รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษาเพื่อหาชนิดของ enzyme UDP-glucuronosyltransferases ที่ใช้ในขบวนการ glucuronidation ของยา lorazepam และการเกิดอันตรกิริยาระหว่าง lorazepam และ valproic acid

In vitro characterization of UDP-glucuronosyltransferases responsible for lorazepam glucuronidation and the lorazepam-valproic acid interaction

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สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษาและ
สำนักงานกองทุนสนับสนุนการวิจัย

ABSTRACT

The anxiolytic agent lorazepam (LZP) and anticonvulsant valproic acid (VPA) are mainly eliminated in humans by glucuronidation. This study aimed to characterize UDP-glucuronosyltransferases responsible for (R,S) lorazepam glucuronidation and quantitatively predict *in vivo* metabolic drug-drug interaction between LZP and VPA. Formation of the glucuronide metabolites (R-LZPG and S-LZPG) were quantified by HPLC. Recombinant human UGT enzymes were expressed in HEK293 or baculovirus-insect cells. Kinetics of R- and S-LZP glucuronidation by HLMs (n=5) exhibited substrate inhibition. Mean derived binding affinity (K_m) and V_{max} of R- and S-LZPG kinetics were 36.7 ± 11.4 μM and 7.3 ± 2.1 pmol/min.mg, and 50.3 ± 18.2 μM and 11.7 ± 5.2 pmol/min.mg, respectively. Substrate inhibition with both metabolites was weak (K_m 10- to 17-fold lower than K_{si}). Twelve recombinant UGTs were screened for LZP glucuronidation activity at LZP concentrations of 10, 50 and 250 μM. UGT 2B4, 2B7 and 2B15 catalyzed S-lorazepam, but the highest activity is observed for UGT2B15. R-lorazepam was catalyzed by UGT 2B4, 2B7 and 2B15. Notably, UGT 1A7 and 1A10 which are expressed in gastrointestinal tract metabolized only R-lorazepam. To identify UGTs responsible for lorazepam glucuronidation in the liver, the kinetic studies of hepatically UGTs which exhibited measurable activities were further investigated. Derived K_m or S_{50} values observed with recombinant UGT 2B4, 2B7 and 2B15 were in the range of 13-46 μM which is comparable to those observed by using HLMs. VPA exhibited non-competitive inhibition on R- and S-lorazepam glucuronidation by HLMs with respective K_i values of 3.9 and 3.2 mM. In conclusion, UGT 2B4, 2B7 and 2B15 are likely to be the major enzymes responsible for human liver microsomal R,S-lorazepam glucuronidation. Based on K_i value, 20% increased of LZP area under the plasma concentration time curve was predicted when co-administered with VPA.

Keywords: lorazepam, valproic acid, glucuronidation, UDP-glucuronosyltransferase, drug-drug interaction

Final Report

Contract Number MRG5180009

In vitro characterization of UDP-glucuronosyltransferases responsible for lorazepam glucuronidation and the lorazepam-valproic acid interaction

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Project Investigator: Assist.Prof. Dr. Verawan Uchaipichat

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1. INTRODUCTION

Lorazepam is a 3-hydroxy-1,4-benzodiazepine derivative which is used widely in clinical practice as its sedative-antianxiety and anticonvulsant properties. The major metabolic pathway in the liver of lorazepam involves glucuronidation via the hydroxyl group at the 3-position, yielding a water-soluble compound (Greenblatt et al. 1976). This metabolite is pharmacologically inactive, and is eliminated from the body mainly by renal excretion. It has been found that about 75% of oral dose of lorazepam can be recovered in urine as the glucuronide metabolite, while the remainder is eliminated as oxidized metabolites, their glucuronides, and unchanged form (Greenblatt et al. 1976; Greenblatt et al. 1979; Greenblatt et al. 1979).

Glucuronidation is a synthetic reaction in phase II drug biotransformation that involves the covalent linkage (or "conjugation") of glucuronic acid, derived from the cofactor UDP-glucuronic acid, to a substrate bearing a suitable functional group (Miners and Mackenzie 1991; Radominska-Pandya et al. 1999; Tukey and Strassburg 2000). This reaction, which proceeds according to a second order nucleophilic substitution mechanism, is catalyzed by the enzyme UDP-glucuronosyltransferases (UGTs; EC 2.4.1.17) (Radominska-Pandya et al. 1999; Tukey and Strassburg 2000). UGTs exist as an enzyme superfamily. To date, nucleotide sequences encoding 21 human UGT proteins of approximately 530 amino acids have been identified (Miners et al. 2004; Mackenzie et al. 2005). Based on sequence identity, UGTs are classified in three families, *UGT1* on human chromosome 2q37, *UGT2* on human chromosome 4q13, and *UGT3* on human chromosome 5p13.2 (Tukey and Strassburg 2000; Mackenzie et al. 2005).

Although the glucuronide conjugation of lorazepam *in vivo* was well established, the information of the UGT isoforms glucuronidating this drug is limited. To date, knowledge of UGT isoform catalyzing lorazepam was implied from the evidences illustrating that lorazepam clearance *in vivo* were reduced by several factors (see literature review) including UGT polymorphism, Gilbert's syndrome and co-administration of other glucuronidated drugs. However the UGT isoform (s) responsible for elimination of lorazepam in the liver have not been identified in a systematic manner. Valproic acid is an anticonvulsant which is primarily eliminated by glucuronidation (Cotariu and Zaidman 1988). It has been shown that valproic acid increased the area under the plasma-concentration time curve (AUC) of several glucuronidated drugs such as lamotrigine (Morris et al. 2000) and zidovudine (Lertora et al. 1994). Co-administration of lorazepam and valproic acid is used clinically both in the treatment of epilepsy and psychiatric disorder, thus the potential exists for inhibitory interaction via glucuronidation pathway between both drugs are likely possible. Indeed, there is evidence to suggest that the apparent clearance of lorazepam through the formation of lorazepam glucuronide was reduced during co-administration of valproic acid (see literature review). This pharmacokinetic interaction is likely to be clinically significance as the severe encephalopathy such as coma was reported from co-administration of both drugs (see literature review).

The development and use of *in vitro* approaches to predict drug clearance and drug interaction *in vivo* (*in vitro-in vivo* extrapolation) has been found increasing acceptance in recent years, not only to assist in the selection of new drug candidate but also for the rationalization and optimization of dosage regimens for established drugs. Due to a limited availability of UGTs selective substrate and inhibitor, a process to identify the UGT isoform(s) responsible for the metabolism of any given drug has most commonly involved screening for activity using a 'panel' of recombinant UGTs (for example; (Court et al. 2003; Stone et al. 2003; Ghosal et al. 2004; Bowalgaha et al. 2005; Di Marco et al. 2005)). Recent studies illustrated that kinetic parameters generated *in vitro* by using human liver microsomes (HLMs) and recombinant human UGTs as the enzyme sources can also predict clearance and magnitude of inhibitory interactions involving glucuronidated drugs such as zidovudine-fluconazole (Uchaipichat et al. 2006) and lamotrigine-valproic acid (Rowland et al. 2006) interactions. Employing such an *in vitro* approach, we aim to investigate UGT isoform (s) responsible for lorazepam glucuronidation in the liver. In addition, UGT isoform (s) and mechanism involving the inhibitory interaction of lorazepam and valproic acid will be further investigated.

2. OBJECTIVES

2.1 To characterize the kinetics of lorazepam glucuronidation by using human liver microsomes and a 'panel' of recombinant human UDP-glucuronosyltransferases

2.2 To characterize the inhibitory constant (K_i) of valproic acid on lorazepam glucuronidation by using human liver microsomes and recombinant human UDP-glucuronosyltransferases

2.3 Comparing kinetic parameters obtained from both enzyme sources, we sought to assess the contribution of known UDP-glucuronosyltransferases to lorazepam hepatic clearance and lorazepam-valproic acid interaction. (Qualitative prediction)

2.4 Comparing the K_i value obtained from *in vitro* data and serum valproic acid level obtained from literature reviews, the magnitude of valproic acid and lorazepam inhibitory interaction *in vivo* is predicted. (Quantitative prediction)

3. LITERATURE REVIEW

Lorazepam is a 3-hydroxy-1,4-benzodiazepine derivative which is used widely in clinical practice as its sedative-antianxiety and anticonvulsant properties. The major metabolic pathway in the liver of lorazepam involves glucuronidation via the hydroxyl group at the 3-position (Figure 1), yielding a water-soluble compound (Greenblatt et al. 1976). This metabolite is pharmacologically inactive, and is eliminated from the body mainly by renal excretion. At clinically relevant concentrations, lorazepam is 91 ± 2% bound to plasma proteins; its volume of distribution is approximately 1.3 L/kg (Greenblatt et al. 1979; Greenblatt et al. 1979). Unbound lorazepam penetrates the blood/brain barrier freely by passive diffusion, a fact confirmed by CSF sampling. Following parenteral administration, the terminal half-life and total clearance averaged 14 ± 5 hours and 1.1 ± 0.4 mL/min/kg, respectively (Greenblatt et al. 1979; Greenblatt et al. 1979). Following a single 2-mg oral dose of ^{14}C-lorazepam to 8 healthy subjects, 88 ± 4% of the administered dose was recovered in urine and 7 ± 2% was recovered in feces. The percent of administered dose recovered in urine as lorazepam-glucuronide was 74 ± 4%. Only 0.3% of the dose was recovered as unchanged lorazepam, and the remainder of the radioactivity represented minor metabolites (Greenblatt et al. 1976).

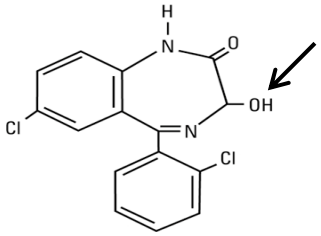

Figure 1 Structure and site of glucuronidation (arrow) of lorazepam

Glucuronidation is a synthetic reaction in phase II drug biotransformation that involves the covalent linkage (or "conjugation") of glucuronic acid, derived from the cofactor UDP-glucuronic acid, to a substrate bearing a suitable functional group, including carboxyl, hydroxyl (phenol or aliphatic alcohol), amino (primary, secondary, or tertiary), acidic carbon atom, or thiol moiety (Miners and Mackenzie 1991; Radominska-Pandya et al. 1999; Tukey and Strassburg 2000). This reaction, which proceeds

according to a second order nucleophilic substitution mechanism, is catalyzed by the enzyme UDP-glucuronosyltransferase (UGT; EC 2.4.1.17) (Radominska-Pandya et al. 1999; Tukey and Strassburg 2000). To date, nucleotide sequences encoding 21 human UGT proteins of approximately 530 amino acids have been identified (Miners et al. 2004; Mackenzie et al. 2005). Based on sequence identity, UGTs are classified in three families, *UGT1* on human chromosome 2q37, *UGT2* on human chromosome 4q13, and *UGT3* on human chromosome 5p13.2 (Tukey and Strassburg 2000; Mackenzie et al. 2005). Twenty-one human UGT proteins have been identified namely UGT 1A1, 1A3, 1A4, 1A5, 1A6, 1A7, 1A8, 1A9, 1A10, 2A1, 2A2, 2A3, 2B4, 2B7, 2B10, 2B11, 2B15, 2B17, 2B28, 3A1 and 3A2. Of these, UGT 1A5, 2A2, 2A3, 2B10, 2B11 and 2B28 exhibit very low, if any, activity (Radominska-Pandya et al. 1999; Tukey and Strassburg 2000; Miners et al. 2004). Available evidence indicates that the individual UGTs exhibit distinct, but overlapping, substrate selectivities (Miners et al. 2004; Miners et al. 2006). It appears that, of the hepatically expressed enzymes, UGT 1A1, 1A3, 1A4, 1A6, 1A9, 2B7 and 2B15 contribute to the glucuronidation of clinically used drugs to the greatest extent (Miners et al. 2004). Of these, UGT2B7 shows the broadest selectivity and is arguably the most important drug glucuronidating enzyme. By way of example, UGT2B7 catalyzes the glucuronidation of many opioids and non-steroidal anti-inflammatory drugs, as well as valproic acid, epirubicin and zidovudine (Jin et al. 1993; Coffman et al. 1998; Radominska-Pandya et al. 1999; Tukey and Strassburg 2000; Court et al. 2003; Kiang et al. 2005).

