Available online at www.sciencedirect.com
ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 43 (2007) 204–212
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

# Combinative method using HPLC quantitative and qualitative analyses for quality consistency assessment of a herbal medicinal preparation

Ying Xie [a], Zhi-Hong Jiang [a], Hua Zhou [a], Xiong Cai [a], Yuen-Fan Wong [a], Zhong-Qiu Liu [a], Zhao-Xiang Bian [a], Hong-Xi Xu [b], Liang Liu [a,*]

[a] *School of Chinese Medicine, Hong Kong Baptist University, Kowloon Tong, Hong Kong, China*
[b] *Hong Kong Jockey Club Institute of Chinese Medicine Limited, Hong Kong, China*

Received 12 May 2006; received in revised form 28 June 2006; accepted 1 July 2006
Available online 21 August 2006

**Abstract**

A selective and efficient quality consistency assessment system was developed for monitoring the manufacturing processes of a Chinese herbal preparation, Qingfu Guanjieshu (QFGJS) capsule, and for assessing its stability over time. This system is based on quantitative determination of four marker compounds, i.e., sinomenine, paeoniflorin, paeonol, and curcumin, and on qualitative fingerprinting analysis of QFGJS using high-performance liquid chromatography–photodiode array detection (HPLC–DAD) method. The separation was performed on a Phenomenex ODS column by gradient elution with acetonitrile and aqueous phase (containing 0.1% phosphoric acid, adjusted with triethylamine to pH $3.5 \pm 0.2$) at a flow-rate of 1.0 ml/min. In fingerprinting analysis, the chemical characteristics of four herbs present in QFGJS (excluding Radix Aconiti Lateralis Preparata) were present in the HPLC chromatographic file. In addition, quantitative determination of hypaconitine was carried out with our published HPLC method as a supplement for quality control of the Radix Aconiti Lateralis Preparata in QFGJS. The results showed that the contents of these five marker compounds and HPLC fingerprint profiles of three batches of QFGJS products collected at 3 months after production in the stability testing were relatively consistent. This well-developed method could be used for quality assessment of the complex preparations of herbal medicine.


*Keywords:* Quality control; Herbal preparation; Quantitative determination; Fingerprinting analysis; HPLC–DAD

## 1. Introduction

The last few decades have seen rapid worldwide growth in the demand for herbal medicines and their proprietary products in the pharmaceutical industry and medicinal markets, especially in China, Japan, and countries in Europe and North America. As demand grows so does the demand for mass production and quality assurance that each batch of medicine meets certain standards both at the time of production and over its shelf life. Quality control for herbal preparations or proprietary products, however, is much more difficult than for synthetic drugs because of the chemical complexity of the ingredients. As herbal preparations comprise hundreds of mostly unique, or species-specific, compounds, it is difficult to completely characterize all of these compounds. It is also equally difficult to know precisely which one is responsible for the herbs, or herbal preparation's therapeutic action because these compounds often work synergistically in delivering therapeutic effects. Thus, maintaining consistent quality in herbal preparations, both from batch to batch and over time, is as problematical as it is necessary and has drawn serious attention recently as a challenging analytical task.

In recent years, significant efforts have been made to devise methods for the quality control of herbal materials as well as herbal preparations by utilizing quantitative methods and/or qualitative fingerprinting technologies [1,2]. Both methods have advantages and disadvantages. Quantitative analysis aims to separate and identify the marker compounds from herbs or herbal preparations and then use them as indicators or standards to assess quality. Determining the effective or principal chemical constituents and the toxic compounds is crucial to the quality control of herbs and herbal preparations [3]. Much attention has been drawn to the development of chromatographic meth-

* Corresponding author. Tel.: +852 3411 2457; fax: +852 3411 2461.
*E-mail address:* liuliang@hkbu.edu.hk (L. Liu).

0731-7085/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2006.07.008

ods in this field, and a great number of such papers have been published [4,5]. But for quality control of complex systems, the determination of only a few compounds cannot, realistically speaking, give a comprehensive and accurate assessment of all active constituents in herbal medicinal products; such an approach is inherently inadequate for quality control and stability evaluation of herbal medicinal products.

Qualitative analysis is typically used to demonstrate the general characteristics of herbal materials or their herbal preparations with regard to quality consistency and stability. Fingerprinting analysis, that is, using chromatographic profiles such as HPLC-UV, HPLC-MS, gas chromatography (GC), and thin-layer chromatography (TLC), is a rapidly developing technique that offers great potential for monitoring the quality of herbal materials, particularly for identifying a particular herb and distinguishing it from closely related species [6,7]. By fingerprinting the entire pattern of components present in one herb or its preparation, the complete information could be acquired. Both the U.S. Food and Drug Administration (FDA) [8] and the European Medicines Agency (EMEA) [9] clearly state that the appropriate fingerprint chromatograms should be used to assess the consistency of botanical products. In 2004, the State Food and Drug Administration (SFDA) of China officially required all injections made from herbal medicine to be standardized by chromatographic fingerprints [10]. Fingerprinting has also been introduced and accepted by the World Health Organization (WHO) as a strategy for identification and quality evaluation of herbal medicinal products [11]. Recently, quite a few reports can be found in which fingerprint chromatograms are used to assess the consistency of herbal preparations in stability testing. Goppel and Franz used HPLC fingerprinting for stability control of senna leaves and senna extracts [12]; Koll et al. reported a validated high performance thin-layer chromatography (HPTLC) fingerprinting method for the study of quality control and stability testing of herbs [13].

Qingfu Guanjieshu (QFGJS) capsule is a pharmaceutical preparation used for the treatment of rheumatoid arthritis. The formula, on which the preparation is based, is composed of five anti-inflammatory and anti-arthritic Chinese medicinal herbs that are all included in the Chinese Pharmacopoeia (edition 2005, volume 1) and have been commonly used for treating rheumatic and arthritic diseases for a long time by Chinese and Japanese doctors. Our recent studies showed that QFGJS has significant suppressive effects on arthritic [14] and acute inflammatory animal models [15]. Five representative bioactive compounds, sinomenine, paeoniflorin, paeonol, cucurmin, and hypaconitine (Fig. 1) derived from five herbs (Caulis Sinomenii, Radix Paeoniae Alba, Cortex Moutan, Rhizoma Curcumae Longae, and Radix Aconiti Lateralis Preparata, respectively) in the original clinical prescription, were selected as chemical markers. Related studies found in early literatures showed that these five compounds are the major effective constituents of the corresponding individual herbs, and all of them possess significant pharmacological actions both *in vivo* and *in vitro*, especially in anti-inflammation, analgesia, anti-arthritis, and immunosuppression [16–20]. Thus, variations of these five compounds can be regarded as important indicators of quality variation and inconsistency in the pharmaceutical processes or of deterioration over time. In our present study, we developed a high-performance liquid chromatography–photodiode array detection (HPLC–DAD) method that could be used both for quantitative determination of four bioactive compounds and for fingerprinting analysis to evaluate the quality consistency of QFGJS during its stability testing. Another HPLC method published in our previous paper was used for determination of hypaconitine in trace content in QFGJS as a supplement of this HPLC–DAD method [21]. In this way, a convenient and efficient quality specification and quality assurance system for evaluating the stability of QFGJS has been established based on HPLC qualitative and quantitative analyses.

