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An Approach for the Quantitative Consideration of Genetic Polymorphism Data in Chemical Risk Assessment: Examples with Warfarin and Parathion

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In recent years, a great deal of research has been conducted to identify genetic polymorphisms. One focus has been to characterize variability in metabolic enzyme systems that could impact internal doses of pharmaceuticals or environmental pollutants. Methods are needed for using this metabolic information to estimate the resulting variability in tissue doses associated with chemical exposure. We demonstrate here the use of physiologically based pharmacokinetic (PBPK) modeling in combination with Monte Carlo analysis to incorporate information on polymorphisms into the analysis of toxicokinetic variability. Warfarin and parathion were used as case studies to demonstrate this approach. Our results suggest that polymorphisms in the *PON1* gene, that give rise to allelic variants of paraoxonase, which is involved in the metabolism of paraoxon (a metabolite of parathion), make only a minor contribution to the overall variability in paraoxon tissue dose, while polymorphisms in the *CYP2C9* gene, which gives rise to allelic variants of the major metabolic enzyme for warfarin, account for a significant portion of the overall variability in (S)-warfarin tissue dose. These analyses were used to estimate chemical-specific adjustment factors (CSAFs) for the human variability in toxicokinetics for both parathion and warfarin. Implications of alternatives in the calculation of CSAFs are explored. Key decision points for applying the PBPK-Monte Carlo approach to evaluate toxicokinetic variability for other chemicals are also discussed.

Key Words: genetic polymorphisms; physiologically based pharmacokinetic (PBPK) modeling; Monte Carlo analysis; toxicokinetic variability; warfarin; parathion; risk assessment.

The increased recognition that there is a genetic basis for variability in xenobiotic response has led to a surge in polymorphism-related research in the toxicology field. A search of Medline in early 2002 identified over 60,000 hits for "polymorphism," of which more than 10,000 were published in 2001 alone. The number of identified alleles for a gene of interest may range from two (e.g., *GSTT1*, reviewed in Eaton and Bammler, 1999) to more than 70 (*CYP2D6* alleles), some with more than one nucleotide change (Ingelman-Sundberg *et al.*, 2002). These research efforts have led to the realization that many (if not all) of the genes that give rise to enzymes that metabolize environmentally relevant toxicants are polymorphic. However, the development of tools and approaches for evaluating the toxicological implications of this genetic variability has not kept pace with the identification of new genetic polymorphisms.

Indeed, the whole question of whether and the degree to which polymorphisms increase human variability in toxic response is not well characterized, although it has been discussed in numerous recent reviews (Ingelman-Sundberg, 2001; Knudsen *et al.*, 2001; Linder and Valdes, 2001; Miller *et al.*, 2001). A wide range in activity between different alleles, or between a null allele and a wild type, might lead to the expectation of large differences in tissue dose arising from similar exposures. This conclusion is supported by epidemiological comparisons of cancer risk between populations with the wild-type and variant alleles that show an increased risk (or decreased risk) among populations harboring different alleles (Uematsu *et al.*, 1991), and observed variability in blood or tissue levels of pharmaceuticals in patients receiving similar administered doses (Furuya *et al.*, 1995). On the other hand, a genetic polymorphism may have minimal or no impact on toxicity. Some genetic polymorphisms may not affect the resulting amino acid sequence (e.g., they may be in a noncoding region of the gene, or in the coding region without altering the encoded amino acid), and so not affect enzyme activity. Conversely, polymorphisms in the regulatory region of a gene may affect gene expression or mRNA stability, and thereby modify the total level of enzyme activity in a tissue, without directly modifying the protein. Other polymorphisms may affect enzyme activity, but the effect may be insignificant at environmental exposure levels, perhaps because other enzymes can carry out the same reaction, or the kinetics of that enzyme are not rate limiting. Other genetic and environmental factors may also affect the enzyme level and activity. Overall, the key question for evaluating the effects of polymorphisms in genes encoding metabolic enzymes is how the polymorphism affects the interindividual variability in the tissue dose of active agent

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resulting from a given administered dose of the parent compound. For risk assessment scientists this question is critical in deriving "safe" or subthreshold dose estimates that are protective for a highly variable human population.

"Safe" or subthreshold doses are determined by health agencies worldwide by identifying a critical effect level, such as a no observed adverse effect level (NOAEL) or benchmark dose (BMD), and dividing by uncertainty factors to account for extrapolations from the available data and for database deficiencies (Barnes and Dourson, 1988; IPCS, 1994; Jarabek, 1994; Meek *et al.*, 1994; U.S. EPA, 1994). A default factor of 10 is used by most organizations to protect sensitive populations. This factor of 10 is applied to the human NOAEL (e.g., a NOAEL measured in humans or extrapolated from an animal NOAEL) and reflects the difference in sensitivities expected between the midrange of the distribution of the overall population and a sensitive individual (Dourson *et al.*, 1996). As reviewed in Haber *et al.* (2002), considerable research has been performed in recent years to refine this approach beyond the use of default uncertainty factors (e.g., Baird *et al.*, 1996; Renwick, 1993). Recent guidance from the International Programme on Chemical Safety (IPCS, 2001) addresses the data needs for replacing default uncertainty factors with chemical-specific adjustment factors (CSAFs). This approach breaks the intraspecies uncertainty factor into toxicokinetic and toxicodynamic components,[2] each of which can be replaced by a CSAF if data are available. Depending on the data available for the chemical, the magnitude of the adjustment factor for human variability in toxicokinetics (HK_{AF}) may be calculated based on an evaluation of human variability in such toxicokinetic factors as the area under the tissue concentration-time curve (AUC) or clearance. Physiologically based pharmacokinetic (PBPK) models can also be used to estimate HK_{AF} based on variability in intrinsic clearance (e.g., from *in vitro* enzyme kinetic data; Lipscomb *et al.*, 2002).

The purpose of the current research effort was to develop an optimal approach for evaluating the variability in tissue dose resulting from polymorphisms in genes that encode enzymes important for xenobiotic metabolism. The ultimate goal is to incorporate information about genetic polymorphisms into the derivation of CSAFs, and thereby enhance noncancer risk assessment by facilitating the movement from default uncertainty factor approaches to data-informed, biologically based methods. This article presents the second phase of a project designed to evaluate the toxicological significance of genetic polymorphisms in some key metabolic enzymes, by conducting case studies evaluating the extent to which the polymorphisms affect tissue dose. In the first phase of the project (Haber *et al.*, 2002), a list of 17 toxicologically significant chemicals was developed that are substrates for polymorphic enzymes. Information on these chemicals was reviewed to identify a subset of chemicals with well-characterized metabolic pathways, and for which allelic frequency data and phenotype data (i.e., kinetic parameters such as the *VMax* and *KM*) were available. More in-depth analyses were conducted in Phase I on four chemicals: methylene chloride (dichloromethane), warfarin, parathion, and dichloroacetic acid. Evaluation of the data for these chemicals identified several common deficiencies that increase the uncertainty in the type of analysis described here (Table 1). These are discussed more fully by Haber *et al.* (2002).

TABLE 1
Common Problems Increasing Uncertainty in the Evaluation of Polymorphisms

Description of problems
Absence of chemical-specific kinetic phenotype data
Uncertainty regarding differences between *in vitro* and *in vivo* kinetic data
Absence of data on the relative contribution of multiple enzyme systems
Absence of data on the allelic frequencies for major ethnic groups
Large numbers of low frequency alleles
Uncertainty regarding the extent of induction/inhibition through coexposure

Note. Adapted from Haber *et al.*, 2002.

The present phase of the effort provides the results from two case studies, for warfarin and parathion, in which the combination of PBPK modeling and Monte Carlo analysis is used to develop a quantitative estimate of the impact of genetic polymorphisms on tissue doses, and hence internal dose metrics for risk assessment. These two case study chemicals provided a useful comparison because they are metabolized by two very different metabolic pathways, involving polymorphisms in two unrelated metabolic enzymes, with different biological implications resulting from the presence of the polymorphisms. These analyses evaluated the impact of different choices of key input data on resulting distributions of tissue doses, and downstream implications for noncancer dose-response assessment when different approaches to calculating CSAFs are used.

MATERIALS AND METHODS

The PBPK models were initially parameterized using *in vivo* time-course data from animals and humans of unknown genotypes. The fitted values for the metabolic parameters were then compared to the metabolic parameters reported in the available *in vitro* studies of all known alleles. Once it was confirmed that the fitted values fell within the range of the reported *in vitro* values for the different alleles, the genotype-specific *in vitro* means and SDs were used to model the tissue dose among the different genotypes. Variability distributions for the area under the concentration curve (AUC) were computed and examined with and without incorporation of the genetic polymorphisms in a Monte Carlo analysis. A sensitivity analysis was also conducted to determine those parameters whose changes most impacted estimates of internal dose metrics.

[2] IPCS (2001) defines toxicokinetics as "the process of the uptake of potentially toxic substances by the body, the biotransformation they undergo, the distribution of the substances and their metabolites in the tissues, and the elimination of the substances and their metabolites from the body." Toxicodynamics is defined as "the process of interaction of chemical substances with target sites and the subsequent reactions leading to adverse effects."

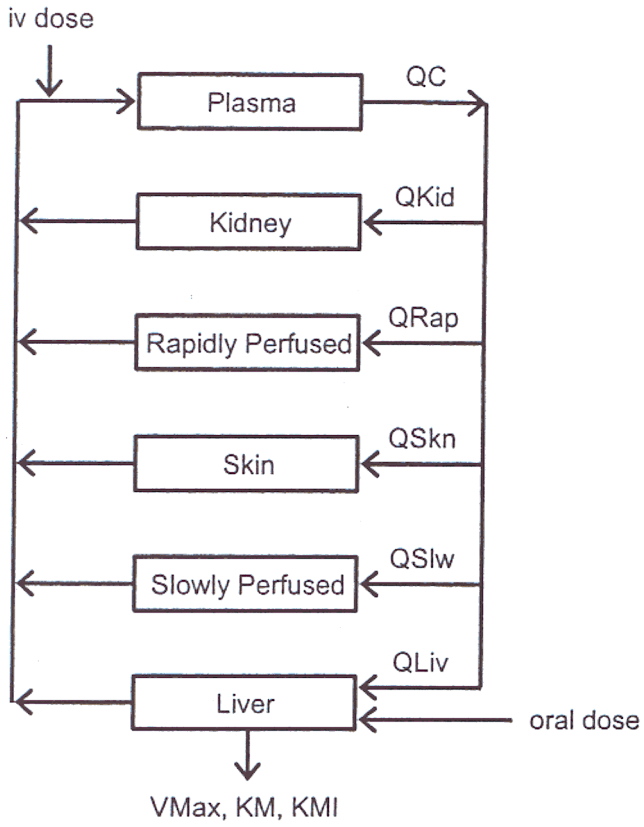

FIG. 1. Diagram of the PBPK model for warfarin, which is an extension of the warfarin model reported by Leucke and Wosilait (1979). Routes of exposure include iv and po dose uptake, binding, and metabolism. Tissue compartments include plasma and liver, plus the skin and kidney, as storage tissues. The remaining storage tissues were combined into either a rapidly (other organs) or slowly (e.g., muscle or bone) perfused tissue compartment.

