SIMULTANEOUS HPLC METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF SALBUTAMOL IN COMBINATION WITH VASICINE

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ABSTRACT
A sensitive high-performance liquid chromatography method has been developed for the simultaneous determination of salbutamol and vasicine isolated from Adhithoda vasia. Isolated vasicine was authenticated by TLC, FT-IR and HPLC analysis compared with reference standard. Salbutamol was obtained as pure compound and further authenticated for purity by FT-IR, UV and HPLC analysis with reference to Indian Pharmacopoeia. Simultaneous estimation was carried out on a reversed-phase C18 column (150 mm × 4.6 mm; 5 μm) using isocratic solvent system consisting of methanol and de-ionized water in ratio of 40:60 as mobile phase at detection web length of 298 nm. Quantification was performed by preparation of calibration curve using standardized vasicine and salbutamol as the internal standard. Calibration curves showed good linearity between concentration of 100-1000 μg/ml for vasicine and 1-10 μg/ml for salbutamol with correlation coefficients higher than 95%. The average recovery rates were between 98.35 to 99.34%. The intra- and inter-day relative standard deviations were below 2%. The lower limit of quantification was found to be 136.25 and 21.69 ng/ml for vasicine and salbutamol respectively. This validated method was sufficiently sensitive, accurate and precise with less than 2% relative standard deviation. The validated HPLC method developed was further optimized for co-estimation of vasicine and salbutamol from in vitro plasma. Specific identifiable peaks were obtained in the optimized HPLC condition with excellent selectivity and sensitivity for the co-analysis of vasicine and salbutamol in the in vitro blood samples.

KEYWORDS: Authentication, Co-estimation, HPLC, Salbutamol, Validation, Vasicine.

SUMMARY
A validated HPLC method was developed and optimized for co-estimation of vasicine and salbutamol from in vitro plasma. Specific identifiable peaks were obtained in developed HPLC condition with excellent selectivity and sensitivity for the co-analysis of vasicine and salbutamol. This method is sensitive, accurate and precise with less than 2% relative standard deviation.

INTRODUCTION
Ayurveda is the most ancient science of life having a holistic health approach. The traditional system of medicine has been largely commercialized and proved as a milestone for treatment of many incurable diseases. The era of traditional system of medicine has been changed and Ayurvedic treatment is now dynamic and progressive with a good profit margin. Adverse drug reactions are associated with almost all allopathic drugs but now-a-days reports on adverse reactions are increasing with herbal treatment also.[1,2] Herbal drugs offer comparatively less or almost no side effects as widely believed although still there are chances of drug interaction between herbal active constituents and allopathic medicines. As a common practice in India patients many times consume both types of medicines without consent of physician.[3,4] A survey was designed with the aim to gather information on extent of self medication practice of Ayurvedic medicines along with allopathic drugs in asthmatic patients. This was a questionnaire based survey conducted on male and female population of all age group in Jabalpur district, M.P., India. Questionnaire included the variables like conditions, reason, system of medicine used, type of medicine consumed and source of information etc. Average 45% population revealed tendency of using both allopathic and Ayurvedic medicines concurrently. The outcome showed that self medication with Ayurvedic anti-asthmatic drugs is practiced mostly by males (71%) favouring the age group of 40-60 years. Maximum participants (58.6%) considered self medication for quick relief, 23.2% said to have lack of time for consulting doctor. The most common herb in Ayurvedic medicine used for chronic asthma was found to be Vasaka, one of the key ingredients of many anti-asthmatic formulations. Concomitant use of herbal and allopathic anti-asthmatics was very common among the middle aged male in respect to the major factors as literacy and economy.
Nausea, insomnia, restlessness, headache, tremors, diarrhoea, excessive sweating and vertigo were the frequently found side effects in patients using allopathic anti-asthmatic treatment. Sweating, restlessness and irregular heartbeat was mostly observed in patients using both allopathic and Ayurvedic combination may be due to additive bronchodilator effect.\(^5\)

