Individual variation in biotransformation: relation to styrene kinetics and solvent-induced neur
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Chapter 10

Summarising discussion

Interindividual variability in the disposition and toxicity of xenobiotics has gained more and more interest in recent years. It has been identified as one of the most important factors determining the efficacy of drugs, adverse effects of xenobiotics, and disease susceptibility [1,2]. Thus, interindividual variability is likely to play a major role in the risk assessment and biological monitoring of exposure to chemicals. Polymorphisms in biotransformation enzymes seem to be an important factor in determining the interindividual variability in kinetics and toxicity. While research in the past two decades has focused mainly on the efficacy of drugs and on susceptibility to environmentally-induced cancer, little is known about the influence of differences in metabolic capacity on the kinetics and neurotoxicity of organic solvents.

This thesis focused on the relation between interindividual differences in metabolic capacity and the toxicokinetics of styrene, as well as the relation between genetic polymorphism in biotransformation enzymes and susceptibility to solvent-induced neurotoxicity. The main study objectives were: (1) interindividual variability in styrene metabolism in vitro and the relationship with genetic polymorphism of the two involved enzymes, (2) the relation between individual metabolic capacity, assessed by phenotyping and genotyping, and interindividual differences in the toxicokinetics of styrene in vivo, and (3) the relation between the genetic polymorphism of several biotransformation enzymes and the risk of chronic toxic encephalopathy.

In addition, in the in vitro and in vivo studies, stereochemical aspects of styrene metabolism were investigated.

Gas chromatographic methods for SO and MA enantiomers (chapters 2 and 5)

In order to measure SO and MA enantiomers, enantiospecific and sensitive gas-chromatographic (GC) methods needed to be developed. In chapter 2, a GC method for the analysis of styrene oxide (SO) enantiomers is described. The method was based on base-catalysed hydrolysis with sodium methoxide. In this way, opening of the epoxide ring was achieved without racemisation, in contrast to other methods which employed acid-catalysed hydrolysis. The resulting methoxy alcohols were derivatised to enable sensitive determination by electron capture detection (ECD). Derivatization proceeded also without racemisation and a good baseline separation was achieved by serial coupling of two columns, a chiral CP
With this method, the rate and enantioselectivity of the CYP450-dependent oxidation of styrene to SO enantiomers in human liver microsomes could be determined.

In chapter 5, a GC method for the analysis of mandelic acid (MA) enantiomers in urine is described. In contrast to generally used methods [3-5], esterification of MA with isopropanol was performed directly in urine, without previous solvent extraction. No racemisation of the enantiomers occurred during esterification, and a good baseline separation of the isopropyl esters of the MA enantiomers was obtained using a Chirasil-Dex column. To achieve more sensitive ECD determination, subsequent acylation of the isopropyl esters of MA was performed. With this GC method, a 24-h follow-up of the excretion of MA enantiomers in urine of volunteers exposed to 104 and 360 mg/m$^3$ was possible.

Interindividual variability in styrene biotransformation in vitro (chapters 3 and 4)

In chapters 3 and 4 the in vitro biotransformation of styrene in the livers of twenty Caucasian males is described. The observed interindividual variability in the enzyme kinetic parameters was correlated to the genetic polymorphism of the enzymes involved in styrene oxidation and styrene oxide hydrolysis.

The oxidation of styrene was shown to be mediated by CYP2E1 (chapter 3), although at higher styrene concentrations other CYP450s also contributed, however, with much lower catalytic efficiency. The $V_{\text{max}}$ and $K_m$ of CYP2E1-mediated styrene oxidation varied 8- and 6-fold, respectively, between the livers. The variability in $V_{\text{max}}$ correlated well with the variation in CYP2E1 activity, as measured by p-nitrophenol hydroxylation. However, the CYP2E1 polymorphisms did not seem to be responsible for the observed variability. This could indicate that the polymorphisms do not change the catalytic activity of the CYP2E1 enzyme, although the number of livers studied and the low frequency of the mutations in Caucasians preclude any definite conclusions.

