Section A-Research paper
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Analytical Quality-by-Design Approach to Stability Indicating RP-HPLC Method Development and Validation for Estimation of Umifenovir in Bulk and Formulation

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ABSTRACT

Method: A simple, specific, accurate, precise and selective stability-indicating reverse phase high performance liquid chromatography method was developed for estimation of Umifenovir in bulk and formulation by analytical quality-by-design paradigm. Cosmosil C-18 column (250 x 4.6 mm x 5µ) at ambient temperature and UV detector at 258 nm wavelength was employed. The mobile phase was methanol:water (65:35 % v/v) adjusted to pH 3.0 and flowrate 0.8 ml/min. The method involved varying three key parameters (composition of mobile phase, flow rate and pH) and evaluating their effects on the responses. Box-Behnken design was employed for method development and optimized using statistical software. ICH guidelines were followed for method validation as well as forced degradation study. The stability of drug in stress conditions of acid/base hydrolysis, oxidation, thermal and photolytic degradation was evaluated.

Results: A linear response was observed over the concentration range of 10 – 50 µg/mL with r^2=0.9992. Limit of detection (LOD) and limit of quantitation (LOQ) for umifenovir were 0.04 µg/mL and 0.13 µg/mL respectively. The developed method was found to be highly robust and efficient. Stability studies showed some degradation in peroxide, alkali and acid, with only minor degradation in heat and photolytic conditions.

Conclusion: The proposed stability indicating analytical QbD method can be used for routine analysis of umifenovir active pharmaceutical ingredient (API) and formulation in quality control laboratories. The method developed by analytical quality-by-design approach is a highly robust, efficient and offers the added advantages of QbD with enhanced quality.

Keywords: Quality-by-Design (QbD), Reverse Phase High Performance Liquid Chromatography (RP-HPLC), Umifenovir, Box-Behnken Design (BBD), Forced degradation.

INTRODUCTION

Quality-by-design is a holistic approach where product specifications, critical process parameters (CPP) and critical quality attributes (CQA) are included along with risk assesment and creation of a robust design space to build quality into the analytical process and drug product. This serves to ease the final approval and ongoing quality control of the drug [1]. In relation to analytical systems, it is termed Analytical Quality-by-Design or AQbD [2-5].

Since QbD technique implements the lifecycle concept for analytical methods and offers the advantage of regulatory flexibility for operation within the design space, it has become an area of interest for many pharmaceutical companies and research institutes [6-9].

The pandemic of Corona was witnessed globally in the year 2020 and 2021. Lakhs of people lost their lives due to the devastating effects of covid-19 virus on human health. Doctors were in dire need of antiviral drugs for treatment of the deadly virus [10]. Umifenovir (UMF), an antiviral drug, approved in Russia and China for the prophylaxis and treatment of influenza, was being tested for the treatment of Covid-19 virus infection. It is 6-bromo-4-[(dimethylamino)methyl]-5-hydroxy-1-methyl-2-[(phenylthio)methyl]-1H-indole-3-carboxylic acid, ethyl ester with molecular formula $C_{22}H_{25}BrN_2O_3S$ and molecular weight 513.9. Umifenovir's ability to bind to the haemagglutinin (HA), a glycoprotein found on the surface of the influenza virus. Once umifenovir binds to the HA protein, this glycoprotein is prevented from binding to sialic acid, present on the surface of the target cells, so the virus cannot penetrate the host cell. The drug also inhibits SARS-CoV-2 infection, wherein it blocks viral entry by preventing viral attachment and release [11]. Its chemical structure is given in figure-1.

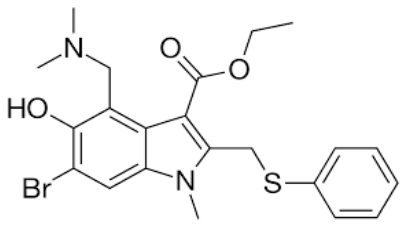


Fig. 1: Structure of Umifenovir

High Performance Liquid Chromatography (HPLC), especially, reverse phase HPLC (RP-HPLC) is the most popular and widely used analytical technique in the pharmaceutical industry. Its quality has gained huge importance with a QbD approach.

Literature reports a bioanalytical UPLC-MS/MS method, very few analytical HPLC methods and an HPTLC method for determination of Umifenovir [12-15].

It is evident from literature that no analytical HPLC methods are available for routine analysis of Umifenovir in pharmaceutical formulations developed through the AQbD approach. The mobile phase systems used in reported methods are also quite complex, expensive and not eco-friendly.

