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Development and Validation of an Analytical Method for the Simultaneous Estimation of Curcumin and Piperine in Bulk and Combination Dosage Form by RP-HPLC

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ABSTRACT

A rapid and precise Reverse Phase High Performance Liquid Chromatographic method has been developed and validated for the simultaneous estimation of curcumin and piperine, in bulk as well as in tablet dosage form. Separation was carried out on Inertsil-ODS C18 (250 x 4.6 mm, 5 µm) column using a mixture of Methanol: Buffer Ph 3:2, (80:20) as mobile phase at a flow rate of 1.0 ml/min. The detection was carried out at 252 nm. The retention times of the curcuminoid and piperine was found to be 3.048; 4.316 ± 0.02 min respectively. The method produces linear response in the concentration range of 5-25 µg/ml for curcumin and 50-250 µg/ml for piperine. The method was precise since the % RSD values of peak areas for five duplicate injection was found to be below "2". The % recovery values for the analyte were found to be 99.7% & 99.9% indicating the method was accurate. The LOD & LOQ of curcumin and piperine were found to be 0.12 µg/ml, 0.36 µg/ml & 0.14 µg/ml, 0.45 µg/ml. A series of trials were attempted to develop a sensitive RP-HPLC method and finally optimized with the mobile phase composition Methanol: Buffer pH 3:2 (80:20). The method was found to be rapid, linear, precise, accurate, sensitive that can be adopted for routine analysis.

INTRODUCTION

Curcumin (diferuloylmethane; 1,7-bis[4-hydroxy-3-meth-oxyphenyl]-1,6-heptadiene-3,5-dione, is the major bioactive component of the spice herb turmeric or *Curcuma longa* L, a widely used natural food product[1]. It's a phytopolyphenol pigment with a variety of pharmacologic properties used traditionally in Indian and Chinese medicine and widely consumed ingredient in Asian diet. It belongs to the category of curcuminoids, a phytochemical responsible for the characteristic yellow color to turmeric. Curcumin has also been demonstrated with benefits in various health issues like cancer[2], immunodeficiencies[3], cardiovascular disorders[4], Alzheimer's[5], diabetes[6], diabetes[7] and Crohn's disease. Its bioavailability is considerably low. As a polyphenol antioxidant curcumin also exhibits neuroprotective[8] and anti-inflammatory[9] activities. Curcumin acts as a scavenger of oxygen species, such as hydroxyl radical, superoxide anion, and singlet oxygen and inhibits lipid peroxidation as well as peroxide-induced DNA damage. Commercially curcumin is a mixture of three curcuminoids i.e. curcumin (75%), dimethoxy curcumin (15%) and bis dimethoxy curcumin (5%)[10]. Piperine is a N-acylpiperidine that is piperidine substituted by a (1E, 3E)-1-(1, 3-benzodioxol-5-yl)-5-oxopenta-1, 3-dien-5-yl group at the nitrogen atom. It is an alkaloid isolated from the plant Piper nigrum. It has a role as a NF-kappa inhibitor, a plant metabolite, a food component and a human blood serum metabolite. It is a member of benzodioxoles, a N-acylpiperidine, a piperidine alkaloid and a tertiary carboxamide. It derives from an (E, E)-piperic acid[11-16].

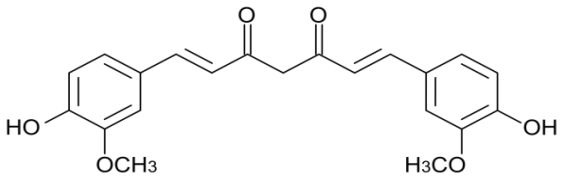

Figure No. 1: Structure of Curcumin

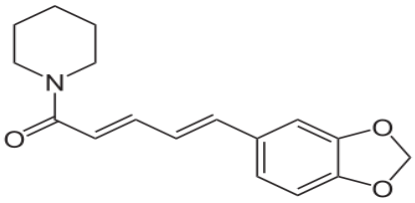

Figure No. 2: Structure of Piperine

Literature reveals that few methods have been reported for the estimation of Curcumin and Piperine individually and in combination with other drugs by HPTLC (Vyas N et al., 2016) and bioanalytical methods by LC-MS (Liu AC et al., 2006), UPLC (Marczylo TH et al., 2009). A few methods have been reported for the estimation of Curcumin and Piperine individually and in combination with other drugs by HPLC (Kalra R, et al., 2016). The present attempt is made to develop a most reliable method for simultaneous estimation of Curcumin and Piperine in bulk and tablet dosage form by RP-HPLC.

