An Automated Method of On-line Extraction Coupled with Flow Injection and Capillary Electrophoresis for Phytochemical Analysis

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Abstract

In this study, an automated system for phytochemical analysis was successfully fabricated for the first time in our laboratory. The system included on-line decocting, filtering, cooling, sample introducing, separation, and detection, which greatly simplified the sample preparation and shortened the analysis time. Samples from the decoction extract were drawn every 5 min through an on-line filter and a condenser pipe to the sample loop from which 20-µL samples were injected into the running buffer and transported into a split-flow interface coupling the flow injection and capillary electrophoresis systems. The separation of glycyrrhetinic acid (GTA) and glycyrrhizic acid (GA) took less than 5 min by using a 10 mM borate buffer (adjusted pH to 8.8) and +10 kV voltage. Calibration curves showed good linearity with correlation coefficients (R) more than 0.9991. The intra-day repeatabilities (*n* = 5, expressed as relative standard deviation) of the proposed system, obtained using GTA and GA standards, were 1.1% and 0.8% for migration time and 0.7% and 0.9% for peak area, respectively. The mean recoveries of GTA and GA in the off-line extract of *Glycyrrhiza uralensis* Fisch root were better than 99.0%. The limits of detection (signal-to-noise ratio = 3) of the proposed method were 6.2 µg/mL and 6.9 µg/mL for GTA and GA, respectively. The dynamic changes of GTA and GA on the decoction time were obtained during the on-line decoction process of *Glycyrrhiza uralensis* Fisch root.

Introduction

Natural medicinal resources provide valuable material for the discovery and development of new drugs of natural origin. Sample preparation is the first crucial step in the analysis of herbal medicines because it is necessary to extract the desired chemical components from herbal plants (1). Several methods found in the monographs of Pharmacopeias and other reports, including distillation (2), decoction (3), microwave (4), and ultrasonic (5) techniques have been reported for the extraction of their bioactive components. Among these different extraction techniques, the decoction method has the advantages of simplicity, no need of organic solvent, and low-cost equipment, so many medicinal preparations of herbs and plants are made by this method.

The concentrations of the bioactive components in the decoction extract will influence their clinical therapeutic effects. For an herbal medicine or formula, the decoction time is one of the most important factors that affect the concentrations of the bioactive components (3,6). The improper decoction time will result in low concentration of the volatile component and expand the toxic side effect of the toxic component. Moreover, some new bioactive components will come into being during the decoction process of herbal medicine (7). So it is essential to study the existence state and the concentration change of the bioactive components of herbal medicines with the decoction time during the decoction process.

The decoction process is as follows: The herbal medicine is firstly immersed into water. Then, the heated water by appropriate fire infiltrates into the inner layer of the herbal plant, and the bioactive components diffuse into the water. Finally, the obtained filtrate is dosed orally for recovery from disease (8). Many analytical methods have been applied to determine the bioactive components of the decoction extract, including spectrophotometry (9), thin-layer scanning (10), high-performance liquid chromatography (11,12), and capillary electrophoresis (CE) (12). However, all these established methods are laborious and time-consuming, and they cannot be used to on-line monitor the dynamic change of the bioactive components and the emergence of new components during the decoction process of herbal medicines.

CE has become a powerful technique in separation and detection because of its high resolution, minimal sample volume requirement, short analysis time, and high separation efficiency. In recent years, more and more attention has been paid to the study of the combination system of CE separation and flow injection (FI) sample introduction (13–19). The advantages of the combined FI-CE system have been demonstrated not only to produce a reliable mode of sample introduction with improved throughput and outstanding reproducibility but also to provide on-line sample pretreatment ability involving on-line sample

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filtration (13), dialysis (14), gas diffusion (15), column sorption (16), ion-exchange pre-concentration (17), and on-line derivatization (18,19). But until now, there is no reference published on applying the FI-CE system to monitor the dynamic changes of the bioactive components during the on-line decoction process of herbal medicines.

