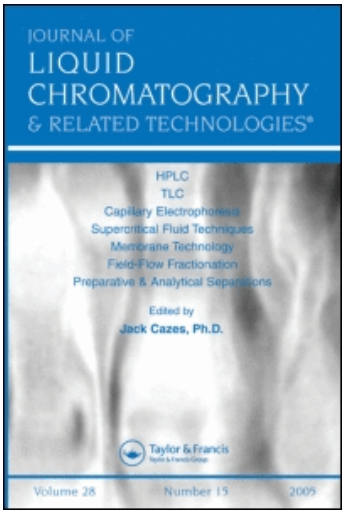

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Separation of Selected Bile Acids by TLC. VI. Separation on Cyano- and Diol-Modified Silica Layers

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Separation of Selected Bile Acids by TLC. VI. Separation on Cyano- and Diol-Modified Silica Layers

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Abstract: Seven selected bile acids: cholic acid (C), glycocholic acid (GC), glycolithocholic acid (GLC), deoxycholic acid (DC), chenodeoxycholic acid (CDC), glycodeoxycholic acid (GDC), and lithocholic acid (LC) were separated on silica gel modified by cyano and diol groups, with the use of a mobile phase: *n*-hexane–ethyl acetate–acetic acid in different volume compositions, at 18°C. The estimation of separation was carried out on the basis of the separation factors values: ΔR_F and R_S. Almost all bile acids, except for CDC/DC, were completely separated on cyano-modified silica plates when a mobile phase *n*-hexane–ethyl acetate–acetic acid in the volume composition 49:49:2 was used; whereas, the optimal separation for all examined bile acids was obtained on diol plates, using a mobile phase *n*-hexane–ethyl acetate–acetic acid in the volume composition 42:42:16.

Keywords: Bile acids, Adsorption HPTLC, CNF_{254} and $DiolF_{254}$-modified silica layers

INTRODUCTION

The division of adsorbents into the polar and non polar ones is rather relative ground for examining the adsorption forces in specified chromatographic systems. In TLC, the forces of adsorption interactions depend on the following parameters: type and number of function groups of the adsorbent

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and its specific surface area, chemical structure of the separated substances, type of used mobile phase and its interaction with the adsorbent.

In the optimization of chromatographic systems, attention is paid to the proper choice of a stationary phase. The adsorbents, which consist of gel matrix precoated with various kinds of chemically connected ligands, e.g., containing the following groups: cyano, diol, and amino,[1] belong to a very important group of polar stationary phases. For example, anilines,[2] flavonoids, favones,[3] nitrosamines,[4] nucleotides,[5] pesticides,[6] phenols,[2,7] plant extracts,[8] and quinolones[9] were separated on cyano-modified silica layers. Conjugates,[10] phenolic acids,[11,12] phenols,[2] and plant extracts[13] were separated on diol-modified silica layers.

In our previous papers,[14–16] we presented the data which allowed us to estimate the usefulness of the examined mobile phases, (*n*-heptane–ethyl acetate–acetic acid and *n*-hexane–ethyl acetate–acetic acid) in different volume compositions, for the separation of all bile acids neighboring pairs, i.e.: cholic acid (C), glycocholic acid (GC), glycolithocholic acid (GLC), deoxycholic acid (DC), chenodeoxycholic acid (CDC), glycodeoxycholic acid, (GDC) and lithocholic acid (LC), on different plates precoated with silica gel 60 and silica gel 60 and Kieselguhr F_{254} mixture. The estimation of examined mobile phases was carried out on the basis of the separation factor values: ΔR_F and R_S. When $\Delta R_F \geq 0.05$ and $R_S > 1$ were obtained for each pair of neighboring bile acids, the used mobile phases were considered the most useful. We also described the chromatographic conditions for the two-dimensional technique of bile acids separation.[15] The similarity analysis was applied to compare the separations of studied bile acids.[17] The similarity analysis showed that the biggest problem was to separate glycocholic acid from glycodeoxycholic acid on the plates precoated with silica gel. In the case of the separation on silica gel 60 and Kieselguhr F_{254} mixture, the biggest problem was to separate cholic acid from glycolithocholic acid. We also determined[18] the influence of temperature (40°C) on the separation of studied bile acids.

The aim of this work was to apply the diol and cyano-modified silica gel and the mobile phase *n*-hexane–ethyl acetate–acetic acid in different volume compositions to separate the studied bile acids.

