notes on methodology

Separation of bile acids of rat bile by thin-layer chromatography

CHARLES M. SIEGFRIED and WILLIAM H. ELLIOTT

Department of Biochemistry, St. Louis University School of Medicine, St. Louis, Missouri 63104

SUMMARY The common bile acids of rat bile (chenodeoxycholic, hyodeoxycholic, cholic, α-muricholic, and β-muricholic acids) are completely separated by a new thin-layer chromatographic system.

KEY WORDS bile acids · separation · thin-layer chromatography · cholic · α- and β-muricholic acids

IN RELATION TO OUR STUDIES on sterol metabolism (1) it became desirable to separate by TLC the common biliary acids obtained from hydrolyzed rat bile. The solvent system described here is similar to that used by Kelly and Doisy (2) for the separation of conjugated bile acids, and in our hands it has been more satisfactory in the separation of certain bile acids in descending or ascending TLC than systems previously reported (3, 4).

Materials. Cholic and hyodeoxycholic acids were a gift of The Wilson Laboratories, Chicago, Ill., and were purified by partition chromatography on Celite with 70% acetic acid as the stationary phase and increasing quantities of benzene in hexane as the mobile phase (5). α-, β-, and ω-muricholic acids and hyocholic acid were prepared in this laboratory (6–8). Chloroform, methanol, and acetic acid were analytical reagent grade obtained from Mallinckrodt Chemical Works. Silica Gel G was obtained from Brinkmann Instruments, Inc., Westbury, N.Y. Rat bile was obtained from adult male rats with cannulated bile ducts, and was hydrolyzed and extracted according to the procedure of Matschiner et al. (5).

Procedure. Glass plates (20 cm × 50 cm or 20 cm × 20 cm) were coated to a thickness of 250 μ with a slurry of 35 g of Silica Gel G in 70 ml of distilled water, air dried for 1 hr, activated in an oven at 110–120°C for 1.5 hr, and stored in a desiccated chamber until used. The sample (20–40 μg in 10 μl of acetone) was applied to the plate with a micropipette in the usual manner (9). In the descending method the longer plates were placed in a cylindrical tank (28.5 cm I.D. × 60 cm) which contained a saturated atmosphere derived from the mixture chloroform–methanol–acetic acid 80:12:3. The same solvent mixture was carried from an elevated reservoir to the top of the plate via a wick of filter paper. The plates were developed for 3–5 hr. In the experiments using material from extracted rat bile, the plates were coated to a thickness of 500 μ, and the sample (about 2 mg, equivalent to 0.25 ml of rat bile) was applied in acetone to the plate. Ascending TLC was carried out with 20 cm × 20 cm plates in the usual manner (9) with the same mixture of solvents. Bile acids were detected after the developed plates had been sprayed with 10% phosphomolybdic acid in 95% ethanol (10) and heated for 30 min at 100–120°C.

Results. The trihydroxycholanoic acids (cholic and α- and β-muricholic acids) and the dihydroxycholanoic acids (chenodeoxycholic and hyodeoxycholic acids) were completely separated by either procedure. Table 1 contains typical R_f values for these acids in the descending system on plates of 250 and 500 μ thickness. By this method ω-muricholic and hyocholic acids could also be distinguished from the common bile acids obtained from hydrolyzed rat bile; deoxycholic acid was well separated from all the acids studied except chenodeoxycholic acid. Relative R_f values in ascending chromatography were about the same for α- and β-muricholic acids. In the descending method the relative R_f values of the dihydroxycholanoic acids increased with longer development time and thicker plates, but the separations within this group of acids remained unchanged. The last column of Table 1

TABLE 1 R_f VALUES OF FREE BILE ACIDS IN DESCENDING TLC

Bile acid	System A* 4.5 hr 250 μ	System A* 4 hr 500 μ	System B† 3.5 hr 500 μ
Cholic acid	1.00	1.00	1.00
β-Muricholic acid	1.19	1.28	0.90
α-Muricholic acid	0.90	0.87	0.82
Chenodeoxycholic acid	1.63	1.93	1.75
Hyodeoxycholic acid	1.42	1.72	1.40
Deoxycholic acid	1.64	1.96	1.78
ω-Muricholic acid	1.10	—	—
Hyocholic acid	1.06	—	—

R_f values are relative to that of cholic acid.
* Chloroform–methanol–acetic acid 80:12:3.
† Benzene–isopropanol–acetic acid 30:10:1 (3).

Abbreviation: TLC, thin-layer chromatography.

