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Validated RP-HPLC Method for the Determination of Clofarabine in Bulk and Tablet Dosage Form

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Abstract

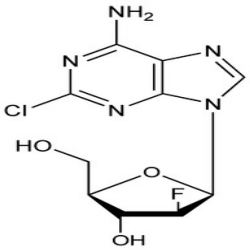
A novel, simple and economic reverse phase high performance liquid chromatography (RP-HPLC) method has been developed for the estimation of Clofarabine in bulk and tablet dosage form with greater precision and accuracy. Separation was achieved on Develosil C18 MG-5 column (150X4.6mm i.d.,5µm) in isocratic mode using Triflouro acetic acid P^H-3.6 buffer, Methanol and Acetonitrile in the ratio of 70:15:15(v/v/v) as mobile phase, pumped in to the column at flow rate of 1.0 mL min−1and the detection of eluent from the column was carried out using variable wavelength UV detector at 263 nm. The total run time was 10 min and the column was maintained at ambient temperature. The retention time of Clofarabine was 5.578 min. The standard curves were linear over the concentration range of 10-30 µg/ml with R^2 0.999 and the LOD and LOQ values for Clofarabine were 0.0007 µg/ml and 0.023 µg/ml , respectively. The percentage recovery was found to be 101.78 – 99.86 %, the % RSD was found to be 0.08. The percentage amount of a marketed tablet formulation of Clofarabine was found to be 101.2 %. The method was validated as per ICH guidelines. Validation studies demonstrated that the proposed RP-HPLC method is simple, specific, rapid, reliable and reproducible. Hence the proposed method can be applied for the routine quality control analysis of Clofarabine in bulk and tablet dosage forms.

Key words: Clofarabine, RP-HPLC, Method Development, Validation, ICH guidelines

INTRODUCTION:

Clofarabine is a next generation deoxyadenosine analogue which is used for the treatment of pediatric leukemia. The mechanism of its anti-cancer activity involves the combination of direct inhibition of DNA synthesis and ribonucleotide reductase and induction apoptosis. This drug is effective against various sub types of leukemia and solid tumors. The Chemical name of Clofarabine is(2R, 3R, 4S, 5R)-5-(6-amino-2-chloro-9H-purin-9yl)-4-fluoro-2-(hydroxymethyl) oxolan-3-ol and chemical formula is $C_{10}H_{11}ClFN_5O_3$ and molecular weight is 303.68 g/mol.

Chemical Structure:



MATERIALS AND METHODS

Instrumentation

List of instruments used

S. No.	Name	Manufacturer
1.	HPLC(empower-2software)	Aliance waters(2487)
2.	HPLC detector	Dual absorbance detector
3.	pH Meter	Lab india
4.	Centrifuge	SV scientific
5.	Ultra Sonicator	SV scientific
6.	UV Spectrophotometer	SHIMADZU
7.	Micro Balance	Mettle Toledo
8.	Water Purifier	Millipore

Chromatographic conditions:

Mobile phase**:** Buffer –trifluoro acetic acid (pH-3.6) and solvent
Mixture (methanol and water) the ratio of 70:15:15(v/v/v)
Column **:** Develosil C-18 MG-5(250x4.6mm), 5µm
Flow rate **:** 1.0 ml/min
Detector wavelength **:** 263 nm
Column temperature **:** 30^0C
Injection volume **:** 10 µl
Run time **:** 10 min
Retention time **:** 5.578 min

Drug:

The reference sample of Clofarabine was gifted by M/s Natco Drugs Limited, Hyderabad.
The Branded formulations of Clofarabine (Lamitor tablets of Torrent Pharma and Lamepil tablets of IPCA laboratories) were procured from the local market.

Chemicals and solvents :

Methanol HPLC grade (Qualigens, India)
Acetonitrile HPLC grade (Qualigens, India)
Tri ethyl amine HPLC grade (Qualigens, India)
Trifluoro acetic acid HPLC grade (Qualigens, India)
Ortho-phosphoric acid HPLC grade (Qualigens, India)
HPLC grade water prepared by using Millipore Milli Q system

Preparation of the P^H 3.6 buffer solution:

Accurately transfer 1 mL of Trifluroacetic acid in 1000 ml of purified water and mix. Adjust the pH of the solution to 3.6±0.05 with triethyl amine.

