Simultaneous Determination of Four Andrographolides in *Andrographis paniculata* Nees by Silver Ion Reversed-Phase High-Performance Liquid Chromatography

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Abstract

A rapid and sensitive silver ion high-performance liquid chromatographic (Ag[I]-HPLC) method is developed for the simultaneous determination of the biologically active diterpenoids andrographolide, 14-deoxy-11,12-didehydroandrographolide, 14-deoxyandrographolide, and neoandrographolide in *Andrographis paniculata* Nees. HPLC is carried out for determining andrographolide and its derivatives with methanol–water (55:45, v/v) as the mobile phase on a C_{18} column (5 µm, 150 mm × 4.6 mm i.d.) with UV detection at 205 nm. Four andrographolides are baseline separated in a novel way: by adding silver ions (0.005 mol L^{-1}) to the previously mentioned mobile phase. Validation of the method challenges specificity, linearity, limit of detection, limit of quantitation, accuracy, and repeatability, and the results met the acceptance criteria for all analytes. The molecular mechanism of retention is demonstrated by comparing partition coefficients (logP) of different andrographolides and andrographolide-Ag(I) complexes. Thus, the method is successfully applied to characterize and determine the four andrographolides in *Andrographis paniculata* Nees extract and its commercial product.

Introduction

Andrographis paniculata Nees (Acanthaceae) is a famous herbal medicine in China and South Asia. The main active constituents of *Andro-graphis paniculata* include labdane diterpenes (1), such as andrographolide, dehydroandrographolide (same as 14-deoxy-11,12-dihydroandrographolide), 14-deoxyandrographolide, neo- andrographolide, and flavonoids.

Extensive research has shown that different diterpenes exhibit different pharmacological effects (2,3). Andrographolide, chemically designated as 2(3H)-furanone, 3-[2-[decahydro-6-hydroxy-5-(hydroxy-methyl)-5,8a-dimethyl-2-methylene-1-napthalenyl] ethylidene] dihydro-4- hydroxy (Figure 1) has anti-inflammatory, immuno-stimulatory, antiviral, anti-microbial, anti-platelet aggregation, hepatoprotective, and anti-cancer activities (4). Dehydroandrographolide demonstrated increased in vitro inhibition against the human immuno-deficiency virus (5). Burgos (6) reported that 14-deoxyandrographolide is a uterine smooth muscle relaxant which produces a selective blockage of voltage-operated calcium channels. Zhang (7) reported that dehydroandrographolide exhibited a greater hypotensive effect in anaesthetized rats and a vasorelaxant activity in isolated rat aorta, compared with 14-deoxyandrographolide. Dehydroandrographolide appeared to have a more potent stimulant effect on nitric oxide production than 14-deoxyandrographolide.

These different effects indicated that qualitative and quantitative analysis of the herbal materials and commercial preparations to ensure its maximal therapeutic value is of great importance. Bioanalytical procedures such as high-pressure liquid chromatography (8), microemulsion electrokinetic chromatography (9,10), and micellar electrokinetic chromatography (11) have been applied to the quantitative determination and routine control of andrographolide, andrographolide analog, and/or metabolites in biological matrices such as blood, serum, plasma, or urine (12,13). Several reversed-phase high-performance liquid chromatography (RP-HPLC) (14,15) assays have been published for the determination of andrographolides in herbal materials or commercial preparations. To the best of our knowledge, there are no reports of simultaneous detection of four andrographolides in herbal and commercial products by RP-HPLC. Minute structural differences between 14-deoxyandrographolide and dehydroandrographolide might be magnified by silver ion complexation (16) in the mobile phase of HPLC, due to the difference in the amounts and positions of double bonds. Using this chromatographic technique (also called 'argentation chromatography'), satisfactory separation was obtained for the quantitative analysis of four major andrographolides in a herbal extract and a commercial preparation.

Materials and Methods

Materials and reagents

Andrographolide, dehydroandrographolide, 14-deoxyandrographolide, and neoandrographolide (the structures are shown in Figure 1) were purchased from the National Institute for Control of Pharmaceutics and Biological Products (Beijing, China).

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Andrographis paniculata Nees extract was provided by Hefei Tuofeng Company of Bioengi-neering Ltd. The Chuanxinlian tablets (Yuxi Pharmaceutical Limited Company, Yunnan, PR China) were purchased from a local herbal store. Methanol of HPLC grade for chromatography was provided by Merck (Darmstadt, Germany). Silver nitrate and all other chemicals were of analytical grade from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China), and used without further purification. Deionized water was obtained from a Milli-Q water filtration/purification system (Millipore, Bedford, MA).

