Prediction of human drug clearance by multiple metabolic pathways – integration of hepatic and intestinal microsomal and cytosolic data

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Text: 28  
Tables: 4  
Figures: 4  
References: 48  
Abstract: 250  
Introduction 641  
Discussion 1,531

Abbreviations used are: CL_{int}, intrinsic clearance; CL_{int,SULT}, intrinsic clearance by sulfation; CL_{int,UGT}, intrinsic clearance by glucuronidation; CL_{int,CYP}, intrinsic clearance by cytochrome P450 (P450) metabolism; CL_{int,h}, hepatic intrinsic clearance; fm_{SULT}, fraction metabolized by sulfation; fm_{UGT}, fraction metabolized by glucuronidation; fm_{CYP}, fraction metabolized by P450 metabolism; fu_{inc}, fraction unbound from protein in the incubation; fu_{b}, fraction unbound in the blood; fu_{p}, fraction unbound in the plasma; HIC, human intestinal cytosol; HLC, human liver cytosol; HLM, human liver microsomes; rmse, root mean squared error; R_B, blood to plasma concentration ratio; SULT, sulfotransferase; UGT, uridine diphosphate glucuronosyltransferase.
Abstract

The current study assesses hepatic and intestinal glucuronidation, sulfation and P450 metabolism of raloxifene, quercetin, salbutamol and troglitazone using different in vitro systems. Fraction metabolized by conjugation and P450 metabolism was estimated in liver and intestine and importance of multiple metabolic pathways on accuracy of clearance prediction assessed. In vitro intrinsic sulfation clearance (CL_{int,SULT}) was determined in human intestinal and hepatic cytosol and compared with hepatic and intestinal microsomal glucuronidation (CL_{int,UGT}) and P450 clearance (CL_{int,CYP}) expressed per gram tissue. Hepatic and intestinal cytosolic scaling factors of 80.7 mg/g liver and 18 mg/g intestine were estimated from published data. Scaled CL_{int,SULT} ranged between 0.7-11.4ml/min/g liver and 0.1-3.3ml/min/g intestine (salbutamol and quercetin were the extremes). Salbutamol was the only compound with high extent of sulfation (51% and 28% of total CL_{int} for liver and intestine, respectively) and also significant renal clearance (26-57% of observed plasma clearance). In contrast, the clearance of quercetin was largely accounted for by glucuronidation. Drugs metabolized by multiple pathways (raloxifene and troglitazone) demonstrated improved prediction of intravenous clearance using data from all hepatic pathways (44-86% of observed clearance) compared to predictions based only on primary pathway (22-36%). The assumption of no intestinal first-pass resulted in under-prediction of oral clearance for raloxifene, troglitazone and quercetin (3-22% of observed, respectively). Accounting for intestinal contribution to oral clearance via estimated intestinal availability, improved prediction accuracy for raloxifene and troglitazone (within 2.5-fold of observed). Current findings emphasize importance of both hepatic and intestinal conjugation for in vitro-in vivo extrapolation of metabolic clearance.
Introduction

The prediction of drug clearance via P450 enzymes is well established. In contrast, much less attention has been given to non P450 reactions, in particular conjugation, or to the situation where multiple enzymes contribute to drug clearance. Prediction of vivo hepatic clearance from in vitro microsomal glucuronidation data results in a general under-prediction trend, but to a similar degree to P450s (Ito and Houston, 2005; Miners et al., 2006; Mohutsky et al., 2006; Cubitt et al., 2009; Kilford et al., 2009). For some drugs (substrates of UGT1A9 and UGT2B7), predictions have been improved by addition of albumin, as a result of sequestration of inhibitory long-chain unsaturated fatty acids released during the incubation. These fatty acids have been reported to competitively inhibit certain, but not all UGT enzymes (e.g. no effect on UGT1A1) (Rowland et al., 2007; Rowland et al., 2009). In addition, clearance of raloxifene (UGT1A9 substrate) was under-predicted even when CLint was determined in the presence of albumin (Kilford et al., 2009). Poor prediction of in vivo clearance from in vitro hepatic microsomal glucuronidation data may be explained by potential contribution of other conjugation pathways (e.g., sulfation).

There is an increasing interest in the sulfotransferases (SULT) and other cytosolic enzymes and their contribution to drug clearance (Zhang et al., 2007; Riches et al., 2008a; Riches et al., 2009b; Pryde et al., 2010; Zientek et al., 2010). Published in vitro sulfation studies (Walle et al., 1993; Pacifici et al., 1997; Honma et al., 2002) generally focus on activity data rather than full kinetic characterization; this is particularly evident with intestinal cytosolic studies. Reported studies show large variation in experimental conditions, including concentration of human cytosolic protein, concentration of the cofactor PAPS, buffer type and pH range. In addition, published in vitro cytosolic studies so far have not addressed in vitro-in vivo extrapolation of generated data, either in isolation or combined with corresponding microsomal data on other contributing metabolic pathways.

A reliable analysis of in vitro clearance data requires accurate scaling factors in order to express clearance to per gram of organ. This has been described previously for hepatic (Barter et al., 2007) and intestinal microsomal scaling factors (Cubitt et al., 2009). To our knowledge, the current study is the first instance where sources of available cytosolic scaling factors have been collated for
both liver and intestine. Limitations in the sources of these data and implications on prediction of in vivo clearance are discussed.

In addition to liver, several conjugative enzymes (UGT1A8, UGT1A10 and SULT1A3) are expressed predominantly in the intestine (Tukey and Strassburg, 2001; Lindsay et al., 2008; Riches et al., 2009b) and the importance of intestinal glucuronidation has been demonstrated for some drugs (Dalvie et al., 2008; Cubitt et al., 2009). In the case of oral clearance, an under-prediction trend may therefore result from ignoring the contribution of extrahepatic glucuronidation or sulfation to the overall clearance.

The overall aim of the current study was to assess multiple metabolic pathways of quercetin, raloxifene, salbutamol and troglitazone and estimate hepatic and intestinal fraction metabolized by SULT, UGT and P450 enzymes for these drugs. To allow this, intestinal and hepatic sulfation was assessed in vitro, using human liver and intestinal cytosol and a substrate depletion method. Scaling of the data was performed using the estimated cytosolic scaling factors for liver and intestine from published sources and data were analyzed in conjunction with previously determined microsomal CL_{int,UGT} and CL_{int,CYP} values (Cubitt et al., 2009). The four drugs selected showed differential specificity for intestinal and hepatic SULT, UGT and CYP enzymes or poor prediction success based solely on hepatic P450 and UGT clearance data. Ultimately, the prediction of intravenous and oral clearance values (in the latter case accounting for intestinal contribution) from in vitro data was explored and the contributing role of the multiple different hepatic and extrahepatic pathways to the clearance prediction was assessed.

