DEVELOPMENT AND VALIDATION OF A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) METHOD FOR THE DETERMINATION OF RELATED SUBSTANCES IN A NOVEL ANTICANCER DRUG

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Abstract:

Azacitidine is a chemotherapeutic agent. Also referred to as Vidaza. It addresses individuals who are ineligible for high-dose stem cell or bone marrow transplants. A novel HPLC methodology for identifying potential active medicinal constituents has been developed and validated. The technique utilizes gradient elution and an ACE C18 column (150 x 4.6 mm, 15 µL). The mobile phases A and B consisted of methanol R and a phosphate buffer solution containing triethanolamine, adjusted to a pH of 7.0. At a flow rate of 1 ml/min, the mobile phase ratio varied gradually during the study. A 240 nm DAD detection was used. The technique was verified according to ICH and Ukrainian State Pharmacopoeia standards. The drug's stability was evaluated at elevated temperatures, strong acids and bases, and oxidizing agents. The solutions were analysed using HPLC. Azacitidine was vulnerable to breakdown by peroxide, alkali, and acid, resulting in the emergence of inexplicable pollutants.

Keywords: Development, Validation, High-Performance Liquid Chromatography and Anticancer Drug.

1 INTRODUCTION

HPLC analysis yielded the most precise results. If the concept is endorsed by regulatory authorities, they are more likely to grant permission. HPLC is frequently employed in the production of generic pharmaceuticals as a result of its ability to identify chemical contaminants and impurities. The chemical constituents under analysis determine the HPLC detector, which is available in a variety of configurations. In order to assess the assay and impurities, a diverse array of detectors is employed in both normal and stressful stability testing. RI, Fluorescence, UV-Visible, ELSD, and Photo Diode Array detectors are among the most common types. In India, there is potential for growth in the commercialization of combination dosage forms and academic research. India enjoys great privileges. According to Sonawane et al. (2010), the majority of methodologies for evaluating combination dosage forms evaluate each constituent individually. This is pertinent to the majority of methodologies (1-3).

Pharmaceutical corporations in every industrialized nation are working assiduously to create a generic prescription that is as effective as the original but more cost-effective. During standard stability

assessments, a variety of degradation products were produced in accordance with ICH standards. It is logical to employ the quality equation technique to address these issues, as it has been proposed as a solution. The proposed method for assessing the quality attributes of the formulation at each time interval necessitates a greater amount of time than a conventional stability study. Numerous medicinal components are being investigated during the current phase of technique development. Stress testing, which is frequently referred to as forced degradation, is employed to accomplish robust process design and specificity demonstration. This functions as a test, in the event that no additional information is provided. This is accurate when there is limited product information available. Additionally, the research concentrates on the consequences of storage-related degradation (4, 5).

Pharmaceutical formulation, production, and packaging may be improved by an understanding of chemical behaviour, thereby enhancing the quality of the ultimate product. The conclusions of the investigation are contingent upon the pharmaceutical supplies and apparatus that were employed. Evaluation of degradation products may not be necessary for the development and assessment of analytical methodologies. This is accurate provided that degradation products are not intentionally produced. The method has the potential to improve the efficacy of time and the capacity to identify the most significant degradation products by expediting the decomposition of each formulation component. The reliability and precision of these methods were demonstrated through the development and evaluation of post-degradation materials. They may be implemented in any API evaluation. This eliminates the necessity for modifications to accommodate new contaminants, thereby saving both time and resources (6, 7).

2 MATERIAL AND METHOD

2.1 Instrumentation and Chromatographic conditions:

Instruments used were mentioned in table 1.

Table 1 Instrumentation

Instruments	Model No.	Manufacturer
HPLC	1260 Infinity II	Agilent
HPLC Column	Eclipse Plus C18 (150mm x 4.6mm, 5µm)	Agilent
Detector	Photo Diode Array	-
UV-Visible Spectrophotometer	UV- 1900	Shimadzu
PH meter	EQ-610	Lab Line
Ultra Sonicator	LMUC 6	-
Water purification system	-	Mili- Q
Analytical Weighing Balance	ME204/A04	Shimadzu

2.2 RP-HPLC RS method development for the estimation of Azacitidine in bulk drug.

2.2.1 Trial 1

In the stability study of the DMF approach, it was noted that the tailing factor for the principal peak is unsatisfactory, as shown by the observed bulge and the theoretical plate counts. (8)

2.2.1.1 Chromatographic conditions:

Instrument	:	HPLC
Column	:	Phenomenax LUNA SCX 4.6 X 50 mm, 5 µm
Injection Volume	:	20 µL
Flow rate	:	2.0 mL/min
Wavelength	:	UV 230 nm
Column	:	30°C
Sample Temperature	:	25°C
Retention Time	:	About 3.0 minute for Azacitidine
Run Time	:	20 minutes Standard and SST
	:	40 minutes for Blank, Placebo and sample
Needle wash	:	Mixture of Acetonitrile and Water in the ratio of 90:10.
Seal wash	:	Mixture of Acetonitrile and Water in the ratio of 10:90

2.2.2 Trial 2

Buffer, gradient protocol, column, and column oven temperature Research to establish a novel approach for the RS test of Azacitidine (9).

