Risk Assessment And Design Of Experiments (Doe) Approach In Rp-Hplc Method Development For The Analysis Of Palbociclib

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Abstract-

A Quality by Design (QbD) approach combined with risk assessment and Design of Experiments (DoE) was applied to develop and validate a simple, accurate, and robust RP-HPLC method for the analysis of Palbociclib in bulk and pharmaceutical dosage forms. Critical method parameters including mobile phase composition, pH, and flow rate were identified through risk assessment. A 2-level factorial design using Design Expert 8 software was used to optimize the chromatographic conditions.
The optimized method employed an acetonitrile:buffer mobile phase, achieving a sharp, symmetrical peak for Palbociclib at a retention time of 3.657 min. Validation was performed as per ICH Q2(R1) guidelines. The method showed linearity in the range of 10–60 µg/mL, with accuracy expressed as % recovery of 99.96–100.18%. Intraday and interday precision had % RSD values below 2%, confirming reproducibility. Robustness testing demonstrated that small deliberate variations in method conditions did not significantly affect retention time or peak response.
The validated method was successfully applied to marketed tablet formulations, with results in agreement with label claim and no interference from excipients. The study demonstrates that integrating risk assessment and DoE into RP-HPLC method development improves method understanding, robustness, and regulatory compliance.[68]

Keywords: Palbociclib, RP-HPLC, Quality by Design, Design of Experiments, Method Validation.

1.Introduction

Pharmaceutical Analysis:

Combination therapy or poly therapy is therapy that uses more than one medication1 . The Letrozole is indicated to treat postmenopausal women with hormone receptor (HR) positive early breast cancer,Palbociclib is indicated in combination with Letrozole as initial endocrine-based therapy for the treatment of human epidermal growth factor receptor type 2 (HER2)-negative and hormone receptor (HR)-positive tumors in adult patients with advanced/metastatic breast cancer. It is as well approved in combination with fulvestrant in patients with disease progression with prior endocrine therapy.This method was validated according to ICH guidelines for specificity, LOD, LOQ, Precision, Accuracy, and Linearity . The method showed good reproducibility and recovery with %RSD less than 2. Hence we had made an attempt to develop a simple accurate and precise RP HPLC method for the simultaneous estimation of Tamoxifen and Palbociclib in bulk and in tablet dosage form.[1]
Since the initial development and approval of palbociclib, firstin-class cyclin-dependent kinase 4/6 (CDK4/6) inhibitor, for estrogen receptor–positive/human epidermal growth factor receptor 2–negative (ER1/HER2–) advanced breast cancer (ABC) on the basis of PALOMA-1 in 2015,1-4 the CDK4/6 inhibitor class has transformed the treatment landscape and, in combination with endocrine therapy (ET), has become

the standard of care for the disease.5 PALOMA-2 confirmed the results of PALOMA-1 with statistically and clinically significant improvement in progression-free survival (PFS) for palbociclib plus letrozole versus placebo plus letrozole in ER1/HER2– ABC. At the time of the final analysis of the primary end point of PFS, overall survival (OS) data were immature. Here, we report results of the final OS analysis and updated safety data.[2]

Purpose A large-panel gene expression analysis was conducted to identify biomarkers associated with the effectiveness of adding palbociclib to fulvestrant.[3]

A phase 2 study showed that progression-free survival was longer with palbociclib plus letrozole than with letrozole alone in the initial treatment of postmenopausal women with estrogen-receptor (ER)–positive, human epidermal growth factor receptor 2 (HER2)–negative advanced breast cancer. We performed a phase 3 study that was designed to confirm and expand the efficacy and safety data for palbociclib plus letrozole for this indication.[4]

Human bone marrow mononuclear cells (hBMNC: a heterogeneous population that includes hematopoietic lineage cells such as lymphocytes, monocytes, stem cells, and progenitor cells) and CD34þ human bone marrow hematopoietic stem cells were primary cells purchased from Lonza. The cells were cultured in the hematopoietic progenitor growth (HPGM) medium (Lonza) supplemented with 10% FBS and in the presence of the following cytokines (R&D Systems): 25 ng/mL stem cell factor (SCF), 3 U/mL erythropoietin (EPO), 10 ng/mL G-CSF, 10 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF), 15 ng/mL thrombopoietin (TPO), 10 ng/mL IL3, 10 ng/mL IL6, and 25 ng/mL Flt3 ligand, in a 37C 5% CO2 and 98% humidity incubator. Human peripheral blood mononuclear cells (hPBMC) were primary cells purchased from Lonza, and cultured in the Roswell Park Memorial Institute media (RPMI). MCF-7 breast cancer cells were purchased from the ATCC and cultured in RPMI media supplemented with 10% FBS, in a 37C 5% CO2 and 98% humidity incubator.[5]

All these studies met their primary end point by markedly prolonging progression-free survival versus ET alone, with overall survival (OS) improvements in some settings[6]

We randomly assigned patients with hormone-receptor–positive, HER2-negative advanced breast cancer who had progression or relapse during previous endocrine therapy to receive palbociclib plus fulvestrant or placebo plus fulvestrant. We analyzed overall survival; the effect of palbociclib according to the prespecified stratification factors of presence or absence of sensitivity to endocrine therapy, presence or absence of visceral metastatic disease, and menopausal status; the efficacy of subsequent therapies after disease progression; and safety[7]

The purpose of this review is to summarize the background and latest evidence for the use of palbociclib, an oral, first-inclass, highly selective cyclin-dependent kinase 4/6 inhibitor, in advanced breast cancer, with a focus on some of the unanswered questions about the performance of this agent in clinical practice.[8]

Palbociclib was clastogenic in an in vitro micronucleus assay using Chinese Hamster Ovary cells and an in vivo rat bone marrow micronucleus assay through an aneugenic mechanism. Palbociclib can cause fetal harm based on its mechanism of action and findings in animals. Reports from rodent carcinogenicity studies were not submitted or required to support this application as the indication includes patients with advanced breast cancer.[9]

A robust and reliable RP-HPLC method was developed and validated for the estimation of Palbociclib in bulk and pharmaceutical dosage forms, in accordance with ICH Q2(R1) guidelines. Method optimization using statistical design revealed that flow rate, buffer pH, and temperature significantly influenced chromatographic responses.[10]

Palbociclib is a selective inhibitor of cyclin-dependent kinases 4 and 6 (CDK4/6) approved for the treatment of some cancers. The main mechanism of action of palbociclib is to induce cell cycle arrest and senescence on responsive cells. Here, we report that palbociclib concentrates in intracellular acidic vesicles, where it can be readily observed due to its intrinsic fluorescence, and it is released from these vesicles upon dilution or washing out of the extracellular medium. This reversible storage of drugs into acidic vesicles is generally known as lysosomal trapping and, based on this, we uncover novel properties of palbociclib. In particular, a short exposure of cells to palbociclib is sufficient to produce a stable cell-cycle arrest and long-term senescence.[11]

In estrogen receptor-positive (ER+) luminal breast cancer cell lines, palbociclib was shown to arrest tumor cell growth, accompanied by an inhibition of retinoblastoma protein hyperphosphorylation, and it also showed synergistic antitumor activity when combined with tamoxifen.[12]

Oral cancer chemotherapy with protein kinases such as CDK inhibitors involves administration of a variety of supportive medications such as analgesics, anti-helminthics, and acid-reducing agents (ARA), such as proton pump inhibitors (PPIs) (Cazzaniga et al., 2019). Supportive medicines are prescribed to alleviate the adverse effects of anticancer treatments.[12]

A critical factor the use of palbociclib in the treatment of brain tumors is achieving.The BBB acts as both a physical and biochemical barrier, limiting the brain delivery of numerous treatments (Abbott, 2013).[13]

Polymer disulfide bonds of NPs cleave in elevated GSH concentrations of the tumor microenvironment, resulting in drug release inside the cells. 22 For the first time in the literature, we have presented a novel approach to drug delivery by the incorporation of TPGS-SH, a redox-sensitive polymer, into PLGA-NPs. So far, no research paper has been reported on PLGA/TPGS-SH or PLGA/PLB or any of these combinations. Additionally, the formulation was designed to be entirely new. This developed PLGA-based formulation of PLB has been formulated and evaluated for the first time in breast cancer therapy through ultrasound and photoacoustic imaging.[14]

Our most recent studies with neratinib, alone or in combi nation with HDAC inhibitors, have demonstrate a unique biochemical action for the drug.3–5 Neratinib was originally developed as an irreversible inhibitor of ERBB2, and was then shown to inhibit ERBB1 and ERBB4.6 We discovered that neratinib was both a kinase inhibitor and that it possessed the ability to cause ERBB1/2/3/4 receptor internalization with their subsequent proteolytic degradation.[15]

Multidrug therapy is a particularly valuable strategy to improve the efficacy of cancer treatments, since it aims to act simulta neously on different disease hallmarks and in the mechanisms responsible for cell resistance to chemotherapeutics.[16]

Breast cancer (BC)is one type of cancer that 3 million people all over the world each year. It is estimated that there were more than 140000 women suffering from metastatic breast cancer (WBC) in2018,whileby2025, it is projected that more than169000 women will have WBC. In particular, future prognostications indicate 3million new breast cancer cases annually.[17]

In the ‘First Workshop on Pharmacology and Management of Cyclin-dependent kinase (CDK) 4/6 inhibitors (CDK4/6i): Consensus about Concomitant Medications’, promoted by the Spanish Breast Cancer Cooperative Group SOLTI, medical oncologists specialized in breast cancer joined physicians specialized in pharmacology, cardiology, psychiatry, infectious diseases, palliative care or radiation oncology in an interdisciplinary discussion forum, at Spanish national level, to address different issues regarding patients treated with palbociclib and ribociclib.[18]

The objectives of the present study were to describe the patient characteristics, real-world treatment patterns, clinical outcomes and selected adverse events in the first group of patients treated with palbociclib as part of the IPP in routine clinical practice.[19]

Activation of phosphatidylinositol 3-kinase (PI3K)–protein kinase B promotes cell survival and proliferation. The most important negative regulator of this pathway is phosphatase and tensin homologue deleted on chromosome 10 (PTEN), which antagonizes PI3K activity by dephosphorylating phosphatidyli nositol (3,4,5)-trisphosphate to phosphatidylinositol (4,5)-bisphosphate.[20]

Pharmaceutical analysis is the branch of science which deals with identification of substances and determination of amount present in particular sample. Pharmaceutical analysis contains the bulk materials, dosage forms and more recently, biological samples for bio-pharmaceutical and pharmacokinetic studies. Analytical chemistry can be divided into areas called qualitative and quantitative analysis. A qualitative analysis gives the information about the nature of sample by knowing about the presence or absence of specific components while quantitative analysis provides numerical information as that the relative amount of one or more of this component. Various analytical methods pharmaceutical formulations and biological fluids are generally being used for analysing the drug samples in bulk.

Conventional and physicochemical properties are used to analyse the sample in non-instrumental methods. For the measurements of some physical property of substance to analyse its chemical composition instrumental methods are used. As compared to classical methods the instrumental methods are simple, precise, and reproducible. So, analytical methods developed have wide applications in assuring the quality

and quantity of raw materials and end product by using sophisticated instruments such as spectrophotometer, HPLC, GC and HPTLC

Combination drug products play vital role in therapeutics as it traditional. Fixed-combination drugs may produce greater convenience, lower cost, and sometimes greater efficacy and safety, when rationally formulated. Analysis of multiple components has become one of the most interesting topics for analytical chemists in the last few years, in fields as clinical chemistry, drug analysis, and pollution control. [2]

Types of analysis:

Qualitative Analysis: To identity the product, i.e., it gives valuable hints from which the molecular or atomic species, the structural features, or the functional group in the sample can be identified.

Quantitative Analysis: To refer the purity of the product, i.e., the results are in the form of numerical data equivalent to the concentration of analytes.

Types of Analytical Methods: The various methods of analysis can be divided into two types. These are:

1. Chemical methods.
2. Instrumental methods.

Chemical Methods: In these methods, volume and mass are used as means of detection.

1. Titrimetric methods such as acid-base, oxidation-reduction, non-aqueous, complexometric and precipitation titrations.
2. Gravimetric and thermo gravimetric methods.
3. Volumetric methods.

Instrumental Methods: Based on principles, different Instrumental methods are available. These methods are depending on the measurement of specific and non-specific physical properties of a substance. Table-1.1 represents the instrumental methods and principle. At present, market is filled with various combinations in dosage forms and the number is increasing rapidly. Similarly, multi-components formulations because of a greater patient acceptability, increased potency, multiple action, fewer side effects and quicker relief are gaining attention. Therefore, it is desired that these formulations meet all the standards related to their quality, safety & efficacy and this can only be possible if they are analysed by various methods. The aim behind this quantitative estimation is to ensure that whether a particular drug contains the similar amount of drug as mentioned because if the dose given will be high then it will cause over dosage, side effects.[1,2]

Table No. 1.1: Table showing list of instrumental methods.

Sr. no.	Principle	Instrumental Method
1	Emission of radiation	X-ray emission spectrometry, Fluorescence spectrometry.
2	Absorption of radiation	UV/Visible, I.R Spectrophotometry, NMR Spectroscopy, ESR Spectroscopy, Atomic absorption spectrometry.
3	Mass to charge ratio	Mass spectrometry.
4	Refraction of radiation	Refractometry.
5	Scattering of radiation	Nephelometry
6	Rotation of radiation	Polarimetry.
7	Electrical potential	Potentiometry.
8	Electrical current	Amperometry, Polarography.
9	Electrical resistance	Conductometry
10	Thermal properties	Differential thermal analysis, Differential Scanning Calorimetry, Thermogravimetry.
11	Partition / Adsorption	Chromatographic Techniques.

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Sometimes with main drugs known to contain other substances which possibly interfere in the assay and if not corrected may impart a systemic error to the assay. The main objective to develop new methods to analyze the drugs simultaneously and without interferences is a basic need. Accordingly, it becomes necessary to develop new analytical methods for such drugs for which no analytical method is still available for estimation. [1][2][3]

Basic reasons for the development of newer methods of drugs analysis are:

- The drug or drug combination may not be official in any pharmacopoeias.
- Analytical methods for the estimation of drug in combination with other drugs may not be available.
- A literature search may not reveal an analytical procedure for the drug or its combination.
- Method may be available for simultaneous estimation but it may be tedious, time consuming & expensive.
- There may be a need for an alternative method to confirm for legal or scientific reasons, analytical data originally obtained by existing method.

The present analytical methods may require costly reagents and solvents. It may also involve clumsy extraction and separation procedure and these may not be reliable.

1.1.Analytical Method Development

Active pharmaceutical substances and the dosage forms having each single or multi-component formulated product are needed to be inspected. The ultimate reasons for this sudden legitimate surge in the newer evolving methodologies in the 'analysis of drug substances' is perhaps due to the incredible growth in the progress of 'medicinal chemistry' concerning achieving one ultimate objective which is to obtain 'better drugs for a better world'

As above huge growth in the design of complicated, specific and highly active drug molecules an equally viable, difficult, accurate and precise analytical methods have been evolved with the passage of time which have now occupied pivotal and important positions in most of the Official Compendia viz USP, BP, EP, IP etc., for the analysis of such compounds both in pure and dosage forms.

Consideration for initiating method development

a. Optimization versus new method
b. Method type
c. API/DP
d. Chemistry
e. Analytical Technique
f. Time constraints
g. Specification

Need for drug analysis:

The number of drugs announced into the market is increasing every year. These drugs may be either new individuals or partial structural modification of the existing one. Very frequently there is a time delay from the date of introduction of a drug into the market to the date of its insertion in pharmacopoeias. This occurs since the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities that results in their withdrawal from the market, development of long-suffering opposition and introduction of better drugs by competitor. It becomes necessary, therefore to develop newer analytical methods for such drugs. Also quality is important in every product or service but it is vital in medicines as it involves life. Quality control is a concept, which strives to produce a perfect product by series of measures designed to prevent and eliminate errors at different steps of production. The decision to release or reject a product is based on one or more type of control action. By means of the growth of pharmaceutical industry during last several years, there has been rapid progress in the field of pharmaceutical analysis involving complex

instrumentation. As long as simple analytical procedure for complex formulation is a matter of most importance.

The aims for the development of newer methods of drug analysis are:

- If any drug or drug combination may not be official in any pharmacopoeias,
- In the literature due to patent regulations, if a proper analytical procedure for the drug may not be available,
- If analytical methods may not be available for the drug in the form of a formulation due to the interference caused by the formulation excipients,
- If analytical methods for the quantitation of the drug in biological fluids may not be available,
- If analytical methods for a drug in combination with other drugs may not be available,
- If the existing analytical procedures may require expensive reagents and solvents.
  It may conjointly involve cumbersome extraction and separation procedures and these might not be reliable.[4][5]

MODERN ANALYTICAL CHEMISTRY:

Modern analytical chemistry is controlled by instrumental analysis. There are various types of instruments today that it can seem like a confusing array of acronyms rather than a unified field of study.

Most modern analytical chemistry is classified by different analytical methods. Analytical methods: -

- Spectrophotometry and colorimetry
  UV-visible spectroscopy
- Chromatography and Electrophoresis
  Commonly used methods are,
  - High Performance Liquid Chromatography (HPLC)
  - High Performance Thin Layer Chromatography (HPTLC)
  - Gas chromatography (GC)
  - Gas chromatography-Mass spectroscopy (GC-MS)
  - Liquid chromatography-Mass spectroscopy (LC-MS)

1.1.1. CHROMATOGRAPHIC METHODS:

Chromatography is a separation technique used to separate mixtures of substances into their components on the basis of their molecular structure and molecular composition. This contains a stationary phase such as a solid, or a liquid supported on a solid and a mobile phase like a liquid or a gas. The mobile phase moves through the stationary phase and carries the components of the mixture with it. Sample components that display stronger interactions with the stationary phase will move more slowly through the column than components with weaker interactions. This difference in rates cause the separation of many components. Chromatographic separations can be carried out using a variety of stationary phases, including immobilized silica on glass plates Stationary phase,

- Mobile phase,
- Sample injection system,
- Solvent delivery system,
- Column (support for stationary phase),

- **Chromatographic Methods Classification:**

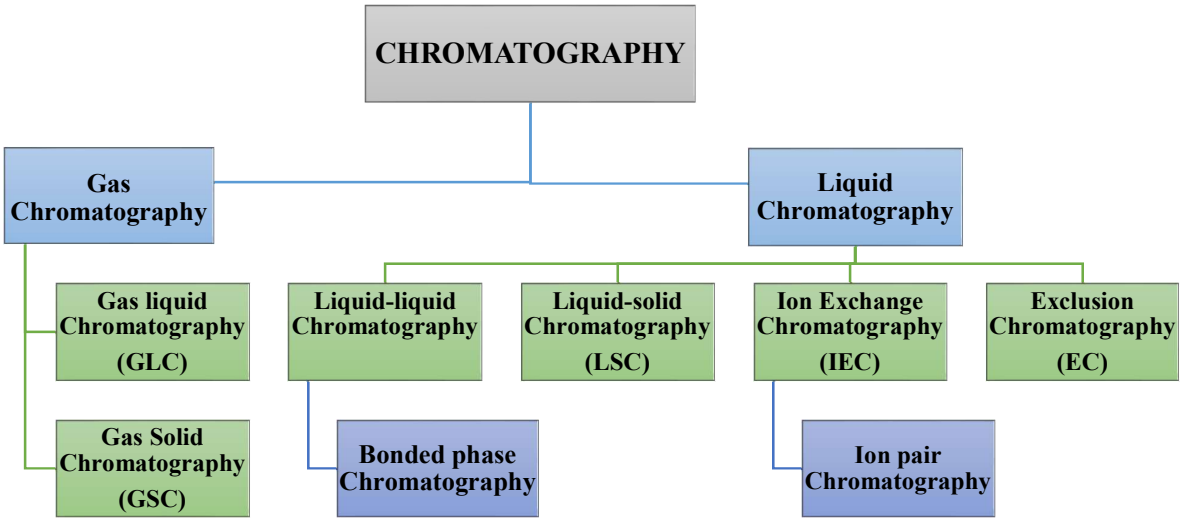


Fig.1.1: Chromatographic Methods Classification

Chromatographic methods can be classified according to the nature of the stationary and mobile phases. The different types of chromatography are:

1. Adsorption chromatography
2. Partition chromatography
3. Ion exchange chromatography
4. Size exclusion or gel permeation chromatography.

