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Qbd Approach High-Performance Liquid Chromatographic Technique For Determination Of Clofarabine Impurities In Clofarabine Api, Clofarabine Parenteral Dosage Form And Reconstitution Studies

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ABSTRACT

Using the quality by design concept (QbD) and a DoE methodology to identify the design space for a technique of Related impurities method for Clofarabine API, Clofarabine parenteral dosage form, and reconstitution studies using 0.9 percent sodium chloride.

Using design expert software, DoE was used to do a simultaneous multivariant approach for pH, buffer strength in the mobile phase, and flow rate. Trials were done in a two-level fractional factorial design (2^{4-1} + 4 canter points = 12 experiments) to establish design space. Method development experiments were performed by using a composite degradation sample with spiked known impurities. Degradation unknown and known impurity pairs were studied statistically to identify the chromatographic parameters essential for their resolution. Various data tables, such as analysis of variance and Pareto charts, were used to assess conclusions made during this process. The 'design space' of the method's chromatographic factor range was shown in the plot of desirability. The procedure is exact, specific, accurate, and reliable all at once. This method is found suitable for the quantification of impurities in Clofarabine API, Clofarabine parenteral dosage form, and reconstitution studies.

KEYWORDS: Clofarabine, injection formulation, HPLC, QbD, Design space, related compounds, degradation impurities.

INTRODUCTION

A purine nucleoside metabolic inhibitor has been approved for the medication of children aged 1 - 21 years with refectory lymphoblastic leukaemia who have undergone at least two prior regimens of Clofarabine (Figure 1). Younger individuals (18-65 years old) with newly diagnosed myeloid leukemia who have a micro-complex karyotype react better to Clofarabine, which increases their chance of cure without relapse[1, 2].

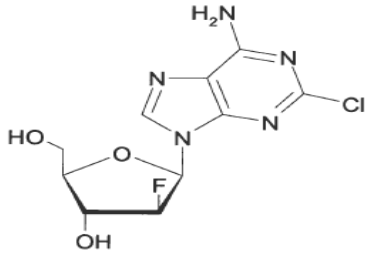


Figure 1. Chemical structure of Clofarabine

Clofarabine injectable concentrate and diluted infusion solutions kept at 25 degrees either 2-8 degrees for least 28 days are stable. Pharmacist-based centralized preparation benefits from physicochemical stability. It is suggested that the items be handled aseptically and kept refrigerated for microbiological reasons[3]. The RP-HPLC method was used to quantify clofarabine and its impurity compounds in the parenteral formulation [4] was published, the method is not suitable for measuring mono benzoate impurity, which is a specific process impurity in Clofarabine drug substance. Due to the impurity's non-polar nature, the run time is inadequate and unable to elute within the required run time. The publication of Clofarabine in the presence of degradation products and process-related impurities of validated reverse phase stability-indicating HPLC method [5], the method has not adequate for Clofarabine injection and reconstitution. Additionally, blank and standard chromatograms show many unknown peaks that interfere with the known impurities. The publication of an Ultra-Performance liquid chromatographic technique for the measurement of Clofarabine-related chemicals in an injectable formulation [6], the method has yet to be proved in reconstitution experiments or to report on the degradation products that form during the process. With HPLC, UV/PDA, UV/PDA detector and ULC with Supercritical fluid assay techniques for Clofarabine quantification was published [7-17], not suitable for the impurities quantification. In order to achieve the multidimensional robust design space, publications on DoE and QbD may be found on the internet [18-26].

Clofarabine's QbD principles of impurity method have not been demonstrated in any published literature. Additionally, the ICH guidelines Q8 (R1) explicitly defined QbD ideas as well as the QbD strategy, which must be applied as a regulatory obligation. In order to prevent findings that are out of trend or out of specification as a consequence of a technique failure in the quality control lab, developing analytical methods based on QbD is necessary procedure.

Using QbD approaches, we want to create an HPLC method that can quantify process-related and deprivation Clofarabine impurities, as well as impurities in the API, injectable dosage form, and reconstitution studies.

In "Quality by Design," the performance characteristics of products and processes are built in order to meet certain objectives, rather than being established by the results of test batches. "Quality by Design" (QbD) Method performance may be assessed using a scientific and risk-based multivariate methodology. DoE (Design of Experiment) is often used to find parameter ranges for instruments, to comprehend sample preparation changes and method precision variations such as MODR terminology for design space (method operable design range). Method performance criteria are aspects of the method's response that may be used in conjunction with the validation of the method. Quality-by-Design (QbD) focuses on developing methodologies that may be used throughout a product's lifecycle. Movements within the "Design Space" of a specified analytical procedure have been granted regulatory flexibility. Quality attributes predictable process inputs (Design space) minimise the failures of release and stability testing of the product.

