In vitro–in vivo correlations for drugs eliminated by glucuronidation: Investigations with the model substrate zidovudine

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Aims To investigate the effects of incubation conditions on the kinetic constants for zidovudine (AZT) glucuronidation by human liver microsomes, and whether microsomal intrinsic clearance (CL_{int}) derived for the various conditions predicted hepatic AZT clearance by glucuronidation (CL_H) *in vivo*.

Methods The effects of incubation constituents, particularly buffer type (phosphate, Tris) and activators (Brij58, alamethacin, UDP-N-acetylglucosamine (UDP-NAcG)), on the kinetics of AZT glucuronidation by human liver microsomes was investigated. AZT glucuronide (AZTG) formation by microsomal incubations was quantified by h.p.l.c. Microsomal CL_{int} values determined for the various experimental conditions were extrapolated to a whole organ CL_{int} and these data were used to calculate *in vivo* CL_H using the well-stirred, parallel tube and dispersion models.

Results Mean CL_{int} values for Brij58 activated microsomes in both phosphate (3.66 ± 1.40 µl min^{-1} mg^{-1}, 95% CI 1.92, 5.39) and Tris (3.79 ± 0.74 µl min^{-1} mg^{-1}, 95% CI 2.87, 4.71) buffers were higher ($P < 0.05$) than the respective values for native microsomes (1.04 ± 0.42, 95% CI 0.53, 1.56 and 1.37 ± 0.30 µl min^{-1} mg^{-1}, 95% CI 1.00, 1.73). Extrapolation of the microsomal data to a whole organ CL_{int} and substitution of these values in the expressions for the well-stirred, parallel tube and dispersion models underestimated the known *in vivo* blood AZT clearance by glucuronidation by 6.5- to 23-fold (3.61–12.71 l h^{-1} *vs* 82 l h^{-1}). There was no significant difference in the CL_H predicted by each of the models for each set of conditions. A wide range of incubation constituents and conditions were subsequently investigated to assess their effects on GAZT formation, including alamethacin, UDP-NAcG, $MgCl_2$, D-saccharic acid 1,4-lactone, ATP, GTP, and buffer pH and ionic strength. Of these, only decreasing the phosphate buffer concentration from 0.1 M to 0.02 M for Brij58 activated microsomes substantially increased the rate of GAZT formation, but the extrapolated CL_H determined for this condition still underestimated known AZT glucuronidation clearance by more than 4-fold. AZT was shown not to bind nonspecifically to microsomes. Analysis of published data for other glucuronidated drugs confirmed a trend for microsomal CL_{int} to underestimate *in vivo* CL_H.

Conclusions AZT glucuronidation kinetics by human liver microsomes are markedly dependent on incubation conditions, and there is a need for interlaboratory standardization. Extrapolation of *in vitro* CL_{int} underestimates *in vivo* hepatic clearance of drugs eliminated by glucuronidation.

Keywords: glucuronidation, *in vitro-in vivo* correlation, UDP-glucuronosyltransferase, zidovudine

Introduction

The use of *in vitro* methodologies to predict aspects of human drug metabolism *in vivo* has found increasing acceptance over the last decade, particularly for drugs metabolized by cytochrome P450 (CYP). The economic imperatives of new drug development and greater aware-

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ness of the need for rational therapeutics have been primary considerations in the development of *in vitro* models. Prediction of human drug disposition parameters potentially decreases attrition of new drug candidates during clinical development by identifying those compounds with unacceptable pharmacokinetic characteristics, and at the same time optimizes clinical utility and market success of newly marketed drugs by selecting for development only those compounds with desirable pharmacokinetics [1]. Knowledge of the potential impact of drug interactions and genetic polymorphism on drug elimination is of importance for rationalizing and optimizing dosage regimens of both newly developed and established drugs [2].

In vitro-in vivo correlations may be qualitative or quantitative. At the qualitative level, identification of the isoform(s) responsible for the biotransformation of any given compound allows prediction of those factors (genetic polymorphism, drug–drug interactions) likely to influence metabolic clearance [2]. Quantitative prediction most commonly involves the scaling of an *in vitro* intrinsic clearance (CL_{int}) for a metabolic pathway, normally calculated from microsomal or hepatocyte kinetic data, to hepatic clearance (CL_H) *in vivo* using a mathematical model of hepatic drug clearance [3–8]. For example, microsomal CL_{int} may be corrected for microsome yield and liver weight to obtain the 'whole organ' hepatic CL_{int}, which subsequently is substituted in the expression for the well-stirred, parallel-tube, distributed or dispersion models.

The success of predictions of *in vivo* CL_H is critically dependent on *in vitro* CL_{int}, and hence how closely the kinetic parameters (V_{max}, K_m) used to derive this parameter reflect enzyme activity *in vivo*. Microsomal incubation components are known to modulate CYP activity and the kinetics of drug metabolite formation may therefore vary with experimental conditions. For example, it has been demonstrated that buffer type (phosphate, Tris) and ionic strength, the presence of certain salts, and stimulators of enzyme activity may variably affect the kinetic constants of CYP3A substrates determined in human liver microsomes [9]. These observations highlight the need to optimize experimental conditions *in vitro* if kinetic data are to be reliably extrapolated to the *in vivo* situation.

Despite the success of *in vitro–in vivo* correlations for drugs eliminated by CYP, few studies have investigated the reliability of extrapolating human liver microsomal kinetic data to an *in vivo* CL_H for drugs metabolized by glucuronidation. UDP-Glucuronosyltransferase (UGT) is quantitatively the most important conjugation enzyme, and drugs from all therapeutic classes are eliminated by glucuronidation [10]. Thus, establishment of an experimental paradigm which allows extrapolation of *in vitro* kinetic data to hepatic clearance *in vivo* assumes considerable importance. Like CYP, microsomal UGT activity is known to be dependent on experimental conditions, and hence the composition of the incubation mixture may influence the derived kinetic constants. Factors affecting UGT activity *in vitro* include the presence of activators (detergents, alamethacin, UDP-N-acetylglucosamine (UDP-NAcG)), metal ions (especially Mg^{2+}), buffer pH and ionic strength, ATP and GTP [11].