Glycyrrhiza uralensis Fisch root is a ubiquitous drug used in Chinese medicinal formula and preparation. Glycyrrhetinic acid (GTA) and glycyrrhizic acid (GA) are its main bioactive components. In this study, *Glycyrrhiza uralensis* Fisch root was used as a model plant to develop an on-line decoction system of herbal plants, and GTA and GA are the model compounds to be determined based on FI-CE technique. The objectives of the study are (a) to develop an on-line system including decocting, filtering, cooling, sample introducing, separation, and detection; (b) to monitor the concentration change of GTA and GA during the on-line decoction process of *Glycyrrhiza uralensis* Fisch root; and (c) to monitor the dynamic change on the appearance of new component or the disappearance of old component during the decoction process of *Glycyrrhiza uralensis* Fisch root.

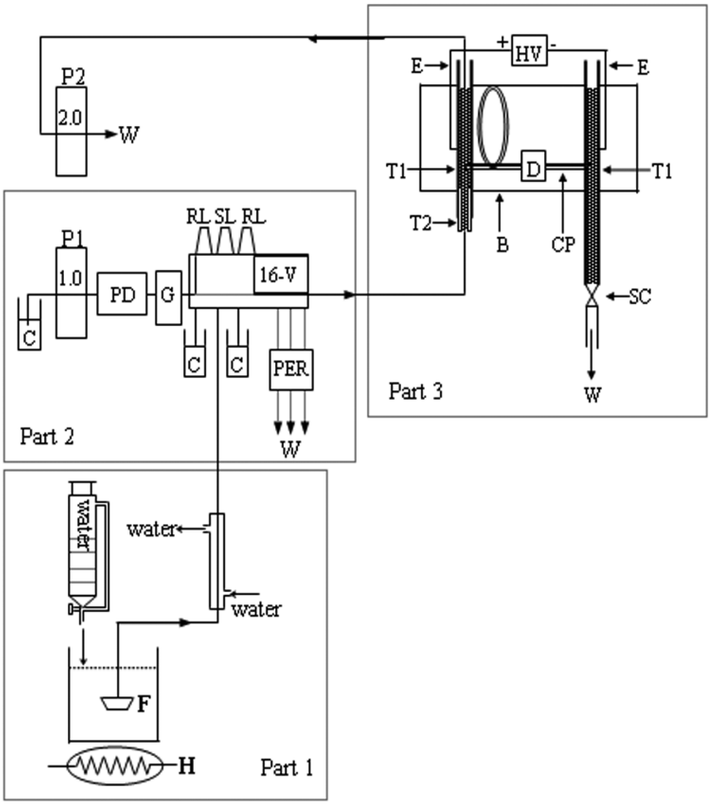

Figure 1. Schematic diagram of on-line system composing decocting, filtering, cooling, sample introducing, separation, and detection (not drawn to scale). Part 1, Part 2, and Part 3 are the decoction system, the FI system, and the CE system, respectively. C, buffer solution; P1 and P2, pumps; PD, pressure damper; G, pressure gauge; RL, reagent loop; SL, sampling loop; 16-V, 16-way valve; PER, peristaltic pump; W, waste; T1 and T2, Tygon tubing; B, planar plastic base; CP, separation capillary column; D, detector; E, electrode; HV, high voltage; SC, screw clamp; H, electric fire; and F, syringe filter.

Experimental

Reagents and chemicals

GTA and GA (purity 98%) were obtained from the Delta Information Centre for Natural Organic Compounds (Anhui, China) and were used as received. The crude drug of *Glycyrrhiza uralensis* Fisch root was purchased from local drug stores.

Ethanol was purchased from Tianjin Secondary Chemical Factory (Tianjin, China). Borate was purchased from Sinopharm Chemical Reagent (Shanghai, China). All chemicals were analytical reagent-grade and used as received.

Stock standard solutions (1 mg/mL) of GTA and GA were prepared in 50% aqueous ethanol. The standard solutions at various concentrations were prepared by appropriate dilution of the stock solution with 50% aqueous ethanol. The electrophoretic running buffer (functioning also as FI carrier solution) was 10 mM borate (pH 8.8), which was prepared daily from 100 mM borate and then adjusted to the desired pH using either 2 M NaOH or 2 M HCl. The final pH of the buffer was checked using a PHS-3B pH meter (Shanghai Precision & Scientific Instrument, Shanghai, China). All solutions were filtered through 0.45-µm syringe filters before use.

