World Journal of Pharmaceutical and Life Sciences
WJPLS
www.wjpls.org
SJIF Impact Factor: 3.347

A SELECTIVE AND SPECIFIC RP HPLC METHOD DEVELOPMENT AND VALIDATION OF CURCUMIN AND STUDY OF ITS APPLICATION IN *IN SITU* FORMING GEL

***Koteswari P[2], Bhargav G C[1], Srinivasababu P, Kishorebabu G[1], Durga Nithya P[1] and Prakash K[3]**

[1]Vignan College of Pharmacy, Vadlamudi, Guntur, Andhra Pradesh, India-522007.

[2]Hindu College of Pharmacy, Amaravathi Road, Guntur, Andhra Pradesh, India-522007.

[3]Priyadarshini College of Pharmacy, Guntur, Andhra Pradesh, India-522007.

Article Received on 13/07/2016 Article Revised on 02/08/2016 Article Accepted on 23/08/2016

***Corresponding Author**
Dr. Koteswari P.
Vignan College of Pharmacy, Vadlamudi, Guntur, Andhra Pradesh, India-522007.

ABSTRACT

For the determination of curcumin in pharmaceutical dosage forms a simple RP HPLC method was developed. The method was simple, accurate, precise, selective and specific, and robust. The mobile phase consists of a mixture of Acetonitrile: water (pH adjusted to 3.3with o-phosphoric acid) in the ratio 51:49. Agilent High Pressure Liquid Chromatograph 1260 series with GI311C QUAT pump, Eclipse XDB-C_{18} 5µm, 4.6×250mm (MADE IN USA) and diode array detector G1315D was used in the study. The total run time and retention time were 10 and 8.77 min. Specificity and selectivity of the method was investigated by subjecting the curcumin to various degradations such as acid, alkali, hydrogen peroxide. The method was validated for various parameters and found linearity in the concentration range 0.25 to 10µg/ml, regression coefficient 0.999, LOD and LOQ 0.73 µg/mL and 2.2 µg/mL respectively. The robustness was checked by changing mobile phase composition and flow rate. Its suitability for routine analysis was tested in *in situ* gel formulation and found to be applicable.

KEYWORDS: curcumin, stability indicating method, forced degradation, *in situ* forming gel, routine analysis, diode array detector.

INTRODUCTION

Curcumin is a polyphenolic compound obtained from rhizomes of turmeric and containing three curcuminoids viz., curcumin I, curcumin II and curcumin III.[1,2 and 3] Curcumin I is therapeutically active and chemically it is a diferuloyl methane molecule [1,7-bis (4-hidroxy-3-mthoxyphenyl)-1, 6-heptadiene-3,5 -dione)] containing two ferulic acid residues joined by a methyl bridge. It has three functionalities: an aromatic o-methoxy phenolic group, α, β-unsaturated β-diketone moiety and a seven carbon linker. It has three pKa values 7.8, 8.5 and 9. Curcumin is also highly instable at all pH range i.e., from acidic to basic.[4] Thence, an analytical method that is specific and selective to curcumin is essential for routine analysis of curcumin in pharmaceutical formulations. Several methods[5], are reported for the estimation of curcumin but the retention time and hence the run time were longer which make the analysis to complete a prolonged period and therefore unsuitable to routine analysis. Prolonged run times also cause wastage of solvents, environmental pollution and not economical. Hence a new method was developed with objectives and scope mentioned below.

- To develop suitable RP HPLC method for the determination of curcumin I and to resolve the three cucrcuminoids i.e., I, II and III.
- Forced degradation study of curcumin under stress condition and to resolve all major impurities generated, in order to determine the selectivity and specificity of the method.
- To validate the developed method according ICH guidelines.
- To study the applicability of the developed method to determine the curcumin content for routine analysis in pharmaceutical formulations such as intra nasal *in situ* forming gel.

MATERIALS AND METHODS

Curcumin was purchased from Laila Pharmaceuticals, Vijayawada, Andhra Pradesh, India. All other materials and solvents used in this method development were HPLC grade and purchased locally in NSB pharmaceuticals, Vijayawada, Andhra Pradesh, India.

Instrumentation

Liquid chromatography was performed with Agilent High Pressure Liquid Chromatograph 1260 series GI311C QUAT pump, diode array detector G1315D and manual injector G1328C. Chromatographic separations were performed with Zorbax eclipse 300 C18 extended column and Eclipse XDB-C_{18} 5µm, 4.6×250mm (MADE IN USA) column. Column backup pressure was maintained between 1200 to 1700 psi.

