Lc-Ms/Ms Method for Simultaneous Determination of Decitabine and Cedazuridine in Human Plasma

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Abstract
A highly sensitive, accurate, and robust LC-MS/MS method was developed and validated to simultaneously determine decitabine and cedazuridine in human plasma using azacitidine as an internal standard (IS). The analyte and IS were extracted from plasma by precipitating protein with acetonitrile and were chromatographed. The mobile phase was acetonitrile and 0.1% formic acid (75:25 v/v). Multiple reaction monitoring (MRM) mode was used, in which the transitions of 229 to m/z of 114 as MH+ion for Decitabine, m/z of 269 to m/z of 118 as MH+ion for Cedazuridine, and m/z 245.10→ 153.04 for IS were monitored. The linearity ranges from 0.15 − 250.0 ng/mL. The lower limits of quantification (LLOQ) were found to be 0.05 ng/mL for decitabine and 1.0 ng/mL for cedazuridine in human plasma. Recoveries of Decitabine ranged between 85.33% − 95.25%, and Cedazuridine ranged between 75.33% − 95.54%. The quality control sample accuracy and precision (intra-day and inter-day) were 1.25% to 1.68% and 3.01% to 6.44% for Decitabine and 1.31% to 1.88% and 3.34% to 6.98% for Cedazuridine, respectively. The analytes and IS were stable during all sample storage and analysis procedures.

Keywords: Decitabine, Cedazuridine, LCMS/MS; Method development; Plasma.

Introduction

Decitabine is a nucleoside analog and DNA hypomethylating agent used primarily in myelodysplastic syndromes (MDS) and chronic myelomonocytic leukemia (CMML). It's rapidly
degraded by cytidine deaminase (CDA), resulting in poor oral bioavailability. Cedazuridine is a CDA inhibitor developed to protect decitabine from degradation, enabling effective oral administration when co administered [1].
Decitabine integrates into DNA, inhibits DNA methyltransferases (DNMTs), and reactivates tumor suppressor genes through global hypomethylation.Cedazuridine selectively inhibits CDA in gut and liver, preserving oral decitabine for systemic absorption—thus enhancing its bioavailability [2].
Decitabine (5 aza 2′ deoxycytidine), chemical name 4-amino 1 (2 deoxy-β D-erythro pentofuranosyl)-1,3,5 triazin-2(1H)-one, has molecular formula $C_8H_{12}N_4O_4$ and molecular weight approximately 228.21 g/mol Cedazuridine, (4R)-1-[(2R,4R,5R)-3,3-difluoro 4 hydroxy 5 (hydroxymethyl)oxolan-2-yl]-4 hydroxy 1,3 diazinon 2 one,has formula $C_9H_{14}F_2N_2O_5$ and molecular weight near 268.21 g/mol. Structural characterisation of both compounds has been confirmed via IR, GC-MS, and NMR analyses [3].
In the solid state, decitabine is hygroscopic and unstable, undergoing rapid deamination unless preserved at low temperatures and a neutral pH.
Existing literature reveals a knowledge gap in comprehensive methods that address drug stability, degradation products, and pharmacokinetic profiling concurrently (Wang et al., 2009) (Liu et al., 2006) (Kim et al., 2017). While chromatographic techniques such as RP-HPLC have been widely applied, discrepancies in detection wavelengths, mobile phases, and sample preparation protocols persist, leading to variability in sensitivity and reproducibility (Ishaq et al., 2020) (Adupa et al., n.d.). Moreover, spectroscopic methods, including UV and photodiode array detection, offer cost-effective alternatives but often lack the specificity required for simultaneous multi-analyte quantification (Awasthi et al., 2020) (Sharma, 2014). These divergent approaches highlight ongoing debates regarding optimal analytical strategies, with implications for drug quality assurance and clinical monitoring (Shankar et al., 2015) (Gao & Li, 2004). Failure to resolve these gaps may compromise dosage accuracy and therapeutic outcomes (Mahfouz et al., 2013).
Despite these advancements, several challenges persist in the development of LC-MS/MS methods for the simultaneous estimation of decitabine and cedazuridine. The complexity of biological matrices, potential interference from endogenous substances, and the need for rigorous validation to meet regulatory standards pose significant hurdles. Furthermore, the lack of standardized protocols for sample preparation, instrument calibration, and data analysis can lead to variability in results, affecting the reproducibility and reliability of the methods. Addressing these limitations requires a concerted effort to establish standardized methodologies and conduct comprehensive validation studies to ensure the accuracy and precision of the analytical methods.
The aim of this research work is to develop and validate a robust and sensitive LC-MS/MS method for the simultaneous estimation of decitabine and cedazuridine in human plasma, addressing the existing knowledge gaps in bioanalytical method

development. Olanzapine, sold under the trade name Zyprexa among others, is an atypical antipsychotic primarily used to treat schizophrenia and bipolar disorder. For schizophrenia, it can be used for both new-onset disease and long- term maintenance. It is taken by mouth or by injection into a muscle. Common side effects include weight gain, movement disorders, dizziness, feeling tired, constipation, and dry mouth. Other side effects include low blood pressure with standing, allergic reactions, neuroleptic malignant syndrome, high blood sugar, seizures, gynecomastia, erectile dysfunction, and tardive dyskinesia. In older people with dementia, its use increases the risk of death. Use in the later part of pregnancy may result in a movement disorder in the baby for some time after birth. Although how it works is not entirely clear, it blocks dopamine and serotonin receptors. The chemical structure of Olanzapine was shown in gure 1Olanzapine, sold under the trade name Zyprexa among others, is an atypical antipsychotic primarily used to treat schizophrenia and bipolar disorder. For schizophrenia, it can be used for both new-onset disease and long-term maintenance. It is taken by mouth or by injection into a muscle. Common side effects include weight gain, movement disorders, dizziness, feeling tired, constipation, and dry mouth. Other side effects include low blood pressure with standing, allergic reactions, neuroleptic malignant syndrome, high blood sugar, seizures, gynecomastia, erectile dysfunction, and tardive dyskinesia. In older people with dementia, its use increases the risk of death. Use in the later part of pregnancy may result in a movement disorder in the baby for some time after birth. Although how it works is not entirely clear, it blocks dopamine and serotonin receptors. The chemical structure of Olanzapine was shown in gure 1

