Simultaneous Determination of Vincristine, Vinblastine, Catharanthine, and Vindoline in Leaves of *Catharanthus roseus* by High-Performance Liquid Chromatography

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Abstract

A simple reversed-phase liquid chromatographic method is developed for the simultaneous quantitation of the anticancerous drugs vincristine, vinblastine, and their precursors catharanthine and vindoline using a Merck Chromolith Performance reversed-phase high-performance liquid chromatography column. A better resolution is obtained in comparison with available particulate-type C_{18} columns. The column provides good reproducibility and peak symmetry. Chromatography is carried isocratically with a mobile phase of acetonitrile–0.1M phosphate buffer containing 0.5% glacial acetic acid (21:79, v/v; pH 3.5) at a flow rate of 1.2 mL/min and UV detection at 254 nm. Parameters such as linearity, limits of quantitation (LOQ) and detection (LOD), precision, accuracy, recovery, and robustness are studied. The method is selective and linear for alkaloid concentration in the range 0.25 µg–25 µg/mL. The LOQ and LOD are 25, 46, 56, and 32 µg/mL and 8, 14, 18, and 10 µg/mL, respectively. The results of accuracy studies are good. Values for coefficient of variation are 2.50, 1.82, 1.33, and 1.13, respectively. The percent recovery of the alkaloids was found to be 96%, 97%, 98%, and 98%, respectively. Peak purity and homogeneity of these compounds in plant extract is studied using a photodiode-array detector. This simple and rapid method of analysis is applied for the determination of these alkaloids in a large number of leaf extracts of *Catharanthus roseus*.

Introduction

Catharanthus roseus (L.) G. Don (Family *Apocyanaceae*), known in trade as Vinca, is a pantropical species occurring chiefly in the West Indies and Madagascar and is extensively cultivated in many states of India. The plant is known to produce more than 200 important compounds (mainly alkaloids). Vinca alkaloids, mainly vincristine (**1**) and vinblastine (**2**) (Figure 1), have extensive use in modern medicine as potential anticancer compounds (1). Other important alkaloids such as catharanthine (**3**) and vindoline (**4**) (Figure 1) are precursors of the vinblastine and vincristine group of alkaloids (1,2). As the two compounds (**1** and **2**) are present in minor quantities in plant leaves, an accurate and rapid method of analysis with reproducible results is required. The major constraint is the lack of a sensitive and accurate analytical procedure because of the inherent complexity of the chemicals in the plant extract. The available high-performance liquid chromatography (HPLC) methods, based on the particulate nature of classical silica gel columns, have been reviewed (3,4). Quantitation of the previously mentioned alkaloids in leaf extract remains a question because of poor resolution and elution of some impurities together with these compounds.

In this paper, an improved analytical procedure for the simultaneous quantitation of the four alkaloids using a Chromolith Performance HPLC column is reported. The analysis provides a better resolution of alkaloids **1**–**4** in comparison to the earlier analytical procedure (3) on a particulate-type C_{18} silica gel column. The resultant back pressure, even at high flow and high polar mobile phase, was very low, which will support the protection of the HPLC pump, column, etc. Other available C_{18} HPLC columns have been made of particulate materials, usually silica. By their very nature, small particles, when packed tightly into HPLC steel columns, create a significant resistance to the mobile phase flow, resulting in a high back pressure that may effect the life of the HPLC pump, its seals, and the column itself. The presently used column, a combination of macropores, allows rapid transit of the eluent and has mesopores to create a large surface area. Owing to the very high porosity of the used column, very high flow rates can be applied with very low back pressure.

The use of the Chromolith column for *Catharanthus* alkaloids analysis provides symmetric peaks with better resolution and reproducible results. The reproducibility of the results was excellent even after the analysis of more than 300 samples on the same column. A photodiode array (PDA) detector has

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been used to check the purity of these alkaloids in a sample HPLC run. Similarity of studied alkaloids with standard alkaloids was satisfactory. The overall PDA results were quite satisfactory and the method is suitable for mass screening of *C. roseus* plants for crop improvement and plant genomics studies. In addition, validation parameters of analysis were also studied. This report is in a series of efforts towards developing liquid chromatographic procedures (5–11) for plant drug analysis.

