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Quantification of Cyclophosphamide Impurities in Cyclophosphamide Drug Substance and formulations by Ion chromatography

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Abstract: ***Non suppressed Ion chromatographic method for quantification of Cyclophosphamide Impurities A, B, D and Propanolamine was optimized validated. In this method, low capacity cation exchange column was used with low strength acidic mobile phase to achieve the optimum resolution between all the impurities which further detected on Conductivity and UV detectors, which were connected in series. Additionally, propanolamine impurity was also quantified in same method simultaneously. LOQ was established at 10 mg/L for Impurity A, B and D while for propanolamine it is 2 .0 mg/L. Also, method found to be linear with correlation coefficient of 0.999 for each impurity. Also, Accuracy results for all the impurities observed between 90 to 110 %. The method validation results showed the perfect precision, accuracy and linearity of the method. Also, same method used to quantify the cyclophosphamide impurities in Cyclophosphamide containing formulations like Sterile powder for Injection and Capsules. Hence the method can be recommended as an alternative for compendial TLC method for simultaneous determination of these Cyclophosphamide Impurities.***

Keywords: Cyclophosphamide Impurities, Non suppressed Ion Chromatography, UV, Sterile Powder, Capsules

1. Introduction

Cyclophosphamide is an anti-cancer agent. It is considered as a first line drug for chemotherapy. Related compounds may arise at any stage of manufacturing or storage condition of drug substances hence these compounds must be detected with accurate analytical method [1, 2,]. Accurate analytical method will assess the quality and safety of the drugs [3]. United States of Pharmacopeia (USP) uses thin layer chromatography (TLC) method for the detection of related compounds in Cyclophosphamide API Monograph [4]. The literature survey reveals that an advance chromatographic technique like Liquid Chromatography-Quadrupole Time of Flight (QTOF) mass spectrometry was used to identify the presence of degradation product in Cyclophosphamide. LC-QTOF is a niche analytical instrument which is unaffordable for many of the quality department. However, there is no LC technique available for the quantification of related compounds present in Cyclophosphamide [5, 6, 7, 8]. Confinement of 20 to 30 mg/L (parts per million) for fresh fruits and 250 mg/L for dried natural products [3].

Hence there was a need to develop it which became the purpose of the further study. In this present study, an attempt has been made to develop an accurate, specific and reproducible method for the quantification of related compounds (Impurity A, B, D and Propanolamine) present in Cyclophosphamide (Figure 1). All these impurities are carcinogenic and hence need to be monitored closely.

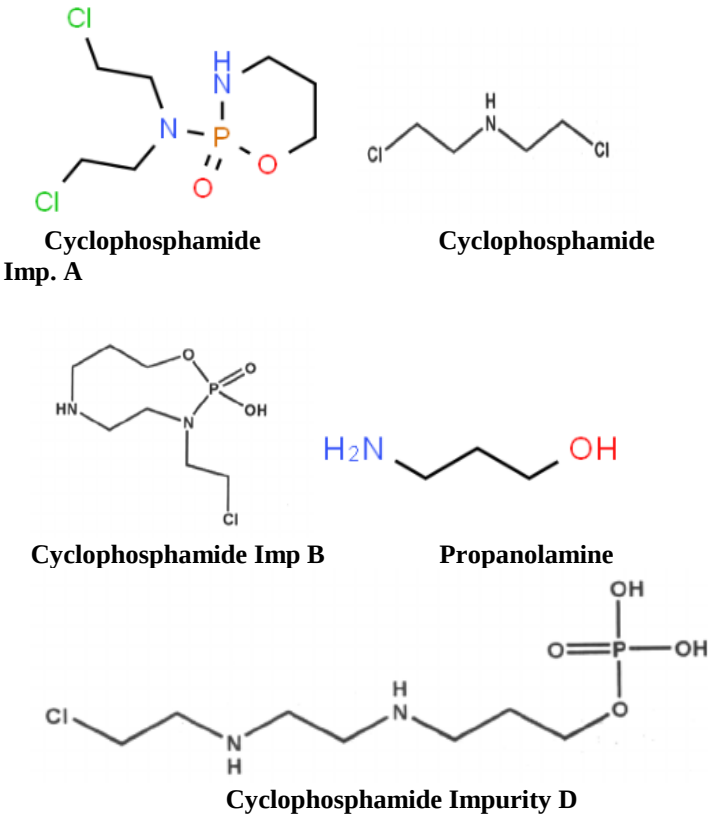


Figure 1: Cyclophosphamide and its Impurity Structures

Ion Chromatography method are capable of reporting precise and accurate results as compared to TLC methods since TLC method are based on visual comparison of spots intensity matching which can be less quantitative in practice.

