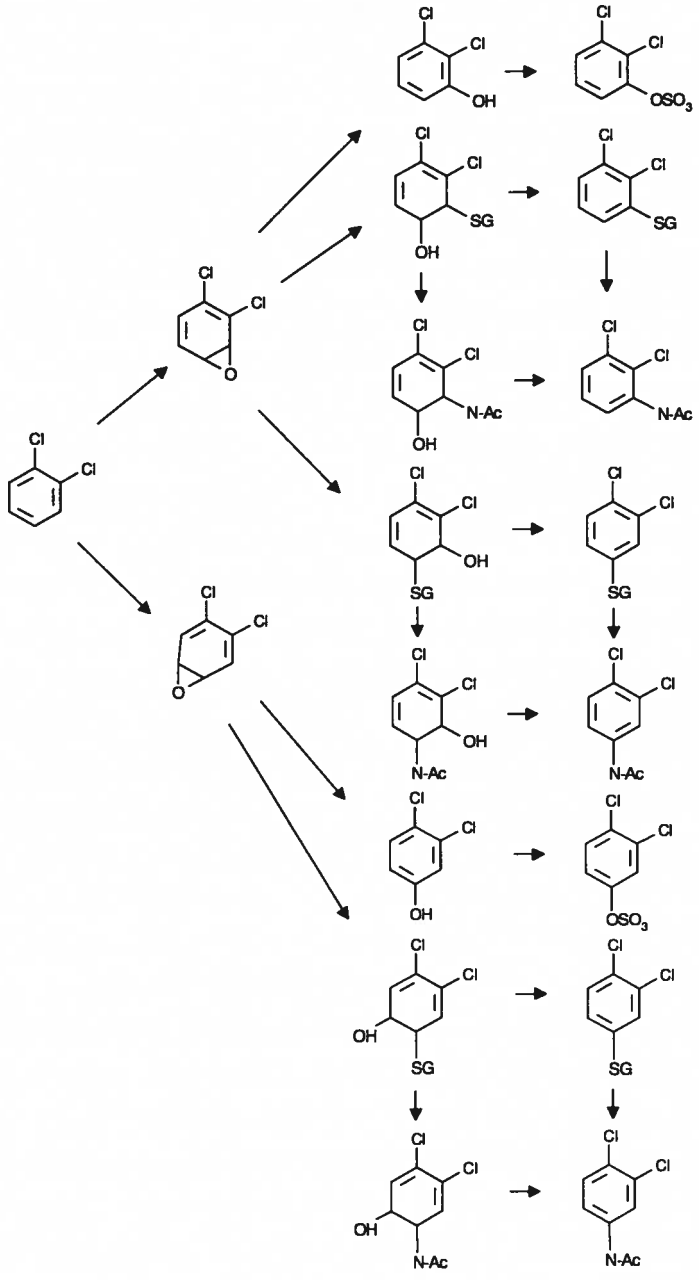

Fig.8. Simplified scheme of the major pathways of the *in vivo* metabolism of 1,2-dichlorobenzene in the rat. SG = glutathione; N-Ac = N-acetyl-cysteinyl.

biotransformation step, in which the arene oxide is formed, enhances toxicity (Reid *et al.*, 1973; Reid and Krishna, 1973; Stine *et al.*, 1991; Gunawardhana *et al.*, 1993; Valentovic *et al.*, 1993). However, recent studies with bromobenzene have indicated that other reactive intermediates, namely secondary quinones, are also involved in covalent protein binding (Narasimhan *et al.*, 1988; Slaughter and Hanzlik, 1991). The present study was undertaken to investigate the influence of a toxic dose level and induction on the kinetics and biotransformation of 1,2-DCB, in order to correlate toxicity with the metabolic profile. In the low and mid dose groups, 1,2-DCB was completely absorbed and metabolized. At the high dose level, the relatively low recovery of total radioactivity (83% of the dose) cannot exclude partial excretion of the (volatile) non-absorbed 1,2-DCB in the faeces, indicating incomplete absorption (see also methods section). A pilot experiment demonstrated that at the high dose level, less than 1% of the administered radioactivity was exhaled (results not shown). Thus, exhalation of the parent compound is not a significant route of elimination.

The tissue distribution study showed that the highest concentrations of radioactivity after absorption of 1,2-DCB were present in the liver, kidneys and fat. The high concentration in the kidneys can be explained by the renal excretion of the metabolites, which is the predominant route of elimination. Apparently, this high renal concentration of metabolites does not result in nephrotoxicity (Den Besten *et al.*, 1991), although Monks and co-workers (1985) demonstrated proximal tubular degeneration by glutathione conjugates of secondary hydroquinone metabolites of bromobenzene. The liver, in which high concentrations of radioactivity are also found, is the primary target organ for bromobenzene as well as 1,2-DCB. The parent compound is easily solubilized in fat, but is not persistent due to the high extent of metabolism, as indicated by the low half life in fat. This extensive metabolism is demonstrated by the bloodkinetics and rapid urinary excretion. At the low dose level, the concentration of 1,2-DCB was just above the limit of detection (0.05 μmol/l) in the first two hours after dosing. Apparently, first pass metabolism was high enough to extract most of the parent compound from the liver. The maximum blood concentration (Cmax) of metabolites after 6 hours (t = 6) is in agreement with t = 6 hours for biliary excretion and the maximum excretion in urine during the first 8 hours. For the 50 mg/kg and the 250 mg/kg dose levels, the concentration of 1,2-DCB in the blood was essentially constant during the first hours, indicating a steady state between absorption, distribution and elimination. At the high dose level, a saturation of metabolism was achieved, as indicated by three observations, namely i) the high dose was excreted much slower than the other two doses ii) the concentration of metabolites in the

blood was similar for the medium and high dose level up to the first 6 hours, and iii) at the high dose level the maximum concentration of radioactivity in blood was reached after 24 hours, whereas the maximum concentration of parent compound was already attained after about 6 hours. Apparently, the saturation of metabolism resulted in temporary storage of the parent compound in fat.

Since the ratio between mercapturic acids, sulfates and free phenols in urine did not change to a major extent with increasing dose levels, it can be concluded that either none of the conjugation routes is rate limiting, or all conjugation routes are rate limiting to the same extent. Since we demonstrated that saturation of the first epoxidation step occurs, the first hypothesis is the most likely. The amount of glutathione available in the liver, taking into account the initial amount and the production rate (based on D'Souza *et al.*, 1988), is approximately equal to the amount of glutathione conjugates formed at the 250 mg/kg dose level, which would explain the similar metabolic profiles at all dose levels. This is confirmed by a study conducted at this high dose level, which showed a depletion of glutathione in the liver of approximately 60% (Hissink, unpublished results). The remaining low glutathione concentration in the liver will result both in a reduced capacity to detoxify epoxides and a reduced defence against oxidative stress. Both mechanisms may be involved in the toxicity of 1,2-DCB.

PF is a known inducer of cytochrome P450 enzymes (mainly the CYP2B isoenzymes), which are responsible for the first biotransformation step of 1,2-DCB (Valentovic *et al.*, 1993). This explains the more rapid excretion after induction, especially at the high dose level at which metabolism seems to be saturated. Also, a shift to a higher urinary excretion is observed after induction. The increase in amount of sulfates may be a consequence of induction of sulfotransferases or, more likely, more formation of 3,4-DCP due to depletion of glutathione. This increased formation of only one isomer (3,4-DCP) after pretreatment with PB has also been found for bromobenzene, which is preferentially metabolized to p-bromophenol (Lau and Zannoni, 1981). Interestingly, both the formation of 3,4-DCP and p-bromophenol occur via the 'para'-epoxidation (see also Fig.8). For bromobenzene, it was shown that the 3,4-epoxide is toxic, whereas the 2,3-epoxide is not (Lau *et al.*, 1980).

Biliary excretion is a major pathway after 1,2-DCB metabolism. Since the renal route was the major route of excretion, most of the biliary compounds must have been reabsorbed from the intestines via the enterohepatic circulation. A mercapturic acid was also detected in bile, the same as was detected in urine. Apparently, the breakdown of the glutathione

conjugates occurs already in the liver and/or in the bile duct. In bile from rats treated with 1,2,4-trichlorobenzene mercapturic acids were also detected (Bakke *et al.*, 1992).

In the case of bromobenzene, a secondary quinone metabolite is thought to be (partly) responsible for the hepatotoxic effects (Narasimhan *et al.*, 1988; Slaughter and Hanzlik, 1991). This mechanism has also been proposed for the toxicity of 1,2-DCB (Den Besten *et al.*, 1992). However, only less than 1% of a 1.5 mmol/kg dose (i.p.) of bromobenzene in rats could be assigned to quinone-derived mercapturic acids, whereas 40% of the dose was excreted as epoxide-derived mercapturic acids (Zheng and Hanzlik, 1992). In addition, we did not find quinone-derived metabolites in urine (peaks >1% of radioactivity were detected and identified), whereas the epoxide-derived mercapturic acids were by far the major urinary metabolites, even more than were found for bromobenzene. This does not exclude a role for quinones in the hepatotoxicity of 1,2-DCB as the reactivity of quinones towards proteins could prevent them from further biotransformation. In fact, the majority of the protein-bound residues from bromobenzene which could be accounted for structurally (10-15%) was quinone-derived, whereas epoxides accounted for <0.5% of total protein covalent binding (Slaughter and Hanzlik, 1991).

In conclusion, our data indicate that GSH-conjugation of the epoxide, resulting in hepatic GSH depletion, is the primary cause of acute hepatic toxicity. At a high dose level, the intracellular GSH-concentration will be lowered to such an extent that the liver cells become susceptible to oxidative damage, and the unconjugated epoxides may react with cellular macromolecules. However, it cannot be excluded that quinone derived covalent binding may be involved in this or other forms of toxicity.

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CHAPTER 3

KINETICS AND METABOLISM OF 1,4-DICHLOROBENZENE IN MALE WISTAR RATS: NO EVIDENCE FOR QUINONE METABOLITES

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Submitted

ABSTRACT

The biotransformation and kinetics of 1,4-dichlorobenzene (1,4-DCB) were studied in male Wistar rats at three oral dose levels (10, 50 and 250 mg/kg). The effect of induction of CYP2E1 by isoniazid on the kinetics and biotransformation was determined.

Excretion was predominantly via the urine (78-85%) and to a small extent via the faeces (2-5%). The relative contributions of these routes were not dose dependent. Excretion via the bile ranged from less than 5% at the low dose level to 30% at the high dose level. The major biliary metabolite was the glucuronide of 2,5-dichlorophenol (2,5-DCP).

The time point at which the plasma concentrations of the parent compound and the metabolites were maximal (T_{Cmax}) as well as the maximum concentrations (C_{max}) increased with higher dose level. Induction by isoniazid resulted in a faster urinary elimination, whereas T_{Cmax} and C_{max} were lower for induced rats. In addition, the area under the blood curve (AUC) was smaller and total clearance was higher for induced rats.

1,4-DCB was mainly metabolized to 2,5-DCP (ca. 90%), which was detected in the urine as its sulfate (50-60%), glucuronide (20-30%) and the free form (5-10%). Minor metabolites were the N-acetyl-cysteine-S-dihydro-hydroxy-1,4-dichlorobenzene and the corresponding dehydrated N-acetyl-cysteine-S-1,4-dichlorobenzene, which comprised ca. 10% of total metabolites. No hydroquinones were observed for the male Wistar rat, not even under conditions of induced oxidative metabolism.

INTRODUCTION

1,4-Dichlorobenzene is mostly known as a moth repellent and as a deodorant in household and industry [1]. It is also an intermediate in the production of dyestuff, pesticides and drugs. The production of 1,4-DCB in the United States in 1990 is estimated to be 65 million kg. and increases yearly [2]. Environmental contamination also leads to human exposure via food and drinking water and via inhalation [3]. Recently the exposure to 1,4-DCB among 1000 adults in the United States has been investigated [4]. It was found that 98% had detectable levels of 2,5-dichlorophenol (2,5-DCP), a metabolite of 1,4-DCB, in their urine and 96% had detectable amounts of 1,4-DCB in their blood.

Various responses to the administration of 1,4-DCB to animals have been reported. 1,4-DCB has been found to be a renal carcinogen in male but not female Fischer-344 rats and a liver carcinogen in B6C3F1 mice [5]. The light hydrocarbon nephropathy induced by 1,4-DCB was observed in male Fischer rats, but not in female rats or in either sex of mice [6,7]. Increased cell proliferation has been found only in male rat kidney and in the livers of mice of both sexes, corresponding with the reported carcinogenic effects of 1,4-DCB in these tissues [8]. In a study with male BALB/c mice and male Wistar rats, only in the former species covalent binding to nucleic acids in various tissues was found, *in vitro* as well as *in vivo* [9]. Moreover, in long-term toxicity and carcinogenicity studies with Wistar rats, no treatment related effects were observed [10]. Apparently, the adverse effects observed in male rats after treatment with 1,4-DCB are species, sex and strain specific.

At present, the renal toxicity and carcinogenicity of 1,4-DCB is thought to be mediated by the binding of 1,4-DCB and 2,5-DCP to a_{2u}-globulin and the accumulation of a_{2u}-globulin in the kidney, a mechanism specific for the male rat [6,7,11]. However, this does not explain the lack of 1,4-DCB-induced toxicity and carcinogenicity in male Wistar rats [10], since this strain does synthesize the α_{2u}-globulin protein [12,13]. Accumulation of protein droplets, associated with binding to α_{2u}-globulin, was observed in male Wistar rats after oral administration of up to 600 mg/kg bw 1,4-DCB, although no distinct nephrotoxic effects were observed [14]. Thus, binding to α_{2u}-globulin may not be the sole factor involved in 1,4-DCB-induced toxicity and carcinogenesis, and an additional, possibly strain-dependent factor may be involved.

For most halogenated benzenes it has been demonstrated that biotransformation is necessary to cause toxicity [15]. The acute hepatotoxicity of 1,2-DCB has been related to the primary

oxidation step, resulting in the formation of epoxides, and the corresponding depletion of glutathione [16]. For e.g. bromobenzene, it has been demonstrated that also the secondary oxidation of the phenols, resulting in the formation of hydroquinones and quinones, plays a role in the covalent binding to proteins [17]. Recently, we showed that quinones derived from 1,4-DCB, but not from 1,2-DCB, play a major role in covalent binding to microsomal protein *in vitro* [18]. Moreover, in this study it was found that Fischer-344 rat microsomes produced more hydroquinones than Wistar rat microsomes. *In vivo*, the formation of hydroquinones derived from 1,4-DCB has been demonstrated in male Fischer-344 rats [19]. Strong evidence exists that the glutathione conjugates of di- and trichlorohydroquinones are responsible for renal toxicity in male Sprague-Dawley rats [20]. Thus, formation of (hydro)quinones may play a role in 1,4-DCB-induced toxicity and carcinogenicity.

We investigated the kinetics and biotransformation of 1,4-DCB at three different oral dose levels in male Wistar rats, as a part of a larger study relating toxicity and biotransformation of 1,2- and 1,4-DCB, conducted in our laboratory. The influence of pretreatment with isoniazid, an inducer of CYP2E1 [21], the main cytochrome-P450 isoenzyme involved in the biotransformation of 1,4-DCB [22] was also studied.

MATERIALS AND METHODS

Chemicals

1,4-Dichloro-[^{14}C]-benzene (1,4-DCB) with a radiochemical purity of >98% and a specific activity of 315 MBq/mmol was obtained from Sigma Chemical Co., St. Louis, MO, USA. The unlabeled 1,4-DCB was from Aldrich (Steinheim, Germany). 2,5-Dichlorophenol (2,5-DCP) was from Merck, Darmstadt, Germany. Isoniazid (isonicotinic acid, purity > 99%) was from Janssen Chimica (Beerse, Belgium). β-Glucuronidase (from E.Coli, sulfatase free) and *Helix pomatia* (100 µl containing 10,000 Fishman units β-glucuronidase and 80,000 Roy units arylsulfatase) were obtained from Boehringer Mannheim GmbH, Germany. All other chemicals used were of reagent grade.

Animals

Adult male Wistar rats (strain Crl: (WI)WUBR) weighing 230-285g and 9 - 11 weeks old, were obtained from Charles River Wiga GmbH, Sulzfeld, Germany. The rats were *ad libitum* provided with the Institute's cereal-based rodent diet (SDS Special Diets Services, Witham, England) and tap water. The animal room was air-conditioned and light controlled, with a light/dark cycle of 12 hours.

Blood kinetics - Control treatment

Untreated rats were cannulated in the jugular vein under light halothane anesthesia. The animals were allowed at least 5 days to recover from the operation. The rats were divided in three groups and were orally dosed by gavage with 10 (n=2), 50 (n=4) or 250 (n=4) mg/kg bw radiolabeled 1,4-DCB (740 kBq/kg for the low dose, 1480 kBq/kg for the other two doses), dissolved in corn oil (3 ml/kg bw). After dosing, the rats were individually housed in glass metabolism cages (Radleys). Blood samples of about 200 ml were taken from the cannula at 1, 2, 3, 4, 5, 6, 8, 12, 24, 48, 72, 96, 120 and 144 hours after administration. The blood samples were gently mixed with 200 µl of 20 mM sodium citrate in a glass vial with a teflonized cap. Aliquots were taken to determine total radioactivity in whole blood. The vials were centrifuged immediately (1560 g; 5 min.) and the red blood cells and plasma were separated and stored at -30 °C until further analysis. During the first 8 hours and from 8-24 hours after administration urine and faeces were collected separately in glass flasks. During the same time intervals the exhaled radioactivity was collected in two successive flasks filled with ethanol. After 24 hours the rats were placed in Techniplast^{tm} metabolism cages. Urine and faeces were collected every 24 hours up to 144 hours. The urine samples were stored at -30 °C; the faeces samples were homogenized with three parts of water and stored at -30 °C. After 168 hours the animals were anesthetized with diethylether and sacrificed by abdominal bleeding. Blood was collected and separated into red blood cells and plasma. The following organs and tissues were collected: liver, kidneys, spleen, lungs, testes, heart, skin and renal fat. All blood samples, organs and tissues were stored at -30 °C until further analysis. The residual carcass was digested by heating in 1.5 M KOH in 20% (v/v) ethanol during 24 hours at 60 °C.

Isoniazid treatment

The pretreated rats received drinking water *ad libitum* with 0.1% (w/v) isoniazid (isonicotinic acid) for 10 days before dosing with 1,4-DCB. Two groups of 4 cannulated rats were

orally dosed by gavage with 50 or 250 mg/kg bw radiolabeled 1,4-DCB (1110 kBq/kg), dissolved in corn oil (3 ml/kg bw). Sampling of blood, urine, faeces and exhaled air was carried out in the same way as described above. At 168 hours after administration of 1,4-DCB, the tissues and organs were removed and stored at -30 °C, and the carcass was digested as described above.

Excretion in urine and faeces

Excretion of radioactivity in urine and faeces was examined in a separate experiment, without possible effects of a surgical operation or blood sampling. Three groups of 3 rats were orally dosed by gavage with 10, 50 or 250 mg/kg bw radiolabeled 1,4-DCB (740 kBq/kg), dissolved in corn oil (3 ml/kg). After dosing, each animal was placed in a Techniplast[tm] metabolism cage. Urine and faeces were collected separately in the first 8 hours after dosing, from 8-24 hours and then every 24 hours up to 6 days. After each time interval the cage was rinsed with 5 ml water to remove remaining urine. The faeces were homogenized with three parts of water. Urine and faeces were stored at -30 °C until further analysis. After 168 hours the animals were sacrificed and organs were collected as described above.

Excretion in bile

The bile ducts of four rats were cannulated under halothane anesthesia. Prior to administration the bile was carried back into the duodenum by a re-entry cannula. The rats were allowed to recover from the operation during several days. Two rats were dosed by gavage with 10 mg/kg bw 1,4-DCB (1327 kBq/kg bw), dissolved in corn oil (3 ml/kg). The other two rats were dosed with 250 mg/kg 1,4-DCB and 1359 kBq/kg bw. For the low dose, bile was collected immediately after dosing every 15 minutes for 2 hours, then every 30 minutes for 3 hours, every hour for 3 hours, from 8-14 hours and from 14-24 hours. One rat was sacrificed after 24 hours, from the other rat bile was collected from 24-48 hours. Urine and faeces were collected from 0-8 hours, 8-24 hours and from 24-48 hours. Bile, urine and faeces samples were analyzed for total radioactivity. For the high dose, bile was collected every 15 minutes for 3 hours, then every 30 minutes for 5 hours, and then up to 24 hours, from 24-48 hours and from 48-72 hours after exposure. Urine and faeces were collected from 0-8, 8-24, 24-48 and from 48-72 hours. Bile, urine and faeces were analyzed for total radioactivity. In addition, bile- and urine samples were treated with b-glucuronidase and arylsulfatase and analyzed on HPLC.

Measurement of radioactivity

Samples of urine (0.5 ml), bile (20 ml) and plasma (0.5 ml) were mixed with 4.5 ml Ultima Gold scintillation cocktail (Packard Instrument Company, Reading, UK), residual carcass (1 ml) was mixed with 15 ml Hionic Fluor (Packard). Radioactivity was measured in a Pharmacia Wallac S1409 Liquid Scintillation Counter (Pharmacia, Uppsala, Sweden) using automatic external standard quench correction, and was counted at efficiencies > 85%. Faeces were homogenized with 3 parts of water. Samples of ± 0.5 g were burned in oxygen using an Automatic Sample Oxidizer (Model 307 Mk.2, Canberra Packard, USA). The combustion products were absorbed into Carbosorb and mixed with Permafluor E scintillator liquid (Packard) for measurement of radioactivity. Samples of whole blood, organs and tissues were weighed in combustible cups, burned in oxygen and analyzed for total radioactivity. The recovery of radioactivity from burnt samples was more than 96.5%.

Enzymatic hydrolysis of metabolites in urine and bile

For determination of glucuronide conjugates, 200 ml urine or 200 ml bile was added to an equal volume of a 200 mM sodium acetate buffer, pH=7, and 50 ml of a β-glucuronidase solution (12000 Fishman units at 37 °C). The mixtures were then incubated at 37 °C for 20 hours. After incubation, the mixtures were stored at -30 °C until analysis on HPLC. The same procedure was followed for control incubations without enzyme. For incubations with sulfatase and *Helix pomatia* (50 ml containing 5000 Fishman units b-glucuronidase and 40000 Roy units arylsulfatase), a 200 mM sodium acetate buffer, pH=5, was used. The same procedure was followed as with β-glucuronidase.

High Pressure Liquid Chromatography

Urinary metabolites were separated using HPLC. This was carried out with a Pharmacia LKB HPLC 2248 Pump (Pharmacia, Sweden), fitted with a Pharmacia VWM 2141 UV-detector operating at 254 nm. Radioactivity was measured on line with a Canberra Packard Radiomatic Detector (type A500, Flo-one Beta). A liquid flow cell of 500 or 2000 ml was used and radioactivity was measured using Flo Scint A (Packard) with a scintillator flow of 2 ml/min. Injections of 100 ml were done automatically by a Pharmacia LKB-Autosampler 2157 at 4 °C. Separations were carried out using a reversed phase column, 250 x 4.6 mm, C18 hypersil ODS 5 mm (Chrompack). Eluting solvents were methanol and 20 mM ammonium acetate, pH=6.7, used

with the following gradiënt: 0-5 min: 15% methanol; 5-10 min: 15->30% methanol; 10-30 min: 30->50% methanol; 30-38 min: 50->95% methanol; 38-45 min: 95% methanol; 45-48 min: 95->15% methanol. The flow rate was 1 ml/min.

Gas Chromatography

The concentration of 1,4-DCB in plasma after oral administration was determined using gas chromatography. Aliquots of plasma were extracted with 3 parts of cyclohexane, containing 2-bromotoluene as internal standard. Extraction efficiency was determined by adding a known amount of 1,4-DCB to plasma; recovery after extraction was at least 95%. Two microliters of the extracts were injected onto a Perkin Elmer 8500 Gas Chromatograph. Separations were accomplished on a DB-5 column (30 m., 0.25 x 0.25μm). The carriergas was helium and the make up gas was nitrogen (20 ml/min). Starting temperature of the oven was 65 °C, and after 5 minutes increased by 5 °C/min to 105 °C. Temperature of the injection port was 200 °C. Detection was accomplished with an electron capture detector (ECD). Detection limit was 0.5 μmol 1,4-DCB/l plasma.

Mass Spectrometry

Metabolites of 1,4-DCB excreted in the urine were collected separately using the HPLC system described above and the fractions were dried under a flow of nitrogen, with minimal loss of radioactivity. The urinary residues were mixed with glycerol as matrix for FAB-MS (Fast Atom Bombardment Mass Spectrometry). A Finnigan-Mat 900 instrument was used with a magnetic scan range of 50-1000 amu. The primary energy of the cesium ionizing beam was approximately 70 eV. Accelerating voltage was 5 kV and the electron multiplier voltage was 1.9 kV. All measurements were performed at a resolution of 1100 (10%) and the source temperature was 50 °C.

Statistics and treatment of data

Statistical analyses (Student's *t*-test) and calculation of kinetic parameters (using linear regression analysis) were performed with Microsoft Excel 5.0.

RESULTS

Toxicokinetics of 1,4-DCB in blood

The concentration of 1,4-DCB in plasma and of total radioactivity in blood as a function of time at three oral dose levels (10, 50 and 250 mg/kg) are shown in Fig. 1A and 1B. For rats pretreated with isoniazid, the concentration-time curves of 1,4-DCB and total radioactivity, determined at 50 and 250 mg/kg bw, are shown in figures 1C and 1D. The various pharmacokinetic parameters, derived from the parent compound curves (1A and 1C), are presented in Table 1. The values for the clearance and the half life of 1,4-DCB did not differ significantly between the different doses, for both control and induced rats. When compared with the control rats, the AUC is smaller (40% and 30% for the 50 and 250 mg/kg group respectively) and the clearance of 1,4-DCB is higher in induced rats (40% and 50% for the 50 and 250 mg/kg group respectively); p-values, calculated with Student's *t*-test, are shown in Table 1. The maximum concentration of 1,4-DCB is reached faster in induced rats and the half life is about 35% shorter than in control rats (p-values in table). For the high dose, the maximum concentration (Cmax) is lower in induced rats than in control rats.

Table 1. Pharmacokinetic parameters calculated from the concentration-time curves of 1,4-dichlorobenzene shown in fig. 1A and 2A (control and induced rats). Mean values and standard deviations (range when $n = 2$) are given for Cmax (maximum plasma concentration), AUC (area under the plasma curve) and clearance. T_{max} is the time point at which the maximum concentration is reached, given as the median with range between parentheses (- = no range observed); k_{el} is the elimination constant and $t_{½}$ is the elimination half-life (mean values and standard deviations). P-values, calculated with Student's t-test, are shown for AUC, clearance, k_{el} and $t_{½}$.

	10 mg/kg (*n=2*)	**50 mg/kg (*n=4*)**		**250 mg/kg (*n=4*)**	
	control	control	induced	control	induced
Cmax (µmol/l)	6.75 ± 0.04	21.3 ± 5.1	22.2 ± 7.8	104 ± 27	76.9 ± 23
t_{max} (hour)	4 (1-6)	6 (3-8)	2 (-)	6 (5-6)	3 (1-6)
AUC (µmol.hour/l)	59.3 ± 11.7	244 ± 48.5	148 ± 39	1181 ± 104	808 ±115
p-value AUC	--	0.011		0.0037	
Clearance (ml/min.kg)	24.1 ± 3.7	23.7 ± 2.9	32.7 ± 9	22.7 ± 2.3	34.0 ± 4.8
p-value clearance	--	0.075		0.0058	
k_{el} ($hour^{-1}$)	0.093 ± 0.037	0.11 ± 0.04	0.17 ± 0.05	0.09 ± 0.02	0.15 ± 0.02
p-value k_{el}	--	0.069		0.012	
$t_{½}$ (hour)	8.124 ± 3.26	7.06 ± 2.38	4.51 ± 1.3	7.62 ± 1.61	4.8 ± 0.55
p-value $t_{½}$	--	0.061		0.041	

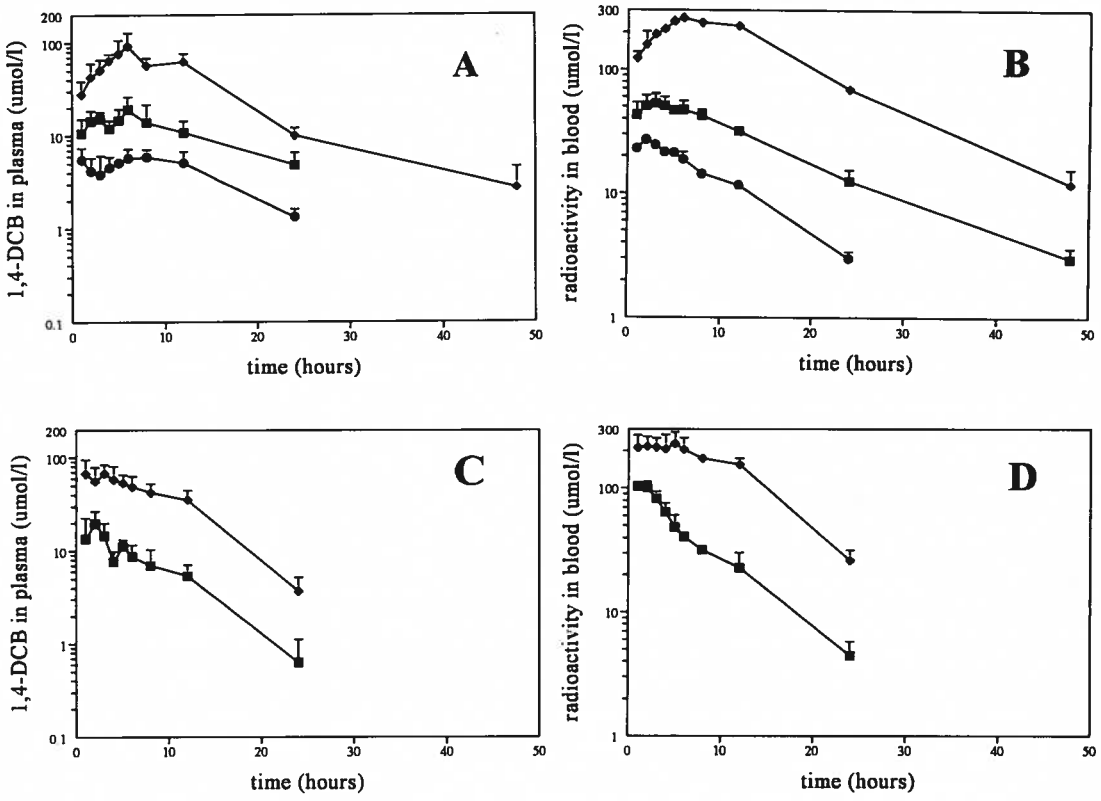

Fig. 1. A. Plasma concentration of 1,4-DCB after a single oral dose of 10 (●), 50 (■) or 250 (◆) mg/kg 1,4-DCB. Mean values and standard deviations (range when $n = 2$) are given for each timepoint and dose ($n = 2$ for the 10 mg/kg dose, $n = 3$ for the other two doses). B. Blood concentration of total radioactivity after a single oral dose of 10 (●), 50 (■) or 250 (◆) mg/kg 1,4-DCB. Concentration is expressed as µmol 1,4-DCB-equivalents/liter blood. Mean values and standard deviations (range when $n = 2$) are given for each timepoint and dose ($n = 2$ for the 10 mg/kg dose, $n = 3$ for the other two doses). C. Plasma concentration of 1,4-DCB after a single oral dose of 50 (■) or 250 (◆) mg/kg 1,4-DCB. Rats were pretreated with isoniazid. Mean values and standard deviations are given for each timepoint and dose ($n = 4$ animals). D. Blood concentration of total radioactivity after a single oral dose of 50 (■) or 250 (◆) mg/kg 1,4-DCB. Concentration is expressed as µmol 1,4-DCB-equivalents/liter blood. Rats were pretreated with isoniazid. Mean values and standard deviations are given for each timepoint and dose ($n = 4$ animals).

