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GEL CHROMATOGRAPHY OF TETRACYCLINE AND DERIVATIVES OF TETRACYCLINE

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SUMMARY

The elution characteristics of tetracycline (TC), anhydrotetracycline (ATC) and 4-*epi*-anhydrotetracycline (EATC) were studied on Sephadex gel under various conditions of pH and salt concentration. The adsorption of the compounds was strong in acid solvents but diminished as the pH was elevated. In the alkaline region of pH 8.5 to 9.5 the ATC and EATC epimer eluted at different rates from the column. An increase in the salt concentration resulted in stronger adsorption of the compounds to the gel, but did not influence their separation efficiency. The differential elution of the compounds appears to be due to a chromatographic effect distinct from the molecular sieve effect commonly associated with Sephadex.

INTRODUCTION

In a previous study[1], the anhydrotetracycline (ATC) and 4-*epi*-anhydrotetracycline (EATC) derivatives of tetracycline (TC) were found to exhibit adsorption to a column of Sephadex G-25 which enabled the derivatives to be separated from the TC. Under the conditions of these experiments, no separation between the ATC and EATC was found, although data did indicate that when the products were passaged separately over a column they displayed slightly different elution rates.

In the present work, the adsorption characteristics of the TC, ATC and EATC on Sephadex were studied under various conditions of pH and salt concentration. The chemical structures of these compounds are outlined in Fig. 1 (A, B and C, respectively).

REAGENTS AND MATERIALS

Compounds of tetracycline and derivatives

Tetracycline hydrochloride of a high degree of purity was used. Purified compounds of anhydrotetracycline hydrate and 4-*epi*-anhydrotetracycline sulfate were supplied by the Bristol Laboratories.

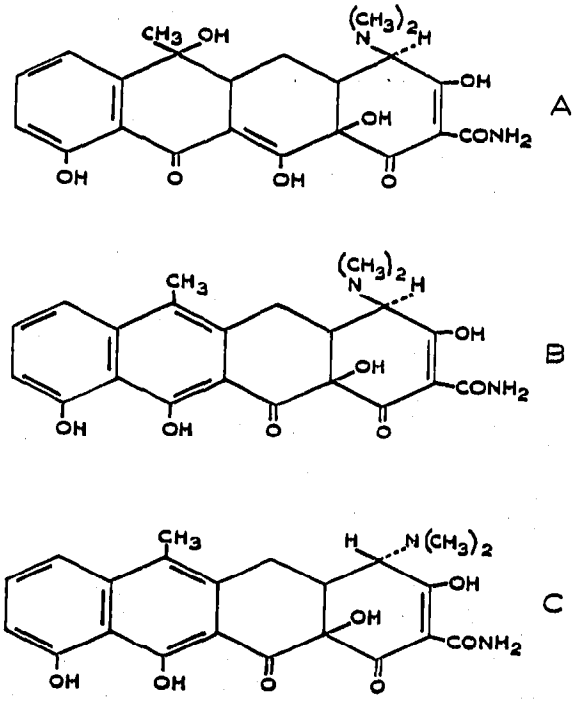

Fig. 1. Diagram of the chemical structures of tetracycline (A), anhydrotetracycline (B) and 4-*epi*-anhydrotetracycline (C).

Gel chromatography

Sephadex G-25 (fine), particle size 20–80 μ, was used to prepare a gel bed 1.8 × 25 cm. Three columns of similar bed dimensions were equilibrated with the following solvents: (1) Dilute HCl, pH 2.5; (2) 0.04 *M* phosphate buffer, pH 7.7; and (3) 0.05 *M* tris buffer, pH 8.5. The column with the tris buffer was re-equilibrated with several solvents of tris buffer of varied pH and/or molarity. Collection of samples was carried out with the LBK Radi Rac fraction collector and 4.4 ml aliquots were collected.

