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The Isolation and Characterization of a Metabolic Product of 3,3'-Carboxymethylenebis-(4-hydroxycoumarin) Ethyl Ester (Tromexan) from Human Urine

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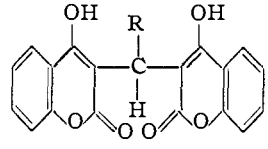
The isolation of a metabolic product of the anticoagulant drug, Tromexan, is described. Degradation studies show that the compound is derived from Tromexan by the introduction of a hydroxyl group into one of the benzene rings.

Tromexan[1] (3,3'-carboxymethylenebis-(4-hydroxycoumarin) ethyl ester) (I), a compound structurally similar to Dicumarol (II), has been recently introduced as an anticoagulant drug in the treatment of thromboembolic diseases. Both Dicumarol and Tromexan are almost completely metabolized in the body,[2,3] but nothing has been reported concerning the nature of their transformation products in man.



I, R = $COOC_2H_5$
II, R = H
III, R = COOH

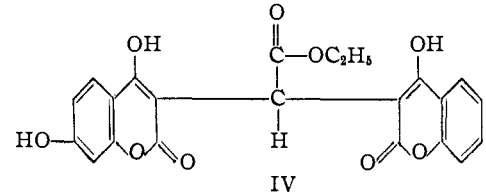
It is the purpose of this communication to report the isolation and characterization of a metabolite of Tromexan from urine of patients who had received the drug.

The metabolite was isolated by countercurrent distribution. Results of elemental analysis indicated that the empirical formula of the isolated compound differed from that of Tromexan by one additional oxygen atom. The ultraviolet absorption spectrum of the metabolite in aqueous alkaline solution differed significantly from that of Tromexan and its de-esterified form, Tromexan acid (III). Potentiometric titration curves obtained for Tromexan and its metabolite were identical, both compounds showing a single inflection point with a pK_a of 3.1. Tromexan acid, on the other hand, had two inflection points with pK_a's of 3.1 and 7.8.[4]

These findings suggested that the metabolite might be derived from Tromexan by the introduction of a hydroxyl group into one of the benzene rings. To determine the position of this group the compound was degraded by fusion with potassium hydroxide under the conditions used by Stahmann, Huebner and Link[5] in their structure proof for Dicumarol. These investigators recovered about two moles of salicylic acid for each mole of Dicumarol submitted to fusion. Tromexan, degraded under the same conditions, yielded about 1.8 moles of salicylic acid. Alkaline fusion of the Tromexan metabolite yielded 0.8 mole of salicylic acid and 0.7 mole of a dihydroxybenzoic acid, indicating the presence of a hydroxyl group on one of the benzene rings.

The ultraviolet absorption spectrum of the unknown dihydroxybenzoic acid was compared with those of authentic samples of the four possible dihydroxybenzoic acids. The spectrum of the unknown dihydroxybenzoic acid was essentially superimposable upon that of a known sample of 2,4-dihydroxybenzoic acid and was different from the spectra of 2,3-, 2,5- and 2,6-dihydroxybenzoic acids in both alkali and ethanol. Further proof of identity was obtained from melting point data. This evidence indicates that the metabolite is derived from Tromexan by the addition of a hydroxyl group in one of the benzene rings meta to the oxygen of the lactone bridge (IV). Tromexan is thus another of a large number of drugs which are metabolized in the body through hydroxylation of an aromatic ring; a few other examples are salicylic acid,[6] the cinchona alkaloids[7] and acetanilide.[8]

Pharmacological studies that will be reported elsewhere indicate that following the administration of Tromexan to man, 10 to 15% of the drug is excreted in the urine as the hydroxy derivative and that the metabolite has no anticoagulant activity.



