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Simultaneous estimation of paclitaxel and curcumin in nano-formulation: Stability analysis of drugs, optimization and validation of HPLC method

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ABSTRACT

To find the therapeutic dose of paclitaxel with curcumin in nano-formulation, it is essential to study the stability of paclitaxel in presence of curcumin. The stability analysis of the drugs individually (paclitaxel/curcumin) and in combination (paclitaxel with curcumin) was carried out at different pH conditions (4.5 and 7.4 pH). Reverse Phase High Performance liquid chromatography method was developed for paclitaxel and curcumin in combined form and used to validate and analyze the amount of drug loaded in the Nano formulation. The method was optimized and developed using the design of experiments. The developed method was analyzed using C-18 Luna, 5 µm, 100Å, and 150 mm column using methanol and water with 0.05% of phosphoric acid as mobile phase (0.8 ml/minutes flow rate) and detected at a wavelength of 370 nm for curcumin and 227 nm for paclitaxel. Results showed that the paclitaxel's stability decreases rapidly in the presence of curcumin. The method was validated according to the International Conference on Harmonization guidelines and found to be reproducible with peaks showing good resolution with short retention time and can be used for simultaneous estimation of the amount of paclitaxel and curcumin loaded in nanoformulations.

INTRODUCTION

Paclitaxel and curcumin are considered to possess anticancer properties (Robles *et al*., 2016; Schwab *et al*., 2014). Various preclinical studies were carried out to find the synergistic effect of paclitaxel with curcumin in the treatment of cancer (Aggarwal *et al*., 2005; Banerjee *et al*., 2010). U.S. National Cancer Institute-funded "Developmental Therapeutic" screening program discovered paclitaxel at 1962 by isolating it from the bark of the Pacific yew also known as *Taxus brevifolia* (Weaver, 2014). Paclitaxel acts on stabilization of microtubules by preventing their depolymerization process which halts or slows down the cell division and induces cell death (Fu *et al*., 2009; Okano and Rustgi, 2001; Zhang *et al*., 2014). Although paclitaxel is considered to be an efficient anticancer drug, there is mounting evidence from researchers that paclitaxel induces the activation of Nuclear Factor Kappa-light-chain-enhancer of activated B cells (NF-κB) and upregulates efflux pump proteins which become the major reason for multidrug resistance in cancer cells (Wei *et al*., 2017). Recent studies show that curcumin, a phytochemical extracted from curcuma longa perennial herb, inhibits the NF-κB by downregulating the Inhibitor of Nuclear Factor Kappa-B Kinase Subunit Beta (IKKβ) which interrupts the cancer cell growth and induce apoptosis (Tang *et al*., 2002; Yallapu *et al*., 2012). The use of curcumin with paclitaxel can help to improve its therapeutic efficiency during the course of treatment (Sreekanth *et al*., 2011; Zhou *et al*., 2015). Although they have a good therapeutic effect for cancer, both paclitaxel and curcumin are associated with poor solubility and low bioavailability and rapid clearance of the drugs (Boztas *et al*., 2013) from the system. There are many methods to improve the bioavailability of hydrophobic drugs; one such approach is the use of polymeric nanoparticles as the drug delivery vehicle (Paramera *et al*., 2011). Testing the stability of the particular drug in the presence of other drugs plays a major role in the combination of drug treatment. The main aim of

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performing stability analysis is to analyze the quality of the drug and their degradation pattern in the presence of other drugs, with respect to time, temperature, and at different pH conditions. Analyzing the stability of the drugs in combined form gives us an insight into the treatment period, storage conditions, and shelf life (Naidu *et al*., 2005). Many stability indicating methods (Bajaj *et al*., 2012) have been reported in the literature for various combinations of drugs (Nalaiya *et al*., 2014; Subramanian *et al*., 2020) but none of the literature has reported the stability analysis of paclitaxel and curcumin in a combined form through Reverse Phase High Performance liquid chromatography (RP-HPLC).

The main objective of this work is to find out the stability of paclitaxel in presence of curcumin. To achieve this, we have developed a RP-HPLC method to analyze paclitaxel and curcumin together and optimized the method using optimization technique. The developed method was validated according to International Conference on Harmonization (ICH) guidelines and used to analyze the amount of drug (paclitaxel and curcumin together) loaded in the nano-formulation. RP-HPLC method developed gives a precise and accurate estimation of the drugs.

The method development stage should include all the parameters such as solubility of the sample, mobile phase, type of column, wavelength, pH, and concentration of the mobile phase. Choosing the right parameter with respect to the HPLC method will always result in a good outcome (Kaushal and Srivastava, 2010; Siddiqui *et al*., 2017). Developing appropriate HPLC conditions for a combination of drugs by conventional trial and error method makes the work more complicated and takes a considerable amount of time. Quality by design (QbD) is the method that utilizes the interaction effect of the combination of independent variables to acquire ideal chromatographic conditions by employing it in the design of experiments (DoE) (ICH, 2009). Many researchers have used the QbD method for optimization of various drugs in single and in combination form (Hasnain *et al*., 2017; Mukthinuthalapati *et al*., 2019; Panda *et al*., 2016; Yadav *et al*., 2016). Thus, the approach of QbD was used for the optimization procedure. Box–Behnken experimental design (BBD) is a response surface method implemented to find the main interaction and quadratic effects of the independent parameters on chromatographic responses. Figure 1A and B shows the chemical structure of the drugs paclitaxel and curcumin.

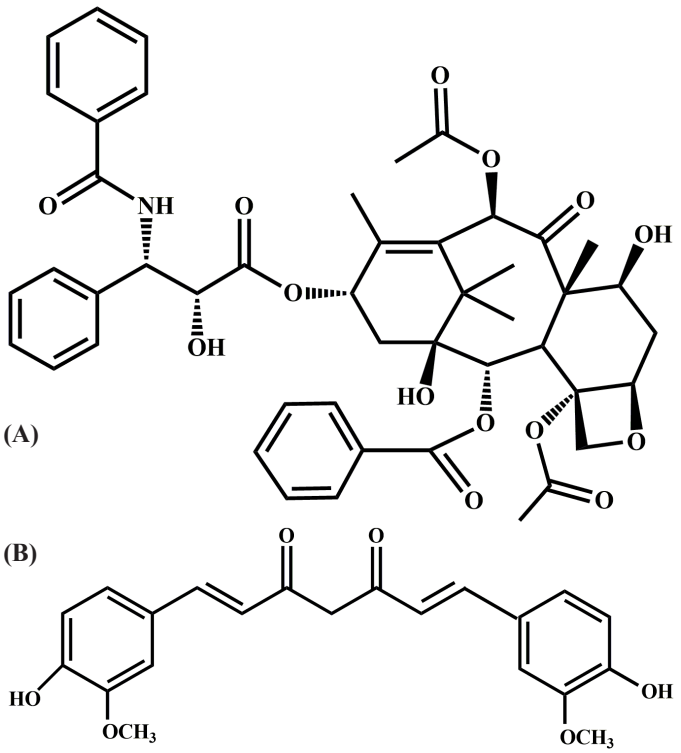

Figure 1. Chemical structure of (A) Paclitaxel (B) Curcumin.

