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QUANTITATIVE ANALYSIS OF CHENODEOXYCHOLIC ACID AND RELATED COMPOUNDS BY A DENSITOMETRIC THIN-LAYER CHROMATOGRAPHIC METHOD

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SUMMARY

A new thin-layer chromatographic (TLC) method was developed for the isolation and determination of chenodeoxycholic acid and related compounds. The separation was performed on pre-coated Kieselgel 60 chromatoplates with chloroform–ethyl acetate–glacial acetic acid–2-methoxyethanol (9:9:2:1). The separated spots were evaluated by densitometry after treatment with *p*-toluenesulphonic acid. The separation of lithocholic, chenodeoxycholic, deoxycholic and cholic acid and their glycine and taurine conjugates was achieved with two chromatographic developments. Overpressured TLC gave a satisfactory separation of bile acids and related compounds by development of a high-performance TLC Kieselgel 60 chromatoplate with a four-component solvent mixture.

INTRODUCTION

Many different thin-layer chromatographic (TLC) systems have been developed for the analysis of bile acids and related compounds (see refs. 1–6 for comprehensive reviews), but the resolution of so-called "critical" pairs is difficult by the methods published earlier[7–11]. The successful separation of deoxycholic and chenodeoxycholic acid and their glycine and taurine conjugates was achieved by Goswami and Frey[12] with six successive chromatographic developments and similar results were subsequently obtained by other authors[13–16]. Reversed-phase partition TLC on octadecyl-bonded silica gel chromatoplates has been successfully applied by Raedsch *et al.*[11] to the separation of individual sulphated bile acid conjugates, but this method cannot be used for the analysis of other conjugates and non-conjugated bile acids. Gas–liquid chromatography (GLC) and high-performance liquid chromatography (HPLC) are less useful for the analysis of bile acids and their conjugates than TLC. The highly polar conjugates are not sufficiently volatile for GLC and the sensitivity of detection is too low for HPLC.

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One of the aims of our work was to develop a TLC method suitable for the analysis of chenodeoxycholic acid in the presence of other bile acids in pharmaceutical raw materials, where the problem is to determine the impurities occurring in low concentrations. The second objective was the separation of bile acids and their glycine and tuarine conjugates, a problem which has not yet been adequately solved. Our third aim was to test the potential of overpressured TLC (OPTLC), a technique which has been recently introduced[17].

Further improvement in separation can be expected from application of the OPTLC technique, which has been recently introduced in the practice of TLC by Tyihák *et al.*[17]. The technique is based on a concept similar to column liquid chromatography; the solvents are passed through an ultramicro chamber by an appropriate pump system and the sorbent layer is completely covered in the chamber with a flexible membrane under an external pressure. In this manner the vapour space over the layer can be excluded, resulting in more reproducible retention, better resolution and faster development compared with classical TLC systems.

EXPERIMENTAL

TLC was performed on pre-coated Kieselgel 60 F_{254} (Merck, Darmstadt, G.F.R.) chromatoplates. For the analysis of chenodeoxycholic acid, chloroform–ethyl acetate–glacial acetic acid–2-methoxyethanol (9:9:2:1) was used as mobile phase.

For the separation of bile acids and conjugates two chromatographic developments were needed. The sample solutions, prepared from the reference substances listed in Table I, were applied twice in 1-cm streaks to the chromatoplate, and solvent A [isooctane–ethyl acetate–glacial acetic acid (5:4:1)] was used for the first development to separate the critical pairs of non-conjugated bile acids. The plate was then dried in a stream of air at room temperature and a narrow channel was cut into the silica gel around the compounds separated by the first development (see Fig. 3). The plate was developed a second time with solvent B [chloroform–*n*-butanol–glacial acetic acid–water (2:16:1:1)].

OPTLC was performed in a Labor MIM (Esztergom-Budapest, Hungary) pressurized ultramicro chamber, the solvents being introduced into the chamber under the flexible membrane by a Micropump Type S13 (Labor MIM). Linear migration of the solvent front in the ultramicro chamber was ensured by impregnating the sides of the layer and by placing a narrow plastic sheet on the layer behind starting line.

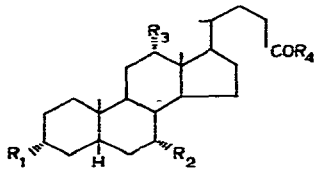
The separation was performed on a high-performance TLC (HPTLC) Kieselgel 60 F_{254} chromatoplate with solvent B as the mobile phase.

The separated spots were evaluated by densitometry after spraying with 20% *p*-toluenesulphonic acid, dissolved in sulphuric acid–ethanol (1:1) in the case of chenodeoxycholic acid and 20% aqueous sulphuric acid in the case of bile acids conjugates. The spots were developed by heating the sprayed plates at 120°C for 20 min. For the analysis, a Zeiss PMQ III chromatogram spectrophotometer (Opton, G.F.R.) was used.