2.2.2.1 Chromatographic conditions:

Instrument	:	HPLC
Column	:	ACE EXCEL 250X 4.6 mm, 3 µm
Injection Volume	:	20 µL
Flow rate	:	1.0 mL/min
Wavelength	:	UV 230 nm
Column Temperature	:	40°C
Sample Temperature	:	25°C

2.2.2.2 Preparation of solutions:

Buffer solution pH-2.5:

After accurately weighing 2.0 g of Tetrabutyl ammonium hydrogen sulfate, incorporate it into two litters of distilled water and stir vigorously until dissolved (pH 2.547 was recorded). Employ a PVDF membrane filter with a pore diameter of 0.45 µm to purify the mixture.

Mobile phase:

Prepare a 95:05 mixture of acetonitrile and buffer solution. Sonicate the solution for fifteen minutes to degas it.

2.2.3 Trial 3

The trial involves the buffer temperature, gradient program, column, and column oven. Investigate the development of a novel Azacitidine RS testing methodology (10).

2.2.3.1 Chromatographic conditions:

Instrument	:	HPLC
Column	:	ACE EXCEL 250X 4.6 mm, 3 µm
Injection Volume	:	5 µL
Flow rate	:	0.6 mL/min
Wavelength	:	UV 230 nm
Column Temperature	:	25°C
Sample Temperature	:	25°C

2.2.3.2 Preparation of solutions:

Buffer solution pH-2.5:

8.0 g of Tetrabutyl ammonium hydrogen sulfate should be accurately weighed and thereafter placed into two litters of filtered water, together with 2.0 mL of triethylamine, and thoroughly agitated to achieve dissolution (observed pH 2.532). Employ a PVDF membrane filter with a pore diameter of 0.45 µm to purify the mixture.

Mobile phase A:

Used Buffer pH 2.532

Mobile phase B:

Used 100% Acetonitrile.

2.2.4 Trial 4

Buffer, gradient protocol, column, and column oven temperature Research to establish a novel approach for the RS test of Azacitidine (11).

2.2.4.1 Chromatographic conditions:

Instrument	:	HPLC
Column	:	ACE EXCEL 250X 4.6 mm, 3 µm
Injection Volume	:	5 µL
Flow rate	:	0.8 mL/min
Wavelength	:	UV 230 nm
Column Temperature	:	50°C
Sample Temperature	:	25°C

2.2.4.2 Preparation of solutions:

Dilute Orthophosphoric acid for buffer pH adjustment preparation:

Introduce 10.0 mL of orthophosphoric acid into a 100 mL volumetric flask holding about 200 mL of water and stir well. Cool and dilute with water to the desired volume, then combine well.

Buffer solution pH-2.5: Accurately weigh about 5.0 g of 1-Heptane sulfonic acid salt and put it into 2 litters of filtered water, then adjust the pH to 2.472 using diluted orthophosphoric acid. Pass the solution over a 0.45 µm PVDF membrane filter.

Mobile phase A:

Used Buffer pH 2.472

Mobile phase B:

Used 100% Acetonitrile.

2.2.5 Trial 5

2.2.5.1 Chromatographic conditions:

Mode	:	HPLC
Column	:	150 X 4.6 mm, 3.0 µm (Inertsil ODS-HL is suitable)
Injection Volume	:	15 µL
Wavelength (dual wave length)	:	UV 260 nm for any unspecified impurities UV 274 nm for Azacitidine Related Compound A and B
Column Temperature	:	35ºC
Sample Temperature	:	25ºC
Flow rate	:	1.0 mL /minute
Retention Time	:	About 28.0 minute for Azacitidine
Run Time	:	80 minute
Seal wash	:	Mixture of acetonitrile and water in the ratio of 10:90

2.2.5.2 Preparation of solutions: (12, 13)

Dilute Acetic acid solution for needle wash:

Prepare a diluted acetic acid solution for needle washing by combining 5 mL of glacial acetic acid with 1000 mL of water and ensuring thorough mixing.

Mobile phase A:

Dissolve 4.33 g of sodium octane sulfonate in 1000 mL of water and stir well using a magnetic stirrer. Introduce 5 mL of glacial acetic acid while stirring, and continue stirring for about 15 minutes. Pass the solution through a 0.2 µm nylon membrane filter and sonicate for about 10 minutes to degas.

Mobile phase B:

Prepare a 50:50 mixture of acetonitrile and methanol, stirring for about 10 minutes on a magnetic stirrer. Sonicate for about 10 minutes to degas.

Mobile phase C:

Acetonitrile.

System suitability solution:

Introduce 6.0 mg of the working or reference standard of Azacitidine into a 50 mL volumetric flask. Bake the sample at 120°C for 10 minutes. The flask must be let to reach room temperature. Introduce 3.0 mL

of 0.1N hydrochloric acid. The solution should be heated in water for 10 minutes. Allow the flask to equilibrate to room temperature, then include 50 mg of the reference or working standard of Azacitidine. To prepare the working standard or reference standard, include 25 mL of diluent and sonicate for 5 minutes. Dilute to achieve homogeneity and increase volume.

2.3 Validation of the RP-HPLC RS Method for the Quantification of Azacitidine in Bulk Drug and Formulations with Forced Degradation Studies.

2.3.1 Specificity:

Specificity refers to the ability to accurately assess the analyse amongst constituents that are expected to be present in the sample matrix. Typically, they may include matrices, degradants, pollutants, etc. (14).

2.3.2 Forced degradation study:

A forced degradation research will be performed on 300 mg tablets, placebo, and Azacitidine active pharmaceutical ingredient (API). The samples will undergo acid, base, oxidation, hydrolysis, photolysis, humidity, and heat degradation. Each degradation study should provide a designated blank space. (15)

2.3.3 Limit of detection (LOD) and limit of quantification (LOQ) precision: (16)

2.3.3.1 Limit of detection (LOD):

The concentration of an analyse at which detection becomes possible but quantification becomes inconsistent is known as the LOD.

