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Original Article

ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF CURCUMIN AND CYCLOSPORINE BY RP-HPLC

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ABSTRACT

Objective: The present work was undertaken with an aim to develop and validate a rapid reverse-phase high-performance liquid chromatography (RP-HPLC) method for the estimation of curcumin and cyclosporine in the capsule dosage form.

Methods: The RP-HPLC method for the simultaneous estimation of curcumin and cyclosporine was developed using Agilent (Infinity 1260) HPLC system and Eclipse XDB-C18 (4.6 x 150 mm i.d., 5µ) stationary phase. The optimized mobile phase comprised of acetonitrile: water: methanol (50: 10: 40 v/v/v) pumped at a flow rate of 0.5 ml/min. Separation of drugs was achieved in an isocratic mode and elution was monitored using PDA detector at 214 nm. The method was validated as per ICH-Q2R1 guidelines.

Results: Retention time of the curcumin and cyclosporine were found to be 3.073 min and 6.373 min with the correlation coefficient (R^2) of 0.9993 and 0.998 respectively. The response of curcumin and cyclosporine was found linear in the concentration range of 8-48 µg/ml and 4-24 µg/ml respectively. The percent recovery values were found in the range of 97-103% indicating satisfactory accuracy of the method. The percent relative standard deviation (% RSD) values for the precision study was less than 2 which suggest that the method is precise.

Conclusion: The proposed method was found accurate, precise and specific for the determination of curcumin and cyclosporine in bulk as well as in capsule dosage form. Thus, the present method can be used for routine analysis and quality control of curcumin and cyclosporine in bulk and capsule dosage form.

Keywords: Curcumin, Cyclosporine, ICH Guidelines, RP-HPLC

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INTRODUCTION

Chemically, curcumin is (1E, 6E)-1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione [1]. Literature survey revealed LC-MS [2], RP-HPLC [2], HPTLC [3], stability indicating HPLC [4], and UV spectrophotometric [1, 5-7] methods for estimation of curcumin alone or in combination with other drugs in bulk, in dosage forms and human plasma. Cyclosporine is an immunosuppressant drug used in post allogenic organ transplant to reduce the activity of patient's immune system. Chemically it is (3S,6S,9S,12R,15S,18S,21S,24S,30S,33S)-30-ethyl-33-[(E,1R,2R)-1-hydroxy-2-methylhex-4-enyl]-1,4,7,10,12,15,19, 25,28-nonamethyl-6,9,18,24-tetrakis (2-methylpropyl)-3,21-di (propan-2-yl)-1,4,7,10,13,16,19,22,25,28,31-undecazacyclotritriacontane-2,5,8, 11,14,17, 20, 23, 26, 29, 32-undecone [8]. Several bioanalytical methods which include RP-HPLC [8], LC-MS [8] and RP-HPLC [8] were also reported for the analysis of cyclosporine in soft gelatin capsule dosage [5]. The molecular structures of curcumin and cyclosporine is given in fig. 1.

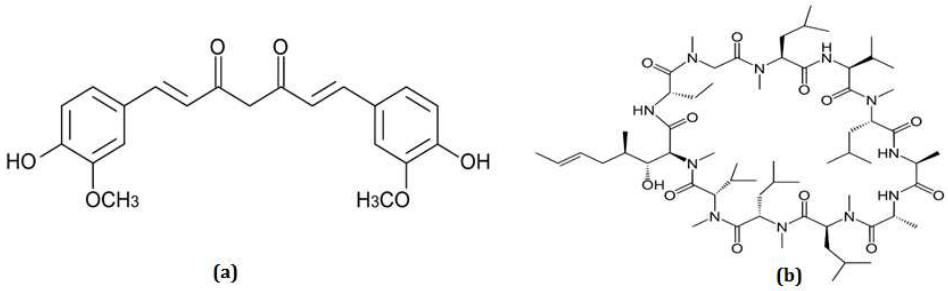


Fig. 1: Molecular structure of (a) curcumin and (b) cyclosporine

Till date, RP-HPLC method was not developed for the simultaneous estimation of curcumin and cyclosporine in the pharmaceutical dosage form. Therefore, the present study was attempted to develop a simple, accurate, precise and specific RP-HPLC method for quantitative determination of curcumin and cyclosporine in capsule dosage form for management of inflammatory bowel disorder (IBD). There is no marketed formulation of curcumin and cyclosporine (pellets in a capsule or any other pharmaceutical dosage form) for the management of IBD. The literature states that curcumin and cyclosporine, when used in combination, can successfully manage the remission of ulcerative colitis [9]. Therefore, a fixed-dose combination capsule formulation containing bioadhesive pellets of

curcumin and cyclosporine was developed in-house. The developed and optimized formulation of bioadhesive pellets containing curcumin and cyclosporine was selected for *in vitro* drug release study.

