A NOVEL RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE DETERMINATION OF AZACITIDINE USING HYDROTROPHIC SOLVENT BY QBD

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Abstract:

Background: Azacitidine is a demethylating agents and an antineoplastic agent used to treat myelodysplastic syndrome.

Method: The RP-HPLC method for analysis of Azacitidine was developed and validated as per ICH guidelines. The separation of Azacitidine was performed on Inertsil C_{18} column (4.6×250mm, 5µ) with PDA detection carried out at 265nm. A box-behnken design with response surface methodology was executed out for optimization of chromatographic conditions of RP-HPLC for finished desired chromatographic estimation of Azacitidine with less number of experimental trials. Three independent factors namely 1%urea composition in the mobile phase, pH of an aqueous phase and flow rate were used to construct a mathematical model and study the effects of these independent factors on responses such as retention time, theoretical plates, tailing factors

Results: Optimized experimental conditions for proposed work consists of 1%urea at pH 9.15 as mobile phase, at flow rate of 1ml/min with retention time was found to be 1.899. Then accuracy study was completed at three different levels and was found in the range 100 to 102%. The percent RSD value for precision was found to be 0.116. LOD and LOQ value for Azacitidine was found to be 0.0013 & 0.00416µg/ml.

Conclusion: The 3D response surface graphs revealed that 1%urea composition and pH of aqueous phase were both most stringent factors affecting the responses. A rapid novel precise and accurate RP- HPLC method was developed and validated and can be used for regular analysis for the estimation of Azacitidine.

Keywords: Azacitidine, Rapid, Box-Behnken design, ICH guidelines, Surface response

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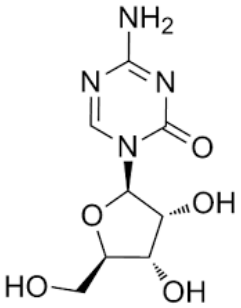
Introduction:

Azacitidine is a chemotherapy drug (Anti neoplastic agent). It is belived to work by helping your bone marrow grow normal blood cells so you will need fewer blood transfusions. Azacitidine also kills abnormal blood cells that have grown too fast and do not work properly by causing hypomethylation of DNA. Analytical methods available for the Azacitidine drug are HPLC, mass spectrometry, high-performance liquid chromatography/tandem mass spectrometric (HPLC-MS/MS), super critical fluid chromatography with tandem mass spectrometry (SFC-MS/MS). In the present study we have used hydrotropes such as urea, sodium citrate, sodium benzoate and sodium acetate etc. So the present study is based on the optimization of Azacitidine by RP-HPLC using Design of experiment. A Box-Behnken response surface design was employed to identify the underlying facts of effects of factors and their interaction effects on selected method responses.



Chemical structure of Azacitidine

Experimental:

Chemicals and reagents:

Azacitidine (Vidaza Manufacturer: CELGENE). 1%Urea,

HPLC instrumentation and chromatographic conditions:

HPLC system Shimadzu Prominence Binary, with PDA detector was used. The system was empowered by Compaq pressario and a Rheodyne injection with a 10µL loop was used for injection of the sample. InertsilC_{18}-ODS-3V (4.6×250mm, 5µm) was used. The mobile phase was composed of 1%urea in the various ratios with flow rate of 1.0ml/min. HPLC system was operated at ambient temperature.

1. Preparation of Standard Stock Solution:

10mg of Azacitidine standard was transferred into 10 ml volumetric flask and 5ml of diluent was added slowly and made up to the mark with diluent to obtain a concentration of 1000µg/ml.

2. Preparation of Working stock solution:

1ml of the standard stock solution was pipetted out and transferred into 10ml of volumetric flask and diluted up to the mark with diluent to obtain a concentration of 100µg/ml.

3. Preparation of Mobile phase:

1gm of urea is weighed and transferred into 100ml volumetric flask and dissolved slowly by adding HPLC grade water and made up to the volume with HPLC grade water to obtain 1% urea mobile phase (Hydrotrope).

