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Research Article

DEVELOPMENT AND VALIDATION OF A REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE ESTIMATION OF CYCLOPHOSPHAMIDE IN BULK DRUG

VITTHAL D. DHAKANE[1], MILIND B. UBALE[1]*

[1]Post graduate Department of chemistry, Vasantrao Naik Mahavidyalaya, Cidco, Aurangabad 431004 (MS), India.
Email: mbubale@yahoo.com

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ABSTRACT

A simple, rapid, and precise method is developed for the related substances determination of Cyclophosphamide in bulk drugs. The method was developed with HPLC, PDA detector and YMC pack ODS – A (250 x 4.6)mm column, Mobile phase-A 900 ml of water 100 ml acetonitrile and mobile phase-B 300 ml of water 700 ml acetonitrile was used as mobile phase. The instrumental settings are flow rate of 1.0 mL min-1, column temperature at 30°C, detector 195 nm and 20 µL sample injection volume. Theoretical plates were 9875. Tailing factor was 1.12. The described method shows excellent linearity over a range of 50 to 150% of related substances concentration. The correlation coefficient was 0.999. The relative standard deviation of six measurements for peak area and retention time less than 5% and 1.0% respectively. The Cyclophosphamide was subjected to stress conditions of acid, base, oxidation (10 % H2O2) and thermal degradation. The degradation was observed for Cyclophosphamide in alkali and partly in oxidative hydrolysis. The developed method was validated with respect to linearity, accuracy, recovery, precision, system suitability, selectivity, robustness and forced degradation studies prove the stability indicating ability of the method.

Keywords: Cyclophosphamide, Stability indicating, RP-HPLC, YMC pack ODS – A and Validation.

INTRODUCTION

N,N-bis(2-chloroethyl)-1,3,2-oxazaphosphinan-2-amine
Cyclophosphamide (the generic name for Endoxan Cytoxan, Neosar, Procytox, Revimmune), also known as cytophosphane, is a nitrogen mustard alkylating agent,from the oxazophorines group.An alkylating agent adds an alkyl group (C_nH_{2n+1}) to DNA[1]. It attaches the alkyl group to the guanine base of DNA, at the number 7 nitrogen atom of the imidazole ring.Cyclophosphamide also decreases the immune system's response to various diseases and conditions. Therefore, it has been used in various non-neoplastic autoimmune diseases where disease-modifying antirheumatic drugs (DMARDs) have been ineffective. Cyclophosphamide (INN, trade names Endoxan, Cytoxan, Neosar, Procytox, Revimmune), also known as cytophosphane, is a nitrogen mustard alkylating agent, from the oxazophorines group[2-4]. An alkylating agent adds an alkyl group (C_nH_{2n+1}) to DNA. It attaches the alkyl group to the guanine base of DNA, at the number 7 nitrogen atom of the imidazole ring[5]. We are gratified to report a stability indicating HPLC method for the analysis and separation of drugs from the degradation products formed under ICH suggested conditions hydrolysis, oxidations, and thermal stress[5-6]. In present article, reversed phase HPLC method was developed for the separation of Cyclophosphamide in bulk drug and the impurities formed from its forced degradation under stress conditions like acid hydrolysis, base hydrolysis, oxidation, heat. [7-8]

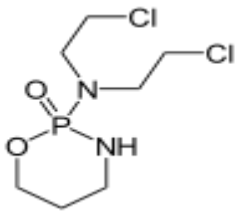

Fig. 1: Chemical structure of Cyclophosphamide

MATERIALS AND METHODS

Material and reagents

Cyclophosphamide were obtained as gift samples from Cure Lab. (Hydrabad, India), acetonitrile (HPLC grade) were purchased from Qualigens Fine Chemicals (Mumbai, India). Sodium hydroxide, hydrochloric acid and hydrogen peroxide was obtained from Merck Laboratories Ltd., (Mumbai, India). The 0.45 - Pump nylon filter was (purchased) from Advanced Micro devices Pvt. Ltd., (Ambala Cantt, India). Double -distilled water was used throughout the experiment. Other chemicals used were analytical or HPLC grade .

