Analytical Method Development And Validation For Determination Of Azacitidine By High Performance Liquid Chromatography

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ABSTRACT
A New simple, accurate rapid and precise isocratic high performance liquid chromatographic (HPLC) method was developed and validated for the estimation AZACITIDINE from its bulk drug. The HPLC method was developed Zorbax bonus RP, 250mm×4.6mm, 5µ column with mobile phase Ammonium acetate buffer and acetonitrile (75:25) in isocratic mode. Flow rate was 1.0ml/min, with injection volume 10µl, detection done by using PDA detector at 242nm. Retention time of azacitidine found to be 8.5min, the calibration was linear in concentration range of 15-225 µg/ml, with regression 0.999. The percentage recovery of v Azacitidine was found to be 99.1% - 100.75%. The LOD and LOQ of the azacitidine were found to be 0.0239 µg/ml and 0.0723µg/ml respectively. All the parameters validated according to ICH guide lines and found to be within the limits

Keywords: Azacitidine, RP-HPLC, Myelodysplastic syndrome, Validation.

INTRODUCTION

Azacitidine (5-azacytidine) is a chemical analogue of the cytosine nucleoside used in the treatment of myelodysplastic syndrome. Azacitidine exhibit antineoplastic activity via two mechanisms, inhibition of DNA methyltransferase at low doses, causing hypo methylation of DNA and direct cytotoxicity in abnormal hematopoietic cells in the bone marrow through its incorporation into DNA and RNA at high doses, resulting in cell death [1-4]. 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 [5–10]. The proposed method was found to be simple, sensitive, rapid and economical for the determination of Azacitidine in pharmaceutical dosage forms. The developed method was checked for the performance characteristics and has also been validated [11-12].

MATERIALS AND METHODS:

Azacitidine working Standard & Reference standards were obtained as gift samples from CIPLA Pharmaceuticals, Mumbai. HPLC grade acetonitrile and methanol were purchased from Sigma Aldrich chemicals, Mumbai, India. Analytical Grade acetic acid and ammonium acetate was purchased from SD fine chemicals, Mumbai, India.

Chromatographic Conditions:

The HPLC system consisted of Shimadzu 2010 CHT liquid chromatography equipped with PDA detector with LC solutions. The wavelength of detection as set at 242nm. Separation was carried out on Zorbax bonus RP, 250mm×4.6mm, 5µ column using ammonium acetate buffer and acetonitrile (75:25) as mobile phase with isocratic elution at a flow rate of 1 ml/min. Retention time of azacitidine found to be 8.5min. The mobile phase filtered through nylon milli pore (0.2µm) membrane filter, purchased from pall life sciences, Mumbai and degassed with Ultrasonicator prior to use. Chromatography was carried out at room temperature 25^0c and maintains the column temperature at 32^0c.

Preparation of Standard stock solutions:

Accurately weighed and transferred 25 mg of Azacitidine standard into a 50 mL volumetric flask. Dissolved and diluted to volume with diluent DMSO:Water (1:1).

Mobile phase preparation*:*

Buffer solution*:* Accurately weighed 1.54 g of ammonium acetate into a beaker, dissolved and diluted to 1000 mL with water and adjust the pH to 4.0 with acetic acid.

Mobile phase: Prepared the mobile phase with a ratio of 75:25(v/v) Buffer solution and acetonitrile. Filtered the mobile phase through a 0.45µm membrane filter and degassed before use

Preparation of Linear Standard Solutions:
Stock solutions of azacitidine working (1mg/ml) and reference (1mg/ml) standards were prepared in acetonitrile. Further dilutions were carried out with diluent DMSO:Water (1:1). Calibration standards were prepared freshly with azacitidine stock solution to give the concentrations of 0.08, 0.5, 1, 1.5 and 2 µg/ml.

Injection sequence:
Separately injected 20 µL each of one diluent sample as blank, five standard solutions and two test solutions into the liquid chromatography, recorded the chromatograms and measured the responses for all peaks excluding the peaks of blank. The formula for calculating test concentration is given below
Calculate the amount of drug by using the followings formula:

$$\%\ ASSAY = \frac{Average\ area\ of\ Test\ solun}{\text{Average area of Standard Solun}} \times \frac{Standard\ Concentration}{Sample\ Concentration} \times \%Assay\ of\ Standard$$

VALIDATION:
System suitability
Prepared standard solution (6 injections) and evaluated system suitability parameters as per test method.

