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A STABILITY-INDICATING UPLC METHOD FOR QUANTIFICATION OF CYCLOPHOSPHAMIDE RELATED COMPOUNDS IN PHARMACEUTICAL DOSAGE FORMS

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ABSTRACT

A specific, precise, rapid and reliable stability indicating UPLC method has been developed and validated for estimation of Cyclophosphamide related compounds in pharmaceutical dosage forms. Chromatographic separation was achieved on an Acquity UPLC BEH C18 (2.1 x 50 mm, 1.7 µm) column using gradient composition of mixer of pH 7.0 buffer as *Mobile Phase A* and mixture of pH 7.0 buffer and Acetonitrile in the ratio of 20:80% v/v as *Mobile Phase B* at a flow rate of 0.5 mL/min and analytes were monitored at 195 nm. The retention times of the Cyclophosphamide was about 4.18 and relative retention times of Cyclophosphamide Related compound A, Cyclophosphamide Related compound B and Cyclophosphamide Related compound D were about 0.91, 0.17 and 0.23 respectively. The results of specificity studies indicate that there was no interference of diluent, excipients, impurities and degradation products at retention time of analyte and it is assured that the peak response was belongs to a single component only. The detector response was linear in the range of LOQ-150% level with respect to test concentration of Cyclophosphamide, Cyclophosphamide Related compound A, Cyclophosphamide Related compound B and Cyclophosphamide Related compound D. Correlation coefficient (R^2) was not less than 0.99 for Cyclophosphamide and its related compounds. The percentage recovery of Cyclophosphamide, Cyclophosphamide Related compound A, Cyclophosphamide Related compound B and Cyclophosphamide Related compound D were meeting the predetermined acceptance criteria. The developed method was validated for specificity, linearity, precision, accuracy, solution stability, ruggedness and stress degradation studies were monitored. Hence, the developed method was specific, rapid

and cost-effective, and it can be used for routine analysis of Cyclophosphamide related compounds in pharmaceutical dosage forms.

KEYWORDS: Cyclophosphamide; Cyclophosphamide Related compound A; Cyclophosphamide Related compound B; Cyclophosphamide Related compound D; UPLC.

INTRODUCTION

Cyclophosphamide is a synthetic anticancer drug. The chemical name is 2-[Bis (2-chloroethyl) amino] tetrahydro-2H-1, 3, 2-oxazaphosphorine 2-oxide monohydrate, molecular formula is $C_7H_{15}Cl_2N_2O_2P$ and molecular weight is 261.1. Its structural formula is represented in Figure.1. It is rapidly absorbed from the gastrointestinal tract after oral administration and distribute widely throughout the body. Onset of action is 2-3 hours. Metabolized mainly in the liver and is excreted exclusively through the urine.[1]

Cyclophosphamide is an alkylating agent that has an effective anticancer activity. It introduces alkyl radicals into DNA strands of cells and stops cancer cells from growing. It has also an immunosuppressive effect – suppress the body's natural immune response, and used to treat some autoimmune diseases. Cyclophosphamide is an inactive drug. With the help of cytochrome P-450 oxidase system in the liver, the inactive drug is converted into phosphoramide mustard and acrolein which are very active compounds. Phosphoramide mustard has an ability to introduces alkyl radicals into DNA strands with interferes DNA replication by forming DNA cross-linkage[1]. Cross-linked cancer cell DNA is unable to complete normal cell division. Thus, it stops cancer cells from growing, causing them to die. Cyclophosphamide also produces immunosuppressive effects possibly through a cytotoxic effect on lymphocytes.[2]

Cyclophosphamide is used to treat in different types of cancer including Non Hodgkin lymphoma, Burkitt's lymphoma, chronic lymphocytic leukemia (CLL), Breast cancer, Bronchial cancer, Prostate cancer, Testicular cancer, malignant pheochromocytoma and Ewing's sarcoma. It is also used to treat autoimmune diseases including systemic lupus erythematosus (SLE), rheumatoid arthritis, multiple sclerosis, myasthenia gravis, Wegener's granulomatosis, Churg-Strauss syndrome, microscopic polyangiitis, polyarteritis nodosa, autoimmune hemolytic anemia, polymyositis, dermatomyositis, mononeuritis multiplex, pemphigus vulgaris, bullous pemphigoid, cicatricial pemphigoid, Goodpasture's syndrome, minimal change disease and membranous nephropathy.[3,4]

