Determination of Anhydrotetracycline and 4-Epianhydrotetracycline in a Tetracycline Mixture

By R. G. KELLY

A method of analysis for anhydrotetracycline and 4-epianhydrotetracycline in the presence of large quantities of tetracycline (about 95 per cent) has been developed. The method involves separation of the components by column chromatography, followed by spectral determination of column eluates. In the presence of 50 mg. of tetracycline, 0.9 mg. of added anhydrotetracycline was determined with the analytical recovery of 100.5 per cent, and 0.9 mg. of added 4-epianhydrotetracycline was determined with the analytical recovery of 92.5 per cent.

RECENTLY, reports have appeared in the literature describing a Fanconi-type syndrome, usually reversible, induced in patients who have ingested outdated or degraded tetracycline (1–5). The qualitative analyses of degraded capsular material by paper chromatographic methods (6) have revealed the presence of three degradation products (Scheme I) typical of what might be expected if tetracycline hydrochloride (I) were exposed to conditions of high temperature and moisture (7, 8). These degradation products are 4-epitetracycline (II), anhydrotetracycline (III), and 4-epianhydrotetracycline (IV). Of these, 4-epianhydrotetracycline in relatively large doses was the causative agent of renal tubular damage in the rat and the dog producing urinary findings suggestive of a Fanconi-type syndrome (9).

Therefore, it was necessary to develop an analytical procedure capable of determining the anhydrotetracyclines in the presence of large quantities of tetracycline.

EXPERIMENTAL

Materials and Methods

Reagents.—Acid-washed diatomaceous earth,[1] 0.1 *M* ethylenediaminetetraacetic acid (EDTA) disodium salt brought to pH 7.8 with ammonium hydroxide, reagent grade chloroform, 0.1 *M* EDTA in 0.2 *M* NH_4OH, and 0.1 *N* HCl were employed.

Procedure.—Equilibrate equal volumes of pH 7.8 EDTA solution and chloroform. To 10 Gm. dry Celite, add 5 ml. of the upper phase in a small conical flask, and shake until the Celite is moistened evenly.

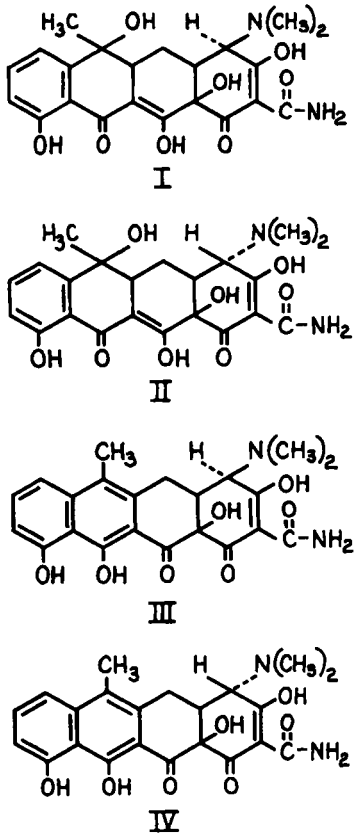
Prepare a column 15 × 1.8 cm. (an ordinary 6-in. test tube with a stopcock) of the moist Celite, using a small circle of filter paper (about 1 cm. diameter) as a plug, tightly packing about 80% of the Celite (10–11 cm.). Dilute an amount of the sample equivalent to 50 mg. of tetracycline to 1 ml. with 0.1 *N* HCl, and mix, effecting as much solution as possible of the sample.

Add 0.25 ml. of the solution (or fine suspension) to 1 Gm. of dry Celite, and mix thoroughly with a small glass stirring rod; add 0.25 ml. of 0.1 *M* EDTA in 0.2 *M* NH_4OH, and mix thoroughly again. Pack the sample Celite mixture on the column. Pack an additional 1 cm. of the EDTA-moistened Celite on top of the sample, and cover with a layer of sand.

Elute with the lower phase of the equilibrated solvent mixture; more rapid elution may be effected by applying a positive pressure to the top of the column after the solvent has passed the tetracycline charge. Collect 5-ml. fractions, and read absorbances at 430 mμ, using lower phase solvent as a blank. Anhydrotetracycline is in the first eluted tube and epianhydrotetracycline in tubes 2–7. (This will vary slightly, according to the precise pH conditions of the column.)

RESULTS AND DISCUSSION

The solubility differences between the anhydro derivatives of tetracycline and tetracycline itself suggested that a method for the determination of these derivatives in the presence of tetracycline



Scheme I

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[1] Marketed as Celite 545 by the Johns Manville Co.

