Detection of 2-Hydroxyiminostilbene in the Urine of Patients Taking Carbamazepine and Its Oxidation to a Reactive Iminoquinone Intermediate

C. JU and J. P. UETRECHT

Faculty of Pharmacy (C.J., J.P.U.) and Medicine (J.P.U.), University of Toronto, Toronto, Canada

Accepted for publication July 27, 1998

This paper is available online at http://www.jpet.org

ABSTRACT

Carbamazepine is one of the most widely used anticonvulsants in North America; however, its use is associated with a range of serious idiosyncratic adverse reactions. These reactions are thought to result from the formation of chemically reactive metabolites. Carbamazepine is extensively metabolized in the liver and one of the major metabolites is 2-hydroxycarbamazepine, which has previously been detected as a urinary metabolite excreted by rats and humans along with its further metabolized product, 2-hydroxyiminostilbene. In this study, we found that the urine of patients taking carbamazepine appeared to contain more of the glucuronide of 2-hydroxyiminostilbene than that of 2-hydroxycarbamazepine. We have also demonstrated that 2-hydroxyiminostilbene can be oxidized readily to an iminoquinone species by HOCl, H₂O₂ or even on exposure to air. The reactivity of this iminoquinone as an electrophile was studied. It was shown to react with sulfhydryl-containing nucleophiles, such as glutathione and N-acetylcysteine. We also found a metabolite with the same molecular weight as 4-methylthio-2-hydroxyiminostilbene, but not the corresponding carbamazepine derivative, in the urine of patients taking carbamazepine and this presumably reflects the formation of a glutathione conjugate of the reactive iminoquinone. This iminoquinone intermediate may play a role in carbamazepine-induced idiosyncratic reactions.

Carbamazepine (5H-dibenzo[b,f]azepine-5-carboxamide) is an effective drug in the treatment of convulsive disorders (Cereghino et al., 1973, 1974). However, carbamazepine-induced adverse reactions have been reported in as many as one-third to one-half of all patients treated with this drug (Pellock, 1987; Durelli et al., 1989; Gram and Jensen, 1989). Among these adverse reactions, 5% of them can be classified as idiosyncratic reactions (Askmark and Wiholm, 1990). These idiosyncratic adverse reactions include skin rash (Crill, 1973), blood disorders (Gerson et al., 1983) and hepatitis (Horowitz et al., 1988). A Swedish survey of 505 reports of 713 idiosyncratic reactions to carbamazepine from 1965 to 1987 reported skin reactions (48%), hematological (12%) and hepatic disorders (10%) to be the most frequent. (Askmark and Wiholm, 1990) Although the mechanism of carbamazepine-induced adverse reactions is not clear, they are thought to result from the formation of chemically reactive metabolites (Shear et al., 1988; Riley et al., 1989). An arene oxide intermediate has been postulated to be responsible for the idiosyncratic toxicity of carbamazepine. This hypothesis is mostly based on the observation that the metabolism-dependent cytotoxicity of carbamazepine in vitro can be enhanced by trichloropropene oxide (TCPO) (Riley et al., 1989), an inhibitor of epoxide hydrolase. However, this evidence is complicated by the fact that TCPO is also known to deplete glutathione (Larrey et al., 1989) and inhibit cytochrome P-450 (Ivanetich et al., 1982). Furthermore, since the arene oxide is chemically reactive, it may not reach targets, such as skin and bone marrow, distant from the liver in sufficient concentrations to induce adverse reactions. It appears that most drugs associated with bone marrow toxicity are metabolized to reactive metabolites by myeloperoxidase or other enzymes present in the bone marrow, such as alcohol dehydrogenase (Uetrecht, 1996). These considerations led us to search for alternative bioactivation pathways of carbamazepine.

