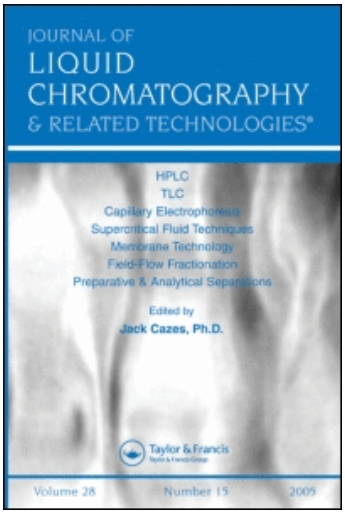

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Separation of Selected Bile Acids by TLC. V. Influence of Temperature on the Separation

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Abstract: Adsorption thin-layer chromatography (TLC) was used to study the influence of temperature, 18°C and 40°C, on retention and separation of selected bile acids, that is, cholic acid (C), glycocholic acid (GC), glycolithocholic acid (GLC), deoxycholic acid (DC), chenodeoxycholic acid (CDC), glycodeoxycholic acid (GDC), and lithocholic acid (LC). The mixture of *n*-hexane–ethyl acetate–acetic acid, in various volume compositions, was used as a mobile phase. Chromatographic experiments were performed on the following stationary phases: glass plates precoated with silica gel $60F_{254}$ without concentrating zone (#1.05715); glass plates precoated with silica gel $60F_{254}$ with concentrating zone (#1.11798); aluminum plates precoated with silica gel $60F_{254}$ (#1.05554), and silica gel 60 (#1.05553), and also on aluminum plates precoated with the mixture of silica gel 60 F_{254} and Kieselguhr F_{254} (#1.05567). It was proven that the temperature of 40°C improved the separation of GC from GDC performed on aluminum plates precoated with silica gel 60 (#1.05715, #1.05554, and #1.05553). However, when this temperature is used, the separation of CDC from DC poses the biggest problem. The obtained results indicate that the separation of some bile acids can be improved by proper choice of temperature. The temperature of 40°C causes the change of bile acids elution order.

Keywords: Bile acids, Adsorption TLC, Temperature of separation

INTRODUCTION

Both the separation and the quantification of individual bile acids from biological samples are very important for diagnosing lipids metabolism and disorders of the gastro-intestinal tract.[1]

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Thin-layer chromatography (TLC) is one of the most attractive methods of bile acids separation and their identification in biological samples. Separation of bile acids with the use of TLC depends on various parameters, for example, ionic strength, pH of a mobile phase, the applied eluent, and temperature. The influence of temperature on bile acids separation has been rarely studied, especially when TLC was used.[2,3] Nonetheless, the work of Rivas-Nass et al.[2] and Zarzycki et al.[3] who investigated the influence of temperature on retention and separation of bile acids using TLC, can be an example of such a study.

Rivas-Nass et al. reported that separation of selected bile acids (using TLC and HPTLC plates) could be improved by varying the temperature, especially in the subambient region (temperature range to -20°C was studied).[2,3] Zarzycki et al. determined the influence of temperature (from 5°C to 60°C) on retention and separation of cholesterol and selected bile acids using reversed-phase TLC. They found that the degree of separation of the examined bile acids in a high region could be increased owing to improving the efficiency of the chromatographic system.

In our previous papers, we presented the data that allowed to estimate the usefulness of the examined mobile phases (*n*-hexane–ethyl acetate–acetic acid in different volume compositions) for the separation of all pairs of neighboring bile acids, that is, 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, as well as silica gel and Kieselguhr mixture at 18°C.[4–6] The estimation of separations was carried out on the basis of the separation factors values, ΔR_F and R_S. When $\Delta R_F \geq 0.05$ and $R_S > 1$ were obtained for all the pairs of neighboring bile acids, the mobile phases were considered the most useful. In this study, the data were a guideline for conducting investigations aimed at estimating the influence of temperature (40°C) on the studied bile acids separation, using mobile phases (*n*-hexane–ethyl acetate–acetic acid) only in such volume compositions for which $R_S \leq 1$ was obtained. When silica gel 60 and Kieselguhr mixture was incorporated, the investigations were limited to five mobile phases in the following volume compositions: 22:20:5, 22:22:5, 25:20:5, 25:20:2, and 25:20:8.

