Inter-individual variability in the interpretation of biological monitoring guidance values

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Inter-individual variability in the interpretation of biological monitoring guidance values

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The incorporation of inter-individual differences in the setting of occupational exposure standards such as biological monitoring guidance values has been studied by investigating the possibility of transferring in vitro measurements of variability in metabolism to the in vivo situation. The generation of generic information on the activity of two major enzymes systems considered important in the metabolism of industrial chemicals, was investigated by using 'probe' substrates. Variability in the activities of cytochromes P450 1A2 and 2E1, and the cytosolic glutathione transferases μ and θ were measured in human hepatocytes, hepatic microsomes and cytosols. Variability in the metabolism of styrene, a chemical of industrial importance, was also measured for comparison. The ability to use in vitro data to simulate in vivo variability was investigated by incorporating in vitro data into a PBPK model for styrene.

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1. INTRODUCTION.................................................................................................................. 1
  1.1 PBPK Modelling and Biological Monitoring ................................................................. 1
  1.2 PBPK Modelling in Chemical Risk Assessment .......................................................... 2
  1.3 PBPK Modelling of Inter-Individual Variability ......................................................... 2
  1.4 Incorporating Inter-Individual Variability in the Interpretation of Biological Monitoring Guidance Values .......................................................................................................................... 3
2. MATERIALS AND METHODS ......................................................................................... 7
  2.1 Chemicals, reagents and biological materials .............................................................. 7
  2.2 Enzyme assays and analysis ....................................................................................... 8
3. RESULTS .......................................................................................................................... 19
  3.1 Variation in enzyme activity ...................................................................................... 19
  3.2 Validation of the PBPK model .................................................................................. 30
  3.3 Incorporation of inter-individual variability ............................................................... 32
4. DISCUSSION .................................................................................................................. 35
  4.1 Variation in enzyme activity ...................................................................................... 35
  4.2 Incorporating inter-individual variability into PBPK models .................................... 38
5. CONCLUSIONS .............................................................................................................. 41
REFERENCES ..................................................................................................................... 43
1. INTRODUCTION

The quantitative estimation of dose following industrial or environmental exposure to chemicals is of fundamental importance in the protection of the health and safety of workers. However, there are difficulties in the estimation of dose or ‘body burden’ which are characterised by the fact that dose often fluctuates considerably over time making any correlation with chronic disease difficult. Also, the ‘target’ dose, which is the concentration of chemical at the site of action, cannot be measured directly because biological targets such as organs and tissues are generally, inaccessible in exposed people. Surrogates of target dose i.e. air exposure concentration or biological monitoring (BM) must therefore be used. The advantages and disadvantages of both air and biological monitoring have been discussed (Droz et al., 1991).

Briefly, air monitoring can introduce a large bias into the estimation of the true target tissue dose. This is because variation in factors such as physical workload, skin absorption, the partition coefficient of the chemical of interest, and individual anatomical, physiological and biochemical differences which determine chemical pharmacokinetics, ensure that air monitoring is, more often than not, a crude indicator of the biologically active dose (Droz et al., 1991). Wide variations in exposure concentrations due to changes in work procedures and practices occurring over periods of months to years can be taken into account during air monitoring. Whereas, short-term within-day and day to day fluctuations are best captured by BM (Droz et al., 1991). Biological monitoring uses measurements of chemicals or their metabolites, known as ‘biomarkers’, in biological media such as blood, urine, breath, saliva, and sometimes tissues, as an index of absorption of chemical (Mason and Wilson, 1999). The strength of BM lies in the potential to define total absorbed dose from all routes of exposure i.e. inhalation, dermal penetration and ingestion (Mason and Wilson, 1999). The results are affected by biological variability and in the past the biological basis of this variability has been characterised as problematic (Droz et al., 1991). More recently however, the introduction of biologically based mathematical modelling techniques, has demonstrated the potential to enhance the value of BM by quantitatively accounting for and explaining the underlying biological variability.

1.1 PBPK Modelling and Biological Monitoring

Many biological factors influence the concentration and delivery of a chemical, or a potentially more toxic metabolite of a chemical, to the blood, urine, target organ, or other biological medium. Therefore, by identifying the relationship between a biomarker concentration in an accessible body fluid and the toxic entity at the target site should improve the application of BM to risk assessment as well as exposure assessment. Models of varying complexity have been used to investigate the possible relationships along the exposure, dose-response continuum. The application of physiologically–based pharmacokinetic (PBPK) models is particularly suited to interpreting BM data. The ability to accurately predict the quantity, or the ‘dosimetry’, of a given foreign chemical at the tissue and organ level in the body, following any route of exposure, is the ‘linchpin’ of a sound risk assessment (Yang et al., 1998). The definition of target tissue dosimetry can be achieved using PBPK modelling which is a powerful means of simulating the factors that determine tissue dose, and consequently, correlation with health effects. A PBPK model is an independent structural model, comprising the tissues and organs of the body with each perfused by, and connected via, the vascular system. Chemical-specific kinetic data, which is dependent on organ and tissue solubility, plasma protein binding, and metabolic rate constants, is overlaid on model predictions. The value of such models is that they are tools for integrating mechanistic pharmacokinetic and toxicologic information through their explicit mathematical description of important anatomical, physiological and biochemical determinants of chemical uptake, disposition and elimination. Model compartments correspond either to specific, localised anatomical structures such as liver, lung, kidney etc. or to types of tissues–such as fat, muscle, viscera and bone–that are distributed throughout the body. Actual
physiological parameters such as breathing rates, blood flow rates and tissue volumes used in
the models to describe the pharmacokinetic process are obtained from the literature or can be
obtained by experiment. These physiological parameters are coupled with chemical-specific
parameters such as partition coefficients (tissue retention of chemicals related to solubility in
biological media and binding to specific proteins in tissues) and metabolic constants to predict
the dynamics of a compound’s movement through a biological system. The advantage of the
physiologically based model is that by simply using the appropriate physiological, biochemical
and metabolic parameters, the same model can be utilised to describe the dynamics of chemical
transport and metabolism in any species, e.g., mice, rats and humans.

1.2 PBPK Modelling in Chemical Risk Assessment
Quantitative chemical risk assessment requires the definition of a toxicologically relevant
measure of dose known as a ‘dose surrogate’. The term dose surrogate or ‘dose metric’ is used
to describe the different way of expressing a target tissue dose estimate calculated using a PBPK
model. The ability to use a dose surrogate in chemical risk assessment ultimately depends on
knowledge of the relationship between the measure of tissue dose and cellular response
involved in a toxic endpoint. There have been many attempts at incorporating pharmacokinetic
data in risk assessments, which have used a measure of dose, such as peak blood concentration
of parent chemical ($C_{\text{max}}$) or total amount metabolised. The underlying principle is the use of the
most up-to-date knowledge on the mode of action of the chemical at the cellular and sub-
cellular level. For example, the neurological effects of short-term exposure to solvents such as
trichloroethylene are rapidly reversible. This suggests that the parent chemical may disrupt
normal lipophilic cellular membrane function via a physicochemical effect. An appropriate
dose metric for such an effect would be the $C_{\text{max}}$ or AUC for the parent chemical in the brain.
Since tissue-blood partition coefficients are relatively uniform across species, $C_{\text{max}}$ in the blood
can be used as a surrogate.

1.3 PBPK Modelling of Inter-Individual Variability
It is well known that individuals do not respond in the same way following exposure to the same
concentration of chemical under identical conditions. The broad range of susceptibilities to the
biological effects of chemical exposure is due to the heterogeneity of the human population.
Sources of inter-individual variability include exposure, hereditary factors such as enzyme
polymorphisms and physiology, and environmental factors, such as diet and lifestyle. The most
obvious factors affecting uptake, distribution and clearance of lipophilic volatile organic
chemicals (VOCs) are body–build, respiratory ventilation rate, blood lipid content and
metabolic capability (Monster, 1979; Monster and Houtkooper, 1979; Fiserova-Bergerova et al.,
1980; Sato et al., 1991; Allen et al., 1996; Clewell III and Andersen, 1996). The inclusion of
biologically realistic parameters, which help explain the basis of observed data, also affords the
ability to incorporate and estimate the impact of inter-individual differences on chemical
disposition.

1.3.1 Monte Carlo Analysis
The variability of the parameters, which contribute to human inter-individual variability in
susceptibility to chemical exposure, can be estimated using Monte Carlo analysis. The Monte
Carlo method is a form of uncertainty analysis that allows the propagation of uncertainty
through a model, which results in an estimate of the variance in model output. A PBPK model
is run with parameter values sampled from distributions that reflect the observed variation in
each pharmacokinetic parameter in the human population. For example, the coefficients of
variation for various mixed-function oxidase enzymes such as the isoforms of cytochrome P450
can be based on reported variations (Bois et al., 1996; Thomas et al., 1996). The distributions
of partition coefficients for many compounds have been reported (Gargas et al., 1989) and the
distributions of physiological and anatomical parameters are also readily available (ICRP, 1975; Arms and Travis, 1988; Brown et al., 1997). Each time the model is run with a sampled set of parameter values, effectively representing a single hypothetical human being, the appropriate dose metric for the toxicity of interest is output. The process is repeated a large number of times (typically 500 to 1000) to generate a distribution of the dose metric for a simulated population.

1.4 Incorporating Inter-Individual Variability in the Interpretation of Biological Monitoring Guidance Values

The Health and Safety Executive has established a system of non-statutory biological monitoring guidance values (BMGVs) as an aid in the interpretation of BM data (HSE, 1997). There are two types of guidance value: Health guidance value (HGV) and Benchmark guidance value (BGV). HGVs are set at a level at which there is no indication from the scientific evidence available that the substance being monitored can cause harm. On the other hand, BGVs are not health based; instead they are achievable levels set at the 90th percentile of available BM results collected from a representative sample of workplaces with good occupational hygiene practices (HSE, 1997). Therefore the impact of inter-individual variability on HGVs, which involves reference to a health effect, is equivalent to variability in BM in general. However, in the establishment of a BMGV no consideration of inter-individual variability is given although there is an assumption of exposure to an invariant concentration of substance by the inhalation or dermal route.