Design of Experiment:

The standard drug sample of Azacitidine was subjected to the design of experiment process. Box-Behnken response surface design was employed to identify the underlying facts of effects of factors and their interaction effects on selected method responses. A total of 17 runs were conducted.

Statistical analysis:

- By using ANOVA the statistical calculations were processed for variables screening and optimisation of the method
- The statistical tools provide the numerical verification of variables and its effect on responses.

Method operable design region:

The different amalgamation and reciprocity of input factors produces the space referred as Design space. The establishment of design space was made by utilizing the contour graphs of Sigma tech software.

Method Verification:

The optimized method conditions were proposed by the software in order to reach the desired method goals. The method was verified to check the predictability of the proposed model.

1. Linearity:

From the working stock solution pipetted out 0.5, 0.75, 1, 1.25 and 1.5ml into a six 10ml volumetric flask and made upto the volume 10ml to get 5, 7.5, 10, 12.5 and 15µg/ml concentrated solutions of Azacitidine was filtered and injected. The calibration curve was plotted between concentration and peak area. Correlation coefficient was determined by regression analysis.

2. Precision:

From the standard stock solution an aliquot of 0.1ml was added into a six 10ml volumetric flasks,

made up to 10ml with diluent. Later it was filtered and six replicates were injected into HPLC.

3. Accuracy:

Preparation of placebo:

As per calculation weighed amount of placebo was taken into 25ml volumetric flask, dissolved in 25ml of mobile phase, sonicated for few minutes, made up to the mark with diluents and filtered.

Accuracy solutions at 50% level:

1ml of placebo solution was transferred into three 10ml volumetric flask. To this add 0.5µg/ml working stock solution and filtered through 0.45 microns membrane filter and inject three samples into HPLC injector.

Accuracy solutions at 100% level:

1ml of placebo solution was transferred into three 10ml volumetric flask. To this add 1µg/ml working stock solution and filtered through 0.45 microns membrane filter and inject three samples into HPLC injector.

Accuracy solutions at 150% level:

1ml of placebo solution was transferred into three 10ml volumetric flask. To this add 15µg/ml working stock solution and filtered through 0.45 microns membrane filter and inject three samples into HPLC injector.

4. LOD and LOQ:

The limit of detection and limit of quantification was calculated by using the average value of slope and standard deviation.

5. Robustness:

It is the capacity of the method to remain unaffected by small deliberate variations in the method parameters. For this method robustness was determined by analysis of the sample under variety of conditions like:

1. Flow rate variations

Flow rate variations:

Standard preparation of 10µg/ml of Azacitidine was prepared and injected into HPLC system with the variation in flow rate, varied by ±0.1ml/min, i.e., 0.6ml/min and 1ml/min.

Assay:

The vial containing the drug substance is constituted with 10ml of double distilled water and the concentration was found to be 100mg/10ml. Further dilutions were made to obtain a concentration of 10µg/ml.

Optimization and development of RP-HPLC-PDA method using Box-Behnken design

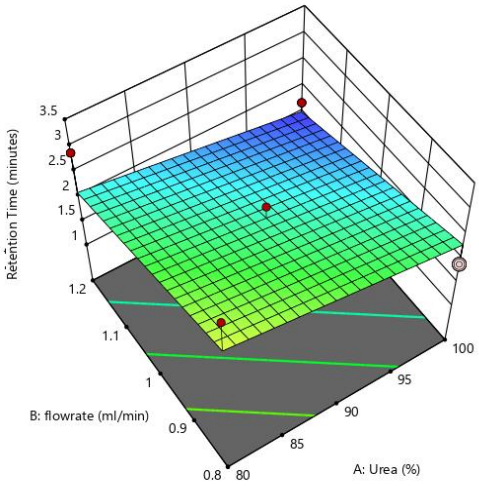
- In the proposed investigation, 17 experimental runs were performed and analysed for obtained results of retention time, theoretical plates, and tailing factors in accord with the Box-Behnken design.
- Further investigation was performed using response surface methodology (RSM) to evaluate the relationship between the dependent responses and independent variables (Factors) using obtained data was reported in Table 1.
- The model was also validated by analysis of variance (ANOVA) using design expert software, and the results are as presented in Table 2. Based on press value, a quadratic model was selected for responses such as retention time, theoretical plates, and tailing factor of APL.
- The significant effects showed p value less than 0.05, while the low standard deviation (% C.V) and a high adjusted R-square value indicated a good relationship between the experimental data and those of the fitted model.
- The predicated R-square value was in acceptance concordance with the adjusted R-square value for all responses.
- The final equation in terms of actual components and factors which can be used to make predictions about the response for given levels of each factor,

