Purification of Hyodeoxycholic Acid and Chenodeoxycholic Acid from Pig Bile Saponification Solution Using Column Chromatography

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The purpose of this study was to purify hyodeoxycholic acid and chenodeoxycholic acid from pig bile saponification solution using column chromatography. The optimal resin was selected through static adsorption experiments. Loading, washing, and elution conditions were optimized. Hyodeoxycholic acid and chenodeoxycholic acid were further purified through crystallization. The best resin was found to be CG161M macroporous resin. After optimization, the sample was loaded at a volume flow of 3 BV h^{-1} to adsorb 5 BV, washed with 40 % ethanol, eluted with 45 % ethanol and 60 % ethanol, respectively. This resulted in purities for hyodeoxycholic acid of 70.34 % and 66.21 % for chenodeoxycholic acid with yields of 86.48 % and 90.57 %, respectively. Crystals of hyodeoxycholic acid with a purity of 91.04 % and chenodeoxycholic acid precipitate with a purity of 80.28 % were obtained through crystallization. Hyodeoxycholic acid and chenodeoxycholic acid can be purified using CG161M resin, and subsequent crystallization yields high-purity hyodeoxycholic acid.

Keywords
hyodeoxycholic acid, chenodeoxycholic acid, column chromatography, separation, purification

Introduction

The pig gall is the gallbladder of a pig (*Sus scrofa domestica* Brisson). It has the ability to clear heat, moisturize dryness, alleviate cough and asthma, and detoxify toxins[1]. Pig bile mainly consists of bile acids, bile pigments, amino acids, lipids, and inorganic substances. Following the saponification of pig bile, hyodeoxycholic acid and chenodeoxycholic acid emerge as the two bile acid components with the highest content[2–5], and their structural formulas are presented in Fig. 1. Hyodeoxycholic acid is known for its effects in reducing blood lipid levels, eliminating phlegm, and inhibiting bacteria, and can be used to treat hyperlipidemia and other diseases[1, 6–8]. Chenodeoxycholic acid exhibits antiasthmatic, anti-inflammatory, antitussive, and expectorant effects, and is primarily utilized to treat cholesterol gallstones[9–13].

The chemical structures and physical properties of hyodeoxycholic acid and chenodeoxycholic acid are similar. Extracting and purifying them from porcine bile and its residues is a challenging task. Currently, methods for extracting hyodeoxycholic acid include the direct crystallization method[14], esterification method[15], alkaline earth metal salt precipitation method[16], and column chromatography method[6]. Methods for extracting chenodeoxycholic acid include esterification acylation[15], alkaline earth metal salt precipitation[17,18], and column chromatography[19]. In industry, many of these methods are often combined to extract hyodeoxycholic acid and chenodeoxycholic acid. However, there are still some disadvantages, such as a complex extraction process, significant solvent consumption, low yield, and high production costs.

Compared to other technologies, column chromatography offers the advantages of easy operation and strong separation capability, and is widely used in the purification of saponins, flavonoids, and alkaloids[20]. The macroporous adsorption resin method

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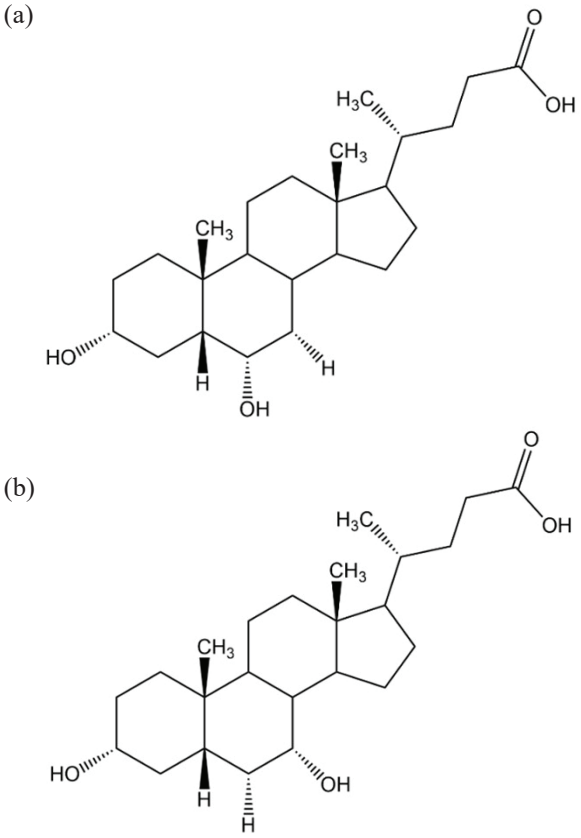

Fig. 1 – *Structural formula of (a) hyodeoxycholic acid and (b) chenodeoxycholic acid*

and ion exchange resin method have been used to separate and purify bile acids[21–23]. For example, Su *et al*. obtained high-purity hyodeoxycholic acid using a silica gel column with a high content of hyodeoxycholic acid as a raw material[6]. Shen *et al.* combined the esterification method and a silica gel column to obtain hyodeoxycholic acid with a purity higher than 99 % [24]. Zhang and Yang used a silica gel column to obtain chenodeoxycholic acid with a purity exceeding 98 % [25]. Zhang *et al*. combined the esterification method and a silica gel column to obtain chenodeoxycholic acid with a purity higher than 99 % [26]. Bi *et al*. combined the calcium salt method and ion exchange resin to obtain chenodeoxycholic acid. However, there is no reported instance simultaneously preparing hyodeoxycholic acid and chenodeoxycholic acid using column chromatography[27].

In this study, porcine bile saponification liquid, obtained after bilirubin extraction, served as a raw material. Simultaneous purification of hyodeoxycholic acid and chenodeoxycholic acid was accomplished through column chromatography. Firstly, the resin type was optimized, followed by optimization of the column chromatographic parameters including the ethanol mass fraction of the sample solution, loading volume, loading volume flow, washing volume, elution ethanol mass fraction, and elution volume. The optimized process was then verified. Finally, the crude products of hyodeoxycholic acid and chenodeoxycholic acid, separated and purified using column chromatography, were utilized as raw materials and further refined to enhance their purity.

Materials and methods

Instruments, materials and reagents

Instruments:

High-performance liquid chromatography (Agilent 1100, Agilent Technology Co., Ltd.); precision electronic balance (XS105, Switzerland Mettler-Toledo); centrifuge (5804 R and 5424 centrifuges, Germany Eppendorf); electric heating constant-temperature blast drying oven (DHG-9146A, Shanghai Jinghong Experimental Equipment Co., Ltd.); peristaltic pump (YZ15, Changzhou Visier Fluid Technology Co., Ltd.); chromatography columns (300 mm × 20 mm, Hangzhou Dingke Biotechnology Co., Ltd.); constant-temperature oscillator (THZ-82 Shanghai Lichen Bangxi Instrument Technology Co., Ltd.); pH meter (SevenMulti, U.S.A Mettler-Toledo); and ultrasonic clearing machine (LMTD15, Beijing Lvjin Technology Co., Ltd.).

Materials and reagents:

Acetonitrile (HPLC grade, Germany Merck Company); phosphate (HPLC grade, U.S.A TEDIA Company); ultrapure water prepared from an ultrapure water preparation system (Milli-Q, Germany Millipore Company); hydrochloric acid (analytically pure, Sinopharm Chemical Reagent Co., Ltd.), and ethanol (analytically pure, Shanghai Lingfeng Chemical Reagent Co., Ltd.) were used. The reference substances hyodeoxycholic acid (purity>98 %, Lot number C11254598) and chenodeoxycholic acid (purity>98 %, Lot number C10741568) were both purchased from Shanghai Maclin Biochemical Co., Ltd. Pig bile saponification solution was prepared following bilirubin extraction (provided by Anhui Chem-Bright Bioengineering Co., Ltd.). The types and properties of the resins used are listed in Table 1.