## 2. Experimental

### 2.1. Chemicals and reagents

Acetonitrile was of HPLC grade (International Laboratory, USA). 95% ethanol (UNI-CHEM, Hong Kong), triethylamine, 85% phosphoric acid (International Laboratory, USA) and hydrochloric acid (MERCK, Germany) were of GR grade. Deionized water was prepared using a Millipore water purification system (Billerica, MA, USA).

Reference chemical standards of sinomenine, paeoniflorin, paeonol, curcumin, and hypaconitine were purchased from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. Bisdemethoxycurcumin and demethoxycurcumin were kindly provided by Prof. Zi-Ren Su of the Guangzhou University of Traditional Chinese Medicine (GUTCM), China. Turmerone (86–87%) was purchased from the International Laboratory, USA. The identity and purity of these chemicals was further validated by LC–MS in our laboratory.

### 2.2. Raw materials and manufacturing procedures of QFGJS pilot products

All five herbs were purchased from the medicinal markets of China and authenticated by Prof. Xiao-Ping Lai of GUTCM, China. The authenticated voucher specimens were kept in the School of Chinese Medicine, Hong Kong Baptist University.

The pilot products of QFGJS (Batch No. 20040612, 20040821, 20040825, and 20040901) were prepared from five herbs in the following amounts: Caulis Sinomenii 12 g, Radix Aconiti Lateralis Preparata 9 g, Rhizoma Curcumae Longae 6 g, Radix Paeoniae Alba 15 g, and Cortex Moutan 9 g. The five herbs were firstly pulverized. Caulis Sinomenii was extracted with water by reflux, and the water extract was spray-dried to obtain extract 1. Radix Aconiti Lateralis Preparata and Radix Paeoniae Alba were refluxed together with 80% ethanol and then the ethanol extract was spray-dried to obtain extract 2. Cortex Moutan was extracted by supercritical $CO_2$ extraction (SFE-$CO_2$) to produce extract 3 and the residue after SFE-$CO_2$ extraction was then refluxed with 80% ethanol and the ethanol extract was spray-dried to obtain extract 4. Similarly, Rhizoma Curcumae Longae was extracted by SFE-$CO_2$ to produce extract

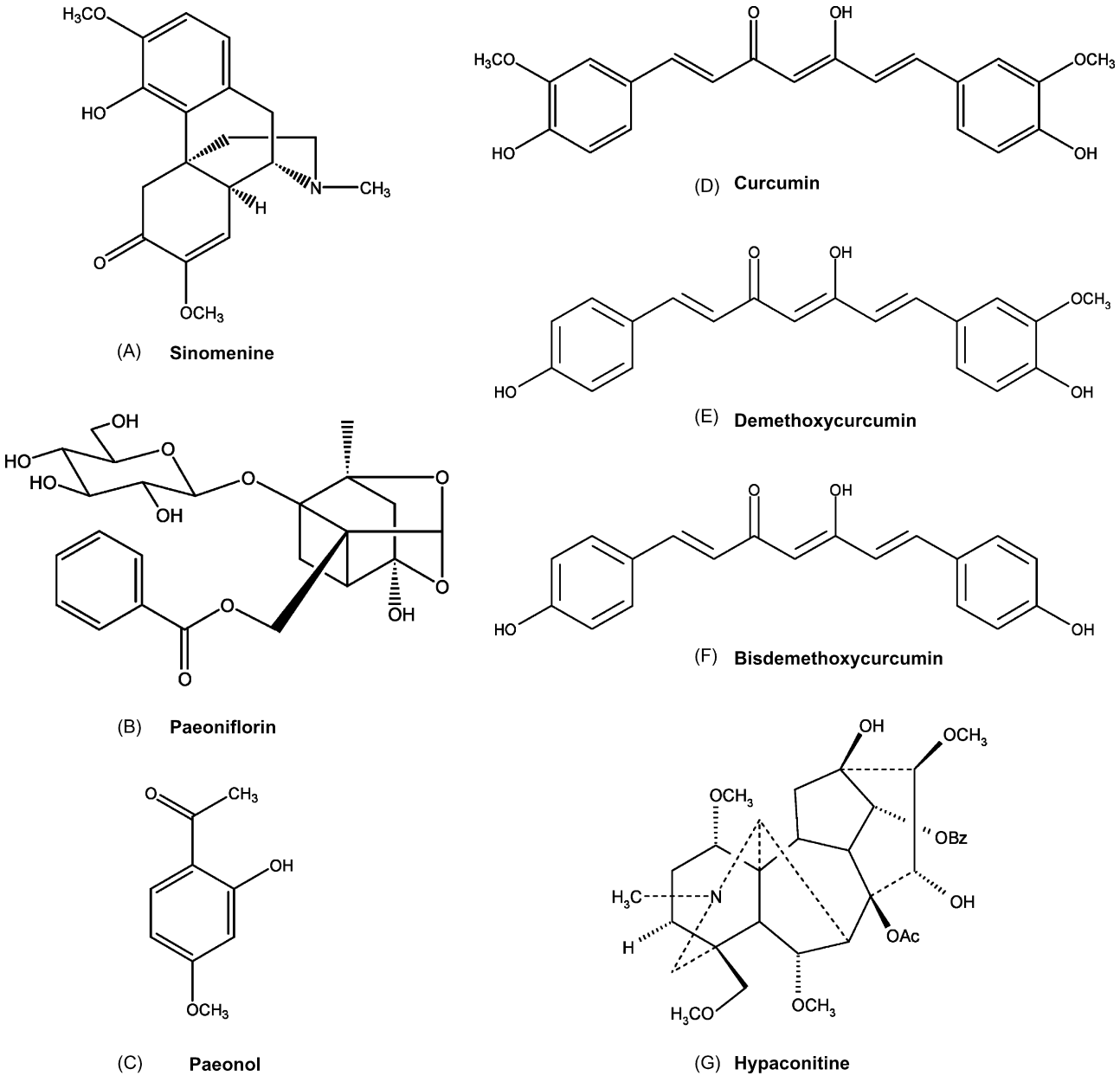


Fig. 1. Chemical structures of sinomenine, paeoniflorin, paeonol, curcumin, bisdemethoxycurcumin, demethoxycurcumin and hypaconitine.

