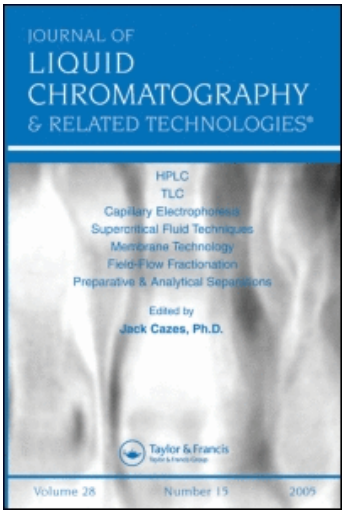

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Separation of Selected Bile Acids by TLC. II. One-Dimensional and Two-Dimensional TLC

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ABSTRACT

The objective of this study was to describe a simple and efficient method to separate unconjugated bile acids and their glycine conjugates. The method described herein involves the use of a two-dimensional thin layer chromatographic (2D TLC) technique. Seven bile acids: chenodeoxycholic (CDC), deoxycholic (DC), cholic (C), glycocholic (GC), lithocholic (LC), glycodeoxycholic (GDC), and glycolithocholic (GLC) were chromatographed on glass plates precoated with silica gel $60F_{254}$ and on aluminum plates precoated with silica gel 60, by developing them with: *n*-hexane–ethyl acetate–acetic acid (25 : 20 : 5, v/v/v) in the first dimension and chloroform–*n*-butanol–acetic acid–water (2 : 32 : 2 : 2, v/v/v/v) in the second dimension. These chromatographic

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conditions, using the 2D technique for bile acids separation, allowed for the complete separation of all the bile acids studied.

Key Words: Bile acids; TLC; One-dimensional; Two-dimensional TLC.

INTRODUCTION

Bile acids are the end products of cholesterol metabolism in the liver. They consist mainly of cholic (C) and deoxycholic (DC) acids and their conjugated forms (glycine, taurine) such as glycocholic (GC), and taurocholic acids. Bile acids play a very important role in the biochemistry of humans. Emulsification of liquid aggregates and solubilization and transportation of lipids in aqueous environment are two important functions of bile acids. 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.[1–4] The effect of the separation of bile acids depends on the kind of bile acids and their chemical structure. Because of their structural similarities, separation of bile acids and their metabolites is difficult.

Bile acids and derivatives of bile acids are analyzed mainly by chromatographic techniques. Various chromatographic methods such as: gas chromatography, high performance liquid chromatography (HPLC), and thin layer chromatography (TLC) are used to analyze bile acids. TLC is a more commonly applied method for the analysis of bile acids and their conjugates than HPLC and gas chromatography, because it is simple, inexpensive, and can be performed directly on biological fluids without prior sample purification.[5–16]

We have previously investigated the optimal conditions for the separation of selected bile acids, which were investigated by TLC on aluminum plates precoated with silica gel $60F_{254}$, with the use of a mixture of *n*-heptane–ethyl acetate–acetic acid in various volume compositions as mobile phases.[16] The aim of this work is to apply one- and two-dimensional TLC (1D and 2D TLC) in order to separate selected bile acids: C, chenodeoxycholic (CDC), DC, GC, glycodeoxycholic (GDC), glycolithocholic (GLC), and lithocholic (LC).

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), chloroform (POCh, Gliwice, Poland), *n*-butyl alcohol

(Reanal, Budapest, Hungry), and distilled water (Department of Analytical Chemistry, Faculty of Pharmacy, Sosnowiec, Poland) were used for the TLC analysis. The commercial samples of C, DC, CDC, LC, GLC, GDC and GC (Sigma Company, St. Louis, 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.

TLC

The 1D and 2D TLC were performed on 20 × 20-cm^2 glass plates precoated with a 0.25-mm layer of silica gel 60F_{254} (#1.05715, E. Merck) and on 20 × 20-cm^2 aluminum plates precoated with a 0.2-mm layer of silica gel 60 (#1.05553, E. Merck). Before they were used, both plates were activated at 120°C for 20 min. Micropipettes (5 μL, Camag, Switzerland) were used to apply the standard solutions and their mixture to the plates. The chromatograms were developed at room temperature (18°C) in a classical chamber (Camag, Switzerland). Mobile phase of 50 mL were used in each case.

