Analytical Method Development and Validation for the Determination of Azacitidine by HPLC

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***Abstract*—For the determination of AZACITIDINE from its bulk medication, a novel isocratic high-performance liquid chromatographic (HPLC) approach that is straightforward, accurate, fast, and precise was created and validated. Column Zorbax RP C18bonus Column 250cm × 4.6mm, 5µm, with a flow rate of 1.0ml/min, and acetonitrile (75:25) and ammonium acetate buffer as the mobile phase are the optimized parameters. The tailing factor and theoretical plates are within the bounds, and the RT of azacitidine was determined to be 7.5 min. Every parameter was verified in accordance with ICH guidelines and was discovered to be within acceptable bounds. The steyex's slope was used to evaluate the limits of quantification and detection.**

***Index Terms*—method development, validation, RP-HPLC, and azacitidine.**

I. INTRODUCTION

Analytical Chemistry [(1)]**:** HPLC is a contemporary analytical method that uses a stationary phase and a liquid mobile phase for separation. Depending upon size of the stationary phase used, separations can be accomplished by partitioning, adsorption, or ion exchange. One of the most adaptable tools in the field of Ph. analysis is HPLC.

II. EXPERIMENTAL MATERIALS

Chemical Compounds: Ammonium acetate, Acetonitrile, Acetic Acid

EquipmentSystem:All spectral measurements were performed using a Schimadzu 2010 CHT liquid chromatograph with a UV detector and LC solutions, a Schimadzu 2010 CHT liquid chromatograph with a PDA detector and LC solutions, and a Schimadzu1800 UV/Vis double beam spectrophotometer with 1 cm matched quartz cells.

Drug Molecules: Azacitidine working Standard & Azacitidine Reference standard (AZC)

SolubilityStudies: The medication underwent appropriate solubility tests in a range of solvents, including DMSO, IPA, and 0.1% DMSO in water.In a mixture of IPA, DMSO, and water, the sample was very soluble.Additionally, it is somewhat soluble in ethanol, methanol, and other solvents.Samples are prepared using the chosen solvents.

Method Development: Column used is ZorbaxRPC18 Bonus (250 mm X 4.6 mm X3.5µ) or equivalent, Flow rate is 1.0 mL/min, Wavelength is 242 nm, Inj. volume10 µL, Run time30 min, Auto sampler cooler temperature5°C, Column oven Temperature35°C, Diluent DMSO: Water (1:1)

Reagents:

- Blank**:** Use diluent as blank.
- Std solution**:** Accurately weigh & transfer about 25.0 mg of Azacitidine std into a 50 mL V.F. Dissolve and dilute to volume with diluent (0.5 mg/mL).
- Test solution**:** Accurately weigh and transfer about 25 mg of sample into a 25 mL V.F. Dissolve and dilute to volume with diluent (0.5 mg/mL).

Preparation of M.P*:*

Buffer sol*:* Weigh about 1.54 g of ammonium acetate into a beaker, dissolve and dilute to 1000 mL with water and adjust the pH to 4.0 with acetic acid. Prepare a mix of Buffer sol: acetonitrile ⇒

75:25(v/v) Filter the m p through a 0.45µm membrane filter and degas prior use.

III. METHOD DEVELOPMENT RESULTS

- UV-spectroscopy-wave length detection

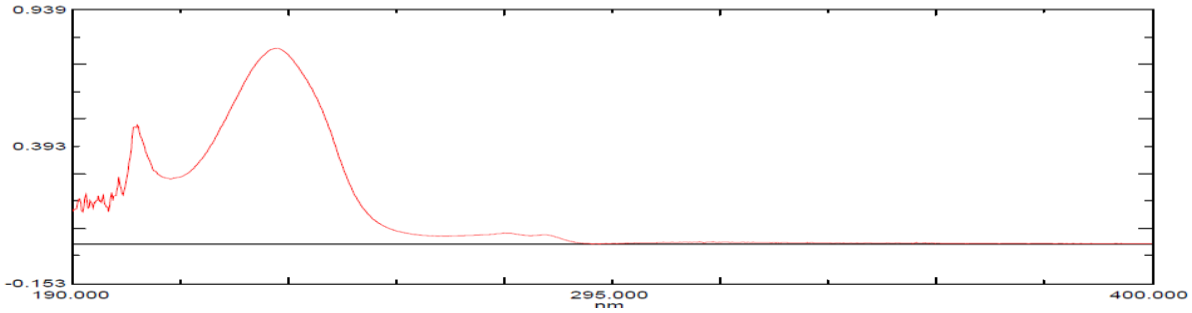

Fig.1: UV Spectrum of Azacitidine (AZC)

