Development and application of Liquid Chromatographic method for determination of Clofarabine in bulk and in parentral dosage forms

[1]Dr.L.Satyanarayana, [2]Dr.Sumalatha. Reddi

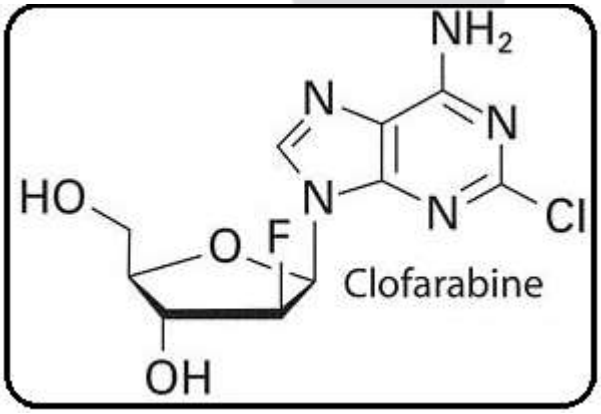
Department Of Pharmaceutical Analysis,
Omega College of Pharmacy, Ghatkesar, Hyderabad, T.S.500034

***Abstract*: Clofarabine is a purine nucleoside antimetabolite that is being studied in the treatment of cancer. It is marketed in the U.S. and Canada as Clolar. In Europe and Australia/New Zealand the product is marketed under the name Evoltra. Clofarabine is used in paediatrics to treat a type of leukaemia called relapsed or refractory acute lymphoblastic leukaemia (ALL), only after at least two other types of treatment have failed. It is not known if the drug extends life expectancy. Some investigations of effectiveness in cases of acute myeloid leukaemia (AML) and juvenile myelomonocytic leukaemia (JMML) have been carried out. Developing a accurate and precise alytical method for the estimation of Clofarabine in a sterile, product for intravenous (IV) infusion a is very challenging, due to the formation of drug-drug and drug-excipient interactions. The present study demonstrates the applicability of chromatographic method to develop a new, sensitive, single HPLC method for the quantitative determination of antifungal agents in freeze dried powder for injection pharmaceutical dosage form. Chromatographic separation active pharmaceutical ingredient was achieved by using a isocratic elution at a flow rate of 1.0 mL/min on Agilent Eclipse XDB Column (250mm×4.6 mm, 5µm particle size, 100Å pore size) at ambient temperature. The contents of the mobile phase were 3.48 gms of Di Potassium hydrogen ortho-phosphate (0.03M) in 1000 ml of water and by adjusting the pH to 2.5 with dilute ortho-phosphoric acid (mobile phase solvent-A) and acetonitrile (mobile phase solvent-B) in a isocratic mode in the ratio of 45:55 (v/v) of separation was used to resolute the Clofarabine. UV detection at 260 nm was employed to monitor the analytes. A linear response was observed for Clofarabine over the concentration range 2-24 µg/ mL. Limit of detection (LOD) and Limit of quantification (LOQ) for Clofarabine were found to be 0.004µg/mL, and 0.012µg/mL respectively.**

***Keywords*: Clofarabine, Isocratic-HPLC, Casporan®, IV infusion**

Introduction: Clofarabine is a Nucleoside Metabolic Inhibitor. The mechanism of action of clofarabine is as a Nucleic Acid Synthesis Inhibitor [1]. Clofarabine is a second generation purine nucleoside analog with antineoplastic activity. Clofarabine is phosphorylated intracellularly to the cytotoxic active 5'-triphosphate metabolite, which inhibits the enzymatic activities of ribonucleotide reductase and DNA polymerase, resulting in inhibition of DNA repair and synthesis of DNA and RNA[2-4]. This nucleoside analog also disrupts mitochondrial function and membrane integrity, resulting in the release of pre-apoptotic factors, including cytochrome C and apoptotic-inducing factors, which activate apoptosis. Clofarabine is a purine analogue and antineoplastic agent used in the therapy of acute lymphoblastic leukemia (ALL) in children. Clofarabine is a purine nucleoside antimetabolite that is being studied in the treatment of cancer. Clofarabine is used in paediatrics to treat a type of leukaemia called relapsed or refractory acute lymphoblastic leukaemia (ALL), only after at least two other types of treatment have failed. It is not known if the drug extends life expectancy. Some investigations of effectiveness in cases of acute myeloid leukaemia (AML) and juvenile myelomonocytic leukaemia (JMML) have been carried out. It is indicated for the treatment of pediatric patients 1 to 21 years old with relapsed or refractory acute lymphocytic (lymphoblastic) leukemia after at least two prior regimens. Chemically Clofarabine is known as (2R, 3R, 4S,5R)-5-(6-amino-2-chloropurin-9-yl)-4-fluoro-2-(hydroxyl methyl)oxolan-3-ol, 2-chloro-2′-arabino-fluoro-2′-deoxy adenosine.