The modern instrumental techniques of GLC and HPLC offer excellent separation and allow precise assay of very low concentrations of wide variety of constituents in complex mixtures.[5,6]

Principles of chromatographic separation:

- Adsorption chromatography: Involves a solid stationary phase and a liquid or gaseous mobile phase.
- Partition chromatography: Includes a liquid stationary phase and a liquid or gaseous mobile phase.
- Ion exchange chromatography: Involves a solid polymeric stationary phase containing replaceable ions.
- Size exclusion chromatography: In this type, an inert gel which acts as a molecular sieve, and liquid mobile phase.

1.2.1 Gas Chromatography:

In gas chromatography, the components of a vaporized sample are parted as a consequence of being partitioned between a gaseous mobile phase and a liquid or solid stationary phase retained in a column. The gaseous mobile-phase in GC is called the carrier gas and it must be chemically inert. gas liquid chromatography (GLC) and gas solid chromatography (GSC) these are two types of gas chromatography. With GLC, the stationary phase is a non-volatile liquid fused to the inside of the column or to a fine solid support, whereas GSC is founded on a solid stationary phase in which retention of analytes occurs because of physical adsorption. Gas chromatography (GC) is one of the useful technique available to analyst. It is mostly used and capable of separating and analysing small quantities of sample even of great complexity series.

1.2.2 Adsorption chromatography:

In adsorption chromatography, the mobile phase containing the dissolved solutes which are passed over the surface of stationary phase, due to the solute affinity towards mobile or stationary phase, solutes gets separated. In normal phase the mobile phase is nonpolar and the stationary phase is polar while in reverse phase the mobile phase is polar and the stationary phase is nonpolar.

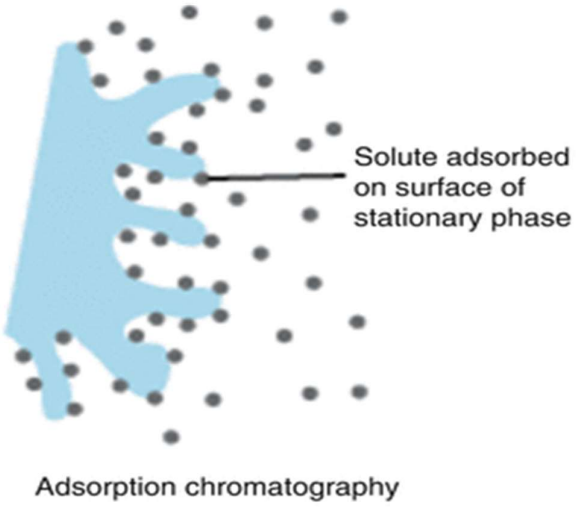


Figure No. 1.2: Principle of Adsorption Chromatography

1.2.3 Partition chromatography:

The principle of separation of partition chromatography is readily understood by considering the partitioning behavior of material between two immiscible liquids. Few substances, when shaken with two immiscible liquids, partition take place completely in to one or other liquid. Instead, most distribute themselves between the liquids such that the partition coefficient (the ratio of concentrations of the substance in each phase) is a constant value independent of the total amount, provided neither phase is saturated with the substance.[7]

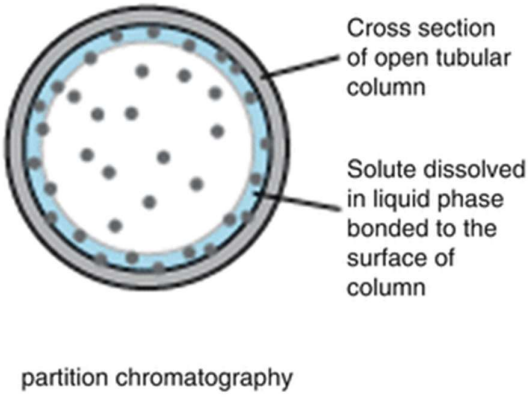


Figure No.1.3: Principle behind Partition chromatography

1.2.4 Mode of chromatographic operations:

There are three modes of chromatographic operation they are as follows:

- **Elution techniques**
  - Isocratic method
  - Gradient method
- **Frontal techniques**
- **Displacement techniques**

Types of chromatography techniques:

- **Planar chromatography**
- **Column chromatography**

HPLC (HIGH PERFORMANCE LIQUID CHROMATOGRAPHY)

HPLC stands for High Performance Liquid Chromatography. It is also called High Pressure liquid Chromatography. Before HPLC was available, LC analysis was carried by gravitational flow of the eluent (the solvent used for LC analysis) thus required several hours for the analysis to be completed. Even the developments added in later time were able to decrease the time of analysis slightly. Those classical/initial LC systems are called "low pressure chromatography" or "column chromatography". [55][56]

In 1970s in the US, Jim Waters founded Waters Corporation and started to sell HPLC instruments. This promoted the use of HPLC in practical analysis regions. The LC systems that Waters Corporation developed used high-pressure pump that generates rapid-flow of eluent, and thus resulted in dramatic enhancement in the time of analysis. By comparing with the “low pressure chromatography” the newer types were called "high pressure liquid chromatography".

Therefore, it was employed to be thought that HPLC stands for High Pressure Liquid Chromatography, however nowadays it is a common contract that HPLC stands for High Performance Liquid Chromatography. Another vast change from Tswett’s date was the data acquisition methods. Detector system was coupled to the LC for observing the changes of layers than visual observation, and results was recorded on paper chart. If we were to determine result of Tswett's analysis on a chart. Initially, HPLC system was referred to Waters Corporation's system. At the present time, Waters Corporation is the HPLC innovator, but there are some other companies that manufacture and sell HPLC systems.

Scientifically speaking, the word LC represents all the Liquid Chromatography, containing low pressure LC, however most LC systems used these days are HPLC thus often the word LC is used as comparable as HPLC.

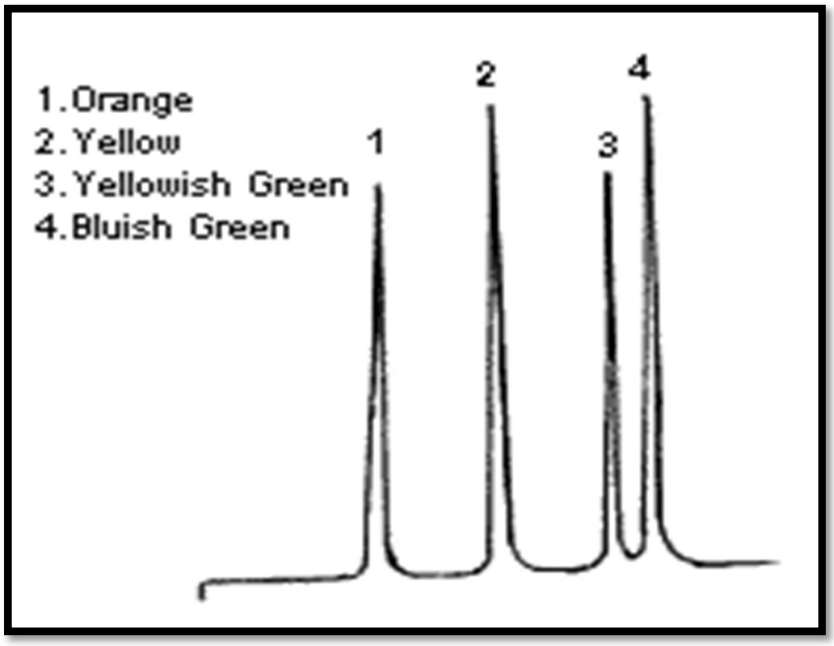


Figure No. 1.4: Representation of Tswett's LC analysis

Many drugs in dosage forms can be evaluated by HPLC technique because of the many advantages like rapidity, specificity, accuracy, precision and ease of automation. HPLC method removes tedious extraction and isolation procedures. [57][58]

Advantages of HPLC:

- Speed of analysis is very less i.e., analysis can be accomplished in 20 minutes or less,
- Having greater sensitivity,
- Resolution is enhanced by using wide variety of stationary phases,
- Expensive columns can be used for many analysis and these columns can be reusable,

- Best for the substances of low volatility,
- Ease of sample recovery, handling and maintenance,
- Instrumentation tends itself to automation and quantitation hence less time and less labour),
- Precise and reproducible,
- Calculations are done by integrator itself,
- Suitable on a widely larger scale for preparative liquid chromatography.

Modes of separation in HPLC

i. Normal phase mode:

Normal phase mode separates compounds on the basis of polarity. In this method, the stationary phase is polar and the mobile phase is nonpolar in nature. Nonpolar compounds travel faster and are eluted first due to lower affinity between the nonpolar compounds and that of the stationary phase. polar compounds are held for longer times due to higher affinity with the stationary phase. These compounds, therefore take more times to elute. Hence this type of chromatographic mode is generally not used for pharmaceutical applications because most of the drug molecules are polar in nature and hence take longer time to elute.

ii. Reversed phase mode:

This method is highly acceptable for analytical and preparative separations of compound of interest in chemical, biological, pharmaceutical, food and biomedical sciences. In this, the stationary phase is nonpolar hydrophobic packing with octyl or octa decyl functional group bonded to silica gel and the mobile phase is polar solvent. Because of aqueous mobile phase, use of secondary solute chemical equilibrium (such as ionization control, ion suppression, ion pairing and complexation) is allowed to control retention and selectivity. The polar compound gets eluted first whereas nonpolar compounds are held for longer time in column. As most of the drugs and pharmaceutical products are polar in nature, hence they are eluting faster. Octa decyl silane (ODS) or C18, C8, C4 are the columns which are used generally.[8][9][10]

i. Ion exchange chromatography:

In this type, the stationary phase encloses ionic groups like NR_3^+ or SO_3^- , which interact with the ionic groups of the sample molecules and so appropriate for the separation of charged molecules. Change in the pH and salt concentration can alter the retention.

i. Ion pair chromatography:

This can be used for the separation of ionic compounds and this method can also substitute for ion exchange chromatography. In general, strong acidic as well as basic compounds can be separated by reversed phase mode by forming ion pairs (columbic association species formed between two ions of opposite electric charge) with suitable counter ions. This method is referred to as reversed phase ion pair chromatography or soap chromatography.

i. Affinity chromatography:

Affinity chromatography is based on highly specific biochemical interactions for separation. The stationary phase contains specific groups of molecules which can adsorb the sample if certain steric and charge related conditions are satisfied. This technique can be used to isolate proteins, enzymes as well as antibodies from complex mixtures.

ii. Size exclusion chromatography:

Size exclusion chromatography usually separates molecules on the basis of their molecular mass. Largest molecules are eluted first and the smallest molecules last. This method is generally employed when a mixture contains compounds with a molecular mass difference of at least 10%. This mode can be further categorized into gel permeation chromatography (with organic solvents) and gel filtration chromatography (with aqueous solvents).

Components of HPLC are:

Following are the components of HPLC.

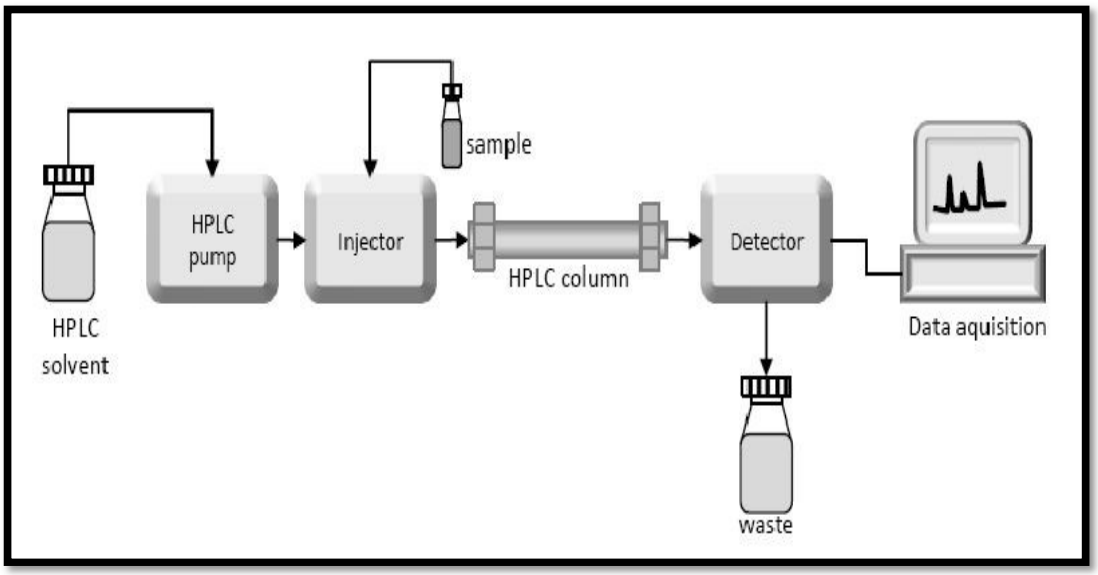


Figure No. 1.5: A schematic diagram of HPLC equipment

i.Reservoir:

Reservoirs are meant to store the mobile phase which is then pumped under pressure and flows through the column at a constant rate. In case of normal phase separation, an eluting power increases with increase in polarity of the solvent but for reversed phase separation, eluting power decreases with increasing polarity.

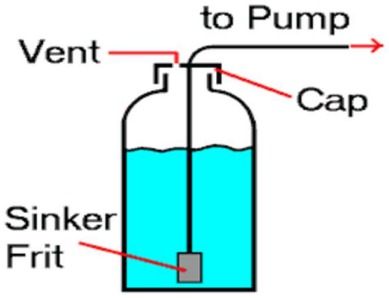


Figure No.1.6: HPLC Solvent Reservoir

ii.Degasser:

A degasser is needed to remove dissolved air with other gases from the solvent. Special grades of solvents are available for HPLC which are purified carefully so as to remove absorbing impurities as well as particulate matter to stop these particles from clogging the column and damaging the pumping system or injection system.

iii. Pumps

The pump is the most important part of HPLC system. Pump was situated in the most upper stream of the LC system and creates a flow of eluent from the solvent reservoir to the system. In the earlier stage of LC development, to be able to generate the high pressure was the one of the most important system requirements. However, at the present time, the high-pressure generation is a "standard" requirement and what is more concerned these days is to be able to provide a constant pressure at any condition, to provide a controllable and reproducible flow rate. So, the change in the flow rate can impact the analysis largely. Performance of pumps directly affects retention time, reproducibility and detector sensitivity, thus pump is important part of HPLC system. There are mainly three types of pumps used in HPLC to propel the liquid mobile phase through the system. These are:

a) **Displacement pump:** Displacement pump generates a flow that tends to independent of viscosity and backpressure and also output is pulse free. But it has limited capacity (250 ml).
b) **Reciprocating pump:** Reciprocating pump has small internal volume 35 to 400 µl, their high output pressure up to 10,000 psi and their constant flow rates, but it produces a pul.

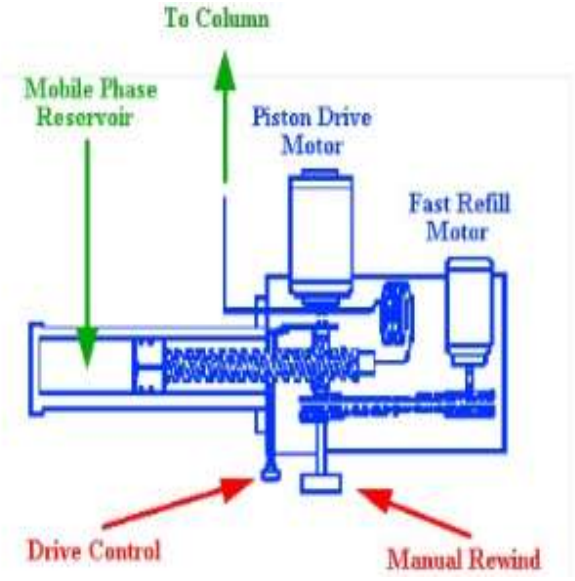


Figure No.1.7: Schematic representation of Displacement Pump

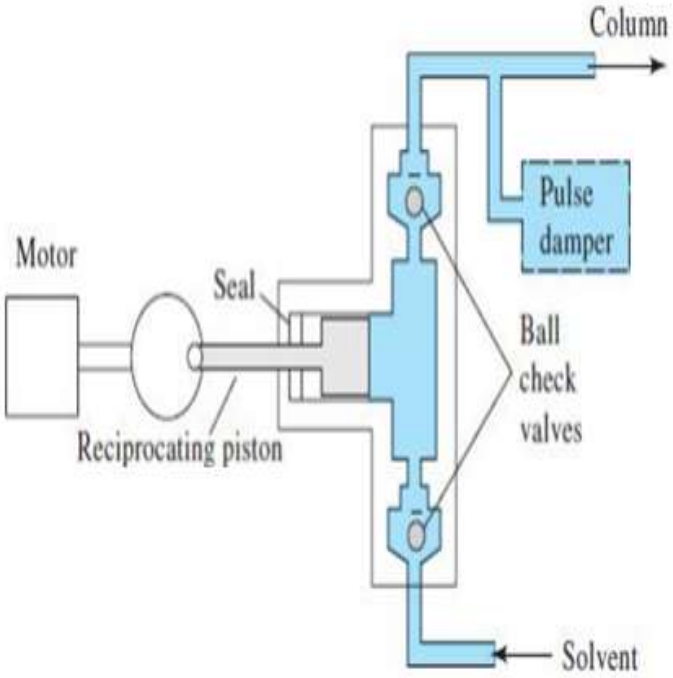


Figure No.1.8: Schematic diagram of Reciprocating pump

c) **Pneumatic or constant pressure pump:** These pumps are pulse free; suffer from limited capacity along with a dependence of flow rate on solvent viscosity and column back pressure and have pressure limit of less than 2000 psi.

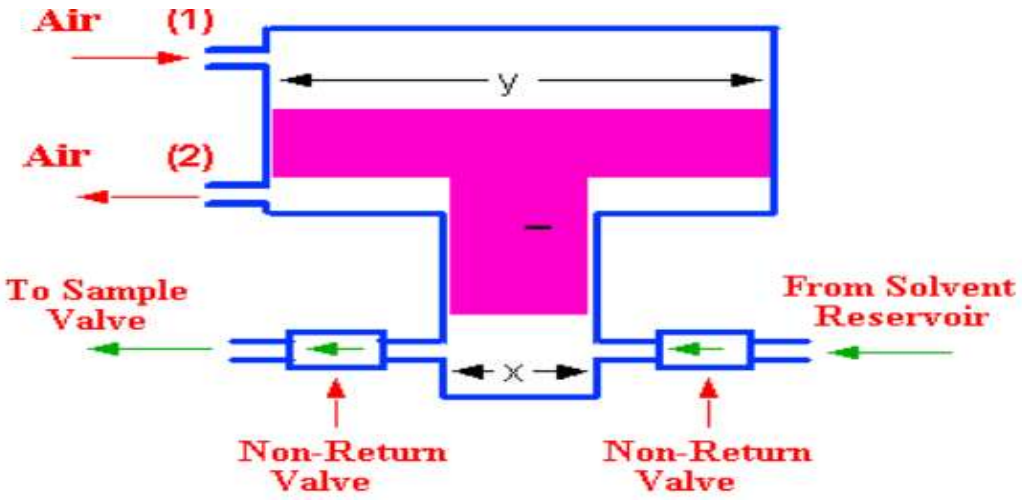


Figure No. 1.9: schematic representation of Pneumatic pump

iv. Sample injection system:

Sample injector is located next to the pump. Use of syringe is the easiest method and by the injector sample is introduced to the flow of eluent. The design of injector is an important factor because the precision of LC measurement is mostly affected by the reproducibility of sample injection. The most widely used injection method is based on sampling loops. The use of autosampler system is also commonly used that permits repeated injections in a set scheduled-timing. The care should be taken about the insertion of sample in column that, sample in form of narrow plug should be inserted so as to avoid attribution to peak broadening. There should be no dead (void) volume in injection system.

Following are the important ways to introduce the sample into injection port:

- Loop injection: With the use of fixed volume loop injector, a fixed amount of volume is introduced.
- Valve injection: With the use of an injection valve, a variable volume is introduced.