Purification using column chromatography

Pig bile saponification solution was used, and 7.4 % diluted hydrochloric acid was added to obtain the precipitate. Subsequently, the precipitate was dissolved with a specific mass fraction of ethanol solution, filtered, and the sample solution was obtained. The resin was then wet-loaded, and the sam-

Fig. 2 – *Column chromatography process*

Table 1 – *Types and properties of resins*

Resin models	Types	Size of resin (μm)	Manufacturer
XAD1600N	nonpolar resin	350–450	Shanhai Anland Biotechnology Company Limited
XAD16N	nonpolar resin	560–710	Shanhai Anland Biotechnology Company Limited
CG161M	nonpolar resin	75	Shanhai Anland Biotechnology Company Limited
ADS-8	nonpolar resin	315–1250	Cangzhou Baoen Adsorbent Material Technology Company Limited
D151	weakly acidic cation exchange resin	315–1250	Cangzhou Baoen Adsorbent Material Technology Company Limited
AB-8	weak-polar resin	300–1250	Zhengzhou Qinshi Technology Company Limited

ples were washed with a certain concentration of ethanol solution to remove impurities. Following this, the samples were eluted with different mass fractions of ethanol solution, and the eluting solution was collected. Crude hyodeoxycholic acid and chenodeoxycholic acid were obtained after drying. A reusable chromatographic column was generated with a 95 % ethanol solution. The process of purifying hyodeoxycholic acid and chenodeoxycholic acid from pig bile saponification solution using column chromatography is presented in Fig. 2.

Preparation of sample solution

The appropriate amount of pig bile saponification solution was measured, and diluted hydrochloric acid was added to adjust the pH to approximately 2.0. The obtained precipitates were then washed with water several times and dissolved in 40 % ethanol solution (w/w) equivalent to 8 times the amount of pig bile saponification solution (w/w). After 30 min of ultrasonication, the sample solution was obtained by filtration.

Pretreatment of resin

Four nonpolar resins, including XAD1600N, XAD16N, CG161M, and ADS-8, cross-linked polymethacrylic acid resin D151, and weakly polar resin AB-8, were selected. The resin was soaked in 95 % ethanol solution for 24 h and washed with water until the ethanol was completely removed. The samples were then soaked in ethanol solution with the same mass fraction as the sample solution for 24 h, dried in surface water to obtain the wet resin, and reserved.

Resin screening

Static adsorption experiment

A total of 4.0 g (wet mass) of pretreated XAD1600N, XAD16N, CG161M, ADS-8, D151, and AB-8 resin were placed into 100-mL conical flasks. Then, 40 mL of sample solution with 40 % ethanol (w/w) was added. The conical flasks were placed in a thermostat and for 12 h at room temperature with a frequency of 140 rpm. After oscillation, the adsorption solution was filtered until the dried adsorbed solution was obtained. The contents of hyodeoxycholic acid and chenodeoxycholic acid were determined by HPLC, and the adsorption capacity was calculated.

The adsorbed resin was washed with a small amount of water to remove surface residues and then collected and dried through suction filtration, after which 80 mL of 80 % ethanol (w/w) was added. The resin was then placed in a thermostat and oscillated for 12 h at room temperature with a frequency of 140 rpm. After oscillating adsorption, the supernatant was utilized to determine the content of hyodeoxycholic acid and chenodeoxycholic acid using HPLC, and the desorption quantity and desorption rate were calculated.

Equations (1)–(3) were employed separately to calculate the adsorption equilibrium, desorption quantity, and desorption rate of the resin for hyodeoxycholic acid and chenodeoxycholic acid:

$$q_e^n = \frac{\left(C_i^n - C_e^n\right)V_i}{W} \quad (n = 1,2) \tag{1}$$

$$q_d^n = \frac{C_d^n V_d}{W} \quad (n = 1,2) \tag{2}$$

$$R_d^n = \frac{C_d^n V_d}{\left(C_i^n - C_e^n\right)V_i} \cdot 100\ \% \quad (n = 1,2) \tag{3}$$

where q_e (mg g^{-1} wet resin) is the adsorption capacity at equilibrium; q_d (mg g^{-1} wet resin) is the desorption capacity; R_d (%) is the desorption rate; C_i (mg mL^{-1}) and C_e (mg mL^{-1}) are the initial concentration and equilibrium concentration of hyodeoxy-

cholic acid and chenodeoxycholic acid in the solution, respectively; V_i (mL) is the volume of the initial solution; W (g) is the wet weight of resin; C_d (mg mL^{-1}) is the concentration of hyodeoxycholic acid and chenodeoxycholic acid in the desorption solution; V_d (mL) is the volume of the desorption solution; and superscript n (n=1,2) separately indicate hyodeoxycholic acid and chenodeoxycholic acid.

Static adsorption kinetics experiment

A total of 2.0 g (wet mass) of pretreated CG161M, XAD16N, and XAD1600N resin was placed into 100-mL conical flasks. Subsequently, 60 mL of sample solution with 40 % ethanol (w/w) was added. The conical flasks were placed in a thermostat and oscillated for 12 h at room temperature with a frequency of 140 rpm. The samples were collected at 0, 3, 5, 10, 20, 30, and 60 min after adsorption had started. The contents of hyodeoxycholic acid and chenodeoxycholic acid were tested by HPLC, and the resin's adsorptive capacity was calculated.

Optimization of process parameters for column chromatography

Investigation of the mass fraction of ethanol in the sample solution

An appropriate amount of pig bile saponification solution was used, and diluted hydrochloric acid was added to adjust the pH to approximately 2.0. Subsequently, the obtained precipitates were washed with water several times. Afterward, 30 %, 40 % and 50 % ethanol solutions (w/w), each equivalent to 8 times the amount of pig bile saponification solution (w/w) were added separately. After 30 min of ultrasonication, the samples were allowed to settle, filtered, and sample solutions with different ethanol mass fractions were obtained. A total of 4.0 g (wet mass) of pretreated CG161M resin was placed into 100-mL conical flasks. Then, 30 %, 40 %, and 50 % ethanol (w/w) were added to the sample solutions, respectively. These conical flasks were placed in a thermostat and oscillated for 12 h at room temperature with a frequency of 140 rpm. Following oscillating adsorption, the supernatant was used to determine the content of hyodeoxycholic acid and chenodeoxycholic acid by HPLC, and the adsorption capacity and adsorption rate of hyodeoxycholic acid and chenodeoxycholic acid were calculated.

Breakthrough curve

A total of 40 mL of pretreated resin was used for wet loading (300 mm×20 mm) at a ratio of diameter to height of 1:10. The volume flow rates of 1, 2, and 3 BV h^{-1} were sampled separately, with a loading amount of 12 BV. Every effluent of 0.5 BV was collected, and the concentrations of hyodeoxycholic acid and chenodeoxycholic acid were determined by HPLC to assess the presence of any leakage.

Investigation of the washing volume

The volume flow rates of sampling, washing, and elution were all maintained at 3 BV h^{-1}. The volume of sampling was 5 BV, the samples were washed with 40 % ethanol at 5 BV, with the eluting solution collected per 1 BV. The eluting solution was used to determine the concentrations of hyodeoxycholic acid and chenodeoxycholic acid to identify any leakage. Subsequently, the eluting solution was used to determine total solids, to calculate the content of washed hyodeoxycholic acid and chenodeoxycholic acid, and, finally, to determine the washing volume of 40 % ethanol.

Investigation of the volume fraction of eluted ethanol

The volume flow rates for sampling, washing, and elution were maintained at 3 BV h^{-1}. The sampling volume was 5 BV, 5 BV of 40 % ethanol was used for washing, and 2 BV was eluted with 40 %, 45 %, 50 %, 60 %, and 70 % ethanol. The concentrations of hyodeoxycholic acid and chenodeoxycholic acid in the eluting solution were calculated as the elution rate and cumulative elution rate. The volume grade of eluted ethanol for hyodeoxycholic acid and chenodeoxycholic acid was determined.

Equation (4) was used to calculate the elution rate and cumulative elution rate for hyodeoxycholic acid and chenodeoxycholic acid:

$$E^n = \frac{C_j^n V_j}{C_i^n V_i} \cdot 100\ \% \quad (n = 1,2) \tag{4}$$

where E (%) is the elution rate; C_i (mg mL^{-1}) and C_j (mg mL^{-1}) are the hyodeoxycholic acid and chenodeoxycholic acid concentrations in the sample solution and eluting solution, respectively; V_i (mL) is the sample volume; and V_j (mL) is the eluting solution volume. The superscript n (n=1,2) indicates hyodeoxycholic acid and chenodeoxycholic acid. The cumulative elution rate is the sum of the elution rates of each part of the eluting solution.

