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 uridine diphosphate glucuronosyltransferase (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 $K_m$ value of 214 $\mu$M and $V_{max}$ value 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.

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 (Shear and Spielberg 1988; Riley et al., 1989); therefore, the study of all CBZ metabolites has important clinical implications.

The metabolism of CBZ is complex and has been widely studied in human 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 product. Glucuronidation is also an important detoxification pathway because 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 (Pirmohamed et al., 1992; Green et al., 1995b). One minor metabolite, 2-hydroxy-CBZ, formed through loss of the carboxamide, could be metabolized to an iminoquinone, which due to its potential chemical reactivity might be the metabolite responsible for the idiosyncratic reactions, although this has not been shown to date (Ju and Uetrecht, 1999). In addition CBZ is also a well known enzyme inducer up-regulating cytochrome P450 (Luo et al., 2002) and UGT activity/expression (Tanaka, 1999).

Carbamazepine is metabolized to an N-glucuronide (Bauer et al., 1976); in addition, glucuronide metabolites have been demonstrated for all 13 of the hydroxylated metabolites of CBZ (Maggs et al., 1997). Formation of N-glucuronides has been principally studied in nonrodent species, because rats and mice do not readily form N-glucuronides, particularly quaternary ammonium glucuronides from tertiary amines (Hucker et al., 1978; Maggs et al., 1997). 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 focused on UGT1A3 (Green et al., 1998).

ABBREVIATIONS: CBZ, carbamazepine (5H-dibenzo[b,f]azepine-5-carboxamide); UGT, uridine diphosphate glucuronosyltransferase; LC/MS, liquid chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; UDPGA, $\beta$-uridine diphosphoglucuronic acid; LC/MS-MS, liquid chromatography-tandem mass spectrometry; LC, liquid chromatography; SRM, single reaction monitoring.
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 toward some drugs and naturally occurring tricyclic compounds (Senafi et al., 1994). It is possible that the failure to identify the isozyme responsible was due to a lack of sensitivity of the methods being used to detect glucuronide formation. This was the case with nicotine, where the UGT isozyme 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 to 1000 times more sensitivity (Casala et al., 2004; Staines et al., 2004) (A. G. Staines, M. W. H. Coughtrie, and B. Burchell, manuscript in preparation) than conventional techniques such as thin layer chromatography/HPLC with radiolabeled β-D-uridine diphosthol glucuronic acid (UDPGA) (Ethell et al., 1998) and therefore is a more suitable technique for detection of low levels of glucuronide conjugates.

Here, we report the characterization 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.

**Materials and Methods**

**Chemicals and Reagents.** Unless stated otherwise chemicals, including UDPGA and carbamazepine, were purchased from Sigma-Aldrich (Gillingham, UK). [Glucuronyl-14C (U)]-UDPGA was purchased from PerkinElmer Life and Analytical Sciences (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 (Jackson et al., 1987; Fournel-Gigleux et al., 1989, 1991; Wooster et al., 1991) except UGT1A4 expressed in the baculovirus/Sf9 cell system (kindly donated by Robert Tukey, Departments of Chemistry & Biochemistry and Pharmacology, University of California, San Diego, La Jolla, CA). 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 of phosphate-buffered saline at pH 7.4. The 200-μl suspension was sonicated for 5 × 5 s (MSE soniprep 150; Sanyo, Gallenkamp, England) on ice, with 1-min cooling on ice between bursts. Microsomes were diluted in phosphate-buffered saline to give 5 to 10 mg/ml and sonicated as described above. Protein concentrations were determined postsonication by the method of Lowry et al. (1951).

**Carbamazepine Glucuronidation Assays.** CBZ (up to 5 mM final concentration in the incubation, from a stock solution in dimethyl sulfoxide) was added to the assay mixture containing 350 μg of sonicated microsomes (or 200 μg of sonicated cell lysate) in 100 mM Tris/maleate buffer (pH 7.5) containing 5 mM MgCl2. The reaction was initiated by addition of UDPGA (10 mM final concentration in the incubation, from 100 mM stock in phosphate-buffered saline) to give a final reaction volume of 100 μl. Samples (assayed in triplicate) were incubated for 3 h at 37°C as a standard condition; a time-course experiment with human liver microsomes indicated that the reaction was linear up to 3 h (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 min, thawed, and centrifuged at 14,000g for 5 min. 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 analyzed 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 (buffer B). LC separation and elution were achieved using a 1-min isocratic segment at 5% buffer B followed by a gradient of 5 to 100% buffer B over 4 min. This was followed by a 2-min wash phase at 95% buffer B and a 3-min re-equilibration step at 5% buffer B. Separations were performed with a Waters Spherisorb (ODS2) 2 μm, 2.1 × 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 min to protect the source from excessive salt. The glucuronide peak from the LC column was analyzed using a single reaction monitoring (SRM) 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 was 15 eV and the collision gas was at 3 mbar. The nebulising gas was set at 100 l/h and the desolvation gas at 700 l/h. 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 [14C]UDPGA as described previously (Staines et al., 2004). 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 s. 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 alkaline lysis at 50°C for 1 h by addition of 250 μl of 30% (v/v) ammonia, followed by neutralization with 130 μl of 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 described above.