EXPERIMENTAL

Materials and Methods

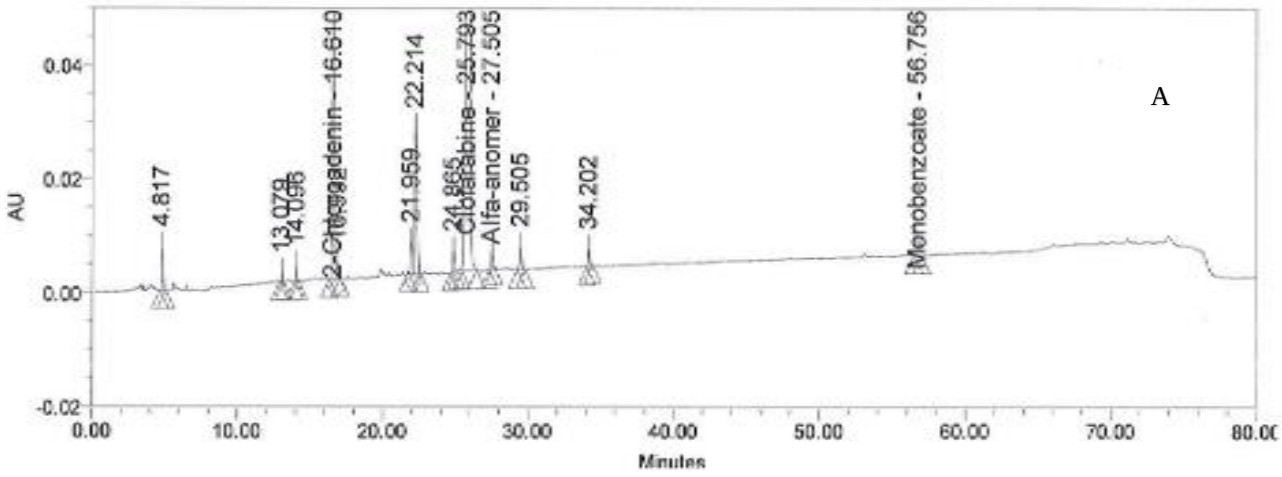
Chemicals and reagents: Manasa life sciences provided the Clofarabine reference standard, whereas Simson in Mumbai provided the Clofarabine impurities. Clofarabine injection (20mg/20mL) was purchased from the market. Analytical grade Potassium dihydrogen phosphate, Acetonitrile, 0.9 percent Sodium chloride, Water and Orthophosphoric acid were purchased from Merck.

Instrumentation and chromatographic conditions: The SHIMADZU i-Prominence HPLC system with auto sampler and UV detector was utilized for method development and validation. An RP-18, 250 x 4.6mm, 5 µm was used for the separations. Empower software was used to do the data analysis.
The chromatographic separation of the drug and its impurities were achieved using Symmetry RP-18, 250 x 4.6 mm, 5 microns. Mobile phase-Mobile phase-A consisted of 10 mM potassium phosphate buffer (adjusted pH to 2.5 with Orthophosphoric acid solution) and mobile phase-B consisted of a mixture of pH 2.5 phosphate buffer and Acetonitrile in the ratio of 30: 70 v/v with gradient method (T/ percent B) was finalised as 0.01/0, 55.0/60, 60.0/100, 70.0/100, 71.0/0, 80.0/0, flow rate of 1.0 mL/minute, and wavelength detection at 254 nm, keeping the column temperature at 25ºC, by using injection volume of 10 µL.

Experiment: The diluent is 0.9 percent sodium chloride. Clofarabine reference standard was used to prepare a 0.002 mg/mL standard solution in diluent. Test sample of API was prepared the concentration of 1 mg/mL in diluent. Test sample of Clofarabine injection 20 mg/20mL, as such solution was used. Clofarabine injection was used to reconstitute a solution for testing. After diluting 20 mg/20 mL with 0.9 percent Sodium Chloride Injection, USP and passing it through a sterile 0.2-micron syringe filter, the final concentration was between 0.15 mg/mL to 0.4 mg/mL. The diluent was used to make individual impurity solutions, which were then injected into the samples using suitable amounts of reference standard materials.

METHOD DEVELOPMENT

Optimisation of buffer pH in mobile phase: Prepared the buffer pH range from 2.0 to 3.0 at three levels pH 2.0, pH 2.5 and pH 3.0 by using 10 mM phosphate buffer and injected the Clofarabine injection composite degradation sample. One unknown impurity is co-eluted with 2-chloro adenine impurity in pH 2.0 phosphate buffer. All known and unknown impurities are well separated in pH 2.5 phosphate buffer. At pH 3.0, Clofarabine and Alfa-anomer co-elute. The chromatograms are shown in Figure 2.