MATERIALS AND METHODS

Curcumin and cyclosporine were procured from Otto Chemie Pvt. Ltd., Mumbai, India and Concord Biotech Limited Ahmedabad, India respectively. HPLC grade acetonitrile and methanol were procured from SD Fine Chemical Ltd., Mumbai, India. Millipore water was used for the preparation of chemicals/solutions.

HPLC instrumentation and chromatographic conditions

Analytical chromatography was performed on a Agilent HPLC system (Infinity 1260) equipped with auto-sampler and photo diode array (PDA) detector. Chromatographic separation of curcumin and cyclosporine was achieved on Eclipse XDB-C18 (4.6 x 150 mm, 5 µ particle size) column at a temperature of 70 °C±0.02 °C. The mobile phase comprised of acetonitrile: water: methanol (50:10:40 v/v/v). The flow rate was at 0.5 ml/min., drug peaks were detected at 214 nm with an injection volume of 20 µl.

Preparation of standard solutions

Preparation of stock solution

Stock solutions were prepared by dissolving 10 mg of curcumin and 10 mg of cyclosporine in 10 ml of methanol separately and subjected to sonication to give an individual concentration of 1000 µg/ml.

Method development

Aliquots of the standard stock solutions were further diluted with mobile phase to get 24 µg/ml of curcumin and 12 µg/ml of cyclosporine. The samples were injected in the HPLC system and chromatograms were recorded. Various combinations of mobile phase components, column temperature, and columns were tried to get desirable resolved peaks.

Method validation

System suitability

The system suitability test is used to verify that the chromatographic system is suitable for the intended analysis or not. The system suitability of the method was checked by injecting six different injections of 24 µg/ml of curcumin and 12 µg/ml of cyclosporine. Various parameters like tailing factor, theoretical plates, peak area and resolution were checked according to USP criteria [10, 11].

Specificity

Specificity is the ability to measure accurately and specifically the analyte of interest in the presence of other components that may be expected to be present in the sample matrix. Specificity was carried by dissolving 500 mg of placebo sample (pellets made up of excipients viz. avicel PH 101, carbopol 940, hydroxypropyl cellulose-H and sodium chloride excluding both the drugs) in 100 ml of mobile phase.

Precision

Precision was studied at three levels: repeatability, intermediate precision, and reproducibility.

Repeatability: Repeatability study was performed by preparing a minimum of six determinations (n=6) of test concentration (curcumin 24 µg/ml: cyclosporine 12 µg/ml) and was analyzed. It is expressed as the percent relative standard deviation (% RSD).

Intermediate Precision: The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. This study was done at two levels (intra-day and inter-day study). Intra-day precision was done by preparing the test concentration (curcumin 24 µg/ml and cyclosporine 12 µg/ml) and assaying for six times (n=6) at 3 different time intervals. Inter-day precision was done by analyzing samples (n=6) for three consecutive days. The percent relative standard deviation (% RSD) values were calculated.

Accuracy

This study was carried out by spiking standard solution of curcumin and cyclosporine to the placebo. Placebo was prepared containing avicel PH 101, carbopol 940, hydroxypropyl cellulose-H and sodium chloride which were further coated with Eudragit S100 as enteric coating polymer using isopropyl alcohol and acetone in the ratio of 1:1 [12-15]. This placebo batch was selected for the accuracy studies since marketed formulation containing both the drugs was not available. Concentrations of curcumin and cyclosporine corresponding to 80%, 100%, and 120% were added to 1 ml solution of pre-analyzed placebo formulation and percent recovery of drug-spiked was determined. The study was performed in triplicates and mean % RSD was determined.

Linearity and range

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. It is generally reported as the variance of slope or regression line. A range is an interval between the upper and lower concentrations of solute that have been demonstrated to be determined with precision, accuracy, and linearity. To establish the linearity of this method 8-48 µg/ml for curcumin and 4-24 µg/ml for cyclosporine was prepared from the stock solution and analysed. Each concentration measurement was performed in triplicate. Peak areas were plotted versus respective concentrations and linear regression analysis was performed on the resultant curves.