So there is a need for development of generic version of Cyclophosphamide. Few analytical methods are available for estimation of cyclophosphamide.[5,6] There are no analytical methods have been reported for the estimation of Cyclophosphamide related compounds in pharmaceutical formulations at the time of commencement of research work. Now a day's all researchers developing UPLC methods to reduce the cost in generic drug development.[7,8] Where as in the present study the author made efforts to develop a stability-indicating RP-UPLC method for the quantification of Cyclophosphamide related compounds. This method has advantages of shorter retention time and higher selectivity.

Experimental

Reagents and materials

Analytical grade reagents such as orthophosphoric acid, triethylamine, barium chloride, hydrochloric acid, sodium hydroxide, hydrogen peroxide, HPLC grade acetonitrile and water were procured from Merck India.

Development and Optimization of the Stability-Indicating UPLC Method

Preparation of mobile phase

Preparation of buffer

Dissolve 1ml of orthophosphoric acid into 1000ml of milli-Q water and adjust the pH 7.0 ±0.05 with diluted triethylamine and filter through 0.22µm membrane filter.

Mobile phase-A

Use pH 7.0 buffer as mobile phase A.

Mobile phase-B

Prepare a mixture of pH 7.0 buffer and Acetonitrile in the ratio of 20:80% v/v.

Preparation of Diluent

Refrigerated water (2-8°C) is used as diluent.

Preparation of Cyclophosphamide Standard stock solution

Accurately weighed and transferred 40mg of Cyclophosphamide monohydrate into a 100ml volumetric flask, add 60ml of diluent and keep on cyclomixer about 2minutes to dissolve and diluted to volume with diluent.

Preparation of Cyclophosphamide Related compound-A Standard stock solution

Accurately weighed and transferred 2mg of Cyclophosphamide Related compound-A into a 10ml volumetric flask, add 6ml of diluent and keep on cyclomixer about 2minutes to dissolve and diluted to volume with diluent.

Preparation of Cyclophosphamide Related compound-B Standard stock solution

Accurately weighed and transferred 2mg of Cyclophosphamide Related compound-B into a 10ml volumetric flask, add 6ml of diluent and keep on cyclomixer about 2minutes to dissolve and diluted to volume with diluent.

Preparation of Cyclophosphamide Related compound-D Standard stock solution

Accurately weighed and transferred 2mg of Cyclophosphamide Related compound-D into a 10ml volumetric flask, add 6ml of diluent and keep on cyclomixer about 2minutes to dissolve and diluted to volume with diluent.

Preparation of Standard solution

Transfer 1.0ml of above Cyclophosphamide standard stock solution and 2.0 ml of Cyclophosphamide Related compound-A, Cyclophosphamide Related compound-B, Cyclophosphamide Related compound-D into a 10ml volumetric flask, dilute to volume with diluent and mixed well.

Preparation of Placebo solution

Weighed and transferred Placebo powder equivalent to 400mg of Cyclophosphamide into 50ml volumetric flask, to this add 20ml of diluent and keep on cyclomixer about 3minutes, mix well. Centrifuge a portion of above solution immediate without any delay for minutes at 4000rpm. Filter the supernant immediately without delay through 0.45 µm PVDF syringe filter by discard the first 3ml of the filtrate using glass syringe, into HPLC vial.

Preparation of Sample Solution

Weighed and transferred sample powder equivalent to 400mg of Cyclophosphamide into 50ml volumetric flask, to this add 20ml of diluent and keep on cyclomixer about 3minutes, mix well. Centrifuge a portion of above solution immediate without any delay for minutes at 4000rpm. Filter the supernant immediately without delay through 0.45 µm PVDF syringe filter by discard the first 3ml of the filtrate using glass syringe, into HPLC vial.

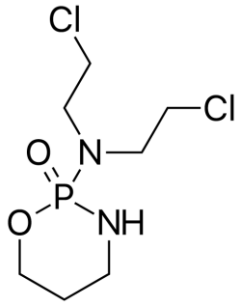

Figure 1: Chemical structure of Cyclophosphamide.