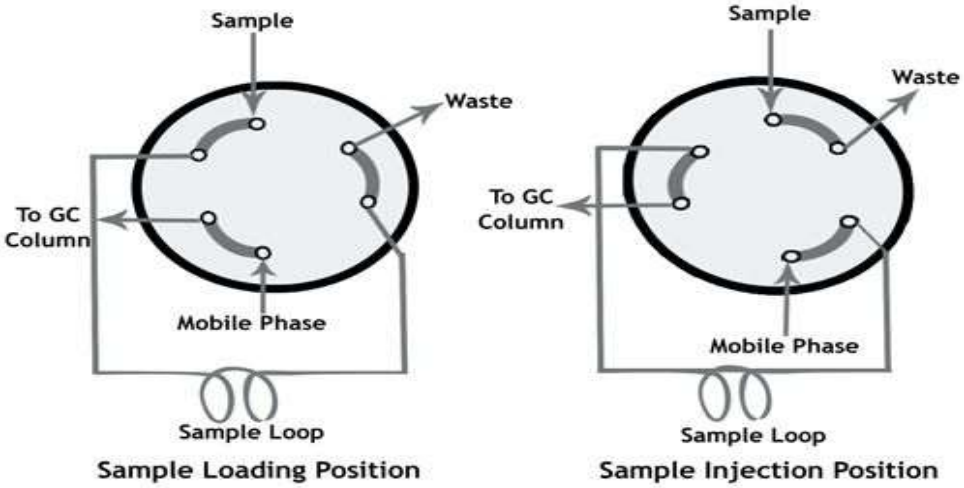


Figure No. 1.10: Schematic representation of sample injection system

v.Chromatographic column:

The chromatographic column is usually made up of heavy glass or stainless-steel tubing so that it withstands high pressure. The columns have length of 10-30 cm and inside diameter of 4-10 mm containing stationary phase at particle diameter of 25 µm or less. Columns having internal diameter of 5 mm give good results due to compromise between efficiency, sample capacity, and the amount of packing and solvent required. The column packing used in modern HPLC consists of small, rigid particles having a narrow particle size distribution.

4 Detector

Separation of compounds is carried out inside the column, while a detector is used to observe the obtained separation. The composition of the eluent is reliable when no component is present. While the presence of

analyte changes the composition of the eluent. The function of detector is to measure these differences. This difference is examined as a form of electronic signal. Various types of detectors are used in HPLC.

5 Recorder

The change in eluent identified by a detector is in the form of electronic signal, and hence it is still not visible to our eyes. In older days, pen (paper)-chart recorder was generally used. These days, computer-based data processor (integrator) is more common. There are various types of data processors; examples include a simple system involving in-built printer and word processor, and a personal computer type containing display monitor, keyboard, and printer. Also, there are software that are specifically designed for LC system. It gives not only data acquisition, but features like peak-fitting, base line correction, automatic concentration calculation, molecular weight determination, etc.

The components introduced so far are the basics of LC system. Below is some optional equipment used with the basic LC system. [11][12][13][14]

Types of column packing in HPLC:

1. Porous, polymeric beds: Porous, polymeric beds based on styrene divinyl benzene co-polymers used in ion exchange and size exclusion chromatography. For analytical purpose these have now been replaced by silica based, packing which are more efficient and more stable.

2. Porous layer beds: Generally consist of a thin shell (1-3 μm) of silica / modified silica on spherical inert core (e.g. Glass).

3. Totally porous silica particles (dia. 20μm are usually dry packed)

RF = Ax / AISTD

On the basis of the response factor and strength of the internal standard (NISTD), the amount of the analyte in the original sample can be calculated using the formula,

X =AS / RF × AISTD × N

After confirming the linearity of the calibration curve for the internal standard and the analytical reference standard of the compound of interest, above described calculations can be used. When more than one component is to be analysed from the sample, the response factor of each component should be determined in the calculations using ISTD similar formula.

Parameters affected by change in chromatographic conditions:

The parameters that are affected by the changes in chromatographic conditions are:

a. Resolution (Rs)
b. Capacity factor (k'),
c. Selectivity (α),
d. Column efficiency (N) and
e. Tailing factor (T)

a. Resolution (Rs)

Resolution describes the separation power of the complete chromatographic system relative to the particular components of the mixture. The resolution, Rs, of two neighbouring peaks is defined as the ratio of the distance between two peak maxima. Resolution is the difference between the retention times of two solutes divided by their average peak width. The ideal value of Rs is 1.5 for the baseline separation. It is calculated by using the formula,

$$RS = \frac{(t_{R2} - t_{R1})}{0.5(w_1 + w_2)}$$

Where tR1 & tR2 are the Retention time of the two compounds and w1 & w2 are the width of the two compounds.

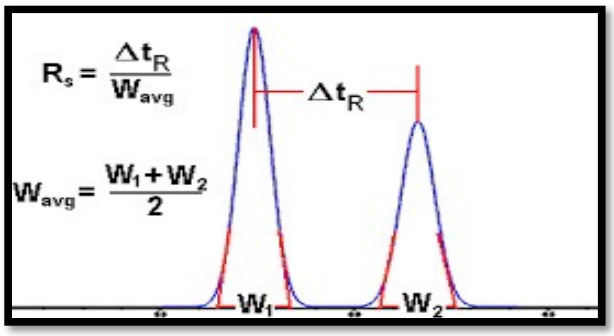


Figure No. 1.11: Resolution Chromatogram

b. Capacity Factor (k')

Capacity factor is the ratio of the reduced retention volume to the dead volume and is defined as the ratio of the number of molecules of solute in the stationary phase to the number of molecules of the same in the mobile phase. It is a measure of how well the sample molecule is retained by a column during an isocratic separation. 2-10 is the range of ideal value of k'. Capacity factor is calculated by using the formula,

$$K = \frac{\textbf{Moles of solute in stationary phase Capacity factor}}{\textbf{Moles of solute in mobile phase}}$$

$$K' = \frac{(t_R - t_0)}{t_0}$$

where t_R is the retention time for the sample peak and
t_0 is the retention time for an unretained peak.

The values of k' of individual bands increase or decrease with changes in solvent strength. In RP-HPLC, solvent strength increases with the increase in the volume of organic phase in water/ organic mobile phase. Usually, an increase in percentage of the organic phase by 10% by volume will decrease k' of the bands by a factor of 2-3.

Adjusting capacity factor (k'):

Good isocratic method usually has a capacity factor (k') in the range of 2 -10 (typically 2 – 5). Lower values may give inadequate resolution. Higher values are usually associated with excessively broad peak and unacceptably long run time. If the shift in k' value is observed with both analyst and the column test solution, the problem is most likely due to change in column, temperature or mobile phase composition. This is true if the shift occurs gradually over series of run.

Capacity factor (k') values are sensitive to:

- Solvent strength
- Composition
- Purity
- Temperature
- Column chemistry
- Sample

c. Selectivity (α)

The selectivity or separation factor (α), is a measure of relative retention of two components in a mixture. It is the ratio of the capacity factors of both peaks, and the ratio of its adjusted retention times. It denotes the separation ability of specific adsorbent to the mixture of these specific components.

Selectivity parameter is independent of the column efficiency; but they only depend on the nature of the components, eluent type, and eluent composition, and adsorbent surface chemistry. If the selectivity of two components is equal to 1, then there is no method to separate them by improving the column efficiency.

The ideal value of α is 2. It can be calculated by using formula,

$$\alpha = \frac{k2}{k1} = \frac{tR2 - t_0}{tR1 - t_0}$$

Where,

t_{R1} is the retention time of compound one
t_{R2} is the retention time of compound two

t_0 is the dead time which refers to the elution time of an unretained compound or the time needed for the solvent to travel through the cartridge.

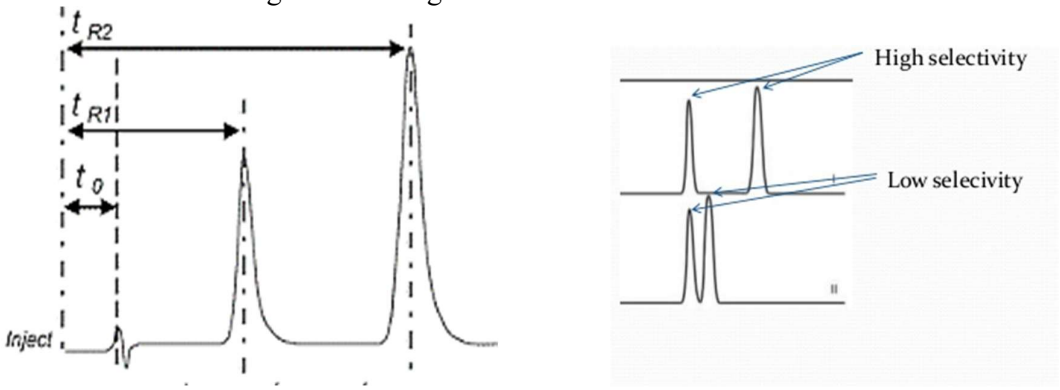


Figure No. 1.12: Determination of retention factor

Adjusting selectivity (α)

When troubleshooting changes in selectivity (α), the approach is similar to the approach used in the capacity factor. If selectivity is affected, the corrective action depends on whether the problem is mobile phase or column related. Be sure to compare results obtained with the test solution to those observed when the column was new.

Selectivity (α) values are sensitive to:

- Variation in mobile phase composition (pH ionic strength)
- Purity
- Temperature

d. Column efficiency/ Band broadening

Column efficiency (N), is measured by the number of theoretical plates per meter. Column efficiency is a measure of band spreading of a peak. Alike the band spread, higher is the number of theoretical plates, indicating good column and system performance. Column with N ranging from 5,000 to 1, 00,000 plates/meter are ideal for a good system.

Efficiency is calculated using the formula,

N = 16 Rt2 / W

Where, Rt is the retention time and W is the peak width.

A decline in measured column efficiency may be due to:

- Age and history of the column
- Extra column band broadening (such as due to malfunctioning injector or improper tubing
- Inappropriate detector setting
- Change in flow rate and solvent viscosity

You can recognize problems in your separation due to a loss of column efficiency when the width or shapes of all peaks are affected.[15,16,17,18]

Methods of measuring column efficiency (N)

Method used for the measurement and calculation of column include (in order to sensitivity to abnormal peak shape):

- Based on Asymmetry (most sensitive to tailing or fronting)
- 5 sigma
- 4 sigma
- Tangent
- 3 sigma
- ½ height
- 2 sigma (least sensitive to tailing or fronting)

Choose the method that best suits your operating requirements. It is significant that the same method always be used and executed reproducibly.

e. Tailing factor (T)

The symmetry of a peak is given by the following equation.
where W0.05 is the peak width at 5% height,
f is the distance from peak front to apex point at 5% height.

$$T = \frac{W0.05}{2f}$$

Ideally, peaks should be Gaussian in shape or totally symmetrical.
The accuracy of quantitation decreases with increase in peak tailing due to the problems met by the integrator in analysing where/when the peak ends and thus the calculation of the area under the peak. Integrator variables are pre-set by the analyst for optimum calculation of the area for the peak of interest. [119,20]

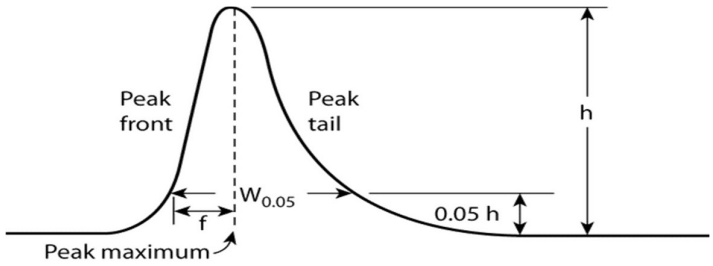


Figure No. 1.13: Asymmetrical chromatographic peak.

Application of HPLC:

The HPLC is applicable for resolution, identification and quantification of a compound. It also helps in chemical separation and purification. The additional applications of HPLC include:

- **Pharmaceutical Applications**

1. To control stability of drug.
2. Tablet dissolution study of pharmaceutical dosages form.
3. Pharmaceutical quality control.

- **Environmental Applications**

1.To detect phenolic compounds in drinking water.
2.For bio-monitoring of pollutants.

- **Forensic Sciences**

1. To quantify drugs in biological samples.
2. To identify steroids in blood, urine etc.
3. Forensic analysis of textile dyes.

- **Food and Flavour**

1. To measure the quality of soft drinks and water.
2. To analyse sugar in fruit juices.

- **Applications in Clinical Tests**

1. Urine analysis, antibiotics analysis in blood.
2. To analyse bilirubin, biliverdin in hepatic disorders.
3. To detect endogenous Neuropeptides in extracellular fluid of brain etc.[21][22]

VALIDATION OF ANALYTICAL METHOD

"Validation of analytical method is an act of proving that any procedure, process, equipment, material, activity or system performs as expected under given set of conditions and also give the required sensitivity, accuracy, precision, ruggedness, etc."

Depending upon the application, if delayed to an analytical procedure, it means that a method works reproducibly, when carried out by same or different persons, in same or different laboratories, using different reagents, different equipments, etc. [43]

The different validation parameters are:

a) Accuracy,
b) Precision,

c) Linearity and Range,
d) Limit of detection (LOD),
e) Limit of quantitation (LOQ),
f) Selectivity/ Specificity,
g) Robustness/ Ruggedness and
h)Stability and System suitability studies

Reasons for Validation

There are two important reasons for validation of assays in the pharmaceutical industry.
1.Assay validation is an integral part of the quality-control system and this is most important reason.
2.And second is current good manufacturing practice regulation requires assay validations

Advantages of Analytical method Validation[44]

- Analytical method validation builds a degree of confidence, not only for the developer but also to the user.
- Validation exercise may look like time consuming and costly, however it results inexpensive, eliminates frustrating repetitions leading to better time management in the end.
- Minor changes in the conditions such as reagent supplier or grade, analytical setup are unavoidable due to obvious reasons but the method validation absorbs the shock of such conditions and pays for more than invested on the process.
- Different performance parameters delivered in a validation exercise, are categorized as follows.

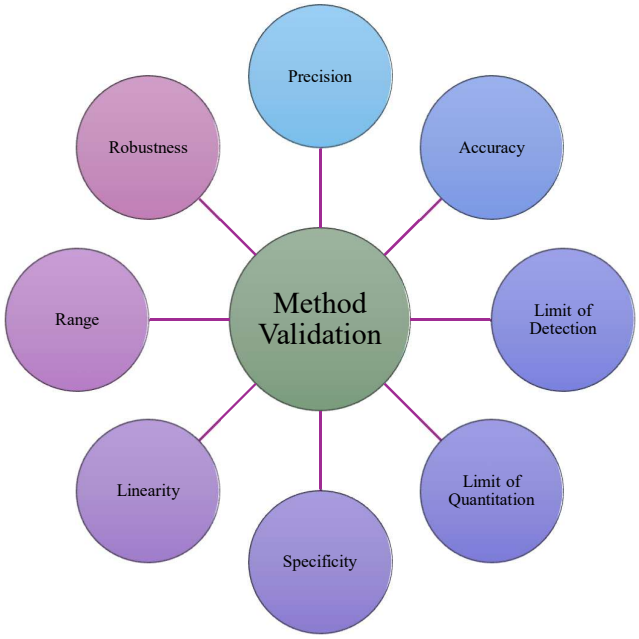


Figure No.1.14: Key parameters of the analytical method validation

Accuracy

The accuracy is the closeness of the measured value to the true value for the sample. The ICH documents suggested that accuracy should be evaluated using a minimum of nine determinations over a minimum of three concentrations levels the specified range i.e., three concentrations and three replicates of each concentration. Accuracy was tested (% Recovery and % RSD of individual measurements) by evaluating samples in triplicate, at each level (80,100 and 120 % of label claim) is advised. For an individual evaluation fresh samples were prepared and assay value is calculated. From regression equation obtained in linearity study recovery was calculated. Accuracy was determined from the mean relative error for a set of replicate analysis i.e. the difference between measured and nominal concentration for spiked samples. [23]

Precision

It is defined as the closeness of agreement between series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. Precision of an analytical method is generally given in terms of the standard deviation, relative standard deviation or coefficient of variations of a series of measurements. The ICH documents mention the repeatability should be determined using a

minimum of nine determinations including specified range of procedure. Precision may be measure of either the degree of reproducibility or of repeatability of the analytical method under normal operating conditions.

$$\boldsymbol{Stanadard\ Deviation} = \sqrt{\frac{\sum(x-\overline{x})^2}{n-1}}$$

Where,

x = sample,

$\bar{x}$ = mean value of samples,

n = number of samples [24]

$$\boldsymbol{Relative\ Standard\ Deviation} = \frac{\boldsymbol{Standard\ Deviation}}{\overline{\boldsymbol{x}}} \times \mathbf{100}$$

Repeatability:

Repeatability says that the precision under the same operating conditions over a short interval of time. Repeatability is also called intra –assay precision.

Intermediate Precision:

Intermediate precision expresses variations in laboratory: different days, different analyst and different equipment.

Reproducibility:

When the experiment is performed by different analyst in different laboratories using different equipment, regents and laboratories setting. Reproducibility was assessed by measuring repeatability and intermediate precision. Reproducibility is determined by an inter-laboratory trial.

Linearity and Range

The linearity of an analytical procedure is its ability to obtain test results that are directly proportional to concentration of analyte in samples. The range of an analytical method can be described as interval between the upper and lower levels of the analyte (including these levels) that have been demonstrated to be determined with precision, accuracy and linearity using the method as written.

$$\boldsymbol{r^2} = \frac{\boldsymbol{n(\sum xy) - (\sum x)(\sum y)}}{\sqrt{\boldsymbol{(n\sum x^2) - (\sum x)^2\,(n\sum y^2) - (\sum y)^2}}}$$

Determination of linearity and range:

These characteristics are determined by application of the procedure to a series of samples having analyte concentration spanning the claimed range of procedure. When the relationship between response and concentration is not linear, standardization may be providing by means of a calibration curve. The ICH guidelines mention that for the establishment of linearity minimum five concentrations are generally used.[25,26]

Limit of detection (LOD)

Limit of detection is the lowest amount of analyte in a sample that can be detected, but not necessarily quantifies as an exact value, under the specified experimental conditions. The detection limit is generally given as the concentration of analyte (percentage parts per million) in the sample. [27]

Determination of detection limit

Detection limit for instrumental and non- instrumental methods is usually determined by the analysis of samples with known concentration of analyte and by establishing the minimum level at which the analyte can be detected.

$$\boldsymbol{LOD} = \frac{\mathbf{3.3} \times \boldsymbol{Standard\ Deviation}}{\boldsymbol{Slope}}$$

Limit of quantification (LOQ)

It is the lowest amount of analyte in a sample that can be analyse with acceptable precision and accuracy under the specified experimental conditions. Quantification limit is given as the concentration of analyte (e.g.: % ppm) in the sample.

Determination of quantification limit

Detection limit for instrumental and non- instrumental methods is usually determined by the analysis of samples with known concentration of analyte and by establishing the minimum level at which the analyte can be recognized with acceptable accuracy and precision.

$$LOD = \frac{10 \times Standard\ Deviation}{Slope}$$

Selectivity and Specificity

The selectivity of an analytical method can be stated as its ability to measure accurately and specifically the analyte of interest in the presence of components that may be expected to be present in the sample matrix. If an analytical procedure is able to separate and resolve the various components of a mixture in addition to identify the analyte qualitatively, then the method is called selective. One fundamental difference in the selectivity and specificity is that, whereas the previous is restricted to qualitative detection of the components of a sample, the latter means quantitative measurement of one or more analytes.[28]

Robustness and Ruggedness

1) Robustness

"The robustness is defined as a measure of its capacity to remain unaffected by small but deliberate variation in method parameters and provides an indication of its reliability during normal usage." The robustness needs that methods characteristic are evaluated when one or more operating parameter varied.

2) Ruggedness

"The ruggedness is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions such as different laboratories, different analysts, using operational and environmental conditions that may differ but are still within the specified parameters of the assay." Testing of ruggedness is advised when the method is to be used in more than one laboratory. It is usually expressed as the lack of the influence on the test results of operational and environmental variables of the analytical method.

The degree of reproducibility of test result is determined as function of the assay variable. This reproducibility can be compared to the precision of the assay under normal condition to obtain a measure of the ruggedness of the analytical method.[29]

Stability and System suitability tests

Stability of the standard, sample as well as reagents is required for a reasonable time to generate reproducible and reliable results.

