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The 'albumin effect' and drug glucuronidation: Bovine serum albumin and fatty acid free human serum albumin enhance the glucuronidation of UGT1A9 substrates but not UGT1A1 and UGT1A6 activities

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Running Title: Scope of the 'albumin effect' on UGT1A enzyme activities

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Abbreviations:

4MU, 4-methylumbelliferone; 4MUG, 4-methylumbelliferone β-D-glucuronide; BSA, bovine serum albumin; FA, fatty acid; FAM, fatty acid mixture (40% C18:1, 40% C18:2, 20% C20:4); HLM, human liver microsomes; HSA, human serum albumin; HSAFAF, human serum albumin, fatty acid free; IV-IVE, in vitro - in vivo extrapolation; PRO, propofol; PRO-Gluc, propofol glucuronide; UDPGA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase.

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ABSTRACT

BSA and HSAFAF reduce the K_m values for UGT2B7 substrates by sequestering inhibitory long-chain fatty acids released by incubations of human liver microsomes (HLM) and HEK293 cells expressing this enzyme. However, the scope of the 'albumin effect' is unknown. This investigation characterized the effects of albumin on the kinetics of 4-methylumbelliferone (4MU) glucuronidation by UGT 1A1, 1A6 and 1A9, and propofol (PRO) glucuronidation by UGT1A9 and HLM. BSA and HSAFAF, but not HSA, reduced the K_m values for 4MU and PRO glucuronidation by UGT1A9. For example, HSAFAF (2%) reduced the K_ms for 4MU and PRO glucuronidation from 13.4 to 2.9μM and 41 to 7.2μM, respectively. Similarly, HSAFAF (2%) reduced the K_m for PRO glucuronidation by HLM from 127 to 10.6μM. Arachidonic, linoleic and oleic acids and mixture of these decreased the rates of 4MU and PRO glucuronidation by UGT1A9. K_m values for these reactions were increased 3- to 6- fold by the fatty acid mixture. Inhibition was reversed by the addition of BSA (2%). Extrapolation of kinetic constants for PRO glucuronidation by HLM in the presence of HSAFAF predicted in vivo hepatic clearance within 15%. Fatty acids had no effect on 4MU glucuronidation by UGT1A1 and UGT1A6 but, paradoxically, all forms of albumin altered the kinetic model for 4MU glucuronidation by UGT1A6 (Michaelis Menten to 2-site). Only BSA caused a similar effect on 4MU glucuronidation by UGT1A1. It is concluded that BSA and HSAFAF reduce the K_m values only of those enzymes inhibited by long chain unsaturated fatty acids.

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INTRODUCTION

Conjugation with glucuronic acid ('glucuronidation') represents both a clearance and detoxification mechanism for a large number of compounds that include drugs from most therapeutic classes, non-drug xenobiotics, and endogenous compounds (Miners and Mackenzie 1991; Tukey and Strassburg 2000; Miners *et al*. 2004). Glucuronidation reactions are catalyzed by enzymes of the UDP-glucuronosyltransferase (UGT) superfamily. The individual UGT enzymes exhibit distinct, but overlapping, substrate and inhibitor selectivities and differ in terms of gene regulation, including tissue distribution (Kiang *et al*. 2005; Kubota *et al*. 2007; Tukey and Strassburg 2000). Although seventeen functional human UGT proteins have been identified to date (Mackenzie *et al*. 2005), only seven of the hepatically expressed enzymes appear to contribute significantly to the elimination of drugs and other xenobiotics; UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B7, and UGT2B15 (Miners *et al*. 2006).

The ready availability of experimental systems (e.g. human liver microsomes (HLM), isolated hepatocyes, and recombinant enzymes) that enable the kinetic characterization of xenobiotic biotransformation *in vitro* has resulted in a steady increase in the number of publications that have investigated the kinetics of drug and chemical glucuronidation. Although K_m values for some substrates, for example bilirubin glucuronidation by UGT1A1 (Udomuksorn *et al*. 2007) and trifluoperazine glucuronidation by UGT1A4 (Uchaipichat *et al*. 2006a), are in the low micromolar range, reported K_m values of many compounds, particularly substrates of UGT2B7, are often in the millimolar range. Notably, K_m values for morphine (3- and 6-), codeine and zidovudine glucuronidation by HLM range from approximately 1.1 to 4.3mM, even when corrected for non-specific binding (Boase and Miners 2002; Court *et al*. 2003). Moreover, the high K_m values

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provide lower than expected estimates of microsomal intrinsic clearance (CL_{int}) that under-predict *in vivo* hepatic CL_{int} and hepatic clearance (CL_H) when used for *in vitro* - *in vivo* extrapolation (IV-IVE) (Boase and Miners 2002; Soars *et al*. 2002; Engtrakul *et al*. 2005).

The high K_m values observed *in vitro* have led to the general perception that glucuronidation is a 'low affinity' metabolic pathway (e.g. Williams *et al*. 2004) and that IV-IVE strategies are unlikely to be successful for glucuronidated substrates, especially for kinetic data obtained with HLM and recombinant enzymes (Engtrakul *et al*. 2005; Soars *et al*. 2002). However, recent work in this laboratory demonstrated that the addition of bovine serum albumin (BSA) or fatty acid free human serum albumin (HSAFAF) to incubations of HLM and recombinant UGT2B7 decreased the K_m for zidovudine glucuronidation by approximately an order of magnitude (Rowland *et al*. 2007). K_m values obtained in the presence of BSA and HSAFAF (with correction for binding) were 40 to 87µM, comparable to the K_m observed for zidovudine glucuronidation by human hepatocytes. In addition, we demonstrated that extrapolation of the inhibitor constants (K_i) generated in the presence of BSA, with both HLM and UGT2B7 as the enzyme sources, correctly predicted the magnitude of the fluconazole – zidovudine and valproic acid – lamotrigine inhibitory interactions *in vivo* (Uchaipichat *et al*. 2006b; Rowland *et al*. 2006).

The mechanism of the 'albumin effect' was shown to involve sequestration of inhibitory long-chain unsaturated fatty acids, particularly oleic, linoleic and arachidonic acids, released from the microsomal membrane (or from HEK293 cell lysate in the case of recombinant UGT2B7) during the course of an incubation (Rowland *et al*. 2007). These fatty acids are potent competitive inhibitors of UGT2B7 (Tsoutsikos *et al*. 2004; Rowland

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et al. 2007), and hence the K_m values of substrates and the K_i values of inhibitors obtained in the absence of BSA or HSAFAF overestimate these parameters. In contrast to the observations with UGT2B7, BSA does not affect the K_m for lamotrigine glucuronidation by UGT1A4 (Rowland *et al*. 2006). This suggests that the effects of long-chain unsaturated fatty acids on the kinetics of drug glucuronidation, and hence implications for IV-IVE, varies from enzyme to enzyme.

Here we report the effects of BSA, crude HSA and HSAFAF on UGT1A1, UGT1A6 and UGT1A9, which, along with UGT1A4, appear to represent distinct clusters of hepatic UGT1A activities. Inhibitory effects of long-chain unsaturated fatty acids on these enzymes were also characterized. Effects on all three enzymes were assessed using the non-selective substrate 4-methylumbelliferone (4MU), while subsequent investigation of human liver microsomal UGT1A9 activity employed the selective substrate propofol (PRO). Inhibition by fatty acids (and reversal by BSA and HSAFAF) was demonstrated only with UGT1A9, although paradoxical effects of albumin on the kinetic models of 4MU glucuronidation by UGT1A1 and UGT1A6 were observed. Importantly, microsomal CL_{int} values for PRO glucuronidation obtained in the presence of BSA and HSAFAF accurately predicted *in vivo* CL_{int}.

