ABSTRACT

Aim: The aim of our present work was to develop and validate a reverse phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous determination of Decitabine (DEC) and Cedazuridine (CED).

Methodology: The developed method was further applied to observe the degradation of analytes under the influence of different forced degradation conditions. Analytes were resolved on C18, 250 x 4.6 mm, particle size 5 μm Xterra column, using a mobile phase combination of 0.1% Ortho Phosphoric Acid buffer pH 6.5: Methanol (40:60v/v) with flow rate of 1mL/min and injection volume of 10 μL. Quantification was carried out with PDA detector at an isosbestic point of 220 nm with a linear calibration curve in the concentration range of 35-175 μg/mL for DEC and 100-500 μg/mL for CED.

Results: Validation of the developed method was performed as per ICH guidelines viz., linearity,
accuracy, precision, and robustness. The limits of detection (LOD) and the limits of quantification (LOQ) for CED were found to be 2.69 µg/mL and 8.15 µg/mL respectively. LOD and LOQ for DEC 1.55 µg/mL and 4.68 µg/mL respectively. Moreover, validated method was applied to study the degradation profile of analytes under various stress degradation conditions. 

**Conclusion:** The proposed method was found to be sensitive, specific and was successfully applied for the simultaneous estimation of Decitabine (DEC) and Cedazuridine (CED) in bulk drug, and tablets.

**Keywords:** Decitabine; degradation; Cedazuridine; Xterra; methanol.

### 1. INTRODUCTION

The DNA methyltransferase (DNMT) inhibitors decitabine and azacitidine are standard drugs used in therapies for myelodysplastic syndromes and chronic myelomonocytic leukemia [1,2]. These cytidine nucleoside analogues are incorporated into DNA during the S phase of the cell cycle and bind covalently to DNMT [1], resulting in reduced methylation of Cytosine followed by Guanine residues, [CpG] in genomic DNA, altered epigenetic pattern and modified gene expression. This process, described as DNMT inhibition or DNA hypomethylation, is believed to be part of the clinical activity of DNMT inhibitors [3].

For the treatment of myelodysplastic syndromes and chronic myelomonocytic leukemia, decitabine and azacitidine are administered parenterally for 5-7 days per treatment cycle [4,5]. More courses of treatment are required to obtain a response; Discontinuation of therapy in responders leads to disease progression. An orally bioavailable DNMT inhibitor would reduce the burden of monthly intravenous or subcutaneous infusions, lasting several days, which could remain on treatment for several months or even years. However, both decitabine and azacitidine are not readily bioavailable when administered orally due to rapid inactivation by cytidine deaminase (CDA), which is highly expressed in the gut and liver [6].

Inhibition of CDA with tetrahydrouridine has been shown to improve the oral bioavailability of decitabine. Cedazuridine (E7727, Astex Pharmaceuticals, Pleasanton, CA, USA), a new CDA inhibitor, has been designed to overcome the instability of tetrahydrouridine. It has been shown to be orally bioavailable safely in preclinical studies [5].

Chemically Decitabine (molecular formula: C8H12N4O4; Mol Wt.: 228.21 g/mol) is a 4-amino-1-\{[(2R,4S,5R)-4-hydroxy-5-(hydroxymethyl)oxolan-2-yl]-1,2-dihydro-1,3,5-triazin-2-one. Decitabine is physically fine white crystalline powder [7]. Cedazuridine (molecular formula: C9H14F2N2O5; Mol Wt.: 268.21 g/mol) is (4R)-1-\{[(2R,4R,5R)-3,3-difluoro-4-hydroxy-5-(hydroxymethyl)oxolan-2-yl]-4-hydroxy-1,3-diazinan-2-one [8]. Fig. 1 (a & b) shows the chemical structures of DEC and CED.

