Chemical Fingerprinting of *Shexiang Baoxin Pill* and Simultaneous Determination of Its Major Constituents by HPLC with Evaporative Light Scattering Detection and Electrospray Mass Spectrometric Detection

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High-performance Liquid Chromatography (HPLC) with evaporative light scattered detection (ELSD) and electrospray ionization mass spectrometric detection (ESI-MS) was employed to establish chemical fingerprint of *Shexiang Baoxin Pill* (SBP) and to simultaneously determinate its seven major constituents, including cholic acid, deoxycholic acid, ursodeoxycholic acid, chenodeoxycholic acid, cinobufagin, recibufogenin, and ginsenoside Rb1. The analysis was performed on a C_{18} column with water–acetonitrile gradient elution, and the investigated constituents were authenticated by comparing their retention times and mass spectra with those of reference compounds. The proposed method was applied to analyze nine SBP samples and produced data with acceptable linearity, precision, stability and accuracy. Both the chemical fingerprints and quantification data were used to evaluate the quality of various SBP products. The proposed method allows obtaining chemical fingerprint and quantification of multi-components in one run, and therefore can be readily utilized as a comprehensive quality control approach for traditional Chinese medicine.

Key words chemical fingerprint; simultaneous determination; traditional Chinese medicine; *Shexiang Baoxin Pill*; quality control

Shexiang Baoxin Pill (SBP) is a well-known composite formula of traditional Chinese medicine (TCM), and is commonly used in clinical practice for the treatment of cardiovascular diseases.[1] It comprises seven medicinal materials or extracts thereof, including *Moschus*, *Radix Ginseng*, *Calculus Bovis*, *Cortex Cinnamomi*, *Styrax*, *Venenum Bufonis* and *Borneolum Syntheticum*. These medicinal materials are often from different origins, sources, cultural manner, harvest time, pretreatment processes, and manufacturing processes, and accordingly will result in significant variances of the quality of SBP produced by different manufacturers or even by the same manufacturer. As a result, quality control of SBP products is very critical to ensure their efficacy and safety. So far, chemical fingerprinting has been internationally accepted as an efficient tool for the integral quality control of TCM.[2—4] On the other hand, the quality of TCM is highly related to its major active constituents, and thus in most cases, quantitative analysis of these components is also necessary.[5] Presently, the quality control of SBP, however, is done mainly according to China Pharmacopoeia 2000 and the draft issued by the Department of Health, which demand neither chemical fingerprint analysis nor quantification.[6] So it is urgently needed to develop the fingerprint of SBP and to simultaneously determine its multiple active constituents so as to ensure the efficacy, safety, and batch-to-batch uniformity.

Up to now, for most medicinal materials of SBP, several methods have already been developed for chemical fingerprinting and quantitative analysis, such as High-performance Liquid Chromatography (HPLC), Gas Chromatography (GC), *etc.* [7—10] However, no proper method has been developed focusing on the formula of SBP yet, since its constituents are much more complicated. Among all the medicinal materials, it is known that the major constituents from *Moschu*, *Styrax*, *Cortex Cinnamomi* and *Borneolum Syntheticum* are volatiles, and in our laboratory, a simple and reliable gas chromatographic method coupled with FID and MS detection has recently been established to simultaneously analyze the multiple volatile components of SBP (to be published elsewhere). Comparatively, analysis of the non-volatile components from other three materials, *Radix Ginseng*, *Calculus Bovis* and *Venenum Bufonis*, is a much more challenging task, because their major bioactive components (listed in Fig. 1), respectively, are ginsenosides, steroids and cardogenanes compounds with low UV–vis absorptivity and can't be determined by common HPLC/UV technique. An alternative methodology is thus highly desirable.

