J Pharm Anal Vol 1, No 2, 119 - 124 (2011)

ORIGINAL ARTICLES

Evaluation preparation technology of Xiaochaihu granules using fingerprint-peak pattern matching

Yu-Qiong Wu[1,2], Yu-Qiang Gou[3], Jing Han[1], Ying-Yan Bi[1], Shi-Lan Feng[1], Fang-Di Hu[1,4]*, Chun-Ming Wang[4]

[1] *School of Pharmacy, Lanzhou University, Lanzhou 730000, China;* [2] *the Second People's Hospital of Gansu Province, Lanzhou 730000, China;* [3] *Center for Disease Control and Prevention, Lanzhou Military Area Command of Chinese People's Liberation Army, Lanzhou 730000, China;* [4] *College of Chemistry and Chemical Engineering, Lanzhou University, Lanzhou 730000, China.*

Abstract: An approach was proposed to evaluate preparation technology by means of fingerprint-peak matching technology of high performance liquid chromatography with diode array detector (HPLC-DAD). Similarity and hierarchical clustering analysis (HCA) were applied to identify the 15 batches of Xiaochaihu granules from different manufacturers and our laboratory, and peak pattern matching between the composite formulae and *Radix Bupleuri* Chinensis, which was one of the main ingredients of Xiaochaihu granules, was utilized to evaluate the preparation technology of Xiaochaihu granules via the indexes of the relative deviation of retention time (RT) and UV spectrum feature similarity of their corresponding peaks. Eleven matching peaks were found between Xiaochaihu granules and *Radix Bupleuri* Chinensis. However, the saikosaponin A and saikosaponin D, which are the important active components in *Radix Bupleuri* Chinensis, were not found in Xiaochaihu granules from any manufacturers. The peak areas of 11 characteristic peaks of Xiaochaihu granules samples formed a matrix of 11×15. The result of HCA showed that Xiaochaihu granules samples were divided into four kinds of category. Xiaochaihu granules samples from the same manufacturer were basically clustered of the same category. The results suggested that the saikosaponin A and saikosaponin D are prone to structural transformation under the condition of decoction and in the presence of the organic acidic components. These active components, existing in raw herb, might transform to a series of non-active secondary saikosaponin due to unfavourable preparation technology. So the conventional decoction-based preparation technology of Xiaochaihu granules might greatly affect its quality and therapeutic effectiveness. This study demonstrates that fingerprint-peak matching technology can not only be used for quality control of this composite formulae, but also provide some guidance for preparation technology of Xiaochaihu granules.

Keywords: Xiaochaihu granules; *Radix Bupleuri* Chinensis; fingerprint-peak matching; hierarchical clustering analysis

1 Introduction

Xiaochaihu granule is the traditional Chinese patent medicines (TCPM) based on Xiaochaihu tang which was first described in the *Shang Han Lun*, a treatise of febrile diseases by the physician Zhong-Jing Zhang during the Chinese Eastern Han Dynasty. The traditional remedy for treatment of chronic liver diseases [1,2] is a mixture of seven herbs, including *Radix Bupleuri* (Chinese thorowax root), *Radix Scutellariae* (huangqin or baical skullcap root), *Rhizoma Pinelliae* (banxia or pinellia tuber), *Radix Ginseng* (renshen or ginseng), *Radix Glycyrrhizae* (gancao or licorice root), *Rhizoma Zingiberis Recens* (shengjiang or fresh ginger), and *Fructus Jujubae* (dazhao or Chinese date). The modern researches demonstrate that there are richly pharmacological effects, such as protective effects on experimental liver injuries [3, 4], preventive and therapeutic effects on experimental hepatic fibrosis via inhibition of hepatic stellate cells and lipid peroxidation of hepatocytes [5-7]. It is contained in ministerial standard Chinese Narikata Volume VIII preparations (Chinese Narikata preparation WS3-B-1498-93). It is well known that the preparation process of TCPM involves a number of links: analysis of Chinese herbal medicine resources, optimum selection of Chinese herbal medicine (including cooked), extraction of Chinese herbs, traditional Chinese medicine preparation technology, and productions (the composite formulae) [8]. The quality control of raw materials is considered to be the most important in the quality control for TCPM. *Radix Bupleuri* Chinensis, which is a monarch ingredient in the Xiaochaihu granules, accounts for one-third amount in whole prescriptions. So, the variety, quality and extraction technology of *Radix Bupleuri* could influence the quality of Xiaochaihu granules significantly. At present, as a currently accepted effective means for the quality control of traditional Chinese medicine (TCM) and natural medicine, TCM fingerprints, with the characteristics of integrity and

