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A comparison of $^{1}H_{8}$- and $^{2}H_{8}$-toluene toxicokinetics in men

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1. To examine the bioequivalence of an isotope-labelled tracer to study toxicant disposition, we conducted 33 controlled human exposures to a mixture of 50 ppm $^{1}H_{8}$-toluene and 50 ppm $^{2}H_{8}$-toluene for 2 h, and measured concentrations in blood and breath, and metabolite levels in urine for 100 h post-exposure.

2. A physiologically based kinetic (PBK) model found that compared with $^{1}H_{8}$-toluene, $^{2}H_{8}$-toluene had a 6.4± 13% (mean± SD) lower AUC, a 6.5± 13% higher systemic clearance (1.46± 0.27 versus 1.38± 0.25 l/h-kg), a 17± 22% larger terminal volume of distribution (66.4± 14 versus 57.2± 10 l/kg) and a 9.7± 26% longer terminal half-life (38± 12 versus 34± 10 h) ($p < 0.05$ for all comparisons).

3. The higher $^{2}H_{8}$-toluene clearance may have been due to an increased rate of ring oxidation, consistent with the 17% higher observed fraction of $^{2}H_{5}$- versus $^{1}H_{5}$-cresol metabolites in urine.

4. The larger terminal volume and half-lives for $^{2}H_{8}$-toluene suggested a higher adipose tissue/blood partition coefficient.

5. Observed isotope differences were small compared with interindividual differences in $^{1}H_{8}$-toluene kinetics from previous studies.

6. The PBK model allowed us to ascribe observed isotope differences in solvent toxicokinetics to underlying physiologic mechanisms.

Introduction

Stable isotope-labelled compounds serve as valuable probes to understand *in vivo* drug and toxicant disposition by obviating contributions from environmental and endogenous sources (Pierce *et al*. 1997). Examination of the toxicokinetics and effects from occupational or controlled exposures to toxicants can be confounded by background contributions. In the case of $^{1}H_{8}$-toluene, studies with controlled human exposures have assumed that blood concentrations of $\geqslant$ 100 nmol/l were well above background and solely due to administered $^{1}H_{8}$-toluene (Veulemans and Masschelein 1978, Nise *et al*. 1989). Occupational studies have attributed blood levels down to 12–110 nmol/l to workplace exposure alone (Kawai *et al*. 1992a–c, 1994, Mannino *et al*. 1995). However, average concentrations in blood from non-occupationally exposed groups have been measured as 3–14 nmol/l, with a range of up to 206 nmol/l (Brugnone *et al*. 1989, Ashley *et al*. 1994, Etzel and Ashley 1994, Brugnone *et al*. 1995, Fustinoni *et al*. 1995, Vrca *et al*. 1995). Background exposures may therefore confound controlled and occupational exposure studies. One way to address this concern is through the use of stable isotope-labelled probes, a technique commonly employed in studying the *in vivo* disposition of drugs (Falconnet *et al*. 1988).

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Studies using isotopic tracers often assume identical disposition kinetics to the native compound, yet this assumption has not often been examined, particularly for toxicologic studies. Previous work has found that a deuterium label did not affect trazodone kinetics following oral dosing (Gammans *et al.* 1984), and that there were no differences in ^{2}H-labelled versus unlabelled lidocaine or bupivacaine kinetics following i.v. infusion (Burm *et al.* 1988). Browne *et al.* (1982) detected no differences in phenobarbital kinetics with the use of ^{15}N and ^{13}C labels. Others, however, have conducted tracer studies assuming, but not testing, kinetic isotope equivalence (Löf *et al.* 1993, Jones *et al.* 1994).

To assess the kinetic differences between $^{1}H_8$-toluene and $^{2}H_8$-toluene, we administered 50 ppm of each compound as a mixture for 2 h to 26 subjects. Blood and breath concentrations of $^{1}H_8$- and $^{2}H_8$-toluene, and urinary concentrations of metabolites were measured in periodic samples taken for up to 100 h post-exposure. By applying a previously developed physiologically based kinetic (PBK) model for $^{2}H_8$-toluene (Pierce *et al.* 1996a), we compared the areas under the blood concentration-time curves, clearances, and terminal volumes of distribution and half-lives of the labelled and native compounds. Urinary excretion rates of the major metabolite hippuric acid, and of the minor cresol metabolites were used to provide an independent assessment of isotopic metabolic differences.

Materials and methods

Chemicals

$^{2}H_8$-toluene (99.6 g-atom%), and $^{2}H_8$-*o*-, *m*-, and *p*-cresols (98 g-atom%, used as standards) were obtained from Cambridge Isotope Laboratories (Woburn, MA, USA). Toluene (distilled in glass grade) was from Burdick and Jackson (Muskegon, MI, USA) and unlabelled metabolites were obtained from Aldrich (Milwaukee, WI, USA). $^{2}H_5$-hippuric acid was synthesized by reacting $^{2}H_5$-benzoyl chloride with glycine ethyl ester (2-fold molar excess based on $^{2}H_5$-benzoic acid). The reaction mixture was extracted with ether, and the ether was dried with Na_2SO_4 and evaporated. The residue was recrystalized from hexane-chloroform and had a m.p. of 53 °C. Mass spectrometry gave the following peaks: m/z 212 ($M^{+\cdot}$, 10%), 166 ([M-$^{2}HCO_2$]$^{+}$, 5%), 139 ([M-$CO_2CH_2H_3$]$^{+}$, 17%), 110 ([$^{2}H_5PhCO$]$^{+}$, 100%), and 82 ([$^{2}H_5Ph$]$^{+}$, 32%).

Subjects

Twenty-six men of ages 20–62 years of age were recruited by advertisement on campus and given a self-administered questionnaire to screen for occupational solvent exposure. Five of these subjects participated in two or three replicate exposures, with at least 2 weeks between exposures, for a total of 33 exposures. Smokers, those with chronic illness or on chronic medication, and those with occupational solvent exposure were excluded from the study. Individuals with anaemia, acute respiratory infection, rhinitis or asthma were given medical evaluations before entering the study; all subjects were Caucasian. Subjects provided written consent, and recruitment, exposure and sampling protocols were approved by the Institutional Review Board of the University of Washington.