Experimental

Plant material and sample preparation

The plant materials of *C. roseus* were obtained from the experimental farm of the Institute at Lucknow (Lucknow, India). Extracts of the leaves were prepared as reported earlier (3). Briefly, powdered leaf (5 g) was extracted thrice with 90% ethanol (3 × 30 mL, 12 h each time) at room temperature. The alcohol extract was filtered, concentrated in vacuo to 10 mL, diluted with water (10 mL), acidified with 3% HCl (10 mL), and washed with hexane (3 × 30 mL). The aqueous portion was basified with ammonia to pH 8.5 and extracted using chloroform (3 × 30 mL); the chloroform extract was washed with water, dried over sodium sulphate, and concentrated under vacuum. The residue was redissolved in 10 mL methanol.

Chemicals

Vincristine and vinblastine were purchased from Sigma (St. Louis, MO). Vindoline and Catharanthine were generously provided by Prof. P. Potier (National Center for Scientific Research Yvette, Cedex, France). The solvents used were of HPLC grade (Merck, Darmstadt, Germany). Sodium dihydrogen orthophosphate was obtained from Glaxo (Mumbai, India). Double distilled water was used after filtering through a 0.45-µm filter before use.

Chromatographic instrument and conditions

A Shimadzu (Kyoto, Japan) LC-8A gradient HPLC equipped with two LC-8A pumps, controlled by a CBM-10A interface module, 7725 I manual injector valve (Rheodyne, Cotati, CA), has been used in the HPLC analysis. SPD-M10Avp PDA detector (Shimadzu) was used for the test of peak purity and similarity of the alkaloids. Solvents were prefiltered before use by a Millipore filtration unit (Millipore, Billerica, MA). An RP-18e reversed-phase Chromolith Performance HPLC column (100- × 4.6-mm i.d.) was used for all the analysis. A constant flow rate of 1.2 mL/min was used during analysis. An optimum mobile phase composition was achieved by using different compositions of acetonitrile–0.1M phosphate buffer containing 0.5% glacial acetic acid. The final composition was optimized as 21:79 (v/v) acetonitrile–0.1M phosphate buffer containing 0.5% glacial acetic acid. The other conditions were pH 3.5, flow rate of 1.2 mL/min, and detector wavelength at 254 nm.

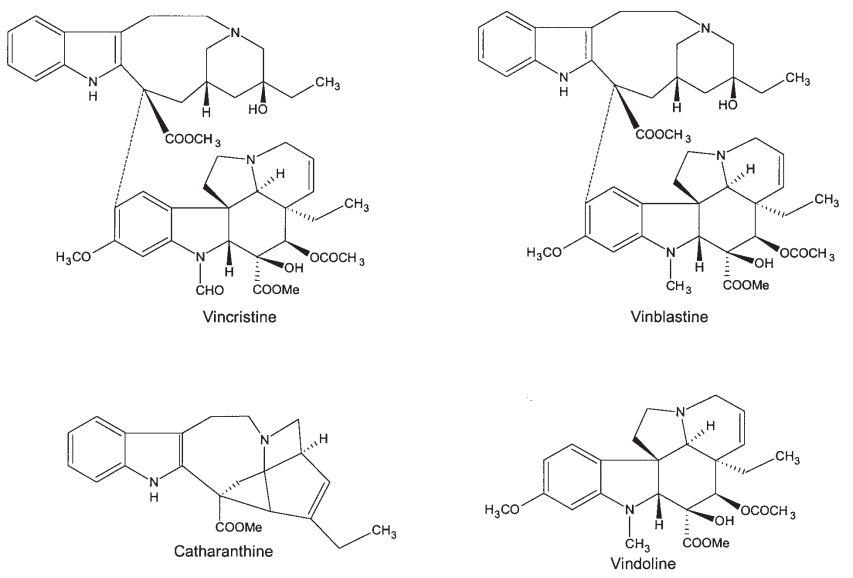

Figure 1. Chemical structures of the alkaloids vincristine, vinblastine, catharanthine, and vindoline.

