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Original Article

Response Surface Methodology as a Tool for Stability-Indicating Method Development and Validation for the Determination of Selective EZH2 Inhibitor

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ABSTRACT

Background: Tazemetostat (TZST) is an efficient first-in-class, selective, EZH2 oral inhibitor which had demonstrated tumor regression and favourable safety in patients with Epithelioid Sarcoma. Methods developed using Analytical Quality by Design (AQbD) are highly robust, cost effective, uses good experimental designs, have shorter run times, optimization can be done by statistical analysis and can be easily validated. **Objectives:** The objective of the present study was to use the Screening designs for selection of initial chromatographic conditions and Response Surface Methodology (RSM) for development of optimized method based on desirability functions approach and validate sthe developed method as per ICH guidelines. **Materials and Methods:** To select the column, organic modifier and buffer, a 2^3 Factorial design was used initially for the experimental plans which majorly affect selectivity. Optimization of the method was done using Central Composite Design (CCD) under RSM. **Results:** Design-Expert software was used for statistical analysis of responses from CCD experimental data using ANOVA and multiple regression analysis. The set of CMP's which shows a maximum composite desirability was taken as optimized chromatographic conditions and evaluated for the responses. **Conclusion:** The present work successfully demonstrated the use of AQbD approach for developing and validating stability-indicating RP-HPLC method for determination of TZST.

Keywords: TZST, AQbD, CCD, RSM, Stability-indicating.

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INTRODUCTION

Tazemetostat (TZST) is a medication used for the treatment of metastatic or locally advanced Epithelioid Sarcoma (ES) which is a rare sub-type of soft tissue sarcoma in adults and pediatric patients aged 16 years and older whose disease cannot be removed by surgery and is sold under the trade name Tazverik. The drug is also used for the treatment of relapsed or refractory follicular lymphoma in adult patients who do not have alternate treatment options.[1] After oral administration, the drug selectively inhibits the activity of both wild and mutated forms of Histone methyl transferase EZH2, thereby preventing the methylation of histone H3 lysine 27 which results in altered gene expression patterns and decreased tumor cell proliferation.[2] The drug was soluble in organic solvents such as ethanol, DMSO, DMF[3] and granted USFDA approval in January 23, 2020 based on overall response rate and duration of response.[4] The drug was developed by Epizyme Inc in collaboration with Eisai is the first of its kind to be approved specifically for the treatment of ES and available as film coated tablets in 200 mg strength.[5] The recommended dose is 800 mg orally twice daily with or without food.[6] TZST structure was shown in Figure 1.

Extensive literature survey revealed that phase studies were performed to determine the safety, efficacy[7-10] and UPLC-MS/MS method has been developed for the estimation of TZST and herb-drug interactions between Plumbagin and TZST were studied in rat.[11] HPLC has become one of the most widely accepted analytical separation techniques because of its performance and reliability especially in Pharmaceutical sector.[12] But in order to develop an optimized method, chromatographic conditions should be properly set especially the critical factors affecting the responses.[13] Therefore to achieve the need, strategy of AQbD was used a present trend in pharmaceutical industry in the method development process which is a part of pharmaceutical product development (ICH Q8), quality risk management (ICH Q9) and pharmaceutical quality system (ICH Q10).[14]

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Response Surface Methodology (RSM) represent superior category of Design of Experiments (DoE) where the factors are studied at more than 2 levels and in order to determine the experimental error the replicated center points are included in the experimental domain. The most commonly used response surface designs are Box-Behnken, Central Composite (CCD) and three-level full factorial designs.[15] Optimization of the chromatographic conditions can be done using Analysis of Variance (ANOVA), multiple regression analysis and desirability functions approach. Factor-response relationships can be described using mathematical models, where optimal responses can be predicted.[16] Hence the objective of current work was to develop a stability-indicating RP-HPLC method for the determination of TZST using RSM and validate as per ICH guidelines.

MATERIALS AND METHODS

Chemical Resources

ACN, Methanol and water of HPLC grade were used in the present study. Ammonium acetate and Ortho Phosphoric Acid (OPA) of AR grade were procured from Merck India Pvt. Ltd., Gift sample of TZST was supplied by Glenmark Pharmaceuticals, Mumbai, India.

Instrumentation

Authenticity of drug was done using FT-IR spectrophotometer and for spectral studies Shimadzu UV-vis spectrophotometer was used. WATERS HPLC 2695 was used provided with 2996 PDA detector, high speed autosampler, column oven, data acquisition and processing were accomplished using Empower 2 software.

Statistical software

Design Expert® (DE) modelling software Version 12 was used for RSM.

Preparation of mobile phase

HPLC grade Acetonitrile (ACN) and 0.1 M Ammonium acetate buffer pH 5 were mixed in 40:60 ratios.

Preparation of TZST Standard stock solution

Exactly weighed 100 mg of TZST was transferred to 100 mL clean and dry volumetric flask, dissolved in few mL of mobile phase, sonicated and made upto the final volume to get 1000 µg/mL. Then the final concentration was made to 50 µg/mL with mobile phase and used for further studies.

Reagents used for forced degradation studies

1N HCl, 1N NaOH, 20% H_2O_2 and 10% Sodium bisulphate solutions were prepared and used for stress study.

Method Development

Authentication of drug by FT-IR spectra

TZST standard was scanned in FT-IR spectrophotometer by using pressed pellet technique preparation where characteristic absorption peaks are found at 3260.79(C-NH), 1652.59(C=O), 1483(C=C Aromatic), 1155.9(C-N) and 1008(C-O) cm^{-1} and the corresponding FT-IR spectra was represented in Figure 2.