In accordance with the recommendations of the International Union of Pure and Applied Chemistry, the following systematic names are given to bile acids referred to in this report: cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid; α-muricholic acid, 3α,-6β,7α-trihydroxy-5β-cholanoic acid; β-muricholic acid, 3α,6β,7β-trihydroxy-5β-cholanoic acid; ω-muricholic acid, 3α,6α,7β-trihydroxy-5β-cholanoic acid; hyocholic acid, 3α,6α,7α-trihydroxy-5β-cholanoic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; hyodeoxycholic acid, 3α,6α-dihydroxy-5β-cholanoic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholanoic acid.

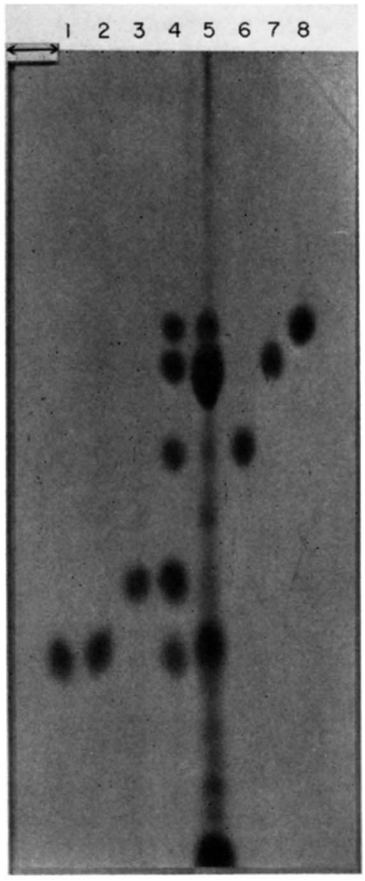

FIG. 1. A typical separation of di- and trihydroxycholanoic acids by descending TLC after 4.0 hr in chloroform–methanol–acetic acid 80:12:3 on a layer 500 μ thick. Arrow, origin; *1*, deoxycholic acid; *2*, chenodeoxycholic acid; *3*, hyodeoxycholic acid; *4*, mixture of α- and β-muricholic, cholic, hyodeoxycholic, and chenodeoxycholic acids; *5*, an acid fraction from hydrolyzed rat bile (2 mg, equivalent to 0.26 ml of rat bile); *6*, β-muricholic acid; *7*, cholic acid; and *8*, α-muricholic acid.

shows R_f values obtained by this procedure but with Eneroth's system (S-VII), benzene–isopropanol–acetic acid 30:10:1 (3).

Fig. 1 shows the migration of several individual acids, a mixture of five acids, and a mixture of acidic components from hydrolyzed rat bile. In the latter case spots corresponding to α-muricholic, cholic, and chenodeoxycholic acids can be seen. A spot with the mobility of β-muricholic acid was also seen on the chromatogram, but is not well distinguished from the streaking background in the photograph. The large spot at the base of the chromatogram has the mobility of free fatty acids, which are usually present in an acid fraction of hydrolyzed rat bile (5).

This work was supported by grants from the National Institutes of Health (HE-07878 and GM-446) and the St. Louis University Biochemistry Committee on Grants.

Manuscript received 27 November 1967; accepted 12 January 1968.

REFERENCES

1. Karavolas, H. J., W. H. Elliott, S. L. Hsia, E. A. Doisy, Jr., J. T. Matschiner, S. A. Thayer, and E. A. Doisy. 1965. *J. Biol. Chem.* **240:** 1568.
2. Kelly, R. L., and E. A. Doisy, Jr. 1964. *Federation Proc.* **23:** 173. (Abstr.)
3. Eneroth, P. 1963. *J. Lipid Res.* **4**· 11.
4. Hofmann, A. F. 1964. *In* New Biochemical Separations. A. T. James and L. J. Morris, editors. D. Van Nostrand Company, Inc., New York. 261.
5. Matschiner, J. T., T. A. Mahowald, W. H. Elliott, E. A. Doisy, Jr., S. L. Hsia, and E. A. Doisy. 1957. *J. Biol. Chem.* **225:** 771.
6. Hsia, S. L., J. T. Matschiner, T. A. Mahowald, W. H. Elliott, E. A. Doisy, Jr., S. A. Thayer, and E. A. Doisy. 1957. *J. Biol. Chem.* **225:** 811.
7. Hsia, S. L., J. T. Matschiner, T. A. Mahowald, W. H. Elliott, E. A. Doisy, Jr., S. A. Thayer, and E. A. Doisy. 1957. *J. Biol. Chem.* **226:** 667.
8. Hsia, S. L., J. T. Matschiner, T. A. Mahowald, W. H. Elliott, E. A. Doisy, Jr., S. A. Thayer, and E. A. Doisy. 1958. *J. Biol. Chem.* **230:** 597.
9. Stahl, E. 1965. *In* Thin-Layer Chromatography. E. Stahl, editor. Springer-Verlag, Berlin. 5–27.
10. Kritchevsky, D., and M. R. Kirk. 1952. *Arch. Biochem. Biophys.* **35:** 346.