Weight (67–129 kg) and height (1.65–1.98 m) were measured, and the fraction of the body that was adipose tissue (0.10–0.39 kg/kg), was estimated by a skin fold method using Lange calipers (Durnin and Womersley 1974). Alveolar ventilation rates (4.5–9.7 l/min), estimated as 70% of total ventilation, and inhaled $^{1}H_8$-toluene concentrations (43–71 ppm) and $^{2}H_8$-toluene concentrations (45–68 ppm) were also measured (Morgan *et al.* 1993). Person-specific $^{2}H_8$-toluene blood/air partition coefficients were determined previously (Pierce *et al.* 1996a) and were used for modelling both the native and deuterated compounds.

Exposures and sampling

Inhaled toluene concentrations were generated using a controlled screw drive which compressed a syringe to inject solvent into the airstream. Subjects inhaled 100 ppm of an approximately equimolar mixture of $^{1}H_8$-toluene and $^{2}H_8$-toluene for 2 h through a gated mouthpiece. A sample of inspired gas from each exposure was collected for measurement of exact $^{1}H_8$- and $^{2}H_8$-toluene concentrations. The exposure system provided real time measurement of inhaled and exhaled concentrations of total toluene, respiratory flow rate and exhaled CO_2 concentrations (Morgan *et al.* 1993).

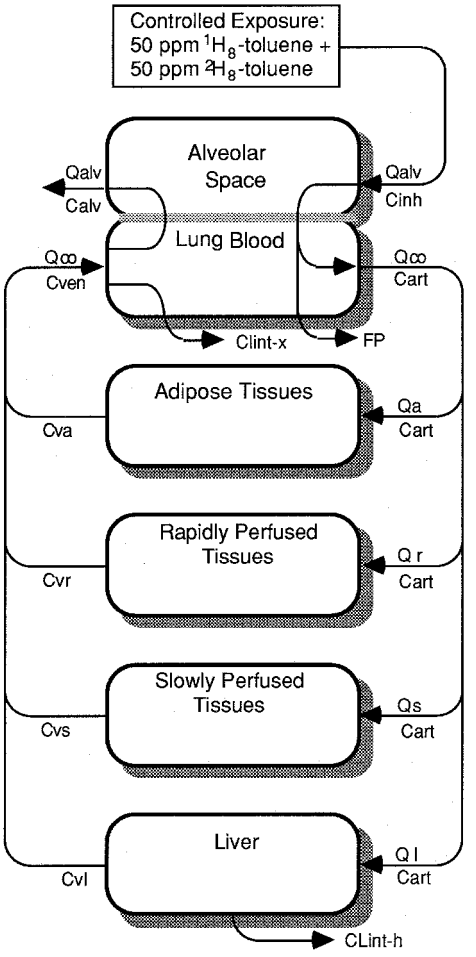

Figure 1. Physiologically based toxicokinetic model for inhaled $^2H_8$-toluene. Q_{alv} = alveolar ventilation (litres air/h); C_{inh} = concentration in inhaled air (ppm); C_{alv} = concentration in alveolar air (ppm); FP = first-pass metabolism (μmol/h); Q_{co} = cardiac output (litres blood/h); C_{art} = concentration in arterial blood (μmol/l); C_{ven} = concentration in venous blood (μmol/l); Cl_{int_x} = intrinsic metabolic clearance in the lung (l/h); Cl_{int_h} = intrinsic metabolic clearance in the liver (l/h); Q_i = blood flow rate to tissue group i (l/h); C_{vi} = concentration in blood leaving tissue group i (mg/l). Subscripts (i) for tissue groups or compartments: a, adipose tissues; r, rapidly perfused tissues; s, slowly perfused tissues; l, liver.

One antecubital venous blood sample and one breath sample were taken prior to exposure for determination of pre-exposure $^1H_8$-toluene levels; 16 simultaneous blood and breath samples were taken after the end of exposure over the following 4 days, at sampling intervals that varied from every 15 min immediately following exposure to every 12 h at times $\geqslant$ 24 h after exposure. Blood samples were collected in 5 ml Vacutainer tubes containing citrate. Exhaled breath samples were collected in 20-litre Tedlar plastic bags prefilled with 10 litres of dry nitrogen. Urine samples were taken prior to, immediately following, and 4 and 8 h after exposure; then with blood and breath samples at times > 24 h after exposure.

Chemical analyses

Blood samples were analysed in triplicate for $^1H_8$-toluene and $^2H_8$-toluene by a headspace method (Dills *et al.* 1991), using gas chromatography with mass spectrometry (GC/MS) detection in selected ion mode. Low-level contamination of blood samples through contact with Vacutainer vials or catheters

Table 1. Values of parameters used in the physiologically based kinetic model.

Physiologic parameter	Tissue group			
	Slowly perfused	Rapidly perfused	Liver	Adipose
Volume (V, litres)	0.95 BW - $V_{adipose}$ *	0.05 BW- V_{liver} *	0.023 BW*	0.10–0.39 BW (measured)
Blood flow (Q, l/h)	0.24 Q_{co}- $Q_{adipose}$ †	0.76 Q_{co}- Q_{liver} †	0.27 Q_{co}*	0.06–0.18 Q_{co} (fitted to data)
K_p	1.54‡	4.64‡	4.64‡	55.9§
K_m (μmol/l)	–	–	5.97†	–
V_{max} (μmol/h)	–	–	52.1 $BW^{0.7}$ †¶ ‖	–

BW = body weight (kg) and Q_{co} = cardiac output (l/h) = 12.92 $BW^{0.74}$, based on similar scaling of cardiac output and alveolar ventilation rate (Tardif *et al.*, 1993) and measurements of ventilation rate in our subjects.

* From Rowland and Tozer (1989).

† From Tardif *et al.* (1993).

‡ From Gargas *et al.* (1989).

§ From Pierce *et al.* (1996b).

¶ Scaling of body weight from (Mordenti 1986).

‖ Maximal extrahepatic rate of metabolism was constrained to a range of 0–70% of V_{max_h} during the model-fitting process.

compromised the $^1H_8$-toluene concentrations in pre-exposure and late post-exposure blood samples, hence the $^1H_8$-toluene blood data were not used. The contents of breath sample bags were drawn through two-section charcoal sorbent tubes using a calibrated personal sampling pump. The charcoal sections were then analysed (US Department of Health 1985). Alveolar $^1H_8$- and $^2H_8$-toluene concentrations were calculated from the exhaled breath concentrations by multiplying the levels in the collection bags by [alveolar CO_2]/[bag CO_2], where the alveolar CO_2 values were measured prior to exposure, or were assumed to be the standard value of 5.26%.

