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Simultaneous determination of nine components in *Qingkailing* injection by HPLC/ELSD/DAD and its application to the quality control

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Abstract

High-performance liquid chromatography coupled with photo diode array detection and evaporative light scattering detection (HPLC/DAD/ELSD) was established to simultaneously determine nine ingredients in *Qingkailing* injection. Four wavelengths at 240, 254, 280 and 330 nm, respectively, were chosen as the monitoring wavelength to determine two nucleosides (uridine and adenosine), geniposide, baicalin and two organic acids (chlorogenic acid and caffeic acid), and an evaporative light scattering detector combined was employed to determine three steroids (cholic acid, ursodeoxycholic acid and hyodeoxycholic acid). This assay was fully validated in respect to precision, repeatability and accuracy. The proposed method was successfully applied to quantify the nine ingredients in 19 different *Qingkailing* injection samples and by principal component analysis (PCA) and hierarchical clustering analysis (HCA), it demonstrated significant variations in the content of these compounds in the samples from different manufacturers and preparation procedures. This method could be readily utilized as a quality control method for traditional Chinese medicine (TCM).

Keywords: *Qingkailing* injection; Evaporative light scattering detection; Quality control; Traditional Chinese medicine

1. Introduction

Qingkailing injection, a well-known composite formula of traditional Chinese medicine (TCM), is commonly used in clinical practice for the treatment of upper respiratory inflammation, viral encephalitis, hepatitis, stoke, cerebal thrombosis, tonsillitis, tracheitis and high fever [1–4]. It comprises eight medicinal materials or extracts thereof, including *Flos lonicerae*, *Fructus gardeniae*, *Radix isatidis*, *Cornu bubali*, *Concha margaritifera*, *Baicalin*, *cholic acid* and *hypodeoxycholic acid* [5]. The combination of these medicinal materials may have several beneficial effects including fewer and less severe side effects and better efficacy through synergistic interaction. However, on the other hand, it enhances the complexity of the constituents and preparation procedures, which makes it difficult to ensure the batch-to-batch uniformity of *Qingkailing* injection. In the practice of quality control, it might be acceptable to quantify some bioactive integrants for a single herbal medicine without complex preparation procedures, whereas for a composite formula of *Qingkailing* injection, it seems necessary to determine not only all available bioactive integrants, but also the marker compounds derived from various medicinal materials to ensure the uniformity of their extraction procedures. From the published literatures [5–8], several components, such as uridine (URI), adenosine (ADE), geniposide (GEN), chlorogenic acid (CHA), caffeic acid (CAA), baicalin (BAI), ursodeoxycholic acid (UCA), cholic acid (CA) and hyodeoxycholic acid (HCA), have been reported to be the biologically active components contributing to the therapeutic effects of *Qingkailing* injection (see Fig. 1 for their chemical structures). Moreover, these components are derived from six different medical materials and are often considered as their marker compounds. Therefore, it is significant to determine the nine components to ensure the quality of *Qingkailing* injection.

These bioactive components belong to five different structural types, namely nucleosides (URI and ADE), iridoid glycoside (GEN), organic acids (CHA and CAA), flavone glycoside (BAI), steroids (CA, HCA and UCA), which have rather different UV absorption properties, and as a result, it is diffi-

