CHARACTERIZATION OF THE METABOLITES OF CARBAMAZEPINE IN PATIENT URINE BY LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY

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ABSTRACT:

The urinary metabolites of carbamazepine (CBZ) in epileptic patients receiving long-term drug treatment have been characterized by LC/MS. CBZ-10,11-epoxide (9.6–15.0 µg/ml), trans-10,11-dihydrodiol-CBZ (273.0–400.00 µg/ml), and CBZ (2.4–3.8 µg/ml) were measured by HPLC. The secondary N-glucuronide of CBZ, four phenolic O-glucuronides (including those of 2- and 3-OH-CBZ), two additional OH-CBZ O-glucuronides, and the N-glucuronide of CBZ-10,11-epoxide constituted the products of either direct conjugation or preliminary monoxygenation. Derivatives of these monoxygenated compounds, which were characterized as O-glucuronides, were represented by dihydroxylated (catechol) CBZ and its putative O-methyl metabolite and by 10,11-dihydrodiol-CBZ. 10,11-Dihydro-10-OH-CBZ O-glucuronide, a metabolite thought to be excreted only by uremic subjects, was not found. More complicated biotransformations of the 10,11-ene moiety were revealed by two carbinol products of azepeine ring contraction: 9-OH-10-carbamoyl acridan and an hydroxylated derivative thereof, which were excreted as O-glucuronides. No polar sulfur-containing metabolites that might serve as indicators of reactive intermediate formation were found in human urine.

Early studies on the urinary metabolites of CBZ\(^1\) (fig. 1) in epileptic patients, which used GC/MS either with or without prior hydrolysis of the conjugated metabolites (1, 2), identified the secondary amine-linked glucuronide of CBZ, the O-glucuronide of trans-10,11-DHD-CBZ, and isomeric O-glucuronides of hydroxy (C-2, C-3, and others), dihydroxy, and hydroxymethoxy CBZ. In addition, several dihydrodiols, other oxygenated products, and four isomers of methylsulfoxoxyhydroxy CBZ—the latter appeared to be trace metabolites—have been described but not quantified (2); certain of these are considered to be derived from arene oxides. However, the principal pathway of CBZ metabolism in humans involves the formation of the chemically stable 10,11-epoxide (3) and its hydrolysis to trans-10,11-DHD-CBZ (4, 5). The O-glucuronide of 10,11-dihydro-10-hydroxy-CBZ has been found only as a minor product in uremic subjects (6).

We have analyzed the urinary metabolites of CBZ in patients by means of LC/MS, which allows direct characterization of polar conjugates (7) and thereby avoids the ambiguities associated with the decomposition of dibenzapines to acridans during derivatization and/or GC analysis (2). Mass chromatograms were examined particularly for any polar sulfur-containing metabolites (i.e. analytes that may have avoided detection by GC/MS) that might serve as indicators of the formation (and deactivation via reaction with glutathione) of arene oxide and other reactive intermediates. These species have been hypothetically implicated in the causation of the toxicities associated with CBZ treatment (8–10).

Materials and Methods

Patients. The study was approved by the local ethics committee. Two male patients (30 and 32 years) and one female (36 years) who had received CBZ monotherapy (1.2 and 1.8 g/day, respectively) for >1 year collected urine for 24 hr; aliquots were stored at \(-20^\circ\)C. None of the patients was receiving other drugs known to be either enzyme inducers or enzyme inhibitors. Urinary concentrations of CBZ, CBZ-E, and trans-10,11-DHD-CBZ were determined by HPLC (5).

Materials. Standards of CBZ and certain of its nonconjugated metabolites were obtained as described previously (7).