In the recent years, the technique of evaporative light-scattering detector (ELSD) has increasingly been used as a credible solution for the analysis of the components with poor UV absorption properties.[11,12] ELSD shows good compatibility to the multi-components of complex analytes such as TCM, and in our former work, HPLC/ELSD method has been successfully established for qualitative and quantitative quality evaluation of *Qingkailing* injection.[5,13] As a series studies on the comprehensive quality control of TCM, we report here, for the first time, the development of HPLC/ELSD method coupled with mass spectrometric detection to develop chemical fingerprint of the complicated formula of SBP, and to simultaneously determine its three types of non-chromophoric constituents, *i.e.*, ginsenosides, steroids and cardogenanes.

Applied the proposed method, the chemical fingerprint of SBP extract was developed for quality control purpose, in which 13 peaks were identified by electrospray tandem mass spectrometry, and seven major non-volatile components, including cholic acid (CA), deoxycholic acid (DCA), ursodeoxycholic acid (UDA), chenodeoxycholic acid (CDA), cinobufagin (CIN), recibufogenin (REC) and ginsenoside Rb1 (Rb1), were further quantified. The proposed method produces chemical fingerprint and quantitative data of multi-components in one run, and therefore can be readily utilized as a comprehensive quality control method for TCM.

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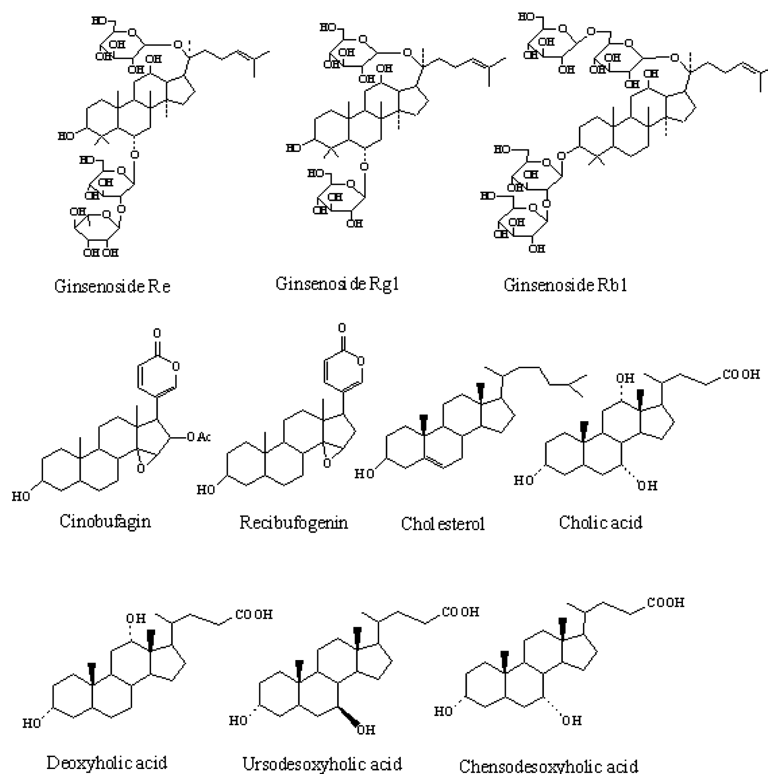

Fig. 1. Chemical Structures of the Some Representative Non-violate Constituents in SBP

Experimental

Reagents and Materials Authentic standards were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, P. R. China). Acetonitrile and formic acid were of HPLC grade (Merck, Darmstadt, Germany). Ultrapure water was prepared from Millipore water purification system (Millipore, Miford, MA, U.S.A.). Other reagents were of analytical grade.

Chromatographic System HPLC/ELSD analysis was performed on a Shimadzu LC2010A liquid chromatograph system (Shimadzu Co., Japan) consisting of a quaternary pump, a column oven, an autosampler and Sedex 75 ELSD detector (Sedere Co., France) coupled with CLASS-VP workstation. ESI-MS-MS analysis was performed on an Agilent-1100 HPLC system with a LC/MSD Trap XCT mass spectrometer (Agilent Corporation, MA, U.S.A.).

