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Simultaneous Determination of Two Effective Components in a *Tractylodes macrocephala* by RP-HPLC

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To establish a RP-HPLC method for simultaneous determination of two effective constituents (atractylenolide II and atractylenolide III) obtained from different district. Two effective constituents were simultaneously determined by RP-HPLC with WATERS XBridge C_{18} column (4.6 mm × 250 mm, 5 μm) by gradient elution (0-40 min, 60-100 % A) using methanol (A) and water (B) as the mobile phase. The flow rate was 1 mL min^{-1}; the detection wavelength was 230 nm (0-17 min) for atractylenolide III and 276 nm (17-25 min) for atractylenolide II with column temperature at 30 ºC. Two effective constituents were separated clearly and respectively in 25 min, the linear ranges of atractylenolide II and atractylenolide III was 0.0301-0.1505 μg (r = 0.9998) and 0.0398-0.1990 μg (r = 0.9999), the average recoveries (n = 3) were 99.08 and 98.88, RSD % were 1.98 and 1.69 %. The method is simple, accurate and can be used for quality control and evaluation of A *tractylodes macrocephala* Koidz.

Key Words: RP-HPLC, A *Tractylodes macrocephala* Koidz, Atractylenolide II, Atractylenolide III.

INTRODUCTION

A *tractylodes macrocephala* Koidz is the dry root of the plants of chrysanthemum family. It is the most widely-used tonic for vital energy, having the medical functions of tonic spleens, eliminating-dampness, alleviation of water retention and sweat-reduction. It is mainly used to treat the diseases of weak spleens, small appetite, distention of abdomen, diarrhea, edema, dizziness, dropsy, spontaneous sweating and painful fetal movement[1,2], *etc*. A *tractylodes macrocephala* Koidz is one of the so-called "characteristic medicines in Zhejiang of China". It has the characteristics of first-rate quality with high yield and good medical effect. Recent years of research has shown that it has the confirmed effect of regulating immune system, antidecrepitude, antitumor, antiinflammation, reducing the blood sugar level and urination-helping, *etc*. It is widely planted in Pan'an of Zhejiang Province, China, Anguo of He'bei Province, China and especially in Lin'an of Zhejiang Province, China with a large-scale, high-quality and standardized planting area. A *Tractylodes macrocephala* Koidz has a strong aroma because of its content of a certain amount of atractylenolide II and atractylenolide III chemical constituents[3-10]. Although there is some report about the content determination of the effective components in A *Tractylodes macrocephala* Koidz[11-15]. To our best of knowledge no literature in available about its research for the lactone of A *Tractylodes macrocephala* Koidz from different region. This article established the RP-HPLC method to determine the contents of atractylenolide II and atractylenolide III in A *Tractylodes macrocephala* Koidz of different growing places and provided the scientific basis for understanding its qualities in different growing places and establishing its quality control standards.

EXPERIMENTAL

Waters 2695 High Efficient Liquid Chromatograph (Online degasser, automatic sample-feeding machine, quarter gradient pump, column oven, American Waters company); Waters 2996 PAD detector (American Waters company); Empower Chromatographic working station; KQ-250B supersonic wave extractor (Kunshan Supersonic Instrument Manufacturing Company); Millipore Simplicity Super Pure Water machine (American Millipore Company); RE-52A rotary evaporator (Shanghai Ya-rong Biochemical Instrument Factory); Model 101-1 Electric Drying Oven; Mettler analyzing scale of the accuracy of one hundred thousandth (EETTLER AE 240 Switzerland); Auto Science solvent Filtration Device; spectrum pure methanol and the other reagents are all analytical pure.

