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Short communication

# RP-HPLC determination of puerarin in Chinese traditional medicinal preparations containing pueraria

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**Abstract**

Puerarin in a Gegen(Pueraria)-based Chinese traditional medicinal preparations, Ganmao Qingre Granules, from four different pharmaceutical manufacturers and in two different dosage forms, were determined using RP-HPLC with methanol-5 mmol $l^{-1}$ $KH_2PO_4$ (pH = 4.0) (V:V = 27:73) as mobile phase and UV detection at 248 nm. Ultrasonication and reflux were compared as pretreatment procedures for the sample. Linear range over 0.2–200 μg $ml^{-1}$ of puerarin was obtained, and the limit of detection was 0.1 μg $ml^{-1}$. Recovery was within 99.7–103%. © 2002 Elsevier Science B.V. All rights reserved.

*Keywords:* Puerarin; Pueraria; RP-HPLC; Ultrasonication; Reflux extraction

## 1. Introduction

Gegen, a commonly used herbal medicine in China, is the fresh or dried roots of plants such as *Pueraria Lobata* (Willd.) Ohwi or *Pueraria thomsonii* Benth. Its active components have been separated and identified as isoflavone compounds, of which puerarin is present in the greatest amount [1,2]. Fig. 1 shows the chemical structure of puerarin. The biomedical effects of puerarin, which have been experimentally or clinically demonstrated [2,3], include improvement of blood circulation, prevention of cardiovascular diseases [1,4–6], control of alcoholism [7–9], treatment for arrhythmia [3], etc.

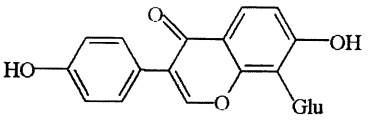


Fig. 1. The chemical structure of puerarin.

Determination of puerarin in Gegen and its medicinal preparations can be accomplished using numerous techniques, such as UV, TLC, and HPLC [10]. Among them, HPLC is the most effective and commonly used one [11–20], and is also the recommended method for quantification of puerarin in Gegen materials and many Chinese

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traditional medicinal preparations (but without Ganmao Qingre Granules) in the Chinese Pharmacopoeia [20].

However, for most Chinese traditional medicinal preparations, extraction of the active components, puerarin in this case, is still a key step for their quantification. Cleaning of the extract prior to chromatography is important for both precise measurement and preservation of chromatographic system. While reflux with methanol or ethanol is a conventional way to extract puerarin from Chinese traditional medicines or herbal medicines [10,11], ultrasonication has recently been used in this purpose [19]. In this paper, an HPLC method was developed for determination of puerarin in a Gegen-based Chinese traditional medicinal preparation, Ganmao Qingre Granules, which is commonly used to treat ailments related to rheum. In the updated Chinese Pharmacopoeia [20] there is no recommended method for quantification of puerarin in this granular dosage. Therefore, different extraction procedures involving ultrasonic and reflux were contrastively investigated in this work. In general, ultrasonication was more effective and simpler than reflux for extraction of puerarin from Gaomao Qingre Granules.

## 2. Experimental

### 2.1. Reagents and materials

Standard puerarin was purchased from the Chinese National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Chromatographic grade methanol was purchased from the Tianjin Shield Company (Tianjin, China). Other reagents were of analytical grade or better and of commercial availability. Double distilled water was used throughout.

A 200 μg $ml^{-1}$ stock solution of puerarin was prepared with 30% ethanol, and diluted with the same solvent when necessary.

### 2.2. .Apparatus

Chromatographic separation was carried out in a Waters 510 HPLC system (MA, USA), consisting of two Waters 510 delivery pumps with a pump control module (PCM), a temperature control module (TCM), a Waters 486 Tunable Absorbance Detector (wavelength range 190–600 nm). A personal computer was used to manipulate the system, and to record and integrate the chromatograms. A Rheodyne 7725i manual injector (CA, USA) was used.

A 150 × 4.6 mm Supelco Discovery RP-Amide C16 column (5 μm particle, PA, USA) was used for separation.