LOD and LOQ

Limit of detection (LOD) and limit of quantification (LOQ) decide the sensitivity of the method. LOD is the lowest detectable concentration of the analyte by the method while LOQ is the minimum quantifiable concentration. ICH defines the detection limit of an individual analytical procedure as the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The LOD is the point at which a measured value is larger than the uncertainty associated with it. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices and is used particularly for the determination of impurities or degradation products. LOD and LOQ were calculated from standard calibration curves of drugs using following equations-

$$\text{Limit of detection (LOD)} \frac{3.3 \times \sigma}{\text{Slope}}$$

Where σ is standard deviation and slope of the calibration curve of respective drug.

$$\text{Limit of quantification (LOQ)} \frac{10 \times \sigma}{\text{Slope}}$$

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during its normal usage. The test concentration of curcumin and cyclosporine (curcumin 24 µg/ml and cyclosporine 12 µg/ml) were injected by changing the chromatographic conditions. Following changes were made to check the reliability of the method. The study was performed in triplicate.

Variation of flow rate: The chromatographic responses were recorded by a change in the flow rate to 0.4 ml/min to 0.6 ml/min.

Variation in temperature: The chromatograms of curcumin and cyclosporine was measured by a change in the temperature to 69 °C to 71 °C.

Drug content

The bioadhesive pellets were triturated using mortar and pestle and the powder blend equivalent to 30 mg of drug (curcumin 20 mg and cyclosporine 10 mg) was dispersed in 10 ml of distilled water in 10 ml amber colored volumetric flask. The resultant dispersion was filtered after an appropriate interval of time (for the drug to come

into the medium) to give a clear solution. An aliquot of 1 ml was withdrawn from this solution and diluted up to 10 ml. The percent of drug content was determined.

***In vitro* dissolution study**

The *in vitro* release of drug from the bioadhesive pellets was investigated using USP Type 1 dissolution test apparatus (Basket type, Electrolab) at a rotational speed of 100 rpm. 500 mg of pellets in size 0 hard gelatin capsule shell equivalent to 30 mg of drug was placed in the basket. The apparatus was maintained at 37 °C±0.5 °C. The dissolution was carried out in 250 ml of 0.1 N HCl with 2% SLS for 2h followed by 250 ml of phosphate buffer (pH 6.8) with 2% SLS for additional 4 h followed by phosphate buffer 7.4 with 2% SLS for additional 18 h. Aliquot (5 ml) was withdrawn from the dissolution apparatus at specific time intervals of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 24 h, filtered through 0.45-micron syringe filter and analyzed using RP-HPLC. Sink conditions were maintained by replenishing the same amount of medium. The study was performed in triplicate (n=3).

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

A mobile phase composition of acetonitrile: water: methanol (50:10:40 v/v/v/) at a flow rate was at 0.5 ml/min and Eclipse XDB-C18 (4.6 x 150 mm, 5 µ) column maintained at 70 °C±0.02 °C were found optimum for to get reproducible peaks with symmetry with limits. The optimized chromatogram of standard curcumin and cyclosporine (100 µg/ml) is given in fig. 2.

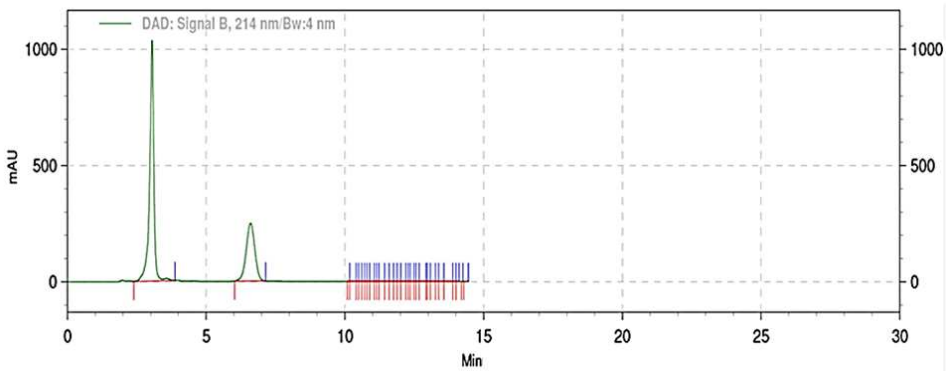


Fig. 2: Chromatogram of standard curcumin and cyclosporine in optimized chromatographic conditions

System suitability

The system suitability parameters like tailing factor were found to be less than 2, theoretical plates were not less than 2000 and resolution was found to be more than 2. All the parameters were fulfilled as per USP acceptance criteria.

