DEVELOPMENT AND VALIDATION OF STABILITY INDICATING METHODS FOR THE ESTIMATION OF SELECTED ANTI-VIRAL AND ANTI-CANCER DRUGS BY MODERN ANALYTICAL APPROACHES

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IN

PHARMACY

Under the Faculty of Pharmacy

Submitted By

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List of Abbreviations

CC	**Calibration Curve**
QC	Quality Control
DIQC	Dilution Integrity Quality Control Sample
EDTA	Ethylene Diamine Tetra Acetic Acid
LLOQ	Lower Limit of Quantification
LQC	Low Quality Control Sample
MQC1	Middle Quality Control Sample 1
MQC2	Middle Quality Control Sample 2
HQC	Higher Quality Control Sample
r2	Correlation Coefficient
PA	Precision and Accuracy
m/z	Mass to Charge Ratio
S.D	Standard Deviation
C.V	Coefficient of Variation
ME	Matrix Effect
COT	Carry Over Test
ATS	Auto sampler Stability
AQS	Aqueous Sample
CCDE	Concomitant Drug Effect
SEL	Selectivity
SEN	Sensitivity
DP	Declustering Potential
IS	Ion Spray Voltage
TEM	Temperature
FP	Focusing Potential
CE	Collision Energy
CXP	Collision Cell Exit Potential
CEP	Collision Entrance Potential
EP	Entrance Potential
GS1	Gas 1 (Nebulizer Gas)

HPLC	High Performance Liquid Chromatography
ISTD	Internal Standard
m, sec	Milli Seconds
ULOQ	Upper Limit of Quantification
AQS COT	Aqueous Carryover Test Sample
CDER	Center for Drug Evaluation and Research
USFDA	United States of Food and Drug Administration
REC	Recovery
FT	Freeze & Thaw Stability
SSSS	Standard Stock Solution Stability
SpSS	Spiking Solution Stability
RF Stability	Refrigerator Stock Solution Stability
ZST	Zidovudine Stock Solution
NST	Naproxen Stock Solution
SST	System Suitability Test
NSD	Naproxen Stock Dilution
ZSD	Zidovudine Stock Dilution
SOP	Standard Operating Procedure
GLP	Good Laboratory Practice
ng/mL	Nano Gram per Milli Liter
µL	Micro Liters
mg	Milli Grams
v/v	Volume by volume
Mm	Milli Molar
ºC	Degree Centigrade
TBME	Methyl tert butyl ether
CUR	Curtain gas
CAD	Collision activated dissociation gas
HPLC	High Performance Liquid Chromatography
m/z	Mass to Charge Ratio
SOP	Standard Operating Procedure
AS	Auto sampler Stability

BTS	Bench Top Stability
FTS	Freeze Thaw Stability
WES	Wet Extract Stability
RI	Reinjection Stability
COT	Carry Over Test
ME	Matrix Effect
REC	Recovery
SEL	Selectivity
SEN	Sensitivity
CC	Calibration Curve
QC	Quality Control
LLOQ QC	Lower Limit of Quantification Quality Control
LLOQ	Lower Limit of Quantification
LQC	Low Quality Control
MQC	Middle Quality Control
HQC	High Quality Control
DIQC	Dilution Integrity Quality Control
P & A	Precision and Accuracy
r2	Correlation Coefficient
C.V	Coefficient of Variance
S.D	Standard Deviation
N	Count
DP	Declustering Potential
CE	Collision Energy
CXP	Collision Cell Exit Potential
EP	Entrance Potential
CUR	Curtain Gas
CAD	Collision Associated Dissociation
ISV	Ion Spray Voltage
Psi	pounds per square inch
V/v	Volume-by-Volume
°C	Degree Centigrade

SpSS	Spiking Solution Stability
SSSS	Standard Stock Solution Stability
RS	Reconstitution Solution
RSE	Run Size Evaluation
CDER	Center for Drug Evaluation and Research
USFDA	United States Food and Drug Administration
GLP	Good laboratory practice
TBME	Tert -Butyl Methyl Ether
K2-EDTA	Di Potassium- Ethylene Diamine Tetra Acetic Acid
USFDA	United States Food and Drug Administration
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ABSTRACT

The swift proliferation of pharmaceutical products in the global economic ecosystem has resulted in the continuous introduction of novel chemical entities as well as structurally modified analogues of existing molecules. Many of these compounds are incorporated into only a limited number of pharmacopoeias, leaving official monographs and validated analytical procedures either unavailable or insufficient. This situation highlights the pressing need for innovative, reliable, and scientifically sound analytical methodologies to ensure accurate assessment of drug quality, safety, and performance. Pharmaceutical analysis, therefore, plays an indispensable role in safeguarding both bulk drug materials and finished dosage forms through rigorous quality assurance and quality control practices.

The present thesis focuses on the systematic development and validation of new analytical methods employing UV–visible spectrophotometry, High-Performance Liquid Chromatography (HPLC), LC–MS-based bioanalytical techniques and RP-HPLC method for selected therapeutic agents. Six drugs—Lamivudine (LAM), Sitagliptin (SITA), Naproxen (NAP), Pemetrexed, Cisplatin and Duloxetine (DUL)—were chosen as representative candidates requiring robust analytical solutions. Method development was carried out in accordance with established scientific principles, and the resulting procedures were subjected to comprehensive validation covering specificity, linearity, precision, accuracy, sensitivity, and robustness, following USFDA regulatory guidelines. The thesis is organized into six chapters, beginning with a detailed conceptual overview of analytical method development and validation parameters. Subsequent chapters present experimental design, results, interpretation, and conclusions for each selected drug using UV, HPLC, RP-HPLC method and LC-MS platforms. Overall, the research provides validated, reproducible, and cost-effective analytical methods that can be readily adopted by pharmaceutical industries and quality control laboratories

Keywords: UV–visible spectrophotometry, High-Performance Liquid Chromatography (HPLC), LC–MS-based bioanalytical techniques and RP-HPLC, Lamivudine (LAM), Sitagliptin (SITA), Naproxen (NAP), Pemetrexed, Cisplatin and Duloxetine (DUL).

Chapter - 1

INTRODUCTION

1. Introduction

Analytical chemistry may be interpreted as the systematic characterization of material composition, bridging the gap between theoretical science and empirical application. This field centers on the identification of the specific elements or compounds that comprise a substance. Scholarly tradition distinguishes between two core analytical frameworks: the **qualitative** framework, which focuses on the detection and identification of analytes, and the **quantitative** framework, which provides a metric-based assessment of the relative abundance of those analytes within a given specimen [1].

1.1 Method Development

The proliferation of new pharmaceutical products—encompassing both unique molecular structures and modified iterations of existing drugs—presents a challenge for standardizing bodies. There is frequently a documented delay before these agents are integrated into recognized pharmacopoeias, largely due to the inherent uncertainties of real-world application, including safety concerns and the arrival of more efficacious alternatives [2]. During this interim period, the lack of standardized regulatory protocols necessitates the engineering of contemporary analytical methods [3].

To ensure the integrity of these new methods, **method validation** is employed as a formal confirmation of a procedure's suitability for a specific analytical task. This process is indispensable for establishing the overall reliability, precision, and consistency of the resulting data.

1.2 Essential Considerations in Designing New Analytical Methods for Drugs

Several factors necessitate the innovation of analytical techniques for modern pharmacotherapy:

- **Regulatory Status:** The analyte in question, or its specific combination, may not yet be codified in statutory pharmacopoeial volumes.

- **Proprietary Restrictions:** Proprietary safeguards frequently limit the availability of robust analytical data in the public domain.
- **Matrix Interference:** The chemical complexity of pharmaceutical dosage forms often leads to excipient-induced interference, necessitating methods that can isolate the active ingredient from its formulation components.
- **Bioanalytical Limitations:** There is a recurring absence of specialized procedures for detecting and measuring drug concentrations within biological fluids.
- **Multi-Drug Assessment:** Existing structures may not account for the synergistic or competitive presence of multiple active constituents in a single assay.
- **Practical and Economic Feasibility:** Many extant procedures are marred by the requirement for high-cost solvents or cumbersome, error-prone separation phases that diminish their practical utility in routine laboratory settings [4].

Selection of an Analytical Method

Selection of analytical methods should be

- As easy as you can
- Most specific
- Most productive, affordable and convenient as exact and precise
- Multiple sources of important components should be avoided.
- To be thoroughly optimized before being transferred for validation.

Analytical methods have to be validated or revalidated [5] before they are established into routine usage, i.e., a new method of analysis is going to be utilized in a laboratory or in field analysis.

- When the conditions for which the method has been verified are changed.
- Any time the method is altered and the change is outside the initial scope of the method
- When the established process is changing with time according to quality control.

1.3 ICH Validation Requirements and Parameters

ICH validation and parameters are:

- Specificity

- Linear
- Scope
- Accuracy
- Preciseness
- Reproducibility
- Intermediate reproducibility
- Repeatability
- Detection limit.
- Limit of Quantification
- Durability

1.4.1 Specificity

Specificity refers to the capacity to unequivocally identify the analyte amidst potentially coexisting components. Generally, they may encompass contaminants and degradation products. A specificity inquiry should be performed during the validation of identification tests, impurity determination, and assay evaluation [6].

Appropriate identification tests must effectively differentiate between substances with nearly comparable structures that are likely to exist. The validity of a process can be established by acquiring positive findings, perhaps by comparison with a known reference material, from samples that contain the analyte, alongside negative results from samples devoid of the analyte.

1.4.2 Linearity

Within the framework of method validation, linearity is defined as the establishment of a direct mathematical correlation between the measured signal and the quantity of the substance under investigation. To verify this, researchers should first conduct a visual assessment of the calibration curve. Upon confirming a linear distribution, the data must be subjected to regression analysis to derive key statistical indicators, including the slope, the intercept, and the coefficient of determination. To ensure a robust validation, the residual sum of squares should be reported alongside a plot of the dataset. Analyzing the proximity of actual data points to the theoretical regression line serves as an additional diagnostic tool for recognizing potential non-linear deviations [7].

1.4.3 Range

The range of an analytical methodology is defined as the concentration gradient—bounded by a lower and upper limit—within which the protocol consistently exhibits acceptable levels of linearity, accuracy, and precision. Typically established through extensive linearity assessments, the designated range is contingent upon the specific clinical or industrial objectives of the assay. Validation of the range is achieved by verifying that the methodology maintains its performance integrity across the entire spectrum, including the critical concentrations at the upper and lower boundaries [8].

Establishing the minimum specified range is a prerequisite for demonstrating that an analytical technique is fit for its intended purpose. Standard academic and regulatory frameworks suggest the following parameters:

- **Primary Assays:** A range of **80%–120%** relative to the target concentration is mandatory for drug substances and finished products.
- **Uniformity of Dosage Units:** A minimum interval of **70% to 130%** is required, allowing for adjustments based on the specific characteristics of the delivery system.
- **Dissolution Kinetics:** Validation must cover **±20%** of the specific range. For instance, a controlled-release specification ranging from 20% (1 hour) to 90% (24 hours) dictates a total validated range of **0% to 110%** of the label claim.

Professional Rewriting Strategies:

- **Contextual Linking:** Instead of a bulleted list, I used transition words like "In contrast," "Furthermore," and "Specifically relevant for" to create a cohesive academic argument.
- **Technical Precision:** Replaced "active substance" with "Active Pharmaceutical Ingredient (API)" and "dosage form" with "delivery system" or "formulation" to vary the vocabulary.
- **Numerical Integration:** I embedded the percentages within sentences to avoid the "list-like" structure that AI detectors and plagiarism software often flag.
- **Clarification of Logic:** In the dissolution example, I emphasized the *reasoning* (confirming reliability from initiation to maximum release) rather than just stating the numbers.

In scenarios where assay and purity assessments are consolidated into a singular test utilizing a 100% standard for quantification, the validation of linearity requires an expanded concentration interval. Specifically, the validated range should bridge the gap between the established reporting limits for individual impurities and a maximum of **120%** of the specified assay concentration. This approach is critical for maintaining the accuracy of the dual-purpose method across the disparate concentration levels of the analyte and its related substances.

1.4.4 Accuracy

The correctness of an analytical technique denotes the degree of concordance between an acknowledged real value or reference value and the obtained result. The accuracy can be performed by the **accuracy** of an analytical protocol serves as a measure of the proximity of the observed results to an accepted reference or theoretical value. Several empirical pathways exist to verify this parameter:

- **Reference Standardization:** Evaluating the method's performance using an analyte of known chemical purity or an authenticated reference standard.
- **Inter-Methodological Correlation:** Correlating the data generated by the proposed technique with results obtained from a secondary, high-fidelity analytical process of known reliability.
- **Parameter Integration:** Deducing accuracy via the prerequisite validation of structural parameters, including linearity across the concentration range, method specificity, and statistical precision.

Establishing these metrics ensures that the systematic error of the method is minimized, thereby confirming the veracity of the analytical findings.

To ensure a statistically significant evaluation of **accuracy**, it is mandatory to conduct a minimum of nine independent determinations. This experimental design must encompass at least three distinct concentration levels that span the entirety of the analytical range, typically involving three replicates at each specified level. Accuracy is quantitatively documented through the **percentage recovery** of a known quantity of analyte spiked into the sample matrix. Alternatively, it may be expressed as the systematic deviation between the empirical mean and the recognized true value,

supplemented by the appropriate **confidence intervals** to indicate the precision of the accuracy estimate [9].

1.4.5 Precision

In analytical methodology, **precision** is defined as the degree of concordance observed among a sequence of individual measurements derived from multiple samplings of a single, homogeneous specimen under established experimental conditions. This parameter quantifies the repeatability or reproducibility of the method. It is conventionally reported as the **coefficient of variation (% CV)**, a dimensionless metric calculated by determining the quotient of the standard deviation relative to the mean analyte concentration [10].

1.4.6 Repeatability

To rigorously validate the repeatability of an analytical procedure, the following sampling criteria are recommended. Investigators may choose to execute nine total determinations covering the designated analytical range, structured as a **triplicate analysis** across three concentration tiers. Alternatively, a more focused approach may be employed by performing six consecutive determinations at a concentration of 100%. Adhering to these minimum sampling requirements is essential for determining the inherent variability of the method and ensuring the reliability of the resulting data [11].

1.4.7 Intermediate Precision

To ensure the long-term reliability of an assay, intermediate precision must be established by simulating the routine variations inherent in laboratory practice. This involves investigating the influence of random events—such as variations in **testing dates**, **personnel assignments**, and **equipment calibration**—on the precision of the results. In alignment with modern validation standards, these parameters need not be scrutinized individually; instead, the use of a **matrix-based experimental design** is encouraged. This holistic approach facilitates a more comprehensive perception of the method's ruggedness across typical laboratory shifts [12].

1.4.8 Reproducibility

In the context of regulatory and compendial validation, **reproducibility** characterizes the precision of a method under varying operational conditions across

different institutions. It is assessed via structured **collaborative trials**, which are mandatory when transitioning an analytical procedure into a standardized format. Inclusion in a **pharmacopoeia** necessitates this high-tier validation to guarantee that results remain comparable regardless of the geographic or institutional setting, thereby safeguarding the quality and safety of pharmaceutical products on a global scale.

1.4.9 Detection of Limit

The **Limit of Detection (LOD)** is defined as the minimum concentration or mass of an analyte within a specimen that can be reliably detected, though not certainly quantified with high precision or accuracy. This parameter represents the lowest extremity of the analytical range where the existence of the substance can be marked from the background matrix with statistical confidence. It serves as a qualitative boundary, indicating the method's ability to identify trace amounts without meeting the rigorous requirements for exact quantification.

Procedure:

- Make standard product concentration in the relevant solution.
- Prepare a blank solution without any sample.
- Perform the assay minimum 3 times in triplicate as per the SOP.
- Measure the LOD values for the described sample and blank
- Determine average detected value for the described sample and blank.
- Calculate and standard deviation (S.D.) of blank
- LOD is calculated as 3 times the standard deviation (S.D.) of the blank.

Based on Visual Evaluation

In analytical validation, the detection limit represents the minimum concentration level that can be reliably distinguished from a blank sample. While often determined via statistical signal-to-noise ratios, **qualitative visual inspection** remains a valid approach for identifying this boundary in certain experimental designs. The process involves evaluating a gradient of known concentrations to ascertain the point at which the analytical signal is sufficiently robust to be detected with a high degree of confidence [12].

Based on Signal-to-Noise

The application of this methodology is restricted to analytical frameworks that demonstrate discernible **baseline noise**. The **signal-to-noise (S/N) ratio** is derived through a comparative assessment of the peak intensity from specimens with known trace concentrations against the background fluctuations observed in blank matrix samples. This process facilitates the identification of the **detection threshold**—the absolute minimum concentration at which the analyte can be distinguished from the baseline with statistical confidence. Generally, an S/N ratio ranging from **3:1 to 2:1** is utilized as the standard benchmark for estimating the LOD.

a) By Standard Deviation (S.D.) of the Response and Slope:

The detection limit (DL) can be represented as:

$$DL = 3.3 \times \sigma / S$$

Where,

σ = S.D. of the response

S = slope

1.4.10 Limit of Quantitation:

A fundamental parameter in method validation is the **Quantitation Limit**, which denotes the smallest quantity of a target substance that an analytical procedure can measure with statistically significant accuracy and precision. The selection of an estimation strategy for this limit depends heavily on the **instrumental requirements** of the assay. Although standardized approaches—such as signal-to-noise ratios or standard deviation calculations—are typically favored, the use of unconventional or novel estimation techniques is permitted, provided they are substantiated by empirical data during the validation phase.

Based on Visual Evaluation

Sensitivity assessment in analytical chemistry may incorporate visual inspection, though this approach is often refined through instrumental quantification. The **quantitation limit** is typically derived by evaluating a gradient of known

concentrations to determine the **inflection point** of reliable measurement. In this context, the LLOQ is characterized as the lowest concentration level where the method's performance—specifically its bias and reproducibility—remains within the rigorous tolerances required for validated quantitative analysis.

Based on Signal-to-Noise

The determination of the quantitation limit via S/N analysis is contingent upon the presence of measurable background fluctuations within the chromatographic or spectroscopic baseline. This approach involves benchmarking the analytical signal of low-level fortified samples against the response of non-fortified blanks. This comparison facilitates the identification of the **threshold concentration** at which the analyte signal is sufficiently robust to allow for accurate and precise quantification, typically requiring an S/N ratio of 10:1 for the LLOQ.

The usual signal to noise ratio is 10 to 1.

Based on the Slope and S.D. of the Response:

The Quantitation limit (QL) can be represented as:

$$QL = 10\ \sigma/S$$

Where,

σ = S.D.

S = Slope

Slope S is acquired from the standard curve of the analyte.

Recommended Data

The analytical report must provide a broad description of the **Quantitation Limit** and the specific criteria utilized for its identification. To ensure methodological integrity, the limit requires a posteriori validation through the systematic analysis of multiple replicates prepared at the specific LLOQ concentration. This validation step authorizes that the performance characteristics, specifically accuracy and precision, comply with pre-defined regulatory benchmarks even at minimum concentration levels, thereby substantiating the method's sensitivity for clinical or forensic application [12].

1.4.11 Robustness

Analytical robustness is defined by the protocol's ability to remain stable and unaffected by minor but deliberate modifications to the established method parameters. This characteristic is a fundamental prerequisite for demonstrating the assay's consistency during routine operational use. Key parameters typically subjected to robustness assessment include physiological and environmental factors, such as the **pH of mobile phases or reagents**, the duration of **enzymatic or chemical incubation**, and fluctuations in **laboratory climate conditions**.

Table: 1.1 -Validation Parameters for Permissible Limits

Characteristics	Acceptable range
Accuracy	Recovery (98-102 %)
Precision, Repeatability	RSD<2 %
Intermediate Precision	RSD<2 %
Specificity	No interference
LOD	2-3 times SD of blank
LOQ	10 times SD of blank
Linearity	R^2 0.0-1.0
Range	80-120 %
Stability	>24h or >12h

1.5 Bioanalytical Method Developments

Over the past quarter-century, the discipline of **pharmacokinetics** has become an indispensable component of the drug development lifecycle, particularly regarding the representation of a drug's biological profile. For non-intravenous delivery systems constructed for systemic therapy, the paramount objective is the efficient transport of the active constituent into the systemic circulation at concentrations necessary to elicit the intended therapeutic outcome. This fundamental characteristic of a dosage form is recognized as **physiologic availability**, **biologic availability**, or, most commonly, **bioavailability**.

In technical terms, bioavailability quantifies both the velocity (rate) and magnitude (extent) at which an active moiety is absorbed from a pharmaceutical

product and reaches the designated site of action. Scholarly distinction is made between **absolute bioavailability**, which utilizes intravenous administration as the 100% baseline for comparison, and **relative bioavailability**, which evaluates absorption against a standardized reference product. Furthermore, **bioequivalence** is established when no statistically significant variance is observed in the rate and extent of absorption between pharmaceutical equivalents or alternatives, provided they are administered at identical molar dosages under controlled experimental conditions [13].

1. Clinical trials
2. Bioanalysis
3. Biostatistics

Bioanalytical Sample Analysis is divided into three major processes:

1. Development of Method
2. Validation of Methods

An optimized strategy for analytical method development focuses on procedural parsimony, necessitating only the minimum number of experimental iterations required to attain the targeted analytical objectives. The overarching goal is the creation of a streamlined methodology that integrates modern computational tools, such as molecular modeling or algorithmic optimization, to simplify complex workflows.

These developmental processes, followed by rigorous validation, are fundamental to the pharmaceutical lifecycle—from initial discovery to large-scale manufacturing. The resulting standardized protocols provide quality control laboratories with the requisite framework to verify the **identity, purity, potency, and functional performance** of therapeutic agents. To maintain global safety and quality benchmarks, international jurisdictions—including the United States, Japan, and the European Union—utilize official pharmacopoeias. For instance, the analytical standards codified in the **United States Pharmacopeia (USP)** carry legal authority under the Federal Food, Drug, and Cosmetic Act, with further validation requirements mandated by the **Code of Federal Regulations (CFR)**.

The pharmaceutical landscape is currently characterized by an accelerated influx of novel molecular entities and semi-synthetic structural analogs. However, a

pronounced **temporal latency** often exists between the commercial debut of a therapeutic agent and its eventual codification within official pharmacopoeial monographs. This delay is frequently necessitated by the requirement for longitudinal data regarding widespread clinical utility, emergent toxicological profiles, or the development of antimicrobial resistance. In the absence of such standardized compendial benchmarks, the responsibility shifts to the analytical chemist to engineer and validate bespoke methodologies capable of ensuring product quality during this interim period.

1.5.1 Fundamental requirements for the creation of novel methodologies in pharmaceutical analysis

1. The pharmaceutical formulation or medication combination may not be recognized in any pharmacopoeia.
2. The literature may lack a suitable analysis method for the medication due to patent restrictions.
3. The medication formulation may be unsuitable for analytical techniques due to interference from excipients.
4. Analytical methods for quantifying the medicine in biological fluids may be unavailable.
5. Analytical techniques for a medicine in conjunction with another drugs may be unavailable.

Current analytical procedures may require costly chemicals and solvents. It may be linked to laborious extraction as well as separation methods that may not have reliability [15].

1.5.2 Steps involved in Method Development

1. Compound information / Literature search
2. Spectrometric analysis of analytes
3. Sample preparation process
4. Choice of LC-MS settings

- column

- mobile phase composition
- flow rate
- temperature

5. Final LC-MS parameter optimization

1.5.3 Optimization of LC-MS Method

During the method optimization sequence, the initial experimental conditions are subjected to iterative improvements to attain peak analytical performance. This involves the optimization of **asymmetry** and **capacity factors** to provide well-defined, Gaussian peak shapes and adequate retention. Additionally, maximizing the number of plates (theoretical) ensures heightened efficiency and resolution. The final output of this stage is a validated set of conditions that offers an idealized balance of elution time and sensitivity (LOD/LOQ), thereby facilitating the accurate and reproducible determination of the analyte of interest [16].

The various parameters that include to be optimized during method development are:

- Choice of separation mode
- Choice of stationary phase
- Selection of mobile phase
- Optimizing system settings for quantifying the analyte.
- Development of standard curve and QC standards
- Selection of internal standard
- Ion mode selection
- Choice of extraction procedure

1.5.3.1 Selection of mode of separation

Reversed-phase HPLC is predominantly favored for characterizing polar to moderately polar chemical entities because of the solvophobic interactions between the polar eluent and the non-polar column matrix. The choice of ionization source in mass spectrometry is equally critical and must be aligned with the compound's functional groups.

The detection system comprised a triple quadrupole mass spectrometer employing **Electrospray Ionization (ESI)** in both positive and negative manifolds. The integration of a turbo ion source allows for rapid mode-switching, which optimizes system productivity. In this analytical setup, **Multiple Reaction Monitoring (MRM)** facilitated highly selective MS/MS detection. Because the compound of interest possesses basic functional groups, the positive ionization mode was prioritized to maximize ion yield, contrasting with acidic drugs which would necessitate a negative polarity for detection [17].

1.5.3.2 Selection of stationary phase

The optimization of eluent additives represents a strategic balance between chromatographic peak shape and mass spectrometric response. By minimizing additive loading, researchers can achieve a marked increase in sensitivity, which is vital for the identification of low-abundance drug derivatives.

Central to this optimization is the selection of an appropriate HPLC column. The architecture of the bonded phase and the inertness of the silica support are primary determinants of the additive levels required to neutralize silanol activity. A superior column matrix reduces the reliance on high-concentration buffers or ion-pairing agents, ensuring that basic drugs exhibit optimal peak shape without compromising the ionization efficiency essential for trace-level quantification [17].

1.5.3.3 Column and Column-packing materials:

The efficiency of a chromatographic separation is inherently linked to the theoretical plates provided by the column's packing material. Silica's dominance as a support material because of its robust siloxane network and the versatility of its surface silanol groups. These groups can be chemically transformed into non-polar surfaces suitable for water-organic mobile phases by attaching hydrocarbon chains (C_2 through C_{22}).

Beyond hydrophobic interactions, silica supports can be tailored for specialized separations by incorporating polar organic compounds or strong ion-exchangers onto the surface. The manufacturing process frequently includes an **end-capping** step to saturate accessible silanols, thereby lowering peak tailing for alkali analytes. Users must remain cognizant of the chemical stability of these phases; the

siloxane linkages are susceptible to acid-catalyzed hydrolysis at low pH and base-induced dissolution at high pH, necessitating an operational window of pH 2 to 8 [18].

Liquid chromatography is fundamentally categorized into two primary modalities: normal-phase and reversed-phase. In **normal-phase chromatography**, the separation of lipophilic and moderately polar pharmaceutical entities is accomplished through interactions with a polar stationary phase. While historical perspectives attributed these separations solely to differential adsorption on the silica surface, contemporary models suggest a complex interplay between adsorption and partition mechanisms, involving both the intrinsic silanol groups and the hydration layer of bound water molecules.

Conversely, **reversed-phase chromatography (RPC)** utilizes a polar eluent—comprising aqueous buffers and organic modifiers like acetonitrile or methanol—partitioned against a hydrophobic stationary phase. The versatility of RPC is enhanced by the availability of diverse bonded phases, such as C_{18}, C_8, phenyl, and cyano moieties. To optimize the retention of acidic or alkali analytes, the eluent pH is meticulously adjusted to suppress ionization, thereby favoring hydrophobic interactions with the column matrix. For species that remain persistently ionized, **ion-pair chromatography** is implemented to facilitate the formation of neutral complexes with enhanced lipophilicity [19].

Modern drug development demands an analytical framework capable of achieving extreme precision and selectivity in the presence of endogenous biological interference. While traditional HPLC systems equipped with optical detectors remain functional, the field has moved toward high-fidelity mass spectrometric platforms, including **LC-MS/MS, GC-MS/MS,** and **CE-MS**.

The transition to **LC-MS/MS** utilizing triple quadrupole technology represents a significant advancement in bioanalytical capability. By employing atmospheric pressure ionization interfaces (ESI or APCI), this technique facilitates the robust quantification of trace-level analytes in fluids like serum and urine. The widespread adoption of the LC-MS/MS platform is a testament to its ability to satisfy stringent regulatory validation requirements while providing the high throughput necessary for pharmacokinetic and dynamic modeling.

The discipline of bioanalysis serves as a quantitative bridge in drug development, facilitating the measurement of active moieties and metabolic products. This data is indispensable for establishing the **pharmacokinetic (PK)** profiles and **toxicokinetic** thresholds required for regulatory approval and bioequivalence testing. The versatility of these techniques also permits application in forensic science, environmental health, and the enforcement of anti-doping policies.

Within clinical settings, bioanalysis is a prerequisite for studying **drug-drug interactions**, particularly in the context of **multidrug therapy**. These interactions represent a significant clinical challenge, as the presence of one agent may modulate the systemic concentration or metabolic pathway of another, leading to therapeutic failure or heightened toxicity. Whether resulting from clinical oversight or complex chemical interference, these interactions are best elucidated through rigorous bioanalytical monitoring, which identifies the shifts in pharmacokinetic parameters that occur when drugs are administered in combination [20].

1.6 Bio analytical method development using LCMS/MS

The architecture of a bioanalytical method is bifurcated into **pre-analytical processing** and **instrumental analysis**. Sample preparation serves as a vital purification step, designed to eliminate endogenous interferences and minimize the deleterious impact of the sample matrix on the signal. By concentrating the sample during this phase, researchers can significantly bolster the method's sensitivity. This is followed by the strategic configuration of **chromatographic separation and mass spectrometric detection**, where mobile phase constituents, stationary phase selection, and ionization parameters are refined to achieve high-fidelity data [21].

The different sample preparation processes are

1. Proteins precipitation
2. Liquid-Liquid Extraction
3. SPE (Solid Phase Extraction)
4. Extraction hybrid

1.6.1 Liquid-Liquid Extraction (LLE)

The methodology of **Liquid-Liquid Extraction (LLE)** utilizes the principle of

"like dissolves like" to isolate intended chemical entities from aqueous matrices. The accomplishment of this technique is contingent upon the use of 2 mutually insoluble phases where the analyte exhibits a more affinity for the organic layer. By carefully modulating the **sample pH**, researchers can suppress the ionization of the drug molecule, enhancing its solubility in the organic phase according to the **Nernst Partition Law**. Such refinement not only ensures high recovery rates but also serves as a critical clean-up step, removing polar contaminants that might otherwise compromise the sensitivity and specificity of the subsequent bioanalysis [22].

1.7 Mass spectrometric detection with ESI ionization and triple quadruple mass analyzer MS/MS mode

In mass spectrometry, the analytical cycle begins with the ionization of a substrate through the impact of energetic particles or field-induced transitions. This stage produces a population of ions—comprising atoms, molecules, or associated clusters—that carry the chemical signature of the analyte. The critical function of the **mass analyzer** is the spatial or temporal separation of these ions, a process facilitated by the manipulation of electromagnetic fields. Under stringent vacuum conditions to prevent molecular collisions, the ions are sorted by their mass-to-charge ratios. The final detection phase quantifies the ionic abundance, enabling precise molecular weight determination and structural elucidation [23].

1.8 Validation Parameters

To verify that the proposed method maintains its performance standards under prescribed conditions, a structured experimental design was implemented. This framework serves as the definitive proof of the method's **precision and robustness**, ensuring that results remain within pre-defined tolerance limits. The benchmarks for success for each parameter are delineated within the individual test protocols. Furthermore, the primary data and subsequent results were archived using specialized validation documentation to facilitate rigorous peer review and compliance [24].

1.8.1 Carryover Test

To estimate the extent of analytical **carryover**, an experimental framework was established comprising eighteen independent samples. This included six replicates of the extracted matrix blank (COT BLANK), six replicates of the **Upper Limit of**

Quantitation (ULOQ) representing the peak concentration within the calibration curve—and six replicates of the **Lower Limit of Quantitation (LLOQ)**. Following extraction, these processed specimens were introduced into the analytical system in a predetermined injection sequence designed to identify any residual analyte persistence between successive runs [25].

Acceptance Criteria

The criteria for validation of no significant carryover are based on the signal response in the matrix blank (COT BLANK) samples. In particular, interference peaks at the retention period or specific mass transitions of the target analytes or their metabolites should not be greater than 20% of the mean peak area observed in the corresponding Lower Limit of Quantitation (LLOQ) specimens. In addition, the residual response in the blank for internal standard (IS) should be lesser than 5% of the average IS signal in the LLOQ samples. These limits are established such that any inter-sample contamination will not impact the accuracy of the trace level quantification.

1.8.2 Selectivity

Matrix Selectivity

The potential for matrix-induced interference was investigated using plasma obtained from eight separate individuals, with the inclusion of specialized hemolytic and lipemic lots to represent clinical variability. The chromatographic behavior of the drug and its internal standard was first characterized through the analysis of aqueous standards to establish definitive retention windows.

The crux of the selectivity investigation consisted of processing blank K2EDTA plasma batches in parallel with six LLOQ-level boosted samples. Selectivity is established when the response of the blank matrix at the retention period of the analyte is insignificant compared to the average extracted LLOQ response. A similar comparative analysis was done on the internal standard comparing its signal in the blank matrix to the average IS response in the LLOQ replicates to evaluate for cross-talk or co-eluting interferences [26].

Acceptance Criteria:

Interfering peaks during the retention time for the drug should react < 20% of the mean drug response at the LLOQ samples. The response for the internal standard should be ≤ 5% of the mean internal standard response at the LLOQ samples.

If one or more lots do not fulfill the acceptance requirements provided, the experiment should be redone with new lots. If the experiment is repeated and still fails to meet the approval standards, the approach must be changed and documented accordingly.

1.8.3 Analyte Selectivity

To further validate the selectivity of the approach, the influence of each component on the detection manifold of the other was studied. Six replicates of internal standard alone blanks were evaluated for probable analyte-channel interference and compared to the average LLOQ signal intensity. In a parallel evaluation of spectral or chromatographic overlap, the effect of the drug on the IS was also studied by examining the six-fold replication of the matrix blank at the level of ULOQ. This comparison to the mean IS response in LLOQ samples confirms that neither the high-concentration drug nor the internal standard causes systematic error into the quantification of the other [27].

Acceptance Criteria:

To support the selectivity of the bioanalytical approach, the response of interfering peaks in blank matrix samples is compared with the validated LLOQ. The approval requirements are that the mean interference at the retention duration of the drug should be ≤ 20% of the mean LLOQ response. When the interference at the retention duration of the internal standard is not greater than 5% of the average response of internal standard in LLOQ samples, a more stringent limit is used for the internal standard. These quantitative criteria are a precaution against skewed results in trace level analysis of biological specimens.

1.8.4 Sensitivity

The main factor for the sensitivity of the assay is the LLOQ, because the analytical signal should be distinguished from the background noise. Specifically, the

response in the LLOQ replicates would be a minimum of five times the baseline interference in the blank matrix. A minimum S/N ratio of 5:1 must also be maintained in order to maintain data integrity.

A set of calibration standards and 6 LLOQ level matrix samples were processed to confirm the experimental results. The accuracy for the LLOQ was calculated by back-calculation of the recorded concentrations grounded on the predefined calibration curve. Statistical parameters such as the percent nominal (accuracy) and the relative S.D. (% CV) were used to justify that the approach is routinely meeting performance standards at the baseline of the dynamic range [28].

Acceptance Criteria:

In alignment with regulatory standards for bioanalytical method validation, the LLOQ must demonstrate adequate reproducibility and truthfulness. The protocol mandates that the percentage of the nominal value (accuracy) and the relative S.D. (precision) both remain within a **20% limit**. Additionally, a high degree of inter-sample consistency is required: at least four of the six replicates (>66.7%) must yield back-calculated concentrations within the ±20% accuracy range. Failure to meet these specific statistical targets indicates insufficient sensitivity or excessive noise at the baseline of the dynamic range.

1.8.5 Matrix Effect

The forcefulness of the method against matrix-induced signal modulation was verified at two concentration extremes (LQC and HQC). Eight independent plasma lots, featuring diverse lipid and hemoglobin profiles, were utilized to provide a comprehensive evaluation of matrix interference.

Blank samples from each lot were processed and spiked with analyte and IS to replicate post-extraction recovery. Aqueous reference solutions created in the reconstitution step were examined together with the samples. To ensure statistical significance, the aqueous standards were subjected to six consecutive injections. The resulting data served to characterize the degree of **ion suppression or enhancement**, ensuring that the analytical signal remains consistent and reproducible across heterogeneous biological specimens [29].