Ion Chromatography method is used to separate impurity A, B and D. This method is also able to analyze one additional impurity from API which is Propanolamine. Propanolamine is a Process Impurity in API and should be monitored as it is toxic above certain level. This paper also describes the method validation activity and its results for this method. Along with Cyclophosphamide API, its formulations like Sterile powder for injection and Capsules were also analyzed with same method for cyclophosphamide impurities. The method involves use of Ion Chromatography with Conductivity as well as UV detector in series. Low capacity Cation exchange column was used with low strength acidic mobile phase. Impurity A, D and Propanolamine was analyzed in using conductivity detector and Impurity B was analyzed on UV detector which will be connected in series.

2. Experimental

2.1 Reagents and Chemicals

All chemicals used for preparation of reagents, standards and mobile phase were of analytical grade. Ultrapure deionized water (18.2 MΩ cm, Milli-Q system) was used for the preparation of mobile phase, standards and samples. Other required Chemicals are Orthophosphoric acid (85 %), Acetonitrile (Merck)

2.2 Instrument

The equipment used was Thermo Fisher Dionex Ion Chromatography system with conductivity and VWD detector having autosampler with a 25 µL loop, IonPac SCS 1 column (2 x 250mm inner diameter) and IonPac SCS 1 Guard Column (2 X 50 mm) was used as separator column. Software used for data acquisition was Thermo Fisher Scientific Dionex Chromeleon (version: 7.2). Chromatograms were monitored simultaneously during analysis.

2.3 Procedure

Preparation of Mobile Phase:
0.2 ml of 85 % Ortho-Phosphoric acid diluted to 1000 ml with water. Sonicated to degas.

Preparation of Diluent:
500 ml of Acetonitrile was mixed with 500 ml of water.

Standard Preparation:
Impurity Stock Solution (Each Impurity 1000 mg/L)

About 5 mg of each of impurity A, Impurity B, Impurity D and Propanolamine was weighed in 5 ml Volumetric flask. Added to it 8 ml of Diluent and Sonicated to dissolve. Volume was made with diluent to 5 ml. Linearity levels for all the impurities were prepared according to Table 1 & 2.

Table 1: Linearity Level Preparation for Impurity A, Impurity B and Impurity D

Linearity Level	Vol pipetted out from 1000 mg/L Impurity Stock Solution (ml)	Diluted to (ml)	Final Concentration (mg/L)
LOQ/Level 1	0.5	50	10
Level 2	1.0	50	20
Level 3	1.5	50	30
Level 4	2.0	50	40
Level 5	2.5	50	50

Table 2: Linearity Level Preparation for Propanolamine Impurity

Linearity Level	Vol pipetted out from 1000 mg/L Impurity Stock Solution (ml)	Diluted to (ml)	Final Concentration (mg/L)
LOQ/Level 1	0.1	50	2
Level 2	0.25	50	5
Level 3	0.5	50	10
Level 4	0.75	50	15
Level 5	1.0	50	20

Sample Preparation
Cyclophosphamide API (S.I. No. IC/CY/01)
About 500 mg of API was transferred to 10 ml Volumetric Flask. Added to it 8 ml of diluent and sonicated to dissolve. Volume was made to the mark with diluent. The sample Solution was filtered through 0.22 µ filter and injected on IC system. Ion chromatography instrument was set as per parameters given in Table 3 [16].

Cyclophosphamide Sterile Powder Formulation (Each 800 mg of Powder contains 500 mg of Cyclophosphamide) (S.I. No. IC/CY/FP01)
About 800 mg of sterile powder was diluted to 100 ml with diluent. Sonicated for 5 mins and filtered through 0.2 µ Nylon Filter. The filtrate was used for injection on IC.

Cyclophosphamide Capsules (Each capsule contains 25 mg of Cyclophosphamide) (S.I. No. IC/CY/FP02)
About 167 mg of capsule powder was diluted to 5 ml with diluent. Sonicated for 5 mins and filtered through 0.2 µ Nylon Filter. The filtrate was used for injection on IC.

