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Note

Separation of conjugated dihydroxy bile acids by thin-layer chromatography

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The complete separation of the conjugated dihydroxy bile acids by thin-layer chromatography (TLC) has not been reported, although slight separations have been accomplished[1,2]. Only recently, glycine conjugates of the dihydroxy pair, deoxycholic and chenodeoxycholic acids, and their respective taurine conjugates were separated by high-pressure liquid chromatography[3–7].

This report describes the separation of glycodeoxycholic acid and glycochenodeoxycholic acid in one TLC mobile phase, and taurodeoxycholic and taurochenodeoxycholic acids in another system. The technique was applied to separation of bile acids from diluted specimens of human bile.

MATERIALS AND METHODS

For separation of the glycine bile acid conjugates, the following TLC system was used: Analtech 10 × 20 cm silica gel G layers (Analtech, Newark, Del., U.S.A.) were scored in 1-cm lanes and dipped into a solution of 5 g potassium dihydrogen phosphate in 150 ml of water and 100 ml of methanol. The plates were placed horizontally in the solution for 1 min, then removed and kept vertically at room temperature until dry. The impregnated plates were activated in an oven for 10 min at 170° before sample application.

Samples of 50–300 ng of the following bile acids were applied across the 1-cm lanes, using Drummond microcapillaries (Drummond Scientific, Broomall, Pa., U.S.A.) (1 to 5 μl depending on the concentration of the samples): glycocholic (GC), taurocholic (TC), glycodeoxycholic (GDC), taurodeoxycholic (TDC), glycochenodeoxycholic (GCDC), and taurochenodeoxycholic (TCDC). In addition, samples of human gallbladder bile diluted 1:100 with methanol were spotted in lanes adjacent to the standards. Continuous development was carried out with the mobile phase chloroform–2-butanone–ethanol (75:15:10), for 3 h. The upper 5 mm of the plate was allowed to protrude from the top of the tank; the lid of the tank was placed against the layer and the edges were sealed with tape. Alternatively, three successive developments to the top edge of the plate may be used.

For separation of the taurine conjugates, Whatman KC_{18} reversed-phase layers (12% carbon load) were developed in the system potassium dihydrogen phosphate (0.2 M, pH 2.5)–ethanol (1:1). These chromatograms were developed in the con-

ventional manner until the solvent front reached 1 cm from the top of the plate (1.5 h).

After the development was completed, the chromatograms were dried in air and then dried for 4 min at 170° in an oven, in order to remove the solvent. The chromatograms were then sprayed lightly with a solution of 10% sulfuric acid in ethanol. After drying in air, the plate was heated in an oven at 170° for 5 min in order to develop the fluorescence. Overheating should be avoided, since this results in charring of the layer and bile acids.

A Schoeffel Model 3000 spectrodensitometer (Schoeffel Instrument Co., Westwood, N.J., U.S.A.), equipped with a computer to convert photomultiplier response to optical density units, was used to scan for fluorescence. This was used in the single-beam mode with a 365 nm excitation wavelength and a Corning 3-72 filter (390–400 nm cutoff) in the viewing mode. The response was channeled into a Schoeffel SDC 300 density computer recorder in order to provide a continuous recording of the photomultiplier response.

RESULTS

The spraying with the sulfuric acid reagent and the short heating time result

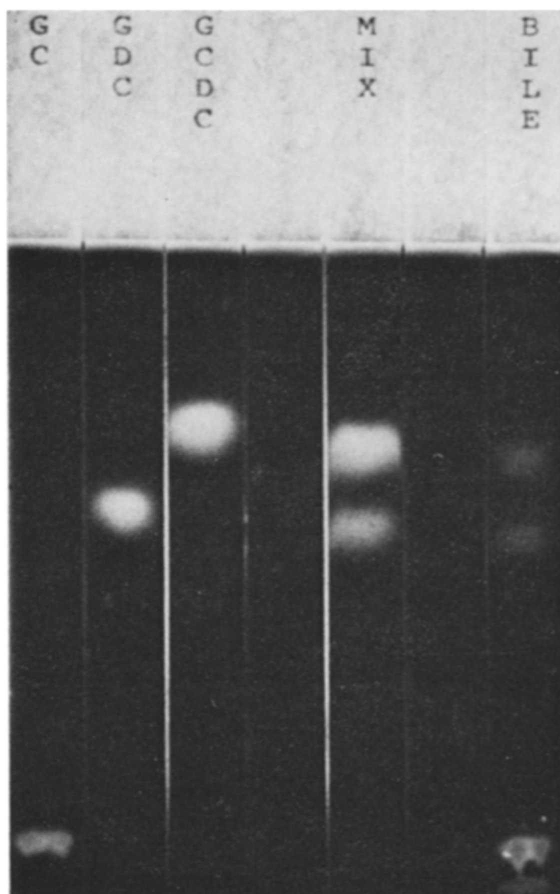

Fig. 1. Chromatogram showing separation of glycocholic acid (GC), glycodeoxycholic acid (GDC) and glycochenodeoxycholic acid (GCDC). Note that in the bile sample the point of sample application is visible, and can be shown to contain the three taurine conjugates.

