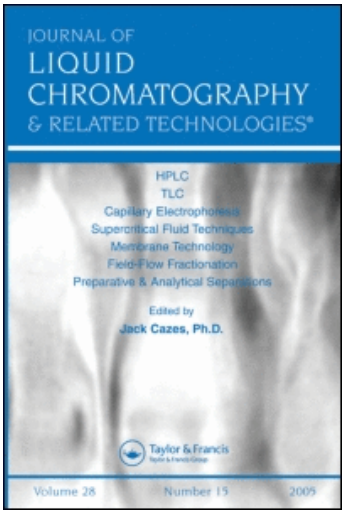

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Separation of Selected Bile Acids by TLC. VIII. Separation on Silica Gel $60F_{254}$ Glass Plates Impregnated with Cu(II), Ni(II), Fe(II), and Mn(II) Cations

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Abstract: The aim of our study was to examine the following bile acids: cholic acid (C), glycocholic acid (GC), glycolithocholic acid (GLC), deoxycholic acid (DC), chenodeoxycholic acid (CDC), glycodeoxycholic acid (GDC), and lithocholic acid (LC). In the present study, to separate a/m bile acids using adsorption thin layer chromatography at 18°C, the glass plates precoated with silica gel $60F_{254}$ (#1.05715), were impregnated with 1%, 2.5%, and 5% aqueous solutions of the following salts: $CuSO_4$, $MnSO_4$, $NiSO_4$, and $FeSO_4$. The mixtures of *n*-hexane–ethyl acetate–acetic acid in the volume compositions: 22:20:5; 25:20:2; 25:20:5, and 25:20:8 were used as mobile phases. These mobile phases were not effective for the separation of bile acids on non impregnated silica gel $60F_{254}$ plates at 18°C. The plates impregnated with the salts whose application resulted in $\Delta R_F \geq 0.05$ and $R_S > 1$ for all neighboring pairs of examined bile acids were considered the most effective for bile acids separation. It was observed that impregnation of silica gel $60F_{254}$ glass plates with aqueous solutions of $CuSO_4$, $MnSO_4$, $NiSO_4$, and $FeSO_4$ improved the separation of GC/GDC and C/GLC, which separated poorly on glass plates precoated with non impregnated silica gel $60F_{254}$ in adsorption TLC at 18°C.

Keywords: Bile acids, Adsorption TLC, Silica gel $60F_{254}$ glass plates, Impregnation with metal cations

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INTRODUCTION

Application of metal cations in TLC is connected with their use in modifying stationary phases, e.g., silica gel, aluminum oxide, polyamide, or chitin, which are generally applied in TLC, by impregnating them with salts of appropriate metals. A variety of impregnation methods and reagents, which can be applied to impregnate adsorbents offers a wide array of possibilities for modification of TLC plates.[1] Modification of adsorbent surface by impregnating is easy and inexpensive and, what is more, the chromatographic plates are very attractive adsorbents.

Scientific literature describes using chromatographic plates precoated with adsorbents impregnated with aqueous solutions of salts of both, organic and inorganic acids to separate different class of organic compounds, e.g., alkaloids, amino acids, mercaptans, sulfonamides, and others.[1–8] Among such impregnated adsorbents, a very special place is occupied by adsorbents impregnated with silver nitrate (V). These adsorbents are widely applied to separate various unsaturated compounds, e.g., unsaturated lipids.[1]

