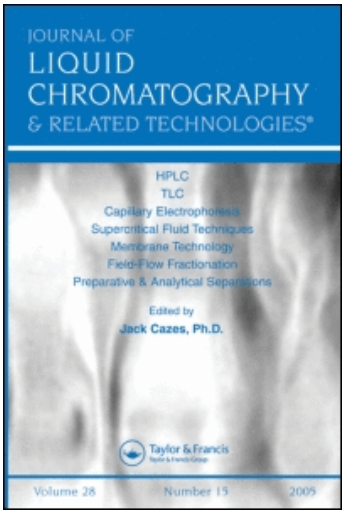

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 15 February 2000

To cite this Article Singh, D. V. , Maithy, A. , Verma, R. K. , Gupta, M. M. and Kumar, Sushil(2000) 'SIMULTANEOUS DETERMINATION OF CATHARANTHUS ALKALOIDS USING REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY', Journal of Liquid Chromatography & Related Technologies, 23: 4, 601 — 607
To link to this Article: DOI: 10.1081/JLC-100101476
URL: http://dx.doi.org/10.1081/JLC-100101476

J. LIQ. CHROM. & REL. TECHNOL., 23(4), 601–607 (2000)

SIMULTANEOUS DETERMINATION OF CATHARANTHUS ALKALOIDS USING REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A rapid and simple reverse phase liquid chromatographic method has been developed for the simultaneous quantitation of anti-cancerous drugs vincristine, vinblastine and their precursors catharanthine and vindoline. Peak purity evaluation of these compounds in plant extract has also been studied using photo–diode array–UV detector.

INTRODUCTION

Periwinkle, Catharanthus roseus (L.) G. Don., known in trade as Vinca, is a pantropical species occurring chiefly in West Indies and Madagascar and is extensively cultivated in many states of India. Vinca alkaloids, mainly vincristine (**1**) and vinblastine (**2**), have extensive use in modern medicine as potential anti-cancer compounds.[1] Catharanthine (**3**) and vindoline (**4**) are precursors of the vinblastine and vincristine group of alkaloids.[1,2] Thus, screening for vindoline and catharanthine would also deserve attention for industrial exploitation of anticancer compounds from C. roseus.[3] No systematic screening of natural population for these alkaloids has been reported.

The major constraint for such studies appears to be the lack of sensitive and accurate rapid estimation methods due to the inherent complexity in the chemical assay of constituents that occur in extremely low quantities. For their

determination, high performance liquid chromatography methods using mobile phase at pH>6.0 are commonly used.[4-10] However, analysis of vincristine and vinblastine with catharanthine and vindoline in a single run remains a question due to elution of some impurities together with these compounds at the same retention times. Knowledge of influence[9] of mobile phase, ionic strength, and pH on the elution of these alkaloids prompted us to separate these alkaloids in catharanthus plant extract using a mobile phase at pH below 5.0. Photo diode array detector (PDA) has been used to check the purity of these alkaloids in a sample HPLC run. Similarity of these alkaloids with standard alkaloids has also been checked using PDA.

PDA results were quite satisfactory and the method is suitable for mass screening of *C. roseus* plants for their important alkaloids under a crop improvement programme. The work is in the series of our efforts towords developing liquid chromatographic procedures for plant drug analysis.[11-15]

EXPERIMENTAL

Plant Material

The plant material of *C. roseus* were obtained from the experimental farm of this Institute at Lucknow. The sample materials of the genotypes used are available in the Gene Bank of this Institute.

Chemicals

Vincristine and vinblastine were from Sigma, USA. Vindoline and Catharanthine were generously provided by Prof P. Potier (National Centre for Scientific Research, Yvette, Cedex, France). Solvents used were HPLC grade (E. Merck, Germany). Sodium dihydrogen ortho-phosphate was from Glaxo, India.

Analytical Procedure

Chromatographic Instrument and Conditions

Chromatographic separations were carried out on a Shimadzu (Japan) LC-10A gradient high-performance liquid chromatography instrument equipped with two LC-10 AD pumps controlled by CBM-10 interface module, a model 7725I manual injector valve (Rheodyne), 20 μL sample loop and a multi-dimensional UV-VIS detector SPD-10A. SPD-M10AVP (Shimadzu) Photo-diode array detector has been used for the peak purity and similarity test of all

the four alkaloids. Data were collected and analysed with a Pentium computer (Datamini Singapore) and HP-Deskjet printer. Solvents were filtered by using a millipore system and analysis was perfomed on a Waters μ Bondapak C_{18} reversed-phase column, 10 μm (30 cm X 3.9 mm I.D.). A constant flow rate of 0.6 mL/min was used during analysis. The composition of mobile phase was optimized by using a different composition of acetonitrile-0.1M phosphate buffer-glacial acetic acid resulting in the following operating condition; acetonitrile: 0.1M phosphate buffer:glacial acetic acid (38:62:0.3); pH 4.14,flow rate 0.6mL/min, column temperature 26°C, detector wave length 254 nm.

Sample Preparation

Extract of the leaves were prepared as reported earlier.[16] In brief, powdered leaf (5 g) was extracted thrice with 90% ethanol (3 X 30 mL, 12hr 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 X 30 mL). The aqueous portion was basified with ammonia to pH 8.5, extracted using chloroform (3 X 30mL); chloroform extract was washed with water, dried over sodium sulphate, and concentrated under vacuum. The extract was redissolved in 10 mL methanol.

Calibration Graphs

Stock solution of vincristine, vinblastine, catharanthine, and vindoline were prepared in methanol (0.25mg/mL) and different amounts of these were used for the preparation of calibration graphs, linear in the range of 0.25 μg-25 μg, the regression equations are given in Table 1.