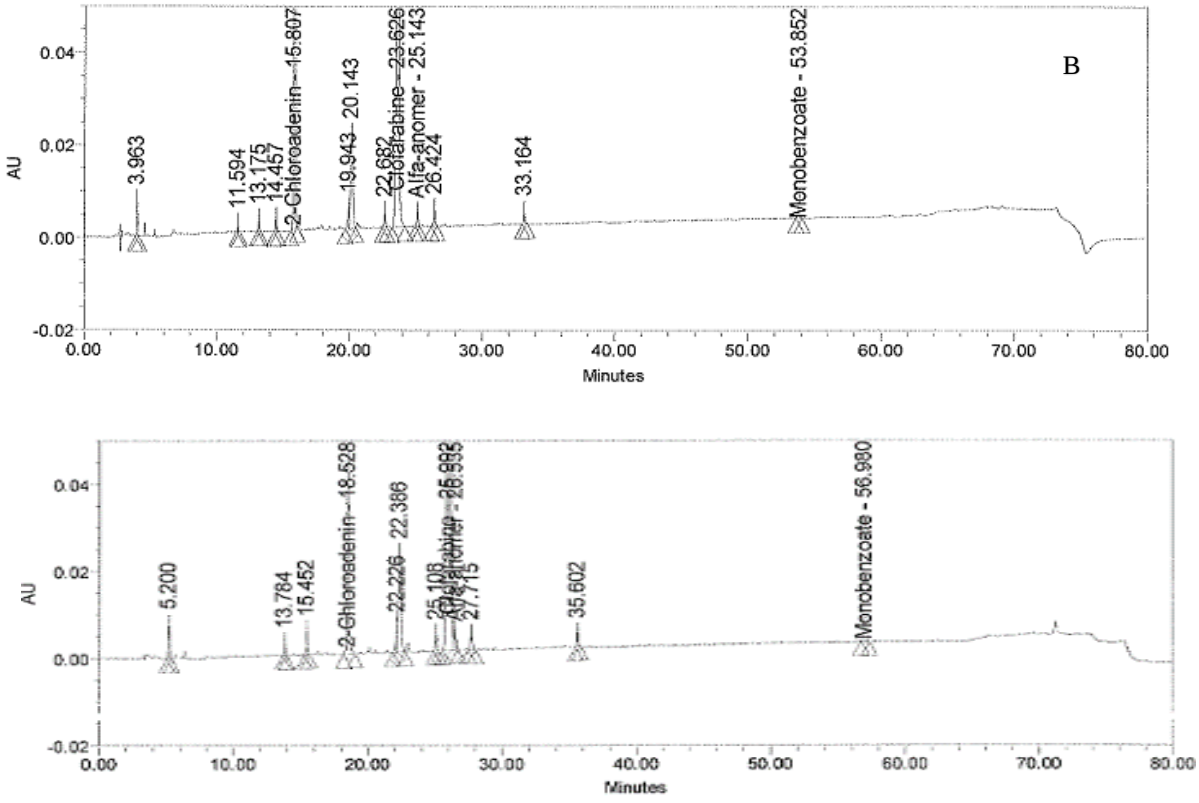


Figure 2. composite degradation sample chromatogram A) pH 2.0 phosphate buffer B) pH 2.5 phosphate buffer C) pH 3.0 phosphate buffer

Infer: Based on the above experiments, pH 2.5 potassium phosphate buffer is found suitable for separation of all impurities of Clofarabine in presence of formulation matrix and in mobile phase-B 70: 30 (Acetonitrile: pH 2.5 buffer) is optimized.

Selection of Diluent (Reference US Product information leaflet): Clofarabine injection formulation contains 0.9 percent sodium chloride. Hence 0.9 percent Sodium chloride was selected as diluent for Clofarabine API, Clofarabine injection and Reconstitution studies related compounds analysis. The response and peak shape of impurities and Clofarabine found satisfactory in this diluent. Hence the same diluent is recommended for API and finished analysis. The specificity of diluent and placebo was demonstrated for stability indicating nature of test method.

Optimization of Wavelength: The detector wavelength was optimized based on the overlay spectra of degradants and Clofarabine peaks. The overlay spectra showed that 254-nm wavelength was found suitable for detection of all impurities of Clofarabine found in stress sample spiked with known impurities. At this wave length the base line drift of the chromatogram was found very stable. The response of standard and impurity peaks across the UV region is observed. At 254-nm, both Clofarabine and the major impurities responses are found satisfactory. Hence the wavelength of 254-nm is suitable for estimation of impurities of Clofarabine. The overlay spectra of stress sample was shown in Figure 3.

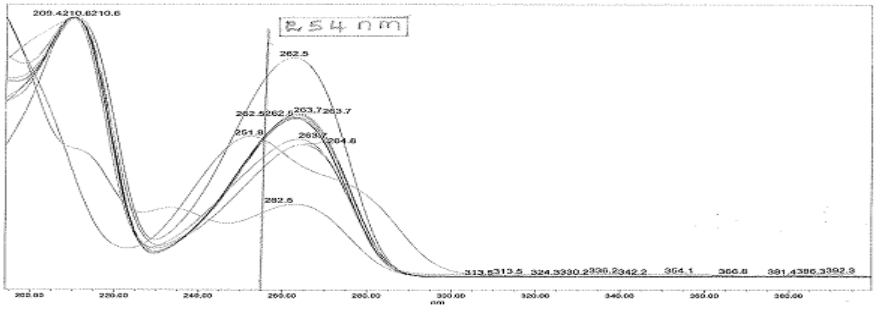


Figure 3. Overlay spectra of impurities and Clofarabine peaks in stress sample

Infer: In the above overlay spectra drawn a vertical line which all impurities are showing absorption maxima. Hence the wavelength 254-nm is optimized for estimation of impurities in Clofarabine API, Clofarabine Injection, and Clofarabine injection reconstitution study (dilution study).

Optimisation of sample concentration and injection volume: Using the above selected chromatographic conditions, sample concentration of 1000 ppm with an injection volume of 10µL is sufficient for detecting its related substances of Clofarabine at reporting threshold level 0.05% and at a lower level. Considering this sample concentration linearity of impurities and Clofarabine was established and data found satisfactory.

Forced degradation

Acid degradation sample preparation (0.1 HCl heat 2 hours at 90°C): The sample solution was heated for two hours at 90°C on a water bath with 0.1 N Hydrochloric acid solution added. kept at ambient temperature for 30 minutes on the counter and diluted to the required amount.

Sample preparation for base degradation (0.1 N NaOH at 90°C for 1 hour): Addition of Sodium Hydroxide Solution (0.1 N) to the sample solution was done out for 60 minutes at 90°C on water bath. Kept on the benchtop for 30 minutes to cool room temperature and made up to the volume with diluent.

Peroxide degradation (Oxidation) sample preparation (3% Peroxide heat 1 hour at 90°C): Added 1 mL of 3% peroxide solution to the sample solution and heated for 1 hour at 90°C on water bath. To make it the appropriate volume, it was reduced with diluent after 30 minutes on the benchtop to bring it to room temperature.

(105°C heat for 6 hours) Thermal degradation sample preparation: For six hours, the sample solution was heated to 105°C.

