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Full Length Research Paper

Enhancement and approval of converse stage HPLC and HPTLC technique for concurrent evaluation of vasicine and vasicinone in SIDA Species

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Quantification of bioactive principles through modern analytical tools is essential for establishing the authenticity and creditability of prescription and usage of herbal drugs. In the present study, simultaneous quantification of vasicine and vasicinone, present in SIDA species (Malvaceae), by reverse phase HPLC and HPTLC methods were developed. In the RP-HPLC method, the extracts were resolved using a mobile phase of acetonitrile - 0.1 M phosphate buffer - glacial acetic acid (15:85:1, V/V/V) with pH 4.0 on a C18-ODS-Hypersil column in isocratic mode. The retention times of vasicine and vasicinone were 5 and 8.7 min, respectively. In the HPTLC method, mobile phase of ethyl acetate, methanol, ammonia (8:2: 0.2, V/V) was used on precoated plate of silica gel 60 F254 and quantified by densitometric method. Validation of the methods was done to demonstrate its selectivity, linearity, precision and accuracy as recommended in the ICH guidelines. Excellent linear behaviors over the investigated concentration ranges were observed with the values of R2 higher than 0.998 for both the analytes. Recovery values between 97 - 101%, and correlation coefficient between (linear dynamic range) 0.977 - 0.9999 shows that the developed methods were accurate and precise. These methods can be employed for the routine analysis of the quality of herbal extracts and formulations.

Key words: Sida cordifolia, Sida acuta, Malvaceae, quantification, validation, HPLC, HPTLC.

INTRODUCTION

Phytochemical evaluation is one of the 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 (Anonymous, 1992; Farnsworth et al., 1985). HPLC and HPTLC both emerged as efficient tool for the phytochemical evaluation. HPTLC is a widely accepted technique for its high accuracy, precision and reproducibility of results. In addition, HPTLC has many advantages because of high sample throughput at low operating cost, easy sample preparation, short analysis time and analytical assurance (Di et al., 2003; Larsen et al., 2004). Chromatographic methods play an important role in the pharmaceutical field, hence, need to validate the method when they are developed and intended to be for routine use.

There are many species of *Sida* which are known by the name *Bala chatushtya*, used in Ayurveda for treating the disorders of the nervous system. According to Ayurveda 'Bala' balances all the doshas – *vata, pitta, kapha*. Rajanighantu describes this herb as extremely bitter (atitikta), yet madhura, and it is beneficial in deranged pitta. The rejuvenating action of this herb extends to the nervous, circulatory, and urinary systems (Chopra et al., 1958). Bala is one of the important ingredients of 'Chyavanprash'. Chyavanprash is a popular ayurvedic preparations placed under 'Rasayana' group of drugs, widely used in improving health and prevention of the diseases as tonic. *Sida cordifolia* Linn. (Family: Malvaceae) is well known in India as Bala (Anonymous, 1998). It grows to a height of 3 - 5 feet and is extensively used as a common herbal drug in

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the Indian subcontinent. The roots, leaves, stems and seeds of *S. cordifolia* are used as traditional medicine against chronic dysentery, asthma and gonorrhea (Yusuf, 1999). Pharmacological investigation carried out with aqueous extract of *S. cordifolia* leaves exhibited an anti-inflammatory and analgesic activity in animal models (Franzotti et al., 2000; Sutradhar et al., 2006). Results of another study showed that *S. cordifolia* has depressant effect on CNS without interfering with motor coordination with a low toxicity (Franco et al., 2005). Another species of *S. acuta* called 'rajbala' or 'brihannagabala'. It is valued as a reliever of stomachache, and is a useful remedy in chronic bowel complaints (Chopra et al., 1958). Phytochemical studies on the roots had shown the presence of ecdysterone, ephedrine, vasicine, vasicinol, vasicinone and N- methyl tryptophan (Asha and Bannerjee, 1985; Ghosh and Dutt, 1930; Gunatilaka et al., 1980). Among the published literature there are no experimental data found which include development, optimization, and validation of vasicine and vasicinone in *Sida* species. The present work reported two new methods for simultaneous determination and validation of vasicine and vasicinone in *S. cordifolia and S. acuta* using HPTLC-densitometry and reverse phase HPLC. The proposed methods were validated as per ICH guidelines. Both methods are simple and suitable for routine determination of two drugs and will reduce the duration of analysis.

