Simultaneous Determination of Nine Active Ingredients in *Belladonna herb* by HPLC

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An HPLC method is established for the simultaneous determination of nine active ingredients in *Belladonna herb* (*B. herb*). HPLC conditions included Agilent Zorbax SB-C₁₈ column (4.6 mm × 250 mm, 5 μm) and the mobile phase was a mixture of methanol and 0.05 % phosphoric acid for gradient elution. The flow rate was 1 mL/min and the column temperature was 30 °C. Nine active ingredients had good linear relationships: scopolinin in the range of 11.08-498.96 ng (R² = 1.000), chlorogenic acid in the range of 14.57-2039.52 ng (R² = 1.000), flavonoid glycoside A in the range of 32.04-4485.60 ng (R² = 0.9999), flavonoid glycoside B in the range of 53.00-1484.00 ng (R² = 1.000), flavonoid glycoside C in the range of 99.84-4992.00 ng (R² = 0.9999), flavonoid glycoside D in the range of 43.20-1484.00 ng (R² = 1.000), umbelliferone in the range of 3.84-133.84 ng (R² = 1.000), scopoletin in the range of 4.45-278.00 ng (R² = 0.9999) and rutin in the range of 25.23-3531.64 ng (R² = 1.000). Their average recoveries were 101.4 % (RSD = 2.51 %), 95.0 % (RSD = 2.63 %), 96.7 % (RSD = 2.04 %), 97.5 % (RSD = 1.89 %), 98.3 % (RSD = 1.87 %), 101.2 % (RSD = 2.09 %), 104.8 % (RSD = 1.57 %), 103.4 % (RSD = 2.00) and 103.3 % (RSD = 2.29 %), respectively. This method was steady with high precision and good repeatability and could be used for the determination of the nine active ingredients and quality control of *B. herb*.

Keywords: HPLC, *Belladonnae herb*, Active ingredients.

INTRODUCTION

Belladonnae herb (*B. herb*) is the entire plant of *Atropa belladonna*, belonging to the family of Solanaceae¹. Original from Europe, the main active components of *B. herb* are mainly tropane alkaloids, possessing pharmacological actions of relieving smooth muscle spasm, inhibition of glandular secretion, etc. Clinically, it is mainly used for the treatment of hyperchlorhydria-induced duodenal ulcer and colicky pains on gastrointestinal tract, biliary colic, renal colic, abdominal pain caused by ureteral calculus, vomiting and diarrhea due to gastritis or gastrospasm, etc.^{2,3}. Modern pharmacological study has found that tropane alkaloids (scopolin, scopolin) possess the function of anti-inflammatory, anticancer, anti HIV, relieving asthma and diuresis⁴⁻⁶. Chlorogenic acid, one kind of organic acids in *B. herb*, has the function of restraining production of reactive oxygen, promoting bioavailability of nitric oxide, depressing oxidative stress, anticoagulation and reducing blood press⁷. In addition, some clinic reports have confirmed chlorogenic acid had antihypertensive effect for moderate hypertension⁸. However, taking in excessive *B. herb* might result in serious toxic effects. Frequently, it has been reported poisoning incidents abroad due to eating the fruits of

B. herb by mistake⁹⁻¹⁵. *B. herb* 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 implan-tation and harvest, the usage and dosage of the herb are of indeterminacy. In addition, there is no report about comparative analysis of the nine active compounds yet. In this research, an HPLC method was developed primarily for the simultaneous determination of scopolin, rutin, chlorogenic acid, flavonoid glycoside A, flavonoid glycoside B, flavonoid glycoside C, flavonoid glycoside D, umbelliferone and scopoletin, which could provide reasonable evidence for the quality control of *B. herb*.

EXPERIMENTAL

Agilent 1100 series HPLC system (Agilent Technologies, USA) was equipped with degasser (G1322), pump (G1312A), automatic sampler (G1313A), UV variable-wavelength detector (1314A-UV) and column oven (G1316A); PA2251 electronic

analytical balance(Sartorius Group, Germany); TW20 constant temperature bath box (Julabo Labortechnik GmbH company, Germany).

Standards of scopolin, rutin, chlorogenic acid, umbelliferone and scopoletin were supplied by National Institute for Food and Drug Control (NIFDC) (Beijing, China) with the batch number of 110768-200504, 100080-200306, 110753-200413, 110713-200911, 110768-200504, respectively. Flavonoid glycoside A, flavonoid glycoside B, flavonoid glycoside C and flavonoid glycoside D were extracted and purified by ourselves (purity > 98 %). *B. herb* was purchased from Luyin Pharmaceutical Co., Ltd. (Yantai, China). Methanol was of HPLC grade and other reagents used were of analytical grade. Deionized water was prepared using a Millipore water purification system.

