Simultaneous Determination of Major Bioactive Components in *Qingkailing* Injection by High-Performance Liquid Chromatography with Evaporative Light Scattering Detection

Shi-Kai YAN,[a] Wen-Feng XIN,[b,c] Guo-An LUO,[b] Yi-Ming WANG,*[,a,b] and Yi-Yu CHENG*[,a]

[a] *Pharmaceutical Informatics Institute, College of Pharmaceutical Sciences, Zhejiang University; Hangzhou, 310027, China:* [b] *Department of Chemistry, Tsinghua University; Beijing, 100084, China: and* [c] *Jiangxi University of Traditional Chinese Medicine; Nanchang, 330006, China.* Received April 27, 2005; accepted June 29, 2005

High-performance liquid chromatography with evaporative light scattering detection (HPLC/ELSD) was established for simultaneous determination of seven major bioactive components of *Qingkailing* injection including adenosine, geniposide, chlorogenic acid, baicalin, ursodeoxycholic acid, cholic acid, and hyodeoxycholic acid. The proposed method was applied to analyze ten various *Qingkailing* injections and produced data with acceptable linearity, repeatability, precision and accuracy having a limit of detection (LOD) of 10—50 ng. In comparison with UV detection, HPLC/ELSD permits the determination of non-chromophoric compounds without prior derivatization, and shows good compatibility to the multi-components of complex analytes. The proposed method is a useful alternative for routine analysis in the quality control of traditional Chinese medicine.

Key words HPLC; evaporative light scattering detection; simultaneous determination; traditional Chinese medicine; *Qingkailing* injection; quality control

The ever-increasing worldwide attention to the therapeutic or pharmaceutical use of traditional Chinese medicine (TCM) has made it absolutely essential to carry out stringent quality control measures.[1—5] So far, it is widely accepted that multiple constituents are responsible for the therapeutic effects of TCM, and to ensure its quality, therefore, it is necessary to quantitatively determine the multi-bioactive components of TCM.[6—8] Over the past decades, various techniques have been applied extensively in the detection of bioactive components of TCM, among which, UV detector is by far the most commonly employed. In quality control, however, it has at least two unavoidable limitations. Firstly, some bioactive components in TCM, especially those in complicate formula, are non-chromophoric compounds and can not be determined unless pre-column or post-column derivatization is applied, which is often undesirable due to its time-consuming and lack of reproducibility and robustness.[9] Secondly, the multi-components of TCM, in general, have rather different UV absorption properties, and it is often difficult to simultaneously determine multi-components with common UV detection at a single fixed wavelength.

Evaporative light scattering detector (ELSD)[10—12] has been increasingly used and seems to offer a useful alternative to conventional methods in the quality control of TCM. Unlike UV detection, ELSD allows the determination of non-chromophoric compounds without preceding derivatization, and thus shows good compatibility to the multi-components of complex analytes such as TCM. In recent years, it has been reported that HPLC/ELSD is an efficient tool for the determination of certain non-chromophoric compounds of TCM, such as saponins,[13—16] terpenes,[17,18] steroids[19,20] and carbohydrates.[21,22] These studies, however, merely focused on the determination of non-chromophoric compounds, which does not comprehensively represent the quality characteristics of TCM.[16] Therefore, there is a need to develop ELSD into a quasi-universal detector for determining all available bioactive components of TCM in quality control of TCM.

Qingkailing injection is a well-known composite formula of TCM and is commonly used in clinical practice.[23,24] It comprises eight medicinal materials or extracts thereof, including *Radix Isatidis*, *Flos Lonicerae*, *Fructus Gardenise*, *Cornu Bubal*, *Concha Margaritifera*, *Baicalinum*, *Acidum Cholicum*, and *Acidum Hyodesoxy-cholicum*. It has been reported that the compounds of adenosine (ADE), geniposide (GEN), chlorogenic acid (CHA), baicalin (BAI), ursodeoxycholic acid (UCA), cholic acid (CA), and hyodeoxycholic acid (HCA) are the major bioactive components synergistically contributing to its therapeutic effects (see Fig. 1 for their chemical structures).[25—28] Moreover, these compounds are commonly used as markers for six important medicinal materials of *Qingkailing* injection. Therefore, it is significant to simultaneously determine these compounds to ensure the efficacy, safety, and batch-to-batch uniformity of *Qingkailing* injection. While UV detection allows simultaneous determination of ADE, CHA, GEN and BAI with multi-wavelength monitoring technique,[29] it is difficult to analyze UCA, CA

