International Journal of Pharmacy and Pharmaceutical Sciences

Print ISSN: 2656-0097 | Online ISSN: 0975-1491 Vol 12, Issue 6, 2020

Original Article

DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF GALLIC ACID, CURCUMIN AND PIPERINE IN AN AYURVEDIC FORMULATION

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Received: 28 Apr 2019, Revised and Accepted: 06 May 2020

ABSTRACT

Objective: The objective of the present work was to establish a simple, precise, accurate and robust method for simultaneous estimation of gallic acid, curcumin and piperine from the marketed ayurvedic formulation by liquid chromatography.

Methods: The separation was carried out on Hemochrom C18 Column (250 mm × 4.6 mm ID, 5 µm pore size) with a mobile phase methanol: acetonitrile: water (pH 3.2adjusted by using orthophosphate acid) in the ratio 70:20:10v/v by isocratic elution modeat 25 °C and the flow rate was setat0.8 ml/min. The analysis was carried out atisoabsorptive wavelength of 295 nm.

Results: The retention time of gallic acid, curcumin and piperine was found to be 3.3(±0.2), 4.7 (±0.2) and 5.6 (±0.2) min, respectively. The linearity range for gallic acid, curcumin and piperine was found to be 10-70 µg/ml, 20-80 µg/ml and 2-14 µg/ml, respectively with the coefficient of linear regression greater than 0.99 for all markers. Mean percent recoveries for gallic acid, curcumin, and piperine were found within the limit of acceptance (99-100%). The percent relative standard deviation (%RSD) for precision and robustness was found less than 2%, which indicates the method is precise and robust. The developed method applied for quantification of these markers from the marketed ayurvedic formulation of Dekofcyn tablet.

Conclusion: The developed method was found to be simple, rapid, precise and reproducible for standardization of Dekofcyn tablet and can be useful for other formulations containing these three markers.

Keywords: Reverse-phase high-performance liquid chromatography (RP-HPLC), Gallic acid, Curcumin, Piperine, Method validation, Ayurvedic formulation, Standardization

DOI: http://dx.doi.org/10.22159/ijpps.2020v12i6.33801. Journal homepage: https://innovareacademics.in/journals/index.php/ijpps

INTRODUCTION

The advance chromatographic separation techniques are used for qualitative and quantitative analysis of multiple constituents present in medicinal plants and herbal formulations.

In the present study, the RP-HPLC method was developed for simultaneous estimation of curcumin, gallic acid and piperine from the Dekofcyn tablet. It is used against chronic cough, cold, sore throat and chronic oral allergies. It consists of haldi (*Curcuma longa*), Talispatra (*Taxus buccata*), Shatavari (*Asparagus racemosus*), Piper(*Piper longum*), Ashwagandha (*Withania somnifera*), Amla (*Emblica officinalis*), Kachura (*Curcuma zedoria*), Vasaka (*Adhatoda vasica*), Galo ghan (*Tinospora cordifolia*),Dagdi Prashanbhed *(Bergenia ciliate*), Suvarna mashik bhasma (A. F. I.-193)10 Puta, Abhrak bhasma (S. Y. S.-159)42 Puta, laghu malinivasanta Rasa (A. F. I.-271) [1]. Gallic acid which is one the constituent of Amlapossess antioxidant, anti-cancer, anti-inflammatory and anti-diabetic properties. Scavenging of superoxide anions, inhibition of myeloperoxidase release activity as well as possible interference with the assembly of active Nicotinamide adenine dinucleotide phosphate hydrogen (NADPH-oxidase) may account for the inhibition of inflammatory process by gallic acid. Structure-activity relationship (SAR) analysis showed that the o-dihydroxy group of gallic acid is important for the inhibitory effect in inflammation [2-4].

Curcumin is the active constituent of *Haldi*, it possesses anti-inflammatory, anti-bacterial, anti-allergic and anti-cancer activities. Possible scenario by which curcumin exerts anti-inflammatory effect is by binding to its own receptor and the ligand-receptor interaction activates signaling pathways leading to the up-regulation of PPAR-γ and the subsequent suppression of inflammatory cytokine release [5, 6].

Piperine is an active constituent of piper, it possesses nervous system depressant, anti-inflammatory, antipyretic, analgesic, antioxidant and bio enhancer properties [7-9]. Piperine enhances absorption from gastrointestinal tract by various mechanisms and reduces gut metabolism of drugs. Due to this piper is one of the ingredients of many ayurvedic formulations. Piperine enhances the bioavailability of curcumin and gallic acid by inhibition of cytochrome p450 enzymes [10].

Literature survey showed that gallic acid, curcumin and piperine were found to possess anti-inflammatory, anti-allergic and anti-oxidant properties, which contributes in therapeutic effect expected in the formulation. Therefore, in the present work gallic acid, curcumin and piperine were selected as a marker.

To the best of my knowledge, no studies have been reported for simultaneous estimation of curcumin, gallic acid and piperine from the ayurvedic formulation. Therefore, an attempt has been made to develop a novel, precise RP-HPLC method for these markers and validate the developed method in accordance with the international council for harmonization (ICH guidelines) Q2 (R1)

MATERIALS AND METHODS

Instrumentation

Chromatographic separation was achieved on HPLC Shimadzu UFLC (LC 2030) system, Auto sampler, SPD-20 A Prominence UV-detector, operated by the Lab Solutions software, Separation was achieved on reverse phase Hemochrom C18 column (250 mm × 4.6 mm ID, 5 µm pore size). Spectrophotometric analysis was carried out on Shimadzu UV-1800 UV spectrophotometer.

Marketed formulation

Marketed formulation of Dekofcyn tablet was used for analysis has been procured from pharmacy of Navi-Mumbai, Maharashtra, India.

Standard and reagents

Standard gallic acid, curcumin, and piperine were procured from Yucca Enterprises, Mumbai, Maharashtra, India. All HPLC and

Analytical grades reagents were purchased from SD Fine Chemicals Ltd, Mumbai, Maharashtra, India.

Preparations of standard stock solution

25 mg of each marker (gallic acid, curcumin, piperine) was transferred individually in three volumetric flasks and volume was made up to 25 ml with methanol to obtain 1000 ppm solutions; these were used as stock solution and stored in the refrigerator.

Preparation of working solutions

Working Solution of each markers having concentration 100 ppm was prepared from the stock solution. From these further dilutions were made to get 10-70 ppm, 20-80 ppm, 2-14 ppm of gallic acid, curcumin and piperine, respectively.

Preparation of sample solutions

5 g powder of Dekofycn tablet was accurately weighed by triturating 12 tablets. The powder was macerated using 25 ml of methanol for 1 h and filtered through Whatman filter paper no.41 and this procedure is repeated again using fresh methanol. Both filtrates were combined and volume was made up to 50 ml with methanol. This solution was then further used for quantification.

Rp-hplc method development

Selection of wavelength

Suitable wavelength for the HPLC analysis was determined using UV Spectrophotometer by recording UV Spectrum in the range of 200-400 nm for individual drug solution of gallic acid, curcumin and piperine then overlaid the spectra. An overlain spectrum of these three markers showed the is the absorptive wavelength at 295 nm.

Chromatographic condition

The mobile phase optimized for the RP-HPLC method was methanol: acetonitrile: water (pH 3.2adjusted by using orthophosphoric acid) 20µlinjection volume was applied at 25 °C and the flow rate was kept at 0.8 ml/min. The retention time of gallic acid, curcumin and piperine was found to be 3.3, 4.7and 5.6 min respectively**.**

Rp-hplc method validation

The method was validated as per the ICH Q2 (R1) Guidelines for parameters such as specificity, linearity, precision, system precision, accuracy, limit of detection, limit of quantification, and robustness [11].

Specificity

Specificity is the ability to assess the analyte in the presence of components which may be expected to be present. It is performed in order to ensure the identification, purity and quantitation of marker compound from ayurvedic formulation under evaluation. It is determined by comparing the retention time and UV spectra of standards with the compound of interest obtained from extract of the marketed formulation.

Linearity

Linearity of an analyte is determined by plotting a graph of area against concentration of analyte and the test results were evaluated by calculating linear regression coefficient (r^2).

Standard stock solution was diluted to obtain 10-70 ppm, 20-80 ppm and 2-14 ppm of gallic acid, curcumin and piperine, respectively. Three sets of each individual solutions were evaluated. Every set was analysed to obtain a calibration curve. The standard deviation (SD), coefficient of determination (r2), slope and intercept of the curves were estimated to determine the method of linearity.

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

Recovery of gallic acid, curcumin and Piperine from the formulation was checked by spiking a known number of markers at three concentration levels (i.e. 80%, 100% and 120% of the quantified amount) to the test samples in triplicate using HPLC. This way accuracy was performed and calculated for nine determination over a specified range and mean recovery was calculated.

Precision

Analytical procedure expresses the closeness of degree between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed condition.

System precision

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

Method precision

Three replicate of quality control (QC) Samples of gallic acid, curcumin and piperine at three different concentration levels (10, 40, 70 ppm), (20, 50, 80 ppm) and (2, 8, 14 ppm) i.e. low-quality control (LQC), mid-quality control (MQC), high-quality control (HQC) respectively, were analysed on three different days (interday precision) and at three different times on a same day (intraday precision).

Limit of detection (LOD)

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value, it is determined on the basis of the standard deviation of response and the slope of final calibration curve.

$$\text{It is expressed as LOD} = 3.3\ \frac{\sigma}{S}$$

σ = standard deviation of response

S = slope of final calibration curve

Limit of quantitation (LOQ)

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low level of compounds in a sample matrix, and is used particularly for the determination of impurities and/or degradation products.

$$\text{It is expressed as LOQ} = 10\ \frac{\sigma}{S}$$

σ = standard deviation of the response

S = slope of final calibration curve

Robustness

Robustness of an analytical method was evaluated by making deliberate changes in mobile phase composition, mobile phase (±0.2), flow rate (±0.2 ml/min) and column temperature (±5 °C). Two concentrations of each marker i.e. 10 and 70 ppmfor gallic acid, 20 and 80 ppm for curcumin and 2 and 14 ppm for piperine were analysed in triplicate.

Quantification and standardization of marketed ayurvedic formulation of Dekofcyn tablet by developed HPLC method

The developed method was applied for standardization and quantification of gallic acid, curcumin and piperine in Dekofcyn tablet. Methanolic extract was used for quantification of gallic acid, curcumin and piperine. The amount of gallic acid, curcumin and piperine present in sample solution was calculated by comparison of the area observed from the sample with the calibration curves constructed from peak areas obtained from standard solution of gallic acid, curcumin and piperine. The result obtained was further used for the recovery experiment.

RESULTS AND DISCUSSION

Selection of wavelength

A UV spectrum of gallic acid, curcumin and piperine of methanolic solutions was scanned in the range of 200-400 nm (fig. 1). An is absorptive wavelength was found to be 295 nm of gallic acid, curcumin and piperine, respectively.

