Separation and Detection of Tetracyclines by High-Speed Liquid Chromatography

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The high-speed liquid chromatographic behavior of 15 tetracyclines on anion- and cation-exchange columns is described and illustrated by typical separations of a number of tetracycline derivatives on several systems, as well as by the resolution of tetracycline from its degradation products epi-, epianhydro-, and anhydrotetracycline. These serve as a basis for a rapid, convenient, and precise method for the direct detection of the tetracycline antibiotics and their degradation products.

Tetracycline has been analyzed by titrimetric (31), polarographic (5, 10), chromatographic (1–4, 8, 9, 11–13, 15, 19, 20, 22–25, 26, 28), spectrophotometric (6, 7, 16–18, 21, 29, 30), and microbiological methods. Many of these, in particular the official (USP and BP) microbiological methods, do not provide precise and accurate means for determining the tetracycline content in the presence of known degradation products, some of which are biologically active in vitro (epitetracycline and anhydrotetracycline). In addition, biological procedures provide no estimation of the microbiologically inactive but pharmacologically toxic degradation product epianhydrotetracycline, which has been implicated in several toxic manifestations, in particular the reversible renal dysfunction (Fanconi-type syndrome) caused by the injection of degraded tetracycline products (see 11 and 13 for pertinent references). The USP and BP have thus found it necessary to prescribe additional chemical tests to augment the official microbiological methods.

Spectrophotometric procedures are more accurate than microbiological methods, but are often time-consuming and intricate since they are usually based on a prior separation by column or thin-layer chromatography or on conversion of the antibiotic to its more stable anhydrotetracycline, or both. A more direct approach has involved the use of direct densitometry on thin-layer chromatograms (22, 26). However, the direct assay of epi- and anhydrotetracyclines in the presence of tetracycline is usually impossible by such methods, because the small amounts (<0.5%) naturally present entail the use of large spotting volumes, with consequent overloading and streaking of the tetracycline.

In an attempt to develop a more rapid, convenient, and precise method for the direct detection and analysis of these degradation products in bulk tetracycline and its formulations, a study of the high-speed liquid chromatographic behavior of tetracycline and related compounds has been initiated. This report describes the high-speed liquid chromatographic identification of 15 tetracyclines. The resolution of tetracycline from the degradation products epi-, epianhydro-, and anhydrotetracycline has also been achieved.

MATERIALS AND METHODS

Tetracycline derivatives. Tetracycline and oxytetracycline were USP reference standards obtained as their hydrochloride salts from USP Reference Standards, Bethesda, Md. Epitetracycline ammonium salt, anhydrotetracycline hydrochloride, epi-anhydrotetracycline hydrochloride, doxycycline hydrochloride, 6-epidoxycycline hydrochloride, and methacycline hydrochloride were BP authentic specimens distributed by the British Pharmacopoeia Commission, London, England. Chlortetracycline hydrochloride and demethylchlortetracycline hydrochloride were purchased from Cyanamid of Canada, Montreal, P.Q. Minocycline, epiminocycline, anhydrochlortetracycline, and epichlortetracycline were provided by the Lederle Division of American Cyanamid, Pearl River, N.Y., and rolitetracycline nitrate was raw drug material donated by Bristol Laboratories of Canada, Candiac, P.Q.

Apparatus. A Varian model 4100 liquid chromatograph (Varian Aerograph, Walnut Creek, Calif.) with a fixed wavelength (254 nm) ultraviolet detector was used throughout the study. The columns (3.2 mm, outer diameter; 1.8 mm, inner diameter; 304 stainless
steeled were coiled (radius, 12 cm) to fit into the water bath of the instrument.

**Chromatographic procedure.** Columns were dry-packed as described elsewhere (A. G. Butterfield, B. A. Lodge, and N. J. Pound, J. Chromatogr. Sci., in press). Three pellicular ion exchangers, Zipax SAX, Zipax SCX (Du Pont de Nemours & Co., Wilmington, Del.), and Pellionex CP-128 (Northgate Laboratories, Inc., Hamden, Conn.) were used in this study.

Sodium and nitrate ions were used as counter ions for cation and anion exchangers, respectively. Mobile phases were prepared by dissolving ethylenediaminetetraacetic acid (EDTA), disodium salt, and sodium hydroxide or sodium nitrate in water to give the required concentration of EDTA and sodium or nitrate ion, adding the required amount of ethanol, then adjusting the pH by the addition of glacial acetic acid or concentrated sodium hydroxide solution. The solution was then brought to final volume with distilled water. The mobile phases and temperatures used in this study are specified in Table 1. A flow rate of 60 ml/h was used throughout.