The root of A *Tractylodes macrocephala* was collected in the Pan'an of Zhejiang Province, China, Lin'an of Zhejiang

Province, China and An'guo of He'bei Province, China in Nov. 2009 and was identified by Professor of Botany Lu-huan Lou of Zhejiang Forestry University. The samples of this plant are mixed and washed clean and then made into powder for later use. Reference substances of atractylenolide II and atractylenolide III (bought from the National Project Institute of Chinese Medicine Solid Reagent Producing Technology affiliated to Jiangxi Bencao Tian-gong Technological Corporation Ltd., the degrees of purity are all greater than 98 % by the method of HPLC area normalization method, with the batch number of 1018-090908 and 1018 -090812, respectively).

Methods

Establishment of HPLC determination method: Chromatographic conditions and system adaptive experiment: Waters XBridge C_{18} column (4.6 mm × 250 mm, 5 μm); mobile phase A; methanol; mobile phase B; distiled water; the process of gradient elution: 0-40 min 60-100 % A; detection wavelength 230 nm (0-17 min timing wavelength) and 276 nm (17-25 min timing wavelength); column temperature 30 ºC; sensitivity 0.2AUFS. Under the above chromatographic conditions, the separation degrees of the chromatographic peaks and the neighboring separation degree of the chromatographic peak of atractylenolide II and atractylenolide III are greater than 2.0; the numbers of the theoretic tower plates are both over 6000.

Preparation of the reference solution: Weigh precisely the reference substances of atractylenolide II 3.01 mg, atractylenolide III 3.98 mg and put them (50 mL) in the flask till the constant volume to scale. Then take 1 mL from the above solution and put it in the 10 mL flask and add methanol till the constant volume to scale. Prepare them into the mixed solution with the separate concentration of 0.00602, 0.00796 mg mL^{-1} in atractylenolide II and atractylenolide III. After filtration through the 0.45 μm sieve, its filtered liquid can be used as the contrast solution.

Preparation of the test solution: Pulverize the three samples of the Chinese medicine (collected in the Pan'an of Zhejiang Province, China, Lin'an of Zhejiang Province, China and An'guo of He'bei Province of A *Tractylodes macrocephala*) into fine powder (through sieve No. 3) and then take about 1.0016 g by weighing precisely and put it into the conical flask. Add methanol 25 mL and make it under the ultrasound for 20 min. Filtrated through the 0.45 μm filtrating film, its filtrated solution can used as the test solution for later use.

Investigation for its linear relationship: Suck up in turn precisely 5, 10, 15, 20 and 25 μL mixed contrast solution and put them into the high efficient liquid phase chromatographic instrument and determine them according to the above chromatographic conditions. Using the comparison substance mass X (μg) as the horizontal axis and peak area value Y as the vertical axis to make the linear regression and get the regression equations of atractylenolide II and atractylenolide III: Y = 5.52 × 106X + 1.51 × 104, r = 0.999824; Y = 2.00 × 106X + 2.89 × 103, r = 0.999997. This shows that a very good linear relationship, with the sampling volume of atractylenolide II between 0.0301-0.1505 μg and the sampling volume of atractylenolide III between 0.0398-0.1990 μg as indicated in Figs. 1 and 2.

