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Original Article

BOX-BEHNKEN DESIGN ASSISTED ECO-FRIENDLY RP-HPLC-PDA METHOD FOR THE QUANTIFICATION OF PACLITAXEL: APPLICATION TO EVALUATE THE SOLUBILITY OF PACLITAXEL-CYCLODEXTRIN COMPLEX

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ABSTRACT

Objective: Paclitaxel (PTX) is one of the oldest chemotherapeutic agents for cancer treatment. However, PTX is a class IV drug under the Biopharmaceutical Classification System (BCS), and its oral administration is restricted due to its low bioavailability. Complexing PTX with Beta-Cyclodextrin (β-CD) is an option to overcome the low solubility and bioavailability. This study aims to optimize and develop an RP-HPLC analytical method for quantifying PTX from the fabricated β-CD complex.

Methods: The HPLC settings were optimized using Design-of-Experiments (DOE) software. The independent variables for the optimization process were buffer ratio, buffer pH, flow rate, and injection volume. The responses were Retention Time (RT), peak area, Tailing Factor (TF), and number of Theoretical Plates (TP) of PTX. The validated method was then used to measure the % entrapment from the PTX-β-CD complex.

Results: The developed and optimized RP-HPLC method was validated as per International Council for Harmonisation (ICH) Q2 (R1) guidelines. The developed method showed linearity R^2 = 0.999 with a 0.5-20 µg/ml range. The Limit of Detection (LOD) and Limit of Quantification (LOQ) were 95 and 125 ng/ml, respectively. The accuracy and precision for the developed method came under the acceptance criteria. The developed method was used to evaluate the enhancement of solubility of the prepared PTX-β-CD complex. The method was also used in the evaluation of % drug loading, % drug release and stability of the PTX-β-CD complex. The study clearly showed that the solubility of PTX increased from 0 to 1.14±0.53 µg/ml at pH 1.2 and 0 to 3.18±0.61 µg/ml at pH 6.8, respectively. The PTX-β-CD complex showed 73±3.75% drug release in 120 min at pH 1.2 and 87±3.51% at pH 6.8. The developed RP-HPLC method was found to be eco-friendly as per the Analytical Greenness (AGREE) metric approach and software analysis.

Conclusion: An eco-friendly RP-HPLC analytical method was successfully developed and optimized for the quantification of PTX from the PTX-β-CD complex.

Keywords: Paclitaxel, HPLC, Design of experiment, Cyclodextrin complex, Solubility

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INTRODUCTION

Cancer is a major health problem and is the leading cause of death. There were 1958310 new cancer cases and 609820 cancer deaths in the year 2023 in the United States as per cancer statistics [1]. Amongst the various options, such as surgery, immunotherapy, radiation therapy, adjuvant therapy, and hormonal therapy available for the treatment of cancer, chemotherapy is one of the most common treatment options, which involves the use of cytotoxic chemicals to eliminate the tumor or at least reduce the invasion of cancer cells to prolong life [2-4]. Paclitaxel (PTX) is an anticancer agent belonging to the class of taxanes derived from the bark of the pacific yew tree *Taxus brevifolia*, primarily used in treating ovarian, breast carcinoma, and acquired immunodeficiency syndrome-related Kaposi's sarcoma. It is an anti-microtubule drug that binds to tubulin and works to depolymerize or stabilize tubulin, thus disrupting the microtubule dynamics, causing mitotic arrest, and preventing cell division, resulting in apoptosis [5, 6].

PTX is categorized under Biopharmaceutical Classification System (BCS) class IV as having low solubility and low permeability, thus making it impossible to administer the drug orally. Intravenous or intramuscular route are the only choices for administering PTX [7]. The solubility of PTX is reported to be less than 0.1µg/ml, which limits its bioavailability [8]. Parenteral administration of drugs has many disadvantages such as pain, anaphylactic reactions, pulmonary infections, sepsis, and patient dislike associated with the injection. To overcome these problems, researchers are exploring the possibility of developing oral formulations for PTX [9]. However, low solubility and poor permeability remain significant concerns and limiting steps in formulation development. The structure of PTX is shown in fig. 1.

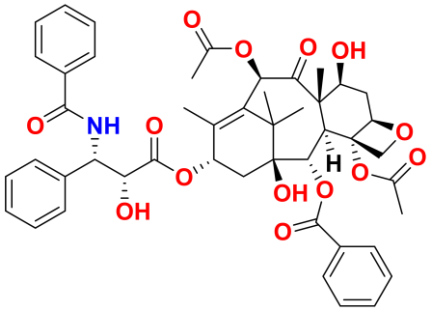

Fig. 1: Pictorial representation of chemical structure of paclitaxel

Beta-Cyclodextrin (β-CD) are complexing agents primarily used to increase the solubility and stability of poorly soluble drugs. β-CD complex is expected to overcome the solubility issue, making the drug administrable orally. The β-CD complex offers additional benefits such as taste masking and shelf-life prolongation of drug products [10]. CDs are toroidal in shape, having a hydrophilic outer surface and hydrophobic inner cavity. Hence, various hydrophobic guest molecules can be incorporated into the hydrophobic cavity, thus forming a stable host-guest complex and increasing the water solubility of the drug [11, 12]. Literature suggests the need for developing the PTX-β-CD complex to overcome the solubility and bioavailability issues of PTX, making it a suitable candidate for oral administration [13, 14].

Formulation development and optimization require determination of drug content from the proposed formulation to evaluate the solubility, % entrapment, stability, % release etc. As an initial step towards formulation development, it is essential to have a sensitive analytical technique to determine the formulation's drug content. Currently, no methods are available for determining the PTX content from the PTX β-CD complex. In this study, we aim to develop a novel and robust Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) method for evaluating the solubility, drug loading, % entrapment, % drug release, and stability of the prepared PTX β-CD complex.

Design-of-Experiments (DOE) is a powerful statistical technique that systemically investigates the relationship and interaction between the independent variables in a method development process. The DOE software aims to identify the critical variables that affect the response. The advantages of the DOE software include efficient experimentation, reduced number of experiments, improved data analysis, increased experiment precision, reduced experiment cost, and improved work quality etc. This work used the DOE technique to optimize the RP-HPLC method development process. The Box-Behnken Design (BBD) was used for the optimization process.

MATERIALS AND METHODS

Materials

PTX was provided as gift sample by Neon Laboratories, Mumbai, India. β-CD was supplied by cyclolab, India. The HPLC-grade methanol and acetonitrile were procured from Finar Chemicals India. All the other reagents, such as potassium dihydrogen phosphate, were obtained from Merck, USA. Orthophosphoric acid was obtained from Finar Chemicals, India. Potassium hydroxide was purchased from Himedia Chemicals, India. HPLC grade water was used to prepare all the solutions after passage through the Milli-Q system obtained from Merck, India. HyperClone Octadecylsilane C_{18} column (5 µm particle size, 120 Å, 250 mm × 4.6 mm) was purchased from Phenomenex (Hyderabad, India).

Instrumentation

HPLC system LC20-AD has a Photo Diode Array (PDA) detector (SPD-20A and SPD-M10A) and LabSolution. The software was from Shimadzu Corporation, Japan. All the chemicals used in the analysis were weighed carefully on the calibrated weighing balance (Sartorius Mechatronics CP225D, India). The prepared buffer solution was filtered through glass vacuum filtration assembly unit, the 0.45 µm membrane filter (Merck India Ltd) was used. Ultra sonicator from Serve Well Instruments, India was used to degas the solvents for 10 min. A calibrated pH meter Systronics India Ltd (Ahmedabad, India) was used to check the pH of the mobile phase.

Preparation of mobile phase, calibrators and quality control samples

Standard stock solution of PTX (1 mg/ml) was prepared in methanol. The 10 mg of PTX was weight accurately and dissolved in the 10 ml of methanol. The solution was vortex for 2 min. and the resultant solution was stored at 8 °CAfter dilution, the working stock solution (100 µg/ml) was prepared from the primary stock solution. The calibration solutions 0.5,1,2,4,8,16,20 µg/ml were prepared using a working stock solution of 0.5–20 µg/ml, respectively. Quality Control (QC) samples were prepared at the concentration of 80%, 100%, and 120% of PTX. All the calibration and QC samples were prepared in methanol.

DOE aided HPLC mothed development using box-behnken design

Initial chromatographic conditions were decided based on the literature review and chemistry of the drug. The mobile and stationary phases were selected based on the pKa (10.9) and Log P (2.3) value of PTX. The phosphate buffer and methanol were chosen for the mobile phase, and the HyperClone Octadecylsilane C_{18} column used as the stationary phase. Isocratic mode was used for all indicated experiments, and the autosampler temperature was set to 10 °C.