Our previous investigations of bile acids focused on determining the influence of stationary and mobile phases, as well as chromatogram development temperature on separation of bile acids using adsorption thin-layer chromatography. Approximately 61 combinations, which included the changes of both, mobile and stationary phase composition and the temperature,[9–13] were examined using adsorption TLC. The similarity analysis was applied to compare the separations of studied bile acids. The similarity analysis showed that on the plates precoated with silica gel, the biggest problem was to separate glycocholic acid from glycodeoxycholic acid. In the case of separation on silica gel 60 and Kieselguhr F_{254} mixture, the biggest problem was to separate C from GLC.[9] It was stated that in the case of non modified adsorbents, the optimal separations of examined bile acids at 18°C was obtained by using *n*-hexane–ethyl acetate–acetic acid in volume compositions: 20:20:5 and 22:21:5 on plates precoated with silica gel.[10] Moreover, it was observed that application of DiolF_{254} plates and the mobile phase *n*-hexane–ethyl acetate–acetic acid in volume composition 42:42:16 (v/v/v) also facilitated the separation of all examined bile acids.[11] In the study of the influence of chromatogram development temperature on bile acids separation, it was suggested that the temperature 40°C would not only improve the separation of some bile acids, but also affect the change of bile acids elution order in the case of plates precoated with the mixture of silica gel 60 and Kieselguhr F_{254}.[12] Moreover, the complete separation of all studied bile acids using adsorption TLC at 18°C by 2D technique was proved possible. The first development used the *n*-hexane–ethyl acetate–acetic acid mobile phase in a volume composition of 25:20:5. The second development was perpendicular to the first one. Chloroform–*n*-butanol–acetic acid–water (2:32:2:2, v/v/v/v) was used as a mobile phase for the second development on glass plates precoated with silica gel 60F_{254} (#1.05715) and on aluminum plates precoated with silica gel 60 (#1.05553).[13]

In the study, the impregnation of TLC glass chromatographic plates precoated with silica gel $60F_{254}$ (#1.05715) with 1%, 2.5%, and 5% aqueous solutions of $CuSO_4$, $NiSO_4$, $MnSO_4$, and $FeSO_4$ was applied. *n*-Hexane–ethyl acetate–acetic acid was used as a mobile phase only in the volume compositions, which were not optimal for the separation of bile acids on non impregnated adsorbent at 18°C.

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 Ślaskie, Poland) was used to prepare a visualizing reagent. The salts of appropriate metals, i.e.: $MnSO_4 \cdot 7\ H_2O$, $FeSO_4 \cdot 7\ H_2O$, $CuSO_4 \cdot 5\ H_2O$, and $NiSO_4 \cdot 7\ H_2O$ (POCh, Gliwice, Poland; pure p. a.) were used to prepare impregnation solutions.

Sample Preparation

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

Impregnation of Applied Stationary Phase with Cu(II), Ni(II), Fe(II), and Mn(II) Cations

Glass plates precoated with silica gel $60F_{254}$ (#1.05715) were dipped for 30 sec. in aqueous solutions of the following salts: $CuSO_4$, $MnSO_4$, $NiSO_4$, and $FeSO_4$ in concentrations: 1%, 2.5%, and 5%. After impregnation, the plates were dried at room temperature (18°C) for 24 h. The modified plates were applied to separate the examined bile acids using adsorption thin-layer chromatography.

Thin Layer Chromatography

Adsorption TLC was performed on 20 × 20 cm glass plates precoated with silica gel $60\ F_{254}$ (E. Merck, #1.05715) and impregnated with the respective cations: Cu(II), Ni(II), Fe(II), and Mn(II). Before use, the plates were

activated at 120°C for 30 min. Micropipettes (5 μL, Camag, Switzerland) were used to apply the standard solutions onto the plates. Solutions of the standard acids were spotted on a chromatographic plate in quantities of 15 μg of each standard acid in 3 μL methanol. The chromatograms were developed at room temperature in a 20 cm × 20 cm classical chamber (Camag, Switzerland) using *n*-hexane–ethyl acetate–acetic acid in the following volume compositions: 22:20:5; 25:20:2; 25:20:5, and 25:20:8 as mobile phases. Fifty mL of mobile phases 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 visualizing reagent. The spots were developed by heating the sprayed plates at 120°C for 20 min. The values of ΔR_F and R_S for the studied bile acids were calculated according to the formulae, which were presented in our previous papers.[9–13] The calculated parameters are the mean values of five analyses.