MATERIALS AND METHODS

Instruments used:

The liquid chromatographic system used was WATERS, software: Empower 2, Alliance 2695 separation module. 996 PDA detectors, Inertsil-C18 ODS (250 x 4.6 mm, 5 µ) column.

Chemicals used:

Samples of Curcumin and Piperine were purchased from Active lab. HPLC grade water, methanol and Acetonitrile and Potassium dihydrogen phosphate ($K_2H_2PO_4$) were purchased from MERCK laboratories, Mumbai.

Method Development:

Preparation of standard solution:

Accurately weighed quantity of 10 mg of Curcumin and Piperine working standards were transferred into 10 ml of clean dry volumetric flasks, about 7 ml of Methanol was added and sonicated to dissolve and removal of air completely. The volume was made up to the mark with the same Methanol.

Further 0.15 ml of the above Curcumin and 1.5 ml of Piperine stock solutions were transferred into 10 ml volumetric flasks and diluted up to the mark with Methanol.

Preparation of Sample Solution:

Equivalent weight of one capsule was taken and crushed in a motor. From this 10 mg equivalent weight of Curcumin and Piperine was transferred into a 10 ml clean dry volumetric flasks and about 7 mL of Diluent was added and sonicated to dissolve it completely. The volume was made up to the mark with the same solvent.

The method development was started by initial chromatographic conditions with liquid chromatographic system WATERS, software: Empower 2, Alliance 2695 separation module. 996 PDA detectors, Inertsil-C18 ODS (250 x 4.6 mm, 5 µ) column. Various compositions of mobile phase were utilized to optimize the method. Initially, varied concentrations of Methanol: Acetonitrile were used for better separation of the analytes. The method was finally optimised by Methanol and Buffer in the ratio of 80:20 V/V, Column Inertsil -ODS C_{18}(250 x 4.6 mm,5µ), flow rate l ml/min, detection wavelength at 252 nm. Retention times

for Curcumin and Piperine were found to be 3.0 min and 4.3 respectively. With this chromatographic conditions, both the analytes were eluted with good resolution. The retention times and theoretical plates were also satisfactory. The Chromatogram is shown in **figure-6.**

The developed method was validated for specificity, accuracy, precision, linearity, LOD & LOQ as per the ICH guidelines.

Method Validation:

System suitability: A Standard solution was prepared by using Curcumin and Piperine working standards as per test method and was injected in replicates for five times into the HPLC system. The system suitability parameters like theoretical plates, tailing factor, resolution were evaluated from standard chromatograms. The results were given in table 2.

Specificity: To ensure zero interference from mobile phase and excipients specificity studies were carried out by injecting sample, standard, and blank and excipients solutions into the HPLC systems. The results were given in figures 7- 8.

Precision: System precision: Standard solutions were prepared as per test method and injected five times in replicates.

Method precision: Sample solutions were prepared as per the test procedure and six injections were given in replicates.

Intermediate Precision: To evaluate intermediate precision, studies were performed on different days by maintaining same conditions. The standard solution was injected for six times in replicates. The peak areas for all six injections were recorded and the %RSD for the same was calculated and reported. The procedure under similar conditions was repeated on day two. The results were given in tables 3 to 5.

Accuracy: The accuracy of the proposed method was evaluated by recovery studies at various concentrations of curcumin and piperine at three different levels equivalent to 50, 100 & 150%. At each level the target concentration was spiked in triplicates and the amount recovered was calculated. The percentage recovery at each level was calculated and reported in table 6 and 7.

