# Simultaneous Determination of Multiple Bioactive Constituents in Total Alkaloid of *Sophora alopecuroides* by HPLC-DAD

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**Abstract:** **Objective** To develop a qualitative and quantitative simultaneous determination of multiple bioactive constituents in total alkaloid in *Sophora alopecuroides* (TASA). **Methods** In the experiment, a new and simple HPLC-DAD method for the simultaneous determination of multiple constituents in TASA was developed. The separation was performed on a Kromasil $C_{18}$ column (250 mm × 4.6 mm, 5.0 μm) eluted with 0.02 mol/L potassium dihydrogen phosphate (adjusted pH 4.3 using 1% glacial acetic acid) and acetonitrile (75 : 25) at a flow-rate of 0.7 mL/min. The detection wavelength was set at 210 nm. **Results** Five constituents (sophoridine, matrine, oxymatrine, aloperine, and lehmannine) were simultaneously analyzed in this study. Four of them were identified and determinated by the developed method. The calibration curves exhibited linear regressions ($r^2 > 0.9995$). The injection precision, the intra-day precision, and the analysis repeatability were validated with the RSD values less than 5.0%. The mean recoveries of the four constituents were ranged from 98.62% to 100.20%, and the RSD values were all less than 3.37%. **Conclusion** This method is convenient, fast, accurate, and is applicable to analyze the multi-constituents in TASA.

**Key words:** alkaloids; HPLC-DAD; qualitative analysis; quantitative analysis; *Sophora alopecuroides*

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## Introduction

Total alkaloid of *Sophora alopecuroides* L. (TASA) is a mixture extracted from the roots or seeds of *S. alopecuroides*, Chinese medicine *Kudouzi*, which has been used for gastrointestinal disorders in Chinese folk medicine for a long time (Mou *et al*, 2005). TASA has been widely used as an ingredient approved by Chinese State Food and Drug Administration for many years, and have pleiotropic effects including anti-inflammation, antivirus, antitumor, immunomodulation, and so on (Han, Zhou, and Liu, 2006; Chen and Deng, 2006; Zhao *et al*, 2009; Zhou, *et al*, 2010; Zhao, Song, and Deng, 2010). They are mainly made up of quinolizidine alkaloids, such as matrine, oxymatrine, sophoridine, sophocarpine, oxysopharpine, sophoramine, aloperine, lehmannine, and so on (Tian *et al*, 2010).

So far, several analytical methods have been described for the determination of quinolizidine alkaloids in TASA, such as acid-based titration, HPLC, HPCE, and GC-MS (Geng *et al*, 2006; Luo *et al*, 2005; Wan *et al*, 2009; Zhang *et al*, 2008). As we know, quinolizidine alkaloids in TASA such as matrine, oxymatrine, sophoridine, sophocarpine, oxysopharpine, sophoramine, aloperine, lehmannine, and so on share the similarity in physical and chemical property, and even biological transformation among these alkaloids. Moreover, matrine and sophoridine, sophocarpine and aloperine, oxymatrine and *N*-oxide-sophoridine belong to isomers. All these evoke great challenge to analysis of TASA. To our knowledge, no more than three alkaloids of TASA have been simultaneously determined by HPLC at present, and few reports about HPLC determination of lehmannine and aloperine in TASA have been published so far. Therefore, it is necessary to develop a method by which the quality control of multi-ingredient in TASA could be fully carried out.

The aim of the present work is to develop a simple, sensitive, and specific HPLC assay for the simultaneous qualitative and quantitative analysis of more alkaloids. The method will improve the quality control of multi-ingredient in TASA.

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## Materials and methods

### Instruments and chromatographic condition

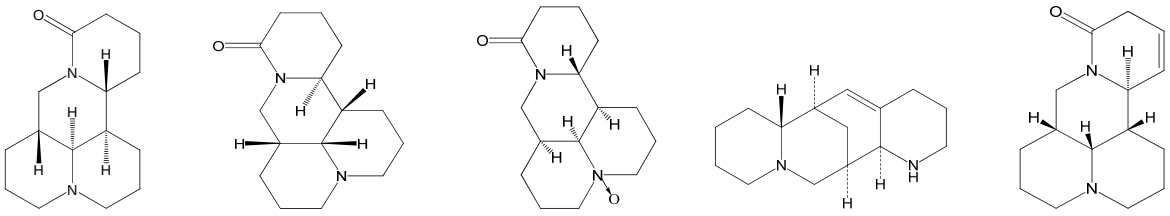
Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with the Chem Station software (Agilent Technologies) and comprised a quaternary pump, an online vacuum degasser, an autosampler, a thermostated column compartment and, a diode array detector (DAD), was used for the chromatographic analysis. Chromatographic separation was carried out on a Kromasil $C_{18}$ column (250 mm × 4.6 mm, 5.0 μm). The solvent system was composed of 0.02 mol/L potassium dihydrogen phosphate (adjusted pH 4.3 using 1% glacial acetic acid) and acetonitrile (75:25) at a flow rate of 0.7 mL/min. The detection wavelength was set at 210 nm. The injection volume was 10 μL. The column temperature was maintained at 30 ℃ and record time was 60 min.