Warfarin model. As described in the previous article (Haber *et al.*, 2002), the stereoisomers of warfarin are metabolized by a variety of cytochrome P450 isozymes, but metabolism of (S)-warfarin is primarily by CYP2C9, and metabolism of (R)-warfarin is primarily by CYP2C19 and CYP1A2. The toxic and therapeutic effects of warfarin are due to the parent compound, and the activity of the (S)-enantiomer is approximately three times the activity of the (R)-enantiomer. Kinetic data are available for the three principal *CYP2C9* alleles, *CYP2C9*1*, *CYP2C9*2*, *CYP2C9*3*. The protein encoded by the wild type (*CYP2C9*1*) allele is the most active, and the *CYP2C9*3* allele results in clinically significant changes in warfarin activity, while the *CYP2C9*2* allele appears to result in a smaller decrease in warfarin metabolism.

The warfarin model (Fig. 1) reported by Luecke and Wosilait (1979) was extended by including the metabolism of both the (S)- and (R)-enantiomers of warfarin, as well as the mutual inhibition of the metabolism of (S)-warfarin and (R)-warfarin (Kunze *et al.*, 1991), and the binding of warfarin in the plasma (Chan *et al.*, 1994). Tissue compartments included the plasma, which included iv dose uptake and binding, the liver, which included oral dose uptake, binding, and metabolism, plus the skin and kidney, as storage tissues. The remaining storage tissues were combined into either a rapidly (other organs) or slowly (e.g., muscle or bone) perfused tissue compartment. The physiological parameters and partition coefficients used in the warfarin model, shown in Table 2, were obtained from the literature. Blood flows were obtained from Clewell *et al.* (2001), tissue volumes were obtained from ICRP (1975), and partition coefficients were taken from Luecke *et al.* (1994). The metabolism of both enantiomers of warfarin was modeled by assuming that the dose of warfarin consisted of equal parts of (S)- and (R)-warfarin. Separate equations and parameter values were included for each enantiomer. Inhibition of (S)-warfarin metabolism by (R)-warfarin was characterized by the addition of an inhibition term in the Michaelis-Menten equation for the rate of metabolism of (S)-warfarin:

$$\frac{dA_{Liv(S)}}{dt} = \frac{VMax_{(S)}C^F_{Liv(S)}}{KM_{(S)}(1 + C^F_{Liv(R)}/KM_I) + C^F_{Liv(S)}}$$

where the subscripts (*S*) and (*R*) denote (S)-warfarin and (R)-warfarin, respectively; the superscript *F* denotes the free (i.e., unbound) concentration; A_{Liv} is the amount in the liver; C_{Liv} is the concentration in the liver; *VMax* is the maximum velocity of metabolism; *KM* is the affinity; and KM_I is the inhibition constant. A similar equation was used to describe the inhibition of (R)-warfarin metabolism by (S)-warfarin. The fraction of total warfarin in the plasma that is not bound was obtained by multiplying the total concentration by *FFPlas*, the fraction of free warfarin in the plasma, as measured by Chan *et al.* (1994).

Parameters describing the metabolism, oral uptake, and fraction of unbound warfarin in humans were estimated by fitting the model output to sets of data in the literature (Table 3); the distributions for the model parameters are shown in Table 4. Time-course data reported in Breckenridge and Orme (1973), Chan *et al.* (1994), and Choonara *et al.* (1986), were used to fit the enantiomer-specific parameters. In the Chan *et al.* (1994) and Choonara *et al.* (1986) studies, time-course concentrations in the plasma of either (S)- or (R)-warfarin were reported in volunteers administered a single po dose of 1.5 or 15 mg. Chan *et al.* (1994) also provided data on free versus bound warfarin in the

TABLE 2
Physiological Parameters and Partition Coefficients for Warfarin PBPK Model

Parameter name	Parameter description	Partition coefficient
Blood flows (fraction of cardiac output)		
QCC	Cardiac output (l/h/kg$^{3/4}$)	12.89[a]
QkidC	Kidney	0.175[b]
QlivC	Liver	0.227[a]
QrapC	Rapidly perfused tissue	0.358[a]
QSknC	Skin	0.182[a]
QSlwC	Slowly perfused tissue	0.058[a]
Tissue volumes (fraction of body weight)		
BW	Body weight (kg)	70.00[c]
VplasC	Plasma	0.070[c]
VkidC	Kidney	0.0044[c]
VlivC	Liver	0.026[c]
VrapC	Rapidly perfused tissue	0.056[a]
VSknC	Skin	0.14[c]
VSlwC	Slowly perfused tissue	0.61[a]
HCT	Hematocrit	0.42[c]
Tissue/blood partition coefficients		
Pkid	Kidney	0.12[d]
Pliv	Liver	0.27[d]
Prap	Rapidly perfused tissue	0.25[d]
PSkn	Skin	0.07[d]
PSlw	Slowly perfused tissue	0.015[d]

Note. Cardiac output (l/h/kg$^{3/4}$) is scaled by (body weight)$^{3/4}$.
[a]Clewell *et al.*, 2001.
[b]Clewell *et al.*, 1999.
[c]ICRP, 1975.
[d]Luecke *et al.*, 1994.

TABLE 3
Binding, Metabolism, and Oral Uptake Parameters in Warfarin PBPK Model

Parameter name	Parameter description	Parameter value
Fraction of free warfarin in tissues		
FFPlas	Plasma	8.00E-03
FFLiv_S	(S)-warfarin in liver	4.00E-02
FFLiv_R	(R)-warfarin in liver	4.50E-02
Metabolism parameters		
KMI_S	Inhibition of (S)-warfarin metabolism by (R)-warfarin (mg/l)	2.00
VMaxC_R	Maximum rate of (R)-warfarin metabolism (mg/h/kg$^{3/4}$)[a]	1.90
KM_R	Affinity for (R)-warfarin metabolism (mg/l)	4.00
KMI_R	Inhibition of (R)-warfarin metabolism by (S)-warfarin (mg/l)	2.00
VmaxC_S	Maximum rate of (S)-warfarin metabolism (mg/h/kg$^{3/4}$)[a]	1.75
KM_S	Affinity for (S)-warfarin metabolism (mg/l)	2.00
Oral uptake		
kAS	Absorption from stomach (/h)	5.00

[a]Scaled by (body weight)$^{3/4}$.

blood. Similar data were reported by Breckenridge and Orme (1973) among patients given either a po or iv dose of 0.5 mg/kg warfarin.

Although the metabolism of (R)-warfarin was considered in the PBPK model, due to the complexity of the metabolism of this enantiomer, the available information on the effect of the *CYP2C9* polymorphism on it was not considered. It has been demonstrated that the (R)-enantiomer of warfarin is metabolized by several cytochrome P450s, but is poorly metabolized by CYP2C9. CYP2C19 has a higher affinity for (R)-warfarin, as does CYP3A4. Attempts to simulate the available time-course data on (R)-warfarin in human plasma (Choonara *et al.*, 1986) using the available kinetic parameters (*KM*, *VMax*) for CYP2C19, CYP2C9, or CYP3A4 (Kaminsky and Zhang, 1997; Sullivan-Klose *et al.*, 1996) individually were unsuccessful, possibly due to an additional, high-affinity metabolic pathway for (R)-warfarin that has not yet been characterized. Therefore, in contrast to the use of allele-specific experimental data for (S)-warfarin, the PBPK model for (R)-warfarin described a single metabolic pathway that represents a combination of multiple metabolic pathways, with the metabolic parameters estimated based on fitting the model to the available kinetic data.

The polymorphism in *CYP2C9* and its impact on the metabolism of (S)-warfarin were examined. There are currently three known human alleles of *CYP2C9* for which the effects on the metabolism of (S)-warfarin have been characterized phenotypically. Information on the variation in the metabolism of (S)-warfarin by the enzymes encoded by the three human alleles (*CYP2C9*1, CYP2C9*2, CYP2C9*3*) was provided in Haining *et al.* (1996), Rettie *et al.* (1994, 1999); Sullivan-Klose *et al.* (1996) and Takahashi *et al.*, 1998a,b). In the PBPK model, the polymorphism was defined by the metabolism parameters, $KM_{(S)}$ and $VMax_{(S)}$ (Table 5). Since the estimates for *Vmax* were derived from *in vitro* systems not necessarily from human cell lines, this parameter was "normalized" based on the available information on the general CYP content per mg protein (0.146 nmol P450/mg protein) in human liver microsomes (Research Diagnostics, Inc., 2001).

Data reported by Black *et al.* (1996) were used to validate the extended warfarin model. In this study, time-course data on concentrations, AUC, and half-life of both (S)- and (R)-warfarin in the plasma were reported in male volunteers given a single po dose of 0.75 mg/kg of a racemic mixture of warfarin.

Parathion/paraoxon model. Parathion is metabolized via CYP3A4 and a desulfuration step to the active form, paraoxon (summarized in Haber *et al.*, 2002). Paraoxon can then exert its toxic effects by reacting with acetylcholinesterases, or it can be detoxified by the polymorphic enzyme paraoxonase (PON1), or by a nonenzymatic reaction with carboxylesterases. PON1 is a polymorphic enzyme, with the high activity *PON1* homozygotes accounting for approximately 41% of the U.S. population, the low activity homozygotes accounting for approximately 15% of the U.S. population, and low/high activity heterozygotes accounting for approximately 45% of the U.S. population (Eckerson *et al.*, 1983; Davies *et al.*, 1996; Diepgen and Geldmacher-von Mallinckrodt, 1986; Haber *et al.*, 2002; Mueller *et al.*, 1983; Sanghera *et al.*, 1998). Since paraoxon is the active agent, low PON1 activity would be expected to result in increased sensitivity.

An existing PBPK model for parathion and paraoxon developed by Gearhart *et al.* (1994) was used. In brief, the model describes the metabolism of parathion to paraoxon by the liver, the inhibition of acetylcholinesterase, butyrylcholinesterase, and carboxylesterase by paraoxon, and the metabolism of paraoxon by paraoxonase in the brain, liver, kidneys, rapidly perfused tissues, and the arterial and venous blood. A schematic of this model with shaded compartments indicating metabolism of paraoxon is shown in Figure 2. The model parameters are given in Gearhart *et al.* (1994) and are reproduced here as the mean values recorded in Table 6. The parathion PBPK model was a modification of a PBPK model for diisopropylfluorophosphate (DFP), another organophosphate whose mechanism of action is thought to be representative of other highly toxic organophosphates. The DFP model was validated in humans based on data in humans repeatedly treated therapeutically with DFP.

Information on the kinetic differences associated with the PON1 polymorphism was provided by Davies *et al.* (1996), Mueller *et al.* (1983), and Smolen *et al.* (1991). The model parameters assumed to be affected by the polymorphism are the Michaelis-Menten metabolism parameters in the arterial and venous blood compartments, KM_{AB}, $VMax_{AB}$, KM_{VB}, and $VMax_{VB}$ (presented as scaled parameters in Table 7).

Monte Carlo analysis. Monte Carlo simulations were used to examine the variability in the area under the blood or plasma concentration curve (AUC) for (S)-warfarin or paraoxon following po exposure to 1 mg/kg warfarin or 0.033 mg/kg parathion, respectively. The warfarin dose was selected because it was within the clinical range of exposures, and the parathion dose was selected because it was similar to human exposures to organophosphates reported in the available literature. For most chemicals, target tissue response is related to cumulative exposure, rather than a peak concentration; therefore, in the absence of other data, the AUC is a reasonable dose metric for these chemicals (IPCS, 2001). The AUC was computed for paraoxon, the metabolite of parathion, instead of the parent compound, because paraoxon is the toxic compound. Similarly, the AUC was calculated for warfarin itself, because the parent form is the active form. As shown in Tables 4 and 6, each parameter in the PBPK models was assigned a variability distribution defined by a mean, a variance, distribution shape (log-normal or normal), and upper and lower bounds based on ± 3 SDs (Clewell *et al.*, 1999, 2000, 2001). For the metabolic parameters affected by the polymorphisms, a separate distribution was defined for each allele. Using Latin Hypercube techniques, 1000 sets of values were determined by sampling from each parameter distribution. Each of the generated sets of values was then used as input to the PBPK models to estimate a corresponding distribution of the AUC.

