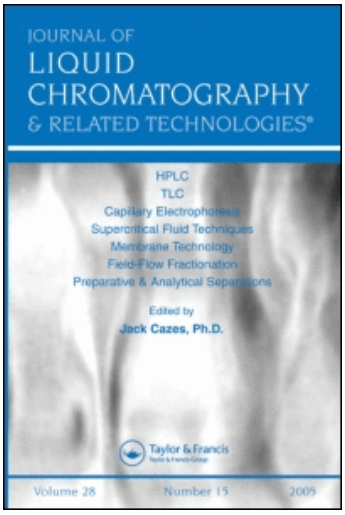

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 04 February 2001

To cite this Article Srivastava, Shaifali , Verma, Ram K. , Gupta, Madan M. , Singh, Subhash C. and Kumar, Sushil(2001) 'HPLC DETERMINATION OF VASICINE AND VASICINONE IN *ADHATODA VASICA* WITH PHOTO DIODE ARRAY DETECTION', Journal of Liquid Chromatography & Related Technologies, 24: 2, 153 — 159
To link to this Article: DOI: 10.1081/JLC-100001478
URL: http://dx.doi.org/10.1081/JLC-100001478

J. LIQ. CHROM. & REL. TECHNOL., 24(2), 153–159 (2001)

HPLC DETERMINATION OF VASICINE AND VASICINONE IN *ADHATODA VASICA* WITH PHOTO DIODE ARRAY DETECTION

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ABSTRACT

A high performance liquid chromatographic method for the determination of the quinazoline alkaloids vasicine (**1**) and vasicinone (**2**) in *Adhatoda vasica* plant extract is reported. Peak purity and similarity of **1** and **2** have been studied using photodiode array detector (PDA). Effects of different solvents have been studied for the extraction of **1** and **2** in *A. vasica* plant, and methanol was found to give the maximum extraction of compounds **1** and **2**. The method is simple, sensitive, rapid, and reproducible for the quantitation of pharmacologically important alkaloids vasicine and vasicinone. The separation of **1** and **2** was performed with acetonitrile–phosphate buffer (pH maintained to 3.9 using glacial acetic acid) (15:85) using a Hibar Merck make C_{18} column.

Key Words: HPLC; *A. vasica*; Leaves; Alkaloid

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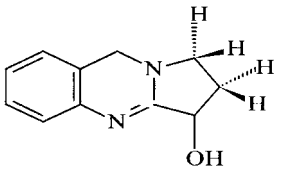

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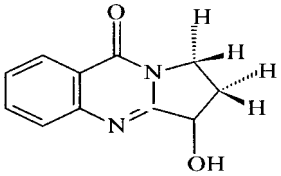

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Figure 1. Structure of compounds **1** and **2**.

INTRODUCTION

Adhatoda vasica (Nees), commonly known as Vasaka in an indigenous system of medicine, is frequently used as an ingredient of a number of proprietary, over the counter (ODC), polyherbal formulations for a variety of respiratory ailments including cough, bronchitis, and asthma (1). Both vasicine (**1**) and vasicinone (**2**) (Fig. 1), formed by oxidation of vasicine at C-8 position, are two major alkaloids of *A. vasica* and known to possess interesting biological activities (2–9). including respiratory, stimulant, bronchodilator, and hypotensive activities. Autooxidation of vasicine to vasicinone takes place in different solvents, in bright daylight or sunlight (10,11). In view of the interesting biological activities associated with vasicine and vasicinone, it was of interest to develop a suitable, simple, rapid, and accurate method with a good degree of peak purity corresponding to compounds **1** and **2**, to assess the quality of plants for their genetic improvement. Since vasicine is readily oxidised to vasicinone and the pharmacological effects of these compounds are different, it is also of importance to develop a sensitive analytical procedure for rapid and simultaneous quantitation of **1** and **2**. For the quantitation of vasicine, titrimetric (12), spectrophotometreic (13), and HPTLC (14) methods are available, but they lack precision and accuracy because of low sensitivity and due to interference by other compounds. Attempts have been made to develop HPLC methods (1,10,11) using a UV detector for the quantitation of **1** and **2**, but they lack a baseline separation of **1** and **2** which may result in impure peaks of **1** and **2**. In our efforts toward developing methods (12–14) using PDA for plant drug analysis here, we report an RPLC method which involves a PDA detector for peak purity/similarity tests of vasicine and vasicinone.