Although the glucuronide conjugation of lorazepam *in vivo* was well established, there has been no systematic investigation of the UGT isoforms contributing to lorazepam glucuronidation. Accumulating evidences suggests that multiple UGTs may involve lorazepam glucuronidation *in vivo* as it was affected by several factors including UGT polymorphism, Gilbert's syndrome and co-administration of other glucuronidated drugs. Chung et al (2005) reported that the pharmacokinetic of lorazepam was significantly affected by the UGT2B15*2 polymorphism in humans. It has been found that systemic clearance and the metabolite AUC ratio of lorazepam were significantly lower in the UGT2B15*2/*2 group by 58% (95% CI, 43%-72%) and 48% (95% CI, 24%-72%), respectively, compared to those in the wild type (UGT2B15*1/*1) group. Data were correlated with those reported previously that the UGT2B15 D85Y(*2) genotype was a major determinant of lorazepam glucuronidation *in vitro* (Court et al. 2004). However, the results from the same author group have been shown recently that UGT2B7 polymorphism also has a minor effect to lorazepam pharmacokinetics in humans (Chung et al. 2007). There was a decreasing tendency in the metabolite AUC ratio as the number of variant alleles (UGT2B7*2) increased, although the difference was insignificant and such a tendency was not observed for systemic clearance. The metabolite AUC ratio of lorazepam were 1.32 ± 0.44, 1.25 ± 0.49 and 0.90 ± 0.33 in UGT2B7*1/*1, UGT2B7*1/*2, and UGT2B7*2/*2 groups, respectively. Apart from the effect of UGT2B polymorphisms, lorazepam glucuronidation is likely to be affected in the presence of Gilbert's syndrome, a familial disorder involving UGT1A1 polymorphism (Herman et al. 1994). Lorazepam clearance was decreased by 20-40% in Gilbert's trait patients.

In addition to UGT polymorphisms, the effect of valproic acid, a known substrate of UGT2B7 (Jin et al. 1993; Ethell et al. 2003) on the glucuronidation of lorazepam has been studied. Anderson et al. (1994) reported that valproic acid (250 mg twice daily) significantly decreased lorazepam plasma clearance by an average of 40%. These effects were accompanied by a reduction in the formation clearance of lorazepam glucuronide in 6 out of 8 subjects. Samara et al. (1997) also showed a decrease in the formation clearance of lorazepam, when valproic acid (500 mg twice daily) was given to healthy volunteers. The apparent clearance of lorazepam through the formation of lorazepam glucuronide was reduced by 31% during co-administration of valproic acid. This interaction was accompanied by increases in steady-state maximum plasma concentration, area under the concentration-time curve, and trough plasma concentration of lorazepam by 8%, 20%, and 31%, respectively. The valproic acid and lorazepam interaction may be clinically significant, as reported by Lee et al. (2002). A 36-year-old woman with intractable seizure was taking valproic acid 1000 mg/day, phenytoin 300 mg/day, and carbamazepine 400 mg/day, when a total of 6 mg of lorazepam was given intravenously over 24 hr. After the last injection of lorazepam, the patient was observed to be unconscious and unresponsive. The serum levels for valproic acid, phenytoin and carbamazepine were in normal range. The other possible causes of inducing coma were excluded. Based on the exclusion and prompt recovery after the withdrawal of valproic acid, it was most likely that the elevated plasma concentration of lorazepam due to lorazepam-valproic acid interaction may induce coma, although serum level of lorazepam was not documented.

The development and use of *in vitro* approaches to predict drug clearance and drug interactions *in vivo* (*in vitro-in vivo* correlation) has found increasing acceptance in recent years, not only to assist in the selection of new drug candidates but also for the rationalization and optimization of dosage regimens for established drugs. The principal drug metabolizing enzymes, cytochrome P450 (CYPs) and UDP-glucuronosyltransferases (UGTs), exist as enzyme 'superfamilies'. The individual enzyme forms appear to exhibit distinct, but overlapping, substrate and inhibitor selectivities, and differ in terms of genetic polymorphism and gene regulation, leading to wide interindividual variability in metabolic clearance. Thus, once the drug interaction profile and regulation of the various enzymes are characterized, identification of the enzyme(s) responsible for metabolism of any given compound allows qualitative prediction of factors likely to alter drug clearance and response in particular patients or population groups (Miners et al. 1994; Rodrigues 1999). Quantitative prediction most commonly involves extrapolation of kinetic parameters generated *in vitro* (CL_{int}, K_i) to predict hepatic clearance and extraction ratio *in vivo* and the magnitude of inhibitory drug interactions (Houston 1994; Iwatsubo et al. 1997; Ito et al. 1998).

In the case of CYP, a number of approaches have been developed to identify the CYP isoform(s) responsible for the metabolism of any given drug, a process often referred to as 'reaction phenotyping' (Miners et al. 1994; Clarke 1998; Rodrigues 1999; Venkatakrishnan et al. 2001). Based largely on the use of human liver microsomes as the enzyme source, the approaches adopted include: (a) inhibition of

metabolism by enzyme-selective chemical and antibody inhibitors; (b) competitive inhibition of the metabolism of enzyme-selective substrates by the compound (with K_m matching K_i); (c) a significant correlation between rates of metabolism of the compound and immunoreactive enzyme contents or enzyme-selective activities in microsomes from a 'panel' of human livers; and (d) comparative metabolism of the compound by a 'bank' of recombinant human enzymes. Used together, these procedures allow identification of the CYP enzyme(s) responsible for the metabolism of a drug with reasonable certainty. Unlike CYP, reaction phenotyping of UGT substrates is still limited due to the limited availability of enzyme-selective inhibitory antibodies and chemicals, although selective substrates are now available for a number of UGTs (Miners et al. 2006). To date, reaction phenotyping of UGT substrates has most commonly involved screening for activity by recombinant enzymes (for example; (Court et al. 2003; Stone et al. 2003; Ghosal et al. 2004; Bowalgaha et al. 2005; Di Marco et al. 2005)). In addition, recent studies illustrated that kinetic parameters generated *in vitro* by using human liver microsomes (HLMs) and recombinant human UGTs as the enzyme sources can predict clearance and magnitude of inhibitory interactions involving glucuronidated drugs, for example zidovudine-fluconazole (Uchaipichat et al. 2006) and lamotrigine-valproic acid (Rowland et al. 2006) interactions.

4. METHOD

4.1 Enzyme sources preparation

Human livers are obtained from the human liver 'bank' of the Department of Clinical Pharmacology, Flinders Medical Centre, Australia. Approval was obtained from the Flinders Medical Centre Research Ethics Committee and from the donor next-of-kin for the procurement and use of human liver tissue in xenobiotic metabolism studies. Microsomes are prepared by differential centrifugation, as described by Bowalgaha et al. (2005). Briefly, liver portions in 0.1 M phosphate buffer (pH 7.4) containing 1.15% w/v potassium chloride are homogenized sequentially with a Janke and Kunkle Ultra Turax (24 000 rpm) and a Potter-Elvehjem homogenizer (mechanical drive at 1480 rpm). The homogenate is centrifuged at 700*g* for 10 min and then at 10 000*g* for a further 10 min. The supernatant fraction is aspirated and centrifuged at 105 000*g* for 60 min at 4ºC. The resulting pellet is re-suspended in 0.1 M phosphate buffer (pH 7.4) containing 1.15% w/v potassium chloride and centrifuge at 105 000*g* for 60 min at 4ºC. The microsomal pellet is suspended in 0.1 M phosphate buffer (pH 7.4) containing 20% glycerol and stored at -80ºC until use. Microsomal protein concentrations are determined by the Lowry method (Lowry et al. 1951) using bovine serum albumin as standard. Prior to use in incubations, human liver microsomes are activated with the pore forming peptide alamethicin (50 µg/mg microsomal protein) by pre-incubation on ice for 30 min (Boase and Miners 2002).

UGT1A cDNAs (viz. 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, and 1A10) were stably expressed in a human embryonic kidney cell line (HEK293). Due to the relatively low activity of UGT 2B4, 2B7, 2B15 and 2B17 expressed in HEK293 cells, baculovirus-expressed UGT2B enzymes (Supersomes)

were employed in this study. The individual UGT1A cDNAs are stably expressed in a human embryonic kidney cell line (HEK293) as described previously (Sorich et al. 2002; Stone et al. 2003). Cells are separately transfected with the individual UGT cDNAs cloned into the pEF-IRES-puro6 expression vector (Hobbs et al. 1998) and incubated in Dulbecco's Modified Eagle Medium (DMEM), which contained puromycin (1.5 mg/l), 10% fetal calf serum, and gentamicin (160 mg/l) or penicillin G sodium (100 units/ml)/streptomycin sulfate (100 µg/ml) in a humidified incubator with an atmosphere of 5% CO_2 at 37°C. After growth to at least 80% confluency, cells are harvested and washed twice in phosphate-buffered saline. The harvested cells are kept in storage buffer (phosphate buffer (0.1M, pH 7.6; PB), 5 mM ethylenediamine tetraacetic acid, and 100 mM dithiothreitol) and lysed by sonication using a Heat Systems-Ultrasonics sonicator set at microtip limit of 4. Cells expressing UGT1A proteins are sonicated with 4 x 2-sec 'bursts', each separated by 3 min cooling on ice.

4.2 Lorazepam glucuronidation assay

Lorazepam glucuronide formation *in vitro* is measured using a modification of the method for zidovudine glucuronide measurements (Boase and Miners 2002). Incubation mixtures, in a total volume 0.2 ml, contain phosphate buffer (0.1 M, pH 7.4), $MgCl_2$ (4 mM), UDP-glucuronic acid (5 mM), lorazepam, and alamethicin-activated HLM or recombinant human UGTs. Reactions are initiated by the addition of UDP-glucuronic acid and performed at 37°C in a shaking water bath. Rates of lorazepam glucuronide formation are optimized for linearity with respect to incubation time and protein concentration. Valproic acid inhibition of lorazepam glucuronidation is investigated in the same condition using human liver microsomes and recombinant human UGTs which the activity can be measured. The reactions are terminated by the addition of 10 µl of 24% $HClO_4$, and then samples are centrifuged (5000*g* for 10 min) and a 30 µl aliquot of the supernatant fraction is injected into the HPLC column.

4.3 Quantification of R- and S- lorazepam glucuronides

High-performance liquid chromatography (HPLC) is performed to quantify R- and S-lorazepam glucuronides. Both metabolites are quantified by comparison of peak areas to those of a lorazepam external standard curve. The lower limit of detection assessed as five times background. Within-day overall assay reproducibility is assessed by measuring lorazepam glucuronide formation in 10 separate incubations of the same batch of pooled HLM. The identity of lorazepam glucuronide is confirmed by enzymatic hydrolysis.

4.4 Kinetic analysis of *in vitro* glucuronidation activity data

Kinetic constants for substrate glucuronidation by human liver microsomes or recombinant human UGTs are performed by fitting untransformed experimental data to the following kinetic models using Enzfitter (Biosoft, Cambridge, UK):

The Michaelis-Menten equation;

$$v = \frac{V_{\max} \times [S]}{K_m + [S]} \qquad \text{Equation 1}$$

where v is the rate of reaction, V_{max} is the maximum velocity, K_m is the Michaelis constant (substrate concentration at 0.5 V_{max}), and [S] is the substrate concentration.

Substrate inhibition model (Houston and Kenworthy 2000),

$$v = \frac{V_{\max}}{1 + (K_m / [S]) + ([S] / K_{si})} \qquad \text{Equation 2}$$

where K_{si} is the constant describing the substrate inhibition interaction.

