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Original Article

A NOVEL, SIMPLE, RAPID RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF FERULIC ACID, QUERCETIN, PIPERINE AND THYMOL IN AYURVEDIC FORMULATION

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ABSTRACT

Objective: A simple, accurate, precise and robust reverse phase high performance liquid chromatography (RP-HPLC) method was developed for simultaneous estimation of ferulic acid, quercetin, piperine and thymol in marketed ayurvedic formulation.

Methods: The selected markers were resolved using shim pack GIST C-18 column, with mobile phase acetonitrile: 0.04 M potassium dihydrogen ortho phosphate buffer (pH 3.0 adjusted with ortho phosphoric acid) in a ratio of 60:40 v/v at a flow rate of 1.0 ml/min. The detection was carried out at 264 nm.

Results: The retention time of ferulic acid, quercetin, piperine and thymol were found to be 2.98, 3.35, 7.83 and 9.72 min respectively. The developed method was validated according to the guidelines provided in ICH Q2 (R1) in term of linearity, precision, limit of detection, limit of quantification, accuracy and robustness. Linear response for all selected markers was obtained in the concentration range of 12-28 µg/ml with a correlation coefficient (r^2) greater than 0.999. The mean % recovery was found to be 99.30 for ferulic acid, 98.77 for quercetin, 100.93 for piperine and 100.25 for thymol.

Conclusion: The developed method was applied for quantification of these marker in marketed ayurvedic formulation. This method can be used to evaluate other formulations containing these selected phytoconstituent, thus conforming the quality and safety of ayurvedic or polyherbal formulations.

Keywords: Ayurvedic formulation, HPLC, Quantification, Ferulic acid, Quercetin, Piperine, Thymol

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INTRODUCTION

Herbal drugs are used extensively in traditional folk medicine in developed as well as developing countries. Biologically active compounds from herbal sources have always been a great interest for scientists working on infectious and non-infectious diseases. 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 [1].

The present study focuses on standardization of an Ayurvedic churna formulation using high performance liquid chromatography. The formulation is indicated mainly against intestinal gas, abdominal gas, pain, cough, ulcer, candidiasis, diarrhea, hyperlipidemia, cancer and microbial infection [2].

The selected ayurvedic churna consist of powder of Ajwain (*Trachyspermum ammi*), black pepper (*Piper nigrum*), myrobalan (*Terminalia chebula*), cumin (*Carm carvi*), asafoetida (*Ferula foetida*), lemon (*Citrus medica*), sodium bicarbonate and other crude drugs. Four chemical markers one from each medicinal herb was selected for the present work namely ferulic acid from *F. foetida*, piperine from *P. nigrum*, quercetin from *T. chebula* and thymol from *T. ammi.* These markers are mainly responsible for the bioactivity of the formulation.

Ferulic acid is the principle compound of asafoetida which exhibits numerous activities such as flatulence, antioxidant and others [3-5].

Quercetin is well known flavonoid used for antioxidant, anticancer, anti-inflammatory and antiviral activity. It is also useful for a variety of cardiovascular diseases [6-7].

Piperine, a principle pungent alkaloid possesses central nervous system depressant, antipyretic, analgesic, anti-inflammatory, antioxidant and hepatoprotective properties [8-10].

Thymol from *T. ammi* is used for dry coughs, bronchitis, laryngitis, indigestion and gastritis [11]. Thymol content in thyme essential oil is much higher than carvacrol content. This compound shows 30 time's higher antiseptic effect and four time's lower toxicity than phenol [12].

To the best of our knowledge, no studies have been reported for simultaneous estimation of ferulic acid, quercetin, piperine and thymol in ayurvedic formulation. Therefore, an attempt was taken to develop a novel RP-HPLC method for simultaneous estimation of ferulic acid, quercetin, piperine and thymol in ayurvedic formulation and validate the developed method in accordance with international council for harmonization (ICH) guidelines.

MATERIALS AND METHODS

Raw materials and marketed formulation

All reagents used in this assay were of high performance liquid chromatography (HPLC) grade purchased from thomas baker. Gashar churna from well-known brand divya pharmacy (Patanjali) used for present analytical work was procured from the local market of Mumbai, Maharashtra, India.

Instrumentation and analytical conditions

HPLC chromatographic separation was performed on a shimadzu liquid chromatographic system LC-2030 with pump P-5000, a fixed injector equipped 20 µl loop and UV-visible detector was used for chromatographic separation Lab solutions software was used for quantification of peak. Chromatographic separation was carried out at flow rate 1.0 ml/min using C18 column (shim pack GIST, 150 mm x4.6 mm, 5µ). The detection was carried out at 264 nm. The mobile phase consists of acetonitrile and buffer (potassium dihydrogen ortho phosphate, pH 3.0 adjusted with ortho phosphoric acid) in a ratio of 60:40 v/v. Ultrasonic bath was used for degassing mixing of the mobile phase. The mobile phase was filtered through a 0.45 µ membrane filter.

Selection of wavelength

A UV spectrum of ferulic acid, quercetin, piperine and thymol in methanol was noted by scanning the solution in a range of 200-400 nm. Ferulic acid, quercetin, piperine and thymol were showing significant absorption at 264 nm. Thus, 264 nm was selected as wavelength for analysis.

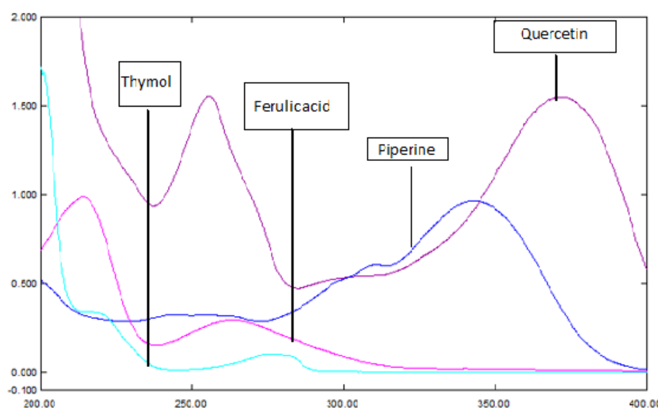

Fig. 1: UV overlain spectrum of quercetin, piperine, ferulic acid and thymol

Preparation of 0.04 M phosphate buffer (pH 3.0)

About 5.44 g of potassium dihydrogen ortho phosphate 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 filtered using membrane filter before use.

Preparation of stock solutions

100 mg of each marker (ferulic acid, quercetin, piperine and thymol) was transferred separately in 100 ml volumetric flask and the volume was made up with methanol to obtain a solution of 1000 µg/ml. These were used as a stock solution.

Preparation of standard solution

A standard solution was prepared from stock solution of the markers, 20 µg/ml each of ferulic acid, quercetin, piperine and thymol was prepared separately in mobile phase.

Preparation of sample solution

Accurately about 1 g of Gashar churna was subjected to soxhlet extraction at 40 °C using methanol as extracting solvent till complete extraction. The extract was filtered through filter paper to obtain a clear solution. The solution was made up to 100 ml using methanol. The stock solution was diluted with the mobile phase for HPLC analysis.

RESULTS AND DISCUSSION

HPLC method development

A reverse phase HPLC method was developed keeping in mind the system suitability parameters, i.e. resolution factor between peaks, tailing factor, run time and cost effectiveness. The retention time of ferulic acid, quercetin, piperine and thymol for this mobile phase were found to 2.98, 3.35, 7.83 and 9.72 min respectively. The total run time was 11 min.

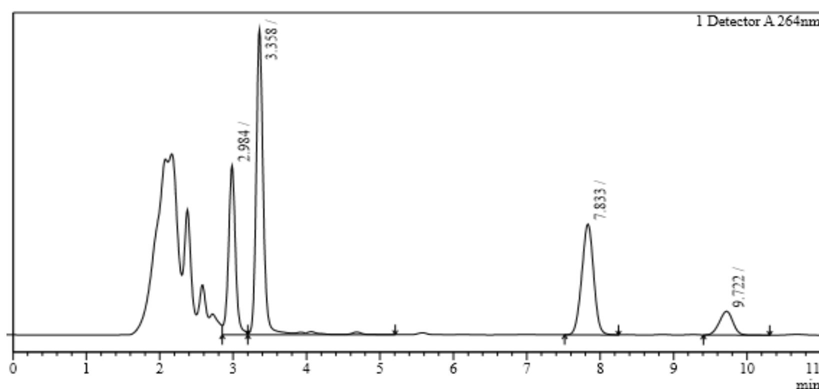

Fig. 2: Chromatogram of extract of gashar churna

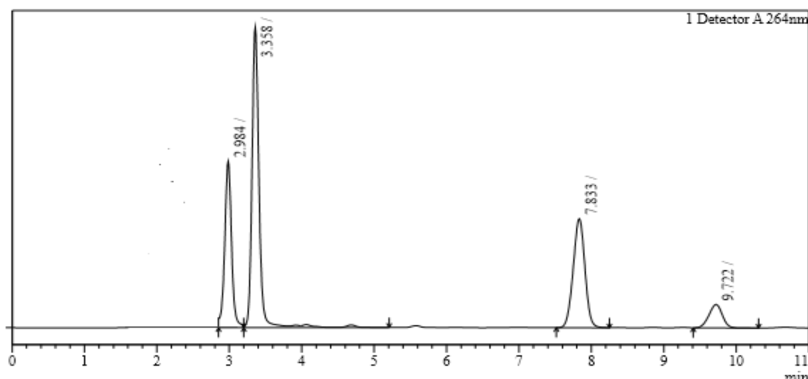

Fig. 3: Chromatogram of mixed standard of ferulic acid, quercetin, piperine and thymol

HPLC method validation

Analytical method validation is the process to substantiate that the analytical procedure used for specific examination is appropriate for its design purpose. The developed method was validated as per ICH guidelines Q2 (R1) for parameters such as system suitability, accuracy, linearity, precision, limit of detection, limit of quantification and robustness [13].

System suitability

System suitability test are an integral part of method development and are used to know reproducibility of the method, the system suitability test was done to establish the parameter such as retention time, peak area, tailing factor and number of theoretical plates. This was performed by injecting the six replicates of standard mixture. The result given in table 1 were within the acceptable limits [14, 15].

Table 1: System suitability results

Drugs	Retention time (min)	Peak area	Theoretical plate	Tailing factor
Ferulic acid	2.98	225063	5287	1.13
Quercetin	3.34	331745	5616	1.15
Piperine	7.76	369676	10437	0.98
Thymol	9.64	78575	12273	0.96

Analysis of gashar churna extract

In order to test the applicability of the developed method to an ayurvedic formulation, Gashar churna extracts were chromatographed and it's shown in fig. 2. The sample peak was identified by comparing the relative retention time with standard markers (fig. 3). For the analysis, of the sample, extract of 1 gm of Gashar churna was injected in triplicate and quantified for four active markers using a linear regression equation. The results of churna extract analysis are reported in table 2.

Table 2: Analysis of ayurvedic churna formulation

Marker	Amount found (n=3)	% Content
Ferulic acid	14.32	0.716
Quercetin	10.92	0.546
Piperine	20.30	1.015
Thymol	17.51	0.875

n= Number of injection (n=3)

System precision

System precision was carried out with 6 replicates (n=6) of standard solution at working concentration of selected four markers. The repeatability of sample applications and measurement of peak area were expressed in term of % RSD, since the % RSD of all the selected marker is less than 2.0 % which indicates the acceptable reproducibility, Hence the developed method was found to be precise [16, 17]. Data obtained from system precision experiments for repeatability studies are summarized in table 3.

Table 3: System precision result

Replicate (n=6)	Peak area of ferulic acid (20 µg/ml)	Peak area of quercetin (20 µg/ml)	Peak area of piperine (20 µg/ml)	Peak area of thymol (20 µg/ml)
1	225063	331745	369676	78575
2	225163	331645	369576	78475
3	224963	331745	369666	78574
4	225063	331746	369676	78575
5	225063	331644	369476	78775
6	225062	331745	369676	78575
mean±SD	225062.8±57.73673	331711.7±47.4962	369624.3±75.36946	78591.5±89.78446
% RSD	0.0256	0.0143	0.020	0.114

n: Number of injection, SD: Standard deviation, % RSD: %Relative standard deviation

Method precision

Method precision was determined by performing the analysis of the sample under the test of repeatability at working concentration. Three injections of the sample from the same homogenous mixture at working concentration showed % RSD less than 2 which indicates that the developed method is precise by the test of repeatability [16, 17].

Data obtained from method precision experiments for repeatability studies are summarized in table 4.

