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Original Article

DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR SIMULTANEOUS QUANTIFICATION OF VASICINE, GLYCYRRHIZIN AND PIPERINE IN POLY HERBAL COUGH SYRUP

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ABSTRACT

Objective: The present study was undertaken to develop a rapid, simple, specific and economic high performance liquid chromatographic (HPLC) method has been developed, validated and used for simultaneous quantification of Vasicine, Glycyrrhizin and Piperine in poly herbal cough syrup

Methods: An Agilent technologies 1200 Series quaternary pump combined with an Agilent 1200 series photo diode array detector (USA), an Agilent 1200 series vacuum degasser (USA) and an Agilent autosampler injector. Chromatographic separation was performed on a Hiber, prepacked column, C_{18}, Size 250x 4.60 mm, 5µ maintained at 25 °C. PJ (solvent A) and HPLC grade Acetonitrile (solvent B).

Results: The HPLC developed for quantization was simple, accurate and specific. The drug follows the beer's lambert's law in the concentration range of of Vasicine in concentration range 25–250 µg/ml, glycyrrhizin in concentration range 100–1000 µg/ml and Piperine in concentration range of 20–100µg/ml and exhibited good correlation coefficient and excellent mean recovery. Percentage RSD for precision and accuracy of the method was found to be less than 2%.

Conclusion: The present standardization provides a specific and rapid tool in the herbal research, permitting to set quality specifications for identity, transparency and reproducibility of these Phytoconsituents in the herbal Cough syrup.

Keywords: HPLC Method, Vasicine, Glycyrrhizin, Piperine in Poly Herbal Cough Syrup

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INTRODUCTION

According to the World Health Organization, mass population (65%–80%) in developing countries depends essentially on plants for primary health care needs owing to poverty and lack of access to modern medicine. (The resurgence of herbal medicines has increased the international trade enormously [1].

Herbal medical database indicates that herbal medicinal product markets in Asia and Japan had reach $2.3 and 2.1 billion, respectively. Pharmaceutical companies have established renewed attention in exploring plants as a major source for new lead molecules and for the development of standardized phytoconsituents agents with assuring efficacy, safety and quality product [2, 3].

The development of reliable and reproducible analytical methods which can dependably profile the phytochemical composition and other major constituents, is a major challenge to manufacturer. In view of the above, standardization is an important step for the establishment of a reliable therapeutic activity, a consistent constituent profile and may systemize a quality element for the manufacturing of an herbal drug [4]. The validation of herbal drugs and identification of adulterants from authentic medicinal herbs are crucial for both pharmaceutical companies as well as community health [5, 6].

Vasicine is a major bioactive pyrroquinazoline alkaloid of the miracle plant vasaka, Adhathoda vasica (L.) Nees. (Family Acanthaceae) have number of therapeutic uses, such as antitussive [7], bronchodilator and anti-inflammatory [8], hypoglycaemic [9], hepatoprotective [10] and antiulcer [11] effects. HPLC analytical methods for quantification of vasicine from A. vasica and other related formulations are reported in the literature [12-14].

The Piperine (1-piperoylpiperidine), a nitrogenous pungent substance, is an alkaloid presents in the fruits of black pepper (Piper nigrum) and other piper species (family: Piperaceae) (Wood AB, 1988). Piperine have anticonvulsant, anti-ulcer, carminative, alternative tonic, laxative, digestive, stomachic, antiseptic, anti-inflammatory and analgesic effect, anti-depressant effect, antioxidant activity and cytoprotective effect [15].

Modern pharmacological studies showed that glycyrrhizin possess multiple activities, such as hypolipidemic, antihyperglycemic, antidepressant, liver regeneration, antimalarial, smooth muscle relaxing, and antioxidant effects [16]. Quantification of glycyrrhizin biomarker in Glycyrrhiza glabra rhizome and herbal formulations by validated HPLC methods [17, 18].

It is important to develop methods for the quantification of the components of herb containing Vasicine, glycyrrhizin and Piperine to ensure quality and customer safety. The constituents of an herb are complex and individual amount was little, therefore, it is more difficult to study herbal preparation than a single herb. The methodologies for the quantification of natural products are currently limited.