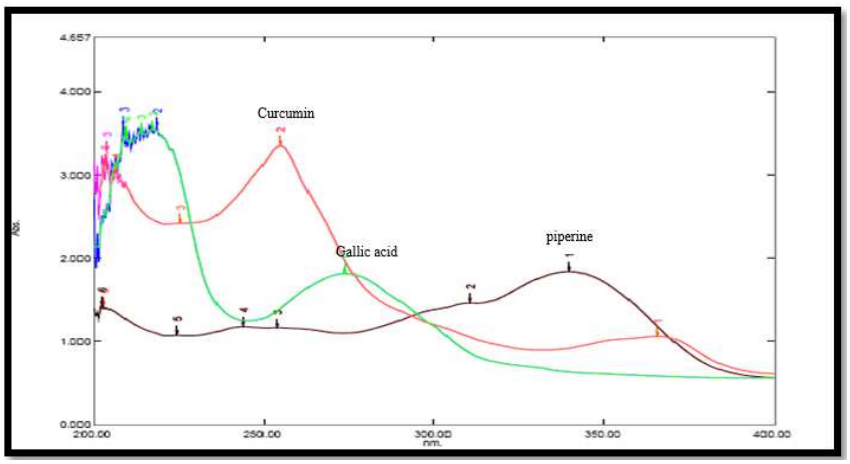

Fig. 1: UV spectrum overlain spectrum of gallic acid, curcumin and piperine

Optimized chromatographic condition

The developed method was finally optimized under the chromatographic conditions mobile phase methanol: acetonitrile: water (pH 3.2 adjusted by using orthophosphoric acid) in the ratio of 70:20:10v/v (fig. 2). The analysis was carried out in an isocratic mode at the flow rate of 0.8 ml/min, at 25 °C and detection was carried out at 295 nm. The retention time of gallic acid, curcumin and piperine were found to be 3.3 (±0.2), 4.7 (±0.2) and 5.6 (±0.2) min, respectively.

Specificity

The developed method was found to be specific because there was no interference of any other component was found at the retention time of gallic acid, curcumin and piperine i.e. 3.3 min. 4.7 min and 5.7 min (fig. 3).

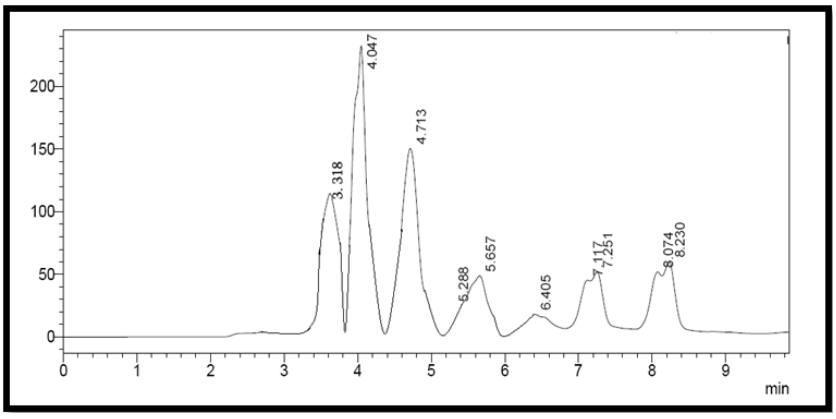

Fig. 2: Chromatogram of extract

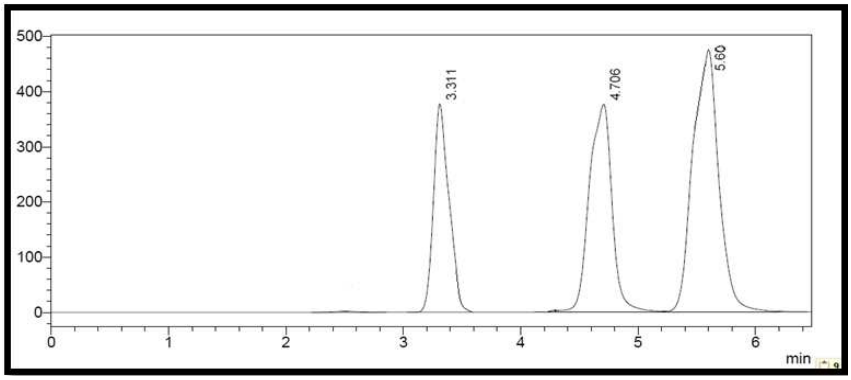

Fig. 3: Chromatogram of standard

Linearity

Standard solutions of gallic acid, curcumin and piperine were found to give linear responses in the concentration range of 10 to 70 ppm, 20 to 80 ppm and 2 to 14 ppm, respectively. The linearity was performed in triplicated and the average area is plotted against the concentration of individual marker (fig. 4. a, 4. b, 4. c). The linearity was validated by the correlation coefficient greater than 0.99 for each marker, which meets the acceptance criteria and hence the method is said to be linear.