RESULTS AND DISCUSSION

According to our previous investigations, it was proven that on glass plates precoated with non impregnated silica gel $60F_{254}$ (#1.05715), at 18°C cholic acid (C) did not separate from GLC when *n*-hexane–ethyl acetate–acetic acid in the volume compositions 25:20:2; 25:20:5, and 25:20:8 was used as a mobile phase, and GC did not separate from GDC in the case of application of the following mobile phases: 25:20:2; 25:20:5, and 22:20:5 (v/v/v) (Table 1).[9,10]

In this work, the glass plates precoated with silica gel $60F_{254}$ (#1.05715) and impregnated with 1%, 2.5%, and 5% aqueous solutions of $CuSO_4$, $NiSO_4$, $MnSO_4$, and $FeSO_4$ were used to separate seven examined bile acids at 18°C. *n*-Hexane–ethyl acetate–acetic acid in the volume compositions 22:20:5; 25:20:2; 25:20:5, and 25:20:8 which were not optimal, i.e., $R_S \leq 1$ was obtained for certain neighboring pairs of bile acids, was used as a mobile phase to separate the examined bile acids on non impregnated adsorbent at 18°C.

The separation factor values, i.e., ΔR_F and R_S, for all pairs of neighboring bile acids were the basis for the estimation of the influence of impregnating chromatographic plates with salts of inorganic acids on the efficiency of examined bile acids separation. The plates impregnated with the cations whose application resulted in $\Delta R_F \geq 0.05$ and $R_S > 1$ for all pairs of neighboring acids were considered the most effective for bile acid separation. The total number of experimental combinations regarding the impregnation of applied stationary and mobile phases used to separate bile acids was 48. Table 2 presents the description of respective pairs of the examined bile acids separation effects on the glass plates precoated with silica gel $60F_{254}$ (#1.05715),

Table 1. The R_F values and separation factors (ΔR_F and R_S) of studied bile acids separated on glass plates precoated with silica gel $60F_{254}$ (#1.05715) with *n*-hexane–ethyl acetate–acetic acid as a mobile phase in different volume compositions (v/v/v), which were not optimal for bile acids separation on non impregnated silica gel at 18°C[9,10]

	n-Hexane–ethyl acetate–acetic acid (v/v/v)											
	22:20:5			25:20:2			25:20:5			25:20:8		
Pair of acids	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	0.01/0.04	0.03	1.00	0.01/0.01	0.00	0.00	0.01/0.05	0.04	1.44	0.02/0.09	0.07	2.24
GDC/C	0.04/0.17	0.13	3.04	0.01/0.06	0.05	1.04	0.05/0.17	0.12	3.40	0.09/0.30	0.22	6.10
C/GLC	0.17/0.24	0.07	1.63	0.06/0.07	0.01	0.35	0.17/0.21	0.04	0.96	0.30/0.34	0.03	1.00
GLC/CDC	0.24/0.54	0.30	5.12	0.07/0.24	0.17	4.00	0.21/0.50	0.29	6.72	0.34/0.69	0.35	8.91
CDC/DC	0.54/0.64	0.10	1.46	0.24/0.29	0.05	1.08	0.50/0.57	0.07	1.52	0.69/0.77	0.09	1.71
DC/LC	0.64/0.93	0.29	6.38	0.29/0.70	0.41	8.85	0.57/0.82	0.25	6.73	0.77/0.94	0.17	5.75

Table 2. Description of separation effects for the respective pairs of studied bile acids on glass plates precoated with silica gel $60F_{254}$ (#1.05715) impregnated with aqueous solutions of inorganic salts developed by using *n*-hexane–ethyl acetate–acetic acid as a mobile phase at 18°C

The volume composition of mobile phase (v/v/v)	Pair of acids					
	GC/GDC	GDC/C	C/GLC	GLC/CDC	CDC/DC	DC/LC
25:20:2	br[a]	br	W[b]	W	1% $CuSO_4$[c] 1% $NiSO_4$	W
25:20:5	W	W	W	W	1% $CuSO_4$ 2.5% $CuSO_4$ 5% $CuSO_4$ 2.5% $MnSO_4$ 1% $NiSO_4$ 2.5% $FeSO_4$ 5% $FeSO_4$	W
25:20:8	W	W	W	W	1% $CuSO_4$ 5% $CuSO_4$ 1% $NiSO_4$ 5% $NiSO_4$ 1% $FeSO_4$ 5% $FeSO_4$	W
22:20:5	W	W except of 2.5% $FeSO_4$	W	W	1% $CuSO_4$ 2.5% $CuSO_4$ 2.5% $MnSO_4$ 5% $FeSO_4$	W

[a]br—Lack of separation.
[b]W—Successful separation of the pair of neighboring bile acids on silica gel impregnated with all applied salt solutions.
[c]Successful separation of the pair of neighboring bile acids on the silica gel impregnated with a/m salts solutions.

impregnated with aqueous solutions of inorganic salts, and developed using *n*-hexane–ethyl acetate–acetic acid at 18°C as a mobile phase.

