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ESTIMATION OF NEWER ANTI-CANCER DRUG CLOFARABINE IN THEIR PHARMACEUTICAL DOSAGE FORM BY STABILITY INDICATING TLC METHOD

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ABSTRACT

A sensitive, accurate, precise and reproducible high performance thin layer chromatographic method has been developed for the estimation of clofarabine in their pharmaceutical dosage form. The objective of this validation of an analytical procedure is to demonstrate that the drug Clofarabine is suitable for its intended purpose. The analytical method development recommends the quality, purity and specificity of the drug. Clofarabine injection form during the manufacturing process and hence the standard of drug may not vary, which produce the desirable therapeutic effect.TLC aluminum plates pre-coated with silica gel $60F_{254}$used as the stationary phase, while toluene: methanol (8:2, v/v) used as mobile phase. The Rf value was observe 0.34± 0.05 for clofarabine. The densitometric analysis was carried out in absorbance mode at 266 nm. The method was linear in the range of 50 – 1000 ng/spot for clofarabine. The method was validated as per ICH guideline. The limit of detection and limit of quantitation were found to be 17.60ng/spot and 53.35 ng/spot, respectively for clofarabine.The proposed method was successfully applied to the estimation of clofarabinein the pharmaceutical dosage form. Clofarabine was subjected to acid and alkali hydrolysis, chemical oxidation, wet hydrolysis, dry heat degradation and sun light degradation. The degradants peaks were well resolved from the pure drug peak with significant difference in their R_f values. Stressed samples were assayed using proposed TLC method.

Keywords: Clofarabine, HPTLC, Validation, Analysis, Force Degradation

1. INTRODUCTION

Clofarabine (CFB) is chemically (2R, 3R, 4S, 5R)-5-(6-amino-2-chloro-9H-purin-9-yl)-4-fluoro-2-(hydroxymethyl) oxolan-3-ol. The emperical formula for CFB is $C_{10}H_{11}ClFN_5O_3$ and molecular weight is 303.68 g/mol. It is a purine nucleoside metabolic inhibitor indicated for the treatment of pediatric patients 1 to 21 years old with relapsed or refractory acute lymphoblastic leukemia after at least two prior regimens.Clofarabine is sequentially metabolized intracellularly to the 5'-monophosphate metabolite by deoxycytidine kinase and mono- and di-phospho-kinases to the active 5'-triphosphate metabolite. Clofarabine has affinity for the activating phosphorylating enzyme, deoxycytidine kinase, equal to or greater than that of the natural substrate, deoxycytidine. Clofarabine inhibits DNA synthesis by decreasing cellular deoxynucleotide triphosphate pools through an inhibitory action on ribonucleotide reductase, and by terminating DNA chain elongation and inhibiting repair through incorporation into the DNA chain by competitive inhibition of DNA polymerases. The affinity of clofarabine triphosphate for these enzymes is similar to or greater than that of deoxyadenosine triphosphate. In preclinical models, clofarabine has demonstrated the ability to inhibit DNA repair by incorporation into the DNA chain during the repair process. Clofarabine 5'-triphosphate 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.CFB is approved by FDA in December 2004.

Literature review reveals that CFB is not official in United State Pharmacopoeia and British Pharmacopoeia. No method has been reported for the estimation of CFB in their pharmaceutical dosage form. In comparison to LC and LC-MS/MS methods, HPTLC method is considered to be a good alternative, and it should be widely explored as an important tool in routine drug analysis. A major advantage of HPTLC is its ability to analyze sample using a small quantity of mobile phase. This reduces the time and cost of analysis[1, 2].

2. EXPERIMENTAL[3-7]

2.1 HPTLC instrument

The samples were applied in the form of a bands of width 8 mm with a Camag 100 µl sample syringe (Hamilton, Switzerland) using Camag Linomat 5 (Switzerland) sample applicator on pre-coated silica gel aluminum plate 60 F_{254} (10 cm x 10 cm with 0.2 mm thickness, E. Merck, Germany). Camag TLC scanner4 was used for the densitometric scanning.

2.2 Chemicals and reagents

Analytically pure CFB and injection formulation (Containing 1 mg/ml of CFB) are procured from reputed pharmaceutical R & D centre as a gift sample. Methanol (AR grade) of SRL Private Ltd. and toluene of Chemdyes Corporation (AR grade) were used.