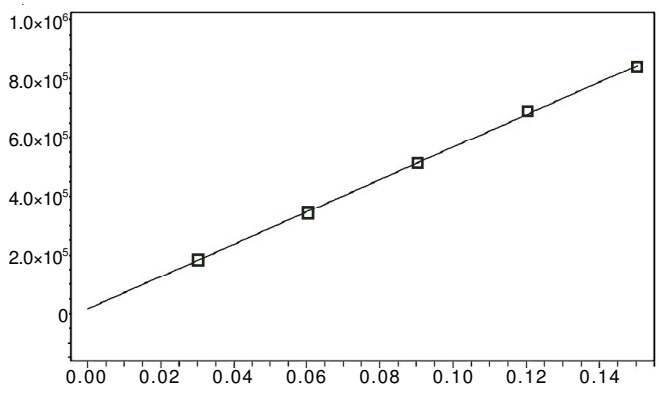

Fig. 1. Standard curve of atractylenolide II

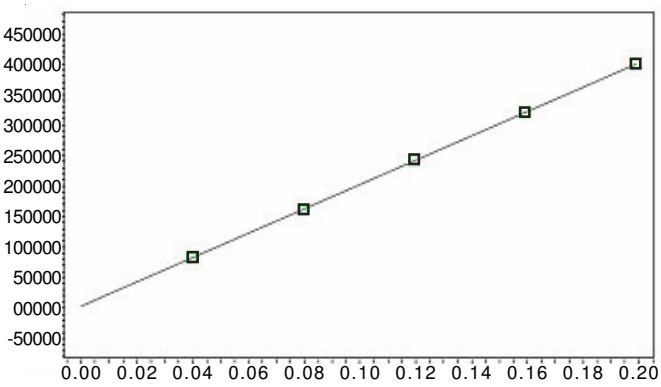

Fig. 2. Standard curve of atractylenolide III

Precision experiment: Take the test solution 10 μL and put it into the liquid phase chromatograph instrument with 6 times of continuous sample-feeding. Then determine their peak area integral quantities and get the peak area integral values of atractylenolide II and atractylenolide III, respectively: 222758, 223068, 225290, 226096, 235627, 234527, with RSD % being 2.51 % in atractylenolide II and the peak area integral values of atractylenolide III are 296119, 294699, 294623, 304826, 299374, 294832, with RSD % being 1.36 %. This shows a good precision in atractylenolide II and atractylenolide III.

Repeatable experiment: Take 6 shares of parallel sampling from the same batch of the sample, each about 1 g. Then weigh them precisely and prepare the test solution according to the method used in preparing the testing solutions. Determine each test solutions for 6 times and then put them into the high efficient liquid chromatograph instrument with 10 μL in each sample-feeding. Finally determine the peak area values of atractylenolide II and atractylenolide III so as to figure out the contents of each component. As can be seen in the Tables 1 and 2, the RSD of contents of atractylenolide II and atractylenolide III are 0.75 and 1.91 %, respectively.

Stability test: Take the same test solution and feed in 10 μL sample at 0, 3, 6, 9, 12, 15 h,and calculate their peak areas. The results are that the peak area values of atractylenolide II are 228008, 223068, 225192, 235141, 226084, 234498, respectively, with its RSD being 2.20 %; the peak area values of atractylenolide III are 294599, 295324, 294522, 298313, 304927, 294134, with its RSD being 1.41 %; this shows that the sample solution is the most stable at 15 h.

Sample-feeding recovery experiment: Weigh precisely five shares of the given amount of the same powder 1 g of

TABLE-1
REPEATED EXPERIMENTS OF ATRACTYLENOLIDE II

Weight of sample (g)	Peak area	Content of atractylenolide II (μg)	RSD (%)
1.0016	237632	0.040	–
1.0017	232899	0.039	–
1.0016	235088	0.040	0.75
1.0017	233411	0.040	–
1.0017	233921	0.040	–
1.0016	233465	0.040	–

TABLE-2
REPEATED EXPERIMENTS OF ATRACTYLENOLIDE III

Weight of sample (g)	Peak area	Content of atractylenolide III (μg)	RSD (%)
1.0016	321529	0.160	–
1.0017	313245	0.156	–
1.0016	331444	0.165	–
1.0017	319082	0.158	1.91
1.0017	317401	0.148	–
1.0016	318421	0.157	–

the A *Tractylodes macrocephala* Koidz. Then add proper amount of the contrast substances of atractylenolide II and atractylenolide III. Prepare them according to the method used in preparing the test solution. Take the sample test solution 10 μL and put them into the liquid phase chromatograph instrument and measure its peak area values. Then calculate the average sample-feeding recoveries of atractylenolide II and atractylenolide III which are 99.081 and 98.875 %, with RSD being 1.98 and 1.69 %, respectively, which are shown in Tables 3 and 4.