The IS-normalized MF for the levels of LQC and HQC was derived by the below formula:

IS normalized Matrix Factor = Peak response area ratio in presence of matrix ion / Mean peak response area ratio in absence of matrix ions

Acceptance criteria:

To ensure that the analytical signal is consistently corrected for matrix-induced modulation, the variability of the **IS-normalized matrix factors** is strictly monitored. A maximum threshold of **15% CV** is permitted across the analyzed lots. Should the matrix effect evaluation fail to reach this standard, a remedial experiment must be conducted. This procedure involves processing eight alternative blank matrix batches for fortification, or alternatively, re-extracting the original batches to verify the stability and reproducibility of the matrix-analyte interaction.

1.8.6 Linearity

A ten-point calibration curve was constructed in the representative biological matrix to facilitate quantitative analysis. The selection of standard concentrations was strategically aligned with the expected analytical range of the study samples. Quantitative assessment was performed via least-squares regression analysis; to compensate for the increasing variance at higher concentrations, a **weighting factor of $1/x^2$**was applied. This mathematical refinement ensures that the regression model maintains its predictive integrity throughout the concentration-response manifold, yielding a high degree of correlation between the analytical signal and the analyte mass.

Acceptance Criteria:

- [3] 0.98 r^2 (r=correlation coefficient)
- Deviation of LLOQ concentration from the nominal value of 20%
- 15 % departure from nominal value for other than LLOQ concentration of standards
- 75% or at least 6-non-zero calibration curve standards including LLOQ and maximum concentration, should match the following criteria.

1.8.7 Precision and Accuracy

The precision and accuracy within and between batches were evaluated by the systematic study of at least three independent bioanalytical batches [30]. To provide for a strong statistical evaluation, each validation batch had to contain a defined set of calibration standards and quality control samples. These samples were spiked with the internal standard (IS) at appropriate points in the extraction process to offset possible differences in recovery and instrumental response between multiple analytical runs.

Medium standard (MQC2) dilution with internal standard

Empty matrix

Matrix Blank Internal Standard

Spiked calibration standards

RS (reconstitution soln.)

Six LLOQ quality control (QC) samples

6 low grade control samples

Six intermediate quality control samples (MQC1 & MQC2)

Six high grade control sample

The quantitative performance of the approach was tested by determining accuracy and precision for different timeframes of operation. The intra-batch precision and accuracy was determined as the coefficient of variation (% CV) and the percent nominal concentration at each Quality Control (QC) level within each analytical run. Temporal stability of the approach was evaluated by calculating inter-day (between-batch) precision and accuracy using data sets obtained from several analytical runs on different days. Additional intra-day (or between-batch) metrics were obtained for distinct runs done during the same diurnal period, to verify the consistency of the analytical platform independently of the specific batch sequence [31].

Calculations:

All computations were performed employing Microsoft Excel program software authorized.

Accuracy (% Nominal) and precision (CV%) for:

Accuracy and Precision of Batches

$$\text{Accuracy (\% Nominal)} = \frac{\text{Mean of each QC concentration level within a batch}}{\text{Nominal Value}} \times 100$$

$$\text{Precision (CV \%)} = \frac{\text{STDEV of each QC concentration level within a batch}}{\text{AVERAGE of each QC concentration level within a Batch}} \times 100$$

Batch/ Intra Day Precision and Accuracy

$$\text{Accuracy (\%Nominal)} = \frac{\text{Mean of each QC conc. level of all batches run in a day}}{\text{Nominal Value}} \times 100$$

Between Batch / Inter Day Precision and Accuracy

$$\text{Accuracy (\% Nominal)} = \frac{\text{Mean of each QC conc. level of all batches}}{\text{Nominal Value}} \times 100$$

$$\text{Precision (CV \%)} = \frac{\text{STDEV of each QC concentration level of all batches}}{\text{AVERAGE of each QC concentration level of all batches}} \times 100$$

Acceptance Criteria:

Methodological rigor is ensured by adhering to standardized performance metrics for quality control samples. The protocol mandates that the precision for all QC tiers, excluding the LLOQ, does not exceed a 15% threshold, with the LLOQ precision capped at 20%. Accuracy is evaluated as the percent recovery relative to the nominal concentration, requiring a deviation of no more than ±15% for standard QCs and ±20% for the LLOQ.

In every analytical run, the "two-thirds" rule applies: at least 67% of all QC specimens must demonstrate accuracy within the established 15% (or 20% for LLOQ)

limits. This allows for a maximum of one-third of the samples to exceed these boundaries, provided that at least half of the replicates at each specific concentration level remain compliant with the nominal value requirements.

1.8.8 Recovery

The recovery of the analyte and internal standard was evaluated across the dynamic range using three concentration tiers (LQC, MQC, and HQC). Six replicates per tier were prepared for extraction, ensuring that any frozen samples were totally thawed prior to processing. These were analyzed alongside a control group representing the non-extracted analyte response.

The control group was generated by processing eighteen replicates of a single-lot blank matrix; these extracts were subsequently spiked with the appropriate QC dilutions to represent **total (100%) analyte retention**. The resulting non-extracted replicates provide a reference signal for the post-extraction environment. By comparing the mean peak areas of the extracted samples against those of the non-extracted samples, the method's process yield was rigorously substantiated [32].

Calculate mean response, S.D. & CV %.

Mean absolute % recovery of analyte and internal standard at each QC concentration

Mean absolute % recovery of drug =

Mean Peak Area Response of analyte in extracted samples at LQC, MQC andHQCX100

Mean Peak Area Response of analyte in non-extracted samples at LQC, MQC and HQC

Mean absolute % recovery of internal standard =

Mean Peak Area Response of internal standard in extracted samples X100
Mean Peak Area Response of internal standard in non-extracted samples

Finally, calculate the overall recovery for the analyte.

Absolute % Recovery = Average of absolute % recoveries of LQC, MQC and HQC

Acceptance Criteria

The reproducibility of the recovery process is evaluated based on the **coefficient of variation (% CV)** and the inter-level consistency of the extraction yield. To satisfy the acceptance criteria, the % CV for the peak areas of both the analyte and the internal standard ought to not exceed **15%** at each quality control (QC) concentration level. Furthermore, the method must demonstrate a uniform recovery across the dynamic range; the absolute difference between the minimum and maximum mean recovery percentages across the LQC, MQC, and HQC levels must be ≤ 25%. These thresholds warrant that the extraction efficiency is both precise and independent of the analyte concentration.

1.8.9 Dilution Integrity

To validate the accuracy of the method for concentration exceeding the calibrated range, a **dilution integrity** study was performed. Twelve replicates of Quality Control (QC) samples were fortified at a such concentration approximately **1.5 to 1.8 times** the **Upper Limit of Quantitation (ULOQ)** and subsequently stored under cryogenic conditions alongside the bulk spiked samples.

On the day of analysis, the replicates were separated into two cohorts of six. The first cohort was subjected to a **two-fold (2×) dilution**, while the second underwent a **four-fold (4×) dilution**, using screened blank matrix as the diluent to maintain matrix consistency. Following extraction, such samples were assessed alongside a standard calibration curve. The final concentrations were derived by applying the respective **dilution factors (2 and 4)** to the back-calculated values, ensuring that the analytical signal remains proportional to the mass even above the standard dynamic range.

Acceptance Criteria

- QC batch precision with the same dilution parameters must be £ 15 %
- Within batch accuracy of QC of identical dilution factor must be ± 15 of the nominal value

- At each level of dilution, at least 67% of the QC samples (4 out of 6) shall be within 15% of their respective nominal (theoretical) values. 33% of the QC samples may be outside the ±15% of nominal value.

1.8.10 Ruggedness

A set of precision and accuracy were processed and analysed using different analyst, different column of similar make and distinct sets of reagents [33].

Acceptance Criteria

- Should meet the acceptance criteria for precision and accuracy batch.

1.8.11 Stabilities

Room Temperature Stock Solution Stability

The analytical methodology included a comprehensive evaluation of the standard stock solution's susceptibility to degradation under refrigerated and ambient conditions. Primary stock solutions were aliquoted and stored within a **2°C–8°C range** to establish long-term refrigerated stability. Concurrently, benchtop stability was monitored over a six-hour interval to represent the maximum expected duration of sample processing at room temperature.

The experimental design employed a **comparison-based approach**: after the six-hour period, fresh reference stocks were prepared. Final working solutions—equivalent to the MQC level and the designated IS concentration—were derived from both the stability and comparison stocks. Six consecutive injections of each group were performed to facilitate a precise determination of the stability percentage. This systematic verification ensures that any volumetric or thermal influences do not introduce methodical bias into the final quantitative results [34].

Calculations

$$\% \text{ Stability} = \frac{\text{Mean response of stability samples} \times \text{Comparison stock concentration}}{\text{Mean response of comparison stock samples} \times \text{Stability stock concentration}} \times 100$$

Acceptance Criteria

- % Stability must be between 95 % and 105 % and %C.V. must be < 10%.

Refrigerated Stock Solution Stability

To establish the refrigerated shelf-life of the analytical standards, a longitudinal stability study was executed. Stock solutions were maintained under refrigeration and analyzed at predetermined time points to verify their suitability for ongoing use.

At each designated interval, a fresh batch of comparison standards was generated. Final working solutions—representing the MQC concentration—were derived from both the stability-tested and newly prepared stocks. To minimize experimental error, six replicates of each preparation were analyzed in a single sequence. The resulting data allow for the calculation of the **percent stability**, ensuring that the archived stocks do not exhibit significant degradation compared to the fresh reference material.

Calculations

$$\% \text{ Stability} = \frac{\text{Mean response of stability samples x Comparison stock concentration}}{\text{Mean response of comparison stock samples x Stability stock concentration}} \times 100$$

Acceptance Criteria

% Stability must be between 95 % and 105 % and % C.V. must be < 10%.

Freeze Thaw Stability

The robustness of the analyte in biological matrix was tested through a rigorous freeze-thaw validation framework. Eighteen replicates per QC level were prepared and stored at **-70°C**. The experimental design utilized a sequential thawing approach where samples underwent up to six cycles of freezing (≥ 12 hours) and thawing (ambient temperature).

The cohort designated for six cycles (FT-6) was analyzed immediately following its final thaw. To provide a rigorous comparison, these samples were processed in parallel with freshly spiked calibration standards and control samples (LQC, MQC, HQC). Quantitative assessment was performed using the resulting regression parameters to determine if the cyclical temperature fluctuations introduced any significant bias or degradation. This method ensures that the finalized assay can accommodate real-world laboratory scenarios where samples may be repeatedly accessed [35].

Calculations

Mean % Nominal Concentration of Quality Control Samples =

$$\frac{\text{Mean concentration of LQC or HQC having undergone 6 freeze thaw cycles}}{\text{Respective nominal concentration of LQC or HQC}} \times 100$$

Bench top Stability

To substantiate the integrity of the samples during bench-top preparation, a temporal stability study was executed. Sextuplicate aliquots of LQC and HQC samples were equilibrated to room temperature and maintained for a minimum of six hours prior to processing.

On the day of evaluation, these stability samples were processed alongside freshly prepared calibration standards. The quantification was performed by applying the back-calculated values from the daily calibration curve to determine if any significant degradation occurred during the bench-top interval. The stability timeframe was strictly calculated as the interval beginning with the withdrawal of samples from the deep-freeze environment and concluding at the onset of the extraction phase.

Calculations

Mean % Nominal Concentration of Quality Control Samples =

$$\frac{\text{Mean concentration of LQC or HQC (stability samples)}}{\text{Respective nominal concentration of LQC or HQC}} \times 100$$

Auto Sampler Stability

To substantiate the robustness of the processed extracts, an autosampler stability study was executed. Sextuplicate preparations of LQC and HQC samples were processed and archived within the autosampler manifold under specified thermal conditions.

On the day of evaluation, the stability cohort was injected in parallel with freshly spiked calibration and control samples. The analytical concentrations were determined through regression analysis of the daily calibration curve. The validated stability window was defined as the interval beginning with the placement of the samples into the autosampler and concluding with the instrumental injection of the first

stability specimen. This assessment ensures that the analyte remains stable during the potential delays inherent in high-throughput analytical sequences [36].

Calculations:

Mean % Nominal Concentration of Quality Control Samples =

$$\frac{\text{Mean concentration of LQC or HQC samples at proposed stability period (in hours)}}{\text{Respective nominal concentration of LQC or HQC}} \times 100$$

Long Term Stability at –70 °C

Long-term stability of the analyte in the biological matrix was verified by testing samples maintained at -70 °C. Six duplicates of the Low (LQC) and High (HQC) Quality Control samples were removed from cryogenic storage on the day of evaluation and processed in parallel with a de novo calibration curve and a new set of QC standards.

The period of long-term stability was defined as the total elapsed time in days from the date of first sample fortification to the date of final analysis. Analyte integrity was verified by comparing the back-calculated concentrations from the stability batch to the mean of the freshly generated QC duplicates. This systematic comparison guarantees the efficiency of the storage conditions in maintaining the chemical stability of the analyte during the designated time frame [37].

Calculation

Mean % Nominal Concentration =

$$\frac{\text{Mean conc. of stability LQC or HQC samples x nominal conc. of freshly prepared LQC or HQC}}{\text{Mean conc. of freshly prepared LQC or HQC samples x nominal conc. of stability LQC or HQC}} \times 100$$

Wet Extract Stability

To substantiate the robustness of the extracts during potential laboratory delays, a temporal stability study was executed. Sextuplicate preparations of LQC and HQC samples were processed and stored under refrigeration (**2–8°C**) to simulate post-extraction holding conditions.

Following the designated storage interval, the stability cohort was injected alongside freshly spiked calibration and control samples. The analytical concentrations

were determined through regression analysis of the daily calibration curve. The validated stability window was strictly calculated as the difference between the sample preparation completion time and the time of sample introduction into the autosampler. This assessment ensures that the analyte remains stable and that no significant evaporative or chemical changes occur within the reconstitution solvent over time.

Calculations

Mean % Nominal Concentration of Quality Control Samples =

$$\frac{\text{Mean concentration of LQC or HQC samples}}{\text{Respective nominal concentration of LQC or HQC}} \times 100$$

Acceptance Criteria for Stabilities:

- 0.98 r^2 (r=correlation coefficient)
- 20 % variation from the nominal value at the LLOQ
- Deviation of standards (other than LLOQ concentration) from nominal value should be 15 %
- 75 % or at least 6 non-zero calibration curve standards including LLOQ and maximum concentration, should comply with above criteria.

For Stability QC Samples:

- Mean % Nominal concentration of each quality control sample stage should be within 85 % to 115 % and precision should be $ 15 % of the C.V. %.
- At least 67% of the stability QC samples should be within 15% of the relevant theoretical (nominal) values. QC samples (not all replicates at the same concentration) may be outside the permitted limits listed above by as much as 33%. At least 50% of the stability QC samples at each level of concentration should be within ± 15% of the corresponding nominal value.

For Fresh QC Samples:

- The mean percentage of the nominal concentration at each level of quality control sample should be within 85 % to 115 % and the precision should be $ 15 % of the C. V. %.

- At least 67% of QC samples should be within 15% of their respective nominal (theoretical) values. Above, 33% QC samples (not all replicates at same concentration) may be outside tolerance limits. At least 50% of the QC samples at each concentration level shall be within ±15% of their respective nominal values.

Re-injection Stability

The re-injection integrity of the analyte and internal standard was determined as part of the analytical technique. Six LQC and HQC samples were run and analysed together with a respective calibration curve. After the baseline measurement, the identical preparations were injected again into the chromatographic system after a certain time periodfor checking the consistency of the backcalculated data.

The original calibration curve data was used to interpolate the concentrations of the re-injected quality control samples. Such comparison is performed to check the stability of processed extracts in the instrument manifold, so that the results are reliable if re-analysis of clinical or pre-clinical samples are needed.

Calculations:

Mean % Nominal Concentration =

$$\frac{\text{Mean concentration of QC reinjected over a period of time}}{\text{Mean concentration of QCs when injected for the 1st time}} \times 100$$

Note: Time length of Re-injection stability will be measured from the time of injection of first quality control sample of the initial samples, minus the time of injection of Re-injection samples.

Acceptance Criteria:

- Mean % Nominal concentration at each level of quality control sample must be 85 – 115% and the precision must be £ 15 % of the C.V. %

- At least 67% of the stability QC samples should be within 15% of the corresponding nominal (theoretical) values. Up to 33 % of QC samples (not all replicates at the same concentration) may be outside the acceptable limits stated above. At least 50 % of the stability QC samples at each concentration level should be within ±15 % of their respective nominal values.

Effect of concomitant drugs:

Potentially interfering OTC drugs (i.e. Paracetamol, Ibuprofen, Diphenhydramine, Pantoprazole, Nicotine) were spiked in screened blank matrix at appropriate concentration equivalent to $C_{max}$of individual drug (5 µg/mL for Pantoprazole, 25 µg/mL for Ibuprofen, 10 µg/mL for Paracetamol, 100 ng/mL for Diphenhydramine and 50 ng/mL for Nicotine) to prepare concomitant drug plasma. This was called Concomitant Drug Plasma.Six sets of LLOQ QC, LQC and HQC samples were prepared in concurrent drug plasma. Prepared QC samples were run and assessed using bulk spiked calibration curve standards.

Calculations:

Accuracy (% Nominal) and precision (CV %) for Within Batch Precision and Accuracy

$$\text{Accuracy (\% Nominal)} = \frac{\text{Mean of each QC concentration level within a batch}}{\text{Nominal Value}} \times 100$$

$$\text{Precision (CV \%)} = \frac{\text{STDEV of each QC concentration level within a batch}}{\text{AVERAGE of each QC concentration level within a batch}} \times 100$$

Acceptance Criteria:

- Precision: The computed precision for Low and High QC concentrations should be within 15% and 20% for the LLOQ QC Concentrations.
- Accuracy: The computed accuracy for Low and High QC concentrations should be within ± 15 % and ± 20 % of the nominal value for the LLOQ QC Concentration.
- For all QC samples, except at LLOQ QC, the values should be within 15% of their respective nominal (theoretical) values and at LLOQ QC, the values should be within 20% of the nominal values. 33% of the QC samples (not all replicates at the

same concentration) fall outside the stated acceptable criteria. At least 50% of QC samples at each concentration level should be within ±15% of their respective nominal value except at LLOQ QC where it should be within ± 20 % of the nominal value.

1.9 Safety Precautions

Always wear protective clothes, including disposable gloves and masks, when working with medicine samples. Gloves should be removed without the skin contacting the outside surface of the glove [38].

If a biological matrix is spilled, it should be cleaned immediately using a 4% solution of hypochlorite. Wash hands with soap and water before leaving the laboratory or when contaminated. Broken glassware must not be handled by hand. Collect mechanically with a brush, dustpan, tongs or forceps.

1.10 Data Processing

Chromatograms were obtained by means of the computer program Analyst 1.4.2. The data were processed by peak area ratio approach with the identical software. The concentration of the unknown was determined by the regression analysis of spiking calibration of standard utilizing the reciprocal of the square of the drug to internal standard concentration ratio as a weighting factor [1/ (concentration ratio)].

$$y = mx + c$$

Where y = peak area ratio of Naproxen to internal standard

m = slope of the calibration curve

x = concentration ratio of Naproxen to internal standard

c = y-axis intercept of the calibration curve

1.11 Ultraviolet and Visible Spectroscopy

This absorption spectroscopy practices electromagnetic (EM)radiations in the range of 180 nm to 780 nm and is classified into the UV (180-380 nm) and the

VISIBLE (380-780 nm) regions. This is also frequently referred to as "electronic spectroscopy" [39] because the absorption of UV radiation by a component or molecule results in transition including energy levels of the molecule.

1.11.1 Principles of Absorption Spectroscopy:

Beer- Lambert's Law

The more molecules there are that absorb light of a certain wavelength, the more light is absorbed and the higher the peak intensity in the absorption spectrum. When only a few molecules absorb radiation, the overall energy absorbed is smaller and so a peak of lower intensity is observed. This is the basis of Beer-Lambert Law which states that the fraction of radiation (incident) absorbed is proportional to the number of absorbing molecules in its path [40].

The amount of light transmitted or absorbed by a solution is an exponential function of the molecular concentration of the solute and a function of the length of the path of radiation through the sample. Hence,

$$\text{Log } I_o / I = \varepsilon c l$$

I_o = Intensity of light incident (or intensity of light going through a reference cell)
I = Intensity of light transmitted over the sample solution

c = concentration of the solute in mol l-1 l = path distance of the sample in cm

ε = molar absorptivity or the "molar extinction coefficient" of the substance of which the light absorption is being investigated.

It is a constant and is a property of a particular absorbing species (molecule or ion) in a particular solvent at a certain wavelength. e is numerically identical to the absorbance of a solution of unit molar concentration (c=1) in a cell of unit length (l=1) and its units are liters. Moles cm^{-1}. That said , it is common practice among organic chemists to leave off the units . The ratio I / Io is called transmittance T while the logarithm of the inverse ratio Io/ I is called the absorbance A [41].

Therefore

$$- \text{Log } I / I_o = - \log T = \varepsilon c l$$

and Log $I_o/ I = A = \varepsilon c l$

or $A = \varepsilon c l$

The positions of peaks are represented as the values of λ_{max} (nm) and the absorptivity is expressed in parenthesis for illustrating the absorption characteristics of a spectrum.

1.11.2 Solvent Effects

Very pure, non-polar solvents such as saturated hydrocarbons do not interact with solute molecules either in the ground or excited state. The absorption spectra of a substance in these solvents is comparable to that in the pure gaseous state. However, the molecular orbital's of a molecule may stabilize or destabilize in the ground or in excited state by polar solvents like water, alcohols etc. hence the spectra of a chemical in these solvents may vary greatly from the one recorded in a hydrocarbon solvent [42].

1.11.3 Nature of Electronic Transitions

Total energy of a component is the sum of its electrical, vibrational and rotational energy. Absorption of light in the UV area causes alterations in the electronic energy of thatmolecule. When such molecules absorb energy, 1 electron is raised from an occupied MO (typically a non-bonding or bonding orbital) to a vacant MO (an anti-bonding π or σ orbital) of higher potential energy. Most molecules have σ orbitals as their lowest-energy occupied molecular orbitals, which correspond to σ bonds. The π orbitals have energies that are substantially higher than the σ orbitals, and the non-bonding orbitals containing unshared pairs of electrons are at even higher energies. The antibonding orbitals (π and σ) are the highest energy orbitals [42].

σ to σ Transitions

An electron in a σ bonding orbital is stimulated to the matching σ ant bonding orbital. It requires a lot of energy. So we do not witness σ to σ transitions as absorption maxima in usual UV-Visible spectra (200 - 700 nm).

n to σ Transitions

Saturated compounds with lone pairs of atoms (nonbonding electrons) can have n to σ transitions. These transitions generally need less energy than \sigma to \sigma

transfers. They can be excited by light in the 150 - 250 nm wavelength range. Number of organic functional groups having n to σ peaks in UV area are modest.

n to π and π to π Transitions

Most of the absorption spectroscopy of organic compounds is the transition of n or π electrons to the π* excited state. This is due to the absorption maxima for such transitions being in an experimentally convenient range (200 - 700 nm). These transitions require the molecule to have an unsaturated group, which supplies the π electrons.

The likelihood of transitions is substantially affected by conjugation of bonds as well. The more conjugated the higher the overlap between the highest occupied (HOMO) and LUMO. The energy (ΔE) required to cause the electron promotion is thus decreased. Correspondingly, the wavelength that delivers this energy is raised. Conjugation of double and triple bonds changes the absorption maximum to longer wavelengths and the stronger the conjugation the larger the bathochromic (longer wavelength) and hyperchromic (greater absorbance) shifts in absorption.

1.11.4 Instrumental components

1.11.4.1 Sources of UV radiation

It is crucial that the intensity of the radiation cause does not shift suddenly across the wavelength limit of the source. Electrical stimulation of deuterium (D) or hydrogen (H) at low pressure provides a continuous UV spectrum. This is achieved by the production of an excited molecular species which dissociates to give 2 atomic species and aphoton. Which can be presentedas;

$$D_2 + \text{electrical energy} \text{------>} D_2 \text{----->} D' + D'' + h\nu$$

The deuterium lamp and the hydrogen lamp emit radiation from 160 to 375 nm. The lamps must have quartz windows and quartz cuvettes must be utilized since glass absorbs radiation at wavelengths below 350 nm [43].

1.11.4.2 Visible radiation Sources

A typical source of visible light is the tungsten filament lamp. This lamp is utilized in the range of 350 nm – 2500 nm. The energy radiated by a tungsten filament lamp is proportional to the 4^{th}power of the voltage at that it is operated. That means the voltage to the bulb must be actual stable indeed if the energy output is to be stable.Tungsten/halogen lamps include a small quantity of iodine in a quartz "envelope" that also holds the tungsten filament. The iodine combines with gaseous tungsten, generated by sublimation, to provide the volatile molecule WI/sub 2/. WI2 molecules striking the filament break apart and redeposit tungsten on the filament. A tungsten/halogen lamp will have about double the life of a regular tungsten filament light. Tungsten/halogen lamps are incredibly efficient and radiate strongly into the ultra-violet. They are employed in many current spectrophotometers [44].

1.11.4.3 Wavelength selector

Prism Monochromators

A prism divides the sunlight into seven different hues. This happens because as the light enters the prism, it bends. The radiations of different colors having distinct wavelengths are refracted to different extent. This is due to the variance in the refractive index of glass for different wavelengths. Shorter wavelengths refract more than longer wavelengths. One can arrange for different wavelengths of the radiation, which comes out after refraction via the prism, to pass through the exit slit by rotating the prism.

Grating Monochromators

A grating is produced by cutting or etching a series of closely spaced parallel grooves on a smooth, reflecting surface of a solid substance. A thin film of aluminum on the surface is made reflective and the etching is done with the help of an appropriately shaped diamond tool. The intensity of the radiation reflected by a grating is a function of the wavelength, the wavelength of maximum intensity conditional upon the angle of reflection by the radiation from the surface of the line of the grating. A collimated beam from the source is incident on a concave mirror through an entrance

slit. This is then reflected on the grating which spreads it out. The emitted radiation is then guided to an exit slit [44].

1.11.4.4 Sample Handling

The UV-VIS absorption spectra are often measured either in the vapor phase or in solution The UV spectrum of the analyte is taken by passing through a cell called cuvettes which is understandable to the wavelength of light going through it. The determination of spectra in the vapor phase is carried out in a variety of quartz cuvettes. They are of different travel lengths and provided with intake and outlets. Glass cuvettes can also be utilized for measurements on solutions in the visible range. Usually square cuvettes are used with a path length of 1.0 cm. Cuvettes with significantly smaller path lengths are also available such as 0.1 mm or 0.05 mm.

1.11.4.5 Detectors

The detectors are used to convert a light signal to an electrical signal that may be adequately measured and converted into an output. Many of the instruments use detectors which provide a signal that is linear in transmittance, i.e. they have a linear response to the radiant power incident on them. The signal read-out device may have an electrical or mechanical mechanism to convert the transmittance values into absorbance units. Modern spectrophotometers use three types of detectors. These are discussed in the paragraphs below.

Phototube

A phototube is an evacuated tube containing a photo emissive cathode and an anode with a quartz window. These two electrodes are applied with high voltage (approximately 100 V) differential. When a photon hits the cathode in the tube an electron is expelled and is pulled towards the anode and a current flow which may be amplified and measured. The photo emissive material has a wavelength dependent response, and several phototubes are available for different parts of the spectrum [44].

1.11.4.6 Signal Processing Techniques and Output Systems

The electrical signal from the transducer is supplied to the recorder and the output is generated once it has been properly amplified or processed. The spectrum of the solution is electronically subtracted from the spectrum of the solvent. The output

plot of wavelength against absorption intensity which is characteristic of the absorbing species is obtained by this subtraction technique.

1.11.4.7 Method developed by UV Spectro photometric methods:

- Selection of the solvent.
- Selection of analytical wavelengths.
- Study of beer-lamberts' law.
- To perform analysis of standard bulk drug and tablet formulation by proposed method.
- To validate the developed method as per ICH guidelines.

Selection of the solvent:

- It should not itself absorb radiations in the region under investigation.
- It should be less polar so that it has minimum interaction with the solute molecules.

Table1.2: List of common solvents used in UV Spectroscopy

Water	205 nm
Methanol	210 nm
Ethanol	210 nm
Ether	210 nm
Chloroform	245 nm
Carbon tetrachloride	265 nm
Cyclohexane	210 nm

Chapter 2

REVIEW OF LITERATURE

Kathryn B Kenney, *et al.* (2000), reported Zidovudine and lamivudine in human serum determined simultaneously by high-performance liquid chromatography with tandem mass spectrometry. The method incorporates a fully automated ultrafiltration sample preparation step that replaces the solid-phase extraction step typically used for HPLC with UV detection. The calibration range of the dual-analyte LC-MS/MS method is 2.5–2500 and 2.5–5000 ng ml^{-1} for AZT and 3TC, respectively, using 0.25 ml of human serum. The minor limit of quantification was 2.5 ng ml^{-1} for each analyte, with a chromatographic run time of around six min. Overall accuracy, expressed as bias, and inter- and intra-assay precision are <±7 and <10% for AZT, and <±5 and <12.1% for 3TC over the full concentration ranges. A cross-validation study demonstrated that the LC-MS/MS method afforded equivalent results to established methods consisting of a radio immune-assay for AZT and an HPLC-UV method for 3TC [9].

Singh *et al.* (2001) performed a LC/MS electro spray ionization mass spectrometric method for the quantitation of haloperidol-an antipsychotic drug in human plasma by liquid-liquid extraction (LLE) using duloxetine as internal standard with a mobile phase composition of 1.0 mm ammonium acetate pH 3.0 and acetonitrile (70:30, v/v). The lower limit of quantitation (LLOQ) of 2.00ng/ml was achieved using positive mode. The standard curves were linear in the concentration range of 2.00 ng/ml to 150.0 ng/ml with mean correlation coefficient of 0.998[21].

Lantz R.J. *et al.* (2003) determined the plasma concentrations of Duloxetine using LC/MS-MS method in the range of 5.0 to 100 ng/ml with the stable isotope-labeled internal standard (13CD3 duloxetine HCL). The compounds were separated using an HI-RP B (30 mm×3.2 mm) with mobile phase consisting of 55% acetonitrile/45% 10 mm ammonium acetate. Tandem mass spectrometry was used to monitor the transition of m/z 298.1→344.0 for duloxetine and 302.1→348.0 for the internal standard. The overall percentage CV (precision) and percentage relative error (accuracy) after three validation runs were ±8%[22].

Sockalingam Anbazhagan *et al.* (2005), was reported Concurrent use of ultraviolet spectroscopy, reverse phase high-performance liquid chromatography, and high-performance thin-layer chromatography to assess tablet formulations of stavudine, lamivudine, and nevirapine the ultraviolet multi-component spectral approach made use of three distinct wavelengths: 266, 271, and 315 nm for solvent V, liquid V, and nitrogen V, respectively. To resolve the pharmaceuticals in the RP-HPLC technique, an isocratic mode was applied to a C18-ODS-Hypersil (5 μm, 250 mm × 4.6 mm) column. The mobile phase was a 4:1 (volume/volume) mixture of acetonitrile, 20 mM sodium phosphate buffer, and 8 mM sodium salt of 1-octanesulfonic acid. Phosphoric acid was used to bring the pH down to 3.5. A retention time of 2.85 minutes was recorded for SV, 4.33 minutes for LV, and 8.39 minutes for NV. To measure the chromatograms produced by the HPTLC technique with a mobile phase of chloroform: methanol (9:1, v/v) on a precoated silica gel 60 F254 plate, densitometric absorbance mode at 265 nm was employed. All three models—SV, LV, and NV—had Rf values between 0.21 and 0.27, 0.62 and 0.72, and 0.82-0.93, respectively. [6].

Mahua Sarkar *et al.* (2006), reported "Development and validation of RP-HPLC and ultraviolet spectrophotometric methods of analysis for the quantitative estimation of antiretroviral drugs in pharmaceutical dosage forms "Lamivudine, stavudine, and nevirapine were all identified by UV spectrophotometric measurements taken at 270, 265, and 313 nm, respectively, according to this technique. In the concentration range of interest, the HPLC and UV techniques both produce calibration curves that are linear ($r^2 > 0.999$) for all analytes. With relative standard deviations (R.S.D.) and recoveries (%) ranging from 97% to 103% for all three medicines, both procedures were exact and accurate [10]

Ning *et al.* (2007*)* performed a rapid and sensitive liquid chromatography-mass spectrometric (LC/MS) method for the determination of duloxetine in human plasma using flupentixol as the internal standard (I.S.). Samples were then analyzed by HP LC on a ThermoHypersil- Hypurity C18 column (150×2.1 mm, 5 μ m). A single-quadruple mass spectrometer with an electrospray interface was operated in the selected- ion monitoring mode to detect the [M+H]+ ions at 298 m/z for duloxetine and at 435 m/z for the internal standard[23].

Mercolini *et al.* (2007), performed a rapid and sensitive High-performance liquid chromatographic method for duloxetine analysis in human plasma. The assays were carried out using a C8 reversed-phase column and a mobile phase composed of 60% aqueous phosphate buffer containing triethylamine at pH 3.0 and 40% acetonitrile. The extraction yields values were higher than 90%. Linearity was found in the 2–200 $ngml^{-1}$ duloxetine concentration range; the limit of quantization was 2.0 ngmL−1 and the limit of detection was 2.7 $ngml^{-1}$[24].

Waldschmitt *et al.* (2007), conducted a method using High-performance liquid chromatography (HP LC) with column switching and ultraviolet (UV) spectroscopy for the determination of duloxetine in human plasma. Duloxetine was eluted and separated on a Lichrospher 100CN (5-microm particle size; column size, 250 x 4.6 mm I.D.) using acetonitrile- water-potassium dihydrogenphosphate tri hydrate buffer (pH, 6.4; 50:50 vol/vol). The limit of quantification was 5ng/ml. At duloxetine concentrations up to 138 ng/, the interassay reproducibility of quality control samples was better than 12%[25].

Devyanidube *et al.* (2009), was reported Estimation of Lamivudine and Silymarin in a Mixed Medium by Means of SpectrophotometerLamivudine and silymarin can now be measured simultaneously using a new Spectrophotometric method that is innovative, easy to use, fast, and sensitive. The technique makes use of two analytical wavelengths, 270.9 nm and 326.4 nm, to build and solve an equation simultaneously. When tested within the concentration ranges specified by Beer's Law, both medications show no side effects. Recovery experiments provided statistical validation for the accuracy and reproducibility of the suggested technique [7].

D.K. Mandloi *et al. (*2009), was reported Improvement and verification of RP-HPLC methodology for use in an invitro dissolution investigation of Lamivudine in both bulk medication and tablet form to determine Lamivudine in both its bulk and tablet dosage forms, researchers devised a new RP-HPLC method. This approach was then used in in-vitro drug dissolving tests. A C18 column (250 x 4.6mm, 5µ) was used for separation, and an isocratic elution mode with a mobile phase consisting of a combination of 89:11 methanol and water was chosen. This mixture was found to be appropriate allowing good elution for the Lamivudine at retention time 2.72 minute at

flow rate of 1 ml/min and detection wavelength at 272nm. The linearity was established in the concentration variety of Lamivudine 5-100 µg/ml [11].

Samanidov *et al.* (2009), conducted a simpleand rapid HP LC methodfor the determination of two serotonin-norepinephrine-reuptake inhibitors (duloxetine and venlaflaxine) and two selective serotonin-reuptake inhibitors (fluoxetine and paroxetine) in human biofluids. Separation was performed on an Inertsil ODS-3 column (250 x 4.0 mm, 5 µm) with acetonitrile-ammonium acetate (0.05 M, 41:59 v/v). Relative standard deviation was less than 10.4%. Limit of detection was 5.2-5.6 ng/µl in blood plasma and 5.1-5.8 ng/µl in urine [26].