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MATERIALS AND METHODS

Materials

Alamethicin (from *Trichoderma viride*), bovine serum albumin (BSA, product number A7906), human serum albumin ('crude' HSA; product number A9511), essentially fatty acid free HSA (HSAFAF; product number A1887), 4-methylumbelliferone (4MU), 4-methylumbelliferone β-D-glucuronide (4MUG), fatty acids (as the free acid), propofol (PRO) and UDP-glucuronic acid (UDPGA; trisodium salt) were purchased from Sigma Aldrich (Sydney, Australia). Solvents and other reagents were of analytical reagent grade.

Human liver microsomes and expression of UGT1A proteins

Pooled human liver microsomes were prepared by mixing equal protein amounts from five human livers (H7, 44yo female; H10, 67yo female; H12, 66yo male; H29 45yo male; and H40, 54yo female), obtained from the human liver 'bank' of the Department of Clinical Pharmacology of Flinders University. Approval for the use of human liver tissue in xenobiotic metabolism studies was obtained from the Flinders Clinical Research Ethics Committee. Human liver microsomes (HLM) were prepared by differential centrifugation, as described by Bowalgaha *et al.* (2005).

cDNAs encoding UGT1A1, 1A6 and 1A9 were stably expressed in a human embryonic kidney cell line (HEK293), as described previously (Sorich *et al*. 2002; Uchaipichat *et al*. 2004). Transfected cells were incubated and harvested according to published methods (Uchaipichat *et al.* 2004). Lysed cells were centrifuged at 12000g for 1min at 4°C, and

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the supernatant fraction was removed and stored at –80°C until use. Expression of UGT protein was demonstrated by immunoblotting with a non-selective UGT1A antibody according to Uchaipichat *et al.* (2004) and measurement of 4MU glucuronidation activity (see below).

4MU glucuronidation assay

Assay conditions for 4MU glucuronidation by each recombinant UGT were optimized for HEK293 cell lysate protein concentration, incubation time and 4MU concentration range: UGT1A1 - 0.5mg/mL lysate protein, 90min and 5-600µM 4MU; UGT1A6 - 0.0025mg/mL lysate protein, 30min and 10-600µM 4MU; and UGT1A9 - 0.03mg/mL lysate protein, 15min and 0.5-40µM 4MU, respectively. Incubations, in a total volume of 200µL, contained phosphate buffer (0.1M, pH 7.4), $MgCl_2$ (4mM), albumin (0-2%), HEK293 cell lysate expressing the UGT1A protein and 4MU (as detailed above). Following a 5min pre-incubation, reactions were initiated by the addition of UDPGA (5mM). Incubations were performed at 37°C in a shaking water bath. Reactions were terminated by the addition of perchloric acid (6µL, 70% v/v). Samples were subsequently centrifuged at 4000g for 10min, and a 25µL aliquot of the supernatant fraction was injected directly into the HPLC column. 4MU β-D-glucuronidation by lysate protein from untransfected HEK293 cells was not detectable. Formation of 4MUG was quantified by HPLC as described by Rowland *et al*. (2007).

PRO glucuronidation assay

Incubations, in a total volume of 200µL, contained phosphate buffer (0.1M, pH 7.4), $MgCl_2$ (4mM), HLM (0.1mg), albumin (0-2%) and PRO (1-500µM). HLM were fully

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activated by the addition of the pore-forming polypeptide alamethicin (50µg/mg protein) with incubation on ice for 30min (Boase and Miners, 2002). Following a 5min pre-incubation, reactions were initiated by the addition of UDPGA (5mM). Incubations were performed at 37°C in a shaking water bath for 30min. Reactions were terminated by the addition of 200µL of ice-cold methanol containing 4% glacial acetic acid. Samples were subsequently centrifuged at 4000g for 10min, and a 5µL aliquot of the supernatant fraction was injected directly into the HPLC column.

For reactions performed using recombinant UGT1A9, incubation mixtures contained HEK293 cell lysate (0.05mg) in place of HLM protein, and the incubation time was decreased to 15 min. Under the reaction conditions employed, PRO glucuronidation was linear with respect to incubation time to 90min, and to a protein concentration of 1.5mg/mL for HLM and 1mg/mL for HEK293 cell lysate. PRO glucuronidation by lysate from untransfected HEK293 cells was not detectable.

Quantification of PRO-Gluc formation

HPLC was performed using an Agilent 1100 series instrument (Agilent Technologies, Sydney, Australia) fitted with Synergie Hydro RP C18 analytical column (150 x 3.0mm 4µm, Phenomenex, Sydney, Australia). PRO-Gluc was separated by gradient elution with a mobile phase containing 20mM phosphate buffer (pH 4.6) (mobile phase A) and acetonitrile (mobile phase B), at a flow rate of 0.6mL/min. Initial conditions were 73% mobile phase A and 27% mobile phase B. These conditions were held for 2min. The proportion of mobile phase B was then increased to 75% over 0.5min, and held for 5.5min. Column eluant was monitored by UV absorbance at 214nm. Retention times for PRO-Gluc and PRO were 3.6 and 8.1 min, respectively. The identity of PRO-Gluc was

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confirmed by hydrolysis with β-glucuronidase (from *E.coli*) and comparison with blank incubations performed in the absence of UDPGA. Briefly, a 1mL sample containing 500μM PRO was incubated overnight in the presence of 2mg/mL HLM. 5IU of β-glucuronidase was added to a 500μL aliquot of this sample, the pH of the sample was reduced to 5.8 and the sample incubated for 120min. Loss of PRO-Gluc and recovery of PRO was determined by comparison with an aliquot of the original sample that was not subjected to β-glucuronidase treatment.

In the absence of an authentic product, PRO-Gluc formation was quantified by comparison of peak areas to those of an PRO standard curve prepared over the concentration range 1 – 40μM. V_{max} and CL_{int} values should therefore be considered 'apparent'. Overall within day assay reproducibility was assessed by measuring PRO-Gluc formation in 8 separate incubations of the same batch of pooled HLM. Coefficients of variation were 3.6% and 2.9% for added PRO concentrations of 2μM and 500μM, respectively. The lower limit of quantitation (assessed as 3 times background noise) for PRO-Gluc was 0.005μM.

Binding of 4MU and PRO to albumin, HLM and HEK293 cell lysate

The binding of 4MU to HEK293 cell lysate, albumin (0.1, 1 and 2%) and mixtures of albumin with HEK293 cell lysate was measured by equilibrium dialysis as described previously (Rowland et al., 2007). The binding of PRO to HLM, HEK293 cell lysate, albumin, and mixtures of albumin with the two enzyme sources was determined according to the general procedure of McLure *et al*. (2000). Binding measurements were performed using Dianorm equilibrium dialysis apparatus comprising Teflon™ dialysis

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cells (capacity of 1.2mL per side) separated into two compartments with Sigma Aldrich dialysis membrane (molecular mass cut off 12kDa).

One side of the dialysis cell was loaded with 1mL of a solution of PRO (2 to 500µM) in phosphate buffer (0.1M, pH 7.4). The other compartment was loaded with 1mL of either a suspension of HLM in phosphate buffer (0.1M, pH 7.4), HEK293 cell lysate in phosphate buffer (0.1M, pH 7.4), albumin (0.1, 1 or 2%) in phosphate buffer (0.1M pH 7.4), or a combination of albumin with each enzyme source in phosphate buffer (0.1M, pH 7.4). The dialysis cell assembly was immersed in a water bath maintained at 37°C and rotated at 12rpm for 4hr. Control experiments were also performed with phosphate buffer or albumin on both sides of the dialysis cells at low and high concentrations of each substrate to ensure that equilibrium was attained. A 200µL aliquot was collected from each compartment, treated with ice-cold methanol containing 4% glacial acetic acid (200µL), and cooled on ice. Samples were subsequently centrifuged at 4000g for 10min at 10°C and an aliquot of the supernatant fraction (5µL) was analysed by HPLC.

