Research Article

Study on the Factors Influencing the Extraction of Chenodeoxycholic Acid from Duck Bile Paste by Calcium Salt Method

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New extraction technology of chenodeoxycholic acid from duck bile paste by calcium salt was investigated. The optimum conditions of extraction were determined by orthogonal experimental design. The results indicated that influencing factors on the extraction efficiency of chenodeoxycholic acid were as follows: hydrogen peroxide, methyl alcohol, glacial acetic acid, and calcium chloride. The optimum extracting conditions of chenodeoxycholic acid were 1000 mL amount of methyl alcohol, 50 mL amount of hydrogen peroxide, 500 mL amount of 20% calcium chloride, and 600 mL amount of 60% glacial acetic acid for a quantity of duck paste. The yield of chenodeoxycholic acid was 30%.

1. Introduction

Chenodeoxycholic acid ($3\alpha,7\alpha$ -dihydroxy- 5β -cholanolic acid, CDCA) is one of the dominant bile acids in human and animals bile. CDCA has significant anti-inflammatory, anti-tussive, and expectorant effects [1]; CDCA is used for the medical treatment of metabolic diseases for it can reduce or dissolve cholesterol gall-stones in vivo [2]. CDCA is also the precursor of ursodeoxycholic acid (UDCA) which is another effective drug for gallstone treatment [3, 4]. UDCA is used to treat a variety of acute and chronic liver diseases [5–8]. The method of synthesizing of UDCA with CDCA is highly efficient. The cost of producing UDCA by this method is the lowest. At present, the UDCA is mainly produced by this method [9–11].

CDCA is the main organic ingredient in the bile of chickens, ducks, geese, and other poultry. It can be obtained from poultry bile by extracting technology [12] or synthesizing from cholic acid, dehydrocholic acid, and so on. Initially, the synthesis of CDCA from CA is the main source of CDCA.

In Europe, chemical synthesis of CDCA with CA as raw material is the main source of CDCA. In the past 10 years in China, the extraction of CDCA from chicken bile was the main source of CDCA. Although the number of chickens and

ducks in China is very large, different techniques are required for extracting CDCA from chicken bile and duck bile because there is a large difference in the composition of chicken and duck bile. Few literatures report on the method of extracting CDCA from duck bile.

A typical process for preparing CDCA is as follows: esterifying of saponified bile acid, acetylating of bile acid ester, removal of intermediate product by using organic solvent, crystallizing of acetylated ester of CDCA, deprotecting, and crystallizing in organic solvent. The entire process is complex, a large number of organic solvents (e.g., ethyl acetate, gasoline) are consumed, and the yield of the final product is low. At present, in China, because of being used as a raw material for CDCA extraction, the price of chicken bile is high. The duck bile is treated as a waste by a slaughterhouse for the lack of related technology, even though the content of CDCA in chicken bile and duck bile is similar. In order to solve the environmental pollution problems of duck bile and make it easy to store and transport, many slaughterhouses turn duck bile into gall ointment which can be used as a feed.

The content of CDCA in duck bile is over 25%. Moreover, extracting CDCA from duck bile can bring considerable economic benefits. This paper is aimed at designing an efficient way to find out the primary factors influencing the

yield of CDCA from duck bile paste. We speculate that this aim could be easily realized through the combination of an orthogonal experimental design method.

2. Materials and Methods

2.1. Materials. Duck bile paste was obtained from Xiayi Kangda Biochemical Raw Materials Co., Ltd. Anhydrous methanol (MeOH), hydrogen peroxide (H_2O_2), calcium chloride (CaCl_2), sodium hydroxide (NaOH), glacial acetic acid, ethanol, chloroform (CHCl_3), and ethyl acetate were supplied by Tianjin Chemical Reagent Company (Tianjin, China), and all of them were of analytical grade; the standard sample of CDCA was from inspection bureau biological project of China.