Preparation of standard and sample solutions of Clofarabine:

Standard Preparation:

Accurately Weigh and transfer accurately 20.0 mg of Clofarabine working Standard into a 100 ml clean dry

volumetric flask, and add about 60 ml of diluents, dissolve and dilute to volume with diluent. Transfer 5.0 ml above solution into 50 ml volumetric flask and dilute to volume with diluents.

Sample preparation:

Accurately weigh 2.0 mL of sample solution in to 100 mL volumetric flask without any loss of solution. Dilute to volume with diluents, shake and mix well.

Preparation of the mobile phase:

Prepare a filtered (0.45µ*)* and degassed mixture of buffer, Methanol and Acetonitrile in the ratio of 70:15:15 v/v respectively

Diluent:

Prepare a mixture of Water Methanol and Acetonitrile in the ratio of 70:15:15 v/v

CHROMATOGRAPHIC CONDITIONS

For developing the method, a systematic study of the effect of various factors was undertaken by varying one parameter at a time and keeping all other conditions constant. The following studies were conducted for this purpose. A Develosil C-18 MG-5(250x4.6mm), 5µm column was chosen as the stationary phase for this study.

The mobile phase and the flow rate

In order to get sharp peak and base line separation of the components, the author has carried out a number of experiments by varying the commonly used solvents, their compositions and flow rate. To effect ideal separation of the drug under isocratic conditions, mixtures of commonly used solvents like water, methanol and acetonitrile with or without different buffers in different combinations were tested as mobile phases on a C_{18} stationary phase. A binary mixture of Buffer –trifluoro acetic acid (pH-3.6) and solvent Mixture (methanol and water) in the ratio of 70:15:15v/v was proved to be the most suitable of all the combinations since the chromatographic peaks obtained were better defined and resolved and almost free from tailing.

A mobile phase flow rate of 1.0 mL/min. was found to be suitable in the study range of 0.5 -1.5 mL/min.

Detection wave length

The UV absorption spectrum of the drug was taken in methanol and the λ max found to be at 263nm. Hence detection of the drug was made at 263 nm.

Retention time of Clofarabine:

A model chromatogram showing the separation of Clofarabine is presented in Fig 2.1.1. Under the above optimized conditions a retention time of 5.578 min was obtained for Clofarabine. After a thorough study of the various parameters the following optimized conditions mentioned in Table 2.1.2 were followed for the determination of Clofarabine in bulk samples and pharmaceutical formulations.

Chromatogram of Clofarabine

Linearity and Construction of calibration curve

The quantitative determination of the drug was accomplished by an external standard method. The mobile phase was filtered through a 0.45µ membrane filter before use. The flow rate of the mobile phase was adjusted to 1mL/min. The column was equilibrated with the mobile phase for at least 30 min prior to the injection of the drug solution.

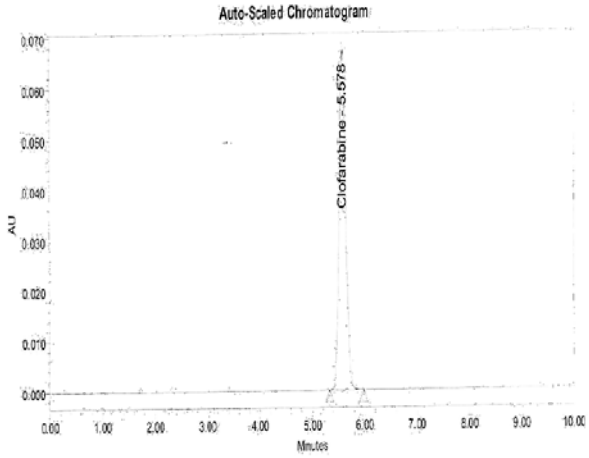


Fig 2.1.1. A Model Chromatogram showing the separation of Clofarabine peak from pure drug.

