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Hepatic UDP-glucuronosyltransferase responsible for eslicarbazepine glucurononidation

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Characterization of ESL glucuronidation

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List of Abbreviations:

ESL, Eslicarbazepine acetate; OXC, Oxcarbazepine; CNS, Central nervous system; AED, Antiepileptic drugs; LTG, Lamotrigine; BSA, Bovine serum albumin; HLM, Human liver microsomes; MLM, Mouse liver microsomes;DMSO, Dimethylsulfoxide; ISTD, Internal standard; HPLC-MS, High performance liquid chromatography-Mass spectrometry; AP-ESI, Atmospheric pressure-electrospray ionization; SIM, Selected ion monitoring; UGT, UDP-glucuronosyltransferase; SPE, Solid phase extraction;UDPGA, Uridine 5'-diphosphoglucuronic acid.

Abstract

Eslicarbazepine acetate (ESL) is a once-daily novel antiepileptic drug approved in Europe for use as adjunctive therapy for refractory partial-onset seizures with or without secondary generalisation. Metabolism of ESL consists primarily of hydrolysis to eslicarbazepine, which is then subject to glucuronidation followed by renal excretion. In the current study, we have identified that human liver microsomes (HLM) enriched with uridine 5'-diphosphoglucuronic acid (UDPGA) give origin to a single Escherichia coli ß-glucuronidase sensitive eslicarbazepine glucuronide (most likely the O-glucuronide). The kinetics of eslicarbazepine glucuronidation in HLM was investigated in the presence and in the absence of bovine serum albumin (BSA). The apparent K_m were 412.2 ± 63.8 µM and 349.7 ± 74.3 µM in the presence and absence of BSA, respectively. Incubations with recombinant human uridinediphosphateglucuronosyltransferases (UGTs) indicated that UGT1A4, UGT1A9, UGT2B4, UGT2B7 and UGT2B17 appear to be involved in eslicarbazepine conjugation. The UGT with highest affinity for conjugation was UGT2B4 (K_m = 157.0 ± 31.2 µM and 28.7 ± 10.1 µM, in the absence and presence of BSA, respectively). There was a significant correlation between eslicarbazepine glucuronidation and trifluoperazine glucuronidation, a typical UGT1A4 substrate; however, no correlation was found with typical substrates for UGT1A1 and UGT1A9. Diclofenac inhibited eslicarbazepine glucuronidation in HLMs with an IC_{50} value of 17 µM. In conclusion, glucuronidation of eslicarbazepine results from the contribution of UGT1A4, UGT1A9, UGT2B4, UGT2B7 and UGT2B17, but the high affinity component of UGT2B4 isozyme may play a major role at therapeutic plasma concentrations of unbound eslicarbazepine.

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Introduction

Eslicarbazepine acetate [(S)-(-)-10-acetoxy-10,11, dihydro-5H-dibenz[b,f]azepine-5-carboxamide; ESL] is a novel once-daily CNS active compound (Almeida and Soares-da-Silva, 2007) that completed the phase III clinical trials as adjunctive therapy in partial epilepsy refractory to standard antiepileptic drugs (AEDs) (Elger et al., 2009; Gil-Nagel et al., 2009; Ben-Menachem et al., 2010). ESL shares with carbamazepine and oxcarbazepine (OXC) the dibenzazepine nucleus bearing the 5-carboxamide substituent, but is structurally different at the 10,11-position (Almeida and Soares-da-Silva, 2007). This molecular variation results in differences in metabolism, namely by preventing the formation of toxic epoxide metabolites such as carbamazepine-10,11 epoxide (Hainzl et al., 2001; Almeida et al., 2009). In humans, ESL is rapidly absorbed and undergoes extensive first-pass hydrolysis to eslicarbazepine (Almeida and Soares-da-Silva, 2007). Following an oral dosing of 800 mg, ESL, R-licarbazepine, OXC and its glucurono-conjugates were clearly minor metabolites (Almeida et al., 2009). The major circulating metabolites were eslicarbazepine and eslicarbazepine glucuronide with a C_{max} of 48.3 µM and 4.3 µM, respectively (Almeida et al., 2008; Maia et al., 2008). The major metabolites recovered in urine were eslicarbazepine and eslicarbazepine glucuronide, the sum of which corresponded to approximately 93% of all metabolites recovered in urine (Maia et al., 2008). The metabolism of ESL is, however, strongly dependent on the species and ESL metabolic may originate different proportions of R-licarbazepine and OXC as minor metabolites (Hainzl et al., 2001).

Glucuronidation reactions are catalysed by the enzymes UDP-Glucuronosyltransferases (UGT) expressed on the inner membrane of the endoplasmatic reticulum. UGT exists as a superfamily of enzymes that exhibit distinct, but overlapping, substrate and inhibitor selectivity (Mackenzie et al., 2005). Seventeen human UGT proteins have been identified and classified into two families (UGT1 and UGT2) based on sequence identity (Mackenzie et al., 2005). The contribution of different isoforms to glucuronidation is dependent on

the relative expression levels in different tissues. UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B7, UGT2B4 and UGT2B17 have been detected in hepatic and extra hepatic tissues (Strassburg et al., 2000). UGT1A9 is one of the major drug glucuronidating enzymes (Ebner and Burchell, 1993) and is the dominant isoform in kidney (Strassburg et al., 2000). The most common human condition relating to UGT activity is Gilbert Syndrome, relating to loss-of-function of UGT1A1 (Strassburg, 2008). UGT2B7 is a very important human UGT isoform that appears to be expressed in many extra-hepatic tissues and catalyses the glucuronidation of a wide range of xenobiotics, including, phenols, aliphatic alcohols, carboxylic acids and tetrazoles (King et al., 2001).

To date, the identity of UGTs involved in eslicarbazepine glucuronidation have not been explored in detail. The present study reports on the enzymes involved in eslicarbazepine glucuronidation and characterized their kinetic parameters using human liver microsomes (HLM) and recombinant UGTs.

Materials and methods

Chemicals

ESL, eslicarbazepine and R-licarbazepine were synthesized in BIAL's Laboratory of Chemical Research with purity >99.5%. OXC was synthesized and provided by Farchemia (Italy). Milli-Q water was used in all steps and solvents were of HPLC grade. All other reagents were obtained from Sigma.

Pooled HLM (from 48 donors), mouse liver microsomes (MLM) and HLM single donor (HG3, HG95, HG64, HH13 and HG93) were purchased from BD Gentest (Woburn, MA). The protein contents were used as described in the data sheets provided by the manufacturers. Recombinant human UGTs expressed in baculovirus-infected insect cells were purchased from Pan Vera-Invitrogen (Carlsbad, CA, UGT: 1A1, 1A3, 1A6, 1A7, 1A10 and 2B7) and from BD Gentest (Woburn, MA, UGT: 1A4, 1A8, 1A9, 2B4, 2B17 and 2B15).