Test of system suitability offer the added assurance that on a specific occasion the method is giving, accurate and precise results. The results obtained of each system suitability test are then compared with defined acceptance criteria and if they pass, the method is deemed satisfactory on that occasion. The nature of the test and the acceptance criteria will be based upon data generated during method development optimization and validation experiments.[30]

INTRODUCTION TO QUALITY BY DESIGN

Quality has been given an importance by all regulatory bodies for pharmaceutical products. Quality indicates customer satisfaction in terms of service, product, and process. Many of these quality related activities reflect requirement for companies to excel in global competition. Customer demands the perfection in quality, reliability, low cost and timely performance. Customer satisfaction can be attained by two ways, first features and second free from deficiencies in goods. The features like performance, trustworthiness, robustness, ease of use, and serviceability have to build in the product and such product should be free from deficiencies. Quality, productivity, cost, cycle time and value are interconnected terms. Quality activities must try to find quality problems early enough to allows actions without demanding compromise in cost, schedule or quality. The emphasis must be on precaution rather than on just correction of quality problems. Quality can be the driving force to empower results in other parameters. Hence the quality has to be built in the product along with services through proper planning, thus the forth coming failure can be avoided. Mere analysis of final product will not work but the quality should be designed in the product. The concept of quality by design was summarized by a well-known quality expert Joseph Moses Juran; he believed that quality could be planned and that most quality related problems have their origin in the way which quality was planned in the first place. The principles of QbD have been used to advance the product and process quality in every industr.[31][32][33][34]

Historical background

In 2007 FDA received 5000 supplements, it was actually a striking raise in the number of manufacturing supplements to applications of New Drug Applications (NDAs), Biological License Applications (BLAs) and Abbreviated New Drug Applications (ANDA's). FDA recognized that there is an increase in lapse of NDA or ANDA submissions by the firms, large number of a supplemental application for every manufacturing change were received. In both original applications and supplements the data mainly focused was on chemistry. [35]

Advantages of QbD:

1.QbD ensures that the designing of a product is made in a way to fulfil the needs of the patient and requirements for better performance.
2.Scientific understanding of pharmaceutical process and method.
3. It includes process development and design of product.
4.Scientific Risk assessment is carried out.
5.Effect on quality of end product is analysed after identification of Critical quality attributes .
6.It offers robust method or process.
7.Method or process becomes flexible.
8.It provides required design space for development.
9.Control strategy can be maintained throughout the analysis.
10.It allows continuous improvement till finished steps of method.[36]

4.1 Elements of pharmaceutical development

QbD includes all elements of pharmaceutical development mentioned in the ICH guideline Q8. To design a quality product and its manufacturing process to constantly deliver the intended performance of product is the goal of pharmaceutical development. The data and knowledge gained from pharmaceutical development studies and manufacturing experience offers scientific understanding to support the establishment of the specifications, and manufacturing controls.

Different elements of pharmaceutical development include,

- Defining an objective
- Determination of critical quality attributes (CQA)
- Risk assessment
- Development of experimental design
- Designing and implementing control strategy
- Continuous improvement.[37]

4.1.1. Define an objective

The basis of QbD is Quality target profile (QTP), which is in relation to the predefined objective criteria stated in the definition of QbD. Considerations for the Quality Target Product Profile could include-

- Intended use in clinical setting,
- route of administration,
- dosage form, delivery Systems.
- Dosage strength(s),
- Container closure system.
- Therapeutic moiety release or delivery and attributes affecting,
- Pharmacokinetic characteristics (e.g., dissolution, aerodynamic performance).

Drug product quality standards like sterility, purity, stability and drug release as appropriate for dosage form the planned for marketing.

QbD needs a Target Product Profile called as Quality Target Product Profile (QTPP) which defines the expectations in final product. In case of analytical method development, it is called as analytical target profile (ATP), also called as Target Product Profile (TPP). The TPP can play a central role in the entire drug discovery and development processes like optimization, planning and decision making, and designing of clinical research strategies. The Target Product Profile (TPP) can be used to design the clinical trials, safety and ADME studies, with drug product design. The TPP will help to recognize critical quality attributes such as potency, purity, bioavailability or Pharmacokinetic profile, shelf life, and sensory properties.[38][39]

4.1.2. Determination of critical quality attributes. (CQA)

According to ICH Q8 R2 ‘‘A CQA is a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality’’. CQAs are usually linked with the drug substance, excipients, intermediates and drug product. The CQAs can additionally include properties like particle size distribution, bulk density that affect drug product. Mostly CQAs are derived from the QTPP and/or prior knowledge is used to guide the product and process development and Subsequently CQAs are accessed for risk management.

In ICH Q9 it is mentioned that in case of Potential drug substance CQAs are used to guide process development. A quality attribute that must be controlled within predefined limits to ensure that the product meets its intended safety, efficacy, stability and performance. [40][41]

4.1.3. Risk assessment

Risk is defined as the combination of the probability of occurrence of harm and the severity of that harm. Risk assessment is useful to increase quality of method or process. Also, it is determinant for effect of input variable on method or processes. From risk assessment one can identify critical attributes that are going to affect quality of end product. A risk assessment is helpful for effective communication between FDA and industry, research/development and manufacturing and among multiple manufacturing sites within company. Risk management for excipients to determine shelf life can be done by statistical parameters. [42]

Methods of risk assessment: Various methods of risk assessment are stated in ICH guideline Q9 as follows:

- 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 (HAZOP);
- Preliminary Hazard Analysis (PHA); Risk ranking and filtering;
  Supporting statistical tools

Risk management and various terminologies associated with it, like Risk Acceptance, Risk Analysis, Risk Assessment, Risk Communication, Risk Control, Risk Evaluation, Risk Identification, and Risk Management are included in ICH Q9 guidelines. Quality management plans should mention procedures and practices to the tasks of assessing, controlling, communicating and reviewing risk. Risk Reduction is actions taken to lessen the probability of occurrence of harm and the severity of that harm.[43]

4.1.4. Development of experimental design

Experimental design is the multidimensional combination and interaction of input variables (e.g., material attributes) and process parameters that have been explained to offer assurance of quality. Design space is proposed by the applicant and is subject to regulatory assessment and approval of ICH Q8 (R2). Pharmaceutical development scientists have begun making use of computer aided process design (CAPD) and process simulation to support process development and optimization of manufacturing. Risk assessment can guide to understand linkage and effect of process parameters and material attributes on product, and ranges for variables within which reliable quality can be achieved. These parameters or attributes are selected for addition in the design space. Information regarding reason for inclusion of some variables in design space as well as exclusion of other variable has to be mentioned. Operation within the design space will result in a product meeting the defined quality. Independent design spaces for one or more unit operations can be applied; a single design space can be applied for multiple operations.[44]

4.1.5. Designing and implementing control strategy

Control strategy is required to confirm that material and process are within the expected lower and upper limits. Parameter and material are routinely controlled during production in order to assure reproducibility. The control space should be within the design space. Mostly, scale up is based on trial and error. During scale, up processes parameters may differ but attributes which affect quality remain the same hence control strategy is essential. QbD gives trace on reproducibility and robustness. Process capability index expresses reproducibility of process.

Process capability index C_pK.

$$\frac{\frac{1}{4}\textit{upper limit of specification lower limit of specification}}{\textit{6 standard deviation}}$$

Control space should be within the design space, it is an upper and lower limit for raw material or a process within which parameter and material are regularly controlled which promises quality of product. Design space cover control space. If control space is smaller than design space it is considered as robust. Usually IPQC tests are performed to examine quality and trace out defects but QbD approach being proactive in the initial steps the potential attributes which could possibly give out of range result and affect the quality are recognized. Deliberate variations in those attributes are studied in design space.[4

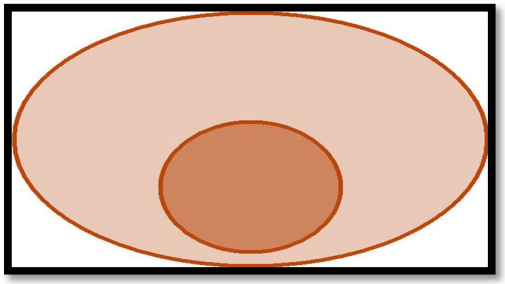


Figure No. 1.15: Control space within the design space.

4.1.6. Continuous improvement throughout product life cycle

Product quality can be improved throughout the product lifecycle; companies have opportunities to opt inventive approaches to improve quality. Process performance can be monitored to make sure consistency in quality. Additional experience and knowledge is gained during routine manufacture which contributes to method/process development. Periodic maintenance can be done within a company's own internal quality system; but design space should be unchanged. The QbD approach avails the continuous improvement throughout products' life cycle this is distinguishing point from the conventional method which is much frozen process.

5.Analytical Quality by Design:

''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. It suggests that approaches like target profile, CQA, design space, and risk assessment are applicable to analytical method also. Though it is not adopted by all pharmaceutical industries it has future perspective because it may become mandatory by regulatory bodies. Voluntary adoption of this concept by industries is possible because of its various benefits, and ease of compliance with regulatory authorities. Pharmaceutical research and manufactures of America (PhRMA), Analytical Technical group (ATG) and European Federation of Pharmaceutical Industries and Association (EFPIA) have given clear ideas about parallel implementation of QbD to analytical method. QbD can be applied for various analytical methods which include,

- 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, UHPLC, and capillary electrophoresis.
- Karl Fischer titration for determination of moisture content.
- Vibrational spectroscopy for identification and quantification of compounds e.g. UV method.
- To biopharmaceutical processes.
- 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

5.1. Aspects of application of QbD to analytical method

Various aspects explained in pharmaceutical development are also put into practice for development of analytical method in QbD paradigm. Some key aspects are discussed hereunder.

5.1.1. Analytical target profile (ATP)

‘‘QbD is a systematic approach to product and process design and development,’’. Hence it begins with determination of goal or method intent. In this emphasis is given on the product and process understanding. ATP is way for method development or it is simply a tool for method development. It describes the method requirements which are expected to be measured. In general, the goal of the chromatographic method is separation, quantification and identification of drug substance impurity or degradants. Impurity is considered to be the critical quality attribute (CQA). While dealing with traces of impurities it will be beneficial to have knowledge of previous synthetic and manufacturing processes and all other possible pathways which lead to the encounter of impurities. The method requirements will be the accuracy precision, robustness, ruggedness and so on as described in ICH guideline.

5.1.2. Method design

Method design is prepared for appropriate availability of material and setting various experimental conditions. In this the reagents required are made available. Regional and geographical conditions are taken into consideration. Feasibility of instruments is checked and experimental design is prepared. In this use of various flowcharts decision tree can be made for correct implementation. In case of HPLC method development scouting is done. In this large number of experimental conditions were tried (pH, temperature, columns, and buffers). Data are collected and software is generated by entering obtained results in terms of values from actual experiments. Then that data base is generated which helps to predict the effect of various chromatographic conditions in large number. This type of software helps to predict outcome without actual experimentation. Response from design also includes resolution and run time. Hence it is cost effective as well as time effective.

An experimental design is an experimental setup to simultaneously evaluate several factors at given numbers of levels in a predefined number of experiments. These experimental designs are as follows,

- full factorial,
- fractional factorial,
- Plackett–Burman designs

5.1.3. Critical quality attributes (CQA)

Factors which directly affect the quality & safety of the product are first sorted out, and its possible effect on method development is studied. Understanding of the product and method will help to sort the CQA. If drug product contains the impurity which may have direct effect on quality and safety of drug product it is being considered the critical quality attribute for the HPLC method development of that particular drug compound. Safety and efficacy can be achieved by demonstrating measurable control of quality attributes i.e. product specification, intermediate specification, and process control.

5.1.4. Risk assessment

It is link between input process variable and CQA. Various tools for risk assessment are,

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

An Ishikawa or fishbone diagram is used to identify all potential variables, such as raw materials, instrumental factors, and environmental factors, which can have an impact on a particular CQA. A FMEA can then be used to rank the variables based on risk (i.e. a combination of probability, severity, and

delectability) and to select the process parameters with higher risks for further studies to gain greater understanding of their effects on CQAs. Main aim of chromatographic method development is separation and identification of compoundPotential noise factors,

a. Factors that can be explored experimentally to determine acceptable ranges.
b. For impurity profiling by HPLC method staggered cross nested design was used and for Karl Fisher Titration (KFT) Method System Analysis (MSA) was found to be useful. Design of experiment was done for the robustness studies

5.1.5. Method qualification

Once the method is designed keeping analytical target profile (ATP) in mind with taking care of the risk involved in development, the next step comes is method qualification this is to ensure that method is being performed as intended. It involves equipment qualification which is part of method qualification. It is divided in, method installation qualification (MIQ), method operational qualification (MPQ), and method performance qualification (MPQ).

For demonstration of instrumental qualification HPLC instrument is considered.

1. Design Qualification
2. Installation Qualification
3. Operational Qualification
4. Performance Qualification

Considering user requirement specifications (URS), design and technical specification of an instrument are defined, it is part of DQ. As HPLC is commercial off the shelf system in this case the users should make sure that the instrument is suitable for their desired applications. User must confirm that the installation site fulfil all vendor specified environmental requirements. Here IQ part begins. Equipment is assembled at the user's site and checked for proper working of all the assembled parts.

5.1.6. Control strategy

It is important that set method performs as intended and consistently gives accurate results, for that purpose control on method is required. A factor identified to have risk has to be controlled. More attention is given to the high-risk factors. System suitability can be checked and verified time to time by having control over it. On ground of practical example; the risk assessment can also help identify a specific control strategy. For example, during robustness studies for an Atomoxetine hydrochloride HPLC impurity profile method, it was found that resolution of the impurities of interest followed the same trend when method parameters such as propanol and temperature were varied. As a result, an early eluting impurity pair was chosen for system suitability and became a convenient method control strategy because the two compounds involved were easily obtained. Validation remains the formality it is done in similar way to that of traditional method development in validation (ICHQ2) but in traditional approach method validated after development i.e. it is like check-box tool, and in QbD the validation parameter in ICHQ2 are consider as method intent.

Figure No 1.16: A QbD tools and life cycle

5.1.7. Life cycle approach

Life cycle approach differs from that of the traditional approach of method development. According to Morefield it includes continuous improvement of method performance and the design space allow flexibility for Continuous improvement in analytical method can be done without prior regulatory approval because of design space made previously. Knowledge gained from risk assessment and data collected from design of experiments can be used as the repos-itory of knowledge to make justified changes wherever re-quired. A complete process analytical method development in QbD environment is summarized in the following flow chart.

Design of Experiment:

Traditional pharmaceutical development approaches are often limited by experiments that test one-at-a-time variability. Comprehensive Design of Experiments uses multidisciplinary teams to design and execute soundly based statistical designs to gain a full understanding of the product and its manufacturing process. The output of DoE confirms CQAs and CPPs that need to be controlled in the manufacturing process.

In DoE, it is ensured that the selected experiments produce the maximum amount of relevant information, keeping costs low by conducting few experiments.

Created by Sir Ronal A. Fisher in the 1920s and 1930s, DoE is defined as a structured and efficient statistical method for planning experiments, so that the data obtained can be analysed to yield valid and objective conclusions and for determining the relationships among the factors affecting a process and its output.

DoE initiates with defining the objectives of an experiment and selecting the process factors for the study. An experimental design is the laying out of a detailed experimental plan in advance of doing the experiment. The statistical theory underlying DoE generally begins with the concept of process models, and the most common it is the process model of the “black box” type, with several discrete or continuous input factors that can be controlled and one or more measured output responses, as shown in Figure. The measured responses describe the properties of the investigated system. By changing the most influential factors the features of the system might be altered according to a response the experiments are affected by a number of uncontrolled factors that may be discrete, such as different machines or operators, and/or continuous such as ambient temperature.[67]

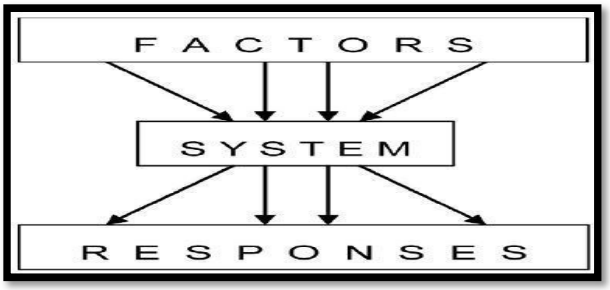


Figure No. 1.17: A “Black Box” Process Model Schematic Frequently

Once factors have been chosen and responses measured, it is desirable to get an understanding of the relationship between them, that is, linking the changes in the factors to the changes in the responses with a mathematical model.

Numerous statistical experimental designs are known. The following list gives the commonly used design types:

1. Full factorial design
2. Box-Behnken design
3. Central composite design
4. Doehlert design

1. **Full two-level factorial designs**

Full two-level factorial design is an experimental matrix that has limited application in RSM when the factor number is higher than 1 because the number of experiments required for this design (calculated by expression N = 2k, where N is experiment number and k is factor number) is very large, thereby losing its efficiency in the modeling of quadratic functions. Because a complete three-level factorial design for more than two variables requires more experimental runs than can usually be accommodated in practice, designs that present a smaller number of experimental points, such as the Box–Behnken, central composite, and Doehlert designs, are more often used. However, for two variables, the efficiency is comparable with designs such as central composite. The majority of applications of three-level factorial designs are in the area of chromatography.

2. **Box–Behnken designs**

Box and Behnken suggested how to select points from the three-level factorial arrangement, which allows the efficient estimation of the first and second-order coefficients of the mathematical model. These designs are, in this way, more efficient
and economical then their corresponding 3k designs, mainly for a large number of variables. Its principal characteristics are:
(1) Requires an experiment number according to N = 2k (k − 1) + cp, where k is the number of factors and (cp) is the number of the central points;
(2) All factor levels have to be adjusted only at three levels (−1, 0, +1) with equally spaced intervals between these levels. This experimental design has been applied for the optimization of several chemical and physical processes; however, its application in analytical chemistry is still much smaller in comparison with central composite design.

3. **Central composite design**

The central composite design was presented by Box and Wilson. This design consists of the following parts:

- A full factorial or fractional factorial design;
- An additional design, often a star design in which experimental points are at a

Fig. (a) and (b) illustrates the full central composite design for optimization of two and three variables. Full uniformly routable central composite designs present the following characteristics:

1. Require an experiment number according to N = k 2 + 2k + cp, where k is the factor number and (cp) is the replicate number of the central point;
2. α- values depend on the number of variables and can be calculated by α = 2(k − p)/4. For two, three, and four variables, they are, respectively, 1.41, 1.68, and 2.00;
3. All factors are studied in five levels (−α, −1, 0, +1, + α).

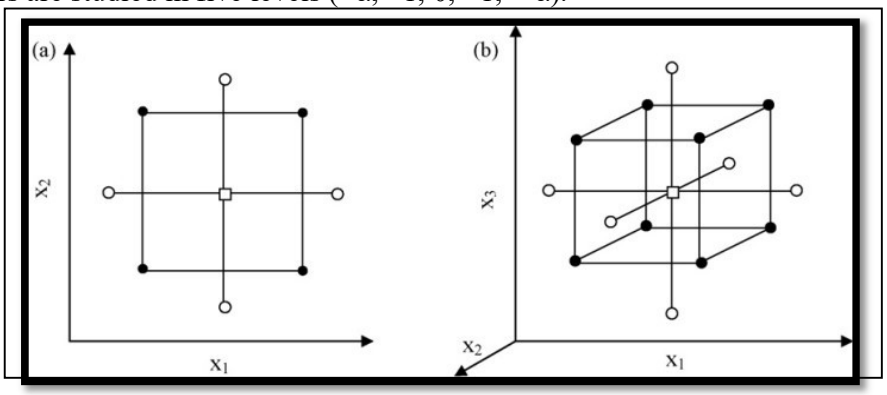


Figure No. 1.18: Central composite designs for the optimization of: (a) two variables (α=1.41) and (b) three variables (α=1.68)

4. **Doehlert design**

Developed by Doehlert, the design is a practical and economical alternative in relation to other second-order experimental matrices. This design describes a circular domain for two variables, spherical for three variables, and hyper spherical for more than three variables, which accents the uniformity of the studied variables in the experimental domain. Although its matrices are not routable as previous designs, it presents

some advantages, such as requiring few experimental points for its application and high efficiency. Other characteristics are presented below:

1. Requires an experiment number according to N = k2 + k + cp, where k is the factor number and (cp) is the replicate number of the central point;
2. Each variable is studied at a different number of levels, a particularly important characteristic when some variables are subject to restrictions such as cost and/or instrumental constraints or when it is interesting to study. [46][47][48][49][50]

2.Aim And Objectives-

Literature survey reveals that several methods such as Stability indicating method, HPTLC method have been developed for Palbociclib but there is no work done on the analytical method development and validation of Palbociclib in bulk and pharmaceutical formulation by applying QbD. Thus, there is need for development of newer efficient and economical analytical methods for Palbociclib.

Aim:

Risk Assessment and Design of Experiments (Doe) Approach in RP-HPLC Method Development for the Analysis of Palbociclib.

Objectives:

The main objectives of the present work are as follows:

- To develop new simple, accurate and economical analytical methods for the estimation of Palbociclib.
- To design the experiment by using Design expert 8 Software.
- To Apply 2 Level Factorial methods..
- To optimize method by using Design expert 8 Software.
- To validate RP-HPLC development for estimation of Palbociclib in bulk and pharmaceutical formulation as per ICH guidelines. [51]

3.Plan Of Work

1. **Selection of Drugs:**

Online Journals, chemical and analytical abstracts were studied to find out drugs for which reported QbD methods or the reported methods were observe, many methods were got costly and time consuming. Market survey was carried to check the availability of these drugs.

