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Development of supercritical fluid (carbon dioxide) based ultra performance convergence chromatographic stability indicating assay method for the determination of clofarabine in injection

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The present study reports the development and validation of a stability indicating assay method for clofarabine in injection on a UPC^{2TM} (ultra performance convergence chromatography) instrument, which utilizes the unrealized potential of supercritical fluid chromatography. The use of UPC^{2TM} provides a single viable technique that is a sustainable, reduced cost, and green technology that lowers the use of organic solvents. Based on this advantage, we explored a simple and robust method in order to increase sample throughput and productivity to quantify clofarabine in the presence of its potential impurities and other degradants. The separation was achieved on a BEH-2-ethyl pyridine (BEH 2EP) column (100 mm × 3.0 mm I.D. with an average pore diameter of 1.7 μm) by using methanol as a co-solvent and carbon dioxide as a mobile phase in the ratio of 30 : 70. The detection is carried out at a wavelength of 254 nm. We are able to achieve the separation of clofarabine from its potential impurities and other degradants in less than 6 minutes with a low amount of solvent consumption. The new method is validated in accordance with the ICH-guidelines and exhibited good intra- and inter-day precision, accuracy and linearity ($r^2 \geq 0.999$) over a range of 50% to 150% of target concentration.

1. Introduction

Various laboratories and industries are generating a large amount of hazardous and non-hazardous waste in the form of organic solvents at present. The management of these waste solvents has become a concern in terms of cost, environmental safety and health. One of the ways to control this is by reducing the usage of these solvents or by applying "*green chemistry*" principles in every process at all stages of manufacturing and testing. In this context, supercritical fluid CO_2 has emerged as a versatile solvent for various chemical separations due to its low toxicity and inflammability. As a result, supercritical fluid chromatography (SFC) has emerged as an alternative and complementary method to HPLC.[1]

The potential of SFC using packed columns for the analysis of impurities and active ingredients in the pharmaceutical industry has been recognized for many years.[2] SFC can offer highly efficient separations with short analysis times and at a low-pressure drop without compromising the resolution, plate counts and tailing. SFC is a reliable technique and can, with appropriate modification, be interfaced readily with standard HPLC detectors.[3]

We have made an attempt to explore the 'green' potential of SFC and developed a simple, short stability indicating assay method for the determination of clofarabine in clofarabine injection, for better waste management and environmental safety.

Clofarabine is a purine nucleoside antimetabolite that is being studied in the treatment of cancer. It is marketed in the U.S. and Canada as Clolar. In Europe and Australia/New Zealand the product is marketed under the name Evoltra. Clofarabine is used in pediatrics to treat a type of leukemia known as relapsed or refractory acute lymphoblastic leukemia (ALL). Clofarabine differs from other purine nucleoside analogs by the presence of chlorine in the purine ring and fluorine in the ribose moiety. Clofarabine prevents cells from making DNA and RNA by interfering with the synthesis of nucleic acids, thus stopping the growth of cancer cells. Its IUPAC name is (2*R*,3*R*,4*S*,5*R*)-5-(6-amino-2-chloro-9*H*-purin-9-yl)-4-fluoro-2-(hydroxymethyl) oxolan-3-ol (Fig. 1).[4]

Currently, clofarabine is not official in any pharmacopoeia and very few studies are available on its chromatographic determination. Zhu reports an LC-MS/MS determination of clofarabine in human urine and human plasma.[5,6] One HPLC method is reported on the determination of the concentration of clofarabine in rat plasma and its pharmacokinetics.[7] This

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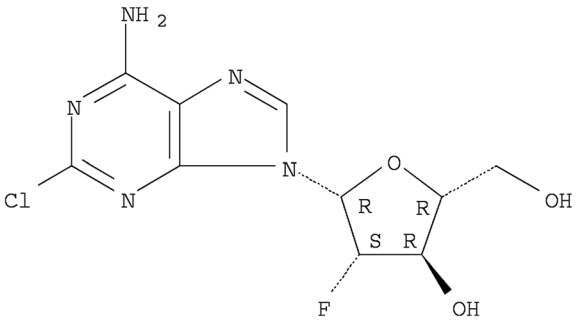

Fig. 1 Chemical structure of clofarabine.

