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Development and Validation of analytical method for Anticancer drug Cyclophosphoamide by UPLC

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ABSTRACT
Cyclophosphamide can be quantified in tablet formulations using the approved Stability Indicating RP-UPLC method, which is simple, quick, accurate, and specific. At the LOQ level, the average recovery for Cyclophosphamide is 95.0%, 90.6%, 86.8%, and 88.7%; for Related compound-A, it is 99.7%, 95.6%, 92.0%, and 94.2% at the LOQ level; for Related compound-B, it is 100.5%, 96.0% at the 50% level, 109.9% at the 100% level, and 88.2% at the 150% level; and for Related compound-D, the LOQ level is 102.8%, 50% is 98.7%, at 100% is 94.9%, and at 150% level is 97.7%. Cyclophosphamide's overall percentage RSD is 3.07, whereas related molecule A, B, and C have respective RSDs of 2.77, 7.76, and 5.29.

Keywords: *Cyclophosphamide, UPLC, Method development, Validation.*

1. INTRODUCTION

Cyclophosphamide, also known as cytophosphane, is classified as nitrogen mustard and an alkylating agent. It is believed to operate by inhibiting the synthesis of ribonucleic acid and the replication of deoxyribonucleic acid [1]. It is an immunosuppressive agent utilized in chemotherapy. It is utilized to treat ovarian cancer, breast cancer, leukemia, small cell lung cancer, neuroblastoma, multiple myeloma, lymphoma, and sarcoma [2]. Cyclophosphamide is employed in the treatment of nephrotic syndrome and granulomatosis with polyangiitis due to its immunosuppressive properties [3].

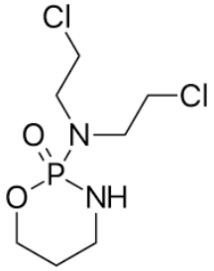

Figure 1: Structure of Cyclophosphamide

2. MATERIALS AND METHODS:

Buffer Preparation:

Dissolve 1 milliliter of orthophosphoric acid in 1000 milliliters of milli-Q water, then employ diluted triethylamine to adjust the pH to 7.0 ±0.05, and subsequently filter through a 0.22 µm membrane filter [4].

Diluent Preparation: As a diluent, chilled water (2–8°C) is utilized.

Standard stock solution Preparation:

40 milligrams of cyclophosphamide monohydrate, accurately measured, was placed into a 100 ml volumetric flask. Sixty milliliters of diluent were incorporated, and the mixture was subjected to a cyclomixer for roughly two minutes to achieve dissolution and dilution to the specified volume with the diluents [5].

Standard Solution Preparation:

Transfer 1.0 ml of the Cyclophosphamide standard stock solution and 2.0 ml of each Cyclophosphamide Related molecule-A, B and C into a 10 ml volumetric flask. Modify the volume to the specified mark using diluent and mix thoroughly [6].

Placebo solution Preparation:

Transfer 1.0 ml of the Cyclophosphamide standard stock solution and 2.0 ml of each Cyclophosphamide Related molecule-A, B and C into a 10 ml volumetric flask. Modify the volume to the specified mark using diluent and mix thoroughly [7].

Preparation of Sample Solution:

Four hundred milligrams of cyclophosphamide powder was precisely measured and transferred into a 50 ml volumetric flask. Twenty milliliters of diluent were added, and the mixture was stirred for roughly three minutes using a cyclomixer. Immediately centrifuge a portion of the specified solution for several minutes at 4000 rpm. Promptly filter the supernatant utilizing a 0.45 µm PVDF syringe filter. Employ a glass syringe to transfer the initial 3 ml of the filtrate into an HPLC vial [8].

SPECIFICITY (SELECTIVITY):

Preparation of Cyclophosphamide stock solution:

10 mg of cyclophosphamide monohydrate was measured, placed into a 10 mL volumetric flask, and subsequently 5 mL of diluent was introduced. The mixture was meticulously combined and sonicated to solubilize the diluents [9].

Preparation of Cyclophosphamide Related molecule -A stock solution: 10 mg of Cyclophosphamide Related molecule - A was weighed, transferred into a 10 mL volumetric flask, and then 5 mL of diluent was added. The liquid was carefully blended and sonicated to dissolve the diluents [10].

Preparation of Cyclophosphamide Related molecule-B stock solution: 10 mg of Cyclophosphamide Related molecule-B was weighed, transferred into a 10 mL volumetric flask, and then 5 mL of diluent was added. The liquid was carefully mixed and sonicated to dissolve the diluents [11].