EXPERIMENTAL

Chemicals

The following components of the mobile phase *n*-hexane (Merck, Germany), ethyl acetate (POCh, Gliwice, Poland), acetic acid 99.5% (POCh, Gliwice, Poland), and distilled water (Department of Analytical Chemistry,

Faculty of Pharmacy, Sosnowiec, Poland) were used for the adsorption 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. Sulfuric acid, 95% (Chempur, Piekary Śląskie, Poland) was used to prepare a visualizing reagent.

Sample Preparation

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

Thin Layer Chromatography

TLC adsorption was performed on 20 × 20 cm aluminum plates precoated with silica gel 60 (E. Merck, #1.05553), silica gel $60F_{254}$ (E. Merck, #1.05554), mixture of silica gel 60 and Kieselguhr F_{254} (E. Merck, #1.05567) as well as on glass plates precoated with silica gel $60F_{254}$ (E. Merck, #1.05715) and silica gel $60F_{254}$ with concentrating zone (E. Merck, #1.11798). 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 standard acids were spotted on a chromatographic plate in quantities of 15 μg of each standard acid in 3 μL methanol. A mobile phase of 50 mL was placed into a classical chamber (Camag). Next, the chamber was saturated with the vapor of mobile phase at 40°C for 120 min (in the incubator). Then the chromatographic plates were developed at 40°C. The development distance was 14 cm.

The chromatographic plates were developed by using a mobile phase comprised of n-hexane–ethyl acetate–acetic acid in the following volume proportions:

22:20:5, 25:20:2, 25:20:5, and 25:20:8 for glass plates precoated with silica gel $60F_{254}$ (#1.05715);

22:20:5, 22:22:5, and 25:20:2 for aluminum plates precoated with silica gel $60F_{254}$ (#1.05554);

22:20:5, 25:20:2, and 25:20:5 for aluminum plates precoated with silica gel 60 (#1.05553);

25:20:2 and 25:20:8 for glass plates precoated with silica gel $60F_{254}$ with concentrating zone (#1.11798);

22:22:5, 22:20:5, 25:20:2, 25:20:5, and 25:20:8 for aluminum plates precoated with silica gel $60F_{254}$ and Kieselguhr F_{254} mixture (#1.05567).

The plates were dried at room temperature using a fume cupboard. The investigated bile acids were detected on the plates using 10% solution of sulfuric acid in water as visualizing reagent. The spots were developed by heating the sprayed plates at 120°C for 20 min.

RESULTS AND DISCUSSION

In this study, we attempted to determine the influence of temperature on bile acids separation using adsorption TLC on glass plates precoated with silica gel $60F_{254}$ without concentrating zone (#1.05715) and with concentrating zone (#1.11798); aluminum plates precoated with silica gel $60F_{254}$ (#1.05554) and silica gel 60 (#1.05553); aluminum plates precoated with the mixture of silica gel $60F_{254}$ and Kieselguhr F_{254} (#1.05567). *n*-Hexane–ethyl acetate–acetic acid was used as a mobile phase in the volume compositions for which the complete separation of studied bile acids was not obtained at 18°C. The chromatographic plates were developed with the use of the a/m mobile phases at 40°C. The results were compared with the ones obtained in our previous investigations conducted at 18°C.[4–6]

Figures 1 and 2 present model variations in R_F values of the bile acids separated on chromatographic plates precoated with silica gel $60F_{254}$ (# 1.05715, and #1.05554) at 18°C and 40°C, developed by using *n*-hexane–ethyl acetate–acetic acid as a mobile phase in respective volume

