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Simultaneous Determination of Reserpine, Yohimbine and Ajmalicine in the Extract of *Rauvolfia* Rootstocks, Branches and Leaves by HPLC

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Rauvolfia [*Rauvolfia verticillata* (Lour) Baill] is a standing indeciduous shrub of *Rauwolfia*. L, Apocynaceae, which was frequently used for cure of numerous diseases, like hypertension, headache, courbature, wind-heat, snake bite diseases, *etc*. A simultaneous high-performance liquid chromatography (HPLC) analysis was developed and validated for the determination of the contents of reserpine, yohimbine and ajmalicine in *Rauvolfia* rootstocks, branches and leaves. HPLC analysis was successfully conducted by using a Hypersil-C_{18} column, gradient elution with the mobile phase methanol and water contained 0.05 % triethylamine, and with a flow rate of 1 mL/min, detected at 280 nm. The developed HPLC method was precise, with relative standard deviation < 2.8 %. The recoveries for the three indole alkaloids in *Rauvolfia* was between 98.3 and 100.5 %. The average contents of reserpine, yohimbine and ajmalicine in root were more than branch and leaf. They were 135.6 ± 0.5, 176.5 ± 0.2, 118.5 ± 0.1 (μg/g), respectively. The method was simple, accurate and reproducible and can provide a quantitative basis for the quality control of *Rauvolfia*.

Keywords: *Rauvolfia*, Reserpine, Yohimbine, Ajmalicine, HPLC.

INTRODUCTION

Rauvolfia [*Rauvolfia verticillata* (Lour) Baill] is a standing indeciduous shrub of *Rauwolfia. L*, Apocynaceae, naturally distributed throughout Burma, China and Vietnam. In China, *Rauvolfia* grow widely in Guangxi, Yunnan, Guizhou and Taiwan and generally grow in the low mountain hills or creek bushes and small trees. They are accustomed to hot and humid weather and can withstand short-term frost. For the past few years, the plants of *Rauwolfia. L* have always been of interest to mankind, in which *Rauvolfia* [*Rauvolfia verticillata* (Lour) Baill], *Rauvolfia yunnanensis* [*R. yunnanensis* Tsiang] and *Rauvolfia vomitoria* [*R. vomitoria* Afzelius] is mainly included[1-3]. The object in this research is *Rauvolfia* [*Rauvolfia verticillata* (Lour) Baill] grown in Pingbian, Yunnan. Many parts of the plant are considered to be a good source of a large number of bioactive substances. The bioactive substances are reputed to have considerable medicinal value and are frequently used for hypertension, headache, courbature, wind-heat, snake bite[4-6]. The indole alkaloids in *Rauvolfia* are main and bioactive constituents and are also the main natural sources of antihypertensive drug reserpine, which are reported to lower blood pressure and have antiarrhythmic, antitumor and sedation properties[7-10], have attracted considerable attentions in the world. Studies found that indole alkaloids existed in *Rauwolfia* roots, stems, branches, leaves and flowers[11-14]. Reserpine, yohimbine and ajmalicine are three major indole alkaloids in the extract of *Rauvolfia* rootstocks, branches and leaves, which have similar molecular structures as shown in Figs. 1-3.

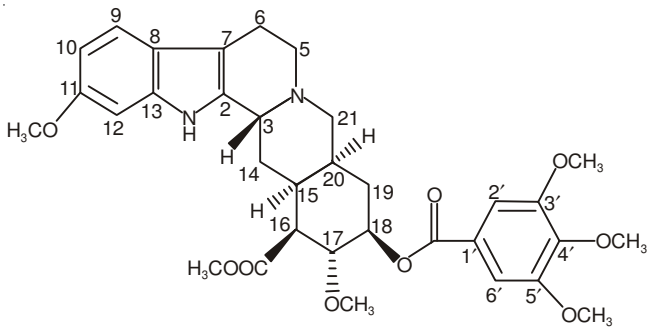

Fig. 1. Chemical structure of reserpine

Reserpine can play an antihypertensive effect by exhuasting the adrenaline around sympathetic nerve endings and at the same time play a sedation effect by acting on the parts of hypothalamus[15]. Yohimbine can be used for the treatment of male sexual dysfunction, and has antiatherosclerotic and antirheumatic properties[16]. Ajmalicine has potent antiarrhythmic

