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Research Article

REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR SIMULTANEOUS ESTIMATION OF CURCUMIN AND QUERCETIN 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 quercetin and to implement the developed method for the estimation of curcumin and quercetin encapsulated in polymeric nanoparticles.

Methods: Method development was carried out with different column, flow rate, elution mode, and buffer-solvent ratio to obtain adequate separation, resolution and symmetrical peak of curcumin and quercetin. 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 quercetin encapsulated in Eudragit E 100 nanoparticles.

Results: The optimum chromatographic condition with adequate resolution for curcumin (10.276 minutes) and quercetin (2.501 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 1.2 $mLmin^{-1}$ 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 (79% & 81%) and quercetin (91% & 98%) 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 quercetin in pharmaceutical formulation.

Keywords: Method Development, Curcumin, Polymeric Nanoparticles, Quercetin, RP-HPLC-PDA.

INTRODUCTION

Curcumin, a hydrophobic polyphenol isolated from *Curcuma Longa* L has been classified as a functional food due to its diverse pharmacological activities including anti-cancer activity. Curcumin is considered as a better alternative for cytotoxic chemotherapeutic agents with severe systemic toxicities as it 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 and exhibit significant anti-cancer potential including up-regulation of p16, p53, p14ARF, CDK inhibitors, Bax, Bad, Bim, lysosomal proteases, phosphatases, lipases and down-regulation of Bcl-2, Bcl-xL, NF-κB, mTOR, VEGF, COX-2, MMP. In spite of its safety and efficacy, the clinical usefulness of curcumin in the treatment of cancer is limited due to certain limitations including poor aqueous solubility, rapid clearance from the systemic circulation, intestinal glucuronidation, and lack of cancer cell targeting. Hence, we have prepared curcumin and quercetin loaded Eudragit E 100 nanoparticles to overcome the limitations of curcumin in the treatment of cancer [1-10]. However, the total amount of curcumin and quercetin encapsulated in the nanoparticles determines the efficacy of the nanoformulation. Therefore, it is mandatory to estimate the amount of curcumin and quercetin encapsulated in the nanoparticles. However, analytical technique for simultaneous estimation of curcumin and quercetin 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 quercetin, 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 quercetin encapsulated in polymeric nanoparticles.

MATERIALS AND METHODS

Materials

Eudragit E 100 was obtained from Degussa, India. Curcumin (97%) and Beta cyclodextrin were purchased from Himedia Laboratories, India. Quercetin (98%) and Poloxamer 188 were purchased from Sigma-Aldrich, India. Analytical grade methanol, acetonitrile, triethylamine and water were purchased from Merck, India. Analytical grade ortho phosphoric acid was purchased from Rankem, India. HPLC grade ethanol was purchased from Brampton, Canada.

Preparation of curcumin and quercetin standard stock solution

Standard stock solution of curcumin and quercetin were prepared in methanol (1 $mgmL^{-1}$) and stored away from light at 4°C.

Instrumentation and analytical method development

Analyses were performed using an Alliance® HPLC (Waters Corp.) equipped with pump, degasser, photodiode array detector, autosampler and the generated signals were monitored and integrated using Empower™ chromatography software. Method development was carried out with different column, flow rate, elution mode, buffer-solvent ratio to obtain adequate separation, resolution and symmetrical peak of curcumin and quercetin. 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.

System suitability parameters were studied to verify the system performance. Six replicate quality control (QC) samples containing curcumin (100μgmL^{-1}) and quercetin (100μgmL^{-1}) were analysed using the developed method. However, theoretical plate count >3000, tailing <1.5, and <2% relative standard deviation (RSD) of peak area and the retention time were considered acceptable.

The accuracy of the developed method was established using recovery studies. Three replicate QC samples containing know amount of curcumin and quercetin at 50, 100 and 150 μgmL^{-1} were added to the pre-analysed samples (100μgmL^{-1} of curcumin and 100μgmL^{-1} of quercetin) and analysed using the developed method. However, percentage recovery within 100±2% and <2% RSD of assay were considered acceptable.

Precision of the developed method was studied using **s**ix replicate QC samples containing curcumin (100µgmL^{-1}) and quercetin (100µgmL^{-1}) were analysed within a short period of time on the same day using the developed method. However, <2% RSD of peak area, plate count, retention time and assay were considered acceptable.

Limit of detection and limit of quantitation of curcumin and quercetin in the developed method were estimated from the signal-to-noise ratio. Signal-to-noise ratio of 3 for estimating LOD and 10 for estimating LOQ were considered acceptable.

Linearity was evaluated at five concentration levels (10, 25, 50, 100 and 150 µgmL^{-1}) of curcumin and quercetin. The linearity was then determined by the least square regression analysis from the peak area against drug concentration plot. The range of the developed method was established by the highest and lowest concentrations of drug where acceptable linearity was obtained.

The robustness refers to its ability to remain unaffected by small but deliberate change to the chromatographic condition which provides an indication of its reliability during normal usage. Analyses were carried out with know concentration of curcumin (100µgmL^{-1}) and quercetin (100µgmL^{-1}) using the developed method with slight 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 [11-15].

