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Original Article

STABILITY BASED HPLC METHOD FOR CYCLOPHOSPHAMIDE RELATED SUBSTANCES IN FINISHED DRUG PRODUCTS: DEVELOPMENT AND VALIDATION

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ABSTRACT

Objective: The current study aimed to develop a simple, sensitive, and precise high-performance liquid chromatographic (HPLC) method for estimating cyclophosphamide and its related substances, as well as to implement the developed method in a capsule product.

Methods: Method development was performed using various solvent and buffer-solvent ratios at different flow rates for better resolution and to decrease the run time. The developed method was validated in accordance with the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines. The developed method was implemented to estimate the amount of cyclophosphamide and its related substances.

Results: Chromatographical conditions were optimised, and the best chromatographical conditions with adequate resolution for cyclophosphamide and its related substances were achieved using the enable X-Bridge C18 column, using a mobile phase combination of phosphate buffer pH 7.0, water, methanol, and acetonitrile at a flow rate of 0.5 ml/min. The detection was monitored at a wavelength of 200 nm. The developed method was validated for system suitability, specificity, limit of detection (LOD), limit of quantitation (LOQ), linearity, precision, accuracy and robustness. The results indicate that the method was sensitive and could detect and quantify lower levels of cyclophosphamide and its related substances. The linearity range was from LOQ to 150 %, and a correlation coefficient ranging from 0.9987 to 0.9999 indicates that at this concentration range, substances were highly linear. This was further supported by satisfactory forced degradation studies.

Conclusion: The developed analytical method is simple, precise, and reproducible and thus can be used for the stability-indicating analysis of cyclophosphamide and its related substances in pharmaceutical formulations.

Keywords: Analytical method development, Cyclophosphamide, and its related substances, Forced degradation studies, and Stability indicating

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INTRODUCTION

Cyclophosphamide, chemically known as N,N-bis(2-chloroethyl)-1,3,2-oxazaphosphinan-2-amine, is a widely utilized deoxyribonucleic acid (DNA) alkylating agent in cancer chemotherapy. Initially, cyclophosphamide exists as an inactive prodrug that requires enzymatic bioactivation to exert its cytostatic effects. Upon activation and the formation of phosphoramide mustard, cyclophosphamide transforms into a bifunctional alkylating agent. Phosphoramide mustard operates by alkylating nucleophiles, a process facilitated through the formation of aziridinyl ion intermediates [1, 2]. Cyclophosphamide has the ability to form both intra-and inter-strand DNA cross-links as well as DNA-protein cross-links. These interactions inhibit DNA replication and lead to cell death through apoptosis [3]. Related substances, which share structural similarities, can emerge at various stages of the manufacturing, storage, and handling of drug substances or drug products. The presence of these substances can significantly impact the quality, safety, and efficacy of the product. Therefore, it is crucial to detect these substances using precise analytical methods. Accurate analytical techniques are essential for evaluating the quality and safety of drugs. Upon reviewing the scientific literature, it has been suggested that there are three potential related substances in cyclophosphamide, namely related substance A, related substance B, and related substance D [4]. Detecting and quantifying these substances is vital for ensuring the integrity and effectiveness of cyclophosphamide-based medications. Through an extensive review of the literature, it has been observed that several methods exist for the *in vitro* estimation of cyclophosphamide: In the analysis of cyclophosphamide in bulk, researchers have employed techniques such as reverse phase (RP) HPLC with a photodiode array (PDA) detector [5], HPLC with an ultraviolet (UV) detector [4], UV Visible Spectrophotometer [6], and ion chromatography [7]. For evaluating cyclophosphamide in finished dosage forms, UV-visible spectrophotometers [6, 7] and ion chromatography [8] have been utilized. In the context of assessing cyclophosphamide in surface waters, researchers have turned to RP HPLC with tandem mass spectrometry [9]. Furthermore, the analysis of cyclophosphamide's related substances in bulk has been conducted using methods such as HPLC with a PDA detector [10] and liquid chromatography combined with quadrupole time-of-flight electrospray ionisation tandem mass spectrometry [11]. This extensive variety of techniques highlights the diverse approaches researchers have taken to accurately estimate cyclophosphamide and its related substances in different ways. Lastly, certain methods have been explored for the *in vivo* estimation of cyclophosphamide: HPLC with UV detector [2], liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) quadrupole mass spectrometer [12, 13], and ultra-performance liquid chromatography-mass spectrometry/mass spectrometry (UPLC-MS/MS) [14, 15]. From the existing literature, it is evident that two methods [10, 11] have been documented for the estimation of related substances in bulk drugs. However, as of now, no method has been reported for assessing related substances in the drug product. It's worth noting that one of the reported methods involves a specialised analytical instrument that might be financially inaccessible for many quality departments. Currently, there is a notable absence of an HPLC technique specifically tailored for quantifying the related substances within cyclophosphamide for drug products. Consequently, there arose a necessity to develop such a method, driving the focus of our further study. HPLC coupled with a UV detector offers excellent sensitivity for light-absorbing substances and is relatively user-friendly compared to other detectors like refractive index detectors, fluorescence detectors, or electron capture detectors. Alkylating agents, including cyclophosphamide, are generally hydrophilic due to the presence of amine groups. Because of their high polarity,

employing a straightforward chromatographic technique is quite challenging [16, 17]. The HPLC/UV technique emerges as a viable option for achieving the utmost sensitivity. This study endeavours to establish a precise, specific, and reproducible method for quantifying the related substances found in cyclophosphamide within drug products.

MATERIALS AND METHODS

Chemicals, reagents and standards

Cyclophosphamide and its related substances A, B, and D were procured from the United States of Pharmacopoeia (USP), India. with a purity of 100 %. HPLC grades of acetonitrile and ultra-HPLC grades of methanol were supplied by J. T. Baker Chemical Company, India. Analytical grades of hydrochloric acid (37 %), SUPRAPUR® hydrogen peroxide (30 %), EMSURE® sodium hydroxide, ACS sodium chloride, and EMPARTA® potassium dihydrogen phosphate were procured from Merck Life Science Private Limited, India. Milli-Q water was obtained from the Milli-pore system at Merck Life Science Private Limited, India.