Investigation of the volume of ethanol eluted by hyodeoxycholic acid

The volume flow rates for sampling, washing, and elution were all maintained at 3 BV h^{-1}. The sampling volume was 5 BV, 5 BV of 40 % ethanol

was used for washing, and elution of 7 BV was achieved with 45 % ethanol. The 45 % ethanol eluting solution was collected per 1 BV and used to determine the concentration of hyodeoxycholic acid. From this, the content in the eluting solution and the cumulative elution rate of hyodeoxycholic acid were calculated. The total solids of the eluting solution were then measured, and the purity of hyodeoxycholic acid was calculated. The cumulative elution rate and purity of hyodeoxycholic acid were used as indices to determine the eluting volume of hyodeoxycholic acid.

Investigation of the volume of ethanol eluted by chenodeoxycholic acid

The volume flow rates for sampling, washing, and elution were maintained at 3 BV h^{-1}. The sampling volume was 5 BV, 5 BV of 40 % ethanol was used for washing, 4 BV was eluted with 45 % ethanol, and elution of 6 BV was achieved with 60 % ethanol. The 60 % ethanol eluting solution was collected per 1 BV, and used to determine the concentration of chenodeoxycholic acid. From this, the content in the eluting solution and the cumulative elution rate of chenodeoxycholic acid were calculated. The total solids of the eluting solution were then measured, and the purity of chenodeoxycholic acid was calculated. The cumulative elution rate and purity of chenodeoxycholic acid were used as indices to determine the eluting volume of chenodeoxycholic acid.

Confirmation experiments

A total of 40 mL of pretreated CG161M resin was used for wet loading (300 mm×20 mm) at a ratio of diameter to height of 1:10. The pig bile saponification solution was dissolved in 40 % ethanol solution, and sample solution with concentrations of hyodeoxycholic acid and chenodeoxycholic acid of 2.25 mg mL^{-1} and 1.64 mg mL^{-1}, respectively, was obtained. A dynamic adsorption of 5 BV was carried out at a volume flow rate of 3 BV h^{-1}, 5 BV was washed with 40 % ethanol, 4 BV was eluted with 45 % ethanol to obtain hyodeoxycholic acid, and 5 BV was eluted with 60 % ethanol to obtain chenodeoxycholic acid. Each washing volume was dried in a vacuum at 60 °C, the weight of the powder was determined, and the content was determined by HPLC. The purity and yield of hyodeoxycholic acid and chenodeoxycholic acid were calculated.

Purification of hyodeoxycholic acid and chenodeoxycholic acid

The crude products of hyodeoxycholic acid and chenodeoxycholic acid separated and purified using column chromatography were used as raw materials for further purification. The crude products of hyodeoxycholic acid were added to the appropriate amount of ethyl acetate, and then heated and stirred at 60 °C for 30 min. A small amount of seed crystal was added, and the mixture was allowed to cool at room temperature, allowing for crystallization over 24 h. The products were dried at 60 °C after crystallization and filtration. The crude products of chenodeoxycholic acid were refined with butyl acetate using the same method.

Analysis method

Determination method of hyodeoxycholic acid and chenodeoxycholic acid content

The reference substance of hyodeoxycholic acid and chenodeoxycholic acid was accurately weighed and then dissolved to create a reference stock solution with a hyodeoxycholic acid concentration of 5.174 mg mL^{-1} and chenodeoxycholic acid concentration of 5.165 mg mL^{-1} with methanol.

Approximately 500 mg of pig bile saponification solution was taken into a 10-mL volumetric flask and accurately weighed. Methanol and a small amount of mobile phase were added to dilute the solution to scale. The mixture was then centrifuged at 12000 rpm for 10 min, and the supernatant was collected to obtain the test solution.

The chromatographic analysis conditions were as follows: ZORBAX SB-C18 chromatographic column (4.6×250 mm, 5 μm); acetonitrile-0.1 % phosphate solution mobile phase (45:55); flow rate of 1.0 mL min^{-1}; detection wavelength of 192 nm; sample size of 10 μL; column temperature of 35 °C. Under these chromatographic conditions, hyodeoxycholic acid and chenodeoxycholic acid were effectively separated from other components.

The test results, which include the chromatograms of reference substances and samples, standard curve, limit of quantitation, limit of detection, precision, repetitiveness, stability, and average recovery of the analysis method, are presented in the supplementary materials.

Calculation of purity and yield

The total solids were determined using the gravimetric method. An appropriate amount of sample was accurately weighed into a weighing bottle and dried to constant weight. The bottle was then placed in a drying oven at 105 °C and dried for 3 h. When completed, the bottle was removed and put into a dryer, cooled to room temperature, and weighed.

Equation (5) was used to calculate the purity of hyodeoxycholic acid and chenodeoxycholic acid in eluting solution, as follows:

$$P_E^n = \frac{C_j^n V_j}{DM} \cdot 100\ \% \quad (n = 1,2) \tag{5}$$

where P_E^n (%) is the purity of hyodeoxycholic acid and chenodeoxycholic acid in eluting solution; DM (mg) is the mass of total solids; C_j (mg mL^{-1}) is the concentration of hyodeoxycholic acid and chenodeoxycholic acid in eluting solution; V_j (mL) is the volume of eluting solution; and superscript n (n=1,2) separately indicates hyodeoxycholic acid and chenodeoxycholic acid.

Equation (6) was used to calculate the purity of hyodeoxycholic acid and chenodeoxycholic acid in solid powder by hypobaric drying, as follows:

$$P_D^n = \frac{M_n}{M_0} \cdot 100\ \% \quad (n = 1,2) \tag{6}$$

where P_D^n (%) is the purity of hyodeoxycholic acid and chenodeoxycholic acid in eluting solution; M_0 (g) is the mass of solid powder; M_n (g) is the mass of hyodeoxycholic acid and chenodeoxycholic acid in solid powder; and superscript n (n=1,2) separately indicates hyodeoxycholic acid and chenodeoxycholic acid.

Equation (7) was used to calculate the yield of hyodeoxycholic acid and chenodeoxycholic acid in eluting solution:

$$Y_E^n = \frac{C_j^n V_j^n}{C_i^n V_i} \cdot 100\ \% \quad (n = 1,2) \tag{7}$$

where Y_E^n (%) is the yield of hyodeoxycholic acid and chenodeoxycholic acid in eluting solution; C_i (mg mL^{-1}) is the concentration of hyodeoxycholic acid and chenodeoxycholic acid in sample solution; C_j (mg mL^{-1}) is the concentration of hyodeoxycholic acid and chenodeoxycholic acid in eluting solution; V_i (mL) is the volume of sampling; V_j (mL) is the volume of eluting solution of each target component; and superscript n (n=1,2) indicates hyodeoxycholic acid and chenodeoxycholic acid.

Equation (8) was used to calculate the yield of hyodeoxycholic acid and chenodeoxycholic acid in solid powder by hypobaric drying:

$$Y_D^n = \frac{M_n}{C_i^n V_i} \cdot 100\ \% \quad (n = 1,2) \tag{8}$$

where Y_D^n (%) is the yield of hyodeoxycholic acid and chenodeoxycholic acid in eluting solution; M_n (g) is the mass of hyodeoxycholic acid and chenodeoxycholic acid in solid powder; C_i (mg mL^{-1}) is the concentration of hyodeoxycholic acid and chenodeoxycholic acid in sample solution; V_i (mL) is the volume of sampling; and superscript n (n=1,2) separately indicates hyodeoxycholic acid and chenodeoxycholic acid.