1.4.1 Measurement of Variance

Risk assessment and the setting of occupational exposure standards would ideally be based on quantitative epidemiological studies, which attempt to define dose-response relationships. As discussed previously, the characterisation of dose is difficult when based on air monitoring. This is also primarily the reason for the large numbers of subjects, characteristic of most epidemiological studies, required to achieve the necessary power (Nieuwenhuijsen et al., 2000). The use of in vitro metabolism data incorporated into ‘population’ PBPK models may offer an alternative approach. Human health risk assessments for chemicals may be refined to reflect variability by characterising the expression of enzymes in the liver, as this organ is the major site of xenobiotic metabolism. This may be achieved by the quantification and extrapolation of metabolic rate constants derived in vitro, combined with their use as input parameters in a PBPK model (Lipscomb and Kedderis, 2002). The determination of in vitro metabolic rate constants in multiple human liver samples can yield valuable qualitative information on human variance. However, such data must be put into a realistic context, i.e., the intact human, in order to yield the most valuable predictions of metabolic differences among humans. For quantitative metabolism data to be most valuable in risk assessment, they must be tied to human anatomy and physiology, and the impact of their variance evaluated under real exposure scenarios. Metabolic constants derived in vitro may be extrapolated to the intact liver, when appropriate conditions are met (Houston, 1994; Kedderis, 1997; Houston and Kenworthy, 2000; Lipscomb and Kedderis, 2002). The limiting rate of metabolism, $V_{\text{max}}$, can be scaled directly to the concentration of appropriate enzyme contained in the liver. Environmental, genetic and lifestyle factors can influence the concentration of the cytochrome P450 enzymes in the liver by affecting the actual expression of the enzyme in the endoplasmic reticulum of the cell or the amount of endoplasmic reticulum itself expressed (Lipscomb and Kedderis, 2002). The endoplasmic reticulum spontaneously forms vesicles, known as microsomes, during tissue homogenisation, which may be isolated by differential centrifugation during sub-cellular fractionation. Therefore, the efficiency of recovery of the microsomal fraction has a major impact on the amount of enzyme activity measured (Houston and Carlile, 1997). The coefficients of variability of the in vitro metabolic rate constants may be included in a PBPK
model to investigate the extent to which human inter-individual variability in enzyme activity can influence the toxification/detoxification routes of xenobiotic metabolism.

1.4.2 The Inherited Basis of Interindividual Differences in Chemical Metabolism

When an allele of a given gene has the same mutation in at least 1% of the population, the gene is said to exhibit a polymorphism (Boobis, 2002). Polymorphisms can be divided into two types. (1) Functional polymorphisms represent a change in the DNA sequence which, results in the altered expression (phenotype) or function of the protein. The presence of functional polymorphisms results in more than one phenotype for that gene in a species (Boobis, 2002). (2) Nonfunctional polymorphisms describe those changes in the DNA sequence which have no effect on the expression or activity of the protein (Boobis, 2002). Inherited differences in individual drug and chemical metabolising enzymes are typically monogenic traits i.e., differences in a single gene (Evans and Johnson, 2001). The influence of such a difference on the pharmacokinetic and pharmacodynamic effects of a xenobiotic is determined by the importance of these polymorphic enzymes on the activation and deactivation rates of such substrates (Evans and Johnson, 2001). Polymorphisms known to affect the pharmacokinetics and pharmacodynamics of xenobiotic chemicals have been described as pharmacogenetic or toxicogenetic polymorphisms (Boobis, 2002). It is likely that almost every gene involved in xenobiotic metabolism is subject to polymorphism. Initially, inherited differences were discovered following clinical observations of marked differences in drug response (Mahgoub et al., 1977). However, subsequent studies have demonstrated that phenotypic consequences may be subtle, such as placing an individual at one end or the other of a distribution of xenobiotic metabolism phenotypes, instead of conferring a complete deficiency of encoded enzyme (Evans and Johnson, 2001). Thus, inactivating polymorphisms can be broadly categorised into two groups, those that confer complete or near-complete loss of activity of encoded proteins and those that confer more subtle changes in function via modest changes in expression, regulation, stability, or catalytic activity, but without complete loss of function (Evans and Johnson, 2001).

1.4.3 Demonstration of Approach Using Styrene as a Model Chemical

Many industrial and environmental chemicals, including carcinogens, may be metabolically activated and deactivated. Styrene (ST) is such a chemical. In humans, more than 80% of inhaled ST is taken up and oxidized (activated) by cytochrome P450 2E1 (CYP2E1) and P450 2B6 (Nakajima et al., 1994) to styrene-7,8-oxide (SO) which exists in two enantiomeric forms, R-SO and S-SO (Delbressine et al., 1981). The enantiomeric forms of SO may be further hydrolysed (deactivated) by microsomal epoxide hydrolase (mEH) to phenylethylene glycol with further oxidation by alcohol dehydrogenase and aldehyde dehydrogenase leading eventually to the urinary metabolites, mandelic acid (MA) and phenylglyoxylic acid (PGA). A minor route of SO deactivation in humans (about 1% of inhaled ST) is conjugation with glutathione (GSH) which eventually leads to the appearance in the urine of phenylethyleneethyl mercapturic acids (PHEMAs) (Hallier et al., 1995). SO is a known non-genotoxic (Sarangapani et al., 2002) carcinogen in mice, therefore the disposition of SO following ST metabolism is a key determinant of the carcinogenic potential of ST. Large differences have been observed in the rates of activation and deactivation processes in vitro using rat, mouse and human tissues. Differences in activation/deactivation (A/D) ratios in rats and mice have been shown to correlate with the risk of cancer from inhalation of butadiene, a chemical metabolised by the same enzymes as ST. Therefore, the concept of an A/D ratio may be applicable to ST toxicity. Individuals who have a high A/D ratio (rapid activation and slow deactivation) and are exposed to high concentrations of ST may have a higher risk of cancer. The aim of this study was to use ST as a model chemical to:
• Determine *in vitro* the coefficients of variation of two important routes of xenobiotic metabolism and their use in a PBPK model to simulate inter-individual variability *in vivo*.

• Investigate how inter-individual variability can be used to interpret and set BMGVs.

A number of *in vitro* techniques employing commercially available human hepatic microsomes, cytosol and cryopreserved hepatocytes were to be explored for their use in the generation of kinetic data.

The specific objectives were to:

• determine the coefficient of variation and specific activity of cytochromes P450 2E1 & 1A2 in a battery of human microsomes,

• determine the coefficient of variation and specific activity of GST μ & θ in a battery of human hepatic cytosol preparations,

• determine the coefficient of variation and specific activity of GST μ & θ in a battery of human blood samples,

• measure the *in vitro* rate of oxidative biotransformation of styrene in human microsomes at physiological pH and temperature,

• measure the *in vitro* rate of oxidative biotransformation of styrene in human hepatocytes and GSH conjugation of styrene in the same preparation at physiological pH and temperature,

• calibrate and validate existing human PBPK models for styrene using available human data,

• to scale up *in vitro* biokinetic data to *in vivo* and input into human PBPK models,

• test models with scaled biokinetic data against human data,

• incorporate measured coefficients of variation for metabolic parameters and perform Monte Carlo uncertainty analysis on PBPK output,

• Propose a range of variation about mean BMGV value for ST.
2. MATERIALS AND METHODS

2.1 Chemicals, reagents and biological materials
Chlorzoxazone, 6-hydroxychlorzoxazone, 3-cyano-7-ethoxycoumarin and 3-cyano-7-hydroxycoumarin (Ultrafine, Manchester, UK). NADP+, glucose-6-phosphate, magnesium chloride, glucose-6-phosphate dehydrogenase and perchloric acid containing phenacetin (Sigma-Aldrich, Poole, UK). Styrene (inhibited with 10-15 ppm 4-tert-butylcatechol) styrene glycol (Sigma Aldrich Gillingham, UK). 1-chloro-2,4-dinitrobenzene (CDNB); 1,2-epoxy-3-(4-nitrophenoxy)-propane (ENPP); and trans-4-phenyl-but-3-en-2-one (tPBO) (Acros Organics, Loughborough, UK). All other chemicals used were of the highest available grade.

Human hepatic microsomes, cytosols, and cryopreserved hepatocytes (Tables 1, 2 and 3) in the form of liverbeads (Liverbeads™) and SoftCell recovery medium were obtained from a commercial supplier (TCS Cellworks Ltd., Botolph Claydon, UK distributors for Biopredic, Rennes, France). Two ampoules (batch L1991) containing 4-4.5 \times 10^6 hepatocytes prepared from a 238g male Sprague Dawley rat with a reported viability of \geq 85\% were obtained from the same supplier and used initially to practice the procedures before using human hepatocytes.

<table>
<thead>
<tr>
<th>Microsome identification</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Disease status</th>
<th>Total CYP content (pmol P450/mg microsomal protein)</th>
<th>Microsomal protein concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC259002</td>
<td>37</td>
<td>M</td>
<td>Steatosis</td>
<td>663</td>
<td>31.1</td>
</tr>
<tr>
<td>MIC259006</td>
<td>53</td>
<td>M</td>
<td>Myocardial infarction</td>
<td>434</td>
<td>31.7</td>
</tr>
<tr>
<td>MIC259007</td>
<td>52</td>
<td>M</td>
<td>Angioma</td>
<td>539</td>
<td>24.8</td>
</tr>
<tr>
<td>MIC259009</td>
<td>74</td>
<td>M</td>
<td>Liver metastasis</td>
<td>629</td>
<td>34.7</td>
</tr>
<tr>
<td>MIC259018</td>
<td>not known</td>
<td>F</td>
<td>-</td>
<td>434</td>
<td>15.3</td>
</tr>
<tr>
<td>MIC259015</td>
<td>62</td>
<td>F</td>
<td>Liver metastasis</td>
<td>659</td>
<td>20.4</td>
</tr>
<tr>
<td>MIC259021</td>
<td>45</td>
<td>F</td>
<td>Angioma</td>
<td>571</td>
<td>14.3</td>
</tr>
<tr>
<td>MIC259011</td>
<td>59</td>
<td>F</td>
<td>Gall bladder adenocarcinoma</td>
<td>556</td>
<td>17.2</td>
</tr>
<tr>
<td>MIC259016</td>
<td>49</td>
<td>M</td>
<td>-</td>
<td>430</td>
<td>16.3</td>
</tr>
<tr>
<td>MIC259017</td>
<td>not known</td>
<td>F</td>
<td>-</td>
<td>410</td>
<td>19.3</td>
</tr>
</tbody>
</table>
Table 2  
Human hepatic cytosol demographics

<table>
<thead>
<tr>
<th>Cytosol identification</th>
<th>Age</th>
<th>Sex</th>
<th>Disease status</th>
<th>Protein content (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I006HL</td>
<td>62</td>
<td>F</td>
<td>Liver metastasis</td>
<td>42.7</td>
</tr>
<tr>
<td>I019HL</td>
<td>45</td>
<td>F</td>
<td>Angioma</td>
<td>36.3</td>
</tr>
<tr>
<td>I016HL</td>
<td>52</td>
<td>M</td>
<td>Angioma</td>
<td>35.7</td>
</tr>
<tr>
<td>CYT259006‡</td>
<td>53</td>
<td>M</td>
<td>Myocardial infarction</td>
<td>30.2</td>
</tr>
<tr>
<td>CYT259007‡</td>
<td>52</td>
<td>M</td>
<td>Angioma</td>
<td>28.4</td>
</tr>
<tr>
<td>CYT259008</td>
<td>64</td>
<td>M</td>
<td>Liver metastasis</td>
<td>16.3</td>
</tr>
<tr>
<td>CYT259009‡</td>
<td>74</td>
<td>M</td>
<td>Liver metastasis</td>
<td>45.7</td>
</tr>
<tr>
<td>CYT259010</td>
<td>40</td>
<td>F</td>
<td>Benign tumor</td>
<td>15.8</td>
</tr>
</tbody>
</table>

