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Quantification of Newer Anti-Cancer Drug Clofarabine in their Bulk and Pharmaceutical Dosage Form

Brijesh D Patel, Usmangani K Chhalotiya* and Dhruv B Patel

Department of Pharmaceutical Chemistry and Analysis, Indukaka Ipcowala College of Pharmacy, New Vallabh Vidyanagar, Anand, Gujarat, India

Abstract

Quantitative determination of Clofarabine in their Pharmaceutical Dosage form was carried out by Liquid chromatographic method. The aim of the present work was to develop a RP-HPLC method for Clofarabine (CFB). The column used was Luna C-18 column (250 mm × 4.6 mm id, 5 m particle size) using Water: Methanol (60:40 v/v) as a mobile phase at a flow rate of 1.0 ml/min. Quantification was achieved with UV detection at 266 nm over the concentration range 0.05-20 µg/ml and % recovery was found to be in the range of 99.58-100.95 for CFB by the RP-HPLC method. The proposed method was validated with respect to linearity, accuracy, precision, selective, sensitive and robustness. The method was fruitfully applied for the estimation of clofarabine in injection dosage forms.

Keywords: Clofarabine; RP-HPLC; Validation

Introduction

Chemically Clofarabine (CFB) is (2R,3R,4S,5R)-5-(6-amino-2-chloropurin-9-yl)-4-fluoro-2- (hydroxymethyl) oxolan-3-ol [1] with an empirical formula of $C_{10}H_{11}C_lFN_5O_3$, and having molecular weight of 303.67 g/mol (Figure 1) [1]. Intracellularly, clofarabine is metabolized to active 5'-monophosphate metabolite by deoxycytidine kinase and 5'-triphosphate metabolite by mono- and di-phospho-kinases. This metabolite inhibits DNA synthesis through an inhibitory action on ribonucleotide reductase, and by terminating DNA chain elongation and inhibiting repair through competitive inhibition of DNA polymerases which leads to the depletion of the intracellular deoxy-nucleotide triphosphate pool and the self - potentiating of clofarabine triphosphate incorporation into DNA, thereby intensifying the effectiveness of DNA synthesis inhibition. In preclinical models, clofarabine has demonstrated the ability to inhibit1 DNA repair by incorporation into the DNA chain during the repair process. Clofarabine 5'- triphosphate is also disrupts the integrity of mitochondrial membrane, leading to the release of the pro-apoptotic mitochondrial proteins, cytochrome C and apoptosis-inducing factor, leading to programmed cell death [2].

A literature survey revealed that no official methods for estimation of CFB, but there are few reported methods for the estimation of CFB, ultra performance convergence chromatographic stability indicating method for determination of CFB in injections [3].

Clofarabine triphosphate is used in leukemia cell by isocratic HPLC [4]. Estimation of clofarabine triphosphate concentration in mononuclear cells has been done by LC-MS/MS [5]. Simultaneous determination of Fludarabine and Clofarabine in human plasma was done by LC-MS/MS [6].

Literature survey revealed that no HPLC method is reported for estimation of clofarabine in their pharmaceutical dosage form. As per literature review, all the reported methods for estimation of clofarabine were carried out biological sample. The intention of present work was to develop a new, precise, accurate, selective and sensitive method RP-HPLC for estimation of Clofarabine. The proposed method was validated according to ICH guidelines [7,8] and its updated international convention ICH guideline on analytical method validation [9].

Experimental

Apparatus

A Series 200 HPLC system (PerkinElmer, Shelton, CT) equipped with a Series 200 UV detector, Series 200 quaternary gradient pump, Series 200 column oven, manual injector rheodyne valve) with 20 µL fixed loop, Total-Chrom navigator software (Version 6.1.1.0.0:K20), and Luna C18 column (250 mm × 4.6 mm id, 5 µm particle size) was used.

Reagents and materials

Pure samples and marketed sample: Analytically pure powder and formulation of Clofarabine was procured as gratis samples from one of the reputed Pharmaceuticals Limited, India. The purity of reference standard is in the range of 99.60%-101%.

Chemicals and reagents: HPLC grade water, Methanol was purchased from E. Merck (Mumbai, India).

Chromatographic conditions

At ambient temperature, The Luna C18 column was used. Water: Methanol (60:40, v/v) is used as mobile phase and the flow rate were maintained at 1 ml/min. The mobile phase was kept in sonicator for 15 min and was passed through nylon 0.45 µm-47 mm membranes filter, degassed before use. The elution was monitored with UV detector at 266 nm and 20 µL was the injection volume.