2.3.3.2 Limit of quantitation (LOQ):

The LOQ is the lowest concentration of an analyse that can be identified quantitatively with a satisfactory level of precision and accuracy.

2.3.4 Linearity:

Analytical procedures are considered linear if the test results are proportionate to the concentration of the sample analyte. Six levels, from LOQ to 200%, will be used for the linearity investigation of designated known contaminants at the specification limit and Azacitidine at standard concentration. Quantitatively diluted Consolidate solutions of contaminants. 1. To get concentrations ranging from the Limit of Quantification (LOQ) to 200% of specified known contaminants and Azacitidine at a standard concentration, combine the impurity solution. Azacitidine Standard stock solution and Azacitidine Standard solution. Each linearity standard solution must be injected three times. (17)

2.3.5 Accuracy:

The accuracy of an analytical procedure is defined as the degree to which its test results match the real value. One common metric for accuracy is the percentage recovery of the known analyte that is added. A complete accuracy metric is used to assess the analytical process. Obtain accuracy at the prescribed limits for the known pollutants, including the Limit of Quantification (LOQ), 50%, 100%, and 200%. We will add the detected pollutants to the sample solution at each stage to measure the recovery rates. Using the given technique to create three control sample solutions, determine the impurity percentage. Take the accuracy levels and remove the detected contaminants to get the recovery. Gather three samples for the accuracy study at each level and combine them into one. (18)

2.3.6 Precision:

The accuracy of an analytical procedure is defined as the degree to which many measurements taken from multiple samples of the same homogenous material under prescribed conditions agree with one another. (19)

2.3.6.1 System precision:

To assess the system's accuracy, add six aliquots of the standard solution to one HPLC vial as per the testing protocol.

2.3.6.2 Method precision (Repeatability):

Accuracy throughout a short period of time under constant operating conditions is measured by reproducibility. For accurate dosing, 300 mg tablets of Azacitidine will be used. Get three 300 mg homogeneous Azacitidine tablet sample solutions and six 300 mg spiked tablet sample solutions ready. For calculations involving related compounds, three solutions will be used as control samples. (20)

2.3.6.3 Intermediate precision:

Intermediate Laboratory intermediate accuracy varies with day, analyst, HPLC system, column manufacturer, and sample batch, as explained in the section on repeatability. With 300 mg of Azacitidine in each tablet, significant precision is required. Create three control sample solutions and six spiked sample solutions on a separate day by a different analyst using unique HPLC equipment, a column of the same brand, and consistent samples of 300 mg Azacitidine Tablets per process. For calculations involving related compounds, three solutions will be used as control samples.

2.3.7 Range:

The range of an analytical methodology is the interval between the maximal and minimum analyte concentrations in a sample, where the method exhibits enough accuracy, precision, and linearity. It is common practice to indicate the range in the same units as the test findings that are derived analytically.

2.3.8 Filter study:

Combine 300 mg of Azacitidine tablets into one sample solution and add a spike to the other.

2.3.9 Solution stability:

One 300 mg Azacitidine tablet sample solution should be prepared according to the specificity of the method, and another 300 mg Azacitidine tablet sample solution should be prepared using the spiked method. All solutions should be kept at room temperature. Find out what happens when you check the answer at different times. Compare the percentage impurity values obtained at various time periods for the sample solution and calculate the overall % relative standard deviation (RSD) of the Azacitidine peak for the standard solution (21).

2.3.10 Robustness:

Wilfully alter all of the chromatographic parameters listed below, and then observe how these changes affect the system's applicability and the percentage values of impurities. Apply the chromatographic conditions specified in the testing process to three solutions of 300 mg Azacitidine Tablets (one each for the control and spiking samples), and use the method variables listed to find the technique's robustness. Evaluate the system's appropriateness and the impurity percentage in each variable circumstance (21, 22).

2.3.11 System suitability:

The suitability of the system must be verified prior to the use of each validation parameter. Assess the system's suitability using the method.

3 RESULT AND DISCUSSION

3.1 Development of an RP-HPLC RS technique for the quantification of Azacitidine in bulk medication and formulations, including forced degradation investigations.

3.1.1 Trial 1

3.1.1.1 Observations-

1. A hump was noticed at the tailing of the main peak.

2. The theoretical plate count and the tailing factor for the principal peak are suboptimal.

3.1.1.2 Conclusion-

A study on buffer, gradient, column, and column oven temperature is necessary.

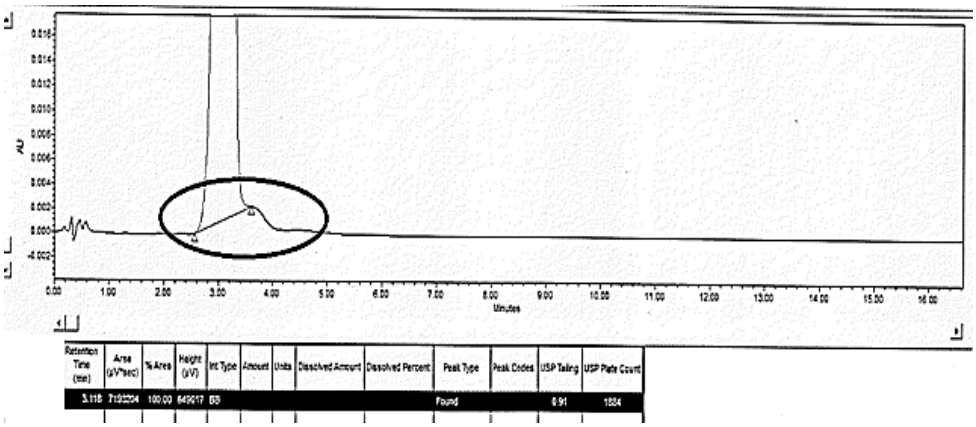

Figure 1 Chromatogram for Trial 1

3.1.2 Trial 2

3.1.2.1 Observations

1 .At tailing of main peak baseline pattern not good

3.1.2.2 Conclusion

Buffer, gradient, column and column oven temperature Study needs to be performed.

