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New RP-HPLC Method for the Determination of Fludarabine in Pharmaceutical Dosage Forms

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ABSTRACT

A simple, precise and accurate RP-HPLC method was developed and validated for rapid assay of fludarabine in tablet dosage form. Isocratic elution at a flow rate of 1ml/min was employed on a kromasil C18 column (250 X 4.6 mm, 5µ) at ambient temperature. The mobile phase consisted of Methanol: Acetonitrile: Phosphate Buffer 50:20:30(v/v) in the ratio of 50:20:30%,v/v/v. The UV detection wavelength was 265 nm. The retention time for fludarabine was 5.97 min. The percentage RSD for precision and accuracy of the method was found to be less than 2%. The method was validated as per the ICH guidelines. The method was successfully applied for routine analysis of fludarabine in tablet dosage form.

Key Words: fludarabine , RP-HPLC, UV detection, recovery, precise.

Introduction

Fludarabine is a drug used to treat hematological malignancies, which are cancers of blood cells such as leukemia's and lymphomas. The drug is also termed as purine analog, which involves in DNA synthesis. The IUPAC name of the drug is [(2*R*,3*R*,4*S*,5*R*)-5-(6-amino-2-fluoro-purin-9-yl)- 3,4-dihydroxy-oxolan-2-yl]methoxyphosphonic acid. The drug is highly efficient and show good efficacy in the treatment of chronic lymphocytic leukemia, which produces higher response rates compared to alkylating agents such as chlorambucil [1].

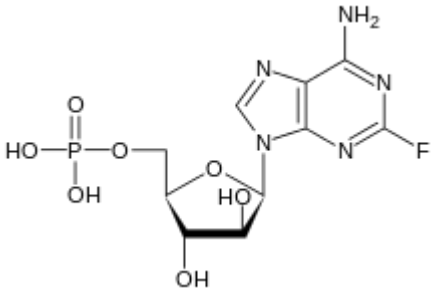

Figure A: Structure of Fludarabine

Some of the side effects of intake of drug were anemia, thrombocytopenia and neutropenia, requiring regular blood count monitoring. Some of patients were associated with autoimmune hemolytic anemia [2].

Literature survey revealed that a few analytical methods were reported for the determination of fludarabine in pure and pharmaceutical dosage forms using HPLC[3,4,5], gradient anion-exchang HPLC[6]. In the present investigation attempts have been made to develop a very fast, accurate and precise method for the analysis of fludarabine in tablet dosage form.

Materials and Methods

Materials

Working standard of Fludarabine was obtained from well reputed research laboratories. HPLC grade Acetonitrile, Water, Methanol, Phosphate buffer was purchased from Merck (Mumbai, India). All the chemicals used in the study were purchased from Rankem.

Instrumentation

Quantitative HPLC was performed on a high pressure gradient high performance liquid chromatography (PEAK LC-7000 liquid chromatography) with two LC-7000 pumps, manual injector with loop volume of 20µL (Rheodyne), programmable variable wavelength PEAK LC-7000 prominence UV-Vis detector and kromasil C-18 Column (4.6 x 250 mm, 5µm).The HPLC system was equipped with "PEAK" software. A Techcomp-2301 model UV/

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Visible spectrophotometry with HITACHI software used for wavelength scanning. Denver electronic analytical balance (SI-234) and pH of the mobile phase was adjusted by using Systronics digital pH meter. Mobile phase consisted of a mixture of Methanol: Acetonitrile (80:20 v/v) at flow rate of 1ml/min using UV detection at 285nm. The mobile phase was filtered through a 0.2 micron membrane filter and degassed. The injection volume was 20 µL and analysis was performed at ambient temperature. Fludarabine showed maximum absorbance at 285nm. So the wavelength selected for the determination of Fludarabine was selected as 265nm.

Preparation of Standard and Sample Solutions

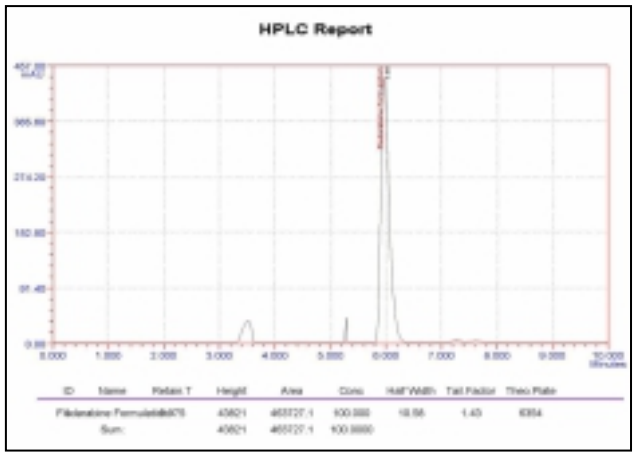
A 10 mg of Fludarabine reference substance was accurately weighed and dissolved in 10 ml mobile phase in a 10 ml volumetric flask to obtain 1000 ppm concentrated solution. Required concentrations were prepared by serial dilution of this solution.

