Quantitative estimation of Vasicine and Vasicinone in *Adhatoda vasica* by HPTLC

A C Suthar 1*, K V Katkar 1, P S Patil 1, P D Hamarapurkar 2, Mridula G 3, V R Naik 1, G R Mundada 1, V S Chauhan 1

1 Herbal Deptt, Piramal Life Sciences Ltd., 1, Nirlon Complex, Off. Western Express Highway, Goregaon (East), Mumbai – 400 063. Maharashtra, India

2 Pharmaceutical Analysis, Principal K. M. Kundnani College of Pharmacy, Jote Joy Building, Rambhau Salgaonkar Marg, Cuffe Parade, Mumbai 400 005. India.

3 Pharmacognosy Deptt., Manipal College of Pharmaceutical Sciences, Manipal University, Manipal, Karnataka, India.

Received on: 22-09-2009; Accepted on: 05-11-2009

ABSTRACT

The present paper deals with development and standardization of HPTLC method used for quantification of vasicine and vasicinone in *Adhatoda vasica* commonly known as Vasaka, a plant of the family Acanthaceae. Today, vasaka is the most widely known herb and widely used in the treatment for a variety of respiratory ailments including cough, bronchitis and asthma. Both vasicine and vasicinone are two major alkaloids of *A. vasica*, which are known to possess biological activities like respiratory stimulant, bronchodilator and hypotensive activity. Hence, a need to develop and standardize a chromatographic method for quantification of vasicine and vasicinone, for better standardization of *Adhatoda vasica*. An attempt has been made to quantify vasicine and vasicinone in *A. vasica* raw herbs, extracts and formulations by HPTLC method. The lowest detectable limit of vasicine and vasicinone was found up to 1 ng and 25 ng respectively with good resolution and separation of vasicine and vasicinone from other constituents of *A. vasica*. Further, recovery values of vasicine and vasicinone were found to be about 95-102 %, which shows the reliability and suitability of the method. The validated HPTLC method was found to be simple, reproducible, accurate and precise. The structure of isolated vasicine and vasicinone was characterized and confirmed by various advanced spectroscopic methods.

Keywords: HPTLC, *Adhatoda vasica*, Vasicine, Vasicinone

INTRODUCTION

*Adhatoda vasica* Nees, indigenous to India, is a member of the Acanthaceae family. It is a well-known plant drug in Ayurvedic and Unani medicine. *A. vasica* is one of the commonest shrubs distributed throughout India and especially in hedges, wastelands and grazing grounds and also over hilly tracts upto a height of 4000 feet above sea level 1. A number of different principles including, alkaloids: vasicine, vasicinone, vasinol, essential oil: betane, vitamins: vitamin C, b-carotene, a non-crystalline steroid: vasakin and a mixture of fatty acids have been identified as contributing to the observed medicinal effects of the plant 2. Vasa is mentioned as an herbal remedy for treating cold, cough, whooping cough and chronic bronchitis and asthma, as sedative expectorant, tuberculosis, inflammation, anti-allergic, antispasmodic and anthelmintic 3-6. The drug is employed in different forms such as fresh juice, decoction, infusion and powder; also given as alcoholic extract and liquid extract or syrup 7.

Vasicine and vasicinone are reported to have bronchodilatory and respiratory stimulation effects and hence regarded as biological markers for standardization of *A. vasica* extract 8-9. Few chromatographic methods are available and reported, such as HPTLC 10-12 and HPLC 13-14, available for estimation of vasicine and vasicinone. The limitation of reported HPTLC methods are use of ammonia in solvent system, which is hazardous, higher limit of detection & quantification and linear range, which demonstrates lower sensitivity and precision and use of water in mobile phase with lower linear range. Similarly, in various HPLC methods, use of buffer may lead to deterioration of stationary phase and lower sensitivity. Hence, the proposed HPTLC method was attempted for fast, precise, sensitive and reproducible method with good recoveries for standardization of extracts of *Adhatoda vasica*.

Materials and methods

The vasaka leaves were collected from two different locations in Maharashtra (coded as AD-1 and AD-2) and used as samples for quantification of vasicine and vasicinone. Similarly, a syrup formul-
tion—in house preparation (coded as IN-1) was also evaluated for the content of vasicine and vasicinone.

Pure vasicine and vasicinone were isolated from the dried leaves of A. vasica. Purity and structure of isolated compounds (vasicine and vasicinone) were confirmed by HPTLC, HPLC and spectral analysis like NMR, IR and MS \(^{15}\). These isolated compounds vasicine and vasicinone were used as working standards for quantification in dried raw herbs, extracts and formulations.