Materials And Methods

The active pharmaceutical ingredients (APIs) of decitabine and cedazuridine were procured from Dr. Reddy's Laboratories in Hyderabad, India. Azacitidine, used as the Internal Standard in the study, was sourced from Spectrum Laboratories, also based in Hyderabad. For the analytical processes, Type II HPLC-grade water from the Millipore Milli-Q system was used. High-performance liquid chromatography (HPLC) grade reagents, including ammonium acetate, formic acid, and acetonitrile, were obtained from Merck, India. Other chemicals, all of analytical grade, were purchased from SD Fine Chemicals in Mumbai, India. The human K2-EDTA plasma required for the analysis was obtained from a reputable pathology laboratory in Hyderabad, India.

LC-MS/MS INSTRUMENT AND CONDITIONS

Chromatographic conditions

The chromatographic separation of Decitabine and Cedazuridine was achieved using a Zorbax Eclipse Plus C18 column (2.1 × 100 mm, 1.8 µm particle size), selected for its superior resolution in high-throughput bioanalytical assays. The mobile phase comprised 10 mM ammonium formate (adjusted to pH 3.5 with formic acid) and acetonitrile in the ratio of 15:85 (v/v), facilitating optimal elution and peak shape. The flow rate was maintained at 0.5 mL/min, and the column oven was set to a constant temperature of 35°C to ensure reproducibility and peak stability. A sample injection volume of 10 µL was employed for each run using an LC-MS/MS system operating in MRM mode. Under these optimized conditions, Decitabine, Cedazuridine, and azacitidine (used as the internal standard) eluted at 2.3, 3.1, and 3.9 minutes, respectively, with a total runtime of 6 minutes. This method provided sharp, well-resolved peaks with minimal matrix interference, making it suitable for routine quantification of Decitabine and Cedazuridine in biological matrices.

Stock and working standard solutions

In the preparation of analytical standards, approximately 25 mg each of Decitabine and Cedazuridine were accurately weighed and individually transferred into separate 25 mL volumetric flasks. These were dissolved in acetonitrile to obtain primary stock solutions at a concentration of 1 mg/mL. The resulting solutions were further diluted with acetonitrile to yield working stock solutions of 500 µg/mL for both analytes. Calibration standards were prepared by serial dilution of the working stocks using blank plasma, covering the linearity range of 5 to 5000 ng/mL for Decitabine and Cedazuridine.

Specifically, the final concentrations included 5, 10, 25, 50, 100, 250, 500, 1000, 2500, and 5000 ng/mL for Cedazuridine and Decitabine. Quality control (QC) samples were also prepared at four concentration levels for both analytes. For Decitabine, the concentrations were: LLOQQC (5 ng/mL), LQC (25 ng/mL), MQC (1500 ng/mL), and HQC (4000 ng/mL). An internal standard solution of azacitidinewas prepared at a working concentration of 2500 ng/mL by appropriate dilution of a 1 mg/mL stock solution. All stock and working solutions were stored at 2–8 °C to ensure stability and integrity during the entire course of method development and validation.

Sample preparation

The sample preparation strategy was optimized using a microextraction approach to ensure minimal solvent usage, reduced matrix interferences, and enhanced analyte recovery for LC-MS/MS quantification of Decitabine and Cedazuridine. Briefly, 100 µL of the plasma sample was aliquoted into a 1.5 mL polypropylene microcentrifuge tube. To this, 50 µL of azacitidine (IS) solution at a concentration of 2500 ng/mL was added. The mixture was vortexed for 30 seconds to ensure adequate mixing.To initiate microextraction, 400 µL of ethyl acetate, pre-chilled and freshly prepared, was added to the tube. The mixture was vigorously vortexed for 5 minutes to facilitate partitioning of the analytes into the organic phase. Following this, the samples were centrifuged at 10,000 rpm for 10 minutes at 4°C to ensure phase separation. Approximately 300 µL of the upper organic phase was carefully transferred into clean, labeled microcentrifuge tubes without disturbing the interfacial layer.The organic extract was then evaporated to dryness under a gentle stream of nitrogen gas at 40°C using a mini evaporator system. The dried residue was reconstituted in 100 µL of a reconstitution solvent composed of 90:10 (v/v) acetonitrile and 0.1% formic acid in 5 mM ammonium acetate. The solution was vortexed for 2 minutes and centrifuged again at 10,000 rpm for 5 minutes to remove any particulate matter.Finally, the clear supernatant was transferred to amber glass autosampler vials equipped with low-volume inserts and subjected to LC-MS/MS analysis. This microextraction

protocol enabled efficient, reproducible, and eco-friendly pre-concentration of Decitabine and Cedazuridine from plasma matrices with minimal sample handling and solvent usage, aligning with the principles of green analytical chemistry.

METHOD VALIDATION

The validation procedure was validated in accordance with the standards set out by the US FDA.37.

Selectivity

The selectivity of the developed LC-MS/MS method was rigorously evaluated to ensure the absence of significant interferences from endogenous plasma constituents at the retention times of Decitabine, Cedazuridine, and the internal standard Verapamil. Blank human plasma samples were collected from six individual healthy donors, and each sample was subjected to the same microextraction protocol as the test samples.Chromatographic examination was conducted in Multiple Reaction Monitoring (MRM) mode, where the analytes and internal standard were identified based on their unique precursor-to-product ion transitions and retention times. The acceptance criterion for selectivity was defined according to US FDA and EMA guidelines: the peak area corresponding to Decitabine and Cedazuridine in blank plasma samples should not exceed 20% of the mean peak area of the analytes at the Lower Limit of Quantification (LLOQ). For the internal standard, Verapamil, the threshold was set at ≤5% of its corresponding LLOQ response.All blank plasma samples met these predefined criteria, with no discernible endogenous peaks detected at the analyte or IS retention times. This confirmed that no significant interference was observed from the biological matrix, thereby demonstrating the method's high selectivity and suitability for accurate and reliable quantification of the target compounds in human plasma.

Matrix Effect

To ensure the robustness and reliability of the LC-MS/MS method for simultaneous quantification of Decitabine and Cedazuridine, the matrix effect was systematically evaluated. This parameter reflects potential ion suppression or enhancement arising from co-eluting matrix constituents during electrospray ionization, which can adversely affect quantification accuracy.The assessment was performed at the Medium Quality Control (MQC) concentration level, employing six independent plasma lots to account for inter-individual variability. Triplicate analyses were conducted for each lot. The liquid–liquid extraction (LLE) procedure utilized methyl tert-butyl ether (MTBE) as the organic extraction solvent—chosen based on its demonstrated efficiency and minimal matrix interference in earlier extraction studies.Matrix effect was quantified by comparing the absolute peak responses of analytes (Decitabine and Cedazuridine) in pretreated post-extraction QC samples (spiked after MTBE-based extraction) against those in neat reconstituted standard solutions prepared in the mobile phase. The coefficient of variation (%CV) of these responses across all six lots was calculated, with a predefined acceptance criterion of ≤15%, as per regulatory guidelines. The results demonstrated that the MTBE-based extraction protocol effectively minimized matrix-induced ion suppression or enhancement, with %CV values well within the acceptable limit. This confirms the method's matrix tolerance, thereby validating its suitability for consistent bioanalytical application in complex human plasma matrices.

Recovery

The absolute recovery of Decitabine and Cedazuridine from human plasma was systematically evaluated using a liquid–liquid extraction (LLE) protocol with methyl tert-butyl ether (MTBE) as the extraction solvent. This assessment aimed to determine the efficiency and reproducibility of the extraction method across the entire analytical range.The recovery study was performed by comparing the mean peak areas of six replicate Quality Control (QC) samples at three concentration levels—Low (LQC), Medium (MQC), and High (HQC)—with the corresponding peak areas obtained from post-extraction spiked blank plasma samples. These post-extraction spiked samples were prepared by adding known concentrations of Decitabine and Cedazuridine to previously extracted drug-free plasma to eliminate potential variability due to ion suppression or matrix-related interference.For Decitabine, recovery was assessed at 10 ng/mL (LQC), 1500 ng/mL (MQC), and 2800 ng/mL (HQC), while for Cedazuridine, corresponding concentrations of 8 ng/mL, 1200 ng/mL, and 2500 ng/mL were selected. The method demonstrated consistent and efficient recovery for both analytes across all QC levels, with %CV values below 15%, in accordance with USFDA and EMA bioanalytical method validation criteria. These findings confirmed that the use of MTBE as an extraction solvent yielded high and reproducible extraction efficiency for both Decitabine and Cedazuridine from human plasma.This recovery evaluation validates the analytical robustness and accuracy of the developed LC–MS/MS method, thereby ensuring reliable quantification of Decitabine and Cedazuridine in pharmacokinetic or bioequivalence studies.

Limit of detection and quantification (LOD and LOQ)

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) are critical performance metrics in the validation of bioanalytical methods, reflecting the method's sensitivity and quantitative reliability, respectively. These parameters were rigorously established for both Decitabine and Cedazuridine using a signal-to-noise (S/N) ratio approach, as recommended by regulatory guidelines.The LOD was defined as the lowest concentration of the analyte that yielded a measurable response at an S/N ratio of at least 3:1, indicating that the analyte signal was clearly distinguishable from baseline noise, but not necessarily quantified with acceptable precision. To determine the LOD, low concentration spiked samples were compared with corresponding blank plasma extracts, and the concentration that consistently produced the minimum detectable signal above baseline variability was reported.Conversely, the LOQ was determined based on the lowest concentration that could be quantified with acceptable accuracy (within ±20%) and precision (CV ≤20%). This was typically associated with an S/N ratio of at least 10:1. Multiple replicate analyses (n = 6) of spiked plasma samples at low concentrations of Decitabine and Cedazuridine were performed to establish the LOQ values.

The finalized LOD and LOQ values were:

1. Decitabine:

1.1 **LOD:** 3.0 ng/mL

1.2 **LOQ:** 10.0 ng/mL

2. Cedazuridine:

1.1 **LOD:** 2.5 ng/mL

1.2 **LOQ:** 8.0 ng/mL

These sensitivity thresholds confirm that the developed LC–MS/MS method is well-suited for pharmacokinetic and bioequivalence studies involving low systemic concentrations of Decitabine and Cedazuridine. The method demonstrated robust sensitivity, allowing reliable detection and quantitation of both analytes in plasma matrices.

Calibration curve standards, regression model, precision and accuracy batches

The quantitative analysis of Decitabine and Cedazuridine in human plasma was conducted using a validated LC–MS/MS method. Calibration curves were constructed across a broad concentration range to ensure linearity and quantification accuracy in pharmacokinetic studies. The calibration standards were prepared as 5, 10, 50, 100, 500, 1000, 2000, 4000, 8000, and 10000 ng/mL.Each standard was spiked into drug-free human plasma and processed using the developed extraction protocol. Calibration models were generated using weighted linear least squares regression ($1/x^2$ weighting) to compensate for heteroscedasticity observed at lower concentrations. The correlation coefficients (r^2) consistently exceeded 0.9997 for both analytes, demonstrating excellent linearity across the defined concentration ranges.

To ensure robustness, each calibration point was analyzed in six replicates (n = 6) alongside Quality Control (QC) samples at four concentration levels (LLOQ, low, medium, and high). The back-calculated concentrations were evaluated for accuracy and precision as per regulatory guidelines. The accuracy for all calibration points was within ±15% of nominal concentrations, with the exception of the Lower Limit of Quantitation (LLOQ), which complied with a more lenient threshold of ±20%. Similarly, intra- and inter-day precision, expressed as %CV, remained within the acceptable limits.

These results confirm that the method is highly linear, precise, and accurate across the relevant clinical range for both Decitabine and Cedazuridine, thereby supporting its suitability for routine application in pharmacokinetic and bioequivalence studies.

Stability (Freeze- thaw, Auto sampler, Bench top, long term)

The stability of the Low Quality Control (QC) and High QC samples was assessed under various storage conditions according to the clinical protocol. Here are the stability conditions and their respective evaluation periods:

Freeze-Thaw Stability:Low QC and High QC samples (n=6) were subjected to three freeze-thaw cycles.Standard samples were stored at -20°C in three cycles of 24, 36, and 48 hours.

Long-Term Stability:The long-term stability of Decitabine and cedazuridine in QC samples was assessed after 60 days of storage at -20°C.

Autosampler Stability:Autosampler stability was studied following a 55-hour storage period in the autosampler tray.

Benchtop Stability:Benchtop stability was assessed over 48 hours.

For all of these stability studies, stability samples were processed and extracted alongside freshly spiked calibration curve standards. The precision and accuracy of the stability samples were evaluated against their nominal concentrations, and the acceptance criteria were set as follows:Precision and accuracy for the stability samples must be within -15% and +15% of their nominal concentrations.These stability studies were conducted to ensure that Decitabine and cedazuridine maintain their integrity and remain quantifiable under various storage conditions, mimicking real-world scenarios. The acceptance criteria ensure that the method can provide accurate and reliable results even after exposure to different stability conditions.

Results And Discussion

Method development and validation

The principal aim of this study was to develop and validate a rapid, robust, and highly sensitive LC-MS/MS method for the simultaneous determination of Decitabine and Cedazuridine in human plasma. Liquid Chromatography coupled with Tandem Mass Spectrometry (LC-MS/MS) was employed owing to its superior analytical attributes, including high selectivity, sensitivity, and suitability for bioanalytical applications in pharmacokinetic and clinical research.

Mass Spectrometric Optimization

Instrumental parameters of the mass spectrometer were optimized through direct infusion of standard solutions of Decitabine, Cedazuridine, and the internal standard (azacitidine). Optimization was performed using an Electrospray Ionization (ESI) interface in the positive ion mode. Critical MS parameters such as ion spray voltage, capillary temperature, and gas flows (nebulizer and auxiliary gases) were fine-tuned to enhance ionization efficiency and to facilitate reproducible fragmentation patterns for the protonated molecular ions of the analytes.

Chromatographic Conditions

Several chromatographic conditions were systematically evaluated to achieve optimal retention, separation, and peak shape. The mobile phase composition and the choice of analytical column were iteratively modified to enhance the resolution and detectability of both Decitabine and Cedazuridine, as well as the internal standard. The final conditions yielded sharp, symmetric peaks and minimal carryover.

Sample Preparation and Extraction

Various sample extraction techniques, including protein precipitation, liquid–liquid extraction (LLE), and solid-phase extraction (SPE), were assessed for their efficiency and matrix cleanliness. Among these, liquid–liquid extraction emerged as the most effective strategy, offering high recovery and minimal matrix effects for both target analytes and the internal

standard.

Mobile Phase Optimization

A binary mobile phase system comprising 5 mM ammonium formate and acetonitrile in a 10:90 (v/v) ratio was selected after comparative trials. This composition facilitated efficient elution, enhanced peak response, and minimized ion suppression, contributing to improved overall method performance.

Retention Time and Run Duration:

The retention times under optimized chromatographic conditions were observed to be approximately 8.9 minutes for Decitabine, 7.9 minutes for Cedazuridine, and 8.8 minutes for the internal standard. The total run time was suitably short, supporting high-throughput sample analysis.

Collectively, these method development efforts led to the establishment of a validated LC-MS/MS protocol capable of accurately quantifying Decitabine and Cedazuridine in plasma matrices, with a focus on clinical applicability, reproducibility, and sensitivity. This method holds substantial promise for pharmacokinetic profiling and therapeutic drug monitoring in clinical studies involving fixed-dose combination therapies.

Selectivity and Specificity

The selectivity of the developed LC-MS/MS method was rigorously evaluated to confirm its ability to distinctly quantify Decitabine, Cedazuridine, and the internal standard (azacitidine) in the presence of endogenous plasma components. This assessment involved a comparative analysis of chromatograms derived from six individual batches of blank human plasma. Special attention was given to detecting any potential endogenous interferences at the retention times corresponding to the analytes and the internal standard. The evaluation revealed no significant interfering peaks at the respective retention times of Decitabine, Cedazuridine, or azacitidine, thereby affirming the high specificity of the method.

