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Ultra-Performance Liquid Chromatographic Method for Quantification of Clofarabine Related Substances in an Injection Formulation

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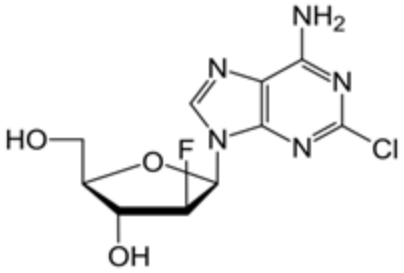
Abstract:-A new and stability-indicatingreversed-phase ultra performance liquid chromatographymethod was developed and validated for simultaneous determination of clofarabine impurities in Injectionformulation. The chromatographic separation was carried out on an ACQUITY UPLC® CSH C18 (50mm x 2.1mm, 1.7µm)using a mobile phase consisting of Ammonium formate buffer with pH 3.0 andacetonitrile at a flow rate of 0.28 ml/min and an injection volume of 1µL. The method was validated for precision, accuracy, specificity, linearity, sensitivity and robustness. The UV detection was performed at 264 nm.The proposed method can be applied for quality control, release and stability analysis of clofarabine and its impurities in Injection formulation.

Keywords:- Clofarabine, Stability-indicating, UPLC, Validation.

I. INTRODUCTION:

Clofarabine, an antimetabolite purine nucleoside used in the treatment of lymphoblastic leukemia. The clofarabine is intracellularly converted to 5'-monophosphate by deoxycytidine kinase and 5'-triphosphate by mono- and di-phosphokinases [101. This metabolite halts DNA synthesis by inhibiting ribonucleotide reductase leading to depletion of intracellular deoxynucleotide triphosphate pool. However, self - potentiation of clofarabine triphosphate incorporation into DNA increases the extent of inhibition of DNA synthesis [Ill Preclinical models has been demonstrated that clofarabine 5'- triphosphate inhibits DNA repair by incorporation into the DNA chain during the repair process [12-13]. It disrupts the mitochondrial membrane integrity and induces apoptosis by releasing cytochrome C and apoptosis-inducing factor [1°1. The IUPAC name of clofarabine is (2R, 3R, 4S, 5R)-5-(6-amino- 2-chloropurin-9- y1)-4-fluoro-2- (hydroxymethyl) oxolan-3-ol empirical formula is CioHi IC1 FN503 and molecular weight is 303.67 g/mol• (Figure 1). Clofarabine developed by Genzyme Corporation, USA, listed on January 11, 2005 by the U.S. FDA expedited procedures, as over the past decade the first approved specifically for the treatment of childhood leukemia drug, mainly used in the treatment of children with refractory or recurrent acute lymphoblastic leukemia (ALL) in children previously at least has received two therapy invalid. Clofarabine is not listed in any pharmacopoeia, however few chromatographic methods are reported on its determination. Recently, LC-MS/MS method was used for determination of clofarabine in human urine and plasma. Of late, HPLC method is reported for the determination of concentration of clofarabine in rat plasma [10]. LC/MS method is reported for analysis and identification of chlorinated impurity of clofarabine. Ultra performance convergence chromatography method was used for identification of two related substances in clofarabine [11]. Over period, several stabilityindicating reverse phase high performance liquid chromatography (RP-HPLC) methods were developed for determination of concentration of clofarabine [14-15].

II. CHEMICAL STRUCTURE OF CLOFARABINE AND ITS IMPURITIES:



a) Clofarabine

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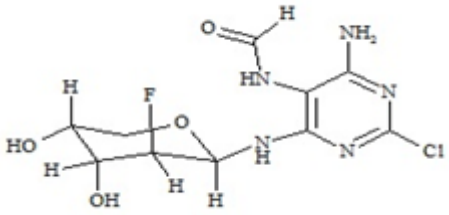
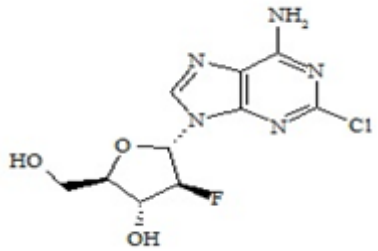
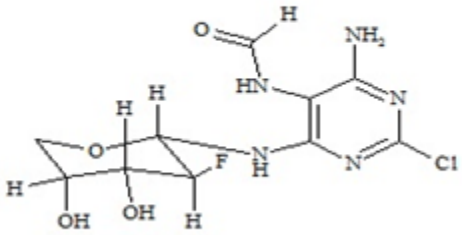


b) Impurity-A:



c) Impurity-B:



d) Impurity-C:

III. MATERIALS AND METHODS

A. Chemicals And Drugs

Ammonium formate (AR Grade) was used to prepare the buffer and obtained from Sigma-Aldrich. Formic acid (AR Grade) used to adjust the pH, was obtained from Merck Specialties, India. The active pharmaceutical ingredient clofarabine and impurities were obtained from internally.

B. Selection of detection wavelength

The sensitivity of a method that uses a UV detector depends on the proper selection of wavelength. An ideal wavelength is that which is maximally absorbed and provides an acceptable response for the drug, which should not interfere with other peaks.

UV spectra of the drug and its impurities were recorded by scanning between 200 and 400 nm.The spectra of drug and its impurities were overlaid and the wavelength 264 nm was selected where the active analyte as well as impurities have sufficient response for detection and quantification. The ultraviolet scans of Clofarabine and the three potential impurities are depicted.

