Development and Validation of RP-HPLC Methods for Estimation of Ondansetron Hydrochloride by Quality by Design Approach.

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Abstract -A Ondansetron Hydrochloride is a widely used antiemetic agent that requires accurate and reliable quantification for quality control in pharmaceutical formulations. The objective of this study was to develop and validate a simple, precise, and cost-effective reverse-phase high-performance liquid chromatography (RP-HPLC) method for the estimation of Ondansetron Hydrochloride, applying a Quality by Design (QbD) approach to ensure method robustness and efficiency. The method was developed using a three-level Box-Behnken design with Design Expert Software (Version 13) for optimization. The independent variables selected were the concentration of acetonitrile in the mobile phase, flow rate, and detection wavelength. Chromatographic separation was achieved using a Qualisil BDS-C18 column (250 mm × 4.6 mm, 5 µm particle size) under isocratic conditions. The mobile phase composition, flow rate, and wavelength were optimized to obtain the best resolution and retention time. Detection was carried out at 239 nm using a UV detector. The optimized method showed a retention time of 2.67minutes. The method exhibited linearity in the range of 2–20 µg/mL with an excellent correlation coefficient. Accuracy was confirmed with percentage recovery between 99.99% and 101%, and precision was demonstrated with relative standard deviation values within acceptable limits. The limit of detection and limit of quantitation were found to be 0.493 µg/mL and 1.495 µg/mL, respectively. The method was validated according to International Conference on Harmonisation (ICH) guidelines and was found to be robust, specific, and reliable.

Key Word: - Ondansetron Hydrochloride, RP-HPLC, Quality by Design, Method Validation, Box-Behnken Design, Analytical Method Development, Chromatography

I. INTRODUCTION

HPLC is the most versatile & commonly used analytical technique utilizing a liquid mobile phase to separate the components of the mixture. This component/analyte is first dissolved in solvent & then forced to flow through a chromatographic column under high pressure. The technique is used to separate & quantify the species in a variety of organic, inorganic & biological materials.

Most commonly used methods in HPLC:

Normal phase chromatography:

Principle: Retention by interaction of the stationary phase polar surface with polar parts of the sample molecule/analyte molecule

Reverse -Phase chromatography:

Principle - Retention by the interaction of stationary phase non-polar Hydrocarbon chain with non-polar parts of the sample molecule

The pharmaceutical industry is constantly in search of new techniques to ensure & enhance product quality in terms of its quality, safety & efficacy. However, still problems such as manufacturing failure, drug recall, scale-up of batch issues & regulatory burdens in the recent past have produced huge challenges for the industry. So the regulatory bodies are focusing on the implementation of QbD, which is a more precise, accurate & science-based approach that improves process understanding, by reducing process variation & enabling a process control strategy (CS).

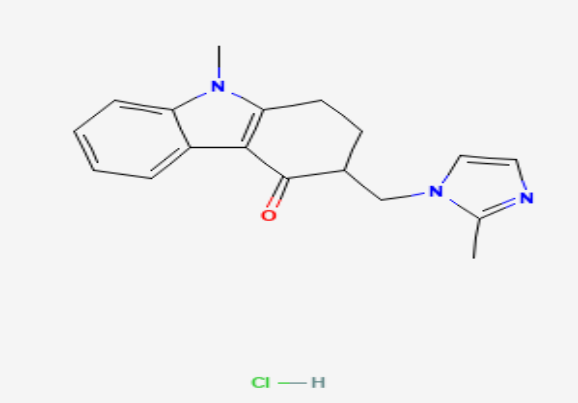
In the traditional approach, product quality & performance are predominantly ensured by product testing, with a limited understanding of the process & critical process parameter (CPP).

Quality by Design (QbD) is a systematic approach to reducing variability and is the traditional approach for method development. QbD approach helps to identify quality issues by analyzing problems & their root cause.

Quality by design identifies critical material attributes

(CMA) & critical process parameters (CPP), to develop the robust method. This leads to identifying a design space Quality - It is the suitability of drug substance or drug product for its intended use, and these terms include attributes, i.e. identity, purity, safety, strength & efficacy.

Ondansetron Hydrochloride (OND HCL):
Chemical Name: (9-methyl-3-[(2-methylimidazol-1-yl) methyl]-2,3-dihydro-1*H*-carbazol-4-one; hydrochloride
Mechanism of action: It is used as Antiemetic. Ondansetron is a selective antagonist of the serotonin receptor subtype, 5-HT3 Cytotoxic chemotherapy and radiotherapy are associated with the release of serotonin (5-HT) from enterochromaffin cells of the small intestine, presumably initiating a vomiting reflex through stimulation of 5-HT3 receptors located on vagal afferents. Ondansetron may block the initiation of this reflex. Activation of vagal afferents may also cause a central release of serotonin from the chemoreceptor trigger zone of the area postrema, located on the floor of the fourth ventricle. Thus, the antiemetic effect of ondansetron is probably due to the selective antagonism of 5-HT3 receptors on neurons located in either the peripheral or central nervous systems, or both.



STRUCTURE OF ONDANSETRON HYDROCHLORIDE.

CHEMICALS AND REAGENTS:
Materials
The reference standard Ondansetron Hydrochloride was received as a gift sample from Ipca Laboratories Ltd. Mumbai, India, and The Zofran tablet containing Ondansetron Hydrochloride (8mg) was purchased from a local pharmacy.
Reagents –Acetonitrile (HPLC grade) Merck Specialties Pvt. Ltd. Mumbai. Water (HPLC grade) (In House supply).

INSTRUMENTATION AND SOFTWARE:
A Jasco HPLC system (Model: PU-2075 Plus) equipped with an isocratic pump and a UV-2080 Plus detector was used for the chromatographic analysis. Chromatographic data were acquired and processed using Borwin software on a Windows-based computer system. The separation was carried out using a Qualisil 5 BDS-C8 column (250 mm × 4.3 mm ID, particle size 5 µm) with an injection volume of 20 µL.

QbD software
Design-Expert® (version 13, trial edition; State-Ease Inc., Minneapolis, MN, USA) was employed for experimental design and data analysis.
To measure absorbance, a Shimadzu model 1800 (Japan) double beam UV/Visible spectrophotometer with a spectral width of 2 nm, wavelength precision of 0.5 nm, and a pair of 10 mm matched quartz cells was employed. The UV-Probe system software generated the spectra automatically.

SELECTION OF DETECTION WAVELENGTH:
The drug solution was scanned over the range of 200 – 400 nm.

PREPARATION OF SOLUTIONS:
Preparation of standard stock solution 1
A standard stock solution of Ondansetron Hydrochloride (500µg/ml) was prepared by dissolving 50 mg of drug in 100ml Acetonitrile: water (50:50) solvent to give a final conc. of 500 µg/ml.
PREPARATION OF STANDARD STOCK SOLUTION 2:
Standard Stock solution of Ondansetron Hydrochloride (100 µg/ml) were prepared by diluting of 20 ml of standard stock solution 1 of Ondansetron Hydrochloride up to 100 ml with solvent to give a final conc. of 100 µg/ml.
PREPARATION OF WORKING STANDARD SOLUTION:
Working standard solution were prepared by diluting of 0.8ml of standard stock solution of Ondansetron Hydrochloride upto 10ml with solvent to give a final conc. of 8 µg/ml.
PREPARATION OF TEST SOLUTION:
Twenty tablets were weighed and average weight was calculated. Tablet powder equivalent to 80mg Ondansetron

Hydrochloride was transferred to 100ml volumetric flask and dissolved in solvent to get 800 µg/ml tablet solution. This solution was sonicated and filtered through 0.22 µ membrane filter.