method uses a Hypersil ODS 150 × 4.6 mm I.D., 5 μm column and a mobile phase of acetonitrile and water (16 : 84). There is a method on the analysis and identification of a chlorinated impurity of clofarabine. This method identifies the chlorinated impurity formed due to triethylamine trihydrofluoride by LC-MS.[8] Another method on related substances in clofarabine crude drug by HPLC is reported by Meng.[9] This method describes the separation of the main peak and related substances on a Shimpak VP-ODS C18 with a mobile phase of acetonitrile and ammonium acetate (10 : 90). On a similar column another method is reported by Zhao for the determination of clofarabine in clofarabine injection.[10] The method consists of acetonitrile : water (10 : 90) as the mobile phase and the detection was performed at a wavelength of 262 nm.

No reference for impurities is available in the method. Shen published a method on the determination of clofarabine and its related substances by HPLC using acetonitrile and water (16 : 84) as a mobile phase.[11] One HPLC method for clofarabine and its enantiomer has been reported by Yang,[12] and Guo[13] published methods using organic solvents in various proportions. In these methods, the concentration of clofarabine was determined by using a stability indicating reverse phase high performance liquid chromatography (RP-HPLC) assay with photodiode array detection. This RP-HPLC method also used 20% methanol as a mobile phase with elution in 5 min.[14–16] Recently, we have developed a method based on supercritical fluid (carbon dioxide) based ultra performance convergence chromatography for the separation and determination of fulvestrant diastereomers.[17] Motivated by this application, we have developed an assay method for clofarabine. There is no literature available on the determination of clofarabine using the green solvent CO_2. Hence, it was felt necessary to develop one such method on a green platform, and this led us to develop and validate this method for clofarabine by UPC^{2TM}.

2. Experimental

2.1 Chemicals and reagents

The samples of clofarabine injection used in the study were obtained from Mylan R&D Center (Hyderabad, India). Reference standards used were in-house standards. The HPLC grade

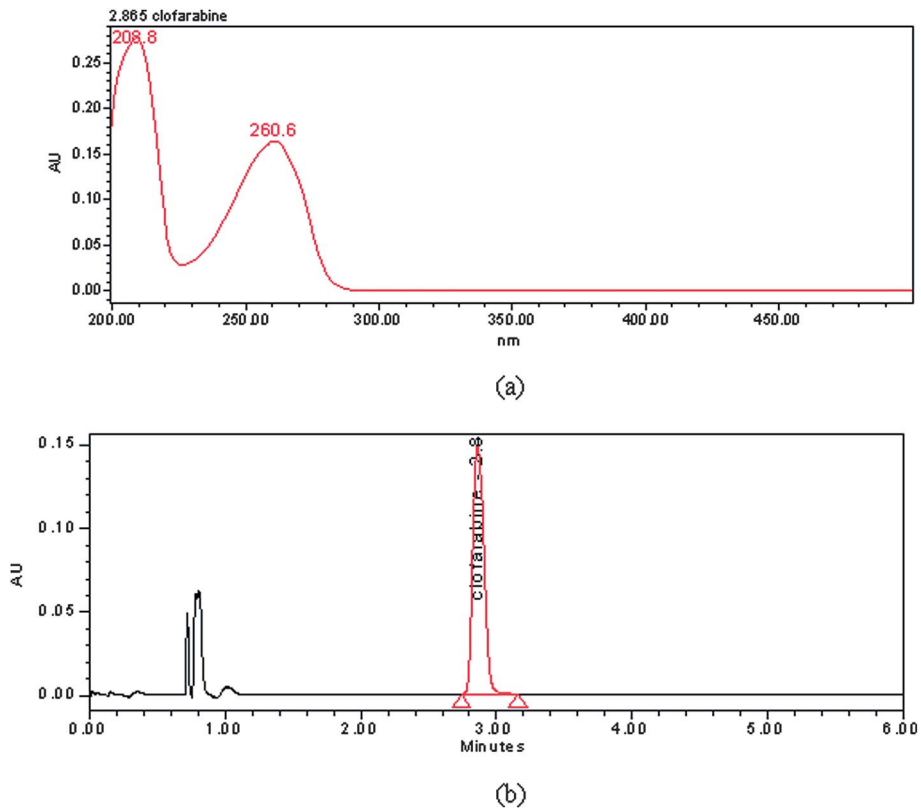

Fig. 2 (a) UV-Visible spectrum of clofarabine, (b) Clofarabine standard chromatogram.

