METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF TOFACITINIB IN TABLET DOSAGE FORM BY USING RP-UHPLC

A Dissertation submitted to

THE TAMILNADU Dr. M.G.R MEDICAL UNIVERSITY
CHENNAI – 600032

In partial fulfillment of the requirements for the award of the degree of

MASTER OF PHARMACY
IN
BRANCH – III → PHARMACEUTICAL ANALYSIS

Submitted by

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Under the Guidance of
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CERTIFICATE

This is to certify that the project entitled "**METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF TOFACITINIB IN TABLET DOSAGE FORM BY USING RP-UHPLC**" was submitted by **JANANI. N (261930002)** in partial fulfillment for the award of the degree of **Master of Pharmacy (Pharmaceutical Analysis)**. It was carried out at Synthiya Research Labs Pvt. Ltd., Villianur, Puducherry – 605 110 and at C.L. Baid Metha College of Pharmacy, Chennai – 600097 under my supervision in the Department of Pharmaceutical Analysis during the academic year 2019 –2021.

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SYNTHIYA RESEARCH LABS PRIVATE LIMITED

27.02.2022

TO WHOM SO EVER IT MAY CONCERN

This is to certify that **Ms. N. JANANI,** Reg. No-261930002, who is studying M.PHARM (PHARMACEUTICAL ANALYSIS) in C.L. BAID METHA COLLEGE OF PHARMACY, Chennai has completed his academic project in our SYNTHIYA REASEARCH LABS Pvt. Ltd., Villianur. Title **"Method development and validation of Tofacitinib in Tablet dosage form by using RP-UPLC".**

We found her conduct was good during the period. We wish her success in all her endeavors.

For SYNTHIYA RESEARCH LABS PRIVATE LIMITED,

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DECLARATION

I hereby declare that this dissertation entitled, **"METHOD DEVELOPMENT AND VALIDATION OF TOFACITINIB IN TABLET DOSAGE FORM BY USING RP-UHPLC"** has been originally carried out by me Synthiya Research Labs Private Limited, Villianur, Puducherry-605 110., and at the Department of Pharmaceutical Analysis, C.L. Baid Metha College of Pharmacy, Chennai-600 097., in the academic year 2021under the guidance and supervision of **Mr. R. Vijayakumar. M.Pharm.,** Associate Professor. This work has not been submitted in any other degree at any other university and that all the sources have used or quoted have been indicated and acknowledged by complete reference.

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INDEX

LIST OF ABBREVATION

CAN	:	Acetonitrile
ICH	:	International Council for Harmonization
mg	:	Milli gram
min	:	Minutes
mL	:	Milliliter
Min	:	Minute
Ml	:	millilitre
N	:	Theoretical Plates
NMT	:	Not More Than
NLT	:	Not Less Than
nm	:	Nanometer
QA	:	Quality Assurance
QC	:	Quality Control
R 2	:	Correlation Coefficient
RP-HPLC	:	Reverse Phase High-Performance Liquid Chromatography
RP-UPLC	:	Reverse Phase Ultra-Performance Liquid Chromatography
RS	:	Related Substance
RSD	:	Relative Standard Deviation
S/N	:	Signal / Noise
SD	:	Standard Deviation
UPLC	:	Ultra- Performance Liquid Chromatography
USP	:	United States Pharmacopoeia
v/v	:	Volume / Volume
WHO	:	World Health Organization
TFB	:	Tofactinib

INTRODUCTION

1. INTRODUCTION

The pharmaceutical analysis is a branch of chemistry, which involves the series of process for the identification, determination, quantitation, and purification. This is mainly used for the separation of the components from the mixture and for the determination of the structure of the compounds. The different pharmaceutical agents are Plants, Minerals Microorganisms and Synthetic compounds.[1]

Pharmaceutical analysis plays a vital role in the Quality Assurance and Quality control of bulk drugs. Analytical chemistry involves separation, identification, and determining the relative amounts of components in a sample matrix; Pharmaceutical analysis is a specialized branch of analytical chemistry that derives its principles from various branches of sciences like physics, microbiology, nuclear science, and electronics etc. Qualitative analysis is required before a quantitative analysis can be undertaken.

A separation step is usually a necessary part of both a qualitative and quantitative analysis. The result of typical quantitative analysis can be computed from two measurements. One is the mass or volume of sample to be analyzed and the second one is the measurement of some quantity that is proportional to the amount of analyte in that sample and normally completes the analysis.

The main steps that are performed during a chemical analysis are the following:

(1) sampling,

(2) field sample pretreatment,

(3) laboratory treatment,

(4) laboratory assay,

(5) calculations, and

(6) Results presentation.

Each must be executed correctly in order for the analytical result to be accurate. Some analytical chemists distinguish between an analysis, which involves all the steps, and an assay, which is the laboratory portion of the analysis[2].

The complete analysis of a substance consists of 5 main steps.

1. Sample preparation / Sampling
2. Dissolution of the sample,
3. Conversion of the analyte into a form suitable for measurement.
4. Measurement
5. Calculation and interpretation of the measurement

Need for pharmaceutical Analysis

1. New Drug Development.
2. Method Validation as for ICH Guidelines
3. Research in Pharmaceutical Sciences
4. Clinical Pharmacokinetic Studies

When promising results are obtained from explorative validation performed during the method development phase, then only full validation should be started. The process of validating a method cannot be separated from the actual development of method condition[3]

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A diversity of analytical techniques such as spectroscopy (UV-Visible), gas chromatography (GC), high performance liquid chromatography (HPLC), Ultra performance liquid chromatography (UPLC) supercritical fluid chromatography (SFC), capillary electrophoresis (CE) coupled with selective detectors (diode-array detector (DAD) and mass spectrometry (MS) are normally used to accomplish the above requirements.

In spite of various techniques existing, HPLC and UPLC has become a universal tool for pharmaceutical and biomedical research, as well as product analysis. The accessibility of fully automated systems, excellent quantitative precision, accuracy, sensitivity, selectivity and increased selection of column stationary phases, applicability to a lane variety of sample, Matrices and ability to hyphenate with several spectroscopic detectors has made HPLC or UPLC the instrument of choice for the analysis of most categories of drugs.

Correspondingly, HPLC or UPLC methods are profusely used in the field of biomedical analysis, viz. therapeutic drug monitoring, pharmacokinetic and bioequivalence studies. The assay of drugs in blood, plasma and tissues presents analytical challenges. The drug substance is typically present at low concentrations, bound to proteinaceous material and endogenous compounds typically present in the samples can interfere with the analysis. For these reasons, the analytical methods usually be highly sensitive to detect analytes at low concentrations and required a sample pre-treatment procedure such as liquid-liquid extraction (LLE) or solid phase extraction (SPE), to isolate the analyte from the complex biological matrix. Hence, high sensitivity and automation of sample processing tools to deal with large number of samples are strong incentives for the consideration of HPLC or UPLC methods in biomedical analysis.

UPLC comes from HPLC .Ultra Performance Liquid Chromatography (UPLC) is a relatively new technique giving new possibilities in liquid chromatography, especially concerning decrease of time and solvent consumption. This enhanced the demand for UPLC methods for the simultaneous determination of drugs in pharmaceutical mixtures[4]

TYPES

Traditionally, analytical chemistry has been split in to two main types, qualitative and quantitative:

Qualitative

• Qualitative inorganic analysis seeks to establish the presence of a given element or inorganic compound in sample.

• Qualitative organic analysisseeks to establish the presence of a givenfunctional group or Organic compound in a sample.

Quantitative

• Quantitative analysis seeks to establish the amount of given element or compound in a sample. Most modern analytical chemistry is categorized by two different approaches such asAnalyticaltargets or analytical methods[5]

By Analytical Targets

• Bio analytical chemistry

• Material analysis

• Chemical analysis

• Environmental analysis

• Forensics

By Analytical Methods

• Spectroscopy

• Mass spectroscopy

• Spectrophotometry and colorimetric

• Chromatography and Electrophoresis

• Crystallography

• Microscopy

• Electrochemistry

Traditional analytical techniques

• Titration

• Gravimetry

• Inorganic qualitative analysis

Instrumental analysis

• Spectroscopy

• Mass spectroscopy

• Crystallography

• Electrochemical Analysis

• Thermal analysis

• Separation

• Hybrid Techniques

• Microscopy

Analytical chemistry research is largely driven by performance (sensitivity, selectivity, robustness, linear range , accuracy, precision, and speed), and cost (purchase, operation, training, time, and space).

Analytical chemistry has played critical roles in the understanding of basic science to a variety of practical applications, such as biomedical applications, environmental monitoring, quality control of industrial manufacturing, forensic science and so on.

Pharmaceutical analysis plays a very significant role in quality control of pharmaceuticals through a rigid check on raw materials used in manufacturing of formulations and on finished products. It also plays an important role building up the quality products through in process quality control. Pharmaceutical analysis is the application of principles of analytical chemistry to drug analysis.

The analytical chemistry may be defined as the science of developing sensitive, relative and accurate methods for determining the composition of materials in terms of elements or compounds which they contain. The most important component aspect analysis is quantitative chemical analysis. In the presence age, the physical, chemical and biological analysis, involve computerized techniques to facilitate better result.[6]

1.1. ANALYTICAL TECHNIQUES[7]

SPECTROSCOPY	APPLICATIONS
Atomic Absorption and Emission spectroscopy(AAS/AES)	To analyse alkali and alkaline earth metals in dilute solution,natural liquids, and extracts at trace levels.
Ultraviolet-visible Spectroscopy (UV/Vis)	To analyse molecular (organic) and ionic species capableof absorbing at UV orVisible wave lengths in dilute solutions.
Fourier Transform Infrared Spectroscopy(FT-IR)	To analyse only molecular compounds (Organic compounds, natural products, polymers, etc.).
Fourier Transform Raman Spectroscopy (FT-Raman)	To analyse molecular (organic) compounds which are not responding well in the IR region and hence, it is an alternative IR.
Nuclear Magnetic Resonance Spectroscopy	To identify and characterize the organic and inorganiccompounds.

Microwave spectroscopy	To analyse simple gaseous molecules in far in region to studytheir stereo chemistry.
Electron spin resonance Spectroscopy (ESR)	To study the information and lifetime of the free radicals formed in organic reactions and all finds applications inbiological works.
Molecular Fluorescence spectroscopy	To study the molecular and ionic compounds in dilutesolutions capable giving fluorescence, applications in vitamin analysis.

1.1.1 CHROMATOGRAPHY

High Performance Liquid chromatography	To separate and analyse complex mixtures or solutions w which include liquids and solids and solids of both organic andinorganic origins.
Gas chromatography	To separate and analyze mixture of volatile organic compunds, solvent extracts and gases.

1.1.2 THERMAL ANALYSIS

Thermo gravimetric analysis (TGA)	To study the mass charges of materials like polymers, glasses,ceramics, etc., such as evaporation ,decomposition gas absorption, desorption, dehydration etc.,
Thermo mechanical analysis (TMA)	To study the expansion coefficient of composite and laminate materials.
Differential thermal analysis (DTA)	To study the exothermic and endothermic behaviour of claymaterials, ceramics, ores, etc.,

1.1.3 X-RAY TECHNIQUE

X-ray fluorescence (XRF) Spectrometry	To identify the elements and their states present in the surfaceof the materials.
X-ray Diffractometry (XRD)	To study the crystalline properties of solid substances.

1.1.4 MICROSCOPY

Scanning electron microscopy (SEM)	To Study the topography, electronic structure and compositions of metals, ceramics, polymers, composites and biological materials.
Transmission electronmicroscopy (TEM)	To study the local structures, morphology, and Dispersion of multi-component polymers, cross sections of crystallizationsof metallic alloys, semiconductors, microstructure of composites, etc.
Scanning probe microscopy (SPM)	To study the hardness and topography of materials likeceramics, polymers, composites, etc. on a nano scale range.

1.1.5 ELECTRO-CHEMICAL TECHNIQUES

Polarography	To study and determine metals, metal complexes, and organic compounds in trace levels.
Capillary electrophoresis (CE)	To study and characterize biologically active compounds like proteins, amino acids and bio-molecules.

1.1.6 MISCELLANEOUS TECHNIQUES

Total organic carbon analyser (TOC)	To monitor pollutants in environmental studies by determining the carbon contents of the trace compounds.
Elemental Analyser(CHN/S)	To estimate percentage compositions of elements like carbon, hydrogen, nitrogen, and sulphur present in newly synthesizedorganic compounds, pharmaceuticals, etc.
Polarimetry	To analyse and quantitative optically active compounds like sugar.
Circular Dichroism (CD) and optical Rotatory Dispersion (ORD)	To get the structural information of optically active compounds like, amino acids, proteins etc.
Vibrational CircularDichroism (VCD) and Vibrational Linear Dichroism (VLD)	Same as above but in the IR region. VLD measurement is employed to study the molecular orientations of thin polymer films.
Mass spectrometry (MS)	To identify the organic compounds. Often used detectors with HPLC and GC.
Laser Light scatteringsystem (LLIS)	In the study of macromolecules like polymers, Gels, proteins,etc. for determining molecular mass and size and their associations.

1.2 CHROMATOGRAPHY

1.2.1 Introduction to Chromatography

Chromatography is relatively a new technique which was first invented by M. Tswett, a botanist in 1906 in Warsaw. In that year, he was successful in doing the separation of chlorophyll, Xanthophyll and several other coloured substances by percolating vegetable extracts through a column of calcium carbonate. The calcium carbonate column acted as an adsorbent and the different substances got adsorbed to different extent and this gives rise to coloured bands at different positions, ion the column. Tswett termed this system of coloured

bands as the chromatogram and the method as chromatography after the Greek words Chroma and graphs meaning “colour” and “writing” respectively. However, in the majority of chromatographic procedures no coloured products are formed and the term is a misnomer.

Chromatography is a non-destructive procedure[8] for resolving a multi-component mixture of trace, minor, or major constituents into its individual fractions. Different variations may be applied to solids, liquids, and gases. While chromatography may be applied both quantitatively, it is primarily a separation tools.

1.2.2 Definition of Chromatograph

Chromatography may be defined as a method of separating a mixture of components into individual components through equilibrium distribution between two phases. Essentially, the technique of chromatography is based on the differences in the rate at which the components of a mixture move through a porous medium (called stationary phase) under the influence of some solvent or gas (called moving phase).

The chromatography method of separation, in general, involves the following steps:

- Adsorption or retention of a substance or separation, in general involves the following steps:
- Separation of the adsorbed substances by the mobile phase.
- Recovery of the separated substances by a continuous flow of the mobile phase, the method

  being called elution.
- Qualitative and quantities analysis of the eluted substances.

1.2.3 Classification of Chromatographic Techniques:

1.2.3.1 Absorption chromatography:

Adsorption chromatography is probably one of the oldest types of chromatography around. It utilizes a mobile liquid or gaseous phase that is adsorbed onto the surface of a stationary solid phase. The equilibration between the mobile and stationary phase accounts forthe separation of different solutes.

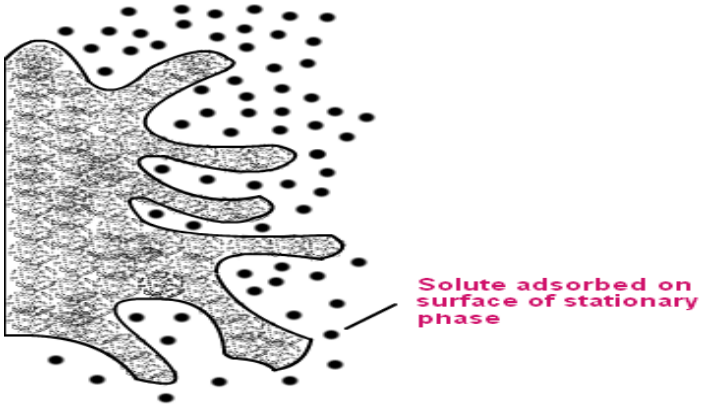


Fig.1 Absorption chromatography

1.2.3.2 Partition Chromatography:

This form of chromatography is based on a thin film formed on the surface of a solid support by a liquid stationary phase. Solute equilibrates between the mobile phase and the stationary liquid.

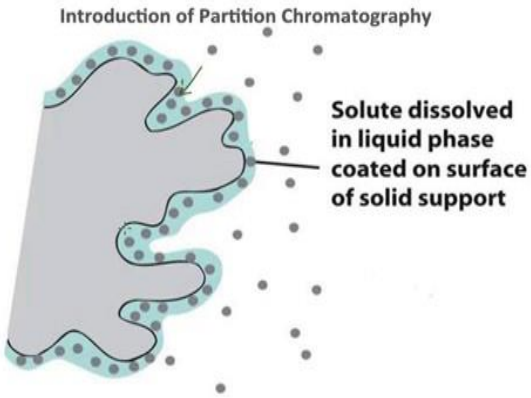


Fig.2 partition chromatography

1.2.3.3 Ion Exchange Chromatography:

In this type of chromatography, the use of a resin (the stationary solid phase) is used to covalently attach anions or captions onto it. Solute ions of the opposite charge in the mobile liquid phase are attracted to the resin by electrostatic forces.

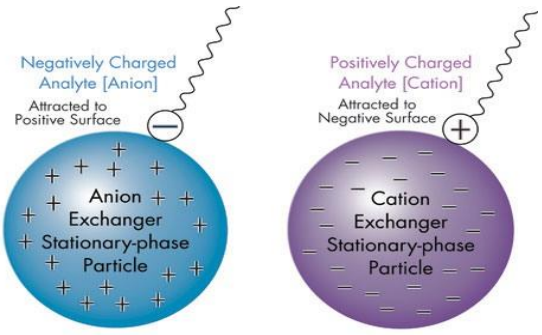


Fig.3 Ion exchange chromatography principle

1.2.3.4 Molecular Exclusion Chromatography:

Also known as gel permeation or gel filtration[8], this type of chromatography lacks an Attractive interaction between the stationary phase and solute. The liquid or gaseous Phase passesthrough a porous gel which separates the molecules according to its size.

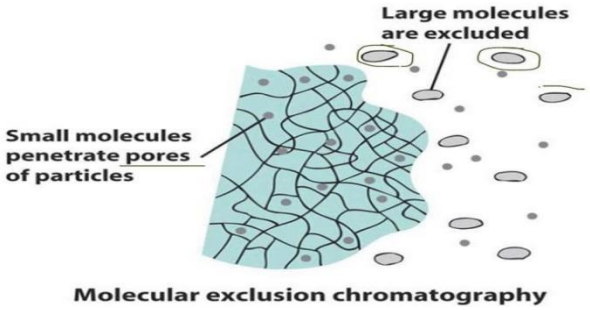


Fig.4 Molecular exclusion chromatography

The pores are normally small and exclude the larger solute molecules, but allow smaller molecules to enter the gel, causing them to flow through a larger volume. This causes the larger molecules to pass through the column at a faster rate than the smaller ones.

