Studies on Quantitative Determination of Total Alkaloids and Berberine in Five Origins of Crude Medicine “Sankezhen”

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Introduction

“Sankezhen” is a commonly used traditional Chinese medicine. The botanical origins are dried roots of many species of Berberis plants in Chinese Pharmacopoeia 2010. Genus Berberis includes ~500 species, and over 200 are spread in the southwest and northwest of China (1, 2). The major chemical constituents of Berberis are alkaloids, such as berberine, berbamine, jatrorrhizine, palmatine and so on (3), which have the effects of antibacterial, anti-inflammatory, chologagogue, antihypertensive, antitussive, immunity enhancing, antineoplastic, antipsoriasis and antituberculous according to the present pharmacological investigation (4). Berberine is mainly extracted from Berberis plants, having the pharmacological activities of antibacterial, anti-inflammatory, antihypertensive, antihyperlipidemic, antiarrhythmic, antineoplastic and antidiabetic materials (5–11). Through market investigation, we found that the “Sankezhen” commodity mixed with lots of stems of the Berberis plants, which were not the traditional medicinal part in China. To evaluate the quality of different origins of “Sankezhen” and ensure the clinical effectiveness, we took the roots and stems of Berberis soulieana (BS) and other four Berberis species as samples and determined the contents of the total alkaloids and berberine. We hope to provide a reliable quality evaluation method of crude drug “Sankezhen” and obtain some basic information of the activity ingredients for developing the stems of Berberis plants as a new resource of “Sankezhen”.

Techniques frequently employed in determination of alkaloids include high-performance liquid chromatography (HPLC), acid–base titration and acid dye colorimetry. HPLC is sensitive and accurate for the determination of one or more individual alkaloid, but do not apply to the total alkaloids of crude medicines and their extracts on account of their various types and complex structures. In addition, it will have an obvious determination error in the content measurement of total alkaloids by the acid–base titration method.

The acid dye colorimetry method is commonly used in the determination of the total alkaloid content. The principle is that certain organic solvent can extract the colored complex (ion pair) quantitatively, which is the combination of an acid dye and a salt ion formed by the reaction of alkaloids and hydrogen ions under some acidic conditions (12). Therefore, the ion pair being formed and extracted completely is the key to this method, while the extraction rate depends on the acidity of aqueous phase and the nature of acid dye and organic solvent. Chloroform is the most commonly used organic solvent for the determination of alkaloids, having the advantages of forming hydrogen bonds with ion pair easily, high extraction rate, good selectivity and poor aqueous solubility (13). Acid dyes chosen for the experiment must be combined with alkaloids quantitatively, while the formation must have great solubility in organic phase and high absorbance at its maximum absorption wavelength. Besides, the dye should not be extracted by the organic solvent. Therefore, bromothymol blue and bromoresol green were compared in this experiment, as well as different acidity and dosage of the buffer solution. Moreover, the chloroform was selected.

Experimental

Materials and reagents

Five origins of “Sankezhen” [B. soulieana (BS), B. henryana (BH), B. triacanthophora (BT), B. gagnepainii (BG) and B. bergmanniae (BB)] were collected from Jianshi and Changyang, Hubei province of China and authenticated by D.W., a professor in Pharmacy College of South-Central University for Nationalities.

The standard berberine hydrochloride was purchased from the National Institutes for Food and Drug Control of China (batch number: 1100713–200911). Bromoresol green and bromothymol blue were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China; batch number: 201201010 and 20120917). Acetonitrile, HPLC grade, was purchased from...
Methods for total alkaloid determination

Preparation of standard solutions and the sample
The stock standard solution, 20 μg/mL, was prepared by dissolving dried powder of berberine hydrochloride in methanol. About 1.0 g of the powdered sample was weighed accurately and extracted under a reflux condenser with 40 mL of methanol (90%, v/v) for 40 min. After being cooled to the room temperature, the extract was weighed again. The loss in weight was supplemented with the same solvent, and then, the solution was shaken and filtered. One milliliter of the subsequent filtrate was pipetted and diluted to a final volume of 25 mL with 90% methanol.

Selection of the detection wavelength
Bromocresol green and bromothymol blue were taken as acidic dyes separately. According to the absorption spectra of the standard and sample solutions, the strong absorption peak of both berberine and the sample solutions had a wavelength of 418 nm. Therefore, 418 nm was chosen as the detection wavelength of total alkaloids.

Optimization of measurement procedures
Three milliliters of bromocresol green and bromothymol blue solutions were pipetted into two separating funnels and were mixed with 4 mL of phosphate buffer (pH 5.0). After a series of experimental operations as described before, each absorbance was measured at a wavelength of 418 nm with an ultraviolet (UV)/Vis spectrophotometer (Mapada, China), and the chloroform was used as blank solvent. It turned out that the absorbance value of the former was much smaller.

