

## Simultaneous Determination of Components in Preparation Naodesheng Injection by High Performance Liquid Chromatograph–Atmospheric Pressure Chemical Ionization Mass Spectrometry (HPLC-MS/APCI)

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A high-performance liquid chromatograph with mass spectrum detection (HPLC-MS/APCI) method has been established for simultaneous determination of ten major bioactive components of Naodesheng injection including safflor yellow A, puerarin, daidzein, ginsenosides (Rg<sub>1</sub>, Rg<sub>2</sub>, Rb<sub>1</sub>, Rd, Re, Rh<sub>1</sub>), and notoginsenoside R<sub>1</sub>. The separations were carried out with a Luna C<sub>18</sub> column (5 μm, 150×4.6 mm, Phenomenex, U.S.A.) with a stepwise gradient elution of the mobile phase consisting of water (0.1% of formic acid, v/v)–methanol (0 min, 70 : 30; 8 min, 30 : 70; 20 min, 10 : 90) at a flow-rate of 0.8 ml/min. The proposed method was applied to analyze five various Naodesheng injections and produced data with acceptable linearity, repeatability, precision and accuracy having lower limits of quantitation (LLOQs) of 0.02–0.2 μg. The calibration curves were linear in respective range for all compounds, all of them with coefficients of determination above 0.9900. The intraday precessions were less than 5.0%. The proposed method is accurate, sensitive and simple, a useful alternative for routine analysis in the quality control of Traditional Chinese Medicine.

**Key words** safflor yellow A; puerarin; ginsenosides; notoginsenoside R<sub>1</sub>; HPLC-MS; Naodesheng injection

The ever-increasing worldwide attention to the pharmaceutical research of Traditional Chinese Medicine (TCM) has made it essential to carry out stringent quality control measures.<sup>1–5)</sup> 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 multiple bioactive components of TCM.<sup>6–8)</sup>

Naodesheng injection is a composite formula of TCM preparation comprising five raw materials or extracts including *Radix Puerariae Lobatae*, *Flos Carthami*, *Radix et Rhizoma Notoginseng*, *Rhizoma Chuanxiong*, and *Fructus Crataegi*, which is efficient in the treatment of cerebral arteriosclerosis, ischemic cerebral stroke, and apoplexy linger effect.<sup>9)</sup> It was reported that safflor yellow A, puerarin, daidzein, ginsenosides (Rg<sub>1</sub>, Rg<sub>2</sub>, Rb<sub>1</sub>, Rd, Re, Rh<sub>1</sub>), and notoginsenoside R<sub>1</sub> are the major bioactive components<sup>10–13)</sup> of Naodesheng injection. Therefore, it is significant to simultaneously determine these compounds to ensure the efficacy, safety, and batch-to-batch uniformity of Naodesheng injection.

Liquid chromatograph combined with mass spectrometry (LC-MS), a relatively new technique with rapidly growing popularity, has been employed to determine the contents of bioactive components, such as flavonoids, puerarin and saponins in herbal medicines.<sup>14–20)</sup> Although mass spectrometry is a more expensive and complex option than other HPLC or TLC detectors, LC-MS can greatly simplify the sample pretreatment procedures and shorten separation times of HPLC due to the high selectivity and sensitivity of MS detection, thus dramatically reducing the total analysis time.

At present, determinations of some of these components have been established by the use of TLC,<sup>21,22)</sup> HPLC-UV spectrometry,<sup>23–29)</sup> and mass spectrometry.<sup>19,30,31)</sup> However, none of these methods made the quick identification and quantification of these components in a single run. Although method to analyze the ginsenosides and notoginsenosides in

*Radix Notoginseng* has been described,<sup>32)</sup> that produced too long a chromatographic run and it appeared that no assay existed for simultaneous determination of the drugs in compound formula using HPLC-MS. In this study, an HPLC-MS method has been developed and validated for the quantitative determination of different kinds compounds including flavones (safflor yellow A, puerarin and daidzein) and sponins (ginsenoside Rg<sub>1</sub>, Rg<sub>2</sub>, Rb<sub>1</sub>, Rd, Re, Rh<sub>1</sub>, and notoginsenoside R<sub>1</sub>) in Naodesheng injection.

### Experimental

Safflor yellow A (>97.0%) was isolated in our laboratory (Shenyang Pharmaceutical University, Shenyang, P.R. China). Ginsenoside Rg<sub>2</sub> (>95.0%), Rd (>96.0%), and Rh<sub>1</sub> (>96.0%) were isolated in Department of phytochemistry (Shenyang Pharmaceutical University, Shenyang, P.R. China). Daidzein (>98.0%) was purchased from Sigma Company Inc. (U.S.A.). Puerarin, ginsenoside Rg<sub>1</sub>, Rb<sub>1</sub>, Re, and notoginsenoside R<sub>1</sub> were ordered from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, P.R. China). HPLC grade methanol was obtained from Dikma Company Inc. (U.S.A.). Analytical grade formic acid was obtained from Chemical Reagent Factory of Shenyang (Shenyang, P.R. China). Water was purified using a Milli-Q purification system (Millipore Co., France).