Table 1 SFC screenings[a]

S. no	Column description	Flow rate (mL min^{-1})	Organic modifier (OM)	Mobile phase composition CO_2 : OM (%)	Result
1st screening					
1	Acquity UPLC BEH 3.0 × 100 mm, 1.7 μm	1.0	No	100 : 0	No observable peaks up to 10 minutes
2	Acquity UPLC BEH 2-EP, 3.0 × 100 mm, 1.7 μm	1.0	No	100 : 0	No observable peaks up to 10 minutes
3	Acquity UPC 2-HSS, C18 SB, 3.0 × 100 mm, 1.8 μm	1.0	No	100 : 0	No observable peaks up to 10 minutes
4	Acquity UPLC BEH 2-EP, 3.0 × 100 mm, 1.7 μm	1.0	Methanol	90 : 10	Peaks of impurities and clofarabine are eluted, with less separation. Resolution between isomer and clofarabine is <1.0
5	Acquity UPC 2-HSS, C18 SB, 3.0 × 100 mm, 1.8 μm	1.0	Methanol	90 : 10	Partial elution of peaks observed
2nd screening					
1	Acquity UPLC BEH 2-EP, 3.0 × 100 mm, 1.7 μm	1.0	Methanol	80 : 20	Isomer impurity not resolved properly
2	Acquity UPLC BEH 2-EP, 3.0 × 100 mm, 1.7 μm	1.0	Methanol	70 : 30	Optimum separation
3	Acquity UPLC BEH 2 EP, 3.0 × 100 mm, 1.7 μm	0.7	Methanol	70 : 30	Optimum separation with resolution of 1.5 between isomer impurity and clofarabine

[a] Other chromatographic conditions, diluent and wavelength, are the same throughout.

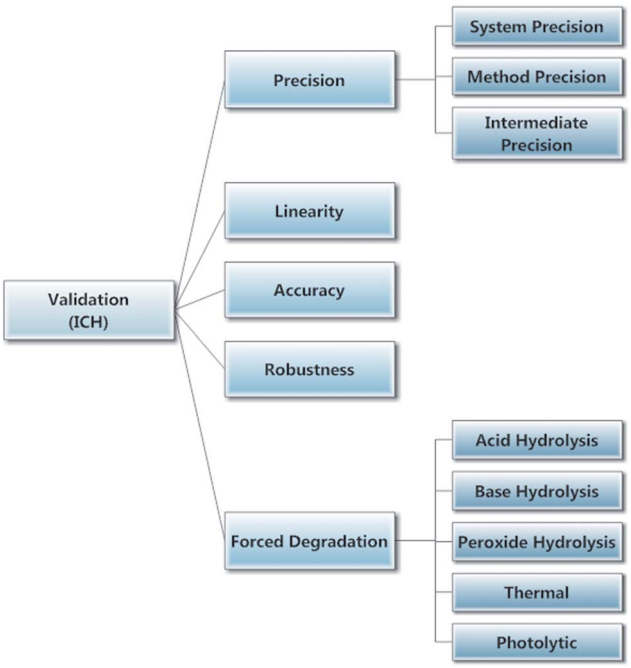

Fig. 3 Validation parameters executed as per current ICH guidelines Q2R1.

methanol was procured from Merck, India. The CO_2 was purchased from Sai Padmaja Oxygen at Hyderabad, India.

2.2 Instrument and chromatographic conditions

An integrated Acquity UPC^2 system from Waters Corporation, Milford, USA equipped with a Waters photodiode array detector (PDA) was used. Data collection and analysis were performed using Empower software 2pro (Waters Corporation). The balance used for weighing the reference standards and impurity standards was from Metler Toledo.