2.2. Apparatus and Detection. FTS135-FTIR Spectrometer (Bio-Rad Instruments) was used to determine the infrared spectra, and the measurements were carried out by the KBr method. AVIII 600 NMR Spectrometer (Bruker A.G) was used to determine the nuclear magnetic spectrum. The conditions were 23°C and 400.4 MHz, and TMS was used as the internal standard. For high-performance liquid chromatograph (Agilent), the chromatographic column was a Waters Symmetry C18 ($4.6\text{ mm} \times 150\text{ mm}$, $5\text{ }\mu\text{m}$). The mobile phase was 0.2% formic acid-methanol (20:80, v/v) and flow rate was 1.0 mL/min. The column temperature was maintained at 30°C . The standard curve was used to determine the purity of the CDCA by HPLC [13–15].

2.3. Orthogonal Experimental Design [16]. Orthogonal experimental design is a popular method to deal with the test, including multiple factors and levels. It has been successfully applied to many fields for acquiring the optimum level group. The key of this method is making an orthogonal design table based on the reasonable and representative levels of the investigated factors. This method can help us to select the representative cases for lowering the number of test cases. In this work, the number of investigated factors is four and they have three levels; an orthogonal design table $L_9(3^4)$ is needed, reducing the number of test cases from 81 to 9.

2.4. Process. After the solution of duck bile paste in CH_3OH was heated to 65°C with stirring for 1 h, the solution was cooled to the room temperature. Insoluble substance was filtered out. The 10% H_2O_2 solution was added to the filtrate with stirring. After 30 min, 20.0% (w/v) CaCl_2 solution was added to the solution; the pH value was adjusted to 11 with 5% (w/v) NaOH solution. The mixture was stirred for 30 min; the precipitation was formed. The precipitate was collected by centrifugation and then was added to 60% glacial acetic acid. The solution was heated and kept reflux until all solid was dissolved. A yellow transparent solution was got. This liquid was poured into water after cooling, and the CDCA solid was obtained by filtration. Finally, CDCA was purified by recrystallizing in EtOH/ CHCl_3 mixed solution. The results show that the amount of H_2O_2 , MeOH, glacial acetic acid, and CaCl_2 added affects the extraction of CDCA in duck

FIGURE 1: Effect of different amount of methanol on the yield of CDCA.

bile paste. The single factor experiment conditions and orthogonal experiment conditions were determined based on the preliminary experimental results.

2.5. Calibration Curves. The solutions were prepared by accurately weighing 50 mg CDCA standard and dissolving it in 50 mL methanol. The working standard solution was prepared by diluting the mixed standard solution with MeOH to a series of proper concentrations. The standard solutions were stored at 4°C until use.

The sample solutions were prepared by accurately weighing 25 mg CDCA sample and dissolving it in 50 mL MeOH. The resultant solution was filtered through a $0.45\text{ }\mu\text{m}$ syringe filter (Type Millipore, USA); $20\text{ }\mu\text{L}$ of the filtrate was injected into the HPLC system. The sample solutions were stored at 4°C . The working standard solutions were brought to room temperature and $50\text{ }\mu\text{L}$ was injected into HPLC for the construction of calibration curves. A linear regression equation was obtained by plotting the logarithms of peak area responses versus logarithms of concentrations, in $\mu\text{g}/\mu\text{L}$.

3. Results and Discussion

3.1. Influence of Different Amount of Reagent on Yield

3.1.1. The Influence of MeOH on Yield. The duck bile pastes 100 g, 100 mL H_2O_2 solution, 300 mL 20.0% CaCl_2 solution, and 600 mL 60% glacial acetic acid were added to the system. The influence of MeOH on yield was shown in Figure 1.

When the amounts of H_2O_2 , CaCl_2 , and glacial acetic acid were fixed, the yield of CDCA increased with the increasing amount of MeOH in a certain range. When the amount of MeOH reached about 1000 mL, the yield reached the maximum value. After that, with the dosage of MeOH kept increasing, the yield remained stable. The results indicated that smaller amount of MeOH could not dissolve the paste completely. When the solvent was more enough, the yield

FIGURE 2: Effect of different amount of hydrogen peroxide on the yield of CDCA.

was not increased. So taking the cost of production into consideration, 1000 mL was suitable.