C. Instrument and Chromatographic condition:

The Integrated Acquity UPLC system used for the study was purchased from Waters Corporation, Milford, USA and equipped with Waters photodiode array detector (PDA). Data collection and analysis was performed using Empower software 2pro (Waters Corporation). The balance used for weighing the reference standards and samples was purchased from Metter Toledo. Separation was achieved on a Waters acquity CSH C18 column with dimensions 50 mm x 2.1 mm I.D and a particle size of 1.7 µm. A simple mobile phase consisting of Ammonium formate buffer (0.01 M, pH 3.0) and Buffer: acetonitrile (Mobile phase B) was pumped into the UPLC chromatograph using a gradient program with varying compositions at a flow rate of 0.28 mL/ min, with a column temperature of 50°C throughout the run. Sample volume of 1µpL was injected into the chromatograph and detected at 264 nm.
The final conditions summarized in the Table.No:1 and 2.

- *Preparation of standard and sample solution*

A mixture of water and methanol in the ratio 20:80 was used as a diluent for preparing the solutions of standard and samples (diluent).

- *Standard stock solution*

A standard stock solution was prepared by dissolving 25 mg of Clofarabine working standard in 100 mL of the diluent.

- *Preparation of standard solution for impurities determination (0.001 mg per mL)*

The standard solution for the determination of impurities was prepared by diluting 5 mL of the standard stock into 50 mL with the diluent and further diluted 2 mL of the solution into 50 mL with the diluent to obtain a concentration of 0.001 mg per mL.

- *Preparation of sample and placebo solution for impurities determination.*

Sample solution was prepared by diluting 2 mL of the pooled Clofarabine injection to 10 mL using the diluents to obtain a concentration of 0.5 mg per mL.

Placebo equivalent to 5 mg of the sample was taken and diluted to 10 mL with the diluents and mixed.

- *Preparation of spiked sample solution for impurities determination*

Stock solution of all impurities was prepared by dissolving an appropriate quantity of each impurity in the diluent to obtain a concentration of 1 mg per mL. An appropriate volume of impurity stock solution was diluted with sample solution to get a final concentration of 0.2% for each impurity.

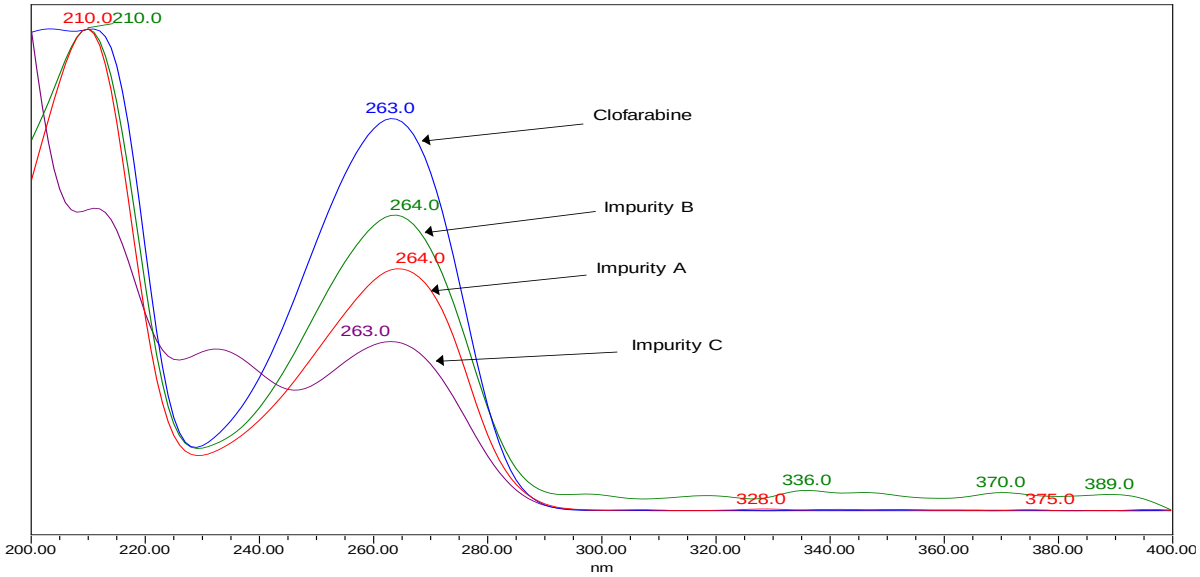


Figure 2.

D. Method development and optimization

Clofarabine has ionizable amino groups, and hence, the retention time of the drug is highly dependent on the pH of the mobile phase. In the present study, the pH of the mobile phase was maintained acidic (pH = 3.0) by the addition of formic acid solution. This yields narrow and symmetrical peak.Before initiating the development activity, information on impurities and their acceptable limits was collected to define sample concentration and the range of the method. The maximum daily dose of Clofarabine is 50mg/day. Based on the daily dose, the qualification threshold did not exceed 0.5%, and the identification threshold was 0.2%. Method development was targeted to cover a range of 50-150% of identification threshold for impurities. A systematic approach was adopted for the method development.