VALIDATION, HPLC METHOD DEVELOPMENT AND OPTIMIZATION OF MOBILE PHASE:

HPLC-grade Acetonitrile and water were selected as solvents. Various mobile phase compositions were prepared and Sonicated.

Mobile Phase Trials

Sr. No.	Composition of mobile phase
1	Acetonitrile: Water (45:55)
2	Acetonitrile: Water (50:50)
3	Acetonitrile: Water (55:45)

The chromatographic conditions were set as follows: The column was allowed to be saturated with the mobile phase. The analytical wavelength was set at 239 nm. The mobile phase flow rate was set at 1.0 ml/min. The chromatogram run time was set at 10 min. Standard Ondansetron Hydrochloride solution (8µg/ml) was injected. The resulting chromatogram was observed for retention time, no. Of theoretical plates, tailing factor, to select suitable mobile phase composition.

SYSTEM SUITABILITY STUDY

For system suitability study std. stock sol.2 of (8µg/ml) was injected in six replicate solution were injected. The chromatogram was observed for system suitability parameters.

HPLC METHOD DEVELOPED BY THE IMPLEMENTATION OF QBD

ANALYTICAL TARGET PROFILE (ATP):

Here method intent was to develop an HPLC method for the estimation of 50mg Ondansetron Hydrochloride which would be robust, accurate, precise, and with a USP tailing factor less than 1.5, number of theoretical plates as per requirement and short analysis time i.e. less than 10 min. As per QbD norms, a robust method should be developed with the help of design space.

CRITICAL QUALITY ATTRIBUTE (CQA):

Quality attributes that would critically impact ATP were mobile phase composition, flow rate, and wavelength.

RISK ASSESSMENT:

For risk assessment, three input variables were selected for method design at three levels i.e. low, central, and high levels.

Chromatographic factors for Box Behnken experimental design

Chromatographic Conditions	Level used		
	Low (-)	Centre (0)	High (+)
ACN Conc.	40	50	60
Flow rate	0.8	1.0	1.2
Wavelength	238	239	241

METHOD DESIGN:

The above three variables were set in the method design software (Box- Behenken design). After that software suggested 17 runs. Evaluation of main factors, their interaction, and quadratic effect on peak tailing factor, theoretical plate, retention time, and area were done. After entering the data in Design Expert Software, the fit summary was applied to the data after which a "quadratic model" was suggested by the software. According to this model polynomial equations were obtained. Polynomial equation in coded terms.

Box Behnken Method

(Where '+' indicates the high value, '- 'indicates the lower value and '0' is the Centre)

Run	Coded	ACN conc. (%)	Flow rate (ml/min)	Wavelength (nm)
1	+ - 0	60	0.8	239
2	0 0 0	50	1	239
3	0 - -	50	0.8	237
4	+ + 0	60	1.2	239
5	+ 0 +	60	1	241
6	- 0 -	40	1	237
7	- + 0	40	1.2	239
8	0 0 0	50	1	239
9	+ 0 -	60	1	237
10	0 0 0	50	1.2	241
11	- - 0	40	0.8	239
12	0 0 0	50	1	239
13	0 + -	50	1.2	237
14	0 0 0	50	1	239
15	0 - +	50	0.8	241
16	- 0 +	40	1	241
17	0 0 0	50	1	239

Software designs in which various CQA combinations were obtained. This CQA combinations were tried on HPLC instrument to achieve ATPs.

ANALYSIS OF ONDANSETRON HYDROCHLORIDE TABLET

ASSAY:

Twenty tablets containing Ondansetron Hydrochloride (8mg) were taken and crushed to a fine powder. Then powder equivalent to 80mg ondansetron hcl was transferred in 100ml volumetric flask and dissolved in ACN and Water. Volume was made up to the mark. It was sonicated for 5-10 min. The resulting 800µg/ml solution was filtered through membrane filter 0.25µ. From this

800µg/ml solution, 0.8 ml was taken and diluted to get 8µg/ml Ondansetron Hydrochloride. The solution was injected six times. The % of Ondansetron Hydrochloride was calculated from the calibration curve.

Analytical Method Validation

Analytical method validation is a component as Specificity, Linearity, Precision, Intermediate precision, Accuracy, Robustness, Ruggedness, Limit of detection, Limit of quantification.

SPECIFICITY:

Mobile Phase, standard solution (80µg/ml), and sample solution (80µg/ml) were injected and the resulting chromatogram was observed for any interference.

LINEARITY:

From the standard stock solution of Ondansetron Hydrochloride working standard solutions of various concentration ranges (2-20 µg/ml) were prepared and injected. The calibration curve was obtained by plotting peak area vs concentration. The regression equation and regression coefficient were obtained.

PRECISION

A) SYSTEM PRECISION:

The system precision was established by analyzing the standard solution of Ondansetron Hydrochloride (8µg/ml) six times. Relative standard deviation was calculated.

B) METHOD PRECISION:

The system precision was established by assaying the sample solution of Ondansetron Hydrochloride (8µg/ml) six times. The relative standard deviation is calculated.

C) INTERMEDIATE PRECISION:

INTRADAY PRECISION: Intraday precision was demonstrated by analyzing Ondansetron Hydrochloride solutions (4, 8 and 12µg/ml) in triplicate, each on the same day at 0, 4 and 8 hrs. The relative standard deviation was calculated.

INTER-DAY PRECISION: Inter-day precision was demonstrated by analyzing Ondansetron Hydrochloride solutions (4, 8, and 12µg/ml) in triplicate each on three consecutive days and relative standard deviation was calculated.

ACCURACY:

Accuracy was evaluated at three different concentrations by spiking standard at 80, 100 and 120% levels of preanalyzing solution and calculating the recovery of Ondansetron Hydrochloride, RSD (%) and standard error (SE) for each concentration. The known amounts (6.4, 8, 9.6ml) of working standard solution of Ondansetron Hcl (100µg/ml) were spiked to 8ml sample solution of Ondansetron Hcl (100µg/ml) in 10ml volumetric flask and diluted upto mark with diluent (mobile phase). Each solution was injected in triplicate and analyzed. % Recovery was calculated to establish the accuracy of the method.

ROBUSTNESS:

The robustness of the method was investigated by making small deliberate changes in the chromatographic conditions at two different levels. The chromatographic conditions selected for deliberate change were flow rate 0.8, 1, 1.2 ml/min and wavelength 237, 239, 241nm. After each change sample solution was injected and the % assay with system suitability parameters was checked.

LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION:

The limit of detection and quantification are calculated for the sensitivity of the method. LOD is the lowest detectable concentration of the analyte by the method while LOD is the minimum quantifiable concentration. It is expressed as the concentration of analyte (e.g. percentage, parts per billion) in the sample.

LOD and LOQ are calculated using the following equation,

LOD=

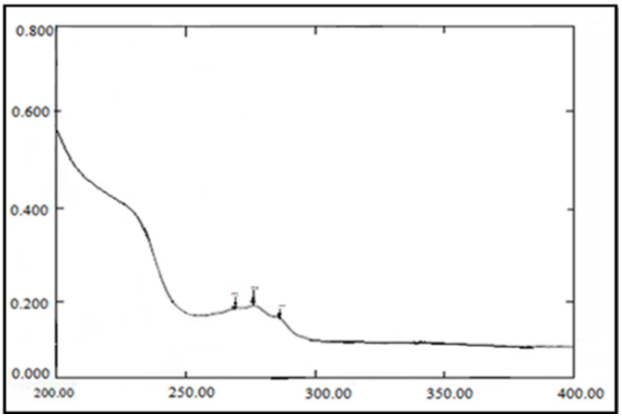
Where,

SD = Response

S Slope of the calibration curve.

