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Research Article

DEVELOPMENT AND VALIDATION OF REVERSE PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR QUANTITATIVE ESTIMATION OF VASICINE IN BULK AND PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

Objective: The aim was to develop and validate a new, rapid, and highly sensitive high performance liquid chromatography (HPLC) method for the quantitative estimation of vasicine in bulk and pharmaceutical dosage form, according to International Conference on Harmonization (ICH) guideline.

Methods: The chromatographic separation was achieved on an agilent 1200 series HPLC system phenyl (250 mm×4.6 mm×5 µm) column packing, using a mobile phase consisting of hexane sulphonic acid-acetonitrile-acetic acid (60:20:1; v/v/v) in isocratic mode. The flow rate was set at 1.0 ml/minute, and ultraviolet detection was monitored at 300 nm.

Results: The method was linear in the concentration range of 3.125-200 ppm/ml with a correlation coefficient of 0.999. The retention time for vasicine was found to be 5.30±0.05 minutes. The main recoveries obtained in the range of 90.476-107.1%, shows that the developed method was accurate and precise (<2% relative standard deviation). The lower limit of detection and limit of quantification were 3.0208 and 9.1541 µg/ml, respectively.

Conclusion: The proposed method met the general requirements with an acceptable performance for validation. This selective method is found to be reliable, accurate, and effectively used for the vasicine. The result showed that the method is achieved a good performance with simple, rapid and accurate characteristics for quantification of vasicine in pharmaceutical preparations. The proposed method can be employed for the routine analysis of the quality of herbal extracts and in formulations.

Keywords: High-performance liquid chromatography, Vasicine, International conference on harmonisation guidelines, Validation, Phenyl column.

INTRODUCTION

Adhatoda vasica, commonly known as vasaka, as Malabar nut tree in English and arusa or adulsa in local Hindi language [1], is a primary herb of ayurvedic system of medicine and has been used in indigenous systems of medicines in India [2]. The plant is widely employed for the treatment of various disorders of respiratory tract [3]. The leaves of the plant, employed in Ayurveda for the treatment of respiratory disorders since a long time. The most interesting point is that the lower concentrations of vasicine induce bronchodilation and relaxation of tracheal muscles whereas higher concentrations offered significant protection against histamine-induced bronchospasms in guinea pigs. Uterine stimulant effects have also been reported for vasicine [4]. The leaves of the plant was reported to contain the quinazoline alkaloids-vasicine, vasicinone, and deoxyvasicine [5], vasicinolone, vasicol, and peganine have also been reported in the roots, whereas bioflavonoid namely quercetin and kaempferol are reported in flowers [6]. Molecular formula and molecular weight of vasicine are $C_{11}H_{12}N_2O$; 188.22, respectively. IUPAC name of vasicine is 1,2,3,9-tetrahydropyrrolo [2,1-b] quinazolin-3-ol (Fig. 1).

High-performance liquid chromatography (HPLC) is one of the important tools for quality assessment, which includes preliminary phytochemical screening, chemoprofiling and marker compound analysis using modern analytical techniques. Use of chromatography for standardization of plant products was introduced by the WHO and is accepted as a strategy for identification and evaluation of the quality of plant medicines [7,8]. Literature survey reveals few analytical methods were reported for the determination of vasicine in bulk and pharmaceutical preparations by HPLC [9-11]. However, most of the available methods have limitations such as long runtimes, low sensitivity, uneconomical, and have poor symmetry. Keeping in view of these, an attempt has been made to develop a simple, accurate, precise and reliable reverse phase-HPLC method for the estimation of vasicine in pharmaceutical dosage forms. In fact, the established method was validated with respect to specificity, range and linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ), and robust as per International Conference on Harmonization (ICH) guidelines [12].

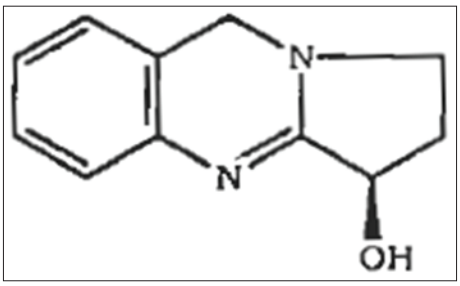

Fig. 1: Chemical structure of vasicine

METHODS

Materials

An analytically pure sample of vasicine standard was procured as gift sample from Natural Remedies Pvt. Ltd., Bangalore (India). All the

chemicals were analytical grade. HPLC grade acetonitrile was procured from Merck Pharmaceuticals Private Ltd., Mumbai, India. Hexane sulfonic acid was purchased from sigma to Aldrich. Acetic acid used was of HPLC grade and purchased from Merck Specialties Private Ltd., Mumbai, India. Commercial vasaka capsules (Himalaya Drugs Company Ltd.) was procured from the local pharmacy.

