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Quantitative determination of vasicine and vasicinone in *Adhatoda vasica* by high performance capillary electrophoresis

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A new method of capillary electrophoresis was developed for the quantitative determination of vasicine and vasicinone from *Adhatoda vasica* (L.) Nees. The electrophoretic separation was performed using a 47 cm × 50 μm ID (38.5 cm effective length) fused silica capillary. The samples were injected by pressure for 3 s at 50 mbar and the running voltage was 19 kV at the injector end of the capillary. The capillary temperature was maintained at 40 °C. The separation of the two alkaloids has been achieved within 11 min with good repeatability. The method was validated in terms of reproducibility, linearity, accuracy and applied for the quantitative determination of vasicine and vasicinone in *A. vasica* plant samples/extracts. Parameters affecting the resolution such as pH, temperature, organic modifier, buffer concentration and capillary dimensions were reported.

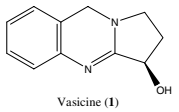
1. Introduction

Adhatoda vasica (L.) Nees (Family Acanthaceae) is a small evergreen sub herbaceous bush widely spread through out the tropical region of Southeast Asia. *Adhatoda zeylanica* Medic. and *Justica adhatoda* L. are used synonymously for *A. vasica*, it is also known as vasaca and Malabar nut tree. The plant has been used in the indigenous Ayurvedic system of medicine in India and Pakistan for more than 2000 years, particularly against respiratory tract ailments (Ubonwan et al. 2000). *A. vasica* leaves contain the major quinazoline alkaloids deoxyvasicine, vasicine and vasicinone. The latter two are respiratory stimulant and have remarkable therapeutic activities (Duke et al. 2002; Keith et al. 1983). The major alkaloid of the plant, vasicine, has been found to be biologically active and was subject of many chemical and pharmacological studies (Atal et al. 1980). Vasicine undergoes auto-oxidation to vasicinone when exposed to bright day light (Mehta et al. 1963), and it has been reported that stability of vasicine varied widely according to the nature of solvent used. The rate of auto-oxidation of vasicine in ethanol and methanol is very low even after UV irradiation (Keith et al. 1983). Vasicine has appreciable bronchodilatory effect and marked respiratory stimulant activities, whereas vasicinone shows relaxation of the tracheal muscle *in vitro* and bronchoconstriction *in vivo*. A detailed preclinical study revealed that vasicinone has a synergetic effect on vasicine in bronchodilation, as well as in increasing ciliary movements. It was also observed that the cardiac depressant effect manifested by vasicine was normalized by vasicinone. Vasicine and vasicinone mixed in 1 : 1 ratio has shown higher bronchodilatory activity and antagonism against histamine induced bronchoconstriction as vasicine alone (Duke et al. 2002; Atal et al. 1980; Gupta et al. 1977).

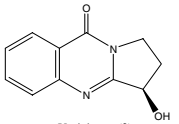
The World Health Organization (WHO) has also considered *A. vasica* as an important medicinal plant with therapeutic utility and non-toxic nature, and included it in the WHO manual, *The Use of Traditional Medicines in Primary Health Care*, which is intended for health workers in South-East Asia to keep them informed of the therapeutic utility of their surrounding flora (Ubonwan et al. 2000).

Information available about the safety of *A. vasica* is insufficient but no adverse effects were reported (Ubonwan et al. 2000; Jellin et al. 1999).

Previous reported methods for the determination of vasicine and vasicinone include HPLC (Keith et al. 1983; Chowdary et al. 1987; Gupta et al. 2001; Srivastava et al. 2001) and HPTLC (Narayana et al. 1995). There was no capillary electrophoresis (CE) method developed for the separation of vasicine and vasicinone compounds. Therefore, a simple, accurate and reliable high performance capillary electrophoresis (HPCE) method for the simulta-



Vasicine (**1**)



Vasicinone (**2**)

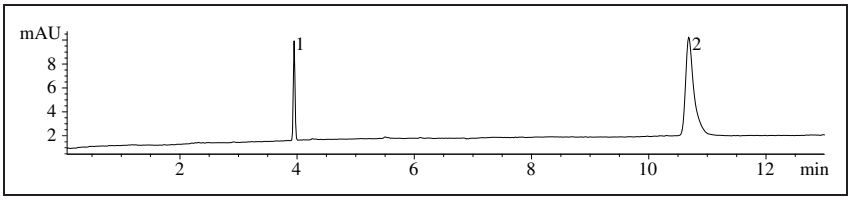


Fig. 1:
Electropherogram of two alkaloidal standard mixture (Conditions: 10 mM sodium borate, 20 mM sodium dihydrogen phosphate, pH 2.1, 19 kV, 40 °C, 210 nm, fused silica capillary 47 cm (38.5 cm to the detector) × 50 μm I.D.)

neous quantitation of the two compounds vasicine (**1**) and vasicinone (**2**) in *A. vasica* extracts has been developed. The method presented is cheaper than the previously reported methods.

