Journal of Chromatography, 116 (1976) 184–187

CHROM. 8730

Note

New improved method for separation of fecal bile acids by thin-layer chromatography

RON SPEARS, DAVID VUKUSICH, SURINDER MANGAT and BANDARU S. REDDY
Naylor Dana Institute for Disease Prevention, Valhalla, N.Y. 10595 (U.S.A.)
(Received July 28th, 1975)

Many solvent systems have been reported for free and methylated fecal bile acid separation by thin-layer chromatography (TLC)[1–4]. None as yet have provided an adequate procedure for removing the fatty acids present in the fecal extract before separating the bile acids. Fatty acids interfere with gas–liquid chromatographic (GLC) analyses of bile acids and can also interfere with spectrophotometric analysis.

To overcome these problems, new and existing TLC methods of both free and methylated bile acids have been modified in our laboratory. Separation of free bile acids was required for a total enzymatic determination using techniques employed by Weber *et al.*[4] and Engert and Turner[5]. The fatty acids were removed by a modification of the TLC solvent system used by Weber *et al.*[4] and the bile acids subsequently separated into three bands according to the amount of hydroxyl functions present using a modification of the TLC solvent system described by Sundaram *et al.*[1].

The solvent system used for removing fatty acids from free bile acid extracts was found to work equally well for removing fatty acids from methylated bile acid extracts. A second solvent system was utilized to complete the separation of the bile acids after the method described by Reddy *et al.*[6].

MATERIALS AND METHODS

The TLC plates used were pre-coated silica gel GF, 250 μm thick, 20 × 20 cm, obtained from Analtech (Newark, Del. U.S.A.; Cat. No. 2011). A standard rectangular tank was used with a heavy glass cover. Cholic acid, deoxycholic acid, lithocholic acid, palimitic acid and their methyl esters were used as standards. All standards were obtained from Applied Science Labs. (State College, Pa., U.S.A.). To allow for solvent saturation of the developing chamber, Whatman No. 1 qualitative filter paper was used to line the walls of the tank.

The free bile acids were extracted from the feces after the methods of Reddy *et al.*[6] and methylated overnight at ambient temperature with a freshly prepared ethereal–alcoholic solution of diazomethane.

A 5-cm vertical portion of the plate was separated by scoring the media for the standard spots so as to insure that no mixing of the standards with the sample could occur. Another portion was scored 2 cm from the upper border of the plate and the solvent front allowed to proceed up to this point. Samples were streaked 2 cm from

the lower border of the plate and placed directly into the developing chamber which had previously been allowed to become saturated with solvent vapor for approximately one hour. The first development for both free and methylated bile acids was carried out in a modification of the system reported by Weber *et al.*[4] and consisted of hexane–chloroform–diethyl ether–*n*-butanol–acetic acid (40:10:10:3:0.5). The plates were removed, dried, and viewed under a short wave ultraviolet lamp to visualize the fatty acid band. A line was scored just below the fatty acids and the plates developed in their respective second baths up to this point. The second development for the free bile acids was carried out in a modification of a solvent system reported by Sundaram *et al.*[1] and consisted of isooctane–ethyl acetate–*n*-butanol–*n*-propanol–acetic acid (20:10:3:3:3) For methylated bile acids, the second system consisted of isooctane–isopropanol–acetic acid (120:40:1). When separating the free bile acids. tailing was often encountered with deoxycholic acid. This was alleviated by increasing the volume of acetic acid from 15 to 20 parts and excluding *n*-propanol. After the second development the plates were dried and sprayed with 0.1 % 8-hydroxy-1,3.6-pyrene-trisulfonic acid trisodium salt in water or methanol when the plates were to be eluted and subsequently analyzed. For photographic purposes, the plates were sprayed with 0.1 % 1,2-dichlorofluorescein in ethanol. Both dyes were obtained from Eastman (Rochester, N.Y., U.S.A.; Cat. Nos. 373 and P728, respectively).

RESULTS AND DISCUSSION

The resolution of the described solvent systems is shown in Fig. 1. Previous methods using a chloroform–benzene (1:1) solvent system for removing the fatty acids in methylated samples required two and sometimes three successive developments to attain the separation that our new system affords us after only one development. The fatty acids in all cases moved a minimum of 10 cm from the origin and usually 12–13 cm.

The actual separation of both free and methylated bile acids was accomplished by the second solvent system. Free and methylated bile acids were separated into three bands corresponding to the number of hydroxyl functions in the molecule. As was mentioned, occasional tailing of deoxycholic acid occurred in both the standard spots and samples causing overlap of deoxycholic and cholic acid. To eliminate this tailing, the solvent system used by Sundaram *et al.*[1] was modified by increasing the volume of acetic acid and excluding *n*-propanol. This variation allowed the deoxycholic acid to migrate up the plate with no significant tailing as shown in Fig. 2.