Eslicarbazepine glucuronidation in pooled and single donor HLM

Glucuronidation activity by HLM was measured using the following assay conditions: the incubation mixture (100 µl total volume) contained 0.5 mg/ml

total protein, 10 mM $MgCl_2$, 2 mM uridine 5'-diphosphoglucuronic acid (UDPGA), 25 µg/ml alamethicin, 5 mM saccharolactone in 50 mM phosphate buffer pH 7.5 and 10-1000 µM eslicarbazepine. Drug was dissolved in DMSO and the final concentration of DMSO in the reaction was below 0.5% (v/v). Experiments were performed in the presence and in the absence of BSA 2%. Reactions were pre-incubated 5 min and were initiated with the drug. Reaction mixtures were incubated for up to 60 min and terminated by the addition of 400 µl 10,11-dihydrocarbamazepine (ISTD) working solution (250 ng/ml in 0.1M phosphate buffer pH 5.6). Samples were subjected to solid phase extraction and injected on a HPLC-MS. All incubations were performed in a shaking water bath at 37ºC. Linearity of product formation with time (0-120 min) was evaluated in HLM. All experiments were performed with duplicates.

Eslicarbazepine glucuronide hydrolysis

It was not possible to use the eslicarbazepine glucuronide standard; therefore, in its absence the compound was quantified by peak area ratio to ISTD. The identity of eslicarbazepine glucuronide was confirmed by hydrolysis with β-glucuronidase (from E. Coli) using HLM and MLM. In brief, eslicarbazepine glucuronide was obtained by incubating 100 µM eslicarbazepine with microsomes (1 mg/ml) in a final volume of 200 µl, during 2 h under the conditions described above. A control without UDPGA was also prepared. After incubation, the remaining eslicarbazepine was removed from the samples by liquid-liquid extraction with chloroform. The chloroform layer containing eslicarbazepine was discarded, and this process was repeated twice. The aqueous layer (100 µl) containing eslicarbazepine glucuronide was then incubated for 2 h with β-glucuronidase (100 000 units/ml) at 37ºC, after adjusting pH to 6.8. The disappearance of eslicarbazepine glucuronide and the subsequent appearance of eslicarbazepine in the sample as compared to an aliquot of the original sample that was not subject to β-glucuronidase hydrolysis identified the compound.

Eslicarbazepine glucuronidation screening by recombinant UGTs

Glucuronidation by recombinant UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15 and UGT2B17 was evaluated with 1000 µM eslicarbazepine and 0.5 mg/ml total protein using the conditions described above for HLM with the exception that reactions did not contain s*accharolactone* and were carried out over 120 min. Kinetics of eslicarbazepine glucuronidation by selected UGTs were determined, as described above with eslicarbazepine concentrations ranging 10-1000 µM and 60 min incubation time .

Inhibition of eslicarbazepine glucuronide formation by typical substrates for UGT isoforms

In experiments designed to evaluate the inhibition of eslicarbazepine glucuronidation, pooled HLM were incubated for 60 min with 100 µM eslicarbazepine in the presence and in the absence of the following substrates: propofol, (substrate for UGT1A9; Hanioka et al., 2001), imipramine (substrate for UGT1A4; Nakajima et al., 2002), diclofenac (substrate for UGT1A1, UGT1A3, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B7, UGT2B15 and UGT2B4; Uchaipichat et al., 2006a) and testosterone, (substrate for UGT2B17, UGT2B7 and UGT2B15; Bowalgaha et al., 2007), at 10, 50, 100, 500 and 1000 µM.

Experiments to determine the inhibition specificity of UGTs were performed by incubation of 500 µM eslicarbazepine with recombinant UGTs and 500 µM propofol, imipramine, diclofenac or testosterone, for 60 min at 37ºC.

Extraction of eslicarbazepine glucuronides

The samples with ISTD were placed on an automatic liquid handler (ASPEC-XL4, Gilson) for solid phase extraction. The solid phase extraction cartridges (Oasis, HLB, 30 mg, 1ml, Waters) were conditioned with 1 ml of acetonitrile and then washed twice with 1 ml of water. Samples (400 *µ*l) were loaded onto the cartridges and the cartridges washed twice with 1 ml of water. After the second wash, the cartridges were flushed with an air push of 10 ml at 6 ml/min. The cartridges were eluted twice with 200 *µ*l of methanol with an air push of 2 ml at 6 ml/min. To the eluted sample 200 µl of water were added and

mixed twice with aspiring dispensing cycles. The eluted samples were injected (5 µl) into an HPLC-MS.

HPLC-MS Analysis

The analysis of samples extracts was performed using HPLC-MS (Agilent, AP-ESI, 1100 Series, Agilent Technologies) with positive ion detection. Briefly, separation was performed on a Waters Symmetry C8, 3.5 µm, 4.6 cm x 150 mm (Waters) using a mobile phase A: water containing 1 % formic acid (v:v) and B: acetonitrile with gradient conditions of 80 % of A and 20 % of B at 1 min; 60 % of A: 40 % of B at 10 min and 80 % of A: 20 % of B at 10.1 min. Selected ion monitoring (SIM) with the detection of m/z 431 for eslicarbazepine glucuronide was used for quantification. For maximal sensitivity, the fragment energy was set to 120 V and further settings were 3500 eV for the capillary voltage, 350ºC nebulizer gas temperature and 40 psi nebulizer pressure.

LC-MS/MS glucuronide identification

The analysis of the extracted samples to identify eslicarbazepine glucuronide was performed using LC (Agilent Technologies 1290 Infinity) coupled with MS/MS detector (Agilent Technologies 6460 Triple Quad LC/MS). It was used electrospray ionisation in positive ion detection with a fragmentor of 120 V and collision energy of 20 eV. The multiple reaction monitoring pair was *m/z* 431.3→237.0 and 431.3>194 m/z. The source parameters were: gas temperature of 200 ºC; gas flow of 10 L/min; nebulizer of 30 psi; sheath gas temperature of 350 °C; sheath gas flow of 11 L/min; capillary of 3500 V; nozzle voltage of 300 V.

Data analysis

Kinetic parameters of glucuronidation were obtained by fitting velocity data to Michaelis-Menten equation with GraphPad Prism (CA, USA).

Results

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Eslicarbazepine glucuronidation by HLM

Incubation of eslicarbazepine with HLM in the presence of UDPGA resulted in the appearance of one peak with an $[M-H]^+$ ion at *m/z* 431 $[M+H-176]^+$ corresponding to m/z of eslicarbazepine glucuronide (**figure 1**). The disappearance of this peak with β-glucuronidase treatment and its absence in control experiments performed without UDPGA (data not shown) suggests that this peak corresponded to eslicarbazepine glucuronide. Furthermore, the product ion spectra from precursor m/z 431 contained an ion at m/z 237.2 for the aglycone (from loss of the neutral glucuronic acid) with an additional peak at m/z of 194.2 for the glucuronic acid and the neutral loss of glucuronide moiety of 176 daltons, yielded a peak corresponding to m/z 431 **(figure 2).** In addition, the fact that this compound formation is time and protein dependent further corroborated its identity as eslicarbazepine glucuronide. When incubation was performed with MLM in the presence of UDPGA, two peaks with an $[M-H]^+$ ion at *m/z* 431 were detected but only one disappeared with β-glucuronidase treatment (**figure 3**), suggesting that in mouse two eslicarbazepine glucuronides were formed (N-glucuronide and O-glucuronide), but only one was hydrolysed by β-glucuronidase from E. Coli.