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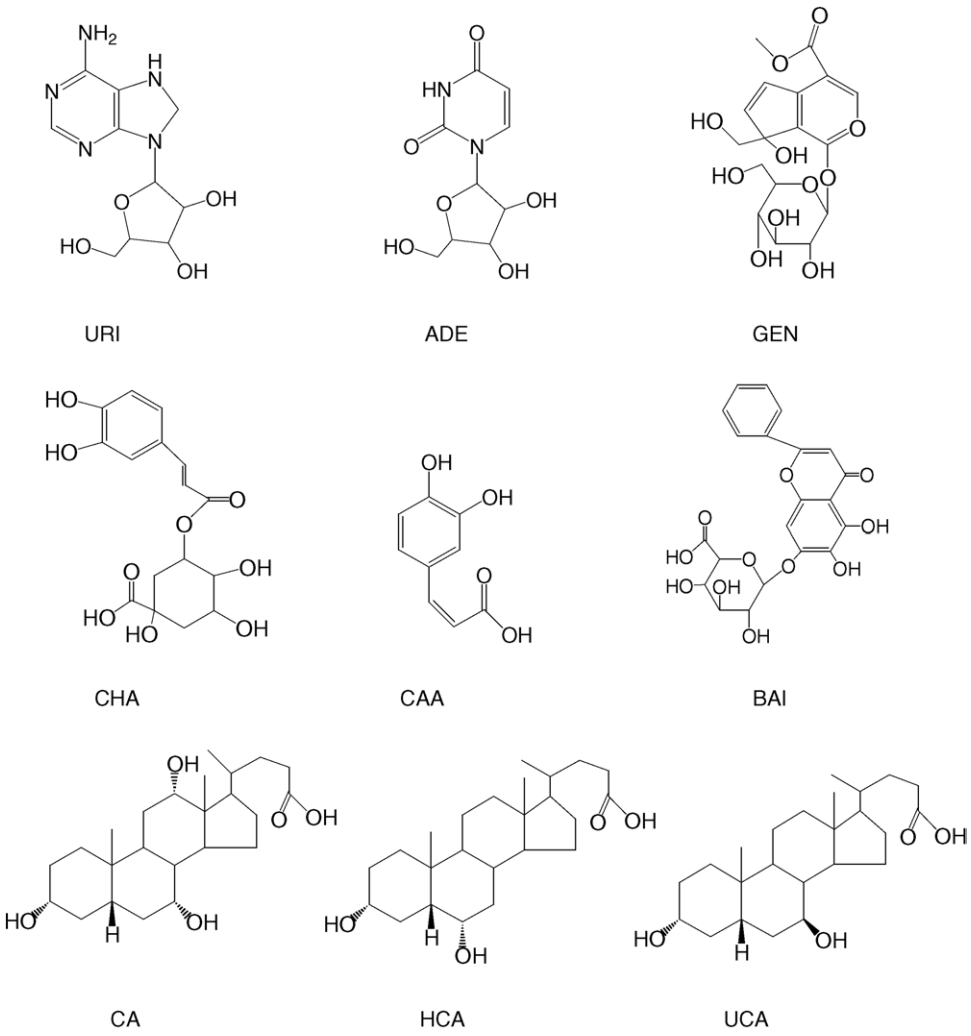

Fig. 1. Chemical structures of nine bioactive compounds determined.

cult to simultaneously determine them by common analytical method. Cao et al. [9] established an approach of HPLC with multi-wavelength UV detection and realized the simultaneous quantification of BAI, GEN and CHA in *Qingkailing* injection, but the components of steroids cannot be determined since their poor UV absorptions. In recent years, evaporative light scattering detector (ELSD) has been increasingly used as an efficient tool to determine the non-chromophoric compounds of TCM [10,11]. In the former studies of our group, ELSD was successfully applied to determine steroid compounds in *Qingkailing* injection [12], and it was further employed to analyze all available bioactive components for quality control purpose [13]. However, the results demonstrated that in comparison with UV detection, ELSD has poor sensitivity and some important bioactive components of low contents, such as URI and CAA, cannot be detected. Therefore, there is a need to develop new approaches for determining all the five structural types of bioactive components of *Qingkailing* injection to ensure its batch-to-batch uniformity.

In this study, an approach of high-performance liquid chromatography coupled with photo diode array detection and evaporative light scattering detection (HPLC/DAD/ELSD) was proposed as an alternative solution, which made it possible to determine five different structural types of components in one run. The proposed method could be readily utilized as a quality control method for traditional Chinese medicine.

2. Experimental

2.1. Instrumentation

The HPLC analysis was performed using a Dionex P580 liquid chromatograph (Dionex Inc., Germany) equipped with a photo diode array UV detector (USD340S), an intelligent quaternary pump, a column oven, a manual injection system with a 20 μL loop, and an Alltech ELSD 2000 detector (USA). DAD and ELSD were connected to the column by a *Y* connector.

