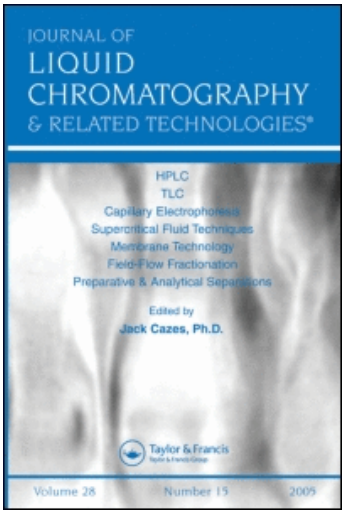

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Dołowy, M. , Bat, B. and Niestrój, A.(2009) 'Application of NP-TLC with Densitometric Detection for Separation and Quantitative Analysis of Unconjugated Bile Acids Presented in Human Bile', Journal of Liquid Chromatography & Related Technologies, 32: 1, 144 — 153
To link to this Article: DOI: 10.1080/10826070802548754
URL: http://dx.doi.org/10.1080/10826070802548754

Journal of Liquid Chromatography & Related Technologies®, 32: 144–153, 2009

ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826070802548754

Application of NP-TLC with Densitometric Detection for Separation and Quantitative Analysis of Unconjugated Bile Acids Presented in Human Bile

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Abstract: A simple and rapid method of separation and quantification of five bile acids such as: cholic acid (C), deoxycholic acid (DC), chenodeoxycholic acid (CDC), lithocholic (LC) and ursodeoxycholic (UDC) with the use of the NP-TLC method has been developed. The five bile acids were satisfactory separated on aluminum and glass plates precoated with silica gel 60 and silica gel $60F_{254}$ (E. Merck, Art. 1.05553, Art. 1.05554, and Art. 1.05715) using mobile phase *n*-hexane–ethyl acetate–acetic acid in different volume compositions. For the optimal conditions of separation the quantitative analysis of studied acids was elaborated on. The quantitative analysis was provided with the use of densitometric detection at $\lambda = 360$ nm. Densitometric analysis has been used to find the linear correlations between the peak area and amount of each bile acid spotted on the chromatographic plates. The statistical parameters of obtained linear regressions indicated that NP-TLC method with densitometric detection is statistically significant and can be used for separation and detection of C, DC, CDC, LC, and UDC in biological samples and also in pharmaceutical formulations.

Keywords: Adsorption TLC, Bile acids, Densitometry, Silica gel

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INTRODUCTION

Bile acids belong to one of the steroid groups. C, DC, CDC, LC, and UDC are usually present in human bile in free and conjugated forms with glycine and taurine.

They play a very important role in the laboratory diagnostics of liver and intestinal diseases, such as gall stones and cholestase. In routine diagnostics the enzymatic method is used to determine the content of all bile acids (not single bile acid) in human serum. Description of a new method for identification and quantitation of single unconjugated bile acids in human bile or serum can be helpful in better clinical diagnostics of digestive diseases. The literature describes many examples of application of chromatographic methods such as HPLC and GC for quantitative analysis of different bile acids, but usually in their conjugated forms or after derivatization.[1–4] We also tried to apply the HPLC technique with UV detection for the separation and detection of free bile acids: C, DC, CDC, LC, and UDC in mixture, but the method wasn't useful for separation of free bile acids without their previous derivatization.

This paper presents a rapid and successful method for separation and quantitative determination of five free bile acids in their mixture by the NP-TLC technique using UV densitometric detection. This analysis was performed on NP-TLC plates precoated with silica gel $60F_{254}$ (Art. 1.05715) and developed with the mobile phase *n*-hexane–ethyl acetate–acetic acid (22:22:5 v/v/v). Visualization of spots with the water solution of H_2SO_4 allowed detection of examined bile acids by densitometry in the UV region ($\lambda = 360$ nm) and quantitative analysis of the bile acids in a mixture. The linear relationships between peak area and amount of each bile acid spotted on chromatographic plates were found. The statistical parameters of linear regressions such as: standard error, correlation coefficient, significance level, F-value of Fisher test, indicates that the provided chromatographic method is statistically significant and can be used in routine analysis of biological samples. The developed method for quantitative analysis of bile acids will be used to analyze UDC in pharmaceutical formulations and human bile.