For each chemical, multiple cases of Monte Carlo simulations were performed. A brief summary of the parameters that were varied and the distributions assigned to the metabolic parameters for each case is given in Table 8. A more detailed discussion of each case is given in the following paragraphs.

Case 1 examined the potential effect of the different homozygous genotypes (i.e., the metabolic variability alone) on the distribution of the AUC if there were no other variability in pharmacokinetic parameters. For this case, separate simulations were performed for each homozygous genotype. The values of KM and VMax for homozygous individuals were randomly generated from the distributions for the allele of interest, and all other model parameters were held fixed at their mean values.

TABLE 4
Distributions for Parameters in the Warfarin Model

Parameter name	Paramter description	Mean	Coefficient of variation	Lower bound	Upper bound
Vmax, maximum rate of metabolism (mg/h/kg$^{3/4}$)[a]					
CYP2C9*1	Haining *et al.*, 1996	1.61			
	Takahashi *et al.*, 1998b	2.13	0.0036	2.11	2.15
	Sullivan-Klose *et al.*, 1996	1.01	0.046	0.88	1.16
	Rettie *et al.*, 1994	3.20			
	Rettie *et al.*, 1994	3.20			
CYP2C9*2	Sullivan-Klose *et al.*, 1996	1.26	0.031	1.15	1.38
	Rettie *et al.*, 1994	0.21			
	Rettie *et al.*, 1994	0.36			
	Rettie *et al.*, 1999	1.10	0.028	0.99	1.23
CYP2C9*3	Haining *et al.*, 1996	0.31			
	Takahashi *et al.*, 1998b	0.51	0.22	0.26	0.97
	Sullivan-Klose *et al.*, 1996	1.37	0.044	1.20	1.57
KM, affinity (mg/l)					
CYP2CP*1	Haining *et al.*, 1996	1.85			
	Takahashi *et al.*, 1998b	0.81	0.12	0.56	1.12
	Sullivan-Klose *et al.*, 1996	3.57	0.078	2.82	4.49
	Rettie *et al.*, 1994	1.26			
	Rettie *et al.*, 1994	1.05			
CYP2C9*2	Sullivan-Klose *et al.*, 1996	3.85	0.056	3.25	4.55
	Rettie *et al.*, 1994	0.52			
	Rettie *et al.*, 1994	0.65			
	Rettie *et al.*, 1999	1.85	0.17	1.11	2.99
CYP2C9*3	Haining *et al.*, 1996	9.24			
	Takahashi *et al.*, 1998b	3.20	0.16	1.94	5.15
	Sullivan-Klose *et al.*, 1996	28.43	0.059	23.74	33.93
Blood flows (fraction of cardiac output)					
QCC	Cardiac output (l/h/kg$^{3/4}$)[b]	12.89	0.22	6.56	24.17
QKidC	Kidney	0.18	0.30	0.07	0.40
QLivC	Liver	0.23	0.32	0.08	0.55
QRapC	Rapidly perfused tissue	0.36	0.30	0.14	0.83
QSknC	Skin	0.18	0.30	0.07	0.42
QSlwC	Slowly perfused tissue	0.06	0.30	0.02	0.13
Tissue volumes (fraction of body weight)					
BW	Body weight (kg)	70.00	0.11	50.07	96.69
VPlasC	Plasma	0.07	0.15	0.04	0.11
VKidC	Kidney	0.00	0.30	0.00	0.01
VLivC	Liver	0.03	0.25	0.01	0.05
VRapC	Rapidly perfused tissue	0.06	0.30	0.02	0.13
VSknC	Skin	0.14	0.30	0.06	0.33
VSlwC	Slowly perfused tissue	0.61	0.16	0.37	0.97
HCT	Hematocrit (RBC/Total = 2200/5200)	0.42	0.30	0.17	0.98
Tissue/blood partition coefficients (from Luecke *et al.*, 1994)					
PKid	Kidney	0.48	0.30	0.19	1.11
PLiv	Liver	1.08	0.30	0.43	2.50
PRap	Rapidly perfused tissue (used gut value)	1.00	0.30	0.40	2.31
PSkn	Skin	0.28	0.30	0.11	0.64
PSlw	Slowly perfused tissue	0.06	0.30	0.02	0.14
PFac	Adjustment for differences in plasma binding between animals and humans	4.00	0.30	1.59	9.24
Fraction of free warfarin in tissues					
FFPlas	Plasma	0.0080	0.3	8.00E-04	1.52E-02
FFLiv_S	Liver for (S)-warfarin	0.040	0.3	4.00E-03	7.60E-02
FFLiv_R	Liver for (R)-warfarin	0.045	0.3	4.50E-03	8.55E-02
Metabolism parameters					
KMI_S	KM for inhibition of (S)-warfarin	2.00	0.50	0.43	7.38
VMaxC_R	Maximum reaction rate (mg/h per 1 kg)	1.90	0.30	0.75	4.39
KM_R	Michaelis-Menten (mg/l)	4.00	0.50	0.87	14.76
KMI_R	KM for inhibition of (R)-warfarin	10,000	0.50	2168	36,898
Uptake parameter					
kAS	Absorption from stomach (/h)	5.00	0.30	1.99	11.55

Note. A missing coefficient of variation indicates that the SD was not reported. For the parameters associated with the fraction of free warfarin in tissues, distribution was assumed to be normal; for all others, it was assumed to be log-normal.

[a] Sampling of this parameter is described in the text.

TABLE 5
Metabolic Parameters for (S)-Warfarin for Three CYP2C9 Alleles

Allele	Reference	Vmax (mg/h/kg$^{3/4}$) Mean	Vmax (mg/h/kg$^{3/4}$) CV	Km (mg/l) Mean	Km (mg/l) CV	Intrinsic clearance (VmaxC/Km)
CYP2C9*1						
	Haining *et al.*, 1996[a]	1.61		1.85		0.87
	Takahashi *et al.*, 1998b[b]	2.13	0.0036	0.81	0.12	2.6
	Sullivan-Klose *et al.*, 1996[b]	1.01	0.046	3.57	0.078	0.28
	Rettie *et al.*, 1994[c]	3.20		1.26		2.5
	Rettie *et al.*, 1994[d]	3.20		1.05		3.0
CYP2C9*2						
	Sullivan-Klose *et al.*, 1996[b]	1.26	0.031	3.85	0.056	0.33
	Rettie *et al.*, 1994[c]	0.21		0.52		0.40
	Rettie *et al.*, 1994[d]	0.36		0.65		0.55
	Rettie *et al.*, 1999[e]	1.10	0.036	1.85	0.17	0.59
CYP2C9*3						
	Haining *et al.*, 1996[a]	0.31		9.24		0.034
	Takahashi *et al.*, 1998b[b]	0.51	0.22	3.20	0.16	0.16
	Sullivan-Klose *et al.*, 1996[b]	1.37	0.044	28.4	0.059	0.048

[a]Baculovirus/insect cell system, purified enzyme.
[b]Yeast expression, microsomes.
[c]Hep G2 cells, cell lysate.
[d]Hep G2 cells, particulate preparation.
[e]Expressed in insect cells, purified enzymes.

For Case 2, Monte Carlo simulations were performed in order to determine the variability distribution that represents a clear majority of genotypes in the population. All of the model parameters were varied and the values of the metabolic parameters were generated from the distribution chosen to represent the "normal" population. For (S)-warfarin metabolism, the most prevalent genotype, homozygous CYP2C9*1, was chosen to represent the normal population since 78% of the population possesses this genotype. However, for paraoxon metabolism, there is no clear wild-type genotype, since the homozygous high activity genotype is present in 15% of the population, the homozygous low activity genotype is in 40% of the population, and 45% of the population is heterozygous. To provide a benchmark population for which meaningful comparisons to a sensitive subpopulation could be made, the "normal" population was arbitrarily defined as the high activity homozygotes plus the heterozygotes, which accounts for 60% of the population. Quantitatively, the population was defined by sampling from the distributions of the high activity and heterozygous genotypes, with the probability of choosing a high activity genotype equal to 25% and the probability of choosing a heterozygous genotype equal to 75%. This corresponds to the 3:1 ratio between the heterozygous and homozygous high-activity populations.

Case 3 was conducted to model the variability in the total population. In Case 3, all parameters were varied and the values for the metabolic parameters were randomly generated from the distributions for all genotypes, with the frequency of sampling from each distribution determined by the prevalence of each genotype in the U. S. population (Haber *et al.*, 2002). The prevalence of the genotypes affecting (S)-warfarin and paraoxon metabolism are shown in Table 9. For both (S)-warfarin and paraoxon, the values of the metabolic parameters representing the heterozygous genotypes were computed by randomly selecting a value from each of the distributions for the homozygous genotypes and averaging the two values, an approach that assumes a gene dosage effect.

For parathion, a fourth case was performed to generate an AUC distribution for a sensitive subpopulation. All parameters were varied and the values for the metabolic parameters were randomly generated from the distribution for the low activity genotype to represent the sensitive subpopulation.

For warfarin, there were considerable differences among the kinetic parameters reported for a given CYP2C9 allele among the different studies and expression systems (Haining *et al.*, 1996; Rettie *et al.*, 1994, 1999; Sullivan-Klose *et al.*, 1996; Takahashi *et al.*, 1998a,b). All of the distributions defined by the different *in vitro* kinetic parameters for a given allele were sampled evenly to represent the full range of the variability and uncertainty in these parameters. This approach was used because there was no *a priori* way to identify one set of data as preferable to another, based on study design, quality, or model fit. The data used for model validation were inadequate for determining if certain sets of parameters were preferred over others, because the relatively small sample sizes for the data sets used for model validation may not have fully represented the variability in the population, even for the wild-type allele.

SDs were reported in only three of the five studies used to describe warfarin kinetics, so simulation of the warfarin metabolic parameters was achieved through a dynamic distribution. For each simulation of the metabolic parameters representing a given homozygous genotype, the studies of that genotype were assigned equal probabilities of being chosen to define the distributions of KM and VMax. If the study that was chosen reported a mean and SD, the definition of the distribution was complete. If the chosen study did not report a SD, a SD was chosen at random from the studies of the same allele that reported standard deviations.

Correlation coefficients were computed as a measure of the sensitivity of the AUC dose metric to the model parameters for each of the Monte Carlo simulations. Pearson correlation coefficients were computed using the SAS System®.

