Tetracyclines I

Separation and Examination by Thin-Layer Chromatography

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A thin-layer chromatographic (TLC) method for the examination of tetracyclines has been developed. This technique provides a rapid means for the separation and identification of tetracyclines and their derivatives in a wide variety of mixtures. Acid-washed diatomaceous earth served as the support. The support was treated with a buffer consisting of 0.1 *M* ethylenediaminetetraacetic acid disodium salt (EDTA) at pH 7.0, glycerin, and polyethylene glycol 400 (PEG 400). The developing solvent was ethyl acetate saturated with 0.1 *M* EDTA at pH 7.0. While the work reported was limited to demethylchlortetracycline (DMCTC), tetracycline (TC), and chlortetracycline (CTC), it has also been successfully used with other tetracyclines.

At this time, a technique is needed for thin-layer chromatography for separating tetracyclines, whether in specifically prepared mixtures or in mixtures resulting from failure of the strains used to produce pure tetracylcines. Such a technique should be applicable not only to separating mixtures of tetracyclines, but also for studying their stability in pharmaceutical formulations.

The application of TLC for the separation of antibiotics was presented by Nicolaus, Coronelli, and Binaghi (1) in which several tetracyclines were examined in many solvents using various adsorption layers. Kapadia and Subba Rao (2) described a circular thin-layer separation employing sequestering agents in a silica gel layer for better resolution. Sonanini and Anker (3) studied the chromatography of tetracyclines using a support impregnated with glycerin, which maintained moisture in the support. A method for quantitation on thin-layer was reported by Simmons, Koorengevel, Kubelka, and Seers (4) using microcrystalline cellulose as a support. These TLC methods used in the separation of tetracyclines as described by the various workers (1–4) were examined in these laboratories. However, more than two tetracyclines could not be resolved by a single system on one chromatogram, and the resolution of the related compounds of each tetracycline could not be accomplished without a great deal of streaking and tailing.

This report presents the results of preliminary work in which various adsorbants, sequestering agents, PEG 400–glycerin mixture, and pH were directed toward obtaining clear separations. Satisfactory separation of the tetracyclines and their related compounds was achieved on diatomaceous earth, impregnated with EDTA at pH 7.0, glycerin–PEG 400, using ethyl acetate as the developing solvent.

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EXPERIMENTAL

Reagents—Disodium ethylenediaminetetraacetate A.C.S., glycerin A.C.S., polyethylene glycol 400 U.S.P., ammonium hydroxide A.C.S., ethyl acetate A.C.S.

Buffer—Mix 5 ml. of 20% v/v PEG 400 in glycerin with 95 ml. of 0.1 *M* EDTA previously adjusted to pH 7.0 with ammonium hydroxide.

Developing Solvent—Equilibrate 120 ml. of ethyl acetate with 20 ml. of 0.1 *M* EDTA previously adjusted to pH 7.0. Use the organic layer to develop the plates.

Preparation of Support—Wash diatomaceous earth[1] with hot 6 *N* hydrochloric acid until the washings no longer contain calcium or iron. Wash with water until a neutral pH is obtained and then dry at 105°.

Preparation of Plates—Prepare glass plates (20 × 20 cm.) according to the method of Lees and DeMuria (5). Place a double thickness of 1-cm. masking tape[2] on opposite edges of the plate. Triturate a slurry of 8 Gm. of acid-washed diatomaceous earth and 16 ml. of buffer in a glass mortar until smooth. Pour this slurry onto the plate and spread over the surface with a stout glass rod using the taped edges of the plate to control the thickness of the layer; press the glass rod against the taped edges at one end and slide to the opposite end in one motion. Air-dry the plate for 35–50 min. or until the layer surface appears whitish gray. Remove the tape from the edges of the plate and use immediately.

Preparation and Application of the Samples—Prepare a methanol solution of each of the tetracyclines to be chromatographed using a concentration of 1 mg./ml. At the same time, prepare standards of tetracyclines to be carried through the same procedure. Apply 1 µl. of each solution at 1-cm. intervals, 1.5 cm. from one edge of the plate. Inscribe a solvent–front line across the surface of the plate 12 cm. from the origin.

[1] Marketed by Desaga/Brinkmann Co. under the trademark of Kieselguhr G.

[2] Scotch brand tapes, cores No. 0300, 3M Co., St. Paul, Minn.

Development of Chromatogram—Line a rectangular glass jar (20 × 8 × 20 cm.) internal dimensions) with Whatman 3 MM chromatography paper. Pour the mobile phase into the glass jar. After the glass jar has equilibrated for 30 min., chromatograph the plates. When the mobile phase reaches the solvent-front line, remove the plate and air-dry for 2–3 min. Rechromatograph the plate for additional resolution. The plates may be developed for a third or fourth time if necessary.

