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Original Article

DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF CURCUMIN AND PIPERINE

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ABSTRACT

Objective: The present study was aimed to develop a simple, sensitive and precise high performance liquid chromatographic (HPLC) method for the simultaneous estimation of curcumin and piperine and to implement the developed method for the estimation of curcumin and piperine in the nanoparticulate formulation.

Methods: Method development was performed using various solvent, buffer-solvent ratios, at different flow rates for adequate separation of both drugs. The developed method was validated in accordance with the international conference on harmonization (ICH) guidelines. The developed method was implemented to estimate the amount of curcumin and piperine in the nanoparticulate formulation.

Results: Chromatographical conditions were optimized, and the best chromatographical conditions with adequate resolution for curcumin and piperine was achieved using enable C18G reverse phase column, using a mobile phase combination of acetonitrile and phosphate buffer (pH 3)in a ratio of 70:30 v/v at a flow rate of 1.0 ml/min. The detection was monitored at a wavelength of 360 nm. The retention time of curcumin and piperine was found to be 7.2 min and 8.5 min respectively.

Conclusion: The developed analytical method is simple, precise, and reproducible and thus can be used for simultaneous estimation of curcumin and piperine in pharmaceutical formulations.

Keywords: Curcumin, Piperine, RP-HPLC, Simultaneous estimation, Nanoparticulate formulation

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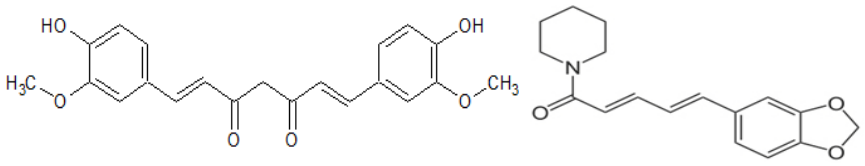
INTRODUCTION

Curcumin is yellowish-orange crystalline phytochemical. It is a hydrophobic polyphenolic substance isolated from the rhizomes of *Curcuma longa Linn.* Family (Zingiberaceae). Structurally, curcumin is 1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione as shown in fig. 1 along with other two de-methoxy compounds which are desmethoxycurcumin and bisdemethoxycurcumin [1, 2]. It possesses antioxidant, anti-inflammatory, anti-microbial and anticancer activity due to its diverse molecular targets [3, 4]. Curcumin is reported to be a potent antibacterial and anti-hepatotoxic agent [5]. Safety of curcumin at very high doses has been proved in various animal and human studies. These studies led to the approval of curcumin as a 'generally regarded as safe (GRAS)' ingredient by the food and drug administration (FDA) of the United States of America, by the natural health products directorate of Canada and the expert joint committee [6].

Piperine is (1-piperoylpiperidine) as shown in fig. 1, an active alkaloid of *Piper longum* and *Piper nigrum (*Piperaceae*)* [7, 8]. It exhibits CNS depressant, antipyretic and anti-inflammatory activity [9]. It has been reported that piperine enhances the bioavailability of several drugs through inhibition of drug metabolism such as curcumin [10, 11]. Methods are available for the analysis of piperine [7, 12].



Curcumin **Piperine**

Fig. 1: Chemical structure of curcumin [1] and piperine [7]

Several HPLC methods have been developed to quantify curcumin and piperine. In reported high-performance liquid chromatography (HPLC) method, the separation was observed at 9 and 9.5 min for curcumin and piperine respectively which is not desired to estimate the drug concentration in the formulation. While other reported method has used 0.1% orthophosphoric acid as mobile phase with pH 2.1 which is less desirable and may damage the column [13].

Hence, phosphate buffer was used, and pH was adjusted to 3 with ortho-phosphoric acid.

In the reported method, the difference between retention time of curcumin and piperine was less, hence simultaneous estimation of this drugs was difficult in the nanoparticulate formulation. Also, the mobile phase used was highly acidic which may damage the column and may not show the desired results. So in the present work, a simple, precise, sensitive method was developed by using low-cost solvent acetonitrile with buffer pH 3 in ratio 70:30, detected by using UV visible detector. The developed method was used for estimation of curcumin from curcumin nanoparticles in which mobile phase was used for extraction of curcumin.

The primary objective of the study was to develop HPLC method for the simultaneous estimation of curcumin and piperine with adequate separation and to validate developed method according to ICH guidelines [14].

MATERIALS AND METHODS

Chemicals and reagents

Curcumin was kindly supplied by VAV life sciences Pvt. Ltd. (Mumbai), India and piperine were supplied from Sigma-aldrich, India. Methanol and acetonitrile used were of HPLC grade and obtained from Rankem and Loba-Chemie, India respectively. Purified water used was of HPLC grade, obtained from Merck Labs (Mumbai), India. Other reagents were of analytical grade including potassium dihydrogen orthophosphate, the orthophosphoric acid obtained from Loba-Chemie, India.

Nanoparticulate formulation

It was prepared by modified hot melt emulsification/ultrasonication technique using lipids and surfactants at Vivekanand Education Society's College of Pharmacy Chembur, Mumbai. Solid lipid precirol ATO5, liquid lipid labrafac lipophile WL 1349 and surfactant gelucire 50/13 used for nanoparticulate formulation were kindly gifted by Gattefosse, India. Surfactant tween 80 was gifted by BASF, India. The prepared nanoparticulate formulation labeled to contain 8 mg curcumin and 4 mg piperine per 10 ml.

Instruments used

Analyses were carried out using shimadzu HPLC-MD2010-UV system equipped with the pump and UV/VIS detector. The generated analytical signals were monitored and integrated using chrompass data software. The chromatographic separation was performed using enable C18G (250 mm x 4.6 mm x 5 µm) reverse phase column.

Methods

The method development for the simultaneous estimation of curcumin and piperine was performed using a different flow rate, elution mode, different mobile phase and buffer ratio [1, 2].

Preparation of stock solution

Stock solutions of curcumin and piperine were prepared by dissolving 10 mg of each in 10 ml of methanol to give 1000 µg/ml. All solutions were prepared in amber glassware as curcumin and piperine have poor light stability.

Preparation of working standard

Different working solutions containing curcumin and piperine were obtained by dilution of stock solutions with methanol.

Mobile phase preparation

Potassium dihydrogen orthophosphate of about 1.7011 g was weighed accurately and solubilized in mill Q water (500 ml), and pH was adjusted with orthophosphoric acid to 3.5 (solution A). A mixture of solution A and acetonitrile (30:70 %v/v) were mixed, degassed and used as mobile phase.

Method validation

The developed method was validated in compliance with ICH guideline for system suitability, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), linearity, range, and robustness in accordance with ICH guideline for analytical procedures Q2(R1) [14].

System suitability

Six replicate samples containing curcumin and piperine were analyzed using the developed method. Theoretical plate count more than 3000, tailing less than 1.5 and percentage relative standard deviation (% RSD) less than 2% of peak area was set as acceptance criteria.

Linearity

The linearity was evaluated at five concentration levels in the range between 5-25 µg/ml for both curcumin and piperine. The calibration curve was plotted by plotting concentration against its corresponding peak area, and linearity was determined using least square regression analysis. The slope value and correlation coefficient (r^2) values were calculated separately for each drug.

Precision (Repeatability)

The precision of the method is determined to check repeatability. Inter-day and intra-day precision studies were carried out at three different concentrations (12, 15 and 18 µg/ml) by injecting each sample three times and % RSD of estimated concentration was determined.

Accuracy (Recovery)

The accuracy of the method was determined by spiking the pre-analysed samples of curcumin and piperine with a known amount of standard solution (80, 100 and 120 %) and its recovery was estimated using the developed method. It was determined as the percentage difference between the expected and measured drug concentrations. Percentage recovery within 100±2% and % RSD of assay less than 2 % was set as acceptance criteria.

LOD and LOQ

LOD is the smallest concentration of analyte that gives response an accurately but not necessarily quantified as an exact value. LOQ is the least concentration of the analyte that gives an accurate response. The LOD and LOQ were calculated by using the standard deviation of response (SD) and slope (S) method with formula as mentioned below:

$$LOD = \frac{3.3 \times SD}{S}$$

$$LOQ = \frac{10 \times SD}{S}$$

Robustness and ruggedness

These parameters were studied by carrying out experiments by changing the chromatographic conditions such as pH, flow rate and column temperature.