This paper describes studies which were performed to characterize the effects of buffer type (phosphate or Tris) and detergent (Brij58) activation on the human liver microsomal glucuronidation of the model glucuronidated drug zidovudine (3′-azido-3′-deoxythymidine; AZT), and whether CL_{int} values calculated using the various experimental conditions predicted hepatic AZT clearance by glucuronidation *in vivo*. While kinetic constants were found to vary with changes in the composition of the incubation mixture, CL_{int} values determined for the various reaction conditions all underestimated *in vivo* metabolic clearance irrespective of the model of hepatic clearance used. Subsequent experiments were thus conducted to investigate the influence of nonspecific microsomal binding, a nondetergent activator (alamethacin), UDP-NAcG, pH, buffer type and ionic strength, $MgCl_2$, D-saccharic acid 1,4-lactone, ATP and GTP, on human liver microsomal AZT glucuronidation.

AZT was employed as the model substrate in this work since it is extensively glucuronidated and has high hepatic clearance (with possible dependence of extrapolated CL_H on the mathematical model of hepatic drug clearance employed). Mean AZT systemic clearance ranges from 77 to 114 l h^{-1} normalized to 70 kg body weight [12–14], giving an average value of 94 l h^{-1} 70 kg^{-1}. Since 75% of the dose is recovered as AZT glucuronide (GAZT; AZT 5′-β-D-glucuronide) in urine [12], the plasma AZT clearance by glucuronidation *in vivo* may be taken as 70.5 l h^{-1} 70 kg^{-1}.

Methods

Chemicals, reagents and human tissue microsomes

Alamethacin, ATP, AZT, Brij58 (polyoxyethylene-20-cetyl ether), creatine kinase, creatine phosphate, GAZT (3′-azido-3′-deoxythymidine 5′-β-D-glucuronide), GTP, 4-methylumbelliferyl-β-D-glucuronide (4-MUG), D-saccharic acid 1,4-lactone, Tris-HCl, UDP-glucuronic acid (UDPGA), and UDP-NAcG were purchased from the Sigma Chemical Co (St Louis, MO, USA). All other chemicals and reagents were of the highest grade available.

Microsomes were prepared from five human livers obtained from the human liver 'bank' of the Department of Clinical Pharmacology, Flinders Medical Centre, as

described previously [15]. Approval was obtained from the Clinical Investigation Committee of Flinders Medical Centre and from the donor next-of-kin for the procurement and the use of human liver tissue in xenobiotic metabolism studies.

Microsomal incubations

GAZT formation by incubations of human liver microsomes was measured by a modification of the method of Sim *et al.* [16]. Incubation mixtures, in a total volume of 0.2 ml, typically contained microsomal protein (0.2 mg), AZT (50–4000 μM), $MgCl_2$ (4 mM), phosphate (prepared from sodium dihydrogen phosphate and disodium hydrogen phosphate) or Tris-HCl buffer (0.1 M, pH 7.4). Duplicate samples were prepared for each added concentration of AZT. Reactions were initiated by the addition of UDPGA, such that the final concentration of cofactor was 10 mM. Reactions were carried out in air at 37 °C (shaking water bath), and terminated after 30 min by the addition of perchloric acid (24% v/v, 10 μl) to precipitate microsomal protein. The assay internal standard, 4-MUG (10 μl of a 2.5 mM aqueous solution), was added to each sample. Mixtures were vortex mixed for approximately 2 min and subsequently centrifuged at $1500 \times g$ for 10 min. A 0.1 ml aliquot of the supernatant fraction was transferred to a 1.5 ml eppendorf tube containing 2 M KOH (2 μl) to raise the mixture pH to approximately 3.5. An aliquot (0.04–0.05 ml) of each sample was injected directly on to the h.p.l.c. column.

Variations to this procedure included: (i) Omission of $MgCl_2$ from the incubation mixture, (ii) Activation of microsomes by the addition of the nonionic detergent Brij58. Microsomes were preincubated with Brij58 on ice for 30 min, using a Brij58 to microsomal protein ratio of 0.15 (w/w). This ratio was shown to maximally activate human liver microsomal GAZT formation (data not shown), (iii) Activation of microsomes by addition of the pore-forming peptide, alamethacin (25–100 mg l^{-1}). Microsomes were preincubated with alamethacin on ice for 30 min, (iv) Activation with UDP-NAcG (1 mM), by preincubation with microsomes on ice for 30 min, (v) Addition of the β-glucuronidase inhibitor, D-saccharic acid 1,4-lactone (8.5 mM), (vi) Phosphate and Tris buffer pH. Incubations were performed using buffers of pH 6.0, 6.5, 7.0, 7.4, 8.0, 8.5 and 9.0 (at a concentration of 0.1 M), (vii) Phosphate buffer concentration. Incubations were performed in buffer over the concentration range 0.02–0.2 mM (at pH 7.4), (viii) Addition of ATP generating system. Incubations were performed in the presence of ATP (4 mM), creatine phosphate (10 mM) and creatine kinase (100 mg l^{-1}) in Tris-HCl buffer (0.1 M, pH 7.4) and (ix) Addition of GTP (0.1 mM).

Experiments performed to determine the Michaelis constant (K_m) and maximal velocity (V_{max}) for GAZT formation by human microsomes included at least 8 AZT concentrations in the range 50–4000 μM, at constant UDPGA concentration (10 mM). Similarly, kinetic constants for UDPGA were determined by measuring GAZT formation by human liver microsomes for 8 UDPGA concentrations in the range 100–4000 μM, at constant AZT concentration (5 mM).