Instrumentation

Liquid chromatography

The analysis was carried out on a Waters e2695 HPLC system equipped with an X-Bridge C18 column (Waters) (250 × 4.6 mm and 5 µm particle size) and a UV detector from Waters (India) Private Limited, India. All the data acquired was processed with Empower 3.0 software by Waters (India) Private Limited, India.

Other instruments and equipment

Various analytical columns, like the Prontosil Ghost Hunter column with a dimension of 53 × 4.6 mm from Bischoff, India, were used during development apart from the X-Bridge C18 column with dimensions of 250 × 4.6 mm and 5 µm particle size of Waters (India) Private Limited, India. A micropipette (Eppendorf, India), an analytical weighing balance (Mettler Toledo, Model XP205 and XP26) of Mettler Toledo, India, and a pH metre (Metrohm, Model 780) of Metrohm India Private Limited, India, were used. The mobile phase was filtered under the vacuum through 0.22 µm membrane filters (Merck, Millipore). A glass microfiber filter, 25 mm GD/X, 0.45 µm pore size, was procured from Whatman, India. 0.20 µm Nylon membrane filter and 0.45 µm polyvinylidene fluoride (PVDF) filter were purchased from Millipore, India. 0.45 µm polytetrafluoroethylene (PTFE) filter was procured from MDI, India.

Methods

Method development

Preparation of solutions

Prepared from 1N sodium hydroxide solution and pH 7.0 phosphate buffer, the pH 7.0 phosphate buffer was used as a mobile phase A, and the degassed water, methanol, and acetonitrile in the ratio of 20:40:40 %v/v, respectively. Prepared the 20.0 g of sodium chloride into 1000 ml of HPLC-grade water and sonicated to dissolve.

Preparation of cyclophosphamide standard stock solution

The equivalent of 43 mg of cyclophosphamide monohydrate (equal to 40 mg of cyclophosphamide) in a vortex machine (Remi, India) for 2 min to ensure complete dissolution. Subsequently, to achieve a concentration of approximately 200 µg/ml.

Cyclophosphamide-related substances A, B, and D stock preparation

Weighed and transferred 5 mg of cyclophosphamide related substance A into a 25 ml volume of HPLC-grade water. The mixture was agitated on a Vortex (Remi, India) until complete dissolution. It was then further diluted with HPLC-grade water to achieve a concentration of approximately 200 µg/ml. The same procedure was followed separately for preparing cyclophosphamide related substances B and D, both resulting in the same concentration.

Preparation of standard solutions for 25 mg and 50 mg strengths

Combine 5 ml of Cyclophosphamide standard stock solution with 5 ml of each impurity stock solution in a 100 ml volumetric flask. Dilute the mixture up to the mark with the diluent, ensuring thorough mixing. This results in a final concentration of approximately 10 µg/ml for cyclophosphamide, cyclophosphamide related substance A, cyclophosphamide-related substance B, and cyclophosphamide-related substance D in the solution.

Placebo solution preparations for 25 mg and 50 mg strengths

First, a sample of not less than 20 capsules were weighed, and the average filled weight was determined. Placebo powder equivalent to 250 mg of cyclophosphamide was weighed and transferred into a 50 ml volumetric flask. Approximately 30 ml of diluent was added, and the mixture was vortexed for 2 min. The volume was then diluted to the mark with diluent and mixed thoroughly. The resulting solution was centrifuged using a Remi centrifuge at 4000 rpm for 2 min. After centrifugation, the solution was filtered through a 0.45 µm glass microfiber filter, discarding the first 2 ml (approximately) of the filtrate.

Test sample solution preparations for 25 mg and 50 mg strengths

A sample of not less than 20 capsules was weighed to determine the average filled weight. Powder equivalent to 250 mg of cyclophosphamide was accurately weighed and transferred into a 50 ml volumetric flask. Approximately 30 ml of diluent was added, and the mixture was vortexed for 2 min. It was then further diluted with diluent and thoroughly mixed. The resulting solution, with a concentration of cyclophosphamide of about 5000 µg/ml, was centrifuged at 4000 rpm for 2 min. After centrifugation, the solution was filtered through a 0.45 µm glass microfiber filter, discarding the first 2 ml (approximately) of the filtrate. The system was purged in the mobile phase first. Then, two separate gradients were run under specific column conditions. Following this, a single injection of the blank solution was made, followed by six replicates of the standard solution. Subsequently, individual injections of the placebo, each test preparation, and the bracketing standard were introduced into the chromatographic system. The resulting chromatograms were carefully recorded for analysis.

Method validation

The developed method was validated in compliance with ICH guidelines for system suitability, specificity, limit of detection (LOD), limit of quantitation (LOQ), linearity, precision, accuracy, range, and robustness in accordance with ICH guidelines for analytical procedures Q2(R1) [18].

System suitability

Six replicate samples, each containing Cyclophosphamide, Cyclophosphamide related substance A, Cyclophosphamide related substance B, and Cyclophosphamide related substance D, were analysed employing the developed method. The acceptance criteria were established as follows: a theoretical plate count exceeding 2000, a tailing factor less than 2.0, and a percentage relative standard deviation (% RSD) of peak area less than 5 %.

Specificity

To confirm the specificity of the related substances, spiked test solutions were then injected into the HPLC system. The resulting chromatogram was compared with that of the blank. Specific acceptance criteria were applied, which included the following conditions:

1. Chromatogram comparison: The chromatograms of the blank and placebo preparations should not exhibit any peaks at the retention times corresponding to cyclophosphamide and its related substances.

2. Retention time comparison: The retention times observed for cyclophosphamide and its related substance peaks in the test solution should be comparable to those of the standard solutions.

3. Purity angle and purity threshold: The purity angle calculated for the peaks should be less than the specified purity threshold value.