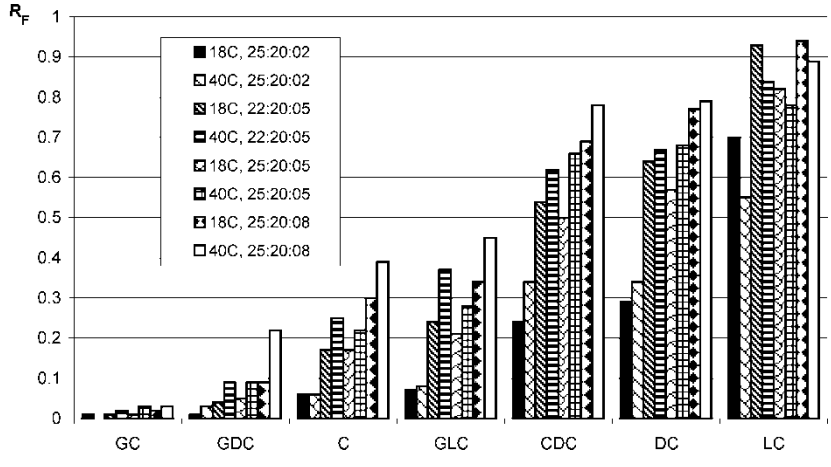

Figure 1. Comparison of R_F values for bile acids separated on glass plates precoated with silica gel $60F_{254}$ (#1.05715) at 18°C and 40°C by using mobile phase *n*-hexane–ethyl acetate–acetic acid in the following volume compositions: 25:20:2, 22:20:5, 25:20:5, and 25:20:8.

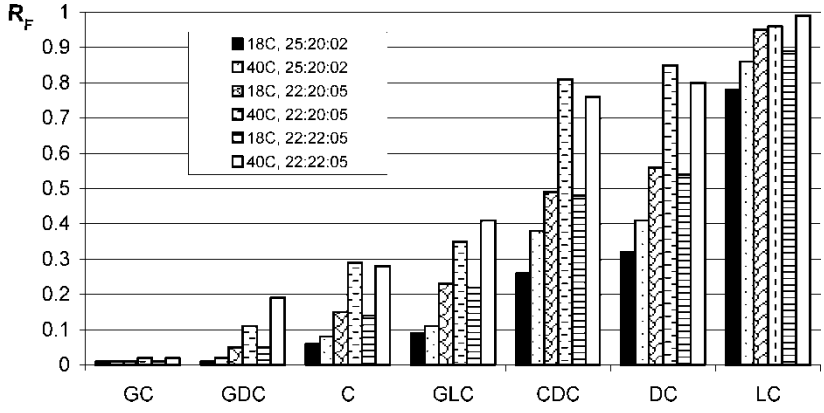

Figure 2. Comparison of R_F values for bile acids separated on aluminum plates precoated with silica gel $60F_{254}$ (#1.05554) at 18°C and 40°C by using mobile phase *n*-hexane–ethyl acetate–acetic acid in the following volume compositions: 25:20:2, 22:20:5, and 22:22:5.

compositions. Diagrams obtained for other chromatographic adsorbents (#1.05553, #1.11798, and #1.05567) were analogical to the ones presented in Figure 2. Generally, the temperature 40°C was proven to increase the R_F values of all examined bile acids when compared with their R_F values obtained at 18°C. Lithocholic acid (LC), investigated on glass plates precoated with silica gel $60F_{254}$ (#1.05715), is an exception since its R_F values are lower at 40°C than the ones obtained at 18°C. At 40°C, the mobile phase, *n*-hexane–ethyl acetate–acetic acid in volume composition 25:20:2, does not improve the separation of examined bile acids on plates precoated with silica gel without concentrating zone (#1.05715, #1.05554, and #1.05553) and silica gel and Kieselguhr mixture (#1.05567).