Chromatographic Conditions

A chromatographic system Waters(USA) Empower software consisting of quaternary solvent delivery pump, a degasser, an auto-injector, column oven and photodiode array detector. The chromatographic column of 250 mm length and internal diameter of 4.6 mm YMC pack ODS – A stationary phase with particle size 5 micron and pore size 100A was used The column is end capped and carbon content of 11%. The instrumental settings were a flow of 1 ml/min. The HPLC gradient was kept as T/ min and %B Composition of mobile phase-B 0/70,20/70,35/80,40/90,50/70.The analysis was performed at 30°C. The injection volume was 20 µL. The peak purity was checked with the photodiode array detector. Detection was performed at 195 nm.

Mobile Phase

The mobile phase containing 900 ml of water 100 ml acetonitrile as mobile phase-A and 300 ml of water 700 ml acetonitrile as mobile phase-B

Preparation of Standard stock solutions

Standard solutions were prepared by dissolving the drugs in the diluents and diluting them to the desired concentration. Diluents used for the standards and sample was prepared as follow: diluent A was composed of water and aceonitrile in the ratio of 50:50(v/v).

A 50-mg sample of Cyclophosphamide standard was accurately weighed, transferred in a 50-mL volumetric flask, and dissolved with the diluent (1000 µg mL-1). Further 1.0 ml above solution transferred in a 100-mL volumetric flask, and dissolved with the diluent (10 µg mL-1).

A 50-mg sample of Monochlorocyclophosphamide impurity standard was accurately weighed, transferred in a 50-mLvolumetric flask, and dissolved with the diluent (1000 µg mL-1). Further 1.0 ml above solution transferred in a 100-mL volumetric flask, and dissolved with the diluent (10 µg mL-1).

A 50-mg sample of Impurity-A standard was accurately weighed, transferred in a 50-mL

volumetric flask, and dissolved with the diluent (1000 µg mL-1). Further 1.0 ml above solution transferred in a 100-mL volumetric flask, and dissolved with the diluent (10 µg mL-1).

Sample solution

A 100-mg sample was accurately weighed, transferred in a 50-mL volumetric flask, and dissolved with the diluent (2000 µg mL-1).

Selectivity

Selectivity is the ability of the method to assess unequivocally the analyte in the presence of components, which may be expected to be present[9]. Typically, these might include degradants, matrix etc. The selectivity of the developed LC method for Cyclophosphamide was carried out in the presence of its degradation products. Stress studies were performed for Cyclophosphamide bulk drug to provide an indication of the stability indicating property and selectivity of the proposed method. Intentional degradation was attempted to stress condition exposing it with acid (1.0 N Hydrochloric acid)Fig-2, alkali (0.5N NaOH)Fig-3, hydrogen peroxide (10%)Fig-4, to evaluate the ability of the proposed method to separate Cyclophosphamide from its degraded products. For heat study, study period was 10 days where as for acid, oxidation 2 hr , for base 2 hour and for hydrogen peroxide 30 min . Related substances studies were carried out for stress samples against Cyclophosphamide reference standard and the mass balance (% assay + % sum of all impurities + % sum of all degraded products) was calculated.