Table: 1 Box - Behnken design experimental runs

Std	Runs	Factors			Responses		
		1%urea	Flow rate ml/min	pH	Retention time	Theoretical plates	Tailing factor
17	1	90	1	9.5	1.893	2111	1.45
14	2	90	1	9.5	1.893	2246	1.47
2	3	100	0.8	9.5	1.899	2173	1.41
13	4	90	1	9.5	1.895	2171	1.42
3	5	80	1.2	9.5	2.881	1802	1.32
4	6	100	1.2	9.5	1.687	1800	1.38
15	7	90	1	9.5	2.43	1980	1.43
10	8	90	1.2	9	1.687	1650	1.42
5	9	80	1	9	2.21	1900	1.32

12	10	90	1.2	10	1.542	1620	1.45
9	11	90	0.8	9	2.772	1604	1.39
16	12	90	1	9.5	1.892	2040	1.45
7	13	80	1	10	1.942	2030	1.48
6	14	100	1	9	2.522	1620	1.41
8	15	100	1	10	1.984	2060	1.53
11	16	90	0.8	10	2.87	1980	1.45
1	17	80	0.8	9.5	3.483	1840	1.45

Retention Time:



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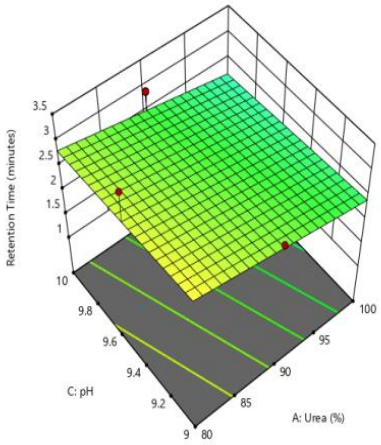

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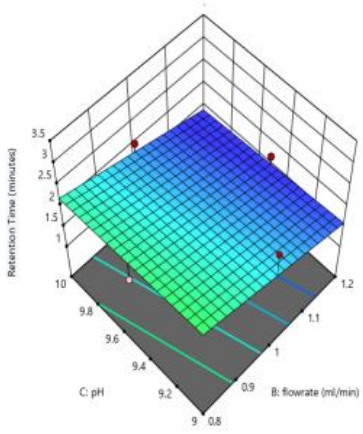

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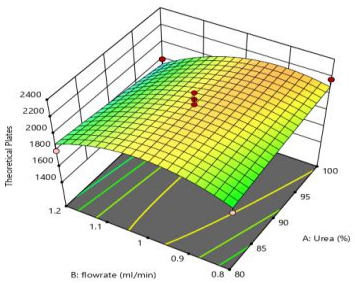
Fig.No.1: 3D RSM plots for retention time

Theoretical Plates:



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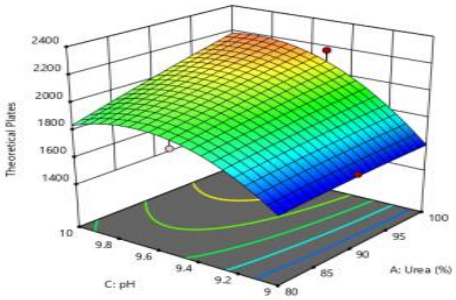