The Hill equation, which describes sigmoidal kinetics (Houston and Kenworthy 2000),

$$v = \frac{V_{\max} \times [S]^n}{S_{50}{}^n + [S]^n} \qquad \text{Equation 3}$$

where substrate concentration resulting in 50% of V_{max} (S_{50}) is analogous to the K_m parameter in Michaelis-Menten equation, and n is the Hill coefficient.

To determine the inhibitor constants (K_i) of selected compound, Dixon plots (which included four or five inhibitor concentrations at each of three substrate concentrations) are constructed. Apparent K_i values are then estimated by fitting experimental data to the expressions for following models using Enzfitter (Biosoft, Cambridge, UK).

Competitive inhibition model (Palmer 1995);

$$v = \frac{V_{\max} \times [S]}{\left(1 + \frac{[I]}{K_i}\right) K_m + [S]} \qquad \text{Equation 4}$$

Non-competitive inhibition model (Palmer 1995);

$$v = \frac{V_{\max} \times [S]}{\left(1 + \frac{[I]}{K_i}\right) K_m + \left(1 + \frac{[I]}{K_i}\right) [S]} \qquad \text{Equation 5}$$

Mixed inhibition model (Palmer 1995);

$$v = \frac{V_{\max} \times [S]}{\left(1 + \frac{[I]}{K_i}\right) K_m + \left(1 + \frac{[I]}{K_i'}\right) [S]} \qquad \text{Equation 6}$$

where [I] is the inhibitor concentration. In the case of mixed inhibition, K_i and K_i' are the dissociation constant of inhibitor to the enzyme and enzyme-substrate complex, respectively. Mixed inhibition is

termed 'competitive-noncompetitive inhibition' (if $K_i < K_i'$) or 'noncompetitive –uncompetitive inhibition' (if $K_i > K_i'$) (Palmer 1995).

Goodness of fit to kinetic and inhibition models is assessed from the F statistic, r^2 values, parameter standard error estimates, and 95% confidence intervals. Kinetic constants are reported as the value ± standard error of the parameter estimate.

4.5 Quantitative prediction of the lorazepam-valproic acid interaction

The extent of inhibition of lorazepam hepatic clearance by valproic acid (determined as the ratio of the areas under the plasma lorazepam concentration – time curves with and without valproic acid co – administration, $R = AUC_{(+fvalproic\ acid)}/AUC_{(control)}$) is predicted using the equation for oral administration of a low hepatic clearance drug (Ito et al. 1998);

$$R = \frac{1}{\left(f_h \times f_m \times \left[\frac{1}{1 + \left(I_u / K_i \right)} \right] \right) + 1 - \left(f_h \times f_m \right)} \qquad \text{Equation 7}$$

where I_u is the unbound valproic acid concentration at the enzyme active site. The f_h and f_m values can be estimated from the urinary recovery of the parent compound and each metabolite, while K_i values can be evaluated by *in vitro* kinetic analysis using human liver microsomes and/or recombinant enzymes.

5. RESULTS AND DISCUSSION

5.1 Quantification of lorazepam glucuronides using HPLC

The analytical condition was modified from the previous method (Franzelius and Besserer 1993). HPLC was performed using an Agilent 1100 series instrument (Agilent Technologies, Sydney, NSW, Australia) fitted with a Zorbax Eclipse XBD-C8 analytical column (4.6 x 150 mm, 5 um; Agilent Technologies). Analytes were separated using a linear gradient with flow rate of 1 ml/min. Initial conditions were 87.5% of 10 mM sodium phosphate buffer (pH 7) (mobile phase A) and 12.5% acetonitrile (mobile phase B). The proportion of mobile phase B was increased to 30% over 20 min and then continued for 7 min. Column eluant was monitored by UV absorbance 230 nm. Under these conditions, retention times of LZPG1, LZPG2 and lorazepam (LZP) were 12.3, 13.4 and 26.3 min, respectively (Figure 1). LZPG1 and LZPG2 were quantified by comparison of peak areas to those of a LZP external standard curve prepared over the concentration range 0.5-5 µM (Figure 2). Within-day overall assay reproducibility was assessed by measuring LZPG1, LZPG2 formation in 9 separate incubations of the same batch of pooled HLM (from HL 7, 10, 12, 13 and 40). Coefficients of variation were less than 2% for added lorazepam concentrations of 25 and 400 µM.

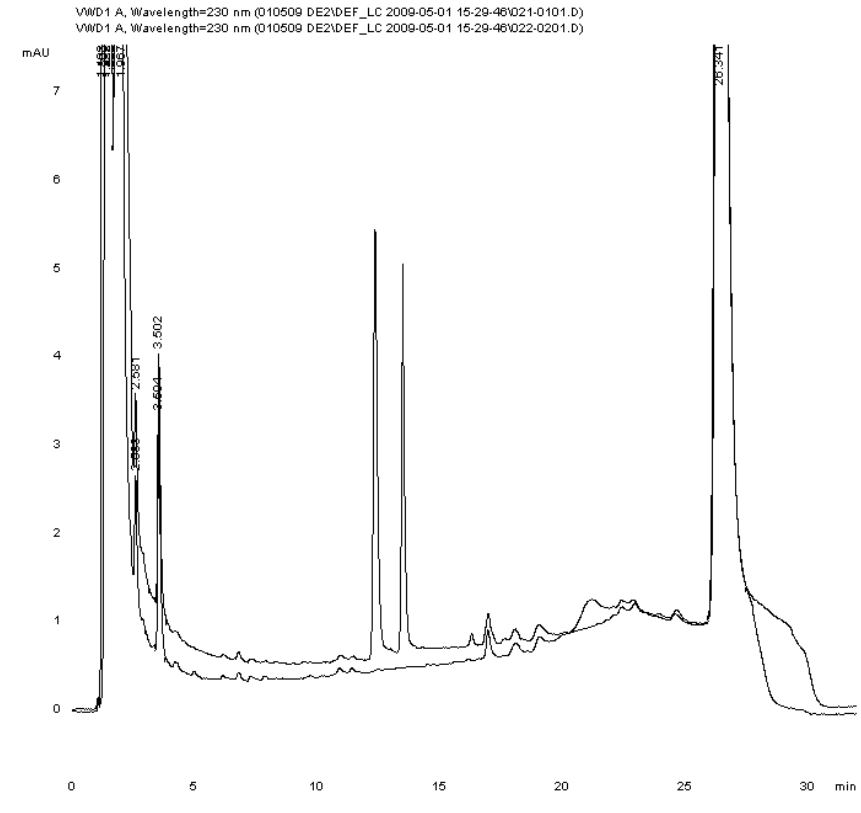

Figure 1 HPLC chromatograms of lorazepam (LZP) and lorazepam glucuronides (LZPG1 and LZPG2)

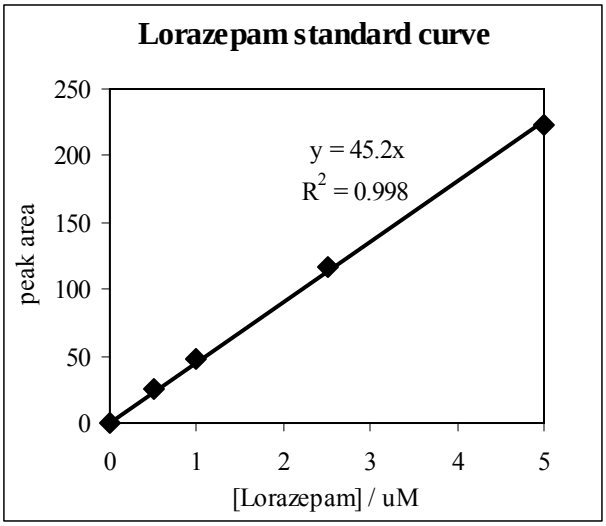

Figure 2 Lorazepam standard curve

5.2 Identification of R- and S-lorazepam glucuronides using *β*-glucuronidase enzyme

The identity of LZPG1 and LZPG2 was confirmed by enzymatic hydrolysis. A 0.2 ml aliquot of the LZP glucuronidation incubation (see section 2.2.4) was terminated with 70% $HClO_4$ (2 µl) and centrifuged. The aqueous sample was decanted and mixed with 2.1 ul of 4M KOH (to raise the pH to 6.5) and 25 units (125 units/ml) of *β*-glucuronidase (from *Escherichia coli*). Control were performed in the same fashion but distilled water were added instead of *β*-glucuronidase enzyme. Sample was incubated at 37°C for 0, 2, 4, 10, 120 min, 19 and 92 hour. After incubation, a 150 µl aliquot was separated and treated with 70% $HClO_4$ (1.5 µl). Following centrifugation (5000*g* for 10 min), 10 µl of the supernatant fraction from each reaction was injected into the HPLC column. As shown in Table 1, the treatment resulted in loss of 50% of the LZPG2 peak area within 10 min and the peak was undetectable at 2 hr incubation. However, the LZPG1 was resistance to cleave by *β*-glucuronidase at 125 units /ml. The LZPG1 and LZPG2 peak area in the control were not changed during the time course of incubation.

It has been reported by Ruelius et al. (Drug metabolism and disposition 1979) that *β*-glucuronidase from *Escherichia coli* hydrolyzed the S-diastereoisomer of benzodiazepine glucuronide 400 times faster than R- diastereoisomer. Given this information, enzyme hydrolysis experiment (see above) was performed by increasing the amount of *β*-glucuronidase (from *Escherichia coli*) from 125 units/ml to 5,000 units/ml. As shown in Table 2, the treatment resulted in the negligible of LZPG1 and LZPG2 peak at 24 and 2 hr, respectively. The result indicates that LZPG1 was resistance to cleave by *β*-glucuronidase enzyme (from *Escherichia coli*) when compare to LZPG2.

In conclusion, the HPLC chromatogram at retention time 12.3 and 13.4 min were both lorazepam glucuronides. Due to the different rate of enzyme hydrolysis, it can be concluded that LZPG1 and LZPG2 were R- and S- diastereoisomer of lorazepam glucuronide metabolites, respectively.

Table 1 Hydrolysis of lorazepam glucuronides by *β*-glucuronidase enzyme (125 units/ml)

Enzyme hydrolysis (Beta-glucuronidase 125 units/ml)				
Time	LZPG1 (RT 12.2 min)		LZPG2 (RT 13.4 min)	
	(Peak area)	% peak area	(Peak area)	% peak area
0 min	47.1	100	45.5	100
2 min	48.6	103	40.3	89
4 min	47.6	101	34.6	76
10 min	48.2	102	22.6	50
120 min	47.6	101	0	0
19 hr	48.0	102	0	0
92 hr	48.7	103	0	0
Control				
Time	LZPG1 (RT 12.2 min)		LZPG2 (RT 13.4 min)	
	(Peak area)	% peak area	(Peak area)	% peak area
0 min	46.2	100	45.8	100
2 min	46.6	101	46.3	101
4 min	46.1	100	45.9	100
10 min	46	100	46.2	101
120 min	46.3	100	46.1	101
19 hr	49.6	107	50	109
92 hr	57.9	125	58.6	128

Table 2 Hydrolysis of lorazepam glucuronides by *β*-glucuronidase enzyme (5,000 units/ml)

Enzyme hydrolysis (Beta-glucuronidase 5,000 units/ml)				
Time	LZPG1 (RT 12.2 min)		LZPG2 (RT 13.4 min)	
	(Peak area)	% peak area	(Peak area)	% peak area
0 hr	31.4	100	38.5	100
2 hr	23.9	76	0	0
24 hr	1.4	4	0	0
Control				
Time	LZPG1 (RT 12.2 min)		LZPG2 (RT 13.4 min)	
	(Peak area)	% peak area	(Peak area)	% peak area
0 hr	39.9	100	47.7	100
2 hr	39.5	99	47.7	100
24 hr	45	113	53	111

5.3 In vitro assay of lorazepam glucuronide formation

Lorazepam glucuronide formation *in vitro* is measured using a modification of the method for zidovudine glucuronide measurements (Boase and Miners 2002, Uchaipichat et al. 2006). Incubation mixtures, in a total volume 0.2 ml, contain phosphate buffer (0.1 M, pH 7.4), $MgCl_2$ (4 mM), UDP-glucuronic acid (5 mM), lorazepam (LZP), and alamethicin-activated HLM. The screening kinetic was performed at lorazepam concentrations of 25, 50, 100, 200, 400 and 600 uM and at the protein concentration of 0.5 mg/ml. Reactions were initiated by the addition of UDP-glucuronic acid and performed at 37°C in a shaking water bath for 120 min. The reaction were terminated with 70% $HClO_4$ (2 µl) and centrifuged at 5000*g* for 10 minute at 10°C. A 20 µl aliquot of the supernatant fraction was injected into the HPLC column. The screening kinetic results presented as rate versus substrate concentration plots and Eadie-Hofstee plots were illustrated in Figure 3. The R- and S- lorazepam glucuronide formation were followed the substrate inhibition kinetic. The respective substrate dissociation constants (Ks) were 86 and 82 uM.

