Deoxycholic (DC) and chenodeoxycholic (CDC) acids are typically C24 polyhydroxy steroids which are formed in the liver from cholesterol. They serve different functions, such as elimination of cholesterol from the body and emulsifying lipids and fat-soluble vitamins in the intestine to form micelles that can be transported via the lacteal system (1). The conjugated bile acid salts with taurine or glycine, respectively, are more efficient especially at emulsifying fats. Determination of bile acids concentration in biological samples (e.g., serum) provides information important in clinical study, for instance in diagnosis of hepatobiliary diseases. The analysis of bile acids including DC and CDC in biological matrices as well as in pharmaceutical samples depends on accurate sample pretreatment. Among various advanced chromatographic techniques, such as gas chromatography (GC) and high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC) is applicable to separation of bile acids (as free and conjugates with taurine and glycine) in preliminary study of bile acid mixture. It is well known that thin-layer chromatography coupled with densitometry is a fast, simple and inexpensive method as opposed to other chromatographic techniques. The main advantage of TLC-densitometry is that a number of bile acids can be handled simultaneously in one step. In addition to this, the bile acids analyzed by TLC with densitometric detection can be visualized directly on chromatographic plate after removing off mobile phase. However, in the case of TLC analysis of described bile acids, the scientists must be confronted with a problem of separation of two optically active bile acids DC, CDC and their conjugates. Literature review indicates that in order to overcome the problem of TLC separation of different classes of optically active compounds, various chiral selectors as impregnating reagents (stationary phase modifiers) as well as mobile phase additives have been used (2-7). Of many chiral selectors which have been presented in the available literature, β-cyclodextrin (β-CD) is often recommended as mobile phase additive or stationary phase modifier for the resolution of...
selected optically active compounds (medical and also pharmaceutical importance), like, for example: propranolol and atenolol (8), ropivacaine (9), mandelic acid (10), some amino acids (11) or cefaclor epimers (12).

According to the best authors knowledge, only few papers have reported the use of β-cyclodextrin for the separation of difficult to separate pair of bile acids: DC and CDC. These studies involved mainly the two dimensional (2D) reversed phase (RP) high performance thin-layer chromatographic technique (HPTLC) and silica gel RP-18F254 as stationary phase (13-15). The present work reports the direct resolution of DC and CDC and their conjugated forms with glycine and taurine by means of β-cyclodextrin as mobile phase additive in one dimensional (1D) RP-HPTLC system coupled with densitometry. Different chromatographic plates have been tested in this study, such as RP-2F254, RP-8F254 and RP-18F254. Effect of variation of β-cyclodextrin concentration in mobile phase: methanol-water was studied to optimize the chromatographic conditions, appropriate for complete bile acid resolution. The results of studies obtained on RP-HPTLC plates were compared with those obtained on HPTLC chiral plates.

EXPERIMENTAL

Chemicals and sample preparations

The reference standards of deoxycholic acid (DC), chenodeoxycholic acid (CDC), and also their conjugated forms as sodium salts, such as taurodeoxycholic acid (TDC), taurochenodeoxycholic acid (TCDC), glycodeoxycholic acid (GDC), glycochenodeoxycholic acid (GCDC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Standard mixture containing 5 mg of each studied bile acid in 1 mL was prepared in methanol (POCh, Gliwice, Poland). β-Cyclodextrin (β-CD, 98%) was from Sigma-Aldrich. Aqueous solutions of β-CD in the following concentrations: 0.5, 1, 2, 3 and 5% were obtained by dissolving of proper amount of this substance in water at higher temperature. The following components of mobile phase: methanol-water was studied to optimize the chromatographic conditions, appropriate for complete bile acid resolution. The results of studies obtained on RP-HPTLC plates were compared with those obtained on HPTLC chiral plates.

Measuring optical rotation of examined bile acids

In order to confirm the optical activity of examined bile acids: DC, CDC, GDC, GCDC, TDC and TCDC, which is not widely described for these compounds in literature, digital polarimeter type P-2000 from Jasco (United Kingdom) was applied. Optical activity of six investigated bile acids, which is the measure of the ability of them to rotate plane polarized light, was expressed as specific rotation (αD). For measuring this parameter for all studied bile acids at 22 ± 1°C, the methanolic solutions of respective compound at concentrations 10 mg/mL were used. The length of the polarimeter tube was 5 cm. The source of light was sodium lamp.

