Separation and Analysis of Degradation Products of Tetracycline by Gel Filtration on Sephadex G-25

By B. W. GRIFFITHS

The anhydrotetracycline and 4-epi-anhydrotetracycline degradation products of tetracycline have been found to separate from tetracycline on a column of Sephadex G-25. The tetracycline elutes as the "fast" component, whereas the degradation products which are not separated from each other elute as the "slow" component. The ease of column preparation, rapidity of analysis, and repetitive use of a single column make the technique a useful screening procedure for gross contamination of tetracycline preparations with anhydro- and 4-epi-anhydrotetracycline degradation products.

In view of recent findings that relate a reversible Fanconi-type syndrome to the ingestion of degraded tetracycline capsules (1–5), interest has developed in the analytical determination of products of degradation of tetracycline (TC). Particular interest has centered on the 4-epi-anhydrotetracycline derivative, since a similar syndrome to the above was induced in rats and dogs on the ingestion of large doses of this compound (6).

A column chromatographic method has been developed by Kelly (7) for the determination of both anhydrotetracycline (ATC) and 4-epi-anhydrotetracycline (EATC) in the presence of large quantities of TC. In the author's experience the method was found workable and reproducible but had the disadvantage of tedious column preparation and the limitation of a single analysis per column.

During a search for an alternative method, it was found that a partially degraded TC compound separated into 2 colored components on a column of Sephadex G-25. The "fast" component was found to have the U.V. characteristics of TC while the "slow" one had those of ATC or EATC. The potential of this observation for use in analysis was studied and a test was developed for the gross analysis of ATC and EATC in pharmaceutical preparations of TC. Although the method is not expected to be a substitute for one providing a separate analysis for ATC and the epimer, the features of ease of column preparation, rapidity of analysis, and repetitive use of the same column make it a technique highly useful for the routine screening of TC preparations for gross ATC and EATC content.

EXPERIMENTAL

Reagents and Materials

Compounds of Tetracycline and Derivatives.—Commercial tetracycline HCl (TC) of a high degree of purity by microbiological assay was used. Anhydrotetracycline (ATC) and 4-epi-anhydrotetracycline (EATC) were obtained from Lederle Laboratories.

Sephadex Columns.—*Acid Column.*—A pledget of glass wool was placed in the outlet of a glass column (1.8 × 35 cm.) over which were placed small glass beads to a depth of 1 cm. The Sephadex G-25 (medium) previously swollen in 0.03 *N* HCl was applied as a slurry to the closed column. At this normality the epimerization rate of tetracycline is small and acid-catalyzed dehydration to anhydrotetracycline does not occur (8). After about 5 min. the outlet was opened and more slurry added until a bed length of 23 cm. was attained. Flow rates of 180–200 ml./hr. were obtained. An overhead volume of about 400 ml. provided continuous delivery of solvent. Fractions were collected usually in 5-ml. portions with the aid of an LKB Radi-Rac fraction collector.

Alkaline Column.—The Sephadex was equilibrated with 0.04 *M* phosphate buffer, pH 7.7–7.8. The column preparation followed the above outline with the exception that the length of the bed was 30.5 cm.

Method for Tetracyline Oral Preparations

The powdered tablet or capsule material is accurately weighed and dissolved in 0.03 *N* HCl to give 20 mg. of TC/ml. The insoluble residue is centrifuged and 0.5 ml. of the supernatant is applied to the acid column and washed in gently with the HCl solvent. The TC passes rapidly through the column while the ATC and EATC elute as a slower single component which is visible as a diffuse, bright yellow band. This band, which is eluted after approximately 115 ml. of solvent has passed over the column, is detected by U.V. analysis at 273 mμ. The routine collection of samples is implemented at a slightly earlier stage in the elution, and the occasional check of samples at 273 mμ indicates the complete elution of the band. The contents of the tubes are transferred to a volumetric flask (100 or 250-ml.) and corrected to volume with the dilute HCl. The absorption is read at 273 mμ, and the content of degradation product is expressed as ATC. The absorptivity (defined as absorbance/mcg. ATC/ml., 1-cm. light path) of ATC at wavelength maximum of 273 mμ was found to be 1.01×10^{-1}. The ATC obeyed Beer's law over the range of 1.0–10 mcg./ml.