Analytical Conditions A C_{18} RP-ODS column (4.6 mm×250 mm, 5 μ, Agilent, U.S.A.) and a C_{18} guard column (4.6 mm×7.5 mm, 5 μ, Merck, U.S.A.) were used. The mobile phases were composed of water/formic acid (100/0.5, A) and acetonitrile (B). The gradient was as follows: 0 min, 80% A, 20% B; 27 min, 42% A, 58% B; 37 min—60 min, 0% A, 100% B. Elution was performed at a solvent flow rate of 0.8 ml/min. The column compartment was kept at the temperature of 25 °C, and the sample injection volume was 10 μl. The drift tube temperature of ELSD was 40 °C, and the gas pressure was set as 3.5 bar.

For HPLC/ESI-MS-MS analysis, 0.2 ml/min portion of the column effluent was delivered into the ion source of mass spectrometry. The ESI-MS spectra were acquired in both the positive ion mode and negative ion mode. The conditions of electrospray ionization source were as follows: drying gas N_2, 10 l/min, temperature 350 °C, pressure of nebulizer 30 psi, capillary voltage 2500 V and scan range 200—1300 u.

Sample Preparation Seven standards, including CA, UDA, DCA, CDA, CIN, REC and Rb1, were accurately weighted, dissolved in acetonitrile and then diluted to appropriate concentration. A mixed stock solution of standards, containing CA 5.60 mg/ml, DCA 1.32 mg/ml, UDA 1.09 mg/ml, CDA 0.44 mg/ml, CIN 1.92 mg/ml, REC 0.88 mg/ml and Rb1 0.61 mg/ml, was finally prepared. The stock solutions were further diluted to make working standard solutions.

Nine batches of SBP samples were kindly offered by Shanghai Hutchison Pharmaceuticals Co. (Shanghai, China), three of which were produced in the year of 2004 (marked as sample 1—3) and the others were produced in 2005 (marked as sample 4—9). Samples were ground into fine powder and 2.00 g of each was accurately weighted. 50 ml 50% ethanol (V/V) added, the samples were ultrasonic extracted (15 min×2) under the same conditions, then centrifuged and filtered. All solutions were stored in the refrigerator at 4 °C, and filtered through a syringe filter (0.45 μm) before HPLC analysis.

Results and Discussion

Chemical Fingerprint of SBP Using the proposed method, HPLC/ELSD chromatograms of the extracts of SBP were acquired, in which there were mainly 26 peaks eluted (shown in Fig. 2) in total. Reduplicate analysis showed that, the 26 peaks represented the common major constituents of different SBPs with consistent retention values (RSDs of retention times lower than 1%, and those of most peak areas lower than 8%). In this work, HPLC/ELSD chromatograms of its medicinal materials were also studied, and by comparing those chromatograms to that of SBP, it suggested that the HPLC/ELSD chromatogram represened the characteristic chemical information of most the non-volatile constituents in SBP, except some volatile constituents from *Moschus sifanisus Przewalski*, *Cortex Cinnamomi* and *Borneolum Syntheticum* not detected. The obtained HPLC/ELSD chromatogram, therefore, can be applied as the chemical fingerprint of SBP for quality control purpose.

In order to identify these peaks in the fingerprint, HPLC/ESI-MS-MS analysis was performed under the same chromatographic condition. Table 1 lists the mass spectrometric analysis results. There were altogether 13 peaks authenticated, including peak 2—5, peak 7 and peak 10—17, most of which were further confirmed by comparing retention times and mass spectra with those of standard compounds; however, in the present work, we were failed to identify the other peaks. In Table 1, it indicates that peak 2 in-