No published reports were found for simultaneous quantification of Vasicine, glycyrrhizin and Piperine by HPLC method. However, most of the available methods have limitations such as long duration, low sensitivity, costly, reproducibility, and symmetry and have poor accuracy. Keeping in view of these, an attempt has been made to develop a simple, accurate, precise and reliable simultaneous HPLC method for the estimation of Vasicine, glycyrrhizin and Piperine from herbal cough syrup.

MATERIALS AND METHODS

Chemicals and reagents

Methanol, acetonitrile and water used were HPLC-grade and were obtained from Qualigens Fine Chemicals (Mumbai, India). All solvents were filtered through 0.5-µm (Millipore, Billerica, MA) membrane and degassed in an ultrasonic bath. Distilled water was prepared by using Milli-Q water purifying system (Millipore). Reference standards Vasicine, glycyrrhizin and Piperine with 99% purity were procured from Fluka and Sigma Aldrich (Albuch, Germany), respectively.

Analytical conditions

An Agilent technologies 1200 Series quaternary pump combined with an Agilent 1200 series photo diode array detector (USA), an Agilent 1200 series vacuum degasser (USA) and an Agilent autosampler injector. Chromatographic separation was performed on a Hiber, prepacked column, C_{18}, Size 250x 4.60 mm, 5µ maintained at 25 ℃. The mobile phase consisted of buffer 0.01M potassium dihydrogen ortho phosphate in 900 ml of HPLC grade water and added 0.5 ml of Orthophosphoric acid. Make up to 1000 ml with water, filter through 0.45µ membrance and degas in a sonicator for 3 min (solvent A) and HPLC grade Acetonitrile (solvent B).

For HPLC separation, the mobile phase consisted of a binary mixture of solvent-A in the ratio of and solvent-B (ACN) in the ratio of (85:15) with a gradient program as follows: 0–35% B (0–20 min), 35-55% B (15-25 min), 85% B isocratic (35 min), and return to 15% B (45 min),

Gradient conditions and finally, reconditioning the column with 5% B isocratic (48–50 min). The analysis was performed at aflow rate of 1.0 ml/min. Injection was manual and the injection volume was 50µl. All samples and standards injected are filtered through 0.45µm membrane filter.

Preparation of stock and and sample solutions

Weigh accurately about 7 mg of vasicine, 12 mg of glycyrrhizin and 8 mg of piperine to a 100 ml volumetric flask Approximately 50 ml methanol were added and the solution was sonicated for 30 min. The flask was filled upto 100 ml with methanol then mixed and filter.

Method validation [19, 20]

Linearity

Six working standard solutions of Vasicine in concentration range 25–250 µg/ml, glycyrrhizin in concentration range 100–1000 µg/ml and Piperine in concentration range of 20–100µg/ml were prepared. Each solution was injected in triplicate in the chromatographic system under optimized conditions. The calibration plot for each standard was obtained by plotting a graph of mean peak areas of that standard against its injected concentration.

Limits of detection and limit of quantitation

The limits of detection (LOD) and limit of quantitation (LOQ) for Vasicine, glycyrrhizin and Piperine were established at signal-to-noise ratios of 3:1 and 10:1, respectively (7,8). The results for both the standards are represented in table I.

Precision

Precision is determined in terms of instrumental precision, intra-assay precision, and inter-assay precision. The instrumental precision for Vasicine, glycyrrhizin and Piperine were studied by separate, repetitive injections (n = 3) of standard solutions of Vasicine (150.00 µg/ml), glycyrrhizin (500.00 µg/ml) and Piperine (50.0 µg/ml).

The intra-assay precision was performed by analysis of replicate injections of sample solutions of three different concentrations on the same day. The intermediate precision was evaluated by replicate analysis of sample solutions of three different concentrations on three different days. The values of percent relative standard deviation of peak areas of Vasicine, glycyrrhizin and Piperine for instrumental, intra-assay, and intermediate precision were determined.