EXPERIMENTAL

Standard Substances

Cholic acid (No. C1129, 98%, Sigma Aldrich); Chenodeoxycholic acid, (No. C9377, 98%, Sigma Aldrich); Deoxycholic acid, (No. D2510, 99%, Sigma Aldrich); Ursodeoxycholic acid, (No. U5127, 99%, Sigma Aldrich); Lithocholic acid, (No. L6250, 98%, Sigma Aldrich); Methanol (POCh, Gliwice, Poland), was used for the preparation of bile acid solutions.

Solvents

The following components of the mobile phase: *n*-hexane (Merck, Germany), ethyl acetate (POCh, Gliwice, Poland), acetic acid 99.5% (POCh, Gliwice, Poland), were used for the NP-TLC analysis. Sulfuric acid, 95% (Chempur, Piekary Śląskie, Poland) was used to prepare a visualizing reagent. All chemicals were analytical grade.

Apparatus

A Desaga (Germany) Model CD 60 densitometer equipped with Windows–compatible ProQuant software was used.

Separation of Bile Acids with the Use of TLC

Separation of investigated bile acids: C, DC, CDC, LC, and UDC was performed on aluminum plates $20 \times 20\,cm^2$ precoated with silica gel $60F_{254}$ (E. Merck, Art. 1.05554), silica gel 60 (E. Merck, Art. 1.05553) and on glass plates precoated with silica gel $60F_{254}$ (E. Merck, Art. 1.05715). Before use the plates were activated at 120°C for 30 minutes. The methanolic solutions of the above mentioned bile acids in concentrations 5 mg/mL (12 mM) were spotted on a chromatographic plate in quantities of 15 μg of each standard in 3 μL methanol with the use of micropipettes (5 μL, Camag, Switzerland). The chromatograms were developed at room temperature in a 20 cm × 20 cm horizontal chamber (Camag, Switzerland) using *n*-hexane–ethyl acetate–acetic acid in volume compositions, which were previously optimal for separation of C, DC, CDC, and LC in mixture.[5,6]

The development distance was 14 cm. Mobile phases of 50 mL were used in all cases. Next the plates were dried at room temperature using a fume cupboard. The spots were visualized by spraying them using 10% water solution of sulfuric acids and then heating the plates at 120°C for 20 minutes.

Quantitative Determination of Bile Acids with the TLC Densitometric Method

The quantitative analysis of bile acids mixture containing: C, DC, CDC, LC, and UDC was performed on $10 \times 20\,cm^2$ glass plates precoated with silica gel $60F_{254}$ (E. Merck, Art. 1.05715). Before use, this plate was activated at 120°C for 30 minutes. For construction of the calibrations plots

for studied bile acids, and to determine their limit of detection (LOD) and quantitation (LOQ), the stock solutions of each at concentration of 12 mM were diluted. Ten methanolic standards solution of C, DC, CDC, LC, and UDC in the range 12 mM ÷ 0.024 mM (12; 6; 3; 1.5; 0.75; 0.375; 0.188; 0.094; 0.047; 0.024 mM) were spotted on a chromatographic plate in quantities of 3 μL with the use of micropipettes (5 μL, Camag, Switzerland) in equivalence: 0.014; 0.0071; 0.0035; 0.0018; 0.00088; 0.00044; 0.00022; 0.00011, and 0.000055 and 0.0000275 mg/spot, respectively. The chromatograms were developed at room temperature in a 20 cm × 20 cm horizontal chamber (Camag, Switzerland) using *n*-hexane–ethyl acetate–acetic acid in volume composition 22:22:5 (v/v/v). The development distance was 14 cm. Mobile phases of 50 mL were used in all cases. Next, the plates were dried at room temperature using a fume cupboard. The spots were visualized by spraying them using a 10% water solution of sulfuric acids and then heating the plates at 120°C for 20 minutes. After visualization, the chromatograms of bile acids were scanned with the use of a densitometer.

Densitometric Analysis of the Chromatograms

Densitometric scanning was performed by a TLC Scanner at maximum for bile acids ($\lambda = 360$ nm). The slit dimensions were 2×0.2 mm. The scanning speed was $20\ \text{nm} \cdot \text{s}^{-1}$. Each analysis was repeated three times. The results were used to determine the effect of the resolution of the mixture for investigated bile acids and to obtain the linear relationships between area of peaks and quantity of studied bile acids.