Preparation of stock solution for Cyclophosphamide Related molecule-C: 10 mg of Cyclophosphamide Related Compound-D was weighed and transferred to a 10 mL volumetric vessel. Subsequently, 5 mL of diluent was added. The diluent was dissolved by vigorously mixing and sonicating the mixture [12].

Spiked Placebo Preparation:

We weighed and deposited the equivalent of 400 mg of cyclophosphamide in placebo powder into a 50-ml volumetric vessel. We thoroughly mixed the mixture on a cyclomixer for approximately three minutes after adding 20 ml of diluent. Centrifuge a portion of the aforementioned solution at 4000 rpm for a few minutes without delay. Use a 0.45 µm PVDF syringe filter to immediately filtrate the supernant. Discard the initial 3 ml of the filtrate using a glass injector. Subsequently, transfer the filtrate to an HPLC vial and inject it into a UPLC [13].

Spiked Solution Preparation:

We weighed 400 mg of cyclophosphamide and added it to a 20-mL volumetric flask. We added 10 mL of diluent and thoroughly mixed the mixture on a mixer for approximately three minutes. Add 0.8 mL of Cyclophosphamide Accuracy stock solution and Cyclophosphamide related molecule A, B, and C accuracy stock solutions, adjust the volume with diluent, and thoroughly mix. Without delay, centrifuge a portion of the aforesaid solution for a few minutes at 4000 rpm. Filter the supernant right away using a 0.45 µm PVDF syringe filter. Use a glass syringe to discard the first 3 ml of the filtrate, then transfer it to an HPLC vial and inject it into a UPLC [14].

PRECISION:

Prepared samples according to the test procedure (six spiked samples and one unspiked sample) to represent a single batch,

demonstrating the method's accuracy. identified the relevant compounds in these samples and calculated the % RSD to assess the method's accuracy [15].

INTERMEDIATE PRECISION:

Purpose: The purpose of this test method is to show that the findings obtained for the variables of instrument to instrument, column to column (different lots), analyst to analyst, and day to day are reproducible.

Procedure: Samples representing a single batch should be prepared using the test technique (six spiked samples and unspiked samples) and performed by two different analyzers on two different days, on two different columns, and on two different instruments in order to conduct the precision research [16].

LOD and LOQ:

Stock solution Preparation for Cyclophosphamide:

We weighed and transferred 5 mg of cyclophosphamide monohydrate to a 25 mL volumetric flask, then added 15 mL of diluent. We properly mixed the mixture and then used a sonicator to dissolve the diluents [17].

Stock solution Preparation for Cyclophosphamide (Related Molecule-A): We weighed and transferred 5 mg of Cyclophosphamide Related Molecule-A into a 25 mL volumetric flask, then added 15 mL of diluent. The mixture was well mixed and sonicated to dissolve the diluents [18].

Stock solution Preparation for Cyclophosphamide (Related Molecule -B): We weighed and transferred 5 mg of Cyclophosphamide Related Molecule-B into a 25 mL volumetric flask, then added 15 mL of diluent. We thoroughly mixed the mixture and sonicated it to dissolve the diluents [19].

Stock solution Preparation for Cyclophosphamide (Related Molecule -C): We weighed 5 mg of Cyclophosphamide **Related Molecule-C**, transferred it to a 25 mL volumetric flask, and then added 15 mL of diluent. We thoroughly mixed the mixture and sonicated it to dissolve the diluents [20].

LOQ PRECISION STUDY:

LOQ Solution preparation: Transferred each 2mL of Cyclophosphamide stock, Cyclophosphamide Related Molecule-A, B, C linearity stock solutions in to 50mL volumetric flask, diluted volume with diluent and mixed well [21].

LOD Solution preparation: Transferred 3.3 mL of LOQ solution into a 10 mL volumetric flask, diluted with diluent, and mixed thoroughly [22].

LINEARITY & RANGE:

LOQ Solution preparation: Cyclophosphamide stock (2 mL) and Cyclophosphamide Related Molecule-A, B, C linearity stock solutions were transferred to a 50 mL volumetric flask, diluted with diluent, and thoroughly mixed [23].

