Journal of Chromatography, 288 (1984) 461–468
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,540

Note

Reversed-phase high-performance thin-layer chromatography of free and conjugated bile acids

L. LEPRI*, D. HEIMLER and P. G. DESIDERI
Institute of Analytical Chemistry, University of Florence, Via G. Capponi 9, 50121-Florence (Italy)
(Received December 20th, 1983)

In recent years many different thin-layer chromatographic (TLC) systems have been developed for the analysis of free bile acids and their glycine and taurine conjugates[1–10]. The individual and class separation of bile acids are very important, *e.g.*, from a clinical point of view the presence of anomalous amounts of one or more bile acids indicate metabolic disorders and diseases[11–14]. Silica gel G has been widely employed as stationary phase[1–7] and recently reversed-phase chromatography on Whatman KC_{18} plates has been used[8,15]. However, these studies concerned only the behaviour of some taurine and glycine conjugates[8] and of sulphated bile acids[15].

In order to have a more complete picture of the separations which can be achieved on reversed-phase plates, we studied the behaviour of the most common bile acids (eight free, four glycine- and six taurine-conjugated) on RP-18 plates with different eluents.

Special attention was given to the two-dimensional technique, using aqueous and non-aqueous mobile phases in the two directions, since this technique gave very good results in the case of dansyl[16] and dinitrophenyl[17] amino acids and of indole derivatives[18]. This technique was employed to study the composition of free and conjugated acids in pig and chicken biles.

EXPERIMENTAL

Standard solutions were prepared by dissolving bile acids (Supelco, Bellefonte, PA, U.S.A.) in methanol; the sample volume used was 0.2–0.5 μl. The migration distance was 6 cm, unless otherwise stated. All the measurements were carried out at 25°C using a Desaga thermostatic chamber. After development, the plates were heated at 170°C for 10 min, then sprayed with a 10% sulphuric acid solution in methanol and again heated at 170°C for 3 min. The spots were visualized under UV light (254 nm).

Abbreviations used are: cholic (C), 3α,7α,12α-trihydroxy-5β-cholanic acid; deoxycholic (DC), 3α,12α-dihydroxy-5β-cholanic acid; chenodeoxycholic (CDC), 3α,7α-dihydroxy-5β-cholanic acid; hyodeoxycholic (HDC), 3α,6α-dihydroxy-5β-cholanic acid; lithocholic (LC), 3α-hydroxy-5β-cholanic acid; hyocholic (HyC), 3α,6α,7α-trihydroxy-5β-cholanic acid; ursodeoxycholic (UDC), 3α,7β-dihydroxy-5β-cholanic acid; dehydrocholic (DhC), 3,7,12-trioxo-5β-cholanic acid.

0021-9673/84/$03.00

TABLE I

R_F VALUES OF FREE AND CONJUGATED BILE ACIDS ON RP-18 PLATES WITH DIFFERENT ELUENTS

Compound	*Acetic acid–methanol–water*		*0.5 M ammonia in 60% methanol*	*Hexane–ethyl acetate–acetic acid*			*Amount (μg)*
	40:30:30	*60:20:20*		*72:27:1*	*72:18:10*	*63:27:10*	
Lithocholic acid	0.02	0.09	0.12	0.97	0.97	0.95	0.2
Deoxycholic acid	0.07	0.18	0.22	0.45	0.86	0.97	0.1
Chenodeoxycholic acid	0.08	0.21	0.25	0.39	0.80	0.96	0.1
Hyodeoxycholic acid	0.16	0.35	0.52	0.27	0.69*	0.85	0.2
Ursodeoxycholic acid	0.20	0.40	0.55	0.44	0.72*	0.85	0.2
Cholic acid	0.18	0.37	0.42	0.05	0.45*	0.90	0.05
Hyocholic acid	0.20	0.40	0.57	0.12	0.52*	0.86	0.2
Dehydrocholic acid	0.66	0.76	0.92	0.69	0.97	0.98	3
Glycodeoxycholic acid	0.16	0.35	0.23	0.00	0.26	0.87	0.2
Glycochenodeoxycholic acid	0.19	0.40	0.26	0.00	0.17*	0.85	0.2
Glycocholic acid	0.33	0.56	0.44	0.00	0.08	0.18	0.05
Glycodehydrocholic acid	0.77	0.80	0.93	0.00	0.11	0.95	1
Taurolithocholic acid	0.23	0.40	0.13	0.00	0.00	0.01	0.5
Taurodeoxycholic acid	0.36	0.54	0.26	0.00	0.00	0.00	0.05
Taurochenodeoxycholic acid	0.42	0.59	0.28	0.00	0.00	0.00	0.05
Taurohyodeoxycholic acid	0.63	0.75	0.55	0.00	0.00	0.00	0.1
Taurocholic acid	0.57	0.72	0.48	0.00	0.00	0.00	0.05
Taurodehydrocholic acid	0.92	0.92	0.79	0.00	0.00	0.00	1

* Slightly elongated spot.