3.1.2. The Influence of H_2O_2 on Yield. The duck bile paste 100 g was dissolved in the system composed of 1000 mL MeOH, 300 mL 20.0% $CaCl_2$, and 600 mL 60% glacial acetic acid. The impact of H_2O_2 amount on yield was shown in Figure 2.

When the amount of the H_2O_2 was added to 25 mL, the yield of CDCA reached the maximum value. After that the yield of CDCA decreased with the increasing of H_2O_2 solution. This situation may be as a result of the large amount of H_2O_2 oxidizing CDCA so as to reduce the yield of CDCA. But this may be less effective toward the decolorization of CDCA if the amount was too small. So the appropriate dosage was 50 mL

3.1.3. The Influence of $CaCl_2$ on Yield. The duck bile paste 100 g was dissolved in the system composed of 1000 mL MeOH, 100 mL H_2O_2 , and 600 mL 60% glacial acetic acid. The influence of $CaCl_2$ amount on yield was shown in Figure 3.

When the amount of 20% $CaCl_2$ solution was 400 mL or less, the yield increased with the increasing amount of $CaCl_2$ solution; when the amount of $CaCl_2$ solution was over 400 mL, the yield decreased with the increasing of $CaCl_2$ solution. It indicated that the yield of CDCA reached the maximum value when 400 mL $CaCl_2$ solution was added. When the amount of $CaCl_2$ was not enough, the CDCA would not form $CaCl_2$. On the contrary, if the amount was too large, it would generate $Ca(OH)_2$, which could adsorb $CaCl_2$ and decrease the yield of CDCA.

3.1.4. The Influence of Glacial Acetic Acid on Yield. The duck bile paste 100 g was dissolved in 1000 mL MeOH, and then 100 mL H_2O_2 and 600 mL 20.0% $CaCl_2$ solution were added with stirring. The influence of glacial acetic acid amount on yield was shown in Figure 4.

FIGURE 3: Effect of different amount of 20% calcium chloride on the yield of CDCA.

FIGURE 4: Effect of different amount of 60% glacial acetic acid on the yield of CDCA.

When the amount of glacial acetic acid added was less than 500 mL, the yield of CDCA increased with the increasing amount of glacial acetic acid. When the amount of glacial acetic acid added was 500 mL or more, the yield of CDCA decreased with the increase of glacial acetic acid amount. A small quantity of glacial acetic acid could neutralize calcium salt and lead to release of CDCA. When the amount of glacial acetic acid reached 500 mL, the calcium salt of CDCA just hydrolyzed completely and the additive amount was suitable. While the amount of glacial acetic acid continuously is increased, CDCA would be dissolved, leading to yield decrease.

3.2. Orthogonal Test. Based on the above experimental data, taking the yield of CDCA as an index, orthogonal experimental design of four factors' three levels was taken to optimize the extracting conditions of CDCA. The factors and levels are shown in Table 1. $L_9(3^4)$ orthogonal test results of intuitive

FIGURE 5: The IR of CDCA of sample product.

TABLE 1: Factors and levels for orthogonal test.

Level	A Methanol (mL)	B H ₂ O ₂ (mL)	C CaCl ₂ (mL)	D Glacial acetic acid (mL)
1	750	25	300	400
2	1000	50	400	500
3	1250	100	500	600

analysis were carried out as in Table 2. Variance analysis of orthogonal experiment results was shown in Table 3. The analysis of variance was performed by statistical software SPSS 12.0.

The results showed that factors influencing the extraction of CDCA from duck bile paste by calcium salt were as follows: hydrogen peroxide, methyl alcohol, glacial acetic acid, and calcium chloride. The optimum extracting conditions of this process were that every 100 g duck bile paste collocated with 1000 mL MeOH, 50 mL H₂O₂, 500 mL 20% CaCl₂ solution, and 600 mL 60% glacial acetic acid. A parallel experiment was performed three times under the conditions of the best combination of factors; the yield was 30%, 29%, and 31%, and average value was 30%; it was significantly higher than single factor tests results.

