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Note

Reversed-phase high-performance liquid chromatographic separations of tetracycline derivatives using volatile mobile phases

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Recent studies in our laboratories have been directed toward photoaffinity labeling of the tetracycline (TC) binding site on the *Escherichia coli* ribosome[1,2], the identification of TC photoproducts[3], and the investigation of the phototoxicity of TC, TC derivatives, and TC photoproducts[4]. A method for efficient separations of TC and TC derivatives and photoproducts has been important for each of these studies, both for the preparation of TC derivatives, sometimes in radioactive form, and for the identification of the products of TC photolysis. The prior uses of high-performance liquid chromatography (HPLC) for such separations have typically employed mobile phases containing salts and other non-volatile solutes that could interfere with subsequent spectral analyses[5–8]. Even when reversed-phase (RP) HPLC has been employed, non-volatile solutes such as oxalic acid and ammonium phosphate were used[7,8]. In this paper we describe RP-HPLC separations of TC derivatives and photoproducts using volatile mobile phases conducive to subsequent spectral analyses.

MATERIALS AND METHODS

TC and 7-chloro-TC hydrochlorides were obtained from Pfizer, Sigma or Lederle Labs. 5,6α-Anhydro-TC (AHTC) and 4-*epi*-AHTC were obtained either by treatment of TC in dilute hydrochloric acid at 60°C for 6 h[9], or as gifts from Pfizer. 4-*epi*-TC was prepared from TC according to McCormick *et al.*[10] or obtained as a gift from Pfizer. Sancycline (6-demethyl-6-deoxy-TC, hereafter abbreviated as SC) derivatives were synthesized by known procedures[11] from SC and obtained as gifts from the laboratory of Dr. Michael Cava (University of Pennsylvania). [^{3}H]Minocycline was prepared by the reductive methylation of 7-amino-SC with formaldehyde and NaB[$^3H_4$] (Amersham)[2]. HPLC analyses were performed either on a Waters 6000 system with a U6K injector, a 660 programmer, and an M440 UV detector set at 280 nm, or on a Spectra-Physics 8100 chromatograph equipped with an SP-8400 UV–VIS detector and an SP-4100 computing integrator. The solvents used (HPLC-grade acetonitrile and trifluoroacetic acid) were obtained from Fisher and the columns used

(μBondapak C_{18}, 150 Å pore size, 250 × 4.1 mm I.D.) and phenyl (150 Å pore size, 250 × 4.1 mm I.D.) were obtained from Waters. Detailed conditions are presented in the figure legends.

RESULTS

Separations of mixtures typically found in our synthetic work are presented in Fig. 1. Fig. 1A shows the chromatogram of a mixture containing [^{3}H]minocycline

