Potency Fingerprint of Herbal Products Danshen Injection for Their Quality Evaluation

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The fingerprint technique has been studied frequently as a useful strategy for quality of traditional Chinese medicine. A novel potency fingerprint that can quantitatively analyze the antioxidant activity of individual constituent and provide the total antioxidant activities of the samples has been developed by high-performance liquid chromatography coupled with ultraviolet and pyrogallol–luminol chemiluminescence detection (HPLC-diode array detection (DAD)-PLD). Hierarchical clustering analysis has been used as a powerful pattern recognition tool to identify and classify Danshen injection from different factories. In addition, the combination use of the chromatographic fingerprint and potency fingerprint with principal component analysis was applied to quality control of Danshen injection. The results demonstrated that the proposed potency fingerprint was a useful means to control the quality and to clarify the possible mechanism of action of herbal products.

Key words potency fingerprint; Danshen injection; phenolic acid; herbal medicine quality control

Nowadays, the consumers' demand of traditional Chinese medicine (TCM) is clearly rising in biological medicine industry. TCM is a complex mixture containing hundreds of chemically different constituents which are usually responsible for the therapeutic effects.[1)] Therefore based on the pharmacological activities or the efficacy, how to control the quality becomes a challenge for research of the traditional Chinese medicines. The chromatographic fingerprint has gained rising attentions in recent years and been internationally accepted as a feasible means for the quality control of TCM or other natural products,[2—7)] but these chromatographic fingerprints only represent chemical information of main active constituents in the herbal medicine, do not exhibit their pharmacological activities or the efficacy. At present, considerable efforts have gone into the development of biological fingerprint to screen the bioactive compounds in TCM or other natural products.[8—11)] In our previous research, it was demonstrated that the activity-integrated fingerprint could be regarded as an advantageous tool for evaluation of quality of herbal medicines. The activity-integrated fingerprint could represent active and chemical information of main active constituents in the herbal medicine.[12)] Since the relationship between biological activities and contents of active constituents was not linear, the whole biological activities of herbal medicine were unequal to the sum of biological activities of all active constituents. To overcome this problem, the herbal medicine potency fingerprint, which was defined as one of activity fingerprints using one effective component as a positive control to evaluate the activities of each chromatographic peak, was proposed for the screening of multiple bioactive compounds and assessing of quality of herbal medicines.

Danshen injection, a traditional Chinese medicine preparation, was widely used to treat coronary heart disease, unstable angina, brain hemorrhage, cerebral thrombosis and cerebrovascular diseases in clinics in China.[13—15)] In addition, it was also applied to treat other diseases, such as liver dysfunction, renal deficiency and diabetic vascular complication.[16,17)] Chemical and pharmacological investigations on Danshen injection showed that phenolic acids are beneficial compounds responsible for the pharmacological activities and therapeutic efficacy.[5,12)] It was demonstrated that the therapeutic effects of Danshen injection have been associated to antioxidant properties.[18—21)] The analysis of antioxidant could be done by on-line HPLC-CL and several methods have been developed to simultaneously determine the antioxidant activities of main active constituents in complex plant extracts.[22—25)] The presence of one or more hydroxylated aromatic rings seems to be responsible for their antioxidant properties as radical scavengers. Given their structure, phenolic acids could inhibit the chemiluminescence (CL) reaction of luminol with oxidant reagents, it was possible to select a simple and reliable HPLC-CL method to develop a potency fingerprint of Danshen injection.

To our knowledge, no potency fingerprint analysis of herbal medicines has been reported in literatures. Therefore, Danshen injection was investigated as an example to develop potency fingerprint for quality control of herbal medicines by high performance liquid chromatography coupled with diode array detection (DAD) and pyrogallol–luminol chemiluminescence detection.

Experimental

Chemicals Acetonitrile was of HPLC grade from Dima Technology Inc. (U.S.A.). Deionized water was purified with a Milli-Q Academic ultra-pure water system (Billerica, MA, U.S.A.) prior to the use as HPLC mobile phase. Phosphoric acid for analysis was of analytical grade from the first chemical company of Nanjing (Jiangsu, China); analytical grade methanol was purchased from Hanbang Science & Technology (Nanjing, P. R. China).

