ORIGINAL RESEARCH PAPER

Chemistry

THE ANALYTICAL PROCEDURE FOR DETERMINATION OF ASSAY IN FINISHED PRODUCT OF CLOFARABINE INJECTION, 1MG/ML IS AN IN-HOUSE PROCEDURE.

KEY WORDS: Clofarabine, HPLC, Method Development, Validation, ICH guidelines

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ABSTRACT

A new and stability-indicating High performance liquid chromatographymethod was developed and validated for simultaneous determination of clofarabine impurities in Injectionformulation.The Chromatographic system consisted of a Shimadzu Class VP Binary pump LC-10ATvp, SIL-10ADvp Auto sampler, CTO-10Avp Column Temperature Oven, SPD-10Avp UV-Visible Detector.The method was validated as per the ICH guidelines Apart from these Chromatographic parameters likeresolution, capacity factor, separation factor, column efficiency and peakasymmetry should also be the ideal for estimation.

INTRODUCTION

Clofarabine is a next generation deoxyadenosine analogue which is used for the treatment of pediatricleukemia. The mechanism of its anti-cancer activity involves the combination of direct inhibition of DNA synthesis and ribonucleotidereductase and induction apoptosis. This drug is effective against various sub types of leukemia and solid tumors. The Chemical name of Clofarabineis (2R, 3R, 4S, 5R)-5-(6-amino-2-chloro-9H-purin-9yl)-4-fluoro-2- (hydroxy methyl) oxolan-3-ol and chemical formula is $C_{10}H_{11}$ ClFN$_5$O$_3$ and molecular weight is 303.68 g/mol.

For many years, the Southern Research Institute has had a programme, supported by the US National Cancer Institute, searching for new nucleoside anticancer drugs. In the early 1980s, two adenine-containing nucleosides, now known as fludarabine (Fludara; Berlex Oncology) and cladribine (Leustatin; Ortho Biotech) were in clinical trials. At the time, it was not clear whether either drug would gain approval by the FDA because some concerns were raised during preclinical and clinical development of these agents. Both drugs were susceptible to glycosidic bond cleavage with fludarabine subject to some phosphorylase cleavage and cladribine subject to both hydrolytic and enzymatic cleavage[1]. In the case of fludarabine, this cleavage resulted in the formation of 2-fluoroadenine, which is converted to the highly toxic 2-fluoro-adenosine triphosphate[2].

Over several years, a number of experiments on mouse tumours, as well as human tumour xenografts, were examined, and it was determined that the 2-chloro, 2-fluoro and 2-bromo analogues all had some activity, but that the 2-chloro analogue had the best activity among the three compounds[3-5]. Clofarabine is a slightly lipophilic prodrug (mLog P = 0.5) that gains entry into cells by facilitative and active nucleoside transporter mechanisms and at higher concentrations and longer exposure times, by passive diffusion across lipid membranes[6].

As expected from its potent inhibition of DNA synthesis, clofarabine demonstrated strong in vitro growth inhibition and cytotoxic activity (IC50 values = 0.028–0.29 μM) in a wide variety of leukaemia and solid tumour cell lines[7]. The anticancer activity of clofarabine was dose- and schedule-dependent, and greater antitumour activity was associated with more frequent administration[8].

Clofarabine administered intraperitoneally had significant activity against a wide variety of human tumourxenografts implanted subcutaneously in athymic nude or severe combined immune deficiency mice [9]. Moderate to excellent sensitivity to tumour growth delays were seen in all eight human colon tumours, three out of four human renal tumours, all four non-small-cell lung tumours, and all three prostatetumours. This spectrum of widespread anticancer activity has been confirmed by other investigators in human tumourxenograft models in mice [10].The anticancer activity of clofarabine was dose- and schedule-dependent, and greater antitumour activity was associated with more frequent administration [11].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 ribonucleotidereductase and DNA polymerase, resulting in inhibition of DNA repair and synthesis of DNA and RNA[12-14].

The aim of the method is to develop an analytical procedure for the determination of Clofarabine in Pharmaceutical Formulations.The analytical procedure for determination of Assay in finished product of Clofarabine Injection, 1mg/mL is an In-House procedure.

The method shall be validated for the following parameters:

A) Accuracy
B) Stability of Analyte in solution
C) Filter compatibility.
D) System Suitability of overall validation study.

