Prediagnostic methods for the hemolysis of herbal medicine injection

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**A R T I C L E   I N F O**

Article history:
Received 7 March 2011
Received in revised form 30 August 2011
Accepted 18 September 2011
Available online 22 September 2011

Keywords:
Ginsenoside
Hemolysis
HPLC
Fuzzy dissemination
Adverse drug reaction

**A B S T R A C T**

*Ethnopharmacological relevance:* The Xue-Sai-Tong injection, a traditional Chinese medicine injection with total saponins extracted from Sanchi Ginseng, has been used for more than half a hundred years to treat coronary artery disease. The study is to establish a prediagnostic method for the hemolytic adverse effect of herbal medicine injection by taking Xue-Sai-Tong injection as an example.

**Materials and methods:** A new method named “fuzzy dissemination” was established to identify the hemolytic ginsenosides in Xue-Sai-Tong injection on the basis of fuzzy changes of individual ginsenosides in the injections altered by re-adding the fractions prepared from the total saponins and statistic analysis between hemolytic degrees and individual ginsenosides. Related substances test, safety tests and fingerprints of the injections in different batches were tested.

**Results:** HD50, P50 and interactions on hemolysis of individual ginsenosides were examined. Experiment indicated that the content of Rg1, Rg2, M1 (an unknown ingredient with retention time at 51 min in HPLC) and M9 in Xue-Sai-Tong injection showed a significant positive correlation with hemolytic degree, and the content of R1, Re, Rb1 and Rd showed a significant negative correlation with hemolytic activity. Furthermore HD50 of injection exhibits superiority to other tests for the hemolysis of injections. Abnormal hemolysis in some batches of injections was observed, but there were no significant differences among injections of different batches in related substances test, safety test and fingerprints.

**Conclusions:** This is an original method to analyze active ingredients of a complicated integrity instead of studying on individual ingredients, it showed that the interactions of some individual ginsenosides and some unknown micro-ingredients in Xue-Sai-Tong injection were the major factors causing hemolysis, and this method could also be utilized in research of corresponding aspects. HD50 of injection can reflect the changes of hemolytic property of injections caused by not only the change of active constituents of injection, but also the auxiliary materials. Thus it was recommended as an index for the hemolytic prediagnosis of the injections in practice.

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**1. Introduction**

Herbal medicine injections are widely used in China and Europe, such as the injections of ginkgo leaf, ginseng or even compound vitamins. The complicated ingredients are the outstanding characteristic of the injections, which make their quality difficult to be controlled. Thus adverse drug reactions (ADR) of herbal injections happened frequently (SFDA, 2006; Xu, 2006; Yi et al., 2009). There are over 700 kinds of herbal injections used clinically, about 120 of which have been approved by China’s State Food and Drug Administration (SFDA) and produced by several hundreds of pharmaceutical manufacturers. In recent decade, more and more reports indicated that the ADR of herbal injections is serious and

SFDA has noticed to stop the production of 7 kinds of herbal injections (SFDA, 2006).

Hemolysis is one of saponins’ properties and can lead to hemolytic ADR of injections containing saponins. Thus, the method establishment for prediagnosing ADR of herbal injections is imperative at present. This paper deals with the establishment of prediagnostic method for hemolysis of herbal injections by taking ginsenosides and injections containing ginsenosides as examples.

Sanqi ginseng, the roots of Panax notoginseng (Burk.) F.H. Chen, is an important ancient herbal medicine widely used for more than two thousands years in traditional Chinese medicine to treat atherosclerosis and cerebral infarction (Wang et al., 2008). Ginsenosides, were considered as the principal ingredients responsible for the pharmacological activities of the drug and also Sanqi ginsenosides with the purity of over 80% are the active components of Xue-Sai-Tong injections generally used as anti-coronary medicine (Yao and Li, 2001). Clinically Xue-Sai-Tong injection was reported
to possess hemolytic ADR and is suitable subject in our research for we have examined the various pharmacological actions of ginsenosides previously (Dou et al., 2001; Yu et al., 2005; Qiu et al., 2009).