Instrumentations Experiments were performed on Agilent 1100 series HPLC system equipped with a binary pump, an autosampler, a column oven and DAD. An Agilent Zorbax Extend reversed-phase C_{18} column (250×4.6 mm) and an Agilent Zorbax C_{18} guard column were used for all chromatographic separation. The CL solutions and oxidant reagent were injected by a BT-200 peristaltic pump (Huxi Analysis Instrument Factory, Shanghai, China). The CL emission was detected by BPCL system (Academia Sinica Biophysics Institute, Beijing, China).

Preparation Reagent Solutions for the Determination of $O_2^{\cdot-}$ Scavenging Activity and Sample Solutions 0.1 M carbonate buffer (pH 11.0) was

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prepared by mixing of appropriate volumes of 0.1 M Na_2CO_3 and 0.1 M $NaHCO_3$. A 1.8×10^{-2} M stock solution of luminol was prepared in 0.1 M Na_2CO_3 and was stored in a refrigerator at least 3 d before dilution. A 2.7×10^{-5} M luminol solution was prepared by mixing 1.8×10^{-2} M stock solution of luminol, 0.1 M carbonate buffer and 6.3×10^{-3} M ethylenediaminetetraacetic acid (EDTA). A 1.1×10^{-2} M stock solution of pyrogallol was prepared in 0.1 mM HCl then stored in dark bottles at 4 °C. Oxidant reagent was an aqueous 1.65×10^{-5} M pyrogallol solution.

Danshen injection was diluted with methanol at the ratio of 1 to 4 and filtrated through 0.45 μm filter. An aliquot of 20 μl of the filtrate was injected into HPLC for analysis.

HPLC-UV-CL Analysis HPLC conditions were used as described previously.[12] The pyrogallol–luminol detection was selected to measure $O_2^{-\cdot}$ scavenging activity of sample solutions. HPLC-CL optimization methods have been discussed in the literature. In brief, 2.7×10^{-5} M luminol and 1.0×10^{-5} M pyrogallol were used as the chemiluminescence reactive reagents to form pyrogallol–luminol detection. The luminol solution and oxidant reagent were performed respectively at the flow rate of 1.1 ml/min and 1.3 ml/min with the peristaltic pumps. The CL detector was equipped with a flat glass coil as detection cell of 80 μl.[26]

Antioxidant Activity The antioxidant activity of main active constituents was measured using HPLC-CL analysis. The relative percentage of inhibition $O_2^{-\cdot}$ was calculated by equation

$$\text{inhibition (I\%)}=(CL_0-CL_1)/CL_0\times100\%$$

Where CL_0 was baseline intensity of CL (without sample) and CL_1 was the inhibited CL intensity of every compound in the injections. If 3,4-dihydroxyphenyllactic acid (danshensu) (1 μg/ml) was presumed as a potency unit, the standard potency curves were prepared using scavenging rate (%) (x) and potency unit according to above method. In order to clarify the relationship between antioxidant activity and contents of antioxidant, the scavenging rates x (%) of 3,4-dihydroxyphenyllactic acid in different concentrations (y) were measured by using HPLC-PLD. Standard potency curves $\text{Log}(y)=3\text{E-}05x^3-0.0041x^2+0.2168x-0.849$ ($r^2=0.9906$) were generated. The relative activities of peaks in difference batches of Danshen injections were calculated according to standard potency curves. Therefore, antioxidant activity of each chromatographic peak was expressed potency unit of 3,4-dihydroxyphenyllactic acid equivalent antioxidant capacity. The total antioxidant activities should be the sums of potency of all peaks.

