ORIGINAL ARTICLE

A Simplex-Optimized Chromatographic Separation of Phytoconstituents in Cardioprotective Polyherbal Formulation and Crude Drugs

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Abstract

Aim: The study aims to develop and validate accurate, precise, and robust reverse phase high performance liquid chromatographic method for determination of phytoconstituents such as ascorbic acid, gallic acid, quercetin, and reserpine in polyherbal formulation and crude drugs. **Materials and Methods**: The adequate chromatographic separation was accomplished on C_{18} column, 250 × 4.6 mm, 5 µmAgilent eclipse using mobile phase 0.05 M sodium dihydrogen phosphate buffer (pH-4 adjusted with 1% Orthophosphoric acid): Acetonitrile with properly resolved gradient program. The flow rate was 1 ml/min, and the ultraviolet detection was monitored at 227 nm. The retention time of ascorbic acid, gallic acid quercetin and reserpine was found to be 3.44 min, 5.26 min, 10.02 min and 13.24 min, respectively. The method was validated as a final verification of method development concerning precision, linearity, accuracy, sensitivity and robustness as per International Council for Harmonization (ICH) guideline Q2 (R1). **Results and Discussion**: A method is considered to be linear as the correlation coefficient was found to be within acceptance criteria. The detector response was linear in the range of 50–250 µg/ml Ascorbic acid, 100-300 µg/ml Gallic acid, 100-300 µg/ml, Quercetin and 50–250 µg/ml Reserpine. The % RSD of peak area response due to Ascorbic acid, Quercetin, Reserpine and Gallic acid in five replicate injections of standard solution was to be less than 2.0%, and system suitability parameters were passed. **Conclusion**: The proposed method was effectively applied for the simultaneous estimation of Ascorbic acid, Gallic acid, Quercetin and Reserpine in Polyherbal formulation and in crude drugs.

Key words: Polyherbal formulation, Phytoconstituents, Ascorbic acid, Quercetin, Reserpine, Gallic acid, RP-HPLC

INTRODUCTION

Herbal remedies are increasingly being used to treat and manage cardiovascular diseases (CVDs). Plants include a plethora of phytoconstituents that have been shown to protect against a variety of diseases. The literature provides scientific evidence and hence justification for the use of phytotherapy in the treatment of CVDs.[1] Herbal medicines are slowly but steadily making their way into evidence-based practice. Rauwolfia serpentina is a natural remedy for hypertension that is both safe and effective. Many doctors in India utilized the plant in the 1940s, and it was used all over the world in the 1950s, including in the United States and Canada. The root has the largest concentration of Reserpine, whereas the stems and leaves have the lowest. Triphala has been used to rejuvenate the body in India for hundreds of years and is regarded one of the most essential Ayurvedic formulae.[2] Triphala is made up of three herbs that work together to generate a powerful combination that has a subtle yet deep effect. The three plants Amla (Emblica officinalis), haritaki (Terminalia chebula), and vibhitaki are

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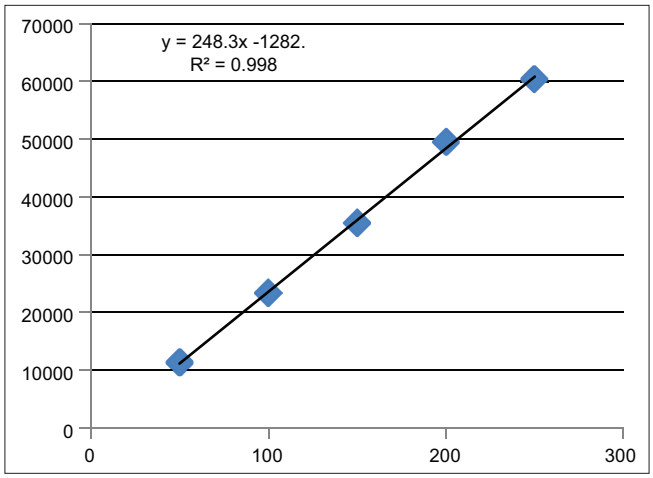