Over the past few years, literature reports have successfully demonstrated the immense applicability of DoE approach for developing effective and economic LC methods for estimation of pharmaceuticals [16].

Forced degradation study (FDS) is a necessary and integral part of drug development. It typically indicates stability of the drug/drug product by subjecting it to various stress conditions as outlined in the ICH Q1A (R2) guidelines. A stability-indicating analytical method should be capable of detecting degradation products and the assay method should be capable of detecting any decrease in drug content, during the product's shelf life [17].

There are very few stability indicating analytical HPLC methods for estimation of Umifenovir in bulk and dosage form. However, not all parameters of stability studies are evaluated [18, 19]. The reported stability-indicating methods are also not focused on any risk assessment, DoE and robustness of the method. Hence, there was a need to develop a simple, rapid, robust, precise, selective and cost-effective stability-indicating RP-HPLC method for determination of Umifenovir in bulk and formulation through the AQbD approach.

MATERIALS AND METHODS

Chemicals and Reagents:

Umifenovir was procured as a gift sample from Glenmark Pharmaceuticals Pvt. Ltd. Chemicals and reagents used were of analytical grade and solvents used were of HPLC grade.

Instruments:

HPLC system of Analytical Technologies Ltd. with UV-3000-M detector and HPLC Workstation software was used.

Chromatographic conditions:

The Column employed was Cosmosil C18 (250 x 4.6 mm id., particle size: 5µ) at ambient temperature and 258 nm wavelength. The mobile phase was methanol:water (65:35) adjusted to pH 3.0 with orthophosphoric acid (OPA), flow rate was 0.8 ml/min. Injection volume was 20 µl.

Preparation of standard solutions:

Buffer (0.1% OPA)

About 1 ml of orthophosphoric acid solution was added in a 1000 ml of volumetric flask, about 100 ml of milli-Q water was added to it and final volume was made up to 1000 ml with milli-Q water. This was used to adjust the pH of the mobile phase to 3.0.

Standard preparation

Accurately weighed and transferred 25 mg of umifenovir standard into a 25 ml clean dry volumetric flask and three-fourth volume of diluent was added to it. This was then sonicated for 5 min, and made up to the final volume with diluent. The concentration of the solution was 1000 µg/ml. From this stock solution, working standard of 100 µg/ml was prepared.

HPLC method development by analytical QbD approach

The method employed is as follows.

Defining the analytical target profile (ATP)

This includes the goals or targets set according to the intended use of the analytical method. For example, quantitative analysis of API, degradation products, etc. [20]. Thus, ATP of present research was estimation of Umifenovir drug, formulation and forced degradation/stress study for assessing its stability.

Risk assessment

Risk assessment was performed for identification of critical quality attributes (CQAs, dependent variables) and critical process parameters (CPPs, independent variables). The CQAs like resolution, peak asymmetry, theoretical plates, area, retention time etc. and CPPs like flow rate, wavelength, column, ratio of mobile phase, pH etc. are crucial method parameters for quality on which the method performance rests [21].

The CPPs for the present method were identified to be ratio of mobile phase, flow rate and pH for a 3-variable Box-Behnken design (BBD), whereas the CQAs were identified to be peak asymmetry, theoretical plates, area, retention time and noted as quadratic responses of BBD.

Optimization of chromatographic conditions and design of experiment

Umifenovir maximum absorbance wavelength was selected at 258 nm by scanning the UV range of 200–400 nm using 30 µg/ml standard solution of the drug. The elution was carried on Cosmosil C18 column at ambient temperature. This column gave a peak shape with good system suitability parameters. The UV detector was used to detect umifenovir. The QbD method was optimized for three different parameters i.e. composition of mobile phase, pH and flow rate, using the design provided by the software. The Box-Behnken design was selected and 17 chromatographic runs were conducted as per DoE design. The responses were recorded under 4 headings of area, retention time, peak asymmetry and theoretical plates. The optimized chromatographic conditions were selected from the desirability indicated by the software.

Design software

Design Expert 10 software (free trial version) was used to plan the experiment's design.

Method validation

The method was ensured to be fit for its intended purpose by validating it as per ICH Q2 (R1) guideline. The parameters tested were linearity, accuracy, percentage recovery, precision, limit of detection (LOD), limit of quantitation (LOQ), assay and robustness [22].