2.3 Chromatographic system

2.3.1 Sample application

Standards and formulation samples of CFB were applied on the HPTLC plates in the form of narrow bands of 6 mm length, 10 mm from the bottom and left edge, and with 9 mm distance between two bands. Samples were applied under a continuous stream of nitrogen gas.

2.3.2 Mobile phase and development

Plates were developed using a mobile phase consisting of toluene: methanol (8:2v/v). Linear ascending development was carried out in a twin-trough glass chamber equilibrated with the mobile phase vapors for 20 min at 25 ± 2^0C. Ten milliliters of the mobile phase (5 ml in the trough containing the plate and 5 ml in the other trough) was used for each development and was allowed to migrate a distance of 80 mm, sample application rate is 200nl/sec. After development, the HPTLC plates were dried completely using continuous stream of nitrogen.

2.3.3 Densitometric analysis

Densitometric scanning was performed in the absorbance mode under control by winCATS planar chromatography software. The source of radiation was the deuterium lamp and bands were scanned at 266 nm. The slit dimensions were 6 mm length and 0.45 mm width, with a scanning rate of 20 mm/s. Concentrations of the compound were determined from the intensity of diffusely reflected light and evaluated as peak areas against concentrations using a linear regression equation.

2.4 Preparation of standard stock solution

CFB (10 mg) was accurately weighed and transferred to 10 ml volumetric flasks and dissolved in few ml of methanol. Volumes were made up to the mark with methanol to yield a solution containing 1000µg/ml of CFB. Aliquot from the stock solutions of CFB appropriately diluted with mobile phase to obtain working standard of 100µg/ml, 50 µg/ml, 5µg/ml of CFB.

2.5 Validation[8]

Validation of the developed HPTLC method was carried out according to International Conference on Harmonization (ICH) guidelines Q2 (R1) for specificity, sensitivity, accuracy, precision, repeatability, and robustness.

2.5.1 Linearity of calibration curves

Linearity of the method was evaluated by constructing calibration curves at six concentration levels over a range of 50–1000 ng/band for CFB by applying 10µl from 5 µg/ml, 2µl and 4 µlfrom 50 µg/ml and 6µl, 8µl, 10µl from 100 µg/mlsolution has been applied on HPTLC plate using sample applicator.The calibration curves were developed by plotting peak area versus concentration (n = 6) with the help of the winCATS software.

2.5.2 Accuracy

The accuracy of the method was determined by calculating recoveries of CFB by method of standard additions.Known amount of CFB(0, 100, 200, 300 ng/spot) were taken from the working standard solutions (50 µg/ml of CFB).It was added to a pre quantified sample and the amount of CFB were estimated by

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measuring the peak area and by fitting these values to the straight-line equation of calibration curve.

2.5.3 Precision

Precision was evaluated in terms of intraday and interday precisions. Working standard solutions of 50 µg/ml of CFB, were prepared and used for the precision study. Intraday precision was determined by analyzing sample solutions of CFB at three levels covering low, medium, and high concentrations of the calibration curve three times on the same day. Interday precision was determined by analyzing sample solutions of CFBat three levels covering low, medium, and high concentrations over a period of 3 days. The peak areas obtained were used to calculate mean and RSD values.

2.5.4 Specificity

The specificity of the method was ascertained by analyzing CFB in presence of preservative (Benzyl Alcohol) commonly used for injection formulations. The bands of CFB were confirmed by comparing Rf values and respective spectra of sample with those of standards. The peak purity of CFBwas assured by comparing the spectra at three different levels, that is, peak start, peak apex and peak end positions.

2.5.5 Sensitivity

Sensitivity of the method was determined with respect to LOD and LOQ. Noise was determined by scanning a blank band (methanol) six times. LOD was calculated as 3 times the noise level, and LOQ was calculated as 10 times the noise level.

2.5.6 Robustness

Small changes in the chamber saturation time, Detection limit and mobile phase composition were introduced and the effects on the results were examined. Robustness of the method was determined in triplicate at a concentration level of 200 ng/band for CFB. The mean and RSD of peak areas were calculated.

2.6 Analysis of marketed formulations

The injection has the strength of 1mg/ml solution. From this solution, 1 ml of aliquot was transferred to 10 ml volumetric flask and then volume was adjusted to the mark with methanol and flask was sonicated for 15 min. A solution containing 200 ng/band CFB were injected as per the above chromatographic conditions and peak areas were recorded.The quantifications were carried out by keeping these values to the straight line equation of calibration curve.

