RESEARCH ARTICLE

# Development and Validation of Stability Indicating RP-HPLC Method and Characterization of Degradation Products of Anti-neoplastic Agent by LCMS-MS

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**ABSTRACT**

In present research a stability indicating reversed-phase high-performance liquid chromatography (RP-HPLC) was developed and validated for quantification of azacitidine and its degradants. The chromatographic isolation of azacitidine carried out using Phenominex C18 (150×4.6 mm, 5 µ) column, movable phase of 0.1% HCOOH in water and acetonitrile (30:70 v/v), at a flowing rate of 1-mL/min analyzed at 210 nm. The %recovery findings ranged between of 99.13–99.41%. The limit of detection (LoD), and limit of quantitation (LoQ)values were assessed from the rectilinear plot and were found to be 3.78 and 15.12 µg/mL. The regression equation of the linearity curve was found to be y = 851.76x + 310.38 and $r^2$ value of 0.9998. The samples were subjected to stress degradation and characterized by three acid degradation products by liquid chromatography-mass spectrometry (LC-MS/MS) - DPI, DPII, and DPIII. The stationary phase and movable system attained excellent resolution as chromatographic isolation and structure identification was accomplished using LC-MS/MS fragmentation.

**Keywords:** Azacitidine, High-performance liquid chromatography, Liquid chromatography-mass spectrometry, Degradation studies.

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## INTRODUCTION

Azacitidine (AZA) is pyrimidine nucleoside analogs of cytidines with anti-neoplastic action. It is chemically 4-amino-1-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl) oxolan-2-yl] -1, 2-dihydro-1,3,5-triazin-2-one, respective to the molecular[1] formula $C_8H_{12}N_4O_5$ (Figure 1). It has the relative mass of the molecule is of 244.20 g/moL. Non-proliferating cells are less sensitive to azacitidine. Adding azacitidine to DNA inhibits DNA methyl transferases, resulting in hypomethylated DNA. A hypomethylation of genes related with cell cycle regulation, differentiation, and death pathway may reactivate genes with cancer-suppressing properties. Yet to be tested is the relative importance of DNA hypomethylation to clinical outcomes, as opposed to cytotoxicity or other sites of action of azacitidine. Few analytical approaches have been described for the determination of AZA and to identify degradation products, including High-performance liquid chromatography (HPLC), Liquid chromatography-mass spectrometry (LC-MS/MS).[2-4]

Moreover, the utilization of novel methods and sophisticated instrumentations (LC-MS/MS) is lacking. Hence it is essential to develop a stability-indicating LC method for the estimation of azacitidine in incidence of degradation components and characterizing of degradation products. In this study, a systematic investigation of degradation products was carried out using the LC-MS/MS technique and stability indicating reversed-phase high-performance liquid chromatography (RP-HPLC) procedure has been developed.[4-6]

The research aims to characterize the unspecified degradation components of selected drugs by LC-MS and develop stability indicating test procedure for quantitation of drug and its degradation constituents.

## MATERIAL AND METHODS

Azacitidine was obtained from Wellona Pharma Pvt. Ltd., Gujarat, India. The solvents acetonitrile and methanol were purchased from Standard Reagents Pvt. Ltd., Hyderabad, India.

### Development of Stability Indicating RP-HPLC Method

To study azacitidine stability under stressed conditions, an RP-HPLC method was developed. The method was optimized for defining the optimal chromatographic conditions based on a suitable response surface design.[7-9]

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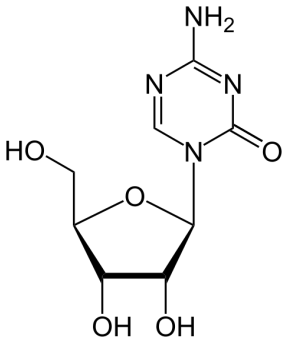


**Figure 1:** Chemical structure of azacitidine.

### Selection of Mobile Phase

Various solvents having varying solubility as well as their combinations with water or buffer at a particular pH are evaluated for the estimation of azacitidine and the mobile phase is adjusted according to the parameters associated with the chromatogram like Rs, Tf, k, N, Rt and so on.