Table 3: Chromatographic condition:

Chromatographic Condition	Chromatographic Parameter
Column	SCS 1 Analytical 2 X 250 mm (P/N 061520) and SCS 1 Guard 2 X 50 mm (P/N 061522)
Eluent	0.2 ml Ortho-phosphoric acid (85 %) in 1 L with Water
Flow rate	0.25 ml/min
Injection Vol	25 µL
Column Temp.	30° C
Detector	Non-suppressed Conductivity Detector for Impurity A, Propanolamine and Impurity D UV Detector for Impurity B at 200 nm
Detector Temperature	35°C
Run time	50 mins
Sample Temperature	10°C

3. Result and Discussions

LOQ for Impurity A, Impurity B, Impurity D observed at 10 mg/L concentration of each with Signal to Noise ratio between 12 to 20. Also % RSD for 6 consecutive impurity injections at LOQ level found less than 5.0. LOQ for Propanolamine Impurity was observed to be at 2.0 mg/L

Concentration with Signal to Noise ratio at about 15. Also % RSD for 6 consecutive injection observed to be below 5 %. Refer chromatogram in Figure 2 and LOQ results in Table 4

LOD for Impurity A, Impurity B, and Impurity D observed at 3.0 mg/L Concentration, while for Propanolamine Impurity it was observed at 0.6 mg/L.

Table 4: RSD and Signal to Noise ratio for Six Consecutive Injections of Cyclophosphamide Impurities at LOQ level

Compound	Amount (mg/L)	Signal to Noise Ratio	% RSD (n=6)
Impurity A	10.0	15	2.2
Impurity B	10.0	12	1.8
Impurity D	10.0	14	1.5
Propanolamine	2.0	11	2.6

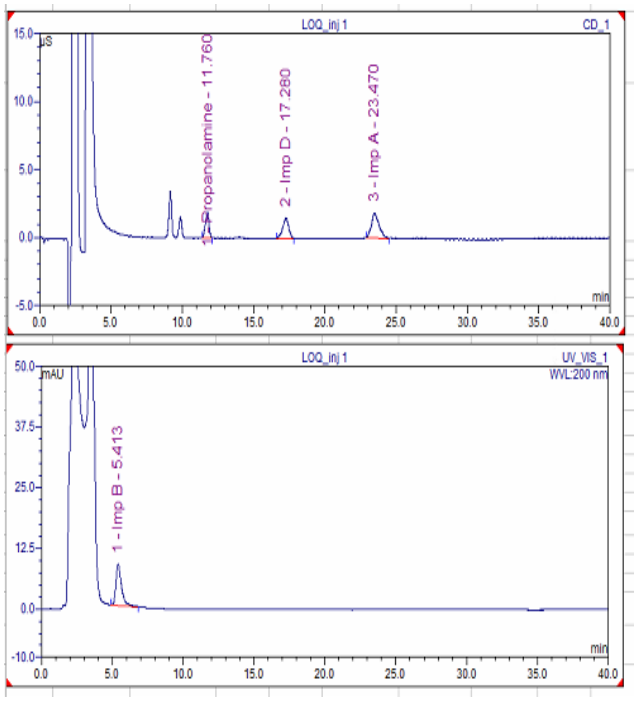


Figure 2: LOQ Chromatogram for all Cyclophosphamide impurities

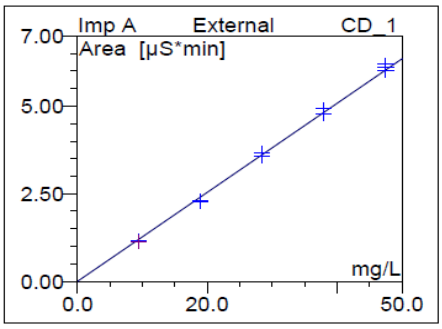
All the impurities found well resolved from each other as Impurity D found to be well resolved from Propanolamine with resolution of 8.2 and from Impurity A with resolution of 5.3

The linearity for Impurity A, Impurity B and Impurity D was studied in concentration range of 10.0 mg/L to 50.0 mg/L while for propanolamine the Study was performed in Concentration range of 2 mg/L to 10 mg/L Concentration. Each level was injected in triplicate. The correlation coefficient, slope and offset for all impurities was observed as given in Figure 3 and Table 5.

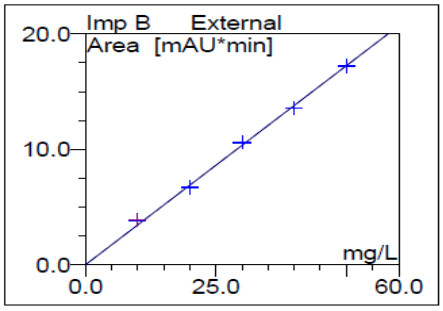
Table 5: Linearity Results for all Cyclophosphamide Impurities.