‡ cytosol preparations came from the same donors listed in Table 1

Table 3  
Liverbeads™ immobilized human hepatocytes demographics

<table>
<thead>
<tr>
<th>Hepatocyte Identification</th>
<th>Age</th>
<th>Sex</th>
<th>Disease status</th>
<th>Viability reported by supplier %</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVB005001</td>
<td>77</td>
<td>M</td>
<td>Liver metastasis</td>
<td>79</td>
</tr>
<tr>
<td>L1545</td>
<td>50</td>
<td>M</td>
<td>Liver metastasis</td>
<td>82</td>
</tr>
<tr>
<td>L1247</td>
<td>45</td>
<td>F</td>
<td>Carcinoma</td>
<td>82</td>
</tr>
<tr>
<td>L1239</td>
<td>43</td>
<td>F</td>
<td>Benign tumour</td>
<td>82</td>
</tr>
<tr>
<td>HUMA136</td>
<td>58</td>
<td>F</td>
<td>Liver metastasis</td>
<td>86</td>
</tr>
<tr>
<td>HUMA048</td>
<td>71</td>
<td>F</td>
<td>Liver metastasis</td>
<td>77</td>
</tr>
</tbody>
</table>

2.2 Enzyme assays and analysis

2.2.1 Microsomal chlorzoxazone hydroxylation

Duplicate samples were prepared in 0.5 ml 50 mM phosphate buffer (pH 7.4) containing 0.5 mg/ml microsomal protein, 1.3 mM NADP⁺, 3.3 mM glucose-6-phosphate, 5 mM magnesium chloride, 0.4 U/ml glucose-6-phosphate dehydrogenase and chlorzoxazone in the concentration range 0 - 600 µmol/l. Incubations were initiated by addition of chlorzoxazone. Samples were incubated for 10 minutes at 37°C in a water bath. The reaction was stopped by addition of 100 µl 6% perchloric acid containing phenacetin as internal standard and mixed by vortexing. Tubes were centrifuged for 5 minutes at 13000 rpm in a bench-top centrifuge. The supernatant was transferred into a glass, screw–cap tube and extracted with 2 ml diethyl ether. The ether fraction was evaporated to dryness under nitrogen and samples were reconstituted in 150 µl 50mM ammonium acetate.

Standards of 6-hydroxychlorzoxazone were prepared in phosphate buffer at 0 - 5 µg/ml (0 - 26.7 µM) and extracted in 2 ml diethyl ether. The ether fraction was evaporated to dryness under nitrogen and samples were reconstituted in 150µl 50mM ammonium acetate. Samples were analysed by HPLC using a Hewlett Packard 1050 fitted with a diode array detector operating at wavelengths of 287 and 246 nm. 6-Hydroxychlorzoxazone was quantified using 287 nm. A guard column filled with Bondapak C18 was used. Separation was performed on a Speroclon ODS(2) 5 µm column (250 mm x 4.6 mm I.D., Phenomenex, Macclesfield, UK) at ambient temperature. The mobile phase composition was ammonium acetate buffer (0.1 M); acetonitrile; tetrahydrofuran (70:24.5:5:5) at a flow rate of 1 ml / min.
Calibration curves were constructed using the peak height ratios of 6-hydroxychlorzoxazone to internal standard. Least-squares linear regression analysis (MS Excel software) was used to determine the slope, intercept and correlation coefficient and to quantify the unknowns. Quality control, was performed by analysing a 0.5 µg/ml (2.7 µM) standard after every 10 samples. The acceptance criterion was ± 15% of nominal concentration (2.3 - 3.1 µM).

*K*<sub>M</sub> and *V*<sub>max</sub> for microsomal hydroxylation of chlorzoxazone were determined using a non-linear regression one-site binding curve fit (GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA).

### 2.2.2 Hepatocytic chlorzoxazone metabolism

Liverbeads<sup>TM</sup> are made of freshly isolated hepatocytes that are immobilised in sodium alginate to form beads approximately 1 mm in diameter (Fremond *et al.*, 1993), which are then frozen and stored in liquid nitrogen. Microencapsulation protects the cells from freeze-thaw damage during cryopreservation and subsequent thawing for use. Liverbeads<sup>TM</sup> therefore have potential as an easily transportable and convenient source of hepatocytes for use in *in-vitro* pharmacotoxicology. It has previously been shown that isolated hepatocytes retain a wide variety of hepatic functions when immobilised in alginate, and many of these functions are well retained following cryopreservation (Guyomard *et al.*, 1996). Liverbeads<sup>TM</sup> metabolised probe substrates at comparative rates to conventional hepatocyte cultures (Hammond *et al.*, 1999).

The probe substrate chlorzoxazone was selected for *in-vitro* incubations with rat liverbeads<sup>TM</sup> in order to:

- Gain experience in handling them and because liver metabolism of this compound is relatively simple and well documented.
- Compare rates of chlorzoxazone metabolism from *in-vitro* studies with published data.

Liverbeads<sup>TM</sup> were handled according to the protocol supplied by Biopredic. An ampoule of liverbeads<sup>TM</sup> was immersed in a water bath at 37°C until almost completely thawed. Beads were then removed using a pipette with the tip cut off to avoid damage to the beads and placed in 10 ml SoftCell recovery medium in a 25 ml Sterilin container. Diluted cells were incubated for 2 minutes at room temperature, gently shaking the tube by hand 3 or 4 times during this period, after which the beads were left to sediment. This typically took 10 - 20 minutes. The supernatant was removed and 5 ml SoftCell Hep medium was added and the beads rinsed by gently shaking. This step was repeated to wash the beads. Cell viability was measured using trypan blue exclusion. Approximately 50 µl drop of bead suspension was removed using a pipette with a cut down tip and placed onto a 35 mm Petri dish. Excess medium was removed from the beads. 100 µl trypan blue diluted to 0.05% in SoftCell Hep medium was added and left at room temperature for 1 minute. Beads were observed under a microscope at ×100 magnification. Dead cells take up the dye and appear blue while live cells exclude the dye. The percentage of live cells was estimated by averaging the number of live and dead cells counted from 3 different beads (one focus per bead).

Liverbeads were seeded into multiwell plates. Either 24– or 48– well plates were used. All medium was removed from sedimented cells and 5.4 ml of SoftCell Hep medium was added for 48–well plates. A bead suspension of 0.5 ml was added to each well whilst continually swirling to keep the beads in suspension. Beads were left to sediment for a few minutes and 300 µl of medium was removed from each well to give a final bead concentration of approximately 1000 beads / ml. The floor of each well should be almost covered (80–90%) with beads. Liverbeads<sup>TM</sup> were left to pre-incubate for 1 hour in an incubator at 37°C. Beads were washed by adding 200 µl SoftCell Hep medium to each well, mixing, then removing 200µl medium after allowing
beads to sediment. Substrate was prepared at 100-fold the desired final concentration in suitable solvent. For chlorzoxazone, acetonitrile was used and final concentration in the incubation mixture ranged from 0–590 µmol/l. Substrate solution (2 µl) was then added to each well and plates were incubated for 1 hour at 37°C. At the end of the incubation, 100 µl of incubation medium was removed and internal standard was added. Samples were extracted and analysed using the same method previously described for microsomes (Section 2.2.1).

2.2.3 3-Cyano-7-ethoxycoumarin O-deethylation
A novel method was developed for measurement of CYP1A2 activity in microsomes using a continuous fluorimetric assay developed on a Cobas Fara centrifugal analyser (Roche, Welwyn Garden City, UK). The substrate 3-cyano-7-ethoxycoumarin (CEC) is de-ethylated to 3-cyano-7-hydroxycoumarin, which is fluorescent at neutral pH values, making it suitable for a continuous assay. The method described below was based on an original assay described by White (White, 1988).

Sample buffer was prepared by dissolving 4.2 mg NADPH and 20.3 mg magnesium chloride in 10 ml 0.15 M phosphate buffer (pH 7.4). CEC substrate solution was prepared by adding 45 µl stock CEC (23.3 mM in DMSO) to 455 µl DMSO. Microsomes were diluted in 0.1 M phosphate buffer (pH7.4) to an approximate microsomal protein concentration of 4 mg/ml. Incubations were performed at 37°C and the reaction was started by addition of CEC. Final concentrations in the incubation mixture were 30 µM CEC, 0.4 mM NADPH, 8.5 mM magnesium chloride and 20 µg microsomal protein in a total volume of 350 µl. Production of the metabolite 3-cyano-7-hydroxycoumarin was followed for 10 minutes using an excitation wavelength of 408 nm and measuring emission at 450 nm. The assay was calibrated using a factor, which was determined by measuring the fluorescence of a 53 µM solution of 3-cyano-7-hydroxycoumarin measured in the same way as a normal sample.

Enzyme kinetic data in human liver microsomes was generated by measuring CEC de-ethylase activity at different substrate concentrations. The data was analysed as described above for chlorzoxazone.

2.2.4 Styrene biotransformation
Standard incubation mixtures for the microsomal metabolism of styrene contained the following components: 0.05M phosphate buffer (pH 7.4); 0.5 mg/ml microsomal protein; 1.3 mM NADPH; 3.3 mM glucose-6-phosphate; 5 mM magnesium chloride; and 0.4 µg/ml glucose-6-phosphate dehydrogenase in a total volume of 0.5 ml. After 3 minutes pre-incubation in a water bath at 37°C, reactions were initiated by addition of 5µl substrate in acetonitrile and mixed by inversion. Acetonitrile has been previously shown to cause the least inhibition to cytochrome P450 activities of the commonly used solvents (Chauret et al., 1998) and does not inhibit CYP2E1 activity at a concentration of 1%. Substrate was prepared in acetonitrile at 100 times the desired final concentration in the incubation mixture. Final substrate concentrations in microsomal incubations were 5-5000 µM for styrene. Duplicate incubations were performed at each substrate concentration. Microsomal incubations were carried out at 37°C for 20 minutes.

Metabolite production was determined to be linear with respect to both microsomal protein concentration and incubation time under these conditions at high and low substrate concentrations. After 20 minutes, incubations were stopped by addition of 50 µl 3M sulphuric acid, cooled on ice and centrifuged to precipitate proteins. Supernatants were removed for analysis of metabolite production. The styrene metabolite, styrene glycol was quantified by HPLC (250 mm x 4.6 mm ID column packed with Speroclide ODS(2) (Phenomenex, Macclesfield, UK); mobile phase, 10% acetonitrile in water containing 0.25% orthophosphoric acid; flow rate, 1 ml/min; detection wavelength, 200 nm).
K_M and V_max values for microsomal metabolism of styrene were determined using non-linear, two-site binding regression analysis (GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego CA, USA).

2.2.5 Variation in glutathione transferase activity

CDNB is a general GST substrate widely used for quantifying GST activity in tissue extracts. In a previous study, ENPP activity was not detectable in human liver cytosol (Schulz et al., 2000), however in rats ENPP is predominantly metabolised by GST Theta (GSTθ). tPBO is a specific substrate for GST Mu (GSTµ).

Cytosols prepared from individual human livers were obtained from TCS Cellworks Ltd. and were characterised for total protein concentration (Table 2). Spectrophotometric assays to determine GST activity of human liver cytosols towards CDNB, ENPP, and tPBO were developed on a Cobas Fara centrifugal analyser (Roche, Welwyn Garden City, UK). Incubation conditions were based on those previously described by Mannervik and Widersten (Mannervik and Widersten, 1995) and are outlined in Table 4. All incubations were performed in 0.1 M sodium phosphate buffer (pH 6.5) at 37°C. Cytosols were pre-incubated for 2 minutes with respective substrates before the reaction was initiated by addition of glutathione. Incubation conditions were chosen so that metabolite production, i.e., the respective glutathione conjugates of the three substrates, was linear with respect to protein concentration and incubation time.