LOQ=

According to the ICH recommendations, the techniques have been validated for linearity, accuracy, precision (method precision, intermediate precision), limit of detection, and limit of quantification.



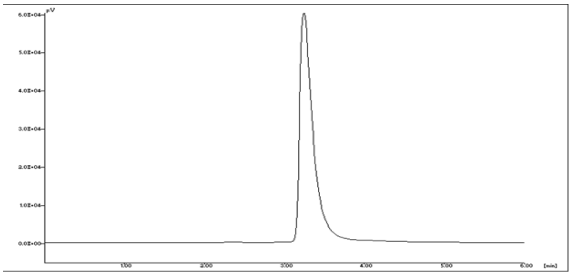
UV SPECTRUM OF ONDANSETRON HYDROCHLORIDE

The absorbance spectrum for Ondansetron Hydrochloride was recorded using a 40µg/ml solution. The maxima absorption was found at 239nm. Hence it was selected as an analytical wavelength.

MOBILE PHASE OPTIMIZATION AND METHOD DEVELOPMENT:

Above mobile phase composition was tried for optimizing mobile phase for analysis of Ondansetron Hydrochloride. The mobile phase composition ACN: Water 50:50%V/V mobile revealed sharp chromatographic peak with RT 3.24 and No of theoretical plate 2465. This mobile phase composition was selected for further analysis.

CHROMATOGRAM OF ONDANSETRON HYDROCHLORIDE WITH OPTIMIZED MOBILE PHASE COMPOSITION



Chromatogram of Ondansetron Hydrochloride with ACN: Water (50:50)

Optimized chromatographic condition

Sr. No.	Parameter	Selected value
1	Conc. of Ondansetron Hydrochloride	80 µg/ml
2	Mobile Phase	ACN: Water (50:50)
3	Flow rate	1.0 ml/min
4	Analytical wavelength	239 nm
5	Column temp.	Ambient
6	Injection volume	20µl

Chromatographic results with the above conditions

Mobile Phase	RT (min)	Flow rate (ml/min)	Tailing Factor	No. of theoretical plates
ACN: Water (50:50)	3.24	1.0 ml/min	1.8	2465

SYSTEM SUITABILITY

System Suitability Study

Sr. No.	RT	Peak area	No. of theoretical plates	Tailing factor
Injection 1	3.23	206148	2477	1.5
Injection 2	3.24	206512	2470	1.5
Injection 3	3.24	206558	2440	1.6
Injection 4	3.23	206969	2441	1.5
Injection 5	3.23	206150	2458	1.7
Injection 6	3.24	206049	2441	1.4
Mean	3.23	206397.7	2454.5	1.533
SD	0.006	318.96	14.908	0.094
%RSD	0.182	0.154	0.607	0.613
Acceptance Criteria		-	NLT 2000	NMT 2

System suitability study revealed the satisfactory values for system suitability parameters such as tailing factor, injection precision, No. of theoretical plates. Retention time value was significant. The system was found suitable for proposed chromatographic analysis.

IMPLEMENTATION OF QBD APPROACH:

1:IDENTIFICATION OF ANALYTICAL TARGET PROFILE (ATP):

HPLC parameters targeted here are retention time, no. of theoretical plates, peak area, and tailing factor.

2: DETERMINATION OF CRITICAL QUALITY ATTRIBUTES (CQA):

The quality attributes which would critically impact on ATP are mobile phase composition, flow rate, and wavelength.

3: RISK ASSESSMENT:

For assessing the risk three input variables were selected for the method design of three levels such as a low, central and high level to assess the risk

Table 10: Chromatographic factors for Box Behnken experimental design

Chromatographic Conditions	Level used		
	Low (-)	Centre (0)	High (+)
ACN Conc.	40	50	60
Flow rate	0.8	1.0	1.2
Wavelength	237	239	241

These three variables were set in the design expert software at three levels. The software suggested the following runs:

Run sheet and found value

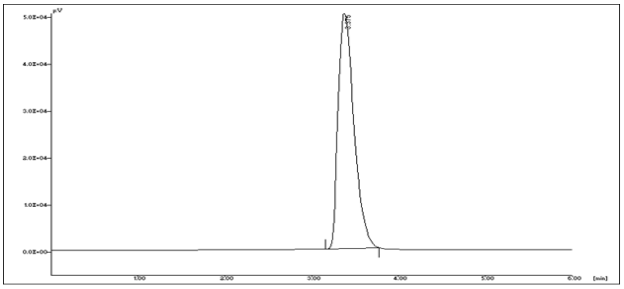
Run no.	ACN conc.	Flow rate	Wavelength	*NTP	*RT	*TF
1	60	0.8	239	2477	3.33	1.56
2	50	1	239	2670	2.66	1.52
3	50	0.8	237	2012	3.09	1.40
4	60	1.2	239	3082	2.50	1.57
5	60	1	241	2240	2.48	1.50
6	40	1	237	2806	3.05	1.42
7	40	1.2	239	2546	2.24	1.54
8	50	1	239	2659	2.67	1.54
9	60	1	237	2669	2.66	1.43
10	50	1.2	241	3045	2.49	1.64
11	40	0.8	239	2545	3.37	1.51
12	50	1	239	2737	2.67	1.55
13	50	1.2	237	2816	2.51	1.44
14	50	1	239	2789	2.67	1.52

15	50	0.8	241	2456	3.08	1.61
16	40	1	241	2654	2.48	1.63
17	50	1	239	2685	2.67	1.54

*NTP: No. of theoretical plates *RT: Retention time
*TF: Tailing factor

RESULTS OF 17 RUNS PROVIDED BY SOFTWARE:

Run 1: I) Mobile phase- ACN: Water (60:20)
II) Flow rate- 0.8ml/min
III) Wavelength- 239nm



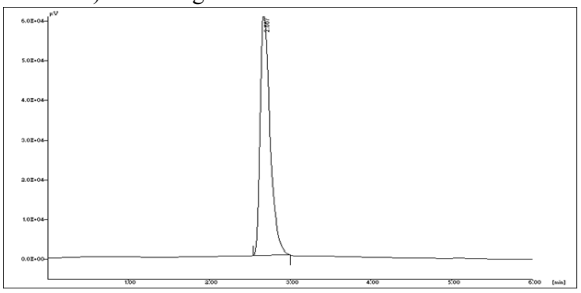
Chromatogram of run 1

* Results of run 1

No. of theoretical plates	Retention time	Tailing factor
2477	3.23	1.56

*Mean of three readings

Run 2: I) Mobile Phase: ACN: Water (50:50)
II) Flow rate: 1 ml/min
III) Wavelength: 239nm



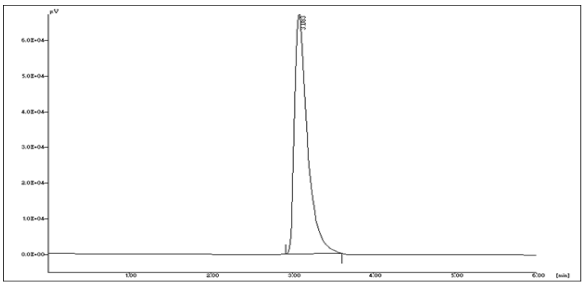
Chromatogram of run 2

Results of run 2

No. of theoretical plates	Retention time	Tailing factor
2737	2.67	1.52

*Mean of readings

Run 3: I) Mobile Phase: ACN: Water (50:30)
II) Flow rate: 0.8ml/min
III) Wavelength: 237nm