Table 1: Summary of Forced degradation results

Stress condition	Time	Degradation of Substance%	Remarks
Acid Hydrolysis (1.0 N HCl)	2 Hrs	6.27	No Degradation
Base Hydrolysis (0.5 N NaOH)	2 Hrs	21.44	Degradation
Oxidation (10% H_2O_2)	48 Hrs	9.56	No Degradation
Thermal (60°C)	7 days	2.88	No Degradation

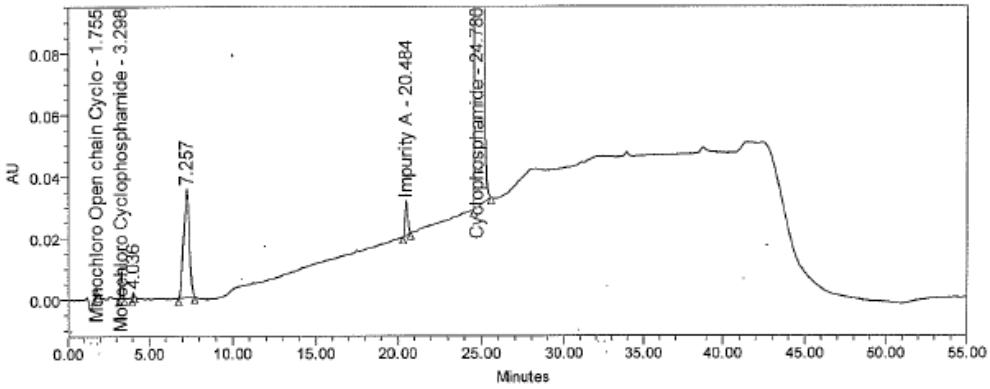

Fig. 2: Chromatogram of Cyclophosphamide in Acid degradation

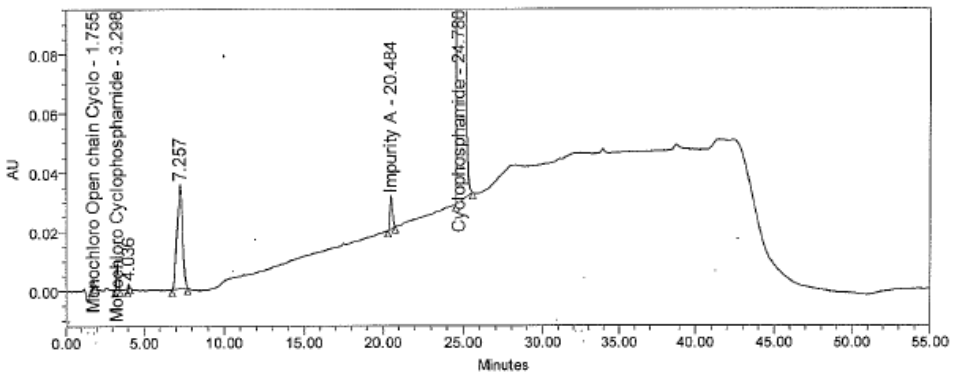

Fig. 3: Chromatogram of Cyclophosphamide in Alkali degradation

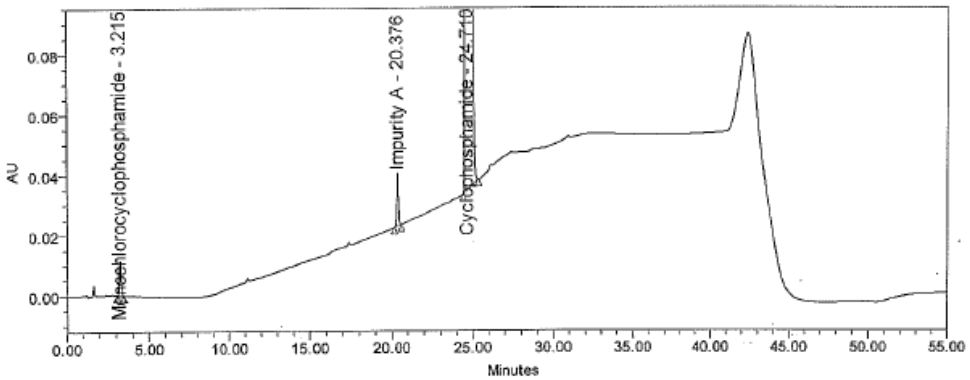

Fig. 4: Chromatogram of Cyclophosphamide in Peroxide degradation

RESULTS AND DISCUSSION

Method Development

The primary target in developing this LC method is to achieve determination of Cyclophosphamide and impurity-A in bulk drugs under common conditions that are applicable for the routine quality control of this product in ordinary laboratories. Taken in to account the instability of Cyclophosphamide in strong acidic and basic media, a mobile phase with water and acetonitrile different combination is preferred[10-11]. To achieve this number of stationary phase like C8, C18, CN and NH2 were employed. In C8 stationary phase using water and acetonitrile the resolution between Cyclophosphamide, and impurity-A was achieved but broad peak shape of Cyclophosphamide was obtained having tailing factor about 2.4. To minimize tailing effect, further NH2 and CN columns were tried but it has been observed that (tailing factor 2.5) but resolution between Cyclophosphamide, Monochlorocyclophosphamide and impurity-A decreased and in case of CN stationary phase peak shape of Cyclophosphamide was improved but peak of impurity-A and monochlorocyclophosphamide was eluted at, 6.3 and 18.7 respectively.