Preparation of sample

Small quantities of the ATC and EATC were weighed and dissolved to give final concentrations of 210 and 200 μg/ml, respectively, (calculated as the hydrochlorides). The tetracycline hydrochloride was prepared in a relatively higher concentration of 6.25 mg/ml to correspond to the approximate sample size analyzed in pharmaceutical preparations. The products (either singly or combined) were dissolved in distilled water and diluted with an equal volume of buffer solution. When the buffer was alkaline, column separation was immediately carried out since the TC on prolonged standing at room temperature developed a precipitate. A volume of 0.5 ml of the solution was applied to the column for analysis.

Determination of the partition coefficient K_d

The calculation briefly follows the formula by GELOTTE[2]:

$$K_d = \frac{V_e - V_0}{V_t}$$

Where $V_t = a \cdot W_r$ and a = dry weight of Sephadex necessary to pack a column bed 1.8 × 25 cm and

W_r = water regain of Sephadex,

V_e = elution volume of test substance at the point of maximum adsorption 273 mμ,

V_0 = eluton volume of haemoglobin measured at 540 mμ.

No correction was made for water of hydration of Sephadex.

RESULTS

In Fig. 2 are shown the elution diagrams of mixtures of tetracycline (TC), anhydrotetracycline (ATC) and 4-*epi*-anhydrotetracycline (EATC) under varied conditions of pH. At pH 2.5 the ATC and EATC displayed equal and maximum retardation on the column but did not show evidence of separation one from the other. Similar elution rates were obtained at pH 1.5. As the pH was raised the elution rates of the ATC and EATC increased, but differentially, so as to effect a separation between the compounds. At pH 9.0 and 9.5, the TC and the derivatives produced constant elution patterns and optimal separation between the ATC and EATC. The effect was eliminated when the compounds were passaged on a column equilibrated with 0.01 *N* NaOH. Under these conditions, the ATC and EATC formed a slight shoulder adjacent to the TC peak (data not shown).

Fig. 3 depicts the elution diagram for the TC and derivatives passaged separately on Sephadex columns equilibrated with phosphate buffer solvent pH 7.7 and tris buffer solvent pH 9.0. It is noted that the positions of elution of the separate compounds agree closely with the admixture elutions in Fig. 2. The sensitivity of the

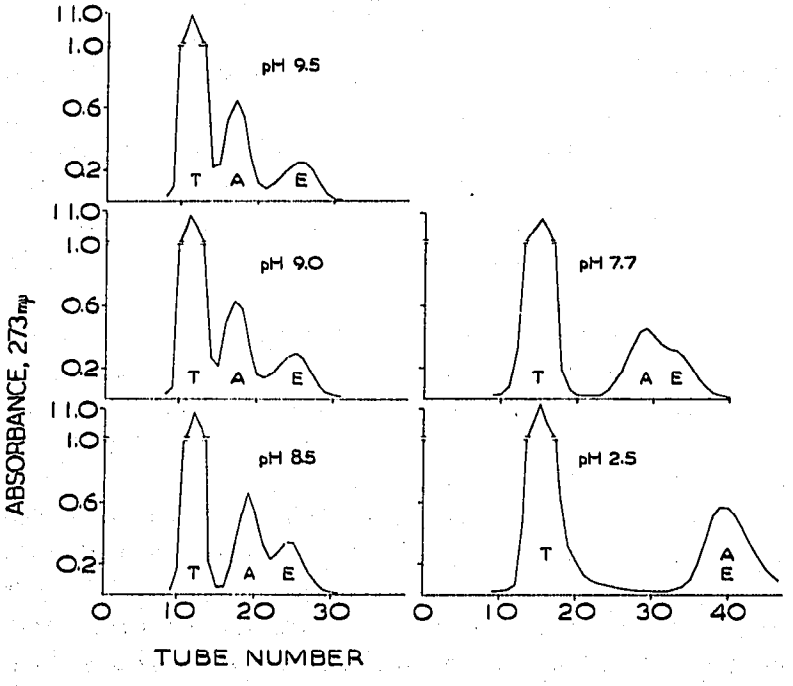

Fig. 2. The gel chromatography of mixtures of tetracycline (T), anhydrotetracycline (A) and 4-*epi*-anhydrotetracycline (E) in solvents of different pH.

