IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)
e-ISSN:2278-3008, p-ISSN:2319-7676. Volume 11, Issue 6 Ver. III (Nov. - Dec.2016), PP 63-72
www.iosrjournals.org

Validated Reverse Phase Stability-Indicating Hplc Method for Clofarabine in the Presence of Degradation Products and its Process-Related Impurities.

JagadeswaraRao K[1,2], Murali Mohan SV [2] and *Rama Rao Malla[1]
[1]*Department of Biochemistry, Cancer Biology Lab, GIS, GITAM University, Visakhapatnam, India*
[2]*Analytical Research and development Department, SHILPA MEDICWERE LIMITED, India*

Abstract: *The study reports simultaneous determination of clofarabine and its related substances of impurities in their pharmaceutical dosage form using validated stability indicating reverse phase high performance liquid chromatography method. The column used was Inertsil ODS 3V, 250x4.6 mm, 5 µm using phosphate buffer pH 3.0: acetonitrile with gradient mixing mobile phase at a flow rate of 1.0 ml/min. Quantification was achieved with UV detection at 250 nm over the concentration range 0.05-20 µg/ml and % recovery was found to be in the range of LOQ to 150 % for clofarabine and related impurities by the RP-HPLC method. The proposed method was validated with respect to precision, linearity, accuracy, selective, sensitive and robustness. The method was successfully applied for the estimation of clofarabine and its related compounds in injection dosage forms.*

Keywords: *Analytical method development, analytical method validation, clofarabine and high performance liquid chromatography.*

I. Introduction

Stability Indicating Method (SIM) is a quantitative analytical procedure measures the potential interferences of degradation products, process impurities, excipients, or other potential impurities in pharmaceutical ingredients[1]. Several stability indicating methods were used for potential impurities including colorimetry[2], capillary gas chromatography[3], HPLC–fluorescence detector in human serum[4], Ultra Performance Liquid Chromatography (UPLC)[5], liquid chromatography with electrochemical detection[6], HPLC–MS–TOF[7], High Performance Thin Layer Chromatography (HPTLC) method coupled with Gas Chromatography and Mass Spectroscopy (GC–MS) detection[8]. Clofarabine, an antimetabolite purine nucleoside used in the treatment of lymphoblastic leukemia [9]. The clofarabine is intracellularly converted to 5'-monophosphate by deoxycytidine kinase and 5'-triphosphate by mono- and di-phosphokinases [10]. 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 [11]. 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 [10]. The IUPAC name of clofarabine is (2R, 3R, 4S, 5R)-5-(6-amino- 2-chloropurin-9-yl)-4-fluoro-2- (hydroxymethyl) oxolan-3-ol empirical formula is $C_{10}H_{11}Cl\ FN_5O_3$ and molecular weight is 303.67 g/mol. (Figure 1). The trade name of clofarabine in the U.S. and Canada is Clolar and Europe and Australia/New Zealand is Evoltra. 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 [14-15]. Of late, HPLC method is reported for the determination of concentration of clofarabine in rat plasma [16]. LC/MS method is reported for analysis and identification of chlorinated impurity of clofarabine [17]. Ultra performance convergence chromatography method was used for identification of two related substances in clofarabine [15]. Over period, several stability indicating reverse phase high performance liquid chromatography (RP-HPLC) methods were developed for determination of concentration of clofarabine [18-20]. Recently, reverse-phase high-performance liquid chromatography (RP–HPLC) method was developed and validated for determination of forced degradation products and process related impurities of bortezomib [21]. The present study is aimed to develop and validate RP-HPLC for determination of five clofarabine related impurities in pharmaceutical dosage forms.

II. Experimental

2.1 Chemicals

The clofarabine and its related impurities such as clofarabine α- anomer, 2-chloroadenine, clofarabine monobenzoate, dichloro derivate and bis-suger clofarabine with > 99% purity were obtained from PS3 LABORATORIES LLP, Hyderabad and India and used in the present study. HPLC grade acetonitrile (ACN)

was purchased from Merck (Darmstadt, Germany). All other chemicals used in the present study were analytical grade and obtained from commercial source.

2.2 Equipment's:

The LC system was used for the method development, forced degradation studies and method validation as described in our previous report [21]. The system consists of Agilent 1200 series binary pump equipped with an auto sampler and a photodiode array detector (Agilent, USA). The output signal was monitored and processed using viewsonic computer with Empower software (Version 3). Photo stability studies were carried out using photo stability chamber (Newtronic life care, India). Thermal stability studies were performed using dry hot air oven (Newtronic life care, India).

2.3 Optimization of chromatographic conditions:

The chromatographic column (ODS 3V, 250x4.6 mm, 5 µm) was used with a mobile phase-A containing 0.01M potassium di hydrogen phosphate, pH 3.0 diluted with ortho-phospharic acid and mobile phase-B containing 100 % acetonitrile. The flow rate was fixed at 1.0 mL/min, column temperature was maintained at 30°C, and detection wavelength was set at 250 nm. The sample was diluted with methanol and water mixture (50:50) and 10µL was injected into system. The sampler-cooling temperature was set at 25°C and total run time was fixed at 55 min.