Separation of clofarabine from its potential impurities, namely chloro impurity, monobenzoate impurity, and alpha anomer impurity, was achieved on a BEH-2EP column (100 mm × 3.0 mm, I.D and 1.7 μm) at 50 °C. A simple mobile phase containing liquid CO_2 and methanol mixed in the ratio 70 : 30 (v/v) respectively was pumped in an isocratic mode at a flow rate of 0.7 mL min^{-1} throughout the run. The injection volume was 2 μL and detection was carried out at a wavelength of (λ_{max}) 254 nm.

2.3 Standard and sample preparations

100% methanol was used as a diluent for preparing the standards and samples. The standard solution was prepared by dissolving an amount of clofarabine in the diluent and suitably

Table 2 System suitability results obtained and validation parameters

System suitability	Method precision	Intermediate precision	Linearity	Accuracy	Forced degradation	Robustness
(%RSD)	0.4	0.5	0.4	0.4	0.4	[a]

[a] The system suitability for robustness experiment is shown in Table 4.

Table 3 Precision and accuracy results

Validation parameter							Mean	STD dev	%RSD
Precision[a]									
System suitability									
	% Clofarabine								
Method precision	98.1	98.4	98	97.9	98.9	97.4	98.1	0.504	0.5
Intermediate precision	97.5	99.1	98.3	98.1	97.9	99	98.3	0.627	0.6
Regression analysis									
Correlation	0.99987								
Slope	6787.5754								
Intercept	−4277.1974								
Residual sum of squares	106 194 783.7								
Accuracy[b]									
	%Recovery						Mean		%RSD
50%	100	100	99.2				99.7		0.5
100%	99.2	98.8	99.2				99.1		0.2
150%	100.3	100	100.3				100.2		0.2

[a] Six replicate sample preparations. [b] Three replicate preparations.

diluting to obtain a concentration of 125 μg mL^{-1}. The sample solution was prepared by diluting 2.5 mL of the formulation to 20 mL with methanol to obtain a final concentration of 125 μg mL^{-1}. Individual impurities were prepared in methanol and spiked at a 1.0% level in the sample. Placebo solutions equivalent to one sample concentration were prepared by suitably diluting with methanol.

2.4 Forced degradation study

Forced degradation studies were conducted on samples and plain placebo to prove the specificity of the method. The specificity studies were carried out by injecting the placebo and stressed sample at a concentration of 125 μg mL^{-1}. Sample and placebo solutions were exposed to various stress conditions (such as acid, base, peroxide, UV, heat and light) as per the ICH guidelines. The clofarabine peak was calculated and the peak purity of clofarabine was checked by using the PDA detector.

2.5 Solution stability

The solution stability of standard and sample solutions was assessed by leaving both test solutions at controlled room temperature for 24 hours.

3. Results and discussion

3.1 Method development and optimization

3.1.1 SFC 1st tier screening. Before going on to develop an assay method for clofarabine, the UV-absorption spectrum was measured to find the absorption maxima for this particular drug. Clofarabine has an absorption maximum at 260 nm, and a clofarabine peak was confirmed in the HPLC at that particular wavelength. Both UV-absorption spectra and HPLC chromatogram are shown in Fig. 2. A way to speed up the screening procedure is to improve the method development capacity using the UPC[2] multicolumn manage Aux.

Table 1 describes the various trials taken in the first screening and the corresponding results obtained. In the first screening, with 100% CO_2 no peak resulted within 10 minutes. A small amount (10%) of organic modifier methanol was introduced and further trials were undertaken. From the first screening, the BEH-2EP column was found to be better than other columns in terms of separation and peak shape. Furthermore, the method was optimized in the second screening.

3.1.2 SFC 2nd tier screening. By slightly modifying the ratio of organic modifier from 10% to 30% and the flow rate to 0.7 mL min^{-1}, an optimum resolution of 1.5 is achieved between the isomer and clofarabine.