Experimental:

Instrumentation, Chromatographic Conditions & Method:

The Chromatographic system consisted of a Shimadzu Class VP Binary pump LC-10ATvp, SIL-10ADvp Auto sampler, CTO-10Avp Column Temperature Oven, SPD-10Avp UV-Visible Detector. All the components of the system are controlled using SCL-10Avp System Controller. Data acquisition was done using LC Solutions software.

The mobile phase consisted of 85:15 (v/v) of buffer solution and acetonitrileoperated on isocratic mode. The flow rate is 1.0 ml/min. Chromatographic Estimation of Clofarabinewas performed onInertsil ODS-2 (150 x 4.6) mm, 5µm column. The wavelength of detection is 263 nm. The injection volume is 25µL.

Chromatographic conditions

A High Performance liquid chromatography equipped with UV detector and an auto sampler or its

Column	:	Inertsil ODS-2 (150 x 4.6) mm, 5µm
Detection wavelength	:	263 nm
Flow rate	:	1.0 mL / min
Injection volume	:	25µL
Run time	:	10 min
Column temperature	:	40°C
Sample cooling rack	:	25°C

5.2.6. Calculation

$$\%\ Assay = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{V} \times \frac{P}{100} \times \frac{100}{LA}$$

Where,

AT : Average peak area of Clofarabine obtained from chromatogram of Sample preparation

AS : Average peak area of Clofarabine obtained from chromatogram of Standard preparation

WS : Weight of Clofarabine reference / working standard in mg

DS : Dilution of standard preparation

DT : Dilution of sample preparation

P : Potency of Clofarabine reference / working standard on as is basis

V : Volume of sample taken

LA : Label amount of Clofarabine in mg/mL.

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value that is accepted either as a conventional true value or an accepted reference value and the value found.

To demonstrate the accuracy of assay test method, drug substance is spiked quantitatively in to placebo from 50% to 150% of working concentration of test concentration at each level with triplicate preparation and analyzed using the test method. The result for Clofarabine is tabulated in the below table. Typical chromatogram of Accuracy at 100 % level for is exhibited below.

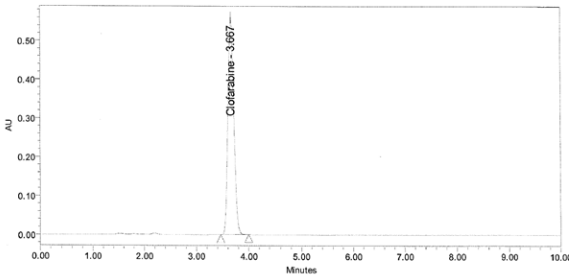
Results of Accuracy for Clofarabine

Accuracy Level	Sample #	% Recovery	Average % Recovery	% RSD
50 %	1	99.9	100.1	0.2
	2	100.2		
	3	100.1		
100 %	1	100.0	100.0	0.1
	2	100.0		
	3	99.9		
150 %	1	98.8	98.8	0.1
	2	98.9		
	3	98.8		
Overall % Recovery				99.6
Overall % RSD				0.61

Chromatogram of Accuracy at 100% Level

Acceptance Criteria:

- Recovery at each level and overall average recovery of assay results should be between 95.0% and 105.0%
- The RSD at each level and overall RSD of % recovery should not be more than 3.0%



Peak Results

	Name	RT	Area	% Area	Height (µV)	USP Plate Count	USP Tailing	Int Type
1	Clofarabine	3.667	4349127	100.00	559586	4913	1.1	BB

CONCLUSION:

The results are well within the acceptance criteria; hence the method is accurate for its intended use.

Stability of Analyte in Solution

Stability of analyte in solution is evaluated for the standard and sample solutions. The standard and sample solutions are prepared and analyzed as per the analytical procedure. A portion of these solutions were preserved at room temperature and analyzed at different time intervals from the time of preparations. The results are calculated from initial versus over a period of time. The results are summarized in the below Table A and Table B

Table A Stability of Standard Solution

Time Interval	Peak area of Clofarabine		% Difference	
	RT	2-8°C	RT	2-8°C
Initial	4366049		-	
24 hours	4350072	4355612	0.37	0.24
48 hours	4349877	4347580	0.37	0.42

Table B. Stability of Sample Solution

Time Interval	Peak area of Clofarabine		% Difference	
	RT	2-8°C	RT	2-8°C
Initial	4419004		-	
24 hours	4408166	4402274	0.25	0.38
48 hours	4405770	4416374	0.30	0.06

Acceptance Criteria:

- % Difference of Clofarabinepeak area obtained from standard solution at each time point should not be more than 2.0 from the initial area.
- % Difference of Clofarabine peak area obtained from sample solution at each time point should not be more than 2.0 from the initial area.

