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Original Research Article

Extraction-condition Optimization of Baicalein and Schisandrin from Hu-gan-kang-yuan Formula Using Orthogonal Array Design

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Abstract

Purpose: *To optimize the extraction conditions for Hu-gan-kang-yuan Formula based on extraction rates of baicalein and schisandrin using an orthogonal array design.*
Methods: *Ethanol concentration (50 - 70 %), ratio of solvent to raw material (8 - 12 mL/g), and extraction time (1 - 3 h) were examined with a three-factor and three-level L9(3)3 orthogonal array design. In addition, analysis of variance (ANOVA) was used to evaluate the statistical significance of the effects of individual factors on extraction rates of baicalein and schisandrin determined by high performance liquid chromatography (HPLC). The number of extractions was further investigated to confirm the extraction rate of target compounds based on the optimized conditions.*
Results: *The optimized conditions were an ethanol concentration of 70 %; ratio of solvent to raw material, 12:1 mL/g; and extraction time of 60 min. Ethanol concentration and ratio of solvent to raw material showed significant effects on the extraction of the two compounds ($p < 0.05$). The number of extraction steps, two, was reasonable. The final optimum extraction conditions resulted in 79.48 ± 1.40 and 88.55 ± 1.85 % of extraction for baicalein and schisandrin, respectively.*
Conclusion: *The optimized extraction conditions for baicalein and schisandrin are practicable and repeatable, and can be upgraded for pilot-scale production of Hu-gan-kang-yuan preparations.*

Keywords: *Hu-gan-kang-yuan Formula, Extract-condition optimization, Orthogonal array design, Baicalein, Schisandrin*

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INTRODUCTION

Hepatitis B virus (HBV) infection remains a major global health threat [1]. Interferon-α and some nucleoside analogues have unavoidable drawbacks. Adefovir has relatively high resistance during long-term treatment [2]. Thus, there is a continuing need to develop further effective and safe drugs for treating HBV infection.

Hu-gan-kang-yuan pill consists of 12 traditional Chinese medicinal materials in a hospital preparation and has been used to treat hepatitis B, decreasing aminopherase and increasing immunity, for many years. However, the processing required, directly mixing all the powders of medicinal material into pills, inevitably results in very large doses (6 g three times per day). Further development of Hu-gan-kang-yuan formula by optimizing extraction conditions is needed. To date, selecting extraction rates of

typical active compounds as indicators for optimizing extraction conditions has been the main extraction scheme in Chinese medicine preparation. Baicalein and Schisandrin are both typical active ingredients with hepatoprotective and antifibrotic effects in *Scutellariae radix* and *Chinensis fructus*, respectively [3-6].

Orthogonal array design is commonly used to optimize conditions for the extraction of Chinese medicinal materials based on a reasonable number of experiments [7-9]. Here, we sought to optimize the extraction conditions of Hu-gan-kang-yuan formula, based on the extraction rates of baicalein and schisandrin. The main process parameters (ethanol concentration, ratio of solvent to raw material, extraction time and numbers of extraction) and their effects on the extraction rates of the target compounds were studied. A simple and efficient simultaneous determination method of baicalein and schisandrin was developed.

EXPERIMENTAL

Materials and reagents

The medicinal materials (*Schisandrae chinensis fructus, Ligustri lucidi fructus, Epimedii folium, Isatidis Radix, Polygoni cuspidate rhizome et radix, Acanthopanacis senticosi radix et rhizoma seu caulis, Bupleuri radix, Moutan cortex, Scutellariae radix, Astragali radix, Hominis placenta, Taxilli herba*) were purchased from Kangsheng Medicinal Company (Guanghzou, China). They were all identified by Associate Professor Zhang Hongwei (Department of Medicinal Plants & Pharmacognosy, Southern Medical University, Guangzhou, China) according to pharmacognostic standards documented in Volume I of 2010 Edition of China Pharmacopoeia. The samples (Table 1) were kept in a desiccator (silica gel as desiccant) at room temperature in Pharmaceutical Laboratory, Department of Chinese Pharmaceutics, Southern Medical University, Guangzhou, China.

Reference standards for baicalein and schisandrin (Fig 1) were purchased from National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China (batch numbers 111595-201306 and110857-201010, respectively). The purities of the standards were 98.8 % and 99.4 %, respectively. Acetonitrile was chromatographic grade, and phosphoric acid, methanol, and ethanol were analytical grade. Ultrapure water was provided by Southern Medical University (Guangzhou, China).