Table I. RP-18e Chromolith Column Performance in the Separation of *Catharanthus* Alkaloids (1–4) from the Extract of *Catharanthus roseus*

Alkaloid	t_R*	No. of theoretical plates	Recovery (%)	Separation factor	Linear regression equation†	Correlation coefficient (r)
1	16.57	3323	96	1.13	$y = 103.14x - 17.38$	0.999
2	26.93	3247	97	1.68	$y = 171.06x - 15.95$	1.000
3	6.81	2359	98	1.09	$y = 135.04x - 24.71$	0.998
4	13.22	1941	98	1.14	$y = 126.20x + 12.67$	0.999

* Retention time (min).
† Number of data points, 5; number of replicates, 3.

Calibration graphs

Stock solutions (0.25 mg/mL) of each of vincristine, vinblastine, catharanthine, and vindoline were prepared in methanol and different amounts of these were used for the preparation of calibration graphs, linear in the range of the working concentration of each standard (0.25–25 µg). The regression equations are presented in Table I.

Results and Discussion

HPLC analysis

Figure 2 is an illustration of the separation of alkaloids (**1**–**4**) in a standard

mixture (B) and a plant sample extract (A). All the peaks corresponding to the alkaloids **1**–**4** were symmetrical with recoveries of 96%, 97%, 98%, and 98%, respectively. The retention times of the four test alkaloids were 16.57, 26.93, 6.81, and 13.22 min, respectively. Increased percentage of water in the mobile phase resulted in a higher back pressure in the earlier used C-18 HPLC column (3). Replacement of the particulate type C-18 column by a Chromolith column allows for work with a mobile phase of increased buffer concentration in acetonitrile without an increase of the back pressure.

An increase in polarity of mobile phase was attempted, resulting in a better separation of the alkaloids compared with the earlier reported method (3). This will allow a long life of the HPLC column, pump, etc. Use of the Chromolith column allowed for an analysis of more than 300 *Catharanthus* samples in comparison to approximately 200 samples with the previously used particulate-type C-18 columns. Between gradient runs, the latter columns require a lengthy flushing or phase equilibration before the next sample injection; the present method is more convenient than the earlier reported multistep variable gradient method (4). Analysis was carried out at a wavelength of 254 nm, close to the absorption maxima of all the four alkaloids. Column performance is reported in Table I.

Recovery

For the estimation of recovery, a known amount of stock solution of the four alkaloids was added to *C. roseus* plant extract. The average of three results is given in Table I.

Precision

Precision or reproducibility of the method was measured by repeating each experiment three times. The coefficient of variation values were 2.50%, 1.82%, 1.33%, and 1.13% for compounds **1**, **2**, **3**, and **4**, respectively.

Evaluation of peak purity

The identity of each peak and its homogeneity was checked by comparing the PDA-generated data with the of the reference compounds using library matching. All the peaks were found pure both at up and down slopes of the peaks (Table II). Similarity of alkaloids **1**–**4** in the samples checked by comparison of data in a library was > 0.99 (Table II).

LOD and LOQ

The LOD and LOQ values were calculated for compounds **1**–**4** based on 3 and 10 times of noise level, respectively. The LOD were found to be 8, 14, 18, and 10 µg/ mL, whereas the LOQ were found to be 25, 46, 56, and 32 µg/mL, for compounds **1**–**4**, respectively.

Robustness

The robustness of the method was determined by measuring the effect of small and deliberate changes in the analytical parameters on the retention time and peak area counts. The parameters selected were mobile phase concentration, flow rate, and temperature. While one parameter was altered, the others were kept constant. The standard deviations (SDs) of retention time and peak area counts were calculated for each parameter and SD% values were found to be less than 2%, which indicated the robustness of the method. The results are presented in Table III.

