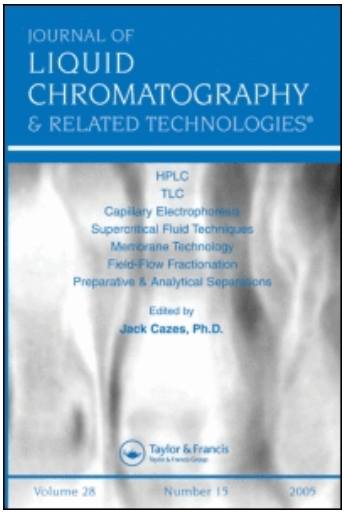

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Separation of Selected Bile Acids by TLC. III. Separation on Various Stationary Phases

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ABSTRACT

The aim of our study was to determine the optimum conditions of the separation of selected bile acids, such as cholic acid (C), glycocholic acid (GC), glycolithocholic acid (GLC), deoxycholic acid (DC), chenodeoxycholic acid (CDC), glycodeoxycholic acid (GDC), and lithocholic acid (LC) using thin-layer chromatography on aluminum plates which are precoated with silica gel 60 (E. Merck, #1.05553), silica gel 60F_{254} (E. Merck, #1.05554), a mixture of silica gel 60 and Kieselguhr F_{254} (E. Merck, #1.05567), as well as on glass plates which are precoated with silica gel 60F_{254} (E. Merck, #1.05715) and on glass plates precoated with silica gel 60F_{254} with a concentrating zone (E. Merck, #1.11798) using *n*-hexane–ethyl acetate–acetic acid in

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various volume compositions as mobile phases. The retardation factor (R_f), and separation factors ΔR_f and R_S of each pair of examined bile acids were obtained. All bile acids have been successfully separated only on glass and aluminum plates precoated with silica gel by developing them with *n*-hexane–ethyl acetate–acetic acid in the following volume compositions: 20 : 20 : 5 and 22 : 21 : 5 as the mobile phases.

Key Words: Bile acids; Adsorption TLC.

INTRODUCTION

Separation and quantification of bile acids (free, glycine, and taurine-conjugated acids) from biological materials are very important diagnostic indicators of liver and gastro-intestinal diseases in humans.[1] Because of their structural similarities, separation of bile acids and their metabolites is difficult. Routinely, a diagnostic enzymatic method is used because it is easy and quick to perform.

Besides enzymatic methods, bile acids are analyzed mainly with the use of electrochemical and chromatographic techniques.[1] Among these techniques, the most commonly used are chromatographic techniques, such as gas chromatography (GC),[2] high performance liquid chromatography (HPLC),[3–6] capillary electrophoresis (CE),[1] supercritical fluid chromatography,[1,7,8] and thin layer chromatography (TLC).[9–12] Chromatography represents the method of choice for detailed analyses of the bile acid profiles in biological materials. However, no single chromatographic method or detection technique is capable of providing the complete determination of the components of the mixture of naturally occurring bile acid.[1] Several analytical techniques for the analysis of bile acids should be used.

We have previously investigated the optimal conditions for the separation of selected bile acids, which were studied by TLC on aluminum plates precoated with silica gel $60F_{254}$ (E. Merck, #1.05554), with the use of a mixture of *n*-heptane–ethyl acetate–acetic acid in various volume compositions as mobile phase.[13] We have also described the chromatographic conditions of the two-dimensional technique for bile acids separation.[14]

The aim of our study was to work out the optimum conditions to separate selected bile acids, such as cholic acid (C), glycocholic acid (GC), glycolithocholic acid (GLC), deoxycholic acid (DC), chenodeoxycholic acid (CDC), glycodeoxycholic acid (GDC), and lithocholic acid (LC) by using adsorption TLC on silica gel, and on a mixture of silica gel and Kieselguhr.

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 above-mentioned bile acids at a concentration of 50 mg/10 mL of each acid were prepared.