RP HPLC Method Development

Selection of column and mobile phase

Trial I: Initially Acetonitrile and methanol, Acetonitrile and water, Acetonitrile, water and methanol in different ratios were tried as mobile phases and chromatographic separation was carried out with Zorbax eclipse 300 C18 extended column at 421 & 419 nm. **Trial II:** As curcumin has three different pKa values initially sodium acetate buffer pH 5 as solvent A and acetonitrile as solvent B in different ratios were used as mobile phase and chromatographic separation was carried out with Zorbax eclipse 300 C18 extended column at 421 & 419 nm. **Trial III:** Sodium acetate buffer pH 4.5 as solvent A and acetonitrile as solvent B in different ratios were used as mobile phase and chromatographic separation was carried out with Zorbax eclipse 300 C18 extended column at 421 & 419 nm. **Trial IV:** Sodium acetate buffer pH 3 as solvent A and acetonitrile as solvent B in different ratios were used as mobile phase and chromatographic separation was carried out with Zorbax eclipse 300 C18 extended column at 421 & 419 nm. **Trial V:** Sodium acetate buffer pH 3 with 1% triethylamine as solvent A and acetonitrile as solvent B as mobile phase was tried. **TrialVI:** Sodium acetate buffer pH 3 with 1% tetra hydro furan as solvent A and acetonitrile as solvent B as mobile phase was tried. **Trial VIII:** In this trial water (pH was adjusted with o-phosphoric acid to 3) as solvent A and acetonitrile as solvent B in different ratios was used as mobile phase. Trial VIII In this trial the chromatographic separation was performed using Eclipse XDB-C_{18} 5µm, 4.6×250mm (MADE IN USA) column mobile phase consisted of water whose pH adjusted to 3.3 with o-phosphoric acid and acetonitrile in various ratios at different flow rates were tried.

Optimization of chromatographic conditions using trial VIII

Chromatographic conditions were optimized by adjusting the ratio of solvent A and solvent B and the flow rates and depicted in **table 1.**

Specificity

The specificity of the method was determined by checking for interference with drug from placebo components. Further the specificity of the method toward the drug was established by means of the interference of the degradation products against drug during the forced degradation study. There was no interference of any peak of degradation product with drug peak.

Forced Degradation of Curcumin

Preparation of standard and sample solutions

Stock solutions of curcumin (10 µg/ml) were prepared by dissolving accurately weighed 10 mg of curcumin in methanol using 10 ml volumetric flask. Standard solutions were prepared by dilution of the diluted stock solution with methanol to obtain solutions in the concentration range of 0.1-10 µg/ml for curcumin in 10 ml volumetric flask.

Preparation of acid- and alkali-induced degradation product

To 1 ml of methanolic stock solution of curcumin 1 ml of 0.1 N HCl and 1 ml of 0.1 N NaOH were added separately and diluted to 10 ml with methanol to give concentrations of 10 µg/ml of curcumin. The acidic mixture was heated for 30 min at 80°C, and basic mixture was heated for 30 min at 80°C. Forced degradation in acidic and basic media was performed in 10 ml amber volumetric flasks in order to exclude the possible degradative effect due to light. The neutralized solutions were injected in triplicate and chromatograms were run as described.

Preparation of hydrogen peroxide-induced degradation product

To 1 ml of methanolic stock solution of curcumin and 1ml of hydrogen peroxide (3% v/v, H_2O_2) was added and diluted to 10 ml with methanol to give concentrations of 10 µg/ml of curcumin.. This solution mixture was heated for 1 h at 80°C. Forced degradation was performed in 10 ml amber volumetric flask in order to exclude the possibility of light induced degradation. The final solution was injected in triplicate and chromatogram was run as described previously.

Heat-induced degradation product

1 ml of methanolic stock solution of curcumin taken in 10 ml amber volumetric flask and diluted to 10 ml with methanol to give concentrations of 10 µg/ml of curcumin and heated for 1 h in water bath maintained at 80°C to study the heat degradation. The resultant cooled mixture was diluted to 10 ml with methanol, injected in triplicate and the chromatogram was run as described previously.

Photochemical degradation product

1 ml of methanolic stock solution of curcumin in 10 ml ambervolumetric flask and diluted to 10 ml with methanol to give concentrations of 10 µg/ml of curcumin. Photochemical stability of drugs was studied by exposing the stock solution to direct sunlight for 30 min.

Validation of The Developed Method

Determination of linearity

Accurately weighed quantity of curcumin was transferred into volumetric flask and drug was dissolved in HPLC grade methanol. Remaining volume was made up to the mark with methanol to get a concentration of 1mg/ml of curcumin. Stock solution was diluted with mobile phase to get a concentration of 100µg/ml of curcumin. The above solution was further diluted with mobile phase to get final concentrations of 0.25, 0.5, 1, 2, 4, 6, 8 and 10 µg / ml. A 20µl volume of each standard was injected into HPLC and the eluent was monitored using PDA detector at a wavelength of 419 nm. The total runtime was 20 min. The areas were noted and linearity was determined by constructing the linear curve using **excel 2007** and regression equation.

LOD and LOQ

Limit of Detection is the lowest concentration in a sample that can be detected, but not necessarily quantified under the stated experimental conditions. The limit of quantification is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy. Limit of Detection and Limit of Quantification were calculated using following formula LOD= 3.3σ/S and LOQ= 10σ/S, where SD=standard deviation of response (peak area) and S= slope of the calibration curve.

Precision

Intra-day precision was investigated by replicate applications and measurements of peak area for curcumin sample preparation for six times on the same day under similar conditions. Inter-day precision was obtained from % RSD values obtained by repeating the assay six times on two different days. The percent relative standard deviation (% RSD) was calculated.

Accuracy/Recovery

Accuracy is the degree of agreement between a measured value and the accepted reference value. The accuracy of the method was assessed by adding 3 different known concentrations equivalent to 80%, 100% and 120% of the active ingredient to *in situ* forming gel sample. The drug was extracted and analyzed. Then the % recovery and % RSD of recovery of each concentration was calculated to determine the accuracy.

Robustness

Robustness is the ability to provide accurate and precise results under a variety of conditions. In order to measure the extent of method robustness, the most critical parameters were interchanged while keeping the other parameters unchanged and in parallel, the chromatographic profile was observed and recorded. The studied parameters were: the composition of mobile phase, flow rate.

Application of the developed method for the determination of curcumin *in situ* forming gel

The developed method was applied to the assay of curcumin *in situ* forming gel. The drug content was calculated as an average of six determinations. A quantity of *in situ* gel equivalent to10mg of curcumin was taken in a 10 ml volumetric flask and drug was extracted with HPLC grade methanol. The extracts were pooled into a 10 ml volumetric flask and then made up the volume to 10 ml. Further dilutions were prepared with mobiles phase to obtain a concentration of about 10µg of curcmin per ml. The solution was filtered with sample filtration unit (the pore size: 0.22µm), degassed and 20 µl volumes were injected into HPLC in triplicate.

$$\% \text{ Drug content} = \frac{\text{Area of sample solution X Standard concentration X Dilution factor X 100}}{\text{Area of standard solution X 1000 X Label claim}}$$

RESULTS AND DISCUSSION

Literature survey revealed that the curcumin and curcumin combinations are under clinical trials for it's/or theirs many therapeutic properties and will be the drug of choice in future in the treatment of many ailments such as cancer, alzheimer's disease and so on. The reported HPLC methods are not validated for the quantification of curcumin and/or curcumin in combination with other drugs. Hence there is an urgent need to develop HPLC method for the determination of curcumin in pharmaceutical dosageforms. Therefore in the present study a RP HPLC method was developed and validated for the determination of curcumin in pharmaceutical formulations such as *in situ* forming gel. The basic chromatographic conditions used for this method were designed to be simple and easy to use and reproduce. The analytical conditions were selected after testing the different parameters that influence LC analysis, such as column, aqueous and organic solvents for mobile phase, mobile phase proportion, wavelength, diluent, concentration of analyte and other chromatographic parameters. Initially Zorbax eclipse 300 C18 extended column was tried at 421 & 419 nm, for

mobile phase selection, the preliminary trials using different compositions of mobile phases consisting of acetonitrile and water, methanol, buffers in different ratios was used. But all these mobile phases gave merged peaks or peaks of poor resolution or single peak for three curcuminoids. All the chromatograms clearly indicated that the peaks are either merged or not symmetrical and value of theoretical plates is lower side. In focus to develop good symmetrical peak of curcumin I and to obtain good resolution between the three curcuminoids, finally acetate buffer was replaced by water whose pH adjusted to 3.3 with o-phosphoric acid as solvent A and acetonitrile as solvent B was used as mobile phase. And Zorbax eclipse 300 C18 extended column was also replaced with Eclipse XDB-C_{18} 4.6×250mm (MADE IN USA) column 5µm, particle size was used because of its advantages of high degree of retention, high resolution capacity, better reproducibility, ability to produce lower back pressure and low degree of tailing. The representative chromatogram for the same is shown in **fig 1.**

Column: Eclipse XDB-C_{18} 5µm, 4.6×250mm (MADE IN USA) column

Validation of the proposed method.

The developed method for the analysis of curcumin was validated as per the ICH guidelines for parameters like specificity, system suitability, linearity, precision, accuracy, robustness and system suitability, limit of detection (LOD) and limit of quantitation (LOQ).

