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RP-HPLC Method Development and Validation for Simultaneous Determination of Decitabine and Cedazuridine in Pure and Tablet Dosage Form

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Abstract

A simple, rapid, accurate and precise isocratic reversed phase high performance liquid chromatographic method has been developed and validated for simultaneous estimation of Decitabine and Cedazuridine in tablet dosage form. The chromatographic separation was carried out on Zorbax C18 column (150 mm x 4.6 mm I.D., 5 µm particle size) with a mixture of 0.01N potassium dihydrogen phosphate buffer and acetonitrile in the ratio of 65:35% v/v as a mobile phase at a flow rate of 1.0 mL/min. UV detection was performed at 245 nm. The retention times were 2.263 minutes and 3.001 minutes for Decitabine and Cedazuridine respectively. Calibration plots were linear (r^2=0.999 for both Decitabine and Cedazuridine respectively) over the concentration range of 8.75-52.5 µg/mL for Decitabine and 25-150 µg/mL for Cedazuridine. The method was validated for linearity, precision, accuracy, ruggedness and robustness. The proposed method was successfully used for simultaneous estimation of Decitabine and Cedazuridine in tablet dosage form. Validation studies revealed that the proposed method is specific, rapid, reliable and reproducible. The high % recovery and low % RSD confirms the suitability of the proposed method for routine quality control analysis of Decitabine and Cedazuridine in bulk and tablet dosage form.

Keywords: Decitabine, Cedazuridine, Validation, HPLC.

Introduction

Decitabine is indicated for the treatment of patients with myelodysplastic syndromes (MDS) including refractory anaemia, refractory anaemia with ringed sideroblasts, refractory anaemia with excess blasts, refractory anaemia with excess blasts in transformation and chronic myelomonocyticleukaemia (1). Chemically it is, 4-amino-1-[(2R,4S,5R)-4-hydroxy-5-(hydroxymethyl)oxolan-2-yl]-1,2-dihydro-1,3,5-triazin-2-one (2) (Fig. 1). It acts as nucleoside metabolic inhibitor, Decitabine is recognized as a substrate by DNA methyl transferase enzymes (DNMTs). This mode of action depletes DNMTs and results in global DNA hypomethylation (3).

Cedazuridine is acytidine deaminase inhibitor co-administered with the hypomethylating agent. Decitabine is indicated for the treatment of variable forms of myelodysplastic syndrome (MDS**)** (4). Chemically it is, (4R)-1-[(2R,4R,5R)-3,3-difluoro-4-hydroxy-5-(hydroxymethyl)oxolan-2-yl]-4-hydroxy-1,3-diazinan-2-one (Fig.2). It acts as DNA methyltransferase (DNMT) inhibitor

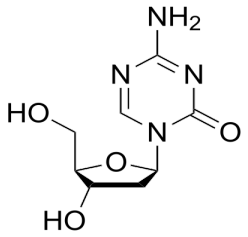

Fig 1. Chemical structure of decitabine

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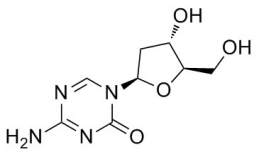

Fig 2. Chemical structure of cedazuridine

which is co-administered with hypomethylating agents like Decitabine. Cedazuridine inhibits major mechanism by which Decitabine is degraded in the gut and liver, and the combination therefore permits the efficient delivery of Decitabine orally (5).

Literature survey reveals that there is only one HPLC method was reported for simultaneous estimation of Decitabine and Cedazuridine in pharmaceutical formulations (6). Therefore, an attempt has been made to develop a novel, rapid, accurate and precise RP-HPLC method for simultaneous estimation of Decitabine and Cedazuridine in tablet dosage form and validated in accordance with ICH guidelines (7).

Materials and Methods

Instrumentation: To develop a high-performance liquid chromatographic method for simultaneous estimation of Decitabine and Cedazuridine using Waters 2695 HPLC system on Zorbax C18 (150 mm x 4.6 mm I.D., 5 µm particle size) column was used. The instrument is equipped with UV-Visible detector. Data was analysed by using Empower 2 software. A Eutech pHmeter was used for pH measurements.

Chemicals and solvents: The marketed formulation of Decitabine and Cedazuridine tablets (Decitabine of 35mg and Cedazuridine of 100mg) were procured from local market. HPLC grade water and acetonitrile were purchased from Rankem Ltd., India. Methanol and potassium dihydrogen phosphate of AR grade was obtained from Rankem Ltd., India.

