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Mechanism for Interference of Carbamazepine in Porter-Silber Reaction for Determination of Urinary 17-Hydroxycorticosteroids[1]

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The mechanism by which dosage of carbamazepine (5*H*-dibenz[*b,f*]azepine-5-carboxamide) interferes with the Porter-Silber reaction for the determination of urinary 17-hydroxycorticosteroids has been investigated. A specimen of human urine collected following oral administration of carbamazepine was processed in the usual manner. The principal metabolites responsible for the Porter-Silber test were separated and characterized to be carbamazepine-10,11-epoxide and dihydrocarbamazepine-10,11-*trans*-diol by usual criteria. Being treated with the incomplete Porter-Silber reagent, these metabolites underwent facile rearrangement yielding acridine-9-aldehyde accompanied with a small amount of acridine. It has been disclosed that the interference in the determination of 17-hydroxycorticosteroids due to the undesirable coloration is ascribable to the formation of acridine-9-aldehyde phenylhydrazone from the drug metabolites.

In running a routine diagnostic laboratory, interferences due to the commonly used medications in the assay procedures have become more of a problem. It is documented in the literatures that many drugs influence the methods measuring steroid hormones in the biological fluid.[4] In a preceding paper of this series we reported the mechanism for a marked increase in the urinary 17-ketosteroids value by the Zimmermann test after ingestion of ethinamate 1(-ethynylcyclohexyl carbamate).[5] Recently it has been found that oral administration of carbamazepine (5*H*-dibenz[*b,f*]azepine-5-carboxamide) (1), an antiepileptic drug, interferes with the determination of 17-hydroxycorticosteroids (17-OHCS) in urine by the Porter-Silber reaction.[6] The present paper deals with the mechanism by which ingestion of carbamazepine produces the Porter-Silber chromogen to elevate falsely the value for urinary 17-OHCS.

Carbamazepine itself, when added to the urine specimen, did not exert any significant influence on the determination of 17-OHCS. This finding suggested that drug metabolites in urine would possibly serve for the coloration with the Porter-Silber reagent. Therefore, it became necessary to elucidate the urinary metabolites in man administered with this drug. Although the metabolic fate of carbamazepine has been previously investigated by Frigerio and his coworkers,[7] we attempted the characterization of the drug metabolites in human urine responsible for the Porter-Silber reaction.

Extraction of the collected urine with ethyl acetate, followed by purification of the extract by column chromatography and further preparative thin-layer chromatography

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[1] Part CXVI of "Studies on Steroids" by T. Nambara; Part CXV: K. Shimada, M. Hasegawa, K. Hasebe, Y. Fujii, and T. Nambara, *Chem. Pharm. Bull.* (Tokyo), **24**, 2995 (1976).

[2] Location: *a*) *Fukushima, Osaka*; *b*) *Aobayama, Sendai.*

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[4] D.S. Young, D.W. Thomas, R.B. Friedman, and L.C. Pestaner, *Clin. Chem.*, **18**, 1041 (1972); D.S. Young, L.C. Pestaner, and V. Gibberman, *ibid.*, **21**, 1D (1975).

[5] T. Nambara, K. Shimada, and R. Hanabuchi, *Chem. Pharm. Bull.* (Tokyo), **23**, 2458 (1975).

[6] C.C. Porter and R.H. Silber, *J. Biol. Chem.*, **185**, 201 (1950); R.H. Silber and C.C. Porter, *ibid.*, **210**, 923 (1954).

[7] *a*) A. Frigerio, R. Fanelli, P. Biandrate, G. Passerini, P.L. Morselli, and S. Garattini, *J. Pharm. Sci.*, **61**, 1144 (1972); *b*) K.M. Baker, J. Csetenyi, A. Frigerio, and P.L. Morselli, *J. Med. Chem.*, **16**, 703 (1973).