Market survey of herbal anti-asthmatics has revealed that many formulations are available in the market containing vasaka as active ingredient. Herb-drug interaction may be due to pharmacokinetic or pharmacodynamic drug interaction resulting in additive or synergistic response. Outcome of the survey has encouraged us to explore in vivo pharmacokinetic interaction between widely used anti-asthmatic bronchodilator salbutamol concurrently administered with vasicine the bioactive phytoconstituents of vasaka. Literature review has revealed that the miracle plant vasaka, *Adhatoda vasica* (L.) Nees. (Family Acanthaceae) have number of therapeutic uses, such as antitussive, bronchodilator and anti-inflammatory, hypoglycaemic, hepatoprotective and antinfluenza effects. The leaves, roots, and flowers of *A. vasica* are extensively used in indigenous medicines as a remedy for cold, cough, bronchitis and asthma for more than 2000 years.\(^11,12\)

Vasicine is a major bioactive pyroquinozoline alkaloid of vasaka present in concentration of 1.3%, along with minor alkaloids like adhatonine, vasicinol and vasicinoline.\(^13\)

Isolation of vasicine from the leaves of *A. vasica* was first reported by Sen and Ghosh\(^14\) and Chopra and Ghosh\(^15\) reported the persistent bronchodilator property of vasicine. Vasicine is reported to have oxicotic, abortifacient, uterine stimulant, bronchodilatory, antioxidant, anti-inflammatory, hypotensive and bradycardiac effects.\(^16-22\) HPLC and HPTLC analytical methods for in vitro determination and quantification of vasicine from *A. vasica* and other related formulations are reported in the literature.\(^23-28\) Ram *et al.*\(^26,27\) reported bioavailability and pharmacokinetic of vasicine on rat. Many herbal formulations containing vasaka as a single herb or in combination are widely manufactured and sold in India. The sale value for salbutamol is reported to grow upto 30% in last 5 years, which is widely prescribed for acute asthmatic attacks. Drug interaction between vasicine, a very commonly used herbal phytocative constituent and other allopathic bronchodilators has not been reported yet. Hence aim of this project was to isolate, identify and develop a validated simultaneous estimation method for co-estimation of vasicine and salbutamol that can be further be implemented for determination of concentration in plasma to evaluate pharmacokinetic drug interaction.

**MATERIAL AND METHODS**

**Collection and authentication of plant material**

Herb was collected from, Jabalpur (M.P.) in the month of August-September 2013. It was made completely clean, dust free and allowed to get dried under the shade. The plant materials were authenticated by Dr. Ziaul Hasan assistant Professor, Department of Botany Saifia Science College, Bhopal and a specimen voucher no. 265/Bot/Saifia/13 was given and was preserved for future reference.

**Drying and extraction of plant material**

The plant material was dried under shade and pulverised to coarse powder with the help of hand grinder. The coarse powder was packed into airtight container and stored in cool and dry place. The powdered plant material was subjected to soxhlet extraction with ethanol, filtered and solvent recovered. Yield of extract was calculated. The crude extracts obtained by solvent extraction were subjected to various qualitative tests to detect the presence of common chemical constituents viz alkaloids, glycosides, carbohydrates, phytosterols, saponins, tannins, phenolics and flavonoids.\(^29-38\)

**Chemical and Reagents**

High performance liquid chromatography (HPLC) grade methanol, acetonitrile and ortho-phosphoric acid were purchased from Merck (Germany). HPLC grade water was generated using a Milli-Q integral water purification system (Millipore, France). Blood was obtained from a human volunteer and frozen at -20°C until analysis. All reagents used are of analytical grade. Standard salbutamol had been kindly provided by Sun Pharmaceutical Industries Ltd., Vadodara, Gujarat, as a gift sample. Vasicine (> 97% pure by HPLC) was purchased from Natural Remedies Pvt. Ltd., Bangalore, India.