DOE is a mathematical technique that helps to understand the different degrees of complexity. Applying DOE in chromatographic method development helps in predicting how the flow rate, mobile phase composition, buffer pH, and injection volume affect the Retention Time (RT), peak area, Tailing Factor (TF), and number of Theoretical Plates (TP). To identify the optimized method parameters DOE has different response surface methods such as Central Composite Design (CCD) and BBD [15, 16]. The BBD design was used for the optimization process. The four independent variables were used in the optimization process: buffer ratio (X1), buffer pH (X2), flow rate (X3) and injection volume (X4). The dependent variables were the RT of PTX (Y1), the peak area of PTX (Y2), TF of PTX (Y3), and TP of PTX (Y4).

Method validation

The optimized RP-HPLC for PTX method was validated as per International Council for Harmonisation (ICH) Q2(R1) guidelines. Six injections of standard solution (1 µg/ml) of PTX were injected and the TF, TP, and RT were calculated to determine the system suitability. A selectivity study was performed to check the interference of any solvent and matrix. The linearity curve of the PTX was plotted between peak area (y) and concentration (x) using the regression model. The calibration curve range was (0.5–20 µg/ml) and injected in triplicates. The accuracy was achieved at 80%, 100%, and 120%, by comparing mean calculated values to nominal concentrations [17, 18]. The precision was performed for inter-day and intra-day. Six samples were injected, and the % CV was calculated. The robustness of the developed method was investigated by changing chromatographic parameters slightly at a time [19–21]. The Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated using equations 1 and 2, respectively.

$$\text{LOD} = \frac{3.3\,\sigma}{s} \quad \text{Equation (1)}$$

$$\text{LOQ} = \frac{10\,\sigma}{s} \quad \text{Equation (2)}$$

Preparation of PTX-β-CD complex

A modified co-solvent lyophilization method was used to prepare the PTX-β-CD complex. The complex was prepared in the different drug and β-CD molar ratios (1:1,1:3,1:5) to obtain maximum complexation leading to optimal solubility enhancement. A respective amount of β-CD per molar ratio was dissolved in 2.5 ml of deionized water in a solubility vial. In another vial, 4.26 mg of PTX was added, followed by 200 µl** of organic solvent (Methanol, acetonitrile, ethanol) separately. Both vials were vortexed to get a clear solution. The above clear drug solution was added dropwise for 10 min to the CD solution with continuous stirring and continued stirring for the next 6-7 h using a magnetic stirrer at 400 rpm. The mixture was filtered through a 0.45 µ Polyvinyl Difluoride (PVDF) filter and kept for lyophilization overnight to get the PTX-β-CD complex. Until further use, the prepared PTX-β-CD complexes were stored in glass vials at 2-8 °C [22-24].

Determination of drug loading in the PTX-β-CD complex

About 6.22 mg of PTX-β-CD complex was dissolved into a 10 ml volumetric flask. The complex was dissolved in a sufficient quantity of diluent containing a mixture of acetonitrile, methanol, and buffer at a ratio of 30:50:20 to make up the final volume of 10 ml. From the above solution, 2 ml solution was pipetted out and diluted to 10 ml with diluent. The solution was filtered with a 0.45 µm PVDF syringe filter, and the filtrate was taken into an autosampler vial [25]. The % drug loading was calculated using equation 3.

$$\%\ \text{Loading} = \frac{\text{Concentration of paclitaxel calculated in complex}}{\text{Weight of paclitaxelMBCD complex taken}} \times 100 \ldots \ldots \text{Equation (3)}$$

Solubility studies of prepared PTX-β-CD complex

A solute apparent solubility is the empirically determined solubility in a solvent system when equilibrium cannot be established or when the system is not given enough time to attain equilibrium. Due to temporary supersaturation, inadequate dissolution, and insufficient time to achieve equilibrium, the apparent solubility may be either higher or lower than the equilibrium solubility. Equilibrium solubility may be defined as the concentration limit; at

thermodynamic equilibrium, a solute can dissolve into a saturated solution when excess solid is present and no longer changes during a certain time frame. Apparent and equilibrium solubility of the PTX-β-CD was checked at stomach pH 1.2 (HCL solution) and intestinal pH 7.4 (phosphate buffer) to determine the improvement in the solubility of the PTX-β-CD complex [26–28].

***In vitro* dissolution studies of PTX-β-CD complex**

The dissolution behavior of the prepared PTX-β-CD complex was compared with the pure PTX and the physical mixture. The dissolution study used the USP type II apparatus (paddle method) at pH 1.2 and 7.4. An amount of PTX-β-CD complex equivalent to 10 mg of PTX was added to the dissolution media. The experiment was conducted at a stirring speed of 50 rpm, while maintaining the temperature at 37±0.5 °C. At specific time intervals (5, 10, 15, 30, 45, 60, 90, and 120 min), 10 ml samples were withdrawn using a syringe and filtered using a 0.45 µm PVDF disc filter. Subsequently, the samples were appropriately diluted, and the concentration of PTX was analyzed using HPLC. During the experiment, 10 ml of fresh dissolution media was added at regular intervals to maintain a constant total volume of the dissolving medium. The dissolution efficiency tests were carried out in triplicate to ensure accuracy [29, 30].

Stress-induced degradation studies of PTX-β-CD complex

The purpose of stress-induced degradation is to assess the stability of the PTX-β-CD complex in stressful circumstances. Acidic, alkaline, oxidative, thermal, and photolytic stress conditions were applied to the samples. For these investigations, an amount PTX-β-CD complex equivalent to 1 mg of PTX was utilized. The developed analytical method was used to assess the drug content in the degradation samples. The assay was done in triplicate, and the percentage of deterioration was determined [31, 32].

We assessed the impact of two different molar concentrations of HCl (0.1 N and 1 N) for the acid hydrolysis process. The complex was combined with twice as much acid, heated at 60 °C, and neutralized by adding NaOH. After further diluting the diluent sample, it was analyzed using HPLC [33, 34].

In an alkaline media, forced degradation of PTX-β-CD complex was tested at two different molar concentrations of NaOH (0.1 M and 1 M). After mixing twice as much alkali with the PTX-β-CD complex, the mixture was heated at 60 °C. The resulting solutions were neutralized using 0.1 M and 1 M HCl. After further dilution, these samples were analyzed using HPLC [34, 35].

The forced degradation by hydrogen peroxide was accomplished by mixing PTX-β-CD complex with 2 ml of 3% w/v H_2O_2 solution and keeping it at room temperature in the dark for 24 h. Samples were then diluted and analyzed using HPLC [36, 37].

PTX-β-CD complex was left in the sun for 24 h to investigate photolytic degradation. The samples were further diluted and analyzed using HPLC [38, 39].

PTX-β-CD complex was mixed with 2 ml of water for the thermal degradation investigation, and the combination was then heated for 24 h at 60 °C. The degradation samples were analyzed using HPLC [40, 41].

Stability on storage of the prepared PTX-β-CD complex

The stability of the prepared PTX-β-CD complex was to check the suitable storage condition. The stability can affect the PTX concentration in the complex. The prepared PTX-β-CD complexes stability study was evaluated for up to 30 days with sampling points 0, 3,7,15, and 30 days, at 0-4 °C, 2-8 °C, and room temperature [42, 43].

Greenness of the analytical procedure

The greenness of the analytical method was performed using the Analytical Greenness (AGREE) metric approach and software [44, 45]. This technique considers twelve factors, each of which is given a score between 0 and 1, with higher numbers denoting a greener approach.

RESULTS AND DISCUSSION

The analytical technique required for the solubility study, release profile, stability, % complexation, and the % drug loading of the PTX-β-CD complex was based on the HPLC technique. DOE was used to reduce the number of trials and to make the analytical method green. The PDA detector was chosen based on the absorbance of PTX at 227 nm. Based on the previously reported literature and chemistry of PTX, the Octadecylsilane C_{18} column was selected as the stationary phase. PTX has the hydrophobic taxane ring in its structure, which interacts with the nonpolar stationary phase. Based on the pKa of PTX, the ideal pH at which the drug will be in an ionized form is 3.0. As a result, the phosphate buffer was chosen as the aqueous phase. Further optimization of chromatographic conditions was done using DOE.

DOE aided optimization of the method using box-behnken design (BBD)

The independent variables in this study, including buffer phase (X1), buffer pH (X2), flow rate (X3), and injection volume (X4), were optimized using a three-level BBD. The upper and lower limits were calculated from the One-Factor at a Time (OFAT) approach. Based on the OFAT trials. The upper limit for buffer was 30% and the lower limit was 20%. The upper limit for buffer pH was 3.1, and the lower limit for buffer pH was 2.8. The upper limit for flow rate was 1.1 ml/min, and the lower limit was 0.8 ml/min. The upper limit for injection volume was 15 µl, and the lower limit was 5 µl, respectively. Accordingly, the DOE suggested 24 different runs of these independent variables, and the center point was 5 for this model.

