Asian Journal of Chemistry Vol. 20, No. 3 (2008), 1907-1914

Determination of Artemisinin by Post-Column Derivatization High Performance Liquid Chromatography

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Artemisinin (an antimalaric compound), is isolated as the active principles of the medicinal plant *Artemisia annua* L. A simple, rapid and high efficient method of extraction was developed. It was extracted by matrix solid phase dispersion (MSPD) and directly analyzed by post-column derivatization high performance liquid chromatography. Quantification was compared the method of MSPD with two conventional liquid solvent extraction processes of Soxhlet and super wave by F-test. The result indicated no significant difference. The production rate of artemisinin during wild plant growth (was tasted in 2 years, from Yunnan provincial yuan yan country) was determined. The recoveries range of determination is 88.1-91.2 %. The relative standard deviation (RSD) is 4.55-6.43%. The limits of detection (LOD) are 0.1 μg/mL and the limits of quantities (LOQ) are 0.5 μg/mL.

Key Words: Matrix solid phase dispersion, Post-column derivatization, HPLC, Artemisinin.

INTRODUCTION

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

Artemisia annua L. (sweet wormwood), a plant in traditional Chinese medicine has been used 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 (ACTs-artemisinin-based combination therapies)[3,4].

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 currently applied technique for artemisinin. Also more complicated extraction techniques such as super critical fluid extraction (SFE), pressurized solvent extraction (PSE) and microwave-assisted extraction (MAE) have been used.

However, traditional methods of extraction may be both time consuming and labour intensive and creating delays in the flow of information from the analysis laboratory to the field or product line. Complicated extraction techniques need expensive apparatus. This is why in a plant development project it is important to have simple, rapid and specific extraction and analytical procedures, which allow the quantity determination of the analysis and possibility of their precursors.

Matrix solid-phase dispersion (MSPD) involves 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 quantitation of Artemisinin, a large array of techniques have been developed including thin layer chromatography (TLC), HPLC, HPLC/MS, gas chromatography (GC), GC/MS, supercritical fluid chromatography (SFC), capillary electrophoresis (CE). A review by Christen *et al.*[9] gives an excellent overview of these techniques.

Among these methods, the HPLC method was widely used, 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] was reported to be one alternative way for analysis of artemisinin. HPLC-UV detection of artemisinin is not straightforward because it lacks a suitable ultraviolet 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] (Fig. 1). The limit of the unstable compound Q260 can be overcome by doing post-column derivatization. There were some methods[19,20] reported on determination of dihydroartemisinin and artesunate in plasma using HPLC with post-column derivatization. Here we reported a HPLC of post-column derivatization directly screened Q292 with MSPD extraction that can be used in the quantitative analysis of artemisinin. This method is fast, simple, sensitive and reliable. We determined the accumulation rate of artemisinin during the plant growth. Plant samples are harvest when they contain the highest amount of artemisinin and their artemisinin content are rapidly screened by this method. We also compare the method of MSPD with two traditional extractions of Soxhlet and Super-wave by F-test.

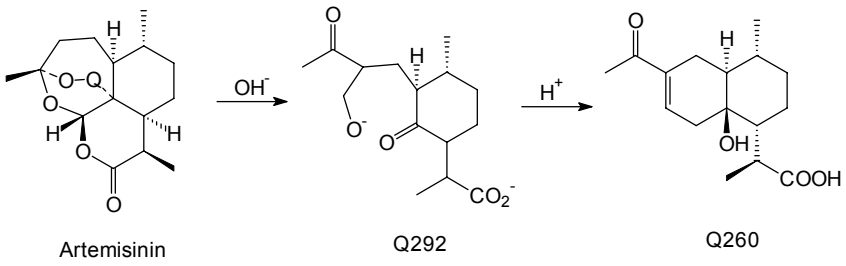

Fig. 1. Pre-column derivatization reaction

EXPERIMENTAL

500 mg of wild seeds of *A. annue* L. (was tasted in two years, from Yunnan provincial yuan yan country) were sown at kunming municipal shuan long agriculture base (one unit of area) in April 5, 2006. Seedlings were later planted in June 1 at 30 cm height above the ground (to analysis 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, plants growth was quick, July 25th, 80-100 cm height; August 15th, 100-150 cm height; August 25th, 200-250 cm height. Flower bud is first observed on Sept. 5th. Plants grow flower bud in Sept. 5th and blossomed in Sept. 21th. 100 g of fresh leafs were harvested on each stage. The fresh leafs were grounded immediately to syrup and stored in refrigeration at -40 °C before instrumental analysis.

Artemisinin (98 %) was purchased from Sigma-Aldrich. All solvents used in this research were of HPLC grade and purchased from Fisher Scientific. Florisil (0.15-0.25 mm, 60-100 mesh ASTM) and octadecyl silica C18 (50 μm, 65A) was obtained from Phenomenex (USA). Purified water of 18.2 mμ/cm was obtained from a Milli-Q system (Millipore, Belgium). LC analyses were performed on an waters 2695 system equipped with an auto-sample, 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, temperature control module (TCM-00418), 510 pump. A waters Nova-Park C18 column (3.9mm × 150 mm, 5 μm) was selected for HPLC separation.