**Isolation of vasicine**

Vasicine was isolated following the reported methods of Ghosal *et al.*,\(^26\) and Das *et al.*\(^21\) with minor modification. Ethanolic extract (15 gm) was acidified with 2N HCl (pH 2-2.5), the final volume adjusted to 200 ml with distilled water and refluxed for 30 min below 50°C temperature. Aqueous acidic solution was then extracted with chloroform (3 × 50 ml) to remove neutral components. After removal of chloroform phase the aqueous acidic layer was basified (pH 8-8.5) with liquor ammonia and repeatedly extracted with chloroform (3 × 50 ml). Chloroform extracts were pooled, evaporated to dryness by removing the solvent under reduced pressure at 50°C and recovered as amorphous residue which is crude alkaloid fraction with 4.25% yield. Monitoring was done at each step for the presence of vasicine and vasicine was identified as the major compound in the crude alkaloid fraction by thin layer chromatography using solvent system ethyl acetate :methanol :ammonia (8 :0.5 :0.2) as per IP.\(^31\) The crude alkaloid fraction was washed with petroleum ether :acetone (1 :1; 3 × 15 ml), filtered and dried to get alkaloid fraction. The alkaloid fraction was dissolved in a minimum quantity of ethanol. Vasicine was crystalized and purified by repeated crystallization with ethanol. Isolated vasicine was obtained as white needles.
Authentication of isolated vasicine

Melting point of isolated vasicine was measured by a digital melting point apparatus.

Thin layer chromatography (TLC)

Chromatographic conditions followed were as per Indian Pharmacopoeia. Freshly coated plates with silica gel G/ was allowed to air dry in room temperature and activated in an oven for 30 min at 110°C. Standard sample of vasicine was used for co-TLC to compare the Rf values with the isolated sample. Vasicine sample and standard was dissolved in methanol and filtered through Whatman filter paper. Mixture of ethyl acetate, methanol and strong ammonia solution in ratio of 8:2:0.2 was employed as mobile phase. The plates were placed into the developing chamber and allowed to run until reaching a height of approximately 10 cm from the point of application. The plates were placed in iodine chamber for detection of spots observed under UV light at 254 nm.

Fourier Transform Infra Red spectroscopy (FT-IR)

The IR spectrum of isolated vasicine was recorded by using FT-IR (Perkin Elmer, 883) spectrophotometer. Sample was prepared in KBr disks (2 mg sample in 200 mg KBr) in the scanning range 400-4000 cm\(^{-1}\), resolution 2 cm\(^{-1}\) and compared with reported spectra of Jain et al. [32]

High Performance Liquid Chromatography (HPLC)

High performance chromatographic analysis was performed on Shimadzu (Japan) liquid chromatography system, equipped with prominence (LC-20AD) pump, SPD-M20A photodiode array UV-Visible detector (PDA-100), a quaternary solvent delivery system, degasser (DGU-20A5) and data analysis system (LC solution). Baseline resolution was obtained at 30 ± 2°C using stainless steel Luna column (150 mm × 4.6 mm) packed with octadecylsilane bonded to porous silica (5 μm, C-18, 100A). An isocratic solvent system consisting of methanol and water (de-ionized) in ratio of 40:60 was passed through 0.45 PVDF filter and degassed before use. The flow rate was kept constant at 0.7 ml/min and effluents were monitored at 298 nm. Standard and isolated vasicine solutions were prepared by dissolving 100 μg/ml in methanol and filtered through a 0.45 μm membrane filter. A volume of 20 μl was injected through a rhodeyn injection valve fitted with a 20 μl injection loop in a run time of 10 min [26,27]

Authentication of standard salbutamol

Melting point of the drug was measured by a digital melting point apparatus. The IR spectra had been recorded by using FT-IR (Perkin Elmer, 883) spectrophotometer and compared with that reported in Indian Pharmacopoeia. Ultra Violet spectroscopy (UV 1800, Shimadzu, Japan) of 0.008% w/v solution of salbutamol in 0.1 N hydrochloric acid was recorded in the range of 230 to 360 nm.

Assay as per Indian Pharmacopoeia

Ten ml of 1.0% w/v solution of salbutamol had been prepared in carbon dioxide free water and then 0.15 ml of methyl red solution and 0.2 ml of 0.01 M sodium hydroxide was added resulting a yellow solution. This solution was titrated with 0.01 M hydrochloric acid and volume recorded. Not more than 0.4 ml of 0.01 M hydrochloric acid is required to change the colour of solution to red in compliance with IP [31,33]

HPLC of salbutamol

Shimadzu HPLC system with prominence (LC-20AD) pump and SPD-M20A photodiode array UV-Visible detector (PDA-100) was used. Salbutamol 100 μg/ml solution in mobile phase was prepared degassed and 20 μl was injected for estimation. Mixture of 0.025 M phosphate buffer (pH adjusted to 3.5 using orthophosphoric acid) and acetonitrile 70:30 was used as mobile phase. Mobile phase flow rate was 1.0 ml/min in the reverse phase Luna 5 μ C-18 column at 25°C and effluent monitored at 207 nm.