Figure-1: Chemical structures of Clofarabine



A survey of literature has revealed only one analytical method for the determination of Clofarabine in biological fluids. These include; high-performance liquid chromatography (HPLC) [4-6]. On the contrary, to the best of our knowledge, there is no method reporting the determination of Clofarabine in pharmaceutical formulation. In this paper, we report the simple precise and accurate RP-HPLC method for the assay of Clofarabine acetate for Intravenous (IV) Infusion in injection dosage form. The new method is capable of separating active ingredient present in the Intravenous (IV) Infusion. Validation of the current method will be performed according to the requirements of USP for assay determination which include accuracy, precision, selectivity, linearity and range.

Experimental:

Chemicals and reagents: Clofarabine was obtained as kind gift sample from Hetro pharma Ltd, Hyderabad. Potassium dihydrogen ortho-phosphate, acetonitrile and *ortho*-phosphoric acid were obtained from Merck, Mumbai, India. All the solutions were prepared in Milli Q water (Millipore, USA). Test samples composed of Clolar® (1 mg/mL) injection is supplied in a 20 mL, single-dose vial, Bayer, India contains 0.2 mg of Clofarabine, is obtained from local market.

HPLC Instrumentation and Chromatographic conditions: Quantitative HPLC was performed on the Waters Alliance 2695 Separations Module is a high performance liquid chromatographic system with a quaternary, low-pressure mixing pump and inline vacuum degassing. Waters Alliance 2695 separation module (Waters Corporation, Milford, USA) equipped with 2489 UV/visible detector or 2998 PDA detector with Empower 2 software was used for the analysis. Flow rates from 50 uL/ min to 5 mL/min can be generated for use with 2.1 mm ID columns and larger. The auto-sampler has a maximum capacity of 120 vials (12x32, 2-mL) with programmable temperature control from 4 to 40°C. A heated column compartment provides temperatures from 5 degrees above ambient to 65°C. The detector is a photodiode array (model 2996) with a wavelength range of 190-800 nm and sensitivity settings from 0.0001-2.0000 absorbance units The HPLC system was equipped with a column compartment with temperature control and an on-line degasser. Agilent Eclipse XDB Column C-18 (250x4.6 mm i.d; particle size 5 µm) was used for separation of Clofarabine. The contents of the mobile phase were 3.48 gms of Di Potassium hydrogen *ortho*-phosphate (0.03M) in 1000 ml of water and by adjusting the pH to 2.5 with dilute *ortho*-phosphoric acid (mobile phase solvent-A) and acetonitrile (mobile phase solvent-B) in a isocratic mode in the ratio of 45: 55 (v/v) of separation was used to resolute the Clofarabine. They were filtered before use through a 0.45 µm membrane filter and degassed by sonication. The flow was adjusted at 1.0 ml/min flow rate and 20 µL injection load volumes were maintained. The eluted compounds were monitored at 260 nm. The column oven temperature was maintained at 25 ◦C. Data acquisition, analysis, and reporting were performed by Empower2 (Waters) chromatography software.

Preparation of Solutions:

Standard and stock solutions: Standard solution of the active pharmaceutical ingredient was prepared in the following manner: Transfer 20 mg of Clofarabine working standard into a 100 ml volumetric flask, dissolve and dilute with Acetonitrile and water in the ratio of 50:50 v/v as diluent. 5 ml of the resulting solution is further diluted up to 50 ml in volumetric flask with diluents. The resulting solution contains 20 µg/mL of Clofarabine as working standard solutions. The prepared stock solutions were stored at 4 ^{0}C and protected from light.