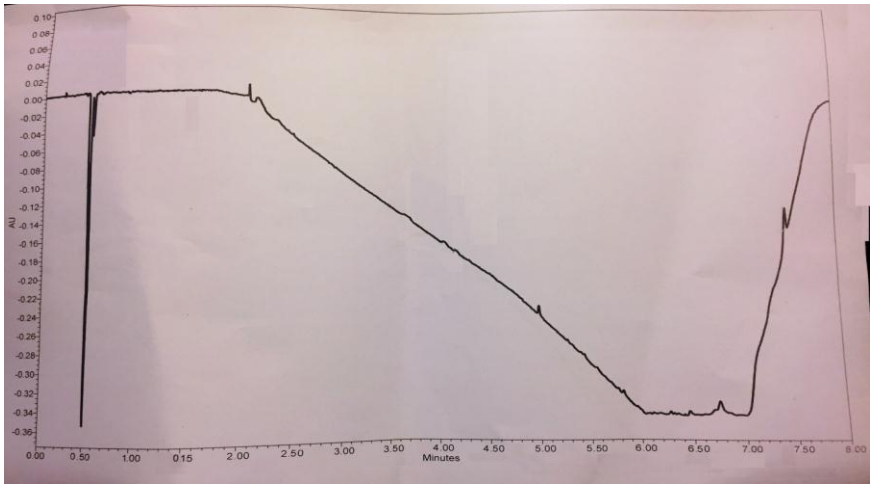

Figure 2: Representative chromatogram of blank.

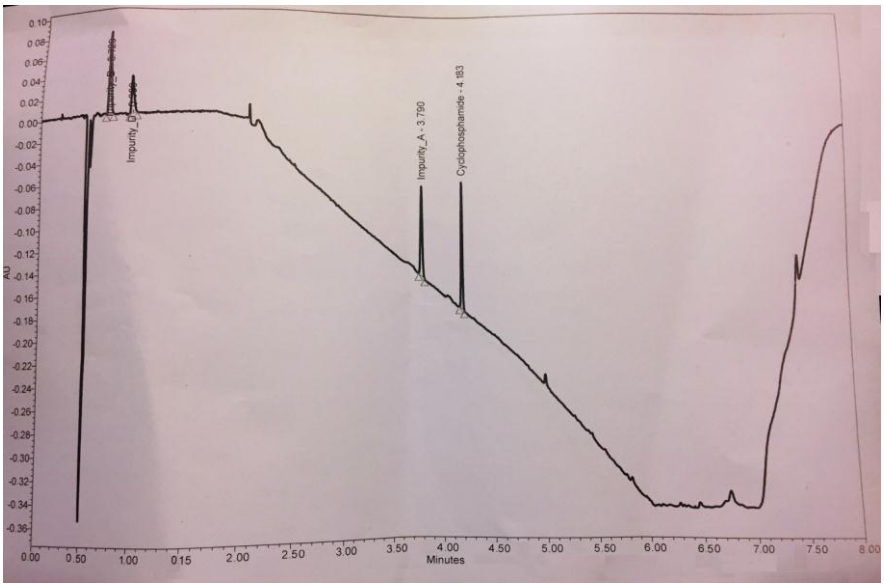

Figure 3: Representative chromatogram of spiked sample.

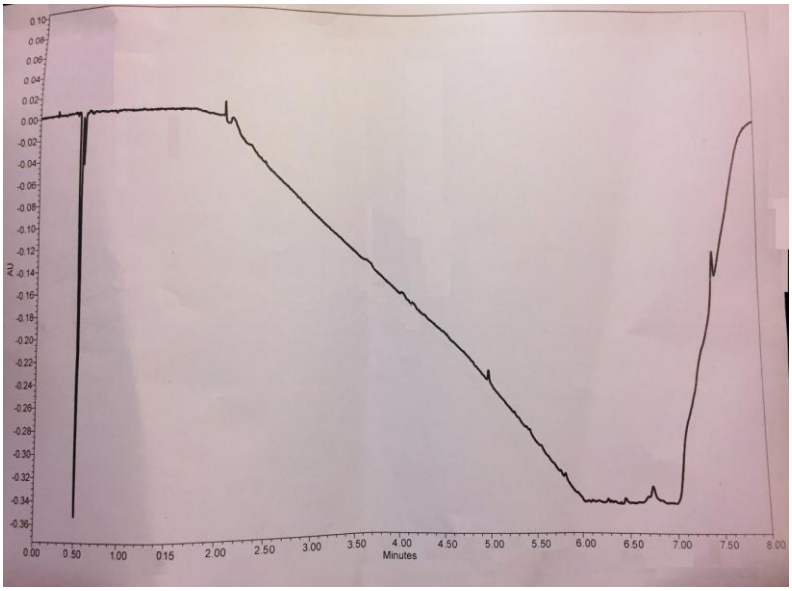

Figure 4: Representative chromatogram of placebo.