2.2. Chemicals

The standards of BAI, GEN, ADE, URI, CAA, CHA and UCA were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China); CA and HCA were purchased from Sigma (St. Louis, MO, USA).

HPLC grade methanol (CH_3OH) and acetonitrile (CH_3CN) were purchased from Tedia Company Inc. (OH, USA). Ultrapure water was prepared from Milli-Q purification system (Millipore Corp., France). Other reagents were of analytical grade.

2.3. Standard solutions

Stock solution of the mixture of nine standards was prepared by dissolving accurately weighted portions of the standards in methanol, transferring it to a 25 mL volumetric flask, and then adding methanol to make up the volume. The stock solution was further diluted to make working solutions. The solutions were brought to room temperature and filtered through a 0.45 μm membrane filter before HPLC analysis.

2.4. Sample solutions

Nineteen *Qingkailing* injection samples (marked as 1–19) were collected from three Chinese medicine manufacturers, manufactures A–C, whose actual names had been removed in order to preserve confidentiality. Samples 1–10, 11–15 and 16–19 were produced from manufacturers A–C, respectively. Two different preparation procedures were applied on these samples: samples 1–4 produced by initial procedures and the others by adjusted procedures. All the samples were diluted to 1/10 (v/v) with ultrapure water and then filtered through a 0.45 μm membrane filter for HPLC analysis. Blank sample was presented by manufacturer A, which was prepared in the absence of two raw materials of *cholic acid* and *hyodeoxycholic acid*.

2.5. Chromatography

The column of C_{18} RP-ODS (250 mm × 4.6 mm, 5 μm, Phenomenex Luna, USA) and another C_{18} guard column (7.5 mm × 4.6 mm, 5 μm, Alltech, IL) were used. The mobile phases were composed of water/formic acid (100/0.1, A) and methanol/acetonitrile (4/1, B). The gradient was as follows: 0 min-100% A; 33 min-34% A; 60 min-12% A. Elution was performed at a solvent flow rate of 1 mL/min. The column compartment was kept at the temperature of 35 °C.

For UV detection, four detection wavelengths of 240, 254, 280 and 330 nm were chosen simultaneously to record chromatograms, and for ELSD detection, carrier gas was nitrogen (99.999%), the drift tube temperature was set at 110 °C, and the gas flow rate was 1.5 L/min.

2.6. Calibration curves

The calibration curves were constructed by analyzing at least six different concentrations of standard solutions. For the components by UV detection, their regression equations were calculated in the form of $Y = A \times X + B$, where Y and X was peak area and sample amount, 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 [14].

2.7. Method validation

2.7.1. Linearity and limit of detection (LOD)

The linearity study was carried out by preparing calibration curves described in Section 2.6. Aliquots of standard solutions, ranging from 1 to 100 ng/mL for each component, were analyzed to obtain LOD values, which was determined when the signal-to-noise ratio of the testing peak of analyte was greater than 5.

2.7.2. Precision, repeatability and accuracy

The intra- and inter-day precision were determined by analyzing calibration samples during a single day and on 3 different days, respectively. To confirm the repeatability, five different working solutions prepared from the same sample were analyzed. The accuracy tests were carried out by spiking known contents of standard samples into a *Qingkailing* injection sample and comparing the determined amount of these standards with the amount originally added. The relative standard deviation (R.S.D.) was taken as a measure of precision, repeatability and accuracy.

3. Results and discussion

3.1. Optimum conditions of detection

Maximally efficient detection can be obtained by selecting the wavelength, where the component has the maximum absorption. Table 1 shows various UV maximal absorption of each component. In this study, four detection wavelengths of 240, 254, 280 and 330 nm were chosen to record chromatograms for four dif-

Table 1
UV maximal absorptions of five types of components

No.	Components	Retention time (min)	Structural types	Wavelength of maximal absorption (nm)
1	URI	8.75	Nucleoside	254
2	ADE	9.10		
3	CHA	20.47	Organic acid	330
4	CAA	21.41		
5	Gen	21.68	Iridoid glycoside	240
6	BAI	34.58	Flavone glycoside	280
7	UCA	51.97		
8	CA	53.05	Steroid	No UV absorptions
9	HCA	54.12		