Accuracy

The accuracy of the method was established by performing a recovery experiment using standard addition method. For zero level, only sample solution was analyzed by HPLC in seven replicates.

To about 100 mg of sample, pure standards of Vasicine, concentration (150 µg/ml) and pure standards of Glycyrrhizin concentration (500 µg/ml) were added. Similarly, pure standards of methyl Piperine concentration (50 µg/ml) were added to same sample. The solutions were prepared as described earlier and were analyzed by HPLC (n = 3) for each level, and mean amounts of Vasicine, glycyrrhizin and Piperine present in each level of sample solution were determined.

The average values of percent recoveries of Vasicine, glycyrrhizin and Piperine were determined at three level addition were found to be 98.37, 98.80 and 99.73% respectively.

System suitability

The system suitability test was carried out by injecting standard solutions of (150.00 µg/ml), glycyrrhizin (500.00 µg/ml) and Piperine (50.0 µg/ml) three in the chromatographic system under optimized chromatographic conditions [21].

The peak areas values and retention times of Vasicine, glycyrrhizin and Piperine were noted for each injected concentration of both the standards. As the values of percent relative standard deviations for peak areas and retention times of both the standards were found to be less than 2%, the system was found to be suitable.

Robustness studies

The effects of small, deliberate variation of the flow rate of mobile phase on the retention time of the drugs were examined. The robustness of the proposed chromatographic method was performed at a concentration of 150 µg/ml for Vasicine, 500 µg/ml for glycyrrhizin aand 50 µg/ml for Piperine. The standard deviation of retention time and % RSD were calculated for each variable parameter.

Specificity

The specificity of the proposed HPLC method was estimated by analyzing the standard marker and sample. Peaks for Vasicine, glycyrrhizin and Piperine were confirmed by comparing the retention time. Excipients present in the herbal formulation did not interfere with the peaks of Vasicine, glycyrrhizin and Piperine.

Analytical solution stability

The stability of Vasicine (150 µg/ml), glycyrrhizin (500 µg/ml) and Piperine standard solutions (50 µg/ml) was performed after 0, 6, 12, 24 and 48 h of storage at room temperature. Solution stability was determined by comparing peak areas at each time point against freshly prepared solutions of standard markers

Sample preparation from Herbal cough syrup

Sample details: Cofnil-H9**.**

Manufactured by: SKM Siddha and Ayurveda Company (India) P(Ltd).

A total of 40 g of syrup was weighed accurately in 250 ml conical flask; 50 ml of methonal was added, sonicated for 10 min and boil on water bath for 15 min at 70-80˚C by fixing condenser. Cool and filter the solution through a 201 filter paper in to a separate 250 ml beaker. Repeate the extraction procedure for 2 more times with 20 ml methonal. Collect all the filtrates and concentrate to less than 100 ml and transfer the extract solution into a 100 ml. volume flask, reinse beaker with methonal make up to the volme to 100 ml with methonal. mix well and filter the solution through 0.45 µ membrane filter paper.

RESULTS AND DISCUSSION

HPLC method optimization

During the optimization of the proposed HPLC method, different HPLC columns, mobile phases of various compositions of acetonitrile, water, methanol, potassium dihydrogen phosphate, sodium dihydrogen phosphate buffer with different molarities and different pH were tried. Finally the mobile phase consisting of 0.01M potassium dihydrogen ortho phosphate in 900 ml of HPLC grade water and added 0.5 ml of Orthophosphoric acid was selected as it gave well resolved peaks. The column used was Hiber, prepacked column, C_{18}, Size 250x 4.60 mm, 5µ and a flow rate of 1.0 ml/min.

The optimum wavelength for detection and quantitation for Vasicine, glycyrrhizin and Piperine were found to be 280 nm, 254 nm, 343 nm were used respectively. Average retention time for Vasicine, glycyrrhizin and Piperine were found to be 7.89, 27.32 and 31.74 min, respectively.