TLC

Adsorption TLC was performed on 20 cm × 20 cm aluminum plates precoated with silica gel 60 (E. Merck, #1.05553), silica gel 60F_{254} (E. Merck, #1.05554), a 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 a 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 the standard acids were spotted on a chromatographic plate in quantities of 15 μg of each standard, in 3 μL methanol. The chromatograms were developed at room temperature in a horizontal chamber (Camag, Switzerland) using *n*-hexane–ethyl acetate–acetic acid in various volume compositions, 20 : 20 : 5, 22 : 20 : 5, 22 : 21 : 5, 22 : 22 : 5, 25 : 20 : 2, 25 : 20 : 5, and 25 : 20 : 8 as the mobile phases. Mobile phases (50 mL) were used in all cases. The development distance was 14 cm. The plates were dried at room temperature using a fume cupboard. The investigated bile acids were evaluated on the plates using 10% solution of sulfuric acid in water as a visualizing reagent. The spots were developed by heating the sprayed plates at 120°C for 20 min.

Densitometry

Densitometric measurements of examined bile acids were made by a densitometer (Desaga, Germany). Densitometric profiles of bile acids were obtained at wavelength $\lambda = 250\,\text{nm}$.

Separation Factors

The separation factors: ΔR_f and R_S characterize the possibility of bile acid separation by TLC.

ΔR_f is calculated according to the formula:

$$\Delta R_f = R_{f1} - R_{f2} \tag{1}$$

where R_{f1} and R_{f2} are the values of two adjacent spots; and $R_{f1} > R_f$. R_S is calculated with formula:[15]

$$R_S = 2 \times \frac{a}{b} \tag{2}$$

where a is a distance between the center of two adjacent spots (cm); b is a sum of widths of two spots in the direction of flow (cm).

RESULTS AND DISCUSSION

According to our previous investigations[13,16] the separation factors, such as ΔR_f and R_S are used to estimate chromatographic separations of studied bile acids. To obtain optimal mobile phase for the separation of bile acids on glass plates precoated with silica gel (#1.05715), differences between R_f of neighboring spots of examined bile acids on chromatograms were calculated. Figure 1 presents ΔR_f values of bile acids neighboring pairs, which were eluted by using the mixture of *n*-hexane–ethyl acetate–acetic acid in different volume compositions. Separation of each investigated pair of bile acids is satisfactory when $\Delta R_f \geq 0.05$ and $R_S > 1$. $\Delta R_f \geq 0.05$ were obtained for the following bile acids: GC and GDC acids using all mobile phases, except for mobile phase in volume compositions: 20 : 20 : 5, 22 : 22 : 5, and 25 : 20 : 2 (v/v/v); GDC and C acids using all mobile phases, except for mobile phases in volume compositions: 25 : 20 : 2 (v/v/v); C and GLC acids using only mobile phases in volume compositions: 20 : 20 : 5, 22 : 21 : 5, 22 : 20 : 5, and 22 : 22 : 5 (v/v/v); GLC and CDC acids using all mobile phases; CDC and DC acids using mobile phases; DC and LC acids using all mobile phases.

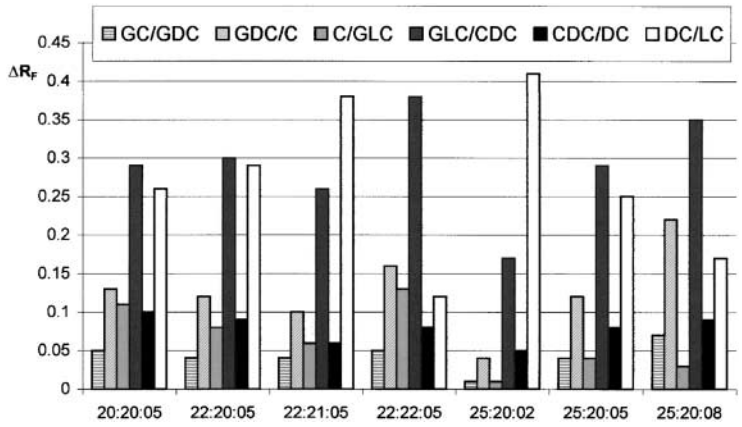

Figure 1. The ΔR_f values for particular pairs of neighboring bile acids separated on glass plates precoated with silica gel $60F_{254}$ (#1.05715) by using: *n*-hexane–ethyl acetate–acetic acid mobile phase in different volume compositions.