A sensitive gas chromatography-electron capture detection assay for phenolic deuterated and unsubstituted *o*-, *m*- and *p*-cresols was developed with a quantitation range of 0.0092–4600 μmol/l (Dills *et al.* 1997). Cresol glucuronides and sulphates in urine were hydrolysed with β-glucuronidase and arylsulphate, extracted with *tert*-butyl methyl ether and benzene, and derivatized with hepta-flurorobutyric anhydride. $^1H_8$-Cresol levels in pre-exposure urine samples were 0.211± 0.32, 0.500± 0.66 and 333± 290 (μmol/l, mean± SD, n = 38) for the *o*-, *m*- and *p*-cresols, respectively. Due to the high level of background *p*-cresol concentrations in urine from food products and amino acid metabolism, only levels of the native and deuterated *o*- and *m*-isomers were compared.

Assays for hippuric acid were performed on duplicate 1 ml aliquots of urine. Samples were spiked with 50 μl 0.2 mg/ml *N*-(3-methylbenzoyl)-alanine in ethanol (recovery standard), acidified to pH 2, and extracted twice with 2 ml ethyl acetate. The extracts were combined and evaporated to dryness under vacuum. Ethyl esters of the hippuric acids were then formed by reaction at 80 °C for 30 min with acidified ethanol (100 μl 1 M acetyl chloride in absolute ethanol). The reaction mixture was cooled and ethanol was removed by evaporation under vacuum. Ethyl acetate (0.5 ml) and internal standard (50 μl 2 mg/ml methyl decanoate) were added. Samples were then analysed by gas chromatography/mass spectrometry using selected ion monitoring (m/z 110 for ethyl $^2H_5$-hippurate and m/z 134 for ethyl $^1H_5$-hippurate). While both $^1H_5$- and $^2H_5$-hippuric acid were measured in urine samples, relatively high contributions of dietary and endogenous sources of $^1H_5$-hippuric acid were expected to preclude its use as a quantitative marker of exposure (ACGIH 1991).

Physiologically based model

A previously described semiempirical PBK model (Pierce *et al.* 1996a) (figure 1) was implemented with SimuSolv software (Dow Chemical Co., Midland, MI, USA) using subject-specific values of body weight, adipose tissue fraction, $^1H_8$-toluene blood/air partition coefficient, exposure concentration and alveolar ventilation rate. Values of fractional tissue compartment volumes, fractional blood flows, tissue/blood partition coefficients and hepatic metabolic constants V_{max_h} and K_m were taken from literature sources (table 1). A minimum number of tissue compartments (five) was used to represent toluene distribution in the body. As a small lipophilic molecule, toluene was expected to rapidly diffuse across tissue membranes. The exchange of toluene between arterial blood and tissue groups was therefore treated as being flow-limited. Cardiac output (CO), and therefore all blood flows, were scaled to (body weight)$^{0.74}$ (Mordenti 1986, D'Sousa and Boxenbaum 1988, Ings 1990, Ritschel *et al.* 1992).

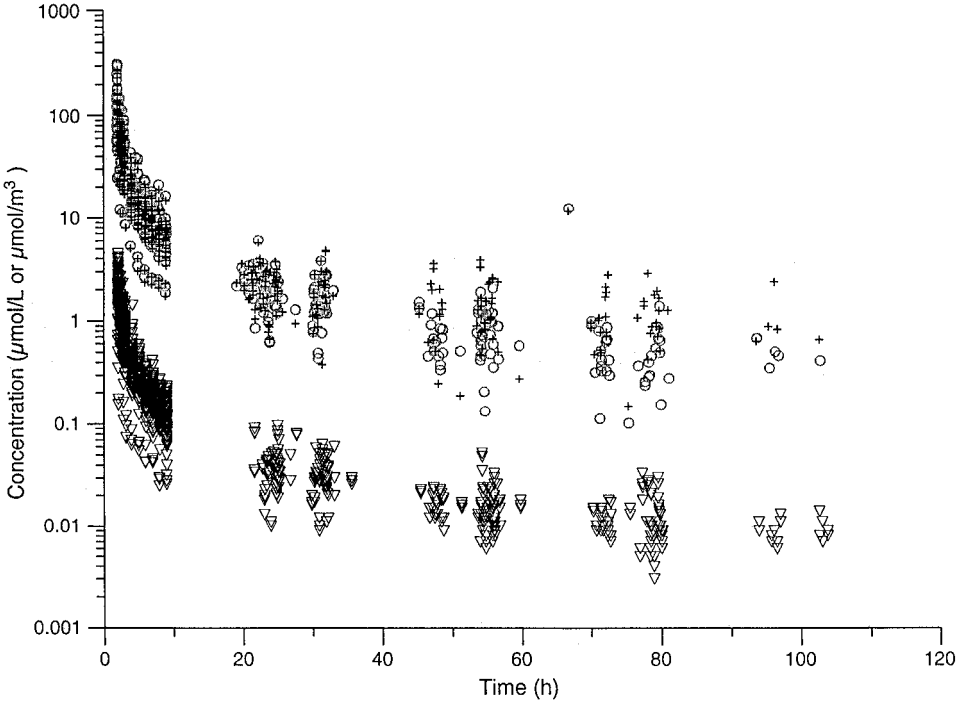

Figure 2. Measured concentrations of $^1H_8$-toluene (+) and $^2H_8$-toluene (O) in alveolar breath, (μmol/m^3) and $^2H_8$-toluene in blood (∇, μmol/l) from 33 exposures to 50 ppm each for 2 h.