Preparation of the Sample solution: Clolar® (clofarabine) Injection contains clofarabine, a purine nucleoside metabolic inhibitor. Clolar (1 mg/mL) is supplied in a 20 mL, single-dose vial. The 20 mL vial contains 20 mg clofarabine formulated in 20 mL unbuffered normal saline (comprised of Water for Injection, USP, and Sodium Chloride, USP). The pH range of the solution is 4.5 to 7.5. The solution is sterile, clear and practically colorless, and is preservative-free. Clolar® should be filtered through a sterile 0.2 micron syringe filter and then diluted with 5% Dextrose Injection, USP, or 0.9% Sodium Chloride Injection, USP, prior to intravenous (IV) infusion to a final concentration between 0.15 mg/mL and 0.4 mg/mL. Use within 24 hours of preparation. Store diluted Clolar at room temperature (15-30ºC). The contents of 5 vials of Clolar® (clofarabine) Injection were collected, uniformly blended and a quantity equivalent to 2 mg was weighed and transferred in to a 10-mL volumetric flask, extracted in diluent by sonication, and filtered through Whatman no. 41 filter paper. The filtrate (5 mL) was quantitatively transferred to a 50-mL volumetric flask, and solution was diluted to volume with the diluents. The resulting solution contains 20 µg/mL of Clofarabine as working sample solutions. The prepared stock solutions were stored at 4 ^{0}C and protected from light.

Solutions for validation study:

Calibration and Quality control samples: Calibration standards (2–24 µg/ mL of Clofarabine were prepared from working standard solutions by appropriate dilution with Acetonitrile and water in the ratio of 50:50 v/v as diluents. Quality control (QC) samples for accuracy studies were prepared at three concentrations of the linearity range (16 µg/ mL, 20 µg/ mL and 24 µg/ mL) for Clofarabine were prepared from the standard solutions.

Method Validation [7-9]: The developed chromatographic method was validated for selectivity, linearity, precision, accuracy, sensitivity, robustness and system suitability.

Specificity: The terms selectivity and specificity are often used interchangeably. The specificity of the developed LC method for quantification of active pharmaceutical ingredient was determined the presence of excipients present in pharmaceutical products. In specificity study, interference between drugs and excipients usually employed in IV infusion were evaluated from the comparison of spectral purity obtained from the analysis for the standard solutions and sample solutions.

System suitability: The system suitability was assessed by six replicate analyses of the drugs at concentrations of 20 µg/ mL for Clofarabine. The acceptance criterion was ±2% for the RSD for the peak area and retention times for all four analytes. The system suitability parameters with respect to theoretical plates, tailing factor, repeatability and resolution between peak and peaks of the other three analytes were defined.

Linearity: Linearity of the method was evaluated at seven equi-spaced concentration levels by diluting the standard solutions to give solutions over the ranges 10–120% target concentration for main analyte of interest. The calibration curves were constructed

at seven concentrations between 2–24 µg/ mL for Clofarabine. These were injected in triplicate and the peak areas were inputted into a Microsoft Excel® spreadsheet program to plot calibration curves. The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method. The peak areas of the analyte to concentration of analyte were used for plotting the linearity graph. The linearity data is reported in Table-3.

Table-3: Linearity Data for Clofarabine

Precision: Precision was evaluated in terms of intra-day repeatability and inter-day reproducibility. The intra-day repeatability was investigated using six separate sample solutions prepared, as reported above, from the freshly reconstructed tablet formulations at 100% of the target level. Each solution was injected in triplicate and the peak areas obtained were used to calculate means and RSD% values. The inter-day reproducibility was, by preparing and analyzing in triplicate sample solutions from the reconstructed formulations at the same concentration level of intra-day repeatability; the means and RSD% values were calculated from peak areas. (Table-4)

Table-4: Intra-day and inter-day precision data for Clofarabine

Accuracy: The accuracy of the method was determined by measuring the recovery of the drug by the method of standard additions. Quality control (QC) samples for accuracy studies were prepared at three concentrations of the linearity range (16 µg/ mL (80% dilution), 20 µg/ mL (100% dilution) and 24 µg/ mL (120% dilution) for Clofarabine were prepared from the standard solutions. Known amounts of 10 % dilution of drug (2 µg/mL of Clofarabine) was added to corresponding to 80%, 100%, and 120% of the target test concentrations were added to a placebo mixture to determine whether the excipients present in the formulation led to positive or negative interferences. Each set of additions was repeated three times at each level. Extraction sample preparation procedure is followed and assayed against qualified reference standard. The accuracy was expressed as the percentage of the analytes re-covered by the assay. (Table-5)

