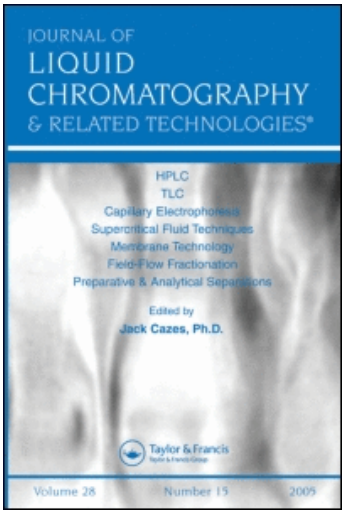

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Determination of Vincristine and Vinblastine in *Catharanthus roseus* Plants by High Performance Liquid Chromatography/ Electrospray Ionization Mass Spectrometry

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DETERMINATION OF VINCRISTINE AND VINBLASTINE IN *CATHARANTHUS roseus* PLANTS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY/ ELECTROSPRAY IONIZATION MASS SPECTROMETRY

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ABSTRACT

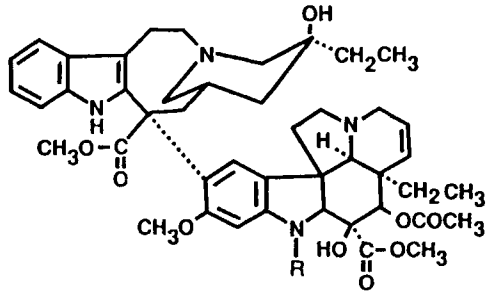
A reversed phase high performance liquid chromatographic/ mass spectrometric (LC-MS) method with an electrospray ion source was used in this study to determine the concentration of two dimeric alkaloids, vinblastine and vincristine, in near-isogenic lines of *Catharanthus* leaves. A linear gradient of 25 mM ammonium acetate in methanol (solution A) and the same concentration in water (solution B) served as the mobile phase. A C_{18} column was used to separate those two compounds from other components in the extracted plant samples. Mass spectra

generated by the electrospray ionization source were dominated by protonated molecules with little or no fragmentation being observed under the experimental conditions applied. Since very little fragmentation occurred, the method exhibited enhanced sensitivity over other LC-MS techniques. Product-ion mass spectra of vincristine and vinblastine were produced in a single quadrupole mass spectrometer through collision-induced dissociation (CID) between the capillary exit and first skimmer of the electrospray source. CID spectra can be used to unequivocally differentiate between vincristine, vinblastine and other alkaloids.

INTRODUCTION

Vincristine (VC) and vinblastine (VB) (Figure 1) are subjects of many biological and pharmaceutical studies, primarily because they exhibit anti-tumor activity. [1-3] Several high performance liquid chromatographic (HPLC) methods have been developed in the past few years[1,4-8] including some very promising HPLC/mass spectrometry (LC-MS) studies that utilized thermospray ionization.[9] Mass spectrometry as a detector offers several advantages over other detection devices for HPLC. These include; (1) molecular weight and/or fragmentation information about the compounds which allow structural elucidation and identification of unknown compounds, (2) the capability for further separation of ions with different mass-to-charge ratios for HPLC peaks that are not very well resolved, and (3) less sample preparation (e.g., destructive derivatization procedures) is required which reduces the possibility for artifacts.

In this study, a LC interfaced to an electrospray ionization (ESI) source was used to quantify by mass spectrometry the amount of VC and VB in near-isogenic lines of *Catharanthus* leaves. Electrospray ionization is recognized as one of the most versatile ionization techniques.[10,11] It is a soft ionization technique, therefore, most ions produced are protonated or otherwise cationized molecules for low molecular weight compounds (in the positive ion mode) or multiply charged ions for large molecules. Thus identification of analytes can be immediately confirmed, by comparison of elution time and the molecular weight. More informative collision-induced dissociation (CID) spectra can be generated, if needed, by electrospray ionization[12] in a single quadrupole mass spectrometer which provides characteristic product ion spectra for structure analysis. A comparison between HPLC-UV and LC-MS (using the full scan mode) was made in this report and the overall sensitivity of the two techniques is discussed.



R = CH_3 (VB)

R = CHO (VC)

Figure 1. Chemical structures of two dimeric indole alkaloids. (A) vinblastine and (B) vincristine.

