Supercritical Fluid Extraction and Liquid Chromatography-Electrospray Mass Analysis of Vinblastine from *Catharanthus roseus*

Young Hae CHOI,[a,c] Ki-Pung YOO,[b] and Jinwoong KIM*,[c]

[a] *GreenTek21 Co., Ltd.; #10–132, Yeomri-dong, Mapo-gu, Seoul 121–870, Korea:* [b] *Department of Chemical Engineering, Sogang University; Seoul 121–742, Korea: and* [c] *College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University; Seoul 151–742, Korea.* Received May 7, 2002; accepted June 3, 2002

Supercritical fluid extraction using carbon dioxide modified with methanol, methanol–diethylamine, or methanol–triethylamine was used to extract vinblastine from the aerial portions of *Catharanthus roseus*. An HPLC-electrospray ionization (ESI)/MS analysis method was also developed to quantify the alkaloids in these extracts. Of the supercritical solvents evaluated, carbon dioxide–methanol–triethylamine (80 : 18 : 2) at 80 °C and 34.0 MPa greatly improved the supercritical fluid extraction (SFE) yield of vinblastine by as much as 76.4% over methanol extraction, while the other solvent conditions extracted the compound at yields less than 25% that of a methanol extraction. These results were confirmed by the robust HPLC-ESI/MS analytical method developed in this study.

Key words *Catharanthus roseus*; vinblastine; HPLC-electrospray ionization (ESI)/MS; supercritical fluid extraction

Catharanthus roseus (L.) G. DON contains a variety of indole alkaloids and is used as antitumor, hypotensive, and antiarrhythmic agents. Among these indole alkaloids, vinblastine (**1**) and vincristine (**2**) (Fig. 1) are currently used to treat a wide variety of neoplasms and is recommended for treatment of Hodgkin's disease, acute leukemia, and choriocarcinoma which is resistant to other types of therapy.[1,2] The unique antitumor activities of these indole alkaloids have resulted in a great demand for vinblastine (**1**) and vincristine (**2**) as anticancer agents. Therefore, several attempts haven been made to develop optimum extraction methods for these alkaloids in order to satisfy the demand. However, it is very difficult to develop a method for the extraction and analysis of these compounds because of their low content, less than 1 g/500 kg. Previously developed extraction methods involved aqueous or alcohol extraction followed by pH control and re-extraction with an organic solvent.[3—5] They involve a long and tedious procedure and the use of large quantities of toxic organic solvents. Conventional analytical methods using HPLC-UV require a pre-purification procedure in order to remove compounds which interfere with the detection of the target compounds.[6,7] Therefore, an alternative method for the extraction and analysis of vinblastine (**1**) and vincristine (**2**) from *C. roseus* would be highly desirable.

We recently developed an supercritical fluid extraction (SFE) method for the extraction of alkaloids such as cephalotaxine, ephedrine, hyoscyamine, and scopolamine from plant materials using a basic modifier, which increases the solubility and degree of desorption from the matrix.[8—10] Therefore, we applied basified supercritical CO_2 as an extraction solvent in SFE for vinblastine (**1**) and vincristine (**2**) and developed a method to analyze these alkaloids using HPLC-electrospray ionization (ESI)/MS.

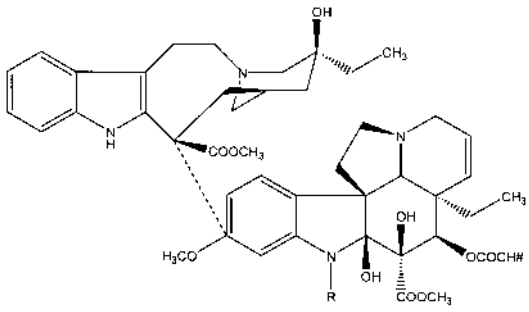
Experimental

Plant Material and Chemicals The aerial portions and roots of *Catharanthus roseus* (L.) G. DON were collected from the Medicinal Plant Garden, College of Pharmacy, Seoul National University in May, 2001. The plant materials were freeze dried for 48 h and then pulverized. HPLC grade acetonitrile, methanol, and water were purchased from Merck (Darmstadt, Germany). Ammonium acetate, vinblastine, and vincristine were purchased from Sigma (St. Louis, MO, U.S.A.).

