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APPLICATION OF QUALITY BY DESIGN QbD IN DEVLOPMENT OF HPLC METHOD FOR FAMCICLOVIR

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ABSTRACT

The development of HPLC Method for Antiviral Drug called Famciclovir was carried out using Quality by Design Approach. The Study Started with the development of UV Method for Famcicolvir followed by its Validation as per ICH Guidelines. Further HPLC Analysis was carried out on a Shimadzu HPLC SILADvp Model Chromatograph equipped with a LC20 AT Isocratic Delivery System (pump) SPD-10Avp Detector. The Analytical Column used was C-18 Column [3.9x300 mm] 10 um particle size. The mobile phase used consist of Buffer (Potassium di hydrogen Phosphate: Acetonitrile) in 90:10 ratio. The factors like wave length and flow rate was found to be critical parameters to maintain the method development of HPLC. Hence Box-Behnken optimization models were applied for the main interactions and quadratic effects of these three factors on the selected response. Furthermore, the effects of these parameters was studied on tailing factor (Resolution) by using the surface diagram, the results of these study were analyzed.

1. INTRODUCTION

1.1 DEFINATION OF QBD

"A systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management"[1]

1.1.2. Introduction of QBD

Quality has been given an importance by all regulatory body for pharmaceutical products. Quality means customer satisfaction in terms service, product and process. Many of these quality related activities reflects need for companies to excel in global competition customer demands the perfection in quality, reliability, low cost and timely performance. Customer satisfaction can be achieved by two ways i.e. features and freedom from deficiencies in goods. The features like performance, trustworthiness, robustness, ease of use, serviceability has to build in the product and such product free from deficiencies. Quality, productivity, cost, cycle time and value are interrelated terms. Quality activities must try to detect quality problems early enough to permit actions without requiring compromise in cost, schedule or quality. The emphasis must be on precaution rather than on just correction of quality problems. Hence the quality has to be built in the product as well as services through proper planning, so that the forth coming failure can be avoided. Mere analysis of final product will not work but the quality should design in the product.

Qualities by Design refers to the achievement of certain predictable quality with desired and predetermine specification. Since first initiated by the U.S. FDA in its pharmaceutical cGMP has become an important concept for the pharmaceutical industry that is further defined in the International Conference on Harmonization (ICH) guidance on pharmaceutical development as, "a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management."

In2005 implementation of QbD for more systematic approach was asked by USFDA to some firms to submit their CMC in QbD format.[1] Question base review (QbR) forms the platform of QbD principle.[2] USFDA had announced that 2013 was deadline for generics to implement QbD in interview with Lawrence Yu Deputy Director, Science and Chemistry, FDA.[3] FDA also states importance of quality of pharmaceutical products by giving Process Analytical Technology (PAT) which is a framework for Pharmaceutical Development, Manufacturing and Quality Assurance.[1]

A very useful component of the QbD is the understanding of the factors and their interaction effects by a desired set of experiments. The present study describes the development of a comprehensive science and risk based HPLC method and subsequent validation for the analysis of active pharmaceutical ingredient. QbD is a systemic process to build into a

product from the inception of final output. QbD requires a thorough understanding of the product and its process of manufacture, necessitating an investment in time and resources up front in the discovery and development of product. For QbD the product and process knowledge base must include an understanding of variability of raw material, the relationship between a process and product critical quality attributes (CQA) and the association between a CQA and products clinical properties.[4] Quality by Design is a concept outlined by well-known quality expert Joseph M. Juran in various publications. QbD principles have been used to advance product and process quality in every industry and particularly the automotive industry. They have most recently been adopted by the U.S. Food and Drug Administration (FDA) as vehicle for the transformation of drugs are discovered, developed and commercially manufactured. Because the need of potent drug with safety profile, pharmaceutical industry investing billions of money in the drug discovery and development process with endeavour to design quality product and that to with consistency in manufacturing process to deliver the intended performance of product. The information and knowledge gained from pharmaceutical studies and manufacturing provides base for scientific understanding to support establishment design space, specification and manufacturing control. Information from pharmaceutical development studies can be a root for quality risk management. Lifecycle management allows making changes in formulatio0n and manufacturing process during development and providing additional opportunities to gain added knowledge and it further establishment of the design space. Design space is planned by the applicant and will undergo regulatory assessment and approval. Working within the design space is not considered as a change. But an operation out of the design space is considered change and hastobe facing a regulatory post approval change process. During the drug development process, the aspects like drug substances, excipients, container closure systems, manufacturing process and quality control tests are critical to product quality.[5]

1.1.3 UNDERSTANDING THE KEY TERMS OF QBD

Risk analysis: Estimation of the risk associated with identified hazards. In a pharmaceutical context, this term is often used interchangeably with risk evaluation – the comparison of the estimated risk to given risk criteria using a quantitative or qualitative scale to determine the significance of the risk.

Knowledge management: Systematic approach to collecting, analyzing, storing, and disseminating information related to products, processes and components.

Design space: Multidimensional combination and interaction of input variables (e.g.material attributes) and process parameters that have been demonstrated to provide assurance of quality.

Critical quality attributes: Physical, chemical, biological or microbiological property or characteristic of the product that should be within an appropriate limit, range or distribution to ensure the desired product quality.

Target product profile: Summary of the quality characteristics of a drug product that ideally will be achieved to ensure that the desired quality, and thus the safety and efficacy, of a drug product is realized. Also referred to as the Quality Target Product Profile.

Drug product: Pharmaceutical product type that contains a drug substance, generally in association with excipients. Also referred to as the dosage form or finished product.

Drug substance: Active pharmaceutical agent which is subsequently formulated with excipients to produce the "drug product".

Design of experiment: Use of statistically designed experimental arrays to determine the effect of multiple variables on an experimental system that take into account experimental variation and are able to determine both the effects of each variable alone and the combined effect (interaction) of multiple variables.[1]

1.1.4 QbD BY PHARMACEUTICALS

Even though the pharmaceutical industry has focus on quality, it has failed to keep up with other industries in terms of manufacturing efficiency and productivity. Currentscenario in the Pharmaceutical Industry: Cost of revalidation Off-line analysis for in-process - need based Product specifications as primary means of control Unpredictable Scaleup issues Inability to understand failures.

Systematic approach to development

That begins with predefined objectives Emphasizes products and process understanding Process control.

Figure 1: Process control.

QUALITY TARGET PRODUCT PROFILE

A summary of the drug development program described in terms of labeling concepts and it mainly focus on the safety and efficacy.

- Description
- Clinical Pharmacology
- Indications and Usage
- Contraindications
- Warnings
- Precautions
- Adverse Reactions
- Drug Abuse and Dependence
- Over dosage
- Dosage and Administration
- How Supplied
- Animal Pharmacology and/or Animal Toxicology
- Clinical Studies

A natural extension of Target Product Profile for product quality – Quality characteristics (attributes) that the drug product should possess in order to reproducibly deliver the therapeutic benefit promised in the label guide to establish formulation strategy and keep the formulation effort focused and efficient. It facilitates identification of what's needed/critical for the patient/consumer in the Quality Target Product Profile (such as Critical Quality Attributes, CQAs). Identifies risks and best approaches to manage.

