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Original Article

DEVELOPMENT AND VALIDATION OF A RP-HPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF QUERCETIN, ELLAGIC ACID AND RUTIN IN HYDROALCOHOLIC EXTRACT OF TRIPHALA CHURNA

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ABSTRACT

Objective: To develop a novel, accurate, precise and linear reverse phase high performance liquid chromatographic (RP-HPLC) method for simultaneous qualitative and quantitative estimation of quercetin, ellagic acid and rutin in an ayurvedic formulation and validate as per international conference on harmonization (ICH) guidelines.

Methods: In the present work, good chromatographic separation was achieved isocratically using a shim-pack HPLC C18 column (4.6 x 250 mm, 5µm) and a mobile phase consisting of 0.02 M potassium dihydrogen orthophosphate buffer (pH adjusted to 3 with orthophosphoric acid) and methanol in the ratio 55:45, at flow rate of 1 ml/min and column temperature maintained at 35 °C. The effluents obtained were monitored at 254 nm with a UV-visible detector.

Results: The retention time of quercetin, ellagic acid and rutin were found to be 7.52 min, 9.10 min and 12.47 min respectively. Linearity of quercetin, ellagic acid and rutin were found in the range of 8-12 ppm, 9-17 ppm and 7-11 ppm respectively. The correlation coefficient for quercetin, ellagic acid and rutin were 0.997, 0.999 and 0.999 respectively. The high recovery values (98 %-102 %) indicate a satisfactory accuracy. The low percent relative standard deviation (% RSD) values in the precision study reveal that the method is precise.

Conclusion: The developed method is novel, simple, precise, rapid, accurate and reproducible for simultaneous quantitative estimation of quercetin, ellagic acid and rutin in an ayurvedic formulation. Hence the developed method can be used for quantitative analysis and quality control of extracts and commercial samples of other plant species and formulation containing these three markers.

Keywords: Quercetin, Ellagic acid, Rutin, Ayurvedic formulation, RP-HPLC, Validation, ICH

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INTRODUCTION

Ayurvedic medicines are polyherbal formulations and every herb consists of an array of chemical constituents. Hence, each ayurvedic formulation is a source of many different phytochemicals, which individuals may have different medicinal properties. The standardization parameters set for such polyherbal formulations are inadequate and therefore quality specifications are not stringent. Standardization is an important parameter for maintaining and accessing the safety and quality of herbal medicines as they are comprised of many chemical constituents and are capable of variation [1] Therefore, the development of various novel analytical techniques for the analysis of medicinally active phytoconstituents has become a crucial step of standardization. The information generated from high-performance thin layer chromatography (HPTLC) and high-performance liquid chromatography (HPLC) fingerprint profiles have a potential application in deciding the identity, purity, and strength of herbal medicines and for fixing standards for herbal formulations [2].

Triphala churna is (1:1:1) combination of three compounds viz. *Terminalia chebula*, *Terminalia belerica* and *Terminalia officinalis* each of which has its own therapeutic value, but in combination, it enhances the overall potential these are used mostly as an antioxidant, anti-aging, anti-inflammatory, mental and memory enhancing the effect. The extract of triphala was obtained by using a soxhlet extraction process and methanol, water in a ratio (70:30). A marker which has been found in triphala by the various method are quercetin, rutin, gallic acid, ellagic acid, ascorbic acid and known for its effect [3]. In the present study, we have selected three chemical markers namely quercetin, ellagic acid and rutin.

The literature survey reveals that various analytical methods for estimation of quercetin, ellagic acid and rutin were reported alone and in combination with other drugs [3-8] also simultaneous estimation of gallic acid, catechin, rutin, ellagic acid and quercetin in flower extract of *Michelia alba*, *Caesalpinia pulcherrima* and *Nelumbo nucifera* by HPLC reported [9] but to the best of our knowledge there is no such reported HPLC analysis method for simultaneous estimation of quercetin, ellagic acid and rutin in triphala churna.

In the present investigation, we have developed a simple, optimized and validated HPLC method for the standardization of triphala churna ayurvedic formulation using three chemical markers namely quercetin, ellagic acid and rutin. The method was validated as per the international conference on harmonization (ICH) guidelines. This novel validated method has applicability in an industry as well as in academia.

MATERIALS AND METHODS

HPLC grade quercetin, ellagic acid and rutin (purity 99%) were procured as gift sample from Yucca Enterprises, Mumbai, India. An ayurvedic preparation Zandu triphala churna (Zandu Pharmaceutical Works Ltd.) used for analysis was procured from local market. HPLC grade solvents were purchased from Thomas Baker. RP-HPLC shimadzu (LC 2030) model with "Lab Solution" software was employed in this method. Analytical column used for the separation of analytes was shim-pack HPLC C18 (250 X 4.6 mm, 5 µm).

Methods

Selection of wavelength

Suitable wavelength for the HPLC analysis was determined by recording UV spectrums in the range of 200-400 nm for individual drug solutions of quercetin, ellagic acid and rutin then overlapped. UV overlain spectra of these three markers showed that the drugs absorb appreciably at 254 nm and hence 254 nm was taken as a detection wavelength for HPLC analysis (fig. 1).

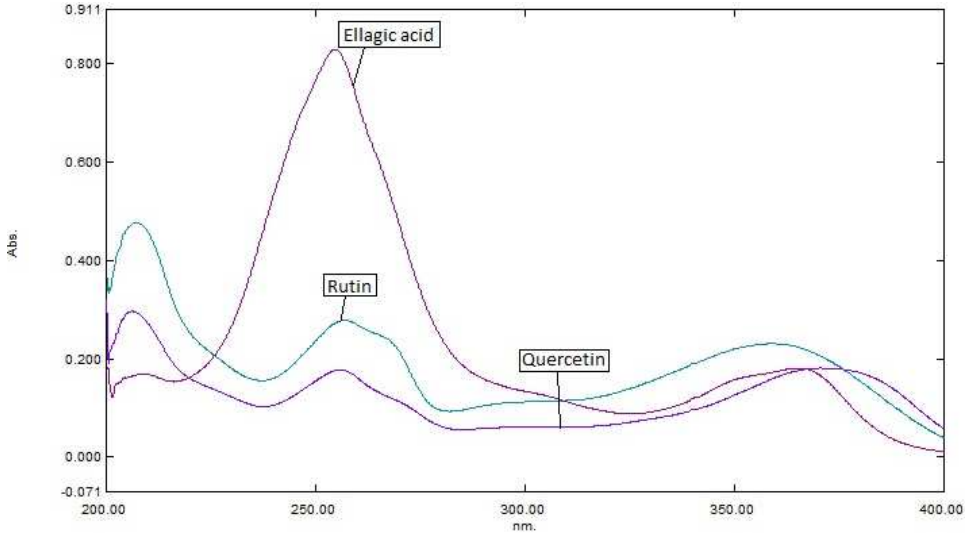

Fig. 1: UV overlap spectrum of quercetin, ellagic acid and rutin

Chromatographic conditions

The method was developed using reverse phase, shim-pack HPLC C18 column (250 X 4.6 mm, 5 µm). The runtime was of 20 min. The mobile phase used was 0.02 M potassium dihydrogen orthophosphate buffer (pH adjusted to 3 with orthophosphoric acid) and methanol in the ratio 55:45 at a flow rate of 1.0 ml/min, column temperature maintained at 35 °C and a detection wavelength of 254 nm using a UV-visible detector.

Preparation of 0.02 M phosphate buffer (pH 3)

About 3.48 g of potassium dihydrogen orthophosphate was accurately weighed and dissolved in 950 ml of water. The pHwas adjusted to 3 with orthophosphoric acid and the volume was made up to 1000 ml in a volumetric flask. The solution was then filtered using 0.45 µ membrane filter.

Preparation of standard solution

100 mg of quercetin, ellagic acid and rutin standard were accurately weighed and transferred into 100 ml volumetric flask respectively. About 70 ml solvent was added, sonicated to dissolve and diluted up to the mark using a solvent (1000 ppm). The final concentration of quercetin, ellagic acid and rutin were made to 10 ppm, 13 ppm and 9 ppm respectively by suitable dilutions.

Sample preparation

Accurately about 500 mg of triphala churna was extracted with 100 ml methanol and water in the ratio (70:30). The sample solution was filtered to obtain a clear solution. The stock solution after suitable dilutions was used for further analysis.

RESULTS AND DISCUSSION

Method development

A reverse phase HPLC method was developed keeping in mind the system suitability parameters, i.e. resolution factor between peaks, tailing factor, number of theoretical plates, runtime and the cost-effectiveness. The developed optimized method resulted in the elution of quercetin at 7.52 min, ellagic acid at 9.10 min and rutin at 12.47 min. fig. 2,3 and 4 represent chromatograms of quercetin, ellagic acid and rutin standard solution respectively. The total run time was 20 min. System suitability tests are an integral part of method development and are used to ensure adequate performance of the chromatographic system. Retention time, a number of theoretical plates, peak resolution and peak tailing factor were evaluated for six replicate injections of the standard working concentration. The results given in table 1 were within the acceptable limits [10, 11].

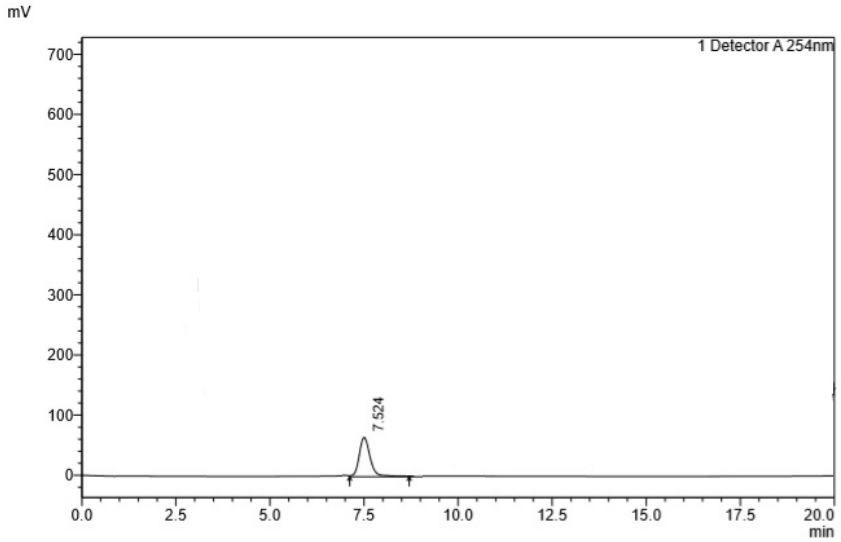

Fig. 2: Typical chromatogram of quercetin standard solution

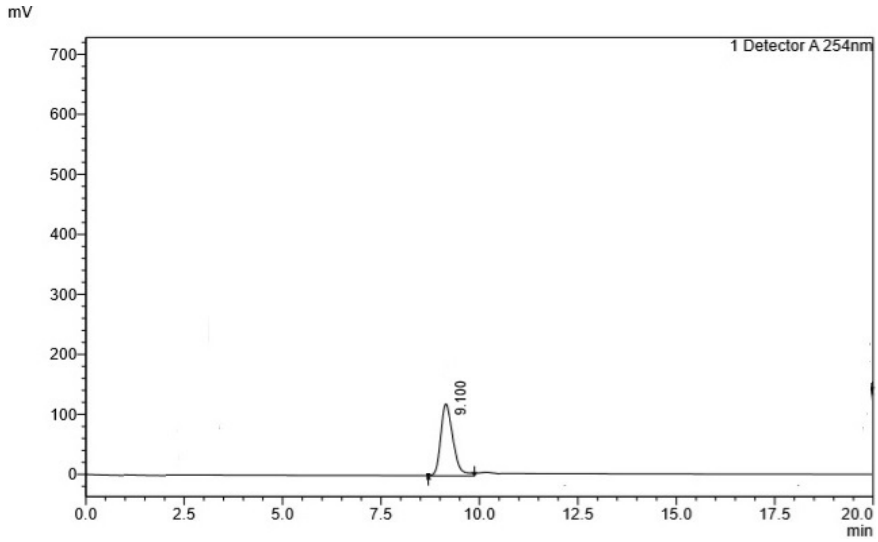

Fig. 3: Typical chromatogram of ellagic acid standard solution

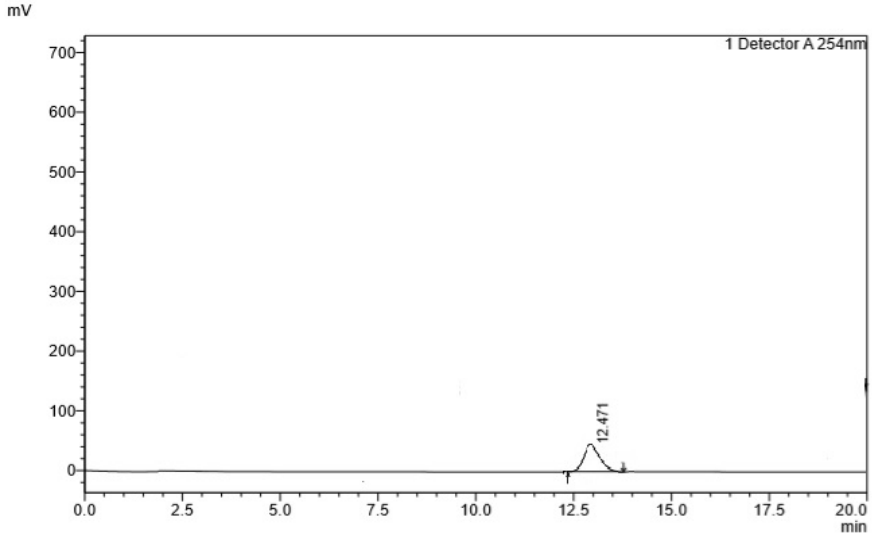

Fig. 4: Typical chromatogram of rutin standard solution

Table 1: Results of system suitability studies

Parameters	Acceptance limits	Quercetin	Ellagic acid	Rutin
Retention time (min)	-	7.52	9.10	12.47
Resolution factor	Not less than 2	-	3.04	7.10
Number of theoretical plates	Not less than 2000	3436	4147	3786
Tailing factor	Not more than 2	1.20	1.35	1.21

To test the applicability of the developed method to an ayurvedic formulation, triphala churna extract was chromatographed and it is shown in fig. 5. The sample peaks were identified by comparing the relative retention times with standard markers (fig. 2-4). System suitability parameters were within the acceptable limits, ideal for the chromatographed sample. Integration of the separated peak area was done, and each marker concentration was determined by using the peak area concentration relationship obtained in the standardization step. For the analysis, of the sample, extract of 1000 ppm of triphala churna was injected in triplicate and quantified for three active markers using a linear regression equation. The results of churna extract analysis are reported in table 2.

Table 2: Analysis of triphala churna extract

Formulation	Marker	Amount found (ppm) n=3	Content (%)
Triphala churna extract (1000 ppm)	Quercetin	9.49	0.94
	Ellagic acid	11.33	1.13
	Rutin	8.12	0.81

