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Studies on the dynamic accumulations of *Sophora alopecuroides* L. Alkaloids in different harvest times and the appropriate harvest time

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ABSTRACT

A sensitive and accurate method for the simultaneous determination of five alkaloids, namely 9α-hydroxymatrine (M1), matrine (M2), sophoridine (M3), oxymatrine (M4), alopecurin A (M5) in different parts (seed, legume, stem, and root) and different harvest times of *Sophora alopecuroides* L. was developed by high performance liquid chromatography (HPLC) with photodiode array detector (PDA) for the first time. The separation by gradient elution was achieved on Scienhome Kromasil C_{18} (4.6 × 250 mm, 5 μm) column at 30 °C with acetonitrile (A)/0.1% phosphatic acid + 0.1% triethylamine (B) as the mobile phase. The detection wavelength was 205 nm. The optimized method provided a good linear relation ($r \geq 0.9993$ for all the target compounds), satisfactory precision (RSD values less than 2.3%) and good recovery (96.4–103.6%). The limits of detection ranged between 0.11×10^{-3} and 4.70×10^{-3} μg for the different analytes. The method was successfully applied to analysis and quality control of alkaloid extracts from the traditional Chinese herbal drugs of *S. alopecuroides* L.

1. Introduction

Sophora alopecuroides L. (Leguminosae, *Sophora* Linn.), a traditional Chinese herbal medicine, is widely distributed in the northwest of china, especially in Xinjiang. Phytochemical investigations show that there exist more than twenty chemical compounds, belonging to alkaloids, flavonoids, volatile oil, organic acid, amino acid, protein, saccharide, *etc*. Among these chemical constituents, the principal bioactive constituents of *S. alopecuroides* L. are quinolizidine alkaloids, which have been shown to exhibit sedative, depressant, analgesic, hypothermic, antipyretic, and cardiotonic activities [1], especially anti-tumor [2] and improve the immunity [3]. Two types of quinolizidine alkaloids, matrine-type and pyridone quinolizidine bases, are found in *Sophora* species and can be used for identification of *Sophora* species in commercial preparations [4]. Hence, sensitive, rapid and specific methods for analyzing quinolizidine alkaloids are of great interest.

Several analytical methods such as high performance liquid chromatography (HPLC), thin-layer chromatography (TLC) and capillary electrophoresis (CE) have been used to analyze this crude drug for the presence of quinolizidine alkaloids [5–10]. However, TLC lacks quantitative precision, presented CE method is applied in non-aqueous phase, which limited their application. HPLC analyses of these alkaloids have been reported elsewhere: under uncontrolled conditions it has not been possible to obtain satisfactory selectivity or reproducibility and also takes longer analyze time. Therefore, it is necessary to establish a rapid and effective method for the quantitative analysis of these alkaloids.

This paper reports an approach that gives an acceptable chromatogram for separation of the major quinolizidine alkaloids. The RP-LC method is suitable for quantitative analysis and can be used as an effective tool to evaluate herbal medicines. In this study, an RP-LC method was developed for simultaneous detection and quantification of the five bioactive compounds, namely 9α-hydroxymatrine (M1), matrine (M2), sophoridine (M3), oxymatrine (M4), alopecurin A (M5) (Fig. 1) in *S. alopecuroides* L., while the samples of its different growing periods were analyzed for identifying the best collecting time. Five alkaloids were separated and quantitatively determined. Up to now, there is no published quality standards for *S. alopecuroides* L., although laboratories in various universities are engaged in the analysis of *S. alopecuroides* constituents. It is necessary to establish standards for the quality control of *S. alopecuroides* L. phytomedicines.