MATERIALS AND METHODS

Instruments

SHIMADZU (Model No LC-20AD, JAPAN) HPLC system attached with Auto-injector (Model No SPD- 20A, JAPAN) and UV detector (Model No SPD-20A, JAPAN), LC solution software was used to record and integrate chromatograms. The stationary phase column used for the analysis was Phenomenex Luna C18, 4.6 × 150 mm, 5 µm, 100A◦ (Model No 00F-4252-E0, USA). Q Sonica Ultrasonication device (Model No Q -700, Newtown, CT); SHIMADZU Electronic balance (Model No AUW 220D, JAPAN); Ultra Bath Sonicator (Model No XUBA1, Grant Instruments, Cambridge, UK); Magnetic stirrer (Model No SLK6, SCHOTT Instrument GmbH, Mainz, GERMANY) were used in this research.

Software and statistical data analysis

The chromatographs were processed using Shimadzu's LC Solutions software. Design expert 11 trial version was used to design the experiments. Percentage relative standard deviation (%RSD) and the linear regression analysis were calculated using the method of least squares.

Materials and reagents

HPLC grade organic solvent Methanol (Catalogue (CAT) No: 60600725001730) was obtained from Merck life science private limited, Mumbai, India; Paclitaxel (Mw 853.91, CAT No: NFA-019) was obtained from Xi'an Natural field Bio-Technique co., Ltd, China; Curcumin (Mw 368.38, CAT No: B21573) was obtained from Alfa Aesar Mumbai, India; Phosphoric acid (CAT No: 05063) was obtained from Lobo Chemie, Mumbai, India; Poly(lactic-co-glycolic acid) (PLGA) 50:50 (MW: 1,00,000-1,20,000 Da, CAT No: B6010-4P) was procured from Durect corporation Birmingham, AL; Polyvinyl alcohol (PVA; molecular weight 1,25,000, CAT No: 130086) was obtained from Thomas Baker, Mumbai, India.

Method development considerations

To develop an RP-HPLC method, the physicochemical characteristics of the analytes (solubility, polarity, wavelength, etc.) were studied. Two stationary phases (C8 & C18) were kept under for consideration, stationary phase was chosen as C18 due to its high hydrophobic nature which helps in strong retention of hydrophobic analytes.

Preparation of mobile phase

As mentioned earlier, selection of the mobile phase was based on the polarity of the sample, availability of the solvents, proper retention time (RT), the sensitivity of the assay, and a short run time of the sample (Hakkimane *et al*., 2017).

Chromatographic conditions

The mobile phase used for this study was methanol with 0.05% of phosphoric acid as solvent (organic phase) and MilliQ Water with 0.05% of phosphoric acid (aqueous phase). The solvents used for the analysis were filtered using a 0.45 µm membrane filter and degassed through an ultra-bath sonicator. Phenomenex Luna C18, 4.6 × 150 mm, 5 µm, 100A◦ was used as the stationary phase. The analysis was carried out in isocratic conditions with 0.8 ml minutes^{-1} flowrate with an injection volume of 20 µl. The wavelengths of the detectors used were 227 nm and 370 nm for paclitaxel and curcumin, respectively.

Preparation of standard stock solutions

About 20 mg of paclitaxel/curcumin was accurately weighed and dissolved in 10 ml of methanol to make a concentration of 2 mg/ml. From that 1 mg/ml concentration was made and stored as stock solution. Further concentrations (0.1, 1, 10, 40, 60, 80, and 100) µg/ml were made by serial dilution to prepare the standard plot.

Design of experiment using BBD

The optimization of the RP-HPLC method was implemented using Box–Behnken design. Three parameters (Independent variables) concentration (%) of organic phase (A), % buffer concentration (B) and flow rate (minute/ml) (C) at three different levels low (−1), medium (0), and high (+1) were implemented to find the optimum combination and is represented in Table 1. The design comprised of fifteen experimental runs, a standard concentration (paclitaxel and curcumin) 10 µg/ml was used for all fifteen experimental runs, which were analyzed for the BBD method. The chromatographic responses like retention time of curcumin [$R_{t(CUR)}$], the retention time of paclitaxcel [$R_{t\,(PTX)}$], the peak height of curcumin, the peak height of paclitaxel Peak height $_{(PTX)}$, area % of curcumin, area % of paclitaxel, resolution of the peak (Rs), and total analysis time (T) were studied and shown in Table 2.

Optimization and model validation

Design expert 11 trial version was used for optimization and model validation. The quadratic polynomial equations were

Table 1. Box-Behnken design table incorporated with independent experimental variables and levels(coded).

Variables	levels		
Independent	−1	0	+1
A—% organic phase (%) methanol	65	70	75
B—Buffer strength (%)	0.01	0.05	0.1
C—Flowrate (ml/minute)	0.6	0.8	1

Dependent
$R_{t\,CUR}$ (minute) = Retention time curcumin
$R_{t\,PTX}$ (minute) = Retention time paclitaxel
Peak height $_{(CUR)}$ = Peak height of curcumin
Peak height $_{(PTX)}$ = Peak height of paclitaxel
Area % $_{(CUR)}$ = Area % purity of curcumin
Area % $_{(PTX)}$ = Area % purity of paclitaxel
Rs $_{(CUR\text{-}PTX)}$ = Resolution of the peak
Total analysis time = Total run time (T)

Table 2. Chromatographic response and measured response values for BBD.

Runs	Factors			Responses							
No of runs	*A*	*B*	*C*	$R_{t\,(CUR)}$	$R_{t\,(PTX)}$	Peak height $_{(CUR)}$	Peak height $_{(PTX)}$	Area % $_{(CUR)}$	Area% $_{(PTX)}$	$R_{s\,(CUR\text{-}PTX)}$	*T*
1	65	0.01	0.8	13.62	17.53	14,277	15,227	42.37	57.60	2.22	20
2	75	0.01	0.8	5.23	5.69	26,881	30,499	44.60	55.47	0.42	12
3	65	0.1	0.8	14.41	18.30	13,017	13,963	42.90	57.09	2.16	20
4	75	0.1	0.8	5.16	5.69	26,459	30,277	43.85	56.14	0.52	12
5	65	0.05	0.6	15.29	19.56	13,046	14,234	42.69	57.31	2.05	21
6	75	0.05	0.6	8.51	9.29	25,904	35,616	43.80	56.19	0.42	12
7	65	0.05	1	10.21	13.37	13,878	14,982	43.21	56.79	1.35	20
8	75	0.05	1	4.08	4.49	27,917	31,700	44.83	55.16	0.25	12
9	70	0.01	0.6	13.91	15.58	18,731	20,702	43.60	56.40	0.92	20
10	70	0.1	0.6	14.15	15.81	18,495	20,780	43.40	56.60	0.90	20
11	70	0.01	1	6.11	7.176	21,743	23,982	43.86	56.30	1.02	12
12	70	0.1	1	7.04	7.98	18,799	19,988	44.23	55.77	0.80	12
13	70	0.05	0.8	8.99	10.19	21,339	17,573	50.52	49.48	0.93	12
14	70	0.05	0.8	8.99	10.18	21,265	17,518	50.56	49.40	0.91	12
15	70	0.05	0.8	8.97	10.16	21,318	17,500	50.70	49.29	0.85	12

A = organic phase (%); *B* = Buffer strength (%); *C* = Flow rate (ml/minutes); $R_{t\,(CUR)}$ = Retention time of Curcumin; $R_{t\,(PTX)}$ = Retention time of Paclitaxel; Peak height $_{(CUR)}$ = Peak height of Curcumin; Peak height $_{(PTX)}$ = Peak height of Paclitaxel; Area % $_{(CUR)}$ = Area % purity of Curcumin; Area% $_{(PTX)}$ = Area % purity of Paclitaxel; R_s = Resolution of the peak; T = Total run time.

framed from the statistical significance of coefficients of both the main effects and interaction effects. The aptness of the designed model was validated by analyzing parameters like the coefficient of correlation (R^2), adjusted (R^2), and adequate precision. The possible interaction effect of the chosen factors was studied from the response surface plot, contour plot, and perturbation plot. The optimum chromatographic conditions were selected based on short analysis time, peak elution time being in range, and percentage area of the peak. Derringer's desirability function and Design space plot was carried out to show the optimum chromatographic conditions.