HPLC methods were depends upon the nature of the sample (ionic or ionizable or neutral molecule), its molecular weight and solubility. In the direction to optimize the chromatographic conditions the effect of chromatographic variables such as mobile phase, pH, flow rate and solvent ratio were studied. The resulting chromatograms

***Corresponding author:** Usmangani K Chhalotiya, Department of Pharmaceutical Chemistry and Analysis, Indukaka Ipcowala College of Pharmacy, Beyond GIDC Phase IV, Vithal Udyognagar, New Vallabh Vidyanagar-388 121, Anand, Gujarat, India, Fax: +912692229520; E-mail: usmangani84@gmail.com

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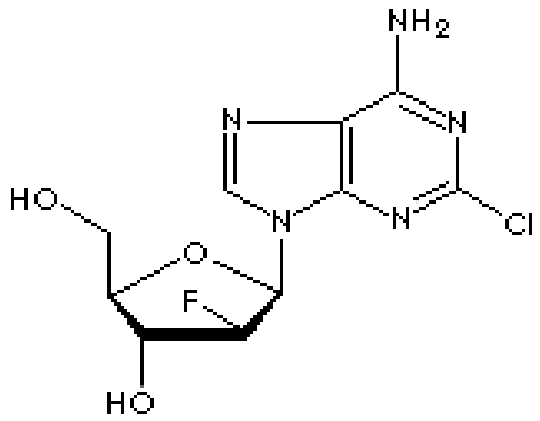

Figure 1: Structure of Clofarabine.

were recorded and the chromatographic parameters such as capacity factor, asymmetric factor, and resolution and column efficiency were calculated. The condition that gave the satisfactory resolution, symmetry and capacity factor was selected for estimation of active drugs.

Preparation of CFB standard stock solutions (100 µg/ml)

Weighed accurately, 25 mg of CFB transferred to a 25 ml volumetric flask containing few ml (10.0 ml) of methanol, swirled to dissolve and diluted up to the mark with methanol to obtain a standard solution of 1000 µg/ml. Pipette out (1.0 ml) of standard solution and transfer it into 10 ml volumetric flask with make up the volume up to the mark with methanol to obtain a Working standard stock solution of 100 µg/ml for the RP- HPLC method.

Preparation of sample solutions

From the above stock solution, appropriate aliquots of CFB working standard solution was transfer into different 10 ml volumetric flasks. The volume was make up to the mark with mobile phase to obtain final concentrations of 0.05, 0.1, 0.5, 1, 5, 10 and 20 µg/ml of CFB, respectively. The solutions were injected using a 20 µL fixed loop system and chromatograms were recorded. Calibration curves were constructed by plotting peak area versus concentrations of the drug and regression equations was computed for CFB.

Method validation

Linearity and range: Calibration curves were constructed by plotting peak areas versus concentrations of CFB, and the regression equations were calculated. The calibration curves were plotted over the concentration range 0.05-20 µg/ml. Aliquots (20 µL) of each solution were injected under the proposed operating chromatographic conditions as described above.

Accuracy (Recovery): The accuracy of the proposed method was determined by calculating % recovery of CFB by the standard addition method. Known amounts of standard solutions of CFB (50, 100 and 150%) were added to prequantified sample solutions of Injection. The amounts of CFB were quantified by applying these values to the regression equation of the calibration curve.

Method precision (repeatability): The precision of the instruments was checked by repeatedly injecting (n=6) solutions of CFB (10 µg/ml) and measure peak area by using proposed RP-HPLC method.

Intermediate precision: By intraday and inter day precision, we can evaluate precision. The intra-day and inter-day precision study of CFB was carried out by estimating the corresponding responses three times on the same day and on three different days for three different concentrations of CFB. The results are reported in terms of percentage relative standard deviation (%RSD).

Robustness: Robustness of the method was studied by observing the stability of the sample solution at 25 ± 2°C for 24 h, change in flow rate at 1 ± 0.1 ml, temperature of working area ± 5°C, and change in mobile phase ratio. Low value of relative standard deviation was indicating that the method was robust.

LOD and LOQ: The detection limit is defined as the lowest concentration of an analyte that can reliably be differentiated from background levels. Limit of quantification is the lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy. LOD and LOQ were calculated using following equation as per ICH guidelines.

$LOD=3.3 \times \sigma/S$, $LOQ=10 \times \sigma/S$

Where σ is the standard deviation of y-intercepts of regression lines and S is the average slope of the calibration curves.