According to the kinetic screening results, low (25 uM) and high (400 uM) lorazepam concentrations were selected to examine the protein and time linearity. Rates of R- and S-lorazepam glucuronide formation are optimized for linearity with respect to incubation time and protein concentration as shown in Figure 4 and 5, respectively. At 90 min incubation time, rate of both metabolite formation at 25 and 400 uM of lorazepam were linear with respect to protein concentrations ranging from 0.25-1.25 mg/ml. At protein concentration of 1.0 mg/ml, rate of R- and S-lorazepam glucuronide formation from both lorazepam concentrations were linear with respect to incubation time range from 30 to 150 min. Incubation condition at protein concentration of 1.0 mg/ml and incubation time 150 min were selected to perform the kinetic study.

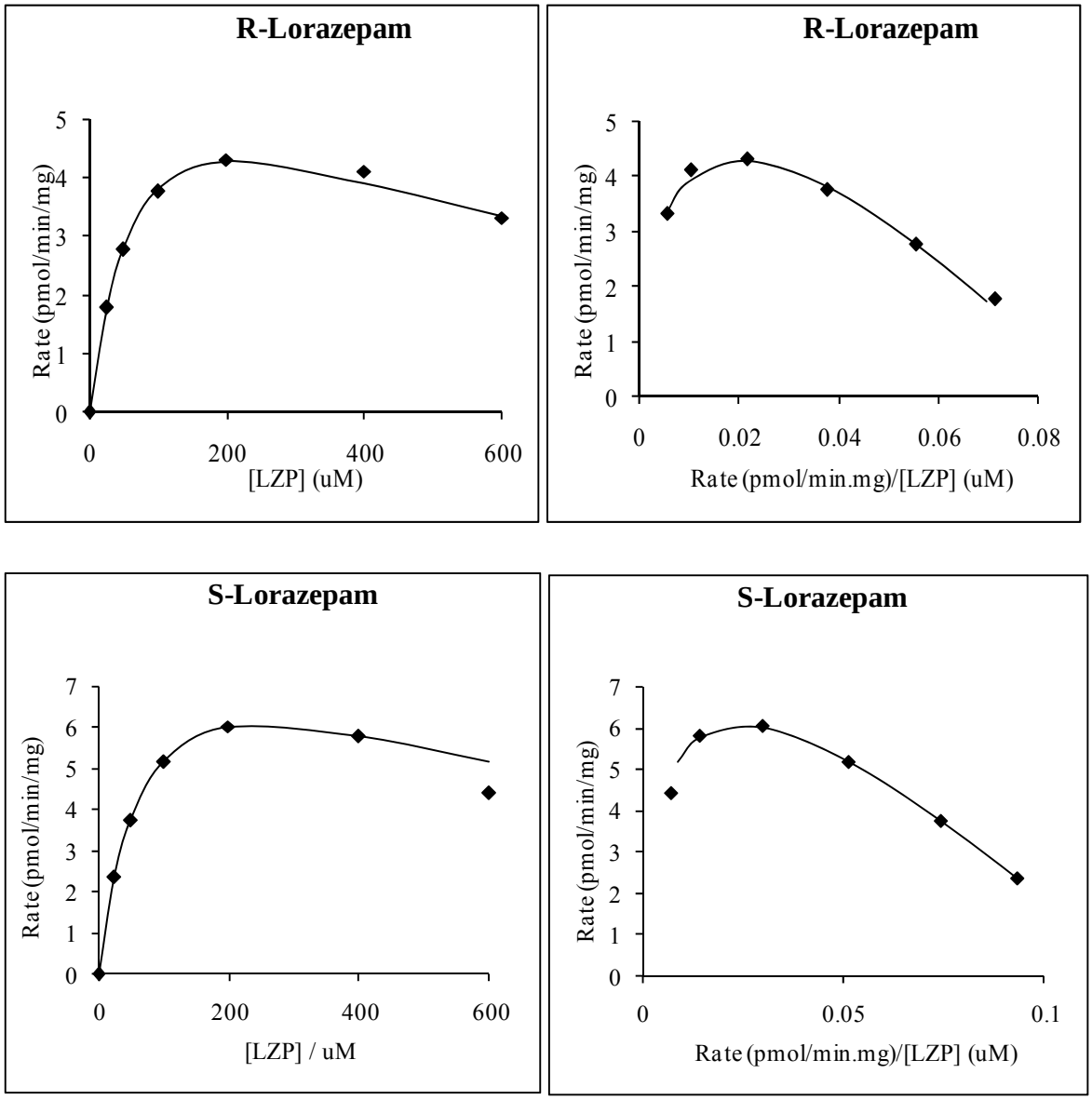

Figure 3 Rate (v) versus [S] plots (left column) and Eadie-Hofstee plots (right column) for lorazepam glucuronidation by pooled HLMs.

Points are experimentally determined values while the solid lines are the computer-generated curves of best fit.

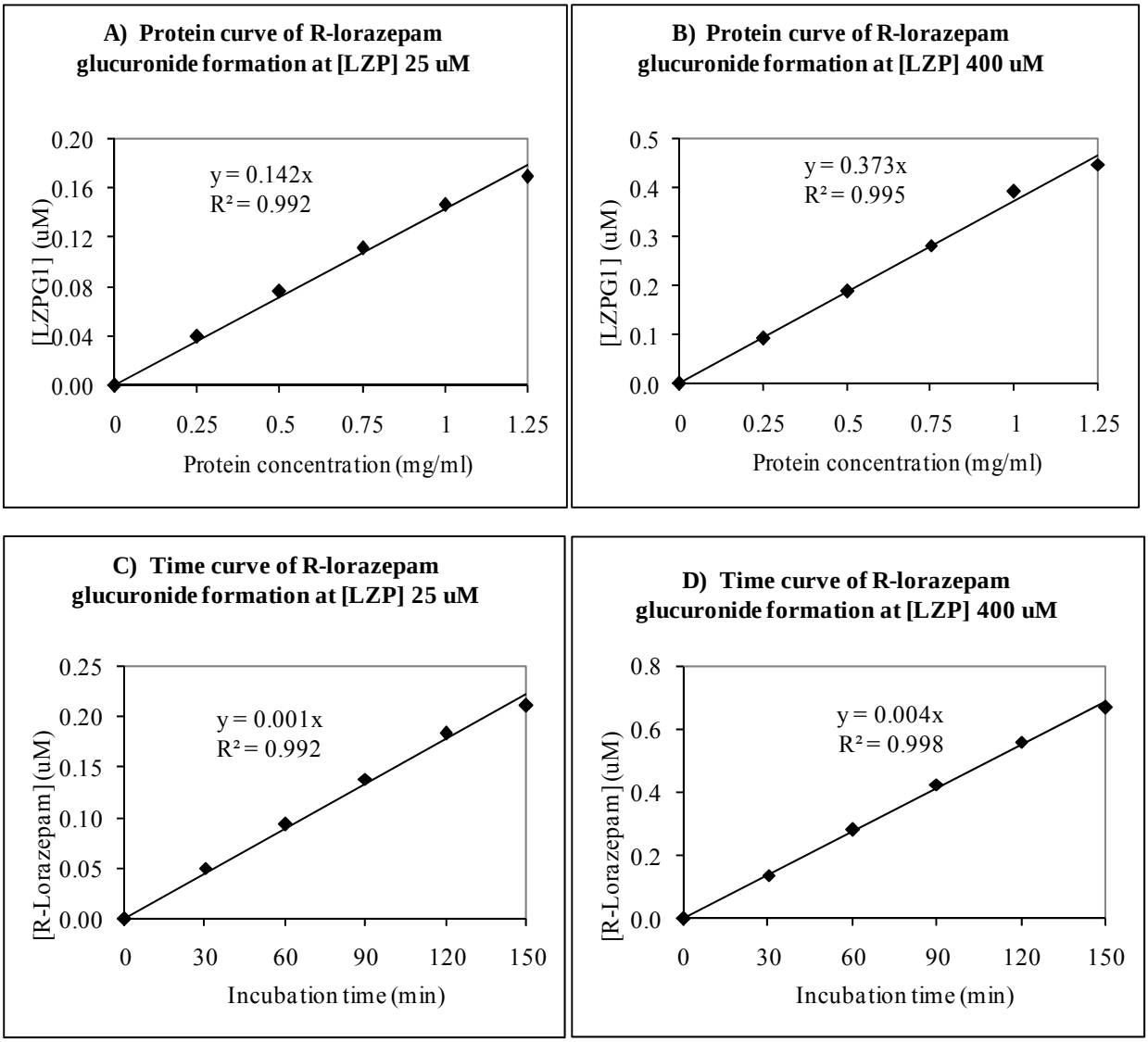

Figure 4 Relationships between R-lorazepam glucuronide formation by pooled human liver microsome and protein concentration and incubation time

Panel A and B: Lorazepam (25 and 400 μM) incubated with pooled human liver microsome (0.25-1.25 mg/ml) for 90 min.
Panel C and D: Lorazepam (25 and 400 μM) incubated with pooled human liver microsome (1.0 mg/ml) for 30-150 min.