Results

Selection of resins

Static adsorption experiments

The results of the adsorption amounts, desorption amounts, and desorption rates of 6 resins for hyodeoxycholic acid and chenodeoxycholic acid are presented in Fig. 3 and Fig. 4. The results reveal that different kinds of resins exhibited distinct adsorption capacities for hyodeoxycholic acid and chenodeoxycholic acid. Among them, XAD1600N,

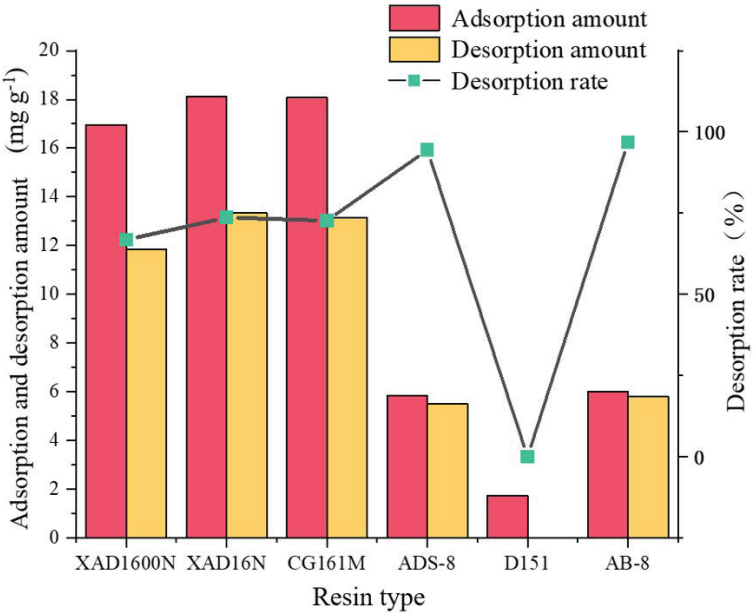

Fig. 3 – *Adsorption amount, desorption amount, and desorption rate of 6 resins for hyodeoxycholic acid*

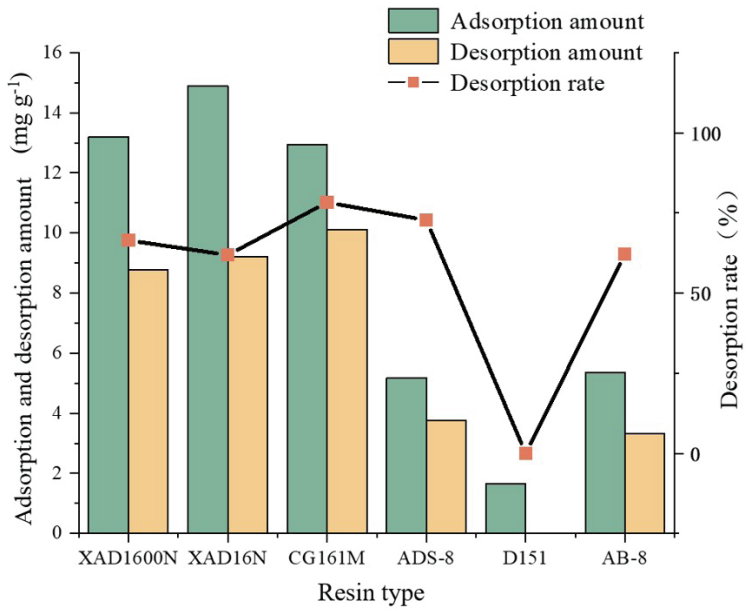

Fig. 4 – *Adsorption amount, desorption amount, and desorption rate of 6 resins for chenodeoxycholic acid*

Table 2 – *Static adsorption kinetic models*

Equation name	Equation form	Meaning of parameters	Reference
Pseudo-first-order kinetic model	$\frac{dq_t}{dt} = k_1(q_e - q_t)$	k_1: Adsorption rate constant of the pseudo-first-order kinetic model (min^{-1}) t: Adsorption time (min) q_t: The amount of adsorption at time t (mg g^{-1}) q_e: Adsorption capacity at equilibrium (mg g^{-1})	29
Pseudo-second-order kinetic model	$\frac{dq_t}{dt} = k_2(q_e - q_t)^2$	k_2: Adsorption rate constant of the pseudo-second-order kinetic model (g mg^{-1} min^{-1}) t: Adsorption time (min) q_t: The amount of adsorption at time t (mg g^{-1}) q_e: Adsorption capacity at equilibrium (mg g^{-1})	30
Elovich equation	$\frac{dq_t}{dt} = \alpha e^{-\beta q_t}$	α: Adsorption rate constant at the start (mg g^{-1} min^{-1}) β: Desorption rate constant (g mg^{-1}) t: Adsorption time (min) q_t: The amount of adsorption at time t (mg g^{-1})	31

XAD16N, and CG161M resins displayed adsorption amounts exceeding 16 mg g^{-1} wet resin for hyodeoxycholic acid, and over 12 mg g^{-1} wet resin for chenodeoxycholic acid. These three resins demonstrated the strongest adsorption capacities. The adsorption amount of ADS-8 and AB-8 resins for hyodeoxycholic acid and chenodeoxycholic acid was less than 6 mg g^{-1} of wet resin, indicating relatively weak adsorption capacity. As a weakly acidic cation exchange resin, D151 resin exhibited the smallest and weakest adsorption capacity.

With the exception of D151 resin, the other five resins demonstrated better desorption capacity for hyodeoxycholic acid and chenodeoxycholic acid, with desorption rates exceeding 60 %. Among them, XAD1600N, XAD16N and CG161M resins had desorption amounts exceeding 11 mg g^{-1} wet resin for hyodeoxycholic acid and over 8 mg g^{-1} wet resin for chenodeoxycholic acid, indicating relatively substantial desorption capacity. Therefore, considering the adsorption capacity, desorption capacity, and desorption rate of the various resins, XAD1600N, XAD16N and CG161M were provisionally selected for the subsequent experiments.

Static adsorption kinetics experiments

The adsorption processes of hyodeoxycholic acid and chenodeoxycholic acid by XAD1600N, XAD16N, and CG161M resins were studied. The pseudo-first-order kinetic model, pseudo-second-order kinetic model, and Elovich equation modeling were used to quantitatively evaluate the relationship among adsorption time, adsorption capacity, and adsorption rate[28]. See Table 2 for an introduction to each model.

The experimental results of the adsorption kinetics of the three resins for hyodeoxycholic acid and chenodeoxycholic acid are presented in Fig. 5 and Fig. 6. The adsorption capacity of these three resins increased with increasing adsorption time. The adsorption amount of CG161M resin for hyodeoxycholic acid and chenodeoxycholic acid approached equilibrium in 10 minutes, while the other two resins required at least 30 minutes to do so. As seen in Table 1, the particle diameter of CG161M resin was 75 μm, notably smaller than that of XAD1600N and XAD16N resins. Thus, the adsorption rate of CG161M resin for hyodeoxycholic acid and chenodeoxycholic acid was higher. The XAD1600N resin and XAD16N resin slightly differed in particle diameter and exhibited similar adsorption rates.

The experimental results were fitted using a pseudo-first-order kinetic model, pseudo-second-order kinetic model, and Elovich equation, as shown in Table 3 and Table 4. The R^2 values of the fitting results of the pseudo-first-order kinetic model and the pseudo-second-order kinetic model exceeded 0.96, and the standard error was less than the fitting value. This suggests that the two models could well fit the adsorption results of the three resins for hyodeoxycholic acid and chenodeoxycholic acid. However, the R^2 value for the fitting result of the Elovich equation was above 0.93, but the uncertainty in the fitting result was relatively high, resulting in a less than ideal fit.

The pseudo-first-order rate constant k_1 values of CG161M, XAD16N, and XAD1600N resins for the adsorption of hyodeoxycholic acid were 0.5328, 0.1718, and 0.1610 min^{-1}, respectively, and the pseudo-second-order rate constant k_2 values were 0.0229, 0.0044, and 0.0035 g mg^{-1} min^{-1}, respectively. For chenodeoxycholic acid, the pseudo-first-order rate constant k_1 values were 0.4675, 0.0895, and 0.0960 min^{-1}, and the pseudo-sec-