2. Experimental

2.1. Plant material

The samples of *S. alopecuroides* L. in different harvest times were collected from Shihezi (Xinjiang, China), and identified by Profes-

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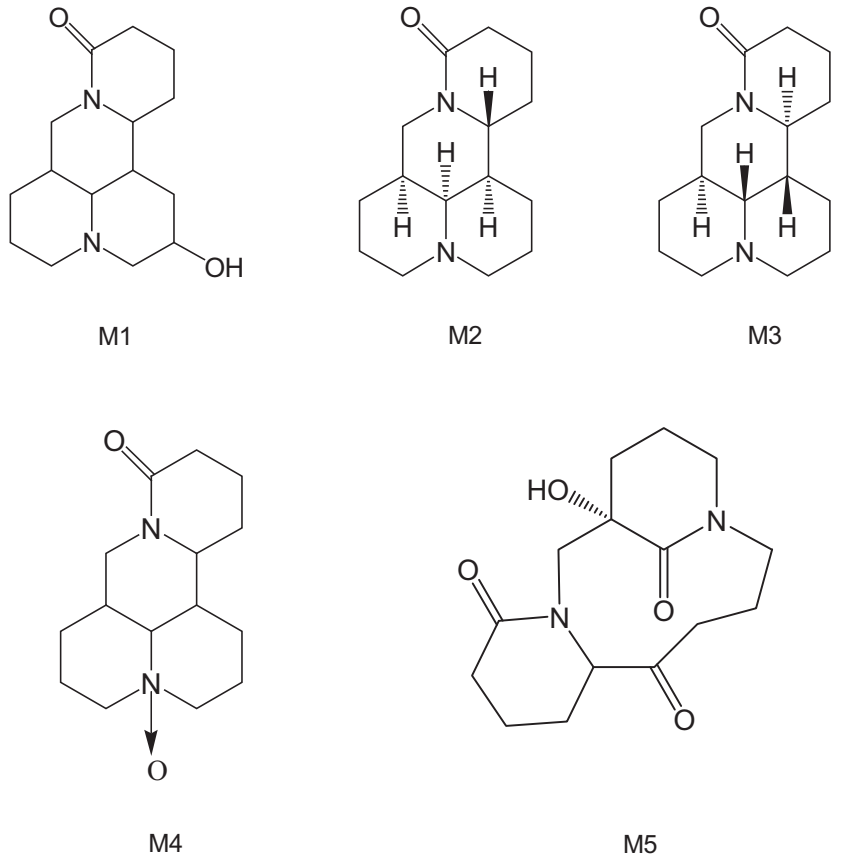

Fig. 1. Structures of compounds M1–M5.

sor Yong Tan (Shihezi University, Xinjiang, China). The voucher specimen (no. 20070816006) is deposited in School of Pharmacy faculty, Shihezi University. For the analysis, samples were dried and pulverized into powder.

2.2. Reference standards and solvents

The reference standard of M1–M5 quinolizidine alkaloids was isolated previously from the total alkaloids of *S. alopecuroides* L. by author, structures of which were elucidated by comparison of spectral data (UV, IR, MS, ^{1}H NMR and ^{13}C NMR) with the literature data [11–13]. The purity of each reference standard was determined to be above 98% by LC analysis based on a peak area normalisation method, detected by HPLC-PDA and confirmed by LC–ESI-TOF-MS and NMR spectroscopy.

Acetonitrile (HPLC-grade) and methanol (HPLC-grade) were purchased from Fisher Scientific Co. (Franklin, USA). LC grade water was prepared using a redistilled water equipment. Phosphoric acid (analytical grade) was purchased from Tianjin Guangfu Chemical Reagent Co. Ltd. (Tianjin, China). Deionised water for LC analysis was purified using a Milli-Q system (Milford, MA, USA). Other solvents from Tianjin Guangfu Chemical Reagent Co. Ltd. (Tianjin, China) were all of analytical grade.

2.3. Instrumentation and chromatographic conditions

A Waters 2695 Alliance HPLC system with Waters 2998 PDA detector was used. The analysis was performed on Scienhome Kromasil C_{18} column (4.6 mm × 250 mm, 5 μm) at a column temperature of 30 °C, using (A) acetonitrile and (B) 0.1% phosphatic acid + 0.1% triethylamine as mobile phase with the gradient elution procedure show in Table 1. The flow rate was 1.0 ml/min and the detection wavelength was 205 nm. The injection volume was 10 μl and the alkaloids derivatives were well separated in chromatographic conditions above.