Kinetic analysis of eslicarbazepine glucuronidation was performed in pooled HLM in the presence and absence of BSA. As shown in **figure 4**, eslicarbazepine glucuronidation displayed typical hyperbolic kinetics; however, the Eadie-Hofstee plot of the data in the presence of BSA was biphasic suggesting the involvement of more than one enzyme in the reaction (Nakajima et al., 2002). The apparent K_m and the V_{max} derived from these curves fitted to the Michaelis-Menten equation are listed in **table 1**. The apparent K_m values were similar for liver microsomes irrespective of the presence of BSA but the V_{max} was significantly higher in the presence of BSA.

Eslicarbazepine glucuronidation by recombinant UGTs

Eleven commercially available UGT enzymes were used to evaluate their ability to conjugate eslicarbazepine (**figure 5**). To maximize product formation a concentration of 1000 µM eslicarbazepine was used. From the tested UGTs only UGT1A9, UGT1A4, UGT2B7, UGT2B4 and UGT2B17 produced

significant amounts of eslicarbazepine glucuronide. No eslicarbazepine glucuronide formation was detected with control baculosomes/supersomes and UGT1A1, UGT1A3, UGT1A6, UGT1A8, UGT1A10 and UGT2B15 over an incubation period of 120 min.

Kinetics of eslicarbazepine glucuronidation by recombinant UGTs

The characterization of eslicarbazepine glucuronidation kinetics was performed for the UGTs with highest activities, namely UGT1A9, UGT2B4, and UGT2B17.The low activities of UGT1A4 and UGT2B7 precluded full kinetic analysis. Each enzyme was incubated 60 min with different concentrations of eslicarbazepine (10-1000 µM) in the presence and in the absence of 2 % BSA. No significant glucuronidation was observed with UGT1A4 and UGT2B7 for low eslicarbazepine concentrations.

Accordingly, the experimental data from UGT1A9, UGT2B4 and UGT2B17 was fitted to the Michaelis-Menten equation. The resulting curves are represented in **figure 6** and the kinetic parameters, derived from these curves, are shown in **table 1**. There is a considerable range of affinities for the conjugation of eslicarbazepine, as shown by difference in K_m values for eslicarbazepine glucuronidation. The enzyme with the highest affinity was UGT2B4 with a K_m of 157.0 ± 31.2 µM, followed by UGT 2B17 with a K_m value of 524.2 ± 195.9 µM. UGT1A9 was the one with the lowest affinity (K_m = 1299 ± 841.8 µM). The presence of BSA did not change the order of affinities of the different enzymes for the glucuronidation of eslicarbazepine; however, a significant increase in the affinities of UGT2B4 and UGT1A9 was observed (K_m values of 28.7 ± 10.1 µM and 577.1 ± 330.1 µM respectively). The K_m of UGT2B17 (279.3 ± 62.9 µM) did not change significantly.

The comparison of affinities between the recombinant UGTs and HLM in the presence and in the absence of BSA suggests the involvement of more than one enzyme in eslicarbazepine conjugation.

Interindividual differences of eslicarbazepine glucuronidation in HLM and correlation analysis

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Eslicarbazepine glucuronidation was measured in the microsomal fraction from 5 different donors (HG3, HG93, HG95, HG64, HH13), chosen to provide differences in their catalytic activities of the UGT1A1, UGT1A4, andUGT1A9 enzymes (Table 2). Eslicarbazepine conjugation activity in these individual HLM was significantly (r^2 =0.65) correlated with trifluoperazine glucuronidase activity (**figure 7**).

Inhibitory effect of propofol, imipramine, diclofenac and testosterone in eslicarbazepine glucuronidation in HLM and UGTs

The effect of propofol, imipramine, diclofenac and testosterone (10, 50, 100, 500 and 1000 µM) on eslicarbazepine (100 µM) glucuronidation activities in pooled HLM was investigated. As shown in **figure 8**, diclofenac completely inhibited eslicarbazepine glucuronidation with an IC_{50} of 17.0 µM. Imipramine and testosterone inhibited eslicarbazepine glucuronidation with IC_{50}s of 642.2 µM and 694.2 µM, respectively. This effect of imipramine was abolished with 500 µM eslicarbazepine and a reduction on the effect of testosterone was also observed. Propofol had a slight inhibitory effect on eslicarbazepine glucuronidation and only at the eslicarbazepine concentration of 100 µM.

To evaluate the UGT isoform selective inhibition, we investigated the inhibitory effects on the eslicarbazepine glucuronidation in recombinant UGT isoforms by incubating 500 µM eslicarbazepine with recombinant UGTs and 500 µM propofol, imipramine, diclofenac or testosterone. Eslicarbazepine glucuronidation by recombinant UGT1A4, UGT2B4, UGT2B17 and UGT1A9 was completely inhibited by diclofenac. Testosterone completely inhibited the eslicarbazepine glucuronidation by UGT1A4, UGT2B17 and partially inhibited glucuronidation by UGT1A9 (50%) and UGT2B4 (50%). Propofol completely inhibited eslicarbazepine glucuronidation by UGT1A9 and had also an effect on the UGT2B4 (45%) and UGT2B17 (35%). Imipramine had no effect on eslicarbazepine glucuronidation at eslicarbazepine concentration of 500 µM. The effect of inhibitors was not evaluated on UGT2B7, due to the low activity found in eslicarbazepine conjugation.

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Discussion

In this study we have identified the main UGT isozymes responsible for eslicarbazepine glucuronidation in the human liver by first investigating this reaction in pooled HLM, in individual HLM and using recombinant UGTs. Eslicarbazepine is conjugated into one glucuronide in human liver microsomesidentified by the fragmentation ion at *m*/*z* 431 $[M + H -176]^+$; the neutral loss of glucuronide moiety, 176 Da in the positive ion mode; the product ion spectrum from precursor m/z 431 in addition to the enzymatic hydrolysis of conjugation product. In opposition to HLM, in MLM two glucuronides of eslicarbazepine were found; however, only one was hydrolysed by Escherichia coli β-glucuronidase, an enzyme that preferentially hydrolyzes O-glucuronides over N-glucuronides (Zenser et al., 1999). The difficulty to obtain standards of eslicarbazepine glucuronides precluded the distinction between the N- or O-glucuronide of eslicarbazepine; however, the selectivity reported for Escherichia coli β-glucuronidase together with the fact that OXC undergoes O-glucuronidation (Flesch, 2004), point to the conjugation of eslicarbazepine in the hydroxyl group (O-glucuronide).