CONCLUSION:

The data indicates that the, Standard solution is stable up to 48 hours and sample solution is stable up to 48 hours at room temperature and 2-8°C for Clofarabine peak.

5.3.9. System suitability of overall validation study

The System suitability is an integral part of analytical procedure. The tests are based on the concept that the equipment, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. The system suitability results are tabulated in the below Table.

System Suitability of Overall Validation Study

Parameter	% RSD	Tailing Factor	Theoretical plates
System Suitability/ System Precision	0.02	1.1	4943
Specificity by diluent, placebo and known impurities	0.02	1.1	4943

Specificity by Forced degradation (Acid, Alkali, Thermal, Peroxide)	0.02	1.1	4983
Specificity by Forced degradation (Alkali)	0.03	1.1	5047
Specificity by Forced degradation (UV)	0.02	1.1	4983
Linearity	0.02	1.1	4943
Method Precision	0.02	1.1	4943
Intermediate Precision	0.04	1.1	6303
Accuracy (Recovery)	0.02	1.1	4943
Robustness-Flow rate: 0.8mL/minute	0.05	1.1	6243
Robustness-Flow rate: 1.2mL/minute	0.02	1.0	3940
Robustness-Column oven temperature: 38°C	0.03	1.1	4914
Robustness-Column oven temperature: 42°C	0.08	1.1	4875
Robustness-Low organic composition(142.5 mL)	0.02	1.1	5489
Robustness-High organic composition(157.5 mL)	0.04	1.1	4545
Stability of Analyte in Solution (Initial)	0.02	1.1	4943
Stability of Analyte in Solution (24 Hours)	0.02	1.1	4943
Stability of Analyte in Solution (48 Hours)	0.02	1.1	5489
Minimum	0.02	1.0	3940
Maximum	0.08	1.1	6243
Average	0.03	1.1	5078

Acceptance Criteria:
System suitability criteria should meet during overall validation studies, otherwise needs to be justified. Report minimum, maximum and average values of system suitability parameters.

The Tailing factor for Clofarabine should be NMT 2.0.
The relative standard deviation for Clofarabine peak from five replicate injections of standard solution should be NMT 2.0 %.
The theoretical plates for Clofarabine peak in standard solution should be not less than 3000.

The Cumulative relative standard deviation for Clofarabine peak from five replicate injections of standard solution and bracketing standard should be not more than 2.0%.

CONCLUSION:
The results for system suitability are well within the acceptance criteria; hence the given chromatography system is acceptable for its intended use.

Overall Summary of Validation Results

Validation Parameters	Acceptance Criteria	Results	
Precision	**1.1 System precision** The RSD of results obtained from six standard NMT 2.0%	**Component name**	**% RSD**
		Clofarabine	0.02%
	1.2 Method Precision The relative standard deviation results obtained from six sample preparations should not be more than 2.0%	**Component name**	**% RSD**
		Clofarabine	0.08%

Validation Parameters	Acceptance Criteria	Results	
	2.1 No interference from diluent, placebo and known impurities No Interference should be observed at the retention time of Clofarabine peak in chromatograms obtained from the diluent, placebo and the impurities	There is no interference is observed at the retention time of Clofarabine peak in the chromatogram obtained from the diluent, placebo and known impurities.	
	2.2 Forced degradation study a. Calculate the % degradation against as such test preparation for each condition in any one of condition degradation should be achieved between 5.0% to 20.0%. b. For each degradation sample, purity angle should less than the purity threshold for Clofarabine peak.	Drug Product (FP)	
		As Such (Unstressed)	0.0
		Acid degradation	-1.2
		Alkali degradation	8.0
		Peroxide degradation	-1.5
		UV degradation	-0.3
		Thermal degradation	-0.5

Validation Parameters	**Acceptance Criteria**	**Results**	
linearity	a. Correlation coefficient should not be less than 0.999 b. Report the slope of regression line c. Report the Y-intercept of regression line d. Y-intercept at 100% level should be between ± 5.0%	**Clofarabine**	
		Correlation coefficient	1.000
		slope of regression line	72366.0
		Y-intercept of regression line	26262.5
		Y-intercept bias at 100% level	0.6