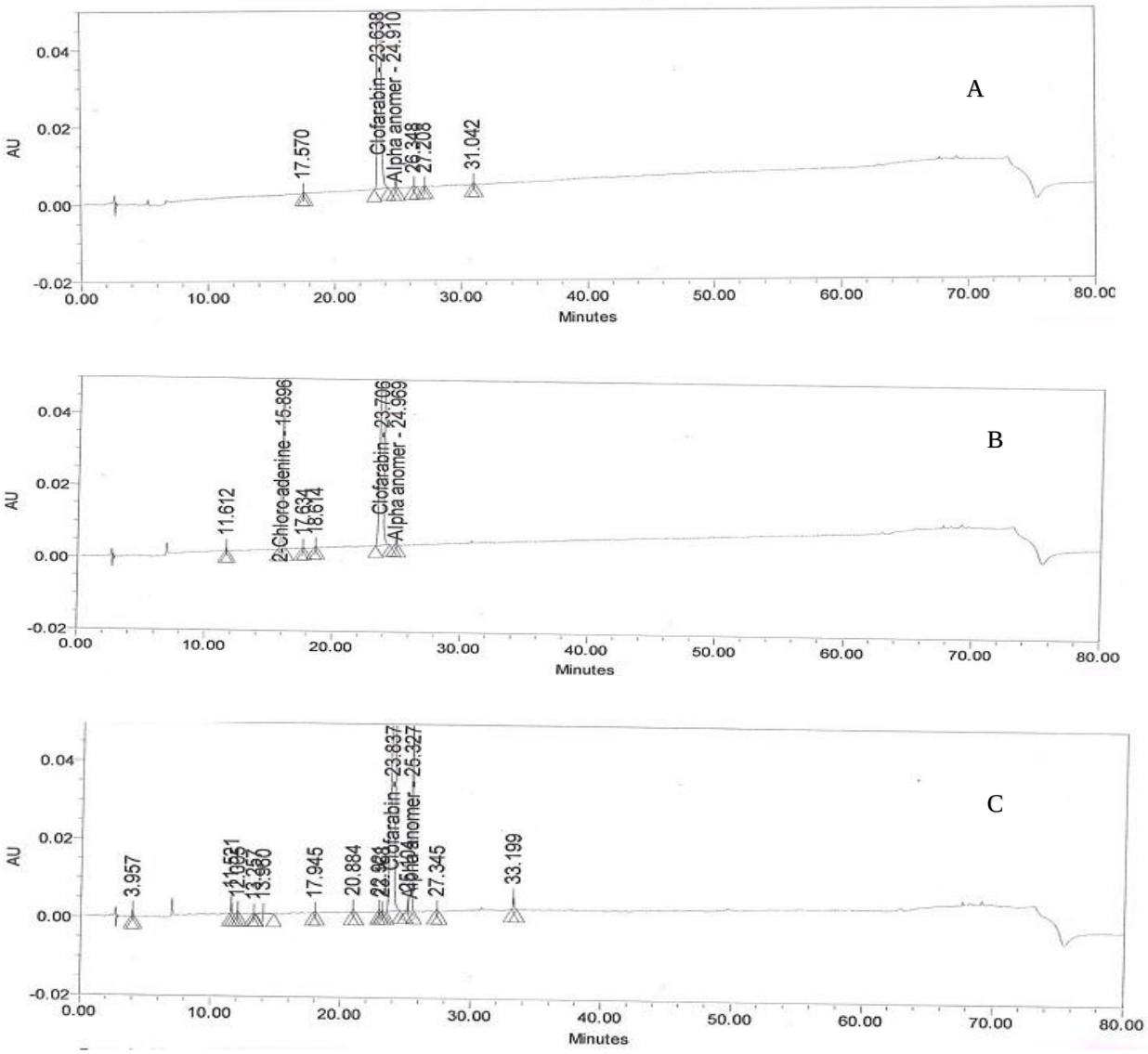
Composite degradation sample preparation: To construct the composite degradation sample, 5 mL of each of the acid degradation, base degradation, oxidation degradation, and heat degradation samples were added to a 20 mL volumetric flask. As illustrated in Figure 4, forced degradation chromatograms were obtained from the analysis of acidic, basic, peroxided, and thermally degraded samples and results were summarised in Table 1.

Table - 1. % Total impurities, Mas balance, and peak purity

Name of impurity	RRT	Un stressed	Acid	Base	Peroxide	Thermal

2-Chloro Adenine	0.668	ND	1.2	ND	1.02	0.1
Alpha Anomer	1.054	0.06	0.07	1.96	0.11	0.07
Mono benzoate	2.281	ND	ND	ND	ND	ND
Individual max impurity	0.464	0.03 (RRT 1.404)	0.06 (RRT 0.76)	0.15 (RRT 0.76)	1.83 (RRT 0.93)	0.4 (RRT 0.58)
Total impurities		0.11	1.33	2.39	5.72	0.9012
% assay		99.3	102.7	95.1	91.3	95.7
Mass balance		99.4	104.0	97.5	97.0	96.6
Purity angle		0.141	0.065	0.069	0.067	0.078
Purity threshold		1.031	1.038	1.043	1.046	1.040
Purity flag		No	No	No	No	No

