J Pharm Anal Vol 1, No 1, 20 - 25 (2011)

ORIGINAL ARTICLES

Quantification of six bioactive compounds in Zhenqi Fuzheng preparation by high-performance liquid chromatography coupled with diode array detector and evaporative light scattering detector

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Abstract: A simple and accurate high-performance liquid chromatography (HPLC) coupled with diode array detector (DAD) and evaporative light scattering detector (ELSD) was established for the determination of six bioactive compounds in Zhenqi Fuzheng preparation (ZFP). The monitoring wavelengths were 254, 275 and 328 nm. Under the optimum conditions, good separation was achieved, and the assay was fully validated in respect of precision, repeatability and accuracy. The proposed method was successfully applied to quantify the six ingredients in 31 batches of ZFP samples and evaluate the variation by hierarchical cluster analysis (HCA), which demonstrated significant variations on the content of these compounds in the samples from different manufacturers with different preparation procedures. The developed HPLC method can be used as a valid analytical method to evaluate the intrinsic quality of this preparation.

Keywords: high-performance liquid chromatography (HPLC); diode array detector (DAD); evaporative light scattering detector (ELSD); Zhenqi Fuzheng preparation; quantification; hierarchical cluster analysis

1 Introduction

Traditional Chinese medicines (TCMs), used for thousands of years in China, have played an important role in clinical therapy[1,2]. Therefor, to ensure the clinical efficacy and safety, overall quality control of TCMs is of significant importance[3]. However, most of the published literature has reported the qualitation or quantification of one or two types of components from only one comprising herb, and it could not be responsible for the overall quality control of the composite formulae[4]. Therefore, it seems necessary to determine the marker compounds of Chinese medicine preparation (CMP)[5].

Zhenqi Fuzheng preparation (ZFP), composed of Radix Astragali (Huangqi in Chinese) and Fructus Ligustri Lucidi (Nüzhenzi in Chinese), which has been listed in "Ministry of Health Drug Standards" of Chinese medicine formulated prescription, is commonly used in clinical practice for the treatment to improve immunity, protect bone marrow and adrenal cortex, increase leukocytes, promote the recovery of normal functions as an accessory of surgical operation, radiotherapy or chemotherapy, and it could also be used as an adjuvant therapy of cancer[6-10].

It is proved that the biologically active components in Huangqi, Nüzhenzi and ZFP include isoflavonoids, triterpene sapogenins, glycosides, saponin and phenolic acids[11-14]. Therefore, developing a simple and sensitive method to determine these ingredients becomes essential for quality control of this preparation. Although many analytical methods have been reported for the qualitative evaluation of the crude drug of Huangqi or Nüzhenzi, or their combined prescriptions, including high-performance liquid chromatography (HPLC)-ultraviolet (UV) or diode array detector (DAD)[15-17], thin layer chromatography scanning (TLCS)[18], chemiluminescence-mass spectrometry (LC-MS)[19-22], and HPLC-evaporative light scattering detector (ELSD)[23], there is no report on simultaneous determination of the six components as yet.

In the present study, we developed the HPLC-DAD-ELSD method to determine six components of ZFP, including adenosine (1), rhodioside (2), chlorogenic acid (3), calycosin (4), fermononetin (5), and astragaloside IV (6), as shown in Figure 1. This approach is potentially ideal for routine analysis and quality evaluation of ZFP. In order to improve the sensitivity and selectivity of the determination, DAD at three different wavelengths was employed for the quantitative analysis of strong UV absorbing compounds such as phenolic acids and isoflavones due to their different UV characteristics, while ELSD was used to determine none or poor UV absorbing compounds such as saponins.

2 Experimental

2.1 Instruments

HPLC analysis was carried out with a Waters Liquid Chromatography (Alliance, USA), 2996 diode array detector

Received 26 July 2010; Accepted 19 December 2010
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and evaporative light scattering detector (Alltech, USA), a Waters 717 automatic injector and M[32] ADD-ON single system, using a Hypersil ODS column (4.6 mm × 250 mm, 5 μm, Dalian Elite Analytical Instruments Co., Ltd., Dalian, China).