Visualization, erythrocytometry and colorimetry are the main methods for determining the hemolytic reaction. In China’s Pharmacopoeia, visualization method was proposed for hemolytic test of injection which exhibited defect of poor accuracy. So colorimetry was established to evaluate the hemolytic adverse effect of TCM injections in our previously study (Zhou et al., 2002; Yang et al., 2008).

This study is designed to investigate hemolytic and hemolytic protection properties of ginsenosides firstly, then the identification method for the hemolytic constituents of Xue-Sai-Tong injections as well as practical prediagnostic methods for TCM injections.

2. Materials and methods

2.1. Materials and chemicals

Xue-Sai-Tong injections were purchased from Harbin Zhenbao Pharmaceutical Co., Ltd. (Batch No. 20091108 43, 20090911 42, 20091217 44 and 20091006 31, Harbin, China), Wanrong-Sanjiu Pharmaceutical Co., Ltd. (Batch No. 0911271 and 0911273, Henan, China), Kunming Xingzhong Pharmaceutical Co., Ltd. (Batch No. 20100312, Kunming, China), Kunming Pharmaceutical Co., Ltd. (Batch No. 0911212, Kunming, China), Harbin Sancity Pharmaceutical Co., Ltd. (Batch No. 20081121, Harbin, China), Yunnan Phytopharmaceutical Co., Ltd. (Batch No. 200609011), Huayuan-Changfu Pharmaceutical Co., Ltd. (Batch No. 20071206) and Livzon Pharmaceutical Co., Ltd. (Batch No. 1001017 and 1002025, Guangdong, China). Saponin from Quillaja bark was purchased from Sigma–Aldrich, Inc. (St. Louis, USA). Ginsenosides-Rg1 was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), ginsenosides Rg1, Rg2, Rb1, and Rd were isolated and identified in our laboratory from ginseng with over 98% of purity. HPLC-grade acetonitrile was purchased from Tedia Company Inc. (Fairfield, OH, USA) and other organic solvents for HPLC analyses were purchased from Kermel Chemical Co. (Tianjin, China) and filtered through 0.45 μm organic membranes prior to use. Water was purified using Milli-Q-plus filter systems (Millipore, Bedford, MA, USA). All other reagents were of either analytical or HPLC grade.

UV-vis spectrophotometer was purchased from Unique Co. (Shanghai, China), Agilent 1100 series HPLC was purchased from Agilent Technologies, Inc. (USA), Olympus BX50 (Japan). Cryostat microtome system Lecia RM-2135 was purchase from LEICA. Co. (Germany). Similarity Evaluation System (SES) for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2004A) was purchase from committee of China’s Pharmacopeia.

Formaldehyde, dimethyl benzene and mineral wax were purchased from Shenglong Chemical Co. (Ningbo, China), hematoxylin and eosin were purchased from Kailiang Biochemical Co. (Shanghai, China).

Animals for experiment were purchased from Dalian Medical University. The animals were fed ad libitum with standard feed and water in the course of the study. All experimental procedures were in accordance with institutional animal care guidelines.

2.2. Hemolytic activity assay

After the hemaleucin in the whole blood of rabbit was discarded, the blood was washed three times with isotonic sodium chloride solution. The washed erythrocytes suspended in physiological saline solution in a final concentration of 1.1% (v/v). Hemolytic degree of ginsenosides or Xue-Sai-Tong injection was assayed by colorimetry, according to the reported method with minor modification (Yang et al., 2008). The total saponins standard solutions of different concentration and isotonic saline solution were added into tubes, which made up to 3.5 mL. The erythrocyte suspension (2.5 mL) was added into above mixed solution and the mixture was incubated for 2 h, at 37 °C. After incubation, the reaction mixture was centrifuged at 3000 rpm for 10 min and the absorbance at 575.0 nm of the supernatant was measured by a UV–vis spectrophotometer. Distilled water was used in place of physiological saline solution, which gave 100% hemolysis (positive control group). Hemolytic degree (HD) of each sample was determined, and the formulation as follows: HD (\\%) = (ODpc-ODnc)/(ODpc-ODnc) × 100, where ODpc, ODnc and ODpc represent the absorbance of experimental group, negative control group and positive control group, respectively. Hemolytic activities of Xue-Sai-Tong injections of different batches were also assayed as above.