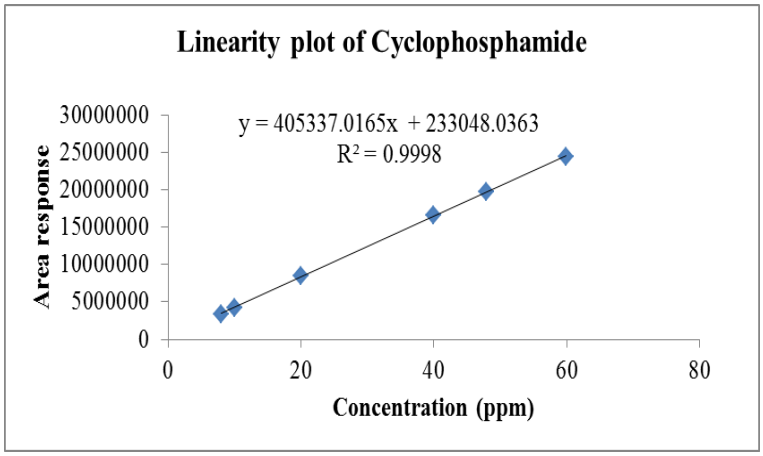

Figure 5: Linearity plot of Cyclophosphamide.

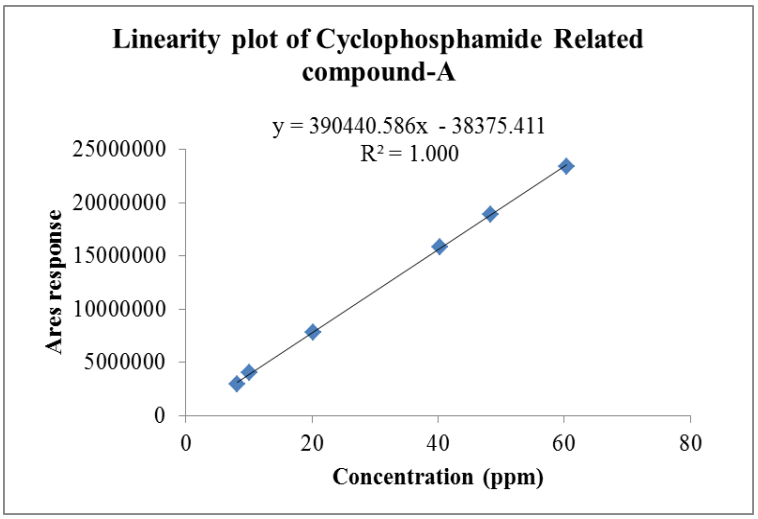

Figure 6: Linearity plot of Cyclophosphamide Related compound A.

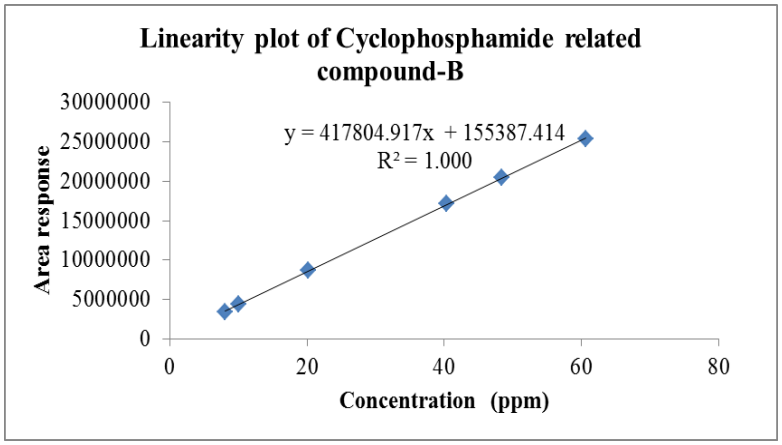

Figure 7: Linearity plot of Cyclophosphamide Related compound B.