Validation of the method

The RP-HPLC method developed for this study was validated according to the ICH guidelines (ICH, 2005). Linearity, Specificity, precision (repeatability), the limit of detection and quantitation of paclitaxel and curcumin in combined form was evaluated using the optimized RP-HPLC method.

Linearity

ICH states linearity as the ability to acquire test results of the dependent variable data being directly proportional to the sample concentration. It is mandatory to analyze within an appropriate range for which the response of the instrument should be proportional to the concentration of the drug (Krier *et al*., 2011; Sharma *et al*., 2020). Normally, the value of co-relation coefficient (R^2) >0.998 is acceptable. The standard solutions of the combination of drugs paclitaxel and curcumin were prepared by diluting the stock solution (1 mg/ml) with methanol. The concentration of standard solutions ranged from 0.1 to 100 µg/ml were analyzed and plotted with peak area value with respect to the concentration of the drug. The linearity was estimated by linear regression analysis which was calculated through the method of least squares.

Precision

Precision is estimated normally with three parameters: repeatability, intermediate precision, and reproducibility. ICH allows exclusion of intermediate precision, provided if the results of reproducibility are proven very well. Three different concentrations (60, 80, and 100 µg/ml) of a combination of paclitaxel and curcumin were analyzed for their intra- and inter-day precision using the optimized method. Standard Deviation (SD) and %RSD values were also calculated.

Specificity

ICH defines specificity as the efficiency to measure unequivocally the analyte even in the presence of other components (Shaalan *et al*., 2013). Test for specificity was carried out by analyzing six replicates of a combination of paclitaxel and curcumin (40 µg/ml) drug standards proceeded with a calculation of %RSD and SD of the peak RT.

System suitability

System suitability test was carried out to confirm that RP-HPLC system and the developed method are acceptable for the analysis. The RP-HPLC system was stabilized for 45 minutes with the developed method followed by a blank run. Six replicates of paclitaxel and curcumin together of known concentration (40 µg/ml) were injected to find the suitability of the developed method. The criteria for testing system suitability include %RSD of peak area (Moorthi and Kathiresan, 2013).

Limit of detection and limit of quantitation

Limit of detection defines the ability to detect the lowest concentration of the analyte from the sample where quantitation is not needed under certain conditions. Limit of quantitation deals with the quantitation of the lowest concentration of the analyte in the sample with precision and accuracy. It is estimated through the slope and SD of the response (Veeragoni *et al*., 2016). The method was validated by analyzing 6 replicates of a combination of paclitaxel and curcumin drug standard (40 µg/ml) and the Limit of Detection (LOD) and Limit of Quantitation (LOQ) was calculated

$$LOD = 3.3\ \sigma/s \text{ and } LOQ = 10\ \sigma/s$$

Where = the SD of the response & S= the slope.

Application of the optimized method in stability analysis and in nano-formulations

The optimized and validated RP-HPLC method for the combination of the Drugs paclitaxel and curcumin were used to find the stability of the drugs in combined form and to find the amount of drug loaded in the polymeric nano-formulation.

Combination of paclitaxel and curcumin stability analysis

About 1 mg of both the drugs were weighed accurately and dissolved in 1 ml of Phosphate Buffer saline (PBS) (4.5 & 7.4 pH) and kept in a rotating shaker for 24 hours at 37°C. The samples were centrifuged at 10,000 rpm and the supernatant was taken as samples and they were analyzed through RP-HPLC using the validated method for a time period of 48 hours.

Preparation and analysis of curcumin and paclitaxel in nano-formulation

Curcumin and paclitaxel together in nano-formulation were prepared using the single emulsion solvent evaporation method as described earlier (Hakkimane *et al*., 2018). Briefly, PLGA 90 mg and the drugs paclitaxel and curcumin 22.5 mg each (total quantity of drugs 45 mg) was weighed and dissolved in 1.8 ml of chloroform. The Polymer-Drug ratio 2:1 was maintained. Emulsification (oil-in-water) of the polymer-drugs solution was carried out with 12 ml of 2 % *w/v* aqueous PVA using probe sonication with 60% amplitude for 8 minutes under 4°C ice bath which was followed by overnight solvent evaporation to remove the chloroform. Repetitive centrifugation wash (four times) at 13,000 rpm at 4°C for 45 minutes was done to remove the PVA. The formed nanoparticle pellet was dispersed in 5–7 ml of deionized water and lyophilized for 48 hours to acquire the powder form of nanoparticles. To find the amount of drug loaded in the nanoparticle, HPLC analysis was performed using an optimized and validated method. The nanoparticles samples were accurately weighed in triplicates (1 mg) followed by the addition of the methanol and placed on a rocker shaker for about 2 days. The samples were centrifuged at 10,000 rpm for about 20 minutes and the supernatant containing the dissolved drug was collected and subjected to the RP-HPLC method to find the amount of paclitaxel and curcumin loaded in 1 mg of the nanoparticle.

RESULTS AND DISCUSSION

Simultaneous estimation of paclitaxel and curcumin RP-HPLC method optimization and validation

The total fifteen experimental runs designed using BBD were analyzed for their chromatographic response. The chromatograms obtained from the above mentioned fifteen runs are given in the Supplementary Information S1a–o. Table 3 is provided with the statistical parameters from the analysis of variance (ANOVA). The probability p lies below < 0.05 for most of the chromatographic responses and the correlation coefficient and adjusted R^2 values were found to be high which infers the model chosen is significant. Both the main and the interaction effect are studied using the full quadratic equations which are shown [Eq. (1–8)] where A: % organic phase, B: buffer strength (%) and C: flow rate (ml minute−1). The positive and negative values in the quadratic equation symbolize the positive and converse effects of the independent parameters and experimental response. From the equations, buffer strength (B) has positive effects on $R_{t\,(CUR)}$, $R_{t\,(PTX)}$, Peak Height$_{(CUR)}$ & Area% $_{(CUR)}$, Organic phase (A) shows favorable response on Peak Height$_{(CU)}$ & Area% $_{(CUR)}$, while flow rate (C) shows a positive effect on Peak Height $_{(CUR)}$ & Area% $_{(CUR)}$. Supplementary Information S2a–h explains how independent variable levels have an impact on chromatographic responses through perturbation plots.