### Method Development

*Selection of chromatographic method*

Azacitidine was estimated by using several solvents and their combinations with water and/or buffer at specific pH values, and the mobile phase was determined by considering the suitability parameters of the system.[10-13]

*Forced degradation studies*

In order to assess the stability of azacitidine, it was subjected to thermal analysis. Acid, base and neutral hydrolysis, oxidation, and photolysis were used. To assess the stability of azacitidine, 25 ppm of the resultant solution was diluted, and 10 µL were taken of the sample.[14-20]

*Acid degradation*

Study results were obtained using hydrochloric acid solutions of different strengths (2M, 1M, 0.5M). Mix 1-mL of azacitidine stock solution with 1-mL of hydrochloric acid. Degradation is conducted at room temperature. Prior to chromatographic analysis, the degraded sample was neutralized in a sodium hydroxide solution of equal strength.

*Alkali degradation*

The degradation was performed in different sodium hydroxide concentrations (2M, 1M, and 0.5M). Add 1-mL of azacitidine stock solution to 1-mL of sodium hydroxide solution and let it degrade at room temperature. Analyzed samples were then neutralized with hydrochloric acid.

*Neutral degradation*

The drug refluxed in water for 24 hours at 25°C.

*Oxidative degradation*

Oxidative degradation study using hydrogen peroxide solutions of varying strengths. To 1-mL of azacitidine stock solution, 10, 20, and 30% $H_2O_2$ was added. It was allowed to stand for two hours.

*Thermal degradation*

The study was performed by exposing solid drug in a petri dish as about 1-mm thickness at 60°C for 72 hours.

*Photo degradation*

In this study, a petri dish was exposed for 24 hours to UV radiation with a drug layer of approximately 1-mm thickness. A degraded powder equivalent to 100 mg of drug was weighed precisely and a sample solution of the desired strength was prepared.

*Validation of proposed method*

The developed chromatographic procedure was validated as per the ICH-Q2(R1) guidelines. The validation constraints linearity, specificity and selective, accurateness, precisions, robustness, LoD and LoQ were elected for this methodology validation.

### Applicability of Stability representing RP-HPLC Methodology on LC-MS/MS

In order to complete the characterizing degradation components by the use of mass detector, an API-5000 triple quadrupoles mass system (MDSSCIEX, Canada) was used. The mass parameters were adjusted after configuring the LC-MS/MS instrument to account for all of the degradation components. This was accomplished by incorporating the acid degradation sample into the LC mobile phase. Ionic source gas1 was fixed to 35 psi, ionic spraying voltage was fixed to 4500V, curtain gas was fixed to 25 psi, and the temperature of the turbo spray was set at 450°C. The optimal mass conditions were as follows: ion source gas-2 was set at 35 psi. Good resolution was achieved by utilizing the same stationary and movable systems as in chromatographic isolation. The structural characterization of every degradation component was conducted by LC-MS fragmenting method.

## RESULTS AND DISCUSSION

### Stability Indicating RP-HPLC Method

The UV spectrum of azacitidine at a concentration of 50 µg per mL was utilized to pick the chromatographic analysis's detection wavelength. At a wavelength of 210 nm, the medication exhibited its highest absorption.

### Mobile Phase

The mobile phase selection was carried out by considering various types of solvent system, including carbinol, water, 0.1M potassium dihydrogen phosphate, ammonium acetate buffer, acetonitrile and 0.1% HCOOH in water. The chromatographic

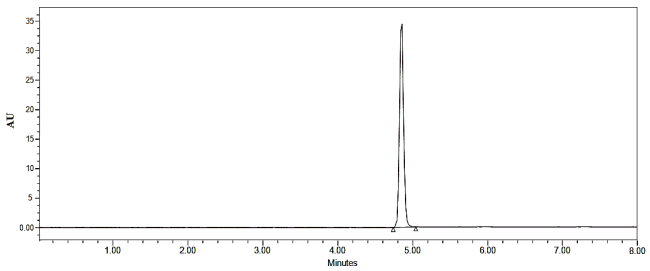


**Figure 2:** Optimized chromatogram of azacitidine

elution of azacitidine was carried out using Phenominex C18 (150 × 4.6 mm 5 µ) stationary phase, movable system of 0.1% HCOOH in water and acetonitrile (30:70 v/v), at flowing rate of 1-mL/min analyzed at 210 nm (Figure 2).