Application of the validated method for the assay of the curcumin-piperine nanoparticulate formulation

Sample preparation

1 ml of the curcumin-piperine nanoparticulate formulation (0.8 µg/ml curcumin and 0.4 µg/ml piperine) transferred into 10 ml of volumetric flask. 7 ml of methanol was added and sonicated for 60 min for complete extraction. The sonicated solution was cooled down to room temperature, and volume was made up with methanol, mixed and filtered through 0.45µ nylon syringe filter. From the above solution, 1 ml was pipetted out and transferred to a 10 ml volumetric flask. Volume was made up with diluents to get a final concentration of 8 µg/ml curcumin and 4 µg/ml piperine. 20 µl of standard solutions was injected into HPLC system, chromatogram was recorded, and percent assay or recovery was calculated.

RESULTS AND DISCUSSION

Method development

The method development for estimation of curcumin and piperine was carried out using various mobile phases including acetonitrile: 0.1% orthophosphoric acid, methanol: water and acetonitrile: water to achieve separation of curcumin and piperine. These systems were unable to show two separate peaks for both the drugs. It was found that separation was best achieved using acetonitrile: phosphate buffer with pH 3. Also, various solvent-buffer ratios were tried such as (45:55, 60:40, 50:50 and 70:30). Combination of acetonitrile and phosphate buffer pH 3 at 70:30 v/v ratios has shown good resolution of curcumin and piperine. The optimum wavelength was selected for the estimation of curcumin and piperine was 360 nm based on the maximum absorption using a UV-Visible spectrophotometer. Initially, 0.8 ml/min flow rate was used but the increase in flow rate from 0.8 to 1 ml showed adequate separation and high theoretical plates. The flow rate of 1.2 ml/min did not show adequate separation of curcumin and piperine. Similarly, isocratic elution mode has shown better separation in comparison with gradient elution mode.

The optimum chromatographic condition with adequate resolution for curcumin and piperine was achieved when the separation was carried using enable C_{18} column (250 mm x 4.6 mm, 5 µ) at 35 ℃ temperature with an isocratic elution mode of mobile phase composed of a degassed mixture of acetonitrile: phosphate buffer pH 3 (70:30 v/v) at 1.0 ml/min flow rate with a total run time of 15 min where retention time for curcumin and piperine was observed at 7.2 and 8.69 min respectively as shown in fig. 2.

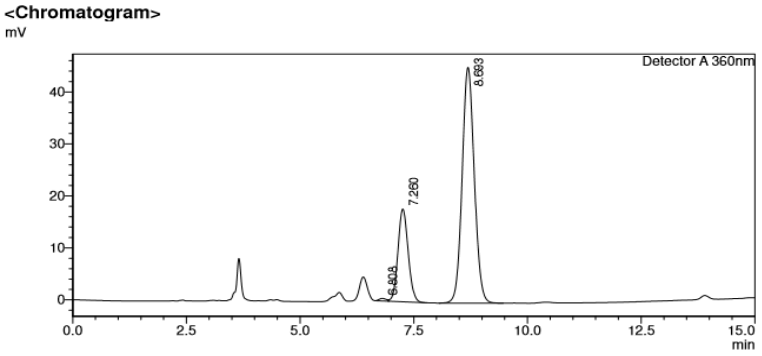


Fig. 2: Chromatogram of curcumin and piperine using optimum chromatographic conditions

Method validation

System suitability

The developed method indicated theoretical plates above 2000 with tailing factor less than 1.5 for both drugs curcumin and piperine. Similarly, the %RSD of peak area and retention time of curcumin was less than 2, which ensures the suitability of the developed method. The results of system suitability study are summarized in table 1.

Acceptance criteria

1. The relative standard deviation of six replicate injections for peak area and retention time should not be more than 2.0%.
2. The tailing factor should not be more than 1.5.
3. The theoretical plates should be more than 3000.

Linearity and range

The correlation coefficient (r^2) of curcumin and piperine was found to be 0.9994 and 0.9993 as given in fig. 3 and 4 respectively which confirms that acceptable linearity was obtained in the range of 5-25 µg/ml. The results of linearity are summarized in table 2.

Precision

The developed method has shown % RSD less than 1.5 for peak area for both intra-day and inter-day precision study, which ensures precision of the developed method. The results of intra-day and inter-day precision study are summarized in table 3 and 4.

Acceptance criteria: The % RSD for retention time and peak area should be NMT 2.0%.