HPLC method development and optimization for co-estimation of vasicine and salbutamol

Chromatographic Conditions

The HPLC method for estimation of vasicine reported by Ram et al. [27] was optimized for co-estimation vasicine and salbutamol to develop a simultaneous assay method. A mixture of methanol and water in the ratio of (40:60 v/v) was used as a mobile phase filtered through 0.45 μm membrane filter and degassed by sonication before use. The flow rate was 0.7 ml/min and detection was carried out at three detection wavelengths 254, 276, 298 nm. The injected volume was 20 μl and run time was 15 min.

Preparation of standard solution

Standard solutions for isolated vasicine were prepared by dissolving 25 mg in 5 ml methanol. The solution was shaken and sonicated for 5 min and finally volume was made up to 25 ml to get a concentration of 1000 μg/ml, and filtered through a 0.45 μm membrane filter. The standard solution of salbutamol was prepared by dissolving salbutamol in mobile phase to make a final concentration of 10 μg/ml.

Working standard solution

Mixed standard stock solution (1000 μg/ml of vasicine and 10 μg/ml of salbutamol) was prepared in mobile phase, sonicated for 10 min. Different aliquots were taken from standard and stock solution and diluted with mobile phase to prepare the series of concentrations.

Selection of detection wavelength

The ultraviolet spectra of salbutamol showed λ max at 276 nm in 0.1 N hydrochloric acid and 257 nm in methanol. [34] Detection web length for vasicine reported in literature is in the range of 233, 254, 281, 292 and 298 nm. [31,35] The mixed standard solutions were tried for three detection web length 254, 276, 298 nm in set HPLC condition.
Analytical method validation for HPLC co-estimation of vasicine and salbutamol

Validation of the optimized HPLC method for analysis of salbutamol and vasicine in combination was carried out in terms of parameters like linearity and range, accuracy, precision, robustness, limit of detection (LOD) and limit of quantification (LOQ) as per ICH guidelines. For all the parameters percentage relative standard deviation values were calculated.

Linearity and range

The mixed stock solution of vasicine and salbutamol was prepared to get concentration in the range of 100-1000 μg/ml for vasicine and 1-10 μg/ml for salbutamol in methanol. Linearity of the method was evaluated by regression analysis. Calibration curve was prepared by injecting six concentrations of each drug prepared in the mobile phase in triplicate into the HPLC system keeping the injection volume constant. The peak areas were plotted against the corresponding concentrations to obtain the calibration graphs.

Accuracy (Recovery study)

The accuracy of the HPLC method developed was determined by the method of standard addition for measuring the recovery of the drugs as per ICH guidelines. Known amount of the extra drug (80%, 100% and 120%) was added and mixed to the standard solution. These solutions are then analyzed by injecting in HPLC system under set chromatographic condition. The accuracy was expressed as the percentage of the analyte recovered along with percentage relative standard deviation (%RSD).

Precision

The precision of the method was verified by repeatability and intermediate precision (intra-day, inter-day) expressed as %RSD. The repeatability indicates the performance of the HPLC method under specified chromatographic conditions. Three replicates of the standard sample (100 μg/ml for vasicine and 1 μg/ml for salbutamol) were prepared, injected and assay value in terms of percent drug recovered and RSD was calculated to measure the repeatability of retention time and peak area. Intraday precision were determined by repeating the assay method at three different time intervals under the same experimental conditions on the same day, while interday precision was determined by analyzing above mentioned concentrations of solutions on three consecutive days. The acceptance criteria are < 2% for the %RSD value for the peak area and retention time of vasicine and salbutamol.