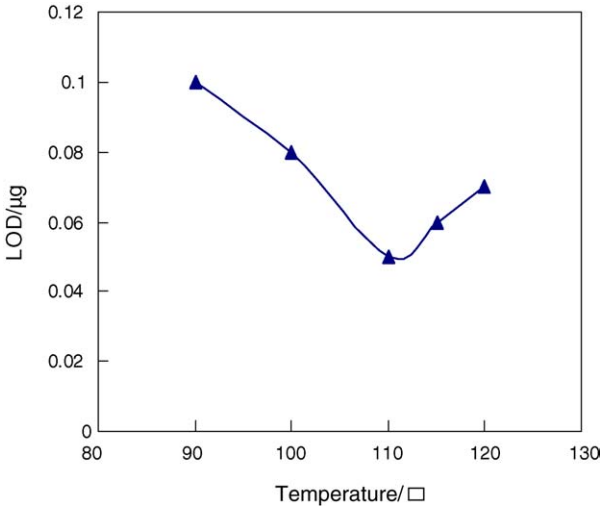

Fig. 2. Plot of LOD of CA at various temperatures.

ferent structural types of components, and ELSD was used to determine three compounds of steroid.

Under fixed chromatographic conditions, nebulizing gas flow rate and evaporating temperature are the two major instrumental adjustments available for maximizing the detector response efficiency. We varied gas flow rate at 1.0, 1.5, 2.0, 2.5 and 3.0 L/min and found that the noise was decreased with the flow rate increased, but the responses would be weakened if the flow rate increased too much. In this study, a moderate flow rate of 2.0 L/min was adopted to achieve the best results. To get the optimal evaporator temperature, *Qingkailing* sample was analyzed at various evaporating temperatures of 90, 100, 110, 115 and 120 °C, and the LOD of CA was calculated. As shown in Fig. 2, 110 °C is the optimal temperature. HPLC/DAD/ELSD chromatograms of *Qingkailing* injection under such detection conditions are shown in Fig. 3.

3.2. Method validation

Chromatographic analysis of the mobile phase shows no interferences in the range of retention times. The purity tests of UV spectra (data not shown) and blank sample analysis confirm that no impurities co-eluted with the compounds determined (as shown in Fig. 4).

Table 2 lists linear equation and its correlation coefficient, linear range and LOD of each compound determined. As a result, good linearity with $R^2 > 0.99$ and LODs between 5 and 20 ng are achieved. Table 3 shows the results of the tests of precision, repeatability and accuracy. It indicates that most the R.S.D.s are less than 5%, and the method is thus acceptable.

3.3. Analysis of samples

The developed HPLC/DAD/ELSD analytical method was subsequently applied to simultaneously determine the nine ingredients in 19 *Qingkailing* injection samples with results shown in Table 4. The results indicate that the concentration of each component in different samples, especially in the samples