Specificity: Specificity is the ability to assess unequivocally the analyte in the presence of components, which may be expected to be present. The specificity was estimated by spiking commonly used excipients (starch, talc and magnesium stearate) into a pre weighed quantity of drug. The chromatogram was taken by appropriate dilutions and the quantities of drugs were determined.

System suitability: A system suitability test was an integral part of the method development to verify that the system is adequate for the quantification of CFB to be performed. The suitability of the chromatographic system was demonstrated by comparing the obtained parameter values with the acceptance criteria of the U.S. Food and Drug Administration, Center for Drug Evaluation and Research guidance document (U.S. Food and Drug Administration). A system suitability test of the chromatography system was performed before each validation run. Six replicate injections of a system suitability/calibration standard and one injection of a check standard were made. Area, retention time (RT), tailing factor, asymmetry factor, and theoretical plates for the six suitability injections were determined.

Stability of standard and sample solutions: Stability of standard and sample solution of CFB was evaluated at room temperature for 48 hr at 2 hr time interval measure the peak area of standard and sample solution. The relative standard deviation was found below 2.0%. It showed that both standard and sample solution were stable up to 48 hr at room temperature.

Determination of CFB in injection: The injection has the strength of 1 mg/ml solution. From marketed injection, 1.0 ml of aliquot was pipette out and transferred into 10 ml volumetric flask and then volume was adjusted upto the mark with mobile phase to obtain concentration of 100 µg/ml CFB. From above solution, 1.0 aliquot ml was transferred to another 10 ml volumetric flask and volume was adjusted up to the mark to obtain 10 µg/ml CFB. The solution was sonicated for 10 min. Solution were injected as per the above chromatographic condition and the peak areas were measure and quantification was carried by keeping these values to straight line equation of calibration curve.

Results and Discussion

Optimization of the chromatographic conditions

HPLC method: Optimization of mobile phase was performed based on resolution of the drug, asymmetric factor and theoretical plates obtained for CFB. Water: Methanol (60:40, v/v) was selected as a mobile phase at a flow rate of 1.0 ml/min was found to be satisfactory and gave well resolved and symmetric peak for CFB. The retention time for CFB was 5.39 min. For the selection of detection wavelength, the spectrum of 10 µg/ml CFB solution revealed that, at 266 nm the drug possesses significant absorbance. So considering above fact, 266 nm was selected as a detection wavelength for estimation of CFB using HPLC. Complete resolution of the peaks with clear baseline separation was obtained (Figure 2). The system suitability test parameters are shown in Table 1. Analytically pure CFB was identified by melting point study and infrared spectroscopy study.

Validation of the proposed methods: The developed method was validated, as described below, for various parameters like linearity and range, accuracy, precision, ruggedness, system suitability, specificity, LOQ, and LOD.

Linearity and range: Linearity of the method was evaluated at seven concentration levels by diluting the standard stock solution to give solutions in the range of 0.05-20 µg/ml. The calibration curve for CFB was prepared by plotting area v/s concentration. Calibration data for CFB was shown in Table 2. The linearity plot of CFB was found to be linear with the linear equation y=60725x+26878 and correlation coefficient 0.998 for CFB. Linearity was observed in the expected concentration range, demonstrating suitability of the method for analysis (Figure 3). This indicates that the method is linear in the specified range for the analysis of CFB in dosage form.

Accuracy: The recovery experiments were carried out by the standard addition method. The method was found to be accurate with % recovery 99.58%-100.95% the recoveries were obtained by the proposed RP-HPLC method for estimation CFB are shown in Table 3.

Precision: Precision was calculated as repeatability and intraday and interday precision for estimation of CFB. The method was found to be precise with %RSD 0.59-1.37 for intraday (n=3) and %RSD 0.87-1.77 for interday (n=3) for CFB. The low value of %RSD (i.e., NMT 2%) has observed for the three level results (Table 4) hence it concluded that the method is precise for the analysis of CFB.

Specificity (Placebo interference): There is no interference of mobile phase, solvent and placebo with the analyte peak and also the peak purity of analyte peak which indicate that the method is specific for the analysis of CFB in their dosage form.

Robustness: The method was found to be robust, as small but deliberate changes in the method parameters have no detrimental effect on the method performance as shown in Table 5. The low value of relative standard deviation was indicating that the method was robust.

Standard and sample solution stability: Standard and Sample solution stability was evaluated at room temperature for 48 hr. The relative standard deviation was found below 2.0%. It showed that both standard and sample solutions were stable up to 48 hr at room temperature.

LOD and LOQ: These data show that the method is sensitive for the determination of CFB. The LOD and LOQ were carried out by visual method 0.01 and 0.05 µg/ml, respectively.