Linearity: A Series of solutions were prepared using Curcumin and Piperine working standards at concentration levels from 20 ppm to 80 ppm of target concentration. Each sample solution was injected into HPLC system in replicates and the peak areas were measured. A graph was plotted with peak areas vs concentrations and the r2 values were calculated. The results were shown in fig 7 & 8, table 8 and 9.

Limit of Detection and Limit of Quantification:

From the linearity data, the limit of detection and quantification were calculated using the following formulae.

$$LOD = \frac{3.3\ \sigma}{S}$$

σ = standard deviation of the response

S = slope of the calibration curve of the analyte

$$LOQ = \frac{10\ \sigma}{S}$$

σ = standard deviation of the response

S = slope of the calibration curve of the analyte

The values were given in results and discussion

Robustness: A study was conducted to determine the effect of variation in flow rate, change in mobile phase composition and detection of wavelength. Standard solution prepared as per the test method was injected into the HPLC system using flow rates, 1.0ml/min and 1.2ml/min. The same studies were also performed by varying mobile phase composition and detection wavelength. The system suitability parameters were evaluated and reported in table 10 and 11.

RESULTS AND DISCUSSION

Method development:

Trial 1: Mobile phase: - Methanol: Water (90:10%V/V)

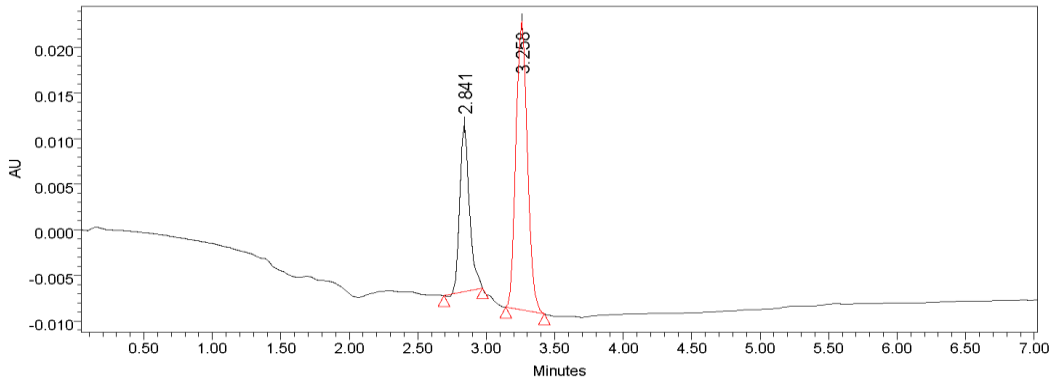

Figure No. 3: Chromatogram of Trial 1

Trail 2 Mobile Phase Methanol: water (40:60% V/V)

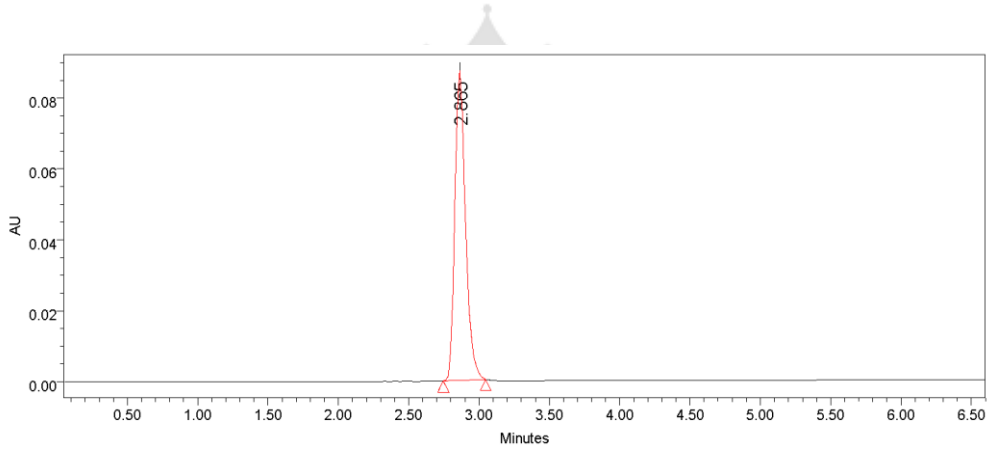

Figure No. 4: Chromatogram of Trial 2

Trail 3: Mobile phase Methanol: Water (70:30%, v/v)