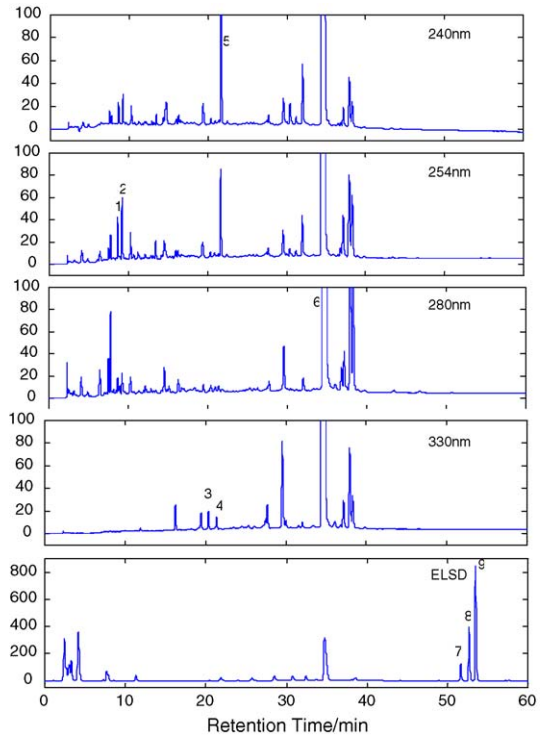

Fig. 3. Typical HPLC/DAD/ELSD chromatograms of *Qingkailing* injection. Peaks are determined by standard references according to their retention times, spectra and blank samples: (1) uridine (8.57 min); (2) adenosine (9.10 min); (3) chlorogenic acid (20.47 min); (4) caffeic acid (21.41 min); (5) geniposide (21.68 min); (6) baicalin (34.58 min); (7) ursodeoxycholic acid (51.97 min); (8) cholic acid (53.05 min); (9) hyodeoxycholic acid (54.12 min).

from different manufacturers, is significantly different. Among the nine compounds, BAI, CA and HCA are the most abundant. Table 4 also lists the R.S.D.s of the samples from the same manufacturer. It can be noted that the samples from manufacturers B and C have much lower R.S.D.s, which reveals that the batch-to-batch uniformity of these products are superior to those of manufacture A.

Moreover, among the nine bioactive components determined, BAI, UCA, CA and HCA have much lower R.S.D.s, probably due to the fact that in the preparation procedures of *Qingkailing* injection, these four components are derived from medicinal extracts with purity higher than 90% of *baicalin*, *cholic acid* and *hyodeoxycholic acid*, and accordingly, their concentrations can be easily controlled. The other three components of ADE, CHA and GEN, however, are directly derived from herbal materials of *Radix isatidis*, *Flos lonicerae* and *Fructus gardeniae*, respec-

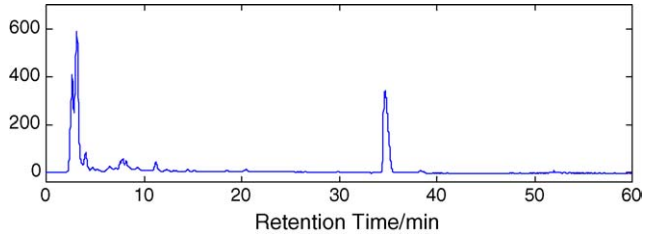

Fig. 4. ELSD chromatogram of blank sample without steroid components.

Table 2
Regression data and LODs for the components determined

Peak no.	Components	Monitoring wavelength (nm)	Prediction function[a]	Correlation factors	Linear range (μg)	Limit of detection (ng)
1	URI	254	$Y = 1820.9X + 3.1551$	0.9991	0.5–70	10
2	ADE	254	$Y = 324.41X + 1.0543$	0.9989	0.4–80	10
3	CHA	330	$Y = 518.2X - 0.1317$	0.9992	0.2–60	5
4	CAA	330	$Y = 1566.6X + 0.2331$	1.0000	0.1–40	5
5	GEN	240	$Y = 389.64X + 7.215$	0.9985	0.1–40	10
6	BAI	280	$Y = 522.11X - 95.003$	0.9976	0.2–1000	10
7	UCA	–	$y = 0.9641x + 6.8665$	0.9991	0.5–150	40
8	CA	–	$y = 0.8648x + 5.7615$	0.9980	1–2000	50
9	HCA	–	$y = 0.8987x + 5.7701$	0.9944	1–2000	50

[a] Y is the peak area, X the concentration, and y, x are the logarithmic values of area and concentration, respectively.