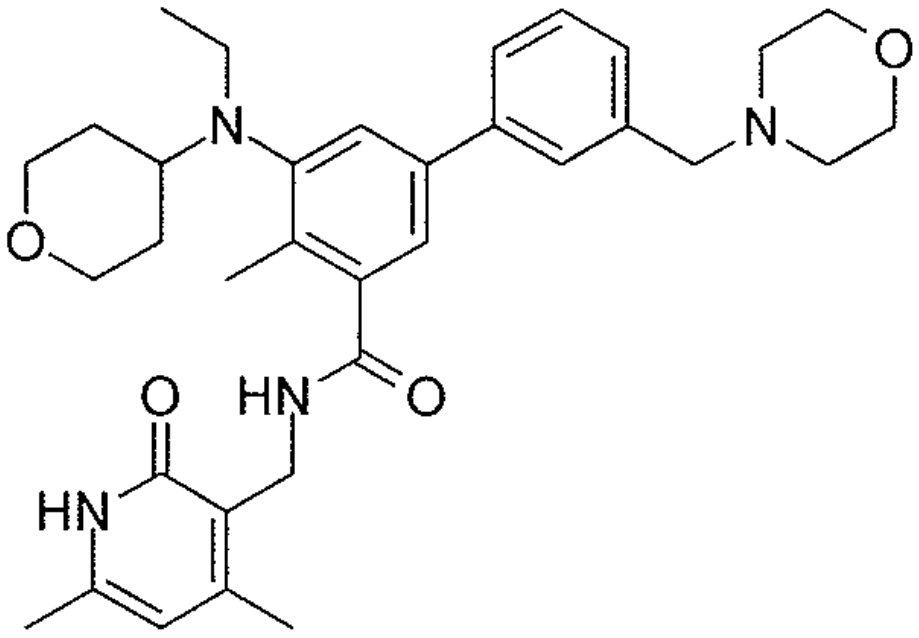

Figure 1: Chemical structure of TZST.

Selection of λ_{max}

UV spectrum in the range of 200-400 nm was recorded for 50 µg/mL TZST which was prepared using ACN. The λ_{max} of the drug was found to be 257 nm represented in Figure 3.

Method development by AQbD

Screening design

For selection of appropriate column, organic modifier and buffer, a 2^3 Factorial design was used.[17] The three factors and two levels chosen for 2^3 Factorial design were shown in the Table 1.

Optimization design

Central composite design under Response surface methodology was used for method optimization which involves identification of CMP's and selection of CQA's.[18]

Optimization of the method by Desirability functions approach

Desirability function was used to predict optimized chromatographic conditions based on required objectives for each response i.e retention time and tailing factor should be minimum and theoretical plates should be maximum. This desirability functions approach range depends on a scale of d=0 for a completely undesirable response, to d=1 for a fully desirable response. Based on the specified goals and boundaries for the chosen CQA's, the set of CMP's which shows a maximum composite desirability was taken as optimized chromatographic conditions and evaluated for the responses.[19]

RESULTS AND DISCUSSION

The chosen screening design generated eight trial runs proposing different combinations for the chosen factors and the obtained results were given in Table 2.

The obtained responses were entered to the software and main effect plots of the three responses were plotted.[20]

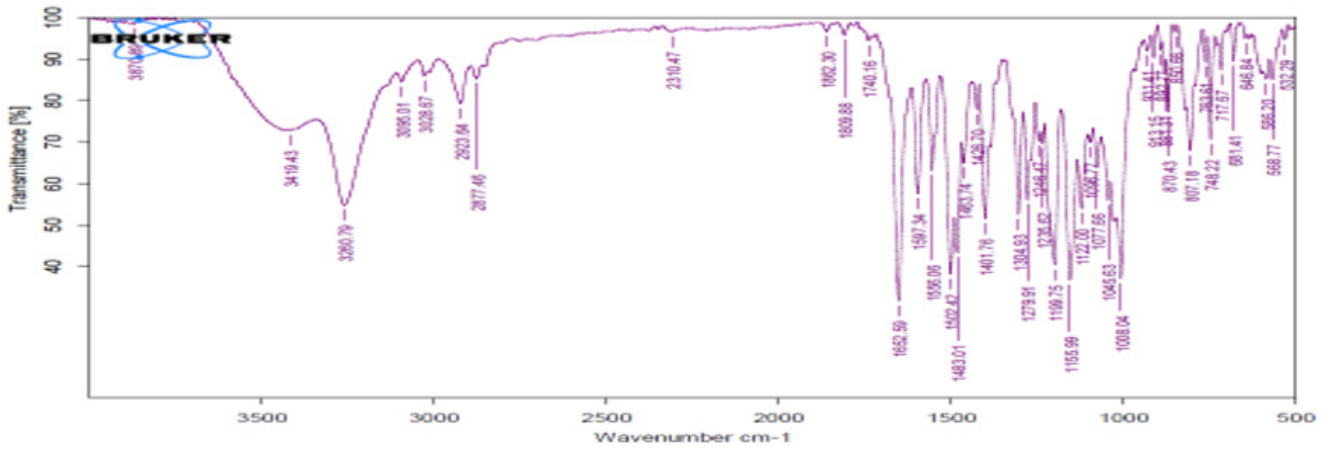

Figure 2: FT-IR spectra of TZST.