MATERIALS AND METHODS

Chemicals and reagents

Reference standard vasicine and vasicinone (purity 98%, *w/w*) were from Natural Remedies pvt. Ltd. Bangalore, kindly provided as a gift sample. All the solvents used in the experiments were of HPLC grade. Purified HPLC grade water was obtained by reverse osmosis and filtration through a Milli-Q® system (Millipore, Milford, MA, USA). Potassium dihydrogen phosphate and ortho-phosphoric acid were obtained from Merck Chemicals Ltd. All the chemicals and solvents used were of analytical grade and purchased from Merck Chemicals, India.

Plant material

Roots of *S. cordifolia* and *S. acuta* were collected from Pune region, India. The samples were authenticated and voucher specimens were deposited in the authors Department of Pharmacognosy and Phytochemistry. The samples were stored at 25ºC in airtight container and powdered to 40 mesh when required.

Sample preparation

Sample of air dried and powdered roots (1 g) were extracted with methanol three times (50 ml each time for 3 h) separately. The extracts were filtered and concentrated under vacuum. The methanolic extract was extracted with hexane treated with 5% acetic acid, to remove fatty substance. Then it was basified with ammonia (pH 9.0) and again extracted with chloroform. The chloroform extracts were concentrated, made up to 1.0 ml in methanol and filtered through a 0.45 µm filter for HPLC and HPTLC analysis.

HPTLC instrumentation

HPTLC was performed on 20 × 10 cm aluminium-backed HPTLC plates coated with silica gel 60 F_{254}, 0.25 mm layer thickness. (E. Merck, Darmstadt, Germany; supplied by Anchrom Technologists, Mumbai, India). Before use the plates were prewashed with methanol and activated at 60°C for 5 min. Samples were applied as 6 mm wide bands, 6 mm apart, by the spray-on technique, by means of a Camag (Switzerland) Linomat V sample applicator fitted with a 100-µL syringe (Hamilton, Bonaduz, Switzerland). A constant application rate of 0.1 µL s^{-1} was used. Plates were developed to a distance of 9 cm, with ethyl acetate: methanol: ammonia (8: 2: 0.2, *v/v*), as mobile phase. The volume of mobile phase was 15 ml. Before development the chamber was saturated with mobile phase for 20 min at room temperature (25 ± 2°C). Chromatography was performed in a controlled humidity chamber; relative humidity was fixed at 60 ± 5%. These conditions resulted in good resolution. Densitometric scanning was performed with a Camag TLC scanner 3, under control of WinCATS software (Camag). The slit dimensions were 5 × 0.45 mm and the scanning speed was 10 mm s^{-1}.

HPLC instrumentation

The HPLC system of Jasco consists of a pump (model Jasco PU2080, intelligent HPLC pump) with injecting facility programmed at 20 µL capacity per injection was used. The detector consists of a UV/ VIS (Jasco UV 2075) model operated at a wavelength of 300 nm. The software used was Jasco Borwin version 1.5, LC-Net II/ADC system. The column was Thermo ODS Hypersil C_{18} (250 x 4.6 mm, 5 µm) in isocratic mode. The separation was achieved using a mobile phase of acetonitrile - 0.1M phosphate buffer - glacial acetic acid (15: 85: 1, *v/v/v*) with pH adjusted to 4.0 using phosphoric acid at a flow-rate of 1.0 ml/min. The effluent was monitored using UV detection at a wavelength of 300 nm. The mobile phase was filtered through 0.45 µm nylon filter prior to use.

Preparation of stock solutions of standards

Stock solution of vasicine and vasicinone (100 µg/ml) were prepared by accurately weighing approximately 10 mg pure standard of vasicine and vasicinone in a 100 ml volumetric flask and making up to volume with HPLC grade methanol. The stock solution was protected from light using aluminium foil. Aliquots of the standard stock solution of vasicine and vasicinone were transferred using bulb pipettes into 10 ml volumetric flasks and the solutions were made up to volume with mobile phase to give final concentrations for calibration curve.

