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Original Article

A NOVEL RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF BERBERINE, QUERCETIN, AND PIPERINE IN AN AYURVEDIC FORMULATION

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ABSTRACT

Objective: The objective of this study was to develop and validate a novel, simple, rapid, precise and accurate reversed-phase high performance liquid chromatographic (RP-HPLC) method for simultaneous quantitative estimation of berberine, quercetin, and piperine in Ayurvedic formulation.

Methods: The chromatographic separation was achieved using a stationary phase C18 shim-pack (150 mm x 4.6 mm, 5µ) column and mobile phase consisted of acetonitrile: 0.04 M potassium dihydrogen phosphate buffer (pH 3.0 adjusted using orthophosphoric acid) in a ratio of 65:35 v/v, with a flow rate of 1 ml/min and UV detection at 255 nm.

Results: The retention time of berberine, quercetin, and piperine were found to be 2.7, 3.0 and 6.3 min respectively. Linearity for berberine, quercetin, and piperine were found in the range of 12-28 µg/ml. All calibration curve showed good linear correlation coefficients (r^2> 0.999) within the tested ranges. Mean percent recoveries for berberine, quercetin, and piperine were found to be within the acceptance limits (98-120%). The percent relative standard deviation (% RSD) for precision was found to be less than 2% which indicates method is precise.

Conclusion: The developed method is novel, simple, precise, accurate and can be used for quantitative analysis and quality control of the raw material as well as other commercial formulations containing these three markers.

Keywords: Berberine, Quercetin, Piperine, Ayurvedic formulation, Validation, RP-HPLC, ICH

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INTRODUCTION

Standardization and analysis of the chemical markers in any ayurvedic formulation or polyherbal formulation is always difficult. Quantitative determination of chemical markers of each ingredient in the polyherbal preparation requires optimal separation techniques by which these markers are separated with high resolution and the least interferences from each other [1]. As herbal preparation comprise hundreds of mostly unique, or species-specific compound, it is difficult to characterise all of these compounds completely. Thus, maintaining consistent quality in herbal preparation, both from batch to batch and over time is as problematical, as it is necessary and has drawn serious attention recently as a challenging analytical task [2]. The advances in chromatographic separation techniques made it possible to quantify the chemical constituents in a mixture with comparatively little clean-up [3-4]. Particularly, methods using high-performance liquid chromatography (HPLC) with reversed phase columns are most commonly applied to the analysis of multiple constituents present in medicinal plants and herbal preparations [5].

Vaidrishi Arshkalp capsule is a well-known ayurvedic formulation used for the treatment of hemorrhoids. The selected ayurvedic formulation contains extract of Berberis aristata, family Berberidaceae, and fine powders of Piper nigrum, family Piperaceae, Azadirachta indica, family Meliaceae, Terminalia chebula, family Combretaceae and other crude drugs. Three bioactive markers were selected for quantification, berberine from B. aristata, quercetin from A. indica and piperine from P. nigrum. The chemical structures are depicted in fig. 1.

The literature survey reveals that spectrophotometric [6-7], high-performance thin layer chromatography (HPTLC) [8], reverse-phase high-performance liquid chromatography (RP-HPLC) [9-10] based method based methods have been reported for analysis of these drugs alone and in combination with other drugs. However, no HPLC method has been reported for simultaneous quantitative estimation of berberine, quercetin, and piperine in any ayurvedic formulation. Therefore, an attempt had been made to develop a novel, rapid and sensitive method for simultaneous determination of berberine, quercetin, and piperine in an ayurvedic formulation and to validate the developed method according to international council on harmonization (ICH) guidelines [11]. This novel validated method has applicability in industry and academia for routine quality control testing.

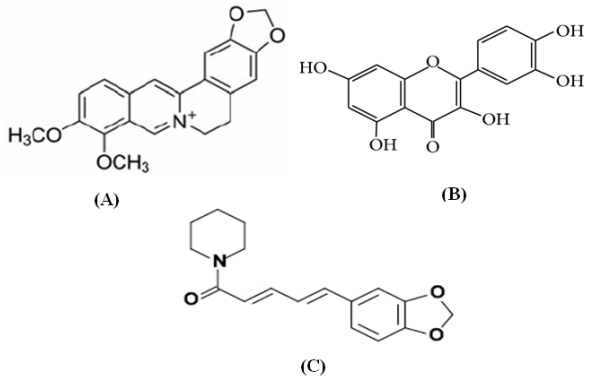

Fig. 1: Chemical structures of (A) berberine, (B) quercetin and (C) piperine

MATERIALS AND METHODS

Chemicals and reagents

Berberine, quercetin and piperine reference standards were procured as a gift sample from Yucca Enterprises, Mumbai, India. All other chemicals and reagents used were of HPLC grade. Vaidrishi arsh kalp capsule (Sagius Lifesciences Ltd, India) was purchased from local market.

Instrumentation

An RP-HPLC (Shimadzu) LC-2030 model equipped with lab solution software, an autosampler and ultraviolet-visible detector was used. The analysis was carried out on C18 shim-pack (150 mm x 4.6 mm 5µ)

column used as the stationary phase. A freshly prepared mobile phase consisting of acetonitrile: 0.04 M potassium dihydrogen phosphate buffer (pH 3.0 adjusted using orthophosphoric acid) in a ratio of 65:35 v/v. The mobile phase was filtered by 0.45 µm Whatmann filter and sonicated before use. The flow rate of the mobile phase was 1 ml/min, column temperature was maintained at 25 °C, detection was carried out at 255 nm, and the runtime was around 10 min

Selection of wavelength

A UV spectrum of berberine, quercetin, and piperine in methanol was noted by scanning the solution in the range of 200-400 nm. Berberine, quercetin, and piperine were showing significant absorption at 255 nm. Thus, 255 nm was selected as wavelength for analysis.

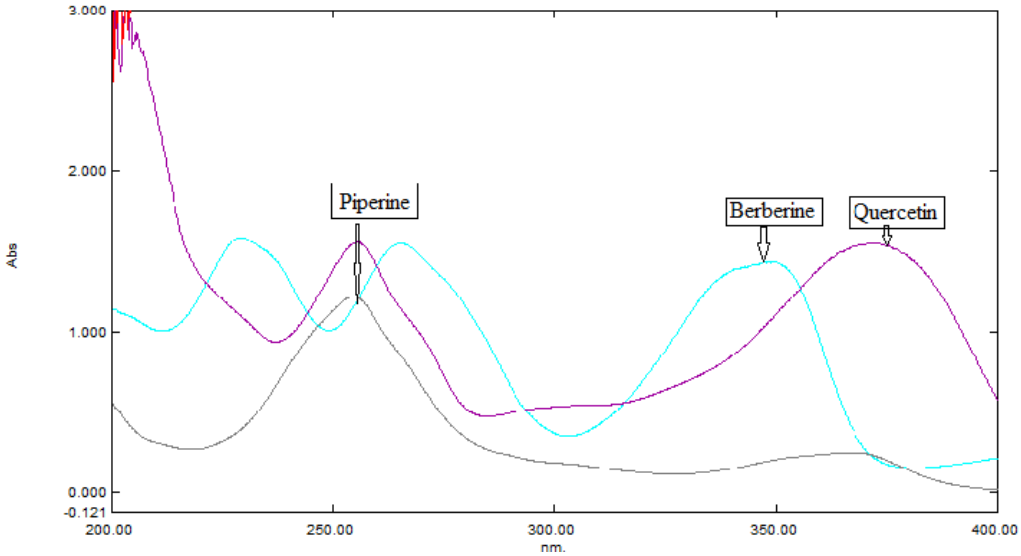

Fig. 2: An overlay UV spectrum of berberine, piperine, and quercetin

Preparation of 0.04 M phosphate buffer (pH 3)

About 5.44 g of potassium dihydrogen orthophosphate was accurately weighed and dissolved in 950 ml of water. The pH was adjusted to 3.0 with orthophosphoric acid, and the volume was made up to 1000 ml in a volumetric flask. The solution was then filtered using 0.45µ membrane filter.

Preparation of standard stock solution

The standard stock solutions containing 100 mg/ml each of berberine, quercetin, and piperine were prepared. A further solution with 20 ppm each of berberine, quercetin, and piperine was prepared separately by diluting the stock solution with the mobile phase.

Preparation of sample solution

A total of ten capsules were weighed, their mean weight was determined. An amount of powder equivalent to 1 g was accurately weighed and taken for extraction. The powder was extracted with methanol using soxhlet apparatus till complete exhaustion. The extract was made up to 100 ml with methanol. The extract was then filtered through Whatman filter paper to obtain a clear solution. Further, the stock solution after suitable dilution with the mobile phase was used for the analysis.

Method validation

System suitability

System suitability tests are a fundamental part of the liquid chromatography method. It ensures that the system is working correctly. System suitability parameters such as a number of theoretical plates, retention time and tailing factor were evaluated. This was performed by injecting a mixture of standard in six replicates.

Linearity

The linearity of the proposed method was determined by quantitative dilution of the standard solution of berberine, quercetin, and piperine to obtain the solution in the concentration range of 12-28 µg/ml for all markers. A graph of peak area *Vs* concentration in µg/ml was plotted for all three drugs in triplicate. The slope, intercept and correlation coefficient of the regression line was determined.

LOD and LOQ

The limit of detection and limit of quantitation represents the concentration of analyte that would yield to signal to noise ratio of 3 for LOD and 10 for LOQ. LOD and LOQ were calculated using the following formula.

$$LOD = 3\delta/S$$

$$LOQ = 10\delta/S$$

Where δ =standard deviation of response (peak area) and S = average of the slope of the calibration curve.

System precision

The system precision was checked by injecting six replicates of the mixed standard solution to ensure that the analytical system is working properly.

Method precision

The method precision of the proposed method was determined by injecting six replicates of sample and standard on the same day and on three different days to ensure that the analytical method is repeatable and reproducible.

Accuracy

The accuracy of this method was determined by calculating recovery of berberine, quercetin, and piperine by the standard addition method. Known amount of each standard drug solution was added to the pre-analyzed sample corresponding to 80, 100 and 120 % of the label claim. At each level, three determination were performed.

Robustness

Robustness is the measure of method capacity to remain unaffected by small, but deliberate variations in method parameters such as

mobile phase flow rate (±0.2 ml/min), wavelength nm (±1 nm) and column oven temperature (±1 ℃).

RESULTS AND DISCUSSION

Optimized chromatographic conditions

The developed method was finally optimized with following chromatographic conditions mobile phase consisting of acetonitrile: 0.04 M potassium dihydrogen phosphate buffer (pH 3.0 adjusted using orthophosphoric acid) in a ratio of 65:35 v/v. The analysis was carried out in an isocratic elution mode using a flow rate of 1.0 ml/min, at 25 ℃ and detection was carried out at 255 nm. The retention time of berberine, quercetin, and piperine in the standard were found to be 2.7, 3.0 and 6.3 min, respectively as shown in (fig. 3, 4). Quantification of the selected three markers in the formulation was done with respect to a linear regression equation. The results of quantification of active markers in the ayurvedic formulation are summarized in table 1.

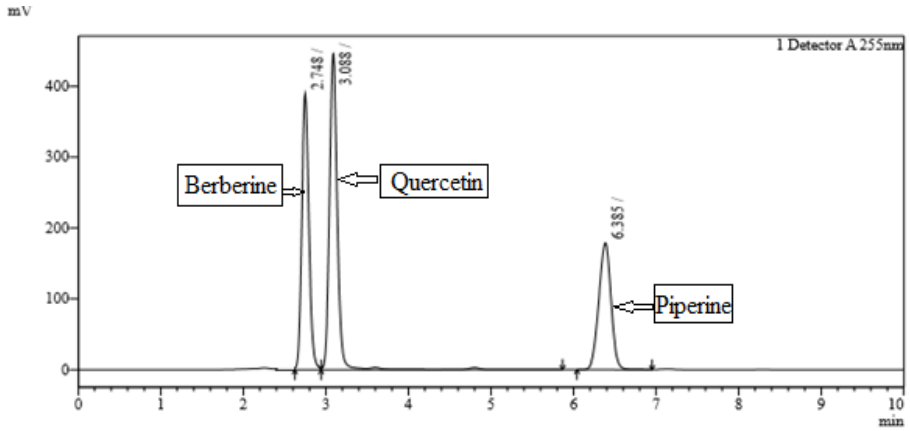

Fig. 3: Typical chromatogram of standard berberine, quercetin and piperine

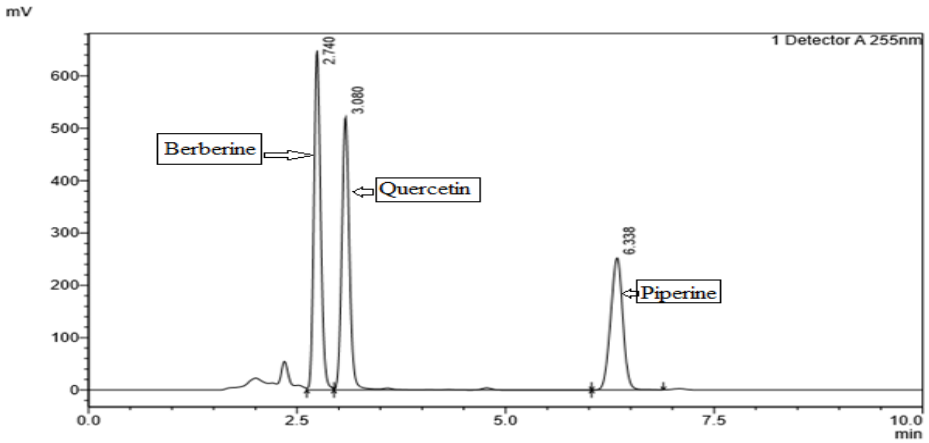

Fig. 4: Typical chromatogram of a marketed ayurvedic formulation

Table 1: Quantification of berberine, quercetin, and piperine in ayurvedic formulation

Formulation	Marker	Amount found ppm (n=3)	% Content
Vaidrishi Arshkalp capsule	Berberine	20	1.0
	Quercetin	19.95	0.997
	Piperine	19.97	0.998

n= No of injection (n=3)

Table 2: Result of system suitability studies

Parameters	Berberine	Quercetin	Piperine
Number of theoretical plates	4211	4738	8053
Tailing factor	1.15	1.12	0.96
Peak area	2323045	2765704	1856059
Retention time (min)	2.7	3.0	6.3

System suitability

The system suitability was performed by injecting a mixed standard solution containing 20 µg/ml each of berberine, quercetin, and piperine in six replicates. The acceptance criteria for evaluating system suitability are % RSD<2, tailing factor 2 and theoretical plate > 1500. The result indicates that the system suitability parameters are within acceptable limits hence ideal for the chromatographed sample [12, 13]. The results are summarized in table 2.

Linearity

The Linearity of the proposed method was determined by constructing a calibration graph between the tested concentration level and corresponding peak areas for all three drugs in triplicate. The results show an excellent correlation between peak areas and concentrations level within the tested concentration range of 12-28 µg/ml for all three selected marker.

The correlation coefficients were greater than 0.999 for all three drugs, which meets the method validation acceptance criteria [14, 15] and hence the method is said to be linear for the drugs (fig. 5-7).