Excretion in urine, faeces and exhaled air

Elimination of 1,4-DCB via the lungs was less than 1% for all dose levels, for control rats as well as for induced rats. In all organs, including perirenal fat, less than 0.05% of the dose was recovered at 168 hours after administration. In Fig. 2, the cumulative excretion of total radioactivity in urine and faeces by control (A) and induced (B) rats is represented. For the control rats, the excretion in the urine and faeces was 80% and 4% respectively. Most of the radioactivity was excreted between 8 and 24 hours. No significant differences between the doses were observed. For

the induced rats, the excretion in urine and faeces was 92-97% and 5% respectively for the 50 mg/kg group and 80-85% and 4% for the 250 mg/kg group. In the first 8 hours, the urinary excretion of radioactivity was much higher for the 50 mg/kg group than for the 250 mg/kg group. In addition, for both doses the urinary excretion by the induced rats was more rapid and higher than by the control rats.

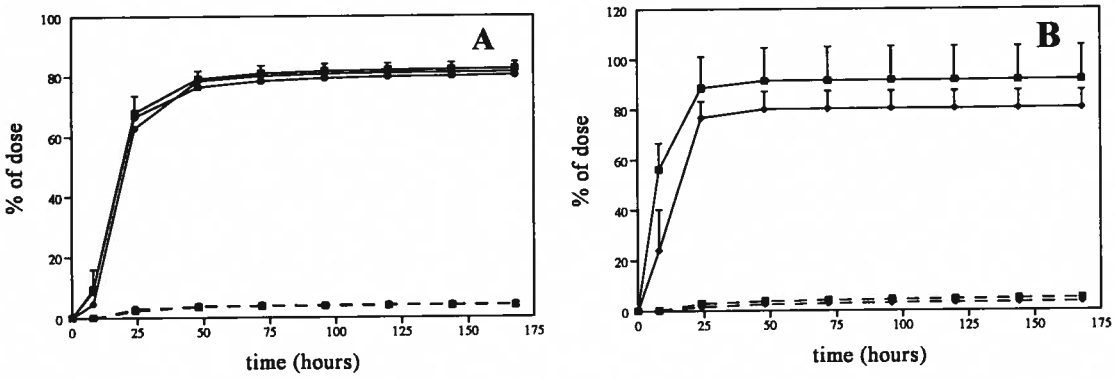

Fig. 2. A. Cumulative excretion of total radioactivity in urine (solid lines) and faeces (dotted lines) as percentage of the dose after an oral dose of 10 (l), 50 (n) or 250 (u) mg/kg 1,4-DCB. Mean values and standard deviations are given for each timepoint and dose (n = 3 animals). B. Cumulative excretion of total radioactivity in urine (solid lines) and faeces (dotted lines) as percentage of the dose after an oral dose of 50 (n) or 250 (u) mg/kg 1,4-DCB in isoniazid pretreated rats. Mean values are given for each timepoint and dose (n = 4 animals).

Excretion in bile

For the 10 mg/kg dose, only 4% of the dose (total radioactivity) was detected in bile, which was excreted mainly in the first 12 hours. Less than 2.5% of the dose was excreted in the faeces and in the urine 40% was found; elimination in urine was probably higher, since during sampling of bile not all of the urine could be collected. For the 250 mg/kg dose, 10-30% of the dose (total radioactivity) was excreted in the bile, 40-50% in the urine and less than 5% was detected in the faeces. Most of the radioactivity was excreted between 8 and 24 hours. The biliary elimination half life for the two rats used for the high dose level was 6.5 and 7.2 hours.

Identification of urinary and biliary metabolites

In Fig. 3A the HPLC profile of urine from rats treated with 50 mg/kg 1,4-DCB is represented; Fig. 3B shows the chromatogram of urine from rats pretreated with isoniazid, at the same dose level. Hydrolysis of conjugates by β-glucuronidase and arylsulfatase from

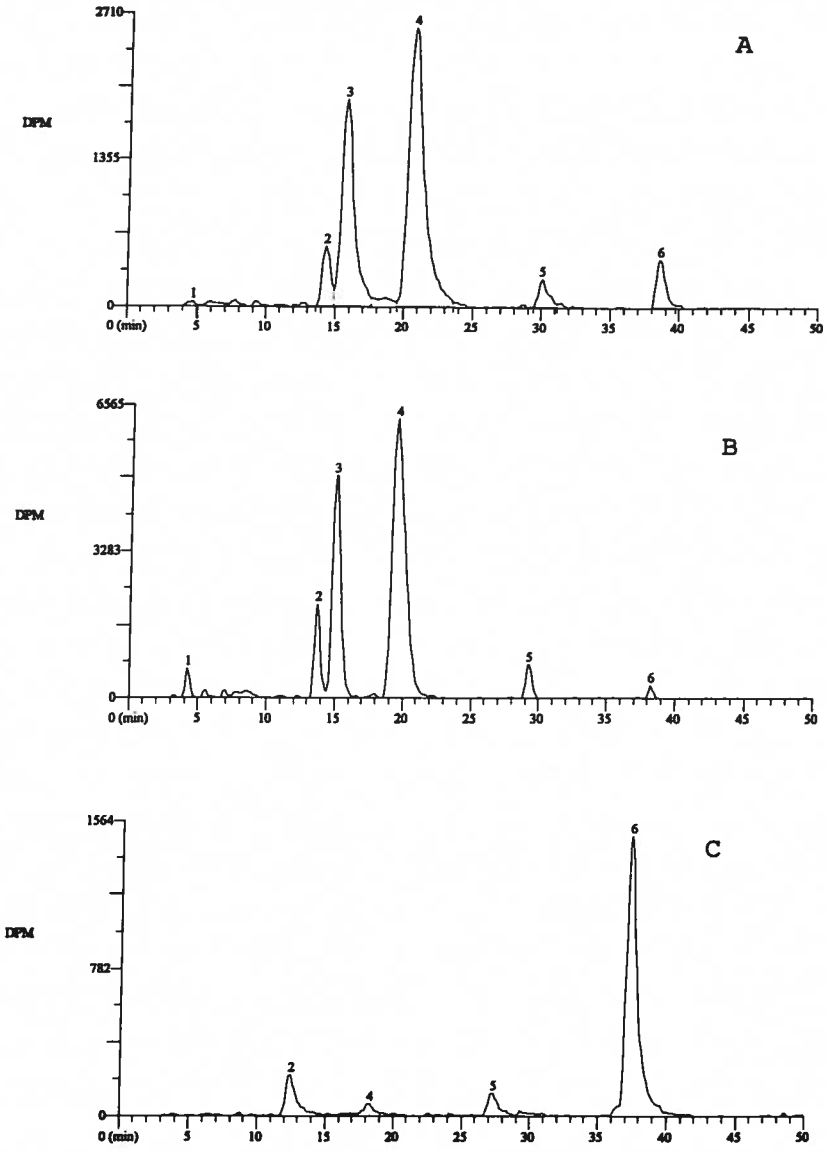

Fig. 3. A. ^{14}C-HPLC chromatogram of urine, collected in the first 8 hours, from a rat dosed with 50 mg/kg 1,4-DCB. Peaks 1 and 4: sulfates; peak 3: glucuronide; peaks 2 and 5: mercapturic acids; peak 6: 2,5-DCP. B. ^{14}C-HPLC chromatogram of urine, collected in the first 8 hours, from a rat dosed with 50 mg/kg 1,4-DCB after induction with isoniazid.C. ^{14}C-HPLC chromatogram of a urine sample treated with arylsulfatase and β-glucuronidase from *Helix pomatia*.

Helix pomatia (shown in Fig. 3C), revealed that the metabolites with peak numbers 1 and 4 are sulfate-conjugates; peak 3 can be assigned to a glucuronide. Hydrolysis of these conjugates resulted solely in the formation of peak 6, which was identified as 2,5-DCP by co-elution with

the reference compound (results not shown). Further identification of the conjugates by mass spectrometry demonstrated that the metabolites with peak numbers 1 and 4 had a molecular ion at *m/z* = 242 with fragments at *m/z* = 208 (- chlorine), 162 (dichlorophenol), 128 (monochlorophenol) and 80 (SO_3) (Fig. 4A). Therefore both of these metabolites could be assigned to the sulfate of 2,5-DCP. However, one of these two metabolites might contain a water molecule in the conjugate (peak 1), i. e. be the sulfate of the dihydrodiol, which easily releases this water molecule during post-HPLC treatment of the fraction (see also mass spectra of peak 2 and peak 5), thus resulting in the same mass as peak 4. The metabolite with peak number 3 was identified as the glucuronide of 2,5-DCP, since the molecular ion had a mass of *m/z* = 338 with a fragment at *m/z* = 162 (dichlorophenol) and 128 (monochlorophenol); this mass spectrum is shown in Fig. 4B. The mass spectra of the metabolites with peak numbers 2 and 5 are represented in Fig. 4C and D. The former was identified as N-acetyl-cysteine-S-dihydro-hydroxy-1,4-dichlorobenzene, since the ions, displaying a two-chlorine isotope pattern with a molecular ion at *m/z* = 325 and fragments at *m/z* = 307 and 178, were attributed to loss of water and fragmentation of the N-acetylcysteinyl side-chain, resulting in the thio-ether. The mass spectrum of the component with peak number 5 showed a molecular ion at *m/z* = 307. The structure of this metabolite can be assigned to the dehydrated metabolite of peak 2, N-acetyl-cysteine-S-1,4-dichlorobenzene; the fragment at *m/z* = 178 can be attributed to the thio-ether.

The identified metabolites were quantified by integrating the different peak areas of the ^{14}C-chromatograms. All peaks accounting for 0.5% or more of the eluted radioactivity were identified; the lower limit of quantification (LLQ) was 5.2 nmol/metabolite for a single run. In Table 2 the mean quantities of the different metabolites are represented as percentages of total excreted urinary metabolites, at different dose levels and for control and induced rats. Sulfates are the summed areas of peaks 1 and 4; mercapturic acids are the summed areas of peaks 2 and 5.

The main difference between the doses was less excretion of sulfates and more excretion of glucuronides with increasing dose. No differences in excretion of mercapturic acids and the unconjugated phenol were observed at the different dose levels. For the induced rats, hardly any unconjugated phenols were detected. For the 50 mg/kg dose, all other metabolites were slightly increased compared to the control rats; for the 250 mg/kg dose less sulfates and more glucuronides and mercapturic acids were observed for the induced rats than for the control rats. For the 10 and 50 mg/kg groups relatively more

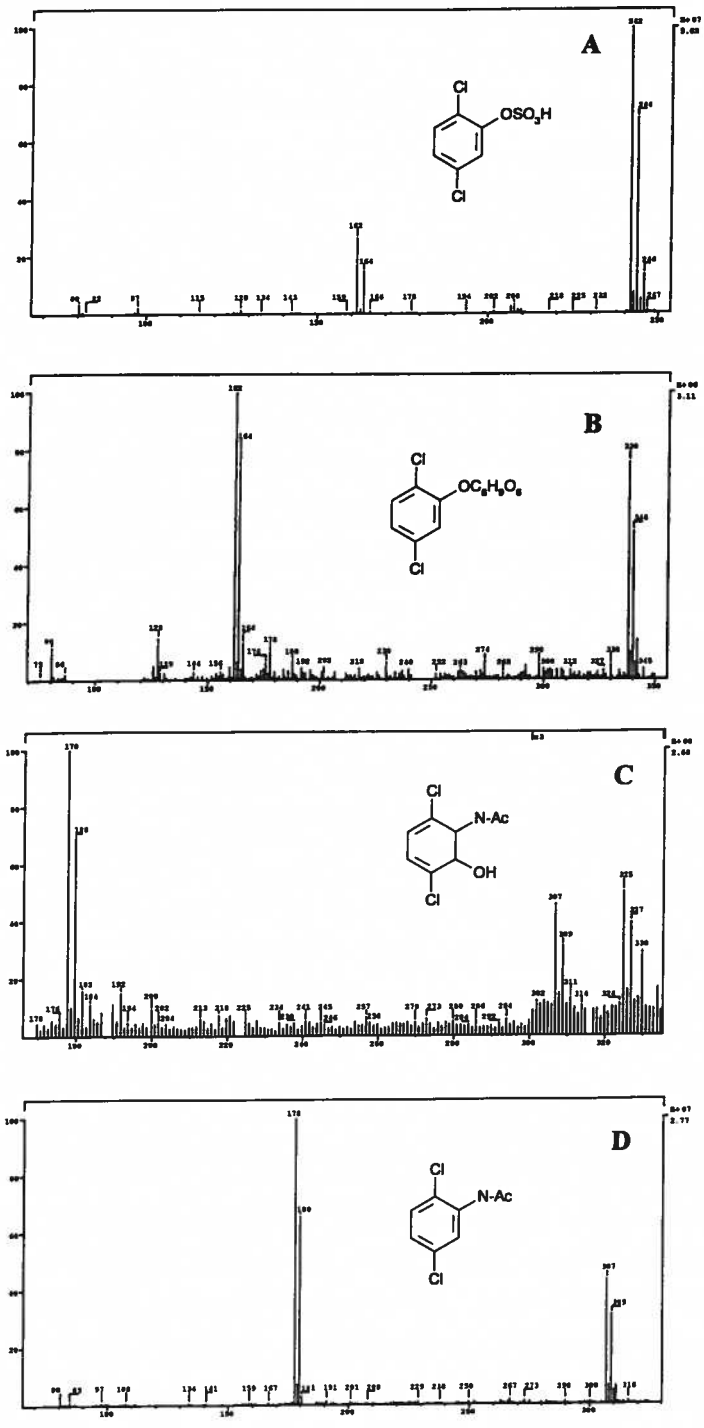

Fig. 4. Mass-spectra of the major urinary metabolites of 1,4-DCB. A. Peaks 1 and 4. B. Peak 3. C. Peak 2. D. Peak 5. Peak numbers refer to those described in Fig. 3. N-Ac = N-acetylcysteinyl.

Table 2. Mean quantities and standard deviations of the different urinary metabolites, expressed as percentage of total excreted urinary metabolites. Ind = induction with isoniazid. (n = 3 for control rats, n = 4 for induced rats).

	Identified metabolites			
Group	Sulfates	Glucuronides	Merc. acids	2,5-DCP
10 mg/kg	62.2 ± 2.3	19.3 ± 2.9	10.2 ± 0.8	8.4 ± 1.5
50 mg/kg	60.1 ± 0.7	21.7 ± 1.7	9.2 ± 0.5	9.0 ± 1.8
50 mg/kg-ind.	63.6 ± 1.9	23.1 ± 2.3	11.3 ± 1.0	2.0 ± 0.7
250 mg/kg	56.7 ± 0.6	25.4 ± 1.3	8.6 ± 0.9	9.3 ± 0.2
250 mg/kg-ind.	54.0 ± 4.6	33.8 ± 4.2	11.2 ± 2.2	1.1 ± 1.3

Table 3. Elimination half lives (hours) of individual metabolites. Mean values and standard deviations (range when n = 2) are given for each metabolite and dose level, for control and induced rats. (n = 2 for 10 mg/kg, n = 4 for all other groups). ND = not determined, LLQ = lower limit of quantification. To compare induced rats with control rats, at the 50 and 250 mg/kg dose level, p-values are calculated with Student's t-test for the different metabolites. The data presented for the control animals are taken from the blood sampling study, however elimination half lives of metabolites calculated from the excretion study (data not shown) did not differ from these values.

	Half life ($t_{½}$)							
Dose	Sulfates		Glucuronides		Mercapt. acids		2,5-Dichlorophenol	
(mg/kg)	control	induced	control	induced	control	induced	control	induced
10	7.8 ± 0.3	ND	7.7 ± 0.5	ND	9.3 ± 0.6	ND	9.2 ± 1.8	ND
50	9.2 ± 3.2	6.2 ± 0.5	8.6 ± 3.0	5.3 ± 0.5	9.6 ± 3.9	7.1 ± 0.6	7.3 ± 2.9	<LLQ
p (t-test)	0.079		0.056		0.15		ND	
250	7.6 ± 1.2	5.3 ± 1.5	8.3 ± 2.6	4.7 ± 1.4	7.2 ± 2.0	4.6 ± 1.7	8.5 ± 0.9	<LLQ
p (t-test)	0.031		0.030		0.046		ND	

glucuronides were excreted in the first 8 hours (ca. 35%) than during the other time intervals (10-20%), at the expense of the sulfates.

The half lives of the elimination of the individual metabolites were calculated for control and induced rats, for all dose levels (Table 3). No dose dependency and no significant differences between the half lives of the various metabolites were observed. The values for the induced rats were smaller than for the control rats; p-values, calculated with Student's t-test, are shown in the table.

The major metabolite in bile eluted at the same retention time as the glucuronide of 2,5-DCP in urine. In addition, this metabolite was hydrolysed by β-glucuronidase and not by arylsulfatase. Therefore the metabolite was assigned as the same glucuronide-conjugate as was identified in the urine. One minor metabolite, which was not affected by enzymatic hydrolysis, was most likely a precursor of the mercapturic acids excreted in the urine, i.e. a glutathione conjugate, since in the urine of the bile-duct cannulated rats only trace amounts of the mercapturic acids were detected. The unknown metabolite was not characterized further.

DISCUSSION

In male Wistar rats, 1,4-DCB is predominantly metabolized to the sulfate and glucuronide of 2,5-DCP, 57-63% respectively 19-25% of the urinary metabolites, together comprising 70% of the dose. Slightly different percentages were found by Hawkins *et al.* in Sprague-Dawley rats, namely 46-54% respectively 31-34% of the urinary metabolites (together 77% of an oral dose of 250 mg/kg after repeated dosing during 10 days) [23]. A distinctly lower amount of conjugates of 2,5-DCP was found by Klos and Dekant in Fischer-344 rats, namely 17% of the administered dose, but their total recovery in the urine was only 40% [19]. We were able to quantify the metabolites derived from glutathione conjugates of the epoxide of 1,4-DCB, i.e. the mercapturic acid, N-acetyl-cysteine-S-1,4-dichlorobenzene, and its precursor, N-acetyl-cysteine-S-dihydrohydroxy-1,4-dichlorobenzene. We found a total percentage of 10%, whereas other authors could not detect mercapturic acids [24] or only reported trace amounts [19,23]. Thus, the acute hepatotoxicity of 1,4-DCB in GSH-depleted rats, found by Stine *et al.* [25] and in GSH-depleted mice, reported by Mizutani *et al.* [26] may be explained by the action of epoxide metabolites. However, the percentage of 10% is still considerably lower than is found for 1,2-DCB, namely 60% [16]. The difference in extent of acute hepatotoxicity between 1,2- and 1,4-DCB can be explained by this difference in GSH conjugation. The formation of the epoxide and resulting depletion of hepatic glutathione and hepatotoxicity is much more pronounced for 1,2-DCB compared to 1,4-DCB.

Regarding the blood kinetics of 1,4-DCB, it is not likely that the P450-mediated oxidation is saturated at the dose levels investigated in this study, since the clearance and half lives of the parent compound are similar for all dose levels. Also the elimination half lives of all individual metabolites are more or less independent of the administered dose, indicating that none of the conjugating routes is saturated. Induction of cytochrome P4502E1 by isoniazid resulted in a smaller AUC, a

corresponding higher clearance of 1,4-DCB and a more rapid urinary excretion of metabolites, confirming that this P450 isoenzyme indeed plays a major role in the oxidation of 1,4-DCB, as reported by Bogaards *et al.* [22]. However, a role for cytochrome P4502B cannot be excluded, since it has been shown that 1,4-DCB decreased hexobarbital sleep times, implying that it is also a phenobarbital-type inducer [27].

We could not identify hydroquinones or hydroquinone-derived metabolites (with a detection limit of 0.5% of the dose), in contrast to Hawkins *et al.*, who found trace amounts (not quantified) of hydroquinone in female Sprague-Dawley rats at a dose level of 250 mg/kg, and Klos and Dekant who also reported trace amounts of conjugated hydroquinones in Fischer-344 rats (1.1% of the dose for males, 1.4% of the dose for females) [19,23]. A possible explanation for the difference could be the dose level used for the Fischer rats (900 mg/kg), at which the conjugation routes of 2,5-DCP could be very well saturated, resulting in the accumulation of more dichlorophenol which is available for secondary oxidation to hydroquinones. However, the significant amount of unconjugated 2,5-DCP and the absence of hydroquinones in the urine of Wistar rats might indicate that this compound is a poor substrate for secondary oxidation, at least *in vivo* in the rat strain we used.

In a recent *in vitro* study we found that Fischer rat microsomes produced more hydroquinones than Wistar rat microsomes [18]. The potential of glutathione conjugates of dichlorohydroquinone to induce nephrotoxicity has been established [20]. Thus, the present results in male Wistar rats, together with the metabolic data obtained in male Fischer-344 rats [19] suggest a role for (hydro)quinone metabolites in the nephropathy of 1,4-DCB, in addition to the well-described role of α_{2u}-globulin in 1,4-DCB-induced nephropathy.

As demonstrated by the various responses to 1,4-DCB by the different species and strains, a straightforward comparison of the toxicity in one species with another is not possible. In extrapolating toxicity data, one has to incorporate differences in toxification and detoxification. For 1,4-DCB, the organ selectivity of carcinogenicity in rats (kidneys) and mice (liver) can possibly be explained by differences in the metabolic pathways. Elucidating these differences may result in a more accurate risk assessment for humans.

ACKNOWLEDGEMENTS

This work was supported, in part, by the Utrecht Toxicological Center (UTOX). The authors wish to thank Dr. Kees Groen for helpful advise with the pharmacokinetic studies.

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CHAPTER 4

SPECIES AND STRAIN DIFFERENCES IN THE CYTOCHROME P450-MEDIATED BIOTRANSFORMATION OF 1,4-DICHLOROBENZENE; IMPLICATIONS FOR HUMAN RISK ASSESSMENT

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Submitted

ABSTRACT

A study on the relationship of the possible species and strain differences in biotransformation with the strain and species differences observed in liver and kidney carcinogenicity and toxicity, the microsomal biotransformation of 1,4-dichlorobenzene (1,4-DCB) was investigated. Hepatic microsomes of humans, of male B6C3F1 mice, Fischer-344-, Sprague-Dawley (SD)- and Wistar rats, and of phenobarbital (PB)- and isoniazid (ISO) pretreated male Wistar rats were used.

The general oxidative metabolic pathway observed was primary oxidation to 2,5-dichlorophenol (with the epoxide postulated as covalently bound intermediate), and subsequent secondary oxidation to the hydroquinone, with benzoquinone-mediated covalent binding as a potential mechanism of toxicity. The oxidation to 2,5-DCP was most likely mainly catalyzed by CYP2E1, whereas formation of hydroquinones was predominantly mediated by CYP2B-enzymes.

Conversion of 1,4-DCB was most extensive by the mouse liver microsomes. Conversion by microsomes from Fischer and Wistar rats was similar, whereas SD-rats showed less biotransformation than the other two strains. Of all species tested, human microsomes showed the least conversion.

Comparing all species, the mouse produced most of the reactive metabolites that interacted with macromolecules (> 20% of total metabolites formed). This covalent binding was nearly completely inhibited by ascorbic acid (AA), with a concomitant increase in hydroquinone formation, indicating that benzoquinones were the reactive metabolites involved. Of all rat microsomes, the PB-microsomes showed the highest covalent binding. Covalent binding was for all rat microsomes partly (33-79%) inhibited by AA. The remaining covalent binding was inhibited by glutathione (GSH), with concomitant formation of the GSH-conjugate of the epoxide. Human microsomes produced least of the reactive metabolites, of which the majority (> 70%) was prevented from covalent binding by GSH.

Correlating the observed species- and strain differences in biotransformation of 1,4-DCB with its reported effects, it can be concluded that liver carcinogenicity in B6C3F1 mice is most likely related to a high formation of benzoquinones and therefore not relevant for man.

INTRODUCTION

1,4-(*para*)-Dichlorobenzene (1,4-DCB) is used worldwide as a space deodorant in toilets, as a moth repellent and as a fungicide. It is also used in the production of dyestuffs and organic solvents. The production of 1,4-DCB in the United States in 1990 is estimated to be 65 million kg. and increases yearly (Chem. Marketing Reporter, 1990). Recently the exposure to 1,4-DCB among 1000 adults in the United States has been investigated (Hill *et al.*, 1995). It was found that 98% had detectable levels of 2,5-dichlorophenol (2,5-DCP), a metabolite of 1,4-DCB, in their urine and 96% had detectable amounts of 1,4-DCB in their blood. In a study with residents of Germany it was shown that 88% of the investigated individuals had significant concentrations of chlorophenols in their urine (Angerer *et al.*, 1992). These reports suggest widespread human exposure to 1,4-DCB and therefore stress the importance of an accurate risk evaluation in order to estimate human risk after (chronic) exposure to 1,4-DCB.

Exposure to 1,4-DCB causes various toxic effects in different species and also in different rat strains. The compound induces liver carcinogenicity in B6C3F1 mice (NTP, 1987). The mechanism of action involved in this carcinogenicity is not clear. In long-term toxicity and carcinogenicity studies with Wistar rats of both sexes, no treatment-related effects were observed (Loeser and Litchfield, 1983). However, 1,4-DCB has been found to be a renal carcinogen in male Fischer-344 rats (NTP, 1987).

At present, the nephropathy of 1,4-DCB is thought to be mediated by the binding of 1,4-DCB and 2,5-DCP to α_{2u}-globulin in the proximal tubules of the kidneys. This binding would result in accumulation of the protein, leading to protein droplet formation and cell proliferation (Swenberg *et al.*, 1989). However, since male Wistar rats also synthesize this protein, but lack the 1,4-DCB-induced nephropathy, an additional factor may be involved.

For most halogenated benzenes it has been demonstrated that biotransformation is necessary to cause toxicity (Reid and Krishna, 1973). For 1,2-dichlorobenzene (1,2-DCB) it has been shown that its toxicity is most likely related to the formation of epoxides, conjugation of these epoxides with glutathione (GSH), followed by depletion of GSH (Hissink *et al.*, 1996). However, in a recent study with 1,4-DCB, only 10% of the urinary metabolites from male Wistar rats were found to consist of epoxide-derived mercapturic acids, whereas for 1,2-DCB 60% of these metabolites were found (Hissink *et al.*, submitted, 1996). Klos and Dekant (1994) found less than 1% mercapturic acids in the urine of Fischer rats. Therefore it is not likely that the epoxide and/or GSH depletion are involved in 1,4-DCB-induced toxicity. For some time the formation of secondary oxidation products has been thought to play a key role in halobenzene toxicity. For bromobenzene, it has been demonstrated that quinone metabolites covalently bind to proteins (Slaughter and Hanzlik, 1991). The glutathione conjugates of such quinones can be responsible for the renal toxicity and/or carcinogenicity of halogenated benzenes, due to the targeting effect of the glutathione moiety (Mertens *et al.*, 1991; Monks *et al.*, 1985; Monks and Lau, 1990), and this may very well be also the case for 1,4-DCB. For 2,5-dichlorobenzoquinone, a secondary oxidation product of 1,4-DCB, it has been shown that its glutathione conjugate causes renal injury in rat (Mertens *et al.*, 1991). Interestingly, 2,5-dichlorohydroquinone has been detected in the urine of male Fischer-344 rats (Klos and Dekant, 1994), the strain in which 1,4-DCB induces nephrotoxicity and -carcinogenicity, but not in the urine of male Wistar rats, a strain not susceptible to 1,4-DCB induced nephropathy (Hissink *et al.*, submitted). Therefore, it is postulated that the glutathione conjugates of 2,5-dichlorobenzoquinone are (partly) responsible for the adverse renal effects observed in male Fischer-344 rats after treatment with 1,4-DCB.

To study the possible role of biotransformation in the species and strain differences regarding the 1,4-DCB-induced toxicity, the microsomal biotransformation of rat, mouse and man was investigated. In addition, the roles of CYP2E1 and 2B1/2 in biotransformation of 1,4-DCB were investigated, using microsomes of induced rats. In a preliminary attempt to assess human risk after exposure to 1,4-DCB on a mechanistic basis, the possible correlation between

rat, mouse and human microsomal biotransformation of 1,4-DCB, and its reported adverse effects is discussed.

MATERIALS AND METHODS

Materials

Microsomes of human liver were obtained from Human Biologics, Inc. (Phoenix, AZ, USA). Microsomes from cell lines transfected with cDNAs expressing human CYP2E1, CYP1A1, CYP1A2, CYP2B6, CYP2C9, CYP2D6, CYP2A6 or CYP3A4 were obtained from the Gentest Corp. (Woburn, MA, USA). 1,4-Dichloro-[^{14}C]-benzene (1,4-DCB) with a radiochemical purity of >98% and a specific activity of 348 MBq/mmol was obtained from Sigma Chemical Co., St. Louis, MO, USA. ^{35}S-glutathione with a radiochemical purity of > 90 % and a specific activity of 3075 GBq/mmol was purchased from DuPont NEN, Dordrecht, the Netherlands. The unlabeled 1,4-DCB (purity >99%) was from Aldrich (Steinheim, Germany). 2,5-Dichlorophenol (2,5-DCP) was from Merck, Darmstadt, Germany, 2,5-dichlorohydroquinone was from Eastman Kodak Company, Rochester, N.Y., USA. Isoniazid was from Janssen Chimica (Beerse, Belgium) and phenobarbital (PB) was from CAV, Utrecht, the Netherlands. Glutathione (GSH) and NADPH were obtained from Boehringer Mannheim GmbH, Germany and S-hexylglutathione, cyclohexene oxide and γ-glutamyltranspeptidase were from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals used in this study were of reagent grade.