Table 3
R.S.D.s of the validation tests for the proposed HPLC/DAD/ELSD method

Peak no.	Compounds	Precision				Repeatability ($n = 5$)		Accuracy[a] ($n = 5$)	
		Intra-day ($n = 6$)		Inter-day ($n = 3$)					
		Mean (μg/mL)	R.S.D. (%)	Mean (μg/mL)	R.S.D. (%)	Mean (μg/mL)	R.S.D. (%)	Mean	R.S.D. (%)
1	URI	1.58	6.48	1.55	6.89	1.65	3.36	96.41 ± 3.63	3.37
2	ADE	4.24	2.30	4.20	2.16	4.18	4.23	102.02 ± 3.83	3.75
3	CHA	1.75	4.22	1.74	4.26	1.73	2.64	101.37 ± 4.70	4.64
4	CAA	0.42	2.53	0.39	4.09	0.41	3.74	98.54 ± 4.48	4.55
5	GEN	140	3.69	142	3.99	135	4.39	100.18 ± 2.70	2.70
6	BAI	2998	1.22	3005	3.29	2986	1.08	100.58 ± 4.88	4.85
7	UCA	24.3	1.27	24.1	3.44	24.5	3.03	98.81 ± 3.10	3.14
8	CA	2484	2.21	2497	1.90	2481	2.84	99.78 ± 2.66	2.67
9	HCA	2589	2.32	2576	2.12	2591	1.67	102.81 ± 3.79	3.69

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

Table 4
Quantitative analytical results of various *Qingkailing* injection samples

Samples manufacturer	URI (mg/mL)	ADE (μg/mL)	CHA (μg/mL)	CAA (μg/mL)	GEN (μg/mL)	BAI (mg/mL)	UCA (μg/mL)	CA (mg/mL)	HCA (mg/mL)
A									
1	2.24	9.33	2.20	0.317	152	2.98	25.8	2.10	2.64
2	1.67	4.20	1.56	0.421	145	3.02	26.5	2.50	2.60
3	1.45	4.25	1.09	0.221	137	1.53	27.0	2.38	2.50
4	0.898	3.47	0.993	0.116	141	1.47	28.1	2.27	2.62
5	6.23	27.4	3.11	1.56	37.5	3.16	16.8	1.26	2.96
6	7.70	36.9	4.58	2.49	51.0	3.09	16.1	1.20	3.22
7	6.14	20.0	0.95	0.37	45.0	3.05	14.0	1.22	3.20
8	4.99	16.7	2.97	1.78	47.2	3.14	14.3	1.23	3.15
9	5.76	24.8	4.58	2.41	61.3	3.14	16.2	1.34	3.14
10	5.62	18.1	1.08	0.33	45.4	3.18	16.6	1.37	3.12
R.S.D. (%)	57.30	68.19	62.10	95.11	57.96	24.34	29.15	32.58	9.96
B									
11	3.77	13.1	3.15	6.97	72.4	3.60	13.9	2.36	2.36
12	4.85	28.6	2.92	8.24	83.3	3.85	15.5	1.97	2.59
13	4.58	31.0	2.80	8.73	80.1	3.72	12.8	2.13	2.26
14	3.72	25.6	2.73	8.81	70.1	3.75	14.0	2.18	2.28
15	7.99	42.4	1.60	6.25	71.1	3.92	14.4	1.89	2.41
R.S.D. (%)	35.18	37.46	22.84	14.58	7.85	3.27	6.89	8.75	5.55
C									
16	9.82	69.1	5.93	3.78	94.1	4.61	22.8	1.89	2.31
17	13.0	56.1	7.63	3.84	92.5	4.99	22.5	1.88	2.32
18	15.0	45.2	7.33	4.98	87.5	4.71	20.1	1.96	2.22
19	13.3	58.3	7.22	4.82	93.1	4.39	20.1	1.91	2.10
R.S.D. (%)	16.91	17.14	10.70	14.54	3.20	5.32	6.91	1.86	4.56

tively, and there are much more factors in the complex extraction procedures affecting the efficiency of extraction which might lead to the variance of these components.

3.4. Quality assessment by PCA and HCA

In this study, principal component analysis (PCA) and hierarchical clustering analysis [15] were performed on the analytical data of all the 19 samples. By the method of PCA, the first two principal components PC1 and PC2 are often used to provide a convenient visual aid for identifying inhomogeneity in the data sets. Fig. 5 shows the principal component projection plot of PC1 and PC2 (over 80% variance explained) of 19 *Qingkailing* samples. From the scatter points, the samples could be classified into four groups (marked as I–IV). Although samples 1–10 are from the same manufacturer, they are divided into two separate groups (groups I and II) due to their different preparation procedures. Samples 1–4 were produced by initial procedures in which *Fructus gardenia* was extracted by ethanol, while an adjusted procedure was applied to produce samples 5–10 and the extraction solvent of *Fructus gardenia* was water. It thus indicates that preparation procedure is highly related to the quality of products. Samples 11–15 and samples 16–19 are from manufacturer B and manufacturer C, respectively, so, they are properly clustered into two separated groups (groups III and IV).