HPLC runs were performed for 24 runs suggested by the DOE tool, and the response obtained from the experiment was used for the method optimization. The parameters used for the optimization of the chromatographic condition were RT of PTX (Y1), area of PTX (Y2), TF of PTX (Y3), and TP of PTX (Y4). Table 1 shows the different runs and the obtained responses. Table 2 shows the Analysis of Variance (ANOVA) with $p<0.001$.

Effect of independent variables on the retention time of PTX (Y1)

The ANOVA analysis showed that the independent variables buffer ratio (X1) and flow rate (X3) had a significant effect on the retention time of PTX (Y1) $p<0.001$. In contrast, buffer pH (X2) and injection volume (X4) do not significantly affect the RT of PTX. The generated quadratic equation (Equation 4) showed the buffer ratio (X1) and flow rate (X3) showed a positive effect on the RT of PTX (Y1). An increase in X1 and a decrease in X3 will increase the RT of PTX. X2 and X4 did not significantly affect the RT of PTX.

$$Y1 = +5.72435 + 1.75792 \times A - 0.022125 \times B - 0.868583 \times C - 0.000791667 \times D - 0.003875 \times AB - 0.268625 \times AC - 0.00825 \times AD + 0.007 \times BC + 0.013 \times BD - 0.006625 \times CD \quad \text{... Equation (4)}$$

The 3D plot, fig. (2a) and the perturbation plot, fig. (3a) showed that RT of PTX was significantly influenced by altering the buffer ratio (A) and flow rate (C).

Effect of independent variables on peak area of PTX (Y2)

The ANOVA analysis's quadratic equation (Equation 5) generated revealed that independent variables X1, X3, and X4 significantly affected the response Y2 with $p<0.001$. As the factors X1 and X3 decrease, the peak area of PTX (Y2) will increase. As the factor X4 increases, the peak area of PTX(Y2) will increase.

$$Y2 = +33182.4 - 3066.25 \times A - 221 \times B - 2627.58 \times C + 13811.5 \times D - 179.5 \times AB + 324.5 \times AC - 1630.75 \times AD - 260.5 \times BC - 465.75 \times BD - 8881 \times CD \quad \text{.... Equation (5)}$$

The 3D plot, fig. (2b), and the perturbation plot, fig. (3b), showed that the area of PTX was significantly influenced by altering the buffer ratio, flow rate, and injection volume.

Effect of independent variables on the tailing factor of PTX (Y3)

The independent variables X2 and X3 have a more significant effect on the TF of PTX, while the independent variables X1 and X4 were less significant. The quadratic equation (equation 6) showed that increased pH of the buffer and decreased flow rate leads to reduced TF of the PTX. Similarly, when the buffer ratio and injection volume decrease, the PTX tailing decreases.

$$Y3 = +1.39271 + 0.009375 \times A - 0.0085 \times B + 0.0822917 \times C + 0.0179167 \times D + 0.046875 \times AB + 0.010875 \times AC + 0.001875 \times AD$$ ..... Equation (6)

The 3D plot, fig. (2c), and the perturbation plot, fig. (3c) showed that tailing factor of PTX was significantly influenced by altering the buffer pH and flow rate.

Table 1: The experimental design suggested by the DOE and their responses

Std	Run	Buffer ratio (%)	Buffer pH	Flow rate (ml/min)	Injection volume (µl)	RT of PTX	Area of PTX	TF of PTX	TP of PTX
16	1	25	3.1	1.1	10	4.5105	25165	1.4915	2619.95
11	2	20	2.95	0.95	15	4.369	55139	1.395	2771.74
4	3	30	3.1	0.95	10	7.947	30720	1.445	3227.15
1	4	20	2.8	0.95	10	4.385	37614.5	1.3335	2505.9
21	5	25	2.8	0.95	5	5.575	15385	1.38	2812.35
15	6	25	2.8	1.1	10	4.5085	25656.5	1.462	2548.36
17	7	20	2.95	0.8	10	4.908	41850	1.453	2902.17
7	8	25	2.95	0.8	15	6.1285	52262.5	1.2925	3486.43
2	9	30	2.8	0.95	10	7.9925	31776.5	1.408	3152.82
13	10	25	2.8	0.8	10	6.178	34469.5	1.304	3352.06
10	11	30	2.95	0.95	5	7.845	15592.5	1.37	3313.85
9	12	20	2.95	0.95	5	4.338	18180	1.3685	2635.86
22	13	25	3.1	0.95	5	5.466	15658.5	1.384	2677.23
18	14	30	2.95	0.8	10	8.9025	34849.5	1.314	3889.2
3	15	20	3.1	0.95	10	4.355	37276	1.183	2967.52
24	16	25	3.1	0.95	15	5.453	46210.5	1.445	3023.17
8	17	25	2.95	1.1	15	4.478	38127.5	1.462	2724.76
14	18	25	3.1	0.8	10	6.152	35020	1.3045	3362.87
20	19	30	2.95	1.1	10	6.4465	25440	1.457	2965.47
23	20	25	2.8	0.95	15	5.51	47800	1.4675	2970.21
6	21	25	2.95	1.1	5	4.4715	38201.5	1.4895	2745.37
12	22	30	2.95	0.95	15	7.843	46028.5	1.404	3341.67
19	23	20	2.95	1.1	10	3.5265	31142.5	1.5525	2327.55
5	24	25	2.95	0.8	5	6.0955	16812.5	1.259	3477.05

RT: Retention Time, TF: Tailing Factor, TP: Theoretical Plates, PTX: Paclitaxel.

Table 2: ANOVA results of box-behnken design

Response	RT of PTX (Y1)		Peak area of PTX (Y2)		TF of PTX (Y3)		TP of PTX (Y4)	
F-value	**19.11**		**9.89**		**2.91**		**11.44**	
p-value	**Model**	**<0.0001**	**Model**	**<0.0001**	**Model**	**0.0366**	**Model**	**<0.0001**
	A	<0.0001	A	0.0678	A	0.6433	A	<0.0001
	B	0.8788	B	0.8881	B	0.6744	B	0.3060
	C	<0.0001	C	0.1116	C	0.0008	C	<0.0001
	D	0.9956	D	<0.0001	D	0.3804	D	0.2147
	AB	0.9877	AB	0.9474	AB	0.1919	AB	0.2054
	AC	0.2955	AC	0.9050	AC	0.7560	AC	0.2509
	AD	0.9738	AD	0.5514	AD	0.7560	AD	0.7159
	BC	0.9778	BC	0.9237			BC	0.8375
	BD	0.9587	BD	0.8640			BD	0.5286
	CD	0.9790	CD	0.0054			CD	0.9194
R^2	0.9363		0.8838		0.5598		0.9174	
Adjusted R^2	0.8873		0.7945		0.3672		0.8539	

Note: The symbols A, B, C, and D stand for buffer ratio, pH, flow rate, and injection volume; AB, AC, AD, BC, BD, and CD denote combinations of these variables. RT: Retention time, PTX: Paclitaxel, TF: Tailing Factor, TP: Theoretical Plates.

Effect of independent variables on the theoretical plate of PTX (Y4)

The ANOVA analysis quadratic equation (Equation 7) revealed that the independent variables X1 and X3 significantly affect the TP of PTX. The buffer ratio increase and flow rate decrease lead to an increase in TP of PTX. The buffer pH and injection do not significantly affect the TP of PTX.

$$Y4 = +2991.7 + 314.952 \times A + 44.6835 \times B - 378.193 \times C + 54.6899 \times D - 96.8226 \times AB - 87.2746 \times AC - 27.0151 \times AD + 15.1964 \times BC + 47.0194 \times BD - 7.49587 \times CD$$ ..... Equation (7)

The 3D plot, fig. (2d), and the perturbation plot, fig. (3d) showed that the TP of PTX was significantly influenced by altering the buffer ratio and flow rate.

Desirability and final optimized method

As per the ANOVA analysis, a desirability plot was generated (fig. 4), and the chromatographic conditions with desirability of 1 were confirmed as optimized conditions. The finalized conditions were Column: Phenomenex HyperClone C_{18} column (5 µm particle size, 120 Å, 250 mm × 4.6 mm); Mobile phase composition and ratio: phosphate buffer: methanol (23:77); Mobile phase pH: 3; Flow rate: 0.9 ml/min; Injection volume 15 µl; Mode of analysis: Isocratic; Column temperature: 25 °C

Under these circumstances, chromatographic runs (n=6) were performed using a Shimadzu quaternary HPLC system that was connected to an autosampler and a PDA detector at wavelengths of 227 nm. After comparing each response's achieved results with the regression model's projected values, it was discovered that each response's relative error was less than 10%. In fig. 5, the chromatogram acquired under ideal circumstances is displayed.