Measurement of GAZT formation

The h.p.l.c. used comprised a LC110 solvent delivery system, LC 1200 u.v.-vis variable wavelength detector (both ICI Instruments, Melbourne, Australia) and a BBC Goetz Metrawatt dual pen recorder, and operated at ambient temperature. The instrument was fitted with a Waters Guard-Pak C18 μBondapak precolumn (Waters Associates, Milford, MA, USA) and a Beckman Ultrasphere C8 analytical column (5 μm particle size, 4.6 mm (i.d.) × 25 cm; Beckman Instruments, Fullerton, CA (USA). The mobile phase, phosphate buffer (0.05 M, pH 3.1)-acetonitrile (89 : 11), was delivered at a flow rate of 1 ml min^{-1}. Column eluant was monitored at 267 nm. Under these conditions, retention times of GAZT, 4-MUG and AZT were 7.3, 15.2 and 16.1 min, respectively.

Five GAZT standards, dissolved in phosphate buffer (0.1 M, pH 7.4), were prepared in the range 5–300 μM and treated in the same manner as incubation samples. Concentrations of GAZT in incubation samples were determined by comparison of GAZT to 4-MUG peak height ratios with those of the standard curve.

Assay validation

Standard curves were linear in the range 5–300 μM ($r^2 > 0.990$), and the coefficient of variation for the slopes of 25 standard curves was 4.7%. Overall assay reproducibility, assessed by measuring GAZT formation in 10 separate incubations of the same batch of pooled human liver microsomes, was 3.7%, 2.1% and 2.5% for added AZT concentrations of 50 μM, 1000 μM and 3000 μM, respectively. The limit of determination for GAZT was 1 μM. Using pooled human liver microsomes, rates of GAZT formation were shown to be linear for incubation times between 15 and 90 min and for microsomal protein concentrations between 0.5 and 3.0 mg ml^{-1} (data not shown).

Non-specific microsomal binding

Non-specific microsomal binding of AZT to human liver microsomes was investigated by equilibrium dialysis, according to the procedure of McLure *et al.* [17]. Briefly,

one side of the dialysis apparatus contained AZT (100, 1000 or 3000 μM), pooled human liver microsomes (1 mg ml^{-1}, native or activated with Brij58), and phosphate buffer (0.1 M, pH 7.4), while the other compartment contained phosphate buffer alone. Duplicate measurements were performed for each AZT concentration. The dialysis cell assembly was immersed in a water bath maintained at 37 °C and rotated at 12 rev min^{-1} for 3 h. The contents of each compartment (1.2 ml) were collected, treated with perchloric acid (0.015 ml of a 24% v/v solution), vortex mixed, and centrifuged (1500 *g* for 10 min). The supernatant fractions were treated with KOH (1 M, 0.1 ml) to raise the pH to approximately 3.5, and an aliquot (0.05 ml) was injected on to the h.p.l.c. The h.p.l.c. system and conditions were essentially identical to those described previously for the measurement of GAZT, except that the proportion of acetonitrile in the mobile phase was increased to 20% and internal standard (4-MUG) was omitted. Under these conditions, AZT eluted at 6.0 min Standards in the concentration range 50–2000 μM were prepared in phosphate buffer (0.1 M, pH 7.4) and treated in the same manner as dialysis samples. AZT concentrations of dialysis samples were determined by comparison of peak heights with those of the standard curve. Standard curves were linear ($r^2 > 0.990$), and the coefficient of variation for the slopes of four standard curves was 2.8%. Assay reproducibility, assessed by measurement of 10 replicates, was 5.7% and 3.6% for AZT concentrations of 100 μM and 1000 μM, respectively. The limit of determination for AZT was 2.5 μM.

Data analysis

Microsomal kinetic data were model-fitted using EnzFitter (Biosoft, Cambridge, UK) to obtain values of apparent K_m and V_{max}. The choice of model (one or two enzyme Michaelis-Menten, Hill function) was confirmed by *F*-test and coefficient of determination. Microsomal intrinsic clearance, CL_{int}, was calculated as V_{max}/K_m (units of μl min^{-1} mg^{-1} microsomal protein) and subsequently scaled to the 'whole' liver CL_{int} assuming a liver weight of 1500 g and a microsome yield of 45 mg microsomal protein g^{-1} of liver [1, 3]. *In vivo* CL_H was then predicted using expressions for the well-stirred, parallel-tube and dispersion models [1, 3–8].

$$\text{Well stirred model:} \quad CL_H = \frac{Q_H.fu.CL_{int}}{Q_H + fu.CL_{int}}$$

where fu is fraction unbound in blood and Q_H is liver blood flow, assumed to be 90 l h^{-1}.

$$\text{Parallel-tube model:} \quad CL_H = Q_H\left(1 - e^{\frac{-CL_{int} \times fu}{Q_H}}\right)$$

Dispersion model:

$$F_H = \frac{4a}{\left[(1+a)^2.e^{(a-1)/2D_N}\right] - \left[(1-a)^2.e^{-(a+1)/2D_N}\right]}$$

$$\text{and } CL_H = Q_H(1 - F_H)$$

D_N, the dispersion number, may be taken as 0.17 [6] and $a = (1 + 4.R_N.D_N)^{1/2}$

R_N, the efficiency, is given by $R_N = \frac{fu.CL_{int}}{Q_H}$.

The fraction of drug unbound in blood may be evaluated as, $fu = fu_p/R_B$, where R_B is the blood to plasma concentration ratio and fu_p is the fraction unbound in plasma. For AZT, fu_p was taken as 0.77 and R_B as 0.86 [18]. Taking into account the blood to plasma concentration ratio, the blood AZT CL_H by glucuronidation may be calculated from the plasma clearance (see Introduction) as 82 l h^{-1}.