RESULTS AND DISCUSSION

Table I lists the chromatographic characteristics of eighteen bile acids (free and conjugated with glycine and taurine) on RP-18 plates eluted with aqueous–organic solutions and non-aqueous mixtures.

Aqueous–organic eluents

The elution time with aqueous–organic eluents increases from 70 to 100 min as the water content increases from 20 to 40%. When eluted with acetic acid–methanol–water (40:30:30), an increase in the R_F values is observed on changing from the free acid to the corresponding glycine and taurine conjugates; the three forms, therefore, are well-separated.

The complete separation of the groups, on the contrary, cannot be achieved even by changing the eluent composition. A decrease of the water content of the eluent (see column 2) results in a general increase of the R_F values and a better resolution in the case of glycochenodeoxycholic and glycodeoxycholic acids, whose separation is difficult on other kinds of layers.

Fig. 1 shows some separations effected on RP-18 plates eluted with the above aqueous–organic solutions; Fig. 1A shows the separation of the six tauro-conjugates and Fig. 1B those of the four glycoconjugates and of the five most common free acids. The number and the positions of the –OH groups play an important rôle in the retention of the acids. With increasing hydroxyl number in the molecule, the affinity of the compounds towards the stationary phase decreases. A similar trend

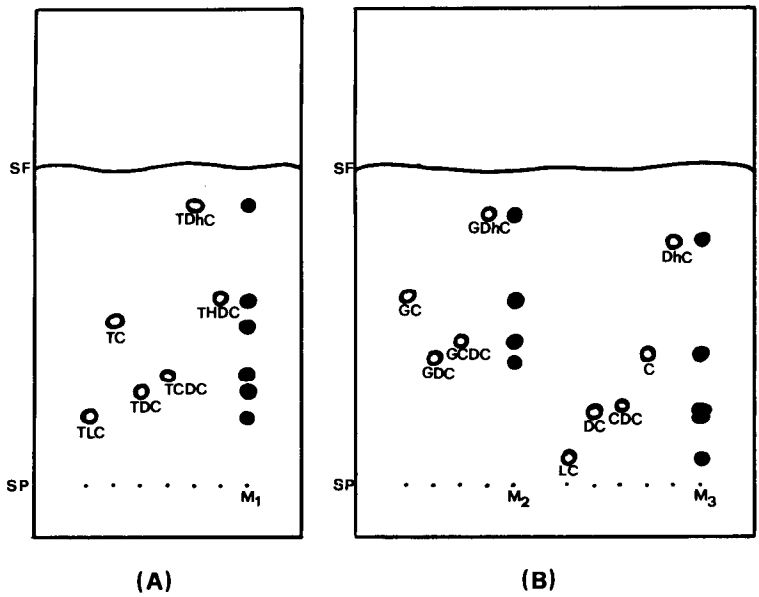

Fig. 1. Thin-layer chromatograms of bile acids. Eluents: A, acetic acid–methanol–water (40:30:30); B, acetic acid–methanol–water (60:20:20). M_1 = Mixture of TLC, TC, TDC, TCDC, TDhC, THDC; M_2 = mixture of GC, GDC, GCDC, GDhC; M_3 = mixture of LC, DC, CDC, C, DhC; SP = starting points; SF = solvent front; T = tauro; G = glyco.

has been observed on μBondapak C_{18} columns[19] eluted with aqueous–organic mixture with apparent pH = 7.0. As regards the position of the –OH groups in the molecule, there is a good correlation between the polarity of such groups and the affinity sequence of the corresponding compounds. For example, the sequence of R_F values for isomers with two hydroxyl groups is

R_F(UDC) >	R_F(HDC) >	R_F(CDC) >	R_F(DC)
3α,7β	3α,6α	3α,7α	3α,12α

The presence of an equatorial 7β-hydroxyl group generally conters a higher polarity on the molecule than does on axial α group. Also, from a comparison of the two isomers with three hydroxyl groups in the molecule it is found that the 6α-hydroxyl group is more polar than the 12α group since hyocholic acid is less strongly retained than cholic acid.