Quantification was achieved by analysis of the above-mentioned samples for both carbamazepine and carbamazepine glucuronide. This was performed using the same LC conditions as described above. Detection was with a dual multiple reaction monitoring method in positive ion mode with dual transitions at 413.3 > 237.0 (cone voltage 25 eV, capillary 3.0 eV, and collision energy 15 eV) and at 237.3 > 194.1 (cone voltage 40 eV, capillary 3.0 eV, and 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 hydrolyzed and nonhydrolyzed samples was analyzed 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 ionization potential between the CBZ glucuronide and the CBZ.

**Determination of Kinetic Parameters.** Kinetic parameters for carbamazepine glucuronide formation were determined using the standard assay described 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, 1000, 5000, and
10,000 μM). Standard Michaelis-Menten curves were used to calculate the kinetic parameters (Kaleidagraph; Synergy Software, Reading, PA).

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, androstene, 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 min 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.

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 (Fig. 1). The limit of detection of this method was estimated at 10 fmol, which translates to a minimum detectable UGT reaction rate of <1 fmol/mg/min (for standard 1-h assay, with 250 μg of protein). This is 8000 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 Fig. 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 Fig. 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 ionization 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

Fig. 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).

Fig. 2. Schematic showing the structure of carbamazepine and its β-D-glucuronide.
forms the CBZ glucuronide. All of the three tissue types analyzed show formation of the CBZ glucuronide. Examples of the rate versus substrate concentration curves for the glucuronidation of CBZ in each different system are shown in Fig. 5, A to 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 Fig. 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 Fig. 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 that 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) (A. G. Staines, M. W. H. Coughtrie, and B. Burchell, manuscript in preparation). Even when using the maximum CBZ concentration, the glucuronidation rate with human liver microsomes is below the limits of detection even for radiolabeled 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 metabolized by liver, kidney, and intestine microsomes and that the only isoform catalyzing 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 (calculated at 1: 2.2). The \( K_m \) value reported here (approximately 200 \( \mu M \)) is typical for UGT2B7 substrates, which are usually between 50 and 500 \( \mu M \) (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 glucu-
ronidation, 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

<table>
<thead>
<tr>
<th>Source of UGT</th>
<th>UGT Activity</th>
<th>Probe Substrate</th>
<th>UGT Activity with Probe Substrate</th>
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<tr>
<td>Human liver</td>
<td>3.2 pmol/mg/min</td>
<td>1-Naphthol</td>
<td>4000 pmol/mg/min</td>
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<tr>
<td>Human kidney</td>
<td>0.45 pmol/mg/min</td>
<td>Morphine</td>
<td>520 pmol/mg/min</td>
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<td>Human intestine</td>
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<td>1-Naphthol</td>
<td>3000 pmol/mg/min</td>
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<td>1-Naphthol</td>
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</tbody>
</table>

**TABLE 1**

Relative rates of CBZ β-D-glucuronide formation by human tissue microsomes and cloned/expressed UGTs.

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 et al., 2003). Morphine glucuronidation rates for human tissues were included to give a better indication of relative UGT2B7 levels, although it should be noted that morphine is not exclusively a UGT2B7 substrate.

**Fig. 5.** A to 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. D, human UGT2B7. The data points are based on the mean of a duplicate experiment.
that valproate has minimal effect on carbamazepine glucuronidation in liver microsomes. This might be predicted, because 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, because UGT2B7 is a major isoform present in the intestine (Strassburg et al., 2000; Staines et al., 2004).

Another anticonvulsant that is often coadministered with CBZ is lamotrigine and this drug, like most amines, 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-metabolizing enzymes, including cytochromes P450 and UGTs (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 metabolizes a wide range of therapeutic drugs.

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References


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