An SB3200 Ultrasonic Generator (50 kHz, 120 W) from the Shanghai Branson Ultrasonics Co. Ltd. (Shanghai, China) was used to extract puerarin from the samples, as well as to degas the mobile phase and sample solutions.

### 2.3. Conditions for chromatographic separation

The mobile phase was methanol-5 mmol $l^{-1}$ $KH_2PO_4$ (pH = 4.0) (V:V = 27:73) at a flowrate of 1.0 mL $min^{-1}$. The column was thermostated at 30 °C. The detection wavelength was set at 248 nm. The injection size was 20 μl.

### 2.4. Experimental procedure

The chromatography system was equilibrated by the mobile phase. When same retention times and peak areas for repetitive injections of standard solution (not less than three times) were observed, separation of sample could then be carried out. If not specified, an average result of triplicate injections for a solution was reported.

### 2.5. Sample Treatment

At first, the pharmaceutical samples were ground in a mortar box to particles, which passed through a 50-mesh screen, and then subject to one of the following procedures.

1) A 3 g ground sample was put into a ground-glass conical beaker containing 25 ml metha-

nol, which was then assembled to a reflux condenser and refluxed on a boiling water-bath. The extract was cooled and filtered with filtering paper, rinsed with 20 ml methanol in three portions. The merged filtrate was evaporated to near dryness on a boiling water-bath. The cooled residue was dissolved and volumed to 100 ml using 30% ethanol, the resulted solution was centrifuged at 4000 rpm for 10 min. The supernatant was at last passed through a 0.45 μm filter membrane and then subject to chromatography analysis.

2) (a) A 3 g ground sample was put into a ground-glass conical beaker containing 25 ml methanol, which was then stoppered and ultrasonicated in a water-bath. The resulted suspension was filtered and rinsed with 30% ethanol, and the merged filtrate was then treated as the steps for the merged filtrate in the procedure (1).
   (b) The steps were same as that in (2a), but 30% ethanol was used instead of methanol.
3) A 3 g ground sample was ultrasonicated as procedure (2a). No significant weight loss was observed of the beaker containing the sample and 25 ml methanol after the ultrasonication. The resulted suspension was first filtered with filtering paper, and the filtrate was then directly subject to centrifugation, membrane-filtration, and at last injected for chromatography analysis as the previous procedures, but without evaporation and re-dissolution.

## 3. Results and discussion

Most of the Chinese traditional medicinal preparations are usually made from dozens of herbal or mineral medicines and involved prolix manufacturing procedures, therefore the complex matrix is often an indispensable problem in analysis of those Chinese traditional medicinal preparations. Taken as an example, the Ganmao Qingre Granules in this work were made from 11 Chinese herbal medicinal materials. Extraction of puerarin from the raw material of Gegen may not be difficult, but problems recur in HPLC analysis of puerarin in most Chinese traditional medicinal preparations. Thus, various procedures of sample extraction and cleaning were compared in this work to seek a practical and simple extraction approach for HPLC measurement of puerarin in the Ganmao Qingre Granules.

Table 1
Comparison between reflux and ultrasonication procedures for puerarin extraction

| Sample No. | Treatment method | Extraction time | Peak area per gram of sample (mean ± SD) ($n = 3$) |
|---|---|---|---|
| R-1 | (1) | Reflux 30min | 744214 ± 742 |
| R-2 | (1) | Reflux 60min | 756665 ± 941 |
| R-3 | (1) | Reflux 90min | 763283 ± 797 |
| R-4 | (1) | Reflux 120min | 771377 ± 902 |
| U-1 | (3) | Ultrasonic 30min | Bad resolution |
| U-2 | (2a) | Ultrasonic 30min | 767180 ± 914 |
| U-3 | (2a) | Ultrasonic 60min | 770245 ± 668 |
| U-4 | (2a) | Ultrasonic 90min | 769735 ± 703 |
| U-5 | (2a) | Ultrasonic 120min | 770399 ± 698 |

Methanol as extractant.