2. **Drug procurement:**
   - Palbociclib
3. **Selection of analytical techniques**:
   - RP-HPLC method
4. **Physicochemical characterization of the drugs:**
   - Melting point
   - UV Spectrum (Individual spectrum as well as overlain spectrum)
   - Selection of wavelength maxima
   - FTIR
5. **Method development and validation:**

   RP-HPLC method development and validation of Palbociclib by applying QbD are is as follows:
   - Selection of different mobile phase and range of pH.
   - Selection of range of proportion of mobile phase with appropriate retention time.
   - Design of experiment by using **2 Level Factorial methods.**
   - Selection of suitable detection wavelength.
   - Optimization by using “Design Expert 8” software.
   - To develop calibration curve of optimized result for Palbociclib drug.
   - Method Validation as per ICH guidelines.[52]

4.Review Of Literature-

1.A Novel RP-HPLC Method Development and Validation for Determination of Palbociclib drug in tablet Formulation

Omkar D. Garje, Sunil S. Hindole et al, 2026

A concise, reliable, and stability-indicating RP-HPLC method was developed for the quantification of Palbociclib in its tablet dosage form. Using a mobile phase consisting of 0.1% sodium hydrogen phosphate buffer and acetonitrile in a 55:45 (v/v) ratio, the separation was carried out on a BDS C18 column (250 × 4.6 mm, 5 µm.

2.Development and Optimization of UHPLC Method for the Quantification of Process-Related Impurities of Palbociclib

Narshima Rao et al,2025

This work mainly focuses on development and validation of ultra-high-performance liquid chromatography (UHPLC) technique for quantifying palbociclib and its related impurities I, II, III, and IV. The method involved a systematic investigation of critical method parameters and their optimization using design of experiments principles.

3.Kuntla J, Shalini S, Reddy A. Quality by design (QBD) based Development and Validation of RP-HPLC method for palbociclib in capsule dosage form. International Journal of Pharmacy and Natural Medicines. 2025 Oct 24;13(02):93-101.

A robust and reliable RP-HPLC method was developed and validated for the estimation of Palbociclib in bulk and pharmaceutical dosage forms, in accordance with ICH Q2(R1) guidelines. Method optimization using statistical design revealed that flow rate, buffer pH, and temperature significantly influenced chromatographic responses.

4.Dhamija P, Mehata AK, Tamang R, Bonlawar J, Vaishali, Malik AK, Setia A, Kumar S, Challa RR, Koch B, Muthu MS. Redox-sensitive poly (lactic-co-glycolic acid) nanoparticles of palbociclib: development, ultrasound/photoacoustic imaging, and smart breast cancer therapy. Molecular Pharmaceutics. 2024 May 6;21(6):2713-26.

Polymer disulfide bonds of NPs cleave in elevated GSH concentrations of the tumor microenvironment, resulting in drug release inside the cells. 22 For the first time in the literature, we have presented a novel approach to drug delivery by the incorporation of TPGS-SH, a redox-sensitive polymer, into PLGA-NPs. So far, no research paper has been reported on PLGA/TPGS-SH or PLGA/PLB or any of these combinations. Additionally, the formulation was designed to be entirely new. This developed PLGA-based formulation of PLB has been formulated and evaluated for the first time in breast cancer therapy through ultrasound and photoacoustic imaging.

5.Design of experiment-oriented development and validation of novel bioanalytical reverse phase high performance liquid chromatography method of palbociclib in rat plasma and tissues, and its application in pharmacokinetic studies

Naveen Rajana et al, 2024

The current research involved the development and validation of an easy, cost-effective, and sensitive bioanalytical reverse-phase high-performance liquid chromatography method for the assessment of palbociclib (PAL) in rat plasma and kidney, liver, spleen and heart .

6.Slamon DJ, Diéras V, Rugo HS, Harbeck N, Im SA, Gelmon KA, Lipatov ON, Walshe JM, Martin M, Chavez-MacGregor M, Bananis E. Overall survival with palbociclib plus letrozole in advanced breast cancer. Journal of Clinical Oncology. 2024 Mar 20;42(9):994-1000

Since the initial development and approval of palbociclib, firstin-class cyclin-dependent kinase 4/6 (CDK4/6) inhibitor, for estrogen receptor–positive/human epidermal growth factor receptor 2–negative (ER1/HER2–) advanced breast cancer (ABC) on the basis of PALOMA-1 in 2015,1-4 the CDK4/6 inhibitor class has transformed the treatment landscape and, in combination with endocrine therapy (ET), has become the standard of care for the disease.5 PALOMA-2 confirmed the results of PALOMA-1 with statistically and clinically significant improvement in progression-free survival (PFS) for palbociclib plus letrozole versus placebo plus letrozole in ER1/HER2– ABC. At the time of the final analysis of the primary end point of PFS, overall survival (OS) data were immature. Here, we report results of the final OS analysis and updated safety data.

7.Vural O, Bugday N, Genc AA, Erk N, Duygulu O, Yasar S. Superior Electrochemical Sensor Application of Co3O4/C Heterostructure in Rapid Analysis of Anticancer Drug Palbociclib in Pharmaceutical Formulations and Biological Fluids. Langmuir. 2024 Sep 28;40(40):21139-51.

Breast cancer (BC)is one type of cancer that 3 million people all over the world each year. It is estimated that there were more than 140000 women suffering from metastatic breast cancer (WBC) in2018,whileby2025, it is projected that more than169000 women will have WBC. In particular, future prognostications indicate 3million new breast cancer cases annually.

8.Palmieri C, Musson A, Harper-Wynne C, Wheatley D, Bertelli G, Macpherson IR, Nathan M, McDowall E, Bhojwani A, Verrill M, Eva J. A real-world study of the first use of palbociclib for the treatment of advanced breast cancer within the UK National Health Service as part of the novel Ibrance® Patient Program. British Journal of Cancer. 2023 Sep 21;129(5):852-60.

The objectives of the present study were to describe the patient characteristics, real-world treatment patterns, clinical outcomes and selected adverse events in the first group of patients treated with palbociclib as part of the IPP in routine clinical practice.

9.Assessment of pH-shift drug interactions of palbociclib by in vitro micro-dissolution in bio relevant media: An analytical QbD-driven RP-HPLC method optimization

Prajakta Harish Patil et al,2022

In the present study, pH-dependent solubility and dissolution of Palbociclib (PB), a weakly basic cyclin-dependent kinase 4/6 inhibitor was investigated by application of analytical quality by design (QbD) approach to an reverse phase high performance liquid chromatography (RP-HPLC) method.

10..Patil PH, Desai M, Rao RR, Mutalik S, Shenoy GG, Rao M, Jagadish PC. Assessment of pH-shift drug interactions of palbociclib by in vitro micro-dissolution in bio relevant media: An analytical QbD-driven RP-HPLC method optimization. Journal of Applied Pharmaceutical Science. 2022 May 5;12(5):078-87.

Oral cancer chemotherapy with protein kinases such as CDK inhibitors involves administration of a variety of supportive medications such as analgesics, anti-helminthics, and acid-reducing agents (ARA), such as proton pump inhibitors (PPIs) (Cazzaniga et al., 2019). Supportive medicines are prescribed to alleviate the adverse effects of anticancer treatments.

11..Method Development and Validation of Degradation Studies of Palbociclib by RP-HPLC

Venkateshwarlu et al, 2021

Background: A simple, economical, authentic, and faithful method was used for the study of palbociclib. The Chromatographic analysis was performed by using a C18 intersil ODS (250 X 4.6 mm, 5µm) and a mobile phase containing 0.5M Ammonium acetate

12.Beula J. Analytical Method Development And Validation For The Simultaneous Estimation Of Letrozole And Palbociclib By Using Rp-Hplc Technique. Jetir. 2021 Jan 1.Beckett A.H., Stenlake J.B, Practical Pharmaceutical Chemistry. 4th Ed. Part 2. Publication Of New Delhi, (2004) Pp.275-300

Combination therapy or poly therapy is therapy that uses more than one medication1 . The Letrozole is indicated to treat postmenopausal women with hormone receptor (HR) positive early breast cancer,Palbociclib is indicated in combination with Letrozole as initial endocrine-based therapy for the treatment of human epidermal growth factor receptor type 2 (HER2)-negative and hormone receptor (HR)-positive tumors in adult patients with advanced/metastatic breast cancer. It is as well approved in combination with fulvestrant in patients with disease progression with prior endocrine therapy.This method was validated according to ICH guidelines for specificity, LOD, LOQ, Precision, Accuracy, and Linearity . The method showed good reproducibility and recovery with %RSD less than 2. Hence we had made an attempt to develop a simple accurate and precise RP HPLC method for the
simultaneous estimation of Tamoxifen and Palbociclib in bulk and in tablet dosage form.

13.Rugo HS, Finn RS, Gelmon K, Joy AA, Harbeck N, Castrellon A, Mukai H, Walshe JM, Mori A, Gauthier E, Lu DR. Progression-free survival outcome is independent of objective response in patients with estrogen receptor-positive, human epidermal growth factor receptor 2-negative advanced breast cancer treated with palbociclib plus letrozole compared with letrozole: analysis from PALOMA-2. Clinical breast cancer. 2020 Apr 1;20(2):e173-80.

A phase 2 study showed that progression-free survival was longer with palbociclib plus letrozole than with letrozole alone in the initial treatment of postmenopausal women with estrogen-receptor (ER)–positive,

human epidermal growth factor receptor 2 (HER2)–negative advanced breast cancer. We performed a phase 3 study that was designed to confirm and expand the efficacy and safety data for palbociclib plus letrozole for this indication. All these studies met their primary end point by markedly prolonging progression-free survival versus ET alone, with overall survival (OS) improvements in some settings

14.Booth L, Roberts JL, Rais R, Cutler Jr RE, Diala I, Lalani AS, Poklepovic A, Dent P. Palbociclib augments Neratinib killing of tumor cells that is further enhanced by HDAC inhibition. Cancer Biology & Therapy. 2019 Feb 1;20(2):157-68.

Our most recent studies with neratinib, alone or in combi nation with HDAC inhibitors, have demonstrate a unique biochemical action for the drug.3–5 Neratinib was originally developed as an irreversible inhibitor of ERBB2, and was then shown to inhibit ERBB1 and ERBB4.6 We discovered that neratinib was both a kinase inhibitor and that it possessed the ability to cause ERBB1/2/3/4 receptor internalization with their subsequent proteolytic degradation.

15.Bellet M, Ahmad F, Villanueva R, Valdivia C, Palomino-Doza J, Ruiz A, Gonzàlez X, Adrover E, Azaro A, Valls-Margarit M, Parra JL. Palbociclib and ribociclib in breast cancer: consensus workshop on the management of concomitant medication. Therapeutic Advances in Medical Oncology. 2019 May;11:1758835919833867.

In the 'First Workshop on Pharmacology and Management of Cyclin-dependent kinase (CDK) 4/6 inhibitors (CDK4/6i): Consensus about Concomitant Medications', promoted by the Spanish Breast Cancer Cooperative Group SOLTI, medical oncologists specialized in breast cancer joined physicians specialized in pharmacology, cardiology, psychiatry, infectious diseases, palliative care or radiation oncology in an interdisciplinary discussion forum, at Spanish national level, to address different issues regarding patients treated with palbociclib and ribociclib

16.Llanos S, Megias D, Blanco-Aparicio C, Hernández-Encinas E, Rovira M, Pietrocola F, Serrano M. Lysosomal trapping of palbociclib and its functional implications. Oncogene. 2019 May 16;38(20):3886-902.

Palbociclib is a selective inhibitor of cyclin-dependent kinases 4 and 6 (CDK4/6) approved for the treatment of some cancers. The main mechanism of action of palbociclib is to induce cell cycle arrest and senescence on responsive cells. Here, we report that palbociclib concentrates in intracellular acidic vesicles, where it can be readily observed due to its intrinsic fluorescence, and it is released from these vesicles upon dilution or washing out of the extracellular medium. This reversible storage of drugs into acidic vesicles is generally known as lysosomal trapping and, based on this, we uncover novel properties of palbociclib. In particular, a short exposure of cells to palbociclib is sufficient to produce a stable cell-cycle arrest and long-term senescence.

17.Turner NC, Liu Y, Zhu Z, Loi S, Colleoni M, Loibl S, DeMichele A, Harbeck N, André F, Bayar MA, Michiels S. Cyclin E1 expression and palbociclib efficacy in previously treated hormone receptor–positive metastatic breast cancer. Journal of Clinical Oncology. 2019 May 10;37(14):1169-78.

Purpose A large-panel gene expression analysis was conducted to identify biomarkers associated with the effectiveness of adding palbociclib to fulvestrant.

18.Serra F, Lapidari P, Quaquarini E, Tagliaferri B, Sottotetti F, Palumbo R. Palbociclib in metastatic breast cancer: current evidence and real-life data. Drugs in context. 2019 Jul 16;8:212579.

The purpose of this review is to summarize the background and latest evidence for the use of palbociclib, an oral, first-inclass, highly selective cyclin-dependent kinase 4/6 inhibitor, in advanced breast cancer, with a focus on some of the unanswered questions about the performance of this agent in clinical practice.

19.Optimization and validation of RP-HPLC method for simultaneous estimation of palbociclib and letrozole

Yuvraj Dange et al, 2018

A simple, rapid, and robust RP-HPLC method have been developed and validated to measure palbociclib (PB) and letrozole (LT) at single wavelength (254 nm).

20..Dosil MA, Mirantes C, Eritja N, Felip I, Navaridas R, Gatius S, Santacana M, Colàs E, Moiola C, Schoenenberger JA, Encinas M. Palbociclib has antitumour effects on Pten-deficient endometrial neoplasias. The Journal of pathology. 2017 Jun;242(2):152-64 Activation of phosphatidylinositol 3-kinase (PI3K)–protein kinase B promotes cell survival and proliferation. The most important negative

regulator of this pathway is phosphatase and tensin homologue deleted on chromosome 10 (PTEN), which antagonizes PI3K activity by dephosphorylating phosphatidyli nositol (3,4,5)-trisphosphate to phosphatidylinositol (4,5)-bisphosphate.

21.Cristofanilli M, Turner NC, Bondarenko I, Ro J, Im SA, Masuda N, Colleoni M, DeMichele A, Loi S, Verma S, Iwata H. Fulvestrant plus palbociclib versus fulvestrant plus placebo for treatment of hormone-receptor-positive, HER2-negative metastatic breast cancer that progressed on previous endocrine therapy (PALOMA-3): final analysis of the multicentre, double-blind, phase 3 randomised controlled trial. The Lancet Oncology. 2016 Apr 1;17(4):425-39.

We randomly assigned patients with hormone-receptor–positive, HER2-negative advanced breast cancer who had progression or relapse during previous endocrine therapy to receive palbociclib plus fulvestrant or placebo plus fulvestrant. We analyzed overall survival; the effect of palbociclib according to the prespecified stratification factors of presence or absence of sensitivity to endocrine therapy, presence or absence of visceral metastatic disease, and menopausal status; the efficacy of subsequent therapies after disease progression; and safety.

22.Tamura K, Mukai H, Naito Y, Yonemori K, Kodaira M, Tanabe Y, Yamamoto N, Osera S, Sasaki M, Mori Y, Hashigaki S. Phase I study of palbociclib, a cyclin-dependent kinase 4/6 inhibitor, in Japanese patients. Cancer science. 2016 Jun;107(6):755-63.

In estrogen receptor-positive (ER+) luminal breast cancer cell lines, palbociclib was shown to arrest tumor cell growth, accompanied by an inhibition of retinoblastoma protein hyperphosphorylation, and it also showed synergistic antitumor activity when combined with tamoxifen

23.Beaver JA, Amiri-Kordestani L, Charlab R, Chen W, Palmby T, Tilley A, Zirkelbach JF, Yu J, Liu Q, Zhao L, Crich J. FDA approval: palbociclib for the treatment of postmenopausal patients with estrogen receptor–positive, HER2-negative metastatic breast cancer. Clinical cancer research. 2015 Nov 1;21(21):4760-6.

Palbociclib was clastogenic in an in vitro micronucleus assay using Chinese Hamster Ovary cells and an in vivo rat bone marrow micronucleus assay through an aneugenic mechanism. Palbociclib can cause fetal harm based on its mechanism of action and findings in animals. Reports from rodent carcinogenicity studies were not submitted or required to support this application as the indication includes patients with advanced breast cancer.

24.De Melo-Diogo D, Gaspar VM, Costa EC, Moreira AF, Oppolzer D, Gallardo E, Correia IJ. Combinatorial delivery of Crizotinib–Palbociclib–Sildenafil using TPGS-PLA micelles for improved cancer treatment. European Journal of Pharmaceutics and Biopharmaceutics. 2014 Nov 1;88(3):718-29.

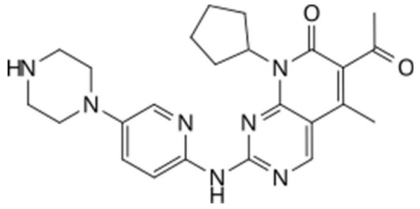
Multidrug therapy is a particularly valuable strategy to improve the efficacy of cancer treatments, since it aims to act simulta neously on different disease hallmarks and in the mechanisms responsible for cell resistance to chemotherapeutics.

25.Parrish KE, Pokorny J, Mittapalli RK, Bakken K, Sarkaria JN, Elmquist WF. Efflux transporters at the blood-brain barrier limit delivery and efficacy of CDK4/6 inhibitor palbociclib (PD-0332991) in an orthotopic brain tumor model.

A critical factorin the use ofpalbociclib in the treatment of brain tumors is achieving effective drug delivery to all tumor cells, including those invasive cells Abbott, 2013)

5.PROFILE

Palbociclib

Name of drug	Palbociclib
Structure	
IUPAC name	6-Acetyl-8-cyclopentyl-5-methyl-2-{[5-(1-piperazinyl)-2-pyridinyl]amino}pyrido[2,3-d]pyrimidin-7(8H)-one
CAS no	571190-30-2
Molecular formula	$C_{24}H_{29}N_7O_2$
Molar mass	447.543 g·mol^{-1}

- **Pharmacokinetics:**
  - ✓ **Bioavailability:** 46%
  - ✓ **Protein binding:** 85%
  - ✓ **Metabolism:** Liver (CYP3A, SULT2A1, glucuronidation)
  - ✓ **Elimination half-life:** 29 (±5) hours
  - ✓ **Excretion:** 74% feces, 18% urine
- **Mechanism of action:**

It is a selective inhibitor of the cyclin-dependent kinases CDK4 and CDK6. In the G1 phase of the cell cycle, mammalian cells must pass a checkpoint, known as the restriction point "R", in order to complete the cell cycle and divide. CDK4 and CDK6 complex with Cyclin D drive the phosphorylation of the retinoblastoma protein, Rb, which allows the cell to pass R and commit to division. Regulation of one or more proteins involved in this checkpoint is lost in many cancers. However, by inhibiting CDK4/6, palbociclib ensures that the cyclin D-CDK4/6 complex cannot aid in phosphorylating Rb. This prevents the cell from passing R and exiting G1, and in turn from proceeding through the cell cycle.[61]

- **Adverse effects**

A majority of patients taking palbociclib experience neutropenia, a condition where a patient has an abnormally low number of neutrophils. This side effect impacts the immune system, and is thus likely responsible for the second most common side effect, infection. Leukopenia and anemia are also frequent among patients taking palbociclib. The FDA further cautions that women should be aware that the medication can have a harmful effect on a fetus, and thus should not be taken while pregnant. [53][54]

6.MATERIALS AND METHODS

6.1.1 Materials:

Table No 6.1: Table showing list of the Materials

Sr. No.	Name	Description
1.	Palbociclib	The Active Pharmaceutical Ingredient In Powder Form Is A Yellow To Orange Solid.
2.	Palbociclib Tablets	Palbociclib (125mg) Natco Pharma Ltd

Table No.6.2 List of Chemicals used in Research work

Sr. No.	Name Of Chemicals	Grade	Manufacturer
1	Methanol	HPLC Grade	Merck Lie Sciences Pvt. Ltd, Mumbai.
2	Acetonitrile	HPLC Grade	Merck Lie Sciences Pvt. Ltd, Mumbai.
3	Water	HPLC Grade	Merck Lie Sciences Pvt. Ltd, Mumbai.
4	Potassium Dihydrogen Phosphate	LR Grade	Research Fine Chem. Indu.