In accordance with previously used equations, [4–6] the values of separation factors ΔR_F and R_S were calculated for the examined bile acids developed on each chromatographic adsorbent, using the mobile phase: *n*-hexane–ethyl acetate–acetic acid in various volume compositions at 40°C. The obtained data are presented in Tables 1–5.

The development of examined bile acids at 18°C on glass plates precoated with silica gel $60F_{254}$ (#1.05715), using *n*-hexane–ethyl acetate–acetic acid in a volume composition 25:20:5; v/v/v, leads to complete separation of almost all neighboring pairs of the studied bile acids, except for the following pairs: C and GLC ($\Delta R_{F(C/GLC)} = 0.04$ and $R_{S(C/GLC)} = 0.96$) and GC and GDC ($\Delta R_{F(GC/GDC)} = 0.04$ and $R_{S(GC/GDC)} = 1.44$). The temperature of 40°C improves the separation of both the pairs ($\Delta R_{F(C/GLC)} = 0.06$ and $R_{S(C/GLC)} = 1.24$, as well as $\Delta R_{F(GC/GDC)} = 0.06$ and $R_{S(GC/GDC)} = 1.63$). However, at 40°C, CDC and DC, for which $\Delta R_{F(CDC/DC)} = 0.02$ and

Table 1. The values of separation factors ΔR_F and R_S for examined bile acids separated on glass plates precoated with silica gel $60F_{254}$ (#1.05715) by using mobile phase *n*-hexane–ethyl acetate–acetic acid in different volume compositions at 40°C

	n-Hexane–ethyl acetate–acetic acid (v/v/v)							
	25:20:2		25:20:5		25:20:8		22:20:5	
Pair of acids	ΔR_F	R_S	ΔR_F	R_S	ΔR_F	R_S	ΔR_F	R_S
GC/GDC	0.03	0.73	0.06	1.63	0.19	3.71	0.07	1.82
GDC/C	0.03	0.67	0.13	2.20	0.17	2.88	0.16	4.89
C/GLC	0.02	0.64	0.06	1.24	0.06	1.00	0.12	2.46
GLC/CDC	0.26	4.90	0.38	7.03	0.33	5.75	0.25	4.00
CDC/DC	0.00	0.00	0.02	0.44	0.01	0.08	0.05	0.90
DC/LC	0.21	5.22	0.10	3.25	0.10	3.11	0.17	4.00

$R_{S(CDC/DC)} = 0.44$ were obtained, do not separate well (Figures 3 and 4). On the same plates and using the mobile phase in a volume composition 22:20:5 at 18°C, GC separates poorly from GDC ($\Delta R_{F(GC/GDC)} = 0.03$ and $R_{S(GC/GDC)} = 1.00$). The separation of these bile acids pairs improves at 40°C ($\Delta R_{F(GC/GDC)} = 0.07$ and $R_{S(GC/GDC)} = 1.82$), but the separation of CDC and DC deteriorates ($\Delta R_{F(CDC/DC)} = 0.05$ and $R_{S(CDC/DC)} = 0.90$). At 18°C and the mobile phase 25:20:8; v/v/v, C separates poorly from GLC ($\Delta R_{F(C/GLC)} = 0.03$ and $R_{S(C/GLC)} = 1.00$). The development of plates at 40°C does not improve the separation of these acids ($\Delta R_{FC/GLC)} = 0.06$ and $R_{S(C/GLC)} = 1.00$). Furthermore, it makes the separation of CDC and DC

Table 2. The values of separation factors ΔR_F and R_S of examined bile acids separated on aluminum plates precoated with silica gel $60F_{254}$ (#1.05554) by using mobile phase *n*-hexane–ethyl acetate–acetic acid in different volume compositions at 40°C

	n-Hexane–ethyl acetate–acetic acid (v/v/v)					
	25:20:2		22:20:5		22:22:5	
Pair of acids	ΔR_F	R_S	ΔR_F	R_S	ΔR_F	R_S
GC/GDC	0.01	0.33	0.09	1.93	0.08	1.63
GDC/C	0.06	1.10	0.18	3.03	0.18	2.74
C/GLC	0.03	0.53	0.06	0.90	0.13	1.90
GLC/CDC	0.27	4.71	0.46	5.91	0.35	5.10
CDC/DC	0.03	0.47	0.04	0.62	0.04	0.67
DC/LC	0.45	8.50	0.11	2.91	0.19	5.31