Preparation of standards for HPLC analysis: About 50 mg of artemisinin was accurately weighed and solved in methanol and placed into a 25 mL volumetric flask. A standard curved (0.05, 0.1, 0.25, 0.5, 1.0 mg/mL) was prepared from the standard stock solution by methanol dilutions.

The equation of standard curve is $y = 6.20e^2X + 9.67e^2$, the correlation of is 0.9998, the line range is 0.05-1.00 mg/mL.

Preparation of samples: Fresh *A. annua* L. leaves chopped in a high speed blending jar and the sample was homogenized for 2 min and stored at -18 ºC.

Matrix solid-phase dispersion (MSPD): 500 mg portion of the stored 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 and fortified with 50 µL of the standard solution. The fortified samples were allowed to stand for 15 min after the introduction of standards.

The samples with 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. 10 mL acetone was added. The eluate was collected in 10 mL gaviation tube. A 10 µL portion of the elution was analyzed by HPLC.

Soxhlet extraction: According to the literature[9], an amount of 10 g samples of the stored sample with 200 mL of *n*-hexane in Soxhlet extraction, heated by 70 ºC evaporator for 5 h, cooled to air room temperature. 1 mL portion of extraction solution accurately transferred to 5 mL tube was compressed by nitrogen gas, dissolved in 1 mL methanol and analyzed by HPLC.

Ultrasonic-wave extraction: An amount of 10 g samples of the stored sample with 100 mL of *n*-hexane in 500 mL cone flask, extracted by ultrasonic-wave for 10 min, filtered, then 50 mL *n*-hexane was added twice, extracted by ultrasonic wave for 10 min, filtered and pooled the extraction solution. 1 mL portion of extraction solution accurately transferred to 2 mL tube was compressed by nitrogen gas, dissolved in 1 mL methanol and analyzed by HPLC.

HPLC chromatographic condition: The HPLC was run on the Water 2695 system with Novapak C18 (3.9 mm × 150 mm, 5 µm). The mobile phase was water (40 %) and methanol (60 %). The flow rate was set at 1.0 mL/min, injection column was 10 µL, run time is 20 min. Post-column derivatives was 0.05 mol/L sodium hydroxide, flow rate was set at 0.5 mL/min. The reaction temperature was set at 60 ºC. The UV max wavelength was 290 nm. Quantification was based on the HPLC peak area of artemisinin and standard curve was used for calculation.

RESULTS AND DISCUSSION

In the present work for the methodology using MSPD extraction, the fruit sample is dispersed over deactivated florisil. This differs from traditional MSPD methods, in which the dispersion step is usually made on octadecyl silica C18. Various tests with other solid supports, such as neutral alumina and florisil, were performed. C18 washed with hexane then eluted with acetone was not successful but only 20 % recovery. This may be due to the analytes and the matrices used in this work and the fact that C18 acts

as a fat dispersant due to its hydrophobic characteristics[21]. Neutral alumina can't completely purify colour-dye, because neutral alumina can't be completely eluted with middling polar solution such as acetone, only the purification of florisil was good.

In order to choose a proper elute for the retained artemisinin, various organic solvents were studied. It was found that, exception *n*-hexane, acetone, diethyl ether and ethyl acetate could elute the artemisinin from cartridge quantitatively. The effect of the various elution for the retained artemisinin was listed in following sequence acetone (recovery 90 %) > diethyl ether (recovery 90 %) > ethyl acetate (recovery 60 %) > *n*-hexane (recovery 0 %). The *n*-hexane cannot elute artemisinin from cartridge, so *n*-hexane was selected as clean solvent. Due to the difficulty of dissolving diethyl ether in de-ionized water, it was unsuitable for direct analysis by RP-HPLC. So it is highly advantageous when using *n*-hexane as pre-elution, then acetone as the elution from matrix solid phase dispersion (MSPD) directly for HPLC analysis. Typical chromatograms of standard sample and fortified artemisinin with MSPD are shown in Fig. 2, respectively.

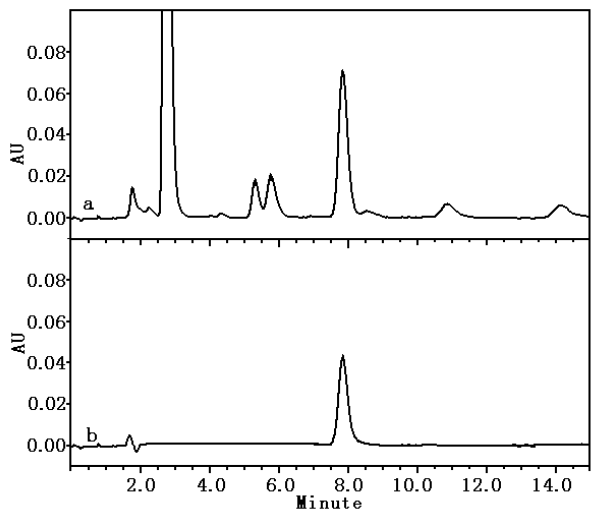

Fig. 2. Chromatogram of standard solution (0.05 mg/mL, a) and sample of *A. annua* L. (the large off scale peak is solvent)

Calculations for the evaluation of method validation data

The extraction method compared MSPD with two traditional methods were investigated to asses the recovery. 15 Equal samples with three extraction methods were prepared, analyzed by HPLC immediately. Table-1 shows the value of arterminin from the same sample with different extraction methods.