Accuracy: recovery data for Clofarabine

Sensitivity: Limits of detection (LOD) and quantification (LOQ) were estimated from the signal- to-noise ratio. The detection limit was determined as the lowest concentration level resulting in a peak area of three times the baseline noise. The limit of detection was determined, by injecting progressively low concentrations of analyte of interest. The quantification limit was determined as the lowest concentration level that provided a peak area with signal-to-noise 10.

Robustness: To determine the robustness of the developed method, experimental conditions were deliberately changed and the relative standard deviation for replicate injections of Clofarabine and the USP resolution factor between and the other two peaks were evaluated. The mobile phase flow rate was 1.0 mL/min. This was changed by ±0.2 units to 0.8 and 1.2 mL/min. The effect of stationary phase was studied by the use of LC columns from different batches at 25°C. The effect of buffer pH was studied at pH 2.3 and 2.7 (± 0.2 units). The chromatographic variations were evaluated for resolution between and the other three analytes in a system suitability solution with respect to retention time RT and % assay of drugs.

Table-6: Robustness data for Clofarabine

Solution stability: To assess the solution stability, standard and test solutions were kept at 25 ◦C (laboratory temperature) for 24 h. These solutions were compared with freshly prepared standard and test solutions.

RESULTS AND DISCUSSION:

HPLC method development: The API solution of analyte of interest i,e., Clofarabine was prepared in diluent at a concentration of 20µg/mL and scanned in UV-Visible spectrometer; and the Clofarabine was found to have UV maxima at around 260 nm. Hence detection at 260 nm was selected for method development purpose. Some important parameters, pH of the mobile phase, concentration of the acid or buffer solution, percentage and type of the organic modifier, etc. were tested for a good chromatographic separation. The main analytical challenge during development of a new method was obtaining adequate retention of the polar compound Clofarabine. Trials showed that acidic mobile phase with reverse phase column gives symmetric and sharp peaks. For this reason, potassium dihydrogen phosphate buffer with pH-3.0 was adjusted with *o*-phosphoric acid was preferred as acidic buffer solution. Acetonitrile and buffer in the ratio of 55:45 (v/v) were chosen as the organic modifier because it dissolves drugs very well. Mobile phase composition in isocratic mode at a flow rate of 1.0 mL per minute was observed for a good resolution. Then method was optimized to separate the active ingredient by changing to isocratic mode. The satisfactory chromatographic separation, with good peak shapes were achieved on Agilent Eclipse XDB-C18 (250 × 4.6) mm with 5 µm particles, using the column temperature as maintained at 35°C and the detection was monitored at a wavelength of 260 nm. The injection volume was 20 µL. Acetonitrile and water in the ratio of 50:50 v/v were used as diluent. In the optimized isocratic conditions, Clofarabine was well separated with a resolution (Rs) of greater than 2 and the typical retention time of about 3.165 minutes, the typical chromatogram of System suitability shown in **Figure 2**.

Method validation: The developed method was validated, as described below, for the following parameters: system suitability, selectivity, linearity, precision, accuracy and LOD/LOQ.

Selectivity: Selectivity of the current method was demonstrated by good separation of the active ingredients. Furthermore, matrix components, e.g. excipients, do not interfere with the four analytes as they have no absorbance. The representative chromatogram (Fig. 5A) of the parenteral dosage form solution containing excipients showed no peak interfering with analytes; moreover the adjacent chromatographic peak was separated with resolution factors >3. Overall, these data demonstrated that the excipients did not interfere with the active ingredients peaks, indicating selectivity of the method

System suitability: The RSD values of peak area and retention time for the analytes are within 2% indicating the suitability of the system.

Figure-2: System suitability chromatogram of working standard solution contains 20 µg/mL of Clofarabine.