RESULTS AND DISCUSSION

Content determining results of the atractylenolide II and atractylenolide III in samples: Feed respectively each of the test solution 10 μL into the high efficient liquid chromatograph instrument and determine their peak area values according to the above-mentioned chromatographic conditions. Figure out the contents of atractylenolide II and atractylenolide III in the test solutions, which are shown in Table-5 and the Figs. 3-6.

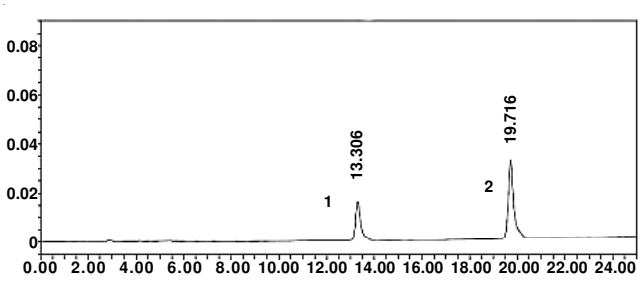

Fig. 3. Chromatogram of reference substances (1: atractylenolide III; 2: atractylenolide II)

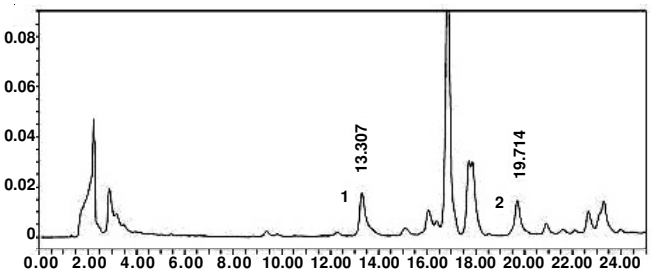

Fig. 4. Chromatogram of sample in atractylodes of Lin'an (1: atractylenolide III; 2: atractylenolide II)

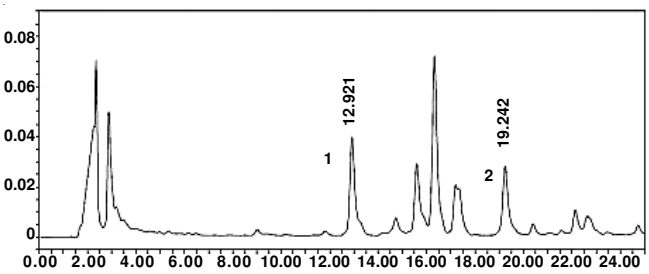

Fig. 5. Chromatogram of sample in atractylodes of Pan'an (1: atractylenolide III; 2: atractylenolide II)

TABLE-3
DETERMINATION RESULTS OF RECOVERIES OF ATRACTYLENOLIDE II

Weight of sample (g)	Content of sample (mg)	Content of reference substances intrant (mg)	Content of determination (mg)	Recoveries rate (%)	Average value (%)	RSD (%)
0.5001	0.0500	0.0501	0.0994	96.63	–	–
0.5002	0.0488	0.0501	0.0966	95.63	–	–
0.4999	0.0500	0.0501	0.1003	100.57	98.53	2.23
0.5001	0.0492	0.0501	0.0983	98.38	–	–
0.5002	0.0496	0.0501	0.0988	98.63	–	–
0.5000	0.0496	0.0501	0.1005	101.35	–	–

TABLE-4
DETERMINATION RESULTS OF RECOVERIES OF ATRACTYLENOLIDE III

Weight of sample (g)	Content of sample (mg)	Content of reference substances intrant (mg)	Content of determination (mg)	Recoveries rate (%)	Average value (%)	RSD (%)
0.5000	0.1975	0.1978	0.3908	97.73	–	–
0.5001	0.1973	0.1978	0.3939	99.63	–	–
0.5002	0.1974	0.1978	0.3874	96.08	98.74	1.80
0.4999	0.1978	0.1978	0.3837	98.96	–	–
0.4998	0.1982	0.1978	0.3935	98.72	–	–
0.5001	0.1980	0.1978	0.3985	101.35	–	–