EXPERIMENTAL

The following components of a mobile phase: *n*-hexane (Merck, Germany), ethyl acetate (POCh, Gliwice, Poland), acetic acid 99.5% (POCh, Gliwice, Poland), distilled water (Department of Analytical Chemistry, Sosnowiec, Poland), and ethanol (POCh, Gliwice, Poland) were used for TLC analysis. The commercial samples of C, DC, CDC, LC, GLC, GDC and GC (St. Louis, Sigma Company, USA) were used as test solutes. Methanol

(POCh, Gliwice, Poland; pure p. a.) was used for the preparation of bile acids solutions. Phosphomolibdic acid (POCh, Gliwice, Poland) was used to prepare a visualizing reagent.

Sample Preparation

The methanolic solutions of the above–mentioned bile acids in concentration 50 mg/10 mL of each acid were prepared.

HPTLC was performed on glass plates precoated with Diol F_{254} (E. Merck, #1.05636) and CNF_{254} (E. Merck, #1.12571) modified silica layers. Before use, the plates were activated at 120°C for 30 min. Micropipettes (Camag, Switzerland) were used to apply the standard solutions to the plates. Solutions of the standard acids were spotted on a chromatographic plate in quantities of 5 μg of each standard acid in 1 μL methanol. The development distance was 8.5 cm. The chromatographic plates were developed by using a mobile phase: *n*-hexane–ethyl acetate–acetic acid in the following volume compositions: a) 49:49:2 (v/v/v), 47.5:47.5:5 (v/v/v), and 37.5:37.5:25 (v/v/v) in the case of CNF_{254} plates (#1.12571), b) 48.7:48.7:2.6 (v/v/v), 47.5 : 47.5 : 5 (v/v/v), 45:45:10 (v/v/v), 42:42:16 (v/v/v), 40:40:20 (v/v/v), and 37.5:37.5:25 (v/v/v) in the case of $DiolF_{254}$ plates (#1.05636). The chromatograms were run in triplicate.

The spots were visualized by dipping HP-TLC plates in 10% ethanol solution of phosphomolibdic acid and then heating them for 15 minutes at 120°C.

From the obtained chromatograms, the values of R_F, ΔR_F, and R_S for studied bile acids were calculated according to the formulas, which were presented in our previous papers.[14–16]

RESULTS AND DISCUSSION

The studied bile acids were separated on cyano-modified silica gel plates using a mobile phase: *n*-hexane-ethyl acetate-acetic acid in the following volume compositions: 49:49:2, 47.5:47.5:5, and 37.5:37.5:25. The R_F, ΔR_F, and R_S values for examined bile acids obtained on CNF_{254} plates at 18°C are presented in Table 1. The influence of volume composition of the a/m mobile phase on the R_F values of separated bile acids is presented in Fig. 1.

Different influences of acetic acid content [%] of a mobile phase on the R_F values of separated bile acids (Fig. 1, Table 1) can be observed. The weakest influence was observed for lithocholic acid (LC), deoxycholic acid (DC), and chenodeoxycholic acid (CDC). The influence increased for C and GLC, and it was the largest for GDC and GC. When the a/m mobile phase in

Table 1. The R_F values and separation factors ΔR_F and R_S of bile acids examined on cyano-modified silica gel CNF_{254} (#1.12571) and developed by using mobile phase: *n*-hexane–ethyl acetate–acetic acid in different volume compositions at 18°C

Pair of acids	*n*-Hexane–ethyl acetate–acetic acid; v/v/v						Pair of acids	*n*-Hexane–ethyl acetate–acetic acid; v/v/v		
	49:49:2			47.5:47.5:5				37.5:37.5:25		
	R_F	ΔR_F	R_S	R_F	ΔR_F	R_S		R_F	ΔR_F	R_S
GC/GDC	0.06/0.38	0.32	7.71	0.26/0.69	0.43	5.20	GC/GLC	0.76/0.85	0.09	2.12
GDC/C	0.38/0.64	0.26	4.00	0.69/0.84	0.15	2.15	GLC/GDC	0.85/0.88	0.03	0.62
C/GLC	0.64/0.76	0.12	2.00	0.84/0.89	0.05	0.90	GDC/C	0.88/0.89	0.01	0.29
GLC/CDC	0.76/0.88	0.12	2.50	0.89/0.92	0.03	0.56	C/CDC	0.89/0.92	0.03	2.00
CDC/DC	0.88/0.91	0.03	0.67	0.92/0.93	0.01	0.29	CDC/DC	0.92/0.93	0.01	1.00
DC/LC	0.91/0.99	0.08	3.11	0.93/0.97	0.04	2.00	DC/LC	0.93/0.96	0.03	0.80