Chromatography

Chromatography was performed on 10 × 10 cm reversed phase plates: RP-18F254, RP-2F254, RP-8F254, and also on HPTLC chiral plates. Samples of mixture containing six examined bile acid: DC, CDC, GDC, GCDC, TDC and TCDC in quantity of 5 µL were applied on the plates.

The chromatograms were developed at 18 ± 2°C in a 10 × 20 cm chromatographic chamber (Camag, Switzerland) which has been previously saturated with solvent vapors during 30 min. The development distance was 8 cm. After developing with methanol-water (40 : 10, v/v) or methanol-water-β-CD (40 : 10 : 5, v/v/v), respectively, the chromatographic plates were dried at 18 ± 2°C using a fume cupboard. Visualization of obtained spots was conducted by dipping the plates in 10% ethanolic solution of sulfuric acid and next, heating them at 110 ± 2°C for 15 min. The plates were scanned by Camag TLC Scanner 3 (Muttenz, Switzerland) which was controlled by WinCATS 1.4.2 software. All densitometric measurements were conducted in reflectance absorbance mode at wavelength of 400 nm. This wavelength was optimal for the examined bile acids and hence it has been selected for further densitometric analysis. The source of radiation was a deuterium lamp. The scanning speed was 20 nm/s and the data resolution was 100 µm/step. The slit dimension was kept at 8.00 × 0.40 mm, Macro. Each analysis was repeated three times. Densitograms obtained under described conditions allowed to estimate the efficiency of separation of six examined bile acids from their mixture.
Figure 1. Chromatograms showing the resolution of examined bile acids on reversed phase plates RP-2F254 developed with the following mobile phases: methanol-water (40:10, v/v) (a), methanol-water-β-CD (0.5%) in volume composition 40:10:5 (b), methanol-water-β-CD (1%) in volume composition 40:10:5 (c) and methanol-water-β-CD (3%) in volume composition 40:10:5 (d)
RESULTS AND DISCUSSION

The purpose of the work reported herein was to develop a simple and rapid TLC-densitometric method suitable for the complete separation and characterizing of two optically active bile acids: deoxycholic and chenodeoxycholic and their conjugates with taurine and glycine (DC, CDC, TDC, TCDC, GDC, GCDC). Optical activity of discussed compounds confirmed the polarimetric measurements. For examined bile acids, the following values of $\alpha_{D}^{22}$ were obtained: +61.2° (DC), +15.4° (CDC), +41.2° (TDC), +22.4° (TCDC), +50.2° (GDC) and +13.4° (GCDC). During preliminary study, various chromatographic conditions (different adsorbents and mobile phases) in normal and reversed phase systems (NP-TLC and RP-TLC) were tested. In order to improve the effect of bile acids separation by NP-TLC technique on silica gel 60 and 60F254, one of the most popular chiral selector, namely $\beta$-cyclodextrin ($\beta$-CD) was applied. Aqueous solution of $\beta$-CD was used as mobile phase additive and also as impregnating agent of silica gel (stationary phase). Unfortunately, the applied chromatographic conditions in NP-TLC system consisted of silica gel impregnated with aqueous solution or aqueous-methanol solution of $\beta$-CD, respectively, could not provide complete resolution of examined bile acids including unconjugated DC and CDC. Similarly, none of prepared mobile phases containing $\beta$-CD in various proportions, such as methanol-water-$\beta$-CD, methanol-acetic acid-$\beta$-CD, chloroform-methanol-acetic acid-$\beta$-CD, methanol-acetic acid-water-$\beta$-CD, which have been tested in preliminary study, did not allow complete separation of studied bile acids. Efforts were continued, and in further steps of this experiment it was decided to use one dimensional (1D) reversed phase system (RP-TLC, RP-HPTLC) and also HPTLC chiral plates. In order to achieve satisfactory resolution of two investigated bile acids (DC, CDC) and their conjugates, different chromatographic plates from E. Merck (RP-18F254, RP-2F254, RP-8F254, chiral plates) and mobile phase consisting of methanol-water-$\beta$-CD in volume composition: 40 : 10 : 5 were used. In this paper, the influence of $\beta$-CD content in mobile phase applied and the type of chromatographic plates on the separation of studied bile acids and their conjugates with glycine and taurine, respectively, by means of 1D RP-HPTLC and also 1D RPTLC system are described.

