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Response Surface Optimization of the Preparation of Baicalin-β-cyclodextrin Inclusion Compound

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***SUMMARY*. The aim of this study was to optimize the preparation process of baicalin-β-cyclodextrin inclusion compound (baicalin-β-CD) by adopting Response Surface Methodology (RSM). On the basis of the single-factor experiment, the conditions of the inclusion process were optimized by adopting RSM with the factors to be investigated *(i.e.* the proportion of β-CD and baicalin, inclusion time and inclusion temperature); the quadratic regression equation model of the inclusion rate of baicalin-β-CD was constructed via simulation. The analysis of the experimental results showed that the optimal inclusion conditions are: proportion of β-CD and baicalin of 2.51, inclusion time of 1.57 h, and inclusion temperature of 63.79 °C. There was a good compliance between the measured result of the inclusion rate (89.83%) and the predictive value (90.22%) of the equation obtained by fitting response surface. The preparation process of baicalin-β-cyclodextrin inclusion compound established by using RSM has a higher inclusion rate, and there is a good coincidence between the regression equation and the actual situation.**

INTRODUCTION

Baicalin (Fig. 1a) is a kind of flavonoids extracted and isolated from *Scutellaria* root and is a glycoside which is formed by the combination of C7-hydroxyl group of baicalein and glucuronic acid. Baicalin has a significant biological activity and has been proved to have bacterial inhibition, clearing heat, antihypertension, sedation, diuresis, cholagogue, anti-inflammatory and anti-allergy, detoxification, anti-cancer and other pharmacological potencies [1-6]; in addition, it can also absorb ultraviolet rays, scavenge oxygen radicals and inhibit melanogenesis [7]. However, it shows water and diluted acid insolubility and poor absorption and it is also easily oxidized and deteriorated due to many phenolic hydroxyl groups; therefore, its application is greatly restricted [8,9]. β-cyclodextrin (β-CD, Fig. 1b) is formed by seven D-glucose units which are linked with α-1,4-glucosidic bond, and has a special and strong hydrophobic cavities in the structure; thus the drug molecules can be included in the molecular of β-cyclodextrin with Van der Waals force and hydrophobic interaction and have a change in the property, result-

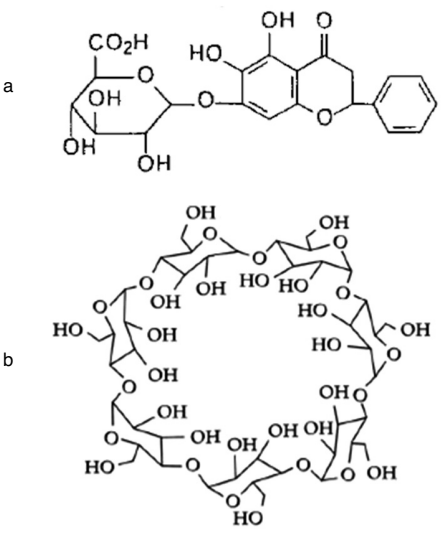

Figure 1. Structures of baicalin (**a**) and β-cyclodextrin (**b**).

KEY WORDS: Baicalin, β-cyclodextrin, Inclusion compound, Preparation process, Response Surface Method.

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ing in a increase of the solubility of the drug molecules and a improvement of the stability and bioavailability [10-12].

Response Surface Methodology (RSM) is a kind of effective method which optimize the process conditions, and use the less data in a range to build the function expression between response value and factors. It can obtain the best combinations and the most accurate results. In this experiment, the saturated aqueous solution was used to prepare baicalin-β-cyclodextrin inclusion compound (referred to as baicalin-β-CD); and the response surface method was adopted to optimize the conditions of the best preparation process; the Differential thermal analyzer (DTA) was employed to characterize the formation, providing a reference for its industrial applications.