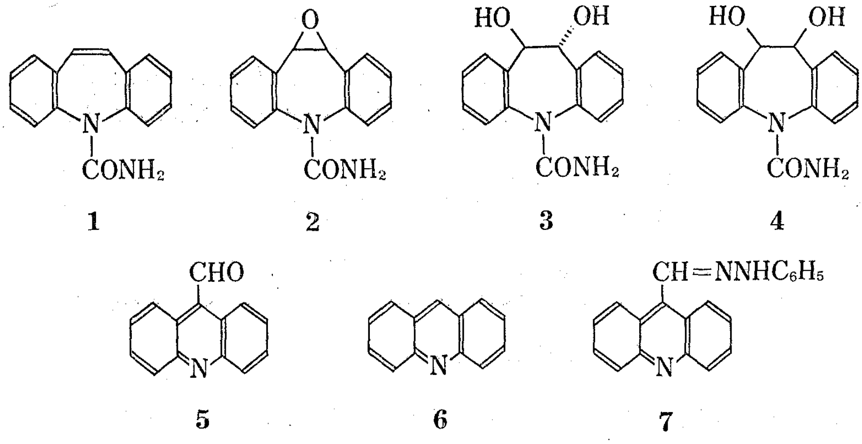

Chart 1

(TLC) afforded two chromogenic metabolites which exhibited promptly purple-red staining on the thin-layer plate with the Porter-Silber reagent. The major metabolite was unambiguously characterized to be 10,11-dihydro-10,11-epoxy-5*H*-dibenz[*b*,*f*]azepine-5-carboxamide (carbamazepine-10,11-epoxide) (2) by direct comparison with the authentic sample.

Repeated purification by high-pressure liquid chromatography (HPLC) provided another metabolite. Unfortunately it was not obtained in the crystalline state, but substantially homogeneous. The metabolite seemed to be a dihydroxylic derivative as judged from the mass spectrum and chromatographic behaviors. For comparison with the metabolite, 10,11-dihydro-10,11-*cis*-dihydroxy-5*H*-dibenz[*b*,*f*]azepine-5-carboxamide(dihydrocarbamazepine-10,11-*cis*-diol) (4) was synthesized from carbamazepine by osmium tetroxide oxidation according to the procedure of Baker, *et al.*[7b] In the mass spectra the metabolite and synthetic *cis*-glycol exhibited apparently the similar fragmentation pattern, but they were somewhat different in the relative intensity of each fragment ion. These two showed the identical mobility on the ordinary thin-layer plate. However, they could be differentiated each other when developed on the boric acid impregnated silica gel plate. In addition the two samples were distinctly separated by HPLC on a reversed phase column. These evidences lent a support to assign the structure 10,11-dihydro-10,11-*trans*-dihydroxy-5*H*-dibenz[*b*,*f*]azepine-5-carboxamide (dihydrocarbamazepine-10,11-*trans*-diol) (3) to the second chromogenic metabolite. To the best of our knowledges it is more reasonable that the 10,11-*trans*-glycol would be formed from the 10,11-epoxide by stereoselective hydrolysis with epoxide hydrase (or epoxide hydrolase).[8] Accordingly, the stereochemical assignment to the *cis*-diol by Frigerio, *et al.*[7b] should be corrected.

The next effort was directed toward elucidation of the structure and formation mechanism of the coloring matter. Brief treatment of carbamazepine-10,11-epoxide with the incomplete Porter-Silber reagent[9] and subsequent purification by preparative TLC provided two main transformation products. One of these exhibited the characteristic staining with phenylhydrazine on the silica gel plate in the manner as the mother epoxide. On the basis of the physico-chemical data, *i.e.* infrared (IR), nuclear magnetic resonance (NMR) and mass spectra, this substance was assumed to be acridine-9-aldehyde (5). In actuality the structure was unequivocally established by direct comparison with the authentic sample prepared by the known methods.[10] Another transformation product, though not responsible for the coloration

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[8] D.M. Jerina, J.W. Daly, B. Witkop, P. Zaltzman-Nirenberg, and S. Udenfriend. *J. Am. Chem. Soc.*, **90**, 6525 (1968); T. Watabe and E.W. Maynert, *Fed. Proc.*, **27**, 302 (1968).

[9] M.L. Lewbart and V.R. Mattox, *J. Org. Chem.*, **29**, 513, 521 (1964).

[10] O. Tsuge, M. Nishinohara, and M. Tashiro, *Bull. Chem. Soc.* (Japan), **36**, 1482 (1963); H. Kaplan, *J. Am. Chem. Soc.*, **63**, 2654 (1941).

with the Porter-Silber reagent, was identified as acridine (**6**) by usual criteria. This might be formed from acridine-9-aldehyde by oxidation, followed by spontaneous decarboxylation.

Being submitted to the Porter-Silber test, acridine-9-aldehyde as well as carbamazepine-10,11-epoxide produced promptly the colored solution whose absorption spectrum (λ_{max} 554 nm) was superimposable with that obtained with the urine specimen collected after oral administration of carbamazepine. These findings strongly implied that the coloring matter would be acridine-9-aldehyde phenylhydrazone. In fact the synthetic phenylhydrazone11) produced the purple-red colored solution having an absorption maximum at 554 nm in an acidic medium.