After the plate has dried under ambient condition for 30 min., examine it under long wavelength ultraviolet light. The sample spots are identified by comparison with individually chromatographed tetracycline standards.

RESULTS AND DISCUSSION

The use of EDTA, glycerin, and pH buffer to improve separation in chromatographic procedures of tetracyclines has been reported (1–3). However, in this investigation these methods were unsuccessful in resolving more than two tetracyclines by a single solvent system on one chromatogram. Also, during the development of these chromatograms, tailing, streaking, and possible degradation occurred which was undesirable.

When Silica Gel G was used as a support on thin layer, the tetracyclines were strongly adsorbed and could not be eluted satisfactorily. On diatomaceous earth, the tetracycline could be developed with various solvent systems, but with pronounced tailing and little resolution of the various tetracyclines.

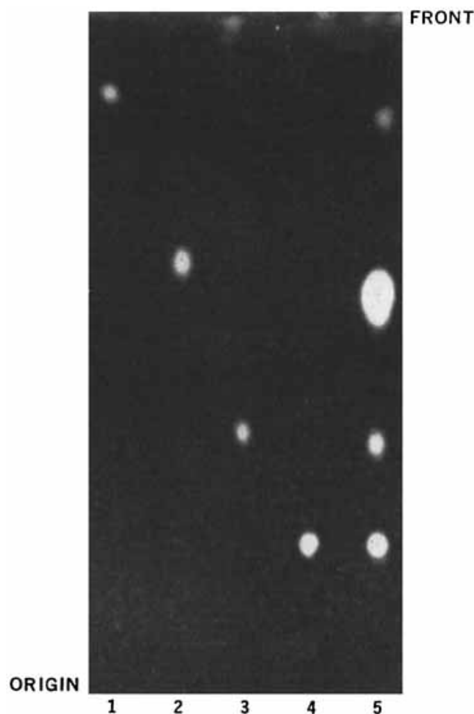

Fig. 2—Thin-layer chromatography of demethylchlortetracycline. Key: 1, ADMCTC; 2, DMCTC; 3, DMTC; 4, EDMCTC; 5, mixture.

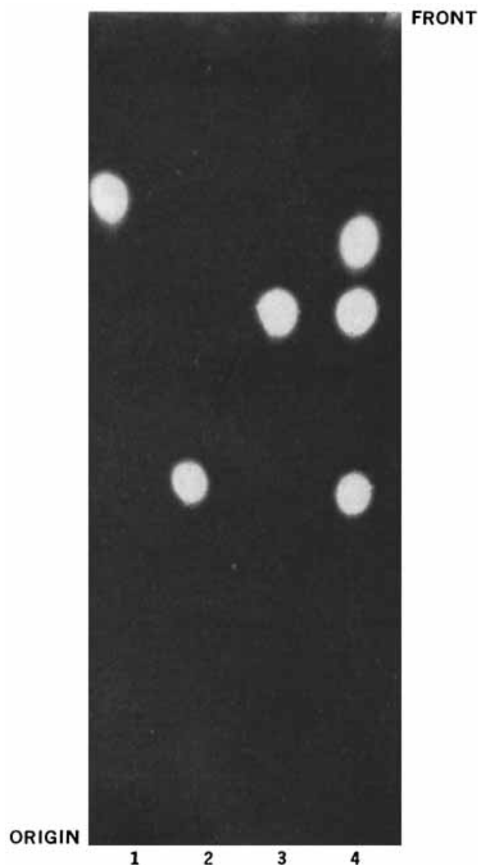

Fig. 1—Thin-layer chromatography of tetracyclines. Key: 1, CTC; 2, TC; 3, DMCTC; 4, mixture.

Kelly and Buyske (6) found that paper impregnated with 0.1 *M* EDTA greatly improved paper chromatograms of tetracyclines. In our work, thin-layer chromatograms were developed without streaking and tailing by using diatomaceous earth which first had the binder removed and then EDTA added.

Sonanini and Anker (3) studied the chromatography of several tetracyclines by impregnating the support with glycerin to maintain a constant water concentration and thus obtain better resolution of the tetracycline. For this reason we used a mixture of glycerin and PEG 400 for the resolution of closely related tetracyclines.

Figures 1–4 show separations using these various techniques—acid-washed diatomaceous earth impregnated with EDTA–PEG 400–glycerin, and ethyl acetate as the solvent. Figure 1 shows the separations and resolution of three tetracyclines, CTC,[3] TC,[4] and DMCTC,[5] which were specifically prepared. Figures 2, 3, and 4 show the separation and resolution of each tetracycline, such as might result from failure of the strains to produce pure tetracycline, as well as possible degradation during storage. In Fig. 2, DMCTC is separated from

[3] Marketed by Lederle Laboratories, Division of American Cyanamid Co., under the trademark Aureomycin.
[4] Marketed by Lederle Laboratories, Division of American Cyanamid Co., under the trademark Achromycin.
[5] Marketed by Lederle Laboratories, Division of American Cyanamid Co., under the trademark Declomycin.