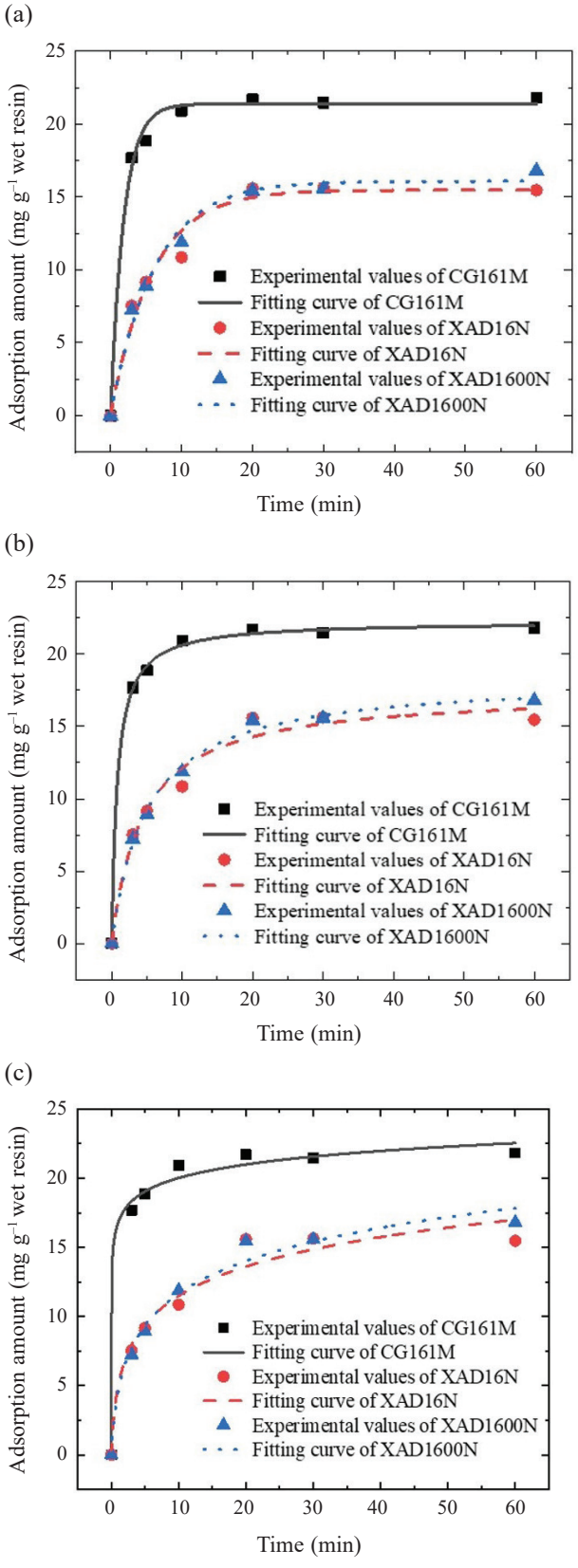

Fig. 5 – *Experimental adsorption kinetics results of three resins for hyodeoxycholic acid (a) Pseudo-first-order kinetic model (b) Pseudo-second-order kinetic model (c) Elovich equation*

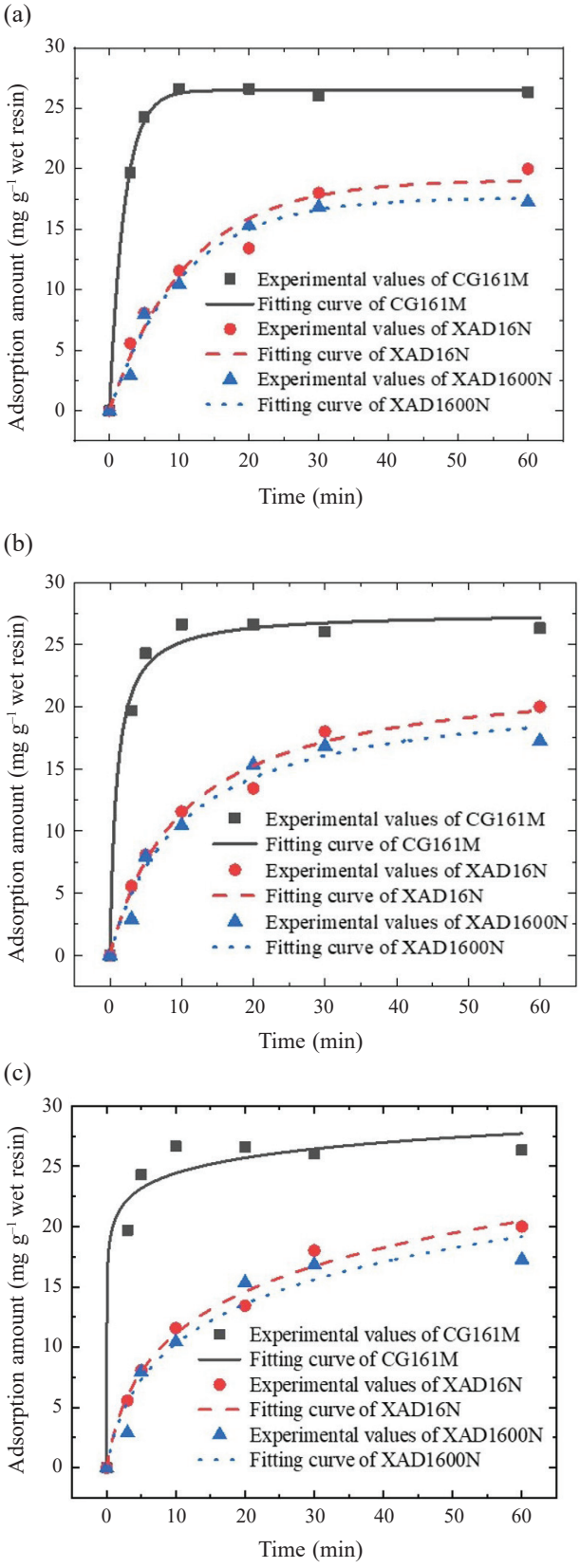

Fig. 6 – *Experimental adsorption kinetics results of three resins for chenodeoxycholic acid (a) Pseudo-first-order kinetic model (b) Pseudo-second-order kinetic model (c) Elovich equation*

Table 3 – *Fitting parameters of the static adsorption kinetic model of hyodeoxycholic acid*

Resin type	Pseudo-first-order kinetic model			Pseudo-second-order kinetic model			Elovich equation		
	q_e (mg g^{-1})	k_1 (min^{-1})	R^2	q_e (mg g^{-1})	k_2 (g mg^{-1} min^{-1})	R^2	α (mg g^{-1} min^{-1})	β (g mg^{-1})	R^2
CG161M	21.38±0.30	0.5328±0.0478	0.994	22.25±0.06	0.0229±0.0038	0.999	$2.15\cdot10^5$±$5.59\cdot10^5$	0.7116±0.1369	0.993
XAD16N	15.47±0.66	0.1718±0.0258	0.966	17.39±0.01	0.0044±0.0008	0.974	12.26±7.15	0.3220±0.0576	0.955
XAD1600N	16.08±0.48	0.1610±0.0164	0.985	18.37±0.01	0.0035±0.0006	0.995	9.32±3.11	0.2849±0.0325	0.980

Table 4 – *Fitting parameters of the static adsorption kinetic model of chenodeoxycholic acid*

Resin type	Pseudo-first-order kinetic model			Pseudo-second-order kinetic model			Elovich equation		
	q_e (mg g^{-1})	k_1 (min^{-1})	R^2	q_e (mg g^{-1})	k_2 (g mg^{-1} min^{-1})	R^2	α (mg g^{-1} min^{-1})	β (g mg^{-1})	R^2
CG161M	26.50±0.18	0.4675±0.0176	0.999	27.59±0.75	0.0385±0.0103	0.987	$1.13\cdot10^5$±$5.46\cdot10^5$	0.5459±0.2088	0.967
XAD16N	19.10±1.20	0.0895±0.0161	0.960	23.03±1.34	0.0043±0.0001	0.982	3.54±0.68	0.1793±0.0181	0.986
XAD1600N	17.62±0.75	0.0960±0.0112	0.982	21.46±1.77	0.0046±0.0015	0.967	3.11±1.29	0.1868±0.0418	0.936

ond-order rate constant k_2 values were 0.0385, 0.0043, and 0.0046 g mg^{-1} min^{-1}. Both the k_1 and k_2 values of CG161M resin were the largest, indicating that CG161M resin exhibited the highest adsorption rate for hyodeoxycholic acid and chenodeoxycholic acid.

For the pseudo-first-order kinetic model, the equilibrium adsorptive quantity q_e values for CG161M, XAD16N, and XAD1600N resins in the adsorption of hyodeoxycholic acid were 21.38, 15.47, and 16.08 mg g^{-1}, respectively, while for the pseudo-second-order kinetic model, the equilibrium adsorptive quantity q_e values were 22.25, 17.39, and 18.37 mg g^{-1}, respectively. The q_e values of the pseudo-second-order kinetic model for the adsorption of chenodeoxycholic acid were 26.50, 19.10, and 17.62 mg g^{-1}, while the q_e values of the pseudo-second-order kinetic model were 27.59, 23.03, and 21.46 mg g^{-1}. The q_e of CG161M resin for the two target components was the highest, indicating that it had the highest adsorption capacity for hyodeoxycholic acid and chenodeoxycholic acid. Considering the equilibrium adsorptive quantity and adsorption rate of the three resins, CG161M resin was selected for the separation and purification of hyodeoxycholic acid and chenodeoxycholic acid from pig bile saponification solution.