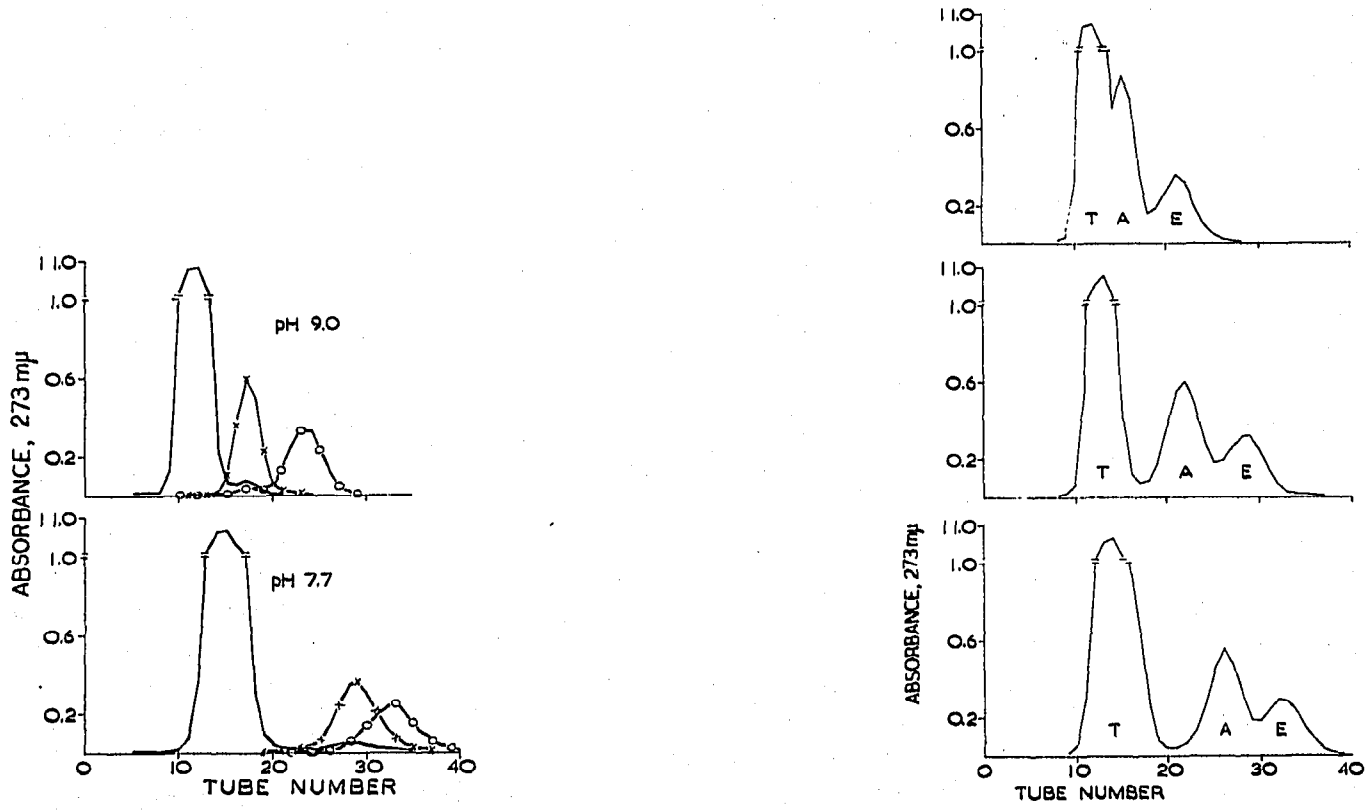

Fig. 3. The gel chromatography of tetracycline (———), anhydrotetracycline (×—×) and 4-*epi*-anhydrotetracycline (○—○) passaged separately on columns of Sephadex at pH 7.7 and 9.0.

Fig. 4. The gel chromatography of tetracycline (T), anhydrotetracycline (A) and 4-*epi*-anhydrotetracycline (E) in solvents of different salt concentration. (A) = 0.025 *M* tris buffer, pH 9.0; (B) = 0.050 *M* tris in 0.025 *M* NaCl, pH 9.0; (C) = 0.050 *M* tris in 0.2 *M* NaCl, pH 9.0.

separation of the components is evident since traces of ATC impurities could be detected in the TC and EATC products.