2. Investigations, results and discussion

The electropherogram of compounds (**1** and **2**) is shown in Fig. 1. The conditions were optimized for a capillary length of 47 cm and a temperature of 40 °C, keeping 19 kV as separation voltage. Under these buffer conditions a mixture of the two compounds were well resolved at pH 2.1.

Parameters such as the separation temperature, pH of the buffer solution, the applied voltage, effective length of capillary and organic modifiers affected the migration times of solutes and the selectivity and resolution of analysis.

2.1. Effect of buffer pH, organic modifiers and cyclodextrin type

The pH of the buffer solution strongly affected the resolution of the two compounds, which needs to be carefully controlled and optimized when ionic compounds are analyzed by HPCE because with a shift in the pH value brought a change in the rate of migration and resolution of the compounds. The migration behavior of the compounds can be related to the structures. The pH between 2.0 and 8.0 were studied. Optimal resolution was achieved at pH 2.1, and above pH 3.0 the compound **2** peak got merged with the solvent peak. The migration times increased with an increase in the amount of organic modifiers. The modifiers did not, however improved the separation selectivity. Temperature played a major role in the separation of compounds. Temperature ranges between 20–45 °C were studied. Capillary length and voltage have a major influence but best separations were achieved at 40 °C, total capillary length 47 cm and voltage 19 kV.

2.2. Validation

For each compound, the linearity was assessed using five standard solutions (each injected in triplicate) in a 10.0–100.0 $\mu g \cdot mL^{-1}$ concentration range. The five-point calibration curves were found to be linear (Table 1) as least squares regression gave good correlation coefficients which ranging from 0.9994 to 0.9996.

Limits of detection (LOD) and quantification (LOQ) were defined, respectively, as signal-to-noise ratio equal to 3 and 10, LOQ of 3.0 $\mu g \cdot mL^{-1}$ for compounds **1** and **2**, respectively, and LOD of 0.5 $\mu g \cdot mL^{-1}$ for compounds **1** and **2**, respectively.

Standard solutions (n = 5) from 10.0–100.0 $\mu g \cdot mL^{-1}$ were injected in triplicate to assess the accuracy of the method. Suitable amounts of the standard compounds were added to the methanolic extract of *Adhatoda vasica* and the mixture was extracted and analyzed with the proposed method. The recoveries of the alkaloids were 96.82–98.85% with relative standard deviation of less than 3.0%. Intra and inter-day variations were determined with the standards. It was performed thrice on three different days and each concentration point was injected in triplicate. The reproducibility (expressed as relative standard deviation (RSD) values of relative migration times (RMTs)) and RSD values of relative peak areas (RPAs) was calculated by analyzing the standard solutions of **1** and **2**. In general RSD values of relative migration times (RMTs) were lower than 3.0%. The RSD values of the relative peak areas (RPAs) were found to be well below 2.0%.

Table 1: Calibration data for standard compounds 1 and 2 including regression equation, correlation coefficient and limits of detection (LOD)

Analyte	Regression equation*	Correlation coefficient (r^2)	LOD (μg/mL)
1	Y = 0.277713*X-0.050729	0.9994	0.5
2	Y = 1.200466*X-1.260014	0.9996	0.5

* Y = the peak area, X = amount of compound in $\mu g \cdot mL^{-1}$

2.3. Plant analysis

The electropherogram separation of the standard mixture of **1** and **2** separated by CE under optimized conditions is shown in Fig. 1. The practical applicability of the CE method was verified by analyzing methanolic extracts of leaves of *Adhatoda vasica.* The peaks were assigned by their UV-spectra and by spiking the samples with reference compounds.

Fig. 2 shows that compounds **1** and **2** were detected in methanolic extracts of plant samples (AV-1 and AV-2). Peaks were identified by the addition of **1** and **2**. The analytical results are summarized in Table 2.

The method was applied to two plant samples of different origin. The plant samples (AV-1 and AV-2) were analyzed using CE. The compounds **1** and **2** were well resolved within 11 min and the concentration of compounds **1** and **2** is shown in Table 2. The concentrations of compounds **1** and **2**, detected in *A. vasica* leaves extracts, were 0.58% and 0.166% for AV-1, respectively and 0.97% and 0.037% for AV-2, respectively.

Intra- and inter-day (Table 3) variation of the assay was determined and showed to be lower than 5.0%, with a maximum RSD of 4.17.

Run to run migration time in CE can often change, which is dependent on the condition of the capillary wall, the composition, pH and viscosity of the buffer and the nature of the sample, hence spiking with standards was necessary for identification of specific compounds.

This method seems to be an efficient way to analyze dietary supplements and various *Adhatoda* species. Furthermore, CE requires a smaller volume of solvents than does the HPLC, which reduces the operating costs (Avula et al. 2005).

The method described in this paper can be applied as an analytical tool for determining the compounds **1** and **2** in dietary supplements and medicinal preparations containing *A. vasica*.