Fabrication of curcumin and quercetin encapsulated Eudragit E 100 nanoparticles

Curcumin and quercetin encapsulated Eudragit E 100 nanoparticles were prepared by nanoprecipitation methods [16] with slight modifications.

Preparation of curcumin and quercetin encapsulated Eudragit E 100 nanoparticles using sonication approach**.** Briefly, curcumin and quercetin 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 quercetin encapsulated Eudragit E 100 nanoparticles using mechanical stirring approach. Briefly, curcumin and quercetin 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.

Amount of curcumin and quercetin encapsulated in nanoparticles were estimated indirectly by measuring the free curcumin and quercetin in the nanoformulations. Prepared curcumin and quercetin 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 quercetin 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 and validation

The optimum wavelength for the estimation of curcumin and quercetin was 292 nm, which was selected based on the maximum area using photo diode array detector. Method development for the simultaneous estimation of curcumin and quercetin was carried out with different column. However, Luna C_{18} column has shown adequate separation and symmetrical peak in comparison with Hibar column, Kromasil column and Eclipse XDB column. Different buffer-solvent ratio (80:20, 60:40 and 50:50) were tried but the mixture of 0.1%v/v ortho phosphoric acid and acetonitrile at 50:50 proportions has shown adequate separation and peak symmetrical for curcumin and quercetin. However, further increase in proportion of 0.1%v/v ortho phosphoric acid does not exhibit adequate separation between curcumin and quercetin. Initially, 0.8 ml flow rate was used but increase in flow rate from 0.8 to 1.2 ml has shown adequate separation and high theoretical plate count. Similarly, isocratic elution mode has shown better separation in comparison with gradient elution mode.

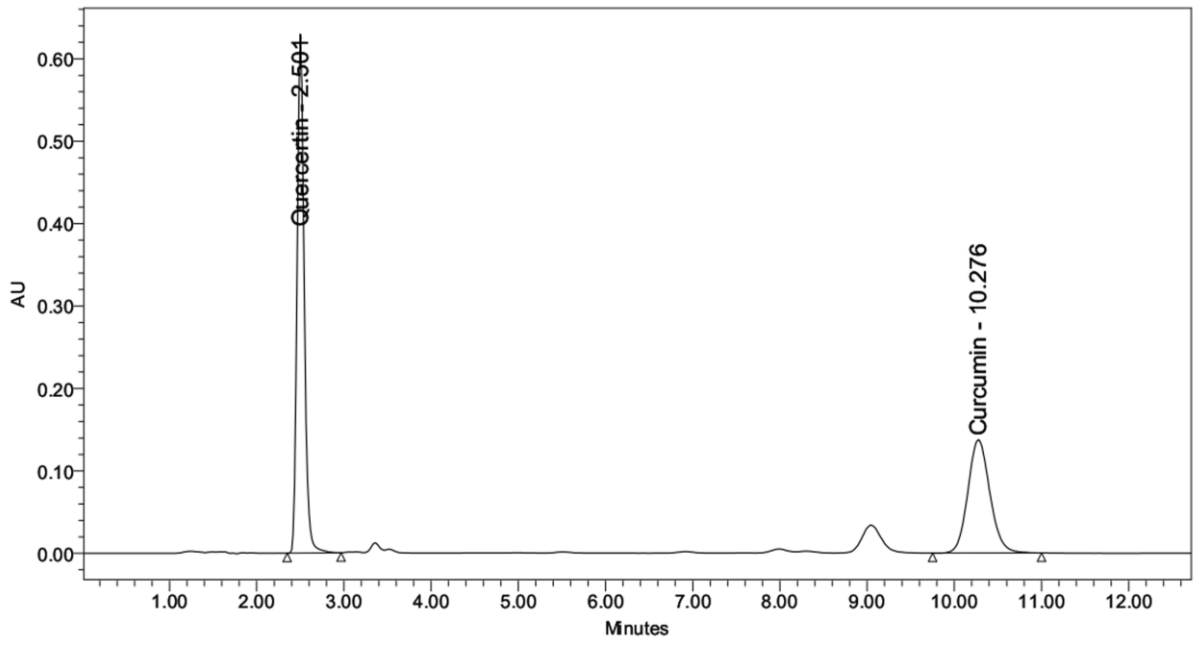

Fig. 1: Chromatogram of curcumin and quercetin using optimized chromatographic condition with adequate separation of curcumin and quercetin

However, after extensive preliminary experimental trials, the best chromatographic conditions to obtain adequate separation, resolution and symmetrical peaks were achieved when 20 µL of sample injected in to a Luna C_{18} column (Reversed Phase, 150 mm x 4.6 mm with 5 micron particle size, Phenomenax) 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 aqueous solution and acetonitrile (50:50v/v) at 1.2 $mLmin^{-1}$ flow rate. Figure 1 shows a typical chromatogram of curcumin and quercetin indicating complete resolution of curcumin at 10.276 minute and quercetin at 2.501 minute.