RESULTS

Warfarin

Model predictions of the plasma concentration of (S)- and (R)-warfarin are compared to the data of Black *et al.* (1996) in

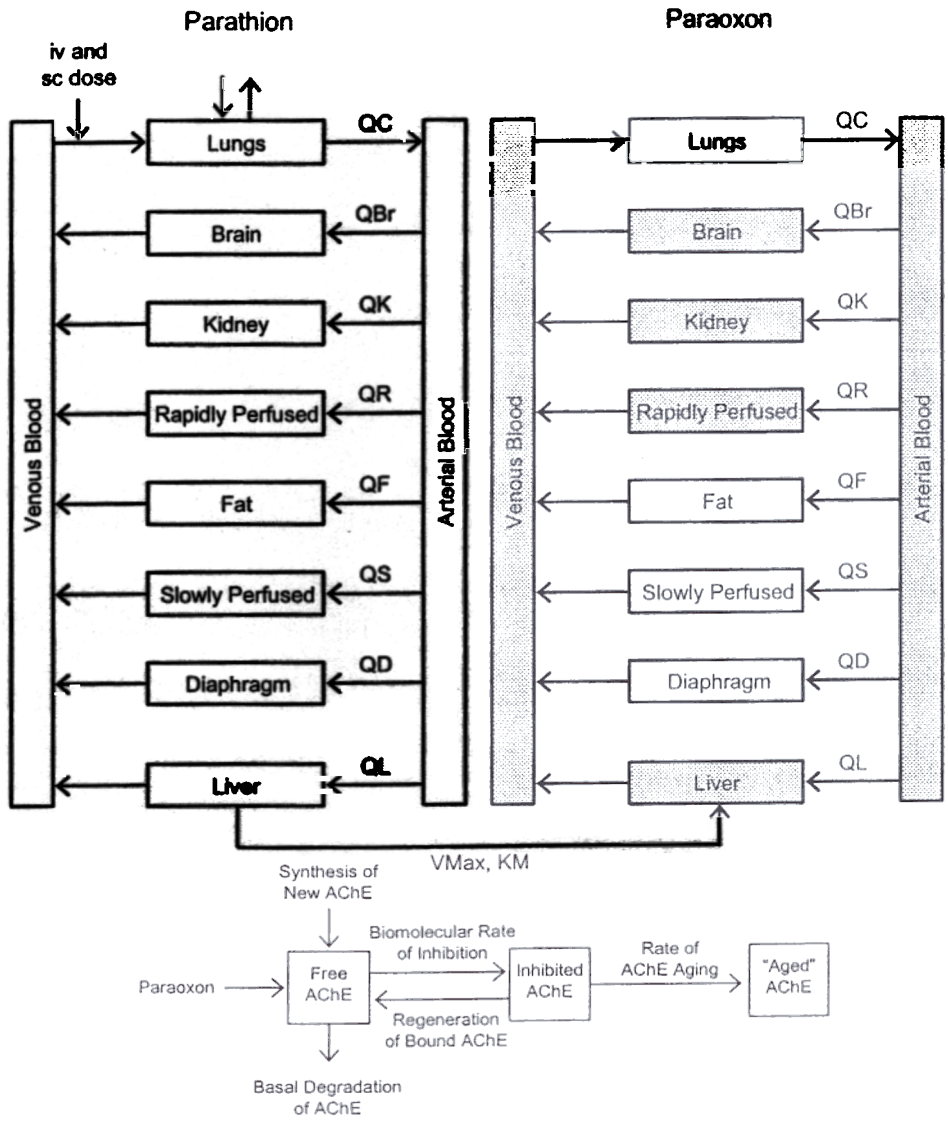

FIG. 2. PBPK model for parathion and paraoxon developed by Gearhart *et al.* (1994). The model describes the metabolism of parathion to paraoxon (shaded areas) in the liver, brain, kidney, rapidly perfused tissues, and both blood compartments. The inhibition of acetylcholinesterase, butyrylcholinesterase, and carboxylesterase by paraoxon was also considered.

Figures 3 and 4, respectively. Excellent agreement between the experimental data and the model predictions was obtained. Once fit to the time-course data of Breckenridge and Orme (1973), Chan *et al.* (1994), and Choonara *et al.* (1986), no further adjustments to the model parameters were necessary to obtain this resulting fit to the validation data set of Black *et al.* (1996).

Figure 5 shows the results for Case 1 for (S)-warfarin, in which the plasma concentration over time was simulated for each of the three homozygous genotypes. Each line in the figure represents one Monte Carlo simulation. The apparent banding of the output is due to differences in the input metabolic data obtained for the same allele in different studies (Table 5). Inspection of the figures demonstrates that the presence of the CYP2C9*3 allele has the greatest impact on the metabolism and clearance of the (S)-enantiomer of warfarin, which is consistent with clinical observations (Aithal *et al.*, 1999; Steward *et al.*, 1997; Takahashi *et al.*, 1998a,b; Taube *et al.*, 2000). However, CYP2C9*3 is present in less than 1% of the population. Approximately 12% of the population is a heterozygous genotype possessing a combination of CYP2C9*1 and CYP2C9*2 alleles, but this heterozygous genotype would not be expected to contribute significantly to the variability of the dose metric among the total population since the protein encoded by CYP2C9*2 is nearly as active as the wild-type CYP2C9*1 protein. The heterozygous genotype possessing a combination of CYP2C9*1 and CYP2C9*3 alleles, however, is present in 9% of the population. Since the CYP2C9*3 protein is far less active than the wild-type, the presence of this heterozygous allele may increase the variability in the AUC dose metric.

The distributions of the AUCs resulting from the different Monte Carlo cases were compared in order to determine the effect of the polymorphisms on the variability of tissue expo-

TABLE 6
Distributions for Parameters in the Parathion/Paraoxon Model

Model parameter	Description	Mean	Coefficient of variation	Lower bound	Upper bound
Paraoxon metabolism parameters defining the polymorphism					
Low activity					
KMABC/KMVBC		132.5	0.50	28.73	488.93
VMABC/VMVBC		339.7	0.24	162.42	671.89
High activity					
KMABC/KMVBC		95.1	0.30	37.75	219.73
VMABC/VMVBC		1619.4	0.20	876.63	2876.53
Partition coefficients					
Paraoxon					
PB	Blood/air	1.0E+06	0.30	397,019.46	2,310,796.54
PBR	Brain/blood	2.3	0.30	0.92	5.34
PD	Diaphragm/blood	3.6	0.30	1.44	8.37
PF	Fat/blood	10.2	0.30	4.06	23.62
PK	Kidney/blood	6.6	0.30	2.63	15.30
PL	Liver/blood	1.5	0.30	0.60	3.47
PLU	Lung/blood	1.0	0.30	0.40	2.31
PR	Rapidly/blood	6.6	0.30	2.63	15.30
PS	Slowly/blood	3.6	0.30	1.44	8.37
PAFCO	Diffusion limitation const. for paraoxon to fat (l/h/kg bw)	3.5	0.30	1.39	8.09
Parathion					
PBP	Blood/air	1.0E+06	0.30	397,019.46	2,310,796.54
PBRP	Brain/blood	4.6	0.30	1.81	10.54
PDP	Diaphragm/blood	0.5	0.30	0.20	1.16
PFP	Fat/blood	40.0	0.30	15.88	92.43
PKP	Kidney/blood	5.2	0.30	2.07	12.04
PLP	Liver/blood	5.2	0.30	2.07	12.04
PLUP	Lung/blood	1.0	0.30	0.40	2.31
PRP	Rapidly/blood	5.2	0.30	2.07	12.04
PSP	Slowly/blood	0.5	0.30	0.20	1.16
PAFPC	Diffusion limitation const. for parathion to fat (l/h/kg bw)	0.3	0.30	0.12	0.69
Esterase inhibition					
Initial concentration of AChE (μmol/kg organ)					
CAABI	Arterial blood	0.001	0.3000	1.12E-04	2.13E-03
CABRI	Brain	0.038	0.3000	3.78E-03	7.18E-02
CADI	Diaphragm	0.005	0.3000	5.33E-04	1.01E-02
CAKI	Kidney	0.000	0.3000	4.76E-05	9.04E-04
CALI	Liver	0.001	0.3000	8.91E-05	1.69E-03
CALUI	Lung	0.002	0.3000	1.94E-04	3.69E-03
CARI	Richly perfused tissue	0.004	0.3000	3.55E-04	6.75E-03
CASI	Slowly perfused tissue	0.007	0.3000	6.61E-04	1.26E-02
CAVBI	Venous blood	0.001	0.3000	1.12E-04	2.13E-03
Initial concentration of BuChE (μmol/kg organ)					
CBABI	Arterial blood	0.005	0.3000	4.80E-04	9.12E-03
CBBRI	Brain	0.013	0.3000	1.27E-03	2.41E-02
CBDI	Diaphragm	0.011	0.3000	1.07E-03	2.03E-02
CBKI	Kidney	0.003	0.3000	2.86E-04	5.43E-03
CBLI	Liver	0.008	0.3000	8.22E-04	1.56E-02
CBLUI	Lung	0.023	0.3000	2.32E-03	4.41E-02
CBRI	Brain	0.099	0.3000	9.91E-03	1.88E-01
CBSI	Slowly perfused tissue	0.006	0.3000	6.06E-04	1.15E-02
CBVBI	Venous blood	0.005	0.3000	4.80E-04	9.12E-03

TABLE 6—*Continued*

Model parameter	Description	Mean	Coefficient of variation	Lower bound	Upper bound
Initial concentration of carboxyl-E (μmol/kg organ)					
CCABI	Arterial blood	4.2	0.3	4.19E-01	7.96E+00
CCBRI	Brain	0.6	0.3	5.50E-02	1.05E+00
CCDI	Diaphragm	2.9	0.3	2.93E-01	5.57E+00
CCKI	Kidney	16.5	0.3	1.65E+00	3.14E+01
CCLI	Liver	45.5	0.3	4.55E+00	8.65E+01
CCLUI	Lung	12.9	0.3	1.29E+00	2.45E+01
CCRI	Richly perfused tissue	213.0	0.3	2.13E+01	4.05E+02
CCSI	Slowly perfused tissue	2.3	0.3	2.27E-01	4.31E+00
CCVBI	Venous blood	4.2	0.3	4.19E-01	7.96E+00
AChE bimolecular rate constant (μmol/l/h)-1					
K1AB	Arterial blood	10.0	0.30	3.97	23.11
K1BR	Brain	10.0	0.30	3.97	23.11
K1D	Diaphragm	10.0	0.30	3.97	23.11
K1VB	Venous blood	10.0	0.30	3.97	23.11
K1	All other tissues	10.0	0.30	3.97	23.11
BuChE bimolecular rate constant (μmol/l/h)-1					
K2AB	Arterial blood	354.0	0.30	140.54	818.02
K2D	Diaphragm	354.0	0.30	140.54	818.02
K2FBR	Fast brain	354.0	0.30	140.54	818.02
K2SBR	Slow brain	30.0	0.30	11.91	69.32
K2VB	Venous blood	354.0	0.30	140.54	818.02
K2	All other tissues	354.0	0.30	140.54	818.02
CarboxylE bimolecular rate constant (μmol/l/h)-1					
K3AB	Arterial blood	0.9	0.30	0.36	2.08
K3D	Diaphragm	0.9	0.30	0.36	2.08
K3FBR	Fast brain	1.1	0.30	0.44	2.55
K3SBR	Slow brain	0.5	0.30	0.21	1.21
K3VB	Venous blood	0.9	0.30	0.36	2.08
K3	All other tissues	0.9	0.30	0.36	2.08
Regeneration rates (/h)					
K5AAB	Arterial blood	0.01	0.30	0.004	0.02
K5ABR	Brain	0.01	0.30	0.004	0.02
K5AD	Diaphragm	0.01	0.30	0.004	0.02
K5AVB	Venous blood	0.01	0.30	0.004	0.02
Aging rates (/h)					
K6AAB	Arterial blood	0.05	0.30	0.02	0.12
K6ABR	Brain	0.05	0.30	0.02	0.12
K6AD	Diaphragm	0.05	0.30	0.02	0.12
K6AVB	Venous blood	0.05	0.30	0.02	0.12
Synthesis rates (μmol/h)					
K7AAB	Arterial blood	0.0001	0.30	0.00002	0.00
K7ABR	Brain	0.0000	0.30	0.00000	0.00
K7AD	Diaphragm	0.0000	0.30	0.00000	0.00
K7AVB	Venous blood	0.0001	0.30	0.00002	0.00
Metabolism parameters					
Paraoxon					
Michaelis-Menten KM (mg/l)					
KMBRC	Brain	439.8	0.50	95.35	1622.77
KMDC	Diaphragm	1000.0	0.50	216.81	3689.79
KMKC	Kidney	134.3	0.50	29.12	495.54
KMLC	Liver	50.1	0.50	10.86	184.82
KMLUC	Lung	1000.0	0.50	216.81	3689.79
KMRC	Richly perfused tissue	50.9	0.50	11.03	187.77
KMSC	Slowly perfused tissue	1000.0	0.50	216.81	3689.79