Uses tools/enablers in an optimized fashion (such as integration of QbD and bio pharmaceutics Generates and enables knowledge sharing. An iterative, learning, life-cycle process for optimizing decision-making and the therapeutic outcomes for the patient benefit.

A drug product designed, developed and manufactured according to Quality Target Product Profile with specification (such as dissolution/release acceptance criteria)consistent with the desired in vivo performance of the product.

CRITICAL QUALITY ATTRIBUTES

It is necessary to identify the quality attributes that are critical, i.e. those defining purity, potency and surrogate for Bioavailability Criticality etc. It is based on the impact of quality attribute/ parameter on the safety, efficacy & quality (manufacturability) of the product. Establish a link between CPP & CQAs: Identification of attribute or parameters that can be used as a surrogate for clinical safety & efficacy (important to patient) (Figure2).Manufacture ability is also an attribute (important to business) that is critical to quality. The level of criticality may differ for an API manufacturing process relative to a drug product manufacturing process API is one component of a drug product and one step further away from the patient compliance of Criticality. Several levels of criticality may be used to describe multiple levels of risk. As attribute or parameter boundaries approach edges of failure, the level of critically increased with the risk.[1]

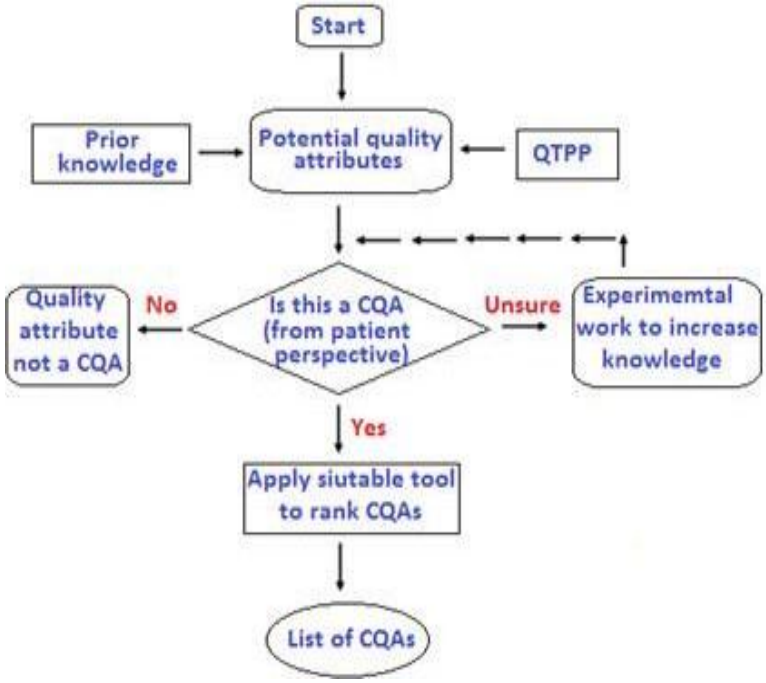


Figure 2: Decision tree to decide CQAs.

1.1.5. TOOLS OF QUALITY BY DESIGN

I] Design of Experiments (DOE)

Design of experiments (DOE) is a structured and organized method to determine the

relationship among factors that influence outputs of a process. It has been suggested that DOE can offer returns that are four to eight times greater than the cost of running the experiments in a fraction of the time. Application of DOE in QbD helps in gaining maximum information from a minimum number of experiments. When DOE is applied to a pharmaceutical process, factors are the raw material attributes (e.g., particle size) and process parameters (e.g., speed and time), while outputs are the critical quality attributes such as blend uniformity, tablet hardness, thickness, and friability. As each unit operation has many input and output variables as well as process parameters, it is impossible to experimentally investigate all of them. DOE results can help identify optimal conditions, the critical factors that most influence CQAs and those who do not, as well as details such as the existence of interactions and synergies between factors.[7]

II] Process Analytical Technology (PAT)

PAT has been defined as "A system for designing, analyzing, and controlling manufacturing through measurements, during processing of critical quality and performance attributes of raw and in-process materials and processes, with the goal of ensuring final product quality". The goal of PAT is to "enhance understanding and control the manufacturing process, which is consistent with our current drug quality system: quality cannot be tested into products; it should be built-in or should be by design." The design space is defined by the key and critical process parameters identified from process characterization studies and their acceptable ranges. These parameters are the primary focus of on-, in- or at-line PAT applications. In principle, real-time PAT assessments could provide the basis for continuous feedback and result in improved process robustness. NIR act as a tool for PAT and useful in the RTRT (Real Time Release Testing) as it monitors the particle size, blend uniformity, granulation, content uniformity, polymorphism, dissolution and monitoring the process online, at the line and offline, thus it reduces the release testing of the product.

III] Risk Management Methodology

Quality Risk Management is defined as "A systematic process for the assessment, control, communication and review of risks to the quality of the drug (medicinal) product across the product lifecycle". Risk assessment tools can be used to identify and level parameters (e.g., process, equipment, input materials) with potential to have an impact on product quality, based on prior knowledge and primary experimental data. The early list of potential parameters can be fairly broad, but can be modified and prioritized by additional studies (e.g.,

through a combination of design of experiments, mechanistic models). Once the considerable parameters are identified, they can be further studied (e.g., through a combination of design of experiments, mathematical models, or studies that lead to mechanistic understanding) to achieve a higher level of process understanding.

The pharmaceutical industry and regulators can evaluate and manage risks by using well-known risk management tools such as,

- Basic risk management facilitation methods (flowcharts, check sheets etc.);
- Failure Mode Effects Analysis (FMEA);
- Failure Mode, Effects and Criticality Analysis (FMECA);
- Fault Tree Analysis (FTA);
- Hazard Analysis and Critical Control Points (HACCP);
- Hazard Operability Analysis (HOP)
- Preliminary Hazard Analysis (PHA);
- Risk ranking and filtering;
- Various Statistical Tools like –
- Acceptance Control Charts
- Design of Experiment (DoE)
- Pareto Charts& Histograms

1.1.6. BENEFITS OF QbD[8, 9, 10, 12]

Proper implementation of QbD can potentially provide three main benefits for development:

- More efficient use of development time and costs.
- Ability to meet FDA submission guidelines and expectations.
- Reduced approval times – and fewer queries – from the FDA.

Likewise, QbD can potentially provide significant benefit in manufacturing. Even after your drug has gained FDA approval, routine QC testing may detect an out of specification (OOS) result. For a company that did not use a QbD approach, an OOS result can mean a seemingly endless quest to find the root cause. Absent the data that QbD provides, test results may be suspect, questions difficult to answer, and long delays inevitable. Without knowing where to look, your team may resort to a trial-and-error approach to resolve any OOS occurrences.

One recent article presented several scenarios that could cause a 4 to 9 fold increase in testing to clear up an OOS investigation – a costly and time-consuming prospect.[17] The impact of

poor quality that spirals out of control into an OOS event can be horrendous.

"For manufacturers, there are potentially huge external costs for delayed product launches or approvals, or severe actions such as consent decrees," notes one editor of an industry journal, plus "the internal costs of wasted raw materials, scrap batches, and the cost of investigation and remediation."

