Research Article

A Novel RP-HPLC Method for the Quantification of Azacitidine and Its Impurities in Active Pharmaceutical Ingredients and Pharmaceutical Dosage Forms

Arun Kumar Kuna*, Seru Ganapaty, G. V. Radha
Department of Pharmaceutical Analysis, Gitam Institute of Pharmacy, Gitam University (Deemed to be University), Rushikonda, Visakhapatnam, Andhra Pradesh, India.
***Corresponding author's E-mail:** kunaarun@yahoo.co.in

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ABSTRACT

A simple, specific, accurate reversed phase high performance liquid chromatographic method was developed for the quantification of Azacitidine impurities and Azacitidine. The effective separation was achieved on a Discovery C18 250 mm x 4.6 mm, 5 µm column using a gradient mode by the mobile phase 3.1g of ammonium acetate dissolved in 1000 mL of water filtered through 0.45µm filter paper and mobile phase Buffer and Acetonitrile taken in the ratio 70:30 v/v. The flow rate of the mobile phase was 1.0 mL/minute and the detection was carried at the wavelength 217 nm. The retention times of Azacitidine and its impurities are 8.016, 2.576 and 3.091 min respectively. Correlation coefficient is 0.9995. The developed method was validated in terms of system suitability, specificity, linearity range, precision, accuracy, limits of detection and quantification. Therefore, the proposed method is suitable for the simultaneous determination of Azacitidine and its two related impurities.

Keywords: Azacitidine, Impurities, Method Development, RP-HPLC.

INTRODUCTION

Azacitidine (4-amino-1-β-D-ribofuranosyl-1, 3, 5-triazin-2(1H)-one) is a chemical analogue of cytidine, a nucleoside present in DNA and RNA. Azacitidine (Figure 1) and its deoxy derivative, decitabine (also known as 5aza-2'deoxycytidine) are used in the treatment of myelodysplastic syndrome.[1] Few HPLC methods for quantitative determination of Azacitidine, Azacitidine impurity A (4-amino-1-methyl-1,3,5-triazin-2-one) (Figure 2), Azacitidine impurity B ([(2R,3R,4R)-5-acetyloxy-3,4-dibenzoyl oxyoxolan-2-yl]methyl benzoate) (Figure 3) in formulations were reported in the literature.[2-10] Aim of this work is to develop and validate a rapid, economical and sensitive HPLC method for quantitative determination of Azacitidine, Azacitidine Impurity-A and Impurity-B in bulk drug samples and pharmaceutical dosage form.

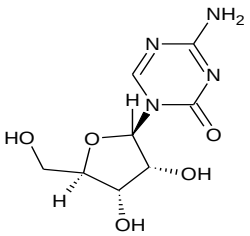


Figure 1: Structure of Azacitidine

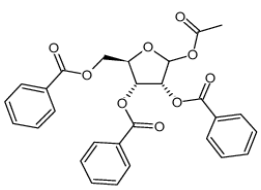


Figure 2: Structure of Impurity-B.

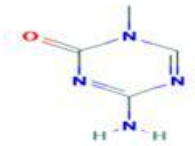


Figure 3: Structure of Impurity-C

MATERIALS AND METHODS

Chemicals and Reagents

Azacitidine, Azacitidine impurity A, Azacitidine impurity B was obtained as a gift samples from Nishka Labs. Methanol and water used were of HPLC grade and were purchased from Spectrochem Pvt. Ltd. Mumbai, India. In addition, Weighing Balance belongs to by Denver, Ultra Sonicator from Fast clean, UV visible Spectrophotometer of Lab India TG 1800, HPLC from Waters 2695 were also used in the study.

Preparation of Mobile Phase and Stock Solutions

(0.01N KH_2PO_4) Buffer: Accurately weighed 0.77gm of ammonium acetate taken in a 1000ml of volumetric flask and 900ml of milli-Q water was added and sonicated, finally volume made with water and pH adjusted to 4 with Ortho Phosphoric Acid.

Mobile phase

Buffer and Acetonitrile taken in the ratio 70:30%v/v

Preparation of Standard solution: 1mg of Azacitidine drug is weighed accurately and transferred to 10ml volumetric flask and dissolved in small amount of diluent. The solution was sonicated for 5 min and the solution was diluted to 1ppm.

Chromatographic conditions

Flow rate : 1 ml/min

Column : Discovery C18 250 mm x 4.6 mm, 5µ.

Detector wave length: 217 nm

Column temperature: 30°C

Injection volume : 10µL

Run time: 13min

Listed Analytical method validation are [11]

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

The LOD and LOQ for Azacitidine and its 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 impurities are prepared by diluting stock solutions to the required concentrations. The solutions are prepared at different concentration levels and injected into the column under the chromatographic conditions developed. The data of peak area versus concentration was subjected to least-square regression analysis.