A composite of 20[OFORTA -10mg] tablets was prepared by grinding them to a fine, uniform size powder 10 mg of Fludarabine was accurately weighed and quantitatively transferred into a 100 ml volumetric flask. Approximately 25 ml mobile phase were added and the solution was sonicated for 15 min. The flask was filled to volume with mobile phase, and mixed. After filtration, an amount of the solution was diluted with mobile phase to a concentration of 60ppm.

Results and Discussion

System Suitability

Having optimized the efficiency of a chromatographic separation, the quality of the chromatograph was monitored by applying the following system suitability tests: capacity factor, tailing factor and theoretical plates. The system suitability method acceptance criteria set in each validation run were: capacity factor >2.0, tailing factor ⩽2.0 and theoretical plates >2500. In all cases, the relative standard deviation (R.S.D) for the analytic peak area for two consecutive injections was < 2.0%. A chromatogram obtained from reference substance solution is presented. System suitability parameters were shown in Table 1. Standard chromatogram was given in below Figure (HPLC Report).



Linearity

Standard curves were constructed in three consecutive days, using six standard concentrations in a range of 20, 40, 60, 80, 100 and 120 for Fludarabine. The linearity of peak area responses versus concentrations was demonstrated by linear least square regression analysis. The linear regression equation was y = 5495.9 + 140551x (r^2= 0.9993). Linearity values can shown in Table 2.

Table - 1

Optimization of parameters for estimation of Fludarabine

Parameter	Condition
Mobile phase	Methanol: Acetonitrile: Phosphate Buffer 50:20:30(v/v)
Pump mode	Isocratic
pH	4.7
Column	kromasil C18 column (250 X 4.6 mm, 5µ)
Column Temp	Ambient
Wavelength	265nm
Injection Volume	20µl
Flow rate	1.0ml/min
Run time	10minutes
Pump Pressure	7.2±5MPa
Retention Time	5.97min
Theoretical plates	6110
Tailing Factor	1.08

Table - 2

Linearity results of Fludarabine

S. No.	Concentration in µg/ml	Peak Area
1	20	252985
2	40	358195
3	60	467006
4	80	587252
5	100	682134
6	120	803992
	Slope: 0.025717 Intercept:0.02207 Correlation Coeffi-cient:0.9991	

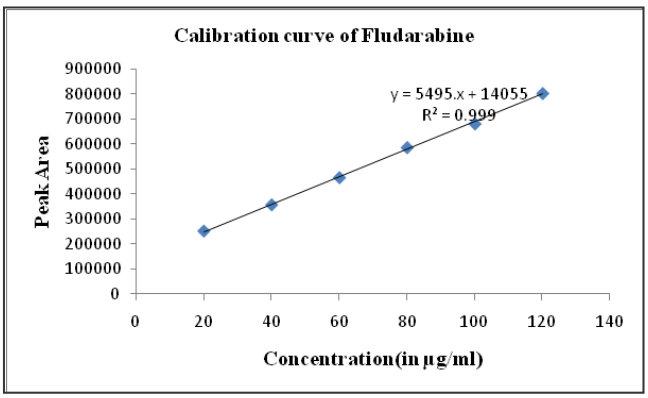


Fig. B : Calibration curve of Fludarabine

Precision

To study precision, six replicate standard solutions of Fludarabine 80µg/ml were prepared and analyzed using the proposed method. The relative standard deviation (% RSD) for peak responses was calculated and it was found that the values were within the acceptance criteria, i.e. RSD of the developed method didn't exceed 2.0%. Results of system precision studies are shown in Table 3 and Table 4.

Table - 3

Intraday Precision Results for Fludarabine

S.NO	Concentration in µg/ml	Peak Area
1	80	467060
2	80	467677
3	80	466978
4	80	476611
5	80	471297
6	80	469043
	RSD: 0.79	

Table - 4

Interday Precision results of Fludarabine

S.NO	Concentration in µg/ml	Peak Area
1	80	470334
2	80	465681
3	80	464910
4	80	466032
5	80	476007
6	80	462203
	RSD: 1.05	

Recovery

The accuracy of the method was determined by standard addition method. A known amount of standard drug was added to the fixed amount of pre-analyzed tablet solution. Percent recovery was calculated by comparing the area values obtained before and after the addition of the standard drug. Recovery test was performed at 3 different concentrations i.e.20, 40 and 60µg/ml. The percent recovery was calculated and results are presented in Table. Satisfactory recovery values were reported ranging from 98.304 to 99.73% by the proposed method. This indicates that the proposed method was accurate. Results are given in table 5.

Robustness

Typical variations in liquid chromatography conditions were used to evaluate the robustness of the assay method. The robustness study was performed by slight modification in flow rate of the mobile phase, composition of the mobile phase and wavelength of the detector. Fludarabine at standard concentration was analyzed under these changed experimental conditions. It was observed that there were no marked changes in chromatograms, which demonstrated that the developed method was robust in nature. The robustness acceptance criteria set in the validation were the same established on system suitability test describe above. Results were shown in table 6.

Ruggedness

Ruggedness was performed by using six replicate injections of standard and sample solutions of concentrations which were prepared and analyzed by different analyst on three different. Ruggedness also expressed in terms of percentage relative standard deviation.