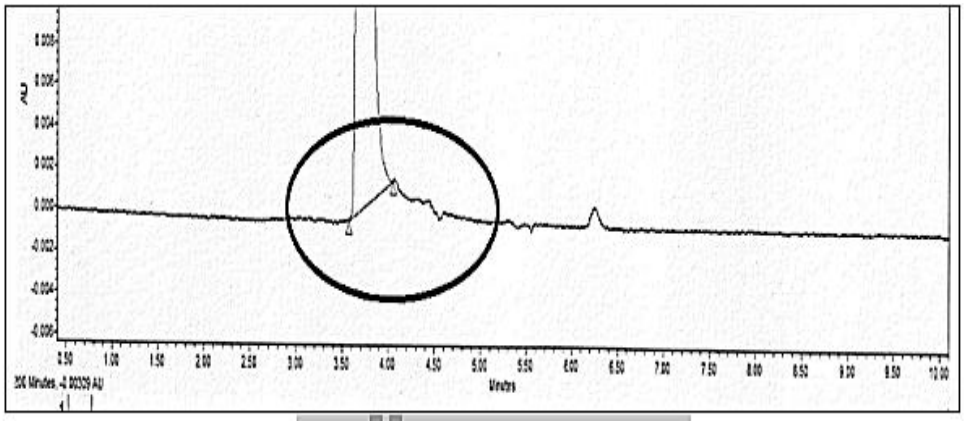

Figure 2 Chromatogram for Trial 2

From observation this methodology can be finalized and Prevalidation study needs to be performed.

3.1.3 Trial 3

3.1.3.1 Observations-

At tailing of main peak baseline pattern not good

3.1.3.2 Conclusion-

Buffer, gradient, column and column oven temperature Study needs to be performed.

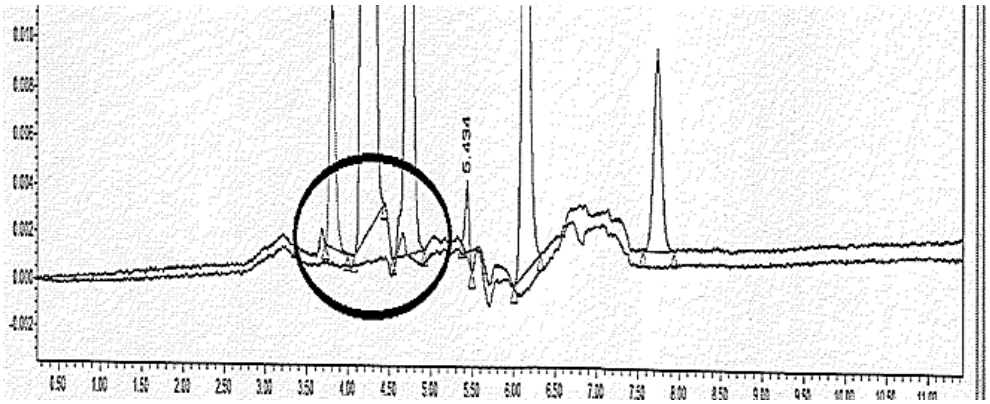

Figure 3 Chromatogram for Trial 3

3.1.4 Trial 4

3.1.4.1 Observations-

1. The major tailing noticed is around 1.64, which is somewhat elevated.

2. All impurity peaks are distinctly separated from the primary peak.

3.1.4.2 Conclusion-

To enhance the tailing gradient, an investigation into column oven temperature and injection volume is necessary.

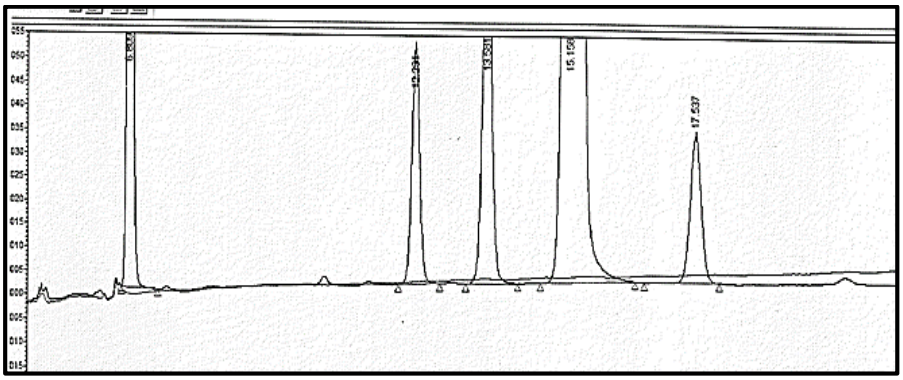

Figure 4 Chromatogram for Trial 4

3.1.5 Trial 5

3.1.5.1 Observation:

1. Absence of interference Blank peak seen during the retention time of the primary peak impurity.

2. All impurity peaks are distinctly separated from one another.

3. The tailing factor for the primary peak is around 5, representing an improvement over the prior experiment result.

3.1.5.2 Conclusion

This approach may be finished based on observation, and a validation study must be conducted.