1.2.3.5 Affinity chromatography:

- Affinity chromatography is a type of liquid chromatography for the separation, purification or specific analysis of sample components.
- It utilizes the reversible biological interaction or molecular recognition called affinity which refers to the attracting forced exerted in different degrees between atoms which cause them to remain in combination.

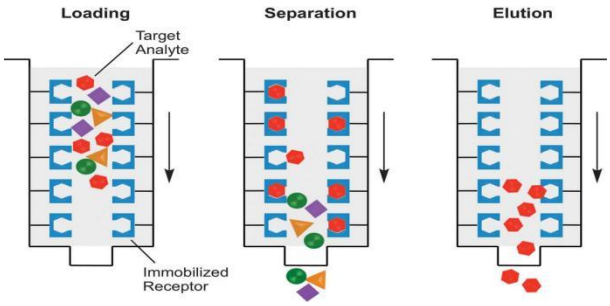


Fig.5 Affinity chromatography

1.2.3.6 Chiral Phase Chromatography:

Separations of optical isomer can be done by using stationary phases different principles operate for different types of stationary phases and different samples. The stationary phases are used for this type of chromatography are mostly chemically bonded silica gel[8].

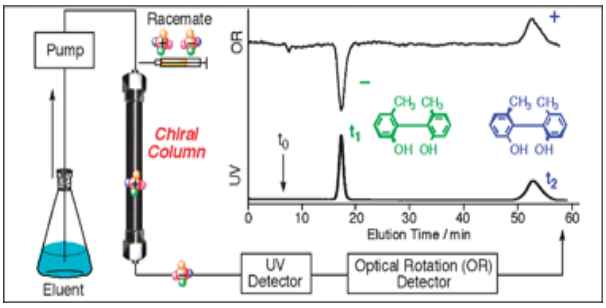


Fig. 6 chiral chromatography

1.2.4 High performance liquid chromatography:[9]

High Performance Liquid Chromotagraphy (HPLC) is an analytical technique used for the separation of compounds soluble in a particular solvent.

Affinities for Mobile and Stationary Phases

All chromatographic separations, including HPLC operate under the same basic principle; every compound interacts with other chemical species in a characteristic manner. Chromatography separates a sample into its constituent parts because of the difference in the relative affinities of different molecules for the mobile phase and the stationary phase used in the separation.

1.2.4.1 Technique

Normal Phase vs. Reverse Phase

If the stationary phase is more polar than the mobile phase, the separation is deemed normal phase. If the stationary phase is less polar than the mobile phase, the separation is reverse phase. In reverse phase HPLC the retention time of a compound increases with decreasing polarity of the particular species. The key to an effective and efficient separation is to determine the appropriate ratio between polar and non-polar components in the mobile phase. The goal is for all the compounds to elute in as short a time as possible, while still allowing for the resolution of individual peaks. Typical columns for normal phase separation are packed with alumina or silica. Alkyl, aliphatic or phenyl bonded phases are typically used for reverse phase separation.

Gradient Elution vs. Isocratic Elution

If the composition of the mobile phase remains constant throughout the HPLC separation, the separation is deemed an **isocratic elution**. Often the only way to elute all of the compounds in the sample in a reasonable amount of time, while still maintaining peak resolution, is to change the ratio of polar to non-polar compounds in the mobile phase during the sample run. Known as **gradient chromatography**, this is the technique of choice when a sample contains components of a wide range of polarities. For a **reverse phase gradient**, the solvent starts out relatively polar and slowly becomes more non-polar. The gradient elution offers the most complete separation of the peaks, without taking an inordinate amount of time. A sample containing compounds of a wide range of polarities can be separated by a gradient elution in a shorter time period without a loss of resolution in the earlier peaks or excessive broadening of later peaks. However, gradient elution requires more complex and expensive equipment and it is more difficult to maintain a constant flow rate while there are constant changes in mobile phase composition. Gradient elution, especially at high speeds, brings out the limitations of lower quality experimental apparatus, making the results obtained less reproducible in equipment already prone to variation. If the flow rate or mobile phase composition fluctuates, the results will not be reproducible.

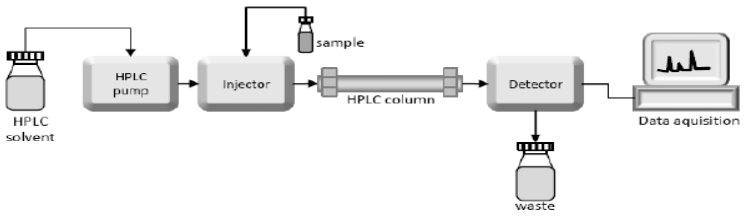


Fig. 7 Schematic presentation of HPLC

(i) Distribution Constant

All chemical reactions have a characteristic equilibrium constant. For the reaction

Aaq+Bs⇌ABs(1)(1)Aaq+Bs⇌ABs

There is a chemical equilibrium constant Keq that dictates what percentage of compound A will be in solution and what percentage will be bound to the stationary compound B. During a chromatographic separation, there is similar relationship between compound A and the solvent, or mobile phase, C. This will yield an overall equilibrium equation which dictates the quantity of A that will be associated with the stationary phase and the quantity of A that will be associated with the mobile phase.

A mobile⇌A stationary(2)(2)A mobile⇌A stationary

The equilibrium between the mobile phase and stationary phase is given by the constant Kc.

Kc=(aA)S(aA)M≈cScM(3)(3)Kc=(aA)S(aA)M≈cScM

Where Kc, the distribution constant, is the ratio of the activity of compound A in the stationary phase and activity of compound A in the mobile phase. In most separations, which contain low concentrations of the species to be separated, the activity of A in each is approximately equal to the concentration of A in that state. The distribution constant indicates the amount of time that compound A spends adsorbed to the stationary phase as the opposed to the amount of time A spends solvated by the mobile phase. This relationship determines the amount of time it will take for compound A to travel the length of the column. The more time A spends adsorbed to the stationary phase, the more time compound A will take to travel the length of the column. The amount of time between the injection of a sample and its elution from the column is known as the retention time; it is given the symbol tR.

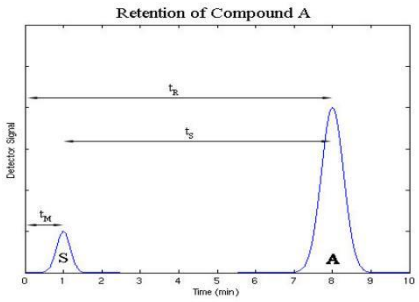


Fig. 8 Chromatogram

The amount of time required for a sample that does not interact with the stationary phase, or has a Kc equal to zero, to travel the length of the column is known as the void time, tM. No compound can be eluted in less than the void time.

(ii) Retention Factor

Since Kc is a factor that is wholly dependent on a particular column and solvent flow rate, a quantitative measure of the affinity of a compound for a particular set of mobile and stationary phases that does not depend on the column geometry is useful. The retention factor, k, can be derived from Kc and is independent of the column size and the solvent flow rate.

kC = KCVSVM(4)(4) kC = KCVSVM

The retention factor is calculated by multiplying the distribution constant by the volume of stationary phase in the column and dividing by the volume of mobile phase in the column.

(iii) Selectivity

In order to separate two compounds, their respective retention factors must be different, otherwise both compounds would be eluted simultaneously; the selectivity factor is the ratio of the retention factors.

α = kBkA(5)(5)α = kBkA

Where B is the compound that is retained more strongly by the column and A is the compound with the faster elution time.

(iv) Band Broadening

As a compound passes through the column it slowly diffuses away from the initial injection band, which is the area of greatest concentration. The initial, narrow, band that

contained all of the sample becomes broader the longer the analyte remains in the column. This band broadening increases the time required for complete elution of a particular compound and is generally undesirable. It must be minimized so that overly broad elution bands do not overlap with one another. We will see how this is measured quantitatively when we discuss peak resolution momentarily.

(v) Separation Efficiency

The overriding purpose of a chromatographic separation is just that, to separate two or more compounds contained in solution. In analytical chemistry, a quantitative metric of every experimental parameter is desired, and so separation efficiency is measured in plates. The concept of plates as a separation metric arose from the original method of fractional distillation, where compounds were separated based on their volatilities through many simultaneous simple distillations, each simple distillation occurred on one of many distillation plates. In chromatography, no actual plates are used, but the concept of a theoretical plate, as a distinct region where a single equilibrium is maintained, remains. In a particular liquid chromatographic separation, the number of theoretical plates and the height equivalent to a theoretical plate (HETP) are related simply by the length of the column

$$N = LH(6)(6)N=LH$$

Where N is the number of theoretical plates, L is the length of the column, and H is the height equivalent to a theoretical plate. The plate height is given by the variance (standard deviation squared) of an elution peak divided by the length of the column.

$$H = \sigma 2L(7)(7)H=\sigma 2L$$

The standard deviation of an elution peak can be approximated by assuming that a Gaussian elution peak is roughly triangular, in that case the plate height can be given by the width of the elution peak squared times the length of the column over the retention time of the that peak squared times 16.

$$H = LW216t2R(8)(8)H=LW216tR2$$

Using the relationship between plate height and number of plates, the number of plates can also be found in terms of retention time and peak width.

$$N = 16(tRW)2(9)(9)N=16(tRW)2$$

In order to optimize separation efficiency, it is necessary in maximize the number of theoretical plates, which requires reducing the plate height. The plate height is related to

the flow rate of the mobile phase, so for a fixed set of mobile phase, stationary phase, and analytes; separation efficiency can be maximized by optimizing flow rate as dictated by the van Deemeter equation.

$$H = A+Bv+Cv(10)(10)H=A+Bv+Cv$$

The three constants in the van Deemeter equation are factors that describe possible causes of band broadening in a particular separation. AA is a constant which represents the different possible paths that can be taken by the analyte through the stationary phase, it decreases if the packing of the column is kept as small as possible. BB is a constant that describes the longitudinal diffusion that occurs in the system. CC is a constant that describes the rate of adsorption and desorption of the analyte to the stationary phase. AA, BB and CC are constant for any given system (with constant analyte, stationary phase, and mobile phase), so flow rate must be optimized accordingly. If the flow rate is too low, the longitudinal diffusion factor (BvBv) will increase significantly, which will increase plate height. At low flow rates, the analyte spends more time at rest in the column and therefore longitudinal diffusion in a more significant problem. If the flow rate is too high, the mass transfer term (CvCv) will increase and reduce column efficiency. At high flow rates the adsorption of the analyte to the stationary phase results in some of the sample lagging behind, which also leads to band broadening.

(vi) Resolution

The resolution of a elution is a quantitative measure of how well two elution peaks can be differentiated in a chromatographic separation. It is defined as the difference in retention times between the two peaks, divided by the combined widths of the elution peaks.

$$RS = 2[(tR)B−(tR)A]WB+WA(11)(11)RS=2[(tR)B−(tR)A]WB+WA$$

Where B is the species with the longer retention time, and tR and W are the retention time and elution peak width respectively. If the resolution is greater than one, the peaks can Usually be differentiated successfully.

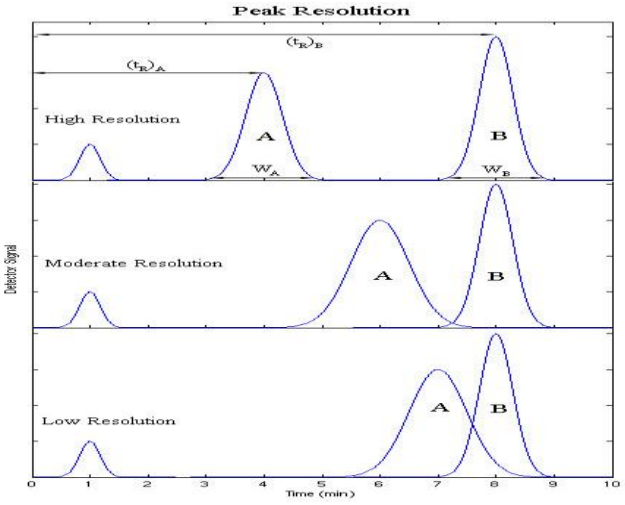


Fig.9 Resolution chromatogram

1.3 ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY

High-performance liquid chromatography (HPLC) is an important liquid chromatography (LC) technique used for the segregation of different components in mixtures. It is also used for the identification and quantification of compounds in the process of drug development and has been used over the world since decades. To further achieve the dramatic increase in resolution, speed and sensitivity in LC, a significant advancement in the instrumentation and column technology (column particle size and column dimension) were made[10]. To achieve the above targets, Ultra Performance Liquid Chromatography (UPLC) which is based upon small, porous particles (sub 2micron particles). Van Deemter equation is the principle behind this evolution which correlates the connection between linear velocity and plate height. The small particles require a high pressure to work with UPLC i.e., 6000 psi which is typically the upper limit of conventional HPLCs. It was observed that when the particle size is decreased below 2.5 µm, there is a remarkable increase in the effectiveness and this effectiveness does not lessen on increasing the linear speed or rate of flow[11]. The use of speed, particles with a small radius and maximum number of resolvable peaks (peak capacity) comprehends the efficiency together with resolution. This method reduces the mobile phase volume consumption by at least 80% compared to HPLC with a shorter runtime of about 1.5 min. The smaller sized particles increase the pressure up to 1000 bars or more which can alone increase the retention factor of the separation. Lower injection volume is required for UPLC which results in higher efficiency and increase in resolution. The higher

column temperature reduces the mobile phase viscosity resulting in the high diffusion coefficient and flow rate without significant loss in efficiency and increase in column back pressure. UPLC is a special version of HPLC which has the advantage of technological strides made in particle chemistry performance, system optimization, detector design, data processing and control[12]. These achievements lead to a very significant increase in resolution, sensitivity and efficiency with faster results and less consumption of solvents which lowers the cost and make the technology environment friendly also[13,14]

1.3.1 PRINCIPLE

The underlying principle of UPLC is based on the van Deemeter relationship which explains the correlation between flow rate and plate height[15]. The van Deemeter equation (i) shows that the flow range with the smaller particles is much greater in comparison with larger particles for good results[16].

$$H = A + \frac{B}{v} + Cv$$

Where H represents height equivalent to the theoretical plate (HETP), A, B & C are the constants and v is the flow rate (linear velocity) of the carrier gas. The aim is to minimize HETP to improve column efficiency. The term A does not depend on velocity and indicates eddy mixing. It is smaller if the columns are filled with small and uniform sized particles. The term B denotes the tendency of natural diffusion of the particles. At high flow rates, this effect is smaller, so this term is divided by v. The term C represents the kinetic resistance to equilibrium during the process of separation. The kinetic resistance is the time lag involved in moving from the mobile phase to the stationary phase and back again. The higher the flow rate of the mobile phase, the more a molecule on the packing material inclines to lag behind molecules in the mobile phase. Thus, this term is inversely proportional to linear velocity. Consequently, it is likely to enhance the throughput, and without affecting the chromatographic performance, the separation can be speeded up. The emergence of UPLC has necessitated the improvement of existing instrumentation facility for LC, which takes the benefit of the separation performance (by decreasing dead volumes) and consistent pressures (about 500 to 1000 bars, compared with 170 to 350 bars in HPLC). Efficiency is proportionate to the length of the column and inversely proportional to the radius of the particles[20]. Consequently, the column length can be reduced by the similar factor as the particle radius without affecting the resolution. The use of UPLC has

helped in the detection of drug metabolites and enhancement of the quality of separation spectra[16].

Fig.10 UPLC

1.3.2 INSTRUMENTATION

The instrumentation of UPLC includes- sample injection, UPLC columns and detectors.

Sample Injection:

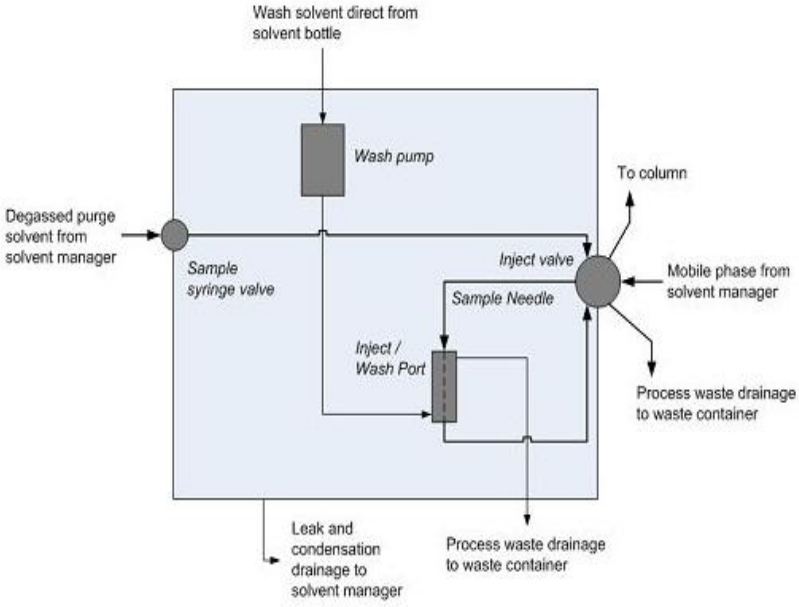


Fig. 11 Flow path through the system

The use of the injector is to add precisely measured, a small volume of solution containing the sample in the mobile phase. The injection must be done reproducibly and accurately. Conventional injection valves may be manual or programmed and to guard the column from extreme pressure instabilities, the injection process must be comparatively pulse-free. To reduce the potential band spreading, the swept volume of the device is desired to be minimal. A quick injection cycle time is required to fully avail the speed afforded by UPLC. To increase the sensitivity, low volume injections with minimal carryover are required. The volume of the sample in UPLC is usually 2-5 µL. Nowadays, direct injection approaches are utilized for the biological samples[17].

UPLC Column

Different types of columns being used in UPLC are packed with particles which are produced through different technologies [18]. These are as follows:

1. Charged Surface Hybrid [CSH] particle technology,
2. Ethylene Bridged Hybrid [BEH] particle technology,
3. High Strength Silica [HSS] particle technology and
4. Peptide Separation Technology (PST).