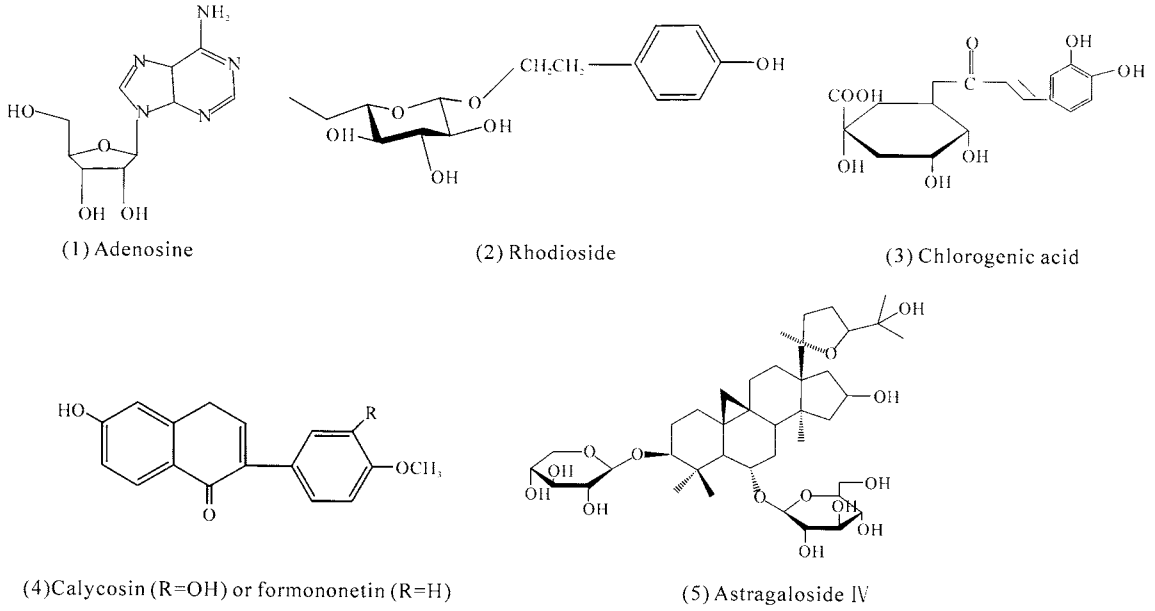

Figure 1 Structures of six standard substances

2.2 Chromatographic conditions

The mobile phase consisted of methanol (A) and water (B) in a gradient elution mode as follows: 0 – 10 min, A : B (10 : 90, v/v); 10 – 30 min, linear gradient from A : B (10 : 90, v/v) to A : B (40 : 60, v/v); 30 – 65 min, linear gradient to A : B (60 : 40, v/v); 65 – 90 min, the linear gradient to A : B (100 : 0, v/v). The column temperature was maintained at 30 ℃ and the flow rate was 1.0 mL/min. An aliquot of 10 μL of each sample was injected. Detection wavelengths were set at 254 nm for adenosine, calycosin, and fermononetin, 275 nm for rhodioside, and 328 nm for chlorogenic acid. The drift tube temperature for ELSD and carrier gas flow rate were 115 ℃ and 3.0L/min, respectively.

2.3 Chemicals and samples

Compounds (4) (99.83% purity) and (5) (99.37% purity) were supplied by Shanghai Institute of Chinese Materia Medica (Shanghai, China), and the other standard substances (1, 2, 3, and 6) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol (HPLC grade) was provided by Yuwang Chemical Co., Ltd. (Shandong, China). All solutions were prepared with double-deionized water. Other reagents were all of analytical grade. Thirty-one batches of ZFP were obtained from different pharmaceutical companies in China.

2.4 Preparation of sample solutions and negative control (NC) solutions

ZFPs were triturated in the particle size of 40 – 60 mesh. The accurately weighed powder (2.0000 g) was extracted three times with aliquots of 30 mL methanol by sonication (400 W, 30 min). Then the pooled filtrate was concentrated to dryness, and the residue was dissolved in 5 mL methanol. The methanol solution was filtered with a 0.45 μm membrane prior to HPLC analysis. According to the prescription and preparation protocol of ZFP recorded in Chinese Ministry of Health Drug Standards, two kinds of NC samples in which the formula contained no Nüzhenzi (B1) and Huangqi (B2), respectively, were prepared to validate the specificity of the method. The NC sample solutions were prepared using the same method as the sample solutions.

