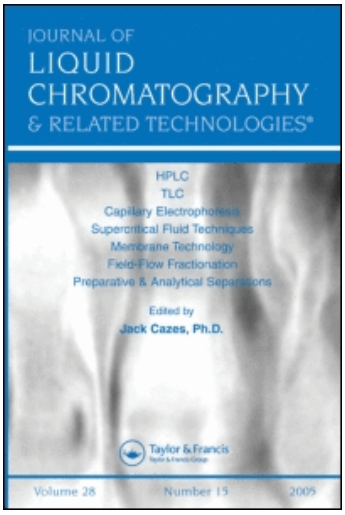


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# HPLC-PDA Determination of Bioactive Diterpenoids from Plant Materials and Commercial Products of *Andrographis paniculata*

Wenkui Li[a]; John F. Fitzloff[a]

[a] Functional Foods for Health (FFH) Core Analytical Laboratory, Department of Medicinal Chemistry and Pharmacognosy (PCRPS), College of Pharmacy, University of Illinois at Chicago, Chicago, Illinois, USA

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# HPLC–PDA Determination of Bioactive Diterpenoids from Plant Materials and Commercial Products of *Andrographis paniculata*

**Wenkui Li and John F. Fitzloff***

Functional Foods for Health (FFH) Core Analytical Laboratory,
Department of Medicinal Chemistry and Pharmacognosy (PCRPS),
College of Pharmacy, University of Illinois at Chicago,
Chicago, Illinois, USA



## ABSTRACT

The present paper describes the development of an HPLC–PDA method for the simultaneous determination of bioactive diterpenoids, andrographolide, isoandrographolide, neoandrographolide, and 14-deoxy-11,12-didehydroandrographolide in plant materials and commercial products of *Andrographis paniculata*. Separations were achieved using a conventional $C_{18}$ column with PDA detection at 200–400 nm for UV spectrum and 225 nm for quantification. The mobile phase consists of water and acetonitrile with acetonitrile varying from 20% to 50% over 40 min.

*Correspondence: John F. Fitzloff, Functional Foods for Health (FFH) Core Analytical Laboratory, Department of Medicinal Chemistry and Pharmacognosy (PCRPS), College of Pharmacy, University of Illinois at Chicago, 833 South Wood Street, Chicago, IL 60612, USA; E-mail: fitzloff@uic.edu.



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The quantification was performed using external standards. The method was validated for linearity, limit of detection (LOD), limit of quantification (LOQ), inter-day and intra-day reproducibility, and recovery.

*Key Words:* *Andrographis paniculata*; Plant materials; Commercial products; HPLC; PDA.



## INTRODUCTION

*Andrographis paniculata* Nees (Acanthaceae) is one of the most important medicinal plants and has been widely used in Chinese and Ayurvedic medicine for the treatment of gastric disorders, infectious diseases, and the common cold.[1,2]

In Europe, standardized *A. paniculata* preparation has been therapeutically used in treating the common cold for more than 20 years. Several randomized, placebo-controlled, double-blind clinical trials have concluded that *A. paniculata* has a preventive effect against the common cold, and has the capacity to significantly shorten the duration of the ailment.[3–6] Pharmacological research has demonstrated that *A. paniculata* possesses anti-inflammatory, antiallergic, immuno-stimulatory, antiviral, antioxidant, hepatoprotective, and cardiovascular activities, etc., and these studies have been well reviewed.[1,2] Diterpenoids, including andrographolide and its analogues, are believed to be responsible for the above biological activities, and more than 20 diterpenoids have been reported from *A. paniculata*.[1,2] However, each one of these diterpenoids might exhibit varying degrees of biological activity. Recent studies showed that andrographolide possesses the properties of preventing oxygen radical production by human neutrophils,[6] anti-HIV,[7] and inhibitory effect on PFA-induced platelet aggregation,[8] while isoandrographolide was reported to possess the same cell differentiating–inducing activity as andrographolide against MI cells, with a phagocytic ratio of more than 30% at the concentration of $5 \times 10^{-6}$ M.[9] Neoandrographolide was reported to possess the strongest protective effect among other andrographolide analogues on hepatotoxicity induced in mice by carbontetrachloride ($CCl_4$) or tert-butylhydroperoxide (*t*BHP) intoxication.[10,11] On the other hand, 14-deoxy-11,12-didehydroandrographolide shows marked hypotensive effects in anesthetized rats and a vasorelaxant activity in isolated rat aorta, by stimulating nitric oxide (NO) release from endothelial cells.[12] These different effects imply that qualitative and quantitative control of the *A. paniculata* plant materials and, consequently, commercial products is of paramount importance to ensure its maximal therapeutic value.