Saponin, purchased from Sigma–Aldrich, Inc, was used as a standard substance which could give 100% hemolysis. Standard curve of content to hemolytic degree of “saponin” was constructed as above description, and half hemolytic dosage (HD50) was also calculated. The hemolysis and hemolytic protection of individual ginsenosides were assayed by HD50 and P50, according to the reported method (Namba et al., 1974). P50 was an indicator of sample which gave 50% protection (P50) of the control hemolysis caused by the HD50 amount of such hemolytic reagent as “saponin”.

2.3. Interactions of individual ginsenosides on hemolysis and hemolytic protection

Ginsenoside Rd at concentration of 350 μg/mL was used as an indicator which gave 50% hemolysis to assay the interactions of other ginsenosides. Rg1 (0→9 μg/mL) or Rg2 (0→400 μg/mL) of different concentration were added into test tubes, respectively, the concentration of Rg1 and Rg2 involved 0 to 100% protective activity against hemolysis caused by “saponin”.

2.4. Preparation of extracts from Xue-Sai-Tong injection

Xue-Sai-Tong injection (100 mL) was extracted with n-butanol saturated with water, the resulting butanol layer was evaporated to dryness to give the total saponins. Then, the total saponins was subjected to column chromatography over silica gel and eluted with a gradient of CHCl3–MeOH (100:0→1:1) to give five fractions (A→E).

2.5. Preparation of standard solutions for saponins content analysis and hemolytic activity assay

Xue-Sai-Tong injection was diluted in isotonic saline solution to produce stock solution containing 10 mg/mL total saponins. Ginsenoside Rg1, a marker for content analysis of other ingredients in Xue-Sai-Tong injection, was dissolved in methanol at concentration of 1.02 mg/mL. Fraction A was not dissolved in isotonic saline solution, so fractions A and B were mixed as the ratio of content in Xue-Sai-Tong injection, the final concentration of this stock solution was 4 mg/mL. All the other fractions were dissolved in isotonic saline solution, respectively, and the final concentrations of the stock solution were 4 mg/mL, respectively. Pyridine was used as solubilizer in a final concentration of 3.0% (v/v). Isotonic saline solution of above stock solutions was replaced by methanol for HPLC analysis.

Stock solutions of fractions (A→E) with different additions were re-added into Xue-Sai-Tong injection stock solution of different volumes on the basis of designed method, respectively. Fractions A and B (0.17, 0.3, 0.6, 0.9, 1.2 and 1.8 mL), fraction C (0.2, 0.9, 1.0 and 1.2 mL), fraction D (0.2, 1.44 and 2.0 mL) or fraction E (0.2, 0.8 and
2.6. Contents analysis by HPLC

2.6.1. HPLC analysis

Content analysis was achieved in an Agilent 1100 series HPLC system with pump (Agilent model G1314A VWD), and a phenomenex-C$_3$$_8$ column (4.6 mm $\times$ 250 mm, 5 $\mu$m particle size) protected by a pre-column from the same company, eluted with water–phosphoric acid (100:0.5) (A) and acetonitrile (B) in gradient at the flow rate of 1 mL/min. The solvent gradient for determining consisted of 19% B at the beginning, 19% at 12 min, 36% at 60 min, 50% at 75 min, 65% at 85 min. UV absorption of the column effluent was determined at 203 nm. The injection volume was 10 $\mu$L for each fraction. Peak identification was performed by comparison of retention times, and thermostated at 25 $^\circ$C.

2.6.2. Development and validation of HPLC method

2.6.2.1. Calibration. Curves were constructed using standard solutions of ginsenoside R$_{g1}$ in the concentration range 0.1–60 mg/mL. Each sample (10 $\mu$L) was determined in triplicate and the average detector responses were used by the software to construct the curve.

2.6.2.2. Linearity. Aliquots (10 $\mu$L) of 6 solutions of ginsenoside R$_{g1}$ was analyzed in triplicate and the average detector responses were used by the software to construct the curve.