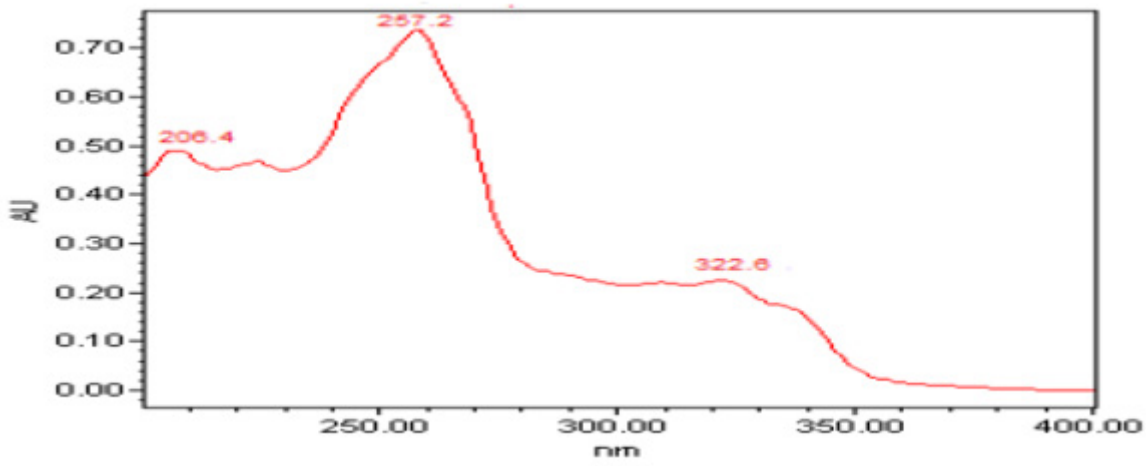

Figure 3: UV spectrum of TZST.

Observation

From the Figure 4, it was found that retention time was less when Waters X-Bridge C18 column, acetate buffer pH 5 and ACN were used in comparison to Inertsil ODS column, OPA pH 3 buffer and methanol.

Observation

From the Figure 5, it was found that theoretical plates were more when acetate buffer pH 5 and ACN were used in comparison to OPA pH 3 buffer and methanol. Theoretical plates are almost similar for both Waters X-Bridge C18 and Inertsil ODS columns.

Observation

From the Figure 6, it was found that tailing factor was less when Waters X-Bridge C18 column, acetate buffer pH 5 and ACN were used in comparison to Inertsil ODS column, OPA pH 3 buffer and methanol.

From the Screening design, based on the above main effect plots, the chromatographic conditions selected initially for the beyond study were Waters X-Bridge C18 column, acetate buffer pH 5 and ACN at which the RT and TF were less and theoretical plates are more.

Optimization by RSD-CCD

The CQA's selected were RT, TP and TF. To optimize the CMP's, CCD was used where each parameter was varied over five levels. Different ranges of CMP's selected were 26.59-43.4% ACN, 0.83-1.16 mL/min flow rate and buffer pH 4.15-5.84 as shown in Table 3.

A Three-factor five-level CCD design generating 20 experimental runs were carried out and the obtained results shown in the Table 4 were analysed by software.

Statistical evaluation of CCD data by DE software

Based on evaluation of results from CCD experimental data, it had been possible to elaborate statistical models and to find out the relationship between them. The model significance obtained for

Table 1: Factors and levels selected for 2^3 Factorial design of TZST.

Factor	Level
Column	Inertsil ODS/Waters X-Bridge C18.
Organic phase	Methanol/can.
Buffer pH	OPA pH 3/Ammonium acetate buffer pH 5.

Table 2: Results of Screening design for TZST.

Run	Column	Organic phase	Buffer pH	Retention time (min)	Theoretical plates	Tailing factor
1	Waters X-Bridge C18	ACN	OPA pH 3	2.108	3887	1.56
2	Inertsil ODS	Methanol	Acetate pH 5	3.512	4546	1.12
3	Waters X-Bridge C18	ACN	Acetate pH 5	2.527	6578	0.72
4	Inertsil ODS	ACN	Acetate pH 5	2.325	6541	0.85
5	Waters X-Bridge C18	Methanol	Acetate pH 5	3.357	4826	1.13
6	Inertsil ODS	Methanol	OPA pH 3	3.954	1620	1.41
7	Inertsil ODS	ACN	OPA pH 3	2.251	5755	0.98
8	Waters X-Bridge C18	Methanol	OPA pH 3	3.628	2108	1.24

Table 3: Design summary of CCD for TZST.

Design Summary							
File version: DX 12.0.12.0 RSM-CCD, Quadratic model			CQA: Retention time, theoretical plates, tailing factor Runs: 20				
CMP	Unit	Type	Min.	Max.	Coded low	Coded High	Mean
A- Flow rate	mL/min	Numeric	0.83	1.16	-1 ↔ 0.90	+1 ↔ 1.10	1.00
B- % Organic content in mobile phase	% v/v	Numeric	26.59	43.41	-1 ↔ 30.00	+1 ↔ 40.00	35.00
C- Buffer pH	-	Numeric	4.15	5.84	-1 ↔ 4.5	+1 ↔ 5.5	5.00

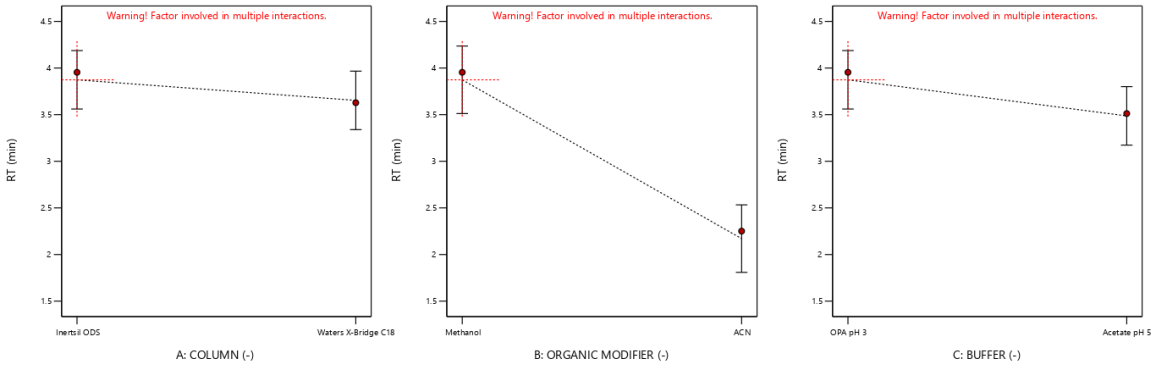

Figure 4: Main effect plots for Retention Time (RT) of TZST.

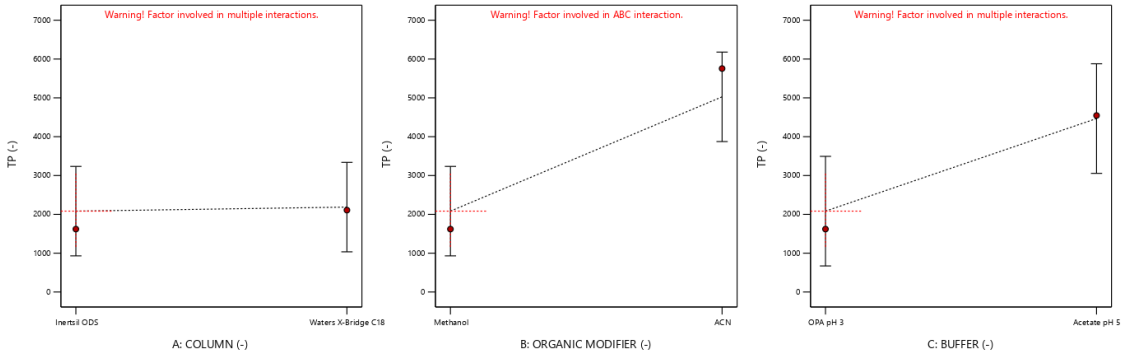

Figure 5: Main effect plots for Theoretical Plates (TP) of TZST.

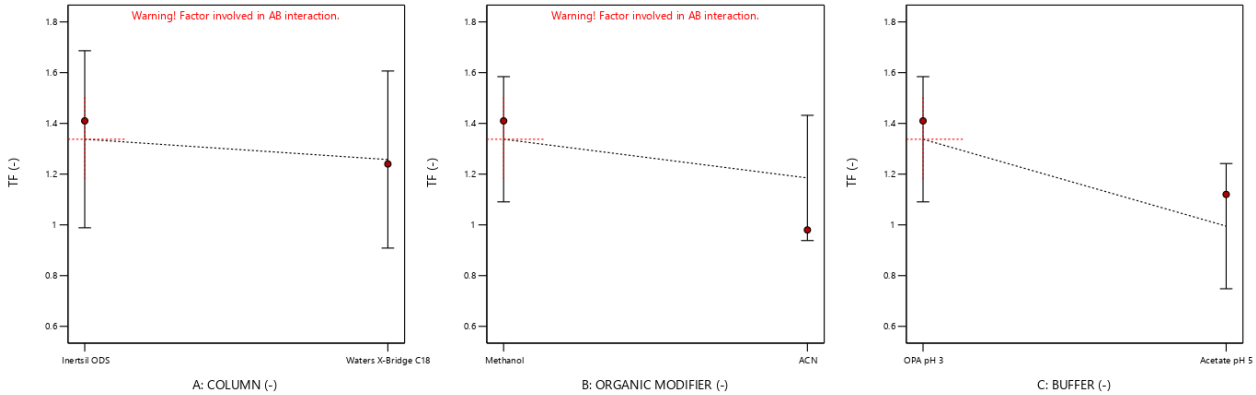

Figure 6: Main effect plots for Tailing Factor (TF) of TZST.

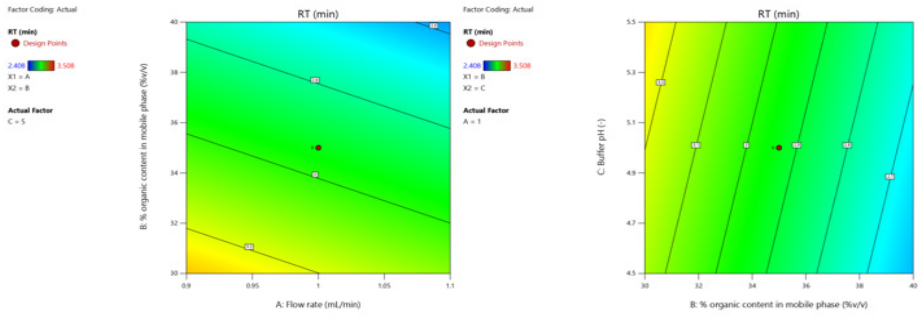

Figure 7: 2D Contour plots of RT as a function of flow rate, % organic content in mobile phase and buffer pH for TZST.

the three responses were determined by applying the ANOVA[21] as shown in Tables 5-7.