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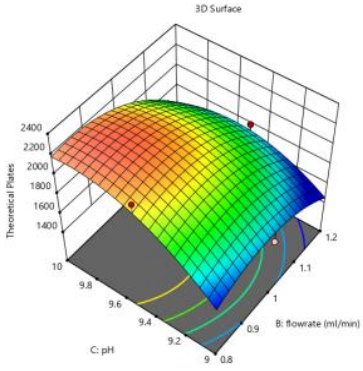

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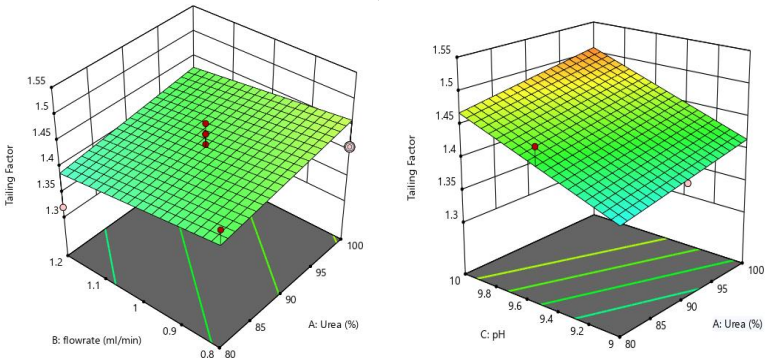
Fig.No.2: 3D RSM plots for Theoretical plates

Taling Factor:



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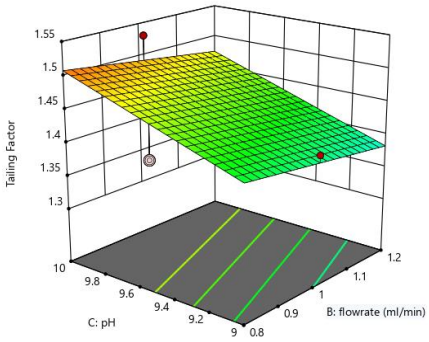

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Fig.No.3: 3D RSM plots for Tailing factor

Table: 2 ANOVA Table.

S.NO	Response	±S.D	Mean	%CV	R^2	Adjusted R^2	Predicated R^2	Adequate precision	P value
1	Retention time	0.4345	2.20	19.71	0.4643	0.3406	0.0290	6.7025	0.0384
2	Theoretical plates	115.67	1919.24	6.03	0.8683	0.6989	0.2140	5.8780	0.0212
3	Tailing factor	0.0417	1.43	2.93	0.4981	0.3822	0.0194	6.5515	0.0258

Coded Equations:

Coded Equations:

Retention time:

+2.20-0.3030A-0.4034B-0.1066C

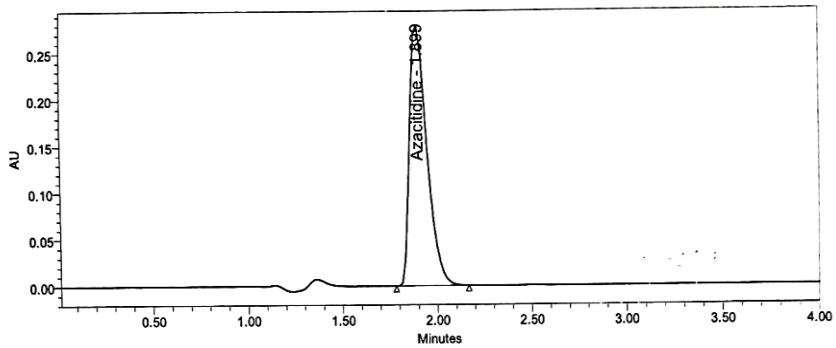
Theoretical Plates:

+2109.60+10.12A-90.62B+114.50C-83.75AB+77.50AC-101.50BC-8.43A^2-197.43B^2-198.67C^2

Tailing Factor:
+1.43+0.0200A-0.0162B+0.0463C

Positive values represent an effect that favours optimisation while a negative value indicates an inverse relationship between the factors and responses. 3-D response surface plots revealed that effect of factors such as 1%urea composition in mobile phase, pH of aqueous phase and flow rate of HPLC system on the responses Retention time, Theoretical plates and Tailing factor.

