Research Article

New Reverse-Phase High Performance Liquid Chromatographic Method for the Determination of Zalcitabine in Pharmaceutical Tablet Dosage Forms

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ABSTRACT

An accurate, sensitive, precise, rapid and isocratic reversed-phase HPLC, (RP-HPLC) method for simultaneous estimation of Zalcitabine in the bulk drug and in Pharmaceutical fixed dosage forms has been developed and validated. The best separation was achieved on a 150 mm × 4.6 mm i.d., 5-µm particle size of Zorbax Eclipse XDB-C18 Column with is 0.1% ortho-phosphoric acid in 1000ml water. Solvent-B is acetonitrile in the gradient mode of elution as mobile phase at a flow rate of 1.0 mL min−1. UV detection was at 265 nm. Retention times were found to be 8.04 min. for Zalcitabine. Response was a linear function of concentration over the range of 3-18 mcg/mL for zalcitabine with correlation coefficient of around 0.9999. The percentage assay was found to be 98.85. The limit of detection (LOD) and limit of quantification (LOQ) for Zalcitabine were found to be 0.015µg/ml and 0.03 µg/ml respectively. The excipients present in the formulation did not interfere with the assay. The method is suitable for application in quality-control laboratories, because it is simple and rapid with good accuracy and precision.

Keywords: Zalcitabine HIVID®, Gradient-RP-HPLC, and Tablet dosage forms.

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INTRODUCTION

The 3'-hydroxyl group in deoxycytidine has been substituted with hydrogen in zalcitabine, a synthetic form of the naturally occurring nucleoside (2003)[1-3]. Zalcitabine is converted to the active form dideoxycytidine 5'-triphosphate intracellularly (ddCTP). Despite the fact that the chemical was first described in 1966 by Bartlett, the antiviral activity of zalcitabine was found in 1986. (1999). The FDA granted zalcitabine, an inhibitor of HIV-1 reverse transcriptase, for use in conjunction with zidovudine (AZT) for the treatment of HIV infection in 1992[4-6]. Zalcitabine is a synthesized dideoxynucleoside[8-10]. After intracellular phosphorylation to its active metabolite, zalcitabine inhibits the gamma form of DNA polymerase present in tumor cell mitochondria, resulting in tumor cell mitochondrial DNA replication inhibition and tumour cell death[11-12].

Chemistry and Mechanism of action

A dideoxynucleoside substance in which the 3'-hydroxyl group on the sugar moiety has been changed by a hydrogen. This variation prevents the formation of 5' to 3' phosphodiester linkages, which are needed for the elongation of DNA chains, therefore leading to the termination of viral DNA growth. The compound is a powerful inhibitor of HIV replication at reduced concentrations, working as a chain-terminator of viral DNA by binding to reverse-transcriptase. Its major harmful side effect is axonal degeneration causing outer neuropathy. Zalcitabine is a nucleoside reverse transcriptase prevention (NRTI) with task versus Human Immunodeficiency Infection Kind 1 (HIV-1). Within cells, zalcitabine is transformed to its active metabolite, dideoxycytidine 5'-triphosphate (ddCTP), by the sequential action of mobile enzymes. ddCTP hinders viral RNA-directed DNA polymerase (reverse transcriptase) by contending for application of the natural substratum deoxycytidine 5'-triphosphate (dCTP), along with incarnating right into viral DNA. Because of it's lack of a 3'-OH team, the development of a 5' to 3' phosphodiester link that is necessary for DNA chain prolongation is inhibited, hence resulting in the termination of viral DNA development. The objective of the work was to develop a precise and efficient RP-HPLC method to estimate the zalcitabine in pharmaceutical dosage forms. This paper also deals with the validation of the developed method, as per USP guidelines.[12-15]

MATERIALS AND METHODS

Chemicals and Reagents

Zalcitabine of 99% pure are acquired from Sigma-Aldrich Chemicals, Mumbai, India. Acetonitrile HPLC Grade and Methanol HPLC Grade from Rankem Fine chemicals of HPLC Grade. Trifluoroacetic acid from Rankem Fine Chemicals AR grade and HPLC Grade water from sigma-Aldrich.

Chromatography Instrument

Quantitative HPLC was performed on the Waters Alliance 2695 Separations Module is a high-performance liquid chromatographic system with a quaternary, low-pressure mixing pump and inline vacuum degassing. Flow rates from 50 µL/min to 5 mL/min can be generated for use with 2.1 mm ID columns and larger. The auto-sampler has a maximum capacity of 120 vials (12x32, 2-mL) with programmable temperature control from 4 to 40°C. A heated column compartment provides temperatures from 5 degrees above ambient to 65°C. The detector is a photodiode array (model 2996) with a wavelength range of 190-800 nm and sensitivity settings from 0.0001-2.0000 absorbance units. Zorbax Eclipse XDB-C18 Column (150x4.6 mm i.d; particle size 5 µm) was used. The HPLC system was equipped with Empower solution software.