Data Analysis Hierarchical clustering analysis (HCA) and principal component analysis (PCA) were performed to analyze the data from HPLC chromatograms and potency fingerprint. All above were implemented by using SPSS software (SPSS for Windows 13.0, SPSS Corporation, U.S.A.). The nearest neighbor and cosine, which is a pattern similarity measure, were selected as measurement for hierarchical cluster analysis. PCA was employed to evaluate the discrimination ability of these common components using their antioxidant activities and chromatographic areas as input data.

Results and Discussion

Potency Fingerprint of Danshen Injections The antioxidant activities of antioxidants including phenolic acids were measured by *in vitro* assays that are based on their ability to scavenge free radicals. Considering the role of $O_2^{-\cdot}$ in cardiovascular disease, the optimized pyrogallol–luminol chemiluminescence detections (PLD) in our previous research was selected to measure $O_2^{-\cdot}$ scavenging activity of sample solutions in this research. Consequently, the two chromatographic and scavenging activity scavenging $O_2^{-\cdot}$ profiles can simultaneously be obtained by using HPLC-DAD-PLD. The representative HPLC-DAD and PLD profiles of Danshen injection were presented in Figs. 1A, B. The results showed that 3,4-dihydroxyphenyllactic acid, protocatechuic acid, protocatechuic aldehyde, caffeic acid, rosmarinic acid, lithospermic acid and salvianolic acid B, salvianolic acid A, salvianolic acid C, salvianolic acid H or I, I or H, D, G, E and B or E isomer possess antioxidant effect and scavenging $O_2^{-\cdot}$ activities according to our previous research.[12]

3,4-Dihydroxyphenyllactic acid was shown to exhibit strong antioxidant activity by scavenging reactive oxygen species (ROS) in previous studies.[27] 3,4-Dihydroxyphenyllactic acid was selected as positive control to control the quality of Danshen injections in current studies. The above standard potency curves showed that the relationship between biological activities and contents of active constituents is not linear, thus the whole biological activities of herbal medicines are unequal to the sum of biological activities of all active constituents. If 3,4-dihydroxyphenyllactic acid (1 μg/ml) was presumed as a potency unit, the relative activities of peaks in antioxidant activity fingerprint were calculated as potency unit according to standard potency curves of 3,4-dihydroxyphenyllactic acid. Therefore the relative percentages of inhibition $O_2^{-\cdot}$ of all common peaks were expressed potency unit of 3,4-dihydroxyphenyllactic acid equivalent antioxidant capacity according to standard potency curves.

Twenty-one batches samples collected from a variety of drugstores were analyzed to simulate the standard potency fingerprint. According to ministry standard of traditional Chinese medicine (WS3-B-3766-98), only protocatechuic aldehyde has been selected as the mark compound for the

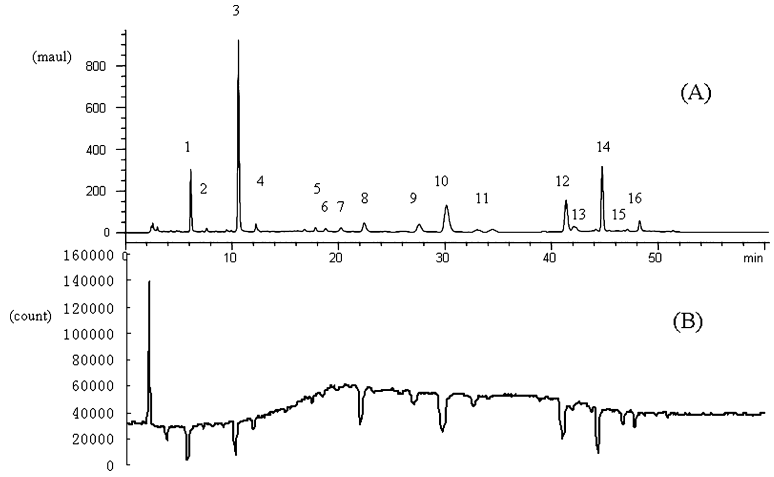

Fig. 1. The Representative HPLC-DAD and PLD Profiles of Danshen Injections

(A) DAD; (B) PLD.