HPLC conditions: The separation of the nine ingredients was carried out on an Agilent Zorbax SB-C₁₈ (250 mm × 4.6 mm, 5 μm). The solvents used for HPLC separation were methanol (A) and 0.05 % phosphoric acid (B) at a flow rate of 1 mL/min with gradient elution (0-15 min, 3-15 % A; 15-60 min, 15-60 % A) and the analysis was monitored at 344 nm with the column temperature of 30 °C and the injection volume was 10 μL.

Preparation of standard solution: Certain amounts of the nine standard compounds were dissolved with 50 % methanol into volumetric flasks, respectively and then diluted to the concentrations of 5.54, 14.57, 32.04, 53.00, 99.84, 43.20, 0.96, 4.45 and 25.23 μg/mL with 50 % methanol.

Preparation of sample solution: *B. herb* were pulverized into fine powder, after passing through the 80 mesh sieve, 0.8 g sample was accurately weighted, then added into a 150 mL conical flask. Accurate 20 mL 50 % methanol were added into the flask, after weighted, 0.5 h ultrasonic extraction was performed. After refrigerating, the losing weight was complemented with 50 % methanol. The solution was ready for chromatographic analysis after passing through a 0.45 μm membrane filter.

RESULTS AND DISCUSSION

Validation of the chromatographic method

Linear range: Linear regression analysis for each component was performed by the external standard method. The above nine compounds solution was accurately injected 1, 2, 5, 10, 20, 30 and 50 μL, respectively. The linearity of each compound was calculated by plotting the peak area (Y) vs. concentration (X) (Table-1). All the nine components showed good linearities in wide concentration ranges (Fig. 1).

Fig. 1. HPLC chromatograms of the nine ingredients mixture (A) and *B. herb* (B). 1: scopolin; 2: rutin; 3: chlorogenic acid; 4: flavonoid glycoside A; 5: flavonoid glycoside B; 6: flavonoid glycoside C; 7: flavonoid glycoside D; 8: umbelliferone; 9: scopoletin

Precision: The standard mixture solution of nine components was injected into HPLC 6 times continuously and the area of each peak was used for the calculation of precision. The results showed that relative standard deviation (RSD) of peak area of each standard was 1.2, 1.1, 0.8, 1.7, 1.0, 1.9, 1.5, 1.5 and 1.5 %, respectively.

Stability: For stability test, the same sample solution and standard solution were analyzed at designated time points in 36 h. The results showed that RSDs of sample solution were 2.55, 2.18, 0.69, 0.91, 0.42, 1.21, 2.02, 1.42 and 0.99 % (Table-2); RSDs of standard solution were 0.34, 0.57, 0.58, 0.56, 0.59, 0.70, 1.49, 0.74 and 0.49 % and both found to be stable for the experiment (Table-3).

Repeatability: Repeatability was carried out using six samples solution after the same treatment procedure. The results showed that the average contents in *B. herb* of scopolin, rutin, chlorogenic acid, flavonoid glycoside A, flavonoid glycoside B, flavonoid glycoside C, flavonoid glycoside D, umbelliferone and scopoletin were 0.1460, 0.3962, 0.9074, 1.2940, 2.381, 1.105, 0.026, 0.115 and 0.687 mg/g, RSD of each peak area was 2.31, 1.83, 0.73, 0.93, 0.69, 0.80, 1.76, 0.67 and 0.90 %, respectively (Table-4).

Recovery test: The sample with known targeted contents was spiked with certain amounts of the nine standards. Then the spiked sample was processed in accordance with the established method for the HPLC detection. The recoveries of the nine standards were 101.4, 95.0, 97.5, 96.7, 98.3, 101.2,

TABLE-1
LINEAR REGRESSION EQUATION AND LINEAR RANGES

Component	Linear equation	R ²	Linear range (ng)
Scopolin	$y = 1.5039x - 0.1392$	1.000	11.08-498.96
Chlorogenic acid	$y = 2.1368x - 3.6174$	1.000	14.57-2039.52
Flavonoid glycoside A	$y = 1.4017x - 2.9278$	0.9999	32.04-4485.60
Flavonoid glycoside B	$y = 1.257x + 2.3491$	1.000	53.00-1484.00
Flavonoid glycoside C	$y = 1.3583x + 24.815$	0.9999	99.84-4992.00
Flavonoid glycoside D	$y = 1.1003x + 4.5157$	1.000	43.20-1484.00
Umbelliferone	$y = 2.894x - 0.9627$	1.000	3.84-133.84
Scopoletin	$y = 3.8236x + 0.9824$	0.9999	4.45-278.00
Rutin	$y = 1.5546x - 7.2712$	1.000	25.23-3531.64