Received 20 July 2010; Accepted 31 December 2010

* Corresponding author. E-mail: hufd@lzu.edu.cn

fuzziness, focus on the gross structure feature and internal rules, and fit the Chinese medicine theory and pharmacodynamic model [9-13]. Some exploring studies on the fingerprint of *Radix Bupleuri* have been carried out. Using principal component analysis and canonical correlation analysis, the spectral information of *Radix Bupleuri* correlated to its pharmacological data was identified by Ni and his coworkers [14]. The canonical correlative variable U_1 of its UV spectra is correlated with what is obtained in pharmacological experiments. Tian [15] utilized HPLC fingerprint and HPTLC fluorescent images to distinguish *Radix Bupleuri* from several major herbal distribution centers. So far we have not found studies on fingerprints of Xiaochaihu granules yet except some qualitative and quantitative analysis of one or two bioactive components [16-18]. The conventional quality control mode does not satisfy the requirement of evaluation of the quality of Xiaochaihu granules. In this study, we applied fingerprint technology with peak pattern matching to analyze the active components (saikosaponin A and saikosaponin D) in Xiaochaihu granules from different manufacturers. It can provide the technical support for further development and improvement of preparation technology of Xiaochaihu granules.

2 Experimental

2.1 Apparatus, reagents and materials

All chromatographic studies were performed with a Waters Alliance 2695 HPLC system (Waters, USA) equipped with a diode array detector (Waters, 2996) and M32 chromatographic software.

Acetonitrile and methanol (chromatographic grade) were purchased from Yucheng Chemicals Inc. (Shandong, China). Glacial acetic acid and phosphoric acid (analytical grade) were obtained from Beijing Chemical Plant (Beijing, China). Double-distilled water was provided by the First Affiliated Hospital of Lanzhou University.

Radix Bupleuri was purchased from the Huanghe Market of medicinal materials, and identified as dried roots of *Bupleurum scorzonerifolium* Willd (Umbelliferae family) by Professor Yin-Suo Zhou (School of Pharmacy, Lanzhou University). Xiaochaihu granules 1, 2, and 3 were homemade in our laboratory, Xiaochaihu granules 4, 5, and 6 were purchased from manufacturer A (batch lots: 20070502, 20070810, 20061101), Xiaochaihu granules 7, 8, and 9 were purchased from manufacturer B (batch lots: 20070507, 20070204, 20061219), Xiaochaihu granules 10, 11, and 12 were purchased from manufacturer C (batch lots: 20071017, 20071103, 20071213), Xiaochaihu granules 13, 14, and 15 were purchased from manufacturer D (batch lots: 20100305, 20091007, 20091025). The names of manufacturers had been removed in order to preserve confidentiality. Reference substances, saikosaponin A and saikosaponin D, were purchased from the National Institute of China for the Control of Pharmaceutical and Biological Products.

2.2 Chromatographic conditions

Analyses were performed on a Waters Alliance 2695 HPLC system consisting of a quaternary delivery system, an autosampler and a DAD detector. All the separation was carried out on a Hypersil ODS column (250 mm × 4.6 mm i.d., 5 μm). A binary gradient elution system with acetonitrile as solvent A and water as solvent B was programmed as follows: 0 – 10 min, 5% – 15% A; 10 – 25 min, 15% – 20% A; 25 – 30 min, 20% – 25% A; 30 – 40 min, 25% 40% A; 40 – 50 min, 40% – 50% A; 50 – 60 min, 50% – 80% A; 60 – 65 min, 80% – 100% A. Each run was followed by an equilibration time of 15 min. The flow rate was 1.0 mL/min and the column temperature was maintained at 30 ℃. The wavelength of DAD ranged from 190 to 400 nm. Aliquots of 10 μL of sample and standard solutions were injected into the elution system. The data were collected and analyzed with M32 software.

2.3 Preparation of Xiaochaihu granules sample solutions

Accurately weighed 10.0 g of Xiaochaihu granules sample, ultrasonic-extracted with 50 mL methanol for 30 min, and then filtered through 0.45 μm nylon membrane to obtain the Xiaochaihu granules sample solution.