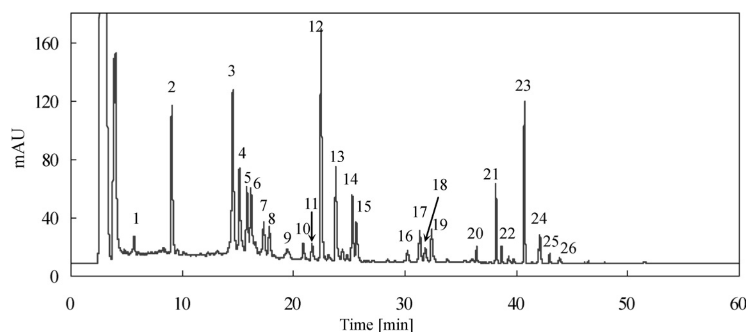

Fig. 2. HPLC/ELSD Fingerprint of the Extract of SBP

Table 1. ESI-MS-MS Ions of the Identified Compounds

Peak No.	t_R (min)	Compound	MW	MS (m/z)	
				Positive mode	Negative mode
2	9.4	Rg1	801	824 [M+Na]$^+$ 662 [823−pyg]$^+$	846 [M+HCOO]$^-$
		Re	947	970 [M+Na]$^+$ 927 [M+H−H_2O]$^+$	946 [M−H]$^-$ 992 [M+HCOO]$^-$
3	14.8	Rb1	1109	1132 [M+Na]$^+$ 462 [M+H−4pyg]$^+$	1108 [M−H]$^-$ 1144 [M+Cl]$^-$
4[a]	15.4	Ra1	1211	1234 [M+Na]$^+$ 1102 [M+Na−tpt]$^+$	1210 [M−H]$^-$ 1078 [M−H−pyr]$^-$
5	16.1	Rc	1079	1102 [M+Na]$^+$ 778 [M+Na−2pyg]$^+$	1114 [M+HCOO]$^-$ 1078 [M−H]$^-$
7	17.7	Rd	947	970 [M+Na]$^+$ 646 [M+Na-2pyg]$^+$ 610 [646−$2H_2O$]$^+$ 750 [M+H−pyg−$2H_2O$]$^+$	946 [M−H]$^-$ 992 [M+HCOO]$^-$
10	21.1	CA (isomer)	409	432 [M+Na]$^+$ 839 [2M+Na]$^+$ 374 [M+H−$2H_2O$]$^+$	408 [M−H]$^-$ 454 [M+HCOO]$^-$ 817 [2M−H]$^-$
11	21.8	Cholesteal	386	795 [2M+Na]$^+$ 387 [M+H]$^+$ 409 [M+Na]$^+$	
12	22.6	CA	409	819 [2M+H]$^+$ 428 [M+H+H_2O]$^+$	817 [2M−H]$^-$ 408 [M−H]$^-$
13	24.0	UCA	392	415 [M+Na]$^+$ 357 [M+H−$2H_2O$]$^+$	391 [M−H]$^-$ 437 [M+HCOO]$^-$
14	25.4	CIN	442	443 [M+H]$^+$ 465 [M+Na]$^+$ 907 [2M+Na]$^+$	
15	25.7	REC	384	385 [M+H]$^+$ 407 [M+Na]$^+$	785 [2M+H_2O−H]$^-$ 831 [2M+H_2O+HCOO]$^-$
16	30.3	CDA	392	357 [M+H−$2H_2O$]$^+$ 415 [M+Na]$^+$	783 [2M−H]$^-$ 391 [M−H]$^-$ 437 [M+HCOO]$^-$
17	31.4	DCA	392	357 [M+H−$2H_2O$]$^+$ 785 [2M+H]$^+$ 415 [M+Na]$^+$	783 [2M−H]$^-$ 391 [M−H]$^-$

a) tpt denotes the compound of tetrahydro-pyran-2,3,4,5-tetraol.