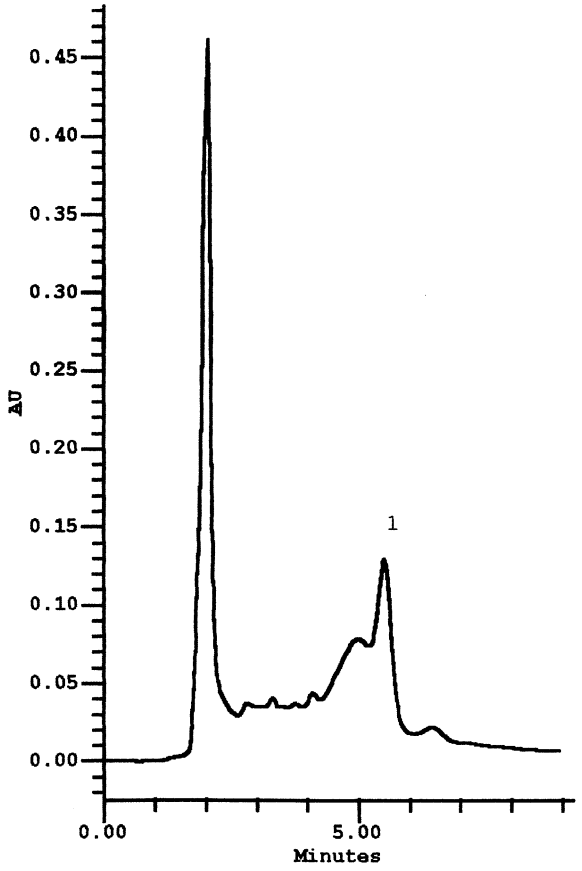


Fig. 2. A typical chromatogram of the sample U-1. 1. Puerarin.

Table 2
Comparison between methanol and ethanol extractant for puerarin extraction

| Sample No. | Extract method | peak area per gram sample | RSD(%) |
|---|---|---|---|
| M-1 | (2a) | 1001309 | 2.9 |
| M-2 | | 998125 | |
| M-3 | | 1050323 | |
| E-1 | (2b) | 870703 | 3.3 |
| E-2 | | 865045 | |
| E-3 | | 821440 | |

Ultrasonication for 60 mins.

### 3.1. Reflux and ultrasonication

Reflux and ultrasonication (both with methanol as extractants) were respectively used to extract puerarin from the Ganmao Qingre Granules. Table 1 shows the results with different extraction times. It could be observed that, the extraction completeness by reflux depended to some extent on the extraction time, whereas that by ultrasonication was almost independent on the extraction time (at least, it was the case when the extraction time was more than 30 min). Moreover, 30-min ultrasonication gave the almost same result as 90-min (if not say 120-min) reflux did. Therefore, in this work, ultrasonication was considered a simpler and more effective method for extraction of puerarin, and consequentially used in the followed tests. Wan and Liu [17] also found that ultrasonication was a more effective method than solvent extract for puerarin.

In Table 1, the sample U-1, which was indicated as bad resolution, was necessary to emphatically discuss as a special case. In fact, the sample U-1 was used to try a simpler procedure, i.e. the method (3) in the Section 2.5, in which the filtrate of the ultrasonicated suspension was directly injected for chromatography analysis. Fig. 2 show a typical chromatogram of the sample U-1. In the chromatogram, an obvious bulge before the puerarin peak was observed and obstructed quantification of puerarin. The bulge couldn't be eliminated by any routine approaches, including gradient elution. In our view, the bulge probably resulted from the effect of the volatile components (such as some compounds of peppermint oil and nepeta oil) in the medicine materials. When the extracts of the samples were heated on a boiling water-bath, those volatile components were evolved and hence did not affect the separation. Thus, in the viewpoints of resolution and column protection, cleaning steps were necessary for the extract prior to chromatography separation.