Accuracy

The recovery study of Azacitidine and its impurities from placebo is conducted. Samples are prepared by mixing placebo with Azacitidine as per the formulation composition and then spiked the known impurities at different spike levels. 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 by using relative response factor and compared against the known amounts of impurities spiked

Precision

Three different concentrations of standard solutions (within the linear range) were analysed six consecutive days. The RSD values were derived for the prepared concentrations.

RESULTS AND DISCUSSION

Linearity

Linearity details derived for Azacitidine and its impurities are detailed (Table 1, Table 2, Table 3, Figure 4, Figure 5 and Figure 6).

Table 1: Linearity details of developed method for 25%, 50% and 75%.

Linearity in %		RT	Area	USP Plate count	USP tailing
25%	Impurity A	2.570	11561	7393	1.49
		2.572	11657	6107	1.13
		2.577	11404	5960	1.34
	Impurity B	3.074	10898	3640	0.99
		3.077	10923	3318	1.40
		3.077	10568	4837	1.21
	Azacitidine	7.945	12161	12746	1.00
		7.957	12254	21064	1.01
		7.959	12164	9516	1.04
50%	Impurity A	2.574	23069	5652	1.33
		2.575	23225	5951	1.52
		2.575	23127	6369	1.59
	Impurity B	3.083	21430	4830	1.19
		3.084	20961	5302	1.22
		3.085	20912	5195	1.06
	Azacitidine	7.958	25465	3843	1.16
		7.959	25009	4975	0.96
		7.964	25171	18152	1.10
75%	Impurity A	2.572	34593	6124	1.32
		2.573	34681	5739	1.36
		2.575	34533	5730	1.40
	Impurity B	3.085	31513	5370	1.17
		3.086	31472	5695	1.18
		3.087	30928	5357	1.21
	Azacitidine	7.950	36588	10412	1.11
		7.962	36407	9411	0.93
		7.963	36053	17331	0.92

Table 2: Linearity details of developed method for 100%, 125% and 150%.

Linearity in %		RT	Area	USP Plate count	USP tailing
100%	Impurity A	2.573	45280	6043	1.38
		2.573	45441	5767	1.34
		2.575	45334	5762	1.38
	Impurity B	3.086	43087	5915	1.31
		3.087	43192	5685	1.16
		3.088	42787	5943	1.28
	Azacitidine	7.955	48726	10026	1.02
		7.955	48805	12443	1.13
		7.969	49062	12206	1.03
125%	Impurity A	2.572	57862	6079	1.40
		2.573	57995	6087	1.50
		2.574	57961	5901	1.50
	Impurity B	3.087	52325	5403	1.24
		3.087	52470	5694	1.26
		3.088	52372	5917	1.24
	Azacitidine	7.954	60370	16965	1.05
		7.960	60954	8345	1.04
		7.962	60902	10961	1.19
150%	Impurity A	2.572	66568	6088	1.39
		2.576	66667	5779	1.39
		2.577	66761	5692	1.45
	Impurity B	3.088	63590	6015	1.18
		3.091	64321	6059	1.23
		3.093	63322	5960	1.21
	Azacitidine	7.960	71637	11070	1.09
		7.963	71897	15156	1.03
		7.966	71820	14406	0.95

Table 3: Linearity details of Azacitidine and its impurities.

Azacitidine			Impurity-A		Impurity-B	
Level	ppm	Area	ppm	Area	ppm	Area
25 %	0.303	12193	0.311	11541	0.313	10796
50 %	0.606	25215	0.622	23140	0.625	21101
75 %	0.909	36349	0.934	34602	0.938	31304
100 %	1.212	48864	1.245	45352	1.250	43022
125 %	1.515	60742	1.556	57939	1.563	52389
150 %	1.818	71785	1.867	66665	1.875	63744