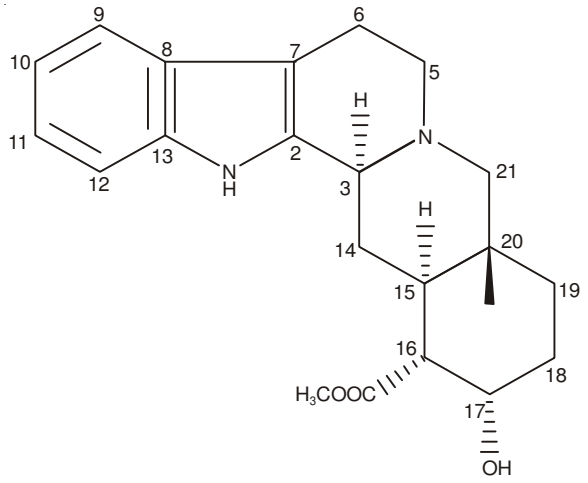

Fig. 2. Chemical structure of yohimbine

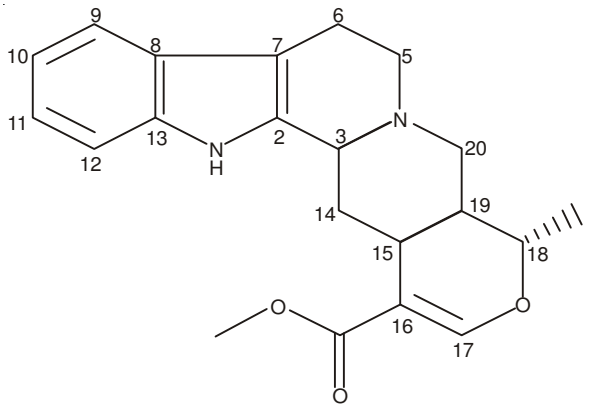

Fig. 3. Chemical structure of ajmalicine

effects as well as antihypertensive effects. The three compounds are major indole alkaloids and bioactive constitutes in the extract of *Rauvolfia* rootstocks, branches and leaves. Therefore, it is important for the determination of the three major indole alkaloids.

The analytical methods for the analysis of indole alkaloids in *Rauvolfia* are nuclear magnetic resonance (NMR), liquid chromatography with mass spectrometry (LC-MS) and reverse phase high performance liquid chromatography (RP-HPLC). NMR and LC-MS have proved to be valuable detection methods due to their sensitivity and tandem mass spectrometry (MS-MS) facilitates exclusive analysis of selected peaks of interest with unequivocal peak identification. These analytical methods require complex sample preparation, highly trained personnel and maintenance, which make these detection methods currently impractical for routine analysis. HPLC is the simplest tool for the quantitative determination of the bioactive constituents in pharmaceutical industry[17-20]. As reserpine, yohimbine and ajmalicine are strong chromophores, it makes UV detection easy and feasible. So far, there's no report on simultaneous determination of the three major indole alkaloids in *Rauvolfia* rootstocks, branches and leaves by HPLC method.

In present study, different extraction methods were compared and optimized for the high extraction yield of the three indole alkaloids. A validated system permitting the separation of reserpine, yohimbine and ajmalicine within 25 min without the need of sample clean-up prior to analysis from the crude extract of *Rauvolfia* has been developed.

EXPERIMENTAL

The chromatographic system consisted of Agilent Chemstation software (Agilent company, USA), Agilent G1379A Degasser (Agilent company, USA), Agilent G1311A Quatpump (Agilent company, USA), Agilent G1313A Autosampler (Agilent company, USA), and Agilent G1314A Ultraviolet Detector (Agilent company, USA), Hypersil-C_{18} column (4.6 mm × 250 mm, 5 µm, Dalian Zhonghuida Corporation, China). Methanol was of HPLC grade (Shandong Yuwang Industry Company, China). Trichloromethane and triethylamine were of analytical grade (Shandong Yuwang Industry Company, China). Deionized water was prepared by a Automatic triple pure water distiller (Shanghai Yarong Biochemical Instrument Factory, China). Reserpine, yohimbine and ajmalicine standards were purchased from Sigma company (USA). Rootstocks, branches and leaves of *Rauvolfia* were collected from the hilly of Pingbian, Yunan province.

Preparation of standard solutions: Standard stock solutions of three indole alkaloids were prepared in methanol, at concentration of 0.101 mg/mL for reserpine, 0.101 mg/mL for yohimbine and 0.102 mg/mL for ajmalicine. All sample solutions were filtered through 0.45 µm membrane filter (Millipore) and injected directly.