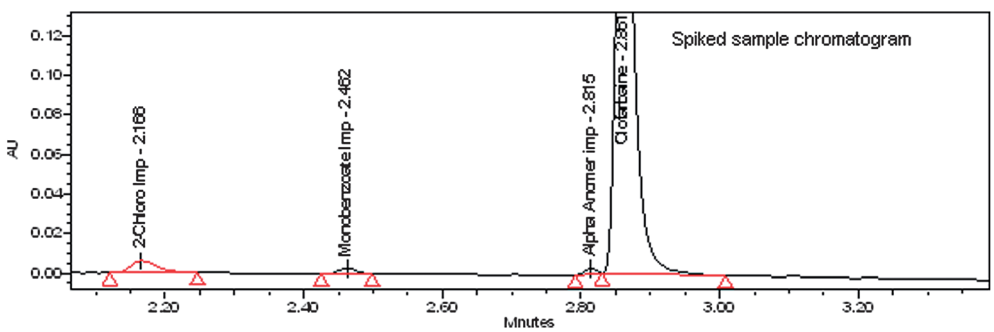

Fig. 4 Chromatogram of impurities spiked in clofarabine formulated sample.

Table 4 The data of stress conditions, assay of clofarabine injection and peak purity

Entry no.	Stress parameter	Stress condition	Assay of degraded sample	Peak purity
1	Controlled sample (no degradation)	N/A	98.1	Pass
2	Spiked sample	N/A	98.6	Pass
2	Acid degradation	2 mL of 2 N HCl for 10 hours	97.8	Pass
3	Base degradation	2 mL of 2 N NaOH for 10 hours	83.3	Pass
4	Hydrogen peroxide degradation	2 mL of 30% H_2O_2 for 10 hours	95.8	Pass
5	Photolytic degradation (UV)	200 watts per unit	93.6	Pass
6	Photolytic degradation (sunlight)	1.2 m lux hours	96.2	Pass

3.2 Method validation

The optimized method was validated in accordance with the current ICH guidelines (Q2R1) on Validation of Analytical Procedures: Text and Methodology.[18] Fig. 3 shows the validated executed parameters which are related to the method development as per ICH guidelines Q2R1.

3.2.1 System suitability. System suitability parameters were measured to verify the system performance. For this, system precision was determined on six replicate injections of standard preparations. %RSD was also evaluated. The system suitability results obtained during the method validation are shown in Table 2.

3.2.2 Linearity. The linearity of the detector response was established by injecting the clofarabine standard solution in concentrations ranging from 62.62 to 187.87 μg mL^{-1} (50% to 150% of the target concentration) and the correlation coefficient was determined. The obtained correlation coefficient was 0.9999. The regression data are summarized in Table 3.

3.2.3 Accuracy. The accuracy of the analytical procedure expresses the degree of the closeness of the obtained results to the true values. The accuracy of the method was evaluated at three different concentrations, namely 62 μg mL^{-1}, 125 μg mL^{-1}, and 187 μg mL^{-1}, of the drug product and assays were calculated for each added amount. The average assays were in the range of 99.1 to 100.2 with %RSD ranging from 0.2 to 0.5. The accuracy results summarized in Table 3 show the method is accurate and precise.

3.2.4 Precision. The HPLC-system precision was established using clofarabine standard (125μg mL^{-1}) by injecting six replicate injections. RSD (%) was calculated for the areas of clofarabine peaks. The repeatability of the method was established by preparing and injecting six samples at 125 μg mL^{-1} and %RSD (%) of the assays were calculated (Table 3). Intermediate precision was carried out by analyzing the samples on the same instrument but by a different analyst on a different day to evaluate the ruggedness of the method. The results of the precision studies are summarized in Table 3.

The precision results show that the method is precise, with assay ranging from 97.9% to 98.4% with an RSD of 0.5%.

3.2.5 Specificity. The specificity of the method was determined by analyzing the placebo solution, the spiked sample and the stressed samples on a PDA detector. The samples were evaluated for assay and peak purity. Fig. 4 shows a enlarged chromatogram obtained with the spiked sample. Table 4 summarizes the assay of the degraded sample and the peak purity of clofarabine.

The result shows that there was a significant drop in the assay of clofarabine in the base and UV stressed samples. In the presence of base, the assay value was 83.3% and 93% in UV-light, indicating that clofarabine is sensitive to base hydrolysis and UV light. In all other stress conditions the assay of clofarabine was ranging from 95.8–98.6%. The purity angle was less than the purity threshold demonstrating the analyte peak homogeneity.

3.2.6 Robustness. The robustness of an analytical method is the measure of its capacity to remain unaffected by small but deliberate changes in the method parameters. To determine the robustness of the method, deliberate variations were made in the parameters such as ABPR (active back pressure regulator), flow rate, column temperature and organic composition. The conditions and results were summarized in Table 5.