Using the mobile phase in volume compositions: 20 : 20 : 5 and 22 : 22 : 5, respectively, and glass plates precoated with silica gel $60F_{254}$ (#1.05715), $\Delta R_f \geq 0.05$ was obtained for all the pairs of examined bile acids. Figure 2 presents the separation factor R_S values for neighboring pairs of separated bile acids on chromatograms.

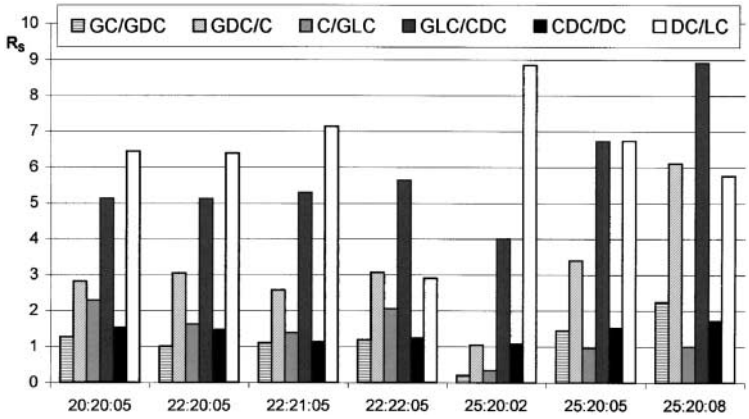

Figure 2. The R_S values for particular pairs of neighboring bile acids separated on glass plates precoated with silica gel $60F_{254}$ (#1.05715) by using: *n*-hexane–ethyl acetate–acetic acid mobile phase in different volume compositions.

From the data presented in Fig. 1, it can be observed that for mobile phases: *n*-hexane–ethyl acetate–acetic acid in volume compositions: 20 : 20 : 5 and 22 : 22 : 5 (v/v/v), respectively, the values of separation factors R_S for the studied bile acids pairs are larger than 1. The mobile phase in volume composition 22 : 21 : 5 was the most efficient one. Using this phase, $R_S > 1$ was obtained in spite of fact that the difference between R_f of glycocholic acid and glycolithocholic acids was only 0.04.

Table 1 presents the data, which estimates the examined mobile phases for the separation of all neighboring pairs of studied bile acids on particular chromatographic plates. The estimation of examined mobile phases usefulness for the separation of studied bile acids was obtained on the basis of ΔR_f and R_S values. The mobile phases, with which $\Delta R_f \geq 0.05$ and $R_S > 1$ were obtained for each pair of neighboring bile acids, were considered the most useful.

An optimum separation for other chromatographic adsorbents was obtained by using one-dimensional developing at 18°C and *n*-hexane–ethyl acetate–acetic acid mobile phases in the following volume compositions: 20 : 20 : 5, 22 : 21 : 5, 22 : 22 : 5, and 25 : 20 : 8 on aluminum plates precoated with silica gel 60 (#1.05553); 22 : 21 : 5 and 25 : 20 : 8 on aluminum plates precoated with silica gel $60F_{254}$ (#1.05554); 20 : 20 : 5 on glass plates precoated with silica gel $60F_{254}$ with concentrating zone (#1.11798).

Using mobile phase in volume compositions: 22 : 22 : 5, 22 : 21 : 5, 22 : 20 : 5, and 25 : 20 : 5, respectively, and glass plates precoated with silica gel $60F_{254}$ with concentrating zone, for all neighboring pairs of bile acids $R_S > 1$ was obtained. Under these conditions, $\Delta R_f \geq 0.05$ was obtained for all pairs of bile acids, except for GC and GDC acids, for which $0.03 < \Delta R_f < 0.05$ was obtained. It can be concluded, that $\Delta R_f \geq 0.03$ is sufficient to complete the separation of GC acid from GDC acid on glass plates precoated with silica gel $60F_{254}$ with concentrating zone (#1.11798). Similarly, using mobile phase in volume compositions 20 : 20 : 5 and 25 : 20 : 5, respectively, and aluminum plates precoated with silica gel $60F_{254}$ (#1.05554), as well as mobile phase in volume composition 22 : 21 : 5 (v/v/v) and glass plates precoated with silica gel $60F_{254}$ (#1.05715), R_S larger than 1 was obtained for all studied bile acids pairs, in spite of the fact that the difference between R_f values of GC and GDC acids was only 0.04.

