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APPENDICS

Phytochemical Analysis
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Phytochemical Analysis

TLC-Densitometric Analysis of Artemisinin for the Rapid Screening of High-producing Plantlets of *Artemisia annua* L.

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Abstract: A simple TLC-densitometric technique has been developed for the rapid and accurate analysis of artemisinin in a large number of *Artemisia annua* plantlets cultured *in vitro*. This new analytical method is based on the structural conversion of artemisinin on a silica gel layer by ammonia vapour to form 10-azadesoxyartemisinin, a chromophore-containing compound (λ_{max} 320 nm) that can be detected by UV-based TLC densitometry. The TLC system was evaluated quantitatively in terms of product stability, precision, accuracy and calibration. Good linearity was obtained in the range of 0.01–0.12 μg artemisinin. The technique appeared to be accurate and sensitive as compared with the complicated pre-column reaction-HPLC technique. Among 90 samples of *A. annua* plantlets, the artemisinin content in the leaves appeared to be highly variable, ranging from 0.02 to 0.67% w/w dry weight. These results demonstrate that densitometric TLC can be a cheap and simple technique for the accurate screening of high-artemisinin-producing plants. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: TLC densitometry; quantitative analysis; artemisinin; 10-azadesoxyartemisinin; *Artemisia annua* L.; Asteraceae.

INTRODUCTION

Although artemisinin (**1**) absorbs light in the UV region between 210 and 220 nm, its extinction coefficient is poor. As a result, the methodology of standard UV detection is ineffective in the quantitative analysis of artemisinin. For this reason, the detection of artemisinin has been accomplished using pre-column derivatisation to convert artemisinin into a UV-active compound that absorbs with a large extinction coefficient at longer wavelengths that are applicable to HPLC-UV methods (Zhao and Zeng, 1986; Zhao, 1987; Thomas *et al.*, 1992). In addition, HPLC employing various detection methods has been reported for the analysis of artemisinin, giving different levels of detection, including chemiluminescence (detection limit 2.5 ng; Green *et al.*, 1995), reductive electrochemical detection (detection limit 1–10 mg; Melendez *et al.*, 1991; Navaratnam *et al.*, 1995; Sandrenan *et al.*, 1997), thermospray mass spectrometry (detection limit of 0.2 ng; Chi *et al.*, 1991) and evaporative light scattering detection (detection range of 6–60 ng; Avery *et al.*, 1999). Other analytical methods have also been developed, such as GC-MS (Sipahimalani *et al.*, 1991), capillary electrophoresis (D'Hulst *et al.*, 1996; Yu *et al.*, 2004) immuno-detection (Jaziri *et al.*, 1993) and tandem MS (Ranasing *et al.*, 1993). These techniques usually require either sophisticated equipment or complicated procedures for the analysis of artemisinin.

Recently, a simpler technique of TLC densitometry has been developed in order to analyse artemisinin and its derivatives, both on a normal silica-gel layer (Gabriels and Plaizier-Vercammen, 2003) and on a reversed phase-layer (Gabriels and Plaizier-Vercammen, 2004). For both TLC systems, spots are visualized by derivatisation with acidified 4-methoxybenzaldehyde reagent in methanol:water. Whilst these methods permit the rapid quantification of artemisinin, their sensitivity is relatively low, with detection in the range from 0.5 to 8 μg. In the present paper, we report an improved TLC-densitometric method that is much more sensitive (10 ng detection limit) and accurate. This new technique has enabled us to screen for high-artemisinin-producing plantlets from a large number of samples.

EXPERIMENTAL

Materials. Authentic artemisinin was obtained from the Plant Biochemistry and Plant Physiobiochemistry Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. Organic solvents were all of reagent grade or better and were supplied either by LAB-SCAN Analytical Sciences (Dublin, Ireland) or Merck (Damstadt, Germany). Water

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was triple deionised. Silica gel Polygram® SIL G/UV254 TLC plates were purchased from Macherey-Nagel (Düren, Germany). For plant material, *Artemisia annua* L. seeds originating from Hanoi, Vietnam, were surface sterilised and then germinated on MS medium for 2 months before their shoots were excised and irradiated with gamma rays (500 rad dose). The exposed shoots were then transferred to fresh MS medium for micro-propagation. The shoot cultures were maintained by sub-culture every 6 weeks. Those plantlets that survived four sub-culture passages were harvested and their leaves were used for the determination of artemisinin as described below.