The first of RP-HPTLC chromatographic systems which has been tested, was consisted of RP-2F254 plates as stationary phase and methanol-water-$\beta$-CD as mobile phase in volume composition 40 : 10 : 5. Different concentration in [% w/w]: 0.5; 1; 2; 3 and 5 of aqueous solution of $\beta$-CD added to mobile phase was applied. The results of analyzed mixture obtained using RP-2F254 plates and mobile phase containing $\beta$-CD at different concentration indicate that the use of RP-2F254 plates and mobile phase containing $\beta$-CD at different concentration indicate that the use of RP-2F254 plates and mobile phase containing $\beta$-CD (40 : 10 : 5, v/v/v) improves the resolution of

![Figure 2](image-url)  
Figure 2. The effect of $\beta$-CD concentration as mobile phase additive on separation factor ($\Delta R_f$) of studied bile acids determined on RP-2F254 plates developed with the mobile phase methanol-water-$\beta$-CD in volume composition 40 : 10 : 5. The concentration of $\beta$-CD was: 1, 2, 3 and 5%, respectively.
The effect of \(\beta\)-cyclodextrin on the resolution of free and conjugated forms of bile acids was studied. Figure 3 shows chromatograms demonstrating the resolution of examined bile acids on reversed phase plates RP-8F254 developed with the following mobile phases: methanol-water (40 : 10, v/v) (a), methanol-water-\(\beta\)-CD (0.5%) in volume composition 40 : 10 : 5 (b), methanol-water-\(\beta\)-CD (2%) in volume composition 40 : 10 : 5 (c).

Examined bile acids significantly. The chromatograms of mixture consisted of DC, CDC, TDC, TCDC, GDC and GCDC acids separated on RP-2F254 plates with the use of this mobile phase, where \(\beta\)-CD concentration was: 0.5, 1, 3%, and also to compare those produced by mobile phase without \(\beta\)-CD are presented in Figure 1. Good separated peaks coming from investigated mixture of bile acids show the
Figure 4. Chromatograms showing the resolution of examined bile acids on reversed phase plates RP-18F254 developed with the following mobile phases: methanol-water (40 : 10, v/v) (a), methanol-water-\(\beta\)-CD (0.5%) in volume composition 40 : 10 : 5 (b), methanol-water-\(\beta\)-CD (2%) in volume composition 40 : 10 : 5 (c).

Exemplary chromatograms presented in Figure 1 c and d. On the basis of Figure 1, it could be observed that addition of \(\beta\)-CD at concentration from 1 to 5% to the mobile phase enabled resolution of all investigated compounds. The best results of separation of studied bile acids, especially DC and CDC can be observed on RP-2F254 plates developed using mobile phase containing 3% \(\beta\)-CD (Fig. 1 d). In this case,
there are two separated peaks coming from both bile acids with regular shape. Moreover, to confirm this complete separation, comparison the values of separation factor ($\Delta R_f$) calculated for each pair of examined bile acids, which have been determined on the basis of chromatograms produced using 1, 2, 3, and 5% solutions of $\beta$-CD and RP-2F$_{254}$ plates was done (Fig. 2). Complete resolution of each pair (two
neighboring bile acids) enables $\Delta R_F = 0.05$. The results of $\Delta R_F$ calculated for five pairs of studied bile acids show that the proposed chromatographic conditions allowed complete resolution of all compounds because the $\Delta R_F$ values obtained for five pairs of examined bile acids: DC/CDC, CDC/GDC, GDC/GCDC, GCDC/TDC and TDC/TCDC are higher than 0.05 in each case. Figure 2 confirms that applied $\beta$-CD as mobile phase additive improves the resolution of free bile acids: DC and CDC and their conjugates, especially CDC/GDC and also GCDC/TDC.

