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Original Article

DEVELOPMENT AND VALIDATION OF STABILITY-INDICATING RP-HPLC ASSAY METHOD FOR AZACITIDINE AND ITS BULK DRUG

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ABSTRACT

Objective: A novel gradient reverse phase High Performance Liquid Chromatographic (HPLC) method was developed for the quantification of Azacitidine impurities and degradation products in the Azacitidine tablets.

Methods: The effective separation was achieved on an YMC ODS AQ-5, (250 x 4.6 mm), 5 µm column using a gradient mode by the mobile phase A: 3.1g of ammonium acetate dissolved in 1000 mL of water filtered through 0.45µm filter paper and mobile phase B: mixture of buffer – A, methanol and acetonitrile In the ratio of 500:300:200 v/v. The flow rate of the mobile phase was 1.0 mL/minute and the total elution time, including the column equilibration was approximately 60 minutes. The UV detection was carried at the wavelength 242 nm and experiments were conducted at 35 ^{0}C.

Results: The retention times of Azacitidine and its impurities are 15.3, 3.9, 4.9, 25.0 and 33 respectively. Azacitidine tablets were subjected to the stress conditions of oxidation, acid, base, hydrolytic, thermal and light degradation. The assay method was found to be linear in the range of 400 µg·mL^{-1} to with 1000 µg·mL^{-1} correlation coefficient is 0.998 and the linearity of the impurities was established from LOQ to 150%. Recoveries of assay and impurities were found between 99.0% and 103.6%.

Conclusion: The developed method was validated in terms of system suitability, specificity, linearity range, precision, accuracy, limits of detection and quantification for the impurities following the ICH guidelines. Therefore, the proposed method is suitable for the simultaneous determination of Azacitidine and its four related impurities.

Keywords: Azacitidine, Impurities, Method Development, YMC ODS AQ-5, and RP-HPLC.

INTRODUCTION

Azacitidine (4-amino-1-β-D-ribofuranosyl-1, 3, 5-triazin-2(1*H*)-one) is sold under the trade name Vidaza, it is a chemical analogue of cytidine, a nucleoside present in DNA and RNA. Azacitidine and its deoxy derivative, decitabine (also known as 5-aza-2′deoxycytidine), are used in the treatment of myelodysplastic syndrome [1].

Both drugs were first synthesized in Czechoslovakia as potential chemotherapeutic agents for cancer.[2] Azacitidine is mainly used in the treatment of myelodysplastic syndrome (MDS), for which it received approval by the U. S. Food and Drug Administration on May 19, 2004. It is marketed as Vidaza. In a randomized controlled trial comparing azacitidine to supportive treatment of MDS, around 16% of people receiving the drug had a complete or partial response blood cell counts and bone marrow morphology returning to normal and 2/3 patients who required blood transfusions before the study no longer needed them after receiving azacitidine[3]. It can also be used *in vitro* to remove methyl groups from DNA. This may weaken the effects of gene silencing mechanisms that occurred prior to the methylation. Methylation events are therefore believed to secure the DNA in a silenced state. Demethylation may reduce the stability of silencing signals and thus confer relative gene activation.[4] Borodovsky, et al. described the dramatic effect of 5-azacytidine on IDH1 mutant glioma xenografts in mice.[5]

Very few analytical techniques have been published for the determination of azacitidine in biological fluids, including high-performance liquid chromatography and liquid chromatography-mass spectrometry [6–13]. So far to our current knowledge there is no method reported in any of the Pharmacopoeia or in the literature for the determination of azacitidine in pharmaceutical formulation for assay and impurities. Literature survey reveals that methods have been reported for estimation of azacitidine individual drug products but none of the reported articles described a single method for estimation of impurities of azacitidine. Instead of following two individual methods, author has developed a single analytical method for estimating impurities for this product.