Compound	Points	Offset	Correlation Coefficient	Slope
Impurity A	15	0.00	0.9995	7.62
Impurity B	15	0.00	0.999	20.70
Impurity D	15	0.00	0.9995	4.63
Propanolamine	15	0.00	0.9997	17.53

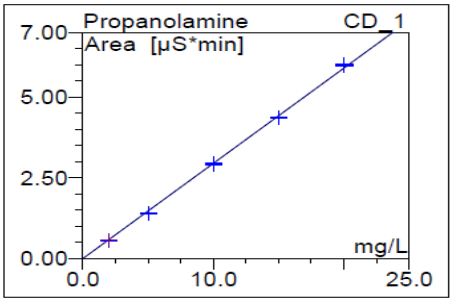
Linearity Plot: Impurity A



Linearity Plot: Impurity B



Linearity Plot: Propanolamine



Linearity Plot: Impurity D

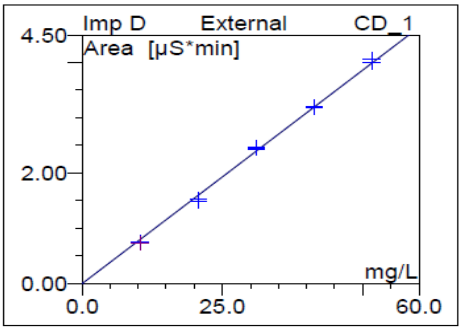


Figure 3: Linearity Plot for Cyclophosphamide Impurity A, B, D and Propanolamine

Method Specificity was also performed by injecting Diluent injection and it was compared with Standard and Sample Chromatograms. No interference from Blank was Observed at RT of any Impurity in standard as well as sample Injection chromatograms. The sample includes the Cyclophosphamide API and its finished products like Sterile powder for injection and capsule. Also, Individual impurity Chromatogram showed no interference among each other. Refer Figure 4 for overlay chromatograms with Conductivity detector.

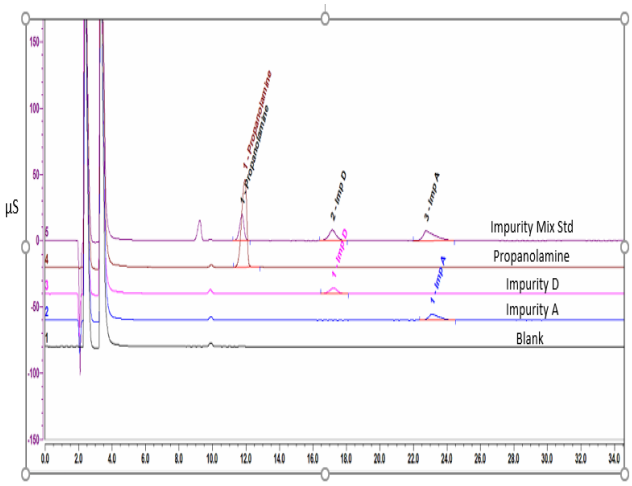


Figure 4: Overlay of Blank and Standard Chromatogram for Impurity A, D and Propanolamine on Conductivity detector

Sample precision and results: The percentage of Cyclophosphamide impurities were analyzed in Cyclophosphamide API as well as finished products like sterile powder and capsule samples. The method precision was performed by injecting 6 homogenous preparations for Cyclophosphamide API samples. In case of API and sterile powder sample, the sample data showed the presence of Impurity B and Impurity D Refer Figure 5 & 7; Table 6 and 7. In case of Cyclophosphamide capsule samples only Imp D was monitored and observed to be increasing as increase in temperature in accelerated stability study samples Refer Figure 6 and Table 8.

Table 6: Sample Results for Cyclophosphamide API Sample

Analyte	Cyclophosphamide (API) (%)
Impurity A	Not Detected
Impurity B	0.019 %
Impurity D	0.014 %
Propanolamine	Not Detected

Table 7: Sample Results for Cyclophosphamide Sterile Powder for injection.

Analyte	Cyclophosphamide Sterile Powder (%)
Impurity A	Not Detected
Impurity B	0.011 %
Impurity D	0.006 %
Propanolamine	Not Detected

Table 8: Sample Results (Imp D)* for Accelerated stability testing of Cyclophosphamide capsules.