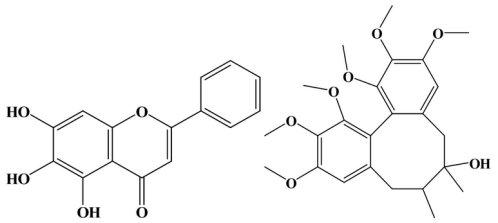

Figure 1: Chemical structure of baicalein (A) and schisandrin (B)

Table 1: Medicinal plants used in the study

No.	Botanical name
1	*Schisandrae chinensis fructus*
2	*Ligustri lucidi fructus*
3	*Epimedii folium*
4	*Isatidis Radix*
5	*Polygoni cuspidate rhizome et radix*
6	*Acanthopanacis senticosi radix et rhizoma seu caulis*
7	*Bupleuri radix*
8	*Moutan cortex*
9	*Scutellariae radix*
10	*Astragali radix*
11	*Hominis placenta*
12	*Taxilli herba*

Preparation of reference/standard solutions

Single-stock solutions of baicalein (6.23 mg/mL) and schisandrin (11.30 mg/mL) were prepared. The final concentrations of baicalein and schisandrin in the mixed standard solution were 62.3 µg/mL and 113.0 µg/mL, respectively. Three quality control standard (QC) samples containing baicalein and shisandrin (LQC: 12.46 µg/mL, 22.6 µg/mL; MQC: 62.3 µg/mL, 113.0 µg/mL and HQC: 112.14 µg/mL, 203.4 µg/mL respectively) were prepared from stock solutions of baicalein and schisandrin with methanol.

Preparation of sample solution

All medicinal materials in Hu-gan-kang-yuan formula were ground into particles. One formulation amount (total, 104 g) was exactly weighed and put into a 2000 mL of round-bottom flask, 824 mL 60 % ethanol was added. The mixture was extracted for 60 min by reflux. Thereafter, 10 mL supernatant was transferred and evaporated to dryness at 60 ^{o}C over a hot water bath. The residue was dissolved in methanol and completely transferred to 5 mL of volumetric flask and the volume made up to scale mark with methanol. Finally, the sample solution was filtered with a 0.45 µm-membrane filter (Supelco Inc., Bellefonte, PA, USA) before injection into the HPLC for analysis. The extraction rate (%) of the two compounds was calculated as in Eq 1.

Extraction rate (%) = $(m/m_0)100$ …….. (1)

where m (mg) is the amount of baicalein or shisandrin in extraction solution, and m_0 is the amount of baicalein or shisandrin in raw material of *Schisandrae chinensis fructus* and *Scutellariae radix*, respectively.

Preparation of negative control sample

All herbal medicines in Hu-gan-kang-yuan Formula, except *Schisandrae chinensis fructus* and *Scutellariae radix*, were weighed exactly and exacted according to the preparation of sample solution. The extraction solution was used as negative control sample solution to evaluate the specificity of analytical method for determining baicalein and schisandrin.

Orthogonal array experimental design

Based on the physicochemical property of baicalein and schisandrin and other medicinal materials in Hu-gan-kang-yuan Formula, a three-factor and three-level orthogonal array design, $L_9(3)^3$, was used. Ethanol concentration, ratio of solvent to raw material and extraction time were the independent variables selected to optimize the extraction conditions of baicalein and schisandrin. The variables and their levels are reported in Table 2. Nine experiments with extraction rates of baicalein and schisandrin used as indexes to evaluate the independent variables are shown in Table 3. The experiments were carried out in random order to minimize any effects of unexplained variability in observed indexes due to systematic errors. All samples were tested in triplicate.

Table 2: Variables and experimental design levels

Independent variable	Level		
	1	2	3
Ethanol concentration (A, %)	50	60	70
Ratio of solvent to raw material (B, mL/g)	12	10	8
Extraction time (C, h)	1	2	3

Number of extractions

Normally, extraction rates increase as number of extractions is increased; however, it will increase energy consumption and take a large amount of time. To avoid an interaction effect between extraction time and number of extractions [9], three different numbers of extraction under the optimized extraction conditions were also studied as a single factor experiment.