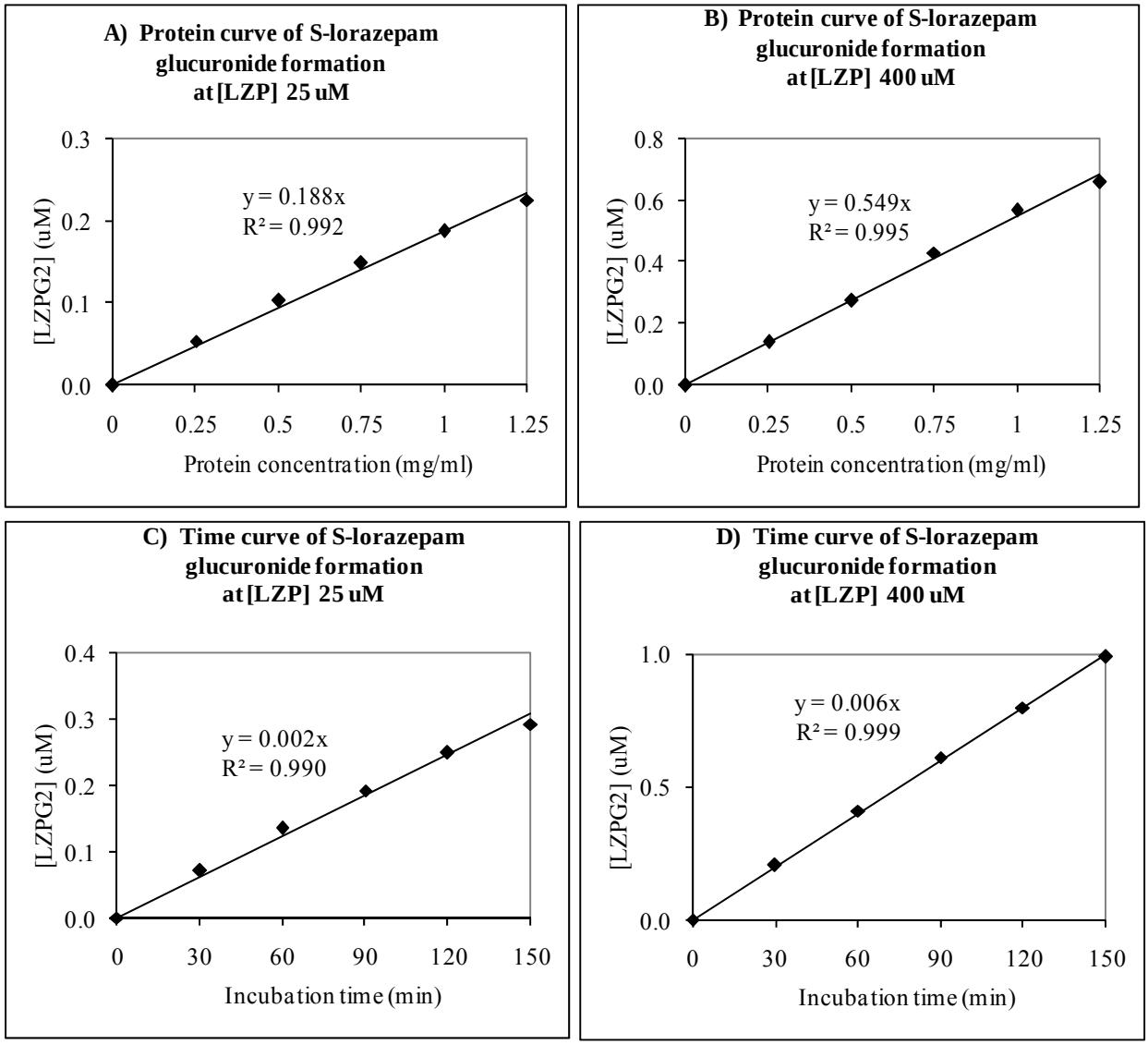

Figure 5 Relationships between S-lorazepam glucuronide formation by pooled human liver microsome and protein concentration and incubation time

Panel A and B: Lorazepam (25 and 400 μM) incubated with pooled human liver microsome (0.25-1.25 mg/ml) for 90 min.
Panel C and D: Lorazepam (25 and 400 μM) incubated with pooled human liver microsome (1.0 mg/ml) for 30-150 min.

5.4 Binding of lorazepam to HLM

The binding of lorazepam to HLM was characterised by equilibrium dialysis as described by McLure et al. (2002). One side of dialysis cell contained lorazepam (10, 50, 100, 300 or 500 µM) in phosphate buffer (0.1M, pH 7.4), while the other side contained a suspension of pooled HLM (1 mg/ml). The dialysis cell assembly was immersed in a water bath maintained at 37°C and rotated at 12 rpm for 4 h. A 200 µl aliquot was collected from each cell and treated with 200 µl of ice-cold methanol containing 4% glacial acid. Samples were chilled on ice for 20 min and subsequently centrifuged at 5000*g* for 10 min at 10 °C. A 10 µl aliquot of the supernatant fraction was analysed by HPLC. The HPLC system and conditions were essentially as described previously for the measurement of R- and S-lorazepam glucuronides (Section 5.1), except that the mobile phase (60% mobile phase A / 40% mobile phase B) was delivered isocratically at a flow rate of 1 ml/min. Under these conditions, lorazepam eluted at 4.5 min. Lorazepam standards in the concentration range 1-250 µM were prepared in phosphate buffer (100 mM, pH 7.4) alone or in the combination of phosphate buffer with pooled human liver microsomes, and treated in the same manner as dialysis samples. The lorazepam concentrations of dialysis samples were determined by comparison of peak areas with those of a standard curve.

The fraction of lorazepam unbound in incubations (fu_{inc}) was calculated as the drug concentration in the buffer compartment divided by the drug concentration in the protein compartment. The binding of lorazepam to HLM was 28%, which was independent to lorazepam concentration. To determine kinetic constants of R- and S- lorazepam glucuronide formation, the concentration of lorazepam was corrected with fu_{inc} value.

5.5 Kinetic of lorazepam glucuronidation by human liver microsomes

Kinetic plots for R- and S-lorazepam glucuronide formation by individual livers (n=5) are shown in Figure 6 and 7, respectively and derived kinetic constants were given in Table 3. R- and S-lorazepam glucuronidation kinetics by HLMs exhibited substrate inhibition (Figure 6 and 7). Mean derived K_m and V_{max} values for R- and S- lorazepam glucuronidation were 29 ± 8.5 µM and 7±1.8 pmol/min.mg, and 42 ± 19.7 µM and 11 ± 4.5 pmol/min.mg, respectively. Substrate inhibition with both metabolites was weak (K_m 8- to 14-fold lower than K_{si}). Similar kinetic model was previously reported for glucuronidation kinetic of other benzodiazepine, S-oxazepam (Court et al., 2002).

Mean ± SD of R- and S- lorazepam glucuronidation kinetics by pooled HLMs, prepared by mixing equal protein amounts of microsomes from the 5 separate livers, exhibited substrate inhibition model as shown in Figure 8. Kinetic constants and microsomal CL_{int} for R- and S-lorazepam glucuronidation by pooled HLM were comparable to the mean data obtained for the separate livers (Table 3). Thus, further experiment to investigate the magnitude of inhibitory interaction between lorazepam and valproic acid will be employed by using pooled HLM as the enzyme source. The CL_{int} for R- and S- lorazepam glucuronidation by pooled HLM are similar (0.21 and 0.27 µl/min.mg protein, respectively). This

indicated that the rate of elimination via glucuronidation pathway in liver for both stereoisomer *in vivo* are likely similar.

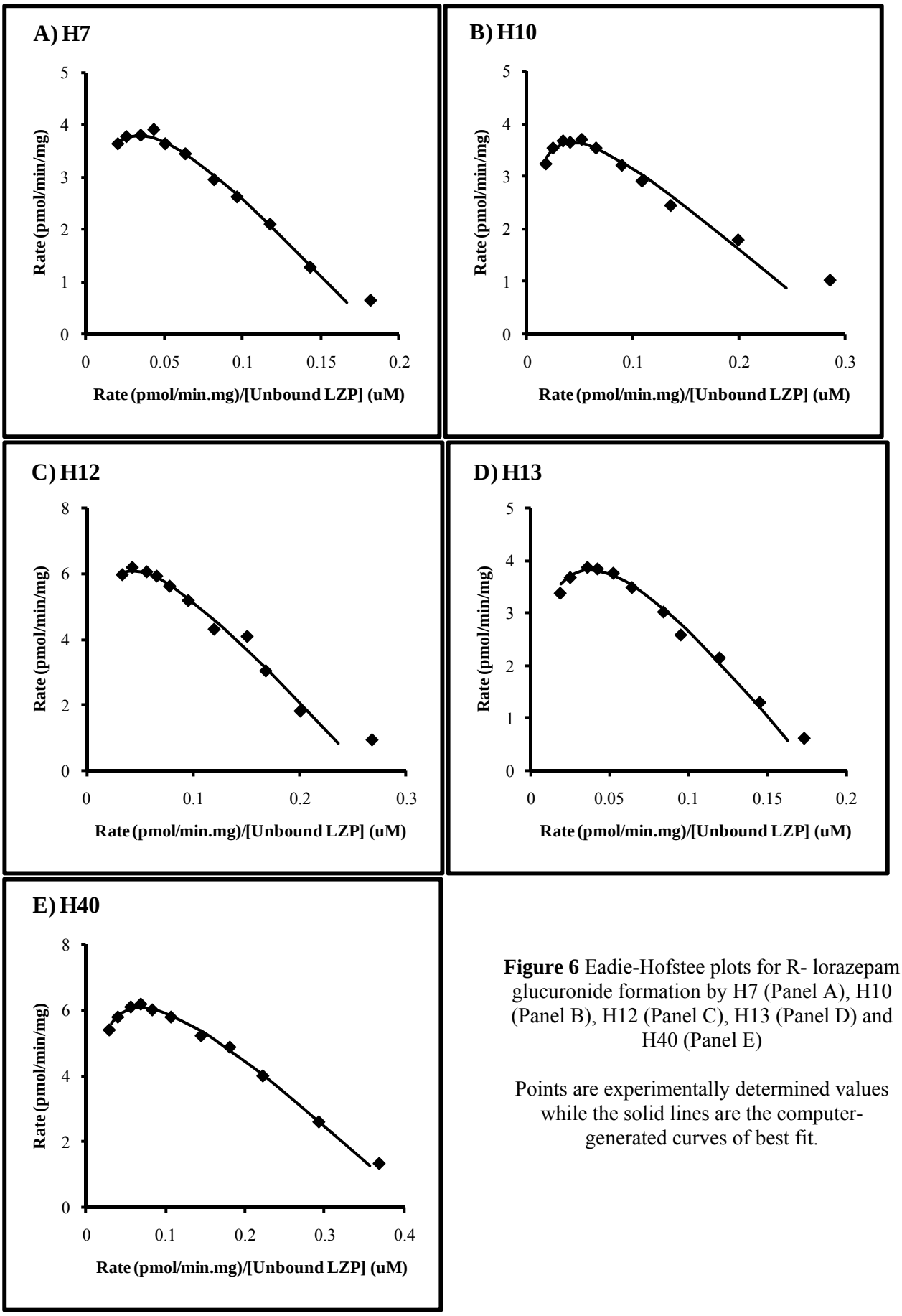

Figure 6 Eadie-Hofstee plots for R- lorazepam glucuronide formation by H7 (Panel A), H10 (Panel B), H12 (Panel C), H13 (Panel D) and H40 (Panel E)

Points are experimentally determined values while the solid lines are the computer-generated curves of best fit.