Animals

Adult male Wistar rats (strain Crl: (WI)WUBR), Fischer-344 rats (strain CDF(F-344)/CrlBR) and Sprague-Dawley rats (strain Crl: CD(SD)BR), weighing 250-300g and 9-10 weeks of age, were obtained from Charles River Wiga GmbH, Sulzfeld, Germany. Adult male B6C3F1 mice of 6-8 weeks old (30-40 g) were obtained from the same supplier as the rats. The animals were provided *ad libitum* with the Institute's cereal-based rodent diet (SDS Special Diets Services, Witham, England) and tap water. The phenobarbital pretreated rats (Wistar) received drinking water with 0.1% (w/v) PB during 4 days prior to sacrifice. The isoniazid pretreated rats (Wistar) received drinking water with 0.1% (w/v) isoniazid

(isonicotinic acid) during 10 days prior to sacrifice. The animal room was air-conditioned and light controlled, with a light/dark cycle of 12 hours.

Preparation of microsomes

Microsomes were isolated from male Wistar rats (untreated, PB and ISO pretreated), from male Fischer-344 and Sprague-Dawley rats and from male B6C3F1 mice. The animals were killed by abdominal bleeding and the livers were removed, washed and homogenized in ice-cold 20 mM KCl-Tris buffer pH = 7.4. Of each species and strain, livers of at least 3 animals were pooled. The liver homogenates were centrifuged for 30 min. at 10,000 g and 2 °C. The supernatants were centrifuged for 90 min. at 105,000 g and 2 °C. The supernatants (cytosol) were stored at -30 °C and the pellets (microsomes) were washed with KCl-Tris buffer and centrifuged again. The pellets were then resuspended in 0.1 M potassium phosphate buffer, pH = 7.4, and stored at -80 °C.

Biochemical assays

Total cytochrome P450 content was determined by the method of Omura and Sato (1964), modified by Rutten *et al.* (1987). The protein concentration of the microsomes was determined by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard.

Incubations with γ-glutamyltranspeptidase (identification of GSH conjugate) were performed by mixing 100 µl of microsomal incubate (described elsewhere) without the addition of HCl with 50 µl 400 mM Tris-buffer (pH 8.25) and 50 µl 400 mM glycylglycine. To this mixture 0.1 mg γ-glutamyltranspeptidase (= 2.5 units) was added and the reaction was performed in a shaking water bath at 37 °C for 20 hours.

Microsomal incubations

All incubations were performed in duplicate. Incubation mixtures contained 0.1 M potassium phosphate buffer pH 7.4, 3 mM $MgCl_2$, 3 mM NADPH and hepatic microsomes from the various species in a final protein concentration of 1 mg/ml (preliminary experiments indicated that product formation was linear with protein concentration, at least up to this concentration), in a final volume of 200 µl. In experiments with human microsomes, microsome samples of 5 individuals were pooled. P450-contents of the mouse, human and rat (control Wistar, ISO, PB, SD and Fischer) microsomes were 0.84, 0.49, 0.63, 0.82, 2.21, 0.74

and 0.62 nmol/mg protein, respectively. Where indicated in the Results section, 5 mM GSH, 1 mM ascorbic acid (AA), 2 mM S-hexylglutathione, 2.5% (v/v) acetone, 500 μM cyclohexene oxide or rat liver cytosol with a final protein concentration of 2 mg/ml. was added to the incubation mixtures. A stock solution of 1,4-^{14}C-DCB was prepared in acetone and BSA (1 mg/ml), with a final concentration of acetone in the incubation mixtures of 0.05%, which was shown not to inhibit cytochrome P4502E1 to a significant amount (Bogaards *et al.*, 1995). Volatility losses of the substrate were accounted for by measuring the actual concentration in each incubation mixture (see Results section, Table 1). In preliminary experiments, it was determined that product formation was linear with substrate concentration. Incubation mixtures contained ca. 5 kBq of radioactivity. The reactions were started by the addition of the substrate and were performed using glass vials with a teflonized cap in a shaking waterbath at 37 °C. For each experiment, control incubations were performed without NADPH. After 15 min., the reactions were stopped by adding 20 μl 6 M. HCl through the cap using a Hamilton™ needle. The vials were immediately put on ice and then frozen at -20 °C. After thawing on ice, the vials were centrifuged for 10 min. at 1560 g and 4 °C to precipitate the protein. The caps were removed and the supernatants were transferred to HPLC-vials with teflonized caps and immediately analyzed by HPLC. The protein pellets were resuspended in 0.5 ml water and stored at -20 °C until further processing, i.e. measuring covalent protein binding.

Measurement of radioactivity

Aliquots of incubation mixtures (20 μl) were mixed with 4.5 ml Ultima Gold scintillation cocktail (Packard Instrument Company, Reading, UK) and radioactivity was measured in a Pharmacia Wallac S1409 Liquid Scintillation Counter (Pharmacia, Uppsala, Sweden) using automatic external standard quench correction, and was counted at efficiencies > 85%.

HPLC analysis

HPLC analysis of incubation mixtures was carried out with a Pharmacia LKB HPLC 2248 Pump (Pharmacia, Sweden), fitted with a Pharmacia VWM 2141 UV-detector operating at 254 nm. Radioactivity was measured on line with a Canberra Packard Radiomatic Detector (type A500, Flo-one Beta). A liquid flow cell of 2000 µl was used and radioactivity was measured using Flo Scint A (Packard) with a scintillator flow of 3 ml/min. Injections of 100-160 µl were done automatically by a Pharmacia LKB-Autosampler 2157 at 4 °C. Separations were carried out using a reversed phase column, 250*4.6 mm, C18 hypersil ODS 5 µm (Chrompack). Eluting solvents were methanol and 20 mM ammonium acetate, pH = 6.7, used with the following gradiënt: 0-5 min: 15% methanol; 5-10 min: 15→30% methanol; 10-30 min: 30→50% methanol; 30-38 min: 50→95% methanol; 38-45 min: 95% methanol; 45-48 min: 95→15% methanol. The flow rate was 1 ml/min.

Covalent binding to protein

Total covalent binding of radioactivity to microsomal protein was measured for all protein pellets obtained from the various incubation mixtures. The vials containing protein suspensions were thawed and centrifuged for 15 min. at 1560 g and 4 °C. Supernatant was removed and 1 ml methanol was added to the pellets. Vials were extensively vortexed and centrifuged for 10 min. at 1560 g. The same procedure was followed with acetone and then n-hexane, until the supernatant contained background radioactivity. The pellets were dried at room temperature, dissolved in 1 ml Soluene-350 from Packard and mixed with 20 ml Hionic Fluor from Packard. This mixture was analyzed for radioactivity as described above. Values were corrected for residual radioactivity as bound in incubations without NADPH.

RESULTS

Identification of metabolites

In Fig. 1 ^{14}C-HPLC chromatograms are shown of incubation mixtures with microsomes of mice (A), rats pretreated with phenobarbital (C) and man (D) in the presence of 5 mM glutathione (GSH) and in the absence of ascorbic acid (AA). Figure 1B shows the chromatogram of an incubation mixture with mouse microsomes without GSH and in the

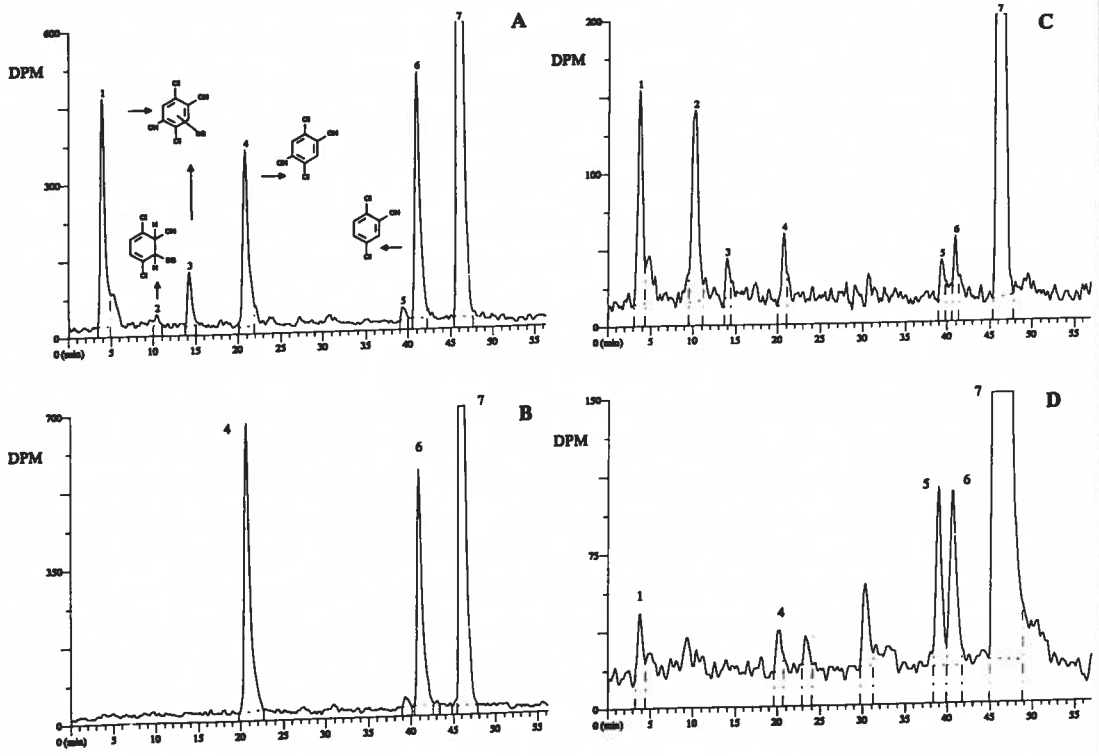

Fig.1. A. ^{14}C-HPLC-chromatogram of an incubation mixture with microsomes of B6C3F1 mice (pool of 3 animals) in the presence of 5 mM glutathione. No ascorbic acid (AA) was added. Peak 1: GSH-conjugate of quinone. Peak 2: GSH-conjugate of epoxide. Peak 3: GSH-conjugate of quinone. Peak 4: 2,5-dichlorohydroquinone. Peak 5. Impurity in label (< 0.25% of 1,4-DCB). Peak 6: 2,5-DCP. Peak 7. 1,4-DCB. B. The same as A, but in the presence of 1 mM AA and no GSH added. C. The same as A, but microsomes of male Wistar rats pretreated with phenobarbital were used (pool of 3 animals). D. The same as A, but human microsomes (pool of 5 samples) were used. Concentrations of 1,4-DCB in A, B, C and D were respectively 26, 26, 20 and 150 μM, with an impurity peak comprising <0.25% of the substrate.

presence of 1 mM AA. Peaks 1 and 3 are metabolites which were mainly produced in the absence of AA, resulting in the formation of benzoquinones, and presence of GSH. Most likely these are GSH conjugates of dichlorobenzoquinone, since a standard obtained by reacting unlabeled GSH and 2,5-dichlorobenzoquinone resulted in a compound with the same retention time as peak 3. Peak 1, if present, was not visible due to the GSH eluting at the same time (results not shown). The high polarity of peak 1 is probably caused by the presence of 2 GSH

molecules, since dichlorobenzoquinone has 2 sites which are easily attacked by a nucleophilic compound like GSH (see also the metabolic scheme in Figure 6), however both metabolites (1 and 3) will be referred to as GSH conjugates of dichlorobenzoquinone. Peak 2 is assigned to the GSH conjugate of the epoxide of 1,4-DCB. This metabolite increased in the presence of excess GSH at the expense of 2,5-DCP, indicating that the epoxide was the intermediate conjugated with GSH. The metabolite completely disappeared when the sample was incubated with γ-glutamyltranspeptidase (this peak was more or less present in all incubation mixtures with rat microsomes due to the presence of a small amount of endogenous GSH). Moreover, incubations of rat microsomes with ^{35}S-GSH and unlabeled 1,4-DCB in the presence of AA (inhibited formation of quinones) resulted in a single metabolite with the same retention time as peak 2 (results not shown). Peak 4 was identified as 2,5-dichlorohydroquinone by co-elution with the reference compound; this peak increased in the presence of AA (Figure 1B) which protected the compound against oxidation to 2,5-dichlorobenzoquinone. Peak 5 was an impurity in the substrate solution (less than 0.25% of 1,4-DCB), peak 6 was identified as 2,5-DCP by co-elution with the reference compound and peak 7 is 1,4-DCB. The unlabeled peaks in Figure 1D were also present in the blank incubations (-NADPH) which were included in the incubation series with human microsomes.

Total conversion of 1,4-DCB by the various microsomes

In Table 1 the total conversion of 1,4-DCB into metabolites and covalent binding by the various microsomes is presented, expressed as percentage of the amount of 1,4-DCB present in the incubation mixtures. No conversion was observed in incubation mixtures without NADPH. Comparing the different species, it is clear that the mouse microsomes are by far the best metabolizers (16% conversion) and human microsomes the poorest (0.3%). Comparing the different rat strains, conversion by the Wistar and Fischer rat was similar (ca. 1.3%), but conversion by the Sprague-Dawley microsomes was only 0.6%. Conversion by microsomes from pretreated Wistar rats was higher compared to control rats, with more than 5% conversion by PB pretreated rats.

Table 1. Total conversion of 1,4-DCB, covalent binding of radioactivity to microsomal protein and formation of the four major metabolites by the various microsomes.

Mic.species[a]	Conc. 1,4-DCB[b]		Conversion[c]	Cov. Binding[d]	Identified Metabolites (% of total conversion)				
			% of total r.a.	% of total conv.	GSH-Epox.[d]		Hydroquinone[d]	2,5-DCP[d]	GSH-Quinone[e]
					- cyt	+ cyt			
Wistar	41 μM	-AA,-GSH	1.30%	8.08%	15.0%	NM	10.5%	50.0%	ND
	41 μM	+AA,-GSH	1.25%	1.62%	14.2%	NM	16.2%	52.6%	ND
	41 μM	-AA,+GSH	1.30%	ND	52.2%	NM	11.5%	33.6%	3.08%
	41 μM	+AA,+GSH	1.37%	ND	48.9%	50.4%	9.28%	34.1%	5.53%
Wistar-ISO	24 μM	-AA,-GSH	1.75%	8.91%	10.4%	NM	21.4%	52.1%	ND
	24μM	+AA,-GSH	1.70%	5.93%	11.2%	NM	21.63%	53.5%	ND
	24 μM	-AA,+GSH	1.65%	1.30%	31.8%	34.3%	14.7%	41.1%	11.1%
	24 μM	+AA,+GSH	1.77%	1.18%	33.9%	36.8%	20.9%	40.7%	3.35%
Wistar-PB	20 μM	-AA,-GSH	5.12%	13.4%	13.0%	NM	30.6%	19.0%	9.0%
	20 μM	+AA,-GSH	5.15%	5.17%	10.6%	NM	48.8%	20.0%	ND
	20 μM	-AA,+GSH	5.20%	ND	35.3%	36.3%	9.07%	7.23%	48.36%
	20 μM	+AA,+GSH	5.24%	ND	37.1%	40.4%	17.6%	7.34%	37.9%
Fischer-344	82 μM	-AA,-GSH	1.10%	6.75%	5.0%	NM	27.1%	56.8%	6.0%
	82 μM	+AA,-GSH	1.15%	2.72%	5.5%	NM	34.5%	55.2%	5.0%
	82 μM	-AA,+GSH	1.20%	0.14%	39.7%	49.3%	13.7%	24.1%	22.4%
	82 μM	+AA,+GSH	1.26%	0.20%	42.5%	50.6%	20.9%	26.8%	9.65%

Table 1. Continued.

Sprague-Daw.	50 μM	-AA,-GSH	0.57%	10.1%	7.3%	NM	10.2%	70.7%	ND
	50 μM	+AA,-GSH	0.61%	4.21%	6.5%	NM	19.6%	58.2%	ND
	50 μM	-AA,+GSH	0.61%	1.39%	49.9%	65.9%	ND	37.2%	11.6%
	50 μM	+AA,+GSH	0.60%	1.50%	65.5%	48.1%	ND	32.9%	ND
B6C3F1-Mouse	26 μM	-AA,-GSH	15%	20.9%	ND	NM	16.1%	50.5%	3%
	26 μM	+AA,-GSH	16%	1.74%	ND	NM	55.4%	42.9%	ND
	26 μM	-AA,+GSH	16%	1.98%	ND	3.7%	25.4%	33.6%	39.0%
	26 μM	+AA,+GSH	17%	0.52%	2.4%	3.1%	45.0%	35.2%	16.2%
Human	150 μM	-AA,-GSH	0.29%	5.81%	ND	NM	16.75%	66.2%	ND
	150 μM	+AA,-GSH	0.28%	4.37%	ND	NM	27.9%	58.9%	ND
	150 μM	-AA,+GSH	0.28%	ND	5.88%	43.1%	15.9%	52.0%	26.3%
	150 μM	+AA,+GSH	0.29%	ND	6.93%	46.5%	14.9%	62.2%	15.9%

Note. [a] Liver microsomes from male Wistar rats were isolated from control, isoniazid (ISO) or phenobarbital (PB) pretreated rats (pools of 3 animals). For Fischer- and SD-rats and mice, pooled microsomes of 3 animals were used. For human, microsomes of 5 individuals were pooled. [b] Incubation volume was 200 μl. Cytosol was only present when stated. All incubations were performed in duplicate, in separate vials, as described under Materials and Methods. Variation in conversion and covalent binding of 2 incubations ranged from 0 to 10%. Presented data are the means of two incubations. Conversion was linear with substrate concentration upto at least at the concentrations used, resulting in similar percentages of conversion at different concentrations (not shown). The concentration for human microsomes was higher compared to the other species, in order to obtain detectable amounts of metabolites. [c] Expressed as percentage of amount of substrate (= r.a, radioactivity) added. Metabolites (including a small percentage of unidentified peaks) quantified with HPLC and covalently bound radioactivity (1,4-DCB equivalents) were summed. [d] Expressed as percentage of total conversion. cyt = cytosol. [e] Expressed as percentage of total conversion. Based on the concentrations used, Fischer rat microsomes produced 46 pmol of these metabolites, and human microsomes 22 pmol; at the same concentration as for the Fischer rats, this would be 12 pmol (conversion was linear with substrate concentration). ND = not detected, NM = not measured.

Covalent binding of radioactivity to microsomal protein

In Table 1 the covalent binding of radioactivity to microsomal protein is presented, expressed as percentage of the total conversion. The mouse microsomes produced much more reactive metabolites binding to liver microsomal protein than the other species. Addition of AA nearly completely inhibited this covalent binding. In all other species, covalent binding was in the same order of magnitude, i.e. 6 to 12% of total conversion, with lowest covalent binding for man and highest covalent binding for the PB pretreated rats. For the different rat microsomes, AA significantly reduced covalent binding (33-79%). For human microsomes, covalent binding was reduced with only 25%. Addition of GSH to the incubation mixtures resulted in a more than 85% decrease of covalent binding for all microsomes studied, independent of the presence or absence of AA.

Relative contributions of the different metabolites to the total conversion

In Table 1 the formation of the four main metabolites expressed as percentage of tal conversion is presented. *The GSH conjugate of the epoxide of 1,4-DCB* (peak 2 in Fig.1). For all rat strains, the GSH-conjugates were mostly formed non-enzymatically, since addition of cytosol had a marginal effect on formation of these metabolites. To determine the contribution of the microsomal glutathione S-transferases (GST), incubations were performed in the presence of 2 mM S-hexylglutathione, which inhibits these enzymes (Mosialou and Morgenstern, 1990). GST-inhibition resulted in a small decrease (ca. 10%) of the conjugate formation, in favour of 2,5-DCP, indicating that for rat microsomal GST's do not play a role of significance (results not shown). In contrast with rat, where the GSH-conjugate comprised more than 50% of total metabolites, mouse produced only 2% of this metabolite. Addition of cytosolic glutathione S-transferases increased GSH conjugate formation in mouse microsomes to 3%. In human microsomes, the non-enzymatic conjugation of the epoxide with GSH was 6%, whereas enzymatic conjugation was 45% of total metabolites. *2,5-Dichlorohydroquinone* (peak 4 in Fig.1). Comparing the different rat strains, the Fischer rat produced considerably more hydroquinones than the other two strains (34% vs. 16% and 19% of total metabolites). The ISO pretreated rats produced slightly more hydroquinones than the control Wistar rats, whereas the PB pretreated rats produced much more hydroquinones, respectively 22% and 49% of total conversion. Comparing the different species, the formation of hydroquinones was most pronounced in mice (55%, see also Fig.1B). The amount of hydroquinones formed by human microsomes was 28% of total conversion. *2,5-DCP* (peak 6 in Fig.1). Formation of 2,5-

DCP by ISO-microsomes was slightly higher compared to control microsomes, whereas PB-microsomes produced much less 2,5-DCP. The percentage obtained with Fischer rat microsomes was smaller compared to that obtained with Wistar and Sprague-Dawley rat microsomes. Production by the mouse was similar to that of the rat. The human microsomes showed the highest conversion into 2,5-DCP (62%). *The GSH-conjugates of 2,5-dichlorobenzoquinone* (summed peaks 1 and 3 in Fig.1). By comparing the different rat strains, it was observed that most of the conjugates were produced by the Fischer rat microsomes. Comparing the three different Wistar microsomes, the PB induced microsomes were by far the most extensive producers of these GSH conjugates (48%). Human microsomes produced relatively more conjugates than the three rat strains, whereas the mouse showed the highest conversion of 1,4-DCB to GSH-conjugates of quinones (39%).

Incubations with microsomes containing human cytochrome P450

Incubations with microsomes derived from different cell lines selectively expressing one human cytochrome P450, revealed that only CYP2E1, and not 1A1, 1A2, 2B6, 2C9, 2D6, 2A6 or 3A4, metabolized 1,4-DCB to a detectable amount of 2,5-DCP. No (hydro)quinones or other metabolites were observed (results not shown).

Effect of inhibition of epoxide hydrolase by cyclohexene oxide

To evaluate the contribution of (microsomal) epoxide hydrolase in the biotransformation of 1,4-DCB and its epoxide, incubations were performed with microsomes from the different species in the presence of 500 μM cyclohexene oxide, which has been shown to inhibit microsomal epoxide hydrolase activity (Gazzotti *et al.*, 1981). No differences were observed between the metabolite profiles of incubation mixtures in the presence or absence of cyclohexene oxide (results not shown).

Effect of inhibition of CYP2E1 by acetone

In order to determine the individual roles of both CYP2E1 and CYP2B1/2 in the oxidation of 1,4-DCB, incubations were performed with PB-microsomes in the presence of acetone, an inhibitor which has been shown to completely inhibit CYP2E1 activity towards chlorzoxazone hydroxylation at a concentration of 2.5% (Bogaards, personal communication). In Fig.2 the formation of 2,5-dichlorohydroquinone, 2,5-DCP and

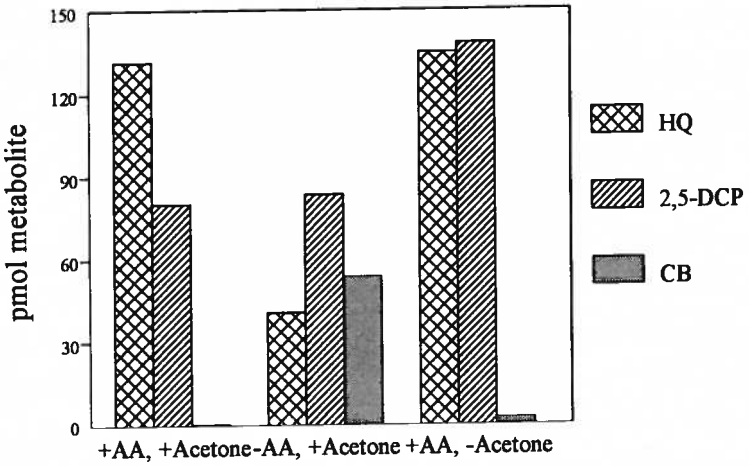

Fig.2. Effect of 2.5% acetone on the conversion of 1,4-DCB into 2,5-DCP, hydroquinone (HQ) and covalently bound metabolites (CB) by hepatic microsomes isolated from male Wistar rats pretreated with phenobarbital (pool of 3 animals), expressed as pmol metabolite produced. AA is ascorbic acid. No GSH was added. Concentration of 1,4-DCB was 85 μM. To determine total conversion, all metabolites formed, including CB, were summed.

covalently bound metabolites is presented for three different incubation conditions. The incubation without acetone resulted in a higher total conversion than the other two conditions (367 pmol vs. 285 pmol). Acetone causes (in the presence of AA) a decrease in the amount of 2,5-DCP, but not in the amount of hydroquinone. In acetone containing incubations, in the absence of AA, the amount of 2,5-DCP is equal to when AA is present, but the amount of hydroquinones is much smaller. This decrease in hydroquinones resulted in a concomitant increase of covalent binding, which was 18% of total conversion.

DISCUSSION

In this study the biotransformation of 1,4-dichlorobenzene by hepatic microsomes from different species (rat, mouse and man) and rat strains (Wistar, Fischer-344 and Sprague-Dawley) has been determined. In Fig. 3 a metabolic scheme is shown, representing the proposed microsomal biotransformation of 1,4-DCB by hepatic microsomes from rat, mouse and man. 1,4-DCB is firstly oxidized to 2,5-dichlorophenol via an epoxide, which is

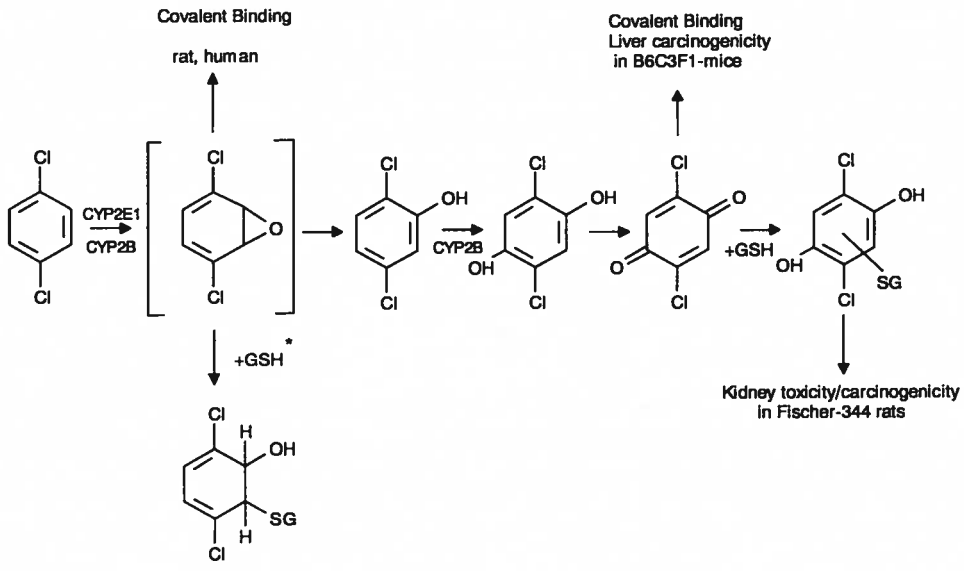

Fig.3. Proposed metabolic scheme of the microsomal biotransformation of 1,4-DCB by rat-, mouse- and human liver microsomes. * GSH-conjugation of the epoxide by human- and mouse liver microsomes needs to be catalyzed by glutathione S-transferases. SG = glutathione.

subsequently oxidized to the hydroquinone, with benzoquinone-mediated covalent binding as a potential mechanism of toxicity. The primary oxidation to 2,5-DCP is most likely mainly catalyzed by CYP2E1, whereas the secondary oxidation to quinones is predominantly mediated by CYP2B-enzymes.

Exposure to 1,4-dichlorobenzene causes various toxic effects in the investigated species and strains. (NTP, 1987; Bomhard *et al.*, 1988; Charbonneau *et al.*, 1989; Loeser and Litchfield, 1983). Our results indicate that biotransformation may indeed play a major role in the liver carcinogenicity observed in B6C3F1 mice. Since it has been shown that mice possess a lower epoxide hydrolase activity as compared to other species (Glatt and Oesch, 1987), it was thought that this lower detoxification activity, resulting in a relatively high epoxide concentration, might play a role in the liver carcinogenicity observed in mice, as has been shown for butadiene (Csanády *et al.*, 1992). However, hydrolysis of the epoxide of 1,4-DCB was not a route of biotransformation since no dihydrodiols were identified and no effect of cyclohexene oxide, an inhibitor of epoxide hydrolase, was observed. Compared to the other species investigated, 1,4-DCB is highly metabolized by the mouse. This presumably results in a high liver concentration of metabolites *in vivo*. Moreover, the metabolites produced are

highly reactive, as evidenced by the 20% covalent binding to liver microsomal protein. This covalent binding was completely inhibited by ascorbic acid (AA) by preventing the oxidation of hydroquinones to benzoquinones. This resulted in a concomitant increase of hydroquinones, indicating that benzoquinones are the most important reactive metabolites, produced by the B6C3F1 mouse *in vitro*. Interaction of the quinones with DNA, by themselves or through reactive oxygen species produced by redox cycling, may result in the liver carcinogenicity observed in B6C3F1 mice (NTP, 1987). The quinones can also be detoxified by glutathione (GSH), since in the absence of AA and presence of GSH covalent binding was reduced with 92%, with concomitant formation of the GSH-conjugates of the hydroquinone. Apparently the epoxide of 1,4-DCB does not play a significant role in covalent binding for the mouse. Conjugation of the epoxide with GSH is much less predominant in the mouse compared to the other species, and in addition, glutathione S-transferases are needed to catalyze the major part of the reaction. The hepatotoxicity of 1,4-DCB observed in mice depleted of GSH (Mizutani *et al.*, 1994) may be a consequence of reduced detoxification by conjugation of the quinones.

Our results also point to a possible mechanism for the renal toxicity and carcinogenicity which is observed in Fischer-344 rats (NTP, 1987), but not in other rat strains (Loeser and Litchfield, 1983). The extent of metabolism by Fischer and Wistar rats was similar, but metabolism by Sprague-Dawley rat liver microsomes was twice as low. Comparing the metabolite profiles of the three rat strains, the microsomes of the Fischer rat are most predominant in producing hydroquinones, and even more importantly, (hydro)quinone conjugates. These data are in agreement with results obtained *in vivo*: hydroquinone conjugates were detected in the urine of Fischer-344 rats (Klos and Dekant, 1994), but not in the urine from Wistar rats (Hissink *et al.*, submitted). Also, these results are in accordance with the observed nephropathy as found only in Fischer rats. Between the different rat strains, no difference in covalent binding as percentage of total conversion was observed *in vitro* in liver microsomes. The nephropathy is presumably not a consequence of binding of quinones or epoxides to macromolecules, since these reactive compounds are not likely to reach the kidneys when produced in the liver, but caused by glutathione conjugates of the quinones which are targeted to the kidneys (Mertens *et al.*, 1991; Monks *et al.*, 1985). The toxicity of the glutathione conjugates is thought to be mediated by hydrolysis of the glutathione moiety by γ-glutamyltranspeptidase, resulting in a labile and readily oxidized cysteine conjugate which may attack macromolecules (Monks *et al.*, 1990).