*A. paniculata* and related products have been previously analyzed by using TLC.[13–15] However, the TLC method normally takes 1–3 hr to

separate components in the extracts with comparatively poor sensitivity and selectivity. Luminol-chemiluminescence method has also been proposed.[16] By forming a complex of luminol–$H_2O_2$–$Co^{++}$, a stable form of light emission for detection is produced. However, due to its complexity, the method is not commonly adopted. More recently, some other methods, such as MEKC,[17] HPLC–ELSD,[18] CE and GC–MS,[19] have been reported. Among these methods, it is HPLC-UV, but not CE, GC–MS, MEKC, or HPLC–ELSD that dominates the techniques in the identification and quantification of bioactive constituents in botanical dietary supplements, crude materials, and related preparations. Although several HPLC-UV methods have been proposed for the QA/QC of *A. paniculata* plant materials, these methods have not been fully validated, especially for reproducibility and recovery.[20,21] In continuing our investigation of botanical dietary supplements,[22,23] we developed an HPLC–PDA method for the simultaneous quantification of andrographolide, neoandrographolide, 14-deoxy-11,12-didehydroandrographolide, and isoandrographolide in the plant materials and commercial products of *A. paniculata*. It is the first time to include isoandrographolide in the QA/QC of *A. paniculata* plant materials and commercial products. In the present work, special attention was focused on the validation of the method, including reproducibility and recovery.



## EXPERIMENTAL

### Reagents

Methanol, acetonitrile, hexane, chloroform, ethyl acetate, and *n*-butanol were HPLC grade from Fisher Scientific Co. (Fair Lawn, NJ). Deionized (DI) water was obtained with an in-house Nano-pure® water system (Barnstead, Newton, MA).

### Chemicals

Due to the lack of commercially purified reference standards of andrographolide, isoandrographolide, neoandrographolide, and 14-deoxy-11,12-didehydroandrographolide, they were isolated from a commercial *A. paniculata* extract. Briefly, 85 g of the standardized extract was re-extracted with methanol (3 × 250 mL) using a sonicator. The combined extracts were evaporated under reduced pressure and the resulting residue was partitioned using hexane, chloroform, ethyl acetate, and *n*-butanol. The combined chloroform and ethyl acetate extracts were chromatographed on a silica gel column (100 g) eluted with gradient chloroform–methanol to yield 14 subfractions.

Andrographolide was obtained from subfractions 6, 7, and 8 as crystals. MS, $m/z$ 349 $[M - H]^-$; $^{13}$C-NMR (deuterated pyridine) $\delta$ (ppm), 37.22 (C-1), 28.93 (C-2), 79.80 (C-3), 43.16 (C-4), 55.21 (C-5), 24.93 (C-6), 38.14 (C-7), 147.88 (C-8), 56.28 (C-9), 39.10 (C-10), 24.31 (C-11), 147.02 (C-12), 130.13 (C-13), 65.90 (C-14), 75.41 (C-15), 170.75 (C-16), 108.80 (C-17), 23.69 (C-18), 64.14 (C-19), 15.17 (C-20).[9]

Isoandrographolide was isolated from subfraction 5 and the mother solution of subfraction 6, and purified by using semi-preparative HPLC. MS, $m/z$ 349 $[M - H]^-$; $^{13}$C-NMR (deuterated pyridine) $\delta$ (ppm), 37.19 (C-1), 28.95 (C-2), 79.86 (C-3), 43.19 (C-4), 55.67 (C-5), 24.42 (C-6), 38.26 (C-7), 148.14 (C-8), 56.29 (C-9), 39.31 (C-10), 23.62 (C-11), 148.36 (C-12), 129.79 (C-13), 69.10 (C-14), 74.30 (C-15), 169.92 (C-16), 108.15 (C-17), 23.72 (C-18), 64.10 (C-19), 15.28 (C-20).[9]