2.7 Forced degradation study[9 – 15]

Stress degradation study using acid and alkali hydrolysis, chemical oxidation, wet hydrolysis exposure to sun light and dry heat degradation was carried out and interference of the degradation products was investigated. CFB was weighed (10 mg) and transferred to 10 ml volumetric flasks and expose to different stress conditions.

2.7.1 Heat induced alkali hydrolysis

To the 10 ml volumetric flask, 10 mg of CFB was taken and 2 ml of 0.1 N NaOH was added to perform heat induced base hydrolysis. The flask was heated at 80ºC for 2 hrs and allowed to cool to room temperature. Solution was neutralized with 0.1 N HCl and volume was made up to the mark with methanol. Pipette out aliquot (0.5 ml) was taken in 10 ml of volumetric flask and diluted with mobile phase to obtain final concentration of 200ng/spot of CFB. The final solutions were analysed under the proposed chromatographic conditions and chromatograms recorded. The amounts of undegraded drugs were computed using regression equation.

2.7.2 Heat induced acid hydrolysis

To the 10 ml volumetric flask, 10 mg of CFB was taken and 2 ml of 0.1 N HCl was added to perform heat induced acid hydrolysis. The flask was heated at 80ºC for 2 hrs and allowed to cool to room temperature. Solution was neutralized with 0.1 N NaOH and volume was made up to the mark with methanol Pipette out aliquot (0.5 ml) was taken in 10 ml of volumetric flask and diluted with mobile phase to obtain final concentration of 200ng/spot of CFB. The final solutions were analysed under the proposed chromatographic conditions and chromatograms recorded. The amounts of undegraded drugs were computed using regression equation.

2.7.3 Heat induced oxidative stress degradation

To heat induced perform oxidative stress degradation, 10mg of CFB was taken in 10 ml volumetric flask and 2 ml of 3% hydrogen peroxide was added. The mixture was heated in a water bath at 80ºC for 2 hrs and allowed to cool to room temperature and volume was made up to the mark with methanol. Pipette out aliquot (0.5 ml) was taken in 10 ml of volumetric flask and diluted with mobile phase to obtain final concentration of 200ng/spot of CFB. The final solutions were analysed under the proposed chromatographic conditions and chromatograms recorded. The amounts of undegraded drugs were computed using regression equation.

2.7.4 Dry heat degradation

Analytically pure 10 mg sample of CFB was exposed in oven at 80ºC for 2 hrs. The solids were allowed to cool and transferred to volumetric flasks (10 ml) and dissolved in few ml of methanol. Volume was made up to the mark with the methanol. Pipette out aliquot (0.5 ml) was taken in 10 ml of volumetric flask and diluted with mobile phase to obtain final concentration of 200ng/spot of CFB. The final solutions were analysed under the proposed chromatographic conditions and chromatograms recorded. The amounts of undegraded drugs were computed using regression equation.

2.7.6 Photolytic (UV light) degradation

Analytically pure 10 mg of drug were exposed to UV-light for 24 hrs. The solids were allowed to cool and transferred to volumetric flask (10 ml) and dissolve in few ml of methanol. Volume was made up to the mark with the methanol. Pipette out aliquot (0.5 ml) was taken in 10 ml of volumetric flask and diluted with mobile phase to obtain final concentration of 200ng/spot of CFB. The final solutions were analysed under the proposed chromatographic conditions and chromatograms recorded. The amounts of undegraded drugs were computed using regression equation.

3. RESULTS AND DISCUSSION

3.1 Optimization of the Mobile Phase

To develop the HPTLC method for analysis of CFBin the pharmaceutical dosage form for routine analysis, selection of the mobile phase was carried out on the basis of polarity. A mobile phase that would give a dense and compact band with an appropriate Rf value for CFBwas desired. Various mobile phases such as methanol, toluene, methanol- toluene were evaluated in different proportions.A mobile consisting of toluene: methanol (8:2v/v) was found to be satisfactory and gave good result for CFB. It was also observed that chamber saturation time and solvent migration distance were crucial in the chromatographic separation. Therefore, toluene: methanol (8:2, v/v) mobile phase with a chamber saturation time of 20 min at 25 ^{0}C and solvent migration distance of 80 mm was used. Densitogram and photograph of developed TLC plate of CFB has been shown in figure 1 - 2 .Three dimensional overlays of HPTLC densitograms of calibration bands of CFBhas been shown in figure-3.