2.4 Preparation of standard solutions and sample solutions:

The standard test solution was prepared by dissolving clofarabine standard in methanol (1mg/ml) using ultrasonic bath. It was diluted further to 0.001 mg/mL with diluent (purity standard). Further, it was diluted to 0.000067 mg/mL (system suitability standard LOD). The test solution for determination of related substance prepared at a concentration of 1 mg/mL. Test solution for assay determination of clofarabine standard was prepared at a concentration of 1 mg/mL. Further, it was diluted to a concentration of 0.3 mg/mL.

III. Method Development

3.1 Method development and optimization

The method development study was initiated with experimental setup 1 using simple reverse phase HPLC with mobile phase A consisting of 0.01 M Phosphate buffer, pH was 3.0, mobile phase B (Acetonitrile), stationary phase (Waters C18 150 X 4.6 mm, 5µm), detection wavelength (250 nm) and column oven temperature (25°C). The injection volume of impurity mixture, 10 µL was analyzed using simple gradient program. Same mobile phase A, mobile phase B, stationary phase and detection wavelength but column oven temperature was set at 30°C in experimental setup 2. In experimental setup 3, same mobile phase A, mobile phase B, but stationary phase (Inertsil ODS 3V, 250x4.6 mm, 5 µm) at a flow rate of 1.0 mL/min, detection wavelength (250 nm) and column oven temperature (30°C) was used for separation of clofarabine related impurities.

1.2 Method validation parameters as per current ICH guidelines Q2R1

3.2.1 Stress studies / Specificity:

The stress/specificity studies were performed to evaluate the capability of the method to resolve possible impurities and degradation products of clofarabine. The impurity standards were spiked to the sample and degradation products 2-chloroadenine, clofarabine -anomer and clofarabine monobenzoate were spiked at a 10 PPM; and bis-suger clofarabine and dichloro derivate were spiked at a 5 PPM in the clofarabine sample. Forced degradation studies were also performed on clofarabine to infer stability-indicating property and specificity of the validating method. The stress conditions used for the degradation study were light (ICH Q1B), heat (40°C), acid hydrolysis (1.0 M HCl), base hydrolysis (1.0 M NaOH) and oxidation (30% Hydrogen peroxide). The samples were exposed to heat and light for 48 h, whereas samples were treated for 2 h for acid and base hydrolysis and for oxidation. The peak purity of the clofarabine, clofarabine α- anomer, clofarabine β isomer, 2-chloroadenine, clofarabine monobenzoate, dichloro derivate and bis-suger clofarabine stressed samples were also validated using a Waters photo diode array detector (PDA). The purity angle was set within the purity threshold limit for all of the stressed samples and content of impurities were calculated for the stress samples against a qualified reference standard. The mass balance (% assay + % of impurities + % of degradation products) was calculated for all of the samples.

3.2.2 System suitability and System precision:

System suitability parameters were tested and measured the system performance. The system precision criteria was determined on six replicate injection of standard preparation and % RSD of the clofarabine peak was evaluated.

3.2.3 Method precision:

The method precision was explored by injecting six individual preparations of clofarabine. The impurity standards were spiked to samples and degradation products such as 2-chloroadenine, clofarabine-anomer and clofarabine monobenzoate were spiked at a 10 PPM and bis-suger clofarabine, dichloro derivate, clofarabine –β-isomer were spiked at a 5 PPM in the clofarabine sample. The % RSD of the individual known impurities, unknown impurities and total impurities were calculated.

3.2.4 Intermediate precision:

The intermediate precision for clofarabine related impurities was investigated by using different instrument, different analyst, different location and different days. The % RSD of the individual known impurities, unknown impurities and total impurities was calculated. The overall % RSD for both method precision and intermediate precision was calculated.

3.2.5 Accuracy:

The accuracy of the related substance method was evaluated in triplicate at five concentration levels, LOQ, 25, 50, 100 and 150% and the percent recovery was calculated for clofarabine and its impurities. The impurities were spiked into clofarabine sample and recovery experiments were performed to determine the accuracy of the related substance method for quantification of impurities. The study was carried out in triplicate for clofarabine -anomer, 2-chloroadenine, clofarabine monobenzoate impurity at 0.05, 0.75, 1.5 and 2.25 % of the analyte concentration (1.0 >mg/ml) respectively. For bis-suger clofarabine, dichloro derivate and clofarabine –β-isomer 0.03, 0.05, 0.1 and 0.15 % of the analyte concentration (1.0 >mg/ml), respectively was used. The percent of recovery for clofarabine was calculated.

3.2.6 Linearity:

Linearity test solutions of clofarabine were prepared from LOQ to 150% for the related substance method. The peak area versus concentration was analyzed with least-squares linear regression. The Linearity test solutions were prepared by diluting the impurity stock solution to the required concentrations for the related substance method. The solutions were diluted at six different concentration levels from the LOQ to 150%. The slope and y-intercept of the calibration curve was observed. The peak area versus concentration data was analyzed using least squares linear regression. The linearity test solutions were prepared by diluting the impurity stock solution to the required concentrations for the related substance method. The slope and y-intercept of the calibration curve were reported.