Apparatus

A model HPE-100 CE system with 12 kV maximum voltage (Bio-Rad, Hercules, CA) was used for all electrophoretic separations, which was connected to a 486 personal computer with a Chroma chromatography collection system (Bio-Rad) for integration and data treatment. The fused-silica capillaries (75 µm i.d., 375 µm o.d., and 30 cm length, 26.5 cm effective length) were purchased from Yongnian Optical Fiber Factory (Baoding, Hebei, China). UV detection was carried out at 268 nm.

A K-1000 FI analyzer (Hitachi, Tokyo, Japan) was equipped with a peristaltic pump, a 16-way auto-switching injection valve, and a plunger pump. Polytetrafluoroethylene (PTFE) tubing (0.5 mm i.d.) was used for connecting all components of the FI system, including a 33-cm length transport line from the valve to the split-flow interface. A sample loop and two reagent loops were made from PTFE. The time period for the injecting sample was defined through man/access mode.

The experimental setup of the on-line system comprising decocting, filtering, cooling, sample introducing, separation, and detection is shown schematically in Figure 1 (not drawn to scale). Part 1, Part 2, and Part 3 in the dotted lines were the extraction system, the FI system, and the CE system, respectively. The detailed description of coupling FI with CE system can be found in literature (20). In Part 1, an electric fire (H) was adapted as a heat source and a beaker was used as the decoction vessel. To compensate the evaporated water and maintain the decoction volume of 200 mL constant, distilled water was added to the decoction system by a dropping funnel at the speed of 2 mL/min, which was controlled by adjusting the plunger of the

addition funnel and measured by an external stopwatch. The PTFE tube acting as the introduced sample was posited in the middle of a condenser pipe to cool the decoction extract, and 0.45-µm syringe filter (F) was posited at the tip of the PTFE tube as the on-line filter. The decoction extract of *Glycyrrhiza uralensis* Fisch root was introduced into the FI sample loop by the impetus of the peristaltic pump after on-line filtering and cooling and then was injected directly into the CE system by FI.

Procedures

Sample preparation

In order to establish an accurate, sensitive, and fast method to separate and detect GTA and GA in *Glycyrrhiza uralensis* Fisch root, an off-line extract of GTA and GA was firstly made with distilled water (2). A 2.5 g sample, which was crushed into powder, was refluxed with 50 mL of distilled water for 1 h. The extract solution was injected directly into the CE system by FI every 2.5 min after filtering and 20-fold diluting with distilled water.

Then, 5.0 g *Glycyrrhiza uralensis* Fisch root was immersed with 200 mL distilled water in the beaker and decocted by the on-line extraction system (Part 1 in Figure 1). The sample solution at different decoction times was injected directly into the CE system by FI every 5 min after on-line filtering and cooling.

FI procedure

The central part of the FI system is a 16-way auto-switching valve, whose operating procedure can also be found in literature (20). Twenty microliters of the sample solution in the middle loop of the valve was sandwiched by the buffer solutions and transported through the connecting conduit into the split-flow interface. Then, a fraction of the sample zone was introduced into the separation capillary by the electrokinetic injection. The charging time (CT) and the injecting time (IT) of the FI system were set manually. A series of samples were injected continuously without interrupting the voltage (10.0 kV).

CE procedure

Prior to first use, the new capillary was conditioned with distilled water for 10 min, 0.1 M NaOH for 10 min, and distilled water for 10 min, followed by the fresh running buffer for 10 min from the capillary outlet reservoir using a water-circulating vacuum pump. At the beginning of each working day, the capillary was flushed separately with distilled water, 0.1 M NaOH, distilled water, and running buffer for 5 min. Moreover, between runs (a single run comprising introducing sample to the CE system three times at least), the capillary was rinsed with distilled water (2 min), 0.1 M NaOH (3 min), distilled water (2 min), and equilibrated with running buffer (3 min) to ensure good repeatability.