Because fractional blood flow to the adipose tissue ($Q_{af/co}$) and maximal rate of extrahepatic metabolism (V_{max_x}) are difficult to measure, may vary widely between individuals, and are important determinants of toluene blood concentration (Pierce *et al*. 1996a), these parameters were fitted to each of the $^1H_8$- and $^2H_8$-toluene data sets. Based upon previous measurements and estimates of $Q_{af/co}$ (Åstrand and Rodahl 1986, Rowland and Tozer 1989, Leggett and Williams 1991, Ferrannini 1992, Davis and Mapleson 1993), this model parameter was expected to vary within the bounds of 0.04–0.17. As a test of the model, $Q_{af/co}$ was fit independently to the $^1H_8$- and $^2H_8$-toluene data for each exposure; no difference was expected for this adipose blood flow value. A search range for values of V_{max_x} was defined using the observation by Wheeler *et al*. (1992) that human lung microsomes contain 10.5% of the liver microsomal activity of CYP2E1. Thummel *et al*. (1993) found about a 7-fold range of CYP2E1 content (49–372 pmol/nmol protein) in 12 human liver samples examined *in vitro*. Because there may be some CYP2E1 activity in locations other than the liver and lung, and because other P450 isozymes (e.g. CYP1A1; Kawamoto *et al*. 1995) may participate in toluene metabolism (Nakajima and Wang 1994) we expected maximal extrahepatic rate of metabolism to range from 0 to 70% of that in the liver. K_m and V_{max_h} in the liver were assumed to be the same for $^1H_8$- and $^2H_8$-toluene, based upon the relative insensitivity of modelled blood concentrations to these parameters because of flow rate-limited clearance by the liver (Pierce *et al*. 1996a). Using published K_m, V_{max_h} and blood flow to the liver (Rowland and Tozer 1989, Tardif *et al*. 1993), hepatic metabolism was found to be much less affected than extrahepatic metabolism to small changes in V_{max}.

Model goodness-of-fit

The model was fitted to the breath ($^1H_8$-toluene) or blood and breath ($^2H_8$-toluene) data from each exposure by varying V_{max_x} and $Q_{af/co}$ to maximize the log likelihood function (LLF) (Steiner *et al*. 1990):

$$LLF = \frac{1}{2}\left[n(\log(2\pi) + 1) + n\log\left[\frac{1}{n}\sum_{i=1}^{n}\frac{(z_i - f_i)^2}{f_i^{\gamma}}\right] + \gamma\sum_{i=1}^{n}\log f_i\right]$$

where n = number of measurements at each time point, z_i = measured value of the ith data point, f

= predicted value of the ith data point and γ = heteroscedasticity (weighting) parameter. γ was optimized over the range 0–2 during model fitting. Almost every fit produced a value of 2, consistent with our constant coefficient of variation in assay error.

The model goodness-of-fit to each of the 33 sets of data was measured by the fraction of variation in the data that was explained by the model. Similar to the calculation of the coefficient of multiple determination (r^2), this value was computed using the following equation (Steiner *et al.* 1990):

$$r^2 = 1 - \frac{\text{model error}}{\text{total error}} = 1 - \frac{\sum_{i=1}^{n} \frac{(z_i - f_i)^2}{f_i'}}{\sum_{i=1}^{n} \frac{(z_i - \bar{z})^2}{f_i'}}, \text{ where } \bar{z} = \frac{\sum_{i=1}^{n} \frac{z_i}{f_i'^{\gamma/2}}}{\sum_{i=1}^{n} f_i^{1/(\gamma/2)}}$$

The model was also used to determine the area under the blood concentration-time curve (AUC), systemic clearance (Cl_s), and the terminal volume of distribution (V_z) and half-life ($t_{1/2\text{-}z}$), using the following equations:

$$AUC = \int_0^{\infty} C_v dt$$

where C_v = modelled venous blood concentration (μmol/l).
Cl_s = absorbed dose/AUC

$$v_z = \sum_{i=1}^{5} A_{t,i} / C_v$$

where i = tissue group, A_t = amount of toluene in tissue in terminal phase (μmol) and C_v = venous blood concentration in terminal phase (μmol/l)

$$t_{1/2\text{-}z} = \frac{C_v \ln 2}{dC_v/dt}$$

V_z and $t_{1/2\text{-}z}$ were determined in the terminal phase (> 40 h post-exposure).

Differences in $^1H_8$-toluene and $^2H_8$-toluene kinetics

To evaluate kinetic differences between $^1H_8$- and $^2H_8$-toluene, two conflicting concerns were addressed: the post-exposure time interval over which the model was fitted had to be long enough to characterize adequately all phases of toluene disposition but short enough to minimize the confounding effect of background exposure on fitting the terminal phase. To this end, we visually examined the data from all subjects *en masse* to determine when the breath concentrations of $^1H_8$- and $^2H_8$-toluene began to diverge, i.e. when background exposure to $^1H_8$-toluene significantly contributed. We also repeatedly varied the model parameter representing background concentration around a value of 50 ppb (Pierce *et al.* 1997) to observe its contribution to predicted $^1H_8$-toluene breath levels.

Once the optimum time interval for fitting the model was determined, the model was optimized to the $^1H_8$-toluene and $^2H_8$-toluene data separately, for each exposure, by varying V_{max_x} and $Q_{a/co}$. Values of these fit model parameters and the kinetic parameters AUC, Cl_s, V_z, and $t_{1/2\text{-}z}$ for $^1H_8$- and $^2H_8$-toluene were compared within exposures using paired t-tests. A significance level of $p < 0.05$ for all tests was chosen.

As a second, model-independent method to evaluate differences in $^1H_8$- and $^2H_8$-toluene clearances, apparent metabolic formation clearances to the *o*-, *m*- and *p*-cresol metabolites were determined using the expressions (Rowland and Tozer 1989),

$Cl_{s\_metabolite}$ = Rate of excretion$_{metabolite,\ t}$/blood concentration$_{toluene,\ t}$ for $^2H_8$-toluene, and

$Cl_{s\_metabolite}$ = Rate of excretion$_{metabolite,\ t}$/breath concentration$_{toluene,\ t}$ for $^1H_8$- and $^2H_8$-toluene.

Use of these expressions required three assumptions: (1) the initial oxidation step in toluene metabolism is slower than subsequent metabolic steps, which is supported by observed reduced rates of metabolism following co-administration with benzene, styrene, *n*-hexane, trichloroethylene or tetrachloroethylene in animal studies (Snyder 1987); (2) toluene metabolite appearance in urine is not governed by glomerular filtration rate (GFR), an assumption which was tested by comparing $Cl_{s\_metabolite}$ values with a standard value for GFR; and (3) toluene metabolite elimination occurs through urinary excretion, which has been determined previously (Snyder 1987). Use of the second equation was based on a rapid equilibration between blood and breath toluene levels (Pierce *et al*. 1996a), and equivalent $^1H_8$- and $^2H_8$-toluene blood/air partition coefficients (Pierce *et al*. 1996b).

Using these expressions to evaluate isotope differences in cresol formation clearances, 157 $Cl_{s\_^1H_7\text{-}o\text{-}cresol}$ -$Cl_{s\_^2H_7\text{-}o\text{-}cresol}$ simultaneous pairs were compared, and 172 $Cl_{s\_^1H_7\text{-}m\text{-}cresol}$ -$Cl_{s\_^2H_7\text{-}m\text{-}cresol}$ pairs were compared, based on the data from all 33 exposures.