3.2.7 Limit of detection (LOD) and limit of quantification (LOQ):

The LOD and LOQ for Clofarabine -anomer, 2-chloroadenine, Clofarabine monobenzoate, bis-suger clofarabine, dichloro derivate, clofarabine –β-isomer and clofarabine at a of 3:1 and 10:1, signal-to noise ratio by injecting a series of dilute solutions with known concentrations. The precision study was also performed at the LOQ level by injecting six individual samples and calculated the % RSD of the areas.

3.2.8 Robustness:

The robustness of the developed method of evaluated at different experimental conditions such as flow rate, pH and column temperature. The flow rate of the mobile phase was maintained 1.0 ml/min. To study the effect of the flow rate on the resolution, the flow rate was changed by 0.1 units from 0.9 and 1.1 ml/min. The effect of pH on the resolution of the impurities was studied by varying the pH by ± 0.2 units from buffer pH 2.8 and 3.2. The effect of the column temperature on the resolution was studied from 35-45°C. On the resolution was studied to change the organic ratio it was changed from ± 2.0% In all these varied conditions, the components of the mobile phase was remain constant during this study.

3.2.8 Solution stability and Mobile phase stability:

The stability of clofarabine sample solution, standard solution and spiked sample solution in the proposed method was carried out by placing the both sample and reference standard solution in a tightly capped volumetric flask at room temperature for 6 h. The same sample solutions were assayed for in a 6 h interval during the study period. The mobile phase stability was evaluated by assaying the freshly prepared sample solutions against freshly prepared reference standard solutions for 6 h intervals. The prepared mobile phase remained constant during the study period. The % RSD of the clofarabine impurities was calculated for the mobile phase as well as solution stability experiments. The amount of clofarabine, clofarabine -anomer, 2-chloroadenine, clofarabine monobenzoate, bis-suger clofarabine, dichloro derivate and clofarabine –β-isomer was determined up to the study period at 6 h intervals. The stability of mobile phase was evaluated for 48 h by injecting the freshly prepared sample solutions at every 6 h interval. The content of clofarabine, clofarabine -

anomer, 2-chloroadenine, clofarabine monobenzoate, bis-suger clofarabine, dichloro derivate and clofarabine – β-isomer was determined in the test solutions. The prepared mobile phase remained same during the study period.

IV. Results and Discussion

Method development and optimization for clofarabine: The clofarabine API and Product official methods are not available in USP, BP and EP pharmacopeia. According to this API route of synthesis there are 5 specified impurities in clofarabine such as clofarabine α- anomer, 2-chloroadenine, clofarabine monobenzoate, dichloro derivate and bis-suger clofarabine. Out of those, clofarabine α- anomer, 2-chloroadenine, clofarabine monobenzoate are potential degradant impurities and dichloro derivate, bis-suger clofarabine are process related impurities. The target of the current study was to separate clofarabine from impurities and degradants in a shorter run time and using a RP-HPLC stability indicative method. The injection sequence of related substance and assay method of clofarabine was depicted in the table 1. The results on experimental setup 1 of method development study was using simple reverse phase HPLC with phosphate buffer, pH 3.0 and acetonitrile as mobile phase, Water C18 column as a stationary phase and 25^{o}C as column oven temperature reveals that the impurity peaks not separated and peak shapes were not satisfactory. Screening in experimental setup-2 with increasing column oven temperature to 30^{o}C was also not satisfactory for impurity peak separation and peak shapes. The replacement of Water C18 stationary phase with Inertsil ODS in experimental setup-3 achieved separation of impurity peaks with sharp peak shapes. These results indicate that Inertsil was found to be better than water C 18 column in terms of separation and peak shapes.

Table1. Injection sequence of related substance and assay method of clofarabine

S.No.	Name of the injection	No. of injections
1	Blank	1
2	Resolution solution	1
2	Assay standard	5
3	Related substance standard	6
4	Sample preparation 1	1
5	Sample preparation 2	1
6	Standard Bracketing	1

Optimization of mobile phase was performed based on resolution of the drug, asymmetric factor and theoretical plates obtained for clofarabine. The gradient mixture of phosphate buffer and acetonitrile as shown in the table 2 was selected as a mobile phase at a flow rate of 1.0 ml/min was found to be satisfactory, achieved good separation and showed symmetric peak for clofarabine and related impurities. The column oven temperature was selected 30°C. The retention time for clofarabine was found to be 18 min. The spectrum clofarabine solution (1mg/ml) exhibited significant absorbance at 250 nm, hence it was selected as detection wavelength for estimation of cloforabine using HPLC. With above parameters, complete resolution of the peaks with clear baseline separation was obtained.

Table 2. HPLC conditions of gradient program for separation clofarabine impurities

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	98	2
5	98	2
55	10	90
58	10	90
60	98	2
66	98	2

4.0.1 Stress studies / Specificity:

The clofarabine stressed samples were subjected to forced degradation studies using light and heat. Significant degradation of the drug substance and its products was detected with thermal, acid and oxidation, which leading to the formation of one major unknown degradation product with RRT of 1.19 (Table 3).