Materials and Methods

Chemicals. All solvents were purchased from VWR International (Lutterworth, UK). All other compounds and reagents were purchased from Sigma-Aldrich Company Ltd (Dorset, UK).

Source of the subcellular fractions. Pooled human liver cytosol (HLC, n = 20 donors) and intestinal cytosol (HIC, n = 8 donors) were purchased from Xenotech, LLC (Kansas, USA). The HIC was prepared by enterocyte elution mainly of the jejunum section. No enzyme activity data were reported for either of the cytosolic fractions. Glucuronidation and P450 clearance values obtained in
the pooled intestinal microsomes ($n=10$), HLM pool A ($n = 30$) for quercetin and salbutamol and HLM pools A, B and C ($n = 30, 22$ and $33$, respectively) for raloxifene and troglitazone were reported previously (Cubitt et al., 2009). The current study included additional CL$_{int,UGT}$ and CL$_{int,CYP}$ data for quercetin and salbutamol obtained in the same HLM pools B and C. All HLM pools were purchased from BD Gentest (Woburn, MA).

**Preliminary assessment of cytosolic experimental conditions.** Experimental protocols for sulfation studies reported in the literature vary in the concentration of the cofactor PAPS (range 0.4-50µM), buffer type (Tris or phosphate) and pH (range pH 6.2-9.5), as detailed in the Supplementary Material. Therefore, incubation conditions in HLC were initially optimized using troglitazone unbound CL$_{int}$ by sulfation in human liver cytosol as a marker. A range of conditions were assessed including different buffer types (50mM Tris-HCl, 50mM phosphate and 100mM phosphate buffer), cytosolic protein concentration (0.5-1.5mg/ml) and concentration of PAPS (5-400µM). Protein concentration of 1.5mg/ml resulted in high average troglitazone CL$_{int}$ (45.8 µl/min/mg), linear depletion profile and the lowest coefficient of variation. Increase in PAPS concentrations between 5-50µM resulted in corresponding increase in the troglitazone CL$_{int}$ values between 12.0–48.9 µl/min/mg. Further increase in PAPS concentration had no effect on troglitazone clearance or resulted in reduced and more variable estimates (up to 62%) and hence the concentration of 50µM was selected. Cytosolic conditions of 1.5mg/ml HLC/HIC protein concentration, 100mM phosphate buffer pH 7.4 and 50µM PAPS resulted in the most reproducible troglitazone CL$_{int,SULT}$ values and were selected for further sulfation studies.

**Incubation procedure.** All cytosols were stored at -80ºC and rapidly thawed just before use. Incubations for all four compounds were carried out in duplicate and on three separate occasions using an Eppendorf thermomixer at 37ºC and 1400rpm. All substrates were preincubated for 5 min at 37ºC with HLC or HIC and 0.1M pH 7.4 phosphate buffer. Reactions were initiated by the addition of PAPS (final concentration 50µM), to give a final incubation volume of 800µl. Substrate concentrations in the final incubation were 1µM and cytosolic protein concentration was 1.5mg/ml for both HLC and HIC. Glucuronidation and P450 incubations for quercetin and salbutamol (HLM pools B and C) were performed under the conditions reported previously (Cubitt et al., 2009). The mean
CL_{int,UGT} value from three microsomal pools was 5011 and 9.3 µl/min/mg for quercetin and salbutamol, respectively. Corresponding salbutamol CL_{int,CYP} was 7.7 µl/min/mg, whereas quercetin had negligible P450 metabolism. The final concentration of organic solvent in the incubation media was <0.1%. No cofactor incubations were performed for each drug in order to account for any potential cofactor independent loss of a drug over the incubation time. For each time point, 100µl of the incubation was removed and the reaction terminated by the addition of 100µl of ice-cold acetonitrile containing the internal standard, as specified previously (Cubitt et al., 2009). The total length of the cytosolic incubations was 60 min for all compounds except quercetin, which only required an incubation of 16 minutes. Samples were centrifuged at 1400g (MSE Mistral 3000i centrifuge, London, UK) for 10 min and the parent compound was analyzed by LC-MS/MS.

**LC-MS/MS.** The LC-MS/MS system used consisted of a Waters 2790 with a Micromass Quatro Ultima triple quadruple mass spectrometer (Waters, Elstree, UK). Varying gradients of four mobile phases were used, the compositions of which were 1) 90% water and 0.05% formic acid with 10% acetonitrile 2) 10% water and 0.05% formic acid with 90% acetonitrile 3) 90% water and 10 mM ammonium acetate with 10% acetonitrile 4) 10% water and 10 mM ammonium acetate with 90% acetonitrile. A Luna C18 column 3µm, 50 X 4.6mm (Phenomenex, Macclesfield, UK) was used for chromatographic separation of analytes. The flow rate was 1ml/min, and this was split to 0.25ml/min before entering the mass spectrometer. Further analytical parameters were as described previously (Cubitt et al., 2009). The ion chromatograms were integrated and quantified using Micromass QuanLynx software (Waters, Elstree, UK).

**Correction for nonspecific protein binding.** In order to correct CL_{int,SULT} for nonspecific protein binding in the incubation, fu_{inc} values for raloxifene, salbutamol and troglitazone were experimentally determined using human liver cytosol. The fu_{inc} values were determined in HLC at protein concentrations of 0.5, 1 and 1.5mg/ml using the high-throughput dialysis method, as described previously (Gertz et al., 2008). The drugs were with 0.1M phosphate buffer at a concentration of 1µM. Dialysis membranes had a 12 to 14 kDa molecular mass cutoff and were purchased from HTDialysis, LLC (Gales Ferry, CT). The fu_{inc} values for quercetin were predicted using an algorithm proposed by Hallifax and Houston (2006) due to compound degradation during duration of
equilibrium dialysis (6h). Correction of intestinal CL$_{\text{int}}$ values for nonspecific binding assumed the equality of $f_{\text{inc}}$ between intestinal and liver cytosol / microsomes.