System precision:
Prepared and injected one blank, standard solution for 6 replicate injections and calculated the % RSD of retention time and area counts of Azacitidine in standard solution.

Detection limit & Quantification Limit:
The limit of detection and Limit of quantitation can also be determined by using steyex/slope method. The LOD and LOQ values of Sodium phenyl acetate can be determined by injecting the linearity standard solution of Azacitidine and determine the values using the following equations as given below.

$$LOD = \frac{3.3 \times S_a}{b}$$

$$LOQ = \frac{10 \times S_a}{b}$$

Where:
S_a= Standard error of the predicted Y value for each X in the regression
b = Slope of the calibration curve from the regression equation

Accuracy and Recovery
The accuracy/recovery study for unknown impurity was performed with the Azacitidine API at concentrations spanning from 50% to 200 % of the proposed specification limit for single maximum unknown impurity of NMT 0.05 % with respect to the test concentration of 2.0mg/mL of Azacitidine. Six preparations were prepared at 100% & 200% levels and three preparations were at 50% & 150% levels. Each solution will be injected once and analyzed.

RESULTS & DISCUSSION:
Under the chromatographic conditions employed, the sample showed sharp peak of azacitidine at retention time of 8.5 min shown in figure-1.

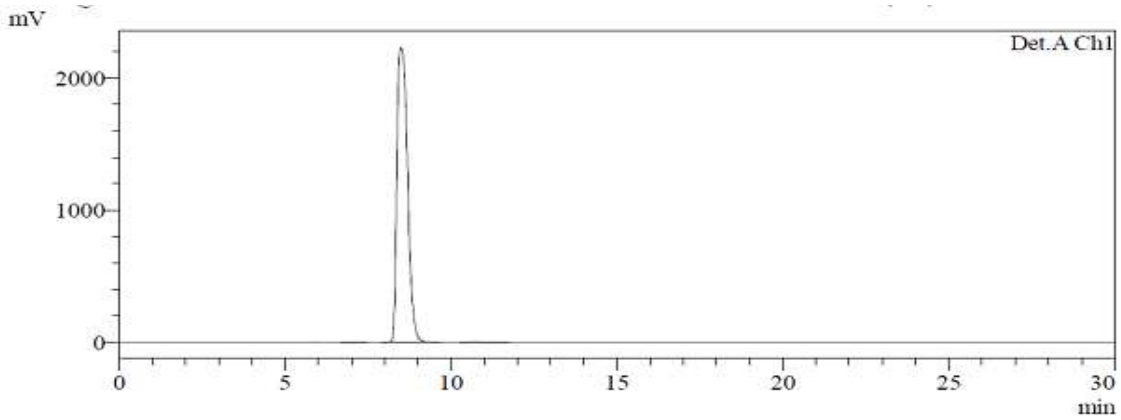

Figure: 1 Chromatogram showing sharp peak of azacitidine retained at 8.5min

Specificity:

The % RSD for retention time of Azacitidine from 6 replicate standard solution injection was 0.1 and the % RSD for area of AZC from 6 replicate standard solution injection was 0.9 (Table 1)

Table 1: Results of specificity

No.of Injections	**GDAHPL22**	
	Retention time (min)	**Area**
Inj-1	8.508	35605
Inj-2	8.500	36197
Inj-3	8.491	36504
Inj-4	8.491	35818
Inj-5	8.485	36037
Inj-6	8.487	36021
AVERAGE	**8.494**	**36030**
SD	**0.0087**	**309.27**
% RSD	**0.1**	**0.9**

Detection Limit & Quantification Limit:

LOD & LOQ of Azacitidine was determined by styex-slope method. LOD was 0.0239 µg/ml (0.001%) and LOQ 0.0723µg/ml, (0.004%) (Table 2)

Table 2: Results of LOD & LOQ:

% W.r.t. test Conc.	**Conc.(µg/mL)**	**Area**
0.00010	0.0020	336
0.00025	0.0050	407
0.00050	0.0100	693
0.00075	0.0150	793
0.00100	0.0200	1239
0.00250	0.0500	2596
0.00500	0.1000	4642
0.00750	0.1500	6242
0.01000	0.2000	8068
0.02500	0.5001	20036
0.05000	1.0002	39242
0.07500	1.5003	59841
0.1000	2.0004	78560
Styex		**283.76**
Slope		**39230.98**
LOD (µg/mL)		**0.0239**
LOQ (µg/mL)		**0.0723**
LOD (%)		**0.001**

LOQ (%)	**0.004**

Accuracy, Recovery, Precision and Linearity of Test Method:

Table 3: Results of accuracy, recovery, precision, linearity of test method

Level (%)	Preparations	Amount found (µg/mL)	Amount added(µg/mL)	% Recovery	Average	% RSD
50% preparation	Prep-1	0.4954	0.5002	99.0	**98.5**	**0.5**
	Prep-2	0.4920	0.5002	98.4		
	Prep-3	0.4912	0.5002	98.2		
100% preparation	Prep-1	0.9584	1.0004	95.8	**96.2**	**0.3**
	Prep-2	0.9627	1.0004	96.2		
	Prep-3	0.9672	1.0004	96.7		
	Prep-4	0.9615	1.0004	96.1		
	Prep-5	0.9633	1.0004	96.3		
	Prep-6	0.9624	1.0004	96.2		
150% preparation	Prep-1	1.4444	1.5005	96.3	**96.1**	**0.3**
	Prep-2	1.4432	1.5005	96.2		
	Prep-3	1.4372	1.5005	95.8		
200% preparation	Prep-1	1.9196	2.0007	95.9	**96.4**	**0.7**
	Prep-2	1.9442	2.0007	97.2		
	Prep-3	1.9441	2.0007	97.2		
	Prep-4	1.9294	2.0007	96.4		
	Prep-5	1.9216	2.0007	96.0		
	Prep-6	1.9107	2.0007	95.5		

Table 4: Linearity of test method

Level%	Average amount added (µg/mL)	Average amount found (µg/mL)
LOQ	0.0800	0.0763
50	0.5002	0.4928
100	1.0004	0.9626
150	1.5005	1.4416
200	2.0007	1.9283
	Correlation coefficient	**0.999973**
	Slope	**0.9608**
	Y-intercept	**0.0038**

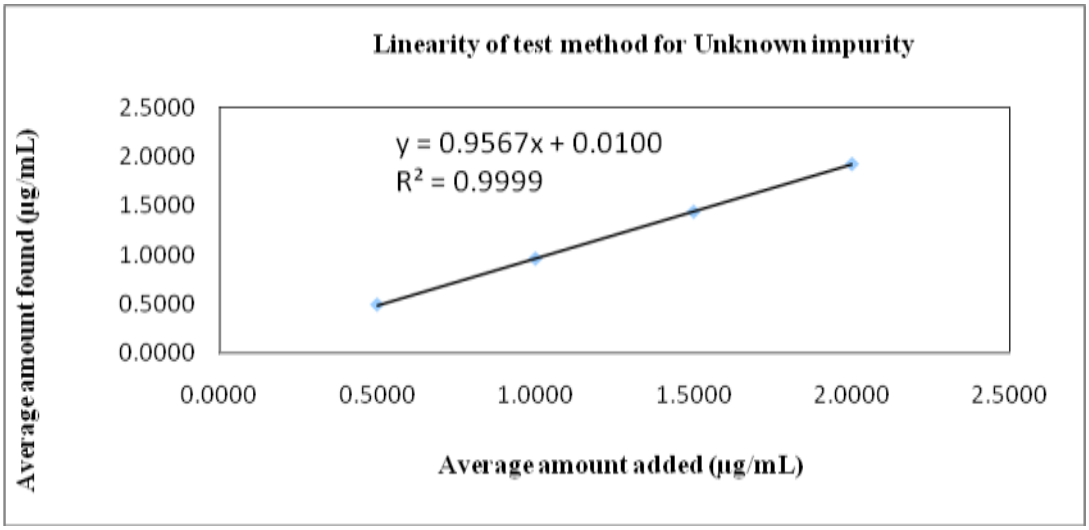

Figure 2: **Linearity of the test method graph**

DISCUSSION:

The solution of 10µg/ml of azacitidine in diluent (1:1 ratio of DMSO and water) was prepared and the solution was scanned in the range of 200-400nm, at 242 nm the drug shows maximum absorbance spectrum and better detector response. The calibration was linear in concentration range of 0.08to 2µg/ml, with regression 0.999, for azacitidine and obeyed the Beer–Lamberts law (Table 4 and figure 2). The percentage recovery of azacitidine was found to be 96.1% to 98.5% which are within the limit. The low % RSD values (≤ 2) indicated that the method was precise and accurate. Accuracy was confirmed by recovery studies by proposed method.

CONCLUSION:

The developed HPLC method was found to be rapid, simple, precise, accurate and economical and can be adopted for routine estimation of azacitidine in quality control laboratories.

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