In contrast with the mouse, conjugation of the epoxide with GSH is a significant route of biotransformation for the rat. Moreover, this conjugation occurs to a large extent non-enzymatically, indicating that the epoxide may react with sulfhydrylgroups of macromolecules as well. For all rat strains, it was shown that ascorbic acid could only partly prevent against covalent binding by reactive metabolites. Most of the remaining covalent binding could be inhibited by GSH. Thus, for the rat, the epoxide as well as quinones play a role in covalent binding. This may explain the hepatotoxicity of 1,4-DCB in GSH-depleted rats, found by Stine *et al.* (1991). *In vivo* however, we found a smaller percentage of the dose as the corresponding mercapturic acids of the epoxide (10% vs. ca. 45% *in vitro*, Hissink *et al.*, submitted).

It has been shown that human CYP2E1 oxidizes 1,4-DCB to 2,5-dichlorophenol (Bogaards *et al.*, 1995), and metabolism was enhanced *in vivo* in male Wistar rats after pretreatment with isoniazid (Hissink *et al.*, submitted). Evidence exists that rat CYP2B1/2 also plays a role in the biotransformation of 1,4-DCB (Carlson and Tardiff, 1976). In order to further investigate the individual roles of the two isoenzymes in biotransformation and toxicity of 1,4-DCB, microsomes from Wistar rats pretreated with phenobarbital (PB), which mainly induces CYP2B1/2 (Burke *et al.*, 1985; Lubet *et al.*, 1985) and isoniazid (ISO), which induces CYP2E1 (Ryan *et al.*, 1984) were used. Since both PB and ISO induced total conversion, cytochrome P4502B1/2 as well as P4502E1 are most likely to be involved in the biotransformation of 1,4-DCB in rat. However, at the same microsomal protein concentration, conversion by PB-microsomes was much higher. In addition, the metabolite profile of the PB-microsomes differed to a large extent from the ISO- and control microsomes. Much more hydroquinones and glutathione conjugates of quinones were produced and the concentration of 2,5-dichlorophenol was notably lower. In addition, the relative contribution of covalently bound metabolites was higher compared to the control- and ISO-microsomes. In the presence of acetone, an inhibitor of CYP2E1, total conversion and formation of 2,5-DCP were reduced, but the formation of hydroquinones was not affected. Moreover, in the absence of ascorbic acid and presence of acetone, covalent binding (by quinones) was 18% of total conversion, whereas 13% binding was observed when CYP2E1 was not inhibited. These data indicate that cytochrome P4502B1/2 is involved in the formation of the hydroquinones and that CYP2E1 seems to be mainly responsible for the formation of 2,5-DCP. Moreover, phenobarbital induction has been shown to significantly decrease CYP2E1, which explains the small amount of 2,5-DCP found for PB-microsomes (Nakajima *et al.*, 1995). In addition, incubations with

microsomes from cell lines expressing human or rat CYP2E1 resulted solely in the formation of 2,5-DCP and not of hydroquinones.

Conversion by human microsomes was much lower compared to the other species, suggesting a lower liver concentration of metabolites *in vivo*. For these microsomes, CYP2E1 was also the most important enzyme involved in the oxidation of 1,4-DCB. From the microsomes containing a single P450-enzyme, CYP2E1 was the only isozyme producing 2,5-DCP. No metabolites could be observed for CYP2B6. The CYP2E1 activity in human microsomes is reflected by the highest percentage of 2,5-DCP (up to 66% of total metabolites) compared to the other species. However, also hydroquinones and glutathione conjugates of quinones were detected. It may be possible that CYP2E1, although less potent than CYP2B1/2, is involved in secondary oxidation. The extensive secondary oxidation observed for mouse, may be explained by 2,5-DCP being a very good substrate for the P450-enzyme involved, possibly a 2B-type enzyme, or a high content of this enzyme.

Covalent binding was least extensive for human microsomes. Ascorbic acid could only inhibit this covalent binding for 25%, indicating that quinones do not play a major role in covalent binding. A striking difference with the other species is the conjugation of the epoxide with GSH: this was as extensive as was seen for the rat, but nearly exclusively catalyzed by glutathione S-transferases whereas conjugation observed for the rat was merely non-enzymatic. This difference in GSH-conjugation of the epoxide for all three species might be a result of a difference in reactivity of the epoxide, however most likely the same epoxide is produced. Therefore it is possible that the P450-enzyme plays a role in the further metabolism of the epoxide. This would be by oxidizing the epoxide or 2,5-DCP rapidly further to a hydroquinone in a second cycle, which would compete with conjugation with GSH (mouse). For man, the epoxide would be released more easily from the active site compared to mouse, but only in the presence of both GSH and glutathione S-transferases. These would then catalyze the subsequent GSH-conjugation.

The data presented in this study stress the importance of elucidating the mechanism(s) of the toxic action(s) of a certain compound and its metabolic profile before one can accurately extrapolate the toxicity data to the human situation. Major differences in *in vitro* biotransformation of 1,4-DCB by different species and rat strains occur. These differences are most likely involved in the organ selectivity of carcinogenicity of the different species and also in the sensitivity of the different rat strains. However, the present *in vitro* experiments have limitations concerning detoxification routes, especially with regard to the role of

glucuronidation and sulfation of 2,5-DCP and hydroquinones which are observed *in vivo* (Hawkins, 1980; Klos and Dekant, 1994; Hissink *et al.*, submitted).

From the differences in biotransformation we can speculate that most likely man is not susceptible to the 1,4-DCB-induced liver carcinogenicity which is observed for mice, since this liver carcinogenicity is probably caused by a high formation of benzoquinones. Regarding the renal toxicity and carcinogenicity which is observed in Fischer-344 rats, one cannot entirely exclude a potential risk for man. Comparing the metabolite profiles of the Fischer rat- and human microsomes, the latter produce relatively more glutathione conjugates of the quinones, the potential metabolites responsible for nephropathy (Mertens *et al.*, 1991). Although the absolute amount of these metabolites is smaller than for mouse and rat due to the lower conversion (12 pmol vs. 46 pmol metabolites for the Fischer rat, see legend of Table 1), chronic exposure to low concentrations of 1,4-DCB might lead to renal damage.

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CHAPTER 5

DIFFERENCES IN CYTOCHROME P450-MEDIATED BIOTRANSFORMATION OF 1,2-DICHLOROBENZENE BY RAT AND MAN: IMPLICATIONS FOR HUMAN RISK ASSESSMENT

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Chemical Research in Toxicology, in press

ABSTRACT

The oxidative biotransformation of 1,2-dichlorobenzene (1,2-DCB) was investigated using hepatic microsomes from male Wistar, Fischer-344 and Sprague-Dawley (SD) rats, phenobarbital (PB)- and isoniazid (ISO) pretreated male Wistar rats and from man. In addition, microsomes from cell lines selectively expressing one cytochrome P450 (P4502E1, 1A1, 1A2, 2B6, 2C9, 2D6, 2A6 and 3A4) were used. The rate of conversion was 0.09 nmol/min/mg. protein for both Wistar and Fischer-344 rat microsomes, 0.04 for SD-microsomes and 0.14 for human microsomes. Induction of Wistar rats with isoniazid (ISO, a P4502E1 inducer) or phenobarbital (PB, a P4502B1/2 inducer) resulted in an increased conversion rate of 0.20 and 0.42 nmol/min/mg. protein, respectively. Covalent binding of radioactivity to microsomal protein was similar for Wistar, Fischer and ISO-pretreated rats (16-17% of total metabolites), whereas induction with PB resulted in an increased covalent binding of 23% of total metabolites. Covalent binding was 31% for SD-microsomes and only 4.6% for human microsomes. Ascorbic acid notably reduced the amount of covalently bound metabolites for the SD-microsomes only, indicating that for these microsomes quinones were likely to be involved in this part of the covalent binding. Conjugation of epoxides with glutathione (GSH) inhibited most of the covalent binding for all microsomes. In the absence of GSH, the epoxides were

hydrolyzed by epoxide hydrolase, resulting in the formation of dihydrodiols. Inhibition of epoxide hydrolase resulted in a decreased conversion and an increased covalent binding for all microsomes tested, indicating a role of epoxides in the covalent binding. Fischer-344 rat liver microsomes showed a lower epoxide hydrolase activity than microsomes from Wistar and Sprague-Dawley rats, which may explain the higher sensitivity to 1,2-DCB induced hepatotoxicity of Fischer rats *in vivo*. Conjugation of the epoxides with GSH was predominantly non-enzymatic for the rat, whereas for man, conjugation was nearly exclusively catalyzed by glutathione-S-transferases. This difference may be explained by the formation of a 'non-reactive' 3,4-epoxide by P4502E1 in human microsomes: incubations with microsomes selectively expressing human P4502E1 as well as human liver microsomes, resulted in the formation of similar amounts of 2,3- and 3,4-dichlorophenol (DCP), as well as two GSH-epoxide conjugates in equal amounts. For rat microsomes, one major GSH-epoxide conjugate was found, and a much higher covalent binding, particularly for the PB-microsomes. Therefore, we postulate that rat P4502B1/2 preferentially oxidizes the 4,5-site of 1,2-DCB, resulting in a reactive epoxide. Postulating these epoxides to be involved in the mechanism(s) of toxicity, human risk after exposure to 1,2-DCB will be overestimated when risk assessment is solely based on toxicity studies conducted in rat.

INTRODUCTION

1,2-Dichlorobenzene (1,2-DCB) is used as a chemical intermediate in the synthesis of pesticides and other chlorinated compounds, as a solvent and as deodorizer. Environmental contamination may lead to human exposure via inhalation and drinking water (*1,2*).

1,2-DCB is a potent hepatotoxicant (*3-6*). The hepatotoxicity of halogenated benzenes is thought to be mediated by the generation of reactive metabolites as a result of their oxidation by cytochrome P450 (P450) (*7-9*). Induction of these P450 enzymes enhances toxicity (*6,10,11*). The ability of 1,2-DCB to deplete glutathione (GSH) and the enhanced toxicity of 1,2-DCB after GSH depletion has been reported by several investigators (*6,12,13*).

Halogenobenzene-induced toxicity and covalent binding to macromolecules have been ascribed mainly to its primary oxidation products, the epoxides (*14*). However, in a recent study, in which the microsomal biotransformation of 1,4-DCB was investigated, we showed that quinones, secondary oxidation products, are the principal metabolites involved in covalent

binding. In addition, studies on bromobenzene-induced hepatotoxicity indicated that secondary quinone metabolites are involved in the alkylation of hepatic proteins (*15,16*). The majority of the protein-bound residues which could be structurally identified, was quinone-derived (10-15%), whereas epoxides only accounted for less than 0.5% of total covalent binding. For 1,2-DCB, it has also been proposed that quinones play a more important role in covalent binding and liver toxicity than epoxides (*17*). However, recently we observed that *in vivo* 1,2-DCB is mainly metabolized to epoxides, which are conjugated with GSH. No quinone-derived metabolites were observed (*18*).

The present study was designed to further evaluate the individual roles of epoxides and quinones in covalent binding *in vitro*, and the roles of P450 enzymes in the formation of these metabolites. This was performed for rat as well as for man.

MATERIALS AND METHODS

Materials

Microsomes of human liver (a pool of 5 individuals) were obtained from Human Biologics, Inc. (Phoenix, AZ, USA). Microsomes from cell lines transfected with cDNAs expressing human P4502E1, 1A1, 1A2, 2B6, 2C9, 2D6, 2A6, 3A4 or human epoxide hydrolase were obtained from the Gentest Corp. (Woburn, MA, USA). 1,2-Dichloro-[^{14}C]-benzene with a radiochemical purity of >98% and a specific activity of 244 MBq/mmol was obtained from Sigma Chemical Co., St. Louis, MO, USA. ^{35}S-Glutathione with a radiochemical purity of >90% and a specific activity of 3075 GBq/mmol was purchased from DuPont NEN, Dordrecht, the Netherlands. The unlabeled 1,2-DCB (purity > 99%) was from Merck, Darmstadt, Germany. 2,3- and 3,4-Dichlorophenol were from Aldrich, Steinheim, Germany. Isoniazid (isonicotinic acid) was from Janssen Chimica (Beerse, Belgium) and phenobarbital was from CAV, Utrecht, the Netherlands. Glutathione (GSH) and NADPH were obtained from Boehringer Mannheim GmbH, Germany. Cyclohexene oxide was from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals used in this study were of reagent grade.

Animals

Adult male Wistar rats (strain Crl: (WI)WUBR), Fischer-344 rats (strain CDF(F-344)/CrlBR) and Sprague-Dawley rats (strain Crl: CD(SD)BR), weighing 250-300 g and 9-10 weeks of age, were obtained from Charles River Wiga GmbH, Sulzfeld, Germany. The animals were provided ad libitum with the Institute's cereal-based rodent diet (SDS Special Diet Services, Witham, England) and tap water. The phenobarbital (PB) pretreated rats (Wistar) received drinking water with 0.1% (w/v) PB during 4 days prior to sacrifice. The isoniazid pretreated rats (Wistar) received drinking water with 0.1% (w/v) isoniazid (isonicotinic acid) during 10 days prior to sacrifice. The animal room was air-conditioned and light controlled, with a light/dark cycle of 12 hours.

Preparation of microsomes

Microsomes were isolated from male Wistar rats (untreated, phenobarbital and isoniazid pretreated) and from male Sprague-Dawley and Fischer-344 rats. The animals were sacrificed by abdominal bleeding and the livers were removed, washed and homogenized in ice-cold 20 mM KCl-Tris buffer (pH 7.4). Livers of at least 3 animals were pooled. The liver homogenates were centrifuged for 30 min. at 10,000 g and 2 °C. The supernatants were then centrifuged for 90 min. at 105,000 g and 2 °C. The resulting supernatants (cytosol) were stored at -30 °C and the pellets (microsomes) were washed with KCl-Tris buffer and centrifuged again. The pellets were then resuspended in 0.1 mM potassium phosphate buffer (pH 7.4), and stored at -80 °C.

Biochemical assays

Total P450 content was determined by the method of Omura and Sato (*19*), modified by Rutten *et al.* (*20*). The protein concentration of the microsomes was determined according to the method of Lowry *et al.* (*21*) using bovine serum albumin (BSA) as standard.

Microsomal incubations

All incubations were performed in duplicate. Incubation mixtures contained 0.1 M potassium phosphate buffer (pH 7.4), 3 mM $MgCl_2$, 3 mM NADPH and hepatic microsomes from rat (pool of at least 3 animals) or man (pool of 5 individuals) at a protein concentration of 0.5 mg/mL, in a final volume of 200 µL. P450-contents of human and rat (control Wistar, ISO, PB, SD and Fischer) microsomes were 0.49, 0.63, 0.82, 2.21, 0.74 and 0.62 nmol/mg protein,

respectively. Where indicated, additions were 5 mM GSH, 1 mM ascorbic acid (AA), 0.6 mM cyclohexene oxide (CO), 2.5% acetone (v/v) or rat liver cytosol as a source of glutathione S-transferases (GST), with a final protein concentration of 2 mg/mL. Substrate concentrations were ca. 15 μM, but exact concentrations were calculated for all incubation mixtures. A stock solution of 1,2-^{14}C-DCB was prepared in acetone and BSA, with a final concentration of acetone in the incubation mixtures of 0.05%, which was shown not to inhibit P4502E1 to a detectable amount (*22*). Incubation mixtures contained ca. 0.85 kBq. The reactions were started by the addition of the substrate and were performed using glass vials with teflonized caps in a shaking waterbath at 37 °C. For each experiment, control incubations were performed without NADPH. After 15 min, the reactions were stopped by adding 20 μL 6 M HCl through the cap using a Hamilton™ needle. The vials were put on ice immediately and then frozen at -20 °C. After thawing on ice, the vials were centrifuged for 10 min at 1560 g and 4 °C to precipitate the protein. The caps were removed and the supernatants were transferred to HPLC-vials with teflonized caps and immediately analyzed by HPLC. The protein pellets were resuspended in 0.5 mL water and stored at -20 °C until further processing, i.e. measuring covalent protein binding.

Covalent binding to protein

Total covalent binding of radioactivity to microsomal protein was measured for all protein pellets obtained with the various experiments. Blank corrections were made for incubations without NADPH. The vials containing protein suspensions were thawed on ice and centrifuged for 15 min at 1560 g and 4 °C. Supernatant was removed and 1 mL of methanol was added to the pellets. Vials were extensively vortexed and centrifuged for 10 min at 1560 g. The same procedure was followed with acetone and then n-hexane, until the supernatant contained background radioactivity. The pellets were dried at room temperature, dissolved in 1 mL Soluene-350 from Packard and mixed with 20 mL Hionic Fluor from Packard. This mixture was analyzed for radioactivity.

Measurement of radioactivity

Aliquots of incubation mixtures (20 μL) were mixed with 4.5 mL Ultima Gold scintillation cocktail (Packard Instrument Company, Reading, UK) and radioactivity was measured in a Pharmacia Wallac S1409 Liquid Scintillation Counter (Pharmacia, Uppsala,

Sweden) using automatic external standard quench correction and was counted at efficiencies > 85%.

HPLC analysis

HPLC was carried out with a Pharmacia LKB HPLC 2248 Pump (Pharmacia, Sweden), fitted with a Pharmacia VWM 2141 UV-detector operating at 254 nm. Radioactivity was measured on line with a Canberra Packard Radiomatic Detector (type A500, Flo-one Beta). A liquid flow cell of 500 or 2000 µL was used and radioactivity was measured using Flo Scint A (Packard) with a scintillator flow of 3 mL/min. Injections of 100-160 µL were done automatically by a Pharmacia LKB-Autosampler 2157 at 4 °C. Separations were carried out using a reversed phase column, 250*4.6 mm, C18 hypersil ODS 5 µM (Chrompack). Eluting solvents were methanol and 20 mM ammonium acetate, (pH 6.7), used with the following gradient: 0-5 min: 15% methanol; 5-10 min: 15 → 30% methanol; 10-30 min: 30 → 50% methanol; 30-45 min: 50 → 95% methanol. The flow rate was 1 mL/min.

LC-MS measurements

Incubation mixtures with microsomes from phenobarbital pretreated rats were directly measured using LC-MS (Liquid Chromatography Mass Spectrometry) to identify the major metabolites in the absence or presence of GSH. A C18 hypersil ODS 5 µM (250*4.6 mm) column was used. The mobile phase A was 20 mM ammonium acetate (pH 6.7), and mobile phase B was 100% methanol. The solvent gradient used was the same as described in the HPLC analysis section. The flow was 1 mL/min. Mass spectrometric analyses were performed using electrospray ionization (Finnigan ESP2) on a Finnigan MAT TSQ 700.

RESULTS

Identification of the major metabolites

In Figure 1, ^{14}C-HPLC chromatograms are shown of incubation mixtures with microsomes from male Wistar rats pretreated with phenobarbital in the absence (A) or presence (B) of glutathione (GSH). Peak 1 is identified as a dihydrodiol, a hydrolyzed epoxide, since in the presence of cyclohexene oxide, an inhibitor of epoxide hydrolase, this metabolite completely disappeared. In addition, in Figure 2A a mass spectrum of this metabolite is shown.

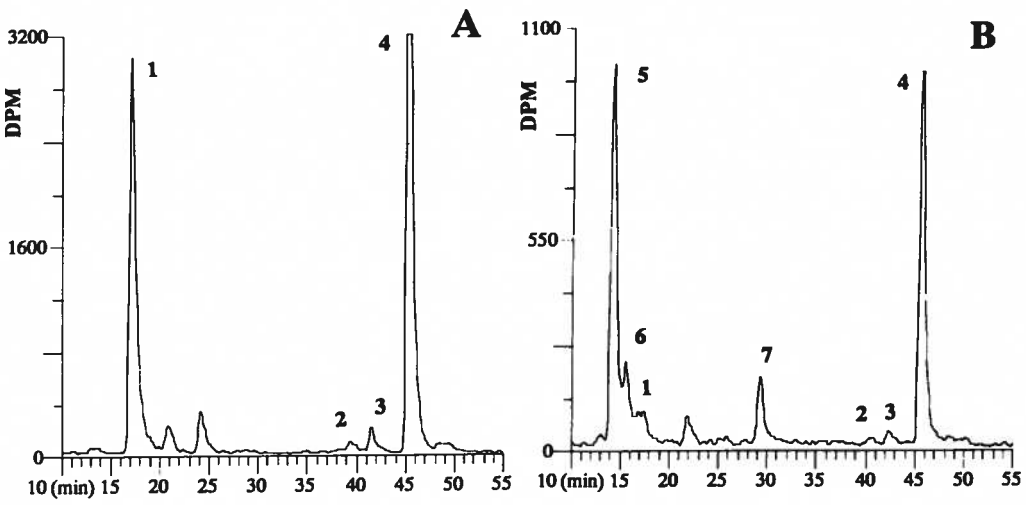

Fig.1. ^{14}C-HPLC-chromatograms of incubation mixtures with microsomes from male Wistar rats pretreated with phenobarbital in the absence (A) or presence (B) of GSH. Peak 1: Dihydrodiol. Peaks 2 and 3: 2,3- and 3,4-Dichlorophenol. Peak 4: 1,2-Dichlorobenzene. Peaks 5, 6 and 7: GSH-conjugates of the epoxides.

The mass at *m/z* = 215 arises from the molecular ion (*m/z* = 180) clustered with a chlorine atom, with a mass of 35. The mass at *m/z* = 239 arises from the dihydrodiol with acetate, which was present in the mobile phase. The mass at *m/z* = 179 originates from a cluster of three acetate molecules. Peak 2 and 3 are assigned to 2,3- and 3,4-dichlorophenol (DCP) respectively, by co-elution with the reference compounds. Peak 4 is 1,2-dichlorobenzene (1,2-DCB). Peaks 5 and 6 in Figure 1B, which appeared in the presence of GSH, are GSH-conjugates of the epoxides of 1,2-DCB. The mass spectrum of these metabolites is shown in Figure 2B: the conjugates have a molecular ion at *m/z* = 470. The metabolite represented by peak 6 disappeared in time, in favour of peak 7 (results not shown). Peak 7 appeared to be formed from peak 6 by loss of a water molecule, since analysis by LC-MS revealed a molecular ion at *m/z* = 452 for peak 7 (Figure 2C).

In Figure 3 ^{14}C-HPLC chromatograms are shown of incubation mixtures with human liver microsomes in the absence of GSH (A), in the presence of GSH (B) and in the presence of both GSH and rat liver GST (C). Peaknumbers correspond with the numbers in Figure 1. Peak 1 and peak 8 both disappeared in the presence of cyclohexene oxide, an inhibitor of epoxide hydrolase, so both metabolites were assigned to dihydrodiol(-derived) metabolites. Peak 2, 3, 4, 5, 6 and 7 constitute the same compounds as in Figure 1.

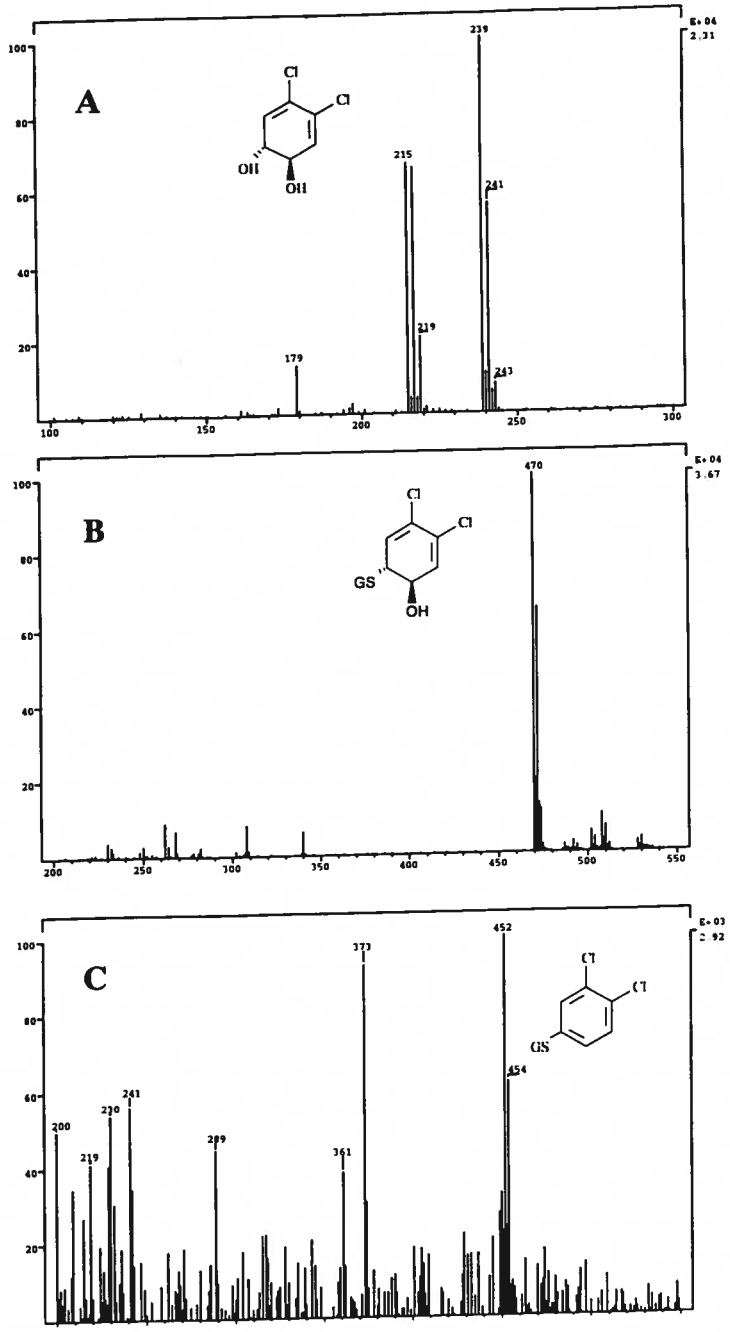

Fig.2. Mass spectra of peak 1, the dihydrodiol (A), peaks 5 and 6, the GSH-conjugates of the epoxides (B, *m/z* = 470) and peak 7, the GSH-conjugate of an epoxide with loss of a water molecule (C, *m/z* = 452). For the chiral metabolites in A and B, two alternative diastereoisomeric structures are possible; the ones with trans vicinal bonds are shown.

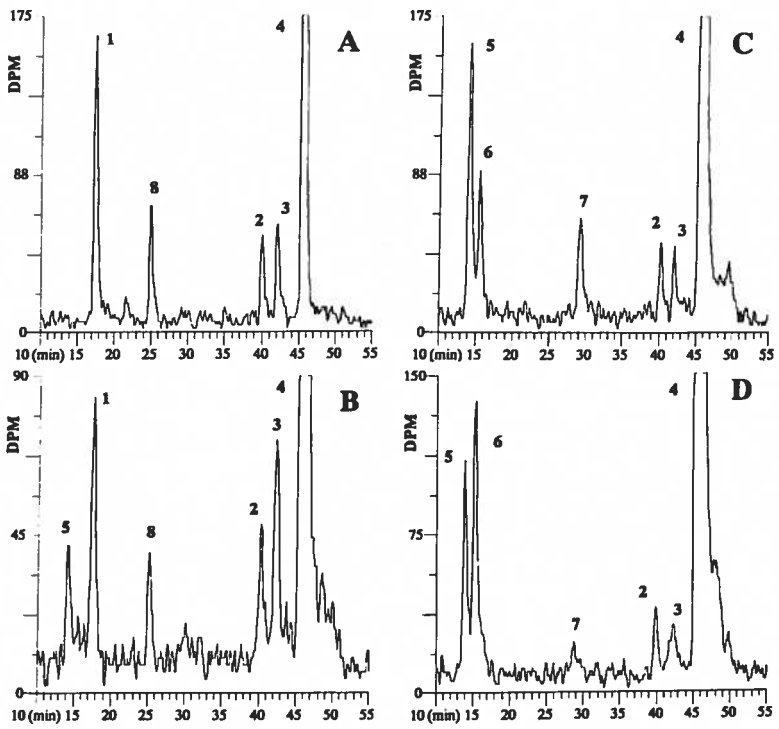

Fig.3. ^{14}C-HPLC-chromatograms of incubation mixtures with human liver microsomes in the absence of GSH (A), in the presence of GSH (B) and in the presence of GSH and rat liver GST (C). Peaks 1 and 8: Dihydrodiols. Peaks 2 and 3: 2,3- and 3,4-Dichlorophenol. Peak 4: 1,2-Dichlorobenzene. Peaks 5, 6 and 7: GSH-conjugates of the epoxides (peak 7 = -H_2O). D: ^{14}C-HPLC-chomatogram of an incubation mixture with microsomes derived from a cell line selectively expressing human P4502E1, in the presence of GSH and rat liver GST.

Rate of conversion of 1,2-dichlorobenzene by the various microsomes

In Table 1, the rate of conversion of 1,2-DCB into metabolites (including covalent binding) by the various microsomes is represented expressed as nmol product per minute per mg protein. Preliminary experiments indicated that the assay conditions were linear with respect to time and protein concentration. No conversion was observed in incubation mixtures without NADPH. Rate of conversion by the control Wistar and Fischer rat was similar, whereas conversion by the Sprague-Dawley rat was lower. For the Wistar rat, both pretreatment with ISO and PB increased the rate of conversion, namely two and four times, respectively. The rate of conversion of 1,2-DCB by human microsomes was higher compared to control rat microsomes, for all strains.

Table 1. Total conversion of 1,2-DCB and formation of the three major metabolites by the various microsomes.

Mic. species[a]		Conversion[b]	Identified Metabolites (% of total conversion)[c]			
			GSH-Epoxide[d]		Dihydrodiol	2,3-+ 3,4-DCP
			- GST	+ GST		
Wistar	- GSH	0.090	ND	ND	49.87% (67)	21.85% (29)
	+ GSH	0.075	72.04% (81)	79.59% (90)	BLQ	14.97% (17)
Wistar-ISO	- GSH	0.196	ND	ND	48.58% (143)	25.97% (76)
	+ GSH	0.254	65.60% (250)	76.92% (293)	2.59% (10)	16.90% (64)
Wistar-PB	- GSH	0.424	ND	ND	42.87% (273)	4.92% (31)
	+ GSH	0.320	58.42% (280)	74.82% (359)	15.31% (73)	4.20% (20)
Fischer-344	- GSH	0.099	ND	ND	23.84% (35)	21.05% (31)
	+ GSH	0.11	56.34% (93)	76.91% (127)	BLQ	8.87% (15)
Sprague-Dawl.	- GSH	0.038	ND	ND	69.31% (40)	BLQ
	+ GSH	0.031	58.66% (27)	68.55% (32)	14.70% (7)	BLQ
Human	- GSH	0.141	ND	ND	56.78% (120)	38.71% (82)
	+ GSH	0.116	8.48% (15)	78.0% (136)	38.87% (68)	40.07% (70)

Note. [a] Liver microsomes from male Wistar rats were isolated from control, isoniazid (ISO) or phenobarbital (PB) pretreated rats (pools of at least 3 animals). For Fischer and SD rats, pooled microsomes of 3 animals were used. For human, microsomes of 5 individuals were pooled. [b] Conversion is expressed as nmol product formed per min per mg protein. All incubations were performed in duplicate, as described under Materials and Methods. Variation in conversion of 2 incubations ranged from 0 to 10%. Presented data are the means of two incubations. [c] Expressed as percentage of total conversion. To determine total conversion, metabolites (including a small (<15%) percentage of unidentified peaks) quantified with HPLC and covalently bound radioactivity (1,4-DCB equivalents) were summed. Of all metabolites eluting from the column, at least 85% was accounted for stucturally. Between parentheses, the absolute amounts (pmol) of metabolites formed in 15 min by 0.1 mg protein (assay conditions) are given. [d] Effect of addition of rat liver GST is represented. ND = not determined. BLQ = below limit of quantification.