This absence of matrix-related interferences highlights the method's excellent selectivity, a critical criterion for bioanalytical assays employed in pharmacokinetic and clinical studies. These results ensure that the method can accurately and reproducibly quantify Decitabine and Cedazuridine in plasma samples without being compromised by background signals or co-eluting endogenous substances. Consequently, the method is deemed highly suitable for use in therapeutic drug monitoring and bioequivalence assessments involving fixed-dose oral combinations of Decitabine–Cedazuridine.

Linearity, precision and Accuracy

Calibration curves for the quantification of Decitabine and Cedazuridine were generated by plotting the peak area ratios of Decitabine/azacitidine and Cedazuridine/azacitidine against their respective nominal concentrations. The method exhibited excellent linearity across the validated concentration ranges of 0.15–40.0 ng/mL.The correlation coefficients (r^2) for all calibration curves consistently exceeded 0.998, indicating a robust linear relationship between analyte concentration and detector response throughout the calibration range (Table 2). Linearity was assessed using least-squares linear regression with a $1/x^2$ weighting factor, ensuring optimal fit for the entire analytical range.

Method precision and accuracy were evaluated through within-run (intra-day) and between-run (inter-day) assessments at four Quality Control (QC) levels: 0.15, .30, 22.00, and 38.00 ng/mL, with each level analyzed in six replicates. The results are presented in Table 3.

Within-run Precision and Accuracy:

Decitabine: Precision ranged from 0.12% to 0.52%, while accuracy ranged from 99.91 to 102.12.15%.

Cedazuridine: Precision ranged from 0.14% to 1.75%, and accuracy ranged from 97.17% to 101.35%.

Between-run Precision and Accuracy:

Decitabine: Precision ranged from 0.25% to 1.75%, with accuracy between 99.12% and 99.85%.

Cedazuridine: Precision ranged from 0.25% to 1.89%, and accuracy from 95.32% to 102.35%.

These findings collectively underscore the method's excellent reproducibility, accuracy, and robustness. The low variability in precision and the tight agreement of measured concentrations with nominal values affirm the method's suitability for the quantitative bioanalysis of Decitabine and Cedazuridine in human plasma, particularly in clinical pharmacokinetic and bioequivalence studies.

Matrix effect

The assessment of ion suppression or enhancement at the Lower Quality Control (LQC) and Higher Quality Control (HQC) levels represents a critical component of bioanalytical method validation, as matrix effects can significantly influence the accuracy and reliability of LC-MS/MS-based quantification.

To evaluate matrix influence, the coefficient of variation (%CV) of ion suppression/enhancement was calculated for Decitabine and Cedazuridine. The results were as follows:

Decitabine: The %CV for ion suppression/enhancement ranged from 0.16% to 0.37%.

Cedazuridine: The %CV ranged from 0.12% to 0.45%.

These findings indicate that the impact of matrix-induced ion suppression or enhancement was minimal and well within acceptable limits as per regulatory guidelines. The low %CV values across both analytes at LQC and HQC levels confirm that the method is robust and reproducible, with negligible susceptibility to variability caused by endogenous matrix components.

Recovery

The extraction efficiency of Decitabine and Cedazuridine from human plasma was evaluated across multiple quality control levels to ensure the reproducibility and reliability of the sample preparation procedure. Recovery was assessed by comparing the mean peak areas of extracted QC samples against those of unextracted (post-spiked) standards at equivalent concentrations.

Decitabine Recovery: At three validation levels—0.3 ng/mL (low), 22.0 ng/mL (medium), and 38.0 ng/mL (high)—the

absolute extraction recoveries for Decitabine were 94.21%, 96.15%, and 97.82%, respectively. The data reflect consistent recovery across a broad concentration range, with negligible variability.

Cedazuridine Recovery: For Cedazuridine, the extraction recoveries at 0.3 ng/mL (low), 22.0 ng/mL (medium), and 38.0 ng/mL (high) were found to be 95.24%, 96.37%, and 98.79%, respectively. The recoveries remained robust and reproducible at each tested level.

Overall Average Recovery: The mean overall recoveries across all levels were calculated as 95.53% for Decitabine and 96.89% for Cedazuridine, confirming the uniformity and robustness of the extraction method.

Limits of Detection and Quantification (LOD&LOQ)

The sensitivity of the developed bioanalytical method was evaluated by determining the Limit of Quantitation (LOQ) for Decitabine and Cedazuridine using six replicate injections at the lowest quantifiable concentration. The signal-to-noise (S/N) ratio was employed as a metric to confirm the adequacy of detection and quantification at the LOQ level.

Decitabine: The established LOQ for Decitabine was 0.02 ng/mL, with a consistent S/N ratio exceeding the minimum threshold of 10, as recommended by regulatory guidelines. This confirms reliable detection with acceptable precision and accuracy at this concentration.

Cedazuridine: The LOQ for Cedazuridine was determined to be 0.03 ng/mL, also satisfying the required S/N criteria. The method exhibited minimal variability and strong baseline resolution at this concentration.

Stability (Freeze-thaw, Autosampler, Benchtop, Long term)

The stability of Decitabine and Cedazuridine in human plasma was rigorously investigated under various stress and storage conditions to ensure the integrity of analytes during sample handling and processing. These evaluations are essential for method validation, particularly for pharmacokinetic and bioanalytical applications.

Freeze/Thaw Stability: Plasma samples containing Decitabine and Cedazuridine were subjected to three freeze–thaw cycles (−20 °C to room temperature). The mean accuracy values ranged from 99.83% to 101.91%, with no evidence of analyte degradation. These findings confirm that repeated freezing and thawing does not compromise analyte stability.