**Preparation of the extract**

The 100 gms of *Adhatoda vasaka* leaves dried powder was extracted with 500 ml solvent (methanol and water) by stirring at 50°C for 1 hr. The filtered extract was concentrated under reduced pressure to remove the solvent. The extraction carried out for two times with the above-mentioned protocol. The extract was obtained by drying the concentrated pooled extract under vacuum. These extracts were used for estimation and comparison of vasicine and vasicinone content.

**Equipment**

A Camag HPTLC system equipped with a sample applicator Linomat V, twin trough plate development chamber, TLC Scanner III and Reprostar, Wincats and integration software 4.02 (Switzerland).

**Sample preparation**

Accurately weighed 20 - 50 mg of aqueous extract and methanol extract of vasaka, 1 gm of dried raw herb or 10 gms cough syrup containing A. vasica were separately extracted with methanol (10 ml x 3) by vortexing and allowed to stand for 5 min. at room temperature. The methanol extract was then filtered through Whatmann no.42 filter paper; extracts were pooled and concentrated to dryness under vacuum. Final volume was made to 10 ml with methanol in volumetric flask. Vasicine and Vasicinone content were then analyzed after subjecting to HPTLC.

**HPTLC method**

Silica gel 60 F\(_{254}\) precoated plates (20 x 10 cm) were used with Chloroform: Methanol (90:10) as solvent system. 1-10 µl of working standard of vasicine, vasicinone as well as test samples were spotted on precoated HPTLC plates. The bandwidth applied on plate was 6 mm and ascending mode was used for development of thin layer chromatography. Saturation time was 20 mins along with humidity level - 65% ± 5% RH and room temperature - 25°C ± 2°C. TLC plates were developed upto 8 cm. The TLC plates were scanned at 280 nm for quantification - in house preparation (coded as IN-1) was also evaluated for the content of vasicine and vasicinone.

**Procedure: -1**

(Preparation curve of standard vasicine and vasicinone)

10 milligram of working standards vasicine and vasicinone were dissolved separately in 10 ml of methanol to yield stock solutions of 1 mg/ml concentration. Calibration curve was obtained by spotting 2 ng to 100 ng of vasicine per spot and 25 ng to 1000 ng of vasicinone per spot. Reproducibility, linearity and validation of the proposed method was checked along with correlation coefficient, coefficient of variance and the linearity curve for both markers.

**Procedure: -2**

(Preparation curve using extract spiked with vasicine and vasicinone)

The content of vasicine and vasicinone in aqueous and methanolic extracts was determined by comparing with the calibration curve of the working standard of vasicine and vasicinone. The aqueous extract, which showed lowest content of vasicine and vasicinone, was then used as blank. This blank was then used to spike extract with the working standard of vasicine and vasicinone. Different samples with varying amount of standard vasicine in range of 25 µg / ml to 75 µg / ml and vasicinone in range of 250 µg / ml to 750 µg / ml were spiked separately in 10 mg of blank extract with pre-determined vasicine and Vasicinone. Procedure for sample preparation was followed as mentioned above. In each sample preparation, 10 µl of spiked solution was then subjected to HPTLC with 10 µl of blank solution for comparison. The percent recovery of vasicine and vasicinone standard was calculated. Reproducibility, precision and validation of the method were achieved by analyzing six replicate spiked sample solutions. Correlation coefficient, coefficient of variance was calculated.

**Linearity along with limit of detection and limit of quantitation**

For a long-term use of the analytical method, a rigorous validation is indicated and requires the following procedures. For the preparation of calibration curve, the stock solution was diluted freshly with methanol to obtain a set of 11 calibration standards. These standards were measured and the integrated peak areas were plotted against the corresponding concentrations of the standards. The complete procedure was repeated on three consecutive days. The so obtained three calibration curves were used to calculate a mean calibration graph. The limit of detection was obtained by analyzing signal to noise ratio and limit of quantification was defined as the lowest concentration of linear range.

**Intraday and interday analysis using vasicine and vasicinone**

Three different concentrations using a different stock solution of vasicine (25, 50 and 75 ng / spot) and vasicinone (250, 500 and 750 ng / spot) were spotted. For the determination of the intraday precision and accuracy, three replicates of the standard solutions were analyzed at the same day in triplicate. The precision and the accuracy of the interday analysis were determined by analyzing the standard solution on 3 different days in triplicate.

**Stability**

Vasicine (1 mg), vasicinone (1 mg) and vasaka methanolic extract (10 mg) were extracted in 10 ml methanol as per the method described in sample preparation. The sample solutions were kept at 4°C in dark and analyzed on consecutive days (24, 48, 72 and 96 hrs) to observe the stability of standard as well as sample solution.
Robustness and Ruggedness studies
Robustness and ruggedness parameters were applied by making small deliberate changes of the conditions (mobile phase composition, mobile phase volume, saturation time, time from application to chromatography, time from chromatography to scanning and analysis) to validate the method.

