Journal of Chromatography, 196 (1980) 150–155
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,839

Note

New solvent systems for separation of free and conjugated bile acids

III. Separation of bile acid methyl ester acetates

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(Received February 12th, 1980)

The examination of bile acid (BA) profiles has received considerable attention in clinical investigation recently because of possible correlation between the altered BA metabolism and certain digestive diseases[1–4] For this reason, there is a general interest in the development of methodology for studying BAs in body fluids of these patients by the use of the combined techniques of thin-layer chromatography (TLC), gas–liquid chromatography (GLC) and mass spectrometry (MS). As a part of our study on BA metabolism in children with cholestatic syndrome, we have developed a new solvent system, N, which gives clean separation of BAs as methyl ester (ME) acetates on the basis of the total number of hydroxyl or keto groups per molecule, using a unidirectional single development system. This system is particularly useful for the determination of chemical structures of atypical BAs by GLC–MS.

MATERIALS AND METHODS

All BA standards were obtained from Steraloids (Pawling, NY, U.S.A.) or Applied Science Labs. (State College, PA, U.S.A.) except 3α, 6β ,7β, 12α-tetrahydroxy-5β-cholanic acid, which was a generous gift from Professor H. B. Kagan, Paris, France. All solvents used were of a reagent grade and obtained from Aldrich (Milwaukee, WI, U.S.A.) or J. T. Baker (Phillipsburg, NJ, U.S.A.). Glass plates (20 × 20 cm), precoated with silica gel G to a thickness of 250 μm, were purchased from Brinkmann (Westbury, NY, U.S.A.). BAs were extracted three times from clinical specimens by warm ethanol–methanol mixture (95:5, v/v) and were solvolyzed according to the procedures of Javitt *et al.*[5]. The solvolyzed products were subjected to hydrolysis in 2 ml of 2.5 *N* NaOH at 110°C overnight, and the free BAs were extracted by diethyl

0021-9673/80/0000–0000/$02.25

ether at pH 1. Free BAs may be further separated by TLC using new solvent systems developed in our laboratory[6,7]. The MEs of BAs were prepared by the method of Yousef *et al.*[8], and the acetates of BA-ME were obtained according to the method of Roovers *et al.*[9] before further separation by TLC using the present system (N). The solvent system (N) was made up of heptane–diethyl ether (anhydrous) (40:60, v/v). All TLC runs were carried out by applying 20–40 μg of the sample in 20–40 μl of diethyl ether or ethyl acetate to the plate with a micropipette or by a sample streaker (Applied Science Labs.). The plate was allowed to dry, placed in a rectangular glass tank (10 × 30 × 25 cm) and developed by the ascending technique at room temperature (23–25°C). The time required for a run was *ca.* 1 h. Areas of TLC plates corresponding to mono-, di-, tri-, and those below trihydroxy (presumably tetrahydroxy) were scraped and extracted with ethyl acetate before injection into a gas chromatograph.

The GLC analyses were done in a Packard Model 420 gas chromatograph with dual flame ionization detectors. A 1.8 m × 1 mm I.D. glass column packed with 1% Poly S-179 on 100–120 mesh Gas-Chrom Q (Supelco, Bellefonte, PA, U.S.A.) was used for the separation of ME acetates of BAs. The operating temperatures were: column 240°C, detector 250°C, and injection port 260°C. The carrier gas was nitrogen, at a flow-rate of 30 ml/min with inlet pressure of 20–40 p.s.i.g. The detailed procedures for GLC analyses were as described previously[1]. The identification of each BA was made by the use of a GLC–chemical ionization mass spectrometer (Biospect, Scientific Research Instruments Corp., Baltimore, MD, U.S.A.) interfaced to a PDP-12 computer (Digital Equipment Corp., Maynard, MA, U.S.A.). Isobutane gas was used for ionization of the BA-ME acetate derivatives. Detailed procedures for GLC–MS analysis of BA-ME acetates were reported elsewhere[3,10,11].