TABLE-5
RESULTS FOR THE DETERMINATION OF SAMPLE (n = 3)

Sample	Weight of sample (g)	Average content of atractylenolide II (%)	Average content of atractylenolide III (%)
A *tractylodes* of Lin'an in Zhejiang Province	1.0006	0.0132	0.040
A *tractylodes* of Pan'an in Zhejiang Province	1.0007	0.0173	0.056
A *tractylodes* of An'guo in Hebei Province	1.0006	0.0160	0.026

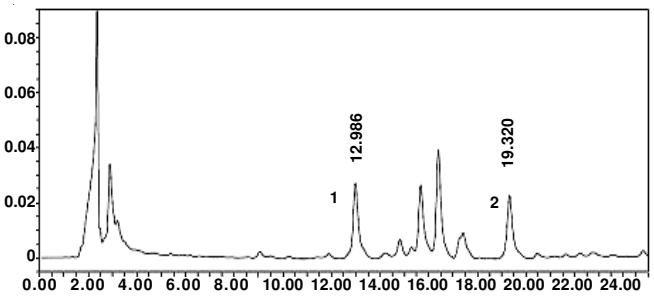

Fig. 6. Chromatogram of sample in atractylodes of An'guo (1: atractylenolide III; 2: atractylenolide II)

The method determination of the preparation for the test solution: Observe in turn the extract solvent (methanol, ethanol and acetone) of the different polarities and the different extrac-ting methods (supersonic method, successive reflux method and reflux method). Then optimize the extracting time. It is found that using methanol to extract 20 min by the supersonic method, the effective composition will be extracted completely.

The choice of the testing wavelength: Because the maximum absorption wavelength of atractylenolide III is 230 nm and the maximum absorption wavelength of atractylenolide II is 276 nm. The retention time in atractylenolide III is before 13 min, while the retention time in atractylenolide II is after 17 min, in order to increase the sensitivity of this method and reduce the outside interference, this experiment sets up the flexible program to determine, with the following results (wavelength being 230 nm between time periods 0-17 min and 276 nm between the time periods 7-25 min).

Choice of the mobile phase: This study compared the eluting systems of methanol-water and acetonitrile-water, finding that the system of mobile phase of methanol-water is helpful to the separation of atractylenolide II and atractylenolide III, with the separation values of both over 1.5. Thus, we use methanol-water as the solvent system in this experiment.

Choice of flow velocity: In the experiment, it is found that the flow velocity has a great influence on the separating effect of atractylenolide II and atractylenolide III. Control the flow speed within 0.5, 1.0 and 1.5 mg mL^{-1} and we will find that when the flow speed is at 1.0 mg mL^{-1}, the separating effect of the atractylenolide II and atractylenolide III is the best. Therefore, we choose the flow speed at 1.0 mL min^{-1}.

Choice of the column temperature: It is found that the column temperature also influences the separating effect of atractylenolide II and atractylenolide III. Control the column temperature within 22, 26, 30 and 34 ºC and conduct the experiment. When the column temperature is at 30 ºC, we adjust the mobile phase ratio and flow speed and it can reach the point of baseline separation. Therefore, we choose the column temperature at 30 ºC.

Analysis of the determinating results: This study use atractylenolide II and atractylenolide III as the observing target and determined the content of the two effective compositions in A *Tractylodes macrocephala* Koidz from three different growing areas. It can be seen from the results that the contents of the two effective compositions are quite different in different growing areas, with the relative high contents in atractylenolide II and atractylenolide III grown in Pan'an, Zhejiang province, the next being those grown in Lin'an, Zhejiang and the lowest in Angu, Hebei Province. In the same growing area, the content of atractylenolide III is much greater than that in atractylenolide II.

The RP-HPLC method established by this research has the characteristics of speediness, accuracy and high efficiency, which can be used the effective way of control the quality of a *Tractylodes macrocephala* Koidz.

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