Calibration curves by HPTLC–densitometric method

The standard solutions were prepared by dilution of the stock solution with methanol to reach a concentration range 320 - 960 and 80 - 400 ng/spot for vasicine and vasicinone, respectively. Each standard solution was spotted on the HPTLC plate. The plates were developed on previously described mobile phase. Then the plates were scanned at 298 nm for vasicine and vasicinone. Calibration curves of vasicine and vasicinone were prepared by plotting peak areas *vs* concentration.

Calibration curves by HPLC method

The standard solutions were prepared by dilution of the stock solution with mobile phase to reach a concentration range 4 - 20 µg/ml for vasicine and vasicinone, respectively. Triplicate 20 µL injections were made for each concentration for vasicine and vasicinone, respectively and chromatographed under the conditions described above. Calibration curves of vasicine and vasicinone were prepared by plotting peak areas *vs* concentration.

Method validation

Precision

Precision of the method was determined with the standard vasicine and vasicinone solution. System repeatability was determined by six replicate applications and six times measurement of a sample solution at the analytical concentration. The repeatability of sample application and measurement of peak area were expressed in terms of relative standard deviation (% RSD) and standard error (SE). Method repeatability was obtained from RSD value by repeating the assay six times in same day for intra-day precision. Intermediate precision was assessed by the assay of two sets on different days (inter-day precision). The intra-day and inter-day variation by HPTLC was carried out at three different concentration levels 320, 480, 640 ng/spot and 160, 320, 400 ng/spot of vasicine and vasicinone respectively. It was carried out at three different concentration levels 8, 16, 20 µg/ml and 8, 16, 20 µg/ml of vasicine and vasicinone, respectively.

Limit of detection and quantification

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. The quantification limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. The quantification limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products. The limit of detection (LOD) and limit of quantification (LOQ) were separately determined at a signal to noise ratio (S/N) of 3 and 10, respectively.

Specificity

HPTLC–DENSITOMETRIC METHOD

The specificity of the method was ascertained by analyzing standard drug and sample. The spots for vasicine and vasicinone in sample were confirmed by comparing the R_f and UV spectra of the spot with that of standard. The peak purity of vasicine and vasicinone was assessed by comparing the spectra at three different levels, i.e., peak start, peak middle and peak end positions of the spot.

HPLC METHOD

The specificity of the HPLC method was determined by the complete separation of vasicine and vasicinone along with other parameters like retention time (Rt), capacity factor (*k*), tailing or asymmetrical factor (*T*), etc.

Recovery studies

According to ICH guidelines, the accuracy of an analytical method expresses the closeness of agreement between the value that is accepted either as a conventional true value or an accepted reference value and the value found. Accuracy is often calculated as percent recovery by the assay of known added amount of the analyte to the sample. Accuracy of the method was tested by performing recovery studies at three levels (50, 100 and 150% addition). To the powdered roots of *S. cordifolia,* known amounts of vasicine and vasicinone standard were added, extracted and estimated as described above. The percent recovery as well as average percent recovery was calculated.

Estimation of vasicine and vasicinone in *SIDA CORDIFOLIA* and *SIDA ACUTA*

To determine the content of vasicine and vasicinone in extracts, 500 mg was transferred into a 100 ml volumetric flask containing 50 ml methanol, sonicated for 30 min and diluted to 100 ml with methanol. The resulting solution was centrifuged at 3000 rpm for 15 min and supernatant was analyzed for drug content. Estimation by HPTLC was done as described in above section. The peak areas were plotted for quantification of markers compound at 298 nm. By HPLC, the eluent was monitored using UV detection at a wavelength of 300 nm. The possibility of interference from other components of extract in the analysis was studied.

RESULT AND DISCUSSION

Optimization of HPTLC–densitometric method and HPLC method

Initially different mobile phase on HPTLC were tried but the developed spots lack compactness and were less persistent. Mobile phase, ethyl acetate and methanol (8:2 *v/v*) with ammonia in different ratios were tried. Then 0.2 ml of ammonia was added to ethyl acetate and methanol (8:2 *v/v*). Total dragging of the spots from the point of sample application was observed. Mobile phase consisting of ethyl acetate: methanol: ammonia (8:2: 0.2, *v/v*) gave good resolution and the spots developed were dense, compact and typical peak nature for both vasicine and vasicinone was achieved. In HPLC method, there was a good resolution in acetonitrile - 0.1 M phosphate buffer - glacial acetic acid with pH adjusted to 4.0 using phosphoric acid (15:85:1, *v/v/v*) and this ratio was selected for validation purpose also.