2.4 Preparation of *Radix Bupleuri* sample solutions based on the different preparation technology

2.4.1 Preparation of *Radix Bupleuri* sample solution 1

Radix Bupleuri was extracted for twice with 10 times volumn of water for 1.5 h each time. The decoctions were concentrated to dryness, and the residue was weighted (equivalent to 10.0 g Xiaochaihu granules). According to the method described in 2.3 section, sample solution 1 was prepared.

2.4.2 Preparation of *Radix Bupleuri* sample solutions 2, 3, 4, and 5

Radix Bupleuri was extracted for twice with 10 times volumn of water for 1.5 h each time. The filtrate was concentrated to dryness, and the residue was weighted (equivalent to 10.0 g Xiaochaihu granules) and followed by redissolving with 50 mL of 40% (2), 60% (3), 80% (4), and 100% (5) alcohol solution, respectively. The alcohol solutions were ultrasonic-extracted for 30 min and then cooled down to room temperature. Each of the sample solutions in the four solvents was prepared in triplicate.

2.4.3 Preparation of *Radix Bupleuri* sample solution 6

Radix Bupleuri samples (equivalent to 10.0 g Xiaochaihu granules) were extracted with 80 mL of petroleum ether under ultrasonic-extraction for 3 times with 30 min for each time. After petroleum ether was removed completely, 50 mL of 5% ammonia methanol was added to the residue and

the mixture was weighted and followed by an ultrasonic-extraction process for 1 h. The extraction solution was evaporated to dryness, and the residue was dissolved with 50 mL of methanol. The sample solution 6 was prepared.

2.5 Preparation of standard solutions

Two stock solutions (0.920 mg/mL saikosaponin A and 1.064 mg/mL saikosaponin D) were obtained by dissolving a certain amount of saikosaponin A and saikosaponin D in 10 mL methanol respectively. The stock solutions were further diluted to appropriate concentrations for subsequent analysis.

3 Results

3.1 Validation of methodology

The precision and repeatability of fingerprint method were assessed by three injections of Xiaochaihu granules (20070204) sample (duplicated 3) according to the HPLC methods described in 2.2 section. The relative standard deviations (RSDs) of retention time (RT) and peak area (PA) of characteristic peaks in the precision test were found in the range of 0.8% − 3.1% and 0.3% − 4.6%, respectively. The RSDs of RT and PA of characteristic peaks in the reproducibility test were also below 1.0% and 3.4%, respectively. Meanwhile, the stability test was evaluated by analyzing the samples at intra-day different time points (0, 2, 4, 8, 24 and 48 h) and the RSDs of RT and PA of characteristic peaks were less than 1.0% and 4.1%, respectively. The results indicated that the method of HPLC fingerprint analysis was valid and feasible.

3.2 Chromatographic fingerprint and similarities of Xiaochaihu granules

To obtain the fingerprints, 15 batches of Xiaochaihu granules from different manufacturers were analyzed using the developed method. The fingerprint similarity of 15 batches of Xiaochaihu granules was calculated and generated with the Computer Aided Similarity Evaluation (CASE) software at 210 nm, which was developed based on chemometrics by the Research Center for the Modernization of Traditional Chinese Medicines (Central South University, Changsha, China). The pattern fingerprints of Xiaochaihu granules are shown in Figure 1. The correlation coefficients (r_1) of Xiaochaihu granules from four pharmaceutical factories (A, B, C and D) and our laboratory were 92.90%, 97.69%, 89.48%, 89.39% and 94.48% at 210 nm, respectively. The congruence coefficients (r_2) of Xiaochaihu granules from four pharmaceutical factories (A, B, C and D) and our laboratory were 93.17%, 97.98%, 92.40%, 93.44% and 94.71% at 210 nm, respectively (Table 1).