Selected ΔR_F values for neighboring pairs of bile acids separated on glass plates precoated with silica gel $60F_{254}$ are presented in Table 2.

When aluminum plates precoated with the mixture of silica gel and Kieselguhr F_{254} (#1.05567) were used, the selection of mobile phases depended on the kind of bile acids which are used for separation. The mobile phase 25 : 20 : 8 (v/v/v) allows separation of all pairs of bile acids, with the exception of the pair of lithocholic and deoxycholic acids. The pair of LC and DC acids can be separated by using mobile phases in other volume compositions. In this case, the separation on silica gel 60 and

Table 1. Separation factors (ΔR_F and R_S) of selected bile acids separated with the mobile phase *n*-hexane–ethyl acetate–acetic acid in different volume compositions (v/v/v) on different stationary phases.

n-Hexane–ethyl acetate–acetic acid (v/v/v)	Chromatographic plates									
	1		2		3		4		5	
	$\Delta R_F \geq 0.05$	$R_S > 1$	$\Delta R_F \geq 0.05$	$R_S > 1$	$\Delta R_F \geq 0.05$	$R_S > 1$	$\Delta R_F \geq 0.05$	$R_S > 1$	$\Delta R_F \geq 0.05$	$R_S > 1$
20 : 20 : 5	+[a]	+	−	+	+	+	−	−	+	+
22 : 20 : 5	−[b]	−	−	−	−	−	−	−	−	+
22 : 21 : 5	−	+	+	+	+	+	−	−	−	+
22 : 22 : 5	+	+	−	−	+	+	−	−	−	+
25 : 20 : 2	−	−	−	−	−	−	−	−	−	−
25 : 20 : 5	−	−	−	+	+	−	−	−	−	+
25 : 20 : 8	−	−	+	+	+	+	−	−	−	−

Note: 1, Glass plates precoated with silica gel $60F_{254}$ (#1.05715); 2, aluminum plates precoated with silica gel $60F_{254}$ (#1.05554); 3, aluminum plates precoated with silica gel 60 (#1.05553); 4, aluminum plates precoated with the mixture of silica gel 60 and Kieselgur F_{254} (#1.05567); 5, glass plates precoated with silica gel $60F_{254}$ with concentrating zone (#1.11798).

[a]$\Delta R_F \geq 0.05$ or $R_S > 1$ for all investigated bile acids.

[b]$\Delta R_F \geq 0.05$ or $R_S > 1$ not for all investigated bile acids.

Table 2. The R_F values and separation factors (ΔR_F and R_S) of selected bile acids separated with the mobile phase *n*-hexane–ethyl acetate–acetic acid in different volume compositions (v/v/v) on silica gel.

Pair of acids	*n*-Hexane–ethyl acetate–acetic acid (v/v/v)											
	1 (20 : 20 : 5)			2 (20 : 20 : 5)			3 (25 : 20 : 8)			4 (25 : 20 : 8)		
	R_F	ΔR_F	R_S	R_F	ΔR_F	R_S	R_F	ΔR_F	R_S	R_F	ΔR_F	R_S
GC/GDC	0.01/0.06	0.05	1.27	0.02/0.07	0.05	4.33	0.02/0.09	0.07	2.50	0.03/0.13	0.10	3.11
GDC/C	0.06/0.19	0.13	2.82	0.07/0.19	0.12	5.67	0.09/0.27	0.18	2.54	0.13/0.34	0.21	5.04
C/GLC	0.19/0.30	0.11	2.30	0.19/0.34	0.15	4.42	0.27/0.36	0.09	2.36	0.34/0.41	0.08	1.91
GLC/CDC	0.30/0.59	0.29	5.12	0.34/0.62	0.28	5.38	0.36/0.66	0.30	6.83	0.41/0.79	0.37	9.45
CDC/DC	0.59/0.69	0.10	1.53	0.62/0.71	0.09	1.62	0.66/0.76	0.10	2.40	0.79/0.84	0.06	1.60
DC/LC	0.69/0.94	0.26	6.44	0.71/0.99	0.28	10.64	0.76/0.99	0.23	10.12	0.84/0.99	0.16	7.30

Note: 1, Glass plates precoated with silica gel $60F_{254}$ (#1.05715); 2, glass plates precoated with silica gel $60F_{254}$ with concentrating zone (#1.11798); 3, aluminum plates precoated with silica gel $60F_{254}$ (#1.05554); 4, aluminum plates precoated with silica gel 60 (#1.05553).