Organic Solvent Extraction The aerial portions and roots (200 mg) were extracted with methanol (50 ml×3) and evaporated *in vacuo*. Each extract was dissolved in 1 ml methanol and filtered through a 0.45 μm PVDF membrane filter (Kenosha, WI, U.S.A.). These experiments were conducted in triplicate.

Supercritical Fluid Extraction SFE was performed using an Isco supercritical fluid extractor, model SFX 3560 equipped with two Isco 260 D syringe pumps (Lincoln, NE, U.S.A.) using CO_2 (99.9%, Seoul Gas Co. Seoul, Korea) in the pressure range of 10.2—34.0 MPa at 40, 60, and 80 °C. In each experiment, 200 mg of aerial portions of *C. roseus* was loaded into an extraction cell (57 mm×20 mm i.d., Isco). The temperature of the restrictor was maintained at 80 °C (±2 °C) and the static extraction time was 50 min. The flow rate was 1.0 ml/min through the extraction vessel during the dynamic extraction time. The total amount of CO_2 consumed was 50 ml during the dynamic extraction at each condition. For the evaluation of the effects of modifiers such as methanol, diethylamine in methanol, and triethylamine in methanol (10%, v/v), each modifier was continuously incorporated into the extraction cell at concentrations of 1, 5, 10, and 20% (v/v) through a syringe pump at 80 °C and 34.0 MPa. All extracted analytes were collected in 20 ml vials containing 10 ml of methanol. After evaporation, each extract was re-dissolved in 1 ml methanol and filtered through a 0.45 μm PVDF membrane filter (Kenosha, WI, U.S.A.). These experiments were conducted in triplicate.

HPLC-ESI/MS Analysis An Agilent 1100 series HPLC system equipped with an autosampler, a photodiode array detector, a column oven, a binary pump and a degasser (Agilent, Waldbronn, Germany) was used. Separation of the vinblastine and vincristine were performed using a Zorbax Bonus RP-18 (150 mm×2.1 mm, particle size 5 μm, Agilent) at 40 °C. The mobile phase system was a gradient of 20 mM ammonium acetate-acetonitrile (0 min, 70 : 30; 30 min, 25 : 75; 45 min, 25 : 75). The flow rate of the mobile phase was 0.2 ml/min and 5 μl of sample solution was injected via the autosampler. The analysis of the vinblastine and vincristine detection



R = CH_3, vinblastine (1)
R = CHO, vincristine (2)

Fig. 1. Chemical Structures of Vinblastine (**1**) and Vincristine (**2**)

* To whom correspondence should be addressed. e-mail: jwkim@snu.ac.kr

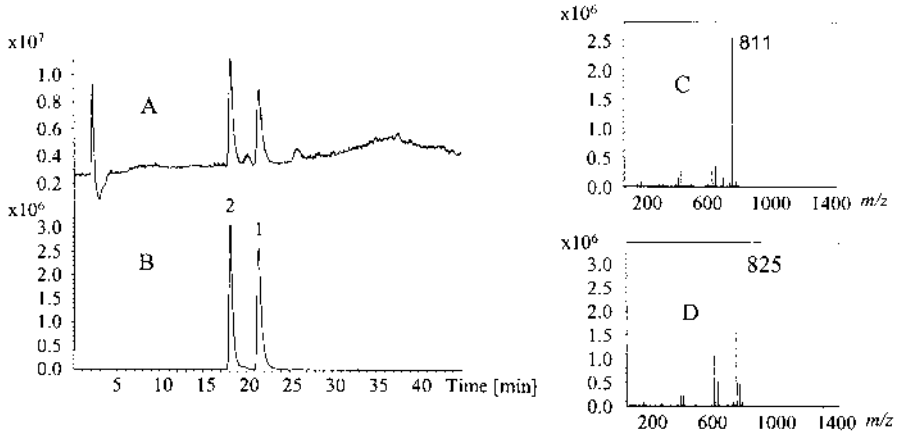


Fig. 2. Total Ion Current (TIC), and Extract Ion Current (EIC) Chromatograms of Vinblastine (**1**) and Vincristine A (**2**), and Their Mass Spectra

(A) TIC of a mixture of vinblastine (**1**) and vincristine (**2**). (B) EIC of a mixture of vinblastine (**1**) at *m/z* 811 and vincristine (**2**) at *m/z* 825. C; Mass spectrum of vinblastine (**1**). (D) Mass spectrum of vincristine (**2**).