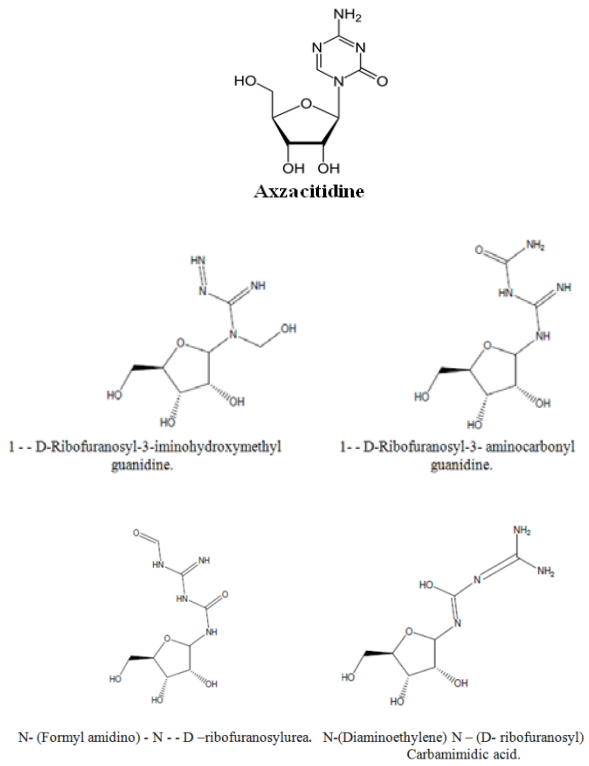


Fig. 1: Chemical structures of azacitidine and impurities

This paper also deals with the validation of the developed HPLC method for the accurate quantification of impurities and degradation products in the azacitidine tablets. The chemical structures of Azacitidine and its impurities were shown in the **Fig.1**

Experimental

Chemicals

Azacitidine and its related impurities are procured from Larous laboratories Ltd, Hyderabad, India. Acetonitrile (HPLC Grade), Methanol (HPLC Grade), and Sodium bisulphate (AR Grade) are procured from Merck, India. Ammonium acetate (HPLC Grade), Acetic acid (AR Grade) and sulfuric acid (AR Grade) are procured from Merck, India. High pure water is from Mill-Q water purification system from Millipore. All samples and impurities used in the study were grater than 99.0% purity.

Equipment

Waters alliance equipped HPLC system with a photo diode array detector is used for the method development and force degradation studies. The HPLC system used for method validation is waters HPLC system with variable wavelength detector (VWD) and Shimadzu 2010 series LC system with UV detector. The data is monitored and processed by using LC-solution Software. The chromatographic column used is YMC pack ODS-AQ, (250 mm x 4.6 mm 5 µm).

Chromatographic conditions

The chromatographic condition follows a gradient program consisting of 3.1g of Ammonium acetate in 1000 mL of water and mixed well. The pH of the solution was adjusted to 6.4±0.05 with dilute acetic acid and mixed well and used as mobile phase A. Mixture of mobile phase A, methanol and acetonitrile in the ratio of 50:30:20 v/v was used as mobile phase B.

The gradient program was: Time/% mobile phase B is 0.0/0, 15/0, 30/20, 45/40, 55/50, 60/0. The flow rate of the mobile phase is 1.0 mL min^{-1}. The gradient programme applied is presented in the **table - 1**. The column temperature is maintained at 35ºC and the detection wavelength is 242 nm. The injection volume is 20µl.

Table 1: Gradient program

Time (Minutes)	Flow (mL/min)	% Mobile phase-A (V/V)	% Mobile phase-B (V/V)
0	1.0	100	0
15	1.0	100	0
30	1.0	80	20
45	1.0	60	40
55	1.0	50	50
60	1.0	100	0

Preparation of solutions

Preparation of standard solutions

A stock solution of azacitidine (0.8 mg·mL^{-1}) was prepared by dissolving an appropriate amount in diluent. A stock solution of impurities (0.8 mg·mL^{-1}) at a concentration of (0.8 mg·mL–1) was also prepared in diluent. Working solutions were prepared from above stock solution for related substances determination and assay determination, respectively.

Preparation of sample solution

Azacitidine tablets containing 20 mg of azacitidine. Ten azacitidine tablets (20 mg) were weighed and the average weight was calculated. The tablets were powdered in a mortar and sample of the powder equivalent to 20 mg of the active pharmaceutical ingredient was transferred into 25 mL and made up with diluents. The flask was placed on rotary shaker for 10 min and sonicated for 10 min to dissolve the material completely. The supernatant solution was collected and filtered through a 0.45 µm pore size Nylon 66-membrane filter.

Analytical Method Validation

The developed chromatographic method was validated for Specificity, Linearity, Precision, Accuracy, Sensitivity, Robustness and System suitability.

Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. Stress testing of the drug substance can help to identify the likely degradation products, which can in turn help to establish the degradation pathways and intrinsic stability of the molecule and validated the stability indicating power of the analytical procedures used. The specificity of azacitidine in the presence of its impurities namely imp-A, imp-B, imp-C, imp-D and degradation products was determined by the developed HPLC method.

Forced degradation studies were also performed on azacitidine to provide an indication of the stability indicating property and specificity of the proposed method. The stress conditions employed for degradation study include light (carried out as per ICH Q1B), heat (60°C), acid hydrolysis (1 N HCl), base hydrolysis (1 N NaOH), water hydrolysis (room temperature at 48 h) and oxidation (3% H2O2). For heat and light studies, the study period was 10 days where as acid, base, peroxide and water hydrolysis the test period was 4h. Peak purity of stressed samples of azacitidine was checked by using Shimadzu 2010 series LC system with UV detector, MA, USA.

Assay studies were carried out for stress samples against qualified reference standard and the mass balance (% as- say +% of impurities +% of degradation products) was calculated.

Precision

Precision (intra-day precision) of the impurities method is evaluated by preparing six different solutions of test sample of azacitidine spiked with known impurities and injected into the developed chromatographic conditions described above. % of impurities is calculated against a qualified azacitidine standard. RSD is then calculated for % of impurities individually obtained for six different preparations. The precision study of diluted azacitidine spiked on placebo is also established. The intermediate precision (inter-day precision) of the method is also evaluated using different HPLC systems and different HPLC columns on different day in the same laboratory.

Limits of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ for azacitidine and its all known impurities are determined at a signal-to-noise ratio of about 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations. Precision study was also carried out at the LOQ level by injecting six individual preparations and % RSD is calculated

Linearity

Linearity test solutions for azacitidine and its known impurities are prepared by diluting stock solutions to the required concentrations. The solutions are prepared at different concentration levels from LOQ to 150% of the specification concentration level for azacitidine and its known impurities. The solutions are then injected into the column under the chromatographic conditions developed. The data of peak area versus concentration is subjected to least-square regression analysis.

Accuracy

A study of recovery of azacitidine and its known impurities from placebo is conducted. Samples are prepared by mixing placebo with azacitidine as per the formulation composition and then spiking the known impurities at different spike levels starting from LOQ and up to 150% of the specification level. Sample solutions are prepared in triplicate for each spike level as described in the test preparation and injected into the chromatographic conditions developed. The % recovery is then calculated against azacitidine diluted standard and by using relative response factor and compared against the known amounts of impurities spiked.

Robustness

To determine the robustness of the developed method, experimental conditions are deliberately altered and the elution patterns, resolution between azacitidine and its impurities are evaluated. The flow rate of the mobile phase is 1.0 ml min-1. To study the effect of flow rate, flow is changed by 0.2 units from 0.8 to 1.2 ml min-1. The effect of the column temperature is studied at 20ºC and 30ºC instead of 35ºC. The effect of the percent organic strength is studied by varying methanol and acetonitrile by −5 to +5% while other mobile phase components were held constant

Solution stability and mobile phase stability

The stability of azacitidine test preparation for the impurities method was determined by leaving solution in a tightly capped volumetric flask at room temperature on bench top and by measuring the amounts of the impurities at different intervals. The stability of mobile phase is also determined by analysing freshly prepared solution of azacitidine and its impurities at 24 hours intervals for 48 hours and by keeping the same mobile phase during the study period.

RESULTS AND DISCUSSION

Method Development and Optimization

The new HPLC method is optimized with a view to develop a stability indicating method of azacitidine and its impurities. 3.1g of ammonium acetate in 1000 mL of water and mixed well. The pH of the solution is adjusted to 6.4±0.05 with dilute acetic acid and mixed well and used as mobile phase A. Mixture of mobile phase A, methanol and acetonitrile in the ratio of 40:40:20 v/v as mobile phase B for initial trail on a inertsil ODS (250x4.6) mm, 5µ stationary phase with a 25 cm length, 4.6 mm ID and 5 µm particle size. Flow rate was 1.0 $mL{\cdot}min^{-1}$ when azacitidine sample spiked with all impurities was injected the resolution between all impurities was >2.0 and retention time (RT) was very high (about 80 mins).