Table No. 6.3 List of Instruments

Sr. No.	Name Of Equipment/Instruments	Model/Specification	Manufacturer
1	HPLC	Series2000	Shimadzu LC 20AD
	Pump	PU2080	
	Sample Injection Port	Rheodyne Injector	
	UV/Visible Detector	UV 2075 Plus	
	Software	Borwin	
2	Double Beam UV-Visible Spectrophotometer	UV-1800 240V	SHIMADZU
3	Ph Meter	EQ-636	EQUIP-TRONICS
4	Balance	BL-220H	SHIMADZU
5	Sonicator	1.5L 50H	ROLEX
6	Refrigerator		Godrej

6.1.2 Methods

Preliminary Analysis of Drug

a) Description

The active pharmaceutical ingredient in powder form is a yellow to orange solid.

b) Solubility

Palbociclib has **pH-dependent aqueous solubility**, categorized as a poorly water-soluble compound under standard physiological conditions.

Palbociclib (a CDK4/6 inhibitor) is practically insoluble in water, but it exhibits good to excellent solubility in select organic solvents.Key solvents used for dissolution and stock preparation include:Methanol: Highly soluble. DMSO (Dimethyl Sulfoxide): Soluble (often requires gentle warming to achieve higher concentrations, (2 mg/mL).Ethanol: Soluble. DMF (N,N-dimethylformamide) and THF (Tetrahydrofuran): Also exhibit relatively high thermodynamic solubility.

c) UV Analysis

UV analysis was carried out by scanning the solution of Palbociclib at 200-400 nm. **Lambda Max was found to be 265 nm.**

2. Design of Experiment

- **2 Level Factorial Design**

- The 2-Level Factorial Design Builder dialog box offers two-level full factorial and fractional factorial designs. You can investigate 2 to 21 factors using 4 to 512 runs. This collection of designs provides an effective means for screening through many factors to find the critical few.
- Full two-level factorial designs may be run for up to 9 factors. These designs permit estimation of all main effects and all interaction effects (except those confounded with blocks.)
- The program offers a wide variety of fractional factorial designs. It calculates detailed information about the alias structure. This evaluation should be inspected to ensure the selected design can cleanly estimate the interactions of interest.
- You will find the resolution, or expected alias pattern, of each fractional factorial by looking at both the color and notation on the 2-Level Factorial Design display. Expect the following consequences:
- Red – Stop and Think: A resolution III design indicates that main effects may be aliased with two factor interactions. Resolution III designs can be misleading when significant two-factor interactions affect the response.
- Yellow – Proceed with Caution: A resolution IV design indicates that main effects may be aliased with three-factor interactions. And two-factor interactions may be aliased with other two-factor interactions. Resolution IV designs are a good choice for a screening design because the main effects will be clear of two-factor interactions.
- Green – Go Ahead: Resolution V (or higher) designs are just about as good as a full factorial, generally at a great savings in the number of runs to perform. Assuming that no three-factor (and higher) interactions occur, all the main effects and two-factor interactions can be estimated.
- You may enter the number of replicates, number of blocks, and number of center points per block. (If you are going to run center points, we recommend adding a around 4 or 5 to get an adequate test for curvature and lack of fit.) Once you have selected a design on the grid you can view the alias structure for this design by pressing Continue. If, after viewing the alias information, you want to make a change simply press the << Back button to return to the design selection screen.[55,56]

Selection of Dependant factors

1. Mobile Phase
2. pH of Mobile phase

Selection of Independent factors

1. Retention Time
2. Area
3. Theoretical Plate
4. Asymmetry

Columns used

- C_{18} Column

Following mobile phases selected

✓ Phosphate buffer : Acetonitrile
✓ Phosphate buffer : Methanol
✓ Change pH Range: 3-5
✓ Change Mobile phase proportion Range: 60-80% (Consider Organic Phase) Software gaves 04 runs for each mobile phase. Total 08 runs

Sr. No	Mobile Phase	pH of Aqueus Phase
1	60.00	5.00
2	80.00	3.00
3	60.00	3.00
4	80.00	5.00

3. Preparation of mobile phase

80 ml of HPLC grade Methanol was added to 10ml of Water i.e. in 80: 20 v/v proportions. The pH was adjusted to 3 and 5 with Triethylamine and orthophosphoric acid. The solution was filtered through 0.45µ membrane filter and then sonicated in sonicator bath for 10 min.

4. Preparation of stock solutions of Palbociclib

Stock solution was prepared by dissolving 10 mg Palbociclib in water and then diluted with Water in 10 ml of volumetric flask to get concentration of 1000 µg/ml. From the resulting solution 0.1 ml was diluted to 10 ml with water to obtain concentration of 10 µg/ml of Palbociclib and labelled as standard stock Palbociclib.

5. Selection of detection wavelength

From the standard stock solution further dilutions were done using water and scanned over the range of 200-400 nm and the spectra were overlain. It was observed that drug showed considerable absorbance at 265 nm.[55,56]

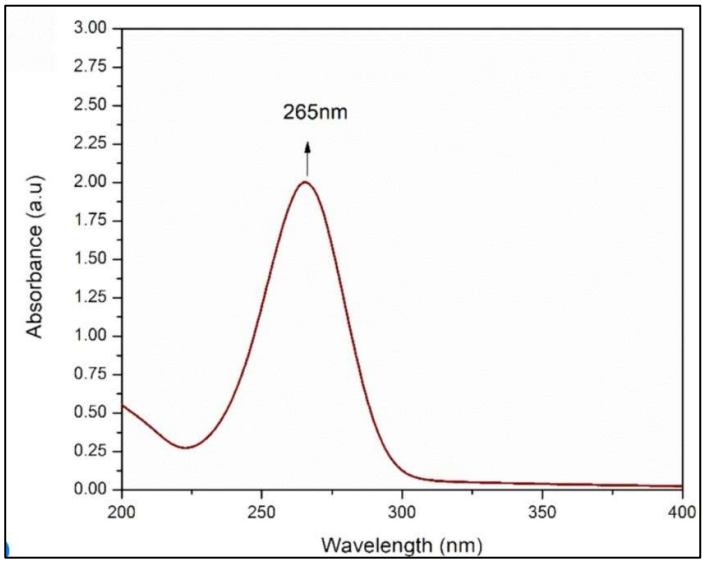


Figure 6.1: UV absorbance at 265 nm

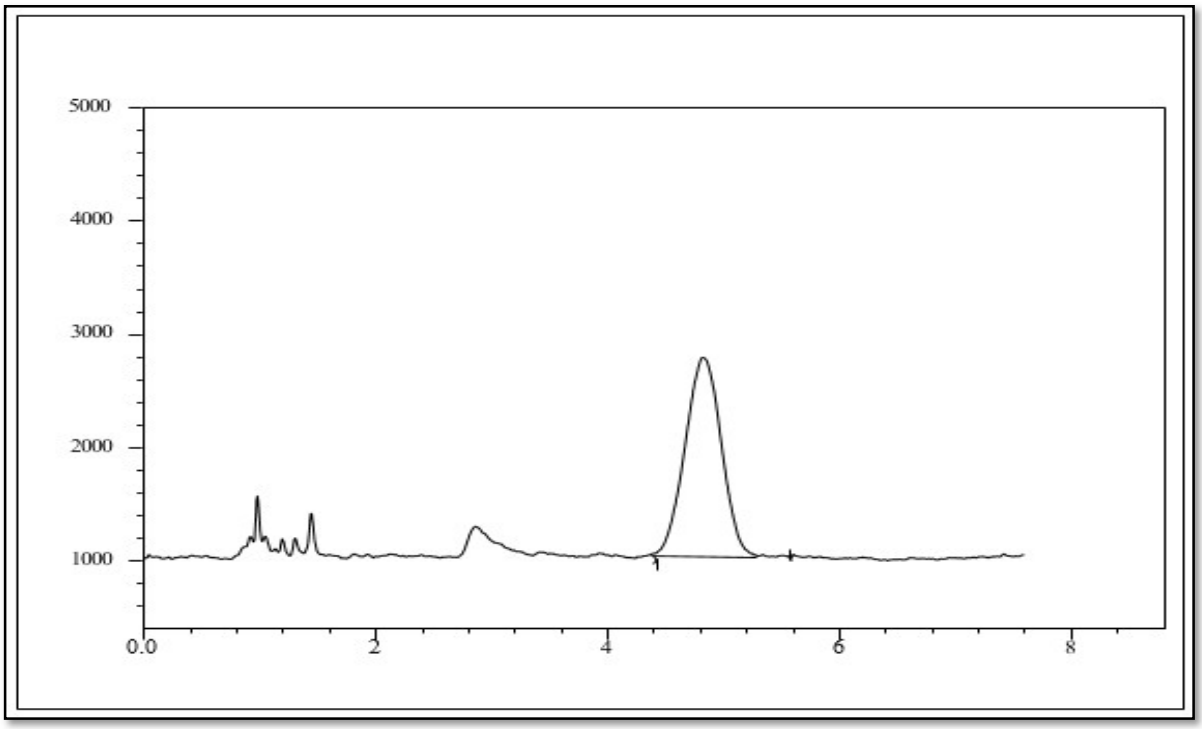
7.Result of Factorial Design

7.1 Trials given by Design Expert Software

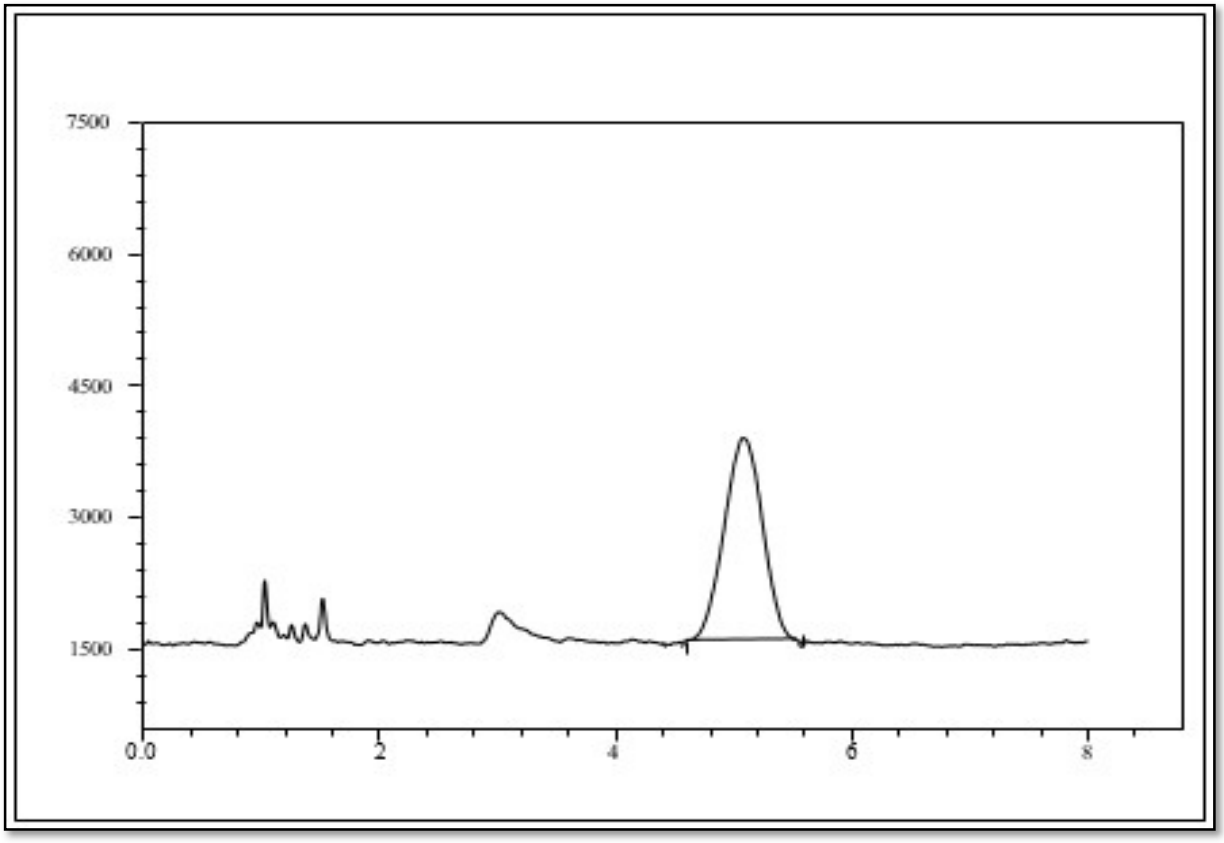
Standard concentration of Palbociclib was taken 50 µg/ml. Miscellaneous Factorial design gave 4 runs at different pH, Solvent proportion 2 Solvent Combination with 8 runs for each Solvent Combination. Software give its 8 runs

Table No.7.1 Trials given by software for full 2 Level Factorial Design Phosphate Buffer: Acetonitrile

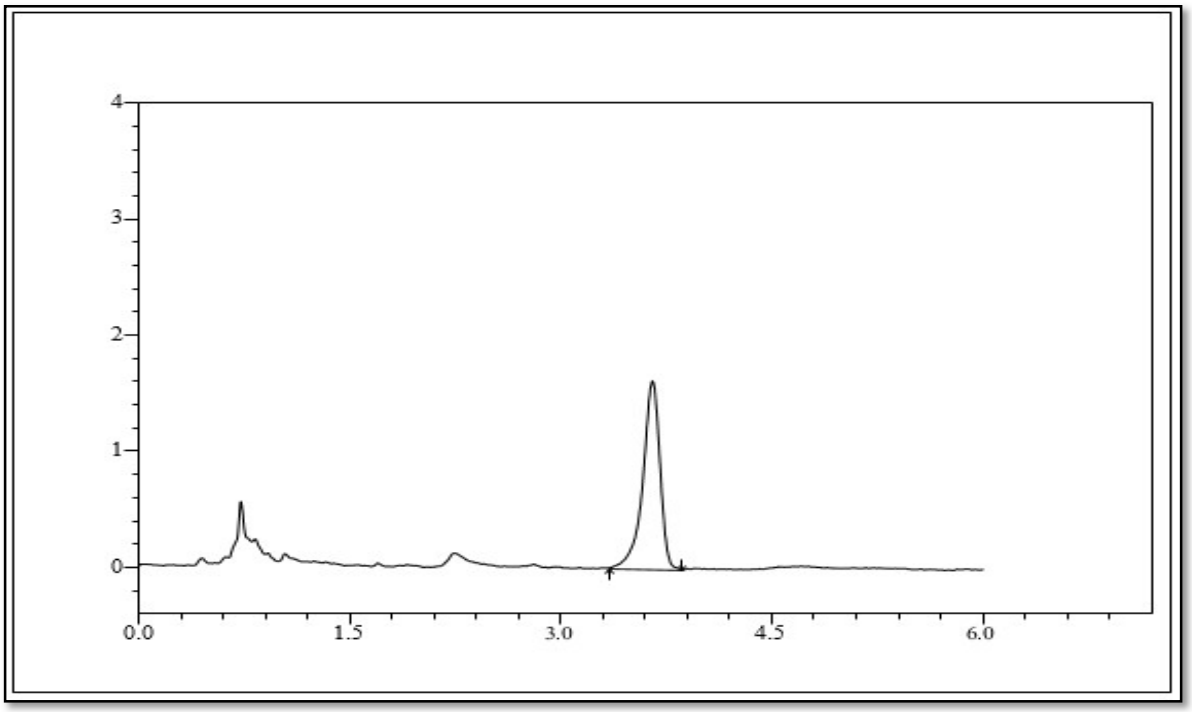
Sr. No	Mobile Phase	pH of Aqueus Phase	Retention Time	Asymmetric Factor	Theoretical Plates
1	60.00	5.00	4.532	1.625	5264
2	80.00	3.00	3.324	1.471	7591
3	60.00	3.00	4.526	1.214	6327
4	80.00	5.00	3.941	1.354	6924



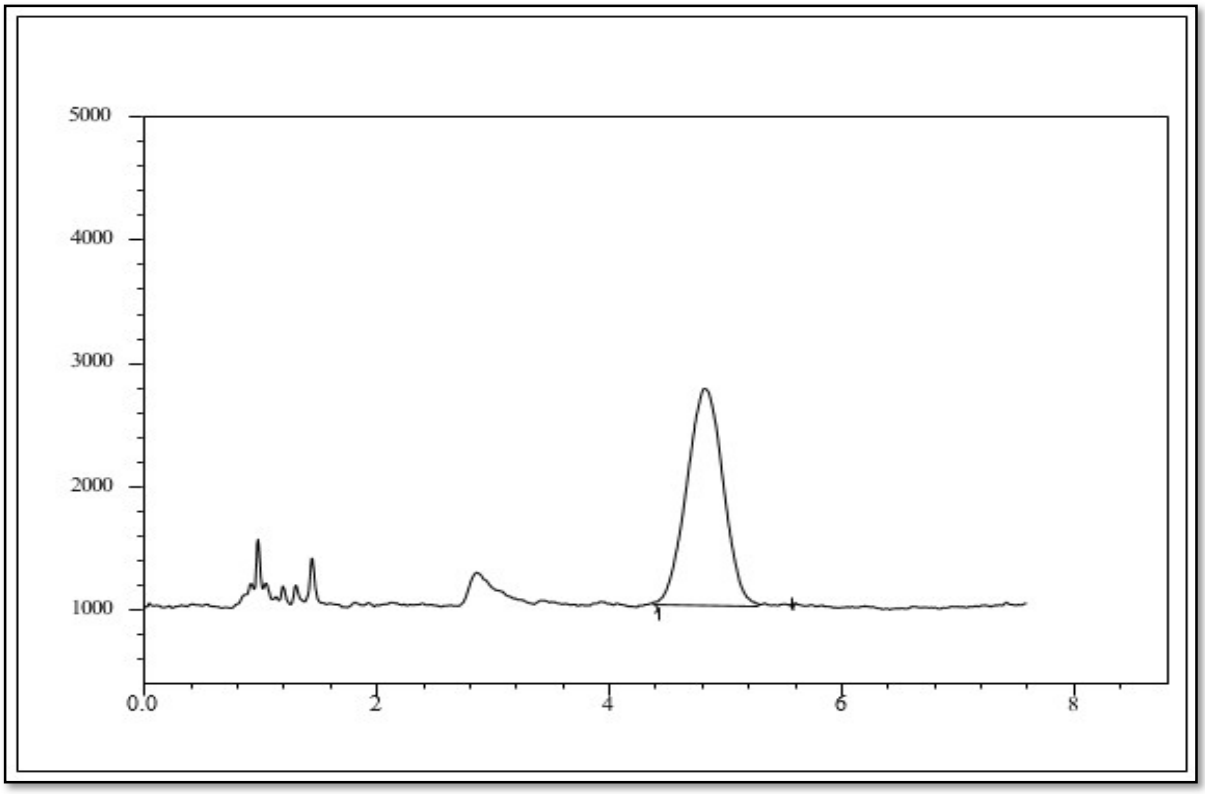
Trials: Phosphate Buffer: Acetonitrile (40: 60), pH: 3
Figure No. 7.1.1 Chromatogram of Palbociclib (40: 60) at pH



Chromatogram of Palbociclib



Chromatogram of Palbociclib
Trials: Phosphate Buffer: Acetonitrile (20: 80), pH: 3
7.1.2 Trials: Phosphate Buffer: Acetonitrile (40: 60), pH: 5



7.1.3 Trials: Phosphate Buffer: Acetonitrile (20: 80), pH: 5

Table No.7.2 Trials given by software for full 2 Level Factorial Design Phosphate Buffer: Methanol

Sr. No	Mobile Phase	pH of Aqueus Phase	Retention Time	Asymmetric Factor	Theoretical Plates
1	60.00	5.00	5.927	1.337	6147
2	80.00	3.00	3.314	0.924	10574
3	60.00	3.00	4.742	1.417	5921
4	80.00	5.00	5.874	1.174	9674