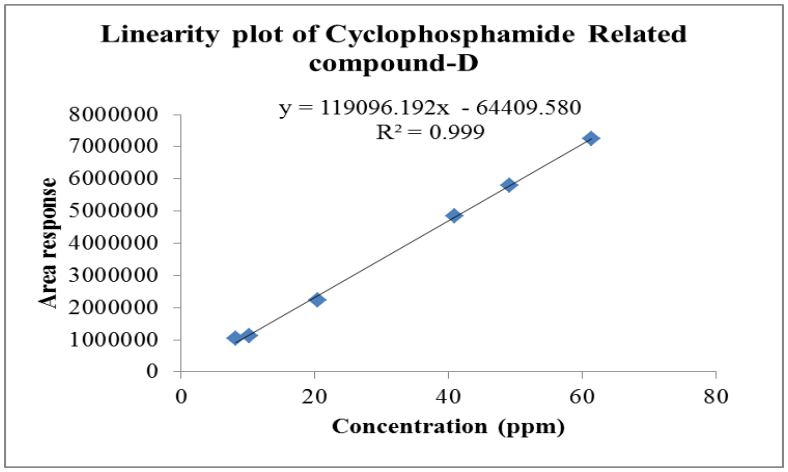

Figure 8: Linearity plot of Cyclophosphamide Related compound D.

Table 1: Gradient programme.

S. No.	Time (minutes)	% A	% B	Curve
1	Initial	100	0	Initial
2	1.0	100	0	6
3	4.0	50	50	6
4	5.5	20	80	6
5	6.5	20	80	6
6	7.0	100	0	6
7	8.0	100	0	6

Table 2: Linearity results for Cyclophosphamide.

% Linearity level	Concentration (ppm)	Response
LOQ	7.9795	3358912
25	9.9744	4215531
50	19.9489	8470691
100	39.8977	16533171
120	47.8773	19642985
150	59.8466	24376905

Table 3: Linearity results for Cyclophosphamide Related compound A.

% Linearity level	Concentration (ppm)	Response
LOQ	8.0949	2961237
25	10.0618	3994140
50	20.1236	7789279
100	40.2472	15857590
120	48.2966	18877246
150	60.3708	23378742

Table 4: Linearity results for Cyclophosphamide Related compound B.

% Linearity level	Concentration (ppm)	Response
LOQ	8.0825	3448407
25	10.1032	4375057
50	20.2063	8659498
100	40.4127	17115589
120	48.4127	20419927
150	60.6190	25392818

Table 5: Linearity results for Cyclophosphamide Related compound D.

% Linearity level	Concentration (ppm)	Response
LOQ	8.1851	1031135
25	10.2314	1118655
50	20.467	2238667
100	40.9254	4841153
120	49.1105	5804698
150	61.3881	7244133

Table 6: Accuracy results for Cyclophosphamide.

Concentration	Sample ID	Actual Impurity Added	Impurity found	% Recovery	Mean
LOQ Level	Sample-1	0.03972	0.03669	92.4	95.0
	Sample-2	0.03972	0.03628	91.3	
	Sample-3	0.03972	0.03793	95.5	
	Sample-4	0.03972	0.03904	98.3	
	Sample-5	0.03972	0.03894	98.0	
	Sample-6	0.03972	0.03764	94.7	
50% Level	Sample-1	0.10030	0.09090	90.6	90.6
	Sample-2	0.10030	0.0983	92.6	
	Sample-3	0.10030	0.08892	88.7	
100% Level	Sample-1	0.20060	0.17679	88.1	86.8
	Sample-2	0.20060	0.17366	86.6	
	Sample-3	0.20060	0.17178	85.6	
150% Level	Sample-1	0.30089	0.26765	89.0	88.7
	Sample-2	0.30089	0.27313	90.8	
	Sample-3	0.30089	0.26709	88.8	
	Sample-4	0.30089	0.26421	87.8	
	Sample-5	0.30089	0.26858	89.3	
	Sample-6	0.30089	0.25961	86.3	

Table 7: Accuracy results for Cyclophosphamide Related compound A.

Concentration	Sample ID	Actual Impurity Added	Impurity found	% Recovery	Mean
LOQ Level	Sample-1	0.04082	0.0407	98.7	99.7
	Sample-2	0.04082	0.03939	96.5	
	Sample-3	0.04082	0.03896	95.5	
	Sample-4	0.04082	0.04356	103.2	
	Sample-5	0.04082	0.04356	106.7	
	Sample-6	0.04082	0.03992	97.8	
50% Level	Sample-1	0.09932	0.09152	92.1	95.6
	Sample-2	0.09932	0.09423	94.9	
	Sample-3	0.09932	0.09920	99.9	
100% Level	Sample-1	0.20319	0.18750	92.3	92.0
	Sample-2	0.20319	0.18692	92.0	
	Sample-3	0.20319	0.18661	91.8	
150% Level	Sample-1	0.30321	0.28717	94.7	94.2
	Sample-2	0.30321	0.28704	94.7	
	Sample-3	0.30321	0.28484	93.9	
	Sample-4	0.30321	0.28643	94.5	
	Sample-5	0.30321	0.28701	94.7	
	Sample-6	0.30321	0.28140	92.8	

Table 8: Accuracy results for Cyclophosphamide Related compound B.

Concentration	Sample ID	Actual Impurity Added	Impurity found	% Recovery	Mean
LOQ Level	Sample-1	0.04011	0.04020	100.2	100.5
	Sample-2	0.04011	0.03954	98.6	
	Sample-3	0.04011	0.03957	98.7	
	Sample-4	0.04011	0.04227	105.4	
	Sample-5	0.04011	0.04097	102.2	
	Sample-6	0.04011	0.03935	98.1	
50% Level	Sample-1	0.10278	0.09904	96.4	96.0
	Sample-2	0.10278	0.10017	97.4	
	Sample-3	0.10278	0.09650	94.2	
100% Level	Sample-1	0.19988	0.22196	111.0	109.9
	Sample-2	0.19988	0.22028	110.2	
	Sample-3	0.19988	0.21690	108.6	
150% Level	Sample-1	0.30358	0.27372	90.2	88.2
	Sample-2	0.30358	0.27383	90.1	
	Sample-3	0.30358	0.27123	89.3	
	Sample-4	0.30358	0.26502	87.2	
	Sample-5	0.30358	0.25938	85.2	
	Sample-6	0.30358	0.26499	87.1	

Table 9: Accuracy results for Cyclophosphamide Related compound D.

Concentration	Sample ID	Actual Impurity Added	Impurity found	% Recovery	Mean
LOQ Level	Sample-1	0.04036	0.04236	104.9	102.8
	Sample-2	0.04036	0.03858	95.6	
	Sample-3	0.04036	0.04300	106.5	
	Sample-4	0.04036	0.03924	97.2	
	Sample-5	0.04036	0.04156	103.0	
	Sample-6	0.04036	0.04156	109.3	
50% Level	Sample-1	0.11423	0.12085	105.8	98.7
	Sample-2	0.11423	0.12085	99.2	
	Sample-3	0.11423	0.11335	91.1	
100% Level	Sample-1	0.20983	0.19965	95.1	94.9
	Sample-2	0.20983	0.20302	96.8	
	Sample-3	0.20983	0.19423	92.7	
150% Level	Sample-1	0.30231	0.31320	103.6	97.7
	Sample-2	0.30231	0.29797	98.5	
	Sample-3	0.30231	0.31013	102.6	
	Sample-4	0.30231	0.30112	99.4	
	Sample-5	0.30231	0.26767	88.3	
	Sample-6	0.30231	0.28442	93.9	

Table 10: Results of Precision.