### Reagents and materials

TASA (Batch No: 1, 2, and 3) was purchased from Ningxia Zijinghua Pharmaceutical Company (Ningxia, China). Voucher samples were deposited in the Insititute of New Drug Development, Guangdong Medical College. Standard substances including sophoridine (Batch No: 110784-200603), oxymatrine (Batch No: 110793-200503), matrine (Batch No: 110798-200603), aloperine (Batch No: 110693-200703), and lehmannine (Batch No: 110794-200503) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol and acetonitrile were of HPLC grade, and both were from Merck (Darmstadt, Germany). Other reagents were all of analytical grade. Deionized water used throughout the experiments was generated by a Milli-Q academic water purification system (Milford, MA, USA).

### Preparation of standard solutions

The standard stock solutions of sophoridine (0.41 mg/mL), oxymatrine (0.15 mg/mL), matrine (0.10 mg/mL), aloperine (0.11 mg/mL), and lehmannine (0.039 mg/mL) were prepared in methanol and stored away from light at 4 ℃. Working solutions of the lower concentration were prepared by appropriate dilution of the stock solution. Chemical structures were shown in Fig. 1.



sophoridine matrine oxymatrine aloperine lehmannine

**Fig. 1 Chemical structures of sophoridine, matrine, oxymatrine, aloperine, and lehmannine**

### Preparation of samples

About 30 mg TASA was extracted with about 30 mL of 20% acetonitrile in a dark brown calibrated flask for 20 min in an ultrasonic bath and then cooled at room temperature, then 20% acetonitrile was added to 50 mL. The solution was injected into HPLC system after filtering through a 0.45 μm syringe filter.

### Qualitative and quantitative HPLC analyses

The identity of each peak in TASA samples was confirmed by comparison of retention time and UV spectrum of each peak with that of five reference compounds. In addition, spiking samples with the reference compounds further confirmed the identities of the peaks.

On basis of the identity of each reference compound in TASA samples, confirmed reference compounds were chosen as quantitative markers to further simultaneously evaluate the quality of TASA.

## Results and discussion

### Optimization of chromatographic conditions

The major compounds in TASA belong to quinolizidine alkaloids, and the maximal UV absorption wavelength of five compounds investigated in the experiment was 210 nm. During this study, various mobile phases including methanol-$H_2O$, acetonitrile-$H_2O$ (containing 1% triethylamine, and adjusted pH 4.3 using 1% glacial acetic acid), potassium dihydrogen phosphate-$H_2O$-methanol, potassium dihydrogen phosphate-$H_2O$-acetonitrile, and acetonitrile mixed with ammonium dihydrogen phosphate as mobile phase were tested. Potassium dihydrogen phosphate (0.02 mol/L, adjusted pH 4.3 with 1% glacial acetic acid) and acetonitrile (75:25) were found to be the best one for

constituents in TASA with good separation and stable baselines. To reduce peak tailing of alkaloids, various concentrations of glacial acetic acid were tested further until excellent resolution of five major constituents was all obtained in TASA.

Different types of chromatographic columns including Hypersil $C_{18}$, Alltima $C_{18}$, and Kromasil $C_{18}$ column were tested to optimize the separation. Good separation was observed on the Kromasil $C_{18}$ column. Column temperatures were at 20, 25, 30, and 40 ℃, and the results showed that the excellent resolution and short analysis time were achieved with 25 ℃.

Representative HPLC chromatograms of the five standard solutions and the real sample solution at 210 nm were shown in Fig. 2 and Fig. 3. Almost no interference was presented in the chromatographic separation.

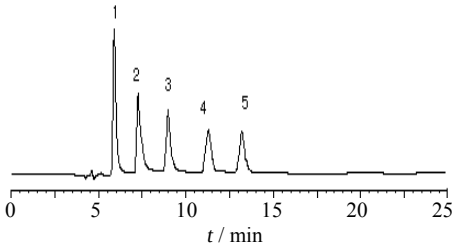



**Fig. 2 Typical chromatogram of standard solution**
1: sophoridine 2: oxymatrine 3: matrine 4: aloperine 5: lehmannine

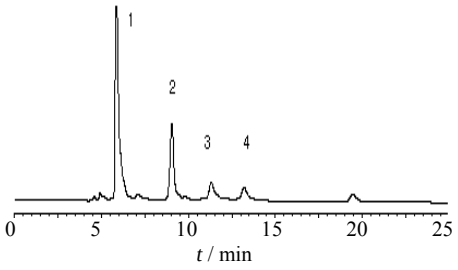



**Fig. 3 Typical chromatogram of the real sample solution**
1: sophoridine 2: matrine 3: aloperine 4: lehmannine

**Optimization of sample preparation**

Ultrasonicator was used and 20% acetonitrile was chosen as extraction solvent for its good solubility for TASA and similarity to mobile phases among five tested solvents: 100%, 80%, 50%, 30%, and 20% acetonitrile. The extraction time tests indicated that all the constituents were almost completely extracted within 20 min.