Table 1: Regression analysis of Calibration Curve.

Parameters	CFB
Linear range (ng/band)	50-1000
Slope	11.13
Intercept	602.77
Regression Co-efficient (r^2)	0.9986
Standard Deviation of slope	0.05
Standard Deviation of intercept	59.38
Limit of Detection	17.60
Limit of Quantification	53.35

3.2 Validation

3.2.1 Linearity and calibration curves

This method was found to be linear for CFBin concentration range of 50–1000 ng/band ($n = 6$). Figure-3 displays a three-dimensional overlay of HPTLC densitograms of the calibration bands of CFB at 266 nm. The regression data shown in Table-1 reveal a good linear relationship over the concentration range studied, demonstrating the suitability of the method for analysis.

3.2.2 Accuracy

Accuracy was determined by the application of analytical procedure to recovery studies, where a known amount of standard is spiked into preanalyzed samples solutions. Results of the accuracy studies from excipients matrix are shown in Table-4. Recovery values demonstrated the accuracy of the method in the desired range.

3.2.3 Precision

In all instances, RSD values were less than 2%, confirming the precision of the method. Repeatability of the scanning device was studied by applying and analyzing sample seven times. RSD was less than 2%, which was well below the instrumental specifications.Summary of validation parameters are shown in table – 2. The RSD values obtained were less than 2% (Table 2), which was under the acceptance criteria of ICH method validation guideline (<2%). The results indicated that the method is repeatable and reproducible.

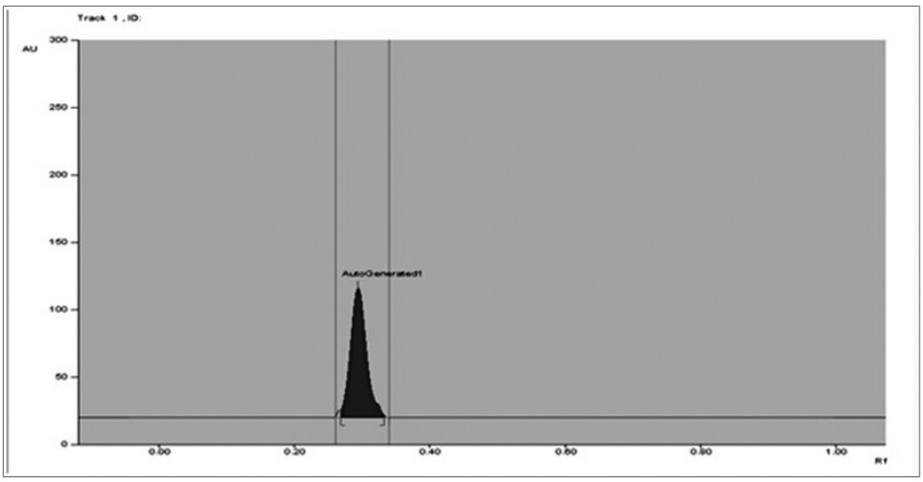

Figure 1: Densitogram of CFB using mobile phase Toluene: Methanol (8:2, v/v).

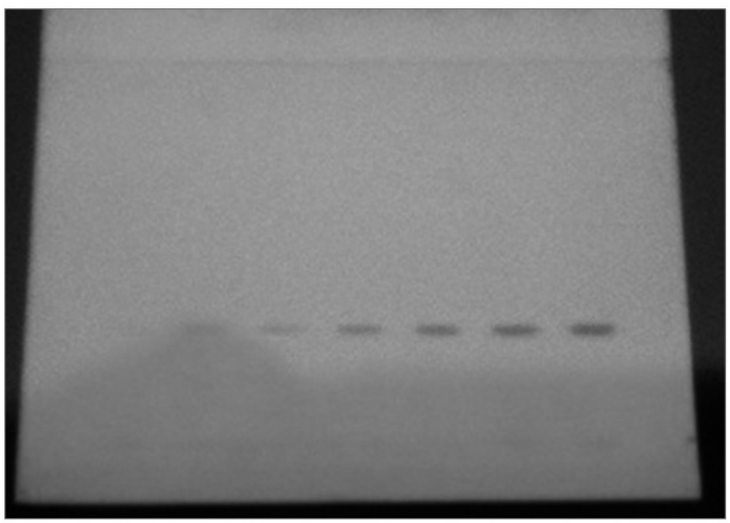

Figure 2: Photograph of developed TLC plate.