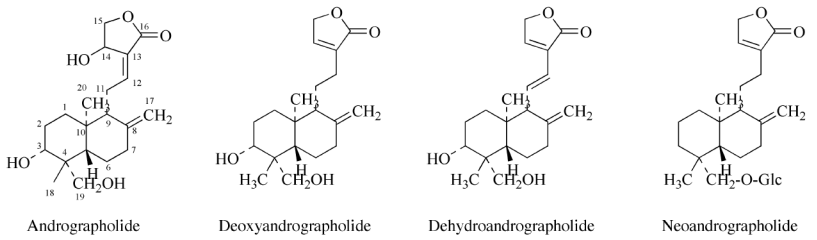

Figure 1. Molecular structures of andrographolide and its analogs.

Table I. Summary of Method Validation for the Determination of Andrographolides in *Andrographis paniculata* Nees Extract

	Andrographolide	Dehydroandrographolide
System suitability USP tailing factor	1.1	1.05
Theoretical plate number	2661	3964
Resolution	> 2	1.5
Detection limit (S/N = 3)	0.05 µg/mL	0.05 µg/mL
Quantitation limit (S/N = 10)	0.25 µg/mL	0.25 µg/mL
Linearity	0.25–200 µg/mL, r = 0.9992, b = 240.6	0.25–200 µg/mL, r = 0.9991, b = 280.2
Accuracy	98.2%, 99.3%, 99.1% for 80%, 100%, 120% levels, respectively	99.2%, 98.5%, 97.9% for 80%, 100%, 120% levels, respectively
Repeatability	Mean%: 99.1%, RSD = 0.5%	Mean%: 98.9%, RSD = 0.6%
Intermediate precision	Mean%: 98.5%, RSD = 1.2%	Mean%: 99.1%, RSD = 0.9%
	14-Deoxyandrographolide	**Neoandrographolide**
System suitability USP tailing factor	1.1	1.2
Theoretical plate number	5107	4058
Resolution	1.7	> 2
Detection limit (S/N = 3)	0.05 µg/mL	0.05 µg/mL
Quantitation limit (S/N = 10)	0.25 µg/mL	0.25 µg/mL
Linearity	0.25–200 µg/mL, r = 0.9983, b = 251.1	0.25–200 g/mL, r = 0.9985, b = 260.2
Accuracy	97.1%, 98.3%, 99.2% for 80%, 100%, 120% levels, respectively	96.1%, 96.7%, 97.3% for 80%, 100%, 120% levels, respectively
Repeatability	Mean%: 98.1%, RSD = 0.9%	Mean%: 99.1%, RSD = 0.8%
Intermediate precision	Mean%: 97.5%, RSD = 1.3%	Mean%: 98.5%, RSD = 1.5%

Chromatographic system

HPLC analysis was performed on an Agilent 1100 series chromatographic system (Agilent Technologies, Palo Alto, CA) comprised of online vacuum degasser, binary gradient pump, built-in variable wavelength detector, and auto sampler. ChemStation software (Rev. A 08.03) was used to control the entire LC system.

The analyte was determined at room temperature on an analytical column (Zorbax SB C18, 150 mm × 4.6 mm, i.d., 5 µm particle size). Elution was isocratic, using a mobile phase consisting of 55% methanol and 45% water (v/v). To this mixture, 0.005 mol L^{-1} Ag(I) was added. The mobile phase was filtered under vacuum through a 0.45 µm membrane filter (47 mm) (Millipore), and degassed prior to use. The analysis was carried out at a flow rate of 0.8 mL/min with UV detection at 205 nm.

Preparations of standards and quality control samples

Stock standard solutions of andrographolide, dehydroandrographolide, 14-deoxyandrographolide, and neoandrographolide of accurately weighed amounts (2 mg each) were prepared with the mobile phase mentioned previously. The stock solutions were stored at –4°C in darkness and brought to room temperature before use.

Six calibrators of standard were freshly prepared by diluting the stock solutions with methanol in appropriate quantities. The calibration range was 0. 5–200.0 µg andrographolide per mL. Three sets of control samples for andrographolide, neoandragrapholide, 14-deoxyandrographolide, and dehydroandrographolide were prepared in the same way for calibration from separate stocks at low (0. 5 µg/mL), medium (100 µg/mL), and high (240 µg/mL) concentrations in the same way for calibration, so as to lie in the lowest, middle, and highest regions of the calibration curves. All samples were injected in triplicate.

Preparations of *Andrographis paniculata* real samples

An appropriate amount of the dried, powdered herbal extract or Chuanxinlian tablets was extracted with methanol at room temperature by sonicating for 10 min, and cooled to room temperature. After centrifugating at 10,000 g, the resulting supernatant was subjected to HPLC analysis.

Calculation of partition coefficients

The logP values of andrographolides and andrographolide-Ag(I) complexes were calculated by ClogP V4.01 (BioByte Corporation, Claremont, CA) (17). The software divides molecules into fragments and uses fragment constants and correction factors taken from its

database as described by Log $K_{ow} = \Sigma n_i f_i$, where n_i is frequency of occurrence of a given group or correction factor and f_i is the contribution of that group or fragment constants.

Molecular structures of four Andrographolides and Andrographolides-Ag(I) complexes were constructed in ChemDraw Ultra V 8.0.1(Cambridge Soft Corporation, Cambridge, MA) (18) and calculated by a built-in ClogP program.

Results

Separation of andrographolides by silver ion LC

Major RP-HPLC separation is based on hydrophobic, electrostatic, or Van der Waals interaction of analytes between mobile and stationary phases. π-Complexation bonds are generally stronger than Van der Waals interaction, which gives rise to high selectivity, and many of these bonds are weak enough to be reversible in HPLC separation processes. π-Complexation has been seriously considered for olefin/paraffin (19) separation and purification by using a solution of cation Ag(I) or Cu(I).

Although the surface of the HPLC stationary phase is hydrophobic, the required Ag(I) can be dissolved in the mobile phase to allow π-complexation with the andrographolides. Primarily, the amount of π electrons in the target absorbate molecule and the ease of donating these π electrons to the S-orbital of the cation (silver ion) helped control the retention of andrographolides. Preliminary experiments indicated that dehydroandrographolide and 14-deoxyandrographolide in *Andro-graphis paniculata* extract were eluted near each other to be unresolved in RP-HPLC. The resolution of dehydroandrographolide and 14-deoxyandrographolide needed to be accomplished because both are key components in these preparations. Dehydroandrographolide has one more double bond than 14-deoxyandrographolide, which allows increased affinity for Ag(I) compared with 14-deoxyandrographolide. Therefore, less retention on the hydrophobic stationary phase was obtained, and baseline separation of dehydroandrographolide and 14-deoxyandrographolide in HPLC was a result.

Validation and application of HPLC method

The total RP-HPLC analysis time was less than 25 min. The specificity of the method was tested by screening different batches of herbal materials. The chromatograms indicated that the method was specific for determining andrographolides under the chromatographic conditions. No interferences were observed for andrographolides in real samples.

The method was validated for its system suitability, linearity, limit of detection, limit of quantification, accuracy, repeatability, and intermediate precision. Table I summarizes the validation results, which demonstrate that the method is suitable for determining the purity of the four andrographolides in herbal materials. Determination of the four andrographolides in herbal extract and a commercial preparation is shown in Table II.

Silver ion complexes with andrographolides and their hydrophobic characteristics

Hydrophobic characteristics of andrographolides and andrographolide-Ag(I) complexes were discussed by logP value, which is the logarithm of the partition coefficient between *n*-octanol and water. The *n*-octanol/water partition coefficient is the ratio of this compound's concentration in *n*-octanol to that in water when the phases are at equilibrium.

In HPLC analysis, logP is related directly to retentive behavior and is a well-established measure of the compound's hydrophilicity or hydrophobicity. Low hydrophilicities and therefore high logP values cause poor solvation in the moblie phase and strong absorption on the stationary phase. Tentative structures of 14-deoxyandrographolide-Ag(I) and dehydroandrographolide-Ag(I) complexes are shown in Figure 2. The logP values of andrographolides and their complexes with silver ions were calculated by ClogP (17), and shown in Table III. According to literature (20), silver ions tend to complex with two olefin bonds, which means that dehydroandrographolide-Ag(I) complex is the favorite form. 11,12 or 13,14 Olefin bonds other than carbonyl or olefin bonds in other parts of andrographolides were emphasized because they are the only difference between dehydroandrographolide and 14-deoxyandrographolide.

