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N-Glucuronidation of Carbamazepine in Human Tissues is Mediated by UGT2B7

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Running title: Carbamazepine is glucuronidated by human UGT2B7

Abbreviations:

CBZ - Carbamazepine (5H-dibenzo*[b,f]*azepine-5-carboxamide)

UGT - Uridine diphosphoglucuronosyltransferase

UDPGA – Uridine diphosphoglucuronic acid

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Abstract:

Carbamazepine (CBZ) is one of the most widely prescribed anticonvulsants despite a high incidence of idiosyncratic side effects. Metabolism of CBZ is complex, and of the more than 30 metabolites identified one of the most abundant is CBZ *N*-glucuronide. To date the UGT isoform responsible for the *N*-glucuronidation of CBZ has not been identified. We have developed a sensitive liquid chromatography/mass spectrometry assay to quantify CBZ glucuronidation, and we report that CBZ is specifically glucuronidated by human UGT2B7. Kinetics of CBZ glucuronidation in human liver, kidney and intestine microsomes were consistent with those of recombinant UGT2B7, which displayed a K_m of 214 μM and V_{max} of 0.79 pmol/mg/min. In addition to revealing the isoform responsible for CBZ glucuronidation, this is the first example of primary amine glucuronidation by UGT2B7.

Introduction:

Carbamazepine (5H dibenzo*[b,f]*azepine-5-carboxamide) is one of the most widely prescribed anticonvulsants, and is used to treat a variety of conditions from epilepsy to muscle spasm and trigeminal neuralgia. However its use is associated with a number of idiosyncratic adverse side effects including skin rash, blood disorders and hepatitis in one third to one half of patients (Ju and Uetrecht 1999). These adverse side effects have been associated with the formation of CBZ metabolites (Riley *et al.,* 1989; Shear and Spielberg 1988) therefore the study of all CBZ metabolites has important clinical implications.

The metabolism of CBZ is complex and has been widely studied in man and in animal models (Madden *et al.,* 1996; Maggs *et al.,* 1997) with over 30 metabolites (Lertratanangkoon and Horning 1982) identified. The major metabolites are the 10,11-epoxide and its hydrolytic *trans*-dihydrodiol product. Glucuronidation is also an important detoxification pathway as the CBZ *N*-glucuronide and glucuronides of the hydroxylated metabolites are significant urinary metabolites, however to date no glucuronide metabolite has been implicated in the incidence of side effects. Epoxide hydrolase has been the focus of most attention as a potential source of toxic metabolites; however the available data do not support a major role for this enzyme in causing side effects (Green *et al.,* 1995b; Pirmohamed *et al.,* 1992). One minor metabolite, 2-hydroxy-CBZ, formed through loss of the carboxamide, could be metabolised to an iminoquinone, which due to its potential chemical reactivity might be the metabolite responsible for the idiosyncratic reactions, though this has not been shown to date (Ju *et al.,* 1999). In addition CBZ is also a well-known enzyme inducer up-regulating CYP (Luo *et al.,* 2002) and UGT activity/expression (Tanaka 1999)

Carbamazepine is metabolised to an *N*-glucuronide (Bauer *et al.,* 1976); in addition glucuronide metabolites have been demonstrated for all thirteen of the hydroxylated metabolites of CBZ (Maggs *et al.,* 1997). Formation of *N*-glucuronides has been principally

studied in non-rodent species, as rats and mice do not readily form *N*-glucuronides, particularly quaternary ammonium glucuronides from tertiary amines (Maggs *et al.,* 1997; Hucker *et al.,* 1978). For this reason rabbit and guinea pig have often been used as model systems to study *N*-glucuronidation. However rats do form the *N*-glucuronide of CBZ (Madden *et al.,* 1996), suggesting that CBZ might be a substrate for a different isoform to those normally responsible for *N*-glucuronidation (i.e. human UGTs 1A4 and 1A3 and their orthologs). Previous attempts to identify the UGT isoform(s) responsible for carbamazepine glucuronidation have failed; however these focussed on UGT1A3 (Green *et al.,* 1998) and UGT1A4 (Green *et al.,* 1995a) (which glucuronidate tertiary amines), UGT1A6 and UGT1A9 which are the major drug glucuronidating enzymes (Ebner and Burchell 1993) and UGT1A1, the bilirubin UGT which also has activity towards some drugs and naturally-occurring tricyclic compounds (Senafi *et al.,* 1994). It is possible that the failure to identify the isoform responsible was due to a lack of sensitivity of the methods being employed to detect glucuronide formation. This was the case with nicotine, where the UGT isoform responsible could not be identified (from among 13 available recombinant isoforms) despite the *N*-glucuronide being the major metabolite in man (Nakajima *et al.,* 2002). Recent studies have indicated that LC/MS may detect glucuronide metabolites with 100-1000 times more sensitivity (Csala *et al.,* 2004; Staines *et al.* 2004; Staines *et al.* in preparation) than conventional techniques such as TLC/HPLC with radiolabelled UDPGA (Ethell *et al.,* 1998) and therefore is a more suitable technique for detection of low levels of glucuronide conjugates.

Here we report the characterisation of the kinetics of CBZ glucuronidation in human tissue microsomes using a very sensitive LC/MS-MS technique. We also report the study of the glucuronidation of CBZ by recombinant UGTs (stably expressed in V79 cells) and demonstrate that CBZ is glucuronidated by UGT2B7.

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Methods:

Chemicals and Reagents

Unless stated otherwise chemicals, including β-D-uridine diphosphoglucuronic acid (UDPGA) and carbamazepine, were purchased from Sigma-Aldrich (Gillingham, UK). [Glucuronyl-^{14}C (U)]-UDPGA was purchased from Perkin Elmer (Beaconsfield, UK). Formic acid and acetonitrile (HPLC grade) were purchased from Merck Eurolab (Poole, UK).

