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Method Development and Validation of Curcumin and Its Nanoformulation by Reverse Phase Ultra Fast Liquid Chromatography (RP-UFLC)

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ABSTRACT

The analysis was performed using mobile phase of acetonitrile and 25mM of phosphate buffer (pH 6.5) at 85:15 ratios and detection was carried out at 418 nm. The optimized conditions showed good peak resolution of curcumin with retention time of 4.5 min of standard and drug loaded nanoparticle. The limit of detection (LOD) and limit of quantitation (LOQ) was found to be 3.5 ng/mL and 12.7 ng/mL respectively. The calibration coefficient was equal to 0.9998 in the range of 25 ng/mL to 300 ng/mL indicating good linearity. The interday and intraday variability and perecentage relative standard daviation (RSD) showed variation of less than 1 in accuracy and precision indicating more accurate and precise method. The samples were also found to be stable for 7 days in mobile phase solution at ambient laboratory temperature and refrigerated temperature with precentage error of less than 0.01%. The presence of polymers and other components did not affect the results. So developed and validated RP-UFLC method can be used for the quantitative and qualitative estimation of curcumin in the nanoformulations as well as other pharmaceutical formulations.

Keywords: RP-UFLC, Curcumin and its nanoparticles, mobile phase stability and degradation studies.

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INTRODUCTION

Curcumin a yellow–orange dye occurs naturally in the rhizome of *Curcuma longa*, which is commercially known as turmeric. Turmeric contains curcumin along with curcuminoids. The major curcuminoids present in turmeric are demethoxycurcumin, bisdemethoxycurcumin and cyclocurcumin. The three principal colouring components of curcumin which are dicinnamoylmethane derivatives present in various proportions[1]. It posses various pharmacological properties to have antispasmodic, anticoagulant, antiangiogenic and antitumor activities[2-4].

Detailed survey revealed several methods such as HPLC[5,6] in plasma and urine, HPTLC[7,8], UV fluorescence detection[9], Capillary electrophoresis[10,11] and LC/MS analysis[12] were developed for estimation of curcumin. Most of these methods revealed only estimation of curcumin. A complete validation of HPLC is also significantly lacking. However only few methods have been developed and validated. Moreover a simple, rapid and sensitive method has been not developed so far. Hence, our study reports a simple, rapid and sensitive RP-UFLC method and was validated as per ICH guidelines[13].

MATERIALS AND METHODS

Materials

Curcumin was purchased from Sigma –Aldrich, India, HPLC grade Acetonitrile (ACN) was purchased from Sigma –Aldrich, USA, potassium dihydrogen ortho phosphate, triethyl amine and ortho phosphoric acid were obtained from Merck, Germany. Triple distilled water was obtained from Milli Q unit.

Instrument and chromatographic conditions

The Ultra Fast Liquid Chromatography (UFLC) consists of Shimadzu LC-20AD solvent delivery system (pump), Photodiode Array Detector (PDA) with a 7725i rheodyne injector with 20µL loop volume (Kyoto, Japan). The LC Solution software was used for integration. Chromatographic separation was done using Hibar C_{18} column (250 X 4.6 mm, 5µ ID). The mobile phase consists of acetonitrile–phosphate buffer pH 6.5 (buffer strength-25mM) in 85:15 ratios at a flow-rate of 1.0 ml/min. Curcumin was quantified at a wavelength of 418 nm. 20µL of injection volume was used for injection. pH meter (Systronics, Mumbai) was used to adjust pH.

Preparation of curcumin standard solution

10 mg standard curcumin was weighed accurately and dissolved in 5 mL of ACN. The solution was again made up to volume with ACN to produce 100 µg/mL solution.

Detection of wavelength

100 µg/mL standard solution of curcumin was scanned in the UV-visible region of 200 – 800 nm by using PDA detector. Maximum absorption was achieved at 418 nm.

Mobile phase preparation

1.7011g of potassium dihydrogen ortho phosphate was weighed accurately and dissolved in 500 mL of mill Q water and the pH of the above solution was adjusted to 6.5 with triethyl amine (buffer solution A). 15% of above buffer solution A and 85% of ACN were kept in a sonicator to remove the air bubbles.

Development of calibration curve

The stock solution (1000 ng/mL) of curcumin was used for the preparation of other working solutions by an adequate dilution using ACN. From stock solution aliquots were pipetted out in 10 ml volumetric flask to obtain the concentration of 25-300 ng/mL[14] and analysed at 418 nm by ultra fine liquid chromatography (UFLC) method. Calibration curve data was subjected to linear regression analysis and obtained the intercept, slope and regression equation. All measurements were performed in triplicate (n=3) and the standard deviation (SD) was recorded.

Preparation of curcumin nanoparticle

The nanoparticles were prepared by modified coacervation method[15]. Chitosan polymer (0.02% w/v) was dissolved in 0.1% w/v of acetic acid; volume adjusted using double distilled water and pH 5.5 was maintained with 0.1M NaOH. Sodium alginate (0.02% w/v) solution was prepared by dissolving in double distilled water and pH adjusted 5.5 using 0.1N hydrochloric acid. The chitosan and sodium alginate solutions were filtered under vacuum. Curcumin drug (5 mg) solution was prepared in tween 80 (0.2% v/v). Prepared drug solution was added in chitosan solution with constant stirring and resultant solution was added dropwise with the help of syringe in sodium alginate solution. The solution was stirred at 3000 rpm for 2 h and formed nanoparticles were collected by centrifugation. Before freeze drying 0.5% w/v of mannitol was added as a lyoprotectant. Freeze dried nanoparticles were stored in desiccator under vacuum.

RP-UFLC assay of curcumin in nanoparticles

For this assay 100 mg of nanoparticles containing 1 mg of curcumin was dissolved in 10 mL of ACN to produce the concentration of 100 µg/mL. 1 mL aliquot of this primary solution was transferred in 10 mL of volumetric flask and ACN added to make up the volume, giving a 100 ng/mL of stock solution. The solution was filtered through 0.22 µm millipore membrane filter and analysed at 418 nm by optimized RP-UFLC method.

METHOD VALIDATION

Validation of the method was carried out after the development of the RP-UFLC methods according to ICH guidelines.

Selectivity/ Specificity

A method is said to be specific when it produces a response only for a single analyte in the presence of other interferences. These parameters were determined by comparing the chromatograms of standard curcumin, curcumin loaded nanoparticles and placebo nanoparticles (without curcumin loaded).

Solution Stability

The short term stability studies were carried out at ambient laboratory temperature protected form sun light (22-25 °C) and at refrigerated temperature (2-8 °C) for 7 days in mobile phase containing acetonitrile and phosphate buffer (pH 6.5) at 85:15 ratios in the range of 100 ng/mL-500 ng/mL. After 7 days the sample were analysed and compared with freshly prepared sample at previously optimized conditions.

Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD and LOQ were determined at a signal-to-noise (S/N) ratio of 3 and 10 respectively.

Linearity

Linearity was established over the range of 25-300 ng/mL using the weight least square regression analysis.

Accuracy

Accuracy of the method was determined by recovery experiments. 25 ng/mL, 50 ng/mL, and 100 ng/mL of the curcumin were added to the nanosuspension of 50 ng/mL to become 75 ng/mL, 100 ng/mL and 150 ng/mL. The average recovery obtained from six injection was reported as percentage nominal of the analyzed concentration.

Precision

Precision was carried out by interday and intraday variations by injecting six injection of three different concentration of curcumin (100, 300, 500 ng/mL) at same optimized chromatographic conditions and percntage RSD was calculated.

Ruggedness

Chromatographic parameters such as retention time, asymmetric factor, capacity factor and selectivity factors were evaluated.

Robustness

Robustness of the method was studied by injecting the standard solutions with slight variations in the optimized conditions, ±1% in the ratio of acetonitrile in the mobile phase and ± 0.1 mL of the

flow rate.