TABLE 6—*Continued*

Model parameter	Description	Mean	Coefficient of variation	Lower bound	Upper bound
Maximum vel. metab. (mg/h/kg bw)					
VMBRC	Brain	26.5	0.30	10.52	61.24
VMKC	Kidney	298.2	0.30	118.38	689.03
VMLC	Liver	31.8	0.30	12.63	73.48
VMRC	Richly perfused tissue	31.9	0.30	12.65	73.65
Parathion					
Michaelis-Menten KM (mg/l)					
KMLUPC	To paraoxon in lung	3.0	0.50	0.64	10.96
KMLEC	To diethyl phosphorothioic acid in liver	15.0	0.50	3.25	55.35
KMLPC	To paraoxon in liver	15.0	0.50	3.25	55.35
Maximum vel. metab. (mg/h/kg bw)					
VMLEC	To diethyl phosphorothioic acid in liver	20.0	0.30	7.94	46.22
VMLPC	To paraoxon in liver	20.0	0.30	7.94	46.22
Flow rates					
QPC	Pulmonary ventiliation rate per kg bw (l/kg bw)	17.0	0.16	10.42	27.05
QCC	Cardiac output per kg bw (l/kg bw)	14.5	0.22	7.38	27.19
Tissue flow rates (fraction of QC)					
QBRC	Brain	0.13	0.30	0.05	0.31
QDC	Diaphragm	0.01	0.30	0.002	0.01
QFC	Fat	0.04	0.30	0.01	0.08
QKC	Kidney	0.22	0.30	0.09	0.52
QLC	Liver	0.27	0.32	0.10	0.66
QRC	Richly perfused tissue	0.20	0.30	0.08	0.46
QSC	Slowly perfused tissue	0.13	0.30	0.05	0.30
Tissue volumes per kg bw (l/kg bw)					
VABC	Arterial blood	0.02	0.15	0.01	0.03
VBRC	Brain	0.02	0.30	0.01	0.05
VDC	Diaphragm	0.003	0.16	0.002	0.00
VFC	Fat	0.17	0.24	0.08	0.34
VKC	Kidney	0.00	0.30	0.00	0.01
VLC	Liver	0.02	0.25	0.01	0.04
VLUC	Lung	0.01	0.30	0.003	0.02
VRC	Richly perfused tissue	0.03	0.30	0.01	0.08
VSC	Slowly perfused tissue	0.55	0.16	0.34	0.88
VVBC	Venous blood	0.06	0.15	0.04	0.09
BW	Human body weight (kg)	60.6	0.11	43.35	83.70

Note. Distribution for parameters associated with esterase inhibition was assumed to be normal; for all others, it was assumed to be log-normal.

sure to (S)-warfarin. Descriptive statistics of the AUC distribution from each Monte Carlo simulation are shown in Table 10. Figure 6 compares the distribution of the (S)-warfarin AUCs from Case 3 (i.e., varying all parameters, with the metabolic parameters sampled from the distributions for all genotypes according to the prevalence of each genotype in the U.S. population) with the AUC distribution from Case 2 (i.e., varying all parameters and generating values for the metabolic parameters from the distributions of the homozygous CYP2C9*1 genotype). In other words, Figure 6 compares the total population variability (including the polymorphisms) with the variability that would be estimated without accounting for the polymorphism. Consideration of all alleles involved in the polymorphism shifts the normal distribution to the right, demonstrating an increase in the AUC, and reflecting the contribution of the less active allelic forms. As shown in Table 10, the polymorphism also extends the right tail of the distribution, producing a greater upper bound on the AUC and greater overall variability. For example, introducing the polymorphism increases the median AUC value from 84 to 104 mg-h/l, and increases the 95th percentile from 731 to 1170 mg-h/l. Thus, the polymorphism in CYP2C9 does increase the overall population variability in warfarin tissue dose. Even though the CYP2C9*3 allele occurs at a low prevalence, the difference in activity, coupled with a 9% prevalence of CYP2C9*1/CYP2C9*3 heterozygotes, is sufficient to increase the population variability.

For each Monte Carlo simulation, correlation coefficients

TABLE 7
High Activity and Low Activity Alleles for Paraoxonase in the Arterial and Venous Blood

Allele	Mean	Coefficient of variation	Intrinsic clearance
Low activity			
Km (mg/l)	132.5	0.50	2.6
Vmax (mg/h/kg$^{3/4}$)	339.7	0.24	
High activity			
Km (mg/l)	95.1	0.30	17.0
VMax (mg/h/kg$^{3/4}$)	1619.4	0.20	

Note. Based on data reported in Mueller *et al.*, 1983; Smolen *et al.*, 1991; Davies *et al.*, 1996.

between the AUC dose metric and the simulated input parameters were computed to determine which parameters had the greatest impact on the AUC. Parameters demonstrating a coefficient greater than 0.1 are presented in Table 11. The estimation of the AUC is most sensitive to changes in the parameters related to the polymorphism for CYP2C9, the VMax and KM for the metabolism of (S)-warfarin in the liver (VMaxC_S and KM_S), illustrating the importance of considering the polymorphism in reducing uncertainty in the dose metric.

Parathion/Paraoxon

The results of the Monte Carlo simulations for Case 1 for paraoxon (Fig. 7) demonstrate how the differences between the high and low activity alleles affect the concentration of paraoxon in human blood. Each line in the figure represents one Monte Carlo simulation. These results illustrate that the presence of the high activity allele reduces circulating paraoxon concentrations, compared to blood concentrations estimated using metabolic parameters for the low activity allele. However, the range of the curves for the two alleles overlap and both allelic forms nearly completely eliminate the paraoxon within 24 h.

The distributions of the AUCs resulting from the different Monte Carlo cases were compared to determine the effect of the PON1 polymorphism on the variability of tissue exposure

TABLE 8
Summary of the Monte Carlo Simulation Cases

Case	Parameters varied	Allele(s) defining distribution for Km and Vmax	Population represented
1	Polymorphic	Homozygous	NA
2	All	Most prevalent[a]	Typical, not sensitive
3	All	All	Total
4[b]	All	Low activity	Sensitive

[a]The most prevalent allele, CYP2C9*1, was used for warfarin. The homozygous high activity and heterozygous genotypes were used for paraoxon.
[b]Case 4 performed for paraoxon only.

TABLE 9
Average Prevalence of CYP2C9 and PON1 Alleles in the U.S. Population

Allele	Prevalence (%)
CYP2C9	
S1 homozygous	78
S1/S2 heterozygous	
S1/S3 heterozygous	
S2 homozygous	
S2/S3 heterozygous	
S3 homozygous	0.5
PON1	
High activity-homozygous	15
Low activity-homozygous	
Heterozygous	45

Note. Based on data reported in Haber *et al.*, 2002.

to paraoxon. Descriptive statistics of the AUC distribution from each Monte Carlo simulation are shown in Table 12. The distribution of the paraoxon AUCs for the total population (Case 3) is compared with the distribution from the simulations of the high activity allele alone (Case 1) in Figure 8 and with the distribution from the simulation of the low activity allele

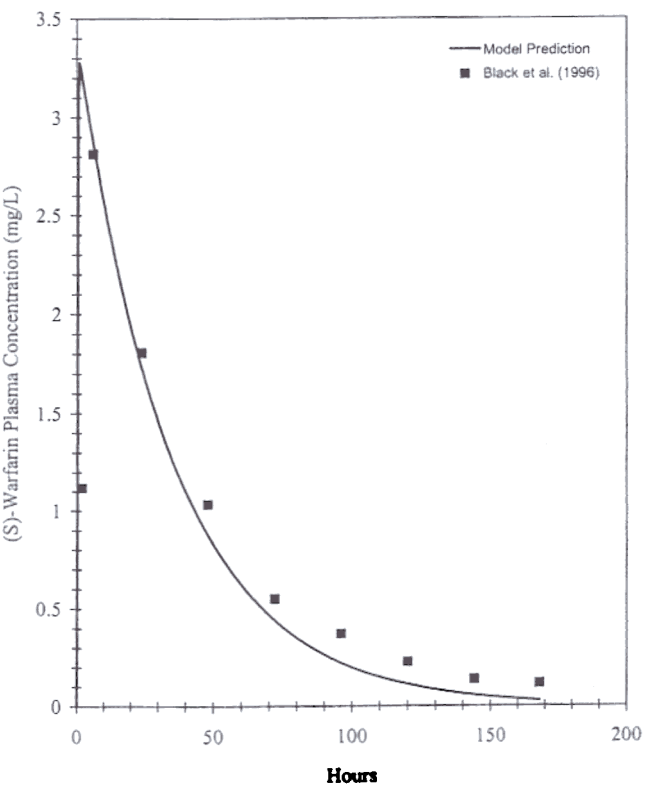

FIG. 3. Model predicted and observed plasma concentrations of (S)-warfarin (Black *et al.*, 1996) in humans following a single po dose of 0.75 mg/kg of a racemic mixture of warfarin.

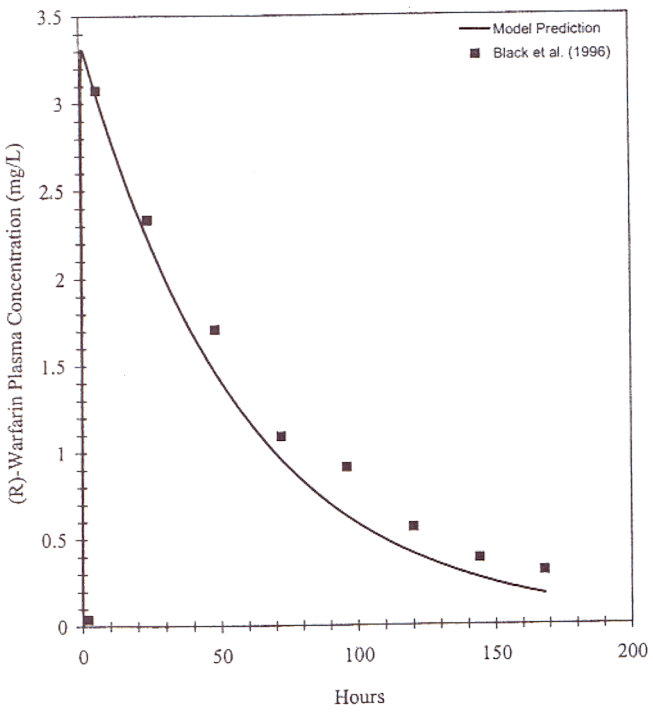

FIG. 4. Model predicted and observed plasma concentrations of (R)-warfarin (Black *et al.*, 1996) in humans following a single po dose of 0.75 mg/kg of a racemic mixture of warfarin.

alone (Case 1). Figure 9 compares the distribution of the paraoxon AUCs for the total population (Case 3), taking into account all sources of the variability, with the distribution for the "normal" population computed by generating the values for the metabolic parameters from the distributions of the high activity homozygotes and heterozygotes (Case 2). Comparison of these two distributions reveals that, while the distribution of the AUC is shifted to the right when the low activity allele is considered in Case 3, the variability associated with the polymorphism does not greatly increase the overall variability compared to what would be expected from variability in other pharmacokinetic parameters. This result is consistent with *in vivo* results in laboratory animals, which suggest that the polymorphism for paraoxonase has little impact on the differences in paraoxon toxicity. Li *et al.* (2000) reported that Pon1 gene knockout mice were not more sensitive to paraoxon than wild-type mice, suggesting that *in vivo*, PON1 is not a major detoxification enzyme. This finding was supported by the absence of a protective effect with injections of rabbit PON1 or human PON1, and by observations of paraoxon-treated transgenic mice over-expressing the human PON1 gene. However, Costa *et al.* (1990) found that PON1 injection did protect rats against paraoxon toxicity, suggesting that the polymorphism may be significant in certain species.