The results of system suitability are expressed in table 1.

Table 1: Results of system suitability parameters for curcumin and cyclosporine

Parameters	Curcumin	Cyclosporine	Acceptance criteria
Retention time	3.047±0.05	6.587±0.01	--
Resolution	9.874±0.01		Rs>2
No. of theoretical Plates	2874±0.06	2589±0.04	N>2000
Tailing Factor	0.764±0.02	0.981±0.01	T<1.5
Peak area	63838533±218	1368407±4291	--

*The data is expressed as mean±SD, *n: 6 i.e. number of injections, *SD: standard deviation

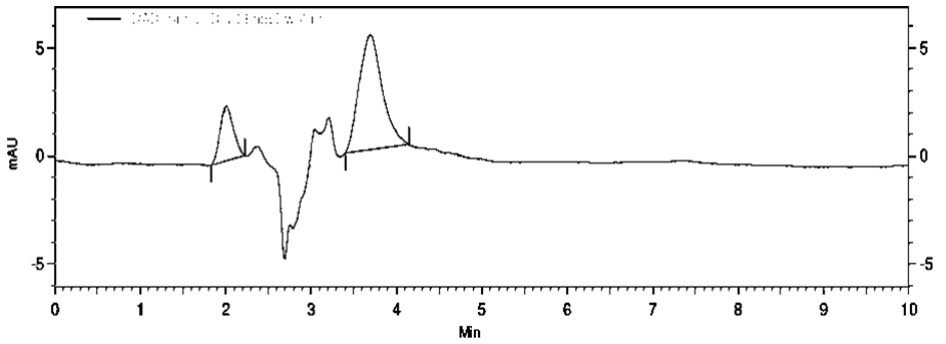


Fig. 3: HPLC chromatogram of placebo (pellets of excipients dissolved in mobile phase)

Specificity

The optimized method was found to be specific as there was no interference of the mobile phase at the retention time of both the drugs. Chromatogram indicating the specificity of the method is shown in fig. 3.

Precision

Repeatability studies, intraday precision, and inter-day precision studies were performed for curcumin and cyclosporine and the % RSD values were found to be<2% for both the drugs. Thus, the

developed method was found to be precise. Results for precision are expressed in table 2.

Table 2: Results of the precision study

Precision parameters		Curcumin	Cyclosporine
Repeatability	Peak area	6408248±5849	1357613±1463
	% RSD	0.9128	1.0782
Intraday study	Peak area: Time 1	6414627	1355923
(n=6)	Peak area: Time 2	6454088	1345019
	Peak area: Time 3	6434833	1345788
	Pooled mean±SD of three-time points	6434516±17649	1348910±5443
	% RSD	0.2742	0.4035
Interday study	Peak area: Day 1	6391294	1351098
(n=6)	Peak area: Day 2	1352103	1352103
	Peak area: Day 3	1362419	1362419
	Pooled mean±SD of three days	6424627±288	1355206±626
	% RSD	0.44931	0.4624

*The data is expressed as mean±SD, *n: 6 i.e. number of injections, *SD: standard deviation, *%RSD: percentage relative standard deviation

Accuracy

The mean recovery for curcumin and cyclosporine was calculated at three different levels. The percentage recovery was found to be between 97-103% for all three-level and % RSD were found to be less than 2. The results obtained for recovery at 80%, 100%, and 120% was found to be within the specified limits.

Hence the method was found to be accurate. Results for accuracy are expressed in table 3 and chromatograms are given in fig. 4.

Table 3: Accuracy studies for curcumin and cyclosporine

	Level	Amount added (mg/ml)	Amount found (mg/ml)	% recovery	%RSD
Curcumin	80%	19.2	19.298	100.67%±0.0020	0.2067
	100	24	24.04	100.09%±0.0009	0.09283
	120%	28.8	28.58	99.73%±0.0075	0.7592
Cyclosporine	80%	9.6	11.93	100.39%±0.0058	0.5862
	100	12	11.92	99.50%±0.0017	0.1011
	120%	14.4	14.43	100.13%±0.0005	0.05765

*The data is expressed as mean±SD, *n: 3 i.e. number of injections, *SD: standard deviation, *%RSD: percentage relative standard deviation

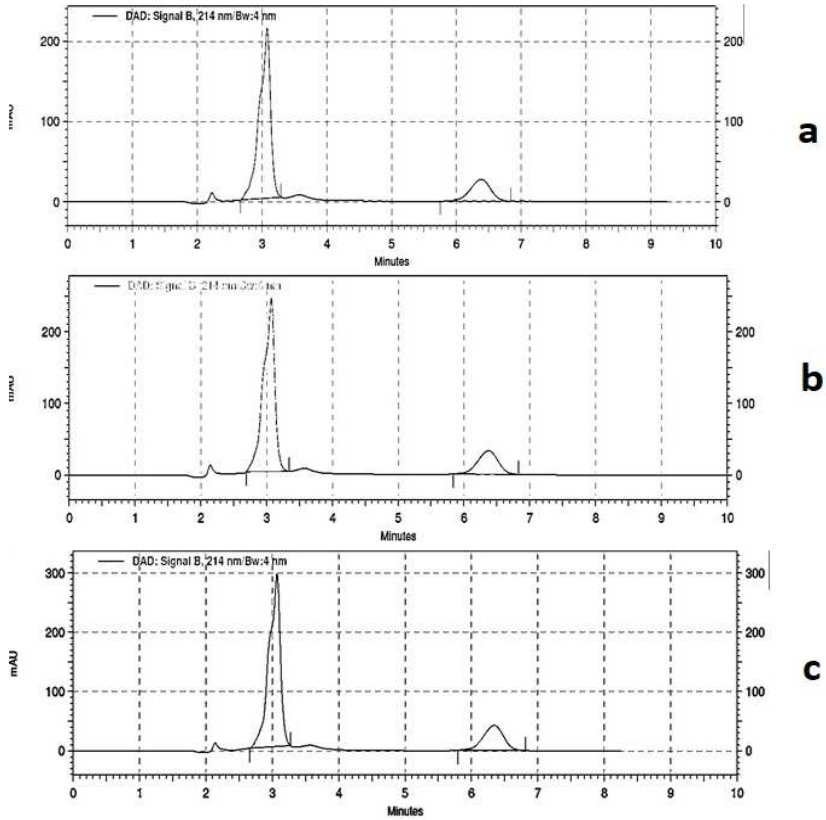


Fig. 4: Chromatogram of curcumin and cyclosporine in accuracy studies at (a) Level 1: 80%, (b) Level 2: 100% and (c) Level 3: 120%

Linearity and range

In order to test the linearity of the method, six dilutions of the working standard solutions for the drugs curcumin and cyclosporine in the range of 8-48 µg/ml and 4-24 µg/ml were prepared. Each of the dilutions was injected for the three times (n=3) into the column. Correlation coefficient (R^2) should be above 0.99 for both the drugs. The correlation coefficients (R^2 value) of developed method for both the drugs were found to be within specified limits. Linearity curves are is depicted in fig. 5 and chromatogram for linearity are shown in fig. 6.

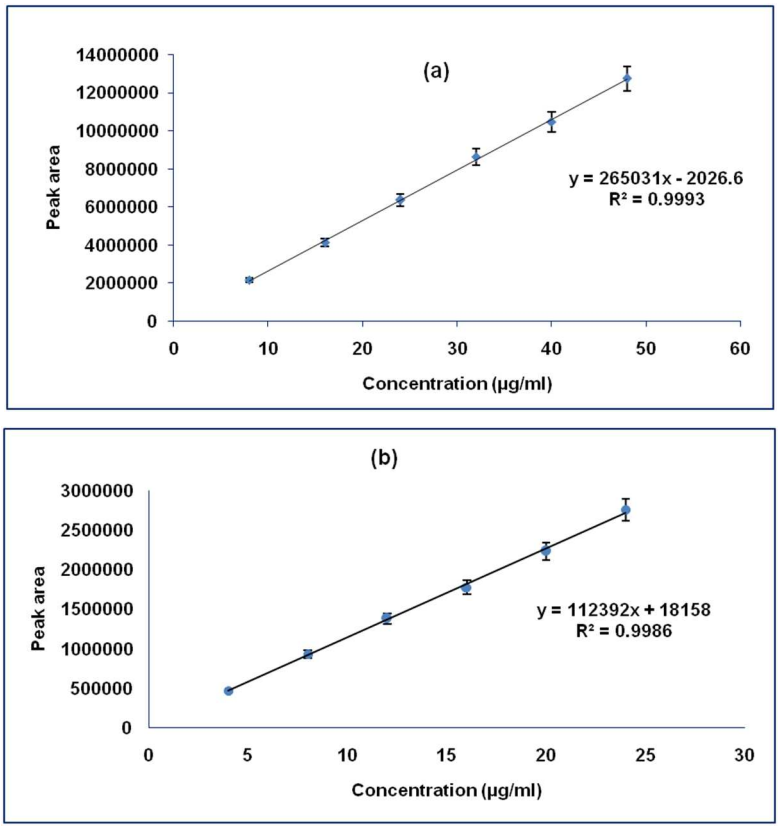
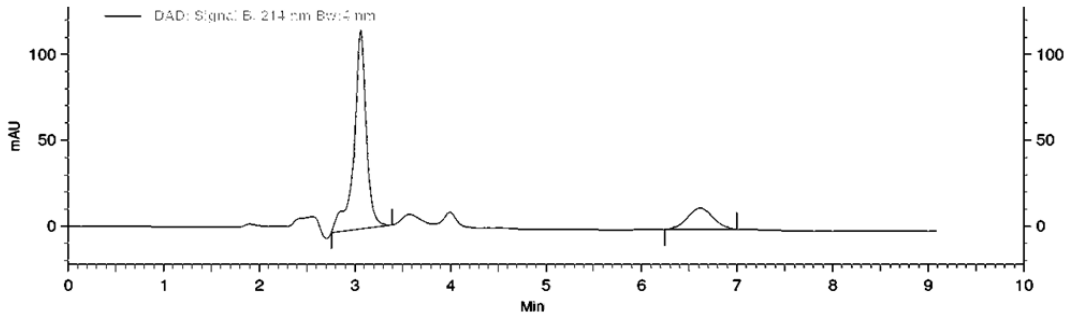
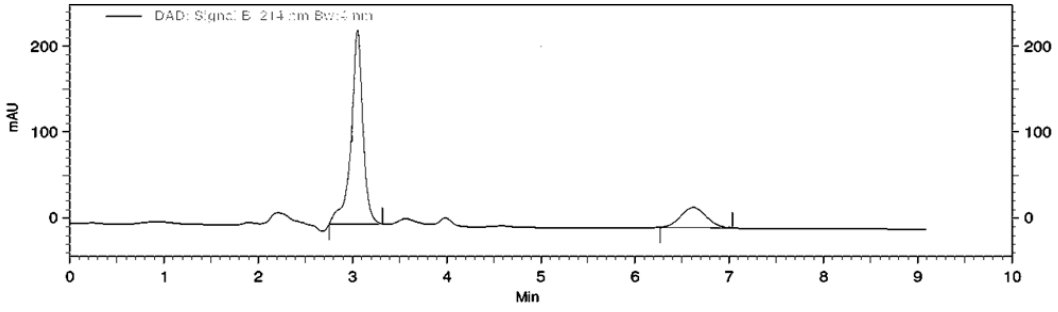


Fig. 5: Linearity curve of (a) curcumin and (b) cyclosporine, (*n: 3 i.e. number of injections of each concentration. The data is expressed in graph as mean±SD, *SD: standard deviation)



(a)



(b)