Sample preparation. Fresh leaves obtained from various irradiated plantlets were dried at 65°C in a hot air oven. Each dried sample was then ground to a fine powder in a grinder supplied with a cool water circulator, and the resulting powder passed through a no. 40 sieve. Powder samples (100 mg) were extracted under reflux with 10 mL hexane for 1 h. After cooling, the extract was filtered and used for the TLC-densitometric analysis.

TLC-densitometric analysis. Crude extracts of various *A. annnua* samples (10 µL each) were spotted onto a pre-coated silica gel 60 F_{254} layer (0.25 mm thickness). Up to 12 samples were applied onto each standard TLC plate, which was then developed using the solvent system hexane:ethyl acetate:acetone (16:1:1) to produce a solvent front height of 8 cm. The plate was dried and exposed for 2 h to saturated ammonia vapour in a closed TLC tank maintained in an hot air oven at 100°C for complete chromophore development of artemisinin. The TLC plate was then air-dried and scanned at 320 nm using a Shimadzu (Kyoto, Japan) TLC densitometer dual wavelength scanner model CS-930.

HPLC analysis. For method validation, the same samples were analysed using the well-established HPLC method with pre-column reaction as described previously (Zhao, 1987). The analysis was performed on a Varian HPLC system (Walnut Creek, CA, USA) equipped with a ternary solvent pump model 9010 and a UV detector model 9050 set at 260 nm. Samples were automatically injected using a Varian model 9095 auto-sampler, and data were recorded with the aid of Varian Star software (version 6.0). The separation was achieved on a reverse-phase C_{18} column (150 × 4 mm i.d.; 5 µm) using an isocratic solvent system of 0.01 M disodium hydrogen phosphate–sodium dihydrogen phosphate buffer (pH 6.7):methanol (38:62) at a flow rate of 1.5 mL/min.

Optimization of structural conversion of artemisinin on the silica gel layer. A 10 µL aliquot of a solution of standard artemisinin (0.4 µg/mL) was applied onto a pre-coated silica gel 60 F_{254} layer (0.25 mm thickness), and the plate (loaded with 10 spots) was developed with a mobile phase of hexane:ethyl acetate:acetone (16:1:1). After drying, the TLC plate was cut into 10 strips of width 2 cm, each with an artemisinin spot at an R_f value of 0.75. Each strip was then exposed separately to ammonia vapour in a closed TLC tank in a hot-air oven and maintained either under a different temperature (from 30 to 125°C) for 2 h, or for different time intervals (from 20 to 180 min) at 100°C. After cooling, each TLC strip was scanned at 320 nm and the intensity of the artemisinin spot determined. The resulting areas under the artemisinin peaks were plotted against either temperature or time of exposure to ammonia vapour. In addition, the artemisinin spot with the highest intensity was also scanned at wavelengths from 200 to 370 nm in order to obtain a UV-absorption spectrum of the chromophore-containing product.

Standard curve. A stock solution containing 1.0 mg/mL artemisinin was diluted to obtain standard solutions with various concentrations of artemisinin ranging from 0.06 to 12 µg/mL, and these were employed in the construction of the calibration curve for artemisinin.

NMR analyses of the product of ammonia-treated artemisinin. An aliquot (1 mL) of artemisinin solution (2 µg/mL in methanol) was streaked onto a silica gel layer and the plate was developed using the solvent system described above. Ten TLC plates were used for product preparation. TLC plates were exposed to saturated ammonia vapour at 100°C for 2 h and the artemisinin band (R_f 0.75) scraped from the plate and eluted with absolute ethanol. The obtained product was further purified by TLC using the solvent system hexane:acetone:methanol (10:3:1), and the component at R_f 0.45 was eluted with absolute ethanol and dried with nitrogen gas. The purified compound was dissolved in deuterochloroform and ^{1}H- and ^{13}C-NMR spectra were recorded on a Jeol (Tokyo, Japan) JNM-A500 NMR at 500 and 125 MHz, respectively. Chemical shifts were determined in ppm as δ values relative to TMS as internal standard.