The decrease in affinity of the bile acids with increasing polarity shows that the retention of these compounds on RP-18 plates, eluted with aqueous–organic solutions, is controlled by a reversed-phase partition mechanism. However, there are some exceptions: taurohyodeoxycholic acid is less strongly retained than taurocholic acid while dehydrocholic acid is less strongly retained than the bile acids with three hydroxyl groups; the last trend is also observed in the case of the corresponding conjugated bile acids. These exceptions demonstrate that the overall polarity of the molecule cannot always be regarded as the sum of the polarities of the different functional groups.

Besides the percentage and kind of organic solvent, the retention of the bile acids is influenced even by the apparent pH of the eluent, which affects the degree of dissociation of the carboxylic group. Comparing the data of column 3 with those corresponding to the elution with 1 *M* acetic acid in 60% methanol, an increase in the R_F values is observed in the case of free and glyco-conjugated bile acids, while in the case of tauro-conjugates there are no large differences in retention with the two eluents.

Therefore a levelling in the retention of the bile acids is achieved by eluting with alkaline solutions which don't exhibit the advantages offered by acidic eluents. However, it should be noted that the separations of the three dehydrocholic acids from all the other compounds and of hyodeoxycholic acid from cholic acid, cannot be effected with acidic eluents.

Non-aqueous eluents

Table I (columns 4, 5 and 6) lists the chromatographic characteristics of bile acids eluted with non-aqueous solutions. The elution time is about 15 min. Compared to the behaviour with aqueous eluents, a completely different affinity sequence of both free and conjugated bile acids is observed. When eluted with *n*-hexane–ethyl acetate–acetic acid (72:27:1), the free acids owing to their higher hydrophobicity can be separated from the conjugated acids which remain at the application point.

The number of hydroxyl groups in the molecule determines the separation of these compounds into three distinct zones. Lithocholic acid (one hydroxyl group) migrates with the solvent front, the four acids with two hydroxyl groups exhibit R_F values between 0.27 and 0.45, while the two acids with three hydroxyl groups have

far lower R_F values (0.05 and 0.12). The chromatographic behaviour of dehydrocholic acid cannot be predicted on the basis of the polarity of its individual functional groups, as has already been pointed out.

As regards the influence of the position of the –OH groups, the R_F values of the dihydroxy bile acids decrease with increasing polarity of the axial α groups:

$$\underset{3\alpha,12\alpha}{R_F(\mathrm{DC})} \geqslant \underset{3\alpha,7\beta}{R_F(\mathrm{UDC})} > \underset{3\alpha,7\alpha}{R_F(\mathrm{CDC})} > \underset{3\alpha,12\alpha}{R_F(\mathrm{HDC})}$$

Ursodeoxycholic acid has an anomalous behaviour with respect to the high polarity of the equatorial β group.

With the present eluent the complete separation of the individual dihydroxy compounds with axial α groups has been achieved. This separation cannot be carried out on RP-18 plates with aqueous eluents and has been found to be difficult in a single development also on silica gel G plates.

In the case of trihydroxy compounds

$$\underset{3\alpha,6\alpha,7\alpha}{R_F(\mathrm{HyC})} > \underset{3\alpha,7\alpha,12\alpha}{R_F(\mathrm{C})}$$

the chromatographic behaviour cannot be explained on the basis of polarity.

As the acetic acid content of the eluent increases (column 5) at a given concentration of the more hydrophobic component (*n*-hexane), the free acids exhibit very high R_F values, the glyco-conjugates move from the starting point and migrate according to their different polarities, while the tauro-conjugates remain at the application point. Such behaviour allows the separation of the bile acids into three homogeneous groups. Some compounds, however, give rise to elongated spots which render their separation more difficult. Keeping the acetic acid concentration constant, a decrease in the *n*-hexane content and a corresponding increase of ethyl acetate content (column 6) results in a further increase in the mobility of both free acids and glyco-conjugates, which migrate with the solvent front with the exception of glycocholic acid. The tauro-conjugates remain at the application point and are completely separated from the other two groups.

As regards the general behaviour of the bile acids with non-aqueous eluents, their retention seems to be controlled by an adsorption mechanism, where the stationary phase behaves as a polar phase owing to the presence of unsilanized hydroxyl groups.