The oral suspensions and syrups are diluted with a volume of solvent to give an estimated tetracycline concentration of 10 mg./ml. A volume of 0.5 ml. of the solution (centrifugation is necessary for clarification of the suspension) is transferred to the column and eluted with either the acid or phosphate buffer solvent. When the analysis is carried out with the latter solvent the absorption of the pooled tube contents from the second U.V. peak is read at wavelength maximum of 269.5 mμ. The ATC absorptivity at this wavelength was found to be 8.2×10^{-2}. The absorptivities for EATC at the wavelength maxima of 273 and 269.5 mμ were slightly lower than those for ATC 9.6×10^{-2} and 7.1×10^{-2}, respectively.

RESULTS AND DISCUSSION

In an initial experiment it was observed that a partly degraded commercial tetracycline preparation separated into 2 colored components on Sephadex

Received November 12, 1965, from the Biologics Control Laboratory, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Ontario, Canada.
Accepted for publication December 22, 1965.
The technical assistance of Mr. D. Roy is appreciated.

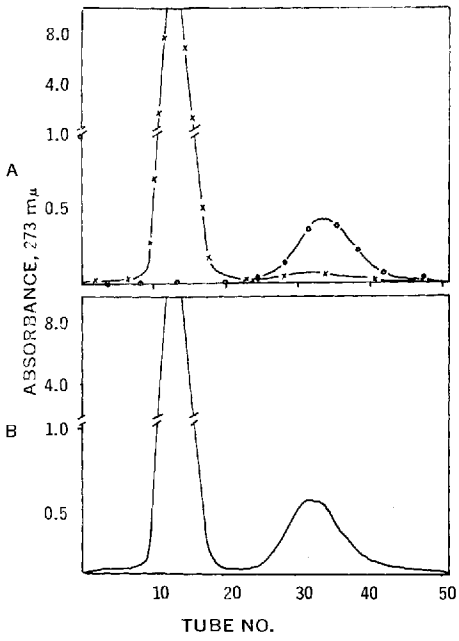

Fig. 1.—*A*. The elution diagram of tetracycline HCl and anhydrotetracycline on Sephadex G-25. Key: ×, tetracycline HCl, applied 1.0 ml. of 5 mg./ml.; O, anhydrotetracycline, applied 1.0 ml. of 200 mcg./ml. Sephadex column, 1.8 × 23 cm. Elution solvent, 0.03 *N* HCl. *B*. The elution diagram of 5 mg./ml. tetracycline HCl containing 200 mcg./ml. anhydrotetracycline: 1.0 ml. solution added to column; solvent and column conditions as above; collected 5-ml. samples.

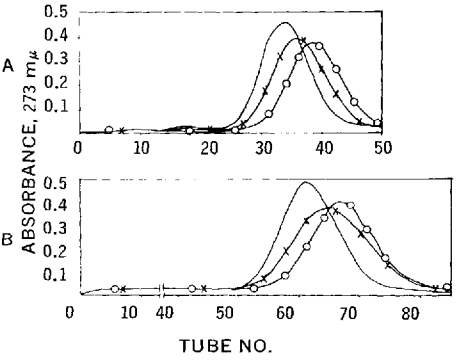

Fig. 2.—Key: ——, the elution diagrams of anhydrotetracycline; O, 4-epi-anhydrotetracycline; ×, an equal mixture of the 2 compounds. All samples were added in 1.0-ml. vol.; ATC = 200 mcg., EATC + ATC = 100 mcg. each and, EATC = 200 mcg., the elution solvent was 0.03 *N* HCl; the Sephadex column in *A* was 1.8 × 23 cm.; the Sephadex column in *B* was 1.8 × 45 cm.; collected 5-ml. samples.

G-25. The first elution band had the U.V. characteristics of tetracycline while the second had those of anhydrotetracycline and/or 4-epi-anhydrotetracycline.