Results and Discussion

Choice of the separation conditions

In order to monitor the dynamic change of GTA and GA during the decoction process of *Glycyrrhiza uralensis* Fisch root, it is necessary to establish a fast on-line CE method with satisfactory separation efficiency and a low limit of detection. The apparent dissociation constant (pKa) values of GTA and GA are 6.5 and 7.1, respectively, and the difference of their effective mobilities is large enough when the buffer pH is higher than 7.5 (2). So, the capillary zone electrophoresis (CZE) mode is the first choice in separating GTA and GA.

Li et al. concluded that 10 mM borate (pH 9.2) was the optimum running buffer for the separation of GTA and GA (2). In our preliminary experiment, 10 mM borate (pH 9.2) was also adapted as the running buffer, and the two analytes were successfully resolved. Further studies, however, suggested that the sensitivities of GTA and GA (expressed by peak area) had a trend of increasing first, followed by decreasing with the increase of buffer pH from 8.2 to 10.2. Therefore, in order to obtain higher sensitivity within enough resolution, pH 8.8 was chosen for further study.

In addition, we studied the effects of the CT of FI system in the range of 5–15 s and the IT of FI system in the range of 1–5 s on the sensitivities of GTA and GA. The optimum experimental conditions were as follows: 10 mM borate buffer (pH 8.8); 10.0 kV of applied voltage; 268 nm UV detection; CT 13 s and IT 3 s, 1.0 mL/min flow rate; and 20-µL sample volume.

Performance of the combined FI-CE system

Calibration graphs were obtained by injecting standard solutions at seven different concentrations. The peak areas were employed for quantification. Each point on the calibration graph corresponded to the mean value obtained from three independent peak-area measurements on a single run. The obtained regression equations, as well as other characteristic parameters for the determination of GTA and GA, are listed in Table I.

The limit of detection was calibrated as the analyte concentration that gave rise to peak areas with a signal-to-noise ratio of 3. The precision test of the proposed FI-CE system was evaluated under optimum conditions using a standard mixture containing 500 µg/mL GTA and GA by the intra-day and inter-day variability. The analytes were injected into the CE system every 2.5 min, and the standard throughput rate was 24 h^{-1}. The electropherogram

Table I. Quantitative Parameters of the Proposed Method For the Analysis of GTA and GA in Standard Solutions

		GTA	GA
LOD (µg/mL) (S/N = 3)		6.2	6.9
Calibration range (µg/mL)		15.6 –1000.0	15.6–1000.0
*Regression equation**			
Intercept*		1224.5	839.2
Slope*		23.2	22.4
Correlation coefficient (R)		0.9992	0.9991
Repeatability (expression as RSD)			
Intra-day†	Migration time	0.7%	0.9%
	Peak area	1.1%	0.8%
Inter-day‡	Migration time	3.9%	2.4%
	Peak area	3.3%	0.8%

* y = a + bx where y = peak area, x = the concentration of standard solution (µg/mL), a = intercept, and b = slope.
† Five replicates. ‡ Three replicates each day for three days.

of five continuous sampling of the standard mixture is shown in Figure 2A. The repeatability was expressed as the relative standard deviation (RSD) values of the migration time and the peak areas obtained from five injections (Table I).

Application of the combined FI-CE system

The accuracy of the established method

The established method was first applied to the detection of the off-line extract of *Glycyrrhiza uralensis* Fisch root in order to demonstrate its accuracy. The typical electropherograms of *Glycyrrhiza uralensis* Fisch root, which were obtained by five continuous sampling, are shown in Figure 2B. Peaks were identified by comparing migration times and spiking samples with known quantities of the standard mixture of GTA and GA. The quantity of each analyte was subsequently obtained from the calibration curve in Table I.

The contents of GTA and GA in dried root of *Glycyrrhiza uralensis* Fisch were 6.2% and 8.9%, respectively. The recovery test was carried as follows: standard solutions with three-level concentration (low, medium, and high) were added into sample solutions. The results showed that the recoveries of GTA and GA were in the range of 99.0–102.3% and 94.5–103.8%, respectively. Compared with previous studies (21), the obtained content of GA in the study was in the reported content range of 5.8–11.4% in dried root of the cultivated licorice from the different growing areas.