Adhering to these criteria ensured the specificity of the analysis, confirming that the identified peaks in the test solution were indeed related to cyclophosphamide and its related substances.

Limit of detection and quantification

The LOD signifies the lowest concentration of an analyte that generates a response accurately, albeit not necessarily quantifiable to an exact value. On the other hand, the LOQ denotes the minimum concentration of the analyte that produces a precise response. In this study, LOD and LOQ values for cyclophosphamide and its related substance peaks were determined through the signal-to-noise (S/N) ratio method. The calculations involved the standard deviation of response (SD) and the slope (S) with the following formulas:

$$LOD = 3.3 \times SD/S$$

$$LOQ = 10 \times SD/S$$

The acceptance criteria established are as follows: The S/N ratio must not be less than 3 for LOD determination. The S/N ratio should be at least 10 for LOQ determination. For LOQ precision, the % RSD should not exceed 10.0 for the area response at the LOQ level. Adhering to these criteria ensures the accuracy and reliability of the LOD and LOQ values, allowing for sensitive and precise quantification of cyclophosphamide and its related substances.

Linearity

Linearity was assessed by measuring the response of standard solutions of related substances over a concentration range from the LOQ to 150 %. The linearity evaluation involved injecting solutions at different levels: LOQ (Level 1), 50 % (Level 2), 80 % (Level 3), 100 % (Level 4), and 150 % (Level 5) of the expected concentration for each substance. A linearity curve was generated by plotting the concentration of related substances at each level against the corresponding peak area. To establish linearity, it is recommended by ICH that a minimum of five concentration levels be used. In this case, five concentration levels (LOQ to 150 %) were employed. The correlation coefficient for cyclophosphamide and each related substance was calculated for each calibration curve. The acceptance criterion for the correlation coefficient was set at not less than 0.99. This stringent criterion ensures a strong linear relationship between the concentration of the analyte and the detector response, indicating the reliability of the analytical method over the specified concentration range.

Precision

The precision of the method was assessed to evaluate its repeatability. Three types of studies-system precision, method precision, and intermediate precision-were conducted at three different concentrations. Each sample was injected six times, and the RSD of the estimated concentration was determined for each study. The acceptance criteria for precision studies are as follows: %RSD should not exceed 5% for system precision; %RSD should not exceed 5% for method precision; and %RSD should not exceed 10% for intermediate precision. Additionally, a 95% confidence interval was calculated for each precision study. Adhering to these criteria ensures that the method is precise and reliable, both within the same laboratory (repeatability) and between different laboratories or analysts (intermediate precision).

Accuracy (recovery)

The accuracy of a method is expressed as the closeness of agreement between the value found and the value that is accepted as a reference value. Accuracy was performed by analysis of three control samples (without spiking) and the sample solution (spiked) in triplicate by spiking the standard solution of related substances of Cyclophosphamide at LOQ, 50 %, 100 %, and 150 % of the specification level for the specified substance, and the sample solution (spiked) in triplicate by spiking the standard solution of Cyclophosphamide at LOQ, 50 %, 100 %, and 150 % of the specification level for unspecified impurity. The acceptance criteria were set as follows: % recovery should be not less than 80 % and not more than 120 % at all spiked levels, including LOQ. Meeting these criteria ensures the method's accuracy and reliability, demonstrating that the results obtained are in close agreement with the true values, even at lower concentration levels.

Robustness

The robustness of the method was evaluated by deliberately introducing variations in method parameters. These experiments involved changing chromatographic conditions, such as flow rate, wavelength, pH, concentration of the mobile phase, and oven temperature. The aim was to assess the method's ability to remain unaffected by small variations in these parameters. The acceptance criteria for robustness testing were established as follows: %RSD should be less than 5 %, tailing factor should not exceed 2 %, and theoretical plates should not be less than 2000. Adhering to these criteria ensures that the method is robust, indicating its ability to produce consistent and reliable results even when minor variations are introduced in the chromatographic conditions.

Stability of mobile phase, standard solution, and sample solutions

In the stability testing of the method, various solutions, including standard solutions and spiked sample solutions, were prepared and injected at different time intervals. The solutions were stored in a refrigerator at a temperature of approximately 5±3 °C. The purpose was to assess the stability of these solutions over time.

Solution Stability: The standard solutions were prepared and injected at different time intervals (initial, 24 h, 48 h, and 72 h). The acceptance criteria for standard solution stability were that the percentage difference between the results obtained at the initial time and different time intervals should not exceed 10 %. Similarly, the sample solutions spiked with the specified related substance of cyclophosphamide at the specified level were prepared and injected at different time intervals (initial and up to 14 h). Similar to the standard solution stability, the percentage difference between the results obtained at the initial time and different time intervals should not exceed 10 %.

Mobile phase stability: The system suitability parameters should meet the acceptance criteria at all-time intervals. These stability studies are crucial to ensuring that the method remains reliable and the results obtained are consistent even when the solutions and mobile phase are stored for specific durations. The acceptance criteria set for each stability test guarantee the method's robustness and suitability for routine analysis.

Application of the validated method: forced degradation study

Acid degradation

The sample stock solution was combined with 5 ml of 5N hydrochloric acid at room temperature for 30 min. Afterward, the resulting solution was neutralised, diluted, filtered, and subsequently injected into the HPLC system. The chromatogram was recorded to analyse the components of the solution.

Base degradation

The sample stock solution was blended with 0.5 ml of 0.5N sodium hydroxide at room temperature for 1 min. Following this, the resulting solution was neutralised, diluted, filtered, and then injected into the HPLC system. The chromatogram was recorded for analysis.

Thermal degradation

The sample stock solution was heated in a hot air oven at 60 °C for 6 h and subsequently cooled. After cooling, the solution was further diluted, filtered, and injected into the HPLC system. The resulting chromatogram was recorded for analysis.

Photolytic degradation

The sample stock solution was exposed to 1.2 million LUX h and 200-watt h per square meter. Following exposure, the solution was diluted, filtered, and injected into the HPLC system. The chromatogram was then recorded for analysis.

