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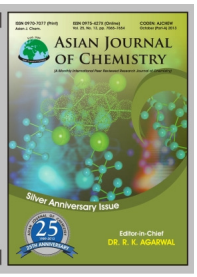

Simultaneous Determination of Tropane Alkaloids in Different Fractions of *Herba belladonnae* Collected in Various Seasons by HPLC Method

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An HPLC method was developed for the simultaneous determination of hyoscyamine, scopolamine and anisodamine to investigate medical parts of *Herba belladonnae* in different harvest time. The solid phase was Shim-pack VP-ODS (4.6 mm × 250 mm, 5 μm), with isocratic elution using 0.1 % phosphoric acid solution (including 0.25 % SDS)-acetonitrile (60:40) as the mobile phase. The flow rate was 1 mL/min and the wavelength was 210 nm. The results of methodology all fit the analytical requests and the recovery were 97.3-103.0 %. Quantitative analysis of the three compounds showed that the contents of hyoscyamine in leaves and flowers were the highest, followed by fruits and roots and the third was stem. The contents of scopolamine in flowers and fruits were the highest. The contents of anisodamine in fruits were the highest. The relative contents of hyoscyamine showed an increasing trend during the growth period, while scopolamine and anisodamine showed an opposite trend, which would reach a balance finally. The method was simple, quick and reliable and could provide a scientific and technical platform for setting up a quality control standard.

Key Words: *Herba belladonnae*, Hyoscyamine, Scopolamine, Anisodamine, HPLC.

INTRODUCTION

Herba belladonnae (HB) is the entire plant of *Atropa belladonna*, belonging to the family of Solanaceae[1]. Original from Europe, the main active components of *Herba belladonnae* are tropane alkaloids, possessing pharmacological actions of relieving smooth muscle spasm, inhabiting the secretion of glandular organs, *etc*. Clinically, it is mainly used for the treatment of hyperchlorhydria-induced duodenal ulcer and colicky pains on gastrointestinal tract, kidney and gall-bladder[3,4]. However, taking in excessive *Herba belladonnae* might result in serious toxic effects. Frequently, it has been reported poisoning incidents abroad due to eating the fruits of *Herba belladonnae* by mistake[5-10]. *Herba belladonnae* has been introduced into China since the 20th century, mainly used as the raw material for extracting belladonna extract and belladonna liquid extract. Because of the limitation of climatic condition in China, there are only certain major regions available for its plantation and cultivation. The researches of alkaloids in different medicinal parts changing along with different growing seasons are almost blank. Because of the lacking of scientific evidence on implantation and harvest, the usage and dosage of the herb are of indeterminacy. In addition, there is no report about the comparative analysis of alkaloids in different medicinal parts and different growing seasons yet. In this research, an HPLC method was developed primarily for the simultaneous determination of hyoscyamine, scopolamine and anisodamine and the changing rules of the above three tropane alkaloids in different medicinal parts and different growing seasons were summarized for the first time, which could provide reasonable evidence for its harvest and collection.

EXPERIMENTAL

Standards of anisodamine, scopolamine and hyoscyamine were supplied by Zhejiang Institute for Food and Drug Control (Hangzhou, Zhejiang Prov., China). *Herba belladonnae* was purchased from Hunan, China. Methanol and acetonitrile were of HPLC grade and other reagents used were analytical grade. Deionized water was prepared using a Millipore water purification system.

HPLC conditions: An Agilent 1200 series LC system was employed in this research, consisting of a G1379B Quaternary Pumps, a G1376B Degasser, a G1316A Diode-Array Detector and a G1376B Autosampler.

The analysis of the alkaloids was carried out on a Shim-pack VP-ODS (250 mm × 4.6 mm, 5 μm), protected by a Security GuardTM RP18 guard column (4 mm × 3.0 mm I.D., Phenomenex, USA).

The solvents used for HPLC separation of the three tropane alkaloids in samples were acetonitrile (A) and buffer solution (B, 0.004 % phosphoric acid solution (including 0.25 % SDS)) at a flow rate of 1.0 mL/min. The mobile phase was isocratic elution with A-B (40:60, v/v) and the analysis was monitored at 210 nm. The column temperature was 30 ºC and the sample injection volume was 10 μL[11].

Preparation of sample solutions: Medical parts of *Herba belladonnae* in different growing time were pulverized into powder, then passed through a 0.45 mm sieve, *ca.* 1.0 g sample was accurately weighted, then added into a 100 mL conical flask. 25.0 mL 50 % methanol solution was added for ultrasonic batch at room temperature for 1 h. The solution was ready for the chromatographic analysis after passing through a 0.45 μm membrane filter.

Preparation of standard solutions: Three standard solutions, reference compounds anisodamine (20.16 mg), scopolamine (16.08 mg) and hyoscyamine (24.40 mg) were dissolved with 50 % methanol and diluted to five different concentrations.