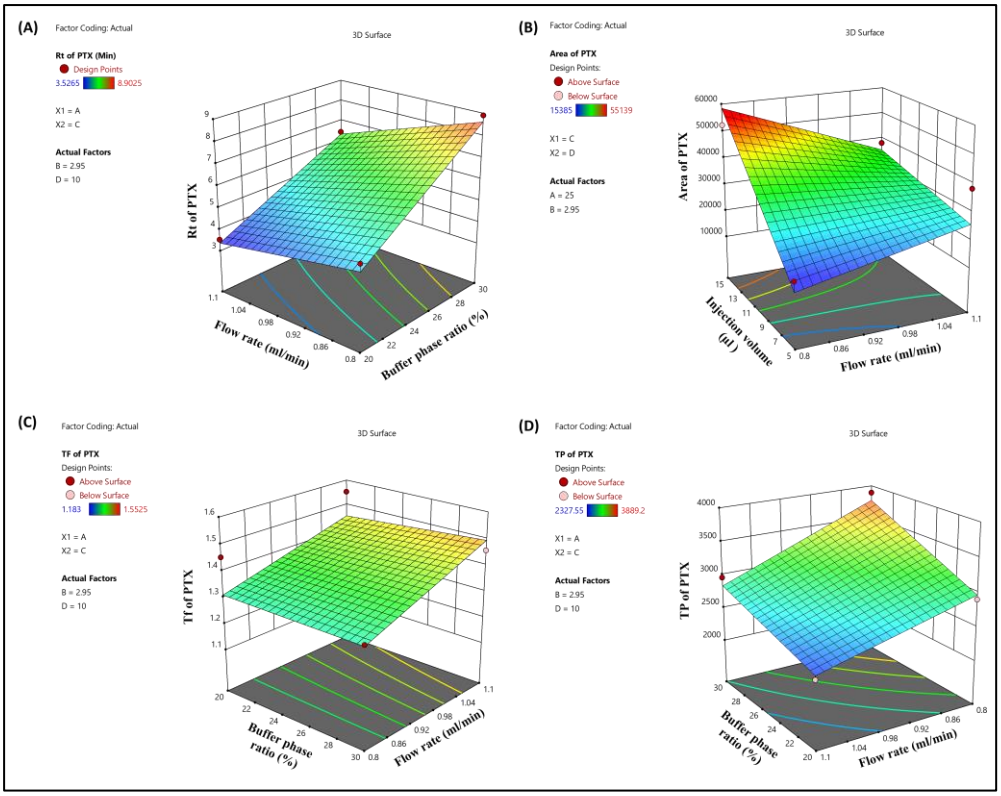

Fig. 2: The 3D surface response plot showing the effect of independent variables: (2a) on the RT of PTX (Y1); (2b) on the peak area of PTX (Y2); (2c) on the TF of PTX (Y3), and (2d) on the TP of PTX (Y4)

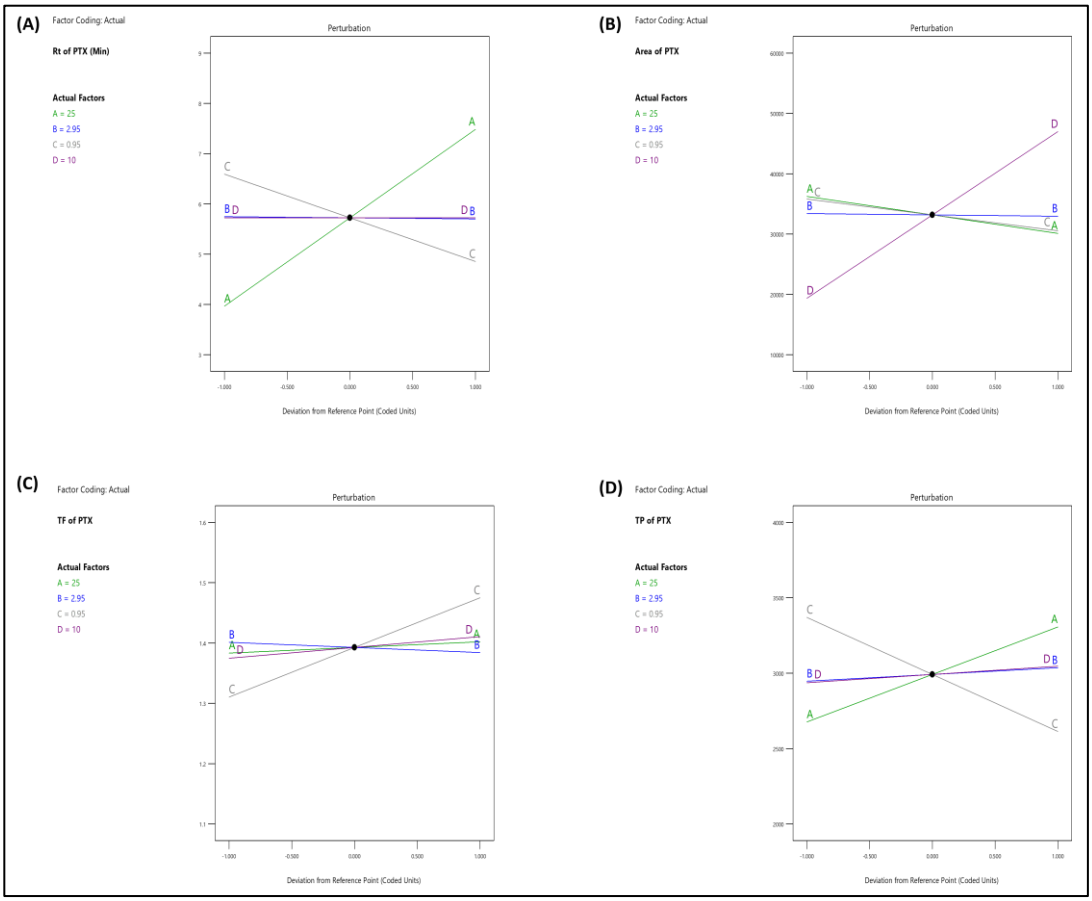

Fig. 3: Perturbation plot depicting the interactions of independent variables: (3a) on the RT of PTX (Y1); (3b) on the peak area of PTX (Y2); (3c) on the TF of PTX (Y3), and (3d) on the TP of PTX (Y4)

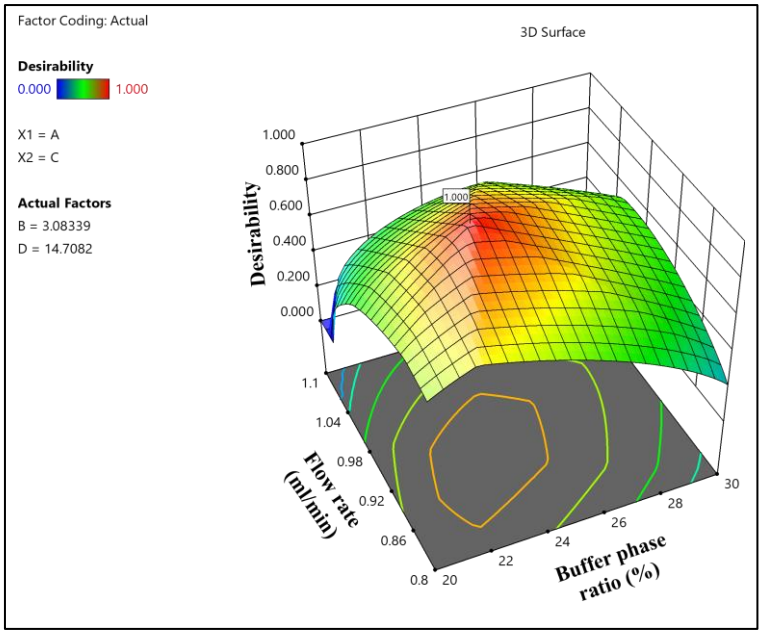

Fig. 4: The 3D surface response desirability plot for the optimized analytical method