Determination of working wavelength (λmax): In simultaneous estimation of two drugsisobestic wavelength was used. Isobestic point is the wavelength where the molar absorptivity is the same for two substances that are inter convertible. So this wavelength was used in simultaneous estimation to estimate two drugs accurately. The wavelength of maximum absorption of the solution of the drugs in mixture of 0.01N KH_2PO_4 buffer and acetonitrile (65:35% v/v) were scanned using PDA detector within the wavelength region of 200-400 nm against 0.01N KH_2PO_4 buffer and acetonitrile (65:35% v/v) as blank. The absorption curve shows isobestic point at 265 nm. Thus 265 nm was selected as detector wavelength for the HPLC chromatographic method.

Chromatographic conditions: 0.01N Potassium dihydrogen phosphate buffer and acetonitrile in the ratio of 65:35% v/v was found to be the most suitable mobile phase for ideal chromatographic separation for simultaneous estimation of Decitabine and Cedazuridine. The solvent mixture was filtered through 0.45 µm membrane filter and sonicated before use. It was pumped through the column at a flow rate of 1.0 mL/min. Injection volume was 10 µL and the column was maintained at a temperature of 25℃. The column was equilibrated by pumping the mobile phase through the column for at least 30 minutes prior to the injection of the drug solution. The detection of the drug was monitored at 245 nm. The run time was set as 5 minutes.

Preparation of standard stock solution: Accurately weighed 17.5 mg of Decitabine, 50 mg of Cedazuridine and transferred to 50 mL volumetric flask separately. $3/4^{th}$ of diluents was added to both of these flasks and sonicated for 10 minutes. Flasks were made up with diluents and labelled as standard stock solution 1 and 2. (350 µg/mL of Decitabine and 1000 µg/mL of Cedazuridine).

Sample solution preparation: 5 tablets were weighed and the average weight of each tablet was calculated, then the weight

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equivalent to 1 tablet was transferred into a 100 mL volumetric flask, 50 mL of diluents was added and sonicated for 25 min, further the volume was made up with diluent and filtered by HPLC filters (350 µg/mL of Decitabine and 1000 µg/mL of Cedazuridine). 1 mL of filtered sample stock solution was transferred to 10 mL volumetric flask and made up with diluent. (35 µg/mL of Decitabine and 100 µg/mL of Cedazuridine).

General Preparations:

0.01N KH_2PO_4 buffer: Accurately weighed 1.36 gm of potassium dihydrogen ortho phosphate in a 1000 mL of volumetric flask add about 900 mL of milli-Q water added and degas to sonicate and finally make up the volume with water then pH adjusted to 5.4 with dil. ortho phosphoric acid solution.

0.1%OPA buffer*:* 1 mL of ortho phosphoric acid was diluted to 1000 mL with HPLC grade water.

Preparation of mobile phase: Mobile phase was prepared by mixing 0.01N KH_2PO_4 buffer and acetonitrile taken in the ratio 65:35% v/v. It was filtered through 0.45 µ membrane filter to remove the impurities which may interfere in the final chromatogram.

Preparation of diluent: Based up on the solubility of the drugs, diluent was selected, acetonitrile and watertaken in the ratio of 50:50% v/v.

Assay: Inject 10 µL of the standard, sample into the chromatographic system and measure the areas for Decitabine and Cedazuridine peaks and calculate the % Assay.

Method Validation

System suitability: System suitability is checked by using standard chemical substance to ensure that the analytical system is working properly. In this peak area and % of drug of six determinations is measured and %RSD should be calculated.

Specificity: Specificity of an analytical method is ability to measure specifically the analyte of interest without interference from blank and known impurities. For this purpose blank chromatogram, standard chromatogram and sample chromatogram were recorded. The chromatogram of blank shows no response at the retention times of drugs which confirms the response of drugs was specific.

Linearity: The linearity of the method was obtained by preparation of the calibration standards of six different concentrations in 6 replicates. The calibration curve plots for Decitabine and Cedazuridine were obtained by plotting the peaks areas on y-axis and concentrations on x-axis over the concentration ranges of 8.75-52.5 µg/mL for Decitabine and 25-150 µg/mL for Cedazuridine. The correlation coefficient should be greater than 0.99.

Range: The range of an analytical method is the interval between the upper and lower levels of analyte (including these levels) that have been demonstrated with precision, accuracy and linearity.