**Data analysis.** Sulfation intrinsic clearance was calculated by Equation 1, using Grafit 5 (Erithacus Software, Horley, UK).

\[
CL_{\text{int}, \text{SULT}} = \frac{k \cdot \text{volume of incubation}}{\text{amount of cytosolic protein in incubation}} \tag{1}
\]

Where $CL_{\text{int}, \text{SULT}}$ is intrinsic clearance by sulfation (microlitre per minute per milligram of protein) and $k$ is the depletion rate constant (minute$^{-1}$). The clearance was corrected for experimentally determined $f_{\text{inc}}$ to give an unbound value for $CL_{\text{int}, \text{SULT}}$.

**Determination of human cytosolic scaling factors.** Hepatic and intestinal cytosolic scaling factors were derived from literature sources quoting cytosolic protein abundance or activity data in both the cytosolic fraction and tissue homogenate (Wynne et al., 1992; Boogaard et al., 1996; Gibbs et al., 1998; Renwick et al., 2002; Mutch et al., 2007). Hepatic $CL_{\text{int}, \text{SULT}}$ values obtained in the current study were scaled using 80.7mg/g liver, whereas intestinal $CL_{\text{int}, \text{SULT}}$ values were scaled using 18mg/g intestine, a value derived from only one literature source available and based on data from 12 donors (Gibbs et al., 1998).

**Comparison of intestinal and hepatic extent of sulfation.** In order to allow valid comparison of sulfation between the intestine and liver, clearance data were expressed per gram of tissue using collated cytosolic scaling factors. Microsomal P450 and UGT clearance data were scaled using hepatic and intestinal microsomal recovery of 40mg/g liver and 20.6mg/g intestine, respectively (Barter et al., 2007; Cubitt et al., 2009). Clearance values scaled to per gram of tissue were used to estimate extent of each metabolic process in vitro; $fm_{\text{SULT}}$ values were calculated from mean $CL_{\text{int}, \text{SULT}}$ and previously reported $CL_{\text{int}, \text{UGT}}$ and $CL_{\text{int}, \text{CYP}}$ using Equation 2. Previously reported $fm_{\text{UGT}}$ and $fm_{\text{CYP}}$ values for the drugs investigated have been reassessed taking into account additional contribution of sulfation process (Equations 3 and 4). For the hepatic estimates of fractions metabolized, $CL_{\text{int}, \text{UGT}}$ and $CL_{\text{int}, \text{CYP}}$ values used represent the mean of three HLM pools (total n=85), whereas in the case of
Data were obtained in a single intestinal microsomal pool. Details of the microsomal pools have been published previously (Cubitt et al., 2009).

\[
fm_{\text{SULT}} = \frac{\text{Scaled} CL_{\text{int. SULT}}}{\text{Scaled} CL_{\text{int. SULT}} + \text{Scaled} CL_{\text{int. UGT}} + \text{Scaled} CL_{\text{int. CYP}}} 
\]

\[
fm_{\text{UGT}} = \frac{\text{Scaled} CL_{\text{int. UGT}}}{\text{Scaled} CL_{\text{int. SULT}} + \text{Scaled} CL_{\text{int. UGT}} + \text{Scaled} CL_{\text{int. CYP}}} 
\]

\[
fm_{\text{CYP}} = \frac{\text{Scaled} CL_{\text{int. CYP}}}{\text{Scaled} CL_{\text{int. SULT}} + \text{Scaled} CL_{\text{int. UGT}} + \text{Scaled} CL_{\text{int. CYP}}} 
\]

**Prediction of intravenous and oral clearance.** $CL_{\text{int}}$ expressed per gram of liver were further scaled using liver weight of 21.4g liver/kg (Ito and Houston, 2005) in order to give a predicted $CL_{\text{int}}$ in units of ml/min/kg. In case of multiple pathways, total in vitro $CL_{\text{int}}$ values were calculated as a sum of unbound $CL_{\text{int}}$ values for all individual metabolic pathways after scaling to per gram of liver. Oral and intravenous clearance values, renal clearances, fraction unbound in plasma ($fu_p$) and blood to plasma concentration ratios ($R_B$) were collated from the literature for all compounds investigated. When multiple clinical studies were available, mean clearance values were calculated weighted for the number of subjects in the study. References for all clinical studies considered are available in the Supplementary material. Hepatic blood clearance ($CL_{hl}$) after i.v. administration was calculated by correcting plasma clearance for renal excretion and $R_B$ (Ito and Houston, 2005; Gertz et al., 2010). Estimated $CL_{hl}$ was used to calculate in vivo hepatic intrinsic clearance ($CL_{\text{int,h}}$) using the well-stirred liver model (Equation 5) and average hepatic blood flow ($Q_h$) of 20.7ml/min/kg. In the case of raloxifene, no observed intravenous clearance data were available and therefore i.v. clearance of 14.7 ml/min/kg was estimated from oral clearance (735 ml/min/kg) and reported bioavailability of 2% (Hochner-Celnikier, 1999; Mizuma, 2009).

\[
\text{Observed} \ CL_{\text{int,h}} = \frac{CL_{hl}}{fu_p} \left( 1 - \frac{CL_{hl}}{Q_h} \right) 
\]
Observed CL_{int,h} after oral administration was calculated as shown in Equation 6.

\[
\text{Observed } \text{CL}_{\text{int,h}} = \frac{D}{\text{AUC} \cdot \text{fu}_b} \cdot F_a \cdot F_G \tag{6}
\]

Where D/AUC represents the hepatic blood clearance obtained from the mean plasma data after correction for renal clearance (when applicable) and R_B. The fu_b represents fraction unbound in blood, F_a fraction absorbed and F_G intestinal availability. The following F_a values were used, 0.54 for quercetin (Petri et al., 2003), 0.60 for raloxifene (Dalvie et al., 2008; Mizuma, 2009) and 0.80 for salbutamol (Mizuma, 2008). In the case of troglitazone, F_a of 0.69 was estimated from animal data (Izumi et al., 1996), due to lack of any human data. The contribution of the small intestine to oral clearance was incorporated using the F_G values estimated from i.v./oral data, as shown in Equation 7.

\[
F_G = \frac{F}{F_a \cdot \left(1 - \frac{\text{CL}_{\text{ii}}}{Q_{\text{u}}}ight)} \tag{7}
\]