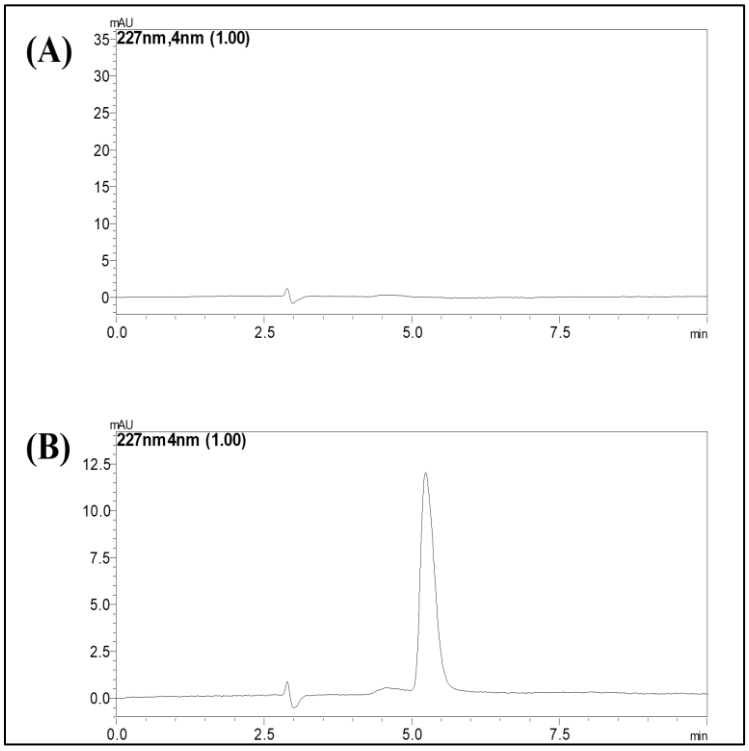

Fig. 5: Chromatogram obtained at optimized conditions (A) Chromatogram of blank formulation, (B) Chromatogram of formulation containing PTX

Method validation

HPLC method was validated as per ICH guidelines. Parameters like RT, TF, resolution, and TP were calculated to assess the system's suitability (table 3). Mohammad *et al.*, developed the RP-HPLC method for the quantification of PTX from the pharmaceutical dosage forms. The author used nucleosil RP-C_{18} column (5 µm particle size, 250 mm × 4 mm) with the RT of 6.17 min. The RT of our developed method was 5.22 which showed that our developed RP-HPLC method is very suitable and effective [6]. The system suitability parameters that were evaluated produced outcomes that were satisfactory. There was no interference in blank and placebo at the RT of PTX. The peak of PTX had a peak purity index of 1.00, indicating the method is specific to the analyte of interest. Badea *et al.*, developed RP-HPLC method for the determination of PTX from the pharmaceutical formulation with the RT of 13.14 min and RT of our developed method was 5.22 min which demonstrated that our developed method is more budget and eco-friendly [46].

The method was linear in the 0.5-20 µg/ml range. The correlation coefficient for PTX was 0.999 and the linear equation was y = 19700x-910.06. Jain *et al.*, developed the RP-HPLC analytical method

for the simultaneous quantification of PTX and topotecan. The method was developed using acetonitrile and water as mobile phase and Phenomenex luna C_{18} (2) column as a stationary phase. The RT of PTX was 14.56 and for topotecan 23.81 min. The our developed RP-HPLC method was optimized using DOE tool with the RT of PTX was 5.22 min and validated as per ICH guidelines [47]. The accuracy was checked by calculating the percentage recovery at three concentrations (80%, 100%, and 120%). The % recovery was found to be between 100-102%, showing good method accuracy. The inter-day and intra-day precision of the developed method was evaluated for PTX. The %RSD was less than 2% for intra-day and inter-day method precision. Choudhury *et al.*, developed the RP-HPLC method to check the oil solubility of PTX. The LOD and LOQ of the method were 100 and 300 ng/ml in triacetin oil. In olive oil LOD and LOQ of the method were 500 and 1500 ng/ml [48]. The LOD and LOQ of PTX were found to be 95 ng/ml and 125 ng/ml, respectively. This showed that our method is more sensitive that previously reported method.

Xavier junior *et al.*, developed HPLC method to quantify and evaluate the solubility of PTX in the copaiba oil-based formulation. Author validated the method as per ICH guidelines. The robustness was performed on the flow rate, column temperature and mobile phase ratio. The % RSD was observed less than 2 for the parameters [49]. In our developed RP-HPLC method small adjustments to the buffer's pH, column temperature, flow rate, injection volume, and absorbance maxima were investigated. The little adjustments made to these parameters did not significantly affect the RT, peak area, TF, and TP. This demonstrates that the existing analytical technique is reliable enough to investigate PTX. The drug was stable at room temperature at bench top and in the autosampler at 15 °C for a period of 24 h.

Table 3: Validation data of the optimized analytical method

Retention time*	**5.221±0.021**
Theoretical plate*	2291.4±2.04
Tailing factor*	1.554±0.012
Linearity range (µg/ml)*	0.5-20
HETP*	50.159±0.016
R^2	0.999
Linearity equation	Y=19700x-910.06
LOD (ng/ml)	95
LOQ (ng/ml)	125
Accuracy (% Recovery)	
80%*	100.753±1.07
100%*	100.289±1.66
120%*	102.708±0.92
Precision (% CV)	
Repeatability	0.187
Inter-day	0.587

*Data represent as mean±SD n =3, HETP: Height Equivalent to a Theoretical Plate, PTX: Paclitaxel, LOD: Limit of Detection, LOQ: Limit of Quantification.

Determination of drug loading of PTX in β-CD complex using the validated method

The % drug loading of PTX was optimized using various trials by changing the ratio of PTX and β-CD as shown in table 4. The optimized PTX-β-CD complex showed a drug content of 145.18±1.95 µg PTX per mg of the complex. Ethanol as a solvent gave the best results. The optimized complex gave 103.28±1.38% assay, indicating no loss of the PTX during the formulation process. Oroumiyeh *et al.*, Checked the solubility of PTX in the different excipients and reported the PTX have more solubility in ethanol. The % loading of PTX was high in ethanol because the PTX more soluble in ethanol than methanol and acetonitrile [50].

Table 4: Different PTX and β-CD molar ratios for the PTX-β-CD complex

Solvent	**Molar ratio of PTX and β-CD**	**Drug loading (µg)**
Methanol	1:1	50.27±1.69
	1:3	85.23±1.36
	1:5	115.24±2.91
Acetonitrile	1:1	65.23±0.84
	1:3	99.91±2.41
	1:5	131.28±1.96
Ethanol	1:1	80.12±2.49
	1:3	115.25±3.51
	1:5	145.18±2.79

Data represent as mean±SD n =3, PTX: Paclitaxel, β-CD: Beta-Cyclodextrin.

Solubility studies of prepared PTX-β-CD complex

Pure PTX and PTX-β-CD solubility studies were performed at pH 1.2 and 6.8. The samples were analyzed using the developed HPLC method. The solubility of PTX-β-CD at pH 1.2 was 1.14±0.53 µg/ml, and at pH 6.8, solubility was 3.18±0.61 µg/ml, respectively. The pH-dependent solubility of pure PTX was not observed as the PTX lacks the ionization group that could affect the solubility. The β-CD increases the solubility of the PTX by reducing the hydrophobic interaction of the PTX with surrounding water molecules [10]. The hydrogen interaction between the drug and the hydroxyl group of β-CD is expected to be the major reason for the solubility enhancement [26]. Velazquez *et al.*, fabricated the PTX and β-CD to increase the solubility, dissolution and bioavailability of the PTX. Author performed the solubility study of the complex and the solubility of complex was found to be $700.0 \pm 93.2\ M^{-1}$ and $1847.5 \pm 26.1\ M^{-1}$ for water and phosphate buffer saline: ethanol, respectively [51].

In vitro dissolution studies of prepared PTX-β-CD complex

The release studies were performed at sink conditions to determine the pattern of PTX release from the complex at pH 1.2 and 7.4. The results of the release profile study are shown in fig. 6. The PTX complex showed 73±3.75% drug release in 120 min at pH 1.2 and 87±3.51% drug release at pH 7.4. The results showed good release of the drug PTX from the complex both at stomach and intestinal pH, which would be helpful for its absorption. The release study showed that the PTX-β-CD complex releases more PTX in the intestinal microenvironment than stomach pH, suggesting that the *in vivo* PTX absorption will be from the intestine [52].