Figure 1: Calibration curve of ascorbic acid (50–250 µg/ml)

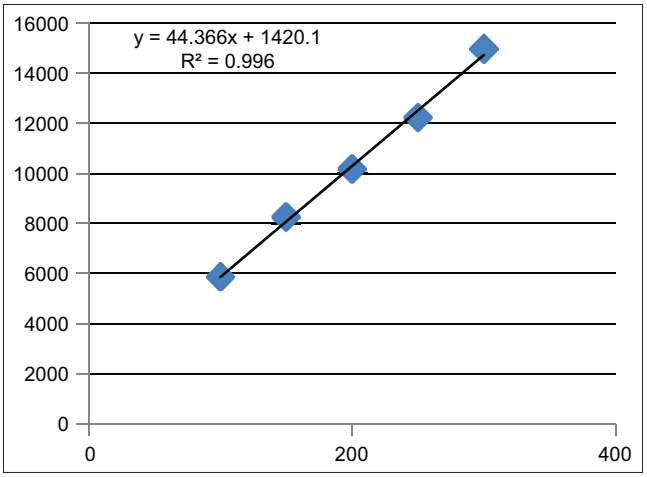

Figure 2: Calibration curve of gallic acid (100–300 µg/ml)

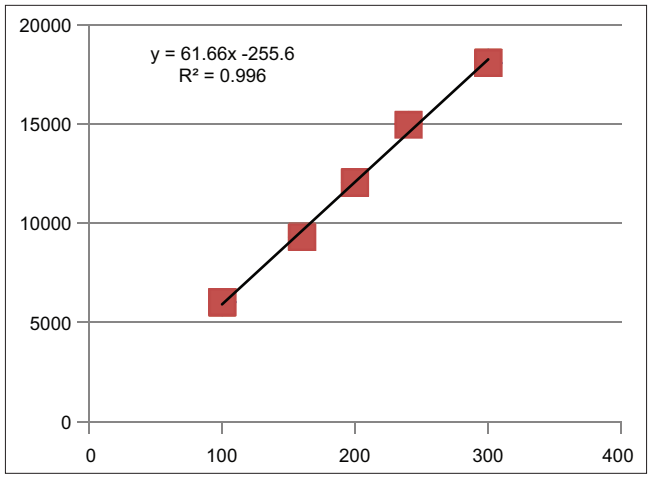

Figure 3: Calibration curve of quercetin (100–300 µg/ml)

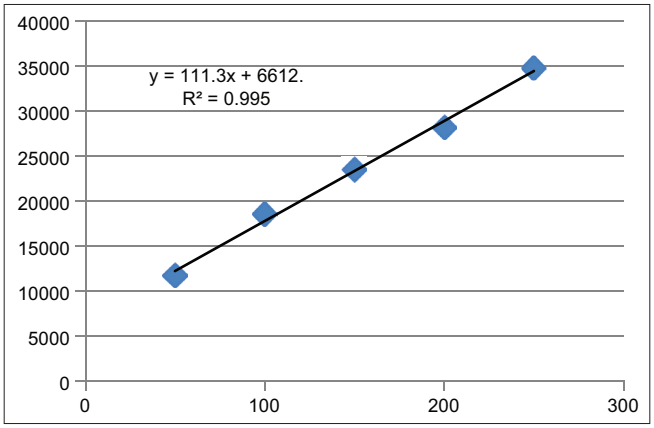

Figure 4: Calibration curve of reserpine (100–300 µg/ml)