MATERIALS AND METHODS

Materials

LC-10A HPLC; BIO-TEK 522 pump; 535 UV detector; WDL-95 chromatographic workstation; Differential thermal analyzer (DTA) (α-alumina as the reference material). *Scutellaria baicalensis* from Changsha Jinsha Chain Drugstores; baicalin reference material from National Institutes for Drug and Food Control (batch number 110715-201112); β-CD from Beijing Aoboxing Biotech Company Ltd (batch number 20100507); anhydrous ethanol from Tianjin Damao Chemical Reagent Factory; other reagents were all of analytical grade.

Extraction and Purification of Baicalin

Decoction pieces of *Scutellaria baicalensis* (200 g) were soaked in 1600 mL of water for 30 min and then was heated until boiling, maintaining the slight boiling for 1.0 h. Then the decoction was filtered with gauze, the dregs of a decoction were re-extracted twice, and the filtrates were merged and then were concentrated by half. The solution obtained above was adjusted to pH 1~2 with 2 M HCl and was kept at 70 °C for 30 min. Then crystals were precipitated, and the centrifugation was performed. The precipitate obtained was added into 8-fold water, and the solution obtained was adjusted to pH 7 with 14.4 M NaOH. The equal amount of ethanol was added, stirring to dissolve and then filtering. The filtrate was adjusted to pH 1~2 with 2 M HCl, kept at 70 °C for 30 min and was allowed to stand for 6 h. After filtered, the filter cake was washed with ethanol to obtain crude baicalin. It was packaged into the filtration paper cylinder and placed in a Soxhlet extractor. Sixty-fold methanol was added, heated to reflux until the extracting solution had very light color, and suction filtration when it was still hot was conducted. The filtrate was concentrated by half under reduced pressure and cooled until the crystals were precipitated. It was allowed to stand for 30 min and filtered. The filter cake was washed with methanol and dried to get Baicalin. Forty-fold methanol was used to recrystallize again, thus obtaining faint yellow Baicalin with high quality [13].

Preparation of Inclusion

The saturated solution method was adopted for preparation. Baicalin (1 g) was dissolved with methanol for use. β-CD was weighed at different feeding ratios and was prepared to be the saturated solution at a certain temperature, which was kept clarified. The different inclusion temperatures were maintained and the baicalin-methanol solution was added into β-CD solution slowly with a dropper. The mixed solution was stirred for a certain period of time on the basis of response surface design, cooled and placed in the refrigerator for 12 h. Suction filtration was performed, and a small amount of water and ethanol was used for washing. The filter cake was dried under vacuum at 60 °C for 10 h to obtain yellowish and loose inclusion compound which was than weighed respectively. The inclusion rate was calculated.

Determination of Inclusion Rate of Baicalin-β-CD

Chromatographic conditions and system suitability Column

CLC-ODS (id 4.6 mm × 150 mm); mobile phase: methanol-water-phosphoric acid (47:53:0.2); detection wavelength: 280 nm; flow rate: 1.0 mL/min; column temperature: 40 °C; injection volume: 20 μL. Theoretical plate number was calculated by baicalin peak and it shouldn't be less than 2,500.

Preparation of the reference solution

Appropriate amount of baicalin reference material dried under reduced pressure at 60 °C for 4 h was taken and weighed accurately. Anhydrous methanol (chromatographic grade) was added to dissolve the sample. Finally, the reference solution (the concentration of baicalin of 60 μg/mL) was prepared.

Preparation of the test solution

Anhydrous methanol (chromatographic grade) was added to dissolve an appropriate amount of baicalin powder to prepare 60 μg/mL of baicalin solution.

