Liquid Chromatographic Microassay for Carbamazepine and Its 10,11-Epoxide in Plasma

Keyphrases □ Carbamazepine—carbamazepine 10,11-epoxide, HPLC □ Carbamazepine 10,11-epoxide—HPLC with carbamazepine

To the Editor:

MacKichan (1) has developed a high-performance liquid-chromatographic assay (HPLC) to measure carbamazepine and one of its active metabolites the corresponding 10,11-epoxide in plasma and saliva using double internal standardization. The double internal standard technique provides a more accurate means of quantitating these substances, since the concentrations of the parent compound and metabolite can vary in the same biological specimen by an order of magnitude or more. The HPLC technique described herein is a micronization of the assay developed by MacKichan and allows carbamazepine and its 10,11-epoxide to be accurately quantitated in biological specimens of 100 μL.

The HPLC system utilized in this procedure consists of a liquid chromatograph with an integrative data system and absorbance detector[1]. Injections were made with an auto injector[2] onto a reverse-phase column[3] equipped with a 70 × 6 mm stainless steel precolumn[4] packed with reverse-phase packing material[5]. A μ-Bondapak phenyl column was employed in these analyses since this column is routinely used in our laboratory. The tribasic sodium phosphate and chloroform used in the extraction procedure were ACS certified[6]. All solvents used in the mobile phase were HPLC grade. The carbamazepine[7], the 10,11-epoxide[7], lorazepam[8], and the *N*-demethyl derivative of diazepam[9] were gifts.

A methanolic solution of carbamazepine and the 10,11-epoxide (400 μg/mL) was employed in the preparation of the following plasma standards: 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, and 30.0 μg/mL. Stock solutions of lorazepam and the *N*-demethyl derivative of diazepam were prepared in methanol at a concentration of 100 μg/mL. These solutions were then combined and diluted with distilled deionized water to create an internal standard solution containing lorazepam and the *N*-demethyl derivative of diazepam at concentrations of 10 and 15 μg/mL.

The extraction procedure is a scaled-down version of the extraction process outlined by MacKichan (1). Samples of plasma (100 μL) and an aliquot of the internal standard solution (50 μL) were added to disposable glass test tubes. Each tube was vortexed to mix the internal standard and sample. To this mixture, 100 μL of an aqueous saturated solution of tribasic sodium phosphate was added and vigorously vortexed for 10 s. Chloroform (2 mL) was then added to the tubes, which were then vigorously vortexed (30 s). The samples were centrifuged (10 min at 1500 rpm) and the aqueous phase was aspirated. The organic phase was decanted into clean test tubes and evaporated to dryness under reduced pressure at 42°C. The residues were reconstituted with 100 μL of mobile phase and ~50 μL was injected onto the column. UV absorbance was monitored at 254 nm. The mobile phase consisted of a deionized water–acetonitrile–methanol mixture (62:35:3). The addition of methanol to the mobile phase rectified some peak broadening which was encountered with the phenyl column. The chromatograms resulting from the injection of blank human plasma, human plasma with internal standards added, as well as human plasma with carbamazepine, the 10,11-epoxide, lorazepam, and the *N*-demethyl derivative of diazepam are presented in Fig. 1. All of the peaks were well resolved from each other and from endogenous components of plasma with the exception of the 10,11-epoxide peak which possessed a minor interference similar to that observed by MacKichan (1). This interfering peak can be seen in Fig. 1A and B and poses no appreciable problem with regard to quantitation of the epoxide, representing the equivalent of 0.2 μg/mL of epoxide. The retention times for the epoxide, carbamazepine, lorazepam, and the *N*-demethyl derivative of diazepam were 4.1, 5.7, 6.7, and 8.9 min, respectively.

The mean extraction recovery was 80.3% for carbamazepine and 73.3% for the corresponding 10,11-epoxide, which is lower than the essentially complete recovery of carbamazepine and the epoxide by the method of MacKichan (1). These lower recoveries may be, in part, related to the greater percentage of tribasic sodium phosphate and methanol in the extraction procedure compared with the original method. The approximate sensitivity limits for the assay are 0.1 μg/mL for carbamazepine and 0.3 μg/mL for the epoxide, based on a signal-to-noise ratio of 2.5. The calibration plot is linear in the 0.5–30 μg/mL range. Intraday and interday variability studies, based on six replicate measurements for a period of 6 d at concentrations of 1.0 and 30.0 μg/mL, have demon-