$$R_{t\,(CUR)} = 74 - 0.37\,A + 46\,B - 51.9\,C - 0.0033\,A^2 + 338\,B^2 + 15.5\,C^2 - 1.23\,AB + 0.162\,AC + 10.7\,BC \quad (1)$$

$$R_{t\,(PTX)} = 290 - 5.96\,A + 39\,B - 68.4\,C + 0.0333\,A^2 + 383\,B^2 + 16.7\,C^2 - 1.17\,AB + 0.346\,AC + 8.0\,BC \quad (2)$$

$$\text{Peak Height}_{(CUR)} = 112783 + 2174\,A + 32948\,B + 23822\,C - 8.09\,A^2 - 439507\,B^2 - 22970\,C^2 + 873\,AB + 295\,AC - 74038\,BC \quad (3)$$

$$\text{Peak height}_{(PTX)} = 632981 - 19012\,A - 48968\,B - 22842\,C + 154.6\,A^2 + 585306\,B^2 + 68427\,C^2 + 751\,AB - 1166\,AC - 103742\,BC \quad (4)$$

$$\text{Area \%}_{(PTX)} = 732.3 + 20.568\,A + 278.0\,B + 124.30\,C - 0.14607\,A^2 - 1755.5\,B^2 - 82.73\,C^2 - 1.386\,AB + 0.1275\,AC + 15.03\,BC \quad (5)$$

$$\text{Area\%}_{(CUR)} = 829.7 - 20.495\,A - 270.0\,B - 124.92\,C + 0.14560\,A^2 + 1773.7\,B^2 + 83.31\,C^2 + 1.283\,AB - 0.1275 - 19.20\,BC \quad (6)$$

$$R_{s\,(CU\text{-}PTX)} = 79.1 - 2.029\,A - 19.7\,B - 2.41\,C + 0.01260\,A^2 + 56.5\,B^2 - 4.91\,C^2 + 0.132\,AB + 0.1310\,AC + 6.02\,BC \quad (7)$$

$$T = 545 - 12.93\,A - 89\,B - 111.9\,C + 0.0850\,A^2 + 938\,B^2 + 53.1\,C^2 + 0.06\,AB + 0.25\,AC - 23\,BC \quad (8)$$

The interaction effect of the independent variables on chromatographic response is studied using three-dimensional response surface plots. Figure 2 shows the effect of the organic phase and buffer strength, Figure 3 shows the effect of the organic phase and flow rate, while Figure 4 shows the effect of buffer strength and flow rate. The interaction effect studied using contour plots are given in Supplementary Information S3a–h, S4a–h & S5a–h.

Optimization

Peak separation within range of all parameters and with short run time is the main criteria for optimization. According to the literature (Mukthinuthalapati *et al*., 2019) Derringer's desirability function, D value nearly 1 indicates that the obtained chromatographic response values are close to the target value. Table 4 shows the criteria for optimization. The criteria are selected based on short analysis time, peak elution time being in range and Percentage Area of the peak. By following the conditions based on the selected criteria, the optimization procedure was conducted. Figure 5 shows the desirability of chromatographic response at the optimized conditions showing the D value 1 confirms the optimized method is in the desirability range. The desirability ramp for the optimized condition with limits and criteria are shown in Supplementary Information S6. Figure 6 indicates that the optimized method is within the range of Design space.

Table 3. ANOVA for the Chromatographic Responses

Variable	$R_{t\,(CUR)}$		$R_{t\,(PTX)}$		Peak height$_{(CUR)}$		Peak height $_{(PTX)}$		Area % $_{(CUR)}$		Area% $_{(PTX)}$		R_s		T	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Model	16.86	0.003	27.19	0.001	434	<0.0001	35.8	0.0005	596	<0.0001	506	<0.0001	31	0.0007	4.2	0.06
A	90.88	0.0002	42.19	0.003	3731	<0.0001	278	0.0001	180	<0.0001	148	<0.0001	238	<0.0001	24	0.02
B	0.35	0.579	0.29	0.548	31	0.02	1.68	0.25	0.01	0.91	0.1	0.049	0.2	0.83	0.00	0.99
C	57.3	0.0006	66.64	0.003	45	0.007	0.06	0.8	39	<0.0001	29	<0.002	9.5	0.1	6.4	0.08
Phase: A^2	0.019	0.89	1.85	0.231	1.6	0.26	25.4	0.003	2150	<0.0001	1802	<0.0001	18.5	0.008	2.9	0.14
B^2	1.3	0.305	1.56	0.266	30	0.002	2.32	0.18	1976	<0.0001	1702	<0.0001	2.3	0.18	2.3	0.19
C^2	1.1	0.342	1.20	0.323	33.2	0.002	12.7	0.016	1766	<0.0001	1510	<0.0001	7.2	0.044	2.9	0.14
AB	0.24	0.644	0.20	0.67	1.66	0.254	0.05	0.827	17.1	0.009	12.3	0.01	0.17	0.69	0.00	0.99
AC	0.08	0.78	0.35	0.58	3.72	0.112	2.51	0.17	2.84	0.153	2.40	0.18	3.4	0.12	0.04	0.84
BC	0.029	0.87	0.015	0.9	19	0.007	1.62	0.25	3.22	0.133	4.43	0.089	0.5	0.47	0.03	0.86
R^2	0.97		0.98		0.99		0.98		0.99		0.99		0.98		0.88	
Adj R^{2a}	0.91		0.94		0.99		0.95		0.99		0.99		0.95		0.67	
Adeq Preci[b]	14.81		18.45		60.71		17.55		66.29		61.01		16.45		6.44	

Adequate precision being greater than 4 indicates that the model is capable to navigate the design space.
p = Probability; F = Fisher ratio, adjusted R^2 values nearer to 1 symbolises the three independent variables are perfect fit for the model.