The Eadie-Hofstee plots show that eslicarbazepine glucuronidation in pooled HLM follows typical Michaelis-Menten kinetics with high and low affinity components (**figure 4**). According to the Eadie-Hofstee plot of the data in the presence of BSA more than one enzyme is involved in eslicarbazepine glucuronidation. All UGTs enzymes are capable of forming O-linked glucuronides (Mackenzie et al., 2005); however, N-glucuronidation of amines was described only for UGT1A4 and to a less extent for UGT1A3 (Green et al., 1998). Recently, UGT2B7 has been described to be involved in the N-glucuronidation of perfluorooctanesulfonamide (Xu et al., 2006) and of carbamazepine primary amine (Staines et al., 2004). The hydroxylated metabolites of carbamazepine are subject to O-glucuronidation; however, the enzymes involved were not yet characterised (Maggs et al., 1997). UGT1A1, UGT1A3, UGT1A4 and UGT1A9 were reported to be involved in the N-glucuronidation of retigabine (Borlak et al., 2006) and UGT1A4, UGT1A1,

UGT1A3, UGT1A6, UGT2B7 and UGT1A7 in the N-glucuronidation of lamotrigine (Rowland et al., 2006).

Glucuronidation of eslicarbazepine was detected in preparations of UGT1A4, UGT1A9, UGT2B4, UGT2B7 and UGT2B17. UGT2B7 and UGT2B4 have been reported to be involved in O-glucuronidation of 1'-hydroxymidazolam, a metabolite of midazolam, whereas 1'-hydroxymidazolam N-glucuronidation is catalysed by UGT1A4 (Zhu et al., 2008). O-glucuronidation of several coumarins, anthraquinones, and flavonoids was observed with UGT2B17 (Turgeon et al., 2003). There is no information in literature regarding the enzymes involved in O-glucuronidation of OXC.

The kinetic analysis of eslicarbazepine glucuronidation by HLM showed that the presence of 2% BSA does not significantly change the affinity of conjugation; however, the capacity was largely increased. The mechanism by which BSA affects the glucuronidation process has been evaluated in several studies (Rowland et al., 2006; Uchaipichat et al., 2006a; Rowland et al., 2007). It has been suggested that BSA is involved in the sequestration of inhibitory long-chain unsaturable fatty acids, particularly linoleic and arachidonic acids released from microsomes membranes during the course of incubation (Rowland et al., 2007). It was demonstrated that, in the presence of BSA, the magnitude of inhibitory interactions of fluconazol-zidovudine and valproic acid-lamotriguine *in vitro*, correctly predicted the in vivo interactions, improving the predictive capacity of the models used. Results herein suggest that adding BSA to the incubation medium may trigger UGTs involved in eslicarbazepine conjugation. It should be noted that in vivo experimental conditions about 30% is bound to plasma proteins (Almeida et al., 2009) and therefore the kinetic constants obtained on the present work are apparent constants.

Since UGT enzymes exhibit distinct, but overlapping, substrates selectivity (Wen et al., 2007), further correlation analysis and chemical inhibition studies were performed to determine the UGT isoforms responsible for eslicarbazepine conjugation. For the eslicarbazepine concentrations of 1000 µM the glucuronidation correlates well with trifluoperazine glucuronidation, which is catalysed mainly by UGT1A4. There was no

significant correlation with propofol and estradiol 3-glucuronide which are typical substrates of UGT1A9 and UGT1A1 respectively. Imipramine, an UGT1A4 substrate (Nakajima et al., 2002) and testosterone a substrate for UGT2B7, UGT2B17 and UGT2B15 (Bowalgaha et al., 2007) were equipotent in inhibiting eslicarbazepine glucuronidation (100 µM substrate concentration) in liver microsomes, while propofol had almost no effect. Diclofenac, on the other hand, was much more potent than imipramine and testosterone in inhibiting eslicarbazepine conjugation (IC_{50} of 17.0 µM, 642.2 µM and 694.2 µM, respectively). Diclofenac at 500 µM was found to inhibit UGT1A1, UGT1A3, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B7, UGT2B15 and UGT2B17 catalysed 4-methylumbelliferon glucuronidation (Uchaipichat et al., 2006b). In the present study, diclofenac markedly inhibited eslicarbazepine glucuronidation through UGT1A4, UGT1A9, UGT2B4 and UGT2B7.Inhibition of UGT1A4, UGT2B17 and partial inhibition of UGT2B4 and UGT1A9 eslicarbazepine glucuronidation was also observed in the presence of testosterone. In addition, propofol was found to inhibit not only UGT1A9 but also partially inhibit UGT2B4 and UGT2B17 mediated eslicarbazepine glucuronidation. Therefore, identification of specific UGTs was demonstrated to be challenging due to lack of isoform-specific probe substrates and inhibitors and overlapping substrate specificities (Uchaipichat et al., 2006b).

A previous study showed that the levels of therapeutic doses of eslicarbazepine ranged from 18.5±15.37 µM for 800 mg/day to 35.1±26.0 µM for 1200 mg/day (Brown and El-Mallakh, 2010). Because eslicarbazepine is 30% bound to serum albumin in vivo (Almeida et al., 2009), plasma concentration of unbound eslicarbazepine was calculated to be less than 25 µM. This suggests that although several UGT are able to metabolize eslicarbazepine the high affinity component of the UGT isozyme may plays a major role in eslicarbazepine glucuronidation. Therefore, the UGT isozyme, UGT2B4 may play the most important role in the eslicarbazepine glucuronidation. Based on the results of the current study, ESL metabolism is

not expected to be altered in patients with Gilbert Syndrome, relating to loss-of-function of UGT1A1 (Strassburg, 2008).

Frequently, AEDs are administered in combination with other anticonvulsants (Riva et al., 1996); therefore, the potential for the drug-drug interaction in the glucuronidation pathway doesfrequently exist between medications that are extensively glucuronidated. Pharmacokinetic interactions have been observed between ESL and phenytoin (Bial data on file), but no significant interaction was observed with topiramate (Nunes et al., 2010), carbamazepine, valproate, and levetiracetam (Bial data on file; see also (CHMP, 2009)). In a study in healthy subjects (Bial data on file, clinical trial BIA-2093-121, see also (CHMP, 2009)), concomitant administration of ESL (1200 mg) and phenytoin (300 mg) resulted in an average decrease of 31-33% in exposure to eslicarbazepine, most likely caused by an induction of glucuronidation, and an average increase of 31-35% in exposure to phenytoin, most likely resulting from inhibition of CYP2C19, which is known to be involved in the first pass metabolism of phenytoin (Nakajima et al., 2007). A population pharmacokinetics analysis of phase III studies in epileptic adult patients indicated that carbamazepine increases eslicarbazepine clearance (Almeida et al., 2009), the probable mechanism being a dose dependent induction of glucuronidation (CHMP, 2009). Recently, phenytoin has been shown to be involved in the inhibition of UGTs (UGT1A6, UGT1A9, and UGT2B15) responsible for detoxifying of acetaminophen through the glucuronidation pathway (Kostrubsky et al., 2005) and valproate increased lamotrigine concentration due to UGT2B7 inhibition (Rowland et al., 2006). Interaction of valproate with carbamazepine glucuronidation has also been described (Bernus et al., 1997). Glucuronidation of valproate (30-70% of which is eliminated via glucuronidation) has been reported to be carried out by UGT1A3, UGT1A6, UGT1A9, UGT2B7 and UGT2B15 (Ethell et al., 2003) and carbamazepine N-glucuronidation is mediated by UGT2B7 (Staines et al., 2004). However, UGTs involved in glucuronidation of the 13 hydroxylated metabolites of carbamazepine were not yet described.