2.6.2.3. Precision. Standard solutions of ginsenoside R$_{g1}$ (1.02 mg/mL) were injected in sextuplicate, respectively, in order to determine the standard deviation of the method on the same day.

2.6.3. Content analysis of ingredients in standard solution

Content analysis of ingredients in standard solution was performed as the reported method using one chemical substance (marker), such as ginsenoside R$_{g1}$, in this experiment, to calculate multi-components simultaneously. The relative correction factors (RCF) of other ingredients to ginsenoside-R$_{g1}$ was approximate as 1 for the similarity of UV absorption of saponins (Wang et al., 2006; Zhu et al., 2008).

2.7. Statistical analysis

Statistical analysis was performed by correlation analysis and stepwise regression analysis using statistical software SPSS 17.0 according to the relationship between content and hemolytic degree in the experiment.

2.8. The effect of pH on the hemolysis of injection

Owing to the solubility of natural constituents, sometimes pH of injections should be adjusted. So, phosphate buffered solutions of different pH ranged from 5.8 to 8.0 and alkaline solutions of physiological saline solution containing NaOH whose pH ranged from 8.0 to 11.0 were selected to substitute saline solution to assay the hemolytic degree of the injection of different pH, and the experimental process was the same as fraction 2.2 “Hemolytic activity assay”.

2.9. Related substances test and safety tests of injection

On the basis of abnormal hemolytic phenomena, the color change was similar to hemoglobin denatured. It has been reported that the injection containing tannin in a certain concentration can make red blood protein precipitation and deformation, degeneration, rupture like flocculent precipitate (Yuan et al., 1994). Thus, injections related substances test, such as resin, tannin and protein examination and safety tests, such as active systemic anaphylaxis test, vascular and muscular irritant tests have been performed on Xue-Sai-Tong injections of different batches according to China’s Pharmacopoeia (Committee of China’s Pharmacopoeia, 2010).

2.10. Fingerprints of Xue-Sai-Tong injection

Fingerprints of Xue-Sai-Tong injection with different batches were also investigated in order to explore the reason of the abnormal hemolysis phenomena. And the experimental conditions as above fraction of “HPLC analysis”. Similarity assay was performed by similarity evaluation system for chromatographic fingerprint of Traditional Chinese Medicine.

3. Results and discussion

3.1. Assay of hemolysis and hemolytic protection of ginsenosides

Standard curve of hemolysis caused by “saponin” was constructed by sigmaplot 10.0 (Fig. 1), HD$_{50}$ of saponin was calculated as 2.23 $\mu$g/mL. As shown in Fig. 1, the concentration of saponin at HD$_{50}$ is the most sensitive to changes of hemolytic degree. So HD$_{50}$ and PD$_{50}$ were used as indicators for assaying hemolytic and hemolytic protection of individual ginsenosides. HD$_{50}$ and PD$_{50}$ of individual ginsenosides were determined as shown in Table 1.

Our result is similar to that in previous research on the hemolytic research of ginsenosides, indicating that saponins always exhibited hemolysis at higher concentration, but showed hemolytic protection at lower concentration.

Then the interactions of individual ginsenosides on hemolysis and hemolytic protection was carried out, showing that Rs at the concentration of 1 $\mu$g/mL, which had no hemolytic protection caused by “saponin”, gave 50% hemolytic protection caused by Rd.
(400 μg/mL). Rg1 at the concentration of 400 μg/mL, which had absolutely protective activity of hemolysis caused by “saponin”, had no protection of hemolysis caused by Rd (400 μg/mL). Thus, we could make a conclusion that the hemolytic degrees caused by mixture of ginsenosides were not the addition or subtraction of hemolytic degrees of individual ginsenosides.