1D TLC

Solutions of the bile acids were prepared in methanol in concentration of 5 mg/mL and 3 μL volumes were spotted on the chromatographic plates. The *n*-hexane–ethyl acetate–acetic acid in various volume compositions of 20 : 20 : 5, 22 : 20 : 5, 22 : 21 : 5, 22 : 22 : 5, 25 : 20 : 5, 25 : 20 : 2, and 25 : 20 : 8 were used as mobile phases. The development distance was 14 cm. The plates were dried at room temperature in a fume cupboard.

2D TLC

A solution of a mixture of bile acids was spotted on chromatographic plates in quantities of 15 μg of each bile acid in 10 μL of methanol. The mixture of *n*-hexane–ethyl acetate–acetic acid in the volume composition of 25 : 20 : 5 was used as mobile phase. After the first development, the plates were dried at room temperature (18°C) for 24 hr to allow for the complete evaporation of the solvent. After 24 hr, the chromatograms were developed in the second direction with the following mobile phase of chloroform–*n*-butyl alcohol–acetic acid–water in the volume composition: 2 : 32 : 2 : 2, as described by Szepesi.[17] The second development was perpendicular to the first one. The development distance was 14 cm in both directions. The plates were dried at room temperature (18°C) in a fume cupboard.

Detection

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

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:

$$\Delta R_F = R_{F1} - R_{F2} \quad (1)$$

where R_{F1} and R_{F2} are values of two adjacent spots, and $R_{F1} > R_{F2}$.

R_S is calculated with formula:[18]

$$R_S = 2 \times \frac{a}{b} \quad (2)$$

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

RESULTS AND DISCUSSION

Table 1 presents the data, which allows an estimation of the usefulness of the examined mobile phases (*n*-hexane–ethyl acetate–acetic acid) in different volume compositions for the separation of all the investigated bile acids. Separation of each investigated pair of bile acids is satisfactory when $\Delta R_F \geq 0.05$ and $R_S > 1$. From the comparison presented in Table 1, it can be concluded that all the bile acids studied on the glass plates precoated with silica gel $60F_{254}$ (#1.05715) have been successfully separated with the mobile phase *n*-hexane–ethyl acetate–acetic acid at a volume composition: 20 : 20 : 5 and 22 : 22 : 5.

On the aluminum plates precoated with silica gel 60 (#1.05553), seven investigated bile acids can be separated with the mobile phase *n*-hexane–ethyl acetate–acetic acid with a volume composition of: 20 : 20 : 5, 22 : 21 : 5, 22 : 22 : 5, and 25 : 20 : 8. The mobile phase *n*-hexane–ethyl acetate–acetic acid in a volume composition of 25 : 20 : 5 was selected to separate the examined bile acids on both types of plates (#1.05715 and #1.05553) in the first development. The pair of acids C and GLC, GDC and GDC on the glass plates #1.05715 precoated with silica gel $60F_{254}$ were poorly separated ($\Delta R_F = 0.04$) under these conditions. This mobile phase is not optimal for separating the

Table 1. The estimation of the examined mobile phase usefulness (*n*-hexane–ethyl acetate–acetic acid) in different volume compositions for the separation of bile acids on glass plates precoated with silica gel $60F_{254}$ (#1.05715) and on aluminum plates precoated with silica gel 60 (#1.05553).

n-Hexane–ethyl acetate–acetic acid in a volume composition (v/v/v)	#1.05715		#1.05553	
	$\Delta R_F \geq 0.05$	$R_S > 1$	$\Delta R_F \geq 0.05$	$R_S > 1$
20 : 20 : 5	+	+	+	+
22 : 20 : 5	−	−	−	−
22 : 21 : 5	−	+	+	+
22 : 22 : 5	+	+	+	+
25 : 20 : 2	−	−	−	−
25 : 20 : 5	−	−	+	−
25 : 20 : 8	−	−	+	+

Note: + indicates that for all investigated bile acids $\Delta R_F \geq 0.05$ or $R_S > 1$; − indicates that not for all investigated bile acids $\Delta R_F \geq 0.05$ or $R_S > 1$.

bile acids on aluminum plates precoated with silica gel 60 (#1.05553). However, when this mobile phase is used, the difference between R_F values of C and GLC is 0.05, but R_S of this pair of bile acids is smaller than 1 ($R_{S(C/GLC)} = 0.96$) (Table 2).