Preparation of Standard Curve

Differente volumnes of the 60 μg/mL reference solution were pipetted (0.33, 0.67, 1.00, 1.33, and 1.67 mL), placed in 10 mL volumetric flasks and diluted to the mark with methanol, mixing well and 20 μL of each sample solution was injected. Finally the standard curve was drawn with the measured chromatographic peak area as Y-coordinate and the concentration of the reference solution as X-coordinate. The results found that there was a linear relationship when the concentration of the reference solution was between 20.0 and 100.0 μg/mL. The regression equation was *Y = 9753.1X+1546, r = 0.9993; (n = 5).*

Determination of Inclusion Rate of Baicalin-β-CD

Appropriate amount (25 mg) of baicalin reference material dried under reduced pressure at 60 °C for 4 h was put it in a 100 mL round bottom flask, added 25.0 mL double distilled water, rotated for 1 h, cooled to room temperature naturally, prefiltrated, and filtrated with a 0.45 μm microporous filter membrane. One mL filtrate was absorbed precisely and put in a 10 mL volumetric flask. It was diluted with double distilled water to the scale. The concentration of baicalin in baicalin-β-CD was calculated and thus the inclusion rate of baicalin-β-CD could be calculated. The inclusion rate = the actual drug content in the inclusion compound/drug amount fed × 100%.

Single-factor Test

The single-factor test was conducted with the factors of the proportion of β-CD and Baicalin (A), inclusion time (B) and inclusion temperature (C); the baicalin inclusion compound was prepared; and finally the inclusion rate of Baicalin was calculated [14].

Impact of the proportion of β-CD and Baicalin on the inclusion rate

Under the inclusion time of 1.5 h and the inclusion temperature of 65 °C, the impacts of the proportion of β-CD and baicalin of 1.5, 2, 2.5, 3, and 3.5 on the inclusion rate were studied.

Impact of the inclusion time on the inclusion rate

Under the proportion of β-CD and Baicalin of 3 and the inclusion temperature of 65 °C, the impacts of the inclusion time of 0.5, 1.0, 1.5, 2, and 2.5 h on the inclusion rate were studied.

Impact of the inclusion temperature on the inclusion rate

Under the proportion of β-CD and baicalin of 3 and the inclusion time of 1.5 h, the impacts of the inclusion temperature of 50, 55, 60, 65 and 70 °C on the inclusion rate were studied.

Response Surface Design

On the basis of the optimal level of the results of the single-factor experiment, according to Box-Behnken design principle [15], the response surface experiment with three factors and three levels was designed with the inclusion rate Y as the response value, and by adopting Design Expert 7.0 software in this test, in which, the proportion of β-CD and baicalin (X_1), inclusion time (X_2) and inclusion temperature (X_3) were selected as the three factors, and the factors coding and levels can be seen in Table 1.

Factors	Codes and Levels		
	-1	0	+1
X_1. Proportion of β-CD and baicalin	2	2.5	3
X_2. Inclusion time (h)	1	1.5	2
X_3. Inclusion temperature (°C)	60	65	70

Table 1. Coded values and corresponding actual values of the optimization parameters used in response surface analysis.

Identification of Baicalin-β-CD by DTA

DTA was performed for baicalin, β-CD, mixture of baicalin and β-CD and baicalin-β-CD, with a heating rate of 10 °C/min and the heating range of 40~400 °C.

RESULTS AND DISCUSSIONS

Extraction of Results from the single-factor experiment

Impact of the proportion of β-CD and Baicalin on the inclusion rate

From Fig. 2 it can be seen that the inclusion rate was rising with the increase of the proportion of β-CD and baicalin, and reached the peak value at the proportion of 2.5 and then began to decline. From the inclusion rate, it was recommended that the proportion should be controlled at about 2.5.

Impact of the inclusion time on the inclusion rate

From Fig. 3 it can be seen that the inclusion rate was increasing with the prolongment of the inclusion time, and reached a certain level when the time was 1.5 h and then did not change significantly with the continuous prolongment of the inclusion time. It was recommended that the inclusion time should be controlled at about 1.5 h by combining with the actual conditions.

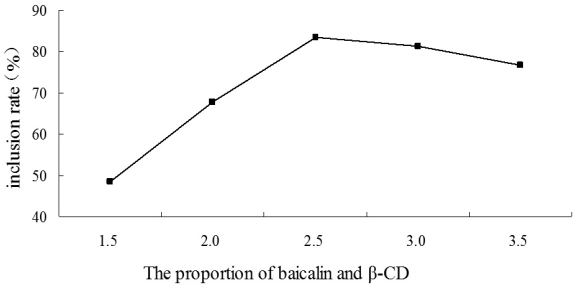

Figure 2. Impact of the proportion of baicalin and β-CD on the inclusion rate.