EXPERIMENTAL

Reagents and Materials

Plant samples were obtained from Goldsmith Seeds, Inc. (Gilroy, CA). Vincristine sulfate, vinblastine sulfate and ammonium acetate were purchased from Aldrich Chemical Co. (Milwaukee, WI). Methanol (HPLC grade) and cyclohexane (spectroscopy grade) were purchased from Baxter Scientific Co. (McGaw Park, IL) and water (HPLC grade) was obtained from Burdick & Jackson Laboratories, Inc. (Muskegon, MI). Glacial acetic acid was obtained from Fisher Scientific Co. (Fair Lawn, NJ).

Glass fiber Acrodisc, 25 mm (Gelman Science, Ann Arbor, MI) and glass fiber syringe filters, 13 mm (Whatman Lab Division, Clifton, NJ) were used for filtration. All chemicals were used without further purification.

Standard Preparation

A 1.0 milligram sample of vincristine sulfate or vinblastine sulfate each was placed into a 25 mL volumetric flask, dissolved and diluted to volume with 0.01 M aqueous acetic acid. The standard solution was sonicated for 1 min.

Analytical standards were prepared by diluting the stock standard solution with 0.01 M acetic acid using an electronic digital pipette (Rainin, Woburn, MA) to give concentrations of 0.92, 2.29, 4.58, 9.17 and 27.51 ppm and 0.92, 2.31, 4.63, 9.27 and 27.80 ppm for VB and VC, respectively.

Sample Preparation

Ground *Catharanthus roseus* leaf samples (ca.0.6g) were accurately weighed into glass scintillation vials. Isopropyl alcohol (4 mL) was added and the vials shaken on a wrist action shaker for 15 min. The extracts were filtered through 25 mm glass fiber Acrodiscs into autosampler vials, taken to dryness under a stream of nitrogen, and reconstituted in 1 mL of 0.01 M acetic acid. The acid solutions were partitioned 3 times with 1 mL of cyclohexane. The cyclohexane solutions were discarded and the acid fraction was taken to dryness under a stream of nitrogen. The samples were finally reconstituted in 200 μL of 0.01 M acetic acid and sonicated for 1 min prior to a final filtration through 13 mm glass fiber syringe filters. The sample preparation should be accomplished as fast as possible, since a reduction in VB concentration was observed as a result of prolonged extraction process.

High Performance Liquid Chromatography

HPLC separations of extracted plant samples were performed on a Water's 600-MS system (Waters, Milford, MA) equipped with a Water's 484 tunable absorbance detector at 254 nm. The column used in this study was a 200 x 4.6 mm with 5 μm HP ODS hypersil (Hewlett-Packard, Palo Alto, CA).

During routine analysis, the flow rate was held at 1 mL/min and split with 0.02 in. i.d. PEEK tubing (Supelco, Bellefonte, PA) to the electrospray source and UV detector. Due to the small i.d. of the electrospray needle, the actual flow rates as determined by measuring the weight of mobile phase collected at the outlet of UV detector over a specified time period, were 400 μL/min and 600 μL/min to the electrospray source and UV detector, respectively.

A linear gradient mobile phase was used for the separation of extracted plant samples. Solution A was 25 mM ammonium acetate in HPLC grade MeOH and solution B contained the same concentration of ammonium acetate in HPLC grade water. Both ammonium acetate solutions were prepared daily and sonicated for 10 min prior to use. Although sodium phosphate is reportedly a better reagent[13] for alkaloid analysis, ammonium acetate was selected because of its volatility and compatibility with electrospray mass

spectrometry. A 20 μL aliquot of each sample was injected onto the HPLC column with a 25 μL syringe. Gradients over a 15 min period were used to separate VC and VB from other components in the plant sample. The HPLC mobile phase ratio was held at 60% A:40% B for 10 min, then the percentage of A was increased to 70% in 15 min and held for another 10 min. Most of the major components in the plant samples studied eluted from the column within 35 min.

Electrospray Ionization Mass Spectrometry

The LC-MS interface was a dual stage electrospray ion source purchased from Analytica of Branford, Inc., (Branford, CT) and recently upgraded to accommodate high flow rates. A HP5988A quadruple mass spectrometer (Palo Alto, CA) operating in the full scan mode with a scan range of 200-900 amu at 0.1 s/scan was used in all experiments. The electrospray needle was maintained at ground potential and the platinum-coated glass capillary at -4.6 kV. Nitrogen served both as drying gas (at 200°C) and sheath gas.