2.4. Preparation of reference standard

The stock reference standard solution was prepared by dissolving an appropriate amount of each reference compound in acetonitrile. Concentrations of stock reference standard solutions for five compounds were (M1) 9α-hydroxymatrine 1, 038 μg ml^{-1}, (M2) matrine 994 μg ml^{-1}, (M3) sophoridine 760 μg ml^{-1}, (M4) oxymatrine 2, 677 μg ml^{-1} and (M5) alopecurin A 1, 088 μg ml^{-1}. These solutions were respectively stored in a refrigerator at 4 °C. Then before LC injection, accurately take 6 μl 9α-hydroxymatrine, 10 μl matrine, 675 μl sophoriding, 351 μl oxymatrine and 2.6 μl alopecurin A were placed in a 2 ml flask with stopper, diluted with acetonitrile to make sure the volume reached 2 ml as the mixed reference standard solution.

Table 1
Time program of the gradient elution.

Time (min)	Flow (ml/min)	Acetonitrile (%)	0.1% phosphatic acid + 0.1% triethylamine (%)
0	1	1	99
10	1	1	99
25	1	8	92
40	1	20	80
50	1	22	78
53	1	95	5
60	1	95	5

2.5. Treatment for samples

The crude dried samples of seeds, legumes, stems and roots for *S. alopecuroides* L. were pulverized and sifting through 40 mesh sieve, respectively. The accurately weighed sample powder (0.5 g) was placed in a 50 ml flask with stopper, and then weighed again correctly. Extracted by ultrasonic method with 25 ml methanol for 30 min. Then standing, cooled down to the room temperature and mended the weight to the incipient weight with methanol. Prior to HPLC analysis, the sample solution was passed through a 0.22 μm millipore filter.

3. Validation of the method

3.1. Calibration curves

Linearity was established by injection of 1, 2, 4, 8, 12, 16 and 20 μl of the mixed reference standard solution prepared, respectively. Calibration graphs were plotted subsequently based on linear regression analysis of the integrated peak (Y) versus content (X μg).

3.2. Limits of detection and quantitation

Mixed standard stock solution was diluted to a series of appropriate concentrations (Diluted 10, 15, 100, 50, 2 times respectively with acetonitrile for M1–M5 compounds to detect LOQ and 30, 40, 200, 100, 4 times for LOD), and an aliquot of the diluted solutions was injected into HPLC for analysis. The limit of detection (LOD) and limit of quantification (LOQ) for each analyte was calculated with corresponding standard solution on the basis of a signal-to-noise ratio (S/N) of 3 and 10, respectively.

3.3. Precision and stability

The precision of the chromatographic system was validated by injecting 10 μl of the mixed reference solution six times during one day. Stability study was performed with sample solution in 48 h (the time-point is 0, 4, 8, 16, 24, 32, 36 and 48 h, respectively). Variations were expressed by relative standard deviations (RSD) of peak area.

3.4. Repeatability and recovery

The repeatability test was analyzed by injecting six independently prepared samples (the concentration and prepared method as Section 2.5). The RSD value of amount was adopted to evaluate repeatability. The recovery tests were studied by adding mixed reference standard solution to untreated sample to yield final concentration. The sample was processed by the above sample preparation procedure. The experiment was repeated six times.

4. Results and discussion

4.1. Optimization of separation conditions

These five major alkaloids are very similar in structure (Fig. 1), and two of them, matrine and sophoridine are structural stereisomers. They are quite similar in terms of chromatographia behavior (retention time) and correspondingly difficult to separate. Thus, this study investigated several sets of conditions in an attempt to optimize separation. Selection an appropriate chromatographic column is the first step in developing the analytical method. In this study, an acceptable separation of the five active compounds was challenging because of their similar molecular structure. Five analytical columns (Scienhome Kromasil NH, Hypersil ODS, Shim-Pack CLC-ODS, ZORBAX ODS, Scienhome Kromasil C_{18}) were selected to separate the five compounds. Optimum separation could be obtained with a Scienhome Kromasil C_{18} column (Merck, Darmstadt, Germany) within 40 min. The Hypersil ODS, Shim-Pack CLC-ODS and ZORBAX ODS column could not resolve compound M2 and M3. Although five compounds could be separated satisfactorily with the Scienhome Kromasil NH stationary phase, the chromatographic peak is dissymmetry. Therefore, the Scienhome Kromasil C_{18} column was selected as analytical column (Fig. 2).