In this trail was less resolution between impurity-C and impurity-D and both impurities retention times are very high. In another trail changes were made on mobile phase-B ratio (50:25:25) and injected sample is then spiked and the gradient was changed to reduce longer run time of azacitidine and other impurities. With this trail less retention time of azacitidine was achieved and peak shapes of impurities were also improved. Based on 2nd trail another trail was carried out by using YMC ODS AQ-5, (250 x 4.6 mm), 5 µm particle size, with column temperature 35°C. The resolution between impurities and azacitidone is <2.0. To further improve the resolution between impurities and azacitidine, again we changed the mobile phase-B (50:30:20) ratio. The all impurities were well resolved the resolution between 2.0 and 3.0. Satisfactory resolution between impurities and symmetry of the azacitidine peak was observed in final trail.

Forced degradation studies: Azacitidine drug was exposed to 1N HCl, 3% H_2O_2, 1N NaOH at 60°C with continuous constant stirring. Azacitidine has shown significant sensitivity towards treatment of 1 N HCl, 3% H_2O_2 and 1 N NaOH. The drug gradually undergone degradation with time in 1 N HCl, 3% H_2O_2 and 1 N NaOH. No major degradation products were observed when azacitidine is stressed in photolytic and thermal conditions after 48 hours. From the degradation studies peak purity test results derived from the PDA detector, confirmed that the azacitidine peak was homogeneous and pure in all the analyzed stress samples. The mass balance of stressed sample was close to 99.2%. No degradants were observed after 60 min in the extended runtime of 60 min of all the azacitidine samples. The developed HPLC method was found to be specific in the presence of all impurities and their degradation products confirm the stability indicating power of the developed method.

Method Validation

Azacitidine and all impurities were prepared in the diluent at a concentration of 100 ppm and scanned in a UV- Visible spectrophotometer all four impurities and azacitidine have maxima at 242 nm, hence detection at 242 nm was selected for quantification of azacitidine and related impurities. In the azacitidine condition of azacitidine of imp – A, imp- B, imp-C and imp – D were well separated with good resolution (**Fig. 2)**.

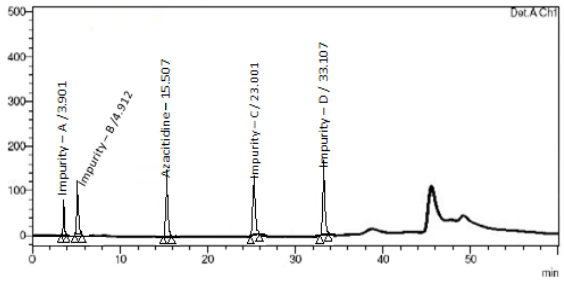


Fig. 2: Typical chromatogram of azactidine spiked with impurities

Precision

The Intra-day and Inter-day precision of the method was evaluated by analyzing six samples of test preparation of azacitidine injection spiked with impurities at 0.3% level. The Intra-day and Inter-day precision for diluted azacitidine was established by spiking on placebo at 0.3% level. % RSD values are presented in **table -2**. % RSD values of less than 5.0% for azacitidine and its known impurities shows that method is precise and works satisfactorily on different day, with different column and HPLC system.

Table 2: Results of Intra-day and Inter day precision of test method for azacitidine known Impurities

	Intra-day precision					Inter-day precision				
Sample No.	**azacitidine**	**Imp-A**	**Imp- B**	**Imp-C**	**Imp-D**	**azacitidine**	**Imp-A**	**Imp-B**	**Imp- C**	**Imp-D**
1	0.329	0.318	0.333	0.345	0.341	0.296	0.286	0.325	0.364	0.368
2	0.315	0.340	0.319	0.352	0.337	0.302	0.288	0.329	0.361	0.361
3	0.338	0.352	0.333	0.361	0.325	0.298	0.294	0.332	0.358	0.356
4	0.346	0.320	0.312	0.342	0.326	0.309	0.295	0.318	0.349	0.352
5	0.326	0.332	0.308	0.367	0.322	0.310	0.299	0.327	0.352	0.359
6	0.322	0.346	0.326	0.355	0.332	0.294	0.286	0.330	0.363	0.372
Avg	0.329	0.335	0.321	0.331	0.331	0.301	0.291	0.327	0.358	0.361
%RSD	3.4	4.1	3.3	2.7	2.3	2.2	1.9	1.5	1.7	2.1

LOQ and LOD

The limit of detection, limit of quantification was determined by following signal to noise ratio method. A precision study is conducted at LOQ level for azacitidine and its known impurities. The results showed that the method is sensitive enough to quantify impurities well below the ICH reporting threshold of 0.1%, as LOQ values are in the range of 0.0062% to 0.014%.

The precision at LOQ for all the impurities and azacitidine is in the RSD range of 1.5-3.7%. The recovery at LOQ level for the impurities is in the range of 99.0–108.3%. The data is summarized in **table -3.**

Table 3: LOD, LOQ and regression data of azacitidine and its impurities

Parameter	Azacitidine	Imp - A	Imp - B	Imp - C	Imp - D
LOD in %	0.0033	0.0024	0.0031	0.0034	0.0030
S/N ratio	3.31	3.26	3.11	2.98	3.41
LOQ in %	0.0082	0.0062	0.0084	0.0090	0.0111
S/N ratio	9.94	9.98	10.56	10.48	10.23
Precision at LOQ(%RSD)	1.5	2.1	1.9	3.4	2.4
Recovery at LOQ in %	100.6	99.0	106.4	108.3	6.1

Linearity

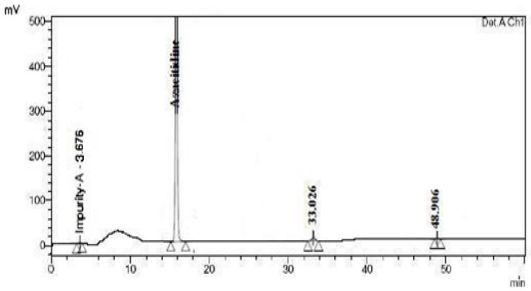
A linear calibration plot for azacitidine and its known impurities is drawn over the calibration range of LOQ to 150% of the precision study levels. Correlation co-efficient for azacitidine and its known impurities is found to be greater than 0.998. The results show that a very good correlation exists between the peak area and concentration of the analyte for azacitidine and its known impurities.

Accuracy

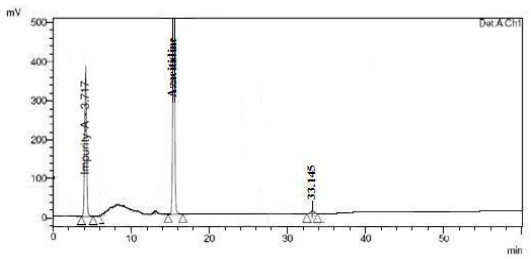
The percentage recovery of azacitidine and its known impurities in presence of placebo matrix of azacitidine injection from LOQ to 150% spike level are in the range of 95.7% to 103.6%. The % recovery values for azacitidine and impurities are presented in **table - 4**. The data shows that the method is having capability to estimate accurately impurities of azacitidine in azacitidine injections. HPLC chromatograms of spiked sample with all four impurities in azacitidine bulk drug sampled are shown in **Fig. 3**.

Table 4: Recovery results of azacitidine impurities in pharmaceutical dosage forms

Spike level	Azacitidine	Imp - A	Imp - B	Imp - C	Imp - D
50%	101.1	99.1	100.1	98.2	96.4
75%	100.8	100.6	102.4	97.9	97.7
100%	99.0	100.2	100.5	99.5	95.7
125%	98.9	101.4	103.6	98.7	97.5
150%	99.1	99.3	99.4	98.2	97.5