HPLC analysis

Quantitative analyses of baicalein and schisandrin were carried out with an Agilent 1260 HPLC system equipped with a diode aray detector (Agilent Technologies, CA, USA). The separation was achieved on a Venusil XBP C18 (250 × 4.6 mm, 5 µm) (dalian, China) at ambient temperature. The mobile phase consisted of acetonitrile (solvent A) and 0.01 % phosphoric acid in water (solvent B). The gradient used was 0 - 5 min, 25 % A; 5 - 20 min, 25 -35 % A; 20 - 40 min, 35 - 50 % A. The flow rate was maintained at 1 mL/min, the injection volume was 10 µL and the wavelength was 280 nm.

Validation of the HPLC method

Specificity is the ability of a method to discriminate between the study analyte(s) and other components in the sample. In this study, the specificity was demonstrated by successively running a mixed reference standard sample of baicalein and schisandrin, a sample and a negative control sample solution under the same conditions. The data for peak area versus injection amount were treated by linear regression analysis. 2, 5, 10, 15 and 20 µl of the mixed standard solution of baicalein and schisandrin was injected. The response area (y) versus injection amount (x), in the range 0.124 - 1.246 and 0.226 - 2.26 µg for baicalein and schisandrin, respectively, was assessed. The intra-day and inter-day precisions (%, RSD) were established by analyzing QC samples on day 1 and on each of three consecutive days in five replicates. QC samples of baicalein and schisandrin at three different concentrations were stored at 4 ºC, analyzed by HPLC after 1, 2 and 3 days, 1 and 2 weeks and 1 month, and then evaluated again by calculating area RSD (%) values of baicalein and schisandrin at the different time points. Accuracy was evaluated by means of recovery assays, carried out by adding known amounts of baicaleina and schisandrin standard solution to the sample at similar concentration in six replicates. The spiked amounts of baicalein and schisandrin reference standards was 6.23 and 11.3 µg, respectively. The original amounts of baicalein and schisandrin in the sample solution was 8.35 µg and 6.83 µg, respectively. Mean recovery was calculated as in Eq 2.

Recovery (%) = {(Ad - Ao)/As} …….. (2)

where Ad, Ao and As are determined amount, original amount and amount spiked, respectively.

Statistical analysis

To assess the statistical significance of the effects of individual factors on extraction rates of baicalein and schisandrin, an ANOVA was used to evaluate the experimental data obtained from the orthogonal array design using SPSS software (ver. 13.0).

RESULTS

HPLC analysis and validation

Typical HPLC chromatograms of the reference standards of baicalein and schisandrin, sample, and a negative control sample are shown in Fig 2. There are no peaks of baicalein or schisandrin in the negative control sample. In addition, their peaks in the reference standard and sample showed good resolution compared to adjacent peaks. The calibration curves of baicalein and schisandrin were linear over the injection amount of 0.124 - 1.246 µg and 0.226 - 2.26 µg, respectively. The calibration curves and coefficients of determination (r) were y = 2715.98x + 17.82 (r = 0.9998) and y = 1721.37x + 10.52 (r=0.9999) for baicalein and schisandrin, respectively. The mean recoveries of baicalein and schisandrin were 98.27 % (RSD = 2.54 %) and 97.18 % (RSD = 1.97 %), respectively. The intra-day precisions (RSD values) was in the range of 0.27 - 1.22 % and 1.02 - 1.27 %, respectively, whereas inter-day precisions were in the range of 0.76 - 1.65 % and 1.02 - 1.89 %. The RSD values of baicalein and shisandrin solution at 4.0 ºC for 1 month were in the range of 1.1 - 2.17 % and 0.86 - 1.98 %.

Analysis of experimental design

Because various parameters can potentially affect the extraction rates, the optimization of experimental conditions is a key step in developing an extraction method for baicalein and schisandrin from Hu-gan-kang-yuan Formula. The three process variables (ethanol concentration, ratio of solvent to raw material, and extraction time) were considered the most important factors. In addition, the focus was on the main effects of the factors and not interactions among different variables in the matrix. The sample obtained from each test was analyzed using the validated HPLC method. The amounts of shisandrin in the raw material of *Schisandrae chinensis fructus* and baicalein in *Scutellariae radix* were determined using the validated method. The experimental and analysis results are shown in Tables 3 and 4, respectively.