Imagine the damage to your brand such an event would have. To add further insult, you may have to spend an enormous amount of money just to get your product back to market. QbD minimizes these risks by mapping all the possible variables of the product attributes and processes into a known control space. This means that if any quality issues occur, your team can use specific methods to quickly pinpoint the scientific variables that are most likely causing the issues.

Potential benefits of adopting QbD for analytical method

- The developed method will be more robust which gives greater level of confidence in case of variations in conditions.
- This approach gives greater transfer success when method is transferred from research level to quality control department.
- It provides a space for invention of new techniques by continuous improvement throughout life cycle.
- It helps for enhanced understanding of the method.
- Design space concept avoids the post-approval changes which may cause to pay a high cost for any of the firm.
- It provides greater compliance with regulatory authorities.

Advantages of QbD

- ✓ Patient safety and product efficacy are focused.
- ✓ It involves product design and process development.
- ✓ It offers robust method or process.
- ✓ Scientific understanding of pharmaceutical process and methods is done.
- ✓ Critical quality attributes are identified and their effect on final quality of product is analysed.
- ✓ Method design concept helps to avoid cost involved with post approval changes
- ✓ Science based risk assessment is carried.
- ✓ Business benefits are also driving force to adopt QbD.

The business benefits includes

- ✓ Fewer lost batches, typically costing $250 - $500K per batch.
- ✓ Fewer manufacturing deviations, saving hundreds of costly hours and $10 - $15K per deviation.
- ✓ Faster time to market and more reliable supply, when each day on the market could mean
  $100K (or more).
- ✓ Fewer inspections of manufacturing sites.
- ✓ A many-fold ROI via cost savings and increased revenue.

Benefits to Industry

- ✓ problems in manufacturing o Reduces number of manufacturing
- ✓ supplements required for post market
- ✓ changes –rely on process and risk
- ✓ understanding and risk mitigation o Allows for implementation of new technology
- ✓ to improve manufacturing without regulatory
- ✓ scrutiny o Allows for possible reduction in overall costs of
- ✓ manufacturing –less waste

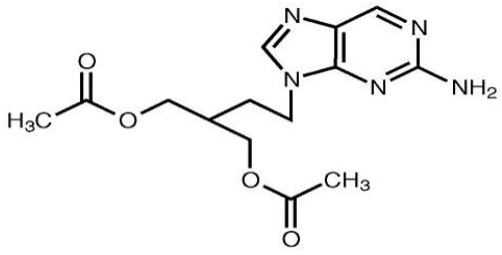
1.1.7APPLICATION OF QBD IN ANALYTICAL METHODS OF MEASUREMENT[13]

‘‘QbD does not necessarily mean less analytical testing’’ rather, it means the right analysis at the right time, and is based on science and risk assessment. Implementation of QbD helps to develop rugged and robust method which helps to comply with ICH guideline hence for that reason pharmaceutical industries are adopting this concept of QbD. Factors which improve robustness are taken into consideration for the development of analytical method in QbD environment. This approach facilitates continuous improvement in method. Parallel opportunities of application of QbD to analytical method as that of manufacturing process are available in the literature.[20] QbD can be applied for various analytical methods which includes.

- Chromatographic techniques like HPLC (For stability studies, method development, and determination of impurities in pharmaceuticals).
- Hyphenated technique like LC–MS.
- Advanced techniques like mass spectroscopy, UPLC and capillary electrophoresis.

- Dissolution studies
- Analysis of genotoxic impurity
- Karl Fischer titration for determination of moisture content.
- Vibrational spectroscopy for identification and quantification of compounds e.g. UV method.

DRUG PROFILE



famciclovir

5.1 FAMCICLOVIR

CAS No. : 104227-87-4

Molecular formula : $C_{14}H_{19}N_5O_4$

Molecular weight : 321.337g/mole

Chemical name : Famvir- Famciclovirum

Physical state : White Powder

LOGP : 0.6

Therapeutic use : Famciclovir is indicated for the treatment of Herpes zoster (shingles) treatment of Herpes Simplex Virus.

Mechanism of action: The mechanism of action of famciclovir is as a DNA polymerase ninhibitor, The chemical Classification of Famciclovir is nucleaoside Analog, Famciclovir is a Nucleoside Analog and Antiviral agent used in therapy of herpes Zoster and simplex virus Infection.Famciclovir undergoes rapid biotransformation to the active antiviral compound penciclovir, which has inhibitory activity against herpes simplex virus types 1 (HSV-1) and 2 (HSV-2) and varicella zoster virus (VZV). In cells infected with HSV-1, HSV-2 or VZV, viral thymidine kinase phosphorylates penciclovir to a monophosphate form that, in turn, is converted to penciclovir triphosphate by cellular kinases. In vitro studies demonstrate that penciclovir triphosphate inhibits HSV-2 DNA polymerase competitively with deoxyguanosine triphosphate. Consequently, herpes viral DNA synthesis and, therefore, replication are selectively inhibited.

Adverse reaction: weakness, confusion, increased thirst, loss of appetite, vomiting, pounding heartbeats or fluttering in your chest; or. swelling, weight gain, feeling short of breath.

Identification of Famciclovir

Solubility: Soluble in, Methanol, Ethanol, Chloroform, soluble in Water,Highly soluble in Phosphate Buffer.

Melting point: 102-104 °C

Determination of UV λmax: 220nm

Linearity on UV: 1-10 µg

6. MATERIALS AND METHODS

6.1 a UV Method Development of Famciclovir

Table 5: Material Used for UV- Spectrophotometry.

Material	Source
Famciclovir (API)	Glenmark Limited, Mumbai Maharashtra.
Water (Distilled grade)	Pharmaceutics Lab, Y.B.C.C.P.A

Table 6: Instrument Used for UV- Spectrophotometric Method.

Instrument	Manufacturing Company
UV-Visible double beam spectrophotometer Shimadzu UV 1800,Wave length range 190-1100 Band width 2nm, 1 cm quartz cells Slit width of 2 nm Instrument scan speed of 600 nm/min UV probe software	Shimadzu, Japan

6.1b Method for UV Spectroscopic Determination

1) Selection of Sampling Wavelength for Analysis and Preparation of Standard Calibration Curve

a) Solvent Used: Phosphate Buffer was used as solvent. Dissolve 6.8gm of Potassium Di hydrogen Phosphate and 1.844 gm of Sodium Hydroxide into a 1000 mL volumetric flask and dilute with Water to produce 1000ml. Adjust the pH 3.0 with Phosphoric Acid. Buffer solution was degassed with Sonicator and filtered prior to use.

b) Preparation of the Standard Stock Solution: Weight accurately 10 mg of Famciclovir and dissolve in 100 ml of Phosphate Buffer then volume is make up to mark to obtain final concentration of 100 µg/ml of the solution.

c) Selection of Analytical Wavelength: By appropriate dilution of standard solution of Famciclovir to 10 µg/ml were prepared and scanned for wavelength region in the spectrum mode and peak observed at 220 nm.

d) Selection of Analytical Concentration Range of Famciclovir: For a drug appropriate aliquots were pipette out from the standard stock solution into series of 10 ml volumetric flask. The volume was made up to the mark with Phosphate Buffer to get a set of solutions.All solutions were measured at selected wavelength and plotted against concentration. The range over which the drug obeyed Beer Lamberts law was chosen.