Enzyme assays

Microsomes were prepared from frozen human tissue (UK Human Tissue Bank, Leicester, UK) by standard methods (Coughtrie *et al.*, 1991). Cell lines (V79 Chinese hamster lung fibroblasts) expressing human UGTs have been created previously in this laboratory (Fournel-Gigleux *et al.*, 1989; Jackson *et al.*, 1987; Wooster *et al.*, 1991; Fournel-Gigleux *et al.*, 1991) except UGT1A4 expressed in the baculovirus/Sf9 cell system (kindly donated by Robert Tukey (La Jolla, California)). UGT-expressing cells were grown, and cell lysates prepared, as described previously (Fournel-Gigleux *et al.*, 1991). Cell membranes were disrupted by sonication. Frozen cell pellets (originally from two 15 cm diameter tissue culture plates) were thawed and resuspended in 200 µl phosphate buffered saline at pH 7.4. The 200 µl suspension was sonicated for 5 x 5 seconds (MSE soniprep 150, Sanyo Gallenkamp) on ice, with 1 minute cooling on ice between bursts. Microsomes were diluted in PBS to give 5-10 mg/ml and sonicated as above. Protein concentrations were determined post sonication by the method of Lowry *et al.* (Lowry *et al.*, 1951).

Carbamazepine Glucuronidation Assays:

CBZ (up to 5 mM final concentration in the incubation, from a stock solution in DMSO) was added to the assay mixture containing 350 µg sonicated microsomes (or 200 µg

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sonicated cell lysate) in 100 mM Tris/maleate buffer (pH 7.5) containing 5 mM $MgCl_2$. The reaction was initiated by addition of UDPGA (10 mM final concentration in the incubation, from 100 mM stock in PBS) to give a final reaction volume of 100 µl. Samples (assayed in triplicate) were incubated for 3 hours at 37 °C as a standard condition; a time course experiment with human liver microsomes indicated that the reaction was linear up to 3 hours (data not shown). The enzyme reactions were terminated by the addition of 100 µl of cold (-20 °C) acetonitrile to the incubation mixtures. Samples were frozen at -20 °C for 20 minutes, thawed and centrifuged at 14,000 x g for 5 minutes. The supernatant was removed and stored at -20 °C until analysis for the presence of CBZ glucuronide.

Sample Analysis by LC/MS-MS

Samples were analysed by LC/MS-MS using a HP1100 LC (Agilent Technologies, Stockport, UK) system connected to a Micromass LC Quattro (Micromass, Manchester, UK) with a 10 µl injection per run. The LC separation used a mobile phase of 0.1 % (v/v) formic acid (buffer A) and acetonitrile containing 0.1 % formic acid (v/v) (buffer B). LC separation and elution were achieved using a 1 minute isocratic segment at 5 % buffer B followed by a gradient of 5-100 % buffer B over 4 min. This was followed by a 2 min wash phase at 95 % buffer B and a 3 min reequilibration step at 5 % buffer B. Separations were performed with a Waters Spherisorb (ODS2) 2 µm, 2.1 mm x 150 mm column at a flow rate of 0.3 ml/min with a 2 cm Hypersil (ODS) guard column.

Mass spectral analysis was performed by direct infusion into the electrospray source, with column diversion during the first 2.5 minutes to protect the source from excessive salt. The glucuronide peak from the LC column was analysed using a SRM (single reaction monitoring) method in positive ion mode. Optimized SRM conditions used the transition from 413.3>237.0 m/z at a cone voltage of 25 eV and capillary voltage of 3.0 eV; collision energy

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was 15 eV and the collision gas was at 3 mbar. The nebulising gas was set at 100 l/hr and the desolvation gas at 700 l/hr. To confirm the peak as CBZ glucuronide a daughter ion scan was performed across the peak at 413.3 m/z parent ion, over a mass range from 50 to 413.3 m/z.

Carbamazepine Glucuronide Quantification

Carbamazepine glucuronide standard was not available and due to lack of sensitivity we could not quantify it using a parallel incubation performed in the presence of [^{14}C]-UDPGA as described previously (Staines *et al.* 2004). In order to quantitate the glucuronide produced, two identical CBZ glucuronide standards were generated by parallel incubation of CBZ with rabbit liver microsomes and UDPGA, using the conditions described above, in a final volume of 500 µl. CBZ was extracted from the samples by addition of an equal volume of chloroform, followed by mixing for 10 seconds. The chloroform layer containing the CBZ was removed and this process was repeated twice. For one sample the aqueous layer, containing the carbamazepine glucuronide, was subjected to alkali lysis at 50 °C for 1 hour by addition of 250 µl of 30% (v/v) ammonia, followed by neutralization with 130 µl formic acid. The parallel control sample had 380 µl of 1 M ammonium acetate added. This experiment was performed in triplicate, and samples were prepared for LC/MS-MS analysis as before.

Quantification was achieved by analysis of the above samples for both carbamazepine and carbamazepine glucuronide. This was performed using the same LC conditions as before. Detection was with a dual MRM (multiple reaction monitoring) method in positive ion mode with dual transitions at 413.3 > 237.0, (cone voltage 25 eV, Capillary 3.0 eV, Collision energy 15 eV) and at 237.3>194.1 (cone voltage 40 eV, Capillary 3.0 eV, Collision energy 20 eV) for CBZ glucuronide and CBZ respectively; collision gas was at 3 mbar. The relative decrease in the carbamazepine glucuronide between the hydrolysed and non-hydrolysed samples was analysed with respect to the consequent increase in carbamazepine levels, and

this was used to calculate the difference in ionization potential between them. A standard curve for each experiment was subsequently generated using a CBZ standard from 0.1 to 200 μM. Levels of carbamazepine glucuronide were then extrapolated from the standard curve using the relative difference in ionisation potential between the CBZ glucuronide and the CBZ.