2.5 Standard calibration curves

Standard stock solutions of the compounds 1 – 6 were prepared by dissolving precisely weighted portions of the standards in methanol to give the concentrations of 0.322, 0.422, 0.502, 0.526, 0.182, and 1.695 mg/mL, respectively. The stock solutions were further diluted with methanol to get 9 different concentrations (equivalent to 1/20, 1/10, 3/20, 1/5, 1/4, 3/10, 7/20, 2/5, and 1/2 of the original concentration). Subsequently, an aliquot of 10 μL of the resulting solutions was injected into HPLC for analysis.

3 Results and discussion

3.1 Development of extraction method

To obtain quantitative extraction, the main experiment factors including extraction solvent (methanol and ethanol), extraction method (Soxhlet extraction and ultrasonic extraction) and extraction time (15, 30, and 45 min) were investigated, and the optimal conditions are presented in detail in Section 2.4.

3.2 Optimization of chromatographic conditions

To optimize the chromatographic conditions, the different stationary phases, such as Kromasil ODS column (5 μm, 4.6 mm × 25 mm) and Hypersil ODS-1 column (5 μm, 4.6 mm × 25 mm), and different mobile phases, such as methanol : water and acetonitrile : water, were attempted during the experiment. As a result, a Kromasil ODS column and the mobile phase of methanol : water were chosen to obtain the desired separation and acceptable tailing factor within the running time of 90 min.

In our previous work (data not shown), these bioactive components have slightly different UV absorption properties, and it is difficult to simultaneously determine them by common analytical approaches. Therefore, three detection wavelengths at 254, 275, and 328 nm were used to record chromatograms for the different structural types of components, and to provide an optimum S/N for simultaneously quantitative analysis. The operating conditions for ELSD were optimized according to the data computed with the ELSD software: The gas flow rate was set at 3.0 L/min, and the drift tube temperature was determined to be 115 ℃. Chromatograms of ZFP under such detection conditions are shown in Figure 2 and Figure 3.

Based on the selected detection mode, all these six compounds had acceptable limits of detection (LOD) and quantification (LOQ) (Table 1). The identity of each peak detected in ZFP samples was confirmed by comparison of retention time, UV spectrum of each peak with those of reference compound and blank samples. In addition, spiking samples with the reference compounds further confirmed the identities of the peaks.

3.3 Validation of the method

3.3.1 Linearity, LOD and LOQ

The construction of calibration curves was carried out according to the section 2.5. For the components by UV detection, their regression equations were expressed by the formula $Y = AX + B$, while by ELSD detection, their regression equations could be described as $Y = aX^b$; so, the calibration curves should be obtained in double logarithmic coordinates[11]. As shown in Table 1, a good linearity ($r > 0.999$) was observed in a relatively wide concentration range.

Working solution was diluted with methanol to get a series of appropriate concentrations, and the LOD and LOQ under the chromatographic conditions were separately determined at the signal-to-noise ratios (S/N) of 3 and 10. LOD and LOQ for each compound are also listed in Table 1.

3.3.2 Precision, repeatability, and accuracy

The precision of this newly developed method was studied by determining intra-day and inter-day variations. The intra-day variation was determined by analyzing the same standard methanol solution in triplicate for three times within one day. While for inter-day variation test, the solution was examined in triplicate for consecutive three days. To estimate the repeatability, five different working solutions, which were prepared from the same sample, were analyzed. As indicated in Table 2, the overall RSDs of the precision test and the repeatability test were lower than 2.63%, which demonstrates good precision and repeatability of the method.