Correlation coefficients between the AUC dose metric and the simulated input parameters were computed to determine which parameters had the greatest impact on the AUC (Table 13). In Cases 2 and 3, over 100 input parameters were simulated; only the coefficients with the ten greatest magnitudes are shown. The estimation of the AUC is most sensitive to changes related to the polymorphism for paraoxonase. Two of the four parameters with the greatest impact on the arterial AUC are the VMax and KM for paraoxonase in the blood compartments, with the affinity for parathion metabolism in the liver (KMLPC and KMLEC) being the second and third most sensitive for estimating the AUC. KMLPC is the affinity for the metabolism of parathion to paraoxon and KMLEC is the affinity for the metabolism of parathion to diethyl phosphorothioic acid. It is reasonable that the paraoxon AUC is highly sensitive to these parameters because they directly influence how much paraoxon is created in the body. However, in contrast to the warfarin case study, many other parameters also had a significant impact on the AUC, reducing the impact of the polymorphism on the total variability.

DISCUSSION

In this article we demonstrate that a PBPK-Monte Carlo based approach is useful for determining the degree to which

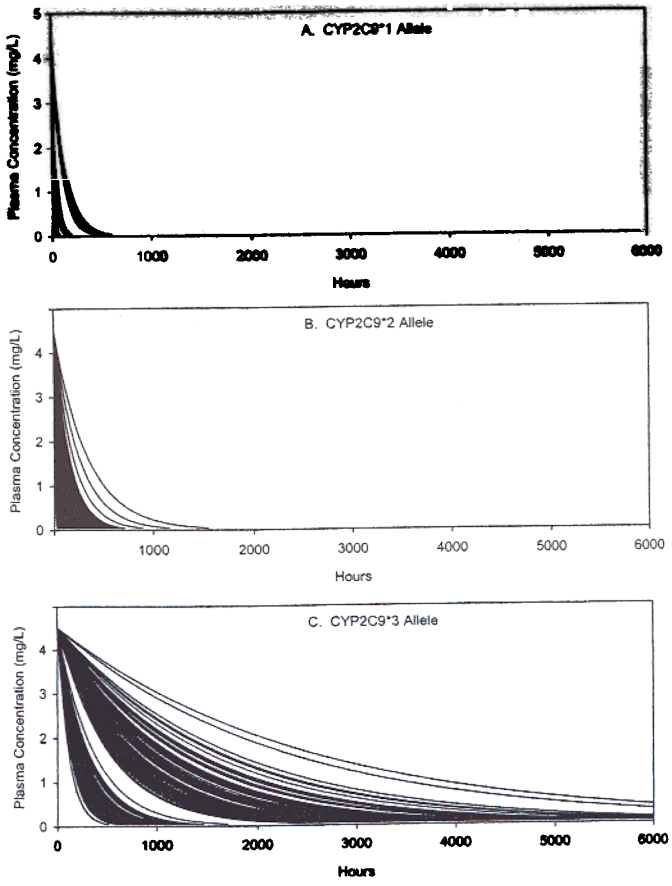

FIG. 5. Case 1 results for (S)-warfarin, in which the plasma concentration over time was simulated for each of the three homozygous genotypes: (A) CYP2C9*1, (B) CYP2C9*2, (C) CYP2C9*3. Each line in the figure represents one Monte Carlo simulation.

TABLE 10
Descriptive Statistics of the AUC (mg-h/l) Distribution for (S)-Warfarin

	Case 1 (CYP2C9*1)[a]	Case 1 (CYP2C9*2)[a]	Case 1 (CYP2C9*3)[a]	Case 2 (normal population)[b]	Case 3 (total population)[c]
Mean	157	273	2670	202	311
SE	5.28	4.73	52.7	11.1	21.9
Median	58.9	252	2680	83.6	104
SD	167	149	1670	351	693
Sample variance	27,900	22,300	2,770,000	123,000	480,000
Kurtosis	0.159	2.48	2.09	99.9	92.1
Skewness	1.34	0.822	1.03	7.44	8.21
Range	578	1180	11,700	6280	9650
Minimum	22.8	20.1	333	10.5	10.1
95th Percentile	509	465	5610	731	1170
99th Percentile	555	692	7670	1380	2670
Maximum	601	1200	12,100	6290	9660
Count	1000	1000	1000	1000	1000

[a]Varying only the metabolism parameters defining the polymorphism, using the allele indicated.
[b]Varying all parameters except those defining the polymorphism.
[c]Varying all parameters, using U.S. population frequencies of each allele.

polymorphisms influence human variability in tissue dose, and for integrating polymorphism data into human health risk assessments. This approach increases the degree to which genetic polymorphisms can be taken into account in assigning uncertainty factor values for variability in human sensitivity—a critical decision point in noncancer risk assessment. Traditionally, uncertainty factors in noncancer risk assessment have considered both interspecies differences and intraspecies variability. Each of these factors is comprised of two components: pharmacokinetics and pharmacodynamics. In recent years, there has been an attempt to develop methodology for the incorporation of more chemical-specific data into the consideration of these factors. As discussed previously, one example of this type of methodology has been developed under the IPCS initiative on the Harmonization of Approaches to the Assessment of Risk from Exposure to Chemicals (Fig. 10).

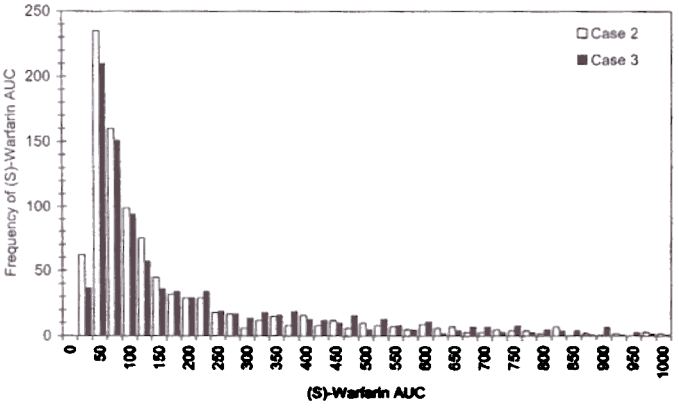

FIG. 6. Comparison of the distributions of (S)-warfarin AUCs from Case 2 (varying all parameters and generating values for the metabolic parameters from the distributions of the homozygous *CYP2C9*1* genotype) and Case 3 (i.e., varying all parameters, with the metabolic parameters sampled from the distributions for all genotypes according to the prevalence of each genotype in the U.S. population).

Based on the IPCS guidance (2001), the current results on human variability in tissue dose for warfarin and parathion could be used to estimate a Chemical Specific Adjustment Factor (CSAF) to replace the standard adjustment factor of 3.2 for intrahuman variability in pharmacokinetics (HK_{AF}), and thus represent a step forward in the goal of incorporating the best available science into the risk assessment process. Sufficient data are available for both warfarin and parathion for the development of CSAFs, based on the criteria developed by IPCS (2001). The active chemical form has been identified (warfarin and paraoxon). For both chemicals, AUC in blood or plasma was considered an appropriate toxicokinetic parameter, and an appropriate PBPK model was available. Kinetic data from the oral route (the route of interest) were used to validate the models developed using *in vitro* kinetic parameters. Information on allelic frequencies in the general U.S. population was used, improving the degree to which the data describe the total population variability. Finally, the major metabolizing tissues for both warfarin and paraoxon are tissues other than the target tissue, so the variability estimated is clearly toxicokinetic variability, not toxicodynamic variability.

The IPCS (2001) CSAF guidelines recommend that HK_{AF} be calculated as the ratio between "given percentiles (such as 95th, 97.5th, and 99th) and the central tendency for the whole population. Alternatively, where there are sensitive subgroups, this ratio is the upper percentile for the sensitive subgroup and the central tendency for the whole population." The guidance does not specify whether the median or mean should be used as

TABLE 11
Significant Correlation Coefficients between (S)-warfarin AUC and Selected Parameters

Parameter name	Parameter description	Case 1 (metabolism parameters)	Case 2 (normal population)	Case 3 (total population)
KM_S	Affinity for metabolism of (S)-warfarin in liver			
		0.98[a]		0.64
		0.54[b]		
		0.27[c]	0.63	
VMaxC_S	Maximum rate of metabolism of (S)-warfarin in liver			
		−0.81[a]		−0.34
		−0.21[b]		
		−0.14[c]	−0.52	
FFLiv_S	Fraction of free (S)-warfarin in liver		−0.27	−0.28
Pfac	Adj. for diff. in plasma binding, animals and humans		0.26	0.17
Pliv	Liver/blood partition coefficient		−0.20	−0.15

[a]CYP2C9*1.
[b]CYP2C9*2.
[c]CYP2C9*3.

a measure of central tendency. Because the IPCS guidance allows different options for calculation of CSAFs, the implications of several different options were considered in our analysis. Specifically, CSAFs were computed as the ratio of the 95th or 99th percentile of the AUC distributions to the mean or median of the AUC distributions. The implications of using the 95th percentile of the total population versus the 95th percentile of the sensitive population were also explored.

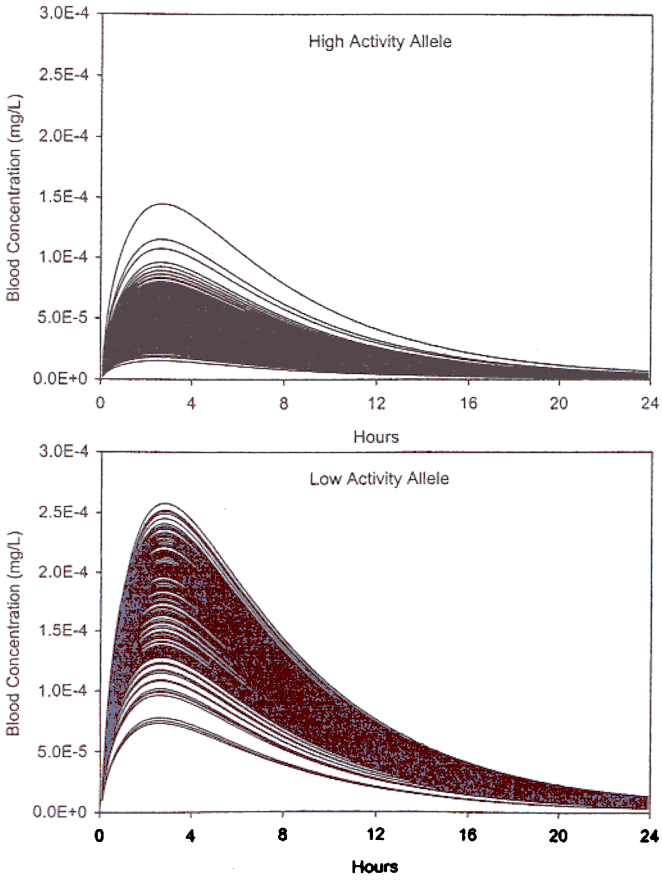

FIG. 7. Case 1 results for paraoxon, in which the plasma concentration over time was simulated for each of the two homozygous genotypes (high activity and low activity). Each line in each figure represents one Monte Carlo simulation.