Ions were accelerated from the electrospray ion source into the mass spectrometer and focused through three electrical lenses and two skimmers. Applied voltages for those electrical elements were optimized while constantly infusing standard solution into the electrospray source. The optimum entrance lens potential (EL) for this experiment was determined by monitoring the ion signal of direct flow injection of a mixture of VC and VB. The areas of VC and VB peaks were plotted against EL to determine the optimum capillary exit potential for this investigation. In some cases where structural information was desired, CID was carried out in the high pressure region between the capillary exit and the skimmer entrance to the mass analyzer by increasing the EL potential to a higher value (250 V in most cases).

Calibration Curves

Five-point calibration curves were constructed for VC and VB to determine the relationship between peak area and the concentration of samples. Standard solutions were prepared in 0.01 M acetic acid ranging from 0.9 to 27.5 ppm and 0.9 to 27.8 ppm for VC and VB, respectively. Calibration curves were generated by plotting the ion intensities versus the standard concentrations used. Each standard was analyzed, in triplicate, on two different days and the average of both sets of triplicate analyses was taken as the ion intensities of standards. The day-to-day relative standard deviations (%RSD) were measured by comparing the ion intensities of VB standard

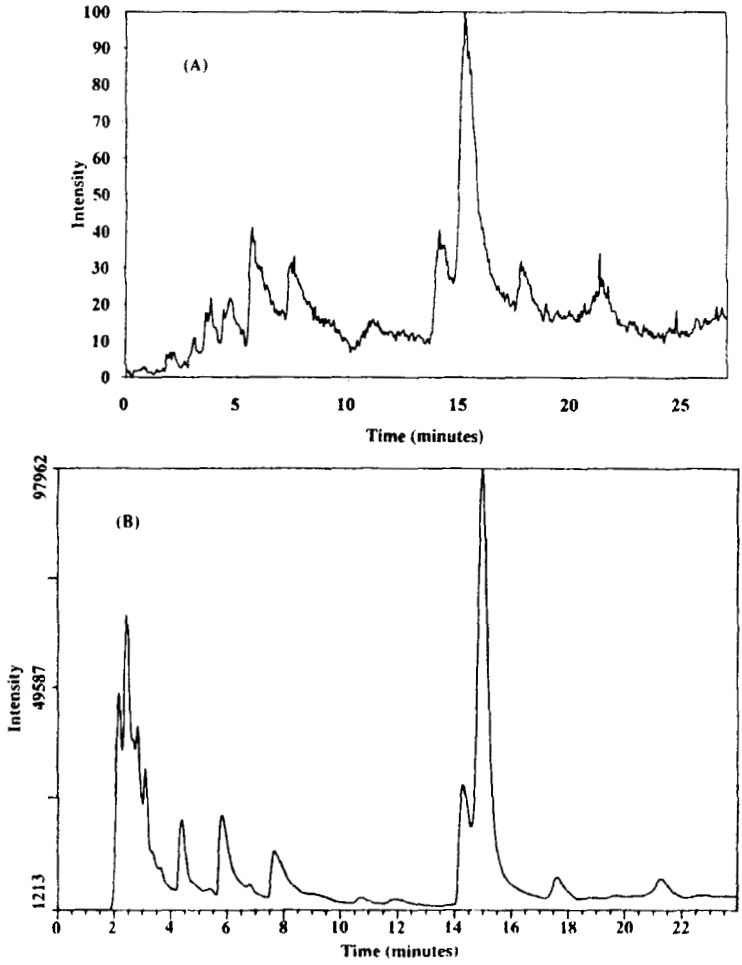


Figure 2. Chromatograms of extracted plant samples obtained by (A) LC-MS (shown as total ion chromatogram) and (B) HPLC-UV. Samples prepared in 0.1 M aqueous acetic acid. Elution times for vincristine and vinblastine are 14.8 and 21.3 min, respectively.

solutions obtained on three different days. The %RSD was approximately 10% for all the samples analyzed. The same set of ion intensity data was re-analyzed on a new calibration curve created after two weeks and similar %RSD were obtained by comparsion of the data with those measured earlier.