Table 5 Variable conditions and results for robustness experiments

S. no	Chromatographic conditions	Actual conditions	Altered conditions	Retention time of clofarabine[b] (minutes)	System suitability[a] (%RSD)
1	Flow rate (mL min^{-1}) ± 10%	0.7	0.63	2.91	1.5
			0.77	2.73	0.9
2	Column temperature ± 5 °C	50 °C	45 °C	2.85	0.8
			55 °C	2.83	0.7
3	ABPR (±100)	2200	2100	2.95	1.1
			2300	2.80	0.9
4	Organic variation (%) ± 10	30	27	2.93	0.9
			33	2.78	0.8

[a] %RSD obtained from actual conditions (method precision) is 0.5. [b] RT of clofarabine in actual conditions is about 2.8 minutes.

The above results showed that the retention time of clofarabine varied with flow rate (−) and ABPR (−). However, those were within the acceptance criteria of ±10% for the retention time. The variation in system suitability, *i.e.*, %RSD, was greatest for flow rate (−) at 1.5%, which is also within the acceptance criteria of ±2% for repeatability of standards. The method is robust under all chromatographic conditions as shown above.

4. Concluding remarks

The rapid isocratic UPC^2 method developed for the quantitative determination of clofarabine is specific, precise, accurate, linear, and robust. The obtained results from the validation studies are satisfactory. This method exhibits excellent performance in terms of sensitivity and speed, and is also cost-effective. This being a green technology is environmentally friendly in terms of waste generated from the system and can be successfully employed for routine assay testing of clofarabine injection samples.

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References

1 M. Koel and M. Kaljurand, *Pure Appl. Chem.*, 2006, **78**, 1993.
2 L. T. Taylor, *J. Supercrit. Fluids*, 2009, **47**, 566.
3 Overview of Acquity UPC^2 System http://www.waters.com/waters/en_US/ACQUITY-UPC2/nav.htm?cid=134658367.
4 Description of clofarabine, http://www.drugbank.ca/drugs/DB00631.
5 B. Zhu and J. F. Y. Huang, *Zhongliu Yaoxue*, 2011, **1**, 140.
6 B. Zhu, L. Zhao, Q. Wang, C. Zhang, X. Chen, J. Huan and Y. Fang, *Zhongguo Yaofang*, 2011, **22**, 2056.
7 Z. Q. Ma, Z. Y. Hong and J. J. Luan, *Zhongguo Linchuang Yaolixue Yu Zhiliaoxue*, 2011, **16**, 852.
8 H. Xiao, Y. Wang and Z. Xia, *Shipin Yu Yaopin*, 2011, **13**, 186.
9 D. Meng, R. Fu, R. Zhang and L. Lu, *Zhongguo Yaofang*, 2009, **20**, 2461.
10 J. Zhao and X. Chen, *Zhongguo Yaoxue Zazhi*, 2009, **44**, 1666.
11 W. Shen, C. Gong and J. Chen, *Zhongguo Yaoxue Zazhi*, 2009, **44**, 1181.
12 L. Yang, *Faming Zhuanli Shenqing*, CN 101206201 A, 2008, p. 0625.
13 H. Guo, Q. Shi and A. Cui, *Faming Zhuanli Shenqing Gongkai Shuomingshu*, CN 1920550 A, 2007, p. 0228.
14 J. Kaiser and I. Krämer, *J. Oncol. Pharm. Pract.*, 2012, **18**, 213.
15 V. Reichelova, J. Liliemark and F. Albertioni, *J. Pharm. Biomed. Anal.*, 1995, **13**, 711.
16 S. L. Berg, P. L. Bonate, J. G. Nuchtern, R. Dauser, L. McGuffey, B. Bernacky and S. M. Blaney, *Clin. Cancer Res.*, 2005, **11**, 5981.
17 G. Venkata Narasimha Rao, G. Gnanadev, B. Ravi, D. Dhananjaya, P. Manoj, B. Indu and R. Venkata Nadh, *Anal. Methods*, 2013, **5**, 4832.
18 ICH, Q2 (R1), *Validation of Analytical Procedures: Text and Methodology*, 2005.