Accuracy: The accuracy of the method was assessed by recovery experiments by adding a known quantity of pure standard drug into the sample solution and recovering the same in terms of its peak areas. The sample was spiked with standard at levels of 50%, 100% and 150% of test concentrations. The resultant spiked sample was assayed in triplicate.

Precision: Precision is the degree of repeatability of an analytical method under normal operation conditions. Precision is of 3types,

1. System precision
2. Method precision
3. Intermediate precision (a. intra-day precision, b. Inter-day precision)

System precision is checked by using standard chemical substance to ensure that the analytical system is working properly. In

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this peak area and % of drug of six determinations is measured and %RSD should be calculated. In method precision, a homogenous sample of single batch should be analyzed 6 times. This indicates whether a method is giving constant results for a single batch. In this analyze the sample six times and calculate the %RSD. The precision of the instrument was checked by repeatedly injecting (n=6) solutions of 35 µg/mL of Decitabine and 100 µg/mL of Cedazuridine.

Robustness: As part of the Robustness, deliberate change in the flowrate, mobile phase composition, temperature variation was made to evaluate the impact on the method.

A. The variation of flow rate: Standard solution 35 µg/mL of Decitabine and 100 µg/mL of Cedazuridine was prepared and analyzed using the varied flow rates 0.9 mL/min & 1.1 mL/min flow rate instead of 1.0 mL/min, remaining conditions are kept constant. On evaluation of the above results, it can be concluded that the variation in flow rateaffected the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±20%.

B. The variation of organic phase ratio: Standard solution of 35 µg/mL of Decitabine and 100 µg/mL of Cedazuridine was prepared and analyzed using the varied in mobile phase ratio.

C. The variation of temperature: Temperature minus (25°C) and temperature plus (35°C) were maintained and samples wereinjected in duplicate manner system.

LOD Sample preparation: 0.25 mL each from two standard stock solutions was pipetted out and transferred to two separate 10 mL volumetric flasks and made up with diluents. From the above solutions 0.1 mL each of Decitabine and Cedazuridine solutions respectively were transferred to 10 mL volumetric flasks and made up with the same diluents.

LOQ Sample preparation: 0.25 mL each from two standard stock solutions was pipetted out and transferred to two separate 10 mL volumetric flask and made up with diluent. From the above solutions 0.3 mL each of Decitabine and Cedazuridine, solutions respectively were transferred to 10 mL volumetric flasks and made up with the same diluent.

Forced Degradation Studies

Decitabine and Cedazuridine standard samples were subjected to degradation under different stress conditions like acidic, alkali, oxidative, thermal, photo stability and neutral conditions. For acidic & alkali degradation samples were refluxed with 2N HCl & 2N NaOH at 60°C for 30 min. For oxidative degradation 20% v/v, H_2O_2 was used and the same was refluxed at 60°C for 30 min. For thermal degradation, sample was placed in oven at 105°C for 1 hr; and for photo stability degradation, drug was exposed to UV light by keeping the sample in UV chamber for 7 days or 00 watt hours/m^2 in photo stability chamber; for neutral degradation, the drugs were refluxed in water for 1 hours at a temperature of 60°C. All the samples were diluted to obtain a final concentration of 35 µg/mL of Decitabine & 100 µg/mL of Cedazuridine. Ten micro liters of the samples were injected into the system and the chromatograms were recorded to assess the stability of the sample.

Results and Discussion

The HPLC procedure was optimized with a view to develop an accurate, precise and reproducible method for simultaneous estimation of Decitabine and Cedazuridine in tablet dosage form using Zorbax C18 column (150 mm x 4.6 mm; 5 µm)) in isocratic mode with mobile phase composition of 0.01N potassium dihydrogen phosphate buffer and acetonitrile in the ratio 65:35% v/v. The use of 0.01N phosphate buffer and acetonitrile in the ratio of 65:35% v/v

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resulted in peak with maximum separation, good shape and resolution. Flow rates between 0.9 to 1.1 mL/min were studied. A flow rate of 1.0 mL/min gave an optimum signal-to-noise ratio with reasonable separation time, the retention times for Decitabine and Cedazuridine were found to be 2.263 minutes and 3.001 minutes respectively. Total run time was 5 minutes. The drug components were measured with UV detector at 245 nm. The results of optimized chromatographic conditions were shown in Table 1.

