The Rapid Determination of Artemisinin by Post-Column Derivatization High-Performance Liquid Chromatography Using Matrix Solid-Phase Dispersion Method

Hongcheng Liu[1], Qiwan Li[1,*], Shaoping Li[2], Yanhong Zou[1], and **Anyun Gu[2]**

[1]Kunming Supervision and Testing Center for Farm Product Quality, Ministry of Agriculture, (Kunming), Yunnan Academy of Agriculture Science, Quality Standard and Testing Technology Institute, Kunming, 650223, China and [2]Medicinal Plants Research Institute, Kunming, 650223, China

Abstract

Artemisinin (an antimalaric compound) is isolated as the active compound of the medicinal plant *Artemisia annua L.* A simple, rapid, and high-efficient method of extraction is developed, in which it is extracted by matrix solid-phase dispersion (MSPD) and directly analyzed by post-column derivatization high-performance liquid chromatography (HPLC). The quantitation results from the MSPD method are compared with two conventional liquid solvent extraction processes, Soxhlet and ultrasonic wave by F-test, and the result indicates no significant difference. The production rate of Artemisinin during wild plant growth (tested over two years, acquired from Yunnan, provincial Yuan Yan country) is determined. The recovery range of determination is 88.1–91.2%. The relative standard deviation (RSD) is 4.55–6.43%. The limits of detection are 0.1 µg/mL, and the limits of quantitation are 0.5 µg/mL.

Introduction

Malaria continues to be a major health problem in many areas of the world, causing about 300 million illnesses and at least one million deaths per year, as reported by the World Health Organization (WHO) (1).

Artemisia annua L. (Sweet Wormwood) has been used in traditional Chinese medicine for centuries for the treatment of fever and malaria (2). Artemisinin, an endoperoxide containing sesquiterpene lactones (also known as qinghaosu) is the main component responsible for this therapeutic effect. The WHO recommends that all countries experiencing resistance to conventional monotherapies should use combination therapies, preferably those containing Artemisinin derivatives [Artemisinin-based Combination Therapies (ACT)] (3,4).

*Author to whom correspondence should be addressed: email liqiwan@vip.sina.com.

The extraction of natural products is essential not only as an evaluation tool for raw materials, but also for the quality control of products. In fact, whatever the analytical method used, an extraction procedure of the plant material is required. Liquid solvent extraction with toluene, hexane, and chloroform or petroleum ether is the most commonly applied technique for extracting Artemisinin. More complicated extraction techniques, such as supercritical fluid extraction, pressurized solvent extraction, and microwave-assisted extraction have also been used.

However, traditional methods of extraction may be both time-consuming and labor-intensive, creating delays in the flow of information from the analysis laboratory to the field or product line. Complicated extraction techniques require expensive apparatus. Therefore, in a plant development project, it is important to have simple, rapid, and specific extraction and analytical procedures, which allow the quantitative determination of the analyte and possible precursors.

Matrix solid-phase dispersion (MSPD) involves the homogenization and dispersion of a small amount of matrix with adsorbent (usually octadecylsilica C18) followed by washing with a small amount of solvent and elution to extract a wide range of compounds. This technique was developed by Barker et al. (5) for isolation of drug residues from tissues and has been widely used for fruit and vegetable samples (6–8).

For the quantitative analysis of Artemisinin, a large array of techniques have been developed, including thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), HPLC-MS, gas chromatography, GC–MS, supercritical fluid chromatography, and capillary electrophoresis. A review by Christen et al. (9) gives an excellent overview of these techniques and discusses some of them in more detail. Among these methods, the HPLC method has been widely used, and HPLC with electrochemical detection (10–12) and chemiluminescence detection (13–14) have been proven to be a sensitive and specific method for Artemisinin analysis. Evaporative light scattering