5 and the residue after SFE-$CO_2$ extraction was then refluxed with 80% ethanol and the ethanol extract was dried with the vacuum drying technique to obtain extract 6. Finally, the extracts 1, 2, 3, 4, 5, and 6 were mixed thoroughly and encapsulated to produce QFGJS capsules. The yield rate of the final product was 11.4% based on all five herbs.

### *2.3. Samples solutions and negative control (NC) samples preparation*

The contents of the hard gelatin capsules of QFGJS were accurately weighed (0.15 g) and then transferred into 25 ml volumetric flasks. Each weighted sample was dissolved with 20 ml of 50% ethanol by sonication for 60 min at room temperature, and added 50% ethanol to 25 ml accurately. Then, 5 ml of this sample solution was transferred into 15 ml centrifuge tube and centrifuged at 3000 rpm for 5 min. The supernatant was further filtered through a 0.45 μm membrane filter before being injected into the HPLC system for analysis.

The negative control (NC) samples for each herb were prepared by evenly mixing the extracts derived from the other four herbs. For example, the NC samples for Caulis Sinomenii were prepared by mixing together extracts 2–6 in proportion as the final products. All NC samples prepared according to the above protocols for HPLC analysis.

### *2.4. Standard solutions preparation*

The standards of sinomenine, paeoniflorin, paeonol, and curcumin were accurately weighed and then dissolved with 50%

ethanol to produce stock standard solutions. The calibration curves were established based on seven concentrations of each standard chemical with a range of 1–400 μg/ml by diluting the stock solutions with 50% ethanol in appropriate quantities. The limits of detection for the marker compounds were determined with a series of standard solutions which were prepared by further diluting the standard solution with lowest concentration in the calibration curves.

### 2.5. Apparatus and chromatographic conditions

An Agilent/HP 1100 series LC system (Hewlett Packard, CA, USA) consisting of a G1311A Quaternary Pumps, a G1322A degasser, a G1315A diode-array detector and a G1313A Autosampler were employed. The chromatographic analysis for determination of sinomenine, paeoniflorin, paeonol, and curcumin was carried out on a Phenomenex ODS (250 mm × 4.6 mm I.D.; particle size 5 μm; Phenomenex, Inc., USA) protected by a Security Guard Cartridge (C18, 4 mm × 3.0 mm I.D.; Phenomenex, Inc., USA). The mobile phase was acetonitrile (A) and solvent (B) (containing 0.1% phosphoric acid, adjusted with triethylamine to pH 3.5 ± 0.2). The conditions of the solvent gradient elution were 8–20% (A) in 0–25 min, 20–40% (A) in 25–30 min, 40–70% (A) in 30–55 min, 70–90% (A) in 55–60 min at a flow-rate of 1.0 ml/min. Detection was conducted with different wavelengths of 230, 240, 270, 262, and 420 nm with the reference wavelength of 550 nm at room temperature. All injection volumes of sample solutions were 5 μl. Because the content of hypaconitine is very low in QFGJS and cannot be detected in current HPLC–DAD method, another HPLC method described in our previous paper [21] was used to determine the content of hypaconitine.

A stability chamber (Binder APT. Line KBF240, Binder Gmbh, Germany) was used in the stability testing. An ultrasonicator (Branson 5210 ultrasonicator) was used for extraction.

### 2.6. Validation of HPLC method

#### 2.6.1. Precision

The intra-daily precision of the HPLC–DAD method was validated with a standard mixture solution of sinomenine, paeoniflorin, paeonol, and curcumin under the selected optimal conditions five times a day. For inter-daily precision, measurements two times a day on three consecutive days were conducted. All of the measurements precision were expressed as relative standard deviations (R.S.D.s).

#### 2.6.2. Repeatability

Six independently prepared sample solutions of QFGJS with the same amount were analyzed and the variations within six measurements were calculated for evaluation of repeatability. The processes of the measurements were in accordance with the "Preparation of sample solutions for HPLC analysis" in parallel.

#### 2.6.3. Recovery

Standards of sinomenine, paeoniflorin, paeonol, and curcumin with the known amounts in solutions were spiked to the QFGJS sample solutions of which the contents of sinomenine, paeoniflorin, paeonol, and curcumin had been determined before the addition of the four chemical standards. Then, four marker compounds in QFGJS sample solutions were extracted, processed and quantified in accordance with the established procedures, and finally the recovery rates were calculated.

### 2.7. Similarity calculation of the HPLC fingerprinting chromatograms

The correlation coefficient was calculated based on the information obtained from entire chromatographic profiles. The software Computer Aided Similarity Evaluation System (CASES), a recently developed computer software based on chemometrics which was used in similarity analysis of chromatographic and spectral patterns, was employed to calculate the correlation coefficients of fingerprint profiles according to the methods described by Liang and co-workers [22–24].