Table 3. The values of separation factors ΔR_F and R_S of examined bile acids separated on aluminum plates precoated with silica gel (#1.05553) by using mobile phase *n*-hexane–ethyl acetate–acetic acid in different volume compositions at 40°C

	n-Hexane–ethyl acetate–acetic acid (v/v/v)					
	25:20:2		25:20:5		22:20:5	
Pair of acids	ΔR_F	R_S	ΔR_F	R_S	ΔR_F	R_S
GC/GDC	0.02	0.67	0.11	2.14	0.08	1.57
GDC/C	0.06	1.15	0.17	2.88	0.16	2.59
C/GLC	0.03	0.58	0.04	0.57	0.09	1.37
GLC/CDC	0.34	5.71	0.42	6.40	0.40	5.78
CDC/DC	0.00	0.00	0.06	1.00	0.04	0.62
DC/LC	0.39	10.27	0.17	13.83	0.19	3.40

($\Delta R_{F(CDC/DC)} = 0.01$ and $R_{S(CDC/DC)} = 0.08$) worse. The last two acids separate very well at 18°C ($\Delta R_{F(CDC/DC)} = 0.09$ and $R_{S(CDC/DC)} = 1.71$).

The GC and GDC separate poorly ($\Delta R_{F(GC/GDC)} = 0.04$ and $R_{S(GC/GDC)} = 1.00$) on aluminum plates precoated with silica gel $60F_{254}$ (#1.05554), using mobile phases in the following volume compositions: 22:20:5 and 22:22:5, at 18°C. Increasing the temperature of the experiment to 40°C enables the separation of these bile acids. However, under such experimental conditions, DC and CDC do not separate completely and incorporating the mobile phase 22:20:5; v/v/v also makes the separation of C and GLC worse.

Table 4. The values of separation factors ΔR_F and R_S of examined bile acids separated on glass plates precoated with silica gel $60F_{254}$ (#1.11798) by using mobile phase *n*-hexane–ethyl acetate–acetic acid in different volume compositions at 40°C

	n-Hexane–ethyl acetate–acetic acid (v/v/v)			
	25:20:2		25:20:8	
Pair of acids	ΔR_F	R_S	ΔR_F	R_S
GC/GDC	0.02	3.50	0.17	6.43
GDC/C	0.10	3.71	0.15	3.17
C/GLC	0.05	1.40	0.04	0.96
GLC/CDC	0.28	6.61	0.38	8.87
CDC/DC	0.02	0.24	0.06	1.65
DC/LC	0.28	7.70	0.07	4.44

Table 5. The values of separation factors ΔR_F and R_S of examined bile acids separated on aluminum plates precoated with silica gel $60F_{254}$ and Kieselguhr F_{254} mixture (#1.05554) by using mobile phase *n*-hexane–ethyl acetate–acetic acid in different volume compositions at 40°C

Pair of acids	*n*-Hexane–ethyl acetate–acetic acid (v/v/v)									
	25:20:2		25:20:5		25:20:8		25:20:5		22:22:5	
	ΔR_F	R_S	ΔR_F	R_S	ΔR_F	R_S	ΔR_F	R_S	ΔR_F	R_S
GC/GDC	0.05	1.03	0.10	1.50	0.19	2.68	0.14	2.35	0.10	1.75
GDC/C	0.12	1.94	0.36	4.21	0.39	4.77	0.33	5.00	0.31	4.09
C/GLC	0.02	0.26	0.02	0.27	0.04	0.63	0.02	0.20	0.06	0.78
GLC/CDC	0.49	8.53	0.40	6.06	0.24	4.57	0.37	5.58	0.29	4.21
CDC/DC	0.02	0.36	0.07	1.36	0.05	1.13	0.10	1.71	0.06	1.12
DC/LC	0.28	9.53	0.00	0.00	0.00	0.00	0.00	0.00	0.13	2.22