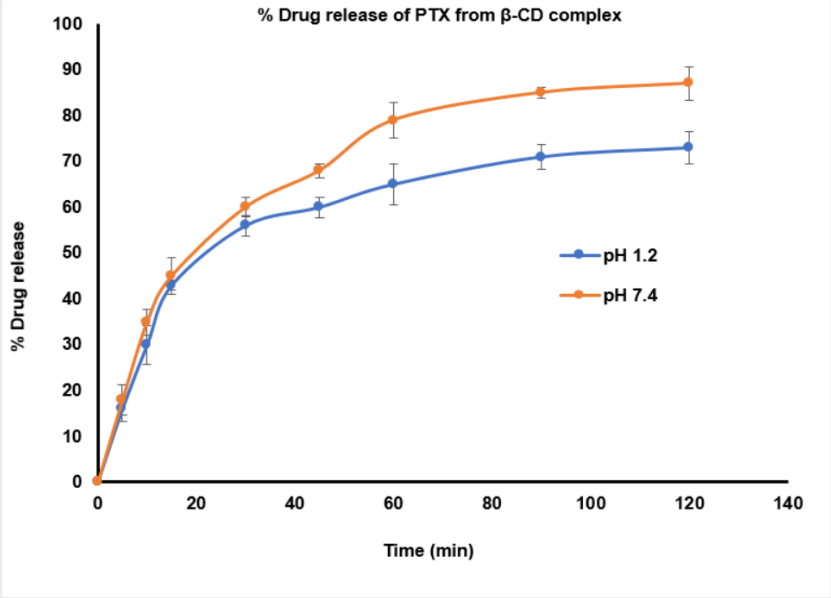

Fig. 6: Pictorial representation of % drug release at pH 1.2 and pH 7.4. Error bars indicate the SD values of 3 replicates

Forced degradation study results

A forced degradation study was conducted to determine the stability of PTX in the complex under various stress conditions. A thorough degradation investigation was thus performed. The effect of various stress conditions and the respective degradation are represented in fig. 7. More than 64% of PTX has been degraded in the 0.1 M HCL solution, and more than 78% of PTX has been degraded in 1 M HCL. PTX showed more than 31% degradation in 0.1 M NaOH and more than 52% degradation in 1 M NaOH solution. Degradation was more than 78% under oxidation conditions with H_2O_2. Degradation was more than 76% in sunlight (UV) conditions and less than 11% in forced temperature. These studies illuminate the intrinsic stability of the PTX in the complex. The high amount of degradation indicates the need to perform future LCMS studies to understand the degradation pathways.

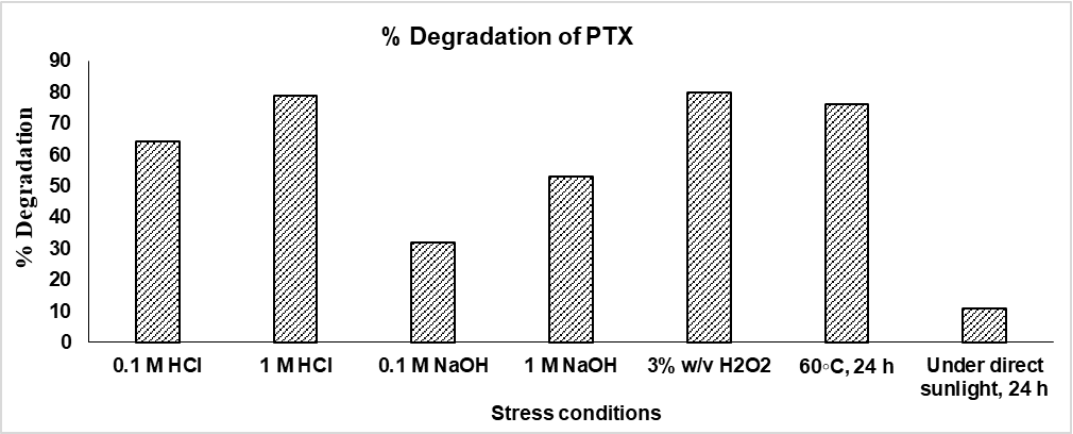

Fig. 7: Column chart representation of forced degradation study of PTX showing the % degradation under each condition

Stability on storage of prepared PTX-β-CD complex

The stability study of the PTX-β-CD was performed at 0-4 °C and 2-8 °C and room temperature. The PTX-β-CD complex exhibited good stability for up to 30 d at 0-4 °C and at 2-8 °C and room temperature (fig. 8). The PTX-β-CD complex was unstable at room temperature with a more than 65.04% loss. This study suggests the need for storing the PTX-β-CD complex at refrigeration temperature (2-8 °C). Bouquet *et al.*, conducted the stability study of the PTX-β-CD complex for 6 mo and reported that the complex was stable at 4 °C. The reason for the complex's stability was hydrogen bonding and Van der Waals interactions between the PTX and β-CD [53]. Parrenne *et al.*, evaluate the stability of the β-CD phenobarbital solution. The observations showed no degradation of the complex at the refrigerator and 20 °C [54].

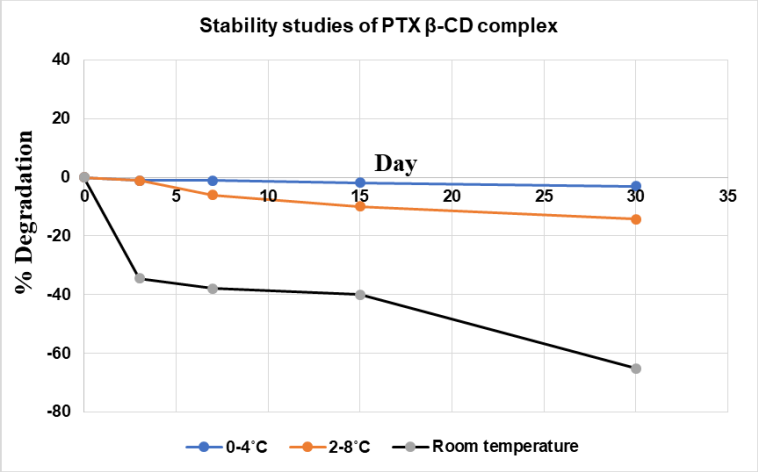

Fig. 8: Pictorial representation of stability of PTX-β-CD complex

Greenness of analytical method

Evaluating the effects of analytical procedures on the environment, operator safety, and health is the aim of Green Analytical Chemistry (GAC), especially when those techniques require the use of organic solvents in the mobile phase. In recent years, there has been a tremendous increase in interest in GAC, making it feasible to rate the greenness of various analytical approaches using a range of green evaluation tools. Demir *et al.*, developed the ecofriendly RP-HPLC method for the quantification of metoclopramide from the pharmaceutical formulation. Author used AGREE software for the evaluation. The AGREE score was 0.75 which was coming under the acceptance criteria more than 0.5 and method was ecofriendly [55]. The results of greenness analysis using the AGREE program is depicted in fig. 9. The overall AGREE score of 0.66 suggests that the developed HPLC technique for PTX quantification is environmentally safe.

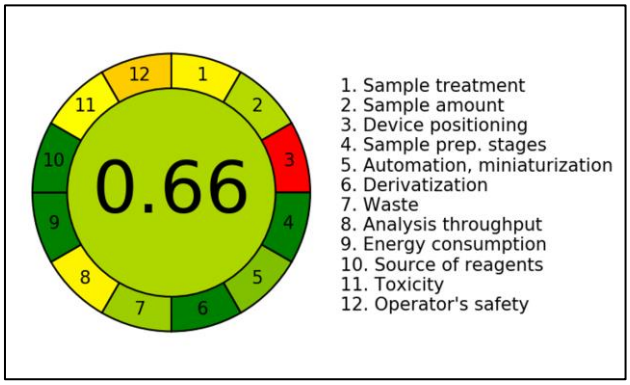

Fig. 9: Pictorial representation for greenness profile assessment of RP-HPLC method

CONCLUSION

An eco-friendly RP-HPLC analytical method was successfully developed and optimized to quantify PTX from the PTX-β-CD. The method was optimized using the three-level BBD. The DOE analysis revealed a desirability of 1. The optimized analytical method was validated as per ICH Q2 (R1) guidelines. The method was used for the *in vitro* characterization of the prepared PTX-β-CD complex. The PTX loading in β-CD was found to be 145.18±1.95 µg PTX per mg of complex. The solubility of PTX-β-CD at pH 1.2 was 1.14 µg/ml, and at pH 6.8 solubility was 3.18 µg/ml, respectively. The result has clearly shown the β-CD complex's ability to improve the solubility of the otherwise insoluble PTX, which could pave the way for an oral dosage form of PTX. The results of the force degradation study have proved the need for further studies to profile the degradation products. The stability study evaluation demonstrated the need to store the complex at 2-8 °C in the refrigerator. Future bioavailability studies in animal models are to be conducted to evaluate the applicability of the PTX-β-CD as a promising option for oral delivery of PTX.

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Nil

AUTHORS CONTRIBUTIONS

Conceptualization, methodology, validation, formal analysis, investigation, and original draft writing were done by Ashutosh Gupta. Methodology, formal analysis, and original draft writing were done by Rima V. Kossambe. Review and editing, supervision of work, finalization, and manuscript draft were conducted by Corresponding author Sudheer Moorkoth.