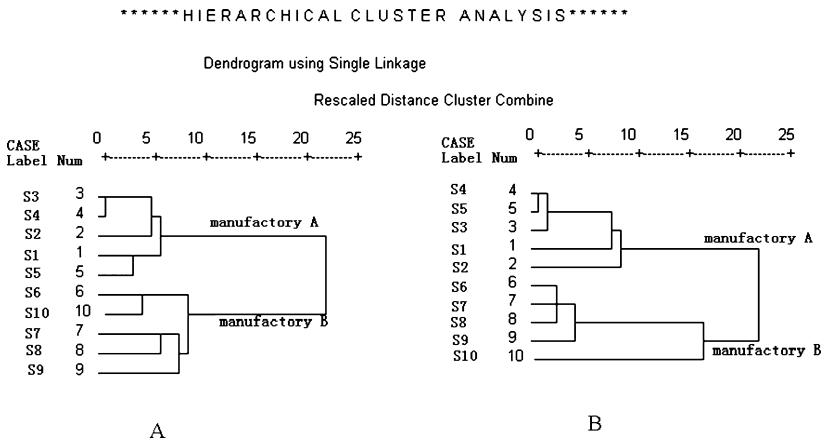

Fig. 2. Dendrogram of Clustering Analysis

(A) Based on the antioxidant activity, (B) based on the chemical information. Samples 1—5 come from the manufactory A and 6—10 come from the manufactory B.

quality control of Danshen injection. The contents of protocatechuic aldehyde in 21 batches samples collected are all higher than that of ministry standard, 21 samples could be regarded as acceptable samples. Peaks that appeared in all HPLC-PLD profiles of 21 batches were assigned as "common active peaks" which represented the bioactivity characteristics of Danshen injection. There were 13 common peaks in the antioxidant activity fingerprints. Thirteen common peaks included peaks 3,4-dihydroxyphenyllactic acid, protocatechuic acid, protocatechuic aldehyde, caffeic acid, salvianolic acid D, salvianolic acid G, salvianolic acid E, rosmarinic acid, lithospermic acid, salvianolic acid B, salvianolic acid B or E isomer, salvianolic acid A and salvianolic acid C. Therefore the relative percentages of inhibition $O_2^{-\cdot}$ of all common peaks were expressed by potency unit of 3,4-dihydroxyphenyllactic acid equivalent antioxidant capacity according to standard potency curves and the antioxidant fingerprint can be converted into potency fingerprint of Danshen injections.

Distinguishing Danshen Injection from Different Origins Using Potency Fingerprint Two dendrograms were prepared using the average linkage between groups (Fig. 2). At the higher rescaled distance of 15, the 10 Danshen injections were respectively divided into two main clusters based on peaks characteristics from the potency and chromatographic profiles of the tested 10 samples, and the samples from manufacturer A (1—5) are different from the samples from manufacturer B (6—10). Their results are all consistent with actual origins. It was concluded that the potency fingerprint can distinguish Danshen injections from different origins as well as HPLC fingerprint. Therefore, the proposed potency fingerprint is more rational to reveal the activity characteristics of the total phenolic acids from Danshen injections, so that it has much more classification and discrimination power in quality control.

Evaluation on the Contribution of Main Antioxidants to Efficacy Using Potency Fingerprint Although main antioxidants could be screened from Danshen injections, it was difficult to calculate the contribution of antioxidant to overall efficacy of samples by above activity fingerprint discussed. In contrast to activity fingerprint, the potency fingerprint could not only represent the antioxidant activity of individual peak, but also provide the total antioxidant activity for Danshen injections. So the contribution of antioxidant to overall efficacy can be obtained by the equation

$$\text{contribution (\%)} = \text{the antioxidant activity of individual peak/the total antioxidant activity}$$