The hydrolysis of styrene oxide to styrene glycol, a detoxification step, proceeded at a high rate in vitro (chapter 4). The measured intrinsic clearance (CLi, $V_{\text{max}}/K_m$) was much higher than the CLi for the CYP450 step, implying that the CYP450 step is the rate-limiting step in the metabolism of styrene. Mendrala et al. [6] showed that humans had the highest capacity to hydrolyse SO and the lowest capacity to form SO, compared to laboratory animals, indicating that humans are less susceptible to styrene oxide toxicity. The variability observed in mEH-mediated SO hydrolysis in the present study was 5-fold for $V_{\text{max}}$ and 3- to 4-fold for $K_m$. No relation with the polymorphisms of the mEH gene was found, possibly due to the low number of livers studied.

From these studies, the conclusion was drawn that a potential for large interindividual variation in styrene metabolism in humans exists. As the rate-limiting step in the biotransformation of styrene seems to be its oxidation, variation in CYP4502E1 could be of crucial importance in susceptibility to styrene.
Interindividual variability in styrene biotransformation in vivo (chapter 7 and 8)

In chapter 7, the interindividual variability in the toxicokinetics of styrene was studied in vivo in relation to individual metabolic capacity. Twenty healthy male volunteers were exposed to 104 and 360 mg/m³ styrene for 1 h and 50 W physical exercise. Individual metabolic capacity was assessed with the probe substrates chlorzoxazone (CYP2E1), caffeine (CYP1A2), dextromethorphan (CYP2D6) and antipyrine (CYP450), and by genotyping for CYP2E1 polymorphism. Although the variation in the kinetics of styrene was quite large for a relatively homogeneous group, no correlation with either one of the model substrates, nor with the genotyping was observed. This is thought to be due to perfusion-limited kinetics of styrene. From the fact that apparent clearance of styrene in vivo (84 l/h) almost equals the blood flow (96 l/h) and that the intrinsic metabolic clearance (580 l/h), calculated from in vitro data, is much larger than the liver blood flow, the conclusion is drawn that styrene is completely metabolised while passing through the liver and that the blood flow limits its extent of metabolism. This implies that induction of CYP2E1 by chronic ingestion of ethanol, previous exposure to other solvents, medication, dietary habits or genetic polymorphism has little or no effect on the clearance of styrene and formation of styrene oxide, as long as the exposure concentration is low. When exposure to styrene increases, reaching internal concentrations that saturate the biotransformation enzymes, the capacity of the enzymes becomes the rate-limiting factor instead of hepatic blood flow. Then, interindividual variations in V_max will become apparent. In this light it is interesting to note that peak exposures have been implicated as an etiological factor in the development of chronic toxic encephalopathy [7]. This means that an increased activity of CYP2E1 could have an impact on the risk of neurotoxicity in the case of peak exposures. However, the increased enzyme activity will be detrimental if styrene oxide is responsible for the neurotoxic effects, but beneficial when styrene itself is the toxic factor.

At low exposure concentrations, inhibition of CYP2E1 will have a more pronounced effect on the clearance of styrene than enzyme induction, because it will decrease the intrinsic clearance, thereby changing metabolism from flow-limited to capacity-limited. Co-exposure to ethanol, other solvents or drugs may inhibit styrene metabolism, although the dose of both styrene and the co-substance determines the extent of interaction. In addition, genetic polymorphisms which significantly reduce or even delete enzyme activity will affect styrene metabolism, if involved in styrene biotransformation. This has been shown for methyl chloride, where subjects with the GSTT1 null genotype have a lower uptake, lower metabolic clearance, higher concentrations in the blood and a slower post-exposure decay compared to subjects with the normal genotype [8].

Other factors can also contribute to the absence of a relation between the model substrates and the kinetic parameters of styrene. Our study was hampered by the fact that SO in blood could not be measured. Therefore, indirect parameters were taken, which were considered to reflect SO formation. Also factors like intraindividual variation in the metabolism of the model substrates and the kinetics of styrene (see below) and the uncertainty
of the enzyme specificity of the model substrates could have contributed to the negative result.