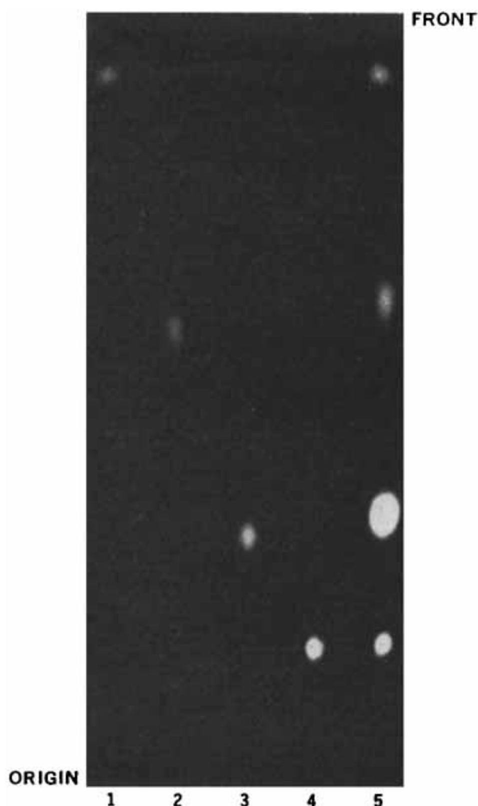

Fig. 3—Thin-layer chromatography of tetracycline. Key: 1, ATC; 2, CTC; 3, TC; 4, ETC; 5, mixture.

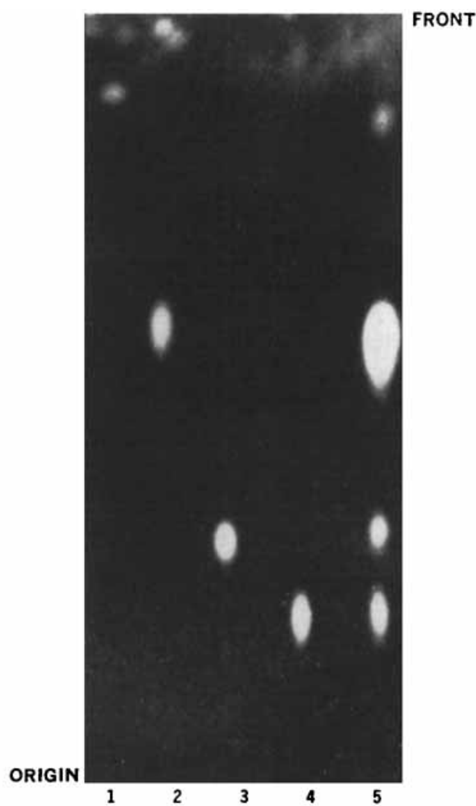

Fig. 4—Thin-layer chromatography of chlortetracycline. Key: 1, ACTC; 2, CTC; 3, TC; 4, ECTC; 5, mixture.

anhydrodemethylchlortetracycline (ADMCTC), demethyltetracycline (DMTC), and epidemethychlortetracycline (EDMCTC); Fig. 3, TC from anhydrotetracycline (ATC), CTC, and epitetracycline (ETC); and Fig. 4, CTC from anhydrochlortetracycline (ACTC), TC, and epichlortetracycline (ECTC).

The apparatus, reagents, developing solvents, plate preparation, and sample application were all evaluated to eliminate unnecessary steps and/or variables for obtaining useful chromatograms. All glassware was scrupulously clean, and only reagents of low native fluorescence were used. The support, diatomaceous earth, was acid-washed to remove the binder and other acid-soluble materials which might contribute to streaking and tailing. The glycerin-PEG 400 mixture maintained the necessary level of moisture that produced well-separated, sharply defined spots. The buffer at pH 7.0 minimized the danger of degradation of the tetracyclines.

Excess moisture due to insufficient drying of the plate reduced the rate of movement of the tetracycline and necessitated chromatographing three or four times. Too little moisture caused elongated spots and poor resolution. Standards were always run for comparative evaluation of samples.

By visual observation under long wavelength ultraviolet light, as minute a quantity as 50 nanograms was easily seen. Additional work is being done in the area of fluorescence analysis using a recording and scanning spectrophotofluorometer which will permit detection of even lower levels of materials, plus quantitation of these tetracyclines and their related compounds.

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