LC/MS. Positive-ion ESP mass spectra of CBZ and its metabolites in urine were obtained by interfacing an Ultracephashe C18 column (25 cm \(\times\) 0.46 cm i.d., 5 µm; HPLC Technology, Macclesfield, UK) to a Quattro II triple quadrupole mass spectrometer (Micromass Ltd., Altrincham, Cheshire, UK). Configuration of the LC/MS system has been described elsewhere (7). Aliquots of untreated urine (100 µl) were eluted with a gradient of methanol (15% for 5 min, 15–20% over 5 min, 20% for 10 min, 20–50% over 20 min) in 1.0% (v/v) aqueous acetic acid at 1.2 ml/min; the split flow to the LC/MS interface was \(-40\) µl/min. Interface temperature was 60°C; capillary voltage, 3.8 \(\times\) 10\(^3\) V; counter electrode (HV lens) voltage, 0.28 \(\times\) 10\(^3\) V; radiofrequency lens voltage, 0.2 V; and skimmer voltage, 1.9 V. Centroided mass spectra were acquired between m/z 100–950 over a scan duration of 4.91 sec;
the cycle time was 5.1 sec. Scans within a chromatographic peak were averaged, and averaged adjacent (background) scans were subtracted from them to obtain the final spectrum. Fragmentation of analyte ions (protonated molecules) during full-scanning acquisitions was achieved by increasing the skimmer cone voltage from 30 to 50 V. Degradation of selected analyte ions was performed by collision with argon \((8 \times 10^{-4} \text{ mbar})\) at energies of 15–20 eV; the daughter spectra were acquired from \(m/z\) 100 at 1 scan/5 sec. SIM (7 channels) was conducted with a dwell time of 2 sec and an interchannel delay of 20 msec. Areas of peaks in computer reconstructed mass chromatograms for protonated molecules were determined at a cone voltage of 30 V to minimize fragmentation. Both photomultipliers were set at 650 V. All data were processed via MassLynx II software (Micromass Ltd).

### Results and Discussion

#### Unconjugated Metabolites.

CBZ-E (9.6, 13.9, and 15.0 \(\mu\)g/ml) and trans-10,11-DHD-CBZ (273.0, 400.0, and 382.9 \(\mu\)g/ml, respectively) were the only unconjugated metabolites of CBZ found in urine; they were identified by matching their retention times (40.0 min and 36.5 min, respectively) and mass spectra with those of authentic standards (table 1). Only small quantities of CBZ (2.4, 2.5, and 3.8 \(\mu\)g/ml), identified by retention time (48 min) alone, were eliminated in urine.

#### Glucuronides of CBZ and Its Monoxygenated Metabolites.

The N-glucuronide of CBZ \((2\) \(\alpha\)) yielded an abundant protonated molecule \([M+1]^{+}\) at \(m/z\) 413 (fig. 2; table 2) and underwent characteristic fragmentations \((7, 11, 12)\) attributable to the loss of the dehydroglucuronic acid moiety \([M+1-176]^{+}\) and cleavage of the carbamoyl group \(((\text{CBZ}+1-\text{NH}_2)^{+}\)). More extensive fragmentation was achieved by CID (fig. 3; table 2).

#### Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ions and Relative Intensities</th>
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<tbody>
<tr>
<td>CBZ-E</td>
<td>(m/z) 253 ([M + 1](^{+}), 100), 210 ([M + 1 – HNCO](^{+}), 70), 180 ([210 – CH(_2)O](^{+}), 45)</td>
</tr>
<tr>
<td>10,11-DHD-CBZ</td>
<td>(m/z) 271 ([M + 1](^{+}), 100), 253 ([M + 1 – HO](^{+}), 55), 210 ([253 – HNCO](^{+}), 38), 180 ([210 – CH(_2)O](^{+}), 15)</td>
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Urine was analyzed by LC/MS. Spectra were acquired at a cone voltage of 50 V.

**Fig. 2.** Q1 mass chromatograms (LC/MS) for the \([M + 1]^{+}\) ions of the glucuronide metabolites of CBZ found in patient urine. Analysis was performed at a low cone voltage (30 V) to minimize fragmentation. Numerals in parentheses refer to structures in fig. 10. Gluc, glucuronide.

**Fig. 3.** CID (LC/MS/MS) spectrum for \([M + 1]^{+}\) of the N-glucuronide of CBZ in human urine.

Parent ion, \(m/z\) 413.

**Fig. 4.** Q2 mass chromatograms (LC/MS/MS) for the parent \((m/z\) 429, \([M + 1]^{+}\)) and daughter ions of the glucuronides of monoxygenated CBZ in human urine.