On the basis of the examined bile acids ΔR_F and R_S values (Table 2), which were obtained for the impregnated adsorbent, it can be concluded that all four applied mobile phases facilitated the separation of both acids, C and GLC, when compared to their separation on non impregnated adsorbent. Whereas, in the case of GC and GDC, the separation improvement on impregnated adsorbent can be observed only when mobile phases 22:20:5 and 25:20:5 (v/v/v) are applied. Unfortunately, even the modification of applied adsorbent by impregnating it with solutions of selected salts did not improve the separation of a/m bile acids, i.e., GC and GDC, which were developed using the mobile phase in a volume composition 25:20:2 (v/v/v). Moreover, there was observed the deterioration of GDC and C separation on adsorbent impregnated with a/m salts, using the volume compositions 25:20:2 and 22:20:5 of a mobile phase on adsorbent impregnated with 2.5% solution of $FeSO_4$ (Table 2). The impregnation of plates with aqueous solutions (1%, 2.5%, and 5% $CuSO_4$; 1%, 2.5% and 5% $MnSO_4$; 1%, 2.5% and 5% $NiSO_4$, and also 1%, 2.5% and 5% $FeSO_4$) allows completion of the separation of GC from GDC and C from GLC chromatographed with the mobile phases in volume compositions: 25:20:5; 25:20:8, and 22:20:5. The remaining pairs of neighboring bile acids, except for CDC/DC, were separated under a/m conditions. The CDC/DC pair of neighboring acids was separated only on a/m adsorbent impregnated with certain solutions of $CuSO_4$, $NiSO_4$, $MnSO_4$, and $FeSO_4$ (Table 2). Thus, the application of glass plates precoated with silica gel $60F_{254}$ and impregnated with salts of inorganic acids causes the separation problem in the case of CDC and DC, in comparison to their separation on non impregnated adsorbent. All pairs of studied bile acids were separated on plates impregnated with the following aqueous solutions of inorganic salts (Tables 3–5):

1% $CuSO_4$, 2.5% $CuSO_4$, 5% $CuSO_4$, 2.5% $MnSO_4$, 1% $NiSO_4$, 2.5% $FeSO_4$, and 5% $FeSO_4$ with a mobile phase in volume composition 25:20:5 (v/v/v);

1% $CuSO_4$, 5% $CuSO_4$, 1% $NiSO_4$, 5% $NiSO_4$, 1% $FeSO_4$, 5% $FeSO_4$, with a mobile phase in volume composition 25:20:8 (v/v/v);

1% $CuSO_4$, 2.5% $CuSO_4$, 2.5% $MnSO_4$, and 5% $FeSO_4$ with a mobile phase in volume composition 22:20:5 (v/v/v).

Figure 1 presents an example of the comparison of ΔR_F and R_S values for bile acids separated on silica gel $60F_{254}$, both non impregnated (#1.05715) and impregnated with 2.5% aqueous solution of $FeSO_4$, using the mobile phase 25:20:5 (v/v/v).

Comparing ΔR_F and R_S values of bile acids presented in Figure 1 separated on non impregnated, precoated with silica gel $60F_{254}$ (#1.05715)

Table 3. The selected values[a] of separation factors ΔR_F and R_S of bile acids examined on glass plates precoated with silica gel $60F_{254}$ (#1.05715) impregnated with salts of inorganic acids developed by using *n*-hexane–ethyl acetate–acetic acid as a mobile phase in volume composition 25:20:5 at 18°C