Method validation

Precision for HPTLC–densitometric method

The repeatability of sample application and measurement of peak area were expressed in terms of percentage RSD and were found to be 0.93, 0.59 and 0.65, 0.94 for vasicine and vasicinone, respectively (Table 3). The percentage RSD values depicted in Table 1 shows that proposed method provides acceptable intra-day and inter-day variation of vasicine and vasicinone.

Table 1. Intra-day and inter-day precision of HPTLC and HPLC method (n = 6).

HPTLC	Intra-day precision			Inter-day precision		
Vasicine	**SD of areas**	**RSD %**	**SE**	**SD of areas**	**RSD %**	**SE**
320	1.84	1.55	0.78	2.24	1.85	1.02
480	1.56	1.33	1.11	2.11	1.44	1.57
640	2.00	1.39	1.22	1.8	1.29	0.56
Vasicinone						
160	0.98	0.45	0.87	1.33	1.09	1.45
320	1.76	1.05	1.34	1.37	1.92	1.77
400	1.56	1.74	1.28	1.74	1.04	0.99
HPLC	**Intra-day precision**			**Inter-day precision**		
Vasicine	**SD of areas**	**RSD %**	**SE**	**SD of areas**	**RSD %**	**SE**
8	1.02	0.88	1.45	1.09	1.55	1.04
16	1.33	0.45	2.00	1.37	1.92	1.06
20	1.33	2.11	1.22	0.67	1.85	0.56
Vasicinone						
8	0.90	1.11	0.87	1.39	1.8	1.29
16	1.51	1.22	1.24	1.57	2.11	1.57
20	1.08	1.43	1.18	1.74	1.44	0.88

Precision for HPLC method

The within-run precision and between-run of the proposed HPLC method were determined by assaying in six times per day for consecutive six days and expressed as percentage RSD (Table 3). The intra -day and inter-day precision has been depicted in Table 1.

Linearity

Vasicine showed good correlation coefficient in concentration range of 320 - 960 ng/spot (r = 0.9994) and 4 - 20 µg/ml (r = 0.999) where as vasicinone in the concentration range of 80 - 400 ng/spot (r = 0.9996) and 4 - 20 µg/ml (r = 0.998) for HPTLC and HPLC, respectively. Linearity was evaluated by determining five standard working solutions containing 320 - 960 ng/spot, 80 - 400 ng/spot by HPTLC and 4 - 20 µg/ml and 4 - 20 µg/ml by HPLC for vasicine and vasicinone, respectively (Table 2). Peak area and concentration was subjected to least square linear regression analysis to calculate the calibration equation.

LOD and LOQ

FOR HPTLC METHOD

The LOD was found to be 80 and 40 ng/spot for vasicine and vasicinone respectively. LOQ was found to be 100 and 60 ng/spot for vasicine and vasicinone, respectively (Table 3).

FOR HPLC METHOD

The LOD was found to be 0.5 and 0.5 µg/ml for vasicine and vasicinone respectively. LOQ was found to be 0.8 and 0.8 µg/ml for vasicine and vasicinone, respectively (Table 3).

Specificity

FOR HPTLC METHOD

The peak purity of vasicine and vasicinone was assessed by comparing their respective spectra at peak start, peak apex and peak end positions of the spot, that is, r (S, M) = 0.9993, 0.9995 and r (M, E) = 0.9992, 0.9997. Good correlation (r = 0.9992 and r = 0.9996) was also obtained between standard and sample spectra of vasicine and vasicinone.

FOR HPLC METHOD

The specificity of the HPLC method found complete separation of vasicine and vasicinone. The average retention time ± standard deviation for vasicine and

Table 2. Linear regression data for calibration curves (n=6), [a]95% confidence limit.