Carbamazepine is extensively metabolized and more than 30 metabolites have been identified in urine of patients taking the drug (Lertratanangkoon and Horning, 1982). The major pathways involve N-glucuronidation on the carbamoyl side chain of carbamazepine; formation of carbamazepine-10,11-epoxide and hydroxylation on the aromatic rings. One
of the major metabolites is 2-hydroxycarbamazepine (Eichelbaum et al., 1984), and it can be further metabolized to 2-hydroxymisostilbene, either in the liver or in other tissues. Both 2-hydroxycarbamazepine and 2-hydroxymisostilbene have been detected as conjugates in the urine of rats and humans (Lertratanangkoon and Horning, 1982). We hypothesized that 2-hydroxymisostilbene can be further oxidized to a reactive iminoquinone intermediate (Fig. 1) which may be responsible for the idiosyncratic toxicity of carbamazepine.

Materials and Methods

Synthesis of the Iminoquinone Intermediate. The iminoquinone was synthesized by oxidation of imostilbene using a modification of the method of Islam and Skibo (1991). Frenal’s salt (2.6 g; Aldrich Chemical Co., Milwaukee, WI) was dissolved in 260 ml of phosphate buffer (pH 7.1). To this solution, 1 g of imostilbene (Aldrich Chemical Co.) in 260 ml of acetone was added. The mixture was stirred at room temperature for 5 min, and the resulting red solution was extracted with 3 × 200 ml portions of chloroform. The combined extracts were washed with 3 × 100 ml portions of water and then dried over magnesium sulfate. The solvent was evaporated by a rotary evaporator. The product was a red solid with a yield of 23%, m.p. 321 (MH+, e1) = 371. 

Synthesis of 2-Hydroxycarbamazepine. The hydroxyl group on the iminoquinone-NAC adduct into ethyl acetate (3 ml) was reacted with 4-nitrophenylchloroformate (40 mg, 0.1 ml). The mixture was stirred for 3 h, diluted with 5% aqueous sodium bicarbonate (0.5 ml), and extracted four times with hexane/dichloromethane (9:1, v/v). The combined extracts were washed with water, dried, and evaporated by a rotary evaporator.

Without further purification, the silylated 2-hydroxycarbamazepine (27 mg, 0.083 mmol) was reacted with 4-nitrophenylchloroformate (50 mg, 0.25 mmol; Aldrich Chemical Co.) in chloroform (1 ml). Triethylamine (36 μl, 0.026 mmol) was added at the beginning of the reaction to neutralize the hydrochloric acid generated from the reaction. The mixture was stirred for 3 days and water (1 ml) was then added to quench the excess 4-nitrophenylchloroformate. The product was extracted three times with chloroform and the solvent was evaporated by a rotary evaporator. The product was redissolved in acetone (1 ml) and the ammonia solution (in excess) was added. The mixture was stirred for 3 days and then neutralized by concentrated hydrochloric acid. The desired product, 2-hydroxycarbamazepine, was extracted into ethyl acetate (3 × 10 ml) and the solvent evaporated. 2-hydroxycarbamazepine was purified by TLC using ethyl acetate:chloroform (8:2, v/v) as the eluting solvent. The product was a white solid and the yield was approximately 20%, m.p. 234–237°C; Rf = 0.35; MS: m/z 253 (MH+) and 210 (MH-HCOO)–;

1H-NMR (chloroform-d): δ 7.05 ppm (H2 or H3, d, J = 7.50 Hz; 1.41 Hz); δ 6.90 ppm (H4 or H5, d, J = 7.57 Hz); δ 6.85 ppm (H6 or H7, d, J = 7.45 Hz; 1.60 Hz); δ 6.55 ppm (H8, dd, J = 8.3 Hz; 2.93 Hz); δ 5.54 ppm (H9 or H10, d, J = 7.61 Hz); δ 6.34 ppm (H11 or H12, d, J = 8.3 Hz); δ 6.32 ppm (H10 or H11, d, J = 12.00 Hz); δ 6.41 ppm (H11, d, J = 2.68 Hz); δ 6.32 ppm (H11 or H12, J = 11.72 Hz) and two broad peaks in the range of 4.9 ppm to 4.4 ppm due to the proton on nitrogen and the proton of the hydroxyl group. The chemical analyses of 2-hydroxycarbamazepine reported in the literature were: m.p. 225–226°C; NMR (chloroform-d/methyl sulfoxide-d6): δ 8.27 ppm (s, 1H, -OH), δ 7.5 to 6.2 ppm (m, 9H, aromatic and olefin), δ 5.41 (s, broad, 1H, -NH); MS: m/z 209 (M+). Chang, 1983).

