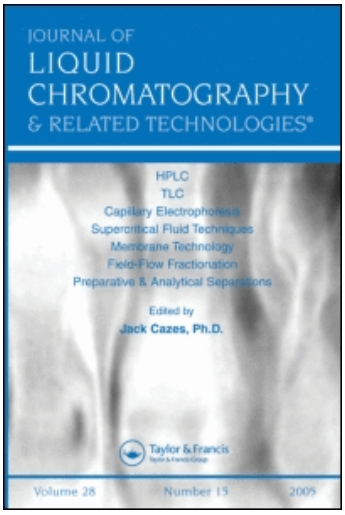

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DETERMINATION OF ANDROGRAPHOLIDE IN COMMERCIAL ANDROGRAPHIS (ANDROGRAPHIS PANICULATA) PRODUCTS USING HPLC WITH EVAPORATIVE LIGHT SCATTERING DETECTION

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ABSTRACT

A reverse-phase high performance liquid chromatographic method is developed for the determination of andrographolide, a characteristic diterpene lactone in commercial Andrographis (*Andrographis paniculata*) products. Samples are analyzed by means of a reverse-phase column (Supelco Discover' C_{18}) using acetonitrile and water, under gradient conditions as the mobile phase, over 40 min. The evaporative light scattering detector (ELSD) used, was set at an evaporating temperature of 61°C and nebulizing gas (compressed air) pressure of 2.9 bars. The detection limit ($S/N > 5$) of andrographolide is 50 ng on the column.

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Key Words: Andrographolide; *Andrographis paniculata*; Commercial products; HPLC; ELSD

INTRODUCTION

Andrographis paniculata Nees (Acanthaceae) is one of the most important medicinal plants having been widely used in Chinese and Ayurvedic medicine for the treatment of gastric disorders, colds, influenza, and other infectious diseases.[1,2] Andrographolide (Figure 1) and related diterpene lactones have been known to be responsible for the reported pharmacological activities, including anti-inflammatory,[3–5] antiallergic,[6] immuno-stimulatory,[7] anti-viral,[8–9] antioxidant,[10] hepatoprotective,[11–12] and cardiovascular[13–16] activities, etc. The identification and quantification of andrographolide have been carried out by using thin-layer chromatography (TLC)[17–19] and high performance liquid chromatography with ultraviolet detection (HPLC-UV),[20–21] and these methods have been successfully applied to the QA/QC of andrographis crude materials and preparations. Other methods, such as gas chromatography coupled with mass spectrometry detection (GC-MS) and capillary electrophoresis (CE), have also been reported.[22] It might be of interest to introduce high performance liquid chromatography with evaporative light scattering detection (HPLC-ELSD) to analyze andrographolide in andrographis products, because up to now there has been no literature regarding this issue.

As a mass detection method, the evaporative light scattering detector (ELSD) is based on the nebulization of LC column effluent into droplets by the nebulizing gas and the entrance of the resulting vapor into a temperature-controlled evaporator tube, where the evaporation of mobile phase takes place.

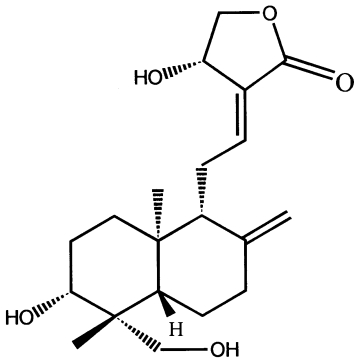

Figure 1. Structure of andrographolide.

The resulting "cloud" of solid microparticles is directed towards a narrow light beam, which is scattered by microparticles and measured using a photomultiplier or photodiode. A plot of detector response versus solute concentration is sigmoidal, and the peak area I is related to the sample size and shape, but not the chemical identity of the residual particles passing through the light beam, by the following relationship: $I = am^b$, where b is the slope of the response line, m is the mass of the compound injected, and a is the response factor. ELSD has been applied to a wide range of analytes, including lipids,[23] peptides,[24] carbohydrates,[25] and diterpene lactones in herbal products.[26]

We recently reported the determination of a characteristic compound, 24 (R)-pseudoginsenoside F_{11} in North American ginseng.[27] It is interesting to us that the ELSD is not a technique that is widely known nor used in the QA/QC of herbal products. This work is based on a project with the aim to evaluate the application of the ELSD in the QA/QC of dietary supplements. The current paper describes the quantitative analysis of andrographolide in andrographis products in a single run by HPLC-ELSD using gradient elution.

EXPERIMENTAL

Chemicals

HPLC grade methanol and acetonitrile were obtained from Fisher Scientific Co. (Fair Lawn, NJ, USA). Deionized water was generated via an in-house Nanopure® water system (Barnstead, Newton, MA, USA). Andrographolide was isolated and identified in the Program for Collaborative Research in the Pharmaceutical Sciences, College of Pharmacy, University of Illinois at Chicago, Chicago, IL 60612, USA.

Commercial Andrographis Samples

Commercial Andrographis products, all in the form of tablets, were obtained from local pharmacies, Chicago, IL. To protect manufacturers' identity, the samples were labeled A-C (Table 1).