To obtain complete separation of the seven examined bile acids on glass plates precoated with silica gel $60F_{254}$ (#1.05715) and on the aluminum plates precoated with silica gel 60 (#1.05553), a 2D technique was used. The first development used the mobile phase *n*-hexane–ethyl acetate–acetic acid in

Table 2. Separation factors ΔR_F and R_S values of the selected bile acids, separated with a mobile phase *n*-hexane–ethyl acetate–acetic acid in volume composition 25 : 20 : 5 on glass plates precoated with silica gel $60F_{254}$ (#1.05715) and on aluminum plates precoated with silica gel 60 (#1.05553).

Pair of acids	#1.05715		#1.05553	
	ΔR_F	R_S	ΔR_F	R_S
GC/GDC	0.04	1.44	0.05	1.62
GDC/C	0.12	3.40	0.14	4.00
C/GLC	0.04	0.96	0.05	0.96
GLC/CDC	0.29	6.72	0.33	7.00
CDC/DC	0.07	1.52	0.09	1.47
DC/LC	0.25	6.73	0.31	6.92

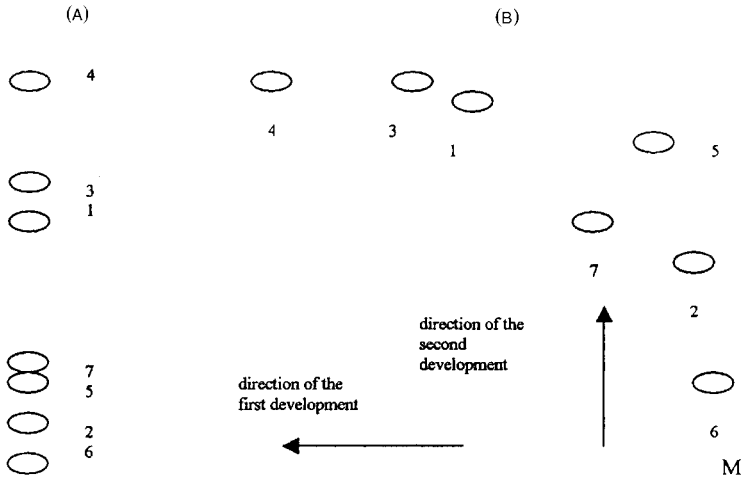

Figure 1. The 1D (A) and 2D (B) chromatograms of the mixture of the seven bile acids investigated on TLC silica gel 60 (E. Merck, #1.05553). I. Eluent: *n*-hexane–ethyl acetate–acetic acid (25 : 20 : 5, v/v). II. Eluent: chloroform–*n*-butanol–acetic acid–water (2 : 32 : 2 : 2, v/v); M—bile acids mixture where: 1—chenodeoxycholic acid, 2—glycodeoxycholic acid, 3—deoxycholic acid, 4—lithocholic acid, 5—cholic acid, 6—glycocholic acid, and 7—glycolithocholic acid.

a volume composition of 25 : 20 : 5. Chloroform–*n*-butanol–acetic acid–water (2 : 32 : 2 : 2, v/v/v/v) as mobile phase was used for the second development. The scheme of the chromatogram of separated bile acids with the use of 2D technique is presented in Fig. 1. The chromatogram indicates that under these conditions, all the bile acids studied were completely separated. Better separation for C and GLC acids was obtained.

The separation conditions used with the 2D technique were better than when a single development with *n*-hexane–ethyl acetate–acetic acid (25 : 20 : 5, v/v/v) on both plates (#1.05715 and #1.05553) was used. The proposed method of separation is useful for the identification and separation of bile acids in biological materials.

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