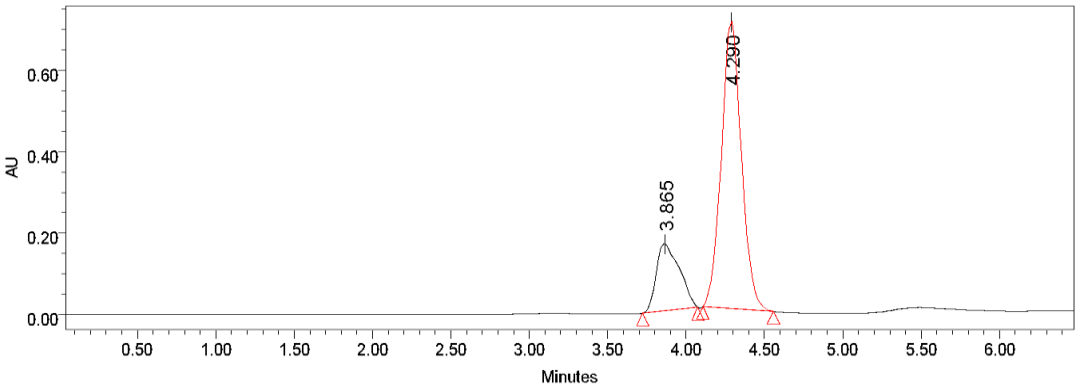

Figure No. 5: Chromatogram of Trial 3

Trail 4: Mobile phase: Methanol: Buffer (80:20% v/v) (Optimized trial)

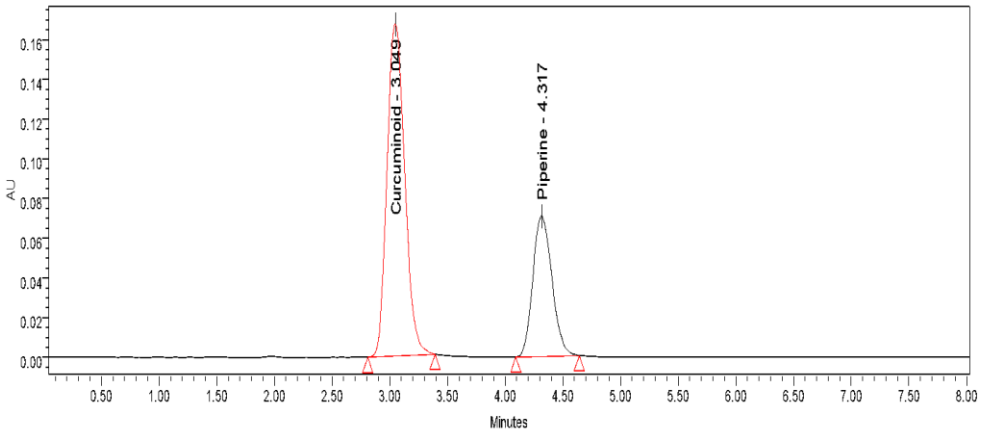

Figure No. 6: Optimized Chromatogram

Table No. 1: Peak characteristics of optimized chromatogram

S.NO	Drug	Retention time(min)	Area	USP Resolution	USP Tailing	USP Plate count
1	**Curcumin**	3.049	2022927	-	1.1257	10060.327091
2	**Piperine**	4.317	322742	4.412624	1.8672	8323.372981

Method Validation:

System suitability: System suitability studies were performed by replicate injections and the parameters like theoretical plates, tailing was recorded. The theoretical plates are more than 2000 and the tailing factor is less than 2 in each injection for both the analytes. The observed values are in accordance with the acceptance criteria.