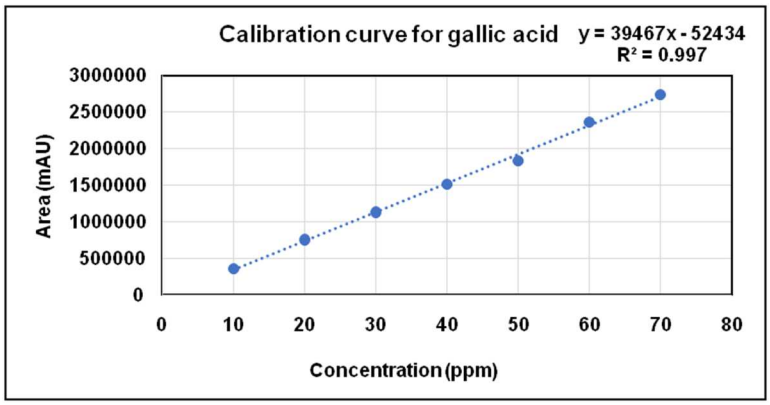

Fig. 4a: Calibration curve of gallic acid

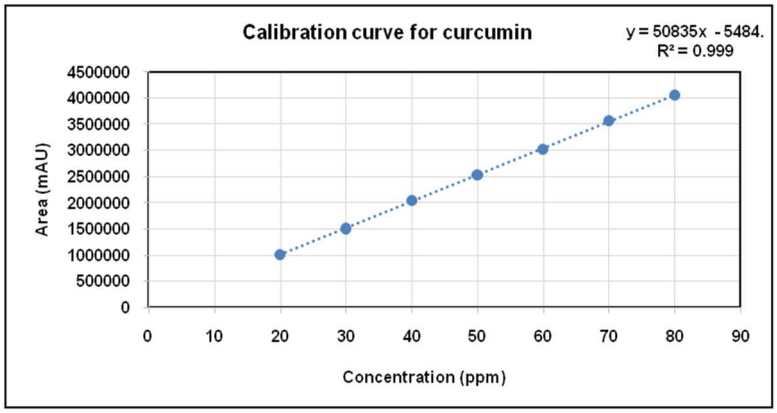

Fig. 4b: Calibration curve of curcumin

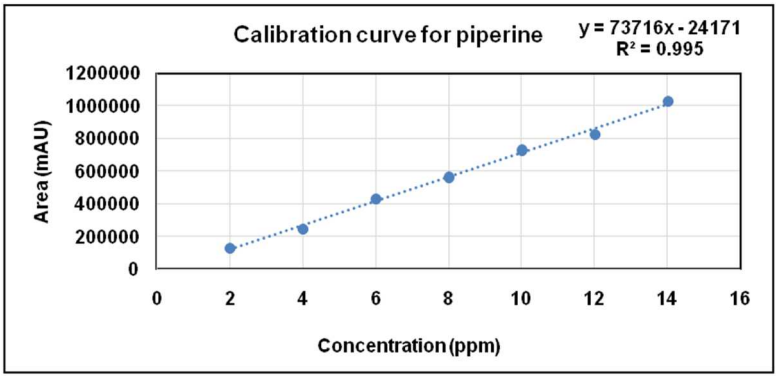

Fig. 4c: Calibration curve of piperine

Table 1: Result for accuracy studies of gallic acid, curcumin and piperine

Selected Markers	Level of recovery %	Theoretical content of marker (µg/ml)	Amount of marker added (µg/ml)	Total Amount of marker (µg/ml)	Amount of marker recovered (µg/ml)	% recovery	Mean recovery	%RSD
	80	120	96	216	215	99.5		
Gallic acid	100	120	120	240	240	100.1	99.66	0.301
	120	120	264	260	260	99.4		
	80	140	112	252	251	99.6		
Curcumin	100	140	140	280	280	100.0	100.2	0.678
	120 %	140 µg/ml	168	308	310	101.2		
	80 %	21 µg/ml	16.8	37.8	36.35	99.5		
Piperine	100 %	21 µg/ml	21	42	41.98	100	99.23	0.764
	120 %	21 µg/ml	25.2	46.2	45.50	98.2		

Accuracy

The accepted limit of mean recovery is in the range of 99-100%, which indicates good recovery values, as depicted in table 1.