RESULTS & DISCUSSION
Mixture of working standards of vasicine and vasicinone showed peak in HPTLC chromatogram along with their respective UV spectra. (Fig I). HPTLC chromatogram of methanolic extract showing the presence of two marker compounds such as vasicine and vasicinone along with their respective UV spectra is shown in fig. II. Spot of vasicine and vasicinone are visible with Rf = 0.1 - 0.11 and 0.45 – 0.48 at 254 nm as shown in photograph 1. The calibration curve of working standards vasicine and vasicinone was obtained by spotting standard vasicine and vasicinone on HPTLC plate after scanning at 280 nm as shown in figure III and IV. Various samples including raw herb, extracts and formulations of *Adhatoda vasica* were analysed by the proposed method and the data are recorded in table I.

**Table I: Percentage of Vasicine and Vasicinone in different samples of Adhatoda vasica by measuring area in HPTLC method**

<table>
<thead>
<tr>
<th>Sr.</th>
<th>Sample name</th>
<th>Vasicine content (% w/w)</th>
<th>Vasicinone content (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AD-1</td>
<td>0.05</td>
<td>0.015</td>
</tr>
<tr>
<td>2</td>
<td>AD-2</td>
<td>0.07</td>
<td>Not detected</td>
</tr>
<tr>
<td>3</td>
<td>Aqueous extract</td>
<td>0.3</td>
<td>0.12</td>
</tr>
<tr>
<td>4</td>
<td>Methanolic extract</td>
<td>0.75</td>
<td>0.45</td>
</tr>
<tr>
<td>5</td>
<td>IN-1</td>
<td>0.40 mg/ 5 ml of syrup</td>
<td>0.25 / 5 ml of syrup</td>
</tr>
</tbody>
</table>

**Fig.I: TLC Chromatogram of working standard vasicine and vasicinone in mixture along with UV spectra**

**Photograph 1: HPTLC Photograph of methanolic extract of Adhatoda vasica along with std. vasicine and vasicinone**

**Chromatographic precision and recoveries from spike sample solution**

Specificity
It was observed that the other phytoconstituents present in the extracts did not interfere with the peak of vasicine and vasicinone. Therefore the method was specific and helps in separation of vasicine and vasicinone from other constituents of herb and hence, helps to get the exact content of both vasicine and vasicinone. Test sample of aqueous and methanolic extract of *Adhatoda vasica* extract showed separated peak of vasicine and vasicinone along with other phytoconstituents as obtained in HPTLC chromatogram (Figure II).

**Limit of Detection**
By applying the proposed method, the minimum detectable limit of vasicine was found to be 1 nanogram / spot at 280 nm and vasicinone was found to be 25 nanogram / spot at 280 nm.
**Limit of Quantification**

By applying the proposed method, the minimum quantification limit of vasicine was found to be 5 nanogram / spot at 280 nm, the lowest concentration in linear range of vasicinone was found to be 50 nanogram / spot at 280 nm.

**Linearity**

The linearity of the method was checked with working standards vasicine with the calibration curve in the concentration range of 5 – 100 ng / spot based on a 0.5 - 10 µl sample volume. The regression equations (Y = 39.932 * X + 157.96) and correlation coefficient were obtained with 6 replicate analysis for each concentration. Correlation coefficients were obtained in the range of 0.9965-0.9986 indicated excellent linearity of the procedure for working standard vasicine analyzed. Calibration curve of working standard vasicine is shown in fig III.

![Vasicine Linearity](image)

**Fig. III: Calibration curve of working standard vasicine with respect to the area under curve at various concentrations.**

The linearity of the method was checked with working standards vasicinone with the calibration curve in the concentration range of 50 – 1000 ng / spot based on a 0.5 - 10 µl sample volume. The regression equations (Y = 5.3032 * X + 301.78) and correlation coefficient were obtained with 6 replicate analysis for each concentration. Correlation coefficients were obtained in the range of 0.9941 - 0.9954 indicated excellent linearity of the procedure for working standard vasicinone analyzed. Calibration curve of working standard vasicinone is shown in fig IV.