No correlation was observed between mEH genotype and styrene kinetics (styrene glycol in blood or urinary excretion of metabolites), this not being mentioned in chapter 7. However, the influence of mEH on styrene kinetics is difficult to assess in vivo. Styrene glycol concentrations in blood are dependent on the hydrolysis of styrene oxide to styrene glycol, but also on the further metabolism of styrene glycol to mandelic acid and conjugated glycol. Since we were unable to measure SO concentrations and since we did not analyse MA concentrations in blood, it is impossible to assess which step determines the SG concentrations in blood. In addition, mEH-mediated hydrolysis is probably not a rate-limiting step in the biotransformation of styrene, therefore, only the absence or a very low enzyme activity of mEH would have an impact on styrene kinetics.

In light of the mechanism of styrene toxicity, i.e. styrene oxide is responsible for the genotoxicity and both styrene and styrene oxide are implicated in the neurotoxicity, we have chosen styrene concentration in blood and the 3-h and 24-h cumulative excretion of MA and PGA as surrogate target doses, in chapter 7. SO concentrations in blood could not be measured. However, Mutti et al. [9,10] implicate PGA as the metabolite responsible for the neurotoxicity of styrene, although much controversy exists regarding this research. They observed a marked depletion of striatal and tubero-infundibular dopamine after dosing rabbits with styrene and mandelic and phenylglyoxylic acid, and, in addition, found that PGA condenses non-enzymatically with dopamine in vitro. If PGA is a neurotoxic metabolite, then interindividual variation in the biotransformation of MA to PGA, which might be capacity-limited, is very important with respect to susceptibility to styrene-induced neurotoxicity. In this light it is interesting to mention that (although not reported in chapter 7) metabolism of antipyrine, caffeine and dextromethorphan at 104 mg/m\(^3\), and metabolism of antipyrine and chlorzoxazone at 360 mg/m\(^3\) were significantly correlated to the formation of PGA. It is evident that much more needs to be known about the mechanism by which styrene induces toxicity, in order to identify biomarkers of susceptibility.

As stated in the introduction, several studies did find a relationship between genetic polymorphism and solvent metabolism. This can be explained in several ways; (1) capacity-limited metabolism of the solvents studied (1,1,1-trichloroethane and perchloroethylene in the study of Berode et al. [11]), (2) ethnic variation, as in the study of Kawamoto et al. [12], where an association was found between ALDH\(_2\) activity and toluene metabolism in Japanese; about 50% of the Orientals lack ALDH\(_2\) activity, but this polymorphism does not exist in Caucasians, and (3) type of gene polymorphism studied; with null-genotypes or gene mutations that lead to absence of enzyme activity, the effect on metabolism will be much more pronounced [8,13].

Despite the fact that the variability in styrene toxicokinetics in vivo in volunteers was not related to oxidative biotransformation capacity in our study, still a relatively large interindividual variation was observed. We currently have no conclusive explanation for the observed variability, but the kinetics of solvents in general depends on many physiological
factors, like body build (body size and body fat), physical activity, tissue blood flow and renal clearance [14,15]. The variability in styrene concentrations in blood observed in our study could perhaps be attributed to physiological variability in liver blood flow. Explaining the variability in urinary excretion presents a bigger challenge, as the intraindividual variability in excretion was also large (calculated by a mixed effects model). Interindividual and day-to-day variation in renal clearance, or changes in behaviour of the volunteers could perhaps explain the intra- and interindividually variability, associated with urinary metabolite excretion. This inter- and intra-variability renders MA and PGA excretion less suitable as biomarkers for individual styrene exposure.

In order to integrate the in vitro and in vivo data on styrene kinetics and variability, pharmacokinetic modelling was applied (chapter 8). For styrene, an existing physiologically-based pharmacokinetic (PBPK) model by Ramsey and Andersen was used [19]. In order to describe metabolite kinetics that model was extended with one-compartment models. The in vitro determined enzyme kinetic parameters of CYP450 and mEH were scaled to the in vivo situation and incorporated in the model. For CYP450, these parameters were in good agreement with the parameters used by Ramsey and Andersen and described the styrene kinetics well, but the $V_{\text{max}}$ of mEH had to be divided by four in order to obtain a good fit of the experimental data. The variability in $V_{\text{max}}$ of styrene oxidation observed in vitro was incorporated into the model, and styrene concentrations in blood and excretion of MA and PGA in urine were predicted. As expected, the interindivdual variation observed in vitro did not result in large changes in styrene or MA and PGA concentrations in vivo, thereby confirming the theory of flow-limited metabolism.