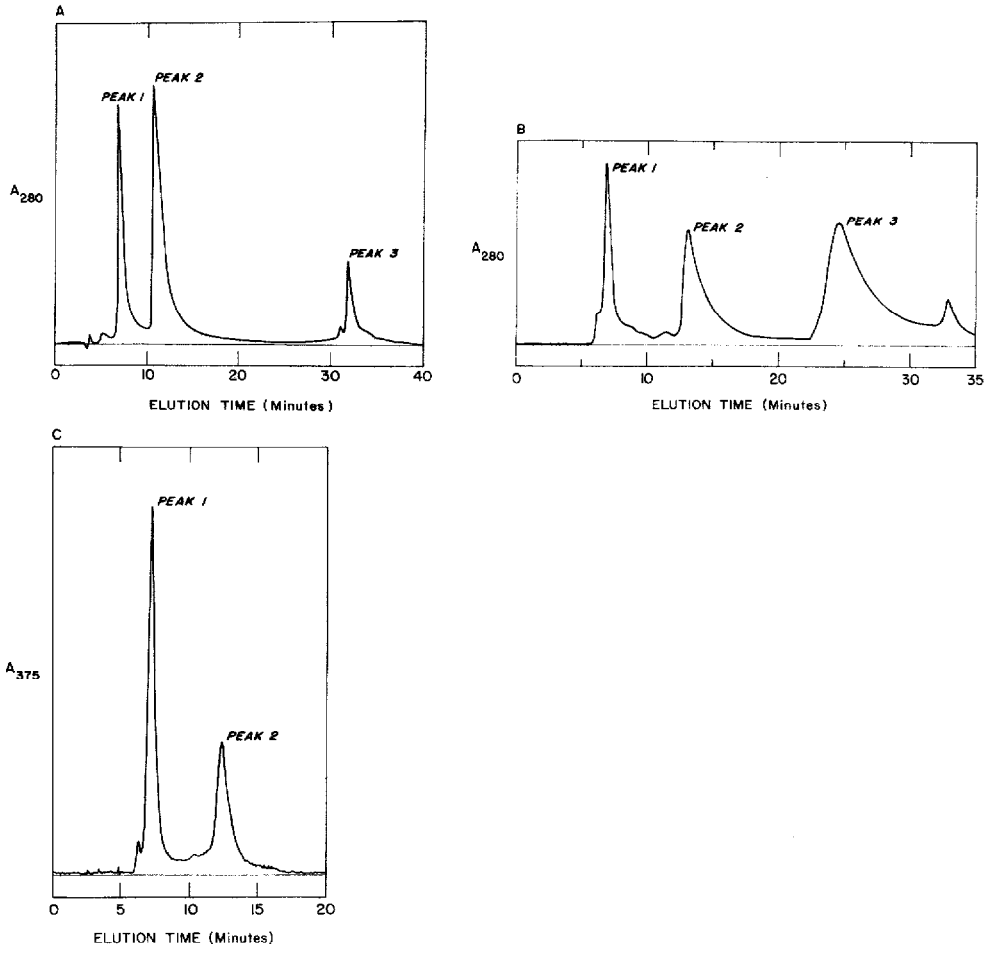

Fig. 1. RP-HPLC separations of typical synthetic mixtures. (A) Minocycline (peak 2) and its synthetic precursors 7-amino-SC (peak 1) and 7-nitro-SC (peak 3). (B) 9-Amino-SC (peak 1) and its synthetic precursors 9-nitro-SC (peak 2) and 7-bromo-9-nitro-SC (peak 3). (C) TC (peak 1) and 7-chloro-TC (peak 2). A μBondapak C_{18} column was used in Fig. 1A and B. Both elutions were run as 25-min concave gradients (curve 10 of the Waters 660 programmer). In A, the gradient was run from 10–80% acetonitrile (90–20% water containing 0.1% (w/v) trifluoroacetic acid) at a flow-rate of 0.6 ml/min. In B, the gradient was run from 24–50% acetonitrile (76–50% water containing 0.1% (w/v) trifluoroacetic acid) at a flow-rate of 0.5 ml/min. In C, a phenyl column was used under isocratic conditions. The solvent was methanol–water (40:60) containing 0.1% trifluoroacetic acid and the flow-rate was 1.0 ml/min.