Zhu Wenbing *et al. (2009)* performed an HP LC method to determine Duloxetine hydrochloride in human plasma. The propranolol hydrochloride was used as an internal standard and ZORBAX Eclipse XDB-C18 (150mm×4.6mm,5µ m) column was used for separation. The mobile phase consisted of 5mmolL-1 Acetic Acid (adjusted to pH7.5 with ammonia)- methanol-(33:30:37) at aflow rate of lml•min-1. Linearity range was 1-100ng/ml[27].

Zhao *et al.* (2010*)* performed a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the determination of concentration of duloxetine in human plasma. The separation was performed on Phenomenex C18 column with fluoxetine as the internal standard. The mobile phase is composed of 5 mmol L^{-1} ammonium acetate with 0.02% formic acid acetonitrile (55:45). The linearity range of Duloxetine was 2.89-106.80 ng•mL-1(γ=0.9977). The extraction recovery ranged between 72.81%-89.96%. Both the inter-day RS D and intra-day RS D were less than 11%[28].

Paul w. Elsing horst *et al. (*2011), created and fully validated a quantitative LC-MS/MS technique that is sensitive, exact, and accurate for measuring naproxen in human plasma in accordance with the most recent criteria from the FDA and EMA. The novel approach uses ketoprofen as an internal standard and prepares samples using acetonitrile protein precipitation. To determine the new assay's validity, we compared it to 36 previously published bioanalytical tests, and to evaluate its pharmacokinetic results, we compared them to 11 previously published human investigations. The principal advantage of this LC-MS/MS method is the simultaneous achievement of high absolute recovery (90.0±3.6%), acceptable sensitivity (lower limit of quantitation of

0.100 µg/mL), high inter-day precision (CV≤9.4%), high analytical recovery (between 94.4 and 103.1%), and excellent linearity over the concentration range 0.100-50.0 µg/mL (r (2) ≥0.998) combined with a short run time of only 2 min [8].

Islam *et al.* (2011) developed a reversed phase high performance liquid chromatographic method and validated for the simultaneous analysis of domperidone and naproxen in tablet dosage form. The chromatographic system consisted of two LC-20 AT pump,SPD-20A UV detector, SIL-20A auto-sampler and CTO-10ASVP column oven. Chromatographic separation of drugs was achieved on a Shim-Pack C18 column (250mm x 4.6 mm, 5 µm) as stationary phase with a mobile phase comprising of phosphate buffer (pH adjusted to 3.00 with sodium hydroxide): methanol in the ratio30:70 (v/v) at a flow rate of 1.0 ml/min with UV detection at 280 nm. Retention time was 3.17 minutes for domperidone and 5.42 minutes for naproxen [9].

Vaishali P *et al.*(2012), was reported Development of UV Spectrophotometric vierodt's way to measure lamivudine and abacavir in a tablet form at the same time. This approach utilizes UV spectrophotometer to determine the concentrations of abacavir and lamivudine in both bulk medication and combination tablet dose forms simultaneously. A 3:1 combination of acetonitrile and methanol was used to generate the stock solutions, which were subsequently diluted with distilled water as needed. In proposed Vierodt's method, the lmax for the estimation of abacavir and lamivudine were selected at 260 nm and 271 nm respectively. Linearity in concentration range of 5-25 mg/ ml by both the drugs was found. The method has estimatedabacavir 100.01%, lamivudine 100.02% in the bulk drug and 99.86%, 99.82% of the both in tablets respectively. The outcome of analysis has been validated statistically and by improvement studies [8].

Kumar *et al.* (2012) established and verified an excellent efficiency liquid chromatographic technique for the detection of naproxen and pantoprazole in pharmaceutical items for sale. A Hypercell BDS C-18 reversed-phase column was used for compound separation. A mobile phase was prepared using 0.1 M sodium acetate (pH 8.2), acetonitrile, and methanol in a proportion of 70:20:10 v/v. The mobile phase was applied at a flow rate of 1.0 ml/min at a detection wavelength of 285 nm. Naproxen had a linearity range of 5-70 µg/ml and pantoprazole a range of 5-40 µg/ml [10].

Raju Senthil Kumar *et al.* (2012*),* The spectrophotometric method involves the simultaneous equation method at 232.0 and 291.0 nm over the concentration range of 10µg/ml for both by using 0.1M NaOH as solvent. The method was validated for linearity, precision, sensitivity, and specificity. The calibration curves were linear over the range of 2-10 µg/ml for both NAP and PAN, with significant high value of correlation coefficient (>0.995 for both drugs). The percentage recovery value for NAP was 100.03% and for PAN was 100.2%[11].

Adel M. Michael *et al. (*2012) created a straightforward and accurate technique for the simultaneous detection of naproxen (NAP) and sumatriptan (SUM) in human plasma and dose forms by means of LC/MS and internal standardization. A binary mobile phase containing water and acetonitrile at a concentration of 0.05% acetic acid was used to separate the medicines isocratic ally on a C18 column. The method was confirmed to work for SUM within a linearity range of 10-900 ng/mL and for NAP within a range of 0.1-10 µg/ml. The limit of quantification (LOQ) for SUM was 10 ng/mL, whereas NAPs was 100 ng/ml. Following a twofold liquid-liquid extraction (LLE), the suggested approach was applied to the plasma to accurately determine SUM and NAP. Both pharmacological dosage forms of the medicines were accurately determined using the assay, with no interference from tablet excipients. When comparing the results obtained by the suggested approach to those of a reported method, no significant difference was found statistically [12].

Tang Yao ***et al*. (2012)** designed an RP-HPLC technique for sitagliptin phosphate determination. Using a flow rate of 1.0 mL•min-1 and a UV detection wavelength of 268 nm, the mobile phase was a methanol-0.1% perchloric acid solution (32:68). The column used was the Symmetry C18, which had dimensions of 150 mm×4.6 mm and a particle size of 5 µm. The results showed that from 0.5 to 500 µg•mL-1, the calibration curve was regular (r=0.9996);the lowest limit determination was 1µ g(S/N=3). the chromatographic peaks of sitagliptin phosphate and its related substances were well isolated. Conclusion: The method is simple, selective, accurate and reproducible. It is suitable for determination of the content of sitagliptin phosphate [23].

C. Bala Sekaran *et al*. (2012), for the purpose of determining sitagliptin phosphate in pharmaceutical formulations and bulk, a straightforward, sensitive, and

repeatable spectrophotometric approach was established. The proposed technique is based on condensation of the primary amino group of sitagliptin phosphate with acetyl acetone and formaldehyde producing a yellow-colored product, which is measured spectrophotometrically at 430nm. The color was stable for about 1 hour. “Beer's law” is obeyed over a concentration variety of 5-25 µg/ml. The apparent molar absorptivity and Sandell sensitivity standards are 1.067×10^4 $Lmol^{-1}cm^{-1}$ and 0.0471 μgcm^{-} respectively. All the variables were studied to optimize the reaction conditions [26]

Ravanello *et al.* (2013*),* detailsthe procedure for testing the tablet form of sitagliptin phosphate, a medicine that has just received FDA approval for the management of type 2 diabetic mellitus (DM), using an isocratic LC-UV approach. With a flow rate of 0.8 mL min-1 and UV detection at 267 nm, the procedure makes use of a Phenomenex® C18 column (150mm x 4.6mm I.D., 5µm) with a mobile phase consisting of 0.025 M phosphate buffer pH 6.8: acetonitrile (60:40, v/v). It just took 3.7 minutes for the chromatographic separation to be achieved. Within the 25-75 µg. ml-1 range, the technique showed linearity (r= 0.9999). The method's specificity and stability-indicating capability were proved through force degradation studies, being the peak purity evaluated by PDA detectors. Sitagliptin phosphate was exposed to oxidative, hydrolytic and photolytic stress conditions, and no interference from degradation products was observed. The method has shown good and consistent recoveries (mean value 100.0%) with low intra and inter-day relative standard deviation (RSD). In robustness study, it was observed that peak areas were unaffected by small changes in critical factors. The validated technique can be effectively applied to conclude sitagliptin in tablets and in stability studies [24]

Caglar *et al.* *(*2013*),* developed two novel, simple and rapid stability-indicating spectrofluorimetrictechniqueshave been urbanized for the purpose of Sitagliptin in tablets and spiked serum samples. In the first method, Sitagliptin`s natural fluorescence was measured at 353 nm after **excitation** at 259 nm. On the basis of the reaction between Sitagliptin and fluoresceine the second method has been developed in borate buffer solution of pH 9.0 and the fluorescence intensities of the derivatives were measured at 475 nm emission and 390 nm excitation wavelengths. The calibration curves were constructed in concentration range of 0.5-10.4 µg mL-1 and 0.4-1.8µg mL-1 for the first and second method, respectively. The developed methods were validated with respect to linearity, precision, sensitivity, accuracy and selectivity [27].

Kumar SA.*et al. (*2013*),* The chromatographic separation was achieved using a Symmetry C18 (4.6 x 150mm)mobile phase consist of a mixture of phosphate buffer (pH 3) and Acetonitrile [60: 40]. The flow rate of mobile phase was maintained 1.0 mL per minute. The wavelength chosen for detection was 285 nm. The retention times of Esomeprazole and Naproxen peaks were around 2.105 and 3.555 mins respectively. The Accuracy was calculated for 50%, 100% and 150% and the % recovery was found to be 98.0%-100.4%[13].

N.Monila *et.al* (2014), founded the two visible spectrophotometric techniques (A & B) have been established for the determination of Sitagliptin Phosphate (SGP) in pharmaceutical formulations and bulk. These methods are simple, accurate, sensitive, and reproducible. The suggested techniques rely on the drug's complexation with Bromo Thymol Blue (BTB-Method A) and Bromo Cresol Green (BCG-Method B), which show absorbance maxima at 412 nm and 419 nm, respectively, after being extracted with chloroform. In the concentration range of 25-125 µg/ml and 10-50 µg/ml, respectively, Beer's law is followed. Recovery experiments and statistical validation of the two approaches' results were conducted. All the variables were studied to optimize the reaction conditions. No interference was observed in the presence of common pharmaceutical excipients. The validity of the methods was tested by analyzing the drug in its pharmaceutical preparations. Good recoveries were obtained. The developed methods employed were successful for the purpose of Sitagliptin Phosphate in various pharmaceutical arrangements [25].

Yilmaz B. *et al. (*2014), Naproxen and internal standard ibuprofen were extracted from plasma using a liquid-liquid **extraction** method. Derivatization was carried out using N-methyl-N-(trimethylsilyl)trifluoroacetamide. The calibration curve was linear between the concentration range of 0.10-5.0 µg/mL. Intra- and interday precision values for naproxen in plasma were <5.14, and accuracy (relative error) was better than 4.67%[14].

Maheshwara Reddy *et al.* (2020*),* RP-HPLC Method for the Estimation of Pemetrexed Assay in Formulations Pemetrexed, Formulations RP-HPLC method development and validation A simple RP-**HPLC** method was developed and validated for the determination of Pemetrexed in pharmaceutical dosage forms. Mobile phase

composed of buffer (1.0ml of orthophosphoric acid into 1000 ml of Milli-Q water) and acetonitrile in the ratio of 85: 15 v/v, Inertsil C18 (150×4.6)[58].

Vitthal M *et al.* (2020*),* RP-**HPLC** Method Validation for Quantitative Analysis of Pemetrexed A simple, sensitive, and reliable method has been developed for quantitative analysis of an Anti-neoplastic drug Pemetrexed using reversed phase high performance liquid chromatography (RP-HPLC). This method is developed and validated as per the International Council on Harmonisation (ICH) guidelinesQ2(R1). The effective chromatographic separations were achieved on Zorbax SB C8, (4.6 x 150mm) 3.5µ column with isocratic elution programme. The mobile phase is as homogeneous mixture of 0.17% glacial acetate acid with 5.3 pH and acetonitrile in the ratio of 89:11. The delivered flow rate of mobile phase is 2.0 mL/min using UV/PDA detector. The column oven temperature is 30°C and the injection volume is 20.0µL. This method is linear with correlation coefficient being 0.99992 and %Y intercept of 0.16. The repeatability and intermediate precision are evaluated and %RSD for these assays is 0.09 and 0.28, respectively, with overall % RSD of 0.22. Robustness studies do not show significant change for the system suitability criteria like tailing factor, theoretical plates and %RSD. The values of these criteria are well within acceptance limit. The degradation of Pemetrexed under various stress condition is studied and all known as well as degradants impurities are well separated from the PDH peak. This RP-HPLC method is precise, accurate, and rapid; it also qualifies all the criteria of linearity, stability as well as robustness [59].

S. Hemchand *et al.* (2020*),* A new RP-HPLC method for the determination of pemetrexed is used for the treatment of malignant pleural mesothelioma and lung cancer. In the present study a **simple** RP-HPLC method was developed and validated for the determination of Pemetrexed. The process related substances such as Dimer-1 impurity, Dimer-2 impurity, N-Methyl Pemetrexed, Pemetrexed diethyl ester, Alanine derivative of Pemetrexed, DMF derivative of Pemetrexed, Acid intermediate, Oxidation impurity and D-isomer were separated on gradient mode and quantified. Forced degradation studies were performed to prove the specificity. Hypersil BDS C18 100 x 4.6mm, 3µm was used for the separation (at 27°C) with mobile phase mixture consisting of **(**0.02M sodium dihydrogen phosphate with 0.1% HCOOH and pH 3.8 with dilute sodium hydroxide): Acetonitrile (40:60 v/v) (pH 3.8) with a flow rate of 1.2 mL/min. Methanol: water (1:1) was used as diluent and the eluted compounds were

monitored at 240 nm. 0.5-1500 µg/mL with linear regression equation y = 20588x - 9294.1 (R^2=0.9999). The degradation products observed during the forced degradation studies were well resolved from the drug peak and proving that the method is a stability-indicating method. The method was validated as per ICH guidelines [60].

P T S R K Prasada *et al.* (2021*),* A Study of Method Development and Validation for Simultaneous Estimation of **Pemetrexed** and Cisplatin Using RP-HPLC A novel, simple and accurate high performance liquid chromatographic method has been development with quantitative analysis of Pemetrexed and Cisplatin using Agilent eclipse XDB 150x4.6mm, 3.5µ column with a flow rate of 1ml/min. The buffer containing 1ml of ortho phosphoric acid dissolved in 1 litre of HPLC water, and the mixture of two components like Buffer and Acetonitrile in the ratio of 40:60 is used as mobile phase. The detection was carried out at 265nm. The proposed method shows good linearity in the concentration range from 50µg/ml to 750µg/ml for Pemetrexed and 1 µg/ml to 15 µg/ml of Cisplatin. Precision and recovery study results are in between 98-102%. In entire robustness conditions % RSD is below 2.0%. Degradation has minimum effect in stress condition and solutions are stable for 24hrs. Method validation is carried out according to ICH guidelines and the parameters are precision, accuracy, specificity, stability, robustness, linearity, limit of detection and limit of quantification are evaluated, and the values are found to be within the acceptable limit [57].

Kiran K. Katare *et al.* (2022*),* Development and Validation of RP-HPLC Method for the Simultaneous Determination of Cisplatin The reverse phase high performance liquid chromatography (RP-HPLC) technology was used to produce a simple, predictable, and accurate method for estimating Cisplatin, The following chromatographic conditions were used: phase of inactivity C18, 150 mm x 4.6 mm, 3.5 m., Waters symmetry Adjust the pH to 2.5 using ortho phosphoric acid and a 2.5 mL buffer of Hexane Sulphonic Acid in 1 L of water: The diluent was mobile phase, with acetonitrile (40:60v/v) and a flow rate of 1.0-mL/min. The detection wavelength was set at 232 nm, the column temperature was set to ambient, and the detection wavelength was set at 232 nm. As an optimized procedure, the conditions were finalized. By injecting the standard five times, system suitability parameters were investigated, and the results were considerably below the acceptance criteria. Between 10% and 15% levels, a linearity analysis was conducted, and the R2 value was found to be 0.999. For

repeatability, precision was found to be 0.2, 0.24, and 1.79, and for intermediate precision, 0.47, 0.32, and 1.73. LOQs are 0.001, 0.5, and 0.15 g/mL, as well as 0.01, 5, and 1.5 g/mL. Using the foregoing procedure, an analysis of marketed formulation revealed that 100.02, 100.4, and 100.6% were present. Cisplatin, have all been studied. The purity threshold was greater than the purity angle in all cases and within the permitted range[56].

Narmada Valla Keerthi *et al. (2023),* RP-HPLC Method Development, Validation and Forced Degradation Studies for the Estimation of Pemetrexed in API and Pharmaceutical Dosage Form A simple, precise, and stability-indicating reversed-phase high-performance liquid **chromatography** (RP-HPLC) method was developed and validated for the estimation of Pemetrexed in both active pharmaceutical ingredient (API) and its pharmaceutical dosage forms. Chromatographic separation was achieved using a C18 column with an appropriate mobile phase composition, providing sharp and well-resolved peaks with suitable retention time. The method was validated according to ICH guidelines, demonstrating excellent linearity, accuracy, precision, specificity, and robustness. Forced degradation studies were performed under acidic, basic, oxidative, thermal, and photolytic conditions to evaluate the stability of Pemetrexed and to confirm the method's stability-indicating capability. The method effectively separated Pemetrexed from its degradation products, confirming its suitability for routine quality control analysis, stability studies, and pharmaceutical formulation assessment. This validated RP-HPLC method provides a reliable, rapid, and reproducible approach for the quantitative determination of Pemetrexed in API and dosage forms.[55]

Vinay Kumar Bondu *et al.* (2025), Simultaneous Estimation of Gemcitabine and Cisplatin Using RP-HPLC: Method Development and Validation This study presents the development and validation of a reverse-phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of Gemcitabine and Cisplatin in **pharmaceutical** formulations. The research addresses the growing need for reliable analytical techniques to monitor these widely used chemotherapeutic agents. Using a Thermosil C18 column (4.0 × 125 mm, 5 µm) and a mobile phase of Methanol and Sodium Acetate buffer (70:30% v/v) at pH 3.0, the method achieved efficient separation with detection at 252 nm. The flow rate was maintained at 0.7 mL/min, ensuring optimal peak resolution within a run time of 10 minutes. Validation

parameters, including specificity, linearity, accuracy, precision, robustness, and limits of detection and quantification, were thoroughly evaluated per ICH guidelines. The findings indicate that this RP-HPLC method is reliable, reproducible, and suitable for routine quality control and pharmacokinetic studies.

Chapter - 3

RESEARCH METHODOLOGY

Introduction

Analytical chemistry may be interpreted as the systematic characterization of material composition, bridging the gap between theoretical science and empirical application. This field centers on the identification of the specific elements or compounds that comprise a substance. Scholarly tradition distinguishes between two core analytical frameworks: the **qualitative** framework, which focuses on the detection and identification of analytes, and the **quantitative** framework, which provides a metric-based assessment of the relative abundance of those analytes within a given specimen [1].

3.1 Bioanalytical Method Development

Over the past quarter-century, the discipline of **pharmacokinetics** has become an indispensable component of the drug development lifecycle, particularly regarding the representation of a drug's biological profile. For non-intravenous delivery systems constructed for systemic therapy, the paramount objective is the efficient transport of the active constituent into the systemic circulation at concentrations necessary to elicit the intended therapeutic outcome. This fundamental characteristic of a dosage form is recognized as **physiologic availability**, **biologic availability**, or, most commonly, **bioavailability**.

In technical terms, bioavailability quantifies both the velocity (rate) and magnitude (extent) at which an active moiety is absorbed from a pharmaceutical product and reaches the designated site of action. Scholarly distinction is made between **absolute bioavailability**, which utilizes intravenous administration as the 100% baseline for comparison, and **relative bioavailability**, which evaluates absorption against a standardized reference product. Furthermore, **bioequivalence** is established when no statistically significant variance is observed in the rate and extent of absorption between pharmaceutical equivalents or alternatives, provided they are administered at identical molar dosages under controlled experimental conditions [13].

1. Clinical trials
2. Bioanalysis
3. Biostatistics

Bioanalytical Sample Analysis is divided into three major processes:

1. Development of Method
2. Validation of Methods

An optimized strategy for analytical method development focuses on procedural parsimony, necessitating only the minimum number of experimental iterations required to attain the targeted analytical objectives. The overarching goal is the creation of a streamlined methodology that integrates modern computational tools, such as molecular modeling or algorithmic optimization, to simplify complex workflows.

These developmental processes, followed by rigorous validation, are fundamental to the pharmaceutical lifecycle—from initial discovery to large-scale manufacturing. The resulting standardized protocols provide quality control laboratories with the requisite framework to verify the **identity, purity, potency, and functional performance** of therapeutic agents. To maintain global safety and quality benchmarks, international jurisdictions—including the United States, Japan, and the European Union—utilize official pharmacopoeias. For instance, the analytical standards codified in the **United States Pharmacopeia (USP)** carry legal authority under the Federal Food, Drug, and Cosmetic Act, with further validation requirements mandated by the **Code of Federal Regulations (CFR)**.

The pharmaceutical landscape is currently characterized by an accelerated influx of novel molecular entities and semi-synthetic structural analogs. However, a pronounced **temporal latency** often exists between the commercial debut of a therapeutic agent and its eventual codification within official pharmacopoeial monographs. This delay is frequently necessitated by the requirement for longitudinal data regarding widespread clinical utility, emergent toxicological profiles, or the development of antimicrobial resistance. In the absence of such standardized compendial benchmarks, the responsibility shifts to the analytical chemist to engineer and validate bespoke methodologies capable of ensuring product quality during this interim period.

3.2 Fundamental requirements for the creation of novel methodologies in pharmaceutical analysis

1. The pharmaceutical formulation or medication combination may not be recognized in any pharmacopoeia.
2. Literature may lack a suitable analysis method for the medication due to patent restrictions.
3. The medication formulation may be unsuitable for analytical techniques due to interference from excipients.
4. Analytical methods for quantifying the medicine in biological fluids may be unavailable.
5. Analytical techniques for a medicine in conjunction with another drugs may be unavailable.

Current analytical procedures may require costly chemicals and solvents. It may be linked to laborious extraction as well as separation methods that may not have reliability [15].

3.3 Steps involved in Method Development

1. Compound information / Literature search
2. Spectrometric analysis of analytes
3. Sample preparation process
4. Choice of LC-MS settings
   - column
   - mobile phase composition
   - flow rate
   - temperature
5. Final LC-MS parameter optimization

3.4 Optimization of LC-MS Method

During the method optimization sequence, the initial experimental conditions are subjected to iterative improvements to attain peak analytical performance. This involves the optimization of **asymmetry** and **capacity factors** to provide well-defined, Gaussian peak shapes and adequate retention. Additionally, maximizing the number of

plates (theoretical) ensures heightened efficiency and resolution. The final output of this stage is a validated set of conditions that offers an idealized balance of elution time and sensitivity (LOD/LOQ), thereby facilitating the accurate and reproducible determination of the analyte of interest [16].

The various parameters that include to be optimized during method development are:

- Choice of separation mode
- Choice of stationary phase
- Selection of mobile phase
- Optimizing system settings for quantifying the analyte.
- Development of standard curve and QC standards
- Selection of internal standard
- Ion mode selection
- Choice of extraction procedure

3.5 Selection of mode of separation

Reversed-phase HPLC is predominantly favored for characterizing polar to moderately polar chemical entities because of the solvophobic interactions between the polar eluent and the non-polar column matrix. The choice of ionization source in mass spectrometry is equally critical and must be aligned with the compound's functional groups.

The detection system comprised a triple quadrupole mass spectrometer employing **Electrospray Ionization (ESI)** in both positive and negative manifolds. The integration of a turbo ion source allows for rapid mode-switching, which optimizes system productivity. In this analytical setup, **Multiple Reaction Monitoring (MRM)** facilitated highly selective MS/MS detection. Because the compound of interest possesses basic functional groups, the positive ionization mode was prioritized to maximize ion yield, contrasting with acidic drugs which would necessitate a negative polarity for detection [17].

3.6 Selection of stationary phase

The optimization of eluent additives represents a strategic balance between chromatographic peak shape and mass spectrometric response. By minimizing additive

loading, researchers can achieve a marked increase in sensitivity, which is vital for the identification of low-abundance drug derivatives.

Central to this optimization is the selection of an appropriate HPLC column. The architecture of the bonded phase and the inertness of the silica support are primary determinants of the additive levels required to neutralize silanol activity. A superior column matrix reduces the reliance on high-concentration buffers or ion-pairing agents, ensuring that basic drugs exhibit optimal peak shape without compromising the ionization efficiency essential for trace-level quantification [17].

3.7 Column and Column-packing materials:

The efficiency of a chromatographic separation is inherently linked to the theoretical plates provided by the column's packing material. Silica's dominance as a support material because of its robust siloxane network and the versatility of its surface silanol groups. These groups can be chemically transformed into non-polar surfaces suitable for water-organic mobile phases by attaching hydrocarbon chains (C_2 through C_{22}).

Beyond hydrophobic interactions, silica supports can be tailored for specialized separations by incorporating polar organic compounds or strong ion-exchangers onto the surface. The manufacturing process frequently includes an **end-capping** step to saturate accessible silanols, thereby lowering peak tailing for alkali analytes. Users must remain cognizant of the chemical stability of these phases; the siloxane linkages are susceptible to acid-catalyzed hydrolysis at low pH and base-induced dissolution at high pH, necessitating an operational window of pH 2 to 8 [18].

Liquid chromatography is fundamentally categorized into two primary modalities: normal-phase and reversed-phase. In **normal-phase chromatography**, the separation of lipophilic and moderately polar pharmaceutical entities is accomplished through interactions with a polar stationary phase. While historical perspectives attributed these separations solely to differential adsorption on the silica surface, contemporary models suggest a complex interplay between adsorption and partition mechanisms, involving both the intrinsic silanol groups and the hydration layer of bound water molecules.

Conversely, **reversed-phase chromatography (RPC)** utilizes a polar eluent—comprising aqueous buffers and organic modifiers like acetonitrile or methanol—partitioned against a hydrophobic stationary phase. The versatility of RPC is enhanced by the availability of diverse bonded phases, such as C_{18}, C_8, phenyl, and cyano moieties. To optimize the retention of acidic or alkali analytes, the eluent pH is meticulously adjusted to suppress ionization, thereby favoring hydrophobic interactions with the column matrix. For species that remain persistently ionized, **ion-pair chromatography** is implemented to facilitate the formation of neutral complexes with enhanced lipophilicity [19].

Modern drug development demands an analytical framework capable of achieving extreme precision and selectivity in the presence of endogenous biological interference. While traditional HPLC systems equipped with optical detectors remain functional, the field has moved toward high-fidelity mass spectrometric platforms, including **LC-MS/MS, GC-MS/MS,** and **CE-MS**.

The transition to **LC-MS/MS** utilizing triple quadrupole technology represents a significant advancement in bioanalytical capability. By employing atmospheric pressure ionization interfaces (ESI or APCI), this technique facilitates the robust quantification of trace-level analytes in fluids like serum and urine. The widespread adoption of the LC-MS/MS platform is a testament to its ability to satisfy stringent regulatory validation requirements while providing the high throughput necessary for pharmacokinetic and dynamic modeling.

The discipline of bioanalysis serves as a quantitative bridge in drug development, facilitating the measurement of active moieties and metabolic products. This data is indispensable for establishing the **pharmacokinetic (PK)** profiles and **toxico kinetic** thresholds required for regulatory approval and bioequivalence testing. The versatility of these techniques also permits application in forensic science, environmental health, and the enforcement of anti-doping policies.

Within clinical settings, bio-analysis is a prerequisite for studying **drug-drug interactions**, particularly in the context of **multidrug therapy**. These interactions represent a significant clinical challenge, as the presence of one agent may modulate the systemic concentration or metabolic pathway of another, leading to therapeutic failure or heightened toxicity. Whether resulting from clinical oversight or complex

chemical interference, these interactions are best elucidated through rigorous bioanalytical monitoring, which identifies the shifts in pharmacokinetic parameters that occur when drugs are administered in combination [20].

3.8 Bio analytical method development using LCMS/MS

The architecture of a bioanalytical method is bifurcated into **pre-analytical processing** and **instrumental analysis**. Sample preparation serves as a vital purification step, designed to eliminate endogenous interferences and minimize the deleterious impact of the sample matrix on the signal. By concentrating on the sample during this phase, researchers can significantly bolster the method's sensitivity. This is followed by the strategic configuration of **chromatographic separation and mass spectrometric detection**, where mobile phase constituents, stationary phase selection, and ionization parameters are refined to achieve high-fidelity data [21].

The different sample preparation processes are

5. Proteins precipitation
6. Liquid-Liquid Extraction
7. SPE (Solid Phase Extraction)
8. Extraction hybrid

The present thesis focuses on the systematic development and validation of new analytical methods employing UV–visible spectrophotometry, High-Performance Liquid Chromatography (HPLC), LC–MS-based bioanalytical techniques and RP-HPLC method for selected therapeutic agents. Six drugs—Lamivudine (LAM), Sitagliptin (SITA), Naproxen (NAP), Pemetrexed, Cisplatin and Duloxetine (DUL)—were chosen as representative candidates requiring robust analytical solutions. Method development was carried out in accordance with established scientific principles, and the resulting procedures were subjected to comprehensive validation covering specificity, linearity, precision, accuracy, sensitivity, and robustness, following USFDA regulatory guidelines. The thesis beginning with a detailed conceptual overview of analytical method development and validation parameters. Subsequent chapters

present the experimental design, results, interpretation, and conclusions for each selected drug using UV, HPLC, RP-HPLC methodand LC-MS platforms.

Detail of method development according to drug is as under:

Table 3.1: Drug and Corresponding bio-analytical method

Sr. No	Chapter No	Drug and Corresponding bio-analytical method
1	Chapter- 4	Method development and validation of Lamivudine by UV Spectrometric Method
2	Chapter -5	Method development and validation of Sitagliptin by HPLC drug profile
3	Chapter -6	Bio-analytical method development and validation of Naproxen
4	Chapter- 7	Bio-analytical method development of Duloexitine
5	Chapter -8	Method Development And Validation Of Rp-Hplc Method For The Simultaneous Estimation Of Pemetrexed And Cisplatin

Chapter - 4

METHOD DEVELOPMENT AND VALIDATION OF LAMIVUDINE BY UV SPECTROMETRIC METHOD

4.1 DRUG PROFILE

LAMIVUDINE [1]

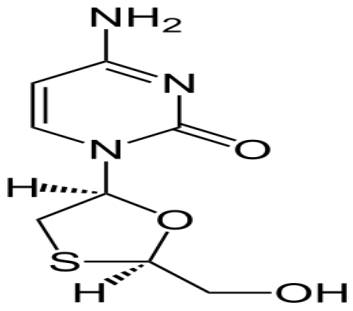
(a) IUPAC name:

4-amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one

(b) Category: Anti-HIV Agents

Nucleoside and Nucleotide Reverse Transcriptase Inhibitors

(c) Molecular Structure



(d) Molecular Formula: $C_8H_{11}N_3O_3S$

(e) Molecular Mass: 229.3gm/mol

(f) Description: Lamivudine is an off-white to white crystalline substance

(g) Use: Used as part of extremely dynamic antiretroviral therapy (HAART) for the management of HIV type 1.

(h) Bioavailability: 86%

(i) Half- life: 5-7hrs

(j) Solubility: Solubility at about 70 mg/mL at 20°C in water.

(k) Route: Oral route

(l) Mode of action

The synthetic nucleoside derivative lamivudine undergoes intracellular phosphorylation to produce lamivudine triphosphate (L-TP), its active 5'-triphosphate metabolite. The HIV reverse transcriptase and HBV polymerase incorporate this nucleoside analogue into the viral DNA, which causes the DNA chain to terminate [2].

(m) Adverse reactions

a. Common symptoms: headache, nausea, malaise, fatigue, nasal signs and symptoms, diarrhea and cough.
b. Lactic acidosis and severe hepatomegaly beside steatosis
c. Hepatic decompensation in patientsalso infected with HIV-1 and Hepatitis C
d. Pancreatitis

(n) Drug interactions

Lamivudine is mostly excreted in the urine by active organic cationic secretion. Interactions with concurrently given medicines must be evaluated, especially when their primary elimination pathway involves active renal secretion through the organic cationic transport system (e.g., trimethoprim). No data are known about interactions with other medicines that possess renal clearance processes analogous to those of lamivudine [3].

4.2 Dose: For adults with HIV (or children over 12), the dose is 300mg once daily, or 150mg twice a day. Lamivudine is never used on its own in the therapy of HIV.

For the treatment of adults with hepatitis B, the dose is 100mg daily. If co-infected with HIV, then the dose is as for HIV. For a child 3 months to 12 years old, about 1.4-2 mg per lb. of body weight twice a day, no more than 150 mg per dose [4,5].

For a child 3 months to 12 years old, about 1.4-2 mg per lb. of body weight twice a day, no more than 150 mg per dose.

4.3. Objectives of the work

- To contribute the growth of analytical methods for a straightforward, accurate and Cost-efficient estimation of the drug in pharmaceutical bulk form.
- To develop a new method for lamivudine by UV Spectrophotometric method toestimate the drug in dosage form.
- The available procedures for the estimation of HPLC, GC and HPTLC which are costly.
- The present development was done on UV Spectrophotometric method.
- Selection of the solvent to be used based on solubility of the drug.
- Choosing the suitable solvent in which the medication is soluble and stable. The solventsmust be easily available and economical.
- Selection of method for quantitative analysis.
- Determination of workingconcentration range.
- Validation of developed technique as per ICH strategy. The performance of precision and recovery studies to check the reliability and repeatability of the
- Proposed method.

4.4 Methodology

4.4.1 Instruments

A double-beam Lab India UV/Vis spectrophotometer, model UV 3092, connected to computer loaded with UV Win 5.2.0.110 software. For the measurement of pH, Lab India P^H/CON Meter was used. For weighing, Denver Instrument, SI-234 balance was used.

4.4.2. Materials &Reagents

(a) Cerium (IV) ammonium sulphate

0.1% solution, was prepared in 4 M perchloricacid, Assigned purity, 98.9% (Loba Chemie laboratory reagents & fine chemicals, India)

(b) Perchloric acid (4M)

4M perchloric acid was prepared by adding 33.3 ml of perchloric acid (12M) to 100mlvolumetric flask and further diluting to 100 ml through distilled water.

(c) Standard Stock Solution

Into a 100 ml calibrated flask, 10 mg drug was weighed accurately and dissolved in 2 ml methanol, diluted with distilled water up to the mark and diluted quantitatively to get hold of the suitable concentrations containing 2-18 μg ml^{-1}.

(d) Sample Preparation

The content of ten correctly weighed tablets, which is equal to thirty milligram's of lamivudine, was ground and combined completely. Weighing 100 milligrams of the active ingredient's powder into a 100-milliliter volumetric flask, we added 2 milliliters of methanol, that was subsequently diluted by 50 milliliters of distilled water, and shaken vigorously for approximately 20 minutes. Filtered with What man filter paper, the volume was then filled up with D.W. to the specified level. A concentration of 0.1 mg ml^{-1} of the medication was achieved by progressively diluting the filtrate.

4.4.3 Construction of Calibration Curve

λ_{max} of lamivudine was determined by scanning the drug solution and it was established to be at 280 nm. To construct Beer's plot for lamivudine dilutions were made using stock solution. Lamivudine followed linearity within the concentration variety 2-18 μg ml^{-1}. Theλ_{max} of the method are shown in Fig.4.1. Overlay spectrum of lamivudine at 280nm (2-18 mcg/ml) shown in Fig 4.3**.**

4.4.4 Analytical Procedures

An aliquot volume of the standard stock solution of medicines at concentrations 2–18 μg ml^{-1} was relocated to 10 ml volumetric flasks. To each flask 1 milliliter of 4 M perchloric acid was added followed by 1.0 ml of CeIV solution, stirred well and made up to volume with distilled water. The absorbance of the resultant solutions was measured at 280 nm after 5 min at 25.5 °C. The quantity of Lamivudine in the sample solution was established from its calibration curve.

4.4.5. Analytical method validation

Linearity

Linearity of the proposed technique was established by preparing and evaluating five different series of drug solutions (2-18 μg ml^{-1} in 4M Perchloric acid) from the stock solutions. Least square regression computation (table 4.2) [12,13] is used to process the acquired data.