Group data are presented as mean ± s.d.. Correlations and statistical analyses were performed with Instat version 3.0 (GraphPad Software Inc). Group data were analysed by nonparametric ANOVA (Kruskal–Wallis test), with Dunn's multiple comparisons test to detect differences between conditions or models.

Results

AZT glucuronidation kinetics by native and detergent-activated microsomes from five human livers were characterized in phosphate and Tris buffers (both 0.1 M, pH 7.4). Derived kinetic parameters are summarized in Table 1, and representative kinetic plots for each set of experimental conditions are shown in Figure 1. Human liver microsomal AZT glucuronidation kinetics followed single enzyme Michaelis-Menten kinetics for all condi-

Table 1 Kinetic data for zidovudine glucuronidation by native and detergent-activated human liver microsomes in phosphate- and Tris-buffered incubations (0.1 M, pH 7.4)[a].

Microsome treatment	K_m *(μM)*	V_{max} *(pmol min^{-1} mg^{-1})*	CL_{int} *(μl min^{-1} mg^{-1})*
Native, phosphate buffered	926 ± 197 (682, 1170)	920 ± 280 (527, 1268)	1.04 ± 0.42 (0.53, 1.56)
Native, Tris-buffered	1235 ± 219 (963, 1506)	1684 ± 394 (1195, 2174)	1.37 ± 0.30 (1.00, 1.73)
Activated (Brij58), phosphate-buffered	1011 ± 261 (687, 1334)	3695 ± 954★ (2311, 4680)	3.66 ± 1.40★ (1.92, 5.39)
Activated (Brij58), Tris-buffered	1390 ± 221 (1116, 1664)	5193 ± 907★ (4068, 6319)	3.79 ± 0.74★ (2.87, 4.71)

[a]Data presented as mean ± s.d., with 95% confidence intervals in parenthesis, for microsomes from 5 human livers.
★$P < 0.05$ compared with values for phosphate- and Tris- buffered native microsomes.

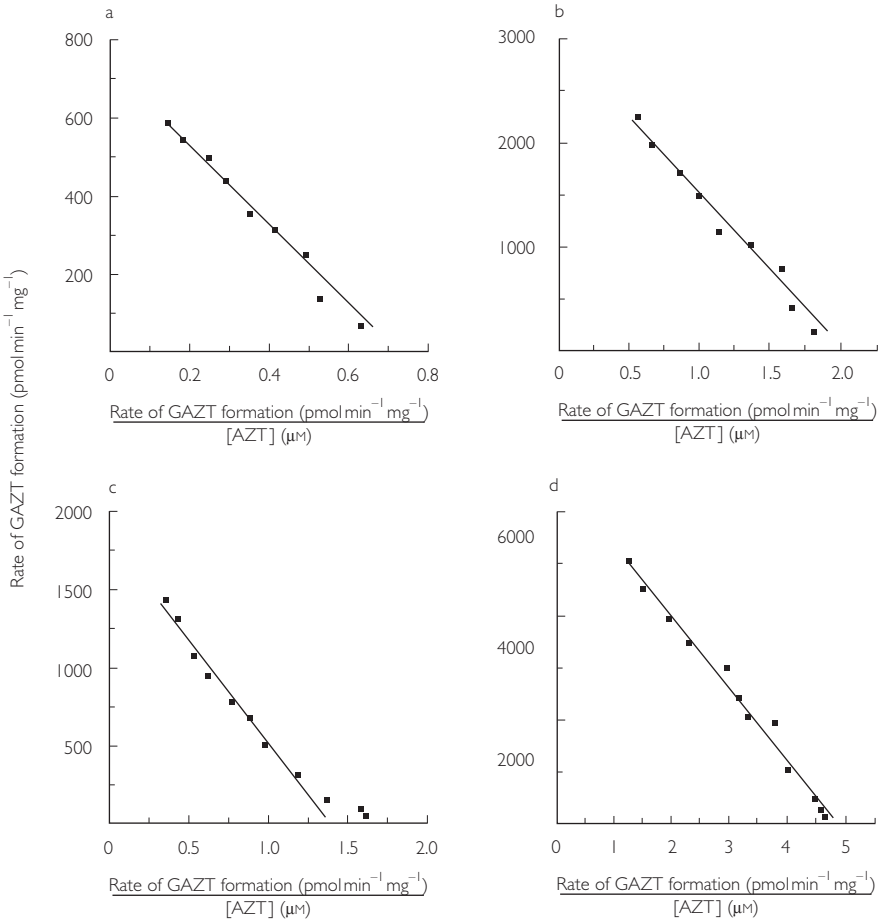

Figure 1 Representative Eadie-Hofstee plots for the conversion of AZT to GAZT using microsomes from a single liver. Incubations were performed at pH 7.4 and a buffer concentration of 0.1 M, with other conditions as described in *Microsomal incubations*. Panel a, native microsomes–phosphate buffer; Panel b, Brij58 activated microsomes–phosphate buffer; Panel c, native microsomes–Tris buffer; Panel d, Brij58 activated microsomes–Tris buffer.

tions in all livers. (Experiments with pooled microsomes using substrate concentrations to 10 μM precluded biphasic kinetics or non-Michaelis Menten kinetics.) Derived kinetic constants are similar to values reported previously for the conversion of AZT to GAZT [16, 19].