Regarding the selection of the mobile phase, gradient elution was employed because the five target compounds have a broad range of polarity. Different mobile phase compositions (methanol, acetonitrile, aqueous phosphatic acid and aqueous triethylamine of different concentration) were tested to resolve the five active compounds in the standard mixture as well as in the extracts of samples. Present researches indicated that better separation and results were obtained using a mobile phase of water and acetonitrile rather than water and methanol. Therefore, in this work, the optimum resolution was achieved using acetonitrile (A) and 0.1% phosphatic acid + 0.1% triethylamine (B) as mobile phase with gradient elution (Table 1), and all five active compounds could be eluted with baseline separation in 40 min. Representative chromatograms for the mixed reference standards and four samples were shown in Fig. 3.

The five standard analytes were well separated and the resolution between any two compounds was greater than 1.0. Other compounds in the sample do not interfere with the analysis of five alkaloids, as shown in Fig. 3.

The chromatographic peaks were identified by comparing their retention time with that of each reference compound, which was eluted in parallel with a series of mobile phases. In addition, spiking samples with the reference compounds showed no additional peaks, which further confirmed the identities of the analytes' peaks (Fig. 3).

4.2. Optimization of extraction method

The optimization of solvent extraction was performed using the seeds of *S. alopecuroides* L. At first, ultrasonic extraction were selected because of their relatively shorter extraction time than refluxing extraction, maceration extraction and Soxhlet extraction. The extraction solvent such as methanol, 75% methanol, dehydrated alcohol, 75% ethanol, chloroform (add ammonia water stronger), China pharmacopoeia method and acetone were selected. Comparing the extraction yields of the target constituents, though the total alkaloids extracted by chloroform (add ammonia water stronger), China Pharmacopoeia method and 75% methanol proved to be more efficient than the other solvent, they could not extract the constituent of M5, so these three solvent were deserted. In the other solvent, methanol showed a good result. Therefore, methanol was chosen as an extractant. Under the optimum extraction solvent, to further optimization the extraction method, compared to Soxhlet extraction, maceration extraction and refluxing extraction, the ultrasonic treatment procedure was found to be more effective and simple for the five bioactive components, while refluxing extraction, maceration extraction and Soxhlet extraction could not completely extract all of the five constituents from the sample. As a result, the optimum extraction method was selected ultrasonic and use methanol as extractant (Table 2).

4.3. Validation of the method

Under the chromatographic conditions adopted in the study, all calibration curves exhibited good linearity ($r \geq 0.9993$) in a rela-

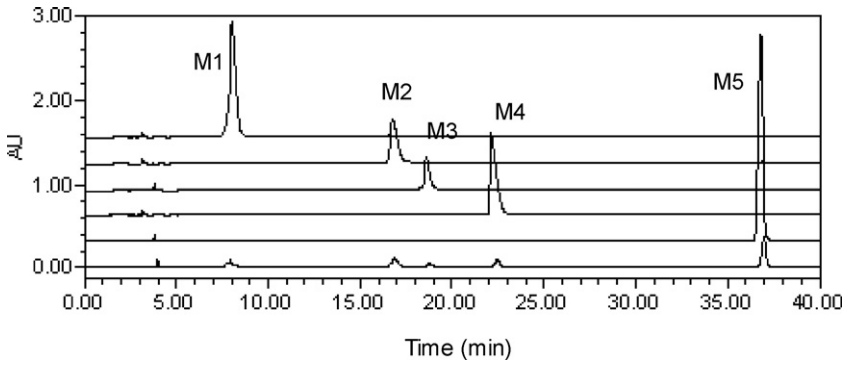

Fig. 2. Stack view of HPLC chromatograms of (a) 9α-hydroxymatrine; (b) matrine; (c) sophoridine; (d) oxymatrine; (e) alopecurin A; (f) mixed reference standards (from up to down). Column: Scienhome Kromasil C_{18} column (4.6 mm × 250 mm, 5 μm), temperature of 30 °C; detector: UV at 205 nm.