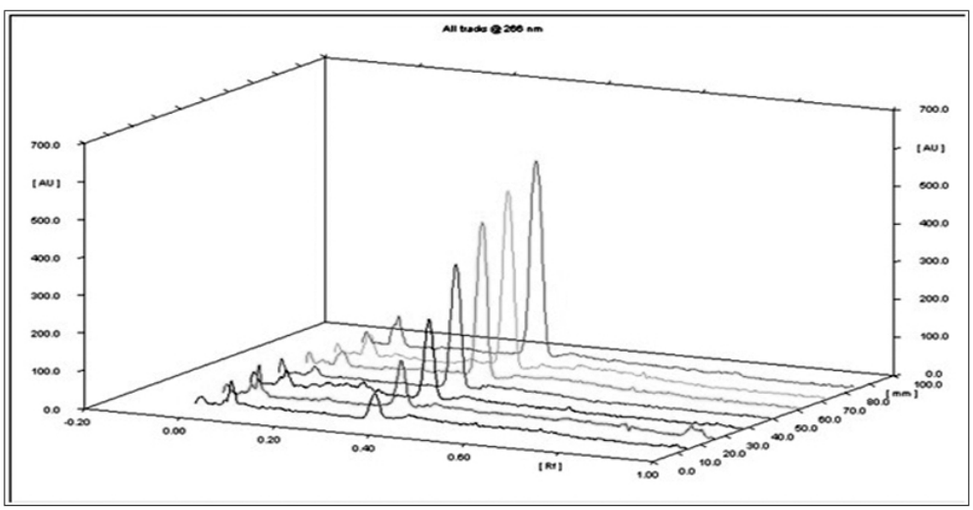

Figure 3: Three dimensional overlay of HPTLC densitograms of calibration bands of CFB.

Table 2: Summary of Validation Parameters.

Parameters	CFB
Range (ng/band)	50-1000
R_f	0.34 ± 0.05
Detection limit (ng/band)	17.60
Quantitation Limit (ng/band)	53.35
Accuracy (%)	99.64-100.60
Precision (%RSD)	
Intra-day (n=3)	0.35-1.49
Inter-day (n=3)	0.45-1.42
Instrumental Precision (%RSD)	
Scanner (n=6)	1.01
Injection (n=6)	1.02

3.2.4 Limit of detection and limit of quantification

Under the experimental conditions used, the lowest amount of drug that could be detected LOD was found to be 17.60ng/bandand LOQ was found to be53.35 ng/band.It indicate that the nanogram quantity of all the drugs can be estimated accurately and precisely which means that the method is sensitive.

3.2.5 Specificity

There was no interfering peak at the Rf value of CFB from preservatives added in the synthetic formulation. In addition, there was no interference from preservatives present in the commercial formulation, thereby confirming the specificity of the method.

3.2.6 Robustness

The low values of RSD (Table-3) obtained after introducing small, deliberate changes in parameters of the developed HPTLC method confirmed its robustness.

Table 3: Robustness Study of Proposed method.

Change in parameters		CFB (200ng/spot)	
		Area ± SD (n =3)	%RSD
Proportion of mobile Phase (toluene: methanol, 8:2, v/v)	8.2-1.8	2914.96 ± 51.01	1.75
	7.8-2.2	2901.33 ± 56.97	1.99
Change in wavelength λ_{max}-266nm	264 nm	2867.73 ± 44.55	1.55
	268 nm	2854.33 ± 38.44	1.77
Chamber saturation time (20 min)	18 min	2861.9 ± 54.66	1.91
	22 min	2854.23 ± 50.63	1.77

3.3 Analysis of marketed formulation

Marketed formulation was analyzed using proposed method which gave percentage recovery of 100.63 ± 0.24% for CFB. No interference from the excipients present in the marketed tablet formulation was observed in figure 4 and data shown in Table-5.

Table 4: Accuracy Study of Proposed method.

% Level	Amount Added (ng/spot)	Amount Recovered (ng/spot) (n=3)	% Recovered ± SD (n =3)
0	200 + 0	201.21	100.60 ± 0.66
50	200+100	301.29	100.50±0.17
100	200+200	399.28	99.64±1.16
150	200+300	499.82	99.9 ± 1.14

Table 5: Analysis of Marketed Formulation.