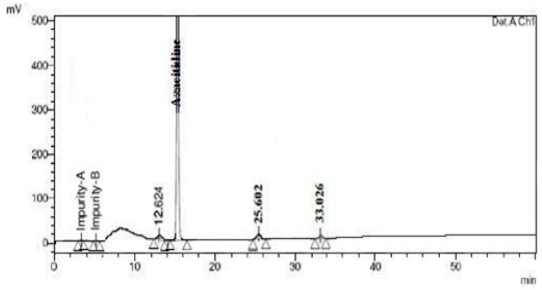
Acid degradation



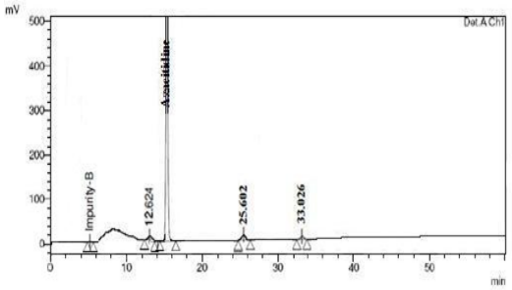
Alkali degradation

Robustness

To determine the robustness of the developed method, experimental conditions were deliberately altered and the resolution between azacitidine and its impurities and tailing factor for azacitidine and its impurities are recorded. The effect of flow rate was studied at 0.8 ml min-1 and 1.2 ml min-1 and compared with method flow rate of 1.0 ml min-1.



H_2O_2 degradation



Water degradation

Fig. 3: Typical Chromatogram of stressed azacitidine sample.

The effect of column temperature is studied at 20°C and 30°C and compared with method column temperature 35°C. The effect of composition of organic solvents is studied by varying acetonitrile and methanol individually by–5 t o +5% while other mobile phase components were held constant and compared with method organic solvent composition.

Table 5: Results of robustness study

Condition	Imp - A	Imp - B	Imp - C	Imp - D
0.8 mL/min	0.12	0.88	0.95	0.98
1.0 mL/min	0.11	0.88	0.94	0.97
1.2 mL/min	0.10	0.87	0.94	0.97
20°C	0.11	0.89	0.94	1.05
30°C	0.11	0.88	0.94	1.05
35°C	0.10	0.87	0.93	1.15
M. P - A 95%	0.12	0.88	0.94	1.05
M. P - A 95%	0.11	0.88	0.94	1.05
M. P - A 95%	0.11	0.89	0.93	1.05
M. P - B 95%	0.12	0.89	0.95	1.05
M. P - B 95%	0.11	0.88	0.94	1.05
M. P -B 95%	0.10	0.87	0.94	1.05

In all the deliberately varied chromatographic conditions (flow rate, column temperature and composition of organic solvent), all analytes are adequately resolved and elution orders remained

unchanged. RRT of all the known impurities for all deliberately varied conditions along with original conditions are summarized in **table-5**. The resolution between all critical pair components is greater than 2.0 and tailing factor for azacitidine and its impurities is found to be less than 1.3.

Solution stability and mobile phase stability

The stability of Azacitidine in diluted standard solution is estimated against freshly prepared standard each time. The stability factor for standard from initial to 2 days found to be within the acceptable limits. Azacitidine test preparation was prepared as per test method and injected at 24 hours intervals up to 48 hours. The results are summarized in **table 6**. The study showed that both standard and test preparations are stable on bench top for 3 days. The stability of mobile phase-A & B on bench top was conducted for a period of about 2 days. Azacitidine test preparation prepared as per test method and injected into the column under the chromatographic conditions developed by preparing the solution freshly each time and by using the stored mobile phase up to 2 days.

The difference in % of known impurities and % of total impurities from initial to 2 days is found to be within the limits. The results are summarized in **table - 7**.

From the above study it is established that the mobile phase is stable for a period of 2 days on bench top.

Table 6: Results of solution stability

Duration In hours	% of the impurity upon storage on bench top			
	Imp - A	Imp - B	Imp - C	Imp - D
0	ND	0.01	ND	0.14
24	ND	0.01	ND	0.14
48	ND	0.01	ND	0.15
Maximum Difference. from zero hrs	0.00	0.00	0.00	0.01

Table 7: Results of mobile phase stability on bench top

Duration	% of impurity for mobile phase stability			
In hours	Imp - A	Imp - B	Imp - C	Imp - D
0	ND	0.01	ND	0.14
24	ND	0.01	ND	0.15
48	ND	0.01	ND	0.14
Maximum Difference. from zero hrs	0.00	0.00	0.00	0.01

CONCLUSION

A precise, sensitive, specific, accurate, validated and well-defined stability indicating HPLC method for the determination of degradation products and its process related impurities is described. The behavior of Azacitidine under various stress conditions is studied. All of the degradation products and process impurities are well separated from each other and from Azacitidine demonstrates the stability- indicating nature of the method. The information presented in this study could be very useful for quality monitoring of Azacitidine pharmaceutical dosage forms and can be used to check drug quality during stability studies.

CONFLICT OF INTERESTS

Declared None

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