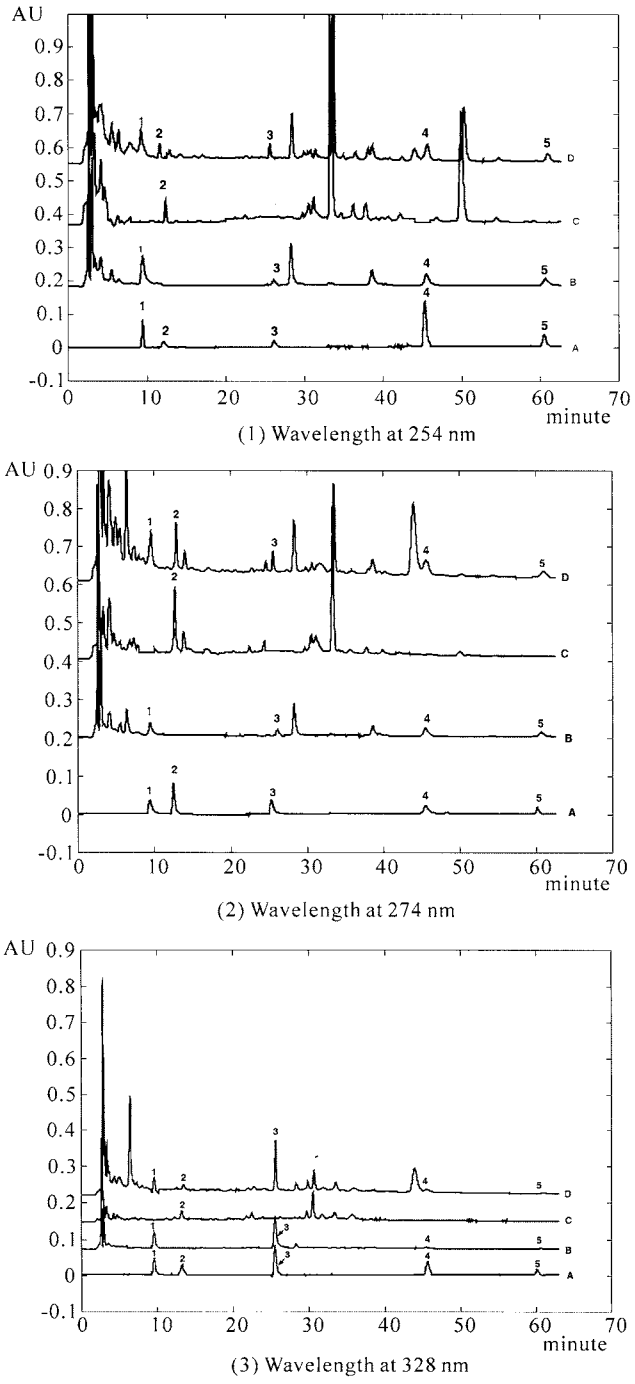

Figure 2 The typical HPLC-DAD chromatographic profiles. A, B, C, and D stand for standard solution, ZFP in absence of Nüzhenzi, ZFP in absence of Huangqi, and ZFP in sequence. Peaks 1, 2, 3, 4, and 5 represent adenosine, rhodioside, chlorogenic acid, calycosin, and fermononetin, respectively. Blank sample analysis confirms that no impurities were co-eluted with the compounds determined.

Table 1 Regression data, LODs and LOQs of six analytes by HPLC-DAD and HPLC-ELSD

Analyte	Calibration curve[a]	r	Linear range (μg)	LOD (ng)	LOQ (ng)
Adenosine	$Y=8.18\times10^4\ X-5.79\times10^4$	0.9999	0.161 - 1.126	10.6	15.1
Rhodioside	$Y=6.08\times10^4\ X-1.11\times10^4$	0.9999	0.211 - 1.266	5.3	8.7
Chlorogenic acid	$Y=7.18\times10^4\ X-1.48\times10^5$	0.9992	0.251 - 1.506	2.9	4.2
Calycosin	$Y=3.24\times10^5\ X-1.03\times10^6$	0.9993	0.263 - 1.578	2.1	3.9
Fermononetin	$Y=9.67\times10^4 X-3.67\times10^5$	0.9990	0.182 - 0.546	2.5	4.2
Astragaloside IV	$A=1.35\ B+3.91$	0.9997	0.848 - 5.085	96	128

[a] Y is the peak area in UV chromatograms monitored at detection wavelengths, X is the compound amount injected, and A, B are the logarithmic values of area and amount injected in ELSD chromatograms.