Parameters	HPTLC densitometry		HPLC	
	Vasicine	Vasicinone	Vasicine	Vasicinone
Linearity range	320 - 960 ng/spot	80 - 400 ng/spot	4 - 20 g/ml	4 - 20 g/ml
R±SD	0.9994 ± 1.31	0.999 ± 1.73	0.999 ± 0.87	0.998 ± 1.37
Slope ± SD	8.351 ± 0.76	19.516 ± 1.23	400535 ± 1.51	520770 ± 2.11
Intercept ± SD	1010.0 ± 2.58	-158.715 ± 0.98	136533 ±1.22	31633 ± 1.57
Confidence limit of slope[a]	7.865 - 8.901	17.62 - 21.11	379905 - 421165	482416 - 559124
Confidence limit of intercept[a]	950.2 - 1161.44	-420.70 - 03.27	68109 - 204956	95573 -158839

Table 3. Summary of validation parameters.

Parameters	HPTLC Densitometric		HPLC	
	Vasicine	Vasicinone	Vasicine	Vasicinone
Precision (RSD % n = 6)				
Repeatability of application	0.93	0.59	0.00	0.00
Repeatability of measurement	0.65	0.94	0.00	0.00
Injection repeatability	0.00	0.00	0.79	0.83
Analysis repeatability	0.00	0.00	1.02	0.99
Limit of detection	80 ng	40 ng	0.5 g/ml	0.5 g/ml
Limit of quantification	100 ng	60 ng	0.8 g/ml	0.8 g/ml
Linearity range	320 - 960 ng	80 - 400 ng	4 - 20 g/ml	4 - 20 g/ml
Average recovery, (%)	99.75	100.123	100.39	99.86.
Robustness	Robust	Robust	Robust	Robust
Specificity	Specific	Specific	Specific	Specific

vasicinone were found to be 5 ± 0.05 and 8.7 ± 0.07 min, respectively, for six replicates. The peaks obtained were sharp and have clear baseline separation.

Recovery studies

Recovery studies at three different levels were done on *S. cordifolia* extract by accurately spiked with various concentrations of reference solutions. The proposed method when used for extraction and subsequent estimation of vasicine and vasicinone from sample after spiking with 50, 100 and 150% of additional vasicine and vasicinone standard gave good results. The percentage recovery by HPTLC at three different levels of vasicine was found to be 99.39, 99.00 and 100.87% with an average of 99.75%, and that of vasicinone was found to be 99.20, 101.38, and 99.00% with an average of 100.123% (Table 4). The percentage recovery by HPLC at three different levels of vasicine was found to be 100.33, 99.84 and 101.01% with an average of 100.39%, and that of vasicinone was found to be 99.33, 100.11 and 100.14% with an average of 99.86%. The results are presented in Table 4.

Estimation of vasicine and vasicinone in herbal extract and marketed formulation

By HPTLC–densitometric method

Two spots at R_f 0.3 (for vasicine) and 0.61 (for vasicinone) were observed in the chromatogram of the sample extract along with other components. There was no interference in analysis from the other components present in the extract (Figure 1). These components appear in the chromatogram at significantly different R_f values. The vasicine and vasicinone content in *Sida cordifolia* and *Sida acuta* were estimated by the proposed method and the results were represented in Table 5.

By HPLC method

Twenty microliter each of suitably diluted different sample solution were injected in triplicate in the HPLC system. The peak areas for each were recorded. The amount of vasicine and vasicinone in the samples extracts were calculated using the linear regression equation derived from the calibration curves. Two peaks at R_t 5 and 8.7

Table 4. Standard addition technique for determination of vasicine and vasicinone by HPTLC and HPLC method.
* Mean ± standard deviation (*n* = 3).

Amount added %	Amount found*	Recovery (%)	RSD (%)	SE
Vasicine (HPTLC method)				
50	0.0165 ± 0.29	99.39	1.88	0.52
100	0.023 ± 0.25	99.00	2.10	0.35
150	0.0285 ± 0.11	100.87	1.55	0.85
Vasicinone				
50	0.0090 ± 0.22	99.20	1.73	1.95
100	0.0125 ± 0.19	101.38	2.13	1.31
150	0.0148 ± 0.28	99.00	1.65	1.75
Vasicine (HPLC method)				
50	1.75 ± 0.19	100.33	1.58	0.92
100	2.28 ± 0.27	99.84	2.21	0.81
150	2.91 ± 0.11	101.01	1.82	0.39
Vasicinone				
50	1.75 ± 0.19	99.33	1.78	0.52
100	2.28 ± 0.27	100.11	2.00	0.39
150	2.91 ± 0.11	100.14	1.72	0.81