Differences in mean apparent K_m and V_{max} values determined from Tris buffered incubations and phosphate buffered incubations using the corresponding activation conditions (i.e. native *vs* native, activated *vs* activated) were not statistically significant (Table 1). Detergent activation of Tris and phosphate buffered microsomes increased V_{max} 3.1- and 3.8-fold ($P < 0.05$), respectively, without changing apparent K_m (Table 1). For detergent activated microsomes, similar relative differences in apparent K_m and V_{max} for Tris buffered microsomes compared to phosphate buffered microsomes resulted in similar values of microsomal CL_{int} for the two buffer types. The mean UDPGA apparent K_m values for detergent activated microsomes ($n = 5$ livers) in phosphate and Tris buffered incubations were 797 ± 62 μM (95% CI: 710–841 μM) and 766 ± 160 μM (95% CI: 638–1005 μM), respectively (data not shown).

Microsomal CL_{int} values determined for GAZT formation by native and detergent activated microsomes in Tris and phosphate buffered incubations were extrapolated to blood AZT glucuronidation hepatic clearances using the expressions for the well-stirred, parallel tube and dispersion models, as described in Data analysis. *In vivo* CL_H values determined using these models are summarized in Table 2. The extrapolated mean *in vitro* CL_{int} values derived for native and detergent activated microsomes underestimated AZT glucuronidation clearance *in vivo* (*viz* 82 l h^{-1}, see Data analysis) by 17–23-fold and 6.5–7.3-fold, respectively.

Given the poor predicability of microsomal CL_{int} for estimating *in vivo* CL_H, factors potentially altering human liver microsomal AZT glucuronidation kinetics were investigated further. Unless stated otherwise, the influence of factors known to influence glucuronide formation *in vitro* were investigated using pooled liver

Table 2 Predicted zidovudine glucuronidation hepatic clearances derived from the well-stirred, parallel tube and dispersion models[a].

	Predicted CL_H (l h^{-1})		
Microsome treatment	*Well-stirred*	*Parallel tube*	*Dispersion*
Native, phosphate-buffered	3.61 ± 1.38 (1.90, 5.32)	3.70 ± 1.44 (1.91, 5.48)	3.67 ± 1.42 (1.90, 5.43)
Native, Tris-buffered	4.69 ± 0.96 (3.50, 5.88)	4.82 ± 1.01 (3.56, 6.07)	4.78 ± 1.00 (3.54, 6.02)
Activated (Brij58), phosphate-buffered	11.27 ± 4.06★ (6.53, 16.34)	12.24 ± 4.37★ (6.81, 16.66)	11.98 ± 4.26★ (6.69, 16.47)
Activated (Brij58), Tris-buffered	11.88 ± 2.00★ (9.54, 15.20)	12.71 ± 2.29★ (9.86, 15.59)	12.46 ± 2.20★ (9.65, 15.36)

[a]Data presented as mean ± s.d., with 95% confidence intervals in parenthesis, for microsomes from 5 human livers.
★$P < 0.05$ compared with values for phosphate- and Tris-buffered native microsomes.

microsomes (1 mg ml^{-1}), AZT (1 mM; the approximate apparent K_m), $MgCl_2$ (4 mM), and UDPGA (10 mM) in phosphate buffered (0.1 M, pH 7.4) incubations. The comparative effects of known activators of UGT and of the β-glucuronidase inhibitor D-saccharic acid 1,4-lactone on AZT glucuronidation are shown in Figure 2. Omission of $MgCl_2$ from incubations decreased GAZT formation by approximately 50%. Detergent (Brij58, 0.15 w/w) produced slightly greater activation than alamethacin (3.9-fold *vs* 3.5-fold). The extent of activation by alamethacin was essentially constant over the concentration range 25–100 mg l^{-1} (data not shown). GAZT formation was activated less than 2-fold by UDP-NAcG, and reduction of the UDPGA concentration to 0.25 mM reversed the effect of UDP-NAcG. Addition of D-saccharic acid 1,4-lactone to incubations did not alter the rate of microsomal AZT glucuronidation.

The effects of incubation pH were assessed for detergent activated microsomes in both phosphate and Tris buffers (0.1 M). Data for phosphate buffered microsomes are shown in Figure 3; an identical trend was observed using Tris buffer. The rate of GAZT formation increased between pH 6.0 and 8.0, and declined thereafter. However, the rates of GAZT formation at pH 7.4 and 8.0 differed by only 7%. Effects of an ATP generating system and added GTP on GAZT formation were investigated in Tris buffer (0.1 M, pH 7.4), with and without detergent or alamethacin (50 mg l^{-1}) activation. The respective rates of GAZT formation in control, ATP-containing and GTP-containing incubations were: 713, 627 and 769 pmol min^{-1} mg microsomal $protein^{-1}$ using native microsomes; 2395, 2443 and 2454 pmol min^{-1} mg^{-1} microsomal protein using detergent activated microsomes; and 2109, 1904 and 2265 pmol min^{-1} mg^{-1} microsomal protein using alamethacin activated microsomes.

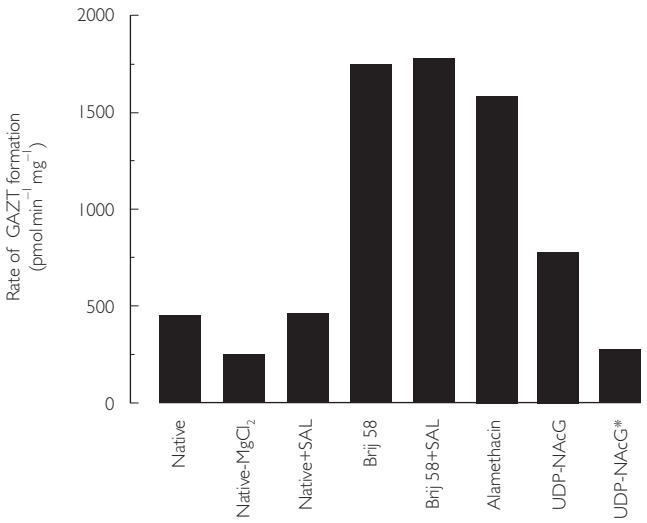

Figure 2 Effects of incubation components on the rate of AZT glucuronidation by pooled human liver microsomes. Incubations were performed in phosphate buffer (0.1 M, pH 7.4) in the presence of AZT (1 mM), UDPGA (10 mM) and, unless indicated, $MgCl_2$ (4 mM). Abbreviations: Native = native microsomes; SAL, D-saccharic acid 1,4-lactone (8.5 mM); Brij58, microsomes activated with Brij58 (0.15 w/w); Alamethacin, microsomes activated with alamethacin (50 mg l^{-1}); UDP-NAcG, microsomes activated with UDP-NAcG (1 mM); UDP-NAcG★, microsomes activated with UDP-NAcG (1 mM) and UDPGA concentration reduced to 0.25 mM.