6.1 c Validation According to ICH Guidelines

a. Linearity: For quantitative analysis ofFamciclovirthe calibration curves were plotted for each concentration ranges. The linearity ranges from 2-10 µg/ml.

b. Limit of Detection (LOD) and Limit of Quantitation (LOQ): The LOD and LOQ of Famciclovir by the proposed methods were determined using calibration standards. LOD and LOQ values were calculated as 3.3 σ/S and 10 σ/S, respectively, where S is the slope of the calibration curve and σ is the standard deviation of y-intercept.

c. Precision: The reproducibility of proposed method was determined by performing tablet assay at different time intervals (3 hour interval) on same day (Intra-day precision) and on three different days (Inter-day precision) at concentration Famciclovirat the concentration 2-10 µg/ml.

6.2 Method for HPLC

Reagents and Chemicals Used

Material	Source
Famciclovir (API)	Glenmark Limited, Mumbai Maharashtra.
Solvents	Badar Chemicals
Water (Distilled grade)	Pharmaceutics Lab, Y.B.C.C.P.A

Reference standard of Famciclovir was obtained from Glenmark Limited, Mumbai. HPLC grade acetonitrile, phosphoric acid of merck were used. All aqueous solutions were prepared with HPLC grade ready water obtained in-house, Milli-Q water purification system (Millipore, USA).

Instrumentation

- HPLC analysis was carried out using a Shimadzu HPLC SILADvp model chromatograph equipped with a LC20 AT isocratic delivery system (pump), SPD-10Avp detector, the analytical column was C-18 column (3.9 × 300 mm), 10 µm particle size). Data acquisition and processing was performed using Class Vp 5.13 software.
- Milli-Q water purification system (Millipore, USA).
- UV visible spectrophotometer (Double Beam), SHIMADZU and wavelength range of 200 to 400 nm.

a) Mobile Phase Preparation

Phosphate Buffer solution was prepared by dissolving 6.8 gm of Potassium Dihydrogen Phosphate and 1.844 gm of Sodium Hydroxide into a 1000 mL volumetric flask and dilute with Water to produce 1000 ml. Adjust the pH 3.0 with Phosphoric Acid. Buffer solution was degassed with Sonicator and Filtered prior to use for HPLC analysis. Dissolve 90% of Phosphate Buffer Solution in 10% of Acetonitrile to produce mobile phase of Buffer: Acetonitrile having concentration 80:20 v/v.

b) Famciclovir Sample Preparation

Famciclovir stock solution for optimization of experiments was prepared by accurately weighing 10mg of Famciclovir and dissolving in 100ml Phosphate Buffer to yield a final concentration of 400µg/ml Famciclovir. Transfer 10 ml standard stock into a 100 mL volumetric flask, Dissolve and make up the volume with Phosphate Buffer. From above stock solution, 4µg/ml sample was prepared for analysis.

c) Wavelength Selection for Analysis

Appropriate dilutions of Famciclovir were prepared and samples were scanned using UV spectrometer in the range of 200nm to 400nm. An absorbance maximum was obtained at 220 nm.

d) Analytical Target Profile

"QbD is a systematic approach to product, process design and development."[6] Hence it begins with determination of goal or method intent. In this emphasis given on the product and process understanding.[1] Here method intent was to develop HPLC method of famciclovir which is robust, accurate, precise and USP Resolution more than 2, 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 help of visualized a design space.

e) Instrument Qualification

Analytical procedures in pharmaceutical analysis are subjected to highly formalized validation procedures in order to demonstrate that they are suitable for the intended use. As a consequence, prior to method validation it is necessary to assure that the equipment or analytical test system itself is adequately designed, maintained, calibrated and tested. These tests are called as analytical instrument qualification (AIQ).

Qualification phases for analytical instrument are

- Design qualification
- Installation qualification
- Operational qualification
- Performance qualification

Here in HPLC system are "of the shelf" equipment, design qualification may be disregarded here. Installation qualification establishes that the instrument is received as designed and that it is properly installed. As far as practical experimentation is considered only operational qualification and performance qualification combine parameters were done as reported by L.Kaminski et al.[27]

Precision of injection volume

It was determined by comparing peak area received with fixed 20µl injection and calibrated dosage loop tolerance limit set was <1%RSD.

Injection carryover

Injection carry over was determined by running a blank test directly after an analysis and measuring possible absorption there should not be any peak from previous analysis.

Flow rate accuracy

It was determine by measuring the volumetric flow rate of mobile phase through the column over a previously set period of time 1.0ml/min for 10 min, 2.0 ml/min for 5 min, 2.5 ml/min for 10 min. RSD should be <1% or tolerance limit is ±3%.

Flow rate precision

A flow rate precision was determined by measuring the RSD of retention times. Limit set was

<1.0% RSD.

Wavelength accuracy

It was done by scanning the compound with known specific maxima. Tolerance limit is specific maxima ±2nm.

Linearity of detector

Linearity of detector was determined by injecting increasing concentration of test substance and tolerance limit set was $R^2 \geq 0.999$.

f) Risk Assessment

It is commonly understood that risk is defined as the combination of probability of occurrence of harm and severity of that harm. Risk assessment helps to increase quality of method or process. Also it is determine for effect of input variable on method or process. From risk assessment one can recognise critical attributes that are going to affect final quality of product. A risk assessment is helpful for effective communication between FDA and industry, research/development, and manufacturing and among multiple manufacturing sites within company.

Various tools for risk assessment are[20],

- Ishikawa or fishbone diagram,
- Failure mode effect analysis(FMEA),
- Pareto analysis.

g) Initial chromatographic Condition

Chromatographic separation was carried out with C18 column, different mobile phases were tried starting with methanol and Phosphate Buffer, The separation was carried on C-18 column (3.9×300 mm, 10-µm particle size) with mobile phase of buffer (pH-3.0): acetonitrile (80:20 v/v) degassed in a sonicator for 10 min and filtered through 0.2µ membrane filter before use. Peak was obtained at retention time of 12.5 min with flow rate of 1 ml/min, Column Temperature of 30°C. Prior to the injection of drug solution, column was equilibrated with mobile phase flowing through the system. Detection was done using UV detector at 245nm. Further changes were done according to optimization model. pH was change by using phosphoric acid.

h) Method Design

i) Screening Method

The screening was done by Plackett-burman Design using Design Expert 9 software. Five factors were selected as following.

1. Flow Rate.
2. Injection Volume.
3. Column Oven Temperature
4. Acetonitrile Concentration
5. Detection Wavelength.

The total runs obtained were 12 in number , the response for the design was resolution between the drug & the impurity. The results were then put in the design to further optimise the method.**(Table9&10)**

Table 7: Chromatographic factors and response variables for Plackett Burman experimental design.

Chromatographic Condition		Level used	
	Low		High
Flow Rate(ml/min)	0.5	1	1.5
InjcVolume(µL)	7	9	13
Column Oven Temperature (°C)	28	30	32
Wavelength (nm)	220	240	400

Table 8: Plackett Burman experimental design for Famciclovir.