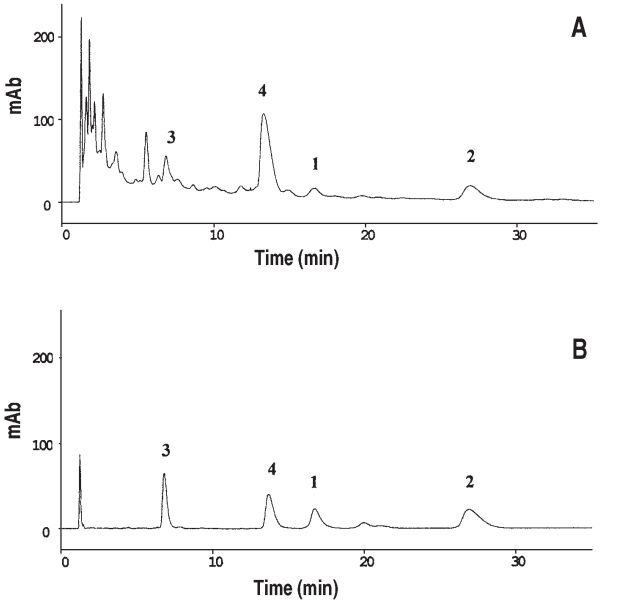

Figure 2. RPLC separation of a standard mixture of the four alkaloids (0.25 mg/mL) (A) and *C. roseus* leaves extract (B). Condition: Chromolith performance RP-18e column; mobile phase, acetonitrile–0.1M phosphate buffer containing 0.5% glacial acetic acid (21:79, v/v); flow rate, 1.2 mL/min; and UV detection at 254 nm. Vincristine (**1**), vinblastine (**2**), catharanthine (**3**), and vindoline (**4**).

Table II. Peak Purity Test Results of *Catharanthus* Alkaloids Using Photodiode-Array Detection

		Peak purity		
No.	Alkaloid	Up	Down	Similarity
1	Vincristine	0.98	0.99	0.99
2	Vinblastine	0.99	0.99	0.99
3	Catharanthine	0.99	0.99	0.99
4	Vindoline	0.99	0.99	0.99

Table III. Robustness Testing for the Four Alkaloids* (*n* = 3)

	SD% of peak area counts				SD% of t_R†			
Parameter	1	2	3	4	1	2	3	4
Mobile phase concentration	1.81	1.71	1.52	0.94	0.58	0.78	1.03	0.88
Flow rate	0.63	0.81	0.76	0.54	0.42	0.34	0.37	0.57
Temperature	0.25	0.23	0.29	0.17	0.18	0.15	0.22	0.27

* Vincristine (**1**), vinblastine (**2**), catharanthine (**3**), and vindoline (**4**).
† Retention time (min).

Table IV. Percent Content of the Alkaloids in the Dried Leaves of *Catharanthus roseus* Accessions

S. no.	Accession no.	Vincristine	Vinblastine	Catharanthine	Vindoline
1	14	0.0001	0.0004	traces	0.0243
2	49	traces	0.0001	0.0002	0.0481
3	76	0.0003	0.0022	0.0004	0.1346
4	98	0.0012	0.0006	0.0004	0.0588
5	112	0.0004	0.0033	0.0363	0.1254
6	151	0.0002	0.0001	0.0002	0.0727
7	176	0.0003	0.0005	0.0002	0.0554
8	183	0.0002	0.0002	0.0002	0.0142
9	243	0.0002	0.0048	0.0005	0.0865
10	260	0.0003	0.0004	0.0003	0.0545

The method described in this paper has been used to determine the alkaloid concentration in a number of samples. The results are reported in Table IV.

Conclusion

In the reported method, alkaloids **1**–**4** were well resolved from each other with symmetrical nature of peaks. The method is suitable for rapid quantitation in vinca plant populations for crop improvement.

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Manuscript received July 19, 2005.