Apparatus

A Waters 2690 Alliance HPLC system (Waters Corporation, Milford, MA, USA), equipped with an on-line degasser and an autosampler, was used for solvent delivery. The column effluent was directed to a Sedex 75 evaporative light

Table 1. Reproducibility over Three Consecutive Days

Group	Spiked Concentration (μg/mL)	Days	Measured Concentration (Mean ± SD, n = 3, μg/mL)	Coefficient Variance (%)	Relative Error (%)
QC-1	13.4	1	14.32 ± 0.52	3.62	6.89
		2	13.61 ± 0.27	1.98	1.56
		3	12.81 ± 0.23	1.75	−4.43
QC-2	33.5	1	33.07 ± 0.35	1.07	−1.27
		2	33.18 ± 0.82	2.48	−0.97
		3	30.98 ± 0.50	1.61	−7.53
QC-3	67.0	1	64.54 ± 0.96	1.49	−3.67
		2	66.34 ± 3.04	4.58	−0.98
		3	62.53 ± 0.25	0.40	−6.67

scattering detector (ELSD) (Cedex 94141, Alfortville, France). The detector output was interfaced, using a SATIN box, to the Waters Millennium 2000® chromatographic manager system (Waters Corporation, Milford, MA, USA) loaded on a Compaq 6400X/10000/CDS computer (Houston, TX, USA) for data handling and chromatogram generation.

Preparation of Standard Solution

In a clean, dry 10-mL volumetric flask, andrographolide reference standard (1 mg) was accurately weighed and dissolved in methanol to make a stock solution. Calibration working standard solutions (5–100 μg/mL) was prepared by diluting the stock solution with methanol in appropriate quantities. Three controls were also prepared so as to lie in the lowest, middle, and highest regions of the calibration curve, i.e., 13.4, 33.5, and 67.0 μg/mL. All working solutions were stored at −20°C and brought to room temperature before use.

Preparation of Sample Solution

One dosage (tablet) of andrographis products was weighed into a PTFE-capped 20-mL sample vial. 50% methanol (15 mL) was added, and the mixture was sonicated at 25–30°C for 60 min. After cooling, the mixture was filtered through filter paper (Whatman # 1) into a 250-mL round-bottom flask, and the

residue was returned to the sample vial. Another 15 mL of 50% methanol was added and the mixture was sonicated at 25–30°C for 30 min. The extract was filtered through filter paper (Whatman # 1) into the same round-bottom flask. The above extraction procedure was repeated one more time before washing the residue with methanol (3×15 mL) while on the filter. The combined methanol extracts were evaporated under reduced pressure at 35–45°C. The residue was re-dissolved and transferred with methanol to a 10-mL volumetric flask and made up to volume with methanol. The sample solution was filtered through 0.2 μm Whatman hydrophilic membrane filter (Whatman Inc., Clifton, NJ, USA) into HPLC sample vial just before HPLC-ELSD analysis.

Chromatography

The chromatographic separations were carried out on a Supelco Discovery C_{18} column (250×4.6 mm, 5 μm particle size, col # 24855-08, bonded phase lot # 3651, silica lot # PS 183) (Supelco, Bellefonte, PA, USA) protected by a Waters Delta-Pak C_{18} guard column (Waters Technological Ireland, Ltd, Wexford, Ireland) and set at 20°C. The mobile phase used for the separation consisted of solvent A (water, deionized) and solvent B (acetonitrile). The elution profile was gradient with solvent B from 20% to 50% over 40 min. The flow rate was set to 1.0 mL/min. The column temperature was fixed at 20°C and the injection volume was chosen to be 10 μL. The peak identification was based on retention time, and comparison to the injected authentic reference standard. The peaks were detected in the ELSD with a gain of 11, the evaporation temperature of 61°C, and the nebulizing gas pressure of 2.9 bars, respectively. Prior to each run, the HPLC-ELSD system was allowed to warm up for 20–30 min, and the pumps were primed using the protocol suggested by the manufacturer. Using freshly prepared mobile phase, the baseline was monitored until stable before the samples were run.

Reproducibility

The precision and accuracy of the method were assessed by within and between run validations. The variation was evaluated by injecting three sets of controls (13.4, 33.5, 67 μg/mL, $n = 3$) on three consecutive days. By substituting the peak area into the calibration curve equation from the same run, the measured concentrations were obtained. By comparing calculated and theoretical concentrations, the relative errors (%) were obtained. The coefficient of variance (%) was calculated by comparing the measured concentrations.

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RESULTS AND DISCUSSION

Optimization of the ELSD Parameters

In ELSD, several factors have influence on the average diameter of the droplets and their distribution, which include density, viscosity, and liquid surface tension. Among these factors, the nebulizing gas flow rate affects the signal responses most significantly. When the gas flow rate is too low, large droplets are formed, resulting in spikes and random noise. However, when the gas flow rate is too high, the droplets decrease in size, which results in a decreased signal response. The optimum nebulizing gas (compressed air) pressure in the current work was determined to be 2.9 bars.