In order to evaluate really the function of individual antioxidant to the global efficacy, 21 samples collected from a variety of sources were analyzed by proposed potency fingerprint. The activity contributions of 13 common peaks analyzed were shown in Fig. 3A. It could be seen that the contributions of common peaks in 21 samples varied markedly, and the highest contributions of 3,4-dihydroxyphenyllactic acid and much higher contributions of protocatechuic aldehyde were generally found in all samples. The total contributions of 3,4-dihydroxyphenyllactic acid and protocatechuic aldehyde fell in the range 58.2—98.3%. With respect to the other eleven common peaks except salvianolic acid B, there were relatively low contributions which are less than 10% to overall efficacy of Danshen injections. Meanwhile, the chemical contributions of common peaks were calculated based on the chromatographic fingerprints of 21 samples (Fig. 3B). The results showed that the contribution of protocatechuic aldehyde is generally higher than 3,4-dihydroxyphenyllactic acid. This result may be related to the ministry standard of traditional Chinese medicine. Rosmarinic acid, salvianolic acid B and salvianolic acid A have relatively high contributions to overall efficacy. The results from two methods are not consistent. Based on the analysis results above, it was concluded that the potency fingerprint is superior to chromatographic fingerprint to exhibit the contributions of active constituents to overall efficacy.

It was also shown that the activities of protocatechuic aldehyde and 3,4-dihydroxyphenyllactic acid are higher than that of other peaks in the samples. Although it was demonstrated that 3,4-dihydroxyphenyllactic acid and salvianolic acid B are efficient radical scavengers and antioxidants, and salvianolic acid B is superior to 3,4-dihydroxyphenyllactic acid,[28] and salvianolic acid B is the most common component in *Salvia* species and the most abundant in their aqueous extracts, it has lower contents and accounts for a little of activity of Danshen injection. This difference may be related to the preparation process. Therefore a potency fingerprint is an economical method for prescreening active constituents in

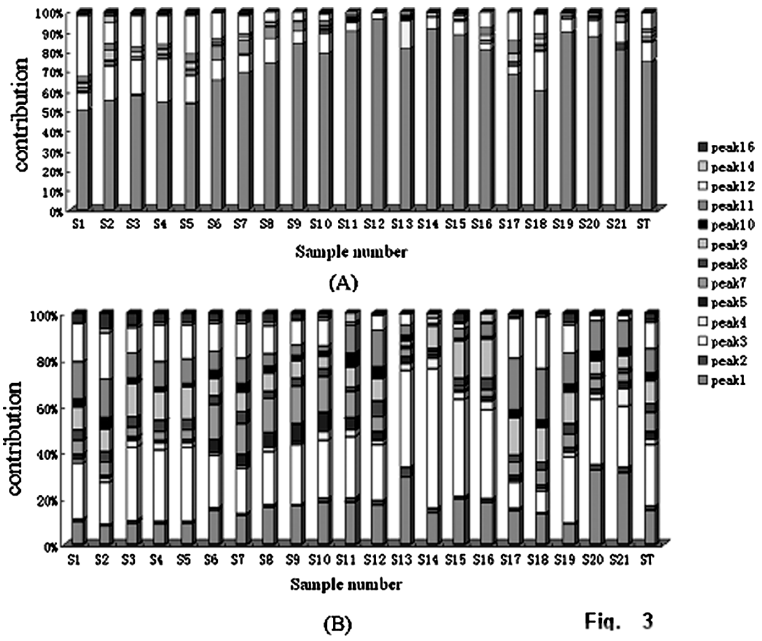

Fig. 3. Contributions of the Phenolic Acids to Danshen Injection

(A) The activity contribution to the total activities; (B) the area contribution to the total areas. Peak 1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 14, 16 respectively stand for 3,4-dihydroxyphenyllactic acid, protocatechuic acid, protocatechualdehyde, caffeic acid, salvianolic acid D, salvianolic acid G, salvianolic acid E, rosmarinic acid, lithospermic acid, salvianolic acid B, Salvianolic acid B or E isomer, salvianolic acid A and salvianolic acid C. S1—21 stand for samples 1—21 come from different factories and ST stands for mean standard sample.

TCMs preparation. It is of great significance for many manufacturers to clarify the possible mechanism of action of herbal products.