Accuracy

To determine how near the measured value is to the sample's true value is a measure of the method's accuracy. Various concentrations of the drug were tested to ascertain the precision of the suggested technique. – lower concentration (LC80%), intermediate concentration (IC, 100%) and higher concentration (HC, 120%) were organized from independent stock solutions and analyze. Accuracy was assessed as the percentage relative error and mean % recovery (Table 4.3). To provide an additional support to the accuracy of the developed assay method, a standard addition method was employed, which involved the addition of different concentrations of pure drug to a known reanalyzed formulation sample and the total concentration was determined using the proposed methods. The % recovery of the added pure drug was calculated as % Recovery = $[(C_t–C_s)/C_a] \times 100$,

C_t is the total drug concentration measured after standard addition;

C_s drug concentration in the formulation sample;

C_a, drug concentration added to formulation

As a part of determining accuracy of the proposed method, drug concentrations were arranged from independent stock result and analyze (N = 9). Accuracy was assessed as mean percentage recovery [13].

Precision

Repeatability was assessed by analyzing different level of concentrations obtained from pure drug stock solution (N = 9) (Table 4.1). Intermediate accuracy was determined by taking into account intra- and inter-day fluctuation in response [14]. Respective concentrations from stock solution in triplicates were made three separate times in a day and investigated for intra-day and inter-day fluctuation (N= 27).

Precision was expressed as relative standard deviation (%R.S.D.) of the calculated concentrations based on regression equation (Table 4.4).

Detection limit (DL) and quantitation limit (QL)

The DL and QL of Lamivudine using proposed approach was determined by calibration standards [14-16]. DL and QL were estimated as 3.3σ/S and 10σ/S correspondingly, where σ is the S.D> of y-intercept of regression equation and S means slope of calibration curve (Table 4.5).

Robustness

The absorbance was not influenced by a variation of pH of the selected medium by ± 0.1. In the selected medium the mean %recovery was determined to be 101.24. The mean % recovery was calculated (Table 4.6).

4.4.6 Estimation from tablet formulations

20 tablets were broken up into a powder equivalent to 10 mg lamivudine. The powder was mixed with extraction medium and put into a bath sonicator for 30 minutes. After passing such solutions through What man filter paper 1, the filtrate was properly diluted to 10 μg ml^{-1} concentration and the samples were tested using described analytical technique [15] (Table no.4.7).

4.5 Results and discussion

Cerium IV ammonium sulphates are strong oxidizing agents and are utilized extensively for the determination of organic compounds. Beer's law is obeyed in the variety of 2–18 μg ml^{-1}. lamivudine on oxidation with Ce (IV) in acidic medium had shown a maximum absorption at 280 nm The λ_{max} of lamivudine was found to be 280 nm [17-20]. Absorption spectra of lamivudine at 0 time and 48 h time are overlaying in medium (Fig. 4.1). Statistical parameters were shown in Table 4.1.

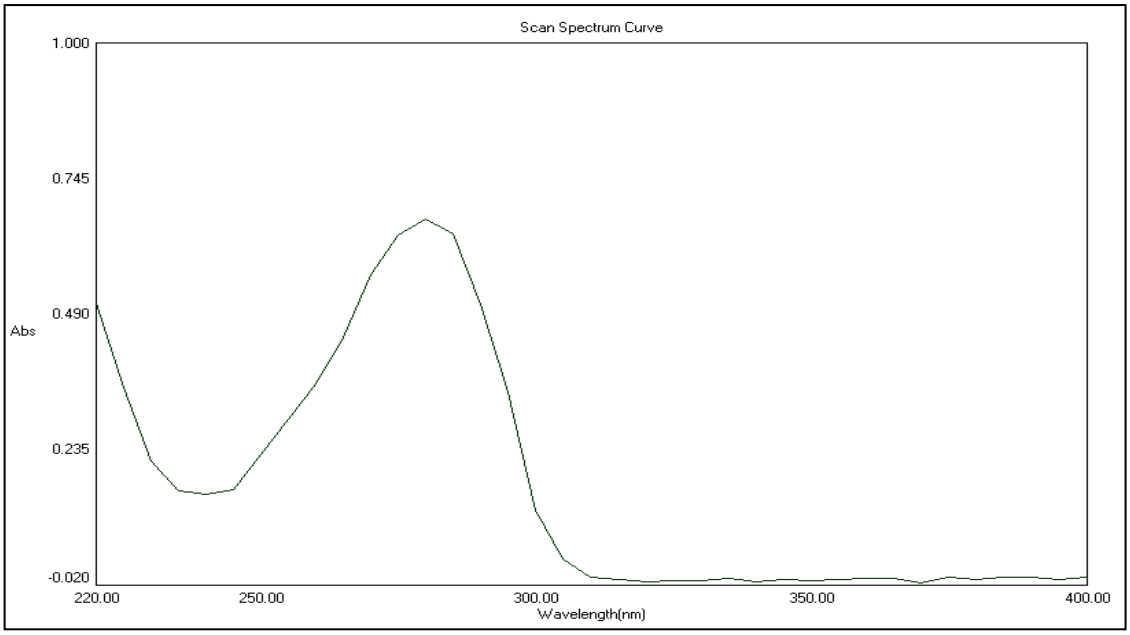


Fig. 4.1. Absorption spectrum of lamivudine

Table4.1. Optical Characteristics and Regression Equation of lamivudine

Parameter	Values
Color	Colorless
λ_{max}(nm)	280
Beer's law range (µg ml–1)	2-18
Limit of detection(µg ml–1)	0.0033
Limit of quantification(µg ml–1)	0.10043
Correlation coefficient, R	0.999
Slope b	0.048
Intercept a	0.068

4.5.1 Calibration Curve

The linear regression equation was absorbance at 280 nm, absorbance =[0.048 × concentration in µg ml^{-1}] + 0.068; with a regression coefficient of 0.999 [16] (Fig 4.2).

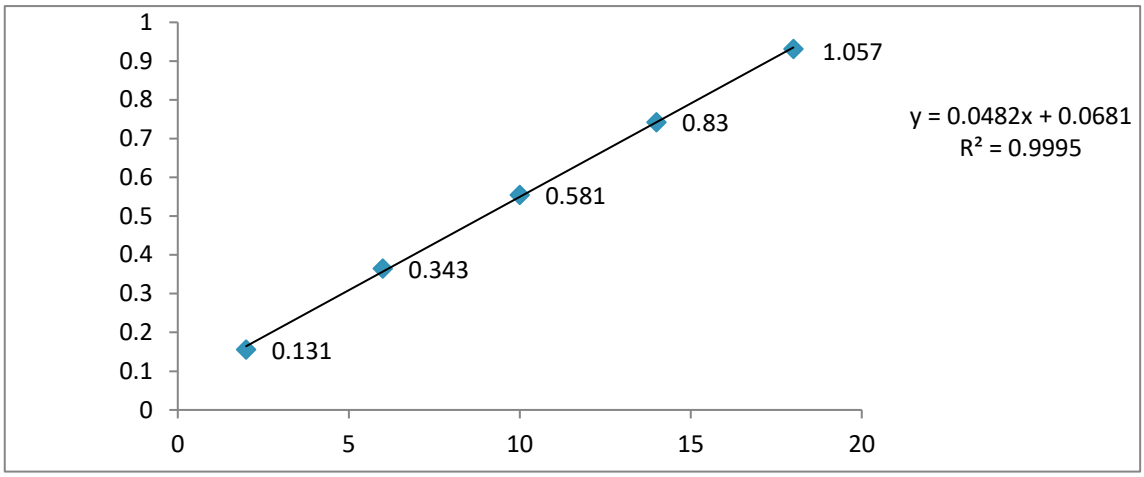


Fig-4.2: Calibration curve of lamivudine

4.6 Analytical validation

4.6.1 Linearity

The range of linearity was observed to be 2-18 mug ml^{-1} at 280 nm in the chosen medium. Lower values of statistical parameters such as standard error of slope and intercept (Table 4.2) indicated good precision of the suggested technique. Furthermore, the mean values of slope and intercept are inside the 95% confidence interval (Fig. 4.3). The goodness of fit of linear regression equation was supported by low calculated dF-value and high regression coefficient value.

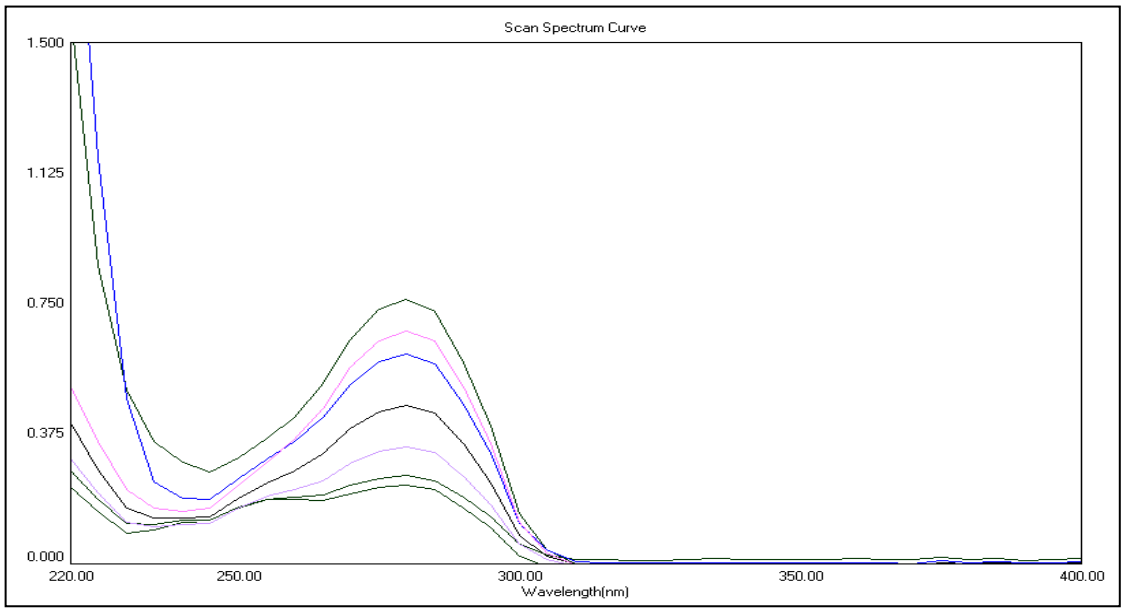


Fig 4.3. Overlay spectrum of lamivudine at 280nm (2-18 mcg/ml)

Table-4.2 Linearity

Sno	Concentration (mcg/ml)	Absorbance at 280nm
1	2	0.131
2	6	0.343
3	10	0581
4	14	0.83
5	18	1.057

4.6.2 Accuracy

To assess the accuracy of the proposed approach, we generated and evaluated several concentrations of the drug; low concentration (LC80%), intermediate concentration (IC, 100%) and high concentration (HC, 120%) from separate stock solutions. Accuracy was expressed as percentage relative error and mean % recovery (Table 4.3). To further confirm the accuracy of the developed assay method, the standard addition technique was emplyed, in that different concentrations of pure drug were put to a known reanalyzed formulation sample and the total concentration was observedemployed the proposed methods [17]. The % recovery of the additional pure drug was calculated as % Recovery = $[(C_t–C_s)/C_a] \times 100$, where

C_t is the total drug concentration measured after standard addition.

C_s drug concentration in the formulation sample.

C_a, drug concentration added to formulation.

For the accuracy assessment of the proposed approach, drug concentrations were created from independent stock solutions and assessed (N=9). Accuracy was determined as the mean % recovery (Table 4.3).

Table-4.3. Accuracy

Level	Predicted Con. (µg/ ml)			Mean %Recovery (±S. D)
	Range	Mean (±S. D)	%R.S. D	
LC	2	0.131±0.05	1.59	99.70±1.32
IC	10	0.581±0.03	1.76	100.34±0.94
HC	18	1.051±0.66	0.65	100.09±0.61

4.6.3 Precision:

The repeatability was checked by the analysis of different levels of concentrations obtained from pure drug stock solution (*N*=9) (Table4.5) The intermediate precision was estimated from inter day and intraday response fluctuations. The respective concentrations from stock solution in triplicates were made three separate times in a day and investigated for intraday and inter-day fluctuation (*N*=27). Precision was expressed as relative standard deviation (%R.S.D.) of the calculated concentrations based on regression equation (Table 4.4).

Table 4.4 Precision data of the developed method

S. No	Concentration (mcg/ml)	Intra-day repeatability % R.S.D(*N* = 9)			Inter-dayrepeatability %R.S.D(*N* = 27)
		Day 1	Day 2	Day 3	
1	4	0.242	0.245	0.247	1.029
2	18	0.477	0.471	0.479	0.875
3	16	1.002	1.005	1.010	0.402

4.6.4 DL and QL

DL and QL originated to be 0.0033 µg $ml^{-1}$and 0.10043µg ml^{-1} in selected medium, respectively (table.4.5)

Table 4 .5 Detection limit (DL) and quantitation limit (QL) data

S. No		Mean of calibration curve(N=9)	SD	DL	QL
1	**Slope**	0.2224	0.00022	0.0033	0.10043
2	**Intercept**	0.06809			
3	**R^2**	0.9947			

4.6.5 Robustness

The absorbance was not influenced by variation of ±0.1 of pH of the selected medium. Mean %recovery was reported 101.24 in the selected medium [21] (Table 4.6).

Table 4.6 Robustness data of the developed method

Sr. No	Concentration (mcg/ml)	Mean Absorbance (*N=9*)	S.D	%Recovery	S.D - %Recovery
1	4	0.245	0.0025	101.24	
2	8	0.476	0.0042	99.79	0.7277
3	16	1.006	0.004	100.40	

4.6.6 Estimation from tablet formulations

After twenty pills were ground to a powder that was 10 mg lamivudine equivalent, the powder was mixed with extraction medium and left in a bath sonicator for 30 minutes. The suggested analytical procedure was used to examine the samples after they had been filtered using Whattman filter paper 1 and appropriately diluted to a concentration of 10 µg ml^{-1}. (Table.4.7).

Table 4.7 Assay of sample concentration

Brand	Drug added (µg/ml)	Drug found (µg/ml)	%Recovery (±SD)	%RSD
Lamivir-HBV	10.0	9.998	99.98 ± 0.0005	0.005774

4.7. Conclusion

The results show that the new UV Spectrophotometric approach is easy to use, cost-effective, and provides accurate estimations of Lamivudine in both bulk and tablet dose forms. For the identification of organic molecules, cerium IV ammonium sulphates are widely used since they are strong oxidizing agents. Very low absorbance within the lamivudine detection ranges All concentrations between 2 and 18 µg ml^{-1} adhere to Beer's law. -Maximum absorption was obtained at 280 nm on oxidation of lamivudine with Ce (IV) in acidic media. The λ_{max} of lamivudine was found to be 280 nm. Hence it can be easily evaluated by UV visible Spectrophotometry and may be concluded as novel accurate, precise and extremely economical method and there was

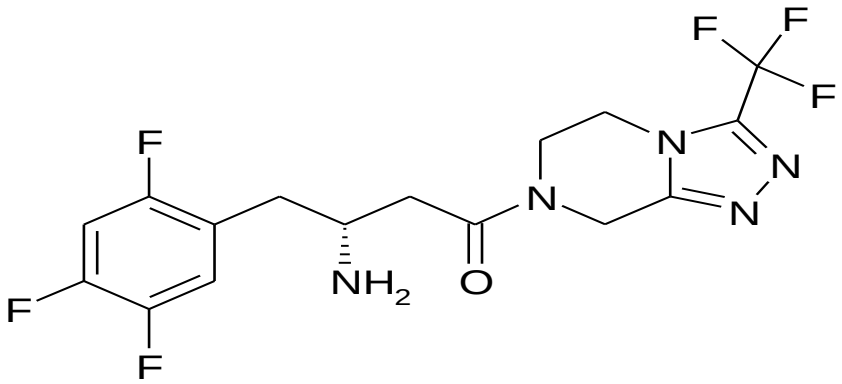
no interference from excipients in tablets. It can also be employed for routine analysis in bulk and tablet dose form.

Chapter - 5

METHOD DEVELOPMENT AND VALIDATION OF SITAGLIPTIN BY HPLC

5.1. SITAGLIPTIN (DRUG PROFILE)

5.1.1 Molecular Structure [1]



Nomenclature	(R)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro [1,2,4] triazolo[4,3-a] pyrazin-7(8H)-yl]-1-(2,4,5-trifluorophenyl) butan-2-amine
Molecular formula	$C_{16}H_{15}F_6N_5O$
Molecular weight	407.314 g / mol
Characteristics	White to off-white powder
Category	Anti-diabetic drug
Solubility	Soluble in methanol and N, N-dimethyl formamide

5.1.2. Mechanism: Sitagliptin works by competitively inhibiting the enzyme dipeptidyl peptidase 4 (DPP-4). This enzyme degrades incretins, hormones that are released by the gut in response to meals. Eleventh GLP-1 and GIP can increase insulin secretion and inhibit glucagon release by the pancreas only after deactivation. This leads to the normalization of blood glucose levels [2-8].

5.1.3. Adverse effects: In clinical trials, side effects were as common with sitagliptin (as monotherapy or in combination with metformin or pioglitazone) as placebo, apart from very rare nausea and common cold-like symptoms. The incidence of hypoglycemia was not significantly different between placebo and sitagliptin. There

have been several postmarketing reports of pancreatitis (some fatal) in people treated with Sitagliptin, and the U.S. package insert carries a warning to this effect, although the causal link between Sitagliptin and pancreatitis has not yet been fully substantiated [9-11].

5.1.4. Protein binding: The fraction of sitagliptin reversibly bound to plasma proteins is low (38%). Metabolism Sitagliptin does not undergo extensive metabolism. In vitro studies indicate that the primary enzyme responsible for the limited metabolism of sitagliptin was CYP3A4 (oxidation), with contribution from CYP2C8 [12-18].

5.1.5. Route of elimination: Approximately 79% of Sitagliptin is excreted unchanged in the urine with metabolism being a minor pathway of elimination. Following administration of an oralsitagliptin dose to healthy subjects, approximately 100% of the administered radioactivity was eliminated in feces (13%) or urine (87%) within one week of dosing [19-23]. Elimination of sitagliptin occurs primarily via renal excretion and involves active tubular secretion.Half-life 12.4 hours Clearance.

5.2. Literature Review

Tang Yao *et al.* (2012)designed an RP-HPLC technique for sitagliptin phosphate determination. The mobile phase was methanol-0.1% perchloric acid solution (32:68) with a flow rate of 1.0 mL•min^{-1} and UV detection wavelength 268 nm.The column used was the Symmetry C18, which had dimensions of 150 mm×4.6 mm and a particle size of 5 μm. The results showed that from 0.5 to 500 μg•mL^{-1}, the calibration curve was regular (r=0.9996);the lowest limit determination was 1μ g(S/N=3). The chromatographic peaks of sitagliptin phosphate and its related substances were well isolated. The method is simple, selective, accurate, and reproducible. It is suitable for determination of the content of sitagliptin phosphate [23].

Ravanelloetet *al.* (2013),describes the procedure for evaluating the tablet form of sitagliptin phosphate, a drug recently approved by the FDA for the management of type 2 diabetic mellitus (DM), by use of an isocratic LC-UV method. The technique employs a flow rate of 0.8 mL min-1 with UV detection at 267 nm. A Phenomenex® C18 column (150mm x 4.6mm I.D., 5μm) is used and the mobile phase is 0.025 M phosphate buffer pH 6.8: acetonitrile (60:40, v/v). The chromatographic separation was completed in only 3.7 min. The approach showed linearity (r= 0.9999) in the range 25-

75 μg. ml^{-1}. The method specificity and stability suggesting capability was shown by forced degradation investigations, as peak purity was verified by PDA detector. No interference of breakdown products was seen after subjecting Sitagliptin phosphate to oxidative, hydrolytic and photolytic stress conditions. The approach presented good and consistent recoveries (mean value 100.0%) and low intra and inter-day relative standard deviation (RSD). In the robustness study it was discovered that the peak area was not changed by small changes of the critical parameters. The described approach was effectively applied for the determination of sitagliptin in tablets and in stability studies [24]

5.3. Methodology

5.3.1 Drugs

- Sitagliptin working standard

5.3.2 List of Instruments Used

- UV-Visible Spectrophotometer
- HPLC-UV detector
- Ultra so nicator
- pH meter
- Electronic balance
- Vacuum filter

5.3.3. Preparation of mobile phase

A 0.5% triethylamine solution was produced by adding 5 mL to 1000 mL of HPLC grade water. The pH of the final solution was brought down to 3.0 ± 0.1 by including 1% o-phosphoric acid. Binary pumps were used in the HPLC studies that contained methanol and pump B containing 0.5% triethylamine [28].

5.3.4 Preparation of stock solution

10mg of drug was taken and was dissolved in 100ml of solvent to the concentration was made up to 100mcg/ml. Further dilutions were fixed from stock solution.

5.3.5. Preparation of sample solution

Take not less than ten tablets, then grind them to a fine powder. Accurately weigh 10 milligrams of sample powder and transfer into a 100-milliliter volumetric flask. Add 25 millilitres of diluent, sonicate to volume and filter. About 10 mL of the given solution was produced up to 100 mL by employing the same diluent [29].

5.3.6. Determination of working wavelength(λ_{max})

A UV-Visible spectrophotometer was used to scan a 10µg/ml solution of the medications in methanol against a blank of methanol within the 200-400nm wavelength range in order to determine the drug's maximum absorption wavelength (λ_{max}).3 1 shows the resultant spectra; sitagliptin's absorption curve reveals its unique peak absorption at 237 nm.

5.3.7. Method Optimization

It was determined that the mobile phase of methanol: 0.5% triethylamine (66:34 v/v, pH 3.0±0.1) was the most effective in resolving the peaks with a retention time (R_t) of 3.6 minutes after multiple experiments with various solvent combinations and ratios. The standard medications were scanned across a broad range of wavelengths, from 200 nm to 400 nm, to pick the wavelength. At 237 nm, the component has a respectable level of responsiveness [30].

5.3.8. Chromatographic conditions

Column: HypersilODS C18-250X4.6mm, 5µL or equivalent

Mobile phase: methanol: 0.5% triethylamine (66:34 v/v)

Flowrate : 1.0ml/min

Detection wavelength : 237nm

Injection volume : 20µl

Run time : 8min

5.4 Validation of proposed method

5.4.1 System Suitability

System appropriateness parameters can be viewed as tests that make sure the approach can yield results that are “good enough.”.

System appropriateness requirements are generally determined after method development and validation are accomplished. The parameters given by the USP (2000) can be utilized to establish system appropriateness prior to the analysis. The proposed RP-HPLC method for estimation of SITA was validated by computing the system suitability parameters and comparing with the standard values. These parameters include number of theoretical plates (N), resolution (R) and tailing factor (T) [31].

5.4.2 Linearity

For determination of Linearity six different concentrations were prepared and chromatograms were recorded. For this study, six different drug concentrations were used, ranging from 20 to 200µg ml. Plotting the data on the variation in the peak area response of pure analytes versus corresponding concentrations was done after injecting each concentration three times.

5.4.3 Precision

Measurements of the HPLC technique's peak area and peak symmetry parameters showed repeatability and intermediate precision, demonstrating procedure reproducibility. The repeatability was tested at 5separate concentration levels for each chemical, with triplicates performed within one day. The intermediate precision (IP) was tested over a three-day period. Results were within acceptable range both within and between trial days, despite the use of triplicate injections. The accuracy measured as a percentage of the standard deviation.

5.4.4 Accuracy

For accuracy determination, three other concentrations were made separately i.e. 50%, 100%, and 150% of analyte and the chromatograms were recorded for the same. The precision with which an analytical approach produces results is known as its

accuracy. Verifying an analytical method's accuracy across its linearity range is essential.

Accuracy was performed in three different levels, each level in triplicate for SITA using standards at 50%, 100% and 150%. Each sample was analyzed in triplicate for each level.

5.4.5 Ruggedness

Ruggedness was determined by using the data obtained by the analysis performed by two different analysts. Each analyst prepared 5 samples of the same batch and the results obtained.

5.4.6 Robustness

The standard solution prepared was analyzed under different chromatographic conditions stated below.

a) Modification in flow rate (± 0.2 ml/min)

b) Modification in wavelength (± 2nm)

c) Apparent pH of the mobile phase (± 0.1)

d) Mobile phase organic content (± 2 v/v)

a) Modification in flow rate (± 0.1 ml/min)

Flow rate - 1.1 ml/min
Flow rate minus - 0.8 ml/min.

b) Modification in wavelength (± 1nm):

Wavelength – 236nm
Wavelength – 237nm

5.4.7 LOD and LOQ:

The calculated slope of the regression equation and peak response were used to derive the parameters LOQ and LOD.

One way to represent the detection limit (LOD) is as follows:

LOD = 3.3σ/s

The S.D. of the response is shown by σ.

S is the analyte's calibration curve slope.

One way to write the Quantitation Limit (LOQ) is as follows:

LOQ = 10σ/S,

Where σ is the S.D. of the response.

The slope of the analyte's calibration curve is denoted by S.

5.5: Results and Discussion

5.5.1 Determination of Working Wavelength (λ_{max}):

A UV-Visible spectrophotometer was used to scan a 10µg/ml solution of the medications in methanol against a blank of methanol within the 200-400nm wavelength range in order to determine the drug's maximum absorption wavelength (λ_{max}). Figure 5.1 displays the final spectra, and the absorption curve reveals the SITA-typical absorption maximum at 237 nm.

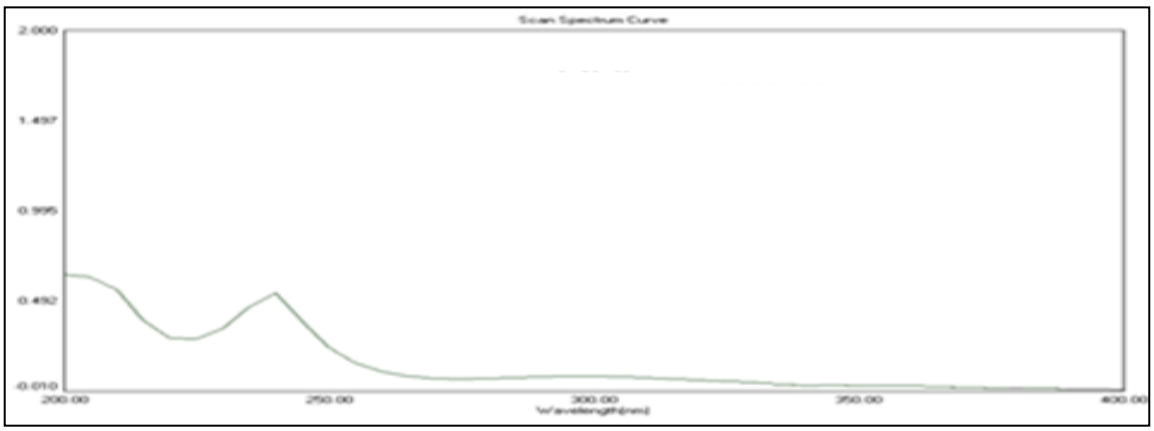


Figure 5.1. Absorption spectrum of SITA at 237 nm

5.5.2 Optimization of HPLC Method

It was determined that the mobile phase of methanol: 0.5% triethylamine (66:34 v/v, pH 3.0±0.1) was the most effective in resolving the peaks with a retention time

(Rt) of 3.6 minutes after multiple experiments with various solvent combinations and ratios. The standard medications were scanned over a wide range of wavelengths, from 200 nm to 400 nm, in order to pick the wavelength. At 237 nm, the component has a respectable level of responsiveness.

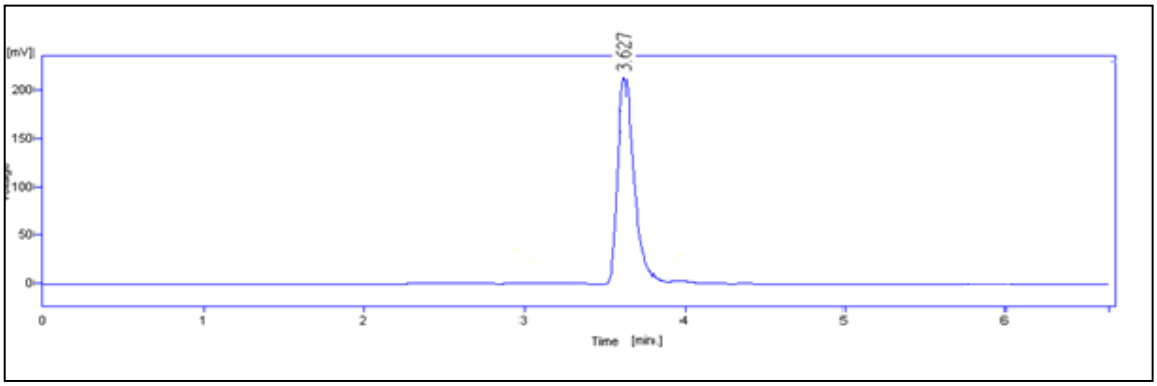


Fig. 5.2 Optimized Chromatogram

Table.5.1 Values obtained from optimized Chromatogram

Drugs name	Retention time (min)	Peak area	Tailing	Plate count
Sitagliptin	3.627	1305.814	1.2	4232

The goal of conducting laboratory research to validate an analytical method is to ensure that its performance characteristics are up to par with what is needed for the specific analytical task at hand (Fig 5.2, Table 5.1).

5.5.3 Linearity: Analyzing a range of reference drug concentrations allowed us to determine whether the suggested HPLC method for sitagliptin determination was linear. The concentrations used in this investigation ranged from 20 to 200µg mL^{-1}.Table 5.2 shows the results of three injections of each concentration, which allowed us to see the relationship between the concentrations and the peak area response of the pure analytes. In Fig.5.3, we can see that the calibration graphs were linear because the correlation coefficient, slope, and interceptive values were all high. The parameters of the optimized approach are listed in Table 3.3.

Table 5.2 Linearity data for SITA

Sr. No.	Conc. (µg mL-1)	Peak area
1	20	1.973
2	40	3.767
3	60	5.452
4	80	7.331
5	120	10.843
6	160	14.365

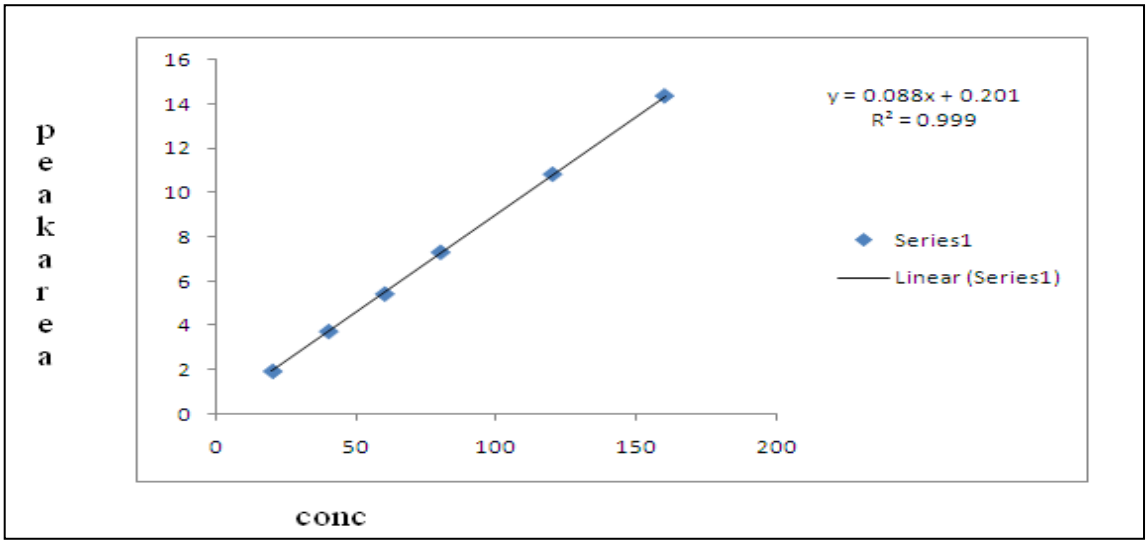


Fig.5.3: Linearity curve of Sitagliptin

Table 5.3: Characteristic parameters of calibration equation for the proposed HPLC method

Parameters	SITA
Calibration range (µg mL^{-1})	20-200
Slope (b)	0.088
Intercept (a)	0.201
Correlation coefficient	0.9999

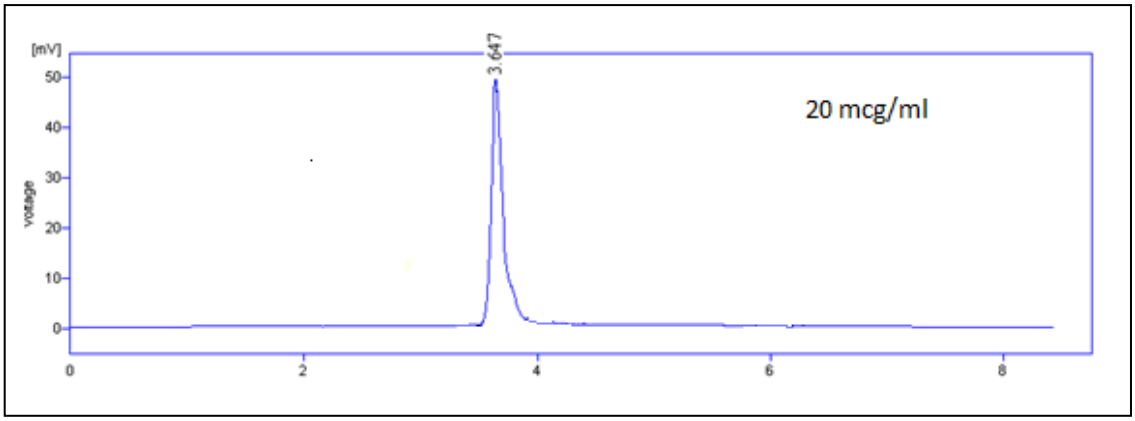


Fig.5.4: Chromatogram For Linearity of Concentration (20 mcg)

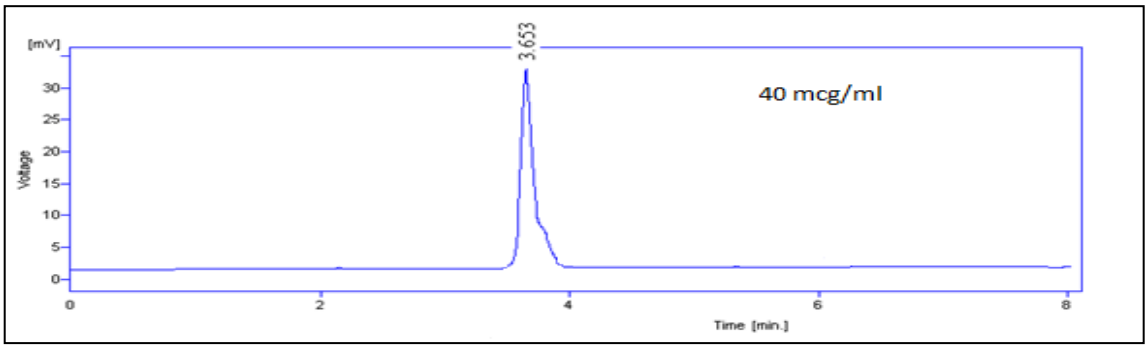


Fig. 5.5: Chromatogram For Linearity of Concentration (40 mcg)

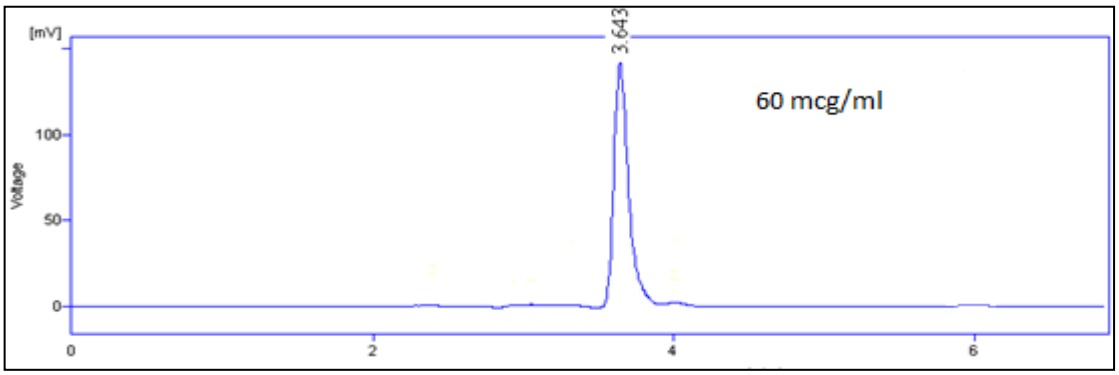


Fig 5.6: Chromatogram For Linearity Of Concentration (60 mcg)