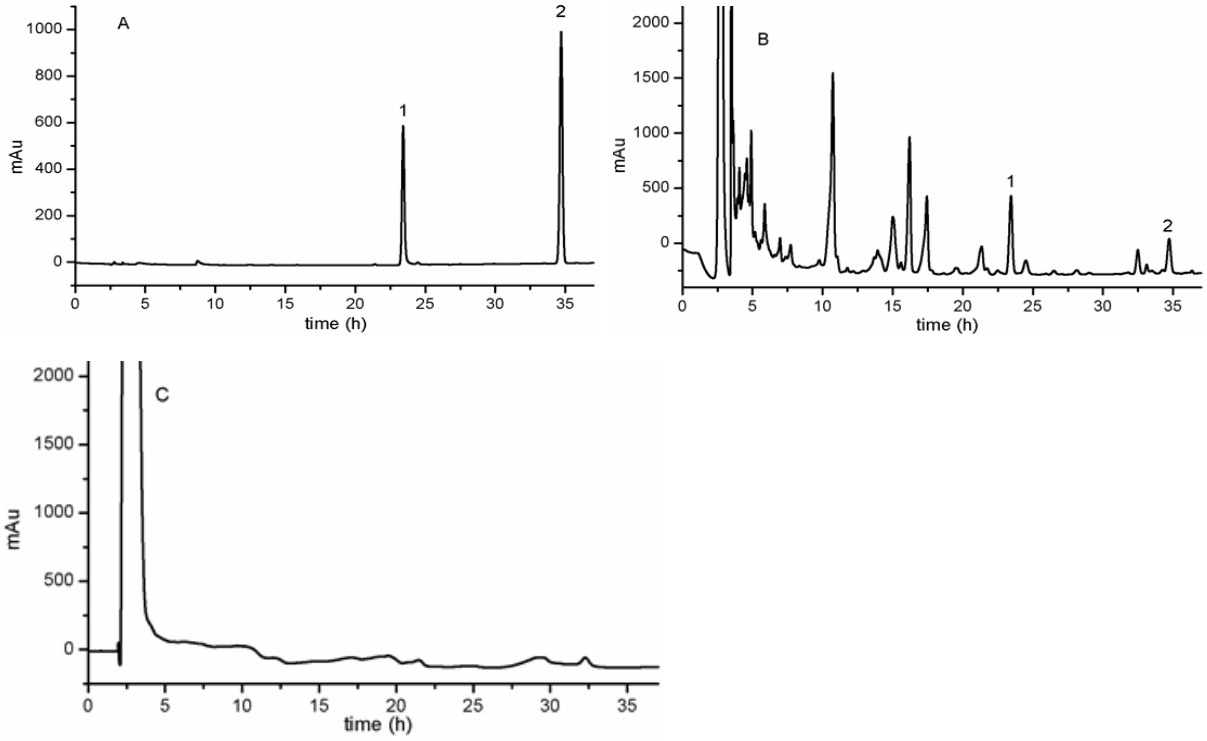

Figure 2: HPLC chromatographic profiles of standards mixture (A) and samples of Hu-gan-kang-yuan Formula (B) and negative control sample (C): (1) baicalein, (2) schisandrin

Table 3: Orthogonal array design and results of the three variables studied

Exp no.	A (%, v/v)	B (mL/g)	C (h)	Baicalein (%)	Schisandrin (%)
1	50	12	1	60.32±0.78	70.29±0.65
2	50	10	2	58.17±0.43	66.55±0.58
3	50	8	3	49.96±0.28	54.99±0.49
4	60	12	2	61.08±0.54	73.76±0.66
5	60	10	3	58.24±0.62	66.01±0.70
6	60	8	1	52.01±0.37	61.00±0.63
7	70	12	3	67.20±0.41	78.42±0.72
8	70	10	1	64.93±0.55	76.54±0.83
9	70	8	2	57.26±0.38	65.76±0.74

Table 4: Analysis of $L_9(3)^3$ test results

Level	Baicalein				Schisandrin		
	A	B	C		A	B	C
L1	168.45	188.60	177.26		191.82	222.46	207.82
L2	171.33	181.34	176.51		200.76	209.10	206.07
L3	189.39	159.23	175.39		220.72	181.74	199.41
R	20.94	29.37	1.87		28.90	40.72	8.41

L = Sum of extraction rates for the factors at each level. R = M_{max} - M_{min}

The maximum extraction rates of baicalein and schisandrin were 67.20 ± 0.41 % and 78.42 ± 0.72 %, respectively (Table 3). Each variable had a different influence on the extraction rates of the target compounds. Based on the results shown in Table 3, it was difficult to select the "best" extraction conditions. Further analysis was subsequently carried out and the results are listed in Table 4. Factor B was the most significant factor, according to the R value, while Factor C was relatively insignificant.