Results

$^1H_8$- and $^2H_8$-toluene breath concentrations

The breath data in figure 2 indicated that after 40 h $^2H_8$-toluene concentrations decreased while $^1H_8$-toluene levels began to plateau, an observation consistent with expected contributions of background exposure (Pierce *et al*. 1997). Model simulations suggested that a nominal 50 ppb background level of exposure was expected to result in a $^1H_8$-toluene breath concentration of ~ 0.5 μmol/m^3, similar to the measured 0.334 ± 225 and 0.764 ± 598 μmol/m^3 found in groups described as non-occupationally exposed (Brugnone *et al*. 1989). The average observed breath levels in the 2–40-h post-exposure period were thus 5–300 times higher than expected background concentrations (figure 2). We therefore considered blood and breath concentrations in the 2–40-h period to be due to the controlled exposure, and optimized the physiologic model to describe these data only. Similarly, the pairing of blood and urine, and breath and urine samples to determine apparent formation clearances to the cresols was done only over the 2–40-h period.

Optimized model parameters

The optimized V_{max_x} averaged 6.7 ± 4.3 μmol/h-kg for $^1H_8$-toluene and 9.6 ± 5.4 μmol/h-kg for $^2H_8$-toluene (table 2); and all were within the expected physiologic bounds (0–70% of V_{max_h}, or 0–36 μmol/kg-h) (Wheeler *et al*. 1992, Thummel *et al*. 1993). The V_{max_x} for $^2H_8$-toluene were significantly higher than those for $^1H_8$-toluene ($p < 0.05$). The fit values of $Q_{a/co}$ were 0.09 ± 0.03 for $^1H_8$-toluene and 0.08 ± 0.03 for $^2H_8$-toluene; they were not significantly different. Two

Table 2. Comparison of $^{1}H_{8}$- and $^{2}H_{8}$-toluene model parameters.

Model parameter	$^{1}H_{8}$-toluene	$^{2}H_{8}$-toluene
Fitted		
$V_{max,x}$ (μmol/h-kg)	6.7± 4.3 [2.3×10^{-4}–14]	9.6± 5.4 [0.34–26]**
$Q_{a/co}$	0.09± 0.03 [0.015–0.15]	0.08± 0.03 [0.03–0.14]
Toxicokinetic		
AUC (μmol-h/l)	12.2± 2.8 [7.75–19.1]	11.3± 2.4 [6.92–19.1]**
Cl_S (l/h)	117± 19 [89.2–170]	123± 18 [97.8–173]*
V_z (l)	4960± 1500 [2170–8890]	5740± 1600 [2520–9270]**
$t_{1/2-z}$ (h)	34.3± 10 [14.1–61.2]	37.5± 12 [13.4–67.6]*
Goodness of fit (%)	94± 2.7% [85–98%]	90± 6.5% [68–98%]**

$p < 0.05$, **$p < 0.01$
Values are mean± SD (range).

of these 66 values were below the expected physiologic range of 0.04–0.17 (Åstrand and Rodahl 1986, Rowland and Tozer 1989, Leggett and Williams 1991, Ferrannini 1992, Davis and Mapleson 1993); for one of these two subjects only limited data (one $^{2}H_{8}$-toluene breath and two blood samples) were available in the terminal phase, where adipose blood flow is most influential on blood and breath concentrations. The model was able to explain 94± 2.7% of the $^{1}H_{8}$-toluene data variability, and 90± 6.5% of the $^{2}H_{8}$-toluene data variability in the 33 exposure sets. This significant difference ($p < 0.05$, paired t-test) was likely a consequence of fitting the model to the breath data for $^{1}H_{8}$-toluene compared with fitting to the blood and breath data for $^{2}H_{8}$-toluene.

Kinetic model parameters

Significant differences between $^{1}H_{8}$- and $^{2}H_{8}$-toluene were observed for all kinetic parameters (table 2). The $^{2}H_{8}$-toluene AUC were on average 6.4± 13% smaller than those for $^{1}H_{8}$-toluene and Cl_S were 6.5± 13% larger. In the evaluation of V_z and $t_{1/2-z}$, the $^{1}H_{8}$-toluene values for one subject were > 2 SD larger than the means, reflecting a final terminal phase breath measurement about half that expected from previous values. V_z and $t_{1/2-z}$ for this subject were therefore not used in the analysis. A typical model fit to $^{1}H_{8}$- and $^{2}H_{8}$-toluene breath concentrations in the same subject is presented in figure 3.

The terminal volume of distribution (V_z) was 17± 22% higher for $^{2}H_{8}$-toluene compared with $^{1}H_{8}$-toluene V_z ($p < 0.01$; table 2); dividing by body weight gave 66.4± 14 versus 57.2± 10 l/kg. Similarly, $t_{1/2-z}$ was 9.7± 26% longer for $^{2}H_{8}$-toluene (38± 12 versus 34± 10 h; $p < 0.05$).

Urinary cresol data

Both peak and 0–40 h average excretion rates (figure 4), and peak urinary concentrations (table 3) were higher for $^{2}H_{7}$- compared with $^{1}H_{7}$-*o*- and *m*-cresols. Intra-subject paired t-tests similarly found higher levels of deuterated cresol metabolites ($p < 0.05$). The data for $^{1}H_{7}$-*p*-cresol were of limited value due to high intra-individual variability, presumably due to dietary and aromatic amino acid