Determination of Kinetic Parameters

Kinetic parameters for carbamazepine glucuronide formation were determined using the standard assay above, performed in duplicate, with variations in the concentration of carbamazepine (final concentrations were 0, 5, 10, 20, 50, 75, 100, 200, 350, 500, 750, 1,000, 5,000, 10,000 μM). Standard Michaelis-Menten curves were used to calculate the kinetic parameters. (Kaleidagraph, Synergy Software, Reading, PA, USA):

Inhibition of Carbamazepine Glucuronidation.

To further investigate the role of various UGT isoforms in the glucuronidation of carbamazepine a competitive inhibition assay was performed with known human UGT2B7 substrates: hyodeoxycholic acid, androsterone and morphine (Coffman *et al.*, 1997; Gall *et al.*, 1999; Soars *et al.*, 2003). Since anticonvulsant drugs are often prescribed in combination, inhibition assays were also performed with a number of common anticonvulsants: valproate, lamotrigine, phenobarbital, oxazepam, temazepam and lorazepam. Assays were performed for 60 minutes with human liver microsomes using the same conditions as for the kinetic experiments, with a carbamazepine concentration of 100 μM and potential inhibitors at 1000 μM.

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Results:

LC/MS Assay

An effective LC/MS assay was developed to detect CBZ glucuronide produced from UGT-expressing cell lines and human tissue microsomes (Figure 1). The limit of detection of this method was estimated at 10 fmoles, which translates to a minimum detectable UGT reaction rate of <1 fmole/mg/min (for standard 1 hour assay, with 250 μg protein). This is 8,000 times more sensitive than the best radioactive LC assay used routinely to detect glucuronides (Ethell *et al.*, 1998).

Confirmation of CBZ Glucuronide

The structure of CBZ and the structure of the glucuronide are shown in Figure 2. CBZ is predicted to form only one glucuronide, through the terminal nitrogen, as previous studies have not indicated the formation of the tertiary ammonium glucuronide salt (Lynn *et al* 1978). The mass spectrum of the CBZ glucuronide is shown in Figure 3. The fragmentation peaks are consistent with the breakdown of a CBZ glucuronide with characteristic peaks at m/z 395 for the parent ion and m/z 237 for the aglycone (from loss of the neutral glucuronic acid) with additional peaks at m/z 220 (deamination of the CBZ aglycone) and m/z 377 (dehydration of the CBZ glucuronide). No peaks for the glucuronic acid can be seen, which is expected in positive ionisation mode. No CBZ glucuronide was observed when control experiments were performed without UDPGA, microsomes or CBZ. CBZ glucuronide was not a substrate for bovine β-glucuronidase.

Glucuronidation of CBZ by Human Microsomes and Recombinant Human UGTs

Figure 4 and Table 1 show the formation of CBZ glucuronide by different human UGT isoforms and human liver microsomes. It is clear from these data that UGT2B7 is the

only isoform studied here that forms the CBZ glucuronide. All of the three tissue types analysed show formation of the CBZ glucuronide. Examples of the rate *vs* substrate concentration curves for the glucuronidation of CBZ in each different system are shown in Figures 5a-d. The kinetic parameters K_m and V_{max} for the three human tissue types and UGT2B7 were calculated from these data and are presented in Figure 5. Quantification was achieved using the relative difference in ionization potential between the CBZ glucuronide and CBZ (calculated at 1 : 2.2).

Inhibition of CBZ Glucuronidation in Human Liver Microsomes.

The result of inhibition of CBZ glucuronidation in human liver microsomes by a variety of other drugs is shown in Figure 6. The UGT2B7 substrates chosen are considered probe substrates for this isoform. The data here show clear inhibition by all the known UGT2B7 substrates. Of the other anticonvulsants drugs used the three benzodiazepines showed significant inhibition, and valproic acid shows a slight inhibition. No inhibition was observed with phenobarbital or lamotrigine.

Discussion:

Studies of the glucuronidation of many compounds have, in the past, been limited by the sensitivity of detection techniques for glucuronides. The use of radiolabeled UDPGA (Ethell *et al.,* 1998) is one solution to this problem, however the practical limit of detection is equivalent to a glucuronidation rate of approximately 10 pmol/mg/min; this is limiting for many compounds which are glucuronidated at low rates, when using low levels of precious proteins or low substrate concentrations for kinetic analysis. LC/MS-MS is now increasingly used to detect glucuronides with a limit of sensitivity in the femtomole range (Staines *et al.* 2004; Staines *et al* in preparation). Even when using the maximum CBZ concentration, the

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glucuronidation rate with human liver microsomes is below the limits of detection even for radiolabelled HPLC. This very low activity observed with CBZ explains why no previous studies have detected CBZ glucuronide formation *in vitro*.

Analysis of CBZ glucuronidation by tissue microsomes and the major human UGT isoforms showed that CBZ can be metabolised by liver, kidney and intestine microsomes and that the only isoform catalysing formation of CBZ glucuronide was UGT2B7. This is consistent with knowledge of the tissue distribution of UGT2B7, which has been shown to be expressed in liver, kidney and intestine (Strassburg *et al.*, 2000). Kinetic analysis showed a similar K_m for all three tissue types and for recombinant UGT2B7 (~200 μM), strongly supporting the data that UGT2B7 is a major isoform responsible for glucuronidation of CBZ in these tissues. The fact that UGT2B7 and not UGT1A4 catalyses CBZ glucuronidation was also supported by the evidence that CBZ is glucuronidated *in vivo* by rats; there is no human UGT1A4 ortholog in rat tissues, whilst there is a UGT2B7 ortholog in rat (UGT2B1). The K_m value reported here (approximately 200 μM) is typical for UGT2B7 substrates, which are usually between 50 μM -500μM (Cheng *et al.*, 1998). The V_{max} values for all tissue types and for UGT2B7 were very low, but similar levels have been observed previously. All three UGT2B7 substrates tested (androsterone, morphine and hyodeoxycholic acid) showed significant inhibition of CBZ glucuronidation, which supports the assertion that UGT2B7 is the major isoform glucuronidating CBZ.