Table 4

Incubation conditions used for measuring human liver cytosol GST activity towards individual substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Substrate concentration range (mM)</th>
<th>GSH concentration (mM)</th>
<th>Protein concentration (µg/ml)</th>
<th>Incubation time (min)</th>
<th>Detection wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDNB</td>
<td>0.03 - 5</td>
<td>1</td>
<td>8</td>
<td>5</td>
<td>340</td>
</tr>
<tr>
<td>ENPP</td>
<td>0.005 - 0.5</td>
<td>5</td>
<td>200</td>
<td>6</td>
<td>360</td>
</tr>
<tr>
<td>tPBO</td>
<td>0.015 - 0.3</td>
<td>0.25</td>
<td>200</td>
<td>8</td>
<td>290</td>
</tr>
</tbody>
</table>

2.2.6 In vitro to in vivo scaling of metabolic rate constants

In order to use in vitro metabolic rate constants to produce quantitative information they must be input within a biologically realistic framework such as a PBPK model. Scaling of in vitro to in vivo was achieved by assuming a hepatic microsomal protein yield of 35 mg g liver⁻¹ (Brian Houston, personal communication) which is similar to the 32.3±2.3 reported by Pacifici et al., (Pacifici et al., 1988) although somewhat lower than the 60.1 mg g liver⁻¹ reported by Carlile et al., (Carlile et al., 1997). However, the value reported by Brian Houston, which is to be published in the peer reviewed literature, is considered more reliable as it has been determined from extensive studies conducted over the past few years. Thus, the in vitro rate constants reported here would be multiplied by the microsomal protein yield to give the number of moles per gram liver. This value can then be multiplied by the weight of the liver to give a V_max per min per liver. Multiplying by 60 converts this value to moles per hour per liver.

Scaling of cytosolic glutathione transferases was conducted in a similar way except that a cytosolic protein yield of 46.5 mg per g liver was used (Pacifici et al., 1988).
2.2.7 Presentation of variability of Michaelis-Menten constants

It is pertinent to emphasise an important point about the form in which variation in metabolic rate constants should be presented. The best measure of enzyme efficiency is $K_{\text{cat}}/K_M$, (Guengerich et al., 1999) where $K_{\text{cat}}$ ($\text{min}^{-1}$) is the intrinsic catalytic activity of an enzyme,

$$\frac{K_{\text{cat}}}{K_M} = \frac{\text{mol min}^{-1}(\text{mg enzyme protein})^{-1}}{\text{mol enzyme (mg microsomal protein)}^{-1}}$$

This parameter is determined by measuring the actual enzyme concentration using an immunochemical assay (Guengerich et al., 1999). However, measurement of enzyme concentration can be difficult, and so, the next best measure of enzyme efficiency, $CL_{\text{int}}$ ($\text{L h}^{-1} \text{g liver}^{-1}$) is most often used. Therefore, the correct way is to express the variability as intrinsic clearance, $CL_{\text{int}}$, which is the ratio $V_{\text{max}}/K_M$ of any enzyme, when substrate concentrations are low and $V_{\text{max}}$ when substrate concentrations are saturating. Reporting the variation in $V_{\text{max}}$ or $K_M$, determined in a number of different preparations, in isolation of each other, may in fact give an erroneous measure of variation. This is because $V_{\text{max}}$ and $K_M$ are inter-related parameters i.e., $K_M$ cannot be determined without $V_{\text{max}}$. This means that the lowest $K_M$ value cannot be matched with the highest $V_{\text{max}}$, and vice versa, which would give more extreme $CL_{\text{int}}$ values than actually measured. However, a more fundamental flaw in doing this would be the fact that one would be using constants derived from different graphs.

2.3 The PBPK model for Styrene and styrene oxide

The PBPK model for ST and SO described by Csanády et al. (Csanády et al., 1994) and Tornero-Velez and Rappaport (Tornero-Velez and Rappaport, 2001) were modified to include uptake of SO by inhalation and the changes in regional blood flow to organs and tissues which occur with changes in work rate (WR). Further, the production, distribution and excretion of mandelic (MA) and phenyglyoxylic (PGA) acids were also added. A diagram of the model is shown in Figure 1. The systemic circulation distributes ST and SO to the liver (the only metabolising organ), fat, muscle and the richly perfused tissues. All the organs and tissues were assumed to be homogeneous, well–mixed compartments with human physiological and biochemical parameters listed in Table 5. The chemical–dependent physicochemical and biochemical constants used in the model are shown in Table 6. Simulation of the changes in regional blood flow occurring at different WRs was achieved by incorporating the appropriate equations for each organ or tissue (Table 5). The latter equations were obtained by fitting a trend line to the changes in fraction of cardiac output perfusing a given organ or tissue against WR in watts (W). These data were adapted from Table 3, p.109 from Fiserova-Bergerova (1983) (Fiserova-Bergerova, 1983) and shown in Figure 2. The distribution of MA and PGA to the liver, fat, muscle and the richly perfused tissue compartments only were described in the sub-models for these metabolites. Exhalation was assumed to be negligible because the saturated vapour pressures of MA and PGA are very low, therefore a lung compartment and blood:air partition coefficients were considered unnecessary. The liver, fat, muscle and richly perfused tissue partition coefficients for the MA and PGA sub-models were predicted using the tissue-composition based algorithm of Baláž and Lukáčová (Baláž and Lukáčová, 1999). This algorithm requires Log P (octanol:water) values as inputs. Calculated Log P values for both MA and PGA were obtained from the SRC on-line Log P calculator (http://esc.syrres.com/interkow/kowdemo.htm). The proportions of MA and PGA measured in the urine relative to total ST metabolism and the first-order urinary excretion rates for the initial rapid phase (0-20 hr for MA and 0-50 hr for PGA) were obtained from Guillemin and Berode (Guillemin and Berode, 1988).
Figure 1. Schematic of the styrene, styrene oxide, mandelic and phenylglyoxylic acid PBPK model
Table 5. Work rate induced changes in physiological parameters used in the PBPK model

<table>
<thead>
<tr>
<th>Physiological parameters</th>
<th>Mathematical relationship describing change in rate due to work rate (WR)</th>
<th>Values for standard man (70 kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiac output (QC) (l/h)</td>
<td>( QC = \frac{-0.0001 \times WR^3 + 0.0202 \times WR^2 + 3.652 \times WR + 350.4}{70^{0.81}} )</td>
<td>Rest 350.4 571 50W</td>
</tr>
<tr>
<td>Alveolar ventilation (QP) (l/h)</td>
<td>( QP = \frac{-0.0005 \times WR^3 + 0.0852 \times WR^2 + 13.544 \times WR + 347.9}{70^{0.74}} )</td>
<td>347.9 1175.6</td>
</tr>
<tr>
<td>Organ and tissue Perfusion rates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat (QF) (l/h)</td>
<td>( QF = -4 \times 10^{-6} \times WR^2 + 0.0006 \times WR + 0.0489 )</td>
<td>17.1 39.3</td>
</tr>
<tr>
<td>Liver (QL) (l/h)</td>
<td>( QL = 6 \times 10^{-6} \times WR^2 - 0.0023 \times WR + 0.2597 )</td>
<td>90.9 91.2</td>
</tr>
<tr>
<td>Muscle tissue (QT) (l/h)</td>
<td>( QT = -2 \times 10^{-5} \times WR^2 + 0.0058 \times WR + 0.2514 )</td>
<td>88.1 280.6</td>
</tr>
<tr>
<td>Richly perfused (QR) (l/h)</td>
<td>( QR = 1 - (QF + QL + QT) )</td>
<td>154.2 159.9</td>
</tr>
<tr>
<td>Compartment volumes as a fraction of body weight (BW)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat</td>
<td>0.19</td>
<td>Richly perfused</td>
</tr>
<tr>
<td>Liver</td>
<td>0.026</td>
<td>Muscle</td>
</tr>
</tbody>
</table>

* Adapted from (Fiserova-Bergerova, 1983)
Table 6
Physiological and biochemical parameters and partition coefficients used in the model

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Symbol</th>
<th>Value (mean±SD)</th>
<th>Population Distributiona</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome P450 (high affinity)</td>
<td>$V_{max}$</td>
<td>0.0024±0.001 b</td>
<td>U</td>
<td>mmol/h/g liver</td>
</tr>
<tr>
<td></td>
<td>$K_{M}$</td>
<td>0.014±0.006 b</td>
<td>U</td>
<td>mmol/l</td>
</tr>
<tr>
<td>Cytochrome P450 (low affinity)</td>
<td>$V_{max}$</td>
<td>0.0069±0.002 b</td>
<td>U</td>
<td>mmol/h/g liver</td>
</tr>
<tr>
<td></td>
<td>$K_{M}$</td>
<td>0.691±0.20 b</td>
<td>U</td>
<td>mmol/l</td>
</tr>
<tr>
<td>Epoxide hydrolase</td>
<td>$V_{max}$</td>
<td>0.0045±0.0025 c</td>
<td>U</td>
<td>mmol/h/g liver</td>
</tr>
<tr>
<td></td>
<td>$K_{M}$</td>
<td>0.001±0.0002 d</td>
<td>U</td>
<td>mmol/l</td>
</tr>
<tr>
<td></td>
<td>$K_{Mapp}$</td>
<td>0.01±0.002 d</td>
<td>U</td>
<td>mmol/l</td>
</tr>
<tr>
<td>Glutathione transferase</td>
<td>$V_{max}$</td>
<td>0.028±0.0084 e</td>
<td>L</td>
<td>mmol/h/g liver</td>
</tr>
<tr>
<td></td>
<td>$K_{M}$</td>
<td>0.1±0.02 d</td>
<td>U</td>
<td>mmol/l</td>
</tr>
<tr>
<td></td>
<td>$K_{M}$</td>
<td>2.5±0.05 d</td>
<td>U</td>
<td>mmol/l</td>
</tr>
<tr>
<td>Initial concentration of cytosolic GSH</td>
<td>$C_{GSH0}$</td>
<td>5.9j</td>
<td>N</td>
<td>mmol/l</td>
</tr>
<tr>
<td>Elimination rate constant for GSH turnover</td>
<td>$K_{GSH}$</td>
<td>0.2±0.06 d</td>
<td>U</td>
<td>l/h</td>
</tr>
<tr>
<td>Urinary excretion rates$^I$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MA</td>
<td>$K_{MA}$</td>
<td>0.074-0.177</td>
<td>U</td>
<td>/h</td>
</tr>
<tr>
<td>PGA</td>
<td>$K_{PGA}$</td>
<td>0.058-0.076</td>
<td>U</td>
<td>/h</td>
</tr>
<tr>
<td>Proportion of Metabolism$^I$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MA</td>
<td>$P_{MA}$</td>
<td>0.05-0.56</td>
<td>U</td>
<td></td>
</tr>
<tr>
<td>PGA</td>
<td>$P_{PGA}$</td>
<td>0.05-0.33</td>
<td>U</td>
<td></td>
</tr>
<tr>
<td>Partition coefficients</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood:air</td>
<td>ST$^b$</td>
<td>48</td>
<td>SO$^b$</td>
<td>MA$^I$</td>
</tr>
<tr>
<td>Fat:blood</td>
<td>2370$^g$</td>
<td></td>
<td>6.1</td>
<td>1.99</td>
</tr>
<tr>
<td>Liver:blood</td>
<td>2.61</td>
<td>2.6</td>
<td>1.03</td>
<td>1.33</td>
</tr>
<tr>
<td>Richly</td>
<td>2.60</td>
<td>2.6</td>
<td>1.03</td>
<td>1.33</td>
</tr>
<tr>
<td>pefused:blood</td>
<td>1.96</td>
<td>1.5</td>
<td>1.06</td>
<td>1.15</td>
</tr>
<tr>
<td>Muscle:blood</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ (N=normal, U=uniform)