Table 1. Yield (μg/g±S.D.)[a] of Vinblastine (**1**) and Vincristine (**2**) from the Aerial Portions and Roots of *Catharanthus roseus* by Methanol Extraction*

Sample	Vinblastine (**1**)	Vincristine (**2**)
Aerial parts	46.60±2.11	Trace amount[a]
Roots	6.30±1.43	Trace amount[a]

* All experiments based on triplicate. *a*) Content of the compound was less than 1 μg/g.

were performed using an Agilent 1100 LC/MSD ion trap mass spectrometer (Agilent, Waldbronn, Germany) equipped with an electron spray ionization interface. Nitrogen was used as a nebulizing gas at a pressure of 20 psi at 5 l/min, temperature at 350 °C and capillary voltage −1.1 kV. HPLC-ESI/MS analyses were carried out in the positive ion mode with the scan range *m/z* 100—1500.

Results and Discussion

We found that a better sensitivity of vinblastine (**1**) and vincristine (**2**) could be achieved in ESI/MS using the positive ion mode compared to a UV detector (254 or 298 nm). Using this method, it was possible to detect levels as low as 1.0 ng of these alkaloids with standard samples.

The ESI/MS spectra of vinblastine (**1**) and vincristine (**2**) in the positive ion mode both displayed $[M+H]^+$ at *m/z* 811 and 825, respectively, as a base peak as shown in Fig. 2. Therefore, MS detection was operated in the positive ion mode in the scan range *m/z* 100 to 1500, and the analysis of these indole alkaloids was performed using the extract ion current (EIC) mode targeted at *m/z* 811 and 825. Concentrations of the standard solution used in this experiment were in the range 0.4—30 μg/ml, and the linear correlation coefficient (γ^2) of vinblastine (**1**) and vincristine (**2**) were determined to be 0.9965 and 0.9951, respectively. Based on this calibration curve, the amount of these alkaloids in *C. roseus* were calculated (Table 1). The content of vinblastine (**1**) is 7 times higher in the aerial portions than the roots, but the vincristine (**2**) was found in trace amounts (<1 μg/g in both plant parts). As shown in Fig. 3, the EIC chromatogram of the extract allowed the separation of these alkaloids without the need for any purification step which is not obtainable by conventional analytical methods. Thus, the HPLC-ESI/MS

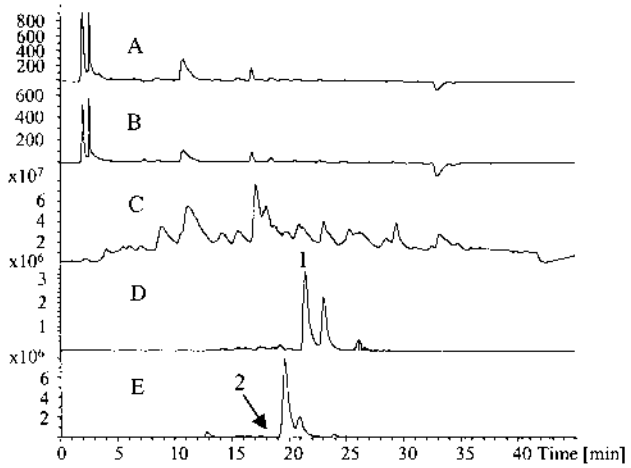


Fig. 3. UV (254, 298 nm), TIC, and EIC Chromatograms of the Aerial Parts of *C. roseus* Obtained by Methanol Extraction

A, UV (254 nm) chromatogram of the extract; B, UV (298 nm) chromatogram of the extract; C, TIC of the extract; D, EIC of the extract at *m/z* 811, E; EIC of the extract at *m/z* 825.

developed in this study is a simple and rapid analytical method with no need for a clean-up step and represents a promising analytical method for the detection of specific compounds from a complex plant matrix.