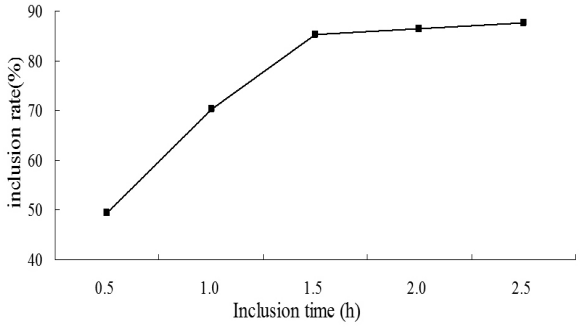

Figure 3. Impact of inclusion time on the inclusion rate.

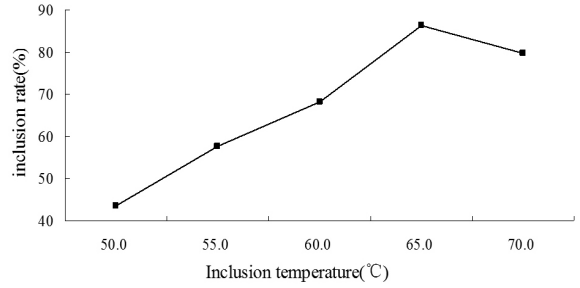

Figure 4. Impact of the inclusion temperature on the inclusion rate.

Impact of the inclusion temperature on the inclusion rate

From Fig. 4, it can be seen that the inclusion rate was increasing with the increase of the inclusion temperature, and reached the peak value when the temperature was 65 °C and then began to decline. It was recommended that the inclusion temperature should be controlled at about 65 °C by considering comprehensively the economic factors and the simplification of the follow-up process.

Response Surface Optimization Test

The values of the response surface optimization test can be seen in Table 2, and there were a total of 15 experimental points. The first 12 were the factorial points, and the independent variable values were at the vertices of the three-dimension composed of A, B and C; the next three were null points which were the central points of the region and used to estimate the experimental error [16]. All the inclusion rates of baicalin-β-CD obtained were shown in Table 2.

Number	X_1	X_2	X_3	Y inclusion rate of baicalin-b-CD (%)
1	2.5	1.0	70	71.26
2	3.0	1.5	60	79.61
3	2.5	2.0.	60	81.23
4	2.0	1.5	60	84.35
5	3.0	1.0	65	77.57
6	2.0	1.0	65	76.47
7	2.5	2.0	70	68.44
8	2.0	2.0	65	78.34
9	2.0	1.5	70	72.54
10	2.5	1.0	60	73.53
11	3.0	1.5	70	77.32
12	3.0	2.0	65	82.31
13	2.5	1.5	65	88.97
14	2.5	1.5	65	89.89
15	2.5	1.5	65	90.18

Table 2. Optimization scheme and results of the optimal extraction process of baicalin-β-CD inclusion compound.

Regression Equation

The proportion of β-CD and baicalin (X_1), the inclusion time (X_2) and the inclusion temperature (X_3) were selected to perform the response surface analysis with three factors and three levels. The optimal conditions for the preparation of the inclusion compound were determined. The results can be seen in Table 2. The software was adopted to perform multivariate regression fit for the experimental data in Table 2, thus obtaining the quadratic polynomial regression equation of the model: $Y = 89.68 + 0.64A + 1.44B - 3.65C + 0.72AB + 2.38AC - 2.63BC - 3.08A^2 - 7.92B^2 - 8.14C^2$.