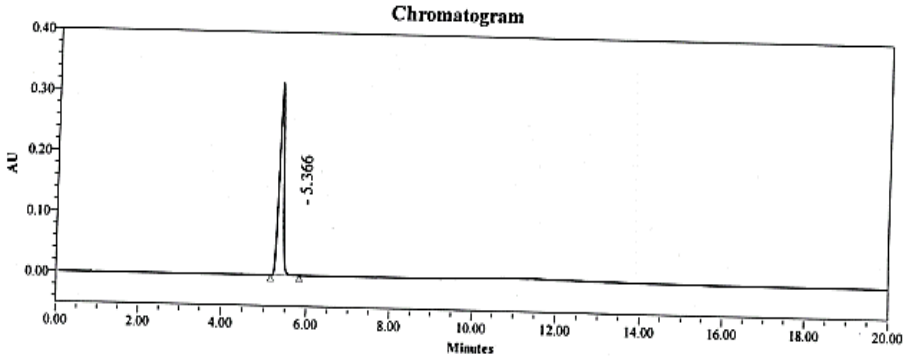

Figure 5 Chromatogram for Trial 5

3.2 RP-HPLC RS method Validation for the estimation of Azacitidine in bulk drug and formulations with forced degradation studies.

Table 2 Validation Summary

Sr. No.	Validation Parameter	Results	Acceptance Criteria
1	**Specificity**		
1.1	**Check for blank, placebo and impurities interference**		
	Interference	There is no any interference due to blank and placebo at the retention time of Azacitidine peak and known impurities peaks.	There should not be any interference due to blank and placebo at the retention time of Azacitidine peak and known impurities peaks.
	Peak purity	Peak purity criteria (Peak purity = purity angle < purity threshold) passes for Azacitidine and known impurities peaks in the mix standard solution, sample solution, placebo spiked solution with known impurities and Azacitidine and sample spiked solution with known impurities.	Peak purity criteria (Peak purity = purity angle < purity threshold) should pass for Azacitidine and known impurities peaks in the mix standard solution, sample solution, placebo spiked solution with known impurities and Azacitidine and sample spiked solution with known impurities.
1.2	**Forced degradation study**		
	Interference	There is no any interference observed due to blank, placebo and degradation products with	There should not be any interference due to blank,

<table>
<tr><td></td><td></td><td colspan="3">the Azacitidine and known impurities.</td><td>placebo and degradation products with the Azacitidine and known impurities.</td></tr>
<tr><td></td><td>Peak purity</td><td colspan="3">Peak purity criteria (Peak purity = Purity angle < Purity threshold) passes for Azacitidine and known impurities peak in all the degraded samples.</td><td>Peak purity criteria (Peak purity = Purity angle < Purity threshold) should pass for Azacitidine and known impurities peak in all the degraded samples.</td></tr>
<tr><td rowspan="6">2</td><td rowspan="6">LOD and LOQ precision</td><td colspan="3">Limit of detection (LOD):
Peak due to Azacitidine and known impurities are detected at LOD level.</td><td rowspan="4">Limit of detection (LOD):
Peak due to Azacitidine and known impurities should be detected at LOD level.</td></tr>
<tr><td>Name</td><td colspan="2">LOD values</td></tr>
<tr><td>Azacitidine</td><td colspan="2">0.038%</td></tr>
<tr><td>Azacitidine Related compound A</td><td colspan="2">0.08%</td></tr>
<tr><td>Azacitidine Related compound B</td><td colspan="2">0.07%</td><td rowspan="5">Limit of quantitation (LOQ):
RSD of peak area response due to Azacitidine and known impurities should not be more than 10.0%.
Signal to noise ratio for Azacitidine and known impurities should not be less than 10.</td></tr>
<tr><td colspan="3">Limit of quantitation (LOQ):</td></tr>
<tr><td>Name</td><td>LOQ</td><td>% RSD</td></tr>
<tr><td>Azacitidine</td><td>0.05%</td><td>4.7</td></tr>
<tr><td>Azacitidine Related compound A</td><td>0.15%</td><td>6.8</td></tr>
<tr><td>Azacitidine Related compound B</td><td>0.14%</td><td>4.3</td><td colspan="2">Signal to noise ratio for Azacitidine and known impurities are found above 10.</td></tr>
<tr><td rowspan="3">3</td><td rowspan="3">Linearity</td><td colspan="2">Name</td><td>Correlation coefficient (R)</td><td rowspan="3">The correlation coefficient (‘R’) value should not be less than 0.99 over the working range.</td></tr>
<tr><td colspan="2">Azacitidine</td><td>0.99998</td></tr>
<tr><td colspan="2">Azacitidine Related compound A</td><td>0.99991</td></tr>
</table>

<table>
<tr><td></td><td></td><td>Azacitidine Related compound B</td><td>0.99994</td><td></td></tr>
<tr><td rowspan="14">4</td><td rowspan="14">Accuracy</td><td colspan="2">Mean % Recovery of Azacitidine Related Compound A:</td><td rowspan="14">Recovery of known impurities at LOQ Level should be between 70.0% and 130.0%.

Recovery and Overall mean recovery of known impurities at each level (except LOQ Level) should be between 80.0% and 120.0%.