3.3. Characterization

3.3.1. Structure Characterization

(1) *IR Spectrogram*. The IR spectrogram of sample was shown in Figure 5.

In FTIR spectra, the appearance of a broad stretching vibration band at 3410 cm⁻¹ for hydrogen groups and C-H groups, C=O stretching vibrations, bend vibration for hydrogen groups, C-H bend vibrations, C-O in carboxyl groups at 2940, 1720, 1450, 1378, and 1250 cm⁻¹ (close to the literature value [17]) determined that the obtained substance was CDCA.

(2) *¹H NMR*. The ¹H NMR spectrogram of sample was shown in Figure 6.

¹H NMR spectra were recorded in CDCl₃ on a AVIII600 NMR Spectrometer (Bruker A.G), using TMS as internal

FIGURE 6: The HNMR spectrum of sample product.

FIGURE 7: The HPLC spectrum of sample product.

standard. Figure 6 showed the characteristic peak value (δ H 0.68 (s, 3H, 18-CH₃), 0.92 (s, 3H, 19-CH₃), 0.95 (d, 3H, 21-CH₃), 3.49 (br, 1H, 3-H), and 3.87 (br, 1H, 7-H)) (close to the literature value [18]). By the ¹H NMR spectrogram and the related literature [19], the obtained substance is determined to be CDCA.

3.3.2. *The Detection Results of HPLC*. The product was determined by HPLC. As shown in Figure 7, the retention time of CDCA was 17.3 min. Small impurity peaks can be seen at 10 min, and some of the corresponding materials were likely contained in the raw material. The regression equation of CDCA was $Y = 0.7229X + 16.908$ ($r = 0.9997$); CDCA in the 0.3 μ g–0.7 μ g/ μ L range showed a good linear relationship. According to the regression equation, the purity of CDCA was 97.2%.

4. Conclusions

The detection data proved that the optimum extracting conditions were that every 100g duck bile paste collocated with 1000 mL MeOH, 50 mL H₂O₂, 500 mL of 20% CaCl₂ solution, and 600 mL of 60% glacial acetic acid. Under the optimal extraction conditions, the yield of CDCA can reach 30%.

Over the years, CDCA is mainly obtained from chicken bile. In this paper, CDCA was extracted from the duck bile paste and the optimal extraction conditions were determined by orthogonal test. The process is simple and suitable for industrial production, what is more, it can enhance the added value of livestock and poultry products.

TABLE 2: Analysis of $L_9(3^4)$ test results.

Test number	A	B	C	D	Productivity %
1	1	1	1	1	24
2	1	2	2	2	24
3	1	3	3	3	17
4	2	1	2	3	29
5	2	2	3	1	28
6	2	3	1	2	18
7	3	1	3	2	24
8	3	2	1	3	25
9	3	3	2	1	13
K1	65.20	76.81	66.53	64.92	
K2	74.83	77.12	66.1	65.61	
K3	60.89	46.99	68.29	70.39	
k1	21.73	25.60	22.18	21.64	
k2	24.94	25.71	22.03	21.87	
k3	20.30	15.66	22.76	23.46	
Range R	4.64	10.05	0.73	1.82	
Order	B > A > D > C				
Combination	$A_2B_2C_3D_3$				

Ki represents the sum of number i of various factors ($i = 1, 2, 3$), ki represents the average of number i of various factors ($i = 1, 2, 3$), R represents the difference between the maximum and the minimum of ki of various factors.

TABLE 3: Variance analysis of orthogonal experiment.

Factors	SS	f	F	F threshold	Significance
A	33.96	2	37.83	19	*
B	199.68	2	222.46	19	*
C	0.90	2	1	19	
D	5.92	2	6.59	19	
Error		2			

* indicates that this factor has significant influence on the experiment.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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