Linearity

Pipette 1, 2, 3, 4, 5 ml of working standard solution into 10 ml volumetric flask and make volume upto the mark with diluent. The concentration of umifenovir in the prepared solution

was 10, 20, 30, 40 and 50 µg/ml. A calibration curve was plotted between concentrations versus peak area.

Precision

A solution of concentration 30 µg/ml was tested for intraday and interday precision. For intraday precision, the solutions were injected in the morning and evening in triplicate. Interday precision was calculated by analysing the solution on two different days in triplicate. The corresponding areas and the % relative standard deviation (RSD) was found.

Accuracy

As per ICH guidelines, accuracy is determined using a minimum of nine determinations over a minimum of three concentration levels covering the specified range of concentration (e.g., three concentrations/three replicates each). Three standards were defined as 10, 30 and 50 µg/ml, from the calibration range. Accuracy should be reported in terms of percent recovery by assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value.

Percentage Recovery

The percentage recovery was determined by calculating recovery of the drug by standard addition method. Known amount of sample (20 µg/ml) was added to standard solutions at 50, 100, 150 % recovery levels (10, 20, 30 µg/ml respectively) in triplicate and the mean area for each reading was calculated. The percentage recovery at every level was found.

Robustness

The robustness of the method was assessed by introducing small intentional variations in the method parameters of wavelength and pH of the mobile phase. The reliability of the method was ensured by evaluating its robustness.

LOD and LOQ

The limit of detection of an analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantified and when quantitated, it is termed as limit of quantitation.

Assay

A total of 20 tablets of umifenovir were taken and ground finely. Assay is reported as percent purity. Solutions of concentration 30 ppm were prepared from formulation as well as standard and injected to record the corresponding areas.

Standard preparation for degradation studies

A standard solution of 50 µg/ml concentration was treated with acid, base, hydrogen peroxide, heat and UV light individually. After degradation, the solutions were injected into LC system.

Forced degradation

Stress degradation studies of umifenovir were carried out under hydrolysis (acid and base), oxidation, thermal and photolytic conditions. The drug was treated with 0.1N HCl for 2 hours at 60°C to perform acid degradation, 0.1N NaOH for 2 hours at 60°C for base degradation and 3 % H_2O_2 for 6, 24 hours at room temperature (R.T.) for peroxide degradation. Standard drug solution was treated thermally at a temperature of 60°C for 24 hours to study degradation by heat and also treated photolytically at R.T. for 24 hours.

RESULTS AND DISCUSSION

Optimization of HPLC method by analytical QbD approach

Trials were performed for optimizing the mobile phase composition by varying the mobile phase ratio of methanol:water at 80:20, 60:40, 70:30, 65:35 and flow rate at 0.8, 0.9 ml/min. The initial chromatographic conditions employed are listed in table 1. The levels used for Box-Behnken experimental design and its layout for 17 QbD trials are given in table 2 and 3 respectively.

Table 1. Initial chromatographic method development conditions

Trial No.	Wavelength (nm)	Mobile phase composition (% Methanol: Water)	pH of mobile phase	Sample volume (µl)	Flow rate (ml/min)	Pressure (MPa)	Run time (min)
1	258	80:20	3.0	20	0.8	9-10	6.8
2	258	60:40	3.0	20	0.8	9-10	13.26
3	258	70:30	3.0	20	0.8	9-10	7.16
4	258	65:35	3.0	20	0.8	9-10	9.41
5	258	65:35	3.0	20	0.9	9-10	10.05

Table 2. Box-Behnken experimental design

Chromatographic Condition	Levels Used		
	Low (-)	Center (0)	High (+)
% Composition	60	65	70
Flow rate (ml/min)	0.8	0.9	1
pH (units)	2.5	3.0	3.5

Table 3. Design of experiment showing factors and responses for 17 QbD trials using BBD

Run No.	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3	Response 4
	A:Composition of mobile phase	B:Flow rate	C:pH	Retention Time (Rt)	Area	Theoretical Plates (N)	Asymmetry Factor