The improvement of examined bile acids separation, in comparison with the separations obtained at 18°C, was not observed at 40°C on aluminum plates precoated with silica gel 60 (#1.05553) using mobile phases in the following volume compositions 25:20:2 and 25:20:5. The separation of GC and GDC ($\Delta R_{F(GC/GDC)} = 0.04$ and $R_{S(GC/GDC)} = 1.00$), which did not separate completely at 18°C, improved at 40°C ($\Delta R_{F(GC/GDC)} = 0.08$ and $R_{S(GC/GDC)} = 1.57$) using the mobile phase 22:20:5; v/v/v. However, the use of the mobile phase 22:20:5; v/v/v at 40°C made the separation of DC and CDC

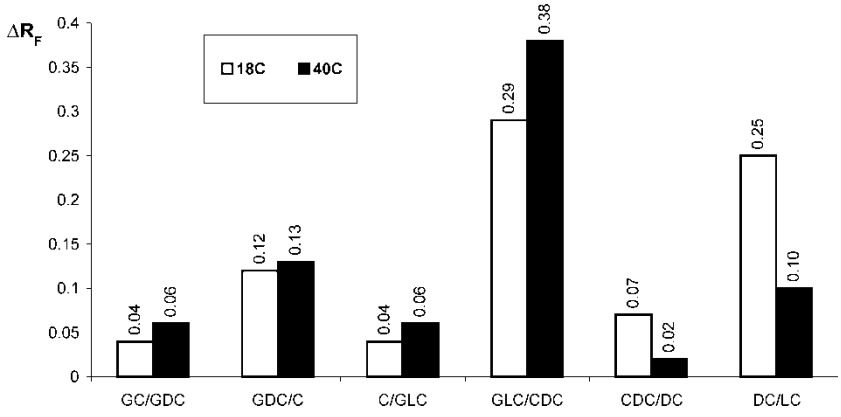

Figure 3. Comparison of ΔR_F values for bile acids separated on glass plates precoated with silica gel $60F_{254}$ (#1.05715) at 18°C and 40°C by using mobile phase *n*-hexane–ethyl acetate–acetic acid in volume composition 25:20:5.

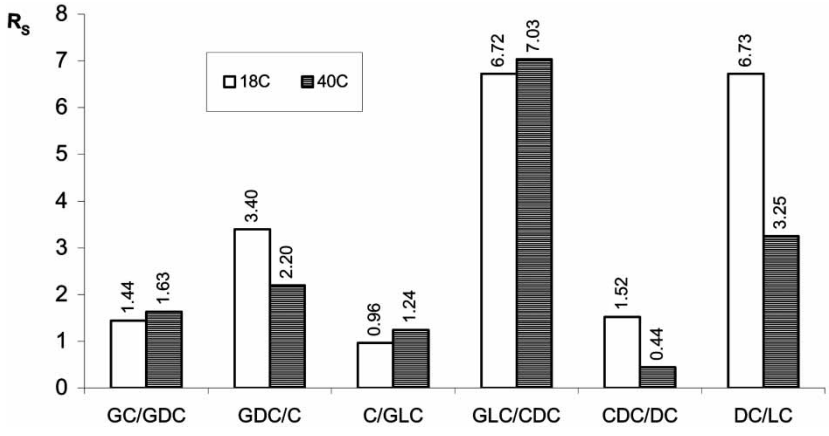

Figure 4. Comparison of R_S values for bile acids separated on glass plates precoated with silica gel $60F_{254}$ (#1.05715) at 18°C and 40°C by using mobile phase *n*-hexane–ethyl acetate–acetic acid in volume composition 25:20:5.