Synthesis of N-acetylcysteine (NAC) Adduct of the Iminoquinone. A methanol solution (4.8 ml) of the iminoquinone (10 mg) was added to NAC (4 g) in 43.2 ml of phosphate buffer (pH 8). The mixture was stirred for 10 min at room temperature and then concentrated on a rotary evaporator. The NAC adduct of the iminoquinone was purified by silica gel thin-layer chromatography (TLC) developed with a solvent of ethyl acetate/methanol (7:3, v/v). The TLC band (Rf = 0.3) that contained the iminoquinone-NAC adduct was scraped off the TLC plate and the adduct was extracted with ethyl acetate. The yield was approximately 33%. The mass spectrum of the iminoquinone-NAC adduct included a MH+ ion at m/z 371.

Synthesis of 2-Hydroxycarbamazepine. The hydroxy group on 2-hydroxymisostilbene was first protected using a modification of the method of Kendall et al. (1979). Imidazole (12 mg, 0.18 mmol; Aldrich Chemical Co.) was dissolved in 260 ml of acetone. The mixture was stirred for 3 days and then neutralized by concentrated hydrochloric acid (3 ml) and the ammonia solution (in excess) was added. The mixture was stirred for 3 days and then neutralized by concentrated hydrochloric acid. The desired product, 2-hydroxycarbamazepine, was extracted into ethyl acetate (3 × 10 ml) and the solvent evaporated. 2-hydroxycarbamazepine was purified by TLC using ethyl acetate:chloroform (8:2, v/v) as the eluting solvent. The product was a white solid and the yield was approximately 20%, m.p. 234–237°C; Rf = 0.35; MS: m/z 253 (MH+) and 210 (MH-HCOO)–;

1H-NMR (chloroform-d): δ 7.47 ppm (H2 or H3, d, J = 7.08 Hz); δ 7.43 ppm (H4 or H5, d, J = 6.83 Hz; 1.71 Hz); δ 7.38 ppm (H6 or H7, d, J = 7.08 Hz); δ 7.34 ppm (H6 or H7, d, J = 6.84 Hz; 1.56 Hz); δ 7.32 ppm (H4 or H5, d, J = 5.84 Hz); δ 6.93 ppm (H6 or H7, d, J = 11.72 Hz); δ 6.84 ppm (H4 or H5, d, J = 8.06 Hz); δ 6.83 ppm (H6 or H7, d, J = 11.32 Hz); δ 6.76 ppm (H1, s); protons on the amide nitrogen, δ 4.5 ppm (2H, s) and a broad peak at δ 5.4 ppm due to the proton on the hydroxyl group. The chemical analyses of 2-hydroxycarbamazepine reported in the literature were: m.p. 239–242°C; NMR (dimethyl sulfoxide-d6): δ 8.25 ppm (s, 1H, -OH), δ 7.5 to 6.6 (m, 9H, aromatic and olefin), δ 5.45 (s, 2H, -CONH); MS: m/z 252 (M+), 209 (M-HCOO) (Chang, 1983).

Treatment of the Urine Sample. Random urine samples were obtained from two male patients (ages 93 and 83, respectively) who had received carbamazepine (250 and 200 mg/day, respectively) for more than 1 year. The urine sample (~100 ml) was concentrated to about 5 ml by lyophilization. The concentrated urine sample was acidified to pH 4.5 and then purified by C-18 Prep-Sep solid-phase extraction column (Fisher Scientific, Unionville, ON). The sample
was loaded onto the column followed by washing with 2 × 10 ml portions of water. The desired urinary metabolites were then eluted from the column by 2 × 10-ml portions of methanol. The methanol effluents were combined and concentrated to ~1 ml. Aliquots (10 μl) were analyzed by liquid chromatography (LC)/MS. Enzymatic hydrolysis was carried out by incubating the urine samples with β-glucuronidase (100 U; Sigma Chemical Co., Oakville, ON) in 0.2 ml of pH 5.1 buffer for 20 h at 37°C. The carbamazepine metabolites were then extracted with ethyl acetate and the extracts were evaporated (N₂ stream), dissolved in methanol, and aliquots of 10 μl were analyzed by LC/MS.