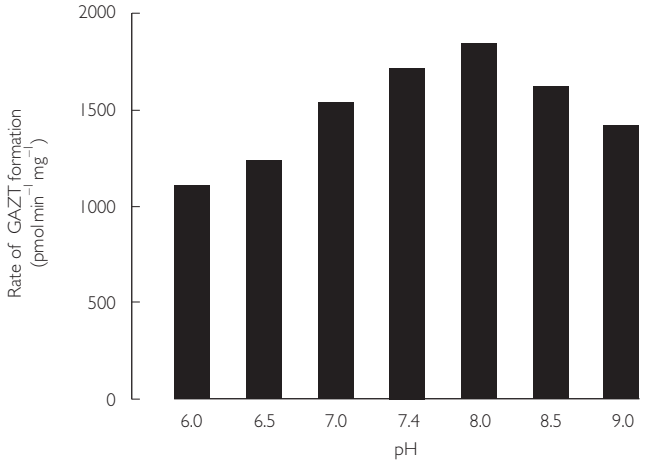

Figure 3 Effects of phosphate buffer (0.1 M) pH on the rate of AZT glucuronidation by Brij58 activated pooled human liver microsomes (1 mg ml^{-1}). Incubations contained AZT (1 mM), UDPGA (10 mM) and $MgCl_2$ (4 mM).

The rate of GAZT formation declined with increasing phosphate buffer (pH 7.4) concentration in the range 0.02–0.2 mM, with both native and detergent activated microsomes (Figure 4). To investigate the mechanism of the effect of buffer concentration on GAZT formation, the kinetics of AZT glucuronidation by native and deter-

gent activated microsomes were determined at phosphate buffer (pH 7.4) concentrations of 0.02 and 0.1 M (Table 3). Apparent K_m decreased while V_{max} remained unchanged at the lower buffer concentration. The microsomal CL_{int} values determined for microsomal incubations performed in 0.02 M phosphate were 62–68% higher than values determined using 0.1 M phosphate buffer.

AZT did not bind nonspecifically to human liver microsomes. For added AZT concentrations of 100, 1000 and 3000 μM, ratios of AZT in the buffer and microsome compartments of the equilibrium dialysis apparatus were 0.97, 1.08 and 0.96, respectively, using native microsomes, and 1.03, 1.10 and 0.99, respectively, using detergent activated microsomes.

Discussion

Despite the widespread use of *in vitro-in vivo* scaling for drugs eliminated by CYP [1, 3, 7, 8], little attention has been paid to the validity of scaling *in vitro* CL_{int} to hepatic clearance *in vivo* for drugs metabolized by UGT. The initial aim of the work described here was to assess whether microsomal CL_{int} for the model glucuronidated drug AZT predicted CL_H *in vivo* using mathematical models of hepatic drug clearance. Since microsomal incubation conditions for the measurement of drug glucuronidation kinetic parameters interchangeably use phosphate and Tris buffers, with or without activation, kinetic constants determined under these various reaction conditions were compared. Mean microsomal CL_{int} for AZT glucuronidation varied 3.6-fold for the different incubation conditions. Depending on the conditions and mathematical model of hepatic clearance employed, predicted CL_H underestimated AZT glucuronidation clearance by 6.5- to 23-fold. Although other factors were subsequently shown to influence AZT glucuronidation *in vitro*, it was apparent that no set of conditions could be developed that gave a microsomal CL_{int} high enough to predict metabolic clearance *in vivo*.

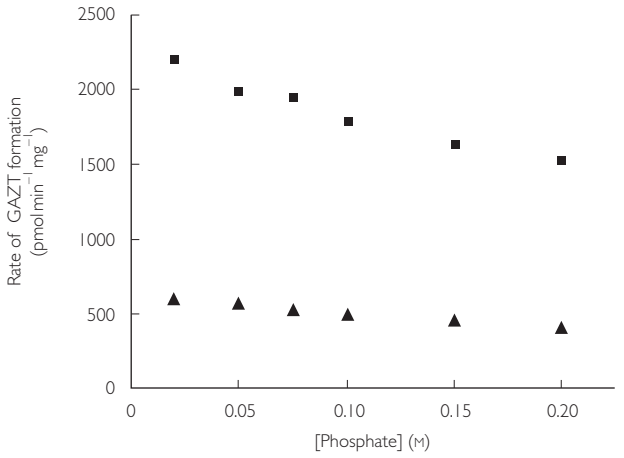

Figure 4 Effect of phosphate buffer (pH 7.4) concentration on the rate of AZT glucuronidation by pooled human liver microsomes. *Native* (▲) refers to native microsomes, and *Activated* (■) to Brij58 treated microsomes (0.15 w/w). Incubations contained AZT (1 mM), UDPGA (10 mM) and $MgCl_2$ (4 mM).