Our previous reports showed that a small addition of a basic modifier could dramatically enhanced the SFE yields of some alkaloids such as cephalotaxine, ephedrine, hyoscyamine and scopolamine.[8—10] This can be attributed to the fact that salts of alkaloids in plant tissues might be converted to free bases by the basic modifiers. This change can enhance the solubility of a compound in carbon dioxide as well as the degree of desorption from the plant matrix. As expected, an increase in temperature and pressure had no influence on the SFE efficiency of vinblastine (**1**) because of its high polarity when pure carbon dioxide was used as a solvent. Moreover, the addition of methanol did not significantly improve the SFE yields. However, the addition of basified modifier such as diethylamine or triethylamine to the mixture of carbon dioxide and methanol resulted in a great increase in the yield of vinblastine (**1**). A mixture of carbon dioxide–

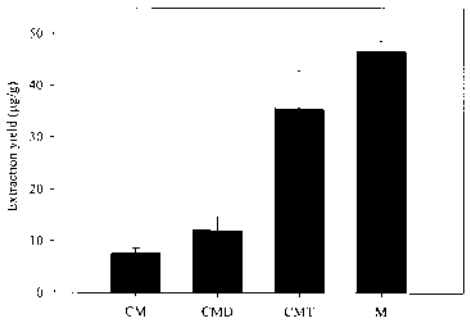


Fig. 4. Extraction Yield of Vinblastine (**1**) from the Extract of the Aerial Portions of *C. roseus* Using Carbon Dioxide–Methanol (80 : 20) at 80 °C and 34.0 MPa (CM), Carbon Dioxide–Methanol–Diethylamine (80 : 18 : 2) at 80 °C and 34.0 MPa (CMD), Carbon Dioxide–Methanol–Triethylamine (80 : 18 : 2) at 80 °C, 34.0 MPa (CMT), and Methanol (M)

methanol–triethylamine (80 : 18 : 2) at 80 °C and 34.0 MPa was especially effective in extracting this compound by as much as 76.6% compared to methanol extraction, while the other supercritical solvents evaluated in this experiment extracted less than 25% of that obtained by methanol extraction. On the basis of these results, SFE using basic modifier such as triethylamine developed in this study appear to be an alternative extraction method for vinblastine (**1**) compared to conventional organic solvent extraction.

References

1) Tyler V. E., Brady L. R., Robbers J. E., "Pharmacognosy," Lea & Febiger, Philadelphia, 1981, pp. 237—239.
2) Lu M. C., "Cancer Antimitotic Agents," ed. by Foye W. O., American Chemical Society, Washington DC, 1995, pp. 345—368.
3) Tillequin F., Michel S., Sequin E., "Methods in Plant Biochemistry," Vol. 8, ed. by Waterman P. G., Academic Press, San Diego, 1995, pp. 309—372.
4) Monforte-Gonzalez M., Ayora-Talavera T., Maldonado-Mendoza I. E., Loyola-Vargas V. M., *Phytochem. Anal.*, **3**, 117—121 (1992).
5) Renaudin J. P., *Physiol. Veg.*, **23**, 281—388 (1985).
6) Verzele M., De Taeye L., Van Dyck J., De Decker G., *J. Chromatogr.*, **214**, 93—99 (1981).
7) Miura Y., Hirata K., Kurano N., Miyamoto K., Uchida K., *Planta Med.*, **54**, 18—20 (1988).
8) Choi Y. H., Kim J., Kim J. Y., Joung S. N., Yoo K. P., Chang Y. S., *Arch. Pharm. Res.*, **23**, 163—166 (2000).
9) Choi Y. H., Kim Y., Kim J., Yoo K. P., *Chromatographia*, **50**, 673—679 (1999).
10) Choi Y. H., Chin Y. W., Kin J., Jeon S. H., Yoo K. P., *J. Chromatogr. A*, **863**, 47—55 (1999).