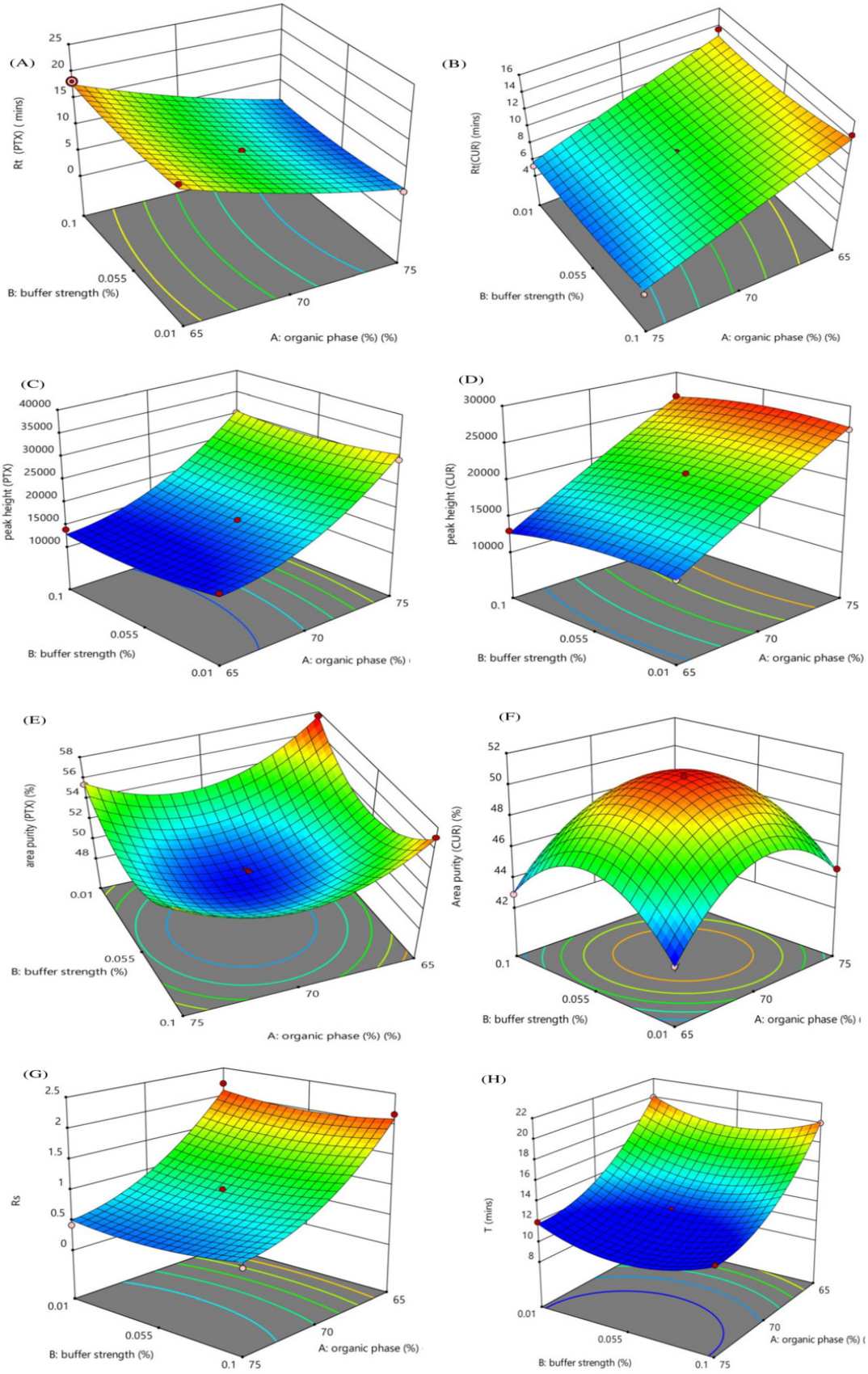

Figure 2. Response surface plots showing the impact of % Organic phase and buffer strength % on (A) $R_{t\,(PTX)}$ (minute), (B) R_{tCUR} (minute), (C) Peak height $_{(PTX)}$, (D) Peak height $_{(CUR)}$, (E) Area % $_{(PTX)}$, (F) Area % $_{(CUR)}$, (G) $R_{s\,(CUR\text{-}PTX)}$, (H) Total analysis time (T).

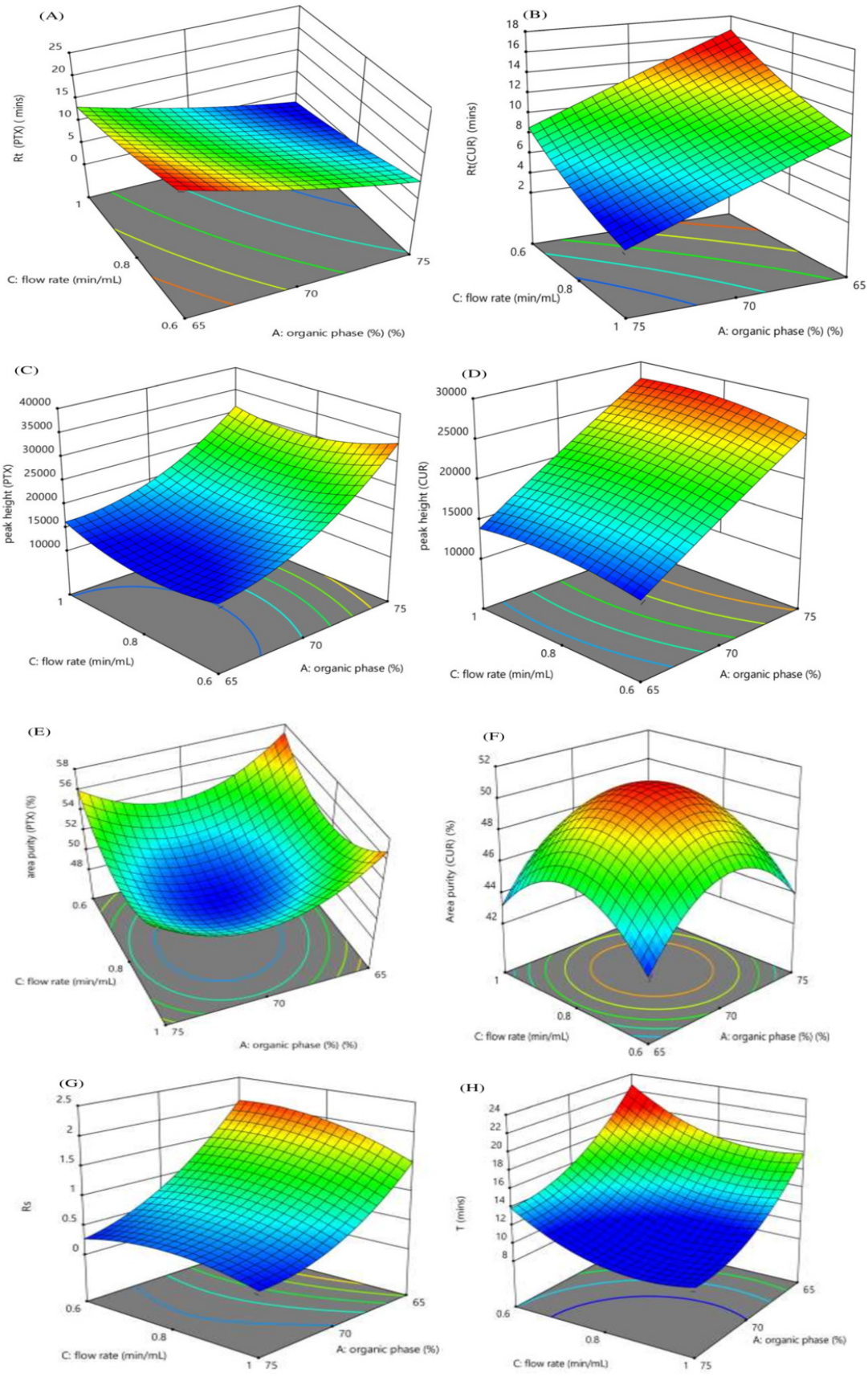

Figure 3. Response surface plots showing the impact of % organic phase and flow rate (minute/ml) on (A) $R_{t\,(PTX)}$ (minute), (B) $R_{t\,(CUR)}$ (minute), (C) Peak height $_{(PTX)}$, (D) Peak height $_{(CUR)}$, (E) Area % $_{(PTX)}$, (F) Area % $_{(CUR)}$, (G) $R_{s(CUR\text{-}PTX)}$, (H) Total analysis time (T).