ND-Not detected



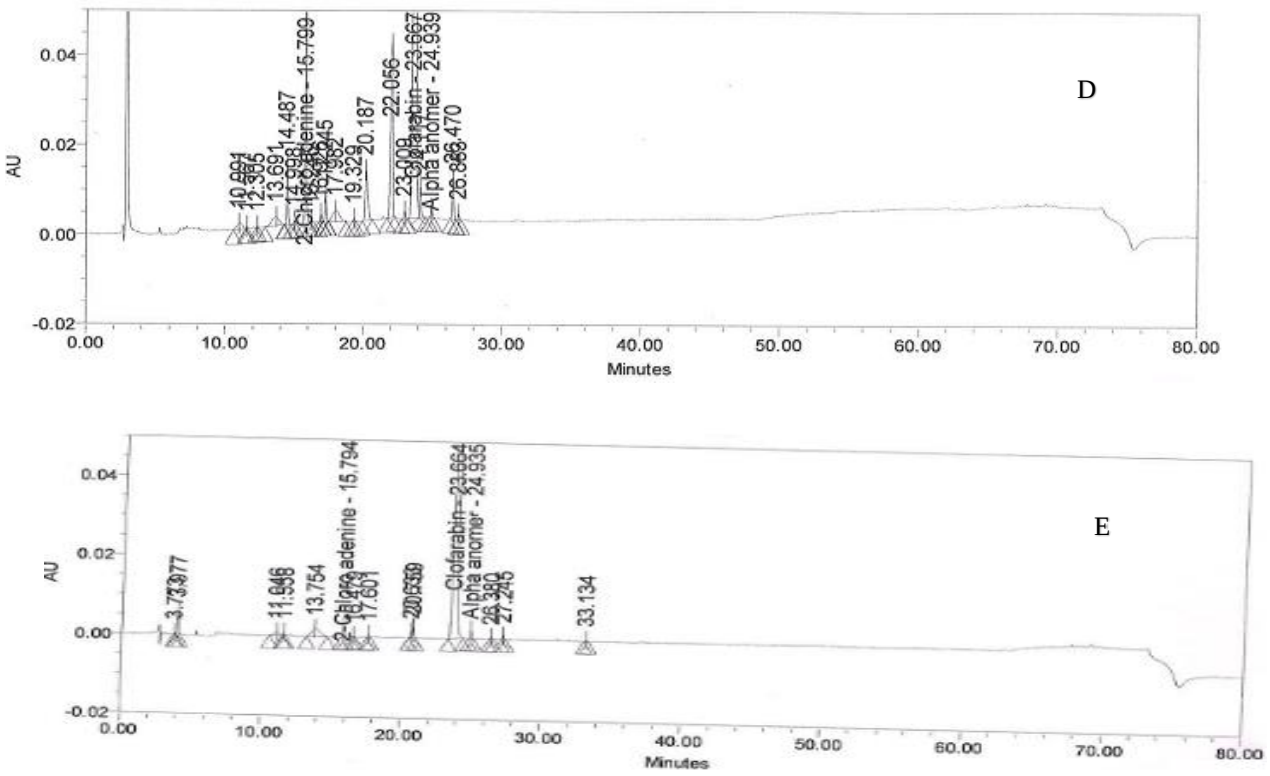


Figure 4. Forced degradation chromatograms: A) Un stressed sample, B) Acid stressed sample, C) Base stressed sample D) Peroxide stressed sample E) Thermal stressed sample

DESIGN of EXPERIMENTS by full factorial design:

For pH, buffer strength in the mobile phase, and flow rate, design expert software was used to do a simultaneous multivariate model. After conducting a two-level fractional factorial design (2^{4-1} + 4 centre points = 12 experiments), the experimental data was analyzed for statistically significant. Responses were tabulated in Table 2.

Total number of experiments: 2^3 (Factorial) + 4 (Center points)

Table - 2. Following lower & higher limits taken for getting the final overlay lots

S. No.	Response	Lower limit	Higher limit
1	Res-1 (Clofarabine to Alpha anomer)	3	4
2	Res-2 (RRT 0.612 to 2-Chloro Adenine)	3	4
3	Res-3 (Alpha anomer to RRT 1.118)	3	4

DOE Results

Experiments were conducted with composite degradation and impurities spiked Clofarabine injection sample in above mentioned factorial design and outcome results were shown in Table 3.

Table - 3. Below results were observed factor vs responses

Std	Run	Factor	Factor	Factor 3	Response 1	Response 2	Response 3

		1A: Flow	2B: pH	C: Buffer strength	Resolution of Clofarabine & Alpha anomer	Resolution of Unknown & 2-Chloro adenine	Resolution of Alpha anomer & unknown
1	1	0.80	2.00	5.00	8.59	1.48	9.59
8	2	1.20	3.00	15.00	2.54	0	4.95
2	3	1.20	2.00	5.00	6.45	2.94	11.31
5	4	0.80	2.00	15.00	7.89	3.55	9.33
12	5	1.00	2.50	10.00	7.8	7.85	6.18
3	6	0.80	3.00	5.00	6.79	9.19	1.26
6	7	1.20	2.00	15.00	5.98	4.72	11.15
7	8	0.80	3.00	15.00	2.78	1.27	4.27
9	9	1.00	2.50	10.00	7.42	7.58	6.24
10	10	1.00	2.50	10.00	7.48	8.25	6.05
11	11	1.00	2.50	10.00	7.46	8.4	6.13
4	12	1.20	3.00	5.00	5.33	9.24	0

Based on the above experiments, Design expert software was given the desirability graph of Design space of the method shown in Figure 5.

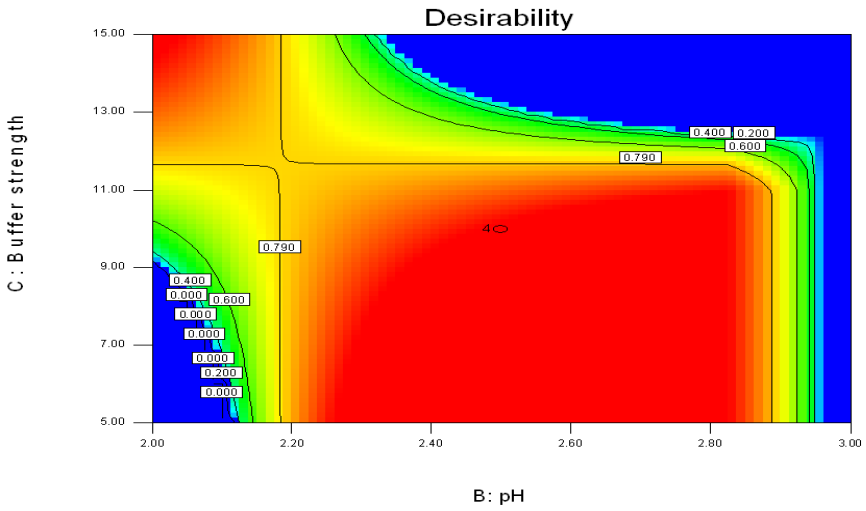


Figure 5. Design space graph Design expert software

Interpretation

Effect of pH: From the above graph, it can be observed that pH effects on the resolution of impurities. Intermediate pH's are found to be more favourable for obtaining satisfactory resolution.