It has been shown that UGT1A3 and UGT1A4 are the principal enzymes responsible for *N*-glucuronidation (Green and Tephly 1998). Only recently has *N*-glucuronidation by another UGT (UGT2B7) been reported, in the glucuronidation of the secondary amine of an indole-2-one group (Zhang *et al.*, 2004). Here we report the first evidence that a UGT other than UGT1A3/1A4 can glucuronidate a primary amine; in addition UGT2B7 is the first isoform to be shown to glucuronidate the carbamoyl group.

Despite the incidence of side effects CBZ is widely used, often in combination with other anticonvulsants and therefore there exists the potential for drug-drug interactions. Valproate, another commonly prescribed anticonvulsant with idiosyncratic side effects has recently been shown by our laboratory to be glucuronidated by UGT2B7 (Ethell *et al.*, 2003), but in this case valproate was also glucuronidated by UGT1A6 and UGT1A9. The contribution of each of these isoforms is dependent on the relative expression levels in different tissues, which are only currently being established; however it is clear from the inhibition data presented here that valproate has minimal effect on carbamazepine glucuronidation in liver microsomes. This might be predicted, as UGT1A6 is a dominant isoform in liver and UGT1A9 is the dominant isoform in kidney. In the intestine however the effects of competitive inhibition of CBZ glucuronidation by valproate would be more pronounced, as UGT2B7 is a major isoform present in the intestine (Strassburg *et al.*, 2000, Staines *et al.*, 2004).

Another anticonvulsant that is often co-administered with CBZ is lamotrigine and this drug, like most amines, has been shown to be a substrate for UGT1A4 (Green *et al.*, 1995a). No other isoform has been tested to date, however valproate has been shown to inhibit lamotrigine glucuronidation, suggesting that UGT1A1, 1A9 and/or 2B7 can also metabolise lamotrigine. The data presented here indicate that lamotrigine and carbamazepine are not metabolised by the same UGT isoform. The three benzodiazepines used in the present study all inhibited CBZ glucuronidation in human liver microsomes, suggesting that they have the potential to disrupt carbamazepine glucuronidation. *R*-oxazepam has been shown to be a substrate for UGT2B7 (though not exclusively) (Court *et al.*, 2002), and from the data presented here it is likely that temazepam and lorazepam are also substrates for UGT2B7. This has implications for the use of these drugs in combination with CBZ. Various anticonvulsants, including carbamazepine, phenobarbital and phenytoin, are potent inducers

of various drug metabolising enzymes, including CYPs and UGTs (Soars *et al.*, 2004). Recent studies demonstrated induction of UGT2B7 by CBZ, though induction of UGT1A6 was also observed (Soars *et al.*, 2004). This demonstration of induction of UGT2B7 in tissue culture suggests that the rate of glucuronidation of CBZ could be increased by induction of UGT2B7.

By applying LC/MS technology we have identified for the first time the UGT isoform responsible for the glucuronidation of CBZ *in vitro*. This is the first example of primary amine glucuronidation by UGT2B7 and of glucuronidation at a carbamoyl group. This specificity of UGT2B7 for CBZ has implications for drug-drug interactions with other therapeutic agents commonly used to treat epilepsy and other disorders, since this isoform metabolises a wide range of therapeutic drugs.

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Footnotes

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FIGURE LEGENDS

Figure 1: LC/MS-MS trace of carbamazepine glucuronide generated from incubation with human liver microsomes sample using SRM detection from 413.3>237.0 m/z. Incubation was made under standard assay conditions with 1000 μM CBZ and 10 mM UDPGA (see Materials and Methods)

Figure 2: Schematic showing the structure of carbamazepine and its β-D-glucuronide.

Figure 3: Mass spectrum of carbamazepine β-D-glucuronide. The carbamazepine glucuronide spectrum was generated from a daughter ion scan at 413.3 m/z of a human liver microsomal incubation. Fragments of the carbamazepine glucuronide correspond to the carbamazepine aglycone (237 m/z), deaminated carbamazepine aglycone (220 m/z) dehydrated carbamazepine glucuronide (395 m/z), doubly dehydrated carbamazepine glucuronide (377 m/z).

Figure 4: Levels of CBZ β-D-glucuronide detected following incubation with microsomes, V79 cell lines and baculovirus infected insect cells expressing human UGTs. Data represent mean of a triplicate experiment; error was less than 10 % in all cases.

Figure 5a-d: Kinetic analysis of CBZ glucuronidation by microsomes or cell lines expressing human UGTs. a) human liver microsomes, b) human kidney microsomes, c) human intestine microsomes and d) human UGT2B7. The data points are based on the mean of a duplicate experiment.

Figure 6: Inhibition of carbamazepine glucuronidation in human liver microsomes by UGT2B7 probe substrates and common anticonvulsant agents: a) androsterone, b) HDCA (hyodeoxycholic acid), c) morphine, d) lorazepam, e) oxazepam, f) temazapam, g) valproic acid, h) phenobarbital and i). lamotrogine Reactions were performed with standard conditions including 100 μM carbamazepine and 1 mM of the potential inhibitor.