## 3. Results and discussion

### 3.1. Optimization of extraction conditions

Sample pretreatment is one of the most important procedures for HPLC fingerprinting analysis as well as for determination of the marker compounds in QFGJS because these compounds are chemically distinct, i.e., sinomenine is an alkaloid; paeoniflorin is a monoterpene glycoside; paeonol is an acetophenone; curcumin is a pigment (Fig. 1). While these chemical markers are different kinds of compounds derived from different herbs, they can be used as indicators for the studies of optimization of extraction solvents and extraction time in both quantitative and fingerprinting analyses. In the preliminary studies for the selection of extraction solvents, ethanol was the preferred choice because it can effectively extract a variety of compounds with different polarities. Extraction efficiencies of ethanol–water at different ratios and of the solution of 0.01 M HCl were examined with ultrasonic extraction. The results shown in Table 1 demonstrated that different ethanol–water ratios significantly affected the extraction efficiencies of sinomenine and cur-

Table 1
Comparison of the extraction solvents for extraction efficiencies of four marker compounds in QFGJS[a]

| Extraction solvents | Contents (mg/g, $\bar{X}$ ± S.D.) | | | |
|---|---|---|---|---|
| | Sinomenine | Paeoniflorin | Paeonol | Curcumin |
| 95% ethanol | 4.65 ± 0.24 | 38.6 ± 1.7 | 22.6 ± 0.4 | 2.99 ± 0.21 |
| 75% ethanol | 5.37 ± 0.02 | 37.1 ± 0.4 | 21.5 ± 0.1 | 2.77 ± 0.06 |
| 60% ethanol | 7.80 ± 0.10 | 37.7 ± 0.3 | 22.2 ± 0.0 | 2.84 ± 0.03 |
| 50% ethanol | 8.28 ± 0.06 | 37.7 ± 0.1 | 22.7 ± 0.3 | 2.81 ± 0.06 |
| 40% ethanol | 8.17 ± 0.06 | 41.2 ± 0.6 | 22.3 ± 0.1 | 2.68 ± 0.04 |
| 20% ethanol | 8.24 ± 0.02 | 39.4 ± 0.3 | 20.0 ± 0.1 | – |
| 0.01M HCl | 8.31 ± 0.24 | 39.1 ± 0.8 | 17.9 ± 0.0 | – |

(–) Under detection limit. Data are expressed as mean ± S.D. of duplicate experiments.

[a] Sample 20040612 was used in this experiment.

cumin. The content of sinomenine was gradually decreased along with increase of ethanol–water ratios, especially when the ethanol–water ratios were above 50%, while the content of curcumin increased at the same time. Considering the relatively satisfactory extraction efficiencies of the four compounds, 50% ethanol was selected as the extraction solvent for the study of QFGJS.

We also compared the influence of the extraction periods, i.e., 30, 60, and 120 min, on the extraction efficiencies of the four compounds in the sample solutions of QFGJS. The results showed that 60 min extraction was optimal.

### 3.2. Optimization of HPLC chromatographic conditions

The HPLC chromatographic conditions for both quantitative determination and qualitative fingerprinting analysis should meet the requirements of providing adequate chemical information, good separation, and repeatability of the assay. The wavelengths of 262, 240, 270, and 420 nm were used for detection of different chemical markers because these wavelengths showed maximum absorption for determination of sinomenine, paeoniflorin, paeonol, and curcumin. However, in fingerprint analysis, most of the compounds in QFGJS showed strong UV absorbance at 230 nm; hence this wavelength was selected for obtaining chromatographic fingerprint profiles, except for 42–48 min in which 420 nm was used for detection of curcumin, demethoxycurcumin, and bisdemethoxycurcumin (Fig. 1).

A reverse-phase $C_{18}$ column was used, and several different elution systems were tracked. It was observed that the resolution of peaks such as curcumin, with neighboring peaks of demethoxycurcumin and bisdemethoxycurcumin, and symmetry factors of peaks such as sinomenine were unsatisfactory when using the acetonitrile–water or methanol–water system. Thus, several aqueous solutions, i.e., the phosphoric buffer solution, acetic acid, formic acid as well as phosphoric acid and phosphoric acid solutions adjusted with triethylamine, were employed for the study of condition optimization with acetonitrile as the organic phase. The results suggest that the elution system composed of 0.1% phosphoric acid adjusted to a pH of $3.5 \pm 0.2$ with triethylamine and acetonitrile in a gradient elution program is an ideal system for separation of various peaks in the chromatograms. The typical HPLC chromatogram for determination of the four marker compounds in QFGJS at four wavelengths was shown in Fig. 2.

Although it is quite difficult to baseline separate all compounds in one HPLC run, the gradient time, gradient shape, and initial composition of the mobile phase were carefully considered in the assessment of gradient optimization. The optimized initial composition of the mobile phase was 8% acetonitrile, and the final concentration of acetonitrile was set at 70% to ensure that most compounds in QFGJS could be eluted in one HPLC run. With the optimized gradient program, the major peaks showed satisfactory resolution from neighbor peaks for chromatographic fingerprint analysis. For example, the resolution factors of curcumin with demethoxycurcumin and bisdemethoxycurcumin are above 2.0.

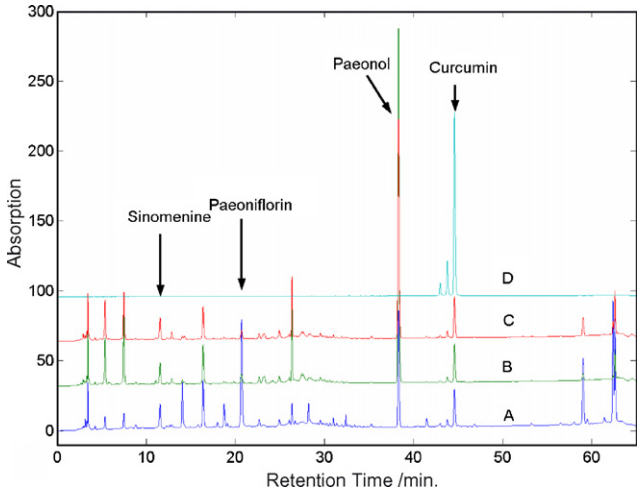


Fig. 2. HPLC chromatograms of the four marker compounds in QFGJS. (A) 240 nm for paeoniflorin detection; (B) 270 nm for paeonol; (C) 262 nm for sinomenine; (D) 420 nm for curcumin. The HPLC chromatographic conditions are described in Section 2.

### 3.3. Method validation for quantitative determination of the four marker compounds

By comparing both the retention times and the UV spectra of the reference standards, four marker compounds (sinomenine, paeoniflorin, paeonol, and curcumin) in QFGJS were well identified. The peak purity was confirmed by studying the DAD data with all peaks of interests in which no indication of impurities of peaks was found. Furthermore, the HPLC chromatograms of different negative control samples, i.e., in the absence of an individual herb in the samples of QFGJS, illustrated that there were no interferences for determination of sinomenine, paeonol, and curcumin at 11.3, 38.4, and 44.7 min, respectively. As paeoniflorin exists in both Cortex Moutan and Radix Paeoniae Alba, a small peak of paeoniflorin in the negative control chromatogram of Radix Paeoniae Alba was observed, which might originate from the extract of Cortex Moutan (Figure not shown).