All solvents and reagents were of analytical-reagent grade (Reanal, Budapest, Hungary). The non-conjugated bile acids were prepared at the Chemical Works of Gedeon Richter (Budapest, Hungary), and the glycine and taurine conjugates of bile acids were supplied by T. Fehér.

TABLE I

COMPOUNDS INVESTIGATED



No.	*Compound*	R_1	R_2	R_3	R_4*
I	Lithocholic acid	OH	H	H	OH
II	Deoxycholic acid	OH	H	OH	OH
III	Chenodeoxycholic acid	OH	OH	H	OH
IV	Cholic acid	OH	OH	OH	OH
V	Glycolithocholic acid	OH	H	H	Gly
VI	Glycochenodeoxycholic acid	OH	OH	H	Gly
VII	Glycodeoxycholic acid	OH	H	OH	Gly
VIII	Taurolithocholic acid	OH	H	H	Tau
IX	Glycocholic acid	OH	OH	OH	Gly
X	Taurochenodeoxycholic acid	OH	OH	H	Tau
XI	Taurodeoxycholic acid	OH	H	OH	Tau
XII	Taurocholic acid	OH	OH	OH	Tau

* Gly = $-NH-CH_2-COOH$; Tau = $-NH-CH_2-CH_2-SO_3H$.

RESULTS AND DISCUSSION

The compounds investigated are listed in Table I.

For the detection of other bile acids in chenodeoxycholic acid, various solvent systems were tried. The chromatograms obtained with three different solvents are shown in Fig. 1.

Chloroform–ethyl acetate–glacial acetic acid–2-methoxyethanol (9:9:2:1) provides the best overall separation, although better resolution can be obtained for deoxycholic acid and chenodeoxycholic acid by using solvent A, previously reported by Fehér and Kazik[7]. Fig. 1 shows that one of the unknown impurities (X_3) is separated satisfactorily from cholic acid only by solvent C (Fig. 2). Fig. 2 shows that a satisfactory separation of the impurities occurring in low concentrations can be achieved when 1 mg of the sample is applied to the plate.

None of the three eluent systems mentioned above can be used for the separation of different conjugates of bile acids because these compounds stay in the neighbourhood of the origin. The less polar non-conjugated bile acids and their more polar glycine and taurine conjugates can be resolved when two eluent systems of different polarities are used. The basic concept is illustrated in Fig. 3 where chromatograms obtained for a model mixture of all the compounds listed in Table I are shown.

The sample solution is applied twice to the same chromatoplate. For the first chromatographic development solvent A is used. This solvent system permits the separation of compounds I–V, while the other compounds stay near the origin. After this development the compounds separated are isolated from the solvents to be used

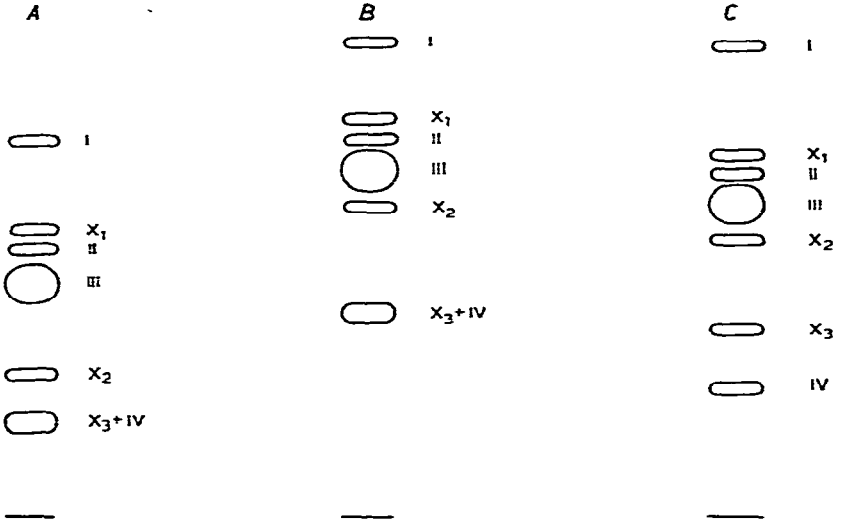


Fig. 1. Separation of chenodeoxycholic acid and related compounds. Plate: Kieselgel 60 F_{254}. Solvent A, isooctane–ethyl acetate–glacial acetic acid (5:4:1); solvent B, dichloromethane–ethyl acetate–glacial acetic acid–2-methoxyethanol (9:9:2:1); solvent C, chloroform–ethyl acetate–glacial acetic acid–2-methoxyethanol (9:9:2:1). Application: 1000–1000 μg of the sample in 1-cm streaks. Detection: 20% *p*-toluenesulphonic acid in sulphuric acid–ethanol (1:1), heated at 120°C for 20 min. Compounds I–IV, see Table I; X_1, X_2, X_3, unknown.