![Chemical structures of DEC and CED](image-url)
In spite of a number of publications describing the synthesis, efficacy, and pharmacokinetics of DEC and CED, information on quantitative analysis and characterization is lacking in the literature. Several HPLC methods of similar nucleoside analogs, including decitabine, zebularine, fluorodeoxycytidine, bromodeoxyuridine and azacitidine, were reported in the literature. Most of these nucleosides have moderate to high polarity and were tested with C18 columns eluted with aqueous buffers containing methanol or acetonitrile as organic modifiers. Many of the methods reported have been developed primarily for bioassays with LC/MS detection and are not suitable for the determination of impurities and degradation products in the analysis of pharmaceutical substances and products. The objective of this study was to develop and validate a Stability Indicator HPLC method for the simultaneous determination of DEC and CED in bulk and tablets according to ICH Q2 (R1) guidelines and evaluate the nature of the stability indicator of the developed method.

2. METHODOLOGY

2.1 Chemicals and Solvents

The pure DEC and CED were procured as a gift sample from Zydus Pharma, India. Tablets of Inqovi® (35 mg decitabine and 100 mg Cedazuridine) were purchased at Mukesh pharmacy, Hyderabad, India. HPLC grade Acetonitrile, Methanol, Di Potassium Hydrogen Ortho phosphate, Ortho phosphoric acid and water were obtained from SD Fine Chem, Mumbai, India. All other chemicals used were of AR grade.

2.2 HPLC–PDA Instrumentation and Chromatographic Conditions

The HPLC system was a Waters LC (Waters, Milford, MA, USA) consisting of a quaternary gradient system (controller 600), an inline degasser (Waters, model AF), a photodiode array detector (Water, model 2998) and an autosampler (Waters, model 717 plus). The data was processed using the Empower Pro software (Waters, Milford, MA, USA). Chromatographic separation assay was performed with an Xterra C-18 analytical column (250 mm × 4.6 mm inner diameter, 5 μm particle size) maintained at ambient temperature. The mobile phase consists of 0.1% Ortho Phosphoric Acid buffer pH 6.5: Methanol (40:60v/v). The mobile phase was pumped at a flow rate of 1.0 mL min⁻¹. The detection wavelength was 220 nm. Mobile phase was used as diluent for the preparation of working standards of analytes.

2.3 Preparation of Standard Solutions

A Mixed standard stock solution of DEC (105 μg/ml) and CED (300 μg/ml) was prepared by accurately weighing 35 mg of DEC and 100 mg of CED and dissolved in 10 mL volumetric flasks containing 5 mL of methanol and flask was sonicated to dissolve the contents and made up to the mark with the same. 0.3 mL of these samples was transferred into 10 mL volumetric flask containing 5 mL of diluent (mobile phase), sonicated for 5 minutes and the remaining volume was made upto the mark with diluent to get final concentration of 105 μg/ml and 300 μg/ml for DEC and CED respectively.

2.4 Analysis of Formulation

Twenty tablets were weighed accurately and ground into fine powder in a mortar. A quantity equivalent to 35 mg of DEC and 100 mg of CED was transferred in a volumetric flask 5ml of diluent was added and sonicated to ensure the solubility. Finally, the volume was made up to 10 ml to achieve the primarily stock solution of the tablets. An aliquot of 0.3 mL was withdrawn and transferred into 10 ml volumetric flask. Volume was made with the mobile phase to achieve 105 μg/ml and 300 μg/ml for DEC and CED respectively. The resulting solution was filtered through a 0.45 μm Millipore nylon filter paper if required.

2.5 Validation of Chromatographic Method

The developed method was validated as per the guidelines of ICH (ICH Guidelines, Q2 (R1), 2005) [16].

2.5.1 System suitability

System suitability parameters were measured to verify system performance. The precision of the system was determined in six repeated injections of standard preparations. All important characteristics were measured, including the peak area, the resolution of the peaks and the theoretical plate number.