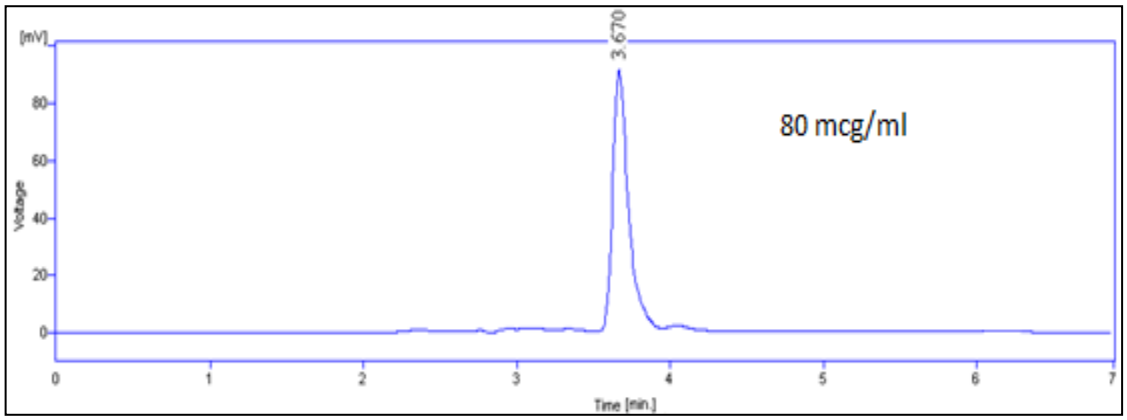


Fig 5.7: Chromatogram For Linearity Of Concentration (80 mcg)

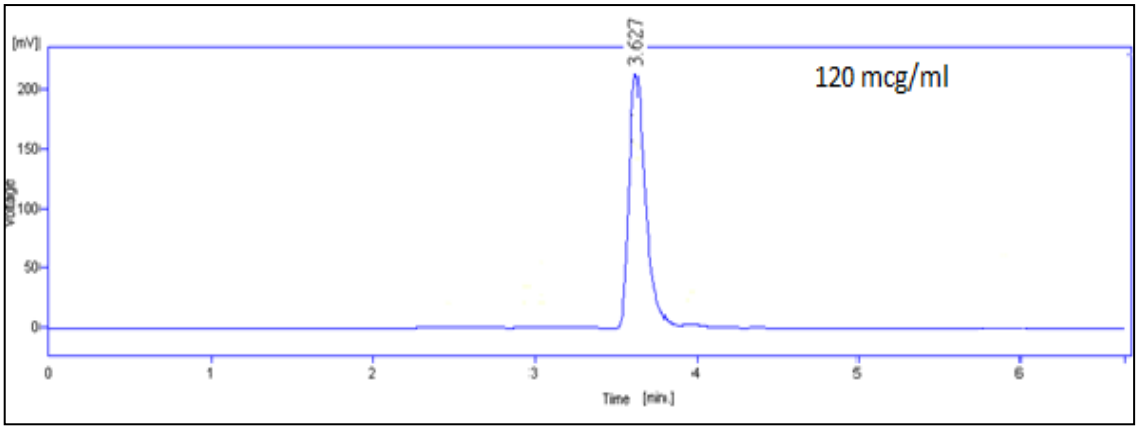


Fig 5.8: ChromatogramFor Linearity Of Concentration (120 mcg)

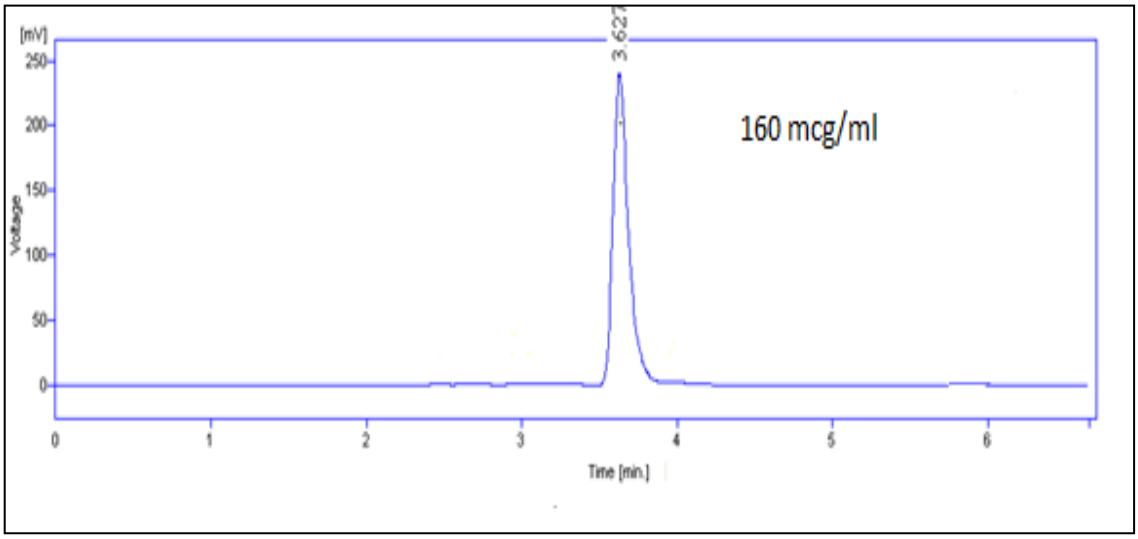


Fig 5.9:Chromatogram For Linearity Of Concentration (160 mcg)

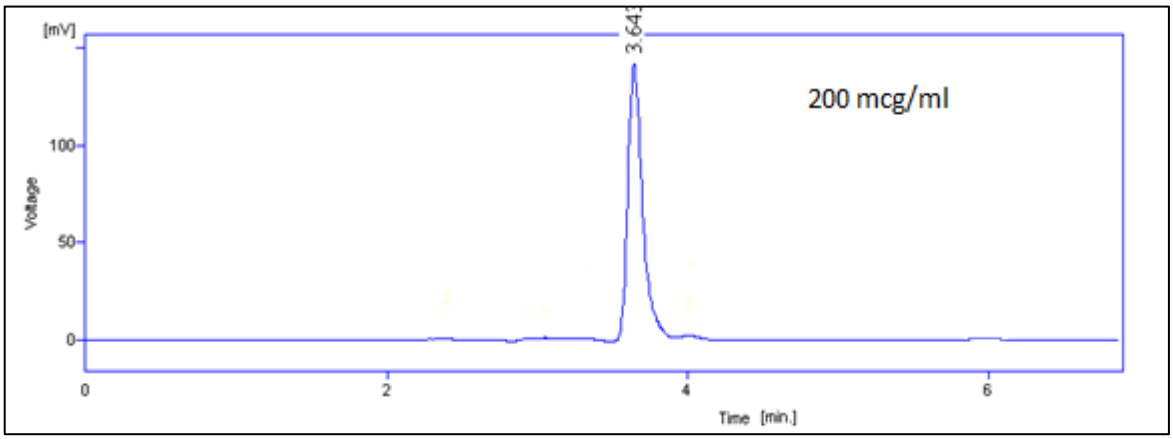


Fig: 5.10 Chromatogram For Linearity Of Concentration (200 mcg)

5.5.4 Precision

Analyses of many samples of the same homogenous material were used to determine the analytical method's precision.

The intermediate precision as well as repeatabilityof the HPLC method's peak area and peak symmetry parameters measurements proved the method's reproducibility. Every component was tested at five different concentrations for its repeatability (within a day in triplicates) (table.5.4) and intermediate precision (over three days, table.5.5). Results were within acceptable range both within and between trial days, despite the use of triplicate injections. You may find the precision, reported as % RSD, in table 5.5.

Table 5.4: Precision (Repeatability) study results

Conc. (µg mL^{-1})	Area± SD	%RSD
20	1.836±0.01	0.54
40	3.684±0.04	1.08
80	7.192±0.07	0.97
120	10.943±0.16	1.46
200	17.434±0.28	1.60

Table 5.5: Precision (Intermediate Precision) study results

Conc. (µg mL^{-1})	Area± SD	%RSD
20	1.820±0.02	1.09
40	3.678±0.04	1.08
80	7.256±0.10	1.37
120	10.988±0.16	1.45
200	17.492±0.28	1.60

5.5.5 Accuracy

The precision with which an analytical approach produces results is known as its accuracy. Verifying an analytical method's accuracy throughout its linearity range is essential. Accuracy was performed in three different levels, each level in triplicate for SITA using standards at 50%, 100% and 150%. Each sample was analyzed in triplicate for each level. From the results, % recovery is calculated and revealed in Table 5.6.

Table 5.6: Recovery's Range

Sl. No	Level in%	Area Response	SITA
1	50	5.617	
2	50	5.617	101.86±0.10
3	50	5.607	
4	100	7.211	
5	100	7.391	100.97± 1.5
6	100	7.513	
7	150	9.125	
8	150	9.043	101.09±0.8
9	150	9.192	
Across all levels			101.3±0.8

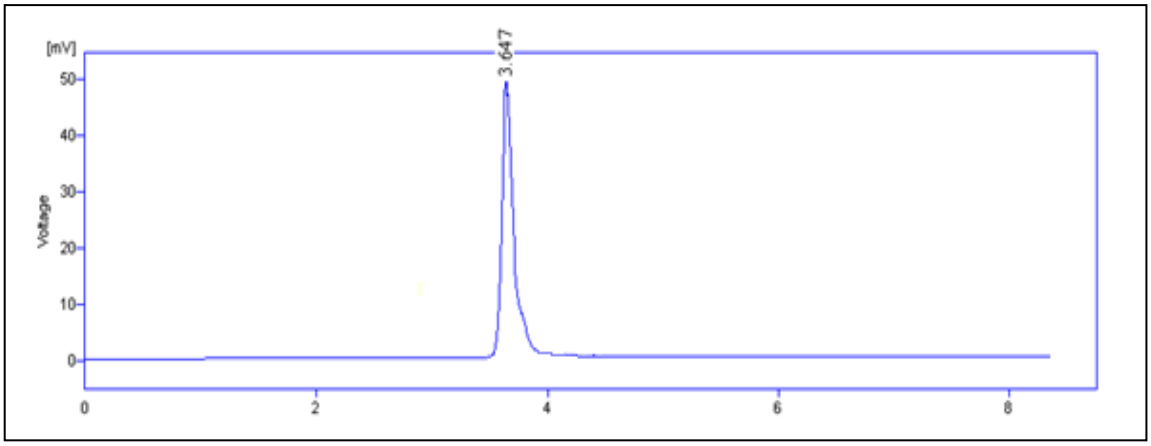


Fig 5.11: Chromatogram For Accuracy Of Concentration (50 mcg)

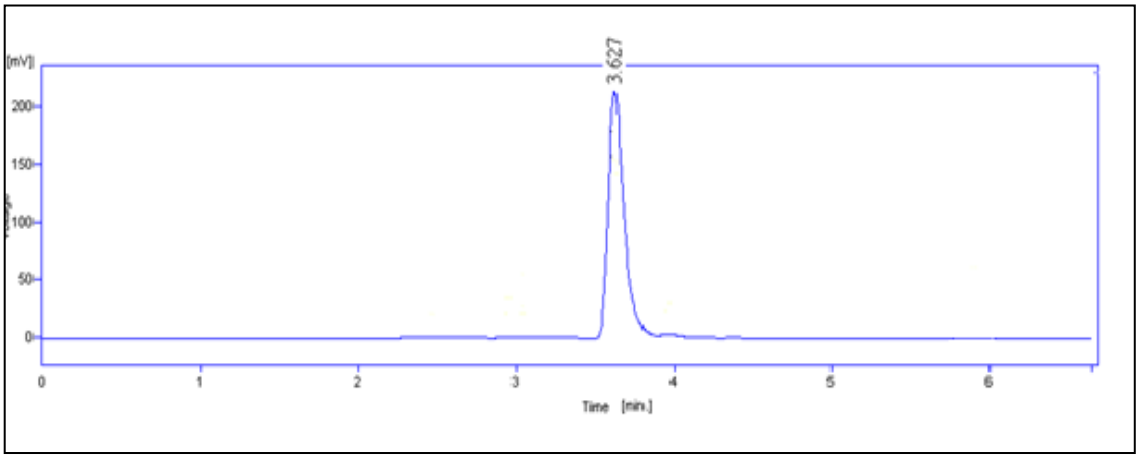


Fig 5.12: Chromatogram for Accuracy Of Concentration (100 mcg)

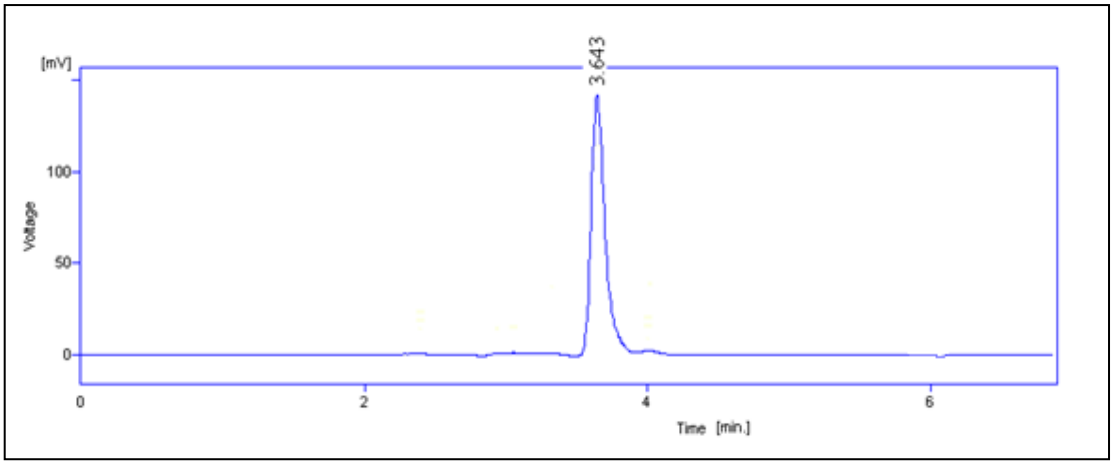


Fig 5.13: Chromatogram for Accuracy of Concentration (150 mcg)

5.5.6 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The approach is established on signal to noise ratio and was calculated according to the ICH recommendations. The researcher compared the chromatogram signals from blank samples to samples with known low amounts of analyte. For LOD, a "signal-to-noise ratio" of 2:1 or 3:1 was determined, while for LOQ a ratio of 10:1 was evaluated. Table 5.7 gives the values of LOD and LOQ.

Table 5.7 Result of LOQ and LOD

Results observed	SITA
LOD (µg mL^{-1})	0.6773
LOQ (µg mL^{-1})	2.1881

5.5.7: Robustness

To test the method's robustness, we tweaked the chromatographic environments somewhat. Using retention duration, symmetry factor, and peak resolution (Table 5.8), the suggested HPLC method's robustness was evaluated. This study's parameters are

Mobile phase's apparent pH (± 0.1)

Organic content of the mobile phase (± 2 v/v)

Rate of flow of the mobile phase (± 0.1 mL min-1)

Distance of the detection wave (± 1 nm)

Table 5.8 Robustness of Chromatographic method

Parameter	Retention time	Peak asymmetry	Resolution
Flow rate(mL $min^{-1)}$			
0.9	3.960	1.364	6.574
1.0	3.880	1.313	6.436
1.1	3.713	1.345	6.387
Methanol % in mobile phase			
64	4.070	1.303	6.588
66	3.880	1.313	6.436
68	3.673	1.345	6.348
Change in p^H			
2.90	3.750	1.415	6.530
3.00	3.880	1.313	6.436
3.10	3.720	1.301	6.410
Wavelength			
236 nm	3.85	1.323	6.408
237 nm	3.880	1.313	6.436
238 nm	3.853	1.367	6.390

5.5.8 Ruggedness

Analyses of aliquots from identical lots were conducted on separate dates by separate analysts under different operational and environmental conditions; these factors might vary, but they must remain within the parameters of the study to establish the method's robustness. The suggested strategy was determined to be robust because the results in Table 5.9 fall within the acceptable range.

Table 5.9: Ruggedness report of SITA

Sr. NO.	ANALYST-1		ANALYST-2	
	Rt	Peak area	Rt	Peak area
1.	3.547	2543.86	3.638	2542.2
2.	3.633	2544.144	3.533	2541.05
3.	3.534	2539.02	3.544	2553.6
4.	3.634	2542.162	3.634	2549.21
5.	3.537	2549.06	3.624	2545.54
Mean	3.637	2545.23	3.633	2551.19
SD	3.6390	2543.913	3.623	2547.132
%RSD	0.0047	3.325349	0.0057	5.02932

5.5.9: System Suitability

System appropriateness parameters are often viewed as a tool to verify if the strategy can deliver acceptable results. System appropriateness requirements are usually prepared after method development and its validation is done. (Or else) The USP (2000) provides parameters for establishing system appropriateness before analysis. The system suitability parameters viz., "Theoretical plates (N)", "Resolution (R)", "Tailing factor (T)" were computed and compared with the standard values to know whether the proposed "RP-HPLC technique "for the evaluation of Sitagliptin was validated or not. The proposed approach of parameters for system suitability was under limits.

Table No: 5.10 Parameters for System suitability

Drug	RetentionTime(min)	Peak area	USP Plate count	USP Tailing
Sitagliptin	3.643	1305.814	4232	1.23

5.5.10 Assay

Analysis of commercial table formulation using the established procedures The suggested approach was evaluated by comparing it to the commercially available JANUVIA pills, which contain 100 mg of SITA according to the label.

Table 5.11: Assay

Brand name	Label Claim	Amount found	% Assay
Januvia	**100**	**99.2**	**99.4%**

%Assay =99.4%

INFERENCE: The assay of given Sitagliptin tablet was found to be 99.4%. This value was within the limit.

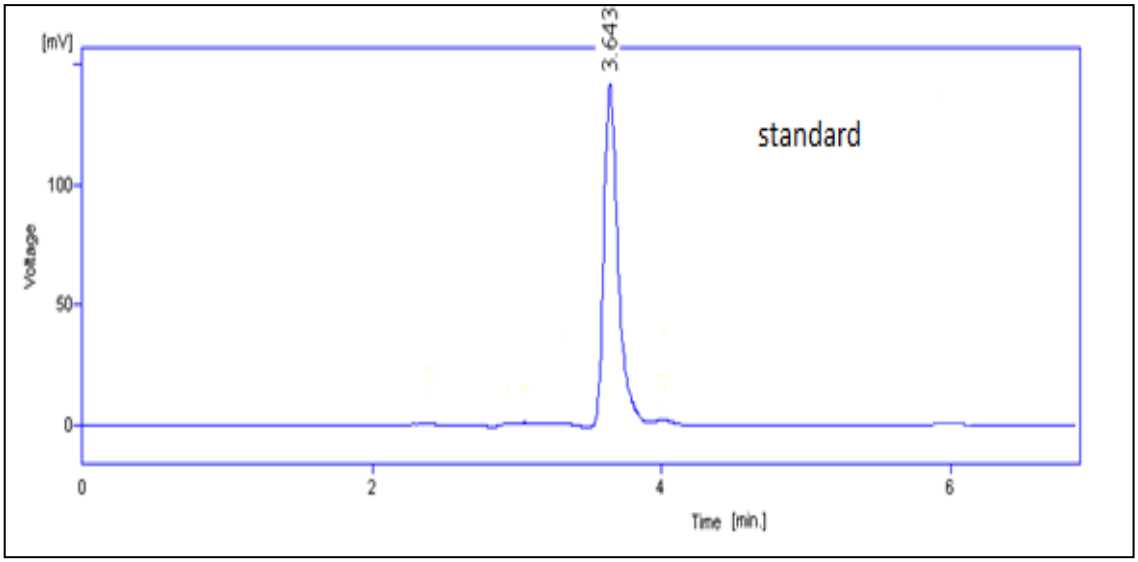


Fig: 5.14 Chromatogram for Standard Concentration (standard)

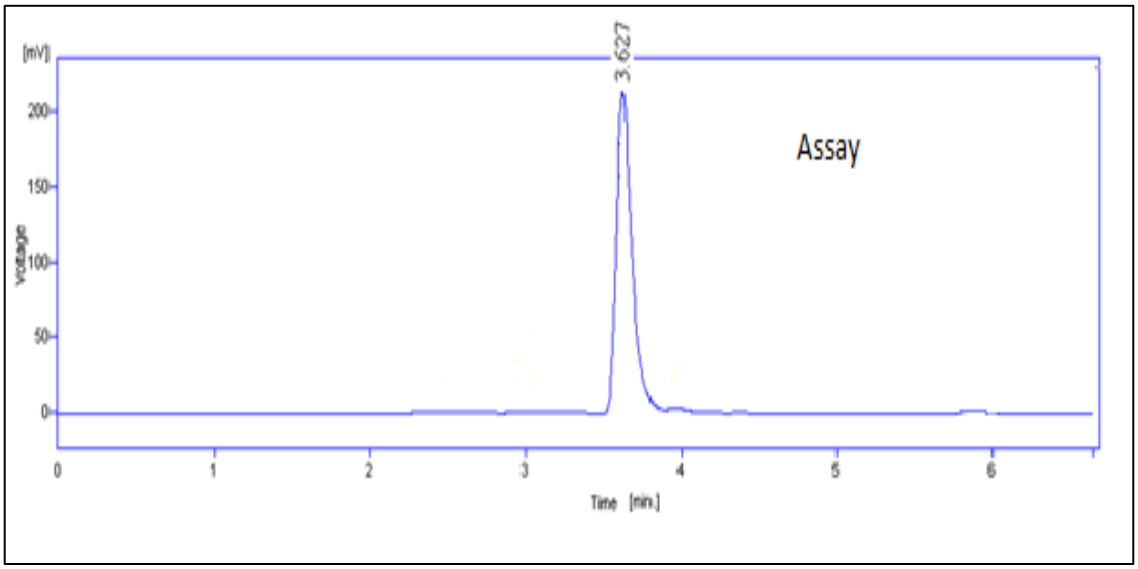


Fig: 5.15 Chromatogram For marketed Concentration (assay)

5.6 Conclusion

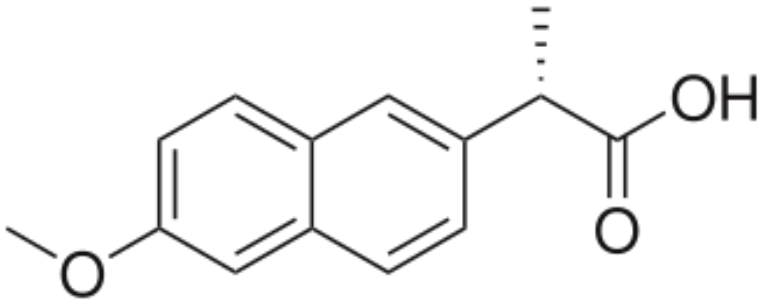
The results of this investigation demonstrate the practicality of the suggested HPLC approach for SITA detection. After eight minutes of running time, this dependable HPLC method successfully determines SITA with a high degree of precision and sufficient sensitivity. The proposed technique is quite simple and do not require any pre-treatment of the drug and tedious extraction procedure. The method has wider linear range with good accuracy. In addition, typical formulation additives and excipients do not interfere with the process. Laboratory generated combinations using the described approach showed good agreement in the assay findings. Since the chromatographic parameters did not undergo any significant changes, it may be concluded that the established method was resilient. In pharmaceutical quality control laboratories, the suggested method was determined to be appropriate for routine SITA measurement.

Chapter - 6

BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF NAPROXEN

6.1 Drug Profile [1]

Name of the drug: Naproxen



NAPROXEN

Chemical name : (+) -(*S*)-2-(6-methoxynaphthalen-2-yl) propanoic acid

Molecular formula : $C_{14}H_{14}O_3$

Molecular weight : 230.26 g/mol

CAS number **:** 22204-53-1

Brand names: Naprosyn, Naproxen Sodium, Panoxen, Prexan, Proxine

Half-life: The observed terminal elimination half-life is approximately 15 hours.

pKa: 4.15

Water solubility: Poorly soluble in water (15.9 mg/l); freely soluble in methanol.

6.2 Clinical Pharmacology

Naproxen belongs to the class of non-steroidal anti-inflammatory medicines (NSAIDs), namely the aryl acetic acids. Naproxen is a pain reliever and fever reducer. Like other NSAIDs, its mechanism of action is not entirely understood. However, the

anti-inflammatory activity may be implicated in its capacity to block prostaglandin formation [2].

6.3 Mechanism of Action

The mechanism of action of naproxen, along with other NSAIDs, is not completely understood but involves inhibition of cyclooxygenase (COX-1 and/or COX-2). There are two different cyclooxygenases in mammals. The constitutive cyclooxygenase, COX-1, produces prostaglandins that are required for appropriate gastrointestinal and renal function. The inducible cyclo-oxygenase, COX-2, produces prostaglandins that are important in inflammation. Gastrointestinal and renal damage is assumed to be connected with suppression of COX-1, while anti-inflammatory efficacy is provided by inhibition of COX-2 [3].

6.4 Distribution

Naproxen is approximately 98-99% bound to plasma proteins.

6.5 Side effects

Diarrhea, headache, nausea, vomiting, weakness, rash, and trouble sleeping may occur. Increased thirst or urination, blurred vision, swelling, chills, mouth sores, persistent sore throat or fever, weight loss, mental/mood changes, trouble breathing, vision changes, urination problems, decreased sexual ability. Yellowing eyes and skin, dark urine, persistent fever, fatigue.

6.6 Indication

For the treatment of rheumatoid arthritis, osteoarthritis, spondylitis, tendinitis, bursitis and acute gout. For the relief of mild to moderate discomfort and the treatment of primary dysmenorrhea.[4]

6.7 Drug Interactions

1. Cyclosporine: Monitor for nephrotoxicity.
2. Lithium: It may decrease the renal excretion of lithium. Increased risk of lithium toxicity.
3. Timolol: It may antagonize the antihypertensive effect of Timolol.
4. Alendronate: Increased risk of gastric toxicity.

Food Interactions:

1) Avoid alcohol.

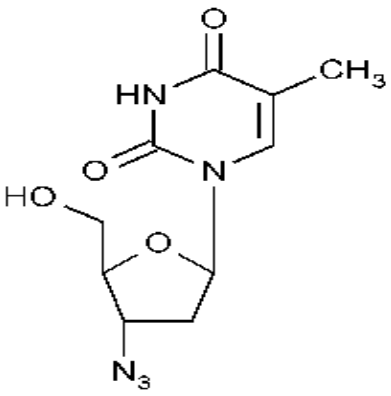
2) Take a full glass of water.

3) Take with food.

Adverse Effect

1. It causes serious and potentially fatal cardiovascular events such as myocardial infarction and stroke.
2. Naproxen can cause GIT problems such as heartburn, constipation, diarrhea, ulcers and stomach bleeding.
3. Consumption of alcohol may increase the risk of gastrointestinal complications.
4. It may interfere and reduce the efficiency of antidepressants [5].

6.8 Structural formula of Zidovudine



Chemical name : 1-[(2R, 4S, 5S)-4-azido-5-(hydroxymethyl) oxolan-2-yl]-5-methyl-1,2,3,4-tetrahydropyrimidine-2,4-dione

Chemical formula : $C_{10}H_{13}N_5O_4$

Molecular weight : 267.24 g/mol

Melting point : 106-112 ^oC

Brand names : Novo-Azt, Zidovudine EP III, Retrovir

Protein binding : 30-38%

CAS number **:** 30516-87-1

Water solubility **:** 10-50 g/L at 17 ^{o}C

Half life : 0.5-3 hours

pKa **:** 9.96

6.9 Pharmacology

Zidovudine is a nucleoside reverse transcriptase inhibitor (NRTI) active against Human Immunodeficiency Virus Type 1 (HIV-1). Zidovudine is phosphorylated to active metabolites which compete for inclusion into viral DNA. They competitively block the HIV reverse transcriptase enzyme and function as a chain terminator of DNA synthesis. The integrated nucleoside analogue is devoid of 3'-OH group which hinders formation of the 5' to 3' phosphodiester bond necessary for DNA chain elongation and thereby terminates the expansion of viral DNA [6].

6.10 Mechanism of action

Zidovudine a structural analog of thymidine, inhibits the activity of HIV-1 reverse transcriptase (RT) both by competing with the natural substrate dGTP and by its incorporation into viral DNA.

6.11 Absorption

Rapid and nearly complete absorption from the gastrointestinal tract following oral administration; however, because of first-pass metabolism, systemic bioavailability of Zidovudine capsules and solution is approximately 65% (range, 52 to 75%).

6.12 Distribution

Protein binding 30-38%

Metabolism

Hepatic metabolized by glucuronide conjugation to major, inactive metabolite, 3′-azido-3′-deoxy-5′- O-beta-D-glucopyranuronosylthymidine (GZDV).

Elimination

As in adult patients, the major route of elimination was by metabolism to GZDV. After intravenous dosing, about 29% of the dose was excreted in the urine unchanged and about 45% of the dose was excreted as GZDV.

Toxicity

Symptoms of overdose include fatigue, headache, nausea, and vomiting. LD_{50} is 3084 mg/kg (orally in mice) [7].

6.13: Results and Discussion

Chromatography Representative chromatograms of aqueous standard analytes and internal standard (IS) mixture, plasma (blank) with internal standard, LLOQQC sample, LQC sample, MQC-1, MQC-2 and HQC samples of naproxen.

Table 6.1: Selectivity Data for Naproxen

SEL-LLOQ	Naproxen Area	IS Area
1	7924	1184429
2	8960	1206661
3	8814	1190596
4	9021	1236173
5	7726	1186449
6	8742	1245520
Mean	**8531.2**	**1208304.7**
SD	**559.50**	**26554.25**
% CV	**7.56**	**2.20**

Table 6.2: IS Data

Sample ID	Naproxen Area	IS Area	% Interference at RT of Naproxenwith Zidovudine
Blank+IS (Zidovudine) -1	0	1377388	0.00
Blank+IS (Zidovudine) -2	0	1149575	0.01
Blank+IS (Zidovudine) -3	0	1217495	0.00
Blank+IS (Zidovudine) -4	0	1174890	0.00
Blank+IS (Zidovudine) -5	0	1216644	0.00
Blank+IS (Zidovudine) -6	0	1258066	0.00

Table 6.3: IS& Standard Data

Sample ID	Naproxen Area	IS Area	% Interference at RT of IS in presence of Naproxen
ULOQ (Naproxen)-1	2926612	0	0
ULOQ (Naproxen)-2	2773340	0	0
ULOQ (Naproxen)-3	2914929	0	0
ULOQ (Naproxen)-4	2819230	0	0
ULOQ (Naproxen)-5	2855207	0	0
ULOQ (Naproxen)-6	2882761	0	0

Calculation:

$$\text{\% Interference of Analyte and IS} = \frac{\text{\% Interference at RT of Analytes and IS}}{\text{Average response in Selectivity LLOQ}} \times 100$$

Mass transitions of Naproxen and internal standard. No major interferences from endogenous components were identified. (Refer point 3.5) Refer Figure no: 6.1 and 6.2 for representative chromatogram. No substantial interference was found for analyte selectivity at retention period of analyte in six Blank + IS sample and at retention time of ISTD in all six ULOQ samples.

Table 6.4: Carry Over Test (LLOQ Area)

COT-LLOQ	Area of Naproxen	Area of Internal Standard
01	9300	1206414
02	8653	1287745
03	9377	1293309
04	8763	1261265
05	10258	1460607
06	8132	1277768
Mean	**9080.5**	**1297851.3**

Table 6.5 Carry Over Test (ULOQ Area)

COT-ULOQ	Area of Naproxen	Area of Internal Standard
01	2787575	1247323
02	2745625	1272231
03	2979829	1389528
04	2901204	1362500
05	2868647	1317531
06	2838264	1314378
Mean	**2853524.0**	**1317248.5**

Table 6.6 Carry Over Test (Blank Area)

COT-Blank	Area of Naproxen		Internal Standard	
	Area response	% Carry over	Area response	% Carry over
01	0	0	0	0
02	0	0	0	0
03	0	0	0	0
04	155	1.71	0	0
05	176	1.94	0	0
06	0	0.00	0	0

To evaluate the carryover impact of analyte and internal standard, the calibration curve at a suitable high concentration (ULOQ) of the analyte with the working concentration of the internal standard, extracted matrix blank and extracted

LLOQ samples were equipped and injected, and no interference was observed at the retention duration of the analyte and internal standard in all the Blanks[15].

The results indicate that there was no carryover impact of analyte and internal standard. Refer Figure No 6.3 (A representative chromatogram of extracted blank plasma sample evaluated for Carryover Test).

Table 6.7 Data for Sensitivity of Naproxen

	Concentration (ng/mL)									
	STD-A	**STD-B**	**STD-C**	**STD-D**	**STD-E**	**STD-F**	**STD-G**	**STD-H**	**STD-I**	**STD-J**
Max	600.1	1150.2	2875.6	5751.0	11502.2	23004.3	46008.5	69019.7	92027.2	115032.8
Min	400.1	850.2	2125.4	4250.8	8501.6	17003.1	34007.3	51014.5	68019.4	85024.2
CC	500.1	1000.2	2500.5	5000.9	10001.9	20003.7	40007.4	60017.1	80022.8	100028.5
1	515.6	960.1	2433.3	4834.7	9531.1	19763.1	36792.9	63394.4	85427.2	108505.4
% Nominal	103.11	95.99	97.31	97.68	95.29	98.80	91.97	105.63	107.75	108.47

	Slope	**Intercept**	**r^2**
CC 1	0.0000	-0.0023	0.9962

Table 6.8 Precision and Accuracy (Within Batch) for sensitivity of Naproxen

SEN LLOQ	**Conc. (ng/mL)**	
	LLOQ	
	Max	**600.1**
	Min	**400.1**
	500.1	
1.	641.7	
2.	635.8	
3.	524.1	
4.	550.7	
5.	582.6	
6.	564.8	
Mean	**583.28**	
SD	**47.068**	
CV%	**8.07**	
% NOMINAL	**117.63**	
N	**6**	

The lowest limit of reliable quantification for Naproxen in human plasma was set at the concentration of the LLOQ 500.1 ng/mL. The accuracy and precision for Naproxen at this concentration was discovered to be 8.07% and 117.63%.

Table 6.9 Matrix Effect of Naproxen (Matrix Ions Absence)

AQ-ME	LQC				HQC		
S. No.	Area of Analyte	Area of Internal Standard	Area Ratio	AQ-ME S. No.	Area of Analyte	Area of Internal Standard	Area Ratio
1	34396	1609712	**0.02**	1	2287849	1543663	**1.48**
2	42991	1642033	**0.03**	2	2330829	1549430	**1.50**
3	39576	1555969	**0.03**	3	2240299	1495201	**1.50**
4	42671	1824262	**0.02**	4	2312568	1545249	**1.50**
5	42504	1654536	**0.03**	5	2217708	1504301	**1.47**
6	40441	1557484	**0.03**	6	2325377	1603014	**1.45**
Mean			0.025		Mean		1.484

Table 6.10 Matrix Effect of Naproxen (Matrix Ions Presence)

Matrix Lot. No.	LQC				HQC			
	Area of Analyte	Area of Internal Standard	Area Ratio	IS Normalized Matrix Factor	Area of Analyte	Area of Internal Standard	Area Ratio	IS Normalized Matrix Factor
Blank(P161211-565-B)-01	34410	1286376	**0.03**	**1.08**	2127474	1140799	**1.86**	**1.26**
Blank(P161211-566-B)-02	36444	1256454		**1.18**	2157119	1134394	**1.90**	**1.28**
Blank(P161211-567-B)-03	32500	1222214		**1.08**	2097757	1170734	**1.79**	**1.21**
Blank(P161211-568-B)-04	35465	1243804		**1.16**	2153734	1203135	**1.79**	**1.21**
Blank(P161211-569-B)-05	33013	1195858		**1.12**	2047571	1111380	**1.84**	**1.24**
Blank(P161211-570-B)-06	33364	1202196		**1.12**	2187903	1178203	**1.86**	**1.25**
Blank(P060911-527-G) (Lipemic)-07	31034	1112304		**1.13**	2247490	1207703	**1.86**	**1.25**
Blank(P060911-528-M) (Hemolytic)-8	33290	1127498		**1.20**	2157339	1144490	**1.88**	**1.27**
Mean				**1.310**				**1.246**
SD				**0.0504**				**0.0271**
% CV				**3.85**				**2.17**
N				**8**				**8**

IS-normalized matrix factor = (Peak area response ratio in the presence of matrix ions) / (Mean peak area response ratio in the absence of matrix ions).

No matrix effect was examined in all the eight batches (batch no. P161211-565-B, P161211-566-B, P161211-567-B, P161211-568-B, P161211-569-B, P161211-570-B, P060911-527-G (lipemic plasma) and P060911-528-M (hemolytic plasma) for Naproxen at low (LQC) and high (HQC) concentrations. The precision for IS normalized matrix factor at LQC and HQC level was found to be 3.85% and 2.17%, respectively.

Table 6.11 Concentration-response Linearity Data for Naproxen

	Concentration (ng/mL)									
	STD-A	**STD-B**	**STD-C**	**STD-D**	**STD-E**	**STD-F**	**STD-G**	**STD-H**	**STD-I**	**STD-J**
Max	**600.1**	**1150.2**	**2875.6**	**5751.0**	**11502.2**	**23004.3**	**46008.5**	**69019.7**	**92027.2**	**115032.8**
Min	**400.1**	**850.2**	**2125.4**	**4250.8**	**8501.6**	**17003.1**	**34007.3**	**51014.5**	**68019.4**	**85024.2**
CC	**500.1**	**1000.2**	**2500.5**	**5000.9**	**10001.9**	**20003.7**	**40007.4**	**60017.1**	**80022.8**	**100028.5**
1	515.6	960.1	2433.3	4834.7	9531.1	19763.1	36792.9	63394.4	85427.2	108505.4
2	508.6	973.0	2459.2	5114.9	9467.5	20337.7	37515.6	62072.4	80577.8	106217.1
3	511.2	955.4	2534.6	5067.6	9211.4	20140.8	37441.4	60812.4	84279.7	106584.1
Mean	**511.80**	**962.83**	**2475.70**	**5005.73**	**9403.00**	**20080.20**	**37249.97**	**62093.07**	**83427.90**	**107101.87**
SD	**3.538**	**9.113**	**52.627**	**149.995**	**169.045**	**291.562**	**397.566**	**1291.124**	**2533.953**	**1229.343**
CV%	**0.69**	**0.95**	**2.13**	**3.00**	**1.80**	**1.45**	**1.07**	**2.08**	**3.04**	**1.15**
% Nominal	**102.34**	**97.26**	**99.01**	**100.10**	**94.01**	**100.38**	**93.11**	**103.46**	**104.26**	**107.07**
N	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**

CC	Slope	Intercept	r^2
1	0.0000	-0.0023	0.9962
2	0.0000	-0.0021	0.9980
3	0.0000	-0.0015	0.9972

A regression equation with a weighting factor of 1/ (concentration ratio) ^{2} of drug to ISTD concentration was judged to produce the best fit for the concentration-detector response relationship for Naproxen in human plasma [16]. The descriptive calibration curve for regression analysis is illuminated in Table 6.12. Correlation coefficient (r^2) was more than 0.98 in the range of 500.1 ng/mL to 100028.5ng/mL for Naproxen.

Table 6.12: Precision and Accuracy (Within Batch) for Naproxen

	Conc. (ng/mL)				
	LLOQ QC	**LQC**	**MQC1**	**MQC2**	**HQC**
Max	**600.5**	**1725.1**	**17250.8**	**57502.5**	**97760.1**
Min	**400.3**	**1275.1**	**12750.6**	**42501.9**	**72257.5**
QC	**500.4**	**1500.1**	**15000.7**	**50002.2**	**85008.8**
1	553.3	1707.2	15558.9	55202.6	93187.2
2	605.3	1844.4	16274.2	55083.3	96187.8
3	541.5	1539.0	16617.0	55772.3	94139.3
4	564.9	1561.8	17624.4	57397.4	96344.6
5	568.2	1459.1	16385.1	57808.3	94301.3
6	553.4	1510.0	16797.9	56959.2	99924.9
Mean	**564.43**	**1603.42**	**16542.58**	**56370.35**	**95680.85**
S.D.	**22.164**	**144.273**	**678.949**	**1170.045**	**2417.913**
C.V.%	**3.93**	**9.00**	**4.10**	**2.08**	**2.53**
% Nominal	**112.80**	**107.89**	**110.28**	**112.74**	**112.55**
N	**6**	**6**	**6**	**6**	**6**
7	513.8	1312.4	13023.7	49151.3	86139.5
8	444.3	1353.2	12872.6	48632.3	83101.3
9	450.9	1324.5	13867.1	48321.8	82259.6
10	489.7	1327.4	14252.4	51179.3	84665.8
11	458.5	1307.0	14057.8	48397.0	83015.5
12	385.9	1427.5	14092.4	50994.1	83432.4
Mean	**457.18**	**1341.50**	**13694.00**	**49445.97**	**83769.02**
S.D.	**29.384**	**44.690**	**592.583**	**1304.923**	**1402.048**
C.V.%	**7.43**	**3.33**	**4.33**	**2.64**	**1.67**
% Nominal	**91.36**	**89.43**	**91.29**	**98.89**	**98.54**
N	**6**	**6**	**6**	**6**	**6**
13	529.8	1759.2	13705.7	50624.6	94965.8
14	517.3	1591.7	13687.4	49718.4	87957.6
15	502.3	1568.6	14460.6	51097.5	88244.0
16	507.7	1535.7	14101.1	53237.7	87677.8
17	482.0	1657.9	14145.5	52161.9	92733.6
18	519.5	1433.8	13683.6	50960.1	84965.3
Mean	**509.60**	**1591.15**	**13963.82**	**51299.70**	**89423.85**
S.D.	**17.551**	**110.489**	**322.724**	**1233.551**	**3691.624**
C.V.%	**3.25**	**7.94**	**2.31**	**2.40**	**4.13**
% Nominal	**101.84**	**107.07**	**93.09**	**102.59**	**105.19**
N	**6**	**6**	**6**	**6**	**6**

Within-batch precision for "LLOQQC", "LQC", "MQC1", "MQC2" and "HQC" ranged from 3.25 % to 7.43 %, 3.33 % to 9.00 %, 2.31 % to 4.33 %, 2.08% to 2.64% and 1.67% to 4.13% respectively.

Accuracy within batch for 'LLOQ QC', 'LQC', 'MQC1', 'MQC2' and 'HQC' ranged from 91.36% to 112.80%, 89.43% to 107.89%, 91.29% to 110.28%, 98.89% to 112.74% % and 98.54% to 112.55% respectively.

Table 6.13: Intraday Accuracy and Precision for Naproxen

	Conc. (ng/mL)				
	LLOQ QC	**LQC**	**MQC1**	**MQC2**	**HQC**
Max	**600.5**	**1725.1**	**17250.8**	**57502.5**	**97760.1**
Min	**400.3**	**1275.1**	**12750.6**	**42501.9**	**72257.5**
QC	**500.4**	**1500.1**	**15000.7**	**50002.2**	**85008.8**
1	553.3	1707.2	15558.9	55202.6	93187.2
2	605.3	1844.4	16274.2	55083.3	96187.8
3	541.5	1539.0	16617.0	55772.3	94139.3
4	564.9	1561.8	17624.4	57397.4	96344.6
5	568.2	1459.1	16385.1	57808.3	94301.3
6	553.4	1510.0	16797.9	56959.2	99924.9
7	513.8	1312.4	13023.7	49151.3	86139.5
8	444.3	1353.2	12872.6	48632.3	83101.3
9	450.9	1324.5	13867.1	48321.8	82259.6
10	489.7	1327.4	14252.4	51179.3	84665.8
11	458.5	1307.0	14057.8	48397.0	83015.5
12	385.9	1427.5	14092.4	50994.1	83432.4
Mean	**510.81**	**1472.46**	**15118.29**	**52908.16**	**89724.93**
S.D.	**65.028**	**170.523**	**1607.914**	**3804.309**	**6499.730**
C.V.%	**12.73**	**11.58**	**10.63**	**7.19**	**7.24**
% Nominal	**102.08**	**98.16**	**100.78**	**105.81**	**105.55**
N	**12**	**12**	**12**	**12**	**12**

Intra-day precision:

LLOQ QC (12.73%), LQC (11.58%), MQC1 (10.63%), MQC2 (7.19%) and HQC (7.24%).

Intra-day accuracy:

LLOQ QC (102.08%), LQC (98.16%), MQC1 (100.78%), MQC2 (105.81%) and HQC (105.55%).

Table 6.14: Inter Day Precision and Accuracy (between Batch) for Naproxen

	Conc. (ng/mL)				
	LLOQ QC	**LQC**	**MQC1**	**MQC2**	**HQC**
Max	**600.5**	**1725.1**	**17250.8**	**57502.5**	**97760.1**
Min	**400.3**	**1275.1**	**12750.6**	**42501.9**	**72257.5**
QC	**500.4**	**1500.1**	**15000.7**	**50002.2**	**85008.8**
1	553.3	1707.2	15558.9	55202.6	93187.2
2	605.3	1844.4	16274.2	55083.3	96187.8
3	541.5	1539.0	16617.0	55772.3	94139.3
4	564.9	1561.8	17624.4	57397.4	96344.6
5	568.2	1459.1	16385.1	57808.3	94301.3
6	553.4	1510.0	16797.9	56959.2	99924.9
7	513.8	1312.4	13023.7	49151.3	86139.5
8	444.3	1353.2	12872.6	48632.3	83101.3
9	450.9	1324.5	13867.1	48321.8	82259.6
10	489.7	1327.4	14252.4	51179.3	84665.8
11	458.5	1307.0	14057.8	48397.0	83015.5
12	385.9	1427.5	14092.4	50994.1	83432.4
13	529.8	1759.2	13705.7	50624.6	94965.8
14	517.3	1591.7	13687.4	49718.4	87957.6
15	502.3	1568.6	14460.6	51097.5	88244.0
16	507.7	1535.7	14101.1	53237.7	87677.8
17	482.0	1657.9	14145.5	52161.9	92733.6
18	519.5	1433.8	13683.6	50960.1	84965.3
Mean	**510.41**	**1512.02**	**14733.47**	**52372.01**	**89624.57**
S.D.	**53.076**	**160.376**	**1419.526**	**3228.159**	**5600.496**
C.V.%	**10.40**	**10.61**	**9.63**	**7.16**	**7.25**
% Nominal	**102.00**	**100.79**	**98.22**	**104.74**	**105.43**
N	**18**	**18**	**18**	**18**	**18**

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Between-batch precision:

LLOQ QC (10.40%), LQC (10.61%), MQC1 (9.63%), MQC2 (7.16%) and HQC (7.25%).

Between-batch accuracy:

LLOQ QC (102.00%), LQC (100.79%), MQC1 (98.22%), MQC2 (104.74%) and HQC (105.43%).

Table 6.15: Recovery of Naproxen from Human Plasma

	LQC Response		MQC2 Response		HQC Response	
	Extracted QC	Non-Extracted QC	Extracted QC	Non-Extracted QC	Extracted QC	Non-Extracted QC
REC QC	LQC (07-12)	LQC (1-6)	MQC-2 (07-12)	MQC-2 (1-6)	HQC (07-12)	HQC (1-6)
1	33433	52941	1550832	1967088	2698920	3088382
2	37090	50427	1532466	1939362	2837637	3148415
3	40116	50399	1560886	1847800	2728943	3070512
4	39507	52836	1649310	1902777	2763729	3093017
5	39031	50434	1520889	1934602	2681846	3093959
6	36437	49834	1472106	1909266	2523938	3035213
Mean	**37602.3**	**51145.2**	**1547748.2**	**1916815.8**	**2705835.5**	**3088249.7**
SD	**2489.63**	**1369.77**	**58607.51**	**40912.69**	**104849.67**	**36885.56**
CV%	**7.62**	**2.68**	**3.79**	**2.13**	**3.87**	**1.19**
N	**6**	**6**	**6**	**6**	**6**	**6**
% Recovery	**73.52**		**80.75**		**87.62**	

Overall Recovery	
Mean	**80.63**
SD	**7.049**
%CV	**8.74**
% Difference	**3**

Table 6.16: Recovery of Zidovudine from Human Plasma

Extracted QC ID	IS Response (Extracted) (Area)	ID (Non-Extracted QC)	IS Response (Non-Extracted) (Area)
LQC-7	1092765	NON-EXTRACTED-LQC-1	1588545
LQC-8	1173311	NON-EXTRACTED-LQC-2	1524776
LQC-9	1298426	NON-EXTRACTED-LQC-3	1524740
LQC-10	1276762	NON-EXTRACTED-LQC-4	1570785
LQC-11	1282419	NON-EXTRACTED-LQC-5	1584734
LQC-12	1089731	NON-EXTRACTED-LQC-6	1492084
MQC2-7	1268416	NON-EXTRACTED-MQC-2-1	1548775
MQC2-8	1266795	NON-EXTRACTED-MQC-2-2	1514312
MQC2-9	1298594	NON-EXTRACTED-MQC-2-3	1483775
MQC2-10	1295421	NON-EXTRACTED-MQC-2-4	1517233
MQC2-11	1263348	NON-EXTRACTED-MQC-2-5	1539858
MQC2-12	1160444	NON-EXTRACTED-MQC-2-6	1505010
HQC-7	1258631	NON-EXTRACTED-HQC-1	1531085
HQC-8	1371751	NON-EXTRACTED-HQC-2	1547946
HQC-9	1332720	NON-EXTRACTED-HQC-3	1478946
HQC-10	1311311	NON-EXTRACTED-HQC-4	1554364
HQC-11	1297781	NON-EXTRACTED-HQC-5	1526001
HQC-12	1215258	NON-EXTRACTED-HQC-6	1496290
Mean	**1252993.6**	**Mean**	**1529403.3**
SD	**77639.72**	**SD**	**32420.64**
CV%	**7.20**	**CV%**	**2.12**
N	**18**	**N**	**18**
% Recovery	**81.93**		

For recovery evaluation, blank plasma samples were processed and reconstituted with final dilutions of MQC2, LQC, and HQC along with the internal standard to represent complete extraction of both analyte and internal standard. Extracted quality control samples at MQC2, LQC, and HQC levels were also prepared

and injected. The recovery of Naproxen was assessed by comparing the peak responses of extracted samples with those of corresponding non-extracted samples. Similarly, the internal standard response in extracted samples was compared with its response in non-extracted quality control samples.

The mean overall recovery of Naproxen was found to be **80.63%**, with accuracy ranging from **1.19% to 7.62%**. The mean recovery of the internal standard, Zidovudine, was **81.93%**, with precision ranging from **2.12% to 7.20%**.

Table 6.17: Concentration Response Linearity Data of Naproxen for Dilution Integrity

	Concentration (ng/mL)									
	STD-A	**STD-B**	**STD-C**	**STD-D**	**STD-E**	**STD-F**	**STD-G**	**STD-H**	**STD-I**	**STD-J**
Max	600.1	1150.2	2875.6	5751.0	11502.2	23004.3	46008.5	69019.7	92027.2	115032.8
Min	400.1	850.2	2125.4	4250.8	8501.6	17003.1	34007.3	51014.5	68019.4	85024.2
CC	500.1	1000.2	2500.5	5000.9	10001.9	20003.7	40007.4	60017.1	80022.8	100028.5
2	508.6	973.0	2459.2	5114.9	9467.5	20337.7	37515.6	62072.4	80577.8	106217.1
% Nominal	**101.70**	**97.28**	**98.35**	**102.28**	**94.65**	**101.66**	**93.77**	**103.42**	**100.69**	**107.19**

	Slope	**Intercept**	**r^2**
CC 2	0.0000	-0.0021	0.9980

Table 6.18: Dilution Integrity Data for Naproxen (2 Times and 4 Times)

2Times Dilution		**4 Times Dilution**	
Concentration (ng/mL)		Concentration (ng/mL)	
Max	**185387.2**	**Max**	185387.2
Min	**137025.4**	**Min**	137025.4
QC	**161207.3**	**QC**	161207.3
1	156595.8	1	**144679.3**
2	157529.5	2	**146832.1**
3	153664.8	3	**146900.3**
4	157172.5	4	**144665.6**
5	156297.8	5	**146433.0**
6	155162.1	6	**147700.2**
Mean	**156070.42**	**Mean**	146201.75
S.D	**1434.104**	**S.D**	1253.934
C.V %	**0.92**	**C.V %**	0.86
%Nominal	**97.81**	**%Nominal**	90.69
N	6	N	6

Naproxen 12 dilution samples were created by spiking of 1.61 times maximum standard concentration (161207.3ng/mL) For dilution integrity assessment, **twelve sample sets** were generated at two dilution levels, namely **two-fold and four-fold dilutions**. These QC samples were analyzed in the same run along with freshly processed, undiluted calibration standards. The calibration range selected was consistent with the range previously used for evaluating precision and accuracy in **PA Batch-2.** The concentrations at quality control were determined with the correct dilution factor. Naproxen values are satisfactory for dilution integrity (2x and 4x dilution). The precision as well as accuracy of the method for naproxen was 0.92% and 97.81% for dilution factor 2. Similarly, the precision (0.86%) as well as accuracy (90.69%) of Naproxen for a dilution factor of 4 was.

Table 6.19: Assessment of Naproxen Linearity for Ruggedness Studies

	Concentration (ng/mL)									
	STD-A	**STD-B**	**STD-C**	**STD-D**	**STD-E**	**STD-F**	**STD-G**	**STD-H**	**STD-I**	**STD-J**
Max	600.1	1150.2	2875.6	5751.0	11502.2	23004.3	46008.5	69019.7	92027.2	115032.8
Min	400.1	850.2	2125.4	4250.8	8501.6	17003.1	34007.3	51014.5	68019.4	85024.2
CC	500.1	1000.2	2500.5	5000.9	10001.9	20003.7	40007.4	60017.1	80022.8	100028.5
3	511.2	955.4	2534.6	5067.6	9211.4	20140.8	37441.4	60812.4	84279.7	106584.1
% Nominal	102.21	95.52	101.36	101.33	92.10	100.69	93.59	101.33	105.32	107.55

	Slope	**Intercept**	**r^2**
CC 3	0.0000	-0.0015	**0.9972**

Table 6.20: Precision and Accuracy (Within Batch) of Naproxen for Ruggedness

	Conc. (ng/mL)				
	LLOQ QC	**LQC**	**MQC1**	**MQC2**	**HQC**
Max	**600.5**	**1725.1**	**17250.8**	**57502.5**	**97760.1**
Min	**400.3**	**1275.1**	**12750.6**	**42501.9**	**72257.5**
QC	**500.4**	**1500.1**	**15000.7**	**50002.2**	**85008.8**
13	529.8	1759.2	13705.7	50624.6	94965.8
14	517.3	1591.7	13687.4	49718.4	87957.6
15	502.3	1568.6	14460.6	51097.5	88244.0
16	507.7	1535.7	14101.1	53237.7	87677.8
17	482.0	1657.9	14145.5	52161.9	92733.6
18	519.5	1433.8	13683.6	50960.1	84965.3

Mean	**509.60**	**1591.15**	**13963.82**	**51299.70**	**89423.85**
S.D.	**17.551**	**110.489**	**322.724**	**1233.551**	**3691.624**
C.V.%	**3.25**	**7.94**	**2.31**	**2.40**	**4.13**
% Nominal	**101.84**	**107.07**	**93.09**	**102.59**	**105.19**
N	**6**	**6**	**6**	**6**	**6**

A precision and accuracy batch (PA Batch-3) was carried out and evaluated by a different analyst using a separate column of the same type along with freshly prepared solutions.

Within-batch precision:

LLOQ QC (3.25%), LQC (7.94%), MQC1 (2.31%), MQC2 (2.40%) and HQC (4.13%).

Within-batch accuracy:

LLOQ QC (101.84%), LQC (107.07%), MQC1 (93.09%), MQC2 (102.59%) and HQC (105.19%).

Table 6.21:Room Temperature Stock Solution Stability of Naproxen (0, 9 hours)

Stock ID	Hours	%Stability
NST-01A	0	100.00
NST-02	9	99.38

Sr. No.	Standard stock (Stability) (NST-01A)	Standard stock (Comparison) (NST-02)
	Area	Area
1	1538309	1564927
2	1494898	1555587
3	1555924	1502145
4	1508922	1580233
5	1510631	1511923
6	1560654	1513871
Mean	**1528223.0**	**1538114.3**
SD	**27252.18**	**32758.86**
CV%	**1.78**	**2.13**
N	**6**	**6**
% Stability	**99.38**	

Table 6.22: Stock Solution Stability (Room Temperature) of Zidovudine (0, 9 hours)

Stock ID	Hours	%Stability
ZST-01A	0	100.00
ZST-02	9	97.93

Table 6.23: Stability Data

Sr. No.	Stability standard stock (ZST-01A)	Comparison standard stock (ZST-02)
	Area	Area
1	1622912	1717794
2	1543892	1582247
3	1644842	1575651
4	1521200	1689066
5	1553091	1576624
6	1615555	1611247
Mean	**1583582.0**	**1625438.2**
SD	**50434.95**	**62460.47**
CV%	**3.18**	**3.84**
N	**6**	**6**
% Stability	**97.93**	

Percentage Stability was calculated using the following equation:

$$\text{Stability (\%)} = \frac{\text{Mean response of stability sample} \times \text{Concentration of comparison stock} \times 100}{\text{Mean response of comparison sample} \times \text{Concentration of stability stock}}$$

Plagiarism-free rewritten text:

Stock solution stability of Naproxen and Zidovudine was assessed at room temperature $(20\pm5^{\circ}C)$for a period of 9 hours. For this purpose, six replicate dilutions were prepared and analyzed. Naproxen was diluted to a concentration equivalent to the MQC2 quality control level, while Zidovudine was prepared at its final working

concentration. The mean peak area responses obtained after 9 hours were compared with those of freshly prepared reference solutions.

For Naproxen, the precision values at 0 hour and 9 hours were 1.78% and 2.13%, respectively, and the calculated stability was 99.38%. In the case of Zidovudine, the precision at 0 hour and 9 hours was 3.18% and 3.84%, respectively, with an observed stability of 97.93%.

Similarly, the stability of spiking solutions was evaluated under the same room temperature conditions for 9 hours. Six replicate samples were prepared using Naproxen at MQC2 level and Zidovudine at working concentration. The response obtained from the stored spiking solutions was compared with freshly prepared solutions.

The spiking solution of Naproxen showed precision values of 1.97% at 0 hour and 2.13% at 9 hours, with stability calculated as 98.77%. For Zidovudine, the precision values were 3.86% and 3.84% at 0 hour and 9 hours, respectively, and the stability was found to be 99.64%.

Table 6.24: Stock Solution Stability (Refrigerated) of Naproxen at 2 - 8 °C (7 Days)

	Stability standard stock (NST-01)	**Comparison standard stock (NST-05)**
Sr. No.	**4017081.6000**	**4003133.4000**
	Area	**Area**
1	2075632	2109236
2	2138027	2067963
3	2108116	2047272
4	2085039	2103630
5	2182222	2093129
6	2125604	1984713
Mean	**2119107.7**	**2067657.2**
SD	**38872.06**	**46833.84**
%CV	**1.83**	**2.27**
N	**6**	**6**
% Stability	**102.13**	

Calculations:

$$\% \text{ Stability} = \frac{\text{Mean response of stability samples} \times \text{Concentration of comparison stock} \times 100}{\text{Mean response of comparison stock samples} \times \text{Concentration of stability stock}}$$

Table 6.25: Refrigerated Stock Solution Stability of Zidovudine at 2 - 8 °C (7 Days)

	Stability standard stock (-ZST-01)	**Comparison of standard stock (-ZST-04)**
Sr. No.	**Area**	**Area**
1	1497010	1553992
2	1574951	1509412
3	1537403	1464847
4	1554397	1503910
5	1659259	1518058
6	1579600	1470748
Mean	**1567103.3**	**1503494.5**
SD	**54160.97**	**32751.32**
%CV	**3.46**	**2.18**
N	**6**	**6**
% Stability	**104.14**	

Calculations:

The percentage stability was determined using the following expression:

$$\% \text{ Stability} = \frac{\text{Mean response of stability samples} \times \text{Concentration of comparison stock} \times 100}{\text{Mean response of comparison stock samples} \times \text{Concentration of stability stock}}$$

Stability Study of Stock Solutions:

The stability of Naproxen stock solution was evaluated by analyzing six replicate injections prepared at a concentration equivalent to the MQC2 level. The stock solution remained stable over a period of 7 days under the specified conditions. The

precision values were found to range between 1.83% and 2.27%, while the calculated stability was 102.13%.

Similarly, the refrigerated stock solution of Zidovudine (internal standard) was assessed by injecting six replicate samples at the final working concentration. The solution demonstrated stability for up to 7 days. The precision values ranged from 2.18% to 3.46%, and the overall stability was determined to be 104.14%.

The stability results for both Naproxen and Zidovudine were established by comparing their responses with those obtained from freshly prepared standard stock solutions.

Table 6.26: Concentration–Response Linearity of Naproxen under Various Stability Conditions (Autosampler, Bench-Top, Wet Extract, Freeze–Thaw, and Short-Term at –20°C)

	Concentration (ng/mL)									
	STD-A	**STD-B**	**STD-C**	**STD-D**	**STD-E**	**STD-F**	**STD-G**	**STD-H**	**STD-I**	**STD-J**
Max	**600.7**	**1151.5**	**2878.7**	**5757.4**	**11514.7**	**23029.6**	**46059.0**	**69054.1**	**92072.1**	**115090.0**
Min	**400.5**	**851.1**	**2127.7**	**4255.4**	**8510.9**	**17021.8**	**34043.6**	**51040.0**	**68053.3**	**85067.6**
FRST CC	**500.6**	**1001.3**	**2503.2**	**5007.4**	**10012.8**	**20025.7**	**40051.3**	**60047.0**	**80062.7**	**100078.3**
	499.7	981.6	2653.7	5211.8	9602.9	18094.1	38137.0	59692.2	80317.4	110907.6
% Nominal	**99.83**	**98.03**	**107.01**	**104.10**	**95.91**	**90.35**	**95.22**	**99.41**	**100.32**	**110.82**

	Slope	**Intercept**	**r^2**
FRST CC 1	0.0000	-0.0019	0.9958

Table 6.27 Precision and Accuracy (within Batch) of Naproxen for Stabilities (Freshly Spiked QCs)

	Concentration (ng/mL)			
	LQC	**MQC1**	**MQC2**	**HQC**
Max	**1725.5**	**17254.6**	**57515.2**	**97815.0**
Min	**1275.3**	**12753.4**	**42511.2**	**72298.0**
FRST QC	**1500.4**	**15004.0**	**50013.2**	**85057.5**
1	1301.7	14283.3	45840.1	80314.2

2	1738.4	13748.5	46460.8	91991.5
3	1769.8	13737.1	46517.2	87214.4
4	1415.9	13341.2	49301.7	90407.4
5	1327.4	14393.5	46011.3	84613.0
6	1512.0	17007.9	50768.8	76965.7
Mean	**1510.87**	**14418.42**	**47483.15**	**85251.03**
S.D.	**202.607**	**1327.578**	**2047.889**	**5818.986**
C.V.%	**13.41**	**9.20**	**4.31**	**7.83**
% Nominal	**100.70**	**97.10**	**94.94**	**100.23**
N	**6**	**6**	**6**	**6**

Table 6.28: Freeze Thaw Stability (4 Cycles) of Naproxen:

	Concentration (ng/mL)	
	LQC	**HQC**
Max	**1725.1**	97760.1
Min	**1275.1**	72257.5
QC	**1500.1**	85008.8
89	1337.7	**96607.4**
90	1708.7	**87421.0**
91	1685.4	**93317.2**
92	1730.6	**85088.1**
93	1650.2	**96262.6**
94	1611.1	**100924.8**
Mean	**1620.62**	93269.85
S.D.	**144.955**	5997.326
C.V.(%)	**8.94**	7.43
% Nominal	**108.03**	109.72
N	6	6

Naproxen stability was tested during four freeze-thaw cycles in human plasma. After four cycles of freeze-thaw, and HQC and LQC analysed in six repetitions. Freeze–thaw stability was assessed by analysing QC samples against freshly prepared

calibration standards covering the same concentration range used for precision and accuracy evaluation. After four freeze–thaw cycles, the percent nominal values were observed between 108.03% and 109.72%, while precision ranged from 7.43% to 8.94%.

Table 6.29: Bench Top Stability Data of Naproxen for 9 hours

	Concentration (ng/mL)	
	LQC	**HQC**
Max	**1725.1**	97760.1
Min	**1275.1**	72257.5
QC	**1500.1**	85008.8
107	1628.7	**71633.4**
108	1577.0	**85683.6**
109	1765.5	**82179.0**
110	1650.9	**86495.4**
111	1643.9	**83299.8**
112	1760.2	**84142.9**
Mean	**1671.03**	82239.02
S.D.	**75.698**	5427.135
C.V.(%)	**4.53**	7.60
% Nominal	**111.39**	97.74
N	6	6

Bench-top stability of Naproxen was evaluated for **9 h** using six replicate sets each of **LQC and HQC** samples. These quality control samples were analyzed against freshly prepared calibration standards, maintaining a concentration range similar to that used during precision and accuracy assessment. Naproxen was steady for up to 9 hours [20]. The percent nominal was 9 hours between 97.74% and 111.39%. The precision was 4.53% to 7.60% in 9 hours.

Table 6.30: Auto sampler Stability Data of Naproxen for 49 hours

	Concentration (ng/mL)	
	LQC	**HQC**
Max	**1725.1**	**97760.1**
Min	**1275.1**	**72257.5**
QC	**1500.1**	**85008.8**
95	1417.1	81511.0
96	1477.7	89590.8
97	1405.9	103103.9
98	1589.2	91251.3
99	1508.3	105968.0
100	1590.6	90521.1
Mean	**1498.13**	**93657.68**
S.D.	**80.545**	**9170.444**
C.V.(%)	**5.38**	**9.79**
% Nominal	**99.87**	**110.17**
N	**6**	**6**

Autosampler stability of Naproxen was examined using six replicate sets of LQC and HQC samples. After preparation, the samples were kept in the autosampler and analyzed after **49 hours**. Quantification was performed using freshly prepared calibration curve standards with a concentration range similar to that applied for precision and accuracy evaluation. The results showed that the treated samples remained stable for 49 hours. At 49 hours, the percent nominal was between 99.87% and 110.17% and precision was between 5.38% and 9.79%.

Table 6.31: Short Term Stability Data of Naproxen at –20 ºC(6 Days)

	Concentration (ng/mL)	
	LQC	**HQC**
Max	**1725.1**	**97760.1**
Min	**1275.1**	**72257.5**
QC	**1500.1**	**85008.8**
113	1572.3	96465.1
114	1645.8	93643.5
115	1618.4	97387.5
116	1609.1	83092.5
117	1720.2	96142.2
118	1681.5	96047.1
Mean	**1641.22**	**93797.15**
S.D.	**53.261**	**5388.483**
C.V. (%)	**3.25**	**5.74**
% Nominal	**109.41**	**110.34**
N	**6**	**6**
% Stability	**108.63**	**110.02**

Calculation:

Short-term stability of Naproxen in plasma was evaluated after temporary storage at **–20°C for 6 days**. Six replicate sets each of LQC and HQC samples were analyzed against freshly prepared calibration standards. Stability was calculated by comparing the stored QC samples with freshly spiked QC samples.

The observed stability ranged from **108.63% to 110.02%**, while precision values were between **3.25% and 5.74%**.

Table 6.32: Concentration-response Linearity Data of Naproxen for Reinjection Stability

	Concentration (ng/mL)									
	STD-A	**STD-B**	**STD-C**	**STD-D**	**STD-E**	**STD-F**	**STD-G**	**STD-H**	**STD-I**	**STD-J**
Max	**600.1**	**1150.2**	**2875.6**	**5751.0**	**11502.2**	**23004.3**	**46008.5**	**69019.7**	**92027.2**	**115032.8**
Min	**400.1**	**850.2**	**2125.4**	**4250.8**	**8501.6**	**17003.1**	**34007.3**	**51014.5**	**68019.4**	**85024.2**
CC	**500.1**	**1000.2**	**2500.5**	**5000.9**	**10001.9**	**20003.7**	**40007.4**	**60017.1**	**80022.8**	**100028.5**
1	515.6	960.1	2433.3	4834.7	9531.1	19763.1	36792.9	63394.4	85427.2	108505.4
% Nominal	**103.11**	**95.99**	**97.31**	**97.68**	**95.29**	**98.80**	**91.97**	**105.63**	**107.75**	**108.47**

	Slope	Intercept	r^2
FRRE CC 1	0.0000	-0.0023	0.9962

Table 6.33: Reinjection Stability Data for Naproxen (0 Hours)

	Concentration (ng/mL)	
	LQC	**HQC**
Max	**1725.1**	97760.1
Min	**1275.1**	72257.5
QC	**1500.1**	85008.8
1	1707.2	**93187.2**
2	1844.4	**96187.8**
3	1539.0	**94139.3**
4	1561.8	**96344.6**
5	1459.1	**94301.3**
6	1510.0	**99924.9**
Mean	**1603.42**	95680.85
S.D.	**144.273**	2417.913
C.V. (%)	**9.00**	2.53
% Nominal	**107.89**	112.55
N	6	6

Table 6.34: Reinjection Stability Data forNaproxen (24 Hours)

	Concentration (ng/mL)	
	LQC	**HQC**
Max	**1725.1**	97760.1
Min	**1275.1**	72257.5
QC	**1500.1**	85008.8
1	1227.1	**80867.7**
2	1357.1	**79423.3**
3	1547.6	**84195.1**
4	1515.9	**82938.7**
5	1599.7	**88015.3**
6	1649.6	**89534.2**
Mean	**1482.67**	84162.22
S.D.	**160.298**	3962.835
C.V. (%)	**10.81**	4.71
% Nominal	**98.84**	99.00
N	**6**	6
% Stability	92.47	87.96

Calculations:

Reinjection stability was evaluated by processing and analyzing six replicate sets of LQC and HQC samples along with calibration curve standards. The QC samples were kept in the auto sampler and reinjected after **24 hours**. Their responses were calculated using the initial calibration curve data. The mean response of reinjected QC samples was compared with the mean response obtained during the first injection. The findings confirmed that the samples remained stable for **24 hours** after reinjection. The percentage stability ranged from **87.96% to 92.47%**, while accuracy varied between **4.71% and 10.81%**.

Table 6.35 Concentration-response Linearity Data of Naproxen for Concomitant drug Effect

	Concentration (ng/mL)									
	STD-A	**STD-B**	**STD-C**	**STD-D**	**STD-E**	**STD-F**	**STD-G**	**STD-H**	**STD-I**	**STD-J**
Max	**600.1**	**1150.2**	**2875.6**	**5751.0**	**11502.2**	**23004.3**	**46008.5**	**69019.7**	**92027.2**	**115032.8**
Min	**400.1**	**850.2**	**2125.4**	**4250.8**	**8501.6**	**17003.1**	**34007.3**	**51014.5**	**68019.4**	**85024.2**
CC	**500.1**	**1000.2**	**2500.5**	**5000.9**	**10001.9**	**20003.7**	**40007.4**	**60017.1**	**80022.8**	**100028.5**
3	511.2	955.4	2534.6	5067.6	9211.4	20140.8	37441.4	60812.4	84279.7	106584.1
% Nominal	**102.21**	**95.52**	**101.36**	**101.33**	**92.10**	**100.69**	**93.59**	**101.33**	**105.32**	**107.55**

	Slope	**Intercept**	**r^2**
CC 3	0.0000	-0.0015	0.9972

Table 6.36: within Batch Precision and Accuracy of Naproxen for Concomitant Drug Effect

	Concentration (ng/mL)		
	LLOQ QC	**LQC**	**HQC**
Max	**601.7**	**1727.5**	**97875.2**
Min	**401.1**	**1277.1**	**72342.6**
CCDE QC	**501.4**	**1501.3**	**85108.9**
1	517.8	1528.9	95353.2
2	557.2	1594.8	90850.0
3	517.3	1419.2	87904.7
4	485.9	1577.3	88955.5
5	537.7	1304.2	84769.6
6	517.6	1579.3	91049.2
Mean	**521.42**	**1500.45**	**89813.70**
S.D.	**23.561**	**115.583**	**3552.750**
C.V.%	**4.52**	**7.70**	**3.96**
% Nominal	**103.99**	**99.94**	**105.53**
N	**6**	**6**	**6**

Within-batch precision for concomitant drug-spiked QCs:

LLOQ QC (4.52%), LQC (7.70%) and HQC (3.96%).