Analysis of variance for the regression model

As shown in Table 3, the analysis of variance was conducted for this regression model, and the results showed $P < 0.0001$ in the model, which indicated the response regression model of this test had a very significant level; $P = 0.2566$ for lack-of-fit is not considered significant; the corrected coefficients of determination of the model $R^2 = 0.9930$ showed a good fit between this model and the actual test; $R^2_{Adj} = 0.9805$ indicated that this model can explain the change of response value of 98.05%, had a small experimental error and can describe well the true relationship of the proportion of β-CD

Source of variance	Quadratic sum	Degree of freedom	Mean square	F value	P Value
Model	634.96	9	70.55	79.27	<0.0001
X_1	3.26	1	3.26	3.67	0.1137
X_2	16.50	1	16.50	18.54	0.0077
X_3	106,29	1	106.29	119.42	0.0001
X_1X_2	2.06	1	2.06	2.31	0.1887
X_1X_3	22.66	1	22.66	25.46	0.0039
X_2X_3	27.67	1	27.67	31.09	0.0026
X_1^2	35.11	1	35.11	39.45	0.0015
X_2^2	231.82	1	231.82	260.247	<0.0001
X_3^2	244.73	1	244.73	274.97	<0.0001
Residual	4.45	5	0.89		
Lack of Fit	3.65	3	1.22	3.05	0.2566
Pure Error	0.80	2	0.40		
Cor Total	639.41	14			
R^2 = 0.9930	*R^2_{Adj} = 0.9930*	*CV = 1.19%*			

Table 3. Analysis of variance for the regression model.

and Baicalin, inclusion time and inclusion temperature with the inclusion rate of Baicalin-β-CD. It can be concluded from the results in Table 3 that all the linear terms (X_2 and X_3) and quadratic terms (X_1^2, X_2^2 and X_3^2) of the equation had a very significant impact on the inclusion rate ($p < 0.01$); the interactive terms X_1 X_3 and X_2 X_3 also had a very significant effect ($p < 0.01$); and other terms did not had a significant impact. From the above, it can be shown that there was not simple linear relationship between each factor and the response value, while the interaction existed. The regression equation model obtained from the optimization test with response surface method can be used to analyze and forecast the results of the inclusion of Baicalin-β-CD.

Response Surface Analysis

Design Expert 7.0 software was adopted to perform quadratic multivariate regression fit for the data in Table 3, thus establishing the response surface. The results can be shown in Fig. 5.

Figure 5a reflected the impact of the proportion of β-CD and baicalin and the inclusion time on inclusion rate of baicalin-β-CD at the inclusion temperature of 65 °C. When the proportion of β-CD and baicalin was lower, the inclusion rate first increased and then decreased with the increase of the inclusion time, and when the proportion was close to 2.5 and the time was close to 1.5 h, the inclusion can reach the peak value.

Figure 5b reflected the impact of the proportion of β-CD and baicalin and the inclusion temperature on inclusion rate of baicalin-β-CD when the inclusion time was 1.5 h. The inclusion rate increased rapidly with the increase of the liquid-to-solid ratio, and when the proportion was about 2.5 and the inclusion temperature was between 62.5 and 65 °C, the response value can reach a highest point.

Figure 5c reflected the impact of the inclusion temperature and the inclusion time on inclusion rate of baicalin-β-CD when the propor-