		Factor 1	Factor 2	Factor 3	Response 1
Std	Run	A: FLOW RATE	B:WAVELENGTH	C:TEMP	TAILING
		MG/ML	NM	Â°C	
10	1	1	225	28	1.223
17	2	1	220	30	1.243
15	3	1	220	30	1.236
1	4	0.8	215	30	1.25
14	5	1	220	30	1.236
6	6	1.2	220	28	1.202
11	7	1	215	32	1.242
3	8	0.8	225	30	1.251
5	9	0.8	220	28	1.249
4	10	1.2	225	30	1.221
13	11	1	220	30	1.239
12	12	1	225	32	1.242
8	13	1.2	220	32	1.225

2	14	1.2	215	30	1.211
7	15	0.8	220	32	1.268
16	16	1	220	30	1.223
9	17	1	215	28	1.208

Optimization was done by response surface methodology, applying a three level Box Behnken design with three centre points **(Table 7)**. Three factors selected were flow rate, acetonitrile concentration and column oven temperature in mobile phase. Evaluation of main factor, their interaction and quadric effect on peak USP Resolution factor were done. Injection volume of 10µl, detection wavelength of 220 nm was kept constant as their effect on Resolution was less significant. Experiments were conducted by making triplicate injections (total17 runs) of standard Famciclovir solution and related substance. The average of USP Resolution was analyzed using Design Expert 9 Software.**(Table 7)**.

Application of multivariate regression analysis resulted in a fitted full quadrate model for the average responses for peak USP Resolution given by the equation: 1

$$Y= \beta_0+\beta_1X_1+\beta_2X_2+ \beta_3X_3+ \beta_{11}X_1^2+ \beta_{22}X_2^2+ \beta_{33} X_3^2 + \beta_{12} X_1 X_2+ \beta_{13} X_1X_3+ \beta_{23}X_2X_3$$

Where Y is the response, β_0 is the arithmetic mean response. B_1 β_2 and β_3 are regression coefficients of the factor X_1, X_2 and X_3 respectively. B_{11}, β_{22} β_{33} are squared coefficients β_{12}, $\beta_{13}$and β_{23} are interaction coefficients.[28,29]

Table 9: Chromatographic 0factors and response variables for Box Behnken experimental design.

Chromatographic		**Level used**	
Condition	**Low**	**Centre**	**High**
Flow rate (X_1)	0.8	1	1.2
Wave length(X_2)	215	220	225
Column Oven Temperature (X_3)	28	30	32

Table 10: Box Behnken method used for Famciclovir optimization(Where '+' indicate the high value, '-' indicates lower value and '0' is the centre).

Run	**Coded (X_1,X_2, X_3)**	**Flow Rate (ml/min)**	**Wavelength (nm)**	**Column Oven Temperature (◦C)**
1	000	100	220	30
2	--0	00.8	215	30
3	000	1	220	30
4	+0+	1.2	220	32
5	+-0	1.2	215	30

6	000	1	220	30
7	-0-	0.8	220	28
8	-0+	0.8	220	32
9	+0-	1.2	220	28
10	000	1	220	30
11	++0	1.2	225	30
12	000	1	220	30
13	0++	1	225	32
14	0--	1	215	28
15	0+-	1	225	28
16	-+0	0.8	225	30
17	0-+	1	215	32

i) Critical Quality Attribute (CQA)

From the software generated result the critical factors which affect the Resolution and capacity factor were determined. Factor such as flow rate, wavelength and ACN concentration in mobile phase were found to be critical. Selection of stationary phase was also critical parameter. The nature of the drug is more retentive on C-18 than C-8.

j) Method validation

The optimized chromatographic method was validated according to the International Conference on Harmonization (ICH) Q2 (R1).[36] guidelines for linearity, range, precision and robustness. For system suitability, standard solution of 10µg/ml of Famciclovir was prepared by diluting and mixing drug with methanol. Six replicate injection of the system standard solution were analysed before sample analysis. The acceptance criteria for Famciclovirwere less than 2% relative standard deviation (RSD)for peak area, retention time, symmetry USP resolution factor more than 2 and number of theoretical plates greater than 2000 for all peaks.

Linearity

As per ICH guidelines the linearity of analytical procedure is its ability (within in a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in sample. Standard calibration curves were prepared with five different concentrations by making serial volume to volume dilution of stock solution with methanol, over the range of 10, 20, 30, 40 and 50µg/ml. Three replicate injections of each concentration were made to determine the linearity of Famciclovir over the concentration range. Linear concentration curves of peak area versus drug concentration were plotted using linear least squares regression and evaluated for linearity.

Precision

According to ICH Q2 guidelines precision is usually reported as the per cent relative standard concentration standard deviation of a set of responses.[36] Precision of the method were evaluated for Famciclovir drug substance by analyzing standard samples prepared daily from stock solution. Three replicate of each low (10µg/ml), intermediate (20µg/ml), high (30µg/ml) standard were analyzed daily over three days as a part of validation and quality control. Precision were determined by analyzing the mean, standard deviation and relative standard deviation of the peak areas and their resultant concentrations. An acceptance criterion for precision is that the RSD of the standards should not be more than 2.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate change in method parameter and provide an indication of its reliability during normal usage.[36] There should be reliability of an analysis with respect to deliberate variation in method parameter such as flow rate (±0.1ml/min), pH (±0.1 units), and mobile phase proportion.

7. RESULT AND DISCUSSION

7.1 UV Spectrometric Determination of Famciclovir

Instrument Used: UV Visible Spectrophotometer (Double Beam)

a) Selection of Sampling Wavelength for Analysis and Preparation of Standard Calibration Curve.

i) Solvent Used: Phosphate Buffer was used as solvent.

ii) Preparation of Standard Stock Solution

Weight accurately 10 mg of Famciclovir and transferred to 100 ml of volumetric flask andand dissolve in 50 ml Phosphate Buffer and then make up the volume with Phosphate Buffer to obtain final concentration of 100 µg/ml.

b) Selection of Analytical Wavelength

By appropriate dilutions of standard solution of Famciclovir to 10µg/ml was prepared and scanned for entire wavelength region in spectrum mode and peak was observed at 270 nm. For a drug appropriate aliquots were pipette out from the standard stock solution into series of 10 ml volumetric flask. The volume was made up to the mark with all Phosphate Buffer to get a set of solution for a drug having the concentration2,4,6,8,10 µg/ml. The absorbance of

each of this solution were measured at the selected wavelength and plotted against concentration. The concentration range which the drug obeyed Beer lamberts law was chosen.(figure 11)

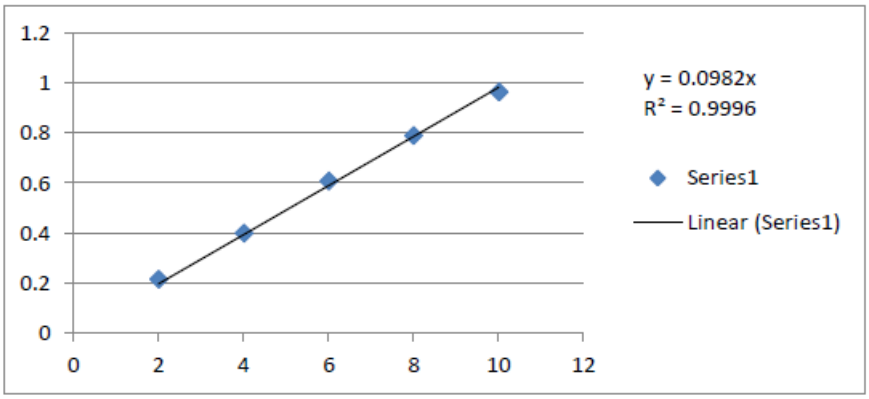


Figure (11): Linearity plot of Famciclovir.