The 10,11-*trans*-dihydroxylic metabolite also exhibited the characteristic coloration with the Porter-Silber reagent and in addition, with sulfuric acid yielded acridine-9-aldehyde and acridine. The acridine derivative may probably be formed by the pinacol-type rearrangement under the acidic conditions.

Based upon these results we arrived at the conclusion that the interference of carbamazepine in the Porter-Silber reaction for the determination of urinary 17-OHCS should be ascribable to the formation of acridine-9-aldehyde from the drug metabolites, the 10,11-epoxide and in part the 10,11-*trans*-glycol, with sulfuric acid, followed by condensation with phenylhydrazine to yield acridine-9-aldehyde phenylhydrazone as a coloring matter.

Experimental12)

Thin-Layer Chromatography (TLC)——TLC was carried out on a silica gel HF_{254} (E. Merck AG, Darmstadt) plate using $CHCl_3$/MeOH (15: 1) (TL-I) or AcOEt (TL-II) as a solvent or on a 5% H_3BO_3 impregnated silica gel HF_{254} plate using $CHCl_3$/MeOH (5: 1) as a solvent (TL-III), and the *Rf* value was given.

High-Pressure Liquid Chromatography (HPLC)——The apparatus used was a Waters Model ALC/GPC 202 R401 high-pressure liquid chromatograph (Waters Associates Inc., Milford) equipped with an ultraviolet detector monitoring the absorbance at 254 nm. A μ-Bondapak C_{18} column (1′×1/4″ i.d.) (Waters Associates Inc.) was used at ambient temperature. The sample was injected with a Model U6K sample loop injector (Waters Associates Inc.) with an effective volume of 2 ml. HPLC was carried out employing MeOH/H_2O (2: 5) as a mobile phase at a flow rate of 1.5 ml/min, and the retention time was given.

Syntheses of Authentic Samples——10,11-Dihydro-10,11-epoxy-5*H*-dibenz[*b*,*f*]azepine-5-carboxamide (Carbamazepine-10,11-epoxide) (**2**): Prepared from carbamazepine (**1**)13) by treatment with *m*-chloroperbenzoic acid according to the procedure of Frigerio, *et al.*7a) mp 194—196.5° (Lit. mp 190—195°). TLC: TL-I 0.54, TL-II 0.27.

10,11-Dihydro-10,11-*cis*-dihydroxy-5*H*-dibenz[*b*,*f*]azepine-5-carboxamide (Dihydrocarbamazepine-10,11-*cis*-diol) (**4**): Prepared from **1** by oxidation with OsO_4 according to the procedure of Baker, *et al.*7b) mp 239—241° (Lit. mp 242—245°). Mass Spectrum *m/e*: 270 (M^+) (3.0), 223 (7.4), 193 (25.3), 180 (100). TLC: TL-I 0.10, TL-III 0.49. HPLC: 4.9 min.

Administration of Carbamazepine (1)——Four hundred milligrams each of **1** was orally administered to two healthy volunteers before sleeping, and urine was collected for a period of following 24 hr.

Separation and Characterization of Metabolites——The urine specimens were combined and extracted with AcOEt (1700 ml) three times. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. The residue obtained was submitted to column chromatography on silica gel (2 g). Elution with $CHCl_3$ gave unchanged **1** (3 mg). TLC: TL-I 0.68. The second eluate obtained with $CHCl_3$/MeOH (10: 1) was submitted to preparative TLC using $CHCl_3$/MeOH (20: 1) as a developing solvent. Elution of the adsorbent corresponding to the spot (^2Rf 0.50) with AcOEt and trituration of the eluate with MeOH gave **2** (1 mg) as colorless prisms. Comparison with the synthetic specimen by TLC and IR spectrum showed

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11) A.E. Porai-Koshits and A.A. Kharkharov, *Bull. Acad. Sci. U.R.S.S., Classe Sci. Chim.*, **1944**, 243 [*Chem. Abstr.*, 39, 1631 (1945)].