the three herbs (Terminalia bellerica). Triphala lowers blood pressure, improves blood circulation, lowers cholesterol and helps to prevent artery atherosclerosis. It has anti-inflammatory qualities, reduces cholesterol, and aids in edema reduction. Through a variety of mechanisms, it aids in the reduction of high blood pressure. By lowering edema and inflammation there's less pressure against vessel walls. Inflammation causes atherosclerosis (cardiovascular disease is now referred to as an inflammatory disease), which narrows blood vessels There is less resistance against artery walls when inflammation is reduced, which decreases vital signs. The work burden on your heart gradually lowers as resistance diminishes.[3] The phytoconstituents of single plants are insufficient to attain the appropriate therapeutic effects. Multiple herbs in polyherbal and herbo-mineral formulations in a meticulous ratio will give an improved therapeutic effect and lessening the toxicity.[4,5] Several chromatographic methods, for example, UV-visible Spectrophotometric,[4-6] HPLC,[7-10] HPTLC,[11-16] and methods are established for the qualitative and quantitative estimation of the Ascorbic acid, Gallic acid, Quercetin, and Reserpine separately or together with other chemical markers. This study primarily reports RP-HPLC method for the straightforward and rapid determination of the Ascorbic acid, Gallic acid, Quercetin, and Reserpine in Polyherbal Formulation. The optimized method is useful for quality control and to know the usage and functions of the herb and its products in research into its antihypertensive and antioxidant activities.

EXPERIMENTAL

In this study, the simultaneous quantification of Ascorbic acid, Gallic acid, Quercetin, and Reserpine in polyherbal formulations and crude drugs was attempted. To create an acceptable and rapid approach for the simultaneous analysis of all four phytoconstituents, several trials were conducted with respect to the mobile phase composition, columns, and UV detector wavelength.

MATERIALS AND METHODS

HPLC grade solvents methanol and acetonitrile were purchased from Merck, Mumbai, India. Deionized water used throughout the experiment was obtained from a Millipore water purification system (Millipore, gradient, 0.22-mmpore size). Reserpine, Quercetin, Gallic acid, and Ascorbic acid were purchased

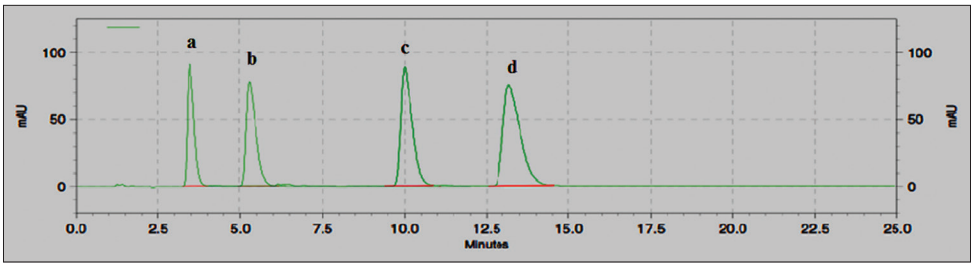

Figure 5: Chromatogram of the standard mixture (a. Ascorbic acid, b. Gallic acid, c. Quercetin, d. Reserpine)

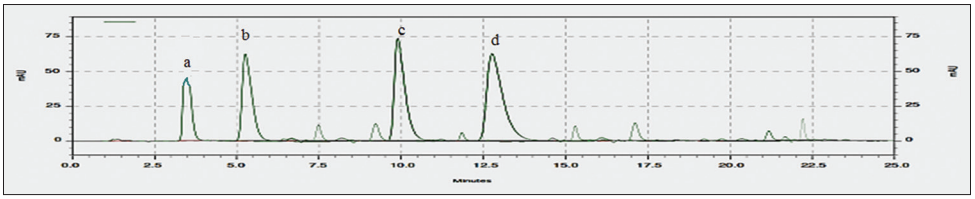

Figure 6: Chromatogram of the formulation (a. Ascorbic acid, b. Gallic acid, c. Quercetin, d. Reserpine)

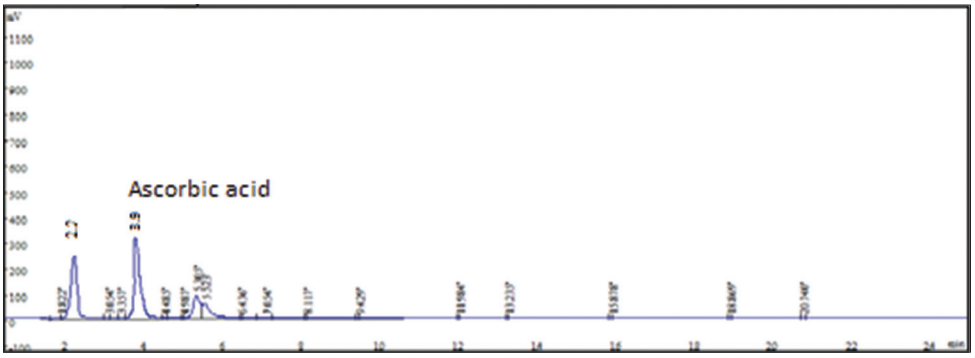

Figure 7: Ascorbic acid (*Emblica officinalis* extract)