Table 14 shows CSAFs calculated for warfarin by applying the IPCS methodology to the results in Table 10. No CSAFs are shown for Case 1 (varying only the metabolism parameters defining the polymorphism and considering only homozygotes), because this case does not consider how variability in other pharmacokinetic parameters affects the AUC. (These considerations were addressed in the full analysis conducted for Case 3.) When all of the parameters (including those for the polymorphism) were allowed to vary (Case 3), the calculated CSAFs ranged from 3.8 to 26. Using the 95th percentile to represent the sensitive individual and the mean to represent the average individual, the warfarin CSAF of 3.8 is approximately equal to the default factor of $10^{0.5}$ (3.2) for pharmacokinetic variability. When the average individual is represented by the median instead of the mean, the calculated CSAF increases to 11. Since the population distribution of S-warfarin tissue dose is highly skewed, rather than being symmetric about the mean (Fig. 6), the median is a more appropriate choice in this case, because it is less sensitive to extreme values.

CSAFs for Case 2 (excluding the polymorphism) were calculated to provide a point of comparison, in order to evaluate the impact of the polymorphism on the CSAFs. The CSAF for warfarin calculated as the 95th percentile/mean for Case 2 is very similar to that calculated for Case 3, but the other Case 2 CSAFs (P95/median, P99/mean, P99/median) were clearly smaller than the corresponding Case 3 values, reflecting the decreased variability in the absence of the polymorphism. Use of the 99th percentile instead of the 95th percentile approximately doubled the calculated CSAF for warfarin for both Case 2 and Case 3.

TABLE 12
Descriptive Statistics of the AUC (mg-h/l) Distribution for Paraoxon

	Case 1 (low activity)	Case 1 (high activity)	Case 2 (normal population)	Case 3 (total population)	Case 4 (sensitive subpopulation)
Mean	2.1E-03	9.2E-04	1.3E-03	1.6E-03	2.2E-03
SE	9.6E-06	6.4E-06	1.9E-05	2.9E-05	3.2E-05
Median	2.1E-03	9.1E-04	1.2E-03	1.4E-03	2.0E-03
SD	3.0E-04	2.0E-04	6.0E-04	9.2E-04	1.0E-03
Sample variance	9.2E-08	4.1E-08	3.6E-07	8.4E-07	1.1E-06
Kurtosis	-0.074	0.29	0.85	1.8	0.74
Skewness	-0.46	0.42	0.78	1.2	0.82
Range	1.6E-03	1.3E-03	4.0E-03	5.9E-03	5.8E-03
Minimum	1.2E-03	3.6E-04	1.9E-04	2.0E-04	3.4E-04
95th Percentile	2.5E-03	1.3E-03	2.3E-03	3.4E-03	4.1E-03
99th Percentile	2.7E-03	1.5E-03	3.0E-03	4.5E-03	5.2E-03
Maximum	2.8E-03	1.6E-03	4.2E-03	6.1E-03	6.2E-03
Count	1000	1000	1000	1000	1000

Table 14 also shows CSAFs calculated for parathion by applying the IPCS methodology to the results in Table 12. Comparison of the results from Cases 1 and 3 show that consideration of all sources of variability actually reduced the CSAF compared to consideration only of variability due to the polymorphism. This is because the variability of the whole population is considered in Case 3, where the variability of only the high activity or low activity genotype is considered in Case 1. All of the CSAFs calculated for parathion were comparable to or smaller than the default of 3.2 for pharmacokinetic variability, reflecting the relatively tight distribution of the paraoxon AUC (Fig. 9). The calculated CSAFs varied by less than 15% when the median was used instead of the mean, consistent with the near-normality of the distribution. Similarly, use of the 99th percentile instead of the 95th percentile increased the CSAF by only 33%. The parathion results were also used to test the implications of using the 95th percentile of the total population versus using the 95th percentile of the sensitive population (Fig. 11). For the latter approach, the normal population was defined as the high activity homozygotes plus the heterozygotes, accounting for 60% of the population; descriptive statistics for this group are shown as Case 2 in Table 12. The sensitive population was represented by the low activity homozygotes, as shown in Case 4 in Table 12. The CSAFs for parathion computed using the 95th percentile of the sensitive population are approximately 50% higher than those calculated using the 95th percentile of the total population.

We also compared the CSAFs calculated using this PBPK-Monte Carlo approach with ones that would be calculated by naively comparing the intrinsic clearance for the different alleles (Tables 7 and 8). Comparing the intrinsic clearance of warfarin obtained with the CYP2C9*1 and CYP2C9*3 proteins in a given report (to avoid interlaboratory variability) results in CSAFs ranging from 5.8 (for Sullivan-Klose *et al.*, 1996) to 26 (for Haining *et al.*, 1996). If these values are

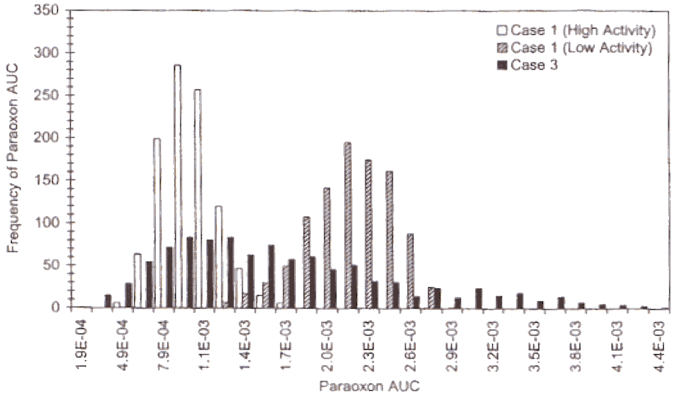

FIG. 8. Comparison of the distribution of paraoxon AUCs for Case 3 (total population) and Case 1 when only the parameters affected by the polymorphism are varied (high or low activity only).

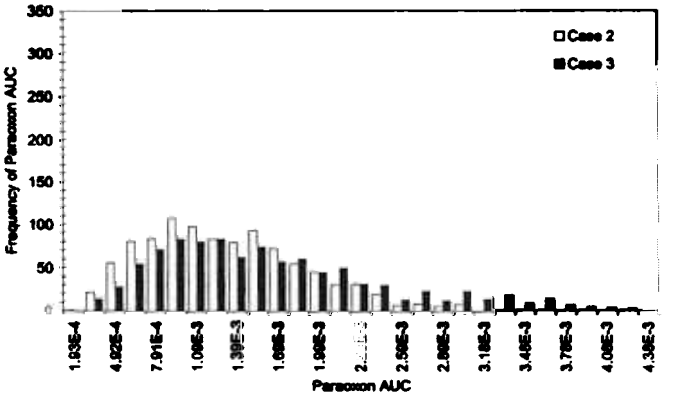

FIG. 9. Comparison of the distribution of the paraoxon AUCs for the total population (Case 3), taking into account all sources of the variability, with the distribution for the "normal" population computed by generating the values for the metabolic parameters from the distributions of the high activity homozygotes and heterozygotes (Case 2).

TABLE 13
Significant Correlation Coefficients between the AUC for Paraoxon and Selected Parameters

Parameter name	Parameter description	Case 1	Case 2	Case 3
KMABC/KMVBC	Affinity for metabolism of paraoxon in arterial and venous blood			
		0.84[a]	0.40	0.30
		0.81[b]		
KMLPC	Affinity for metabolism of parathion to paraoxon in the liver		−0.40	−0.34
KMLEC	Affinity for metabolism of parathion to diethyl phosphorothioic acid in the liver		0.35	0.33
VMABC/VMVBC	Maximum rate of metabolism of paraoxon in the arterial and venous blood			
		−0.56[a]	−0.32	−0.49
		−0.45[b]		
K3	Bimolecular rate constant for the binding of paraoxon to carboxylesterase[c]	—	−0.27	−0.21
VMLEC	Maximum rate of metabolism of parathion to diethyl phosphorothioic acid in the liver		−0.27	−0.21
VMLPC	Maximum rate of metabolism of parathion to paraoxon in liver		0.26	0.24
CCLI	Initial concentration of carboxylesterase binding sites in the liver		−0.20	−0.26
QLC	Fraction of cardiac output to the liver		0.18	0.23
VLC	Fractional volume of the liver		−0.17	−0.18

Note. Case 1, metabolic parameters; Case 2, high activity and heterozygous; Case 3, total population.
[a]High activity allele.
[b]Low activity allele.
[c]In the kidney, liver, lungs, slowly perfused tissues, and rapidly perfused tissues.

compared to the CSAFs presented in Table 14, they are similar in magnitude. However, if a similar comparison is conducted for parathion (Table 8), the ratio of high activity to low activity intrinsic clearance is approximately 7, which is roughly a factor of 3 greater than the CSAF of 2.1 (P95/mean, Case 3). The danger in calculating CSAFs directly from the *in vitro* data is that it ignores the interplay of the polymorphism with other pharmacokinetic factors. For example, if metabolism is flow-limited, variation in enzyme activity may have minimal effect on tissue dose (Clewell *et al.*, 2001; Lipscomb *et al.*, 2002). Due to the lack of these considerations, using comparisons of intrinsic clearance may not adequately describe the *in vivo* variability resulting from polymorphisms. Therefore, the robust analysis provided using a PBPK-Monte Carlo approach is more appropriate for developing CSAFs.

In our analysis, warfarin and parathion provide two rather different case studies for evaluating the implications of various choices made in calculating CSAFs. For warfarin, there is significant *in vivo* variability in toxicokinetics (primarily due to the CYP2C9*3 allele), and the calculated CSAF was markedly different with the use of mean versus median, or 95th versus 99th percentile. By contrast, total human kinetic variability is rather small for the critical metabolite of parathion, and the paraoxonase polymorphism appears to contribute minimally to this variability; alternative definitions of the CSAF for parathion resulted in only minor quantitative differences. Based on these analyses, the default CSAF of 3.2 for human variability in toxicokinetics is adequate or slightly overprotective for parathion. By contrast, a subfactor of 3.2 is not adequately protective for human variability in S-warfarin toxicokinetics. Focusing on the ratio of the 95th percentile and the median, the default of 3.2 may underestimate the "correct" adjustment factor by a factor of approximately 3.4.

Issues, Uncertainties, and Implications for Study Design

The analysis presented here demonstrates that PBPK modeling can be combined with Monte Carlo analyses to evaluate total variability in tissue dose and implications of different definitions of CSAFs. Due to the large number of uncertainties in this analysis, however, we consider this article to be more a demonstration of an approach, rather than deriving definitive CSAFs for the development of RfDs for these chemicals.