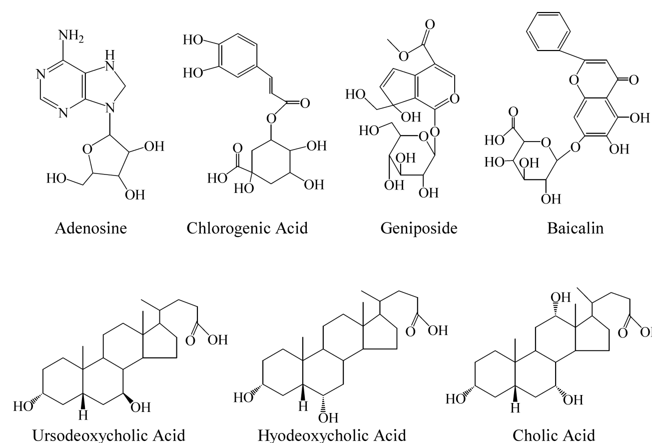

Fig. 1. Chemical Structures of the Bioactive Compounds Determined in *Qingkailing* Injection

* To whom correspondence should be addressed. e-mail: chengyy@zju.edu.cn; wangym@mail.tsinghua.edu.cn © 2005 Pharmaceutical Society of Japan

and HCA because of their poor UV absorption.[30] In this study, coupled HPLC/ELSD was established to allow the simultaneous determination of these seven bioactive components. The method provides a useful alternative for the analysis of multi-bioactive components in TCM for quality control purpose.

Experimental

Chemicals and Reagents Analytical standards of ADE, BAI, GEN, 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, U.S.A.). The HPLC-grade methanol (CH_3OH) was purchased from Tedia Inc. (OH, U.S.A.) and ultrapure water was prepared from a Milli-Q purification system (Millipore Co., France). Other reagents used were all of analytical grade.

Chromatographic System The HPLC analysis was performed using a Dionex P580 liquid chromatograph (Dionex Inc., U.S.A.) equipped with an Alltech ELSD 2000 detector (Alltech, U.S.A.), a USD340S diode array detector (DAD) (Dionex Inc., U.S.A.), an intelligent quaternary pump, a column oven and a manual injection system with a 20 μl loop.

Chromatographic Conditions The C_{18} RP-ODS column (250 mm× 4.6 mm, 5 μ, Phenomenex Luna, U.S.A.) and a C_{18} guard column (7.5 mm× 4.6 mm, 5 μ, Alltech, IL, U.S.A.) were used. The mobile phases were composed of water/formic acid (100/0.1, A) and methanol (B). The gradient was as follows: 0 min, 100% A, 0% B; 33 min, 34% A, 66% B; and 60 min, 12% A, 88% B. Elution was performed at a solvent flow rate of 1.0 ml/min. The column compartment was kept at the temperature of 30 °C.

Detection Conditions For UV detection, monitoring wavelength was set at 254 nm and for ELSD detection, the carrier gas was nitrogen (99.999%), the drift tube temperature was set at 110 °C, and the gas flowrate was 2.0 l/min.

Sample Preparation Stock solution of the mixture of seven standards was prepared by dissolving accurately weighted portions of the standards in methanol (2.0 mg of ADE, CHA, GEN, UCA; 12.5 mg of BAI, CA, HCA), transferring the solution to a 25-ml volumetric flask, and then adding methanol to volume. The stock solutions were further diluted to make working standard solutions. Ten *Qingkailing* injection samples (marked as 1—10) were collected from two Chinese medicine manufacturers. Samples 1—4 were from manufacturer A and 5—10 were from manufacturer B (the names of the two manufacturers were removed in order to preserve confidentiality). All the samples were diluted ten times with ultrapure water. All solutions were filtered through a 0.45 μm membrane filter before HPLC analysis.

Results and Discussion

Optimization of Detection Conditions Under fixed chromatographic conditions, nebulizing gas flow rate (or flow pressure) and evaporating temperature are two major instrumental adjustments available for maximizing the detector response efficiency. HPLC/ELSD analysis of the same sample at different gas flow rates of 1.0, 1.5, 2.0, 2.5 and 3.0 l/min were performed respectively, and it was noted that as the gas flow rate was increased, both the noises and the signals of the chromatogram were decreased. In this study, a moderate flow rate of 2.0 l/min was adopted to gain the best S/N ratio. Assays at the evaporating temperatures including 90, 100, 110, 115 and 120 °C were also performed, and optimal evaporator temperature was then determined by the limit of detection (LOD) of components analyzed (CA was selected as the representative). It was found that the least LOD was achieved at the evaporator temperature of 110 °C.

Comparison of ESLD and UV Detection In this study, both HPLC/ELSD and HPLC/UV analysis of *Qingkailing* injections were performed. The representative HPLC/UV (254 nm) and HPLC/ELSD chromatograms of *Qingkailing* injection are shown in Figs. 2-A and B respectively. Peaks of the components determined were observed by their retention times in comparison with those of reference standards (Fig.