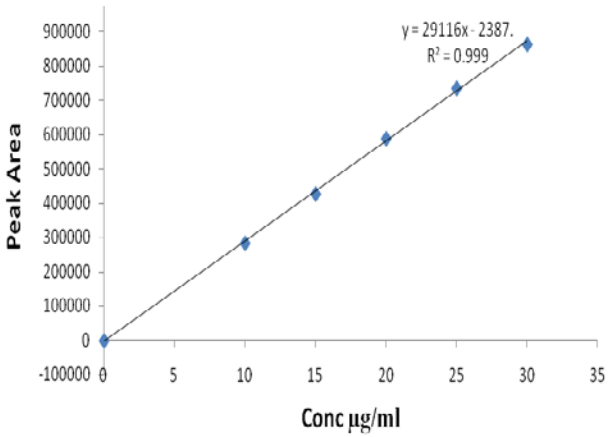


Fig 2.1.2. Linearity Plot for the drug Clofarabine

Linearity of the peak area response was determined by taking measurements at seven concentration points (six replicates at each point). Working dilutions of Clofarabine in the range of 10- 30 µg/mL were prepared by taking suitable aliquots of the working standard solutions in different 10 mL volumetric flasks and diluting up to the mark with the mobile phase. Twenty microlitres of the dilutions was injected each time into the column at a flow rate of 1 mL/min. Each dilution was injected six times into the column. The drug in the eluents was monitored at 263 nm and the corresponding chromatograms were obtained. From these chromatograms, the mean peak areas were noted and a plot of concentrations over the peak areas was constructed. The regression of the plot was computed by least square regression method. The linear relationship was found to be of 10-30 µg/mL between the concentration of Clofarabine and peak area. This regression equation was later used to estimate the amount of Clofarabine in pharmaceutical dosage forms. The linearity plot was shown in the Fig. 2.1.2 and the calibration data and regression parameters are reported in Table 2.1.3 and 2.1.4

Table 2.1.3. Calibration data of the proposed HPLC Method

Concentration of Clofarabine (µg/mL)	Mean Peak Area(n=6)
10	283892
15	427215
20	588407
25	735108
30	862609

Table 2.1.4. Regression characteristics of the linearity plot of Clofarabine

Parameter	Value
Linearity Range (µg)	10-30
Slope (a)	29306
Intercept (b)	-6684.6
Correlation coefficient (γ)	0.9999
Regression equation	y = 29306x-6684.6

VALIDATION OF THE PROPOSED METHOD

The method was validated in compliance with ICH[27] guidelines. The parameters determined for validation are specificity, precision, accuracy, robustness, LOD, LOQ, system suitability and stability of analytical solution.

Specificity

The method specificity was assessed by comparing the chromatograms and UV scans obtained from the drug and the most commonly used excipient mixture with those obtained from blank solution. The blank solution is prepared by mixing the excipients in the mobile phase without the drug. The drug to excipient ratio used was similar to that in the commercial formulations. The commonly used excipients in formulations like lactose, starch, microcrystalline cellulose, ethyl cellulose, hydroxyl propyl methylcellulose, magnesium stearate and colloidal silicon dioxide were used for the study. The mixtures were filtered through 0.45µ membrane filter before injection. The absence of additional peaks in the chromatogram indicates non interference of the commonly used excipients in the tablets and hence the method is specific.

Precision

Precision is the degree of repeatability of an analytical method under normal operational conditions. The precision of the method was studied in terms of repeatability (intra-day assay) and intermediate precision (inter-day assay). The intra-day and inter-day variation for determination of Clofarabine was carried out at three different concentration levels 10, 20, 30 µg/mL level. The percent relative standard deviation (% RSD) values as presented in the Table 2.1.5 shows that the method provides acceptable (<2) intraday and inter-day variation.