Specificity

The specificity of the method was determined by checking for interference with drug from placebo components. Further the specificity of the method toward the drug was established by means of the interference of the degradation products against drug during the forced degradation study. There was no interference of any peak of degradation product with drug peak. To perform the forced degradation study drug was subjected to acidic, alkaline, oxidizing, thermal and photolytic conditions. After the degradation treatments were completed, the stress content solutions were allowed to equilibrate to room temperature and diluted with the mobile phase to attain 10 µg/ml concentrations and injected into HPLC. Specific conditions and figures were described as under.

Acidic condition: In acidic condition the drug was heated under reflux with 1 N HCl at 80° C for 30 min and the solution was diluted with mobile phase to yield 10 µg/ml concentrations and injected into HPLC. The curcumin was found to be degraded up to 99 % in acidic condition. The chromatogram was represented in **fig** 2.

Alkaline condition: In alkaline condition the drug was heated under reflux with 0.1N NaOH at 80° C for 30 min and the solution was diluted with mobile phase to yield 10 µg/ml concentrations and injected into HPLC. The curcumin was found to be degraded up to 95 % in alkaline condition. The chromatogram was represented in **fig 3.**

Oxidative condition: In oxidative degradation the drug was heated under reflux with 3 % hydrogen peroxide at 80° C for 1 h. In oxidative degradation, it was found that around 99.8 % of the drug was degraded. The chromatogram was represented in **fig 4.**

Thermal condition: Thermal degradation was performed by exposing solid drug at 70° C for 72h. Curcumin was found to be degrading up to 33 % in thermal condition. The chromatogram was represented in **fig 5.**

Photolytic condition: Photolytic degradation was performed by exposing the drug content in sunlight for 30 min curcumin was found to be degrading up to 14% in photolytic condition. The chromatogram was represented in **fig 6.**

System suitability

The chromatographic systems used for analysis must pass the system suitability limits before sample analysis can commence. The chromatographic system was set up by allowing the HPLC system to stabilize for 40 min. Blank preparation and standard preparation (six replicates) and was injected and recorded chromatograms were evaluated for system suitability parameters like resolution, tailing factor, theoretical plate count and % RSD for peak area of six replicate injections of curcumin standard. The system suitability data was reported in **Table** 1.

Linearity

The linearity plot was prepared with eight concentration levels (0.1, 0.5, 1, 2, 4, 6, 8 and 10 µg/ml of curcumin). The response of the drug was found to be linear in the investigation concentration range and the linear regression equation was y = 29003x +13701 where x is the concentration in µg/ml and y is the peak area in mv; the regression coefficient was 0.999 given in **fig 7.**

LOD and LOQ

The LOD and LOQ values are presented in **Table 1**. The results of LOD and LOQ supported the sensitivity of the developed method. Method validation following ICH guidelines

indicated that the developed method had high sensitivity with LOD of 0.73 µg/ml and LOQ of 2.2 µg/ml.

Precision

Intra-day precision was investigated by replicate applications and measurements of peak area for curcumin for three times on the same day under similar conditions. Inter-day precision was obtained from % RSD values obtained by repeating the assay three times on two different days. The percent relative standard deviation (% RSD) was calculated which is within the acceptable criteria of not more than 2.0. The intra-day and inter-day precision results were shown in **Table 2** and confirmed the good precision.

Accuracy/Recovery and robustness

The recovery experiments were carried out by standard addition method. The percentage recoveries were depicted in table 3 and found to be good. The robustness of the method was tested by varying the flow rate of mobile phase (1 ml/min) and mobile phase composition (50:50). The results revealed that there was a change in the retention time but the area of the peak was not changed. Chromatograms obtained during robustness studies were showed in **figure 8.**

Application of the proposed method

The method was applied for the determination of curcumin in formulation *in situ* forming gel and drug content was calculated as 98.09 ±2.3% to 104.06±1.59%. The results revealed that the proposed method was suitable for routine analysis of curcumin in various pharmaceutical formulations.

Table 1: Optimized chromatographic conditions and system suitability parameters of proposed RP HPLC method for curcumin.

Instrument	**Agilent high pressure liquid chromatograph 1260 series**
Pump	GI311C QUAT pump
Detector	diode array detector G1315D
Mobile phase	Acetonitrile: water (pH adjusted to 3.3with o-phosphoric acid (51:49)
Diluent	Acetonitrile: water (pH adjusted to 3.3with o-phosphoric acid
Column	Eclipse XDB-C_{18}5µm,4.6×250mm(MADE IN USA)
Coulmn backup pressure	1200 to 1700 psi
Flow rate	1.1mL/ min
Detection Wavelength	419 nm

Injection volume	20µl
Run time	10 min
Retention time	8.77 min
LOD	0.73 µg/mL
LOQ	2.2 µg/mL
Theoretical plates (USP)	9047
Asymmetry	1.13040

Table 2: Summary of precision study.