Preparation of Standard drug solution

A standard stock solution of the drug was prepared by dissolving 0.750 mg (750 µg) of Zalcitabine in 50 ml volumetric flask containing 30 ml of mobile phase, sonicated for about 15 min and then made up to 50 ml with mobile phase to get approximately 15µg/mL.

Preparation of Sample solution

20 tablets of Zalcitabine (**HIVID® 0.75mg**, Film coated tablets, Roche Labs) were and then powdered. A sample of the powdered tablets, equivalent to 0.75 mg of the active ingredient, was mixed with 30 ml of mobile phase in 50 ml volumetric flask. The mixture was allowed to stand for 1 hr with intermittent sonication for complete solubility of the drug, and then filtered through a 0.45 µm membrane filter, followed by addition of mobile phase up 50 ml to obtain a stock solution of 15µg/mL.

Optimized chromatographic conditions

Column- Zorbax Eclipse XDB-C18 Column (150x4.6mm, 5µm)

Mobile phase: Solvent-A is 0.1% *ortho*-phosphoric acid in 1000ml water. Solvent-B is acetonitrile and Diluent is Water: Acetonitrile 30:70 (v/v)

Flow rate – 1.0 ml/min.

Run time—15 min

Temperature- ambient.

Injection volume--20 µL

Detection wavelength--265 nm

Retention time—8.08 min

Preparation of Mobile phase

The contents of the mobile phase were aqueous 0.1 % v/v o-phosphoric acid (mobile phase solvent-A) and acetonitrile (mobile phase solvent-B) in a gradient mode of separation was used to resolute the Zalcitabine. They were filtered before use through a 0.45µm membrane filter and degassed by sonication. The gradient program has been shown in Table-1.3

Time in minutes	Mobile phase solvent-A	Mobile phase Solvent-B
0	70	30
2	70	30
5	20	80
10	20	80
11	70	30
15	70	30

Procedure

Initially the mobile phase was pumped for about 30 min, to saturate the column thereby to set the baseline corrected. Then 20 µl of Zalcitabine standard and sample solution were injected separately. A quantitative determination of the active ingredient was made by compare the peak area of a sample injection to the corresponding peak area of a standard injection. The amount of Zalcitabine present in the sample was calculated through the standard calibration curve.

Linearity

Aliquots of standard Zalcitabine stock solution were taken in different 10 ml volumetric flasks and diluted up to the mark with the mobile phase such that the final concentrations of Zalcitabine are in the range of 15-180 µg/ml. Each of these drug solutions (20 µL) was injected three times into the column, and the peak areas and retention times were recorded. Evaluation was performed with PDA detector at 260 nm and a Calibration graph was obtained by plotting peak area versus concentration of Zalcitabine (Fig 1.8). The linearity Chromatograms presented in Fig-2.

Accuracy

The accuracy of the method was determined by measuring the recovery of the drugs by the standard addition method. Known amounts of each drug (10% standard drug solution) corresponding to 80%, 100%, and 120% of the target test concentrations were added to a formulation mixture to determine whether the analytes present in the formulation led to positive or negative interferences. Each set of additions was repeated three times at each level. Extraction sample preparation procedure is followed and assayed against qualified reference standard. The accuracy was expressed as the percentage of the analytes re-covered by the assay. The results of accuracy studies from standard solution and process related impurity were shown in Table-; recovery values demonstrated that the method was accurate within the desired range (Table-2).

Precision

Intra-day and inter-day precision were evaluated by analyzing quality-control samples present in 100% dilution. For intra-day variation, sets of 6 replicates of the target

concentrations were analyzed on the same day; for inter-day variation, six replicates were analyzed on three different days. The low value (≤1%) of RSD indicates the repeatability of the method (Table 3).

Limits of Detection and Quantification

Limit of detection (LOD) of the method was determined as the lowest concentrations of active pharmaceutical ingredients producing a signal-to-noise (S/N) ratio of about 3. The limit of quantification (LOQ) was determined as the lowest concentrations of active pharmaceutical ingredients capable of being quantitated with acceptable accuracy and precision producing signal-to-noise (S/N) ratio of about 10.

Method Applicability

The present developed method was evaluated by applying to pharmaceutical metered dose aerosols for the estimation of Zalcitabine by our research group.