Table 1

Slopes and Linear Regression Data Obtained for VB and VC Calibration Curves by UV and LC-MS

	LC-MS		UV	
	Slope	**R^2**	**Slope**	**R^2**
VB	234.7	0.9839	24852	0.9998
VC	188.17	0.9995	37663	0.9987

Recoveries were determined by spiking plant samples with both vincristine and vinblastine, followed by extraction with the same procedure as the samples and comparison with authentic standards prepared in the acetic acid solution. The percent recovery ranged from 90-100% for both compounds.

RESULTS

The LC-MS total-ion-chromatograms (TIC) and the HPLC-UV absorbance chromatogram obtained for all the samples studied (e.g. Figure 2) were similar. The relatively high background in the TIC compared to that of the UV absorbance chromatogram, is the result of ammonium acetate cluster ions. VC, which has one more polar group (CHO) than VB (Figure 1) eluted earlier in the reversed phase HPLC system as expected. No indications of interference from either the sample matrix or mobile phases were observed, since the retention times (RT) and mass spectra of VC (RT = 14.8 min, $[MH]^+$ = 825.8) and VB (RT = 21.3 min, $[MH]^+$ = 811.5) in the spiked plant samples were essentially the same as that of the authentic standard compounds.

Linear regression parameters for the calibration curves of VC and VB measured by both UV and LC-MS are shown in Table 1. Six sample sets were analyzed by HPLC-UV and LC-MS to compare the two techniques. The results (Table 2) showed no appreciable differences between concentrations obtained by the two methods in any of the six samples investigated. Also, detection limits between these two detection methods were comparable. However, at lower sample concentration (especially at concentration <0.01 ppm), the detection limit of the UV suffered considerably from interferences of higher concentration components eluting in close proximity to VC and VB. Since little was known about the actual concentrations of these two dimeric alkaloids in the plant samples, we believed that mass spectrometry would be a more

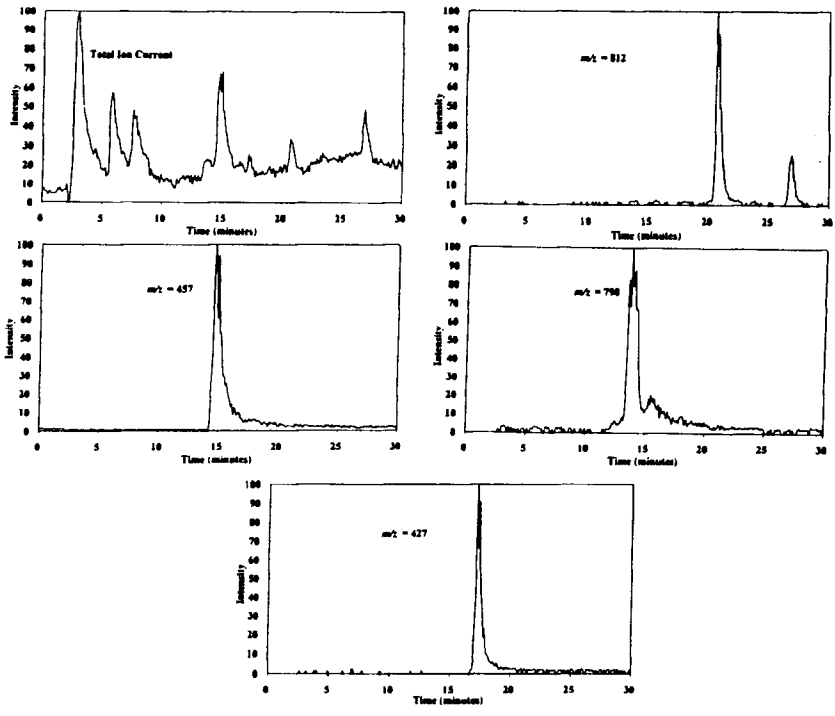


Figure 3. Selected ion chromatograms of protonated molecules for other compounds eluting from the column at close proximity to vinblastine and vincristine.

Table 2

Comparison of UV and LC-MS Concentrations (μg/g) for VB in Six Plant Samples (Mean Conc. ± S.D.)