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Table 1 : Relative rates of CBZ β-D-glucuronide formation by human tissue microsomes and cloned/expressed UGT's

Source of UGT	**UGT Activity (pmol/mg/min)**	**Probe Substrate**	**UGT Activity with Probe Substrate (pmol/mg/min)**
human liver	3.2	1-naphthol morphine	4000 520
human liver			
human kidney	0.45	1-naphthol morphine	3000 140
human kidney			
human intestine	0.32	1-naphthol morphine	1000 135
human intestine			
1A1 (from V79)	0	octylgallate	500
1A3 (from V79)	0	2-aminobiphenyl	70
1A6 (from V79)	0	1-naphthol	2500
1A9 (from V79)	0	propofol	1000
2B4 (from V79)	0	morphine	4.5
2B7 (from V79)	0.5	morphine	350
1A4 (from Baculovirus)	0	2-aminobiphenyl	400

The control substrates used to confirm activity at a concentration of 2 mM. All values are within the normal range expected for these substrates (Gaiser *etal* 2003). Morphine glucuronidation rates for human tissues were included to give a better indication of relative UGT2B7 levels, though it should be noted that morphine is not exclusively a UGT2B7 substrate.

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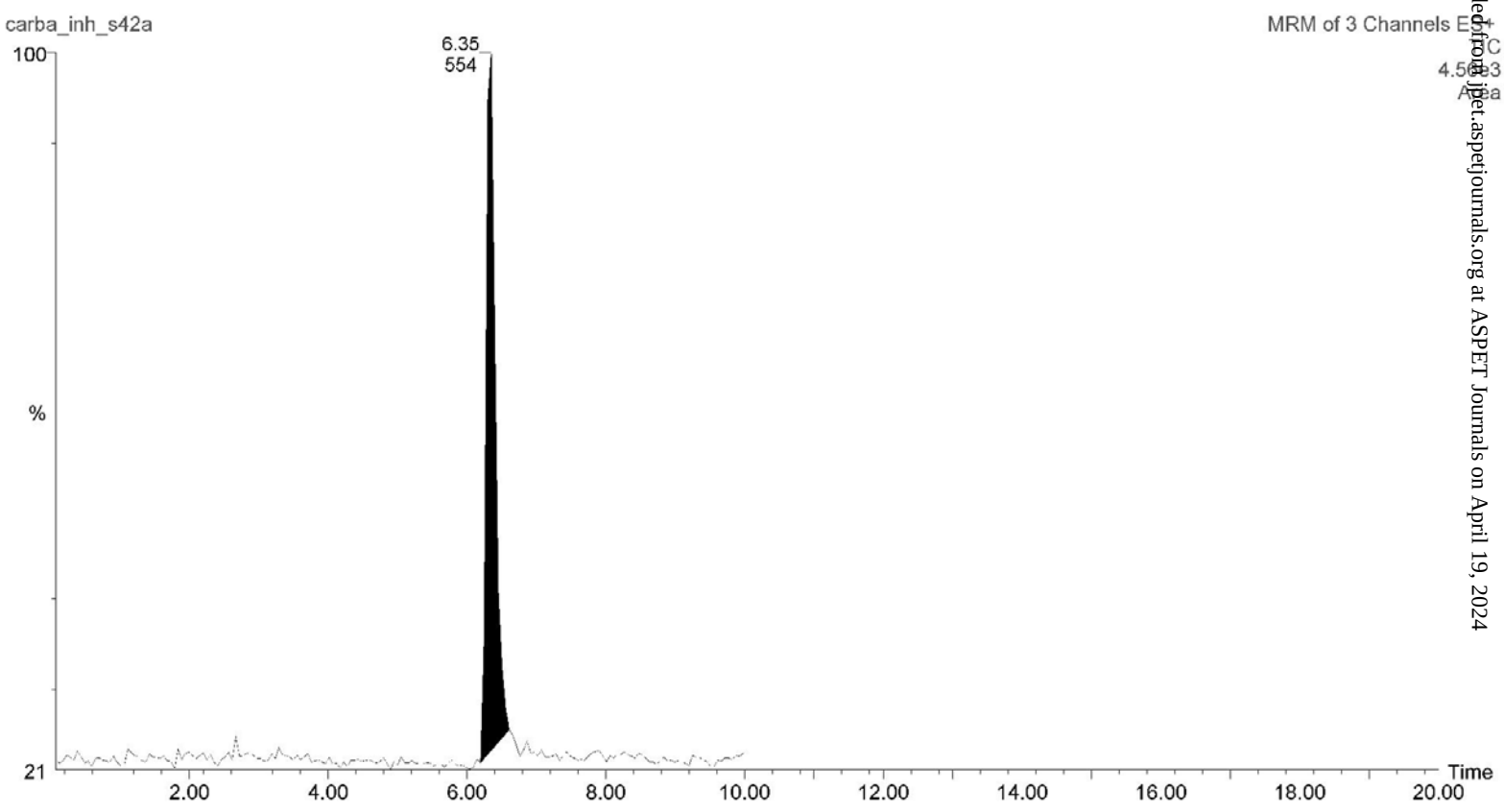
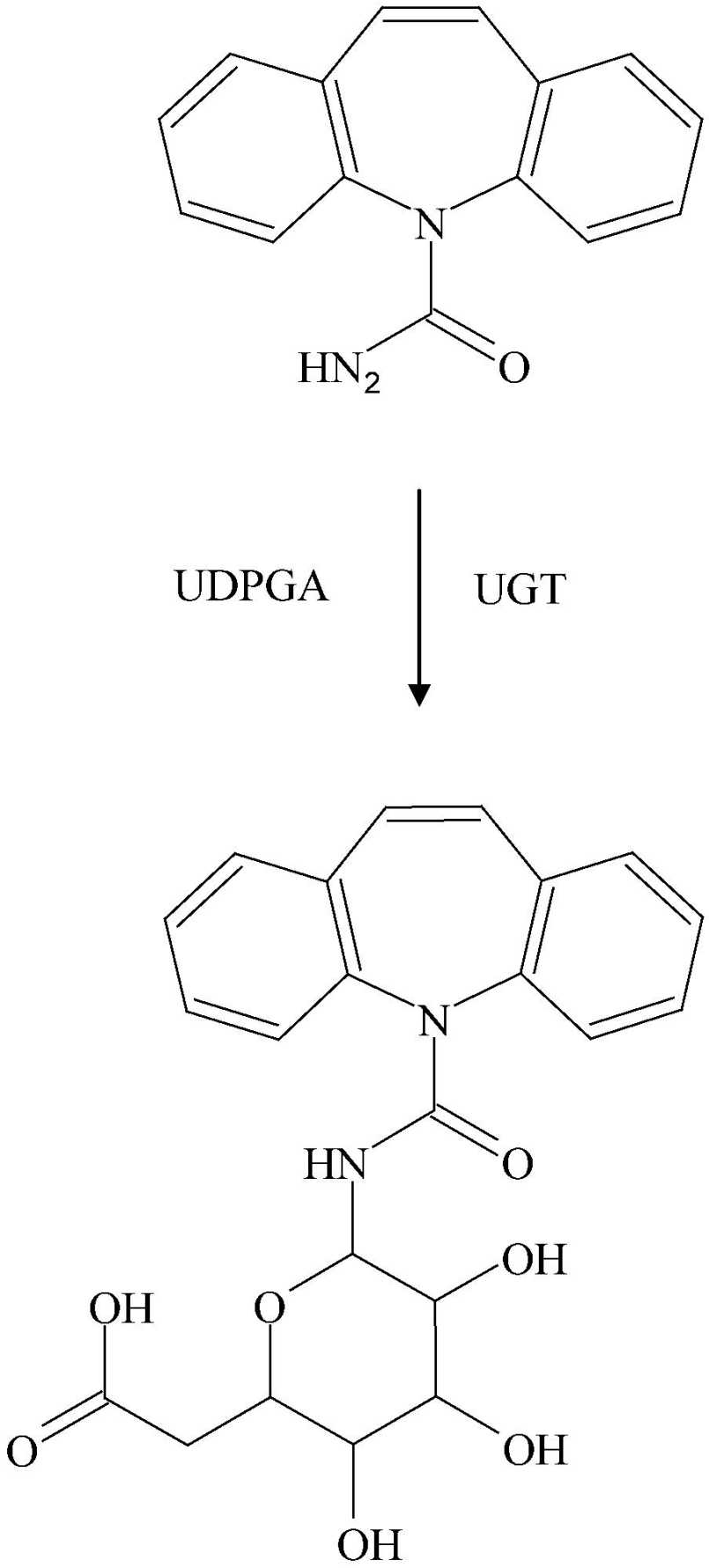
Staines et al., Figure 1