Neoandrographolide was obtained from combined subfraction 10, 11, 12, and 13 using semi-preparative HPLC. MS, $m/z$ 479 $[M - H]^-$; $^{13}$C-NMR (deuterated pyridine) $\delta$ (ppm), 39.05 (C-1), 19.39 (C-2), 36.42 (C-3), 39.82 (C-4), 55.20 (C-5), 24.71 (C-6), 38.77 (C-7), 148.19 (C-8), 56.70 (C-9), 38.65 (C-10), 22.11 (C-11), 25.01 (C-12), 134.16 (C-13), 145.41 (C-14), 70.66 (C-15), 174.65 (C-16), 106.98 (C-17), 28.19 (C-18), 72.61 (C-19), 15.47 (C-20), 105.53 (G-1), 75.34 (G-2), 78.73 (G-3), 71.75 (G-5), 62.83 (G-6). [9]

14-Deoxy-11,12-didehydroandrographolide was purified from combined subfractions 2 and 3 with semi-preparative HPLC. MS, $m/z$ 331 $[M - H]^-$; $^{13}$C-NMR (deuterated pyridine) $\delta$ (ppm), 38.65 (C-1), 28.52 (C-2), 81.25 (C-3), 43.38 (C-4), 55.08 (C-5), 23.38 (C-6), 36.97 (C-7), 148.49 (C-8), 62.07 (C-9), 38.98 (C-10), 136.40 (C-11), 121.50 (C-12), 129.67 (C-13), 143.36 (C-14), 70.05 (C-15), 172.73 (C-16), 109.60 (C-17), 23.05 (C-18), 64.60 (C-19), 16.31 (C-20).[9]

The structure of the above compounds is shown in Fig. 1, and their purity was determined to be more than 99% by using reverse-phase HPLC–PDA as described below.

## Standard Solutions

Reference standards, andrographolide, isoandrographolide, neoandrographolide, and 14-deoxy-11,12-didehydroandrographolide were accurately weighed (1 mg each) into a 10-mL volumetric flask and dissolved in methanol to make a stock solution. The stock solution was stored at $-20^{\circ}$C and brought to room temperature before use. Calibration standard working solutions were freshly prepared by diluting the stock solution with methanol in appropriate quantities. In the same way, three sets of controls for andrographolide, isoandrographolide, neoandrographolide, and 14-deoxy-11,12-didehydroandrographolide