	(%)	(ml/min)	(Units)	(min)	(Area Unit)	(Units)	(Units)
1	70	1	3	3.045	596186	7166	1.04
2	60	1	3	6.493	640155	7314	1.29
3	60	0.9	3.5	7.066	722517	7317	1.27
4	65	0.9	3	4.228	615920	7973	1.07
5	65	0.9	3	4.228	615920	7973	1.07
6	65	0.9	3	4.228	615920	7973	1.07
7	60	0.9	2.5	6.897	668119	7255	1.3
8	70	0.9	3.5	3.481	687363	7192	1.35
9	65	1	3.5	3.848	574792	8130	1.17
10	65	0.9	3	4.228	615920	7973	1.07
11	70	0.9	2.5	2.942	718436	6631	1.01
12	60	0.8	3	7.571	770913	7238	1.25
13	70	0.8	3	3.318	849214	7486	1.17
14	65	1	2.5	3.817	571699	7068	1.17
15	65	0.9	.3	4.228	615920	7973	1.07
16	65	0.8	2.5	4.677	652116	7982	1.02
17	65	0.8	3.5	4.672	698852	8196	1.09

Statistical analysis of method responses for peak asymmetry

Box-Behnken multifactor response surface quadratic model was adopted for the USP tailing factor of peak. The data was statistically analysed by analysis of variance (ANOVA) to evaluate the significance of the variables and interaction effects on the peak asymmetry response. The independent variables of the BBD selected are % composition of mobile phase, flowrate and pH. The statistical values provided by the software generated report are given in table 4.

The model F-value of 6.0585 and p-value less than 0.05 indicated that model terms were significant for optimization. The significant factors found were % composition (p=0.0111), pH (p=0.0469), interaction effects of % composition x pH (p=0.0129) and % composition x % composition (p=0.0033). A positive relationship of these factors and their interaction effects on tailing of peaks could be predicted. “Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 7.476 indicated an adequate signal. This model fit well for optimization. The effect of factors on responses and their interaction effects could be further studied from the 3-dimensional response surface and contour plots. The 3D response surface and contour plots could be utilized for navigation of design space (method operable design region) in development of a robust AQbD method.

Table 4. ANOVA table (partial sum of squares) for peak asymmetry response

Source	Sum of Squares	Df	Mean Square	F Value	p-value	
Model	0.169812	9	0.018868	6.058524	0.0134	Significant
A -	0.03645	1	0.03645	11.70413	0.0111	Significant

Composition						
B - Flowrate	0.00245	1	0.00245	0.786697	0.4045	
C - pH	0.01805	1	0.01805	5.795872	0.0469	Significant
AB	0.007225	1	0.007225	2.319954	0.1715	
AC	0.034225	1	0.034225	10.98968	0.0129	Significant
BC	0.001225	1	0.001225	0.393349	0.5504	
A^2	0.059375	1	0.059375	19.06537	0.0033	Significant
B^2	6.58E-06	1	6.58E-06	0.002113	0.9646	
C^2	0.008059	1	0.008059	2.58782	0.1517	
Residual	0.0218	7	0.003114			
Lack of Fit	0.0218	3	0.007267			
Pure Error	0	4	0			
Cor Total	0.191612	16				

ANOVA Summary					
Std. Dev.	0.0558	PRESS	0.3488	Adeq Precision	7.4764
R^2	0.8862	Adj R^2	0.7400	Pred R^2	-0.8203

The desirability value was high for the software suggested chromatographic conditions as shown in table 5. Therefore, conditions given in table 5 were selected as optimum chromatographic conditions for method development by AQbD approach to be reliably used for routine analysis of Umifenovir and the chromatogram for the optimized condition is given in Figure 2.

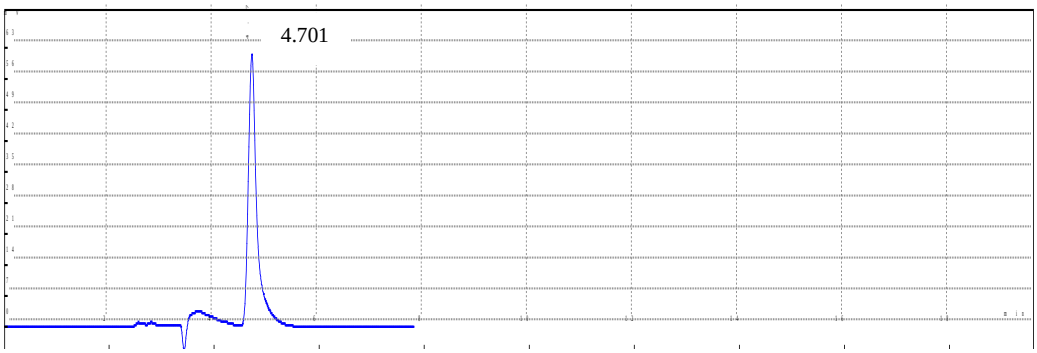


Fig. 2: Chromatogram for optimized condition of Umifenovir

Table 5. Suggested optimized chromatographic condition by AQbD approach

Flow rate (ml/min)	**% Composition (methanol: water)**	**Wave length (nm)**
0.8	65:35	258