$^b$ Measured in vitro

$^c$ Coefficient of variation taken from (Fennell and Brown, 2001)

$^d$ Coefficient of variation taken from (Tornero-Velez and Rappaport, 2001)

$^e$ Variability measured in vitro using 1-chloro-2,4-dinitrobenzene

$^f$ Coefficient of variation not available

$^g$ (Tornero-Velez and Rappaport, 2001)

$^h$ (Csánády et al., 1994)

$^i$ (Guillemin and Berode, 1988)
Calculated (Baláž and Lukácová, 1999)

Figure 2. Change in rate of perfusion with changing work rate. The symbols represent the fraction of cardiac output perfusing various organs and tissues (adapted from (Fiserova-Bergerova, 1983)) as follows: (■) (QFC) fat compartment, (▲) (QLC) liver, (○) (QTC) muscle compartment.

The changes in cardiac output (QCC) and alveolar ventilation (QPC) rates were calculated in a similar manner (data not shown). To ensure the model did not violate mass balance the flow rate to the richly perfused compartment (QRC) was set equal to the difference between the cardiac output and the flows to the remaining tissues (Table 5). The fitting of trend lines was conducted in Microsoft EXCEL 97 SR-2 (Microsoft Corporation). The model was coded in Berkeley Madonna version 8.0.1 (http://www.berkeleymadonna.com/) for initial visualisation and exercising. For Monte Carlo analysis the model was coded in MCSIM version 4.2.0 (http://www.ibiblio.org/pub/gnu/mcsim/).

2.3.1 Model validation
The PBPK model was validated using published measured in vivo data on ST and SO pharmacokinetics. The data sets for ST were taken from Ramsey and Young and Wigaeus et al (Ramsey and Young, 1978; Wigaeus et al., 1983) and for SO from Korn et al (Korn et al., 1994). The latter were occupational exposure data where time-weighted average exposure concentrations of ST in ambient air of 10 to 73 ppm were measured during periods of 248-325 min (4.13-5.42 h). In order to generate model predictions for this data, various exposure concentrations ranging from 10-73 ppm over a period of 4.13 and 5.42 h were simulated. Linear regression analysis of the predicted data was performed in GraphPad Prism version 3 (San Diego, CA 92121 USA).

Urinary excretion data for MA and PGA were obtained from internal HSL data, some of which was published in the peer-reviewed literature (Wilson et al., 1983). The MA data was obtained from 4 volunteers, aged 45, 45, 39 and 43 weighing 68.9, 64.4, 75 and 86 kg exposed to 50 ppm styrene in the HSL controlled atmosphere exposure facility for 6 h. Urine samples were taken every hour during and following exposure for up to 14 h. Data were expressed as mol per hour. The PGA data was obtained from 2 volunteers, aged 45 and 43 weighing 64.4 and 86 kg exposed to 50 ppm styrene in the HSL controlled atmosphere
exposure facility for 10 h. Urine samples were taken every hour during and following exposure for up to 19 h. Data were expressed as mol per hour.

### 2.3.2 Monte Carlo analysis

The PBPK model was modified for Monte Carlo analysis. The anatomical, physiological, biochemical and physicochemical parameters comprising the PBPK model were assigned distributions (Table 6). The basic PBPK model structure was modified to permit re-sampling of model parameters from within the assigned distributions. Each run essentially generates a hypothetical human being exposed to the same exposure conditions where model output should reflect inter-individual variability.

The variation in metabolic rate constants reported as mean±standard deviation (SD) (Table 6) were determined as follows: the high and low affinity cytochrome P450 rate constants were measured *in vitro* as reported in Section 2.2.4. The SD for EH was calculated by multiplying the $V_{maxeh}$ value of 0.0045 mmol h$^{-1}$ g$^{-1}$ by the *in vitro* CV of 56% measured by Fennell and Brown (Fennell and Brown, 2001). The SD for $K_{Mm}$ and $K_{Mapp}$ was calculated by multiplying the values reported by Csanády et al (Csanády *et al.*, 1994) by the CVs reported by Tornero-Velez and Rappaport (Tornero-Velez and Rappaport, 2001). The SD for $V_{maxGST}$ was calculated by multiplying the value of 0.028 mmol h$^{-1}$ g$^{-1}$ reported by Csanády et al (Csanády *et al.*, 1994) by the CV determined *in vitro* using 1-chloro-2,4-dinitrobenzene. In the absence of appropriate information on allelic frequencies and the corresponding expression levels of enzyme polymorphisms, uniform distributions were used, in most cases, in order to capture the possibility that some individuals will have little or no enzyme activity. In such cases the uniform distribution was set from 0 to mean+SD.

Variation in the urinary excretion of MA and PGA, the two major metabolites of ST, during and after constant exposure to 50 ppm ST for 6 and 10 h in the HSL controlled atmosphere exposure facility was investigated.

Sensitivity analysis of the model determined that $V_{maxmo}$, for the high affinity CYP450 metabolism (probably CYP2E1) was the most important parameter in the activation of ST, and $V_{maxeh}$, the most important parameter in the deactivation of SO. The activation/deactivation (A/D) ratio is the rate of activation of a parent chemical to a toxic entity versus the rate of deactivation of the toxin to a non-toxic entity. In this case it is the ratio of the intrinsic clearance of activation versus the intrinsic clearance of deactivation at low exposure concentrations, which changes to $V_{maxmo}$ versus ($V_{maxeh} + V_{maxGST}$) as exposure concentrations approach saturation of the enzymes involved. Therefore, the A/D ratio was expressed as the net clearance of SO. This was obtained by calculating the intrinsic clearances ($CL_{int}$) i.e., $V_{max}/K_M$ for each enzyme system described in the model. Therefore, $CL_{int}$ for the bioactivation of ST to SO by both high and low affinity CYP450 activity ($CL_{mo}=V_{maxmo}/K_{Mmo}+V_{max2}/K_{M2}$), SO to phenylethylene glycol ($CL_{eh}$) and the gutathione conjugation of SO ($CL_{gst}$) were calculated by importing the model parameters obtained from the Monte Carlo analysis into Microsoft Excel 2000 where the appropriate calculations were effected. The net clearance, $CL_{int}-(CL_{eh}+CL_{gst})$ was plotted against area-under-the-curves of venous SO concentrations ($SO_{AUC}$). This would allow the investigation of the frequency and consequences of various A/D ratios with regard to $SO_{AUC}$, and therefore, potentially the numbers of people at higher risk in any given size of simulated cohort. In this exercise each run essentially generates a hypothetical human being exposed to 50, 100, 200 ppm ST for 8 h per day, 5 days per week with a work load ranging from 40-100 W. A work rate of 50 W is about equivalent to a moderate stroll. 3000 runs were performed generating a hypothetical population of 3000 different people. The dose metric chosen as an output from the model was the area-under-the-curve of venous concentration of SO after an 8 h daily exposure, 5 days a week ($SO_{AUC}$).
3. RESULTS

3.1 Variation in enzyme activity

3.1.1 Kinetics of chlorzoxazone 6-hydroxylation in human liver microsomes

Chlorzoxazone was incubated with human liver microsomes from 10 individuals and the production of 6-hydroxychlorzoxazone measured as an estimate of cytochrome P450 2E1 activity. Operating under linear conditions with respect to incubation time and microsomal protein concentration, the formation of 6-hydroxychlorzoxazone was readily detectable at substrate concentrations of 3 µM. Rate of formation against substrate concentration is shown in Figure 3.

![Figure 3. Hydroxylation of chlorzoxazone by 10 human liver microsomes (mean ± SEM; n=3).](image)

In the 10 individual human liver microsomes used in this study, the mean $K_M$ was 45 µM (range 25–73; CV = 36%) and mean $V_{max}$ was 1.536 nmol 6-hydroxychlorzoxazone minute$^{-1}$ mg microsomal protein$^{-1}$ (range 0.690 - 4.833; CV = 83%). Inter-assay variability was assessed using results obtained for QC samples. Mean QC concentration over 10 runs was 2.9 µmol l$^{-1}$ (SD = 0.35) (Table 7). The method CV was calculated to be 12%. The variation in $V_{max}$ was 7.03 fold whereas CLint varied 3.36 fold (Table 7).

3.1.2 Kinetics of chlorzoxazone 6-hydroxylation in rat cryopreserved hepatocytes

Percentages of live cells recovered from thawed rat Liverbeads™ ranged from 20 to 65%. Typically, cell viabilities obtained were around 60%. One experiment gave very low viability (20%) and the results from this preparation were discounted.

It was assumed that using the Biopredic protocol, 48 well plates contained 333,333 cells per well since one vial containing 4 million hepatocytes was sufficient for 12 wells. This number was multiplied by the percentage of live cells measured by trypan blue exclusion in order to give the number of viable cells per well. Typically, this number was approximately 200,000 live cells per well. 0.2 ml incubation mixtures in each well consisted of 0.05 ml beads and 0.15 ml medium. From the concentration of 6-hydroxychlorzoxazone (6-OHCZX) measured in each sample of incubation medium, the amount of 6-OHCZX in 0.15 ml was calculated. This was the amount of 6-OHCZX produced per hour by the number of live cells in the well.
This concentration was corrected to give the amount of 6-OHCZX nmoles (100,000 hepatocytes)⁻¹ hour⁻¹. Rates of metabolism of chlorzoxazone to 6-hydroxychlorzoxazone by rat Liverbeads™ are presented in figure 4.

Figure 4. Metabolism of chlorzoxazone by rat Liverbeads™ in 3 separate incubations (mean ± SEM; n = 2 or 3).

Data from one of these incubations were fitted to a non-linear regression model to predict Michaelis-Menten kinetic parameters in Figure 5. Using this curve fit, the predicted value for $K_M$ was $38 \mu$mol l⁻¹ and $V_{max}$ was 1.63 nmoles 6-OHCZX (100,000 hepatocytes)⁻¹ hour⁻¹.