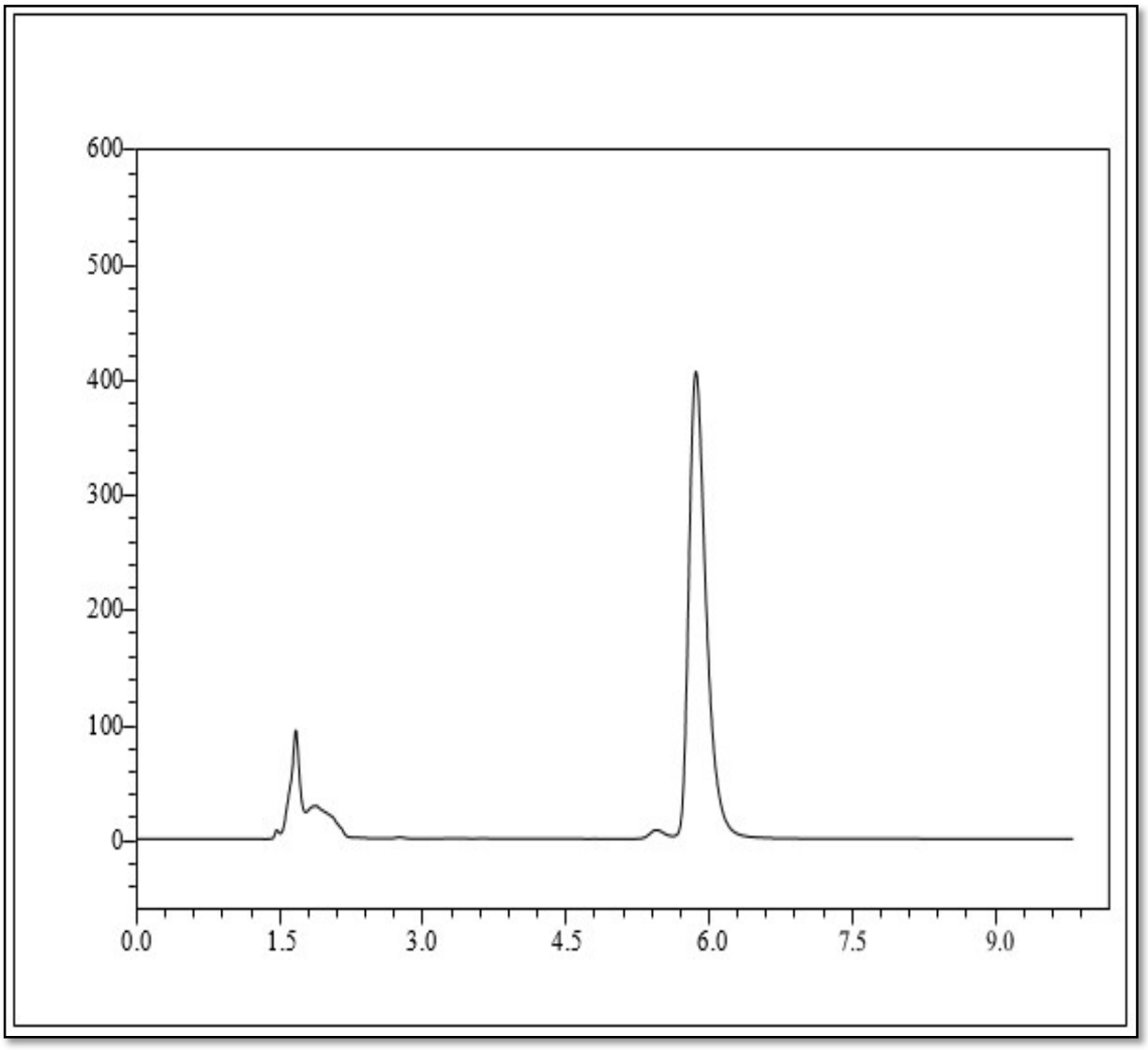


Fig.7.1.4 Trials: Phosphate Buffer: Methanol (40: 60), pH: 3

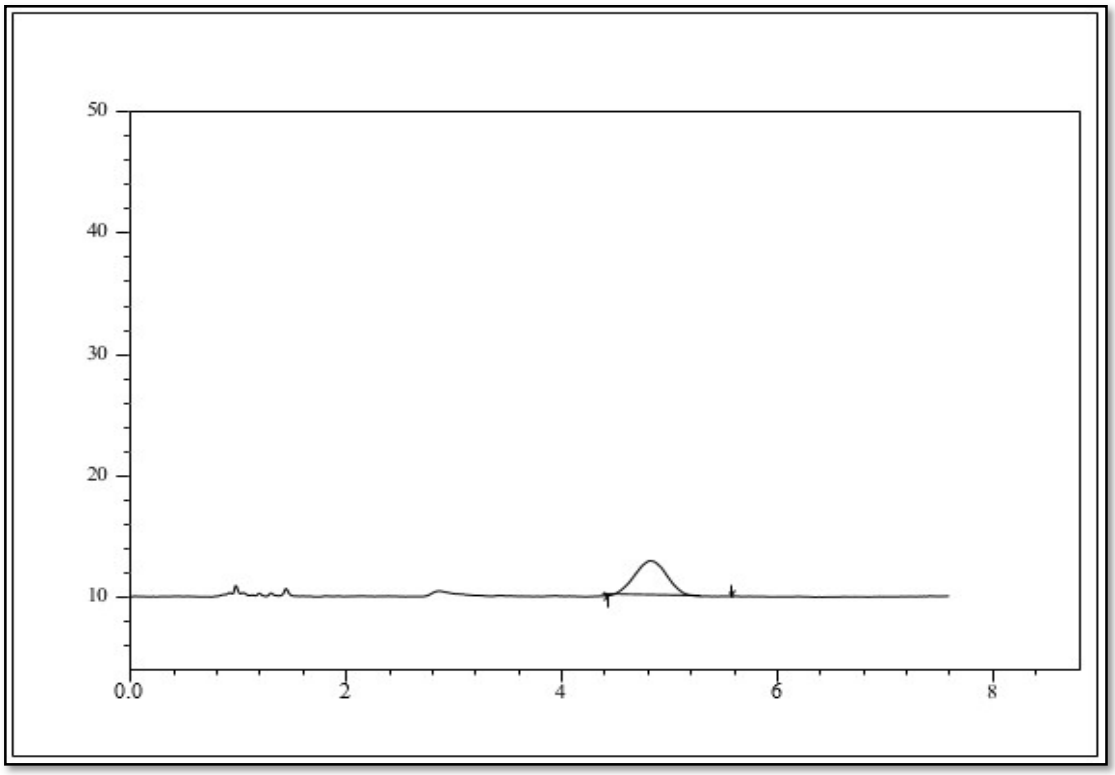


Fig 7.1.5 Trials: Phosphate Buffer: Methanol (40: 60), pH: 5

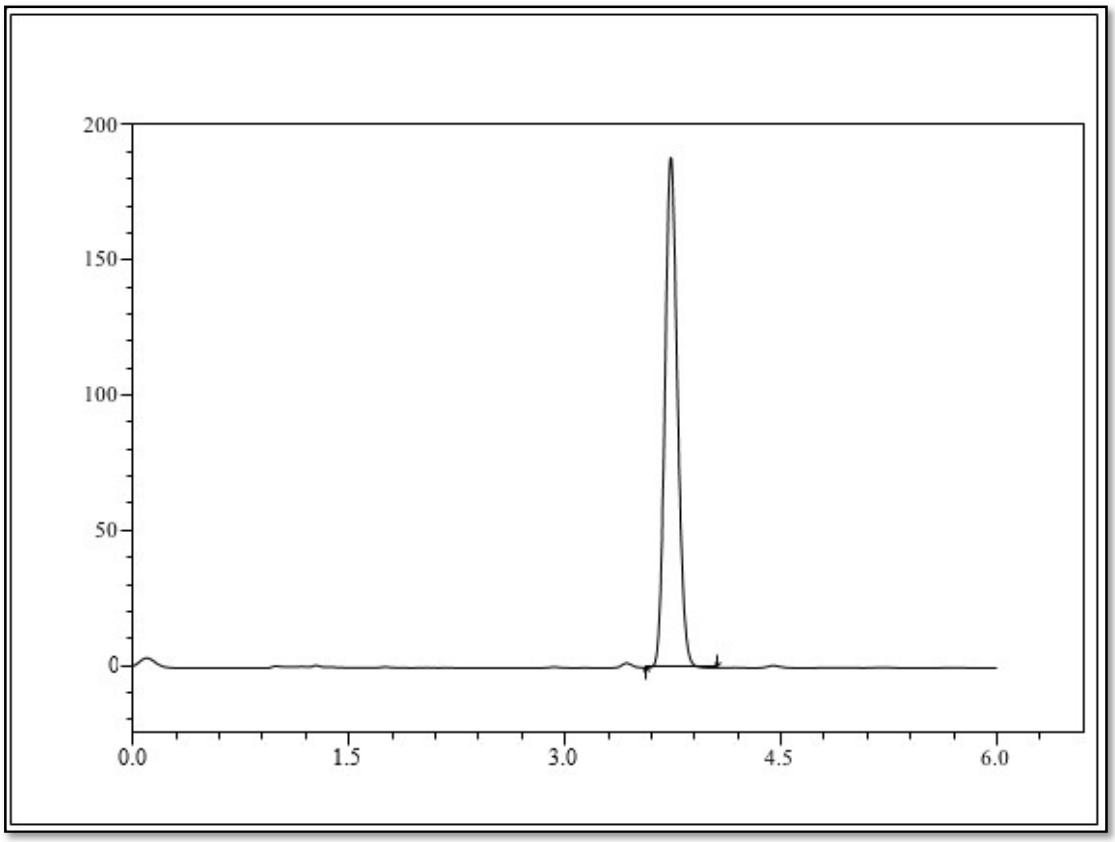


Fig 7.1.6 Trials: Phosphate Buffer: Methanol (20: 80), pH: 3

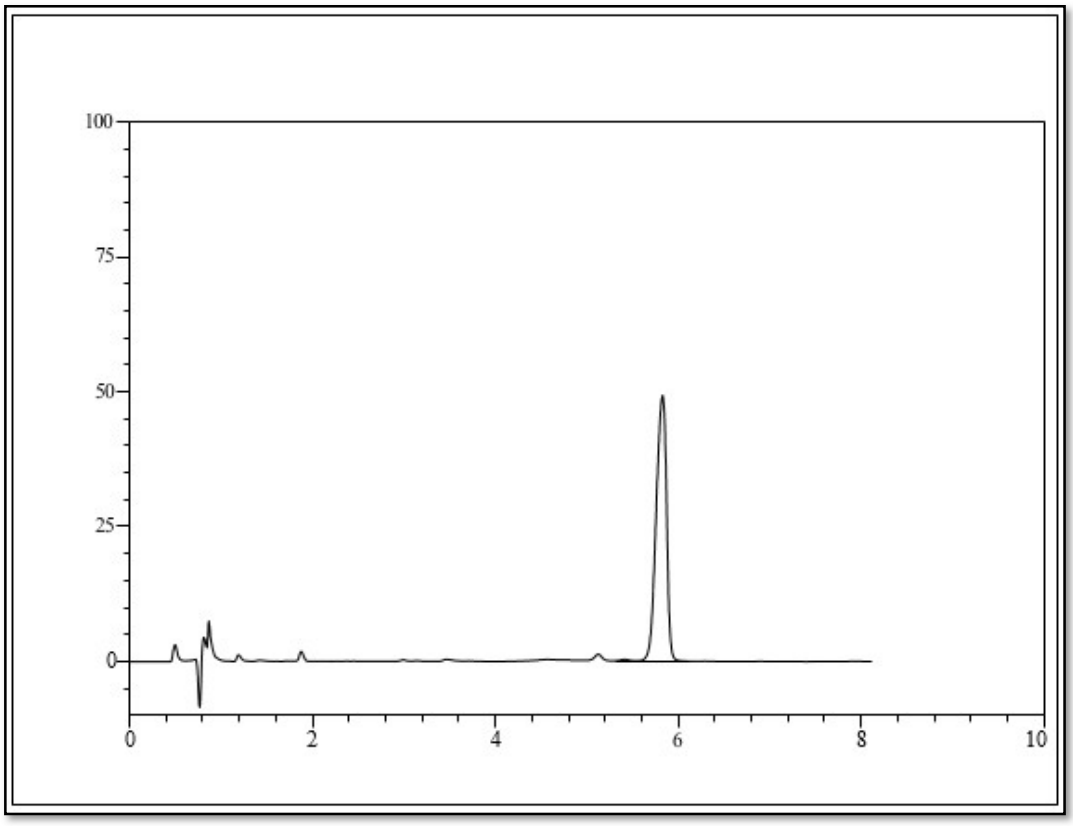


Fig7.1.7 Trials: Phosphate Buffer: Methanol (20: 80), Ph: 5

7.2 Optimization

1. Optimization Result

Screening design for suitable chromatographic condition:

Trials performed on C18 column Mobile phase (Phosphate Buffer: Methanol) with Different pH of Mobile Phase. Design expert has optimized the following chromatographic conditions with respect to desirability value.

This methodology is initially based on constructing a desirability function for each individual response. The scale of individual desirability function ranges between i= 0, for completely undesirable response and i =1, for fully desired response. Selection of trial was based on maximum desirability value. Therefore, first trial which was having desirability one (i=1) selected for method optimization.

Table 7.2.1 Optimized trials suggested by software based on desirability value

Sr. no.	Amount of Methanol	pH of buffer	Flow rate	Retention time	Tailing factor	Theoretical plates	Desirability
1	80.00	3.0	1.0	3.65775	1.0065	10292.5	0.879

Sr. No	Mobile Phase	pH of Aqueus Phase	Retention Time	Asymmetric Factor	Theoretical Plates
1	60.00	5.00	5.927	1.337	6147
2	80.00	3.00	3.314	0.924	10574
3	60.00	3.00	4.742	1.417	5921
4	80.00	5.00	5.874	1.174	9674

Optimized chromatographic conditions

Mobile phase: Phosphate buffer: Methanol (20: 80 v/v), pH of buffer: 3, Analytical column: C_{18} column Waters XBridge (4.6× 250mm id. particle size 5µm), UV detection: 265nm, Injection volume: 10 µL, Flow rate: 1.00 mL min $^{-1}$, Temperature: Ambient, Run time: 10 min

Effect of independent variables on retention time (X):

After applying experimental design, suggested Response Surface Linear Model was found to be significant with model F value of 4.29, *p* value less than 0.005 and R^2 value of 0.8956. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of % C.V. and adjusted R^2 were 13.85 and 0.6868 respectively. The model for response X (Retention time) is as follows:

The equation for response surface quadratic model is as follows

Retention Time =+3.81100-0.037025 * Mobile Phase+0.93625* pH of Aqueous Phase

Fig.3 (b) shows a graphical representation of pH of buffer (B) and amount of Methanol (A), while flow rate (C) is maintained constant at its optimum of 1 mL min^{-1}. Change in pH of buffer showed slightly change in retention time (X), also increase in amount of Methanol showed decreases the retention time.

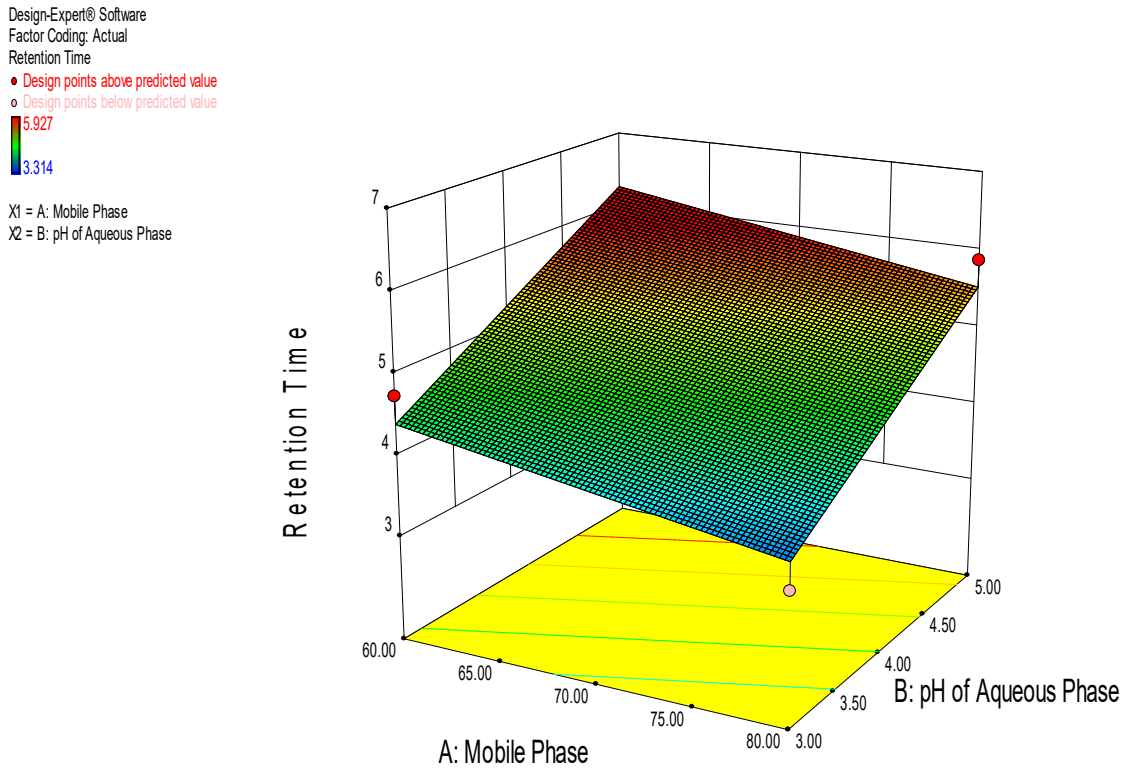


Fig. 7.2.1 Three-dimensional plot for retention time as a function of pH of buffer and Organic Phase Constant factor (flow rate- 1mL min^{-1})

Fit summary: Linear model was suggested by the software.

ANOVA: ANOVA of developed Full three level factorial model for retention time (Y_1).

Values of "Prob > F" (p- value) less than 0.0500 indicate model terms are significant. In this case A and B are significant model terms.

Table 7..2 2 Significance of *p* value on model terms of retention time

Model terms	***p* value**	**Effect of factor**	**Remarks**
A	0.0471	1.16	Significant
B	0.2240	7.42	Insignificant
Overall model	**0.03231**		**Significant**

Effect of independent variables on tailing factor (Y):

After applying experimental design, suggested Response Surface Linear Model was found to be significant with model F value of 2.11, *p* value less than 0.005 and R^2 value of 0.8083. There is only a 43.78% chance that a "Model F-Value" this large could occur due to noise. Values of % C.V. and adjusted R^2 were 13.60 and 0.4250 respectively. The model for response

Asymmetric Factor =+2.19100-0.016400 * Mobile Phase+0.042500* pH of Aqueous Phase

Fig.6.(b) shows a graphical representation of pH of buffer (B) and amount of Methanol (A), while flow rate (C) is maintained constant at its optimum of 1.0 mL min^{-1}. An decrease in pH of buffer decrease the tailing factor, as Increases the amount of Methanol decreases the asymmetric factor.

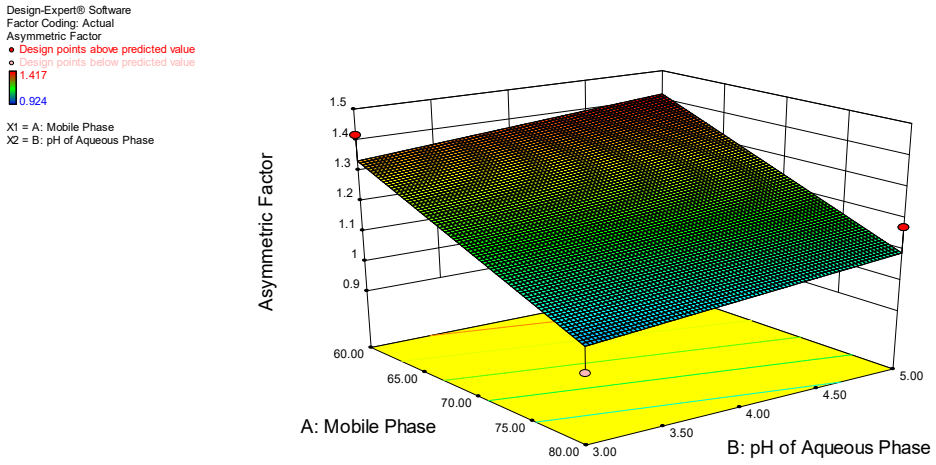


Fig.7.2.2 Three-dimentional plot for tailing factor as a function of pH of buffer and Amount of Methanol. Constant factor (flow rate- 1mL min^{-1})

Fit summary: Response Surface Linear Model was suggested by the software.

ANOVA: ANOVA of developed 2FI model for tailing factor (Y_2).

Values of "Prob > F" (p- value) less than 0.0500 indicate model terms are significant. In this case B is significant model terms.