![Vasicinone linearity](image)

**Fig. IV: Calibration curve of working standard vasicinone with respect to the area under curve at various concentrations.**

**Accuracy and precision**

The method was applied to determine concentration of spiked vasicine and vasicinone in test sample with the range of 25 – 75 ng / spot for assessing the accuracy & precision of the procedure. Table II represents the mean values and coefficient variance (C.V.) results indicate the levels in the above range can be estimated with accuracy and precision. (Table II & III)

<table>
<thead>
<tr>
<th>Amount added (µg / spot)</th>
<th>Amount found (µg / spot, Mean ± S.D., n=6)</th>
<th>Precision / Reproducibility (C.V.)</th>
<th>Mean Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>24.74 ± 1.11</td>
<td>3.09</td>
<td>98.96</td>
</tr>
<tr>
<td>50</td>
<td>46.44 ± 3.26</td>
<td>2.26</td>
<td>95.33</td>
</tr>
<tr>
<td>75</td>
<td>73.75 ± 4.18</td>
<td>3.46</td>
<td>98.33</td>
</tr>
</tbody>
</table>

**Table II: Precision & accuracy of the method applied to spiked vasicine samples**

**Table III: Precision & accuracy of the method applied to spiked vasicinone samples**

<table>
<thead>
<tr>
<th>Amount added (µg / spot)</th>
<th>Amount found (µg / spot, Mean ± S.D., n=6)</th>
<th>Precision / Reproducibility (C.V.)</th>
<th>Mean Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>255.45 ± 18.41</td>
<td>5.25</td>
<td>102.18</td>
</tr>
<tr>
<td>500</td>
<td>475.84 ± 35.00</td>
<td>1.83</td>
<td>95.16</td>
</tr>
<tr>
<td>750</td>
<td>713.30 ± 50.13</td>
<td>1.64</td>
<td>95.10</td>
</tr>
</tbody>
</table>

**Intraday and interday analysis using vasicine and vasicinone**

Furthermore the precision and accuracy of the intraday and interday analysis were investigated on the basis of a set of standard solution. The results given in Table IV & V stands for a quite good trueness of the proposed method particularly considering interday and intraday analysis.

**Table IV: Intraday and interday precision & accuracy of the method applied to vasicine**

<table>
<thead>
<tr>
<th>Amount added (µg / spot)</th>
<th>Intraday Precision (R.S.D., %)</th>
<th>Intraday Accuracy (%)</th>
<th>Interday Precision (R.S.D., %)</th>
<th>Interday Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>4.89</td>
<td>94.39</td>
<td>4.28</td>
<td>95.23</td>
</tr>
<tr>
<td>50</td>
<td>3.24</td>
<td>96.32</td>
<td>3.92</td>
<td>95.62</td>
</tr>
<tr>
<td>75</td>
<td>1.32</td>
<td>98.46</td>
<td>4.26</td>
<td>104.9</td>
</tr>
</tbody>
</table>

**Table V: Intraday and interday precision & accuracy of the method applied to vasicinone**

<table>
<thead>
<tr>
<th>Amount added (µg / spot)</th>
<th>Intraday Precision (R.S.D., %)</th>
<th>Intraday Accuracy (%)</th>
<th>Interday Precision (R.S.D., %)</th>
<th>Interday Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>3.43</td>
<td>96</td>
<td>4.01</td>
<td>95.87</td>
</tr>
<tr>
<td>500</td>
<td>0.73</td>
<td>100.83</td>
<td>3.13</td>
<td>96.82</td>
</tr>
<tr>
<td>750</td>
<td>0.71</td>
<td>100.77</td>
<td>2.09</td>
<td>102.42</td>
</tr>
</tbody>
</table>

**Stability**

In the current assay, analyses of stability samples in methanol on consecutive days (24, 48, 72 and 96 hr) revealed that the major constituents, vasicine and vasicinone either in standard solution or in the methanolic extract of *Adhatoda vasica* are stable in solution.
form with relative standard deviation (RSD (%)) - 3.87 and 2.52 (n = 6) and for vasicine and vasicinone at 4°C respectively.

Robustness and Ruggedness studies
The method was found to be re-producible from one analyst to another. The low values of R.S.D. (2.97% - 3.54%) obtained after small deliberate changes of the conditions (mobile phase composition, mobile phase volume, saturation time, time from application to chromatography, and time from chromatography to scanning) used for the method indicated its robustness.

CONCLUSION
The lowest detectable limit of vasicine was found up to 1 ng / spot and of vasicinone was found to be 25 ng / spot. Good resolution and separation of vasicine and vasicinone from other constituents of Adhatoda vasica. Further, recovery values of vasicine and vasicinone were found to be about 95 – 102%, which shows the reliability and suitability of the method. The proposed HPTLC method is simple, rapid, reproducible, accurate and precise for quantitative monitoring of vasicine and vasicinone in Adhatoda vasica samples.

REFERENCES: