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Research Article

SIMULTANEOUS ESTIMATION OF CURCUMIN AND SILIBININ USING VALIDATED RP-HPLC-PDA METHOD AND ITS APPLICATION IN PHARMACEUTICAL NANOFORMULATION

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ABSTRACT

Objective: The present study was aimed to develop a high performance liquid chromatographic method for simultaneous estimation of curcumin and silibinin and to implement the developed method for the estimation of curcumin and silibinin encapsulated in polymeric nanoparticles.

Methods: Method development for simultaneous estimation of curcumin and silibinin was performed using various solvent, buffer-solvent ratio, flow rate and elution mode. The developed method was validated in accordance with international conference on harmonisation guideline. The developed method was implemented to estimate the amount of curcumin and silibinin encapsulated in Eudragit E 100 nanoparticles.

Results: The optimum chromatographic condition with adequate resolution for curcumin (11.44 minutes) and silibinin (2.92 minutes) was achieved when the separation was carried using **C_{18} column** at a column oven temperature of 35°C with an isocratic elution mode of mobile phase composed of a degassed mixture of 0.1% ortho phosphoric acid and acetonitrile (50:50 v/v) at 1.0 mLmin^{-1} flow rate with a total run time of 15 minutes. The developed method was validated for system suitability, accuracy, precision, limit of detection, limit of quantitation, linearity, range and robustness. The encapsulation efficiency of curcumin (94% & 79%) and silibinin (85% & 73%) was estimated using the developed method.

Conclusion: The developed analytical method is simple, precise, and reproducible and thus can be used for routine analysis of curcumin and silibinin in pharmaceutical formulation.

Keywords: Analytical Method Development, Curcumin, Polymeric Nanoparticles, RP-HPLC-PDA, Silibinin

INTRODUCTION

Curcumin, a hydrophobic polyphenol isolated from powdered rhizomes of turmeric has been studied extensively and found to have wide range of pharmacological activities and exhibits significant therapeutic potential in the treatment of arthritis, atherosclerosis, diabetes mellitus, fever, gastric ulcer, inflammatory bowel disease, lung diseases, malaria, multiple sclerosis, myocardial infarction, osteoporosis, pancreatitis, psoriasis, wound and cancer [1-5]. In particular, curcumin inhibits the cancer cell proliferation, invasion, angiogenesis and metastasis through various mechanism and effective against wide spectrum of cancers [2, 6]. Besides its therapeutic potential, curcumin is well tolerated in humans up to 12,000 mg/day for 3 months and has been declared as 'generally regarded as safe' by United States Food and Drug Administration [1, 2, 7].

In spite of its therapeutic efficacy and safety, the clinical usefulness of curcumin in the treatment of cancer is limited due to its poor aqueous solubility, rapid clearance from the systemic circulation, intestinal glucuronidation, and lack of cancer cell targeting [1. 2]. To overcome these limitations, we have prepared curcumin and silibinin encapsulated Eudragit E 100 nanoparticles [2, 8]. However, the amount of curcumin and silibinin encapsulated in Eudragit E 100 nanoparticles determines the effectiveness of the prepared nanoformulations [9]. Therefore, it is mandatory to estimate the amount of curcumin and silibinin encapsulated in the nanoparticles. However, analytical technique for simultaneous estimation of curcumin and silibinin was not yet reported. Hence, the present study was aimed to develop a simple, precise, and reproducible high performance liquid chromatographic (HPLC) method for simultaneous estimation of curcumin and silibinin, to validate the developed method in accordance with international conference on harmonisation (ICH) guideline and to implement the developed method for simultaneous estimation of curcumin and silibinin encapsulated in polymeric nanoparticles.

MATERIALS AND METHODS

Materials

Silibinin (≥98%) and Poloxamer 188 were purchased from Sigma Aldrich (Bangalore, India). Cationic polymer Eudragit E 100 was obtained from Degussa (India). Curcumin (97%) and β-cyclodextrin were purchased from Himedia Laboratories (Mumbai, India). Analytical grade ethanol was purchased from Brampton (Ontario, Canada), Analytical grade ortho phosphoric acid, methanol, acetonitrile, triethylamine and water were purchased from Merck (Mumbai, India). Sodium hydroxide was purchased from Merck (Mumbai, India).

Preparation of curcumin and silibinin standard stock solution

Standard stock solution of curcumin was prepared in methanol (1 mgmL^{-1}) and silibinin was prepared by dissolving initially in 0.1N sodium hydroxide and the volume was made up with methanol (1 mgmL^{-1}). The stock solutions were stored away from light at 4°C.