The linearity of the plot concentrations ($X$, μg/ml) versus peak areas ($Y$) was investigated in the range of 1–400 μg/ml for these four marker compounds. The results expressed as the values of the correlation coefficient ($R^2$) were shown in following: $Y = 4.362X + 0.279$ ($R^2 = 0.9999$, for sinomenine); $Y = 4.994X + 3.616$ ($R^2 = 0.9996$, for paeoniflorin); $Y = 19.357X + 10.939$ ($R^2 = 1.000$, for paeonol); $Y = 38.272X$-19.234 ($R^2 = 0.9998$, for curcumin).

The limits of the detection, calculated as the amount of the compounds needed to produce a signal that is at least three times stronger than the noise signal, were determined to be 0.51, 1.01, 0.13, and 0.08 μg/ml for sinomenine, paeoniflorin, paeonol, and curcumin, respectively.

For validation of the assay, the results of precision, repeatability and recovery rates are shown in Tables 2 and 3. Analysis of the precision of the intra-daily (five times a day) and inter-daily (two times a day for three consecutive days) determinations was indicated by R.S.D.s that were less than 1.08% ($n = 6$) for all determination of the four compounds (Table 2). This result also

Table 2
Precision of the intra-daily and inter-daily HPLC measurements for four marker compounds in QFGJS

| Compounds | Intra-daily[a] | | Inter-daily[b] | |
|---|---|---|---|---|
| | Contents (mg/g, $\bar{X} \pm$ S.D.) | R.S.D. (%) | Contents (mg/g, $\bar{X} \pm$ S.D.) | R.S.D. (%) |
| Sinomenine | 8.08 ± 0.04 | 0.47 | 8.05 ± 0.07 | 0.83 |
| Paeoniflorin | 39.1 ± 0.1 | 0.33 | 39.5 ± 0.4 | 1.08 |
| Paeonol | 22.3 ± 0.1 | 0.18 | 22.3 ± 0.1 | 0.28 |
| Curcumin | 2.71 ± 0.01 | 0.31 | 2.72 ± 0.01 | 0.37 |

[a] Samples (Batch No. 20040621) were analyzed five times a day.
[b] Samples (Batch No. 20040621) were analyzed twice a day for over three consecutive days.

Table 3
Repeatability and recovery tests for four marker compounds in QFGJS[a]

| Compounds | Contents (mg/g) | R.S.D.s (%) | Added amount (mg) | Recorded amount (mg) | Recovery rates (%) | R.S.D.s (%) |
|---|---|---|---|---|---|---|
| Sinomenine | 8.02 ± 0.05 | 0.61 | 0.256 | 0.263 ± 0.003 | 102.72 ± 1.07 | 1.05 |
| Paeoniflorin | 38.1 ± 0.6 | 1.63 | 0.877 | 0.835 ± 0.020 | 95.21 ± 2.30 | 2.41 |
| Paeonol | 22.2 ± 0.1 | 0.34 | 0.420 | 0.415 ± 0.009 | 98.84 ± 2.04 | 2.06 |
| Curcumin | 2.77 ± 0.05 | 1.76 | 0.069 | 0.066 ± 0.001 | 95.75 ± 1.08 | 1.13 |

Data are expressed as mean ± S.D. of six measurements.
[a] Sample 20040612 was used in this experiment ($n = 6$).

implied that these marker compounds were stable in 50% ethanol solution within three days at least. The R.S.D.s of the repeatability test were less than 1.76% ($n = 6$) for all determination of the four marker compounds (Table 3).

### 3.4. Method validation for HPLC fingerprinting method

The reproducibility and repeatability of the HPLC fingerprinting method in the present study were evaluated by analysis of the chromatograms in the precision and repeatability tests. Fifteen peaks were selected as characteristic peaks in the chromatogram of the QFGJS sample solutions as they have relatively larger areas (Fig. 3). The relative peak areas (RPA) and relative retention times (RRT) of each characteristic peak related to the reference peak were calculated for semi-quantitative expression of the chemical properties in the HPLC fingerprint patterns of QFGJS. The detection wavelength of 230 nm was used for calculating the RPA of peaks 1–9, 14, and15, while 420 nm was used for peaks 11–13. The peak 7 (paeoniflorin) was used as the reference peak as it has a high and stable content. Precisions of the RRT and RPA of all characteristic peaks were in the ranges of 0.03–0.98% and 0.38–2.53% of R.S.D.s ($n = 6$), respectively. The R.S.D.s of RRT and RPA in the repeatability tests were found to lie in the range of 0.04–0.65% and 0.52–3.33% ($n = 6$), respectively. These results indicate that the conditions used in the qualitative chromatographic fingerprint analysis are satisfactory.

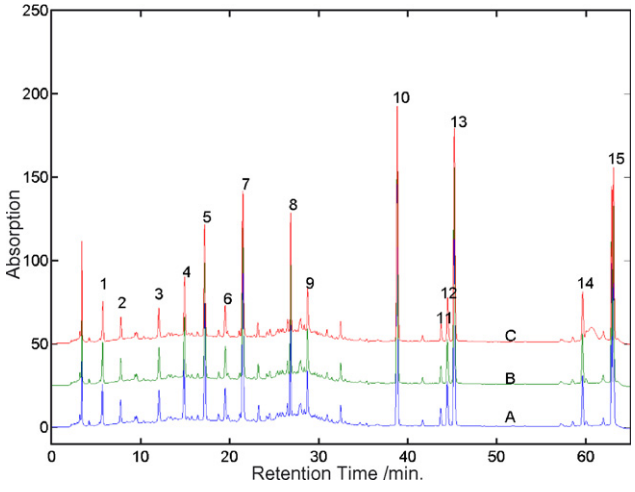


Fig. 3. HPLC chromatographic fingerprint profile of QFGJS with (A) Batch No. 20040821; (B) Batch No. 20040825; (C) Batch No. 20040901. The analytical conditions refer to Section 2.