n: number of injections

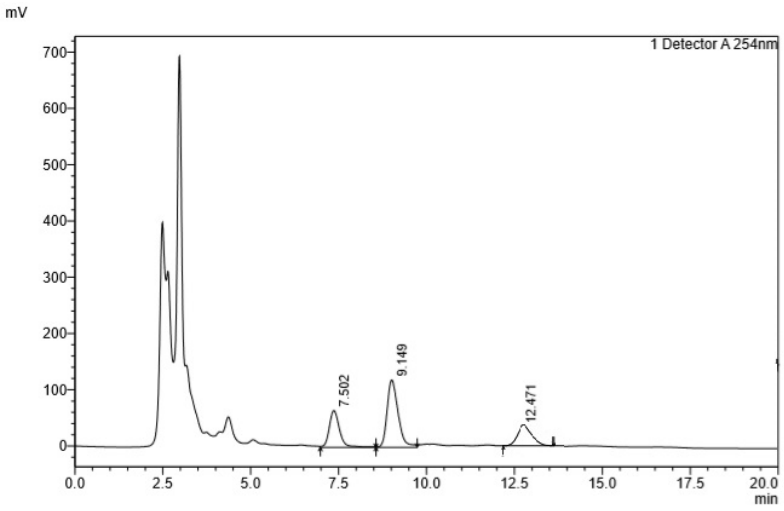

Fig. 5: Typical chromatogram of triphala churna extract

Method validation

Validation of the analytical method is the process that establishes by laboratory studies in which the performance characteristics of the method meet the requirements for the intended analytical application. The developed HPLC method was validated according to ICH guidelines [12] for validation of analytical procedures. The method was validated for the parameters like linearity, accuracy, system precision, method precision, robustness, limit of detection (LOD) and limit of quantitation (LOQ).

Specificity

Fig. 2-5 for standard drug solutions and sample chromatogram reveals that the peaks obtained in the standard solutions and sample solution at working concentrations are only because of the drugs as blank has no peak at the retention time of quercetin, ellagic acid and rutin. Accordingly, it can be concluded that the method developed is said to be specific [13, 14].

Precision

System precision

Six replicate injections of the standard solutions at working concentration showed percent relative standard deviation (% RSD) less than 2 concerning the peak area for each marker, which indicates the acceptable reproducibility and thereby the precision of the system [15, 16]. System precision results are tabulated in table 3.

Method precision

Method precision was determined by performing the analysis of the sample under the test of repeatability at working concentration. Six injections of the sample from the same homogeneous mixture at working concentration showed % RSD less than 2 concerning the content of three markers indicate that the method developed is precise by the test of repeatability [15, 16] and hence can be understood that the method gives consistently reproducible results (table 4).

Table 3: System precision results

S. No.	Peak area of quercetin (10 ppm)	Peak area of ellagic acid (13 ppm)	Peak area of rutin (9 ppm)
1	1327837	2604837	1234879
2	1328687	2618638	1208688
3	1347656	2628765	1218766
4	1332596	2608767	1215678
5	1328677	2618638	1226875
6	1387632	2610876	1218766
mean±SD	1342181±23485	2615087±8661	1220609±9123
%RSD	1.75	0.33	0.75

SD: Standard deviation, # %RSD: Percent relative standard deviation

Table 4: Method precision results

Marker	Intra-day		Inter-day	
	Content (ppm) n=3 mean±SD	% RSD	Content (ppm) n=3 mean±SD	%RSD
Quercetin	9.49±0.02	0.21	9.46±0.04	0.45
Ellagic acid	11.30±0.03	0.26	11.34±0.01	0.13
Rutin	8.11±0.01	0.12	8.14±0.02	0.30

n: number of injections, # SD: Standard deviation, # %RSD: Percent relative standard deviation

Linearity

Standard solutions of quercetin, ellagic acid and rutin at different concentration level were prepared in triplicates. Calibration curves were constructed by plotting the concentration level versus corresponding peak areas for each marker. The results show an excellent correlation between peak areas and concentrations level within the tested concentration range of 8-12 ppm, 9-17 ppm and 7-

11 ppm for quercetin, ellagic acid and rutin respectively (table 5). The correlation coefficients were greater than 0.99 for each marker, which meets the method validation acceptance criteria [11, 15, 16] and hence the method is said to be linear.

Table 5: Data from linearity studies

Marker	Concentration range (ppm)	Regression equation	R^2
Quercetin	8-12	Y=96471x+360471	0.997
Ellagic acid	9-17	Y=146896x+689023	0.999
Rutin	7-11	Y=108009x+290274	0.999

Accuracy

Accuracy was determined by means of recovery experiments, by the determination of % mean recovery of each compound in the formulation at three distinct levels (80%, 100% and 120%). At each level, three determinations were performed. Percent mean recovery was calculated as shown in table 6. The accepted limits of mean recovery are 98%-102% and all observed data were within the required range, which indicates good recovery values, affirming the accuracy of the method developed [15, 16].