LCMS 2010 EV liquid chromatograph mass spectrometers (Shimadzu, Japan) equipped with an LC-10ADvp liquid chromatograph, DGU-14AM Degasser, SIL-HTC Auto sampler, and an atmospheric pressure chemical ionization (APCI) interface were employed. LCMS Solution 3.0 software was utilized.

The Separations were carried out with a Luna C<sub>18</sub> column (5 μm, 150×4.6 mm, Phenomenex, U.S.A.). The mobile phase was a stepwise gradient of water (0.1% of formic acid, v/v)–methanol (0 min, 70 : 30; 8 min, 30 : 70; 20 min, 10 : 90). Chromatography was performed at a flow-rate of 0.8 ml/min and the injection volume was 2 μl.

The detection conditions were as follows: Interface Temperature, 400 °C; CDL Temperature, 200 °C; CDL Voltage, +10.0 kV; Heat Block Temperature, 200 °C; Detector Voltage, 1.40 kV; Nebuling Gas Flow Rate, 2.5 l/min.

Sample Preparation Stock solution of the mixture of ten standards was prepared by dissolving accurately weighted portions of the standards in solution of methanol–water (1 : 1, v/v) (2.0 mg of safflor yellow A, ginsenoside Rg<sub>2</sub>, and notoginsenoside R<sub>1</sub>; 20.0 mg of puerarin; 1.6 mg of daidzein; 16.0 mg of ginsenoside Rg<sub>1</sub>; 20.0 mg of ginsenoside Rb<sub>1</sub>; 8.0 mg of ginsenoside Rd, and Rh<sub>1</sub>; 12.0 mg of ginsenoside Re), transferring the solution to a 10-ml volumetric flask, and then adding solution of methanol–water (1 : 1,

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v/v) to volume. The stock solutions were further diluted to make working standard solutions with methanol. All solutions were filtered through a 0.45  $\mu\text{m}$  membrane filter before HPLC analysis.

## Results and Discussion

The addition of 0.1 % of formic acid into the mobile phase was found to insure the stability of production of molecular ion  $[\text{M}+\text{HCOO}]^-$  and symmetry of peaks.

Under fixed chromatographic conditions, the kind of ionization interface is a major adjustment available for maximizing the detector response efficiency and improving the peak appearance. We have tried to apply with electronic spray ionization (ESI) interface to analysis the TCM Naodesheng injection. But in the TIC with ESI interface there are too many small fragments with the same ratio of  $m/z$  ( $m/z < 500$ ) of every component, which requests more stringent separation than using APCI interface. More than that, we have compared APCI interface in positive and negative mode. Negative mode can ameliorate resolution and peak appearance.

The standards of these components were analyzed by direct-flow injection to optimize the APCI-MS conditions. The spectra of these ten components showed different behaviors in their MS fragmentation. In the MS spectra obtained from fragmental molecule of these components, the most abundant fragment ion for each component was chosen for selective ion monitoring (SIM) quantification. The molecular fragments of them were  $[\text{M}-\text{C}_6\text{H}_{11}\text{O}_5]^-$  for safflor yellow A ( $m/z$ , 431.15),  $[\text{M}-\text{H}]^-$  for puerarin ( $m/z$ , 415.05) and daidzein ( $m/z$ , 253.05), and  $[\text{M}+\text{HCOO}]^-$  for ginsenoside  $\text{Rg}_1$  ( $m/z$ , 845.40),  $\text{Rg}_2$  ( $m/z$ , 829.40),  $\text{Rb}_1$  ( $m/z$ , 1153.75),  $\text{Rd}$  ( $m/z$ , 991.45),  $\text{Re}$  ( $m/z$ , 991.45),  $\text{Rh}_1$  ( $m/z$ , 683.35), and notoginsenoside  $\text{R}_1$  ( $m/z$ , 977.55), respectively. The HPLC-MS/APCI in the SIM mode provided a highly selective method for the determination of these ten components. The retention time of safflor yellow A, puerarin, daidzein, ginsenoside  $\text{Rg}_1$ ,  $\text{Rg}_2$ ,  $\text{Rb}_1$ ,  $\text{Rd}$ ,  $\text{Re}$ ,  $\text{Rh}_1$ , and notoginsenoside  $\text{R}_1$  was 5.904, 5.194, 9.929, 10.403, 15.773, 15.769, 15.773, 10.243, 14.275, and 9.823 min, respectively.

The sample injection needle was washed by methanol before each injection, this could prevent sample-to-sample contamination; the carryover between injected samples was investigated: the areas of the standard solutions for each component were very stable, and there were no impurity peaks or contamination in each chromatogram.

The assay linearity was determined by the analysis of six different concentrations of the standard solutions. Calibration was performed by a least-squares linear regression of the peak-area versus the respective standard concentration.