3.2. Method protocol for the identification of hemolytic components of injections

The hemolytic interaction rules of individual ginsenosides are complex in that an individual ginsenosides can exhibit both hemolytic and hemolytic protection in the nonlinear type way. So the component identification for hemolysis and hemolytic protection of injection should be explored from their behaviors in total saponins. Although statistical analysis has been widely used in the evaluation and assay of herbal medicine (Kitano, 2002; King et al., 2004), the chemical constituents in the samples obtained from different places exhibited discrimination. And in most cases only the part fractions or individual constituents from a herbal medicine are assayed, the interactions caused by other constituents will be overlooked (Yu et al., 2005). Thus, a new method named “fuzzy discrimination” was established to identify the hemolytic ginsenosides in the injection. The basic principle of the method is to examine the hemolytic behavior of individual ginsenosides in total saponins over the 5% hemolytic degree so as to reflect the hemolytic contribution of individual saponins. The basic protocol for this method is designed as follows:

1. Test the hemolytic degree of the total saponins or the injection solution to find the concentration for 5% or less hemolytic degree.
2. Separate the total saponins or the injection solution into different fractions, but try to make main constituents higher in a single fraction.
3. Add the fractions with different concentrations to the solution with 5% hemolytic degree to make different test samples.
4. Assay the hemolytic degree of the test samples.
5. Identification of the hemolytic constituents can be performed by correlation and regression methods.

3.3. Validation of HPLC method

The HPLC method was validated as follows. The calibration curves for ginsenoside Rg1 y = 344.265x – 70.067 (r=0.9998, n=6, 0.1–6.0 mg/mL). The variation coefficient of ginsenoside Rg1 was 0.16% for the intra-day assays. The accuracy of the method was examined by performing recovery experiments according to the method of standard additions. Ginsenoside Rg3 stock solution was added before the extraction at different concentration levels around half of the analyzed amounts in Xue-Sai-Tong injection. Samples were prepared in triplicate at each level. Mean recovery of Ginsenoside Rg1 was 101.23% (RSD% = 1.64), which presented good accuracy for the analysis.

Contents of all the ingredients in the standard solutions with different content of individual ginsenosides were calculated on the basis of the content of marker (ginsenoside Rg1) by HPLC. The HPLC chromatogram of Xue-Sai-Tong injection as follows (Fig. 2).

3.4. Hemolytic assay of Xue-Sai-Tong injection

The hemolysis of Xue-Sai-Tong injection was determined by colormetry. Standard curve of concentration to hemolytic degree was constructed by the software of Sigmaplot 10.0 (Fig. 3). $R^2$ of the equation of the standard curve was 0.9985. Half hemolytic dosage (HD50) of the injection was 8.856 ± 0.329 mg/mL (mean ± SD). Variation coefficients of HD50 of the curve were 3.71%. The results from Fig. 3 showed that erythrocytes were almost not dissolved until the concentration of Xue-Sai-Tong injection was 4.875 mg/mL (HD5).

Hemolytic degrees of standard solutions with different contents of individual ginsenosides were determined according to the method showed in the experiment fraction. In this experiment, the five fractions (A – E) isolated from Xue-Sai-Tong injection were re-added into the reaction system which contained Xue-Sai-Tong injection, so that all the ingredients in the injection could be included.

The contents of standard solutions with different contents of individual ginsenosides were determined by HPLC. And the hemolytic activities of the above standard solutions were also examined by colormetry. The contents and hemolytic activities of standard solutions were obtained, respectively. So, the relationship between content and hemolytic activity of ginsenosides was built up by statistics.

The experiment data were analyzed by SPSS 17.0, and the results of correlation analysis indicated that the content of Rg3, Rg2, M51

<table>
<thead>
<tr>
<th>Ginsenosides</th>
<th>$P_{50}$ (μg/mL)</th>
<th>HD50 (μg/mL)</th>
</tr>
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<tbody>
<tr>
<td>Rb1</td>
<td>43.01</td>
<td>Non-hemolysis (480 μg/mL)*</td>
</tr>
<tr>
<td>Rb2</td>
<td>24.66</td>
<td>Non-hemolysis (2000 μg/mL)*</td>
</tr>
<tr>
<td>Rg1</td>
<td>7.42</td>
<td>Non-hemolysis (543.2 μg/mL)*</td>
</tr>
<tr>
<td>Rd</td>
<td>73.01</td>
<td>356.78</td>
</tr>
<tr>
<td>Re</td>
<td>53.60</td>
<td>Non-hemolysis (420 μg/mL)*</td>
</tr>
<tr>
<td>Rg2</td>
<td>38.88</td>
<td>Non-hemolysis (420 μg/mL)*</td>
</tr>
<tr>
<td>20(R) Rg2</td>
<td>8.79</td>
<td>257.81</td>
</tr>
<tr>
<td>20(S) Rg2</td>
<td>81.73</td>
<td>732.99</td>
</tr>
<tr>
<td>Rh1</td>
<td>100.39</td>
<td>490.68</td>
</tr>
<tr>
<td>Ro</td>
<td>10.97</td>
<td>Non-hemolysis (1230 μg/mL)*</td>
</tr>
</tbody>
</table>