TABLE-2
STABILITY TEST OF SAMPLE SOLUTION

	0 h	2 h	5 h	10 h	18 h	36 h	Mean	RSD (%)
Scopolin	95.79	91.66	98.29	94.20	95.16	93.12	94.70	2.55
Chlorogenic acid	343.13	354.81	342.64	337.05	337.66	336.48	341.96	2.18
Flavonoid glycoside A	524.02	524.21	521.38	523.19	530.94	531.78	525.92	0.69
Flavonoid glycoside B	674.44	670.25	667.02	677.21	682.95	684.36	676.04	0.91
Flavonoid glycoside C	1352.93	1353.49	1360.69	1362.78	1365.89	1368.85	1360.77	0.42
Flavonoid glycoside D	612.04	617.65	615.64	620.34	631.75	632.47	621.65	1.21
Umbelliferone	29.59	29.90	30.23	30.81	31.06	30.66	30.38	2.02
Scopoletin	179.36	180.27	183.59	181.25	185.72	187.79	183.00	1.42
Rutin	432.47	435.66	435.55	438.29	444.09	445.16	438.54	0.99

TABLE-3
STABILITY TEST OF STANDARD SOLUTION

	0 h	2 h	5 h	10 h	18 h	36 h	Mean	RSD (%)
Scopolin	80.91	81.75	81.34	81.93	81.94	81.87	81.62	0.34
Chlorogenic acid	302.36	305.83	305.66	306.45	307.43	306.52	305.71	0.57
Flavonoid glycoside A	438.04	442.13	442.50	443.44	444.84	443.89	442.47	0.58
Flavonoid glycoside B	655.40	661.53	662.59	663.13	665.13	664.12	661.98	0.56
Flavonoid glycoside C	1349.88	1360.82	1364.15	1367.42	1370.93	1369.88	1363.85	0.59
Flavonoid glycoside D	570.86	574.86	576.87	579.68	581.10	581.12	577.42	0.70
Umbelliferone	25.92	26.85	25.96	26.22	26.51	26.54	26.33	1.49
Scopoletin	165.74	167.86	167.08	168.12	169.03	168.20	167.67	0.74
Rutin	379.92	382.50	383.33	384.15	384.72	384.70	383.22	0.49

TABLE-4
RESULTS OF REPEATABILITY TEST (n = 6)

Component (mg/g)	1	2	3	4	5	6	Mean	RSD (%)
Scopolin	0.1420	0.1490	0.1415	0.1447	0.1489	0.1475	0.1460	2.6
Chlorogenic acid	0.3779	0.3683	0.3793	0.3687	0.3730	0.3800	0.3962	1.4
Flavonoid glycoside A	0.9026	0.9031	0.9119	0.9160	0.8992	0.9115	0.9074	0.73
Flavonoid glycoside B	1.284	1.285	1.295	1.304	1.282	1.311	1.294	0.93
Flavonoid glycoside C	2.375	2.362	2.383	2.389	2.369	2.408	2.381	0.69
Flavonoid glycoside D	1.100	1.097	1.106	1.117	1.097	1.1143	1.105	0.80
Umbelliferone	0.0263	0.0259	0.0258	0.0262	0.0265	0.0271	0.0271	1.8
Scopoletin	0.1143	0.1139	0.1141	0.1157	0.1147	0.1157	0.1157	0.67
Rutin	0.6818	0.6820	0.6879	0.6963	0.6817	0.6917	0.6917	0.90

104.8, 103.4 and 103.3 %, RSD were 2.51, 2.63, 2.04, 1.89, 1.87, 2.09, 1.57, 2.00 and 2.29 % (Table-5).

Application of the HPLC method for quantitation studies:

The experiment determined 29 samples of different batches

indifferent parts of *B. herb* by the above method. The contents of the 9 components were showed in Table-6.