detection (15,16) has also been reported to be one alternative method for analysis of Artemisinin. HPLC–UV detection of Artemisinin is not straightforward because it lacks a suitable UV chromophore. However, with pre-column derivatization, Artemisinin can be converted to a reproducible UV-absorbing compound, Q292 in alkaline solution, which was acidified to compound Q260, therefore detectable by HPLC–UV (17,18) (Figure 1). However, interference with other constituents in the extracts at the absorption wavelength (9) and the unstable derivatized compound may render these technologies unsatisfactory. There have been some methods reported on determination of Artemisinin (19) in *Artemisia annua* L., dihydroArtemisinin and artesunate (20,21) in plasma using HPLC with post-column derivatization. Here an HPLC method is reported using post-column derivatization, using directly screened Q292 with MSPD extraction, that can be used in the quantitative analysis of Artemisinin. This method is fast, simple, sensitive, and reliable, and it was found to have satisfactory repeatability. The accumulation rate of Artemisinin during the plant growth was determined. Plant samples are harvested when they contain the highest amount of Artemisinin, and their Artemisinin content is rapidly screened by the MSPD. The MSPD method is compared with two traditional extractions: Soxhlet and Ultrasonic-wave by F-test.

Experimental

Plant materials

Five hundred milligrams of wild seeds *of A. annue L.* (tested over two years, acquired from Yunnan, provincial Yuan Yan country) were sown at Shuan Long Agriculture Base, Kunming Municipal (one unit of area) on April 5, 2006. Seedlings were planted June 1 (to analyze the content of *Artemisinin*). The plant growth from June 1st to July 5th was slow, only from 10 to 40 cm. From July 5th to August 25th, plant growth was quick: July 25th, 80~100 cm height; August 15th, 100~150 cm height; August 25th, 200~250 cm height. Flower buds were first observed on September 5th. Plants grew flower buds on September 5th, and blossomed on September 21st. One hundred grams of fresh leaves was harvested at each stage, immediately ground to syrup, and stored in refrigeration at –40°C before instrumental analysis.

Reagents and chemicals

Artemisinin (98%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All solvents used in this research were of HPLC grade and purchased from Fisher Scientific (Pittsburgh, PA, USA). Florisil (0.15~0.25 mm, 60~100 mesh) and octadecylsilica C18 (50 µm, 65A) were obtained from Phenomenex (Torrance, CA, USA). Purified water of 18.2Mµ/cm was obtained from a Milli-Q system (Millipore, Belgium). LC analyses were performed on a Waters (Milford, MA) 2695 system equipped with an autosample, a quaternary pump system, a 996 diode array detection set at 290 nm, thermostated column compartment, a degasser and Empower software, post-column derivatization including post-column reaction module (RXN 1000 coil, 10 mm × 260 mm), temperature control module (TCM-00418x), 510 pump. A Waters Nova-Park C18 column (3.9 mm × 150 mm, 5 µm) was selected for HPLC separation.

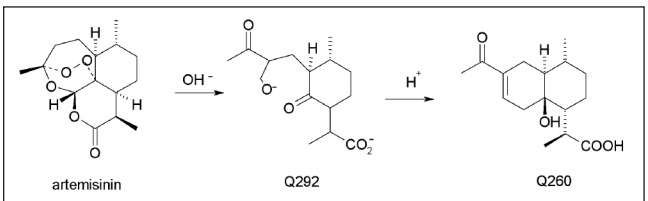

Figure 1. Precolumn derivatization reaction.

Preparation of standards for HPLC analysis

Approximately 50 mg of Artemisinin was accurately weighed, solved in methanol, and placed into a 25-mL volumetric flask. A standard curve (0.05, 0.1, 0.25, 0.5, and 1.0 mg/mL) was prepared from the standard stock solution by methanol dilutions.

Preparation of Samples

Fresh *A. annua L.* leaves and leaves on the third stage were dried under air for 5 days, chopped in a high speed blending jar, homogenized for 2 min, and stored at –18°C.

Matrix solid-phase dispersion

A 500 mg portion of the dried sample was separated, put into a 50-mL beaker, and 1.5 g of Florisil was added. The mixture was then blended with a glass pestle until homogeneous, after which the samples were allowed to stand for 15 min.

The samples containing absorbent were introduced onto the cartridge (6 mL volume capacity). This column was prepared in the laboratory and conditioned with 10 mL hexane, without collection. Ten milliliter acetone was added, and the elution was collected in 10-mL graduated tube. A 10 µL portion of the elution was analyzed by HPLC.