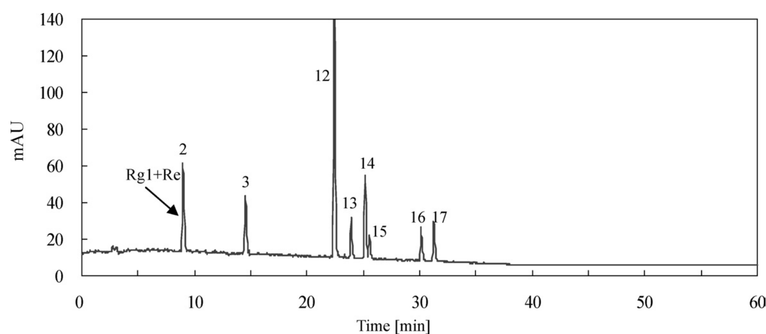

Fig. 3. HPLC/ELSD Chromatogram of Standard Chemicals

2: Rg1+Re; 3: Rb1; 12: CA; 13: UDA; 14: CIN; 15: REC; 16: CDA; 17: DC.

volves two compounds, ginsenoside Rg1 and ginsenoside Re, though it is a symmetrical Gaussian peak. Further study on the chromatograms of standards shows that, under the proposed chromatographic condition, Rg1 and Re have the same retention time and can't be well separated (HPLC/ELSD chromatogram of standards is shown in Fig. 3).

Validation The assay linearity was determined by analysis of five different concentrations of the standard solutions. It is well known that ELSD gives no direct linear response, and the calibration curves can be constructed by partial least squares method on the analytical data of peak area and concentration in double logarithmic co-ordinates.[14] The limit of detection (LOD) was determined as the concentration resulting in a peak height greater than three times the baseline

noise level (S/N>3). Table 2 shows the regression data and LODs of the components determined, and each has a regression coefficient over 0.995.

The intra-day and inter-day precision was determined by analyzing five sets of calibration samples during a single day and on four different days respectively. The stability tests were performed by analyzing the same analyte during periods of 2, 4, 8, 16, 24 h. The accuracy tests were carried out by spiking known contents of standard samples into a SBP sample and comparing the determined amount of these standards with the amount originally added. The relative standard deviation (RSD) was taken as a measure. Table 3 lists the validation results of precision, stability and accuract tests. It shows that most RSDs are less than 5%, and the method is thus acceptable.

Sample Analysis and Quality Evaluation Using the proposed method, nine SBP samples were analyzed, including HPLC/ELSD fingerprint analysis and quantitative determination of seven constituents. Table 4 lists the determination results of seven major constituents of these samples.

Similarity measurement between the fingerprint of a test sample and that of a reference sample is often employed to quantitatively conduct quality evaluation. Similarity value is most commonly calculated by the congruence coefficient,[15] as expressed by the following formula:

$$r = \sum_{i=1}^{n} x_i x_i^0 \Big/ \sqrt{\sum_{i=1}^{n} x_i^2 \sum_{i=1}^{n} x_i^{02}}$$

where r is the similarity value between the fingerprint of sample x and that of reference sample x^0, and x_i, x_i^0 denote the ith peak areas of these two fingerprints respectively. In this study, Similarity Evaluation System for Chromatographic Fingerprint of TCM (Chinese Pharmacopoeia Committee, version 2004A) was used to achieve the similarity values as follows: 0.975, 0.920, 0.934, 0.979, 0.958, 0.983, 0.991, 0.974 and 0.984 (the mean data of the nine fingerprints was used as a reference). All similarity values vary in the range of 0.92—0.99, and accordingly it concludes that there are no obvious differences among these products.

However, the data of Table 4 gives rather a different conclusion. For each component determined, the content is evidently different among various samples, especially CIN and CDA having the highest RSDs, which suggests the large variations in their qualities. In this study, principal components analysis (PCA) on the quantitative analysis data was performed.[16—17] Figure 4 is the score plot of the first two principal components of PC1 and PC2 (data normalized before PCA, and over 94% variance explained). From the scatter points of Fig. 4, it is clear that samples 1—3 (produced in 2004) depart from other samples produced in 2005. The reason might be that the originations of some medicinal materials are different in two years. All samples produced in 2005 (samples 4—9) stay close together in Fig. 4, which indicates qualities of the products in 2005 are much more consistent and reliable.