RESULTS AND DISCUSSION

HPLC Method Development and Optimization

In response to lack of simple, reliable and quick method of analysis for the determination of zalcitabine concentrations in pharmaceutical matrices, a gradient reversed-phase HPLC method was developed for quantification of above mentioned active pharmaceutical ingredients. We examined several HPLC method variables with respect to their corresponding effects on the result of analysis. To optimize the chromatographic conditions, different combinations of methanol-water and acetonitrile-water and acetonitrile-dipotassium phosphate buffer were tested. 0.1 % v/v o-phosphoric acid (mobile phase solvent-A) and acetonitrile (mobile phase solvent-B is preferred because it resulted in greater resolution of active pharmaceutical ingredients after several preliminary investigatory runs, compared with other mobile phases. The other parameters in this factorial design were temperature, flow rate, detection wavelength and volume of injection. *O*-PA was selected as 0.01M on the basis of theoretical plate number with symmetrical peak shape. At 265 nm, UV response of both the active pharmaceutical analyte was good and free form interferences. Under these conditions, the analyte peaks were well defined and free from tailing. Considering the whole body of the data obtained from this extensive study, the set of conditions indicated earlier in this article was selected for further validation.

Method Validation Tests

Recommended method validation characteristics including method precision (RSD, %), method accuracy (recovery % and RSD, %), linear range (correlation coefficient), and LOD & LOQ, were investigated.

Linearity

The plot of peak areas of each sample against respective concentrations was found to be linear in the range of 3-18 µg/mL for zalcitabine with correlation coefficient of 0.9999 (Table 4). Linear regression least square fit data obtained from the measurements are given in Table I. The respective linear regression equation being y = 651183x for Zalcitabine. The regression characteristics, such as slope, intercept, and %RSD were calculated for this method and given in Table 1**.** These results show there was an excellent correlation between peak areas and analyte concentration.

Accuracy

Recovery of the individual substances at 80%, 100%, and 120% of specified concentrations were between 97.25% and 104.25%, which proves the accuracy of the method. From these data, RSD was always less than 1%, which indicates it is obvious that the method is remarkably accurate, produces reliable results (Table 2).

Precision

The intra-day and inter-day variability or precision data are summarized in Table 3. The low value (<1%) of RSD indicates the repeatability of the method. These data indicate a considerable degree of precision and reproducibility for the method both during one analytical run and between different runs (Table 3).

Robustness

Robustness was studied out to evaluate the effect of small but deliberate variations in the chromatographic conditions at three different levels, i.e. –2, 0, +2. To determine the robustness of this method, the experimental conditions were deliberately altered at three different levels and retention time and chromatographic response were evaluated. One factor at a time was changed to study the effect. Variation of the stationary phase and mobile phase flow rate by 1.0 mL min−1 (0.8- and 1.2-mL min^{-1}) had no significant effect on the retention time and chromatographic response of the method, indicating that the method was robust.

Limit of Detection and Limit of Quantifications

The LOQ can be determined by a signal-to-noise ratio of 10:1 or approximated by multiplying the LOD by 3.3. This method is commonly applied to analytical methods that exhibit baseline noise. The limit of detection (LOD) and limit of quantification (LOQ) for Zalcitabine were found to be 0.015µg/ml and 0.03 µg/ml respectively. These values reflect the high sensitivity of the method, which is of great importance in most studies and also indicating the method can be used for detection and quantification of analytes in a very wide concentration range.

Specificity

From the typical chromatogram, shown in Fig-1, the retention times were found to be 8.04 for zalcitabine. No evidence of signals, in the corresponding times of the chromatogram were monitored as a sign of potential interfering peaks, was found when the pharmaceutical fixed dosage forms were tested. Hence, this method can

be used reliably for the estimation of respected active pharmaceutical ingredients in a variety of dosage forms.

Table 1: Results from regression analysis and System Suitability data:

Parameter	Zalcitabine
Retention Time (min)	8.04
Tailing Factor	1.05
Peak areas	9371985
Percentage of peak areas	99.65
Theoretical Plates	22282
Linear range in (µg/mL)	3-18
Limit of Detection (LOD) (mcg/mL).	0.003
Limit of Quantification (LOQ) (mcg/mL)	0.0099
Correlation Coefficient (r)	0.9999
% of Assay	97.85

Table 2: Summery of the calibration data / Linearity studies.