Confirmation of this observation was made by the application of pure products of TC, ATC, and EATC to Sephadex G-25 (Figs. 1 and 2). In Fig. 1, *A*, TC and ATC, when applied separately to the same acid column, were found to elute as 2 distant peaks. It may be seen that the TC contained a small percentage of ATC as evidenced by the significant absorption in the region of the ATC elution. The TC showed full recovery of biological activity by microbiological plate assay. Figure 1, *B*, shows the identical elution pattern for a mixture of TC and ATC.

Figure 2, *A*, outlines the elution diagrams of ATC, EATC, and an equal mixture of the latter from the same acid column of Sephadex. It is of interest that the ATC and EATC exhibited slightly different elution rates when they were applied separately to the column, whereas an admixture eluted as a single band with an elution rate which was an apparent mean of that for the separate components. Figure 2, *B*, shows a similar effect on a longer column of Sephadex.

The time required for the elution of the TC degradation products from either the acid or alkaline columns was about 1 hr., and continuous sampling allowed for the analysis of several TC preparations in 1 day on a single column. In 2 experiments in which 200 mcg. of ATC was applied to a column, the recoveries were 92.5 and 97%. The recovery of EATC under the same conditions was 97.1%. No significant changes in the absorptivities were found for ATC and EATC in either 0.03 *N* HCl or 0.04 *M* phosphate buffer for periods of up to 4 hr.

During the analysis of certain syrups of TC containing amaranth dye on acid columns, it was found that the dye contaminated the ATC band to produce false high results. An examination of several solvents of varied pH revealed that the amaranth was retarded at pH 7.7 so as to completely separate from the "faster" ATC band. The slight overlap of the

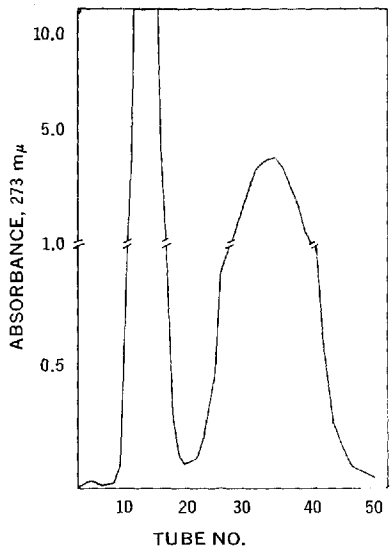

Fig. 3.—The gel filtration of tetracycline syrup on Sephadex G-25. Sephadex column, 1.8 × 23 cm.; elution solvent, 0.03 *N* HCl; 0.5 ml. of syrup (25 mg./ml. tetracycline HCl) added to column; collected 5-ml. samples.

TC band with the ATC band under these conditions required that the column be lengthened for their adequate separation.

Although the main effect of Sephadex is to separate molecules with respect to size, some secondary effects have been noted. Porath (9) and Gelotte (10) have found that heterocyclic and aromatic compounds interact with the Sephadex bed material which results in their delayed elution. The differences in elution rates between TC and ATC (as well as ATC and EATC) are presumed to be due to a similar adsorption phenomenon.

Of a group of about 50 tetracycline preparations analyzed on Sephadex, only about 7 of these contained degradation products in excess of 1% of the labeled amount of tetracycline. This figure of 1% represents the arbitrary amount (tentative) above which the samples were analyzed for both ATC and EATC by the partition chromatography method (7). Figure 3 shows the elution diagram of a tetracycline syrup containing an unusually high quantity of degradation products. The amount expressed as ATC was found to be 4.35 mg./ml. of syrup. The analysis of the same product by the partition chromatography method gave a gross value of 4.10 mg./ml. syrup, most of which was in the form of EATC. In general, the analysis of the TC degradation products by Sephadex analysis has been in close agreement with that by partition chromatography when appreciable quantities were present.