Optimization of HPLC separation conditions: In order to get a separation with better resolution of targeted compo-

TABLE-5
RECOVERY RESULTS OF THE NINE ACTIVE INGREDIENTS (n = 9)

Component	Contents in samples (mg)	Added (mg)	Found (mg)	Recovery (%)	Mean (%)	RSD (%)
Scopolin	0.1649	0.0792	0.2435	99.2	101.4	2.51
	0.1689	0.0792	0.2523	105.2		
	0.1631	0.0792	0.2437	101.8		
	0.1697	0.1584	0.3245	97.8		
	0.1672	0.1584	0.3225	98.1		
	0.1704	0.1584	0.333	102.6		
	0.1697	0.2376	0.4127	102.3		
	0.1702	0.2376	0.4156	103.3		
	0.1736	0.2376	0.4168	102.4		
Chlorogenic acid	0.1502	0.0727	0.2199	95.9	95.0	2.63
	0.1539	0.0727	0.2252	98.1		
	0.1486	0.0727	0.2194	97.4		
	0.1546	0.1453	0.29	93.2		
	0.1523	0.1453	0.2902	94.9		
	0.1553	0.1453	0.2969	97.5		
	0.1546	0.218	0.3583	93.5		
	0.1551	0.218	0.3522	90.4		
	0.1582	0.218	0.3642	94.5		

Flavonoid glycoside A	0.3769	0.1602	0.5287	94.7	97.5	2.04
	0.3627	0.1602	0.5221	99.5		
	0.364	0.1602	0.5199	97.3		
	0.3695	0.3024	0.6897	99.9		
	0.3867	0.3024	0.6998	97.7		
	0.3885	0.3024	0.6901	94.1		
	0.368	0.4806	0.8311	96.3		
	0.3998	0.4806	0.8725	98.4		
Flavonoid glycoside B	0.4006	0.4806	0.8691	97.5	96.7	1.89
	0.5374	0.256	0.7883	94.7		
	0.5171	0.256	0.7792	98.9		
	0.5189	0.256	0.7765	97.2		
	0.5268	0.53	1.0491	98.6		
	0.5514	0.53	1.0652	97		
	0.5538	0.53	1.0487	93.4		
	0.5247	0.795	1.2837	95.5		
Flavonoid glycoside C	0.57	0.795	1.3472	97.8	98.3	1.87
	0.5712	0.795	1.3422	97		
	0.989	0.4992	1.47	96.3		
	0.9517	0.4992	1.4547	100.8		
	0.955	0.4992	1.4518	99.5		
	0.9695	0.9984	1.9622	99.4		
	1.0148	0.9984	1.9891	97.6		
	1.0193	0.9984	1.9664	94.9		
Flavonoid glycoside D	0.9657	1.4976	2.4312	97.9	101.2	2.09
	1.049	1.4976	2.5368	99.3		
	1.0512	1.4976	2.5303	98.8		
	0.4591	0.216	0.6705	97.9		
	0.4418	0.216	0.6646	103.1		
	0.4433	0.216	0.6639	102.1		
	0.45	0.432	0.8948	102.9		
	0.471	0.432	0.9032	100		
Umbelliferone	0.4731	0.432	0.896	97.9	104.8	1.57
	0.4483	0.648	1.1043	101.2		
	0.487	0.648	1.1529	102.8		
	0.488	0.648	1.1511	102.3		
	0.0109	0.0048	0.0154	101		
	0.0105	0.0048	0.0155	104.6		
	0.0105	0.0048	0.0158	104		
	0.0107	0.0096	0.0208	105.8		
Scopoletin	0.0112	0.0096	0.0214	106.3	103.4	2.00
	0.0113	0.0096	0.0214	105.8		
	0.0107	0.0143	0.0257	104.7		
	0.0116	0.0143	0.0268	105.9		
	0.0116	0.0143	0.0267	105.2		
	0.0477	0.0222	0.0706	103.2		
	0.0459	0.0222	0.0697	107		
	0.046	0.0222	0.0693	104.6		
Rutin	0.0467	0.0445	0.0932	104.5	103.3	2.29
	0.0489	0.0445	0.0941	101.5		
	0.0491	0.0445	0.0935	99.8		
	0.0465	0.0667	0.1148	102.3		
	0.0506	0.0667	0.12	104		
	0.0507	0.0667	0.1196	103.3		
	0.2853	0.1261	0.4087	97.8		
	0.2746	0.1261	0.4055	103.9		
Rutin	0.2755	0.1261	0.4013	99.7	103.3	2.29
	0.2797	0.2523	0.542	104		
	0.2928	0.2523	0.553	103.2		
	0.2941	0.2523	0.5457	99.7		
	0.2786	0.3784	0.6537	99.1		
	0.3026	0.3784	0.6926	103.1		
	0.3033	0.3784	0.6876	101.6		

TABLE-6
CONTENTS DETERMINATION OF THE NINE ACTIVE INGREDIENTS IN 29 BATCHES (mg/g)