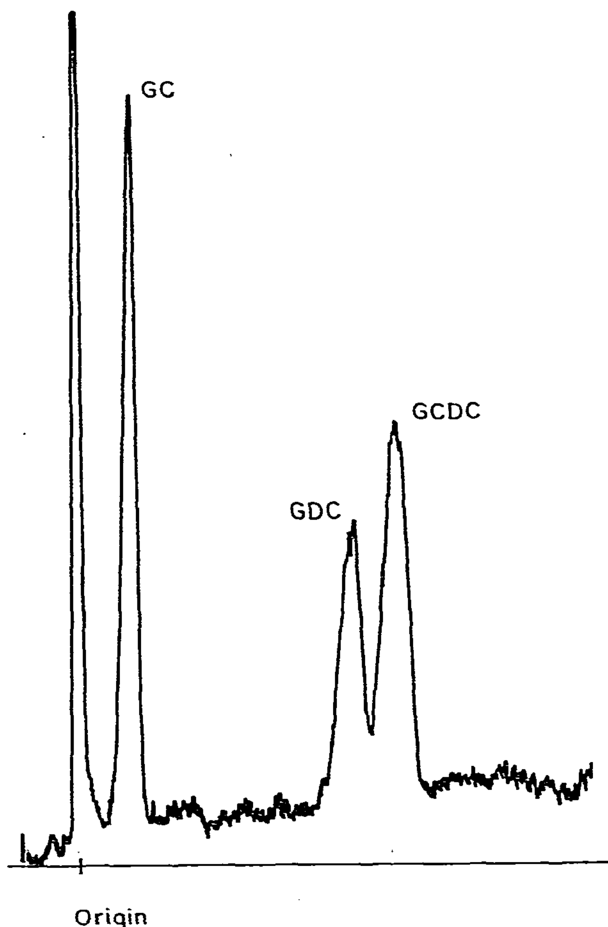

Fig. 2. Scan of the lane containing a bile sample as shown in Fig. 1. Glycocholic acid (GC), glycodeoxycholic acid (GDC) and glycochenodeoxycholic acid (GCDC) are well separated. The peak marked "origin" contains the taurine conjugates.

in the formation of stable fluorescence derivatives of the bile acids. Scanning of the layers could be performed months after chromatography with excellent reproducibility of the results. The limit of the detection was as low as 25 ng, depending on the bile acid. In general, the conjugates of cholic acid gave the best detectability. Fig. 1 shows a photograph of the glycine conjugates of the two dihydroxy bile acids, separated as described. As shown by Fig. 2, the resolution is such that densitometric scans return to baseline between the peaks of each bile acid, which is important for quantitative analysis by densitometry.

Fig. 3 shows the separation of the two taurine conjugates by the method described, and Fig. 4 gives the results of scanning this chromatogram by using densitometry. For glycocholic, glycodeoxycholic, and glycochenodeoxycholic acids the distance traveled was 5, 52, 57 mm, respectively, from the site of application. For taurocholic, taurochenodeoxycholic, and taurodeoxycholic acids the R_F values were 0.68, 0.43 and 0.37, respectively.

The sensitivity of the detection method, as determined by using known standards, was 25 ng. Therefore, bile samples were diluted (1:100), and 1–2 μl were applied