Effect of Buffer strength: From the above graph it can be concluded that higher intermediate buffer concentration results at intermediate pH are found to be favourable for obtaining satisfactory resolution**.**

Effect of Flow: From the above graph it can be concluded that Flow is not affecting the resolution

Allowable variations:

Based on the above **design space** diagram the following are the allowable variations

Potassium dihydrogen phosphate strength is allowed from 5mM to 12mM

The Mobile phase flow is allowed from 0.8mL to 1.2mL

pH ranges from 2.2 to 2.8 for the buffer in the mobile phase.

METHOD VALIDATION

According to ICH guidelines, the procedure was validated. Specificity, linearity, precision, accuracy, limit of detection (LOD), and limit of quantification (LOQ), were all investigated and found to be adequate.

a) System suitability

A diluted standard solution was prepared and injected six times into the chromatographic apparatus as part of the analytical test procedure. Figure 6 illustrates the chromatograms of the system suitability parameters, which were observed to be in the appropriate ranges.

Clofarabine diluted standard was eluted at retention time of 23.36, tailing factor was observed 1.1, and related standard deviation of six consecutive injections for diluted standard was found 0.7%.

Acceptance criteria:

The area six replicate injections of Clofarabine peak in diluted standard are not more than 5.0 percent apart in terms of RSD and not more than a 2.0 tailing factor while performing the diluted standard preparation.

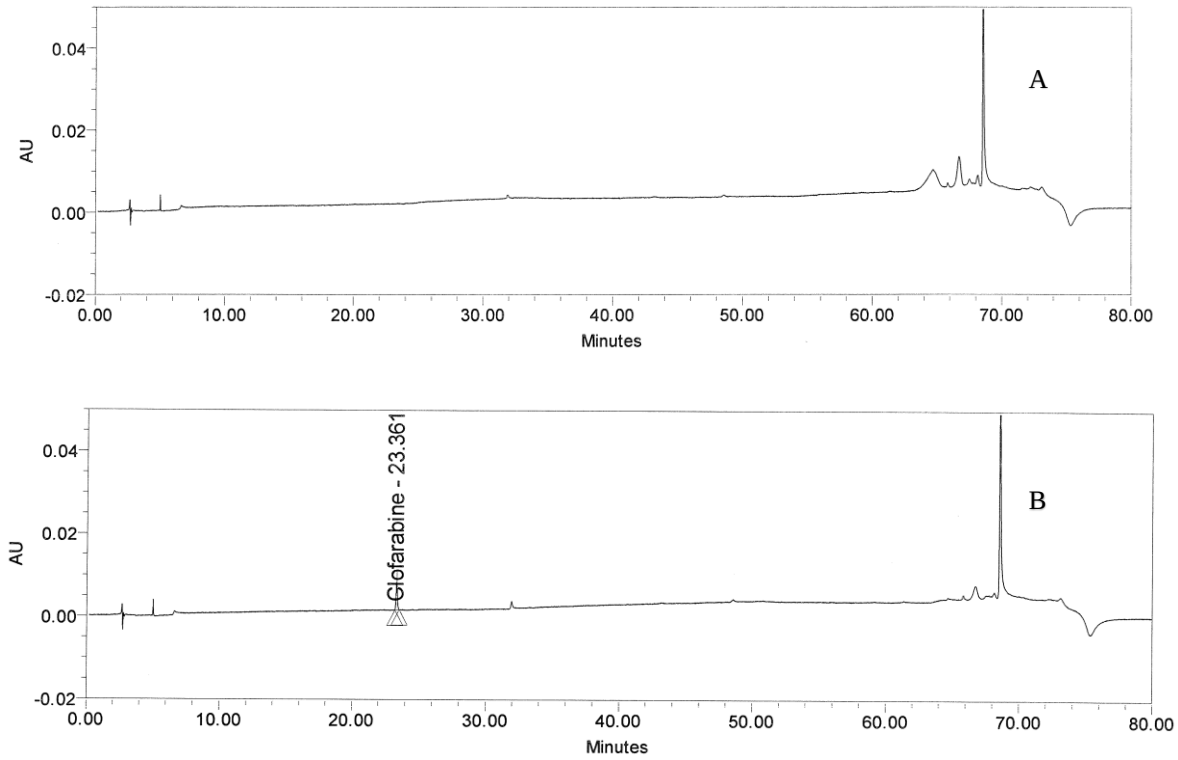


Figure 6. A) Blank chromatogram B) diluted standard chromatogram

b) Specificity

The specificity was demonstrated by injecting the blank solution, Clofarabine standard solution, placebo solution, individual impurity solution of Clofarabine impurity solutions and chromatograms were checked for the interference. As shown in Figure 7, there was no interference in the analysis of the chromatograms.