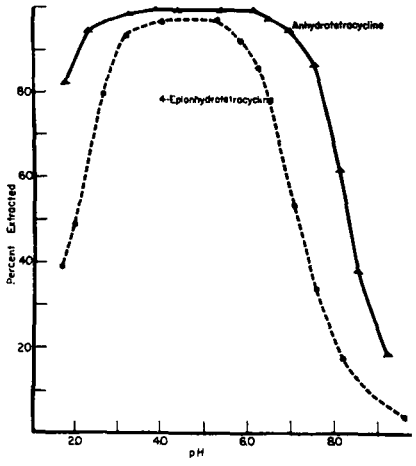

Fig. 1.—Percentage of anhydrotetracycline and 4-epianhydrotetracycline extracted from water into chloroform at various pH's.

might be evolved from a preliminary separation of the various components, followed by a spectroscopic analysis of the purified compounds. In the course of work involving the development of an analytical method for the determination of tetracycline in rat bone (10), the extraction of anhydrotetracycline from an aqueous medium into chloroform was maximal at pH 4.5. Work done subsequently indicated that the same pH maximum for extraction occurred with epianhydrotetracycline. However, in the construction of pH *versus* distribution coefficient curves, at certain pH values differences in the curves derived from the two anhydro compounds were apparent (Fig. 1). These differences occur in the pH ranges 1.5–2.5 and 6.5–8.5. On the basis of this information, column chromatography, using a pH 7.8 chloroform–water solvent system on an inert carrier, was attempted for the separation of anhydrotetracycline from epianhydrotetracycline.

Figure 2 shows the results obtained on a column run on a mixture of 50 mg. of tetracycline hydrochloride, 910 mcg. of anhydrotetracycline hydrochloride, and 894 mcg. of 4-epianhydrotetracycline hydrochloride. The column eluates were read in a Beckman model B spectrophotometer at 430 mμ. For the anhydrotetracycline hydrochloride, an absorptivity of 1.755×10^{-2} was used for calculation of concentration; for 4-epianhydrotetracycline, an absorptivity of 1.62×10^{-2} was used. The absorptivity is defined as absorbance per microgram per milliliter of eluate using a 1-cm. light path. These absorptivities were determined by dissolving (or suspending) an accurately weighed amount of the compound under consideration in a 10-ml. volume of the solvent equilibrated aqueous phase of the pH 7.8, 0.1 *M* EDTA solution and extracting with successive small volumes of the chloroform phase until all of the yellow color was in the chloroform extracts. These were pooled and diluted to a specific volume, then read in the spectrophotometer at 430 mμ.

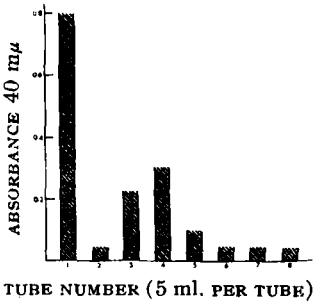

Fig. 2.—Column chromatography of a mixture of tetracycline, anhydrotetracycline, and 4-epianhydrotetracycline.

In the column described by Fig. 2, anhydrotetracycline was in tube 1, and epianhydrotetracycline was in tubes 2–5. The slight absorption in tubes beyond the fifth tube was shown by reading absorbances at 360 mμ to be due to tetracycline, which has an absorptivity of approximately 1.6×10^{-4} at 430 mμ.[2]

Although anhydrotetracycline is in the first eluted fraction in spite of variations in pH over the range 7.5–8.0, epianhydrotetracycline and tetracycline elutions are somewhat affected by such variations. In agreement with the curve in Fig. 1, at a pH more acid than 7.8 the epianhydrotetracycline will be eluted more rapidly than desirable; conversely, at pH's higher than that recommended, the elution of this compound will be slower and extend over a larger number of fractions. Rather than to impose a precise control of pH on the methodology, it is simpler to permit a variable elution rate. The fractions to be estimated as epianhydrotetracycline are easily selected on the basis of their yellow color.

For the column shown in Fig. 2, the tubes 2–5 were chosen in this manner. The recovery of added epianhydrotetracycline was determined as 92.5%; the recovery of added anhydrotetracycline, all of which was in tube 1, was 100.5%. In the original mixture, each of these components represented less than 2% of the total tetracycline content.

In two other experiments performed, recoveries of 95.5 and 102% were obtained on added anhydrotetracycline, and recoveries of 86.0 and 101.0% were derived for epianhydrotetracycline in mixtures of the two compounds. These columns were run without added tetracycline. In one, epianhydrotetracycline was eluted in tubes 2–10 and in the other in tubes 2–7.

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[2] Dr. L. Leeson, Pharmaceutical Product Development Department, Lederle Laboratories, has determined the optimal wavelength for the assay procedure to be 438 mμ. At this wavelength, the absorptivity of highly purified anhydrotetracycline hydrochloride was 1.87×10^{-2}, and that of highly purified 4-epianhydrotetracycline hydrochloride was 1.85×10^{-2}. Furthermore, the absorptivity of tetracycline hydrochloride is lower at 438 mμ than at 430 mμ.