CONFLICT OF INTERESTS

The authors state that the manuscript does not include any conflicts of interest.

REFERENCES

1. Siegel RL, Miller KD, Wagle NS, Jemal A. Cancer statistics 2023. CA Cancer J Clin. 2023;73(1):17-48. doi: 10.3322/caac.21763, PMID 36633525.

2. Nygren P, SBU Group. Swedish council on technology assessment in health care what is cancer chemotherapy. Acta Oncol. 2001;40(2-3):166-74. doi: 10.1080/02841860151116204, PMID 11441929.
3. Sudhakar A. History of cancer ancient and modern treatment methods. J Cancer Sci Ther. 2009;1(2):1-4. doi: 10.4172/1948-5956.100000e2, PMID 20740081.
4. Gupta A, Nishchaya K, Saha M, Naik GA, Yadav S, Srivastava S. Recent advancements in nanoconstructs for the theranostics applications for triple negative breast cancer. J Drug Deliv Sci Technol. 2024 Jan 25;93:105401. doi: 10.1016/j.jddst.2024.105401.
5. Barbuti AM, Chen ZS. Paclitaxel through the ages of anticancer therapy: exploring its role in chemoresistance and radiation therapy. Cancers. 2015 Dec 3;7(4):2360-71. doi: 10.3390/cancers7040897, PMID 26633515.
6. Mohammadi A, Esmaeili F, Dinarvand R, Atyabi F, Walker RB. Development and validation of a stability indicating method for the quantitation of paclitaxel in pharmaceutical dosage forms. J Chromatogr Sci. 2009;47(7):599-604. doi: 10.1093/chromsci/47.7.599, PMID 19772735.
7. Gala UH, Miller DA, Williams RO. Harnessing the therapeutic potential of anticancer drugs through amorphous solid dispersions. Biochim Biophys Acta Rev Cancer. 2020;1873(1):188319. doi: 10.1016/j.bbcan.2019.188319, PMID 31678141.
8. Sharma S, Verma A, Teja BV, Shukla P, Mishra PR. Development of stabilized paclitaxel nanocrystals: *in vitro* and *in-vivo* efficacy studies. Eur J Pharm Sci. 2015;69:51-60. doi: 10.1016/j.ejps.2014.11.012, PMID 25559064.
9. Vijayakumar A, Sharon EV, Teena J, Nobil S, Nazeer I. A clinical study on drug-related problems associated with intravenous drug administration. J Basic Clin Pharm. 2014;5(2):49-53. doi: 10.4103/0976-0105.134984, PMID 25031500.
10. Tiwari G, Tiwari R, Rai AK. Cyclodextrins in delivery systems: applications. J Pharm Bioallied Sci. 2010;2(2):72-9. doi: 10.4103/0975-7406.67003, PMID 21814436.
11. Iacovino R, Caso J, Donato C, Malgieri G, Palmieri M, Russo L. Cyclodextrins as complexing agents: preparation and applications. Curr Org Chem. 2016;21(2):162-76. doi: 10.2174/1385272820666160909111842.
12. YE YJ, Wang Y, Lou KY, Chen YZ, Chen R, Gao F. The preparation characterization and pharmacokinetic studies of chitosan nanoparticles loaded with paclitaxel/dimethyl-β-cyclodextrin inclusion complexes. Int J Nanomedicine. 2015;10:4309-19. doi: 10.2147/IJN.S83508, PMID 26170666.
13. Shah M, Shah V, Ghosh A, Zhang Z, Minko T. Molecular inclusion complexes of β-cyclodextrin derivatives enhance aqueous solubility and cellular internalization of paclitaxel: preformulation and *in vitro* assessments. J Pharm Pharmacol (Los Angel). 2015 Jan 10;2(2):8. doi: 10.13188/2327-204X.1000011, PMID 25950011.
14. Liang N, Yan C, Liu Z, HU W, Jiang K, Li G. Functionalized hydroxypropyl-β-cyclodextrin derivative as a versatile nanovehicle for delivery of paclitaxel. Journal of Polymer Science. 2024;62(11):2399-409. doi: 10.1002/pol.20230833.
15. Mutalik SP, Mullick P, Pandey A, Kulkarni SS, Mutalik S. Box-behnken design aided optimization and validation of developed reverse phase HPLC analytical method for simultaneous quantification of dolutegravir sodium and lamivudine co-loaded in nano liposomes. J Sep Sci. 2021 Aug;44(15):2917-31. doi: 10.1002/jssc.202100152, PMID 34076952.
16. Naik S, Mullick P, Mutalik SP, Hegde AR, Lewis SA, Bhat K. Full factorial design for development and validation of a stability indicating RP-HPLC method for the estimation of timolol maleate in surfactant based elastic nano vesicular systems. J Chromatogr Sci. 2022 Jul 12;60(6):584-94. doi: 10.1093/chromsci/bmab101, PMID 34435614.
17. Gupta A, Navti PD, Mutalik S, Saha M, Moorkoth S. DoE guided development of an HPLC method for evaluation of amoxicillin and metronidazole co-loaded mucoadhesive GRDDS formulation for H pylori eradication. Chromatographia. 2023 Dec 1;86(11-12):729-42. doi: 10.1007/s10337-023-04290-z.
18. Kothawade SN, V Pande VV. Development and validation of an RP-HPLC method for deferiprone estimation in pharmaceutical dosage form. Asian J Pharm Clin Res. 2023 Apr 7:100-3. doi: 10.22159/ajpcr.2023.v16i4.47000.
19. Salwa, Kumar L. Quality by design driven analytical method (AQbD) development and validation of HPLC–UV technique to quantify rivastigmine hydrogen tartrate in lipidic nanocarriers: forced degradation, and assessment of drug content and *in vitro* release studies. Micro Chemical Journal. 2023 Oct;193:108944. doi: 10.1016/j.microc.2023.108944.
20. Pavani Y, Aravind S, Annam C, Subba Rao MS. Quantification and quality control of triple drug combination employed for gastric ulcer using stability indicating selective RP-HPLC technique. Int J App Pharm. 2024 May 7;16(3)140-8. doi: 10.22159/ijap.2024v16i3.50320.
21. Saha M, Gupta A, Shetty S, Mutalik S, Nandakumar K, Raghu Chandrashekar H. DoE-aided optimization of RP-HPLC method for simultaneous estimation of amoxicillin and tinidazole loaded mucoadhesive GRDDS formulation for the treatment of H. pylori. Chromatographia. 2024 Jul;2:1-16. doi: 10.1007/s10337-024-04346-8.
22. Huang J, PU J, Yang Z, Zhang S, Zhang Z, LU Q. Development of a syzygium aromaticum L. essential oil/hydroxypropyl-β-cyclodextrin inclusion complex: preparation characterization and evaluation. Ind Crops Prod. 2024 Aug 1;214:118500. doi: 10.1016/j.indcrop.2024.118500.
23. Zhang Y, LI F, Guo G, Xiu Y, Yan H, Zhao L. Preparation and characterization of betulin/methyl beta-cyclodextrin inclusion complex electrospun nanofiber: improving the properties of betulin. Ind Crops Prod. 2024 Mar 1;209:117974. doi: 10.1016/j.indcrop.2023.117974.
24. Singh RK, Deora AS. An adduct: 5-fluorouracil incorporated β-cd inclusion complex for solubility and stability enhancement in dosage forms. Int J App Pharm. 2022 Mar 7:14(2)286-92. doi: 10.22159/ijap.2022v14i2.43602.
25. Hussein K, Turk M, Wahl MA. Drug loading into β-cyclodextrin granules using a supercritical fluid process for improved drug dissolution. Eur J Pharm Sci. 2008 Mar 3;33(3):306-12. doi: 10.1016/j.ejps.2008.01.003, PMID 18282694.
26. Saokham P, Muankaew C, Jansook P, Loftsson T. Solubility of cyclodextrins and drug/cyclodextrin complexes. Molecules. 2018 May;23(5):1161. doi: 10.3390/molecules23051161, PMID 29751694.
27. Marques HM, Hadgraft J, Kellaway IW. Studies of cyclodextrin inclusion complexes I. the salbutamol cyclodextrin complex as studied by phase solubility and DSC. International Journal of Pharmaceutics. 1990 Sep 30;63(3):259-66. doi: 10.1016/0378-5173(90)90132-N.
28. Bennani I, Yachi L, Chefchaouni AC, Hafidi Y, Moukafih B, El Marrakchi S. Solubilization improvement using non-ionic surfactants and phospholipids optimized by an experimental design: case of albendazole. Res J Pharm Technol. 2024 May 15;17(5):1995-2000. doi: 10.52711/0974-360X.2024.00316.
29. Dua K, Pabreja K, Ramana MV, Lather V. Dissolution behavior of β-cyclodextrin molecular inclusion complexes of aceclofenac. J Pharm Bioallied Sci. 2011;3(3):417-25. doi: 10.4103/0975-7406.84457, PMID 21966164.
30. Pardhi VP, Patel P, Vaish A, Jain K. Inclusion complexes of bedaquiline fumarate with β-cyclodextrin and its derivatives: in silico *in vitro* and *in vivo* evaluation. J Drug Deliv Sci Technol. 2024 Jan 1;91:105253. doi: 10.1016/j.jddst.2023.105253.
31. Hamid MH, Elsaman T. A stability indicating RP-HPLC-UV method for determination and chemical hydrolysis study of a novel naproxen prodrug. J Chem. 2017 Oct 25;2017:1-10. doi: 10.1155/2017/5285671.
32. Aiassa V, Garnero C, Zoppi A, Longhi MR. Cyclodextrins and their derivatives as drug stability modifiers. Pharmaceuticals (Basel). 2023 Jul 28;16(8):1074. doi: 10.3390/ph16081074, PMID 37630988.
33. Zaman B, Hassan W, Khan A, Mushtaq A, Ali N, Bilal M. Forced degradation studies and development and validation of HPLC-UV method for the analysis of velpatasvir copovidone solid dispersion. Antibiotics (Basel). 2022 Jul 5;11(7):897. doi: 10.3390/antibiotics11070897, PMID 35884151.
34. Alsarra IA, Ahmed MO, Alanazi FK, ElTahir KE, Alsheikh AM, Neau SH. Influence of cyclodextrin complexation with NSAIDs on