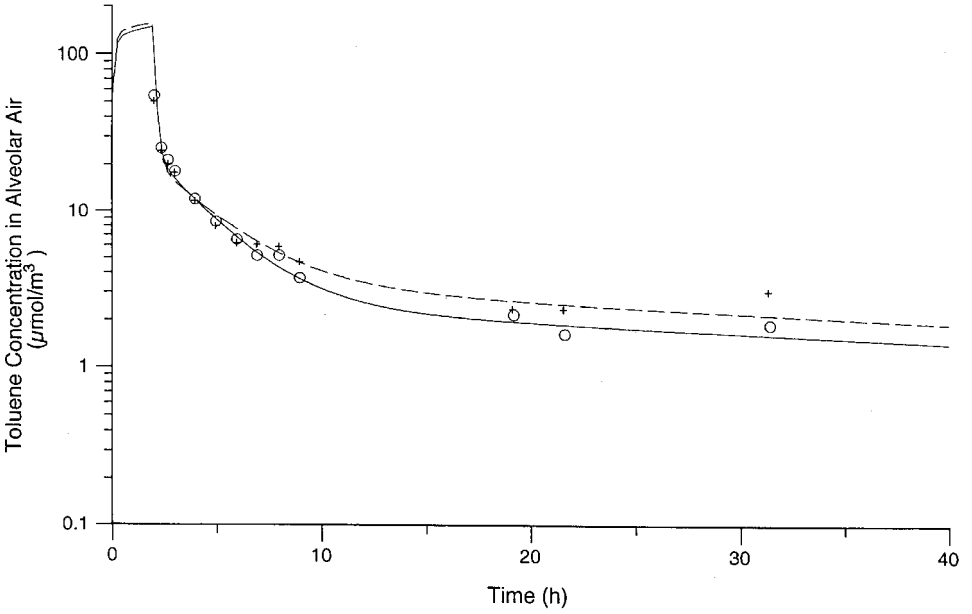

Figure 3. Representative measured and modelled concentrations of $^{1}H_{8}$-toluene (+, ---) and $^{2}H_{8}$-toluene (O, ——) in alveolar breath from an exposure of 50 ppm each for 2 h.

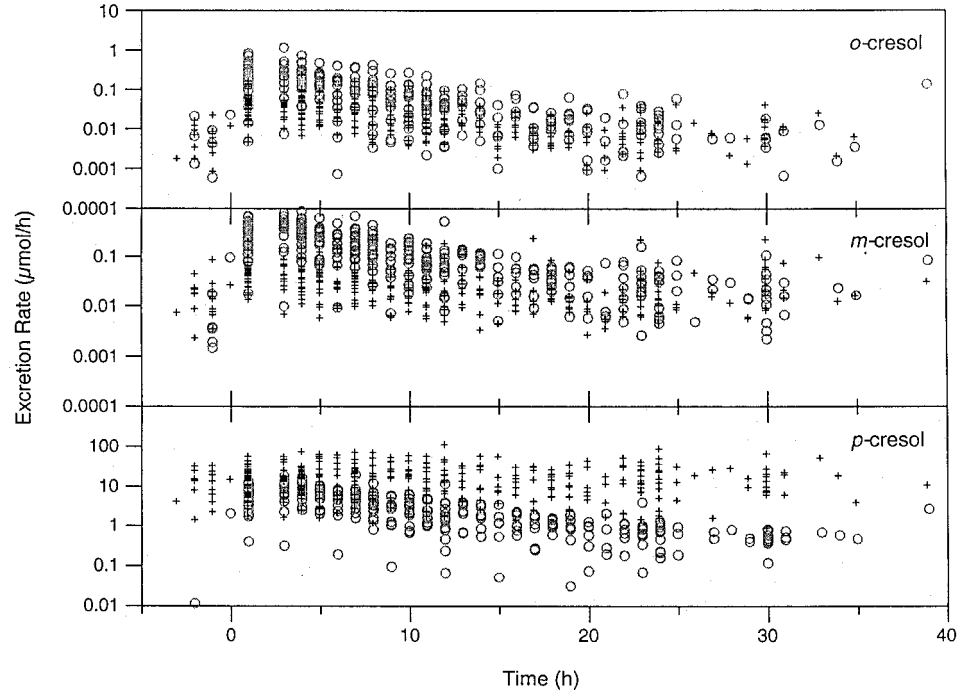

Figure 4. Rates of urinary excretion of $^{1}H_{7}$-(+) and $^{2}H_{7}$-cresols (O) from 33 exposures to 50 ppm of $^{1}H_{8}$-toluene and $^{2}H_{8}$-toluene each for 2 h. Samples are plotted at mid-point collection times relative to exposure.

Table 3. Urinary cresol sample concentrations and formation clearance rates.

Metabolite Urinary concentration	$^1H_8$-toluene mean± SD [range]	$^2H_8$-toluene
o-Cresol (μmol/l)		
Background	0.21± 0.32	–
Peak	1.03± 0.70 [0.24–3.72]	3.86± 2.1 [0.65–10.8]
m-Cresol (μmol/l)		
Background	0.50± 0.66	–
Peak	1.15± 1.0 [0.081–5.14]	6.37± 2.9 [1.15–16.1]
Formation clearance rate ($CL_{s\text{-}metabolite}$)	mean± SD, [*n*]	
o-Cresol (litres blood/h)	–	0.55± 0.41 [137]
(litres breath/h)	4.80± 6.3 [175]	10.6± 8.7 [160]
m-Cresol (litres blood/h)	–	0.95± 0.66 [151]
(litres breath/h)	9.83± 21 [176]	18.8± 15[173]
p-Cresol (litres blood/h)	–	24.5± 15 [153]
(litres breath/h)	–	474± 320 [178]

All $^1H_8$- versus $^2H_8$-toluene differences were significant ($p < 0.05$).

catabolism sources of these metabolites (figure 4). Neither the apparent formation clearance of toluene to *o*- and *m*-cresol (0.55 and 0.95 l/h; table 3) or to *p*-cresol (24.5 l/h, suggesting tubular secretion) was governed by an assumed glomerular filtration rate of 7.5 l/h.

The combined use of breath concentrations of $^1H_8$- and $^2H_8$-toluene paired with urinary excretion rates of $^1H_7$- and $^2H_7$-*o*- and *m*-cresol metabolites revealed $Cl_{s\text{-}^2H_7\text{-}o\text{-}cresol}$ that were 3.59± 3.92 times those of $Cl_{s\text{-}^1H_7\text{-}o\text{-}cresol}$ ($p < 0.05$), and $Cl_{s\text{-}^2H_7\text{-}m\text{-}cresol}$ that were 4.71± 3.87 times those of $Cl_{s\text{-}^1H_7\text{-}m\text{-}cresol}$ ($p < 0.05$). The average formation clearances to the labelled metabolites were about twice as high as to unlabelled metabolites (table 3). Using the paired values, these differences amounted to an increase of 0.40 litres blood/h to *o*-cresol and 0.75 litres blood/h to *m*-cresol for $^2H_8$-toluene compared with $^1H_8$-toluene. Using the observed $Cl_{s\text{-}^2H_7\text{-}p\text{-}cresol}$ of 24.5 l/h and assuming that $^2H_8$-toluene is cleared 4.15 ([3.59 +4.71]/2) times more rapidly than $^1H_8$-toluene to *p*-cresol, an 18.7 l/h increase would be expected. Thus the net increase in $^2H_8$- versus $^1H_8$-toluene Cl_s based on cresol formation was 19.9 l/h (0.40 +0.75 +18.7), a 17% increase over the $^1H_8$-toluene Cl_s of 117 l/h.