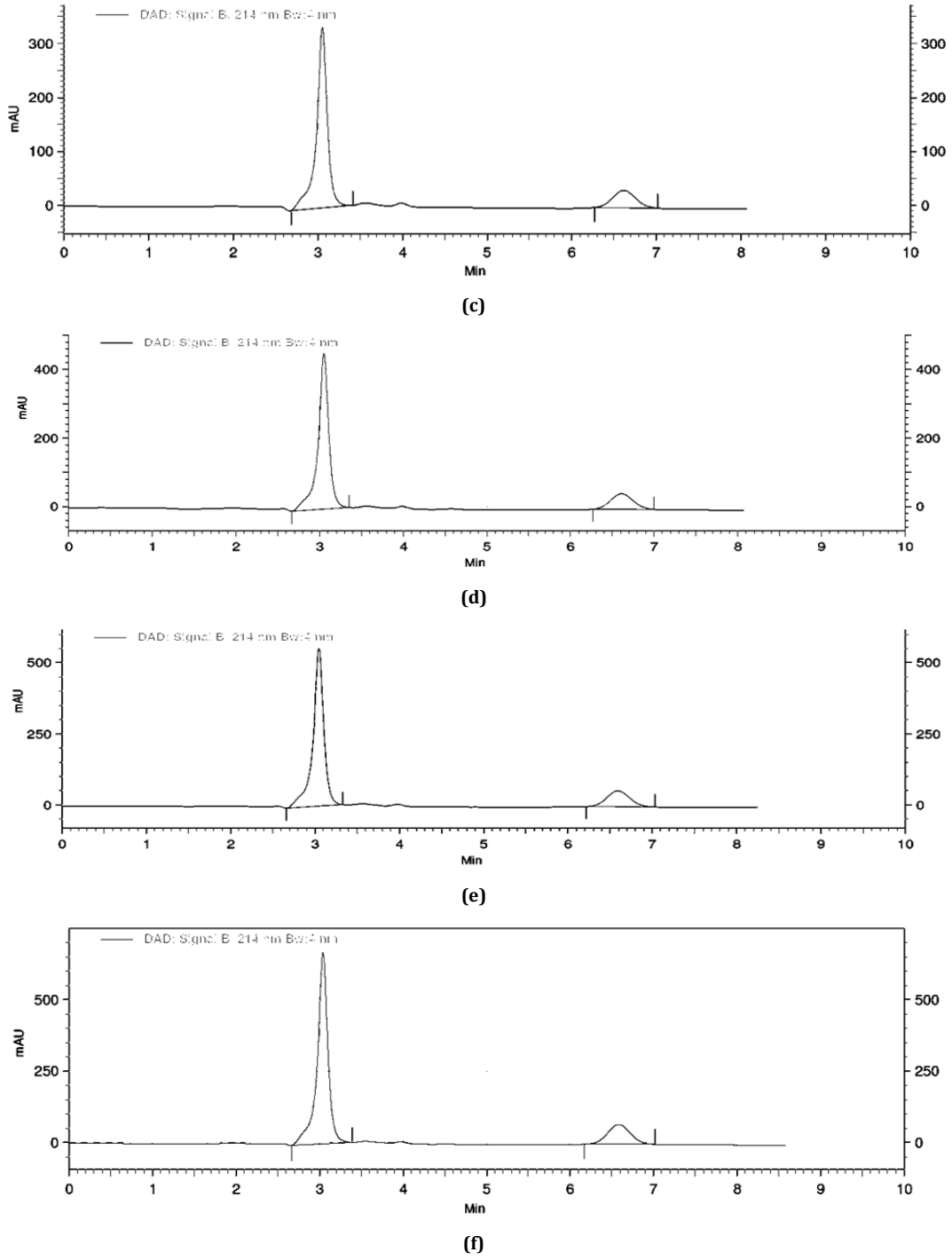


Fig. 6: Linearity study chromatogram of (a) curcumin 8 µg/ml and cyclosporine 4 µg/ml, (b) curcumin 16 µg/ml and cyclosporine 8 µg/ml, (c) curcumin 24 µg/ml and cyclosporine 12 µg/ml, (d) curcumin 32 µg/ml and cyclosporine 16 µg/ml, (e) curcumin 40 µg/ml and cyclosporine 20 µg/ml, and (f) curcumin 48 µg/ml and cyclosporine 24 µg/ml

LOD and LOQ

On the basis of peak response and slope of the regression equation, LOD and LOQ was calculated. The LOD of curcumin and cyclosporine was found to be 0.3594 µg/ml and 0.1839 µg/ml respectively. The LOQ of curcumin and cyclosporine was found to be 1.0892 µg/ml and 0.5575 µg/ml respectively.

Robustness

The method was found to be robust enough that the peak area was not apparently affected by small variation in the chromatographic conditions. On the evaluation of the results, it was concluded that the variation in the flow rate and variation in the temperature does not affect the method significantly. Hence, it was indicated that the method was robust even by a change in these two parameters. The system suitability parameters were within the limits and results for robustness is shown in table 4.

Drug content

Drug content of the formulation was found to be 97.34±0.5% for curcumin and 95.68±0.63% for cyclosporine. Hence it complies with the Pharmacopoeial limit of assay of drugs (90%-110%).