REFERENCES

(1) Gross, J. M., *Ann. Intern. Med.*, **58**, 523(1963).
(2) Frimpter, G. W., *J. Am. Med. Assoc.*, **184**, 111(1963).
(3) Ehrlich, L. I., and Stein, H. S., *Pediatrics*, **31**, 339 (1963).
(4) *Ibid.*, **31**, 698(1963).
(5) Rosenthal, S. M., *ibid.*, **31**, 697(1963).
(6) Benitz, K. F., and Diermeier, H. F., *Proc. Soc. Exptl. Biol. Med.*, **115**, 930(1964).
(7) Kelly, R. G., *J. Pharm. Sci.*, **53**, 1551(1964).
(8) McCormick, J. R. D., Fox, S. M., Smith, L. S., Bitler, B. A., Reichenthal, J., Origoni, V. E., Muller, W. H., Winterbottom, R., and Doerschuk, A. P., *J. Am. Chem. Soc.*, **79**, 2849(1957).
(9) Porath, J., *Biochim. Biophys. Acta*, **39**, 193(1960).
(10) Gelotte, B., *J. Chromatog.*, **3**, 330(1960).

Synthesis of 1,2-Diethyl-4-(2-hydroxyethyl)pyrazolidine

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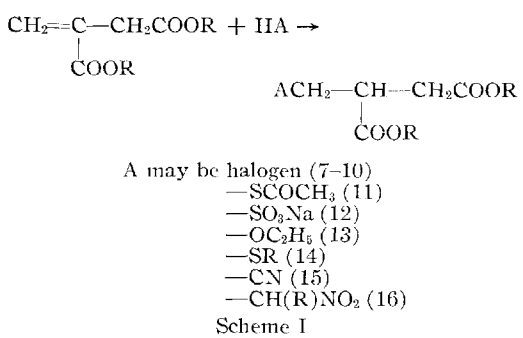
The reaction of 1,2-diethylhydrazine with monomethyl itaconate has been investigated and found to give a mixture of 1,2-diethyl-4-carbomethoxymethyl-3-pyrazolidinone and its hydrolysis product, the corresponding acid. Reduction of either the ester or the acid affords 1,2-diethyl-4-(2-hydroxyethyl)pyrazolidine.

THE AUTHOR'S interest in compounds which contain both an alcoholic hydroxyl group and an alkylated hydrazine group as necessary intermediates in the synthesis of new medicinals prompted the preparation of the title compound. Recently the synthesis of 1,2-diethyl-3-hydroxymethylpyrazolidine, a molecule which embodies the above structural features, was accomplished. Esterification of the latter alcohol with several aromatic acids afforded esters whose hydrochloride salts exhibited local anesthetic activity (1). As the first step in the synthesis of 1,2-diethyl-4-(2-hydroxyethyl)pyrazolidine, it was decided to investigate the reaction of 1,2-diethylhydrazine with an itaconic acid derivative.

A large number of reagents have been added to itaconic acid and its esters (2–16). Unsymmetrical reagents add contrary to Markovnikoff's rule and the addition may be represented by Scheme I.



Scheme I

Itaconic acid reacts with primary amines to give 1-substituted-4-carboxy-2-pyrrolidinones (17). The latter are formed by the addition of the amine to the β-carbon of the double bond followed by ring closure to the 5-membered ring with the elimination of a molecule of water.

In the reaction of 1,2-diethylhydrazine, with an itaconic acid derivative, ring closure to either a 5- or 6-membered ring is possible. Because of this possibility monomethyl itaconate was utilized. Separation of products could be more easily achieved since a mixture would consist of a 5-membered ring methyl ester and a 6-membered ring carboxylic acid. Treatment of 1,2-diethylhydrazine (I) with monomethyl itaconate (II) afforded a mixture of 43.9% of 1,2-diethyl-4-carbomethoxymethyl-3-pyrazolidinone (IV) and 44.6% of its hydrolysis product, 1,2-diethyl-4-carboxymethyl-3-pyrazolidinone (IV*a*). Both compounds gave crystalline picrate derivatives. Compound IV can be visualized as arising from an addition of I to the β-carbon of the double bond of II followed by ring closure *via* the carboxyl group with the elimination of water. The acid (IV*a*) undoubtedly arises from the hydrolysis of IV during the course of the reaction. Elemental analyses and infrared spectra are in agreement with the proposed structures for IV and IV*a*. That the isomeric acid, (III) which would arise from the addition of I to the β-carbon of the double bond of II followed by ring closure *via* the ester carbonyl with simultaneous

Received November 15, 1965, from the College of Pharmacy, University of Kentucky, Lexington.
Accepted for publication December 17, 1965.