Overall % RSD for recovery of known impurities (Except LOQ Level) should be not more than 5.0</td></tr>
<tr><td>% Level</td><td>Mean % Recovery</td></tr>
<tr><td>LOQ</td><td>109.2</td></tr>
<tr><td>50</td><td>102.5</td></tr>
<tr><td>100</td><td>100.4</td></tr>
<tr><td>200</td><td>102.2</td></tr>
<tr><td>Overall Mean (except LOQ Level)</td><td>101.7</td></tr>
<tr><td>Overall % RSD (except LOQ Level)</td><td>2.6</td></tr>
<tr><td colspan="2">Mean % Recovery of Azacitidine Related Compound B:</td></tr>
<tr><td>% Level</td><td>Mean % Recovery</td></tr>
<tr><td>LOQ</td><td>114.5</td></tr>
<tr><td>50</td><td>100.0</td></tr>
<tr><td>100</td><td>103.1</td></tr>
<tr><td>200</td><td>103.0</td></tr>
<tr><td>Overall Mean (except LOQ Level)</td><td>102.0</td></tr>
<tr><td>Overall % RSD (except LOQ Level)</td><td>2.7</td></tr>
<tr><td>5</td><td colspan="3">Precision</td></tr>
<tr><td>5.1</td><td>System precision</td><td>Tailing factor = 1.0

Theoretical plates = 120263</td><td>Tailing factor: Tailing factor of Azacitidine peak obtained from 1st injection of mix standard solution at UV 260</td></tr>
</table>

		% RSD Azacitidine = 0.7% Azacitidine Related compound A = 1.3% Azacitidine Related compound B = 0.8%	nm should not be more than 2.0 **Theoretical Plates:** Theoretical plates of Azacitidine peak obtained from 1st injection of mix standard solution at UV 260 nm should not be less than 2000 **% RSD:** Relative standard deviation of Azacitidine peak area obtained from six replicate injections of mix standard solution at UV 260 nm should not be more than 5.0%.
5.2	**Method precision**	**RSD of known impurities in six spiked solution:** **For Azacitidine Tablets 300 mg:** Azacitidine Related Compound A = 1.6% Azacitidine Related Compound B = 1.1%	RSD for % w/w of known impurities in six spiked sample solutions should not be more than 10.0%.
5.	**Intermediate precision**	**For Azacitidine Tablets 300 mg:** **RSD of known impurities in six spiked sample solution:** Azacitidine Related Compound A = 2.8% Azacitidine Related Compound B = 2.4% **Overall RSD for % w/w of known impurities in twelve spiked sample solutions from method precision and intermediate precision:** Azacitidine Related Compound A = 4.2% Azacitidine Related Compound B = 4.2%	RSD for % w/w of known impurities in six spiked sample solutions should not be more than 10.0%. Overall RSD for % w/w of known impurities in twelve spiked sample solutions from method precision and intermediate precision should not be more than 15.0%.
6	**Range**	Range was established based on	Range should be established

<table>
<tr><td></td><td></td><td colspan="2">the validation data from linearity, accuracy and precision.</td><td>based on the validation data from linearity, accuracy and precision.</td></tr>
<tr><td></td><td></td><td>Impurities Name</td><td>Established Range</td><td></td></tr>
<tr><td></td><td></td><td>Azacitidine Related Compound A</td><td>0.15% to 200% w.r.t. Impurity specification limit</td><td></td></tr>
<tr><td></td><td></td><td>Azacitidine Related Compound B</td><td>0.14% to 200% w.r.t. Impurity specification limit</td><td></td></tr>
<tr><td>7</td><td>Filter study</td><td colspan="2">Absolute difference in the impurities results:
For Azacitidine Tablets 300 mg
Controlled sample:
0.45 µm RC syringe filter:
Azacitidine Related compound A = NA
Azacitidine Related compound B = NA
Any individual unspecified Impurity = 0.002
Total impurities = 0.02
0.45 µm Nylon syringe filter:
Azacitidine Related compound A = NA
Azacitidine Related compound B = NA
Any individual unspecified Impurity = 0.001
Total impurities = 0.001</td><td>Results obtained with centrifuged sample and filtered sample are;
Between LOQ to 0.5%, Absolute difference should not be more than 0.05,
Between 0.5% to 1.0%, Absolute difference should not be more than 0.1 and
More than 1.0%, Absolute difference should not be more than 0.2.</td></tr>
<tr><td></td><td></td><td colspan="2">For Azacitidine Tablets 300 mg
Controlled sample:
0.45 µm pre-filter + PVDF syringe filter:
Azacitidine Related compound A = NA
Azacitidine Related compound B = NA</td><td>Results obtained with centrifuged sample and filtered sample are;
Between LOQ to 0.5%, Absolute difference should not be more than 0.05,</td></tr>
</table>

		Any individual unspecified Impurity = 0.007 Total impurities = 0.007 **0.45 µm pre-filter + PTFE syringe filter:** Azacitidine Related compound A = NA Azacitidine Related compound B = NA Any individual unspecified Impurity = 0.005 Total impurities = 0.005 **Sample spiked solution:** **0.45 µm RC syringe filter:** Azacitidine Related compound A = 0.03 Azacitidine Related compound B = 0.01 **0.45 µm Nylon syringe filter:** Azacitidine Related compound A = 0.01 Azacitidine Related compound B = 0.02	Between 0.5% to 1.0%, Absolute difference should not be more than 0.1 and More than 1.0%, Absolute difference should not be more than 0.2.
8	**Solution Stability**		
		Standard solution: Overall RSD of Azacitidine peak area up to 94 hours at room temperature = 1.6% Overall RSD of Azacitidine related Compound A peak area up to 94 hours at room temperature = 2.1% Overall RSD of Azacitidine related Compound B peak area up to 94 hours at room temperature = 1.2%	Overall RSD of Azacitidine, Azacitidine related Compound A and Azacitidine related Compound B peak areas in mix standard solution obtained at different time interval should not be more than 5.0%.
		For Azacitidine Tablets 300 mg	Results obtained from sample solution and sample spiked solution at different time interval,