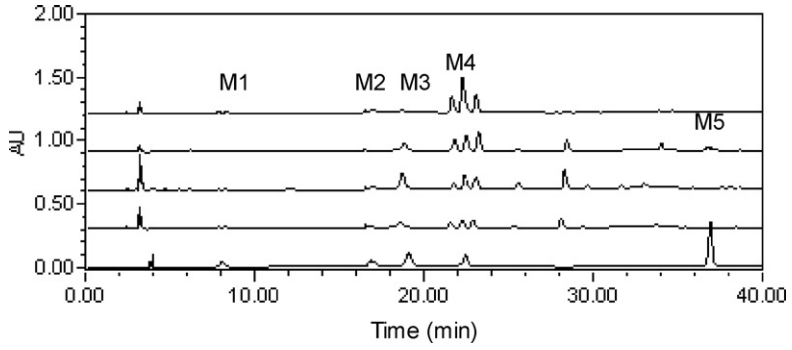

Fig. 3. Stack view of HPLC chromatograms of (A) ultrasonic extract from the root of *Sophora alopecuroides* L.; (B) ultrasonic extract from the seed of *Sophora alopecuroides* L.; (C) ultrasonic extract from the Stem of *Sophora alopecuroides* L.; (D) ultrasonic extract from the Legume of *Sophora alopecuroides* L.; (E) mixed reference standards (from up to down).

Table 2
Optimization of extraction method of *Sophora alopecuroides* L. (mg/g).

Extraction method	Extractant	M1	M2	M3	M4	M5	Total
Ultrasonic	Ch.P.	ND	0.095	3.506	18.614	ND	22.215
	Chloroform (add ammonia water stronger)	ND	0.098	3.074	18.298	ND	21.470
	75% methanol	ND	0.064	3.051	15.911	ND	19.026
	Methanol	ND	0.054	3.103	16.162	0.027	19.346
	75% ethanol	ND	0.135	2.091	13.64	0.025	15.891
	Dehydrated alcohol	ND	0.097	1.065	4.123	0.007	5.292
	Acetone	ND	0.143	1.887	ND	ND	2.030
Soxhlet	Methanol	ND	0.074	3.395	18.731	ND	22.200
Refluxing	Methanol	ND	0.072	3.118	18.312	ND	21.502
Maceration	Methanol	ND	0.181	2.426	17.816	ND	20.423

ND, not detected.

Table 3
Regression equations, correlation coefficients and linear range for five analytes in *Sophora alopecuroides* L.

Analytes	Linear regression				
	Calibration curves	Correlation coefficients γ	Linear range (μg)	LOD (10^{-3} μg)	LOQ (10^{-3} μg)
M1	$Y = 1.78e+006X + 9.49e+003$	0.9993	$3.18 \times 10^{-3} \sim 6.35 \times 10^{-2}$	0.11	0.32
M2	$Y = 4.85e+005X + 5.08e+004$	0.9997	$4.92 \times 10^{-3} \sim 9.84 \times 10^{-2}$	0.12	0.33
M3	$Y = 2.32e+006X + 6.25e+004$	0.9998	$2.57 \times 10^{-1} \sim 3.08$	1.29	2.57
M4	$Y = 8.57e+005X + 3.62e+004$	0.9998	$4.70 \times 10^{-1} \sim 9.40$	4.70	9.40
M5	$Y = 3.22e+006X - 5.41e+002$	0.9999	$1.41 \times 10^{-3} \sim 2.83 \times 10^{-2}$	0.35	0.71

Table 4
Precision, stability, recovery and repeatability data of the five analytes in *Sophora alopecuroides* L.

Analytes	Precision	Stability		Recovery		Repeatability	
	Concentrations (mg/ml)	R.S.D. (%)	R.S.D. (%)	Average (%)	R.S.D. (%)	Average amounts (%)	R.S.D. (%)
M1	3.18×10^{-3}	1.2	1.8	96.4	1.0	0.003	1.9
M2	4.92×10^{-3}	0.8	1.5	100.0	2.0	0.022	2.3
M3	2.57×10^{-1}	0.8	1.2	99.5	1.3	0.510	1.9
M4	4.70×10^{-1}	0.7	0.8	102.8	0.8	3.023	1.5
M5	1.41×10^{-3}	0.8	0.7	103.6	1.1	0.004	1.7

R.S.D. refers to relative standard deviation.