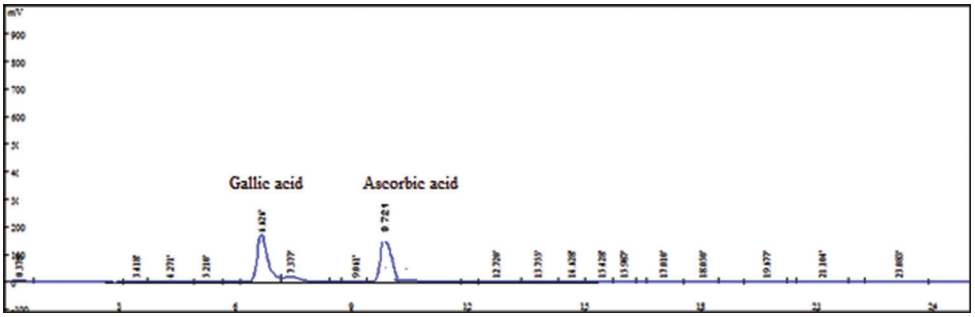

Figure 8: Gallic acid and quercetin (triphala churna extract)

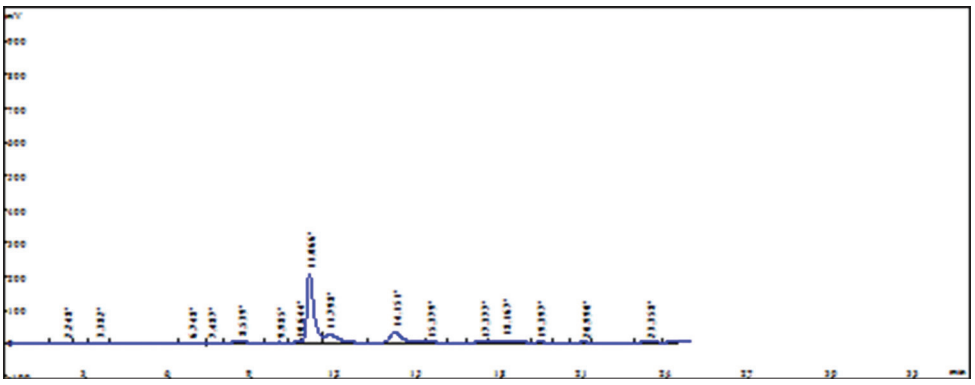

Figure 9: Reserpine (*Rauwolfia serpentina* extract)

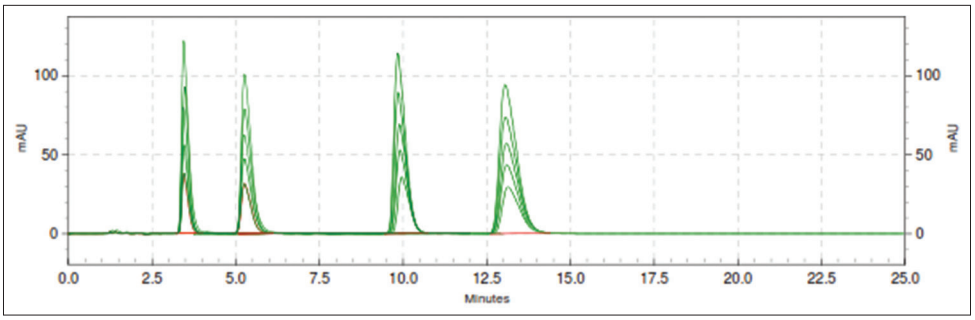

Figure 10: Chromatogram of linearity (ascorbic acid 100–300 µg/ml, gallic acid 100–300 µg/ml, quercetin 100–300 µg/ml, reserpine 100–300 µg/ml)

from Yucca laboratories, Mumbai, India. The authenticity of reference compounds (Reserpine, Quercetin, Gallic acid and Ascorbic acid) was confirmed by recording their IR-spectra.