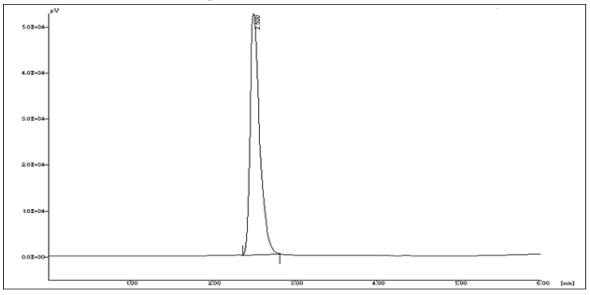
Chromatogram of run 3

Results of run 3

No. of theoretical plates	Retention time	Tailing factor
2012	3.09	1.40

*Mean of three readings

Run 4: I) Mobile Phase: ACN: Water (60:60)
II) Flow rate: 1.2 ml/min
III) Wavelength: 239nm



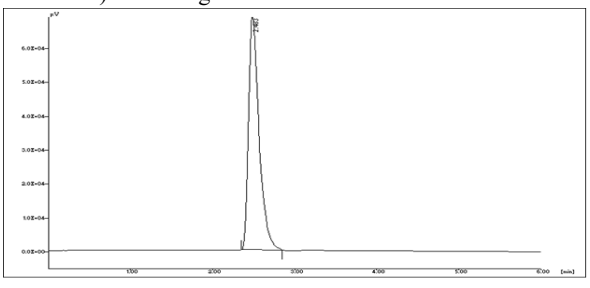
Chromatogram of run 4

Results of run 4

No. of theoretical plates	Retention time	Tailing factor
3082	2.50	1.57

*Mean of three readings

Run 5: I) Mobile Phase: ACN: Water (60:40)
II) Flow rate: 1 ml/min
III) Wavelength: 241nm



Chromatogram of run 5

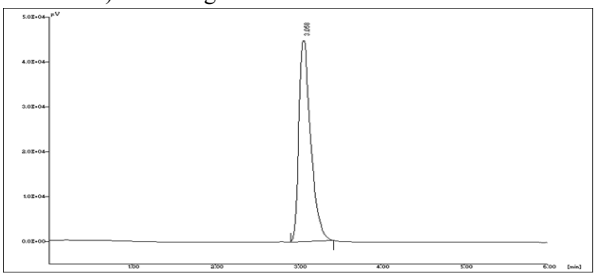
Results of run 5

No. of theoretical plates	Retention time	Tailing factor
2240	2.48	1.50

*Mean of three readings

Run 6: I) Mobile Phase: ACN: Water (40:60)

II) Flow rate: 1 ml/min
III) Wavelength: 237nm



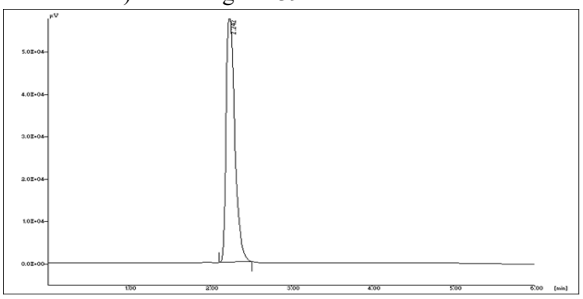
Chromatogram of run 6

Results of run 6

No. of theoretical plates	Retention time	Tailing factor
2806	3.05	1.42

*Mean of three readings

Run 7: I) Mobile Phase: ACN: Water (40:80)
II) Flow rate: 1.2 ml/min
III) Wavelength: 239nm



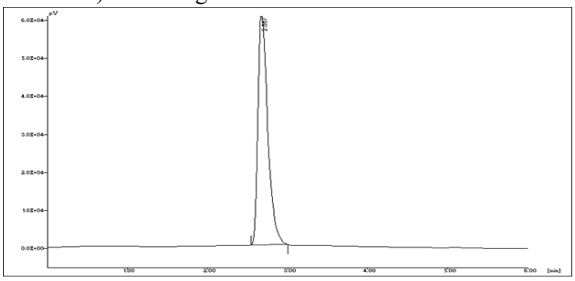
Chromatogram of run 7

Results of run 7

No. of theoretical plates	Retention time	Tailing factor
2546	2.24	1.54

*Mean of three readings

Run 8: I) Mobile Phase: ACN: Water (50:50)
II) Flow rate: 1 ml/min
III) Wavelength: 239nm



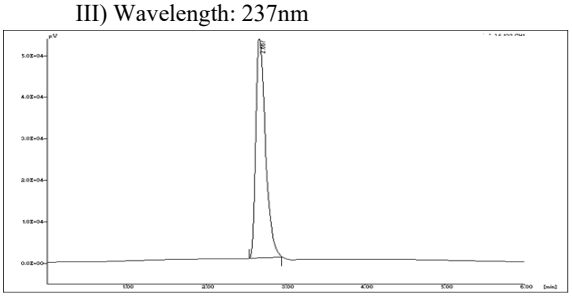
Chromatogram of run 8

Results of run 8

No. of theoretical plates	Retention time	Tailing factor
2670	2.66	1.54

*Mean of three readings

Run 9: I) Mobile Phase: ACN: Water (60:40)
II) Flow rate: 1 ml/min
III) Wavelength: 237nm



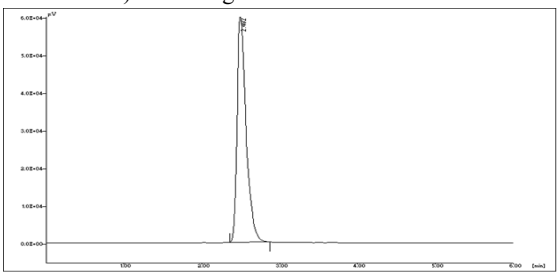
Chromatogram of run 9

Results of run 9

No. of theoretical plates	Retention time	Tailing factor
2806	2.66	1.43

*Mean of three readings

Run 10: I) Mobile Phase: ACN: Water (50:80)
II) Flow rate: 1.2ml/min
III) Wavelength: 241nm



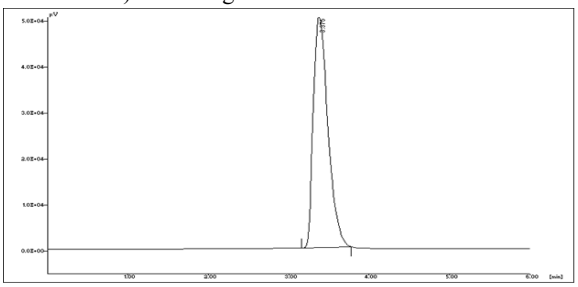
Chromatogram of run 10

Results of run 10

No. of theoretical plates	Retention time	Tailing factor
3045	2.49	1.64

*Mean of three readings

Run 11: I) Mobile Phase: ACN: Water (40:40)
II) Flow rate: 0.8ml/min
III) Wavelength: 239nm



Chromatogram of run 11

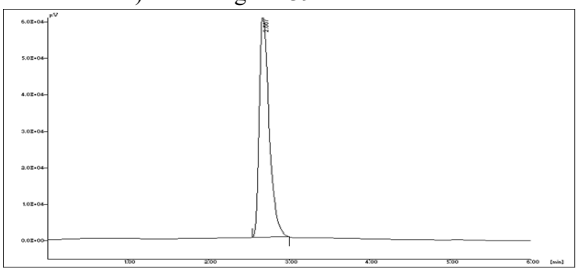
Result of run 11

No. of theoretical plates	Retention time	Tailing factor

2694	3.37	1.51

*Mean of three readings

Run 12: I) Mobile Phase: ACN: Water (50:50)
II) Flow rate: 1 ml/min
III) Wavelength: 239nm