CSH Particle Technology[18]

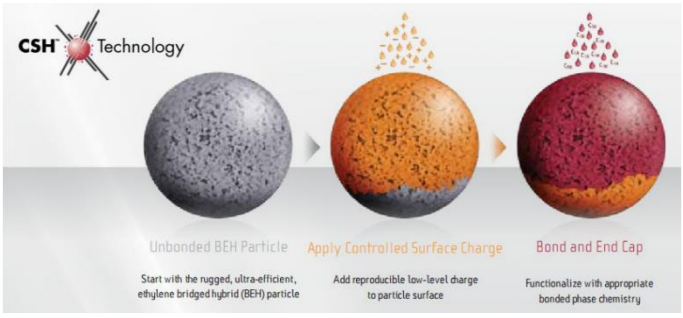


Fig .12 CSH column

CSH Technology is the newest methodology in the development of hybrid materials which utilizes low-level surface charged particles for the enhancement of the selectivity and sharpness of the peaks. Hybrid based packing material approach provides sharp peaks specially for basic compounds under low pH with higher efficiency and chemical stability. CSH C_{18}, CSH Phenyl hexyl, and CSH Fluoro phenyl are the different types of CSH particles being widely used.

These columns have the advantage of exceptional peak shape, increased loading capacity (CSH C_{18}); complementary selectivity to straight chain alkyl phases (CSH-phenyl-hexyl); selectivity for positional isomers, halogenated and polar compounds (CSH-fluoro phenyl). The other advantages include- higher stability at a wide range of pH, improved batch to batch reproducibility and fast column equilibration after any change in the pH of the mobile phase.

Applications of CSH technology based columns include the analysis of basic compounds even in their ionized form. While analyzing the basic compounds under low pH and reversed phase conditions, poor peak shape and retention often result. Whereas, CSH Phenyl hexyl columns provide exceptional peak shape for basic drugs under acidic mobile phase conditions.

BEH Particle Technology[18]

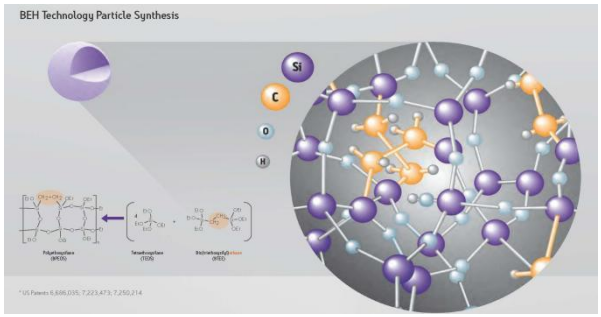


Fig .13 BEH Technology

For more than a decade, hybrid particle technology [HPT] has delivered incomparable versatility and performance, enabling chromatographers to push the limits of LC separations. The XTerra particle was the first commercially available option to improve the issues (poor peak shape for basic compounds and column longevity due to chemical instability) without the drawbacks of unpredictable selectivity produced by alternative materials such as zirconia, organic polymers, and graphitic carbon. With the commercialization of 2.5 µm XTerra particles, the concept of fast HPLC with small particles was born, improving the productivity of chromatographic laboratories globally.

Straight chain alkyl columns (BEH C_{18} and C_8), Embedded polar group column (BEH Shield RP18) and UPLC BEH Phenyl (phenyl group tethered to the silyl functionality with a C_6 alkyl) are the different types of BEH particle technology based columns which are being widely used.

These columns provide higher retention, selectivity (C_{18}); exceptional efficiency, peak symmetry, chemical stability (C_8); enhanced peak shape and higher compatibility with 100%

aqueous mobile phase (RP18); chemical stability, reproducibility and peak shape (phenyl). Applications include the rapid assay of cytochrome p450 isoenzymes which are responsible for more than 90% of drug metabolism on the market today. Cytochrome p450 isoenzymes are used to determine the level of drug inhibition, induction or drug-drug interaction that takes place. The ultra-low dispersion and system dwell volume of UPLC technology enables the rapid analysis of these enzymes in less than 30 seconds. shows the chromatogram of an assay of cytochrome P450.

HSS Particle Technology[18]

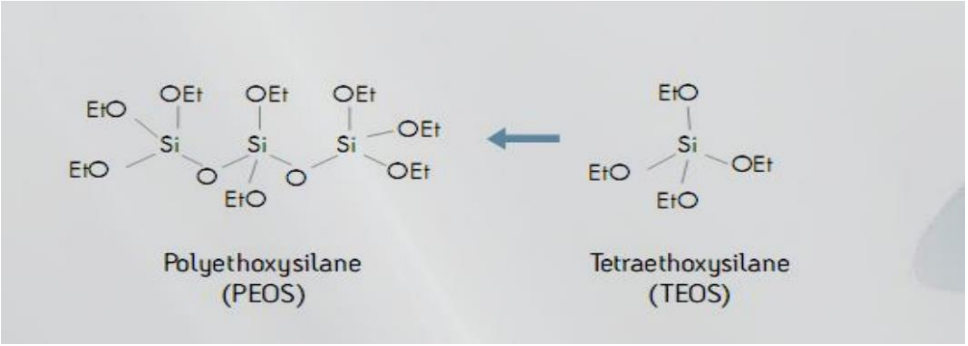


Fig.14 HSS Particle Technology

HSS particle technology is the advanced technology, born from an innovative synthetic process in which the mechanical stability is improved while the pore volumes remain similar to that of HPLC silica-based materials. This results in an advanced technique which provides higher retention in comparison to the hybrid particles. HSS T3, HSS C_{18}, HSS C_{18} SB, HSS PFP and HSS CN are the different types of HSS particles being widely used.

These columns provide balanced retention of polar and hydrophobic molecule (T3); exceptional peak shapes, increased retention (C_{18}); greater retention of basic compounds (CS $_{18}$ SB); ideally suited for planar aromatic, positional, halogenated compounds (PFP); ultra stable retention and compatible with both reversed-phase and normal-phase techniques (CN).

Applications of this technology include the analysis of tetracycline antibiotics which are commonly prescribed for the treatment of bacterial infections. The ACQUITY UPLC HSS C8 column enables a single UV-based method for the simultaneous separation of

oxytetracycline including its degraded and related products (which are produced due to drug fermentation) as well as additional veterinary antibiotics.

PST Columns[18]

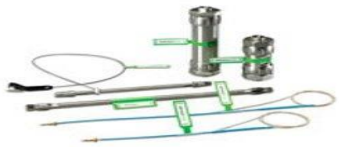


Fig.15 PST column

PST utilizes the C_{18} BEH Technology ^{TM} particles whose particles sizes range from 1.7 µm to 10 µm and the column dimension ranges from 75 µm to 30 mm internal diameter (i.d) and column length from 50 mm to 250 mm. They are used in all kind of research and development that involves analysis and isolation of peptides. The PST columns provide sharp symmetrical peaks.

Solvent Delivery System

The solvent delivery system must perform reproducible high pressure pumping with a smooth and constant flow of solvents. UPLC systems routinely operate at 8000-15000 psi. The delivery system must also remunerate for a variety of solvents used in isocratic, linear & nonlinear gradient elution and solvent compressibility for a wide range of pressures. The Acquity UPLC binary solvent manager has two solvent delivery modules operating in parallel for high pressure merging of two solvents in <140 µL internal system volume. The dissolved gases are removed by vacuum up to four eluents plus two wash solvents[19,20]

The Detector

The detector employed for the UPLC should be able to give a high sampling rate with narrow obtainable peaks (<1 s half-height peak width) and the dispersion of the peaks should be minimum so that the wastage of the separated solute is less on the column. The UPLC technique provides the sensitivity of separation two to three times more than the previous analytical method HPLC, which is also due to the method employed for the detection. The detectors employed in the UPLC are Acquity photodiode array (PDA) and Tunable Vis-UV (TUV) in which Teflon AF is used which provides an internally reflective surface and enhances the light transmission efficiency by eliminating the internal absorptions. These have

path lengths 10 nun, acquisition rates 20 (PDA) and 40 (TUV) points, and total internal volume 500 nL. Mass spectrometric detection has also been used with UPLC[21,22].

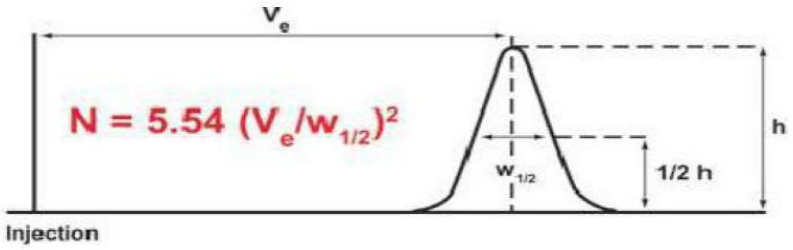
1.3.3 SYSTEM SUITABILITY PARAMETERS

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated.[23] The parameters that are affected by the changes in chromatographic conditions are,

1. Number of theoretical plates or Efficiency (N).
2. Capacity factor (K).
3. Separation or Relative retention (α).
4. Resolution (Rs).
5. Tailing factor (T).
6. Relative Standard Deviation (RSD).

1. Number of theoretical plates / Efficiency (N)

In a specified column, efficiency is defined as the measurement of the degree of peak dispersion and it should have the column characteristics. The efficiency is conveyed in terms of number of theoretical plates". The formula of calculation of N is illustrated bellow in the following,



Where, N = Efficiency / Number of theoretical plates.

Ve = Retention time of analyte.

h = Height of the peak.

W ½ = Gaussian function of the peak width at the half- height.

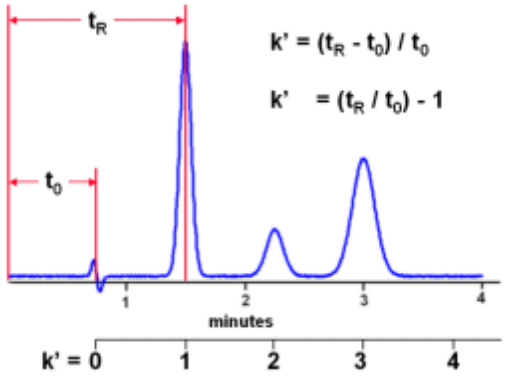
The plate number depends on column length. Theoretical plate number is the measure of column efficiency. As stated by plate theory, the analyte will be in instant equilibrium with stationary phase and column has to be divided into number of\ hypothetical plates and each plate consists of a fixed height and analyte spends finite time in the plate. Height equivalent to theoretical plate (HETP) is given by following formula:

$$\mathbf{HETP = L/N}$$

Where, L = length of column.

N = plate number.

2. Capacity ratio (or) Capacity factor (k)



It is a measure of the retention of a peak that is independent of column geometry or mobile phase flow rate. The capacity factor is calculated as

$$\mathbf{k' = (t_R - t_0)/t_0}$$

Where,

t_R is the retention time of the peak

t_0 is the dead time of the column.

3. **Relative retention (or) separation factor (α)**

$$\boldsymbol{\alpha = t_2\text{-}t_a/t_1\text{-}t_a}$$

Where,

α = Relative retention.

t_2= Retention time calculated from point of injection.

t_a= Unretained peak time (Retention time of an inert component not retained by the column).

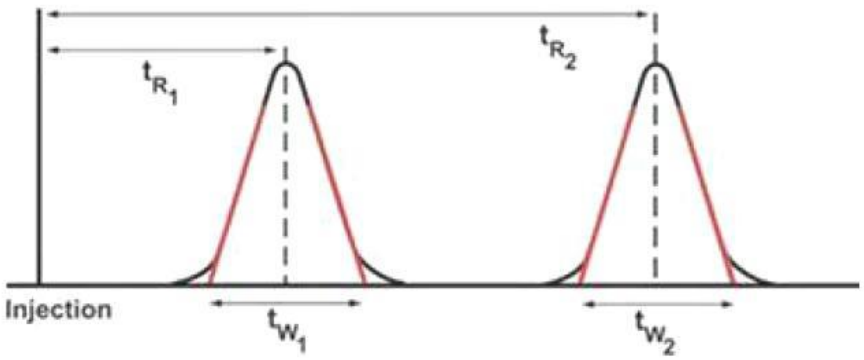
t_1= the retention time from the point of injection of reference peak defined

(Suppose no reference peak is found, value would be zero).

4. **Resolution (Rs)**

Resolution is the capability of the column to separate 2 drugs in 2 individual peaks or chromatographic zones and it is improved by enhancing column length, reduction of particle size and rising temperature, altering the eluent or stationary phase. It can be told in terms of ratio of separation of the apex of two peaks by the tangential width average of the peaks. By using the following formula resolution is calculated.

$$\mathbf{RS = (tR_2\text{-}tR_1)/0.5(tW_1+tW_2)}$$



Where, tR1 and tR2 are the retention times for the two peaks of components tw1 and tw2 are the baseline lies between tangents drawn to the sides of the peaks. If the peaks are correctly symmetric, provided the valley between the two peaks should touch the baseline Rs is 1.5. Generally good value of resolution is Rs ≥2 should be adequate and preferred normally.

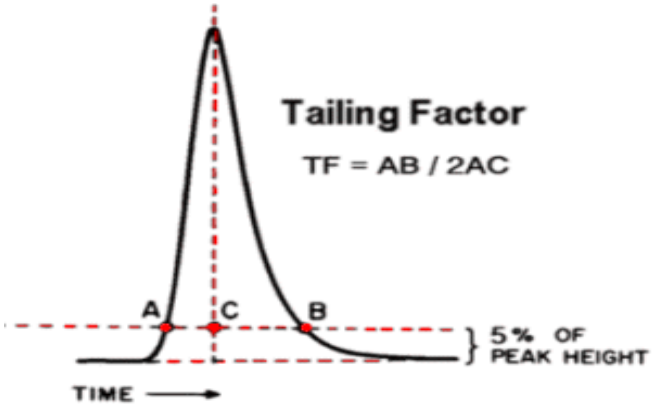
5. Resolution factor (R)

Resolution is a function of capacity factor, function of selectivity and a function of efficiency (or) number of theoretical plates (N). In order to separate any two peaks you must have right capacity factor ideally between 2 and 10, but appropriate selectivity is required i.e., ideally 1.2 and enough efficiency i.e., number of theoretical plates (more than 2000 theoretical plates). Resolution should be ≥ 1.5.

$$\mathbf{R=k'/1+k'\ (\alpha\text{-}1/\ \alpha)\ (\sqrt{\ (N/4)}}$$

6. Tailing factor (or) Asymmetry factor

The tailing factor is a measure of peak tailing. It is defined as the distance from the front slope of the peak to the back slope divided by twice the distance from the centre line of the peak to the front slope, with all measurements made at 5% of the maximum peak height. The tailing factor of a peak will typically be similar to the asymmetry factor for the same peak, but the two values cannot be directly converted.



7. **Relative standard deviation (RSD)**

Relative standard deviation is a measure of the spread of data in comparison of the data. It is simply the standard deviation divided by the mean value.

$$\mathbf{RSD} = \frac{\mathbf{S(xi)}}{\mathbf{X}}$$

1.3.4. STATISTICAL PARAMETERS[24]

i) Regression equation

The linear relationship is characterized by at tendency of the points of the scattered diagram to cluster along a straight line known as the regression line.

$$\mathbf{Y = a + bX}$$

It is used to describe the dependence of one characteristic (Y) up on the other characteristic (X), both X,Y represent values of two characters a, b are two constants it will be evident that two regression lines can be computed for every set of data-one each to describe the dependence of one character to another. b is known as regressive coefficients which shows change expected in Y for unit change in X, it is dependence of Y & X; b is the regressive coefficient of Y& X. The regressive coefficient of b is estimated,

$$b = \frac{\sum\left(x - \bar{x}\right)\left(y - \bar{y}\right)}{\sum\left(x - \bar{x}\right)^2}$$

b = the slope of the regression line and is calculated by this formula

x = an arbitrarily chosen value of the predictor variable for which the corresponding value of the criterion variable is desired.

ii) Correlation coefficient

A measure of the strength of the relationship between two variables is provided by the coefficient of correlation denoted by r, if the relationship between the two variables is of the linear form. It is also called the coefficient of linear correlation.

iii) Pearson's correlation

The correlation coefficient calculation for data values should be +1 or -1 where the values of Correlation coefficient is +1 – positive

Correlation coefficient is -1 – negative.

$$r = \frac{\sum XY - \frac{\sum X \sum Y}{N}}{\sqrt{\left(\sum X^2 - \frac{(\sum X)^2}{N}\right)\left(\sum Y^2 - \frac{(\sum Y)^2}{N}\right)}}$$

Where, X – value of one character

Y – Value of another character

Iv) Standard Deviation

It is the square root of the average of the squared deviations of the observations. From the arithmetic mean, it is used for measures of dispersion. It is denoted by

Standard Deviations = R.S.D (%) = 100 x S.D

Where,

SD = Sum of observations

x = Mean or arithmetic average (x / n)

x = Individual observed value

x – x = Deviation of a value from the mean

n = Number of observations

1.3.5 Method Development and Optimization of Chromatographic Conditions[25]

Methods for analysing drugs in multi component dosage forms can be developed if the nature of the sample, namely, its molecular weight, polarity, ionic character and the solubility parameter are in hand. Until and unless considerable trial and error procedures have not performed, an exact recipe for HPLC cannot be provided .

The most difficult problem usually is where to start, what type of column is worth trying with what kind of mobile phase. In general one begins with reversed phase chromatography, when the compounds are hydrophilic in nature with many polar groups and are water soluble**.**

Selection of stationary phase/ column:

Selection of the column is the initial and the most noteworthy step in method development.

The proper choice of separation column includes three different approaches.

1. Selection of separation system
2. The particle size and the nature of the column packing
3. The physical parameters of the column i.e. the length and the diameter.

Some of the crucial parameters considered while selecting chromatographic Columns. They are length and diameter of the column, packing material, Shape of the particle, Size of the particles, % of Carbon loading, Pore volume, Surface area and End capping. The column is selected depending on the nature of the solute and the information about the analyte, Reversed phase mode of chromatography facilitates information about the analyte.

Reversed phase mode of chromatography[12] facilitates a wide range of columns like dimethylsilane (C2), butylsilane (C4), octylsilane (C8), octadecylsilane (C18), base deactivated silane (C18) BDS, cyanopropyl (CN), nitro, amino etc.

Selection of mobile phase:

The primary objective in selection and optimization of mobile phase[12] is to achieve optimum separation of all the individual impurities and degrades from one other and from analyte peak. In liquid chromatography, the solute retention is governed by the solute distribution factor, which reflects the different interactions of the solute-stationary phase, solute-mobile phase and the mobile phase-stationary phase.

For a given stationary phase, the retention of the given solute depends directly upon the mobile phase, the nature and the composition of which has to be judiciously selected in order to get appropriate and required solute retention. The mobile phase has to be adapted in terms of elution strength (solute retention) and solvent selectivity (solute separation).

Solvent polarity is the key word in chromatographic separations since a polar mobile phase will give rise to low solute retention in normal phase and high solute retention in reverse phase LC.

The selectivity will be particularly altered if the buffer PH is close to thepKaof the analytes; the solvent strength is a measure of its ability to pull analyte from the column. It is generally controlled by the concentration of the solvent with the highest strength. Buffers, pH of the buffer, mobile phase composition are the parameters, which shall be taken into consideration while selecting and optimizing the mobile phase.

Firstly, Buffer and its strength play an important role in deciding the peak symmetries and separations. Some of the most, commonly employed buffers are Phosphate buffer (Potassium di hydrogen phosphate, Di-potassium hydrogen phosphate, Sodium dihydrogen phosphate, Disodium hydrogen phosphate), Phosphoric acid buffers prepared using O-Phosphoric acid, Acetate buffers (Ammonium acetate, Sodium acetate) and Acetic acid buffers prepared using acetic acid.

The retention time[12] also depends on the molar strengths of the buffer. Molar strength is increasingly proportional to retention times. The strength of the buffer can be increased, if necessary, to achieve the required separations. The solvent strength is a measure of its ability to pull analytes from the column. It is generally controlled by the concentration of the solvent with the highest strength. Secondly, pH plays an important role in achieving the chromatographic separations as it controls the elution properties by controlling the ionization characteristics.

Ware conducted using buffers having different pH to obtain the required separations. It is important to maintain the pH of the mobile phase in the range of 2.0to8.0 as most

columns do not withstand the pH which is outside this range. This is due to the fact that the Siloxane linkage area gets cleaved below pH 2.0, while pH valued is above 8.0, silica may dissolve.

Finally, by choosing the optimum mobile phase composition most of the Chromatographic separations can be achieved. This is due to the fact that fairly large amount of selectivity can be achieved by choosing the qualitative and quantitative composition of aqueous and organic portions. Most widely used solvents in reverse phase chromatography are methanol and acetonitrile. Experiments were conducted with mobile phases having buffers with different pH and different organic phases to check for the best separations between the impurities. A mobile phase which gives separation of all the impurities and degrades from the analyte peak and which is rugged with variations of both aqueous and organic phase by at least ±0.2% is preferred.

The low solubility of the sample in the mobile phase can also cause bad peak shapes. It is always advisable to use the same solvents for the preparation of sample solution for the preparation of sample solution as the mobile phase to avoid precipitation of the compounds in the column injector.

Optimizations are often started only after a reasonable chromatogram has been obtained. A reasonable chromatogram means symmetrical peaks in the chromatogram and detection of all the compounds. By slight modification of the mobile phase composition, the position of the peaks can be predicted within the range of investigated changes. An optimized chromatogram is the one in which all the peaks are symmetrical and are well separated in less run time.

1.3.6 Validation of method[26]

Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for its intended use. The methods validation process for analytical procedures begins with the planned and systematic collection by the applicant of the validation data to support analytical procedures. All analytical methods that are intended to be used for analyzing any clinical samples will need to be validated. The validation of analytical methods is done as per ICH guidelines.

Components of method validation

The following are typical analytical performance characteristics which may be tested during methods validation:

1. Accuracy
2. Precision
3. Repeatability
4. Intermediate precision
5. Linearity
6. Detection limit
7. Quantitation limit
8. Specificity
9. Range
10. Robustness
11. Solution stability studies

ACCURACY

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness.

PRECISION

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution.

The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

Intermediate precision

Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

Reproducibility

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).

DETECTION LIMIT

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

QUANTITATION LIMIT

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

LINEARITY

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

RANGE

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

ROBUSTNESS

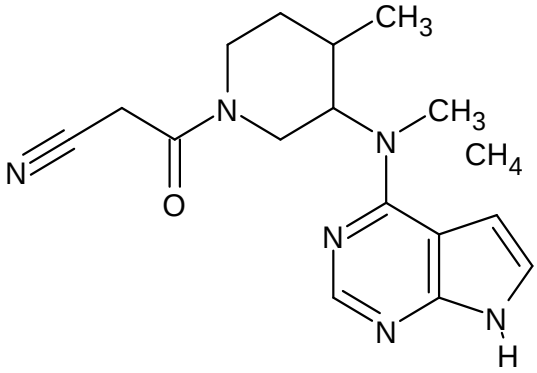
The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

DRUG PROFILE

2. DRUG PROFILE

Tofacitinib

Structure:



IUPAC Name	**:**3-[(3R,4R)-4-methyl-3-[methyl({7H-pyrrolo[2,3-d]pyrimidin4yl})amino]piperidin-1-yl]-3Oxopropanenitrile
Molecular formula	**:** $C_{16}H_{20}N_6O$
Molecular weight	**:** 312.4 g/mol
Synonym	**:** Tofacitinib
CAS No	**:** 477600-75-2
Description	**:** Tofacitinib is a Janus Kinase Inhibitor.
Therapeutic group	**:** Rheumatoid arthritis, psoriatic arthritis and inflammatory bowel disease.
Pharmaceutical form	**:** Tablet
Route Of Administration	**:** Oral[27].

2.1 Physical & Chemical Properties:

Appearance	**:** White to off-white powder.
Solubility	**:** Soluble in *N*,*N*-Dimethylacetamide, insoluble or very slightly soluble in water.
Melting point	**:** 199-206 °C
LogP	**:** 1.808
Dissociation constant	**:** pKa1 = 8.46 (cyano); pKa2 = 13.56 (secondary amine)
Storage conditions	**:** Keep container tightly closed in a dry and well ventilated place [28].

2.2 Pharmacokinetic Properties:

The absorption of tofacitinib was rapid, with plasma concentrations for both tofacitinib and TRA peaking at around 1 hour after oral administration.

Absorption **:** Rapid (74%)

Distribution **:** 87 L

Protein binding **:** ~40% (predominantly to albumin)

Bioavailability **:** 74%

Metabolism **:** CYP3A4 and CYP2C19 to inactive metabolites

Excretion **:** Primarily urine (30%) as unchanged drug

Half-life elimination **:** ~3 hours (IR solution, tablet); ~6 to 8 hours (extended release)

2.3 Pharmacodynamic Properties:

Tofacitinib targets inflammation present in rheumatoid arthritis by inhibiting the Janus kinases involved in the inflammatory response pathway. In placebo controlled trials of rheumatoid arthritis patients receiving 5mg or 10mg of tofacitinib twice daily, higher ACR20 responses were observed within 2 weeks in some patients (with ACR20 being defined as a minimum 20% reduction in joint pain or tenderness and 20% reduction in arthritis pain, patient disability, inflammatory markers, or global assessments of arthritis by patients or by doctors, according to the American College of Rheumatology (ACR) response criteria list), and improvements in physical functioning greater than placebo were also noted.

2.4 Mechanism of Action:

Tofacitinib is a partial and reversible Janus kinase (JAK) inhibitor that will prevent the body from responding to cytokine signals. By inhibiting JAKs, tofacitinib prevents the phosphorylation and activation of STATs. The JAK-STAT signaling pathway is involved in the transcription of cells involved in hematopoiesis, and immune cell function. Tofacitinib works therapeutically by inhibiting the JAK-STAT pathway to decrease the inflammatory response.

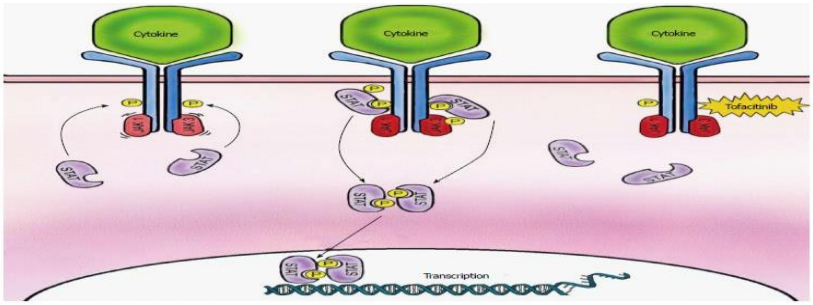


Fig.16 Mechanism of action of Tofacitinib

2.5 Adverse effects:

The most common side effects of tofacitinib include upper respiratory tract infection, **cold-like symptoms, diarrhea, rash**, herpes zoster infection (shingles) and headache. The medication can also cause more severe problems such as serious infections, heart problems, cancer, blood clots, and death.

Common side effects may include:

- ✓ skin rash, shingles;
- ✓ increased blood pressure;
- ✓ abnormal blood tests;
- ✓ fever;
- ✓ headache;
- ✓ nausea, vomiting, diarrhea; or.
- ✓ cold symptoms such as stuffy nose, sneezing, sore throat [30].

2.6 Uses:

- ✓ Tofacitinib is approved for medical use in the United States with an indication "to treat adults with moderately to severely active rheumatoid arthritis who have had an inadequate response to, or who are intolerant of, methotrexate.
- ✓ It has demonstrated effectiveness in the treatment of psoriasis in phase III studies. It is being studied for treatment of disease, and other immunological diseases, as well as for the prevention of organ transplant rejection[31].

LITERATURE REVIEW

3. Review of literature

Sampath Kumar Reddy Govind A et Al.(2014) developed a reverse phase liquid chromatographic (RP-LC) for the quantification of the related impurities of Tofacitinib citrate drug substance. The method was optimized using buffer (prepared by dissolving 2.72g potassium di hydrogen phosphate and 1.0gm of 1-Octane sulphonoic acid sodium salt anhydrous taken in 1000mL milli-Q-water and then pH was adjusted to 5.5 with dilute potassium hydroxide solution) along with Acetonitrile 90:10v/v as mobilephase-A, and Acetonitrile: Buffer in the ratio of 70:30v/v as mobile phase-B. The flow rate was set at 1.0 mL min-1, wavelength at 210nm and the column temperature was maintained at 25°C. The capability of stability indicating method developed was demonstrated by studying the degradation products generated during the forced degradation studies under the following conditions i) water hydrolysis, ii) at 75% relative humidity, iii) oxidative, iv) thermal v) sunlight, vi) acid, vii) base, and viii) photolytic degradation. The developed method can be used for the determination of synthetic and degradation impurities in the regular quality control analysis for the drug substance.[32]

Dhiman V et al. (2015) developed a novel, simple, specific, sensitive and reproducible high-performance liquid chromatography (HPLC) assay method for the development and validation for the estimation of tofacitinib in rat plasma. The bioanalytical procedure involves extraction of tofacitinib and itraconazole (internal standard, IS) from rat plasma with a simple liquid-liquid extraction process. The chromatographic analysis was performed on a Waters Alliance system using a gradient mobile phase conditions at a flow rate of 1.0 mL/min and C18 column maintained at 40 ± 1 °C. The eluate was monitored using an UV detector set at 287 nm. Tofacitinib and IS eluted at 6.5 and 8.3 min, respectively and the total run time was 10 min. Method validation was performed as per US Food and Drug Administration guidelines and the results met the acceptance criteria. The calibration curve was linear over a concentration range of 182-5035 ng/mL ($r(2) = 0.995$). The intra- and inter-day precisions were in the range of 1.41-11.2 and 3.66-8.81%, respectively, in rat plasma. The validated HPLC method was successfully applied to a pharmacokinetic study in rats [33].

Yanjiao, Wang et al.(2015) established a HPLC method for the determination of four enantiomers in tofacitinib citrate. A Chiralpak IC column was used with the mobile phase of methyl tertbutyl ether: ethanol: diethylamine (70: 30: 0.1) at the detection wavelength of

280 nm. The resolutions of tofacitinib citrate enantiomers were greater than 2.0. The calibration curves of four enantiomers were all linear in the ranges of 0.1 - 120 μg/ml. The recoveries were above 99.0 %, with RSDs less than 1.5 %. Their low limits of quantification were all 0.1 μg/ml.[34]

Adnan A Kadi et al. (2016) developed a simple, adequately sensitive, and practical liquid chromatographic-mass spectrometric method for simultaneous quantification of three tyrosine kinase inhibitors, viz, tofacitinib (TOF), cabozantinib (CBZ) and afatinib (AFB) after their extraction from both human plasma and urine. Blood and urine samples were obtained from healthy volunteers who admitted to not being on any medications. The investigated analytes were chromatographically separated on a C18 column (Luna®-PFP 100Å column, 50 mm × 2.0 mm i.d., 3.0 μm) with the aid of a mobile phase containing A; acetonitrile (ACN) and B; 0.01 M ammonium formate buffer (pH 4.1) pumped at a rate of 0.3 mL.min-1 in the ratio A:B, 50:50 v/v. Analyte monitoring was achieved by tandem mass spectrometry interfaced with an electrospray ionization source with the aid of multiple reaction monitoring (MRM) mode for analytes quantification. The proposed method permitted a specific and sensitive determination of the investigated TKIs in the linear range of 1.0 - 100 ng mL-1 with correlation coefficient (r2) of 0.9991, 0.9997, and 0.9998 for TOF, CBZ and AFB, respectively. The method was validated with regard to its limits of quantification (ranging from 0.91 to 1.24 ng mL-1 for the 3 analytes), intra- and inter assay accuracy (in the range -1.85 to 1.22 %) and precision (0.71 - 5.12 %). The method was also validated in terms of recovery from both studied matrices, robustness and matrix effect [35].

N.V. Thakariya et al.(2017) developed a simple, sensitive and economical UV spectrophotometric method has been developed for the estimation of Tofacitinib Citrate in bulk drug and laboratory prepared mixture. The proposed method obeyed Beer's law in the concentration range of 5-25 μg/mL with correlation coefficient=0.9998. Accuracy was confirmed by recovery studies and mean recovery was found to be 100.28±0.65. The intraday and inter day precision were found to be within limits. The proposed methods have adequate specificity, sensitivity and reproducibility for quality control assay of Tofacitinib Citrate in bulk and laboratory prepared mixture [36]

XiaoWu et al. (2017) developed LC-MS techniques for identification of related substance for synthetic optimization process. The separation was achieved on a LiChrospher C18 column (250 mm × 4.6 mm, 5μm) by linear gradient elution of 0.1% ammonium acetate solution (pH adjusted to 4.0 by formic acid) and acetonitrile at a flow rate of 1.0 mL/min. Forced degradation studies were conducted under hydrolytic (acidic, basic), oxidative, photolytic and thermal stress conditions as described in ICH. It was found that tofacitinib was stable under photolytic condition, but degraded obviously in acidic, basic, thermal and oxidative conditions. The high resolution TOF-MS and MS/MS were used for determination and structural identification of the related substances. Eleven major related substances were detected and identified as five process-related substances and six degradation products, and three of them were further synthesized and characterized by NMR spectroscopy. The most plausible mechanisms involved in the formation of the related substances were also proposed [37].

A.S.K. Sankar et al.(2017) Developed a simple validated analytical method for analysis of Tofacitinib by UV Spectroscopy and to studied the forced degradation and stress conditions used to detect the stability of Tofacitinib. Tofacitinib was estimated at 285.9nm. Linearity range was found to be 10-50 mcg/ml. The correlation coefficient was found to be 0.9996. The molar absorptivity was found to be 12468.77mol/cm. The proposed method Sandell's sensitivity was found to be 0.040410 μg cm2/0.001AU. The limit of detection and quantification were found to be 0.8169 and 2.4755 μg/ ml respectively. The degradation behavior of Tofacitinib was carried out as per the standard procedures and guidelines. Forced acid hydrolytic degradation, alkali degradation and oxidative degradation of was performed in bulk Tofacitinib and laboratory prepared admixtures using 1M Hydrochloric acid up to 48 hrs, in 10 % Hydrogen peroxide up to 48 hrs and for 1.0 M Sodium hydroxide up to 10 min at room temperature. The resulting solutions were analyzed for content by UV spectrophotometry at the maximum absorption of 285.9 n. The assay value of Tofacitinib in bulk and physical admixture was calculated at different time intervals for intraday and interday experiments. Results and Conclusions: The proposed method was successfully applied for the determination of tofacitinib in pure and laboratory prepared physical mixtures. The % RSD value of Tofacitinib in bulk and physical admixture was calculated at different time intervals for recovery , precision (Iintraday and Interday experiments) and quantification studies were found to be less than 2 %.[38]

Badithala Siva Sai Kiran et al. (2018) developed a validated method for estimation of tofacitinib Chromatography was carried out on Phenomenex Luna C18 (250 x 4.6mm, 5µm) column using a mobile phase was methanol: water (45:55% V/V) at a flow rate of 1.0mL/min. The analyte was monitored using a UV detector at 254 nm. The retention time was found to be 4.35 minutes. The proposed method was found to be linear in the concentration range of 15-90µg/mL with a correlation coefficient of 0.999. The mean recovery was found to be 99.24 %. The developed method has been validated according to ICH guidelines and found to be selective, precise and accurate with the prescribed values. Thus, the proposed method was successfully applied for the estimation of Tofacitinib in routine quality control analysis [39].

Prathyusha Naik C.N et al. (2018) developed a novel, selective, reliable and sensitive reverse phase high performance liquid chromatography (RP-HPLC) method for the detection and quantification of Tofacitinib in pure form as well as in its dosage form. The chromatographic method was carried out using isocratic elution programme on C18 Phenomenex Luna (250 x 4.6 mm x 5µm) column with a mobile phase proportion of water and Methanol in the ratio of 50:50 (% v/v). The flow rate was set to 1.0 ml/min with 20 µl injection volume. The eluted components were monitored at 254 nm and ambient column oven temperature was maintained. The developed analytical method was validated according to the ICH guidelines. The developed method was also subjected to various stress conditions like acidic and alkaline hydrolysis, oxidation, photolysis and thermal degradation. The method showed linearity across the concentration range of 10- 60µg/ml. Limit of detection and quantification was found to be 1.45 and 4.40 µg/ml respectively. The developed method is specific, precise, accurate, robust and stability indicating which can be successfully applied for routine analysis, quality control analysis and also suitable for stability analysis of assay of Tofacitinib in pure form and its formulation as per the regulatory requirements [40].

Kirtikumar D. Bharwad et al. (2018) has reported a highly sensitive, selective and rapid ultra-performance liquid chromatography–tandem mass spectrometry method for the quantification of a Janus kinase (JAK) inhibitor, tofacitinib (TOF). The assay employed liquid–liquid extraction with methyl-tert butyl ether to extract tofacitinib and tofacitinib-13C3 15 N (as internal standard) from human plasma. The samples were analysed on a UPLC BEH C18 (50 × 2.1 mm, 1.7µm) column using acetonitrile and 10.0 mm ammonium acetate, pH 4.5 (75:25, v/v) as the mobile phase within 1.4 min. The precursor/product ion transitions were monitored at m/z 313.3/149.2 and 317.4/149.2 for tofacitinib and tofacitinib-13C3 15 N,

respectively, in the positive electrospray ionization mode. The calibration curves were linear ($r2 \geq 0.9978$) across the concentration range of 0.05–100 ng/mL. The mean extraction recovery of tofacitinib across quality controls was 98.6%. The intra- and inter-batch precision (CV) and accuracy ranged from 2.1–5.1 and 96.2–103.1%, respectively. All validation results complied well with the current guidelines. The method is amenable to high sample throughput and was applied to determine TOF plasma concentration in a pharmacokinetic study with 12 healthy Indian subjects after oral administration of 5 mg tablets [41].

Dixit A et al.(2018) developed a simple, sensitive and rapid assay method and validated as per regulatory guideline for the estimation of tofacitinib on mice dried blood spots (DBS) using liquid chromatography coupled to tandem mass spectrometry with electro spray ionization in the positive-ion mode. The method employs liquid extraction of tofacitinib from DBS disk of mice whole blood followed by chromatographic separation using 5 mM ammonium acetate (pH 6.5):acetonitrile (20:80, v/v) at a flow rate of 0.60 mL/min on an X-Terra Phenyl column with a total run time 2.5 min. The MS/MS ion transitions monitored were m/z 313→149 for tofacitinib and m/z 316→149 for the internal standard (13C3, 15N-tofacitinib). The assay was linear in the range of 0.99-1980 ng/mL. The intra- and inter-day precision was in the range of 1.17-10.3 and 3.37-10.9%, respectively. Stability studies showed that tofacitinib was stable on DBS cards for one month. This novel method has been applied to analyze the DBS samples of tofacitinib obtained from a pharmacokinetic study in mice.[42]

Hitaishi Panchal et al. (2020) presented a simple, precise, accurate and cost effective RP-HPLC method for estimation of Tofacitinib from Tofacitinib Citrate extended release tablet dosage form. The chromatographic method was carried out using Inertsil ODS-3V (150 mm × 4.6 mm, 5 µm) column with mobile phase Phosphate buffer (pH 5.5) and Acetonitrile in ratio of 65:35 %v/v. The flow rate was set 1.0 ml/min with 5 µL injection volume. Total run time 7 min. Detection was carried out at the wavelength of 287 nm. The developed analytical method was validated according to the ICH guideline. The Develop method was also subjected to various stress condition like acid and alkali hydrolysis, oxidation, photolysis, humidity and thermal degradation. The detector response was linear in the concentration range of 21.86– 174.94 µg/ml. The developed method is successfully applied for estimation of Tofacitinib from Tofacitinib Citrateextended release tablet dosage form.[43]

Ji Eun Kim et al.(2020) developed method for quantification of tofacitinib, a JAK inhibitor, in rat plasma, urine and tissue homogenates by HPLC. Hydrocortisone was used as an internal standard. The mobile phase was an isocratic system of acetonitrile: 10 mM ammonium acetate, pH 5.0 (30.5:69.5, v/v), and the flow rate was 1.0 mL/min. Chromatograms were monitored by a UV detector at 287 nm. The retention times for tofacitinib and hydrocortisone were 7.21 and 11.3 min, respectively. The lower limits of quantification for tofacitinib in rat plasma and urine were 0.01 and 0.1μg/mL, respectively. The intraday assay precisions (coefficients of variation) were generally low; 3.69–5.88% for rat plasma and 4.21–6.18% for rat urine. The corresponding values of interday assay precisions were 5.06% and 5.46%, respectively. Accuracies ranged from 92.9 to 107%, with no interference by endogenous substances. Tofacitinib has a short half-life (39.0 min) and was widely distributed in rat tissues [44].

Srividya Gorantla et al. (2021) developed stability-indicating HPLC method for quantification of tofacitinib in topical nano formulations and different matrices (adhesive tape, and skin layers, i.e., stratum corneum, viable epidermis, and dermis). The major objective was to avoid use of instruments like LC–MS/MS and to ensure a widespread application of the method. The calibration curve showed regression coefficient (R2) 0.9999 and linearity in the concentration range of 50 to 15,000 ng/mL, which is suitable for the analysis of conventional dosage forms and nano formulations. Method validation was performed as per ICH guideline Q2 (R1). The accuracy by recovery studies ranged between 98.09 and 100.82%. The % relative standard deviations in intraday and interday precisions were in the range of 1.16–1.72 and 1.22–1.80%, respectively. Forced degradation studies indicated the specificity of method and showed stability-indicating potential for tofacitinib peak.[45]

Thaticherla Kaleswararao et al (2021) has reported development and validation for cleaning estimation of tofacitinib citrate from tofacitinib tablets using RP-HPLC method. The manufacturing equipment considered in assessment of cleaning has been verified and found the tools assembled to the equipment are made up of Stainless steel, Glass, Teflon and plastic. Hence, these surfaces of manufacturing equipment that come in contact with the drug product during manufacturing are considered for evaluation of the cleaning procedure. By developing and validating an analytical method for residue estimation, the manufacturing equipment can be evaluated for efficient cleaning and to release the manufacturing equipment for further intended use by minimizing the cross contaminations. The stationary phase suited for the well

separation of components is CAPCELL PAK C18 150 x 4.6 mm, 3µm; 0.4 % perchloric acid and acetonitrile in the ratio of 85:15 % v/v is the mobile phase pumped at a flow rate of 1.2 mL/min through the column at temperature of 40 ºC. Each run extended for 10 min as the Tofacitinib peak elutes at RT of 5.2 min. The method has been validated successfully for Specificity, Precision, Linearity, Accuracy, Ruggedness and Filter validation of both rinse and swab methods. The LOD, LOQ concentrations found to be 0.006, 0.019 µg/mL for swab method and 0.03 and 0.1 µg/mL for rinse method respectively. The correlation coefficient is 0.999 and method found linear from LOQ to 500% for swab method and LOQ to 200% for rinse method. Solution stability has been established to ensure the test solution get tested within the stable time (4 Days). Based on the filter validation data, it is concluded that PVDF filter is not suitable for cleaning sample analysis and 2 mL sample should be discarded when 0.45 µm Nylon filter is used for cleaning sample analysis [46].

Liu P et al.(2021) investigated the metabolism of tofacitinib in mouse, rat, monkey, and human liver microsomes fortified with β-nicotinamide adenine dinucleotide phosphate tetrasodium salt and uridine diphosphate glucuronic acid. The biotransformation was executed at a temperature of 37°C for 60 min, and the samples were analyzed by ultra-high performance liquid chromatography combined with high-resolution mass spectrometry (UHPLC-HRMS) operated in positive electrospray ionization mode. The structures of the metabolites were elucidated according to their retention times, accurate masses, and MS/MS spectra. Under the current conditions, a total of 13 metabolites, including 1 glucuronide conjugate, were detected and structurally proposed. Oxygenation of the pyrrolopyrimidine ring, oxygenation of piperidine ring, N-demethylation, oxygenation of piperidine ring side chain, and glucuronidation were the primary metabolic pathways of tofacitinib. Among the tested species, tofacitinib showed significant species difference. Compared with other species, rat showed similar metabolic profiles to those of humans. The present study provides some new information regarding the metabolism of tofacitinib in animals and humans, which would bring us considerable benefits for the subsequent studies focusing on the pharmacological effect and toxicity of this drug.[47]

Anne mette handler et al.(2020) conducted skin penetration studies of tofacitinib by HPLC-MS/MS analysis using extracts of heat-separated epidermis and dermis to estimate the amount of drug penetrated. In this study, MALDI-MSI enabled qualitative skin distribution analysis of endogenous molecules and the drug molecule, tofacitinib and quantitative analysis of the amount of tofacitinib in the epidermis. The delivery of tofacitinib to the skin was investigated

in a Franz diffusion cell using three different formulations (two oil-in-water creams, C1 and C2 and an aqueous gel). Further, in vitro release testing (IVRT) was performed and resulted in the fastest release of tofacitinib from the aqueous gel and the lowest from C2. In the ex vivo skin penetration and permeation study, C1 showed the largest skin retention of tofacitinib, whereas, lower retention and higher permeation were observed for the gel and C2. The quantitative MALDI-MSI analysis showed that the content of tofacitinib in the epidermis for the C1 treated samples was comparable to HPLC-MS/MS analysis, whereas, the samples treated with C2 and the aqueous gel were below LOQ. The study demonstrates that MALDI-MSI can be used for the quantitative determination of drug penetration in epidermis, as well as, to provide valuable information on qualitative skin distribution of tofacitinib.[48]

Yilmaz H (2021) conducted Forced Degradation Studies to Assess the Stability of a Janus Kinase Inhibitor Using RPLC because it is important to study the retention behavior of the compounds containing the ionizable functional groups under the intended chromatographic conditions. In this study, the influence of pH and acetonitrile (ACN) composition in the mobile phase on chromatographic behavior of tofacitinib (TOF), a Janus kinase (JAK) inhibitor, was investigated in details. First, the chromatographic conditions were optimized using retention factors and pKa values. Then, the developed method was used for the stability studies under various stress conditions, and for the estimation of TOF concentration in tablets. Finally, the method was validated using the International Conference on Harmonization (Q2) guidelines and it was successfully used to separate the TOF degradation products. A linearity range, limit of detection and limit of quantification were determined as 2.0-12.0, 0.416, and 1.260 μg/mL, respectively. Between-day and within-day precisions were found to be as 0.290 and 0.462 for 4 μg/mL, respectively. The result indicates that the developed method is rather effective to separate the parent drug from the degradation products.[49]

Qiong wang et al(2022) developed and validated ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method to simultaneously detect plasma concentrations of tofacitinib and its metabolite M9, and to study the pharmacokinetic profiles of the two compounds in beagle dogs. After rapid precipitation of protein by adding acetonitrile, the chromatographic separation of tofacitinib was completed, as well as M9 and upadacitinib (internal standard, IS) by using an Acquity BEH C18 (1.7 μm,

2.1 mm × 50 mm) column. A Xevo TQ-S triple quadrupole tandem mass spectrometer was employed to determine their concentrations under the positive ion pattern. Selective reaction monitoring (SRM) was used with ion transitions at m/z 313.12 → 148.97 for tofacitinib, m/z 329.10 → 137.03 for M9, and m/z 380.95 → 255.97 for IS, respectively. This assay demonstrated excellent linearity, and the ranges of calibration curves for both tofacitinib and M9 were 0.5–400 ng/mL. The new UPLC-MS/MS assay can reach the values (0.5 ng/mL) of lower limit of quantification (LLOQ) for both tofacitinib and M9. Both intra-day and inter-day accuracy of all analytes ranged from −12.0% to 14.3%, while the precision was ≤13.2%. The recovery rate of all analytes was >88.5%, and more importantly there was no conspicuous matrix effect. In addition, the stability was consistent with the quantificative requirements of plasma samples under all conditions. Finally, the assay on UPLC-MS/MS is able to be employed to determine the pharmacokinetic characteristics of tofacitinib and its metabolite M9 in the plasma of beagle dogs after taking orally a dose of tofacitinib at 2 mg/kg.[50]

AIM AND OBJECTIVE

4. AIM AND OBJECTIVE

Tofacitinib, a first oral non-biologic disease-modifying anti-rheumatic drug (DMARD) for treating adults with moderate or severe rheumatoid arthritis. As per ICH guidelines, every pharmaceutical drug compound (API of the drug substance) must be estimated within the limit. So, it is important to detect and quantify the API in pharmaceutical dosage form for safety and efficacy of the patients as per ICH.

AIM:

The aim of the present research work is to develope simple, accurate, sensitive, rapid and economic method for estimation of Tofacitinib in pharmaceutical dosage form by ultra high performance liquid chromatography (UHPLC).

To best of my knowledge, no analytical method was reported for estimation of tofacitinib by UHPLC method. Thus the present work focuses on development of new analytical method for estimation of Tofacitinb in pharmaceutical dosage form and Confirmation of the applicability of the developed method was done by validating according to the International Conference on Harmonization (ICH).

OBJECTIVE:

- To develop a novel RP-UHPLC method for estimation of tofacitinib in tablet dosage form.
- To optimize the chromatographic conditions for quantification of tofacitinib using RP-UHPLC
- To validate the developed method as per ICH Q2 (R1) guidelines for its accuracy, precision, linearity, selectivity, robustness, etc.
- To ensure that the developed method can be utilised for routine analysis.

PLAN OF WORK

5. PLAN OF WORK

Plan of work was designed as follows:

Literature collection.

↓

Study of physicochemical properties of drug (pH, pKa and solubility).

↓

Procurement of chemicals and API.

↓

Method development and optimization of chromatographic conditions

Selection of diluent

Selection of wavelength

Choice of chromatographic method

Selection of initial separation

Optimize the chromatographic variables.

↓

Development of UPLC method for the quantification of Tofacitinib in tablet dosage form.

↓

Validation of proposed method as per ICH Q2 (R1) guidelines.

MATERIALS AND INSTRUMENT

6. MATERIALS AND INSTRUMENTS

INSTRUMENT USED:

INSTRUMENTS / EQUIPMENTS USED	MAKE
UV-spectrophotometer	SHIMADZU
UPLC	AGILENT TECHNOGLOIES
Ultrasonicater	PCI ANALYTICS
Analytical balance	METTLER TOLEDO
PH meter	HANNA INSTRUMENT
Pipettes and Burettes, Volumetric(Standard) Flask	BOROSIL (CLASS A)
Glass Beakers, Measuring Cylinder	BOROSIL

DRUGS AND CHEIMCALS USED:

Materials	Make
Tofacitinib Tablet 5mg	MSN laboratories Batch No: DRB10131A MFD DATE: OCT 2021 EXP DATE: SEP 2023
Working standard for Tofacitinib	Synthiya research lab pvt.ltdvillianur
Potassium dihydrogen phosphate	Rankem
Acetonitrile (ACN), and UPLC grade water	Merck
Methanol	Rankem

METHOD DEVELOPMENT

7. METHOD DEVELOPMENT AND OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS

A good method development strategy should require only as many experimental runs as are necessary to achieve the desired final result. Finally, method development should be as simple as possible, and it should allow the use of sophisticated tools such as computer modelling. The important factors, which are to be taken into account to obtain reliable quantitative analysis, are

- ✧ Careful sampling and sample preparation.
- ✧ Precise sample injection.
- ✧ Appropriate choice of the column.
- ✧ Choice of the operating conditions to obtain the adequate resolution of mixture.
- ✧ Reliable performance of the recording and data handling systems.
- ✧ Suitable integration/peak height measurement techniques.
- ✧ The mode of calculation best suited for the purpose.
- ✧ Validation of the developed method.

7.1 Selection of chromatographic mode:

Reverse phase chromatography is the most preferred mode of separation for polar compounds since it has high resolution. In reverse phase chromatography, non polar stationary phase used for separation. The reversed phase UPLC was selected for the separation because of its simplicity and suitability.

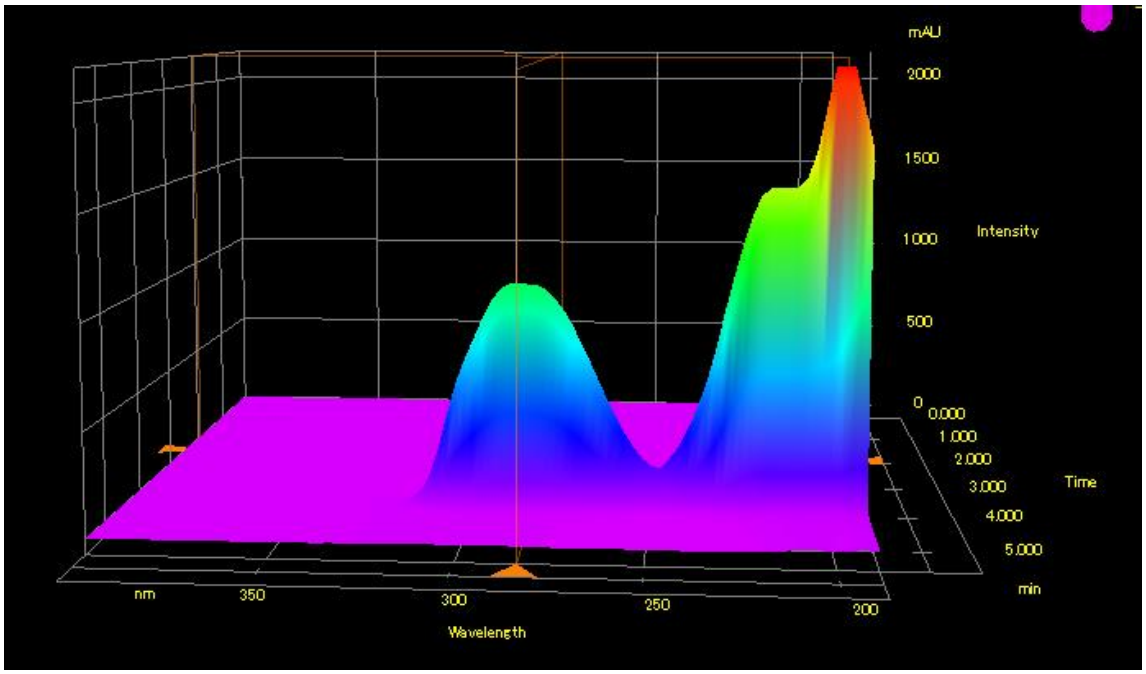
7.2 Selection of Diluent:

Diluent is selected initially based on solubility of the substance. Tofacitinib showed high solubility in methanol and water in the ratio of 50:50 and used as diluent.

7.3 Detection method & Selection of wavelength (λ max):

Known concentration of Tofacitinib working standard was taken and dissolved in methanol and water (50:50) such that the standard solution contains about 1mg/ml. Placebo and blank solutions were also prepared. All these solutions were scanned between 200 to 400nm using HPLC. From the UV spectrum at the drug shows good absorbance 290 nm.

After reviewing the chromatograms and peak purity chromatograms a wavelength of 290 is selected as the optimum wavelength for this drug.



WAVELENGTH SELECTION OF TOFACITINIB

7.4 Optimization of mobile phase:

On the basis of retention property study results of the drug and reviewing the results "phosphate buffer at pH 5.3" is decided as the buffer preparation to be used.

7.5 Selection of pH of the buffer:

'pH' plays very an important role in achieving the chromatographic separation as it controls the elution properties by controlling ionization characteristics.

At pH 5.3 peak shape, peak tailing and theoretical plate count was found to be satisfactory and hence 3.5 is decided as the pH of the Buffer.

7.6 Mobile phase composition:

Many trials on composition of Buffer, Acetonitrile & methanol were made to decide the ultimate composition of the mobile phase.

After reviewing many trials good peak shape, retention time, tailing factor, theoretical plates are obtained with the mobile phase composition Buffer: ACN : Methanol (4:4:2). Hence it was finalized.