INSTRUMENTATION AND CHROMATOGRAPHIC CONDITIONS

Chromatographic conditions

The Chromatographic separation system consisted of Shimadu HPLC (LC – 2010C HT, Shimadzu), detector PDA (SPD - M20A, Shimadzu), injection system (Rheodyne System 20 µl loop), and oven (CTO -10AS, Shimadzu). An Agilent Eclipse C_{18} RP-Column (250×4.6 mm, 5 µ). The elution was carried with flow rate of 1ml/min using Gradient quaternary pump.

Mobile phase

Two solvents used were Phosphate buffer pH 4 (solvent A) and 100% ACN (solvent B). The elution conditions were as follows:

Time (min.)	Mobile phase A	Mobile phase B
0.00	60	40
5.00	60	40
8.00	70	30
10.00	70	30
15.00	60	40
20.00	60	40
25.00	Stop	Stop

Preparation of standard solutions and calibration curve

Quantification of Reserpine, Quercetin, Gallic acid and Ascorbic acid through Calibration Curve.

An accurately weighed 10 mg of standard Ascorbic acid, Gallic acid, Quercetin, and Reserpine were dissolved in 10 ml of methanol to get stock solution of about 1000 µg/mL separately in 10 ml volumetric flask. This solution was filtered through 0.45 µm membrane filter paper and sonicated for10 min and used as a standard stock solution. The 0.5 ml of stock solution of Ascorbic acid and Reserpine were quantitatively transferred into a 10 mL volumetric flask. In the same flask 1 ml of stock solution of Gallic acid and Quercetin were transferred and made up to the mark with methanol to get concentration of working standard solutions of Ascorbic acid 50 µg/ml, Gallic acid 100 µg/ml, Quercetin 100 µg/ml and Reserpine 50 µg/ml. Similarly, further dilutions where made from standard stock solution to construct calibration curves for Ascorbic acid (50–250 µg/ml), Gallic acid (100–300 µg/ml), Quercetin (100–300 µg/ml)and Reserpine (50–250 µg/ml) [Figures 1-4].

Extraction and preparation of sample solutions

About 20 tablets were taken and crushed in mortar pastel. From that, accurately weighed and transferred 1.1 g tablet powder to 50 mL volumetric flask. 20 ml of hexane was added to defat the powder and Boiled for 15 min at 50°C on a water bath. The resulted solution was filtered to get the residue. 50 ml of methanol was added to the dried residue and boiled for 20 min at 50°C in a water bath. The solution was filtered through 0.22 µ filter to obtain sample working solution. Sample solution was analysed for the estimation of Ascorbic acid, Gallic acid, Quercetin and Reserpine.[Table 1 and Figures 5 and 6].

Amla powder: Extracted 1g crude powder with 50 ml of methanol for 30 min by reflux method at 40-50 °C on water bath. The extract was filtered by whatman filter paper no.41 and used for detection [Figure 7].

Triphla Churna: Accurately weighed 1 g crude powder was extracted with 100 ml of methanol for 30 min by heating under reflux condition. The extract was filtered by whatman filter paper no.41 and used for detection [Figure 8].

Rauwolfia root powder: Extracted 3 g crude powder with 50 ml of methanol for 45 min by reflux method. The extract was Filtered by whatman filter paper no.41 and used for detection [Figure 9].