Table No. 2: Data of System Suitability for Curcumin

	Curcumin				**Piperine**			
Injection	RT	Peak Area	USP Plate count	USP Tailing	RT	Peak Area	USP Plate count	USP Tailing
1	3.048	2022356	10238	1.045	4.316	322689	8325	1.145
2	3.048	2021546	10105	1.087	4.315	322187	8325	1.156
3	3.046	2023781	10368	1.065	4.312	322100	8314	1.147
4	3.042	2023852	10272	1.058	4.309	3.22846	8372	1.148
5	3.048	2024854	10846	1.057	4.317	322445	8392	1.152

Precision: System precision and method precision were performed in six replicate injections and the % RSD of the peak areas were calculated. The % RSD for the peak areas of six standard injections for system precision were 0.0877 and 0.073 and for method precision 0.108 and 0.85for curcumin and piperine respectively which were within the limits. The results are observed in table 3 & 4.

Intermediate precision was also performed on two different days and the results were observed in table 4. The % RSD for the peak areas of six standard injections were found to 0.091 and 0.084 for piperine and curcumin respectively, which were in agreement with acceptance criteria. The results were tabulated and presented in table 5.

Table No. 3: Precision: Data of Repeatability (System precision) for Curcumin and Piperine

	Curcumin		Piperine	
Injection	**Peak Areas of Curcumin**	**%Assay**	**Peak Areas of Piperine**	**%Assay**
1	2023987	100.23	322124	100.05
2	2024578	100.26	322689	100.22
3	2028545	100.46	322356	100.12
4	2025346	100.30	322564	100.19
5	2024587	100.26	322484	100.16
6	2023655	100.21	322784	100.25
Mean	2025116	100.29	322500	100.17
SD	1777.04		237.8406	
% RSD	0.0877		0.073	

Table No. 4: Data of Repeatability (Method precision) of Piperine and Curcumin

Injection	**Peak Areas of Piperine**	**% Assay**	**Peak Areas of Curcumin**	**% Assay**
1	322546	100.18	2024568	100.26
2	322840	100.27	2024875	100.28
3	322894	100.29	2024875	100.36
4	322156	100.06	2028655	100.46
5	322968	100.32	2028655	100.38
6	322228	100.08	2028455	100.45
Mean	322605		2026681	
SD	351.4898		1725.19	
% RSD	0.108		0.85	

Table No. 5: Data of Intermediate precision for Curcuminoid

Injection	Peak Areas of Curcumin	% Assay	Peak Areas of Piperine	% Assay
1	2026885	100.37	322045	100.3
2	2028854	100.47	322265	100.09
3	2027845	100.42	322678	100.22
4	2023846	100.22	322145	100.06
5	2027845	100.42	322356	100.12
6	2025375	100.30	322701	100.13
Mean	2026775		322365	
SD	1852.73		272.6705	
% RSD	0.091		0.084	

Accuracy: Accuracy of the method was evaluated by recovery studies. Three target concentrations 50%, 100%, 150% were prepared with respect to target assay and injected into HPLC system in triplicates. At each spike level the mean recovery values are between 98 to 102 % which were in agreement with the acceptance criteria. The recovery values indicate the method is accurate. The results are observed in table 6 & 7.

Table No. 6: Data of Accuracy for Curcumin

Concentration (%) of spiked level	Amount added (ppm)	Amount found (ppm)	% Recovery	Mean recovery
50% Injection 1	20	20.16	100.81	100.76
50% Injection 2	20	20.13	100.68	
50% Injection 3	20	20.15	100.80	
100 % Injection 1	40	40.08	100.21	100.29
100 % Injection 2	40	40.12	100.32	
100% Injection 3	40	40.13	100.34	
150% Injection 1	60	60.07	100.12	100.21
150% Injection 2	60	60.14	100.23	
150% Injection 3	60	60.17	100.28	