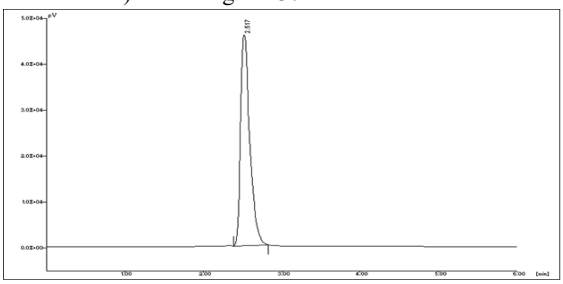
Chromatogram of run 12

Results of run 12

No. of theoretical plates	Retention time	Tailing factor
2789	2.67	1.55

*Mean of three readings

Run 13: I) Mobile Phase: ACN: Water (50:70)
II) Flow rate: 1.2ml/min
III) Wavelength: 237nm



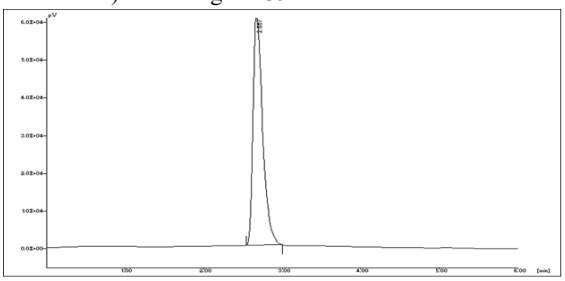
Chromatogram of run 13

Results of run 13

No. of theoretical plates	Retention time	Tailing factor
2816	2.51	1.44

*Mean of three readings

Run 14: I) Mobile Phase: ACN: Water (50:50)
II) Flow rate: 1 ml/min
III) Wavelength: 239nm

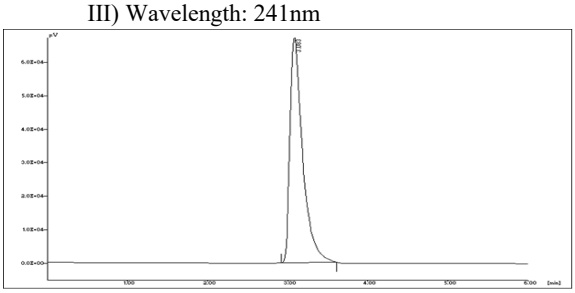


Chromatogram of run 14

Results of run 14

No. of theoretical plates	Retention time	Tailing factor
2789	2.67	1.52

Run 15: I) Mobile Phase: ACN: Water (50:30)
II) Flow rate: 0.8ml/min
III) Wavelength: 241nm



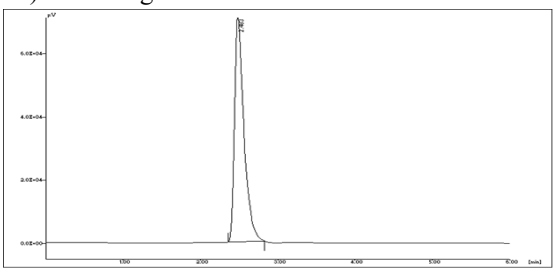
Chromatogram of run 15

Results of run 15

No. of theoretical plates	Retention time	Tailing factor
2456	3.08	1.61

*Mean of three readings

Run 16: I) Mobile Phase: ACN: Water (40:60)
II) Flow rate: 1 ml/min
III) Wavelength: 241nm



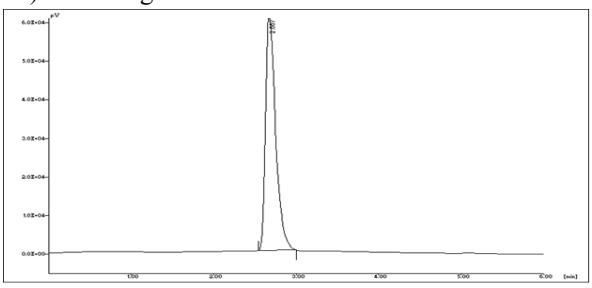
Chromatogram of run 16

Results of run 16

No. of theoretical plates	Retention time	Tailing factor
2654	2.48	1.63

*Mean of three readings

Run 17: I) Mobile Phase: ACN: Water (50:50)
II) Flow rate: 1ml/min
III) Wavelength: 239nm



Chromatogram of run 17

Results of run 17

No. of theoretical plates	Retention time	Tailing factor
2685	2.67	1.54

*Mean of three readings

RESULTS FOR THEORETICAL PLATES OF DOE:

A) ANALYSIS OF VARIANCE (ANOVA) FOR THE THEORETICAL PLATES RESPONSE AS DEPENDENT VARIABLES:

The analysis of variance (ANOVA) was performed to identify the significant and insignificant factors. The results of ANOVA for the theoretical plates of DoE were as follows:

ANOVA for theoretical plates

Source	Sum of squares	df	Mean square	F-value	p-value	
Model	4.061	9	4.512	2.97	0.0824	Not significant
A-Methanol conc.	6.982	1	6.982	4.60	0.0691	
B-Flow rate	1.328	1	1.328	0.8754	0.3806	
C-Wavelength	84750	1	84750	0.5585	0.4792	
AB	4.362	1	4.362	2.87	0.1338	
AC	1.962	1	1.962	1.29	0.2929	
BC	3.792	1	3.792	2.50	0.1579	
A^2	5.630	1	5.630	3.71	0.0954	
B^2	1.521	1	1.521	1.00	0.3501	
C^2	1.391	1	1.391	9.17	0.0192	
Residual	1.062	1	1.517			
Lack of fit	1.062	3	3.541			Significant
Pure error	0.0000	4	0.0000			
Cor total	5.123	14				

The Model F-value of 1.25 implies the model is not significant relative to the noise. There is a 39.24% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case B is a significant model term. Values greater than 0.1000 indicate the model terms are not significant.

B) MODEL ASSESMENT FOR THE THEORETICAL PLATES RESPONSE AS DEPENDENT VARIABLES:

After entering the data in Design Expert Software, fit summary applied to data after which "quadratic model" was suggested by the software. According to this model following polynomial equation was obtained. Polynomial equation in coded terms:

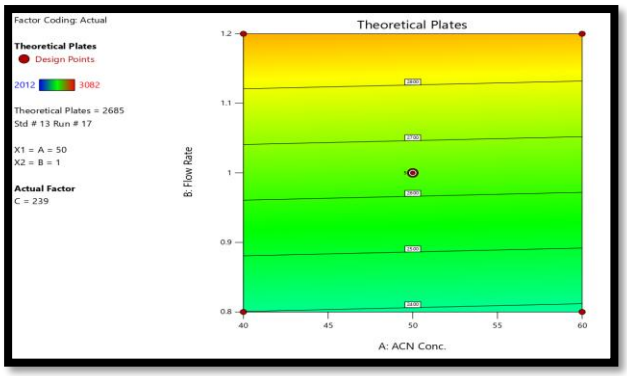
FINAL EQUATION IN TERMS OF CODED FACTOR:

TNP =

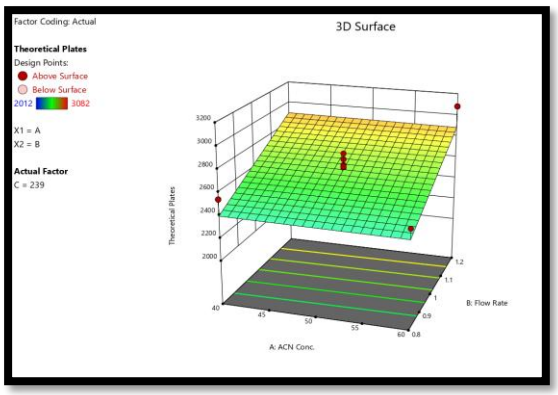
$+2708.00-7.00A+249.87B+8.13C+151.00AB-76.00AC-53.75BC-14.37A^2-31.12B^2-94.63C^2$

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high level of factor were coded as +1 and the low level of the factors were coded as -1. The coded equation was useful for identifying the relative impact of the factors by compairing the factor coefficients.

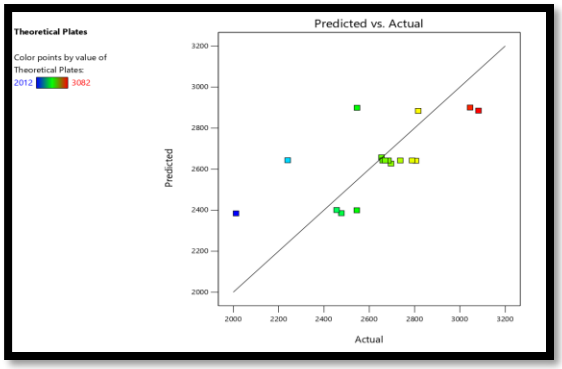
C) GRAPHICAL PRESENTATION:



Contour plot revealed ACN Conc. and flow rate effect on theoretical plates.



Effect of ACN and flow rate on theoretical plate



Predicted V/S Actual for DOE of theoretical plates

Practical data obtained for trials approximately matched

with the data provided by software which showed the authenticity of the software.

The responses obtained after carrying out trial runs were entered back to DOE software and counter plot, 3D graph and prediction graph of theoretical plates were plotted as shown in fig. 31, 32, 33.

Counter plot, 3D graph and prediction graph displayed the relationship in two dimensions with methanol conc. and flow rate plotted on x and y scales and response value represented by counters. A counter plot was like a topographical map in which methanol conc. and flow rate values were plotted instead of longitude, latitude and elevation.

RESULTS FOR RETENTION TIME OF DOE:

A) ANALYSIS OF VARIANCE (ANOVA) FOR THE RETENTION TIME RESPONSE AS DEPENDENT VARIABLES:

The analysis of variance (ANOVA) was performed to identify the significant and insignificant factors. The results of ANOVA for the retention time of DoE were as follows:

ANOVA for retention time

Source	Sum of squares	Df	Mean square	F-value	p-value	
Model	1.48	9	0.1645	6.08	0.0133	Significant
A-ACN Conc.	0.0036	1	0.0036	0.1335	0.7256	
B-Flow rate	1.22	1	1.22	45.26	0.0003	
C-Wavelength	0.0760	1	0.0760	2.81	0.1375	
AB	0.0225	1	0.0225	0.8316	0.3921	
AC	0.0380	1	0.0380	1.41	0.2745	
BC	0.000	1	0.000	0.0009	0.9766	
A^2	0.0047	1	0.0047	0.1746	0.6885	
B^2	1.1058	1	1.1058	3.91	0.0885	
C^2	0.0049	1	0.0049	0.1799	0.6842	
Residual	0.1894	7	0.0271			
Lack of fit	0.1893	3	0.0631	3155.45	<0.0001	Significant
Pure error	0.0001	4	0.0000			
Cor Total	1.67	16				

The Model F-value of 6.08 implies the model is significant. There is only a 1.33% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case B is a significant model term. Values greater than 0.1000 indicate the model terms are not significant.

B) MODEL ASSESSMENT FOR THE RETENTION TIME RESPONSE AS DEPENDENT VARIABLES:

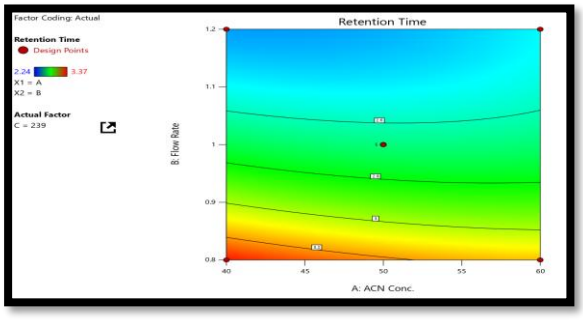
After entering the data in Design Expert Software, fit summary applied to data after which “quadratic model” was suggested by the software. According to this model following polynomial equation was obtained. Polynomial equation in coded terms:

Final equation in terms of coded factor:

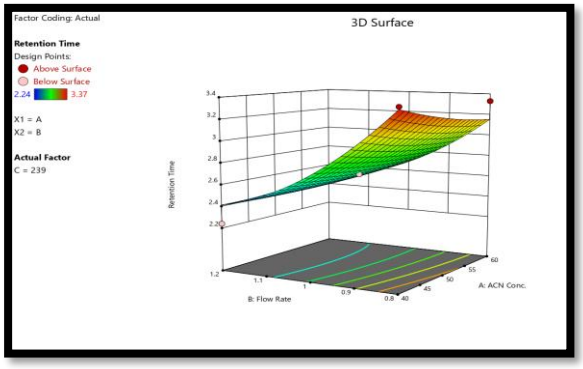
$RT=+2.67-0.0212A-0.3912B-0.0975C+0.0750AB+0.0975AC-0.0025BC+0.0335A^2+0.1585B^2-0.0340C^2$

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high level of factor was coded as +1 and the low level of the factors were coded as -1. The coded equation was useful for identifying the relative impact of the factors by comparing the factor coefficients.

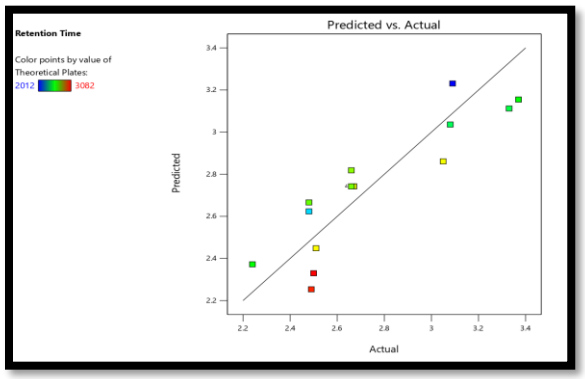
C) GRAPHICAL PRESENTATION:



Contour plot demonstrated methanol conc. And flow rate effect on retention time



Effect of ACN concentration and flow rate on Rt



Predicted V/S Actual for DOE of retention time

Practical data obtained for trials approximately matched with the data provided by software which showed the authenticity of the software.

The responses obtained after carrying out trial runs were entered back to DOE software and counter plot, 3D graph and prediction graph of theoretical retention time were plotted as shown in fig. 34, 35, 36.

Counter plot, 3D graph and prediction graph displayed the relationship in two dimensions with methanol conc. and flow rate plotted on x and y scales and response value represented by counters. A counter plot was like a topographical map in which methanol conc. and flow rate values are plotted instead of longitude, latitude and elevation.