Sample #	1	2	3	4	5	6
UV	5.9 ± 1.3	3.3 ± 0.2	9.4 ± 1.3	3.9 ± 0.3	4.4 ± 0.3	2.4 ± 0.1
LC-MS	7.0 ± 1.1	3.3 ± 0.3	8.1 ± 0.7	4.2 ± 0.1	4.8 ± 0.1	2.4 ± 0.1

reliable quantitative tool for our purpose. In addition, ions of different *m/z* can be distinguished by plotting selected ion chromatograms (Figure 3) or by selective ion monitoring (SIM), which will both greatly increase the detection limits of LC-MS. For this reason, LC-MS was selected for determination of VC and VB in all the plant samples investigated.

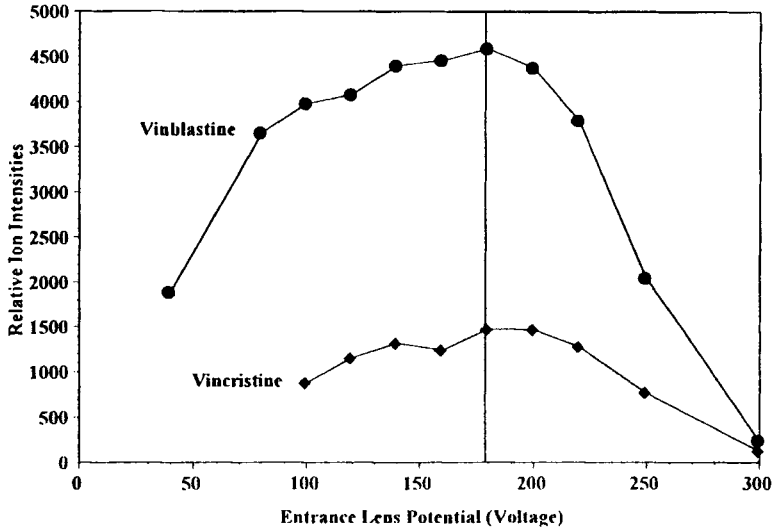


Figure 4. Plot of ion intensities vs. capillary exit potential for vinblastine and vincristine.

The optimum EL potentials for both VB and VC were determined to be approximately 180 V as shown in Figure 4. A higher EL value (250V) was used to provide more information-rich product-ion spectra for some samples which exhibited mass spectra similar to the alkaloids of interest.

DISCUSSION

The success in introducing a high gas flow coaxially around the emerging liquid stream has greatly increased the flow rate feasible for electrospray.[14] Flow rates as high as 1 mL/min are routinely employed in our laboratory so that conventional HPLC conditions can be applied to LC-MS without modification. Similarities between HPLC chromatograms obtained from LC-MS and UV (Figure 2) make direct comparison between these two techniques rather easy. Although the relative peak height is detection-principle-dependent, identification of unknown HPLC-UV peaks can be readily made by examination of the peak eluting at the same retention time in the total ion chromatogram of the LC-MS. This approach allows a more accurate selection of standard compounds than those based solely on retention time.

Similar detection limits were observed between the UV detector and the mass spectrometer for the molecules being studied. Since mass spectrometry has the potential to further distinguish eluted compounds by selecting mass chromatograms, peaks can be presented without background interference from solvent or other molecules (Figure 3). Therefore, more reliable quantitative results can be obtained.

Since very little heating is involved in the electrospray ionization process, most ions produced are protonated molecules (positive ion mode), which is one of the advantages of electrospray ionization over other ionization techniques. For clarification, a comparison between the mass spectra of a VC standard analyzed by both electron-impact ionization (EI) and ESI is presented in Figure 5. As can be seen, two series of small fragments which represent loss of *m/z* 13-14 were observed at *m/z* <149 in the EI spectrum. This could be the consecutive loss of CH or CH_2 groups from one of the monomers of the main VC skeleton. No molecular ion and no significant ions above *m/z* 200 were detected. On the other hand, in the LC-MS spectrum, the protonated molecular ion is the dominant species. Thermospray ionization, which is also a soft ionization technique, produced fairly extensive fragmentation for VB,[13] while essentially no fragmentation of this compound occurred in ESI-MS (Figure 6A). Also, some adduct-ion peaks were observed which resulted from either incomplete desolvation or the addition of alkali-metal ions in the electrospray ion source.