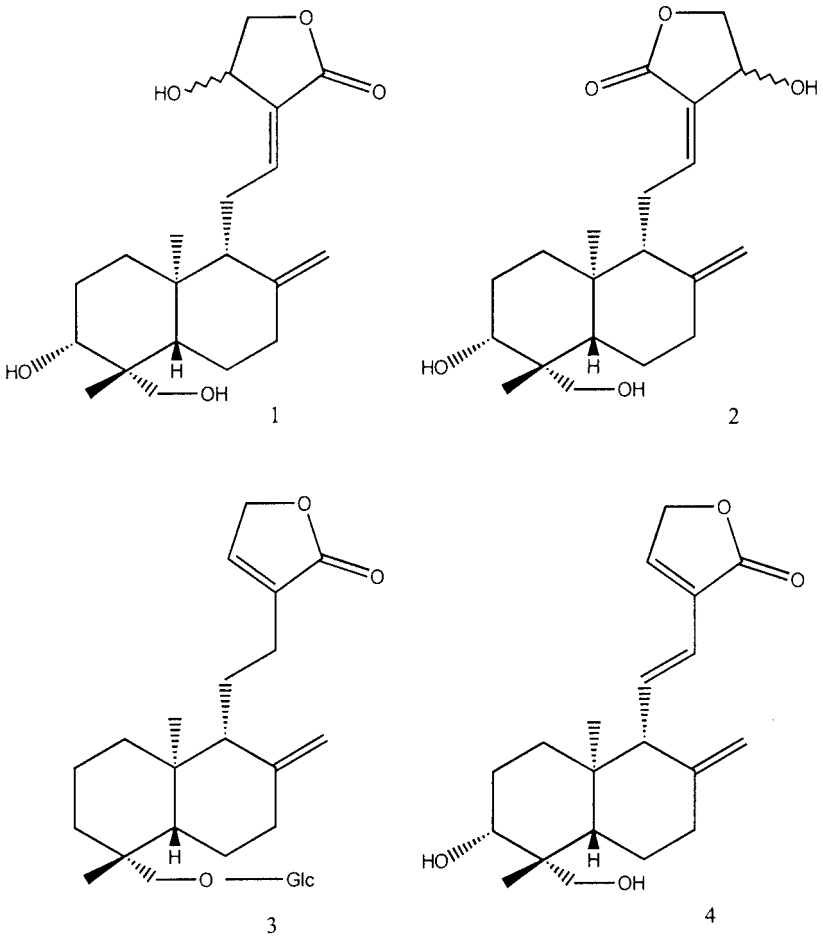


***Figure 1.*** Structure of andrographolide (1), isoandrographolide (2), neoandrographolide (3), 14-deoxy-11,12-didehydroandrographolide (4).

were prepared from a separate stock, so as to lie in the lowest, middle, and highest regions of the calibration curves.

## Sample Preparation

Authentic *A. paniculata* plant materials were collected from Indonesia, Hong Kong, and Mainland China. Three brands of commercial products

(tablets) of *A. paniculata* were obtained from local pharmacies. The dose of these products is one tablet. To prepare methanolic extract of the plant material and commercial products, 0.5–1.0 g of the dried, powdered, aerial parts of the plant, or one dose of finely pulverized commercial product, was extracted with methanol (3 × 18 mL) in room temperature by using a sonicator. The combined extracts were evaporated under reduced pressure at 40–45°C, and the resulting residue was dissolved in methanol into a 10-mL volumetric flask, and made up to the volume with methanol. After centrifugation, the resulting supernatant was subjected to HPLC–PDA analysis.

## HPLC–PDA Analysis

The HPLC–PDA analysis was carried out using a Waters Alliance 2690 liquid chromatograph and a 996 photodiode array detector (Waters, Milford, MA). The chromatographic data were recorded and processed using Waters Millenium 2000 software. The measurements were carried out on a Supelco Discovery RP-18 column (250 × 4.6 mm 5 μm particle size, Supelco, Bellefonte, PA), protected by a Waters Delta-Pak $C_{18}$ guard column, and set at 20°C. The solvents used were water and acetonitrile with acetonitrile from 20% to 50% over 40 min. All injections were 10 μL in volume. The detection was carried out with detection wavelength set 200–400 nm for UV spectrum and 225 nm for quantification of the compounds of interest.



## Identification and Peak Purity

Peaks in HPLC–PDA were tentatively identified by comparison of the retention times and UV spectra of the peaks in the sample solutions with those of reference standards, and by the method of reference standards addition to the sample solutions. The purity of each peak was checked by Waters Millenium PDA software routines.

## Quantification

The concentrations of diterpenoids identified were measured using the external standard method by calibration curves (peak area at 225 nm versus concentration) obtained for these compounds over the range of concentrations observed.

## RESULTS AND DISCUSSIONS

### Method Validation

Under the current HPLC gradient condition, all constituents in *A. paniculata* were eluted within 40 min. Figure 2 shows a typical chromatogram of reference standard mixture at 225 nm. The method was validated for linearity, sensitivity, reproducibility, and recovery.

The linearity was based on the duplicate analysis of calibration working standard solutions, at nine concentration levels on three consecutive days, for andrographolide, isoandrographolide, neoandrographolide, and 14-deoxy-11, 12-didehydroandrographolide (0.5–100 μg/mL) with regression coefficients ($r^2$) better than 0.999.

Under the current chromatographic condition, the limit of detection (LOD) and limit of quantification (LOQ) were determined to be 100 ($S/N > 5$) and 200 ng/mL ($S/N > 10$), respectively, for andrographolide, isoandrographolide, neoandrographolide, and 14-deoxy-11,12-didehydroandrographolide, respectively.