### 3.5. Quantitative determination of the marker compounds in the samples of QFGJS

For evaluating quality consistency in the manufacturing processes of QFGJS, the contents of sinomenine, paeoniflorin, paeonol, and curcumin in three batches (Batch No. 20040821, 20040825, and 20040901) of the pilot products were determined. No remarkable variations in the contents of the four marker compounds were found in different batches of the QFGJS product assessed immediately after production (0 month in Table 4). This indicates that the pharmaceutical engineering technologies, conditions and processes employed in manufacturing QFGJS capsules effectively maintain quality consistency of the production. A second important requirement for an herbal medicinal preparation is stability over time under the influences of varying environment factors, such as temperature and humidity. To evaluate product quality as it changes over time and to establish shelf life with recommended storage condi-

Table 4
The contents of four marker compounds in three batches of the QFGJS product and their stability testing samples

| Months | Batch No. | Contents (mg/g, $\bar{X} \pm$ S.D.)[c] | | | |
|---|---|---|---|---|---|
| | | Sinomenine | Paeoniflorin | Paeonol | Curcumin |
| 0Mon | 20040821 | 5.89 ± 0.05 | 25.3 ± 0.1 | 19.1 ± 0.0 | 6.17 ± 0.09 |
| | 20040825 | 5.90 ± 0.06 | 25.3 ± 0.1 | 19.1 ± 0.2 | 6.19 ± 0.10 |
| | 20040901 | 5.86 ± 0.05 | 25.4 ± 0.0 | 18.9 ± 0.1 | 6.18 ± 0.01 |
| 1Mon[a] | 20040821 | 5.87 ± 0.08 | 24.6 ± 0.1 | 18.5 ± 0.1 | 5.89 ± 0.06 |
| | 20040825 | 6.10 ± 0.06 | 24.7 ± 0.1 | 19.2 ± 0.0 | 6.07 ± 0.09 |
| | 20040901 | 5.80 ± 0.01 | 24.4 ± 0.0 | 18.6 ± 0.1 | 6.05 ± 0.24 |
| 2Mon[a] | 20040821 | 6.00 ± 0.05 | 24.8 ± 0.4 | 18.2 ± 0.2 | 6.39 ± 0.07 |
| | 20040825 | 6.12 ± 0.05 | 24.9 ± 0.1 | 18.4 ± 0.2 | 6.46 ± 0.08 |
| | 20040901 | 5.95 ± 0.03 | 24.8 ± 0.1 | 18.2 ± 0.1 | 6.40 ± 0.07 |
| 3Mon[a] | 20040821 | 5.92 ± 0.12 | 25.0 ± 0.5 | 18.3 ± 0.1 | 6.23 ± 0.17 |
| | 20040825 | 5.84 ± 0.03 | 24.8 ± 0.12 | 18.2 ± 0.1 | 6.02 ± 0.12 |
| | 20040901 | 5.72 ± 0.02 | 24.7 ± 0.1 | 18.5 ± 0.2 | 5.96 ± 0.05 |
| 3M-L[b] | 20040821 | 5.94 ± 0.03 | 25.3 ± 0.3 | 18.9 ± 0.1 | 6.34 ± 0.07 |
| | 20040825 | 5.95 ± 0.01 | 25.1 ± 0.0 | 18.8 ± 0.1 | 6.31 ± 0.05 |
| | 20040901 | 5.89 ± 0.08 | 25.1 ± 0.1 | 18.6 ± 0.1 | 6.23 ± 0.05 |

[a] The samples were stored in the stability chamber for stability test with accelerated conditions.
[b] The samples were stored under room temperature for stability test with long-term conditions.
[c] Data are expressed as mean ± S.D. of triplicate experiments for 0 month and duplicate experiments for others.

tions, stability testing was performed in accordance with the guidelines of the International Conference on Harmonisation (ICH). An accelerated conditional stability test was carried out with four time points (i.e., 0, 1, 2, and 3 months after production of QFGJS). These tests were conducted in a stability chamber at 40 ± 2 °C with the humidity of 75 ± 5%. The contents of the four marker compounds in the product were determined at these four time points, while chromatographic fingerprints analyses were also simultaneously conducted to characterize the changes in other compounds in the product. The results, showed in Table 4, indicate that the contents of sinomenine, paeoniflorin, and curcumin were quite stable during the periods tested; only paeonol showed a slight decrease which is no more than 5% of the content immediately after production. Another stability test (long-term testing) was also carried out at room temperature under normal storage conditions (25 ± 2 °C with the humidity of 60 ± 5%). The results showed (Table 4) that the contents of four marker compounds in QFGJS at three months after production (marked as 3M-L) were almost the same as the contents immediately after production (marked as 0 month). Further stability testing for quality consistency of QFGJS over longer periods is still going on in our lab.

Radix Aconiti Lateralis Preparata, processed *Aconite* (*Aconitum*) root, is a Chinese herb known to have very strong anti-inflammatory and analgesic actions [25]. Chinese doctors have used this plant to treat joint pain and rheumatic diseases for almost 2000 years, even though the dosage of the herb must be carefully monitored and controlled to ensure its safe use because it contain high toxic *Aconitum* alkaloids such as aconitine, mesaconitine, and hypaconitine [26]. It is, therefore, very important to determine the levels of these alkaloids in QFGJS. However, it is quite difficult to identify the characteristic peaks of Radix Aconiti Lateralis Preparata in the HPLC fingerprint profiles of QFGJS due to trace amounts of *Aconitum* alkaloids in the product. In our previous paper, using another HPLC method, determination of the contents of aconitine, mesaconitine, and hypaconitine (Fig. 1) in the *Aconite* roots as well as in the preparation of QFGJS was conducted. It was observed that the total contents of the *Aconitum* alkaloids in QFGJS were lower than the upper limit for the content of aconitine stipulated in the Chinese Pharmacopoeia (Edition 2005, volume 1) [21]. In the current studies, we found that the content of hypaconitine in QFGJS was consistent in three batches of QFGJS product and that it remained consistent over time, as seen in the stability testing (Table 5).

Table 5
The contents of hypaconitine in three batches of QFGJS product and their stability testing samples

| Batch No. | Hypaconitine contents (μg/g, $\bar{X} \pm$ S.D.)[c] | | | | |
|---|---|---|---|---|---|
| | 0Mon | 1Mon[a] | 2Mon[a] | 3Mon[a] | 3M-L[b] |
| 20040821 | 12.29 ± 0.23 | 12.87 ± 0.31 | 12.22 ± 0.26 | 12.48 ± 0.94 | 12.84 ± 0.76 |
| 20040825 | 11.97 ± 0.10 | 12.00 ± 0.06 | 12.51 ± 0.12 | 12.48 ± 0.27 | 12.61 ± 1.37 |
| 20040901 | 11.94 ± 0.16 | 11.47 ± 0.05 | 11.57 ± 0.19 | 12.36 ± 1.74 | 12.85 ± 0.52 |

[a] The samples were stored in the stability chamber for stability test with accelerated conditions.
[b] The samples were stored under room temperature for stability test with long-term conditions.
[c] Data are expressed as mean ± S.D. of triplicate experiments for 0 month and duplicate experiments for others.

