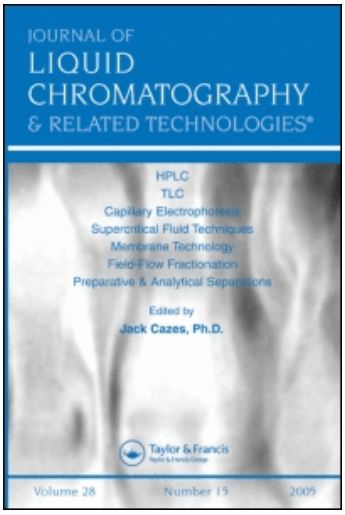

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Separation of Selected Bile Acids by TLC. IV. Comparison of Separation of Studied Bile Acids by the Use of Cluster Analysis

A. Pyka[a]; M. Dolowy[a]
[a] Department of Analytical Chemistry, Faculty of Pharmacy, Silesian Academy of Medicine, Sosnowiec, Poland

Online publication date: 20 September 2004

To cite this Article Pyka, A. and Dolowy, M.(2005) 'Separation of Selected Bile Acids by TLC. IV. Comparison of Separation of Studied Bile Acids by the Use of Cluster Analysis', Journal of Liquid Chromatography & Related Technologies, 27: 19, 2987 — 2995
To link to this Article: DOI: 10.1081/JLC-200032622
URL: http://dx.doi.org/10.1081/JLC-200032622

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES®
Vol. 27, No. 19, pp. 2987–2995, 2004

Separation of Selected Bile Acids by TLC. IV. Comparison of Separation of Studied Bile Acids by the Use of Cluster Analysis

A. Pyka* and M. Dolowy

Department of Analytical Chemistry, Faculty of Pharmacy, Silesian Academy of Medicine, Sosnowiec, Poland

ABSTRACT

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) were separated on both, silica gel and a mixture of silica gel 60 and Kieselguhr F_{254} by using adsorption thin-layer chromatography. The similar analyses were used to compare the separations of studied bile acids. Both analyses showed that on the plates precoated with silica gel, the biggest problem was to separate glycocholic acid from glycodeoxycholic acid. In the case of the separation on silica gel 60 and Kieselguhr F_{254} mixture, the biggest problem was to separate C from GLC. The obtained results indicate that similar analysis can be an

*Correspondence: A. Pyka, Department of Analytical Chemistry, Faculty of Pharmacy, Silesian Academy of Medicine, Jagiellońska 4, PL-41-200 Sosnowiec, Poland; E-mail: alinapyka@wp.pl.

DOI: 10.1081/JLC-200032622 1082-6076 (Print); 1520-572X (Online)

alternative method of the estimation of chromatographic separations of studied bile acids.

Key Words: Adsorption TLC; Bile acids; Cluster analysis.

INTRODUCTION

Bile acids belong to the group of natural substances of steroid structure. They are present in bile in a conjugated form. Bile acids reveal specific physicochemical, biochemical, and physiological properties. In human organisms they perform a lot of functions, e.g., they facilitate digestion and absorption of fat and vitamins soluble in fat (A, E, D, and K) in the intestines. Therefore, separation and quantification of bile acids in biological samples is very important for diagnosis of many diseases. Bile acids are analyzed mainly with the use of chromatographic techniques.[1–7]

An important method for planning and optimization of the bile acids separation by means of chromatographic methods can be cluster analysis (CA). The aim of CA is to find sets (subsets) in the complete set of data obtained by chromatographic techniques (e.g., R_f), which group similar objects. These sets are called clusters. A graphic image of CA is a diagram (tree). Leaves represent separate objects and roots include all objects as one cluster. The basic parameter of CA is the distance between the objects, which is a measure of their similarity. There are many methods of these distances calculation. The most commonly used are: the method of single linkage and the method of complete linkage.[8]

Our previous investigation of bile acids referred to the separation of selected bile acids by using adsorption thin-layer chromatography with the use of one and two-dimensional techniques of developing chromatographic plates.[9–11]

The aim of our work was to apply CA to estimate the separation of selected bile acids such as: cholic acid (C), glycocholic acid (GC), glycolithocholic acid (GLC), deoxycholic acid (DC), chenodeoxycholic (CDC), glycodeoxycholic acid (GDC), and lithocholic acid (LC) by using adsorption thin-layer chromatography.

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 TLC adsorption 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 above-mentioned bile acids in 50 mg/10 mL concentration of each acid were prepared.