Table 2 Precision and repeatability of the six analytes

Analyte	Precision				Repeatability ($n=5$)	
	Intra-day ($n=3$)		Inter-day ($n=3$)			
	Mean (mg/g)	RSD (%)	Mean (mg/g)	RSD (%)	Mean (mg/mL)	RSD (%)
Adenosine	0.3156	1.36	0.3157	1.82	0.0437	2.32
Rhodioside	0.4303	1.32	0.4298	2.41	0.4783	2.58
Chlorogenic acid	0.4970	1.35	0.4975	1.57	0.0571	1.85
Calycosin	0.5207	1.10	0.5309	2.36	0.3503	2.63
Fermononetin	0.1784	1.78	0.1798	2.13	0.2809	2.27
Astragaloside IV	1.7201	0.95	1.6778	1.18	0.6450	1.54

The overall RSD of the intra- and inter-day is in the range of 0.95% - 1.36% and 1.18% - 2.41%, respectively. And the RSD of repeatability test is <3%.

The accuracy of the method was evaluated by spiking known amounts of standard samples into a ZFP sample and comparing the amount determined of these standards with the amount originally added. And as shown in Table 3, the overall RSD of the recovery of ZFP was lower than 3% for all these analyses. The recovery obtained was in the range of 95.4% - 103.5%, indicating that the developed method was sufficiently accurate for determining the six bioactive components in ZFP, and analysis of NC samples confirmed that no impurities were co-eluted with the compounds determined (Figure 2 and Figure 3).

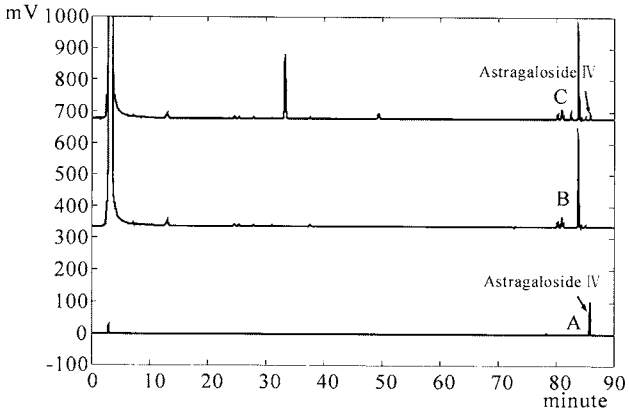

Figure 3 The typical HPLC-ELSD chromatographic profiles. A, B, and C stand for standard solution, ZFP in absence of Nüzhenzi, and ZFP in sequence. NC sample analysis confirms that no impurities were co-eluted with the compounds determined.

3.4 Analysis of samples

The developed method was subsequently applied to simultaneous determination of the six active compounds in 31 commercial ZFP samples from different pharmaceutical companies in China. All the contents of six ingredients in 31 batches of ZFP samples are summarized in Table 4. The results showed that the quality of this preparation was relatively uniform and demonstrated that the chemical variation was obvious among the different companies and/or preparations of ZFP.

Table 3 Recovery of the six analytes

Analyte	Original mean (mg)	Spiked mean (mg)	Found mean (mg)	Recovery mean (%)	RSD (%) ($n=3$)
Adenosine	0.0874	0.0866	0.1726	98.4	2.95
Rhodioside	0.9566	0.9065	1.8738	101.2	1.76
Chlorogenic acid	0.1142	0.0902	0.2024	98.3	2.82
Calycosin	0.7006	0.6882	1.3930	100.6	1.56
Fermononetin	0.5618	0.5534	1.0896	95.4	2.93
Astragaloside IV	1.2825	1.2794	2.6067	103.5	2.79

Table 3 shows the accuracy of repeatability test with RSD <3%.
Recovery mean (%) = [(Found mean-Original mean)/Spiked mean] × 100

The results showed that the content of astragaloside IV (6), a characteristic and active constituent whose presence forms part of the quality assurance of ZFP, is relative high in samples compared with the other target compositions. The contents of the compounds were found lower in granule, and higher in capsule, except compound 2. Therefore, the analytical results obtained also indicated that a multiple-component assay might be a rational strategy to elucidate the synergic effects and much more comprehensive control for the quality of this preparation.