RESULTS AND DISCUSSION

Regression equations: Linear regression analysis for each of the three tropane alkaloids was performed by the external standard method. Calibration curves were established based on six points for anisodamine with concentrations of 80.64, 161.28, 322.56, 483.84 645.12 and 725.76 μg/mL; six points for scopolamine with concentrations of 96.48, 192.96, 385.92, 578.88, 771.84 and 868.32 μg/mL; six points for hyoscyamine with concentrations of 195.2, 390.4, 780.8, 1366.4, 1561.6 and 1756.8 μg/mL. The calculated results were given in Table-1. All the alkaloids showed good linearity in a relatively wide concentration range.

TABLE-1
LINEAR REGRESSION EQUATION AND LINEAR RANGES

Alkaloids	Regression equation	Correlation coefficient (R^2)	Linear range (mg/mL)
Anisodamine	Y=1080.2X-1742.5	0.9997	80.64-725.76
Scopolamine	Y=1000.6X-3122.2	1.0000	96.48-868.32
Hyoscyamine	Y=122.2X+53.6	0.9995	195.2-1756.8

Note: X denoted the concentrations and Y denoted the peak areas.

Precision: The standard mixture solution of anisodamine hydrobromide, Scopolamine hydrobromide and hyoscyamine sulfate was injected into HPLC for six times continuously and the area of each peak was used for the calculation of precision. The results showed that relative stand deviation (RSD) of peak area of each standard was 0.1, 0.2 and 0.5 %, respectively.

Repeatability: Repeatability was carried out taken six samples solution with the same treatment procedure. The results showed that RSD of each peak area was all 0.9 %.

Stability: For stability test, the same sample solution was analyzed at the designated time points for 48 h. The results showed that RSD of peak area were 0.2, 0.5 and 0.2 %, stable for the experiment.

Recovery test: The sample with determined targeted contents was spiked with certain amounts of the 3 standards. Then the spiked sample was processed in accordance with the established method for HPLC detection. The average recoveries for anisodamine hydrobromide, scopolamine hydrobromide and hyoscyamine sulfate determined were 97.3-103.0 % (Table-2). Determination of tropane alkaloids in different fractions of *Herba belladonnae* are given in Table-3.

Application of the HPLC method for quantitation studies: 10 μL sample solution was injected into the instrument. The representative HPLC chromatograms were shown in Fig. 1, respectively. Peaks in the obtained chromatograms were identified by comparing the retention time and on-line UV spectra with those of the standards.

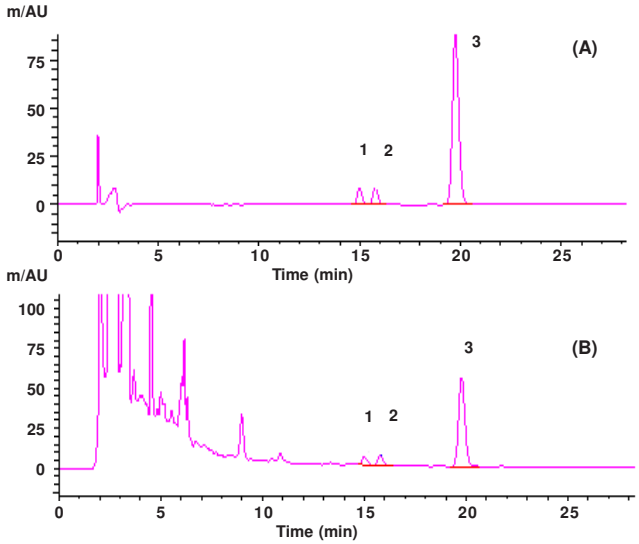

Fig. 1. Typical HPLC chromatograms of standard solution (A) and sample solution (B). 1. Anisodamine hydrobromide. 2. Scopolamine hydrobromide. 3. Hyoscyamine sulfate

Optimization of HPLC separation conditions: In order to get a separation with better resolution of targeted components in a shorter analytical time, we compared three different column temperature: 20, 30 and 40 ºC, then we found that the higher temperature, the shorter retention time with almost the same resolution, so we chose 30 ºC finally for protecting the lifespan of the column.

Besides, reflux extraction and ultrasonic extraction were investigated in the experiments. Considering the advantages of high efficiency and easy operation, we chose ultrasonic extraction as the way to prepare sample solution. In the meantime, we compared the efficiency of 50 % methanol with ethyl acetate which was used for ultrasound, it showed that the former was better. We also tried different ultrasonic time *i.e.*, 30, 60 and 90 min. Finally, taking the determination of targeted components into consideration, ultrasonic extraction 1 h with 50 % methanol was better.