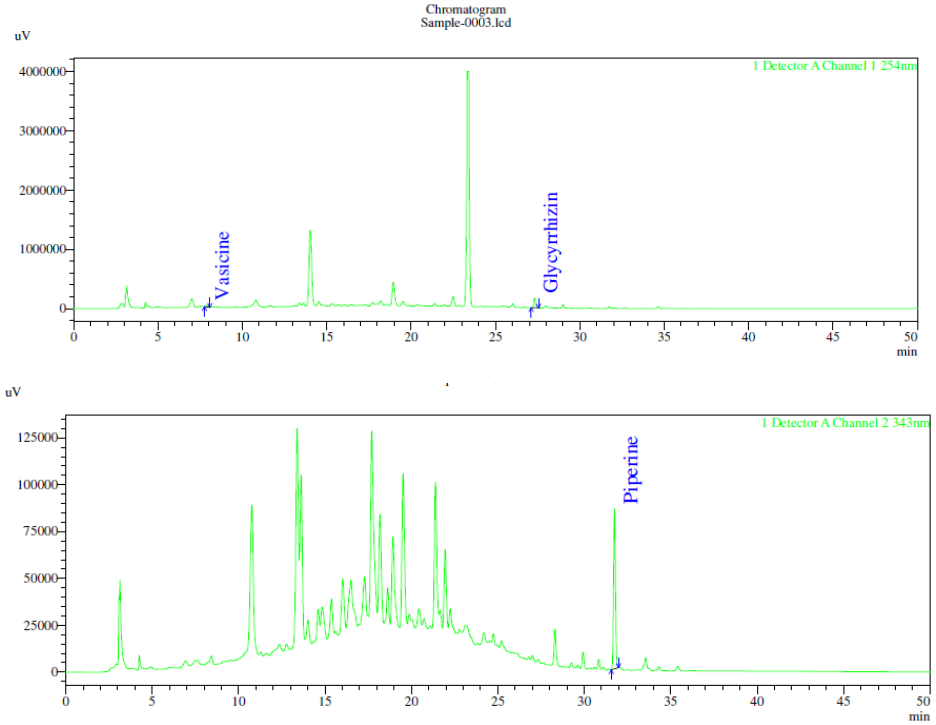

Fig. 1 and 2: Chromatogram of vasicine, glycyrrhizin and piperine in the formulation

Linearity and range

Linearity was performed by injecting stock solutions in the range of 25-250 µg/ml for Vasicine, 100-1000 µg/ml for glycyrrhizin and 200-500 µg/ml for Piperine. Peak areas obtained were processed and calibration curves were generated by Microsoft Excel software. To prove linearity, residual analysis was also performed along with correlation coefficient. Each standard solution of six different concentrations was injected in six replicates and chromatographed using the chromatographic conditions mentioned above. Linear regression data for the calibration curves were presented in table 1.

Table 1: Linear regression data for the calibration curves (n = 6)

Validation parameters	Vasicine	Glycyrrhizin	Piperine
Linearity range (µg/ml)	25–250	100–1000	20–100
Correlation coefficient (r^2)	0.997	0.998	0.998
Regression equation	Y=70067x+40243	Y=4737(k)x+444.2	Y=46885x+54581
LOD (ppm)	4.6	20	3.2
LOQ (ppm)	14.0	60	10
Method precision (RSD %)	1.34	1.76	1.49

Specificity

Peaks for Vasicine, Glycyrrhizin and Piperine were confirmed by comparing the retention time. It was found that, the base line did not show any significant noise and there were no other interfering peaks around the retention time of Vasicine, Glycyrrhizin and Piperine, indicating specificity of the proposed chromatographic method.

Sensitivity

Sensitivity of the proposed HPLC method was illustrated by determination of the limit of detection (LOD) and limit of quantitation (LOQ). As per ICH recommendations, the standard deviation of the response and the slope of the calibration plots were used to determine detection and quantification limits.

Precision

The developed simultaneous HPLC method was found to be precise (table 2), with % RSD values for repeatability and intermediate precision studies below 2 % as recommended by ICH Q2 (R1) guideline.