Next applied reversed phase plates were RP-8F$_{254}$. The exemplary chromatograms obtained by means of this stationary phase and mobile phases: methanol-water without adding $\beta$-CD and also these containing 0.5% and 2% aqueous solution of $\beta$-CD confirm that $\beta$-cyclodextrin improves separation of examined bile acids, in particular glycine and taurine conjugates of DC and CDC, such as GDC/GCDC and TDC/TCDC (Fig. 3 a, b, c). Unfortunately, none of applied modification of mobile phase by use of $\beta$-CD at concentration: 0.5, 1, 2, 3 and 5% did not allow for successful separation of all examined pairs of six bile acids. The biggest problem in the case of discussed silica gel RP-8F$_{254}$ was to achieve complete separation of DC from CDC.

Continuing this research on bile acids separation by 1D RP-TLC system and $\beta$-CD as mobile phase component, next type of commercially available reversed phase plates RP-18F$_{254}$ was applied. Chromatograms of separated bile acids obtained on RP-18F$_{254}$ plates developed using mobile phase containing $\beta$-CD at concentration 0.5, 1, 2, 3 and 5% indicate that addition of $\beta$-CD to applied mobile phase was suitable to improve the separation of glycine and taurine conjugates of examined bile acids, such as GDC from GCDC and also TDC from TCDC, but it did not allow to solve the problem of separation of difficult to separate pair of free bile acids DC/CDC (Fig. 4 a, b, c). Thus, it could be concluded that RP-18F$_{254}$ plates allow to obtain very similar results of separation of investigated mixture like above described RP-8F$_{254}$ plates and these plates may be applied as an alternative to RP-8F$_{254}$ in bile acid separation.

In the third part of this study, the commercially available HPTLC chiral plates were applied. Addition of $\beta$-CD at concentration from 0.5 to 5% to the mobile phase: methanol-water (40 : 10, v/v) in quantity of 5 mL improved the resolution of the following bile acids: GDC/GCDC and TDC/TCDC but the effect of separation of free DC and CDC was not so satisfactory like in the case of previously presented RP systems (Fig. 5 a, b and c). Applied chromatographic conditions did not enable separation of DC and CDC. Thus, it could be suggested that of two 1D TLC systems tested in this work, RP is more efficient than NP for the separation of optically active bile acids, such as DC and CDC and their conjugated forms. Developed 1D RP-HPTLC system with the use of RP-2F$_{254}$ plates and methanol-water-$\beta$-CD (40 : 10 : 5, v/v/v) as mobile phase is the most suitable for the separation and characterizing of examined bile acids, including unconjugated forms of DC and CDC. Other applied chromatographic plates (RP-8F$_{254}$, RP-18F$_{254}$ and also HPTLC chiral plates) and described mobile phase can improve only the separation of glycine and taurine conjugates of studied bile acids: GDC, GCDC, TDC and TCDC.

**CONCLUSIONS**

In conclusion, the authors of presented TLC-densitometric study confirmed that described components of both applied TLC systems, like mobile phase content (especially $\beta$-CD concentration) and also the type of chromatographic plates can improve the resolution of proper pair of free and also conjugated with glycine and taurine deoxycholic and chenodeoxycholic acids. Of all applied chromatographic conditions, RP-2F$_{254}$ plates and one dimensional (1D) system with the use of mobile phase: methanol-water-$\beta$-cyclodextrin in volume composition 40 : 10 : 5 at different concentration of aqueous solution of $\beta$-cyclodextrin (1, 2, 3, 5%) are useful to complete resolution and characterizing of mixture containing all examined bile acids including difficult to separate free deoxycholic and chenodeoxycholic acids. The conjugated with taurine (TDC, TCDC) and with glycine (GDC, GCDC) bile acids can be satisfactory separated also on other chromatographic plates described in this paper like, for example, RP-8F$_{254}$ or RP-18F$_{254}$, respectively, developed by proposed mobile phase consisting of $\beta$-CD.

The developed RP-HPTLC procedure in one dimensional system is simple to use and rapid, thus, it can be used in preparative step of medical or pharmaceutical analysis of studied bile acids by modern chromatographic techniques, such as high-performance liquid chromatography or gas-liquid chromatography.

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