Three chromatographic columns were compared in the experiment: Agilent Zorbax SB-C_{18} (250 mm × 4.6 mm, 5 μm), Kromasil 100-5C_{18} (250 mm × 4.6 mm, 5 μm) and Shim-pack VP-ODS C_{18} (4.6 mm × 250 mm, 5 μm), according to the effect of separation, Shim-pack VP-ODS C_{18} (4.6 mm × 250 mm, 5 μm) was used for further research of the methodology.

TABLE-2
RECOVERY OF THE THREE TROPANE ALKALOIDS (n = 9)

Component	Contents (mg)	Added (mg)	Determined (mg)	Recovery (%)	Mean (%)	RSD (%)
Anisodamine	0.0927	0.0403	0.040	98.7	97.3	1.4
	0.0947	0.0403	0.040	100.2		
	0.0926	0.0403	0.039	97.2		
	0.0946	0.0806	0.0781	96.9		
	0.095	0.0806	0.0784	97.2		
	0.0946	0.0806	0.0773	95.9		
	0.0942	0.1210	0.116	95.9		
	0.0963	0.1210	0.117	96.7		
	0.0947	0.1210	0.118	97.5		
Scopolamine	0.1254	0.0482	0.0490	100.7	103.0	1.5
	0.1278	0.0482	0.0490	104.5		
	0.1225	0.0482	0.0500	100.6		
	0.1207	0.0965	0.101	104.5		
	0.1211	0.0965	0.101	104.9		
	0.1206	0.0965	0.0990	103		
	0.1201	0.145	0.147	101.6		
	0.1228	0.145	0.150	103.6		
	0.1207	0.145	0.150	103.4		
Hyoscyamine	1.042	0.448	0.475	97.3	99.8	1.8
	1.063	0.448	0.491	100.6		
	1.018	0.448	0.47	96.4		
	1.003	0.976	0.996	102.0		
	1.007	0.976	0.986	101.0		
	1.003	0.976	0.968	99.2		
	0.998	1.464	1.464	100.0		
	1.021	1.464	1.478	101.0		
	1.003	1.464	1.475	100.8		

TABLE-3
DETERMINATION OF TROPANE ALKALOIDS IN DIFFERENT FRACTIONS OF *Herba belladonnae* IN DIFFERENT SEASONS (n = 2)

Sample No	Growth time (days)	Different parts	Hyoscyamine (mg/g)	Scopolamine (mg/g)	Anisodamine (mg/g)
1	120	Root	2.249	0.169	0.146
		Stem	1.561	0.592	0.0680
		Leaf	1.377	0.678	0.0780
		Herb	1.503	0.602	0.0790
2	140	Root	1.484	0.251	0.125
		Stem	1.559	0.127	0.0130
		Leaf	3.068	0.962	0.128
		Flowers	3.061	1.396	0.159
		Fruits	3.728	1.587	0.504
		Herb	2.063	0.447	0.0781
3	150	Root	0.383	0.190	0.0752
		Stem	1.005	0.217	0.0772
		Leaf	1.855	0.763	0.135
		Herb	1.219	0.413	0.0961
4	160	Root	2.867	0.236	0.182
		Stem	1.811	0.197	0.129
		Leaf	3.852	0.743	0.206
		Flowers	3.305	0.585	0.162
		Fruits	2.501	0.446	0.0740
		Herb	2.534	0.348	0.1540
5	170	Root	1.788	0.178	0.0980
		Stem	1.129	0.149	0.0710
		Leaf	3.249	0.834	0.215
		Flowers	4.058	0.733	0.211
		Fruits	2.821	0.707	0.197
		Herb	1.872	0.349	0.118
6	180	Root	2.554	0.200	0.298
		Stem	1.222	0.128	0.095
		Leaf	4.168	0.939	0.375
		Flowers	5.111	0.727	0.344
		Fruits	2.539	0.561	0.205
		Herb	2.268	0.362	0.204

Conclusion

In this study, three main tropane alkaloids were investigated in different fractions of *Herba belladonnae* collected in various seasons. To the best of our knowledge, it is the first report which simultaneously determined the three main tropane alkaloids in different fractions of *Herba belladonnae* collected in various seasons, not only quantitatively but also qualitatively. Quantitative analysis showed that the contents of three main tropane alkaloids mainly distributed in leafs, flowers and fruits, the contents of hyoscyamine in leaves and flowers were the highest, followed by fruits and roots and the third was stem; the contents of scopolamine in flowers and fruits were the highest; the contents of anisodamine in fruits were the highest and the contents of the 3 components appeared an upgrade trend during the growing period and reached to a balance in the harvest time. The study on dynamic changes of the three main tropane alkaloids in different parts of *Herba belladonna* in different growing periods could provide a guidance for the plantation, harvest and scientific evaluation of *Herba belladonna*.

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