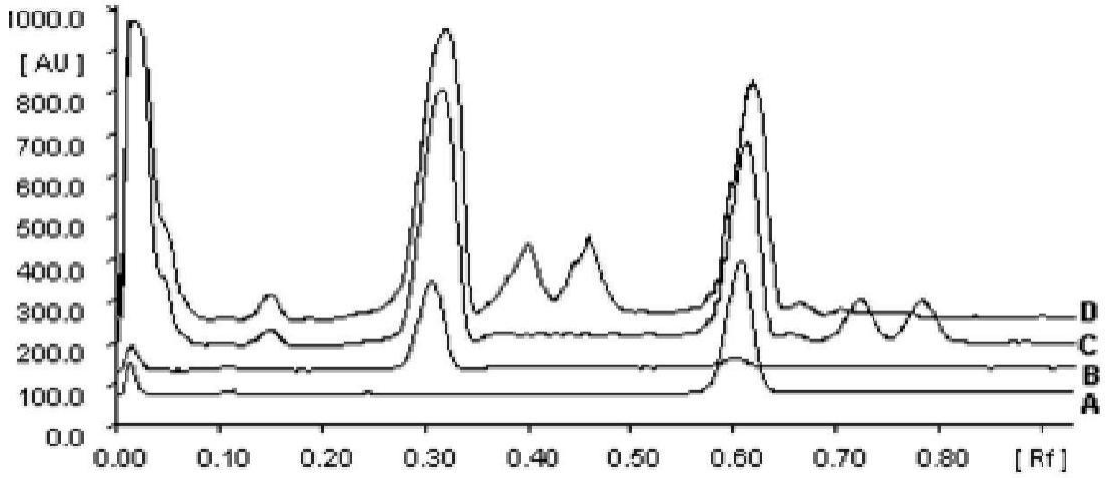

Figure 1. HPTLC densitograms of standard along with extract of *Sida cordifolia* and *Sida acuta.* (A) Vasicinone standard, (B) Vasicine standard (C) *Sida acuta* (D) *Sida cordifolia* extract at 298 nm.

(Figure 2) were observed in the chromatograms of the extracts along with other components. There was no interference in analysis of vasicine and vasicinone from the other components present in the extract. These components appear in the chromatogram at significantly different R_f values. The vasicine and vasicinone content in *S. cordifolia* and *S. acuta* were estimated by the proposed method and the results were represented in Table 5.

Conclusion

The proposed HPTLC and HPLC methods provide simple, accurate and reproducible quantitative analysis for simultaneous determination of vasicine and vasicinone. Both the methods were validated as per ICH guidelines. Statistical tests indicate that the proposed HPTLC and HPLC methods reduce the duration of analysis and appear to be equally suitable for routine