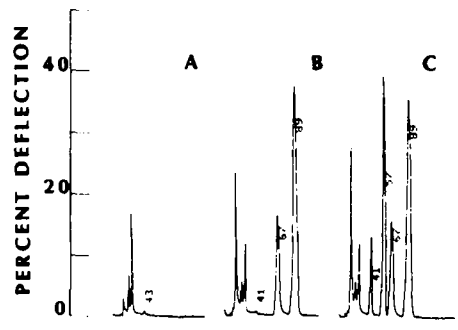

Figure 1—*Chromatograms of a blank human plasma extract (A), a plasma extract with 500 ng of lorazepam (peak 67) and 750 ng of the N-demethyl derivative of diazepam (peak 89) added (B), and a plasma extract containing 10 μg/mL of the 10,11-epoxide of carbamazepine (peak 41) and carbamazepine (peak 57) and 500 ng of lorazepam (peak 67) and 750 ng of the N-demethyl derivative of diazepam (peak 89) added (C). Detector and recorder responses for each chromatogram are 10 mV and 0.005 A full scale. The numbers beside each peak represent its retention time in tenths of minutes.*

[1] Models SP 8000B and SP 8300; Spectraphysics, Santa Clara, Calif.
[2] WISP 710B; Waters Associates, Milford, Mass.
[3] μ-Bondapak phenyl column (30 cm × 2.9 mm i.d., 10 μm particles; Waters Associates.
[4] Crawford (Swagelok-316).
[5] Bondapak C_{18}/Corasil; 37–50 μm.
[6] Fisher Scientific, King of Prussia, Pa.
[7] Tegretol; Ciba-Geigy Corporation, Summit, N.J.
[8] Wyeth Laboratories, Philadelphia, Pa.
[9] Hoffmann-LaRoche, Inc., Nutley, N.J.

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strated assay coefficients of variation of <8.5% for both compounds.

In summary, this micronized assay provides a means for simultaneous analysis of carbamazepine and its 10,11-epoxide from small volumes of plasma. This is of particular value when analyzing samples from pediatric patient populations. In addition, this assay makes possible quantitation of these compounds in the biological fluids of many laboratory animals, where constraints may exist on the volume of blood that can be collected.

(1) J. J. MacKichan, *J. Chromatogr.*, **181**, 373 (1980).

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The Ulcerogenic Potential of Copper Aspirinate Seems to be More Imaginary than Real

Alich *et al.*[1] interpreted the appearance of an intravenously injected protein-bound dye in the gastric mucosa and submucosa of rats given $Cu(II)SO_4$, $Cu(II)_2$(aspirinate)$_4$, or $Cu(II)SO_4$ plus aspirin administered with or without dihydroxyaluminum glycinate and magnesium carbonate buffer as erosions. Leakage of the protein-bound dye from the vasculature was suggested as due to damaged blood vessels. Appearance of the protein-bound dye in the mucosa and submucosa in association with the above treatments does not provide evidence of either erosions or vascular damage.

Appearance of the protein-bound dye in the mucosa and submucosa of copper compound-treated rats is more likely due to leakage associated with absorption-induced hyperemia, as shown for the vehicle control, and the quantity of protein-bound dye found in these two tissues is more likely due to the amount of copper-containing compounds absorbed and their subsequent formation of quaternary dye-protein-copper complexes which produced colorations of varying intensity. Copper sulfate, which is not lipid soluble, was poorly absorbed in the time course of this experiment while more of the lipid-soluble copper aspirinate was absorbed[2]. The combination of aspirin plus copper probably produced an aspirin- and/or salicylate-copper complex as a result of the 10-min sonication, and these complexes were nearly equally well absorbed following instillation. Appearance of the dye-protein-copper complexes in the mucosa and submucosa in association with absorption of administered copper compounds is also consistent with the poor agreement between the two "lesion" grading techniques found when aspirin was administered without a copper-containing compound.

Alich *et al.*[1] suggested that, in general, the presence of buffer had no significant effect on the extent of "damage" produced under these experimental conditions. Exception is also taken to this suggestion since it seems clear that there was a statistically significant reduction in coloration in the group given the buffered copper aspirinate preparation. This reduction in coloration is consistent with a reduction in absorption of copper complex following the formation of an insoluble form of copper, $CuCO_3$, or some other nonabsorbable form of copper during the 10-min sonication of a suspension containing 27 μmol of $Cu(II)_2$(aspirinate)$_4$, 71 μmol of $MgCO_3$, and 22 μmol of dihydroxyaluminum glycinate/mL. The formation of $Cu(II)$(glycinate)$_2$, $Cu(II)$(salicylate)$_2$, and $Cu(II)_2$(aspirinate)$_4$ during the 10-min sonication of buffer, aspirin, and $CuSO_4$ may have only partially overcome the effects of $MgCO_3$ when the copper plus aspirin preparation was used. The absorption of copper when it was given as $CuSO_4$ also appeared to have been impeded by the buffer.