Table 6: Recovery study for three markers in triphala churna

Compounds	Sample content [ppm]	Standard added [ppm]	Actual amount [ppm]	Total area found [n=3]	Amount recovered [ppm]	% Recovery
Quercetin	4.74	3.79	8.53	1197828	8.67	101.75
		4.74	9.48	1276479	9.49	100.15
		5.68	10.42	1347890	10.23	98.22
Ellagic acid	5.66	4.53	10.19	2210231	10.35	101.62
		5.66	11.32	2357891	11.36	100.36
		6.79	12.45	2515343	12.43	99.86
Rutin	4.06	3.24	7.30	1097539	7.47	102
		4.06	8.12	1158998	8.04	99.05
		4.87	8.93	1257656	8.95	100.29

n: Number of injections

Robustness

To determine the robustness of the developed method, experimental conditions were deliberately altered, and the system suitability parameter tailing factor and peak area were evaluated. The solution was prepared as per the test method described earlier and injected at different variable conditions like column temperature (33 °C and 37 °C) and detection wavelength (253 nm and 255 nm). Robustness data clearly show that the proposed method is robust at small but deliberate change [15, 16]. Robustness data are given in table 7.

Table 7: Robustness data for quercetin, ellagic acid and rutin

Parameters	Quercetin (10 ppm)		Ellagic acid (13 ppm)		Rutin (9 ppm)	
	Peak area	Tailing factor	Peak area	Tailing factor	Peak area	Tailing factor
Minus temp [33 °C]	1327876	1.17	2604837	0.98	1232794	1.03
Plus temp [37 °C]	1329968	1.09	2618768	0.97	1208778	0.99
Minus wavelength [253 nm]	1337567	0.99	2628767	0.93	1213434	1.17
Plus wavelength [255 nm]	1318767	1.37	2606787	0.82	1229697	1.09

Sensitivity

The sensitivity of measurement of quercetin, ellagic acid and rutin by use of the proposed method was estimated in terms of the LOQ and LOD. LOQ and LOD were calculated using the equations LOD = $3.3\sigma/S$ and LOQ = $10\sigma/S$ where σ is the standard deviation of intercepts of calibration plots and S is the average of the slopes of the corresponding calibration plot (table 8).

Table 8: LOD and LOQ for quercetin, ellagic acid and rutin

Compound	LOD (ppm)	LOQ (ppm)
Quercetin	0.80	2.43
Ellagic acid	0.19	0.58
Rutin	0.27	0.84

#LOD: limit of detection, #LOQ: limit of quantitation

In previous report [9] HPLC method was developed for simultaneous estimation of gallic acid, catechin, rutin, ellagic acid and quercetin using Luna C18 column (4.6 x 250 mm, 5µm) with gradient elution, whereas this study uses a novel column shim-pack HPLC C18 column (4.6 x 250 mm, 5µm) with isocratic elution. In HPLC method development isocratic elution is simpler than gradient elution because in the gradient elution column has to re-equilibrate to original conditions after each run and thus takes additional time. Weersak S. *et al.* in 2007 for their study uses water-acetic acid and methanol as mobile phase, whereas the present investigation uses novel mobile phase consisting of 0.02 M potassium dihydrogen orthophosphate buffer (pH adjusted to 3 with orthophosphoric acid)

and methanol in the ratio 55:45. Moreover, various analytical methods for these three markers alone or in combination with other drugs exist [3-8] but the method developed in this report is first of its kind and is useful in the qualitative and quantitative analysis of quercetin, ellagic acid and rutin from complex herbal mixture formulation.

CONCLUSION

The results indicate that the selected ayurvedic formulation contains a number of markers that may be responsible for its therapeutic activity. The developed HPLC method will assist in the standardization of triphala churna using biologically active chemical markers. The developed HPLC method for simultaneous determination of quercetin, ellagic acid and rutin from triphala churna is accurate, precise, reproducible and repeatable. Triphala churna also contains a number of other constituents, which are currently the subject of further investigation, apart from those standards studied. With the growing demand for herbal drugs and increased belief in the use of herbal medicine, the development of a standardization tool will help in maintaining the quality of this important ayurvedic preparation.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally

CONFLICT OF INTERESTS

Declared none

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