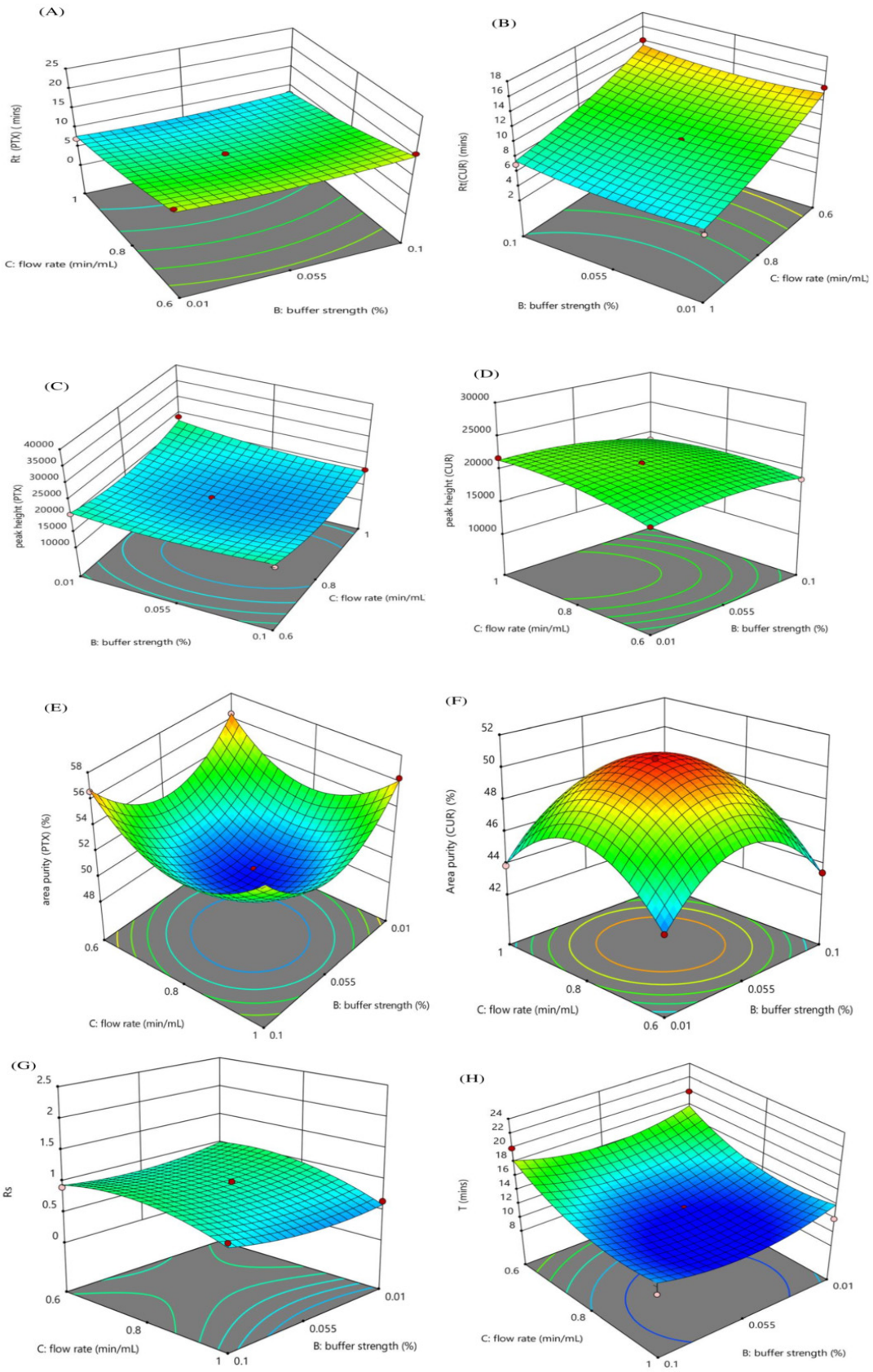

Figure 4. Response surface plots showing the impact of buffer strength % and Flow rate (minute/ml) on (A) $R_{t\,(PTX)}$ (minute), (B) $R_{t\,(CUR)}$ (minute), (C) Peak height $_{(PTX)}$, (D) Peak height $_{(CUR)}$, (E) Area % $_{(PTX)}$, (F) Area % $_{(CUR)}$, (G) $R_{s\,(CUR\text{-}PTX)}$, (H) Total analysis time (T).

Table 4. Desirability criteria.

Name	Goal	Lower limit	Upper limit
$R_{t\,(PTX)}$ (minutes)	In range	4.49	19.56
$R_{t\,(CUR)}$ (minutes)	In range	4.08	15.29
Peak height $_{(PTX)}$	In range	13,963	35,616
Peak height $_{(CUR)}$	In range	13,017	27,917
Area% $_{(PTX)}$	In range	49.29	57.6
Area % $_{(CUR)}$	In range	42.37	50.7
$R_{s(CUR\text{-}PTX)}$	In range	0.25	2.22
Total analysis Time (T)	minimize	12	21

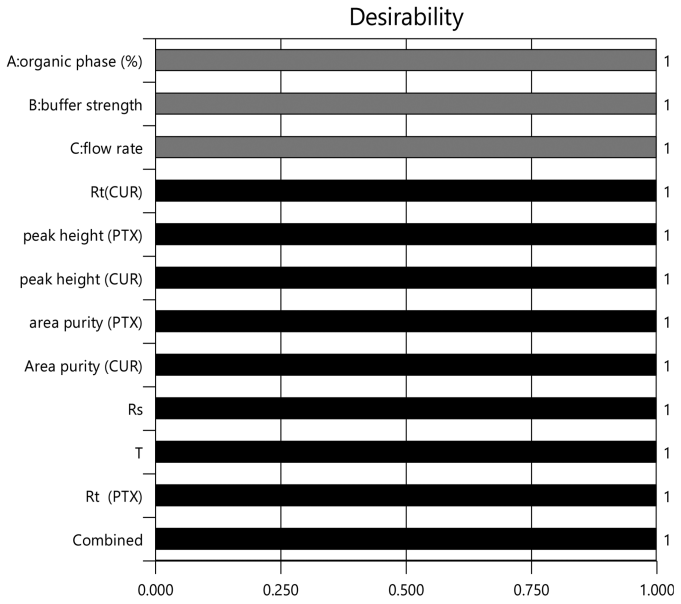

Figure 5. Desirability bar graph of responses for optimized chromatographic condition.