Table (11): Absorbance for Calibration of Famciclovir.

Concentration(µg/ml)	Absorbance
2	0.213
4	0.398
6	0.607
8	0.788
10	0.964

a. Procedure for Analysis of Bulk Drug

Powder mixture of Famciclovir having composition 100 µg/ml made in Phosphate Buffer was analyzed for getting the absorbance at 220 nm. After getting the absorbance at 220 nm, the concentration of Famciclovir was calculated by putting the value of absorbance of mixture in above formula.

7.1 B)Validation According To ICH Guidelines

1. **Linearity:** For quantitative analysis of Famciclovir, the calibration curves were plotted for each concentration ranges. The linearity ranges from 2-10 µg/ml. Regression coefficient was obtained for the drug within analytical range r^2=0.997 for Famciclovir.
2. **Limit of detection (LOD) and Limit of Quantitation (LOQ):**The LOD and LOQ of Famciclovir by the proposed methods were determined using calibration standards. LOD and LOQ values were calculated as 3.3σ/S and 10σ/S respectively, where S is the slope of the calibration curve and σ is the standard deviation of y-intercept. LOD was found to be 0.345 and LOQ was found to be 1.044.

3. **Precision:** The reproducibility of proposed method was determined by performing tablet assay at different time intervals 2hour interval on same day (Intra-day precision) and on three different days (Inter-day precision) at concentration of 2-10µg/ml.

Table 12: Intra-day and Inter-day Precision.

Day 1							
Concentration (µg/ml)	**Absorbance**						
	Morning	**Afternoon**	**Evening**	**Sum**	**Mean**	**SD**	**%RSD**
2	0.213	0.215	0.218	0.646	0.215333	0.000408	0.18947662
4	0.391	0.393	0.397	1.181	0.393666	0.000816	0.20728232
6	0.612	0.614	0.617	1.843	0.614333	0.000408	0.06641382
						Sum	0.46317276
						Average	0.15439092
Day 2							
Concentration (µg/ml)	**Absorbance**						
	Morning	**Afternoon**	**Evening**	**Sum**	**Mean**	**SD**	**%RSD**
2	0.210	0.212	0.216	0.638	0.212666	0.000816	0.38371109
4	0.387	0.393	0.395	1.175	0.391667	0.001633	0.41693582
6	0.611	0.614	0.616	1.841	0.613667	0.000408	0.06648557
						Sum	0.86713248
						Average	0.28904416
Day 3							
Concentration (µg/ml)	**Absorbance**						
	Morning	**Afternoon**	**Evening**	**Sum**	**Mean**	**SD**	**%RSD**
2	0.206	0.213	0.217	0.636	0.212	0.001225	0.57783019
4	0.395	0.396	0.398	1.189	0.39633	0.000408	0.10294452
6	0.616	0.614	0.625	1.855	0.61833	0.000694	0.11223781
						Sum	0.79301252
						Average	0.264337506

Table 13: Summary of Validation Parameters.

Sr No	Parameter		Results	ICH Standard
1.	Precision:		0.056633	RSD < 2 %
	Intraday precision			
	Interday precision	Day 1	0.15439092%	
		Day 2	0.28904416%	
		Day 3	0.264337506%	
2.	LOD		0.344	≤ 2 µg/ml
3.	LOQ		1.044	≤ 2 µg/ml
4.	Linearity		2-10 µg/ml	
6.	Regression coefficient		$R^2 = 0.997$	$R^2 \geq 0.995$
7.	Std. regression equation		Y=x139.07	

7.2 Determination of Famciclovir by HPLC.

Preliminary studies

Famciclovir is used as an Antiviral agent. Different mobile phases were tried starting with methanol and water, the separation was carried on C-18 column (3.9 × 300 mm, 10µm particle size) with mobile phase of Disodium hydrogen phosphate buffer (pH 3.0) : Acetonitrile (80:20 v/v) Peak was obtained at retention time of 12.5 min, with flow rate of 1ml/min, Column Temperature of 30°C, at 242 nm wavelength. Further Screening was done using Plackett-Burman design & Optimization was done by carrying runs as by Box-Behnken design.

Instrument Qualification: Instrument qualification was done by considering combine parameter for operational qualification and performance qualification as it is mentioned in method section, result are given in **(Table 11)**

Method design

1) Plackett Burman Design

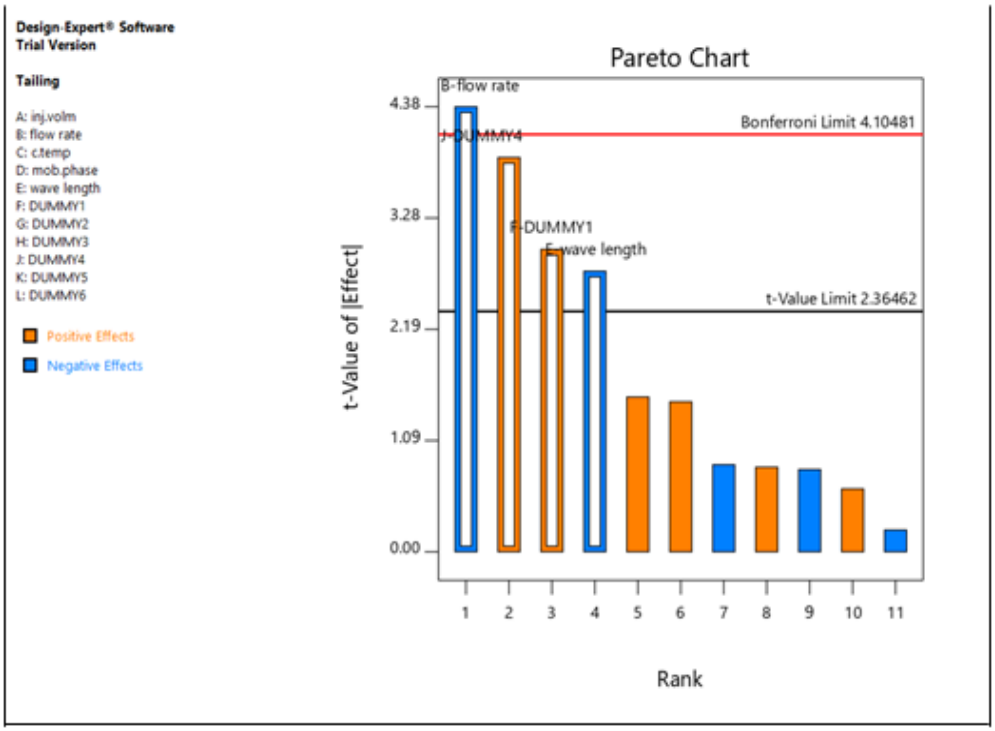


Figure 12: Pareto Chart.