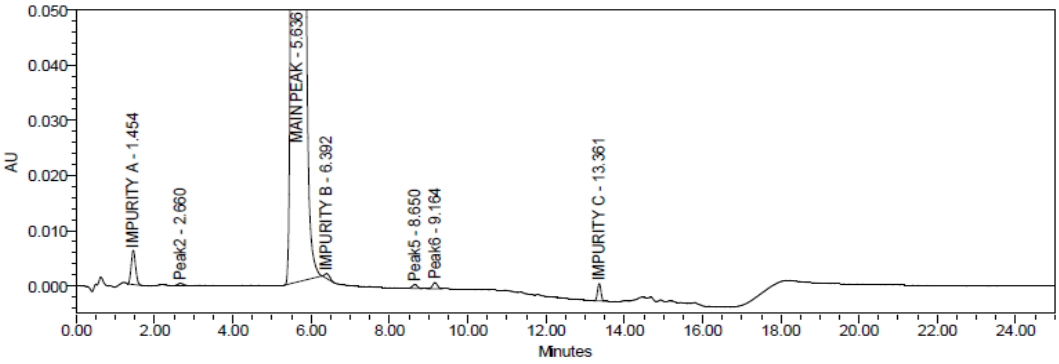


Fig. 3. Chromatogram obtained from Trial-1.

ISSN No: - 2456 – 2165

Another attempt was made by changing the gradient program and keeping other parameters unchanged. The patterns obtained from both trials did not differ significantly. The pattern obtained for trial 2 is depicted in Figure 4.

The next trial was made by changing the column to Waters CSH C18, 50 x 2.1 mm, 1.7 µm. The remaining chromatographic parameters were unchanged. All impurities were appropriately separated from each other. The resolution between impurity B and the Clofarabine is further improved by modifying the gradient program. The optimized chromatographic conditions are listed in Table:1 and 2. A specimen chromatogram obtained from the final method parameters is illustrated in Figure 5.

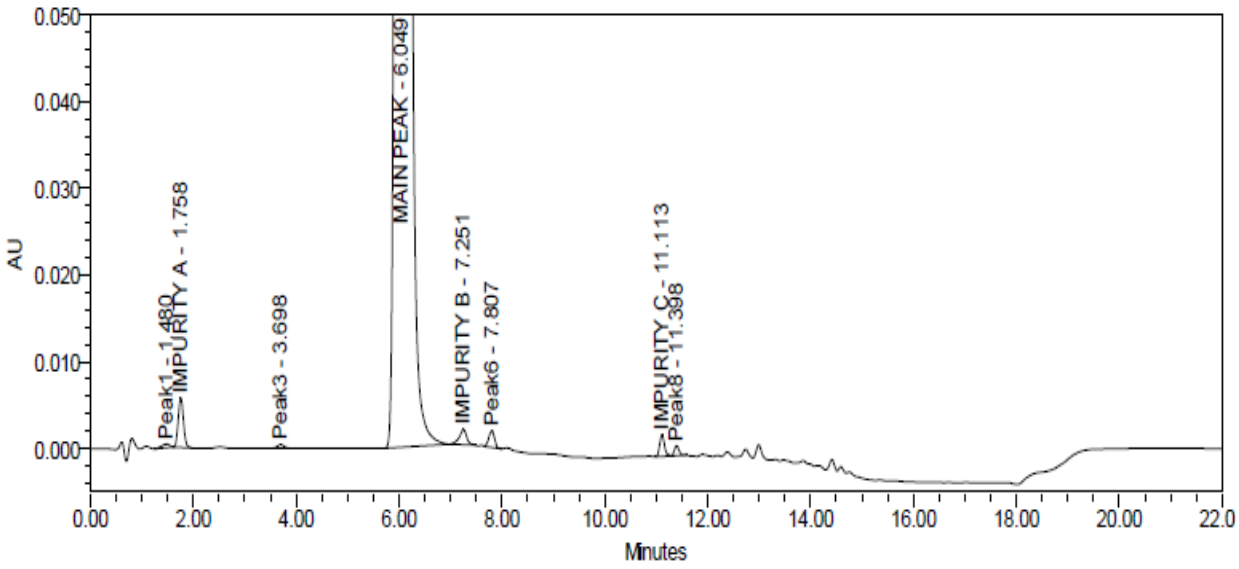


Fig. 4. Chromatogram obtained from Trial-2.

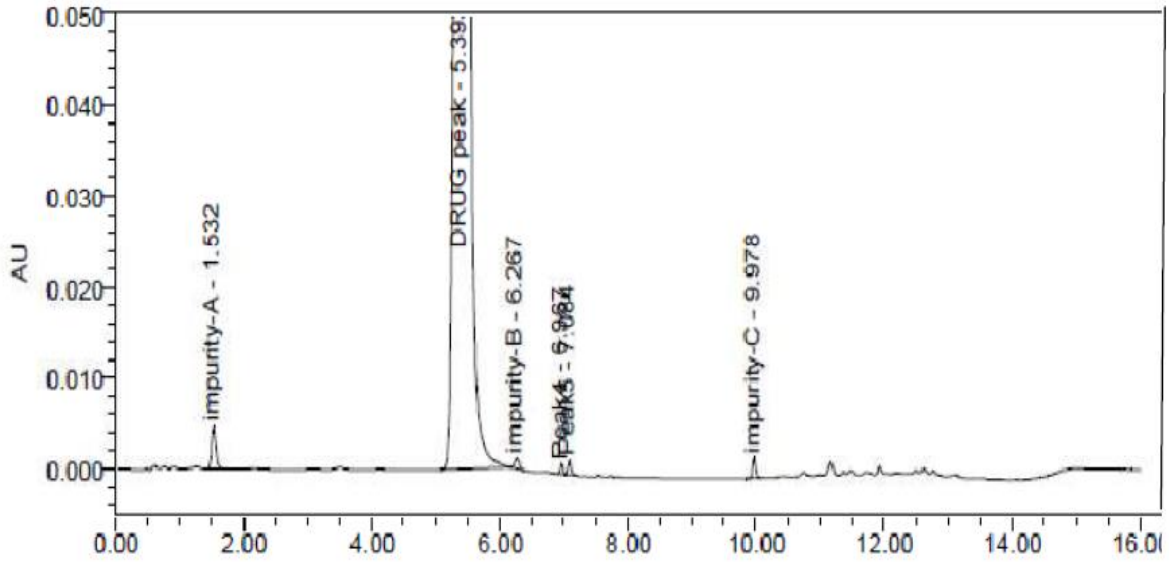


Fig. 5. Specimen Chromatogram obtained from final method:

Table No. : 1. Optimized Chromatographic Conditions.