ACCURACY:

Preparation Stock solutions for Accuracy:

Preparation of Accuracy stock solution for Cyclophosphamide:

20 mg of cyclophosphamide monohydrate was measured, placed into a 25 mL volumetric flask, and subsequently 15 mL of diluent was introduced. The mixture was meticulously combined and sonicated to solubilize the diluents [24].

Accuracy stock solution Preparation for Cyclophosphamide (Related Molecule-A):

We weighed and transferred 20 mg of Cyclophosphamide Related Molecule-A to a 25 mL volumetric flask, then added 15 mL of diluent. We thoroughly mixed the mixture and then used a sonicator to dissolve the diluents [25].

Accuracy stock solution Preparation for Cyclophosphamide (Related Molecule-B):

We weighed and transferred 20 mg of Cyclophosphamide Related Molecule-B to a 25 mL volumetric flask, then added 15 mL of diluent. We properly mixed the mixture and then used a sonicator to dissolve the diluents [26].

Accuracy stock solution Preparation for Cyclophosphamide (Related Molecule-C):

We weighed 20 mg of Cyclophosphamide Related Molecule-C, transferred it to a 25 mL volumetric flask, and then added 15 mL of diluent. The mixture was thoroughly mixed and sonicated to dissolve the diluents [27-32].

RESULTS AND DISCUSSION:

Chromatographic conditions:

Table 1: Chromatographic conditions

Parameter	Condition
Column	Acquity UPLC BEH C18 (2.1 x 50 mm, 1.7 µm)
Detector wavelength	UV, 190nm
Flow rate	0.5 ml/minute Sample cooler temperature: 5°C
Run Time	8 minutes

Table 2: Gradient programme

Sr. No	Time in min	% of Mobile Phase-A	% of Mobile Phase-B	Curve
1	Initial	100.0	00	Initial
2	01.0	100.0	00	6.0
3	04.0	50.0	50.0	6.0
4	05.5	20.0	80.0	6.0
5	06.5	20.0	80.0	6.0
6	07.0	100.0	0.0	6.0
7	08.0	100.0	0.0	6.0

Observations:

Table 3: Retention Times, Purity Angle & Purity Threshold for Principle peak and Known Impurity peaks in Spiked Sample is given below

Sr. No	Component	RT	RRT	Purity Angle	Purity Threshold	Peak purity
1	Cyclophosphamide	4.18	1.00	0.25	0.72	Passes
2	Molecule A (Related)	3.80	0.91	0.27	0.76	Passes
3	Molecule B (Related)	0.71	0.17	0.32	0.83	Passes
4	Molecule C (Related)	0.96	0.23	0.28	0.70	Passes

PRECISION:

Table 4: Method precision of Unspiked Sample

Sr. No	Component	Observation
1	Molecule A (Related)	ND
2	Molecule B (Related)	0.03
3	Molecule C (Related)	ND
4	Total Impurity	0.96

Table 5: Method precision of Spiked Sample

Sr. No.	% Impurity found			Total Impurities
	Molecule A (Related)	Molecule B (Related)	Molecule C (Related)	
1	0.17	0.22	0.20	0.59
2	0.17	0.22	0.20	0.59
3	0.17	0.22	0.19	0.58
4	0.16	0.22	0.19	0.57
5	0.16	0.22	0.20	0.58
6	0.16	0.22	0.19	0.57
Avg	0.17	0.22	0.20	0.58
Std. Dev.	0.005	0.000	0.005	0.009
%RSD	3.32	0.00	2.81	1.54

The % RSD of known impurities in six test preparations for Related Molecule-A is 3.32, Related Molecule-B is 0.00 and for Related Molecule-C is 2.81. The % RSD of total impurities in six test preparations is 1.54. The study concludes repeatability (precision) of test results obtained by this method.

INTERMEDIATE PRECISION:

Intermediate precision Results: (in %)

Table 6: Intermediate precision of Unspiked Sample

Sr. No	Component	Observation
1	Molecule A (Related)	ND
2	Molecule B (Related)	0.02
3	Molecule C (Related)	ND
4	Total Impurity	BQL

Table 7: Intermediate precision of spiked Sample

	Sr. No.	Spiked Sample- Unspiked Sample			Total Impurities
		Molecule A (Related)	Molecule B (Related)	Molecule C (Related)	
PRECISION STUDY (Day-1,System-1, Analyst-1, Column-1)	1	0.17	0.22	0.20	0.59
	2	0.17	0.22	0.20	0.59
	3	0.17	0.22	0.19	0.58
	4	0.16	0.22	0.19	0.57
	5	0.16	0.22	0.20	0.58
	6	0.16	0.22	0.19	0.57