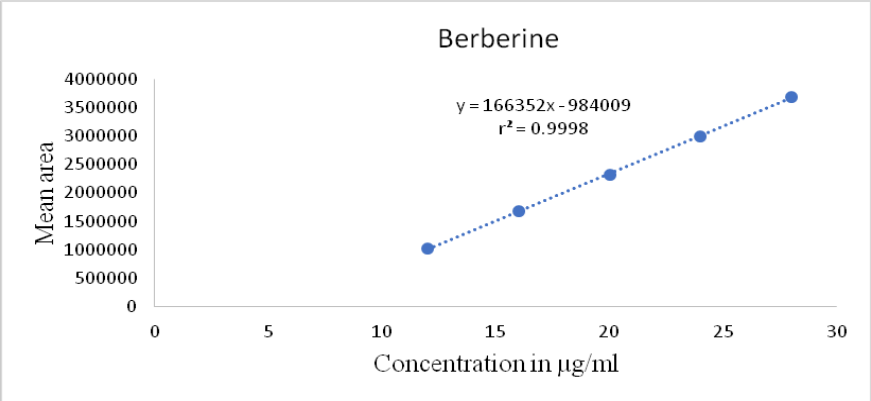

Fig. 5: Graph representing the calibration curve of berberine

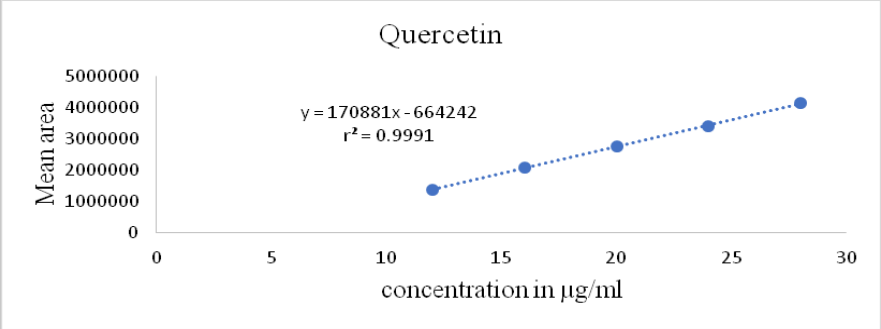

Fig. 6: Graph representing the calibration curve of quercetin

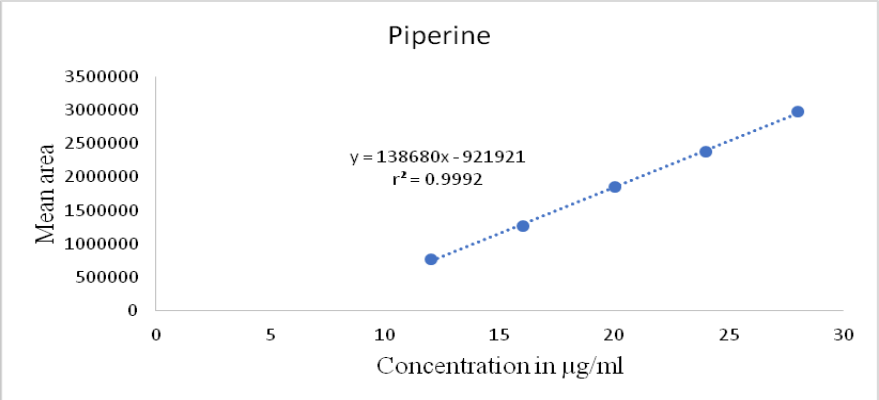

Fig. 7: Graph representing the calibration curve of piperine

LOD and LOQ

The LOD and LOQ for berberine were found to be 0.0037 µg/ml and 0.012 µg/ml, 0.023 µg/ml and 0.077 µg/ml for quercetin and 0.019 µg/ml and 0.064 µg/ml for piperine, which indicates that the method is sensitive. The results are summarized in table 3.

System precision

System precision was determined by measuring the peak area of each marker of six replicate injections of standard solution. The repeatability of sample applications and measurement of peak area were expressed in term of % RSD. The value of % RSD was found to be less than 2, which ensure the analytical system is working properly and reproducible [14, 15]. The results of system precision are tabulated in table 4.

Method precision

The % RSD value as determined by six replicates injection of sample and standard at working concentration was injected on the same day were found to be less than 2 %, which indicates that the method is repeatable and reproducible [14, 15]. The results for method precision are given in table 5.