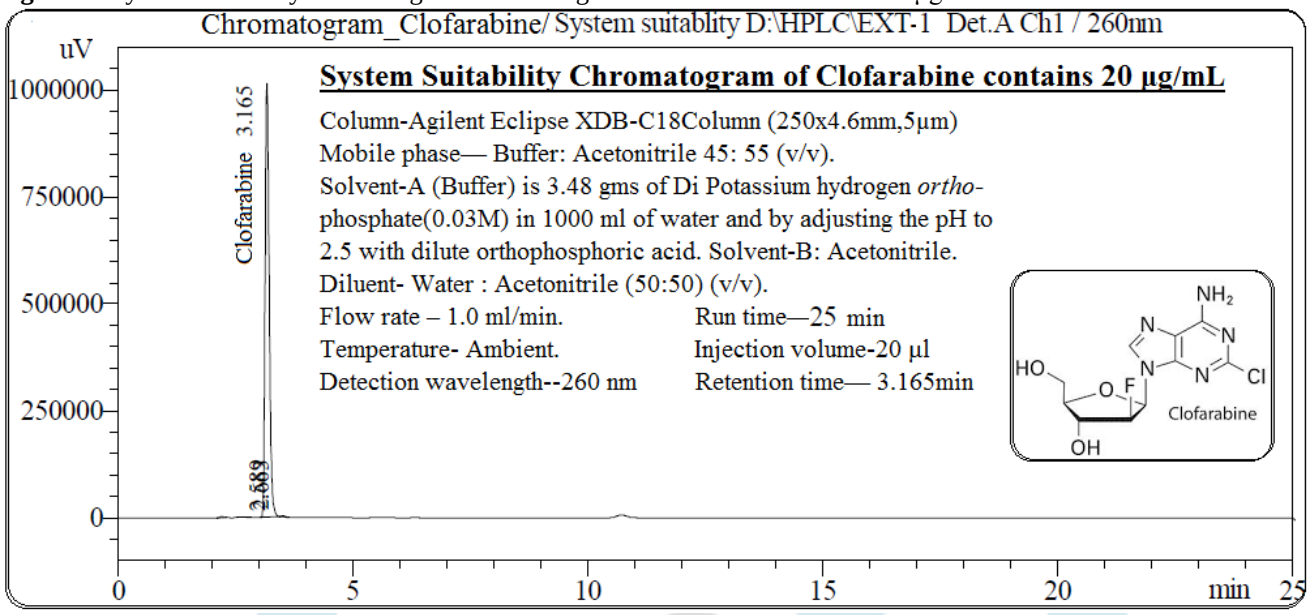


Table-2: Results of System suitability study.

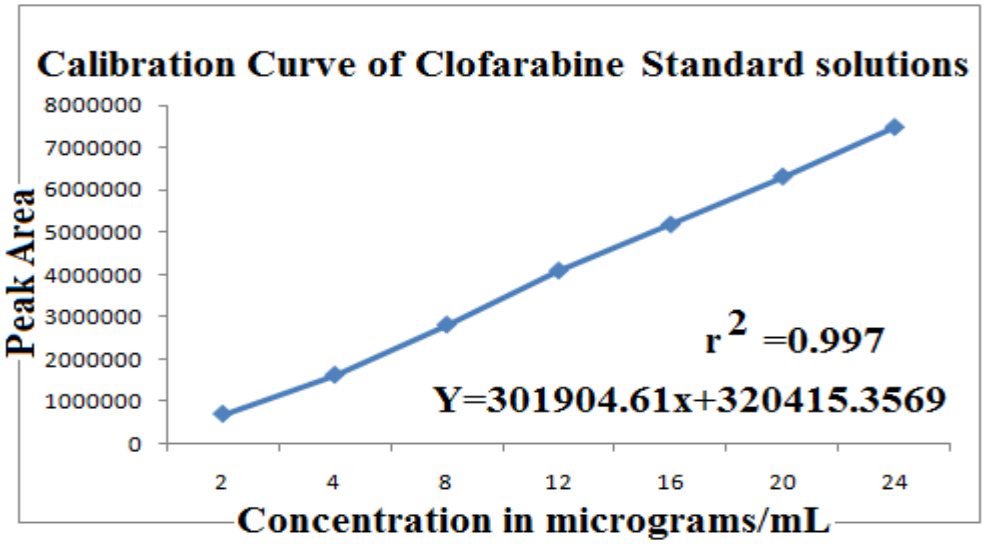
Parameter	Clofarabine
Retention time	3.166
Theoretical plates	8818.144
Tailing Factor	1.386
HETP	2.8350×10^{-5}
USP plates/meter	352726
Resolution	2.125
Peak area	6230528
% of Peak area	99.271