Chromatograph	WatersAcquity UPLC system
Mobile phase	Mobile Phase A: pH3.0, 10mM Ammonium formate Buffer Mobile Phase B: Buffer : Acetonitrile (20:80v/v) (80:20%v/v)
Column	Acquity UPLC CSH C_{18}, (50mmx2.1mm) 1.7µm size)
Detector	PDA
Flow rate	0.28 ml/min
Wavelength detection	264 nm
Injection volume	1µL
Column Oven Temperature	50°C
Run time	16 min
Diluent	Methanol: Water (80:20 v/v)

Table No. : 2. Gradient Programmed.

Time(mins)	Flow(ml/min)	%A	%B
Initial	0.28	95.00	5.00
5.00	0.28	95.00	5.00
7.00	0.28	75.00	25.0
9.00	0.28	60.00	40.0
12.00	0.28	15.00	85.0
14.00	0.28	95.00	5.00
16.00	0.28	95.00	5.00

ISSN No: - 2456 – 2165

IV. METHOD VALIDATION

A. *Results and Discussion*

The optimized method was fully validated for determination of impurities as per ICH guidelines, (Q2A (R1) validation of analytical procedures. The method was validated for specificity, precision, accuracy, linearity,Limit of Detection (LOD), Limit of Quantification (LOQ) and Robustness.

- *System Suitability.*

System suitability parameters were measured to verify the system performance for the intended analysis. Hence, system precision was determined on six replicate injections of standard preparations and the relative standard deviation (% RSD) was evaluated. In addition to the % RSD, USP tailing, and USP plate count were also evaluated and found to be satisfactory, as per current USP requirements for a chromatographic peak (USP General Chapter<621>). The system suitability results obtained are presented in Table 3.

- *Precision*

Precision of the test method was evaluated by injecting six individual samples spiked with all three impurities into the chromatograph. The % RSD values from the six individual test preparations were found to be below 2% for all three impurities. The ruggedness (intermediate precision) of the method was determined using another system and column for the analysis in a different day. The precision data are listed in Table 3. The data indicate that a low % RSD, concluding that the method is precise for impurities determination.

Table No. : 3. System suitability data and Precision data.

Preparation	**Peak Area count**			
	Clofarabine	**Impurity-A**	**Impurity-B**	**Impurity-C**
Peak Tailing	1.1	1.0	1.2	1.1
Theoretical plates	7271	10984	6542	12549
%RSD	**1.9**	**0.8**	**1.4**	**0.8**

- *Accuracy*

Accuracy of the analytical procedure expresses the degree of closeness of the obtained results with true values. Samples for accuracy were prepared in triplicate by spiking impurities at different levels.. The covered levels for impurities were LOQ, 50% , 100% and 150% of the Identification Threshold (0.2%) with respect to the sample concentration (0.5mg/ mL)• From the response of the analyte peaks, the amounts recovered (in %) and % RSD were reported. Accuracy results are summarized in Table 4.

The data indicate that the % recovery of impurities lies in the range of 89.5-106.4 with an RSD of between 0.8 and 3.4%. This indicates that the method is accurate and precise.

Table No. : 4. Recovery of Impurities data

Impurity Name	**Recovery Level (%)**							
	LOQ	**%RSD**	**50**	**%RSD**	**100**	**%RSD**	**150**	**%RSD**
Impurity-A	104.7	3.4	95.5	2.3	97.7	3.1	95.3	1.7
	99.7		99.7		93.8		92.2	
	106.4		96.2		99.7		93.3	
Impurity-B	94.0	2.5	98.7	2.2	103.2	0.9	95.2	2.8
	89.5		95.3		102.2		96.5	
	92.3		99.4		104.0		100.5	
Impurity-C	104.2	3.0	93.3	0.8	100.2	1.0	95.5	2.3
	98.5		92.5		101.8		96.7	
	103.2		94.0		102.1		92.4	

- *Limit of Detection and Limit of Quantization.*

The LOD and LOQ values were established by Signal to Noise ratio. Results are shown in Table no.5

Table No. : 5. Impurities LOD and LOQ Concentrations.

Impurity name	Data based on signal to noise ratio (S/N)	
	LOQ Concentration (%w/w)	LOD Concentration (%w/w)
Impurity A	0.0381	0.01143
Impurity B	0.0524	0.01572
Impurity C	0.0421	0.01263

- *Linearity*

The linearity of the analytical method was tested to check its ability to elicit test results that are directly proportional to the concentration of analyte in samples within a given range. Hence, different concentrations of individual impurities and standard working solution of Clofarabine were prepared and injected into the UPLC, and the chromatograms were recorded. The linearity of detector response was determined by plotting a graph of peak areas versus concentrations. Plots of linearity experiments are illustrated in Figure 6. The correlation coefficients, >0.998 for impurities and 0.997 for Clofarabine, indicate that the method satisfies a linear relationship between the concentration and peak response. The linearity range is between the limit of quantification (LOQ) and 150% of the sample concentration for all impurities. The linearity data for all four impurities and Clofarabine are listed in Table 6 to 9.