Figure 5. Metabolism of chlorzoxazone by rat Liverbeads™ fitted to the Michaelis-Menten equation. Individual data points are shown.
<table>
<thead>
<tr>
<th>Microsomal Preparation</th>
<th>Chlorzoxazone hydroxylation</th>
<th>Styrene high affinity metabolism</th>
<th>Styrene low affinity metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{\text{max}}$ (nmol/min/mg)</td>
<td>$K_{\text{M}}$ (µmol/l)</td>
<td>CL_{int} (l/min)</td>
</tr>
<tr>
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<td>0.6872</td>
<td>34.76</td>
<td>1.98×10^{-5}</td>
</tr>
<tr>
<td>MIC259017</td>
<td>0.8374</td>
<td>39.01</td>
<td>2.15×10^{-5}</td>
</tr>
<tr>
<td>MIC259006</td>
<td>0.8447</td>
<td>34.9</td>
<td>2.42×10^{-5}</td>
</tr>
<tr>
<td>MIC259018</td>
<td>0.913</td>
<td>24.61</td>
<td>3.71×10^{-5}</td>
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<tr>
<td>MIC259007</td>
<td>1.016</td>
<td>54.48</td>
<td>1.86×10^{-5}</td>
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<tr>
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<td>39.19</td>
<td>2.77×10^{-5}</td>
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<td>MIC259015</td>
<td>1.245</td>
<td>73.39</td>
<td>1.69×10^{-5}</td>
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<tr>
<td>MIC259009</td>
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<td>2.98×10^{-5}</td>
</tr>
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<td>MIC259016</td>
<td>2.618</td>
<td>39.24</td>
<td>6.67×10^{-5}</td>
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<td>MIC259002</td>
<td>4.833</td>
<td>72.7</td>
<td>6.65×10^{-5}</td>
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<tr>
<td>Mean</td>
<td>1.53</td>
<td>45.5</td>
<td>3.29×10^{-5}</td>
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<td>SD</td>
<td>1.28</td>
<td>16.3</td>
<td>1.87×10^{-5}</td>
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<tr>
<td>Variation (× fold)</td>
<td>7.03</td>
<td>-</td>
<td>3.36</td>
</tr>
</tbody>
</table>
3.1.3 Kinetics of 3–cyano–7–ethoxycoumarin O–de–ethylation in human liver microsomes

Contrary to rat liver microsomes, human liver microsomes appeared to exhibit product inhibition at 30 µmol l⁻¹ 3–cyano–7–ethoxycoumarin. Therefore, this concentration was omitted from kinetic calculations. Rates of formation of 3–cyano–7–hydroxycoumarin against substrate concentration for 10 individual human liver microsome preparations are shown in Figure 6.

![Figure 6](image_url)

Figure 6. O–de–ethylation of 3–cyano–7–ethoxycoumarin by 10 human liver microsomes (mean ± SEM; n=3).

The mean $K_M$ for the 10 individual liver microsome preparations was 8.2 µmol l⁻¹ (range 5.2 - 10.4; CV = 19%) and the mean $V_{max}$ was 1168 pmol 3–cyano–7–ethoxycoumarin min⁻¹ mg microsomal protein⁻¹ (range 536–2213; CV = 42%). Variation in $V_{max}$ was 4.13 fold and $CL_{int}$ was 3.45 fold (Table 8).
Table 8
Summary of variation in metabolic rate constants and parameters for 3-cyano-7-ethoxycoumarin O-deethylation

<table>
<thead>
<tr>
<th>Microsomal Preparation</th>
<th>V_{max} (nmol/min/mg)</th>
<th>K_{M} (µmol/l)</th>
<th>CL_{int} (l/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC259016</td>
<td>0.5328</td>
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<td>7.68×10^3</td>
</tr>
<tr>
<td>MIC259007</td>
<td>0.9309</td>
<td>10.25</td>
<td>9.08×10^3</td>
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<td>MIC259017</td>
<td>0.8338</td>
<td>8.088</td>
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<td>MIC259011</td>
<td>1.139</td>
<td>9.459</td>
<td>1.20×10^4</td>
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<td>MIC259006</td>
<td>1.172</td>
<td>9.433</td>
<td>1.24×10^4</td>
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<td>MIC259015</td>
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<td>MIC259021</td>
<td>2.201</td>
<td>8.302</td>
<td>2.65×10^4</td>
</tr>
<tr>
<td>Mean</td>
<td>1.16</td>
<td>8.208</td>
<td>1.43E-04</td>
</tr>
<tr>
<td>SD</td>
<td>0.483</td>
<td>1.57</td>
<td>5.68E-05</td>
</tr>
<tr>
<td>Variation (× fold)</td>
<td>4.13</td>
<td>-</td>
<td>3.45</td>
</tr>
</tbody>
</table>

3.1.4 Biotransformation of styrene in human liver microsomes

Styrene was incubated with human liver microsomes from 10 individuals and the production of styrene glycol measured as an estimate of cytochrome P450 2E1 activity. Operating under linear conditions (5-5000 µmol l^{-1}, for 20 minutes, 0.5 mg ml^{-1} protein) with respect to incubation time and microsomal protein concentration, the formation of styrene glycol was readily monitored. Rate of formation against substrate concentration is shown in Figure 7. In vitro ST kinetics were described using a 2-binding site hyperbola equation consistent with high and low affinity metabolism. Microsomal preparations MIC259002 and MIC259016 were more satisfactorily described using a single-binding site equation. MIC259002 gave K_{M}=59.95 µmol l^{-1} and V_{max}=4.929 nmol min^{-1} mg^{-1} and MIC259016 gave K_{M}=23.13 µmol l^{-1} and V_{max}=2.483 nmol min^{-1} mg^{-1}.

Figure 7. Hydroxylation of styrene by 10 human liver microsomes (mean ± SEM; n=3).

The V_{max} values for CHZ and high affinity ST biotransformation determined in the same microsomal preparations were matched and plotted in order to investigate potential correlation (Figure 8).
Figure 8 Correlation of $V_{\text{max}}$ for CHZ hydroxylation and high affinity ST biotransformation ($r^2=0.9732$, $Sy.x=0.2259$).

However, if the highest $V_{\text{max}}$ values for both CHX and ST biotransformation are excluded then the correlation deteriorates (Figure 9).

Figure 9 Correlation of $V_{\text{max}}$ for CHZ hydroxylation and high affinity ST biotransformation: Exclusion of two highest values. ($r^2=0.1625$, $Sy.x=0.2194$).

High affinity styrene versus chlorzoxazone CLint also shows no significant correlation (Figure 10).
Figure 10. Correlation of $\text{CL}_{\text{int}}$ for the high affinity component of styrene biotransformation with the $\text{CL}_{\text{int}}$ for the biotransformation of the CYP2E1 probe substrate, chlorzoxazone ($r^2 = 0.1070, \text{Sy.x}=0.04220$).

It interesting to note that the Eadie-Hofstee plots for the determination of $V_{\text{max}}$ for ST biotransformation in the microsomal samples giving the two highest values suggest that the contribution to metabolism is predominantly from a single enzyme, whereas those of the other microsomal samples indicate that there are at least two. A linear decrease in rate of reaction versus clearance indicates that one enzyme is operating MIC259002 (Figure 11b), whereas MIC259007 (Figure 11a) shows two different phases, indicating a contribution from two enzymes to metabolism.
Figure 11. Eadie-Hofstee plots for the rate of production of styrene glycol from styrene in a) human microsomal preparation MIC259007 which is representative of 8 from 10 of the microsomal preparations and b) MIC259002 which represents the one of the two preparations with very high activity.
Although the main focus of the study was on cytochrome P450 2E1, which is an inducible enzyme and considered to be the high affinity component of ST metabolism, the Michaelis-Menten rate constants for the low affinity component were also determined. It is interesting to note that the variation in CL\textsubscript{int} measured for the low affinity component was 23.5, which is higher than that of 2.53 observed for the high affinity component (Table 7). The enzyme(s), which comprise the low affinity component of ST metabolism, are not known at this time although preliminary results indicate a contribution from more than one enzyme.

3.1.5 Kinetics of glutathione transferase

3.1.5.1 Glutathione transferase activity towards 1-chloro-2,4-dinitrobenzene

The mean \( V_{\text{max}} \) of glutathione transferase activity to CDNB was \( 441.1 \pm 186.5 \) nmol min\(^{-1}\) mg cytosolic protein\(^{-1}\) (range 156.9-757 nmol min\(^{-1}\) mg cytosolic protein\(^{-1}\); CV=42.3\%) (Figure 12). The mean \( K_M \) was 399.4±241.7 µmol l\(^{-1}\). \( V_{\text{max}} \) varied 3.93 and CL\textsubscript{int} 4.94–fold (Table 9).

![Figure 12. Glutathione conjugation with broad-spectrum substrate CDNB. The mean rate of glutathione conjugation was measured in 8 human cytosolic preparations.](image)

3.1.5.2 Glutathione transferase-\( \theta \) activity towards 1,2-epoxy-3-(4-nitrophenoxy)-propane

The mean catalytic activity of GST-\( \theta \) activity to ENPP was \( 3265.51 \pm 1427 \) pmol min\(^{-1}\) mg cytosolic protein\(^{-1}\) (range 1977-5846 pmol min\(^{-1}\) mg cytosolic protein\(^{-1}\); CV=43.7\%) (Figure 13). The mean \( K_M \) was 19.8±8 µmol l\(^{-1}\). \( V_{\text{max}} \) varied 2.96 and CL\textsubscript{int} 6.61–fold (Table 9).
3.1.5.3 Glutathione transferase activity towards \textit{trans}-4-phenyl-but-3-en-2-one

The mean catalytic activity of GST-\(\mu\) activity to tPBO was 1609.4\(\pm\)1861 pmol min\(^{-1}\) mg cytosolic protein\(^{-1}\) (range 18.24-4966 pmol min\(^{-1}\) mg cytosolic protein\(^{-1}\); CV=115.7\%) (Figure 14). The mean \(K_M\) was 125.1\(\pm\)107.7 \(\mu\)mol l\(^{-1}\). \(V_{\text{max}}\) varied 272 and CL\(_{\text{int}}\) 141-fold (Table 9) (Figure 14).

Figure 13. GST-\(\theta\) activity. GST-\(\theta\) activity was measured by determining the rate of glutathione conjugation with ENPP. The mean rate of glutathione conjugation was measured in 8 human cytosolic preparations.

Figure 14. GST-\(\mu\) activity. GST-\(\mu\) activity was measured by determining the rate of glutathione conjugation with tPBO. The mean rate of glutathione conjugation was measured in 8 human cytosolic preparations.
<table>
<thead>
<tr>
<th>Cytosolic Preparation</th>
<th>1-chloro-2,4-dinitrobenzene (CDNB)</th>
<th>1,2-epoxy-3-(4-nitrophenoxy)-propane (ENPP)</th>
<th>trans-4-phenyl-but-3-en-2-one (tPBO)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(V_{\text{max}}) (nmol/min/mg)</td>
<td>(K_{\text{M}}) (µmol/l)</td>
<td>(CL_{\text{int}}) (l/min)</td>
</tr>
<tr>
<td>CYT6B</td>
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</tr>
<tr>
<td>CYT16</td>
<td>500.9</td>
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<td>CYT19</td>
<td>370.8</td>
<td>343.3</td>
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<td>CYT10</td>
<td>304.7</td>
<td>215.6</td>
<td>1.41×10^{-4}</td>
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<td>CYT9</td>
<td>528</td>
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<td>1.47×10^{-4}</td>
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<tr>
<td>CYT7</td>
<td>616.9</td>
<td>398.9</td>
<td>1.55×10^{-3}</td>
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<tr>
<td>CYT8</td>
<td>757</td>
<td>401.3</td>
<td>1.89×10^{-3}</td>
</tr>
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<td>CYT6</td>
<td>156.9</td>
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<td>Mean</td>
<td>441.13</td>
<td>399.4</td>
<td>1.41×10^{-3}</td>
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<td>SD</td>
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<td>241.7</td>
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<tr>
<td>Variation (× fold)</td>
<td>3.93</td>
<td>-</td>
<td>4.94</td>
</tr>
</tbody>
</table>
3.2 Validation of the PBPK model
The PBPK model output was very similar to that reported by Csanády et al (Csanády et al., 1994). Model output for simulation of ST and SO kinetics under controlled experimental and occupational exposure conditions are shown in figures 15a & b and 15c & d, respectively.