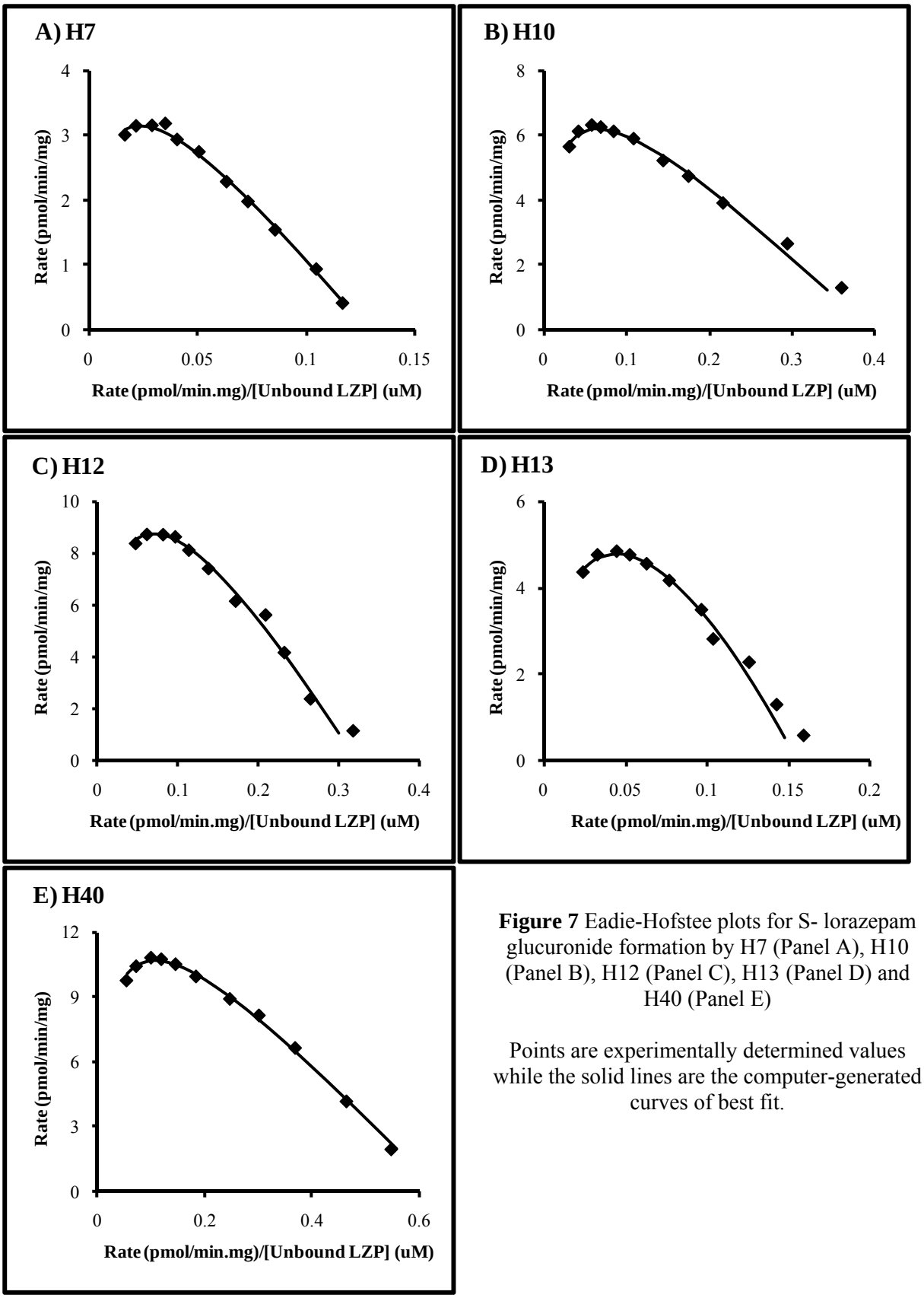

Figure 7 Eadie-Hofstee plots for S- lorazepam glucuronide formation by H7 (Panel A), H10 (Panel B), H12 (Panel C), H13 (Panel D) and H40 (Panel E)

Points are experimentally determined values while the solid lines are the computer-generated curves of best fit.

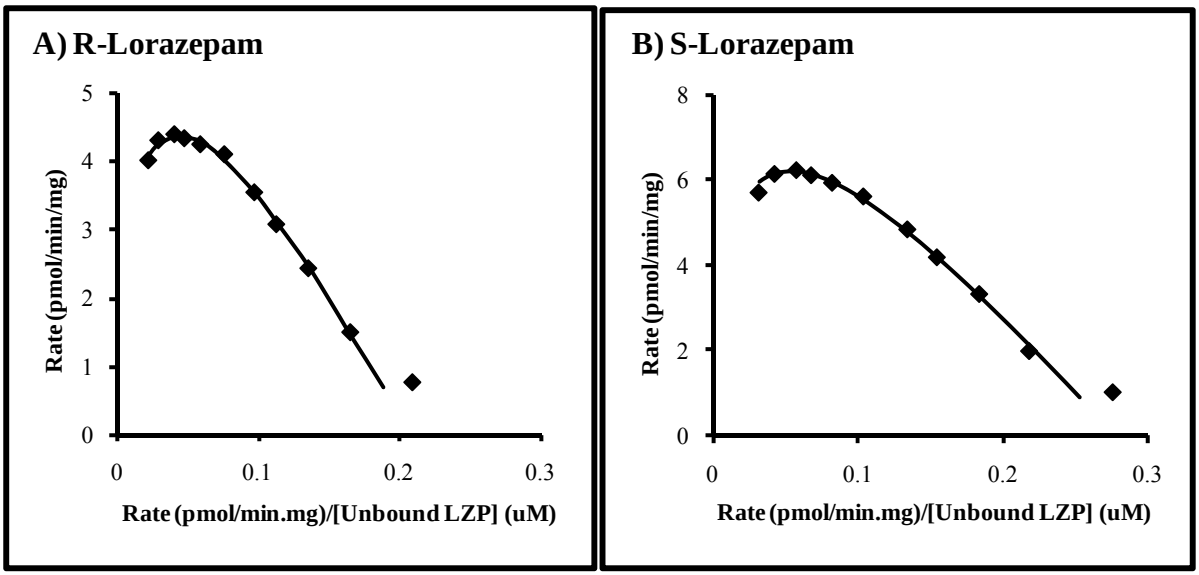

Figure 8 Eadie-Hofstee plots for R- and S- lorazepam glucuronide formation (Panel A and B) by pooled human liver microsomes

Points are experimentally determined values while the solid lines are the computer-generated curves of best fit

Table 3 Kinetic parameters of lorazepam glucuronidation by individual and pooled human liver microsomes (n=5)[a,b]

	R-Lorazepam				S-Lorazepam			
	K_m (µM)	**V_{max} (pmol/min/mg protein)**	**K_{si} (µM)**	**CL_{int}[c] (ul/min/mg)**	**K_m (µM)**	**V_{max} (pmol/min/mg protein)**	**K_{si} (µM)**	**CL_{int}[c] (ul/min/mg)**
H7	32 ± 0.9	5.9 ± 0.1	408 ± 23	0.18	39 ± 0.04	5 ± 0.01	447 ± 1.2	0.13
H10	17 ± 2	5 ± 0.3	424 ± 93	0.29	24 ± 1.7	9.3 ± 0.3	381 ± 51	0.39
H12	35 ± 3.6	9 ± 0.5	588 ± 155	0.26	48 ± 4.3	15 ± 0.9	322 ± 52	0.31
H13	37 ± 0.1	6.6 ± 0.01	275 ± 0.76	0.18	72 ± 8.3	11 ± 0.9	162 ± 0.3	0.15
H40	22 ± 1.2	9.1 ± 0.3	358 ± 40	0.41	25 ± 0.07	16 ± 0.01	395 ± 2.5	0.64
Mean ± SD	29 ± 8.7	7 ± 1.9	411 ± 115	0.27 ± 0.1	42 ± 20	11 ± 4.5	341 ± 110	0.32 ± 0.2
Pooled HLMs	37 ± 0.6	7.6 ± 0.06	263 ± 6.8	0.21	37 ± 1.4	10 ± 0.2	354 ± 30	0.27

[a] Data presented as mean ± standard error of parameter fit
[b] Data were best fitted to substrate inhibition model
[c] CL_{int} calculated as V_{max}/K_m

5.6 Screening of lorazepam glucuronidation by human UGTs

Twelve recombinant UGTs were screened for lorazepam glucuronidation activity at three substrate concentrations; 10, 50 and 250 μM (Figure 9). UGT 2B4, 2B7 and 2B15 were the only hepatically expressed enzymes that markedly catalysed R-lorazepam glucuronidation. These enzymes also catalyzed S-lorazepam glucuronidation. However, highest activity was associated with UGT2B15. At an S-lorazepam concentration of 250 μM, UGT2B15 activity was about 4-times higher than those observed with UGT 2B4 and 2B7. Thus, it appears that S-lorazepam is a relatively selective substrate for UGT2B15. Previous study also reported that UGT2B15 is the sole enzyme glucuronidated S-lorazepam (Court et al., 2005). The finding highlights the potential of using S-lorazepam as a probe substrate for UGT2B15 in the reaction phenotyping study.

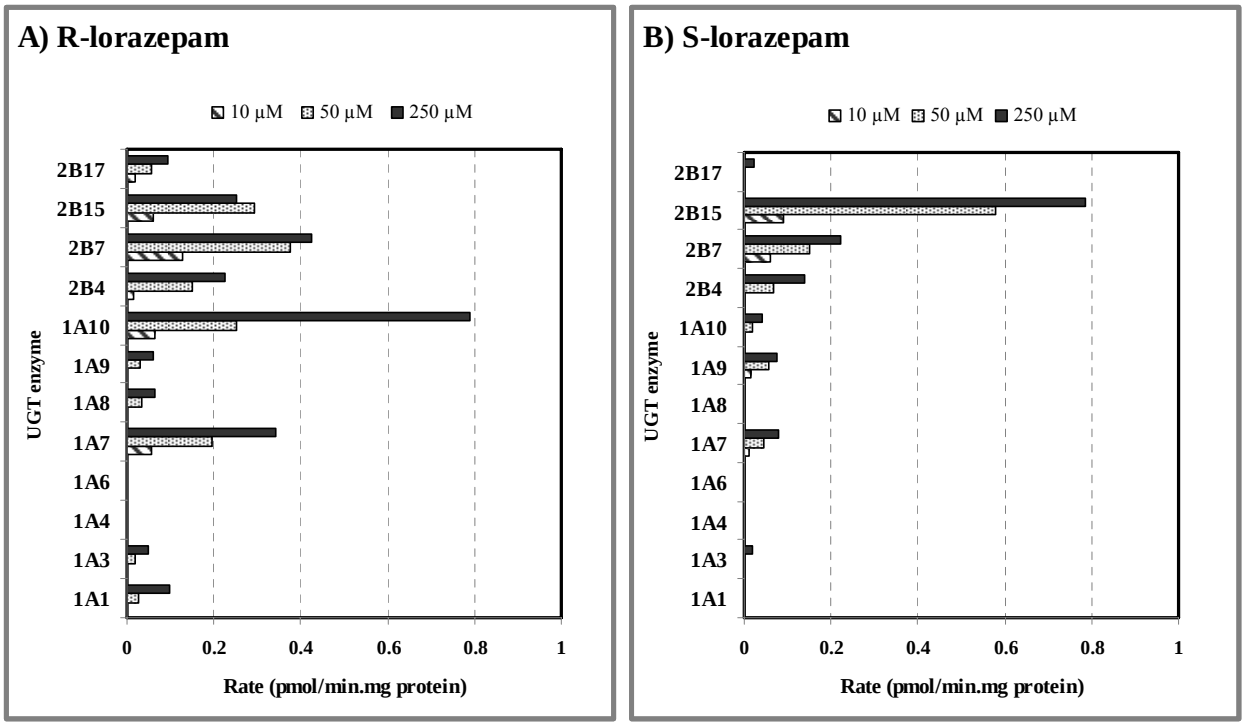

Figure 9 Formation of R- and S- lorazepam glucuronides by recombinant human UDP-glucuronosyltransferases at lorazepam concentration of 10, 50, and 250 μM.

Results represent the means of duplicate estimations

5.7 R- and S- lorazepam glucuronidation kinetics by human UGTs

Further kinetic studies of recombinant UGTs exhibited measurable activities were performed. Kinetic models and parameters of R- and S- lorazepam glucuronidation were shown in Figure 10 and 11, and Table 4 and 5, respectively. R-Lorazepam glucuronidation by UGT 2B4, 2B7 and 2B15 was well described by the Hill, Michaelis-Menten, and substrate inhibition equations, respectively (Figure 10). The kinetic of R-lorazepam glucuronidation by UGT1A7 and 1A10, enzymes which only expressed in gastrointestinal tract, were well described by Michaelis-Menten, and substrate inhibition equations, respectively (Figure 10). In contrast, the kinetics of S-LZPG formation by UGT 2B4, 2B7 and 2B15 were well modelled using the Hill equation (Figure 11). It should be noted that atypical kinetic of glucuronidated drugs (i.e., morphine, codeine and oxazepam) by UGT 2B4, 2B7 and 2B15 has been reported previously (Court et al., 2002, Stone et al., 2003, Raungrut et al., 2009). In particular, the interaction between two-identical binding sites was assumed as the mechanistic explanation for glucuronidation of UGT2B7 substrate exhibiting atypical kinetic (Uchaipichat et al., 2008).

Regarding to kinetic parameters of hepatically expressed UGTs, derived K_m or S_{50} values for UGT 2B4, 2B7 and 2B15 catalyzed R-lorazepam were 43, 13 and 44 μM, while the respective S_{50} values for S-LZP glucuronidation were 46, 17 and 23 μM. Derived K_m or S_{50} values observed with recombinant UGTs and HLM were comparable which suggested that UGT 2B4, 2B7 and 2B15 are all likely to contribute human liver microsomal R and S-lorazepam glucuronidation. This result is consistent with previous evidences showing that UGT2B polymorphism, in particular UGT2B15, is a major determinant of *in vitro* and *in vivo* lorazepam glucuronidation (Court et al. 2004, Chung et al. 2007). However, due to the lack of relative protein expression data, percent contribution of UGT 2B4, 2B7 and 2B15 to lorazepam hepatic clearance cannot be elucidated.