IV

Experimental

Isolation of a Metabolite of Tromexan from Urine.—Six human subjects were given 1.5 g. of Tromexan orally and urine was collected for the subsequent 24 hours. The pooled urine (9 liters) was acidified with 300 ml. of 12 *N* hydrochloric acid and shaken with 25 l. of heptane containing 3% isoamyl alcohol. Acidic material in the heptane phase was returned to an aqueous phase by shaking with 200

(1) Supplied through the courtesy of Geigy Pharmaceuticals, New York, N. Y.

(2) M. Weiner, S. Shapiro, J. Axelrod, J. R. Cooper and B. B. Brodie, *J. Pharmacol. Expl. Therap.*, **99**, 409 (1950).

(3) B. B. Brodie, M. Weiner, J. J. Burns, G. Simson and E. K. Yale, *ibid.*, **106**, 453 (1952).

(4) The marked acidity of Tromexan should be noted. For comparative purposes, the pK_a of Dicumarol and 4-hydroxycoumarin were measured under the same conditions and found to be 5.7 and 5.8, respectively. The high acidity of Tromexan, compared to Dicumarol, may be due to the stabilizing effect of the ester carbonyl group on the enolate ion of Tromexan. The acid dissociation constant of Tromexan is the same as the first dissociation constant of its de-esterified derivative, Tromexan acid. It is not known whether the first dissociation constant of Tromexan acid is due to the ionization of an enolic group or the carboxyl group. Irrespective of which group first dissociates, the anion formed is probably stabilized by hydrogen bonding between the enolic groups and the neighboring carboxyl group.

(5) M. A. Stahmann, C. F. Huebner and K. P. Link, *J. Biol. Chem.*, **138**, 513 (1941).

(6) E. M. Kapp and A. F. Coburn, *ibid.*, **145**, 549 (1942).

(7) B. B. Brodie, J. E. Baer and L. C. Craig, *ibid.*, **188**, 567 (1951).

(8) B. B. Brodie and J. Axelrod, *J. Pharmacol. Exp. Therap.*, **94**, 29 (1948).

ml. of 2.5 *N* sodium hydroxide. The absorption spectrum of this solution was examined with a Beckman model DU spectrophotometer and revealed considerable amounts of material with an absorption peak at 325 mμ. The alkaline solution, when acidified with 12 *N* hydrochloric acid and allowed to stand at 5° for 12 hours, yielded a precipitate. The filtered material was subjected to countercurrent distribution according to the method of Craig, *et al.*[9] The distribution was effected in a series of separatory funnels between equal volumes of 40% isoamyl alcohol in heptane, and 0.1 *M* phosphate buffer, *p*H 7.6. After countercurrent distribution the partition ratio of the material in each separatory funnel was determined. The relative concentration of the material was measured by its absorption at 325 mμ in 2.5 *N* sodium hydroxide. The contents of funnels 3 to 7 contained material with almost identical partition ratios and were pooled. The aqueous phase was acidified and the total acidic material was extracted into the organic phase by shaking. The organic phase was then extracted three times with 20-ml. portions of 2.5 *N* sodium hydroxide. The combined alkaline extract was acidified with 12 *N* hydrochloric acid. The resulting brownish precipitate was recrystallized from methanol. Colorless needles (250 mg.) were obtained and after drying under vacuum melted at 245–247°.[10] In comparison, Tromexan melts at 177–178° and Tromexan acid at 214–215°.

Anal. Calcd. for $C_{22}H_{16}O_9$: C, 62.26; H, 3.80. Found: C, 62.25; H, 3.90.

Potentiometric titration curves were obtained for the metabolite, Tromexan and Tromexan acid. Twenty milligrams of each substance was dissolved in 10 ml. of 90% ethanol. The solutions were titrated with 0.025 *N* sodium hydroxide using a Beckman *p*H meter with a glass electrode. The acid dissociation constants were found to be as follows: Tromexan, pK_a 3.1; metabolite, pK_a 3.1; Tromexan acid, pK_a's 3.1 and 7.8.