Discussion

Stable isotope labels have been used in a wide variety of studies, including the evaluation of zinc, iron, selenium and copper absorption (Couzy *et al*. 1993, Boza *et al*. 1995); the measurement of gastrointestinal transit time (Heine *et al*. 1995); the determination of steroid pharmacokinetics (Kasuya 1994); the evaluation of transdermal nitroglycerin absorption (Kochak *et al*. 1992); and the toxicokinetics of lead in the rat (Smith *et al*. 1992). While less of a concern for higher mass isotopes, most of these studies have assumed, but not tested, a kinetic equivalence between labelled and unlabelled substances. Jones *et al*. (Jones *et al*. 1994) suggested the use of $^2H_8$-toluene as a toxicokinetic tracer without consideration of possible isotope effects, and Löf *et al*. (Löf *et al*. 1993) conducted human exposures to $^2H_8$-toluene assuming kinetic equivalence with the native toxicant. This is the first study of

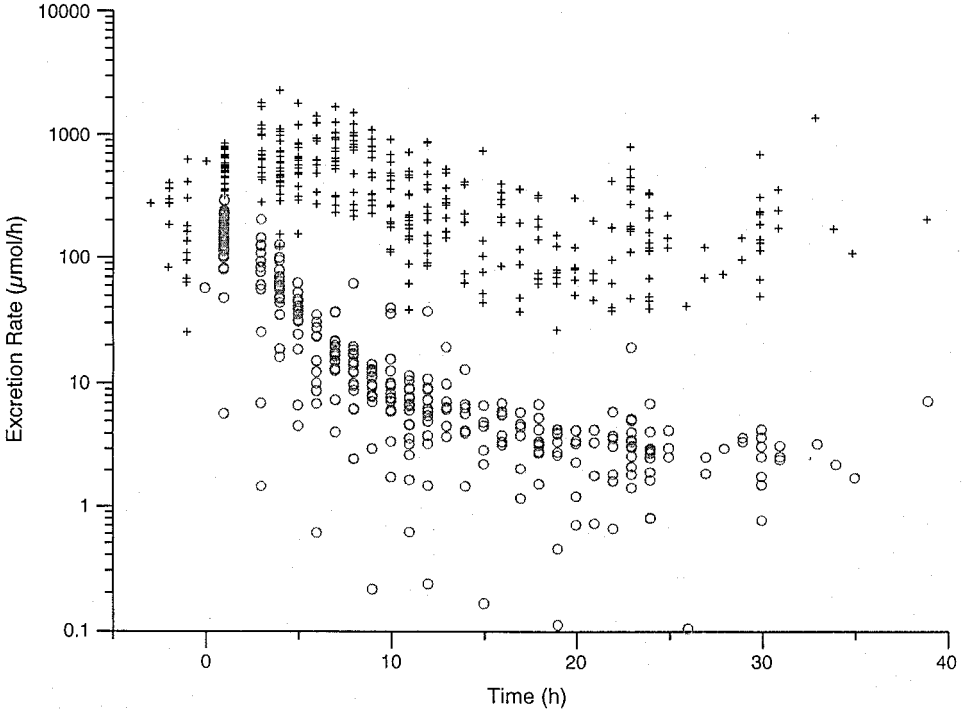

Figure 5. Rates of urinary excretion of $^{1}H_{5}$-hippuric acid (+) and $^{2}H_{5}$-hippuric acid (O) from 33 exposures to 50 ppm of $^{1}H_{8}$-toluene and $^{2}H_{8}$-toluene each for 2 h. Samples are plotted as mid-point collection times relative to exposure.

which we are aware, aside from our preliminary observations (Morgan *et al*. 1993), to compare the kinetics of a stable isotope-labelled and unlabelled toxicant in humans.

Model fitting to the data indicated a 6.5% higher systemic clearance of $^{2}H_{8}$-toluene compared with $^{1}H_{8}$-toluene, a small difference compared with the 2-fold interindividual variability observed. The estimated increase in metabolic clearance based on formation of cresol metabolites was 17%; the difference in the two estimates may have been due to a decrease in formation clearance of $^{2}H_{8}$- relative to $^{1}H_{8}$-toluene, to hippuric acid. While we were not able quantitatively to evaluate this difference due to endogenous and dietary sources of urinary $^{1}H_{5}$-hippuric acid, an examination of excretion rates supports this explanation. In figure 5, the increase from background to peak levels of $^{2}H_{5}$-hippuric acid excretion is $\sim$ 150 μmol/h (0–150), whereas the increase of $^{1}H_{5}$-hippuric acid is $\sim$ 400 μmol/h (200–600).

Dividing by each subject's body weight, our clearances were 1.46± 0.27 l/h-kg for $^{2}H_{8}$-toluene and 1.38± 0.25 l/h-kg for $^{1}H_{8}$-toluene, both very similar to 15 previous studies which found an average clearance of 1.54± 0.44 l/h-kg (range 0.9–2.3 l/h-kg) in groups of men experimentally exposed to $^{1}H_{8}$-toluene (Wallén *et al*. 1984, 1985, Wallén 1986, Hjelm *et al*. 1988, Löf *et al*. 1990, Hjelm *et al*. 1994). In these previous studies, there was an average intersubject coefficient of variation of 38%, with a range of 10–50%. This degree of imprecision suggests that isotopic differences in human toluene clearance are small compared with other sources of variability.

Deuteration has been shown to reduce the rate of alkyl C-H bond oxidation by P450 enzymes, primarily due to the increased isotopic mass and a more stable C-^{2}H bond (Langenhove 1986). White and McCarthy (1986) found a deuterium isotope effect of 2.6 in the hydrogen abstraction from toluene using an *in vitro* rabbit liver system. A pronounced decrease in P450-mediated formation of carbon monoxide following inhalation of $^2H_2$- versus $^1H_2$-dichloromethane in mouse has also been observed (Andersen *et al.* 1994). Van Beerendonk *et al.* (1995) have reported that a completely deuterated form of the flame retardant tris (2,3-dibromopropyl) phosphate showed a > 3-fold decrease in cytochrome P450 metabolism in the isolated perfused rat liver; there was no significant change in the P450 independent glutathione *S*-transferase detoxification pathway. Similar *in vitro* results were seen with deuterated ethylbenzene, where deuteration of the alkyl side chain resulted in an approximate 55% decrease in formation of phenyl ethanol (McMahon and Sullivan 1966). The apparent partial shift from hippuric acid to cresol metabolites observed in this study for the deuterated compound compared with the proteated analogue is consistent with these observations, given that methyl group oxidation is expected to be the rate-limiting step in hippuric acid formation.

Cytochrome P450-mediated ring oxidation occurs by addition of oxygen through an arene oxide mechanism, where C-H bond cleavage is no longer the rate-limiting step (Langenhove 1986). Based on deuteration experiments with a number of aromatic compounds, Tomaszewski *et al.* (1975) suggested that oxidation of the aromatic ring would not be affected by deuteration, since this occurs by direct addition of oxygen to the C-C bond rather than by breakage of the C-H bond, as would occur during oxidation of a methyl side chain. A study in humans using labelled and unlabelled carbamazepine (Browne *et al.* 1994), where ring oxidation is the rate-limiting step in metabolism, revealed no pharmacokinetic differences. In our study, the increased $^2H_5$- versus $^1H_5$-(*o*- and *m*-) cresol production rates were indicative of a higher fractional rate of ring oxidation.

There is some previous evidence of increased overall rate of toluene metabolism following deuteration. Previous research (Ling and Hanzlik 1989) determined that stepwise deuteration of the toluene methyl group *in vitro* led to a stepwise increase in overall oxidation with a decrease in the yield of the benzyl alcohol metabolite relative to cresol. Complete substitution of the methyl group led to a 48% increase in overall oxidation. This suggested that benzyl alcohol release is much slower than cresol release from the enzyme-product complex. Thus, the reduced rate of $^2H_8$- versus $^1H_8$-toluene binding to the enzyme for benzyl alcohol formation shifts more substrate to the cresol pathway, resulting in a net increased rate of toluene metabolism. Our observations of increased clearance and increased fraction of cresol metabolites are consistent with this explanation - the average value of V_{max_x} for $^2H_8$-toluene was 82% higher, the AUC 6.4% lower, and the systemic clearance 6.5% higher than the non-deuterated counterpart. While our modelling approach assumed that differences in extrahepatic metabolism (V_{max_x}) accounted for the observed isotopic differences in clearance, shifts in hepatic V_{max_h} and K_m, which because of flow-limited hepatic clearance have smaller effects on total clearance, may have also contributed.

A potential minor contributor to the observed differences in clearance is an isotopic shift in the blood/air partition coefficient ($K_{b/a}$), which could result in a changed rate of exhalation. Recent analysis of pre-exposure blood samples from 29 experiments using a vial equilibration technique (Pierce *et al.* 1996b) in our

laboratory suggested that the $K_{b/a}$ for $^2H_8$-toluene is about 3% lower than that for $^1H_8$-toluene ($p < 0.05$). However, using average anthropometric and model-fit values, this difference would result in only about a 0.5% difference in Cl_s.

$^2H_8$-toluene demonstrated a 17± 22% larger V_z compared with $^1H_8$-toluene, a small difference compared with the 4-fold interindividual variability seen (table 2). While the $t_{1/2-z}$ for $^2H_8$-toluene was 9.6± 26% longer than for $^1H_8$-toluene, this difference was much smaller that the 4-fold interindividual range. The $t_{1/2-z}$ of 38± 12 h for $^2H_8$- and 34± 10 h for $^1H_8$-toluene were within the wide range of values found in previous studies: 10 h (Monster *et al.* 1993), 10.2–15.7 h (Hjelm *et al.* 1994), 12–65 h (Carlsson and Ljungquist 1982), 15.8 h (Droz and Guillemin 1986), 19.2–22.1 h (Brugnone *et al.* 1983), 21.4 h (Matsumoto *et al.* 1992), ~ 72 h (Nise and Ørbæk 1988), 77 h (Cohr and Stokholm 1979), and 90 and 118± 86 h (Nise *et al.* 1989). As with the clearances, this range suggests that the observed kinetic differences in $t_{1/2-z}$ due to isotopic labelling are small compared with other sources of variation.

Due to its high lipophilicity, ⩾ 96% of toluene is expected to be in adipose tissue in the terminal phase of disposition (~ 30 h) (Pierce *et al.* 1996a). The rate-limiting step in terminal phase elimination is release from adipose tissue, the rate of which is mediated by the volume of tissue, tissue blood flow and the tissue/blood partition coefficient. The fit fractional blood flows to adipose tissue were not different between $^1H_8$- and $^2H_8$-toluene data sets. Nor was there a difference in tissue volume, since the isomers were co-administered to each subject. The observed differences in terminal volume and half-life may thus be due to an increased $^2H_8$-toluene adipose tissue/blood coefficient. However, we have not found a significant difference in the human adipose tissue/air partition coefficient for $^2H_8$- versus $^1H_8$ toluene (1017± 162 versus 962± 298, mean± SD), nor in the blood/air partition coefficient (18.1± 1.40 versus 18.3± 1.39; Pierce *et al.* 1996b). In addition, previous reports have found decreased lipophilicity with deuteration, assessed using octanol/water partition coefficients or reverse-phase HPLC, for a range of aliphatic and aromatic hydrocarbons (Falconnet *et al.* 1988). Thus, the reasons for the larger terminal volume of distribution and longer half-life for $^2H_8$- compared with $^1H_8$-toluene are not currently understood.

Overall, our results indicated that $^2H_8$-toluene was cleared ~ 6% more rapidly than $^1H_8$-toluene; an example of this difference is presented in figure 3. $^2H_8$-toluene also exhibited a 17% larger terminal volume of distribution and a 10% longer terminal half-life. These small distinctions are unlikely to be significant in the practical evaluation of toluene toxicokinetics, given the large intersubject and interstudy differences previously reported. The stable isotope label was therefore a valuable tool to elucidate toluene kinetics.

This investigation extended the use of physiologically based modelling to test the use of stable isotope-labelled tracers to represent the unlabelled compound *in vivo*. Such an approach allowed us to ascribe observed isotope differences in solvent toxicokinetics to underlying physiologic mechanisms.

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