Table 4: Method precision result

Marker	Intra-day		Inter-day	
	Average content (ppm) n=3 mean±SD	% RSD	Average content (ppm) n=3 mean±SD	% RSD
Ferulic acid	14.32±0.005	0.034	14.38±0.005	0.040
Quercetin	10.91±0.005	0.045	10.98±0.005	0.051
Piperine	20.31±0.011	0.054	20.37±0.011	0.056
Thymol	17.51±0.005	0.028	17.57±0.005	0.032

n: Number of injection, % RSD: % Relative standard deviation, SD: Standard deviation

Linearity

The linearity of an analyte procedure is its ability to obtain test results which are directly proportional to the concentration of analyte in the sample. Linearity was evaluated by analyzing area under the curve as a function of analyte concentration. The result shows an excellent correlation between peak areas and concentration level within the tested concentration range of 12-28 µg/ml for ferulic acid, quercetin, piperine and thymol (table 5). The results were evaluated by calculating of regression coefficient (r^2). The correlation coefficient was greater than 0.99 for each marker, which meet the method validation acceptance criteria [16, 17] and hence the method is said to be linear (fig. 3-6).

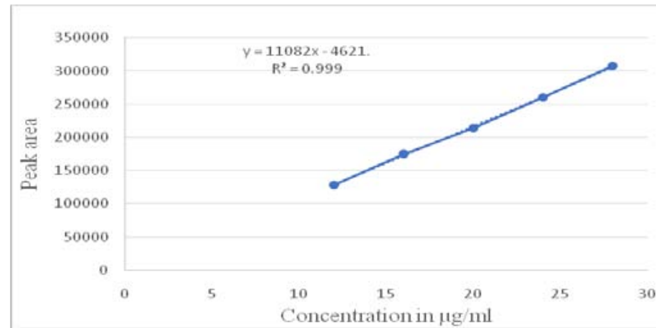

Fig. 3: Calibration curve of ferulic acid

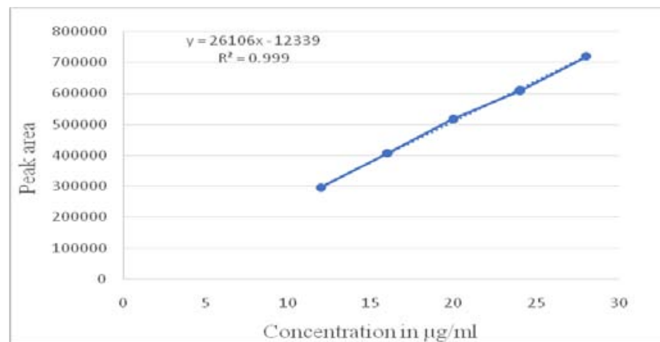

Fig. 4: Calibration curve of quercetin

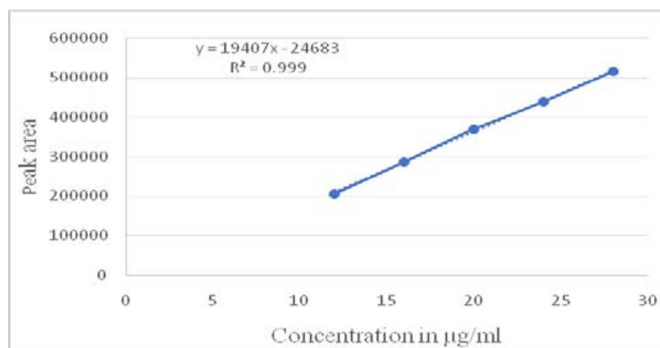

Fig. 5: Calibration curve of piperine

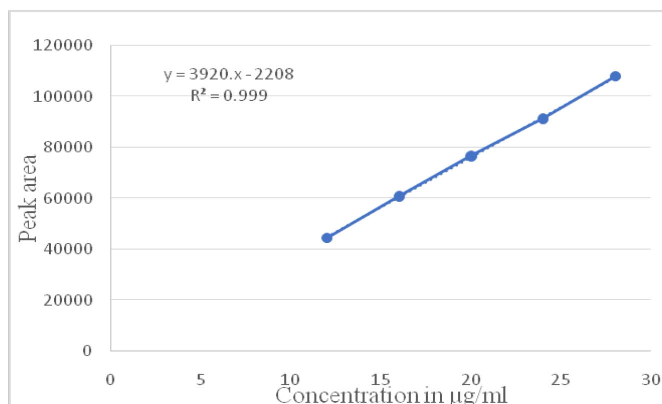

Fig. 6: Calibration curve of thymol

Table 5: Data for linearity studies

Marker	Concentration range (µg/ml)	Regression equation	Slope	Intercept	Correlation coefficient (r^2)
Ferulic acid	12-28	y = 11082x-4621.6	11082	4621.6	0.999
Quercetin	12-28	y = 26106x-12339	26106	12339	0.999
Piperine	12-28	y = 19407x-24683	19407	24683	0.999
Thymol	12-28	y = 3920.1x-2208	3920.1	2208	0.999