RESULTS FOR TAILING FACTOR OF DOE:

A) ANALYSIS OF VARIANCE (ANOVA) FOR THE TAILING FACTOR RESPONSE AS DEPENDENT VARIABLES:

The analysis of variance (ANOVA) was performed to identify the significant and insignificant factors. The result of ANOVA for the tailing factor of DoE were as following:

Table 31: ANOVA for tailing factor

Source	Sum of squares	df	Mean square	F-value	p-value	
Model	0.0719	9	0.0080	1.56	0.0088	Significant
A-ACN conc.	0.0002	1	0.0002	0.3297	0.6827	
B-Flow rate	0.0015	1	0.0015	0.3297	0.2862	
C-Wavelength	0.0595	1	0.0595	0.1077	0.0002	
AB	0.0001	1	0.0001	1.63	0.7752	
AC	0.0049	1	0.0049	0.2153	0.0763	
BC	0.0000	1	0.0000	4.36	0.8862	
A^2	0.0003	1	0.0003	0.0625	0.6307	
B^2	0.0016	1	0.0016	6.79	0.2794	
C^2	0.0040	1	0.0040	0.3484	0.1032	
Residual	0.0079	7	0.0011			
Lack of Fit	0.0072	3	0.0024	13.38	0.0149	Significant
Pure Error	0.0007	4	0.0002			
Cor Total	0.0798	16				

The Model F-value of 7.04 implies the model is significant. There is only a 0.88% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case C is a significant model term. Values greater than 0.1000 indicate the model terms are not significant.

The Lack of Fit F-value of 13.38 implies the Lack of Fit is significant. There is only a 1.49% chance that a Lack of Fit F-value this large could occur due to noise. Significant lack of fit is bad -- we want the model to fit.

B) MODEL ASSESSMENT FOR THE TAILING FACTOR RESPONSE AS DEPENDENT VARIABLES:

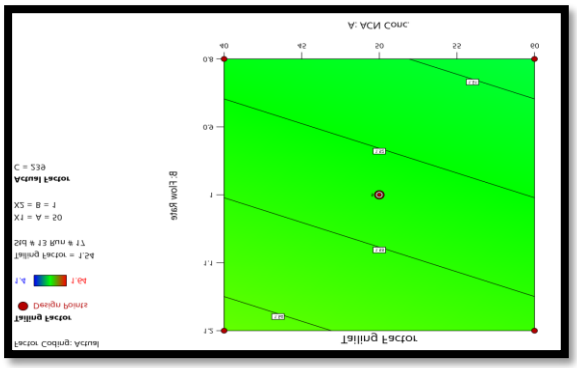
After entering the data in Design Expert Software, fit summary applied to data after which “quadratic model” was suggested by the software. According to this model following polynomial equation was obtained. Polynomial equation in coded terms:

Final equation in terms of coded factor Tailing factor:

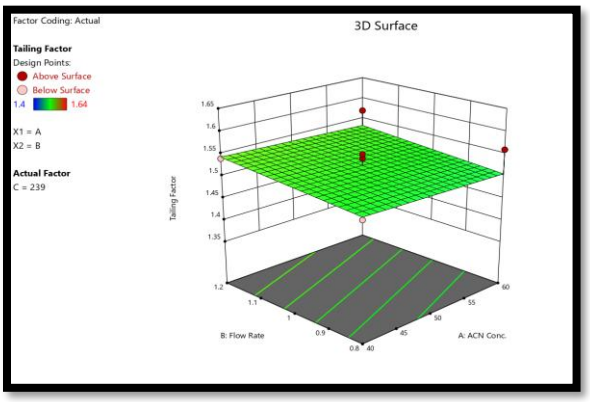
$1.53-0.0050A+0.0138B-0.0863C-0.00050AB-0.0350AC-0.0025BC-0.0083A^2+0.0193B^2-0.0308C^2$

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high level of factor were coded as +1 and the low level of the factors were coded as -1. The coded equation was useful for identifying the relative impact of the factors by comparing the factor coefficients.

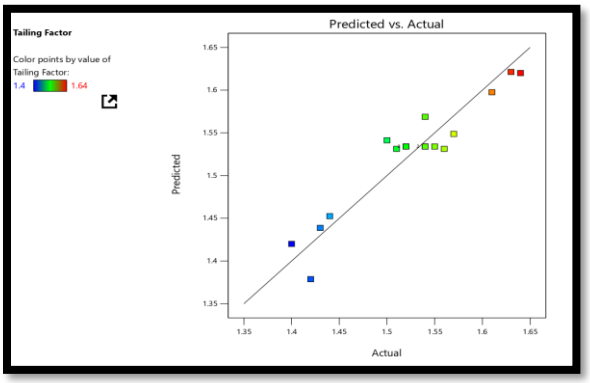
C) GRAPHICAL PRESENTATION:



Contour plot revealed ACN Conc. and flow rate effect on tailing factor



Effect of ACN concentration and flow rate on tailing factor



Predicted V/S Actual for DOE of tailing factor

Practical data obtained for trials approximately matches with the data provided by software which showed the authenticity of the software.

The responses obtained after carrying out trial runs were entered back to DOE software and counter plot, 3D graph and prediction graph of theoretical tailing factor were plotted as shown in fig. 37, 38, 39.

Counter plot, 3D graph and prediction graph displayed the relationship in two dimensions with methanol conc. and flow rate plotted on x and y scales and response value represented by counters. A counter plot was like a topographical map in which methanol conc. and flow rate values are plotted instead of longitude, latitude and elevation.

QUANTIFICATION OF ONDANSETRON HYDROCHLORIDE TABLET BY DEVELOPED METHOD

ASSAY OF ONDANSETRON HYDROCHLORIDE TABLET

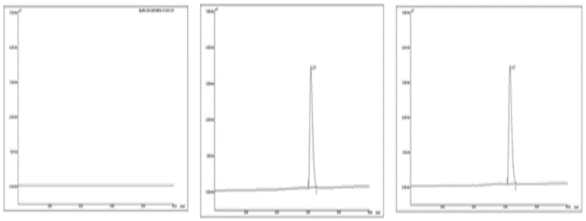
Table 32: Result of Quantification of Ondansetron Hydrochloride tablet

Sr.No.	Peak area std.	Peak area sample	Amount found (µg/ml)	% Assay
1	206562	206675	8.003	100.05
2	206523	206650	8.004	100.06
3	206689	206611	7.998	99.96
4	206626	206798	8.006	100.08
5	206713	206853	8.004	100.06
6	206491	206690	8.007	100.09
Mean	206700	206712	8.0025	100.05
SD (±)	82.278	84.823	0.0057	0.4243
%RSD	0.039	0.041	0.071	0.4240

The concentration of the Ondansetron Hydrochloride tablet was calculated from the regression equation. The percent content of the Ondansetron Hydrochloride tablet was observed as per the standard limit of 90-110 %w/w. It was an acceptable limit.

METHOD VALIDATION

SPECIFICITY:



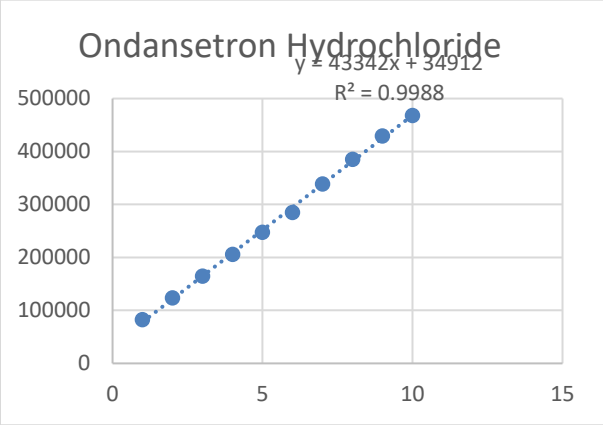
A) Chromatogram of blank sol. B) Chromatogram of std. sol. c) Chromatogram of sample sol

The chromatogram of blank, standard and sample revealed that there was no interference due to mobile phase components and excipients.