RESULTS AND DISCUSSION

The structural conversion of artemisinin on a silica gel layer following exposure to ammonia vapour was investigated by NMR. The resulting ^{1}H-NMR [($CDCl_3$) δ (ppm), 2.97 (1H, ddd, J = 14, 7, 4, CH-8), 6.20 (1H, brs, CH-10), 5.06 (1H, d, J = 3, CH-11), 1.40 (3H, s, CH_3-2), 0.86 (3H, d, J = 7, CH_3-5), 1.06(3H, d, J = 7, CH_3-8)] and ^{13}C-NMR [($CDCl_3$) δ (ppm), 107.5 (C-2), 34.6 (C-3),

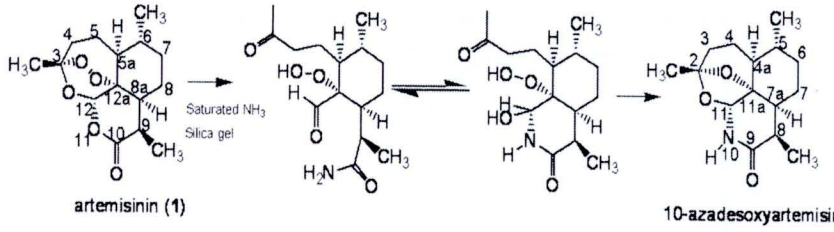

Figure 1 Proposed structural conversion of artemisinin to 10-azadesoxyartemisinin on a silica gel layer following exposure to ammonia vapour for 2 h at 100°C.

22.6 (C-4), 45.6 (C-4a), 35.3 (C-5), 33.6 (C-6), 24.3 (C-7) 43.5 (C-7a), 32.9 (C-8), 173.6 (C-9), 81.5 (C-11), 82.2 (C-11a), 22.2 (CH_3-2), 18.5 (CH_3-5), 11.9 (CH_3-8)] were very similar to those of 10-azadesoxyartemisinin (**2**) described previously by Torok and Ziffer (1995a, b). It was clear that the characteristic C-10 ester (δ 171.9) of artemisinin was converted into the C-9 amide (δ 173.6) characteristic of **2**, with the signal at C-11 of **2** being at lower field than the original C-12 of artemisinin. These data confirmed the conversion of artemisinin to **2** (Torok and Ziffer, 1995a, b). In these previous reports, the structural rearrangement of artemisinin was observed in a reaction mixture containing methanolic ammonia and sulphuric acid:silica gel, which produced a mixture of 11-azaartemisinin (45%) and 10-azadesoxyartemisinin (9%). In our case, however, the latter compound appeared to be the major product (>90%) of rearrangement of artemisinin. This might be due to the different conditions of the reaction. Our reaction took place on a silica gel layer with a spot of artemisinin reacting with ammonia vapour under a temperature of 100°C. It is likely that the silica gel-catalysed reaction of artemisinin involves an intermediate that can react with the ammonia molecule to form the final product of 10-azadesoxyartemisinin as shown in Fig. 1.

After performing a UV scan at different wavelengths on the compound, the resulting UV absorption spectrum showed clearly a single λ_{max} at 320 nm (Fig. 2). It can be seen that the UV absorption characteristic of 10-azadesoxyartemisinin was completely different from that of artemisinin itself. Thus, it is clear that natural artemisinin with no UV-chromophore in the molecule is converted into a chromophore-containing compound of 10-azadesoxyartemisinin (**2**) under the ammonia:silica gel-catalysed reaction. This allowed us to use the wavelength of 320 nm for artemisinin analysis with this TLC-densitometric method. In order to maximise the conversion of artemisinin to 10-azadesoxyartemisinin, the optimum conditions of temperature and time used for the ammonia exposure of artemisinin spot on the TLC plate were studied. The results demonstrated that the amount of 10-azadesoxyartemisinin produced by the reaction