Thin Layer Chromatography

TLC adsorption was performed on $20 \times 20\,cm^2$ 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 the use, the plates were activated at 120°C for 30 min. Micropipettes (5 μL, 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 15 μg of each standard in 3 μL methanol. The chromatograms were developed at room temperature in a 20 cm × 20 cm horizontal in a classical 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 of 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.

RESULTS AND DISCUSSION

The bile acids were separated on chromatographic plates precoated with silica gel and silica gel 60 and Kieselguhr F_{254} mixture by using *n*-hexane–ethyl acetate–acetic acid as a mobile phase. The volume composition of

the mobile phase was modified in order to obtain the best separation of all studied bile acids. The obtained results were presented as relationships between R_f values and various volume compositions for all used chromatographic adsorbents. Chosen graphs only, for glass plates precoated with silica gel $60F_{254}$ (#1.05715) without concentrating zone (Fig. 1), with concentrating zone (#1.11798) (Fig. 2), and aluminum plates precoated with silica gel 60 and Kieselguhr F_{254} mixture were presented (Fig. 3).

On the change of R_f values in separated bile acids, the different influences of volume composition of mobile phase (*n*-hexane–ethyl acetate–acetic acid) can be observed. The smallest influence of volume composition of applied mobile phase on the separation for GC and GDC was observed. The influence increased for C, LC, and GLC and it was the largest for CDC and DC. From the data presented in Figs. 1 and 2, it can be concluded that GC and GDC, as well as C and GLC, do not practically separate on the chromatogram, which was separated by using the mobile phase in volume composition: 25 : 20 : 2. A weak separation of DC and CDC was observed in this mobile phase, whereas this mobile phase separates DC from LC best of all. The order of elution of investigated bile acids at the applied mobile phase: *n*-hexane–ethyl acetate–acetic acid (regardless of applied volume composition) is the following: LC, DC, CDC, GLC, C, GDC, and GC. Similar results were obtained on aluminum plates precoated with silica gel 60 and $60F_{254}$ (#1.05553 and #1.05554).

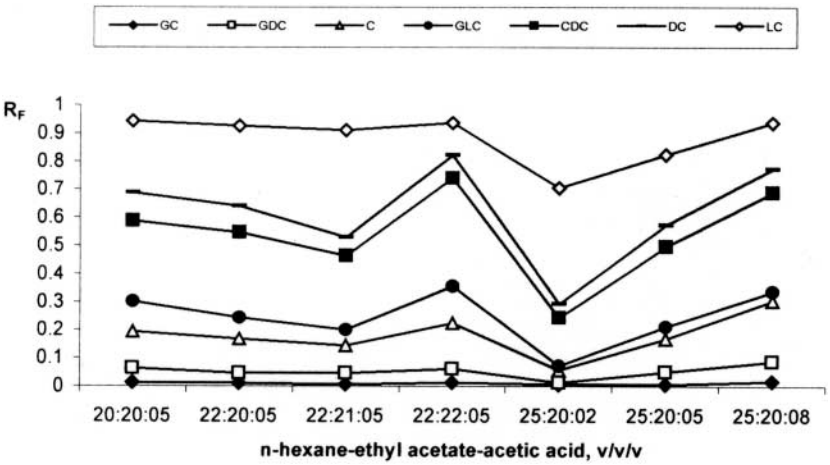

Figure 1. The dependence between R_f and the volume composition of the mobile phase: *n*-hexane–ethyl acetate–acetic acid of bile acids separated on glass plates precoated with silica gel $60F_{254}$ (#1.05715).

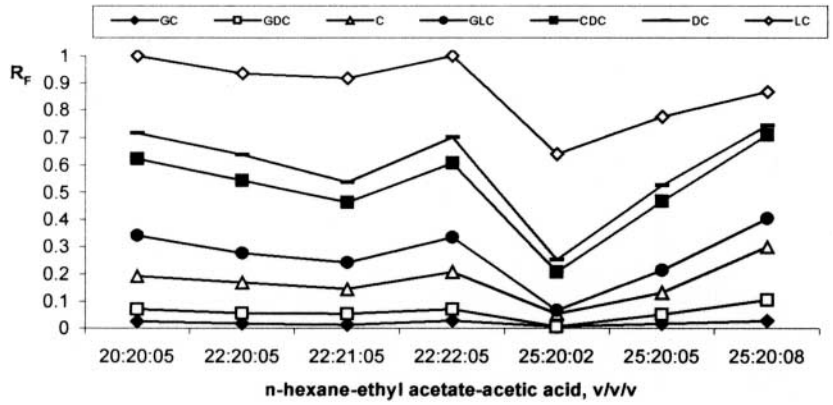

Figure 2. The dependence between R_f and the volume composition of the mobile phase *n*-hexane–ethyl acetate–acetic acid of bile acids separated on glass plates precoated with silica gel $60F_{254}$ with concentrating zone (#1.11798).