		Sample solution: Absolute difference in the impurities results obtained at initial and at 75 hours at room temperature: Azacitidine Related compound A = NA Azacitidine Related compound B = NA Any individual unspecified Impurity = 0.001 Total impurities = 0.001 **Sample spiked solution:** Absolute difference in the impurities results obtained at initial and at 75 hours at room temperature: Azacitidine Related compound A = 0.02 Azacitidine Related compound B = 0.01	Between LOQ to 0.5% Absolute difference should not be more than 0.05, Between 0.5% to 1.0% Absolute difference should not be more than 0.1 and More than 1.0% Absolute difference should not be more than 0.2.
9	**Robustness**		
	Change in column oven temperature (± 5°C) of 35°C	**High Column Oven Temperature (HT): 40°C** Tailing factor = 1.0 Theoretical plates = 116386 % RSD Azacitidine = 0.8% Azacitidine Related compound A = 1.9% Azacitidine Related compound B = 1.0% **RSD of three spiked**	**Tailing factor:** Tailing factor of Azacitidine peak obtained from 1st injection of mix standard solution at UV 260 nm should not be more than 2.0 **Theoretical Plates:** Theoretical plates of Azacitidine peak obtained from 1st injection of mix standard solution at UV 260 nm should not be less than 2000. **% RSD:** Relative standard deviation of Azacitidine peak area obtained from six replicate injections of mix standard solution at UV 260 nm should not be more than 5.0%. **% RSD:** Relative standard deviation of Azacitidine related Compound A and Azacitidine related Compound B area obtained from six replicate injections of mix standard solution at UV 274 nm should not be more than 5.0%. RSD for % w/w of known impurities in three spiked

		sample solution: Azacitidine Related compound A = 0.7% Azacitidine Related compound B = 2.0% **Overall RSD:** Azacitidine Related compound A = 4.6% Azacitidine Related compound B = 2.3%	sample solution of each variable condition should not be more than 10.0%. Overall RSD for % w/w known impurities in six spiked sample solutions from method precision and three sample spiked solutions of each variable condition should not be more than 15.0%.
		Low Column Oven Temperature (LT): 30°C Tailing factor = 1.0 Theoretical plates = 80920 % RSD Azacitidine = 0.2% Azacitidine Related compound A = 0.7% Azacitidine Related compound B = 0.8% **RSD of three spiked sample solution:** Azacitidine Related compound A = 2.3% Azacitidine Related compound B = 1.2% **Overall RSD:** Azacitidine Related compound A = 1.8% Azacitidine Related compound B = 2.0%	**Tailing factor:** Tailing factor of Azacitidine peak obtained from 1st injection of mix standard solution at UV 260 nm should not be more than 2.0 **Theoretical Plates:** Theoretical plates of Azacitidine peak obtained from 1st injection of mix standard solution at UV 260 nm should not be less than 2000. **% RSD:** Relative standard deviation of Azacitidine peak area obtained from six replicate injections of mix standard solution at UV 260 nm should not be more than 5.0%. **% RSD:** Relative standard deviation of Azacitidine related Compound A and Azacitidine related Compound B area obtained from six replicate injections of mix standard solution at UV 274 nm should not be more than 5.0%. RSD for % w/w of known impurities in three spiked sample solution of each variable condition should not be more than 10.0%. Overall RSD for % w/w known impurities in six spiked sample solutions from method precision and three sample spiked solutions of each variable condition should not be more than 15.0%.

4 SUMMARY AND CONCLUSION

This work sought to provide an analytical approach for identifying degradation impurities arising from forced degradation tests and process impurities produced during the synthesis of Azacitidine. A novel RP-HPLC-UV method was developed for the quantitative analysis of Azacitidine and its impurities based on these properties. The process included assessing elements vital to separation efficiency, such as chromatographic columns, elution techniques, mobile phase composition, solvents, and detection wavelength. The methodology fulfilled the criteria for method validation by adhering to ICH recommendations and demonstrating adequate sensitivity, specificity, accuracy, linearity, repeatability, and robustness. This investigation provides significant new insights for the quality control of Azacitidine (23).