Covalent binding of radioactivity to microsomal protein; effect of GSH and AA

Figure 4 presents the covalent binding of radioactivity to microsomal protein, expressed as percentage of the total amount of metabolites formed. Covalent binding was most extensive for the SD-microsomes (31% of total metabolites) and least extensive for human microsomes (4.6% of total metabolites formed). Covalent binding by Wistar and Fischer rat microsomes was similar (ca. 16%), whereas pretreatment with PB (and not with ISO) increased the formation of reactive metabolites, namely 23% of total metabolites. Addition of ascorbic acid (AA) reduced the amount of covalently bound metabolites only for the SD-microsomes. This is because AA scavenged reactive benzoquinones which are produced by oxidation of hydroquinone metabolites. This indicates that quinones were only involved in part of the covalent binding observed in SD-microsomes. Non-enzymatic conjugation of epoxides with GSH inhibited covalent binding for all microsomes to a considerable extent. Addition of rat liver GST further decreased covalent binding for all Wistar and human microsomes, in favour of the GSH-conjugates of the epoxides. This decrease was larger for the ISO-microsomes compared to the PB-microsomes. Thus, for all microsomes, covalent binding was predominantly mediated by the epoxides of 1,2-DCB.

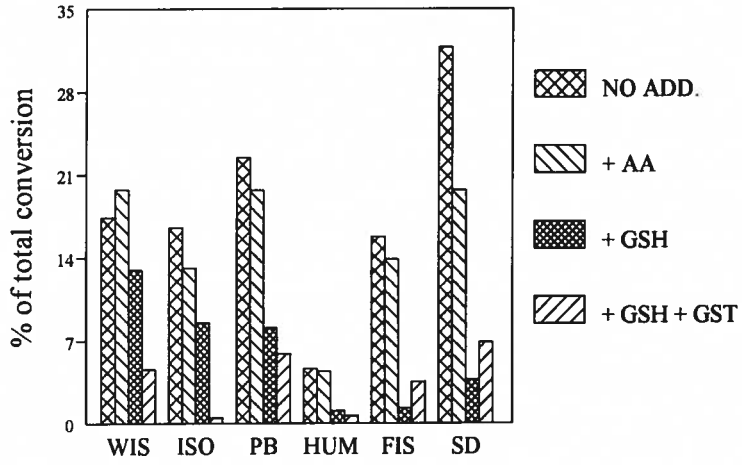

Fig.4. Covalent binding of radioactivity to microsomal protein, expressed as percentage of the total amount of metabolites formed. The effect of 1 mM ascorbic acid (AA), 5 mM GSH or 5 mM GSH + rat liver GST (2 mg protein/mL) is represented in separate bars. Presented data are the means of two incubations; variation between duplicates was 0-10% .

Relative contributions of different metabolites

The formation of the dihydrodiol metabolites, the GSH-conjugates of the epoxides and the dichlorophenols, relative to the total amount of metabolites formed, is shown in Table 1, for all microsomes. Relative formation of dihydrodiols was rather similar for all microsomes, except for the Fischer microsomes, that produced less of these metabolites. The amount of GSH-epoxide conjugates was similar for all microsomes. The conjugation occurred non-enzymatically for all rat microsomes, whereas for human microsomes conjugation nearly completely depended on catalysis by glutathione S-transferases. Formation of dichlorophenols was most extensive for the human microsomes. For the SD-microsomes no dichlorophenols could be detected (the lower limit of quantification was ca. 4% of total metabolites). Induction with phenobarbital resulted in a significant relative decrease of the formation of dichlorophenols, compared to the control microsomes.

Incubations with microsomes containing human cytochrome P450 and epoxide hydrolase

Incubations with microsomes derived from different cell lines selectively expressing one human P450, revealed that only P4502E1, and not 1A1, 1A2, 2B6, 2C9, 2D6, 2A6 or 3A4 metabolized 1,2-DCB to detectable amounts of 2,3- and 3,4-dichlorophenol (DCP). The isomers were produced in equal amounts (not shown). Incubation of microsomes containing P4502E1 together with microsomes derived from a cell line selectively expressing human epoxide hydrolase resulted in a single extra metabolite, which corresponded with a metabolite which was also present in the incubation mixtures with human microsomes, peak 1 in Figure 3 (not shown). Figure 3D shows a HPLC-chromatogram of an incubation mixture with human P4502E1-microsomes, GSH and rat liver GST. The metabolites formed were the same as shown in Figure 3C, with peak 6 being converted to peak 7 in time. Without the addition of rat liver GST, no conjugates were produced.

Effect of inhibition by cyclohexene oxide and acetone

In Table 2, the effect of acetone and cyclohexene oxide on total conversion, covalent binding and on the formation of three metabolites is given for the different microsomes. SD and Fischer microsomes were not included in this experiment. Both acetone and cyclohexene oxide inhibited total conversion for all microsomes. Addition of acetone increased covalent binding for all microsomes except for PB-microsomes. Addition of cyclohexene oxide resulted in increased covalent binding for all microsomes. The dihydrodiol was hardly formed in the

Table 2. Effect of addition of 2.5% acetone or 0.6 mM cyclohexene oxide (CO) to microsomal mixtures on total conversion, covalent binding and amount of three metabolites formed.[a]

WIS	Conv.[b]	Cov.bind.[b,c]	DHD[c]	2,3-DCP[c]	3,4-DCP[c]
+ AA	100%	100%	72.99%	6.35%	12.11%
+ Acetone	29.9%	285%	71.95%	<LLOQ[d]	10.38%
+ CO	48.1%	144%	4.95%	35.97%	27.08%

HUM	Conv.	Cov.bind.	DHD	2,3-DCP	3,4-DCP
+ AA	100%	100%	74.34%	10.32%	13.91%
+ Acetone	31.7%	822%	62.91%	10.69%	14.66%
+ CO	25.2%	578%	<LLOQ	49.38%	42.36%

ISO	Conv.	Cov.bind.	DHD	2,3-DCP	3,4-DCP
+ AA	100%	100%	60.90%	4.99%	9.78%
+ Acetone	19.8%	515%	60.15%	4.66%	7.35%
+ CO	38.5%	247%	5.0%	28.65%	22.78%

PB	Conv.	Cov.bind.	DHD	2,3-DCP	3,4-DCP
+ AA	100%	100%	68.46%	1.49%	3.09%
+ Acetone	26.0%	100%	58.95%	0.40%	4.18%
+ CO	57.7%	167%	8.18%	18.11%	10.09%

Note. [a] Concentration of 1,2-DCB was ca. 80 μM. [b] Conversion (% of substrate present) and covalent binding are set at 100% in the presence of ascorbic acid (AA) only, which was present in all mixtures. [c] Expressed as % of total metabolites formed. [d] <LLOQ = below the lower limit of quantification.

presence of cyclohexene oxide, whereas the formation of the DCP's increased, with slightly more 2,3-DCP than 3,4-DCP. Addition of cyclohexene oxide to the incubation mixtures with PB-microsomes, resulted in the formation of a relatively high amount of unidentified metabolites.

DISCUSSION

In the present study, the biotransformation of 1,2-dichlorobenzene (1,2-DCB) by hepatic microsomes from man, male Wistar, Fischer-344 and Sprague-Dawley rats and from male Wistar rats pretreated with phenobarbital (PB) or isoniazid (ISO), has been examined. In Figure 8 a metabolic scheme is presented, depicting the proposed biotransformation pathways of 1,2-DCB. In this scheme, the predominant routes of biotransformation for rat and man are shown. For man, it has been shown that P4502E1 is the major cytochrome-P450 enzyme involved in oxidation of 1,2-DCB (*22*). Our results indicated that this isozyme preferentially, or even selectively, oxidizes 1,2-DCB at the 3,4-position of the chlorine atoms, since incubations with microsomes selectively expressing human P4502E1, resulted in the formation of equal amounts of 2,3- and 3,4-DCP. Moreover, in the presence of GSH and glutathione S-transferases, two GSH-epoxide conjugate peaks with comparable peak area were observed in the chromatograms. Incubations with human microsomes resulted in a very similar metabolic profile, compared to microsomes selectively expressing human P4502E1, with equal amounts of DCP's and GSH-epoxide conjugates. The covalent binding as percentage of total metabolites formed by human microsomes was much smaller compared to that of each of the rat microsomes. Therefore, we conclude that the metabolite(s) produced by the human microsomes are much less reactive than those produced by the rat microsomes. For rat microsomes, one particular GSH-epoxide conjugate was formed. This conjugate did not need glutathione S-transferase catalysis for its formation. The same non-enzymatically formed GSH-conjugate was detected only in very small amounts in human microsomes. By addition of GSH, most of the covalent binding was inhibited for both human and rat liver microsomes. Since from the 'para'-4,5-epoxide only a single GSH-conjugate can be formed (see Figure 5), we speculate that rat microsomal enzymes preferentially oxidize the 4,5-site of the 1,2-DCB molecule, resulting in a reactive epoxide. The relative high formation of this metabolite in rat microsomes compared to human microsomes is responsible for the higher covalent binding to rat microsomal protein. In agreement with these data, we found a selective increase in the formation of 3,4-DCP *in vivo* by male Wistar rats after pretreatment with phenobarbital, an inducer of P4502B1/2 (*18*). In several reports it has been described that 1,2-DCB-induced hepatotoxicity was to a large extent increased by phenobarbital (*6,8,10,11*). Based on the results of the present study, this may be a consequence of the selective formation of the reactive 4,5-epoxide. The speculated difference in reactivity and toxicity of different epoxides

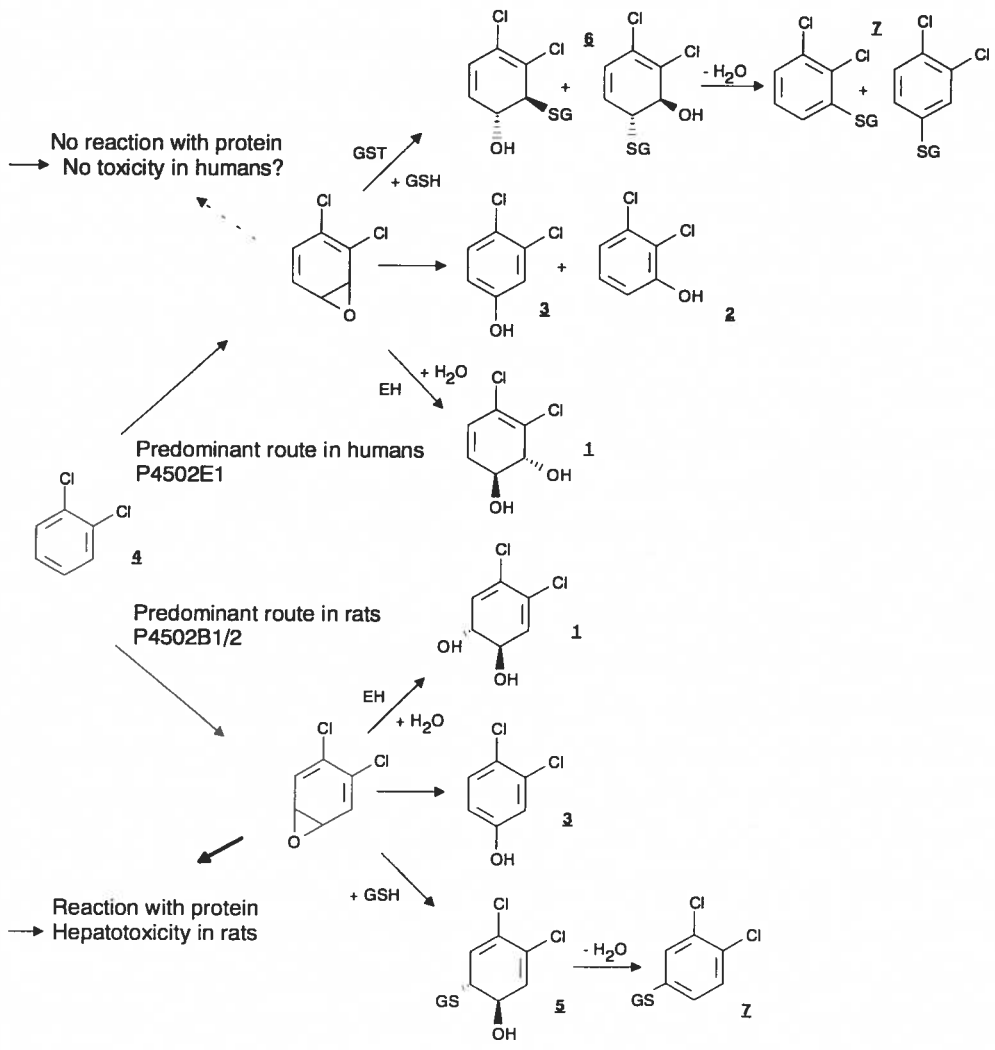

Fig.5. Metabolic scheme, representing the proposed oxidative biotransformation of 1,2-DCB by rat and human liver microsomes. The numbers correspond to the peak numbers in Figure 1. SG = glutathione derivative, EH = epoxide hydrolase, GST = glutathione S-transferase. Absolute stereochemistry is not implied in the structures. Trans metabolites with trans vicinal bonds are depicted since they are mechanistically most likely.

of 1,2-DCB has also been demonstrated for bromobenzene (*23-25*). Bromobenzene was preferentially metabolized to *para*-bromophenol by the phenobarbital pretreated rabbit via the toxic 3,4-epoxide, whereas the formation of the non-toxic 2,3-epoxide was induced by 3-methylcholanthrene and β-naphthoflavone in rats. Similar to bromobenzene metabolism, the two possible epoxidation pathways of 1,2-DCB appear to require specific P450 enzymes. The 4,5-epoxidation would then preferentially be catalyzed by the phenobarbital inducible P4502B-enzymes, whereas human P4502E1 most likely selectively oxidizes the 3,4-site of 1,2-DCB. Conversion by rat liver microsomes was induced by both phenobarbital and isoniazid (a P4502E1 inducer, (*26*)), indicating that rat P4502E1 is also involved in 1,2-DCB oxidation. Covalent binding as percentage of total metabolites formed was increased to a small extent for the PB-microsomes, but not for the ISO-microsomes, confirming the prevalenced formation of reactive metabolites by P4502B1/2. Rat P4502E1 would thus not be explicitly involved in the formation of reactive metabolites. However, it has been shown *in vivo* that 1,2-DCB-induced hepatotoxicity is potentiated by acetone and other low molecular weight ketones, which are associated with induction of P4502E1 (*27*). Possibly rat P4502E1, a significant constitutive enzyme, is involved in the oxidation of both the 3,4- and 4,5-site of the molecule, to the same extent. This is confirmed by the 2:1 ratio of 3,4- and 2,3-DCP, which is shown in Table 2 for the rat.

Inhibition of P450-enzymes with acetone resulted in a significant decrease in conversion for all microsomes. For control and ISO-rat microsomes and human microsomes, relative covalent binding was considerably increased by the addition of acetone. By inhibiting P4502E1 in these microsomes, the activity of P4502B-enzymes will become relatively more important, resulting in the formation of a relatively larger amount of reactive metabolites. These data suggest that for human microsomes, another type of P450 enzyme (possibly 2B6) becomes involved after inhibition of P4502E1. For PB-microsomes, no increase in covalent binding was observed, probably because the major isoenzyme involved in 1,2-DCB oxidation in these microsomes is already P4502B1/2 and not P4502E1, since phenobarbital induces P4502B1/2 and significantly decreases the amount of P4502E1 (*28*). Hence, inhibition of P4502E1 will have no effect.

As mentioned earlier, covalent binding was much higher for all rat microsomes compared to human microsomes. In order to elucidate the identity of the metabolites giving rise to covalent binding, GSH and ascorbic acid (AA) were used to scavenge epoxides and prevent hydroquinones from oxidation to benzoquinones, respectively. Ascorbic acid inhibited

covalent binding for the SD-microsomes to some extent only, indicating that generally quinones do not play a significant role in covalent binding *in vitro*. The fact that no DCP's could be quantified in the experiment with the SD-microsomes, is in line with the assumption that these microsomes are more active in secondary oxidation of DCP's into quinones compared to the other microsomes.

The high amount of dihydrodiols in the incubation mixtures, which were formed by epoxide hydrolase, points to the extensive formation of epoxides. Addition of GSH resulted in a shift from the formation of the dihydrodiols to the GSH-conjugates of the epoxides and also in a significant decrease of covalent binding, indicating that epoxides were responsible for this part of the covalent binding. To check the involvement of microsomal GST, S-hexylglutathione, an inhibitor of (microsomal) glutathione S-transferases (*29*) was added and showed to have a negligible effect on GSH-conjugation (results not shown), indicating that the large amount of GSH-conjugates found for rat liver microsomes were indeed formed non-enzymatically. For human microsomes, GSH-conjugation was nearly completely catalyzed by cytosolic GST. However, the small amount of non-enzymatically formed conjugates (8% vs. 78% enzymatically formed conjugates) seemed to be responsible for the major part of the decrease in covalent binding. In the absence of GSH, epoxide hydrolases play an important role in the detoxification of epoxides *in vitro*. However, we could not identify dihydrodiols *in vivo* (*18*), most likely due to the favored conjugation of the epoxides with GSH. It is noteworthy that for 1,4-DCB no dihydrodiols were found, *in vitro* as well as *in vivo*.[2,3] Apparently, not all types of epoxides are suitable substrates for epoxide hydrolase. Inhibition of epoxide hydrolase by cyclohexene oxide resulted in a decreased conversion of 1,2-DCB for all microsomes tested. Most likely this decrease is caused by inactivation of the P450-enzymes involved in oxidation, by the epoxides of 1,2-DCB formed. It has been shown *in vivo* that 1,2-DCB reduced the amount of P450 to a large extent (*30,31*), and, therefore, it has been proposed that 1,2-DCB is a suicide substrate. Covalent binding increased in the presence of cyclohexene oxide for all microsomes, confirming the involvement of epoxides in covalent binding. In addition, for all microsomes the dihydrodiols nearly completely disappeared, in favor of the two isomers of DCP. These isomers appeared in more or less equal amounts, with even slightly more 2,3-DCP, indicating that these DCP's were derived from the 3,4-epoxide. The 'para'-4,5-epoxide might thus be responsible for the inactivation of the P450 enzymes, resulting in the decreased conversion, which confirms the higher reactivity of the 4,5-epoxide. Stine and co-workers (*6*) found a dramatic difference in 1,2-DCB-induced hepatotoxicity between F344-rats and SD-

rats. They proposed a higher susceptibility of the Fischer rat due to a lower epoxide hydrolase activity of the F344-rat compared to the SD-rat, which was found by Glatt and Oesch (*32*). We observed indeed relatively less dihydrodiol formation for the Fischer rat, compared to the other two rat strains and human microsomes, whereas the other metabolites were found in similar quantities. The *in vivo* detoxification route via hydrolysis of the epoxides appears not to be relevant (*18*), but at the dose levels used by Stine and co-workers (1.8 and 5.4 mmol/kg) GSH might be depleted, which would result in a more important role for epoxide hydrolases *in vivo* in detoxification. However, the overall lower conversion (and thus toxification) of 1,2-DCB by SD-microsomes might also play a role in the lower susceptibility of this rat strain.

The present results have revealed a striking difference in the oxidative biotransformation of 1,2-DCB between rat and human liver microsomes, with a resultant difference in covalent binding. Rat liver microsomes produced much more reactive metabolites, whereas the total rate of conversion by human microsomes was higher. The observed difference in covalent binding seems to arise from different P450 enzymes involved in oxidation of 1,2-DCB, and the subsequent formation of different metabolites with different reactivity. From these data it is tempting to suggest that man would be less susceptible to 1,2-DCB-induced hepatotoxicity compared to the rat, and that human risk assessment of 1,2-DCB solely based on toxicity studies conducted in rats will lead to an overestimation of human risk. However, this speculation would go beyond the scope of the present *in vitro* results. Many other pharmacokinetic and -dynamic factors need to be considered before one can make an accurate risk estimate for man. Nevertheless, we emphasize that elucidating the molecular mechanism of toxicity of xenobiotics is essential in accurate risk assessment, and that the animal models used for assessing the risk to man have to be selected carefully.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. E.R. Verheij from the Division of Analytical Sciences, for performing the LC-MS measurements. This work was supported, in part, by the Utrecht Toxicological Center (UTOX).

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CHAPTER 6

DIFFERENTIAL TIME DEPENDENT HEPATOTOXICITY AND GLUTATHIONE DEPLETION AFTER SINGLE ORAL ADMINISTRATION OF 1,2- AND 1,4-DICHLOROBENZENE IN THE MALE WISTAR RAT

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Submitted

ABSTRACT

The time dependent hepatotoxicity, depletion of glutathione (GSH) and plasma concentration of parent compound were determined after oral administration of 50 or 250 mg/kg BW 1,2- or 1,4-dichlorobenzene (DCB).

Plasma concentrations were much higher for 1,4-DCB than for 1,2-DCB, for both dose levels, corresponding with a much higher area under the plasma curve (AUC) and a much lower total clearance for 1,4-DCB compared to 1,2-DCB. For both isomers a saturation of metabolism was observed at 250 mg/kg.

The liver toxicity parameters plasma alanine amino transaminase activity (ALT) and plasma asparagine amino transaminase activity (AST) were significantly increased compared to control values at 24 and 48 hours after administration of 250 mg/kg 1,2-DCB, with less pronounced toxicity at 48 hours. For the 50 mg/kg dose level of 1,2-DCB and for both dose levels of 1,4-DCB, no increase in ALT or AST activity was observed.

In accordance with the ALT and AST values, treatment related histopathological changes were only detected in the liver of animals dosed with 250 mg/kg 1,2-DCB, at 24 hours and less pronounced at 48 and 72 hours after dosing. These changes were characterized by cytoplasmatic alterations and increased eosinophilia in the perivenous hepatocytes, but not in

the periportal hepatocytes. 1,4-DCB produced no histopathological changes at the dose levels used.

Glutathione (GSH, including the oxidized form, GSSG) was depleted in the liver for both dose levels of 1,2-DCB at 4, 8 and 24 hours after dosing, with maximum depletion of 54% at 24 hours for the high dose level. An overshoot of GSH synthesis was observed at 48 and 72 hours, particularly for the 250 mg/kg dose level. For 1,4-DCB, the GSH concentration in the liver was slightly lower compared to the control values at 24 and 72 hours after dosing, for the 50 mg/kg dose level only.

The relative liver weights (liver/BW ratio) were significantly increased at 48 and 72 hours after dosing with 250 mg/kg BW 1,2-DCB. No increase was observed after dosing with 1,4-DCB.

In conclusion, 1,2-DCB induces hepatotoxicity at the 250 mg/kg dose level only, most likely due to a reactive intermediate which depletes GSH, whereas 1,4-DCB is not metabolized to a GSH scavenging metabolite, and therefore does not induce acute hepatotoxicity at 250 mg/kg.

INTRODUCTION

Dichlorobenzenes (DCB's) are used extensively in the industrial synthesis of organic compounds and as solvents, insecticides and deodorizers and therefore represent significant environmental pollutants (Morita and Ohi, 1975; Zoeteman *et al.*, 1980; Oliver and Nicol, 1982; U.S. EPA, 1985, 1987; Angerer *et al.*, 1992; Hill *et al.*, 1995). 1,2-DCB is a potent hepatotoxin, but not a carcinogen in rat (Cameron *et al.*, 1937; Hollingsworth *et al.*, 1958; Brondeau *et al.*, 1986; NTP, 1987; Robinson *et al.*, 1991; Stine *et al.*, 1991). 1,4-DCB is not hepatotoxic, but it induces liver carcinogenicity in B6C3F1-mice and kidney toxicity and carcinogenicity in Fischer-344 rats, but not in other rat strains (Loeser and Litchfield, 1983; NTP, 1987; Bomhard *et al.*, 1988; Charbonneau *et al.*, 1989; Umemura *et al.*, 1992). The hepatotoxicity of 1,2-DCB is thought to be mediated by the generation of reactive metabolites as a result of their oxidation by cytochrome P450 (Brodie *et al.*, 1971; Reid and Krishna, 1973; Jollow *et al.*, 1974). In a recent *in vivo* study with male Wistar rats, we demonstrated that 1,2-DCB is mainly metabolized to epoxides, which are conjugated with glutathione (GSH) (Hissink *et al.*, 1996). In a subsequent *in vitro* study, using hepatic microsomes from male

Wistar rats, we demonstrated that the epoxides were the main reactive metabolites involved in covalent binding to microsomal protein (Hissink *et al.*, 1996-*b*).

About the mechanism of the toxicity and carcinogenicity of 1,4-DCB two hypotheses exist. The first is that 1,4-DCB binds to α_{2u}-globulin in the proximal tubules of the kidneys. This binding would result in accumulation of the protein, leading to protein droplet formation and cell proliferation (Swenberg *et al.*, 1989). However, cell proliferation is not sufficient to lead to 1,4-DCB-induced carcinogenesis (Umemura *et al.*, 1992). The second hypothesis involves bioactivation of 1,4-DCB. For 2,5-dichlorobenzoquinone, a secondary oxidation product of 1,4-DCB, it has been shown that its glutathione conjugate causes renal injury in rat (Mertens *et al.*, 1991). Male Fischer-344 rats, the strain in which nephropathy is observed, excreted 2,5-dichlorohydroquinone in the urine (Klos and Dekant, 1994). In a recent *in vivo* study with male Wistar rats, we could not identify these metabolites in the urine, and only 10% of the urinary metabolites consisted of epoxide-derived mercapturic acids. (Hissink *et al.*, submitted-1). Moreover, in an *in vitro* study, in which we compared the microsomal biotransformation of 1,4-DCB by different rat strains, we demonstrated that epoxide-mediated covalent binding was much less pronounced compared to 1,2-DCB-epoxides, and that F344-rat microsomes produced the highest amount of 2,5-dichlorohydroquinone metabolites (Hissink *et al.*, submitted-2).

In order to correlate our *in vivo* kinetic and metabolism studies with 1,2- and 1,4-DCB, which were conducted in male Wistar rats, with relevant toxicity data, we investigated the time dependent toxicity and GSH depletion in liver and kidneys in this rat strain after oral administration of two dose levels of 1,2- and 1,4-DCB.

MATERIALS AND METHODS

Materials

1,2-Dichlorobenzene (purity > 99%) was purchased from Merck, Darmstadt, Germany and 1,4-dichlorobenzene (purity > 99%) was from Aldrich, Steinheim, Germany. The reduced form of glutathione was obtained from Boehringer Mannheim GmbH, Germany. The oxidized glutathione, N-ethylmaleimide and o-phthalaldehyde were from Sigma Chemical Co., St. Louis, MO, USA. 1-Bromohexane (used as internal standard for GC-measurements) was from

Janssen Chimica, Geel, Belgium. Cyclohexane was from Merck, Darmstadt, Germany. All other chemicals used in this study were of analytical grade.

Animals

Adult male Wistar rats (strain Crl: (WI)WUBR), weighing 250-300g and 9-10 weeks of age, were obtained from Charles River Wiga GmbH, Sulzfeld, Germany. The animals were provided *ad libitum* with the Institute's cereal-based rodent diet (SDS Special Diets Services, Witham, England) and tap water. The animal room was air-conditioned and light-controlled, with a light/dark cycle of 12 hours.

Dosing and sacrifice of the animals

Five groups of 15 male Wistar rats were weighed and orally dosed with 3.3 ml cornoil/kg bw (group A, control), 50 mg 1,2-DCB/kg bw (group B), 250 mg 1,2-DCB/kg bw (group C), 50 mg 1,4-DCB/kg bw (group D) or 250 mg 1,4-DCB/kg bw (group E). For all groups, DCB was dissolved in 3.3 ml cornoil/kg bw. All groups of 15 animals were divided in 5 groups of 3 animals; these groups were weighed and sacrificed after 4 hours (groups A1-E1), 8 hours (groups A2-E2), 24 hours (groups A3-E3), 48 hours (groups A4-E4) or 72 hours (groups A5-E5). The animals were anaesthetized with a controlled flow of diethylether and sacrificed by exsanguination via the abdominal aorta. The blood was collected in glass tubes containing heparine. The bloodsamples were centrifuged immediately (15 min, 2000 g) and the plasma was removed and stored in glass tubes at -30 °C.

Histology

After exsanguination, livers and kidneys were removed, blotted dry and weighed. Of each kidney and liver one half was preserved in 4% buffered formaline (pH = 7.4) and the other half was stored at -80 °C. Sections of the preserved liver and kidneys were stained with hematoxylin and eosin for microscopic examination.

Biochemical assays

Plasma alanine amino transaminase activities (ALT) and plasma asparagine amino transaminase activities (AST) were measured employing a Cobas-Bio centrifugal analyzer and commercially available kits.

Determination of GSH and GSSG concentrations in liver and kidneys

Concentrations of GSH and GSSG in livers and kidneys were determined according the fluorometric method of Hissin and Hilf (1976). Of each liver and pair of kidneys (stored at -80 °C), ca 250 mg tissue was homogenized in 0.1 M sodium phosphate buffer (pH = 8), containing 5 mM EDTA, and 5% (w/v) HPO_3 (metaphosphoric acid). The homogenates were centrifuged for 45 min. at 105,000 g at 2 °C. The resulting supernatants were stored at -80 °C until assayed. For the GSH-assay, 50 µl of the supernatants was mixed with 950 µl of the phosphate-EDTA buffer. Of this mixture, 50 µl was mixed with 900 µl buffer and 50 µl of an o-phtalaldehyde (OPT) solution (1 mg/ml methanol). After thorough mixing and incubation at room temperature for 15 min, the fluorescence was determined at 420 nm with activation at 350 nm, using a Shimadzu fluorescence detector. GSH-concentrations were calculated using a GSH calibration curve. For the GSSG-assay, 100 µl of the supernatants was mixed with 40 µl 0.04 M N-ethylmaleimide (NEM) and incubated for 30 min at room temperature to interact with GSH present in the tissue. To this mixture, 860 µl 0.1 M NaOH was added. Of this mixture, 50 µl was mixed with 900 µl 0.1 M NaOH and 50 µl of the OPT-solution. After 15 min. at room temperature, the fluorescence was determined as with the GSH-assay. GSSG-concentrations were calculated using a GSSG calibration curve.