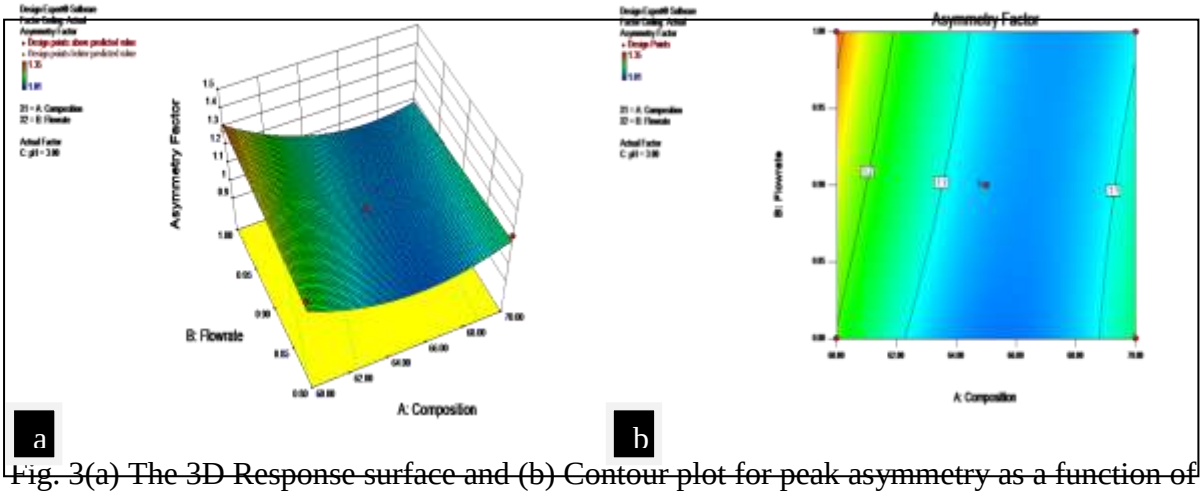


Fig. 3(a) The 3D Response surface and (b) Contour plot for peak asymmetry as a function of composition and flowrate (constant pH 3.0).

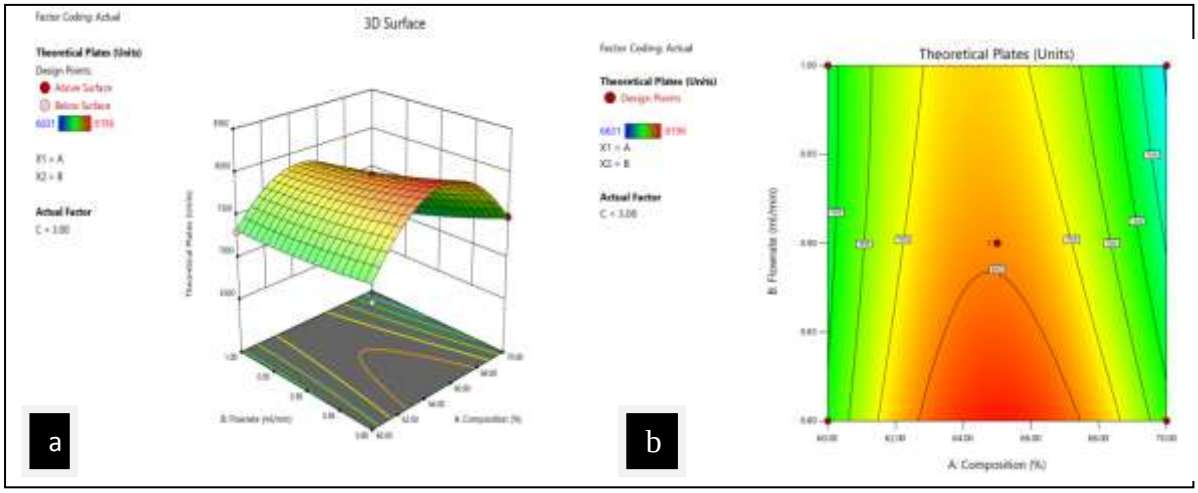


Fig. 4(a) The 3D Response surface and (b) Contour plot for theoretical plates as a function of composition and flowrate (constant pH 3.0).