Optimization of column chromatography process parameters

Investigation of the ethanol mass fraction of the loading solution

Ethanol solutions of 30 %, 40 %, and 50 %, equivalent to 8 times the amount in pig bile saponification fluid, were used to treat acid precipitation products from the pig bile saponification fluid. It was observed that the 40 % and 50 % ethanol solutions could completely dissolve the acid precipitation products, while the 30 % ethanol solution could not. As seen in Table 5, there was little difference between the concentrations of hyodeoxycholic acid and chenodeoxycholic acid in the 40 % and 50 % ethanol solutions, but both concentrations were significantly higher than the concentration in the 30 % ethanol solution. A higher concentration of the loading solution is advantageous for reducing the loading volume and shortening the loading time.

Table 5 – *Concentrations of hyodeoxycholic acid and chenodeoxycholic acid in different loading fluids*

Mass fraction of ethanol in loading fluids (%)	Concentrations of hyodeoxycholic acid (mg mL^{-1})	Concentrations of chenodeoxycholic acid (mg mL^{-1})
30	1.34	0.74
40	2.16	1.49
50	2.16	1.50

The effect of ethanol mass fraction on the adsorption behavior of hyodeoxycholic acid and chenodeoxycholic acid was investigated through static adsorption experiments, with the results presented in Fig. 7. As the ethanol mass fraction increased, the adsorption rates of CG161M resin for hyodeoxycholic acid and chenodeoxycholic acid steadily decreased. Excessively high ethanol mass fraction led to low adsorption rates, potentially leading to the premature appearance of leakage points. Considering the loading efficiency and the adsorption rate of hyodeoxycholic acid and chenodeoxycholic acid, the 40 % ethanol solution was chosen as the loading solution.

Breakthrough curve

The effects of various volume flow rates on the adsorption behavior of hyodeoxycholic acid and

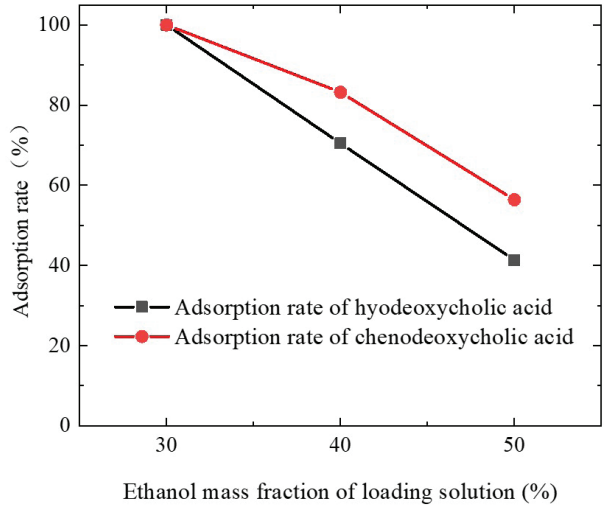

Fig. 7 – *Investigation of the ethanol mass fraction of the loading solution*

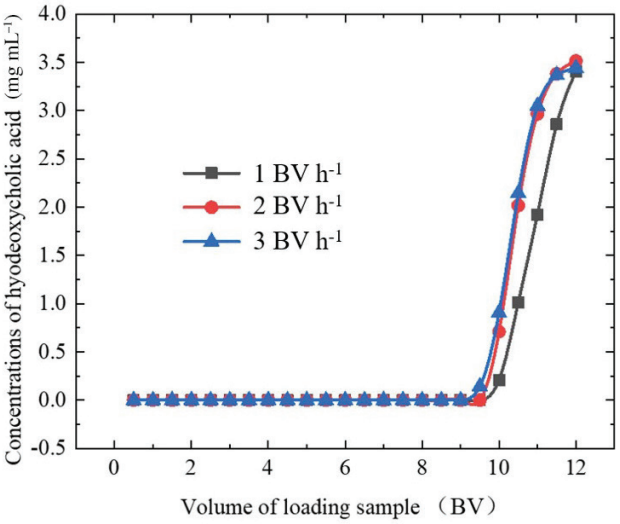

Fig. 8 – *Breakthrough curves of hyodeoxycholic acid loaded with different volume flow rates*

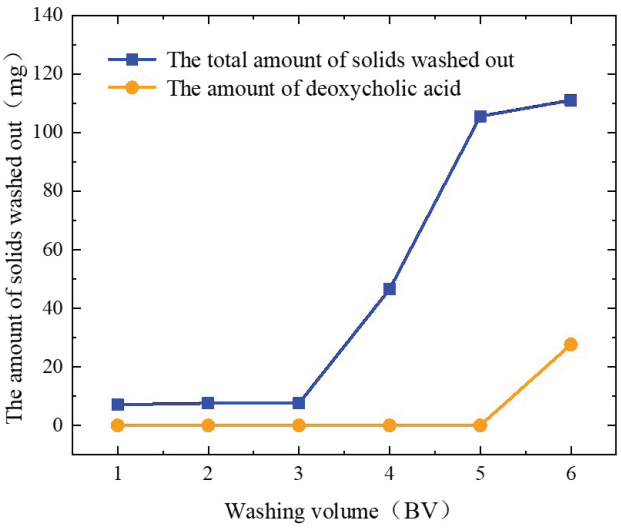

Fig. 9 – *Amount of solids washed out by 40 % ethanol*

chenodeoxycholic acid were investigated, and the results are presented in Fig. 8. As observed in the figure, when the volume flow rates were set at 1 BV h^{-1} and 2 BV h^{-1}, hyodeoxycholic acid began to leak after the addition of a 10 BV sample, whereas with the volume flow rate of 3 BV h^{-1}, hyodeoxycholic acid began to leak after adding a 9.5 BV sample. Chenodeoxycholic acid, on the other hand, exhibited no leakage. Differences in the leakage points of the three volume flow rates were minimal. To reduce the loading time and improve the loading efficiency, the volume flow rate of 3 BV h^{-1} was chosen at. In the experiment, excessive loading could affect the subsequent removal of impurities and separation of hyodeoxycholic acid and chenodeoxycholic acid, so the loading sample volume was established at 5 BV.

Investigation of washing volume

When 6 BV was washed with 40 % ethanol, hyodeoxycholic acid began to leak, while chenodeoxycholic acid did not leak. The amount of solids washed out by 40 % ethanol, including the total amount of solids washed out and the amount of deoxycholic acid, was determined. The results are presented in Fig. 9. The total amount of solids washed out increased with the increase in the washing volume, and when washing 6 BV, hyodeoxycholic acid began to leak; therefore, the washing volume of 40 % ethanol was set at 5 BV.

Investigation of the elution volume fraction

The elution rates and cumulative elution rates of different mass fractions of ethanol solutions for hyodeoxycholic acid and chenodeoxycholic acid are presented in Table 6. The results reveal that hyodeoxycholic acid was primarily concentrated in the eluates of 40 %, 45 %, and 50 % ethanol, with the elution capacity of 45 % ethanol being stronger than that of 40 % ethanol. Additionally, 50 % ethanol could elute chenodeoxycholic acid. Therefore, 45 % ethanol was chosen as the eluent for hyodeoxycholic acid. Chenodeoxycholic acid was mainly concentrated in the eluates of 50 % and 60 % ethanol, with the elution capacity of 60 % ethanol being stronger than that of 50 % ethanol; thus, the 60 % ethanol was chosen as the eluent.