In their discussion of the existing literature pertaining to effects of $Cu(II)_2$(aspirinate)$_4$, Alich *et al.*[1] further reduce the credibility of their interpretation of their results as indicating gastric erosions and vascular damage. They did point out that Williams *et al.*[2] found no gastric erosions with $Cu(II)_2$(aspirinate)$_4$, but failed to mention that Williams *et al.*[2] had used doses of 115, 345, 690, and 1380 mg of $Cu(II)_2$(aspirinate)$_4$/kg of body weight, doses much greater than the 115-mg/kg dose used in their study[1].

Alich *et al.*[1] did point out that Williams *et al.*[2] reported "some evidence" of hemorrhage without gastric erosions in rats given $Cu(II)_2$(aspirinate)$_4$, but failed to mention that this hemorrhage which was observed at 345, 690, and 1380 mg/kg was not observed at 115 mg/kg. Alich *et al.*[1] offered no rationale as to how this hemorrhage, which occurred in the absence of gastric erosions, was relevant to their gastric study or in any way supported their interpretation of their results. We have determined the $LD_{50/7}$ of $Cu(II)_2$(aspirinate)$_4$ to be 895 ± 222 and 977 ± 297 mg/kg, respectively, in male and female rats using doses ranging up to 1500 mg/kg. Doses of 1000 and 1500 mg/kg did not produce gastric ulcers but did produce diarrhea. A diarrheal-inducing bolus may cause intestinal bleeding, but this cannot be mistaken for gastric bleeding, and these doses are much greater than the 115-mg/kg dose used by Alich *et al.*[1]. We have also reported results of our chronic toxicity study of $Cu(II)_2$(aspirinate)$_4$ in rats given 100 mg/kg *for 5 d/week* for 3 months[3]. No gastric histopathology was found at the light microscopic level with this more challenging treatment regimen.

Alich *et al.*[1] incorrectly indicated that we had reported that aspirin was an active antiulcer agent in the Shay rat[4]. They also failed to notice that we reported a reduction in gastric acid secretion in response to treatment of the Shay rat with copper complexes and not an accumulation of gastric acid following pyloric ligation as they indicated. The antisecretory effects of copper complexes have been confirmed and extended by others[5-8]. These results support the suggestion that antisecretory activity, in part, accounts for their mechanism of antiulcer activity. Alich *et al.*[1] also overlooked the report by Hayden *et al.*[9] that $Cu(II)_2$(aspirinate)$_4$ prevents aspirin-potentiated ulcers in the Shay rat.

Alich *et al.*[1] questioned the significance of data obtained in nonfasted rats in comparison with their data, but failed to recognize that our Shay rat preparation, like theirs, includes fasting.

Alich *et al.*[1] did point out that Rainsford and Whitehouse[10] reported the production of ulcers in rats with $Cu(II)_2$(aspirinate)$_4$ (200 mg/kg), but overlooked the response to that report which pointed out that those studies were flawed by the use of an inappropriate suspending agent, which has the potential of removing the copper from the administered complex, and the solution of copper complexes in 20 mM hydrochloric acid, which has the potential of destroying the "dissolved" complex and freeing the ulcerogenic ligand[11].

Alich *et al.*[1] did point out that Boyle *et al.*[12] found an antiulcer effect for $Cu(II)_2$(aspirinate)$_4$, but failed to mention that the dose was 345 mg/kg, a dose 3 times as large as their 115-mg/kg dose, and dismissed their results for specious reasons, the inclusion of both number and size of lesions in their assessment of the ulcerogenicity of aspirin.

Alich *et al.*[1] also pointed out that the results reported by Lewis[13,14] and Brown *et al.*[15] were "about the same for free aspirin and $Cu(II)_2$(aspirinate)$_4$" and indicated that the method used for quantitation of was less than state of the art, but failed to mention that doses of 300 mg/kg were used.

The significance of $Cu(II)_2$(aspirinate)$_4$-induced gastric irritation, even if it did occur with doses of 100 mg/kg and higher, remains obscure since the antiulcer dose of this compound is 5–10 mg/kg[4,9,13]. The 10-mg/kg dose has also been shown to markedly increase the rate of interstitial tissue repair in the surgically induced ulcer using histochemical techniques and light microscopy[13].

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