Preparation of sample solution: The extraction was carried out using 1 g of powdered rootstocks,branches and leaves, respectively with 30 mL of trichloromethane in an ultrasonic extraction device for 30 min,repeated for twice and wash the residue with 30 mL trichloromethane. The extract and washing liquid were combined and filtered, then evaporated to dryness under reduced pressure in a rotary evaporator. The dried extract was dissolved in methanol, diluted to a 10 mL volumetric flask. After filtering through a filter paper and a 0.45 µm membrane filter (Millipore), the extract was injected directly.

Chromatographic conditions: Chromatographic analysis was carried out by Hypersil-C_{18} column reversed-phase column (ø 4.6 × 250 mm) packed with 5 µm diameter particles, the temperature of the column was set at 30 °C. The mobile phase was methanol (A) -water (B) containing 0.05 % triethylamine. The gradient elution was programmed as follows: 0-12 min, 30 % B; 12-30 min, 30-10 % B. This mobile phase was filtered through a 0.45 µm membrane filter (Millipore), then deaerated ultrasonically prior to use. Reserpine, yohimbine and ajmalicine were quantified by UV following RP-HPLC separation at 280 nm. Flow rate and injection volume were 1 mL/min and l0 µL, respectively. The chromatographic peaks of the analytes were confirmed by comparing their retention time and UV spectra with those of the reference standards. Quantification was carried out by the integration of the peak using external standard method.

RESULTS AND DISCUSSION

Reproducibility: A standard mixture solution of 0.101 mg/g reserpine, 0.101 mg/mL yohimbine, 0.102 mg/mL ajmalicine was analyzed six times to determine the reprodu-

cibility of the peak areas and retention time under the optimum conditions in this experiment. The relative standard deviations (R.S.D.) of the peak areas were 4.2 % for reserpine, 3.4 % for yohimbine and 3 % for ajmalicine.

Precision and stability: The precisions (expressed as the relative standard deviation) for peak area were determined for all three compounds standards by repeated analysis (n = 6). The relative standard deviations of the peak areas were, 2.2 % for yohimbine, 2.5 % for ajmalicine and 2.8 % for reserpine. The results show that relative standard deviations for peak area were quite low and the precision is good. For stability test, a sample solution (sticks sample solution) was analyzed 0, 2, 4, 8, 12 and 24 h in 1 days and the sample solution was found to be rather stable within 24 h (RSD < 3 %).

Linearity and detection limit: A series of the standard mixture solutions of these three compounds were tested to determine the linearity between the standard mixture concentration and peak areas. The results of regression analysis on calibration curves and detection limits were presented in Table-1. The detection limits were evaluated on the basis of a signal-to-noise ratio of 3 (S/N = 3), the detection limits was between 0.000088 and 0.00018 mg/mL for three compounds.

Recovery: The recovery experiments of the three compounds were performed by adding 80, 100 and 120 % yohimbine, ajmalicine, reserpine standards of the extract of Yunnan Pingbian *Rauvolfia* branches, peak areas were recorded to calculate contents of three compounds of the test solution. With measured value of three components and the original amount that the test solution contained to calculate the recovery. The recoveries for the three indole alkaloids in *Rauvolfia* was that yohimbine was 100.5 % (RSD, 3.7 %), ajmalicine was 98.3 % (RSD, 2.3 %) and reserpine was 98.9 % (RSD, 3.1 %).

Sample analysis: Solutions in the extract of Yunnan Pingbian *Rauvolfia* rootstocks, branches and leaves were injected directly and separated under the condition mentioned earlier. The result was shown in Fig. 4 (1-yohimbine, 2-ajmalicine, 3-reserpine). The calculated contents of the three compounds were given in Table-2.

TABLE-2
CONTENTS (μg/g) OF THREE INDOLE ALKALOIDS IN RAUVOLFIA FROM DIFFERENT RESOURCES

	Contents of compounds (μg/g)[a]		
Analyte	Yohimbine	Ajmalicine	Reserpine
Root	135.6 ± 0.5	176.5 ± 0.2	118.5 ± 0.1
Branch	77.0 ± 0.1	84.6 ± 0.2	76.6 ± 0.2
Leaf	17.5 ± 0.2	11.8 ± 0.1	10.9 ± 0.1

[a]Mean ± S.D., n = 3

The maximum absorption wavelength of indole alkaloids was about 280 nm, this study used HPLC-UV method for the simultaneous determination of the content of several herbs major indole alkaloids and compared the content of the differences between different parts. The method is simple, duplicate, no interference of impurities and the measurement time is short. *Rauwolfia* alkaloids such as reserpine and yohimbine have been determinated, but because medicine concented *Rauwolfia* rhizome mainly and determination of the main component parts was roots mostly. So far, determination of the three components in *Rauwolfia* roots, branches and leaves at the same time has not been reported.

As a natural complex systems, Chinese herbal medicines contained a large number of structural analogs and increased difficulty of the separation and analysis. In this paper, three compounds separated completely in 25 min by gradient elution method and without interference. Methanol -0.05 % triethylamine was used as the mobile phase, the method was economic and rational. Triethylamine can be a good reagents for the peak shape of each compound and the method is simple and reproducible.

At the same time, combined with the literature reports , the preparation of the test solution methods were investigated. 75 % ethanol, chloroform and methanol were examed as extraction solvent. The extraction method was reflux extraction along with ultrasonic extraction. The results show that ultrasonic chloroform extraction method is simple and rapid, three compounds can be extracted more completely and with good reproducibility.

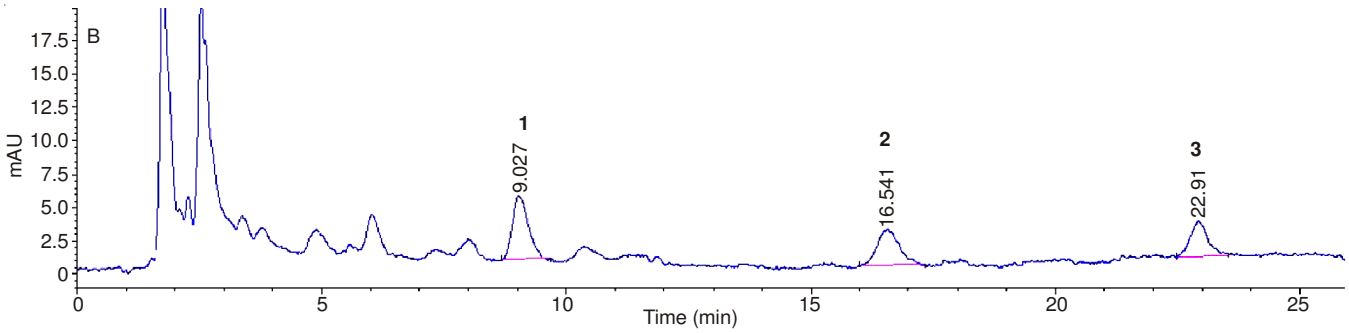

Fig. 4. Chromatograms of sample solution (1-yohimbine, 2-ajmalicine, 3-reserpine)

TABLE-1
CALIBRATION CURVE, CORRELATION COEFFICIENT, TEST RANGE, INSTRUMENTAL LODS AND LOQS

Analyte	Calibration curve	r	Range (μg/g)	LOQ (μg/g)	LOD (μg/g)
Yohimbine	y = 12.22x + 10.88	0.9990	1.01-40.4	0.32	0.088
Ajmalicine	y = 14.90x + 8.06	0.9997	1.02-40.8	0.43	0.150
Reserpine	y = 10.07x + 3.52	0.9998	1.01-40.4	0.54	0.180

In this paper, the indole alkaloids of Yunnan Pingbian on *Rauwolfia* roots, branches, leaves. Yohimbine, ajmalicine, reserpine using the analytical method established to determinate are the mainly alkaloids of *Rauwolfia* and also major effective components. So the three substances were determined for the more comprehensive quality control of medicines. Test showed that the accumulation of the active ingredient in different parts was in different levels. Alkaloids in roots were the highest, followed by branches, while leaves was the least. The content of reserpine in branches and leaves was similar.

Conclusion

The RP-HPLC method mentioned here represented an excellent technique for simultaneous determination of yohimbine, ajmalicine and reserpine in the extract of *Rauvolfia* rootstocks, branches and leaves, with good sensitivity, precision and reproducibility. The method gives a good resolution among yohimbine, ajmalicine, reserpine with the analysis time (25 min). Furthermore, the method can be used as quality control of alkaloids in *Rauvolfia* rootstocks, branches and leaves and will play a reference role on the determination of alkaloids in other medicinal plants or pharmaceutical preparations.

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