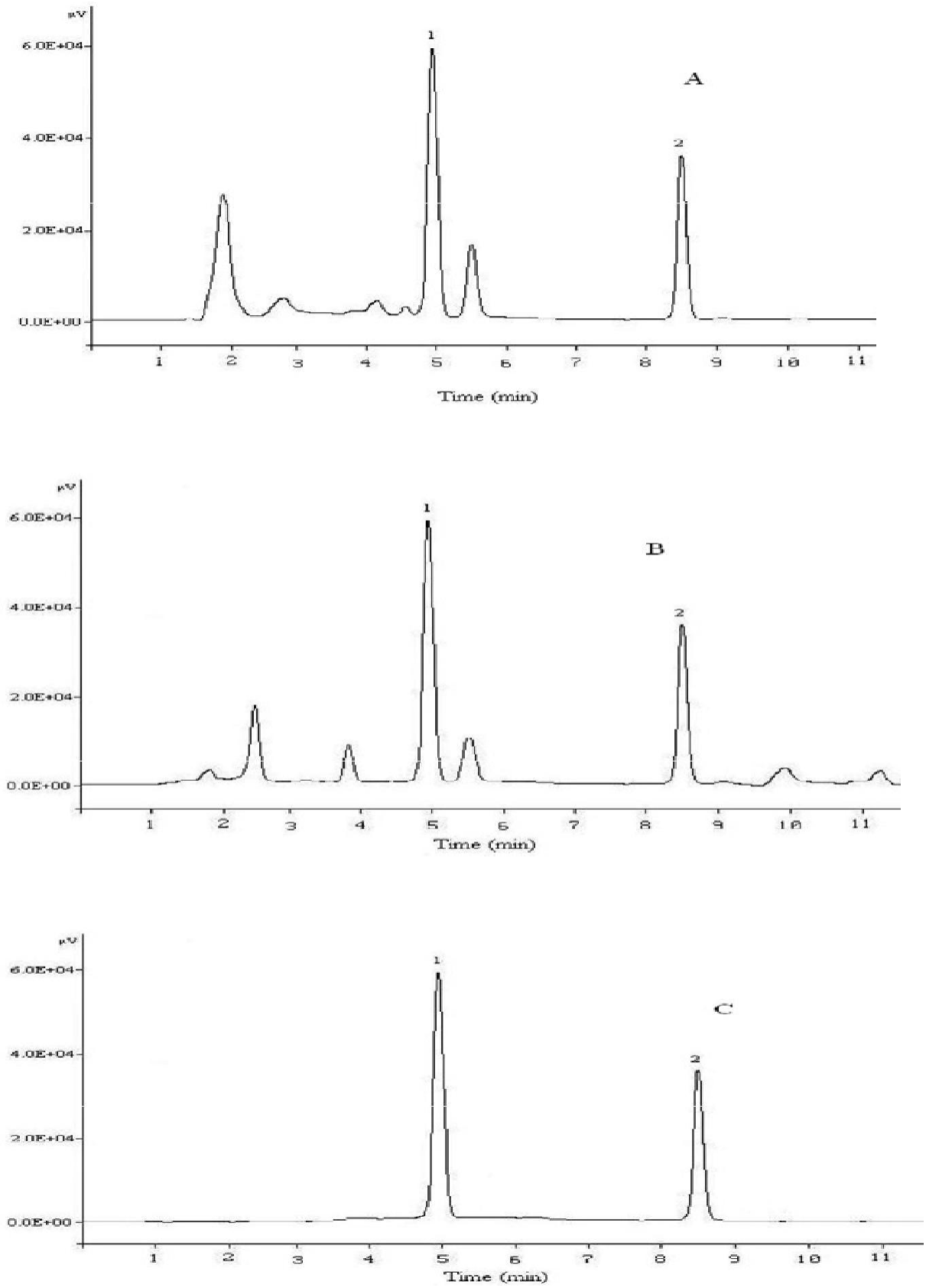

Figure 2. HPLC chromatograms of *Sida cordifolia* (A), *Sida acuta* (B) and Reference standard (C); Peak 1 is of vasicine (R_t: 5.0), peak 2 is of vasicinone (R_t: 8.7), and unknown peaks are of other components present in the extract.

Table 5. Applicability of the proposed methods for the determination of vasicine and vasicinone in *Sida cordifolia* and *Sida acuta.* [a]Mean ± standard deviation (*n* = 3).

	Content % ±SD[a]	% RSD	SE	t-value	F- value
HPTLC densitometry (*S. cordifolia*)					
Vasicine	0.011 ± 0.031	2.0	0.21	0.053	1.093
Vasicinone	0.0065 ± 0.022	2.1	0.29	0.115	1.263
HPLC (*SIDA CORDIFOLIA*)					
Vasicine	0.010 ± 0.031	1.1	0.26	0.215	1.255
Vasicinone	0.0061 ± 0.022	1.5	0.50	0.244	1.278
HPTLC densitometry (*S. ACUTA*)					
Vasicine	0.008 ± 0.031	2.1	0.23	0.305	1.215
Vasicinone	0.0026 ± 0.022	1.3	0.26	0.224	1.988
HPLC *(S. ACUTA*)					
Vasicine	0.008 ± 0.031	1.2	0.36	0.295	1.265
Vasicinone	0.0023 ± 0.022	1.9	0.40	0.264	1.178

determination of vasicine and vasicinone simultaneously in herbal extract and formulation.

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