12) All melting points were taken on a micro hot-stage apparatus and are uncorrected. The visible and IR spectral measurements were run on Hitachi Model 124 and JASCO Model IR-S spectrophotometers, respectively. Mass spectra were obtained on a Hitachi Model RMU-7 spectrometer at the following conditions: ionization voltage, 70 eV; ion source temperature, 250°; accelerating voltage, 3.5 kV. NMR spectra were recorded on a JEOL PS-100 spectrometer at 100 MHz. The chemical shifts are quoted as ppm downfield from tetramethylsilane used as an internal standard. Abbreviation used s=singlet, q=quartet, and m=multiplet.

13) Pure material was kindly donated by Ciba-Geigy Ltd. (Basle, Switzerland).

the identity of the two samples in every respect. The third eluate obtained with $CHCl_3$/MeOH (10: 1) was purified by preparative TLC on silica gel HF_{254} using $CHCl_3$/MeOH (10: 1) as a developing solvent. Elution of the adsorbent corresponding to the spot (^2Rf 0.61) with AcOEt/MeOH (4: 1), followed by further purification by HPLC employing MeOH/H_2O (2: 5) as a mobile phase gave 10,11-dihydro-10,11-*trans*-dihydroxy-5*H*-dibenz[*b*,*f*]azepine-5-carboxamide (dihydrocarbamazepine-10,11-*trans*-diol) (3) (1 mg) as colorless oil. Mass Spectrum *m*/*e*: 270 (M^+) (5.9), 223 (17.3), 193 (9.1), 180 (100). TLC: TL-I 0.10, TL-III 0.43. HPLC: 5.3 min. This metabolite was evidently differentiated from 4 by TLC and HPLC. In the conjugate fraction obtained by incubation with beef-liver β-glucuronidase the presence of 3 was also demonstrated.

Transformation of Carbamazepine-10,11-epoxide (2) with Incomplete Porter-Silber Reagent——To a solution of 2 (105 mg) in CH_2Cl_2 (10 ml) was added the incomplete Porter-Silber reagent (21 N H_2SO_4/EtOH (3: 1)) (8 ml) and shaken vigorously. After removal of the upper layer the remaining solution was heated at 60° for 30 min. The resulting solution was neutralized with 20% NaOH under ice-cooling and then extracted with ether. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated. The residue obtained was submitted to preparative TLC using hexane/AcOEt (20: 1) as a developing solvent. The Porter-Silber chromogen was located by spraying the edges of the plate with the phenylhydrazine–H_2SO_4 reagent, and the adsorbent corresponding to the spot (^3Rf 0.72) was eluted with AcOEt. The eluate was purified by HPLC using MeOH/H_2O (2: 1) as a mobile phase at a flow rate of 1.5 ml/min and recrystallized from EtOH to give acridine-9-aldehyde (5) (5 mg) as pale yellow needles. mp 145—148.5°. IR $\nu_{\max}^{\mathrm{CHCl_3}}$ cm^{-1}: 1684 (–CHO). NMR (1% solution in $CDCl_3$) δ: 7.70 (4H, m, 2,3,6,7-H), 8.30 (2H, q, $J=10$, 2 Hz, 4,5-H), 8.70 (2H, q, $J=10$, 2 Hz, 1,8-H), 11.57 (1H, s, –CHO). Mixed melting point on admixture with the authentic sample[10] showed no depression. TLC, HPLC, IR, NMR, and mass spectra of two samples were entirely identical in every respect. Phenylhydrazone (7): dark purple needles (from MeOH). mp 193—196° (Lit. mp 194°).[11] A solution of the phenylhydrazone dissolved in 21 N H_2SO_4/EtOH (3: 1) exhibited an absorption max. at 554 nm. Elution of the adsorbent corresponding to the spot (^3Rf 0.83) in the preparative TLC with AcOEt gave a yellow oily residue. The eluate was purified by HPLC using THF/H_2O (1: 1) as a mobile phase at a flow of 1.5 ml/min to give acridine (6) (1 mg) as yellow oil. This substance proved to be identical with the authentic sample by TLC, HPLC, IR, and mass spectral comparison.

Transformation of Dihydrocarbamazepine-10,11-*trans*-diol (3) with Incomplete Porter-Silber Reagent——A trace amount of 3 was dissolved in 21 N H_2SO_4/EtOH (3: 1) (2 ml) and heated at 60° for 30 min. The resulting solution was neutralized with 20% NaOH under ice-cooling and then extracted with ether. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated. The residue obtained was submitted to TLC employing hexane/AcOEt (4: 1) as a developing solvent, whereby **5** (*Rf* 0.40) and **6** (*Rf* 0.45) were unequivocally characterized.

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