Table 4: Robustness study by varying flow rate and temperature

		Curcumin		Cyclosporine	
Variable	**Level (±)**	**Mean Retention time (min)±SD**	**% RSD**	**Mean Retention time (min)±SD**	**% RSD**
Flow rate (ml)	0.4 ml	3.14±0.005	0.184	6.61±0.005	0.087
	0.6 ml	2.9±0.005	0.199	6.55±0.005	0.088
Temperature (°C)	69 °C	3.043±0.001	0.056	6.58±0.005	0.087
	71 °C	3.04±0.0005	0.018	6.60±0.005	0.087

*The data is expressed as mean±SD, *n: 3 i.e. number of injections at each level, *SD: standard deviation, *%RSD: percentage relative standard deviation

In vitro dissolution study

In vitro dissolution studies were performed 20% weight gain of pellets after coating with Eudragit S100. Curcumin and cyclosporine showed better drug release at 20% weight gain of pellets after coating with pH-sensitive polymer Eudragit S100. The *in vitro* dissolution profile of curcumin and cyclosporine in capsule formulation estimated using developed HPLC method is shown in fig. 7. During the acidic stage (gastric stage), cyclosporine and curcumin did not show any release at the end of 2 h. At the buffer stage (Intestinal stage), less than 15% release of both the drugs was observed at the end of 6h. At the end of 24h, almost 80% of curcumin and cyclosporine was released which was desirable. Thus, the developed HPLC method was successfully applied for the determination of drug release of curcumin and cyclosporine from the capsule dosage form in a dissolution study.

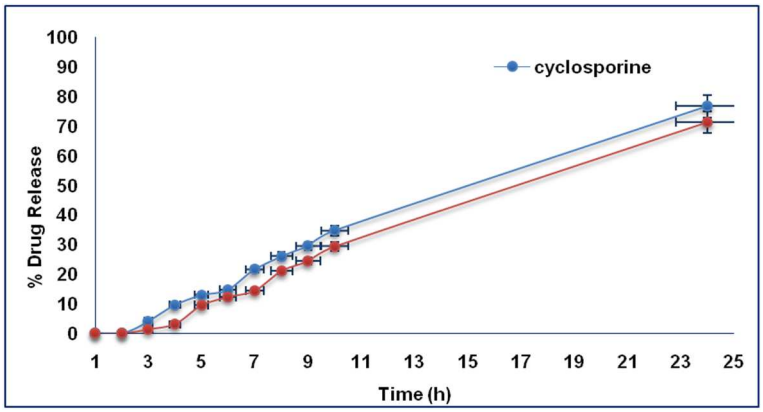


Fig. 7: *In vitro* drug release study of curcumin and cyclosporine in a capsule formulation using developed RP-HPLC method (*n: 3 i.e. no. of capsule formulation tested at each time interval. The data is expressed in graph as mean±**SD, *SD: standard deviation)**

DISCUSSION

A novel, rapid and reproducible RP-HPLC method for simultaneous estimation of curcumin and cyclosporine in capsule dosage form has been developed and validated as per ICH Guidelines. The validation acceptance criteria were satisfied in all cases as per USP/ICH guidelines. Application of this method for estimation of curcumin and cyclosporine from capsule dosage form showed that the excipients did not interfere in the estimation of both the drugs. Hence, this method was specific. The sample recoveries in the formulation were in good agreement and they suggested non-interference of formulation excipients in the estimation. The results obtained from the above set of observations prove that the present RP-HPLC method developed for the quantitative determination of curcumin and cyclosporine in the formulation was simple, sensitive, accurate, precise and robust. Moreover, various analytical methods for estimation of curcumin and cyclosporine alone were reported and in combination with other drugs [8]. However, there is no RP-HPLC method for simultaneous estimation of curcumin and cyclosporine in combination along with drug release analysis from the capsule dosage form and the novel method developed in this report is the first of its kind. The developed method is based on the use of a very economical solvent, had short chromatographic time and hence can be performed with ease.

CONCLUSION

A simple, specific, accurate, precise analytical method was developed. The proposed method presented the ability to separate curcumin and cyclosporine when used in combination in a dosage form using RP-HPLC method. It was successfully validated as per ICH-guidelines. Hence it can be concluded that this method can be easily adopted for routine analysis and quality control of curcumin and cyclosporine fixed-dose combination pharmaceutical dosage forms and API.

AUTHORS CONTRIBUTIONS

All authors have contributed equally to the manuscript

CONFLICTS OF INTERESTS

Authors declare no conflicts of interest

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