Two-dimensional technique

The separation of all the bile acids can be effected with the two-dimensional technique using non-aqueous and aqueous–organic eluents in the two directions. Fig. 2 reports (A) the theoretical chromatogram of the eighteen compounds on the basis of their R_F values (B) the experimental separation of a mixture of the eight free acids and (C) the experimental chromatogram of a mixture of all the bile acids eluted in the first direction with hexane–ethyl acetate–acetic acid (72:27:1) and in the second direction with acetic acid–methanol–water (60:20:20). After the first elution the plate was dried at room temperature for 1 h to allow the complete evaporation of the

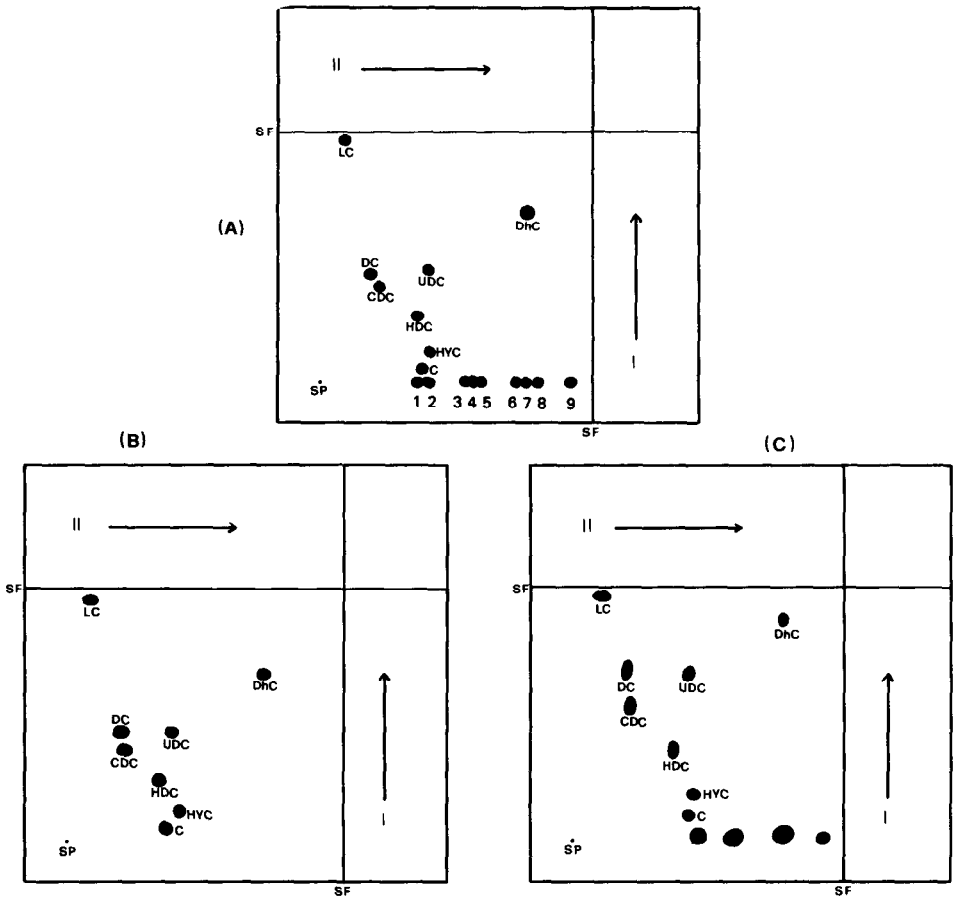

Fig. 2. Two-dimensional chromatogram of bile acids. Eluents: first direction, *n*-hexane–ethyl acetate–acetic acid (72:27:1); second direction, acetic acid–methanol–water (60:20:20). A, Theoretical chromatogram: 1 = GDC; 2 = GCDC + TLC; 3 = TDC; 4 = GC; 5 = TCDC; 6 = TC; 7 = THDC; 8 = GDhC; 9 = TDhC. B, Experimental chromatogram of free acids. C, Experimental chromatogram of free, glyco- and tauro-conjugated bile acids.

solvents. All the eight free acids can be separated independently of the presence of their conjugates. It should be noted that the experimental separation differs increasingly from the theoretical one the higher the number of compounds in the mixture. The individual components of the mixtures of Fig. 2B and 2C were successively deposited at the application point.