Humidity degradation

The sample stock solution was subjected to 85 % relative humidity (RH) at room temperature for 7 d. After the exposure period, the solution was diluted, filtered, and injected into the HPLC system. The resulting chromatogram was recorded for analysis.

Oxidative degradation

The sample stock solution was combined with 5 ml of 30 % hydrogen peroxide at room temperature for 30 min. Following this

step, the resultant solution was neutralised, further diluted, filtered, and injected into the HPLC system. The resulting chromatogram was then recorded for analysis. Calculated the mass balance by using the following formula:

$$\text{Mass balance} = \frac{(\%\ \text{Assay of degradation sample} + \%\ \text{RS of degradation sample})}{(\%\ \text{Assay of as such sample} + \%\ \text{RS of as such sample})} \times 100$$

Acceptance criteria encompass the following conditions: Firstly, there should be no interference at the retention time corresponding to cyclophosphamide and its related substances in the chromatogram. Secondly, a comparable mass balance for both the assay and related substances is essential. Additionally, the purity angle should be below the specified purity threshold, indicating a more homogeneous peak. Lastly, the mass balance needs to fall between not less than 90 % and not more than 110 %. These stringent criteria ensure the accuracy and reliability of the analysis, confirming the absence of unwanted substances and the purity of the substances being measured.

RESULTS AND DISCUSSION

Method development

Various compositions of mobile phases were experimented with during the method optimisation process. Eventually, an appropriate mobile phase composition was identified for analysing cyclophosphamide and its related substances. The chosen mobile phase consisted of phosphate buffer pH 7.0 as mobile phase A and a mixture of water, methanol, and acetonitrile in a ratio of 20:40:40 %v/v, respectively, with degassed components as mobile phase B. This specific combination of mobile phases resulted in well-defined peaks, notably enhancing the peak shape of the related substances. Prior to use, the mobile phase was both filtered and degassed to ensure optimal performance in the analysis. Cyclophosphamide and its related substances were subjected to analysis using various columns. After experimenting with different options, the X-Bridge C18 column (Waters) emerged as the most suitable choice. A column temperature of 25 °C was selected for the separation of these related substances, with a sample cooler temperature of 5 °C. The flow rate of the mobile phase was maintained at 0.5 ml/min to achieve optimal resolution between the peaks. Additionally, an injection volume of 50 µl was set for the analysis, and the analyte was analysed at a wavelength of 200 nm. In addition to adjusting the flow rate, modifications were made to the gradient program. Initially, at 12 min, the composition of mobile phase A to mobile phase B was set at 85:15% v/v. Towards the end of the analysis, the composition was changed to 90:10 %v/v from 12 to 60 min. This change decreased the polarity, ensuring that the components were retained in the column for a longer duration, thus aiding in better separation and analysis.

Method validation

System suitability

System suitability parameters were calculated according to USP guidelines to confirm the adequacy of the chromatographic system for analysis. A system-suitability solution was prepared using the standard mixture solution of cyclophosphamide and its related substances, following the established method. This solution was injected six times into the HPLC system, and the results are presented in table 1. The developed method demonstrated theoretical plates exceeding 2000 and a tailing factor less than 2 for cyclophosphamide and its related substances. Additionally, the %RSD of the peak area was below 5, indicating the suitability of the developed method and the excellent performance of the chromatographic system.

Table 1: System suitability results of the developed HPLC method for cyclophosphamide and its related substances

Parameter	Area of peak			
	Cyclophosphamide	CRS-A	CRS-B	CRS-D
N	6	6	6	6
Mean	127180.367	140316.340	222115.195	61082.318
SD	2620.987	2205.047	557.766	266.206
% RSD	2.1	1.6	0.3	0.4
TP	147492	167658	10035	17803
TF	1.1	1.0	1.4	1.5

CRS-A: Cyclophosphamide related substance A; CRS-B: Cyclophosphamide related substance B; CRS-D: Cyclophosphamide related substance D; N: number of injections; SD: standard deviation; % RSD: percentage relative standard deviation; TP: theoretical plates; TF: tailing factor;

Specificity

The specificity parameter data demonstrates that in both blank and placebo preparations, no peak was observed at the retention time corresponding to the cyclophosphamide peak. The retention times for cyclophosphamide and its related substances obtained from the standard solution were comparable to those from the spiked sample solution. Additionally, the peak purity of cyclophosphamide and its related substances met the acceptance criteria. Therefore, the method was found to be specific, making it suitable for the determination of related substances of cyclophosphamide in cyclophosphamide capsules using HPLC. The retention time, purity angle, and threshold of the cyclophosphamide and its related substances peaks from the standard solution, individual standard solution, spiked sample, and sample solutions are detailed in table 2 and fig. 1.

Table 2: Retention time, purity angle, and threshold for cyclophosphamide and its related substances

Substance	Parameter	Standard solution	Individual standard solution	Spiked sample	Sample solution	RRT
Cyclophosphamide	RT	35.498	34.878	34.856	34.842	1.00
	PA	1.103	-	0.038	0.043	
	PT	2.019	-	0.294	0.296	
CRS-A	RT	30.603	30.599	30.586	ND	0.88
	PA	0.336	-	0.532	ND	
	PT	0.748	-	1.115	ND	
CRS-B	RT	6.945	6.971	6.981	6.975	0.20
	PA	0.244	-	0.498	1.185	
	PT	0.542	-	0.646	3.099	
CRS-D	RT	9.174	9.226	9.255	9.274	0.27
	PA	0.684	-	0.734	12.463	
	PT	1.835	-	2.740	42.816	

CRS-A: Cyclophosphamide related substance A; CRS-B: Cyclophosphamide related substance B; CRS-D: Cyclophosphamide-related substance D; RT: Retention time (min); PA: Purity angle; PT: Purity threshold; RRT: relative retention time; ND: Not detected.