Optimized Chromatogram:



Peak name	Retention time	Peak area	Theoretical plates	Tailing factor
Azacitidine	1.899	1752930	2173	1.41

Figure.No.4: Chromatogram of optimized method

Discussion: Azacitidine eluted with good peak shape, retention time and tailing factor.

Method Validation:
Table.No.3: Linearity Concentration data

S.NO	Concentration (µg/ml)	Area
1	5	864424
2	7.5	1286568
3	10	1721432
4	12.5	2130823
5	15	2575247

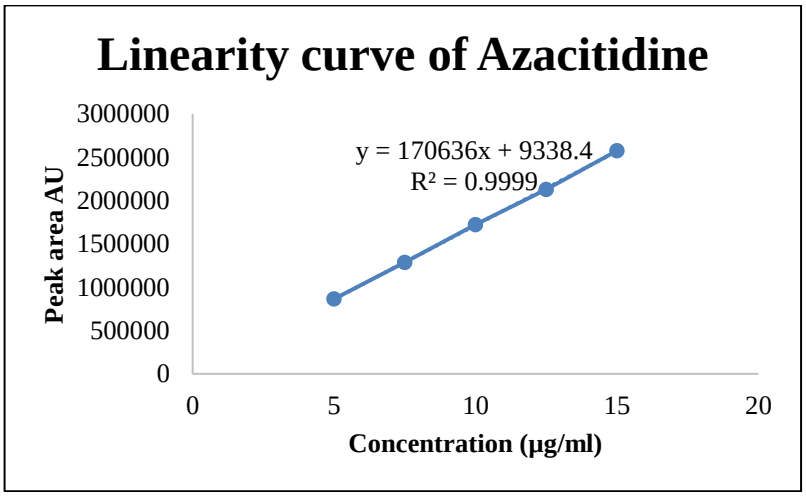
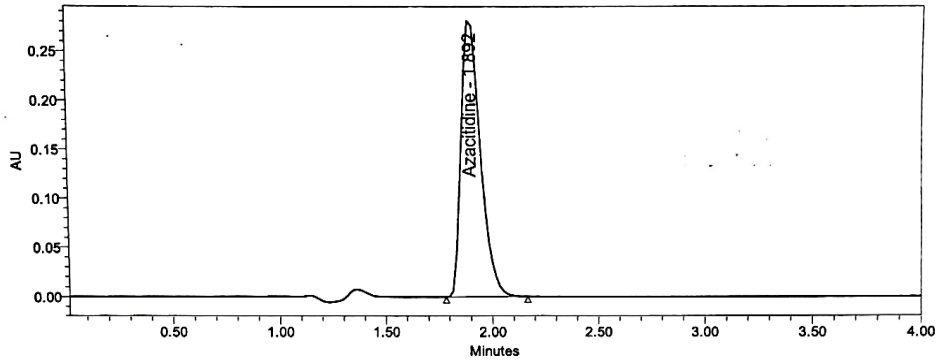


Figure.No.5: Calibration plot of Azacitidine

Discussion: The correlation coefficient value, $R^2 = 0.9999$.
Precision: As per method development six standard injection of precision were prepared and injected.



Peak name	Retention time	Peak area	Theoretical plates	Tailing factor
Azacitidine	1.892	1742203	2110	1.45

Figure.No.6: Chromatogram of Precision

Table.No.4: Precision data

S.NO	Concentration (µg/ml)	Concentration Found(µg/ml)	Percentage Recovery	Mean %	Standard Deviation	%R.S.D
1.	10	10.11	101.1	101.33	0.118	0.116
2.		10.15	101.5			
3.		10.17	101.7			
4.		10.11	101.1			
5.		10.15	101.5			
6.		10.11	101.1			