RESULTS AND DISCUSSION

The TLC separations of the BA-ME acetates utilizing the present solvent system (N) and another system (1)[12] are shown in Fig. 1, and the $R_F \times 100$ values of BA-ME acetates for these two systems are compared in Table I. It is seen from Fig. 1 and Table I that our solvent system (N) gives generally better separation of BA-ME acetates according to the number of substituents (OH or keto) in the molecule than the other system (1). In addition, our system (N) also provides clean separation for the BA-ME acetates and cholesterol acetates. This is an advantage over the other system (1) because cholesterol and its metabolites are the principal sterols in many biological specimens[1,2], and the separation of these neutral sterols from BAs would be very important for the analyses of both steroids. Fig. 2 shows the GLC profile of an unfractionated urine sample from a child with intrahepatic cholestasis, and Fig.s 3–6 show the GLC profiles for each of the fractions from the same sample isolated from TLC plates by using the present system (N). It is clear that the system (N) does give satisfactory separation of BA-ME acetates in the biological sample according to the number of substituents in the molecule. We have found this system (N) to be quite suitable for the isolation of BA fractions in clinical specimens as ME acetates, a derivative used extensively for structural studies of BAs by GLC–MS[3,10,11]

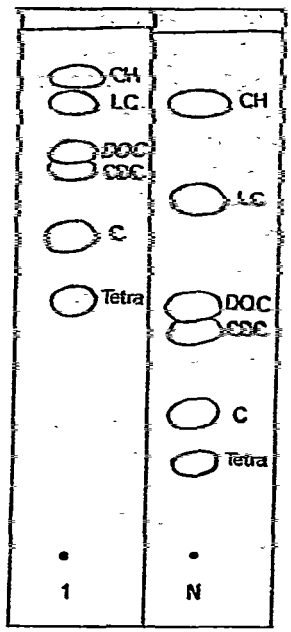

Fig. 1. Thin-layer chromatogram of bile acid methyl ester acetates developed in the solvent system (N), heptane–diethyl ether (40:60, v/v), and in system (1), benzene–acetone (92:8, v/v). The abbreviations used are: CH = cholesterol (as acetate); LC = lithocholic acid; DOC = deoxycholic acid; CDC = chenodeoxycholic acid; C = cholic acid; Tetra = 3α,6β,7β,12α-tetrahydroxy-5β-cholanic acid.

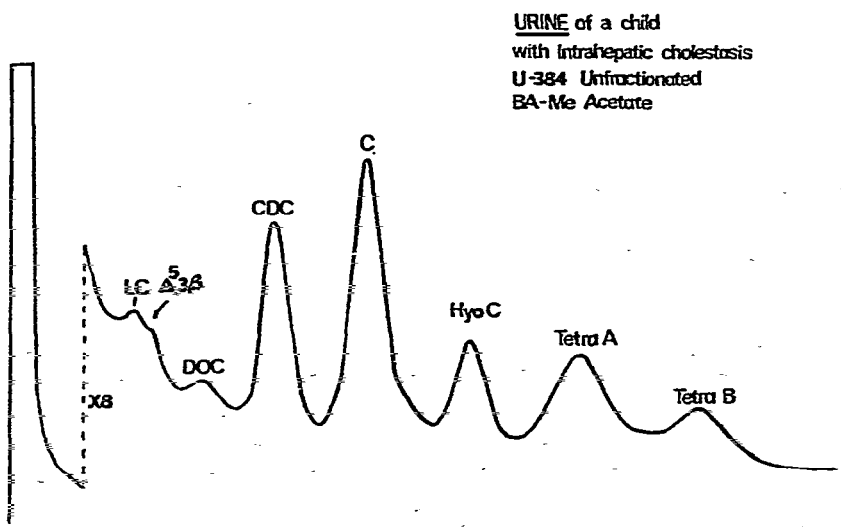

Fig. 2. Gas–liquid chromatogram of the bile acids in the urine (unfractionated) from a patient (F.M.) with intrahepatic cholestasis (as methyl ester acetates on 1% Poly S-179 at 240°C). See Fig. 1 for explanatory notes on the abbreviations used. Δ^5,3β = 3β-hydroxy-5-cholenoic acid; Hyo C = hyocholic acid; Tetra A and Tetra B = tetrahydroxy bile acid compounds of unknown structures.