Removal of fatty acids from samples which are to be analyzed later by GLC is essential as they would interfere with bile acid analysis. Separation of bile acids according to those modified methods and separate elution of each band allowed us to use TLC and GLC procedures to identify the bile acids. The system used for free bile acids was designed to obtain adequately pure bile acids for a total enzymatic determination using a combination of methods used by Weber *et al.*[4] and Engert and Turner[5]. One can assay each band separately or altogether, the decision being left up to the individual investigator. We felt that these new systems allow for a rapid and precise method for separating free and methylated bile acids from fecal extracts in adequately pure form to be taken right to a GLC or enzymatic analysis.

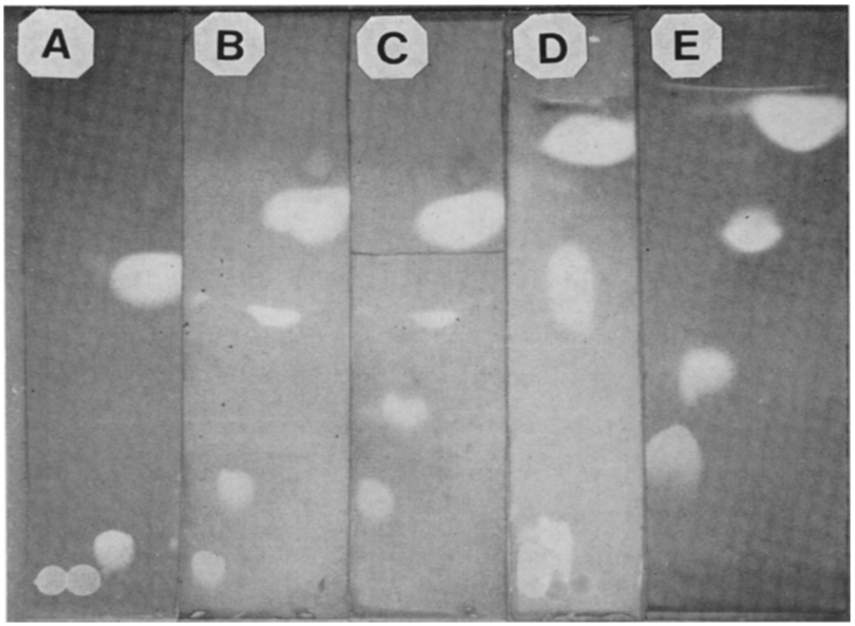

Fig. 1. Separation in one development of (A) methylated bile acids with chloroform–benzene (1:1); (B) methylated bile acids with hexane–diethyl ether–chloroform–*n*-butanol–acetic acid (40:10:10:3:0.5), (C) methylated bile acids with isooctane–isopropanol–acetic acid (120:40:1); (D) free bile acids, solvent system as in (B); (E) free bile acids with isooctane–ethyl acetate–*n*-butanol–*n*-propanol–acetic acid (20.10:3:3:3).

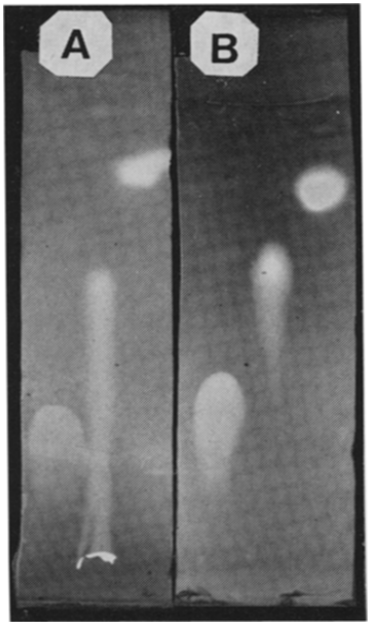

Fig 2. Separation of free and methylated bile acids with (A) Isooctane–ethyl acetate–*n*-butanol–acetic acid (20·10:3:3) (tailing of deoxycholic acid spot) and (B) Isooctane–ethyl acetate–*n*-butanol–acetic acid (20:10·3:4) (tailing of deoxycholic acid reduced).

ACKNOWLEDGEMENTS

This work was supported by PHS grant CA-16382-04 through the National Large Bowel Project and contract CP-33208 from the National Cancer Institute.

REFERENCES

1 G. S. Sundaram, H. Singh and H. S. Sodhi, *Clin. Chim. Acta*, 34 (1971) 425
2 A. Bruusgaard, *Clin. Chim. Acta*, 28 (1970) 495.
3 A. F. Hofmann, *J. Lipid Res.*, 3 (1962) 127.
4 A. M. Weber, L. Chartrand, G. Doyon, S. Gordon and C. C. Roy, *Clin. Chim. Acta*, 39 (1972) 524.
5 R. Engert and M. D. Turner, *Anal. Biochem.*, 51 (1973) 399.
6 B. S. Reddy, J. Weisburger and E. L. Wynder, *J. Nutrition*, 105 (1975) 878