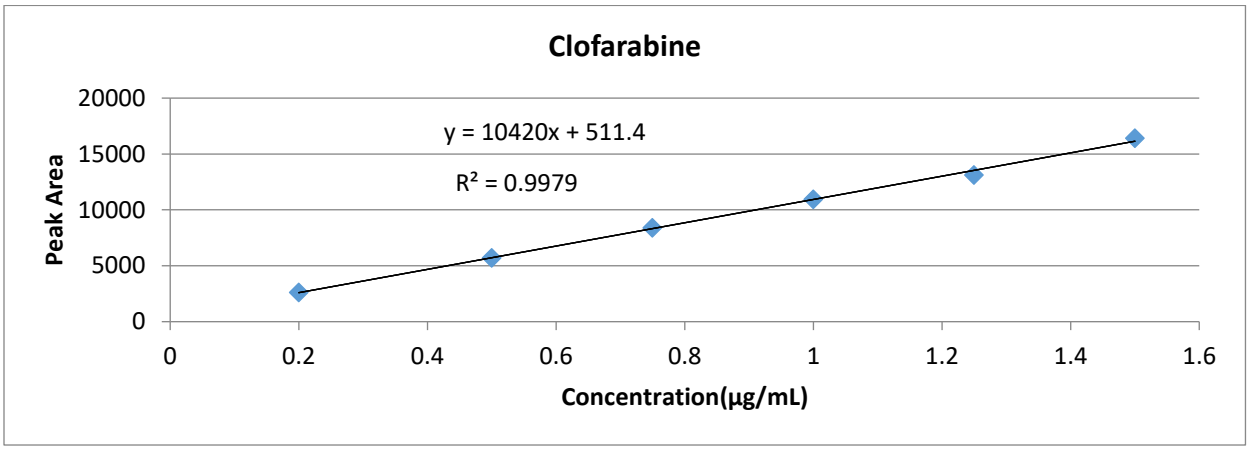


Fig.6. Clofarabine

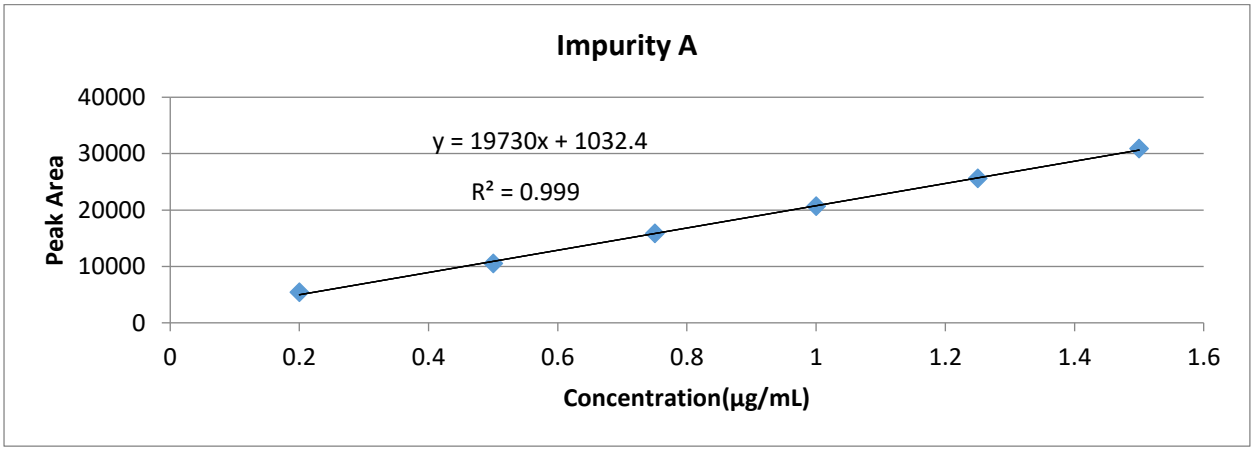


Fig.7 Impurity A.

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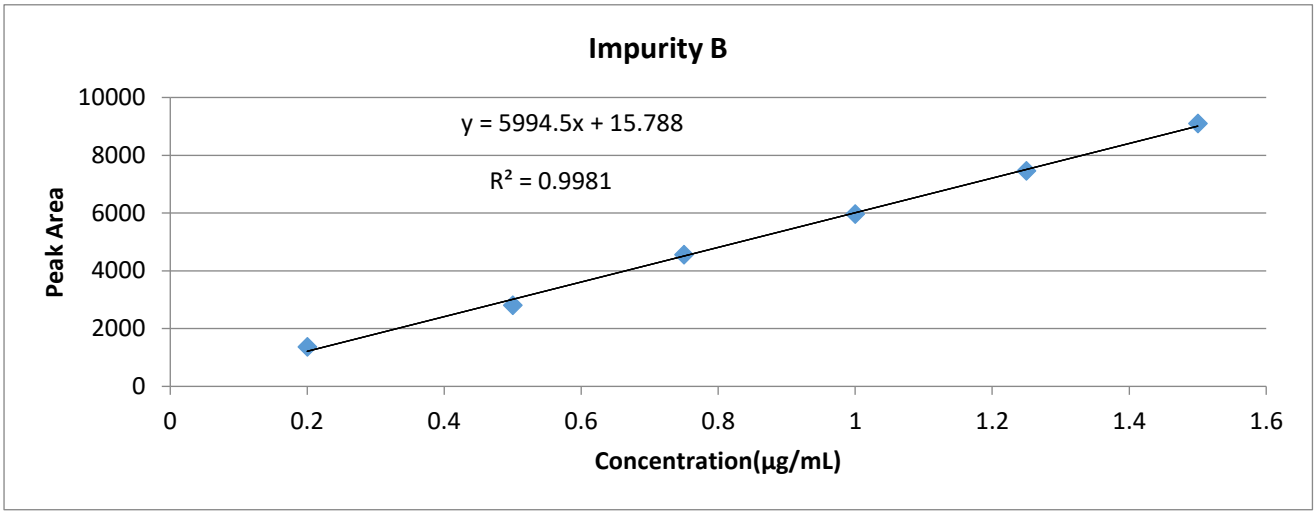