TABLE I

SEPARATION OF BILE ACID METHYL ESTER ACETATES BY TLC*

Bile acid	*Substituents*	*R_F × 100 values*	
		N	*1*
Monosubstituted			
Lithocholic (LC)	3α-OAc	68	86
3β-Hydroxy-5-cholenoic (Δ^5, 3β)	3β-OAc	68	86
Disubstituted			
Deoxycholic (DOC)	3α-OAc, 12α-OAc	48	76
12-Ketolithocholic (12-keto LC)	3α-OAc, 12 keto	45	74
Chenodeoxycholic (CDC)	3α-OAc, 7α-OAc	43	73
Ursodeoxycholic (Urso DOC)	3α-OAc, 7β-OAc	42	75
Trisubstituted			
Hyocholic (Hyo C)	3α-OAc, 6α-OAc, 7α-OAc	28	63
Cholic (C)	3α-OAc, 7α-OAc, 12α-OAc	27	61
7-Ketodeoxycholic (7-keto DOC)	3α-OAc, 7-keto, 12α-OAc	22	57
Tetrasubstituted			
3α,6β,7β,12α-Tetrahydroxy-5β-cholanic (Tetra)	3α-OAc, 6β-OAc, 7β-OAc, 12α-OAc	18	49
A, B, C	—	<15**	—
ΔR_M × 100 values***			
Mono (LC) — CH		50	22
Di (DOC) — mono (LC)		36	29
Tri (Hyo C) — di (Urso DOC or CDC)		27	20
Tetra — tri (7-keto DOC)		11	14

* Solvent systems: N, heptane–diethyl ether (40:60, v/v); 1, benzene–acetone (92:8, v/v). See ref. 12.

** Estimated by the mobility of the band under the trisubstituted fraction of the urine sample (U-384), and the structures of the tetrasubstituted compounds in this region were established by GLC–MS. R_F × 100 value for cholesterol acetate is 87 for our system (N), 91 for System 1.

*** $\Delta R_M = R_{M2} - R_{M1}$ where $R_M = \log [(1/R_F) - 1]$.

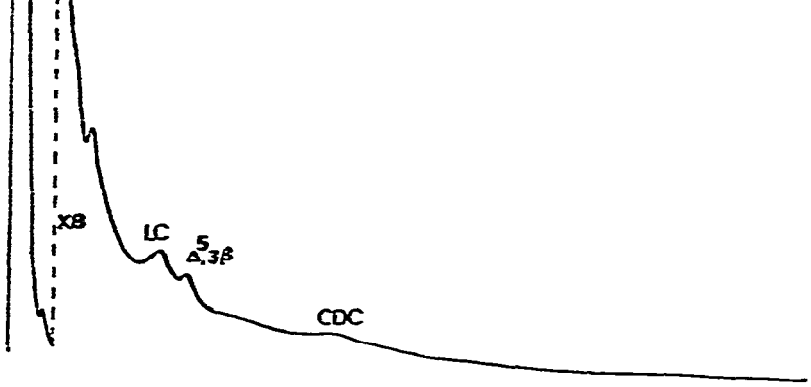

Fig. 3. Gas–liquid chromatogram of the monohydroxy bile acid fraction as isolated from the TLC plate of the urine sample from the same patient (F.M.) (as methyl ester acetates on 1% Poly S-179 at 240°C). The solvent system (N) was used and the abbreviations are the same as in Fig. 1.

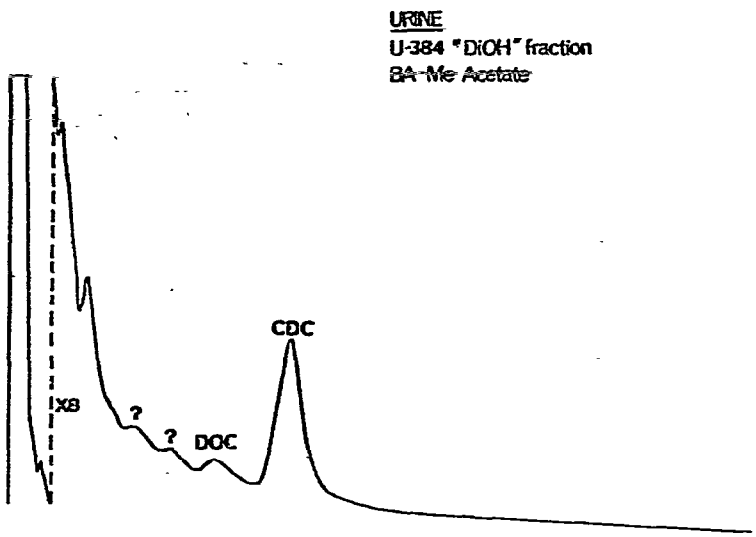

Fig. 4. Gas–liquid chromatogram of the dihydroxy bile acid fraction as isolated from the TLC plate of the urine sample from the patient F.M. (as methyl ester acetates on 1% Poly S-179 at 240°C). The solvent system (N) was used and the abbreviations are the same as in Fig. 1.