Sample	Percentage Impurity D in Cyclophosphamide Capsules
Sample 25°C/60% RH_3M	0.033
Sample 30°C/65% RH_3M	0.050
Sample 40°C/75% RH_3M	0.310
Sample 25°C/60% RH_6M	0.025
Sample 30°C/65% RH_6M	0.075
Sample 40°C/75% RH_6M	0.784

*Impurities A, B and propanolamine are not detected in these samples.

The % RSD for Impurity B and Impurity D in six homogeneous sample preparations found 1.90 and 1.81 % respectively. Refer Table 9 (Impurities A and propanolamine are not detected in mentioned sample).

Table 9: Method precision data for Cyclophosphamide API

Sample	(Area µS*Sec) for Impurity D	(Area µS*Sec) for Impurity B
Preparation 1	64.0000	148.8700
Preparation 2	65.8816	152.2200
Preparation 3	67.1300	155.4090
Preparation 4	65.7388	155.1049
Preparation 5	64.1200	155.4457
Preparation 6	65.3781	156.8762
% RSD	1.80	1.91

Recovery Study: Recovery study was performed by spiking the impurities in Cyclophosphamide API samples from LOQ to 150 % level. It was spiked at 10 mg/L (LOQ), 30 mg/L (100 % Level) and 45 mg/L (150 % Level) for Impurity A, B, C and D. For Propanolamine impurity it was spiked at 2 mg/L (LOQ level), 10 mg/L (100 % level) and 15 mg/L (150 %). The recovery value found between 90 % to 110 % as per given in Table 10 below. Also refer Figure 5 for overlay of spiked and un spiked sample chromatogram at 100 % level.

Table 10: Accuracy Study results for Cyclophosphamide API samples.

Impurity	LOQ	100 % Level	150 % Level
Impurity A	97.55	98.20	98.90
Impurity B	98.13	99.01	99.00
Impurity D	97.30	98.44	101.30
Propanolamine	95.59	97.34	98.23

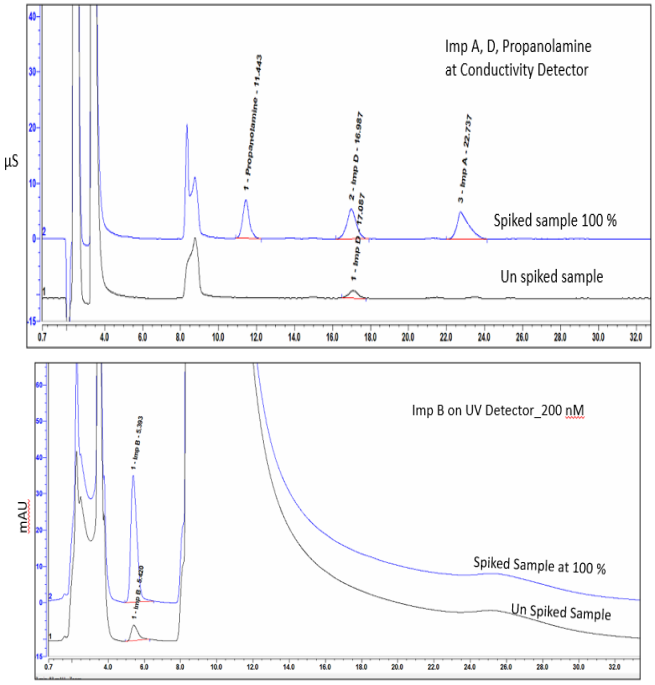


Figure 5: Spiked and Unspiked sample Overlay for Cyclophosphamide API sample on Conductivity and UV detector.

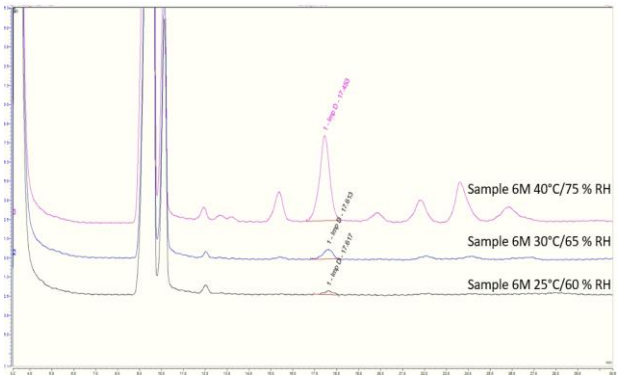


Figure 6: Sample Chromatogram for Stability samples of Cyclophosphamide Capsule samples