Certain differences in chromatographic separations of the studied bile acids on aluminum plates precoated with silica gel 60 and Kieselguhr F_{254} mixture (#1.05567) were observed in comparison with the separations obtained on plates precoated with silica gel (Fig. 3). General conclusions,

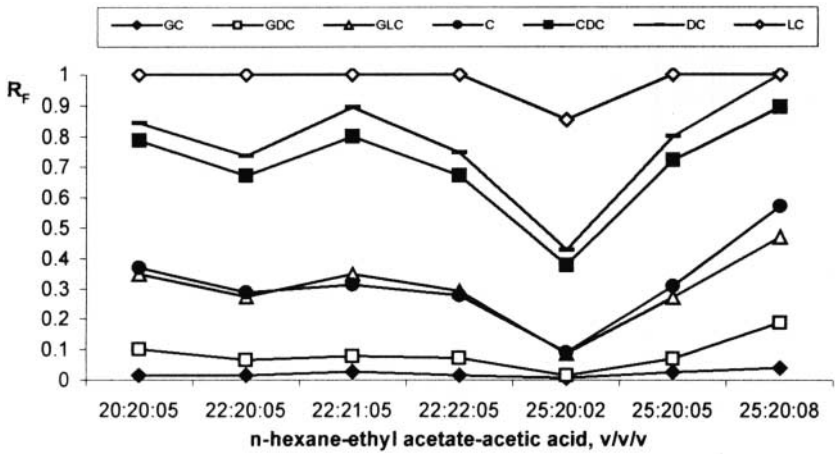

Figure 3. The dependence between R_f and the volume composition of the mobile phase *n*-hexane–ethyl acetate–acetic acid of the bile acids separated on aluminum plates precoated with a mixture of silica gel 60 and Kieselguhr F_{254} (#1.05567).

arising from the results obtained on aluminum plates precoated with silica gel 60 and Kieselguhr F_{254} (#1.05567) mixture are similar to the conclusions based on results obtained on the rest of adsorbents. The differences concern separation of GLC from C. Adsorption of bile acids by using the mobile phase: *n*-hexane–ethyl acetate–acetic acid in volume composition 22 : 21 : 5 and 22 : 22 : 5 is identical to the one when glass plates precoated with silica gel $60F_{254}$ without concentration zone (#1.05715) and with concentrating zone (#1.11798) are used. The adsorption does not change when aluminum plates precoated with silica gel $60F_{254}$ (#1.05554) and on aluminum plates precoated with silica gel 60 (#1.05553) are employed. However, the adsorption of bile acids by using the mobile phase: *n*-hexane–ethyl acetate–acetic acid in volume compositions 20 : 20 : 5, 22 : 20 : 5, 25 : 20 : 5, 25 : 20 : 2, and 25 : 20 : 8 increases in the following order: LC, DC, CDC, C, GLC, GDC, and GC.

The CA for comparison of chromatographic separations of studied bile acids was later applied in the study. Figure 4 presents a dendogram of CA of R_f parameters for the separation on glass plates precoated with silica gel $60F_{254}$ (#1.05715), with application of *n*-hexane–ethyl acetate–acetic acid in different volume compositions. The greatest similarity in R_f values was stated for the following acids: GDC and GC, next for GLC and C, also for DC and CDC. The LC shows the greatest similarity in R_f to DC and CDC.