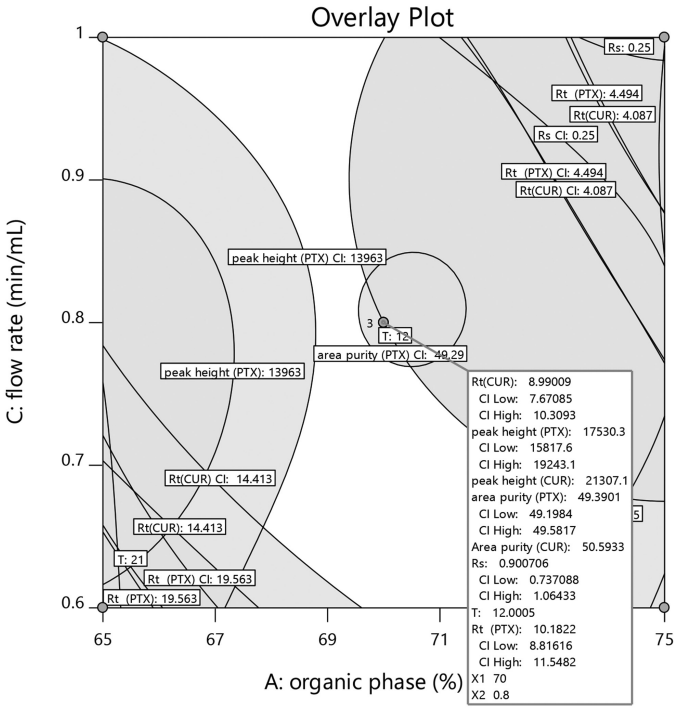

Figure 6. The design space of the optimized method.

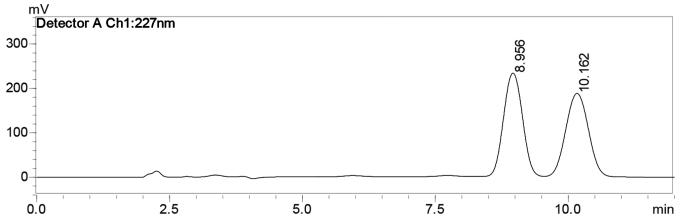

Figure 7. HPLC peak of simultaneous estimation of curcumin and paclitaxel 100 µg/ml RT 8.9 for curcumin 10.1 for paclitaxel respectively.

The optimized chromatographic condition for the chromatographic separations of the drugs paclitaxel and curcumin was achieved using reverse-phase phenomenex® Luna C18, 4.6* 150 mm, 5 µm, 100A° column. The drugs were eluted with a mobile phase consisting of methanol with 0.05% of O-phosphoric acid and Milliq water with 0.05% of O-phosphoric acid (70:30) at a flow rate of 0.8 ml/minutes. The drug curcumin eluted with a RT of 8.9 minutes which was detected at 370 nm, while paclitaxel eluted with a RT of 10.1 minutes which was detected at 227 nm. The chromatogram Figure 7 shows two distinct clear peaks with no additional peaks. The RP-HPLC method can segregate clearly at low concentrations as well as in mixture of compounds (Hakkimane *et al*., 2017).

Method validation

Linearity

The linearity of the optimized RP-HPLC method for the combination of drugs paclitaxel and curcumin was established by plotting the area value of the chromatogram with respect to different concentrations ranging from 0.1 -100 µg/ml. Table 5 shows the R^2 (correlation coefficient) of drugs paclitaxel and curcumin to be linear with a value of 0.999 exhibiting a good relationship between concentration range and peak area. Supplementary Information S7 shows chromatograms of different concentrations of paclitaxel and curcumin were having the same RT.

Table 5. Calibration results.

Test	Acceptance criteria	Results CUR	PTX
Linearity (n = 3)			
Correlation coefficient	$R^2 > 0.998$	0.999	0.999
Slope		71,994	50,596
Linearity range (µg/ml)		0.1–100	0.1–100

Precision

Analysis of the intra- and inter-day precision of the combination of paclitaxel and curcumin standards of three different concentrations (60,80 and 100 µg/m) revealed a %RSD value less than 1% inferring an acceptable level of precision for the developed method. Table 6 shows the intra and intermediate precision of the drug standards.

Table 6. Intra and inter day precision of RP HPLC method for Combination of Drugs

Actual value (µg ml^{-1})	60 curcumin	60 paclitaxel	80 curcumin	80 paclitaxel	100 curcumin	100 paclitaxel
Intraday repeatability mean (n = 3)	4,367,743	3,064,407	4,367,743	4,014,257	7,260,292	5,056,586
SD	193.84	30,199.48	4,051.98	1,509.22	14,123.1	4.709.67
%RSD	0.04	0.98	0.07	0.03	0.19	0.09
Interday repeatability mean (n = 3)	4,373,042	3,013,332	5,735,735	4,015,769	7,224,408	5,039,213
SD	1,926.41	4,805.92	27,430.12	6,429.97	8,333.14	9,555.83
%RSD	0.04	0.16	0.48	0.16	0.11	0.19

RT: Retention time, SD: Standard Deviation, RSD: Relative standard deviation. a: Analysis of 3 determinations.

Specificity and system suitability

Analysis of specificity and system suitability studied with 6 replicates of a combination of paclitaxel and curcumin standard (40 µg/ml) shown in Figure 8 has clear separation of the drugs with no Supplementary peaks seen. Table 7 shows that the developed method was found to be reproducible with %RSD for the RT and the chromatogram area to be < 1 %.

LOD and LOQ

From the calculation, the lowest detectable concentration was found to be 1.02 & 1.1 µg for curcumin and paclitaxel respectively. But the experiment was conducted and analyzed using standard solutions from 0.1 to 100 µg/ml concentrations. The chromatogram of the lowest detectable concentration of 0.1 µg/ml is shown in Supplementary Information Figure S8. The limit of quantitation was found to be 3.41 and 3.84 µg for curcumin and paclitaxel respectively.

Combination of paclitaxel and curcumin stability analysis

The combination of the drug paclitaxel and curcumin's stability indicating analysis was carried out to check how the drug behaves in combined form when it is induced in different pH conditions (4.5 & 7.4) with respect to time and temperature at 37°C. Studying the stability of the drugs gives an insight into the therapeutic dosage during *in-vitro* drug studies. The initial run (0th hour) of the samples showed two distinct intense peaks, which then decreased with the time period. Figure 9 shows the stability analysis peaks for a combination of drugs at 0th and 48th hour in 4.5 pH condition. Figure 10 shows the stability analysis peaks for a combination of drugs at 0th and 48th hour in 7.4 pH condition. The stability of the drugs was calculated in percentage using slope and was found approximately 8.7% of paclitaxel and 1.4% curcumin were stable at 4.5 pH and around 13% of paclitaxel and 2% of curcumin were stable at 7.4 pH at the end of 48 hours.

The stability analysis of the single drug paclitaxel/ curcumin was also conducted for a time period of 96 hours by using the optimized method, the data [Supplementary Information S9 (a–d) & S10 (a–d)] reveals that at 4.5 pH 62% of paclitaxel and 70% of curcumin were stable and at 7.4 pH around 19% of paclitaxel and 51% of curcumin were stable at the end of 96 hours. Batrawi *et al*. (2017) had shown that the combination of two antibiotic drugs under different pH conditions is stable and they do not degrade in presence of the other drug. But our results show that the stability of paclitaxel and curcumin in combination under different pH conditions decreased substantially compared to pure paclitaxel or curcumin in the same condition.