A better colorimetric condition was selected, including the dosage of acidic dye and phosphate buffer and the acidity of the buffer solution. The results showed that the extraction rate was the highest when 3 mL of bromocresol green and 3 mL of phosphate buffer solution of pH 4.5 were used.

Optimization of extraction procedures
A root of BS was used for the investigation of the extraction procedure. Similarly, five main factors including different extraction solvents, extraction process, exacting time, the concentration of acidic dye and phosphate buffer and the acidity of the buffer solution were investigated by using the single factor analysis method. For the extraction process, heated reflux and ultrasonic methods, as well as different extracting time (20, 40, 60 and 80 min), were investigated. The suitable solvent was selected according to the choice solvent experiments, including choices between methanol and ethanol; among 80%, 90% and pure of methanol; and among solvent volume of 30, 50, 70 and 90 mL.

Optimization of chromatographic conditions
HPLC analysis was performed on a Dionex Ultimate 3000 HPLC system that comprised a quaternary pump, an autosampler, a column thermostat, temperature-controlled sample trays, an online degasser and a UV detector. The analytical column was a Waters Symmetry C18 (4.6 × 250 mm, 5 μm).

Based on the results of the full wave scanning of the five medicinal Berberis species and the absorbing wavelength of the standard solution of berberine, 265 nm was chosen as the detection wavelength. A series of solutions were analyzed with different ratios of acetonitrile–0.02% potassium dihydrogen phosphate solution to choose the mobile phase. Finally, the ratio of 26:74 (v/v) was selected for the mobile phase with a flow rate of 1.0 mL/min. The temperature of the column was maintained at 30°C, and the injection volume was 10 μL.

The chromatographic conditions were optimized to separate the primary marker peaks of each sample with good resolution (R > 1.5) and theoretical plate numbers (berberine: n > 8,000). The berberine peaks were identified by comparing their retention times of the peaks with that of the standard (Figure 1). The concentration of the berberine was calculated according to the equation of the calibration curves. Three parallel operations were performed for each sample.

Methods for HPLC determination of berberine

Preparation of standard and sample solutions
The stock standard solution, 50 μg/mL, was prepared by dissolving standard berberine hydrochloride in methanol. Sample solutions were prepared in the same way as they were prepared in the experiment for determination of total alkaloids. Each solution was filtered through a 0.45-μm nylon membrane filter before injection.

Optimization of extraction procedures
A root of BS was used for the investigation of the extraction process. Similarly, five main factors including different extraction solvents, extraction process, exacting time, the concentration of the solvent were investigated by using the single factor analysis method. For the extraction process, heated reflux and ultrasonic methods, as well as different extracting time (20, 40, 60 and 80 min), were investigated. The suitable solvent was selected according to the choice solvent experiments, including choices between methanol and ethanol; among 80%, 90% and pure of methanol; and among solvent volume of 30, 50, 70 and 90 mL.

Optimization of measurement procedures
A certain amount of the test solution was accurately pipetted and evaporated, and the residue was dissolved in 3 mL of phosphate buffer solution of pH 4.5. The solution was transferred into a separatory funnel and was thoroughly mixed with 3 mL of bromocresol green solution (0.03%). Thirty minutes later, 5 mL of chloroform was added into it and shaken for 2 min. The lower layer was separated after 10 min. The extraction was continued for three times, and the extracts were mixed in a volumetric flask. The same step was repeated, and the same volume of methanol (90% v/v) was used as solvent blank. The extracts were analyzed by using a UV–Vis spectrophotometer at a wavelength of 418 nm. There were three parallel operations for each sample.

Results

Experiment for the determination of total alkaloids by UV–Vis spectrophotometry

Method validation
The calibration curve was drawn with six standard solutions at the concentration ranged from 1.16 to 11.57 μg/mL. The
calibration curve showed a good linearity with the correlation coefficient \(r = 0.9996\). The regression was calculated using the formula \(Y = aX + b\), where \(Y\) and \(X\) correspond to the absorbance and concentration, respectively. The regression equation was \(Y = 0.0743X - 0.0076\).

The sample of BG root was used for the validation. The relative standard deviation (RSD) was calculated for each determination and taken as a measure of precision, repeatability, stability and recovery.

For the precision test, a sample solution was used as described above and then determined for six times. In order to evaluate repeatability of the developed assay, six identical sample powders were analyzed according to the above method from preparation to determination. For tests of precision and repeatability, the RSD values of relevant measurement data were 0.21\% (\(n = 6\)) and 2.52\% (\(n = 6\)), respectively, indicating a high degree of precision with the UV–Vis spectrophotometer and the reproducibility of the method.

Stability of the sample solution was tested at room temperature. The solution was analyzed at 0, 2, 4, 6, 8, 10, 12 and 24 h to assess the stability. The RSD of relevant measurement data was 1.22\%, which showed that the sample solution was stable in 24 h.

The recovery test was performed in six replicates. The recoveries ranged from 96.63 to 102.14\%. The mean recovery was 99.33\% and the RSD was 2.23\%, confirming the accuracy of this method (Table I).