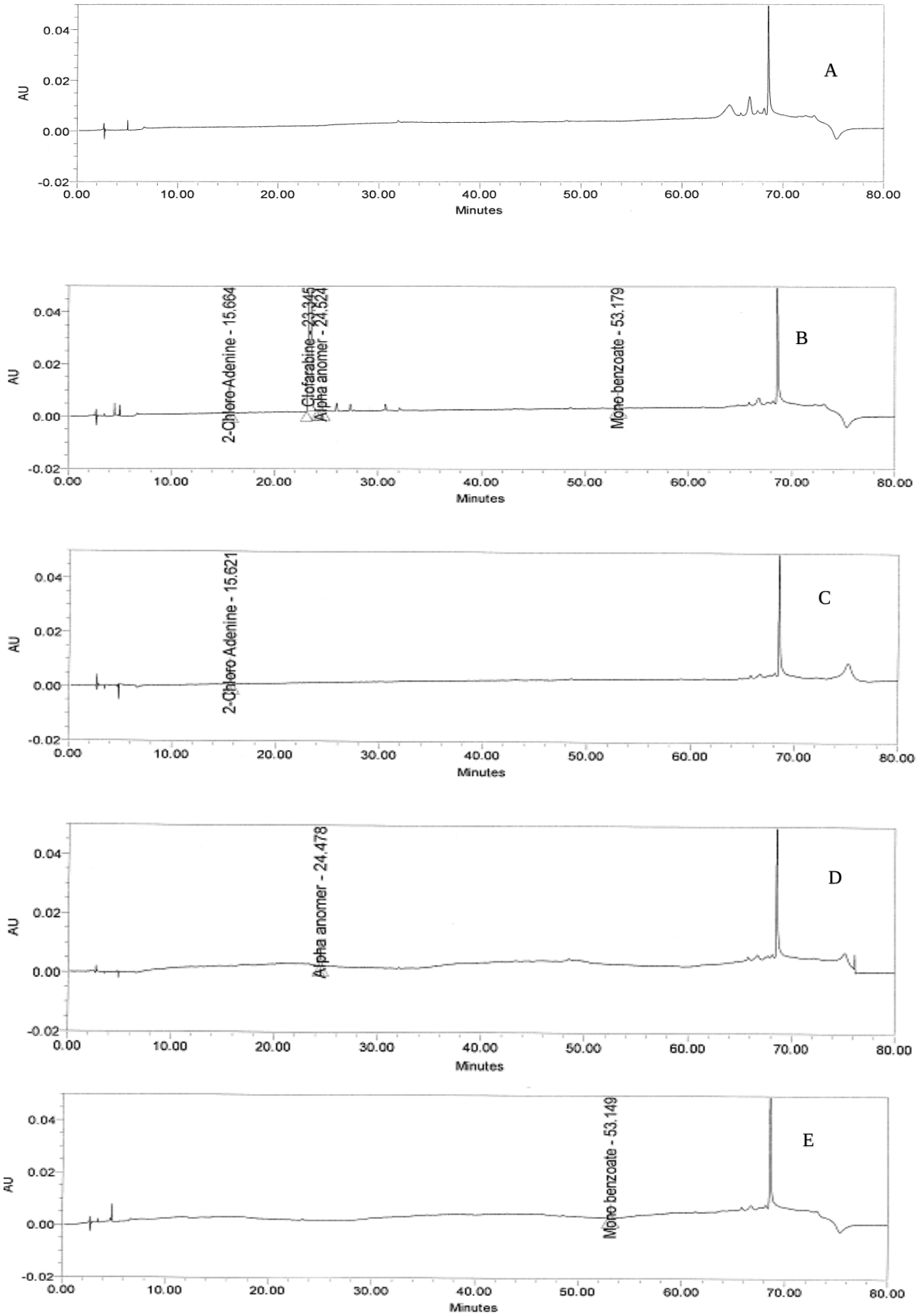

A
AU
Minutes
B
2-Chloro Adenine - 15.664
Clofarabine - 23.345
Alpha anomer - 24.524
Mono benzoate - 53.179
C
2-Chloro Adenine - 15.621
D
Alpha anomer - 24.478
E
Mono benzoate - 53.149

Figure 7. Specificity chromatograms: A) Placebo B) Spiked sample C) 2-Chloro adenine D) Alfa anomer E) Mono benzoate.

c) Linearity

Using a correlation coefficient and bias at 100% response, the linearity of Clofarabine, 2-Chloro Adenine, Alpha Anomer and Clofarabine Mono benzoate was assessed by plotting concentration versus area. Clofarabine, 2-Chloro adenine, Alpha anomer, and Clofarabine Mono benzoate solutions were produced and injected into the system in concentrations ranging from approximately Limit of Quantification level to 150 percent level of target concentration (0.20 %). As shown in Table 4, the detector response was determined to be within the appropriate range.

Table - 4. Results of Linearity study

Compound Name	Linearity (µg)	Slope	Intercept	Correlation coefficient
Clofarabine	0.213 - 3.21	252030	984	0.9999
2-Chloro adenine	0.221 – 3.22	376560	2179	0.9998
Alpha anomer	0.209 – 3.16	151063	281	0.9998
Monobenzoate	0.264 – 3.28	185909	145	0.9997

d) Precision

Clofarabine Mono benzoate, Alpha anomer, and 2-Chloro adenine were injected at 0.20 percent in the test preparation of the target concentration (1000 ppm) and analyzed as per analytical test technique to evaluate the test method's precision. Table 5 shows that the relative standard deviation was within the appropriate range.

Table 5. Results of Precision study

S. No	2-Chloro adenine	Alpha anomer	Monobenzoate
1	0.21	0.20	0.21
2	0.20	0.20	0.22
3	0.22	0.21	0.20
4	0.21	0.20	0.22
5	0.21	0.22	0.20
6	0.22	0.20	0.19
Mean	0.21	0.21	0.21
SD	0.008	0.008	0.012
%RSD	3.56	4.08	5.86

e) Limit of detection (LOD) and Limit of quantification (LOQ)

Clofarabine, 2-Chloro adenine, Alpha anomer, and Clofarabine Mono benzoate were all tested to check their limit of detection and limit of quantification. On the basis of signal-to-noise ratio, a detection limit and a quantification limit were set. Analytical testing methods called for the production and injection into the chromatographic system of a series of solutions containing Clofarabine and its contaminants. Each impurity's detection limit was established by measuring the signal-to-noise ratio (SNR) at a concentration of around three and quantification limit was established signal-to-noise ratio around 10 was found by selecting the concentration that gave the best results. Table 6 shown that results of limit of detection and limit of quantification.

Table 6. Limit of detection and Limit of quantification data

Compound name	Limit of detection			Limit of quantification		
	ppm	percentage	S/N ratio	ppm	percentage	S/N ratio
Clofarabine	0.07	0.007	3.1	0.213	0.021	10.7
2-Chloro adenine	0.07	0.007	3.4	0.221	0.022	10.2
Alpha anomer	0.06	0.006	3.1	0.209	0.021	10.2
Monobenzoate	0.08	0.008	3.6	0.264	0.026	10.5

f) Accuracy

Specimens were taken in triplicate by spiking 2-Chloro adenine, Alpha anomer, and Clofarabine Mono benzoate in test preparation, with the limit of quantification set at 150 percent of the sample target concentration (0.20 %) (1000ppm). The average percent recoveries of 2-Chloro adenine, Alpha anomer, and Clofarabine Mono benzoate to 150 percent of specification (0.20 percent) were found to be within the limits, as shown in Table 7.