Instrumentation

The system used was an agilent 1200 rapid resolution liquid chromatography with a 1200 series binary pump SL and vacuum degasser, a 1200 series high-performance auto-sampler, a 1200 series thermo stated column compartment SL, a 1200 series DAD SL for up to 80 Hz operation which were controlled by chemstation B.02.01. SR1 data acquisition and evaluation.

Chromatographic conditions

An isocratic separation was carried out using a mobile phase consisting of Hexane sulfonic acid-acetonitrile-acetic acid (60:20:1; v/v/v) was used at a flow rate of 1.0 ml/minutes with ultraviolet (UV) detection at 300 nm with 8.0 minutes runtime. The column was heated to 25°C, and an injection volume of 10 µl was used. The mobile phase was filtered through 0.45 µm nylon filters and degassed in an ultrasonic bath prior to use.

Preparation of reagents and standard

Preparation of mobile phase

The mobile phase was prepared by mixing of hexane sulfonic acid, acetonitrile and acetic acid (all of HPLC grade) in the ratio of 60:20:1; v/v/v. It is filtered through 0.45 µm nylon membrane filter and then sonicated for degassing (Table 1).

Preparation of stock and working standard solutions

Accurately 10 mg of vasicine was weighed and transferred to a 10 ml clean volumetric flask, and mobile phase was added and sonicate to dissolve. The volume was made up to the mark with mobile phase. The stock standard solution of vasicine with concentration of 1000 ppm/ml. Prepare seven working standard solutions for calibration plot by adding defined volumes of the stock standard solution and diluting the standard with mobile phase. The concentrations of vasicine are 3.125, 6.25, 12.5, 25.0, 50.0, 100, and 200 ppm/ml, respectively.

Procedure for sample preparation/capsule analysis

Sample details: Vasaka 250.
Manufactured by: Himalaya Drug Co.

20 capsules were weighed; contents were finely powdered. A quantity of powder equivalent to 25 mg vasicine was weighed transferred to a 25 ml calibrated volumetric flask. 20 ml mobile phase was added to the same flask and sonicated for 10 minutes. The volume was made up to 25 ml with mobile phase. The solution was first filtered using Whatmann filter paper No. 41 and then through 0.45 µ filter paper in order to remove the recipients. From the filtrate, appropriate dilution was done in the mobile phase to get the concentration approximately of 100 ppm/ml. Such five replicates were made and injected into the system.

Selection of detection of wavelength

The UV spectrum of diluted solutions of various concentrations of vasicine in the mobile phase was recorded using UV spectrophotometer. The wavelength of maximum absorbance was observed at 300 nm. This wavelength was selected for detection of vasicine (Fig. 2).

Calibration curve of vasicine

Seven dilutions at concentration of stock solution ranging from 3.125 to 200 ppm/ml of vasicine were injected in triplicate. The linearity of peak area responses versus concentrations was demonstrated by linear least square regression analysis. A linear regression equation was y=16963x+23701. Linearity values were shown in Table 2, and the calibration curve is given in Fig. 3. A regression equation and co-efficient of correlation (r^2) was derived and shown in Table 3. The standard chromatograms of vasicine have been depicted in Figs. 4-10.