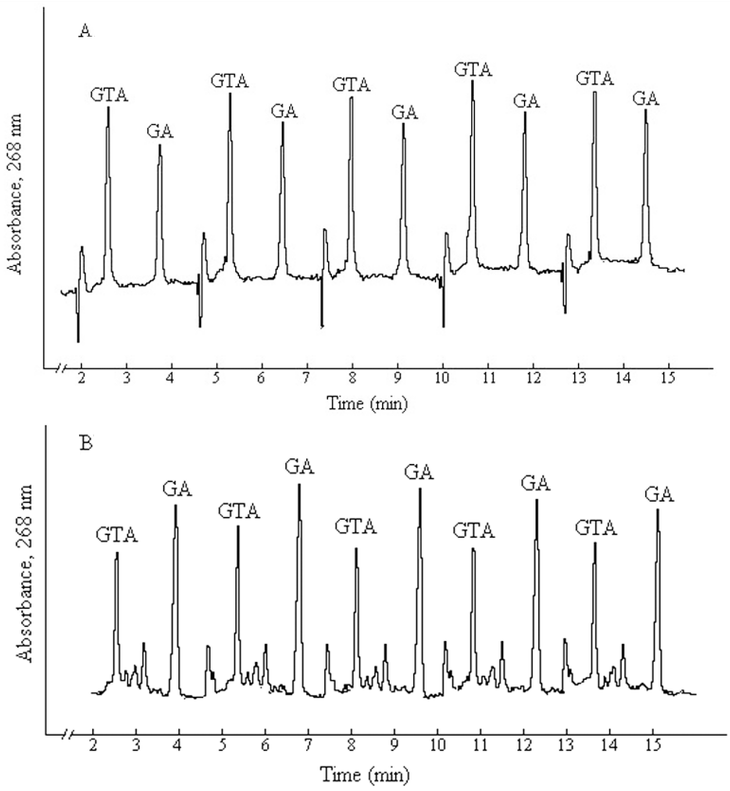

Figure 2. Electrochromatograms of the standard mixture solution and the real sample: (A) the standard mixture of 500 µg/mL GTA and GA; (B) the decoction extract of *Glycyrrhiza uralensis* Fisch root after 20-fold diluting with distilled water. Separation conditions: 10 mM borate (pH 8.8), 10.0 kV; uncoated separation capillary (75 µm i.d. × 30 cm length, 26.5 cm effective length); detection wavelength, 268 nm. Conditions of K-1000 FIA: man/access mode; CT 13 s; IT 3 s; sample volume, 20 µL; carrier flow rate, 1.0 mL/min; frequency of injecting sample, 2.5 min; room temperature.

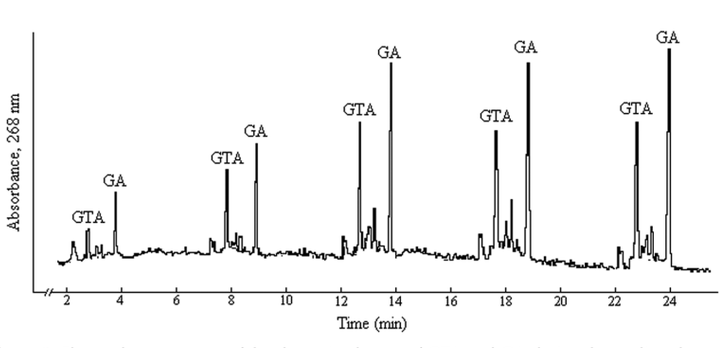

Figure 3. Electrochromatogram of the dynamic change of GTA and GA during the on-line decoction process of *Glycyrrhiza uralensis* Fisch root. Frequency of injecting sample, 5 min. The other CE and FI conditions are as stated previously.