Linearity and range: Seven concentration levels within 10–120% of the target concentration range for analytes were considered to study the linearity. The calibration curves were prepared by plotting the peak area of the drug to the respective concentrations, which were linear in the range of 2–24 µg/ mL for Clofarabine. Peak areas of the active ingredients and concentrations were subjected to least square linear regression analysis to calculate the calibration equations and correlation coefficients. The mean regression equations were found as Y= 301904.61 x+ 320415.3569 for Clofarabine. The square of the correlation coefficient ($r^2 >$ 0.999) demonstrated a significant correlation between the concentration of analytes and detector response. The results show that there is an excellent correlation between the peak area ratios and the concentrations of drugs in the range tested.

Table-3: Linearity data for the Clolar®- 0.2 mg- Film coated tablets.

Concentration	Peak Area	Parameter	Clofarabine
2 µg/ mL	698762	Concentration Range	2-24 µg/ mL
4 µg/ mL	1534217	Regression equation	Y=301904.61 x+ 320415.3569
8 µg/ mL	2791236	Correlation Coefficient	0.999
12 µg/ mL	4089902	0.95 Confidence interval	Lower-Limit-0.993/ Upper Limit-1
16 µg/ mL	5180679	0.95 Confidence interval	Lower-Limit-0.987/ Upper Limit-1
20 µg/ mL	6315827	Limit of Detection(LOD)	0.004 µg/ mL
24 µg/ mL	7486081	Limit of Quantification(LOQ)	0.012 µg/ mL

Figure-3: Calibration Curve of Clolar® 20 mg/20 mL injection



Precision: Precision of this method was determined by injecting the standard solution of the three analytes six times. The R.S.D. of peak area of six replicates was found to be less than 2. The results obtained are shown in Table 4. In all instances the %RSD values were less than 2%.

Table-4: Intra-day and inter-day precision data for Clofarabine

Precision data of Clofarabine	**Inter-day precision**		**Intra-day precision**	
Analyte-conc. (20 µg/ml)	Retention time in min.	Peak Area	Retention time in min.	Peak Area
Clofarabine injection-1	3.166	6241725	3.168	6232590
Clofarabine injection-2	3.164	6233791	3.168	6233698
Clofarabine injection-3	3.165	6232983	3.169	6234841
Clofarabine injection-4	3.167	6236755	3.168	6234773
Clofarabine injection-5	3.168	6235465	3.167	6228530
Clofarabine injection-6	3.168	6242724	3.168	6229812
Mean	3.166	6237240	3.168	6232374
% RSD.	0.043	0.066	0.016	0.042
Std. Deviation	0.001	4089	0.001	2645

Accuracy: Percentage recovery of the active ingredient using this method was determined using Clolar (clofarabine) Injection contains clofarabine, a purine nucleoside metabolic inhibitor. Clolar (1 mg/mL) is supplied in a 20 mL, single-dose vial. The 20 mL vial contains 20 mg clofarabine formulated in 20 mL unbuffered normal saline (comprised of Water for Injection, USP, and Sodium Chloride, USP). The pH range of the solution is 4.5 to 7.5. The solution is sterile, clear and practically colorless, and is preservative-free. The results of accuracy studies from standard solution and excipient matrix were shown in Table 5; recovery values demonstrated that the method was accurate within the desired range.

Table-5: Accuracy study and recovery data for Clolar® 20 mg/20 mL injection

S. No	Recovery at 80% dilution Level Peak areas		Recovery at 100% dilution Level Peak areas		Recovery at 120% dilution Level Peak areas	
	Standard	Spiked	Standard	Spiked	Standard	Spiked
1	5127921	5677448	5677448	6717889	7012659	7724222
2	5131411	5680902	5680902	6709953	7012132	7770160
3	5128800	5686669	5686669	6717883	7018299	7786289
Avg	5129377.3	5681673.0	5681673.0	6715242	7014363.3	7760224
Std.Dev	1815.22	4659	4659	4580	3418.56	32204
%RSD	0.035	0.082	0.082	0.068	0.049	0.415
% Recovery	97.78		99.36		112.36	
Clolar® (1 mg/mL) is supplied in a 20 mL, single-dose vial working sample solution was spiked -at 80% level (16 µg/ml was spiked with 10% of mixed standard solution of API's(2 µg/ml) -at 100% level (20 µg/ml was spiked with 10% of mixed standard solution of API's(2 µg/ml) -at 120% level (24 µg/ml was spiked with 10% of mixed standard solution of API's(2 µg/ml)						