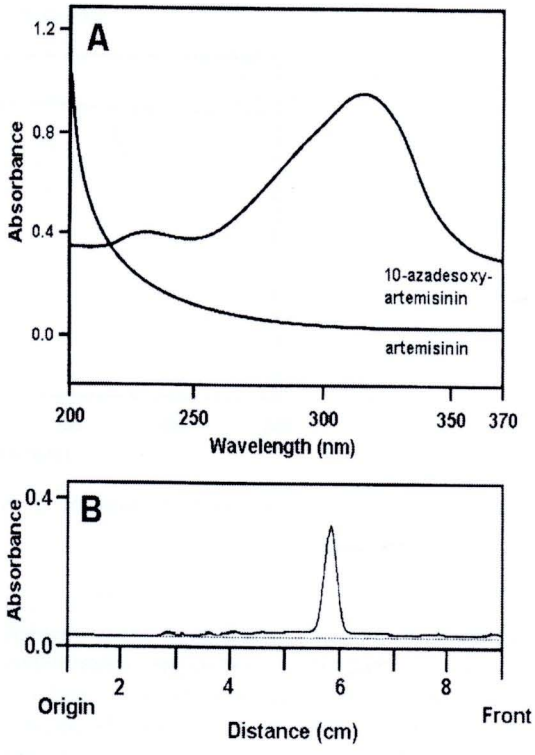

Figure 2 (A) UV-absorption spectra of artemisinin and 10-azadesoxyartemisinin obtained by scanning spots on a silica gel layer. (B) A typical TLC-chromatogram (scanned at 320 nm) of standard artemisinin after being converted into 10-azadesoxyartemisinin using the developed TLC process.

increased from room temperature to reach its highest level at 75°C, and remained at that level up to a temperature of 125°C [Fig. 3(A)]. In the time-course study, it was found that the conversion of artemisinin increased continuously from 20 to 120 min [Fig. 3(B)], but there was no further increase of product after this time. These results clearly suggested that the minimal conditions for the complete conversion of artemisinin to 10-azadesoxyartemisinin would involve treatment of the TLC plate with ammonia vapour at 75°C for 2 h. However, for consistency and reproducibility, a temperature of 100°C was employed in the remainder of the investigation.

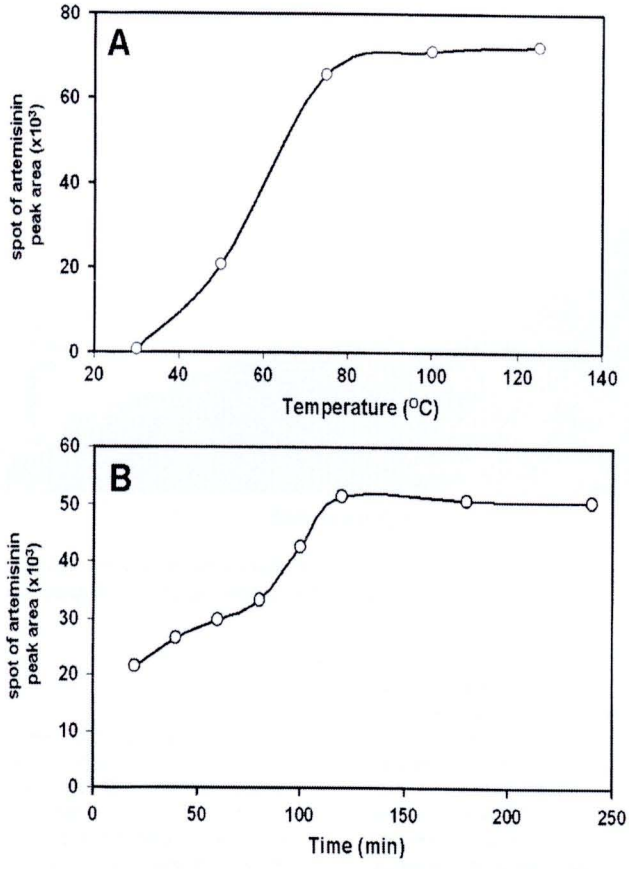

Figure 3 The effects of time (A) and temperature (B) of exposure to ammonia vapour on the conversion of artemisinin to 10-azadesoxyartemisinin on a silica-gel layer.