Table 3 Retention time of clofarabine and its impurities and degradants

S.No.	Name of the Impurity	Retention time in min	RRT
1	bis-suger clofarabine	46.1	2.60
2	dichloro derivate	35.3	2.00
3	Clofarabine α- anomer	18.4	1.03
4	2-chloroadenine	14.2	0.80
5	Clofarabine monobenzoate	31.3	1.76
6	Clofarabine –β-isomer	17.7	Not Applicable

The peak purity test derived from the PDA detector confirmed that the cloferabine peak and the degraded peaks were homogeneously pure in all the analyzed stress samples. The degradation studies were carried out for the stress samples at 100>g/ml against a qualified reference standard of clofarabine. The mass balance of the stressed samples was close to 99.5%. These results indicate that assay of clofarabine was unaffected by the presence of clofarabine and clofarabine -anomer, 2-chloroadenine, clofarabine monobenzoate, bis-suger clofarabine, dichloro derivate and clofarabine –β-isomer and its degradation products, confirming the stability-indicating power of the developed method (Table 4).

Table 4. Effect of different stress conditions on clofarabine

S.No.	Conc. (PPM)	Stress Condition	% of clofarabine						
			BC	BS	DD	CA	CD	Clofarabine monobenzoate	Unknown Impurity of clofarabine
1	1000	Acidic degradation,HCl (1 M)	96.13	0.00	0.00	0.13	0.26	0.22	0.09
2	1000	Acidic degradation, H_3PO_4 (85%)	98.96	0.00	0.00	0.02	0.06	0.00	0.12
3	1000	Basic degradation, NaOH (1 M)	94.21	0.00	0.00	0.00	5.66	0.05	0.07
4	1000	Oxidative stress, H_2O_2 (30%)	99.80	0.00	0.00	0.00	0.00	0.09	0.04
5	1000	Oxidative stress, $KMnO_4$ (0.1N)	88.79	0.00	0.00	0.49	7.80	0.00	1.27
6	1000	Heat degradation, 120°C, 15 days	90.78	0.00	0.00	0.00	7.87	0.08	0.00
7	1000	Light degradation	99.84	0.00	0.00		0.02	0.08	0.08

BC: β-Clofarabine, **BS:** bis-suger clofarabine, **DD**: dichloro derivate, **CA**: Clofarabine α- anomer, **CD**: 2-chloroadenine **CM**: Clofarabine monobenzoate

4.0.2 System suitability and System precision:

The clofarabine resolution solution and standardard solution were injected into the respective chromatographic condition and recorded the clofarabine peak areas. The observed resolution between the clofarabine and clofarabine –α-anomer was found to be 4.5 and % RSD for six replicate injections was observed as 3.0% (Table 5).

Table 5. Data of system suitability and system precision

S.No.	**Clofarabine peak area (at 250nm)**
1	102569
2	102356
3	105649
4	100261
5	108974
6	105642
Average	104241.8333
STDEV	3115.630236
% RSD	**3.0**

4.0.3 Method precision:

The method precision study was carried out within 10.0 % RSD. The % RSD of six individual spiked sample solutions impurities were spiked at specification level. The results show that % RSD of clofarabine -anomer, 2-chloroadenine, clofarabine monobenzoate, bis-suger clofarabine, dichloro derivate and clofarabine –β-isomer were 5.1, 5.1, 4.9, 5.3 and 3.2, respectively (Table 6).

Table 6. Method precision for separation of clofarabine and its impurities

S.No.	% bis-suger clofarabine	% dichloro derivate	%Clofarabine α-anomer	%2-chloroadenine	%Clofarabine monobenzoate
1	0.10	0.10	1.46	1.46	1.51
2	0.10	0.09	1.40	1.40	1.57
3	0.09	0.09	1.46	1.46	1.42
4	0.09	0.09	1.55	1.55	1.48
5	0.10	0.09	1.56	1.56	1.39
6	0.09	0.09	1.38	1.38	1.56
Average	**0.10**	**0.09**	**1.47**	**1.47**	**1.49**
STDEV	**0.0051**	**0.0029**	**0.0745**	**0.0745**	**0.0731**
%RSD	**5.3**	**3.2**	**5.1**	**5.1**	**4.9**

4.0.4. Intermediate precision:

The intermediate precision study was carried out within 10.0 % RSD. The overall RSD for both method precision and intermediate precision within 15%. The results show that % RSD of clofarabine -anomer, 2-chloroadenine, clofarabine monobenzoate, bis-suger clofarabine, dichloro derivate and clofarabine –β-isomer were 4.9, 5.1, 5.1, 4.9, 5.3 and 3.2, respectively (Table 7).

Table 7. Intermediate precision for separation of clofarabine and its impurities

S.No.	% bis-suger clofarabine	% dichloro derivate	%Clofarabine α- anomer	%2-chloroadenine	%Clofarabine monobenzoate
1	0.09	0.10	1.51	1.45	1.46
2	0.09	0.09	1.57	1.56	1.40
3	0.10	0.09	1.42	1.42	1.46
4	0.10	0.10	1.48	1.54	1.55
5	0.10	0.10	1.39	1.36	1.56
6	0.10	0.11	1.56	1.45	1.38
Average	**0.09**	**0.10**	**1.49**	**1.46**	**1.47**
STDEV	**0.0041**	**0.0054**	**0.0731**	**0.0750**	**0.0745**
%RSD	**4.3**	**5.6**	**4.9**	**5.1**	**5.1**

4.0.5 Accuracy:

The percent recovery of clofarabine impurities in the drug substances was ranged from 110 to 95.6, 106.7 to 95.6, 93.3 to 96.7, 93.3 to 98.5, 93.9 to 96.7 and 110 to 95.6 for bis-suger clofarabine, dichloro derivate, clofarabine α- anomer, chloroadenine, clofarabine monobenzoate and clofarabine respectively. The HPLC chromatograms of spiked samples at the 100% level of all impurities in the clofarabine drug substance sample were shown in Figure 3. These results indicate the accuracy of method (Table 8).

Table 8. Data derived from of clofarabine by proposed HPLC method

S.No.	Level	% bis-suger clofarabine	% dichloro derivate	%Clofarabine α-anomer	%2-chloroadenine	%Clofarabine monobenzoate	Clofarabine
1	**LOQ**	0.03	0.03	0.05	0.05	0.03	0.03
2		0.04	0.04	0.04	0.04	0.04	0.04
3		0.03	0.03	0.046	0.046	0.03	0.03
1	**50%**	0.05	0.05	0.86	0.86	0.86	0.05
2		0.06	0.06	0.84	0.84	0.84	0.06
3		0.05	0.05	0.76	0.76	0.76	0.05
1	**100%**	0.09	0.10	1.46	1.46	1.51	0.09
2		0.10	0.09	1.40	1.40	1.57	0.10
3		0.09	0.09	1.46	1.46	1.42	0.09
1	**150%**	0.14	0.14	2.15	2.2	2.15	0.14
2		0.14	0.14	2.18	2.15	2.18	0.14
3		0.15	0.15	2.20	2.30	2.20	0.15
Average	**LOQ**	**0.03**	**0.03**	**0.05**	**0.05**	**0.03**	**0.03**
STDEV		0.0026	0.0053	0.0031	0.0031	0.0053	0.0026
%RSD		**8.0**	**16.5**	**6.5**	**6.5**	**16.5**	**8.0**
Average	**50%**	**0.05**	**0.05**	**0.82**	**0.82**	**0.82**	**0.05**
STDEV		0.0012	0.0012	0.0529	0.0529	0.0529	0.0012
%RSD		**2.1**	**2.1**	**6.5**	**6.5**	**6.5**	**2.1**
Average	**100%**	**0.10**	**0.09**	**1.44**	**1.44**	**1.50**	**0.10**
STDEV		0.0065	0.0032	0.0341	0.0341	0.0755	0.0065
%RSD		**6.8**	**3.5**	**2.4**	**2.4**	**5.0**	**6.8**
Average	**150%**	0.14	0.14	2.18	2.22	2.18	0.14
STDEV		0.0058	0.0058	0.0252	0.0764	0.0252	0.0058
%RSD		**4.0**	**4.0**	**1.2**	**3.4**	**1.2**	**4.0**
Recovery at LOQ %		**110**	**106.7**	**93.3**	**93.3**	**93.9**	**110**
Recovery at 50 %		**109**	**109**	**109**	**109**	**109**	**109**
Recovery at 100 %		**95.6**	**92.7**	**96.0**	**96.0**	**100.0**	**95.6**
Recovery at 150 %		**95.6**	**95.6**	**96.7**	**98.5**	**96.7**	**95.6**

4.0.6 Linearity:

The linear calibration plot of the method was obtained in the tested calibration range of 50-150% level and the correlation coefficient obtained was > 0.999. These results indicate significant correlation between the peak areas and analyte concentration. The linear calibration plot for the related substance was determined in the calibration range for clofarabine and impurities was 150% with respective to LOQ, a correlation coefficient of greater than 0.999 was obtained. In this range, the linearity was checked for the related substance at same concentration range for three consecutive days. The % RSD values of the slope and y-intercept of the calibration curves were achieved within 10%. These results showed significant correlation between the peak areas and concentrations of clofarabine and impurities. The residuals were within ± 10% scattered with respect to 100% concentration response. The sensitivities were scattered within ± 10% with respect to 100% concentration sensitivity (Table 9).

Table 9. Regression and precision data analysis of clofarabine related impurities

Name of the Impurity	**BS**	**DD**	**CA**	**CD**	**CM**	**BC**
LOD %	0.003%	0.004	0.002	0.003	0.003	0.003
LOQ %	0.03	0.03	0.006	0.01	0.01	0.01
Slope (m)	10845	11458	10755	10845	11256	10548
Intercept (C)	-32.4	-31.56	21.56	23.47	-21.45	-32.4
Correlation coefficient	0.989	0.996	1.000	0.999	0.995	0.998
Precision (%RSD)	4.6	3.8	4.2	2.5	3.5	3.6

BC: β-Clofarabine, **BS:** bis-suger clofarabine, **DD**: dichloro derivate, **CA**: Clofarabine α- anomer, **CD**: 2-chloroadenine **CM**: Clofarabine monobenzoate

4.0.7 Limit of detection (LOD) and limit of quantification (LOQ):

The LOQ precision study was carried out within 10.0 % RSD. The results show that % RSD of clofarabine -anomer, 2-chloroadenine, clofarabine monobenzoate, bis-suger clofarabine, dichloro derivate and clofarabine –β-isomer were 10.8, 11.5, 12.5, 13.6, and 12.5 respectively.

4.0.8 Robustness:

At deliberately, modified chromatographic conditions such as flow rate, pH, solvent ratio and column temperature etc., the resolution between the closely eluting impurities, clofarabine and clofarabine -anomer, 2-chloroadenine, clofarabine monobenzoate, bis-suger clofarabine, dichloro derivate and clofarabine –β-isomer, resolution achieved was greater than 3.5. The variability of clofarabine and the impurities area response was within ±5% and ±5%, respectively. These results emphasize the robustness of the method.

4.0.9 Solution stability and Mobile phase stability:

The series of solution stability and mobile phase stability indicate the % RSD of the related substances method of clofarabine and was less than 1%. These results indicate that no significant changes in the content of clofarabine and clofarabine -anomer, 2-chloroadenine, clofarabine monobenzoate, bis-suger clofarabine, dichloro derivate and clofarabine –β-isomer during the solution stability and mobile phase stability experiments The results of the solution and mobile phase stability experiments confirm that the sample solutions and mobile phase used during the related substance determinations were stable up to120 h. The mobile phase was proved stable up to one-week days.

V. Conclusion

The degradation pathway of clofarabine was established as per ICH recommendations [17-21]. The gradient LC method was developed and used for stress studies also fit for quantification of impurities of clofarabine drug. The behavior of clofarabine under various stress conditions was studied. The thermal and all of the degradation products and process impurities were well separated from the clofarabine and related impurities, demonstrates the stability-indicating power of the method. This method is sensitive and can be detected up to 0.002% impurities. This method was precise, accurate and stability indicative as per ICH recommendations. The developed method can be used to determine the impurities in clofarabine injection in the routine as well as and stability sample analysis [17-21].

Acknowledgements

The authors wish to thank the management of PS3 LABORATORIES LLP, Hyderabad and India for supporting this work. We thank management of GITAM University for supporting research work.

Figure. 1: UV Specturm of clofarabine

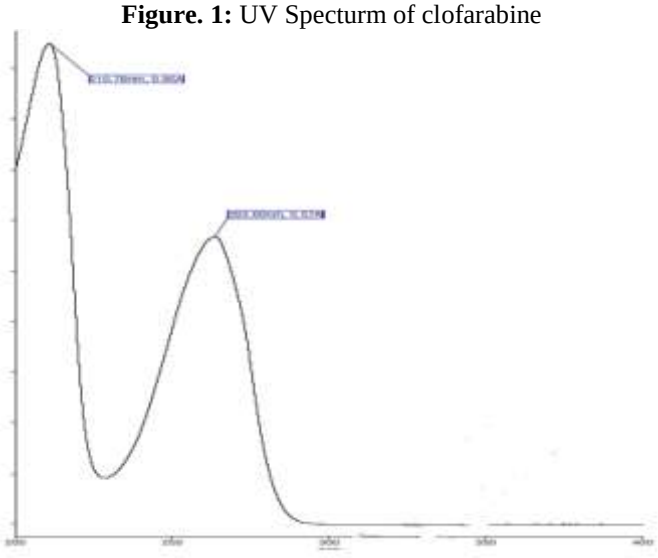
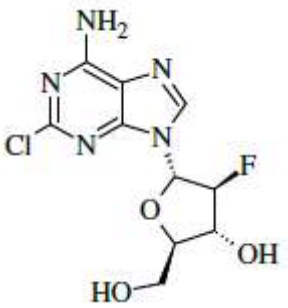
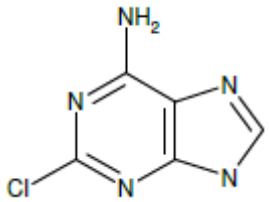


Figure. 2: Chemical structure of clofarabine and its impurities.

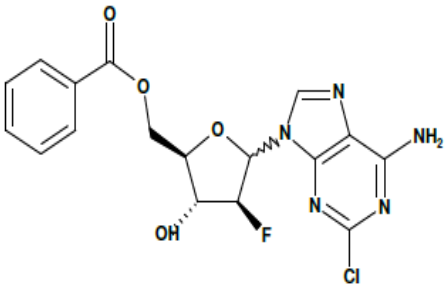
a) Clofarabine –anomer



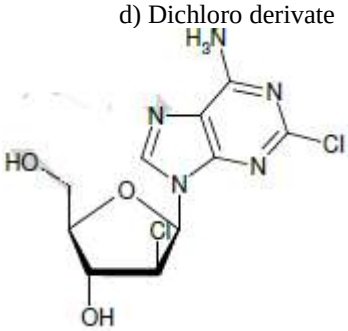
b) 2-Chloroadenine



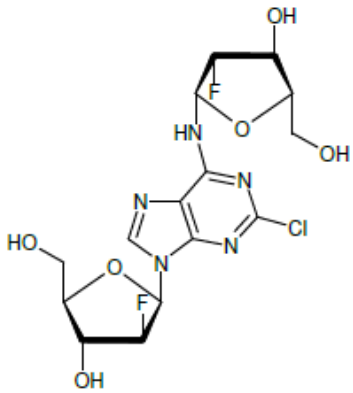
c) Clofarabine monobenzoate



d) Dichloro derivate



e) Bis-sugar Clofarabine



f) Clofarabine Please adjust side by side

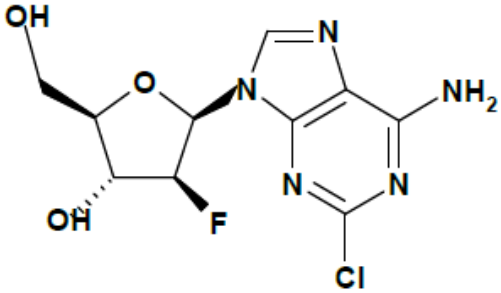
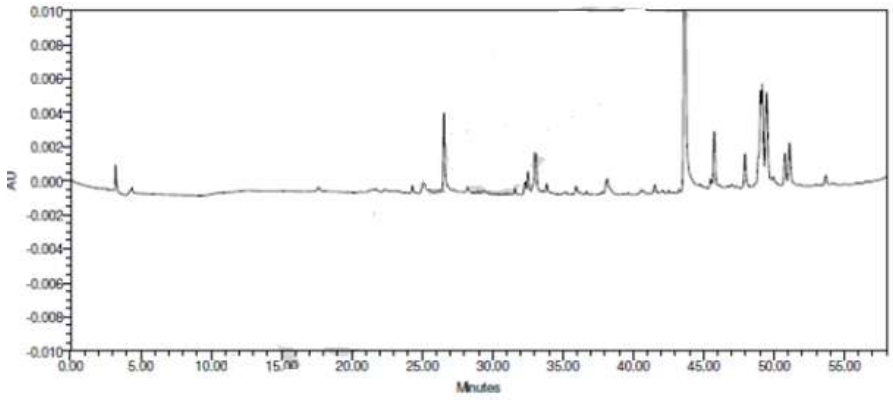
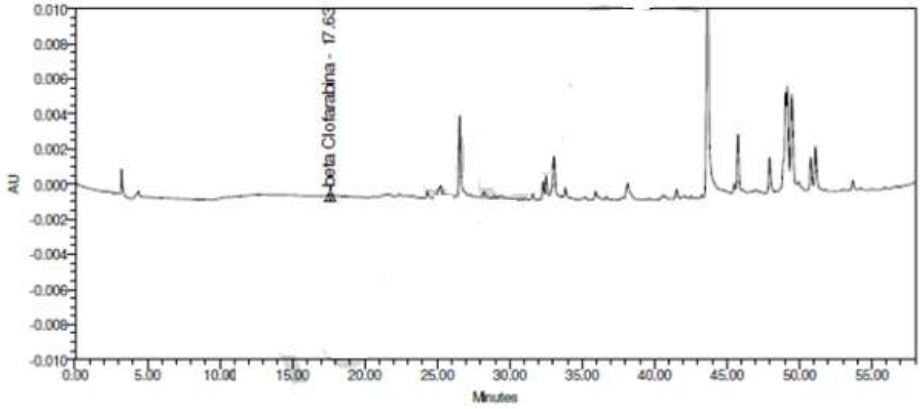


Figure 3. Typical HPLC chromatograms of clofarabine and its impurities under different stress conditions.