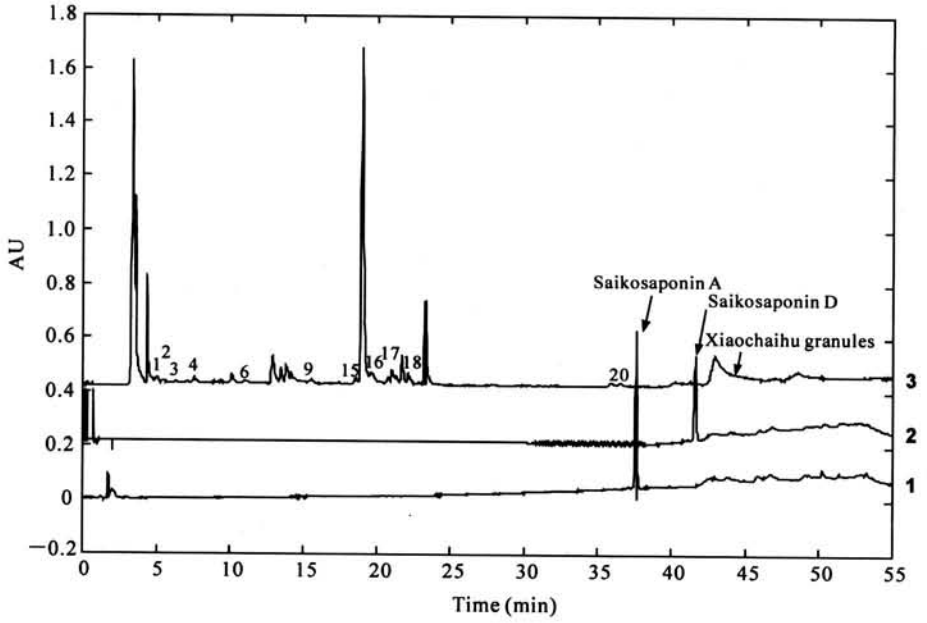

Figure 1 The chromatographic fingerprint of Xiaochaihu granules, saikosaponin A and saikosaponin D. Both saikosaponin A and saikosaponin D were not found in fingerprint of Xiaochaihu granules. The marked peaks were common peaks between *Radix Bupleuri* and Xiaochaihu granules, and serial numbers of peaks were correspondence with *Radix Bupleuri*. **1**, saikosaponin A; **2**, saikosaponin D; **3**, chromatogram of Xiaochaihu granules.

3.3 The fingerprint-peaks matching between saikosaponin A, saikosaponin D and *Radix Bupleuri* Chinensis

To obtain the matching peak of reference components (saikosaponin A and saikosaponin D), the relative deviation of retention times of matching peak corresponding the saikosaponin A and saikosaponin D in *Radix Bupleuri* samples extracted with different methods (1, 2, 3, 4, 5 and 6) was calculated using the Eq. (1).

$$\Delta t_i = \frac{t_{R(P)_i} - t_{R(H)_i}}{t_{R(P)_i}} \quad (1)$$

where Δt_i represents the relative deviation of retention times corresponding common peaks, $t_{R(P)_i}$ is the retention time of peak saikosaponin A or saikosaponin D, $t_{R(H)_i}$ represents the

retention time of matching peak with saikosaponin A or saikosaponin D in fingerprint of *Radix Bupleuri*. As Δt_i approaches 0, the peak is selected as matching peak.

Angle cosine value of matching peak is carried out with Eq. (2).

$$\cos\theta = \frac{X \times Y}{|X| \times |Y|} \quad (2)$$

where X_t and Y_t ($t = 1, 2, 3, \cdots, n$) represent the corresponding spectrum response values (above half-peak width) of peak $t_{R(P)_i}$ and $t_{R(H)_i}$ at selected wavelength, respectively, n is the number of collective wavelength. As the value of $\cos\theta$ approaches 1, the peak is defined as the peak pattern matching.

Table 1 The correlation coefficient (r_1) and congruence coefficient (r_2) of Xiaochaihu granules from four pharmaceutical factories (A, B, C and D) and our laboratory at 210 nm

Sample No.	Correlation coefficient (r_1)	Congruence coefficient (r_2)	Sample No.	Correlation coefficient (r_1)	Congruence coefficient (r_2)
Sample 1	0.9444	0.9497	Sample 9	0.9807	0.9832
Sample 2	0.9473	0.9524	Sample 10	0.8964	0.9069
Sample 3	0.9426	0.9411	Sample 11	0.9877	0.9789
Sample 4	0.9282	0.9364	Sample 12	0.8895	0.8862
Sample 5	0.9293	0.9286	Sample 13	0.8773	0.9754
Sample 6	0.9295	0.9302	Sample 14	0.9013	0.9143
Sample 7	0.9862	0.9878	Sample 15	0.9030	0.9136
Sample 8	0.9638	0.9683			

The chromatographic fingerprint of *Radix Bupleuri* samples with different extracted methods is shown in Figure 2.

The results of chromatogram peak pattern matching between saikosaponin A, saikosaponin D and all *Radix Bupleuri* samples are listed in Table 2. The 21th peak in Table 2, compared to saikosaponin A, the values of Δt approach 0 for all of *Radix Bupleuri* samples, but the value of $\cos\theta$ does not approach 1 except for the *Radix Bupleuri* samples 3 and 6. The 25th peak, compared to saikosaponin D, the values of Δt approach 0 for all of *Radix Bupleuri* samples, but the value of $\cos\theta$ does not approach 1 except for the *Radix Bupleuri* sample 6.

