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NOVEL RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF RESERPINE AND PIPERINE IN POLYHERBAL FORMULATION

U. D. Jaiswar, R. M. Patil and V. N. Jain *

Department of Quality Assurance, Oriental College of Pharmacy, Sector 2, Sanpada, Navi Mumbai - 400705, Maharashtra, India.

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Correspondence to Author:
Dr. Vandana Jain

Professor,
Department of Quality Assurance, Oriental College of Pharmacy, Sector 2, Sanpada, Navi Mumbai - 400705, Maharashtra, India.

E-mail: vandana.jain@ocp.edu.in

ABSTRACT: A novel, simple, accurate, precise, robust High-Performance Liquid Chromatography method was developed to simultaneously estimate reserpine and piperine. This method was applied to standardize polyherbal formulation containing these markers as one of its ingredients. The markers were resolved using Prontosil C-18 column, with mobile phase acetonitrile and water containing 0.05% ortho-phosphoric acid in the ratio 50:50, flow rate of 1 ml/min and detector set at 225 nm. Retention time of reserpine and piperine were found to be 5.4±0.2 min and 13.5 ± 0.2 min, respectively. Linear response was obtained in the tested concentration range of 1 to 20 µg/ml for reserpine and 10 to 100 µg/ml for piperine, with correlation coefficients of 0.999 and 0.998 for reserpine and piperine, respectively. This method can be used to evaluate other formulations containing reserpine and piperine as markers, thus conforming to the need of ensuring safety and quality of herbal formulations.

INTRODUCTION: Herbal medicines are polyherbal formulations, and each herb consists of different types of phytoconstituents that may have different medicinal attributes [1]. In recent years there has been a rapid increase in herbal product use in developed and developing countries. It is important to ensure herbal medicines' safety and efficacy [2]. Standardization is essential in maintaining and assessing the quality of herbal products [3]. Modern analytical techniques such as High-Performance Liquid Chromatography (HPLC), Gas Chromatography (GC), High-Performance Thin-Layer Chromatography (HPTLC), Mass Spectrometry (MS) and others are used in assessing the quality of herbal products [4].

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The present study illustrates a novel RP-HPLC method development and validation for detection, quantification, and standardization of reserpine and piperine in marketed polyherbal formulation. The literature survey found that various analytical methods have been developed for the standardization of reserpine and piperine individually or in combination with other components; however, no method is reported for simultaneous estimation of both components (reserpine and piperine). Hence, it was thought worthwhile to develop a novel HPLC method that can be used to standardize polyherbal formulations containing these markers [5-9].

The marketed formulation used in the present study contains several ingredients, mainly Kamla (*Nelumbo nucifera*), Ashwagandha (*Withania somnifera*), Arjuna (*Terminalia arjuna*), Maricha (*Piper nigrum*), Sarpagandha (*Rauwolfia serpentina*), Pippali (*Piper longum*) and others. The selected active constituent reserpine from *R. serpentina* is a well-known anti-hypertensive

constituent [10]. Piperine is an alkaloid found in *Piper* species of Piperaceae family. It increases bioactivity of various therapeutic drugs and phytochemicals because of its inhibitory action on enzymatic drug biotransformation in liver. It also has liver protective activity [11].

MATERIALS AND METHODS:

Marketed Formulation: A commercial preparation Navhridaya Kalpa (Sriveda Sattva Pvt. Ltd) used for analysis was procured from the local market of Mumbai, Maharashtra, India. Each tablet contains 23.26 mg of Sarpagandha, 34.89 mg of Maricha, and 23.26 mg of Pippali.

Requisites: All the chemicals of HPLC grade used were procured from Thermo Fisher Scientific Pvt. Ltd, India. Standards of reserpine and piperine were procured from Sigma-Aldrich Chemicals Pvt. Ltd, India. HPLC-grade water was used for analysis.

Preparation of Standard Solutions: 100 mg of both markers *i.e.,* reserpine and piperine were weighed accurately and transferred to a volumetric flask of 100 ml and volume was then made up with methanol to obtain a solution of 1000 µg/ml. The stock solution was injected in HPLC after suitable dilutions.

Preparation of Sample Solution: 10 tablets of the formulation were triturated and 2 g powder of this tablet mixture was weighed accurately. The weighed powder was subjected to extraction with methanol for 30 minutes using reflux assembly. The refluxed solution was filtered through the Whatman filter paper. The solution was injected after suitable dilutions.