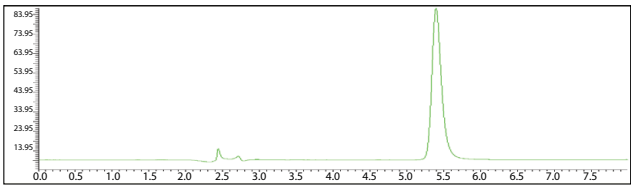

Figure 2: Chromatogram of CFB (RT 5.39 min) on C_{18} Luna column using Water: Methanol (60:40, v/v) as the mobile phase.

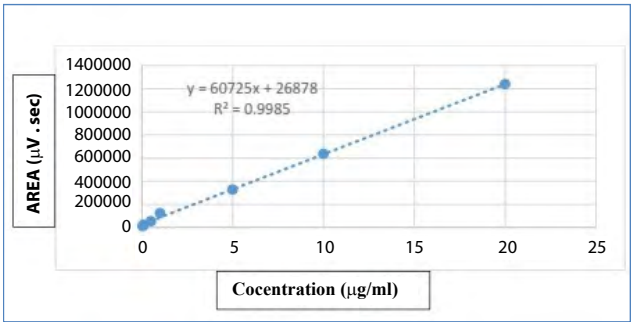

Figure 3: Calibration Curve of CFB (0.05-20 µg/ml).

Parameter	*Clofarabine*
Retention times (R_T)	5.39 Min
HPLC Plate Count	8292
Tailing factor	1.14
Capacity factor	1.20
Base width (sec)	14.22

Table 1: System suitability test parameters for CFB the proposed HPLC method.

Parameter	*Clofarabine*
Linearity (µg/ml)	0.05-20
Correlation co-efficient (r)	0.998
Slope of Regression (S)	60725
Intercept of Regression	26878
Standard deviation of slope	1080.58
Standard deviation of intercept	2471.16

Table 2: Regression analysis of CFB by proposed HPLC method.

Analysis of a formulation: The proposed method was applied for the determination of CFB in injection of Clolar. The percentage amount of drugs was found to be 99.59% of the label claim for the formulation. The results of the assay indicate that the method is selective for the assay of CFB without interference from excipients used in the injection (Table 6).

Conclusions

The proposed validated RP-HPLC analytical method has been developed for the quantification of CFB in bulk and injectable dosage form. Validation of method undertaken according to the ICH guidelines revealed that the method is selective. The proposed method is sensitive, accurate, precise, and specific. The method is suitable for the routine analysis of CFB in injection and other pharmaceutical dosage form.

Amount of Sample (µg/ml)	Sets	Amount drug of spiked (µg/ml)	Area (n=3)	Average amount recovered (µg/ml)	% Recovery	Mean % Recovery	% RSD[c]
5	1	0	329568.26	4.97	99.69	99.58	1.13
	2	0	332514.87		100.66		
	3	0	325649.82		98.40		
5	1	2.5	481698.54	7.48	99.79	99.71	0.64
	2	2.5	479365.96		99.03		
	3	2.5	483245.71		100.30		
5	1	5	632569.41	10.04	99.48	100.95	1.65
	2	5	635964.09		100.60		
	3	5	642568.55		101.78		
5	1	7.5	791563.67	12.51	101.85	100.29	1.47
	2	7.5	786354.22		100.13		
	3	7.5	782589.38		98.89		

[c]RSD=Relative standard deviation

Table 3: Data derived from accuracy of Clofarabine by proposed HPLC method.

Parameters	Clofarabine
LOD (µg/ml)[d]	0.01
LOQ (µg/ml)[e] (n=5)	0.05
Accuracy, %	99.58-100.95
Repeatability, (% RSD, n=6)	1.27
Precision (% RSD) Interday (n=3) Intraday (n=3)	0.87-0.1.77 0.59-1.37

[d]LOD=Limit of detection
[e]LOQ=Limit of quantitation

Table 4: Summary of validation parameters.

Parameters	Normal condition	Change in condition	Change in % RSD of Peak area
Flow Rate	1.0 ml/min	0.8 ml/min	1.45
		1.2 ml/min	1.30
Detection wavelength	266	264	1.36
		268	1.80
Mobile phase ratio	60:40	58:42	1.21
		62:38	1.41

Table 5: Robustness study of Clofarabine by proposed HPLC method.

Injection	Concentration (µg/ml)	Amount recovered (µg/ml)	Amount of Drug Found ± SD[a] (*n*=3), %
A	10	9.95	99.59 ± 0.88

[a]SD=Standard deviation

Table 6: Assay Results of marketed formulation.

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