Linearity was obtained in the range of 8.75-52.5 µg/mL for Decitabine and 25-150 µg/mL for Cedazuridine. The correlation coefficient (r^2) was found to be 0.999 for both Decitabine and Cedazuridine respectively. The regression equation of the linearity plot of concentration of Decitabine over its peak area was found to be y=37806x+10196, where x is the concentration of Decitabine (µg/mL) and y is the corresponding peak area. The regression equation of the linearity plot of concentration of Cedazuridine over its peak area was found to be y=31736x+21050, where x is the concentration of Cedazuridine (µg/mL) and y is the corresponding peak area. The results show that an excellent correlation exists between peak area and concentration of drugs within the concentration range indicated. The linearity results were shown in Table 2.

The % RSD for intra-day precision and inter-day precision for Decitabine were found to be 0.6% and 1.0% respectively (limit % RSD<2.0%). The % RSD for intra-day precision and inter-day precision for Cedazuridine were found to be 0.7% and 1.0% respectively (limit % RSD<2.0%) and hence the method is precise. The precision data of Decitabine and Cedazuridine were furnished in Table 3 & Table 4.

The % recovery of the drugs Decitabine and Cedazuridine were found to be 99.71 to 100.17% and 99.91 to 100.50% respectively and the high percentage of recovery of Decitabine and Cedazuridine indicates that the proposed method is highly accurate. The results of accuracy studies of Decitabine and Cedazuridine were shown in Table 5 & Table 6.

The retention times for the drugs Decitabine and Cedazuridine was 2.263 minutes and 3.001 minutes respectively. The number of theoretical plates calculated for Decitabine and Cedazuridine was 6633 and 11974 respectively. The tailing factor for Decitabine and Cedazuridine was 1.52 and 1.305 respectively, which indicates efficient

Table 1. Optimized chromatographic conditions

S. No.	Parameters	Conditions
1	Instrument used	Waters HPLC 2695 System
2	software	Empower 2.0 version
3	Injection volume	10 µL
4	Mobile Phase	0.01N KH_2PO_4Acetonitrile (65:35% V/V)
5	Column	Zorbax C 18 (150 mm x 4.6 mm, 5 µm)
6	Detection Wavelength	245 nm
7	Flowrate	1 mL/min
8	Runtime	5 min
9	Temperature	Ambient (25°C)
10	Diluent	Water and Acetonitrile (50:50% V/V)

RP-HPLC Method Development

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performance of the column. The limit of detection (LOD) and limit of quantification (LOQ) for Decitabine were found to be 0.58 µg/mL and 1.92 µg/mL; 0.99 µg/mL and 3.01 µg/mL for Cedazuridine respectively, which indicate the sensitivity of the method. The summary of system suitability parameters and validation parameters were shown in Table 7.

Table 2. Results of linearity for decitabine and cedazuridine

S. No.	Decitabine		Cedazuridine	
	Conc. (µg/mL)	Peak area	Conc. (µg/mL)	Peak area
1	0	0	0	0
2	8.75	338918	25	809643
3	17.5	668059	50	1614417
4	26.25	1010116	75	2409485
5	35	1362686	100	3225114
6	43.75	1669573	125	4026519
7	52.5	1968823	150	4723591
Regression Equation	y = 37806x + 10196		y = 31736x + 21050	
Slope	37806		31736	
Intercept	10196		21050	
R2	0.999		0.999	

Table 3. Intra-day precision for decitabine and cedazuridine

S. No.	Area for Decitabine	Area for Cedazuridine
1	1338456	3118026
2	1354590	3155597
3	1352438	3180969
4	1333754	3169152
5	1336688	3163536
6	1342507	3178497
Average	1343072	3160963
S.D	8597.6	23038.0
%RSD	0.6	0.7

The robustness studies indicated that no considerable effect on the determination of the drugs. Therefore, the test method is robust for the quantification of the drugs. In all deliberately varied conditions, the %RSD for replicate injections of Decitabine and Cedazuridine were found to be within the acceptable limits.

