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Note

Separation of digoxin and dihydrodigoxin by thin-layer or paper chromatography

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Dihydrodigoxin is the major dihydro metabolite of digoxin in man[1]. Although the activity of this metabolite is unknown in man, experiments in animals show it to be approximately 1/10 as active as digoxin[2,3]. In addition, the production of the dihydro metabolite varies from patient to patient[1]. Quantitation of dihydrodigoxin and digoxin in plasma and urine is clinically important for the monitoring of patients on digoxin maintenance therapy and for the diagnosis of digoxin intoxication.

Current techniques for the assay of digoxin include inhibition of ^{86}Rb transport by the red blood cells[4], radioimmunoassay[5], and enzymatic isotope displacement[6]. None of these methods differentiate between digoxin and dihydrodigoxin. Cross reaction of dihydrodigoxin in digoxin assays may vary widely depending upon the nature of the assay, making determination of true digoxin levels difficult. Researchers have used gas chromatography–mass spectroscopy as well as combining mass spectrometry with other assays to determine the contribution of dihydrodigoxin[7,8]. Previous attempts to separate digoxin from its reduced metabolite, dihydrodigoxin, by solvent partitioning, column chromatography, paper chromatography and thin-layer chromatography (TLC) have proven unsuccessful[7,8]. We wish to report three chromatographic systems which give satisfactory separation of digoxin and dihydrodigoxin.

EXPERIMENTAL

Spray reagents

Reagent 1. 25% trichloroacetic acid solution in chloroform, containing 4 drops of 30% hydrogen peroxide in each 50 ml. Chromatograms are sprayed and heated at 90–100° for 2 min.

Reagent 2. Acetic anhydride–sulfuric acid–absolute ethanol (5:5:100).

Reagent 3. 0.05 ml *p*-anisaldehyde, 0.2 ml concentrated sulfuric acid, 10 ml glacial acetic acid. The plates are sprayed with freshly prepared reagent and heated at 110° for 4–6 min.

Reagent 4. 20 mg ascorbic acid, 19 ml methanol, 30 ml conc. hydrochloric

acid and 2.1 μl 30% hydrogen peroxide. The plates are sprayed and heated at 110° for 4 min.

Reagent 5. 10 ml of a freshly prepared 3% aqueous solution of chloramine T and 40 ml of 25% trichloroacetic acid in ethanol are mixed prior to spraying. The plates are heated at 110° for 8 min.

TLC on silica gel

Silica gel F_{254} plates (0.25 mm × 20 cm × 20 cm; E. Merck, Darmstadt, G.F.R.) were heat-activated at 100° for 1 h and stored in a desiccator. Plates were developed two times to 18 cm using chloroform–methanol–ammonia (9:1:1). The methanol used in this mixture had previously been saturated with silver nitrate. Chromatography tanks were fitted with saturation pads and allowed to equilibrate for 1 h. Plates were sprayed with the reagents described and visualized under long-wavelength UV light.

TLC on cellulose

Precoated cellulose plates (0.1 mm × 20 cm × 20 cm cellulose MN-300; Macherey, Nagel & Co., Düren, G.F.R.) were activated at 110° for 10 min and then dipped into a 25% formamide in acetone solution and air dried for 1 h. The plates were developed twice in a sandwich apparatus (Chromagram Developing apparatus No. 6071; Eastman-Kodak, Rochester, N.Y., U.S.A.) using chloroform saturated with formamide as the mobile phase. Plates were sprayed lightly with reagent 5 and visualized under long-wavelength UV light.

Paper chromatography

Paper chromatograms were run using Whatman No. 1 filter paper cut into 12 × 40 cm strips. The strips were impregnated with formamide by drawing them through a freshly prepared 30% solution of formamide in acetone contained in a chromatography trough. They were air dried for about 5 min. After spotting the sample, the strip was placed in a cylindrical chromatography tank (45 × 15 cm I.D.) containing a trough assembly 35–40 cm above the bottom of the tank. The tank was allowed to equilibrate for 30 min with 25 ml of the mobile phase (chloroform saturated with formamide) in the bottom. Then 20 ml of mobile phase were added to the trough. The chromatogram is developed to the bottom of the paper (about 1½ h), and allowed to drip for about 4 h. The paper was removed and air dried (10 min) and in a 100° oven with free air circulation, until it was dry to the touch (15–20 min). Then it was sprayed heavily with reagent 1 and visualized under long-wavelength UV light.