Assay of vasicine capsules

The developed method was applied to the assay of vasicine capsules. The drug content was calculated as an average of six determinations, and assay results were shown in Table 4. The results were very close

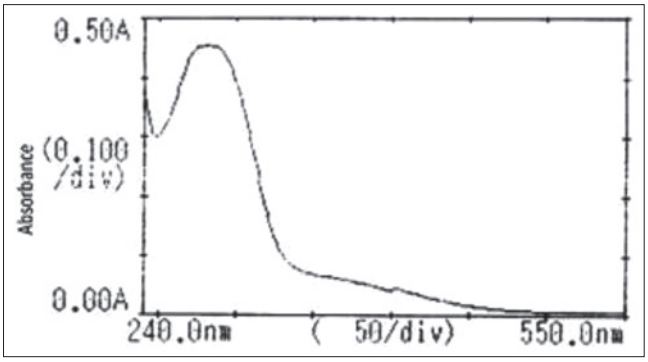

Fig. 2: Absorption spectra of vasicine

Table 1: Chromatographic conditions of vasicine

S. No.	Test conditions	Results
1	Elution	Isocratic
2	Wavelength	300 nm
3	Mobile phase	Hexane sulfonic acid-acetonitrile-acetic acid (60:20:1; v/v/v)
4	Column	Phenyl column
5	Retention time	5.30 minute
6	Flow rate	1 ml/minute
7	Run time	8.0 minute

Table 2: Linearity results for vasicine

S. No.	Linearity level	Concentration in ppm	Average area	Retention time
1.	I	3.125	61,338	5.300
2	II	6.25	116,354	5.300
3	III	12.5	229,531	5.293
4	IV	25	490,993	5.293
5	V	50	870,184	5.273
6	VI	100	1,714,704	5.267
7	VII	200	3,415,043	5.227

Table 3: Linear regression data of the proposed HPLC method of vasicine

Parameter	Method
Detection of wavelength	UV at 300 nm
Linearity range	3.125-200 ppm/ml
Regression equation (Y=a-bX)	Y=16963X+23701
Slope (b)	23701
Intercept (a)	16963
Correlation coefficient	0.999

UV: Ultraviolet, HPLC: High performance liquid chromatography

Table 4: Assay results of vasicine in formulation

Label claim mg/cap	Percentage of label claim	±SD	% RSD	SE
250	100.74	0.88	0.83	0.14