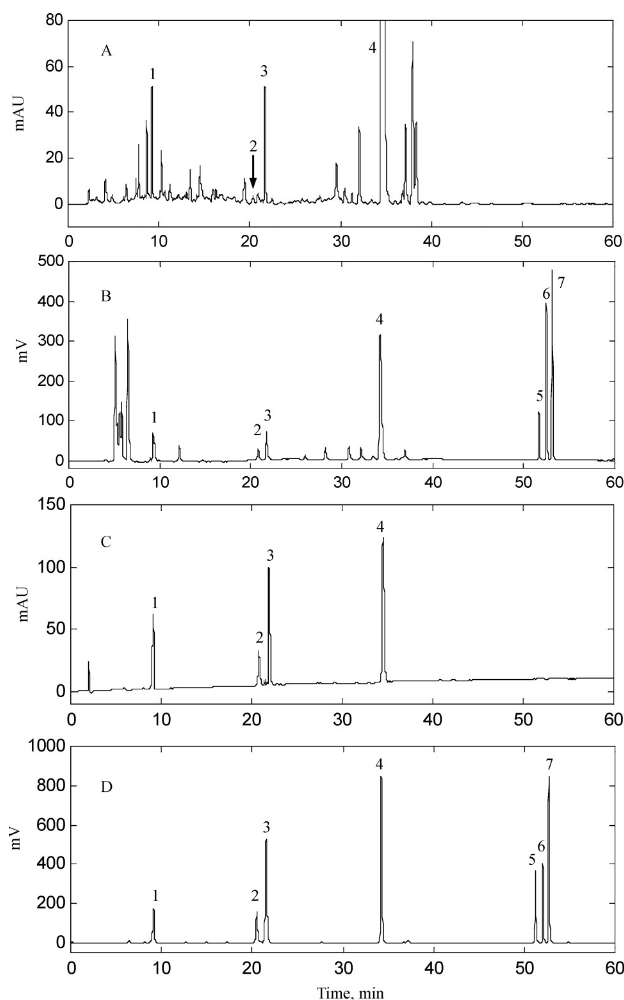

Fig. 2. Chromatograms of Samples of *Qingkailing* Injection and Reference Chemicals

(A) HPLC/UV chromatogram of *Qingkailing* injection (254 nm), (B) HPLC/ELSD chromatogram of *Qingkailing* injection, (C) HPLC/UV chromatogram of reference chemicals, (D) HPLC/ELSD chromatogram of reference chemicals. 1: ADE (9.10 min), 2: CHA (20.47 min), 3: GEN (21.68 min), 4: BAI (34.58 min), 5: UCA (51.95 min), 6: CA (53.05 min), 7: HCA (54.12 min).

2-C, D), and also by the method of standard addition to the sample. Additionally, in the UV chromatogram of Fig. 2-A, peaks 1—4 were further identified by their DAD spectra.

It is found that, as shown in Fig. 2-A, ADE, CHA, GEN and BAI can be detected simultaneously with UV detection, although CHA has too weak a response to be sensitively quantified. UCA, CA and HCA, however, have no responses in the UV chromatogram. Furthermore, DAD spectra suggests that these compounds have no UV absorption at any other wavelengths in the range of 200—590 nm. Thus it is impossible to simultaneously determine these bioactive components using UV detection. Fig. 2-B indicates that all the seven components have been detected simultaneously by ELSD detector. Therefore, ELSD detection shows clear advantages over conventional UV detection and should be accepted as a tool in the quality control of *Qingkailing* injections.

Validation of the Method The assay linearity was determined by the analysis of five different concentrations of the standard solutions. It is well known that ELSD gives no direct linear response, and the calibration curves could be

constructed by partial least squares method on the analytical data of peak area and concentration in double logarithmic co-ordinates.[31,32] 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 1 shows the regression data and LODs of the components determined. In this study, LODs of ADE, CHA, GEN and BAI with UV detection were also determined with the results of 5, 5, 10 and 10 ng, respectively (CHA was determined at 330 nm). It is demonstrated that for the same analyte, the LOD of ELSD is 2—6 times higher than UV detection.

The intra-day and inter-day precision were determined by analyzing four sets of calibration samples during a single day and on four 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. Table 2 shows these results of validation. The relative standard deviation (RSD) was taken as a measure. It indicates that all the RSDs are less than 5%, and the method is thus acceptable.