\(m/z\) 253 is \([M + 1 – 176]^{+}\). \(V =\) CBZ-E N-glucuronide.
The Q1 and Q2 mass chromatograms for glucuronides of monoxygenated metabolites (m/z 429; fig. 2 and fig. 4) each contained four consecutive peaks (I-IV) that by virtue of yielding only the protonated aglycone (m/z 253) upon cone-voltage fragmentation (table 2) and CID (fig. 5), respectively, were identified as the four possible phenolic glucuronides (5). Four OH-CBZ O-glucuronides found in patient urine during an earlier study were characterized as their permethylated derivatives, but the positions of functionalization could not be determined (1). Two chromatographically distinct monoxygenated glucuronides (VI and VII, R<sub>T</sub> = 37 and 37.5 min) and their aglycone fragments were more clearly observed in the Q2 mass chromatograms (fig. 4). In common with metabolites I-IV, they yielded only the protonated aglycone when subjected to CID (fig. 5). By process of elimination, they are the two that can be the diastereoisomeric O-glucuronides of 10-OH-CBZ, conjugates previously characterized as biotransformation products of 10,11-dihydro-10-oxo-CBZ (oxcarbazepine) in human urine (12, 13). The fifth and most intense peak in the Q1 mass chromatogram for m/z 429 corresponded to the N-glucuronide of CBZ-E (3). It coeluted with the CBZ-E glucuronide found in the urine of rats administered CBZ-E (7) and yielded diagnostic fragments (table 2; fig. 4 and fig. 6). CBZ-E glucuronide was not identified in a study that depended on derivatization before GC/MS (1). It was not possible to assign the positions of functionalization (C-1 to C-4) of O-glucuronides I-IV, but two of the monooxygenated aglycones liberated by enzymic hydrolysis (beef liver β-glucuronidase) and detected by SIM (m/z 253) during LC/MS analysis of the hydrolysate coeluted with authentic 2- and 3-OH-CBZ (R<sub>T</sub> = 39 and 41 min, respectively; fig. 7). Two other peaks in the mass chromatogram for m/z 253 corresponded to CBZ-E (40 min) and an unidentified compound (43 min). Studies that used either GC/MS (2) or direct-inlet MS (14) for the analysis of fractions isolated from human urine resolved four monohydroxy compounds, of which two were identified as 2- and 3-OH-CBZ. The C-1 and C-4 isomers (the latter being only a minor product) are reported to be urinary metabolites of CBZ (14), but were not located during the present study. They may have coeluted with the C-2 isomer (2). In agreement with previous observations (7, 14), CBZ N-glucuronide was found to be refractory to hydrolysis by β-glucuronidase; no CBZ was detected by SIM analysis of the hydrolysate. The approximate proportions of glucuronides I-IV, as determined by
integration of the peaks in the Q1 mass chromatogram for m/z 429, were 0.69, 0.37, 0.37, and 1.0, respectively.

Glucuronides of Dioxygenated Metabolites. The Q1 mass chromatograms (fig. 2) for glucuronides of di-OH-CBZ ([M + 1]+ at m/z 445) and OH-methoxy-CBZ (m/z 459) both contained only one fraction that was identifiable as a metabolite of CBZ: when fragmented, each conjugate gave rise to a peak in the mass chromatogram for its respective protonated aglycone moiety (m/z 269 and m/z 283 respectively; table 2) that was coincident with the peak of the protonated conjugate. These metabolites, which appeared to be present at relatively low concentrations, are presumed to be glucuronides of a catechol (6) and O-methylated catechol (7). Three di-OH-CBZ (catechol) O-glucuronides and their O-methylated analogs found in human urine have been characterized as permethylated derivatives (1).

Independent analysis of CBZ metabolites isolated from enzymically hydrolyzed human urine found four dihydroxy compounds (two of which were assigned to catechols) and two O-methylated catechols (2, 14).

Glucuronides of Dihydrohydroxy and Dihydrodihydroxy Metabolites. Only one dihydro-OH-CBZ glucuronide (m/z 431) was found in each of the three urine samples (fig. 2). At higher cone
voltages, the conjugate fragmented by loss of dehydroglucuronic acid but the protonated aglycone (m/z 255) was resistant to scission of the carbamoyl group (fig. 8a; table 2), a spectral pattern considered diagnostic of an O-glucuronide. Two possible aglycones were considered: 10,11-dihydro-10-OH-CBZ and 9-HM-10-CA (aglycone of 8). However, the former was excluded by LC/MS analyses of hydrolyzed urine (fig. 7) that established that none of the peaks in the mass chromatogram for m/z 255 coeluted with the authentic standard (RT = 38 min). The predominant dihydrohydroxyl aglycone detected by SIM (RT = 44 min) eluted after CBZ-E and 3-OH-CBZ in the manner of 9-HM-10-CA (2). These findings support an earlier contention that the O-glucuronide of 10,11-dihydro-10-OH-CBZ, which is the major urinary metabolite of oxcarbazepine in humans (12), is not normally eliminated in human urine as a product of CBZ (6). Nevertheless, because 10,11-dihydro-10-OH-CBZ can be converted to 10,11-DHD-CBZ in humans (13), and thereby constitutes an alternative to CBZ-E as an intermediate in the biotransformation of CBZ to its principal metabolite, it is possible that 10,11-dihydro-10-OH-CBZ is formed but not excreted by healthy subjects. In which case, elimination of the O-glucuronide by uremic subjects might arise from a deficiency of C-11 hydroxylation. The glucuronide of 9-HM-10-CA was previously known only in the form of a decarbamylated artifact of derivatization and GC/MS (1).