Precision

System precision

Six replicates injection at working concentration of standard solutions showed that percent relative standard deviation (% RSD) less than 2% for each marker, which indicates the result under acceptance limit; hence analytical system is working properly as depicted in table 2.

Method precision

The statistical analysis of the results proved that percent relative standard deviation (%RSD) of the peak areas of the individual marker was obtained less than 2% hence the developed method was found to be precise as depicted in table 3. a, 3. b.

Table 2: Result for system precision

Parameter (Replicates (no. of injections) n=6)	Peak area of gallic acid (40 ppm)	Peak area of curcumin (50 ppm)	Peak area of piperine (8 ppm)
1	1586421	2568952	5636952
3	1568234	2643521	5623462
4	1568924	2684682	5694865
5	1589743	2658452	5682451
6	1589365	2658942	5695225
Average	1577528	2629673	5670862
SD (standard deviation)	11219.22	49494.48	29315.98
%RSD (relative standard deviation)	0.71%	1.76%	0.51%

*Mean for six independent analysis, SD= standard deviation, RSD-relative standard deviation, n= no. of injections

Table 3a: Result for method precision (intra-day)

Parameter	Intra day								
	Markers								
Level of Conc	Gallic acid			Curcumin			Piperine		
	LQC	MQC	HQC	LQC	MQC	HQC	LQC	MQC	HQC
Avg area (ppm) n=3	341650	1571705	2721663	1015694	2569243	4049321	131982	5539207	1015243
SD	5014.40	8741.08	45786.04	2828.41	44707.45	3535.53	960.86	80000.42	23899.74
%RSD	1.46%	0.55%	1.68%	0.27%	1.74%	0.08%	0.72%	1.44%	0.23%

Table 3b: Result for method precision (inter-day)

Parameter	Inter day								
	Marker								
Levelofconc	Gallic acid			Curcumin			Piperine		
	LQC	MQC	HQC	LQC	MQC	HQC	LQC	MQC	HQC
Avgarea of 3 d (ppm) n=3	331984	1557301	2731204	1017293	2558416	4058928	131769	5467634	1019232
SD	685.04	17936.95	23716.71	1555.69	26873.14	10073.34	2472.42	50775.23	6672.10
%RSD	2.0%	1.15%	0.86%	0.15%	1.05%	0.24%	1.87%	0.92%	0.65%

*conc = concentration, SD = standard deviation, mean for three independent analyses, %RSD = relative standard deviation, n= No. of injections

Table 4: Result for LOD and LOQ values calculated from the calibration curve

Parameters	Gallic acid	Curcumin	Piperine
LOD (ppm)	0.093	0.025	0.083
LOQ (ppm)	0.28	0.076	0.25

*LOD = Limit of detection, LOQ = Limit of quantification

Table 5: Results for robustness

Parameter	Variation	% RSD					
		Gallic acid		Curcumin		Piperine	
		Area		Area		Area	
		10 ppm	70 ppm	20 ppm	80 ppm	2 ppm	14 ppm
Column temperature	35 °C	0.17	0.11	0.05	0.49	1.25	1.00
	45 °C	0.25	0.06	0.52	1.01	1.19	0.11
Mobile phase composition	60:25:15	1.24	1.15	1.43	1.95	0.19	0.86
	60:20:20	1.22	1.14	0.66	1.28	1.46	1.82
Mobile phase change in pH	pH 3.0	1.29	1.66	1.45	1.52	1.25	1.02
composition	pH 3.4	1.46	1.86	1.65	1.76	1.40	0.66
Flow rate (ml/min)	0.6 ml/min	0.98	0.66	0.34	0.55	0.28	0.23
	1.0 ml/min	0.15	0.85	0.59	0.27	0.34	0.56

*****mean for three independent analyses, SD= standard deviation, % RSD= relative standard deviation

LOD and LOQ

LOD and LOQ were calculated with the aid of standard deviation (σ) and slope (s) from the calibration curve (n=3), by using the formula LOD = 3.3 σ/s and LOQ = 10 σ/s as depicted in table 4 respectively. Which indicates the good sensitivity of the method towards the analyte.