The reproducibility of the method was evaluated by analyzing three sets of controls ($n = 3$) on three separate days ($n = 3$), and calculating the RSD (%) and relative errors (%). As shown in Table 1, the RSD (%) and the relative errors (%) were found to be less than 3.89 and 4.80, respectively. Meanwhile,

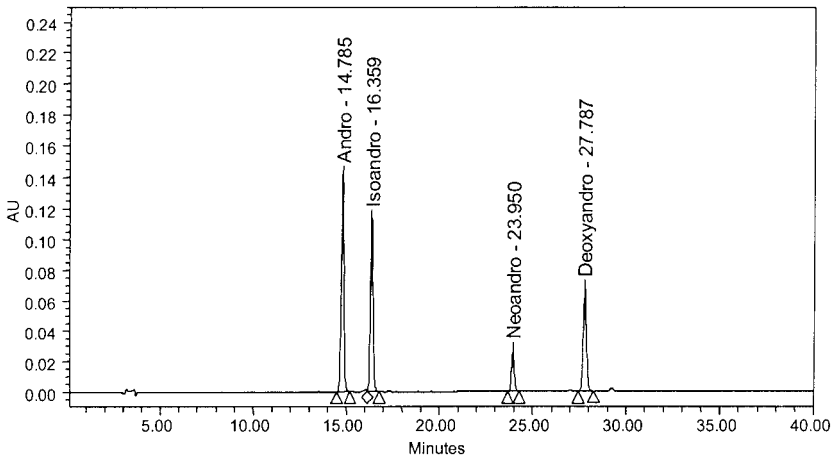


***Figure 2.*** A typical HPLC–PDA chromatogram of a standard mixture of andrographolide, isoandrographolide, neoandrographolide, and 14-deoxy-11,12-didehydroandrographolide.

***Table 1.*** Reproducibility.

| Compound | Spiked concentration (μg/mL) | Day-1 Observed value (μg/mL)[a] | Day-1 RSD (%) | Day-1 RE (%) | Day-2 Observed value (μg/mL)[a] | Day-2 RSD (%) | Day-2 RE (%) | Day-3 Observed value (μg/mL)[a] | Day-3 RSD (%) | Day-3 RE (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| Anhydrographolide | | | | | | | | | | |
| QC-1 | 25 | 24.10 ± 0.54 | 0.51 | −3.59 | 24.08 ± 0.19 | 0.79 | −3.67 | 24.16 ± 0.10 | 0.43 | −3.36 |
| QC-2 | 50 | 48.78 ± 0.12 | 0.24 | −2.44 | 49.59 ± 1.61 | 3.25 | −0.82 | 49.10 ± 0.66 | 1.35 | −1.80 |
| QC-3 | 75 | 77.74 ± 0.12 | 0.70 | 3.65 | 78.03 ± 3.03 | 3.88 | 4.05 | 78.56 ± 0.76 | 0.96 | 4.75 |
| Isoanhydrographolide | | | | | | | | | | |
| QC-1 | 25 | 24.37 ± 0.13 | 0.53 | −2.54 | 24.14 ± 0.20 | 0.81 | −3.44 | 24.20 ± 0.11 | 0.46 | −3.21 |
| QC-2 | 50 | 48.97 ± 0.15 | 0.31 | −2.05 | 49.57 ± 1.62 | 3.27 | −0.85 | 49.12 ± 0.65 | 1.32 | −1.75 |
| QC-3 | 75 | 77.89 ± 0.39 | 0.50 | 3.85 | 78.06 ± 3.04 | 3.89 | 4.08 | 78.60 ± 0.68 | 0.87 | 4.80 |
| Neoanhydrographolide | | | | | | | | | | |
| QC-1 | 25 | 24.15 ± 0.10 | 0.40 | −3.39 | 24.19 ± 0.20 | 0.85 | −3.25 | 24.21 ± 0.17 | 0.70 | −3.17 |
| QC-2 | 50 | 48.86 ± 0.12 | 0.25 | −2.28 | 49.59 ± 1.64 | 3.30 | −0.81 | 49.15 ± 0.66 | 1.34 | −1.70 |
| QC-3 | 75 | 77.78 ± 0.46 | 0.60 | 3.71 | 77.98 ± 2.99 | 3.83 | 3.97 | 78.49 ± 0.74 | 0.94 | 4.66 |
| 14-Deoxy-11,12-didehydroandrographolide | | | | | | | | | | |
| QC-1 | 25 | 24.11 ± 0.16 | 0.65 | −3.57 | 24.05 ± 0.21 | 0.87 | −3.82 | 24.13 ± 0.19 | 0.79 | −3.46 |
| QC-2 | 50 | 48.74 ± 0.12 | 0.25 | −2.52 | 49.52 ± 1.66 | 3.35 | −0.97 | 49.03 ± 0.68 | 1.38 | −1.94 |
| QC-3 | 75 | 77.69 ± 0.56 | 0.72 | 3.59 | 78.02 ± 2.95 | 3.78 | 4.03 | 78.45 ± 0.81 | 1.04 | 4.61 |