In conclusion, (genetic) polymorphism in styrene oxidation or styrene oxide hydrolysis does not seem to influence the kinetics of styrene and, therefore, is unlikely to modify susceptibility to styrene-induced toxicity, or compromise biological monitoring. Physiological factors and genetic and environmental factors that interfere further down in the metabolic pathway apparently determine the interindividual variability in styrene kinetics. However, interindividual variability in styrene biotransformation keeps attracting considerable attention, as witnessed from ongoing studies in this field [16,17].

The oxidative metabolism of many commonly used solvents, e.g. toluene, xylene, trichloroethylene, methylene chloride, is perfusion-limited [18], thus interindividual variation in biotransformation will probably play a minor role in the overall variability observed in the disposition of, and susceptibility to, these chemicals.

Stereochemistry of styrene metabolism in vitro and in vivo (chapters 3, 4, 6 and 8)
In the in vitro studies (chapters 3 and 4) the enantioselectivity of the styrene biotransformation reactions was described. Enantioselectivity is an important aspect, because enantiomers can differ in their interaction with enzymes, receptors, DNA and proteins, and therefore kinetics and toxicity can differ appreciably between enantiomers. This is the case with styrene oxide, with the (R)-enantiomer being four times as mutagenic to Salmonella
typhimurium TA100 as the (S)-enantiomer. In chapter 3, we have shown that (S)-SO is preferentially formed, with a S/R ratio of 1.3, but that this enantioselectivity can change towards a preference for the (R)-enantiomer at high concentrations of styrene, probably because of involvement of different CYP450 enzymes. In chapter 4, the hydrolysis of SO was shown to be enantiospecific as well as enantioselective, with $V_{\text{max}}$ and $K_m$ for (S)-SO being five times higher than for (R)-SO. This means that the intrinsic clearance ($V_{\text{max}}/K_m$) of (R)- and (S)-SO hydrolysis is similar, when incubated separately at non-saturating concentrations. However, (R)-SO inhibited the hydrolysis of (S)-SO when incubated together in a racemic mixture. The stereokchemical metabolism of styrene was also measured in vivo in the volunteer study (chapter 6). Concentrations of SO could not be measured. The maximal concentration and area under the curve (AUC) of unbound (R)-SG in blood proved to be higher than the concentration of (S)-SG, indicating either the inhibitory effect of (R)-SO on (S)-SO hydrolysis, or enantioselective metabolism of SG to MA. The shorter half-life found for (S)-SG compared to (R)-SG points towards the latter explanation. For MA, an excess of the (S)-enantiomer was observed in cumulative 24-h urine (S/R ratio of 1.6). In theory, the cumulative excretion of MA represents the ratio of SO enantiomers formed in the liver. However, the S/R ratio of MA enantiomers in the volunteers was 1.6, approaching the 1.3 found in vitro. This could mean that either the ratio of SO enantiomers formed in vivo is 1.6 instead of 1.3, or that enantioselective metabolism of MA to PGA occurs. Either way, the data show that the less toxic (S)-SO is the preferred enantiomer formed in vivo. However, as long as the enantioselectivity of several steps in styrene metabolism remains unknown, measurement of the enantiomeric ratio of metabolites like MA cannot be used to predict the enantiomeric ratio of styrene oxide.

The model in chapter 8 was also used to describe stereokchemical metabolism. The S/R ratio of 1.3 found in vitro for the SO enantiomers and the enantioselective parameters of the SO hydrolysis (without inhibition because, amongst others, the inhibition constant was unknown) were incorporated. With these assumptions, the model indeed predicted enantioselective metabolism of SG to MA, with (S)-SG being preferentially metabolised. Without assuming enantioselective oxidation of MA to PGA, the S/R ratio of MA enantiomers observed with the model was indeed the 1.3 of the SO enantiomers.

The enantioselective variation in vivo is somewhat smaller than the variation in total metabolite excretion, described in chapter 7. Therefore, we conclude that the ‘additional’ susceptibility to styrene-induced toxicity associated with its stereokchemical metabolism is relatively small.