Staines et al. Figure 2



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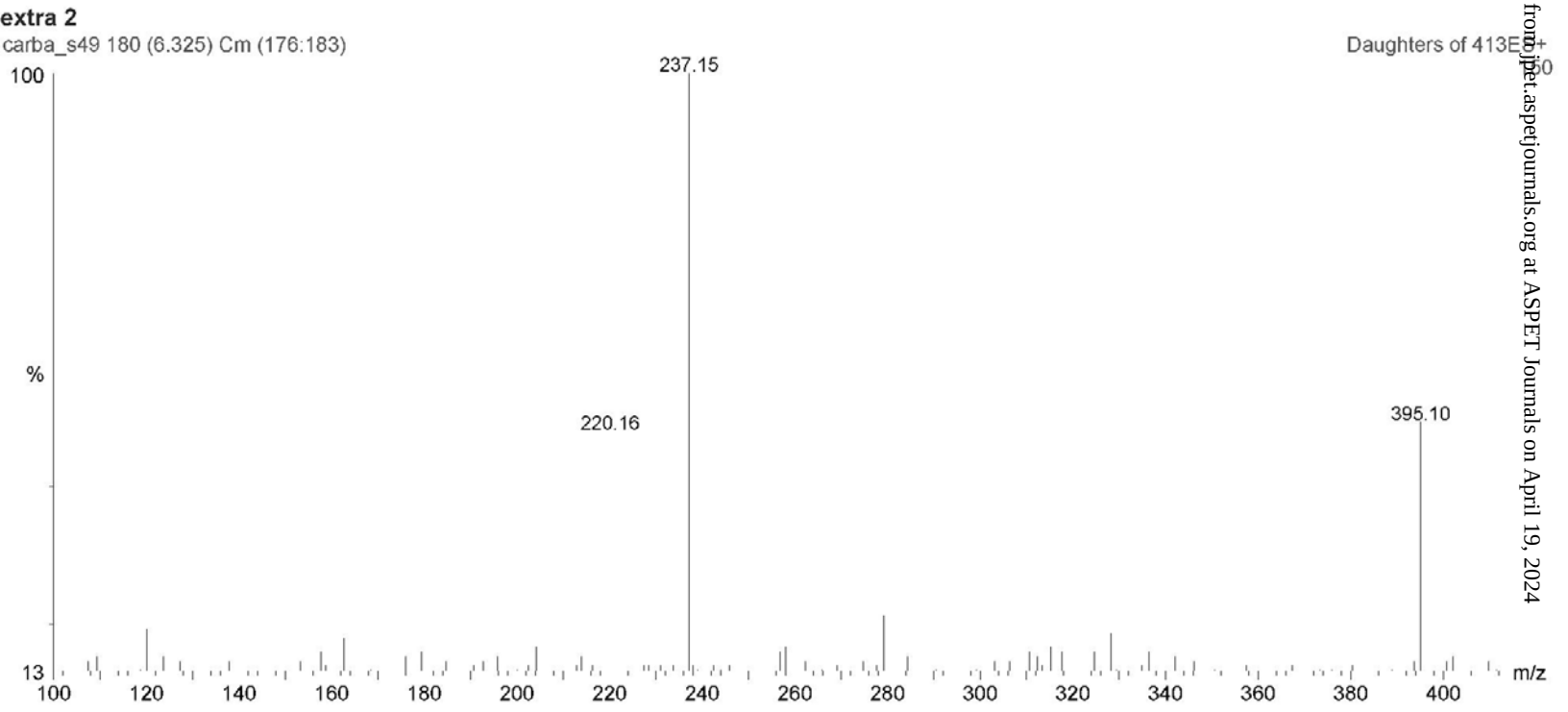
Staines et al., Figure 3