% of Impurity found								
S. No.	Intraday Precision				Interday Precision			
	Cyclophosphamide Related compound A	Cyclophosphamide Related compound B	Cyclophosphamide Related compound D	Total Impurity	Cyclophosphamide Related compound A	Cyclophosphamide Related compound B	Cyclophosphamide Related compound D	Total Impurity
1	0.17	0.22	0.20	0.59	0.16	0.22	0.19	0.59
2	0.17	0.22	0.20	0.59	0.17	0.21	0.20	0.58
3	0.17	0.22	0.19	0.58	0.16	0.22	0.19	0.57
4	0.16	0.22	0.19	0.57	0.16	0.22	0.20	0.59
5	0.16	0.22	0.20	0.58	0.16	0.21	0.20	0.57
6	0.16	0.22	0.19	0.57	0.17	0.22	0.20	0.57
Mean	0.17	0.22	0.20	0.58	0.16	0.22	0.20	0.58
SD	0.005	0.000	0.005	0.009	0.005	0.005	0.005	0.010
%RSD	3.32	0.00	2.81	1.54	3.16	2.38	2.63	1.70
Overall Mean	NA				0.16	0.22	0.20	0.58
Overall SD					0.005	0.004	0.005	0.009
Overall % RSD					3.14	1.78	2.63	1.55

Table 11: Forced degradation data.

Degradation Condition	Impurity (%w/w)				
	Cyclophosphamide Related compound A	Cyclophosphamide Related compound B	Cyclophosphamide Related compound D	Total Impurity	Mass balance
Control sample	ND	0.03	ND	NA	NA
Acid Stress / 0.1N HCl for 3hours at 50°C	ND	0.75	1.86	5.4	99.4
Base Stress / 0.1N NaoH for 3housr at 50°C	ND	4.44	0.15	5.0	95.2
Oxidative stress/ 0.05M KMNO4 at RT	ND	3.65	0.05	7.6	100.1
Thermal stress/40°C 60 days	ND	0.05	ND	0.0	95.1
Photolytic stress/ 1.2million LUX hours and 200 watt-hours/Sq. mts	ND	0.88	0.03	1.0	97.8
Humidity stress/90% RH, 168 hours	ND	0.08	ND	0.1	97.7

Chromatographic conditions

The UPLC system used for method development, degradation studies, and validation was Waters 2695 separation module consisting of binary pump plus autosampler, autoinjector; SM4 E 07 SM 4094 A (Singapore), online degasser, column oven, and 2996 photodiode array (PDA) detector. The output signal was monitored and processed using Empower software, Waters Corporation, Milford, USA (Database Version 6.10.01.00). An Acquity UPLC BEH C18 (2.1 x 50 mm, 1.7 µm) column was used for LC studies and to develop the SIM (Stability Indicating Method). The flow rate of mobile phase was 0.5 mL/min. The analyte was separated in gradient mode; details are represented in Table 1. The column and autosampler temperature was maintained at 30°C and 5°C respectively, and the detection was monitored at a wavelength 195 nm. The injection volume was 0.5 µL.

RESULTS AND DISCUSSION

Method Validation Parameters

The optimized HPLC method was validated in accordance with the ICH Q2 (R1) guidelines and reported.

Specificity

The results of forced degradation studies of Cyclophosphamide in the presence of their degradation products indicated a high degree of specificity of this method. No interference was observed with blank, placebo and known impurities at the retention time of analyte peak. Typical chromatograms of Blank, spiked sample and placebo are presented in Figure. 2, Figure. 3 and Figure. 4.

Linearity and Range

The linearity was established over the range of LOQ-150% level of standard concentration for Cyclophosphamide and its related compounds. Correlation coefficients (*R*) were found not less than 0.990 for Cyclophosphamide and its related compounds. Typically, the means of the regression equations were y = 405337.0165x + 233048.0363, y = 390440.586x - 38375.411, y = 417804.917x + 155387.414 and y = 119096.192x - 64409.580 for Cyclophosphamide, Cyclophosphamide Related compound-A, Cyclophosphamide Related compound-B and Cyclophosphamide Related compound-D respectively and results are represented in Table 2, Table 3, Table 4 and Table 5. Linearity Plots are presented in Figure. 5, Figure. 6, Figure. 7 and Figure. 8.

Accuracy

Accuracy of the method was determined by performing the recovery experiment for spiked related substances to sample at LOQ level, 50% level, 100% Level and 150% Level of specification limit and the recovery was found good. The recoveries of Cyclophosphamide, Cyclophosphamide Related compound-A, Cyclophosphamide Related compound-B and Cyclophosphamide Related compound-D were found within acceptable ranges of 100 ± 15% except for LOQ where it is 100 ± 20%. The results are presented in Table 6, Table 7, Table 8 and Table 9.