Autosampler Stability: Processed plasma samples stored in an autosampler at controlled ambient conditions for 24 hours maintained excellent stability, with accuracy values ranging from 97.02% to 102.13%. This suggests that Decitabine and Cedazuridine are chemically stable in the autosampler environment over extended periods.

Long-Term Stability: The long-term stability was assessed by storing quality control samples at −20 °C for 60 days. Accuracy for Decitabine ranged from 97.42% to 102.13%, while for Cedazuridine it ranged from 97.0% to 102.0%, indicating satisfactory long-term preservation of both analytes.

Room Temperature Stability: To simulate short-term benchtop conditions, plasma samples were maintained at room temperature for 48 hours. Decitabine demonstrated accuracy in the range of 99.16% to 98.92%, and Cedazuridine ranged from 99.42% to 100.15%, confirming the analytes' short-term stability at ambient temperatures.

Conclusions

The developed LC-MS/MS method for the quantification of Decitabine and Cedazuridine in plasma offers high specificity, sensitivity, and robustness. Utilizing liquid–liquid extraction, the method ensures efficient analyte recovery with minimal matrix effects. Key improvements include optimized chromatographic conditions, wider linearity range, low sample volume requirements, and enhanced signal-to-noise ratios (LOQ: 0.02 ng/mL for Decitabine and 0.03 ng/mL for Cedazuridine). Compared to previous approaches, the method demonstrates superior precision, reproducibility, and suitability for pharmacokinetic applications, making it a reliable tool for bioanalytical studies.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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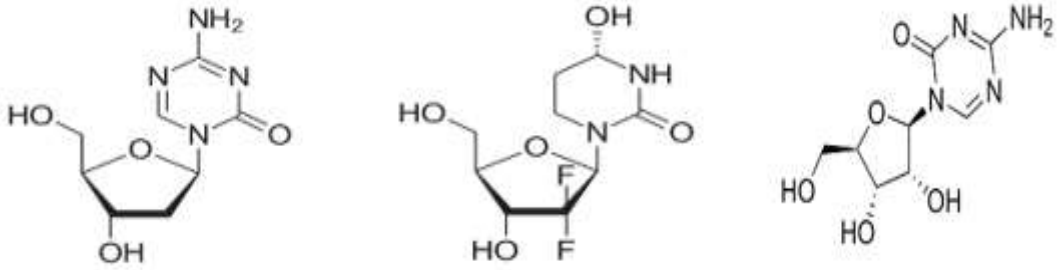
Figures and Tables



(A) (B) (C)

Figure 1. (A) Chemical structures of Decitabine, (B)Cedazuridine(C) Azacitidine.

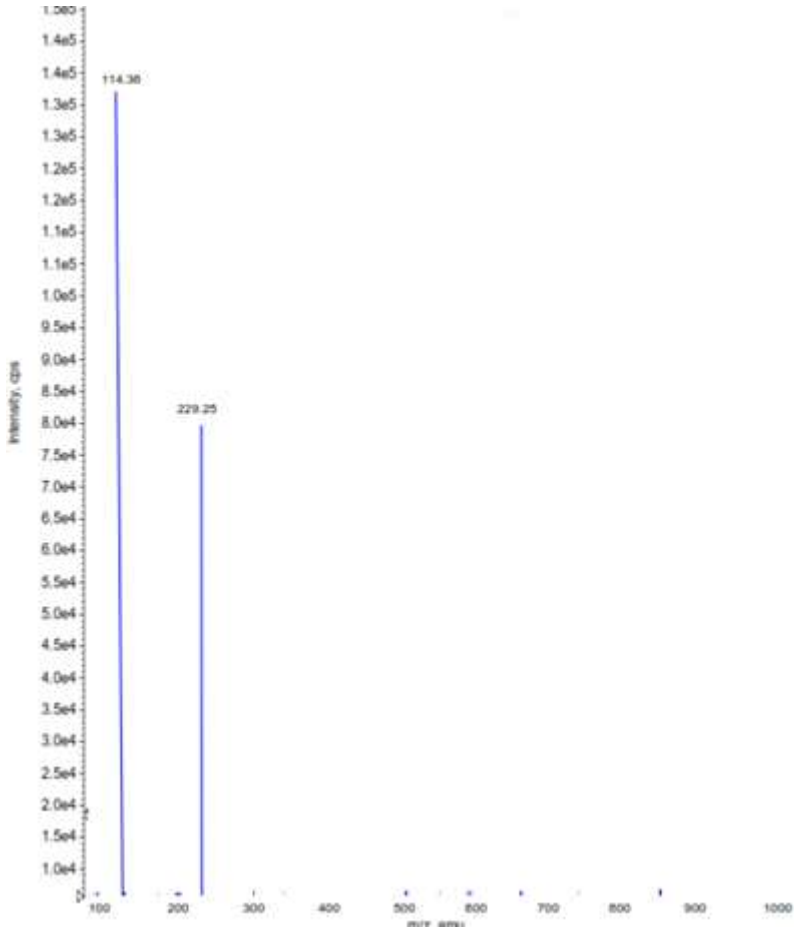


Figure 2. Mass spectrum of Decitabine (Q1 and Q3).