From the ANOVA Table 5 for RT, the model was found to be statistically significant with F-value of 42.30. *p*-value of 0.093 suggests insignificant lack of fit. In the suggested linear model, all the terms were significant with *p*-values <0.05. In the 2-D Contour plots, dark blue colour represents lower values and dark red represents higher values. The remaining regions represent intermediate values.

From the contour plots represented in Figure 7, it was seen that RT was less at a higher level of organic content, flow rate and lower level of pH.

From the ANOVA Table 6 for TP, the model was found to be statistically significant with F-value of 10.13. *p*-value of 0.075 suggests insignificant lack of fit. In the suggested linear model A and B are significant terms with *p*-values <0.05.

From the contour plot represented in Figure 8, it was seen that TP are more at a higher level of organic content, flow rate and lower level of pH.

From the ANOVA Table 7 for TF, the model was found to be statistically significant with F-value of 27.29. *p*-value of 0.065 suggests insignificant lack of fit. In the suggested 2-Factor Interaction model A, B and AB are significant terms with *p*-values < 0.05.

From the contour plot represented in Figure 9, it was seen that TF was less at a higher level of organic content, flow rate and lower level of pH.

Method Optimization by Desirability functions approach

The highest desirability of 0.903 was achieved using mobile phase consisting of ACN: 1 mM Ammonium acetate buffer pH 4.5 in 40: 60% v/v pumped at 1.1 mL/min flow rate. The CMP's which gave the maximum desirability was shown as a flag in the overlay contour plot represented in Figure 10. To confirm the optimized chromatographic conditions, three replicates of 50 µg/mL TZST were injected into HPLC system in order to find out if the obtained values were within the desirable limit as shown in Table 8 and the respective optimized chromatogram was shown in Figure 11.

Optimized chromatographic conditions

Column: Waters X-Bridge C18 (150×4.6 mM, 3.5 µ).

Mobile phase: ACN: 1 mM Ammonium acetate buffer pH 4.5 (40: 60 %v/v).

Table 4: Results of CCD for TZST.

Run	Flow rate (mL/min)	% Organic content in mobile phase (%v/v)	Buffer pH	Response 1 (RT) (min)	Response 2 (TP)	Response 3 (TF)
1	1	35	5	2.952	5568	1.02
2	0.9	40	5.5	2.754	5172	1.08
3	1.1	40	4.5	2.408	6527	0.64
4	1	26.59	5	3.508	4243	1.21
5	1	35	5	2.958	5547	1.04
6	1.1	30	5.5	3.173	5523	1.02
7	1	35	5	2.954	5563	1.01
8	1	35	5	2.956	5551	1.05
9	0.83	35	5	3.247	4785	1.21
10	1	43.40	5	2.600	6485	0.77
11	0.9	30	5.5	3.213	4896	1.15
12	1	35	5.84	3.082	5147	1.05
13	1.1	30	4.5	3.091	5052	1.08
14	1	35	4.16	2.872	5736	1.00
15	1	35	5	2.952	5578	1.03
16	0.9	40	4.5	2.689	4874	1.05
17	1	35	5	2.951	5553	1.01
18	1.16	35	5	2.754	5896	0.92
19	0.9	30	4.5	3.034	5421	1.11
20	1.1	40	5.5	2.557	5877	0.84

Table 5: ANOVA for RT of TZST.

ANOVA for Response surface linear model						
Analysis of variance table [Partial sum of squares-Type III]						
Source	**Sum of squares**	**d_f**	**Mean square**	**F value**	***p*-value**	**Inference**
Model	1.14	3	0.3790	42.30	<0.0001	Significant
A-Flow rate	0.1219	1	0.1219	13.60	0.0020	Significant
B-%Organic content in mobile phase	0.9649	1	0.9649	107.68	<0.0001	Significant
C-Buffer pH	0.0502	1	0.0502	5.60	0.0309	Significant
Residual	0.1434	16	0.0090			

df: degrees of freedom, F: Fischer's ratio, *p*: Probability value.

Table 6: ANOVA for TP of TZST.

ANOVA for Response surface linear model						
Analysis of variance table [Partial sum of squares-Type III]						
Source	**Sum of squares**	**d_f**	**Mean square**	**F value**	***p*-value**	**Inference**
Model	3.694E+06	3	1.231E+06	10.13	0.0006	Significant
A- Flow rate	1.473E+06	1	1.473E+06	12.12	0.0031	Significant
B- % Organic content in mobile phase	2.079E+06	1	2.079E+06	17.11	0.0008	Significant
C-Buffer pH	1.428E+06	1	1.428E+06	1.18	0.2944	-
Residual	1.944E+06	16	1.215E+06			

Table 7: ANOVA for TF of TZST.

ANOVA for Response surface 2FI model						
Analysis of variance table [Partial sum of squares-Type III]						
Source	**Sum of squares**	**d_f**	**Mean square**	**F value**	***p*-value**	**Inference**
Model	0.3306	6	0.0551	27.29	<0.0001	Significant
A- Flow rate	0.1233	1	0.1233	61.07	<0.0001	Significant
B- %Organic content in mobile phase	0.1626	1	0.1626	80.51	<0.0001	Significant
C-Buffer pH	0.0063	1	0.0063	3.14	0.1000	-
AB	0.0300	1	0.0300	14.86	0.0020	Significant
AC	0.0006	1	0.0006	0.3033	0.5911	-
BC	0.0078	1	0.0078	3.87	0.0709	-
Residual	0.0263	13	0.0020			

pH of the buffer: 4.5.

Flow rate of the mobile phase: 1.1 mL/min.

Wavelength: 257 nm.

Injection volume: 10 µL.

Run time: 5 min.

Method Validation

ICH Q2 (R1) guidelines were used to validate the final optimized method.[22] The method developed was found to be linear over 12.5-75 µg/mL with 0.999 correlation coefficient and the corresponding overlay linearity chromatogram was shown in Figure 12. For accuracy studies the % recovery of the drug was found to be within 98-102%. The method was found to be precise with % RSD values <2%. The limit of detection and limit of

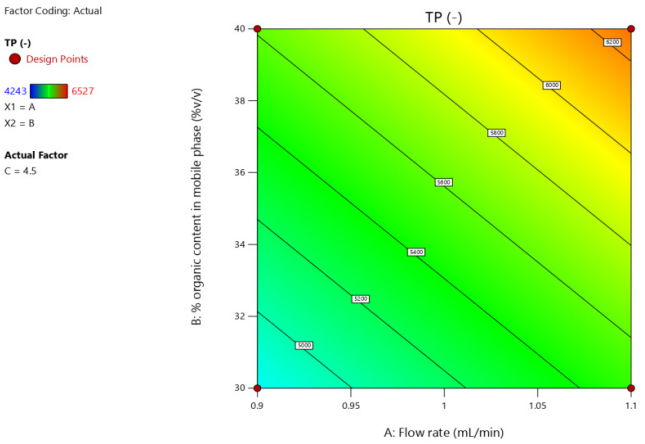

Figure 8: 2D Contour plot of TP as a function of flow rate and % organic content in mobile phase for TZST.

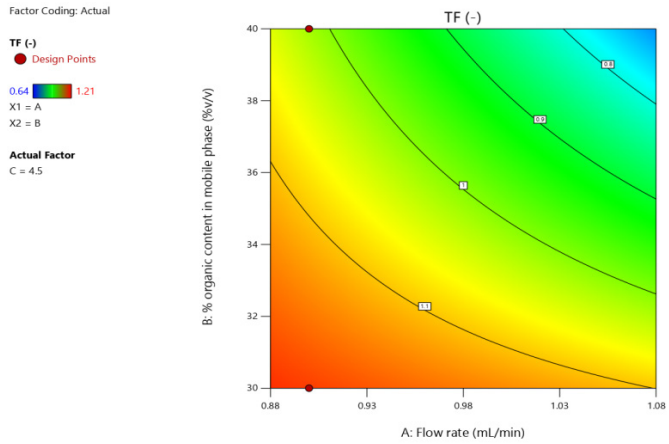

Figure 9: 2D Contour plot of TF as a function of flow rate and % organic content in mobile phase for TZST.

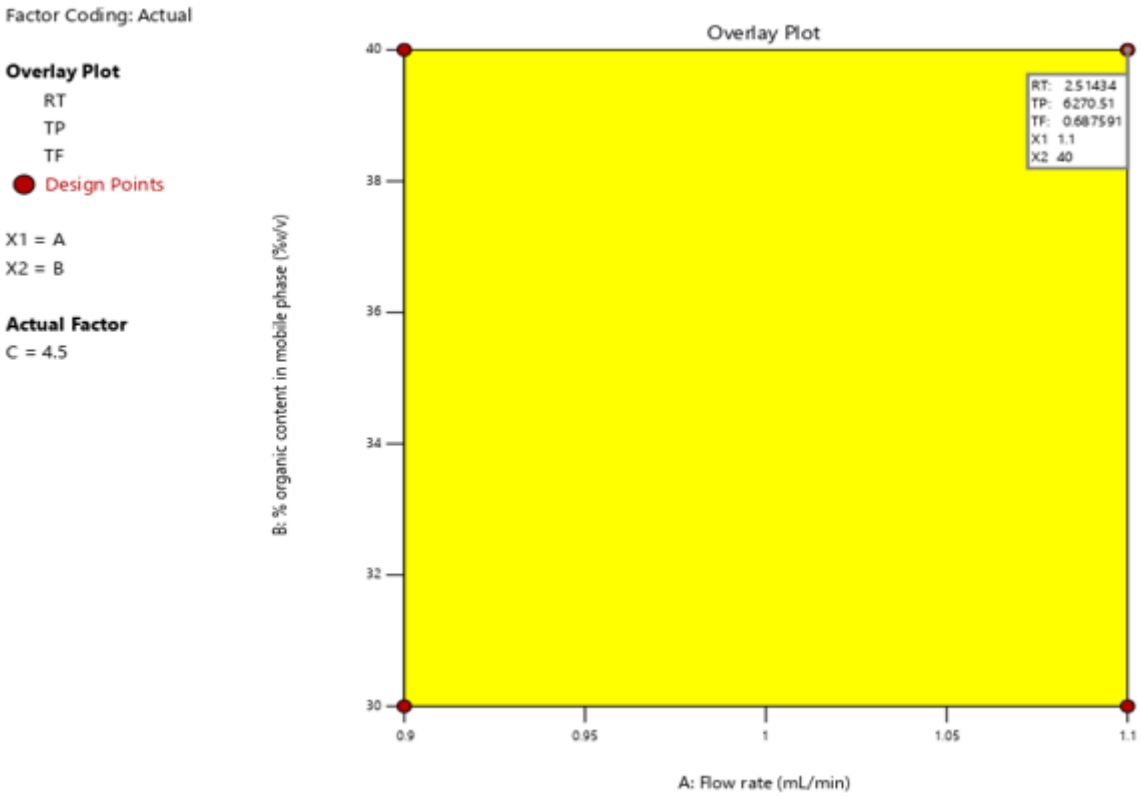

Figure 10: Overlay Contour plot supported by responses for TZST.