RSD: Relative standard deviation, SD: Standard deviation, SE: Standard error

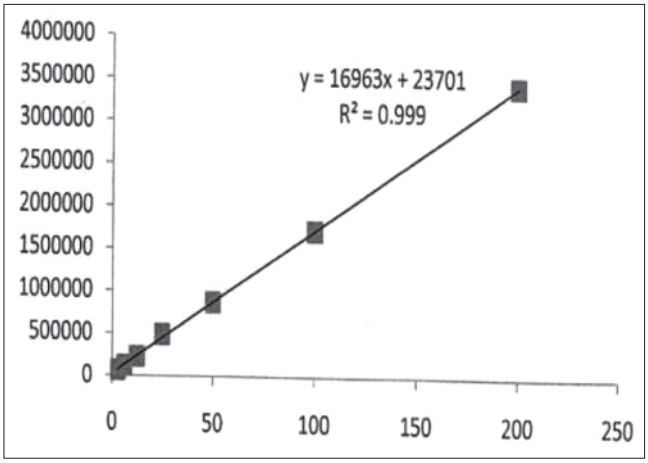

Fig. 3: Calibration plot of vasicine

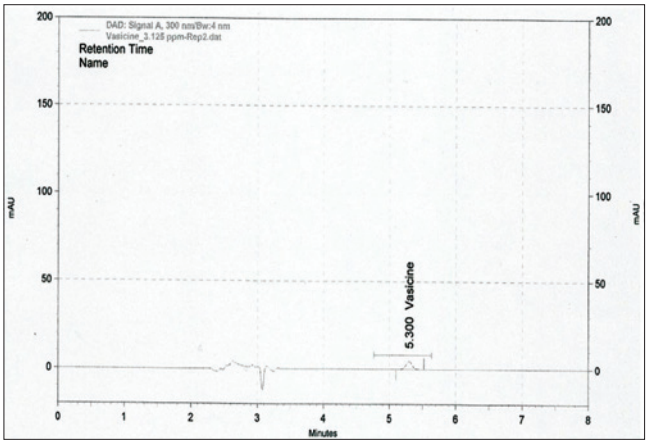

Fig. 4: Standard chromatogram of vasicine 3.125 ppm/ml

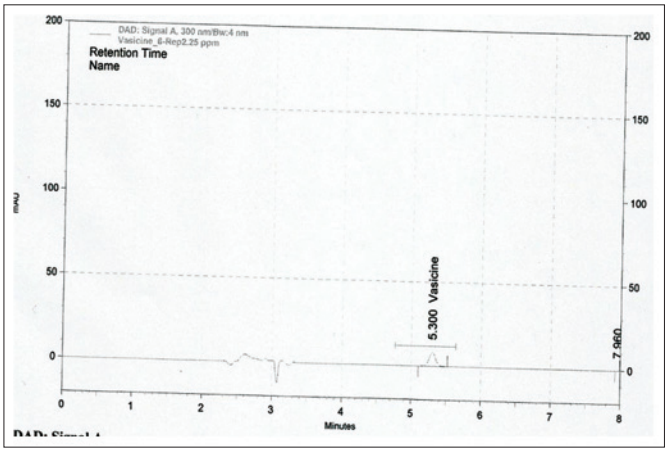

Fig. 5: Standard chromatogram of vasicine 12.5 ppm/ml

to the labeled value of commercial capsules. The representative sample chromatogram of vasicine is shown in Fig. 13.

Method validation

The method was validated with respect to parameters, laid out by the (ICH 2002), including system suitability, linearity, accuracy, precision, robustness, LOD, and LOQ.

System suitability

A system suitability test was performed to evaluate the chromatographic parameter (retention time, capacity factor, theoretical plate number,

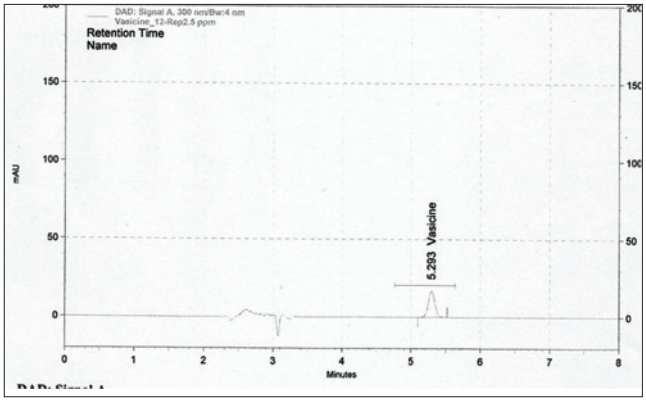

Fig. 6: Standard chromatogram of vasicine 6.25 ppm/ml

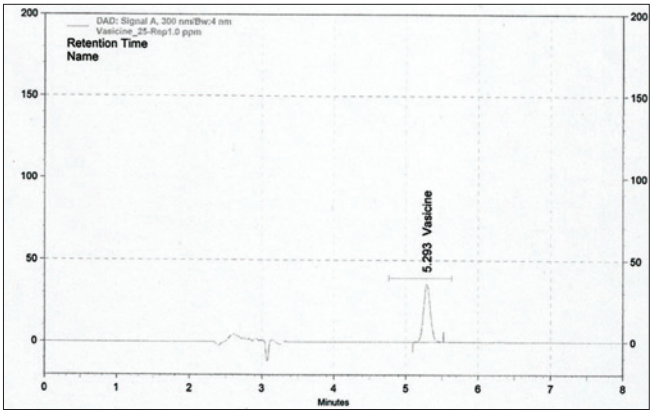

Fig. 7: Standard chromatogram of vasicine 25 ppm/ml

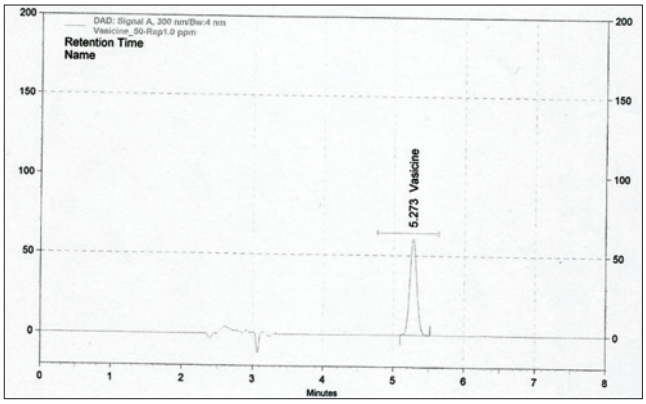

Fig. 8: Standard chromatogram of vasicine 50 ppm/ml

and tailing factor) before the sample analysis. The system suitability data are reported in Table 5.