The on-line decoction process of Glycyrrhiza uralensis Fisch root

It can be seen from the previous experiments that the proposed method manifested good precision and accuracy. Next, the on-line system was applied to monitor the dynamic changes of GTA and GA during the decoction process of *Glycyrrhiza uralensis* Fisch root. Figure 3 is the typical electropherograms of on-line decocting *Glycyrrhiza uralensis* Fisch root, which were obtained by continuous sampling. Owing to the complexity of the instrumental operation, the on-line decoction extract was injected into the CE system every 5 min, and the sample throughput rate reached 12 h^{-1}. Obviously, the on-line system can successfully complete all the operations composing of decocting, filtering, cooling, sample introducing, separation, and detection. The whole analytical time was about 70 min, including the separation time of 5 min and the decoction process of 65 min, which meant a fast and automated analysis process with dramatic improvement in sample throughput. In addition, it can be concluded that no new component appeared and that no old component disappeared during the decoction process.

The decoction solution at different times was injected directly into the CE system by FI every 5 min after on-line filtering and cooling. The concentrations of GTA and GA at different time in the decoction progress are calculated by the calibration curve in Table I and are depicted in Figure 4. From it, it was clear that the concentration of GTA and GA changed gradually with increasing the decoction time during the decoction process of *Glycyrrhiza uralensis* Fisch root. At 50 min, the concentration reached dynamic

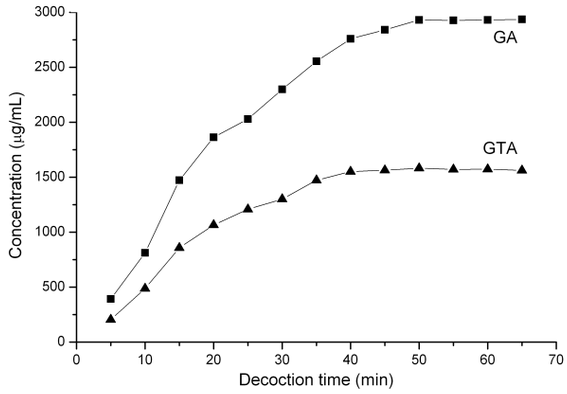

Figure 4. The concentration change of GTA and GA with different time during the decoction process of Glycyrrhiza uralensis Fisch root.

balance, and the longer decoction time seemed to be in vain. Therefore, the appropriate decocting time of 50 min was obtained, which was close to the two-time decocting steps of 15–20 min and 10–20 min in Chinese medicine. This information is very important for the extraction of *Glycyrrhiza uralensis* Fisch root and is a reference to decocting other herbal medicines with stable effective components.

Conclusion

The coupling of an FI system integrating on-line extraction with CE equipment has been successfully used to monitor the dynamic changes of GTA and GA in the decoction process of *Glycyrrhiza uralensis* Fisch root. Excellent separation efficiency, high sampling frequency, and good precision were achieved using borate as running buffer. On-line filtering and cooling coupled to FI in a flow arrangement is a suitable technique for automated pre-treatment of the decoction system. The results indicate that the proposed FI-CE system is very suitable for the determination of the dynamic changes of GTA and GA during the decoction process of *Glycyrrhiza uralensis* Fisch root. It also affords a useful method for any other decoction system of herbal medicines. The technique should have large potential in scientifically understanding the clinical therapeutic effect of herbal medicines.