Table 1. Regression Data and LODs of the Multi-components Determined

Compounds	Retention time (min)	Regression equation[a)]	Correlation coefficient (r^2)	Linear range (μg)	LOD (μg)
ADE	9.10	$y=0.9028x+6.6132$	0.9974	0.4—80	0.01
CHA	20.47	$y=0.9982x+7.2132$	0.9957	0.2—80	0.03
GEN	21.68	$y=1.0336x+6.6745$	0.9965	0.1—80	0.02
BAI	34.58	$y=1.2549x+6.0745$	0.9983	0.5—500	0.05
UCA	51.97	$y=0.9641x+6.8665$	0.9991	0.5—80	0.03
CA	53.05	$y=0.8648x+5.7615$	0.9980	1—500	0.05
HCA	54.12	$y=0.8987x+5.7701$	0.9944	1—500	0.05

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

Sample Analysis The method was applied to analyze ten *Qingkailing* samples with the results shown in Table 3. The concentration of each component in different samples, especially in the samples from different manufacturers, is significantly different. Among the seven compounds, BAI, CA and HCA are the most abundant. The majority of the components of *Qingkailing* samples by manufacturer B have higher concentrations than those by manufacturer A with the exception of CA, which is lower in samples by manufacturer B, and HCA, which has similar concentration in sample by both manufacturers.

Figure 3 shows the RSDs of the determination, which represents the batch-to-batch uniformity of the injection products. It can be noted that there are no obvious differences between the two manufacturers on the uniformity of products. Among the seven bioactive components determined, BAI, UCA, CA and HCA have much lower RSDs (<10%) 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

Table 2. Precision, Repeatability and Accuracy Data of the Proposed HPLC/ELSD Method

Peak No.	Compounds	Precision Intra-day ($n=6$) Mean (μg/ml)	Intra-day R.S.D. (%)	Inter-day ($n=6$) Mean (μg/ml)	Inter-day R.S.D. (%)	Repeatability $n=5$ Mean (μg/ml)	Repeatability R.S.D. (%)	Accuracy[a)] $n=5$ Mean (μg/ml)	Accuracy R.S.D. (%)
1	ADE	18.6	1.63	18.9	2.05	26.8	1.14	96.33	4.65
2	CHA	5.76	1.44	5.53	3.89	2.81	2.05	99.18	4.09
3	GEN	135	1.27	140	1.98	50.1	0.986	97.50	2.51
4	BAI	4225	1.66	4198	3.46	2608	2.55	99.81	2.63
5	UCA	24.3	1.27	24.1	3.44	13.7	3.03	98.81	2.36
6	CA	1917	2.21	1897	1.90	2071	2.84	99.78	1.74
7	HCA	2309	2.32	2276	2.12	2292	1.67	102.81	3.02

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

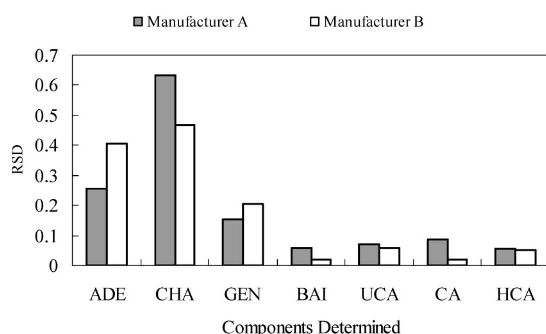

Fig. 3. Relative Standard Derivations of the Determination

Table 3. Quantitative Analytical Results of Various *Qingkailing* Injection Samples (ml^{-1})

Manufacturer	No.	ADE (μg)	CHA (μg)	GEN (μg)	BAI (mg)	UCA (μg)	CA (mg)	HCA (mg)
A	1	15.8	4.96	59.3	2.27	13.9	2.36	2.36
	2	19.1	0.67	48.3	2.33	15.5	1.97	2.59
	3	26.4	2.75	40.2	2.52	12.8	2.13	2.26
	4	17.2	2.93	45.5	2.55	14.0	2.18	2.28
	5	14.3	1.53	55.3	2.59	14.4	1.89	2.41
B	6	47.0	6.51	109	4.46	21.2	1.85	2.40
	7	23.3	6.19	96	4.24	22.8	1.89	2.31
	8	18.0	5.81	148	4.31	22.5	1.88	2.32
	9	49.5	14.8	92.6	4.43	20.1	1.96	2.22
	10	46.9	7.07	102	4.40	20.1	1.91	2.10

three components of ADE, CHA and GEN, however, are directly derived from herbal materials of *Isatidis*, *Flos Lonicerae* and *Fructus Gardeniae*, respectively, 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.

Conclusions

The proposed analytical method makes it possible to simultaneously determine seven bioactive components of different structural types, including three compounds without UV absorption. Acceptable linearity, precision, repeatability and accuracy were achieved. The proposed HPLC/ELSD method provides a useful alternative for the analysis of multi-bioactive components in TCM for quality control purpose.

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