RUGGEDNESS STUDY (Day-2,System-2, Analyst-2, Column-2)	1	0.16	0.22	0.19	0.59
	2	0.17	0.21	0.20	0.58
	3	0.16	0.22	0.19	0.57
	4	0.16	0.22	0.20	0.59
	5	0.16	0.21	0.20	0.57
	6	0.17	0.22	0.20	0.57
Average		**0.17**	**0.22**	**0.20**	**0.58**
Std dev		0.005	0.000	0.005	0.009
%RSD		**3.32**	**0.00**	**2.81**	**1.54**
Overall Average		**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**

The % RSD of known impurity from six test preparations is less than 15.0. i.e., for Related Molecule-A is 3.16, Related Molecule-B is 2.38 and Related Molecule-C is 2.63. The % RSD of total impurities from six test preparation is less than 15.0. i.e.,

1.70. Cumulative %relative standard deviation of Intermediate precision study along with the precision study for known impurities is less than 15.0. i.e., for Related Molecule-A is 3.14 Related Molecule-B is 1.78, Related Molecule-C is 2.63 and for total impurities is less than 15.0. i.e., 1.55. The study demonstrates that the approach is robust to variables such as two distinct instruments, two distinct columns, and two distinct analysts on two distinct days.

LOD and LOQ:

Table 8: LOD and LOQ Concentrations

Name	**LOD Conc. (ppm)**	**S/N**	**LOQ Conc. (ppm)**	**S/N**
Cyclophosphamide	2.6598	3.5	7.9795	9.8
Molecule A (Related)	2.6983	3.1	8.0949	10.6
Molecule B (Related)	2.6942	3.3	8.0825	11.1
Molecule C (Related)	2.7284	3.4	8.1851	10.6

From this study, it is shown that limit of detection of Cyclophosphamide is 2.6598ppm, S/N ratio is 3.5, Related Molecule-A is 2.6983ppm, S/N ratio is 3.1, Related Molecule-B is 2.6942ppm, S/N ratio is 3.3 and Related Molecule-C is 2.7284ppm, S/N ratio is 3.4.

Limit of quantitation of Cyclophosphamide is 7.9795ppm, S/N ratio is 9.8, Related compound-A is 8.0949ppm, S/N ratio is 10.6, Related Molecule-B is 8.0825ppm, S/N ratio is 11.1 and Related compound-D is 8.1851ppm, S/N ratio is 10.6.

From this study, Limit of Detection and Limit of Quantitation of known impurities and unknown impurities in terms of Cyclophosphamide are evaluated.

LOQ PRECISION STUDY:

Table 9: LOD Solution Areas

Sr. No	**Peak Areas**	
1	Cyclophosphamide	1119623
2	Molecule A (Related)	987079

3	Molecule B (Related)	1149483
4	Molecule C (Related)	343716

Table 10: LOQ Precision Results

Injection	**Peak Areas**			
	Cyclophosphamide	**Molecule A (Related)**	**Molecule B (Related)**	**Molecule C (Related)**
Injection-1	3378922	2961238	3448407	1091105
Injection-2	3225991	3012159	3365421	991546
Injection-3	3355983	2945249	3521836	1070103
Injection-4	3569891	2959419	3532156	1000252
Injection-5	3289691	3078119	3431161	1012902
Injection-6	3332995	2811235	3391463	1020904
Average	**3358912**	**2961237**	**3448407**	**1031135**
Std Dev.	116663.917	88333.4	67575.784	40182.26
%RSD	**3.47**	**2.98**	**1.96**	**3.90**

The % RSD of peak areas from six injections of LOQ level for Cyclophosphamide is 3.47, for Related Molecule-A is 2.98, for Related Molecule-B is 1.96 and Related Molecule-C is 3.90.