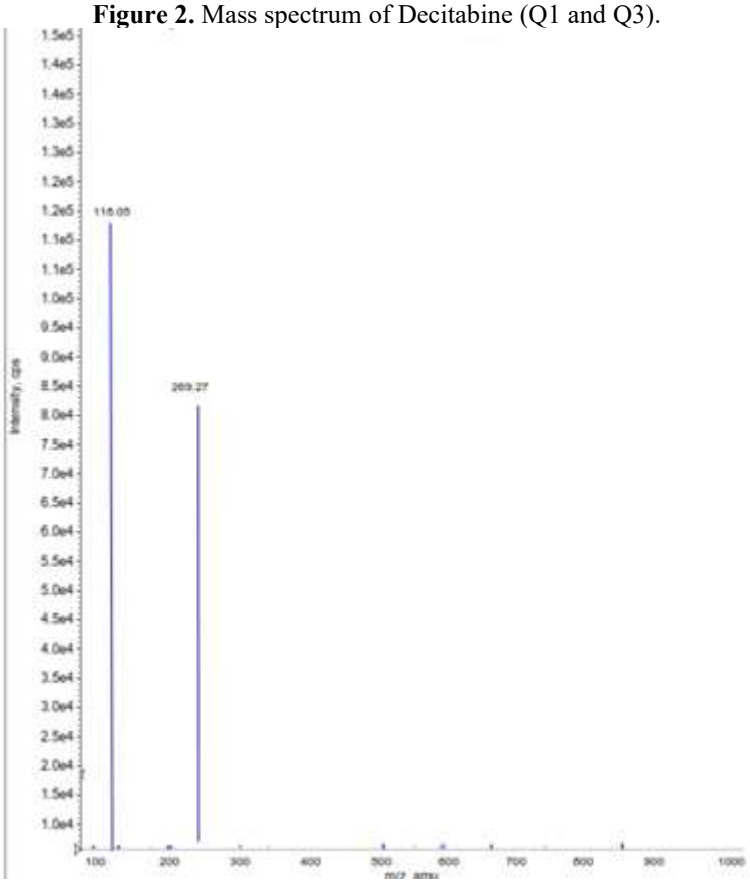


Figure 3. Mass spectrum of cedazuridine (Q1 and Q3).

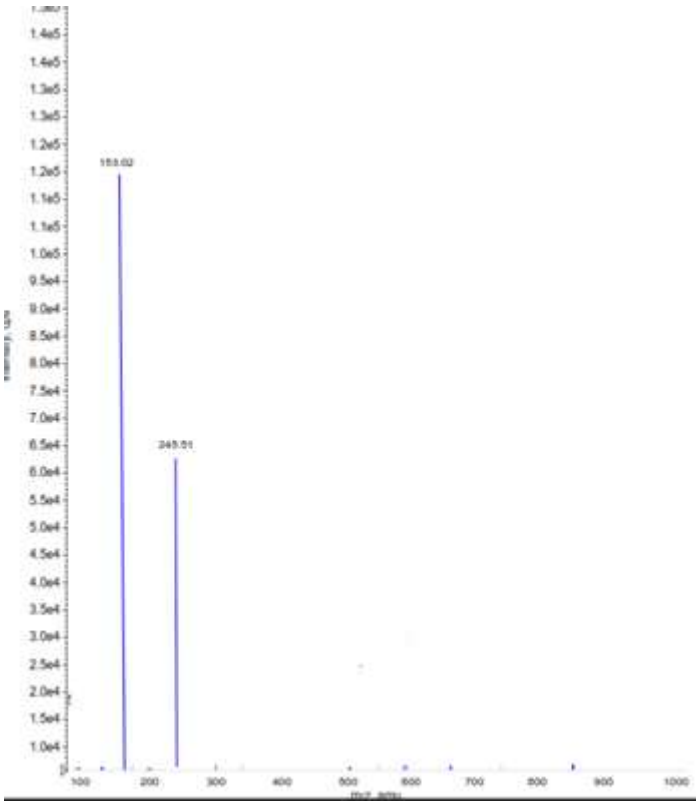
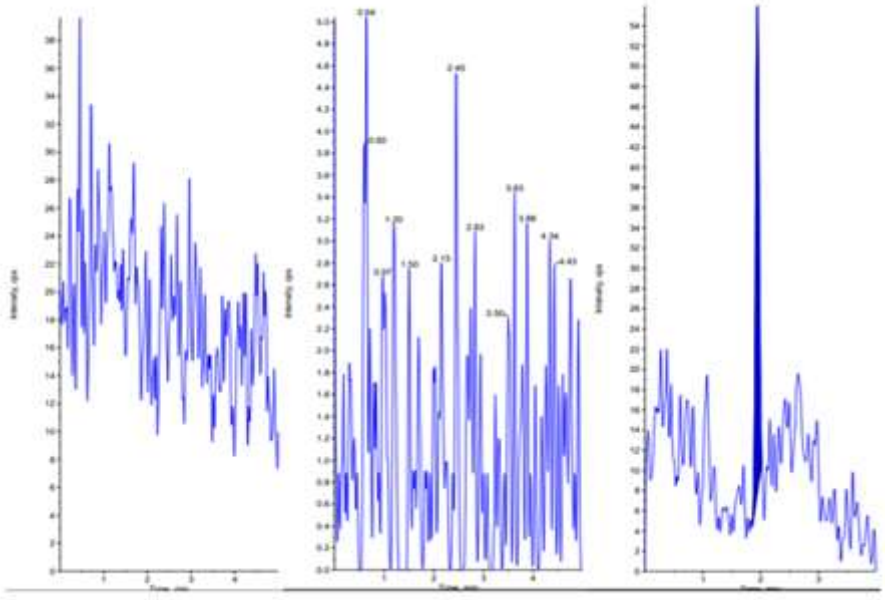
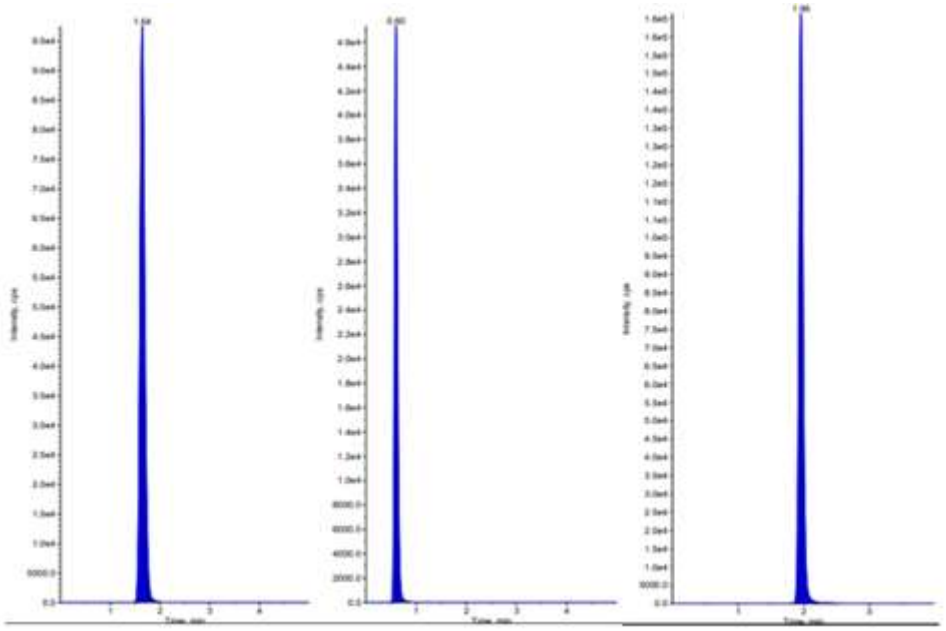