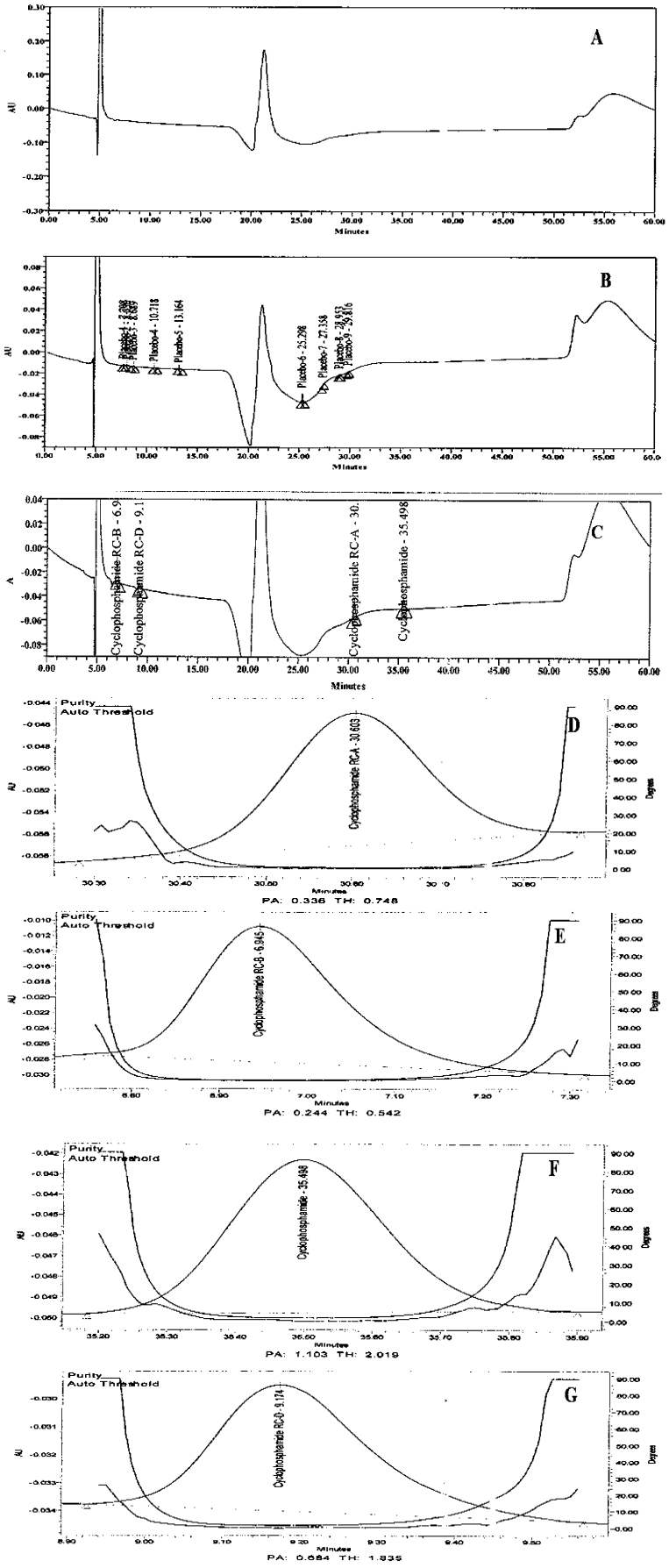

Fig. 1: Chromatogram of A. Blank solution; B. Placebo solution; C. Standard solution; D. Cyclophosphamide related substance A; E. Cyclophosphamide related substance B; F. Cyclophosphamide-related substance D; and G. Cyclophosphamide

Limit of detection and quantification

The LOD and LOQ were determined using the signal-to-noise (S/N) method. For LOD, the signal-to-noise ratio (S/N) should be not less than 3, and for LOQ, it should be not less than 10. The S/N ratios for each related substance were presented in table 3. The reported LOQ values for cyclophosphamide and its related substance peaks were found to be below 50 % of the specification concentration, meeting all the required acceptance criteria. The LOQ precision data for Cyclophosphamide and its related substances peaks indicated that the method is precise for the detection and quantitation of related substances of Cyclophosphamide in Cyclophosphamide Capsules by HPLC, as detailed in table 4.

Linearity

The correlation coefficients for cyclophosphamide and its related substances were observed to range from 0.9987 to 0.9999, as indicated in fig. 2. This confirms the achievement of acceptable linearity within the range from LOQ to 150 %. The summarised results of linearity can be found in table 5.

Precision

The developed method demonstrated precision for both system, method, and intermediate analyses, as the % RSD values fell within the acceptance criteria. Detailed information is provided in tables 6 and 7 below.

Table 3: The LOD, LOQ and S/N ratio for cyclophosphamide and its related substances

Substance	LOQ*	S/N	LOD*	S/N
Cyclophosphamide	0.0322	15	0.0096	4
CRS-A	0.0319	17	0.0095	4
CRS-B	0.0198	21	0.0059	5
CRS-D	0.0522	15	0.0156	4

*With respect to sample concentration (% w/w); CRS-A: Cyclophosphamide related substance A; CRS-B: Cyclophosphamide related substance B; CRS-D: Cyclophosphamide related substance D; LOQ: quantification limit; LOD: limit of detection.

Table 4: The LOQ precision data for cyclophosphamide and its related substances peaks

Parameter	Area of peak			
	Cyclophosphamide	CRS-A	CRS-B	CRS-D
N	6	6	6	6
Mean	20312.599	20006.185	20524.414	17264.487
SD	909.410	574.677	530.275	250.156
%RSD	4.5	2.9	2.6	1.4

LOQ: quantification limit; CRS-A: Cyclophosphamide-related substance A; CRS-B: Cyclophosphamide-related substance B; CRS-D: Cyclophosphamide-related substance D; N: Number of injections; SD: standard deviation; %RSD: percentage relative standard deviation.