[a]The data represent the mean ± SD of three observations.

six sample solutions prepared from the same batch were analyzed by HPLC–PDA. The content of constituents of interest was evaluated to calculate the RSD (%). The following data were obtained: andrographolide 2.15%, isoandrographolide 3.87%, neoandrographolide 4.23%, and 14-deoxy-11,12-didehydroandrographolide 2.24%.

For the recovery test, *A. paniculata* plant material powder (5.0 g) was extracted in a 50-mL flask with 25 mL of methanol by means of sonication for 30 min. After filtration, the residue was put back in the same flask and 25 mL of fresh methanol was added to the flask. The flask was sonicated for another 30 min before the filtration. The above extraction procedure was repeated until no peaks were detected in the filtrate by HPLC–PDA described as above. The residue was dried before use. A portion of the above dried residue powder (0.5 g) was spiked with 1 mL of recovery working solution, containing andrographolide, isoandrographolide, neoandrographolide, and 14-deoxy-11,12-didehydroandrographolide at 225, 500, and 800 μg, respectively. After dryness, the mixture was extracted with 18 mL of methanol



***Table 2.*** Determination of recovery.

| Compound | Spiked concentration (μg/mL) | Observed value (μg/mL)[a] | RDS (%) | RE (%) |
|---|---|---|---|---|
| Anhydrographolide | | | | |
| RE-1 | 22.5 | 23.98 ± 0.18 | 0.75 | 6.60 |
| RE-2 | 50 | 49.49 ± 0.34 | 0.68 | −1.02 |
| RE-3 | 80 | 79.06 ± 0.35 | 0.44 | −1.18 |
| Isoanhydrographolide | | | | |
| RE-1 | 22.5 | 24.19 ± 0.26 | 1.31 | −3.25 |
| RE-2 | 50 | 49.54 ± 0.39 | 0.79 | −0.91 |
| RE-3 | 80 | 78.50 ± 0.32 | 0.34 | 4.67 |
| Neoanhydrographolide | | | | |
| RE-1 | 22.5 | 24.25 ± 0.20 | 0.83 | −3.01 |
| RE-2 | 50 | 49.52 ± 0.34 | 0.68 | −0.96 |
| RE-3 | 80 | 78.26 ± 0.60 | 0.76 | 4.34 |
| 14-Deoxy-11,12-didehydroandrographolide | | | | |
| RE-1 | 22.5 | 23.95 ± 0.13 | 0.56 | −4.18 |
| RE-2 | 50 | 49.51 ± 0.36 | 0.72 | −0.98 |
| RE-3 | 80 | 78.06 ± 0.48 | 0.62 | 4.08 |

[a]The data represent the mean ± SD of three observations.

(3 × 18 mL) in room temperature by using a sonicator. The combined extracts were evaporated under reduced pressure and the resulting residue was dissolved in methanol into a 10-mL volumetric flask, and made up to the volume with methanol. Triplicate recovery samples were prepared. Meanwhile, a blank recovery sample (without adding standards) was prepared and analyzed for the comparison. The recovery rates were determined to be between 96.75 and 106.6% (Table 2).