Concentration of drug (µg/mL)	Retention time	Peak Area
3	8.10	1964756
6	8.04	3915815
9	8.05	5925174
12	8.10	7841027
15	8.0	9648128
18	8.04	11766063

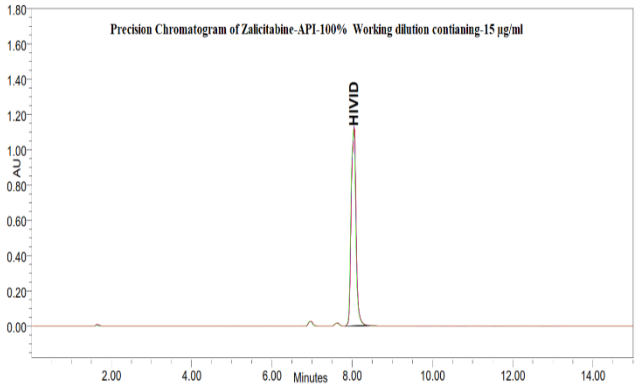

Figure 1: Typical Chromatogram of Zalcitabine (15µg/mL) analyzed by optimized Isocratic RP-HPLC method

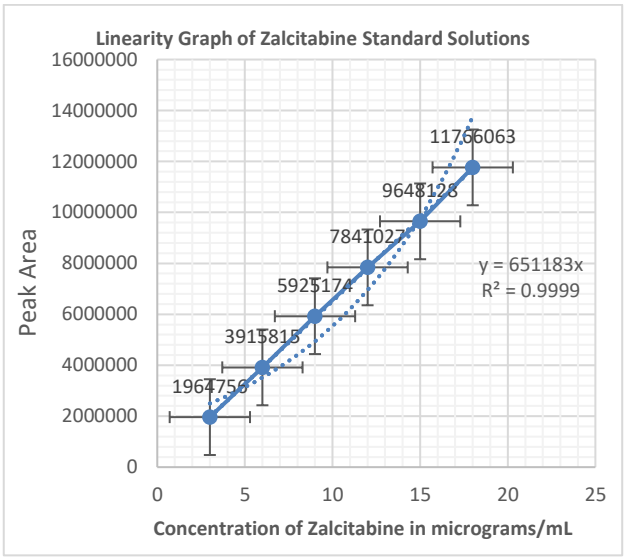

Figure 2: Calibration Curve of Zalcitabine standard dilutions:

Table 3: Precision Studies of Zalcitabine in Standard & Sample dilutions with statistics.

Precision of Standard drug with statistics			
Injection No.	**Name of the drug & conc. (15 µg/ml)**	**Retention time in min.**	**Peak Area**
1	Zalcitabine injection-1	8.05	9362310
2	Zalcitabine injection-2	8.03	9368131
3	Zalcitabine injection-3	8.04	9375054
4	Zalcitabine injection-4	8.04	9383751
5	Zalcitabine injection-5	8.05	9398693
6	Zalcitabine injection-6	8.05	9383511
Mean		8.0	9378575.0
% RSD.		0.0	12976.1
Std. Deviation		0.1	0.1
Precision study of Sample Solution (HIVID® 0.75 mg tablets)			
1	HIVID® injection-1	8.09	9238092
2	HIVID® injection-2	8.08	9269521
3	HIVID® injection-3	8.08	9313302
4	HIVID® injection-4	8.09	9247866
5	HIVID® injection-5	8.08	9226975
6	HIVID® injection-6	8.09	9254200
Mean		8.1	9258326.1
% RSD.		0.0	30549.8
Std. Deviation		0.1	0.3

Table 4: Accuracy studies of Zalcitabine

S. No	Recovery at 80% dilution Level Peak areas		Recovery at 100% dilution Level Peak areas		Recovery at 120% dilution Level Peak areas	
	Standard	Spiked	Standard	Spiked	Standard	Spiked
1	7955319	8938880	9365986	10367636	11864569	12852313
2	7985295	8943586	9371985	10344617	11873919	12876817
3	7987428	8927689	9389118	10340060	11859578	12871581
Avg	**7976014**	**8936718.3**	**9375696.3**	**10350771.0**	**11866022**	**12866903.67**
Std. Dev	17954.1	8166.0	12004.3	14782.2	7280.1	12904.2
%RSD	**0.2**	**0.1**	**0.1**	**0.1**	**0.1**	**0.1**

CONCLUSION

A simple and easily available HPLC method was developed in this study for the quantification of Zalcitabine in pharmaceutical matrices. The main advantages of this method are its considerably shorter run times, easy-to-use and its simplicity. All of these properties are very important in practice, particularly when a large number of samples are to be analyzed. The results of validation tests were, collectively, indicative for a method with a relatively wide linear range, acceptable precision and accuracy and practically reliable sensitivity. The method enables simple, selective, sensitive, and specific analysis of Zalcitabine and can be used for routine analysis in pharmaceutical quality control.

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