Two dihydrodi-OH-CBZ glucuronides (m/z 447) were located at RT = 27 and 35 min (fig. 2) in the relative proportions of 0.24:1.0 (mean; N = 3), but only the latter was unambiguously identified as a conjugate of a dihydrodiol (4); it corresponded to a metabolite of CBZ-E in rats (5), gave ESP and CID spectra (fig. 8c; fig. 9; table 2) containing a base peak at m/z 253 attributable to facile dehydration of a dihydrodiol fragment, and yielded on hydrolysis an aglycone that coeluted with trans-10,11-DHD-CBZ. The aglycone fragment (m/z 271) of the other dihydrodi-OH-CBZ glucuronide did not dehydrate (fig. 9, table 2), although greater fragmentation was achieved by CID (fig. 8b). The aglycone liberated by enzymic hydrolysis eluted immediately after trans-10,11-DHD-CBZ (fig. 7). Whereas the possibility that this metabolite is a conjugate of a benzenoid dihydrodiol [i.e. a product of an arene oxide (9)] cannot be discounted, the absence of facile dehydration constitutes evidence against such a structure. Instead, it is tentatively identified as a glucuronide of hydroxylated 9-HM-10-CA (9). The aglycone’s chromatographic behavior resembled that of a metabolite previously characterized only as an acridan artifact of OH-9-HM-10-CA (2). It was originally presumed that the ring-contracted carbinol acridans were formed via the epoxide-diol pathway (1, 2). However, 9-HM-10-CA is only a minor metabolite of CBZ-E in humans (4) and neither carbinol nor its glucuronide was detected by LC/MS in the urine or bile of rats given radiolabeled CBZ-E (7) intravenously. It has been suggested that 9-HM-10-CA is derived, via a carboxaldehyde, from a monooxygenated intermediate that is common to the epoxidation and ring-contraction pathways (15).

The glucuronide metabolites of CBZ found in human urine are represented in fig. 10. Based on the areas of the peaks in their parent-ion ([M + 1]⁺) chromatograms (fig. 2), the relative proportions of 8, 4, and 5 (I–IV combined) and 2, 3, 9, and 5 (VI and VII) were 1.0, 0.5, 0.4, 0.33, 0.18, 0.13, and 0.05, respectively (mean for three samples). Although these values are only uncorrected approximations—largely because no account could be taken of variations in the instrument response factor between analytes—the rank order of 4, 5, 2, and 3 does correspond to that derived from the radiometric assay of isolated metabolite fractions (14). The notable misalignment was in respect of 8, which was a minor urinary metabolite in two volunteers.

Fig. 10. Glucuronide metabolites of CBZ (1) found in human urine.

Arrows and broken arrows indicate, respectively, major and minor metabolites [relative proportion >0.2 and <0.2, respectively; (8) = 1.0] as estimated from the areas of peaks in mass chromatograms (fig. 2); the O-glucuronides of the six monohydroxylated CBZ derivatives were taken collectively.

2 J. L. Maggs, unpublished observation.
given a single dose of [10,11-14C]CBZ (14). However, in comparing the metabolite profile of a single administration and that of chronic therapy, it should be noted that the metabolism of CBZ via the epoxide-diol pathway in humans is subject to dose-dependent autoinduction, which attains a maximum during the first 4 weeks of therapy (16, 17).

The relevant mass chromatograms were examined for any peaks that might have corresponded to protonated molecules of cysteinylglycine, cysteiny1, and N-acetyl cysteinyl derivatives of CBZ, which, together with the derivatives’ α-hydroxy and dihydroxy analogs (2), were potential urinary catabolites of glutathionyl dihydro-OH-CBZ [i.e. the product(s) of the reaction of glutathione with CBZ’s arene oxide intermediate(s)] (7). Diepoxide formation and sequential epoxidation of CBZ were taken into account by scanning for the indicative tetrahydrothiohydroxy-thioether adducts (18). None of these derivatives of epoxides and diepoxides was found, nor the nonpolar thioethers, sulfoxides, and sulfones identified previously in either rat (2) or human urine (2, 14). Recent findings on the bioactivation of CBZ by mouse hepatic microsomes have suggested that the catechol metabolites of CBZ may be oxidized to chemically reactive CBZ by mouse hepatic microsomes (1). The catechol metabolites to quinoid-glutathione conjugates in rat (15) and human urine (16, 17).


References