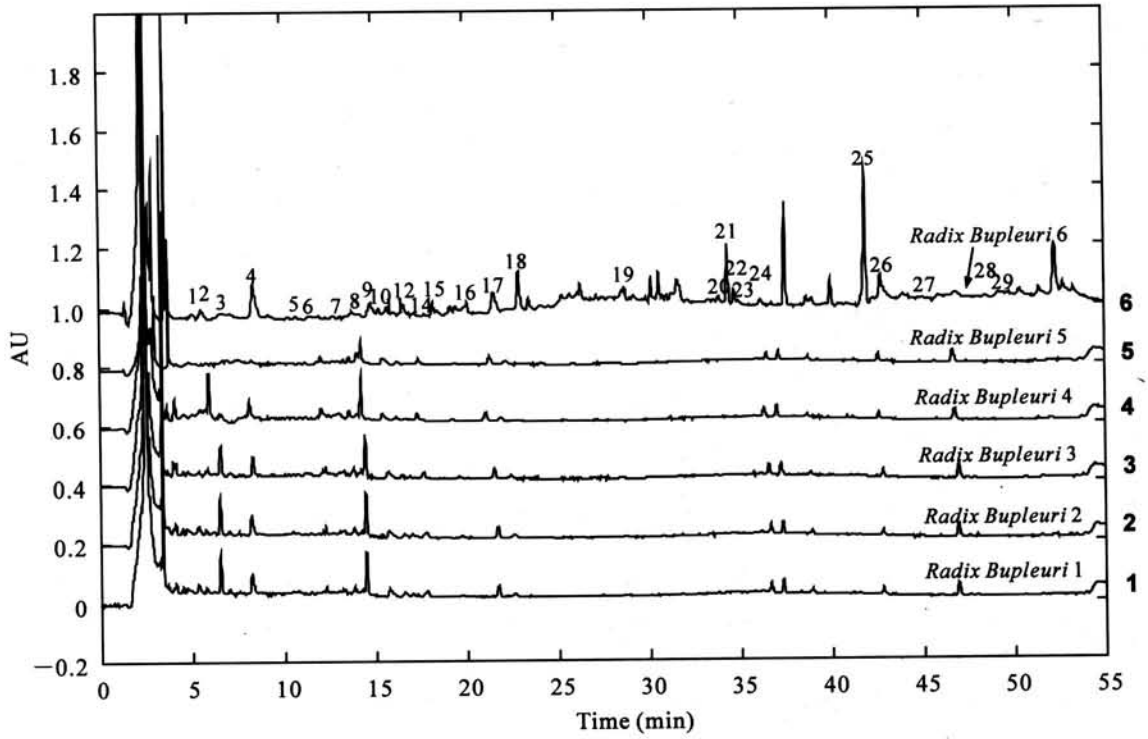

Figure 2 The chromatographic fingerprint of *Radix Bupleuri* samples with different extracted methods. **1**, *Radix Bupleuri* 1; **2**, *Radix Bupleuri* 2; **3**, *Radix Bupleuri* 3; **4**, *Radix Bupleuri* 4; **5**, *Radix Bupleuri* 5; **6**, *Radix Bupleuri* 6 (the peaks of 21th and 25th were saikosaponin A and saikosaponin D, respectively).

3.4 Hierarchical clustering analysis

HPLC-based hierarchical clustering analysis (HCA) method is a well-known method for distinguishing preparation technology for TCPM. In the analysis, we found 11 common peaks between Xiaochaihu granules and *Radix Bupleuri* sample 1, and the PA of these 11 characteristic peaks of Xiaochaihu granules samples (1 − 15) formed a matrix of 11×15. The result of HCA is shown in Figure 3.