Gas Chromatography

The concentration of 1,2- and 1,4-dichlorobenzene (DCB) in plasma was determined using gas chromatography. Aliquots of plasma were extracted with 3 parts of cyclohexane, containing 1-bromohexane as internal standard. Extraction efficiency was determined by adding a known amount of 1,2- or 1,4-DCB to plasma; recovery after extraction was at least 95%. One microliter of the extracts was injected onto a Perkin Elmer 8500 Gas Chromatograph. Separations were accomplished on a DB-5 column (30 m, 0.25 x 0.25 µm). The carriergas was helium and the make up gas was nitrogen (20 ml/min). Starting temperature of the oven was 65 °C, and after 5 minutes increased by 5°C/min to 105 °C. Temperature of the injection port was 200 °C. Detection was accomplished with an electron capture detector (ECD). Concentrations of 1,2- or 1,4-DCB were calculated using calibration curves for both isomers.

Statistic analyses

Data were analyzed by an analysis of variance (ANOVA), followed by a two-sided Dunnett's test.

RESULTS

Relative liver and kidney weights

In Table 1 the organ/body weight ratios are presented for the different test groups, for the liver and the kidneys. Liver weights were significantly increased for the 250 mg/kg dose level of 1,2-DCB, after 48 and 72 hours. Exposure to 1,4-DCB did not result in an increased relative organ weight.

Histopathology

Treatment related histopathological changes were only detected in the liver of animals dosed with 250 mg/kg 1,2-DCB, at 24, 48 and 72 hours after administration of the compound. The histopathological changes observed were characterized by slight cytoplasmatic alterations in perivenous hepatocytes. In contrast with the periportal hepatocytes, the perivenous hepatocytes demonstrated homogeneous cytoplasm, accompanied by increased eosinophilia. These changes were generally not accompanied by evidence of degeneration or necrosis. The cytoplasmatic changes observed seemed to be slightly less pronounced after 48 and 72 hours than after 24 hours. No histopathological changes were observed in the kidneys, for both isomers.

Liver toxicity parameters

In Table 2, plasma alanine amino transaminase activities (ALT) and plasma asparagine amino transaminase activities (AST) are presented for the different test groups. Both liver toxicity parameters were significantly increased at 24 hours after administration of 250 mg/kg 1,2-DCB, and at 48 hours ALT was still increased. For the 50 mg/kg dose level of 1,2-DCB and for both dose levels of 1,4-DCB, no increase in ALT or AST activity was observed.

Table 1. Organ/body weight ratios at different time points after oral administration of 50 or 250 mg/kg BW 1,2- or 1,4-DCB, for the liver and the kidneys, expressed as g/kg BW. A = control group, B = 50 mg/kg 1,2-DCB, C = 250 mg/kg 1,2-DCB, D = 50 mg/kg 1,4-DCB, E = 250 mg/kg 1,4-DCB. Mean values and standard errors of the mean (sem) are presented (n = 3). Values which were significantly different from control rats (Anova and Dunnetts test, two-sided) are marked with * ($p<0.05$) or ** ($p<0.01$).

	4 hr		8 hr		24 hr		48 hr		72 hr	
	liver	*kidneys*	*liver*	*kidneys*	*liver*	*kidneys*	*liver*	*kidneys*	*liver*	*kidneys*
control	35.3 ± 0.32	7.19 ± 0.13	32.9 ± 0.99	7.55 ± 0.2	37.1 ± 0.5	7.6 ± 0.22	37.8 ± 0.9	7.38 ± 0.16	37.9 ± 0.6	7.22 ± 0.07
50 mg/kg 1,2	34.5 ± 0.61	7.26 ± 0.11	32.0 ± 0.82	7.15 ± 0.09	38.8 ± 2.1	7.73 ± 0.18	38.7 ± 0.3	7.44 ± 0.11	37.2 ± 0.9	7.43 ± 0.11
250 mg/kg 1,2	32.9 ± 0.12	7.36 ± 0.27	33.4 ± 0.55	7.59 ± 0.27	35.3 ± 0.3	7.37 ± 0.1	44.1** ± 1.3	7.53 ± 0.09	41.0* ± 0.5	7.29 ± 0.01
50 mg/kg 1,4	36.0 ± 0.61	7.05 ± 0.06	32.9 ± 0.18	7.49 ± 0.12	37.0 ± 0.8	7.29 ± 0.1	36.4 ± 0.5	7.55 ± 0.09	36.7 ± 0.6	7.08 ± 0.08
250 mg/kg 1,4	35.1 ± 0.76	7.07 ± 0.16	31.3 ± 0.24	7.58 ± 0.07	41.2 ± 1.3	8.0 ± 0.3	39.7 ± 0.5	7.68 ± 0.18	38.5 ± 0.4	7.29 ± 0.05

Table 2. Plasma alanine amino transaminase activities (ALT) and plasma asparagine amino transaminase activities (AST) at different time points after oral administration of 50 or 250 mg/kg BW 1,2- or 1,4-DCB, expressed as Units/liter. A = control group, B = 50 mg/kg 1,2DCB, C = 250 mg/kg 1,2-DCB, D = 50 mg/kg 1,4-DCB, E = 250 mg/kg 1,4-DCB. Mean values and standard deviations are presented (n = 3). Values which were significantly different from control rats (Anova and Dunnetts test, two-sided) are marked with * ($p<0.05$) or ** ($p<0.01$).

	4 hr		8 hr		24 hr		48 hr		72 hr	
	ALT	*AST*	*ALT*	*AST*	*ALT*	*AST*	*ALT*	*AST*	*ALT*	*AST*
control	40 ± 3	62 ± 2	34 ± 2	61 ± 4	39 ± 2	69 ± 6	40 ± 2	67 ± 3	47 ± 8	78 ± 14
50 mg/kg 1,2	36 ± 3	60 ± 2	43 ± 16	76 ± 18	44 ± 3	69 ± 7	51 ± 8	76 ± 6	40 ± 4	67 ± 5
250 mg/kg 1,2	40 ± 3	62 ± 2	40 ± 2	65 ± 5	137** ± 35	197** ± 35	79* ± 27	93 ± 27	42 ± 20	68 ± 6
50 mg/kg 1,4	38 ± 1	61 ± 2	34 ± 1	58 ± 2	42 ± 5	70 ± 6	40 ± 3	70 ± 2	40 ± 1	81 ± 12
250 mg/kg 1,4	38 ± 1	60 ± 3	32 ± 3	59 ± 6	44 ± 5	71 ± 2	43 ± 5	68 ± 7	43 ± 3	67 ± 7

Total GSH and GSSG in liver and kidneys

In Figure 1 the summed GSH and GSSG levels in the liver at different time points after administration of 50 or 250 mg/kg bw 1,2-DCB (A) or 1,4-DCB (B) are represented as percentage in relation to the control values (from animals receiving only cornoil = 100%). These control values were not constant during the twenty-four hours' period (see insert in Fig. 1). For 1,2-DCB, GSH was significantly depleted for both doses after 4, 8 and 24 hours (up to 54% depletion for the high dose level after 24 hours). An 'overshoot' of GSH was observed after 48 and 72 hours, particularly for the 250 mg/kg dose level. No depletion of GSH was observed in the kidneys (results not shown). For 1,4-DCB, the GSH concentration in the liver was slightly lower compared to the control animals after 24 and 72 hours, for the 50 mg/kg dose level only. No GSH 'overshoot' was observed. In the kidneys, no GSH depletion was observed (results not shown).

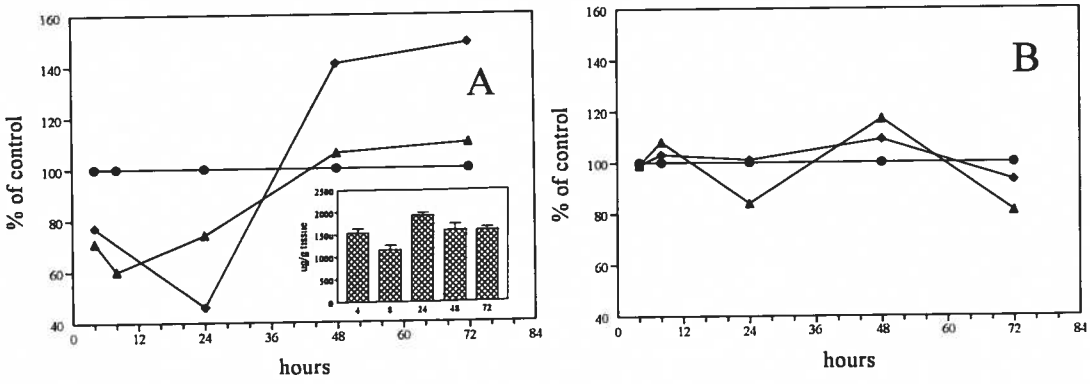

Fig.1. Total GSH and GSSG levels in the liver at different time points after administration of 50 mg/kg (triangles) or 250 mg/kg (diamonds, t=8 hours was not determined in fig.1A) 1,2-DCB (A) or 1,4-DCB (B). Data are represented as percentage of the control values (circles, from animals receiving cornoil), which were set at 100%. *n* = 3 animals. Insert shows control GSH concentrations in the liver, expressed as µg/g tissue.

Plasma concentrations of 1,2- and 1,4-DCB

In Figure 2 the plasma concentrations at different time points after administration of 50 or 250 mg/kg 1,2-DCB (A) or 1,4-DCB (B) are represented. In Table 3 the AUC (area under the plasma curve), clearance and elimination half life are represented for the two isomers and

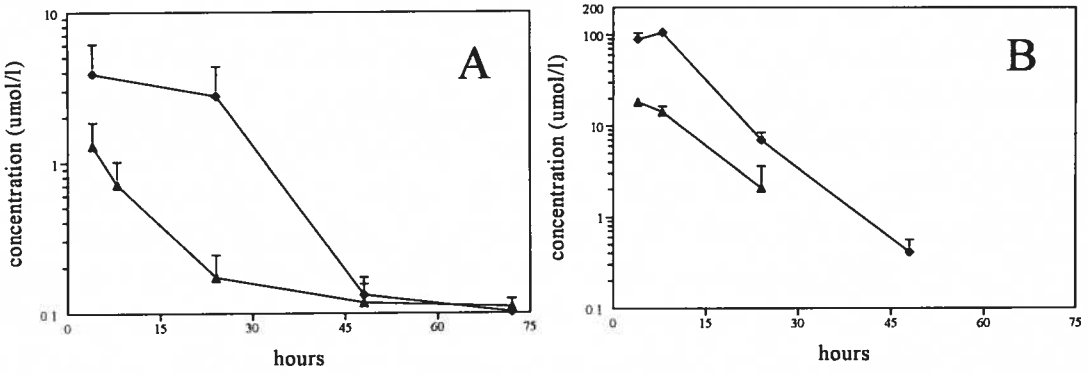

Fig.2. Plasma concentrations at different time points after administration of 50 mg/kg (triangles) or 250 mg/kg (diamonds) 1,2-DCB (A, t=8 hours was not determined for the high dose level) or 1,4-DCB (B). Mean values and standard deviations are represented (n = 3).

two dose levels. For the high dose level, the maximum concentration (Cmax) of 1,4-DCB was more than 20 times as high compared to 1,2-DCB. In addition, the AUC was much higher and the clearance much lower for 1,4-DCB, for both dose levels. For both isomers, the clearance was slightly lower for the 250 mg/kg dose level, indicating a saturation of metabolism.

Table 3. Area under the plasma curve (AUC), total clearance and elimination half life, calculated from plasma curves obtained after administration of 50 or 250 mg/kg 1,2- or 1,4-DCB (see also Figure 2). For 50 mg/kg 1,4-DCB, the AUC was calculated over 24 hours, for the other test groups over 48 hours.

	AUC (μmol.hr/l)	**Clearance (ml/min.kg)**	**half-life (hr)**
50 mg/kg 1,2-DCB	17.2	330.5	7.13
250 mg/kg 1,2-DCB	109.5	258.8	8.80
50 mg/kg 1,4-DCB	232.5	24.4	6.15
250 mg/kg 1,4-DCB	1561.0	18.2	5.04

DISCUSSION

In the present study, the time dependent toxicity and GSH depletion in the liver and kidneys of male Wistar rats, after oral administration of 50 or 250 mg/kg BW 1,2- or 1,4-dichlorobenzene (DCB), are described.

Liver toxicity was exclusively observed in rats treated with 250 mg/kg 1,2-DCB, as evidenced by increased plasma alanine amino transaminase- and asparagine amino transaminase activities, and cytoplasmatic alterations in perivenous hepatocytes. These changes were most pronounced at 24 hours after administration, for both enzyme activities and histopathological changes. In addition, maximum depletion of glutathione (GSH) coincided with the hepatic toxicity parameters, which indicates a correlation between hepatotoxicity and GSH depletion. These data are in agreement with the metabolic data, previously obtained in our laboratory, which showed a high formation of GSH-conjugates of the epoxides of 1,2-DCB, *in vivo* as well as *in vitro* (Hissink *et al.*, 1996-*a,b*). These epoxides also appeared to be highly reactive *in vitro*, so it is likely that with the 54% depletion of GSH observed at 24 hours after dosing with 250 mg/kg 1,2-DCB, epoxides are, at least partly, responsible for the hepatotoxicity observed. The 50 mg/kg dose level appeared not to elicit hepatotoxicity, and in accordance to this, depletion of GSH in the liver was less pronounced after 24 hours than for the high dose level. After 4 hours, GSH depletion was similar for both dose levels, indicating an equal conjugation with GSH, which, in turn, may be a consequence of saturated metabolism. This is in agreement with our *in vivo* kinetic study, which showed similar blood concentrations of metabolites in the first 6 hours after dosing of 50 and 250 mg/kg 1,2-DCB (Hissink *et al.*, 1996). In addition, this study showed a maximum concentration of metabolites at 24 hours after administration of the 250 mg/kg dose level, which coincides with the maximum toxicity observed at that time point. Stine and co-workers (1991) found also maximum plasma ALT activity 24 hours after ip administration of 2.7 mmol/kg 1,2-DCB. This toxicity corresponded with a maximum amount of covalent binding of radioactivity to hepatic proteins *in vivo*, 24 hours after dosing. In order to explain this delayed timepoint, they suggested accumulation of secondary metabolites, nonhepatic degradation of these metabolites and redistribution to the liver. Kimura *et al.* (1984) reported peak concentrations of dichlorophenylmethyl sulfoxide and methyl sulfone metabolites of 1,3-DCB in blood at 24 hours after dosing, which might indeed be further activated to reactive species in the liver. However, a saturation of metabolism, together with the depletion of GSH, could result in a delayed toxic response. A difference

between our study and that of Stine *et al.* is that with a comparable dose level, Stine *et al.* found a much higher plasma ALT activity. Most likely this is related to the rat strain used, since in their study a striking difference in susceptibility to 1,2-DCB mediated hepatotoxicity between Fischer-344 and Sprague-Dawley rats was observed, with the former eliciting a much higher liver injury. Fischer-344 rats would then also be much more susceptible than Wistar rats, the strain used in this study. In addition, Allis *et al.* (1992) found more pronounced degenerative changes in the liver of Fischer-344 rats at dose levels below 250 mg/kg, compared to the changes we observed. It has been proposed that the Fischer rat is more susceptible to 1,2-DCB induced toxicity due to a lower epoxide hydrolase activity, as reported by Glatt and Oesch (1987). Indeed we found less dihydrodiols (epoxides hydrolized by epoxide hydrolase) for Fischer rat microsomes compared to Wistar and Sprague Dawley rat microsomes, and under GSH depleted conditions this would result in a higher amount of epoxides (Hissink *et al.*, 1996-*b*). For 1,2-DCB, an 'overshoot' of GSH in the liver compared to control levels was observed at 48 and 72 hours after dosing. This overproduction of GSH, after being depleted in the liver, was also reported for 1,2,4-trichlorobenzene (Den Besten *et al.*, 1991), ethyl acrylate (Frederick *et al.*, 1992) and ethylene dichloride (D'Souza *et al.*, 1988). The extensive production of GSH we found, coincided with an increased relative liver weight at 48 and 72 hours after dosing, for the high dose level of 1,2-DCB only. This increase may be ascribed to regenerative processes in the liver.

In control animals, we found decreasing GSH levels during day time, which was also found by Stine *et al.* (1991), and which is in accordance with the circadian rhythm described by Schnell *et al.* (1983). In turn, this decrease in GSH levels coincided with decreasing relative liver weights for control animals during the day, varying from ca. 38 g/kg BW in the early morning to ca. 32 g/kg BW in the afternoon. This will be a consequence of the higher activity and food consumption of the rats during the night, which requires a higher capacity of the liver.

For 1,4-DCB, no treatment-related effects were found in the present acute toxcity study conducted with male Wistar rats, for both the liver and kidneys. This is in agreement with the results described by Loeser and Litchfield (1983), who found no treatment-related effects in long-term toxicity- and carcinogenicity studies conducted with the same rat strain. Previously, we compared the *in vitro* biotransformation of 1,4-DCB by hepatic microsomes from different rat strains, and significantly more glutathione conjugates of hydroquinones, the potential metabolites involved in 1,4-DCB-induced nephropathy in Fischer-344 rats, were

produced by the Fischer-344 rat liver microsomes compared to the Wistar rat microsomes (Hissink *et al.*, submitted-2). Furthermore, no depletion of GSH was found in the liver or the kidneys after administration of 1,4-DCB. This is consistent with our *in vivo* metabolism study, in which epoxide-derived mercapturic acids comprised only 10% of total urinary metabolites. In addition, epoxides were hardly responsible for covalent binding to hepatic protein in the *in vitro* study previously performed in our laboratory (Hissink *et al.*, submitted-1;2).

Treatment related histopathological changes, which occurred as from 24 hours after dosing with 250 mg/kg 1,2-DCB, were restricted to the perivenous region of the liver. This was also reported by Reid *et al.* (1973) and Allis *et al.* (1992). We found previously that reactive metabolites of 1,2-DCB, produced in hepatic microsomes from male Wistar rats, are most likely generated by cytochrome-P4502B1/2 isozymes, and possibly P4502E1 (Hissink et al., 1996-*b*). In various studies it has been described that the expression of these cytochrome-P450 isozymes is predominant in or even restricted to the perivenous area of the liver (Bühler *et al.* 1992; Oinonen *et al.*, 1993; White *et al.* 1993; Wolf *et al.* 1984), which corresponds with the occurrence of histopathological changes observed in this region of the liver after treatment with 1,2-DCB.

In conclusion, the results presented in this study are consistent with the metabolic data previously found in our laboratory. We showed that 1,2-DCB induced hepatotoxicity at the 250 mg/kg dose level, which correlated well with the depletion of GSH in the liver. These results correspond with the high formation of epoxide-derived mercapturic acids by male Wistar rats, and with the extensive covalent binding of epoxides to microsomal protein. However, large differences have been reported for 1,2-DCB-induced hepatotoxicity in different rat strains. 1,4-DCB did not induce hepatotoxicity or nephrotoxicity in male Wistar rats at the dose levels used, whereas this isomer has been shown to induce nephropathy in Fischer-344 rats. Fischer-344 rats have been shown to produce hydroquinones *in vivo*, whereas no such metabolites could be identified for the Wistar rat. This confirms the hypothesis that the nephropathy observed in Fischer-344 rats is a consequence of the formation of glutathione conjugates of quinones, and not of the binding to renal α_{2u}-globulin, a mechanism which is thought to be specific for male rats, independent of strain. For both isomers, it appears to be of importance to understand the underlying mechanism of toxicity in order to perform accurate risk assessment.

ACKNOWLEDGEMENT

The authors wish to thank Dr. M.V.W. Wijnands from the Department of General Toxicology for histopathological examination of the tissue samples. This work was supported, in part, by the Utrecht Toxicological Center (UTOX).

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CHAPTER 7

A PHYSIOLOGICALLY BASED PHARMACOKINETIC (PB-PK) MODEL FOR 1,2-DICHLOROBENZENE LINKED TO TWO POSSIBLE PARAMETERS OF TOXICITY

A.M. Hissink, B. Van Ommen, J. Krüse and P.J. Van Bladeren

ABSTRACT

A physiologically based pharmacokinetic (PB-PK) model was developed for 1,2-dichlorobenzene (1,2-DCB), for the rat. This model was then extended to man, using human *in vitro* parameters, including a V_{max} and K_m determined with human microsomes. For comparison, the V_{max} and K_m values from the rat were also scaled allometrically to the human. The model was used in two ways:

1). Acute hepatotoxicity was related to the amount of reactive metabolites (epoxides) formed *in vitro*: For rats, the hepatic concentration of epoxide metabolites *in vivo* was predicted from *in vitro* parameters and then related to a toxic dose level (250 mg/kg bw). Then the dose level was predicted, needed to obtain the same toxic liver concentration of reactive metabolites in man as in rat, assuming a concentration-effect relationship in the liver. It could be concluded that this concentration will not be reached, even after induction of the oxidation step, due to saturation of metabolism and a concomitant accumulation of 1,2-DCB in fat.

2). Hepatotoxicity was related to depletion of glutathione (GSH) in the liver: In the model, the consumption of hepatic GSH by metabolism (based on *in vivo* and *in vitro* data) and normal turn-over was described, not including increased resynthesis after depletion. *In vivo* validation was conducted by comparing the predictions of the model with the results of a GSH depletion study performed at two dose levels (50 and 250 mg/kg bw). Subsequently, the GSH consumption by 1,2-DCB metabolites was estimated for man using human *in vitro* metabolic

data. GSH turn-over in human liver was assumed to be the same as in rat. It appeared that at a dose level of 250 mg/kg, hepatic GSH was completely depleted after 10 hours for man, whereas for the rat a maximum depletion of 75% was predicted, after 15 hours. The presented model provides a quantitative tool for evaluating human risk for two different toxicity scenarios, namely covalent binding of reactive metabolites and depletion of GSH.

INTRODUCTION

Mathematical modeling of physiological systems to determine the kinetic behavior of compounds, commonly known as physiologically based pharmacokinetic (PB-PK) modeling, has been introduced several years ago in the field of risk assessment. For many, mainly volatile organic compounds, PB-PK models have been developed (Ramsey and Andersen, 1984; Medinsky *et al.*, 1989; Corley *et al.*, 1990; Reitz *et al.*, 1996). PB-PK modeling provides a means of estimating the tissue doses of chemicals and their metabolites over wide ranges of exposure conditions in different animal species. It can thus also be used to predict effects in man, if the tissue dose of parent compound or metabolite has a clear-cut quantitative relationship with the effect. To illustrate this possibility, in the present paper, a PB-PK model is developed for 1,2-dichlorobenzene (1,2-DCB) in rat and man, which is subsequently extended to predict hepatotoxicity and depletion of hepatic glutathione.

1,2-DCB is used as a chemical intermediate in the synthesis of pesticides and other chlorinated compounds, as a solvent and as deodorizer. Environmental contamination may lead to human exposure via inhalation and drinking water (Oliver and Nicol, 1982; U.S. EPA, 1985). 1,2-DCB is a potent hepatotoxicant (Cameron *et al.*, 1937; Hollingsworth *et al.*, 1958; Robinson *et al.*, 1991; Stine *et al.*, 1991). The hepatotoxicity of 1,2-DCB is thought to be mediated by the generation of reactive metabolites, presumably epoxides, as a result of their oxidation by cytochrome P450 (Hissink *et al.*, 1996*a,b*). Induction of these cytochrome-P450 enzymes enhances toxicity (Stine *et al.*, 1991; Gunawardhana *et al.*, 1993; Valentovic *et al.*, 1993). The ability of 1,2-DCB to deplete glutathione (GSH) and the enhanced toxicity of 1,2-DCB after GSH depletion has been reported by several investigators (Stine *et al.*, 1991; Reid, 1972; Den Besten *et al.*, 1991). Therefore, 1,2-DCB-induced hepatotoxicity may be a combined effect of reactive metabolites covalently binding to macromolecular proteins and oxidative stress as a consequence of depletion of GSH.

Thus, a physiologically based pharmacokinetic model for 1,2-DCB is developed for rat and man, which can be used to predict the concentration of reactive metabolites in the liver as well as the hepatic depletion of GSH, possible determinants for acute hepatotoxicity. The manner in which such a model can be used to extrapolate toxicity information from animal to human for risk assessment is illustrated.

MATERIALS AND METHODS

Materials

Microsomes of human livers (a pool of 5 individuals) were obtained from Human Biologics, Inc. (Phoenix, AZ, USA). 1,2-Dichloro-[^{14}C]-benzene with a radiochemical purity of >98% and a specific activity of 244 MBq/mmol was obtained from Sigma Chemical Co., St. Louis, MO, USA.

Determination of V_{max} and K_m values in vitro

The Michaelis-Menten parameters V_{max} and K_m of the saturable P450-epoxidation pathway for rat and man were determined using hepatic microsomes. For the human microsomes, a pool of samples of 5 individuals was used. The liver microsomes of male Wistar rats were prepared as described elsewhere (Hissink *et al.*, 1996-b). All incubations were performed in duplicate, with a variation in conversion of less than 10%. Briefly, incubation mixtures contained 0.1 M potassium phosphate buffer (pH 7.4), 3 mM $MgCl_2$, 3 mM NADPH, 1 mM ascorbic acid and hepatic microsomes at a protein concentration of 0.5 mg/ml, in a final volume of 400 µl. Substrate concentrations were varied, in a range of 5 µM - 300 µM. A stock solution of 1,2-^{14}C-DCB was prepared in acetone and BSA, with a final concentration of acetone in the incubation mixtures of 0.05%, which was shown not to inhibit CYP2E1 activity towards 1,2-DCB to a detectable extent (Bogaards *et al.*, 1995). The methods of incubation, measuring covalently bound radioactivity to microsomal protein and ^{14}C-HPLC analysis of the incubation mixtures are described elsewhere (Hissink *et al.*, 1996-b). To determine total product formation (nmol product/min/mg protein), all metabolites eluting from the HPLC-column and the radioactivity (1,2-DCB equivalents) bound to microsomal protein (corrected for incubations without NADPH) were summed. (For identification of the metabolites, we refer to Hissink *et al.*, 1996-b). V_{max} and K_m values were calculated using the EZ-FIT enzyme

kinetics program (Perella, 1988), and are shown in Table 1. The rat parameters were incorporated in the model, in order to examine their potential to describe the *in vivo* data.

PB-PK model development.

The basic structure of the PB-PK model is similar to that developed by Ramsey and Andersen (1984) for styrene, utilizing a lumped-tissue group approach. Compartments are the "rapidly perfused" tissue group which comprises such tissues as the lung, kidneys and spleen; the "slowly perfused" tissue group, comprising muscle and skin, and a fat compartment. Metabolism was assumed to take place solely in the liver, the fourth compartment. To adapt this inhalation model for oral exposure (three dose levels, 5, 50 and 250 mg/kg bw), uptake of 1,2-DCB from the gastro-intestinal tract was simulated as a first-order process, depositing 1,2-DCB directly into the liver compartment. The first-order constants used for the three dose levels are shown in Table 1. The first-order constant used to describe the uptake of 1,2-DCB was dose dependent. This dose dependency of first-order uptake rate constants from the gastro-intestinal tract has also been reported by Frederick and co-workers (1992).

Partition coefficients

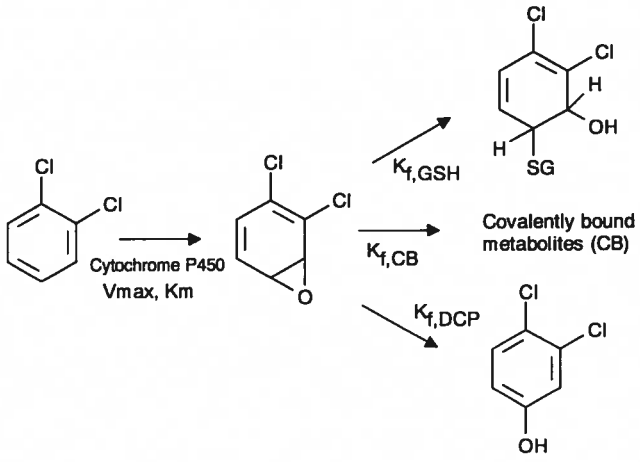
Partition coefficients (PC) were calculated according to the equations proposed by Droz *et al.* (1989), using the water/air (PC_{water} = 9.0), oil/air (PC_{oil} = 39920) and blood/air (PC_{blood} = 423) partition coefficients for 1,2-DCB calculated by Fiserova-Bergerova (1983). This method has also been applied by Kumagai and Matsunaga (1995), for a PB-PK model for chlorobenzene. Tissue/blood partition coefficients were obtained by dividing the tissue/air partition coefficients by the blood/air partition coefficient. The partition coefficients are shown in Table 1.

Physiologic parameters

Values for organ volumes and blood flows were taken from Gargas *et al.* (1986) and are shown in Table 1. Human organ volumes were taken from Andersen *et al.* (1987). Values for cardiac output (QC) and ventilation rate (QP) were scaled to the body weights of rat and man according to the equation QC or QP = 15 liters/hr $(BW)^{0.74}$.

Modeling 1,2-DCB metabolism

Metabolism of 1,2-DCB is described by a saturable P450-mediated oxidation, which results in the formation of an epoxide. This epoxide may either be 1) converted into dichlorophenol (DCP), 2) result in covalent binding (CB) or 3) conjugated with GSH (Scheme 1).



Scheme 1. Simplified metabolic scheme of 1,2-dichlorobenzene (1,2-DCB), representing the saturable oxidation by cytochrome P450 into an epoxide, and the subsequent formation of dichlorophenol (described with $K_{f,DCP}$), covalently bound metabolites ($K_{f,CB}$), and conjugates of glutathione ($K_{f,GSH}$). The three different routes are described as first-order reactions in the PB-PK model. Of the epoxide, DCP and GSH conjugate, only one of the possible isomers is represented.