Formulation	Drug	Amount Taken (ng/band) (n=3)	Amount Found (ng/band) (n = 3)	% Amount of Drug Found (n = 3)
Clolar (Injection)	CFB	200	201.66	100.63 ± 0.24

Table 6: Result of Forced Degradation Study by HPTLC.

Condition	Time	% Amount of drug found	Rf values of degradation products
Acid 0.1 N HCl (70-80°C)	2 Hrs	94.94	0.42
Base 0.1 N NaOH (70-80°C)	2 Hrs	68.86	0.41
Oxidative degradation 3% H_2O_2, (70-80°C)	2 Hrs	101.00	--
Dry heat (70-80°C)	2 Hrs	100.73	--
Photolytic (Room temperature)	24 hrs	101.54	--

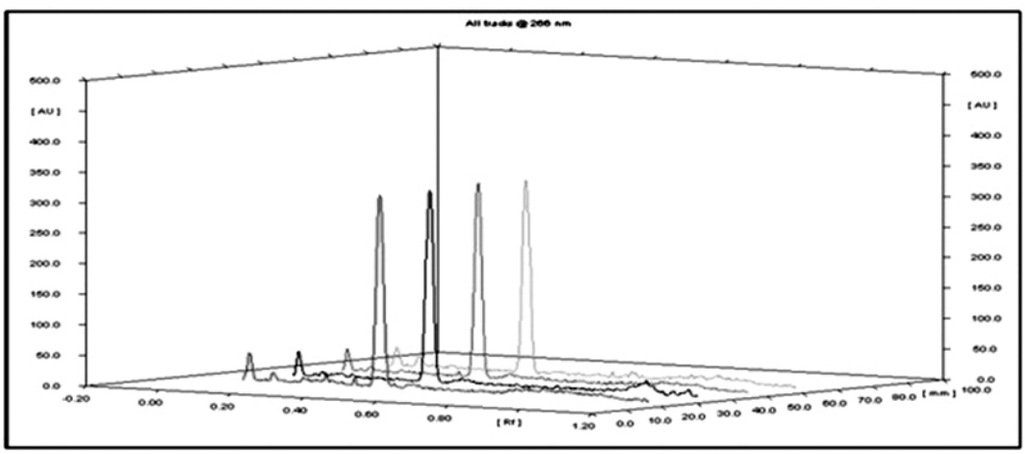

Figure 4: Densitogram of marketed formulation analysis of CFB.

3.4 Forced degradation study

Chromatogram of acid hydrolysis performed at 80^{0}C for 2 hrs reflux showed degradation of CFB with degradation product peak at retention factor (Rf) 0.69 (Figure 5). Chromatogram of base hydrolysis performed at 80^{0}C for 2 hrs reflux showed degradation of CFB with degradation product peak at retention factor (Rf) 0.35 (Figure 6).

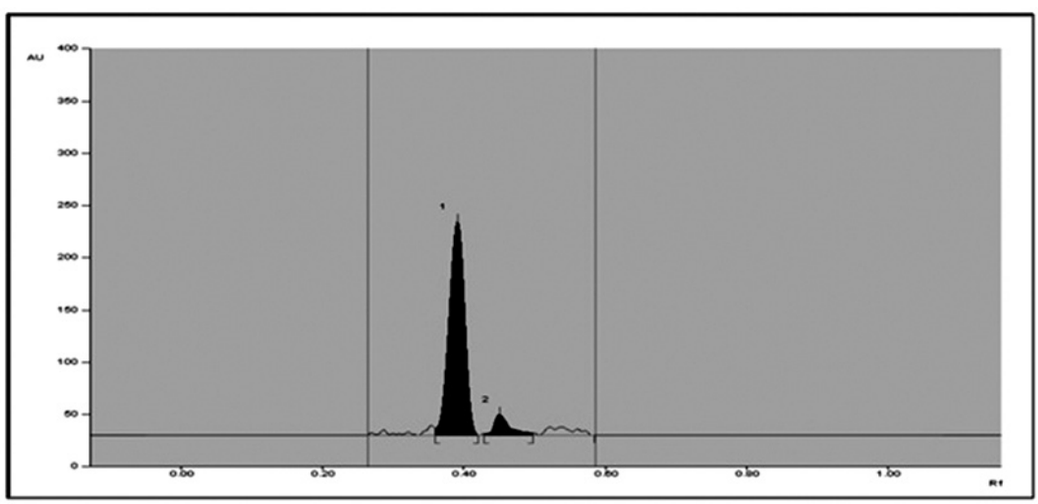

Figure 5: Densitogram of acid hydrolyzed (0.1 N HCl) sample of CFB (200 ng/band) under reflux condition (70 – 80 °C).