Fig.8. Impurity B

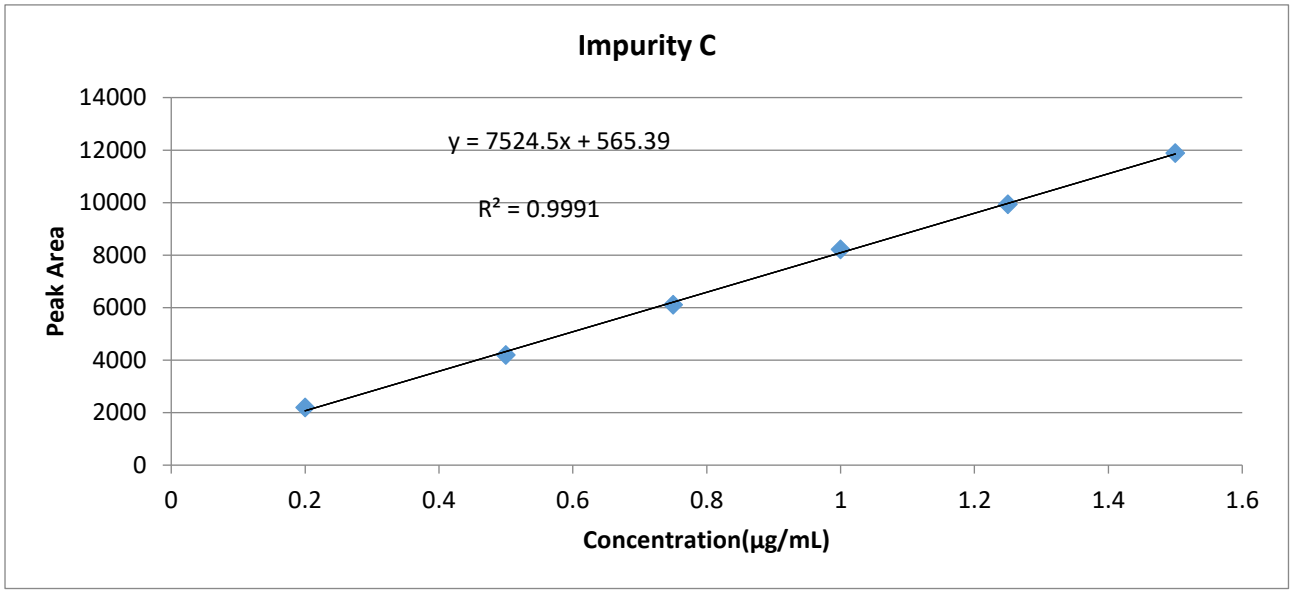

Fig.9. Impurity C

- *Specificity*

The specificity of the RP-HPLC method was checked by comparison of chromatogram obtained from standard, placebo and sample. The Placebo did not should not show any interference at retention of main analyte peak and at impurities.

➢ *Forced degradation study.*

Forced degradation studies were conducted on samples and on the plain placebo to prove the specificity and stability-indicating power of the method. Specificity was determined by exposing test solution to oxidation by hydrogen peroxide, acid hydrolysis, base hydrolysis, heat and photolytic stress. A detailed procedure has been reported.

The stressed samples were then further diluted with the diluent and chromatographed as per the proposed method.
The peak purity of Clofarabine peak was evaluated using the PDA. The purity angle should be less than the purity threshold. The results are summarized in Table.No:6

ISSN No: - 2456 – 2165

Table No. : 6. Degradation Data.

Stress condition	%degradation	Peak purity		Purity flag
		Purity Angle	Purity Threshold	
Treated with 5N HCL solution for 24hrs on bench top.	5.42	0.10	0.24	NO
Treated with 5N NaOH solution for 24hrs on bench top.	11.7	0.11	0.23	NO
Treated with 30% H_2O_2 solution for 24hrs on bench top.	0.40	0.10	0.24	NO
Treated with heat at 60°C for 72hrs.	0.38	0.27	0.52	NO
Exposed to sunlight 1.2 million lux hrs for 6days.	0.09	0.24	0.62	NO
Exposed to UV-Light 200 watts/square meter for 6days.	0.05	0.18	0.32	NO