Note: the results are calculated from Sigmaplot 10.0.

* The highest concentration in this experiment.
3.5. The effect of pH on the hemolysis of injection

Our results revealed that acid solution of PBS whose pH less than 7.0 or alkaline solution whose pH over 8.0, which could not destroy the erythrocyte by itself, could obvious enhanced the hemolysis of the injections containing saponins. So, pH of injection is an important index and should be adjusted correctly (Table 2).

3.6. HD50 as a prediagnostic index for injections

As shown above, some minor constituents of injections or pH change can induce the hemolysis of injections. However the change of minor constituents is not easy to be identified for they are difficult to be identified and quantified in most cases. So HD50 can be applicable for the prediagnostic index for injection hemolysis. The HD50 of 11 batches of injections were determined and the results are in Table 3. But some batches of injections showed abnormal hemolytic reactions at higher concentrations of 10.0 mg/mL with reaction system turned into brown color when it came to about 1.5 h after water-bathing as shown in Fig. 4. The maximum absorbance wavelength of the supernatant whose color turned into brown was not at 575.0 nm, so the absorbance of supernatant could not be measured correctly at 575.0 nm.

According to China’s Pharmacopoeia, the safety test including anaphylaxis, muscle and vein irritating and related substances tests were further explored, indicating that the Xue-Sai-Tong injections which showed abnormal hemolysis in vitro experiments had no significant differences among injection of different batches. All the injections were qualified according to the China’s Pharmacopoeia. But the tissue sections showed that there was slightly muscular stimulation. One of the external rabbits slight hyperemia quadriceps, and striated muscle fibers of one rabbit arranged in some abnormal tissue, muscle bundle fracture, cell degeneration and necrosis, accompanied by inflammatory cells infiltration. The results also enlighten us that the methods for related substances tests of herbal injections should be modified to improve their sensitivity for there must be any changes of chemical constituents for the injections with abnormal hydrolysis.

As usual fingerprint is an important method for the qualitative control of herbal injections. However the results of fingerprints showed that all the injections exhibited good similarity in HPLC chromatographs with over 90% similarity which is up to the standards of China’s Pharmacopoeia. And all the components in the injection had no difference from each other (Fig. 5). So, fingerprints of injection could not reveal the quality change of injections in this case.

Thus HD50 of injections can reflect the change of not only minor constituents but also the factors with no peak exhibited in the chromatogram.
Table 3

<table>
<thead>
<tr>
<th>Similarities of Xue-Sai-Tong injections with different batches.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZBD-20091108</td>
</tr>
<tr>
<td>0.99</td>
</tr>
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</table>

Fig. 5. Fingerprints of Xue-Sai-Tong injections with different batches.

4. Conclusions

It is for the first time that fuzzy dissemination was reported and it can be applied to analyze active ingredients with synergy in a complicated integrity instead of studying on individual ingredients. The method emphasized to reflect the individual situation within range of playing action (0–100%) and with consideration of individual interactions. Thus, it is recommended in not only ADR of traditional Chinese medicine injection, but the key indexes of active substances of traditional medicine with multi-ingredients. In addition, HD50 of injections is also an original indicator in supervising adverse reaction of herbal injections, it could be a supplementary in the evaluation of herbal medicine injection. The application of HD50 to other injections is in progress.

Acknowledgements

Thanks for the National Natural Science Foundation of China (30973859) and Doctoral Fund of Ministry of Education of China (2009213310001) as well as the Outstanding Scholar supporting Plan of Liaoning Education Department (2008RC34).

Appendix A. Supplementary data


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