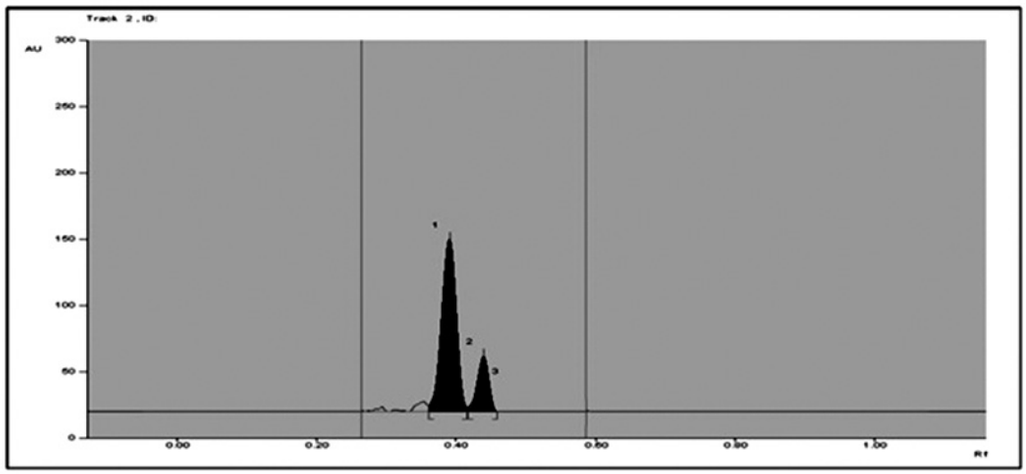

Figure 6: Densitogram of alkali hydrolyzed (0.1 N NaOH) sample of CFB (200 ng/band) under reflux condition (70 – 80 °C).

The chromatogram of oxidized CFB with 3% hydrogen peroxide at 80^0C for 2 hrs reflux do not showed any degradation of CFB and found stable (Figure 7). The chromatogram of CFB exposed to dry heat at 80^0C for 2 hrs do not showed any degradation of CFB and found stable (Figure 8). The chromatogram of CFB expose to UV light for 24 does notshowed degradation of CFB (Figure 9).

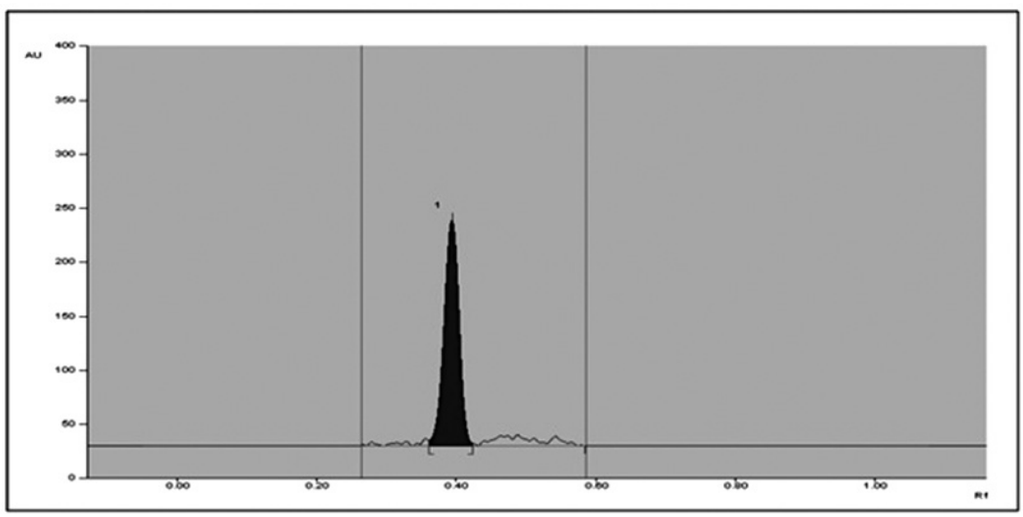

Figure 7: Densitogram of oxidative stressed (3% H_2O_2) sample (200 ng/band) of CFB under reflux condition (70 – 80 °C).