Table 14: Regression coefficients and associated probability values (p-values) for USP Resolution of Famciclovir (Plackett Burman design).

Source	Sum of	Mean	F-value	p-value	
Model	0.029835	0.007459	12.65844	0.002544	Significant
B-flow rate	0.011285	0.011285	19.15242	0.003249	
E-wave length	0.004485	0.004485	7.61209	0.028136	
F-DUMMY1	0.005208	0.005208	8.839098	0.020718	
J-DUMMY4	0.008856	0.008856	15.03014	0.006077	
Residual	0.004125	0.000589			
Cor Total	0.03396				

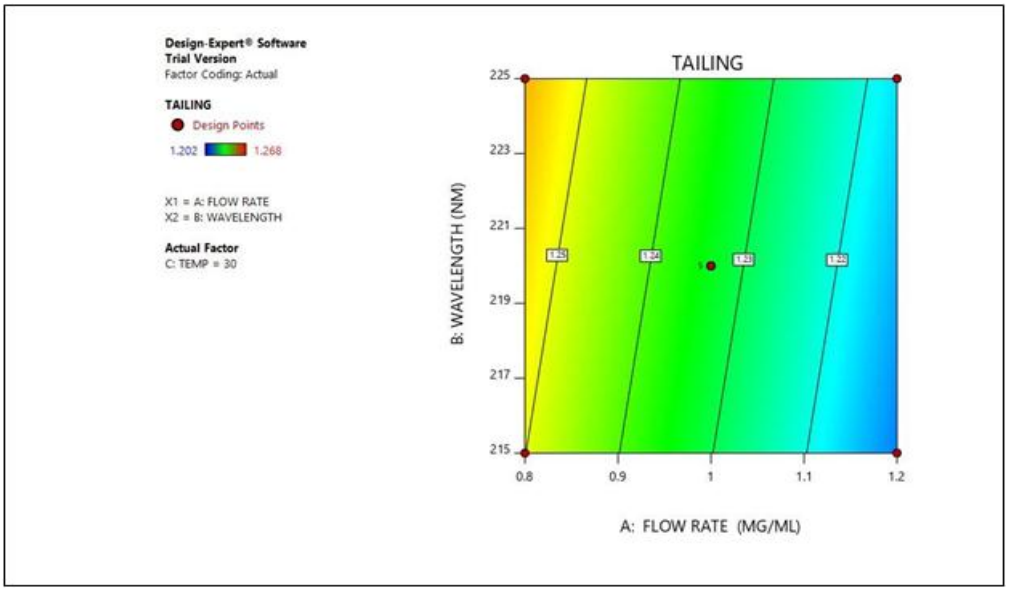


Figure 13: Usp Tailing.

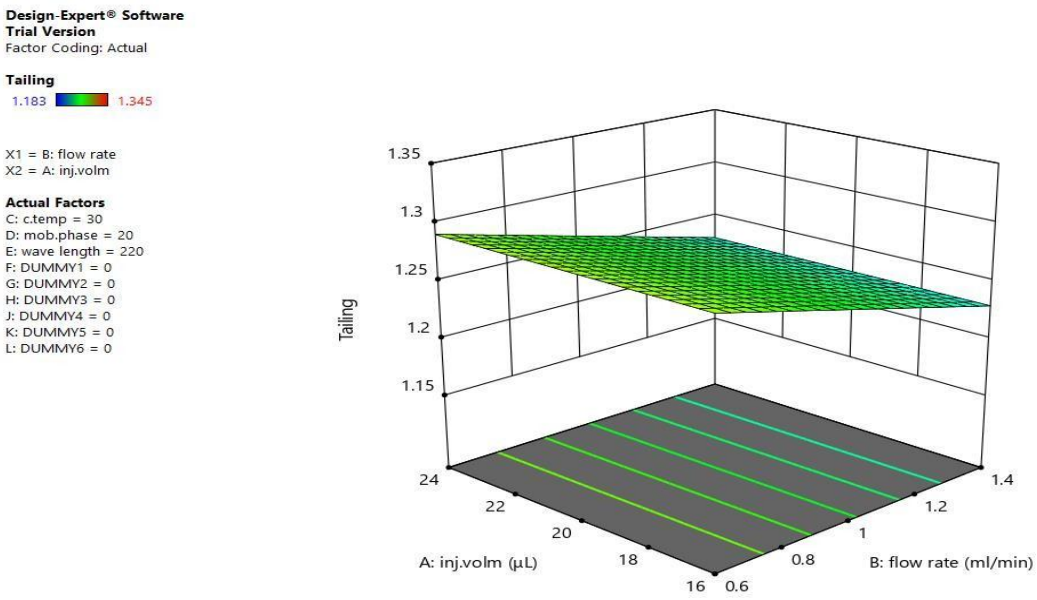


Figure 14: Response surface (3D) and contour plot showing the effects of Injection volume and Flow Rate on USP Tailing factor of Famciclovir (PlackettBurman design).

2) Box Behnken Design

Multivariate regression analysis was applied and fitted full quadratic model was obtained for the USP Resolution factor of peak. Factor considered here are Column Temperature, Acetonitrileconcentration., and Wavelength. Regression coefficient and p-values obtained from software generated report are given in **(Table 15**).

Table 15: Regression coefficients and associated probability values(p-values) for USP Resolution of Famciclovir (Box Behnken Design).

Source	Sum of	Mean	F-value	p-value	
Model	0.004373	0.001458	37.04619	1.23686E-06	Significant
A- FLOW RATE	0.00316	0.00316	80.31829	0.00	
B- WAVELENGTH	8.45E-05	8.45E-05	2.147667	0.16655189	
C-TEMP	0.001128	0.001128	28.67262	0.00013093	
Residual	0.000511	3.93E-05			
Cor Total	0.004884				

Analysis of variance (ANOVA) was perform to study the significance of the factors and interaction terms on the response i.e. USP Resolution of the peak, p-value simply provide the cut-off beyond which we assert that the findings are 'statistically significant' by convention, it is $p<0.05$. A value of Prob > F was found to be less than 0.05, hence model was found to be significant for prediction of response. Entire model was fitted well for optimization. A lack fit was not significant. Significant factors were found From this, Wavelength was found to be most significant Two of the factors were found to affect the Resolution from their respective coefficients. ACN concentration, Flow rate is showing inverse relationship with Resolution.

Response surface and contour plot were studied to visualize effect of factor so as to develop design space for robust method 3D graph are given below in **Figure.15.**

From the graph some facts about effect of the factors and their interaction on the response can be found. Curvatures in the contour plot show linear relationship between factors. From **Figure.15** showing effect of ACN concentration& Flow Rate where wavelength is constant at 265 nm,. ACN concentration should be between 8-12 % the Resolution was in limit and above and below this limit Resolution factor get increased. If Column Temperature and ACN concentration. gets increased then the Resolution gets affected.

To obtain optimum set of condition to achieve desired goal composite desirability parameters were applied. Response was set to Maximum Resolution between Famciclovir and the

impurity above target value of 2. Optimum condition having desirability was chosen from obtained runs i.e. Column Temperature of 30 °C , ACN concentration of 8% and Flow Rate 0.8ml/min. Set of conditions were analyzed to compare predicted response with actual response.