Since relative ion intensities are inversely proportional to the degree of fragmentation; the detection limit of electrospray ionization for these dimeric alkaloids is likely to be lower for LC-MS than that of thermospray techniques. However, no direct comparison was made. The capability for generating predominantly molecular ions by LC-MS allowed us to separate molecules which have similar proton affinities but differ in molecular weights in a complex mixture. This was accomplished by simply examining their protonated molecules without further time-consuming HPLC separations.

As previously mentioned, CID for the compounds under investigation, can be generated between the capillary exit and skimmer entrance of the ESI source. The effect of EL on the molecular ion intensities was investigated and the results are shown in Figure 4. Both VB $[M+H]^+$ (*m/z* 811) and VC $[M+H]^+$ (*m z* 825) ion intensities start low at EL = 40 V, reach a maximum at EL of ~180 V and then decreased dramatically at EL > 220V. The initial increase in the ion signals as EL increases is probably due to the fact that the ions are gaining sufficient energy for more efficient transfer to the mass analyzer by increasing the potential difference between capillary exit and the skimmer entrance. When this potential difference exceeds a certain value (in this case,

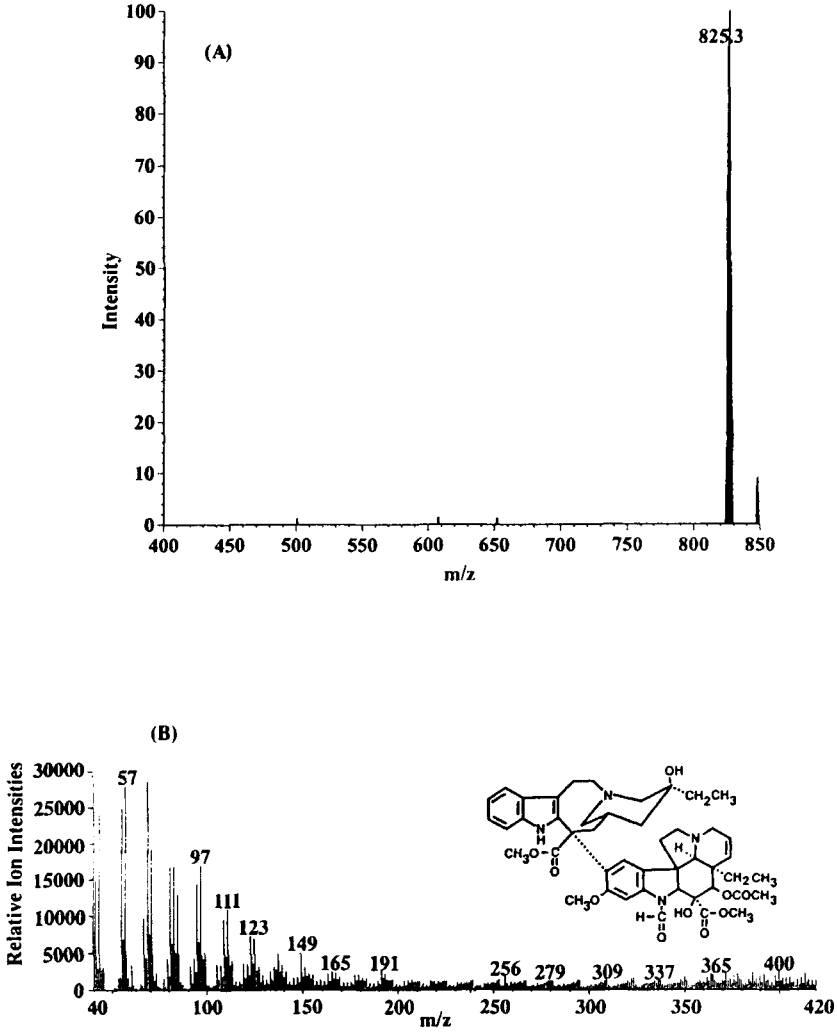


Figure 5. Mass spectra of vincristine by (A) electrospray and (B) electron-impact ionization mass spectrometry. Electrospray conditions: drying gas (N_2) at 200°C and capillary exit potential at 180 V.