Soxhlet extraction

In accordance with the method in the literature (9), 10 g samples of the dried sample with 200 mL of *n*-hexane in Soxhlet extraction were heated to 70°C in an evaporator for 5 h, and then cooled to air room temperature. One milliliter portion of extraction solution (accurately transferred to a 2 mL tube) was compressed by nitrogen gas, dissolved in 1 mL methanol, and analyzed by HPLC.

Ultrasonic-wave extraction

A ten-gram sample of the dried sample with 100 mL of *n*-hexane in a 500 mL cone flask was extracted by ultrasonic-wave for 10 min and filtered. Then 50 mL *n*-hexane was added twice, extracted by ultrasonic wave for 10 min, filtered, and pooled the extraction solution. One milliliter portion of extraction solution (accurately transferred to a 2 mL tube) was compressed by nitrogen gas, dissolved in 1 mL methanol, and analyzed by HPLC.

HPLC conditions

The chromatogram was run on a Waters Alliance 2695 system with Novapak C18 (3.9 mm × 150 mm, 5 µm). The mobile phase consisted of water (40%) and methanol (60%). The flow rate was set at 1.0 mL/min; injection column was 10 µL; run time was

20 min. Temperature, flow rate, and concentration of alkaline were interrelated variables, which had to be simultaneously optimized. According to literature (19), 70°C, 1M KOH (methanol–water, 9:1), flow rate of 0.2 mL/min was compared with 60°C, 0.05M NaOH (water), flow rate of 0.5 mL/min. The result was similar. Therefore, the post-column derivitization condition was 0.05 mol/L sodium hydrate (NaOH), and the flow rate was set at 0.5 mL/min. The reaction temperature was set at 60°C. The UV max wavelength was 290 nm. Quantitation was based on the HPLC peak area of Artemisinin and standard curve was used for calculation.

Results and Discussion

In the present work, MSPD is used for extraction, and the plant sample is dispersed over deactivated Florisil. Various tests with other solid supports, such as neutral alumina and Florisil, were performed. When the samples and C18 were blended, which involved washing with hexane then elution with acetone, the result was not successful, with only 20% recovery. Neutral alumina cannot successfully purify color-dye because Artemisinin cannot be completely absorbed by neutral alumina with no polar solution, such as hexane or toluene; thus, only the purification of Florisil was good.

In order to choose a proper elution for the retained Artemisinin, various organic solvents were studied. It was found that, with the exception of *n*-hexane, acetone, diethyl ether, and ethyl acetate could elute the Artemisinin from the cartridge quantitatively. The effect of the various elution conditions for the retention of Artemisinin is listed in the following sequence: acetone (recovery 90%) > diethyl ether (recovery 90%) > ethyl acetate (recovery 60%) > *n*-hexane (recovery 0%). The *n*-hexane cannot elute Artemisinin from the cartridge, so *n*-hexane was selected as clean solvent. Due to the difficulty of dissolving diethyl ether in deionized water, it was unsuitable to directly analyze by reversed-phase-HPLC. So it was highly advantageous to use *n*-hexane for pre-elution, then to use acetone as the elutent for MSPD directly before HPLC analysis. Typical chromatograms of standard sample and fortified Artemisinin with MSPD are shown in Figure 2, respectively.