TABEL-1
METHOD OF EXTRACTION OF ARTEMISININ IS COMPARED MSPD WITH SOXHLET, ULTRASONIC-WAVE

The method of extraction	Determination value (%)					AV (%)	SD (%)	RSD (%)
MSPD	0.511	0.505	0.5621	0.515	0.485	0.502	0.019	3.09
Soxhlet	0.502	0.494	0.428	0.465	0.449	0.468	0.031	5.43
Ultrasonicwave	0.504	0.533	0.485	0.550	0.482	0.518	0.030	4.89

AV = stands for average value, SD = stands for standard deviation,
RSD = stands for relative standard deviation

We used F-test to calculate the evaluation of the difference in MSPD and Soxhlet.

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

for a = 0.05 (95 % confidence level) the critical value is F (0.05, 4.00, 4.00) = 6.39 > 1.61.

Result indicated that the methods between MSPD and Soxhlet are not significantly different.

The same calculation was between MSPD and super-wave extraction.

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

for a = 0.05 (95 % confidence level) the critical value is F (0.05, 4.00, 4.00) = 6.39 > 2.75.

Result also indicated that the methods between MSPD and Soxhlet are not significantly different.

So the coefficient of MSPD was the same as the Soxhlet and ultrasonic-wave extraction, but compared with the Soxhlet and ultrasonic extraction, the method of MSPD was more rapid, simple and lesser amount of solvent used (only with 10 mL acetone, but Soxhlet and ultrasonic extraction with 200 mL acetone) was consumed.

Accuracy, precision, limit of detection (LOD) and limit of quantity (LOQ)

18 Equal samples of fresh *A. annua* stem were prepared, 5 of which were spike 0.1 mg of standard solution, 5 of which were spike 0.3 mg of standard solution, other 5 of which were spike 0.5 mg of standard, the other 3 of which were unspiked sample. Table-1 showed 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 RSD was between 6.43 and 4.55 % (Table-2).

TABLE-2
RECOVERIES AND RSD OF IN SAMPLE (n = 5)

Sample	Blank (mg)	Addition (mg)	Determination value (mg)					Recovery (%)	RSD (%)
Artemisinin	0.006	0.1	0.102	0.089	0.095	0.096	0.097	91.2	6.43
	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

The 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 LOQ was defined as the lowest concentration for the analyte with a respond signal 10 times the system noise in the matrix. The LOD was 0.1 μg/mL and LOQ was 0.5 μg/mL. The artemisinin was determined by HPLC in one day to research its stability; 2 h determined one times, totally determined 8 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.

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 Fig. 3, the fresh leafs were harvested on 7 stages, the content of artemisinin on seven stages were determined 2 times, the average content on first stage was little (0.005%), from second to fourth stage, the content of artemisinin rise quickly, reach the highest content (0.384 %) at the forth stage. When flower bud is first observed, the content of artemisinin was rapidly turned down (0.168 %).

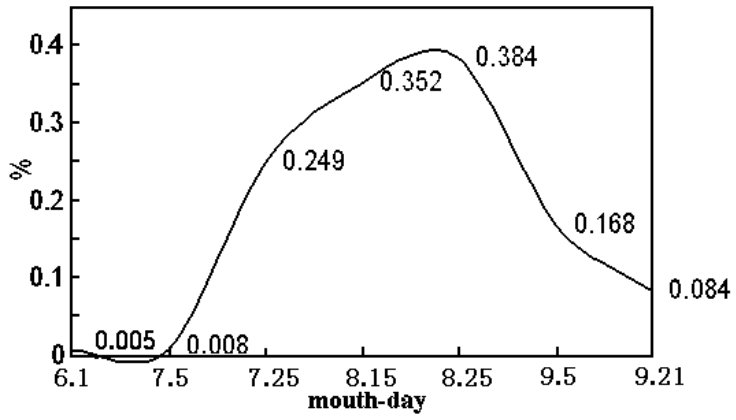

Fig. 3. Content rate of artemisinin in plant growth process

Application to different samples: This method has been applied to evaluate the content of artemisinin from the fresh leaves and dry leaves of artemisinin sample (Shuang Long agriculture base, Long Chuan agriculture base, Pan Long agriculture base and Guan Du base) according to the general procedure. On the most harvested stage, the content of artemisinin was shown that in fresh leaves, it is 0.3-0.5 %, while in dry samples, it is 0.6-0.9 %.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (20471051) and the Key Natural Science Foundation of Yunnan Province (2005K021).

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(*Received*: 5 March 2007; *Accepted*: 8 November 2007) AJC-6060