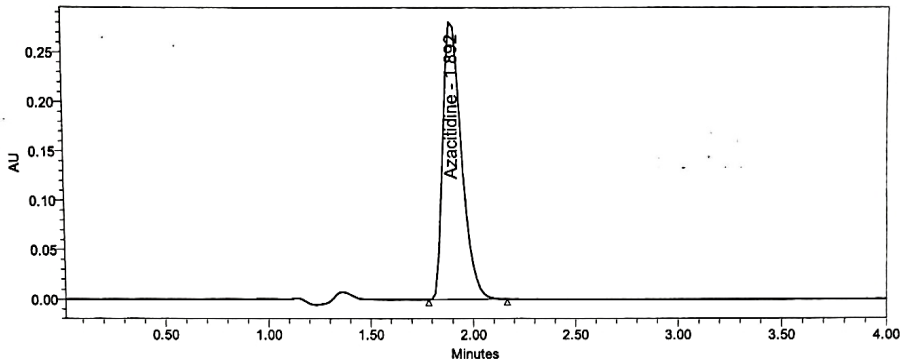
Discussion: The %RSD of six standard injection results was found to be 0.116.

Table.No.5: Accuracy data

S.NO	% Level	Concentration added (µg/ml)	Concentration Found (µg/ml)	% Recovery	Mean %	SD	%RSD
1.	50%	5	5.032	100.64	101.166	0.4432	0.438
2.			5.093	101.86			
3.			5.050	101			
4.	100%	10	10.033	100.33	100.28	0.0324	0.0323
5.			10.027	100.27			
6.			10.024	100.24			
7.	150%	15	15.18	101.2	101.39	0.2053	0.2024
8.			15.26	101.73			
9.			15.19	101.26			

Discussion: The percent recovery for 50%, 100% and 150% spiked concentration was found to be 101.1%, 100.28% and 101.39%.

Assay:



Peak name	Retention time	Peak area	Theoretical plates	Tailing factor
Azacitidine	1.892	1712090	2149	1.45

Figure.No.7: Chromatogram of Assay

Table.No.6: Assay studies of Azacitidine

S.NO	Drug name	Label claim	Amount Found	% Purity
1.	Azacitidine	100mg	99.7mg	99.7%

Discussion: Percentage purity of Azacitidine was found to be 99.7%

Summary & Conclusion:

Table.No.7: Summary data of validation parameter

S.NO	VALIDATION PARAMETERS		ACCEPTANCE CRITERIA	RESULTS
1.	System suitability		The %RSD for five replicate injections of standard solution NMT 2.0%	0.185
2.	Linearity		The correlation coefficient should be NLT 0.999	R^2=0.9999
3.	Precision		The %RSD of peaks obtained from the 6 replicate injections should be NMT 2.0%	0.116
4.	Accuracy		The % recovery at each level should be NLT 98.0% and NMT 102% of the amount added.	100.94%
5.	LOD		-	0.0013 µg/ml
6.	LOQ		-	0.00416 µg/ml
7.	Robustness	Variation in flow rate (0.6ml/min-1ml/min)	%RSD should be NMT 2	0.6ml/min-0.113 1ml/min-0.334
.	Assay		The amount of assay % should be NLT 98.0% and NMT 102% of the amount added	99.7%

Conclusion Conclusion

The proposed RP-HPLC method, which uses a hydrotropic solution as the mobile phase eliminates the need for organic solvents, avoiding issues like volatility, pollution and cost. Further more, the majority of the organic solvents in analysis can be reduced by selecting hydrotropic solvents as the mobile phase. DOE approach was carried out by applying Box-Behnken design and assignment of independent factors and mutually interaction between factors was carried out and a rapid, novel, precise accurate cost effective and robust RP-HPLC method has been developed for the estimation of Azacitidine in bulk and dosage form. According to response surface plots the hydrotropic solvents (1%urea composition) was found to be more deliberate factor for optimization of the method it is understood that the utilization of DOE approach is an adaptable practice to decrease the total experimental runs required for method developed and in short duration of time. Excellent % recovery of the drug in presence of placebo was obtained the proposed method was found to be accurate, precise, robust and novel.

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