Quantification of PRO

The HPLC instrument and column used to measure PRO binding was as described for the measurement of PRO-Gluc formation. Separation of PRO was achieved using a 25:75 mixture of mobile phases A and B, as utilized for the PRO-Gluc assay. The mobile phase flow rate was 0.6mL/min. Column eluant was monitored at 214nm. The retention time for PRO under these conditions was 3.6min. PRO concentrations in dialysis samples were determined by comparison of peak areas to those of a standard curve, in the concentration range 2 to 500µM. Within day assay variability was assessed by measuring PRO (10 and 500µM) (n=5 for each concentration) in samples containing

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phosphate buffer (0.1M, pH 7.4) or BSA in phosphate buffer (0.1M, pH 7.4). Coefficients of variation were less than 5% in all cases.

Data analysis

Kinetic data are presented as mean values derived from duplicate experiments with either pooled HLM or recombinant UGT1A proteins. Kinetic constants (K_m and V_{max}) for PRO glucuronidation by HLM and recombinant UGT1A9 in the presence and absence of albumin were generated by fitting experimental data to the Michaelis-Menten equation. Similarly, kinetic constants (K_m and V_{max}) for 4MU β-D-glucuronidation by recombinant UGT1A1 and UGT1A9 in the presence and absence of albumin were generated by fitting experimental data to the Michaelis Menten equation, except for UGT1A1 in the presence of BSA, where kinetic constants (K_m, V_{max}, α and β) were obtained by fitting experimental data to the two-site model described by Houston and Kenworthy (2000) and Uchaipichat *et al*. (2004):

$$\frac{v}{V_{max}} = \frac{\frac{[S]}{K_s} + \frac{\beta[S]^2}{\alpha K_s^{\,2}}}{1 + \frac{2[S]}{K_s} + \frac{[S]^2}{\alpha K_s^{\,2}}}$$

where K_s represents binding affinity and α and β are modifying factors that reflect changes in K_s and K_p (the catalytic rate constant), respectively.

Similarly, kinetic constants (K_m, V_{max} and α) for 4MU β-D-glucuronidation by recombinant UGT1A6 in the presence and absence of albumin were generated by fitting experimental

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data to the two-site model with β set to 2 (autoactivation; Uchaipichat *et al*. 2004). In all cases, fitting was based on unbound substrate concentrations (i.e. corrected for albumin and non-specific binding) in incubations and performed with EnzFitter (Biosoft, Cambridge, UK). For reaction exhibiting Michaelis-Menten kinetics, intrinsic clearance (CL_{int}) was determined as V_{max}/K_m.

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RESULTS

Binding of PRO to albumin

The binding of PRO to incubation components was calculated as the concentration of drug in the buffer compartment divided by the concentration of drug in the protein compartment and expressed as the fraction unbound in incubations ($f_{u,inc}$). In the absence of albumin, the binding of PRO to HEK293 cell lysate (0.25mg/mL) was negligible (<5%). However, PRO bound significantly to HLM and all forms of albumin. The binding of PRO to HLM was independent of substrate concentration, but varied with protein concentration; in the presence of 0.5mg/mL HLM, the mean f_u for PRO was 0.70. Similarly, the binding of PRO to albumin did not vary with substrate concentration or albumin form, but varied with albumin concentration. In the presence of 0.1, 1.0 and 2.0% albumin, mean f_u values for PRO were 0.70, 0.25 and 0.20, respectively. Interestingly, the binding of PRO to albumin plus HLM (0.25mg/mL) and albumin plus HEK293 cell lysate (0.25mg/mL) was identical to values observed for binding of PRO to the individual albumins alone, indicating that the binding is not additive. Non-additive binding to BSA and HLM has been observed previously with lamotrigine (Rowland *et al*. 2006). Recovery of PRO was >95% in all cases. Confirmatory binding studies were also performed with 4MU. The $f_{u,inc}$ values for 4MU binding to 0.1, 1 and 2% BSA, HSA and HSAFAF were 0.89, 0.49 and 0.27, 0.79, 0.37 and 0.14, and 0.76, 0.34 and 0.09, respectively. These values are in agreement with previous results from this laboratory (Rowland *et al*. 2007). Where binding was observed, the concentration of PRO and 4MU present in incubation mixtures was corrected in the calculation of kinetic parameters.

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Effect of albumin on 4MU glucuronidation by recombinant UGT1A enzymes

The kinetics of 4MU β-D-glucuronide (4MUG) formation by recombinant UGT1A1, in the presence and absence of albumin, were best described by the Michaelis-Menten equation with the exception of UGT1A1 in the presence of BSA where kinetic data were consistent with a two site model that assumes the simultaneous binding of substrate to two equivalent 'catalytic' sites (Houston and Kenworthy 2000; Uchaipichat *et al*. 2004). The K_m and V_{max} values for 4MU glucuronidation by recombinant UGT1A1 in the absence of albumin were 59μM and 374pmol/min/mg, respectively. Addition of BSA (0.1, 1 and 2%) altered the kinetics of 4MU glucuronidation by recombinant UGT1A1 from Michaelis Menten to two site (Figure 1). In contrast, neither HSA nor HSAFAF had an effect on the kinetic model of 4MUG formation by UGT1A1. HSA caused a concentration dependant increase in the K_m for this pathway with no effect on V_{max}, while HSAFAF did not appreciably affect any of the kinetic parameters (Table 1).

Although the kinetics of 4MUG formation by UGT1A6 in the absence of albumin were adequately described by the Michaelis Menten equation (K_m 59μM and V_{max} 86682pmol/min.mg), inspection of kinetic plots indicated increasing autoactivation in the presence of all forms of albumin and hence data were analysed using the two site model. By way of example, Figure 2 shows the kinetics of 4MU glucuronidation by UGT1A6 in the presence of increasing concentrations of BSA. In the absence of albumin the K_s, V_{max} and α values for 4MUG formation by recombinant UGT1A6 were 78μM, 82151pmol/min/mg and 0.46 respectively. All forms of albumin caused a concentration dependant decrease in the value of α, consistent with increasing autoactivation. With the

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exception of higher concentrations of HSAFAF, K_s tended to increase with increasing albumin concentration (Table 1).

4MU glucuronidation kinetics by recombinant UGT1A9, in the presence and absence of albumin, were best described by the Michaelis-Menten equation. In the absence of albumin, the K_m and V_{max} values for 4MUG formation by recombinant UGT1A9 were 13.4μM and 8362pmol/min/mg, respectively. BSA and HSAFAF increased the rate of 4MU glucuronidation by recombinant UGT1A9 in a concentration dependant manner (Figure 3) by decreasing the K_m for this pathway, without an appreciable effect on V_{max} (Table 1). In contrast, crude HSA decreased the rate of 4MU glucuronidation by recombinant UGT1A9, due largely to an increase in K_m.

Effect of albumin on PRO glucuronidation by HLM and recombinant UGT1A9

Kinetic data for PRO glucuronidation by HLM and recombinant UGT1A9, in the presence and absence of albumin, were obtained by fitting experimental data to the Michaelis-Menten equation. Kinetic parameters for PRO glucuronidation by HLM and recombinant UGT1A9 (in the presence and absence of albumin) are shown in Table 2. In the absence of albumin, the respective K_m and V_{max} values for PRO glucuronidation by HLM and recombinant UGT1A9 were 127μM and 967pmol/min.mg and 41μM and 2285pmol/min.mg. BSA and HSAFAF (0.1, 1 and 2%) increased the rate of PRO glucuronidation by both human liver microsomal and recombinant UGT1A9 in a concentration dependant manner (Figure 3) by decreasing the K_m for this pathway (Table 2). In contrast, crude HSA decreased the rate of PRO glucuronidation by HLM and recombinant UGT1A9 via an increase in K_m (Table 2).

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Inhibition of 4MU and PRO glucuronidation by fatty acids in the presence and absence of albumin.

Potential inhibition of UGT 1A1, 1A6 and 1A9 catalyzed 4MU glucuronidation by oleic acid (C18:1), linoleic acid (C18:2) and arachidonic acid (C20:4) was measured in the presence and absence of BSA (2%). When added at one twentieth of the concentration observed in HLM (Rowland *et al*. 2007), C18:1 (3µM), C18:2 (3µM) and C20:4 (1.5µM) inhibited the glucuronidation of 4MU by UGT1A9 by 28%, 31% and 68%, respectively (Figure 4A). The addition of BSA (2%) to incubations eliminated the inhibitory effect of each fatty acid (< 2% difference to control values). Inhibition of UGT1A1 and UGT1A6 catalyzed 4MUG formation was negligible (<5%) for all fatty acids, both in the presence and absence of BSA. Inhibition of PRO glucuronidation by C18:1, C18:2 and C20:4 was also measured in the presence and absence of BSA (2%) using recombinant UGT1A9 as the enzyme source. C18:1 (3µM), C18:2 (3µM) and C20:4 (1.5µM) inhibited the UGT1A9 catalyzed glucuronidation of PRO by 25%, 26% and 44%, respectively (Figure 4B). As with HLM, the addition of BSA (2%) in incubations eliminated the inhibitory effects of all fatty acids (< 3% difference to control values).

The effect of a mixture of fatty acids (FAM; comprising 3µM C18:1, 3µM C18:2 and 1.5µM C20:4) on the kinetics of 4MU and PRO glucuronidation by recombinant UGT1A9 was characterized in the presence and absence of BSA (2%). In the absence of BSA (2%), the FAM caused a 6-fold increase in the K_m for 4MU glucuronidation by recombinant UGT1A9, from 13µM to 89µM with only a minor effect on V_{max} (8920 versus 9304pmol/min.mg) (Figure 5). In contrast, the FAM had no effect on the kinetics of 4MU glucuronidation by recombinant UGT1A9 in the presence of BSA (2%). Respective K_m

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and V_{max} values obtained for incubations containing BSA were 3.8 and 3.8μM and 9817 and 10208pmol/min.mg in the absence and presence of the FAM (Figure 5).

Similarly, the FAM increased the K_m for PRO glucuronidation by recombinant UGT1A9, from 41μM to 127μM, with only a minor effect on V_{max} (2285 versus 2101pmol/min.mg) (Figure 5). In the presence of BSA (2%), the FAM had a negligible effect on the kinetics of PRO glucuronidation by recombinant UGT1A9; respective K_m and V_{max} values for experiments performed in the absence and presence the FAM were 7.2 and 7.5μM, and 2280 and 2316pmol/min.mg (Figure 5).

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DISCUSSION

Addition of BSA and HSAFAF to incubations of UGT1A9 (expressed in HEK293 cells) caused a concentration dependent decrease in the K_m values for 4MU and PRO glucuronidation. At an albumin concentration of 2%, K_m values were decreased by approximately 70 – 80%, with little or no effect on V_{max}. Since 4MU is a non-selective UGT substrate, kinetic studies with HLM as the enzyme source were performed only with PRO. Results were generally similar to those observed with recombinant UGT1A9. K_m values for PRO glucuronidation by HLM were 88% and 92% lower than control values in the presence of 2% BSA and HSAFAF, respectively, while V_{max} remained essentially unchanged. In contrast, addition of 'crude' HSA to incubations increased the K_m for 4MU and PRO glucuronidation by UGT1A9.

We have reported previously that BSA and HSAFAF, but not HSA, decrease the K_m values for zidovudine glucuronidation by UGT2B7 and HLM and for phenytoin hydroxylation by CYP2C9 and HLM. The decrease in K_m values, approximately an order of magnitude, arises from sequestration of long-chain unsaturated fatty acids released from microsomes or cell lysate during the course of an incubation (Rowland *et al.* 2007 and 2008). Long-chain unsaturated fatty acids, particularly arachidonic, linoleic and oleic, were shown to be potent competitive inhibitors of UGT2B7 and CYP2C9 (Tsoutsikos *et al.* 2004; Rowland *et al.* 2007 and 2008). The present work indicates that the same mechanism is responsible for the enhanced glucuronidation of UGT1A9 substrates observed in the presence of BSA and HSAFAF. Arachidonic, linoleic and oleic acids, individually and as a mixture (at concentrations corresponding to approximately one twentieth of the content of these fatty acids present in HLM), inhibited UGT1A9 catalyzed 4MU and PRO glucuronidation. K_m values for 4MU and PRO

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glucuronidation were increased 3- to 6- fold by the FAM without a change in V_{max}, consistent with competitive inhibition. Inhibition was abolished by the addition of BSA (2%) to incubations. As with UGT2B7 and CYP2C9 (Rowland *et al*. 2007 and 2008), long chain unsaturated fatty acids are substrates of UGT1A9 (P Gaganis, KM Knights and JO Miners, unpublished data).

As noted in the Introduction, there is a perception that glucuronidation is 'low affinity' metabolic pathway. However, when taken together with previously published data for zidovudine glucuronidation and the zidovudine – fluconazole and valproic acid – lamotrigine interactions (Uchaipichat *et al.* 2006; Rowland *et al.* 2006 and 2007), the present study suggests that published K_m and K_i values of UGT1A9 and UGT2B7 substrates and inhibitors may be overestimated by as much as an order of magnitude due to the inhibitory effects of fatty acids which are 'released' during an incubation. It is noteworthy that low K_m values (< 5μM) have been reported for substrates of enzymes not subject to inhibition by long- chain unsaturated fatty acids, for example bilirubin glucuronidation by UGT1A1 (Udomuksorn *et al.* 2007) and trifluoperazine glucuronidation by UGT1A4 (Uchaipichat *et al.* 2006a). The present study further demonstrates that K_m (and K_i) values generated using recombinant enzymes are generally lower than those obtained with HLM (Court 2005), presumably due to the lower fatty acid content of expression systems (Rowland *et al.* 2007 and 2008).

Extrapolation of kinetic constants (CL_{int}, K_i) generated in the presence of BSA have been reported to improve the prediction of *in vivo* CL_H for zidovudine (Rowland *et al.* 2007) and phenytoin (Rowland *et al.* 2008), and the magnitude of the fluconazole - zidovudine (Uchaipichat *et al.* 2006b) and valproic acid – lamotrigine interactions (Rowland *et al.* 2006). PRO disposition *in vivo* is well characterized. The mean systemic clearance,

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generally assumed to be almost entirely due to metabolism, taken from four studies is 108L/hr (Gepts *et al.* 1987; Servin *et al.* 1988; Morgan *et al.* 1990; Bailie *et al.* 1992). Glucuronidation accounts for 53% of PRO metabolism in humans (Simons *et al.* 1988), hence clearance via glucuronidation is 57 L/hr.

CL_{int} values obtained for PRO glucuronidation by HLM were extrapolated to a 'whole liver' CL_{int} employing a microsome yield of 38 mg/g (Rowland *et al.* 2008) and a liver weight of 1500g. Extrapolated whole liver CL_{int} values were then substituted in the expression for the well stirred model of hepatic clearance (Houston 1994) assuming liver blood flow is 90L/hr and that the fraction of PRO unbound in blood is 0.364. It should be noted that measured fraction of PRO unbound in blood is 0.014 (Mazoit and Samii, 1999). However, the binding affinity (K_D) of the component 'free' within erythrocytes (0.35) is very low, approximately 2mM (Mazoit and Samii, 1999). If it is assumed that this component is diffusible, then the 'available' fraction unbound in blood is 0.364. Predicted hepatic clearances based on microsomal CL_{int} values generated in the absence of albumin and in the presence of BSA (2%) and HSAFAF (2%) were 9, 44 and 50 L/hr, respectively.

A previous report from this laboratory demonstrated that lamotrigine glucuronidation by UGT1A4 was unaffected by BSA (Rowland *et al.* 2006). Here, rates of 4MU glucuronidation by UGT1A1 and UGT1A6 were similarly not enhanced by BSA and HSAFAF. Unexpectedly, BSA, but not HSA or HSAFAF, altered the Eadie Hofstee plot for 4MU glucuronidation by UGT1A1 from Michaelis Menten to one resembling substrate inhibition. However, the fit of kinetic data to the substrate inhibition equation was poor (data not shown). Rather, the kinetics of 4MU glucuronidation by UGT1A1 in the presence of BSA were best described by a model that assumes two equivalent

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interacting substrate binding sites (Houston and Kenworthy, 2000). We have shown previously that the 'atypical' (substrate inhibition and sigmoidal) kinetics observed for 4MU and 1-naphthol glucuronidation by several UGT enzymes were well described by the two site model (Uchaipichat *et al.* 2004).

Although 4MU glucuronidation by UGT1A6 was adequately modelled by the Michaelis Menten equation, consistent with Uchaipichat *et al.* (2004), closer inspection of Eadie Hofstee plots (Figure 2) revealed non-linearity, which became increasingly evident in the presence of increasing concentrations of albumin. Data were consistent with autoactivation, and values of the interaction factor α decreased with increasing albumin concentration. With the exception of HSAFAF (0.1 and 1%), binding affinity tended to decrease (i.e. K_s increased) in the presence of albumin. The mechanism by which the various albumin preparations alter the kinetics of 4MU glucuronidation by UGT1A1 and UGT1A6 is unknown. Interestingly, UGT1A1 has been reported recently to immuno-coprecipitate with HSA (Ohta *et al.* 2005), suggesting the possibility of a direct protein – protein interaction with some UGTs. The current unavailability of selective drug substrates for UGT1A1 and UGT1A6 in vivo precludes assessment of IV-IVE for compounds metabolized by these enzymes.

In summary, BSA and HSAFAF reduced the K_m values for 4MU and PRO glucuronidation by UGT1A9 and for PRO glucuronidation by HLM by sequestering inhibitory long-chain unsaturated fatty acids. The approximate ten-fold decrease in the K_m for human liver microsomal PRO glucuronidation is similar to the reduction observed for the UGT2B7 substrate zidovudine. Extrapolation of the microsomal CL_{int} obtained in the presence of HSAFAF underpredicted PRO hepatic clearance via glucuronidation by

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only 13%. In contrast to the effects of BSA and HSAFAF on UGT1A9 and UGT2B7 activities, neither form of albumin enhanced UGT1A1 and UGT1A6 catalyzed 4MU glucuronidation. Paradoxically, the albumin preparations variably altered the kinetic model of 4MU by UGT1A1 and UGT1A6, and the effects of albumin on reactions catalyzed by these enzymes require careful interpretation. However, available evidence indicates that inclusion of BSA or HSAFAF in incubations is likely to improve the accuracy of hepatic clearances predicted from microsomal CL_{int} values for substrates of UGT1A9 and UGT2B7, arguably the most important enzymes involved in drug glucuronidation in humans, and indeed for substrates of cytochromes P450 inhibited by long-chain unsaturated fatty acids (Rowland *et al*. 2008).

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ACKNOWLEDGEMENTS

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FOOTNOTES

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FIGURES

Figure 1 – Eadie-Hofstee plots (V versus V/[S]) for 4MU glucuronidation by recombinant UGT1A1 in the presence and absence of BSA (2%). Points are experimentally determined values, while curves are from model fitting.

Figure 2 – Eadie-Hofstee plots (V versus V/[S]) for 4MU glucuronidation by recombinant UGT1A6 in the presence of increasing concentrations of BSA (0 to 2%). Points are experimentally determined values, while curves are from model fitting.

Figure 3 – Eadie-Hofstee plots (V versus V/[S]) for 4MU and PRO glucuronidation by recombinant UGT1A9 in the presence and absence of albumin. Points are experimentally determined values, while lines are from model fitting.

Figure 4 – The effects of oleic acid (C18:1), linoleic acid (C18:2) and arachidonic acid (C20:4) on the rates of UGT1A9 catalyzed 4MU (panel A) and PRO (panel B) glucuronidation. Incubations were performed as described in Materials and Methods, at 4MU and PRO concentrations of 15 µM and 40 µM, respectively (which correspond to the approximate K_m values of these substrates). Bars show the mean of duplicate measurements and 'control' refers to the activity in the absence of added fatty acid.

Figure 5 - Eadie-Hofstee plots (V versus V/[S]) for 4MU and PRO glucuronidation by recombinant UGT1A9 in the presence and absence of a fatty acid mixture, with and without added BSA. Points are experimentally determined values, while lines are from model fitting.

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Table 1 – Kinetic parameters for 4MU β-D-glucuronide formation by recombinant UGT1A enzymes

	Parameter	No albumin	BSA			HSA			HSAFAF		
			0.1%	1.0%	2.0%	0.1%	1.0%	2.0%	0.1%	1.0%	2.0%
UGT1A1	K_m or K_s	59	249[a]	350[a]	393[a]	92	153	174	51	50	48
	V_{max}	374	722[a]	687[a]	960[a]	406	363	380	407	398	390
	CL_{int}	6.3	n/a[b]	n/a[b]	n/a[b]	4.4	2.4	2.2	8.0	8.0	8.1
UGT1A6	K_s[a]	78	71	95	93	81	102	176	91	64	65
	V_{max}[a]	82151	81036	80826	80766	86483	87060	76432	72850	70023	73002
	α[a]	0.46	0.44	0.47	0.33	0.36	0.38	0.078	0.46	0.52	0.26
UGT1A9	K_m	13.4	10.8	7.6	3.8	16.6	20.5	22.8	9.8	4.2	2.9
	V_{max}	8362	7860	11262	9817	8422	8632	8649	8067	10265	9765
	CL_{int}	624	728	1482	2583	507	421	379	823	2444	3367

Units: K_m, μM; V_{max}, pmol/min/mg; CL_{int}, μL/min/mg. Kinetic constants were generated from fitting experimental data to the Michaelis Menten equation, except UGT1A1 in the presence of BSA and all kinetic data for UGT1A6.

[a] Data from fitting to a two site model (Uchaipichat *et al*. 2004). The two site model provides an estimate of binding affinity (K_s) rather than K_m. Alpha and beta values for 4MU glucuronidation by recombinant UGT1A1 in the presence of 0.1, 1 and 2% BSA were 0.20 and 0.37, 0.034 and 0.45, and 0.063 and 0.27, respectively. The value of β was set at 2 for the modeling of 4MU glucuronidation by UGT1A6 since for autoactivation V_{max} is equivalent to $2K_p[E]_t$ (Uchaipichat *et al*. 2004).

[b] n/a: not applicable

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Table 2 – Kinetic parameters for PRO glucuronidation by HLM and recombinant UGT1A9

	Parameter	No albumin	BSA			HSA			HSAFAF		
			0.1%	1.0%	2.0%	0.1%	1.0%	2.0%	0.1%	1.0%	2.0%
HLM	K_m	127	19.4	15.9	15.5	155	155	264	15.8	10.1	10.6
	V_{max}	967	1009	1048	1048	970	1013	892	964	926	970
	CL_{int}	8	52	66	68	6	7	3	61	92	92
UGT1A9	K_m	41	17.6	9.1	7.2	97	98	99	18.1	8.8	7.2
	V_{max}	2285	2105	2213	2280	2694	2352	2196	2380	2150	2322
	CL_{int}	56	120	243	317	28	24	22	131	244	323

Units: K_m, µM; V_{max}, pmol/min/mg; CL_{int}, µL/min/mg.

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Figure 1

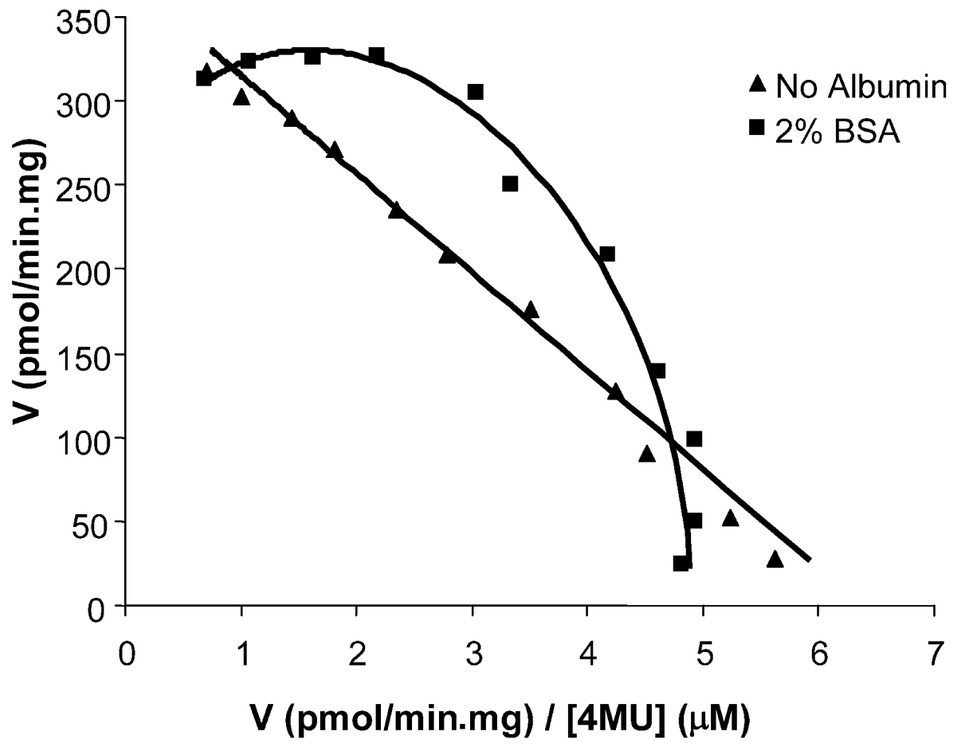

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Figure 2

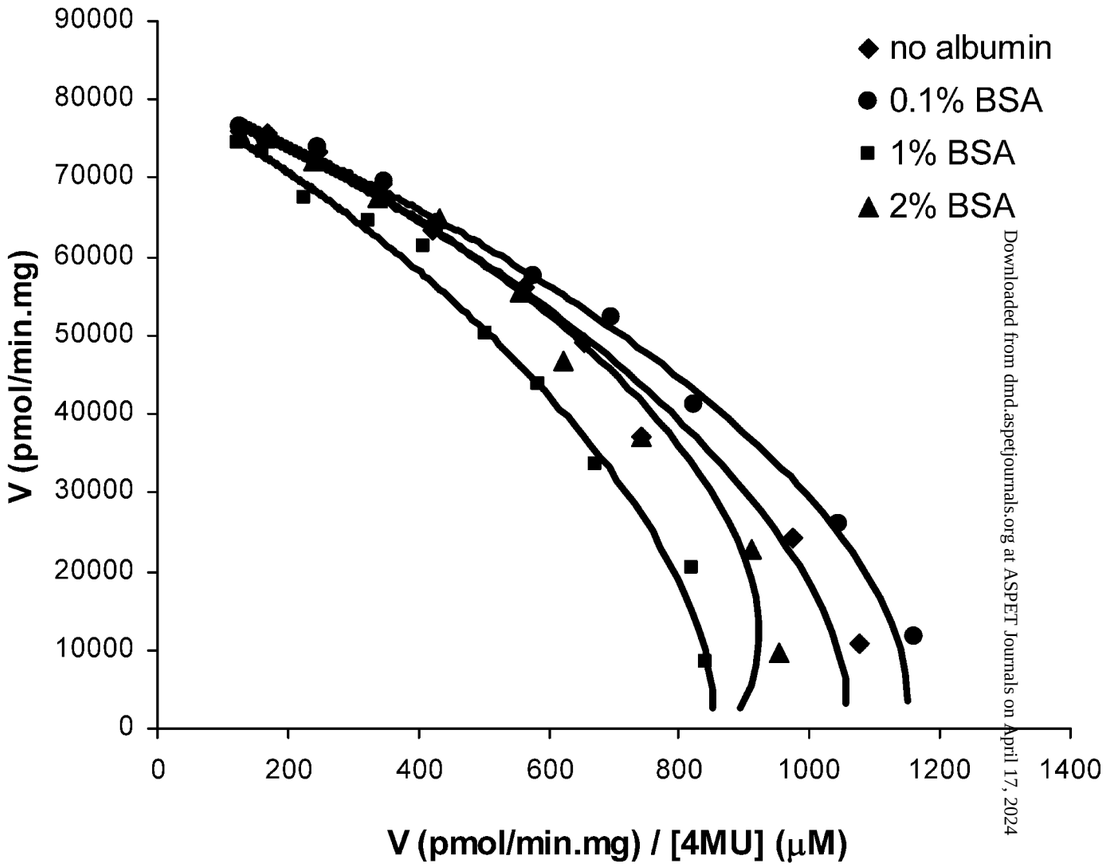

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Figure 3

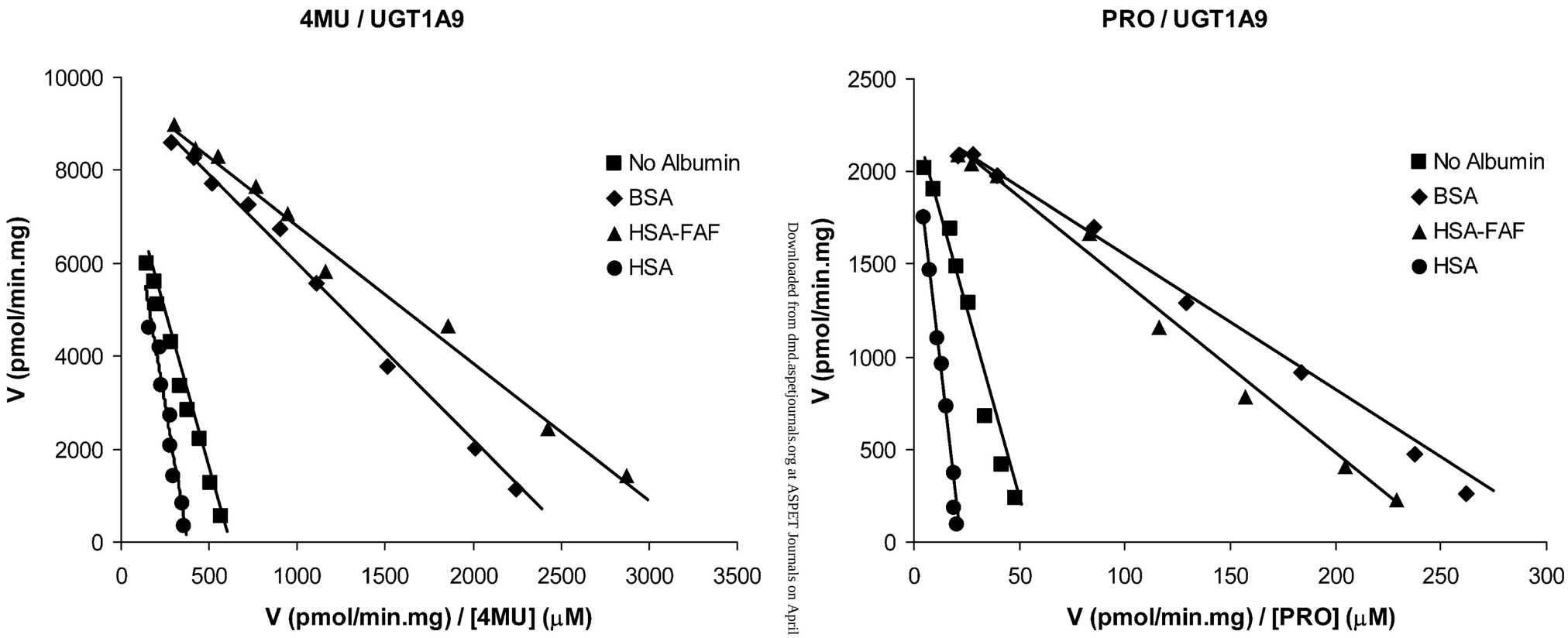

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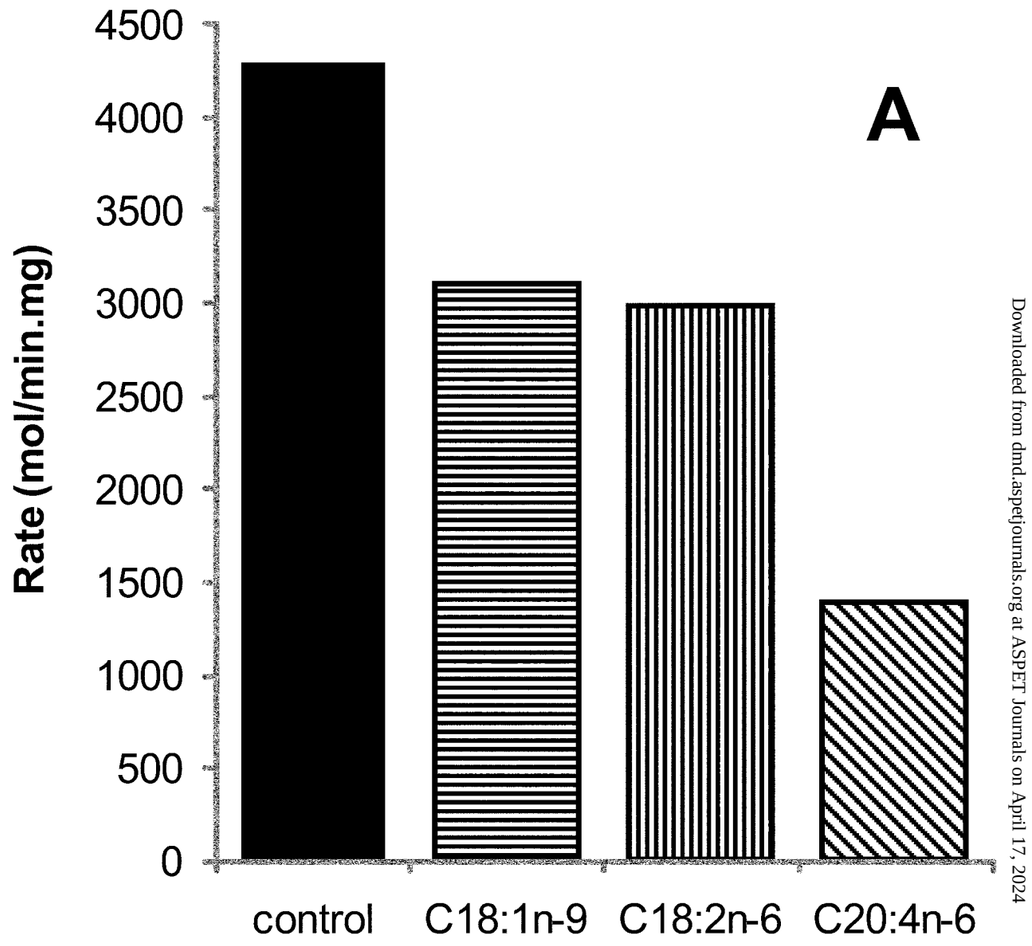
Figure 4



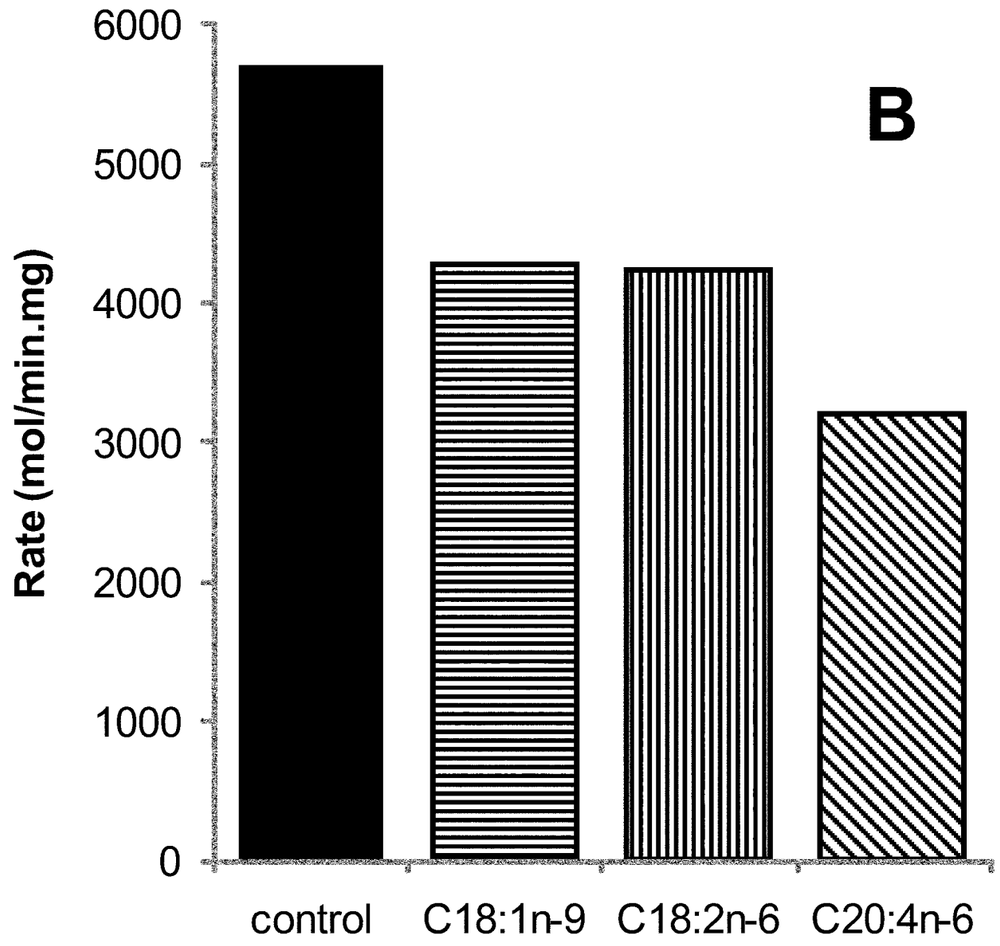


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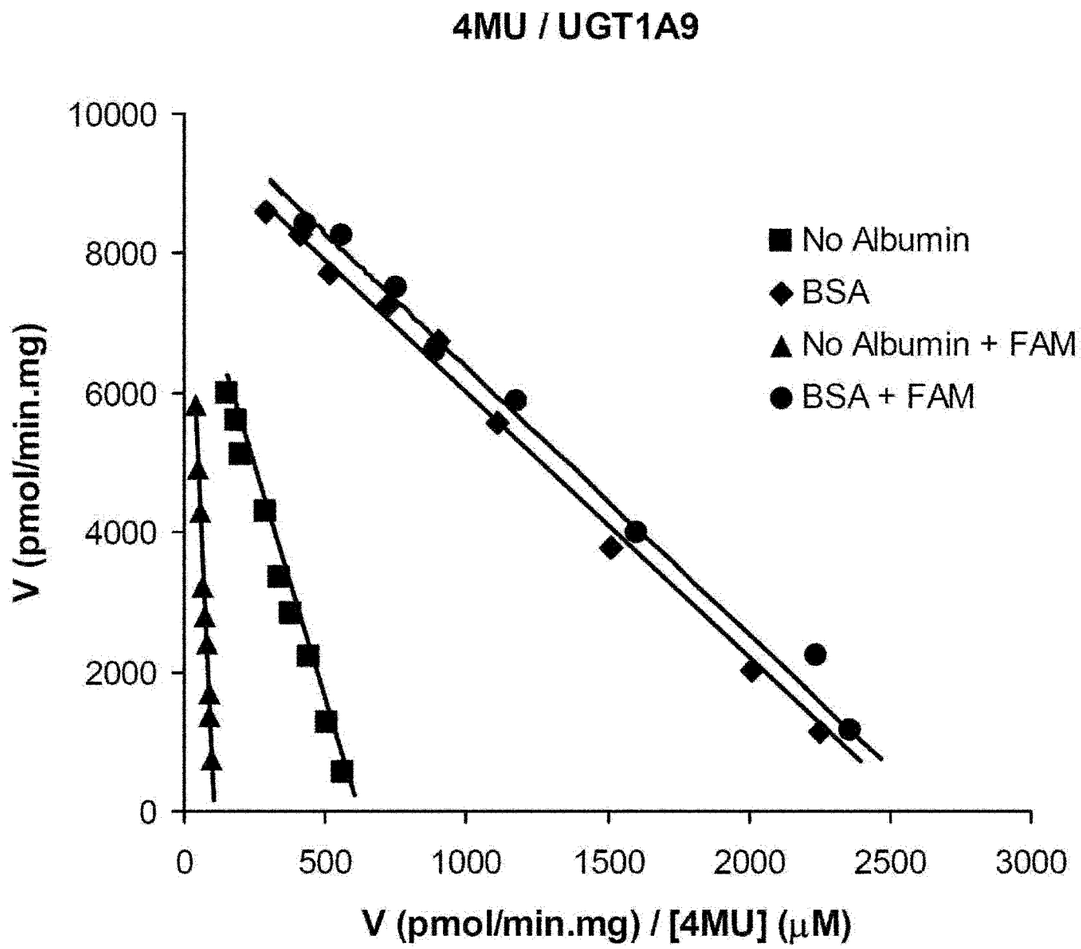
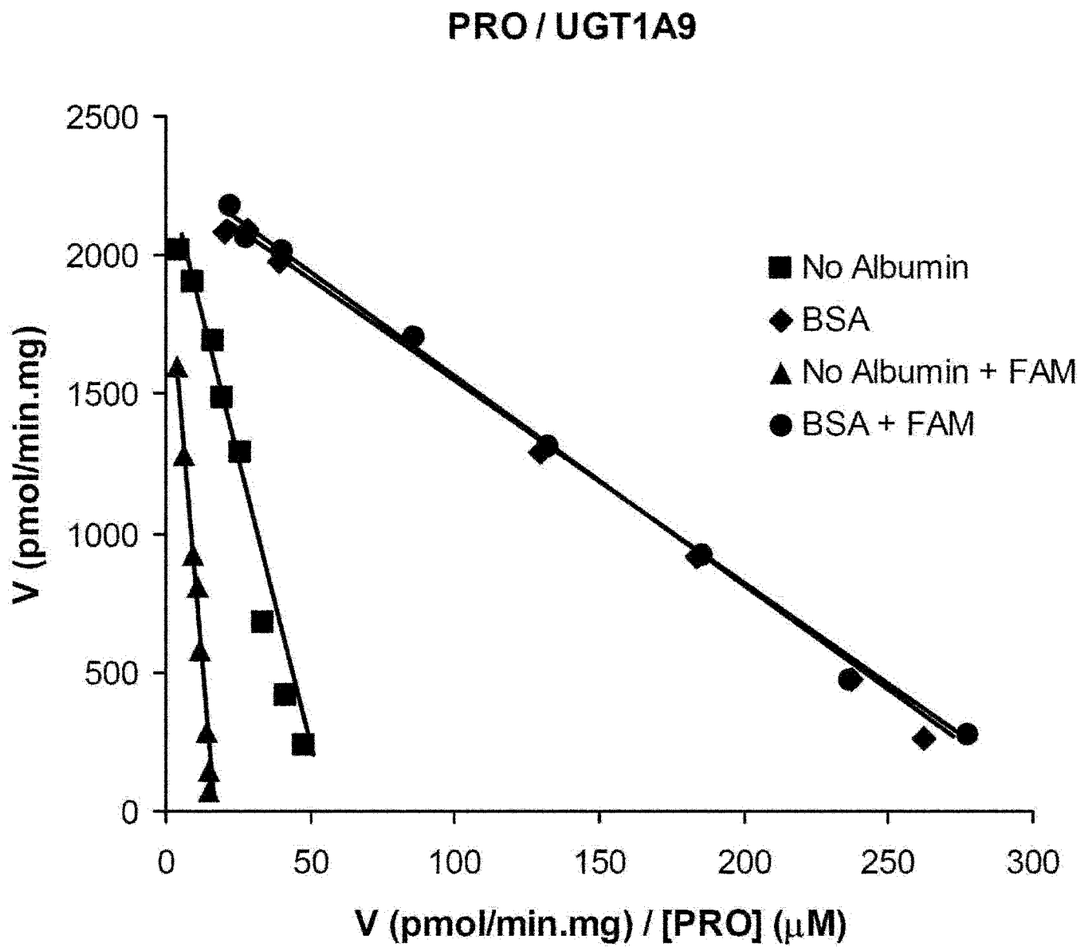
Figure 5





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