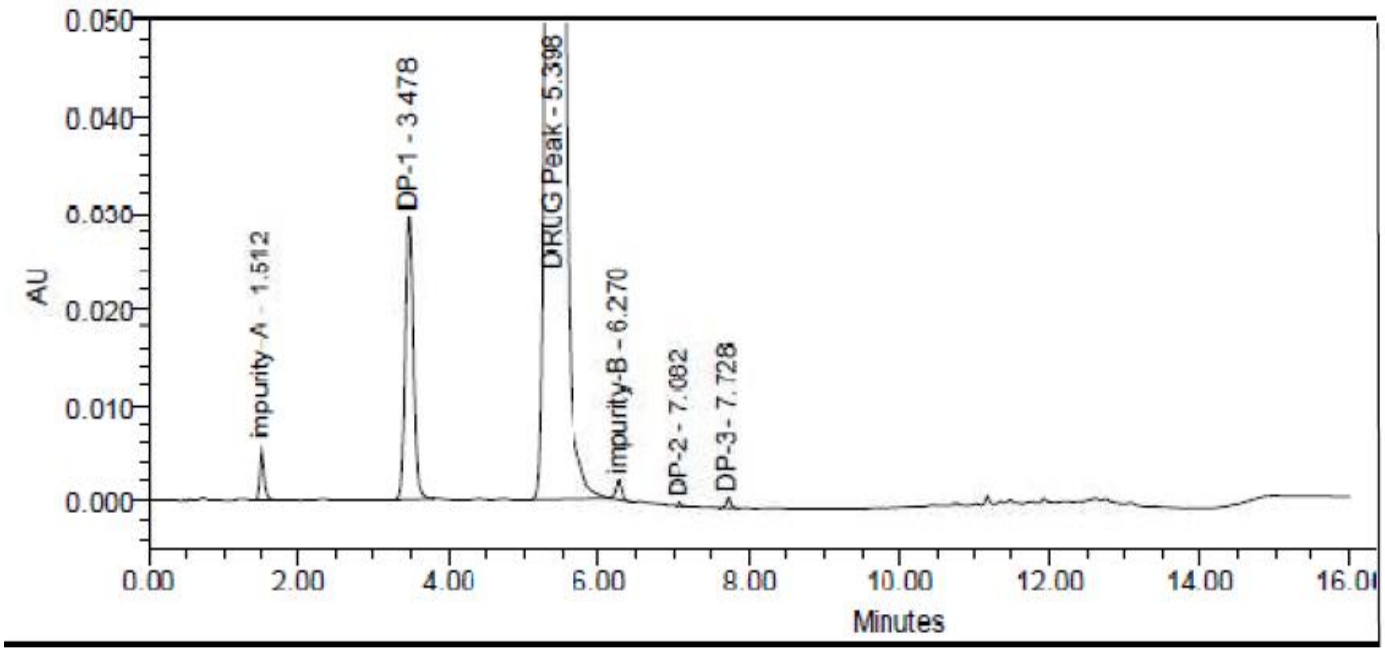

Fig. 10. Acid Sample.

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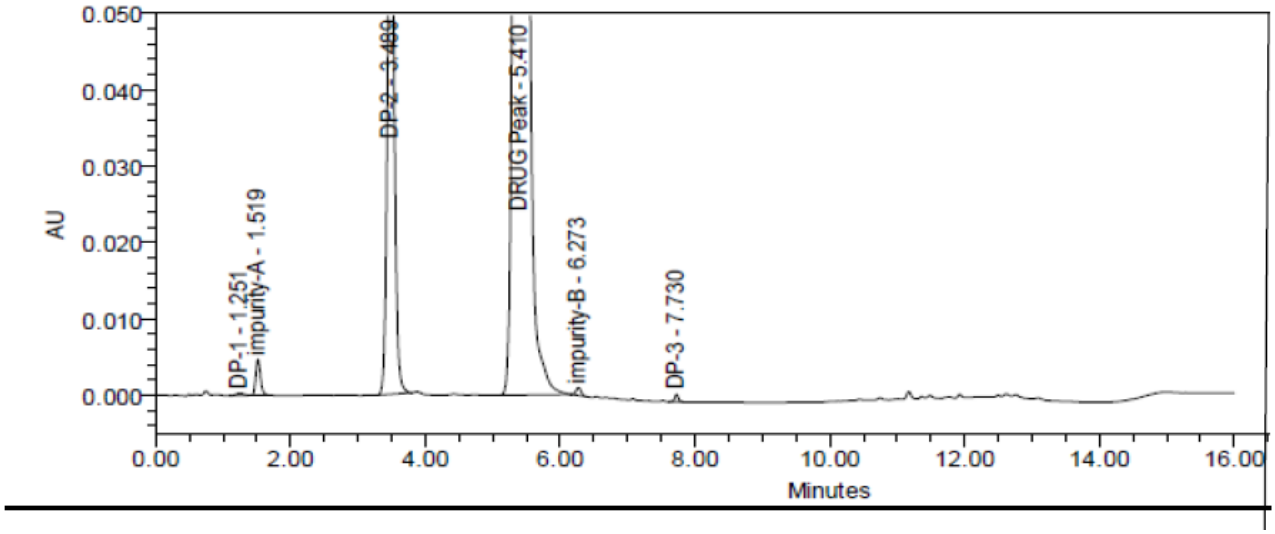

Fig. 11. Base Sample.