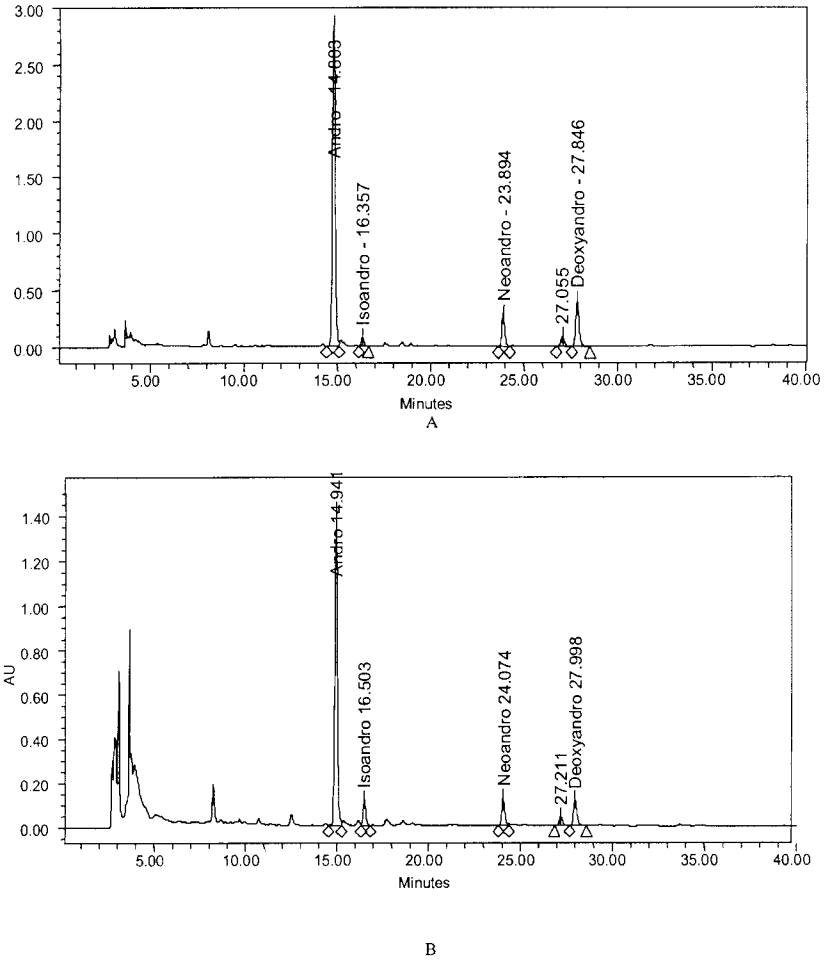


***Figure 3.*** Typical HPLC–PDA chromatograms of methanolic extract of *A. paniculata* plant materials (A), and commercial products (B).

***Table 3.*** Content of diterpenoids in plant materials and commercial products of *A. paniculata*.

| | Indonesia (%, w/w) | Hong Kong (%, w/w) | Mainland China (%, w/w) | Product 1 (mg/dose) | Product 2 (mg/dose) | Product 3 (mg/dose) |
|---|---|---|---|---|---|---|
| Anhydrographolide | 1.35 | 0.53 | 1.05 | 11.33 | 7.75 | 12.12 |
| Isoanhydrographolide | 0.05 | 0.03 | 0.05 | 0.65 | 0.25 | 1.09 |
| Neoanhydrographolide | 0.76 | 0.44 | 0.39 | 3.11 | 0.74 | 4.27 |
| 14-Deoxy-11,12-di-dehydroandrographolide | 0.41 | 0.43 | 0.60 | 1.14 | 1.25 | 1.92 |

## Application

Typical HPLC–PDA chromatograms of methanolic extracts of plant materials and commercial products are shown in Fig. 3A and B. Triplicate samples of *A. paniculata* plant materials and commercial products (each) were prepared and analyzed according to the method as described above. The average content of andrographolide, isoandrographolide, neoandrographolide, and 14-deoxy-11,12-didehydroandrographolide was summarized in Table 3. As shown in the table, the plant materials from Indonesia contain, generally, higher contents of diterpenoids than that obtained from Hong Kong and Mainland China. Our investigation of commercial *A. paniculata* products showed that these products contain higher contents of andrographolide than isoandrographolide, neoandrographolide, and 14-deoxy-11, 12-dide-hydroandrographolide. The ratios of andrographolide to isoandrographolide, neoandrographolide, and 14-deoxy-11,12-didehydroandrographolide are similar to that obtained from plant materials. All the three commercial products claim to contain 12 mg of andrographolide per tablet. However, one of these products was found to contain only 7.7 mg of andrographolide per tablet, indicating variability of diterpenoid content among these products.



## CONCLUSION

A high-performance liquid chromatography method has been developed for the identification and quantitation of four diterpenoids in the plant materials and commercial products of *A. paniculata* with photodiode array detection. With this method, andrographolide, isoandrographolide, neoandrographolide, and 14-deoxy-11,12-didehydroandrographolide was successfully quantitated, using standard calibration curves. The current method was found to be specific and suitable for routine analysis because of its simplicity, specificity, accuracy, and reproducibility.

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