Table 6
The attribution of the characteristic peaks in the HPLC chromatographic fingerprints

| Peak No. | Compound names | Intermediate products | Corresponding herbs |
|---|---|---|---|
| 3 | Sinomenine | Extract 1 | CS |
| 5 | – | Extract 1 | CS |
| 2, 4, 6 | – | Extract 2 | RPA |
| 7 | Paeoniflorin | Extract 2 and 4 | RPA and CM |
| 1, 8, 9 | – | Extract 2 and 4 | RPA and CM |
| 10 | Paeonol | Extract 3 | CM |
| 11 | Bisdemethoxycurcumin | Extract 6 | RCL |
| 12 | Demethoxycurcumin | Extract 6 | RCL |
| 13 | Curcumin | Extract 6 | RCL |
| 14 | Turmerone | Extract 5 | RCL |
| 15 | – | Extract 5 | RCL |

(–) Unknown. CS: Caulis Sinomenii; RPA: Radix Paeoniae Alba; CM: Cortex Moutan; RCL: Rhizoma Curcumae Longae.

### 3.6. Contribution of the characteristic peaks from individual herbs

The fingerprinting chromatograms of three batches of pilot products as well as of samples collected from the stability testing were obtained with the given chromatographic parameters using detection wavelength of 230 nm and on-line UV spectrum from 190 to 600 nm. In addition to the known peaks of the marker compounds of sinomenine, paeoniflorin, paeonol, and curcumin (3, 6, 8, and 13, respectively), peaks 1, 2, 4, 5, 7, and 9–15 were also chosen as characteristic peaks as they showed relatively high contents (Fig. 3).

In order to identify the origins of these characteristic peaks from each herb, a comparative study was carried out with and without certain kind(s) of herb(s) by using various extracts of herbs and negative control samples (Sections 2.2 and 2.3). Accordingly, HPLC fingerprinting analysis of the possible individual contributions from the corresponding herbs to the general chromatographic profile were carried out. For example, as shown in Table 6, the peaks 3 (sinomenine) and 5 were attributed to extract 1; while they disappeared in the negative control sample without extract 1, indicating that these two peaks were contributed by the herb Caulis Sinomenii. The characteristic peaks (2, 4, and 6) in the extract 2 came mainly from Radix Paeoniae Alba. Peak 10 was contributed by Cortex Moutan while peaks 1, 7, 8, and 9 were contributed by the common constituents of Cortex Moutan and Radix Paeoniae Alba. Lastly, peaks 11–15 were determined to be contributed by Rhizoma Curcumae Longae. No characteristic peak was found in the fingerprint profiles of QFGJS corresponding to Radix Aconiti Lateralis Preparata. In summary, the general HPLC fingerprint profile of QFGJS derives mainly from four herbs, while the chemical information of Radix Aconiti Lateralis Preparata is not detected in this analysis.

### 3.7. Analysis of the stability and consistency of QFGJS products by HPLC fingerprint

Three batches of QFGJS (0 month) were examined immediately after production using the fingerprinting analysis, and their HPLC–DAD chromatograms are showed in Fig. 3. The correlation coefficients of each chromatogram (Batch No. 20040821, 20040825, and 20040901) as compared to the simulative mean chromatogram were 0.9919, 0.9962, and 0.9771, respectively, with S.D. value of 0.0056; these results indicate that the manufacturing of QFGJS was highly consistent in quality from batch to batch in these three pilot products.

This HPLC fingerprint was further used for examining the stability of QFGJS products at 0, 1, 2, and 3 months after production in accelerated stability testing, and 3 months in the long-term stability testing. Using one batch (No. 20040821) of the product as an example, the correlation coefficients of each chromatogram as compared to the primary chromatogram of the product just after production (0 month) were 0.9659, 0.9560, 0.9365, and 0.9562 for the samples collected at the time points of 1, 2, and 3 months in the accelerated testing and 3 months in the long-term testing, respectively. These results indicate that the QFGJS capsules remained relatively stable for at least three months after production under different environmental conditions. The HPLC fingerprints were further semi-quantitatively analyzed and expressed as values of the RPA and RRT of the characteristic peaks, as showed in Table 7. Ratios comparing RPA values of each peak in the stability testing sample chromatograms with the initial ones all fell in the range of 80–120%. These results clearly indicated that the QFGJS product was relatively stable for at least three months after production under conditions of the accelerated testing and the long-term testing. The R.S.D. values of RRT in the stability test fell in the range of 0.07–2.44%, which also indicate that this HPLC fingerprinting method is reproducible.

Table 7
The relative peak areas (RPA) and relative retention time (RRT) of characteristic peaks in stability testing samples of QFGJS[a]

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| RRT[b] | 0.27 | 0.36 | 0.56 | 0.69 | 0.80 | 0.91 | 1.00 | 1.25 | 1.31 | 1.81 | 2.04 | 2.07 | 2.11 | 2.78 | 2.94 |
| 0 Mon | 0.21 | 0.12 | 0.21 | 0.36 | 0.68 | 0.20 | 1.00 | 0.45 | 0.22 | 1.58 | 0.10 | 0.28 | 1.38 | 0.35 | 1.65 |
| 1 Mon | 0.20 | 0.10 | 0.23 | 0.39 | 0.67 | 0.22 | 1.00 | 0.45 | 0.21 | 1.52 | 0.09 | 0.26 | 1.38 | 0.38 | 1.35 |
| 2 Mon | 0.22 | 0.12 | 0.23 | 0.35 | 0.69 | 0.19 | 1.00 | 0.41 | 0.23 | 1.56 | 0.08 | 0.24 | 1.23 | 0.39 | 1.50 |
| 3 Mon | 0.20 | 0.10 | 0.22 | 0.38 | 0.66 | 0.23 | 1.00 | 0.41 | 0.21 | 1.51 | 0.08 | 0.24 | 1.32 | 0.40 | 1.34 |
| 3 M-L | 0.20 | 0.11 | 0.22 | 0.38 | 0.65 | 0.23 | 1.00 | 0.41 | 0.20 | 1.52 | 0.09 | 0.25 | 1.35 | 0.40 | 1.37 |

[a] The stability testing data of samples of Batch No. 20040821 were shown.
[b] The relative retention time (RRT) was calculated based on the 0 month data of samples of Batch No. 20040821.