Accuracy

The accuracy of an analytical method is the closeness of the results obtained by that method to the true value of the sample. It is expressed as percent recovery, which is determined by the standard addition method. The accuracy was performed by spiking 80, 100 and 120 % amount of standard drug externally added to the pre-analyzed sample. The acceptance limit for recovery studies ranges from 98-102%. The mean % recovery was found to be 99.30 for ferulic acid, 98.77 for quercetin, 100.93 for piperine and 100.25 for thymol and all the observed data were within the required range, which indicates good recovery values, affirming the accuracy of the method development [16, 17].

Table 6: Recovery data for ferulic acid, quercetin, piperine and thymol

Marker	Sample amount in (µg/ml)	Standard amount in (µg/ml)	Total amount in (µg/ml) (n=3)	Amount found (µg/ml)	% recovery
Ferulic		5.72	12.88	12.08	99.86
acid	7.16	7.16	14.32	14.21	99.98
		8.59	15.75	15.31	98.1
Quercetin		4.36	9.82	9.76	99
	5.46	5.46	10.92	10.46	99
		6.55	12.01	12.0	99.3
Piperine		8.12	18.21	18.10	98.9
	10.15	10.15	20.3	20.11	101
		12.18	22.33	22.10	98.96
Thymol		7	15.75	15.32	99.67
	8.75	8.75	17.5	17.19	98.42
		10.5	19.25	19.12	98.75

n= No of injection (n=3)

Limit of detection (LOD)

The LOD of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected, but not necessarily quantified as an exact value.

Limit of quantification (LOQ)

The LOQ of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

LOD and LOQ are expressed as:

$LOD = 3.3\ \sigma/S$

$LOQ = 10\ \sigma/S$

Where, σ is the standard deviation of the responses and S is the slope of the calibration curves.

A low LOD and LOQ values indicate that the method is sensitive. The result of LOD and LOQ are summarized in table 7.

Table 7: LOD and LOQ for ferulic acid, quercetin, piperine and thymol

Drug	LOD (µg/ml)	LOQ (µg/ml)
Ferulic acid	0.0004	0.001
Quercetin	0.015	0.04
Piperine	0.015	0.046
Thymol	0.008	0.026

LOD: Limit of detection, LOQ: Limit of quantification

Robustness

The robustness of the method was determined by assessing the ability of the developed method to remain unaffected by the small changes in the parameters such as flow rate, wavelength and oven temperature. A deviation of ±1 nm in the detection wavelength ±0.2 ml/min in the flow rate and±0.1 °C in the oven temperature. It was observed that there was no marked change in analytical method which indicates good reliability during normal usage. Robustness data clearly shows that the proposed method is robust at small but deliberate change [16, 17]. Robustness data are given in table 8.

Table 8: Robustness data for ferulic acid, quercetin, piperine and thymol

Parameter	Ferulic acid		Quercetin		Piperine		Thymol	
	Peak area	Tailing factor	Peak area	Tailing factor	Peak area	Tailing factor	Peak area	Tailing factor
Minus temp (24 °C)	225063	1.13	331745	1.15	369676	0.98	78575	0.96
Plus temp (26 °C)	225076	1.16	331749	1.17	369679	0.99	78577	0.97
Minus flow rate (0.8 ml/min)	225079	1.17	331748	1.17	369677	0.98	78571	0.96
Plus flow rate (1.2 ml/min)	225078	1.17	331747	1.16	369673	0.97	78579	0.97
Minus Wavelength (263 nm)	226076	1.16	331750	1.17	369681	0.98	78573	0.96
Plus wavelength (265 nm)	225080	1.18	331746	1.15	369678	0.98	78574	0.96

CONCLUSION

A novel validated RP-HPLC method has been developed for the determination of ferulic acid, quercetin, piperine and thymol in an ayurvedic formulation. The developed method is validated according to the guidelines provided in ICH Q2 (R1) in term of linearity, precision, limit of detection, limit of quantification, accuracy and robustness. The chromatographic run time is less than 11 min which allows the analysis of large number of samples in a short period of time, hence developed method can be used for routine analysis of ferulic acid, quercetin, piperine and thymol in any polyherbal or ayurvedic formulation containing selected markers as one of the ingredient.

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AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICT OF INTERESTS

All authors are declared no conflict of interest

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