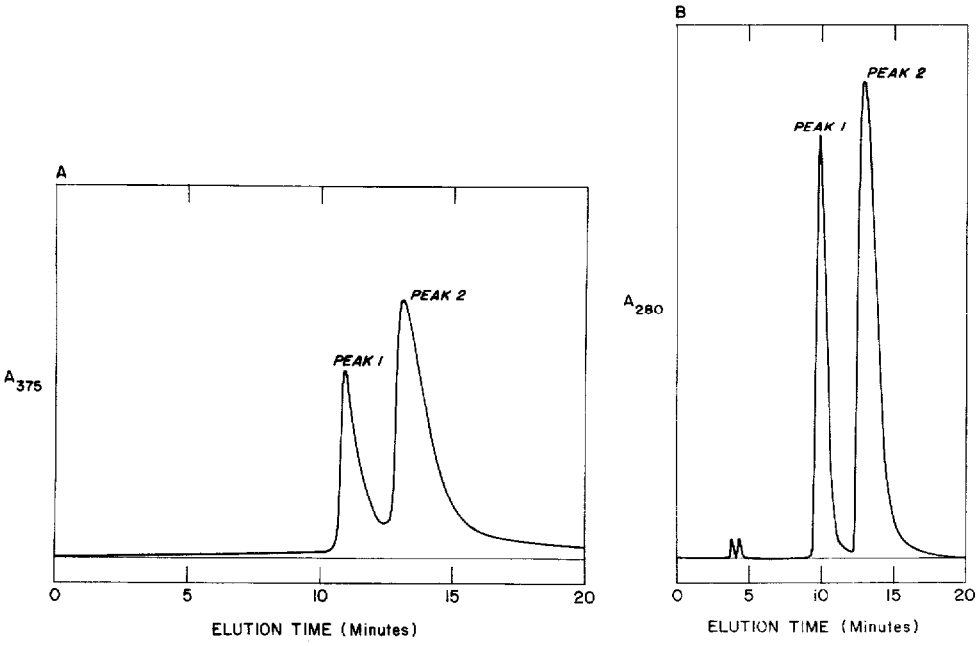

Fig. 2. RP-HPLC separations of epimeric mixtures. (A) 4-*epi*-TC (peak 1) and TC (peak 2). (B) 4-*epi*-AHTC (peak 1) and AHTC (peak 2). Both elutions were isocratic. In A, a phenyl column was used, the solvent was the same as in Fig. 1C, and the flow-rate was 0.5 ml/min. In B, a C_{18} column was used, the solvent was acetonitrile–water (27:73) containing 0.1% trifluoroacetic acid, and the flow-rate was 1.3 ml/min. The two small shorter retention time peaks in B represent *epi*-TC and TC.

and its synthetic precursors, 7-amino-SC and 7-nitro-SC. The only major peak of radioactivity was found with peak 2, corresponding to minocycline. The individual peaks were collected and their UV spectra were found to be identical to those of authentic samples. It should be noted, however, that under these conditions only a marginal separation was obtained of 7-nitro-SC from its synthetic precursor, 7-bromo-SC. Similar separations, using somewhat different chromatographic conditions, were obtained for 9-amino-SC from its synthetic precursors 9-nitro-SC and 7-bromo-9-nitro-SC (Fig. 1B), and for 7-chloro-TC from TC (Fig. 1C).

Both TC and SC, and their derivatives, are subject to fairly rapid epimerization at the 4-position[10]. The ability to separate the enantiomeric pairs efficiently is especially important because of the large differences in their biological properties[2]. Fig. 2A and B show two clear HPLC separations of TC from 4-*epi*-TC, and of AHTC from 4-*epi*-AHTC, respectively. Previously, such separations had been achieved on Kieselguhr plates following the more laborious procedure of McCormick *et al.*[10]. More recently, these compounds have been separated using RP-HPLC in the presence of ammonium phosphate[7] and oxalic acid[8]. The separations achieved by us are either better or comparable to those reported.

The application of HPLC for the analysis of the products of TC photolysis is illustrated in Fig. 3. The major product formed, peak 2, has been identified by several criteria (including ^{1}H NMR) as AHTC[3]. Peak 1 is 4-*epi*-AHTC, which is formed during the workup procedure.

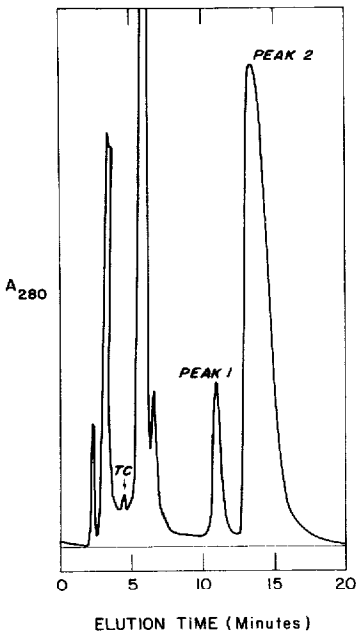

Fig. 3. RP-HPLC separation of the components of a TC photolysis mixture. TC at a concentration of 50 μM was photolyzed in a Tris (50 mM, pH 7.6), magnesium chloride (10 mM), potassium chloride (50 mM), β-mercaptoethanol (0.1%) buffer at 4°C with a Rayonet RPR 3500 Å lamp[1]. The reaction mixture was concentrated by lyophilization and the concentrate was subjected to HPLC under conditions identical to those used in Fig. 2B. The major photoproduct, peak 2, was spectrally characterized as AHTC, and peak 1, as *epi*-AHTC.

The HPLC separations described in this paper are adequate for semi-micro and microscale preparations, and are improvements over previously used methods in that they allow facile recovery of uncontaminated products. We have employed completely volatile, easily removable mobile phases without any sacrifice of resolution. Typically recovered yields were in the 75–90% range. Separations of similar synthetically related compounds are in progress and will be reported in subsequent publications.

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