System suitability analysis (robustness)

The system suitability parameters like theoretical plate (N), resolution (R) and tailing factor (T) were calculated, and compared with the standard value to ascertain validity of the proposed HPLC method for simultaneous estimation of vasicine and salbutamol at 298 nm. The system suitability was determined by replicate 20 μl injections at a fixed concentration of 100, 600 and 1000 μg/ml for vasicine and 1, 6 and 10 μg/ml for salbutamol respectively. To establish the robustness of the method the variables evaluated are variation in ratio of mobile phase (± 10%) and flow rate (± 0.2 ml/min).

Detection and quantization limits (sensitivity)

Limit of detection (LOD) and limit of quantification (LOQ) were determined by signal-to-noise ratio. The LOD and LOQ of the developed method were determined by injecting progressively low concentrations of the drug solutions under set chromatographic condition of the HPLC method compared with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3:1 is generally considered acceptable for estimating the detection limit and values were derived using following equation as per ICH guidelines.

$$\text{LOD} = 3.3 \times \sigma / S$$  
$$\text{LOQ} = 10 \times \sigma / S$$  
where, $$\sigma$$ = standard deviation of the y-intercept and $$S$$ = mean slope of the calibration curve. The concentration range tested for sensitivity varies from 175-25 ng/ml for vasicine and 90-5 ng/ml for salbutamol.

Co-estimation of vasicine and salbutamol from in vitro plasma

HPLC chromatogram of blank plasma

Blood was collected from human volunteer in a clean tube containing anticoagulant and shaken well. Plasma was separated by freezing microcentrifuge at 2000 g for 10 min at 4°C. The plasma was separated out and 0.4 ml was treated with 15 μl of 10N hydrochloric acid at room temperature to adjust the pH 6.2 for the precipitation of plasma proteins. The mixture was vortexed in a cyclo-mixer for 10 S and 10 ml of chloroform was added. The content was centrifuged for 10 min at 2000 g, the organic layer was separated and evaporated under nitrogen at room temperature. The residue was reconstituted in 200 μl of methanol and filtered through 0.45 μm filter and 20 μl was injected into the set conditions of HPLC system for determination of chromatogram of blank plasma.

HPLC chromatogram of vasicine and salbutamol in spiked plasma

Plasma 0.4 ml was taken in a clean tube and 0.2 ml of mixed standard stock solution (100 μg/ml of vasicine and 1 μg/ml of salbutamol in mobile phase) of drug was added. To this 20 μl of 10N hydrochloric acid was added at room temperature to adjust the pH 6.2 for the precipitation of plasma proteins. Content of the tube was vortexed in a cyclo-mixer for 10 S and then vasicine and salbutamol was extracted by adding 10 ml of chloroform. Drugs were extracted by centrifugation for 10 min at 2000 g and the organic layer was separated and evaporated under nitrogen at room temperature. The remaining residue was reconstituted in 200 μl of methanol and filtered through 0.45 μm filter. The methanolic sample 20 μl was injected into the set
conditions of HPLC system. The extraction process was repeated three times and chromatogram was recorded to ascertain the accuracy of the method.

RESULTS
Isolation and phytochemical test of A. vasica and isolation of Vasicine
Percentage yield of ethanolic extract of A. vasica was 13.25% and showed the presence of alkaloidal contents, essential oils and saponins. Percentage yield of isolated vasicine was found to be 2.50%, which gave positive test for alkaloids. Isolated vasicine was freely soluble in chloroform, methanol, ethanol, water and tween 80.

Authentication of isolated Vasicine
Isolated vasicine showed melting point at 209-211°C. TLC with reference to Indian Pharmacopoeia showed Rf value 0.67 for isolated vasicine closely same to reference standard Rf value 0.68 [Figure 1]. FT-IR spectra show broad peaks at 3436 cm⁻¹ for hydroxyl group and N-H bond stretching at 2983 cm⁻¹ as reported in literature[32] represented in Figure 2. HPLC showed Rt of isolated vasicine at 3.734 min [Figure 3] as that of standard Rt 3.692 min.[30]

Authentication of Salbutamol Sulphate
Melting point of salbutamol sulphate had been found to be 157-158°C. FT-IR spectra showed O-H bending peaks at 1616 cm⁻¹ and C=O stretching peaks at 907 cm⁻¹ identical to standard spectra of salbutamol Sulphate as per IP [Figure 4]. UV spectra of salbutamol sulphate showed absorption maximum at 276.5 nm,[31] Identification assay performed with reference to Indian Pharmacopoeia showed that 0.3 ml of 0.01 M hydrochloric acid had been required to change the colour of solution from yellow to red within the range of specification reported in IP, i.e. “not more than 0.4 ml.” HPLC of salbutamol sulphate showed Rt at 2.85 min very close to standard Rt reported in literature[30], i.e. 2.76 min [Figure 5].