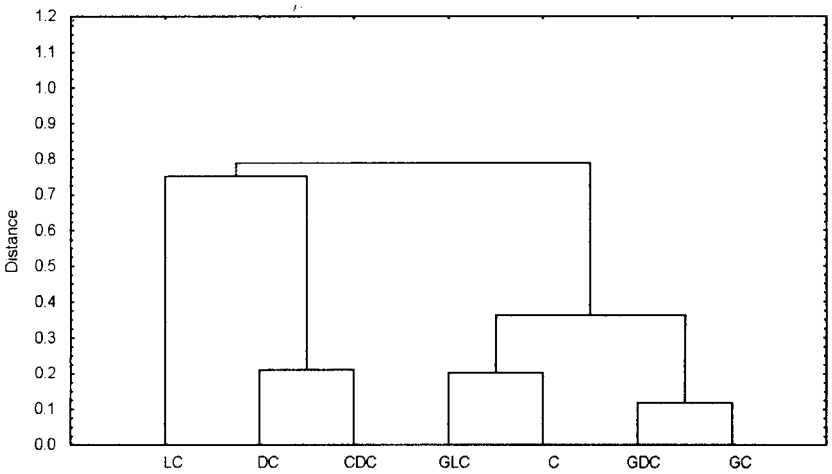

Figure 4. Cluster analysis of R_f parameters of bile acids separated on glass plates precoated with silica gel $60F_{254}$ (#1.05715) by using mobile phase: *n*-hexane–ethyl acetate–acetic acid in different volume compositions (Euclidean distance, single bond method).

The CA permits grouping the studied bile acids into four subgroups: The first subgroup: DC and CDC. The second subgroup: GLC and C. The third subgroup: GDC and GC. The fourth subgroup: LC.

A similar dendrogram is for the studied bile acids separated on aluminum plates precoated with silica gel 60 (#1.05553). Figure 5 presents a dendogram of CA of R_f values for the separation of studied bile acids on silica gel $60F_{254}$ (#1.05554). This dendogram shows four well separated clusters which correspond to respective bile acids: the first cluster makes GDC and GC, the second one: GLC and C, the third one: DC and CDC, and the fourth cluster is LC. Simultaneously, the biggest similarity in chromatographic separations (the greatest similarity in R_f values) for GDC and GC was stated. An analogical dendogram was obtained for bile acids separated on glass plates precoated with silica gel $60F_{254}$ with concentrating zone (#1.11798). Figure 6 presents a dendogram of CA of R_f values for bile acids separated on silica gel 60 and Kieselguhr F_{254} mixture. Figure 6 shows three clusters: the first one makes C and GLC, the second one GDC and GC, and the third one DC, CDC, and LC. The greatest similarity in R_f values for C and GLC was obtained in the case of silica gel 60/Kieselguhr F_{254}.

It was observed that on silica gel (#1.05715, #1.05553, #1.05554, #1.11798) and silica gel 60 and Kieselguhr F_{254} mixture (#1.05567) (Figs. 4–6) similar separation of GLC from C was obtained. GLC is better separated from C on silica gel in comparison with both acids separation on the mixture

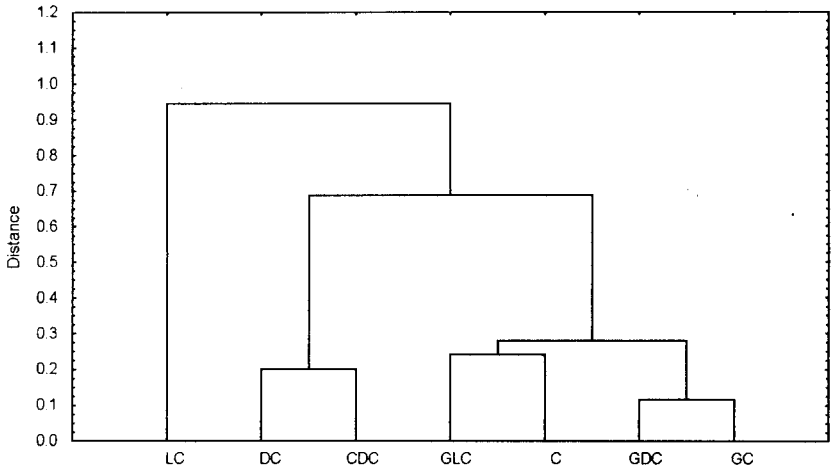

Figure 5. CA of R_f parameters of bile acids separated on aluminum plates precoated with silica gel $60F_{254}$ (#1.05554) by using mobile phase: *n*-hexane–ethyl acetate–acetic acid in different volume compositions (Euclidean distance, single bond method).