2.5.2 Specificity

To assess the strategy specificity, working placebo solution (blank) in the absence of the
DEC and CED and standard solution having a concentration of 105 µg/ml and 300 µg/ml for DEC and CED respectively, as well as formulations were introduced into the HPLC system and analyzed chromatograms.

2.5.3 Accuracy (recovery)

Recovery studies were performed to determine Accuracy. In this process, it was tested at three different levels that were 50, 100 and 150% and analyzed chromatogram.

2.5.4 Precision

Precision (Intraday and Interday) of the analytical technique was proven by using optimized concentration of DEC and CED by six replicate injections. Average and % Relative Standard Deviation (RSD) of peak area and Assay were determined from chromatograms.

2.5.5 Linearity

Linearity was confirmed by preparing and analyzing the pure analytical standards at five different concentrations. The developed method shows ideal linearity over a range of 35 to 175 µg/ml and 100 to 500 µg/ml for DEC and CED respectively.

2.5.6 Limit of detection (LOD) and Limit of quantitation (LOQ)

The LOD and LOQ of DEC and CED were determined by using the standard deviation (SD) of the response and the slope. LOD = 3.3*SD/Slope and LOQ = 10*SD/Slope.

2.5.7 Robustness

Robustness as a measure of the method's ability not to be affected by small but deliberate changes in chromatographic conditions was investigated by testing the influence of small changes in mobile phase flow rate (± 0.2 units), change in column temperature (± 2°C) and wavelength variation (± 2%).

3. RESULTS AND DISCUSSION

3.1 System Suitability Study

To ensure the validity of the analytical procedure, a system suitability test was established. The following parameters like theoretical plat number (N), Resolution, Retention time (RTs) and tailing factor were analyzed by using working standard solution containing DEC (105 µg/mL) and CED (300 µg/mL) injecting six times into HPLC system. The results are presented in Table 1.

3.2 Specificity

To assess the strategy specificity, blank solution, DEC and CED standard having a concentration of 105 and 300 µg/mL respectively as well as formulations were injected into the HPLC system. No peaks were found in blank; the RTs reported in standard and samples were similar. Fig. 2(a-c) shows the representative chromatograms of blank, Standard and sample.

3.3 Accuracy (Recovery)

Accuracy was determined at three different levels that were 50, 100 and 150%. The results are shown in Table 2. Mean % Recoveries at 50, 100 and 150% for DEC were found to be 99.85, 100.21 and 100.95% respectively. Similarly for CED 99.38, 99.78 and 100.88% at 50, 100 and 150% respectively.

3.4 Precision

Precision of the analytical method were established for both intra and interday by using concentration of 105 and 300 µg/mL of DEC and CED six replicate injections. The results are shown in Table 3.

3.5 Linearity

Linearity curves were constructed by plotting peak areas on X axis versus drug concentration along Y axis and the regression equations were computed. The curves were plotted over the concentration ranges of 35-175 µg/mL and 100-500 µg/mL for DEC and CED Respectively. Fig. 3a and 3b shows the linearity curves of DEC and CED.

3.6 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD and LOQ values of DEC were found to be 1.55 and 4.68 µg/mL respectively. For CED LOD and LOQ were found to be 2.69 µg/mL and 8.15 µg/mL respectively.

3.7 Robustness

The study reveals that there was no much deviation in the robust chromatograms in assessment with optimized one. The results were shown in Table 4.
Table 1. System suitability parameters of DEC and CED

| S. No | Analyte      | RT(min) | Area    | Height (µV) | USP resolution | USP (min) | USP (min) |
|-------|--------------|---------|---------|-------------|----------------|-----------|-----------|
| 1     | Decetabine   | 3.097   | 170698  | 14786       | --             | 1.12      | 2137.25   |
| 2     | Cedazuridine | 4.224   | 346853  | 26148       | 3.78           | 1.14      | 2657.72   |

Fig. 2. Representative chromatogram of DEC and CED a) Blank, b) Standard and c) Sample