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References

1. C.H. Deng, N. Liu, M.X. Cao, and X.M. Zhang. Recent developments in sample preparation techniques for chromatography analysis of traditional Chinese medicines. *J. Chromatogr. A* **1153:** 90–96 (2007).
2. G.B. Li, H.Y. Zhang, Y.Q. Fan, L. Zhao, and Z.D. Hu. Migration behavior and separation of active components in Glycyrrhiza uralensis Fisch and its commercial extract by micellar electrokinetic capillary chromatography. *J. Chromatogr. A* **863:** 105–114 (1999).
3. W. Zhu, X.M. Ruan, H.L. Wu, and K.J. Chen. Effect of preparation's time on contents of ferulic acid and paeoniflorin in Buyanghuanwu decoction. *Chin. J. Basic Med. Trad. Chin. Med.* **12:** 552–554 (2006).
4. S.Z. Hao, H. Zhang, L. Liu, Z.E. Li, and Z.H. Xu. Application of microwave technique to extraction of free anthraquinones in Rheum emodi. *Chin. Trad. Herbal Drugs* **33:** 23–26 (2002).
5. L.H. Liu, L.Y. Fan, H.L. Chen, X.G. Chen, and Z.D. Hu. Separation and determination of four active anthraquinones in Chinese herbal preparations by flow injection-capillary electrophoresis. *Electrophoresis* **26:** 2999–3006 (2005).
6. Y.Q. Sun, X.H. Xiao, Y.G. Ma, X.Y. Xing, and D. Yan. Study on dynamic variation patterns of anthraquinones of Radix et Rhiroma Rhei at different decocting time. *Pharm. J. Chin. PLA* **22:** 281–283 (2006).
7. D.R. Xu, K.C. Wang, Z.T. Wang, C.J. Wang, and J. Hu. Primary study on the original problems of Danshensu and protocatechuic aldehyde of Salvia miltiorrhiza. *Chin. J. Nat. Med.* **3:** 148–150 (2005).
8. *Pharmacopoeia of People's Republic of China* (2000 edition). Chemical Industry Publishing, 2000.
9. L.S. Huang, W.C. Zhao, and Y.F. Xia. Effect of preparation methods on the content of alkaloids and glycyrrhetic acid in Sini injection. *J. China Pharm. Univ.* **30:** 332–334 (1999).
10. S.G. Fan and X.Y. Wu. Determination the variety of the alkaloids amount of Radix Aconiti in different decocting time. *Nat. Prod. Res. Dev.* **17:** 645–647 (2005).
11. X.A. Wu, H.X. Wang, Y.H. Wei, Z.W. Liu, and R.H. Wang. Study on the content changes of free and combined anthraquinones in the decocting process of rhubarb powder. *Chin. Hosp. Pharm. J.* **27:** 1387–1390 (2007).
12. W. Li, F.R. Song, Z.Q. Liu, and S.Y. Liu. Studies on change of chemical composition in Coptis-Scute herb couple by using HPCE and HPLC. *Acta Pharmaceutica Sinica* **43:** 191–194 (2008).
13. H.W. Chen and Z.L. Fang. Combination of flow injection with capillary electrophoresis. Part 4. Automated multicomponent monitoring of drug dissolution. *Anal. Chim. Acta* **376:** 209–220 (1998).
14. P. Kuban, and B. Karlberg. On-line dialysis coupled to a capillary electrophoresis system for determination of small anions. *Anal. Chem.* **69:** 1169–1173 (1997).
15. P. Kuban and B. Karlberg. On-line coupling of gas diffusion to capillary electrophoresis. *Talanta* **45:** 477–484 (1998).
16. H.W. Chen and Z.L. Fang. Combination of flow injection with capillary electrophoresis. Part 3. On-line sorption column preconcentration capillary electrophoresis system. *Anal. Chim. Acta* **355:** 135–143 (1997).
17. L. Arce, P. Kuban, A. Ríos, M. Valcárcel, and B. Karlberg. On-line ion-exchange preconcentration in a flow injection system coupled to capillary electrophoresis for the direct determination of UV absorbing anions. *Anal. Chim. Acta* **390:** 39–44 (1999).
18. N. Dantan, W. Frenzel, and S. Küppers. Flow injection analysis coupled with HPLC and CE for monitoring chemical production processes. *Chromatographia* **54:** 187–190 (2001).
19. H.L. Chen, K.T. Wang, Q.S. Pu, X.G. Chen, and Z.D. Hu. On-line conversion and determination of artemisinin using a flow-injection capillary electrophoresis system. *Electrophoresis* **23:** 2865–2871 (2002).
20. L.Y. Fan, Y.Q. Cheng, Y.Q. Li, H.L. Chen, X.G. Chen, and Z.D. Hu. Head-column field-amplified sample stacking in a capillary electrophoresis-flow injection system. *Electrophoresis* **26:** 4345–4354 (2005).
21. H.Y. Gu, L.D. Gong, and J.H. Yu. Measurement and comparison of glycyrrhizic acid contents in root of licorice (Glycyrrhiza uralensis Fisch.) from different cultivating areas. *J. Forestry Res.* **13:** 141–143 (2002).

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