### 3.2. Methanol and ethanol as extractants

We also compared methanol and ethanol as ultrasonic extractants for puerarin. Table 2 gives the results of 60-min ultrasonication with metha-

Table 3
The reproducibility and recovery[a]

| Sample No. | Samples mass (g) | Added puerarin (mg) | Measured puerarin (mg) mean ± SD ($n = 3$) | Measured puerarin content (mg g$^{-1}$) mean ± SD ($n = 3$) | RSD (%) for contents of P-1 to P-4 | Recovery (%) |
|---|---|---|---|---|---|---|
| P-1 | 3.1842 | 0 | 1.1054 ± 0.005 | 0.3470 ± 0.0016 | 2.8 | – |
| P-2 | 3.0235 | 0 | 1.0421 ± 0.005 | 0.3447 ± 0.0016 | | – |
| P-3 | 3.0690 | 0 | 1.0232 ± 0.005 | 0.3334 ± 0.0016 | | – |
| P-4 | 3.0314 | 0 | 1.0824 ± 0.005 | 0.3570 ± 0.0016 | | – |
| S-1 | 1.0336 | 1.0560 | 1.4496 ± 0.006 | – | | 103 |
| S-2 | 1.0197 | 1.5840 | 1.9320 ± 0.008 | – | | 99.7 |

[a] The samples S-1 and S-2 were treated and volumed to 25 ml.

nol and ethanol as extractants, respectively. A significant discrimination could be found visually or via a $t$-test for those results. Methanol could extract more puerarin from the samples than ethanol. Zhang and coworkers [18] also compared methanol and ethanol as reflux extractants for puerarin in a Chinese traditional medicinal capsule, which was used to treat rhinitis. A polyamide powder cartridge was used to clean the extracts with 10% ethanol as eluent in their work. Finally they chose ethanol as the reflux extractant, because of the two drawbacks of methanol extract: (i) the methanol extract was deeper in color than the ethanol extract, and (ii) the solution resulted from the polyamide cartridge cleaning was not clear enough for be suitability of chromatographic analysis. In this work, the deep color of the methanol extract was also observed, but in our consideration, the color difference should be related to the polarity difference between methanol and ethanol. Therefore, methanol was chosen as the ultrasonication extractant for puerarin in the followed work.

### 3.3. Choice of separation conditions

Various detection wavelengths for puerarin in HPLC have been reported from 248 to 305 nm, among which 250 nm was the most frequently used [10–19]. In this work, 248 nm experimentally gave the highest peak and hence was used for puerarin detection.

When the separation was carried out with mobile phase flowrate at 1.0 ml min$^{-1}$ and 30 °C column temperature, methanol-5 mmol l$^{-1}$ $KH_2PO_4$ (pH = 4.0) (V:V = 27:73) gave satisfactory resolution ($R > 1.5$) between puerarin and its adjacent peaks. For the sample U-1, other solvents and gradient model were also tried, but failure was the final result as above described.

### 3.4. Linearity and sensitivity

Good linearity was obtained in the tested puerarin concentration range of 0.2–200 μg ml$^{-1}$ with regression equation as follows

$$A = 96594C + 23646$$

($r = 0.9999; n = 7$, RSD for the slope = 0.3%; RSD for the intercept = 15%)

where $A$ is the peak area ($\mu V \cdot s^{-1}$), and $C$ the concentration of puerarin solution ($\mu g\ ml^{-1}$).

The limit of detection, defined as signal-to-noise ratio of 3, was 0.1 $\mu g\ ml^{-1}$.

### 3.5. Robustness and recovery of the method

Four samples from a same bag of Ganmao Qingre Granules were treated in parallel as the sample treating method (2a) and separated under the above-determined conditions. The results were listed in Table 3. The RSD for the four samples was 2.8%. Meanwhile, another three weighed samples, in which different amounts of standard puerarin were spiked respectively, were also treated and separated as the same methods that for the above four samples. The average content of puerarin in the first four samples was used as the 'real value' for calculation of the spike recovery, which was within 99.7–103% (also showed in Table 3).

The puerarin solution and sample solution showed great stability under 4 °C storage. The RSDs of the peak areas were found less than 0.2 and 0.5% for the 80 $\mu g\ ml^{-1}$ standard puerarin solution and the sample solutions, respectively, during a tested 48 h, where the solution was repeatedly injected at an interval of 4-h.

### 3.6. Applications of the Method

The ultrasonication-methanol extraction-HPLC separation method was used to determine puerarin in Ganmao Qingre Granular samples from four different manufacturers and several different lots or in different dosage forms. Fig. 3 shows a typical chromatogram of those samples. Table 4 gives the measurement results. The significant differences of puerarin content among the different manufacturers' samples or different lots from same manufacturer were probably caused by the different raw material sources and the fluctuation of production procedure.