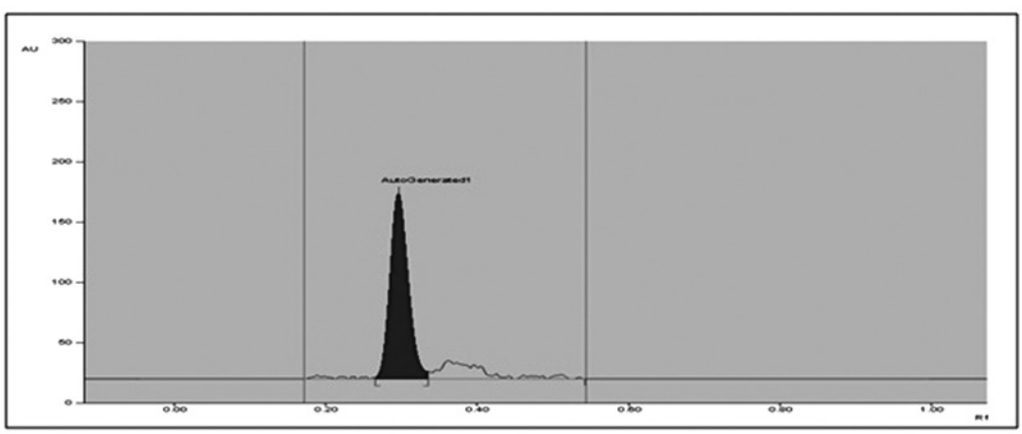

Figure 8: Densitogram of dry heat condition sample of CFB (200 ng/band).

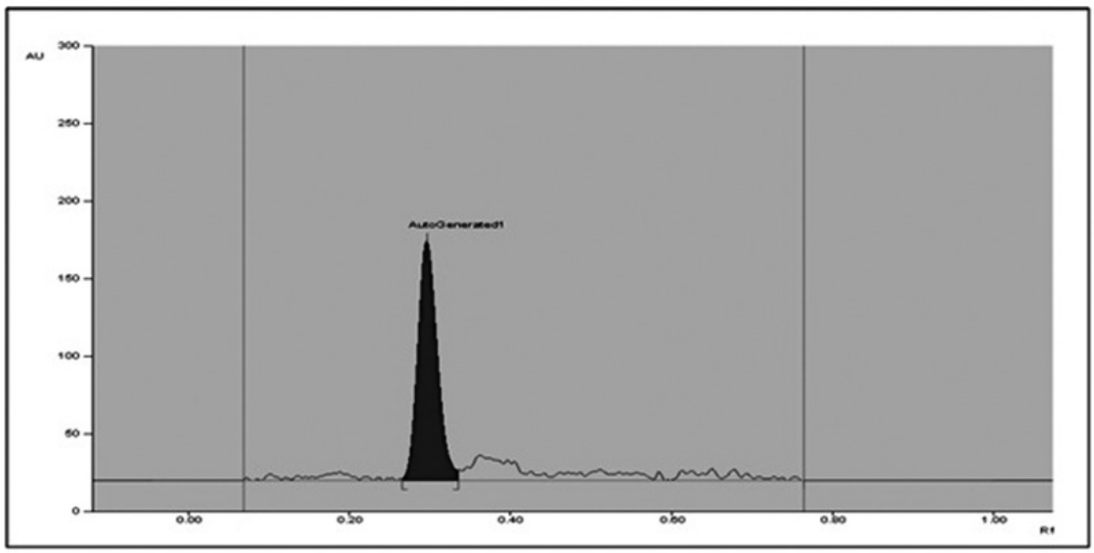

Figure 9: Densitogram of Photolytic degraded sample (200 ng/band) of CFB.

The degradation study thereby indicated that CFB was found to be stable to oxidation (3% hydrogen peroxide), dry heat degradation and Photolytic degradation while it was susceptible to base hydrolysis, acid hydrolysis, (Table 6). The degradation peaks were well resolved from the drug peak and no degradation products from different stress conditions affected determination of CFB which indicate that the method is selective and specific.

4. CONCLUSIONS

Proposed study describes stability indicating TLC method for the estimation of CFB in bulk and their pharmaceutical dosage form. The proposed method shows the degradation of Clofarabine in alkali hydrolysis, acid hydrolysis.A simple accurate and precise TLC method has been developed for the identification and quantification of CFB in bulk and its pharmaceutical dosage form. The method was successfully validated in accordance with ICH guidelines. It can be conveniently used for routine quality control analysis of CFB in marketed Injection without any interference from preservatives.

5. ACKNOWLEDGEMENTS

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