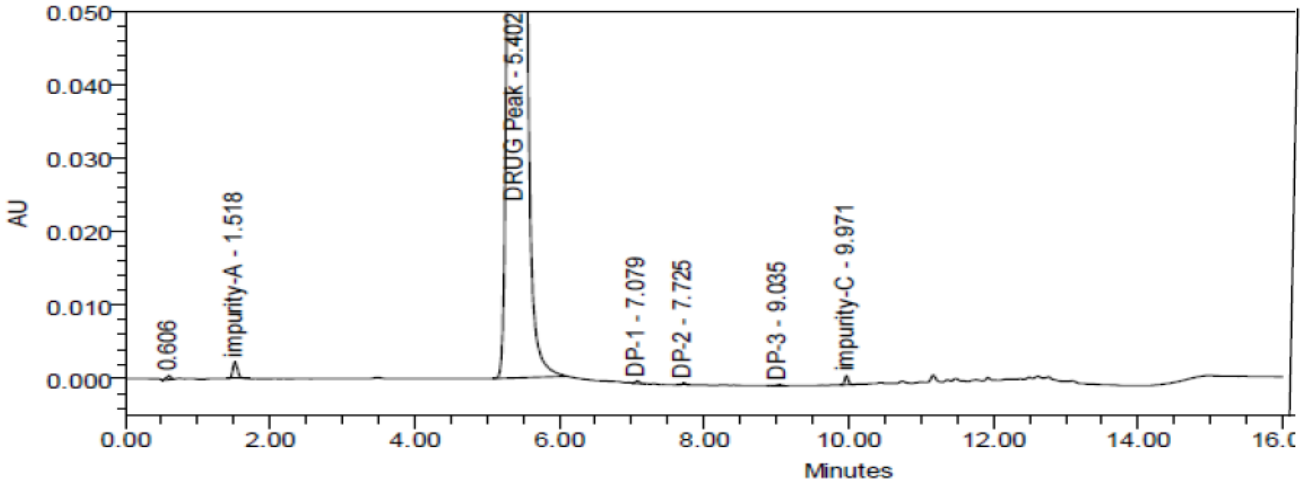

Fig.12. Thermal Sample.

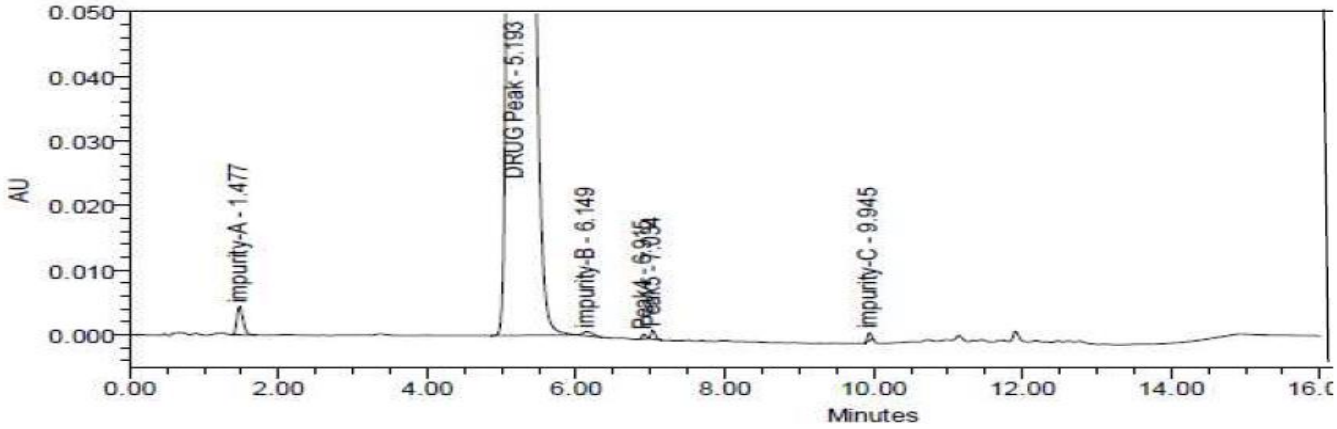

Fig.13. Sunlight Sample.

- Robustness.

Reliability during normal usage. The robustness of a method is evaluated by varying method parameters such as mobile phase composition, pH, etc., and determiningthe effect (if any) on the results of the method. Result are shown in Table no.8 The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its

Table No. : 7. Robustness conditions.

Parameters	**Change of conditions**	
Flowvariation (ml/min)	High(+)	0.31
	Actual	0.28
	Low(-)	0.25
Buffer pH variation	High(+)	3.2
	Actual	3.0
	Low(-)	2.8
Column oven temp. variation (°C)	High(+)	55
	Actual	50
	Low(-)	45
Organic phase (%) Mobile phase B	High(+)	88
	Actual	80
	Low(-)	72

Table No. : 8. Robustness data.

Impurity Name	**Relative Retention Time (RRT)**								
	Control	**pH(+)**	**pH(-)**	**Flow(+)**	**Flow(-)**	**Temp(+)**	**Temp(-)**	**Organic(+)**	**Organic(-)**
Impurity A	0.28	0.27	0.28	0.28	0.28	0.28	0.28	0.28	0.28
Impurity B	1.18	1.12	1.16	1.16	1.16	1.16	1.16	1.16	1.16
Impurity C	1.92	1.75	1.86	1.86	1.86	1.86	1.86	1.86	1.86

V. CONCLUSIONS

The rapid gradient reverse phase UPLC method, developed for the quantitative analysis of clofarabine related substances in pharmaceutical dosage forms, is precise, accurate, linear, specific, and robust. Satisfactory results were obtained from validation of the method. The method is stability-indicating and can be used for routine analysis of production samples, and checking the stability of samples of Clofarabine Injection.

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