Table 7. Results of Accuracy study

Level	2-Chloro adenine		Alpha anomer		Monobenzoate	
	µg added	µg found	µg added	µg found	µg added	µg found
LOQ	0.212	0.209	0.221	0.218	0.263	0.253
		0.201		0.216		0.249
		0.210		0.213		0.246
50 %	1.083	1.076	1.126	1.098	1.341	1.261
		1.073		1.086		1.275
		1.079		1.089		1.283
100 %	2.132	2.076	2.262	2.239	2.643	2.564
		2.096		2.243		2.593
		2.069		2.234		2.547
150 %	3.196	3.182	3.391	3.296	3.963	3.820
		3.169		3.249		3.761
		3.172		3.267		3.821
Level	Individual	Average	Individual	Average	Individual	Average
LOQ	98.6	97.5	98.6	97.6	96.2	94.8
	94.8		97.7		94.7	
	99.1		96.4		93.5	
50 %	99.4	99.4	97.5	96.9	94.0	94.9
	99.1		96.4		95.1	
	99.6		96.7		95.7	
100 %	97.4	97.6	99.0	99.0	97.0	97.2
	98.3		99.2		98.1	
	97.0		98.8		96.4	
150 %	99.6	99.3	97.2	96.5	96.4	95.9
	99.2		95.8		94.9	
	99.2		96.3		96.4	

g) Ruggedness

System to system/Column to column/ analyst to analyst/Day to Day variability study was conducted on six samples of Clofarabine Injection were prepared by spiking 2-Chloro adenine, Alpha anomer and Clofarabine Mono benzoate at specification limit (0.20%) in test solution of sample target concentration (1000ppm) and analyzed as per the analytical test method. There were no issues with the system suitability parameters for both the systems and columns on various days. Clofarabine Monobenzoate and 2-Chloro adenine percent relative standard deviations were found to be within the limits in table 8.

Table 8. Comparison table results of 2-Chloro adenine, Alpha anomer and Clofarabine Mono benzoate between two analysts

Sample	2-Chloro adenine		Alpha anomer		Mono benzoate	
	*Analyst-1	Analyst-2	*Analyst-1	Analyst-2	*Analyst-1	Analyst-2
01	0.21	0.22	0.20	0.24	0.21	0.19
02	0.20	0.23	0.20	0.21	0.22	0.18
03	0.22	0.20	0.21	0.22	0.20	0.21
04	0.21	0.21	0.20	0.21	0.22	0.20
05	0.21	0.20	0.22	0.20	0.20	0.19
06	0.22	0.22	0.20	0.20	0.19	0.21
Average	0.21	0.21	0.21	0.21	0.21	0.20
%RSD	3.56	5.68	4.08	7.06	5.86	6.16

*****For Analyst-I, System-1 and Column-I and day -1 refer to results from precision of test method
%RSD-Percentage Relative standard deviation

h) Robustness

The effect of mobile phase change was tested for robustness. In mobile phase-B, organic phase composition, column oven temperature, and buffer pH each play a role. To prepare the diluted standard and test solution, the analytical test methodology requires that 2-Chloro adenine be surged with Alpha anomer and Clofarabine Mono benzoate at a concentration of 0.20 percent. For the preparation of the standard and test solution, a flow rate of 0.8 mL/min was used for the standard and 1.2 mL/min was used for the test solution at a column temperature of 20°C and 30°C, respectively, with an organic phase composition of 10% in mobile phase B (Acetonitrile) and a buffer pH change of 2%. Evaluations were carried out on system suitability parameters and findings were summarized in Table 9.

Table 9. System suitability results of Robustness

System suitability	As such	Flow rate (mL/min)		Column oven temperature		Organic composition in MP-B		pH of Buffer	
		0.8 mL	1.2 mL	20°C	30°C	-10%	+10%	pH 2.0	pH 3.0
% RSD of Clofarabine peak	0.2	0.7	0.2	0.3	0.7	1.1	1.1	0.8	0.7
Tailing factor	1.1	1.1	1.1	1.1	1.1	0.2	0.2	1.0	1.0
RRT of 2-Cl adenine	0.670	0.694	0.651	0.669	0.664	0.659	0.681	0.597	0.688
RRT of Alpha anomer	1.054	1.054	1.055	1.055	1.057	1.056	1.061	1.052	1.053

RRT of Monobenzoate	2.273	2.195	2.351	2.278	2.307	2.269	2.274	2.290	2.263

RRT: relative retention time, RSD: Relative standard deviation

RESULTS:

Clofarabine and 2-Chloroadenine, Alf-anomer, and monobenzoate were successfully separated with well resolution. According to the rules established by the international conference on harmonisation, the procedure was considered to be accurate. It was discovered throughout the testing process that the procedure is accurate, linear and specific, and reliable.

DISCUSSION:

Clofarabine API, Clofarabine injection, and Clofarabine injection reconstitution, related compound analysis was established using a precise, accurate, and robust method. At present, only Related compounds method Clofarabine API and Clofarabine injection related compounds method were reported. No impurities method was reported by QbD approach for Clofarabine API, Clofarabine injection, and Clofarabine injection reconstitution study impurities. The created technique met all of the ICH validation parameters. From the LOQ level to 150 percent of impurity specification limits, the approach proved appropriate for all impurities.

CONCLUSION:

Successful separation and determination of Clofarabine and its impurities were achieved by the proposed method. Analysis of Clofarabine and its impurities in the API, the injection, and the reconstitution studies may be performed using this approach.

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