In the Chinese Pharmacopoeia [20], an HPLC method with reflux as sample treatment was applied to determine puerarin in herbal materials, such as *P. Lobata* (Willd.) Ohwi or *P. thomsonii* Benth. In the present work, the Pharmacopoeia method was used to determine puerarin in some of the Ganmao Qingre Granular samples. The results (Table 4) showed good agreement between the presented ultrasonication-HPLC and the Pharmacopoeia methods.

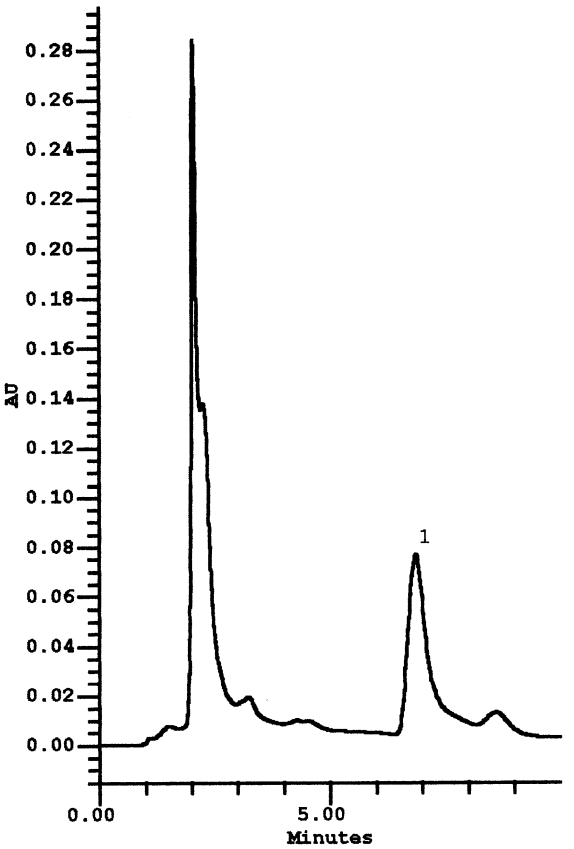


Fig. 3. A chromatogram of Ganmao Qingre Granules sample. 1. Puerarin.

## 4. Conclusion

The developed method with ultrasonication-methanol extraction and HPLC separation was

Table 4
The measurement results of puerarin in the Ganmao Qingre Granules samples

| Sample No. | Manufacturer[a] | Lot No. | Puerarin Content (mg $g^{-1}$) (Mean ± SD) | |
|---|---|---|---|---|
| | | | Present method ($n = 5$) | Pharmacopoeia method [20] ($n = 3$) |
| 1-1 | I | 991201 | 0.5141 ± 0.028 | 0.5012 ± 0.027 |
| 1-2 | I | 990520 | 0.7902 ± 0.031 | |
| 1-3 | I | 981015 | 0.3370 ± 0.020 | |
| 2-1 | II | 980603 (no sugar addition) | 0.9778 ± 0.038 | 0.9671 ± 0.052 |
| 2-2 | II | 980915 | 1.696 ± 0.072 | |
| 3-1 | III | 980415 | 0.6864 ± 0.033 | 0.6747 ± 0.039 |
| 4-1 | IV | 981105 | 0.1740 ± 0.010 | |

[a] I: Guangdong Yihe Pharmaceutical Co., Ltd., Guangzhou, China; II: Beijing Tongrentang Pharmaceutical Manufacturer, Beijing, China; III: Tianjin Aikang Pharmaceutical Co., Tianjin, China; IV: Zhanjiang Manufacturer of Traditional Chinese Medicine, Zhanjiang, China.

successfully used to the determination of puerarin in the Chinese traditional medicinal preparation Ganmao Qingre Granules. The method was sensitive, robust, precise, and rather agreed with the Chinese Pharmacopoeia method. Ultrasonication extraction was more effective, speedy, and simpler than reflux as a sample treatment procedure. However, in the viewpoint of the authors, cleaning steps were strongly recommended for the extract prior to the chromatography analysis.

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