**Forced Degradation Studies**

The drug displayed significant degradation at higher concentrations of acid and bases (Figure 3 and Table 1). At 0.5 M hydrochloric acid for 24 hours at room temperature, the drug displayed < 20% degradation, while about 10% degradation was observed in 0.5 N NaOH for 24 hours at room temperature.

**Risk Assessment**

Based on the results of degradation studies of azacitidine, it is evident that the acid degradation conditions have a major impact on the stability of the drug. It is also evident that the appearance of three major critical impurity peaks and the drug peaks risks the method's performance. It means majority of method failures were related to the method's selectivity. So the appearance of critical impurity peaks made the developed method less selective. The critical technique parameters mainly affected the selected approach's selectivity. The critical method parameters of the developed method were identified and listed in Table 2.

**Method Validation**

*Calibration curve*

The calibration curve of azacitidine was studied in a range of 25 to 125 µg/mL. The regression equation of the linearity curve was found to be y = 851.76x + 310.38 (Figure 4) and $r^2$ value of 0.9998 (Table 3).

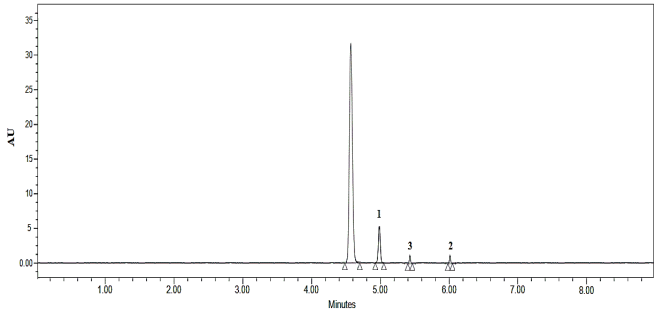


**Figure 3:** Optimized chromatogram of acid degradation of azacitidine

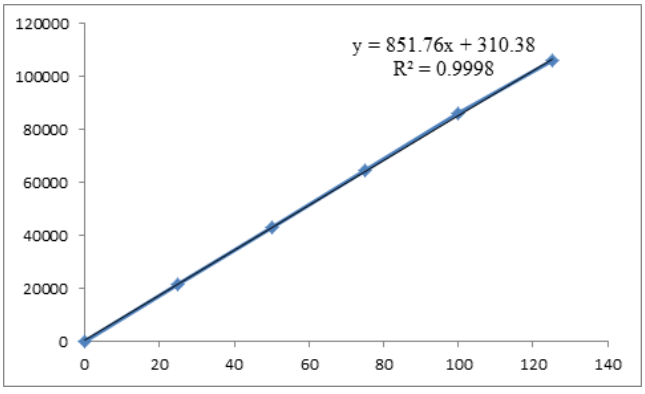


**Figure 4:** Linearity of azacitidine

**Table 1:** Percentage degradation of azacitidine under different stress conditions

| *Condition* | *Initial assay (%)* | *%Assay post degradation* | *%Degradation* |
|---|---|---|---|
| Acid | 100 | 84.27 | 15.23 |
| Alkaline | 100 | 92.63 | 7.37 |
| Neutral | 100 | 98.39 | 1.61 |
| Oxidation | 100 | 98.87 | 1.13 |
| Thermal | 100 | 98.65 | 1.35 |
| Photolytic | 100 | 98.59 | 1.41 |

**Table 2:** Critical method parameters

| *S. No* | *Condition* | *Criticality* |
|---|---|---|
| 1 | pH of mobile phase | Variable |
| 2 | %of aqueous in mobile phase | Variable |
| 3 | Flowrate of mobile phase | Variable |