The use of phosphate or Tris buffer in microsomal incubations is known to variably affect CYP3A activities [9]. Although both apparent K_m and V_{max} for AZT glucuronidation tended to be higher for incubations buffered with Tris compared with phosphate (with and without detergent activation), differences were not statistically significant. Moreover, K_m and V_{max} tended to increase in parallel resulting in similar values of CL_{int} for the two buffer types. As expected from studies with other glucuronidated substrates (for example [20]), the nonionic detergent Brij58 increased V_{max} approximately 3- to 4-fold. In both the presence and absence of detergent, the rate of AZT glucuronidation increased with decreasing phosphate concentration in the range 0.2-0.02 M. This effect was due to a lowering of apparent K_m rather than an increase in V_{max}. Although lowering the phosphate buffer concentration resulted in an approximately 60% increase in the microsomal CL_{int} obtained for detergent activated microsomes (Table 3), the extrapolated CL_H still underestimated AZT glucuronidation clearance by a factor of 4.3–4.8. (CL_H values obtained using the well-stirred and parallel tube models were 17.26 and

Table 3 Kinetic data for zidovudine glucuronidation by native and detergent activated pooled human liver microsomes in phosphate buffer of concentration 0.02 M.

Microsome treatment	K_m *(μM)*	V_{max} *(pmol min^{-1} mg^{-1})*	CL_{int} *(μl min^{-1} mg^{-1})*
Native, phosphate buffer 0.1 M	890	957	1.08
Native, phosphate buffer 0.02 M	551	999	1.81
Activated (Brij58), phosphate buffer 0.1 M	1018	3708	3.64
Activated (Brij58), phosphate buffer 0.02 M	672	3960	5.89

19.01 l h^{-1}, respectively.) Interestingly, the trend observed for the effect of phosphate concentration on AZT glucuronidation is opposite to that observed for most CYP-catalysed reactions, where activity tends to increase in the buffer concentration range 0.01–0.2 M [9]. In the case of CYP, it has been proposed that this effect may arise from changes in protein conformation [21].

No other treatment increased rates of AZT glucuronidation more than Brij58. The pore-forming agent alamethacin activated AZT glucuronidation by 3.5-fold, which is similar to observations with other glucuronidated substrates [22]. UDP-NAcG, an endogenous activator of UGT [11], increased AZT glucuronidation less than 2-fold. It has been suggested that effects of UDP-NAcG are maximal at physiological concentrations of cofactor (UDPGA) [23], but rates of AZT glucuronidation decreased in the presence of UDP-NAcG (1 mM) and UDPGA (0.25 mM) compared with conditions where the cofactor concentration (*viz* 10 mM) was not rate-limiting. Omission of $MgCl_2$ decreased the rate of AZT glucuronidation, confirming the stimulatory effect of Mg^{2+} on human liver microsomal UGT. A dependence of AZT glucuronidation rate on incubation pH was also confirmed, although activity was near maximal at pH 7.4, which is most commonly employed for investigations of microsomal xenobiotic glucuronidation (at least for those substrates forming an ether glucuronide). Significant β-glucuronidase activity has been reported for human liver microsomes [24], and hydrolysis of product would result in underestimation of the rate of AZT glucuronidation. However, addition of D-saccharic acid 1,4-lactone was without effect on AZT glucuronide formation.

Available evidence suggests that the UGT active site may be located on the lumenal side of the microsomal membrane and that cofactor availability may be dependent on carrier mediated transport [25]. There are previous reports of stimulation of UGT activity by ATP and GTP [11, 26], although effects are variable and may involve mechanisms other than facilitation of active transport [11]. In this work, the rate of AZT glucuronidation was unaffected by both ATP and GTP. Moreover, the pore-forming peptide alamethacin, which apparently facilitates free diffusion of substrate and product between the UGT active site and the cytosol without affecting enzyme activity [22], increased AZT glucuronidation only to the same extent as detergent activation.

Given the poor correspondence between the known clearance of AZT by glucuronidation and the CL_H predicted from *in vitro* kinetic data, *in vitro-in vivo* comparisons were additionally performed for the glucuronidated drugs naloxone, propofol, morphine, 5,6-dimethylxanthenone-4-acetic acid, paracetamol, amitriptyline, lamotrigine, clofibric acid, valproic acid and naproxen, using published data (Table 4). Blood drug clearances by glucuronidation for these drugs are in the range 0.20–77 l h^{-1}. *In vitro* kinetic data used to calculate CL_{int} and CL_H

Table 4 Comparison of *in vivo* blood drug clearances by glucuronidation and hepatic clearances predicted from published *in vitro* kinetic data[a].

Drug	In vivo *blood clearance by glucuronidation*[a,b] *(l h^{-1})*	*Hepatic clearance by glucuronidation predicted by dispersion model*[c,d] *(l h^{-1})*	*References for* in vitro *and* in vivo *data*
Zidovudine	82.0	12.46	This study, 12–14
Naloxone	76.72	1.20	31–33
Propofol	56.28	0.16	33–36
Morphine (3- and 6-glucuronidation)	44.40	4.92	33, 37, 38
DMXAA[e]	19.75	2.49	39–41
Paracetamol	11.21	2.19	42–46
Amitriptyline	7.40	1.63	47–49
Lamotrigine	2.08	0.45	50–52
Clofibric acid	0.35	0.28	53–55
Naproxen	0.26	0.04	33, 56
Valproic acid	0.20	0.09	33, 57

[a]Clearance by glucuronidation was determined as $CL_{s,blood} \times f_{gluc}$, where f_{gluc} is the fractional urinary recovery of glucuronide. For the low hepatic clearance drugs clofibric acid, naproxen, and valproic acid, CL/F was used due to the unavailability of CL_s values.

[b]$CL_{s,blood}$ values were available for propofol and amitriptyline. R_B values reported for zidovudine, morphine, DMXAA and paracetamol were used to calculate $CL_{s\ blood}$ as: $CL_{s,blood} = CL_{s,plasma}/R_B$. R_B was taken as 1 for naloxone and lamotrigine, and 0.7 for clofibric acid, naproxen, and valproic acid [58].