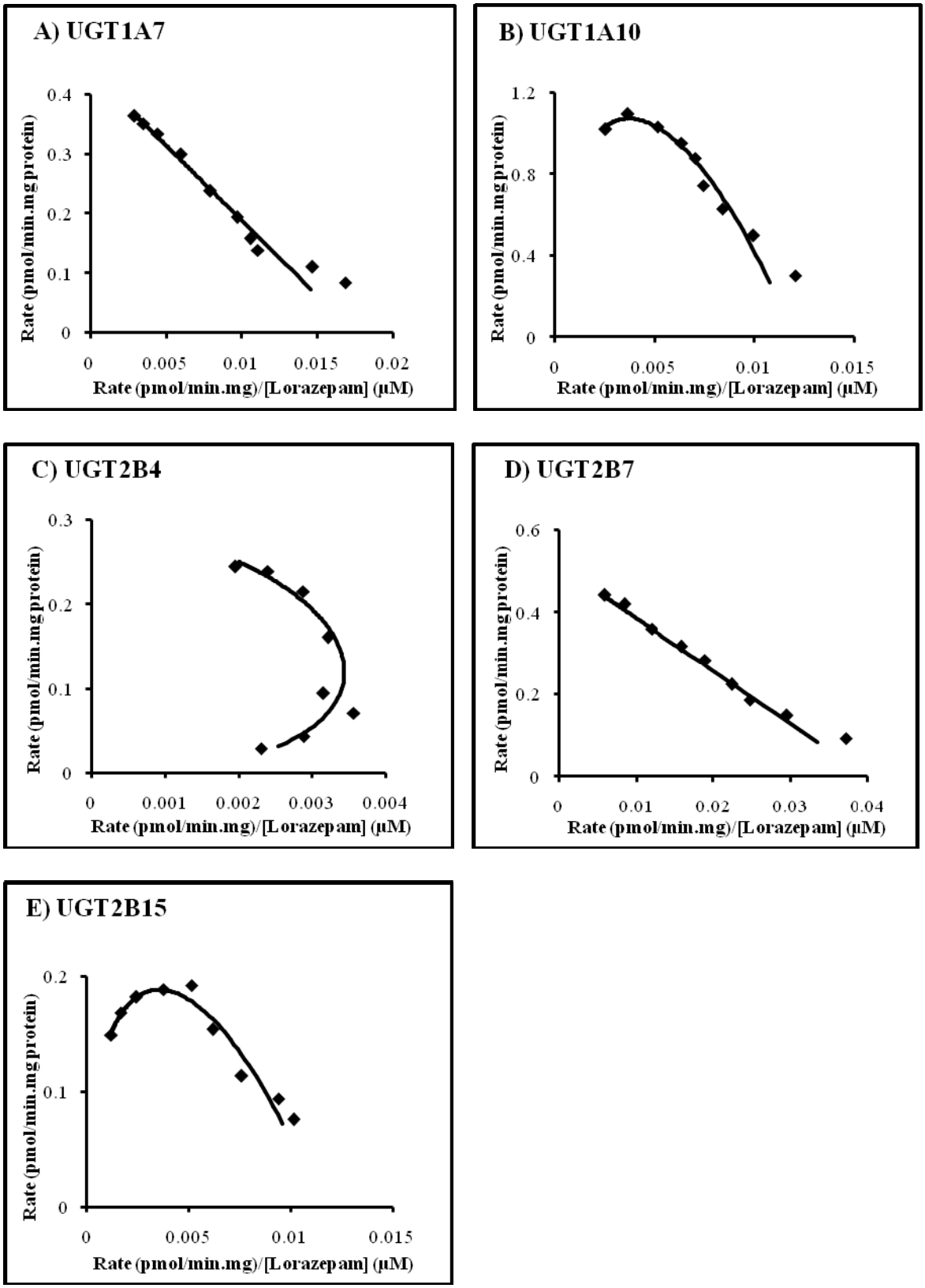

Figure 10 Eadie-Hofstee plots for R-lorazepam glucuronide formation by recombinant human UGT 1A7 (Panel A), 1A10 (Panel B), 2B4 (Panel C), 2B7 (Panel D) and 2B15 (Panel E)

Points are experimentally determined values while the solid lines are the computer-generated curves of best fit.

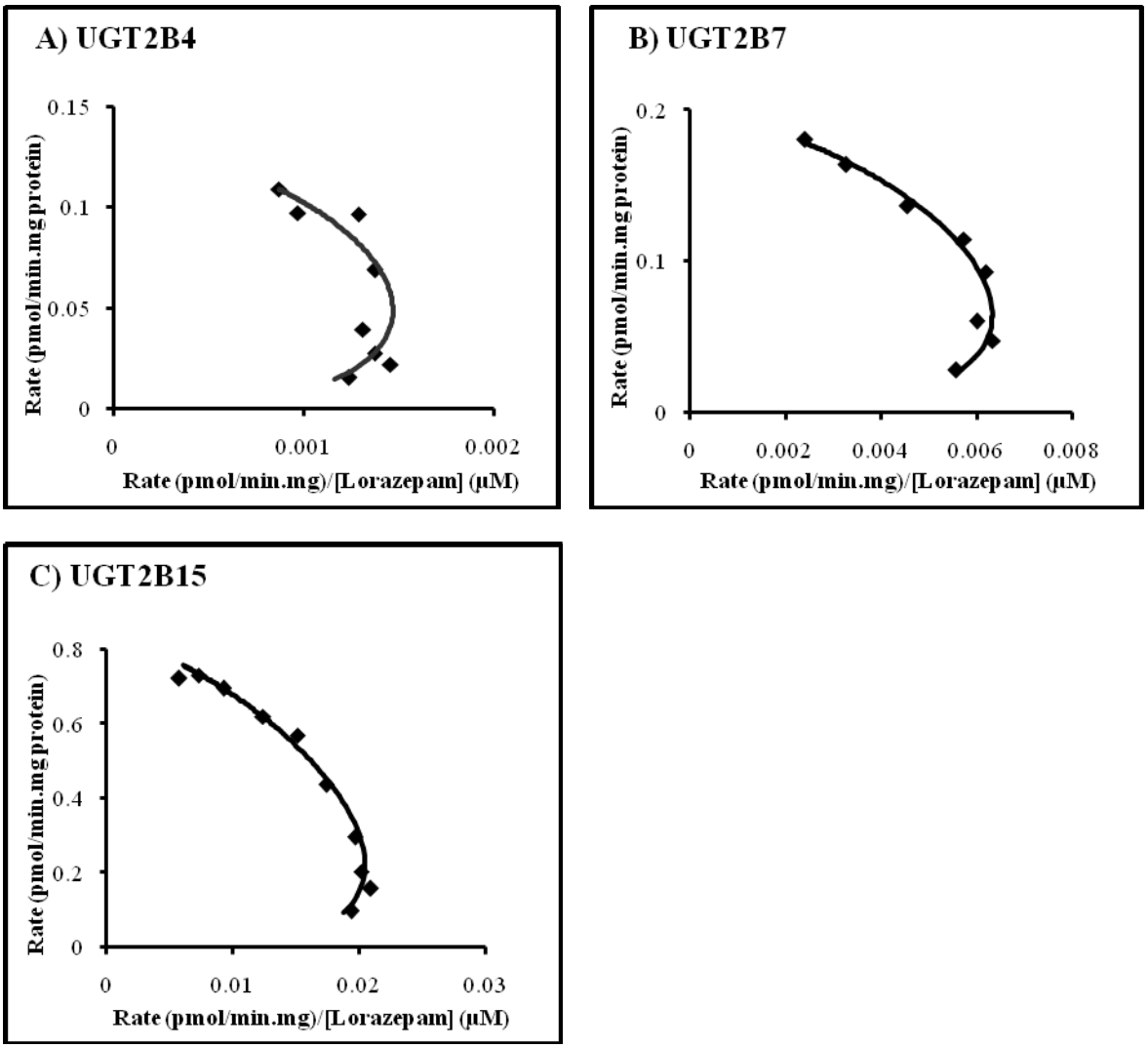

Figure 11 Eadie-Hofstee plots for S-lorazepam glucuronide formation by recombinant human UGT 2B4 (Panel A), 2B7 (Panel B) and 2B15 (Panel C)

Points are experimentally determined values while the solid lines are the computer-generated curves of best fit.

Table 4 Kinetic models and constants generated for R-lorazepam glucuronidation by human UGTs [a]

UGT	Kinetic models	K_m or S_{50} (μM)	K_{si} (μM)	V_{max} (pmol/min.mg protein)
1A7	Michaelis-Menten	25 ± 1		0.44 ± 0.01
1A10	Substrate inhibition	236 ± 59	350 ± 121	2.8 ± 0.5
2B4[b]	Hill equation	43 ± 4.2		0.29 ± 0.02
2B7	Michaelis-Menten	13 ± 0.05		0.52 ± 0.01
2B15	Substrate inhibition	44 ± 8.5	65 ± 15	0.5 ± 0.07

[a] Data presented as mean ± standard error of parameter fit
[b] n = 1.7 ± 0.2 from Hill equation

Table 5 Kinetic models and constants generated for S-lorazepam glucuronidation by human UGTs [a]

UGT	Kinetic models	S_{50} (μM)	n	V_{max} (pmol/min.mg protein)
2B4[b]	Hill equation	46 ± 8	1.6 ± 0.2	0.13 ± 0.01
2B7	Hill equation	17 ± 0.7	1.5 ± 0.06	0.20 ± 0.01
2B15	Hill equation	23 ± 0.8	1.4 ± 0.04	0.83 ± 0.01

[a] Data presented as mean ± standard error of parameter fit

5.8 Inhibition of valproic acid on R- ans S-lorazepam glucuronidation kinetics

Using R- and S- lorazepam at K_m concentration, valproic acid inhibition was screened at concentrations of 100, 500, 1000, 2500, 5000 and 7500 μM. As shown in Figure 12, inhibition of valproic acid on R- and S-lorazepam glucuronidation is in a concentration-dependent manner with respective IC_{50} values of 3,575 ± 32and 3,200 ± 19 μM. To determine the inhibitor constants (K_i), Dixon plots which included four or five valproic acid concentrations at each of three R- or S- lorazepam concentrations are constructed. Using pooled human liver microsomes, valproic acid non-competitively inhibited R- and S- lorazepam glucuronidation with K_i values of 3,900 and 3,200 μM, respectively (Figure 13). The extent of inhibition of lorazepam hepatic clearance by valproic acid (determined as the ratio of the areas under the plasma lorazepam concentration – time curves with and without valproic acid co – administration, $R = AUC_{(+fvalproic\ acid)}/AUC_{(control)}$) is predicted using Equation 7 for oral administration of a low hepatic clearance drug (Ito et al. 1998). The predicted AUC increased of lorazepam when co-administered with valproic acid is about 20% which is comparable to *in vivo* data reported previously (Samara et al., 1997).