Specificity

The specificity of the method was determined by observing interference of any encountered ingredients present in the formulations. The test results obtained were compared with the results of those obtained for standard drug. It was shown that those ingredients were not interfering with the developed method. Furthermore, the well-shaped peaks also indicate the specificity of the method. The results for specificity are tabulated in Table 6. The chromatogram for placebo indicating the specificity of developed method is presented in Fig. 12.

Precision

The precision was examined by performing the intra-day and inter-day assays of six replicate injections of the mixture of standard solutions

Table 5: System suitability

S. No.	Injection of standard	Retention time	Area of vasicine	Average area	% RSD	Number of theoretical plates	Percentage of RSD	Tailing factor
1	1	5.300	877,875			13,503		1.16
2	2	5.300	879,400			13,499		1.18
3	3	5.307	880,455	879211.33	0.10	13,583	0.24	1.19
4	4	5.307	879,745			13,506		1.19
5	5	5.307	878,803			13,520		1.12
6	6	5.307	878,988			13,505		1.12

RSD: Relative standard deviation

Table 6: Specificity study for vasicine

Name of the solution	Retention time
Mobile phase	No peak
Placebo	No peak
Vasicine 25 ppm/ml	5.293 minutes

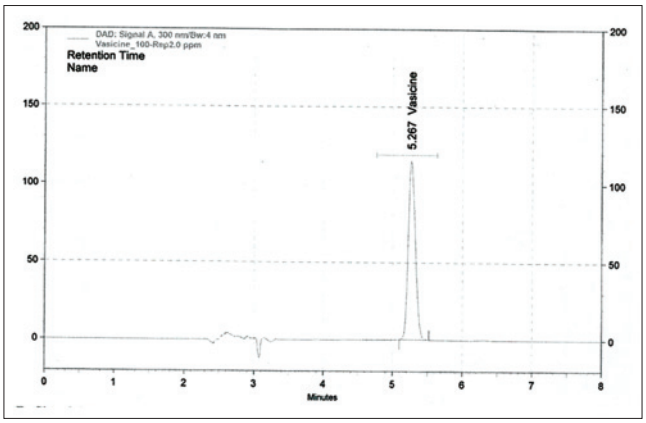

Fig. 9: Standard chromatogram of of vasicine 100 ppm/ml

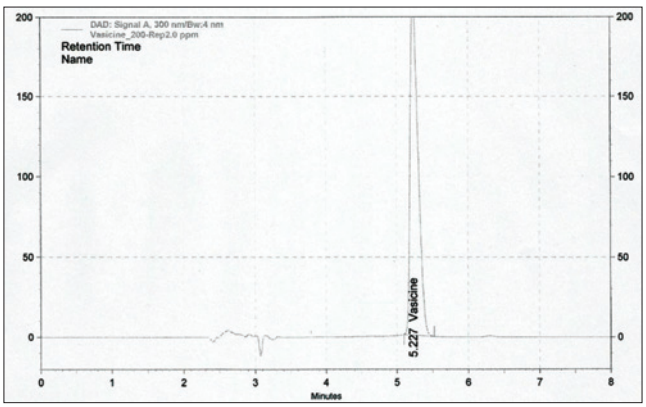

Fig. 10: Standard chromatogram vasicine 200 ppm/ml

at two concentration levels (50 and 200 ppm/ml). The intra-day assay precision was performed with the interval of 4 hrs in 1 day while the inter-day assay precision was performed over 2 days. The percent relative standard deviation (% RSD) was calculated which is within the acceptable criteria of not more than 2.0. The intra-day and inter-day precision results are shown in Table 7.

LOD and LOQ

The LOD and LOQ were determined by kD/S where k is constant (3.3 for LOD and 10 for LOQ), SD is the standard deviation of the analytical signal, and s is the slope of the concentration/response graph (Table 8).