From Table III, we find that the addition of silver ions has changed the retention time of andrographolides in different ways. From andrographolides to andrographolide-Ag(I) complexes, logP of 14-deoxyandrographolide did not decrease as dramatically as that of dehydroandrographolide. Thus, differential values of log P explained how silver ions affect the HPLC elution and suggested that π-complexation has a notable influence on the resolution of structurally related terpenes. Relying on this

Table II. The Content of Andrographolides in *Andrographis paniculata* Nees Extract and a Medicine Preparation

	Andrographis paniculata Extract		Chuanxinlian Tablets	
	Content (%)	RSD (%, *n* = 3)	Content (%)	RSD (%, *n* = 3)
Andrographolide	0.89	2.13	0.52	2.27
Dehydroandrographolide	1.58	1.47	2.87	1.24
14-Deoxyandrographolide	0.57	1.96	0.17	2.89
Neoandrographolide	0.48	2.71	ND	ND

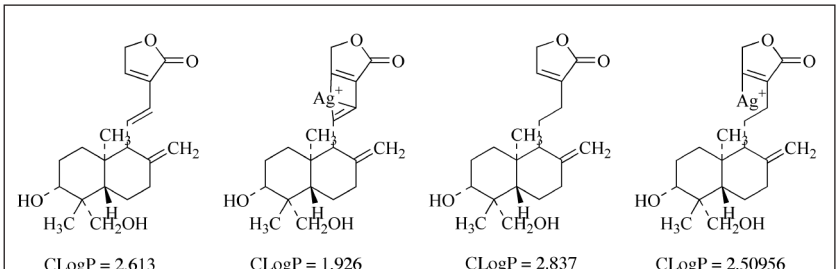

Figure 2. Tentative structures of dehydroandrographolide-Ag(I) and 14-deoxyandrographolide-Ag(I) complexes and their partition coefficients calculated by CLogP.

method, we provide a shortcut to model and predict the selectivity of Ag(I)-HPLC for olefins.

These results were consistent with retention times of dehydroandrographolide and 14-deoxyandrographolide, shown in Figure 3. Baseline resolution of the four andrographolides was obtained. In particular, resolution of dehydroandrographolide and 14-deoxyandrographolide was above 1.5 in the condition where silver ions existed in mobile phase.

Conclusions

This paper describes a specific and sensitive HPLC method for the determination of four andrographolides in an herbal extract and a commercial preparation. The HPLC profiles of andrographolide, dehydroandrographolide, 14-deoxyandrographolide, and neoandrographolide were baseline separated in the presence of 0.005 mol L^{-1} of silver ions. By comparing andrographolide-Ag(I) interaction by partition coefficient, we successfully elucidated the retention mechanism of Ag(I)-HPLC.

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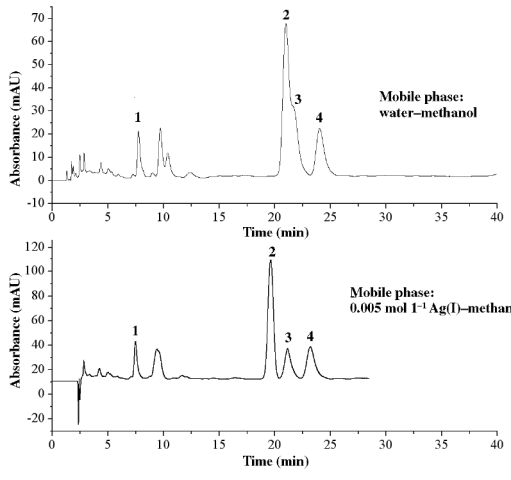

Figure 3. HPLC chromatogram showing the separation of andrographolides. Water–methanol in ratio of 45:55(%, v/v) (A), 0.005 mol L^{-1} Ag(I)–methanol in ratio of 45:55 (%, v/v) (B). For other details, please see "Materials and Methods". Peaks: andrographolide, 1; dehydroandrographolide, 2; 14-deoxyandrographolide, 3; neoandrographolide, 4.

Table III. Partition Coefficients of Dehydroandrographolide, 14-Deoxyandrographolide, Dehydroandrographolide-Ag(I), and 14-Deoxyandrographolide-Ag(I) Complexes

	Dehydroandrographolide		Deoxyandrographolide		
	RT (min)	$logP_1$	RT (min)	$logP_2$	$logP_2$–$logP_1$
Blank	20.98	2.613	21.67	2.837	0.224
Ag(I) complex	19.56	1.926	21.12	2.509	0.583

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