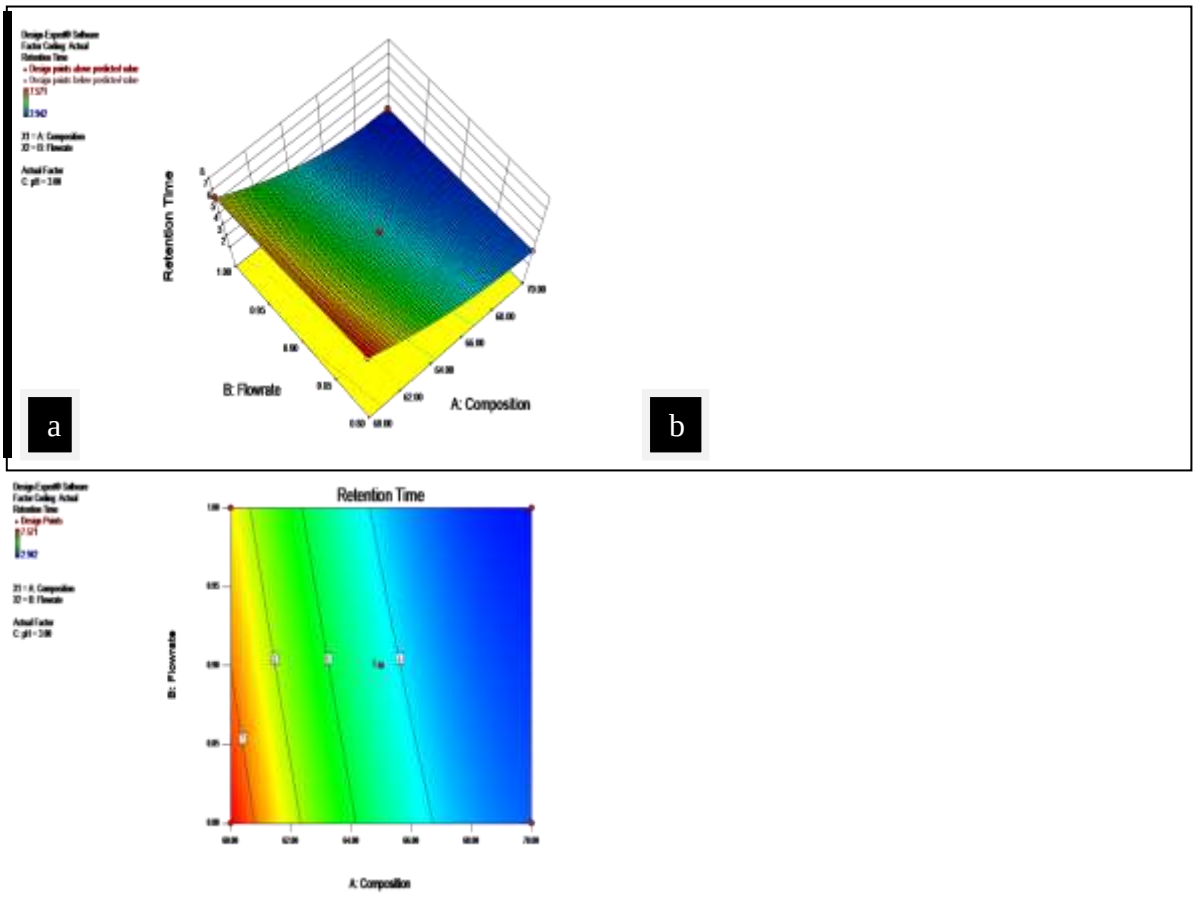


Fig. 5(a) The 3D Response surface and (b) Contour plot for retention time as a function of composition and flowrate (constant pH 3.0).