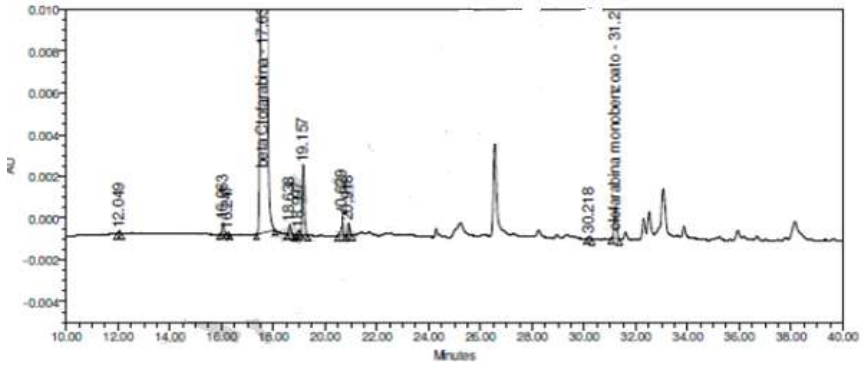
a) Chromatogram of blank solution



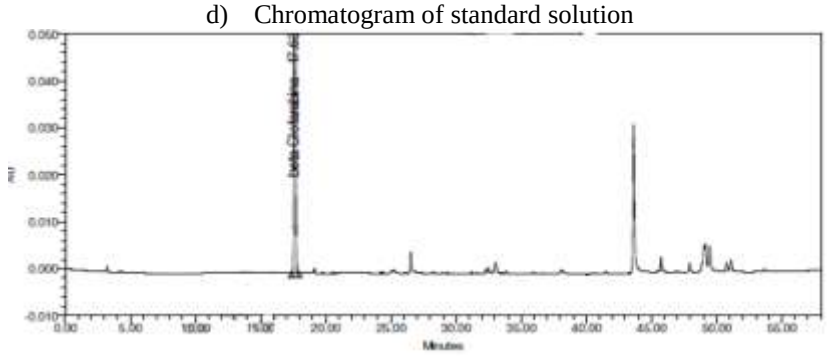
b) Chromatogram of standard solution



c) Chromatogram of resolution solution



d) Chromatogram of standard solution



References

[1]. A. Shrivastava, and P. Saxena, Stability indicating reverse phase high performance liquid chromatography method for the estimation of capsaicin, Pharm Methods, 2(2), 2011, 135–142.
[2]. N. J. Bayonee, Colorimetric determination of capsaicin in oleoresin of capsicum, Analy Chem., 21, 1949, 934-936.
[3]. S.H. Wooderck, H. Jaeho, H. Jinbong, N.Youngjung, Effective separation and quantitative analysis of major heat principles in red pepper by capillary gas chromatography, Food Chem., 49, 1994, 99–103.
[4]. J. Lu, M.J. Cwik, B, Chromatogr, Determination of capsaicin and zucapsaicin in human serum by high-performance liquid chromatography with fluorescence detection, Biomed Sci., 701(1), 1997, 135-139.
[5]. J. Ha, H.Y. Seo, Y.S.Shim, D.W. Seo, H. Seog, M.Ito, Hiroaki Nakagawa, Determination of capsaicinoids in foods using ultra high performance liquid chromatography, Food Sci Biotech., 19, 2010,1005-1009.
[6]. G.H.Chiang, HPLC Analysis of Capsaicins and Simultaneous Determination of Capsaicins and Piperine by HPLC-ECD and UV, J Food Sci., 51, 2006, 499–503.
[7]. A. Garcés-Claver, M.S. Arnedo-Andrés, J. Abadía, R.Gil-Ortega, A. Alvarez-Fernandez, Determination of capsaicin and dihydrocapsaicin in capsicum fruits by liquid chromatography-electrospray/time-of-flight mass spectrometry, J Agric Food Chem., 54(25), 2006, 9303-9311.
[8]. M.I. Moise, C. Marutoiu, D.N. Badea, C.A. Gavrila, C. Patroescu, Application of TLC and GC-MS to the detection of capsaicin from hot peppers (Capsicum annuum), J Planar Chromatograph., 17, 2004,147–148.
[9]. L. J. De Gennero, E. Raetz, Acute lymphoblastic leukemia (Leukemia and Lymphoma Society, 2014, pp 1-52).
[10]. C.H.Pui, S. Jeha, P.Kirkpatrick, Clofarabine, Nature Reviews Drug Discovery, 4, 2005, 369-370.
[11]. A. Zhenchuka, K. Lotfib, G. Juliussond, F. Albertionia, Mechanisms of anti-cancer action and pharmacology of clofarabine, Biochemical Pharmacology, 78(11), 2009,1351–1359.
[12]. A. M. Moufarij, D. Sampath, M. J. Keating and W. Plunkett, Fludarabine increases oxaliplatin cytotoxicity in normal and chronic lymphocytic leukemia lymphocytes by suppressing interstrand DNA crosslink removal, Blood, 108, 2006, 4187-4193.
[13]. B.D.Patel, U. K.Chhalotiya, D.B.Patel, Quantification of Newer Anti-Cancer Drug Clofarabine in their Bulk and Pharmaceutical Dosage Form. J Chromatogr Sep Tech, 7(3), 2016, 328-832.
[14]. B.Zhu, L. Zhao, Q. Wang, C. Zhang, X. Chen,J. Huan and Y.Fang, Zhongguo Yaofang, 22, 2011, 2056.
[15]. N.R.Venkata NR, D. Jalandhar, G. Gnanadey, R. Bandari, P. Manoi, Development of supercritical Fluid (Carbon dioxide) based ultra-performance convergence chromatographic stability indicating assay method for determination of clofarabine in injection. Analytical methods, 5, 2013, 7008-7013.
[16]. M.A. Zhang-Qing, H.Zong-Yuan, L. Jia-Jie. Determination of clofarabine in rat plasma and the pharmacokinetic research, Acta Metallurgica Sinica, 16(8), 2011, 852-855.
[17]. L. Huang, P. Lizak, C. Dvorak, J. Long-Boyle, Simultaneous determination of Fludarabine and Clofarabine in human plasma by LC-MS/MS, Journal of Chromatography B Analyt Technol Biomed Life Sci., 960, 2014, 194-199.
[18]. L. Peng, T. Farkas, Analysis of basic compounds by reversed-phase liquid chromatography electrospray mass spectrometry in high-pH mobile phases, J Chromatogr A., 1179, 2008,131–144.
[19]. Bakshi M, Singh S, Development of validated stability indicating assay methods critical review. J Pharm Biomed. 2002: 28:1011-1040.
[20]. Brijesh D Patel, Usmangani K Chhalotiya, Dhruv B Patel Quantification of Newer Anti-Cancer Drug Clofarabine in their Bulk and Pharmaceutical Dosage Form.J Chromatogr Sep Tech 2016, 7:3
[21]. K.Jagadeswara Rao, B. Mohan, N.V.S. Venugopal, S. V. Murali Mohan, Ramarao Malla, Avalidation reverse phase stability-indicating HPLC method for bortezomib in the presence of degradation products and its process related impurities, *International journal of pharmaceutical chemistry and analysis* , 3(3), 2016, 150-161.