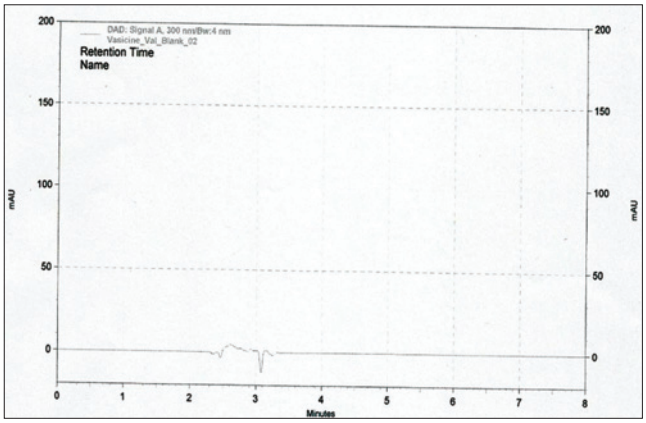

Fig. 11: Chromatogram of placebo

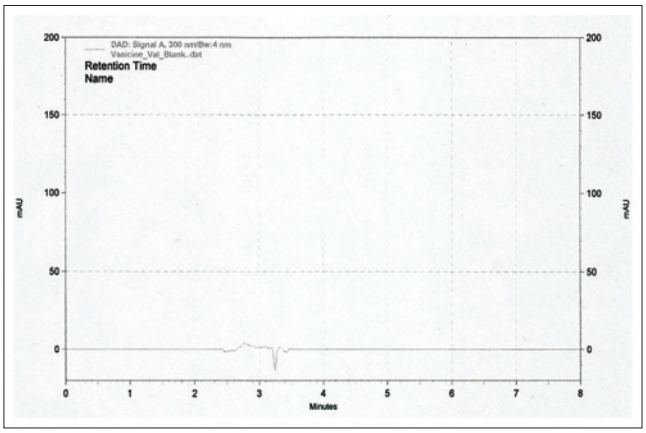

Fig. 12: Chromatogram for blank

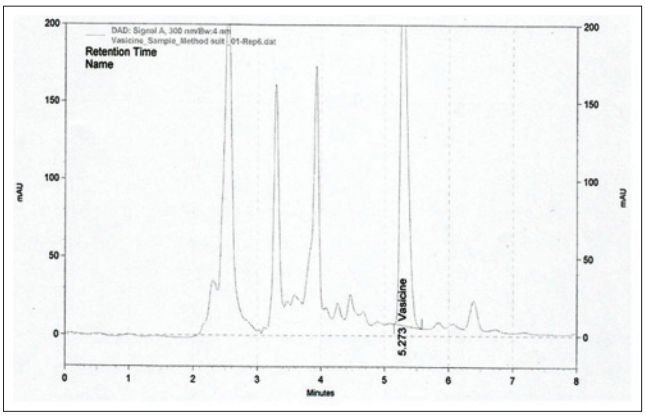

Fig. 13: Chromatogram of vasicine in market formulation

Table 7: Results of precision study for vasicine

S. No.	Injection	Retention time	Peak area	Average	SD	% RSD
System precision						
1	Injection 1	5.3	877,875	879,211	879.96	0.100
2	Injection 2	5.3	879,400			
3	Injection 3	5.3	880,455			
4	Injection 4	5.3	879,747			
5	Injection 5	5.3	878,803			
6	Injection 6	5.3	878,988			
Method precision						
1	Replicate 1	5.2	3,957,897	3985703.667	44781.09	1.123
2	Replicate 2	5.2	3,956,130			
3	Replicate 3	5.2	3,938,170			
4	Replicate 4	5.2	4,037,274			
5	Replicate 5	5.2	4,044,291			
6	Replicate 6	5.2	3,980,460			

Intermediate precision

S. No.	Analyst	Retention time	Area 1	Area 2	Average area	SD	% RSD*
1	1	5.3	881,726	881,524	887,340	8252.4047	0.9300
2	2	5.3	886,987	889,123			

*Acceptance criteria<2.0. RSD: Relative standard deviation, SD: Standard deviation

Table 8: LOD and LOQ

LOD	3.0208552
LOQ	9.1541074

LOD: Limit of detection, LOQ: Limit of quantification

Accuracy

The accuracy of the method was ascertained by recovery experiments. The known concentration of working standard was added to the fixed concentration of the pre-analyzed extract solution. Percent recovery was calculated by comparing the area before and after the addition of the working standard. The recovery studies were performed in triplicate. The standard addition method was performed at 50, 100, 150 ppm/ml, and percentage recovery was calculated. Results are given in Table 9.

Robustness

The robustness of the method was evaluated by analyzing the system suitability standards and evaluating system suitability parameter data after varying, individually, the HPLC pump flow rate (±0.1), wavelength (±2), and temperature (±2). Solution was injected 3 times for each change. Mean and SD were calculated for each peak. % RSDs were calculated for each component during each change equation of the calibration curve.