**Estimation of enterocytic drug concentration.** The concentration of drug present in the enterocytes was estimated using Equation 8 (Obach et al., 2006; Galetin et al., 2007), based on typical in vivo doses for quercetin, raloxifene, salbutamol and troglitazone (500mg, 60mg, 4mg and 400mg, respectively). F_a values used were as outlined above. A mean value of 0.3L/min (Yang et al., 2007; Galetin et al., 2008) was used for enterocytic blood flow (Q_{ent}). Absorption rate constants (k_a) of 0.009min^{-1} were available for quercetin and raloxifene (Czock et al., 2005; Moon et al., 2008), whereas the value of this parameter in the case of troglitazone (0.004min^{-1}) was estimated from animal data and (Izumi et al., 1996). There were no published k_a data available for salbutamol and the generic value of 0.03min^{-1} was used (Dalvie et al., 2008). All references associated with in vivo data are listed in the Supplementary material.

\[
\text{Concentration in enterocyte } s = \frac{k_a \cdot F_a \cdot \text{dose}}{Q_{\text{ent}}} \tag{8}
\]

Estimated enterocytic drug concentrations were 31µM, 2 µM, 1µM and 9µM for quercetin, raloxifene, salbutamol and troglitazone, respectively.
Results

Depletion profiles. Figure 1 shows sulfation depletion profiles for all four compounds in HLC and HIC using substrate concentrations below the expected $K_m$ (1µM). Monoexponential time profiles were observed for the intestinal and hepatic sulfation of raloxifene and the hepatic sulfation of both quercetin and troglitazone. The hepatic sulfation of salbutamol and the intestinal sulfation of both quercetin and troglitazone followed a biphasic profile. In these instances, the initial linear phase was used to estimate $k$ and obtain $\text{CL}_{\text{int},\text{SULT}}$. Salbutamol and quercetin had the lowest and highest unbound $\text{CL}_{\text{int},\text{SULT}}$, respectively in both organs, with a range between 8.7-141µl/min/mg HLC and 8.0-186µl/min/mg HIC (Table 1). Values of cytosolic $\text{CL}_{\text{int},\text{SULT}}$ were based on triplicate determinations using a pool of 8 and 20 donors for HIC and HLC, respectively and were subject to experimental variability of 11-44% (quercetin and troglitazone, respectively). The average coefficient of variation of $\text{CL}_{\text{int},\text{SULT}}$ for the four drugs were comparable between cytosol from both tissues.

Correction for nonspecific cytosolic protein binding. $\text{CL}_{\text{int},\text{SULT}}$ values were corrected for nonspecific cytosolic protein binding using experimentally determined $f_u^{\text{inc}}$ values in human liver cytosol (Table 1). The extent of nonspecific protein binding in cytosol differed to that reported previously in microsomes at the same protein concentration (Cubitt et al., 2009). Considering the small dataset no particular trends could be identified, as experimental $f_u^{\text{inc}}$ was higher in the cytosol for raloxifene and troglitazone, whereas opposite was seen for salbutamol. Therefore, direct use of microsomal $f_u^{\text{inc}}$ is not ideal for the correction of cytosolic kinetic data. Experimental $f_u^{\text{inc}}$ could not be determined for quercetin due to the instability of this compound during the equilibrium dialysis procedure and was therefore estimated using the Hallifax and Houston algorithm (2006). For comparison, predicted cytosolic $f_u^{\text{inc}}$ for the remaining drugs are also shown in Table 1. While discrepancies are evident, in particular for raloxifene, the use of a predicted value for quercetin was felt not to be of concern in subsequent calculations of unbound $\text{CL}_{\text{int}}$. Unlike drugs like raloxifene, quercetin is not particularly lipophilic ($\text{LogP} = 1.6$) and minimal binding is to be expected.

Human cytosolic scaling factors. In order to compare sulfation clearance in the intestine and liver, $\text{CL}_{\text{int},\text{SULT}}$ values were scaled from per mg cytosolic protein to per gram of tissue. To achieve this, intestinal and hepatic cytosolic scaling factors were collated from sources reporting measurement
of a specific marker protein activity (glutathione-S-transferase or alcohol dehydrogenase) in both tissue homogenate and subcellular fraction. Data from 52 donors in total were collated from five independent sources and considered in the current analysis, as illustrated in Table 2. The hepatic cytosolic scaling factor ranged from 45-134 mg cytosolic protein per gram of liver. Mean value weighted according to the number of livers used in each study (80.7 mg cytosolic protein per gram of liver) was used in the current study for scaling of hepatic cytosolic data. Estimates obtained from the study by Wynne et al. indicated higher cytosolic recovery from male donors; however, overall data on the gender of donors, as well as age, were limited across studies to ascertain any impact on the estimated cytosolic recovery. In the case of the intestine, data from only one study in 12 donors were available (Gibbs et al., 1998), resulting in a cytosolic scaling factor of 18 mg cytosolic protein per gram of intestine. No information on the potential variation of the scaling factor along the intestine was available, as reported for intestinal microsomal recovery (ranges from 16.6-30.6 mg protein/g intestine for ileum and duodenum, respectively (Cubitt et al., 2009)).

Comparison of intestinal and hepatic scaled intrinsic clearance by sulfation, glucuronidation and P450 metabolism. Scaled CL_{int,SULT} (expressed per gram of tissue) were compared for the intestine and liver and also relative to microsomal glucuronidation and P450 clearance data. Unbound microsomal CL_{int,UGT} and CL_{int,CYP} were scaled as reported previously (Cubitt et al., 2009). Comparison of scaled intestinal and hepatic CL_{int,SULT}, CL_{int,UGT} and CL_{int,CYP} values is shown Figure 2. Scaled CL_{int,SULT} ranged from 0.70 to 11.4 ml/min/g liver and 0.14 to 3.34 ml/min/g intestine for salbutamol and quercetin, respectively. Mean scaled hepatic CL_{int,SULT} values were 3- (quercetin and troglitazone) and 5-fold (raloxifene and salbutamol) higher than the intestinal value. Salbutamol was the only compound predominantly cleared by sulfation in both the liver and intestine. Out of the four drugs, raloxifene and troglitazone showed higher clearances by both glucuronidation and P450 metabolism than by sulfation in both the intestine and liver (Figure 2). Quercetin had the highest hepatic scaled CL_{int,UGT}, negligible P450 metabolism and sulfation clearance of 11.4 and 3.3 ml/min/g liver and intestine, respectively. Raloxifene had the highest intestinal CL_{int,UGT} (88 ml/min/g intestine) of the drugs investigated; a value that was >40-fold higher than its intestinal CL_{int,SULT} and CL_{int,CYP}.
Comparison of individual metabolic pathways in the liver and intestine. In vitro fmSULT, fmUGT and fmCYP estimates were calculated from mean scaled CL_{int,SULT}, CL_{int,UGT} and CL_{int,CYP} values. A comparison of these fractions for each of the drug investigated is represented in Figure 3A and B, for the liver and intestine, respectively. Small contribution of sulfation to overall CL_{int} was evident for quercetin in the liver and for raloxifene in the intestine (<5%), whereas up to 51% and 28% was seen for the respective hepatic and intestinal metabolism of salbutamol. The extent of glucuronidation ranged from 27% and 40% (hepatic and intestinal metabolism of salbutamol, respectively) up to 97% (intestinal metabolism of raloxifene). Mean fm_CYP ranged from zero in either organ for quercetin, up to 42% and 32% (liver and intestine for troglitazone and salbutamol, respectively).

Quercetin was the compound with the highest CL_{int,SULT} in both liver and intestine (Figure 2). However, the extent of sulfation was fairly marginal relative to glucuronidation of this drug and resulted in fmSULT <15% in both liver and intestine (Figure 3). Quercetin was the only drug where fm_{SULT} was higher in intestine than in liver (13% and 5%, respectively) and this could be a consequence of the reported high affinity (K_m of 2µM) for SULT1E1 (Teubner et al., 2007; Riches et al., 2009a). Salbutamol was predominantly cleared by sulfation in comparison to glucuronidation and P450 metabolism (Figure 3) in both the liver and the intestine. Salbutamol is reported to be a substrate for SULT1A3 (Mizuma, 2008) and therefore higher intestinal CL_{int,SULT} would be expected, in contrast to current findings. However, there are no recombinant SULT data available to support specificity for this particular SULT. The intestinal fm_{CYP} of salbutamol was comparable to the extent of sulfation, in contrast to liver where contribution of P450 was lower (22% of the total clearance). The contribution of sulfation to the total in vitro metabolism of troglitazone was 23% and 9% in the liver and intestine, respectively (Figure 3). The scaled CL_{int,SULT} of troglitazone was half of the scaled CL_{int,UGT} and CL_{int,CYP} (Figure 2) in both organs, in contrast to in vivo data suggesting that troglitazone sulphate is the main metabolite (Loi et al., 1999). Hepatic P450 metabolism (42%) was comparable to glucuronidation (35%) for this compound, whilst in the intestine glucuronidation was the dominant process (67%); the intestinal fm_{CYP} of troglitazone was only half of the hepatic value. Glucuronidation was the predominant intestinal pathway for raloxifene (>90%), whereas hepatic fm_{UGT} represented only half of the intestinal value and approximately 30% of the hepatic in vitro clearance was
attributed to the P450 metabolism and 17% to sulfation. Both intestinal fmSULT and fmCYP were negligible and represented <5% of the hepatic clearance (Figure 3). This is surprising considering that the recombinant SULT data suggest high affinity for SULT1A1 and 1A3 (<3µM), comparable to recombinant UGTs (5-59µM) (references in the Supplementary material).

**Prediction of in vivo clearance from cytosolic and microsomal in vitro data.** In vivo CL\textsubscript{int,h} values used for the assessment of prediction accuracy were calculated from a number of reported i.v. and oral studies using well-stirred liver model. Intravenous clearance data were generally less available, with the exception of salbutamol. Oral clearance data were available from >1000 individuals in the case of raloxifene (single study) and from 4-7 different studies for quercetin, salbutamol and troglitazone (Table 3 and Supplementary Material). Salbutamol had the lowest in vivo observed CL\textsubscript{int,h} (4.7 and 8.1 ml/min/kg from intravenous and oral clearance data, respectively) of the four compounds and was the only drug in the dataset with a substantial contribution of renal elimination. Raloxifene and quercetin CL\textsubscript{int} after both i.v. and oral administration were >100-fold higher in comparison to salbutamol, as shown in Table 4.

Further analysis assessed the accuracy of predicted intravenous or oral in vivo clearance, using in vitro data from both cytosolic and microsomal sources. Multiple metabolic pathways contributing to clearance of the selected drugs (CL\textsubscript{int,SULT}, CL\textsubscript{int,UGT} and CL\textsubscript{int,CYP}) were considered once expressed per g liver. In the case of quercetin, accuracy of prediction based on the combined SULT, UGT and P450 hepatic in vitro clearances (Figure 4) showed no improvement relative to the use of only glucuronidation data. This was consistent with high estimated contribution of glucuronidation to the total hepatic clearance (hepatic fm\textsubscript{UGT} of 95%, Figure 3). Predicted quercetin CL\textsubscript{int,h} was 6-fold higher than the observed intravenous CL\textsubscript{int,h}. Accounting for intestinal glucuronidation (F\textsubscript{G} estimated as 0.03 from i.v. and oral data) resulted in comparable degree of over-prediction of oral CL\textsubscript{int,h} to intravenous data. In contrast, raloxifene, salbutamol and troglitazone, with lower primary pathway involvement (<60% of total in vitro hepatic metabolism), showed a significant difference in predictions performed combining clearances from all metabolic pathways in comparison to those using data from the primary pathway in isolation. Prediction of intravenous clearance of both raloxifene and troglitazone was poor (22-36% of observed, respectively) when using in vitro hepatic
CL_{int} data corresponding to their primary pathway. Incorporation of additional SULT, UGT and P450 hepatic in vitro data improved prediction accuracy of intravenous clearance in particular for troglitazone, as the predicted clearance from combined data represented 86% of the observed value. In the case of raloxifene, predicted clearance from the combined approach was marginally outside of the 2-fold of in vivo i.v. clearance (44% of the i.v. clearance, Figure 4). In contrast to the success achieved with troglitazone and raloxifene, over-prediction of salbutamol intravenous CL_{int,h} was observed using data from all metabolic pathways. For this drug, in vitro CL_{int} data corresponding to the primary sulfation pathway over-predicted intravenous clearance by 3-fold and so incorporation of additional pathways increased further this error (626% of observed). The oral clearance of this compound was predicted well from in vitro hepatic SULT CL_{int} (within 2-fold), whereas inclusion of other pathways reduced the accuracy (364% of observed).