HPLC method development for co-estimation of vasicine and salbutamol
Organic solvent-rich mobile phases are typically used for their rapid elution property and strong retention in reversed-phase chromatography. Solubility nature of vasicine and salbutamol is similar with good solubility in most of the organic solvents. Mixtures of de-ionized water with a common HPLC organic modifier methanol were used as the mobile phase for co-estimation. Based on the molecular structures and solubility of salbutamol HPLC assay was performed for estimation at 100 μg/ml concentration in methanol, by using chromatographic conditions reported Ram et al.[27] for vasicine and scanned at three detection web length 254, 276, 298 nm [Figure 6, 7 and 8]. The optimum detection wavelength was selected as 298 nm where both the drugs showed significant absorbance with acceptable Rt, theoretical plates and good resolution. Sharp peaks were obtained at Rt 2.852 min (salbutamol) and Rt 3.584 min (vasicine).

The flow rate was 0.7 ml/min and sample injection volume was 20 μl where the mobile phase offered better peak symmetry at detection web length of 298 nm and was selected for subsequent validation.

Method Validation
Linearity and range
Linearity has been evaluated by preparing calibration curve in the concentration range of 100-1000 μg/ml for vasicine and 1-10 μg/ml for salbutamol. Calibration curve were plotted against the corresponding concentrations as presented in Figure 9 and Figure 10 for salbutamol and vasicine respectively. Salbutamol and vasicine showed correlation coefficient above 95% in given concentration range.

Accuracy (Recovery study)
The method when used for extraction of salbutamol and vasicine after adding 80, 100, 120% extra drug showed recovery in the range between 98.35 to 99.34%. Table 1 showed the relation between amount of drug added, % recovered and %RSD.

Precision
The precision of the HPLC method for co-estimation was expressed in terms of % recovery and %RSD. The results obtained show high precision of the method with good repeatability and reproducibility [Table 2]. The intra and inter day precision data showed that the RSD values for repeatability were < 2% as recommended for good precision in ICH guidelines. Intra-day study also indicated the standard and sample solutions were stable on estimation for longer time.

System Suitability Parameters
System suitability analysis had been performed for establishing parameters like Theoretical plate (N), Resolution (R) and Tailing factor (T). Salbutamol showed tailing factor less than 2.0% at flow rate 0.6 ml/min or above, whereas for vasicine it was the flow rate of 0.7 ml/min or less [Table 3]. Every factor selected was changed three times one factor at a time to estimate the effect. Insignificant differences in retention time and % recovery were observed with change in mobile phase ratio.

Sensitivity (Limit of detection and limit of quantization)
Limit of detection (LOD) and limit of quantification (LOQ) were determined by signal-to-noise ratio by injecting minimum possible concentrations of the drug. Signal-to-noise ratios of 4:1 and 6:1 were obtained for the LOD and LOQ respectively. The LOD and LOQ were found to be 7.15 and 21.69 ng/ml for salbutamol, and 68.90 and 136.25 ng/ml for vasicine [Table 4].

HPLC simultaneous estimation of vasicine and salbutamol from in vitro drug plasma sample
Efficient extraction solvent was selected using a recovery test in whole blood sample in various extraction solvents, such as methanol, acetonitrile, chloroform and acetone,
and methanol was found to have better recovery. Acetonitrile is commonly used as optimal protein precipitation solvent due to good protein precipitation efficiency and extraction efficiency. Hydrochloric acid was used for precipitating plasma protein in place of acetonitrile due to good solubility of both the drugs in acetonitrile.