Investigation of the ethanol elution volume for hyodeoxycholic acid

The elution results of 45 % ethanol for hyodeoxycholic acid are presented in Fig. 10. With the increase in elution volume, the cumulative elution rate of hyodeoxycholic acid continued to rise, reaching over 88 % when eluted to 4 BV; thereafter, the increase became more gradual. With increasing

Table 6 – *Elution capacity of hyodeoxycholic acid and chenodeoxycholic acid in ethanol solutions with different mass fractions*

Mass fractions of ethanol (%)	Elution rate of hyodeoxycholic acid (%)	Cumulative elution rate of hyodeoxycholic acid (%)	Elution rate of chenodeoxycholic acid (%)	Cumulative elution rate of chenodeoxycholic acid (%)
40	22.30	22.30	0.00	0.00
45	51.60	71.89	0.00	0.00
50	26.89	98.79	35.09	35.09
60	0.00	98.79	54.47	89.56
70	0.00	98.79	10.01	99.58

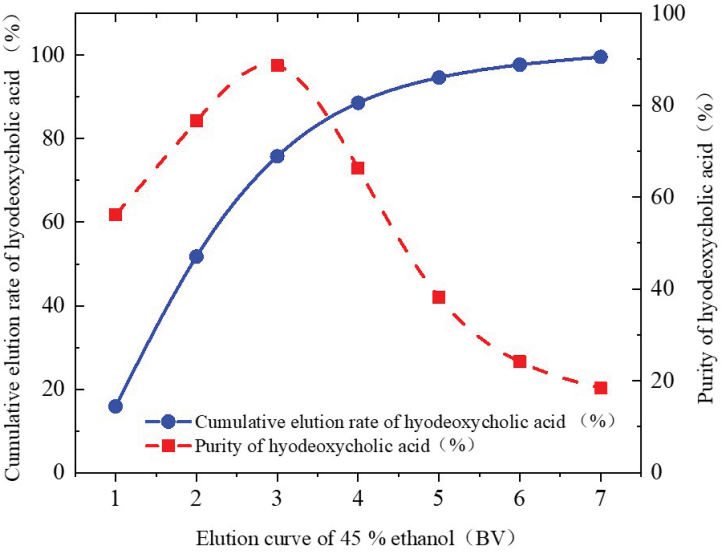

Fig. 10 – *Elution curve of 45 % ethanol*

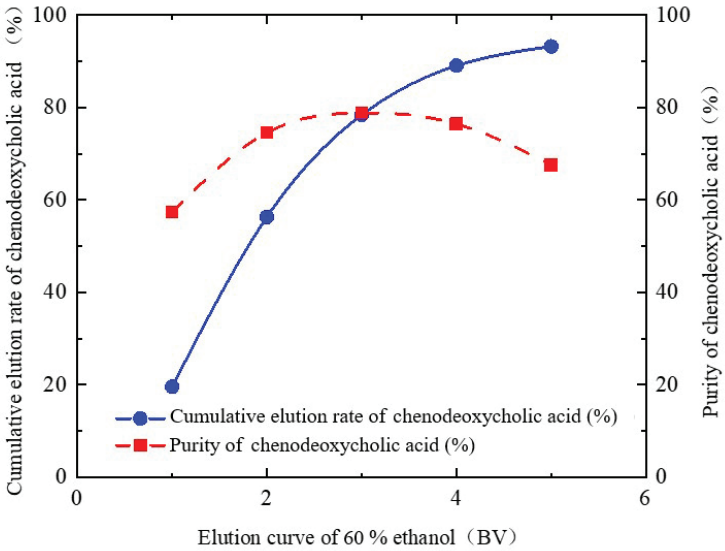

Fig. 11 – *Elution curve of 60 % ethanol*

elution volume, the purity of hyodeoxycholic acid initially increased, and then decreased. Using 45 % ethanol to elute 1–4 BV, the purity of hyodeoxycholic acid ranged from 55 % to 89 %. When eluted to 5 BV, the purity of hyodeoxycholic acid dropped below 40 %. Based on the elution rate and purity of hyodeoxycholic acid, the elution volume of 45 % ethanol for hyodeoxycholic acid was determined to be 4 BV.

Investigation of the ethanol elution volume for chenodeoxycholic acid

The elution results of 60 % ethanol are presented in Fig. 11. As the elution volume increased, the cumulative elution rate of chenodeoxycholic acid increased continuously, reaching more than 93 % when the elution volume reached 5 BV. With the increase in elution volume, the purity of chenodeoxycholic acid initially increased and then decreased, while the purity of chenodeoxycholic acid in each elution volume ranged from 57 % to 79 %. Based on the elution rate and purity of chenodeoxycholic acid, the elution volume of 60 % ethanol for chenodeoxycholic acid was determined to be 5 BV.

Validation experiment

The results of the verification experiments are presented in Table 7. The purities of hyodeoxycholic acid and chenodeoxycholic acid were 70.34 % and 66.21 %, respectively, and the yields were 86.48 % and 90.57 %, respectively. The process demonstrated good repeatability. The contents of hyodeoxycholic acid and chenodeoxycholic acid in the acid precipitation products of pig bile saponification fluid were 33.74 % and 24.59 %, respectively. After column chromatography, the purities of hyodeoxycholic acid and chenodeoxycholic acid were significantly improved.

Refinement of crude products of hyodeoxycholic acid and chenodeoxycholic acid

The crude products of hyodeoxycholic acid and chenodeoxycholic acid separated by column chromatography were further refined using three solvents: ethyl acetate, butyl acetate, and methyl isobutyl ketone. These were dissolved by heating and then cooled to obtain an oily substance, but they could not be directly crystallized into a solid. This suggests that the purity of the substance before crystallization was low. Therefore, eluates with higher purities of hyodeoxycholic acid and cheno-

Table 7 – *Validation experiments (n=3)*

Experiment number	Purity of hyodeoxycholic acid (%)	Yield of hyodeoxycholic acid (%)	Purity of chenodeoxycholic acid (%)	Yield of chenodeoxycholic acid (%)
1	70.83	85.94	69.14	94.90
2	71.46	89.08	64.30	87.28
3	68.73	84.40	65.19	89.53
Average value	70.34	86.48	66.21	90.57
RSD/%	2.03	2.76	3.89	4.33

Fig. 12 – *(a) Crystallization of hyodeoxycholic acid and (b) the precipitation of chenodeoxycholic acid*

deoxycholic acid in column chromatography were collected for refining experiments. The 3rd BV eluted by 45 % ethanol was selected in the hyodeoxycholic acid crude product, and the content of hyodeoxycholic acid in the crude product was 86.62 %. The 2nd–4th BV eluted by 60 % ethanol was selected in the chenodeoxycholic acid crude product, and the content of chenodeoxycholic acid in the crude product was 70.02 %.

The results indicate that hyodeoxycholic acid could crystallize into a white solid in ethyl acetate, while chenodeoxycholic acid could yield a white precipitate in butyl acetate. The crystallization of hyodeoxycholic acid and the precipitation of chenodeoxycholic acid are presented in Fig. 12. The purity of the hyodeoxycholic acid crystals was 91.04 %, and the purity of the chenodeoxycholic acid precipitate was 80.28 %.

Conclusion

In this study, we established an HPLC method for the determination of hyodeoxycholic acid and chenodeoxycholic acid in pig bile. Initially, the best resin, CG161M, was selected from among six resins, namely, XAD1600N, XAD16N, CG161M, ADS-8, D151, and AB-8. Despite the higher cost of this resin, it boasts a large adsorption capacity, high adsorption rate, and reusability, all of which contribute to cost reduction.

CG161M resin was used to separate hyodeoxycholic acid and chenodeoxycholic acid. The technological parameters of column chromatography, such as the ethanol mass fraction of the sample solution, loading volume, loading volume flow, washing volume, elution ethanol volume fraction and elution volume, were optimized. The optimized column chromatography process was verified. The purity of crude hyodeoxycholic acid was approximately 70.34 %, with yield of approximately 86.48 %. The purity of crude chenodeoxycholic acid was approximately 66.21 %, with yield of approximately 90.57 %. The process demonstrated good repeatability. The CG161M resin enables the simultaneous purification of hyodeoxycholic acid and chenodeoxycholic acid. Using the crude products of hyodeoxycholic acid and chenodeoxycholic acid separated and purified by column chromatography as raw materials, further cooling and crystallization can yield hyodeoxycholic acid crystals with a purity of 91.04 %. Crude chenodeoxycholic acid was precipitated by cooling, and the purity of the precipitate was increased to 80.28 %.

AUTHOR CONTRIBUTIONS

Liu B. and Gong X. C. designed the study and developed the process method. Liu B., Chen T. L., Chen X. Y. and Li X. N. completed the experiments and data analysis. Liu B., Chen T. L., Chen X. Y., Li X. N., Abudureheman K., Hu S. F., and Gong X. C. jointly wrote the manuscript.

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Supplementary materials

Chromatograms of reference substances and samples

Chromatograms of reference substances and samples are presented in attached Fig. 1.