Ignoring intestinal first-pass resulted in significant under-prediction of oral CL_{int} (predicted estimates were 5 and 31% of the observed data for raloxifene and troglitazone, respectively). F_{G} estimated from i.v./oral data were 0.03, 0.12, 0.76 and 0.35 for quercetin, raloxifene, salbutamol and troglitazone, respectively. Accounting for F_{G} in the oral clearance decreased the overall degree of under-predictions seen for these drugs, resulting in good comparability in prediction accuracy between oral and intravenous data, as illustrated in Figure 4.

Discussion

Contribution of more than one metabolic elimination mechanism (hepatic and extrahepatic) is increasingly becoming a more common scenario, with implications on the in vitro-in vivo extrapolation of clearance and assessment of drug-drug interaction potential (Mohutsky et al., 2006; Rostami-Hodjegan and Tucker, 2007; Zhang et al., 2007; Hinton et al., 2008; Cubitt et al., 2009; Kilford et al., 2009; Gan et al., 2010; Zientek et al., 2010). So far, little attempt has been made to include in vitro data corresponding to cytosolic enzymes such as SULTs in prediction of in vivo clearance in combination with microsomal P450 or UGT data. The current study aimed to compare extent of metabolism by sulfation in the intestine and liver in conjunction with previously published microsomal UGT and P450 data for four selected drugs. A standardized experimental design and
appropriate scaling of in vitro cytosolic clearance data were used. Sulfation clearance obtained in the current study covered a 16 to 23-fold range in hepatic and intestinal cytosol, respectively. In both tissues, salbutamol and quercetin had the lowest and highest CL<int>SULT, respectively. Experimental variability in cytosolic CL<int>SULT estimates of the four drugs, although comparable between liver and intestinal cytosol, was more pronounced in comparison to microsomal CL<int>UGT and CL<int>CYP (Cubitt et al., 2009); this could be partly due to instability of the cofactor PAPS and inhibition of SULT activity by PAP (Novakova et al., 2004).

Appropriate scaling factors are particularly important when accounting for data on multiple metabolic pathways obtained in different subcellular fractions in order to avoid propagation of error in the combined approach. Consideration of inter-individual and experimental variability in scaling factors is important and this has been addressed for hepatic and to less extent for intestinal microsomal recovery (Barter et al., 2007; Cubitt et al., 2009). The human hepatic cytosolic scaling factors used here were estimated from several literature sources reporting measurement of a marker protein in both tissue homogenate and subcellular fraction (Table 2). Hepatic cytosolic scaling factors were subject to variation across five studies investigated (52 donors in total), with a coefficient of variation of 38%. No individual data were available in order to distinguish between the experimental and inter-individual variability. No information on variability was reported in the study quoting intestinal cytosolic data in 12 donors (Gibbs et al., 1998). As was the case for intestinal microsomes (Paine et al., 1997), the intestinal cytosolic scaling factor was obtained from cytosols prepared by mucosal scraping. Use of this method rather than enterocyte elution may influence activity and protein yield of cytosolic enzymes, as was reported previously for a range of P450s (Galetin and Houston, 2006), and hence the estimate of intestinal cytosolic scaling factor. Distribution of sulfation activity along the length of small intestine has been reported to be highly variable and was also associated with substrate and donor differences (Chen et al., 2003). However, the section of intestine used in Gibbs et al. was not reported; hence, the use of a single intestinal cytosolic scaling factor and lack of any information on the regional differences in these factors may affect the analysis of the contribution of intestinal sulfation relative to hepatic. Therefore, further refinement of cytosolic scaling factors in
the liver and intestine is required using larger numbers of individuals, more consistent protocol and analysis for covariates.

In the current study, scaled CL\textsubscript{int,SULT} per gram of organ was generally higher in the liver than intestine, with raloxifene and salbutamol showing the largest difference (>4-fold). This trend was also seen for most compounds in the glucuronidation dataset (Cubitt et al., 2009) with the exception of raloxifene and troglitazone. If CL\textsubscript{int,SULT} were corrected for protein abundance of the main SULT enzyme responsible for the sulfation of each drug (e.g., SULT1E1 for troglitazone and SULT1A3 for salbutamol) (Honma et al., 2002; Riches et al., 2009b) an increased apparent importance of intestinal sulfation relative to the liver was observed (data not shown). Protein abundance data for a range of SULTs reported by Riches et al. (2009) in various human tissues were obtained using cDNA-expressed protein as a positive control (abundance units of ng SULT/mg cytosolic protein). However, SULT abundance data obtained using purified SULT as a positive control (units of pmol SULT/mg cytosolic protein) (Honma et al., 2002) are limited, in contrast to the data for P450s. In addition, corresponding UGT absolute abundance data in the liver and intestine are currently lacking, affecting the assessment of the relative contribution of conjugation pathways in the liver and intestine to the overall clearance.

In vitro fraction metabolized by each metabolic pathway was calculated from mean scaled CL\textsubscript{int,SULT}, CL\textsubscript{int,UGT} and CL\textsubscript{int,CYP} for each drug in both intestine and liver (Figure 3). Intestinal mean fm\textsubscript{UGT} values were within 20% of hepatic for only one of the four drugs analyzed (quercetin). The difference between hepatic and intestinal fm\textsubscript{CYP} was consistently >40% for all compounds. Consideration of the sulfation clearance provided a more accurate estimate of the contribution of the individual pathways to the total clearance in comparison to the previous analysis performed using only glucuronidation and P450 metabolism data for the drugs investigated (Cubitt et al., 2009). In addition, discrepancy between high enterocytic concentrations estimated in vivo for some drugs in the dataset (≥9µM for quercetin and troglitazone- see Methods) and current in vitro conditions (at 1µM) may impact estimated extent of intestinal glucuronidation and sulfation. Finally, any potential contribution of renal elimination is not considered when estimating fractions metabolized in vitro and
hence their interpretation needs to be done with caution; in the current study this would only affect salbutamol.