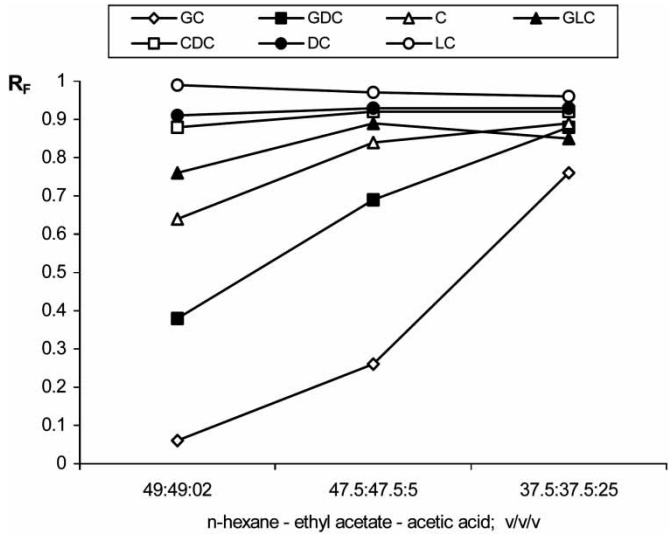

Figure 1. The dependence between R_F values of examined bile acids and the volume composition of mobile phase: *n*-hexane–ethyl acetate–acetic acid on cyano-modified silica gel plates (#1.12571).

volume composition 37.5:37.5:25 is used, GC separates very well from GLC ($\Delta R_{F(GC/GLC)} = 0.09$, $R_{S(GC/GLC)} = 2.12$). However, weak separation was observed for other pairs of bile acids. When the mobile phase *n*-hexane–ethyl acetate–acetic acid is applied in the following volume compositions: 49:49:2 and 47.5:47.5:5, the adsorption of studied bile acids increases in the order: LC, DC, CDC, GLC, C, GDC, and GC. The mobile phase in the volume composition 47.5:47.5:5 allowed separation of the following pairs of neighboring bile acids: GC/GDC, GDC/C, and DC/LC, while the mobile phase in the volume composition 49:49:2 separates almost all pairs of bile acids, except for CDC/DC (Fig. 2). Thus, it can be concluded that the application of the mobile phase *n*-hexane–ethyl acetate–acetic acid on cyano-modified silica gel plates hinders the separation of CDC from DC. However, these conditions facilitate the separation of GC from GDC when compared to their separations on non-modified silica gel and a mixture of silica gel 60 and Kieselguhr F_{254}.[16]

The R_F, ΔR_F, and R_S values for studied bile acids, which were obtained on DiolF_{254}-modified silica gel plates at 18°C are listed in Table 2. Fig. 3 presents the dependence between the R_F value of studied bile acids and the volume composition of a mobile phase *n*-hexane–ethyl acetate–acetic acid for the bile acids separated on Diol F_{254} plates (#1.05636). Different influence of the mobile phase volume composition on the change of separated bile acids R_F values was observed. The adsorption of examined bile acids on diol

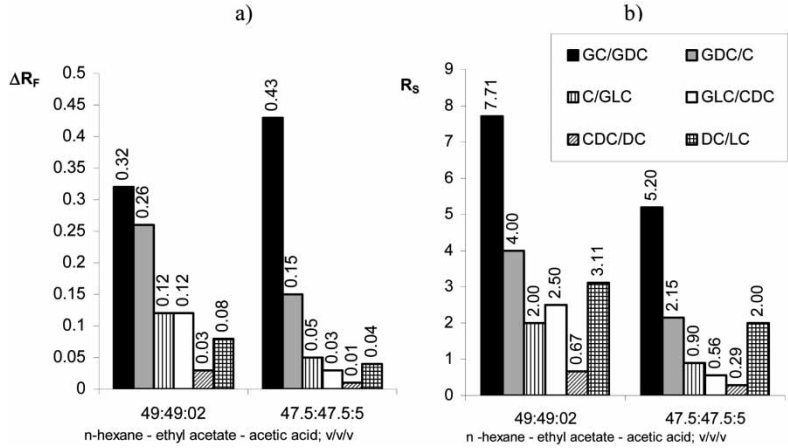

Figure 2. The separation factors ΔR_F (a) and R_S (b) for particular pairs of neighboring bile acids separated on CNF_{254} plates (#1.12571) by using: *n*-hexane–ethyl acetate–acetic acid mobile phase in the volume compositions: 49:49:2 and 47.5:47.5:5.