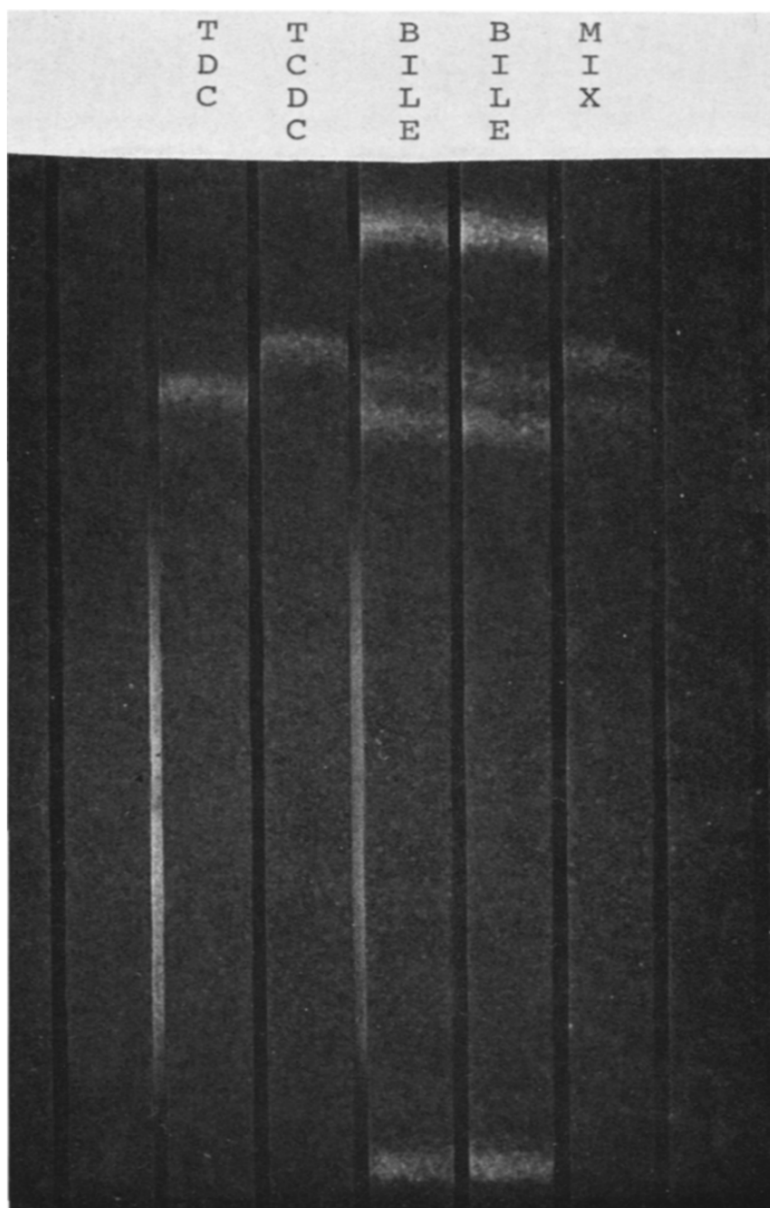

Fig. 3. Chromatogram showing separation of taurodeoxycholic acid (TDC) and taurochenodeoxycholic acid (TCDC). In the lanes where bile was applied, taurocholic acid (TC) may be seen at the top of the lanes.

directly to the layer. With a conventional 20 × 20 cm plate, it was possible to analyze 15 samples along with the reference standards.

DISCUSSION

The chromatographic systems reported herein accomplish for the first time, clear resolution of both glycine and taurine conjugates of the two major dihydroxy

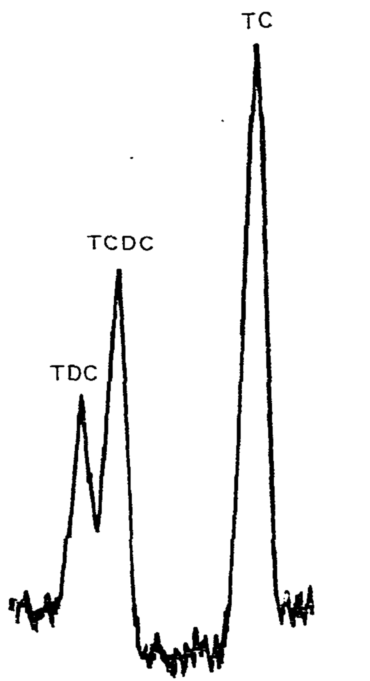

Fig. 4. Scan showing resolution of taurocholic acid (TC), taurodeoxycholic acid (TDC) and taurochenodeoxycholic acid (TCDC) by photodensitometry.

bile acids, deoxycholic and chenodeoxycholic acid. Baseline separations between the peaks of each bile acid were obtained. The TLC system for glycine conjugates is specific because taurine conjugates do not move from the origin. However, the reversed-phase system is not specific because both glycine and taurine conjugates migrate. The separation of the taurine and glycine conjugates, as groups, on silica gel layers with the mobile phase isopropanol–glacial acetic acid (93:7) is a prerequisite to separation in the systems described. The sensitivity of the method is such that bile acids in samples obtained by duodenal intubation of human subjects are easily detectable, and the detectability is within the range of concentrations in human serum. These separations should provide the basis for a simple, rapid, and inexpensive technique for analysis of bile samples.

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