A primary source of uncertainty for the warfarin analysis was the surprising degree of variability in the data reported for a given isoform using different expression systems (Table 5). Because no clear basis could be identified for choosing one set of kinetic values over another, all of the data were used together, as an indication of the uncertainty regarding the true enzyme kinetics. This approach, however, meant that the distribution of *VMax* and *KM* included inter- (and intra-) laboratory reproducibility as one component of the uncertainty. Information on which expression systems are most representative of *in vivo* enzyme kinetics could help to inform the choice of expression system, both for laboratory scientists, and for risk assessors/modelers conducting similar exercises in the future. Kinetic data from enzymes in *in vitro* expression systems may also be distorted by differences in the degree of expression of the enzyme of interest. For example, in the available literature the *VMax* data for warfarin are expressed in terms of activity per nmol P450 (Haber *et al.*, 2002). Because the activity was not normalized to the amount of CYP2C9, differences between expression systems in the amount of CYP2C9 protein present

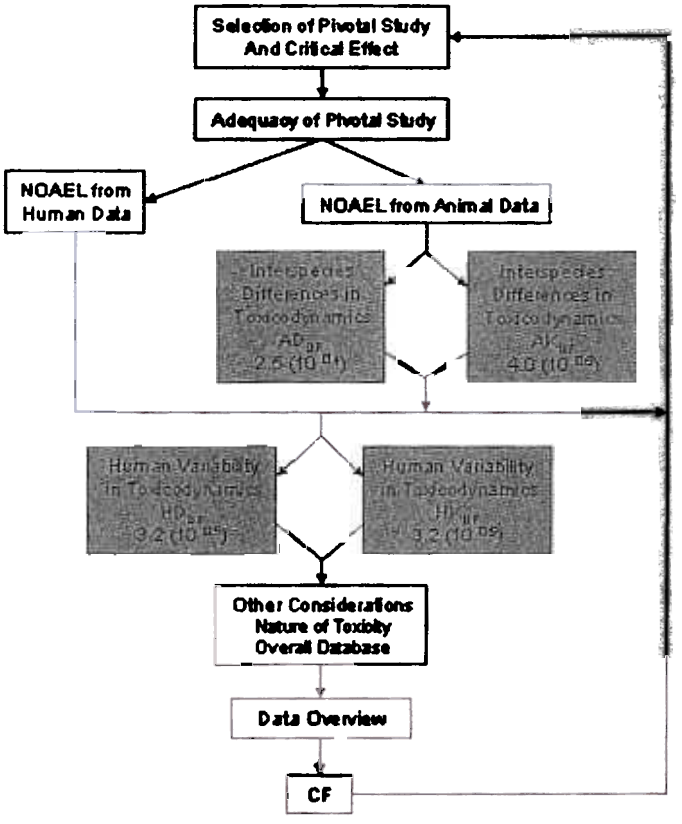

FIG. 10. Methodology currently recommended under the IPCS initiative on the Harmonization of Approaches to the Assessment of Risk from Exposure to Chemicals (IPCS, 2001) for incorporation of quantitative pharmacokinetic and pharmacodynamic data into dose/concentration-response assessments. AD_{UF}, animal to human dynamic uncertainty factor. AK_{UF}, animal to human kinetic uncertainty factor. HD_{UF}, human variability dynamic uncertainty factor. HK_{UF}, human variability kinetic uncertainty factor. CF, composite factor. Chemical-specific data can be used to replace a default uncertainty factor (UF) by an adjustment factor (AF). In the absence of appropriate data, the subdivision of the 10-fold factors collapses back to the 100-fold factor.

TABLE 14
Chemical Specific Adjustment Factors (CSAFs) for Warfarin and Parathion

	Estimator			
	P95/Mean	P95/Median	P99/Mean	P99/Median
Warfarin				
Normal population (Case 2)	3.6	8.7	6.8	17
Total population (Case 3)	3.8	11	8.6	26
Parathion				
Low activity/high activity (Case 1)	2.7	2.8		
Total population (Case 3)	2.1	2.4	2.8	3.2
Sensitive/remaining population (Case 4/Case 2)	3.2	3.4		

Note. P95, 95th percentile; P99, 99th percentile.

may account for some or all of the differences among expression systems. Using an antibody specific to CYP2C9 (e.g., a monoclonal antibody), and normalizing the enzyme activity to the amount of CYP2C9 protein present would provide a more uniform measure of enzyme activity. Kinetic data developed directly from human tissues with known genotypes provides the ideal form of input data for the sort of analysis conducted here, in which enzyme data (*VMax* and *KM*) are used as a parameter in the PBPK-Monte Carlo analysis. In the absence of human tissue data, enzyme kinetics determined for the allelic form of interest can be used. The data should be collected using the substrate of concern, and normalized to the amount of the enzyme that is present. However, further departures from the ideal data set increase the uncertainty in the use of kinetic data on polymorphisms to define CSAFs for risk assessment.

An additional source of uncertainty in the warfarin analysis was that information on variability (SD or similar measure) was not available for several of the *in vitro* studies providing metabolic parameters. As described in the Methods section, the SDs from other *in vitro* studies were applied randomly to the studies for which no SDs were available. This approach could be avoided if experimental reports of enzyme kinetics include a measure of variability.

Several assumptions and simplifications were also needed for the warfarin analysis. The enzymatic activity of the heterozygotes was assumed to be the average of the activity of the homozygotes for each of the two alleles represented in the heterozygote, under an assumption of a gene dosage effect. The analysis was conducted only for (S)-warfarin, the more active enantiomer; good model fit could not be obtained for the PBPK model for (R)-warfarin using available *in vitro* activities. Fi-

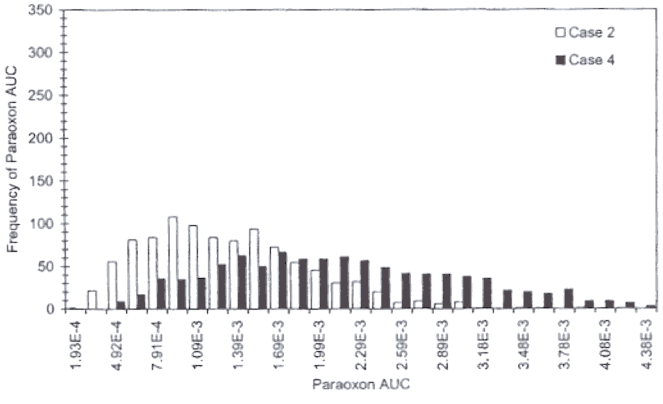

FIG. 11. Comparison of the distribution of paraoxon AUCs for a sensitive population (Case 4), represented by those with the low activity homozygotes, to the distribution of paraoxon AUCs for the "normal" population (Case 2), defined as the high activity homozygotes plus the heterozygotes.

TABLE 15
Minimal Data Needs for PBPK/Monte Carlo Analysis of Effect of Polymorphisms

Description of needs
Well-characterized metabolic pathway, with relevant isozyme identified for all major steps
Allelic frequency data available for all major polymorphic enzymes
Phenotype data for the chemical of interest for each major variant allele
Existing PBPK model or development of an adequate model to describe polymorphism data

nally, only variability in the major metabolic pathway for (S)-warfarin, via CYP2C9, was considered; metabolism by other CYPs was not included in the model.

There are also several uncertainties in the parathion analysis, particularly with regard to characterization of its metabolism. Metabolism of paraoxon by PON1 was included in the PBPK model, but variability in the generation of paraoxon was based on observed variability in kinetic parameters for other chemicals (Clewell *et al.*, 1999, 2000, 2001). Insufficient data were available to quantitatively include data on polymorphism of *CYP3A4* in the analysis of variability in the generation of paraoxon. In addition, genotype does not fully account for the variability in PON1 metabolic capacity. Clearance among individuals with the same low metabolism genotype ranged over 13-fold, at least partially due to differences in protein levels (Furlong *et al.*, 1993). These differences in protein levels could not be accounted for in the current analysis, which rely on *in vitro* enzyme kinetic data to represent the impact of the *PON1* polymorphism. This resulting uncertainty emphasizes the value of kinetic data for human tissues as an ideal input for the PBPK modeling. Nonetheless, we believe that the kinetic parameters used based on the *in vitro* data do adequately represent the situation *in vivo* (although maybe not the full *in vivo* variability), based on our ability to replicate *in vivo* data.

Implications for Future Work

This article, together with our previous companion article (Haber *et al.*, 2002), identifies the minimal data needed to conduct a chemical-specific analysis of the effect of polymorphism on tissue dose. The minimal data needed are summarized in Table 15. The minimal data identified are very similar to the criteria initially identified in choosing the case studies for this analysis (Haber *et al.*, 2002). The primary difference is that we found that chemical-specific phenotype data are necessary; phenotype data using other related substrates is not sufficient. Our initial hope in beginning this analysis was that we would be able to reach generalized conclusions regarding the effects of a specific polymorphism for a class of related chemicals. However, we found that there are marked differences in the effects of a polymorphism among related chemicals. For example, Li *et al.* (2000) found that the two human plasma $PON1_{192}$ isoforms differed by a factor of approximately 9-fold in the intrinsic clearance (*VMax*KM) of paraoxon, but the intrinsic clearance of the two forms was nearly identical for diazoxon hydrolysis (although *VMax* and *KM* individually differed by approximately 3-fold). For hydrolysis of chlorpyrifos oxon, the intrinsic clearance of the two polymorphic forms differed by a factor of about 1.7. These data show that a given amino acid change can have very different quantitative effects on the kinetics of an enzyme towards closely-related substrates.

Combining PBPK modeling and Monte Carlo analysis provides a powerful (although labor-intensive) approach for quantitatively characterizing the effects of polymorphisms on human variability in tissue dose. Because this is a labor-intensive process, key questions need to be addressed in considering this approach. These key questions could be incorporated into the future development of an overall decision framework for integrating polymorphism data into risk assessments.

The first level of questions in this framework would address whether the approach is likely to impact estimates of human variability. Has a polymorphism(s) been identified in a key metabolic enzyme for the chemical of interest? Are there other (unquantified) major contributors to variability? Our case study of parathion highlights this latter point, where other sources of variability likely minimized the impact of the polymorphism on variability in tissue dose. As another example, using an analysis of this type to address variability for a chemical metabolized by CYP2E1 would not be fruitful (even assuming that a polymorphism affecting CYP2E1 activity were known), unless information was included on the large degree of environmentally related variability in CYP2E1 activity. Identification of the active agent (e.g., parent vs. metabolite) is also desirable at this stage. It is possible, however, to conduct the analysis in the absence of such knowledge. In that case, one would need to calculate the effect of the polymorphism on both the tissue dose of the parent and of the metabolite, recognizing that the polymorphism may increase or decrease toxicity. Determination of the active agent would be needed before the results of such an analysis were incorporated into a risk assessment, such as for calculation of a CSAF.

The second level of questions might address whether the available input data are adequate. These questions address whether the criteria listed in Table 15 are met, with particular attention to chemical-specific phenotype data. If the criteria listed in Table 15 are met, the approach described in this article can be used. If the criteria are not met, additional data would need to be generated, or a PBPK model developed, in order to apply this approach. As a long-term goal, it may become possible to take the lessons learned from this current analysis, as well as future analyses presented in the literature, to develop more fully a general decision framework for incorporating polymorphism data into risk assessments. This would be a useful tool as risk assessment scientists will need to address the

impact of a continually growing list of genetic polymorphisms in conducting chemical risk assessments.

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