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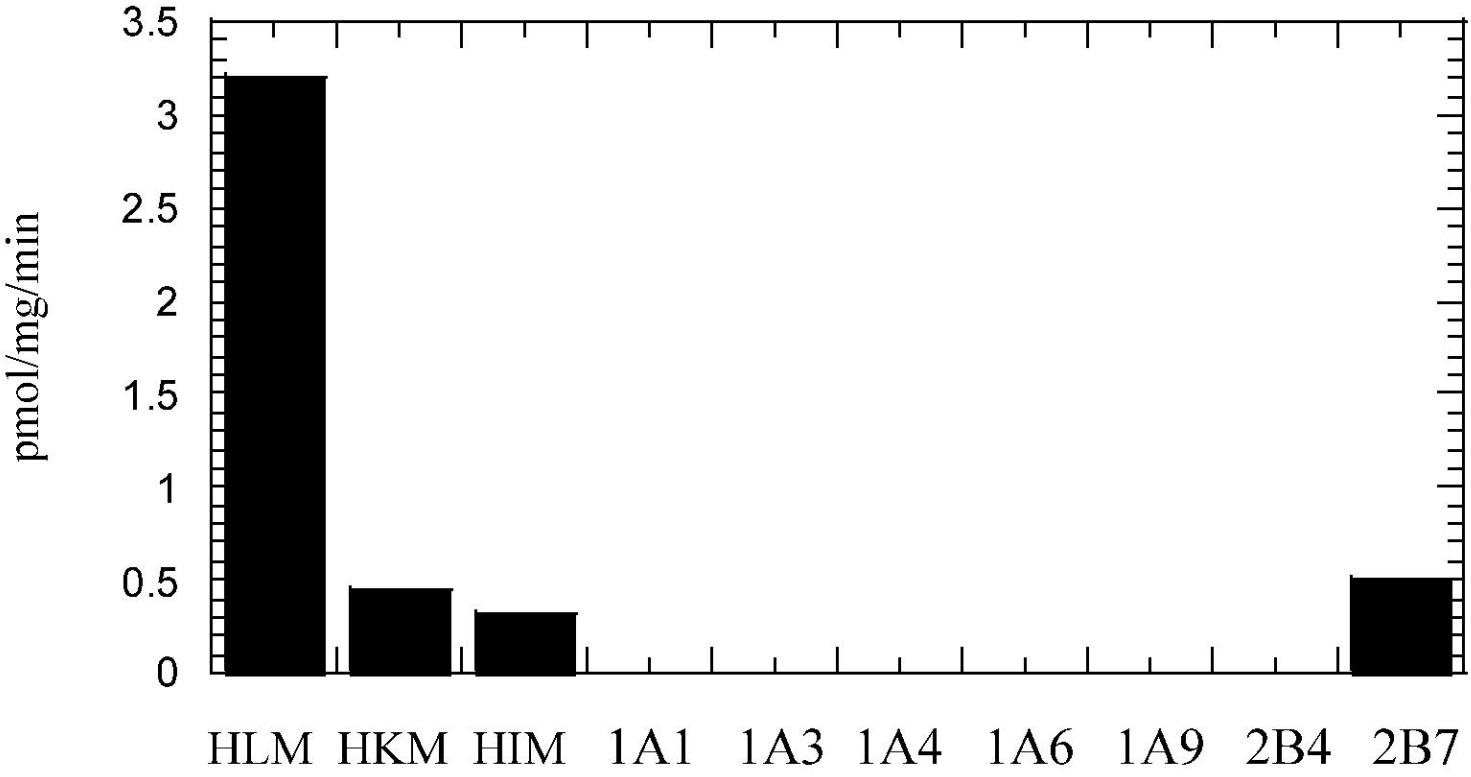
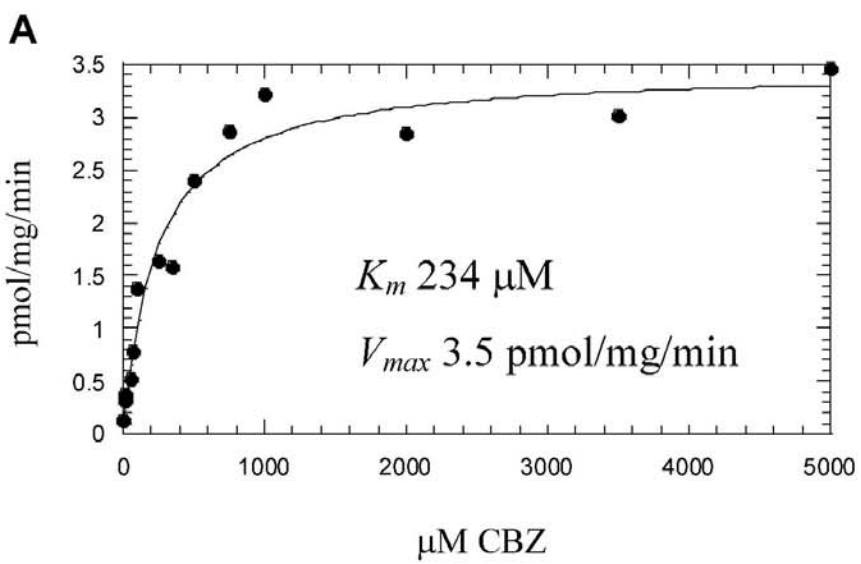
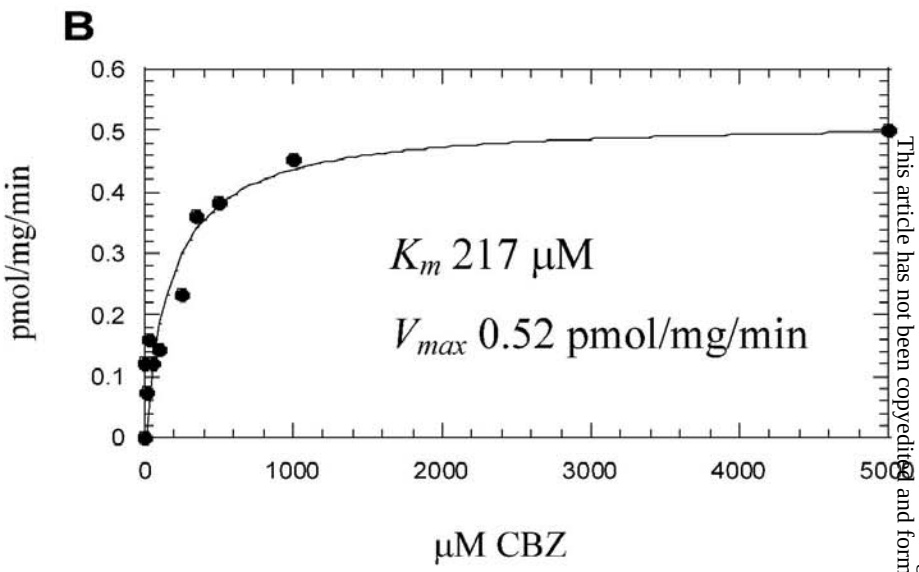
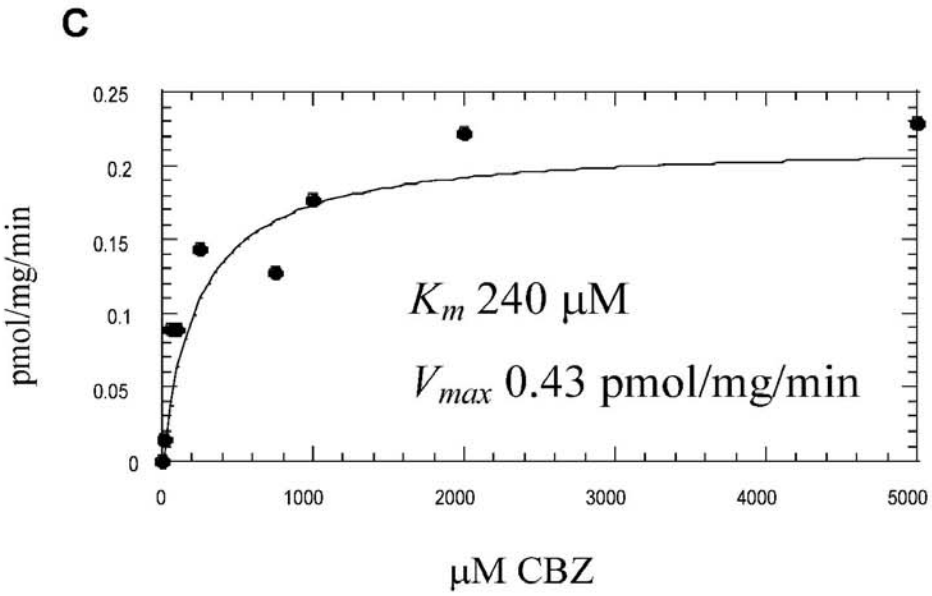
Staines et al., Figure 4