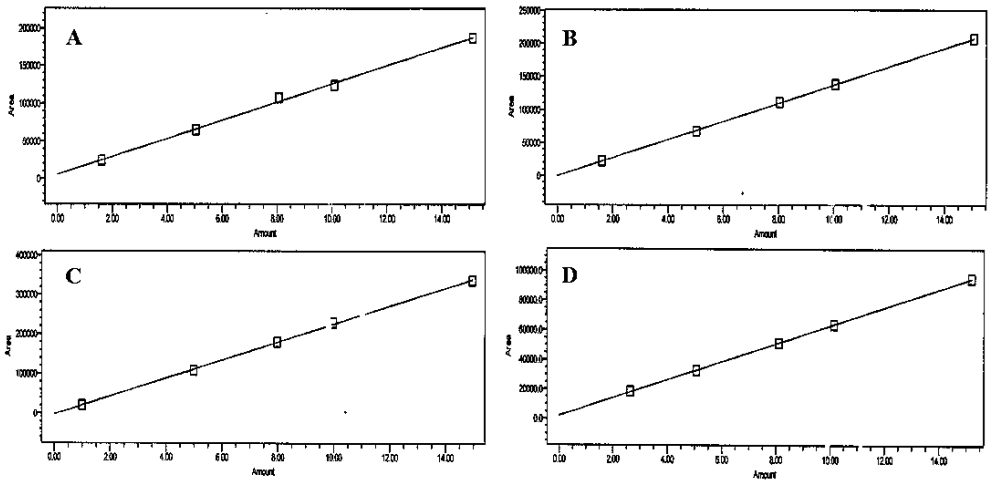

Fig. 2: Linearity curve for A. cyclophosphamide; B. cyclophosphamide related substance (A); C. cyclophosphamide related substance (B); and D. cyclophosphamide related substance (D)

Table 5: Linearity for cyclophosphamide and its related Substances [Concentrations range from 1.6 ppm to 15 ppm]

Substance	Cyclophosphamide	CRS-A	CRS-B	CRS-D
Intercept	5304.471	-225.495	-2790.41	1953.658
Slope	12025.69	13729.79	22753.76	6110.757
CC	0.9999	0.9987	0.9997	0.9999
#	4.3	-0.2	1.2	3.1

CRS-A: Cyclophosphamide related substance A; CRS-B: Cyclophosphamide related substance B; CRS-D: Cyclophosphamide related substance D; CC-correlation coefficient; #: %Y intercept at 100% level; ppm: parts per million.

Table 6: Precision (system, method, and intermediate) data of the developed method for cyclophosphamide and its related substances

Parameter	Area of peak			
	Cyclophosphamide	CRS-A	CRS-B	CRS-D
System precision				
N	6	6	6	6
Mean	127180.367	140316.340	222115.195	61082.318
SD	2620.987	2205.047	557.766	266.206
% RSD	2.1	1.6	0.3	0.4
95 % CI	2751.11	2314.52	585.46	279.42
Method precision				
N	6	6	6	6
Mean	0.2139	0.2002	0.2117	0.1972
SD	0.0030	0.0019	0.0051	0.0029
% RSD	1.4	0.9	2.4	1.5
95 %CI	0.003	0.002	0.005	0.003
Intermediate precision				
N	6	6	6	6
Mean	0.2215	0.1807	0.2123	0.2052
SD	0.006	0.0012	0.0032	0.0012
% RSD	2.7	0.7	1.5	0.6
95 %CI	0.006	0.001	0.003	0.001

CRS-A: Cyclophosphamide related substance A; CRS-B: Cyclophosphamide related substance B; CRS-D: Cyclophosphamide related substance D; N: number of injections; SD: standard deviation; %RSD: percentage relative standard deviation; CI: confidence interval.

Table 7: Overall statistical analysis from method precision and intermediate precision developed method for cyclophosphamide and its related substances

Parameter	Cyclophosphamide	CRS-A	CRS-B	CRS-D
Mean	0.2177	0.19049	0.21205	0.20124
SD	0.0060	0.0102	0.0040	0.0046
% RSD	2.8	5.4	1.9	2.3
95 %CI	0.004	0.007	0.003	0.003

CRS-A: Cyclophosphamide related substance A; CRS-B: Cyclophosphamide related substance B; CRS-D: Cyclophosphamide related substance D; SD: standard deviation; %RSD: percentage relative standard deviation; CI: confidence interval.

Accuracy (Recovery)

An accuracy study was conducted by spiking related substances into the sample at various concentration levels. The resulting % recoveries were calculated and presented in tables 8. These recovery results demonstrate that the test method exhibits an acceptable level of accuracy across the range from LOQ to 150 % of the specification level. This accuracy study confirms the method's reliability for determining cyclophosphamide and its related substances in cyclophosphamide capsules using HPLC.

Table 8: Accuracy of the developed method for cyclophosphamide and cyclophosphamide related substances A, B, and D

Substance	Levels	Average (%)	SD	% RSD
Cyclophosphamide	LOQ	115.2	3.0761	2.7
	50 %	109.9	1.0143	0.9
	100 %	106.2	0.4996	0.5
	150 %	101.75	1.6249	1.6
CRS-A	LOQ	86.5	2.2676	2.6
	50 %	91.8	1.0161	1.1
	100 %	93.3	1.2539	1.3
	150 %	93.1	0.9968	1.1
CRS-B	LOQ	82.5	1.5994	1.9
	50 %	105.3	1.3426	1.2
	100 %	106.5	1.8922	1.8
	150 %	113.6	4.2281	3.7
CRS-D	LOQ	101.4	1.0852	1.1
	50 %	105.6	1.9563	1.9
	100 %	101.7	0.2499	0.2
	150 %	100.0	0.7047	0.7

CRS-A: Cyclophosphamide related substance A; CRS-B: Cyclophosphamide related substance B; CRS-D: Cyclophosphamide related substance D; LOQ: quantification limit; SD: standard deviation; %RSD: percentage relative standard deviation.