Table 1: System suitability of the developed method

Sample	Retention time (min)	%RSD*	USP tailing factor	USP theoretical plates
Curcumin	7.20	0.86	1.026	7103
Piperine	8.70	0.93	1.094	8087

Data given in this table is presented as mean, n=3, RSD: Relative standard deviation.

Table 2: Linearity and range of the developed method

Conc (µg/ml)	Peak area (mAU*sec) (mean±SD)	
	Curcumin	Piperine
5	80566±854.40	232297±1024.80
10	286716±987.72	843803±959.18
15	513435±706.06	1470932±1219.52
20	753277±734.18	2162255±637.99
25	965423±906.49	2725927±988.75
Linearity equation	y = 44726x–150999	y = 126114x–404671
Correlation coefficient	r^2 = 0.9994	r^2 = 0.9993

Data given in this table is presented as mean±SD, n=3, SD: Standard deviation.

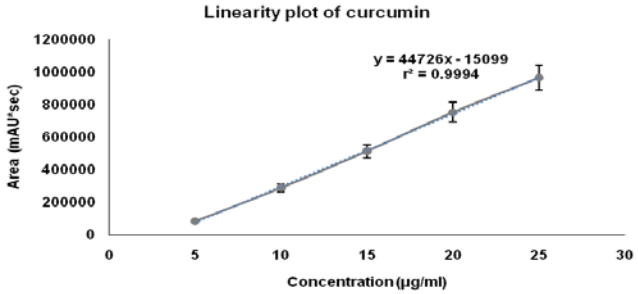


Fig. 3: Linearity plot of curcumin. (Data given in mean±SD) (n=3), SD: standard deviation

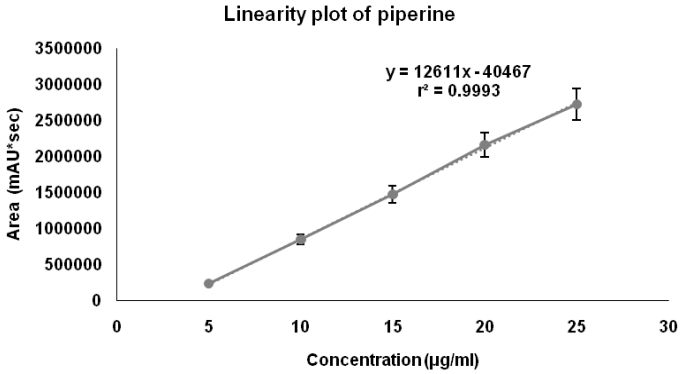


Fig. 4: Linearity plot of piperine, (Data given in mean±SD) (n=3), SD: standard deviation

Table 3: Intra-day precision of the developed method

Conc (µg/ml)	Curcumin			Piperine		
	retention time (min)	Area (mAU*sec) (mean±SD)	% RSD	retention time (min)	Area(mAU*sec) (mean±SD)	% RSD
12	7.30	552024.7±10606.31	1.57	8.72	1712079±4273.05	0.27
15	7.26	673719.7±5563.48	0.83	8.69	2092002±2883.76	0.14
18	7.28	800087±444.16	0.06	8.73	2460887±1304.81	0.05

Data given in this table is presented as mean±SD, n=3, SD: Standard deviation, RSD: Relative standard deviation.

Table 4: Inter-day precision of the developed method

Conc (µg/ml)	Curcumin			Piperine		
	retention time (min)	Area (mAU*sec) (mean±SD)	% RSD	retention time (min)	Area (mAU*sec) (mean±SD)	% RSD
12	7.30	558752.3±801.78	0.12	8.74	1717727±1654.13	0.03
15	7.28	674443±4636.57	0.74	8.75	2066546±1673.01	0.08
18	7.26	805796.7±6696.03	0.83	8.72	2449613±5243.75	0.21

Data given in this table is presented as mean±SD, n=3, SD: Standard deviation, RSD: Relative standard deviation.

Accuracy

The percentage recovery of the spiked curcumin and piperine was within 100±2% which ensures the accuracy of the developed method. The results of recovery studies were summarized in table 5 and 6.

Acceptance criteria: % Recovery should be between 98-102%.