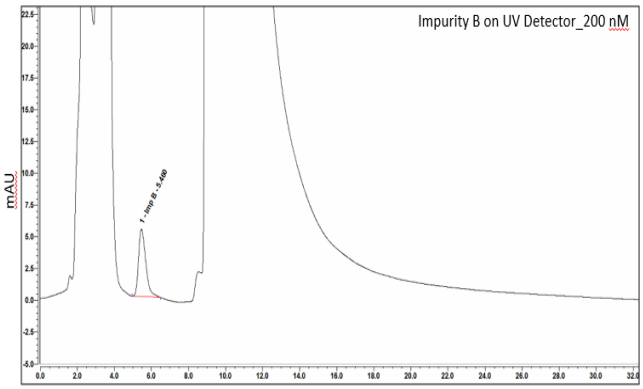


Figure 7: Sample Chromatogram for Cyclophosphamide Sterile powder

4. Conclusion

The method for quantitative determination of Cyclophosphamide related substances found precise, Linear and accurate with significant sensitivity. One additionally studied propanolamine impurity also found to be well resolved from all other impurities and showed promising results in all validation parameters. Also, method found to be feasible for cyclophosphamide formulations like Sterile powder and capsules. This method can be better alternative for TLC method, which is currently recommended in Cyclophosphamide API USP monograph for its impurity analysis. This method is advantageous in determining degradant impurities A, B and D along with Propanolamine simultaneously which otherwise requires two different methods as per USP monograph of Cyclophosphamide API.

References

[1] Novack SN, Pearson CM. (April 1971). "Cyclophosphamide therapy in Wegener's granulomatosis". New England Journal of Medicine 284 (17): 938–42. 193.
[2] http://cyclophosphamide.net/.
[3] Kalhorn, T. F.; Ren, S.; Howald, W. N.; Lawrence, R. F.; Slattery, J. T. Analysis of cyclophosphamide and five metabolites from human plasma using liquid chromatography-mass spectrometry and gas chromatography-nitrogen-phosphorus detection. J. Chromatogr. B 1999, 732, 287–298.
[4] Milind B Ubale. "Development and Validation of a Reverse Phase High Performance Liquid Chromatographic Method for the estimation of Cyclophosphamide in bulk drug" IJPPS (March 2013) Vol 5 Suppl 2 2013.
[5] Gudlawar Shivakumar, Jaya Dwivedi. Identification of Degradation Products in Cyclophosphamide API by LC-QTOF Mass Spectrometry. Journal of liquid chromatography and related technologies 38 : 190-195, 2015.
[6] KK ankalla, N. R. Gollapalli. A Stability indicating UPLC method for quantification of Cyclophosphamide related Compounds in Pharmaceutical Dosage forms. Vol 6, Isuue 7, 973- 987.
[7] Nicolini A, Mancini P, Ferrari P, et al (2004). "Oral dose cyclophosphamide in metastatic hormone refractory prostate cancer (MHRPC).". Biomed Parmacother. 58: 447–50.
[8] Terry TK, Robert JB, Dana B. High-Performance Liquid Chromatographic Analysis of Cyclophosphamide. J Phar Sci., 1979; 68(2): 175-177.
[9] FDA: Guidance for Industry, Analytical Procedures and Methods Validation, August 2000.
[10] International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH) Q2B, Validation of Analytical Procedures, Methodology. (1996).
[11] ICH Q2B: Validation of Analytical Procedures: Methodology
[12] Validation of Compendial Methods, United States Pharmacopeia Convention: Rockville. United States Pharmacopeia (2002)
[13] http://www.uspbpep.com/usp31/v31261/usp31nf26s1_c621.asp
[14] ICH Guideline Q3A (R2), Impurities in New Drug Substances,
[15] https://online.uspnf.com/uspnf/document/GUID-022A9663-92EE-473D-87BA-C43F0F3E60B1_5_en-US?highlight=cyclophosphamide
[16] https://online.uspnf.com/uspnf/document C235395-M21360-CHM32015, rev. 00 20191025 Cyclophosphamide for Injection

Author Profile

Chetan Chavan had completed B.Sc (Chemistry). and M.Sc (Chemistry) from Mumbai University and completed his Ph.D in year, 2018., He had joined Dionex India Pvt. Limited, which is now Thermo Fisher Scientific Pvt. Ltd., as Applications Manger for Ion Chromatography (IC) and HPLC and currently working as Product Manager – IC/SP. His role is to provide technical support coordination with factory for India team requirements, provide guidance to sales, application and service team for IC and sample preparations.

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