**Analytical Methods.** The analyses of carbamazepine urinary metabolites were performed by interfacing an Ultracarb C-18 column (2 × 100-mm; Phenomenex, Torrance, CA) to a Sciex API III mass spectrometer (Perkin-Elmer, Sciex, Thornhill, Ontario, Canada). Aliquots of a urine sample (10 μl) were eluted with a solvent consisting of water, acetonitrile, and acetic acid (49:50:1, v/v) including 1 mM ammonium acetate. A splitter was used to decrease the flow to the LC/MS interface at 0.2 ml/min. When the metabolites with shorter retention times (e.g., the metabolite with MH⁺ ion at m/z 432) were analyzed, the eluting solvent consisted of water, acetonitrile, and acetic acid (74:25:1, v/v) including 1 mM ammonium acetate. When β-glucuronidase conjugates were analyzed, the eluting solvent consisted of water, acetonitrile, and acetic acid (84:15:1, v/v) including 1 mM ammonium acetate. A splitter was used to decrease the flow to the LC/MS interface at ~10 μl/min. The collisional activation spectra were obtained by using the LC/MS/MS mode, with argon as the target gas.

1H NMR spectra were recorded at 500 MHz with a Brucker AM 500 spectrometer (Brucker Canada, Milton, Ontario, Canada). Spectra were obtained in chloroform-d except for that for the iminoquinone-NAC adduct, which was obtained in deuterium oxide (D₂O).

**Results**

**Oxidation of 2-Hydroxyiminostilbene.** 2-Hydroxyiminostilbene was readily oxidized to the iminoquinone intermediate by HOCl. When 2-hydroxyiminostilbene was reacted with HOCl at equal concentrations, the reaction mixture changed instantaneously from yellow to red and a new peak due to the iminoquinone was observed by high-performance liquid chromatography analysis. Figure 2 shows the UV absorption spectra of 2-hydroxyiminostilbene and the reaction mixture after HOCl was added. The UV absorption spectrum of the oxidation product was similar to that of the iminoquinone. H₂O₂ was also able to oxidize 2-hydroxyiminostilbene to the iminoquinone species, however, at a much slower rate than HOCl. It was also observed that 2-hydroxyiminostilbene underwent autoxidation by air at room temperature to the iminoquinone intermediate. The half-life of 2-hydroxyiminostilbene was approximately 2 h in phosphate buffer (pH 7.4).

**Reactivity of the Iminoquinone Intermediate.** The reactivity of the iminoquinone as an electrophile was studied. It was found to react with sulphydryl-containing nucleophiles, such as glutathione (GSH) and NAC, to form conjugates. The iminoquinone-NAC conjugate was isolated and analyzed by NMR (Fig. 3). The 1H NMR (D₂O) consisted of: δ 7.17 ppm (H₇ or H₆, d, J = 7.9 Hz; 1.70 Hz); δ 6.99 ppm (H₆ or H₇, d, J = 7.48 Hz); δ 6.97 ppm (H₅ or H₉, d, J = 7.80 Hz; 1.65 Hz); δ 6.91 ppm (H₅ or H₉, d, J = 2.77 Hz); δ 6.89 ppm (H₆ or H₇, d, J = 7.56 Hz); δ 6.51 ppm (H₁₀ or H₁₁, d, J = 11.72 Hz); δ 6.44 ppm (H₉, d, J = 2.78 Hz); δ 6.40 ppm (H₉ or H₁₀, d, J = 11.75 Hz); NAC-CH₃, δ 4.28 ppm (1H, dd, J = 7.05 Hz, 3.66 Hz); NAC-CH₂-d, δ 3.34 ppm (1H, dd, J = 14.11 Hz, 3.84 Hz); NAC-CH₃,-d, δ 3.13 ppm (1H, dd, J = 14.10 Hz, 7.05 Hz); NAC-CH₃, δ 1.67 ppm (3H, s). The 1H NMR data, combined with the findings from the 1H-1H and 1H-13C correlation experiments, confirmed the structure of the conjugate where the sulfur of NAC was substituted in the 4-position on the aromatic ring (Fig. 4).

**Patient Urine Sample Analysis.** When the urine sample was analyzed by LC/MS, neither the GSH nor the NAC conjugate of iminoquinone was detected. However, a metabolite with MH⁺ ion at m/z 432 (Rₗ = 5.7 min) was detected. This metabolite has the same molecular weight as the glucuronide conjugate of 2-hydroxyiminostilbene, which is a probable further metabolite of the iminoquinone-GSH conjugate. The MS/MS of the metabolite with MH⁺ ion at m/z 432 showed a fragment ion at m/z 256 which corresponded to the loss of dehydroglucuronic acid moiety ([M – H⁻] = 429 and 10,11-dihydrodiol-carbamazepine with a MH⁺ ion at m/z 413, the glucuronide conjugates of monohydroxylated carbamazepines as well as the N-glucuronide of carbamazepine 10,11-epoxide with MH⁺ ions at m/z 429 and 10,11-dihydrodiol-carbamazepine with a MH⁺ ion at m/z 271. The ratios of the TIC of 2-hydroxyiminostilbene glucuronide to that of other urinary metabolites are shown in Table 1.

The MS/MS spectrum of 2-hydroxyiminostilbene glucuronide conjugate showed that it underwent characteristic fragmentation with the major fragment ion at m/z 210 which represents the loss of the dehydroglucuronic acid moiety ([M + 1–176]+) (Fig. 7). Interestingly, the iminoquinone intermediate was also observed with LC/MS and it had the same retention time (Rₗ = 8.4 min) and MS/MS fragmentation pattern as the synthesized iminoquinone. When NAC was
added to the sample, the peak due to the iminoquinone disappeared, suggesting that it reacted with sulfhydryl-containing nucleophiles. When the urine samples were hydrolyzed by \( \beta \)-glucuronidase, the peak of the iminoquinone increased dramatically (Fig. 8). In addition, when the glucuronide conjugates of monohydroxylated carbamazepines were hydrolyzed, a peak due to 2-hydroxycarbamazepine was observed, which has the same retention time and MS/MS spectrum as the synthesized 2-hydroxycarbamazepine.

**Discussion**

The mechanism(s) of the idiosyncratic reactions associated with carbamazepine therapy are poorly understood. It has been postulated that bioactivation to a reactive arene oxide metabolite is a prerequisite to toxicity (Shear et al., 1988; Pirmohamed et al., 1992) and a risk factor for the adverse reactions is a deficiency of epoxide hydrolase (Spielberg et al., 1981). However, two good studies have failed to find a consistent mutation, or pattern of mutations, in the microsomal epoxide hydrolase gene which is common in patients with a history of carbamazepine hypersensitivity reactions (Gaedigk et al., 1994; Green et al., 1995). Carbamazepine has been shown to be bioactivated to a protein-reactive metabolite by human liver microsomes; however, the covalent binding of \(^{14}\)C carbamazepine to human liver microsomes was relatively low (Pirmohamed et al., 1992). The formation of the arene oxide metabolite has only been inferred from the urinary excretion of unquantified phenols and catechols of...
TABLE 1
The ratios of the TIC of 2-hydroxyiminostilbene-glucuronide (m/z 386) to that of carbamazepine-glucuronide (m/z 413; monohydroxycarbamazepine-glucuronide (m/z 429) and 10,11-dihydrodiosil-carbamazepine (m/z 271)

<table>
<thead>
<tr>
<th>Samples</th>
<th>TIC Ratios m/z 386</th>
<th>TIC Ratios m/z 429</th>
<th>TIC Ratios m/z 271</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>1.76</td>
<td>0.64</td>
<td>0.41</td>
</tr>
<tr>
<td>Patient 2</td>
<td>4.16</td>
<td>1.23</td>
<td>0.47</td>
</tr>
<tr>
<td>Patient 3*</td>
<td>7.60</td>
<td>2.70</td>
<td>0.66</td>
</tr>
</tbody>
</table>

* The third urine sample was collected from a patient in chronic use of carbamazepine. However, detailed information about this patient was not obtained.

Fig. 7. MS/MS spectrum of the glucuronide conjugate of 2-hydroxyiminostilbene.

One of the major routes of carbamazepine metabolism in the liver is hydroxylation of the aromatic ring to yield one of the major carbamazepine metabolites, 2-hydroxy carbamazepine, which can be further hydrolyzed to 2-hydroxyiminostilbene, either in the liver or in other tissues (Lertratanangkoon and Horning, 1982). In this study, we were able to detect the glucuronide conjugate of the 2-hydroxyiminostilbene by LC/MS. Two of the hydroxyiminostilbenes had been detected in human urine during an earlier study in which they were characterized as their trimethylsilyl (TMS) derivatives after enzymatic hydrolysis of the urine sample with Glusulase (Lertratanangkoon and Horning, 1982). One of the TMS derivatives of the hydroxyiminostilbenes was observed to have the same GC/MS properties as the TMS derivative of 2-hydroxyiminostilbene (Lertratanangkoon and Horning, 1982). Although the amount of the metabolite was too small for us to identify the structure by NMR, the fact that we also observed a peak due to the iminoquinone in the same sample suggested that 2-hydroxyiminostilbene glucuronide conjugate must be excreted in the urine because other hydroxyiminostilbene isomers would not generate the iminoquinone intermediate upon oxidation. In addition, when the sample was hydrolyzed by β-glucuronidase to liberate 2-hydroxyiminostilbene, a much larger peak due to the iminoquinone was observed instead of a peak due to 2-hydroxyiminostilbene (Fig. 8). This was not unexpected since we had shown that 2-hydroxyiminostilbene glucuronide conjugate was presumably oxidized to iminoquinone by HOCl, H2O2 or even by air. The free 2-hydroxyiminostilbene released from enzymatic hydrolysis of the glucuronide conjugate was presumably oxidized to iminoquinone readily before it was detected by LC/MS. We also detected a urinary metabolite with the same molecular weight as the glucuronide conjugate of 4-methylthio-2-hydroxyiminostilbene. However, we did not obtain enough material to identify its structure.

In this work, we have also demonstrated that the iminoquinone reacted with sulffhydryl-containing nucleophiles, such as GSH and NAC, to form conjugates. The sulfur was substituted on the aromatic ring in the meta position to the hydroxy group. This result suggested that the iminoquinone is an reactive electrophil, and it may bind to macromolecules (i.e., proteins) in vivo to cause direct toxicity or act as a hapten to modulate the immune system. It could also generate reactive oxygen species by redox cycling.

In summary, we have demonstrated that the β-glucuronide conjugate of 2-hydroxyiminostilbene was excreted in the urine of patients taking carbamazepine. We have also shown that 2-hydroxyiminostilbene was readily oxidized to a reactive iminoquinone intermediate that can be trapped by GSH.
and NAC. The ease of oxidation of 2-hydroxyiminostilbene makes the iminoquinone an attractive candidate for the reactive metabolite of carbamazepine responsible for idiosyncratic reactions in the bone marrow and skin. Specifically the multistep metabolic pathway could explain the relatively low covalent binding of carbamazepine in human hepatic microsomes and the 2-hydroxyiminostilbene could be readily oxidized in target organs, such as bone marrow and skin, by peroxidasases or even by oxygen.

Acknowledgments

We would like to thank Dr. Robert A. McClelland for helpful suggestions on the synthesis of 2-hydroxycarbamazepine and on NMR spectral interpretations.

References


Send reprint requests to: Chris J. van Koppen, Doctor of Philosophy, Institut für Pharmakologie, Universitätsklinikum Essen, D-45122 Essen, Germany. E-mail: van_koppen@uni-essen.de.