The tetracycline derivatives were dissolved in 0.01 N hydrochloric acid (except rolitetracycline, which was dissolved in water), and 5-μlitter samples, containing approximately 10 μg of each compound, were injected directly onto the column with a 10-μlitter syringe (Hamilton Co., Whittier, Calif.) by use of a stop-flow injection technique.

**RESULTS AND DISCUSSION**

Figures 1, 2, and 3 show typical separations of a number of tetracycline derivatives on several of the systems employed in this study. Perhaps the most significant separation is that shown in Fig. 3. Here, a sample (15 μg) of tetracycline containing 4% epitetracycline and 0.5% of each of anhydrotetracycline and epianhydrotetracycline has been chromatographed. These concentrations represent the upper BP limits for these degradation products in tetracycline formulations. It is possible to separate all four compounds in less than 8 min. This represents a significant saving in time over the present methods and enables one to monitor easily the content of the toxic epianhydrotetracycline in pharmaceutical formulations. Figures 1 and 2 illustrate the use of HSLC for the identification of various tetracyclines as well as serving as a basis for assays of specific tetracyclines in the presence of their epimers.

Since tetracyclines exist as zwitter ions, the use of either cation- or anion-exchange chromatography is applicable to their separation. Table 1 lists both the adjusted retention volumes (Vₐ,; 27) and relative adjusted retention volumes (relative to tetracycline; Vₑᵥₑᵥₑᵥₑᵥₑ) for a number of tetracyclines. Vₐ is measured from the solvent peak to the apex of the solute peak, and Vₑᵥₑᵥₑᵥₑ is the ratio of the Vₐ for the solute to the Vₑᵥₑᵥₑᵥₑ of tetracycline. These data provide a basis from which suitable column and mobile phase parameters can be selected for the analysis and separation of the compounds listed.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Zipax SAX (1)</th>
<th>Pellionex CP-128 (2)</th>
<th>Pellionex CP-128 (3)</th>
<th>Zipax SCX (4)</th>
<th>Zipax SCX (5)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Vₑᵥₑᵥₑᵥₑ</td>
<td>Vₑᵥₑᵥₑᵥₑᵥₑᵥₑ</td>
<td>Vₑᵥₑᵥₑᵥₑᵥₑᵥₑ</td>
<td>Vₑᵥₑᵥₑᵥₑᵥₑ</td>
<td>Vₑᵥₑᵥₑᵥₑᵥₑ</td>
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<tr>
<td>Tetraacycline</td>
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<td>1.0</td>
<td>4.9</td>
<td>1.0</td>
<td>4.9</td>
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<td>Epitetracycline</td>
<td>3.8</td>
<td>1.7</td>
<td>9.3</td>
<td>1.9</td>
<td>1.7</td>
</tr>
<tr>
<td>Anhydrotetracycline</td>
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<td>NP</td>
<td>NP</td>
<td>NP</td>
<td>NP</td>
</tr>
<tr>
<td>Epianhydrotetracycline</td>
<td>22.0</td>
<td>10.0</td>
<td>7.3</td>
<td>1.5</td>
<td>8.1</td>
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<tr>
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<td>13.2</td>
<td>9.9</td>
<td>2.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Epichlorotetracycline</td>
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<td>8.0</td>
<td>7.8</td>
<td>1.6</td>
<td>0.9</td>
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<tr>
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<td>NP</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Demethylchlorotetracycline</td>
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<td>1.0</td>
<td>3.2</td>
<td>0.7</td>
<td>0.5</td>
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<td>Oxytetracycline</td>
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<td>9.5</td>
<td>11.7</td>
<td>2.4</td>
<td>1.0</td>
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<tr>
<td>Doxytetracycline</td>
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<td>1.4</td>
<td>6.7</td>
<td>1.4</td>
<td>0.7</td>
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<tr>
<td>6-Mepoxytetracycline</td>
<td>14.9</td>
<td>6.8</td>
<td>15.1</td>
<td>3.1</td>
<td>1.2</td>
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<tr>
<td>Minocycline</td>
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<td>4.0</td>
<td>NP</td>
<td>9.7</td>
<td>14.0</td>
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<tr>
<td>Epiminocycline</td>
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<td>3.2</td>
<td>NP</td>
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<td>Rolitetracycline</td>
<td>NP</td>
<td>NP</td>
<td>NP</td>
<td>NP</td>
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</table>