RESULTS AND DISCUSSION

RP-UFLC separation optimization

Sakaria *et al.*, 2002[16] described eastimation of curcumin using mobile phase of methanol, 2% AcOH, and acetonitrile, with detection at 425 nm. There are various reasons for rejecting methanol in comparision to ACN. Methanol increased the pressure above 4000 psi at more than 70%. Where less noise, rapid, high sensitivity and low constant pressure made ACN appropriate for estimation of curcumin by RP-UFLC analysis.

For RP-UFLC analysis ACN was tried with phsophate buffer in different ratios, different pH strength and at different flow rate. The mobile phase consisting of ACN: Phosphate buffer of pH 6.5 in 85:15 ratios at a flow rate of 1.0 mL/min was found to be an appropriate mobile phase for eastimation of curcumin.

System suitability test

Specificity and Selectivity

Specificity and selectivity describe the capacity of the analytical method to measure the drug in presence of other components[17] were determined by comparing the chromatograms of the standard curcumin, drug loaded nanoparticle and placebo nanoparticle. The chromatograms of standard curcumin and curcumin loaded nanoparticle showed peak at retention time of 4.5 min. No peak was observed in the chromatogram of placebo nanoparticle which means the component of nanoparticles do not interfere with the analysis (Figure 1).

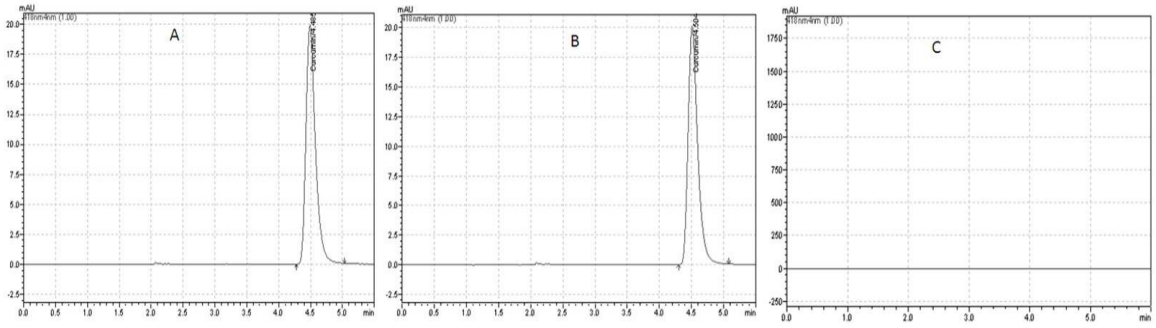

Figure 1. Typical chromatogram of (A) standard curcumin, (B) curcumin loaded nanoparticle, (C) placebo nanoparticle

Solution stability studies

Curcumin in mobile phase were found to be stable in ambient laboratory temperature and at refrigerated condition when compared with freshly prepared samples with % error below 0.01 in standard and in nanoformulation both. Therefore, curcumin standard and in noformulation

samples solutions were stable for up to 7 days when stored both at room temperature or under refrigeration (Table 1).

Table 1. Stability studies at ambient laboratory temperature and at refrigerated temperature

Concentration (ng/mL)	Freshly prepared Samples (Peak area)	Samples after 7 days (Peak area) at refrigerated temperature (2-8 °C)	Percentage error (%)	Samples after 7 days (Peak area) at room temperature (22-25 °C) protected from light	Percentage error (%)
50	3762	3761	0.001	3760	0.002
100	6827	6827	0.00	6826	0.001
150	10723	10723	0.000	10722	0.011
200	15081	15081	0.000	15080	0.001
250	20722	20721	0.001	20722	0.000
300	25987	25987	0.000	25985	0.002
350	32679	32679	0.000	32678	0.001
400	39026	39025	0.001	39025	0.001
450	58431	58431	0.000	58431	0.000
500	72902	72902	0.000	72900	0.002

Linearity and Detection limit

Linearity was checked by preparing standard solution of curcumin at concentration range of 25 ng/mL to 300 ng/mL and the spectrum was measure three times for each concentration. The equation and correlation coefficient was Y = 153.7x - 3381 and 0.9998 respectively, indicating good linearity at lower concentration range (Figure 2). The detection limit and quantification limit were presented on the basis of signal to noise ratio of 3 and 10 (S/N), found to be 3.5 ng/mL and 12.7 ng/mL.