Table No. 7: Data of Accuracy for Piperine

Concentration (%) of spiked level	Amount added (ppm)	Amount found (ppm)	% Recovery	Mean recovery
50% Injection 1	20	20.05	100.25	100.49
50% Injection 2	20	20.10	100.54	
50% Injection 3	20	20.12	100.68	
100 % Injection 1	40	40.01	100.03	100.09
100 % Injection 2	40	40.03	100.09	
100% Injection 3	40	40.06	100.15	
150% Injection 1	60	60.06	100.07	100.07
150% Injection 2	60	60.10	100.16	
150% Injection 3	60	60.00	100.00	

Linearity: Linearity of the method was evaluated by injecting various concentrations of both the drugs into HPLC system. A graph was plotted with peak area versus concentration and the correlation coefficient was calculated. The r^2 values of both the drugs were found to 0.999 which were within the limits. The r^2 values confirmed the method was linear and the results were shown in table 8 & 9 and figures 7 & 8.

Table No. 8: linearity data of curcumin

Concentration (ppm)	Average Area	Statistical Analysis	
0	0	Slope	50529
20	1012458	y-Intercept	-1945
30	1516384	Correlation Coefficient	0.999
40	2022586		
50	2500874		
60	3033956		
70	3539425		
80	4045986		

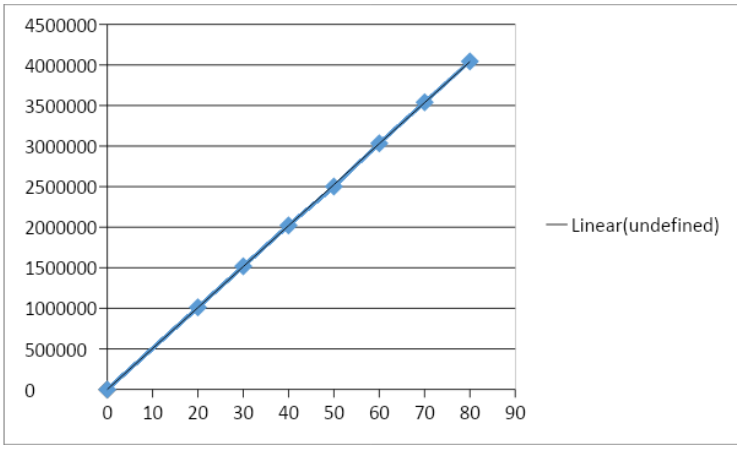

Figure No. 7: Linearity Plot of Curcuminoid

Table No. 9: linearity data of Piperine

Concentration (ppm)	Average Area	Statistical Analysis	
0	0	Slope	8057
20	161274	y-Intercept	-331.8
30	241911	Correlation Coefficient	0.999
40	322548		
50	398456		
60	483822		
70	564459		
80	645096		

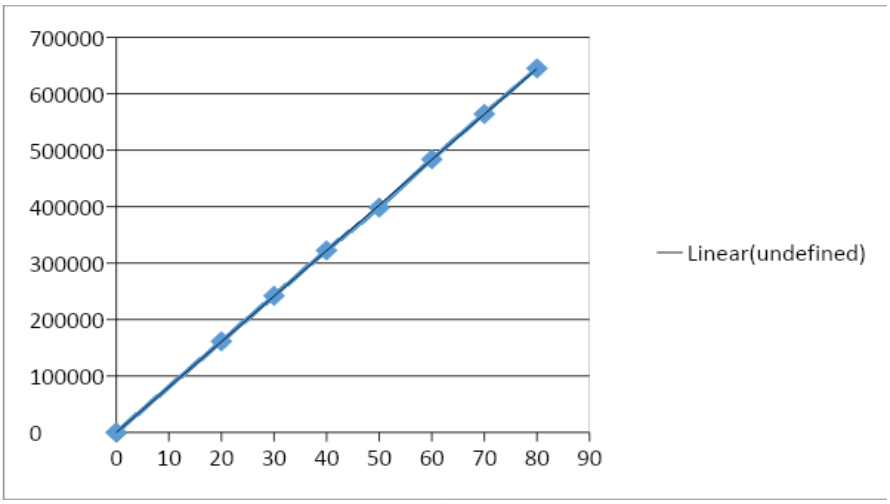

Figure No. 8: Linearity Plot of Piperine

Specificity: There is no interference observed in blank. The chromatograms of Standard and Sample were identical with same retention time.