*NP = no peak after 40 ml; = compound not chromatographed. Columns and mobile phases: (1) Zipax SAX; 100 cm; 9.6% (vol/vol) ethanol, 0.046 M NaNO₃, 0.004 M EDTA²⁻, pH 9.03; 25 C; (2) Pellionex CP-128; 225 cm; 10.0% (vol/vol) ethanol, 0.05 M Na⁺, 0.002 M EDTA²⁻, pH 4.00; 50 C; (3) Pellionex CP-128; 225 cm; 30.0% (vol/vol) ethanol, 0.10 M Na⁺, 0.002 M EDTA²⁻, pH 4.60; 50 C; (4) Zipax SCX; 200 cm; 12.5% (vol/vol) ethanol, 0.08 M Na⁺, 0.002 M EDTA²⁻, pH 4.00; 50 C; (5) Zipax SCX; 100 cm; 13.5% (vol/vol) ethanol, 0.143 M Na⁺, 0.004 M EDTA²⁻, pH 4.45; 50 C.*
SEPARATION AND DETECTION OF TETRACYCLINES

Fig. 1. Separation of tetracycline (TC), epitetracycline (ETC), demethylchlortetracycline (DMCTC), chlortetracycline (CTC), epichlortetracycline (ECTC), epiminocycline (EMC), and minocycline (MC). Column: 100 cm by 1.8 mm (inner diameter) Zipax SAX. Mobile phase: 9.6% (vol/vol) ethanol, 0.046 M NO₃⁻, 0.004 M EDTA²⁻, pH 9.03. Flow rate: 60 ml/h; 23 C. Detector attenuation: 0.32 absorbance units full scale.

The tetracyclines respond in a conventional manner to changes in the operating temperature, ionic strength, and pH of the mobile phase.

The V₁ and the column back pressure decrease as the column temperature is increased. Column efficiency varies markedly from solute to solute and is generally improved at elevated temperatures. As shown in Table 2, height equivalent to theoretical plate values of 1.5 to 33.3 mm (i.e., 650 to 33 plates/meter) were observed for the tetracyclines under one particular set of parameters.

Increasing the ionic strength of the mobile phase produces a decrease in the V₁. Similarly, as the pH is increased on the cation exchangers or decreased on the anion exchanger, the V₁ decreases. Changes in pH also produce variations in V₁, however, the isocratic separation of tetracycline, epitetracycline, anhydrotetracycline, and epi-anhydrortetracycline, the derivatives of particular interest in this study, could not be achieved solely by the adjustment of pH because of the highly retentive nature of the anhydro derivatives. These four compounds were successfully separated (Fig. 3) by the addition of ethanol to the mobile phase.

An increase in the alcohol concentration pro-
duced a reduction in $V_n$ and significant changes in $V_n^1$. As shown in Table 1, the effect on $V_n$ for Pellionex CP-128 resin was more pronounced for the more retenive derivatives.

Although the ethanol concentration is restricted to approximately 10% for the Zipax materials (E. I. DuPont de Nemours & Co. Product Bulletins), the Pellionex exchangers may be used with higher concentrations of organic modifier (Northgate Laboratories, Inc., Product Bulletin). The Pellionex CP-128 resin has been used with up to 50% ethanol with no indication of degradation. These changes in $V_n$ are not surprising, since organic ions are sorbed both by ionic interactions with the exchange groups and by other interactions with the resin matrix itself (14). The matrices of Zipax SAX, Zipax SCX, and Pellionex CP-128 are methacrylate, fluorocarbon, and polystyrene polymers, respectively. These differences are significant since the selection of the resin provides another parameter for the separation of the tetracyclines.

EDTA was found to be required for the elution of the tetracycline derivatives on both anion and cation exchangers. Although high ionic strengths will elute most derivatives, severe tailing results when EDTA is not present. The use of low concentrations (0.002 to 0.005 M) of EDTA in the mobile phase eliminates this tailing.

The quantitative aspects of these separations and their application to the quality control of tetracycline antibiotics in pharmaceutical formulations are under further investigation.

**LITERATURE CITED**