Table 2 The result of fingerprint peak matching between *Radix Bupleuri* and saikosaponin A, saikosaponin D

Sample No. of *Radix Bupleuri*	21th peak		25th peak	
	cosθ	Δt	cosθ	Δt
	0.4304	0.002	0.5103	0.024
Sample 1	0.4215	0.002	0.5023	0.023
	0.4462	0.0017	0.5244	0.023
	0.2303	0.004	0.3301	0.150
Sample 2	0.2400	0.003	0.3211	0.090
	0.2256	0.004	0.3614	0.140
	0.9701	0.003	0.4302	0.040
Sample 3	0.9655	0.002	0.4125	0.030
	0.9718	0.004	0.4371	0.020
	0.5102	0.003	0.4178	0.095
Sample 4	0.5122	0.003	0.4256	0.096
	0.5236	0.003	0.4101	0.095
	0.2303	0.001	0.2506	0.026
Sample 5	0.2135	0.001	0.2612	0.024
	0.2416	0.002	0.2531	0.019
	0.9986	0.002	0.9998	0.001
Sample 6	0.9978	0.002	0.9996	0.001
	0.9988	0.001	0.9989	0.002

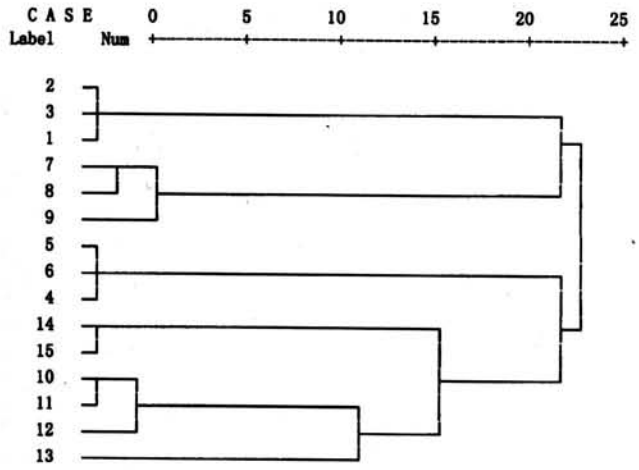

Figure 3 The clustering analyses of chromatograms of 15 Xiaochaihu granules samples (No.1 − 15). Samples 1, 2, and 3 were home-made in our laboratory, samples 4, 5, and 6 belonged to pharmaceutical factory A, samples 7, 8, and 9 belonged to pharmaceutical factory B, samples 10, 11, and 12 belonged to pharmaceutical factory C, samples 13, 14, and 15 belonged to pharmaceutical factory D.

It was clearly shown that the Xiaochaihu granules samples might be divided into four clusters: with samples 1, 2, 3 in cluster 1, samples 4, 5, 6 in cluster 2, samples 7, 8, 9 in cluster 3 and samples 10 − 15 in cluster 4. Group 1 including three samples turned out to be home-made according to preparation technology recorded in the Pharmacopeia of P.R. China. Group 2 including three samples turned out to be from pharmaceutical factory A. Group 3 including three samples turned out to be from pharmaceutical factory B. Group 4 including six samples turned out to be from pharmaceutical factory C and D. HCA provided a quantitative comparison of the samples. The HCA results indicated that Xiaochaihu granules from different pharmaceutical factories could be distinguished clearly.

4 Discussion

The saikosaponin D was not found in all of Xiaochaihu granules and *Radix Bupleuri* samples (1, 2, 3, 4 and 5) except *Radix Bupleuri* sample 6. The saikosaponin A was not found in all of Xiaochaihu granules and *Radix Bupleuri* samples (1, 2, 4 and 5) except *Radix Bupleuri* samples 3 and 6. The *Radix Bupleuri* sample solutions 1, 2, 3, 4, and 5 were prepared based on the preparation technology of Xiaochaihu granules (water decoction). The result demonstrated that saikosaponin A and saikosaponin D in *Radix Bupleuri* might be easily subjected to thermal degradation in the conventional preparation technology of Xiaochaihu granules, which coincides with previous literature [19-21].

Radix Bupleuri is one of the well-known TCM herbs and is often used as a significant ingredient for many Chinese multi-herb remedies. It is proved that there are many forms of saikosaponins, such as saikosaponin A and D, which have been recognized as pharmacologically active compounds and possess of immunomodulatory, hepatoprotective, anti-tumor and anti-viral activities [22,23]. Xiaochaihu granules manufactured with conventional preparation technology do not contain the two important active components, so it might affect the therapeutic effects of Xiaochaihu granules. The study developed fingerprint-peak matching technology and elucidated the causes that Xiaochaihu granules do not contain both saikosaponin A and saikosaponin D. Furthermore, the fingerprint-peak matching technology can not only be used for quality control of the composite formulae, but also provide some guidance for further development of preparation technology of Xiaochaihu granules.

Acknowledgments

This work was supported by the Fundamental Research Funds for the Central Universities.

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