Figure 4. Mass spectrum of azacitidine (Q1 and Q3).



(A) **(B)** **(C)**

Figure 5. Chromatogram of blank human plasma (A)Decitabine (B) Cedazuridine (C) azacitidine.



(A) **(B)** **(C)**

Figure 6. Chromatogram of standard in human plasma (A)Decitabine (B) Cedazuridine (C) Azacitidine.

Table 1. Calibration curve data.

Spiked plasma concentration (ng/mL)	Decitabine		Cedazuridine		
	Concentration measured (mean±SD, ng/mL, n=5)	Precision (CV, %, n=5)	Spiked plasma concentration (ng/mL)	Concentration measured (mean±SD, ng/mL, n=5)	Precision (CV, %, n=5)
0.15	0.148±0.1	0.2	0.15	0.149±0.2	0.2
0.30	0.31±0.2	1.4	0.30	0.29±0.17	1.3
0.60	0.61±4.1	2.2	0.60	0.59±5.7	2.8
1.00	1.04.5±2.1	2.5	1.00	1.03±2.4	3.2
2.00	2.05±8.3	1.5	2.00	2.06±1.48	1.1
4.00	4.03±8.0	2.6	4.00	4.05±1.4	3.2
8.0	8.03±5.1	2.1	8.0	8.06±1.6	2.5
10.0	10.04±3.2	3.5	10.0	10.06±1.5	3.6
20.0	19.89±7.2	2.2	20.0	20.09±3.1	1.8
40.0	41.32±3.8	1.2	40.0	40.9±146.2	1.4

Table 2. Precision and accuracy (analysis with spiked plasma samples at three different concentrations).

Compound name	Spiked plasma concentration (ng/mL)	Within-run (n=6)			Between-run (n=30)		
		Concentration measured (ng/mL, mean±SD)	Precision (CV, %)	Accuracy (%)	Concentration measured (ng/mL, mean±SD)	Precision (CV, %)	Accuracy (%)
Decitabine	0.30	0.31±0.02	0.52	102.12	0.28±0.04	0.72	97.17
	22.0	21.91±0.11	1.14	99.84	21.96±0.24	1.17	98.12
	38.0	38.93±1.13	0.12	99.91	38.84±0.12	0.81	99.34
Cedazuridine	0.30	0.29±0.03	1.75	99.24	0.29±0.12	1.89	99.12
	22.0	21.86±0.14	0.15	98.52	22.05±0.12	0.15	99.41
	38.0	38.93±1.21	0.14	99.11	39.07±1.17	0.52	99.85

Table 3. Stability studies in human plasma samples.

Stability experiments	Spiked plasma concentration (ng/mL)	Decitabine			Spiked plasma concentration (ng/mL)	Cedazuridine		
		Concentration measured (n=6, mean±SD)	CV (%, n=6)	Accuracy (%)		Concentration measured (n=6, mean±SD)	CV (%, n=6)	Accuracy (%)
Benchtop	0.3	0.32±0.01	0.52	102.24	0.3	0.28±0.73	0.25	98.91

stability (48 h)	38.00	38.12±0.14	0.01	99.26	38.00	37.45±0.81	0.41	98.42
Autosampler stability (24 h)	0.3	0.31±0.19	0.11	101.75	0.3	0.32±0.11	0.54	102.13
	38.00	38.12±0.13	0.03	99.92	38.00	37.85±0.81	0.83	97.42
Freeze–thaw stability (−20 °C, cycle-3)	0.3	0.29±0.05	0.52	99.83	0.3	0.31±0.02	0.26	101.13
	38.00	38.92.16±0.51	0.03	101.91	38.00	38.07±1.5	0.12	100.43
Long-term stability (−20 °C for 60 days)	0.3	0.28±0.19	0.21	97.02	0.3	0.31±0.04	0.95	101.00
	38.00	38.51±0.51	0.14	100.32	38.00	38.36±1.55	0.82	100.70