5 REFERENCES

1. Vossler, D. G., Weingarten, M., Gidal, B. E. American epilepsy society treatments committee. Summary of antiepileptic drugs available in the United States of America: working toward a world without epilepsy. Epilepsy Currents. 2018; 18.4_suppl: 1-26.
2. Vogt, V.L., Äikiä, M., Del Barrio, A., Boon, P., Borbély, C., Bran, E., Braun, K., Carette, E., Clark, M., Cross, J.H., Dimova, P., Fabo, D., Foroglou, N., Francione, S., Gersamia, A., Gil-Nagel, A., Guekht, A. Current standards of neuropsychological assessment in epilepsy surgery centers across Europe. Epilepsia. 2017; 58(3): 343-355.
3. Janmohamed, M., Brodie, M.J., Kwan, P. Pharmacoresistance - Epidemiology, mechanisms, and impact on epilepsy treatment. Neuropharmacology. 2020; 168: 107790
4. Severina, A. I., Georgiyants, V. A., Shtrygol, S. Yu., Kavraiskyi, D. P. Synthesis of N-substituted derivatives of 1-(4-methoxyphenyl)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one as potential anticonvulsants. Scripta scientifica pharmaceutica. 2016; 3(2): 36-40.
5. Severina H. I., Kavraiskyi D. P., Shtrygol S. Yu., Georgiyants V. A. National University of Pharmacy. Declared 06.07.2015 № a 2015 06662. Published 26.02.2018. Bulletin 4.
6. Jain, D., Basniwal, P. K. Forced degradation and impurity profiling: recent trends in analytical perspectives. Journal of Pharmaceutical and Biomedical Analysis. 2013; 86: 11-35.
7. ICH Harmonised Tripartite Guideline. Impurities in new drug substances Q3A (R2). In: Proceedings of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, Geneva, Switzerland. 2006.
8. Roy, J. Pharmaceutical impurities—a mini-review. AAPs Pharm SciTech, 2002; 3.2: 1-8.
9. Venkannaa, G., Madhusudhan, G., Mukkanti, K., Sampath Kumar, Y. Identification synthesis of process-related impurities (substances) ethyl-4-(1-hydroxy-1-methylethyl)-2-propyl-imidazole-5-carboxylate [key intermediate of Olmesartan medoxomil (Antihypertensive drug)]. Asian Journal of Research in Chemistry. 2015; 8(5): 307-317.
10. Yamgar, R., Sawant, S. Synthesis and characterization of novel impurities in Paroxetine Hydrochloride hemihydrate and adopting QbD principles to build in quality in process of final drug substance. Asian Journal of Research in Chemistry. 2012; 5(3): 329-335.
11. Li, Y., Liu, D. Q., Yang, S., Sudini, R., McGuire, M. A., Bhanushali, D. S., Kord, A. S. Analytical control of process impurities in Pazopanib hydrochloride by impurity fate mapping. Journal of Pharmaceutical and Biomedical Analysis. 2010; 52(4): 493-507.
12. Pirrone, G. F., Mathew, R. M., Makarov, A. A., Bernardoni, F., Klapars, A., Hartman, R., Limanto J., Regalado, E. L. Supercritical fluid chromatography-photodiode array detection-electrospray ionization mass spectrometry as a framework for impurity fate mapping in the development and manufacture of drug substances. Journal of Chromatography B. 2018; 1080: 42-49.

13. Kjell, D. P., Hallberg, D. W., Kalbfleisch, J. M., McCurry, C. K., Semo, M. J., Sheldon, E. M., Spitler T. J., Wang, M. Determination of the source of the N-methyl impurity in the synthesis of pemetrexed disodium heptahydrate. Organic Process Research & Development. 2005; 9(6): 738-742.
14. Hongliu Wu. Pemetrexed intermediate and preparation method thereof. China Unexamined APPLIC, open to Public inspection CN20071024889. 06 Jul 2007.
15. Liu, D. Q., Chen, T. K., McGuire, M. A., Kord, A. S. Analytical control of genotoxic impurities in the pazopanib hydrochloride manufacturing process. Journal of Pharmaceutical and Biomedical Analysis. 2009; 50(2): 144-150.
16. Vadgaonkar, A. R., Kasture, V. S., Gosavi, S. A., Pawar, S. S., Jadhav, G. P. (2014). Synthesis, isolation, characterisation and quantification of process related impurity of zidovudine. Rasayan Journal Chemistry. 2014; 7(2): 181-189.
17. Guidance for Industry: Q1A(R2) Stability Testing of New Drug Substances and Products, United States Department of Health and Human Services, Food and Drug Administration, November 2003.
18. Gupta, A., Rawat, S., Gandhi, M., Yadav, J.S. Method development and acid degradation study of doxofylline by RP-HPLC and LC-MS/MS. Asian Journal of Pharmaceutical Analysis. 2011; 1(1): 10-13.
19. Holm, R., Elder, and D. P. Analytical advances in pharmaceutical impurity profiling. European Journal of Pharmaceutical Sciences, 2016; 87: 118-135.
20. Liu, M., Wang, J., Liu, P. HPLC method development, validation, and impurity characterization of a potent antitumor nucleoside, T-dCyd (NSC 764276). Journal of Pharmaceutical and Biomedical Analysis, 2016; 131: 429-435.
Swathi, P., Rajeswar Dutt, K., V Rao, K. N., Alagar Raja, M. RP-HPLC Method development and validation for estimation of Sofosbuvir in pure and tablet dosage form. Asian Journal of Pharmacy and Technology. 2017; 7 (3): 153-156.
21. Prakash, S. S., Kalyan, K., Raju, S.A., Danki, L.S. Development and validation of spectrophotometric methods for the estimation of Gemcitabine in bulk drug and its pharmaceutical formulations. Research Journal of Pharmacy and Technology. 2011; 4(6): 951-954.
22. Annapurna, M. M., Baswani R., Pradhan D.P. New Stability Indicating RP-UFLC Method for the Determination of Flucytosine – An Anti-Fungal Agent. Acta Scientific Pharmaceutical Sciences, 2019; 3(5): 84-90.
23. Satyanarayana, L., Naidu, S.V., Narasimha Rao, M., Reddy Suma Latha. The estimation of Nilotinib in capsule dosage form by RP-HPLC. Asian Journal of Pharmaceutical Analysis. 2011; 1(4): 100-102.