LINEARITY & RANGE:

Table 11: Preparation of Linearity solutions

Level	**Each Stock solution to be added in ml**	**Dilute with diluent**	**Concentration in ppm**			
	Cyclophosphamide and its impurities		**Cyclophosphamide**	**M-A**	**M-B**	**M-C**
LOQ	2.0	50	7.9795	8.0949	8.0825	8.1851
25%	1.0	20	9.9744	10.0618	10.1032	10.2314
50%	2.0	20	19.9489	20.1236	20.2063	20.467
100%	2.0	10	39.8977	40.2472	40.4127	40.9254
120%	2.4	10	47.8773	48.2966	48.4127	49.1105
150%	3.0	10	59.8466	60.3708	60.6190	61.3881

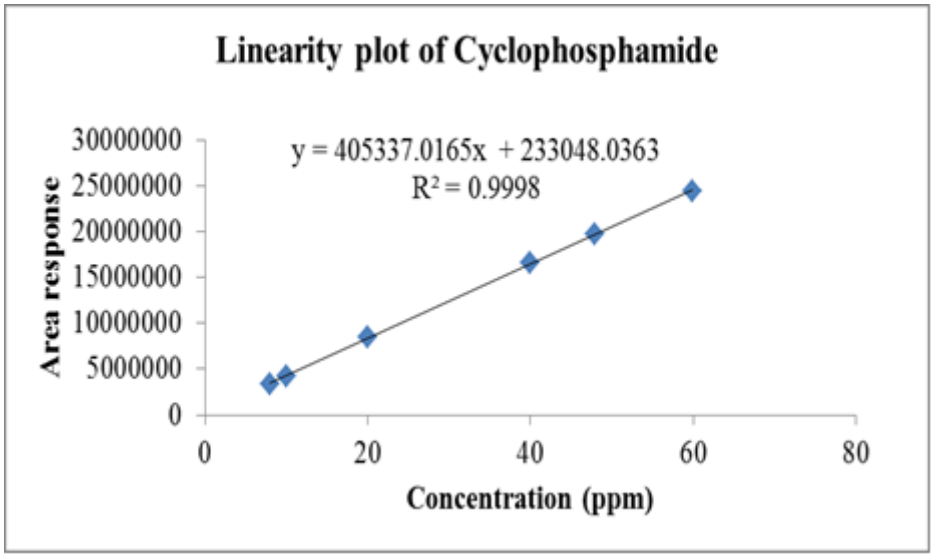

Figure 2: Cyclophosphamide linearity curve

Table 12: Correlation Coefficient (R) values

Name	Cyclophosphamide	Molecule A (Related)	Molecule B (Related)	Molecule C (Related)
R (Correlation Coefficient)	0.9998	1.0000	1.0000	0.999
The % RSD of Lower level (LOQ)	3.82	2.70	3.10	1.33
The % RSD of Higher Level (150%)	1.36	1.61	0.65	0.57
% Intercept	1.41	-0.24	0.91	-1.33

The Correlation Coefficient for Cyclophosphamide is 0.9999, for Related Molecule A is 0.9999, for Related Molecule B is 1.0000 and Related Molecule C is 0.9995 % RSD of peak area responses at LOQ level for Cyclophosphamide is 3.47, for Related Molecule A is 2.98, for Related Molecule B is 1.96 and Related Molecule C is 3.90.

% RSD of peak area responses at 150% level for Cyclophosphamide is 1.36, for Related Molecule A is 1.61, for Related Molecule B is 0.65 and Related Molecule C is 0.57.

ACCURACY:

Table 5.13: Preparation of Accuracy solutions

Level	Wt. of sample to be taken (in mg)	Each Stock solutions to be added in mL	Dilute with diluent	Concentration in ppm			
		Cyclophosphamide impurities		Cyclophosphamide	M-A	M-B	M-D
LOQ	400	0.2	20	8	8	8	8
50%	400	0.5	20	20	20	20	20
100%	400	1.0	20	40	40	40	40

150%	400	1.5	20	60	60	60	60

LOQ Solution preparation: Transferred each 0.2mL of Cyclophosphamide stock, Cyclophosphamide Related Molecule A, B, C linearity stock solutions in to 20mL volumetric flask, diluted volume with diluent and mixed well.

Table 13: Accuracy (% Recovery) of Cyclophosphamide

Sr. No.	**% Recovery of Cyclophosphamide**			
	Accuracy- LOQ	**Accuracy-50%**	**Accuracy-100%**	**Accuracy-150%**
T1	92.4	90.6	88.1	89.0
T2	91.3	92.6	86.6	90.8
T3	95.5	88.7	85.6	88.8
Average	93.1	90.6	86.8	89.5
Std.Dev.	2.178	1.950	1.258	1.102
%RSD	2.34	2.15	1.45	1.23
Over all % RSD of Cyclophosphamide is 3.07				