Mode of chromatography	:	Reversed phase chromatography
Mode of elution	:	Isocratic
Column	:	Prontosil C-18 column, 5µ (4.6×250 mm)
Flow rate	:	1.0 ml/min
Column oven temperature	:	28°C
Wavelength	:	225 nm
Column	:	C18 (5µm Particle size, Dimensions: 4.6 x 250 mm)
Run time	:	15 min
Injection volume	:	20 µl
Mobile Phase	:	Acetonitrile and water containing 0.05% ortho-phosphoric in the ratio (50:50)
Diluent	:	Methanol

HPLC Method Development:

HPLC Method Development and Validation: This study aimed to validate the HPLC method for quantification of reserpine and piperine as per the ICH Guidelines [12].

Instrumentation: Analysis was performed on Shimadzu prominence i-3D LC 2030 HPLC consisting of a quaternary low-pressure gradient solvent delivery pump, UV detector and an autosampler. Stationary phase of the column was Prontosil C18 (250 × 4.6 mm) SH 5.0 µm, operated at 28°C. Responses of peak area were recorded and integrated using Lab Solutions software. The wavelength maximum (λ max) was obtained by using a UV-Visible spectrophotometer.

Optimized Chromatographic Conditions: The optimized mobile phase was acetonitrile and water containing 0.05% ortho-phosphoric acid in the ratio 50:50, flow rate was kept 1 ml/min, column temperature was set at 28°C and detection wavelength was at 225 nm using a UV-visible detector.

HPLC Method Validation [12]: The developed method was validated as per ICH guidelines Q2 (R1) for the parameters such as specificity, linearity, precision, accuracy, robustness, the limit of detection, and limit of quantification.

Specificity: Specificity is the ability of any analytical method to assess distinctly the analyte in the presence of components that may be presumed to be present.

Linearity: The linearity of any analytical procedure is its ability to obtain test results that are directly proportional to the concentration (amount) of analyte in the sample.

Precision: System precision and method precision were evaluated using six replicates of standard and sample solution of reserpine and piperine.

The solutions were analyzed, and percent relative standard deviation (% RSD) was calculated.

Accuracy: Recoveries of reserpine and piperine from the formulation were checked by spiking known quantity of standards *i.e.,* 80 %, 100 % and 120 % of the quantified marker amount to the sample.

Robustness: The robustness of the developed method was evaluated by making deliberate changes in flow rate of the mobile phase (± 0.2 ml/min), column temperature (± 1°C) and wavelength (± 1 nm).

Limit of Detection (LOD): LOD of an individual analytical technique is the lowest amount of analyte in a sample which can be detected but not necessarily quantified as an exact value. LOD is determined by the formula given below.

$$LOD = 3.3\ \sigma / S$$

Limit of Quantification (LOQ): LOQ of an individual analytical process is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

$$LOQ = 10\ \sigma / S$$

The slope (S) and standard deviation (σ) were estimated from the calibration curve of each marker.

Quantification of Markers: The concentration of two markers reserpine and piperine were estimated from marketed formulation using linear regression analysis.

RESULTS AND DISCUSSION:

Specificity: According to the established optimized chromatographic conditions, retention time (RT) of reserpine and piperine at this mobile phase was found to be 5.4 min and 13.5 min, respectively as shown in **Fig. 1** and **Fig. 2**.