NSAID/cold stress-induced gastric ulceration in rats. Int J Med Sci. 2010 Jul 5;7(4):232-9. doi: 10.7150/ijms.7.232, PMID 20617127.

35. Goyal PK, Jaimini M. Development and validation of stability indicating RP-HPLC method for quantitative estimation of metoprolol succinate and azelnidipine from synthetic mixture. J Pharm Neg Res. 2023 Feb 6;14(3):772-9. doi: 10.47750/pnr.2023.14.03.101.
36. Gupta A, Navti PD, Mutalik S, Saha M, Moorkoth S. DoE guided development of an HPLC method for evaluation of amoxicillin and metronidazole co-loaded mucoadhesive GRDDS formulation for H. pylori eradication. Chromatographia. 2023 Oct 28;86(11-12):729-42. doi: 10.1007/s10337-023-04290-z.
37. Diamantis DA, Ramesova S, Chatzigiannis CM, Degano I, Gerogianni PS, Karadima KE. Exploring the oxidation and iron binding profile of a cyclodextrin encapsulated quercetin complex unveiled a controlled complex dissociation through a chemical stimulus. Biochim Biophys Acta Gen Subj. 2018;1862(9):1913-24. doi: 10.1016/j.bbagen.2018.06.006, PMID 29886278.
38. Emanuelli J, Eva Scherman Schapoval E. Stability indicating HPLC method for estimation of omarigliptin in tablets oxidative and photolytic kinetics and degradation products formed under oxidative conditions. Microchemical Journal. 2020;157(16):105084. doi: 10.1016/j.microc.2020.105084.
39. Carlotti ME, Sapino S, Ugazio E, Caron G. On the complexation of quercetin with methyl β-cyclodextrin: photostability and antioxidant studies. J Incl Phenom Macrocycl Chem. 2011 Jun 1;70(1-2):81-90. doi: 10.1007/s10847-010-9864-7.
40. Vianna RF, Bentley MV, Ribeiro G, Carvalho FS, Neto AF, DE Oliveira DC. Formation of cyclodextrin inclusion complexes with corticosteroids: their characterization and stability. International Journal of Pharmaceutics. 1998 Jun 1;167(1-2):205-13. doi: 10.1016/S0378-5173(98)00068-4.
41. Peraman R, Lalitha KV, Raja NM, Routhu HB. Identification of degradation products and a stability indicating RP-HPLC method for the determination of flupirtine maleate in pharmaceutical dosage forms. Sci Pharm. 2014;82(2):281-93. doi: 10.3797/scipharm.1310-01, PMID 24959399.
42. Connors KA. The stability of cyclodextrin complexes in solution. Chem Rev. 1997 Aug 1;97(5):1325-58. doi: 10.1021/cr960371r, PMID 11851454.
43. Huang J, Fang Z, Bai C, MO Y, Liu D, Yang B. Novel nano encapsulated limonene: utilization of drug in cyclodextrin in liposome formulation to improve the stability and enhance the antioxidant activity. Int J Pharm. 2024 Mar 25;653:123914. doi: 10.1016/j.ijpharm.2024.123914, PMID 38373597.
44. Pena Pereira F, Wojnowski W, Tobiszewski M. AGREE analytical GREEnness metric approach and software. Anal Chem. 2020;92(14):10076-82. doi: 10.1021/acs.analchem.0c01887, PMID 32538619.
45. Raveendran A, Gupta A, Lewis LE, Prabhu K, Moorkoth S. A comprehensive approach for detection of biotin deficiency from dried blood spot samples using liquid chromatography mass spectrometry. Future Sci OA. 2024 Dec 31;10(1):2355038. doi: 10.1080/20565623.2024.2355038, PMID 38963009.
46. Badea I, Ciutaru D, Lazar L, Nicolescu D, Tudose A. Rapid HPLC method for the determination of paclitaxel in pharmaceutical forms without separation. J Pharm Biomed Anal. 2004 Feb 18;34(3):501-7. doi: 10.1016/s0731-7085(03)00628-9, PMID 15127805.
47. Jain A, Gulbake A, Jain A, Shilpi S, Hurkat P, Kashaw S. Development and validation of the HPLC method for simultaneous estimation of paclitaxel and topotecan. J Chromatogr Sci. 2014 Aug 1;52(7):697-703. doi: 10.1093/chromsci/bmt101, PMID 23843442.
48. Choudhury H, Gorain B, Karmakar S, Pal TK. Development and validation of RP-HPLC method: scope of application in the determination of oil solubility of paclitaxel. J Chromatogr Sci. 2014 Jan 1;52(1):68-74. doi: 10.1093/chromsci/bms206, PMID 23293041.
49. Xavier Junior FH, Gueutin C, DO Vale Morais AR, DO Nascimento Alencar E, DO Egito ES, Vauthier C. HPLC method for the dosage of paclitaxel in copaiba oil: development validation application to the determination of the solubility and partition coefficients. Chromatographia. 2016 Apr 1;79(7-8):405-12. doi: 10.1007/s10337-016-3058-4.
50. Sadeghi Oroumiyeh A, Valizadeh H, Zakeri Milani P. Determination of paclitaxel solubility and stability in the presence of injectable excipients. Pharm Chem J. 2021 Dec 1;55(9):983-7. doi: 10.1007/s11094-021-02526-6.
51. Velazquez NS, Ferreyra MG, Mengatto LN, Santagapita PR, Buera MP, Luna JA. Paclitaxel/β-Cyclodextrin interactions a perspective from pulsed NMR spectroscopy experiments. Carbohydr Res. 2019;486:107828. doi: 10.1016/j.carres.2019.107828, PMID 31648123.
52. Weng J, Tong HH, Chow SF. *In vitro* release study of the polymeric drug nanoparticles: development and validation of a novel method. Pharmaceutics. 2020 Aug 4;12(8):732. doi: 10.3390/pharmaceutics12080732, PMID 32759786.
53. Bouquet W, Ceelen W, Fritzinger B, Pattyn P, Peeters M, Remon JP. Paclitaxel/β-cyclodextrin complexes for hyperthermic peritoneal perfusion formulation and stability. Eur J Pharm Biopharm. 2007 Jun 1;66(3):391-7. doi: 10.1016/j.ejpb.2006.11.025, PMID 17240125.
54. Parrenne L, Ribier Z, Abisror J, Cadix J, Benoit G, Bordenave J. Formulation and stability study of an oral paediatric phenobarbital 1% solution containing hydroxypropyl-β-cyclodextrins. Eur J Hosp Pharm. 2024 Jul 1;31(4):363-8. doi: 10.1136/ejhpharm-2022-003487, PMID 36754621.
55. Demir I, Bulduk I, Darwısh IA, Enginar H. A green approach for metoclopramide quantification in pharmaceutical products: new HPLC and spectrophotometric methods. Sci Rep. 2024 Apr 16;14(1):8765. doi: 10.1038/s41598-024-59149-6, PMID 38627518.