RESULTS AND DISCUSSION

The first step in NP-TLC quantitative analysis of free bile acids presented in bile such as C, DC, CDC, LC, and UDC was to find the optimum conditions for complete separation of above mentioned bile acids. For the separation of studied bile acids the same chromatographic conditions were applied (kind of plates and volume compositions of *n*-hexane–ethyl acetate–acetic acid as mobile phases), which were previously described for resolution of their conjugates with glycine.[5,6]

— The mixture of *n*-hexane–ethyl acetate–acetic acid in volume compositions: 22:21:5 and 25:20:8 for separation on aluminum TLC plates precoated with silica gel $60F_{254}$ (E. Merck, Art. 1.05554)
— The mixture of *n*-hexane–ethyl acetate–acetic acid in volume compositions 22:21:5; 22:22:5, 20:20:5 and 25:20:8 for separation on aluminum TLC plates precoated with silica gel 60 (E. Merck, Art.1.05553)

— The mixture of *n*-hexane-ethyl acetate–acetic acid in volume compositions: 22:22:5 and 20:20:5 in the case of the separation on glass plates precoated with silica gel $60F_{254}$ (E. Merck, Art.1.05715).

On the basis of R_F values obtained for all applied chromatographic conditions, the resolution parameters for each pair of studied bile acids like ΔR_F and R_S were calculated. The ΔR_F and R_S values for all pairs of bile acids are listed in Table 1.

According to Table 1, it can be observed that all used chromatographic conditions allow for complete separation of five studied bile acids. For each investigated pair of bile acids the $\Delta R_F \geq 0.05$ and $R_S > 1$ were obtained. Figure 1 presents the UV spectrum at $\lambda = 360$ nm nm of the mixture of bile acids separated on glass plates (Art. 1.05715) with *n*-hexane–ethyl acetate–acetic acid mixture in the volume composition 22:22:5 as a mobile phase.

This figure indicates that the applied chromatographic conditions allow for complete separation of five bile acids. The best separation was obtained for C and LC. The observations of the effect of bile acids resolution are similar to the conclusions based on separation factors presented in Table 1. The provided cluster analysis (CA) of R_F values for studied bile acids at all chromatographic conditions indicates that the poor similarity in separation of C and LC determines their good resolution. Figure 2 presents CA of R_F parameters for bile acids separated on glass plates (Art. 1.05715) with the use of mobile phase *n*-hexane–ethyl acetate–acetic acid in volume compositions 20:20:5 and 22:22:5.

The next part of the provided studies involved a search for the chromatographic method enabling, not only the separation of C, DC, CDC, LC, and UDC mixture presented in human bile, but also determination of their quantity. For determining the quantity of each bile acid in mixture, which corresponded with human bile content, the NP-TLC with densitometric detection was applied. A quantitative analysis of studied bile acids was performed on glass plates (Art. 1.05715) and developed with *n*-hexane–ethyl acetate–acetic acid in volume composition 22:22:5. This mobile phase allowed obtaining good resolution of investigated bile acids and was applied in the quantitative analysis. For construction of calibrations plots for five bile acids, the stock solutions (12 mM) of C, DC, CDC, LC, and UDC were diluted to the concentration in the range: 12 mM–0.024 mM. Next, we scanned the plates at $\lambda = 200$–360 nm to find the maximum of UV detection. The maximum of UV spectrum for each bile acid was exactly 360 nm according to Figure 3, which presents an example of UV-spectrum for DC at $\lambda = 200$–360 nm.

Table 1. The R_F values and separation factors (ΔR_F and R_S) of studied bile acids separated on aluminum plates precoated with silica gel 60 (E. Merck, Art. 1.05553), silica gel $60F_{254}$ (E. Merck, Art. 1.05554) and also on glass plates precoated with silica gel $60F_{254}$ (E. Merck, Art.1.05715) and developed with *n*-hexane–ethyl acetate–acetic acid as a mobile phase in different volume compositions (v/v/v), which were optimal for bile acids separation on non impregnated adsorbent at 18°C

	n-hexane–ethyl acetate–acetic acid (v/v/v) Art. 1.05553								*n*-hexane–ethyl acetate–acetic acid (v/v/v) Art. 1.05554				*n*-hexane–ethyl acetate–acetic acid (v/v/v) Art. 1.05715			
	22:21:5		20:20:5		22:22:5		25:20:8		25:20:8		22:21:5		22:22:5		20:20:5	
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	ΔR_F	R_S
C/DC	0.52	9.29	0.48	13.50	0.51	12.35	0.55	10.07	0.58	14.00	0.52	15.16	0.49	11.25	0.45	10.25
DC/CDC	0.10	2.00	0.07	1.82	0.07	1.67	0.10	2.08	0.07	1.48	0.08	1.83	0.09	11.47	0.07	1.59
CDC/LC	0.38	9.09	0.34	10.33	0.31	9.26	0.19	5.30	0.20	5.60	0.37	9.71	0.30	7.08	0.35	9.52
LC/UDC	0.42	9.91	0.37	10.74	0.35	9.00	0.27	5.62	0.26	6.00	0.40	11.10	0.36	7.48	0.41	9.74