plates, using the mobile phase: *n*-hexane–ethyl acetate–acetic acid (regardless of the applied volume composition), increases in the same direction as when the plates precoated with silica gel are used,[14–16] i.e., LC, DC, CDC, GLC, C, GDC, and GC. ΔR_F larger than 0.05 and R_S larger than 1 were obtained for the following pairs of neighboring bile acids: glycocholic (GC) and glycodeoxycholic (GDC) by using mobile phases in volume compositions: 45:45:10, 42:42:16, 40:40:20, and 37.5:37.5:25 (v/v/v), glycodeoxycholic (GDC) and cholic (C) by using mobile phase in volume compositions: 47.5:47.5:5, 45:45:10, and 42:42:16 (v/v/v), cholic (C) and glycolithocholic (GLC) by using all mobile phases, glycolithocholic (GLC) and chenodeoxycholic (CDC) by using almost all mobile phases, except for 48.7:48.7:2.6 and 45:45:10 (v/v/v), chenodeoxycholic (CDC) and deoxycholic (DC) by using only the mobile phases: 45:45:10 and 42:42:16 (v/v/v), deoxycholic (DC) and lithocholic (LC) by using all mobile phases.

Thus, we can conclude that by using the mobile phase in the volume composition 42:42:16, the optimal separation of all examined bile acids was obtained ($\Delta R_F \geq 0.05$ and $R_S > 1$ for all pairs of neighboring bile acids). When the mobile phase in the volume composition 45:45:10 was applied, very good separations were obtained for almost all examined bile acids, except for GLC and CDC ($\Delta R_{F(GLC/CDC)} = 0.08$ and $R_{S(GLC/CDC)} = 1.00$) (Fig. 4).

The mutual interactions in the system: stationary phase–mobile phase–separated compounds affect the separated substance retention. When cyano plates were used as a stationary phase, the retention properties of

Table 2. The R_F values and separation factors ΔR_F and R_S of bile acids examined on diol-modified silica gel $DiolF_{254}$ (#1.05636) and developed by using mobile phase: *n*-hexane–ethyl acetate–acetic acid in different volume compositions at 18°C

	Pair of acids					
	GC/GDC	GDC/C	C/GLC	GLC/CDC	CDC/DC	DC/LC
n-Hexane–ethyl acetate–acetic acid; v/v/v						
48.7:48.7:2.6						
R_F	0.02/0.04	0.04/0.07	0.07/0.21	0.21/0.27	0.27/0.32	0.32/0.69
ΔR_F	0.02	0.03	0.14	0.06	0.05	0.37
R_S	0.75	1.20	2.30	0.92	0.82	5.73
47.5:47.5:5						
R_F	0.02/0.08	0.08/0.16	0.16/0.42	0.42/0.52	0.52/0.59	0.59/0.90
ΔR_F	0.06	0.08	0.26	0.10	0.07	0.31
R_S	0.95	1.08	3.00	1.10	0.80	5.20
45:45:10						
R_F	0.02/0.12	0.12/0.20	0.20/0.54	0.54/0.62	0.62/0.71	0.71/0.94
ΔR_F	0.10	0.08	0.34	0.08	0.09	0.23
R_S	1.52	1.04	4.38	1.00	1.07	4.71
42:42:16						
R_F	0.07/0.39	0.39/0.49	0.49/0.74	0.74/0.82	0.82/0.87	0.87/0.96
ΔR_F	0.32	0.10	0.25	0.08	0.05	0.09
R_S	5.68	1.80	4.67	2.00	1.33	3.56
40:40:20						
R_F	0.14/0.47	0.47/0.55	0.55/0.74	0.74/0.82	0.82/0.86	0.86/0.95
ΔR_F	0.33	0.08	0.19	0.08	0.04	0.09
R_S	4.22	0.97	2.91	1.40	0.52	2.00
37.5:37.5:25						
R_F	0.14/0.49	0.49/0.54	0.54/0.81	0.81/0.88	0.88/0.90	0.90/0.98
ΔR_F	0.35	0.05	0.27	0.07	0.02	0.08
R_S	4.29	0.50	3.29	1.08	0.27	1.44