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Lamotrigine glucuronidation by UGT1A4 and UGT2B7 account for approximately 70% of compound elimination (Rowland et al., 2006); however, no significant pharmacokinetic interaction between ESL and lamotrigine was observed (Almeida et al., 2010), which indicates that eslicarbazepine does not interfere with lamotrigine glucuronidation. Conversely, lamotrigine interacts with retigabine, which undergoes predominantly N-glucuronidation by UGT1A1, UGT1A3, UGT1A4 and UGT1A9, and its renal excretion (Hermann et al., 2003).

In conclusion, glucuronidation of eslicarbazepine results from the contribution of UGT1A4, UGT1A9, UGT2B4, UGT2B7 and UGT2B17, but the high affinity component of UGT2B4 isozyme may play a major role at therapeutic plasma concentrations of unbound eslicarbazepine.

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Authoship contribution

Participated in research design: *Loureiro, Bonifácio, Wright, and Soares-da-Silva.*
Conducted experiments: *Loureiro, Fernandes-Lopes, and Bonifácio.*
Contributed new reagents or analytic tools: *Loureiro, Fernandes-Lopes, and Bonifácio.*
Performed data analysis: *Loureiro, Fernandes-Lopes, Bonifácio, Wright, and Soares-da-Silva.*
Wrote or contributed to the writing of the manuscript: *Loureiro, Fernandes-Lopes, Bonifácio, Wright, and Soares-da-Silva.*

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Legends

Figure 1. HLPC-MS/MS representative chromatogram of human liver microsomes (HLM) incubated with eslicarbazepine. The incubation mixture contains pooled HLM and eslicarbazepine in the presence of UDPGA.

Figure 2. MS/MS spectra for eslicarbazepine glucuronide. a) Representative product ion spectrum for m/z of 431 and b) neutral loss spectra for the glucuronide moiety 176 Da. The incubation mixture contains pooled human liver microsomes (HLM) and eslicarbazepine in the presence of UDPGA.

Figure 3. HLPC-MS/MS representative chromatogram of mouse liver microsomes (MLM) incubated with eslicarbazepine. The incubation mixture contains pooled MLM and eslicarbazepine a) in the absence b) in the presence of UDPGA and c) in the presence of UDPGA after treatment with β-glucuronidase.

Figure 4. Kinetics of eslicarbazepine glucuronidation in human liver microsomal pools. Eslicarbazepine concentrations ranged from 10 to 1000 µM, in the presence and in the absence of 2 % BSA. A) The Michaelis-Menten equation was used to fit eslicarbazepine glucuronidation data. B) Eadie-Hofstee plots for the same data. Values represent means ± sem of duplicates. Velocities are expressed as relative units (peak area ratio/mg protein/min).

Figure 5. Eslicarbazepine glucuronidation catalysed by recombinant human UGT enzymes. Each recombinant enzyme (total protein 0.5 mg/ml) was incubated for 120 min with 1000 µM eslicarbazepine at 37ºC. Values represent mean ± sem of 2 determinations. Results are expressed as relative units (peak area ratio)

Figure 6. Kinetics of eslicarbazepine glucuronidation by recombinant human UGT1A9, UGT2B4 and UGT2B17 enzymes (total protein 0.5 mg/ml) in the presence and in the absence of 2 % BSA. Eslicarbazepine concentrations ranged from 10 to 1000 µM. Values represent mean ± sem of duplicates. Lines represent the fitting curves to Michaelis-Menten equation. Velocities are expressed as relative units (peak area ratio/mg protein/min).

Figure 7. Correlation analysis between eslicarbazepine glucuronidation and specific substrates glucuronidation in single donor human liver microsomes. Liver microsomes from 5 donors (HG3, HG93, HG95, HG64, HH13) were incubated with 1000 µM of eslicarbazepine for 60 min at 37 ºC. Values represent mean ± sem of duplicates. Results are expressed as relative units (peak area ratio)

Figure 8. Inhibitory effect of imipramine, diclofenac, testosterone and propofol on eslicarbazepine glucuronidation. Eslicarbazepine glucuronidation was evaluated in pooled HLM at the concentration of 100 µM eslicarbazepine in the

presence of inhibitors (10, 50, 100, 500 and 1000 μM). Values represent mean ± sem of duplicates.

TABLE 1

Apparent kinetic parameters of eslicarbazepine glucuronidation in HLM and recombinant UGT enzymes

	K_m (*μM*)		V_{max} (*peak area ratio/mg protein/min*)	
	No BSA	2% BSA	No BSA ($x10^4$)	2% BSA ($x10^4$)
HLM	412.2 ± 63.8	349.7 ± 74.3	27.1± 1.8	98.7± 8.7
UGT1A9	1299.0 ± 841.8	577.1 ± 330.1	43.0± 1.8	48.0±1.3
UGT2B4	157.0 ± 31.2	28.7 ± 10.1	1.7± 0.2	0.9± 0.07
UGT2B17	524.2 ± 195.9	279.3 ± 62.9	1.5± 0.25	1.4±0.2

Values represent mean ± sem of duplicate samples.

TABLE 2

Activities of UGTs in single donor human liver microsomes. Enzyme activity is expressed as pmol product per mg protein per min. Data is from BD Biosciences.