**Table 3:** Linearity data for azacitidine

| *S. No* | *Conc. µg/mL* | *Average Peak area* | *Slope* | *Correlation coefficient* |
|---|---|---|---|---|
| 1 | 25 | 21523 | | |
| 2 | 50 | 42941 | | |
| 3 | 75 | 64836 | 851.76 | 0.9998 |
| 4 | 100 | 85951 | | |
| 5 | 125 | 106023 | | |

**Table 4:** Purity of peak and method specificity findings

| *Analyte/ degradation components* | *Purity of peak* | *Threshold at single point* | *Resolution* |
|---|---|---|---|
| DP1 | 0.9982 | 0.9988 | 1.87 |
| DP2 | 0.9985 | 0.9976 | 7.18 |
| DP3 | 0.9938 | 0.9979 | 4.03 |
| Azacitidine | 0.9988 | 0.9928 | - |

**Table 5:** Precision data of azacitidine

| *Level* | *Peak area of azacitidine* | | |
|---|---|---|---|
| | *Standard* | *Intraday precision* | *Inter day precision* |
| Mean* | 42883 | 42735 | 42737 |
| S.D | 109.887 | 140.870 | 119.223 |
| % RSD | 0.26 | 0.33 | 0.28 |

**N=6*

*Specificity*

The proposed HPLC method has specificity related to each peak respective to the purity of peak achieved by utilizing PDA detection (Table 4).

*Precision*

The intraday precision study and the interday precision study had RSD values of less than 1.0%. The inter-day and intra-day precision data were 0.28 and 0.33%, respectively (Table 5).

**Table 6:** Accuracy results

| *80% level* | | | | *100% level* | | | *120% level* | | |
|---|---|---|---|---|---|---|---|---|---|
| Amount Added (µg) | 40 | 40 | 40 | 80 | 80 | 80 | 120 | 120 | 120 |
| Amount found (µg) | 39.79 | 39.69 | 39.81 | 79.36 | 78.74 | 79.82 | 119.64 | 118.56 | 119.23 |
| %Recovery | 99.475 | 99.225 | 99.525 | 99.2 | 98.425 | 99.775 | 99.7 | 98.8 | 99.35833 |
| AVG | 99.41 | | | 99.13 | | | 99.29 | | |
| SD | 0.160 | | | 0.677 | | | 0.454 | | |
| RSD | 0.161 | | | 0.68 | | | 0.46 | | |

*Accuracy*

The accuracy research was conducted at concentration levels of 40, 80, and 120 µg/mL, and the data revealed that the technique had a higher degree of accuracy for the determination of azacitidine. The %recovery findings ranged between of 99.13 to 99.41% (Table 6).

*Limit of quantification and limit of detection*

The LoD and LoQ values were determined by analyzing the linearity graph, and they were found to be 3.78 and 15.12 µg/mL, respectively (Figures 5 and 6)

*Robustness*

The robustness of the method was tested by making modest, intentional changes to the flow rate (0.2 mL/min) and composition (± 5%) of the mobile phase. Additionally, the relative standard deviation (RSD) was calculated for chromatographic parameters such as theoretical plates and tailing factor in relation to the alterations. The results, which may be seen in Table 7, were considered to be acceptable.

*System suitability*

System suitability and stability of solution (at 2–8°C) were processed and %RSD values were calculated. All the findings were within the limits and the values were tabulated in Table 8. The standard solution was stable for more than 24 hours.