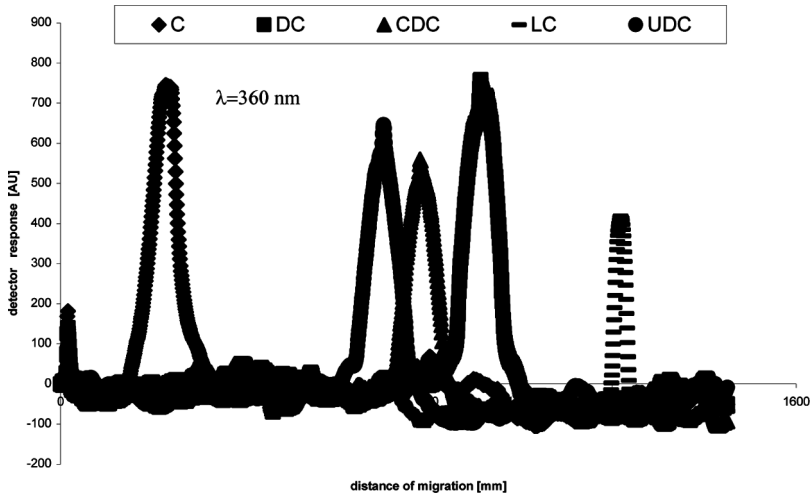

Figure 1. Densitogram of C, DC, CDC, LC, and UDC mixture separated on glass plates (Art. 1.05715) with the use of *n*-hexane–ethyl acetate–acetic acid in volume composition 22:22:5.

To prepare the calibrations plots, we measured the area of spots (AU) containing from 0.014 mg to 0.0000275 mg/spot of respective bile acid. The range of the linearity for examined bile acids is different:

- 14.71-0.92 μg/spot for C
- 7.07-0.44 μg/spot for DC
- 3.53-0.44 μg/spot for CDC

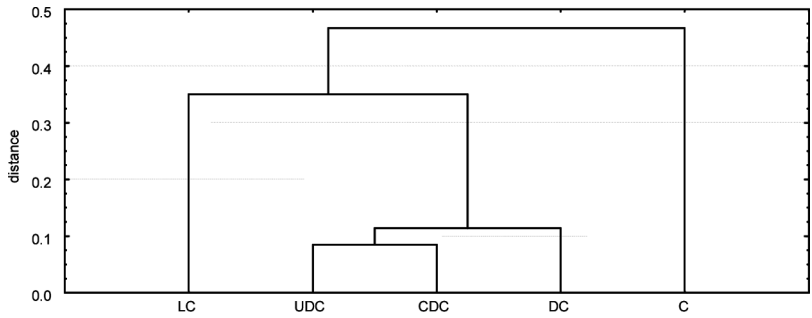

Figure 2. Cluster analysis of R_F parameters of bile acids separated on glass plates precoated with silica gel $60F_{254}$ (E. Merck, Art. 1.05715) by using *n*-hexane–ethyl acetate–acetic acid in volume compositions: 20:20:5 and 22:22:5 as mobile phases (Euclidean distance, single bond method).

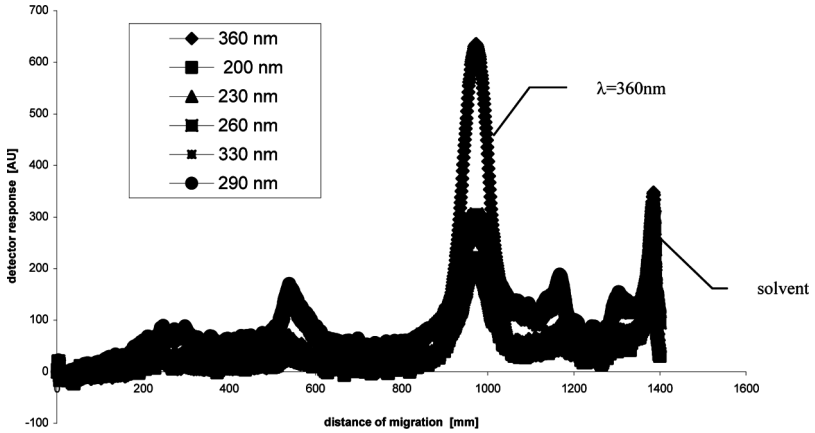

Figure 3. Densitogram of DC separated on glass plate (E. Merck., Art. 1.05715) with the use of *n*-hexane–ethyl acetate–acetic acid in volume composition 22:22:5.