7.7 Selection of flow rate:

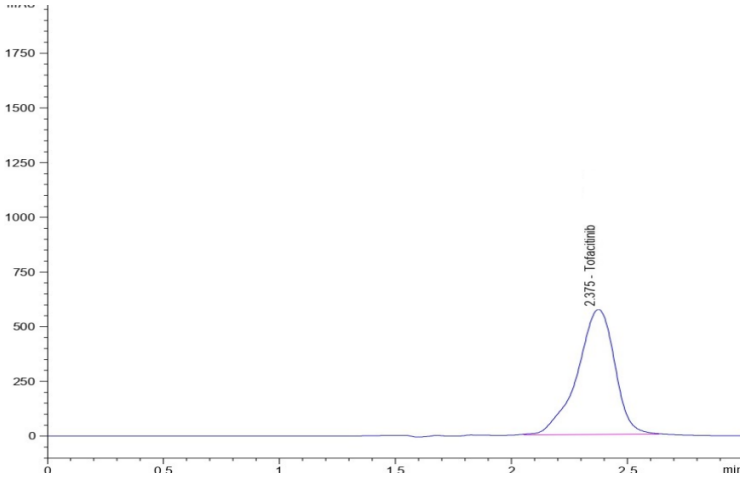
Flow rate selection is done depending on the retention time, peak symmetry, hence many trials were made to decide the flow rate and 0.7 ml /min was found to be acceptable as the peak is sharp. Hence 0.7 ml/ min flow rate was selected for this project.

7.8 Method development trials:

7.8.1 TRIAL 1:

Chromatographic Conditions:

Mobile phase	: Buffer: Methanol(80:20)
Column	: X-bridge C18 (2.1X50mm, 1.7µm)
Flow rate	: 0.5 ml
Wavelength	: 290 nm
Column Temperature	: Ambient
Injection Volume	: 10µl
Diluent	: Methanol : water(50:50)



Chromatogram -1

Observation:

From the above chromatogram it was observed that the peak is broad and shows tailing.

7.8.2 TRIAL 2:

Mobile phase : Buffer: Acetonitrile: Methanol(40:40:20)

Column : X-bridge C18 (3.0X50mm, 2.5µm)

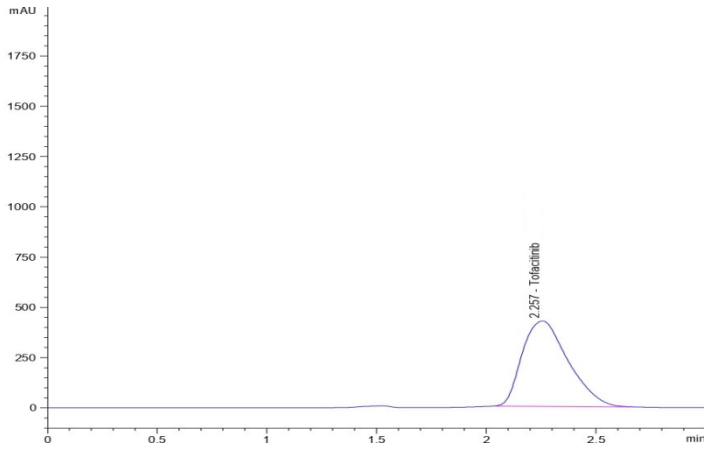
Flow rate : 0.5 ml

Wavelength : 290 nm

Column Temperature : Ambient

Injection Volume : 10µl

Diluent : Methanol : water(50:50)



Chromatogram -2

Observation:

From the above chromatogram it was observed that the peak shape and tailing factor is not upto the aim of the project.

7.8.3 TRIAL 3:

Mobile phase : Buffer: Acetonitrile: Methanol(40:40:20)

Column : X-terra MS C18(4.6X100mm, 3.5µm)

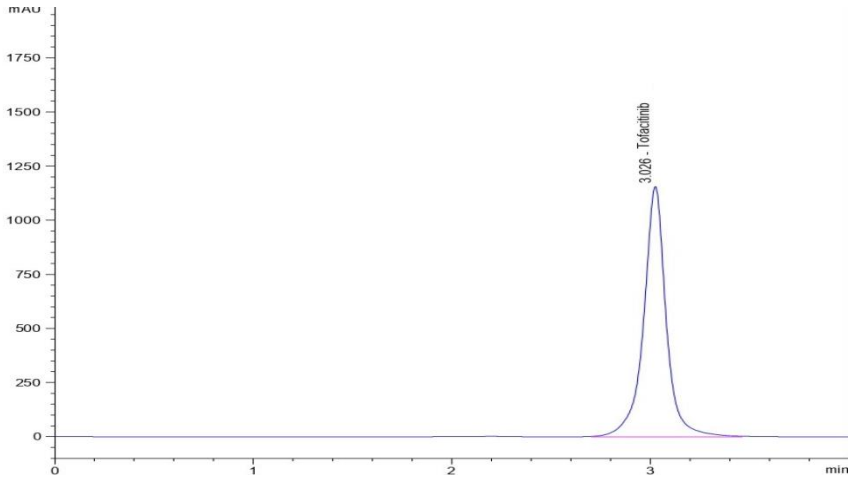
Flow rate : 0.7 ml

Wavelength : 290 nm

Column Temperature : Ambient

Injection Volume : 10µl

Diluent : Methanol : water(50:50)



Chromatogram -3

Observation:

From the above chromatogram the peak showing both fronting and tailing and retention time is not upto the aim of the project.

7.8.4 TRAIL 4:

Mobile phase : Buffer: Acetonitrile: Methanol(40:40:20)

Column : Thermo scientific (4.6mmX 3mm,5µm)

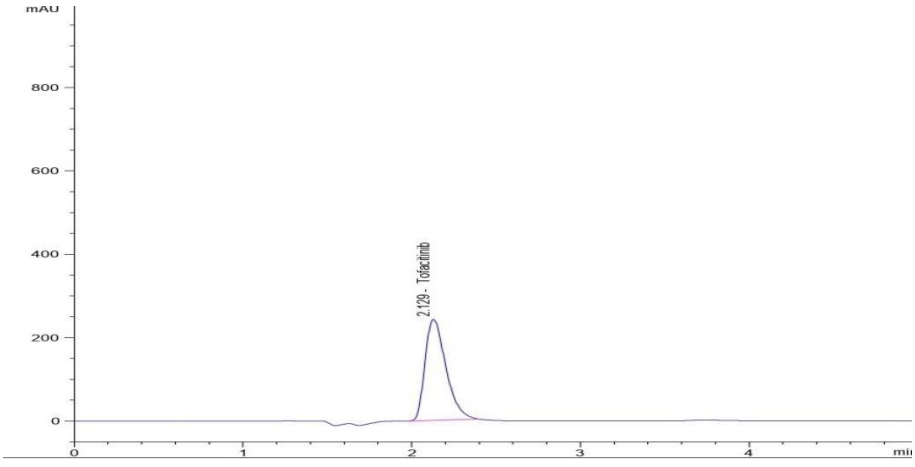
Flow rate : 0.7 ml

Wavelength : 290 nm

Column Temperature : Ambient

Injection Volume : 10µl

Diluent : Methanol : water(50:50)



Chromatogram -4

Observation:

From the above chromatogram it was observed that the obtained Retention time, peak shape, tailing factor, theoretical plates,satisfies the aim of the project.

Drug	RT	Area	TP	TF
Tofacitinib	2.129	2096.937	2210	1.4

Fixed chromatographic conditions:

Instrument : UHPLC AGILENT TECHNOLOGIES with UV detector and auto sampler.

Column : Thermo scientific (4.6mmX 3mm,5µm)

Flow rate : 0.7 mL/min

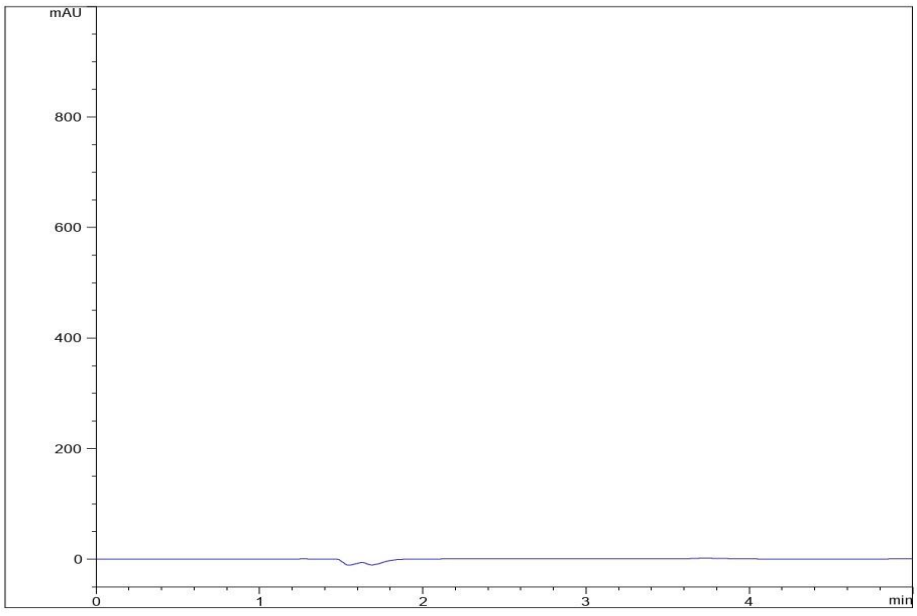
Wavelength : 290nm

Temperature : Ambient

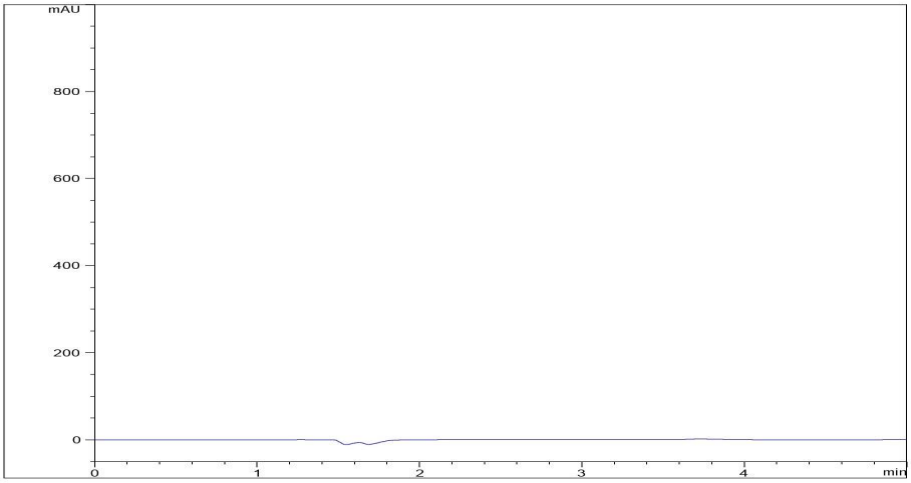
Injection Volume : 10µL

Mobile phase composition : pH 5.3 Buffer: Acetonitrile: Methanol (40:40:20 v/v)

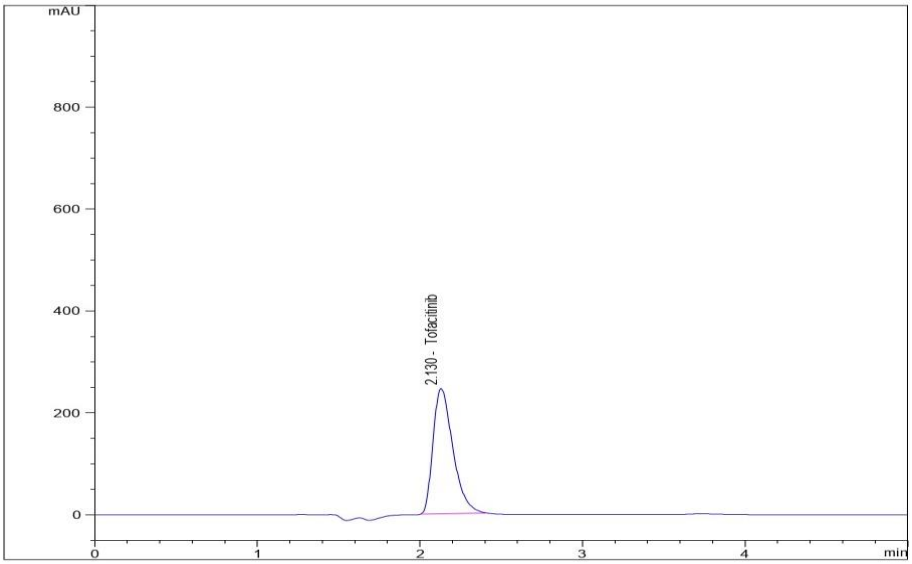
Run time : 5minutes



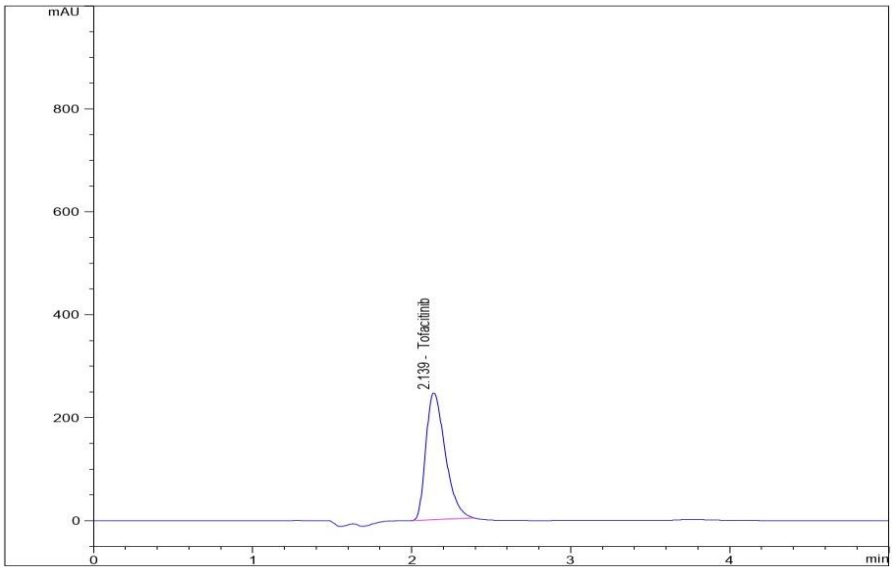
Chromatogram -5 Blank



Chromatogram -6 Placebo



Chromatogram -7 Standard



Chromatogram - 8 Sample chromatogram

Name of the injection	RT	Area	Theoretical plates	Tailing factor
Blank	0.00	0.00	0	0
Placebo	0.00	0.00	0	0
Standard solution	2.130	2090.937	2547	1.4
Sample solution	2.129	2101.160	2687	1.4

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METHOD VALIDATION

8. METHOD VALIDATION

The main objective of the validation is to demonstrate that the analytical method is suitable for its intended purpose, is accurate, specific and precise over the specified range that an analyte will be analyzed. The developed method was validated according to ICH guidelines.

8.1 Specificity:

Specificity Is The Ability Of The Method To Measure The Analyte In Presence Of Matrix Components.

8.1.1 Procedure:

The specificity of the method was demonstrated by injecting Blank (diluent), placebo solution and sample solution. Evaluated the interference of matrix and blank and placebo peaks at the retention time of Tofacitinib.

8.1.2 Acceptance criteria:

8.1.3 Interference:

- There should not be any interference from blank, placebo and impurity peaks at the Retention Time of Tofacitinib peak.
- The resolution between Tofacitinib peak and closely eluted peak should not be less than 1.5.

8.1.4 Analytical results:

Table 01: Specificity

Name	Interference
	RT(min)
Blank	Nil
Placebo	Nil
Standard	2.130
Sample	2.129

8.1.5 Conclusion

The data demonstrates that the retention time of tofacitinib are comparable in standard and sample. There is no interference observed from blank and placebo solution at the retention time of main analyte peaks. The results obtained are found within the acceptance criteria.

8.2 System suitability:

Prepared blank and standard preparation as per test procedure and made five replicate injections evaluated the system suitability parameters as per the test procedure.

8.2.1 Acceptance criteria:

- The relative standard deviation of peak areas of Tofacitinib from five replicate injections of standard preparation-2 should not be more than 2.0%.
- Symmetry factor for Tofacitinib from first injection of standard preparation should not be less than 0.8 and not more than 2.0.
- Theoretical Plates for Tofacitinib from first injection of standard preparation should not be less than 2000.

8.2.2 Analytical Results:

Table 02: system suitability

No of injection	Area	RT	Theoretical plates
Standard injection-01	2090.937	2.129	2673
Standard injection-02	2096.766	2.130	2841
Standard injection-03	2096.895	2.132	2798
Standard injection04	2089.145	2.130	2737
Standard injection-05	2091.971	2.139	2719
Mean	2094.479	2.132667	
SD	0.003983	4.538515	
% RSD	0.186775	0.216689	

8.2.3 Conclusion:

System Suitability Parameters Met The Acceptance Criteria For All Validation Parameters. Hence The System And Method Found Suitable For Tofacitinib Tablets.

8.3 Linearity and range:

The Linearity of analytical method is its ability to elicit test results that are directly, or by well defined mathematical transformation, proportional to the concentration of analyte in

samples within a given range Performed the linearity with Tofacitinib in the range of 50%, 75%, 100%, 125%, 150% of working concentration.

Recorded the area response at each level and calculated slope, intercept, correlation coefficient (r) and regression coefficient (r square). Plotted a graph of concentration (ppm) on X-axis and area response under the curve on y-axis.

8.3.1 Preparation of 50% Solution:

Dilute 2.5 ml of stock solution 50 ml volumetric flask and make up the volume up to the mark with same diluent.

8.3.2 Preparation of 75% Solution:

Dilute 3.75 ml of stock solution 50 ml volumetric flask and make up the volume up to the mark with same diluent.

8.3.3 Preparation of 100% Solution:

Dilute 5ml of stock solution 50 ml volumetric flask and make up the volume up to the mark with same diluent.

8.3.4 Preparation of 125% Solution:

Dilute 6.25ml of stock solution 50 ml volumetric flask and make up the volume up to the mark with same diluent.

8.3.5 Preparation of 150% Solution:

Dilute 7.50ml of stock solution 50 ml volumetric flask and make up the volume up to the mark with same diluent.