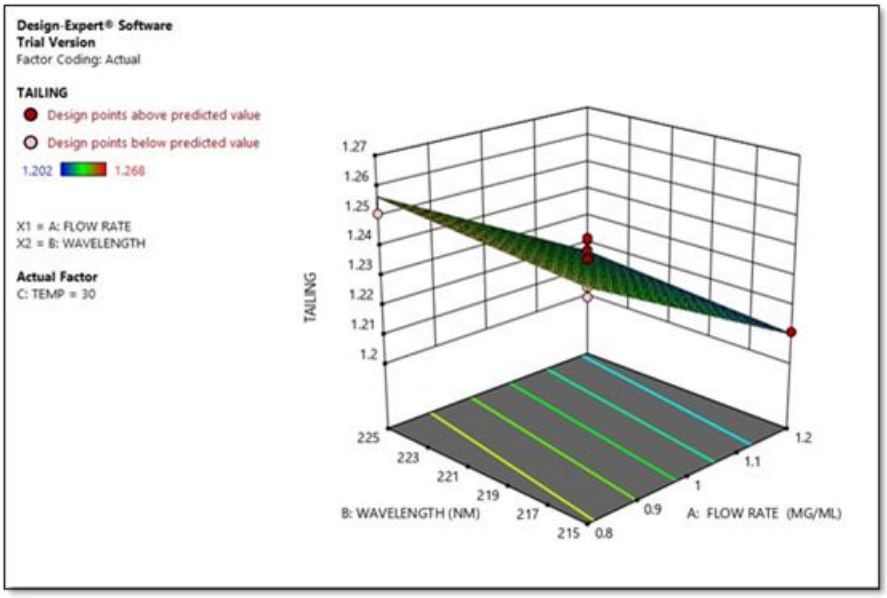


Figure 15: Response surface (3D) and contour plot showing the effects of Flow Rate and Wavelength on USP Tailing factor of Famciclovir (Box Behnken Design).

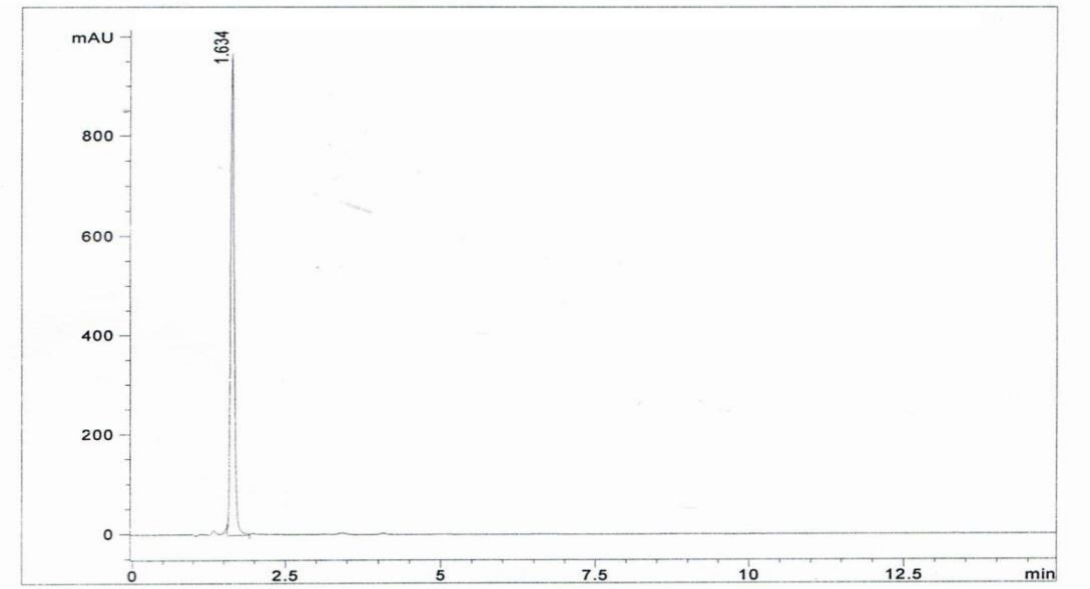


Figure 16: Chromatogram of Famciclovir. Method Validation.

Method validation was done according to the ICH guideline Q2.[36] Results were within the specified limit. Method was found to be accurate, precise and robust. Validation results are

given below in **(Table16).**

Table 16: Validation of method in term of linearity and precision of Famciclovir.

Validation parameter	Result	Acceptance criteria
Linearity (10 - 50 µg/ml)	Coefficient of Correlation-0.9987	Coefficient of Correlation- >0.999
Precision : Repeatability	RSD: 1.7320%	RSD less than 2%

Linearity

A set of five solution of Famciclovir at concentration ranging from 10-50 µg/ml were prepared. Each sample was analysed in triplicate, calibration curve was constructed by plotting the peak area verses the concentration using linear regression analysis. The correlation coefficient was found to be 0.997(**Table16) (Figure 17)**

Table 17: Linearity of Famciclovir.

Standard Concentration(µg/ml)	Peak area of Famciclovir0
10	1456
20	2927
30	4123
40	3973
50	5184
Regression equation	Y=139.07x
Regression coefficient	0.9972

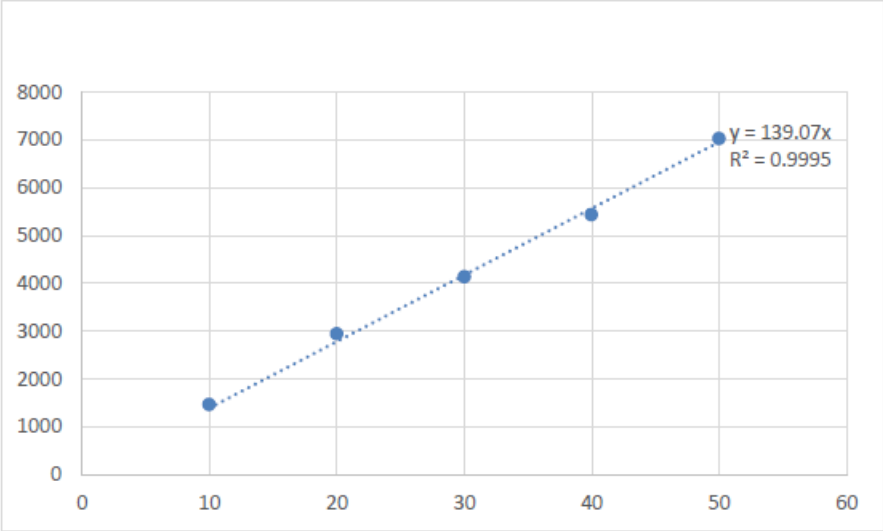


Figure 17: Linearity plot of Famciclovir Repeatability.

Repeatability was determined by running six replicates of samples and evaluating the average and %RSD for sample by comparing peak area. **(Table18).**

Table 18: Repeatability of Famciclovir.

Sr. No	Concentration (µg/ml)	Peak Area
1.	10	1428
2.	10	1432
3.	10	1489
4.	10	1499
5.	10	1508
6.	10	1526
Average		1480.33
%RSD		0.9421

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