The significance of each variable was determined based on a *p*-value of < 0.05, and the results are summarized in Table 5, along with analysis of variance (ANOVA) results. Ethanol concentration and the ratio of solvent to raw material were significant factors for the extraction of baicalein and schisandrin ($p < 0.05$), while extraction time had no significant effect ($p > 0.05$).

The extraction efficiency significantly increased as the numbers of extractions increased from 1 to 2 (Table 6), whereas there was only a slight increase when the number of extractions was increased from 2 to 3 (Table 6).

Table 6: Effect of numbers of extraction (n = 3)

No. of extraction	Baicalein (%)	Schisandrin (%)
1	68.47±1.78	79.84±1.56
2	79.48±1.40	88.55±1.85
3	80.16±2.28	89.37±2.09

DISCUSSION

The extraction rates of baicalein and schisandrin increased with increasing ethanol concentration. Ethanol was selected at the highest concentration considering the hepatoprotective effect and immunity-enhancing effects of Hu-gan-kang-yuan formula. There are many polysaccharides in this formula, such as *Astragalus* polysaccharides from *Astragali radix*, that display hepatoprotective properties [10], immunity-enhancing function [11,12], anti-inflammatory and antioxidant effects [13-15]. Polysaccharides are largely water-soluble and their contents decrease with increasing ethanol concentration as extraction solvent. Considering the maximum extraction rates of baicalein of 67.20 ± 0.41 % and schisandrin of 78.42 ± 0.72 % under the optimized extraction conditions,

Table 5: F-values from ANOVA results

Factor	Baicalein			Schisandrin		
	Sum of squares	*F*-value	*P-value*	Sum of squares	*F*-value	*P-value*
A	85.88	173.92	0.006*	145.84	50.13	0.02*
B	156.01	315.95	0.003*	287.21	98.72	0.01*
C	0.58	1.18	0.458	13.12	4.51	0.18
Error	0.49			2.91		

**P < 0.05*

70 % ethanol was selected as the extraction solvent to balance the extraction amounts of target compounds and polysaccharides.

The results of ratio of solvent to raw material between 8:1 and 12:1 mL/g indicated that the solvent volume in the studied range played an important role in the extraction of baicalein and schisandrin. Generally, a larger solvent volume will dissolve constituents more effectively, leading to improved extraction yields of target compounds. However, from an economic perspective, using a large amount of solvent is not cost-effective due to the high costs of solvents and extra energy consumption. Thus, a ratio of solvent to raw material 12 mL/g was selected.

Time is a major factor for the evaluation of extraction efficiency. A shorter extraction time could cause incomplete extraction, while a longer extraction time could waste time and solvent. Based on the reports of baicalein and schisandrin extractions, extraction times of 1, 2 and 3 h were assessed. The extraction rates were only slightly affected as the extraction time was increased from 1 to 3 h. Thus, a 1 h extraction time was chosen. Considering the maximum extraction rates of baicalein and schisandrin, the cost of energy and the feasibility of experiment, the optimum extraction conditions were determined to be an ethanol concentration 70 %, ratio of solvent to raw material 12 mL/g, and two extractions of 1h.

CONCLUSION

The effect of variables assessed on the extraction rates of baicalein and schisandrin was in the rank order: ratio of solvent to raw material > ethanol concentration > extraction time. The process variables (ethanol concentration and ratio of solvent to raw material) were the two major factors affecting the extraction rates. Moreover, an efficient HPLC method has been developed for the simultaneous determination of baicalein and schisandrin in the extraction solution of Hu-gan-kan-yuan Formula (composed of 12 Chinese herbal medicines) with good sensitivity, precision and repeatability. The results obtained can be used to enhance pilot-scale production of Hu-gan-kang-yuan Formula and for the quality control of baicalein and schisandrin in the preparation.

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