	Assay	HG3	HG93	HG95	HG64	HH13
UGT1A1	Estradiol-3-glucuronidation	360	180	750	690	2600
UGT1A9	Trifluoroperazine-glucuronidation	7200	4300	4000	680	6400
UGT1A4	Propofol-glucuronidation	890	600	400	570	250

Figure 1

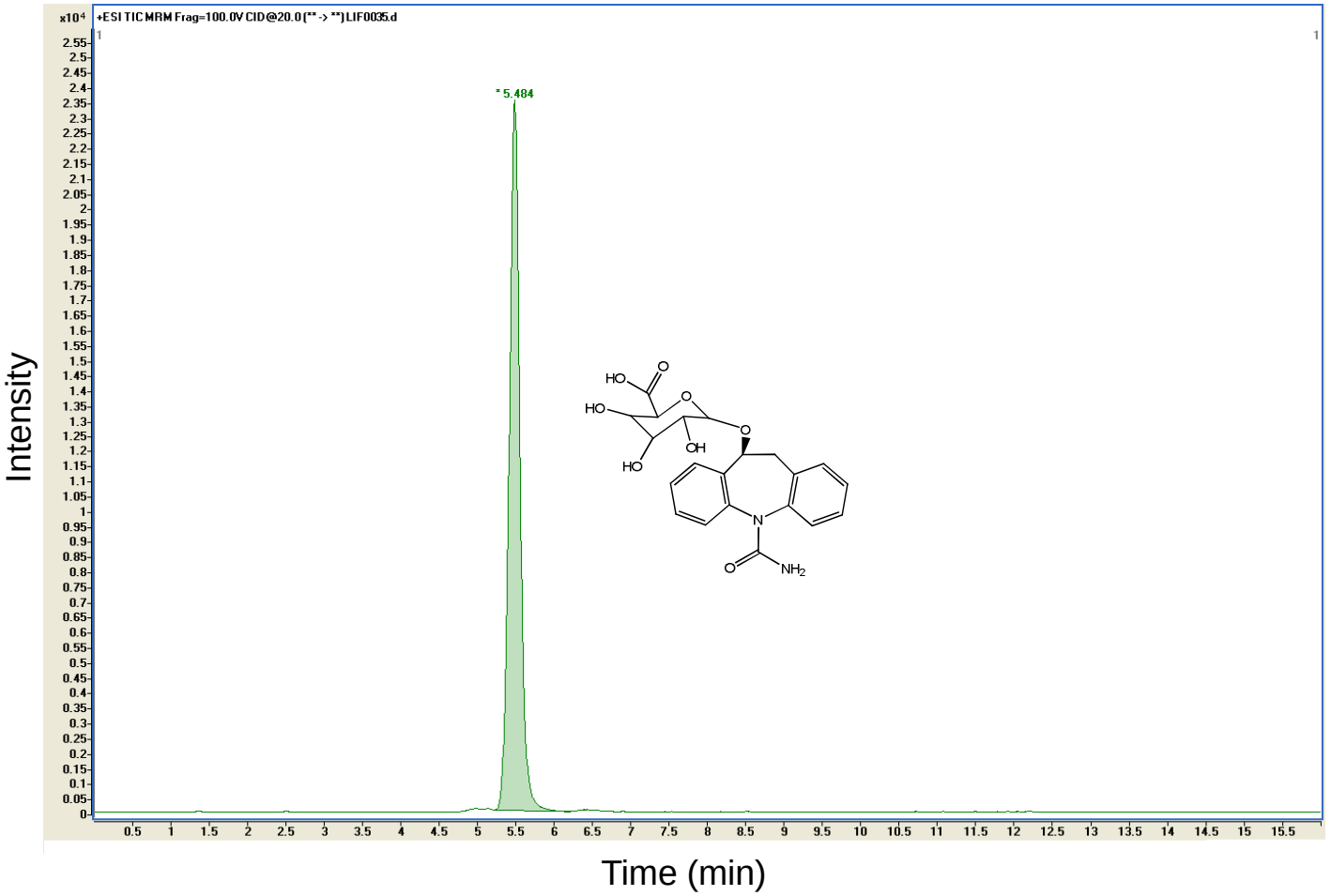
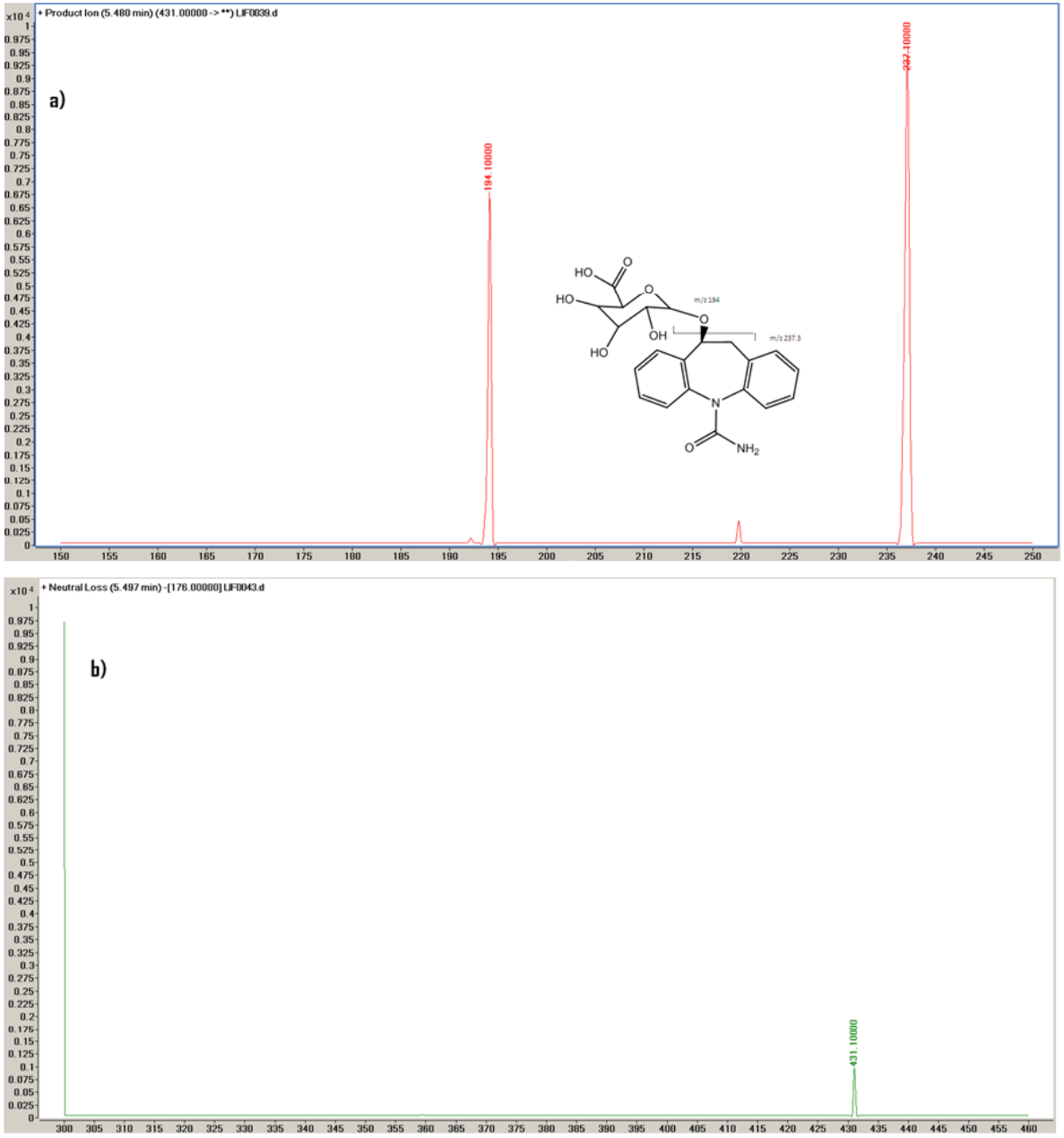