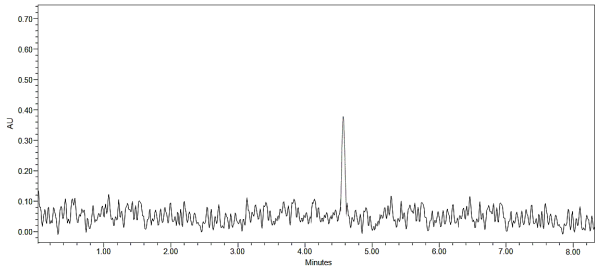


**Figure 5:** LoD of chromatogram of azacitidine

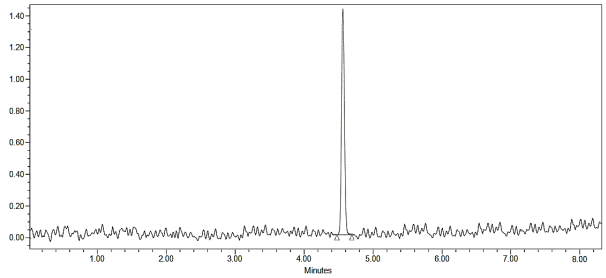


**Figure 6:** LoQ of the chromatogram of azacitidine

**Application of Stability Indicating RP-HPLC Method on LC-MSMS**

The circumstances of LC were transferred to LC-MS/MS, which led to the detection of azacitidine RT in around 6.8 minutes; all of the chromatographic variables remained the same, with the exception of the infusion volume, which was set to 0.3 µL due to the very high signal intensity that was detected while employing MS/MS detection. Because the negative mode did not produce any ionization, the ESI source had to be run in the positive ion mode in order to function properly. The mass parameters were adjusted after configuring the LC-MS/MS system to account for all of the degradation products. This was accomplished by including the acid degradation sample into the chromatographic movable phase.

**Azacitidine Degradation Behavior**

Figure 7 shows the proposed degradation behavior of Azacitidine under acid stress. Total three degradants were produced from the drug. The degradation product DP-I, generated in acidic stress condition is a product of triazine ring opening reaction. DP-II and DP-III might be generated from the ring opening reaction via acid hydrolysis of triazine of Azacitidine.

**Table 7:** Robustness data for azacitidine

| *Parameter* | | *Retention time in min* | *Theoretical plates N* | *Tailing factors* |
|---|---|---|---|---|
| Mobile phase: 0.1% HCOOH in water-Acetonitrile | 35:65 v/v | 4.695 | 7845 | 1.064 |
| | 30:70 v/v | 4.775 | 9658 | 1.035 |
| | 25:75 v/v | 3.474 | 9563 | 1.078 |
| Flow rate | 1.2 mL/min | 4.489 | 9421 | 1.045 |
| | 1.0 mL/min | 4.709 | 9637 | 1.062 |
| | 0.8 mL/min | 7.332 | 8654 | 1.054 |

**Table 8:** Robustness solution stability data for azacitidine

| *Parameter* | *%RSD** |
|---|---|
| System suitability | 0.89 |
| Post 1-hour | 1.05 |
| Post 4 hours | 0.87 |
| Post 8 hours | 1.27 |
| Post 16 hours | 1.07 |
| Stability after 24 hours | 1.08 |

*Relative standard deviation for 6 injections

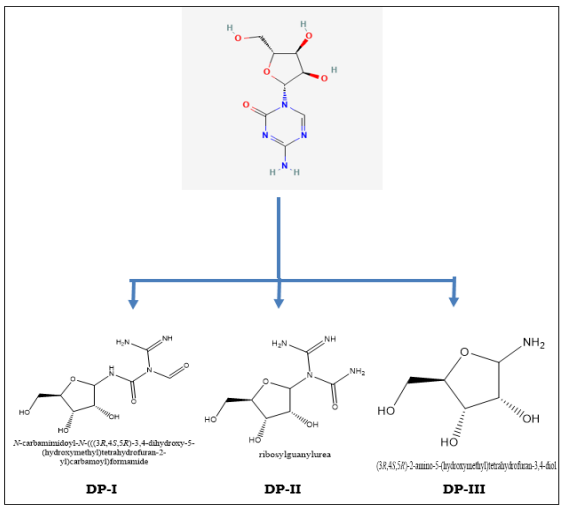


**Figure 7:** Structure of impurities

## Identification of Degradation Products by LC-MS/MS

*DP-I (m/z 263.10)*

The ESI MS/MS represented a peak at $[M + H]^+$ ions of DP-I (Rt= 4.97 minutes) corresponding to m/z 263.10 with formula $C_8H_{15}N_4O_6^+$ indicating the breakage of triazine ring of the azacitidine. Further, the fragmentation of DP1 produced fragment ions at m/z 148.15, 134.20 and 116.10 (Figure 8).