The lower limits of quantitation (LLOQs) were at the lowest concentration level among the linear ranges. Table 1 shows these results of regression data and LLOQs.

The intraday precisions of injection were evaluated using the results of five replicate injections of the standard solutions containing the ten components at a middle concentration. The repeatability of the quantitative procedure was based on the results of five analyses of one batch of Naodesheng injection sample. The relative standard deviations (RSD) of precision and repeatability of ten components were both less than 5.0%.

The recovery studies were carried out by spiking the same batch of Naodesheng injection sample five times with safflor

Table 1. The Regression Data and LLOQs of the Multi-Components Determined

Components	Linear range ( $\mu\text{g}/\text{ml}$ )	Correlation coefficients ( $r^2$ )	LLOQ ( $\mu\text{g}$ )
Safflor yellow A	10.0—200	0.9922	0.02
Puerarin	100—2000	0.9997	0.2
Daidzein	8.0—160	0.9992	0.02
$\text{Rg}_1$	80.0—1600	0.9995	0.16
$\text{Rg}_2$	10.0—200	0.9971	0.02
$\text{Rb}_1$	100—2000	0.9984	0.2
$\text{Rd}$	40.0—800	0.9938	0.08
$\text{Re}$	60.0—1200	0.9951	0.12
$\text{Rh}_1$	40.0—800	0.9910	0.08
$\text{R}_1$	10.0—200	0.9967	0.02

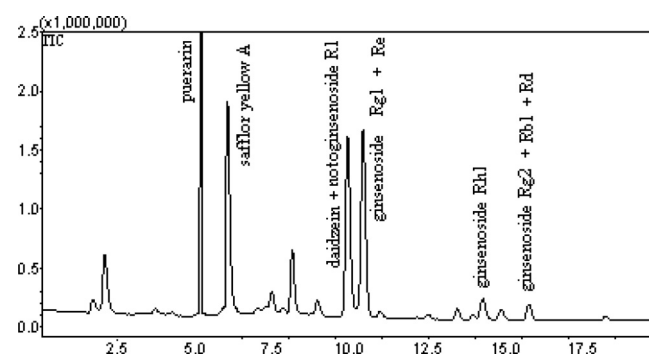


Fig. 1. Typical Chromatogram of Naodesheng Injection Sample

yellow A, puerarin, daidzein, ginsenosides ( $\text{Rg}_1$ ,  $\text{Rg}_2$ ,  $\text{Rb}_1$ ,  $\text{Rd}$ ,  $\text{Re}$ ,  $\text{Rh}_1$ ), and notoginsenoside  $\text{R}_1$  at the concentration level in the vicinity of the respective concentration of Naodesheng injection and comparing the determined amount of these standards with the amount originally added. The mean recovery rates of safflor yellow A, puerarin, daidzein, ginsenoside  $\text{Rg}_1$ ,  $\text{Rg}_2$ ,  $\text{Rb}_1$ ,  $\text{Rd}$ ,  $\text{Re}$ ,  $\text{Rh}_1$ , and notoginsenoside  $\text{R}_1$  were 96.1%, 101.3%, 99.7%, 100.1%, 97.2%, 98.5%, 99.3%, 98.7%, 97.5% and 99.4% with RSD of 3.20%, 2.75%, 1.95%, 1.78%, 1.68%, 1.02%, 1.83%, 1.67%, 2.04% and 2.31%, respectively. The relative standard deviation (RSD) was taken as a measure. It indicates that all the RSDs are less than 5.0%, and the method is thus acceptable.

The method was applied to analyze five batches of Naodesheng injection samples. The LC-MS/SIM chromatogram of a typical sample indicating the retention time of each component is shown in Fig. 1. The mean contents of safflor yellow A, puerarin, daidzein, ginsenoside  $\text{Rg}_1$ ,  $\text{Rg}_2$ ,  $\text{Rb}_1$ ,  $\text{Rd}$ ,  $\text{Re}$ ,  $\text{Rh}_1$  and notoginsenoside  $\text{R}_1$  are 69.08, 1211, 32.42, 482.8, 37.82, 614.2, 164, 356.6, 224.4, 79.82  $\mu\text{g}/\text{ml}$ , respectively. It was found that the concentration of each component in different samples is minutely different.

## Conclusions

Compared with other methods, HPLC-MS improved the resolution, specificity and sensitivity, shortened the analytical time of the samples. The main aim of the study was to establish a HPLC-MS method that was suitable for simultaneous determination of safflor yellow A, puerarin, daidzein, ginsenosides ( $\text{Rg}_1$ ,  $\text{Rg}_2$ ,  $\text{Rb}_1$ ,  $\text{Rd}$ ,  $\text{Re}$ ,  $\text{Rh}_1$ ) and notoginsenoside  $\text{R}_1$  in TCM Naodesheng injection. The method described

here has been found to be specific and accurate in application which is also suitable for the determination of each of the drugs studied. The proposed HPLC-MS method provides a useful alternative for the analysis of multi-bioactive components in TCM for quality control purpose.

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