	n-Hexane–ethyl acetate–acetic acid (v/v/v) (25:20:5)													
	1% $CuSO_4$		2.5% $CuSO_4$		5% $CuSO_4$		2.5% $MnSO_4$		2.5% $FeSO_4$		5% $FeSO_4$		1% $NiSO_4$	
Pair of acids	ΔR_F	R_S	ΔR_F	R_S	ΔR_F	R_S	ΔR_F	R_S	ΔR_F	R_S	ΔR_F	R_S	ΔR_F	R_S
GC/GDC	0.06	1.38	0.05	1.08	0.17	3.27	0.06	1.64	0.08	2.09	0.07	1.83	0.07	1.50
GDC/C	0.12	2.13	0.10	2.07	0.09	1.04	0.09	1.92	0.08	1.59	0.08	1.47	0.12	2.27
C/GLC	0.18	3.25	0.28	5.52	0.29	3.73	0.14	3.15	0.18	2.73	0.18	2.63	0.15	2.27
GLC/CDC	0.18	2.94	0.10	1.88	0.15	2.10	0.17	3.43	0.12	1.89	0.14	1.81	0.25	4.55
CDC/DC	0.09	1.37	0.07	1.05	0.09	1.37	0.06	1.24	0.10	1.65	0.06	1.06	0.08	1.20
DC/LC	0.32	6.85	0.35	7.69	0.17	4.98	0.22	6.00	0.29	7.00	0.23	5.91	0.26	6.27

[a]In table presents only the values of optimal separation of the studied bile acids for given impregnated concentrations.

Table 4. The selected values[a] of separation factors ΔR_F and R_S of bile acids examined on glass plates precoated with silica gel $60F_{254}$ (#1.05715) impregnated with salts of inorganic acids developed by using *n*-hexane–ethyl acetate–acetic acid as a mobile phase in volume composition 22:20:5 at 18°C

	n-Hexane–ethyl acetate–acetic acid (v/v/v) (22:20:5)							
	1% $CuSO_4$		2.5% $CuSO_4$		2.5% $MnSO_4$		5% $FeSO_4$	
Pair of acids	ΔR_F	R_S	ΔR_F	R_S	ΔR_F	R_S	ΔR_F	R_S
GC/GDC	0.09	2.17	0.17	2.42	0.07	1.26	0.10	2.24
GDC/C	0.11	2.00	0.20	2.67	0.08	1.48	0.06	1.12
C/GLC	0.28	5.00	0.22	3.43	0.19	3.60	0.20	3.80
GLC/CDC	0.19	3.48	0.14	2.52	0.15	2.71	0.15	2.39
CDC/DC	0.06	1.21	0.06	1.06	0.06	1.09	0.06	1.03
DC/LC	0.19	4.91	0.13	3.50	0.25	5.23	0.25	6.57

[a]In table presents only the values of optimal separation of the studied bile acids for given impregnated concentrations.

glass plates developed by using *n*-hexane–ethyl acetate–acetic acid in volume composition 25:20:5 (v/v/v) as a mobile phase at 18°C with the values of a/m separation factors obtained on adsorbent impregnated with 2.5% aqueous solution of $FeSO_4$, leads to the conclusion that impregnation of plates with 2.5% aqueous solution of $FeSO_4$ especially facilitated the separation of respective pairs of bile acids, i.e., GC/GDC and C/GLC, which separated more poorly on non impregnated adsorbent. The values of separation factors $\Delta R_F \geq 0.05$ and $R_S > 1$ obtained for each pair of studied bile acids under these conditions confirm the conclusion.

CONCLUSION

In the present study, to separate bile acids using adsorption thin layer chromatography at 18°C the glass plates precoated with silica gel $60F_{254}$ (#1.05715) were impregnated with 1%, 2.5%, and 5% aqueous solutions of the following salts: $CuSO_4$, $MnSO_4$, $NiSO_4$, and $FeSO_4$. *n*-Hexane–ethyl acetate–acetic acid was used as a mobile phase, but only in the following volume compositions: 22:20:5; 25:20:2; 25:20:5, and 25:20:8. These mobile phases were not optimal for bile acids separation on non impregnated silica gel $60F_{254}$ at 18°C.