A study of the TLC separation of artemisinin in hexane extracts of *A. annua* leaves was carried out using a number of solvent systems. By varying the ratio of hexane, ethyl acetate and acetone in the mobile phase, it was found that complete separation of artemisinin could be achieved with the solvent system containing these components in the ratio 16:1:1. Figure 4 shows the TLC-densitometric chromatograms of hexane extracts obtained from some leaf samples. These chromatograms were obtained after the developed silica gel layer had been exposed to ammonia vapour and scanned at 320 nm. It can be seen that the position of artemisinin (R_f 0.75) was well defined and separate from other components present in the crude extracts. The product peak showed a symmetrical shape with very low baseline noise. The chromatograms also exhibited different peak heights of artemisinin with different samples, suggesting that the developed TLC-densitometric method would be relatively sensitive for detecting the artemisinin product.

A calibration curve was constructed for artemisinin using the described conditions for TLC separation and UV detection. The resulting standard curve showed linearity in the range 0.5 to 12 μg/mL of artemisinin concentration (equivalent to 5–120 ng of analyte). The limit of detection (0.5 μg/mL of artemisinin) was determined using the criteria of the lowest detectable amount that would produce a peak three times the peak-to-peak baseline noise. Linear regression analysis of the area under the curve (x) vs the concentration of calibration standards (y) gave the equation $y = 3159 + 15{,}743x$ with an $r^2 = 0.9938$.

In order to determine the accuracy and precision of the developed TLC-densitometric method, a comparison was conducted using a method involving pre-column reaction coupled with HPLC-UV detection (Zhao, 1987). It was found that the values of artemisinin content in various leaf samples determined by TLC densitometry were similar to those determined by the HPLC method (Table 1). The values of the relative standard deviations between replicate analyses of the artemisinin contents were found to be within 1.4% ($n = 3$). These values indicated that the developed TLC-densitometric method provided high accuracy and precision for the determination of artemisinin in *A. annua* leaves. In terms of stability, the rearranged product of 10-azadesoxyartemisinin appeared to be

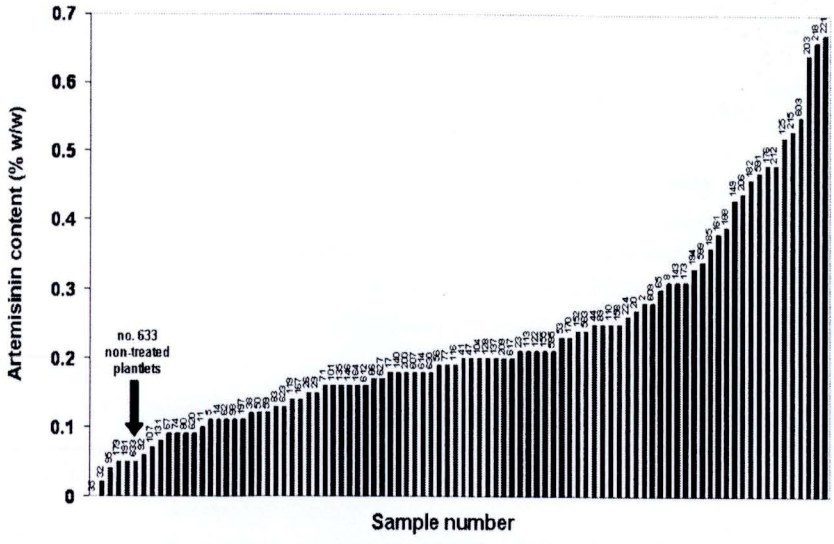

Figure 5 Variation of artemisinin content in various plantlets of *A. annua* that had been exposed to a 500 rad dose of gamma rays. The values were obtained using the TLC-densitometric method described.

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5. PUBLICATION

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6. RESEARCH HIGHLIGHTS

- Plant tissue culture technique, Transfer of *in vitro* plantlets to *ex vitro* plants technige, *Agrobacterium*-Mediated transformation techniqe
- Gene cloning, over-expression of recombinant protein, SDS-PAGE, Western Blotting and other molecular biology techniques.
- Radio-tracer technique for biosynthetic pathway
- Experience with HPLC, GC, GC-MS, TLC-desitometry, TLC-radioscanner, DNA sequencing, ELISA
- Natural product analysis for xanthophylls, alliin, allicin, artemisinin, capcicine, plunotol