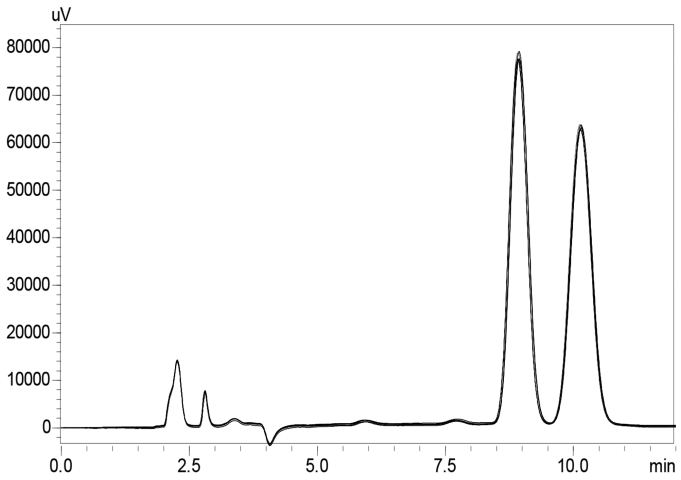

Figure 8. Replicates of curcumin & paclitaxel (40 µg/ml) showing specificity..

Drug loading analysis of combination of curcumin and paclitaxel nanoparticle by RP-HPLC

Curcumin and paclitaxel loaded nanoparticles were prepared using a single emulsion solvent evaporation with similar polymer-drug ratio and other experimental conditions as mentioned in (Hakkimane *et al*., 2018). The formulated nanoparticles were analyzed for their drug loading content using RP-HPLC with the help of the validated method. Table 8 represents the amount of the drug loaded per mg of nanoparticles. Around 101 µg of paclitaxel and 62 µg of curcumin were found to be loaded for every 1 mg of nanoparticles. This confirms the loading of the drugs into the nanoparticle was similar to the results obtained (Swaminathan *et al*., 2013).

Table 7. Specificity and system suitability.

Samples* µg/ml	$R_{t(CUR}$	$R_{t(PTX)}$	Area under curve curcumin	Area under curve paclitaxel
Mean ± SD	8.923333 ± 0.009	10.13833 ± 0.0106	2,764,425 ± 24,573.8	2,035,408 ± 19,432.06
RSD	0.001057	0.001053	0.008889	0.009547
%RSD	0.10	0.10	0.89	0.95

SD = Standard Deviation; RSD = Relative standard deviation.

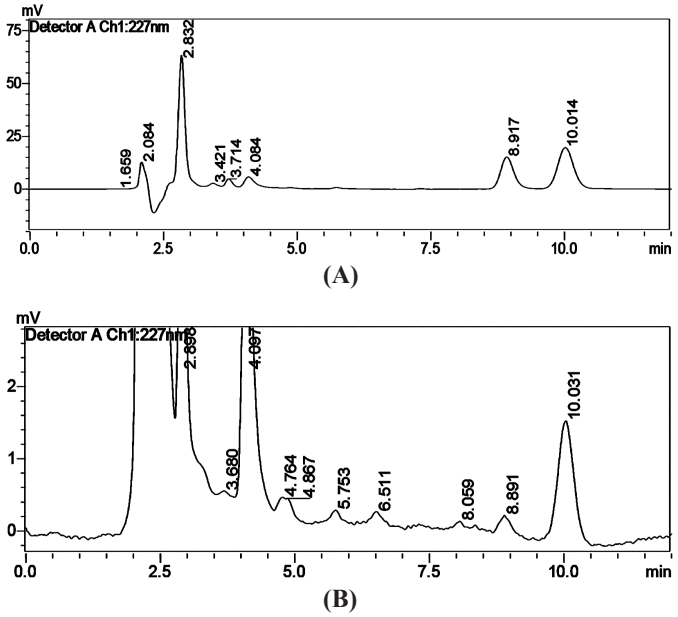

Figure 9. Stability analysis of combination of drugs at (A) 0th and (B) 48th hour in 4.5 pH.

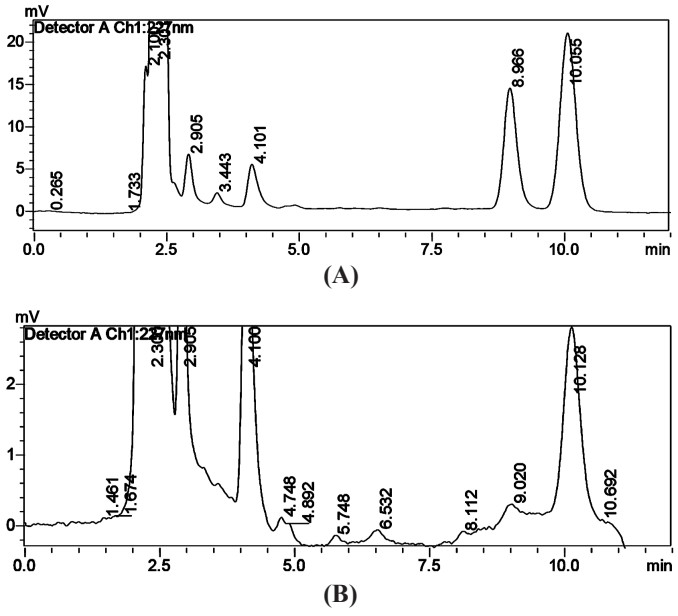

Figure 10. Stability analysis of combination of drugs at (a) 0th and (b) 48th hour in 7.4 pH.

Table 8. Drug loading of combination of paclitaxel and curcumin loaded PLGA nano formulation.

Batch	Area (units) paclitaxel	Area (units) curcumin	Drug loading of paclitaxel nanoparticles (amount of drug per mg of nanoparticle) (in µg)	Drug loading of curcumin nanoparticles (amount of drug per mg of nanoparticle) (in µg)
1	5,115,924	4,533,624	101	62
2	5,164,085	4,528,598	102	62
3	5,161,969	4,551,175	102	63

Average = 101 µg/mg for paclitaxel & 63 µg/mg for curcumin; PLGA = Poly (lactic-co-glycolic acid).

CONCLUSION

A simple RP-HPLC method for simultaneous estimation of paclitaxel and curcumin was developed and optimized. Optimization of the method for the combination of drugs by the conventional trial and error method would have been a tedious job. Design of Experiments (DOE) made it simpler with a minimum number of experiments. The applied BBD design gave more information about the interactional effect of the independent variables to accomplish the desired chromatographic response. The optimized RP-HPLC method developed for the simultaneous analysis of paclitaxel and curcumin was found to be simple, precise, and reproducible. The validation of the RP-HPLC method was carried out according to ICH guidelines. The absence of significant interfering peaks and lower %RSD values (<1%) shows that the developed method was sensitive. Considerable low LOD and LOQ values determine the method is suitable for quantifying and the detection of low concentrations of drugs. The method developed was effectively applied for the stability analysis of paclitaxel and curcumin in combined form. Results showed that the degradation of paclitaxel in presence of curcumin is faster. More research work on this area is needed to figure out the reason behind the fast degradation of paclitaxel in the presence of curcumin. The optimized method was used to analyze the drug loading content in polymeric nanoparticles. Hence the developed method can be implemented for the simultaneous analysis of paclitaxel and curcumin in pharmaceutical dosage forms in quality control.

AUTHOR'S CONTRIBUTION

The concept and design of this study was done by Dr Bharath Raja Guru & Joyceline Praveena. Acquisition of data, statistical analysis and manuscript writing was done by Joyceline Praveena. The acquired data analysis and the written manuscript was supervised and approved by Dr Bharath Raja Guru**.**

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

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GRAPHICAL ABSTRACT