Robustness

The method's robustness was assessed by intentionally introducing minor variations in specific parameters, including changes in flow rate and temperature. The study aimed to evaluate the method's stability in light of these deliberate modifications. The results obtained from the system suitability injections were found to be highly satisfactory. Therefore, it can be confidently affirmed that the method is robust, as supported by references [20, 21]. The system suitability data demonstrated the method's robustness even with variations in flow rate (+10 %), wavelength (±5 nm), buffer pH (±0.2), mobile phase B composition (±10 % absolute organic content

for acetonitrile and methanol), and column oven temperature (+5 °C), all these Variations meet the acceptance criteria for system suitability. This robustness has been confirmed for the accurate determination of cyclophosphamide and its related substances in cyclophosphamide capsules using HPLC.

Stability of mobile phase, standard solution, and sample solutions

Based on the solution stability data, it has been established that the mobile phase remains stable for up to 72 h when kept at the benchtop. Furthermore, the standard solution maintains its stability for a similar duration of 72 h when stored at approximately 5±3 °C. In the case of the sample solution, its stability lasts up to 5 h under the same storage conditions at around 5±3 °C. These findings support the method's reliability for determining cyclophosphamide and its related substances in cyclophosphamide capsules using HPLC. No particles or haziness were observed in the mobile phase during the initial period, after 24 h, after 48 h, and after 72 h intervals study.

Application of the validated method: forced degradation study

The samples subjected to various forced degradation conditions showed well-separated chromatograms of cyclophosphamide and its related substances at different retention times (fig. 3 and 4). Cyclophosphamide and its related substances peaks are homogeneous, and there are no co-eluting peaks. No interference is observed at the retention time of peaks due to cyclophosphamide and its related substances. Comparable mass balance is achieved for assays and related substances (tables 9 and 10).

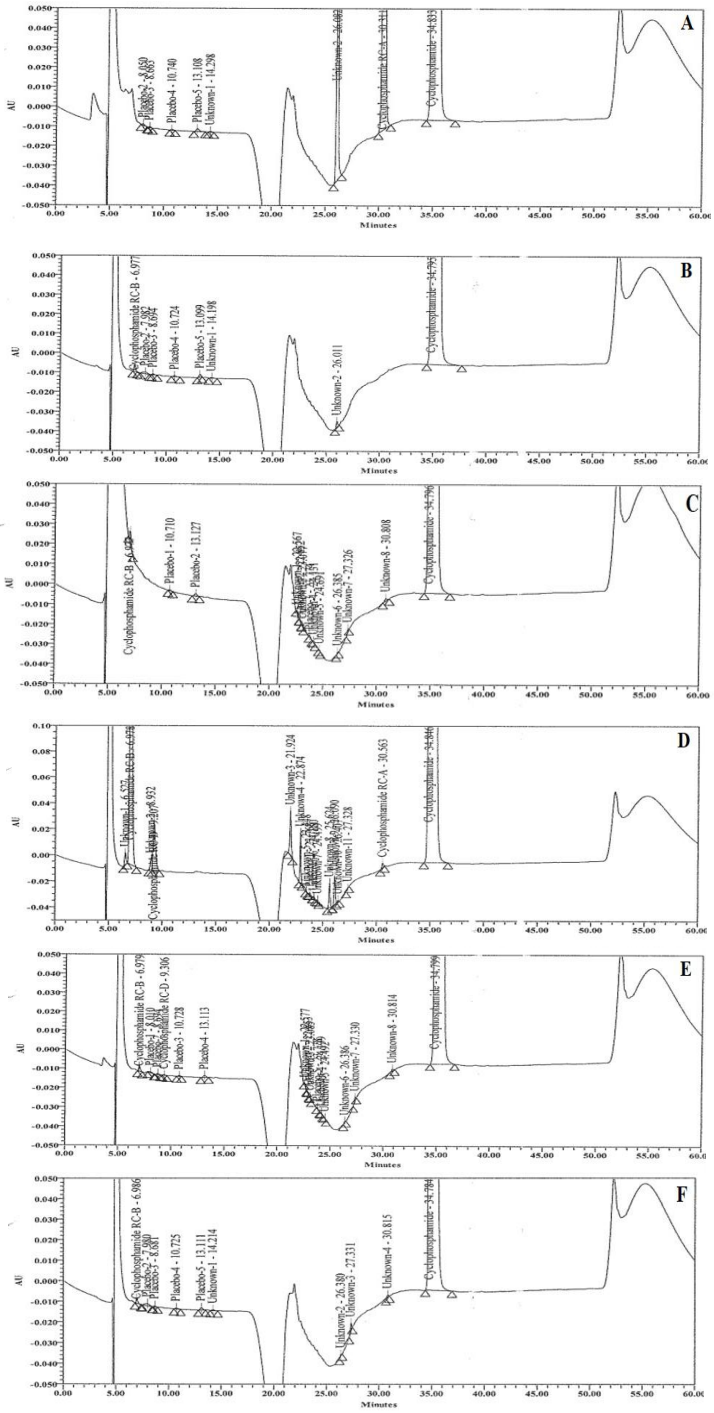

Fig. 3: Chromatogram of sample solution under A. acid; B. base; C. peroxide; D. thermal; E. photolytic; and F. humidity degradation

Table 9: Assay and mass balance of cyclophosphamide and its related substances in a forced degradation study

Stress condition	% Assay	% Degradation	Total related substance (%w/w)	% Mass balance	PA	PT
As Such	103.130	-	0.0511	-	0.050	0.381
Acid	89.798	12.927	12.5042	99.1	0.048	0.431
Base	102.482	0.628	0.1140	99.4	0.054	0.378
Thermal	91.908	10.881	9.3222	98.1	0.056	0.421
Photolytic	102.406	0.702	0.1224	99.4	0.053	0.411
Humidity	103.559	-	0.119	100.5	0.058	0.331
Peroxide	103.892	-	0.1525	100.8	0.042	0.346

PA: Purity angle; PT: Purity threshold

Table 10: Forced degradation data of cyclophosphamide and its related substances detected by the developed method