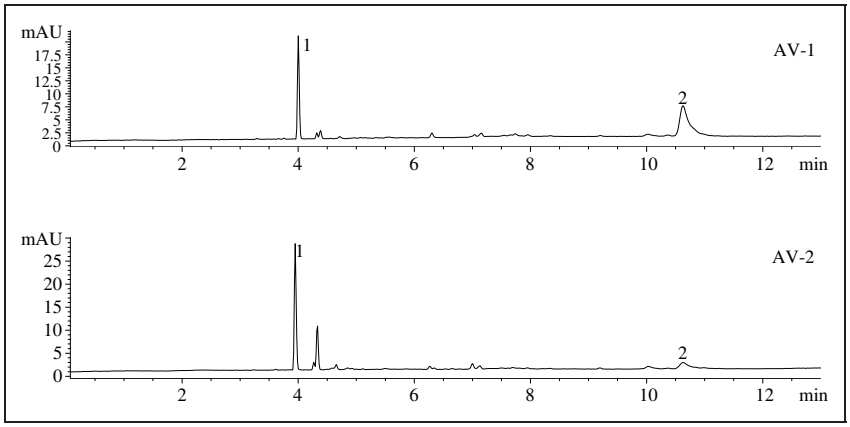


Fig. 2:
Electropherogram of *A. vasica* extracts AV-1 and AV-2 (Conditions: 10 mM sodium borate, 20 mM sodium dihydrogen phosphate, pH 2.1, 19 kV, 40 °C, 210 nm, fused silica capillary 47 cm (38.5 cm to the detector) X 50 μm I.D.)

Table 2: Percentage (%) of alkaloids 1 and 2 found in A. vasica samples of different origin

Sample	1	2
AV-1	0.58	0.166
AV-2	0.97	0.037

* All % RSD were below 5.0

Table 3: Intra- and inter-day precision of plant extract (AV-2) assayed under optimized conditions for compounds 1 and 2 by CE

AV-2	Intra-day (n = 5)					Inter-day (n = 3)
	Day 1	Day 2	Day 3	Day 4	Day 5	
1	0.951	0.995	0.951	0.974	0.938	0.968 (2.309)
2	0.037	0.040	0.037	0.039	0.038	0.037 (4.166)

Values in mg/100 mg of plant sample; relative standard deviation are given in parentheses

3. Experimental

3.1. Instrument

All HPCE experiments were performed on a Hewlett-Packard 3D-CE (Agilent Technologies, Palo Alto, CA, USA) equipped with PDA detector operated at 210 nm. An HP Chemstation software system controlled all the equipment and carried out the data processing and recording of electropherograms.

3.2. Materials

Standards compounds **1** and **2** were purchased from Chromadex (Santa Ana, CA, USA). Sodium phosphate and sodium borate were purchased from Sigma (St Louis, MO, USA). Fused silica tubing (50 μm ID, 360 μm OD) was purchased from Polymicro Technologies (Phoenix, AZ, USA). All buffer and sample solutions were filtered through 0.45 μm Nylon filters (Phenomenex, Torrance, CA, USA) prior to experiments. Plant materials were identified and procured from Hamdard University, Pakistan and Vadik Herbs, USA. Voucher specimens of all are deposited at the National Center for Natural Products Research (NCNPR), University of Mississippi.

3.3. Sample and standard preparation

Stock solutions of compounds were prepared in methanol at concentration of 1.0 $mg \cdot mL^{-1}$. Further dilutions were made in the same solvent ranging in the concentration from 10.0–100.0 $\mu g \cdot mL^{-1}$ for **1** and **2**.
Ground plant material (200.0 mg) was weighed and sonicated in 2.5 mL of methanol for 20 min, followed by centrifugation for 20 min at 4000 rpm. The supernatant was transferred to a 10.0 mL volumetric flask. The procedure was repeated thrice and respective supernatants were combined. The final volume was adjusted to 10 mL with methanol. Prior to use all samples were filtered through a 0.45 μm nylon membrane filter.

3.4. Procedure

The separations were performed in fused silica capillaries of varying lengths. The effective separation distance was 38.5 cm to the detector. Samples were injected by pressure for 3 s at 50 mbar. The injection end was the anode (+). The applied voltage was 19 kV and observed current was always less than 100 μA. The capillary temperature was maintained at 40 °C. The running electrolyte was prepared by mixing a 10 mM solution of sodium borate with 20 mM sodium dihydrogen phosphate solution in water and pH adjusted to 2.1 with pH meter (IQ Scientific Instruments, Inc., San Diego, CA, USA) by adding dilute sodium hydroxide or dilute phosphoric acid. Prior to use, the buffer was filtered through 0.45 μm Nylon membrane filter. After every run the capillary was preconditioned with 0.1 N NaOH (3 min), and water (3 min), followed by an equilibration period with buffer (5 min).
At the beginning of each day and whenever the buffer solution was changed, the capillary was conditioned with an initial wash cycle, consisting of 0.1 N NaOH for 20 min and deionized water for 20 min. Daily wash cycles after finishing experiments were 0.1 N NaOH for 5 min, deionized water for 5 min and air for 5 min.
In conclusions, the presence of organic modifiers did not have influence on the separation of alkaloids. Quantitative analysis has shown good results in terms of linearity, accuracy and repeatability. The assay described has proved to be accurate and precise for the determination of vasicine and vasicinone in *A. vasica* samples.

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