Genetic polymorphism and risk of chronic toxic encephalopathy (chapter 9)
A possible relationship between differences in metabolic capacity and solvent-induced neurotoxicity was investigated by genotyping CTE patients and an exposed control group for several polymorphic biotransformation enzymes (chapter 9). A higher risk of CTE was demonstrated in persons with the CYP2E1 mutated alleles. The mechanism behind this increased risk is unclear. CYP2E1 is involved in the metabolism of many solvents and the
CYP2E1*5B mutated allele has been shown to increase transcriptional activity and mRNA expression and increase the elimination rate of ethanol and paracetamol [20-22]. Thus, the polymorphism could increase the biotransformation rate of solvents to reactive intermediates. However, our conclusions from chapter 7 and 8 are contradictory to this theory; with perfusion-limited kinetics an increase in the activity of biotransformation enzymes has no effect on metabolism. One possible explanation for the observed association is that the exposure of the patients might have consisted mainly of capacity-limited solvents. The majority of the patients and the controls were painters; exposure in painters consists mainly of mixtures and the assessment of individual solvents is difficult. Therefore, we can neither confirm nor deny this explanation. Another possible explanation is that the CYP2E1 polymorphisms might be linked to other mutations that affect CTE risk. Yet another possibility is that the increased risk might be due to an increased amount of reactive metabolites formed directly at the target site, the brain [23]. The presence of CYP450 enzymes has been demonstrated in the brain, although in much smaller quantities than in liver (making brain metabolism probably capacity-limited) [24-26]. Metabolism of parathion [27], n-hexane [28], imipramine [29] and m-dinitrobenzene [30] has been demonstrated in the brain in vitro, using brain slices or brain microsomes. In vitro and in vivo studies have shown metabolism of toluene and, consequently, generation of reactive oxygen species in the brain [31,32]. Indirect evidence has been obtained for metabolism of styrene in the brain; after i.p. injection of radioactive styrene in rats, the liposoluble fraction of radioactivity in the brain decreased at a faster rate than the water soluble fraction [33]. This may indicate metabolism of styrene in the brain, because with passive redistribution both fractions would decrease at the same rate.

Before the CYP2E1 alleles can be used as biomarkers of susceptibility to CTE, the results should be confirmed in other studies, and the rational behind the association should be elucidated. However, because of ethical, social and legal issues, care should be taken in implementing genetic screening as a tool in occupational health practice [34,35].

**Further research**

Organic solvents are still extensively used in the industrialised world, and much concern has been raised about the interindividual variation in the disposition of solvents and their metabolites and the possibility of individual susceptibility to solvent-induced toxicity.

Variability in styrene toxicokinetics was observed in this study, although this variation seemed not to be related to individual biotransformation capacity. The focus in studying interindividual variability in styrene, or perhaps perfusion-limited solvents in general, should shift from metabolism to physiological factors affecting absorption, distribution and excretion. More knowledge should be gathered about the interindividual variation in these physiological factors. In order to improve biological monitoring of styrene, sources of the large intra- and interindividual variation should be determined. The metabolism of SG to MA and PGA should be studied in more detail. This is of particular importance if PGA is a metabolite responsible for the neurotoxic effects of styrene. Studies with genetic
polymorphisms of biotransformation enzymes can best be performed by studying either capacity-limited solvents (although metabolic clearance normally accounts for only a few percent of total elimination with these solvents), or studying solvents metabolised by enzymes showing ‘null’ polymorphisms. \textit{In vitro} studies and PBPK modelling can be of assistance in determining whether a solvent exhibits capacity-limited or perfusion-limited kinetics.

Research on the mechanism by which solvents cause (neuro)toxicity has been limited so far in toxicology. This is, however, of vital importance as it provides information on whether the parent compound or the metabolites (and which metabolite) are responsible for the toxicity. This information can be used in studies investigating susceptibility, in refining PBPK models and in the risk assessment of the solvent. One of the mechanistic points to be studied, is to determine whether brain metabolism plays an important role in the toxicity of organic solvents and whether interindividual variability in brain metabolism is an important aspect in susceptibility.

In the study of susceptibility to the toxic effects of chemicals, interindividual variation in toxicokinetics is usually highlighted. However, as the toxicity of any chemical is determined by both toxicokinetics \textit{and} toxicodynamics, the importance of interindividual variation in the toxicodynamics of organic solvents should be emphasised.
References