3.5 Comparison of different batches of ZFP

To evaluate the variation of ZFP, hierarchical cluster analysis (HCA) was performed based on the investigated components' characteristics from HPLC profiles of 31 test samples. The dendrogram of HCA is shown in Figure 4, and the quality characteristics are revealed more clearly. Supposing an appropriate distance level (Level I) is chosen,

the samples can be classified into seven quality groups, thereby indicating that preparation procedure is related to the quality of products. If a higher distance level (Level Ⅱ) is adopted, samples 1 – 19 tend to be classified into the same group, which indicates that the samples from the same manufacturer are more similar, and then the samples can be divided into five quality groups, among which there is an abnormal sample (No.28). Thus, HCA is an effective way to discover the abnormal sample rapidly.

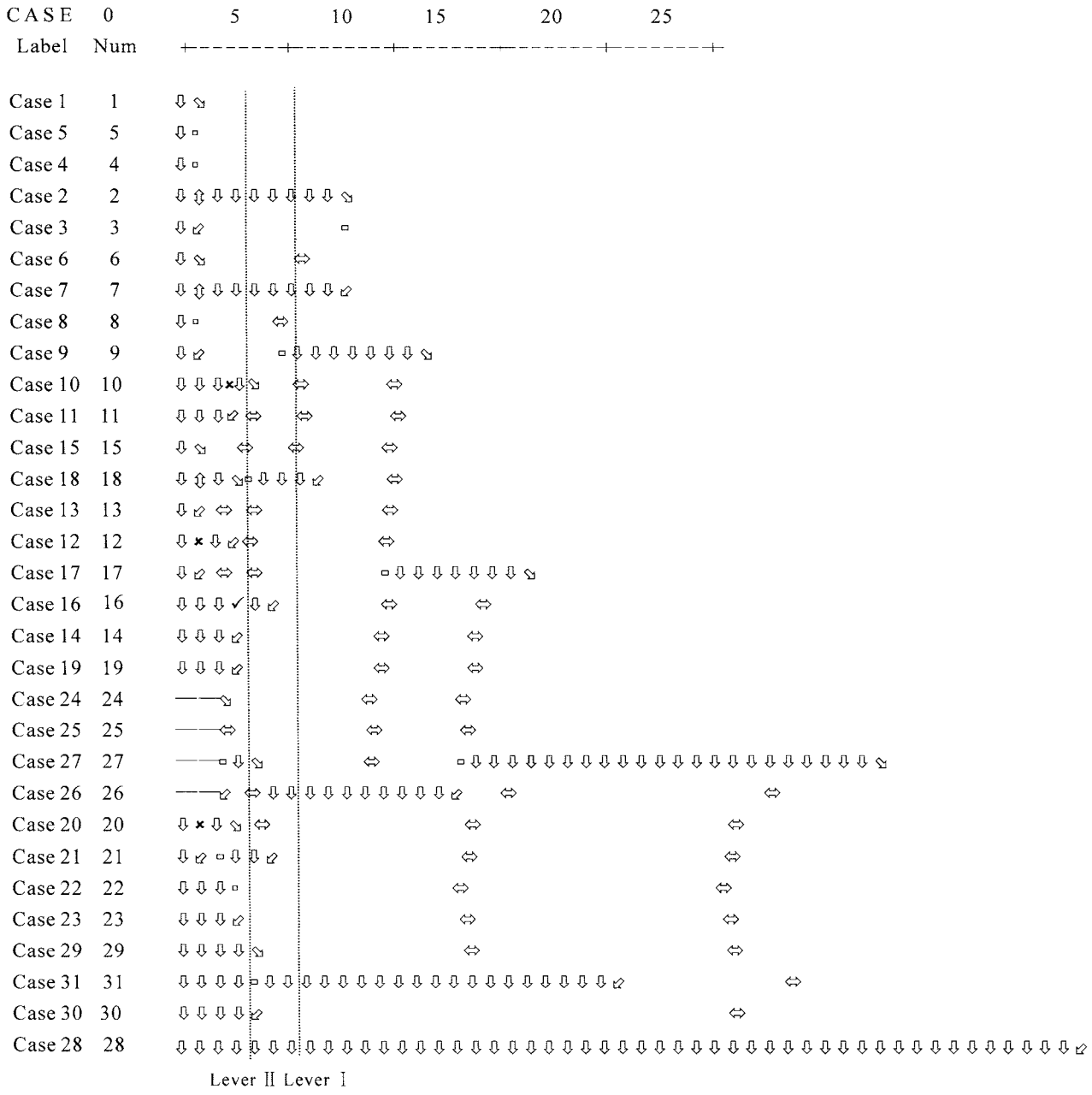

Figure 4 The cluster analysis of chromatograms of 31 ZFP samples. Level Ⅰ was used to distinguish different dosage forms of preparation. Level Ⅱ was used to distinguish different manufacturers of samples 1 – 19. An abnormal sample (No.28) can be discovered obviously.

4 Conclusion

We reported a simple and sensitive HPLC method coupled with DAD and ELSD, and these two detectors are complementary. The proposed HPLC-DAD-ELSD method makes it possible to evaluate the quality of ZFP through a determination of multi-components with different structures. This method has been applied successfully to quantify six bioactive compounds in 31 different batches of ZFP samples. The results prove that the present method is suitable for routine quantitative analysis and intrinsic quality control of ZFP.