Table 8: Responses of the Optimized method for TZST.

Sl. No.	Responses	Predicted value	Observed value	Desirable range
1	Retention time (min)	2.514	2.412	2.288-2.740
2	Theoretical plates	6270.51	6527	5437.78-7103.23
3	Tailing factor	0.687	0.61	0.563-0.812

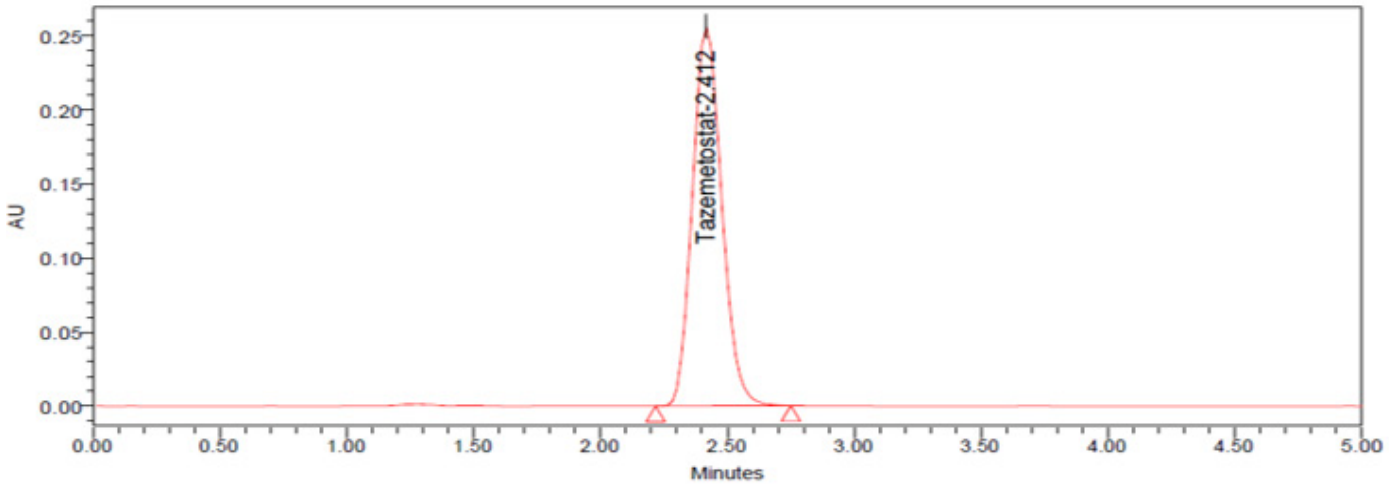

Figure 11: Chromatogram of the Optimized method for TZST.

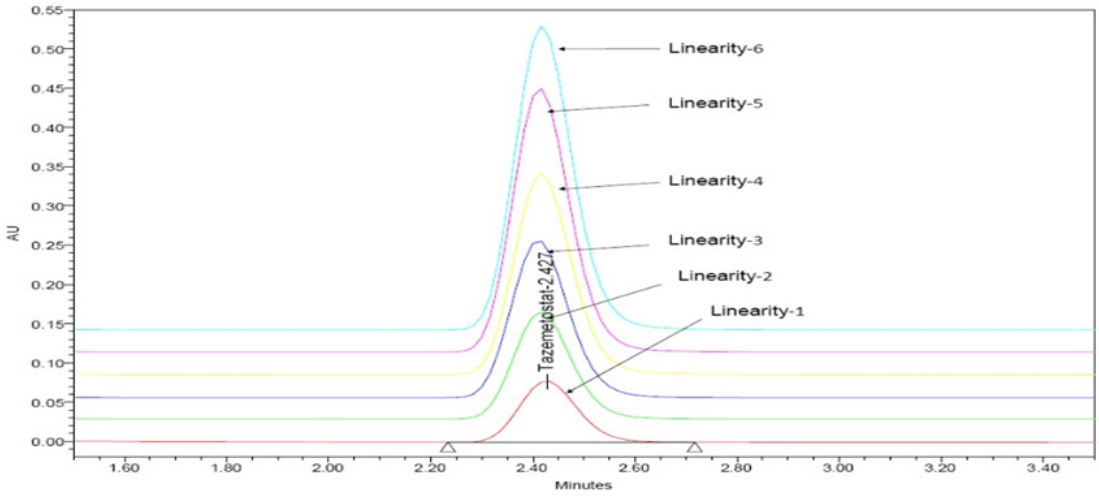

Figure 12: Overlay Linearity chromatogram of TZST over 12.5-75 µg/mL concentration.

Table 9: Summary of the Method Validation parameters.

Sl. No.	Validation parameter		Result
1	Linearity	Linearity Range (µg/mL) Correlation Coefficient Regression equation.	12.5-75 0.999 y=74280x+16091
2	Accuracy (% recovery)	50%, 100%, 150% levels.	99.7-100.52
3	Precision (% RSD of peak area)	Intermediate precision Repeatability.	0.79 0.67
4	Sensitivity	LOD (µg/mL) LOQ (µg/mL).	0.062 0.207
5	Robustness (% RSD of peak area)	Flow rate (±0.1 mL/min) Organic phase (±5%).	0.478 0.315
6	System suitability	Retention time (min) Tailing factor Theoretical Plate count.	2.424 0.63 6541

Table 10: Stress degradation studies for TZST.