The evaporating temperature is another important parameter, which affects the signal response. At low temperature solvent evaporation is not complete, and at high temperature the detector response is decreased, owing to the decrease in particle size by improper vaporization of the nebulized analytes in the drift tube. The signal-noise ratio (S/N) was improved when the temperature was lowered to 61°C. Also, the gain in ELSD was set at 11 in order to obtain the best sensitivity.

Chromatography

Figure 2 shows a typical HPLC-ELSD chromatogram of the methanolic extract of commercial andrographis product with retention time of andrographolide at about 18.6 min, within a 40-min gradient elution. The detection limit

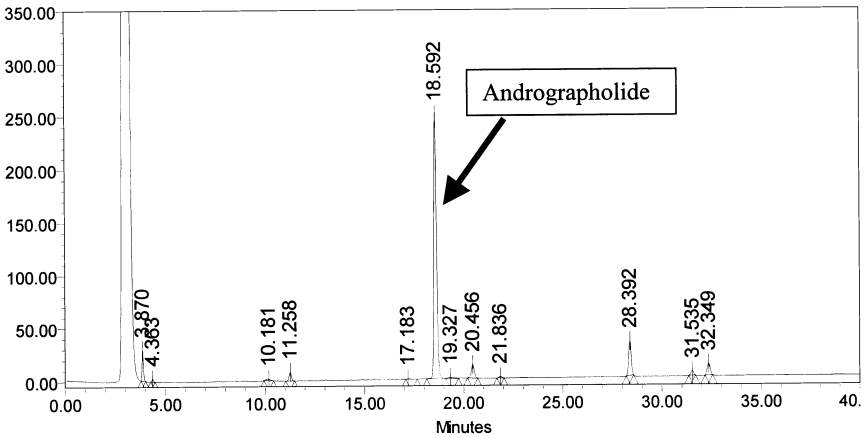

Figure 2. Typical HPLC-ELSD chromatogram of methanolic extract of a commercial *Andrographis* product.

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(S/N > 5) of the described method was observed for andrographolide at 50 ng on the column in the current assay (Figure 3).

Linearity and Reproducibility

The linearity was examined by applying the calibration working standard solutions for three consecutive days. The calibration curve, log-transformed peak area versus log-transformed concentration, was calculated according to the least squares methods ($y = a + bx$) for andrographolide tested with regression better than 0.998.

The reproducibility of the method was evaluated by analyzing a set of three controls (13.4, 33.5, 67 μg/mL, $n = 3$) on three separate days ($n = 3$), and calculating the coefficient variance (%) and relative error (%). As shown in Table 1, the coefficient variances (%) and the relative errors (%) were found to be less than 4.58 and 7.53%, respectively.

Sample Analysis

As shown in Figure 2, three commercial andrographis product methanolic extracts were analyzed in duplicate according to the method as described above. The average content of andrographolide (mg/serving ± SD) was shown in Table 2. Based on the current analysis, one of three commercial andrographis products does not contain as much of andrographolide as the manufacturer claimed.

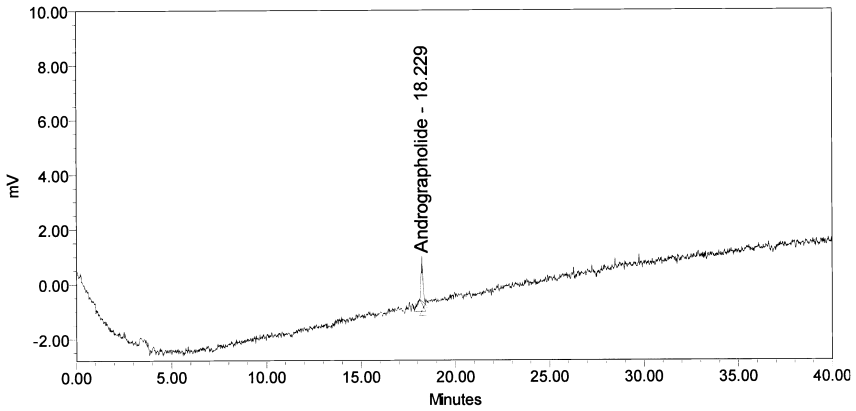

Figure 3. Typical HPLC-ELSD chromatogram of andrographolide reference standard with 50 ng on the column.

Table 2. The Content of Andrographolide in Three Commercial Andrographis Products

Sample	Amount/ Serving	Label Claim/ Serving	Andrographolide Found (mg +/− SD)/ Serving
A	300 mg leaf extract	12 mg andrographolide	9.06 +/− 0.36
B	60 mg root extract	12 mg andrographolide	13.71 +/− 0.82
C	300 mg extract	12 mg andrographolide	13.92 +/− 0.09

CONCLUSION

A high performance liquid chromatographic method has been developed for the determination of andrographolide in commercial andrographis products using an evaporative light scattering detector. With this method, andrographolide was successfully quantified, using the calibration curve with a detection limit of 50 ng on the column.

Validation of the current HPLC-ELSD method included inter-and intra-day precision and accuracy. All of the validation parameters studied were found to have coefficient variances (%) less than 7.53% and did not show a bias toward a single direction.

The current HPLC-ELSD method was found to be rapid, relatively inexpensive, straightforward, and reproducible.

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