Accuracy

Accuracy of the method was evaluated by standard addition method. An amount of the pure drug in its solution form corresponding to 80, 100 and 120 % levels has been added to the pre analysed working dilution (25 µg) of the drug. The sample solutions were analysed in triplicate at each level as per the proposed method. The percent individual recovery and %RSD for recovery at each level are calculated. The results are tabulated (Table 2.1.6). Satisfactory recoveries ranging from 100.78 to 99.86% were obtained with the proposed method. This indicates that the method is accurate.

Table 2.1.5. Precision of the proposed method

Concentration of Clofarabine (µg/mL)	Intra-day Precision			Inter-day Precision		
	Mean Amount found	Percent Amount found	Percent RSD	Mean Amount found	Percent Amount found	Percent RSD
10	284317	933.43	0.3	284442	1006.1	0.3
20	589233	587.69	0.09	588702	345.26	0.05
30	859393	4226.8	0.491	860990	825.90	0.09

Table 2.1.6. Accuracy data (Triplicate values at 80, 100 and 120 percent levels)

Spike Level	Conc µg/ml *Amount added(pure)*	Conc µg/ml *Amount added (formulation)*	Amount found	%Recovery	Mean % recovery
80%	8	10	7.851	101.88	101.78
	8	10	7.864	101.77	
	8	10	7.862	101.75	
100%	10	10	10.030	99.68	99.77
	10	10	10.023	99.72	
	10	10	10.022	99.77	
120%	12	10	12.176	98.55	99.86
	12	10	12.144	98.81	
	12	10	12.158	98.70	

Robustness

A study was conducted to determine the effect of deliberate variations in the optimized chromatographic conditions like composition of the mobile phase, flow rate, column oven temperature and mobile phase pH. The system suitability parameters were compared at the effected changes keeping all other parameters constant. The assay, tailing factor and the number of theoretical plates were evaluated. The results were found to be within the allowed limits which indicate that the method is specific.

Variations in the pH of the mobile phase

The effect of variation in the pH of the mobile phase was evaluated at ±0.2 levels. The system suitability results were found to be within the limits as shown in the table 2.1.7.

P^H of the mobile phase	*Peak area*	*average*	*% RSD*
3.4	588677	588718	0.01%
	588689		
	588788		
3.8	588766	588807	0.01%
	588778		
	588879		

Variations in flow rate

A study was conducted to determine the effect of variation in flow rate (± 10% of optimum flow). The system suitability parameters were evaluated at 0.9 mL/min. and 1.1 mL/min. The results were within the acceptance criteria. Hence the allowable variation in flow rate is 0.9 mL/min to 1.1 mL/min.

Variations in column temperature

A study was conducted to determine the effect of variation in column oven temperature at 28^0 C and 31^0 C. The system suitability results were found to be within the limits for both column temperatures.

Temperature of the column (^{0}c)	*Peak area*	*Average*	*% RSD*
28	588678	588780	0.18%
	588772		
	588892		
31	588645	588797	0.31%
	588772		
	588974		

Limit of Detection and Limit of Quantification

Limit of detection (LOD) is defined as the lowest concentration of analyte that gives a measurable response. LOD is determined based on signal to noise ratio (S/N) of three times typically for HPLC methods. The limit of quantification (LOQ) is defined as the lowest concentration that can be quantified reliably with a specified level of accuracy and precision. It is the lowest concentration at which the precision expressed by an RSD of less than 2%. In this study the analyte response is 10 times greater than the noise response. For this study six replicates of the analyte at lowest concentration in the calibration range were measured and quantified. The LOD and LOQ of Clofarabine obtained by the proposed method were

Average Baseline Noise obtained from Blank = 38.56 µV

Signal Obtained from LOD solution = 0.00896 µV

LOD = $3.3\times\sigma/s$

$= 3.3\times0.00896/38.56$

$= 0.000766$

$LOQ = 10\times\sigma/s$

$= 10\times0.00896/38.56$

$= 0.02323$

System precision and System suitability

System precision and system suitability studies were carried out by injecting six replicates of the working standard solution. The % RSD for the areas obtained was calculated. The data presented in Table 2.1.9 establishes reproducible performance of the instrument. The system suitability parameters are given in Table 2.1.10. The percent RSD for peak area response for six replicate injections should not be more than one.