Table 2. Recovery study

| Analyte | Accuracy level* | Peak area* | Amount added (mg)* | Amount found (mg)* | % Recovery* | Mean % Recovery* |
|---------|-----------------|------------|--------------------|--------------------|-------------|-----------------|
| DEC     | 50%             | 85620      | 17.5               | 16.85              | 99.85       | 100.34          |
|         | 100%            | 171845     | 35                 | 35.01              | 100.21      |                 |
|         | 150%            | 259676.0   | 52.5               | 52.45              | 100.95      |                 |
| CED     | 50%             | 172505.0   | 50                 | 49.97              | 99.38       | 100.01          |
|         | 100%            | 346412     | 100                | 99.98              | 99.78       |                 |
|         | 150%            | 525309.0   | 150                | 150.23             | 100.88      |                 |

*Mean of three determinations at each level

Table 3. Precision study

| Precision | Mean Peak area* | % RSD* | Mean Assay* | % RSD* |
|-----------|-----------------|--------|-------------|--------|
| DEC       | CED             | DEC    | CED         | DEC    |
| Intraday  | 178302.2        | 342329.0 | 0.6        | 0.3    |
| Interday  | 178688.7        | 348783.8 | 0.3        | 0.4    |

*Mean of six determinations

Fig. 3. a) Linearity of DEC and b) Linearity of CED
Table 4. Results of robustness

| Parameter       | Condition | Decitabine |             | Cedazuridine |             |
|-----------------|-----------|------------|-------------|--------------|-------------|
|                 |           | RT Peak    | % Assay     | RT Peak      | % Assay     |
| Flow            | 0.9       | 3.867      | 177367.3    | 98.96        | 4.567       | 348578.3    | 99.67        |
|                 | 1.0       | 3.097      | 178302.2    | 99.86        | 4.224       | 348783.8    | 99.85        |
|                 | 1.1       | 2.172      | 178563.9    | 99.88        | 3.879       | 348629.4    | 99.74        |
| Column Temperature | 25       | 2.782      | 178106.3    | 99.58        | 4.182       | 351673.0    | 99.98        |
|                 | 30        | 3.097      | 178302.2    | 99.86        | 4.224       | 348783.8    | 99.85        |
|                 | 35        | 3.267      | 178082.8    | 99.17        | 4.262       | 348294.2    | 98.99        |
| Wave length     | 218       | 3.026      | 178312.7    | 99.87        | 4.221       | 3486325.9   | 99.79        |
|                 | 220       | 3.097      | 178302.2    | 99.86        | 4.224       | 348783.8    | 99.85        |
|                 | 222       | 3.106      | 178320.5    | 99.96        | 4.227       | 348790.3    | 99.90        |

3.8 Method Applications

The validated method was applied for the determination of DEC and CED in commercially available tablets. Fig. 2(b-c) shows two typical HPLC chromatograms obtained later the test of the standard DEC and CED reference solution and of the tablets sampling solution, respectively. The results of the trial (n = 6) produced more than 99% (RSD = 0.14%) of the label declaration for DEC and CED in Inqovi® tablets, respectively (Table 3). The average retention time of DEC and CED was found to be 3.097 and 4.242 minutes respectively. The test results indicate that the method is specific for the analysis of DEC and CED without interference from the excipients used to formulate and produce these tablets.

4. CONCLUSION

The developed Rp-HPLC method provides reliable, reproducible, accurate and specific for the quantification of DEC and CED in bulk and their tablets. The newly developed method has been validated as per the regulatory requirements and has shown the acceptable accuracy and precision with adequate sensitivity. This method can be used for the routine analysis of the above titled drugs in Quality control laboratories.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENTS

Authors are thankful to Torrent Pharma, Ahmedabad, India for providing gift sample of APIs and for the management of RGM & Santhiram Group of Institutions for supporting this work.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/63354