Combination Use of the Chromatographic Fingerprint and Potency Fingerprint with PCA It was reported that the differences of the samples were visually analyzed by principal components analysis (PCA).[29] The 3D-PCA scores plot (shown in Fig. 4A) demonstrated that chemical information obtained from the 13 common components was enough for discriminating the unacceptable samples from 21 batches samples. It can be seen that 21 samples and one simulate standard sample 22 can be divided into three clusters, and the samples 13, 20 and 21 were far away from simulate standard sample. So they could be regarded as unacceptable samples for quality control based on chemical information. Seen from Fig. 4B samples were divided into two main clusters. All samples except for 1, 13 and 21 were clustered together with the standard fingerprints firstly, which suggested that these products were superior to samples 1, 13 and 21. Samples 1, 13, 21 were different from other samples, which were unacceptable samples for quality control according to antioxidant activity. Therefore, the combination use of the chromatographic fingerprint and potency fingerprint can represent the "similarities" and "differences" of not only chemical constituents but also efficacy of Danshen injections.

Conclusion

In this paper, the potency fingerprint has been successfully developed to control TCM preparation Danshen injections by HPLC-UV-PLD methods. The proposed potency fingerprint could provide not only the antioxidant activity of the individual chromatographic peaks but also the total antioxidant activity of whole sample. It has been shown to be a suitable tool for Danshen injection classification or identification pur-

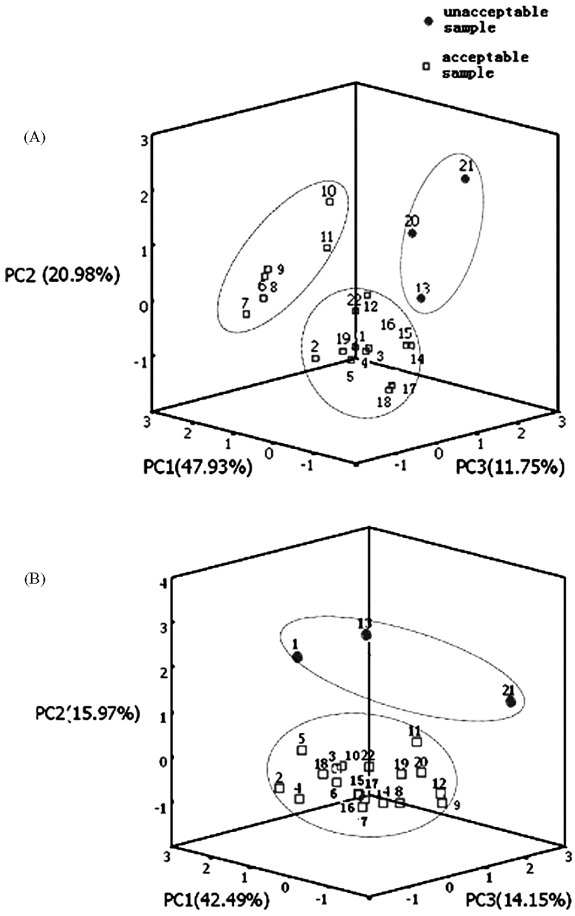

Fig. 4. The 3D-PCA Scores Plot of Danshen Injections Showing Differentiation According to Different Fingerprints

(A) Based on chromatographic fingerprint, (B) based on the potency fingerprint. Number 1—21 stand for samples 1—21 come from different factories and 22 stands for mean standard fingerprint.

poses. It could be applied to the quality control of the extract of other herbal medicines. Meanwhile, the potency fingerprint could directly reveal the contribution of the main active constituents to the global efficacy of TCM preparations. It could be concluded that the combination of a chromatographic fingerprint with potency fingerprint was a preferred strategy to control the quality and to clarify the possible mechanism of action of herbal products. Considering the bioactivity diversity of ingredients in herbal drugs, multiple potency fingerprints may be required for adequately assessing quality in the future.

Acknowledgements This research was financially supported by Ministry of Science and Technology of China (No. 973: 2005CB523404).

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