the examined bile acids became similar due to the increase in acetic acid concentration in a mobile phase, which resulted in poorer bile acids separation. On the CNF_{254} plates and 2% acetic acid content in a mobile phase, the distance of the investigated bile acids separation is the longest ($\Delta R_{F(LC/GC)} = 0.85$). When the content of acetic acid in a mobile phase is 5%, the separation distance of bile acids is shorter ($\Delta R_{F(LC/GC)} = 0.67$). The shortest distance is obtained ($\Delta R_{F(LC/GC)} = 0.17$) at 25% acetic acid content in a mobile phase; the R_F values of the examined bile acids are similar ($0.76 \leq R_F \leq 0.96$) when the highest content of acetic acid is applied. Under a/m conditions, the complete separation of bile acids is not possible

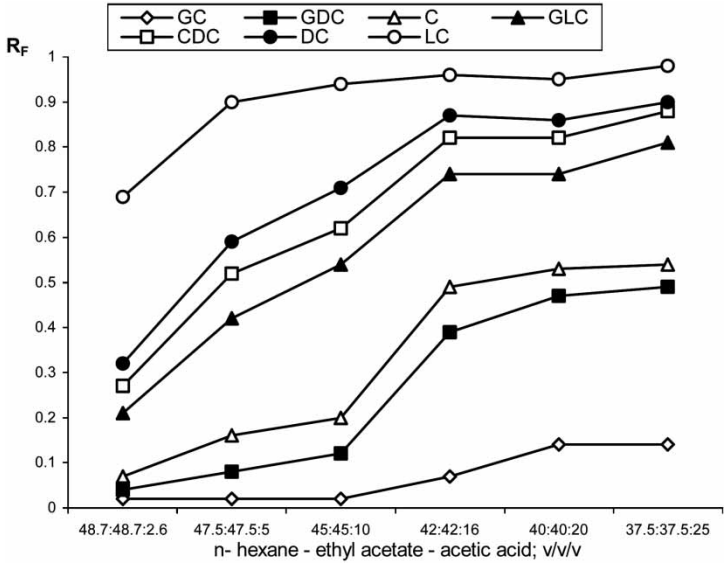

Figure 3. The dependence between R_F values of examined bile acids and the volume composition of mobile phase: *n*-hexane–ethyl acetate–acetic acid separated on diol-modified silica gel plates (#1.05636).

and it is worse in comparison to the separations in which lower content of acetic acid was used in a mobile phase.

However, the bile acids separation improves when diol plates are applied and the acetic acid content in a mobile phase increases to 16%. Then, the

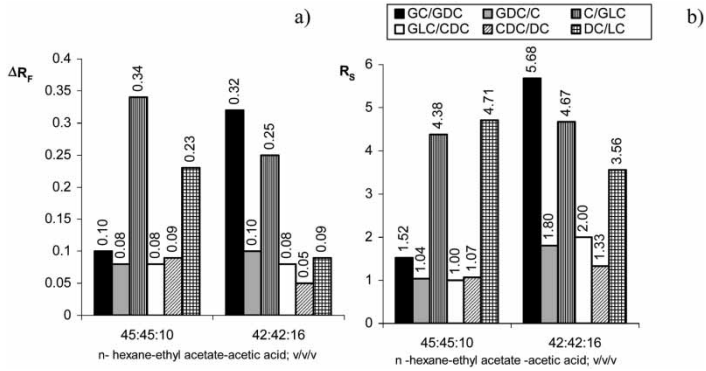

Figure 4. The separation factors ΔR_F (a) and R_S (b) for particular pairs of neighboring bile acids separated on $DiolF_{254}$ plates (#1.05636) by using: *n*-hexane–ethyl acetate–acetic acid mobile phase in the volume compositions: 45:45:10 and 42:42:16.

examined bile acids separation distance is the longest ($\Delta R_{F(LC/GC)} = 0.80$). While, further increase in acetic acid content in a mobile phase hinders the separation of bile acids.

Further investigations of bile acids separation are continued and they concern the separation by HP-RPTLC technique and adsorption TLC on silica gel impregnated by salts of inorganic acids.

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