Study	Set No.	Conc (µg/ml)	Area	Std. dev	RSD %
Intraday precision	1	10	4019235	53717.78	1.36
	2	10	3913883		
	3	10	3948324		
Inter day precision	1	10	3920027	49733.34	1.2
	2	10	3978134		
	3	10	3879167		

Table 3 percentage recovery of curcumin in accuracy studies.

Amt added (mg)	Amt recovered (mg)	% recovered	Average percent recovered ± SD	% RSD
10	9.964	99.64	99.95±1.23	1.23
10	9.89	98.9		
10	10.13	101.3		
15	15.237	101.58	102.70±2.53	2.46
15	15.84	105.6		
15	15.14	100.93		
20	19.94	99.7	100.00±0.36	0.36
20	20.08	100.4		
20	19.978	99.89		

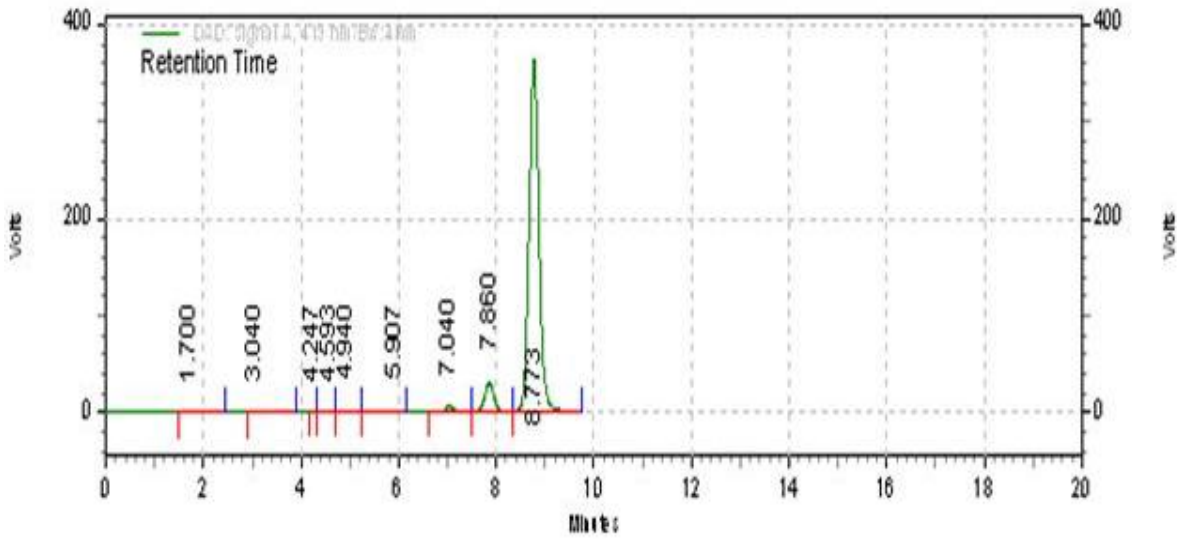

Figure 1.

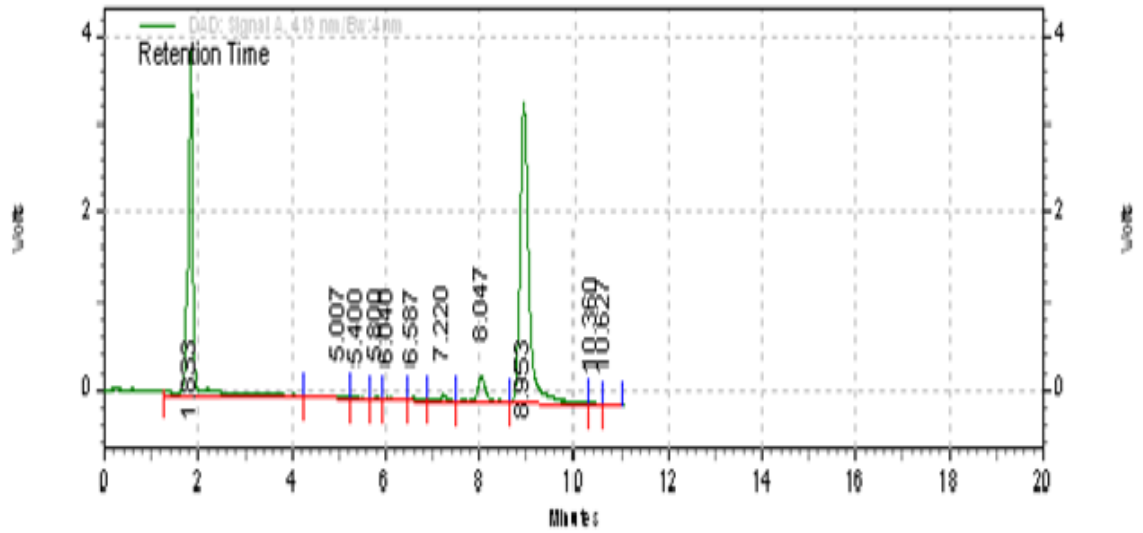

Figure 2.