With low molarity of salt and constant pH of 9.0, the ATC and EATC showed weak adsorption to the column (Fig. 4). As the salt concentration was increased, the ATC and EATC exhibited stronger adsorption as evidenced by their delayed elution (Fig. 4, Table I). Evidence of broadening of the TC peak in the tris–NaCl solvent (C) was observed (Fig. 4).

The TC is subject to oxidation in the alkaline region of pH 9.0. The column eluates of the TC after standing at room temperature for several hours developed a brown discoloration which may be evidence of oxidation. The narrow elution bands obtained for the TC in the region of pH 9.0 as opposed to the broad bands at pH 7.7 and 2.5 may be further evidence of oxidation of the TC.

The ATC and EATC which are important from the analytical point of view were found to be stable by U.V. absorption criteria for periods up to 4 h at pH 9.0. The absorptivities of the compounds (calculated as the hydrochlorides) at pH 9.0 agreed with previous values determined at pH 7.7 (ref. 1). Also in agreement with the reported values, the absorptivity of the EATC was significantly lower than that of the ATC. Higher concentrations of 1 mg/ml of the ATC and EATC were recovered quantitatively with K_d values similar to those of the lower concentrations. Con-

TABLE I

THE INFLUENCE OF pH AND MOLARITY OF SOLVENTS ON THE ADSORPTION OF TC, ATC AND EATC ON SEPHADEX

An increase in numerical magnitude of the K_d values indicates stronger adsorption to the gel matrix. Figures in brackets in top portion of the Table indicate the salt concentration (in molarity) and those in the bottom part indicate the pH of the solutions.

pH	K_d		
	TC	*ATC*	*EATC*
2.5* (<0.01)	2.0	6.9	7.1
7.7* (0.04)	2.0	4.7	5.7
8.5 (0.05)	1.4	2.8	3.8
9.0 (0.05)	1.0	2.4	4.0
9.5 (0.05)	1.0	2.4	4.2
Molarity	*TC*	*ATC*	*EATC*
0.025 (9.0)	1.4	2.0	3.2
0.075 (9.0)	1.6	3.4	4.7
0.250 (9.0)	1.8	4.2	5.3

* The K_d values at these pH readings were calculated from the elution volumes of the individual compounds chromatographed separately.

versely, a dilute solution of TC (200 μg/ml) showed no difference in elution rate from the relatively high quantities routinely passaged on the column.

DISCUSSION

The adsorption effect as it applies to tetracycline (TC) and its derivatives is distinct from the molecular sieve effect which is commonly associated with gel filtration on Sephadex. The separation of the compounds appears to be related to a pure chromatographic effect since they differed only in minor chemical or stereochemical properties.

The TC although retarded in its elution from the column was relatively refractory to the diverse conditions of pH and salt concentration. The anhydro- and 4-*epi*-anhydrotetracycline derivatives (ATC and EATC) exhibited strong and similar adsorption at low pH with K_d values of 6.9 and 7.1, respectively (Table I). With an increase of the pH to the alkaline range, the adsorption of the ATC and EATC diminished and their respective elution rates were almost constant at pH 9.0 and 9.5. In this narrow range, the ATC and EATC eluted at different rates and could thus be separated. As the salt concentration of the solvent was increased at the constant pH of 9.0, the K_d values of the ATC and EATC increased (Table I), but the separation efficiency between the compounds was unchanged. This indicates that the pH is the important variable in separating the compounds.

GELOTTE[2] has studied the secondary adsorption properties of Sephadex. A distinction was drawn between substances which were weakly adsorbed to Sephadex in the absence of electrolyte and those adsorbed in the presence of electrolytes. The tetracycline derivatives fall into the latter category which applies to aromatic and heterocyclic compounds.

The findings on the separation of the ATC and EATC epimer products on Sephadex are of theoretical and practical importance since they reveal a refined chromatographic mechanism operative under optimal solvent conditions. The results are of immediate practical interest since the toxic nature of the EATC (ref. 3) requires its analytical determination in pharmaceutical preparations for human use.

ACKNOWLEDGEMENT

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