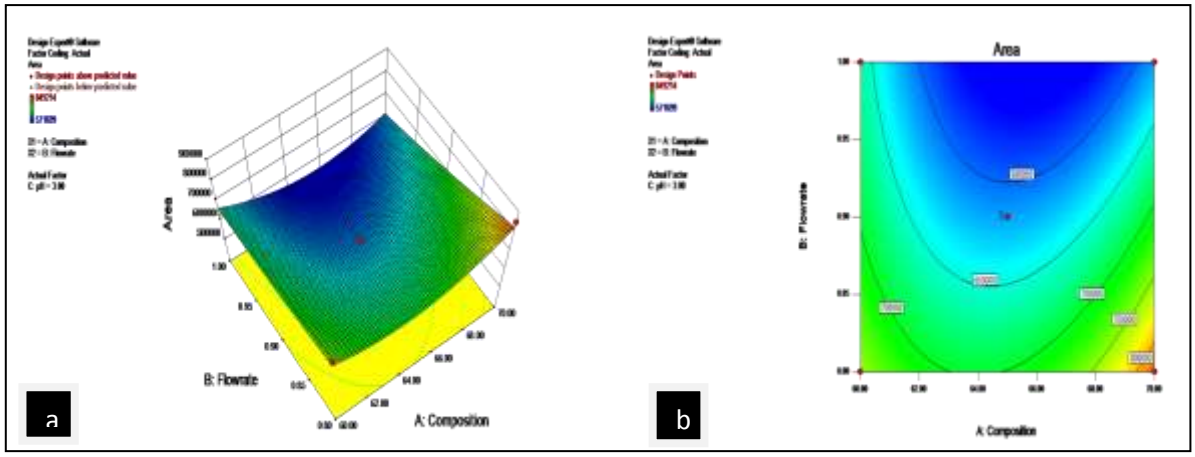


Fig. 6(a) The 3D Response surface and (b) Contour plot for area as a function of composition and flowrate (constant pH 3.0).

Method Validation

Linearity

From the observations of linearity studies as shown in table 6, a calibration curve was plotted between drug concentration versus area, refer figure 7. A straight line was obtained with a correlation coefficient $r^2 = 0.9992$. The equation of line showing slope m and y-intercept c was obtained in the form of y = mx + c. A linear correlation between drug concentration and

area was found within the range of 10-50 µg/ml. Therefore, this method could be used for estimating the concentration of Umifenovir quantitatively.

Table. 6 Data obtained from linearity study by HPLC

Concentration (ppm)	Area
10	199847
20	613334
30	1058794
40	1538798
50	2001984

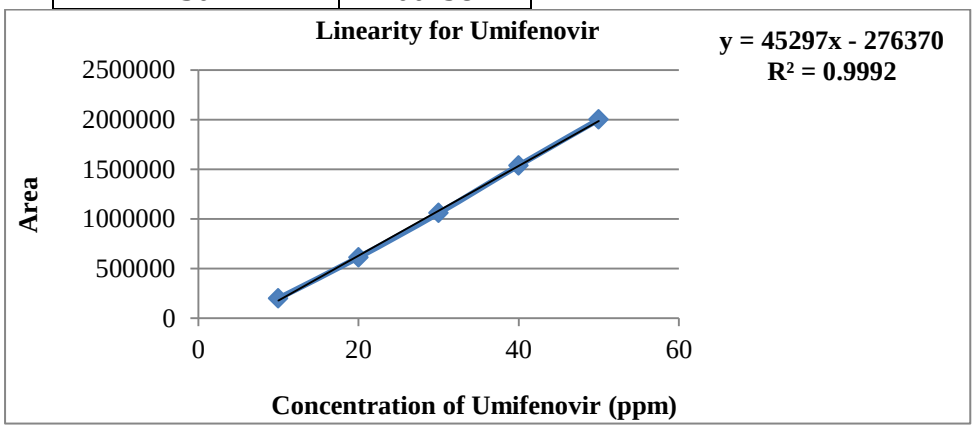


Fig. 7 Linearity for Umifenovir

Precision

Table 7 shows the results of intraday and interday precision. The mean area of the readings, standard deviation (SD) and relative standard deviation (% RSD) was calculated. The results complied with the limits for precision (% RSD < 2), proving the method was precise.

Table 7. Intraday and interday precision

Intraday Precision			**Interday Precision**		
Concentration (µg/ml)	Mean Area ± SD	% RSD	Concentration (µg/ml)	Mean Area ± SD	% RSD
30	1052514	0.58	30	1054353	0.46

Accuracy

Table 8 shows results of accuracy studies. Test was passed with specification RSD < 2 %.

Table 8. Accuracy

Sr. No.	Concentration (µg/ml)	Area	Mean	SD	% RSD
1	10 10 10	199847 199216 198669	199244	589.49	0.2958
2	30 30 30	1058794 1050874 1060601	1056756.33	5173.75	0.4895
3	50 50 50	2001984 1988904 1992884	1994590.66	6704.93	0.3361

% Recovery

The results of recovery studies are given in table 9. The recovery of Umifenovir was within the compendial limits.