Report: Azacitidine maximum absorbance observed at 242nm

- IR Spectrum Of Azacitidine

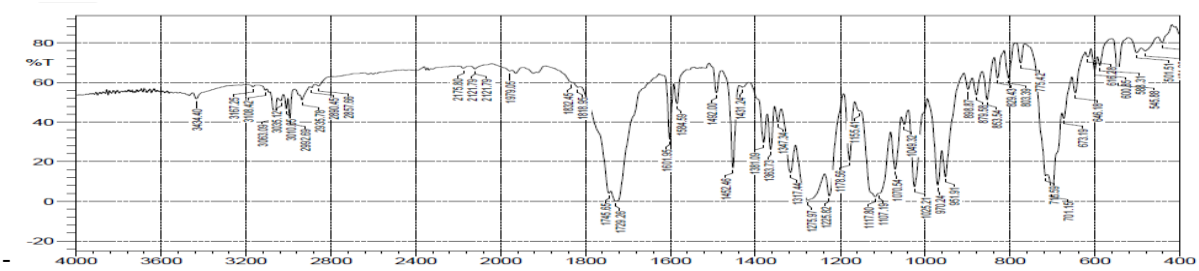

Fig.2: IR Spectrum of Azacitidine (AZC)

Table 1: Optimized Chromatographic Parameters:

Peak#	Ret. Time	Area	Area %	RRT
1	1.13	28392	0.131	0.00
2	1.30	5824	0.027	0.00
3	1.56	3064	0.014	0.00
4	1.62	2044	0.009	0.00
5	5.74	21698926	99.819	0.00
Total		21738251	100.000	

Report: Azacitidine peak retention time is 5.74 min.

IV. I. SPECIFICITY

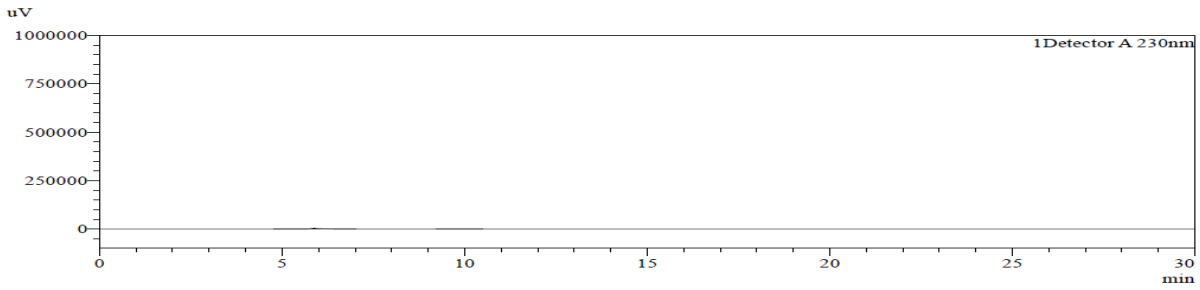

Fig. 3: Specificity-Typical blank chromatogram

Table 2: Results of specificity

S.No.	Sample ID	R_t (min)	Remarks
1	Blank	ND	No interference of blank peak with analyte peak.
2	AZC reference standard	7.52	No interference with blank & other related compound peaks.
3	AZC API	7.48	No interference with blank & other impurities

Table 3: Results of specificity

No. of 'Injections'		
	RT (min)	'Area
1	'8.508'	'35605'
2	'8.500'	'36197'
3	'8.491'	'36504'
4	'8.491'	'35818'
5	'8.485'	'36037'
6	'8.487'	'36021'
AVG	'8.494'	'36030'
SD.	'0.0087'	'309.2777'
% RSD.	'0.1'	'0.9'

Report: % RSD for RT of Azacitidine from 6 'replicate std solution 'inj was 0.1% RSD for area of AZC from 6 replicate std sol inj was 0.9. Tailing factor for AZC is found to be 1.2 and Theoretical plates is 15860 all found to be within the limit.

Table 4: 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'		'283.7621'
'Slope'		'39230.9823'
'LOD (µg/mL)'		'0.0239'
'LOQ (µg/mL)'		'0.0723'
'LOD (%)'		'0.001'
'LOQ (%)'		'0.004'

Report: '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 5: 'Results of accuracy at LOQ'

'Amount found'(µg/mL)	'Amount added' (µg/mL)	% -Recovery	Mean Recovery (%)	%RSD
0.0764	0.0800	95.5	95.3	1.1
0.0763	0.0800	95.3		
0.0768	0.0800	96.0		
0.0746	0.0800	93.2		
0.0769	0.0800	96.1		
0.0767	0.0800	95.9		

Report: % Mean recovery of LOQ level (0.0723µg/ml) injection was 95.3%and % RSD was 1.1

Table.6: '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	Prep1	'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-7: 'Linearity test method'

Level%	Avg. amt added- (µg/mL)	Avg. amt 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'