Method validation

Linearity

Linearity of the method was executed by linear regression and was linear in range 50–250 µg/ml Ascorbic acid, 100–300 µg/ml Gallic acid, 100–300 µg/ml Quercetin and 50–250 µg/ml Reserpine, three phase's validation done. The graph was plotted as the mean peak area versus the concentration of each analyte. The R^2 value was found to be 0.998 for Ascorbic acid, 0.996 for Gallic acid, 0.996 Quercetin. and 0.995 for Reserpine. Calibration curve of Ascorbic acid, Gallic acid, Quercetin and Reserpine is shown in Figures 1-4 and 10 respectively.

Selectivity

The selectivity for interference of any peak with main peak of interest is checked. No other peak appeared at the retention times of Ascorbic acid, Gallic acid, Quercetin and Reserpine, (3.44, 5.26, 9.96 and 12.6 min, respectively). Furthermore, the interaction studies reported that the analyte did not interact with each other and were well below the 2.0% acceptance level of RSD.

Accuracy

Three replicate injection containing known amount of Ascorbic acid, Gallic acid, Quercetin and Reserpine at 80%, 100%, and 120% with respect to assay concentration. The developed method satisfies the acceptance criteria %RSD < 2 and ensures accuracy of method.

Precision

Repeatability [Table 2]

Interday precision

Assay method was analyzed by three independent sample solutions. Concentration of samples was calculated from the area obtained and results were expressed as %RSD [Table 3].

Table 1: Analysis of Formulation :(n=3)

Cardostab tablet	Content (%w/w) ±SD	% RSD	Triphala churna	Content (%w/w) ±SD	% RSD	Rauwolfia root powder extract	Content (%w/w) ±SD	% RSD
Ascorbic acid	0.17±0.005	1.33	Ascorbic acid	0.54±0.004	1.61	----------	----------	------
Gallic acid	1.16±0.014	1.53	Gallic acid	2.64±0.03	1.01	----------	----------	------
Quercetin	0.92±0.007	1.32	Quercetin	1.02±0.01	1.32	----------	----------	------
Reserpine	0.16±0.009	1.76	----------	----------	----------	Reserpine	0.15±0.004	1.14

Table 2: Repeatability study

Conc. of ascorbic acid (µg/ml)	Area	Conc. of Gallic acid (µg/ml)	Area	Conc. of quercetin (µg/ml)	Area	Conc. of reserpine (µg/ml)	Area
150	22409	200	10167	200	12045	150	23052
	21365		10123		12057		23445
	22036		10113		12101		23765
	21853		10334		12067		23126
	22456		10003		12034		22998
	22354		10345		12267		23014
Mean	22079	Mean	10180	Mean	12095	Mean	23233
SD	421.7	SD	134	SD	87	SD	308
% RSD	1.91	% RSD	1.31	% RSD	0.72	% RSD	1.32

SD: Standard deviation, RSD: Relative standard deviation

Table 3: Interday precision of the method

Ascorbic acid			Gallic acid			Quercetin			Reserpine		
Conc.	Area	%RSD	Conc.	Area	%RSD	Conc.	Area	%RSD	Conc.	Area	%RSD
50	11447±213	1.86	100	5188±91	1.75	100	6163±93	1.5	100	11544±125	1.08
150	23444±432	1.75	200	10256±186	1.82	200	12223±224	1.83	200	23433±368	1.57
250	61226±956	1.56	300	15534±245	1.57	300	18254±277	1.52	300	33969±569	1.67

RSD: Relative standard deviation

Table 4: Intraday precision of the method

Ascorbic acid			Gallic acid			Quercetin			Reserpine		
Conc.	Area	%RSD	Conc.	Area	%RSD	Conc.	Area	%RSD	Conc.	Area	%RSD
50	11314±181	1.60	100	5136±61	1.18	100	6103±71	1.16	50	11357±163	1.43
150	23433±285	1.22	200	10353±172	1.66	200	12131±78	0.64	150	23186±234	1.01
250	60981±663	1.09	300	15453±213	1.38	300	18213±228	1.25	250	34480±452	1.31

Table 5: Results of LOD and LOQ

Parameter	Ascorbic acid	Gallic acid	Quercetin	Reserpine
S.D. of intercept	162	349	23.56	18.90
Mean of slope	248.4	43.7	12.76	45.02
LOD (µg/ml)	2.15	27.3	6.11	8.4
LOQ (µg/ml)	6.5	82.95	18.53	25.6