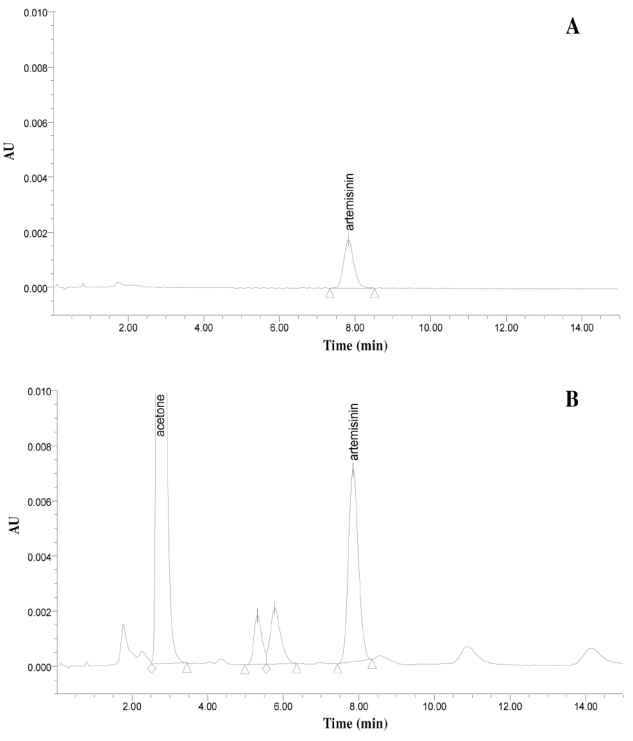

Figure 2. The chromatogram of standard solution 0.05 mg/mL (A) and sample of *A. annua* L. [the large off-scale peak is solvent peak (acetone)] (B).

The calculations for the evaluation of method validation data

The extraction method compared MSPD with two traditional methods to assess the extraction efficiency. Fifteen equal dried samples with three extraction methods were prepared and immediately analyzed by HPLC. Table I shows the value of Artemisinin from the same sample using different extraction methods. The F-test was used to calculate the evaluation of the difference in MSPD and Soxhlet.

$$F(a,f1,f2) = S1^2/ S2^2 = 1.61$$

Where a = 0.05 (95% confidence level), and the critical value is F (0.05, 4, 4) = 6.39 > 1.61. Results indicated that the methods between MSPD and Soxhlet are not significantly different. The same calculation was between MSPD and ultrasonic-wave extraction.

$$F(a,f1,f2) = 2.75$$

Where a = 0.05 (95% confidence level), and the critical value is F (0.05, 4, 4) = 6.39 > 2.75. Results also indicated that the methods between MSPD and Soxhlet are not significantly different. The coefficient of MSPD was the same as that of the Soxhlet and ultrasonic-wave extraction, but *compared with the Soxhlet and ultrasonic extraction,* the method of MSPD was more rapid, simple and used less solvent (only with 10 mL acetone, but Soxhlet and ultrasonic extraction used 200mL hexane).

Table I. The Method of Extraction of Artemisinin: MSPD Compared MSPD to Soxhlet and Ultrasonic-Wave

The method of extraction	Determination value %					AV* %	S.D.† %	RSD‡ %
MSPD	0.502	0.494	0.428	0.465	0.449	0.468	0.031	5.43
Soxhlet	0.504	0.533	0.485	0.550	0.482	0.518	0.030	4.89
Ultrasonic	0.511	0.505	0.5621	0.515	0.485	0.502	0.019	3.09

* AV stands for average value.
† S.D. stands for standard deviation.
‡ RSD stands for relative standard deviation.

Accuracy, precision, limit of detection, limit of quantity, and quantitation

Eighteen equal samples of fresh *A. annua* leaves were prepared, five of which were spike 0.1 mg of standard solution, five of which were spike 0.3 mg of standard solution, five of which were spike 0.5 mg of standard, and three of which were unspiked samples. Table I shows the mean of the recoveries for the different spike samples. Within the range of the standard curve, recovery was between 91.2% and 88.1% for all analytes, and the RSD was between 6.43% and 4.55%. (Table II)

The limit of detection (LOD) was defined as the lowest observable peak response for an analyte above the background noise, 3 times the system noise in the matrix. The limit of quantity (LOQ) was defined as the lowest concentration for the analyte with a response signal 10 times the system noise in the matrix. The LOD was 0.1 µg/mL (1 ng), and the LOQ was 0.5 µg/mL. The reported method determined Artemisinin in *A. annua* leaves by HPLC–UV with precolumn derivatization (18), postcolumn derivatization (19), and HPLC–ELSD (16) to be 5, 25, and 6 ng, respectively. The Artemisinin was determined by HPLC in one day to research its stability; 2 h determined one times, totally determined eight times, calculated the variation of the area of Q292 in eight times, the relative standard deviation (RSD) was 3.21%, so the compound of Artemisinin derivative was stable at room temperature. Injection 10 µL of standard curved (0.05, 0.1, 0.25, 0.5, and 1.0 mg/mL) was performed in the HPLC for quantitative analysis. A five point calibration curve was constructed from the peak area; the line range was 0.05–1.0 mg/mL. The equation of standard curve was $y = 45.812X + 71.452$, and the correlation of was 0.9998.