Table 1

C_{18} Column Performance in the Separation of Catharanthus Alkaloids from the Extract of *Catharanthus Roseus*

Alkaloid	RT	No. of Theoretical Plate Counts (N)	Capacity Factor (K)	Recovery (%)	Separation Factor	Linear Regression Equation
Vincristine	10.15	3544	1.05	97	1.22	Y = 78.7X + 7.4 (r = 0.999)
Vinblastine	11.34	3268	1.30	97	1.13	Y = 99.9X + 3.8 (r = 0.999)
Catharanthine	12.32	4829	1.50	96	1.15	Y = 99.9X + 3.3 (r = 0.999)
Vindoline	13.54	4886	1.74	97	1.16	Y = 56.8X + 0.5 (r = 1.000)

RESULTS AND DISCUSSION

The composition of mobile phase was optimized using different proportions of acetonitrile,0.1M phosphate buffer and acetic acid, the final result being acetonitrile – 0.1M phosphate buffer–glacial acetic acid (38:62:0.3). Figure 1 illustrates the separation of alkaloids (**1-4**) in a standard mixture (A) and a plant sample extract (B). Peaks corresponding to compounds (**1-4**) were symmetrical. Recoveries of compounds **1**,**2**,**3** and **4** were 97,97,96,97%, respectively.

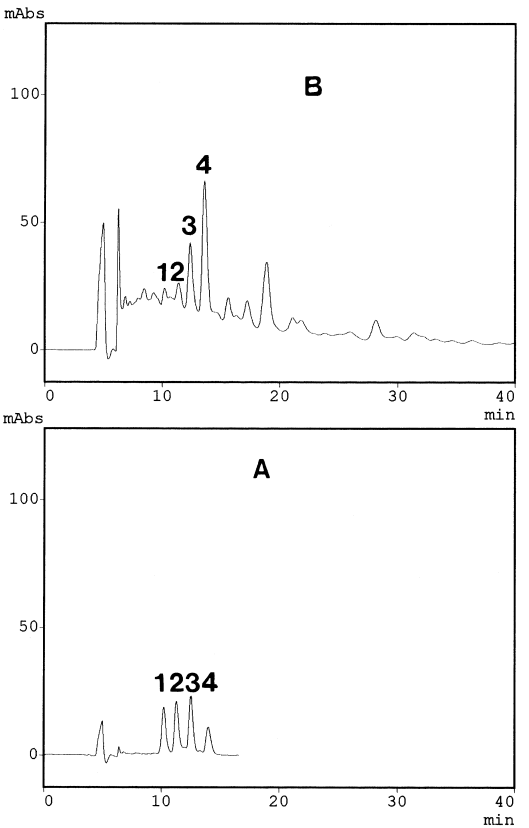

Figure 1. HPLC separation of catharanthus alkaloids in an artificial mixture of pure compounds (A) and a *Catharanthus roseus* leaf extract (B). **1**: Vincristine; **2**: Vinblastine; **3**: Catharanthine; **4** : Vindoline.

Table 2

Peak Purity Test Results of Catharanthus Alkaloids Using Photo Diode Array Detector

Alkaloid	Peak Purity		
	Up	Down	Similarity
Vincristine	0.99	0.99	0.99
Vinblastine	1.00	1.00	1.00
Catharanthine	1.00	1.00	1.00
Vindoline	1.00	1.00	1.00

For the examination of recovery, known amounts of stock solution of pure compounds (**1-4**) were added in the *C. roseus* plant extract and the quantitation was repeated thrice. Selected detection wavelength (254 nm) was closed to absorption maxima of all the four alkaloids (**1-4**). Column performance report for **C. roseus** plant extract has been presented in Table 1.

Table 3

Percent Content of Alkaloids (1-4) in the Leaves of Catharanthus Roseus Accession

S. No.	Accession No.	Vincristine (1)	Vinblastine (2)	Catharanthine (3)	Vindoline (4)
1	CON	0.0018	0.0003	0.0063	0.0052
2	M_3 2	0.0024	0.0015	0.0288	0.0069
3	M_3 14	n.d.	0.0016	0.0424	0.0069
4	M_3 20	0.0005	0.0017	0.0327	0.0406
5	M_3 28	0.0004	0.0006	0.0116	0.0122
6	M_3 36	0.0004	0.0016	0.0036	0.0087
7	M_3 51	n.d.	0.0007	0.0006	0.0087
8	M_3 53	0.0001	0.0002	0.0026	0.0102
9	M_3 72	0.0003	0.0001	0.0007	0.0067
10	M_3 91	n.d.	n.d.	0.0005	0.0068

n.d. = not detectable.

Evaluation of Peak Purity

Peak purity test of compounds **1-4** has been performed using SPD-M10AVP photodiode array detector. All the peaks were found pure both at up and down the peaks (Table 2). A similarity test of compounds **1-4** in sample extract was performed by comparing similarity of peaks in sample track to that of a library maintained for alkaloids **1-4**. Similarity of all the compounds were >0.99 (Table 2). The peak homogeneity was tested by examining the UV spectra at different points of the resolving peaks.

In the method applied here, alkaloids **1-4** were well separated from each other, and peaks were symmetrical and suitable for the rapid screening purpose of vinca plant populations for crop improvement. Results of a few accessions are given in Table 3 (average of three readings).

ACKNOWLEDGMENT

The authors are indebted to Prof. P. Potier (National Centre for Scientific Research, Yvette, Cedex, France), for the generous gift of vindoline and catharanthine standards.

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Received March 3, 1999 Author's Revisions October 18, 1999
Accepted April 3, 1999 Manuscript 5032