Table 3. The R_F values and separation factors (ΔR_F and R_S) of selected bile acids separated with the mobile phase *n*-hexane–ethyl acetate–acetic acid in different volume compositions (v/v/v) on mixture of silica gel 60 and Kieselgur F_{254} (#1.05567).

	n-Hexane–ethyl acetate–acetic acid (v/v/v)					
	25 : 20 : 5			25 : 20 : 8		
Pair of acids	R_F	ΔR_F	R_S	R_F	ΔR_F	R_S
GC/GDC	0.02/0.07	0.05	1.13	0.04/0.19	0.15	3.65
GDC/GLC	0.07/0.27	0.20	3.93	0.19/0.47	0.28	6.58
GLC/C	0.27/0.31	0.04	0.61	0.47/0.57	0.10	2.44
C/CDC	0.31/0.72	0.41	5.20	0.57/0.89	0.32	7.20
CDC/DC	0.72/0.80	0.08	1.19	0.89/0.99	0.11	2.13
DC/LC	0.80/0.99	0.19	7.10	0.99/0.99	0.00	0.00

Kieselguhr F_{254} mixture, the biggest problem was to separate glycolithocholic and cholic acids. Separation of these acids was possible only by using mobile phase in volume composition 25 : 20 : 8. Selected ΔR_f values of neighboring pairs of bile acids separated on aluminum plates precoated with the mixture of silica gel 60 and Kieselguhr F_{254} are presented in Table 3.

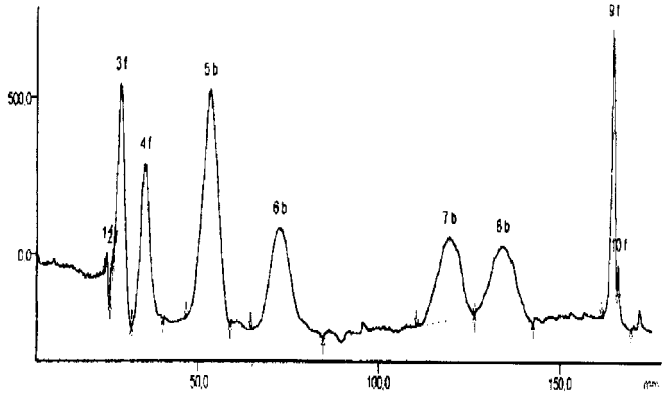

Figure 3. Densitometric profiles obtained for the investigated bile acids separated on glass plates precoated with silica gel with concentrating zone (E. Merck, #1.11798) using *n*-hexane–ethyl acetate–acetic acid in volume composition 20 : 20 : 5 as mobile phase where: 1f and 2f, pollution; 3f, glycocholic acid; 4f, glycodeoxycholic acid; 5b, cholic acid; 6b, glycolithocholic acid; 7b, chenodeoxycholic acid; 8b, deoxycholic acid; 9f, lithocholic acid; and 10f, front of the mobile phase.

Figure 3 presents densitogram of bile acids separated on glass plates precoated with silica gel $60F_{254}$ with concentrating zone (#1.11798) by using the mobile phase: *n*-hexane–ethyl acetate–acetic acid in volume composition 20 : 20 : 5.

The data presented in Table 1 can be a guideline for a further investigation aimed at the estimation of both the temperature and the impregnation of the inorganic acids salts of silica gel and the mixture of silica gel and Kieselguhr, to separate the studied bile acids, while using mobile phase: *n*-hexane–ethyl acetate–acetic acid only in the volume compositions in which $R_S \leq 1$ was obtained.

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