*DP-II (m/z 235.20)*

The molecular $[M + H]^+$ ion peak at (Rt = 6.08 minutes) corresponds to m/z 235.20 with formula $C_7H_{15}N_4O_5^+$ specifies the triazine ring opening of azacitidine and hydrolytic cleavage of amide bond. Other peaks include m/z 173.75, 148.15 and 65.40 (Figure 9).

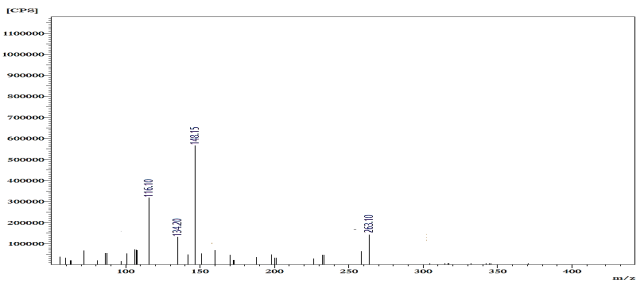


**Figure 8:** Mass spectra of DPI in LC-MSMS study

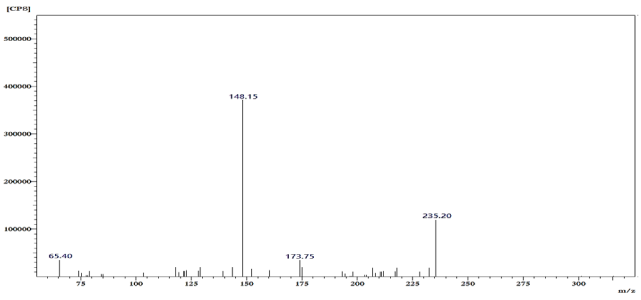


**Figure 9:** Mass spectra of DPII in LC-MSMS study

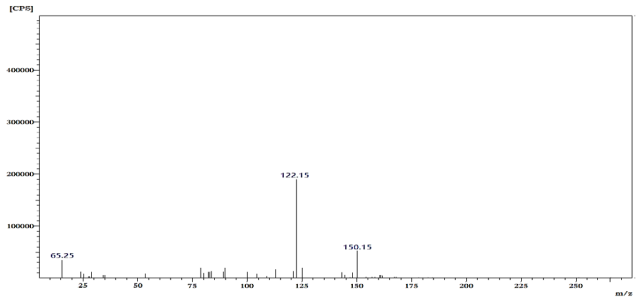


**Figure 10:** Mass spectra of DPIII in LC-MSMS study

*DP-III (m/z 150.15)*

The molecular $[M + H]^+$ ion at (Rt = 5.42 minutes) corresponding to m/z 150.15 with formula $C_5H_{12}NO_4^+$ discloses the complete cleavage of triazine ring from the robosyl part and breakdown amide bond that finally produces a simple amine attached to the ribosyl part of azacitidine. Other fragmentations include em/z 122.15 and 65.25 (Figure 10).

## CONCLUSION

The antitumor effects of azacitidine are analogous to those of cytidine. The chromatographic separation of azacitidine was carried out using Phenominex C18 (150 × 4.6 mm × 5 µ) column, a mobile phase of 0.1% HCOOH in water-acetonitrile (30:70 v/v), at flowing rate of 1-mL/min analyzed at 210 nm. The samples were subjected to stress degradation conditions and the drug displayed maximum degradation of 15.23% under acidic conditions. We characterized three acid degradation products by LC-MS/MS – DPI, DPII, and DPIII. The excellent resolution was attained by using the same stationary phase and movable system as in chromatographic isolation and the identification of this structure was identified using LC-MS/MS fragmentation. Three major compounds were detected based on the LC-MS/MS analysis of acid degradation products. Analyses of the fragmentation pattern of azacitidine and its degradation products have identified the probable mechanisms by which degradation products are formed. An accurate, precise, robust and reproducible method was developed, RP-HPLC, for detecting stable degradation products. In accordance with ICH guidelines, RP-HPLC has been validated. LC-MS/MS was performed to determine azacitidine's degradation products.

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