Table II. The Recoveries and RSD of Sample (n = 5)

Sample	Blank (mg)	Addition (mg)	Determination value (mg)					Recovery %	RSD %
	0.006	0.1	0.102	0.089	0.095	0.096	0.097	91.2	6.43
Artemisinin	0.006	0.3	0.269	0.289	0.262	0.257	0.275	88.1	4.60
	0.006	0.5	0.435	0.482	0.463	0.471	0.437	90.3	4.55

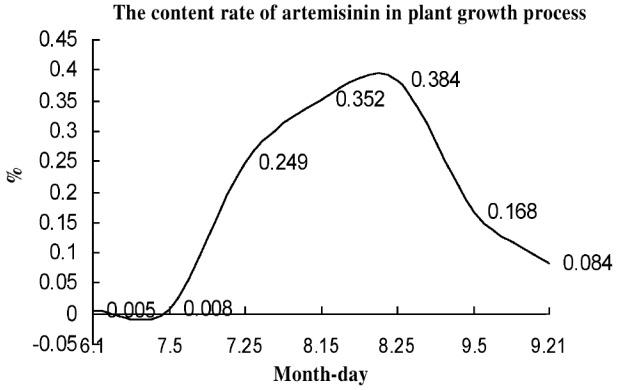

Figure 3. The content rate of Artemisinin in plant growth process.

Table III. The Determination Value of Artemisinin and RSD in Plant Growth Stage (n = 5)

Determination value date	AV (%) ± SD	RSD (%)
6.1	$0.005 \pm 2.9\times10^{-4}$	5.70
7.5	$0.008 \pm 3.3\times10^{-4}$	4.14
7.25	0.249 ± 0.012	4.99
8.15	0.352 ± 0.023	6.48
8.25	0.384 ± 0.021	5.61
9.5	0.168 ± 0.0098	5.83
9.21	0.084 ± 0.0040	4.71

The rate of Artemisinin content in plant growth process

The content of Artemisinin was analyzed in the plant growth process from June 1 to September 21. From Figure 3, the fresh leaves were harvested over seven stages, and the content of Artemisinin at every stage was determined five times (the average value and RSD shown Table III). The average content at first stage was small (0.005%); from second to fourth stage, the content of Artemisinin rose quickly, and reached the highest content (0.384%) at the forth stage. When flower bud was first observed, the content of Artemisinin was rapidly turned down (0.168%).

Application to different samples

We further applied this method to evaluate the content of Artemisinin from the fresh leaves and dry leaves of Artemisinin samples (Shuang Long Agriculture Base, Long Chuan Agriculture Base, Pan Long Agriculture Base and Guan Du Base), according to the general procedure. At the most harvested stages, the content of Artemisinin in fresh leaves is shown to be 0.3~0.5%, and while in dry samples, it is 0.6~0.9%.

Acknowledgments

We thank Yang Tian (Center for Pharmaceutical Biotechnology MBRB, Department of Medicinal Chemistry and Pharmacognosy University of Illinois at Chicago) for his discussion and revision of this paper. We are grateful for support funded by the Yunnan Academy of Agriculture Science and Technology Breakthrough Prgram of Yunnan Province (No: 2006SG18).

Reference

1. B. Greenwood and T. Mutabingwa. Malaria in 2002. *Nat.* **415:** 670–672 (2002).
2. D.L. Klayman. An antimalarial drug from China. *Sci.* **228:** 1049–1055 (1985).
3. P.L. Olliaro and W.R. Taylor. Developing Artemisinin-based drug combinations for the treatment of drug resistant falciparum malaria: A review. *J. Postgrad. Med.* **50(1):** 40–44 (2004).
4. T.M. Davis, H.A. Karunajeewa, and K.F. Ilett. Artemisinin-based combination therapies for uncomplicated malaria. *Med. J. Aust.* **182(4):** 181–185 (2005).