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EXPERIMENTAL

Materials and Reagents

Compounds **1** and **2** were isolated from *A. vasica* leaves. Powered leaves, 1.5 kg of *A. vasica*, collected from experimental farms CIMAP, LKO, were soaked with ethanol for 24 h, the extract was filtered and concentrated. Extraction was repeated three times. The concentrated material was treated with 5% acetic acid (100 mL) and warmed for 15 min and filtered. The filtrate was defatted with hexane, basified with ammonia (pH 9.0), and then extracted with chloroform (250 mL × 3 times) to yield 4.0 g of crude alkaloids. Pure vasicine and vasicinone were obtained by column chromatography over Si gel (200 g) using chloroform:methanol: ethyl acetate mixtures with increasing polarity.

Vasicine **1**: Fractions (351–370) of the $CHCl_3$:MeOH:EtOAc (60:20:10) afforded compound **1**, crystallized in methanol-dichloromethane, 1.5 g, m.p. 210–212°C structure was confirmed by its spectral analysis (2).

Vasicinone **2**: Fractions (77–85) of the $CHCl_3$:MeOH:EtOAc (85:15:10), gave compound **2**, crystallized in methanol, 0.5 g, m.p. 198–200°C, structure was confirmed by its spectral analysis (2).

Chromatographic Apparatus and Conditions

A Shimadzu (Japan) gradient high performance liquid chromatography instrument equipped with two LC-8A pumps, a Model 7725 I manual injector valve (Rheodyne), CBM-10A interface module to control the system, LC-10 work station to analyze the results, and a SPD M 10 AVP (Shimadzu) was used. A photodiode array detector for peak detection, analysis, peak purity, and similarity tests was also used. Solvents were filtered through a Millipore system and analysis was performed on a Merck Hibar C_{18} column (250 mm × 4.0 mm I. D. 10 μm), (Fig. 2). The composition of the mobile phase was optimized by varying the percent of acetonitrile in phosphate buffer and observation of peak purity and similarity results of **1** and **2**, using a photodiode array detector; this resulted in the following operating conditions: acetonitrile–0.1 *M* phosphate buffer–glacial acetic acid (15:85:1, v/v/v), pH 3.9; flow rate 0.7 mL/min; column temperature, 26°C; detector wavelength, 300 nm, absorption maxima close to both the compounds **1** and **2**.

Sample Preparation

Samples of air dried and powdered leaves (1 g) was extracted with methanol three times (10 mL each time for 3 h) and the combined extract was filtered,

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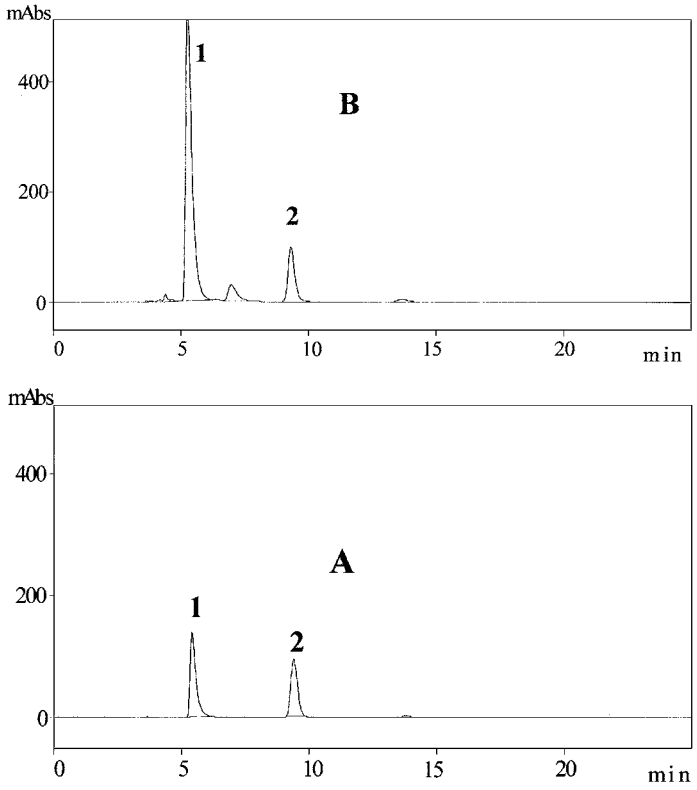

Figure 2. RPLC separation of artificial mixture of pure vasicine and vasicinone (1 mg/ml) (A) and *Adhatoda vasica* leaves extract (B). Conditions: Merck Hibar ODS column (250 × 4.0 mm); UV detection at 300 nm; mobile phase, acetonitrile–0.1 *M* phosphate buffer–glacial acetic acid (15:85:1); flow rate 0.7 mL/min. (**1**) vasicine and (**2**) vasicinone.

concentrated under vacuum, extracted with 5% acetic acid (5 mL × 3 times), defatted using hexane (10 mL × 3 times), basified with ammonia (pH 9.0) and extracted with chloroform (10 mL × 3 times), concentrated under vacuum, and made up to 1.0 mL in methanol and filtered through a Millipore filter for HPLC analysis. The content of compounds **1** and **2** was calculated using an external standard.