**Experiment for determination of berberine by HPLC**

**Method validation**

Standard solutions with different concentrations were prepared by pipetting accurately the stock standard solutions (0.4, 1, 3, 5, 7 and 10 mL) and then diluting to a final volume of 10 mL with methanol, followed by shaking. The standard solutions with different concentrations \(X\) were injected into the HPLC system in duplicate, and the average peak areas \(Y\) were calculated. As a result, the regression equation was \(Y = 75.585X + 0.0185\) \((r = 0.9998)\). Therefore, the standard curve was linear within the range of 0.02–0.5 \(\mu g\).

In HPLC analysis, the stock standard solution and powder of BS root were used for the validation. Precision was evaluated by analyzing six replicated injections of the sample solution, and the RSD of the determination results was 0.24\% (\(n = 6\)). The repeatability was evaluated by operating the sample powder from preparation to determination simultaneously in six replicates, and the RSD value for the results of the content was 0.88\% (\(n = 6\)). These results showed that the precision and repeatability of this method were accepted. In addition, the sample solution was assayed for 24 h, and the results indicated that the analyte was stable during 24 h with an RSD value of 0.30\%. As shown in Table II, the recovery for berberine ranged from 95.81 to 102.60\% and the

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<th>Table I</th>
<th>Recovery of Berberine in the Samples (n = 6)</th>
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<td>Alkaloid in the samples (mg)</td>
<td>Spiked berberine amount (mg)</td>
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<th>Table II</th>
<th>Recovery of Berberine in the Samples by HPLC (n = 6)</th>
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<tr>
<td>Berberine in the samples (mg)</td>
<td>Spiked berberine amount (mg)</td>
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Figure 1. HPLC chromatograms of reference substance and sample. (1) B. soulieana Schneid. root and (2) berberine.
average was 100.38%, while its RSD value was 2.76%, demonstrating that the method possessed a good accuracy.

Sample analysis
Total alkaloids and berberine (Figures 2 and 3) were determined by the above-mentioned methods and are listed in Table III.

Discussion
The extraction process of HPLC in Chinese Pharmacopoeia (2010) has been improved with the concentration of solvent changing from “methanol” to “90% (v/v) methanol” and the way of extraction changing from “ultrasonic for 1 h” to “reflux for 40 min”.

The source of “Sankezhen” is dry roots of several *Berberis* plants and the content of berberine should not be <0.6% determined by HPLC, as per the Chinese Pharmacopoeia (2010) records. The results of the study showed that berberine contents determined in the roots of five *Berberis* plants had matched the provision. Besides, the contents of total alkaloids in most of the roots were obviously higher than those of total alkaloids in stems, indicating that the stipulation about the source of “Sankezhen” in the Pharmacopoeia was reasonable. Furthermore, as berberine, an active ingredient having antibacterial and anti-inflammatory properties, was the main component of total alkaloids in roots and stems, it was significant to stipulate the content

Figure 2. HPLC chromatograms of the root samples. (1) *B. soulieana* Schneid., (2) *B. henryana* Schneid., (3) *B. gagnepainii* Schneid., (4) *B. bergmanniae* Schneid and (5) *B. triacanthophora* Fedde.

Figure 3. HPLC chromatograms of the stem samples. (1) *B. soulieana* Schneid., (2) *B. henryana* Schneid., (3) *B. gagnepainii* Schneid., (4) *B. triacanthophora* Fedde and (5) *B. bergmanniae* Schneid.
determination of berberine in the Pharmacopoeia to control the quality of “Sankezhen”.

There were three to four time differences between the maximum and minimum of content of berberine and total alkaloids in root samples, which indicated that there were significant differences in quality among crude drugs with multiple origins. To ensure the stable quality and the effectiveness for clinical use, it was necessary to specify the source of “Sankezhen”. With higher contents of total alkaloids and berberine, we considered that the roots of BS, BG and BB were good sources of “Sankezhen”.

As the experimental data shown, the total alkaloid content of the BS root was significantly higher than other species, while the content of berberine was much lower than the BG and BB roots. The results indicated that the higher content of total alkaloids did not always mean higher specific alkaloid in one medicinal material with different sources. However, the content of berberine was the only index to evaluate the quality of “Sankezhen” in Chinese Pharmacopoeia. It might be more rational to add the index of total alkaloids for reflecting the complex chemical composition and extensive pharmacological effects and evaluating the quality of the medicinal material with different sources.

It was found from the study that the stems of BS, BG and BB had higher alkaloid contents, accounting for almost two-third of their roots. Besides, >0.6% (as it recorded in the Pharmacopoeia accordingly) of the berberine was present in the stems of BH, BG and BB. Thus, these stems can act as important medicinal resources. It is possible for part of the Berberis plant stems to be alternative resources on the basis of further pharmacological investigation, and as a result, it is of great significance for protecting the perennial woody plants of Berberis, the resources of Sankezhen.

### Acknowledgments

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