[c]Literature values of K_m and V_{max} were all determined using activated (detergent or sonication) human liver microsomes.

[d]Predicted CL_H was determined using the dispersion model (*Data analysis*). Literature values of R_B were used to calculate fu from fu_p, as described in *Data analysis*. Where R_B could not be obtained from the literature (naloxone, lamotrigine, clofibric acid, naproxen, valproic acid), a value of 1 or 0.7 was assumed (see footnote b).

[e]5,6-Dimethylxanthenone-4-acetic acid.

were obtained using microsomal incubation conditions similar to those employed in this work; incubations were performed in phosphate or Tris buffer (0.1 M, pH 6.8–7.5) with activated (detergent or sonication) human liver microsomes. It is apparent from Table 4 and Figure 5 that predicted CL_H underestimated blood clearance by glucuronidation for all compounds. Ratios of predicted CL_H to *in vivo* clearance ranged from 0.003 (propofol) to 0.80 (clofibric acid). Interestingly, predicted CL_H and clearance *in vivo* were significantly correlated (Figure 5: $P = 0.042$; $r = 0.62$; slope = 0.078, 95% CI 0.003, 0.140). Exclusion of the data for naloxone and propofol, which show the greatest discrepancies, greatly improved the correlation ($P < 0.0001$; $r = 0.98$; slope = 0.143, 95% CI 0.124, 0.163). Mistry and Houston [28] reported similar findings in rat for morphine, naloxone and buprenorphine, where hepatic microsomal CL_{int} values were approximately 20–to 30-fold lower than their *in vivo* counterparts but rank orders were the same. Clearly, the approaches adopted currently for the scaling of *in vitro* kinetic data do not accurately predict *in vivo* CL_H for drugs eliminated by glucuronidation. The possibility remains, however, that assessment of a larger data set may validate the use of a scaling factor for the extrapolation of *in vitro* CL_{int}.

Taken together, these data suggest that the CL_{int} determined from microsomal kinetic studies underestimates *in vivo* CL_{int}, or perhaps extrahepatic glucuronidation is a major contributor to drug elimination *in vivo*. The latter seems unlikely, at least with respect to renal AZT glucuronidation. Assuming kidney weight of 300 g and a renal microsome yield of 6 mg g^{-1} [28], the 'whole' kidney CL_{int} for AZT glucuronidation determined from published kinetic constants [19] is 0.017 l h^{-1}. The corresponding 'whole' liver CL_{int} calculated from kinetic constants derived in the present work, using the same incubation conditions (native microsomes, Tris buffer), is 5.55 l h^{-1}.

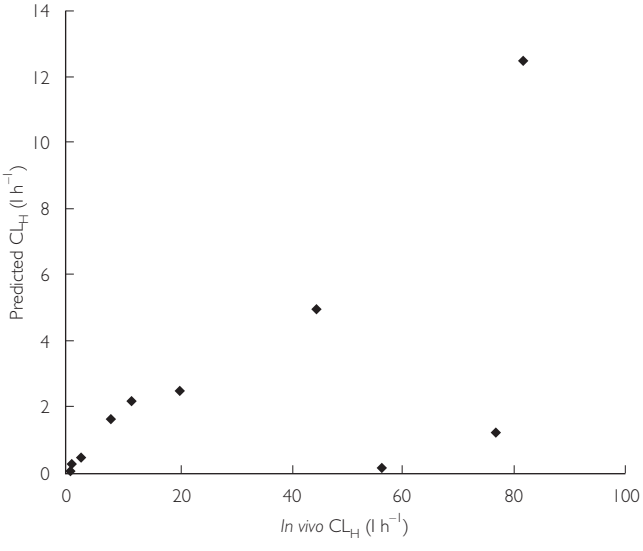

Figure 5 Correlation between the *in vivo* blood clearances by glucuronidation and hepatic clearances predicted from published *in vitro* kinetic data for the drugs listed in Table 4.

The possibility remains that the assumptions underpinning the mathematical models of CL_H used throughout this work are not applicable to glucuronidation. As noted previously, UGT is believed to be localized on the lumenal side of the microsomal membrane and this may give rise to diffusional 'barriers'. Another potential confounding factor, nonspecific microsomal binding of substrate which may result in underestimation of CL_H [17, 29], was discounted here for AZT and is also negligible for morphine (Stone and Miners, unpublished data). In addition, nonspecific binding may normally also be discounted for acidic drugs, such as naproxen, 5,6-dimethylxanthenone-4-acetic acid, valproic acid and clofibric acid [17]. However, extensive binding to the microsomal membrane is known to occur for amitriptyline [17], and cannot be excluded for other bases listed in Table 4 (naloxone, lamotrigine) or for the highly lipophilic compound propofol.

In conclusion, it has been confirmed that the kinetic parameters for AZT glucuronidation by human liver microsomes are markedly dependent on incubation incubations. Burchell *et al.* [34] have previously noted that investigations of UGT activity *in vitro* employ widely differing reaction conditions. The present work highlights the need for standardization of incubation conditions if there is to be meaningful interlaboratory comparison of drug glucuronidation kinetic data. It was also shown that none of the reaction conditions investigated predicted AZT hepatic clearance by glucuronidation *in vivo*. Calculations based on published kinetic data further demonstrated that extrapolated hepatic clearance invariably underestimated *in vivo* clearance by glucuronidation. Importantly, known high hepatic clearance drugs were predicted to have hepatic clearances in the 'low' range. Although a statistically significant correlation may exist between predicted and actual hepatic clearance, caution should be exercised when extrapolating *in vitro* kinetic data to the *in vivo* situation for drugs eliminated by glucuronidation.

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