5. S.A. Barker, A.R. Long, and C.R. Short. Isolation of drug residues from tissues by solid-phase dispersion. *J Chromatogr.* **475:** 353–61 (1989).
6. S.A. Barker. Matrix solid-phase dispersion. *J. Chromatogr. A* **885(1–2):** 115–27 (2000).
7. M.P. Fernandez and J.Y. Manes. Determination of carbamate residues in fruits and vegetables by matrix solid-phase dispersion and liquid chromatogram–mass spectrometry. *J. Chromatogr. A* **871(1–2):** 43–56 (2000).
8. M.P. Navarro and J.Y. Marin. Application of matrix solid-phase dispersion to the determination of a new generation of fungicides in fruits and vegetables. *J. Chromatogr. A* **968(1–2):** 201–209 (2002).
9. P. Christen and J.L. Veuthey. New trends in extraction, identification and quantification of Artemisinin and its derivatives. *Curr. Med. Chem.* **8:** 1827–39 (2001).
10. K. Na-Bangchang, K. Congpuong, L.N. Hung, P. Molunto, and J. Karbwang. Simple high-performance liquid chromatographic method with electrochemical detection for the simultaneous determination of artesunate and dihydroArtemisinin in biological fluids. *J. Chromatogr. B: Biomed. Sci. Appl.* **708(1–2):** 201–207 (1998).
11. M.A. Van Agtmael, J.J. Butter, E.J.G. Portier, and C.J. Van Boxtel. Validation of an improved reversed-phase high-performance liquid chromatography assay with reductive electrochemical detection for the determination of Artemisinin derivatives in man. *Ther. Drug Monit.* **20(1):** 109–116 (1998).
12. J.F.S. Ferreira, D.J. Charles, K. Wood, J. Janick, and J.E. Simon. A comparison of gas chromatography and high-performance liquid chromatography for Artemisinin analyses. *Phytochem. Anal.* **5:** 116–20 (1994).
13. M.D. Green, D.L. Mount, G.D. Todd, and A.C. Capomacchia. Chemiluninescent detection of Artemisinin. Novel endoperoxide analysis using luminal without hydrogen peroxide. *J. Chromatogr. A* **695(2):** 237–242 (1995).
14. A. Amponsaa-Karikari, N. Kishikawa, Y. Ohba, K. Nakashima, N. Kuroda. Determination of Artemisinin in human serum by high-performance liquid chromatography with on-line UV irradiation and peroxyoxalate chemiluninescence detection. *Biomed. Chromatogr.* **20(11):** 1157–1162 (2006).
15. C.A. Peng, J.F. Ferreira, A.J. Wood. Direct analysis of Artemisinin from *Artemisia annua* L. using high-performance liquid chromatography with evaporative light scattering detector, and gas chromatography with flame ionization detector. *J. Chromatogr. A* **1133(1–2):** 254–258 (2006).
16. B.A. Avery, K.K. Venkatesh, and M.A. Avery. Rapid determination of Artemisinin and related analogs by high-performance liquid chromatography and an evaporative light scattering detector. *J. Chromatogr. B: Biomed. Sci. Appl.* **730(1):** 71–80 (1999).
17. S.S. Zhao and M.Y. Zeng. Studies on the analysis of qinghaosu by high-pressure liquid chromatography and spectrometry (HPLC). *Planta Med.* **51:** 233–37 (1985).
18. S.S. Zhao. High-performance liquid chromatography determination of Artemisinine (qinghaosu) in human plasma and saliva. *Analyst.* **112(5):** 661–65 (1987).
19. H.N. ElSohly, E.M. Croom, and M.A. ElSohly. Analysis of the antimalarial sesquiterpene Artemisinin in *Artemisia annua* by high-performance liquid chromatography (HPLC) with postcolumn derivatization and ultraviolet detection. *Pharm Res.* **4(3):** 258–60 (1987).
20. R.B. Taylor, M.I. Awad, R.G. Reid, and R.R. Moody. Determination of sodium artesunate in plasma using ion-pairing high-performance liquid chromatography. *J. Chromatogr. B: Biomed. Sci. Appl.* **744(2):** 415–21 (2000).
21. P.O. Edlund, D. Westerlund, J. Carlquist, and Y.H. Wang. Determination of artesunate and dihydroArtemisinine in plasma by liquid chromatography with post-column derivatization and UV-detection. *Acta. Pharm. Suec.* **21(4):** 223–34 (1984).

Manuscript received November 9, 2006;
revision received March 12, 2007.