Accuracy

Satisfactory accuracy and recoveries for Vasicine, Glycyrrhizin and Piperine were obtained and reported in table 2 and table 3 respectively, which indicate that the proposed chromatographic method is reliable for the simultaneous quantification of selected phytoconsitutnes in this herbal formulation.

Analytical solution stability

Solution stability of Vasicine, Glycyrrhizin and Piperine was estimated at room temperature for 48 h. Low percentage relative standard deviation (below 2.0 %), indicated that both standard and sample solution was stable up to 48 h at room temperature.

System suitability

Higher number of theoretical plates (≥2000), peak symmetry (≤ 2), high resolution between the peaks (R≥2.0), and proper retention time indicated suitability of the proposed HPLC method for simultaneous quantification of Vasicine, Glycyrrhizin and Piperine.

Robustness studies

As shown in table 4, retention time of the selected phytoconstituents remained unaffected (% RSD<2), indicating robustness of the HPLC method.

Table 2: Intra-day and inter-day precision and accuracy of phytoconsituents

Drug concentration	Intra-day			Inter-day		
	Mean	%CV	% Accuracy	Mean	%CV	% Accuracy
Vasicine 150µg/ml	149.23	1.16	99.28	149.75	1.46	98.81
Glycyrrhizin 500µg/ml	499.14	1.19	98.86	499.12	1.74	98.72
Piperine 50µg/ml	49.67	1.62	99.35	49.33	1.83	98.43

Table 3: Results of recovery studies (n=3)

Phyto constituents	Drug amount taken (µg/ml)	Amount added (µg/ml)	Amount found (µg/ml)	% Recovery±% RSD
Vasicine	150	100	248.87	98.12±1.34
	150	150	297.64	98.56±1.12
	150	200	347.58	98.44±1.76
Glycyrrhizin	500	400	897.43	99.02±1.21
	500	500	996.68	98.78±1.19
	500	600	1096.35	98.61±1.45
Piperine	50	25	74.26	98.47±1.66
	50	50	99.12	98.53±1.27
	50	75	124.75	99.19±1.74

SD = Standard deviation; RSD = Relative standard deviation

Table 4: Robustness study

Flow rate (ml/min)	Parameter (ml/min)	Retention time (min)	%CV
Vasicine 150µg/ml	0.8	7.83	1.68
	1.0	7.87	1.73
	1.2	7.88	1.91
Glycyrrhizin 500µg/ml	0.8	27.32	1.52
	1.0	27.31	1.86
	1.2	27.34	1.45
Piperine 50µg/ml	0.8	31.71	1.28
	1.0	31.74	1.89
	1.2	31.77	1.45

Analysis of marketed herbal formulation

The present study was targeted to SKM Cofnil-H9 (Cough syrup), a polyherbal formulation which integrates an outstanding blend of herbs like Ocimum sanctum, solanum xanthocarpum, Adhathoda vasaka, Solanum trilobactum, Trikatu churna, Inula racemosa, Glycyrrhiza glabra**,** Taxus buccata, Alpinia galangal, Myristica fragrans, Coleus aromaticus, Piper betle, Curcuma longa, Viola odorata that have been used for decades to treat Cough, Cold and Bronchitis.

Validity of the proposed HPLC method was applied to standardization of herbal dosage form in six replicate determinations. The percent content of Vasicine, Glycyrrhizin and Piperine in marketed herbal Cough syrup was found to be 0.016, 0.024 and 0.001 % w/w, respectively.

CONCLUSION

The validated HPLC method employed proved to be simple, rapid, precise, accurate, robust and thus can be intended for routine simultaneous quantitation of Vasicine, Glycyrrhizin and Piperine in the herbal Cough syrup has been developed and validated for its linearity, precision, specificity, and accuracy, providing a suitable and practical analytical method for the routine quality control analyses necessary for providing herbal medicines with high safety and efficacy. The present standardization provides a specific and rapid tool in the herbal research, permitting to set quality specifications for identity, transparency and reproducibility of these phytoconsituents in the Cough syrup.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

We declare that we have no conflict of interest.

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