System suitability study has shown the theoretical plate count in the range of 7922-8197 for curcumin and 4389-4638 for quercetin. The tailing factor was in the range of 1.10-1.11 for curcumin and 1.15-1.18 for quercetin. Similarly, the %RSD of peak area and retention time of both curcumin and quercetin were <0.6 and <1.7, respectively, which ensure the suitability of the developed method and the results are summarized in table 1.

Accuracy study has shown a percentage recovery in the range of 98.17-100.29% for curcumin and 98.19-101.64% for quercetin, which ensure the accuracy of the developed method and the results are summarized in table 2.

Precision study has shown the %RSD of peak area, plate count, retention time and assay was less than 1.8%, which ensure precision of the developed method and the results are summarized in table 3.

Table 1: Summary of system suitability study of the developed method

Sample	Peak Area		Plate Counts		Tailing		Retention Time	
	Curcumin	Quercetin	Curcumin	Quercetin	Curcumin	Quercetin	Curcumin	Quercetin
1	2438107	3597398	7922	4485	1.11	1.18	10.16	2.48
2	2416665	3567769	8059	4445	1.10	1.18	10.14	2.49
3	2437362	3584621	7922	4448	1.11	1.18	10.08	2.48
4	2449319	3599775	8054	4511	1.11	1.18	09.91	2.45
5	2440859	3593069	8197	4638	1.11	1.17	10.01	2.48
6	2415364	3551967	7969	4389	1.10	1.15	09.73	2.43
Average	2432946	3582433	8021	4486	1.11	1.17	10.01	2.47
SD	13791	18899	106	85	0.01	0.01	0.16	0.02
%RSD	0.57	0.53	1.32	1.90	0.47	1.03	1.64	0.83

Table 2: Summary of recovery study of the developed method

Analyte	Level	Sample	Spiked (μgmL^{-1})	Recovered (μgmL^{-1})	%Recovery	%RSD
Curcumin	50%	1	50	50.04	100.07	0.11
		2	50	50.07	100.15	
		3	50	50.15	100.29	
	100%	1	100	98.17	98.17	0.89
		2	100	99.86	99.86	
		3	100	99.42	99.42	
	150%	1	150	148.59	99.06	0.72
		2	150	148.03	98.68	
		3	150	150.11	100.07	
Quercetin	50%	1	50	50.82	101.64	1.47
		2	50	49.45	98.89	
		3	50	49.68	99.35	
	100%	1	100	99.04	99.04	0.17
		2	100	99.38	99.38	
		3	100	99.25	99.25	
	150%	1	150	147.96	98.64	0.74
		2	150	147.29	98.19	
		3	150	149.45	99.63	

Table 3: Summary of precision study of the developed method

Sample	Peak Area		Plate Counts		Retention Time		Assay	
	Curcumin	Quercetin	Curcumin	Quercetin	Curcumin	Quercetin	Curcumin	Quercetin
1	2412276	3646635	7749	4515	09.95	2.46	99.15	101.79
2	2425803	3610050	8005	4446	10.30	2.50	99.71	100.77
3	2426896	3631731	8135	4538	10.23	2.50	99.75	101.38
4	2452627	3615222	7984	4387	10.07	2.47	100.81	100.92
5	2411060	3651579	8112	4561	10.01	2.47	99.10	101.93
6	2420952	3601335	8087	4504	09.93	2.45	99.51	100.53
Average	2424936	3626092	8012	4492	10.08	2.47	99.67	101.22
SD	15100	20454	142	64	0.15	0.02	0.62	0.57
%RSD	0.62	0.56	1.77	1.43	1.49	0.82	0.62	0.56

Table 4: Linearity and range of the developed method

Parameter	Curcumin	Quercetin
Correlation Coefficient (R^2)	0.9991	0.9992
Equation	Y = 25908x + 51003	Y = 38021x + 96758
Range (μgmL^{-1})	10-150	10-150

The LOD were 0.05 ppm for curcumin and 0.1 ppm for quercetin at a signal-to-noise ratio of 3. LOQ were 0.2 ppm for curcumin and 0.3 ppm for quercetin at a signal-to-noise ratio of 10. The correlation coefficient of both curcumin and quercetin were 0.999, which confirms its linearity in the range of 10-150 μgmL^{-1}. The results are summarized in table 4.

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.

Estimation of curcumin and quercetin encapsulated in Eudragit E 100 nanoparticles

Developed method was implemented in the estimation of curcumin and quercetin encapsulated in Eudragit E 100 nanoparticles. The encapsulation efficiency of curcumin was around 79% in sonication approach and around 81% in mechanical stirring approach. Similarly, the encapsulation efficiency of quercetin was around 91% in sonication approach and around 98% in mechanical stirring approach. The results are summarized in table 5.

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

Parameters	Curcumin		Quercetin	
	Sonication	Stirring	Sonication	Stirring
Amount found (mg)	3.97	4.08	4.57	4.92
Assay (%)	79	81	91	98

CONCLUSIONS

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

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