Degradation of Metabolite.—A 25-mg. sample of the metabolite was fused with potassium hydroxide according to the method of Stahmann, Huebner and Link.[5] The fusion products were subjected to a countercurrent distribution involving eight transfers. The solvents used were equal volumes of ethylene dichloride and 0.4 *M* phosphate–citrate buffer, *p*H 3.4. Under these circumstances salicylic acid was distributed about equally between the two phases. Salicylic acid was analyzed by its absorption at 305 mμ in 2.5 *N* sodium hydroxide. After countercurrent distribution, the partition ratio of the apparent salicylic acid in each separatory funnel was determined. The material in funnels 5 to 8 had a partition ratio and ultraviolet absorption spectrum identical with salicylic acid. The aqueous phase of funnel 6 was acidified with hydrochloric acid and the total apparent salicylic acid of the funnel was transferred to the organic phase by shaking. Evaporation of the organic solvent yielded colorless crystals which melted at 153.5–155°. A mixed melting point with authentic salicylic acid showed no depression. From the countercurrent data it was calculated[11] that about 0.80 mole of salicylic acid[12] is formed from one mole of metabolite.[13]

Funnels 1 and 2 contained a more water-soluble material which had absorption peaks at 270 and 300 mμ in 2.5 *N* sodium hydroxide. The contents of these funnels were pooled. The aqueous phase of the pooled sample was made alkaline with sodium hydroxide and the acidic material in the organic phase was transferred to the aqueous phase by shaking. The aqueous phase was acidified with hydrochloric acid and the material extracted into ether. The ether was evaporated and the material was subjected to an eight-transfer countercurrent distribution between 40% isoamyl alcohol in heptane and 0.1 *M* phosphate–citrate buffer, *p*H 4.5. Under these conditions, the material with absorption peaks at 270 and 300 mμ in 2.5 *N* sodium hydroxide distributed about equally between the two phases. After countercurrent distribution, the partition ratio of the material in each separatory funnel was determined by measuring its concentration in each phase. The contents of funnels 2 to 7, which represented the major part of material subjected to countercurrent distribution, had identical partition ratios. The material in each of these funnels gave identical ultraviolet absorption curves with peaks at 270 and 300 mμ. The results of this countercurrent distribution indicated the presence of only one major component.

The contents of funnels 4 to 6 were pooled. The aqueous phase of the pooled sample was acidified with hydrochloric acid and the total acidic material transferred to the organic phase by shaking. Evaporation of the organic solvent yielded colorless crystals which melted at 205–206°. An authentic sample of 2,4-dihydroxybenzoic acid melted at 206–208°. The mixed melting point of the two compounds showed no depression. From the above countercurrent data, it was calculated[11] that about 0.70 mole[14] of dihydroxybenzoic acid was formed for each mole of the metabolite subjected to the fusion.

The ultraviolet absorption spectra of the dihydroxybenzoic acid obtained from fusion and the authentic samples of the four possible dihydroxybenzoic acids were measured in a Beckman model DU spectrophotometer. The spectrum of the unknown compound was identical with that of 2,4-dihydroxybenzoic acid.

Acknowledgment.—The authors wish to thank Dr. H. B. Wood of the National Heart Institute for authentic samples of 2,3-dihydroxybenzoic acid, 2,4-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid and 2,6-dihydroxybenzoic acid.

NEW YORK, N. Y.
BETHESDA, MD.

(9) L. C. Craig, C. Golumbic, R. Mighton and E. Titus, *J. Biol. Chem.*, **161**, 321 (1945).

(10) All melting points were determined on a micro melting point stage and are uncorrected.

(11) B. Williamson and L. C. Craig, *J. Biol. Chem.*, **168**, 687 (1947).

(12) Appropriate correction was made for the finding that only 90% of authentic salicylic acid carried through the fusion procedure could be recovered.

(13) When Tromexan was submitted to an alkali fusion and the fusion products subjected to a countercurrent distribution about 1.8 moles of salicylic acid was recovered from each mole of Tromexan.

(14) This value was corrected for recovery since only 75% of authentic 2,4-dihydroxybenzoic acid carried through the fusion procedure could be recovered.