These three pathways are assumed to independently follow (pseudo) first-order kinetics. This seems appropriate, since the epoxide will be converted almost instantaneously into either of the three metabolites, and the three routes will presumably not be saturated because of the saturable formation of the epoxide. Moreover, it was shown *in vivo* that both the GSH- and phenolroute were not saturated up to a dose level of 250 mg/kg (Hissink *et al.*, 1996-a). The ratio of the three routes thus is taken to be constant and was estimated as follows. The rate constants for the formation of reactive metabolites ($K_{f,CB}$) were derived from an *in vitro* study, previously performed in our laboratory (Hissink *et al.*, 1996-b), since no *in vivo* data were available. For rat microsomes, 5% of total metabolites formed was covalently bound to protein. This value was obtained in the presence of GSH and glutathione S-transferases, since this resembles most closely the *in vivo* situation, and was independent of substrate concentration. The relative amounts of DCP and GSH conjugates observed *in vitro* (25 and 70%) were quite similar to the relative amounts observed *in vivo* (30 and 60%). Therefore, the

ratio used for the three pathways was assumed to be 5:30:65. In order to simulate virtually instantaneous reactions while maintaining this ratio, the first-order rate constant for formation of reactive metabolites ($K_{f,CB}$) was arbitrarily set to 50 hr^{-1}, the rate constant for formation of DCP ($K_{f,DCP}$) was set to 300 hr^{-1}, and the rate constant for formation of GSH conjugates ($K_{f,GSH}$) was set to 650 hr^{-1}. For man, no *in vivo* data were available, and therefore the three rate constants were derived from *in vitro* data. The value for $K_{f,CB}$ was estimated from a similar *in vitro* study as was used for the rat, and was set to 5 hr^{-1} (corresponding with 0.5% covalent binding in the presence of GSH and glutathione S-transferases). The amount of GSH conjugates of epoxides formed *in vitro* was identical for rat and man, and therefore $K_{f,GSH}$ was set to 650 hr^{-1}. The *in vitro* formation of DCP was somewhat higher for man compared to rat (30 resp. 25%), and $K_{f,DCP}$ was set to 360 hr^{-1} (30/25*300). The first-order rate constants used in the rat and human model are shown in Table 1.

Modeling GSH turnover

The model for GSH turnover and consumption of GSH by metabolism is rather similar to that reported for GSH depletion by ethyl acrylate (Frederick *et al.*, 1992). The value for the rate of GSH turnover in the liver was abstracted from Potter and Tran (1993), and the rate of GSH synthesis was then calculated, since the concentration of GSH at steady state is determined by the ratio of the rate of synthesis to the rate of turnover. The increased rate of GSH synthesis after GSH depletion can in principle be estimated from the increased GSH liver concentrations observed *in vivo*, by fitting these data using the model. However, this phenomenon was not taken into account since we were interested in acute effects. The constants used to describe the GSH depletion are shown in Table 1. The value for GSH turnover was assumed to be equal for rat and man.

Mathematical construction of the model

Mass-balance differential equations were written for the various compartments in the model (see Appendix). Briefly, for the non-metabolizing compartments differential equations were written to describe the influx, solubility, and efflux of 1,2-DCB. The equations for the liver compartment also accounted for 1,2-DCB metabolism and GSH synthesis, turnover and consumption. These equations were solved simultaneously by numerical integration, using Gear's algorithm for stiff systems with the ACSL[1] computer program.

[1] Advanced Continuous Simulation Language, Mitchell and Gauthier, Concord, MA.

In vivo data

The rat model was used to describe blood concentration time curves obtained after oral dosing of three dose levels of 1,2-DCB (5, 50 and 250 mg/kg bw, n = 3), which was performed previously in our laboratory (Hissink *et al.*, 1996-a). The concentration of parent chemical as well as the concentration of total metabolites was predicted. The model was also validated with *in vivo* GSH depletion data, obtained from a study performed at two oral dose levels (50 and 250 mg/kg bw, n = 3), which is described elsewhere (Hissink *et al.*, submitted).

Table 1. Parameters used in the PB-PK model for 1,2-DCB.

Rat		Human	
Physiologic parameters			
Body weight			
0.258 kg		70 kg	
Percentages of body weight[a]			
Liver	4	Liver	3.14
Fat	7	Fat	23.1
Rapidly perfused	5	Rapidly perfused	2.66
Slowly perfused	75	Slowly perfused	62.1
Flows[b]			
Cardiac output (QC)	5.50 (l/hr)	Cardiac output	348.0 (l/hr)
Alveolar ventilation (QP)	5.50 (l/hr)	Alveolar ventilation	348.0 (l/hr)
Percentages of cardiac output[a]			
Liver	25	Liver	25
Fat	9	Fat	9
Rapidly perfused	51	Rapidly perfused	51
Slowly perfused	15	Slowly perfused	15

Table 1. (continued)

	Rat		Human
Partition coefficients[c]			
Blood:air	423	Blood:air	423
Liver:blood	2.7	Liver:blood	2.7
Fat:blood	66.4	Fat:blood	66.4
Rapidly perfused:blood	2.7	Rapidly perfused:blood	2.7
Slowly perfused:blood	1.3	Slowly perfused:blood	1.3
Biochemical parameters			
1,2-DCB oxidation			
V_{max}	0.142 nmol/min.mg (4.3 μmol/hr)[d]	V_{max}	0.27 nmol/min.mg (2742 μmol/hr)[d]
K_m	4.8 μM	K_m	7.5 μM
$V_{max,fit}$	17 μmol/hr[e]	$V_{max,h}$ (scaled)[f]	10,840 μmol/hr
		$V_{max,r}$ (scaled)[g]	859 μmol/hr
GSH conjugation of epoxide[h]			
$K_{f,GSH}$	650 hr^{-1}	$K_{f,GSH}$	650 hr^{-1}
Formation of dichlorophenol[h]			
$K_{f,DCP}$	300 hr^{-1}	$K_{f,DCP}$	360 hr^{-1}
Formation of reactive metabolies[h]			
$K_{f,CB}$	50 hr^{-1}	$K_{f,CB}$	5 hr^{-1}
GSH turnover rate[i]			
K_{to}	0.14 hr^{-1}	K_{to}	0.14 hr^{-1}
Absorption rate constants[j]			
K_a 5 mg/kg	0.5 hr^{-1}		
K_a 50 mg/kg	0.18 hr^{-1}		
K_a 250 mg/kg	0.06 hr^{-1}	K_a 250 mg/kg	0.06 hr^{-1}

[a] Organ volumes and blood flows were taken from Gargas *et al.*, 1986. Human organ volumes were taken from Andersen *et al.*, 1987.
[b] Values for QC and QP were scaled to the body weights of rat and man according to the equation QC or QP = 15 liters/hr $(BW)^{0.74}$.
[c] PC for liver and rapidly perfused tissues was calculated according to PC = $0.0281*PC_{oil}$ + $1.12*PC_{water}$; PC for fat was calculated according to PC = $0.7*PC_{oil}$ + $0.3*PC_{blood}$; PC for slowly perfused tissues was calculated according to PC = $0.0133*PC_{oil}$ + $1.36*PC_{water}$. The calculated PC values were divided by the blood/air PC, to determine the tissue/blood partition coefficients, which are shown in Table 1.
[d] V_{max}: maximum rate of biotransformation by the microsomes. Values between parentheses are scaled values used in the PB-PK model, based on a scaling factor of 45 mg microsomal protein per g liver for the rat (Houston, 1994), and 77 mg microsomal protein per g liver for man (Bäärnhielm *et al.*, 1986).
[e] $V_{max,fit}$, $K_{m,fit}$: Best fitted parameters for the rat model.
[f] $V_{max,h}$: Scaled from the human *in vitro* parameter, using the scaling factor 3.95 which was derived from the rat *in vitro* and best fitted parameter.
[g] $V_{max,r}$: Scaled from the best fitted parameter for the rat, based on: $V_{max} = V_{max,c}*BW^{**0.7}$.
[h] First-order rate constants for formation of glutathione conjugates (GSH), dichlorophenol (DCP) and covalently bound metabolites (CB).
[i] GSH turnover rate was abstracted from Potter and Tran, 1993.
[j] Absorption rate constants were estimated by fitting the parameters to the experimental data, obtained with three dose levels.

RESULTS

In vitro determined V_{max} and K_m values

In Figure 1, the activity towards 1,2-DCB of rat and human liver microsomes is shown. The Michaelis-Menten parameters V_{max} and K_m calculated from the presented curves were 0.14 nmol/mg protein/min. and 4.8 μM for the rat, and 0.27 nmol/mg/min. and 7.5 μM for the human microsomes (Table 1).

In vitro - in vivo scaling for the rat

In Figure 2, simulated blood concentrations of parent 1,2-DCB and total amount of metabolites are shown for a 5 mg/kg (A), 50 mg/kg (B) and a 250 mg/kg oral dose level (C), for the male Wistar rat. Experimental data are presented in the same figures. In order to obtain adequate fits for all three dose levels, a K_m of 4.8 μM and a V_{max} of 17 μmol/hr were used. This V_{max} value of 17 μmol/hr is a factor 4 higher than the (scaled) *in vitro* determined V_{max} (see Table 1), which is based on a scaling factor of 45 mg microsomal protein per g liver (Houston, 1994). Apparently, only taking into account liver metabolism is not sufficient to describe total metabolism in the rat, and the factor of 4 is necessary to include e.g. extrahepatic metabolism. The *in vitro* determined K_m (4.8 μM) appeared to be adequate to describe the *in vivo* data.

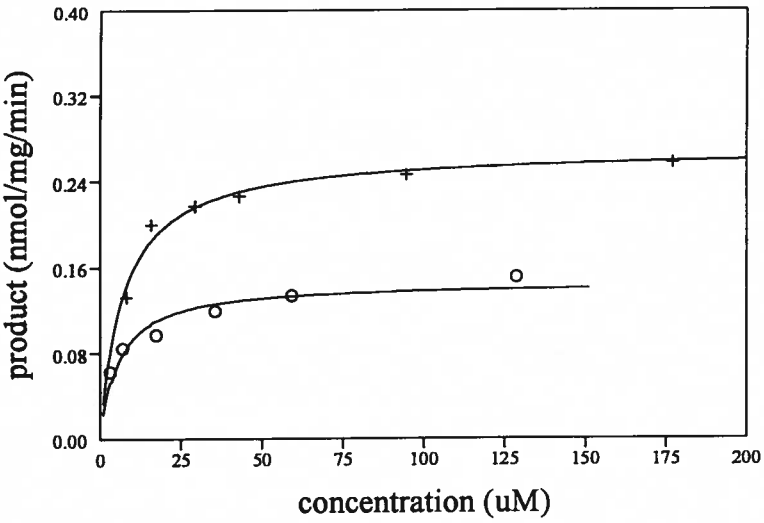


Fig.1. Activity towards 1,2-DCB of rat (O) and human (+) microsomes. Incubation conditions are described in Materials and Methods. The Michaelis-Menten parameters V_{max} and K_m were determined using the EZ-FIT program. 'Product' is the sum of covalently bound metabolites, dichlorophenol and dihydrodiol.

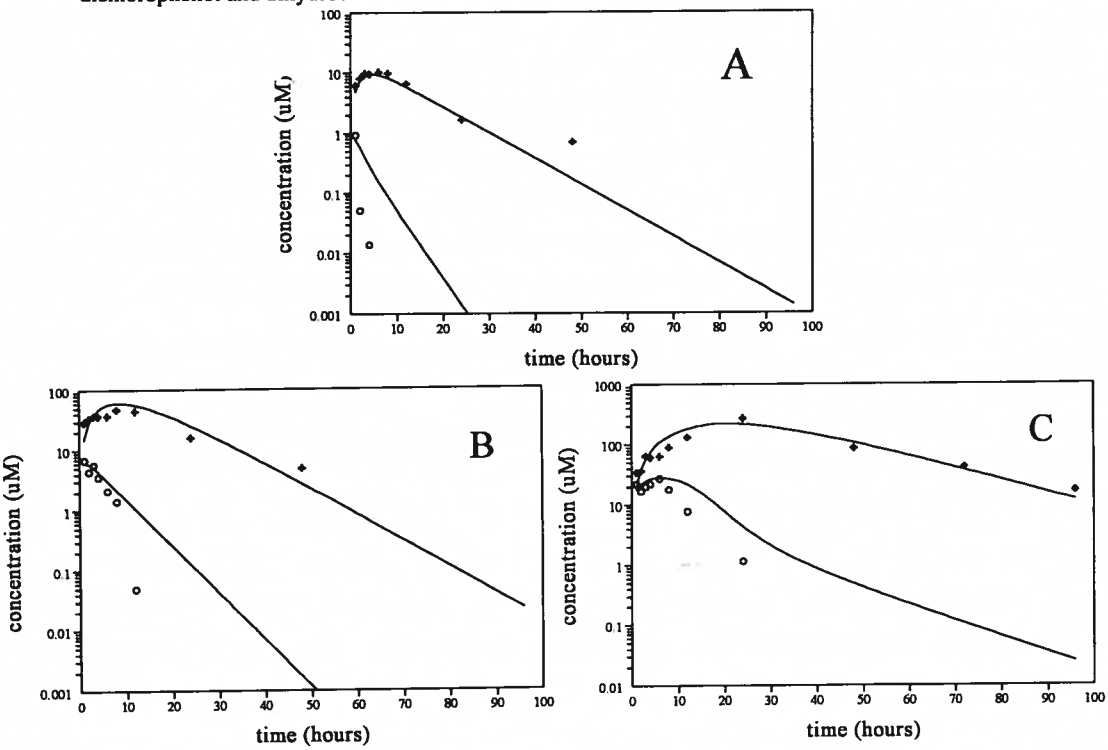


Fig.2. Simulated blood concentrations of parent 1,2-dichlorobenzene and total amount of metabolites following an oral dose of 5 (A), 50 (B) or 250 (C) mg/kg bw (see text). Experimental data (n = 3), obtained with male Wistar rats, are presented in the same figures, for parent chemical (O) and total metabolites (+).

Since the covalently bound metabolites (CB) were the products of interest, the hepatic concentration of these metabolites was predicted. In Figure 3A, the predicted concentration of covalently bound metabolites in the rat liver following an oral dose of 250 mg/kg bw 1,2-DCB (a dose level which elicits acute hepatotoxicity, Hissink *et al.*, submitted), is shown. Since hepatotoxicity has been shown to be most pronounced at 24 hours after dosing, this time point was chosen as the 'effect' time point. The liver concentration of covalently bound metabolites at 24 hours was 1459 μM. In Figure 4A, the predicted glutathione concentration in the rat liver is represented, together with experimental data, after an oral dose of 50 or 250 mg/kg bw. Maximum predicted depletion of glutathione (75%) after a toxic dose level of 250 mg/kg bw 1,2-DCB was reached at 15 hours after dosing.

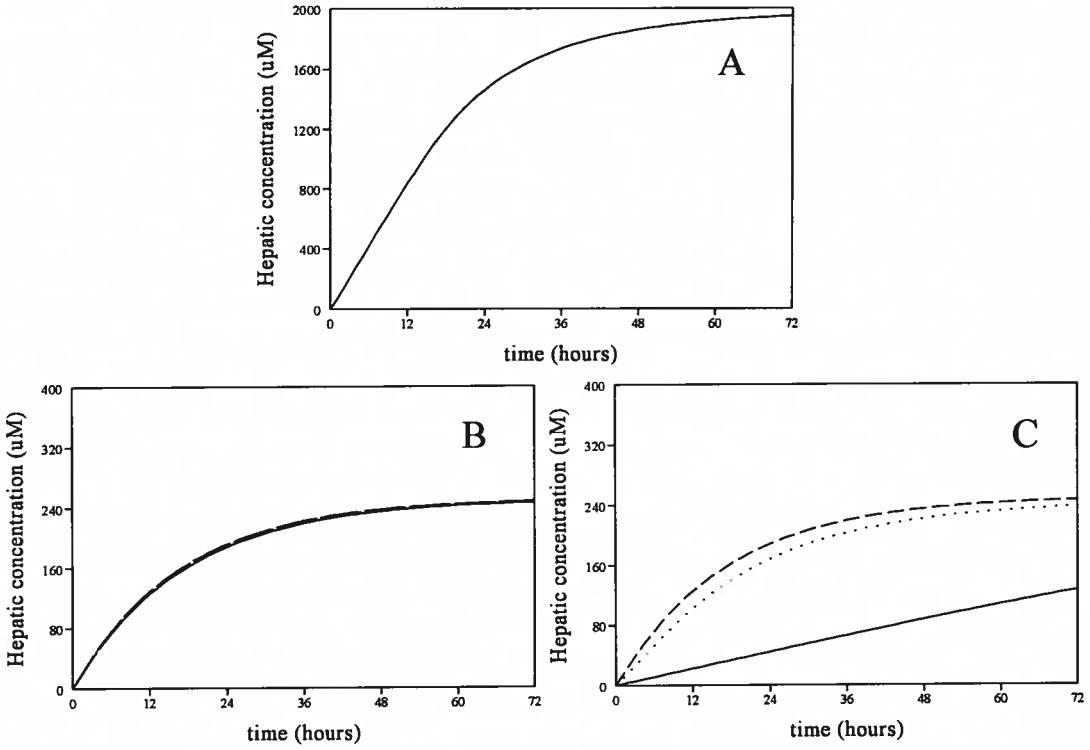


Fig.3. A. Predicted concentration in the rat liver of covalently bound metabolites following an oral dose of 250 mg/kg bw 1,2-DCB. The concentration was calculated using the K_m of 4.8 μM and V_{max} of 17 μmol/hr. The concentration at 24 hours was 1459 μM. B. Predicted concentration of covalently bound metabolites in the human liver following an oral dose of 250 mg/kg bw 1,2-DCB. The concentration was calculated using the human scaled *in vitro* V_{max}. In addition, concentrations are shown after increasing V_{max} with a factor 5 or 10, to account for possible interindividual differences. Curves are nearly identical. C. Predicted concentration of covalently bound metabolites in the human liver following an oral dose of 250 mg/kg bw 1,2-DCB. The concentration was calculated using the allometrically scaled parameters (solid line). In addition, concentrations are shown after increasing V_{max} with a factor 5 (dotted line) or 10 (stripes), to account for possible interindividual differences.

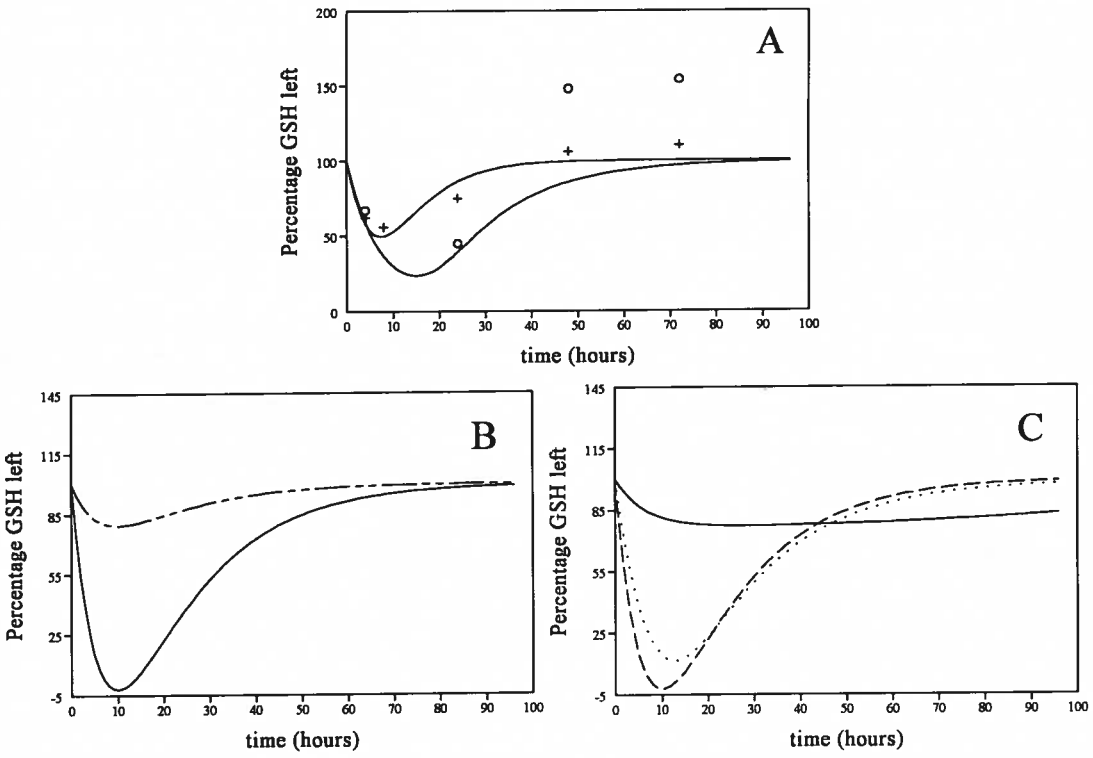


Fig.4. A. Predicted concentration of glutathione (GSH) in the rat liver after an oral dose of 50 (O) or 250 (+) mg/kg bw 1,2-DCB. The symbols represent experimental data (mean of n = 3). The concentrations are represented as percentage left of the native GSH level, 6.5 mM. B. Predicted concentration of GSH in the human liver (represented as percentage left) following an oral dose of 50 (dotted line) or 250 (solid line) mg/kg bw 1,2-DCB. V_{max} is 10,840 μmol/hr and K_m is 7.5 μM. C. Predicted concentration of GSH in the human liver (represented as percentage left) following an oral dose of 250 mg/kg bw 1,2-DCB. The allometrically scaled parameters were used (solid line). In addition, the effect of increasing the V_{max} with a factor 5 (dots) or 10 (stripes), is shown.

In vitro - in vivo scaling for human

The PB-PK model for the rat was scaled to the human by correcting for known physiological differences between the rat and human. To describe the P450-mediated oxidation, the human Michaelis-Menten parameters determined *in vitro* were used, and V_{max} was scaled to whole liver based on a microsomal protein content of 77 mg protein per g liver (Bäärnhielm *et al.*, 1986). In addition, to account for the *in vitro - in vivo* difference which was observed for the V_{max} of the rat, the same scaling factor was applied as for the rat (4), resulting in a V_{max} of 10,840 μmol/hr (see Table 1). The time dependent blood concentration of 1,2-DCB following an oral dose of 5, 50 or 250 mg/kg bw was simulated (Figure 5). In addition, in Figure 3B the predicted liver concentration of covalently bound metabolites is shown, for the

highest dose level. To account for interindividual differences (the expression level of P4502E1, the major human isoenzyme involved in oxidation of 1,2-DCB, may vary with at least a factor 7, Bogaards *et al.*, 1995), the values for V_{max} were increased with a factor 5 or 10. The resulting concentrations of reactive metabolites are also displayed in Figure 3B, however they were nearly identical. In order to examine at which dose level the 'critical' concentration of 1459 μM covalently bound metabolites would be reached, the model was run at different dose levels. However, it was not possible to achieve this concentration at 'acute' time points, even after induced oxidation (10x V_{max}), since the saturable oxidation and the relatively high fat content of human resulted in an accumulation of 1,2-DCB in fat.

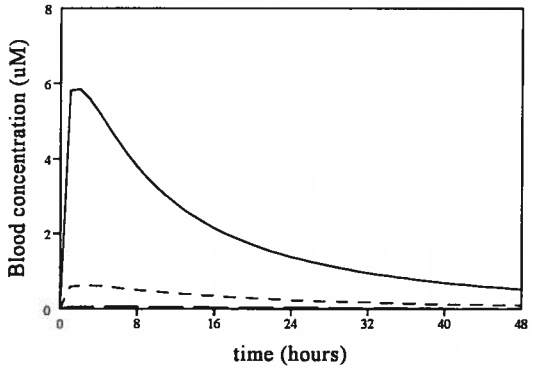


Fig.5. Predicted time dependent blood concentration of 1,2-DCB following an oral dose of 5 (stripes), 50 (dots) or 250 (solid) mg/kg bw for man, using the scaled human *in vitro* V_{max} of 10,840 μmol/hr. The K_m was 7.5 μM.

In Figure 4B, the predicted glutathione concentration in the human liver is shown, after an oral dose level of 50 or 250 mg/kg bw 1,2-DCB. The 'native' GSH concentration was assumed to be the same as in the rat liver, namely 6.5 mM. For the high dose level, GSH was completely depleted after 10 hours, whereas for the rat a maximum depletion of 75% was reached after 15 hours (Figure 4A). Increasing the human V_{max} with a factor 5 or 10 resulted in equal curves as shown in Figure 4B.

Allometric scaling from rat to man

To compare the method of *in vitro - in vivo* scaling with the 'classic' method of allometric scaling from rat to man, the V_{max} of the rat model was scaled to the human situation

based on allometric scaling principles ($V_{max} = V_{max,c}$*BW$^{**0.7}$, Dedrick *et al.*, 1973), which resulted in a V_{max} of 859 μmol/hr (Table 1). The K_m value of 4.8 was used. In Figure 3C, the resulting hepatic concentration of covalently bound metabolites is shown. In the same figure, the effect of increasing the allometrically scaled V_{max} with a factor 5 or 10, is presented. For the 'normal' V_{max}, the concentration of reactive metabolites is underestimated compared to the *in vitro - in vivo* scaling method (Figure 3B). For the higher V_{max} values, curves are rather similar. Figure 4C shows the GSH depletion in the human liver, using the allometrically scaled parameters, also for increased V_{max} values. As for the covalently bound metabolites, GSH depletion is underestimated compared with the *in vitro - in vivo* scaling method (Figure 4B), at the normal value for V_{max}.

DISCUSSION

A physiologically based pharmacokinetic (PB-PK) model for 1,2-dichlorobenzene (1,2-DCB) was developed for the rat. An attempt was made to extend this model to the human situation, adjusting the appropriate biochemical and physiological parameters.

Generally, biochemical parameters in PB-PK models to describe metabolism of volatile organic compounds in rodents are derived from *in vivo* gas uptake studies (Andersen *et al.*, 1987; Gargas *et al.*, 1986; Vinegar *et al.*, 1994). To describe metabolism in humans, usually these parameters are scaled using allometric relationships according to Dedrick (1973). However, this method does not account for mechanistic differences in biotransformation between rodent and man. For instance different isoenzymes of the P450 family may be involved in oxidation of a chemical, possibly resulting in different metabolites with different toxicity. Therefore, the use of *in vitro* parameters would be convenient, since in this way differences between animal and man may be more accurately described. The use of *in vitro* kinetic parameters to describe metabolism in a PB-PK model has successfully been applied for furan by Kedderis *et al.* (1993), who used rat hepatocytes to determine V_{max} and K_m. Andersen *et al.* (1987) used kinetic constants determined *in vitro* in liver and lung of different species, including man, in order to distinguish between these organs with respect to their relative contribution to total metabolism. We incorporated the Michaelis-Menten constants V_{max} and K_m, measured in rat liver microsomes, in our model to describe oxidation of 1,2-DCB. The *in vitro* determined K_m could very well be used to describe the *in vivo* data. The V_{max} appeared to

be too small to adequately describe the blood concentration of 1,2-DCB after exposure to three different dose levels of the compound. The best fitted V_{max} was a factor 4 higher compared to the *in vitro* determined V_{max}. This difference may be explained by the fact that the liver is not the only organ involved in 1,2-DCB metabolism, and that a factor of 4 is necessary to describe 'whole body' metabolism. However, a difference of a factor 4 between measured and best fitted V_{max} is relatively small and thus promising with respect to the use of *in vitro* determined parameters in PB-PK modeling.

Using the fitted V_{max}, depletion of GSH in the rat liver was also predicted very well. It is also noteworthy that due to the easily saturable oxidation of 1,2-DCB, maximum depletion of GSH appears relatively late (15 hours after dosing), which also holds for the maximum concentration of metabolites in the blood. In agreement with this last observation, it was observed that the maximum toxic response to 1,2-DCB occurred from 24 hours after dosing (Hissink *et al.*, submitted).

For man, no *in vivo* data were available which could be used to validate the *in vitro* obtained parameters. We assumed that the same *in vitro - in vivo* scaling factor was necessary for the human V_{max} as for the rat V_{max}. It appeared that in the human liver an acute effect concentration of covalently bound metabolites, as predicted in rat, could not be reached. The human model predicted more accumulation of 1,2-DCB in fat compared to rat, due to a higher fat content in man. In accordance to this, 1,2-DCB has been found in 70% of samples of breast milk from a national survey of 210 Canadian women (Mes *et al.*, 1986).

In order to study the effect of interindividual differences in enzyme (P4502E1) expression, the V_{max} was increased up to a factor 10 for the human model. However, even after increased oxidation, the 'critical' concentration of covalently bound metabolites, as determined in rat, was not attained.

The time-dependent depletion of GSH in the liver was predicted to be more extensive compared to the rat, with a complete depletion at 10 hours after exposure. Since the total conjugation of epoxides with GSH is similar for rat and human (Hissink *et al.*, 1996-b), this will be a consequence of a more rapid initial oxidation, which results in a faster exhaustion of the GSH supply. It has been reported that for the liver, 80-90% depletion of GSH can induce peroxidative damage (Biaglow *et al.*, 1986).

Comparing the method of *in vitro - in vivo* scaling with the method of allometric scaling in the extrapolation process of rat to man, it is clear that the allometric scaling method results in an underestimation of covalent binding as well as GSH depletion. Since for both

methods the concentration of reactive metabolites remains well below the critical effect concentration, even after induced oxidation, this has no consequences for the conclusion. However, with respect to depletion of GSH, the allometric scaling method might lead to a considerable underestimation of risk, emphasizing the advantage of the use of human *in vitro* parameters.

In conclusion, in the present study two parameters are discussed which both may play a role in 1,2-DCB-induced acute hepatotoxicity in male Wistar rats. In the worst case, when depletion of GSH would be the only factor involved in 1,2-DCB-induced acute hepatotoxicity, risk would appear to be higher for man than for the rat, at a same dose level. However, it has been reported that 40% depletion of GSH has no hepatotoxic effects, and in another study it was shown that 80-90% depletion in the liver is necessary to induce peroxidative damage (Yeung *et al.*, 1994; Biaglow *et al.*, 1986). Hence, it is likely that for the rat, covalent binding to hepatic proteins, mediated by reactive epoxides, or a combination effect of covalent binding and GSH depletion, is a much more significant determinant in hepatotoxicity. Since male Wistar rat liver microsomes produced much more reactive metabolites than human microsomes (most likely due to the involvement of different P450 isoenzymes, Hissink *et al.*, 1996-b), resulting in a predicted liver concentration of these metabolites which could not be achieved for man, it may be concluded that with respect to covalent binding of reactive metabolites, man is possibly less susceptible to 1,2-DCB-induced acute hepatotoxicity than rat. Moreover, in the present study the role of the glutathione S-transferases has not been taken into account, their effect is included in the covalent binding results. However, previous work suggests that man is more effective in detoxifying epoxides than the rat (Hissink *et al.*, 1996-b).

In conclusion, differences in enzyme kinetics between species can easily be evaluated using PB-PK models, and extending such models to include parameters for toxic effects may provide a valuable tool for more accurately addressing the process of risk assessment.

APPENDIX

Mass balance equations

Mass balance differential equations for influx and efflux in noneliminating compartments were similar to those for methylene chloride (Andersen *et al.*, 1987). First-order uptake from the gastro-intestinal tract is described by:

$$dDOSE/dt = -K_a * DOSE$$

where

K_a	First-order rate constant for the transport of 1,2-DCB from the gastrointestinal tract into the liver (hr^{-1}).
DOSE	Amount of 1,2-DCB in the gastrointestinal tract (μmol).