LINEARITY:

Linearity study

Sr.No.	Conc. (µg/ml)	Peak area 1	Peak area 2	Peak area 3	Average Peak area	SD (±)	%RSD
1	2	82501	82691	82457	82549.67	101.53	0.122
2	4	123690	123712	123790	123730.7	42.90	0.034
3	6	165231	165173	165270	165224.7	39.85	0.024
4	8	206051	206158	206199	206136	62.39	0.030
5	10	247478	247491	247593	247520.7	51.42	0.021
6	12	285311	285221	285232	285254.7	40.08	0.014
7	14	338886	338678	338751	338771.7	86.16	0.024
8	16	387651	387796	387751	387732.7	60.59	0.015
9	18	429835	429893	429821	429843	31.16	0.007
10	20	468146	468196	468110	468146	36.23	0.007
Regression equation			Y= 43342 x + 34912				
Slope			43342				
Intercept			34912				
R^2			0.9988				



Calibration curve of Ondansetron Hydrochloride

Calibration curve of Ondansetron Hydrochloride revealed that peak area increase linearly as concentration increased from 2-20 µg/ml with correlation coefficient of 0.9998. The analytical range was found from 2-20µg/ml. The method linearity was established.

SYSTEM PRECISION:

Table 34: For system precision

Sr. No.	The peak area of Ondansetron Hydrochloride std. (8µg/ml)
1	206523
2	206713
3	206491
4	206562
5	206626
6	206689
Mean	206600.7
SD (±)	82.2731
%RSD	0.2275
Acceptance criteria	%RSD ≤2

System precision study revealed that standard deviation and %RSD value were well within the limits.

METHOD PRECISION:

For method precision

Sr. No.	Concentration (µg/ml)	% Assay
1	8.004	100.06
2	8.004	100.06
3	8.007	100.09
4	8.004	100.05
5	8.006	100.08
6	7.996	99.96
Mean	8.0025	100.05
SD (±)	0.0057	0.0424
%RSD	0.0712	0.4237
Acceptance Criteria	%RSD ≤ 2	

Method precision study revealed the standard deviation and %RSD value were well within the limits.

INTERMEDIATE PRECISION:

A) INTRADAY PRECISION:

For intraday precision

Conc. of Ondansetron Hcl (µg/ml)	Time Interval	*Conc. (µg/ml)	#% Assay	SD (±)	%RSD
4	At 0 hr.	4.001	100	0.05	0.05
	After 4 hr.	3.998	99.96		
	After 8 hr.	4.002	100.07		
8	At 0 hr.	8.035	100.44	0.03	0.029
	After 4 hr.	8.041	100.52		
	After 8 hr.	8.037	100.47		
10	At 0 hr.	11.914	99.29	0.002	0.002
	After 4 hr.	11.906	99.22		
	After 8 hr.	11.914	99.26		

* Mean of three conc.

Mean of three %assay

B) INTERDAY PRECISION:

Table 37: For interday precision

Conc. Of Ondansetron Hcl (µg/ml)	Day	*Conc. (µg/ml)	#% Assay	SD (±)	%RSD
4	Day 1	4.001	100	0.021	0.02
	Day 2	4.004	100.01		
	Day 3	4.002	100		
8	Day 1	8.036	100.8	0.020	0.199
	Day 2	8.039	99.77		
	Day 3	8.040	101.8		
12	Day 1	50.22	100.4	0.00125	0.0012
	Day 2	49.86	99.72		
	Day 3	50.75	101.5		

* Mean of three conc.

Mean of three % assay

Precision was established by conducting system precision, method precision, and intermediate precision methods. The results for the same were well within the acceptance limit.

ACCURACY:

Table 38: For accuracy study

Level of Recovery	Amt. of drug From sample (µg/ml)	Amt. of Std. added (API) (µg/ml)	Total Amt of (µg/ml)	Area of Spiked sample A	Area of unspiked sample B	Area of std. C	Amt. Recovered (µg/ml)
80 %	8	6.4	14.4	375679	204142	171537	14.4
	8	6.4	14.4	375649	204141	171508	14.4
	8	6.4	14.4	375735	204141	171594	14.4
100 %	8	8	16	408282	204141	204141	16.01
	8	8	16	408314	204142	204172	16.01
	8	8	16	408301	204141	204160	16.01
120 %	8	9.6	17.6	434377	204141	230235	17.6
	8	9.6	17.6	434501	204141	230359	17.59
	8	9.6	17.6	434800	204141	230658	17.58

Table 39: For statistical evaluation of the recovery study

Level of recovery	% Recovery	Avg. of % recovery	SD (±)	% RSD
80%	100.001	99.97	0.03346	0.0337
	99.93			
	100.001			
100%	100.12	100.12	0.00471	0.0470
	100.13			
	100.12			
120%	100.01	99.89	0.08993	0.0900
	99.89			
	99.79			
Acceptance Criteria: % Recovery 98- 102 % and %RSD ≤ 2				

The results of the recovery study showed that the range for % recovery from 99.99-101%. The recovery study revealed that the %RSD value was within the acceptance criteria. It indicated that the analytical method was accurate.

ROBUSTNESS:

A) BY CHANGE IN FLOW RATE

Table 40: Robustness study: Flow rate

B) BY CHANGE IN WAVELENGTH:

Table 41: Robustness study- Wavelength

Robustness Parameter		RT	Tailing factor	Peak area	Theoretical plate
Change in wavelength (nm)	237	2.28	1.61	206954	2916
		2.27	1.54	206821	2907
		2.27	1.59	206914	2966
	239	2.24	1.62	206851	3005
		2.24	1.61	206952	2994
		2.25	1.55	206906	2954
	241	2.26	1.58	206894	3041
		2.26	1.57	206975	3002
		2.26	1.59	206927	3068
Mean		2.26	1.58	206932	3037
SD		0.012867	0.025906	47.51655	53.23533
%RSD		1.63	1.63	0.022	1.752
Acceptance criteria: %RSD ≤ 2					

The robustness of proposed analytical method was performing by deliberate changes in flow rate and wavelength. No deviation was observed in the results. % RSD values were well within the limits, which gave significant indication of its reliability during normal usage.

RUGGEDNESS:

Table 42: Ruggedness

Parameter	Conc. (µg/ml)	Peak area	Mean	SD (±)	%RSD
Analyst 1	8	207105	207143	31.874	0.015
		207183			
		207141			
Analyst 2	8	207145	207144.7	33.885	0.016
		207103			
		207144.7			
Acceptance Criteria: %RSD ≤ 2					

The %RSD was found well within acceptable limits < 2%RSD percentage.

LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTITATION (LOQ)

Table 43: Result of LOD and LOQ

Drug	LOD	LOQ
Ondansetron Hydrochloride	0.493 µg/ml	0.495 µg/ml

The LOD & LOQ for Ondansetron Hydrochloride were found to be 3.02359 µg/ml & 9.16238 µg/ml. These values show the method is suitable for determining the lower concentration & confirm the sensitivity of the proposed method.

II. CONCLUSION

A robust and efficient RP-HPLC method for the estimation of Ondansetron Hydrochloride was successfully developed using a Quality by Design approach. The method utilized optimized chromatographic conditions that enabled rapid separation and efficient analysis. Critical method variables, including mobile phase composition, flow rate, and detection wavelength, were systematically optimized using Design Expert software, leading to the establishment of a reliable Method Operable Design Region (MODR).

The developed method was validated according to ICH guidelines and demonstrated satisfactory linearity, accuracy, precision, sensitivity, robustness, and ruggedness. System suitability parameters confirmed the reliability of the chromatographic system. Furthermore, the method offered advantages such as reduced solvent consumption, shorter analysis time, and cost-effectiveness, contributing to its environmental friendliness.

Thus, the proposed RP-HPLC method is simple, selective, and suitable for routine analysis of Ondansetron Hydrochloride in pharmaceutical research and quality control laboratories.

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