RESULTS AND DISCUSSION

Chromatographic methods for the separation of digoxin and dihydrodigoxin were investigated. Digoxin and dihydrodigoxin appeared as symmetrical well separated spots with R_F values of 0.65 and 0.79 on cellulose plates and 0.33 and 0.41 on silica gel plates. Separation was also achieved on paper with the relative migrations of dihydrodigoxin to digoxin being 1.09 when the solvent flow is sufficient to bring dihydrodigoxin to the edge of the chromatogram.

To demonstrate the degree of separation achieved by the two TLC methods, the plates were scanned using a Schoeffel Spectrodensitometer (Model SD 3000) with

a reflectance mode attachment. Total fluorescence above 405 nm was measured using an excitation wavelength of 350 nm. The chromatograms which were sprayed with reagent 4 and the resulting scans are shown in Fig. 1. Chromatography using cellulose or paper gives greater separation of the two components than is obtained using silica gel. However using the former methods, R_F values are critically dependent on the percentage of formamide in the dipping solution and the drying times, whereas the silica gel method is simpler to carry out and more reproducible.

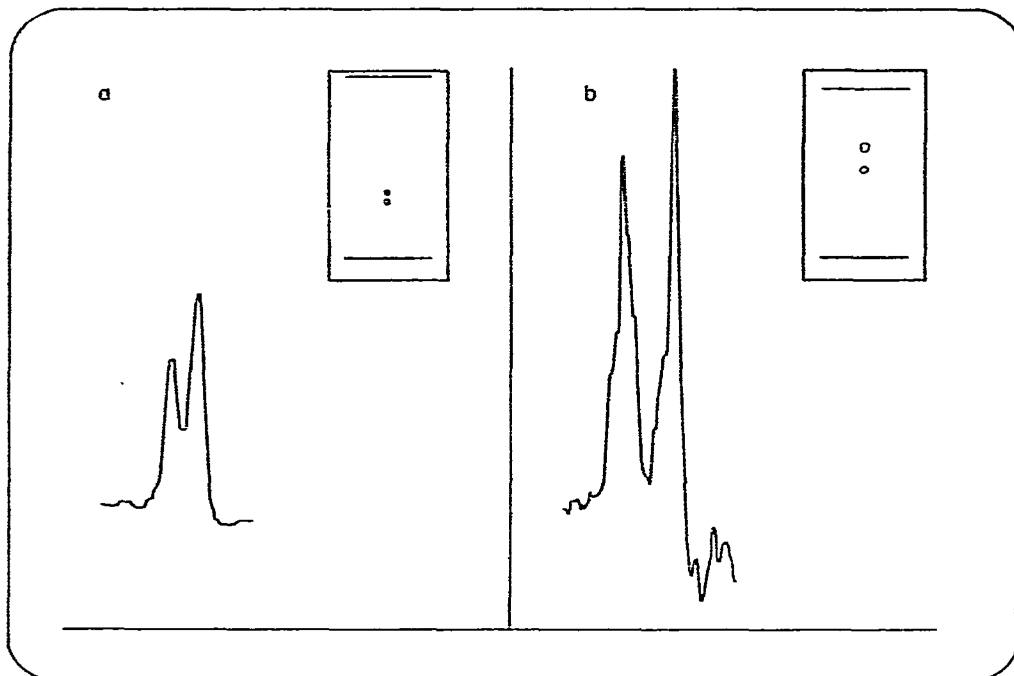

Fig. 1. Diagram of the TLC plate and fluorescence scan of digoxin–dihydrodigoxin separation using (a) silica gel plates and (b) cellulose plates.

These separations would be useful in conjunction with assays in which cross reaction is significant. It may be possible in the future to use these TLC methods for quantitation of digoxin and its metabolites using plate scanning methods. The limiting factor at present is a spray reagent that will allow detection of very low levels of digoxin and dihydrodigoxin. Using the Schoeffel spectrodensitometer reagent 4 was the most sensitive reagent tested with lower limits of 1 ng for digoxin and 10 ng for dihydrodigoxin.

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