Limit of Detection and Quantitation (LOD and LOQ): The LOD of Curcuminoid and Piperine were found to be 0.120 and 0.364 respectively and the LOQ values were found to be 0.149 and 0.40 respectively. The results meet the acceptance criteria and also indicates that the method was sensitive.

Robustness: A study was carried out with variation in flow rate to evaluate the robustness of the method. The standard solutions were injected in the selected robust conditions and the system suitability parameters like theoretical plates, tailing factor and resolution were observed. The results showed that the theoretical plate count was more than 2000, tailing factor was less than 2 and resolution was found more than 2. The results of the study indicated that the method was robust and the results were shown in table 10 & 11.

Table No. 10: Effect of variation in flow rate -Curcumin

Flow rate	Peak Area	Tailing factor	Flow rate	peak Area	Tailing factor	Flow rate	Peak Area	Tailing factor
0.8 ml/m	2008698	1.011	**1 ml/m**	2026578	1.096	**1.2 ml/m**	2054876	1.056
	2003945	1.013		2027564	1.078		2056842	1.075
	2005682	1.011		2022796	1.053		2053896	1.089
	2004265	1.007		2023658	1.048		2057841	1.061
	2008645	1.016		2027894	1.086		2058646	1.077
	2003575	1.010		2024505	1.095		2054784	1.036
Avg	2005801		**Avg**	2025499		**Avg**	2056147	
SD	2334.70		**SD**	2137.66		**SD**	1904.91	
%RSD	0.116		**%RSD**	0.105		**%RSD**	0.092	

Table No. 11: Data for Effect of variation in flow rate -Piperine

Flow rate	Peak Area	Tailing factor	Flow rate	Peak Area	Tailing factor	Flow rate	Std Area	Tailing factor
0.8ml/m	318456	1.145	**1ml/m**	322654	1.110	**1.2ml/m**	326844	1.123
	318860	1.153		322865	1.134		326598	1.135
	318078	1.142		322554	1.120		326870	1.145
	318679	1.133		322784	1.148		326487	1.139
	318522	1.144		322326	1.139		326602	1.130
	318974	1.129		322680	1.128		326395	1.136
Avg	318594		**Avg**	322643		**Avg**	326632	
SD	320.268		**SD**	189.241		**SD**	190.176	
%RSD	0.100		**%RSD**	0.058		**%RSD**	0.058	

CONCLUSION

A Rapid and Precise Reverse Phase High Performance Liquid Chromatographic method has been developed and validated for the estimation of curcumin and piperine in its pure form as well as in tablet dosage form. Chromatography was carried out on Inertsil-ODS C18 (250 x 4.6mm, 5µm) column. The method was optimized using a mixture of Methanol: Buffer (80:20) as the mobile phase at a flow rate of 1.0ml/min, the detection was carried out at 252nm. The retention times of the curcuminoid and piperine were 3.048 & 4.316 respectively. The method produced linear responses in the concentration range of 20-80µg/ml. The method was precise since the %RSD values of peak areas were found to be below " 2". The % recovery values for both the analytes were found to be “99.7% & 99.9%” indicating the method was accurate. The specificity of the method was assessed by injections of standard, sample and blank solutions separately and the chromatograms were recovered. The LOD values of curcumin and piperine were found to be 0.12 µg/ml, 0.36 µg/ml & LOQ values were found to be 0.14 µg/ml, 0.45 µg/ml respectively.

When compared to the previous methods reported the present method is rapid due to less Rt and economical since the present method consumes a minimum organic phase for elution.

The accountability of the method was assessed and documented by validation as per ICH guidelines. The results of validation were in agreement with acceptance criteria. This indicates that the method is suitable and can be adopted for routine analysis of these analytes as a part of regular quality control analysis.

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