Table 2.1.9. System precision data

Injection No.	Peak area	Average	SD	%RSD
1	587847	588786	467.225	0.08%
2	587794			
3	587805			
4	588546			
5	588642			
6	588782			

Table 2.1.10. System suitability parameters

Parameters	Values
Theoretical plates (n)	4960
Plates per meter (N)	9248.4
Tailing factor (T)	1.11
LOD (µg/mL)	0.000766
LOQ (µg/mL)	0.02323

ESTIMATION OF THE DRUG FROM TABLET DOSAGE FORMS

Satisfactory results obtained with the method development for the determination of Clofarabine has prompted the author to attempt its applicability for the estimation of the drug from its formulations.

Assay calculation-:

$$\%Assay = \frac{TA}{SA} \times \frac{SW}{100} \times \frac{5}{50} \times \frac{100}{2} \times \frac{P}{100} \times \frac{100}{L.A}$$

Where,

TA = peak area response due to Clofarabine from sample

SA = peak area response due to Clofarabine from standard

SW = Weight of Clofarabine working standard taken in mg

P = purity of Clofarabine working standard taken on as is basis

%Assay

=588407/587843x 20/100x5/50x100/2x99.2/100x100

= 99.2%

CONCLUSION

The present study was aimed at developing a simple, precise and accurate HPLC method for the analysis of Clofarabine from tablet dosage forms. The method should be developed mainly based on pk_a concept of drug and also different mobile phase composition, flow rate, λ_{max}, different columns and column temperature. Clofarabine has two pk_a values that are 12.7and 1.3. Generally pH of buffer solution should be select ± 1 of pk_a value of drug. In this method value pk_a 2.3 of Clofarabine was selected because in HPLC, column may damage solutions with pH more than 10. So pk_a 12.7 of Clofarabine was eliminated. Then select 2.3 pk_a value of drug and pH should adjust ±2 of pka and change mobile phase composition also. Finally good peak was obtained at pH 4.5 of buffer and retention time also less than compared to other trials. So the method was optimized at these conditions. A non-polar C_{18} analytical chromatographic column was chosen as the stationary phase for the separation and determination of Clofarabine. For the selection of the mobile phase a number of eluting systems were examined. Mixtures of commonly used solvents like water, methanol and acetonitrile with or without different buffers in different combinations were tested as mobile phases on a Develosil C_{18} MG-5(250x4.6mm), 5µm stationary phase. The choice of the optimum composition is based on the chromatographic response factor, a good peak shape with minimum tailing. A P^H 3.6 buffer Methanol and Acetonitrile in the ratio of 70:15:15 v/v respectively was proved to be the most suitable of all the combinations since the chromatographic peak obtained was better defined and resolved and almost free from tailing. The retention time of the drug was found at 5.578 min.

A good linear relationship (r=0.9999) was observed between the concentrations of Clofarabine and the corresponding peak areas. The linearity range was found to be 10-30 µg/mL. The regression equation of the calibration curve between concentration of Clofarabine over its peak area was found to be y = 29306x-6684.6 (where y is the peak area and x is the concentration of Clofarabine). The intra-day and inter-day variations in the range of study showed a low coefficient of variation (Table 2.1.5). This reveals that the method is quite precise. The percent recoveries of the drug solutions were studied at three different concentrations levels. The percent individual recovery and the percent RSD values at each level were (100.78 to 99.86%) within the acceptance limits. The drug content in the tablets was quantified by using the proposed method of analysis. The absence of additional peaks in the chromatogram indicates non interference of the commonly used excipients in the tablets and hence the method is specific.

The deliberate changes in the method have not much affected the peak tailing, theoretical plates and the percent assay. This indicates that the present method is robust. The lowest values of LOD and LOQ as obtained by the proposed method indicate the method is sensitive. The working solution of the drug was stable up to 24 hours.

The tailing factor, the number theoretical plates and HETP are in the acceptable limits. Therefore, the proposed method can be used for routine quality control and analysis of the drug in bulk samples and in tablet dosage forms.

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