Table 5
Contents of five analytes in *Sophora alopecuroides* L. (mg/g).

Sample	Analytes						
	Collection date	M1	M2	M3	M4	M5	Total
Seed of *Sophora alopecuroides* L.	20090725	ND	0.233	4.043	18.911	0.011	23.198
	20090806	ND	0.254	4.634	19.393	0.021	24.302
	20090816	ND	0.259	5.006	24.839	0.033	30.137
	20090826	ND	0.216	5.891	31.091	0.044	37.242
	20090907	ND	0.219	5.228	26.297	0.036	31.780
	20090917	ND	0.215	4.554	13.015	0.015	17.799
	20070809	ND	0.253	5.867	9.857	0.014	15.991
Legume of *Sophora alopecuroides* L.	20090725	0.054	0.033	2.097	2.180	ND	4.364
	20090806	0.063	0.081	2.352	1.399	ND	3.895
	20090816	0.071	0.126	2.411	2.498	ND	5.106
	20090826	0.074	0.107	4.450	3.590	ND	8.221
	20090907	0.069	0.110	2.743	3.335	ND	6.257
	20090917	0.066	0.106	2.412	2.859	ND	5.443
Stem of *Sophora alopecuroides* L.	20090725	0.023	0.089	3.578	3.065	ND	6.755
	20090806	0.023	0.091	4.088	3.924	ND	8.126
	20090816	0.024	0.107	4.401	4.764	ND	9.296
	20090826	0.044	0.099	5.547	6.233	ND	11.923
	20090907	0.034	0.097	4.913	5.754	ND	10.798
	20090917	0.009	0.080	4.270	4.115	ND	8.474
Root of *Sophora alopecuroides* L.	20090917	0.105	0.038	2.315	9.446	ND	11.904

ND, not detected.

tively wide linear range as show in Table 3. Table 4 showed the results of precision, stability, recovery and repeatability of the five analytes. It was indicated that the RSD of the precision variations were less than 1.2% for all five analytes. Further, validation studies of the method proved that this assay had good reproducibility with RSD also less than 2.3% for all the analysis and the sample solutions were stable during 48 h at room temperature with an RSD less than 1.8%.

The recovery results of the five analytes displayed in Table 4 showed that the developed analytical method had good accuracy with the overall recovery from 96.4% to 103.6% for the analytes concerned.

4.4. Quantitative determination of S. alopecuroides L.

Alkaloids in *S. alopecuroides* L. have a significant effects in anthelminthic and sterilization, especially in anti-tumor and improve the immunity. These five alkaloids were adopted as chemical markers to establish a method for the quality control study of *S. alopecuroides* L. The method was utilized to analyze the five alkaloids in twenty samples to compare the different concentrations of the five alkaloids for discussing.

In terms of the chromatographic results show in Table 5, the concentration of compounds M1–M5 in the seed of *S. alopecuroides* L. were higher than those in legume and stem. In addition, the sample concentration of compounds M1–M5 which collected in August 26th were higher than those collected in other period. The results indicate that contents of alkaloids in *S. alopecuroides* L. had vast differences in the same regions from different harvest times. It shows that the synthesis of such component is closely related to its growth cycle. Dynamic analysis and evaluation shows that the appropriate harvest period of *S. alopecuroides* L. (Xinjiang, China) is from the middle of August to the end of August. The results showed in Table 5.

5. Conclusions

A new analytical method for qualitation and quantification of alkaloids in *S. alopecuroides* L. was evaluated to be precise and accurate. Materials of *S. alopecuroides* L. of varieties harvest periods were assayed with this method successfully and it also indicates that the appropriate harvest period of the *S. alopecuroides* L. is from the middle of August to the end of August. The method provides a new tool for the assessment of quality of *S. alopecuroides* L. This result implied that the collection time has significant influence on the amounts of the chemical constituents in *S. alopecuroides* L.

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