Finally used high carbon loading, double end capped C18 (YMC ODS-A C18, 25-cm)

column. Mobile phase was selected in terms of its components and proportions. This work began with a binary mixture of acetonitrile and water. Finally a mobile phase-A 900 ml of water 100 ml acetonitrile and mobile phase-B 300 ml of water 700 ml acetonitrile was used as mobile phase, which produces good resolution and reasonable retention and acceptable for drug the chromatographic analysis time was 50 min. A typical chromatogram for a standard solution is shown in Fig 2 and 3. The retention time is 20.40 for impurity-A, 3.23 for monochlorocyclophosphamide and 24.72 for Cyclophosphamide, respectively.

Method Validation

System suitability

For system suitability studies, six replicate injections of acid, base and oxidative degraded solutions were used and the RSD of peak area ratio, resolutions, tailing factor and number of theoretical plates of the peak were calculated. The system suitability results are shown in Table 2.

Table 2: System suitability reports

Compound (n=3)	Retention Time	% RSD	USP tailing	Theoretical plates
Cyclophospha--mide	24.72	2.28	1.12	9875

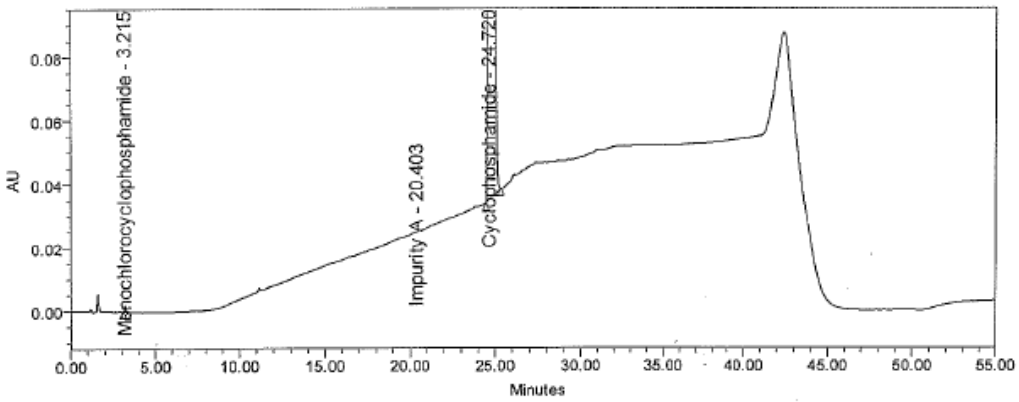

Fig. 5: A chromatogram of the Cyclophosphamide diluted standard

Precision

The precision of the method was studied by determining the concentrations of the drug Cyclophosphamide in the tablet for six times[12-13]. The results of the precision study (Table 4) indicate the reliability of the method (RSD %< 2).