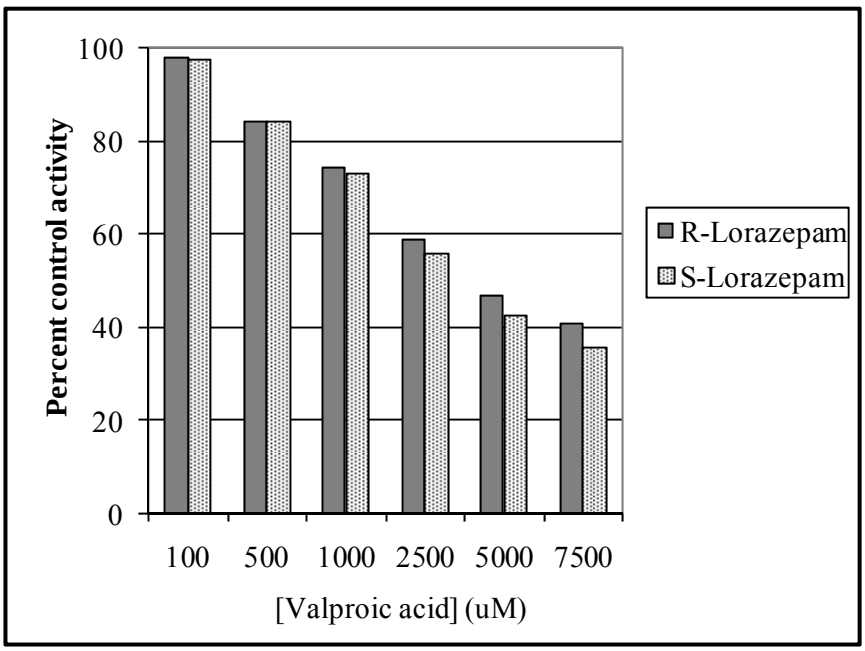

Figure 12 Inhibition of valproic acid on R- and S- lorazepam glucuronidation by using pooled human liver microsomes (n=5)

Lorazepam was used at concentration of 100 μM. Each bar represents the mean percentage activity relative to control from duplicate measurements.

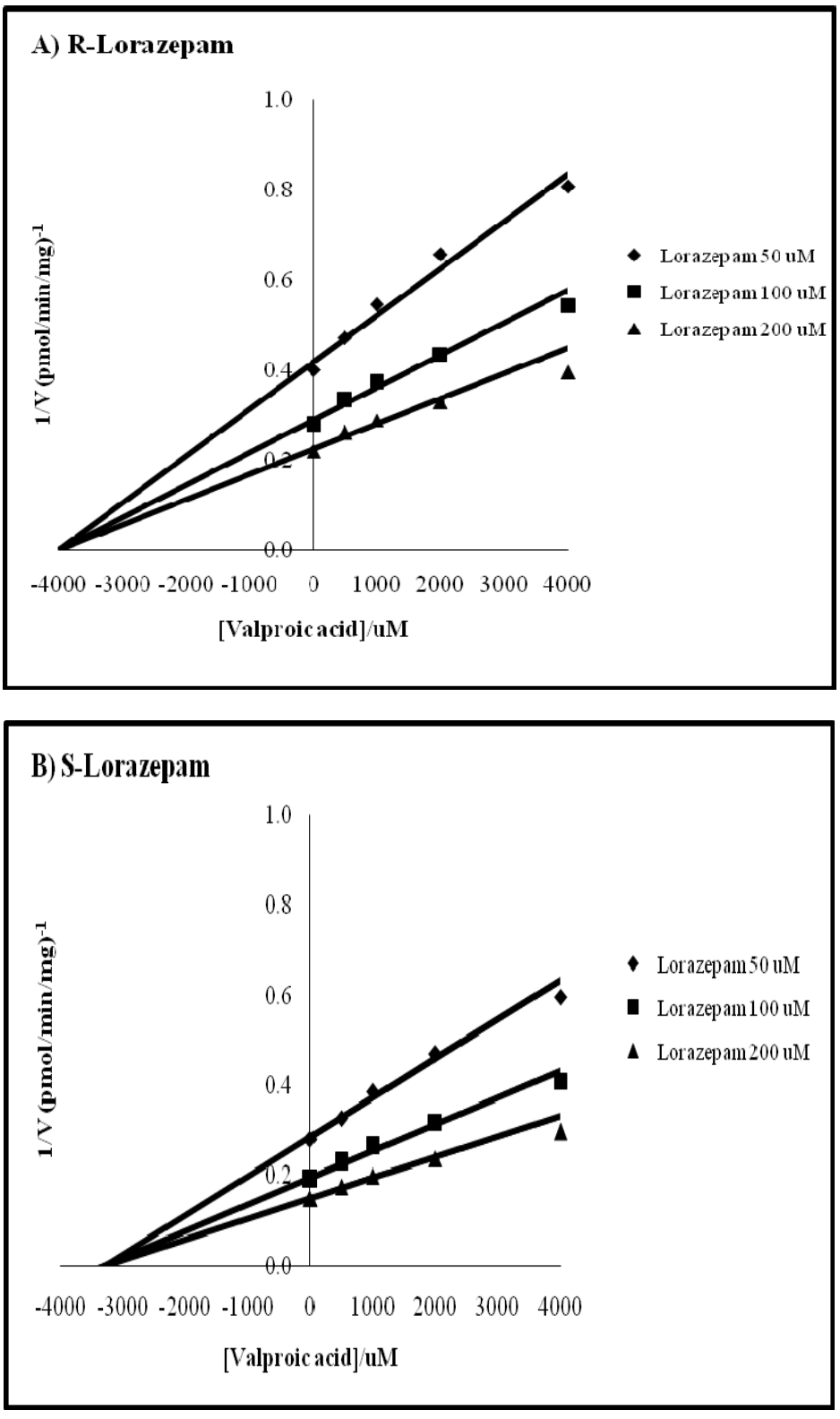

Figure 13 Dixon plots of valproic acid inhibition on R- and S- lorazepam glucuronidation (Panel A and B) by using pooled human liver microsomes (n=5)

Points are experimentally determined values while the solid lines are the computer generated curves of best fit.

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RESEARCH OUTPUT

1. Poster presentation การประชุม นักวิจัยรุ่นใหม่พบเมธีวิจัยอาวุโส สกว./โรงแรมฮอลิเดย์อินน์ รีสอร์ท รีเจนท์ บีช ชะอำ จังหวัดเพชรบุรี 15-17 ตุลาคม 2552

Characterization of lorazepam glucuronidation kinetics using human liver microsomes and human UDP-glucuronosyltransferases

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Abstract

The anxiolytic agent lorazepam (LZP) are mainly eliminated in humans by glucuronidation. This study aimed to characterize the glucuronidation kinetics of (R,S)-LZP by human liver microsomes (HLMs) and to screen the human UDP-glucuronosyltransferase (UGT) enzyme activities toward lorazepam. Formation of the glucuronide metabolites (R-LZPG and S-LZPG) were quantified by HPLC. Recombinant human UGT enzymes were expressed in HEK293 or baculovirus-insect cells. Kinetics of R- and S-LZP glucuronidation by HLMs (n=4) exhibited substrate inhibition. Mean derived binding affinity (K_s) and V_{max} of R- and S-LZPG kinetics were 36.7 ± 11.4 μM and 7.3 ± 2.1 pmol/min.mg, and 50.3 ± 18.2 μM and 11.7 ± 5.2 pmol/min.mg, respectively. Substrate inhibition with both metabolites was weak (K_s 10- to 17-fold lower than K_{si}). Twelve recombinant UGTs were screened for LZP glucuronidation activity at LZP concentrations of 10, 50 and 250 μM. UGT 2B4, 2B7 and 2B15 catalyzed S-lorazepam, but the highest activity is observed for UGT2B15. R-lorazepam was catalyzed by UGT 2B4, 2B7 and 2B15. Notably, UGT 1A7 and 1A10 which are expressed in gastrointestinal tract metabolized only R-lorazepam. To identify UGTs responsible for lorazepam glucuronidation in the liver, the kinetic study of hepatically UGTs which exhibited measurable activities will be further investigated.

Keywords: lorazepam, glucuronidation, UDP-glucuronosyltransferase

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2. Poster presentation The 9th International Meeting of International Society for the Study of Xenobiotics; Istanbul, Turkey September 4-8 2010

Kinetic and enzymatic characterization of human hepatic R- and S- lorazepam glucuronidation

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The hypnosedative-anxiolytic agent lorazepam (LZP) is eliminated in humans by glucuronidation. This study aimed to characterize the glucuronidation kinetics of R- and S-LZP by human liver microsomes (HLM) and identify the human UDP-glucuronosyltransferase (UGT) enzyme(s) responsible for hepatic glucuronidation. LZP glucuronides (R- and S-LZPG) were quantified by a stereo-selective HPLC method. Recombinant human UGT1A (viz. UGT 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10) were expressed in HEK293 cells whereas UGT 2B4, 2B7, 2B15 and 2B17 were from a commercial source (Supersomes). R- and S-LZP glucuronidation by HLM (n=4) exhibited substrate inhibition kinetics. Mean (±SD) K_m and V_{max} values were 29 ± 8.5 μM and 7 ± 1.8 pmol/min.mg for R-LZPG, and 42 ± 20 μM and 11 ± 5.0 pmol/min.mg for S-LZPG. Substrate inhibition with both metabolites was weak; K_{si} values were approximately 8- to 14-fold higher than K_ms. Of the twelve recombinant human UGTs screened for activity, UGT 2B4, 2B7 and 2B15 were the only hepatically expressed enzymes that catalyzed R-LZP glucuronidation. S-LZP glucuronidation was also catalyzed by UGT 2B4, 2B7 and 2B15. However, highest activity was associated with UGT2B15. At an S-LZP concentration of 250 μM, UGT2B15 activity was about 4-times higher than those observed with UGT 2B4 and 2B7. R-LZP glucuronidation by UGT 2B4, 2B7 and 2B15 was well described by the Hill, Michaelis-Menten, and substrate inhibition equations, respectively. In contrast, the kinetics of S-LZPG formation by UGT 2B4, 2B7 and 2B15 were well modelled using the Hill equation. Derived K_m or S_{50} values for UGT 2B4, 2B7 and 2B15 catalyzed R-LZP were 43, 13 and 44 μM, while the respective S_{50} values for S-LZP glucuronidation were 46, 17 and 23 μM. Derived K_m or S_{50} values observed with recombinant UGTs and HLM were comparable. It is concluded that UGT 2B4, 2B7 and 2B15 are likely to be the major enzymes responsible for human liver microsomal R,S-lorazepam glucuronidation. S-Lorazepam appears to be a relatively selective substrate for UGT2B15.

3. <u>Oral presentation</u> การประชุม นักวิจัยรุ่นใหม่พบเมธีวิจัยอาวุโส สกว./โรงแรมฮอลิเดย์อินน์ รีสอร์ท รีเจนท์ บีช ชะอำ จังหวัดเพชรบุรี 14-16 ตุลาคม 2553

In vitro characterization of UDP-glucuronosyltransferases responsible for lorazepam glucuronidation and prediction of the lorazepam-valproic acid interaction

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Abstract

The anxiolytic agent lorazepam (LZP) and anticonvulsant valproic acid (VPA) are mainly eliminated in humans by glucuronidation. This study aimed to characterize UDP-glucuronosyltransferases responsible for (R,S) lorazepam glucuronidation and quantitatively predict *in vivo* metabolic drug-drug interaction between LZP and VPA. Recombinant human UGT enzymes were expressed in HEK293 or baculovirus-insect cells. Using HLMs as the enzyme source, mean derived binding affinity (K_s) and V_{max} of R- and S-LZPG kinetics were 36.7 ± 11.4 μM and 7.3 ± 2.1 pmol/min.mg, and 50.3 ± 18.2 μM and 11.7 ± 5.2 pmol/min.mg, respectively. Of the twelve recombinant human UGTs screened, UGT 2B4, 2B7 and 2B15 were the only hepatically expressed enzymes that catalyzed R and S-LZP glucuronidation. Derived K_m or S_{50} values observed with recombinant UGTs were in the range of 13-46 μM which is comparable to those observed from HLMs. VPA exhibited non-competitive inhibition on R and S-lorazepam glucuronidation by HLMs with respective K_i values of 3.9 and 3.2 mM. In conclusion, UGT 2B4, 2B7 and 2B15 are likely to be the major enzymes responsible for human liver microsomal R,S-lorazepam glucuronidation. Based on K_i value, 20% increased of LZP area under the plasma concentration time curve was predicted when co-administered with VPA.

Keywords: lorazepam, valproic acid, glucuronidation, UDP-glucuronosyltransferase, drug-drug interaction

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