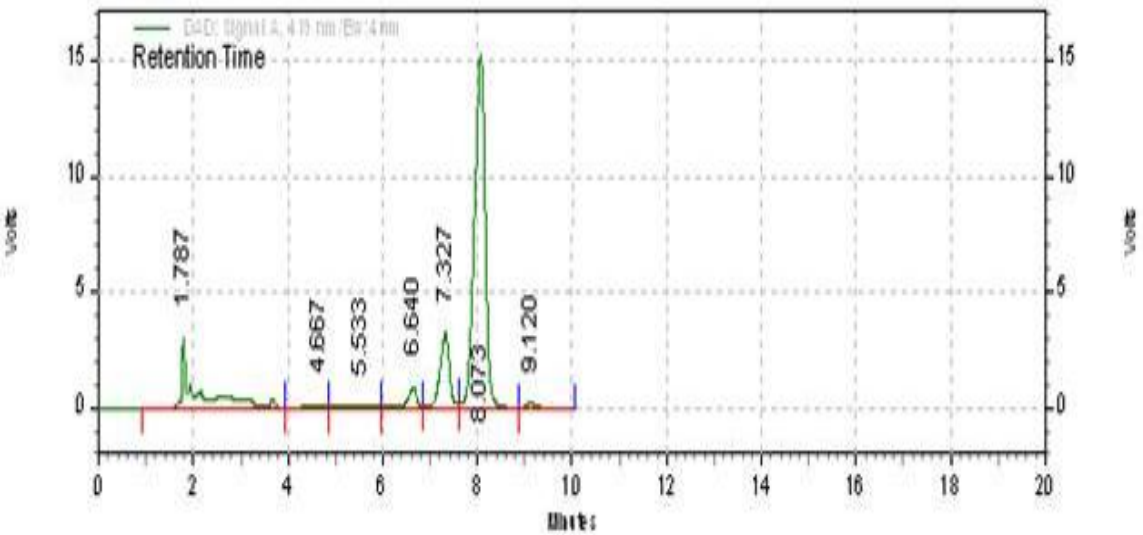

Figure 3.

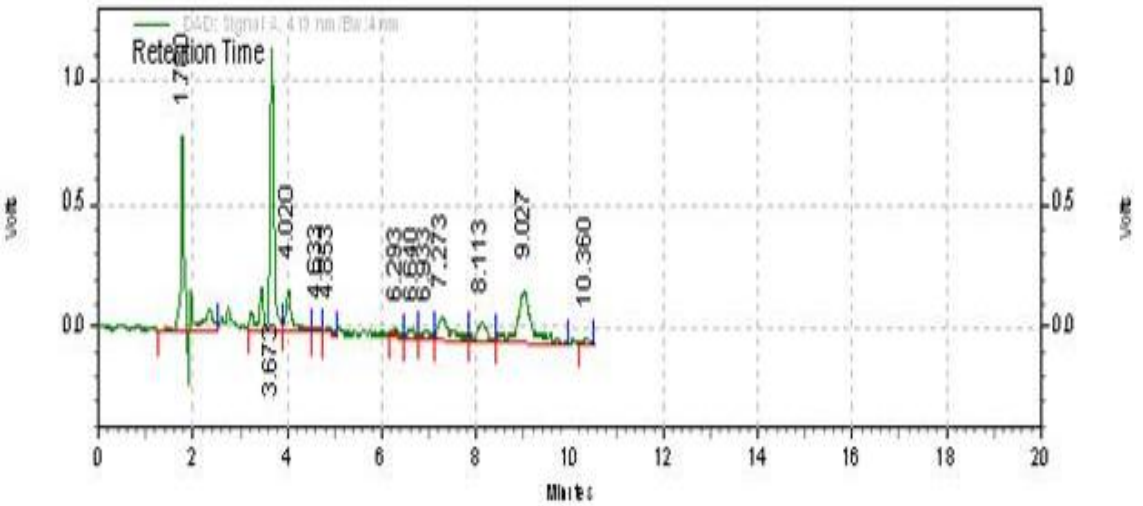

Figure 4.