**Qualitative and quantitative HPLC validation**

The five marker compounds with their UV spectrums were used in this study for qualitative analysis of TASA. Four of them including sophoridine, matrine, aloperine, and lehmannine were qualitatively determined in the test sample solution, while oxymatrine could not be detected in TASA samples in this study, although it could be detected in the herbs of *S. alopecuroides* due to isomerization during extraction. Further research is going on. Spiking samples with the reference constituents further confirmed the identities of the peaks.

**Linearity, work range, and limits of detection**

The linearity calibration curves of the detected four constituents were constructed by five concentration assays. The regression equation was calculated in the form of $Y = aX + b$, where $Y$ and $X$ were the values of peak area and concentration of each reference compound, respectively. The four calibration curves exhibited linear regressions of at least $\gamma > 0.9995$, the limit of detection (LOD) was in the range of 0.0001 to 0.0023 mg/mL, and results were shown in Table 1.

**Precision, repeatability, and stability**

The injection precision was tested by six repeat injections using the standard solutions, while the analytical repeatability was tested by six different samples prepared with the same procedure. The intra-day variability was determined by analyzing the six replicates within 24 h. The results of the tests indicated that most of the RSD values were less than 5%, and the method was thus acceptable (Table 2).

**Recovery test**

The recoveries of the four quantitative constituents were determined by the method of standard addition. Suitable amounts (about 50% of the content) of the four standard substances were spiked into the known sample of TASA. The mixture was extracted and analyzed by the proposed method. The mean recoveries of the four constituents were ranged from 98.62% to 100.20%, and the RSD values were all less than 3.37%, thus the method was deemed to be accurate (Table 3).

**Determination of four constituents in TASA**

The four constituents in three batches of TASA were simultaneously determined by the developed HPLC-DAD method at the conditions described above. The results were shown in Table 4.

**Table 1 Regression equation, correlation coefficients, linearity ranges, and LOD for the markers of TASA**

| Constituents | Regression equation | $r^2$ | Linear range / (mg·mL$^{-1}$) | LOD / (mg·mL$^{-1}$) |
|---|---|---|---|---|
| sophoridine | $Y$ = 33 714$X$ + 42.075 | 0.9999 | 0.041－0.328 | 0.0001 |
| matrine | $Y$ = 31 035$X$ − 55.408 | 0.9995 | 0.010－0.080 | 0.0023 |
| aloperine | $Y$= 27 238$X$ + 20.141 | 0.9998 | 0.011－0.088 | 0.0003 |
| lehmannine | $Y$ = 22 286$X$ − 25.989 | 0.9996 | 0.008－0.039 | 0.0020 |

All the analytes showed good linearity ($r^2$ > 0.9995) in the concentration ranges

**Table 2 Precision, repeatability, and stability of the developed method ($n$ = 6)**

| Constituents | Precision | | Stability | | Repeatability | |
|---|---|---|---|---|---|---|
| | Average peak area | RSD / % | Average peak area | RSD / % | Average contents / % | RSD / % |
| sophoridine | 5568.36 | 1.08 | 5248.86 | 1.12 | 25.03 | 1.71 |
| matrine | 1147.88 | 0.97 | 2116.72 | 1.55 | 12.11 | 1.82 |
| aloperine | 1232.16 | 1.15 | 483.08 | 2.63 | 2.94 | 2.32 |
| lehmannine | 335.89 | 2.77 | 275.16 | 2.55 | 2.05 | 2.91 |

**Table 3 Statistical results of recovery ($n$ = 6)**

| Constituents | Added / (mg·mL$^{-1}$) | Found / (mg·mL$^{-1}$) | Recovery / % | RSD / % |
|---|---|---|---|---|
| sophoridine | 0.1121 | 0.1105 | 98.62 | 1.71 |
| matrine | 0.0392 | 0.0389 | 99.19 | 2.22 |
| aloperine | 0.0116 | 0.0116 | 100.20 | 2.40 |
| lehmannine | 0.0091 | 0.0089 | 99.30 | 3.37 |

**Table 4 Contents of the four markers in TASA ($n$ = 2)**

| No. | Sophoridin% | Matrine/% | Aloperine/% | Lehmannine/% |
|---|---|---|---|---|
| 1 | 25.04 | 12.52 | 2.96 | 2.03 |
| 2 | 28.13 | 13.46 | 2.28 | 2.36 |
| 3 | 26.82 | 10.18 | 3.23 | 1.97 |

## Conclusion

For the first time, a specific and sensitive HPLC method was developed for simultaneous analysis of multi-ingredient in TASA extract. Precision, accuracy, and repeatability were fully shown in the validation procedure. The method provided a sound assurance and improvement for the safety and efficacy for TASA and its preparation.

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