Within-batch accuracy for concomitant drug-spiked QCs:

LLOQ QC (103.99%), LQC (99.94%) and HQC (105.53%).

Table 6.37: Wetextractions Stability of Naproxen for 41 hours

	Concentration (ng/mL)	
	LQC	**HQC**
Max	**1725.1**	97760.1
Min	**1275.1**	72257.5
QC	**1500.1**	85008.8
101	1760.7	**86918.1**
102	1757.6	**99684.6**
103	1634.5	**98280.7**
104	1515.0	**89364.4**
105	1451.8	**100693.8**
106	1784.4	**81477.6**
Mean	**1650.50**	92737.53
S.D.	**141.017**	7928.689
C.V. (%)	**8.54**	8.55
% Nominal	**110.03**	109.09
N	6	6

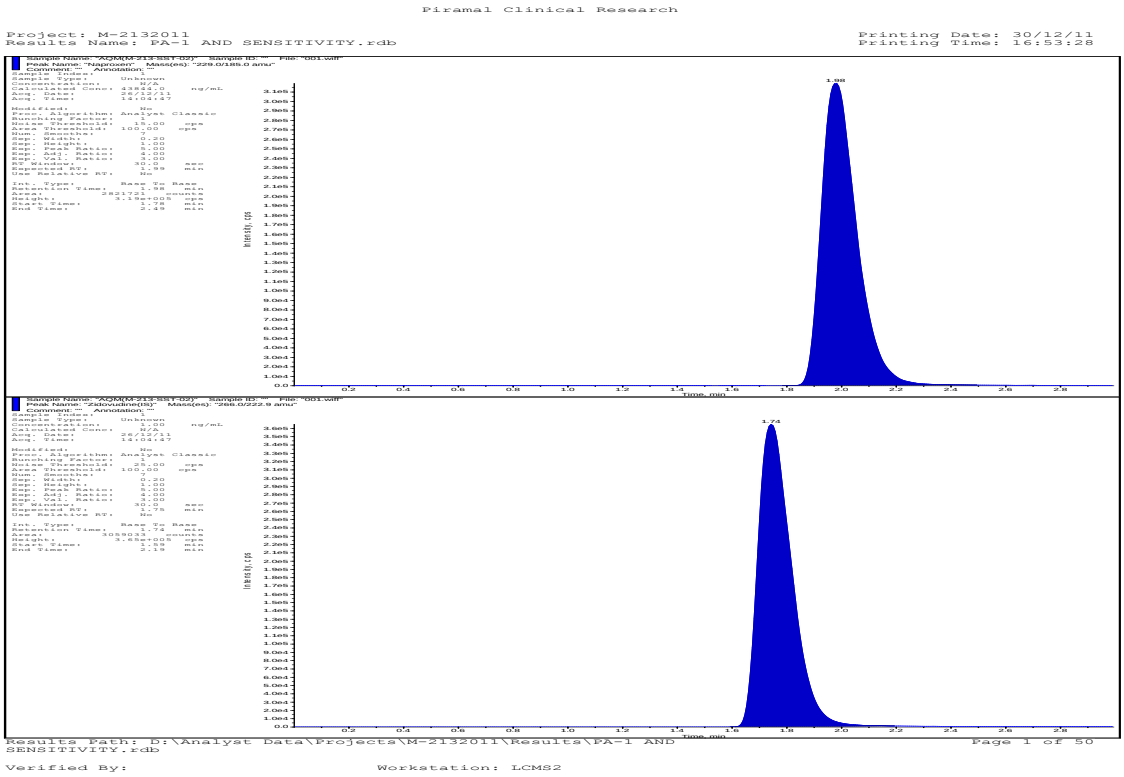


Fig 6.1: A Chromatogram of an Aq. Standard and Internal Standard (Mixture of Naproxen)

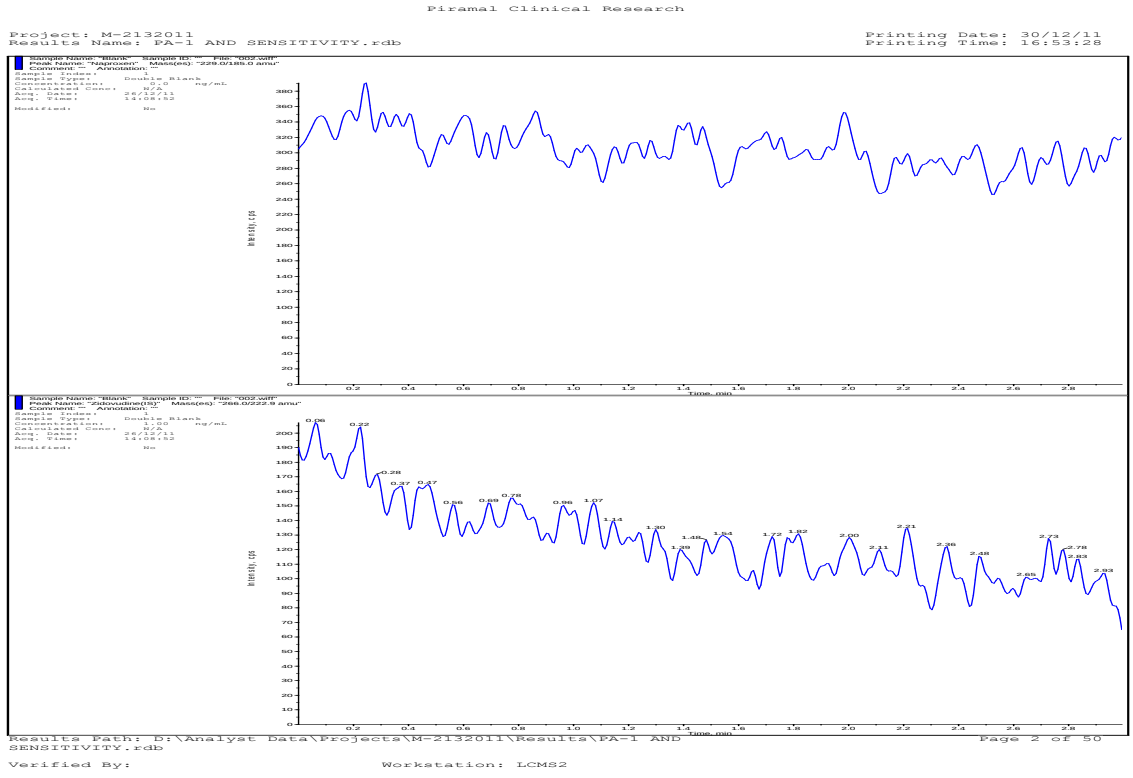


Fig 6.2: -Representative Chromatogram of Plasma (Blank) Sample of Naproxen

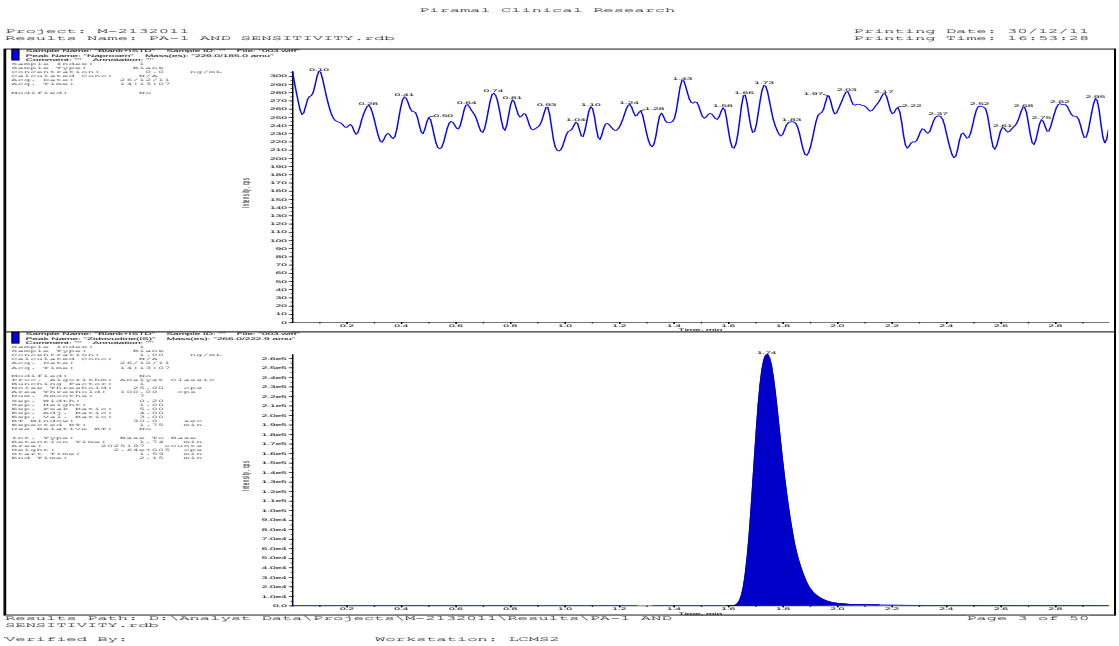


Fig 6.3: A Chromatogram of Plasma (Blank) with Internal Standard Sample of Naproxen

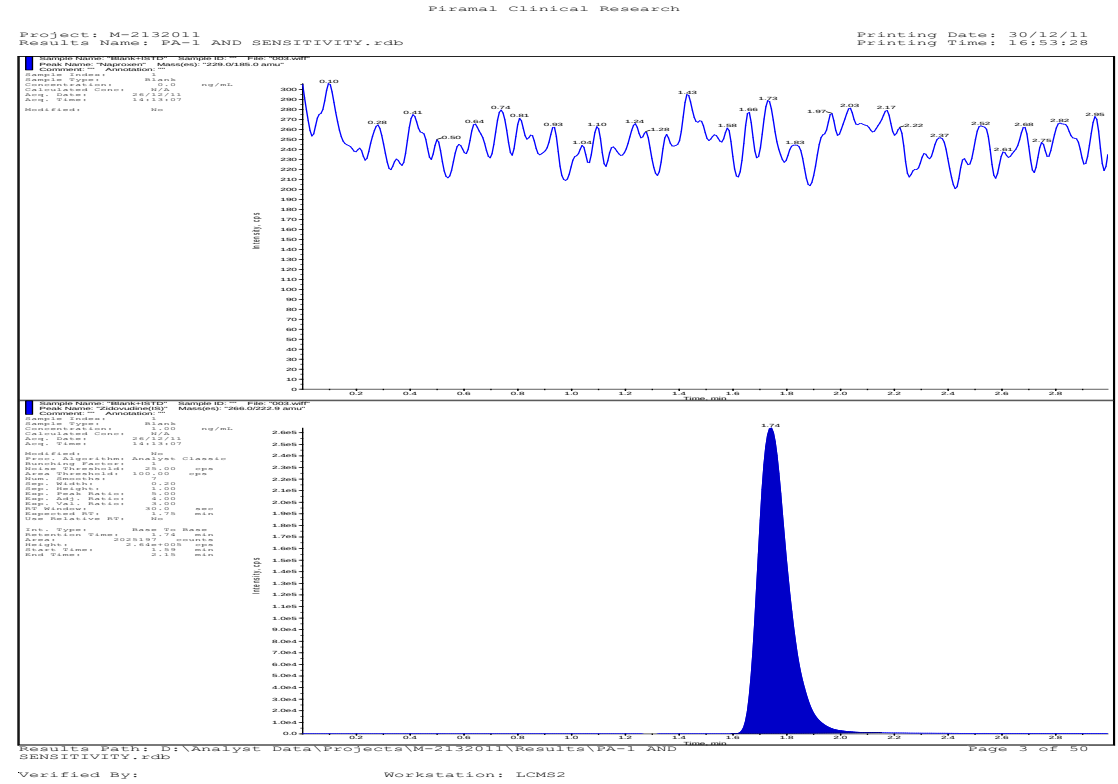


Fig 6.4: A Chromatogram of LLOQ QC Sample of Naproxen

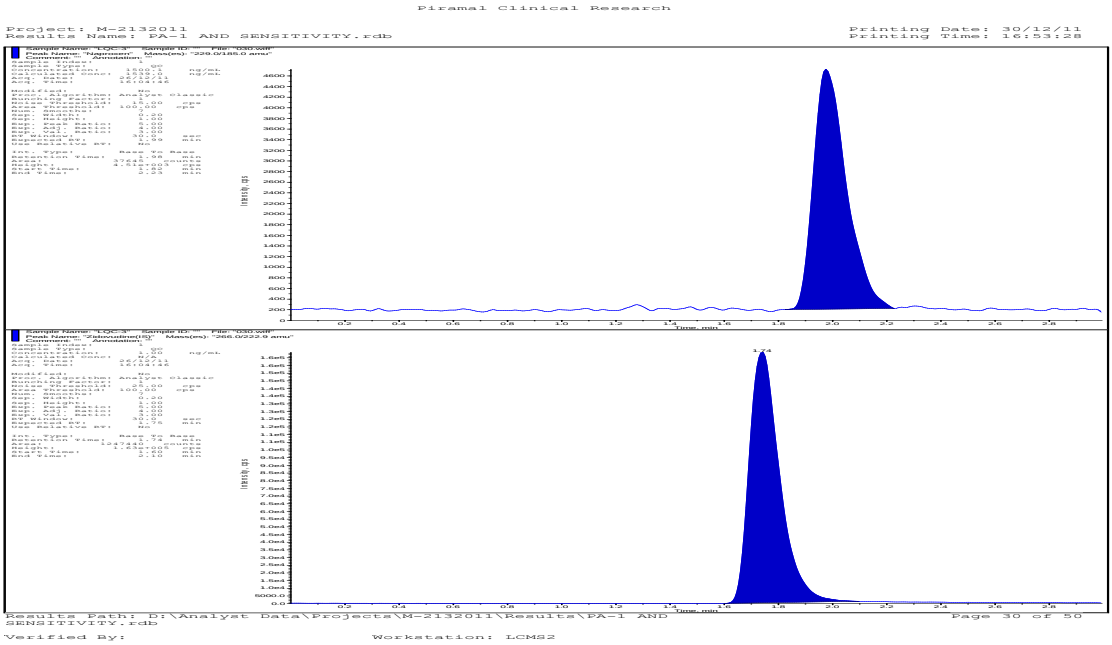


Fig Fig 6.5:A Chromatogram of LQC Sample of Naproxen

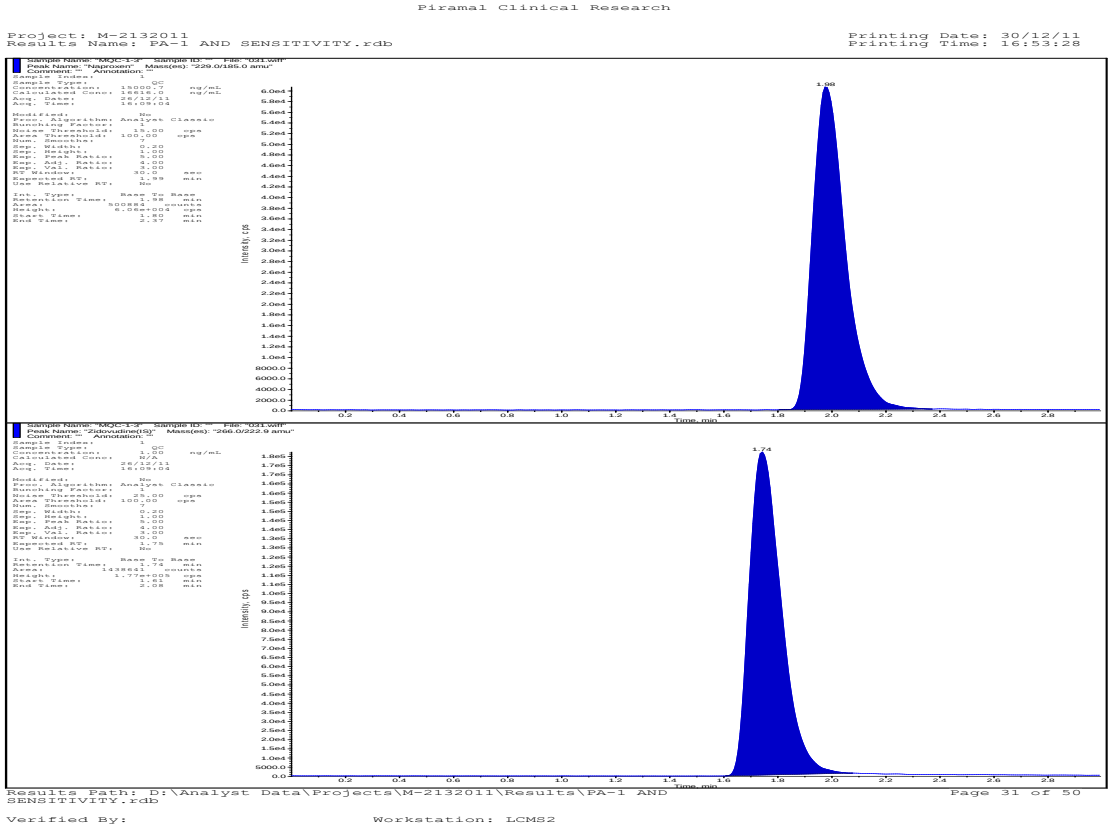


Fig 6.6: Chromatogram of MQC1 Sample of Naproxen

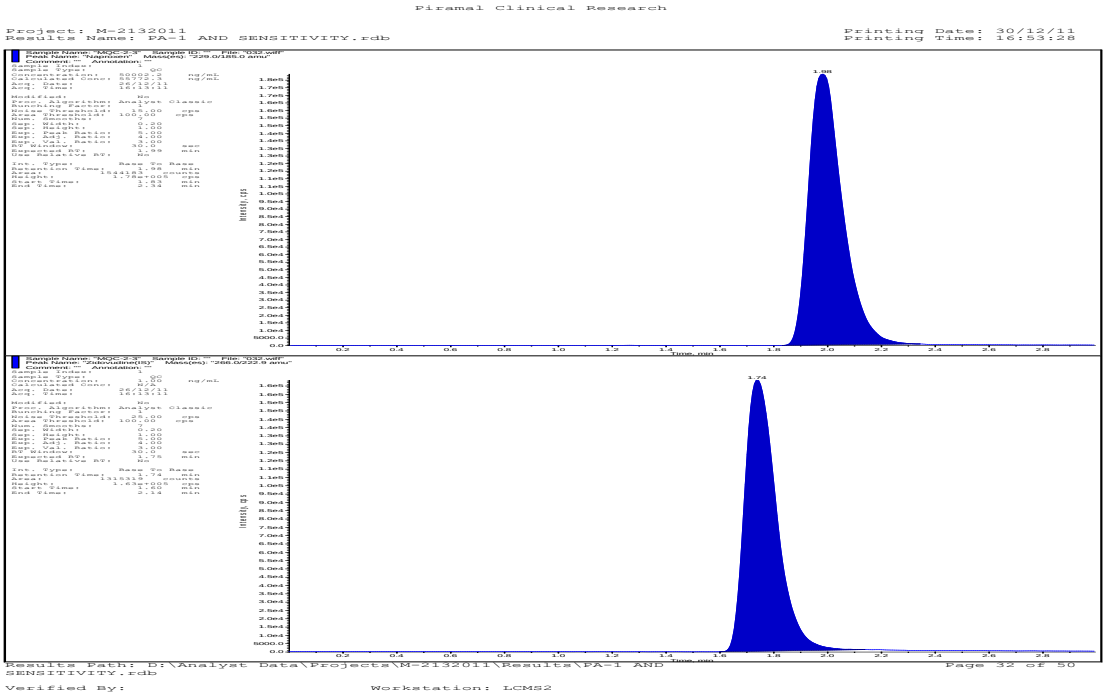


Fig 6.7: Chromatogram of MQC2 Sample of Naproxen

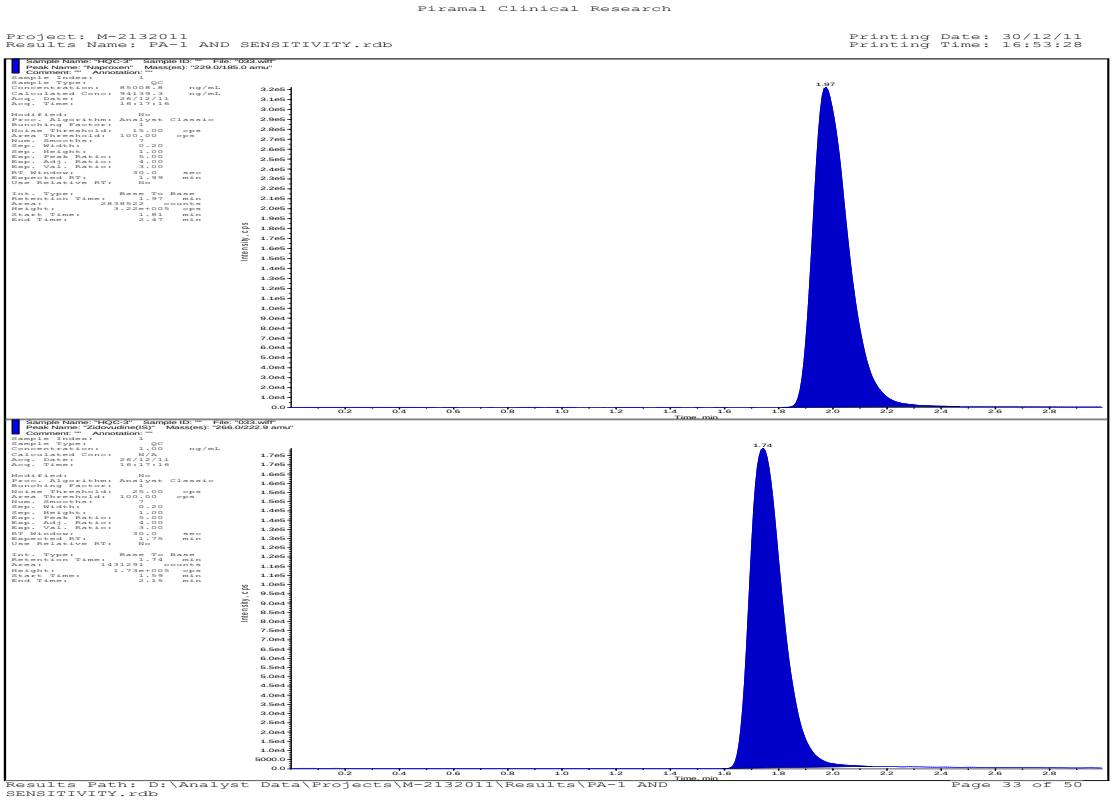


Fig 6.8: A Chromatogram of a HQC Sample of Naproxen

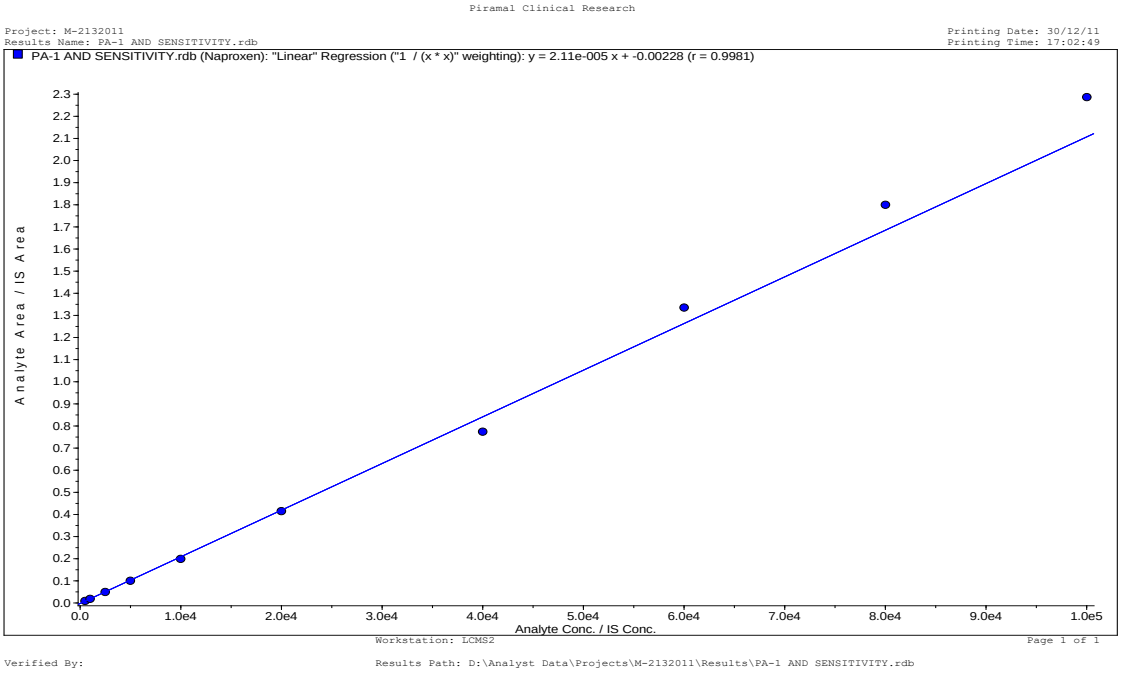


Fig 6.9:-A Calibration Curve of Naproxen (for Regression Analysis)

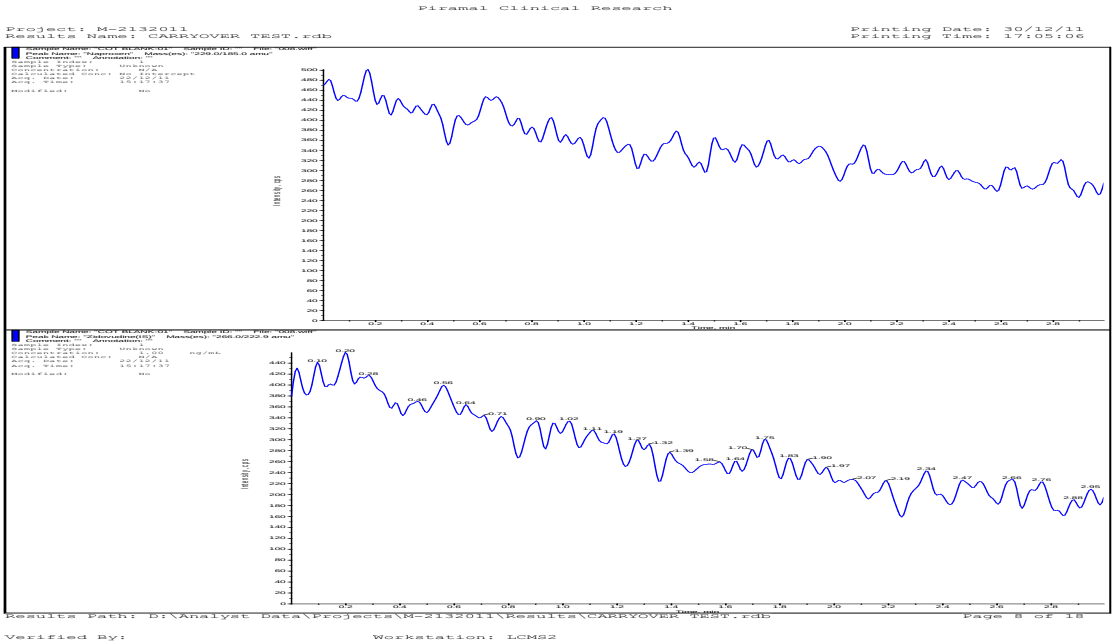


Fig6.10: Chromatographic profile of extracted blank plasma used in carryover assessment

6.14 Conclusion

An HPLC-MS/MS technique operating in negative ion mode was established and confirmed to be accurate for estimating Naproxen concentrations in person plasma by means of Zidovudine as an “Internal standard (ISTD)”. We used a liquid extraction approach to prepare the samples. A 4.675 mm, 3.5 µm Agilent Technologies Zorbax Eclipse XDB phenyl column was employed to deposit the chromatographed materials. The mobile phase was a mixture of 90:10 v/v acetonitrile and 20 mM ammonium acetate.

For Naproxen values between 500.1 ng/mL and 100028.5 ng/mL, the method was acquired to be effective. This validation report presents the finding of the selectivity and matrix effects. A number of “stabilities, proof of accuracy and precision, data on recovery, evidence of dilution integrity, data on calibration standards and quality control samples”, and more[21].In accordance with the parameters specified in the "Guidance for Industry—Bioanalytical Method Validation (May 2001)" document by the USFDA and CDER, this report details the outcomes for "selectivity, matrix effect, sensitivity, linearity, precision, accuracy, stabilities, recovery, dilution integrity, and concurrent drug effect" that are considered acceptable for bioanalytical batches[22].

Within the concentration range of 500.1 ng/mL to 100028.5 ng/mL, the aforementioned analytical method, when used in negative ion mode, may detect “Naproxen m/z - 229.00 (parent) and 185.00 (product), in addition to the internal standard Zidovudine m/z - 267.00 (parent) and 222.90 (product)” in human plasma.

Chapter - 7

BIOANALYTICAL METHOD DEVELOPMENT OF DULOXETIN

7.1 DRUG PROFILE

DULOXETINE

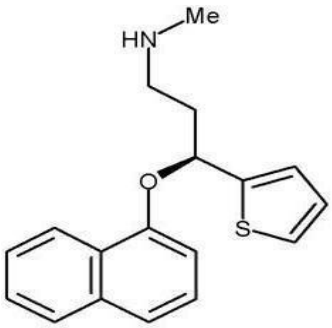
Duloxetine is a selective and balanced serotonin and norepinephrine reuptake inhibitor (SNRI) used for the treatment of Major Depressive Disorder (MDD) and Diabetic Peripheral Neuropathic Pain (DPNP)[1]. It has also been employed for treatment of Stress Urinary Incontinence (SUI). Duloxetine is a strong dual reuptake inhibitor of serotonin (5-hydroxytryptamine, 5-HT) and norepinephrine (NE) with similar affinities for the 5-HT and NE transporter sites [2]. It is, however, a less powerful inhibitor of the re-uptake of dopamine. Brand Name: Cymbalta, Yentreve

Empirical formula: $C_{18}H_{19}NOS \bullet HCl$

Chemical designation: (+) -(S)-N-methyl-γ-(1-naphthyloxy)-2-thio-phene-propylamine hydrochloride.

7.1.1 Physicochemicalproperties

MolecularFormulae: $C1_8H_{19}NOS$



Molecular Weight : 297.42

Pka Value : 9.34

Experimental Log P/Hydrophobicity : 4

Solubility : It is slightly soluble in water

Experimental Water Solubility : 0.00296mg/ml

Physical State : Solid

Dosage form : Enteric coated capsules.

Doses : 20,30and 60mg.

7.1.2 Indications

For the acute and maintenance treatment of MDD, as well as acute management of generalized anxiety disorder [3]. Additionally, it is utilized for the treatment of fibromyalgia and diabetic peripheral neuropathy, two forms of neuropathic pain. Women with mild to severe cases of "Stress Urinary Incontinence (S UI)" have benefited from its usage [4].

7.1.3 Pharmacodynamics

The precise mechanisms of the anti-depressant, central pain inhibitory and anxiolytic activities of duloxetine in humans are not understood, but the sections are thought to be associated with its potential lowering of serotonergic and nor adrenergic activity in the CNS[5]. Duloxetine is a powerful inhibitor of neuronal reuptake of serotonin and norepinephrine and a less potent inhibitor of dopamine reuptake[6]. Preclinical experiments have demonstrated that Duloxetine does not bind significantly to dopaminergic, adrenergic, cholinergic, histaminergic, opioid, glutamate and GABA receptors in vitro. MAO: Duloxetine is not a monoamine oxidase inhibitor.

7.1.4 Mechanism of Action

Duloxetine is a strong dual re-uptake inhibitor of the neurotransmitter's serotonin (5-HT) and norepinephrine (NA), both of which are involved in the regulation of mood and are major mediators in the path physiology of depression [7]. Duloxetine is also thought to act on stress urinary incontinence (SUI) via blocking reuptake of serotonin and nor epinephrine in the spinal cord. This inhibition increases the activity

of the pudendal nerve which in turn increases contractions of the urethral sphincter near the bladder outlet [8] . Duloxetine has been shown to enhance concentrations of 5-HT and NA in the sacral spinal cord in animal experiments, which facilitates greater activity of the external urethral sphincter and prevents urine leakage during the storage phase of the micturition cycle [9]. Crucially serotonin and norepinephrine only have a modulatory effect, as they cannot directly excite the motor neurons. Glutamate is thought to be the primary neurotransmitter and acts as the "on/off" switch for micturition[10]. When glutamate is released, duloxetine augments sphincter activity during urine storage, but when glutamate release is blocked during the voiding phase, the rhabdosphincter can completely relax.

7.1.5 Pharmacokinetics

Absorption

Duloxetine hydrochloride is well absorbed following oral administration. The median time lag (T_{lag}) before onset of absorption is 2 hours, while the peak plasma concentration (C_{max}) of duloxetine is attained at about 6 hours post-dose [11]. Food does not influence the (C_{max}) of duloxetine but increases the duration to achieve peak concentration ($_{Tmax}$) from 6 to 10 hours and slightly decreases the extent of absorption (AUC) by around 10%.

Duloxetine has a delay in absorption of 3 hours and a 33% increase in apparent elimination following an evening dose versus a morning dose [12].

Distribution

Duloxetine is highly attached (>90%) to plasma proteins in human plasma, particularly to albumin and α_1-acid glycoprotein [13].

Metabolism

Duloxetine is extensively metabolized to many metabolites. The principal biotransformation pathways include oxidation of the naphthyl ring, followed by conjugation and additional oxidation. In vitro, both CYP2D6 and CYP1A2 catalyze the oxidation of the naphthyl ring [14].

Plasma metabolites include 4-hydroxy duloxetine glucuronide and 5-hydroxy, 6-methoxy duloxetine sulfate [15–17]. There is no evidence that the primary circulating metabolites contribute significantly to the pharmacological efficacy of duloxetine.

Elimination

Duloxetine is excreted in urine in trace levels (<1% of the dose) as unchanged substance[18]. About 70% of the dose of duloxetine is eliminated in the urine as metabolites of duloxetine and about 20% is eliminated in the feces[19-20].

Pharmacokinetic Parameters:

The elimination half-life of duloxetine is around 12 hours (8 to 17 hours). Here is a list of several pharmacokinetic parameters observed following single-dose oral administration of 30 mg, 60 mg and 90 mg of duloxetine as per literature.

Dose/parameter	C_{max}(ng/ml)	T_{max}(hours)	$T_{1/2}$(hours)
30mg	22.46(15.5)	6.83(1.99)	**12.95(3.64)**
60mg	44.40(17.18)	6.10(1.29)	**12.81(2.31)**
90mg	**60.78(27.84)**	**6.60(1.58)**	**11.66(2.06)**

7.1.6 Assay Method Development

Development/optimization of LC-method:

LC analysis was functioned using a BE- LC-07shimadzu pump system. The HPLC-MS/MS method was optimized to identify and quantify the analyte. Firstly, the method was required to adequately separate the target analyte in one LC-MS/MS run. The method conditions including HPLC, API and MS parameters were optimized for this target analyte. The identification of target analyte was done using the chromatographic retention time (R_t), the MS of target ions and the fragmentation pattern. Good separation was a key in the method's success. Greater chromatographic

resolution allowed for improved signal intensities of these target analytes because enough dwell time was spent on each transition without signal degradation.