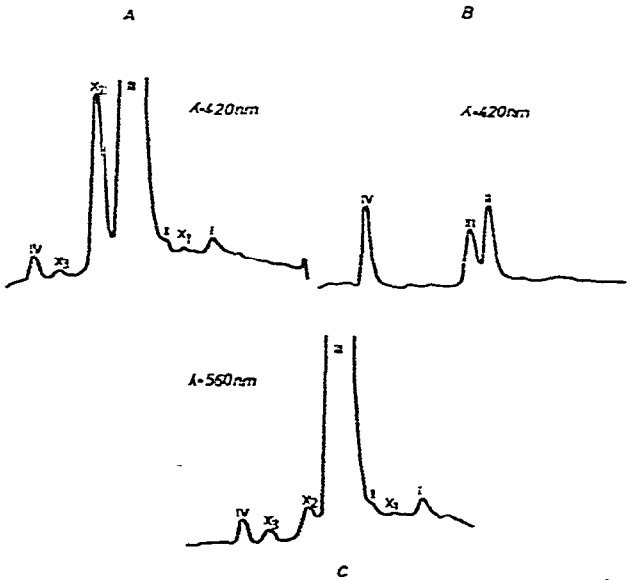


Fig. 2. Densitogram of the separated compounds. Developed with solvent C and assayed with a Zeiss PMQ III chromatogram spectrophotometer at 420 nm (A, B) and 560 nm (C). Application: 1000–1000 μg of the sample (Fig. 2A and 2C) and 10–10 μg of reference compounds (Fig. 2B). For other conditions see Fig. 1.

in the second development by cutting a narrow channel into the silica gel. The more polar compounds (VI–XII) are then separated by a second development with solvent B. No separation of compounds II and III, however, can be achieved owing to the more polar solvent system used.

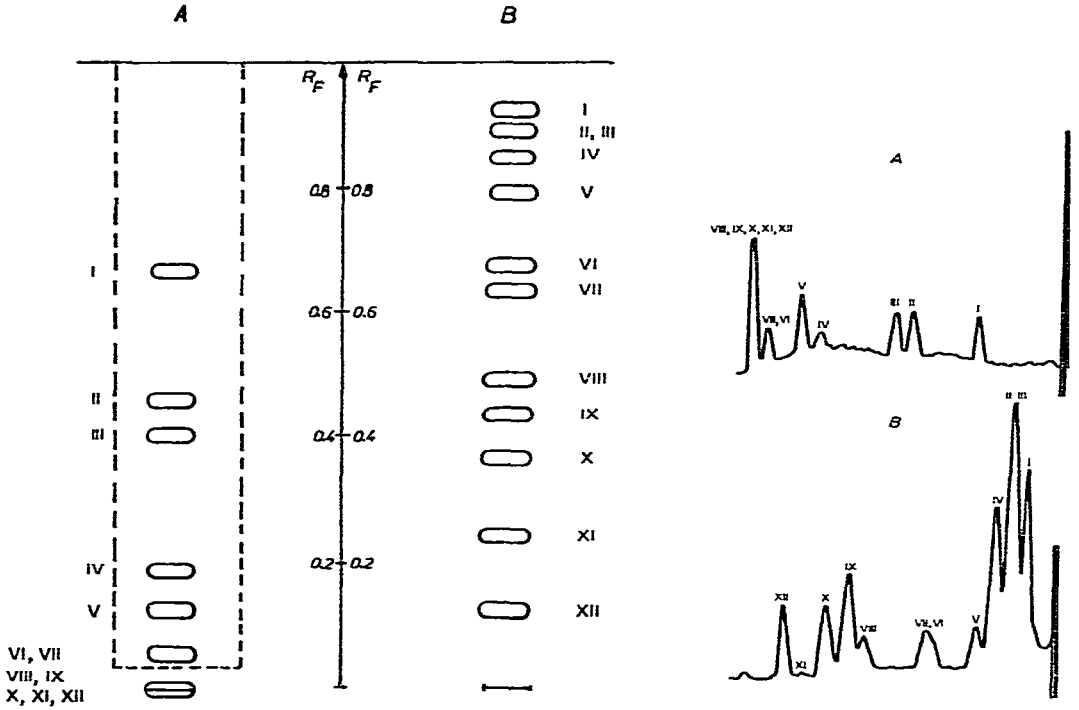


Fig. 3. Chromatogram of bile acids and their conjugates. Solvent A, isooctane–ethyl acetate–glacial acetic acid (5:4:1); solvent B, chloroform–1-butanol–glacial acetic acid–water (2:16:1:1) after first development with solvent A. Detection: 20% sulphuric acid, heated at 120°C for 20 min. For other conditions see Fig. 1. For compounds see Table I.