Table 3: LOD and LOQ value calculated from the calibration curve

Parameters	Berberine	Quercetin	Piperine
LOD	0.0037	0.023	0.019
LOQ	0.012	0.077	0.064

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

Table 4: System precision data for berberine, quercetin and piperine

Replicates (n=6)	Berberine (peak area)	Quercetin (peak area)	Piperine (peak area)
1.	2359465	2712642	1877621
2.	2375421	2723158	1856041
3.	2368418	2715645	1832544
4.	2354712	2711542	1836520
5.	2359842	2715235	1842156
6.	2345261	2717433	1843247
mean±SD	2360520.33±10512.92	2715942.5±4126.30	1848022±16549.81
% RSD	0.4	0.5	0.8

n: Number of injections, # SD: standard deviation, # % RSD: percent relative standard deviation

Table 5: Method precision data for berberine, quercetin and piperine in marketed ayurvedic formulation

Marker	Intra-day		Inter-day	
	Average content (ppm) n=3 mean±SD	% RSD	Average content (ppm) n=3 mean±SD	% RSD
Berberine	19.71±0.0251	0.011	19.81±0.0321	0.011
Quercetin	19.95±0.0571	0.010	19.70±0.0278	0.009
Piperine	19.99±0.0404	0.010	19.84±0.0369	0.011

n: Number of injections, # SD: standard deviation, # % RSD: percent relative standard deviation

Accuracy

The accuracy of this method was determined by calculating the percent mean recovery of each compound in the formulation at three different levels (80-120 %). The % recovery obtained was found to be in the range 100.05-100.15 for berberine, 99.44-99.79 for quercetin and 99.72-99.84 for piperine. The acceptable recovery ranges are from 98-102 %, and all observed data were within the required range, which indicates good recovery values, affirming the accuracy of the method developed [14, 15].

The results are summarized in table 6.

Table 6: Recovery study for berberine, quercetin and piperine

Compounds	Level of recovery (%)	Sample amount (µg/ml)	Standard added (µg/ml)	Actual amount (µg/ml) (n=3)	Amount Recovered (ppm)	% Recovery
Berberine	80	10	8	18	18.09	100.5
	100		10	20	20.01	100.05
	120		14	24	24.05	100.08
Quercetin	80	9.98	7.98	17.96	17.91	99.72
	100		9.98	19.96	19.85	99.44
	120		11.97	23.95	23.90	99.79
Piperine	80	9.99	7.99	17.98	17.93	99.72
	100		9.99	19.98	19.95	99.84
	120		11.98	23.97	23.92	99.79

n= No of injection (n=3)

Robustness

The method was found to be robust when subjected to minor changes in the chromatographic condition such as oven temperature (±1 °C), mobile phase flow rate (±0.2 ml/min) and wavelength nm (±1 nm). It was observed that there was no marked change in an analytical method which indicates good reliability during normal usage. Robustness data clearly shows that the proposed method is robust at small but deliberate change [14, 15]. Robustness data are given in table 7.

Table 7: Robustness evaluation of the method

Chromatographic factors	Variations	Berberine		Quercetin		Piperine	
		Peak area	Tailing factor	Peak area	Tailing factor	Peak area	Tailing factor
Flow rate (ml/min)	0.8	2364231	1.07	2746290	1.11	1854289	0.93
	1.2	2373561	1.09	2738322	1.13	1849621	0.95
Oven temperature (°C)	24	2359169	1.12	2745281	1.09	1848629	0.98
	26	2385421	1.13	2739271	1.10	1851926	1.0
Wavelength (nm)	254	2372594	1.08	2746872	1.09	1842637	0.89
	256	2368231	1.18	2749327	1.02	1843760	0.96

CONCLUSION

The developed HPLC for simultaneous estimation of berberine, quercetin, and piperine in ayurvedic formulation is novel, simple, rapid, accurate and precise. The developed method was validated as per ICH guidelines and the results obtained were within the acceptance limits. Hence, the proposed method was found to be satisfactory and can be applied for routine qualitative and quantitative analysis of berberine, piperine, and quercetin in polyherbal and or ayurvedic formulation containing these markers one of the ingredients.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICTS OF INTERESTS

All authors declare no conflicts of interests

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