The relative role of different metabolic pathways (glucuronidation, sulfation and P450) in the clearance prediction was investigated using either in vitro data corresponding to the primary metabolic pathway in isolation or a combined approach where all contributing metabolic pathways were taken into account. In each case the ability to predict i.v. and oral clearance was used to delineate the role of intestinal conjugation processes relative to hepatic (Figure 4). The use of combined SULT, UGT and P450 data significantly improved accuracy of predicted in vivo clearance for raloxifene and troglitazone and resulted in predictions within 2.5-fold of observed. Raloxifene has a low and variable bioavailability (approximately 2%) and i.v. clearance was indirectly estimated from oral data (obtained in >1000 healthy subjects); therefore, any inaccuracy in this method will affect the assessment of prediction accuracy. In the case of quercetin, the inclusion of additional hepatic pathways resulted in negligible differences in prediction accuracy, considering the predominant contribution of glucuronidation (>90%) to the total hepatic clearance. Over-prediction of quercetin clearance is partially associated with a large variability of its clearance in vivo (20- and >1000-fold range for intravenous and oral in vivo clearance, respectively, Table 3), in contrast to other drugs. Data from cellular systems for the drugs investigated are not available to allow any direct comparison; the only exception is salbutamol where depletion clearance reported in human hepatocytes is approximately 10-fold lower when expressed per g liver in comparison to the estimate obtained by adding different metabolic pathways from different systems performed here. Over-estimation of in vivo clearance by sulfation may also be associated with differences in PAPS concentration in cytosolic in vitro systems and in vivo. In vivo PAPS concentrations have been reported to range from 20 to 30µM (Coughtrie, 2002) and rapid depletion of the cofactor may limit the capacity of SULT enzymes; in particular this might be the case in the enterocytes during the absorption phase.

High intestinal extraction of troglitazone, raloxifene and quercetin (for the latter two F<sub>G</sub>&lt;0.1) has been estimated from in vivo data, supporting indirectly the extent of intestinal conjugation seen in vitro for these compounds. The estimated enterocytic concentration of raloxifene and troglitazone are
below reported $K_m$ for intestinal UGTs contributing to their metabolism, so saturation of intestinal UGTs is not expected. However, potential saturation of SULTs, in particular in the case of troglitazone, cannot be ruled out. Accounting for intestinal extraction using corresponding $F_G$ significantly improved the prediction accuracy of oral clearance data and reduced under-prediction trend reported previously for raloxifene and troglitazone. In addition, incorporating $F_G$ in the oral clearance resulted in comparable prediction accuracy to intravenous data, as expected (Figure 4). These findings are consistent with recent analysis of a large range of CYP3A substrates (Gertz et al., 2010), illustrating indirectly the importance of intestinal metabolism for some drugs in the current dataset. In contrast to CYP3A, the current ability to predict $F_G$ by conjugation from in vitro data is confounded by a number of factors, including differential contribution of UGT and SULT enzymes, lack of absolute enzyme abundance data and its variability along the intestine.

In conclusion, the current study has illustrated integration of cytosolic and microsomal metabolic data for the assessment of multiple metabolic pathways. Appropriate cytosolic scaling factors have been identified for both liver and intestine and limitations discussed. The need to account for all metabolic processes and sites of metabolism in the in vitro-in vivo extrapolation was evident, in particular for raloxifene and troglitazone. Extensive intestinal conjugation contributes to the under-prediction of oral clearance for some drugs and emphasizes the need for the incorporation of extrahepatic processes into clearance prediction using appropriate physiologically-based pharmacokinetic modeling approaches.

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**Authorship Contributions.** Participated in research design: Galetin, A. Houston, J. B. and Cubitt, H. Conducted experiments: Cubitt, H. Performed data analysis: Cubitt, H. Wrote or contributed to the writing of the manuscript: Cubitt, H. Galetin, A. and Houston, J. B.
References:


Mizuma T (2009) Intestinal glucuronidation metabolism may have a greater impact on oral bioavailability than hepatic glucuronidation metabolism in humans: a study with raloxifene, substrate for UGT1A1, 1A8, 1A9, and 1A10. Int J Pharm 378:140-141.


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Figure Legends

Figure 1. Comparison of sulfation depletion profiles in human liver and intestinal cytosol for four drugs: ▲ and ○ represent hepatic and intestinal sulfation, respectively. A, Quercetin B, Raloxifene C, Salbutamol D, Troglitazone. Data points represent mean±sd of three separate sulfation experiments in pooled liver and intestinal cytosol.

Figure 2. Comparison of scaled unbound CL_int by sulfation, glucuronidation and P450 (expressed per g organ) in the intestine and liver for four drugs. ■ represents CL_int,SULT, □ CL_int,UGT and ■ CL_int,CYP. A, Liver. B, Intestine. Data represent mean±sd of three separate sulfation experiments using the same pool of HLC/HIC. Glucuronidation and P450 metabolism represent either mean±sd of CL_int,UGT and CL_int,CYP obtained in three different HLM pools or data from a single intestinal microsomal pool.

Figure 3. Comparison of estimated in vitro intestinal and hepatic fraction metabolized by sulfation, glucuronidation and P450 metabolism for quercetin, raloxifene, salbutamol and troglitazone. ■ represents fm,SULT, □ fm,UGT and ■ fm,CYP. A, Liver. B, Intestine. Contribution of each metabolic process in vitro was estimated from mean clearance values scaled to per gram of tissue, as defined in Equations 2-4.

Figure 4. Comparison of predicted and observed CL_int,h values for quercetin, raloxifene, salbutamol and troglitazone. A, Clearance prediction accuracy using intravenous data for four drugs investigated. B, Comparison of observed and predicted CL_int values from oral data before (■) and after (▲) correction for in vivo FG. Sum of scaled CL_int,SULT, CL_int,UGT and CL_int,CYP values per g liver was used. Observed i.v. CL_int values were calculated using Equation 6, whereas Equation 7 was applied for oral data, assuming either no intestinal first-pass (FG = 1) or accounting for estimated FG from iv/oral data.
TABLE 1

SULT specificity, nonspecific protein binding and sulfation clearance obtained in liver and intestinal cytosol for quercetin, raloxifene, salbutamol and troglitazone

<table>
<thead>
<tr>
<th>Drug</th>
<th>Major SULT Specificity</th>
<th>Predicted $f_{inc}$ at 1.5mg/ml (Hallifax and Houston, 2006)</th>
<th>Experimentally derived $f_{inc}$ (1.5mg/ml HLC)</th>
<th>Unbound CL$_{int,SULT}$ $^{b}$ (µl/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HLC</td>
</tr>
<tr>
<td>Quercetin</td>
<td>SULT1E1, SULT1A1</td>
<td>0.86</td>
<td>ND</td>
<td>141±27</td>
</tr>
<tr>
<td>Raloxifene</td>
<td>SULT1E1</td>
<td>0.03</td>
<td>0.14</td>
<td>45±26</td>
</tr>
<tr>
<td>Salbutamol</td>
<td>SULT1A3</td>
<td>0.84</td>
<td>0.59</td>
<td>8.7±4.4</td>
</tr>
<tr>
<td>Troglitazone</td>
<td>SULT1E1, SULT1A1</td>
<td>0.05</td>
<td>0.23</td>
<td>36±14</td>
</tr>
</tbody>
</table>

$^a$References in Supplementary Material.