The validated HPLC method developed was also optimized for co-estimation of vasicine and salbutamol in plasma. To demonstrate the selectivity of the method and to screen for interfering substances, three replicate analyses of six blank whole blood samples and blood spiked with vasicine and salbutamol were extracted and injected for analysis using the developed HPLC method. A typical chromatogram of chloroform extract of blank plasma reconstituted in methanol showed a sharp peak at Rt 2.222 min. Spiked plasma sample containing 100 μg/ml of vasicine and 1 μg/ml of salbutamol in mobile phase was extracted for simultaneous estimation. Sharp peaks were obtained at Rt 2.239 min (plasma), Rt 2.850 min (salbutamol) and Rt 3.654 min (vasicine) as in Figure 11 and 12 with no significant interfering endogenous peaks at the retention times. According to the guidelines for industrial bioanalytical method validation the developed method can be termed selective for estimation of vasicine and salbutamol. Method selectivity is defined as the ability of the bioanalytical method to measure a substance unequivocally and to discriminate between the analytes and other components that may be present. The optimized HPLC conditions yielded excellent selectivity and sensitivity for the co-analysis of vasicine and salbutamol in the blank whole blood samples.

Table 1: Recovery studies for salbutamol and vasicine

<table>
<thead>
<tr>
<th>Level of recovery (%)</th>
<th>% Recovered*</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Salbutamol</td>
<td>Vasicine</td>
</tr>
<tr>
<td>80</td>
<td>98.35</td>
<td>99.34</td>
</tr>
<tr>
<td>100</td>
<td>98.52</td>
<td>99.17</td>
</tr>
<tr>
<td>120</td>
<td>99.04</td>
<td>99.04</td>
</tr>
</tbody>
</table>

*Mean of three estimates. % RSD = percentage relative standard deviation.
### Table 2: Precision studies for salbutamol and vasicine

<table>
<thead>
<tr>
<th>Concentration added</th>
<th>Concentration measured ± SD</th>
<th>Interday</th>
<th></th>
<th></th>
<th>Intraday</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Retention time ± SD</td>
<td>Peak area ± SD</td>
<td>% Recovered*</td>
<td>% RSD</td>
<td>Retention time ± SD</td>
<td>Peak area ± SD</td>
<td>% Recovered*</td>
</tr>
<tr>
<td>Salbutamol (1μg/ml)</td>
<td>0.952 ± 0.35</td>
<td>2.872 ± 0.06</td>
<td>107935 ± 463.50</td>
<td>98.14</td>
<td>0.74</td>
<td>0.982 ± 0.51</td>
<td>2.897 ± 0.08</td>
</tr>
<tr>
<td>Vasicine (100 μg/ml)</td>
<td>99.06 ± 0.82</td>
<td>3.670 ± 0.12</td>
<td>214824 ± 223.60</td>
<td>99.12</td>
<td>0.59</td>
<td>99.65 ± 0.37</td>
<td>3.642 ± 0.09</td>
</tr>
</tbody>
</table>

*Mean of three estimates. % RSD = percentage relative standard deviation.

### Table 3: System suitability analysis for salbutamol and vasicine

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Volume</th>
<th>Salbutamol</th>
<th>Vasicine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Retention time</td>
<td>Tailing factor</td>
</tr>
<tr>
<td>Flow rate (ml/min)</td>
<td>0.5</td>
<td>3.35</td>
<td>2.50</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>3.12</td>
<td>1.43</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>2.45</td>
<td>1.12</td>
</tr>
<tr>
<td></td>
<td>0.9</td>
<td>2.06</td>
<td>1.41</td>
</tr>
<tr>
<td>Mobile phase ratio (methanol : water)</td>
<td>30 : 60</td>
<td>2.10</td>
<td>1.17</td>
</tr>
<tr>
<td></td>
<td>35 : 65</td>
<td>2.52</td>
<td>1.05</td>
</tr>
<tr>
<td></td>
<td>45 : 55</td>
<td>3.56</td>
<td>1.42</td>
</tr>
<tr>
<td></td>
<td>50 : 50</td>
<td>4.25</td>
<td>1.56</td>
</tr>
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</table>

*Mean of three estimates.
Table 4: Summary of validation parameters Salbutamol and vasicine