Fig. 4. Densitogram of bile acid conjugates. Densitometer, Zeiss PMQ III chromatogram spectrophotometer at 366 nm. For other conditions see Fig. 3.

Fig. 4 shows the densitogram of the separated compounds. In Fig. 4B the peaks of compounds VI and VII as well as those of compounds X and XI are not resolved, but these compounds can be distinguished at 366 nm. The separation of compounds investigated by OPTLC using solvent B is shown in Fig. 5.

Comparing the densitograms in Figs. 3 and 5, it can be concluded that the separation efficiency is improved by using OPTLC and that a satisfactory separation is achieved for the "critical" pairs of conjugates. The R_F values of the compounds obtained in different solvent systems are summarized in Table II.

CONCLUSIONS

The separation of chenodeoxycholic acid and related compounds has been studied by TLC. For detecting the presence of impurities in bulk chenodeoxycholic

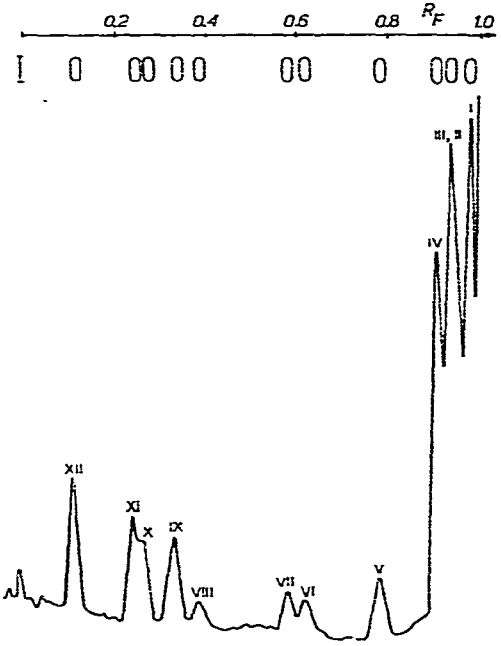


Fig. 5. Separation of bile acids and their conjugates by OPTLC. Labor MIM pressurized ultramicro chamber with Micropump S13, Zeiss PMQ III chromatogram spectrophotometer at 366 nm. Mobile phase: chloroform–1-butanol–glacial acetic acid–water (2:16:1:1); flow-rate 26 ml/h; pressure 1.2 MPa; 16 cm development in 28 min; plate: HPTLC silica gel 60 F_{254} with impregnated edges. For compounds see Table I.

TABLE II

R_F VALUES OF THE COMPOUNDS INVESTIGATED

Plates: 1–4, Kieselgel 60 F_{254}; 5, HPTLC silica gel 60 F_{254}. Solvents: 1, dichloromethane–ethyl acetate–glacial acetic acid–2-methoxyethanol (9:9:2:1); 2, chloroform–ethyl acetate–glacial acetic acid–2-methoxyethanol (9:9:2:1); 3, isooctane–ethyl acetate–glacial acetic acid (5:4:1); 4 and 5, sol ent D, chloroform–1-butanol–glacial acetic acid–water (2:16:1:1). Other conditions: 1–3, see Fig. 1; 4, see Fig. 3; 5, see Fig. 5. Compounds I–XII, see Table I; X_1, X_2, X_3, unknown.

No.	*1*	*2*	*3**	*4***	*5****
I	0.84	0.83	0.66	0.92	0.98
X_1	0.70	0.71	0.50		
II	0.66	0.64	0.46	0.89	0.94
III	0.62	0.59	0.41	0.89	0.94
X_2	0.54	0.40	0.24		
X_3	0.35	0.26	0.16		
IV	0.35	0.16	0.16	0.84	0.90
V			0.13	0.79	0.78
VI			0.05	0.67	0.61
VII			0.05	0.63	0.58
VIII			0	0.48	0.38
IX			0	0.43	0.33
X			0	0.36	0.26
XI			0	0.25	0.23
XII			0	0.15	0.11

* TLC, after first development.
** TLC, after second development.
*** OPTLC.

acid, solvent C was found to be optimal. For the separation of bile acids and their glycine and taurine conjugates, the OPTLC technique with a HPTLC silica gel chromatoplate and solvent D was found to be optimal. The use of classical TLC can also resolve the conjugated and non-conjugated bile acids, but the "critical" pairs of conjugated bile acids (glycochenodeoxycholic acid–glycodeoxycholic acid and taurochenodeoxycholic acid–taurodeoxycholic acid) can be distinguished only at 366 nm. The proposed method seems to be promising for clinical and biological analysis because a satisfactory separation for non-conjugated and conjugated bile acids was obtained.

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