$^b$The data represent the mean and standard deviation of three separate experiments using the same pool of HLC or HIC.

ND- No experimental value could be determined due to compound degradation during equilibrium dialysis.
TABLE 2

Hepatic cytosolic scaling factors collated from published literature sources and the corresponding information on the liver donors

<table>
<thead>
<tr>
<th>Number of donors in liver pool</th>
<th>Age of donors</th>
<th>Hepatic cytosolic scaling factor (mg cytosolic protein/g liver)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>36-65</td>
<td>45</td>
<td>(Boogaard et al., 1996)</td>
</tr>
<tr>
<td>23</td>
<td>unknown</td>
<td>80</td>
<td>(Gibbs et al., 1998)</td>
</tr>
<tr>
<td>3</td>
<td>10-50</td>
<td>97</td>
<td>(Renwick et al., 2002)</td>
</tr>
<tr>
<td>16 (8M, 8F)</td>
<td>45-88</td>
<td>92 (M) 66 (F)</td>
<td>(Wynne et al., 1992)</td>
</tr>
<tr>
<td>4</td>
<td>unknown</td>
<td>134</td>
<td>(Mutch et al., 2007)</td>
</tr>
</tbody>
</table>

M and F represent male and female donors, respectively, where this information was stated.
TABLE 3

Mean intravenous and oral plasma clearance data, number of subjects, blood/plasma ratios and fraction unbound in plasma for drugs investigated.

References for all the clinical data are listed in the Supplementary material

<table>
<thead>
<tr>
<th>Observed in vivo plasma clearance (ml/min/kg)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Number of subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intravenous</td>
</tr>
<tr>
<td></td>
<td>Intravenous</td>
</tr>
<tr>
<td><strong>Quercetin</strong></td>
<td>5.2 (0.61 – 12.01)</td>
</tr>
<tr>
<td><strong>Raloxifene</strong></td>
<td>14.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Salbutamol</strong></td>
<td>8.4 (7.6 – 9.3)</td>
</tr>
<tr>
<td><strong>Troglitazone</strong></td>
<td>2.5</td>
</tr>
</tbody>
</table>

<sup>a</sup>Weighted mean (range). In the case of salbutamol, weighted renal clearance was 4.8 and 4.3 ml/min/kg after i.v. and oral administration, respectively. Renal clearance was negligible for other drugs.

<sup>b</sup>Assumed to be 1 for basic drugs and 0.55 for acidic drugs.

<sup>c</sup>Calculated from oral clearance data (735 ml/min/kg) and reported bioavailability of 2% (Hochner-Celnikier, 1999; Mizuma, 2009).

NA - No subject information available.
### TABLE 4

*Observed and predicted hepatic intrinsic clearance for quercetin, raloxifene, salbutamol and troglitazone*

Predicted clearance was obtained using either individual or combined hepatic sulfation, glucuronidation and P450 in vitro data

<table>
<thead>
<tr>
<th></th>
<th>Predicted $\text{CL}_{\text{int,h}}$ (ml/min/kg)</th>
<th>Observed <em>in vivo</em> $\text{CL}_{\text{int,h}}$ (ml/min/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sulfation(^a)</td>
<td>Glucuronidation(^b)</td>
</tr>
<tr>
<td>Quercetin</td>
<td>243 ± 47</td>
<td>4289 ± 2528</td>
</tr>
<tr>
<td>Raloxifene</td>
<td>78 ± 45</td>
<td>228 ± 176</td>
</tr>
<tr>
<td>Salbutamol</td>
<td>15.1 ± 7.6</td>
<td>8.0 ± 8.0</td>
</tr>
<tr>
<td>Troglitazone</td>
<td>61 ± 24</td>
<td>96 ± 68</td>
</tr>
</tbody>
</table>

\(^a\) SULT predictions were based on mean ± sd of 3 separate experiments using pooled HLC (n = 20).
\(^b\) Based on mean ± sd of in vitro hepatic $\text{CL}_{\text{int,UGT}}$ and $\text{CL}_{\text{int,CYP}}$ from three HLM pools A-C (n = 85). Quercetin and salbutamol values are defined in Methods. $\text{CL}_{\text{int,UGT}}$ and $\text{CL}_{\text{int,CYP}}$ values for raloxifene and troglitazone from Cubitt et al. (2009). ND: not detected.
\(^c\) Sum of $\text{CL}_{\text{int,SULT}}$, $\text{CL}_{\text{int,UGT}}$ and $\text{CL}_{\text{int,CYP}}$ values (expressed per g liver) was scaled using liver weight of 21.4 g liver/kg.
\(^d\) Calculated from oral clearance data using Equation 6. $F_a$ values for all the drugs are listed in Materials and Methods. $F_G$ estimated from i.v./oral data are $0.03, 0.12, 0.76$ and $0.35$ (for quercetin, raloxifene, salbutamol and troglitazone, respectively).
Figure 1

A

B

C

D

Fraction of Quercetin Remaining

Fraction of Raloxifene Remaining

Fraction of Salbutamol Remaining

Fraction of Troglitazone Remaining

Time (mins)

Time (mins)

Time (mins)

Time (mins)
Figure 2
Figure 3
Figure 4

A

B

Predicted / Observed Intravenous CL\textsubscript{int,h} (%)

Predicted / Observed Oral CL\textsubscript{int,h} (%)

Quercetin  Raloxifene  Salbutamol  Troglitazone

Quercetin  Raloxifene  Salbutamol  Troglitazone