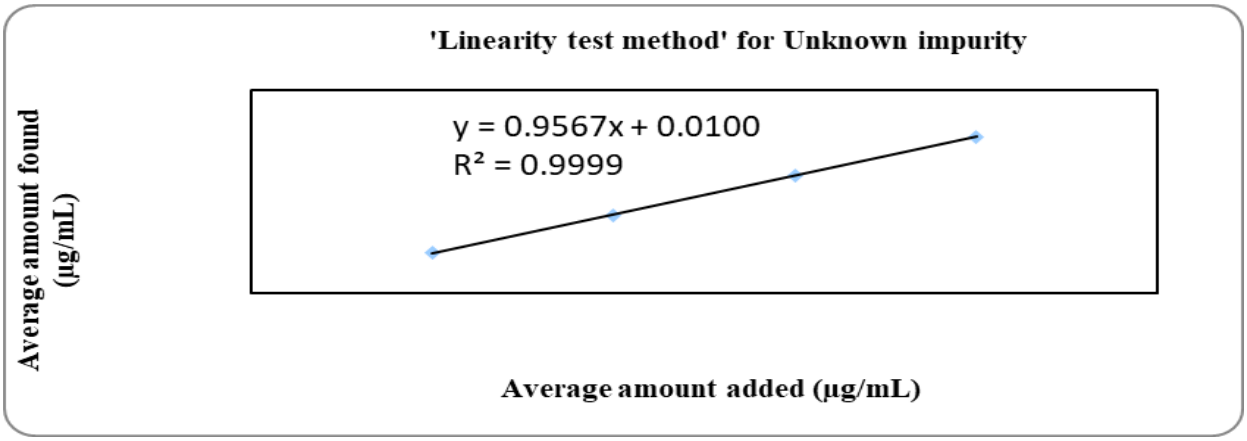

Fig. 4: Linearity of the test method graph

Table 8: Results for linearity of detector response

Level. (%)	Conc (µg/mL)	Area
LOQ.	'0.0800'	'3608'
50.	'0.5001'	'20545'
75.	'0.7501'	'30443'
100.	'1.0001'	'41243'
125.	'1.2501'	'50397'
150.	'1.5002'	'60110'
200.	'2.0002'	'80655'
'Correlation coefficient'		'0.999906'
'Slope'		'39998.7465'
'Y-Intercept'		'540.0523'
'% Y-Intercept'		'1.3'

Table 9: Forced Degradation peak purity and similarity index results

S. No	Sample	% Single maximum unknown impurity	% Total impurities
1	Control	0.006(RRT-0.31)	0.01
2	Dark control	0.006 (RRT-0.31)	0.01
3	Light exposed	0.067 (RRT-0.96)	0.15
4	Dry heat-80°C-7days	0.050(RRT-0.96)	0.07
5	Hygroscopicity	0.007(RRT-0.96)	0.02

Table 10: Acceptance criteria and results-Forced degradation

S. No	Sample	Peak purity	Similarity index
1	Control	0.999999	1.000000
2	Dark control	0.999999	0.999955
3	Light exposed	0.999983	0.999993
4	Dry heat-80°C-7days	0.999999	0.999996
5	Hygroscopicity	0.999996	0.996191

Table 11: Method precision, Intermediate precision & Ruggedness
Results on control sample

Properties	Method precision		Intermediate precision	
	%SMUI	*%Total impurities*	*%SMUI*	*%Total impurities*
Preparation	0.006	0.02	0.006	0.02
Avg	0.006	0.02	0.006	0.02
STDV	0.00003	0.0001	0.00002	0.0006
%RSD	0.5	0.5	0.3	3.7

Table 12: Solution stability results for standard solution

Standard	2-8°C		Room temperature	
	Area	% variation from initial	Area	% variation from initial
Standard solution-0hrs	34977	NA	34846	NA
Standard solution-06hrs	35051	0.1	35065	0.6
Standard solution-12hrs	35067	0.3	35012	0.5
Standard solution-24hrs	34895	0.2	34895	0.1
Standard solution-Day-2	34851	0.4	34940	0.3
Standard solution-Day-3	34747	0.7	34891	0.1
Standard solution-Day-7	33509	4.2	34041	1.4

V. CONCLUSION

The present study shows that an efficient, simple, precise, fast, and accurate RP-HPLC technique was created & checked to use in measuring Azacitidine. In developing this method, the best settings for the chromatography were tested. They looked at things like the best wavelength to use, the type of mobile phase, the mix of the m p, and the flow-rate. A best resultwas found when using a wavelength of 242 nm, with the m p made up of buffer and Acetonitrile in a 75:25 mix, flowing at 1 mL per minute. The analysis worked well with a Zorbax Bonus RP C18 column (250×46mm, 5µm). They also checked the system's performance, which included measuring the number of theoretical plates, how well the peaks were separated, and the tailing factor. For validation, they tested several things like system precision, system performance with control samples, method precision, linearity, limits of detection and quantification, accuracy at the lowest quantification level, precision at the lowest quantification level, overall accuracy, how the detector responds linearly, and the method's robustness. All of these results were found to be within the guidelines set by ICH.

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