Figure 15a. Concentration-time course of ST in arterial blood of male volunteers (mean body weight of 69 kg) during and after exposure to 69 ppm ST for 2 h during light physical exercise (50 W work load) (Wigaeus et al., 1983). The symbols represent the mean of seven subjects.

Figure 15b. Concentration-time course of ST in venous blood of male volunteers (mean body weight of 83 kg) during and after a 6 h inhalation exposure to 80 ppm ST (Ramsey and Young, 1978). The symbols represent the mean of four subjects.
Figure 15c. Correlation of styrene-7,8-oxide concentration in venous blood with styrene concentration in ambient air. ST concentrations in ambient air were given as time-weighted averages. Venous blood concentrations of styrene-7,8-oxide were determined at the end of the exposure period. Each dot represents a measured value for a single person (Korn et al., 1994). The solid line represents the model predictions at various reported time points and ambient exposure concentrations. The broken lines represent the 95% confidence interval for the predicted mean values of SO (See Materials and Methods section 2.3.1 Model validation).
Figure 15d. Correlation of measured versus predicted styrene-7,8-oxide concentration in venous blood. Venous blood concentrations of styrene-7,8-oxide were determined at the end of the exposure period. Each dot represents a measured value for a single person (Korn et al., 1994). The solid line was calculated by regression analysis through the origin: \( y = (0.8313 \pm 0.07096)x \), \( S_y.x = 0.8002 \). The broken lines represent the 95% confidence interval for the predicted mean values of SO (See Materials and Methods section 2.3.1 Model validation).

3.3 Incorporation of inter-individual variability

The urinary excretion data for MA and PGA, the two main metabolites of ST biotransformation, were expressed as mole per hour excreted rather than moles per mol creatinine. The reason for this is because creatinine normalisation, more often than not, increases rather than decreases variability in urinary data. Figures 16a and 16b show that experimentally determined values for urinary excretion of both MA and PGA fall within the envelope bounded by the predicted maximum and minimum rates of excretion.

The venous concentration of SO versus the A/D ratio is shown in figure 17. Positive ratios or differences correspond to the range where the rate of activation of ST to SO exceeds the rate of deactivation of SO. It is interesting to note that the higher venous concentrations of SO observed in some people could not solely be explained by the absolute value of the A/D difference.

Figure 16a. Urinary excretion of Mandelic Acid. Four volunteers were exposed to 50 ppm ST for 6 h in the HSL controlled atmosphere exposure facility. Urine samples were taken every hour up to 14 h after the beginning of exposure. The symbols represent measured concentrations of MA and the solid lines are model simulations.
Figure 16b. Urinary excretion of Phenylglyoxylic Acid. Two volunteers were exposed to 50 ppm ST for 10 h in the HSL controlled atmosphere exposure facility. Urine samples were taken every hour up to 19 h after the beginning of exposure. The symbols represent measured concentrations of MA and the solid lines are model simulations.

Figure 17. Venous concentration of styrene oxide versus the Activation/Deactivation ratio for styrene metabolism. Each symbol represents the model prediction for a single person. 3000 iterations, simulating a cohort of 3000 people, were performed at 0, 50, 100 and 200 ppm exposure concentrations to ST with a work rate varying from 40-100 W. The vertical dotted line at 0 along the x axis corresponds to the point at which the rate of activation is equal to the rate of deactivation. A positive A/D ratio reflects the situation where the rate of production of SO exceeds the rate of removal. Calculation of A/D ratios is described in section 2.3.2 Monte Carlo analysis.
4. DISCUSSION

4.1 Variation in enzyme activity

4.1.1 Cytochrome P450 2E1

CYP2E1 is constitutively expressed in the liver, where the highest concentration is found in the centrilobular region, specifically in the four to five layers of hepatocytes surrounding the terminal hepatic venules (Ingelman-Sundberg et al., 1988). In general, CYP2E1 accounts for approximately 8% of the total P450 in liver (Shimada et al., 1994). CYP2E1 is an inducible enzyme with a complex regulation governing variability (Johansson et al., 1990). CYP2E1 activities, as measured by the rate of chlorzoxazone hydroxylation in this study, were similar to previously reported values in the literature. Peter et al (Peter et al., 1990) reported $V_{\text{max}}$ values between 0.58 and 2.3 nmol 6-hydroxychlorzoxazone/min/mg microsomal protein and an apparent $K_M$ of 39 ± 7 µmol/l (mean ± SD) in human liver microsomes from 14 individuals. In the 10 individual human liver microsomes used in this study, two individuals had significantly higher $V_{\text{max}}$ values than the other eight. This would be expected as CYP2E1 is highly inducible (Kadlubar and Guengerich, 1992). Importantly, the lowest rate of chlorzoxazone hydroxylation measured in this study (0.69 nmol/min/mg) was very close to the lowest rate reported by Peter et al (Peter et al., 1990). This would be expected assuming both of these rates correspond to uninduced enzyme expression. Iyer and Sinz (Iyer and Sinz, 1999) reported a 4.8 fold variation in the $V_{\text{max}}$ for CYP2E1 using $p$-nitrophenol as probe substrate which compares favourably with variation in $V_{\text{max}}$ and $CL_{\text{int}}$ calculated from Peter et al (Peter et al., 1990) which were 5.36 and 3.75 fold respectively, and with the 7.03 and 3.36, respectively obtained in this study (Table 7).

The highest activity measured and presented in Figure 3 was obtained with microsome preparation MIC259002, which came from a donor with steatosis; a condition characterised by fatty liver, and known to induce the activity of CYP2E1. Although the disease status of the other microsomal preparation with high CYP2E1 (MIC259016) activity was not reported, it is likely also to have come from a donor with a condition which induced this enzyme (Table 1).

The inter-batch CV of the method is acceptable. However, the value of 12% only accounts for analytical variation. Some degree of experimental error would also be expected in the microsomal incubations, although the use of a standard protocol should minimise these errors. This is supported by the low standard errors of replicate data points. Knowing the total error (incubation and analytical) would be a useful parameter to help interpret variability of the kinetic parameters measured. An estimate of this error could be achieved by replicating the incubation of microsomes from the same batch with a fixed substrate concentration.

Cell viabilities obtained from rat Liverbeads™ were lower than manufacturers specification of at least 70%. Hammond et al (Hammond et al., 1999) reported cell viabilities between 74 and 95% in their studies using Liverbeads™. They also reported wider variability in viability of Liverbeads™ compared to freshly isolated cells. This is probably largely due to day-to-day variations in the thawing and processing of the Liverbeads™. Thawing and dilution of Liverbeads™ needs to be as rapid as possible to prevent damage to the cells by the cryopreservant, dimethyl sulphoxide. Typical cell viability of thawed Liverbeads™ was 60–65% in this study. With the exception of one preparation, viability was fairly constant (>60%). Therefore, Liverbeads™ appear to be suitable for use in future metabolism studies. Cell viability may improve with experience of handling Liverbeads™.

In a comparative study of chlorzoxazone hydroxylation across a range of species using liver microsomes (Court et al., 1997), Michaelis-Menten kinetic parameters were similar for both human and rat. It this study, human microsomal chlorzoxazone hydroxylation was shown to
have a $K_M$ of 45 $\mu$mol/l and a $V_{max}$ of 1.536 nmol/min/mg microsomal protein. It may therefore be expected that similar results would arise using the same incubation conditions for rat liver microsomes. Given this assumption, $K_M$ for chlorzoxazone hydroxylation by rat liverbeads was 38 $\mu$mol/l. Since this reaction is catalysed by CYP2E1, then it is purely microsomal. Therefore it is reasonable to expect that $K_M$ should be similar in both microsomes and intact hepatocytes. This is supported by the experimental data. Similarly, $V_{max}$ for chlorzoxazone hydroxylation could be expected to be similar in both microsomes and intact hepatocytes. However, the two experimental systems cannot be directly compared because $V_{max}$ is expressed in different units. However, the use of scaling factors allows data from the two systems to be compared by scaling each $V_{max}$ to a common term, based on liver weight. Using scaling factors determined by Carlile et al (Carlile et al., 1997) the two in-vitro systems are compared in Table 10.

<table>
<thead>
<tr>
<th></th>
<th>$V_{max}$</th>
<th>Scaling factor$^a$</th>
<th>Corrected $V_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human liver microsomes</td>
<td>1.536</td>
<td>60.1</td>
<td>5539</td>
</tr>
<tr>
<td>Rat liverbeads</td>
<td>1.63</td>
<td>109 $\times$ 10^6</td>
<td>1777</td>
</tr>
</tbody>
</table>

$^a$ Scaling factors taken from (Carlile et al., 1997).

This demonstrates that $V_{max}$ for chlorzoxazone determined in rat liverbeads was of similar magnitude to $V_{max}$ determined in human liver microsomes. Scaling factors have a significant impact on the final value obtained. (A value of 60.1 mg protein per g liver was used in this case, however, further work on microsomal protein recovery during subcellular fractionation and differential centrifugation indicates values of 35-45 mg microsomal protein per g liver are more common (B Houston, personal communication)). It would be preferable to use scaling factors determined in-house for our own incubation systems, however this crude method of comparison still demonstrates that $V_{max}$ for chlorzoxazone hydroxylation calculated from rat liverbeads is approximately what would be expected. This finding further supports the use of cryopreserved hepatocytes in the form of liverbeads in in-vitro metabolism studies.

Cryopreserved hepatocytes, in the form of Liverbeads™, have been successfully used in this study. They are a convenient source of hepatocytes and are the only practical one since the scarcity and unpredictable availability of fresh liver prevents routine preparation of fresh hepatocytes. However, the process of alginate encapsulation and cryopreservation is fairly new and there is therefore limited data comparing cryopreserved hepatocytes to freshly prepared hepatocytes. In particular, it is important to determine whether cryopreservation alters expression or activity of metabolic enzymes and also if preservation or recovery media contain compounds that could inhibit metabolic enzyme activity. A few studies have attempted to investigate the effects of cryopreservation (Guyomard et al., 1996; Hammond et al., 1999; Li et al., 1999; Rialland et al., 2000), however this is an area which requires substantial further research.
4.1.2 Cytochrome P450 1A2

CYP1A2 expression is largely restricted to the liver, where it represents, on average, about 12% of total hepatic P450 content (Shimada et al., 1994). It is involved in the metabolism of a large number of environmental and dietary chemicals, in some cases converting them into toxic or carcinogenic intermediates (Guengerich, 1988; Guengerich and Shimada, 1991; Guengerich et al., 1999). CYP1A2 is inducible by many environmental agents such as polycyclic aromatic hydrocarbons (Sesardic et al., 1990), polyhalogenated aromatic hydrocarbons (Lake et al., 1996), dioxins (Xu et al., 2000) and indoles contained in cruciferous vegetables (Schrenk et al., 1998). Evidence for genetic polymorphism in human CYP1A2 has been presented based upon in vivo caffeine N³-demethylation pharmacokinetics (Kadlubar et al., 1992; Lang et al., 1994). Some of the 40-fold variation observed may be related to the regulation of expression levels (Guengerich et al., 1999).