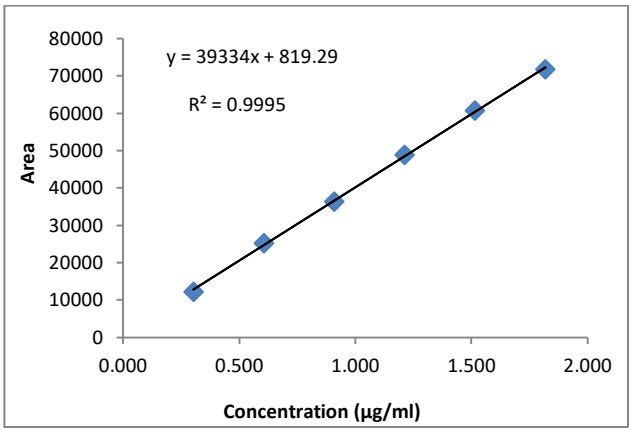


Figure 4: Standard Calibration Curve of Azacitidine

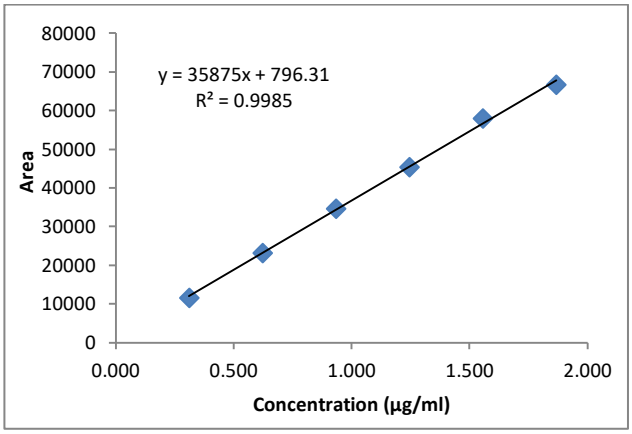


Figure 5: Standard Calibration Curve of Impurity A

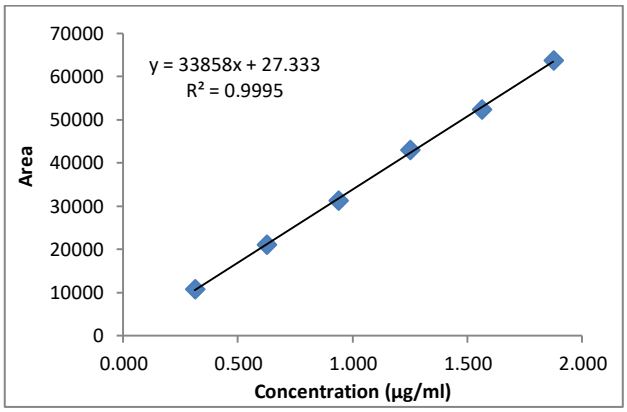


Figure 6: Standard Calibration Curve of Impurity B

Accuracy

The accuracy results of Azacitidine and its impurities are detailed (Table 4)

Table 4: Accuracy details of Azacitidine and its Impurities

Accuracy Level in %		RT	Area	USP Plate count	USP tailing
50%	Impurity A	2.573	22639	6413	1.34
		2.573	22287	6331	1.33
		2.574	22331	5614	1.33
	Impurity B	3.085	26801	5550	1.17
		3.088	26955	5636	1.30
		3.089	26662	6382	1.26
	Azacitidine	7.961	14101670	11105	1.20
		7.966	13934471	11263	1.20
		7.970	13446155	11515	1.19
Accuracy Level in %		**RT**	**Area**	**USP Plate count**	**USP tailing**
100%	Impurity A	2.572	44853	6109	1.54
		2.574	45685	6483	1.35
		2.575	44993	6428	1.28
	Impurity B	3.083	42934	3942	1.16
		3.084	42834	5462	1.23
		3.084	42783	5148	1.08
	Azacitidine	7.953	15281055	11229	1.20
		7.954	15155263	11151	1.20
		7.965	14561384	11312	1.18

Accuracy Level in %		RT	Area	USP Plate count	USP tailing
150%	Impurity A	2.570	66956	6084	1.37
		2.572	67504	5771	1.33
		2.572	67002	5988	1.36
	Impurity B	3.085	60152	5066	1.22
		3.085	60208	6215	1.31
		3.087	60173	6215	1.48
	Azacitidine	7.944	13308494	11236	1.20
		7.945	13122031	11265	1.20
		7.946	13061152	11291	1.20

Recovery Details

The accuracy was established by recovery studies and the recovery studies were found satisfactory. The percentage recovery and average percentage recovery for impurity was found to be within the limit (Table 5 and Table 6).

Table 5: Recovery details of Impurity-A

% Spike level	sample wt (mg)	Area	Amount found (%w/w)	Amount recovered (%w/w)	% Recovery	Mean % Recovery	% RSD
50	10	22639	0.050	0.050	100.67	99.69	0.9
	10	22287	0.049	0.049	99.10		
	10	22331	0.049	0.049	99.30		
100	10	44853	0.099	0.099	99.72	100.44	1.0
	10	45685	0.101	0.101	101.57		
	10	44993	0.100	0.100	100.03		
150	10	66956	0.148	0.148	99.24	99.54	0.5
	10	67504	0.149	0.149	100.05		
	10	67002	0.148	0.148	99.31		

Table 6: Recovery details of Impurity-B

% Spike level	sample wt (mg)	Area	Amount found (%w/w)	Amount recovered (%w/w)	% Recovery	Mean % Recovery	% RSD
50	10	26801	0.063	0.038	99.69	99.72	0.9
	10	26955	0.063	0.038	100.64		
	10	26662	0.062	0.037	98.83		
100	10	42934	0.101	0.076	99.70	99.44	0.2
	10	42834	0.100	0.075	99.39		
	10	42783	0.100	0.075	99.23		
150	10	60152	0.141	0.116	101.94	101.99	0.1
	10	60208	0.141	0.116	102.05		
	10	60173	0.141	0.116	101.98		

Precision

The precision studies of Azacitidine and its Imputies are detailed (Table 7).