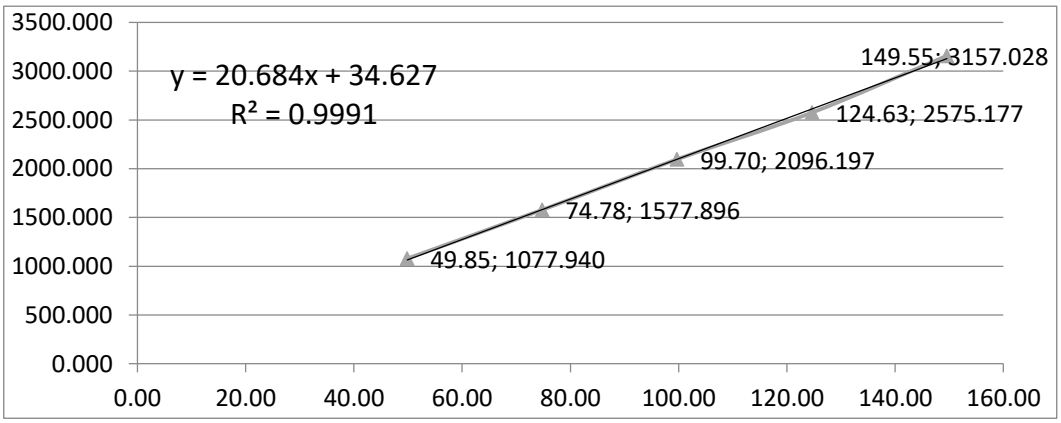
8.3.6 Analytical result:

Table 03: Linearity and range

Concentration (mcg/ml)	Concentration (%)	Areas
49.85	50	1077.940
74.78	75	1577.896
99.70	100	2096.160
124.63	125	2575.177
149.55	150	3157.028

Y = mX+c	
Intercept (c)	20.684
Slope (m)	+34.627

LINEARITY GRAPH OF TOFACITINIB



8.3.7 Acceptance criteria:

- Correlation coefficient and regression coefficient shall be NLT 0.999 and NLT 0.998 respectively.

8.3.8 Conclusion

From the statistical treatment of the linearity data of Tofacitinib, it is clear that the detector response of Tofacitinib is linear 50% to 150% of the assay method working concentration for Tofacitinib Tablets 5 mg. The correlation and regression coefficient found as 0.9991. In addition, the value of the intercept is within the ± 2 % of the area response at 100% level. Hence, the method was found Linear and within the range for Tofacitinib Tablets.

8.4 ACCURACY:

The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value and the found value.

8.4.1 Procedure:

The accuracy was calculated the present method to the analysis of tablet and standard at low, medium, and high concentration level. The accuracy was estimate from three replicate injection and calculated as the µg/mL drug recovered from the drug matrix.

8.4.2 Acceptance criteria:

The percentage Recovery for each level should be between 98.00 to 102.00%.

8.4.3 Analytical result:

Table 04: Accuracy

Spiked Level	Concentation in ppm	% Recovery	%Recovery mean
50%	50	99.40	**99.46%**
	50	99.80	
	50	99.20	
100%	100	99.60	**99.66%**
	100	99.80	
	100	99.60	
150%	150	98.20	**98.86%**
	150	99.20	
	150	99.20	
	Mean	99.28	
	SD	0.480	
	%RSD	0.484	

8.4.4 Conclusion:

The mean % Recovery and % Relative Standard deviation for Tofacitinib found within the limits.

8.5 Precision:

8.5.1 Method precision

In method precision, a homogenous sample of a single batch should be analysed six times. This indicates whether a method is giving consistent results of a single batch and analysed the sample of Tofacitinib Tablets 5 mg.% assay was calculated for Tofacitinib tablets 5 mg with respect to standard preparation

8.5.1.1 Acceptance criteria

- All the results should be not less than 90.0 % and not more than 110.0 % of labeled amount of Tofacitinib.
- The % RSD for % assay of 6 replicate sample preparations should be NMT 2.0.

8.5.1.2 Analytical Results:

Table 05: Method Precision

No. of Sample	% Assay
	Tofacitinib Tablets 5mg
01	99.60
02	99.80
03	99.60
04	99.60
05	99.80
06	99.80
Mean	**99.70**
SD	**0.1095**
% RSD	**0.1099**

8.5.1.3 Conclusion

The relative standard deviations for % assay of 6 replicate preparations were meeting the acceptance criteria as per protocol.

8.5.2 Ruggedness:

In Intermediate precision(ruggedness) six samples are prepared from homogeneous batch sample used for precision study and %RSD for 6 determinations is reported. From the intermediate precision it is ensured that the analytical results remains unaffected due to changes in the environmental conditions like change in instrument, analyst, column, day etc. Intermediate precision set was repeated by using the different analyst, different column, different instrument and different day.

8.5.2.1 Acceptance criteria:

- All the results should be not less than 90.0 % and not more than 110.0 % of labeled amount of Tofacitinib.
- % RSD for 6 determination of assay value for Intermediate precision shall be NMT 2.0
- The % Variation between average assay value obtained in Intermediate precision study and Method Precision study should not be more than 3.0.

8.5.2.2 Analytical results:

Table 06: Intermediate precision

No. of Sample	% Assay
	Tofacitinib Tablets 5mg
01	100
02	99.80
03	99.80
04	100.20
05	99.80
06	99.80
Mean	**99.90**
SD	**0.17**
% RSD	**0.17**

Table 07: Comparison between method prsecision and intermediate precision

No. of Sample		Overall % RSD
Method precision	01	99.60
	02	99.80
	03	99.60
	04	99.60
	05	99.80
	06	99.80
Intermediate precision	07	100
	08	99.80
	09	99.80
	10	100.20
	11	99.80
	12	99.80
Overall Mean (n=12)		99.8
SD		0.17
% RSD		0.17

8.5.2.3 Conclusion:

The relative standard deviations for % assay of 6 replicate preparations were meeting the acceptance criteria as per protocol. Hence, method found rugged for Tofacitinib Tablets.

8.6 Robustness:

Robustness of the method was checked by small deliberate changes in the method parameters such as wavelength (±2nm) and flow rate (±0.025ml) which shall not much affect in theoretical plates and peak asymmetry.

8.6.1 Acceptance criteria:

- All the results should be not less than 90.0 % and not more than 110.0 % of labeled amount of Tofacitinib.
- % Relative difference between average assay value obtained in robustness condition and test condition should not be more than 3.0.

8.6.2 Analytical result:

Table 08: Robustness

Parameter	RT			Area		
	Mean	Std dev	% RSD	Mean	Std dev	% RSD
Low flow variation 0.5mL/min	2.400	0.0016	0.068	2254.222	0.4595	0.02
High flow variation 0.9mL/min	1.908	0.0009	0.046	1888.341	1.33	0.07
Low Wavelength variation 288	2.132	0.0015	0.069	2150.522	1.083	0.05
High Wavelength variation 292	2.130	0.0012	0.054	2133.551	1.53	0.07

8.6.3 Conclusion:

The robustness parameter was performed as per protocol with chromatographic conditions and mobile phase slight variation namely flow variation (Low and high flow), Column temperature variation (High column temperature) and wavelength variation (Low and high) all the robustness condition was meeting the acceptance criteria. Hence, the method found robust for flow variation (Low and high flow), Column temperature variation (High column temperature) and wavelength variation (Low and high pH) for Tofacitinib Tablets.

8.7 Solution stability:

Evaluated the stability of analytical solution by injecting the standard and sample solution at initial, 24 hours and 48 hours time intervals.

8.7 .1 Acceptance criteria:

- % Relative difference between initial assay value & assay value at the each predetermined time intervals should not be more than 3.0
- It should meet system suitability criteria.

Table 09: Solution stability for Standard

Time in hours	% Assay	% Difference
Initial	100	-
24 Hours	100.1	0.1
48 Hours	100.5	0.5

Table 10: Solution stability for Sample

Time in hours	% Assay	% Difference
Initial	99.70	-
24 Hours	99.80	0.1
48Hours	100.1	0.4

8.7.2 Conclusion:

The % of assay difference between the initial and respective time intervals of Standard and sample solutions met the acceptance limit for 48hrs at room temperature (25°C). From the above results, it is concluded that the standard solution and sample solution are stable for 48hrs at room temperature (25°C).

METHOD USED FOR ASSAY OF TOFACITINIB

STRENGTH: 5mg

Chromatographic parameters:

Instrument	: UHPLC AGILENT TECHNOLOGIES with UV detector and auto sampler.
Column	: Thermo scientific (4.6mmX 3mm,5µm)
Flow rate	: 0.7 mL/min
Wavelength	: 290nm
Temperature	: Ambient
Injection Volume	: 10µL
Mobile phase composition	: pH 5.3 Buffer: Acetonitrile: Methanol (40:40:20 v/v)

Preparation of pH 5.3 phosphate buffer:

Weigh and dissolve 1.3909 gm of potassium dihydrogen phosphate in 1000ml of distilled water. pH was adjusted to 5.3 using orthophosphoric acid.

Preparation of mobile phase:

1. Mix pH 5.3 buffer, Acetonitrile and methanol in the ratio of 40:40:20.

2. Filtered through 0.45µm membrane and degassed it for 10mins.

Preparation of standard stock solution:

Weigh accurately and transfer about 50 mg Tofacitinib citrate working standard in 50 ml of volumetric flask, added about 25ml of diluent, sonicate to dissolve the material completely, dilute to volume with diluent and mixed well. Stock concentration of standard solution is about 1mg/ml.

Preparation of standard solution:

1. Pipette out 5ml of stock solution into 50ml volumetric flask dilute the solution with diluent and mixed well.

2. The final concentration of standard solution is 100µg/ml.

Test solution preparation:

20 tablets are accurately weighed and found average weight. Weigh accurately and transfer about 1515.3 mg of tofactinib in 50ml volumentric flask, add about 25ml diluent,

sonicate to dissolve the material completely, dilute to volume with diluent and mixed well. Pipetted out 5ml of stock solution into 50ml volumetric flask dilute the solution with diluent and mixed well. The final concentration of sample solution is 100µg/ml.

System suitability:

1. Inject about 10 µL portion of standard solution into the chromatographic system and measure the response of major peak.
2. The tailing factor for Tofacitinib peak should be NMT 2.0
3. The RSD for the area of Tofacitinib peak obtained from the 5 replicates injections of standard preparation should be NMT 2.0%.

Procedure:

Inject about 10 µL portion of diluent and test preparation into the chromatograph, record the chromatogram and measure the response of major peak.

Calculations:

$$\frac{\text{Test area}}{\text{Standard area}} \times \frac{\text{STD wt}}{50} \times \frac{5}{50} \times \frac{50}{\text{Test wt}} \times \frac{\text{Purity}}{5} \times \frac{\text{Average weight}}{100}$$

LABEL CLAIM	AVERAGE WEIGHT	AREA	PERCENTAGE ASSAY
5mg	151.53mg	2101.160	99.70%

Acceptance criteria:

Assay value should be in the range of 90% to 110% .

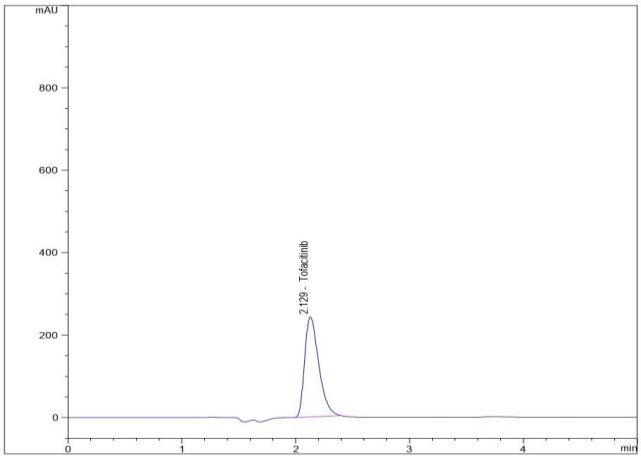
Result :

Test result is showing that the test method is precise. The percentage assay of tofacitinib was found to be 99.70%. Results are within the limits.