Instrumentation, method development and validation

Analyses were carried out using an Alliance® HPLC (Waters) equipped with pump, degasser, photo diode array (PDA) detector and auto-sampler. The generated analytical signals were monitored and integrated using Empower™ chromatography data software. The chromatographic separation was performed using C_{18} column (Reversed Phase, 150 mm x 4.6 mm with 5 micron particle size, Phenomenex Luna) at a column oven temperature of 35°C. The method development for simultaneous estimation of curcumin and silibinin was performed using (a) Various solvent [i.e. acetonitrile and methanol]; (b) Various buffer-solvent ratio [i.e. solvent A (0.1%v/v ortho phosphoric acid aqueous solution) and solvent B (acetonitrile) at the ratio of 40:60, 50:50, 60:40 and 70:30]. 0.1%v/v ortho phosphoric acid aqueous solution was prepared by adding 1mL of ortho phosphoric acid in 1000 mL beaker and about 900 mL of Milli-Q water was added, which was sonicated for 5 minutes and volume was made up to 1000 mL to get 0.1%v/v ortho phosphoric acid and the pH was adjusted to 3.0 by Triethylamine; (c) Various flow rate [i.e. 0.8, 1.0 and 1.2 mLmin^{-1}]; and (e) Various elution mode [i.e. isocratic and gradient]. The developed method was validated for system suitability, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), linearity, range and robustness in accordance with ICH guideline [9-13].

System suitability of the developed method

System suitability parameters were studied to verify the system performance. Six replicate quality control (QC) samples containing curcumin (100µgmL^{-1}) and silibinin (100µgmL^{-1}) were analysed using the developed method. Factor such as theoretical plate count, tailing factor, percentage relative standard deviation (%RSD) of peak area and retention time was taken in to consideration for testing system suitability.

Accuracy of the developed method

The accuracy of the developed method was established using recovery studies. Three replicate QC samples containing known amount of curcumin and silibinin at 50, 100 and 150µgmL^{-1} were spiked in to pre-analysed samples (100µgmL^{-1} of curcumin and 100µgmL^{-1} of silibinin), which were analysed using the developed method and the percentage recovery was taken in to consideration for testing accuracy.

Precision of the developed method

Precision of the developed method was studied using six replicate QC samples containing curcumin (100µgmL^{-1}) and silibinin (100µgmL^{-1}), which were analysed using the developed method within a short period of time on the same day and repeatability was taken in to consideration for testing precision.

Limit of detection and limit of quantitation of the developed method

LOD and LOQ of curcumin and silibinin in the developed method were studied using the signal-to-noise ratio of 3 for estimating LOD and 10 for estimating LOQ.

Linearity and range of the developed method

Linearity was evaluated at five concentration levels in the range between 10-150 µgmL^{-1} for curcumin and silibinin. The concentration was then plotted against its corresponding peak area and the linearity was determined using least square regression analysis. The analytical range was established by the highest and lowest concentrations of analyte where acceptable linearity was obtained.

Robustness of the developed method

The robustness of a method is a measure of its ability to remain unaffected by negligible but deliberate change to the chromatographic condition. Analyses were performed with known concentration of curcumin (100µgmL^{-1}) and silibinin (100µgmL^{-1}) using the developed method with negligible but deliberate change in the buffer-solvent ratio, flow rate, column temperature and significant change in the retention time, peak area, and tailing factor were taken in to consideration for testing robustness.

Preparation of curcumin and silibinin encapsulated Eudragit E 100 nanoparticles

Curcumin and silibinin encapsulated Eudragit E 100 nanoparticles were prepared by nanoprecipitation methods [9] with slight modifications.

Preparation of curcumin and silibinin encapsulated Eudragit E 100 nanoparticles using sonication approach**.** Briefly, curcumin and silibinin along with Eudragit E 100 were added to ethanol and vortexed to dissolve, which was then emulsified with aqueous phase containing poloxamer 188 and β-cyclodextrin under sonication for 10 minutes to form nanoparticles and the sonication process was further continued up to 1 hour to remove the residual organic solvent present in the nanoformualtion.

Preparation of curcumin and silibinin encapsulated Eudragit E 100 nanoparticles using mechanical stirring approach. Briefly, curcumin and silibinin along with Eudragit E 100 were added to ethanol and vortexed to dissolve, which was then emulsified with aqueous phase containing poloxamer 188 and β-cyclodextrin under mechanical stirring for 10 minutes to form nanoparticles and the stirring process was further continued up to 3 hours to remove the residual organic solvent present in the nanoformualtion.