Standard curve

0.40 mL, 0.80 mL, 1.20 mL, 2.00 mL, 3.00 mL, and 4.00 mL of the stock solution of the reference substance for each component were accurately measured and placed into 10-mL volumetric flasks, and added methanol to dilute them to scale to obtain a series of standard solutions. Ten microliter samples were injected according to the chromatographic conditions described in Determination method of hyodeoxycholic acid and chenodeoxycholic acid content. Linear regression analysis was carried out with the peak area (A) as the ordinate and the reference concentration (C) as the abscissa to establish the linear regression equation. For the standard curve of hyodeoxycholic acid, A=549.81C–8.86, with R^2=0.9999; for the standard curve of chenodeoxycholic acid, A=670.43C–17.68, with R^2=0.9998. The linear ranges of hyodeoxycholic acid and chenodeoxycholic acid were 0.207~2.070 and 0.207~2.066 mg mL^{-1}, respectively. The linear relationship of each component was good within the determination range.

Limit of quantitation and detection

The reference solution was diluted step by step and injected for analysis according to the chromatographic conditions described in Determination method of hyodeoxycholic acid and chenodeoxycholic acid content. The peak area was recorded, the limit of quantitation was measured with a signal-to-noise ratio S/N=10, and the detection limit was measured with a signal-to-noise ratio S/N=3. The quantitative limit of hyodeoxycholic acid was 112.46 μg mL^{-1}, and the quantitative limit of chenodeoxycholic acid was 132.34 μg mL^{-1}. The detection limit of hyodeoxycholic acid was 56.23 μg mL^{-1}, and the detection limit of chenodeoxycholic acid was 66.17 μg mL^{-1}.

Injection precision test

The test solution was taken and injected 6 times continuously according to the chromatographic conditions described in Determination method of hyo-

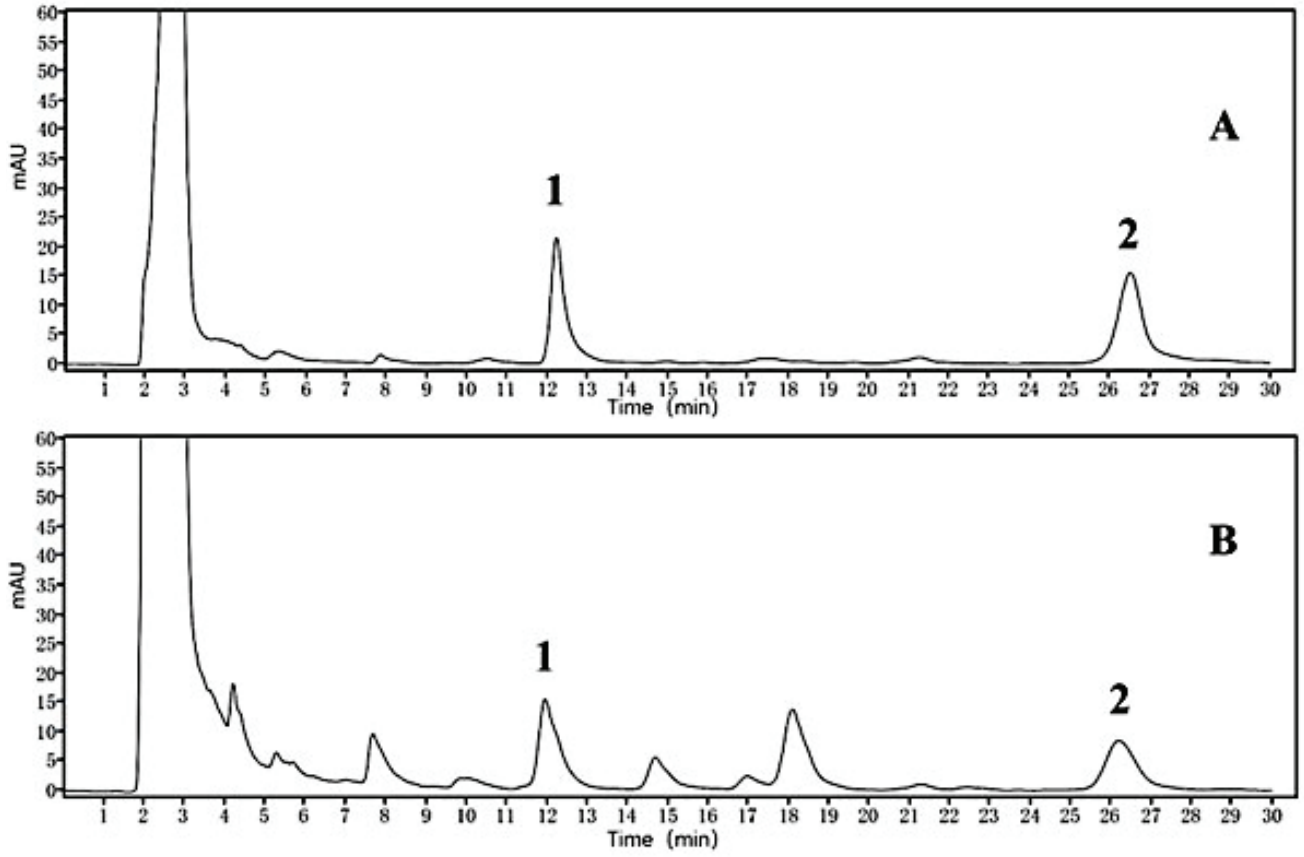

Fig. 1

Attached Table 1 – *Recovery rate of sample addition for the determination of hyodeoxycholic acid and chenodeoxycholic acid*

Compound	Content in sample (mg)	Amount of addition (mg)	Measured amount (mg)	Recovery rate (%)	Average value (%)	RSD (%)
Hyodeoxycholic acid	5.31	2.60	7.91	99.80	99.99	1.85
	5.36	2.60	8.01	101.67		
	5.77	2.60	8.36	99.37		
	5.44	5.21	10.49	97.06		
	5.56	5.21	10.78	100.17		
	5.33	5.21	10.39	97.21		
	5.30	7.81	13.25	101.72		
	5.83	7.81	13.72	100.89		
	5.50	7.81	13.47	102.00		
Chenodeoxycholic acid	3.85	1.90	5.74	99.31	99.39	1.82
	3.88	1.90	5.74	97.43		
	4.18	1.90	6.08	99.82		
	3.94	3.81	7.82	101.92		
	4.03	3.81	7.86	100.75		
	3.86	3.81	7.73	101.62		
	3.84	5.71	9.39	97.24		
	4.23	5.71	9.88	99.05		
	3.99	5.71	9.55	97.36		

deoxycholic acid and chenodeoxycholic acid content. The peak area was recorded, and the relative standard deviation (RSD) was calculated. The RSD of the peak areas of hyodeoxycholic acid and chenodeoxycholic acid were 0.84 % and 0.93 %, respectively. The precision of the instrument was good.

Stability test

The test solution was left at room temperature for 0, 3, 6, 12, 18, and 24 h for injection analysis. The peak area was recorded, and the RSD was calculated. The RSD for the peak areas of hyodeoxycholic acid and chenodeoxycholic acid were 0.86 % and 0.72 %, respectively. The sample solutions of each substance were stable for 24 h.

Repeatability test

Using the same batch of pig bile saponification solution, 6 test solutions were prepared in parallel. The sample was injected according to the chromatographic conditions described in Determination method of hyodeoxycholic acid and chenodeoxycholic acid content. The contents of hyodeoxycholic acid and chenodeoxycholic acid in the sample were determined, and the RSD was calculated. The average contents of hyodeoxycholic acid and chenodeoxycholic acid were 21.05 mg g^{-1} and 15.26 mg g^{-1}, respectively, with RSD of 0.88 % and 0.97 %, respectively, with good repeatability.

Sample addition recovery test

The recovery rates of sample addition for the determination of hyodeoxycholic acid and chenodeoxycholic acid are presented in Attached Table 1. Approximately 250 mg of pig bile saponification solution with a known content was placed into a 10-mL volumetric flask and accurately weighed. The corresponding reference solution was added at ratios of 1.5:1.0, 1.0:1.0, and 0.5:1.0 with respect to the reference substance. The amount of hyodeoxycholic acid and chenodeoxycholic acid in the test substance was used to prepare three parts of high-, medium-, and low-concentration test solutions with sample recovery. The sample was injected for analysis according to the chromatographic conditions described in Determination method of hyodeoxycholic acid and chenodeoxycholic acid content. The sample recovery was calculated. See attached Table 1 for the average recovery rates of hyodeoxycholic acid and chenodeoxycholic acid. The average recovery rates were 99.99 % and 99.39 %, with RSD values of 1.85 % and 1.82 %. This method is accurate and reliable.