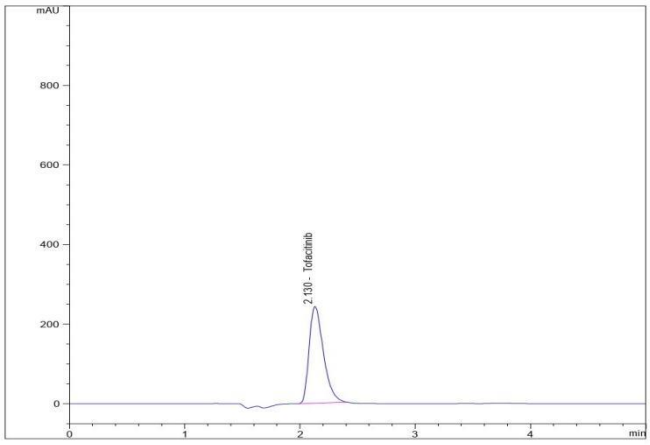
VALIDATION CHROMATOGRAM

VALIDATION CHROMATOGRAM

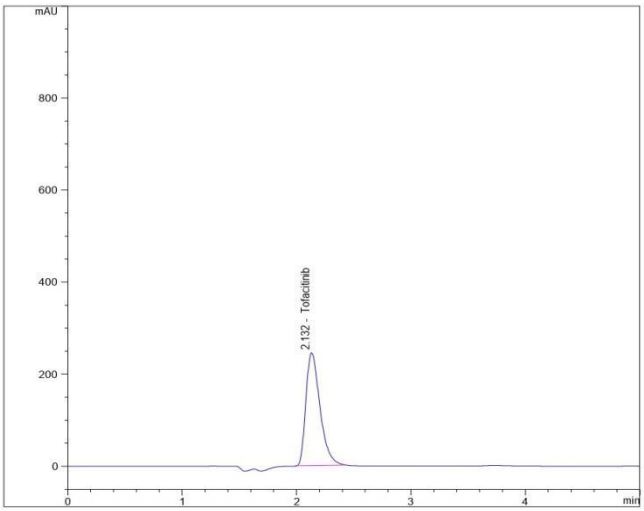
Chromatogram- 9 System suitability



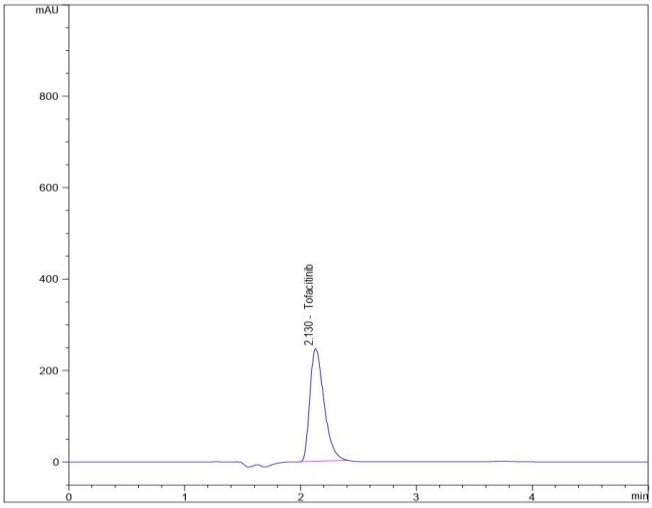
System suitability 01



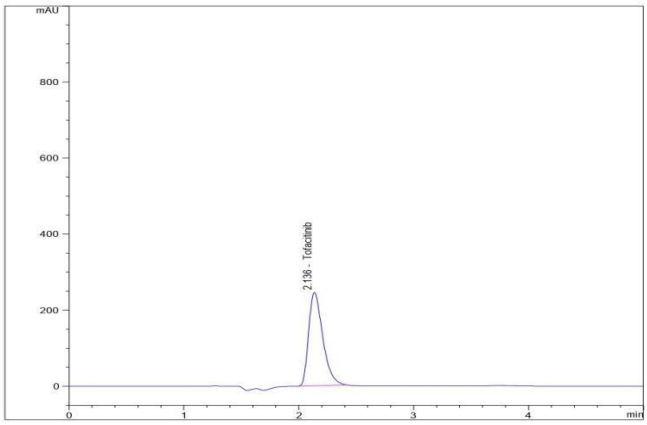
System suitability 02



System suitability 03

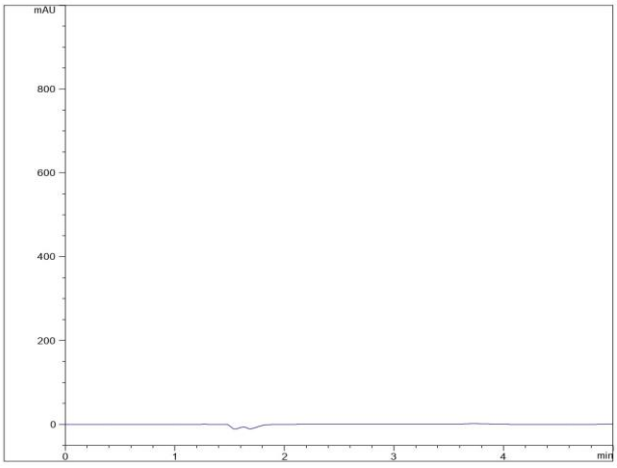


System suitability 04

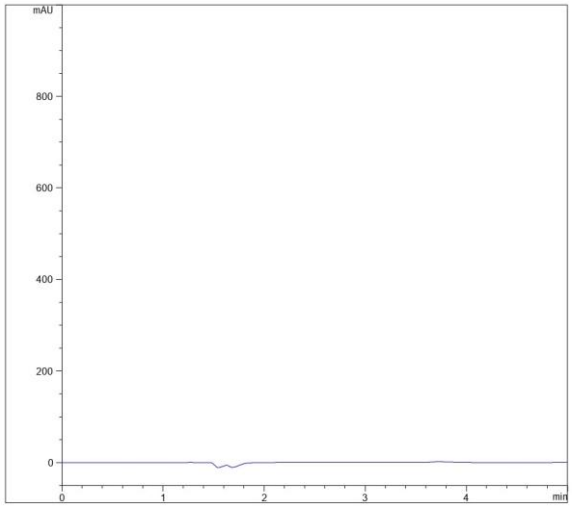


System suitability 05

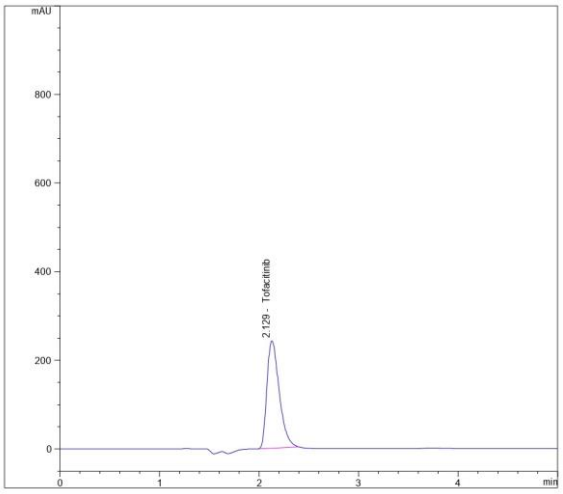
Chromatogram- 10 Specificity



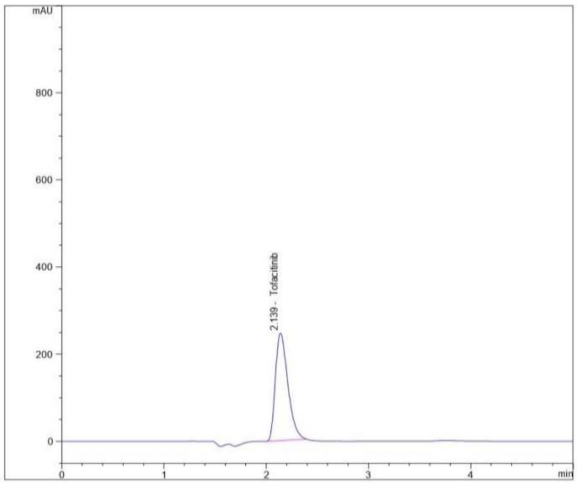
Blank chromatogram



Placebo chromatogram

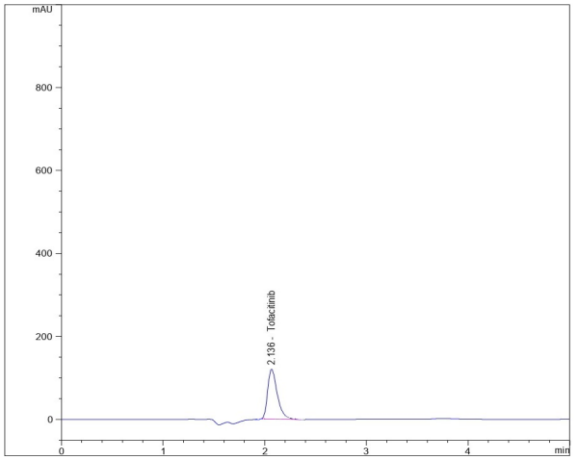


Standard chromatogram

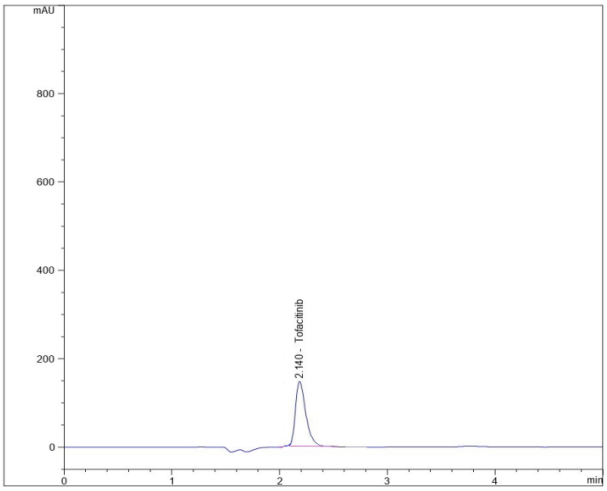


Sample chromatogram

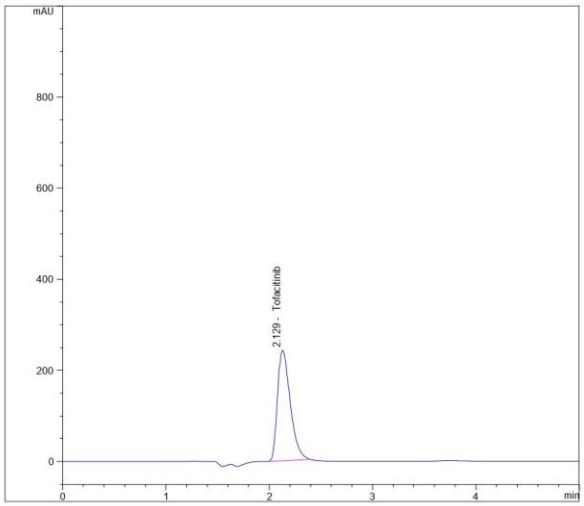
Chromatogram - 11 Linearity



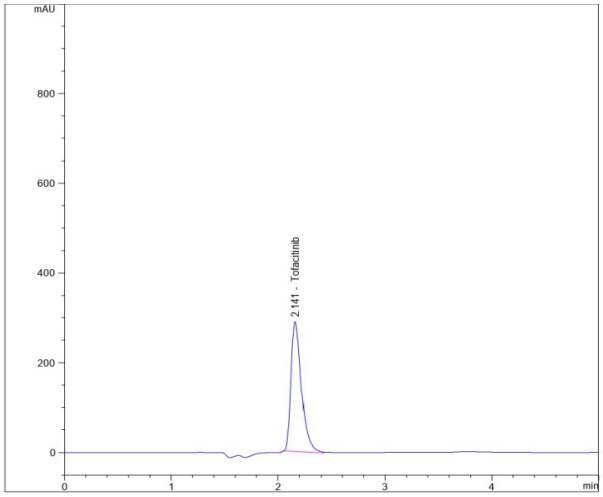
Linearity 50%



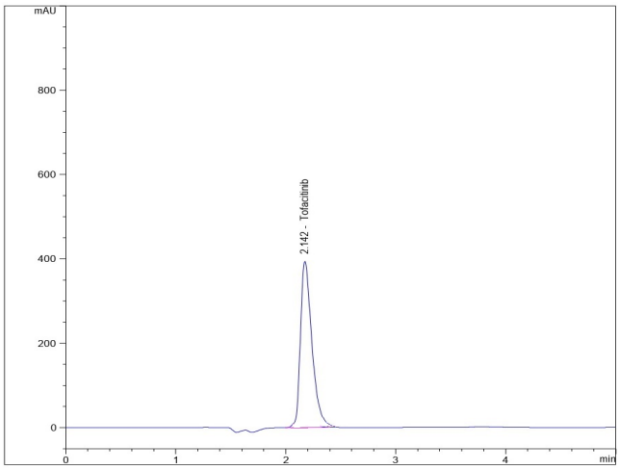
Linearity 75%



Linearity 100%

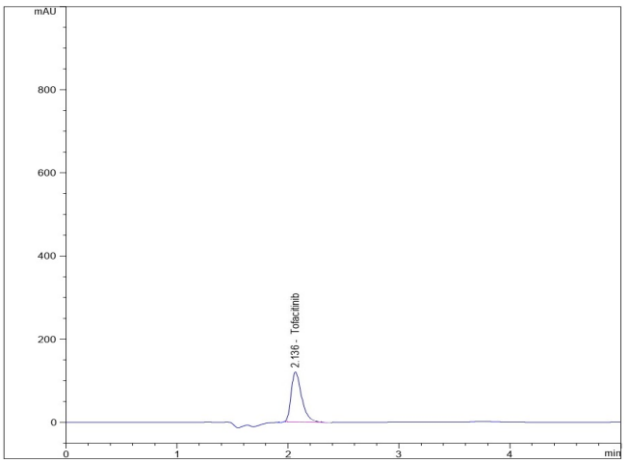


Linearity 125%

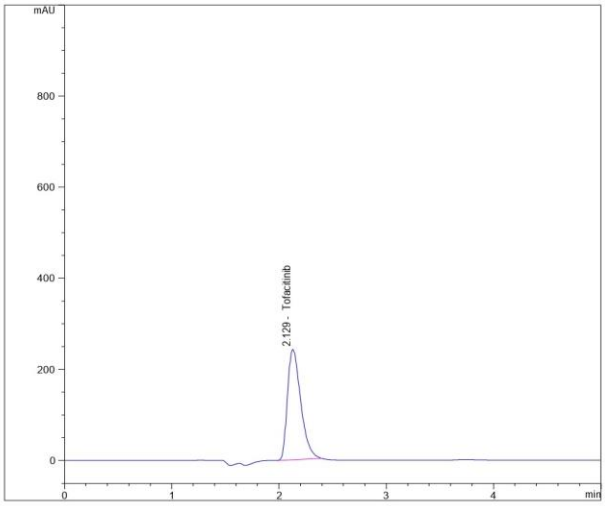


Linearity 150%

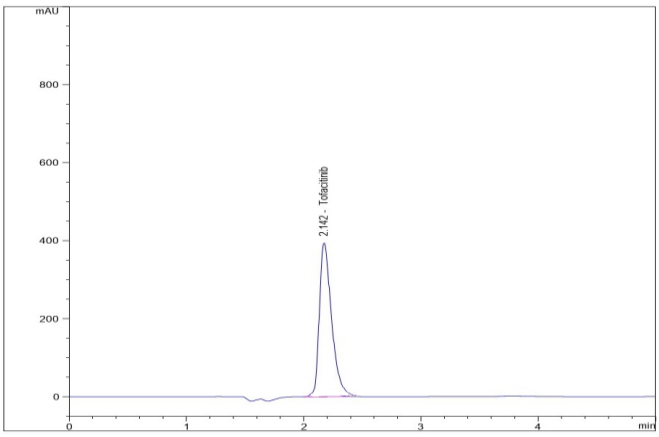
Chromatogram 12 Accuracy



Accuracy 50%

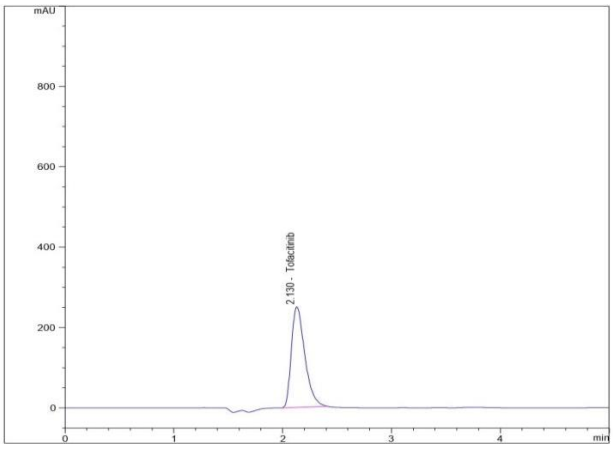


Accuracy 100%

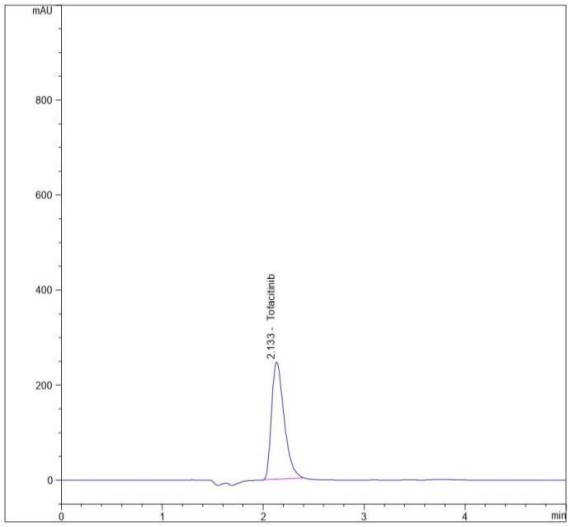


Accuracy 150%

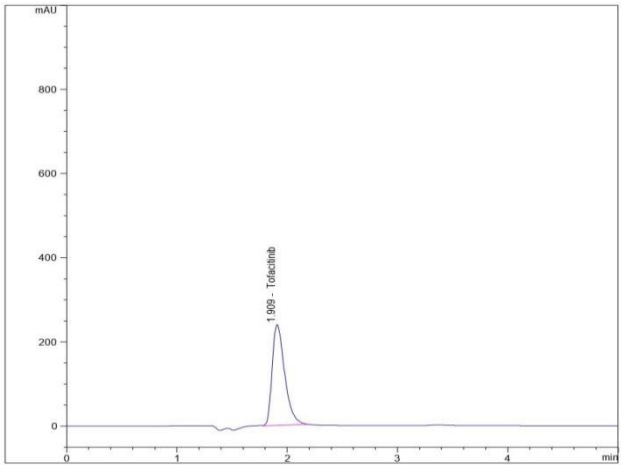
Chromatogram 13 Robustness



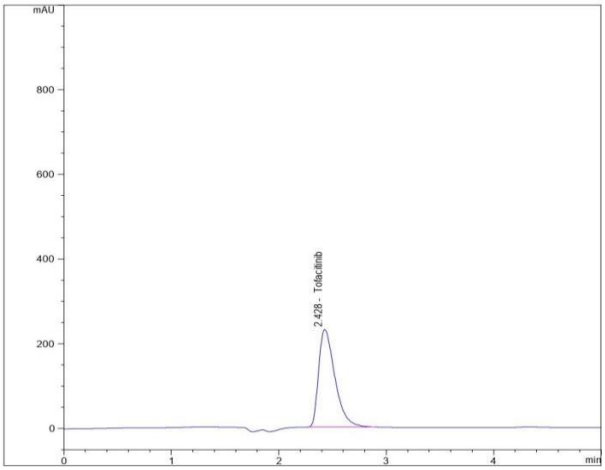
Wavelength increase



Wavelength decrease

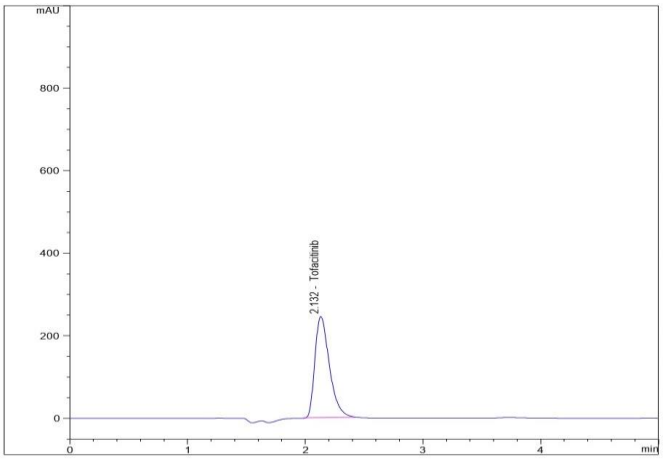


Flowrate increase

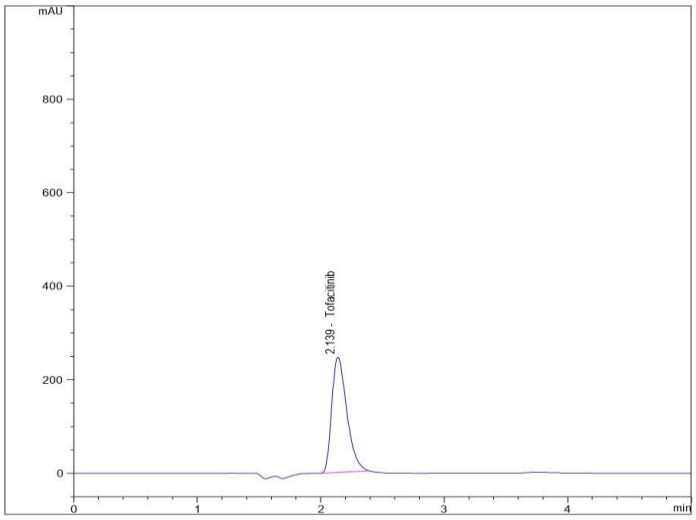


Flowrate decrease

Chromatogram 14 solution stability



Solution stability standard



Solution stability sample

Table 11: Results of Validation Parameters

Validation Parameter	Acceptance Criteria	Observation	Remarks
		Tofacitinib	
Specificity	The peaks of diluent and excipients should not interfere with the main peak and peak of standard and sample should be identical with near retention time.	Complies	-
System Suitability	Asymmetry NMT 2.0	1.4	Complies
	Theoretical plates NLT 2000	2269	
	%RSD of area NMT 2.0	0.21	
Linearity	Correlation Coefficient NLT 0.997	0.9991	Complies
Accuracy	The % recovery at each spike level shallbe NLT 98.0% and NMT 102.0% of the added amount.	98.80-99.50%	Complies
Precision	The % RSD of area for the six determinations shall be NMT 2.0.	0.17	Complies
	The % RSD of assay for the six determinations shall be NMT 2.0.	0.1	
Robustness	Wavelength increase and decrease	Complies	-
	Flowrate increase and decrease	Complies	
Solution Stability	Assay value not more than 3.0	Complies	

RESULT AND DISCUSSION

9. Result and discussion

The new analytical method for the UHPLC method was established for Tofacitinib then optimized and then applied on pharmaceutical dosage forms.

Various mobile phase systems were prepared and used to provide an appropriate chromatographic separation, but the proposed mobile phase comprising of Buffer, Acetonitrile and methanol in the ratio 40:40:20 gave a better resolution and sensitivity.

The detection was carried out by using UV detector at 290nm using Thermo scientific (4.6mmX 3mm,5µm). Among these several flow rates tested, the flow rate of 0.7ml was found to be the best for Tofacitinib with respect to retention times and theoretical plates.

The retention time is 2.129 for Tofacitinib. The asymmetry factor or the tailing factor was found to be 1.4 for tofacitinib, which indicates symmetrical nature of the peak.

System suitability parameters such as retention time, tailing factor, capacity factor and number of theoretical plates were calculated. The number of theoretical plates was found to be around 2210 for Tofacitinib, which indicates efficient performance of the column.These parameters represent the specificity of the method.

Linearity range was evaluated by the visual inspection of plot of peak area as a function of analyte concentration and the corresponding calibration graphs were shown in figure and results are shown in table.

From the linearity studies, the specified concentration range was determined. It was observed that Tofacitinib was linear in the range of 50% to 150% for the target concentrations.

The validation of the proposed method was verified by system precision and method precision. The %RSD for system precision and method precision of Tofacitinib was tabulated.

Placebo interference studies were made by injecting placebo alone, then the standard and the placebo along with the standard. They did not show any interference of placebo at the RT of the analyte peak

Robustness studies were made by varying the flow rate and also by performing filter validation studies on to types of filters. The analytical data and results for filter validation were tabulated, hence the developed method was found to be robust.

Study of ruggedness was made by conducting the study on different system and by two analysts. The results were found to be in limits and were tabulated and hence the developed method is found to be rugged.

SUMMARY AND CONCLUSION

10. Summary and conclusion

A simple, reproducible and efficient reverse phase Ultra High Performance Liquid Chromatography (RP-UHPLC) method has been developed for estimation of Tofacitinib in its tablet dosage form. Separation was done by using mobile phase consists of Phosphate buffer (pH 5.3): Acetonitrile: methanol (40:40:20, v/v). Chromatography separations were carried out on Thermo scientific (4.6mmX 3mm,5µm) at a flow rate of 0.7ml/min and UV detection at 290nm and the retention time for Tofacitinib is 2.129 minutes. The linear dynamic response was found to be in the concentration of 50µg-150µg/ml. The slope, intercept and Correlation coefficient was found to be 0.9991 respectively. Proposed methods were found to be simple, accurate, precise and rapid and could be used for routine analysis.

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