Table 4 Content of six compounds in 31 batches of ZFP samples (mg/g)

Sample No.	Adenosine	Rhodioside	Rhlorogenic acid	Calycosin	Fermononetin	Astragaloside Ⅳ
1	0.0437	0.4783	0.0571	0.3503	0.2809	0.6450
2	0.0400	0.3953	0.0554	0.3394	0.3214	0.6384
3	0.0500	0.4082	0.0502	0.3252	0.2484	0.6294
4	0.0474	0.4217	0.0576	0.3701	0.3120	0.6755
5	0.0450	0.4323	0.0539	0.3459	0.2718	0.6602
6	0.0265	0.4772	0.0545	0.3189	0.2749	0.6625
7	0.0283	0.4533	0.0595	0.3070	0.2650	0.6767
8	0.0294	0.4773	0.0640	0.3265	0.2000	0.6416
9	0.0268	0.3935	0.0633	0.3291	0.2380	0.6606
10	0.0318	0.6395	0.0349	0.2601	0.3006	0.6275
11	0.0310	0.7387	0.0344	0.2599	0.2113	0.6780
12	0.0234	0.4137	0.0348	0.2347	0.2188	0.6167
13	0.0297	0.3600	0.0219	0.2650	0.2165	0.6405
14	0.0294	0.1648	0.0214	0.1956	0.2547	0.6306
15	0.0352	0.2885	0.0302	0.2890	0.2540	0.6620
16	0.0435	0.3852	0.0339	0.2271	0.2539	0.6029
17	0.0344	0.2711	0.0353	0.1958	0.1999	0.6346
18	0.0300	0.3191	0.0306	0.2677	0.2368	0.6865
19	0.0387	0.3022	0.0255	0.2170	0.1933	0.7406
20	0.0450	0.2715	0.0332	0.3341	0.3489	0.8776
21	0.0447	0.1678	0.0370	0.3594	0.3286	0.8484
22	0.0266	0.1693	0.0343	0.3673	0.2986	0.8890
23	0.0254	0.1897	0.0383	0.3442	0.3721	0.9180
24	0.0052	0.3154	0.0616	0.3978	0.3115	0.7820
25	0.0052	0.2927	0.0598	0.3679	0.3039	0.7685
26	0.0050	0.3540	0.0414	0.3665	0.3278	0.7969
27	0.0010	0.3381	0.0625	0.3491	0.3073	0.8012
28	0.0088	1.4742	0.0883	0.3216	0.2571	0.6030
29	0.0101	0.0747	0.0226	0.2171	0.1247	0.7024
30	0.0153	0.1576	0.0122	0.1544	0.1291	0.5575
31	0.0133	0.3806	0.0217	0.2045	0.1671	0.6367

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