- 1.96-0.21 μg/spot for LC
- 7.07-0.44 μg/spot for UDC

For the linear correlations the statistical parameters like: correlation coefficient (R), significance level (p), standard error (s), and the value of the Fisher test (F) were calculated. The values of the above mentioned parameters indicated that all obtained linear equations (given in Table 2) are statistically significant and can be used to determine the content of each bile acid in mixture.

Based on slope and standard deviation for five calibration plots, the limit of detection (LOD and also limit of quantification (LOQ) of examined bile acids were obtained (Table 2).

The lowest limit detection (LOD) can be observed for LC–0.119 μg/spot. The biggest one was obtained for C–2.085 μg/spot. The same situation was observed in the case of the quantification limit. The lowest value of LOQ obtained for LC 0.396 μg/spot and the biggest for C–6.951 μg/spot. The LOD and LOQ range in micrograms for all bile acids indicates that the provided method of separation and detection for studied bile acids can be applied in their clinical and pharmaceutical analysis.

CONCLUSIONS

The chromatographic conditions such as: mobile phase *n*-hexane–ethyl acetate–acetic acid in volume compositions 22:21:5 and 25:20:8 and

Table 2. Statistical parameters of linear correlations for the quantitation of bile acids separated on glass plates precoated with silica gel $60F_{254}$ (Art. 1.05715) with *n*-hexane–ethyl acetate–acetic acid as a mobile phase in volume composition 22:22:5 (v/v/v)*

Parameters	C	DC	CDC	LC	UDC
Wavelength [nm]	360	360	360	360	360
LOD [μg per spot]	2.085	1.274	0.133	0.119	0.946
LOQ [μg per spot]	6.951	4.248	0.443	0.396	3.154
Linearity range [μg/per spot]	14.71–0.92	7.07–0.44	3.53–0.44	1.96–0.21	7.07–0.44
Slope	618.13 ± 246.54	451.36 ± 237.09	32.16 ± 57.28	72.63 ± 31.82	-61.30 ± 234.22
Intercept	523.82 ± 246.54	824.72 ± 64.99	1504.59 ± 28.15	934.91 ± 32.65	1097.50 ± 64.20
Correlation coefficient (*R*)	0.9943	0.9908	0.9996	0.9988	0.9949
N	4	4	3	3	4
p-value	0.0005	0.0011	0.0003	0.0012	0.0004
Standard error (*s*)	364.13	350.35	66.66	37.04	346.12
F-ratio	260.19	161.05	2856.25	819.71	292.22

Note: *n*-number of points used to construction the particular regressions; *R*-correlation coefficient; *p*-significance level; *s*-standard deviation; *F*-value of the Fisher test; LOD-limit of detection; LOQ-limit of quantitation.

*99%-confidence level.

aluminum TLC plates precoated with silica gel $60F_{254}$ (E. Merck, Art. 1.05554), the mixture of *n*-hexane–ethyl acetate–acetic acid in volume compositions: 22:21:5; 22:22:5, 20:20:5 and 25:20:8 as mobile phase and aluminum TLC plates precoated with silica gel 60 (E. Merck, Art. 1.05553), and also the mixture of *n*-hexane–ethyl acetate–acetic acid in volume compositions: 22:22:5 and 20:20:5 and glass plates precoated with silica gel $60F_{254}$ (E. Merck, Art. 1.05715) allow for complete separation of the mixture of C, DC, CDC, LC, and UDC. The mobile phase *n*-hexane–ethyl acetate–acetic acids in volume composition: 22:22:5 and chromatographic plates Art. 1.05715 can be useful in the quantitative analysis of bile acids in biological samples and pharmaceutical formulations. The TLC densitometric analyses with UV detection of free bile acids such as: C, DC, CDC, LC, and UDC are possible only after visualization of spots.

ACKNOWLEDGMENT

This research was financed by Silesian University of Medicine.

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Received March 27, 2008
Accepted June 19, 2008
Manuscript 6352