Table 7: Precision details of Azacitidine and its Impurities

S. No	Peak Name	RT	Area USP	Plate Count	USP Tailing
1	Impurity-A	2.571	45344	5215	1.31
2		2.572	45488	5875	1.34
3		2.572	45324	6112	1.23
4		2.573	45395	6156	1.41
5		2.574	45652	6036	1.41
6		2.575	45057	5725	1.30
Mean		--	45377	--	--
Std. Dev.		--	197.26	--	--
% RSD		--	0.43	--	--
S. No	**Peak Name**	**RT**	**Area USP**	**Plate Count**	**USP Tailing**
1	Impurity-B	3.068	42321	4669	1.11
2		3.071	42612	5041	1.09
3		3.078	42163	5115	0.96
4		3.080	42589	5087	1.01
5		3.080	42093	4860	1.21
6		3.081	42320	4646	0.96
Mean		--	42350	--	--
Std. Dev.		--	213.8	--	--
% RSD		--	0.5	--	--
S. No	**Peak Name**	**RT**	**Area USP**	**Plate Count**	**USP Tailing**
1	Azacitidine	7.944	14866934	11315	1.16
2		7.947	14689274	11308	1.18
3		7.947	15090792	11320	1.18
4		7.954	14970204	11414	1.17
5		7.954	14970204	11414	1.17
6		7.958	14237937	11337	1.18
Mean		--	14804224	--	--
Std. Dev.		--	308236.1	--	--
% RSD		--	2.1	--	--

LOD and LOQ

Limit of Detection and Limit of Quantification are evaluated to determine the sensitivity of the analytical method. The results of Azacitidine and its Impurities are details (Table 8a and Table 8b).

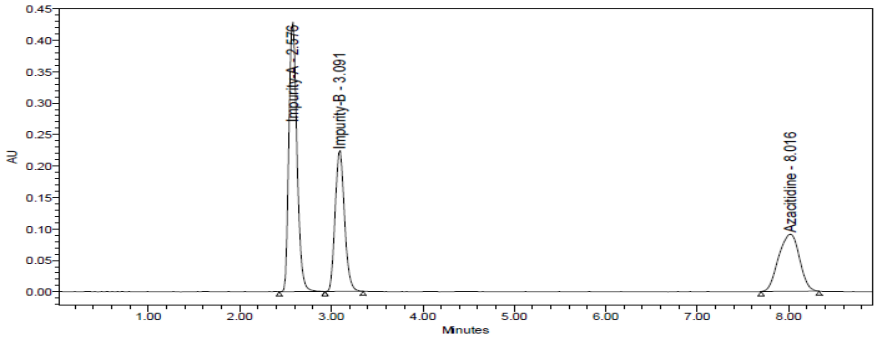
Table 8a: LOD results of Azacitidine and its Impurities

S. No	Peak name	RT	Area	s/n	USP Plate count	USP tailing	s/n
1.	Impurity-A	2.570	1304	32.5	8349.0	1.2	32.501994
2.	Impurity-B	3.074	1398	27.8	3640.1	1.0	27.795093
3.	Azacitidine	7.945	1457	12.7	10062.7	1.1	12.718436

Table 8b: LOQ results of Azacitidine and its Impurities

S. No	Peak name	RT	Area	s/n	USP Plate count	USP tailing	s/n
1.	Impurity-A	2.574	4469	69.0	5652.2	1.3	68.963987
2.	Impurity-B	3.084	4761	44.8	5301.6	1.2	44.780121
3.	Azacitidine	7.959	4967	18.0	5392.2	1.0	17.954909

The retention times of Azacitidine and its impurities are 8.016, 2.576 and 3.091 min respectively (Figure 7).



	Peak Name	RT	Area	% Area	USP Plate Count	USP Tailing	USP Resolution
1	Impurity-A	2.576	2639251	46.54	4100	1.25	
2	Impurity-B	3.091	1595444	28.14	4342	1.15	2.9
3	Azacitidine	8.016	1435828	25.32	5728	0.96	16.1

Figure 7: Chromatogram for Azactidine and its Impurities.

CONCLUSION

A precise, sensitive, specific, accurate, validated and well-defined stability indicating RP-HPLC method for the determination of Azacitidine products and its process related impurities are described. 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 is very useful for quality monitoring of Azacitidine and its Impurities in Active pharmaceutical Ingredient and Pharmaceutical dosage forms. This method can be used for routine analysis of Azacitidine and its impurities.

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