($\Delta R_{F(CDC/DC)} = 0.04$ and $R_{S(CDC/DC)} = 0.62$) worse, but which separated completely at 18°C ($\Delta R_{F(CDC/DC)} = 0.07$ and $R_{S(CDC/DC)} = 1.14$).

The following pairs: GC and GDC, as well as GLC and C do not separate on glass plates precoated with silica gel $60F_{254}$ with concentrating zone (#1.11798), using the mobile phase *n*-hexane–ethyl acetate–acetic acid in volume composition 25:20:2 at 18°C. The temperature of 40°C improves the separation of neighboring pairs of the following acids: GC from GDC ($\Delta R_{F(GC/GDC)} = 0.02$ and $R_{S(GC/GDC)} = 3.50$) and C from GLC ($\Delta R_{F(C/GLC)} = 0.05$ and $R_{S(C/GLC)} = 1.40$), but the separation of CDC from DC is poorer ($\Delta R_{F(CDC/DC)} = 0.02$ and $R_{S(CDC/DC)} = 0.24$). At 18°C and the mobile phase 25:20:8; v/v/v, CDC separates poorly from DC ($\Delta R_{F(CDC/DC)} = 0.03$ and $R_{S(CDC/DC)} = 0.95$). These acids separate completely at 40°C ($\Delta R_{F(CDC/DC)} = 0.06$ and $R_{S(CDC/DC)} = 1.65$). However, at 40°C the separation of C and GLC ($\Delta R_{F(C/GLC)} = 0.04$ and $R_{S(C/GLC)} = 0.96$) is poor in comparison with the separation of these acids at 18°C ($\Delta R_{F(C/GLC)} = 0.10$ and $R_{S(C/GLC)} = 2.42$).

On aluminum plates precoated with the mixture of silica gel 60 and Kieselguhr F_{254} (#1.05567), using mobile phases at volume compositions: 22:20:5; 25:20:5; 22:22:5, and 25:20:2 at 18°C, the GLC did not separate from C. Unfortunately, increasing the temperature to 40°C does not improve the separation of the a/m pairs of acids. The mobile phase 25:20:8; v/v/v at 40°C makes the separation of C from GLC ($\Delta R_{F(C/GLC)} = 0.04$ and $R_{S(C/GLC)} = 0.63$) worse in comparison with their separation at 18°C ($\Delta R_{F(GLC/C)} = 0.10$ and $R_{S(GLC/C)} = 2.44$). At 18°C and the mobile phase 22:20:5, the CDC does not separate completely from DC

($\Delta R_{F(CDC/DC)} = 0.07$ and $R_{S(CDC/DC)} = 1.00$). Their separation improves at 40°C ($\Delta R_{F(CDC/DC)} = 0.10$ and $R_{S(CDC/DC)} = 1.71$). However, DC does not separate from LC at 40°C, using the mobile phases 25:20:5, 25:20:8, and 22:20:5 (v/v/v). The pair of acids DC/LC separates at 18°C using the mobile phases 25:20:5 and 22:20:5 (v/v/v). In the case of the neighboring pair of GLC and C (developed by using the mobile phases at volume compositions: 25:20:2, 22:20:5, 25:20:5, and 25:20:8), it was observed that the increase in temperature inversed the order of these acids relative positions on the chromatogram in comparison with the temperature of 18°C, that is, the order is identical to other adsorbents order at 18°C and 40°C.

CONCLUSIONS

It was observed that temperature at 18°C and 40°C influences the effect of separation of selected bile acids. The right choice of temperature can improve the separation of some bile acids and it also causes the change of their relative positions on aluminum plates precoated with the mixture of silica gel 60 and Kieselguhr F_{254} (#1.05567). Generally, it can be stated that the temperature of 40°C improves the separation of GC from GDC on silica plates (#1.05715, #1.05554, and #1.05553). However, under the a/m conditions the problem concerning the separation of CDC from DC arises.

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