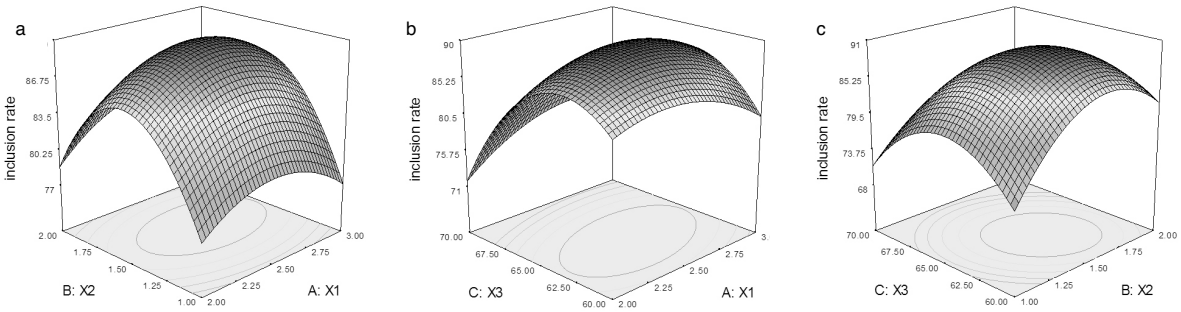

Figure 5. Response surface of the impact of each two-factor interaction on the inclusion rate of baicalin-β-CD. **a**) inclusion time and the proportion of baicalin and β-CD, **b**) inclusion temperature and the proportion of baicalin and β-CD, **c**) inclusion temperature and inclusion time.

tion of β-CD and baicalin was 2.5. The inclusion rate increased rapidly with the increase of the inclusion time, and the inclusion can reach the peak value when the temperature was about 65 °C and the inclusion time was between 1.25 and 1.75 h.

Determination of the optimal conditions and confirmatory experiment

The optimal conditions for baicalin-β-CD obtained through the analysis with the software were: inclusion temperature: 63.79 °C, inclusion time: 1.57 h, proportion of β-CD and baicalin: 2.51, so the predicted inclusion rate of the model was 90.22% under these conditions above. Considering the actual operations, the experimental conditions were modified as follows: inclusion temperature: 64 °C, inclusion time: 1.6 h, proportion of β-CD and baicalin: 2.5. The confirmatory experiment was conducted parallelly for three times under the modified conditions, and the mean inclusion rate of baicalin-β-CD was 89.83% (RSD: 0.47%) with a error of 0.39% compared to the theoretical predicted value (90.22%).

Identification of Baicalin-β-CD

The spectrum of each sample with differential scanning calorimetry was obtained by the results of DTA analysis. It can be seen in Fig. 6 that the curve for the physical mixture (baicalin+β-CD) of β-CD and baicalin was formed by the simple superposition of baicalin and β-CD, while the curve for baicalin-β-CD was significantly different from the other three curves, that is, there was absorption peaks at about 90 and 130 °C, and exothermic peak at about 290 °C; however, completely contradict peaks appeared at 80, 150, and 320 °C for β-CD and baicalin+β-CD, which indicated that baicalin had been included in β-CD and its structure had changed, thus forming a new phase, which confirmed the formation of inclusion compound.

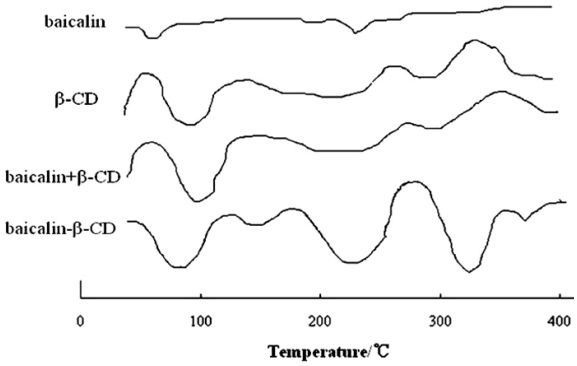

Figure 6. DTA curves of baicalin, β-CD, baicalin+β-CD and baicalin-β-CD.

CONCLUSIONS

Box-Behnken model of response surface method is adopted to design the experimental scheme for the preparation of baicalin-β-CD with saturated solution method, and the inclusion process is optimized. Thus the optimal process conditions are obtained as follows: inclusion temperature: 63.79 °C, inclusion time: 1.57 h, proportion of β-CD and baicalin: 2.51. The results from the confirmatory experiment conducted under the optimal conditions show that the inclusion rate of Baicalin-β-CD is 89.83%. This test results show that the preparation process of Baicalin-β-CD which is optimized by adopting response surface method can obtain the maximum inclusion rate with accurate and reliable parameters and practical value.

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