Estimation of curcumin and silibinin encapsulated in Eudragit E 100 nanoparticles

Amount of curcumin and silibinin encapsulated in nanoparticles were estimated indirectly by measuring the free curcumin and silibinin in the nanoformulations [9, 14]. Prepared curcumin and silibinin encapsulated Eudragit E 100 nanoformulation was centrifuged for 45 minutes at 19,000 rpm and the supernatant was separated. To 1 mL of supernatant, equal volume of methanol was added and sonicated followed by filtration through 0.22 µm membrane and analysed using the developed method. The amount of curcumin and silibinin encapsulated in nanoparticles were estimated and encapsulation efficiency (EE) was calculated as follows EE = [(Total amount of drug added to the formulation) – (Total amount of drug in the supernatant)] / (Total amount of drug added to the formulation) x 100.

RESULTS AND DISCUSSION

Method Development

The optimum wavelength for the estimation of curcumin and silibinin was 292 nm, which was selected based on the maximum area using photo diode array detector. To get an optimum resolution between curcumin and silibinin, we have tried various ratio of buffer-solvent ratio (40:60, 50:50, 60:40 and 70:30). However, combination of 0.1% ortho phosphoric acid and acetonitrile at 50:50 v/v has shown good resolution between curcumin at 11.44 minutes and silibinin at 2.92 minutes (Fig.1) but further increase in the ratio of buffer from 50 to 70 has shown a shift in retention time of silibinin from 2.92 minutes to 14.21 minutes and decrease in the ratio of buffer from 50 to 40 has not shown peak for silibinin. Similarly, change of solvent from acetonitrile to methanol has not shown any peak for both curcumin and silibinin. Initially, we have tried 1.0 mLmin^{-1} flow rate but further increase or decrease in flow rate has not shown adequate separation between curcumin and silibinin. Change of elution mode from isocratic to gradient has shown a shift in retention time from 2.92 minutes to 7.2 minutes for silibinin and from 11.44 minutes to 10.51 minutes for curcumin. However, the optimum chromatographic condition with adequate resolution for curcumin and silibinin was achieved when the separation was carried using C_{18} column (Reversed Phase, 150 mm x 4.6 mm with 5 micron particle size, Phenomenex Luna) at a column oven temperature of 35°C with an isocratic elution mode of mobile phase composed of a degassed mixture of 0.1% ortho phosphoric acid and acetonitrile (50:50 v/v) at 1.0 mLmin^{-1} flow rate with a total run time of 15 minutes.

System suitability of the developed method

The developed method has produced theoretical plate above 3000 for both curcumin and silibinin with tailing factor less than 1.5 for both curcumin and silibinin. Similarly, the %RSD of peak area and retention time of both curcumin and silibinin were less than 2, which ensures the suitability of the developed method. The results of system suitability study were summarized in table 1.

Accuracy of the developed method

The percentage recovery of the spiked curcumin and silibinin were well within 100±2% and %RSD of assay was less than 2%, which ensures the accuracy of the developed method. The results of recovery studies were summarized in table 2.

Precision of the developed method

The developed method has shown <1.3 %RSD of peak area, tailing factor, retention time and assay, which ensures precision of the developed method. The results of precision study were summarized in table 3.

Limit of detection and limit of quantitation of the developed method

The LOD were 0.3 ppm for curcumin and 0.9 ppm for silibinin at a signal-to-noise ratio of 3. LOQ were 0.4 ppm for curcumin and 1.1 ppm for silibinin at a signal-to-noise ratio of 10.

Linearity and range of the developed method

The correlation coefficient (R^2) of curcumin and silibinin were around 0.999, which confirms its linearity in range of 10-150 µgmL^{-1}. The results of linearity were summarized in table 4.

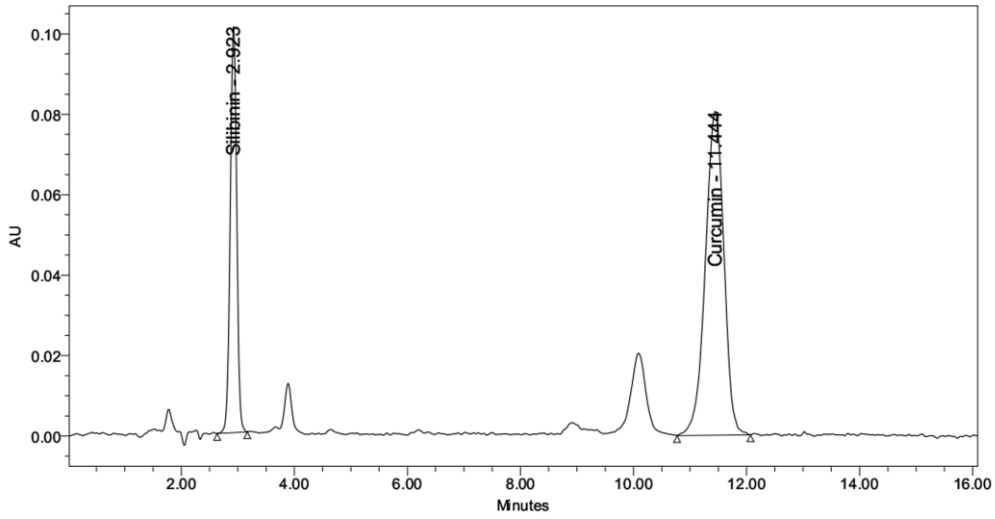

Fig. 1: Chromatogram of curcumin and silibinin using optimized chromatographic condition