Precision

Prepared six samples by spiking its related compounds at specification level and calculated the assay for Cyclophosphamide related compounds and determined the %RSD for assay was within ±15.0. The %RSD values for the intraday and interday precision were ≤15% confirming that the method was precise. The results are presented in Table 10.

Stability Studies

The International Conference on Harmonization (ICH) guideline,[9, 10] entitled stability testing of new drug substances and products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. In this study, the drugs were exposed to different chemical and physical degradation conditions such as 0.1N HCl (acid hydrolysis), 0.1N NaOH (base hydrolysis), 0.05M KMNO4 (oxidation), heat (thermal decomposition) photolytic degradation (200-watt hours/square meter & 1.2 million lux hours) and Humidity (90%rh at 25°C) for specified time, and then diluted as similar as standard dilution, and then chromatograms were obtained under the similar chromatographic conditions, the percent of degradation was calculated from the peak area of the chromatograms. In the study of acid hydrolysis, an amount of fine powdered sample equivalent to 400 mg of cyclophosphamide was transferred into 50 mL volumetric flask and added 5 mL of freshly prepared 0.1N HCl shaken well and heated on water bath at 50°C for 3 hours, cooled to room temperature and neutralized with 0.1N NaoH and added about 20 mL of diluent and keep on cyclomixer about 3minutes, mix well. Makeup to volume with diluent and mix well. Centrifuge a portion of above solution immediate without any delay for minutes at 4000rpm. Filter the supernant immediately without delay through 0.45 µm PVDF syringe filter by discard the first 3ml of the filtrate using glass syringe, into HPLC vial.

In the study of base hydrolysis, an amount of fine powdered sample equivalent to 400 mg of cyclophosphamide was transferred into 50 mL volumetric flask and added 5 mL of freshly prepared 0.1N NaOH shaken well and heated on water bath at 50°C for 3 hours, cooled to room temperature and neutralized with 0.1N HCL and added about 20 mL of diluent and keep on cyclomixer about 3minutes, mix well. Makeup to volume with diluent and mix well. Centrifuge a portion of above solution immediate without any delay for minutes at 4000rpm. Filter the supernant immediately without delay through 0.45 µm PVDF syringe filter by discard the first 3ml of the filtrate using glass syringe, into HPLC vial.

In case of oxidative degradation, an amount of fine powdered sample equivalent to 400 mg of cyclophosphamide was transferred into a 50 mL volumetric flask and added 5 mL of freshly prepared 0.05M KMNO4 shaken well and keep on bench top and added about 20 mL of diluent and keep on cyclomixer about 3minutes, mix well. Makeup to volume with diluent and mix well. Centrifuge a portion of above solution immediate without any delay for

minutes at 4000rpm. Filter the supernant immediately without delay through 0.45 µm PVDF syringe filter by discard the first 3ml of the filtrate using glass syringe, into HPLC vial.

In the study of thermal or Humidity or photolytic degradation, an amount of fine powdered sample equivalent to 400 mg of cyclophosphamide was transferred into a clean and dry watch glass, placed in an oven at 40˚C for 60 days, Photo degradation 200-watt hours/square meter & 1.2 million lux hours and Humidity (90%RH at 25°C) for 168 hrs. Then removed from the oven, photolytic chamber, Humidity chamber and allowed to stand for some time at room temperature The substance was accurately transferred into a 50 mL volumetric flask and added about 20 mL of diluent and keep on cyclomixer about 3minutes, mix well. Makeup to volume with diluent and mix well. Centrifuge a portion of above solution immediate without any delay for minutes at 4000rpm. Filter the supernant immediately without delay through 0.45 µm PVDF syringe filter by discard the first 3ml of the filtrate using glass syringe, into HPLC vial Injected into UPLC and chromatograms were obtained under optimized conditions. A study of forced degradation was carried out to evaluate the stability of the drugs in formulations and the results of degradation and stability of drugs are presented in Table 11.

CONCLUSION

The present developed Stability Indicating RP-UPLC method was found to be simple, rapid, accurate and specific for the determination of cyclophosphamide related compounds in pharmaceutical dosage forms. Finally the simplicity of sample preparation and the shorter chromatographic runtime gives the method capability for high sample throughput. From the results of all the validation parameters we can conclude that the present method can be used for routine analysis of cyclophosphamide related compounds in pharmaceutical dosage forms.

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