Table 9. Data obtained from recovery studies

Recovery level	Concentration of test (µg/ml)	Concentration of standard added (µg/ml)	Amount found (µg/ml)	% recovery
50% Recovery	20	10	30.27	100.88
100% Recovery	20	20	40.24	100.60
150% Recovery	20	30	50.42	100.84

Robustness

Table 10 shows the results of robustness studies. Despite small variations in the experimental parameters, the limit of % RSD < 2 indicated that the method was not affected significantly.

Table 10. Results of robustness studies

Parameter varied	Concentration (µg/ml)	Area	Mean	SD	% RSD
Wavelength (nm)					
256 258 260	20 20 20	612576 613334 611757	612556	788.70	0.13
pH of mobile phase					
2.8 3.0 3.2	20 20 20	340178 339210 340538	612940	355.70	0.06

LOD and LOQ

The formula for calculation of LOD and LOQ according to ICH guidelines is

LOD = 3.3 x σ/S and LOQ = 10 x σ/S

where σ = Standard deviation of the peak area response and S is the slope of the calibration curve obtained from linearity.

From the above formula, the LOD was found to be 0.04 µg/ml and LOQ was 0.13 µg/ml.

Assay

Assay results are shown in table 11. The results comply with the compendial standards.

Table 11. Assay of Umifenovir

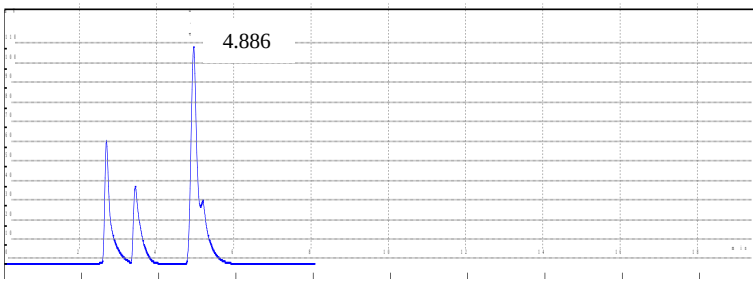
Test	Area of Standard	Area of Sample	% Assay
% Assay	1058794	1053628	99.51

Forced degradation

The forced degradation studies of umifenovir were performed. From the percent degradation shown in table 12, the drug was found to be relatively stable under thermal and photolytic stress conditions. The drug was subjected to acid and base hydrolysis, and it showed some degradation. In 3% hydrogen peroxide, the chromatogram showed two degradant peaks which coeluted with the drug peak. Refer figure 8. It is evident from the values that oxidation, alkaline and acid hydrolysis stress conditions had a significant effect on the stability of drug.

Table 12. Degradation percentage of Umifenovir under various stress conditions

Degradation parameter	% Assay after degradation	% Degradation
Acid	89.35	10.65
Base	87.81	12.19
Peroxide	85.96	14.04
Photolytic (UV)	98.83	1.17
Thermal	98.37	1.63



(a)

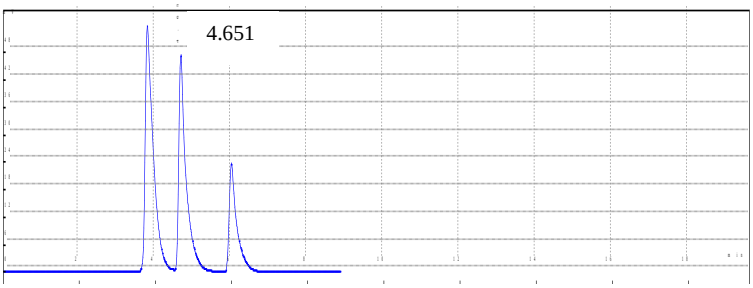
(b) 

Fig. 8 Forced degradation chromatogram of Umifenovir (a) Peroxide degradation: In 3 % H_2O_2 at R.T. for 6 hr (b) Peroxide degradation: In 3 % H_2O_2 at R.T. for 24 hr

CONCLUSION

The AQbD approach employing a Box-Behnken multifactor quadratic response surface model was successfully developed for routine analysis of Umifenovir in bulk and formulation. Statistical analysis of results was provided by the software to aid method development in a novel way. The resultant method was not only efficient but also robust. The forced degradation studies further led to generation of a stability indicating RP-HPLC method. The scientific approach of QbD to analytical method development with strategies of risk assessment, establishing CQAs, CPPs and design of experiment leads to creation of a design space offering regulatory flexibility for changes in the approved design space. The novel analytical QbD approach thus gives an edge to analysis of drugs over the traditional method development approach and insulates against method failures during method transfer.

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