Table 1: System suitability study of the developed method

Sample	Peak Area		Retention Time		Tailing Factor		Plate Counts	
	Curcumin	Silibinin	Curcumin	Silibinin	Curcumin	Silibinin	Curcumin	Silibinin
1	1740664	801350	11.93	2.98	0.95	1.00	6633	3614
2	1762741	795341	11.52	2.96	0.96	0.99	6733	3723
3	1771444	805490	11.95	2.97	0.96	1.01	6705	3747
4	1734753	793669	11.91	2.97	0.95	1.03	6859	3766
5	1726158	798344	11.85	2.97	0.97	1.03	7057	3742
6	1716268	802400	11.55	2.95	0.98	1.04	7280	3756
Average	1742005	799432	11.78	2.97	0.96	1.02	6878	3725
%RSD	1.22	0.56	1.67	0.35	-	-	-	-

Acceptance criteria: Plate Counts >3000, tailing <1.5, Peak area and retention time <2%RSD

Table 2: Recovery studies of the developed method

Analyte	Level	Sample	Spiked (µgmL^{-1})	%Recovery	%RSD
Curcumin	50%	1	50	100.44	0.34
		2	50	100.25	
		3	50	099.78	
	100%	1	100	099.56	0.98
		2	100	099.57	
		3	100	101.27	
	150%	1	150	101.69	1.42
		2	150	099.88	
		3	150	098.89	
Silibinin	50%	1	50	100.79	0.80
		2	50	099.98	
		3	50	101.60	
	100%	1	100	099.19	0.10
		2	100	099.12	
		3	100	098.99	
	150%	1	150	099.33	0.11
		2	150	099.37	
		3	150	099.17	

Acceptance criteria: Recovery within 100±2% with <2% RSD

Table 3: Precision study of the developed method

Sample	Peak Area		Retention Time		Tailing		Assay	
	Curcumin	Silibinin	Curcumin	Silibinin	Curcumin	Silibinin	Curcumin	Silibinin
1	1725171	807689	11.47	2.92	0.98	1.04	98.24	99.82
2	1732096	824266	11.81	2.97	0.97	1.08	98.64	101.87
3	1730984	801945	11.68	2.97	0.96	1.06	98.57	99.11
4	1736558	805460	11.81	2.96	0.98	1.06	98.89	99.55
5	1747832	807553	11.78	2.96	0.97	1.06	99.53	99.80
6	1732850	808420	11.73	2.95	0.98	1.07	98.68	99.91
Average	1734249	809222	11.71	2.96	0.97	1.06	98.76	100.01
%RSD	0.44	0.96	1.10	0.65	0.84	1.25	0.44	0.96

Acceptance Criteria: %RSD <2

Robustness of the developed method

Slightly variation in buffer-solvent ratio, flow rate, and column temperature has not shown any significant changes in validation parameter. However, major deliberate variations have shown significant effect on retention time, peak area and tailing factor.

Table 4: Linearity and range of the developed method

Parameter	Curcumin	Silibinin
Correlation Coefficient (R^2)	0.9997	0.9993
Equation	Y = 8036x - 3645.9	Y = 14991x + 25184
Range (μgmL^{-1})	10-150	10-150

Estimation of curcumin and silibinin encapsulated in Eudragit E 100 nanoparticles

Developed method was successfully implemented in the estimation of curcumin and silibinin encapsulated in Eudragit E 100 nanoparticles. The encapsulation efficiency of curcumin was around 93% in sonication approach and around 79% in mechanical stirring approach. Similarly, the encapsulation efficiency of silibinin was around 85% in sonication approach and around 73% in mechanical stirring approach. The results are summarized in table 5.

Table 5: Encapsulation efficiency of curcumin and silibinin in Eudragit E 100 nanoparticles

Parameters	Curcumin		Silibinin	
	Sonication	Stirring	Sonication	Stirring
Amount found (mg)	4.67	3.96	4.25	3.66
Assay (%)	94	79	85	73

CONCLUSIONS

In present study, a simple, precise, and reproducible HPLC method has been developed for simultaneous estimation of curcumin and silibinin. The developed method was validated as per ICH guideline. The developed method was successfully implemented in the estimation of curcumin and silibinin encapsulated in polymeric nanoparticles. Thus developed method can be used for routine analysis of curcumin and silibinin in pharmaceutical dosage forms.

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