It was observed that impregnation of silica gel $60F_{254}$ glass plates with aqueous solutions of $CuSO_4$, $MnSO_4$, $NiSO_4$, and $FeSO_4$ facilitated the separation of GC from GDC, and also C from GLC, which separated more poorly

Table 5. The selected values[a] of separation factors ΔR_F and R_S of bile acids examined on glass plates precoated with silica gel $60F_{254}$ (#1.05715) impregnated with salts of inorganic acids developed by using mobile phase *n*-hexane–ethyl acetate–acetic acid in volume composition 25:20:8 at 18°C

	n-Hexane–ethyl acetate–acetic acid (v/v/v) (25:20:8)											
	1% $CuSO_4$		5% $CuSO_4$		1% $NiSO_4$		5% $NiSO_4$		1% $FeSO_4$		5% $FeSO_4$	
Pair of bile acids	ΔR_F	R_S	ΔR_F	R_S	ΔR_F	R_S	ΔR_F	R_S	ΔR_F	R_S	ΔR_F	R_S
GC/GDC	0.06	1.36	0.18	4.08	0.09	1.79	0.18	3.33	0.11	3.20	0.19	3.06
GDC/C	0.14	2.82	0.07	1.46	0.11	2.21	0.21	3.16	0.09	2.00	0.10	1.40
C/GLC	0.26	4.87	0.25	5.07	0.20	3.00	0.26	3.80	0.27	5.92	0.24	3.72
GLC/CDC	0.11	1.82	0.06	1.07	0.24	3.78	0.10	1.65	0.17	3.31	0.18	3.12
CDC/DC	0.09	1.33	0.07	1.18	0.08	1.33	0.08	1.53	0.08	1.29	0.06	1.23
DC/LC	0.28	7.33	0.25	5.00	0.23	6.00	0.12	4.60	0.26	5.83	0.13	3.70

[a]In table presents only the values of optimal separation of the studied bile acids for given impregnated concentrations.

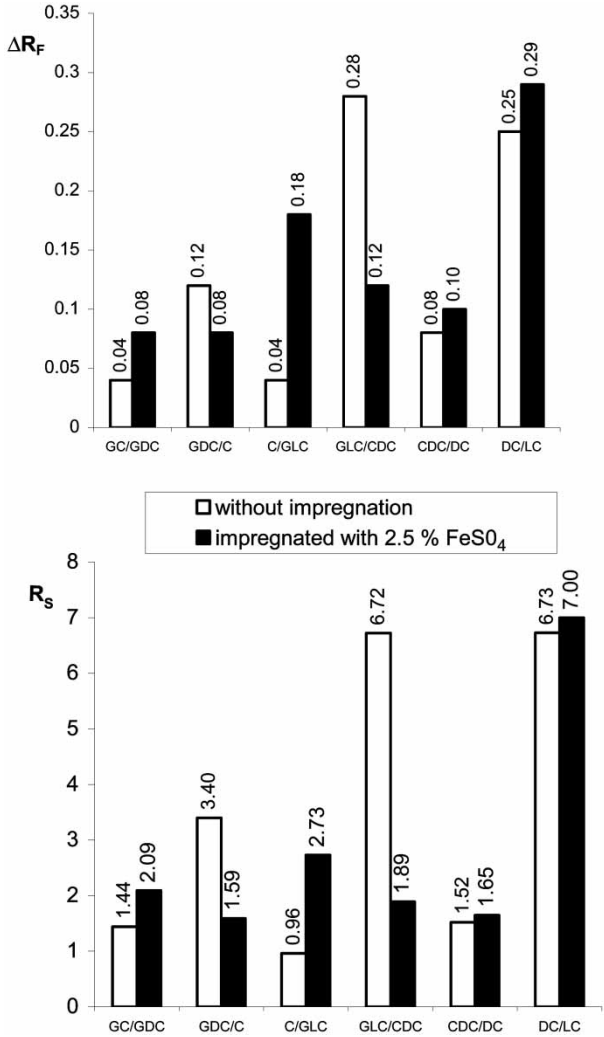

Figure 1. Comparison of the ΔR_F and R_S values for studied bile acids separated on glass plates precoated with non impregnated and impregnated silica gel $60F_{254}$ (#1.05715) with 2.5% aqueous solutions of $FeSO_4$ and developed by using *n*-hexane–ethyl acetate–acetic acid in a volume composition 25:20:5 (v/v/v) as a mobile phase.

using adsorption TLC on non impregnated adsorbent at 18°C. However, the application of glass plates precoated with silica gel $60F_{254}$ causes the problem in the case of the separation of CDC from DC, in comparison to their separation on non impregnated plates.

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