Condition	Parameter	Impurity			
		CRC-A	CRC-B	CRC-D	Total (%w/w)
As such sample	RRT	ND	0.2005	ND	0.0463
	%w/w	ND	0.0228	ND	
Acid	RRT	0.8701	ND	ND	12.2002
	%w/w	5.6513	ND	ND	
Base	RRT	ND	0.2005	ND	0.1002
	%w/w	ND	0.0382	ND	
Thermal	RRT	0.877	0.2002	0.2642	9.2878
	%w/w	0.0545	5.5257	1.8795	
Photolytic	RRT	ND	0.2005	ND	0.1137
	%w/w	ND	0.027	ND	
Peroxide	RRT	ND	0.2005	ND	0.1525
	%w/w	ND	0.0586	ND	
Humidity	RRT	ND	0.2008	ND	0.1127
	%w/w	ND	0.0245	ND	

CRS-A: Cyclophosphamide-related substance A; CRS-B: Cyclophosphamide-related substance B; CRS-D: Cyclophosphamide-related substance D; RRT: relative retention time; ND: Not detected.

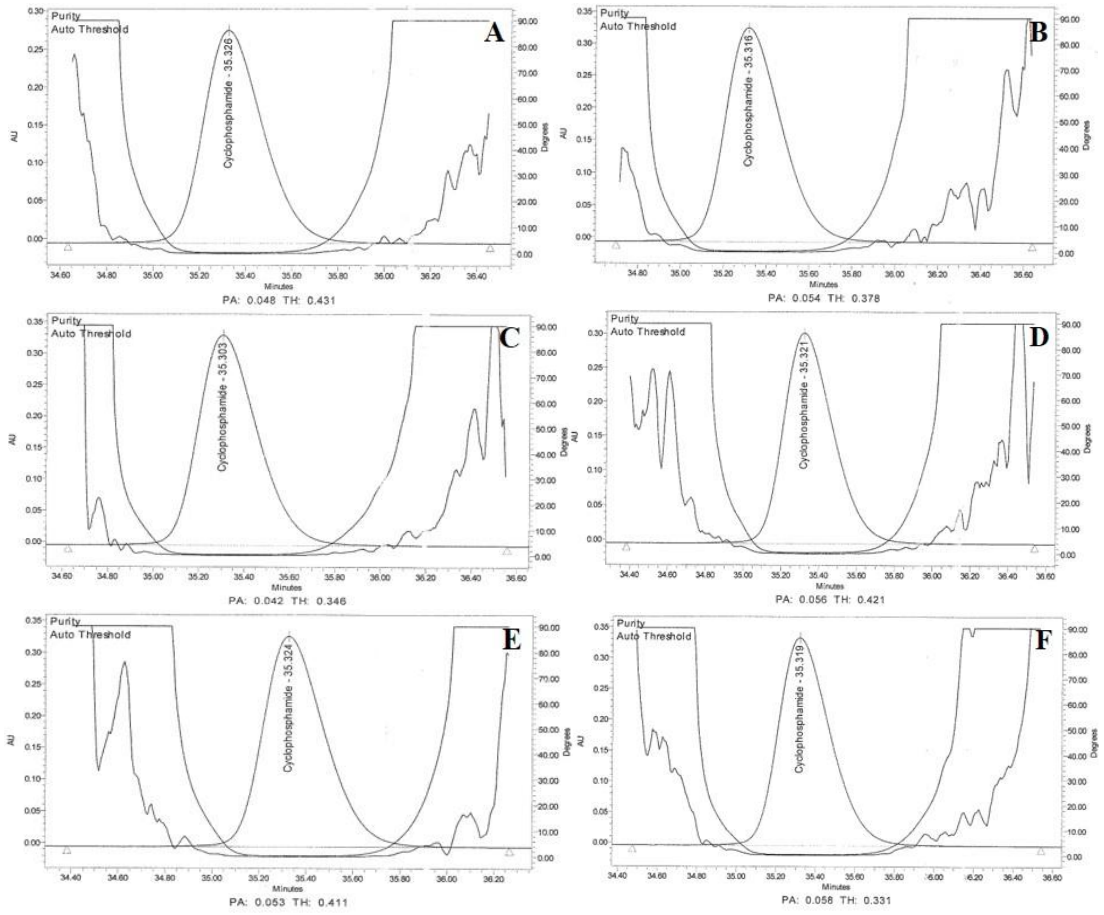

Fig. 4: Chromatogram of peak purity and threshold of diluted sample solution under A. acid; B. base; C. peroxide; D. thermal; E. photolytic; and F. humidity degradation

The forced degradation data underscores the stability-indicating nature of the methodology, highlighting its ability to provide reliable results even under stressful conditions. The consistent mass balance observed in both assays and related substances further demonstrates the method's reproducibility. Under the applied stress conditions, the product exhibited stability in photolytic, humidity, peroxide, and base degradation. However, it was found to be susceptible to degradation in acidic and thermal stress conditions. Despite these challenges, the peak purity of the cyclophosphamide peak remained well within the acceptance criteria, indicating the method's robustness and suitability for identifying and quantifying cyclophosphamide and its related substances.

CONCLUSION

The HPLC method described proves to be a convenient and reliable technique for quantifying related substances in cyclophosphamide. This method, developed and validated in accordance with ICH guidelines, is straightforward and easy to implement. The results obtained from the method's validation study have been highly satisfactory. Due to its accuracy, the HPLC method ensures the precise determination of related substances in drug substances. Therefore, this method can be readily applied for quality control purposes, serving to minimise both the cost and time associated with the analysis of cyclophosphamide and its related substances.

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ETHICAL APPROVAL

The authors confirm that no ethical approval is applicable or required for this study.

AUTHORS CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be authors as per the International Committee of Medical Journal Editors (ICMJE) requirements and guidelines.

CONFLICT OF INTERESTS

The authors assert that they have no conflicts of interest related to this study.

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