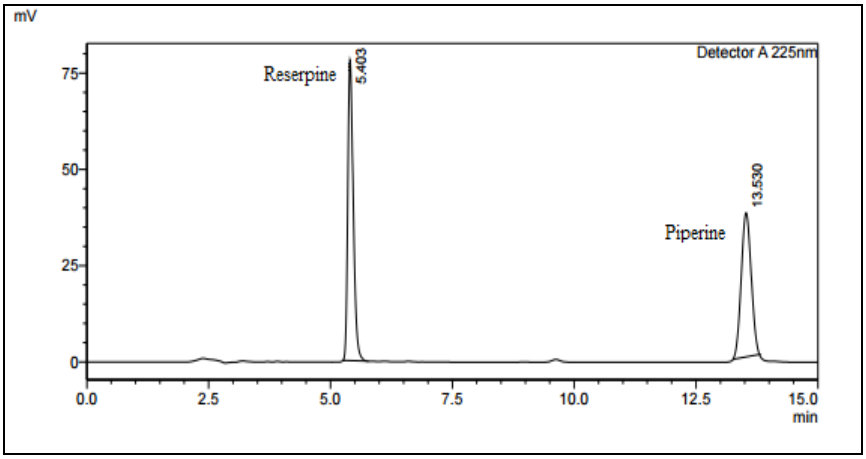

FIG. 1: CHROMATOGRAM OF MIXED STANDARD SOLUTION OF RESERPINE AND PIPERINE USING OPTIMIZED CONDITIONS

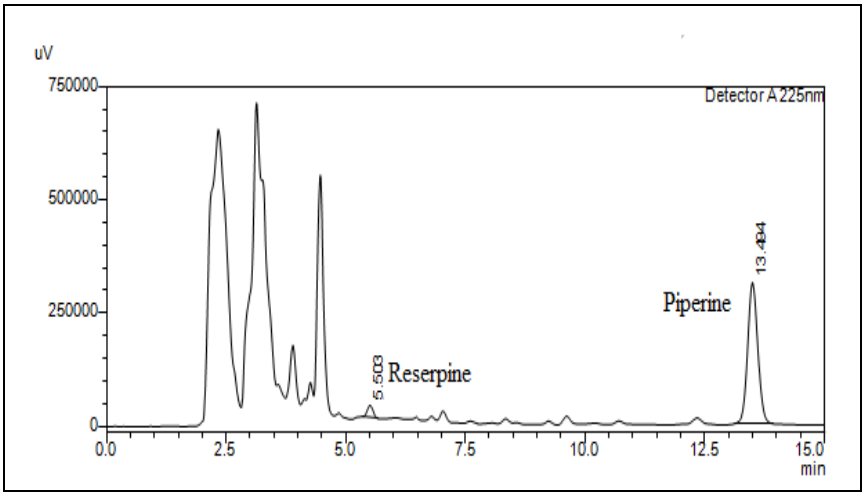

FIG. 2: CHROMATOGRAM OF EXTRACT OF MARKETED FORMULATION USING OPTIMIZED CHROMATOGRAPHIC CONDITIONS

The developed method was found to be specific as there was no interference of any other constituents at the retention time of reserpine and piperine.

Linearity: Reserpine and piperine showed linear responses in the concentration range from 1 to 20 µg/ml and 10 to 100 µg/ml as shown in **Fig. 3**.

The method was found to be linear as the coefficient of correlation (r^2) values were found to be within the limit. The results are depicted in **Table 1.**

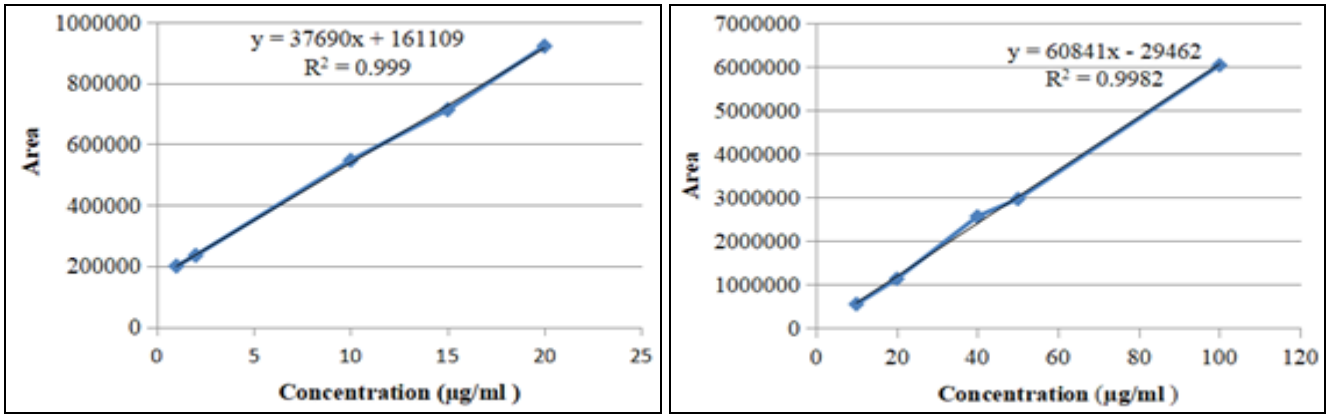

FIG. 3: CALIBRATION CURVE OF RESERPINE AND PIPERINE OBTAINED USING HPLC

TABLE 1: RESULTS OBTAINED FROM CALIBRATION CURVES OF RESERPINE AND PIPERINE

Parameters	Reserpine	Piperine
Linearity range (µg/ml)	1-20 µg/ml	10-100 µg/ml
Equation of regression line	y = 37690x + 16110	y = 60841x - 29462
(r^2)	0.999	0.998
Slope	37690	60841
Intercept	16110	29462

Precision: The statistical analysis of the result proved that the % RSD of the peak areas obtained was less than 2, hence the developed method is found to be precise as depicted in **Table 2**.