Fig. 6 is the dendrogram of HCA, from which the quality characteristics are revealed more clearly. Supposing an appropriate distance level (Level 1) chosen, the samples can also be classified into four quality groups, just as PCA does. If a higher distance level (Level II) is adopted, samples 11–19 tend to be classified into the same group, which indicates that the samples from manufacturer B and those from manufacturer C are more similar, and if we use Level III, all the samples will be divided into two groups according to their preparation procedures (samples of groups II–IV are all produced in the same procedures). Therefore, we can conclude that the difference of preparation procedure is the more important factor to influence the quality of *Qingkailing* injection.

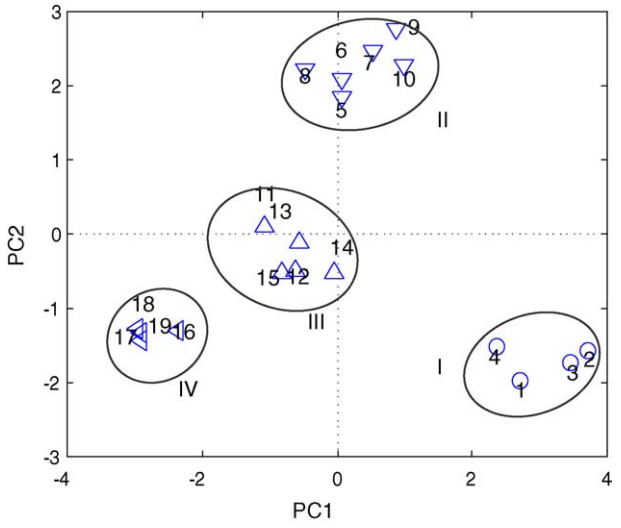

Fig. 5. Representation of integrated quality of various *Qingkailing* injections on PC1 and PC2 (80.2% variance explained): (I) samples of manufacturer A by initial procedures; (II) samples of manufacturer A by adjusted procedures; (III) samples of manufacturer B; (IV) samples of manufacturer C.

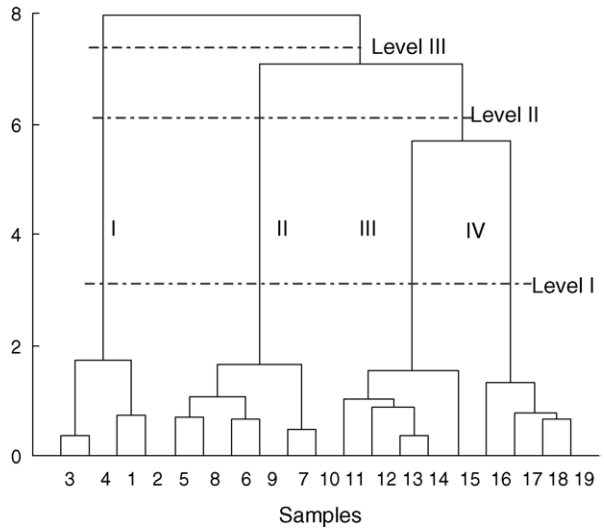

Fig. 6. Dendrogram for various *Qingkailing* Injections from Ward's clustering analysis of the quantitative data: (I) samples of manufacturer A by initial procedures; (II) samples of manufacturer A by adjusted procedures; (III) samples of manufacturer B; (IV) samples of manufacturer C.

4. Conclusions

The proposed method makes it possible to simultaneously determine different structural multi-components in one run with acceptable levels of linearity, precision, repeatability and accuracy. The method has been applied successfully to simultaneously quantify nine bioactive components in *Qingkailing* injection samples. The results demonstrate that the proposed method could be readily utilized as a quality control method for traditional Chinese medicine.

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