Table 14: Accuracy (% Recovery) of Chloroacetanilide Related Molecule-A

Sr. No.	**% Recovery of Related Molecule -A**			
	Accuracy- LOQ	**Accuracy-50%**	**Accuracy-100%**	**Accuracy-150%**
T1	98.7	92.1	92.3	94.7
T2	96.5	94.9	92.0	94.7
T3	95.5	99.9	91.8	93.9
Average	96.9	95.6	92.0	94.4
SD	1.637	3.951	0.252	0.462
%RSD	1.69	4.13	0.27	0.49
Over all % RSD of Related compound-A is 2.77				

Table 15: Accuracy (% Recovery) of related Molecule -B

Sr. No.	**% Recovery of Related Molecule -B**			
	Accuracy- LOQ	**Accuracy-50%**	**Accuracy-100%**	**Accuracy-150%**
T1	100.2	96.4	111.0	90.2
T2	98.6	97.4	110.2	90.1
T3	98.7	94.2	108.6	89.3
Average	99.2	96.0	109.9	89.9
SD	0.896	1.637	1.222	0.493

%RSD	0.90	1.71	1.11	0.55
Over all % RSD of Related compound-B is 7.76				

Table 16: Accuracy (% Recovery) of Cyclophosphamide Related Molecule C

Sr. No.	**% Recovery of Related Molecule C**			
	Accuracy- LOQ	**Accuracy-50%**	**Accuracy-100%**	**Accuracy-150%**
T1	104.9	105.8	95.1	103.6
T2	95.6	99.2	96.8	98.5
T3	106.5	91.1	92.7	102.6
Average	102.3	98.7	94.9	101.6
Std. Dev.	5.886	7.363	2.060	2.702
%RSD	5.75	7.46	2.17	2.66
Over all % RSD of Related compound-A is 5.29				

Table 17: Accuracy (% Recovery) at Lower level (LOQ)

Accuracy (% Recovery) at Lower level (LOQ)				
Samples	**Cyclophosphamide**	**RC-A**	**RC-B**	**RC-D**
1	92.4	98.7	100.2	104.9
2	91.3	96.5	98.6	95.6
3	95.5	95.5	98.7	106.5
4	98.3	103.2	105.4	97.2
5	98	106.7	102.2	103.0
6	94.7	97.8	98.1	109.3
Avg	**95.0**	**99.7**	**100.5**	**102.8**
SD	**2.852**	**4.330**	**2.814**	**5.359**
% RSD	**3.00**	**4.34**	**2.80**	**5.22**

Table 18: Accuracy (% Recovery) at higher level (LOQ)

Accuracy (% Recovery) at Higher level (150%)				
Samples	**Cyclophosphamide**	**M-A**	**M-B**	**M-C**
1	89.0	94.7	90.2	103.6
2	90.8	94.7	90.1	98.5
3	88.8	93.9	89.3	102.6

4	87.8	94.5	87.2	99.4
5	89.3	94.7	85.2	88.3
6	86.3	92.8	87.1	93.9
Avg	**88.7**	**94.2**	**88.2**	**97.7**
Std.Dev.	**1.512**	**0.760**	**2.001**	**5.747**
% RSD	**1.71**	**0.81**	**2.27**	**5.88**

The average recovery for Cyclophosphamide at LOQ level is 95.0%, at 50% level is 90.6%, at 100% level is 86.8% and at 150% level is 88.7%, for Related compound Molecule -A at LOQ level is 99.7%, at 50% level is 95.6%, at 100% level is 92.0% and at 150% level is 94.2% and Related Molecule-B at LOQ level is 100.5%, at 50% level is 96.0%, at 100% level is 109.9% and at 150% level is 88.2%, for Related Molecule C at LOQ level is 102.8%, 50% is 98.7%, at 100% is 94.9% and at 150% level is 97.7%. The overall %RSD of Cyclophosphamide is 3.07, Related Molecule-A is 2.77, Related Molecule-B is 7.76 and Related Molecule-C is 5.29

CONCLUSION:

This report offers a concise summary of the findings obtained from the validation. For each and every parameter, it has been determined that the findings satisfy the acceptance criteria. Specific, precise, linear, and accurate are the characteristics that have been determined to be linked with the technique that has been validated for the estimate of associated chemicals of cyclophosphamide tablets.

DECLARATIONS:

Ethics approval and consent to participate:

Not applicable.

Consent for publication:

All the authors approved the manuscript for publication.

Availability of data and material:

All required data is available.

Competing interests:

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