TABLE 2: RESULTS OF PRECISION OF RESERPINE AND PIPERINE

	System precision	Method precision	Intermediate precision	
	% RSD	%RSD	%RSD	
Markers			**Analyst 1**	**Analyst 2**
Reserpine	0.78	1.07	0.42	0.75
Piperine	0.76	0.81	1.2	1.31

Accuracy: The method was found to be accurate since the percent recovery was found to be within the limits as depicted in **Table 3**.

TABLE 3: RESULTS OF ACCURACY STUDIES FOR RESERPINE AND PIPERINE

Markers	Level of recovery (%)	Sample (µg/ml) n=3	Standard added (µg/ml)	Theoretical amount (µg/ml)	Amount recovered (µg/ml)	% Recovery
Reserpine	80	7.6	6.0	13.6	13.48	99.15
	100	7.6	7.6	15.2	14.9	98
	120	7.6	9.1	16.7	16.66	99.79
Piperine	80	376.3	301.04	677.34	666.56	98.11
	100	376.3	376.3	752.60	744.59	98.9
	120	376.3	451.56	827.86	819.74	99.01

Robustness: The statistical analysis of the robustness results proved that the % RSD of the peak areas obtained was less than 2, hence the developed method was found to be robust. The results of robustness are depicted in **Table 4**.

TABLE 4: ROBUSTNESS RESULTS OF RESERPINE AND PIPERINE

Parameter	Deviation n=3	% RSD			
		Reserpine		Piperine	
		Area	RT	Area	RT
Flow rate (ml/min)	0.8 ml	0.52	0.23	0.79	0.20
	1.2 ml	0.85	0.83	1.31	0.87
Column temperature	27°C	0.95	0.65	0.55	0.63
	29°C	1.36	0.53	0.99	0.25
Wavelength	224 nm	0.88	0.38	0.66	0.40
	226 nm	0.78	0.69	0.76	0.31

LOD and LOQ: LOD and LOQ of reserpine were found to be 0.16 and 0.48 µg/ml and LOD and LOQ of piperine were found to be 0.48 and 1.42 µg/ml. Low LOD and LOQ values indicate that the method is sensitive.

Quantification of Markers: Markers were quantified in the sample using linear regression analysis and the results are depicted in the **Table 5.**

TABLE 5: QUANTIFICATION OF MARKERS

Markers	% w/w content
Reserpine	0.0019
Piperine	0.0940

Solution Stability: The solution stability of the drug substance should be evaluated since most laboratories utilize autosamplers with overnight runs.

The sample solution was injected at a different time interval and % RSD was calculated. The solution was found to be stable for 48 hours.

Summary of Results and Validation: A summary of all the results and validation obtained is given in **Table 6**.

TABLE 6: SUMMARY OF RESULTS OBTAINED

Sr. no.	Parameters	Levels	Results	
			Reserpine	Piperine
1	Linearity range (µg/ml)	-	1 - 20 µg/ml	10 - 100 µg/ml
2	Correlation coefficient (r^2)	-	0.999	0.998
3	Accuracy (% recovery)	80 %	99.15	98.11
		100 %	98	98.9
		120 %	99.79	99.01
4	System precision (% RSD)	-	0.78	0.76
5	Method precision (% RSD)	-	1.07	0.81
6	Intermediate precision (Ruggedness) (% RSD)	Analyst 1	0.42	1.2
		Analyst 2	0.75	1.31
7	Robustness (Wavelength) (% RSD)	224 nm	0.88	0.66
		226 nm	0.78	0.76
8	Robustness (Flow rate) (% RSD)	0.8 ml/min	0.52	0.79
		1.2 ml/min	0.85	1.31
9	Robustness (Column temperature) (% RSD)	27ºC	0.95	0.55
		29ºC	1.36	0.99
10	Solution stability	-	The solution stability of sample solution was found to be 48 hours	
11	LOD (µg/ml)		0.16	0.48
12	LOQ (µg/ml)		0.48	1.42

CONCLUSION: In the present research work, a novel, simple, accurate, precise, and robust HPLC method is developed to simultaneously estimate reserpine and piperine. The developed method was applied for the standardization of a marketed polyherbal formulation. The method was validated as per the guidelines provided in ICH Q2 (R1) in terms of specificity, linearity, precision, accuracy, robustness, limit of detection, and limit of quantification. This novel developed HPLC method can be used to quantify reserpine and piperine in

different herbal formulations containing Rauwolfia and Pepper as one the ingredients.

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CONFLICTS OF INTEREST: Nil

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