The kinetics of 3-cyano-7-ethoxycoumarin O-de-ethylation was investigated in human liver microsomes as a measure of CYP1A2 variability. The K_M measured in this study is close to the K_M reported by Crespi et al (Crespi et al., 1997) of 3.5 µmol/l using cDNA expressed CYP1A2. Unfortunately, V_max data reported by Crespi et al (Crespi et al., 1997) could not be converted to the units used in this study for comparison. A 4- and 3.45-fold variation in V_max and CL_int, respectively, were measured in this study, which differ markedly from the 180-fold in vitro variation (Sesardic et al., 1988) and 6-fold in vivo variation (Schrenk et al., 1998) in CYP1A2-dependent activity reported elsewhere. The choice of substrate however does determine the extent of variation in activity measured. For example, in non-smokers CYP1A2-dependent caffeine metabolic ratio varies 6-fold (Schrenk et al., 1998), whereas the CL_int of phenacetin varies approximately 10-fold (Kahn et al., 1985). Also, substantial intra-individual variation of the CYP1A2 urinary metabolic ratio has also been noted (Nordmark et al., 1999).

4.1.3 Styrene biotransformation

The major biotransformation pathway of ST is via oxidation to SO which is present in two enantiomeric forms (R–SO and S–SO). Many in vitro studies have been conducted to examine the enzymatic processes involved in this process. The in vitro conversion of ST to SO is catalysed by CYP2E1 (Guengerich et al., 1991; Shimada et al., 1994; Kim et al., 1996) although there is evidence that other isoforms, particularly CYP2B6, are also involved at higher concentrations (Nakajima et al., 1994; Kim et al., 1997) including, more recently, CYP2F1 (Cruzan et al., 2002). The data obtained in this study are consistent with previous reports which indicate that there are other isoenzymes of CYP P450 contributing to ST metabolism e.g., a 2-binding site hyperbola equation to fit the data (Figure 7) and the Eadie–Hofstee plots showing the difference between one and two-enzyme activity (Figure 11). Attempts to correlate ST high affinity V_max with CHZ V_max showed that in most cases (8 from 10) there was a very weak correlation (Figure 9). The two microsomal preparations with the highest V_max showed very good agreement with CHZ metabolism (Table 7), which together with the Eadie–Hofstee plot (Figure 11b), suggests that CYP2E1 content and contribution to ST biotransformation, relative to other CYP isoforms, was high. This result supports the view that the measurement of variability in metabolism should be compound specific i.e., variability measured using a probe substrate cannot be transferred to another CYP2E1 substrate (Guengerich et al., 1999; Gentry et al., 2002). This is because most substrates are metabolised by more than one enzyme whereas a probe substrate may be metabolised predominantly by a single enzyme. It is interesting to note that the K_M values for low-affinity ST biotransformation for microsomal preparations MIC259016 and MIC259002 were very much higher, and the CL_int values lower, than in the other 8 preparations suggesting that the amount of low-affinity isoenzymes available to contribute to ST metabolism was lower in these microsomes.
4.1.4 Kinetics of glutathione transferase

Glutathione transferases (GSTs) function as detoxification enzymes and are generally considered to play a prominent role in cellular defence against electrophilic chemical species, of endogenous as well as xenobiotic origins. Human GSTs can be divided into two main categories: membrane-bound and soluble GSTs, with the latter often referred to a cytosolic GSTs although nuclear and mitochondrial forms are included in the soluble category. The soluble GSTs are subdivided into classes based on sequence similarities that reflect evolutionary branches from an ancestral protein (Mannervik, 1985). The classes are designated by the names of Greek letters: $\alpha$ (alpha), $\mu$ (mu), $\pi$ (pi), $\theta$ (theta) etc., and are abbreviated A, M, P, T, and so on. However, all GST polymorphisms so far reported are limited to the soluble GSTs and it is for this reason that this category has received most interest.

Human tissues, like the rat, show differential expression of the multiple soluble forms of GST. Most tissues have different isoenzyme profiles. In the liver, alpha class GSTs, in particular GST A1–1, are predominant and may represent 2-3% of total cytosolic protein (van Ommen et al., 1990; Rowe et al., 1997). GST M1–1 and GST T1–1 are also major hepatic forms, in individuals expressing these isoenzymes (Sherratt et al., 1998; Thier et al., 1998; Landi, 2000; Sherratt et al., 2002; Ross and Pegram, 2003). GST P1–1, which is present in most human tissues, is not expressed in normal adult hepatocytes, and its presence in the liver appears to be restricted to epithelial cells such as those of the bile duct (Kano et al., 1987; Cowell et al., 1988; Islam et al., 1989; Morrow et al., 1989).

GST M1–1, GST M3–3, GST T1–1 AND GST P1–1 have well-established polymorphisms in the human population. GST M1–1 and GST T1–1 both have a frequently occurring null phenotype (Warholm et al., 1980; Board, 1981; Pemble et al., 1994). Approximately half the human population is homozygous for the $GSTM1$ null allele and consequently does not express the GST M1–1 protein. The GSTT1 null phenotype is due to the deletion of the complete $GSTT1$ gene (Pemble et al., 1994).

In this study three glutathione transferase substrates were used to measure the $in vitro$ variation in activity of these enzymes. However, all three substrates are preferentially metabolised by various GST enzymes to varying degrees. CDNB is considered to be a non-specific substrate for alpha-, mu-, and pi-class GSTs (Takamatsu and Inaba, 1994) whereas tPBO is more specific for GST M1–1 and ENPP for GST T1–1 (Hayes and Pulford, 1995). The results listed in Table 9 show that measured variability is dependent upon substrate used, which presents difficulties when trying to draw general conclusions. CDNB and ENPP show similar variation where the first reflects the activity of alpha, mu and pi class GSTs and the second is believed to be more specific for GST T1–1. The activities measured using tPBO suggests that about 50% of the samples came from GST M1 null phenotypes, which is consistent with reported frequencies in various ethnic groups (58% in Chinese, 52% in English, 48% in Japanese and 43% in French nationals) (Hayes and Pulford, 1995). This finding supports previous results with other enzyme systems, which suggest that reliable measurement of variability can only be compound specific. Of course, this may well constitute a major obstacle to the determination of generic enzyme variability data for use in chemical risk assessment.

4.2 Incorporating inter-individual variability into PBPK models

The incorporation of human inter-individual variation in anatomical, physiological, biochemical and physicochemical parameters has been achieved in this study using Monte Carlo sampling linked to PBPK modelling. The form of the data required is the mean and standard deviation of each parameter, which implies that much existing data can be used in this approach. The results indicate that the measurement of variation in $in vitro$ parameters may adequately reflect the variation observed in $in vivo$ data, although more simulations of $in vivo$ data than are presented
in this study are required to further support this observation. However, the power of PBPK modelling has been typically demonstrated by the integration of such a wide range of parameters i.e., biological, chemical and statistical into a single platform i.e., a PBPK model. Specifically, this process represents a means of encapsulating as much of the existing knowledge of a given chemical, in this case styrene, and using it to understand, quantitatively and qualitatively, the behaviour of the chemical in the body. Altogether, this should provide a more transparent and justifiable approach to chemical risk assessment. However, the study also highlighted a problem with the form in which some biological monitoring data is expressed, in particular the rate of urinary excretion of a marker expressed against the rate of urinary excretion of creatinine. Normalisation of urinary excretion data against creatinine has always been controversial and is dependent on the assumption that the rate of urinary creatinine excretion is constant. However, in reality, urinary creatinine excretion is variable to a considerable degree (Harris et al., 2000; LeBeau et al., 2002). HSL routinely measures urinary creatinine and most individual data varies up to 50%. Therefore, a parameter such as creatinine would introduce variability into a PBPK model. The consequence of this would be to obscure the variability of other parameters, which determine the pharmacokinetics of the chemical under study, and make the quantification of variance difficult.

PBPK models can be exercised in many different ways, such as, simulating different work patterns, constant and pulsatile exposure concentrations, and the effects of exercise. In this study we also investigated the effects of variation in the activation and deactivation enzymes of ST metabolism with different work rates. Although this was predominantly a theoretical exercise the assumptions made were realistic. It is reasonable to assume that different individuals will have different metabolic capacities governed by the phenotypic expression of relevant enzymes. One extreme may be those individuals with highly active CYP2E1 and low mEH activity; the opposite extreme may be low CYP2E1 activity and high mEH, with most people having A/D ratios in between. Individuals with high CYP2E1 and low mEH activity would rapidly transform ST to SO but slowly remove SO. This would imply that such individuals would be more susceptible to ST toxicity. Figure 16 shows that relatively few people, less than 100 in 3000, would have a reduced potential to deactivate SO even after exposure to 200 ppm ST for 8 hr per day 5 days per week. This figure also indicates that the A/D difference, which is based on Cl\textsubscript{int}, alone cannot explain the causes underlying the higher venous SO concentrations predicted in these few individuals. The absolute values of V\textsubscript{max} better correlate with the ability to metabolise chemicals at the higher exposure concentrations, which is consistent with classical enzyme behaviour. Exposure concentrations of 100-200 ppm are very high and unlikely to be encountered in the work place in developed countries. A more accurate model is being developed which can estimate the concentrations of SO in the cells of the lung where tumours are observed following chronic exposure of mice to ST. In addition, the model will be capable of incorporating specific distributions and expression levels of enzyme polymorphisms according to known allelic frequencies. Such a model could be used to interpret epidemiological data.
5. CONCLUSIONS

Commercially available human hepatic microsomes and cytosols are ideal for the determination of variation in chemical metabolism. Commercially available cryopreserved human hepatocytes show promise although further development is required in thawing and cell viability protocols in order to improve reproducible yields of viable cells. Variability measured using probe substrates cannot be transferred to other chemicals because probe substrates are by definition almost always exclusively metabolised by one enzyme, whereas other chemicals often have contributions from more than one enzyme. Enzyme activity should be expressed as intrinsic clearance, $\text{CL}_{\text{int}}$, at sub-saturating and $V_{\text{max}}$, at saturating, substrate concentrations. Variability in the urinary excretion of phenylglyoxylic and mandelic acids in human volunteers exposed to styrene was successfully simulated using in vitro metabolism data in a PBPK model. However expression of urinary biomarkers against creatinine increases, rather than decreases, variability, which confounds biological monitoring strategies. Finally, in vitro data were used in a Monte Carlo approach to generate a population PBPK model for describing the variability in the ratio of the activation of styrene to the suspect, non-genotoxic carcinogen styrene oxide, relative to the deactivation of styrene oxide. Population models such as this could help improve epidemiological studies.