## 4. Conclusions

As herbal medicinal preparations and proprietary products are being used more and more widely throughout the world, evaluating and ensuring their quality becomes increasingly urgent. In the present studies, the quality in the pilot manufacturing processes and the short-term stability of an anti-arthritic herbal preparation, QFGJS, was successfully examined by utilizing a combinative HPLC evaluation system. The system employs: (1) quantitative HPLC–DAD method to determine the representative marker compounds, i.e., sinomenine, paeoniflorin, paeonol, and curcumin in the preparation, (2) HPLC fingerprinting analysis to characterize the entire chromatographic pattern of the compounds present in the herbal preparation, and (3) another HPLC method for determination of hypaconitine content in QFGJS. The HPLC–DAD analytical methods determining the marker compounds were demonstrated to be reliable and accurate with validated repeatability, reproducibility, and recovery testing. Quantitative determination showed that the contents of these five marker compounds in three batches of QFGJS products and in QFGJS samples collected at 1, 2, and 3 months after production in the accelerated stability testing and at three months in the long-term stability testing were consistent. However, this quantitative analysis could not provide information on other active compounds in QFGJS. HPLC fingerprinting analysis of the entire pattern of chromatographic profiles of the herbal products could serve as a complementary tool for quality control and for evaluation of quality consistency of herbal preparations. Satisfactory similarity was obtained in three different batches of QFGJS product and in samples of the product collected from the stability tests using HPLC fingerprinting analysis. Furthermore, contributions of the individual herbs in the original herbal formula of QFGJS to the corresponding peaks of the chromatographic fingerprint profiles were intensively investigated. As a supplement to fingerprinting analysis and quantification of the four compounds, quantitative determination of the content of a toxic *Aconitum* alkaloid (hypaconitine) that exists in minute quantities in QFGJS was also conducted; the level of hypaconitine is important for safe clinical use of QFGJS.

In summary, this combinative approach, using both quantitative and qualitative HPLC methods, can provide a systematic and feasible mean to evaluate the quality consistency of herbal preparations in the stability testing, and to monitor the intermediate stages of the manufacturing processes. As herbal medicinal preparations have chemical complexity, it is very difficult to identify and determine all of their chemical components. Using both quantitative and qualitative methods would be a complementary approach for the quality control and stability assessment of the herbal preparations.

## Acknowledgements

This research was funded by the Hong Kong Jockey Club Charities Trust. The authors wish to thank Dr. Martha Dahlen for her professional English editing for this paper.

## References

[1] G. Lin, P. Li, S.L. Li, S.W. Chan, J Chromatogr. A 935 (2001) 321–338.
[2] L.W. Yang, D.H. Wu, X. Tang, W. Pang, X.R. Wang, Y. Ma, W.W. Su, J. Chromatogr. A 1070 (2005) 35–42.
[3] F. Li, S. Sun, J. Wang, D. Wang, Biomed. Chromatogr. 12 (1998) 78–85.
[4] P. Drašar, J. Moravcova, J. Chromatogr. B 812 (2004) 3–21.
[5] S.J. Lin, H.H. Tseng, K.C. Wen, T.T. Suen, J. Chromatogr. A 730 (1996) 17–23.
[6] S. Zschocke, J.H. Liu, H. Stuppner, R. Bauer, Phytochem. Anal. 9 (1998) 283–290.
[7] R.W. Jiang, K.M. Lau, H.M. Lam, W.S. Yam, L.K. Leung, K.L. Choi, M.M.Y. Waye, T.C.W. Mak, K.S. Woo, K.P. Fung, J. Ethnopharmacol. 96 (2005) 133–138.
[8] U.S. Food and Drug Administration, Guidance for Industry Botanical Drug Product, Rockville, 2004.
[9] The European Agency for the Evaluation of Medicinal Products, Note for Guidance on Quality of Herbal Medicinal Products, London, 2001.
[10] State Food and Drug Administration of China, Guidance for Experiment Research on HPLC Fingerprint of Traditional Chinese Injections (Draft), Beijing, 2002.
[11] World Health Organization, Guidelines for the Assessment of Herbal Medicine, WHO, Geneva, 1991.
[12] M. Goppel, G. Franz, Planta Med. 70 (2004) 432–436.
[13] K. Koll, E. Reich, A. Blatter, M. Veit, J. AOAC Int. 86 (2003) 909–915.
[14] X. Cai, H. Zhou, Y.F. Wong, Y. Xie, Z.Q. Liu, Z.H. Jiang, Z.X. Bian, H.X. Xu, L. Liu, Biochem. Biophys. Res. Commun. 337 (2005) 586–594.
[15] H. Zhou, Y.F. Wong, X. Cai, Z.Q. Liu, Z.H. Jiang, Z.X. Bian, H.X. Xu, L. Liu, Biol. Pharm. Bull. 29 (2006) 253–260.
[16] L. Liu, E.B. Bunchner, K. Emrich, R. Kinne, Int. J. Immunopharmacol. 8 (1996) 529–543.
[17] H. Hikino, C. Konno, H. Takata, J. Pharmacobiodyn. 3 (1980) 514–525.
[18] J. Liang, A. Zhou, M. Chen, Eur. J. Pharmacol. 183 (1990) 901–902.
[20] S. Rafatullah, M. Tariq, M.A. Al-Yahya, J.S. Mossa, A.M. Ageel, J. Ethnopharmacol. 29 (1990) 25–34.
[21] Y. Xie, H.Z. Jiang, H. Zhou, H.X. Xu, L. Liu, J Chromatogr. A 1093 (2005) 195–203.
[22] G.H. Lu, K. Chan, Y.Z. Liang, K. Leung, C.L. Chan, Z.H. Jiang, Z.Z. Zhao, J. Chromatogr. A 1073 (2005) 383–392.
[23] Y.Z. Liang, P.S. Xie, K. Chan, J. Chromatogr. B 812 (2004) 53–70.
[24] F. Gong, Y.Z. Liang, P.S. Xie, F.T. Chau, J. Chromatogr. A 1002 (2003) 25–40.
[25] Y. Suzuki, T. Oyama, A. Ishige, Planta Med. 60 (1994) 391–394.
[26] N.G. Bisset, J. Ethnopharmacol. 4 (1981) 247–336.