Degradation condition	% Drug degradation	Purity angle	Purity threshold	Pass/Fail
Control	--	0.152	0.356	Pass
Acidic (1N HCl, 60°C, 30 m).	13.6	0.164	0.361	Pass
Alkali (1N NaOH, 60°C, 30 m).	12.6	0.149	0.417	Pass
Neutral (H_2O, 60°C, 30 m).	0.8	0.166	0.375	Pass
Oxidative (20% H_2O_2, RT, 30 m).	15.4	0.171	0.429	Pass
Reduction (10% Sodium bisulphate, 60°C, 30 m).	16.5	0.182	0.453	Pass
UV light (24 hr).	12	0.159	0.352	Pass
Thermal (60°C, 24 hr).	12.2	0.171	0.468	Pass

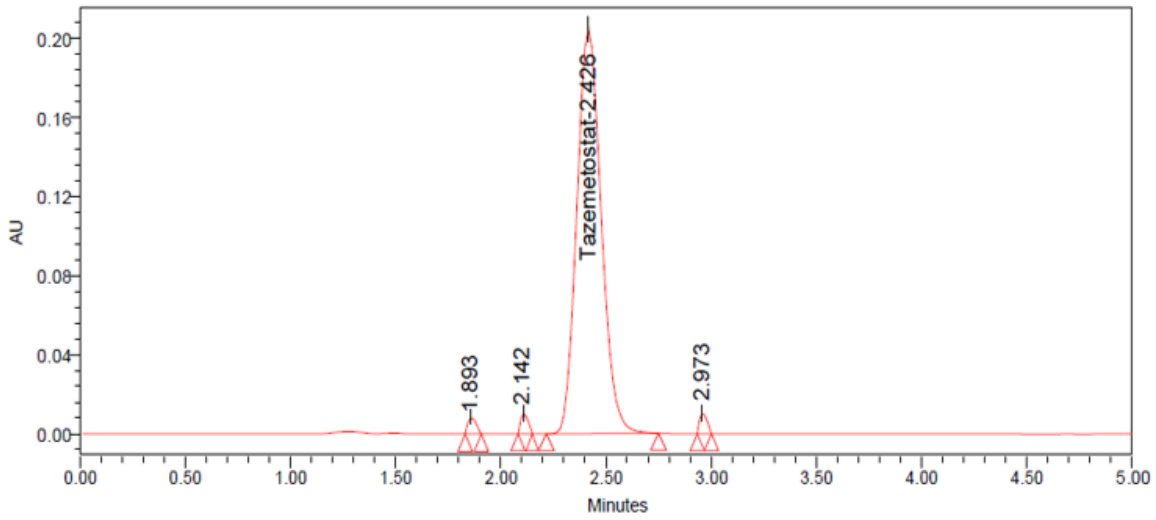

Figure 13: Chromatogram of Stability studies under Reductive condition for TZST.

quantification values were found to be 0.062 and 0.207 µg/mL respectively. The developed method was found to be robust by making minute variations in the experimental parameters and the concise validation parameters were shown in Table 9.

Forced degradation study

Degradation studies were done as per ICH Q1A (R2) guidelines to demonstrate that the optimized method was stability indicating.[23] The samples subjected to different stress conditions showed well separated peak of the analyte and degradation peaks at different retention times. In some conditions, separate degradation peaks are not observed, instead height and area of the analyte peak decrease was observed and the obtained degradation results were shown in Table 10. From the results, it was found that TZST was more susceptible to reductive degradation than other applied stress conditions and the analogous degradation chromatogram was represented in Figure 13.

CONCLUSION

Response surface methodology was used to develop and validate a new, accurate, robust, precise, specific and stability-indicating analytical method for the estimation of Selective EZH2 Inhibitor, TZST. A 2^3 Factorial design was used to select the initial chromatographic conditions. The chromatographic separation was achieved on Waters X-Bridge C18 (150×4.6 mm, 3.5 µM) column which gave the best peak shape and low baseline noise. The UV detection was set at 257 nm and the total run time was 5 min. Optimization was done using CCD under RSM for selection of CMP's and CQA's where factors are studied at five levels. The highest desirability of 0.903 was achieved using ACN: 1 mM Ammonium acetate buffer pH 4.5 in 40: 60 %v/v ratio which is pumped at a flow rate of 1.1 mL/min. The RT of TZST was 2.412 min. ICH Q2 (R1) guidelines were used to validate the final optimized method and all the validation parameters were found to be within the limits. Degradation studies were performed

according to ICH Q1A (R2) guidelines and the developed method was found to be stable in various stress conditions. RSM was considered as a tool for method development where robustness can be tested early in method development stage rather than in validation and statistical analysis of the responses can be done using ANOVA.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

TZST: Tazemetostat; **AQbD:** Analytical Quality by Design; **CCD:** Central Composite Design; **RSM:** Response Surface Methodology; **ANOVA:** Analysis of Variance; **CMP:** Critical Method Parameters; **CQA:** Critical Quality Attributes; **RT:** Retention time; **TP:** Theoretical Plates; **TF:** Tailing Factor; **ICH:** International Council for Harmonisation; **RSD:** Relative Standard Deviation; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification.

SUMMARY

CCD under RSM was employed to develop and validate a new, accurate, robust, precise, specific and stability-indicating analytical method for the estimation of TZST. Analytical Quality by Design assisted developed methods could significantly enhance quality control processes in the pharmaceutical industry by increasing the efficiency, accuracy, substantial cost savings, better compliance with regulatory standards and improved product quality. The developed analytical method may be extended to bioanalytical applications in different biological matrices like blood, tissue and cell lines by modifying sample preparation and analytical techniques and also for performing pharmacokinetic studies.

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