Table 7.2.3 Significance of *p* value on model terms of tailing factor

Model terms	*p* value	Effect of factor	Remarks
Model	0.4378	2.11	Insignificant
A	0.02967	3.95	Significant
B	0.05972	0.27	Significant
Overall model	0.4378	1.26	Insignificant

Effect of independent variables on theoretical plates (Z):

After applying experimental design, suggested Response Surface Linear Model was found to be significant with model F value of 26.57, *p* value less than 0.005 and R^2 value of 0.9815. There is only a 13.59% chance that a "Model F-Value" this large could occur due to noise. Values of % C.V. and adjusted R^2 were 6.97 and 0.9446 respectively. The model for response Z (theoretical plates) is as follows:

Theoretical Plates =-5562.00000+204.50000 * Mobile Phase -168.50000 * pH of Aqueous Phase

Fig.9.(b) shows a graphical representation of amount of Methanol (A) and pH of buffer (B), while flow rate (C) is maintained constant at its optimum value 1mL min^{-1}. A decrease in pH of buffer showed not a significant effect on number of theoretical plates (Z), while increase in amount of Methanol showed increases response.

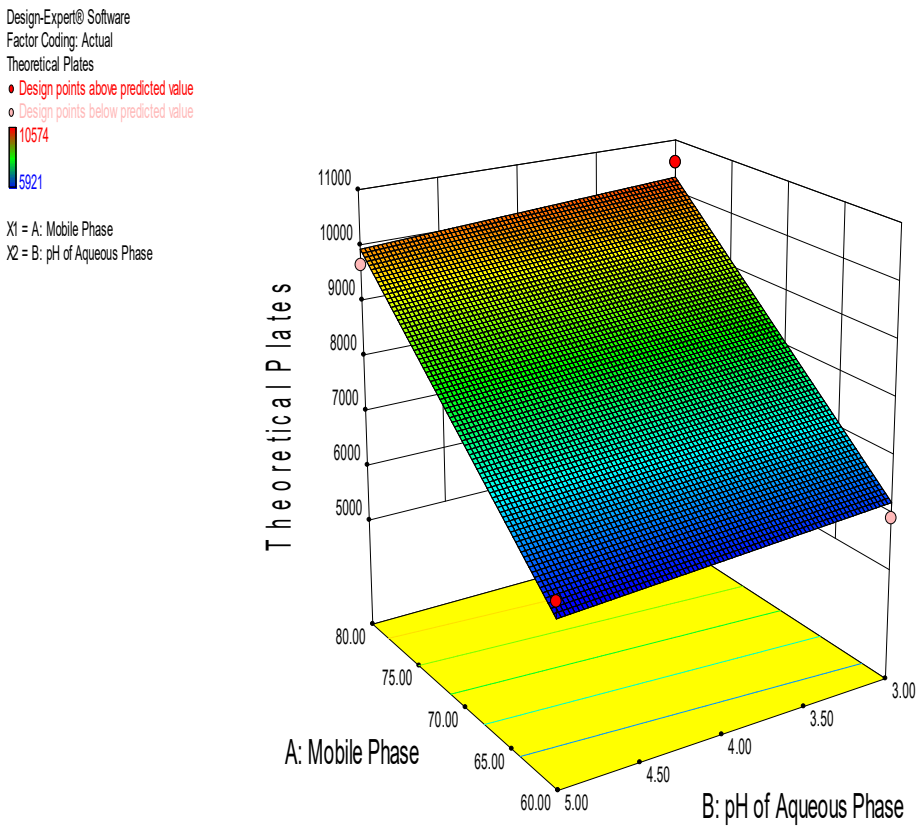


Fig.7.2.3 Three-dimentional plot for theoretical plates as a function of pH of buffer and Methanol. Constant factor (flow rate- 1 mL min^{-1})

Fit summary: Linear model was suggested by the software

ANOVA: ANOVA of developed CCD model for theoretical plates (Y_3).

Values of "Prob > F" (p- value) less than 0.0500 indicate model terms are significant. In this case A value is significant model terms.

Table 7.2.4 Significance of *p* value on model terms of theoretical plates

Model terms	***p* value**	**Effect of factor**	**Remarks**
A	0.1359	26.57	Insignificant
B	0.0871	52.78	Significant
Overall model	0.6566	0.36	Insignificant

7.3 Validation

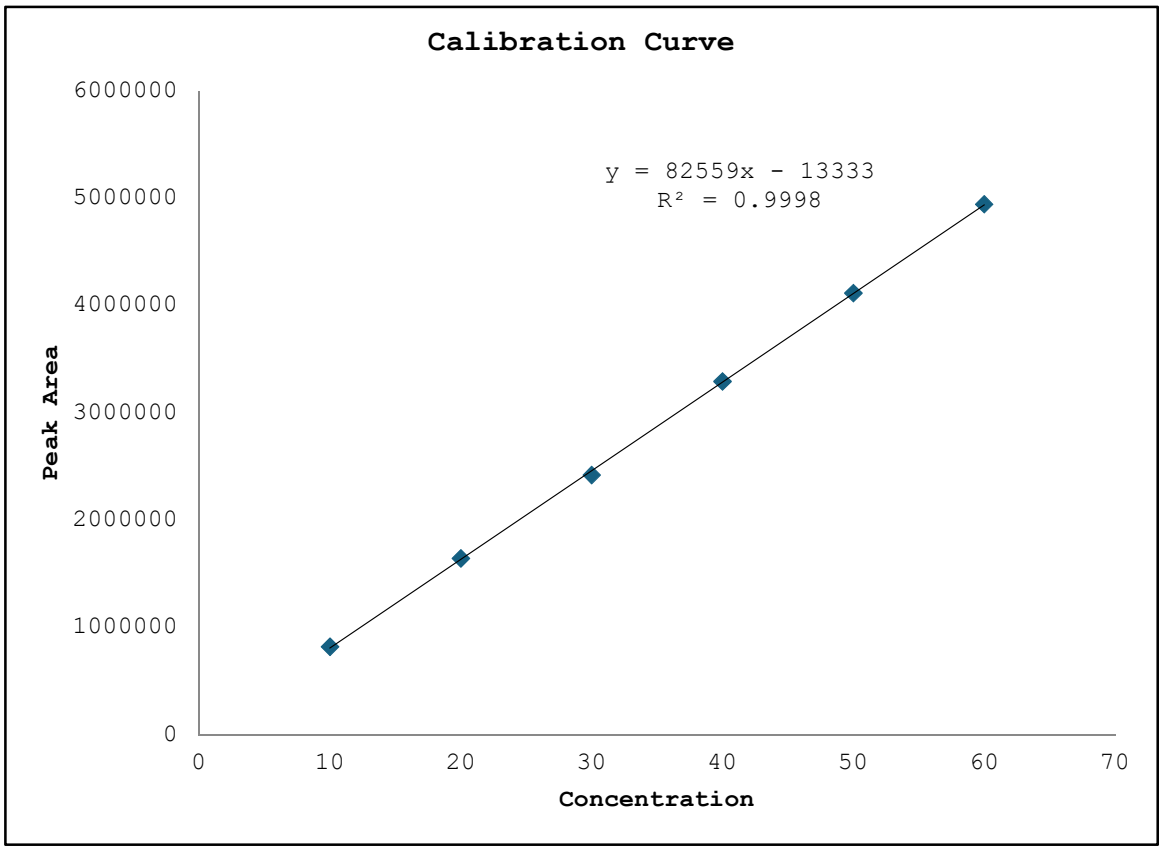
The proposed HPLC method was validated in terms of system suitability, specificity, precision, accuracy and robustness as per the International Conference on Harmonization (ICH) guidelines (7).

1. Linearity:

The linearity of peak area response for Palbociclib was determined from 20%-120% level of working concentration of Palbociclib. The stock solutions of standard Palbociclib was diluted to six different known concentrations. Linearity graph of concentration (as x-value) versus area (as y-value) were plotted and correlation coefficient, y-intercept and slope of the regression were calculated.

Table No. 7.3.1 Linearity Result of Palbociclib

Sr. No	Concentration (µg/mL)	Peak Area
1	10	824157
2	20	1648314
3	30	2422471
4	40	3296628
5	50	4120785
6	60	4944942
Slope		82558.56
Standard Error		22625.31



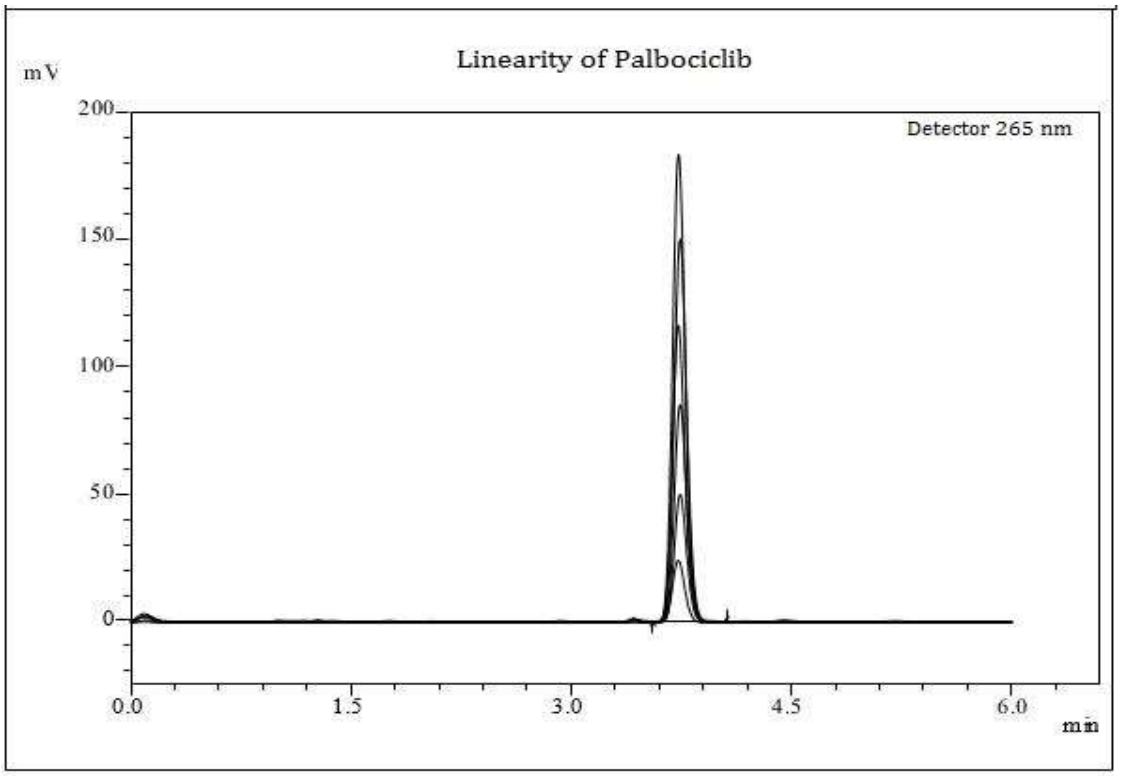


Fig No. 7.3.2 Overlain of Palbociclib

Table No. 7.3.2 Characteristic parameters of Palbociclib for the proposed HPLC method.

System Suitability:

System-suitability tests are an integral part of method development and are used to ensure adequate performance of the chromatographic system. Retention time (Rt), number of theoretical plates (N) and tailing factor (T) were evaluated for six replicate injections of the drug at a concentration of 40 µg/ml. The results which are given in Table No 6.15 were within acceptable limits.

Table No 7.3.2 System suitability studies of Palbociclib by HPLC method.

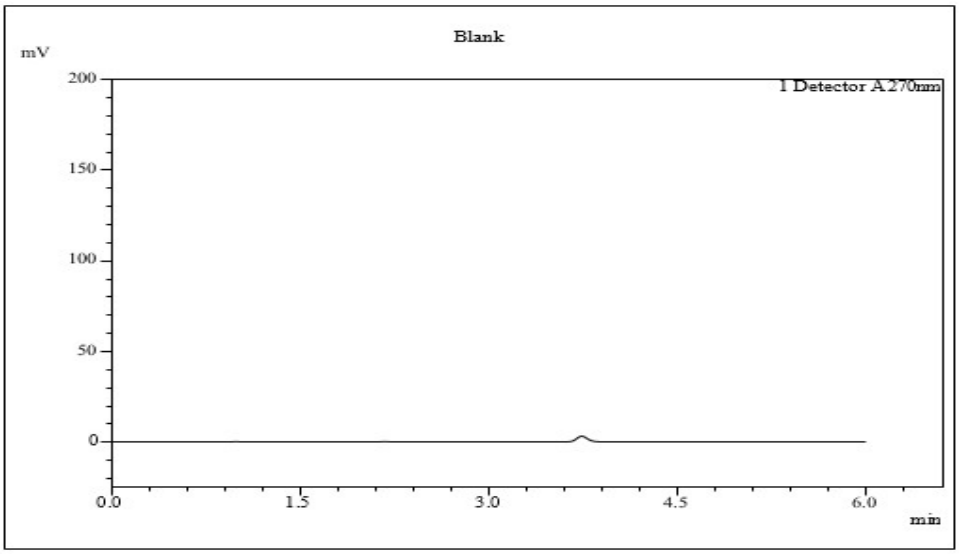
Retention time (min)	**Concentration (µg/mL)**	3.657
Peak area	10	824157
Theoretical plates		10292
Asymmetric Factor		1.0065

Specificity:

Chromatogram of blank was taken as shown in Fig No.6.25. Chromatogram of Palbociclib showed peak at a retention time of 6.223 min. The mobile phase designed for the method resolved the drug very efficiently. The Retention time of Palbociclib was 6.223 ± 0.0098min. The wavelength 265 nm was selected for detection because; it resulted in better detection sensitivity for the drug. The peak for Palbociclib from the tablet formulation was Palbociclib.

Table No 7.3.3. Specificity of Palbociclib by HPLC method

Sample	**Label Claim (mg)**	**Amount Found**	**Recovery**	**Retention Time**
Capsule	125	124.91	99.928	3.657



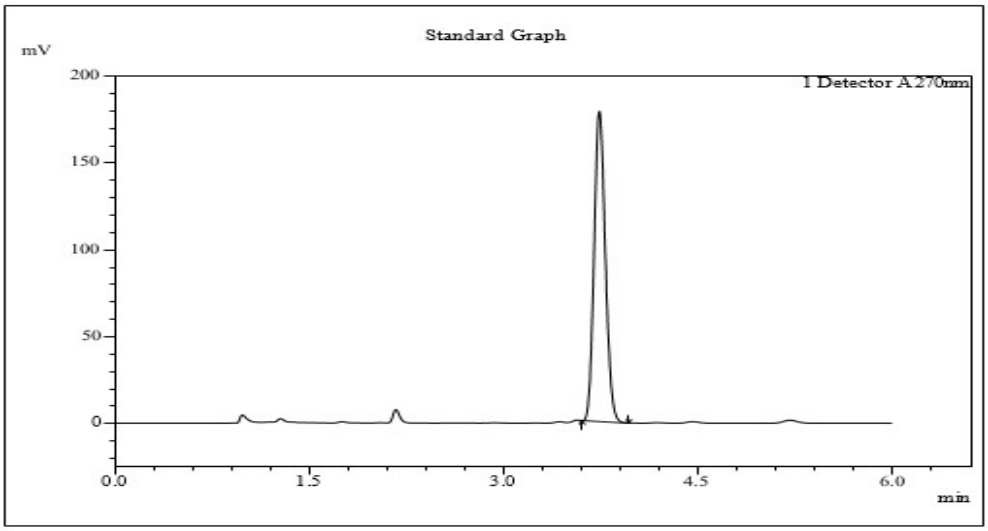


Fig. No. 7.3.3. A typical chromatogram of Blank

Sensitivity:

The sensitivity of measurement of Palbociclib by use of the proposed method was estimated in terms of the limit of detection (LOD) and the limit of quantification (LOQ). The LOD and LOQ were calculated by the use of signal to noise ratio. In order to estimate the LOD and LOQ values, the blank sample was injected six times and the peak area of this blank was calculated as noise level. The LOD was calculated as three times the noise level, while ten times the noise value gave the LOQ. LOD and LOQ were found to be and respectively.

1	LOD (µg/mL)	0.9044
2	LOQ (µg/mL)	2.7405

Precision:

Demonstration of precision was done under two categories. The injection repeatability (System Precision) was assessed by using six injections of the standard solution of Palbociclib and the % RSD of the replicate injections was calculated

Table No. 7.3.4. Intraday Precision of Palbociclib at 270 nm

Sr. No	Concentration	0 hr	2hr	4hr	6hr	Average
1	40	3301867	3302554	3388341	3291656	3321104.5
2	40	3391854	3281333	3473930	3237399	3346129
3	40	3387746	2486740	3476082	3340400	3172742
4	40	3400381	3223680	3396655	3291720	3328109
5	40	3369284	3308023	3395619	3366530	3359864
6	40	3301964	3337295	3303600	3390402	3333315.25
Average						3310211
Standard Deviation						62750.9
RSD						1.8957

Table No. 7.3.5 Interday Precision of Palbociclib at 270 nm

Sr. No	Concentration	1st Day	2nd Day	3rd Day	Average
1	40	3327514	3424661	3176226	3309467
2	40	3342075	3500642	3208415	3350377
3	40	3426385	3639438	3241547	3435790
4	40	3071103	3444808	3130349	3215420
5	40	3180237	3523786	3184967	3296330
6	40	3337035	3428315	3020803	3262051
Average					3311572.56
Standard Deviation					69366.48
RSD					2.095

Accuracy:

Recovery studies by the standard addition method were performed with a view to justify the accuracy of the proposed method. Previously analysed samples of Palbociclib (20 µg/ml) were spiked with 80, 100, and 120 % extra Palbociclib standard and the mixtures were analysed by the proposed method.

Standard deviation of the % recovery and % RSD were calculated and reported in Table No. 6.19.

Table No. 7.3.6 Accuracy of Palbociclib at 270 nm.

Sr. No	Concentration (µg/mL)	Peak area	Found Concentration (µg/mL)	% Recovery
1	32	2662906.4	31.99	99.96
2	40	3328633	39.99	99.98
3	48	1997179.8	48.09	100.18

Robustness:
Robustness is a measure of capacity of a method to remain unaffected by small, but deliberate variations in the method conditions, and is indications of the reliability of the method. A method is robust, if it is unaffected by small changes in operating conditions.

Table No. 7.3.7 Robustness of Palbociclib at 270 nm

Sr. No	**Parameter**		**Response**	**Parameter**	**Response**
Acetonitrile: Buffer (V/V)			**Retention Time (min)**	**Detection Wavelength** (nm)	**Peak Area**
1	79	21	3.56	263	3238501
2	80	20	3.657	265	3296816
3	81	19	3.756	267	3330080
Average			3.658	Average	3288466
Standard Deviation			0.080	Standard Deviation	37850.36
RSD%			2.188	**RSD%**	1.151
Flow Rate (mL/min)			**Retention Time (min)**	**pH of Buffer** (mmol/L)	**Peak Area**
1	0.9		3.788	2.8	3324888
2	1		3.657	3	3296989
3	1.1		3.543	3.2	3221368
Average			3.663	Average	3281082
Standard Deviation			0.1001	Standard Deviation	43733.13
RSD%			2.733	**RSD%**	1.3329

8.Conclusion

From literature review few analytical methods appeared for the determination of Palbociclib includes HPLC, HPTLC and UV- Visible spectrophotometric methods. In view of the above fact, some simple analytical methods were planned to develop with sensitivity, accuracy, precision and economical. In the present investigation RP-HPLC method (Using Quality by Design) for the quantitative estimation of Palbociclib in bulk drug and pharmaceutical formulations as per ICH guidelines has been developed. It was concluded that, the proposed RP-HPLC method applying QbD developed for the Palbociclib in bulk and in its formulations was simple, selective, sensitive, Accurate, precise and rapid. The proposed method was sufficiently sensitive and reproducible for the Analysis of Palbociclib within a short analysis Time. The method was proved to be superior to most of the reported methods. The mobile phases was simple to prepare and economical. The sample recoveries in the formulation were in good agreement with their respective label claims and they suggested non-interference of formulation excipients in the estimation. Hence these methods can easily be adopted as an alternative method to reported ones for the routine determination of Palbociclib depending upon the availability of chemicals and nature of other ingredients present in the sample.
The validation study shows that the developed method is accurate, rapid, precise, reproducible and inexpensive with acceptable correlation co-efficient, RSD (%) and standard deviations which make it versatile and valuable for simultaneous determination of Palbociclib in pharmaceutical dosage forms.
A new method was developed and then validated of UV and RP-HPLC for Palbociclib. The analytical procedure was validated as per ICH guidelines. This method represents an economic analytical procedure. The method is amenable to the routine analysis of large numbers of samples with good precision and

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