RESULT AND DISCUSSION

A simple HPLC method was adopted for the determination of vasicine in herbal formulation. To optimize the proposed HPLC method, all of the experimental conditions were investigated. Different mobile phases were tried to optimize but satisfactory separation, well-resolved and good symmetrical peaks were obtained with the mobile phase consisting hexane sulfonic acid, acetonitrile, and acetic acid (60:20:1; v/v/v) as compared to other mobile phase. The absorption spectrum of vasicine is shown in Fig. 2. The wavelength 300 nm was used for quantification of sample. The retention time of vasicine was found to be 5.30±0.05 minutes, which indicates a good baseline. This indicates that the present HPLC method is rapid, easy, and convenient. The correlation coefficient (0.999) of regression was found almost equal to 1 in the range of 3.125-200 ppm/ml with % RSD value <2 which states that the method was linear to the concentration versus peak area responses. A system suitability test was performed to evaluate the chromatographic parameters (retention time, theoretical plate number and tailing factor) before the validations run (Table 5). The accuracy was studied by the standard addition technique. Three different levels of standard were added to the previously analyzed samples, each level being repeated thrice. The recovery of vasicine was found to be in the range of 90.47-107.1 in herbal formulation as shown in (Table 9). Robustness of the method reflects the reliability of an analysis with respect to deliberate variations in the flow rate, wavelength and temperature were slightly changed to lower and higher sides of the actual values to find if the change in the peak area, plate count, retention time, and tailing factor were within limits which indicated that the method is robust. The results obtained with changes in the parameters are as shown in Table 10. The comparison of chromatograms of placebo, standard, and sample, there was no interference observed from the peaks of placebo, standard, and sample. It shows that the method is specific. The precision result of the solution at medium concentration is presented in Table 7, and it was shown that the RSD values of retention time were <1%, while the RSD values of peak area were <2 % both for intra-day assay and inter-day assay precision (intra 4 hrs six injections, inter 2 days). The LOD and LOQ were found to be 3.02 and 9.154 µg/ml, respectively, for vasicine. Hence, the proposed method was found to be precise.

CONCLUSIONS

The proposed method was found to be simple, precise, accurate, rapid, and specific for the determination of vasicine from pure and its dosage forms. This method was isocratic, and the mobile phase does not contain any buffer and validated for the determination of purity and assay of vasicine. The sample recoveries in the formulation were in good agreement with their respective label claims and suggested non-interference of formulation excipients in the estimation. The drug solutions employed in the study were stable up to 48 hrs. These attribute the high quality of the method. Furthermore, the lower solvent consumption along with a significantly reduced run time leads to an environmentally friendly economically analytical procedure that allows for the analysis of a large number of samples over a short period. The proposed method can be used for the routine analysis of vasicine in bulk preparations of the drug and pharmaceutical dosage forms.

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Table 9: Recovery study of vasicine added to preanalyzed sample using the proposed HPLC method (n=3)

Level/ppm	Replicates	Area	Average practical area	Area of standard	Concentration of standard	Concentration of test	% Recovery
50/75	Replicate 1	1401221	1399629.5	879211	0.25	2.7	107.1
	Replicate 2	1398038					
100/100	Replicate 1	1783144	1773041	879211	0.25	1.8	90.476
	Replicate 2	1762938					
150/125	Replicate 1	2227001	2246937.5	879211	0.25	1.6	101.918
	Replicate 2	2266874					

HPLC: High-performance liquid chromatography

Table 10: Robustness

S. No.	Condition varied	Changed conditions	Retention time	Peak area	Theoretical plates	Tailing factor	Average SD	% RSD
1	Wavelength (nm)	298	5.3	1007712	13930	1.1	16720.95	1.68
		300	5.3	984065	13406	1.1		
		302	5.3	995888	13668	1.1		
2	Temperature (°C)	23	5.4	907140	12741	1.1	1678.67	0.18
		25	5.4	909514	12516	1.1		
		27	5.5	908327	12967	1.1		
3	Flow rate (ml/minute)	0.9	5.9	1005837	13864	1.1	824561.5	0.04
		1.0	5.3	116354	12650	1.1		
		1.1	4.8	824348	12642	1.1		

RSD: Relative standard deviation, SD: Standard deviation

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