Figure 2



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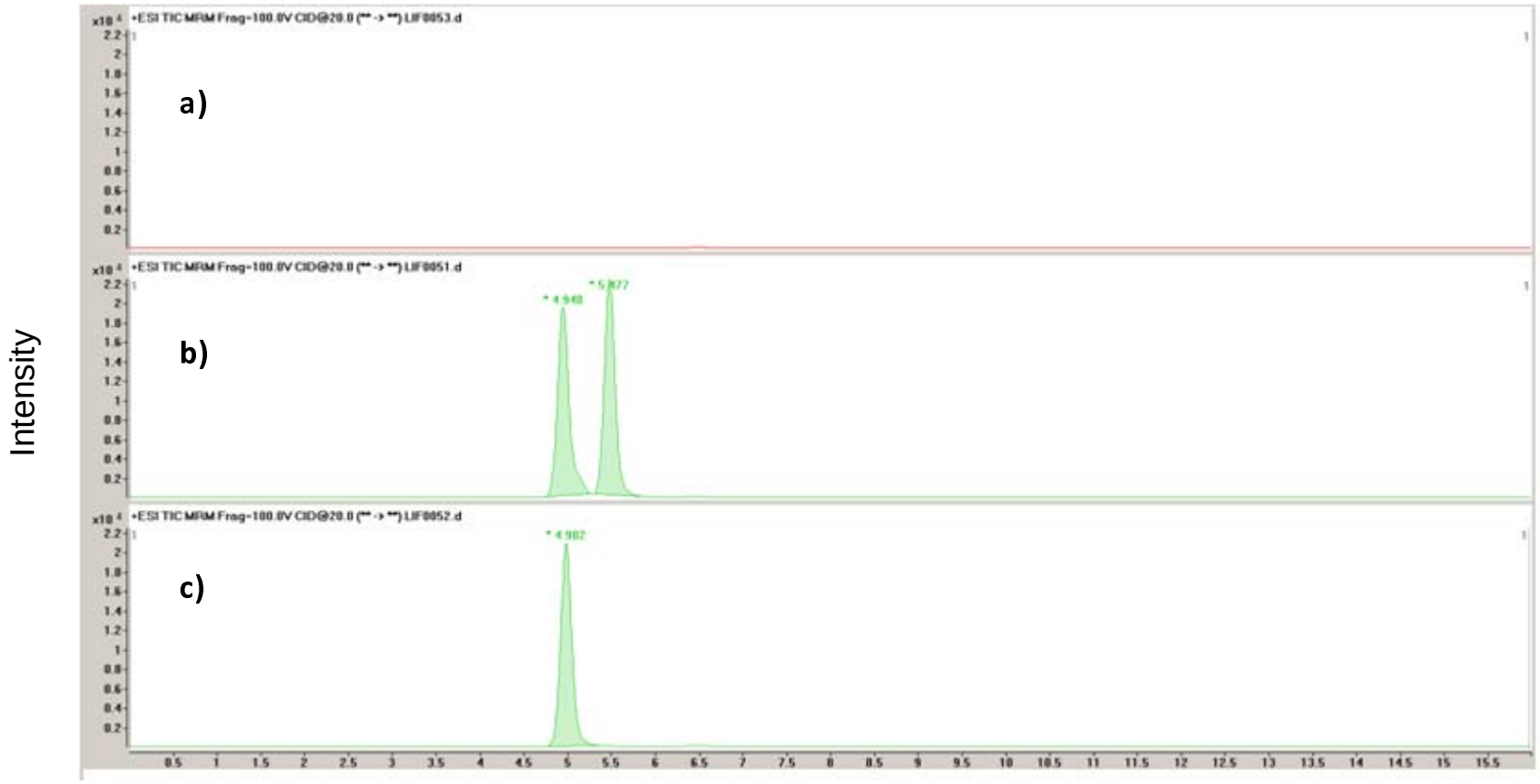