Differential equations describing 1,2-DCB metabolism:

$$dAM/dt = V_{max} * CVL/(K_m + CVL)$$
$$dAMM/dt = dAM/dt - dACB/dt - dADCP/dt - dAGSH/dt$$
$$dACB/dt = K_{f,CB} * CMM * VL$$
$$dADCP/dt = K_{f,DCP} * CMM * VL$$
$$dAGSH/dt = K_{f,GSH} * CMM * VL$$

where

dAM/dt	Rate of formation of epoxide (μmol/hr).
V_{max}	Maximum velocity of oxidative pathway (μmol/hr).
K_m	Michaelis constant for oxidative pathway (μM).
CVL	Liver concentration of 1,2-DCB available for metabolism (μM).
AMM	Amount of epoxide present at time t (μmol).
ACB	Amount of reactive metabolites formed (μmol).
ADCF	Amount of dichlorophenol formed (μmol).
AGSH	Amount of GSH conjugate formed (μmol).
CMM	Concentration of epoxide at time t (μM).
VL	Volume of liver (l).
$K_{f,CB}$	First-order rate constant for formation of reactive metabolites (hr^{-1}).
$K_{f,DCP}$	First-order rate constant for formation of dichlorophenol (hr^{-1}).
$K_{f,GSH}$	First-order rate constant for formationof GSH conjugates (hr^{-1}).
d/dt	Differential.

PB-PK model for 1,2-dichlorobenzene

Differential equations describing the rate of change in amount of GSH in the liver:

$$dGSHL/dt = VL*K_{SYN} - VL*K_{TO}*GSH - K_{f,GSH}*CMM*VL$$

where

GSHL	Amount of GSH in the liver (μmol).
GSH	Concentration of GSH in the liver (μM).
K_{SYN}	Rate of GSH synthesis in the liver (μmol/l/hr).
K_{TO}	First-order rate of GSH turnover in the liver (hr^{-1}).

The concentration of total metabolites in blood was determined by the influx of DCP and GSH conjugates from the liver (all reactive metabolites formed were assumed to bind to liver proteins) and the efflux of these metabolites from the blood into the urine. These processes were assumed to follow first-order kinetics, and the rate constants were varied manually to fit the experimental data.

$$dBLDCP/dt = K_{BL,DCP}*DCPL - KE_{BL,DCP}*BLDCP$$
$$dBLGS/dt = K_{BL,GS}*GSL - KE_{BL,GS}*BLGS$$
$$TOT = (BLDCP + BLGS)/VB$$

where

BLDCP	Amount of DCP in blood at time *t* (μmol).
DCPL	Amount of DCP in liver at time *t* (μmol).
BLGS	Amount of GSH conjugate in blood at time *t* (μmol).
GSL	Amount of GSH conjugate in liver at time *t* (μmol).
$K_{BL,DCP}$	Rate constant for transport of DCP from liver into blood (100 hr^{-1}).
$K_{BL,GS}$	Rate constant for transport of GSH conjugate from liver into blood (100 hr^{-1}).
$KE_{BL,DCP}$	Rate constant for transport of DCP from blood into urine (0.1 hr^{-1} for 5 and 50 mg/kg, 0.06 hr^{-1} for 250 mg/kg).
$KE_{BL,GS}$	Rate constant for transport of GSH conjugate from blood into urine (0.1 hr^{-1} for 5 and 50 mg/kg, 0.06 hr^{-1} for 250mg/kg).
TOT	Total blood concentration of metabolites.
VB	Apparent volume in which metabolites are distributed (0.6 l).

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CHAPTER 8

SUMMARIZING DISCUSSION

A major difficulty in the process of estimating human risk after exposure to xenobiotics, is the nearly complete lack of human *in vivo* data since for ethical reasons almost no experiments can be performed in humans. Indirect approaches have been developed to overcome this problem, namely testing in animals and extrapolation to the human situation. Large safety margins are applied in this extrapolation process, since significant inter- and intraspecies differences may exist in bioavailabilty and susceptibility to a certain compound. Biotransformation plays an important role in either detoxication or activation of xenobiotics and thus in the mechanism of action. Interindividual as well as species differences in susceptibility may for a major part be a consequence of different amounts or types of enzymes involved in the bioactivation and detoxication processes. Thus, including the mechanism of toxic action in the extrapolation process would make it possible to much more rationalize the safety margins applied in risk assessment. However, elucidation of the enzymes involved in the bioactivation and detoxication processes is only one step in the extremely complicated extrapolation process. Reactive metabolites have to be identified, as well as the resulting concentrations of these metabolites in the target organ(s), in which also the availability of active transport systems plays a role. Lastly, the processes at the cellular level in the target organ, e.g. interaction with proteins or DNA or even the availability of targets (the *dynamics* of the reactive species), may vary between species and individuals.

Since several years, physiological models have been developed to describe the kinetics and dynamics of xenobiotics, the so called physiologically based pharmacokinetic or -dynamic (PB-PK/PD) models. An example of an extensively elaborated PB-PK model is the case of methylene chloride (Andersen *et al.*, 1987).

In this thesis, the emphasis is on the differential biotransformation of 1,2- and 1,4-dichlorobenzene by different species, including man. For both compouds, the comparisons *in vivo* rat - *in vitro* rat and *in vitro* rat - *in vitro* man are made, and conclusions for the human situation are drawn from these. In addition, for 1,2-dichlorobenzene the biotransformation

processes are incorporated in a PB-PK model, for the rat as well as man (*Chapter 7*). The dynamics of the processes are included: the hepatic concentration of reactive metabolites is predicted, as well as the depletion of glutathione in the liver, for both rat and man.

The studies performed with the two isomers of dichlorobenzene will be discussed separately in the next two paragraphs.

8.1 1,4-Dichlorobenzene.

The *para*-isomer of DCB is of toxicological interest since it is commonly used as a space deodorant and in moth repellents. As a consequence, widespread exposure has been demonstrated (Angerer *et al.*, 1992; Hill *et al.*, 1995). The adverse effects found in different species are divers, and therefore make extrapolation to the human situation a complicated issue. The compound does not induce acute hepatotoxicity in rats, as does 1,2-DCB. Chronic exposure has been shown to result in nephrotoxicity and -carcinogenicity, but only in Fischer-344 rats and not in other rat strains or mice. Furthermore, the chemical induced liver carcinogenicity in B6C3F1 mice, but not in rats. These widely varying effects in different species and rat strains, tempted us to compare biotransformation patterns, and man was included in the *in vitro* studies.

In *Chapter 3*, the *in vivo* biotransformation and kinetics of 1,4-DCB in male Wistar rats is described. The design of the study was similar to an *in vivo* study performed with 1,2-DCB (described in *Chapter 2*). 1,4-DCB was mainly (90%) metabolized to 2,5-dichlorophenol (2,5-DCP), which was conjugated by glucuronic acid (25%) and sulfate (60%). Only 10% of the metabolites consisted of epoxide-derived mercapturic acids. This low capacity of the epoxide to conjugate with glutathione (for 1,2-DCB, 60% is conjugated with GSH) is in consonance with the inability of 1,4-DCB to induce acute hepatotoxicity or to deplete GSH in the liver (*Chapter 6*). In the *in vivo* study, no hydroquinone metabolites were detected. This is of particular interest, since in an *in vivo* study performed with Fischer-344 rats, the rat strain in which nephrotoxicity and -carcinogenicity has been demonstrated, hydroquinone-derived metabolites were detected (Klos and Dekant, 1994). Since the glutathione conjugates of dichlorohydroquinone have been associated with nephropathy (Mertens *et al.*, 1991), Wistar rats might not be susceptible to 1,4-DCB-induced nephropathy due to their inability to produce hydroquinones.

To further investigate the metabolic differences between Wistar and Fischer-344 rats, an *in vitro* study was designed, using hepatic microsomes, which also included microsomes of Sprague-Dawley rats, B6C3F1 mice, and humans (*Chapter 4*). In addition, microsomes expressing a single human cytochrome P450 enzyme (2E1, 1A1, 1A2, 2B6, 2C9, 2D6, 2A6, 3A4), were studied. Of the human P450 enzymes, only P4502E1 appeared to be capable of oxidizing 1,4-DCB, with 2,5-DCP as the single metabolite. This enzyme is a major catalyst in the oxidation of many low molecular weight cancer suspects (Guengerich *et al.*, 1991). The mouse liver microsomes extensively metabolized 1,4-DCB, and moreover, highly reactive metabolites were produced. These metabolites were identified as quinones, and are most likely involved in 1,4-DCB-induced liver carcinogenicity in B6C3F1 mice. Rat liver microsomes were less extensive in metabolizing 1,4-DCB compared to mouse. Moreover, less reactive metabolites were produced, which consisted of quinone metabolites as well as epoxides. In accordance with the *in vivo* studies discussed above, Fischer rat microsomes produced significantly more hydroquinone metabolites compared to the Wistar and Sprague-Dawley rat, confirming their possible role in nephrotoxicity. Finally, the results obtained with the mouse and rat liver microsomes were compared with the metabolic profile of the human liver microsomes. Biotransformation was much less extensive compared to mouse as well as rat, and less reactive metabolites were formed, which consisted mainly of epoxides. From these observations we speculate that the liver carcinogenicity observed in B6C3F1 mice is not relevant to the human situation. However, for the human microsomes the ratio hydroquinone to epoxide-derived metabolites was higher than observed for the Fischer rat microsomes. This implicates that chronic exposure to 1,4-DCB embodies a certain risk to man in respect to nephrotoxicity. However, other factors might be involved in the development of nephropathy, e.g. the presence of α_{2u}-globulin, a protein present in the proximal tubules of the kidneys of male rats, but not of humans. If this protein is essential in the 1,4-DCB-induced nephropathy, then this is not relevant for man.

In a human volunteers study, which was performed parallel to the studies described in this thesis, six subjects have been exposed by inhalation to 25 ppm 1,4-DCB (Krüse *et al.*, manuscript in prep.). A PB-PK model was developed to describe the kinetics and metabolism of 1,4-DCB in man. The metabolic clearance parameters resulting from the modeling approach were compared with the clearance parameters derived from the rat *in vivo* study which is described above (scaled to body weight). It appeared that the rat values were well within the range of values obtained from PB-PK modeling of the human data.

8.2 1,2-Dichlorobenzene.

1,2-Dichlorobenzene (1,2-DCB) is of interest, since it is widely used as an intermediate in the production of pesticides and chlorinated compounds, as a solvent, and as a deodorizer. Residues have been detected in human fat tissue (Mes *et al.*, 1986). The compound has been demonstrated to elicit acute hepatotoxicity in mice and rats (*Chapter 6*), but not carcinogenicity. Hepatotoxicity is potentiated after induction of certain P450 enzymes. Minor effects have been observed in spleen and thymus after exposure to 1,2-DCB.

In *Chapter 2*, the dose dependent *in vivo* biotransformation and kinetics of 1,2-DCB in male Wistar rats is described. At the highest dose level investigated (250 mg/kg bw), a saturation of metabolism was observed. Of the metabolites formed, 60% consisted of epoxide-derived mercapturic acids. This corresponded with the depletion of GSH in the liver following exposure to 1,2-DCB, which is described in *Chapter 6*. Also, the high potential of the epoxides to conjugate with GSH, particularly in comparison with 1,4-DCB, implied a reactivity of epoxides towards macromolecules as well. The remaining amount of metabolites consisted of 2,3- and 3,4-dichlorophenol (DCP), for the major part conjugated with sulfate. Induction with phenobarbital (an inducer of the P4502B-type enzymes, also potentiating hepatotoxicity of 1,2-DCB) *in vivo* resulted in a selective increase of 3,4-DCP. Similarly for bromobenzene, it has been demonstrated that rabbits pretreated with phenobarbital preferentially metabolized the compound to *para*-bromophenol, via a toxic 3,4-epoxide (Lau and Zannoni, 1981).

To compare biotransformation of 1,2-DCB by rat and man, an *in vitro* study was designed using hepatic microsomes, similar to the study with 1,4-DCB. The results of this study are described in *Chapter 5*. Liver microsomes of male Wistar, Fischer-344 and Sprague-Dawley rats were used. In addition, to investigate the possible roles of P4502E1 and P4502B, microsomes of induced Wistar rats were used. For man, human liver microsomes were used, as well as microsomes expressing a single human P450 enzyme. As for 1,4-DCB, P4502E1 was the only human isoenzyme which produced detectable amounts of metabolites, 2,3- and 3,4-DCP. No major differences in extent of metabolism were observed between rat and man. However, a pronounced difference in the formation of reactive metabolites (covalent binding) was observed between rat and man: rat liver microsomes produced much more reactive metabolites, particularly the P4502B-induced microsomes. For all microsomes investigated, these metabolites consisted mainly of epoxides, which corresponded with the extensive formation of epoxide-derived mercapturic acids *in vivo*. Moreover, it appeared that the

epoxides produced by the rat microsomes conjugated spontaneously with GSH, whereas for the epoxides produced by human microsomes, including the epoxides produced by the individually expressed P4502E1, glutathione S-transferases were needed to catalyze the conjugation. It was hypothesized that human cytochrome P4502E1 selectively metabolizes 1,2-DCB to the 3,4-epoxide, which apparently is much less reactive than the 4,5-epoxide. In addition, rat P4502B enzymes seemed to be responsible for the preferred formation of the reactive 4,5-epoxide; these results corresponded with the increased formation of 3,4-DCP *in vivo* after pretreatment with phenobarbital (*Chapter 2*), as well as the increased hepatotoxicity after induction with phenobarbital (Stine *et al.*, 1991). This differential formation of epoxides by different P450 isoenzymes has also been demonstrated for bromobenzene (Lau and Zannoni, 1981).

In *Chapter 7*, the difference in formation of reactive metabolites between rat and man, has been incorporated in a PB-PK model. With this model, the resulting concentration of reactive metabolites in the liver following exposure to 250 mg/kg 1,2-DCB (a toxic dose level for the rat, as described in *Chapter 6*) could be predicted, for both rat and man. In this way, the difference in kinetics between rat and man is accounted for. In addition, the depletion of GSH in the liver was predicted and compared with experimental data (*Chapter 6*). It appeared that the concentration of reactive metabolites, which would be toxic for the rat (effect concentration), could not be reached in human liver, even at very high dose levels. Maximum depletion of GSH was higher for man than for the rat, however it is not likely that this would be the only factor involved in hepatotoxicity. In *Chapter 5*, it was already observed that conjugation with GSH was similar for rat and man, whereas the covalent binding was much more extensive for rat liver microsomes than for human liver microsomes. With the PB-PK model, it was kinetically and quantitatively supported that the less extensive formation of reactive metabolites by human microsomes *in vitro* as compared to rat, also *in vivo* would not result in a critical (effect) concentration. Hence, the currently applied quantifications of toxicological effects (e.g. the Lifetime Health Advisory) which are based on the NOAEL derived from studies performed in rats (USEPA, 1987), most likely overestimate the risk for man.

The data presented in this thesis emphasize that elucidating the metabolic profile and the molecular mechanism of toxicity of xenobiotics is essential in accurate risk evaluation, and that the animal models used for risk assessment have to be selected carefully. However, more processes are involved between first exposure to a xenobiotic compound and the ultimate

adverse effect, and PB-PK/PD modeling can be a useful tool in gaining insight in these processes.

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SAMENVATTING

Het belangrijkste aspect van de toxicologie betreft de schadelijke effecten van lichaamsvreemde stoffen (xenobiotica) op de gezondheid van de mens. Om ethische redenen kan de schadelijkheid van deze xenobiotica vrijwel niet getest worden in de mens zelf. Daarom is een indirecte methode noodzakelijk: proefdieren worden blootgesteld aan de te onderzoeken stof en de resultaten van deze dierproeven worden vertaald naar de humane situatie. Echter, bij deze 'extrapolatie' worden vaak grote veiligheidsfactoren ingebouwd. Deze zijn nodig om eventuele verschillen in gevoeligheid tussen verschillende species te ondervangen. Ook moet er rekening mee worden gehouden, dat de proefdieren waarin wordt getest meestal een genetisch vrij homogene groep vormen, terwijl de humane populatie zeer inhomogeen is, waardoor grote interindividuele verschillen kunnen optreden. De veiligheidsfactoren die worden toegepast bij de risicoschatting worden echter arbitrair vastgesteld. Om deze veiligheidsfactoren beter te kunnen onderbouwen, kan het *mechanisme* van toxiciteit worden meegenomen in het extrapolatie proces. Daar voor veel xenobiotica biotransformatie noodzakelijk is voor het tot stand komen van een toxisch effect (toxificatie), vormt dit een belangrijk aspect van de risicoschatting.

Een eerste stap in het mechanistische onderzoek van de bioactivatie van xenobiotica is de opheldering van de enzymen die betrokken zijn bij de biotransformatie. De mate van toxiciteit van een stof wordt uiteindelijk bepaald door het evenwicht tussen de vorming en ontgifting van toxische metabolieten, waarbij verschillende enzymen betrokken (kunnen) zijn. Doordat grote verschillen kunnen bestaan in concentratie en aktiviteit van deze enzymen, zowel tussen species als tussen individuen, kunnen dus grote verschillen in gevoeligheid voor xenobiotica bestaan. Naast de opheldering van de betrokken enzymen, is het ook van belang om de identiteit van de reaktieve metaboliet(en) te achterhalen, de concentratie van deze metabolieten in het doelorgaan en hun werking op cellulair niveau. Voor deze laatste processen spelen de kinetiek en dynamiek van de uitgangsstof en de metabolieten een belangrijke rol.

Sinds een aantal jaren worden fysiologische modellen ontwikkeld om de kinetiek en dynamiek van stoffen en hun metabolieten te beschrijven, de zogenaamde 'physiologically based pharmacokinetic of -dynamic' (PB-PK/PD) modellen. Een voorbeeld van zo'n PB-PK model is het methyleenchloride model ontwikkeld door Andersen *et al.* (1987), waarin het

metabolisme in vier verschillende species (rat, muis, hamster en mens) wordt beschreven, en waarin tumor incidentie wordt gecorreleerd aan species afhankelijk metabolisme.

In dit proefschrift wordt de kinetiek, het metabolisme en de toxiciteit van 1,2- en 1,4-dichloorbenzeen (DCB) beschreven. De nadruk ligt op het verschil in biotransformatie tussen proefdier en mens, en de potentiële gevolgen hiervan voor de (humane) toxiciteit. De modelstoffen zijn gekozen op basis van de verschillende effecten die zijn waargenomen in verscheidene proefdieren. Het belangrijkste effect van 1,2-DCB is acute levertoxiciteit in de rat en muis. Voor 1,4-DCB is geen acute levertoxiciteit gevonden, maar wel levercarcinogeniteit in de muis en niertoxiciteit en -carcinogeniteit in de Fischer-344 rat, echter niet in de Wistar rat. Voor beide dichloorbenzeen isomeren wordt onder meer de *in vivo* - *in vitro* biotransformatie in de rat vergeleken, en wordt de *in vitro* rat - *in vitro* mens biotransformatie vergeleken. Voor 1,2-DCB worden de biotransformatie data eveneens in een PB-PK model verwerkt, voor zowel de rat als de mens, met als doel om uiteindelijk een meer nauwkeurige risico-evaluatie van 1,2-DCB te kunnen maken.

In hoofdstuk 2 wordt de dosis afhankelijke *in vivo* kinetiek en biotransformatie van 1,2-DCB in mannelijke Wistar ratten beschreven. Metabolisme was verzadigd bij de hoogste orale dosering (250 mg/kg lichaamsgewicht). Van de gevormde metabolieten bestond 60% uit epoxide-afgeleide mercaptuurzuren. Dit komt overeen met de depletie van glutathion (GSH) in de lever die is gevonden na orale blootstelling aan 1,2-DCB (beschreven in hoofdstuk 6). De resterende hoeveelheid metabolieten bestond uit 2,3- en 3,4-dichloorfenol (DCP), voor het grootste deel geconjugeerd met sulfaat. Inductie van cytochroom P4502B enzymen in de ratten door middel van phenobarbital, een stof die ook de levertoxiciteit van 1,2-DCB versterkt, resulteerde in een selectieve toename van de vorming van 3,4-DCP. Dit is te vergelijken met broombenzeen, waarvoor is aangetoond dat inductie met phenobarbital resulteert in een selectieve toename van de vorming van *para*-broomfenol via een toxisch 3,4-epoxide (Lau en Zannoni, 1984).

In hoofdstuk 3 wordt de *in vivo* kinetiek en biotransformatie van 1,4-DCB in mannelijke Wistar ratten beschreven. De opzet van de studie was vergelijkbaar met de studie beschreven in hoofdstuk 2. 1,4-DCB werd voor 90% gemetabolizeerd tot 2,5-dichloorfenol (2,5-DCP), hetgeen voor 25% werd geconjugeerd met glucuronzuur en voor 60% met sulfaat. Slechts 10% van de gevormde metabolieten bestond uit epoxide-afgeleide mercaptuurzuren.

Deze lage reaktiviteit van het epoxide ten opzichte van GSH, in vergelijking met de 60% mercaptuurzuren gevonden voor 1,2-DCB, is in overeenstemming met de afwezigheid van acute levertoxiciteit en depletie van GSH in de lever na blootstelling aan 1,4-DCB (hoofdstuk 6). In deze *in vivo* studie werden geen hydrochinon-afgeleide metabolieten gevonden. Dit in tegenstelling met de resultaten van een *in vivo* studie met Fischer-344 ratten, waarin wel hydrochinonen werden aangetoond (Klos en Dekant, 1994). Dit is van belang, omdat alleen in de Fischer-344 rat niertoxiciteit en -carcinogeniteit is aangetoond. Aangezien GSH conjugaten van dichloorhydrochinon worden geassocieerd met nierschade (Mertens *et al.*, 1991), zou dit kunnen verklaren dat Wistar ratten niet gevoelig zijn voor 1,4-DCB geïnduceerde nierschade.

Om het verschil in biotransformatie van 1,4-DCB door Wistar en Fischer ratten verder te onderzoeken werd een *in vitro* studie uitgevoerd, waarin levermicrosomen werden gebruikt van de Wistar, Fischer-344 en Sprague Dawley rat, de B6C3F1 muis, en de mens. Deze studie wordt beschreven in hoofdstuk 4. De muis microsomen vertoonden zowel de hoogste omzetting van 1,4-DCB als de meeste vorming van reactieve intermediairen. De reaktieve metabolieten werden geïdentificeerd als chinonen, en zijn waarschijnlijk verantwoordelijk voor de levercarcinogeniteit in de B6C3F1 muis. Levermicrosomen van de verschillende rattestammen produceerden minder reaktieve metabolieten, en werden geïdentificeerd als zijnde epoxides en chinonen. In overeenstemming met de *in vivo* studie werden beduidend meer hydrochinon metabolieten gevonden voor de Fischer rat vergeleken met de Wistar en Sprague Dawley rat, hetgeen de potentiële rol in niertoxiciteit bevestigt. Het metabolietprofiel van de humane levermicrosomen werd vergeleken met dat van de rat en muis. De totale omzetting was lager, als ook de vorming van reaktieve intermediairen, die voornamelijk uit epoxides bestonden. Dit maakt het onwaarschijnlijk dat de levercarcinogeniteit in de B6C3F1 muis relevant is voor de humane situatie. Echter de ratio hydrochinon:epoxide was groter vergeleken met de Fischer rat, hetgeen impliceert dat langdurige blootstelling aan lage concentraties 1,4-DCB ook voor de mens tot nierschade zou kunnen leiden.

In hoofdstuk 5 wordt de *in vitro* microsomale biotransformatie van 1,2-DCB voor de rat en mens vergeleken. Levermicrosomen van de Wistar, Fischer-344 en Sprague-Dawley rat werden gebruikt, als ook levermicrosomen van de Wistar rat, geïnduceerd met isoniazid (induceert P4502E1) of phenobarbital (induceert P4502B). Ook werd de omzetting door humaan cytochroom P4502E1 bestudeerd. De totale omzetting van 1,2-DCB was iets hoger voor de mens vergeleken met de rat. Echter, de rat microsomen produceerden veel meer reaktieve (covalent gebonden) metabolieten dan de humane microsomen, met name de P4502B

geïnduceerde microsomen. Deze metabolieten bestonden bijna volledig uit epoxides, wat overeenkomt met de *in vivo* resultaten beschreven in hoofdstuk 2. Het bleek dat de epoxides die geproduceerd werden door de rat microsomen, spontaan conjugeerden met GSH, terwijl door de humane microsomen een gelijke hoeveelheid epoxides gevormd werden, die echter bijna uitsluitend onder invloed van glutathion S-transferases met GSH conjugeerden. Dit fenomeen werd ook gevonden voor humaan P4502E1. Het is gepostuleerd dat humaan P4502E1 bij voorkeur of zelfs selectief het 3,4-epoxide vormt, terwijl rat P4502B met name het veel reaktievere 4,5-epoxide van 1,2-DCB produceert. Deze resultaten zijn in overeenstemming met de selectieve toename van 3,4-DCP *in vivo* na inductie met phenobarbital (hoofdstuk 2), evenals de toename van levertoxiciteit na inductie met phenobarbital.

In hoofdstuk 6 wordt een studie beschreven waarin de lever- en niertoxiciteit van 1,2- en 1,4-DCB is onderzocht, alsmede de eventuele depletie van GSH in de lever en nieren. Het bleek dat alleen de hoogst onderzochte dosis van 1,2-DCB (250 mg/kg) acute levertoxiciteit veroorzaakte. Voor geen van beide isomeren werd niertoxiciteit geconstateerd. Depletie van GSH werd alleen voor 1,2-DCB gevonden, in de lever maar niet in de nieren.

In hoofdstuk 7 wordt een PB-PK model voor 1,2-DCB beschreven, voor de rat zowel als de mens. Met behulp van dit model en de gegevens uit de verschillende *in vivo* en *in vitro* studies werd de leverconcentratie van reaktieve metabolieten voorspeld, na blootstelling aan een voor de rat toxische dosis van 250 mg/kg. Ook werd de depletie van GSH in de lever voorspeld, en de resultaten werden vergeleken met verkregen experimentele data. De effectieve concentratie van reaktieve metabolieten in de lever, zoals vastgesteld voor de toxische dosis in de rat, bleek niet bereikt te kunnen worden in de humane lever, zelfs niet bij zeer hoge doses. Depletie van GSH was hoger voor de mens dan de rat. Het is echter niet waarschijnlijk dat depletie van GSH de enige factor van betekenis is in acute levertoxiciteit, omdat voor de rat de gevonden depletie waarschijnlijk niet tot toxiciteit leidt, terwijl de door de rat gevormde metabolieten van 1,2-DCB zeer reaktief zijn.

Op basis van de in dit proefschrift beschreven resultaten kan worden geconcludeerd dat opheldering van het metabolisme patroon en het moleculaire mechanisme van toxiciteit van xenobiotica essentieel is voor een nauwkeurige risicoschatting, en dat het proefdiermodel dat gebruikt wordt voor het testen van een bepaalde stof zorgvuldig gekozen dient te worden. PB-PK/PD modellen kunnen een belangrijke bijdrage leveren in het extrapolatie proces van dier naar mens.

CURRICULUM VITAE

Erna Hissink werd op 27 november 1965 te Vorden (Gld) geboren. Na het voltooien van het ongedeeld VWO op het Stedelijk Lyceum te Zutphen, werd in 1984 begonnen met de studie Levensmiddelentechnologie aan de Landbouwuniversiteit te Wageningen. Als specialisatie-richting binnen de studie werd de oriëntatie "Toxicologie" gekozen. Tijdens de doctoraalfase werden de afstudeervakken Levensmiddelenchemie (Prof. Dr. Ir. A.G.J. Voragen), Toxicologie (Prof. Dr. J.H. Koeman) en Biochemie (Prof. Dr. C. Veeger) gevolgd. Een stage Toxicologie werd voltooid bij de afdeling Biologische Toxicologie van het TNO Instituut voor Toxicologie en Voeding (ITV). In augustus 1990 heeft zij het doctoraalexamen afgelegd.

Op 1 mei 1991 trad zij in dienst als assistent in opleiding bij de faculteit Diergeneeskunde van de Universiteit Utrecht. Het in dit proefschrift beschreven onderzoek werd uitgevoerd bij de Divisie Toxicologie van TNO Voeding te Zeist, onder leiding van Prof. Dr. P.J. van Bladeren. Vanaf juli 1996 is zij aldaar werkzaam als wetenschappelijk onderzoeker op een project, gefinancierd door de Stichting Technische Wetenschappen van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO).

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NAWOORD

Zoals iedereen inmiddels wel weet, is een proefschrift een produkt waaraan velen hebben gewerkt. Zo ook dit proefschrift.

Allereerst wil ik Peter van Bladeren bedanken voor het voortreffelijk overnemen van de rol van directe(ur) begeleider. Ik heb het zeer op prijs gesteld dat je ondanks een overvolle agenda mijn manuscripten toch altijd snel en scherp van opbouwend commentaar hebt voorzien.

Ben van Ommen, mede door jouw nimmer aflatende enthousiasme en optimisme is het onderzoek goed op de rails gekomen. Ook vanuit het verre en zonnige Zuiden ben je betrokken gebleven bij het afronden van mijn onderzoek. E-mail heeft ook voor wetenschappelijke doeleinden zijn nut bewezen. Alleen jammer van dat ene kabeltje... Het is maar goed dat de ouderwetse postbode ook nog bestaat.

Willem Seinen wil ik bedanken voor zijn interesse en het regelen van alle promotie-aangelegenheden.

Een belangrijk deel van het labwerk is uitgevoerd door de studenten Maarten van Donk en Ramon Dunnewijk, en door Leonore Blok-Tip. Bedankt voor jullie inzet, vooral op de tijdstippen ver buiten de kantoortijden!

Alle medewerkers van de Divisie Toxicologie dank ik voor de prettige werksfeer, vooral de fijne (ex)collega's van de werkgroep Kinetiek en Metabolisme. Bert, bedankt voor je hulp bij de *in vivo* experimenten in de beginfase van het onderzoek, en vooral bedankt voor het me op de hoogte houden van de gebeurtenissen rond Rits en Broek in de B. en de B. Martin, zonder jouw uitstekende hulp bij de "mic's" van hoofdstuk 4 en 5, waren deze hoofdstukken er niet gekomen. En Jan, bedankt voor het 's ochtends klaar hebben staan van de lekkerste koffie!

Els Gevers en haar medewerkers wil ik bedanken voor de hulp bij de GC-analyses en de mensen van "Massa" voor het produceren van de gezochte massa-spectra. De medewerkers van de diervleugel dank ik hartelijk voor het uitvoeren van allerlei biotechnische handelingen en de computer-jongens voor het uitvoeren van allerlei compu-technische handelingen.

En natuurlijk ben ik mijn vriend(inn)en bijzonder dankbaar voor de vele steuntjes in de rug, in Goede en in Slechte tijden. Mijn twee trouwe huisdieren bedank ik voor het feit dat ze elke avond onvoorwaardelijk op mij (of op eten?) wachten.

En iedereen die bedankt had willen worden en die ik niet genoemd heb: ook heel erg bedankt!

En als laatste wil ik mijn familie "oerent hart" bedanken voor alle aanmoedigingen. Toch nog twee promoties in één jaar!

Erna.