Robustness

In the statistical analysis data, it is observed that there was no change in the analytical method, which indicates good reliability under normal usage. The result shows that % RSD of the peak areas obtained was less than 2%, hence the developed method was found to be robust the as depicted in table 5.

Quantification of marker

Content of gallic acid, curcumin and piperine in the marketed ayurvedic formulation i.e. Dekofycn tablet was found to be 0.59% w/w, 0.75%w/w, 0.21% w/w respectively.

DISCUSSION

A novel, precise and robust HPLC method was developed for the simultaneous estimation of gallic acid, curcumin and piperine present in the marketed formulation using reverse-phase Hemochrom C18 column with a mobile phase composed of methanol: acetonitrile: water (pH 3.2 adjusted by using orthophosphoric acid) in the ratio of 70:20:10v/v/v. The analysis was carried out in an isocratic mode at the flow rate of 0.8 ml/min, at 25 ℃ and detection was carried out at 295 nm. The method is highly specific as there was no interference observed between chromatograms of blank, standard and sample. Good linearity with the coefficient of correlation 0.99 indicated that the proposed method was linear within the range of 10 to 70 ppm, 20 to 80 ppm and 2 to 14 ppm respectively for gallic acid, curcumin and piperine. Accuracy results displayed good reproducibility with % RSD below 2%. The method was found to be accurate as the values observed for gallic acid, curcumin and piperine 99.66%, 100.2% and 99.23% respectively which was well within the range of 98-102%. as shown in table 1. The results for system precision were expressed in %RSD and it was observed as for gallic acid 0.71%, curcumin 1.76% and for piperine 0.51% as tabulated in table 2. Whereas, The %RSD for intra and inter-day precision of gallic acid, curcumin and piperine were observed below 2%. The low values of % RSD indicates that the method is precise. As the individual percentage estimation of gallic acid, curcumin and piperine in other polyherbal formulation extracts were found to be 2.99%, 71.57% and 3.1%w/w respectively [12-14]. According to the method described herein, the percentage of gallic acid, curcumin and piperine were determined to be 0.59%, 0.75% and 0.21% w/w respectively. The LOD and LOQ values showed the method was sensitive for the simultaneous estimation of gallic acid, curcumin and piperine at low concentrations also. The method is validated as per the ICH guidelines.

CONCLUSION

A developed HPLC chromatographic method for simultaneous determination of gallic acid, curcumin and piperine from Dekofcyn tablet was found to be simple, rapid, reproducible, precise and robust. The peaks of individual markers are well resolved in the optimized mobile phase under the set parameters. The method was validated according to the ICH (Q2) R1 guidelines in terms of linearity, specificity, precision, robustness, LOD and LOQ. All the results were obtained were within the acceptance limit. Thus, it also ensures the need of quality and safety of an ayurvedic formulation. Therefore, this method can also be applied for the evaluation of the phytoconstituents from other ayurvedic formulations.

ACKNOWLEDGMENT

The authors extend their gratitude to the Bharati Vidyapeeth's College of Pharmacy, NaviMumbai, for giving an opportunity to carry out work in their college.

FUNDING

Nil

AUTHORS CONTRIBUTIONS

The submitted research work is a part of the Master of Pharmacy in Quality Assurance specialization. This project was guided by Dr. Aruna. P. Jadhav and laboratory research work, development and validation of the method was carried out by Ms. Mehjabeen Shaikh.

CONFLICTS OF INTERESTS

Authors declare no conflicts of interest

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