Table 6: System suitability study

System suitability parameters	Phytoconstituents			
	Ascorbic acid	Gallic acid	Quercetin	Reserpine
Retention time (min) ±SD	3.44±0.002	5.26±0.001	9.96±0.005	13.24±0.001
Tailing factor (T) ±SD	0.90±0.039	1.13±0.006	1.19±0.004	1.35±0.0235
Number of theoretical plates (N) ±SD	5405±23.1	5263±71.6	79472±19.98	13862±36.23
Resolution (R_s)	-----	3.89±0.003	12.2±0.211	7.09±0.173

SD: Standard deviation

Table 7: Results for accuracy of the method

Phytoconstituents	%of Std added	% Recovery (Mean±SD)	%RSD
Ascorbic acid 101.3 µg/ml	80	98.5±1.1	1.0
	100	98.7±1.3	1.09
	120	99.4±1.5	1.46
Gallic acid 130.5 µg/ml	80	100.3±1	1
	100	99.7±1.5	1.5
	120	98.7±1.2	1.24
Quercetin 125.9 µg/ml	80	98.9±1.6	2.0
	100	99.9±1.9	1.9
	120	99.9±1.2	1.15
Reserpine 96.6 µg/ml	80	98.8±1.4	1.4
	100	100.1±1.3	1.3
	120	99.5±1.7	1.71

SD: Standard deviation, RSD: Relative standard deviation

Intraday precision

The method was analyzed by carrying out the experiment on different days and concentration of samples were calculated from the area obtained and results were expressed as %RSD [Table 4].

LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTIFICATION (LOQ)

Limit of detection and limit of quantification were determined by formula 3.3 × σ/slope and 10 × σ/slope, respectively [Table 5].

Where

σ = standard deviation of intercept

Slope = mean slope of calibration curve.

Robustness

Robustness of the method was substantiated by the application of minor but deliberate changes in the investigational parameters, here: (i) Flow rate: ±0.2 mL/min, (ii) Wavelength: ±2 nm (iii) Mobile phase pH: ±0.2. These modifications were done to see how they affected the process. The obtained data was evaluated by calculating percent RSD and percentage of recovery for each parameter.

System suitability

The parameters evaluated were asymmetry of chromatographic peak, Theoretical plates, and retention time [Table 6].

Analysis of formulation: (n=3)

RESULTS AND CONCLUSION

Estimation of Ascorbic acid, Gallic acid, Quercetin and Reserpine in Cardostab tablet is found to be accurate gradient elution [Table 7] with a flow rate of 1 mL min^{-1} was used. A column oven was conditioned at 25°C. The injection volume was 20 µl and analysis time was 25 min. The standard curve showed a linear response in a concentration range 50–250 µg/ml for Ascorbic acid, 100–300 µg/ml Gallic acid, Quercetin 100–300 µg/ml and Reserpine 50–250 µg/ml with correlation coefficient 0.998, 0.996, 0.996 and 0.995, respectively. The result of accuracy studies indicated high recovery values 98.5–99.4% for Ascorbic acid, 98.7–100.4% for Gallic acid, 98.9–99.9 for Quercetin and 98.8–100.1 for Reserpine. The low coefficient of variation values of intraday and interday precision showed the developed method is précised. LOD and LOQ were found to be 2.15 µg/ml and 6.5 µg/ml for Ascorbic acid, 27.3 µg/ml and 82.9 µg/ml for Gallic acid, 6.1 µg/ml and 18.5 µg/ml for Quercetin and 8.4 µg/ml and 25.6 µg/ml for Reserpine, respectively. The method of analysis varied the flow rate, mobile phase and pH to evaluate and measure the capacity of the method to remain unaffected by such variations for which % RSD was found to be less than 2 hence the method was proven to be robust. The developed method can be adopted for the routine analysis and quality control of Ascorbic acid, Gallic acid, Quercetin and Reserpine in Polyherbal formulation and crude drugs.

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