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Figure 4

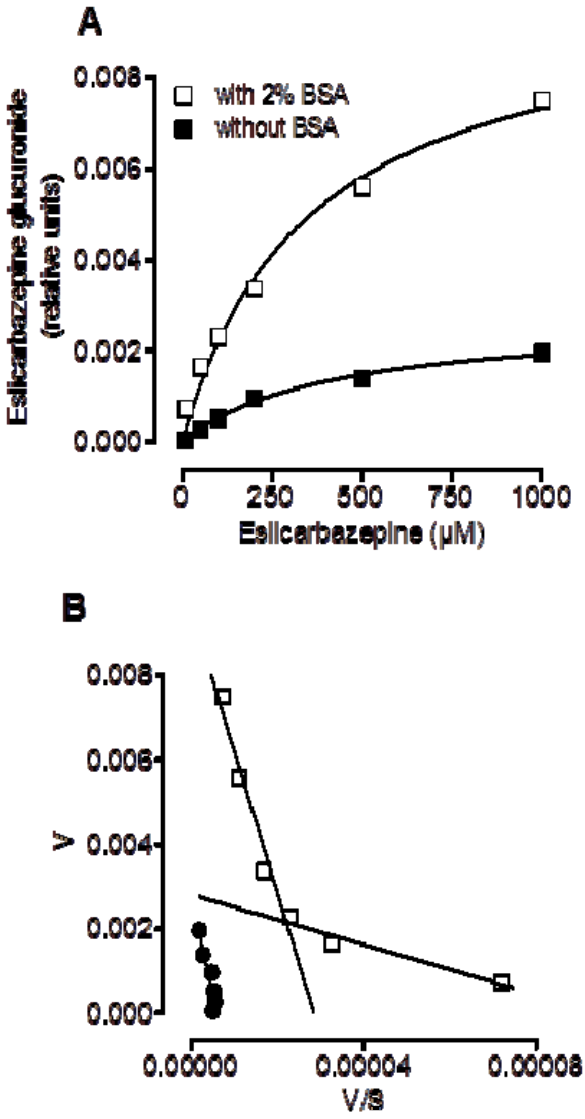

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Figure 5

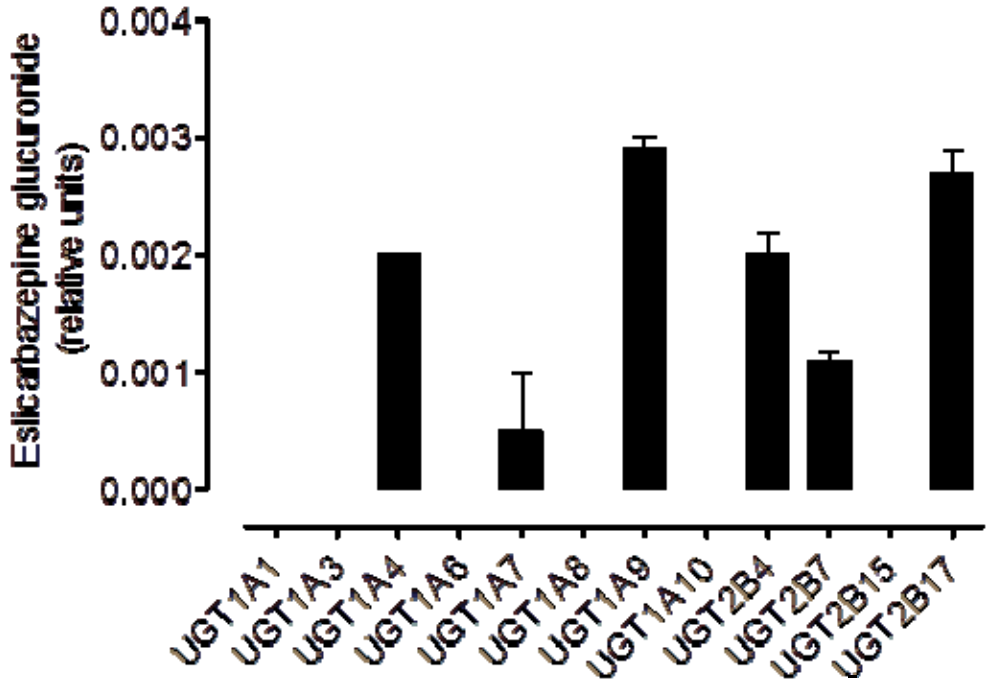

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Figure 6

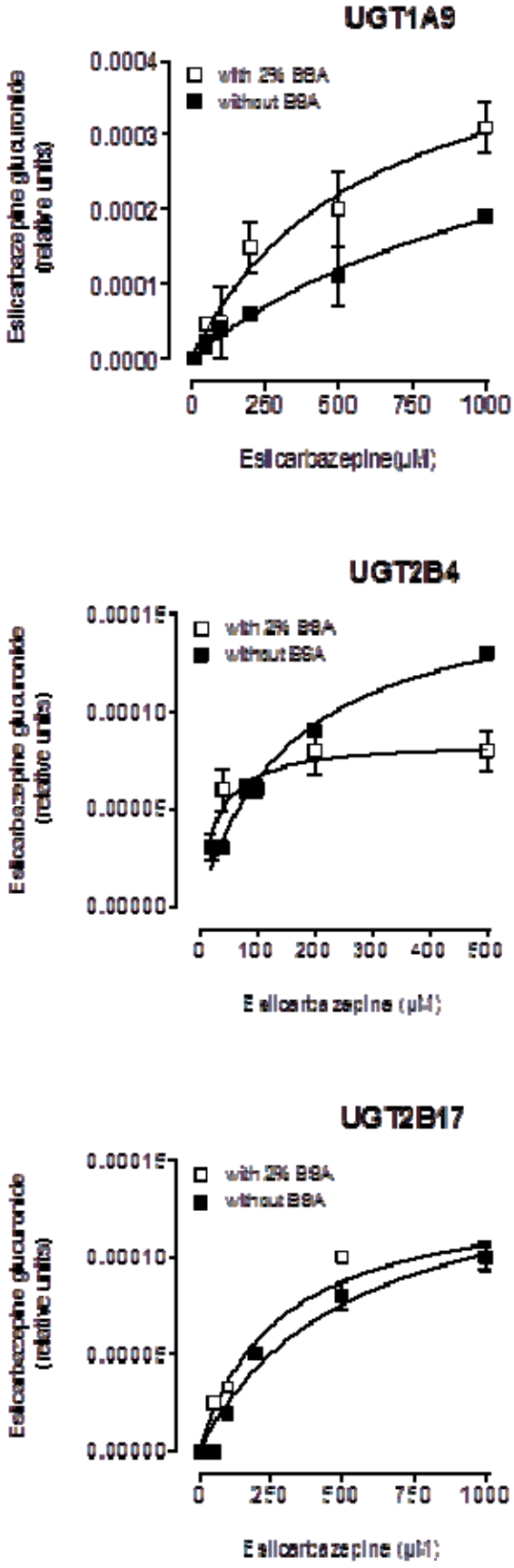

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Figure 7

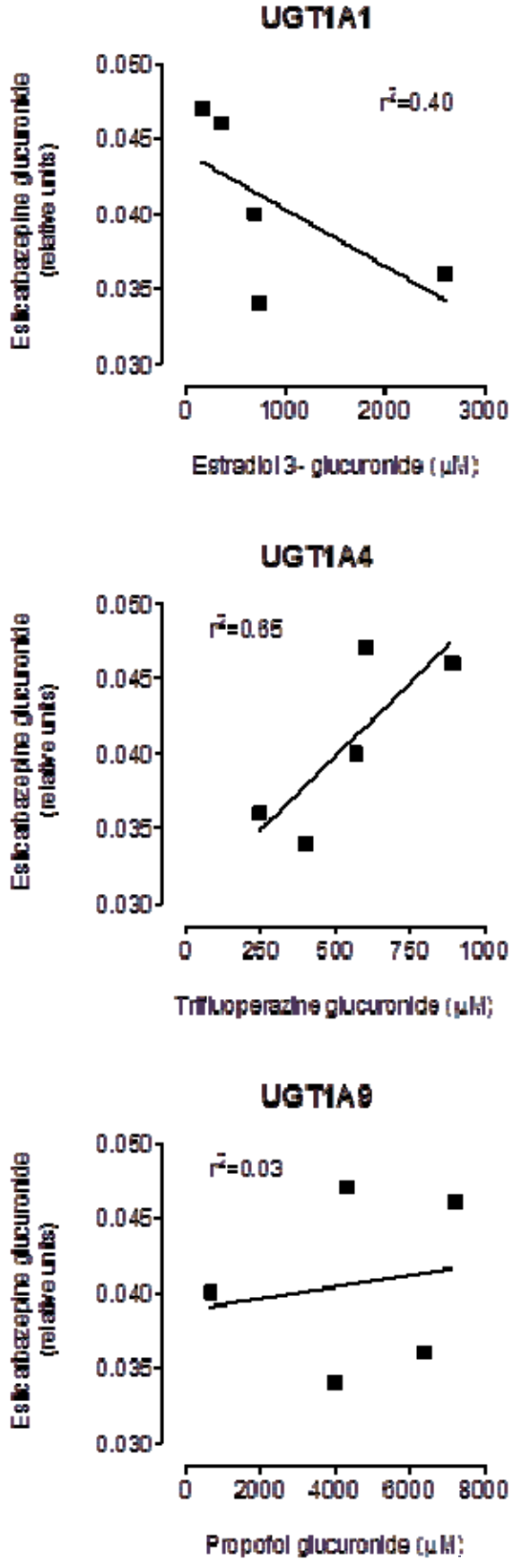

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Figure 8