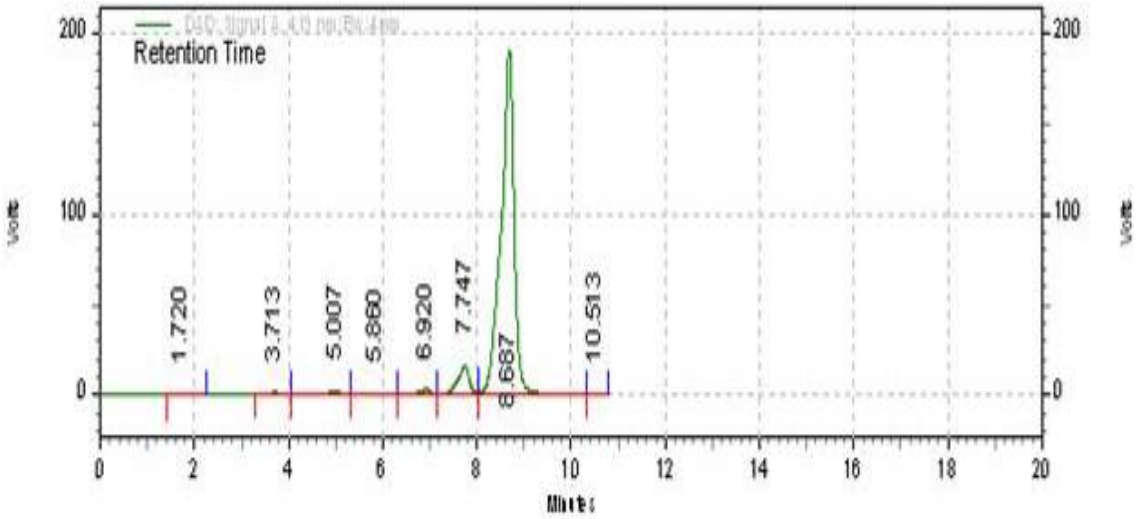

Figure 5.

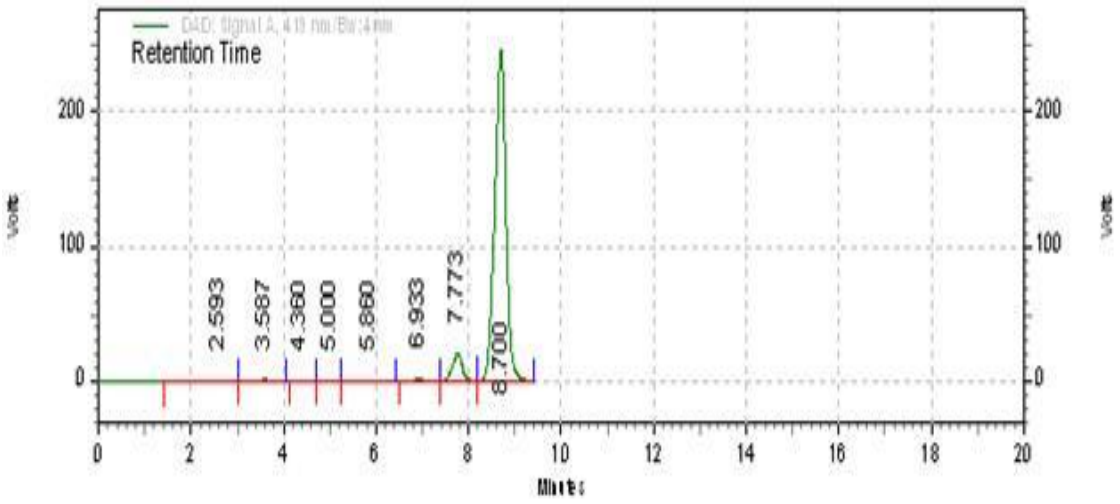

Figure 6.