The tauro-conjugates have been separated in the presence of the other compounds, by eluting in the first direction with hexane–ethyl acetate–acetic acid (63:27:10) and in the second direction with acetic acid–methanol–water (40:30:30). This separation is not sensibly affected by the presence of other compounds, as in the first elution the free acids and their glyco-conjugates are completely separated from the tauro-conjugates which remain at the starting point. The second development, therefore, can be regarded, in the case of tauro-conjugates, as a one-dimen-

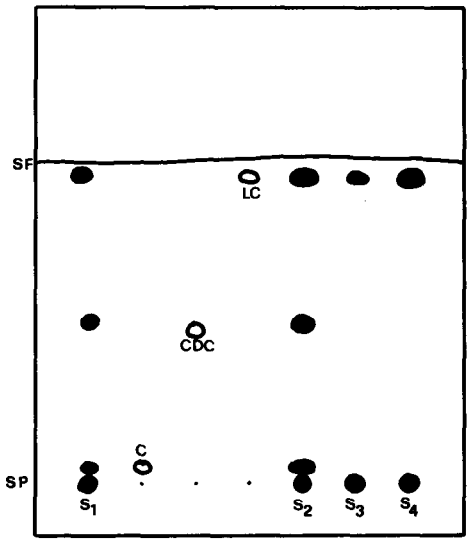

Fig. 3. Thin-layer chromatogram of chicken and pig biles. Eluent: *n*-hexane–ethyl acetate–acetic acid (72:27:1). S_1 = Sample of chicken bile (diluted 1:50 in methanol); S_2 = S_1 + C + CDC + LC; S_3 = sample of pig bile (diluted 1:10 in methanol); S_4 = S_3 + LC. Volume of chicken and pig bile samples deposited on the layer: 1 μl.

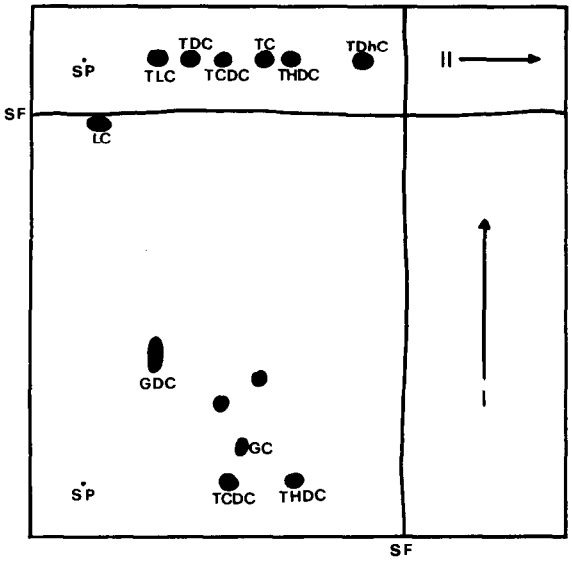

Fig. 4. Two-dimensional chromatogram of pig bile. The sample was diluted 1:50 in methanol to obtain definite spots. Volume deposited on the layer: 1 μl. Eluents: first direction, *n*-hexane–ethyl acetate–acetic acid (72:18:10); second direction, acetic acaid–methanol–water (40:30:30).

sional development with the advantages correlated with the possible use of a reference standard.

The separation of the glyco-conjugates from each other and from all the other compounds can be obtained by eluting in the first direction with hexane–ethyl acetate–acetic acid (72:18:10) and in the second direction with acetic acid–methanol–water (40:30:30). In this case the presence of the other compounds significantly affects the final position of the four glyco-conjugated acids.

Applications

These separation methods have been used for the identification of bile acids in pig and chicken biles. The chromatogram of Fig. 3, for chicken and pig bile samples indicates the presence of the following free acids: (1) cholic, chenodeoxycholic and lithocholic acids (chicken); (2) lithocholic acid (pig). The samples of chicken and pig biles were diluted 1:50 and 1:10 in methanol respectively in order to obtain spherical spots. Therefore in the chicken bile the free acids, besides being more numerous, are more concentrated than in the pig bile.

The presence of the above free acids has also been revealed by the two-dimensional technique with the eluents of Fig. 2. As regards the bile acid conjugates, some of them were identified with the two-dimensional technique using the eluents suitable for glyco- and tauro-conjugates. The chromatogram of Fig. 4 demonstrates the presence of taurohyodeoxycholic, taurochenodeoxycholic, glycocholic, glycodeoxycholic and two other unidentified compounds which may probably be included, owing to their position, in the glyco-conjugated group. In the chicken bile the following conjugated acids were identified: glycodeoxycholic, glycochenodeoxycholic, glycocholic, taurocholic, taurohyodeoxycholic and one unidentified acid (probably a glyco-conjugated).

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