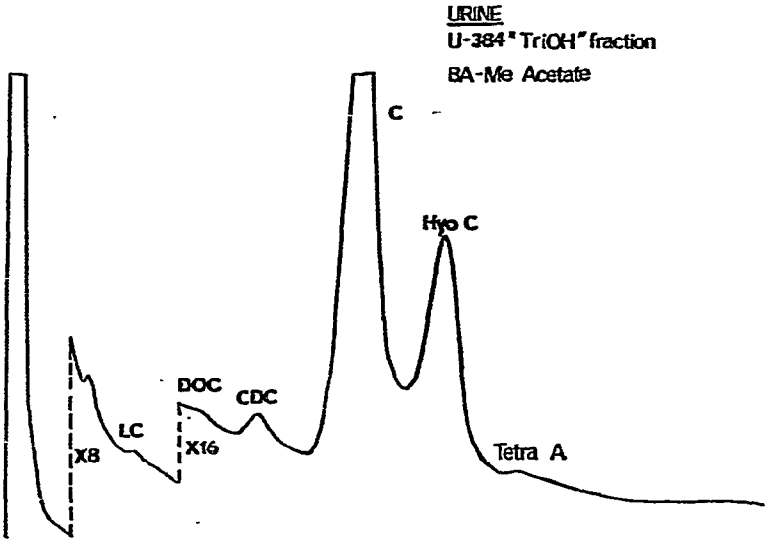

Fig. 5. Gas–liquid chromatogram of the trihydroxy bile acid fraction as isolated from the TLC plate of the urine sample from the patient F.M. (as methyl ester acetates on 1% Poly S-179 at 240°C). The solvent system (N) was used and the abbreviations are the same as in Figs. 1 and 2.

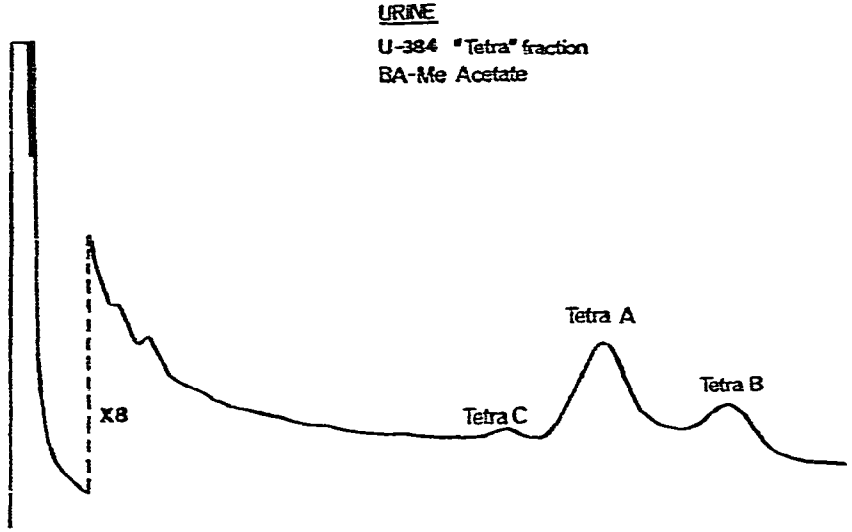

Fig. 6. Gas–liquid chromatogram of the tetrahydroxy bile acid fraction as isolated from the TLC plate of the urine sample from the patient F.M. (as methyl ester acetates on 1% Poly S-179 at 240°C). The solvent system (N) was used and the abbreviations are the same as in Figs. 1 and 2. Tetra C = tetrahydroxy bile acid of unknown structure. Note the separation of Tetra C from Hyo C by using this solvent system (N) (see Fig. 2).

ACKNOWLEDGEMENTS

This study was supported by a NASA Skylab Grant No. NASA-12728 from National Aeronautics and Space Administration, L. B. Johnson Space Center, Houston, TX, and by NIH-5-T22-DE-0035, HEW 06-D-00178, U.S. Department of Agriculture—Children's Nutrition Center and U.S. Department of Energy Contract No. W-31-109-ENG-38 and NIH-GCRC Program. Administrative support and a special travel fund for C.T.L.H. came from the Clinical Research Center, Texas Children's Hospital, Houston, TX, under the NIH grant 2-MO1-RR-00188. The authors thank Miss Elaine Potts, Mrs. D. Curlin, Dr. A. Strickland and Mrs. Ying-Ping Hsieh for their technical assistance.

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