S. No.	Scopolin (mg/g)	Chlorogenic acid (mg/g)	Flavonoid glycoside A (mg/g)	Flavonoid glycoside B (mg/g)	Flavonoid glycoside C (mg/g)	Flavonoid glycoside D (mg/g)	Umbelliferone (mg/g)	Scopoletin (mg/g)	Rutin (mg/g)
1	0.429	0.347	0.236	0.410	0.532	0.337	0.017	0.510	0.056
2	0.101	0.173	0.217	0.200	0.731	0.206	0.015	0.166	0.458
3	0.243	0.059	0.097	0.176	0.394	0.228	0.013	0.279	0.051
4	0.109	0.011	0.000	0.000	0.000	0.000	0.000	0.095	0.000
5	0.308	0.131	0.124	0.225	0.531	0.312	0.018	0.320	0.060
6	0.000	0.010	0.000	0.000	0.000	0.000	0.000	0.014	0.000
7	0.114	0.037	0.079	0.123	0.437	0.209	0.011	0.043	0.063
8	0.013	0.035	0.000	0.000	0.053	0.019	0.016	0.009	0.033
9	0.116	0.037	0.070	0.074	0.260	0.083	0.008	0.161	0.191
10	0.190	0.063	0.089	0.149	0.291	0.143	0.010	0.135	0.061
11	0.120	0.016	0.024	0.042	0.089	0.049	0.007	0.160	0.043
12	0.063	0.017	0.059	0.096	0.301	0.156	0.009	0.030	0.038
13	0.217	0.103	0.134	0.148	0.738	0.256	0.012	0.240	0.145
14	0.174	0.040	0.064	0.096	0.261	0.133	0.009	0.208	0.073
15	0.228	0.026	0.043	0.057	0.169	0.067	0.010	0.309	0.073
16	0.264	0.151	0.447	0.625	2.408	0.900	0.031	0.178	0.223
17	0.146	0.396	0.907	1.294	2.381	1.011	0.026	0.115	0.687
18	0.134	0.036	0.064	0.052	0.300	0.052	0.004	0.130	0.280
A1	0.016	0.375	0.357	0.295	2.665	0.799	0.023	0.146	0.686
A3	0.392	0.300	0.434	0.318	2.030	0.487	0.014	0.470	0.508
A4	0.281	0.163	0.425	0.247	1.941	0.419	0.013	0.336	0.565
A5	0.135	0.296	0.638	0.654	1.831	1.810	0.015	0.171	0.515
A6	0.184	0.282	0.314	0.286	0.590	0.249	0.009	0.053	0.594
A9	0.166	0.192	0.246	0.231	1.136	0.359	0.012	0.138	0.595
B1	0.054	0.096	0.288	0.368	1.699	0.697	0.018	0.073	0.361
B2	0.476	0.235	0.492	0.989	1.264	0.964	0.033	0.157	0.557
B3	0.132	0.287	0.089	0.174	0.174	0.067	0.003	0.078	0.022
B4	0.653	0.210	0.168	0.223	0.585	0.270	0.012	0.186	0.132
B5	0.034	0.032	0.022	0.020	0.112	0.029	0.003	0.024	0.181

nents with shorter analytical time, we compared three different column temperature: 25, 35 and 40 °C, then we found that the higher temperature, the shorter retention time with almost the same resolution, so we chose 35 °C 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, we chose reflux extraction as the way to prepare sample solution. In the meantime, we compared the efficiency of different elution solvents, namely methanol-phosphoric acid and methanol-phosphoric acid. The results indicated that the latter was slightly better. We also tried different reflux time: 1, 2 and 3 h. Finally, taken the determination of targeted components into consideration, 2 h reflux extraction was better.

Nine chromatographic columns of the same specification were compared in the experiment: Phenomenex Luna-C18, Agilent Extend-C18, Agilent Eclipse XDB-C18, Waters Sunfire-C18 and Agilent Extend-C18, according to the effect of separation, Agilent Zorbax SB-C18 (250 mm × 4.6 mm, 5 µm) was used for the further research of the methodology.

Conclusion

In this study, 9 active components were quantitatively determined in *B. herb*. On the whole, this developed method was simple, accurate for the determination of scopolin, rutin, chlorogenic acid, aloe-flavonoid glycoside D, flavonoid

glycoside B, flavonoid glycoside C, flavonoid glycoside D, umbelliferone and scopoletin simultaneously and reliable for the quality control and further efficacy study of *B. herb* in clinic.

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