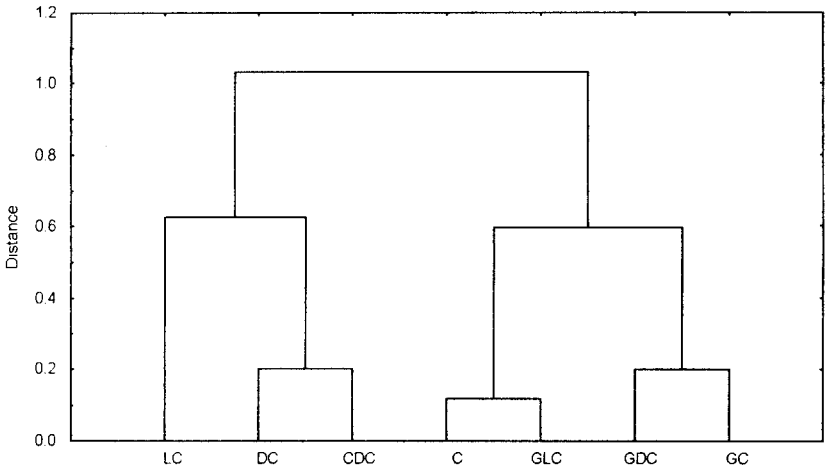

Figure 6. CA of R_f parameters of bile acids separated on aluminum plates precoated with mixture of silica get 60 and Kieselguhr F_{254} (#1.05567) by using mobile phase: *n*-hexane–ethyl acetate–acetic acid in different volume compositions (Euclidean distance, single bond method).

of silica gel 60/Kieselguhr F_{254}, whereas, GDC is better separated from GC on the mixture of silica gel 60 and Kieselguhr F_{254}, when compared with the acids separation on silica gel. It can be concluded, that for the plates precoated with silica gel the biggest problem is the separation of GC from GDC, whereas the biggest problem in the case of the mixture of silica gel 60 and Kieselguhr F_{254} is the separation of C from GLC.

The obtained results indicate that CA can be an alternative method of chromatographic estimation of studied bile acids. The conclusions arising from separations of studied bile acids obtained by using CA, are identical to the ones arising from the results obtained by using separation factors (ΔR_f and R_s).[11]

REFERENCES

1. Scalia, S. Bile acids separation. J. Chromatogr. B **1995**, *671*, 299–317.
2. Batta, A.K.; Salen, G. Gas chromatography of bile acids. J. Chromatogr. B **1999**, *723*, 1–16.
3. Lin, D.L.; Chang, H.Ch.; Chen, Ch.Y. Identification and quantitation of bile acids in bear bile by HPLC. J. Food Drug Anal. **2000**, *8* (4), 283–288.

4. Sasaki, T.; Iida, T.; Nambara, T. High performance ion-pair chromatographic behavior of conjugated bile acids with di-*n*-butyloamine acetate. J. Chromatogr. A **2000**, *888*, 93–102.
5. Sakkaura, H.; Kimura, N.; Takeda, H.; Komatsu, H.; Ishizaki, K.; Nagata, S. Simultaneous determination of bile acids in rat liver tissue by high-performance liquid chromatography. J. Chromatogr. B **1998**, *718*, 33–40.
6. Momose, T.; Mure, M.; Iida, T.; Goto, J.; Nambara, T. Method for the separation of the unconjugates of chenodeoxycholic acid and deoxycholic by two-dimensional reversed -phase thin -layer chromatography with methyl β-cyclodextrin. J. Chromatogr. A **1998**, *811*, 171–180.
7. Gaica, S.B.; Opsenica, D.M.; Šolaja, B.A.; Tešić, Ž.Lj.; Milković-Opsenica, D.M. The retention behavior of some cholic acid derivatives on different adsorbents. J. Planar Chromatogr. **2002**, *15*, 299–305.
8. Massart, D.L.; Vandeginste, B.G.M.; Deming, S.N.; Michotte, Y.; Kaufman, L. *Data Handling in Science and Technology*; Elsevier: Amsterdam, 1988; Vol. 2, 371–383.
9. Pyka, A.; Dołowy, M. Separation of selected bile acids by TLC. I. J. Liq. Chromatogr. Relat. Technol. **2003**, *26* (7), 1095–1108.
10. Pyka, A.; Dołowy, M. Separation of selected bile acids by TLC. II. One-dimensional and two-dimensional TLC. J. Liq. Chromatogr. Relat. Technol. **2004**, *27* (13), 2031–2038.
11. Pyka, A.; Dołowy, M. Separation of selected bile acids by TLC. III. Separation on various stationary phases. J. Liq. Chromatogr. Relat. Technol. **2004**, *27* (16), 2609–2619.

Received June 26, 2004
Accepted July 29, 2004
Manuscript 6423