Validation Parameters	Acceptance Criteria	Results
Intermediate Precision	The cumulative %RSD of method precision and intermediate precision results obtained from twelve sample preparations should not be more than 2.0%.	0.42%

Validation Parameters	**Acceptance Criteria**	**Results**		
Accuracy	% Recovery at each level and overall % recovery should be between 95.0% and 105.0% for Clofarabine. The %RSD at each level and overall %RSD of %recovery should not be more than 3.0%.	**Accuracy Level**	**Average % Recovery**	**% RSD**
		50 %	100.1	0.2
		100 %	100.0	0.1
		150 %	98.8	0.1
		Overall % Recovery	99.6	
		Overall % RSD	0.6	

Validation Parameters	Acceptance Criteria	Results			
Robustness	System suitability criteria defined in test procedure should meet in each condition. 1. The Tailing factor for Clofarabine	Condition	Clofarabine		
			% RSD	Tailing factor	Theoretical plates
		Flow rate:0.8 mL/min	0.05	1.1	6243

	should be NMT 2.0 2. The relative standard deviation for Clofarabine peak from five replicate injections of standard solution should be NMT 2.0 %. 3. The Theoretical plates for Clofarabine peak in standard in standard solution should be not less than 3000.	Flow rate:1.2 mL/min	0.02	1.0	3940
		Flow rate:1.2 mL/min	0.02	1.0	3940
		Column oven temperature: 38°C	0.03	1.1	4914
		Column oven temperature: 40°C	0.08	1.1	4875
		Low organic composition (142.5 mL)	0.02	1.1	5489
		High organic composition (157.5 mL)	0.04	1.1	4545

Validation Parameters	Acceptance Criteria	Results		
Stability of analyte in solution	% Difference of Clofarabine peak area obtained from standard solution at each time point should not be more than ±2.0 from the initial area.	% Difference of area		
		Time Interval	Standard Solution	
			RT	2-8°c
		Initial	0.0	0.0
		24 hrs.	0.37	0.24
		48 hrs.	0.37	0.42

		% Difference of area		
	% Difference of Clofarabine peak area obtained from sample	Time Interval	Sample Solution	
			RT	2-8°c
		Initial	0.0	0.0
		24 hrs.	0.25	0.38
		48 hrs.	0.30	0.06
		Standard solution is stable up to 48Hours and sample solution is stable up to 48Hours at room temperature and 2-8°C for Clofarabine		

Validation Parameters	Acceptance Criteria	Results			
System suitability	System suitability criteria should meet during overall validation studies, otherwise needs to be justified. Report minimum, maximum and average values of system suitability parameters. · The Tailing factor for Clofarabine should be NMT 2.0 · The relative standard deviation for Clofarabine peak from five replicate injections of standard solution should be NMT 2.0 %. · The Theoretical plates for Clofarabine peak in standard in standard solution should be not less than 3000.	System suitability criteria	Minimum	Maximum	Average
		% RSD	0.02	0.08	0.03
		Tailing factor	1.0	1.1	1.1
		Theoretical plates	3940	6243	5078

CONCLUSION

The analytical procedure for Assay is validated and found suitable for its intended use and it meets the acceptance criteria for:

Specificity:
No Interference should be observed at the retention time of peak in the chromatograms obtained from the diluent and the placebo solution.

Forced Degradation:
The method is specific and stability indicating for its intended use.

Linearity:
The analytical procedure is linear within the concentration range from 50 % to 150 % (30.24µg/mL to 90.72µg/mL) for Clofarabine peak.

Intermediate Precision:
The method is precise and rugged with respect to analyst to analyst, day to day, column to column and equipment to equipment for its intended use.

Accuracy:
The analytical test procedure is accurate for its intended use.

Robustness:
The test method is robust enough as demonstrated by altering the Flow rate, Column oven temperature and Organic composition.

Stability of analyte in solution:
The Standard solution is stable up to 48 hours and sample solution is stable up to 48 hours at room temperature and 2-8°C for Clofarabine peak.

The data for each validation characteristic described in this report meets the acceptance criteria with respect to Specificity, Forced degradation, Stability of analyte in solution, Linearity, Precision, Intermediate Precision, Accuracy and Robustness.

The validation results reveal that the analytical procedure is suitable for determination of Assay in ClofarabineInjection, 1mg/mL. The method is stability indicating for determination of Assay of Clofarabine in ClofarabineInjection, 1mg/mL.

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