Calibration Graph

Freshly prepared solutions of **1** and **2** in methanol (1 mg/mL) were used for the preparation of calibration graphs.

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RESULTS AND DISCUSSION

The HPLC analysis was performed using a UV detector in the earlier reported methods without considering the peak purity which may result in impure peak identification of vasicine and vasicinone. Also, there was not a base line separation of peaks in the reported procedures. Here, we have tried a number of solvent systems in different compositions resulting in optimum acetonitrile–0.1 *M* phosphate buffer–glacial acetic acid (15:85:1, v/v/v) to give symmetrical and baseline separated peaks of vasicine and vasicinone (Fig. 2). Compounds **1** and **2** were eluted at retention times 5.27 min and 9.31 min, respectively.

The peak purity of compounds **1** and **2** was tested using a photodiode array detector: compound **1**, purity up 0.9986, down 0.9980; compound **2**, purity up 0.9986, down 0.9993. The similarity of compounds **1** and **2** in the sample and standards was also checked and found to be 0.998 and 0.997, for compounds **1** and **2**, respectively. Peak purity test results and similarity of data of compounds **1** and **2** were satisfactory.

Recoveries of compounds **1** and **2**, calculated by addition of vasicine and vasicinone in plant extract, were $98.2 \pm 1.8\%$ and $97.4 \pm 2.0\%$, respectively.

Linearity was determined in a working range of 2–20 μg using a number of data points, 5, number of replicates, 3. Linear regression equation and correlation coefficient (r) for both the compounds **1** and **2** have been presented in Table 1. Calibration plots of peak areas versus concentration were linear with r values 0.999 for both the compounds. These values indicated a good linearity in the examined concentration range.

Detection limits, a measure of minimal mass of compounds **1** and **2** that can be quantified, were 0.05 μg/injection and 0.08 μg/injection, respectively.

Column performance results in sample analysis have also been presented in Table 1. As a measure of column performance, the number of theoretical plate counts (N) for compounds **1** and **2** were 2065 and 5770, respectively.

Experiments have been performed to select the suitable solvent for the maximum extraction of vasicine and vasicinone. Different solvents, viz., hexane, acetone, chloroform, ethyl acetate, methanol, and ethanol, have been used for the

Table 1. Column Performance and Linear Regression Data for Vasaka Alkaloids (**1** and **2**)

Compound	*Rt*	Recovery (% ± SD)	Capacity Factor	No. of Theoretical Plate Counts	Linear Regression Equation[a]	*r*
1	5.27	98.2 ± 1.8	0.44	2065	$Y = 3539.6x - 108.4$	0.999
2	9.31	97.4 ± 2.0	1.54	5770	$Y = 2505.7x - 488.0$	0.999

[a]Number of data points, 5; number of replicates, 3.

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Table 2. Vasicine (**1**) and Vasicinone (**2**) Content Using Different Solvents for Extraction

	% Content of	
Extracting Solvent	Vasicine	Vasicinone
Hexane	0.0064 ± 0.0003	0.0024 ± 0.0003
Acetone	0.0400 ± 0.0020	0.0042 ± 0.0004
Chloroform	0.0160 ± 0.0030	0.0023 ± 0.0003
Ethyl Acetate	0.0080 ± 0.0004	0.0014 ± 0.0002
Methanol	0.1200 ± 0.0100	0.0340 ± 0.0030
Ethanol	0.0780 ± 0.0040	0.0024 ± 0.0004

extraction of plants and the results of vasicine and vasicinone content using the above procedure are presented in Table 2. Methanol was found to be a suitable solvent for extraction of maximum vasicine and vasicinone.

CONCLUSION

The analytical method reported here for the quantitation of vasicine and vasicinone in *A. vasica* plant extract is suitable for the rapid screening purpose of different genetical and agronomical field experiments.

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Received May 31, 2000
Accepted July 14, 2000
Author's Revisions January 5, 2001
Manuscript 5319

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