EL >180V), high-voltage collision-induced dissociation (HV-CID) will occur (Figure 4) and the relative ion intensity of $[M+H]^+$ decreases, with an onset of fragment ions. Structural information can be obtained through HV-CID. For example, the mass spectrum of VB obtained at EL = 250V (Figure 6B) shows

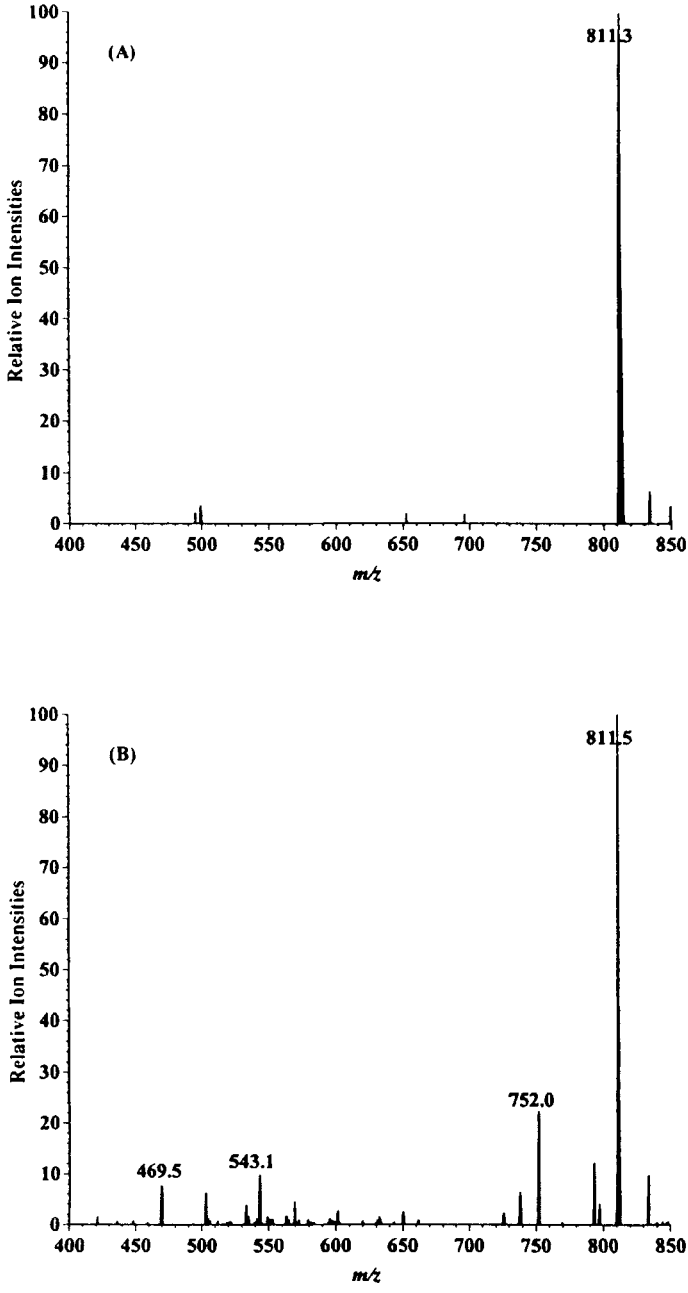


Figure 6. Electrospray mass spectrum of vinblastine at (A) EL = 180 V and (B) EL = 250 V.