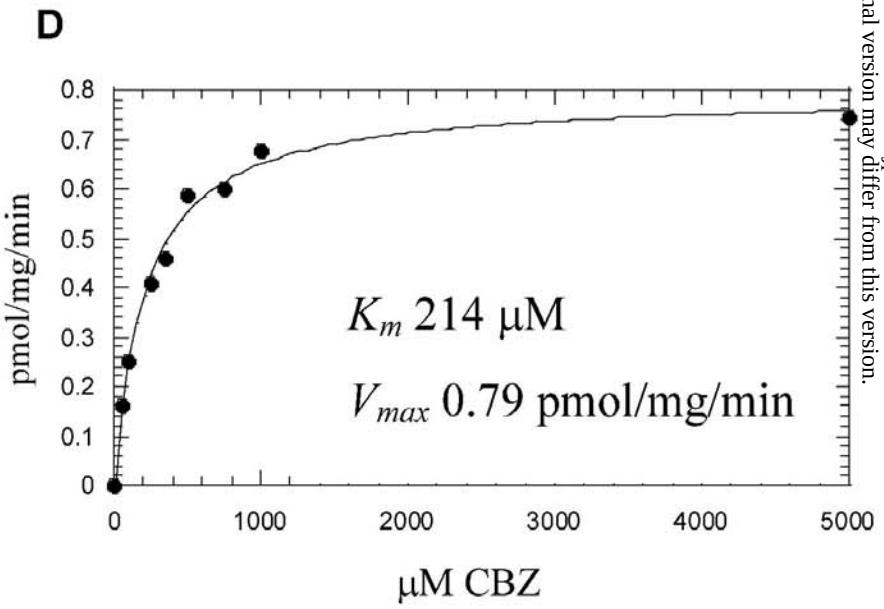


Staines et al., Figure 5









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Staines et al., Figure 6