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Salbutamol</th>
<th>Vasicine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibration range (μg/ml)</td>
<td>1-10</td>
<td>100-1000</td>
</tr>
<tr>
<td>Regression equation</td>
<td>y = 43213x + 44783</td>
<td>y = 51009x - 28053</td>
</tr>
<tr>
<td>Correlation coefficient (r²)</td>
<td>0.963</td>
<td>0.986</td>
</tr>
<tr>
<td>Retention time (minutes)</td>
<td>2.85</td>
<td>3.68</td>
</tr>
<tr>
<td>Theoretical plates (N)</td>
<td>19266</td>
<td>12468</td>
</tr>
<tr>
<td>Resolution (R)</td>
<td>1.92</td>
<td>3.78</td>
</tr>
<tr>
<td>Tailing factor (T)</td>
<td>1.01</td>
<td>1.24</td>
</tr>
<tr>
<td>% Recovery</td>
<td>98.63</td>
<td>99.18</td>
</tr>
<tr>
<td>Repeatability (% RSD)</td>
<td>0.7497*</td>
<td>0.8428*</td>
</tr>
<tr>
<td>LOD (ng/ml)</td>
<td>7.15</td>
<td>68.90</td>
</tr>
<tr>
<td>LOQ (ng/ml)</td>
<td>21.69</td>
<td>136.25</td>
</tr>
<tr>
<td>Robustness</td>
<td>Robust</td>
<td>Robust</td>
</tr>
</tbody>
</table>

*Denotes mean of 6 determinants. LOD = Limits of detection, LOQ = Limits of quantification and % RSD = percentage relative standard deviation.

Fig. 1: TLC of profile of isolated (I; Rf 0.67) and standard (S; Rf 0.68) vasicine in ethyl acetate: methanol: ammonia (8:2:0.2 v/v).

Fig. 2: FTIR spectra of isolated vasicine.

Fig. 3: HPLC chromatogram of standard vasicine (A: Rt 3.692) and isolated vasicine (B: 3.734) in methanol: water (40:60) at 298 nm.

Fig. 4: FTIR spectra of salbutamol.
Fig. 5: HPLC chromatogram of salbutamol (Rt 2.852) in 0.025 M phosphate buffer (pH adjusted to 3.5 using orthophosphoric acid) and acetonitrile 70:30 at 207 nm.

Fig. 6: HPLC chromatogram of salbutamol (Rt 2.559) and vasicine (Rt 2.906) combination in methanol: water (40:60) at 276 nm in methanol: water (40:60) at 254 nm.

Fig. 7: HPLC chromatogram of salbutamol (Rt 2.943) and vasicine (Rt 3.443) combination in methanol: water (40:60) at 276 nm in methanol: water (40:60) at 276 nm.

Fig. 8: HPLC chromatogram of salbutamol (Rt 2.852) and vasicine (Rt 3.684) combination in methanol: water (40:60) at 298 nm.

Fig. 9: Calibration curve of salbutamol in methanol: water (40:60) at 298 nm.

Fig. 10: Calibration curve of vasicine in methanol: water (40:60) at 298 nm.

Fig. 11: HPLC chromatogram of blank plasma sample scanned at 298 nm.

Fig. 12: HPLC chromatogram of salbutamol (Rt 2.85) and vasicine (Rt 3.65) combination extracted from in-vitro plasma sample in methanol: water (40:60) at 298 nm.
CONCLUSION
In this study, a sensitive and accurate reverse phase HPLC method was developed and validated for determination of vasicine and salbutamol in combination and in vitro whole blood. The assay and validation results confirmed that the contents of vasicine and salbutamol estimated is sensitive, precise and reproducible. The validated method was sufficiently sensitive with low LOQ, accurate with over 98% recovery and precise with less than 2% relative standard deviation. Successful application of the developed HPLC method for co-estimation of vasicine and salbutamol from in vitro blood plasma sample suggested its suitability and sufficiency for use in pharmacokinetic studies. The developed HPLC method for simultaneous estimation of vasicine and salbutamol in combination is simple, economical, accurate and specific, and can be conveniently adopted for the routine quality control analysis as well as bioanalysis in pharmacokinetic, bioequivalence and bioavailability studies.

REFERENCES