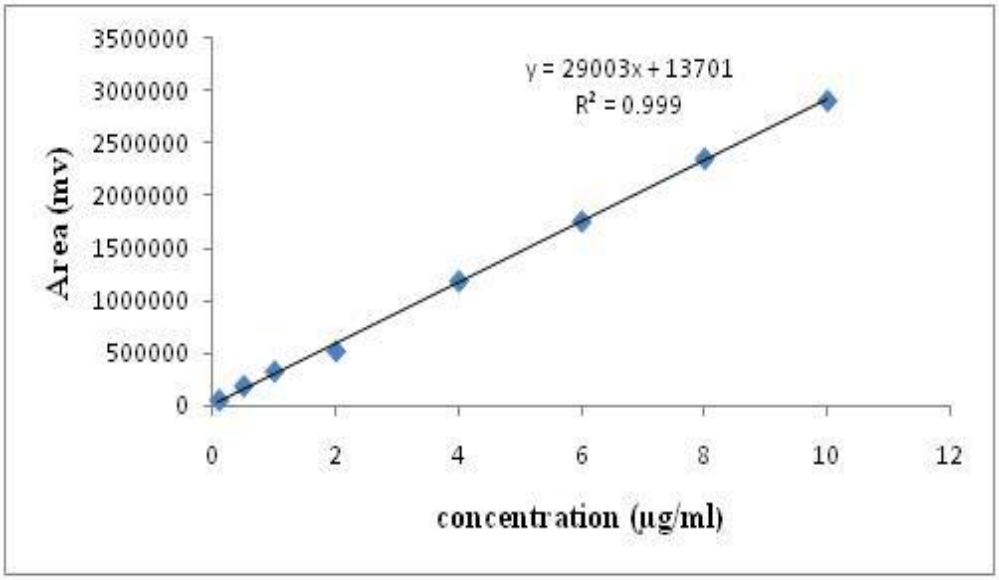

Figure 7.

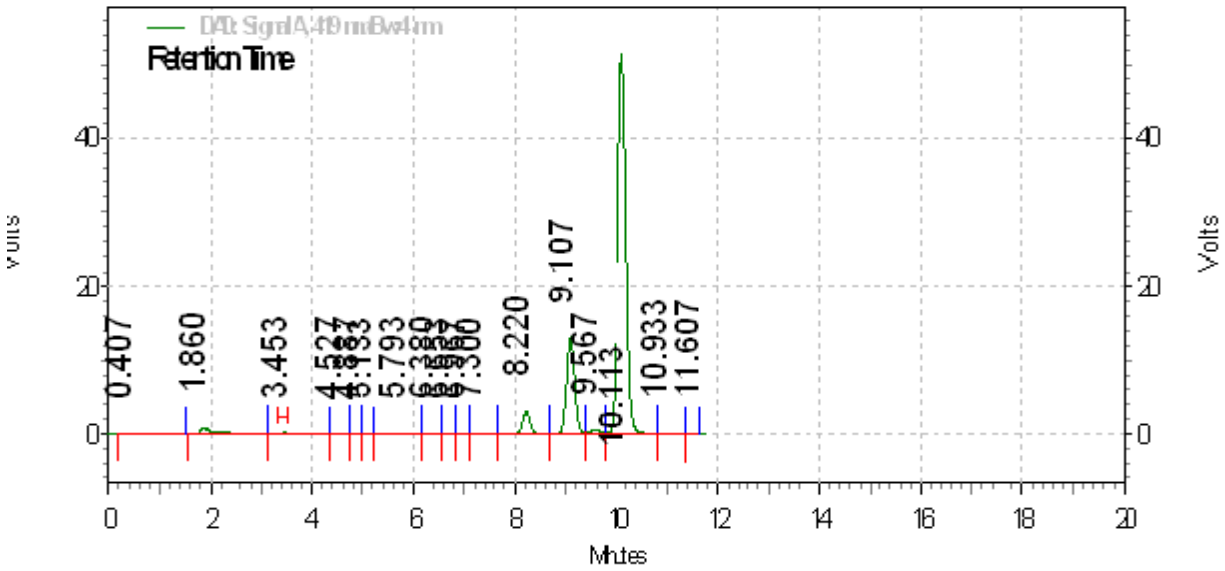

Figure 8.

CONCLUSION

The developed method was simple, sensitive, specific, robust, precise and accurate. It can be applied for routine analysis of pharmaceutical samples.

FINANCIAL SUPPORT

The authors acknowledge All India Council for Technical education for providing research grant under RPS scheme.

ACKNOWLEDGEMENTS

The authors acknowledge management of Vignan pharmacy college management for providing facilities to carry out this work.

REFERENCES

1. Hiroshi A, Yasunari T, Seiji K, Raymond S, Bharat BA and Yasuko K. Evidence that curcumin suppresses the growth of malignant gliomas in vitro and in vivo through induction of autophagy: role of akt and extracellular signal regulated kinase signaling pathways. Molecular pharmacology, 2007; 72: 29-39.
2. Liu J, Chen S, Lv L, Song L, Guo S and Huang S. Recent progress in studying curcumin and its nano preparations for cancer therapy. Current pharmaceutical design, 2013; 19: 1974-93.
3. Christian S, Margareth P, Maike P, Volker S, Donat K and Jakob W. The nontoxic natural compound curcumin exerts anti-proliferative, anti-migratory and anti-invasive properties against malignant gliomas. BMC cancer., 2010; 10: 491.

4. Lisa M C and Zena W. Inflammation and cancer. Nature, 2002; 420: 860–867.
5. Dalapathi BG and Vandana BP. A new stability indicating HPLC method for simultaneous determination of curcumin and celecoxib at single wavelength: an application to nanoparticulate formulation. Pharmaceutical analytica acta, 2012; 3: 157.