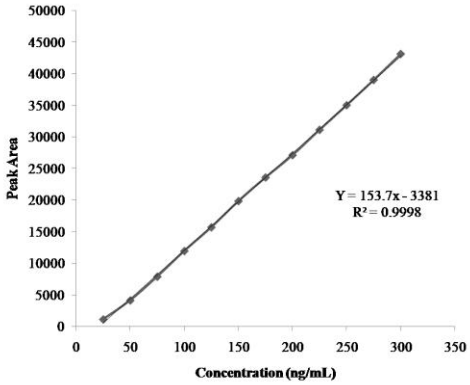

Figure 2. Calibration curve of curcumin

Accuracy and Precision

The measurement of percentage recovery after addition of known amount of curcumin in the nanoformulation was utilized to determine the accuracy of the developed method. The percentage recovery was found to be 100.33, 99.56 and 100.53% after six injections (Table 2).

The three concentrations (100, 300, 500 ng/mL) solutions of standard curcumin were prepared and subjected for intra and interday variability to determine precision of the developed assay method. The prepared solutions were analysed six times within the same day to determine intra-day variability and percentage RSD was found to be in the rage of 0.091 to 0.469. Inter-day precision of the method was tested for three samples in two days for six times. The % RSD values were found to be less than one in all concentrations indicating more precise method (Table 3).

Table 2. Accuracy study of curcumin

S.N.	Curcumin loaded nanoformulation (ng/mL)		
	Measured concentration	Nominal concentration	% Recovery (± RSD)
1	74.61	75	100.53±0.93
2	100.44	100	99.56±0.71
3	149.50	150	100.33±0.68

Table 3. Precision studies: Intraday and Interday precision of day 1 and day 2 for curcumin

S.N.	Curcumin (ng/mL) (Inter Day 1)			Curcumin (ng/mL) (Inter Day 2)			Curcumin (ng/mL) (Intra Day)		
	100	300	500	100	300	500	100	300	500
1	99.4	299.6	499.7	98.4	299.3	499.7	98.9	300	500.2
2	99.6	299.3	499.9	99.9	297.4	498.5	100.1	299.6	499.5
3	98.3	299.9	499.3	99.1	298.3	499.4	100	300.4	499.9
4	99.7	299.7	499.2	98.3	298.8	499.9	99.2	300.1	500
5	99.3	299.8	499.6	98.9	299.7	499.1	100.2	300	500.2
6	98.7	299.3	499.9	98.4	299.1	499.3	100.1	300.2	500.2
Mean	99.0	299.7	499.7	98.7	298.3	499.5	100.1	300.6	499.7
SD	0.368	0.656	0.643	0.433	4.091	3.356	2.891	5.685	8.941
%RSD	0.469	0.211	0.128	0.434	0.271	0.097	0.243	0.169	0.091

Table 4. System Suitability studies

S.N.	Parametes	Curcumin
1	Linearity range	25-300 (ng/mL)
2	Regression Equation Y=mx+c	Y = 153.7x - 3381
3	Correlation coefficient	0.9998
4	Theoretical plate/meter	32469.956
5	Resolution factor	1.05
6	Asymmetric factor	1.00
7	Retention time	4.5 min
8	LOD	3.5 (ng/mL)
9	LOQ	12.7 (ng/mL)

Ruggedness and Robustness

On changing the instrument parametes, instrument, operator and chromatographic conditions

(pH, mobile phase ratio and flow rate) no marked changes in the chrmatogram were observed. The column efficiency, resolution and peak asymmetric factors were calculated (Table 4) which demonstrated the suitability of the system for analysis of curcumin in the nanoformulation.

CONCLUSION

It is first known analytical method for determination and validation of curcumin and in nanoformuation by RP-UFLC method. The developed method was found to be more precise, simple, sensitive, selective and rapid with good system suitability can be used for the quantitative and qualitative estimation of curcumin in different formulations.

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