Table 5: Accuracy of the developed method for curcumin

Level in (%)	Amount spiked (µg)	Amount recovered (µg)	% Recovery	% RSD
80	12	12.070	100.26	0.46
80	12	12.26	100.97	
80	12	12.31	101.13	
100	15	15.58	101.93	0.86
100	15	15.11	100.36	
100	15	15.15	100.50	
120	18	17.60	98.78	0.68
120	18	17.93	99.80	
120	18	18.02	100.06	

RSD: Relative standard deviation

Table 6: Accuracy of the developed method for piperine

Level in (%)	Amount spiked (µg)	Amount recovered (µg)	% Recovery	% RSD
80	12	11.93	99.73	0.19
80	12	11.86	99.48	
80	12	11.83	99.36	
100	15	14.71	99.04	0.01
100	15	14.72	99.06	
100	15	14.72	99.06	
120	18	17.40	98.19	0.19
120	18	17.29	97.85	
120	18	17.39	98.15	

RSD: Relative standard deviation

LOD and LOQ

The LOD for curcumin and piperine were found to be 0.73 µg/ml and 0.82 µg/ml respectively, while LOQ for curcumin and piperine were found to be 2.21 µg/ml and 2.49µg/ml respectively.

Robustness and ruggedness

The method is found to be rugged and robust since there were no changes in the chromatogram by changing the chromatographic conditions such as slight changes in flow rate (0.8-1.2 ml/min), column oven temperature (30-40^{0}C) and pH of mobile phase (2.8-3.2). These changes did not show influence on retention time or area of both drugs.

Application of the validated method for the assay of the curcumin-piperine nanoparticulate formulation

The developed method was successfully implemented in the estimation of curcumin and piperine in the nanoparticulate formulation. Assay of curcumin and piperine was found to be 98.3 and 105.1 % respectively. The results are summarized in table 7.

Table 7: Assay of curcumin and piperine

Parameter	Curcumin	Piperine
Amount recovered (mg)	7.86	4.20
%RSD*	0.22	0.24
Assay (%)	98.30	105.10

Data given in this table is presented in mean, n=3, RSD: Relative standard deviation.

In order to develop a suitable RP-HPLC method for the estimation of curcumin and piperine, different buffer ratios at different flow rate were applied. The reported methods were costly due to the use of highly sophisticated instruments like reverse phase ultrafast liquid chromatographic (RP-UFLC) system [8], HPLC-MS/MS [15] and detectors like fluorescence detectors [10]. An expensive solvent such as trifluoroacetic acid [1] was replaced by the combination of acetonitrile and phosphate buffer pH 3 in this study. The LOD and LOQ for curcumin were found to be 0.7303 and 2.2130 µg/ml, respectively whereas LOD and LOQ for piperine were found to be 0.8204 and 2.4862 µg/ml, which indicates that the method was sensitive, and can detect and quantify at lower levels of curcumin and piperine. Linearity range was from 5-25 µg/ml for both curcumin and piperine with regression coefficients of 0.9994 and 0.9993 respectively indicates that at this concentration range both curcumin and piperine were highly linear. Percent assay of curcumin and were found to be 98.3% and 105.1 respectively. The developed RP-HPLC assay method was found to be appropriate for the analysis of the drug in their pure form and in the nanoparticulate formulation.

CONCLUSION

In this study simple, accurate and cost-effective reversed-phase HPLC method was developed for the simultaneous estimation of curcumin and piperine with mobile phase degassed mixture of acetonitrile: phosphate buffer pH 3 (70:30 v/v) at 1.0 ml/min flow rate which is suitable for column efficiency. The retention time difference found 7.2 and 8.69 min for curcumin and piperine respectively which ensures precise estimation of both drugs in the nanoparticulate formulation. The developed method was validated as per ICH guidelines and statistical analysis also proved that the method is linear, precise, accurate and specific for the analysis of curcumin and piperine. The method is linear with a correlation coefficient 0.999 for both drugs. The accuracy of the method is in the range of 98.68 % to 102.93 %. The developed method was successfully implemented in the estimation of curcumin-piperine in the nanoparticulate formulation. Thus, the developed method can be used for routine analysis of curcumin and piperine in pharmaceutical dosage forms.

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AUTHORS CONTRIBUTIONS

Experimental design, execution, data generation and writing of manuscript were done by Ankita Khismatrao and Srinivas Bhairy. Support to draft manuscript design, data interpretation and corrections were done by Srinivas Bhairy. The design, guidance for work and manuscript review was done by Dr. (Mrs.) Rajashree Hirlekar.

CONFLICT OF INTERESTS

Declared none

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