Accuracy (Recovery test)

The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value and the value found. Accuracy of the method was studied by recovery experiments. The recovery experiments were performed by adding known amounts of the drugs in the placebo. The recovery was performed at three levels, 80%, 100% and 120%. The recovery samples were prepared as aforementioned procedure[14]. The solutions were then analyzed, and the percentage recoveries were calculated from the calibration curve. The recovery values for Cyclophosphamide ranged from 85% to 101% (Table 3).

Table 3: Results of the Recovery Tests for the Cyclophosphamide

Level of Addition (%)	Amount added (n = 3) (ppm)	% Recovery*	% Average recovery^
80	50	96.45	95.66
100	100	99.85	100.12
120	150	100.57	100.76

* RSD shown in parenthesis.

^ Average recovery = the average of three levels, nine determinations

Calibration and linearity

Linearity test solutions for the method were prepared from Cyclophosphamide stock solutions at six concentrations levels from tested from 80% to 120% of the targeted level of the related substances concentration Cyclophosphamide. Standard solutions containing 80-120 µg/ml of Cyclophosphamide in each linearity level were prepared. Linearity solutions were injected in triplicate[15]. The calibration graphs were obtained by plotting peak area verses the concentration data was treated by least-squares

linear regression analysis, the calibration graphs were found to be linear in the mentioned concentrations the slopes and correlation coefficients are shown in Table –4.

Robustness

To determine the robustness of the developed method experimental condition were purposely altered and the resolution between Cyclophosphamide and acid degraded product were evaluated. The flow rate of the mobile phase was 1.0 ml/min. To study the effect of flow rate on the resolution, it was changed by 0.2 unit from 0.8 to 1.2ml/min while the other mobile phase component were held as stated in chromatographic conditions. The effect of column temperature on resolution was studied at 25 and 35°C instead of 30°C while the other mobile phase components were held constant stated in chromatographic condition. The results are shown in table-5

Table 4: Results of the Linearity study and Precision

Ingredient	Precision (% RSD)	Linearity (µg/ml)	Slopes* (n= 3)	Coefficients of correlations
Cyclophospha-mide	2.28	80-120	3675.1	0.9998

*Standard deviation shown in parentheses

Table 5: Results of robustness study

Sr. No.	Parameters	Variations		Resolutions between Cyclophosphamide and Impurity-A
1	Temperature	2.	at 25 °C	11.23
		3.	at 35 °C	9.36
2	Flow rate	1.	0.8 ml/min	10.12
		2.	1.2 ml/min	9.57

LOD and LOQ (Sensitivity)

A series of solutions in the range 0.2–1.0% of the related substances concentration (40 µg mL−1) were prepared by dilution of the standard solutions. Each solution (20 µL) was were injected five times, the areas were measured for the drug peak, and the standard deviation for the five injections was calculated for each concentration[16]. On the basis of data obtained, the standard deviation at concentration zero was calculated and this value was used for calculation of the LOD and LOQ. The results are shown in table-6

Table 6: Results of the LOD and LOQ

Name	%LOD	%LOQ
Impurity-A	0.14	0.05
Monochlorocyclophosphamide	0.17	0.10
Cyclophosphamide	0.11	0.08

Stability of analytical solution

The stability of the standard solutions and the sample solutions was tested at intervals of 24, 48 and 72 h. The stability of solutions was determined by comparing results of the assay of the freshly prepared standard solutions. The RSD for the related substances results determined up to 72 h for Impurity-A and Monochlorocyclophosphamide was 2.23 and 2.28 %. The related substances values were within + 2 % after 72 h. The results indicate that the solutions were stable for 72 h at ambient temperature.

CONCLUSION

The method developed for quantitative determination of Cyclophosphamide is rapid, precise, accurate and selective. The method was completely validated showing satisfactory data for all method-validated parameters tested. The developed method is stability indicating and can be used for assessing the stability of Cyclophosphamide as bulk drugs. The developed method can be conveniently used for the assay determination of Cyclophosphamide in bulk drugs and pharmaceutical dosage form.

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