Sensitivity: Limit of detection (LOD) for Clofarabine was 0.004 µg/mL and limit of quantification (LOQ) for Clofarabine was 0.012µg/mL. The results of LOD and LOQ were indicating a high sensitivity of the method.
Robustness: The HPLC parameters were deliberately varied from normal procedural conditions including the mobile phase flow rate was 1.0 mL/min. This was changed by ±0.2 units to 0.8 and 1.2 mL/min. The effect of stationary phase was studied by the use of LC columns from different batches at 35°C. Under these variations, all analytes were adequately resolved and elution orders remained unchanged. The testing solution maintained a signal-to-noise ratio over 10 in all varied conditions. The peak resolution was all larger than 1.5 under each variation.

Table-5: Robustness study of Clolar® 20 mg/20 mL injection solution at 100 % level (20 µg/mL):

Parameter	**Clofarabine** in Flow increase study		**Clofarabine** in Flow decrease study		**Clofarabine** in Variable column Study	
	Run time	Peak Area	Run time	Peak Area	Run time	Peak Area
Injection-1	2.883	5843702	3.511	7060432	3.168	6262054
Injection-2	2.884	5844134	3.509	7050012	3.167	6261908
Injection-3	2.885	5824122	3.506	7056954	3.169	6261804
Mean	2.884	5837320	3.509	7055800	3.168	6261922
% RSD	0.041	0.196	0.068	0.075	0.025	0.002
Std. Dev	0.001	11432	0.002	5305	0.001	126

Analysis of the fixed dose combination tablet:

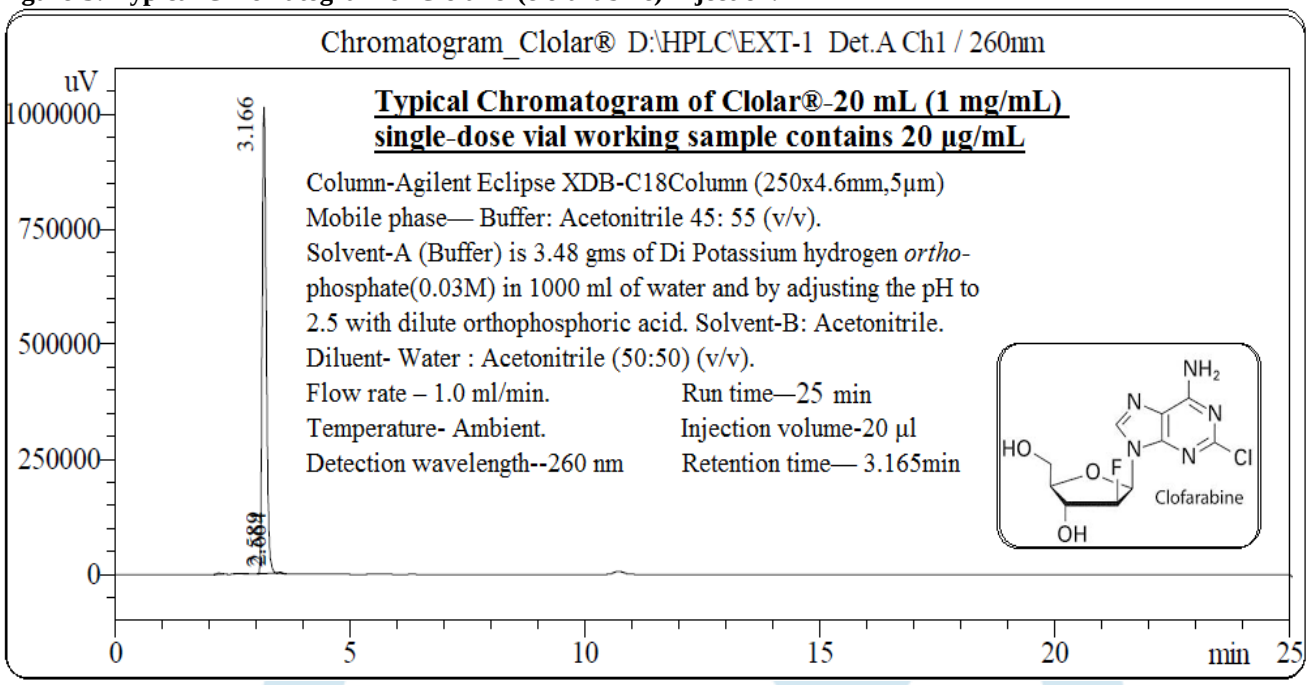
Clolar® (clofarabine) Injection contains clofarabine, a purine nucleoside metabolic inhibitor. Clolar® (1 mg/mL) is supplied in a 20 mL, single-dose vial. The 20 mL vial contains 20 mg clofarabine formulated in 20 mL unbuffered normal saline (comprised of Water for Injection, USP, and Sodium Chloride, USP). The pH range of the solution is 4.5 to 7.5. The solution is sterile, clear and practically colorless, and is preservative-free. Clolar® should be filtered through a sterile 0.2 micron syringe filter and then diluted with 5% Dextrose Injection, USP, or 0.9% Sodium Chloride Injection, USP, prior to intravenous (IV) infusion to a final concentration between 0.15 mg/mL and 0.4 mg/mL. Use within 24 hours of preparation. Store diluted Clolar at room temperature (15-30ºC). An amount of the homogenous sample blend equivalent to 2 mg was transferred into a 10 ml volumetric flask, added 40 ml of diluents (Acetonitrile and water in the ratio of 50:50 v/v), sonicated for 30 min, diluted to 100 ml with diluents. 50ml sample taken from this solution was centrifuged at 3000 rpm for 15 min. A 5-ml aliquot from supernatant was then decanted to another 50-ml volumetric flask. Test solutions were then made up to volume with the diluent. The filtrate (5 mL) was quantitatively transferred to a 50-mL volumetric flask, and solution was diluted to volume with the diluents. The resulting solution contains 20 µg/mL of Clofarabine as working sample solutions. The prepared stock solutions were stored at 4 ^{0}C and protected from light. The amount of Clofarabine in standard mixtures or dosage forms were individually calculated using the related linear regression equations.

On the basis of above results, the proposed method was applied to the determination of antifungal agent Clofarabine present in tablet dosage forms. Figure-3 shows representative chromatograms obtained from the analysis of Clolar® (clofarabine) Injection. The differences between the amount claimed and those assayed were very low and the R.S.D. values were within the acceptable range mentioned by pharmacopoeias. The mean percentage recoveries obtained after six repeated experiments were found between 98 and 108.2 (Table 6), indicating that the results are accurate and precise and there is no interference from the common excipients used in the pharmaceutical dosage forms.

Table-6: Assay results of Clolar® (clofarabine) Injection.

Formulation	Label Claim (mg/vial)	Amount found in (mg/vial)
injection	1	0.99

Figure-5: Typical Chromatogram of Clolar® (clofarabine) Injection.



Conclusion: In this study, a validated simple and reliable RP-HPLC-PDA procedure was described for the assay of a Clolar® (clofarabine) Injection contains clofarabine, a purine nucleoside metabolic inhibitor. Clolar (1 mg/mL) is supplied in a 20 mL, single-dose vial, which is indicated is indicated as empirical therapy for presumed fungal infections in febrile, neutropenic adult and pediatric patients. To our present knowledge, no attempts have yet been made to estimate these tablets by analytical procedure. The active pharmaceutical ingredient was successfully resolved and quantified using Agilent Eclipse XDB-C18 Octadecyl column (250×4.6mm, 5µm) in a relatively short run time of 8 minutes in isocratic mode of chromatographic method. The proposed method provides a good resolution between active ingredients. The developed method reported herein was validated by parameters as described in ICH-Q2B guideline. System suitability, specificity, linearity, LOD, LOQ values, within- and between-day precision and accuracy of the proposed technique were obtained during the validation studies. The proposed method has the advantages of simplicity, repeatability, sensitivity and requires less expensive reagents.

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