Assay of marketed formulation

To determine the content of Fludarabine in conventional tablet (Brand name: OFORTA ,10mg Fludarabine twenty tablets were weighed, their mean weight determined and finely powdered. The weight of the tablet triturate equivalent to 10mg of Fludarabine into a 50 mL volumetric flask containing 30mL methanol, sonicated for 30 min and diluted upto 50mL with methanol. The resulting solution was determined with the concentration (1000µg/ml). Supernatant was taken and after suitable dilution the sample solution was then filtered using 0.45-micron filter (Millipore, Milford,

MA). The above stock solution was further diluted to get sample solution of 10ppm. A 20µl volume of sample solution was injected into HPLC, six times, under the conditions described above. The peak areas were recorded at 265nm and concentrations in the samples were determined using multilevel calibration developed on the same HPLC system under the same conditions using linear regression equation.

Table - 5

Recovery results of Fludarabine

	Target Conc., (µg/ml)	Spiked conc., (µg/ml)	Final Conc., (µg/ml)	Conc., Obtained	% of Assay
50%	40	20	60	59.47	99.11
	40	20	60	59.50	99.16
	40	20	60	59.32	98.86
100%	40	40	80	79.14	98.93
	40	40	80	78.43	98.04
	40	40	80	78.77	98.46
150%	40	60	100	98.92	98.92
	40	60	100	99.47	99.47
	40	60	100	99.73	99.73

Table - 6

Robustness results of Fludarabine

S.NO	Parameter	Change	Area	% of Change
1	Standard	……	467006	……
2	MP 1	55:15:30	471290	0.46
2	MP 2	45:25:30	466599	0.53
3	pH 1	4.8	473193	0.87
4	pH 2	5.0	469476	0.079
5	WL 1	260nm	476277	1.52
6	WL 2	270nm	466718	0.51

Table - 7

Ruggedness results of Fludarabine

S.NO	Concentration in µg/ml	Peak Area
1	80	479865
2	80	476685
3	80	478161
4	80	475599
5	80	473482
6	80	474060
	RSD: 0.51	

Table - 8

Formulation Analysis

S.NO	Brand name	Available form	Label claim	Concentration	Amount found	% Assay
1	OFORTA	Tablet	10mg	60µg/ml	59.58µg/ml	99.30

Discussion of the Results

The study was aimed to develop a stability indicated RP-HPLC method for the determination of Fludarabine in bulk and pharmaceutical dosage forms. The initial trails were conducted based upon the peak symmetry and time reduction in the chromatographic analysis. The C18 column was selected to conduct the method development study based on the polarity of Fludarabine. All the trails were done using kromasil C18 (250 X 4.6mm, 5 µm) column as stationary phase. The mobile phase was assessed after conducting the various trails using the solvents of methanol, acetonitrile and phosphate buffer. The mobile phase of Methanol: Acetonitrile: Phosphate Buffer 50:20:30 (v/v) was used and the result showed a good peak shape with limits of system suitability. The UV spectrum of the drug shows an absorption band at 265nm; therefore, the wavelength of detection was fixed at 280 nm. The optimized conditions are showed in table 3.A. The results of the specificity were showed there was no interference and co-elution of any other peaks with the retention of Fludarabine. The peak purity of Fludarabine and sample was found within the limit which proved that there was no interference between the blank and placebo peaks. Hence the developed method was specific.

The analytical method was validated according to International Conference on Harmonization (ICH) guidelines. The method was validated for accuracy, precision, specificity, detection limit, quantitation limit and robustness. The accuracy of the method was determined by calculating recovery of Fludarabine by method of standard additions.

The instrument precision was evaluated by injecting the different five concentrations three times and peak area was measured. The results are reported in terms of relative standard deviation. The intraday and interday precision study of Fludarabine was carried out by estimating the corresponding responses 3 times on the same day and on 3 different days and the results are reported in terms of relative standard deviation (RSD). %RSD was found to be 0.75 for Intraday and 1.05 for Interday precision (Table 3.C, 3.D). This found to be the method was precise. Robustness of the method was studied by deliberately changing the experimental conditions like Mobile phase ratio, Wavelength of the detector and the pH of the mobile phase. It was observed that there were no marked changes in chromatograms, which demonstrated that the developed method was robust in nature (Table 3.F).

Limit of detection (LOD) and limit of quantification (LOQ) were found to be 0.05µg/ml and 0.16µg/ml respectively. This vales conforms the sensitivity of the developed method. The formulation analysis of the method was expressed by using commercially available marketed formulation (OFORTA-10mg). The % assay was found to be 99.30, conforms that the method can be successfully applied for the estimation of Fludarabine in pharmaceutical dosage forms.

Conclusion

The proposed RP-HPLC method is rapid, simple, specific, accurate, economical and precise for the estimation of Fludarabine in Pharmaceutical dosage form. There were few analytical methods i.e. HPLC and spectrophotometer reported so far for this estimation. The excipients of the commercial sample analyzed did not interfere in the analysis, which proved the specificity of the method for this drug. Hence the developed RP-HPLC method can be conveniently adopted for the routine analysis.

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