Validated method was applied for the simultaneous estimation of Decitabine and Cedazuridine in commercial tablet dosage

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forms. The % Assay of Decitabine and Cedazuridine were found to be 101.60% and 100.54% respectively. The results for the drugs assay showed good agreement with label claims. No interfering peaks were found in the chromatogram of the tablet formulation within the run time indicating that excipients used in tablet formulation did not interfere with the simultaneous estimation of the drugs Decitabine and Cedazuridine by the proposed

Table 4. Inter-day precision for decitabine and cedazuridine

Day	Aea for Decitabine	Area for Cedazuridine
1	1310462	3123728
2	1314676	3094017
3	1305798	3154633
4	1338991	3082297
5	1314841	3128306
6	1332908	3076085
Average	1319613	3109844
SD	13221.2	30622.4
%RSD	1.0	1.0

Table 5. Accuracy results of decitabine

% Concentration (at specification level)	Amount Added (µg)	Amount Found (µg)	% Recovery	Mean Recovery
50%	17.5	17.52	100.14	100.01%
100%	35	34.89	99.71	
150%	52.5	52.59	100.17	

Table 6. Accuracy results of cedazuridine

% Concentration (at specification level)	Amount Added (µg)	Amount Found (µg)	% Recovery	Mean Recovery
50%	50	49.95	99.91	100.29%
100%	100.0	100.38	100.38	
150%	150.0	150.87	100.50	

Table 7. System suitability parameters for decitabine and cedazuridine

S. No.	Parameter	Decitabine	Cedazuridine
1	Retention time (min)	2.252	2.979
2	Plate count	6633	11974
3	Tailing factor	1.52	1.305
4	Resolution	----	6.317
5	%RSD	0.9	0.5

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Table 8. Assay of decitabine and cedazuridine

S. No.	Assay of Decitabine			Assay of Cedazuridine		
	Standard Area	Sample Area	% Assay	Standard Area	Sample Area	% Assay
1	1331358	1338456	101.25	3160242	3118026	99.17
2	1331197	1354590	102.47	3144319	3155597	100.36
3	1330607	1352438	102.30	3151862	3180969	101.17
4	1307769	1333754	100.89	3140695	3169152	100.80
5	1312164	1336688	101.11	3129029	3163536	100.62
6	1310781	1342507	101.55	3119742	3178497	101.09
Avg	1320646	1343072	101.60	3140982	3160963	100.54
SD	11492.4	8597.6	0.6504	14795.6	23038.0	0.7327
%RSD	0.9	0.6	0.6	0.5	0.7	0.7

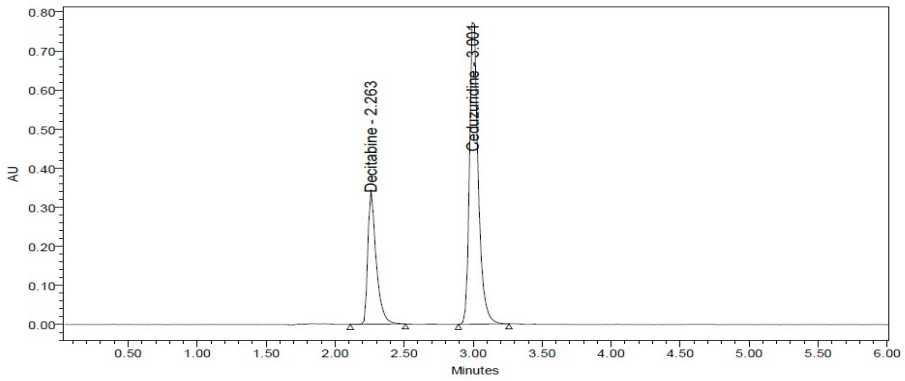

Fig 3. Optimized chromatogram of decitabine and cedazuridine

HPLC method. The assay results are shown in Table 8.

The chromatograms were checked for appearance of any extra peaks under optimized conditions, showing no interference from common tablet excipients and impurities. Also the peak areas were compared with standard and were found to be within limits. As shown in chromatogram, two analytes are eluted by forming symmetrical peaks. The typical chromatogram of Decitabine and Cedazuridine standard were shown in Fig. 3.

Conclusion

The developed RP-HPLC method for the estimation of selected drugs is simple, rapid, accurate, precise, robust and economical. The mobile phase and solvents are simple to prepare and economical, reliable, sensitive and less time consuming. The sample recoveries were in good agreement with their respective label claims and they suggested non-interference of formulation recipients in the estimation and can be used in laboratories for the routine analysis of selected drugs. The present work concluded that stability

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indicating assay method by RP-HPLC was simple, accurate, precise, and specific and has no interference with the placebo and degradation products. Hence these can be used for routine analysis of Decitabine and Cedazuridine.

Conflict of interest

The authors declare that no conflict of interest.

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