Table 2. Linear Regression Data and LODs of the Compounds to Be Qualified

Compound	Linear function[a]	Regression coeffient (R^2)	Linear range (μg/ml)	LOD (ng/ml)
CDA	y=1.2479x+3.8625	0.9919	0.5—100	20
DCA	y=1.2418x+3.9349	0.9885	0.5—100	20
UDA	y=1.2435x+3.9942	0.9985	1—100	30
CA	y=1.2464x+3.7935	0.9971	1—1000	30
CIN	y=1.3628x+3.8855	0.9975	1—100	40
REC	y=1.366x+4.2843	0.9954	1—100	40
Rb1	y=1.4638x+4.0478	0.9951	0.5—80	20

a) y and x, respectively, denote the logarithmic value of content and peak area.

Table 3. Precision, Repeatability and Accuracy Data of the Proposed Method

Peak No.	Compounds	Precision				Stability (n=5)		Accuracy[a] (n=5)	
		Intra-day (n=5)		Inter-day (n=4)					
		Mean (μg/ml)	RSD (%)	Mean (μg/ml)	RSD (%)	Mean (μg/ml)	RSD (%)	Mean	RSD (%)
1	Rb1	24.48	5.01	25.53	4.09	34.01	2.05	95.72	3.09
2	CA	44.43	1.79	42.40	2.51	72.52	2.98	97.50	2.42
3	UDA	25.17	2.49	25.72	2.63	31.01	2.55	102.73	5.02
4	CIN	20.17	2.05	19.21	2.36	21.62	3.03	96.01	4.92
5	REC	20.84	1.53	19.97	1.74	8.12	2.84	95.68	2.14
6	CDA	8.13	2.20	7.76	3.02	8.90	2.84	99.18	1.39
7	DCA	19.47	3.18	18.97	4.65	13.70	1.67	104.20	4.03

a) Accuracy (%)=[1−(mean concentration measured−concentration spiked)/concentration spiked]×100.

Table 4. Quantitative Analysis Data of Seven Components in Various SBP Samples (μg/ml)

Components	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	RSD (%)
Rb1	31.45	34.06	19.57	33.19	30.62	29.65	24.83	30.01	25.81	16.0
CA	63.61	73.92	84.09	95.06	91.17	79.92	61.64	90.08	84.93	14.8
UDA	29.50	31.31	23.59	35.52	32.66	30.49	22.84	33.28	28.75	14.2
CIN	19.96	22.49	41.53	25.03	23.68	19.33	14.94	23.06	23.46	31.0
REC	7.141	8.412	9.373	12.64	12.42	10.23	8.863	11.55	10.50	18.4
CDA	7.145	8.903	9.629	10.48	9.079	3.506	6.330	11.55	9.236	28.7
DCA	11.39	13.90	12.41	19.25	17.29	15.96	11.22	17.18	16.55	19.2

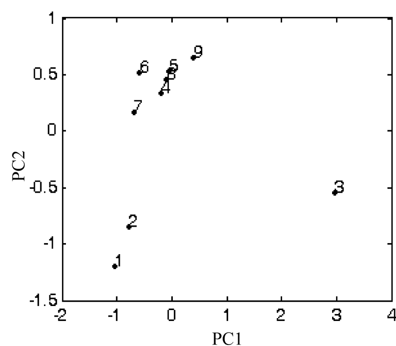

Fig. 4. Representation of the Quantitative Data of Nine Samples on PC1 and PC2

From the above analysis, it concludes that although the integral qualities of various SBP products are similar in general, the content of some major constituents might be rather different. The approach of similarity measurement is commonly used in quality evaluation, but in some cases it is not enough to reveal the quality characteristics in detail especially for these products having very similar qualities, and under this circumstance, quantitative analysis is necessary.

Conclusions

The proposed method allows obtaining chemical fingerprint and quantitative data of multiple constituents of TCM in one run. The method has been applied to develop fingerprints of various SBP samples and to simultaneously quantify their seven non-volatile components for quality control purpose. The results demonstrate that both chemical fingerprint and quantitative analysis are necessary for the quality evaluation of TCM products especially those having very similar qualities, and the proposed method could thus be readily utilized as an approach for the comprehensive quality control of TCM.

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