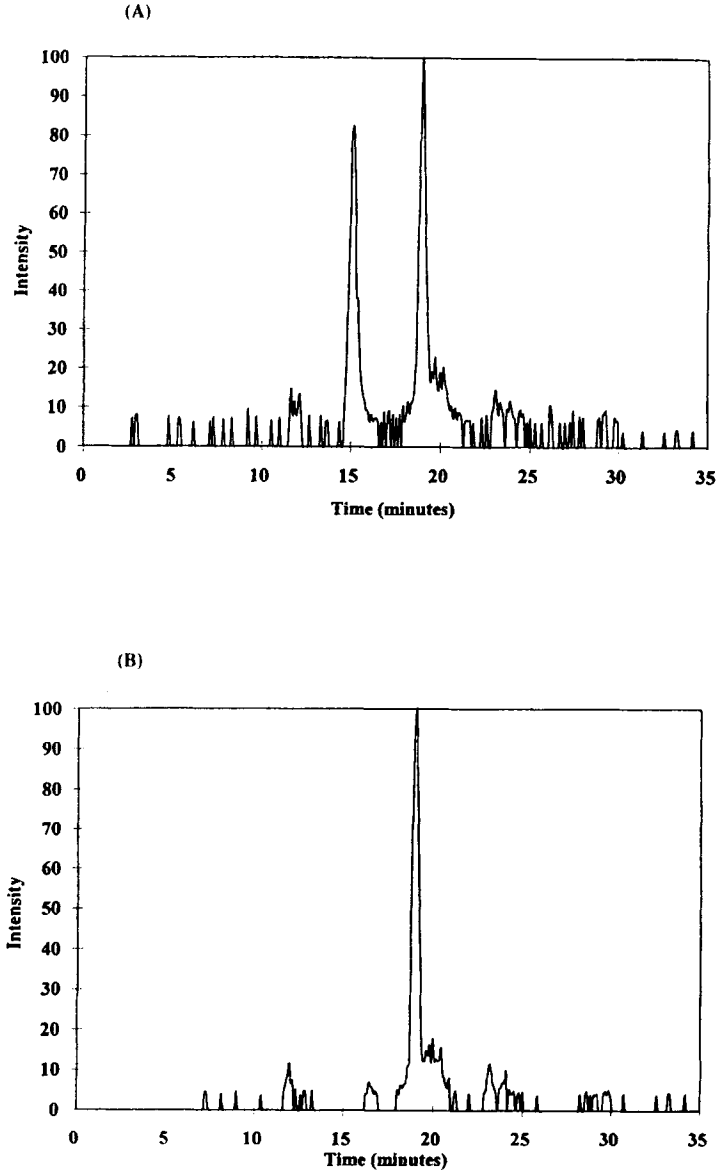


Figure 7. LC-MS ion chromatograms of m/z = 825 (A) plant extract sample spiked with vincristine and (B) unspiked plant extract sample.

fragment ions corresponding to the loss of H_2O (*m/z* 793) and $C_2H_3O_2$ (*m/z* 752) from the parent ion as were observed previously by desorption CI mass spectrometry.[16]

In some of the LC-MS chromatograms of extracts, a peak of *m/z* 825 eluted at a RT approx. 4.9 min. later than the standard VC peak (RT = 14.8 min.). In order to determine the nature of this peak, two approaches were taken. First, the samples were spiked with VC to determine if matrix effect might contribute to a change in RT for this compound. As shown in Figure 7A, a peak eluted at the same RT as the authentic VC standard in the spiked solution with $[M+H]^+$ of *m/z* 825. This indicated that the peak which eluted at RT = 19.7 (Figure 7B)is a different alkaloid than VC, possibly the product-ion of *m/z* 847, (depending on whether the ion at *m/z* 847 is a protonated molecular ion or a sodium adduct $[M+Na]^+$ ion. Next, we analyzed the spiked sample again with an EL of 250 V instead of 180 V to produce CID. The product-ion spectra of the two compounds were distinctly different. The VC standard, like VB, showed a loss of water (*m/z* 807) and $C_2H_3O_2$ (*m/z* 766) from the parent ion. It also had fragment ions at *m/z's* 798 and 780 which represent the $[M+H]^+$ - CHO and $[M+H]^+$ - CHO - H_2O. The other alkaloid (compound eluting later), had a major peak at *m/z* 790 in the product ion spectrum which was not detected in the VC standard. Since the goal of this study was to determine the concentration of VC and VB in near-isogenic lines of plant samples, identification of other possible alkaloids, which is the subject of continuing effort, will not be presented here.

SUMMARY

In this study, the potential of using an HPLC/ESI-MS technique for the analysis of weakly bonded dimeric alkaloids such as vincristine and vinblastine was investigated. These two structurally similar dimeric alkaloids were successfully separated on a C_{18} column from other possible alkaloids in the plant samples by gradient elution with 25 mM ammonium acetate in MeOH and water. Protonated molecules were always the dominant species produced in the electrospray ion source even though more informative spectra could be obtained by collision-induced-dissociation of the molecular ion.

The actual detection limit of this technique is dependent on the nature of the compound (e.g. ionization potential, proton affinity, etc.), but because of the "softness" of the electrospray ionization, the sensitivity of this technique is believed to be better than other LC-MS techniques.

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