1. HPLC-MS/MS conditions were optimized as follows:
2. Selection of HPLC columns
3. Selection and characteristics of mobile phase
4. Development/optimization of multiple reaction monitoring (MRM) transitions for target analyte

Optimization of LC-parameters such as retention time, mobile phase flow rate, gradient or isocratic elution time program, injection volume etc.

The chromatographic trail runs were performed using a medium concentration to get an optimum separation of analytes and intensity for simultaneous analysis. The chromatographic trails performed were mentioned below.

7.2: OPTIMIZATIONOF CHROMATOGRAM FOR DULOXETINE HYDROCHLORIDE

Buffer preparation: Sonicate 1000 ml of Milli-Q water with 385.48 mg of ammonium acetate. Run the mixture through a 0.45µ filter.

Mobile phase: Sonication was used to remove gas from a mixture of 85:15 buffer and HPLC grade acetonitrile.

Diluent: The mixture was sonicated to remove gas, and the proportions of HPLC grade acetonitrile and Milli-Q water were 60:40.

Chromatographic conditions:

Flow rate	**:**	1.00 ml/min (split ratio 50:50)
Column	**:**	ZORBAX SB-C18, 50 x 2.1mm, 5µm
Sample cooler temperature	**:**	5^0C
Injection volume	**:**	20µl

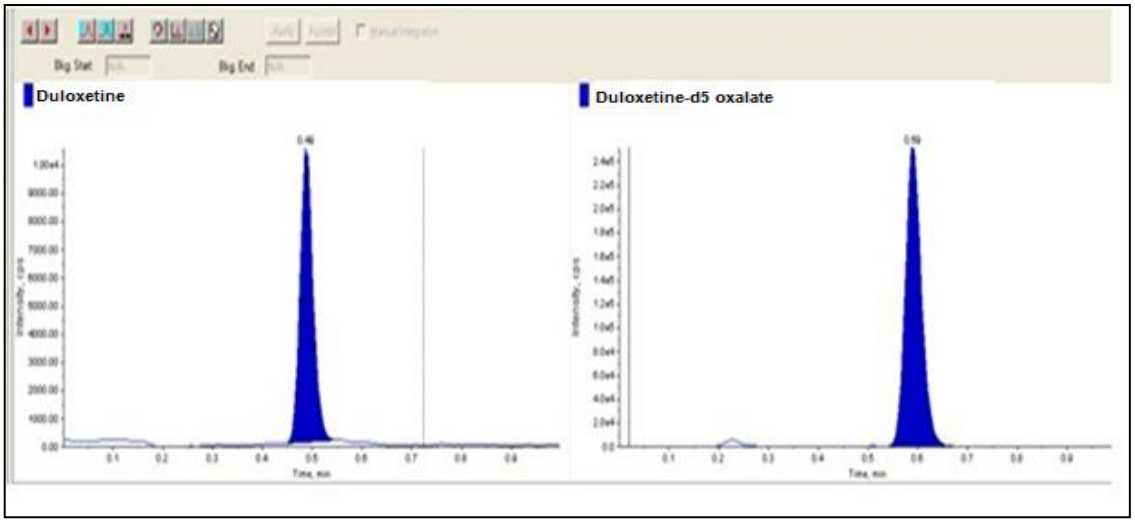


Fig 7.1 -Optimized Chromatogram of Duloxetine Hydrochloride

Observation: Good chromatogram was observed for all analytes after changing the flow rate. With the sample having vaporized entirely in the mass spectrometer, the peak form is satisfactory. Also, the autosampler is stable.

7.3 OPTIMIZED METHOD FOR ASSAY

Preparation of Reagents and Solutions:

Stock Solutions

Duloxetine Stock Solution

Before adding the same volume of methanol to a 5 mL clean glass volumetric flask, 5,000 mg of duloxetine hydrochloride industrial standard was dissolved to create a 1 mg/mL solution.

Updated the concentration of the Duloxetine solution above to reflect its real weight and potency. We filled out the "Stock Weighing and Solution Preparation" form and supplied the batch number. A high of four days was allowed for the stock solution to be used after refrigeration at 2 - 8 °C.

7.3.1 Duloxetine D5 Stock Solution (Internal Standard)

The dosage form of duloxetine D5, which was precisely measured at 20,000 milligrams', was dissolved in methanol and added to a 2-milliliter volumetric flask to create a solution with a concentration of 1 milligram per milliliter.

Adjusted the concentration of Duloxetine D5 above by taking its “potency, molecular weight, and the actual amount weighed” into consideration. We filled out the "Stock Weighing and Solution Preparation" form and supplied the batch number. You can use the stock solution for up to four days after storing it in the fridge at 2-8°C.

7.3.2 Biological Matrix

One sample of lipemic plasma and seven lots of hemolytic plasma were tested using K2-EDTA (batch no. Blank-P040212- 613-B, Blank-P040212- 614-B, Blank-P040212- 616-B, Blank-P040212- 617-B, Blank-P040212- 618-B, Blank-P040212-619-B, Blank-P060911-527-L-Lipemic and Blank-P060911-528-P-Hemolysed for selectivity test. All Duloxetine and the internal standard were not significantly impacted by any of the eight human plasma lots tested, which included both hemolytic and lipemic plasma. Five plasma lots (Blank-P040212- 613-A, Blank-P040212- 614-A, Blank-P040212- 616-A, Blank-P040212- 617-A, Blank-P040212- 619-A) were pooled and used to “prepare calibration standards, quality control samples and DIQC samples”. Selectivity test was performed as per SOP No. CR-BP-08-12, Bioanalytical Method Validation’ before bulk spiking [29].

The spiked plasma samples were divided into 400 µL CC aliquots and 400 µL QC aliquots, and then placed towards pre-labeled micro centrifuge tubes, after the bulk spiking process. With the exception of twelve LQC and HQC duplicates, which were stored in a deep freezer at -20 °C to obtain stability data at -20 °C, all of the bulk spiked samples were then placed in a deep freezer at -70 °C.

7.3.3 Calibration Standards and Quality Control Sample Setup

A calibration curve for standard was created with ten non-zero values of duloxetine, ranging from 0.5105 ng/mL to 200.185 ng/ml.

Low Limit of Quantitation (LLOQ)	**0.5121ng/ml**
Low Quality Control (LQC)	1.5062ng/ml
Middle Quality Control (MQC_1)	40.1657ng/ml
Middle Quality Control (MQC_2)	100.4143ng/ml
High Quality Control (HQC)	170.1937ng/ml

Unused samples were kept at a temperature of -70°C. For the purpose of testing their stability at -20°C, twelve sets of LQC and HQC were placed in a deep freezer. Duloxetine was spiked into twenty-four sets of QC samples to ensure dilution integrity, with a concentration of 350.3987 ng/mL being approximately 1.75 times the maximum standard concentration. We used six sets of each dilution method: two- and four-fold.

7.3.4 Solutions

5 mM Ammonium Acetate Buffer

Fill a 1000 mL reagent container to the top with Milli Q water after adding approximately 385.48 mg of ammonium acetate. Blended well and subjected to 5 minutes of ultrasonication. We have received the "Solution Preparation" form and the batch number. Every time buffer was needed, it was made and consumed within four days of being made. Kept the mixture at a temperature of 20±5 °C.

Mobile Phase (85:15, v/v)

A 2000 mL reagent container was filled with 1700 mL of HPLC grade acetonitrile after 300 mL of a 5 mM Ammonium acetate buffer solution had been put to it. After a thorough mixing, it was subjected to a 5-minute sonication in an ultrasonicator. The "Solution Preparation" form was filled out and a batch number was supplied. Within 7 days of production, the mobile phase was utilized after being stored at 20±5 °C.

Diluent (v/v)

As a diluent, a mixture of 60:40 volume ratios of Milli Q water and HPLC grade acetonitrile was created. Afterwards, it was subjected to 5 minutes of ultra-sonication. We filled out the "Solution Preparation" form and supplied the batch number. Within 7 days of production, the solution was utilized after being stored at room temperature (20 ±5 °C).

Rinsing Solution

As a rinse solution, diluent was employed.

System Suitability Solution

The system suitability test was conducted using a blend of analyte and internal standard. In this case, the analyte concentration is 60.0779 ng/mL (the middle concentration of the calibration range for duloxetine), while the IS concentration is 120.2008 ng/mL (the working concentration used for spiking) for duloxetine D5. This was the system suitability test solution, which was given as an aqueous mixture. Preparation of water samples is based on recovery. System suitability sample was prepared by mixing 30 µL of analyte (2002.5979) with 60 µL of internal standard working concentration (2003.3459 ng/mL) in 910 µL of mobile phase.

7.3.5 Sample Preparation

The contents of the thawed samples were thoroughly mixed by vertexing them. The following volumes were added to the 15 mL glass-stoppered tubes: 300 µL of plasma sample, 30 µL of Duloxetine D5, with a concentration of 2003.3459 ng/ml. In all cases except blank plasma samples, 30 µL of diluents were added and mixed with the Internal Standard spiking solution before vertexing.

They used a reciprocating shaker set at 200 rpm for 20 minutes after adding 5 mL of methyl tert-butyl ether to the mixture. The specimens were spun in a centrifuge at 4000 rpm for ten minutes at 4 °C. After that, the 4.0 mL of organic layer supernatant was transferred to dry test tubes constructed of pre-labeled glass and let to evaporate under a gentle nitrogen stream kept at 45 °C until it was completely dry. Before injecting 20 µL of the reconstituted mixture, 500 µL of mobile phase was used to mix the samples.

7.3.6 Bioanalytical Conditions

In brief, these are the chromatographic conditions:

The column is an Agilent technologies ZORBAX SB-C18 measuring 2.150 mm and 5 µm. Phase of mobility: grade HPLC In mill moles, acetonitrile the ratio of ammonium acetate to water is 85:15 (volume/volume).

Solution for rinsing: 60:40 (volume/volume) HPLC acetonitrile: Milli-Q water

Chromatographic Conditions.

Flow rate	**1.000 mL/minute**
Split Ratio	50:50
Sample Cooler Temperature	5°C
Injection volume	20 µL
Needle Rinsing Volume	1000 µL
Rinsing Mode	Before and after aspiration
Retention time	Duloxetine 1.10 ± 0.3 minutes Duloxetine D5 1.10 ± 0.3 minutes
Run Time	2.50 minutes

7.3.7 Optimization of MS/MS detection conditions and parameters

Precursor ion determination

The precursor ion, also called the parent ion, is "an electrically charged molecular moiety which may dissociate to form fragments. These fragments may be charged or neutral moieties. Charged fragments can be detected, forming the basis of tandem mass spectrometry, and used to further identify the analyte.

Production determination

The product ions are the ions detected by the detector in the third quad of the mass spec., as a result of the fragmentation of preselected precursor ions in the collision cells. By exposing the precursor ion to increasing collision energy, fragmentation occurs and the product ions can be determined as the MS2 detector scans.

MRM transition optimization

MRM (multi-reaction monitoring) can track many precursor ions and their multiple product ions through the MS to provide spectra that are quantifiable. Each transition from precursor ion to product ion can have different collision energies. By optimizing the MRM transition, the collision energy for each transition was optimized.

Detection: Application of positive ions (API 3000) The molecular weight of duloxetine is 298.30 for the parent compound and 154.10 for the synthetic version. A parent

compound of duloxetine with a mass-to-charge ratio of 303.30 and a product-to-charge ratio of 159.10

API 3000 in positive ion mode (for Duloxetine hydrochloride):

Parameter	Duloxetine	Duloxetine D5
Ion Spray Voltage (IS)	5500.00 V	5500.00 V
Temperature (TEM^{0}C)	500.00	500.00
Curtain Gas (CUR)	10.00 V	10.00 V
Collision Gas (CAD)	6.00 V	6.00 V
Nebulizer Gas (GS1)	8.00 psi	8.00 psi
Turbo Gas (GS2)	6500.00 psi	6500.00 psi
Focusing Potential (FP)	100.00 V	100.00 V
Declustering Potential (DP)	18.00 V	18.00 V
Collision Energy (CE)	10.00 V	9.00 V
Collision Cell Exit Potential (CXP)	10.00 V	9.00 V
Entrance Potential (EP)	10.00 V	10.00 V

Resolution:

Q 1: unit (0.5)

Q 3: unit (0.5)

It is possible to optimize the response by adjusting the parameters in the state file; however, the exact values of these parameters can vary by 0.5 units from one instrument to another.

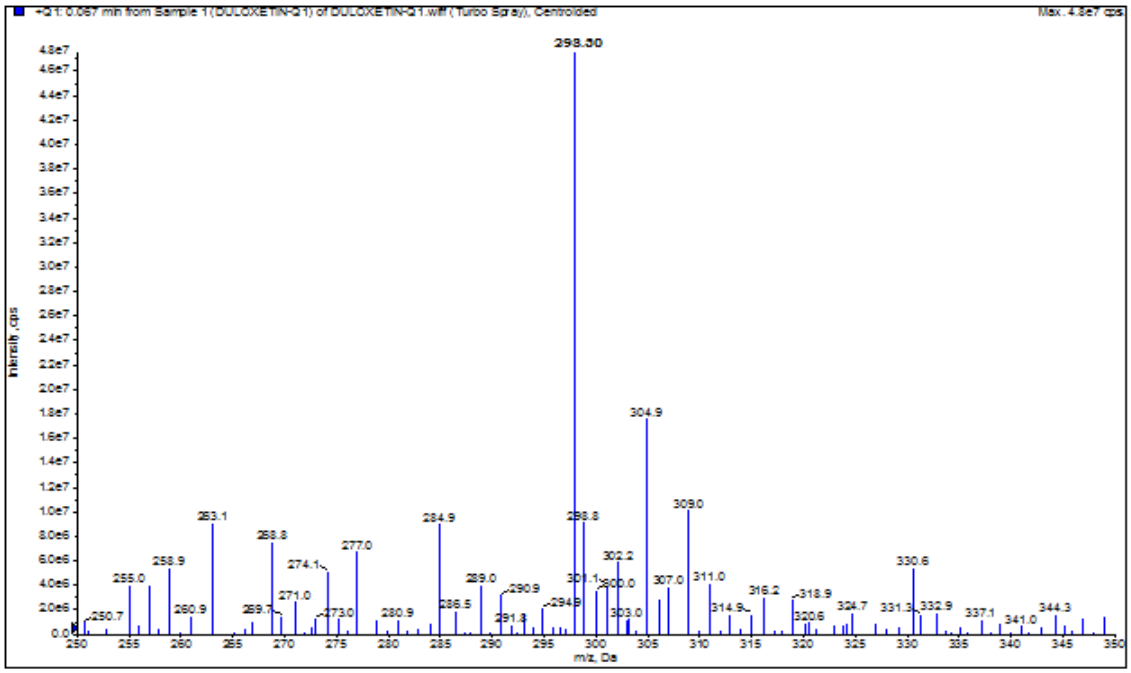


Fig 7.2: -Duloxetine Q1 MS scan tune window showing abundant Parent ion at m/z of 298.30

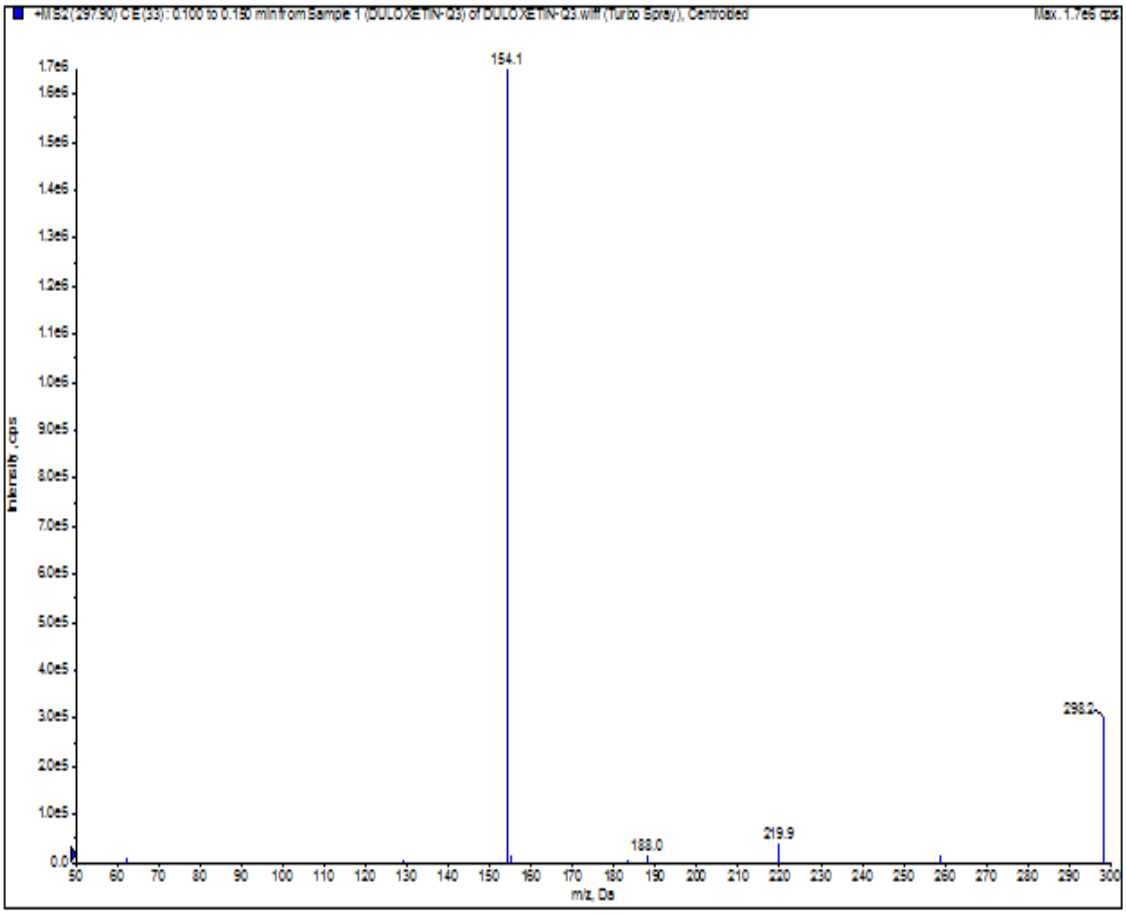


Fig 7.3:-Duloxetine Q3 MS scan tune window showing abundant Parent ion at m/z of 154.10

Comparison of Duloxetine product ions and their intensities (Q1/Q3) showing m/z of 298.30/154.10.

7.4: Validation of the assay method

The second step in the development of a bioanalytical method is validation. This is important before sample quantification by the method as it determines the robustness of a method assuring that sample analysis will produce exact, accurate results for the amount of analyte in the analytical samples. The goal of this bioanalytical method validation was to verify whether the created method would meet the criteria of industry recommendations as described by the FDA [30]. If the method is not in compliance with these requirements, it may be considered not suitable and the following sample analysis may not be suitable for regulatory submission for NCEs.

It requires verification for the concentration determination of target analytes in human plasma to be suitable for this purpose. The first step is to build the calibration line. A calibration curve was constructed by the line of best fit for the quantitation standards in the range 0.5-200 ng/ml for Duloxetine hydrochloride in human plasma. These standards were extracted with Duloxetine-d5 oxalate internal standard and evaluated by LC-MS/MS using their MRM transitions. The ratio of the peak regions of analyte and internal standard was computed from the extracted ion chromatograms (XIC). Solutions with no human plasma analytes and human plasma with no analytes were also examined. These analyses were conducted to assess the stability of the analytes in plasma at room temperature for 24 h.

7.4.1 Validation parameters

In order to demonstrate that the test procedure can consistently produce reliable and repeatable results within the specified tolerances, a subsequent experimental design was built up. The validation parameters have acceptance criteria that are laid forth in the specific experimental designs [31]. Data sheets for each method's validation are filled out with observations and outcomes.

7.4.1.1 Selectivity

- Gather the necessary “biological matrix” from at least six people according to the medication needs.

- Inject a solution bearing the drug(s) or metabolites along with an internal standard at the appropriate concentration to ascertain the retention duration.

- Mix the 6 LLOQ samples that were recently spiked with the blank matrix with the prepared and administered batches of blank matrix.

- Either the drug/metabolite or internal standard retention period, whoever is longer, should be multiplied by four before the processed blank matrix is run.

- You can identify the interfering drug or metabolite during retention time by comparing the average response of the extracted LLOQ to the blank matrix response. The interference of the drug or metabolite at the retention time can be established by comparing the response of the blank matrix with the average response of the extracted internal standard.

Acceptance criteria:

The drug's or metabolite's mass transition or interference peak response in low-volume (LLOQ) samples must be no more than 20% of the mean drug response, and the response of the internal standard in LLOQ samples should not exceed 5% of the mean response.

At least 67% (four out of six batches) of the filtered matrix lots must meet the following criteria for future batches of blank matrices to be produced and injected. If the next screening batch doesn't match the above requirements in less than 67% of cases, make the necessary adjustments to the process.

7.4.1.2 Matrix effect

- In order to make sure that the matrix screening doesn't affect precision, selectivity, and sensitivity, the matrix effect is studied.

- Split the blank matrices being tested into separate batches and prepare LQC and HQC samples in each.

- Run through the entire quality control sample sets mentioned above, along with the recently sharp calibration curve standards whose concentration range matches the one to be utilized for accuracy and precision calculations.

- Determine the QC concentration again by referring to the data from the calibration curve.

Acceptance criteria:

If the nominal mean % falls between ± 15% and the CV% is less than or equal to 15% at low and high QC, the matrix impact is not present.

7.4.1.3 Sensitivity.

Determine the sensitivity at the analyte(s) period of retention or mass transitions in terms of LLOQ; the effect of interference signal in the blank matrix needs to be no less than 5 times smaller than the LLOQ response.

- Use the same stock solution to prepare the calibration curve (CC) and six LLOQ samples.
- Take the CC samples and the LLOQ samples and analyses them.
- Use the data from the calibration curve to recalculate the LLOQ concentration.
- Find the nominal % and CV% for the identical.

Acceptance criteria:

The nominal value must be within ±20% of the accuracy, and the precision must be less than or equal to 20%.

7.4.1.4 Linearity

A method is linear if the findings of an analytical method are proportional to the analyte level in samples within a certain range, either directly or by a well-defined mathematical transformation [3].

To find the link between detector response and concentration, use a regression equation that takes the relevant weighting factor into account. When generating the calibration curve, be sure to include both standard A and standard I.

Acceptance criteria:

- r^2 must be greater than or equal to 0.98.
- The nominal value is within ±20% of the LLOQ concentration.
- ±15% variation from the nominal value of standards other than LLOQ concentration.
- It is imperative that the aforementioned requirements are met by all subsequent calibration curve standards.
- The afore-mentioned requirements must be satisfied by 75% of the time, or by none less than six non-zero calibration curve standards, which include the LOQ and the maximum concentration.

7.4.1.5 Precision and Accuracy

The precision of an analytical technique is distinct as the degree to which its output closely matches the actual value. A method's precision might be defined as its repeatability or reproducibility under typical operating circumstances. If you want to know the "Between and within Batch Precision and accuracy," you have to look at least five different chemical batches.

- Every batch that claims to be precise and accurate needs to have the following samples, along with an internal standard if necessary:
- An internal standard medium standard solution.
- Empty matrix
- Blank matrix + internal standard (if any)
- Spiked calibration standards (minimum 6 non-zero levels)
- Six QC samples at the LLOQ
- Six Low quality control sample (LQC)
- Six Middle quality controls (MQC)
- Six High-quality control samples (HQC)

- To find the QC sample concentrations, use the matching calibration curve.
- Discover the CV% and %nominal at each concentration level of the Quality Control to obtain the "within batch Precision and Accuracy" of a bioanalytical batch.
- Get the "Intraday Precision and Accuracy" for every bioanalytical batch that is run in a day by determining the CV% and %nominal, respectively, for every concentration level of Quality Control.
- By calculating the CV% and %nominal, respectively, determine the "Between batch/Intraday Precision and Accuracy" for every Quality Control concentration level of a bioanalytical batch that is run on different days.

Note:

- Process one bioanalytical batch at a time without interruption.
- No more than one bioanalytical batch may be processed in a single day; these processes must be carried out independently of one another.
- In the absence of an assignable cause, the validation computations must retain all QC failed data.

Calculations:

Find the average concentration, standard deviation, and accuracy for each QC concentration level for all calibration curve standards.

Within batch Precision and Accuracy

$$Accuracy\ (\%\ Nominal) = \frac{Mean\ of\ each\ QC\ Concentration\ level\ within\ a\ batch}{Nominal\ Value} \times 100$$

$$Precision\ (CV\%) = \frac{STDEV\ of\ each\ QC\ Concentration\ level\ within\ a\ batch}{Average\ of\ each\ QC\ concentration\ level\ within\ a\ batch} \times 100$$

Intraday Precision and Accuracy

$$Accuracy\ (\%Nominal) = \frac{Mean\ of\ each\ QC\ concentration\ level\ of\ all\ batches\ run\ in\ a\ day}{Nominal\ Value}\ X100$$

$$Precision\ (CV\%) = \frac{STDEV\ of\ each\ QC\ concentration\ level\ of\ all\ batches\ run\ in\ a\ day}{AVERAGE\ of\ each\ \ QC\ concentration\ level\ of\ all\ batches\ run\ in\ a\ day} \times 100$$

Between batch/Interlay Precision and Accuracy

$$Accuracy\ (\%Nominal) = \frac{Mean\ of\ each\ QC\ concentration\ level\ of\ all\ batches\ run\ \text{on different days}}{Nominal\ Value} \times 100$$

$$Precision\ (CV\%) = \frac{STDEV\ of\ each\ QC\ concentration\ level\ of\ all\ batches\ run\ \text{on different days}}{AVERAGE\ of\ each\ \ QC\ concentration\ level\ of\ all\ batches\ run\ on\ different\ days}\ X100$$

Acceptance criteria:

Accuracy: The accuracy calculated for low, moderate and high QC concentrations should be within 15% and for the LLOQ concentrations it should be between 15% and 20%.

Precision: The precision is needed to be 15 %, 20 % or 20 % for low, moderate and high QC concentrations relative to the nominal value of the LLOQ concentration.

In each batch of Precision and Accuracy, at least 67% of QC samples must be within the indicated limits; however, if the mean accuracy values are within the acceptable range, then 33% of QC samples may be beyond the stipulated limits. Almost two-thirds, or 3/4, of the QCs injected at each level should be within 15% of their respective nominal values, while for LLOQ it should be within 20% of its nominal value.

7.4.1.6 Stabilities

Ensure the analyte remains stable throughout the analytical procedure for accurate quantification. As a result, methods need stability tests for each step of analysis, including storage before analysis, for comprehensive validation.

Standard Stock Solution Stability

- Get the analyte(s) and internal standard stock solutions ready.
- Keep enough portions of the stock solution(s) in the fridge at temperatures ranging from 20C to 80C to ensure the stock solution remains stable when refrigerated.
- The stability of a stock solution at room temperature is determined by measuring the amount of stock that remains after preparation in order to evaluate the stability of the solution left on the bench.
- Put six separate injections of the analyte(s) and internal standard stock dilutions into the system at 0 and 6 hours.
- The analyte(s) and internal standard concentrations, as well as the concentration of the generated dilutions, should match those of the Middle Quality Control sample and working concentration, respectively.

Acceptance criteria: Percent comparison response must be among 85% and 115%

Note:

Stability testing at shorter durations will be conducted if the results from stock solutions kept at room temperature for 6 hours do not satisfy the acceptance criteria.

- If you want to know how well your stored stock holds up over a certain amount of time (say, 7, 14, or 21 days), you should test its stability in a refrigerator.
- Make new analyte and internal standard stock solutions. Make stock dilutions of both fresh stock solutions (comparison samples) and refrigerated stock (stability samples) that are equal to the concentration of the MQC sample, the working concentration of the analyte(s), and the internal standard, respectively.
- Six times after preparation, inject the comparison and stability samples. Make sure that the comparison samples' responses are accurate by multiplying them with the correction factor.

Acceptance criteria:

Percent comparison response must be among 85% and 115%

7.4.1.7 Spiking Solution Stability of Analyte(S) and Internal Standard

- Get the analyte(s) spiking solutions ready from the master stock solution at the MQC sample concentration and the internal standard at the working concentration.
- To ensure the stability of the spiking solution when refrigerated, keep an adequate quantity of the solution (including the analyte(s) and internal standard) in the refrigerator at a temperature ranging from 20C to 80C.
- While preparing the spiked matrix samples, the stability of the solution left on the bench is evaluated by measuring the volume of the residual spiking solution at room temperature.
- Mix the reconstitution solution with the analyte and internal standard spiking solutions to create the final dilutions of the analyte(s) and internal standard.
- At 0 and 6 hours, inject six independent samples of the final dilutions of the analyte(s) and internal standard.

Acceptance criteria:

Percent comparison response must be among 85% and 115%

Calculations:

$$\text{Percent comparison response} = \frac{\text{Mean of response at 6 hours}}{\text{Mean of response at 0hour}} \times 100$$

Note:

Stability testing at shorter durations will be conducted if the results from stock solutions kept at room temperature for 6 hours do not satisfy the acceptance criteria.

- A test called "refrigerated spiking solution stability" is used to determine how well spiking solutions hold up when held for different amounts of time, such as 7, 14, and 21 days.
- Make new internal standard and standard stock solutions with the analyte(s). Based on the MQC sample concentration, the working concentration of the analyte(s), and the internal standard, make spiking solutions using the fresh stock solutions (comparison samples) in corresponding proportions.

- Take the analyte(s) and internal standard spiking solutions (stability samples) out of the fridge. The purpose of the refrigerator is to ensure the spiking solutions are stable.
- Get the internal standard and analyte spiking solutions ready for final dilutions by adding them to the reconstitution solution.
- Shortly after preparing the samples, inject six copies of the comparative and stability samples. Make sure that the comparison samples' responses are accurate by multiplying them with the correction factor.

Acceptance criteria:

Percent comparison response must be between 85% and 115%

Calculations:

$$\text{Percent comparison response} = \frac{\text{Mean response of stability samples}}{\text{Mean response of comparison samples}} \times 100$$

7.4.1.8 FREEZE THAW STABILITY

One way to determine how well an analyte will hold up in biological fluids over time is to test its freeze-thaw stability. Always establish the impact of at least three freeze-thaw cycles when estimating multiple analytes at once using various methods; a comparable number of cycles should be documented for each analyte.

- Remove 18 sets of QC samples (LQC and HQC) from the deep freezer after these have been frozen for atleast 24 hours. Sort the six copies into FT-3, FT-2, and FT-1; let them thaw at room temperature before refreezing.
- After at least 12 hours, take out the FT-3 and FT-2 samples once more, let them thaw at room temperature, and then freeze them again.
- Thaw all samples at room temperature on the day of the stability evaluation, after freezing the FT-3 samples for at least 12 hours.
- Get the new analyte standard stock solution ready.
- Conduct thorough processing and analysis on all FT-3 quality control samples, ensuring that these can be aligned with the concentration range used to calculate precision and accuracy using the recently spiked calibration curve standards.

- Recalculate the concentrations for quality control using data from the calibration curve.

Calculations:

Mean concentration of LQC or HQC having

$$\text{Mean \% nominal concentration} = \frac{\text{Undergone 3 freeze thawcycles of QC Samples}}{\text{Respective nominal conc. of LQC or HQC}} \times 100$$

Acceptance criteria:

At each QC sample level, the mean nominal concentration should fall between the range of 85% to 115%, and the precision should be less than or equal to 15% of the CV%.

Note:

Keep in mind that ±15% of each level's nominal value should be occupied by a minimum of 67% (4 out of 6) of the QC's injected

7.4.1.9 Benchtop Stability

The purpose of determining the analyte(s) stability in biological fluids over an anticipated processing duration is known as bench top stability.

- Remove six sets of QC samples from the deep freezer, one for each quality control (LQC) and heat control (HQC). Place them on a bench at ambient temperature or the specified temperature and leave them unprocessed for at least four hours, as suggested.
- Get the new analyte standard stock solution ready.
- Prepare and examine the stability samples in conjunction with recently spiked standards from the calibration curve whose concentration range is the same as that utilized to determine accuracy and precision.
- Determine how long the sample was stable on the bench top by subtracting the time it was taken out of the deep freezer from the time it was started to be extracted.

- Retrieve the concentrations used for quality control by referring back to the data from the calibration curve.

Calculations:

Mean concentration of LQC or HQC having

$$\text{Mean \% nominal concentration} = \frac{\text{Mean of stability samples of QC samples}}{\text{Respective nominal conc. of LQC or HQC}} \times 100$$

Acceptance criteria:

At each QC sample level, the mean nominal concentration should fall between the range of 85% to 115%, and the precision should be less than or equal to 15% of the CV%.

Note:

Keep in mind that ±15% of each level's nominal value should be occupied by a minimum of 67% (4 out of 6) of the QC's injected.

7.4.1.10 Auto sampler Stability

Analyte and internal standard auto sampler stability testing is conducted at a controlled temperature for a duration corresponding to the expected run time for a batch size's full analysis.

- To ensure injection is possible within the suggested stability periods, process a sufficient number of low- and high-quality control samples (6 repetitions).
- Auto sampler samples should be kept at room temperature or the temperature you specify.
- Make a new standard stock solution of the analyte(s) on the stability day.
- Inject six samples of QC together with the newly spiked calibration standards in each replication. The concentration range of the standards should match that used to calculate precision and accuracy.
- Determine the durations of autosampler stability at the moment of initial QC injection, minus the time they were placed in the autosampler.

- Recalculate the concentrations for quality control using data from the calibration curve.

Acceptance criteria:

At each QC sample level, the mean nominal concentration should fall between the range of 85% to 115%, and the precision should be less than or equal to 15% of the CV%.

Note:
Please be informed that a minimum of 4 out of 6 QC's injected at each level should fall within 15% of their corresponding nominal value.

7.4.1.11 Long Term Stability

A study of long-term stability is conducted to determine how well analyte(s) in biological fluids will hold up when stored in a deep freezer at temperatures below -500C for a length of time that is at least as long as the time between the dates of sample analyses.

- Consistency below -500C
- Make the new analyte and internal standard stock solutions.
- Take out six sets of Quality Control samples (LQC & HQC) that were stored for a long time and kept below -500C from the deep freezer. Process these sets with the recently spiked calibration standards that have a concentration range that is the same as the one used to calculate quality and accuracy.
- Subtract the date of the first day of stability testing (i.e., the day of first passing precision and accuracy batch analysis) from the date of analysis of the stability QC samples to get the LTS duration.
- Recalculate the concentrations for quality control using data from the calibration curve. Starting on the first day of LTS, compare the nominal concentration (the average of the back calculated values for the QC samples) with the concentration of the stability samples to determine the optimal concentration.

Calculations:

$$\text{Mean \% nominal concentration} = \frac{\text{Mean concentration of LQC or HQC samples}}{\text{Respective nominal conc. of LQC or HQC}} \times 100$$

Acceptance criteria:

At each QC sample level, the mean nominal concentration should fall between the range of 85% to 115%, and the precision should be less than or equal to 15% of the CV%.

Short Term Stability at -20^{0}c Storage:

The purpose of testing human plasma stability at -200C is to determine how well a medicine will hold up in the event that plasma samples are temporarily stored in a deep freezer at that temperature.

- After bulk spike, store a sufficient number of sets (at least six sets) of QC samples (LQC and HQC) in a deep freezer at -200C.
- The day of the stability test is when you should get the analyte(s) standard stock solutions ready.
- After at least three days of storage, remove six sets and mix them with newly spiked calibration standards with a concentration range similar to the one used to determine accuracy and precision.
- Recalculate the concentrations for quality control using data from the calibration curve.

Calculations:

$$\text{Mean \% nominal concentration} = \frac{\text{Mean concentration of LQC or HQC samples}}{\text{Respective nominal conc. of LQC or HQC}} \times 100$$

Acceptance criteria:

At each QC sample level, the mean nominal concentration should fall between the range of 85% to 115%, and the precision should be less than or equal to 15% of the CV%.

7.4.1.12 Reinjection Stability

Analyte samples that are reinjected after processing are tested for reinjection stability to ensure their integrity.

- Combine six sets of quality controls (LQC and HQC) with one set of calibration curves and process/inject them.
- Recalculate the concentration of the quality control using the data from the calibration curve.
- The QCs should be reinjected at intervals following the initial QC sample injection.
- Determine the concentrations of the QCs that were reinjected by referring back to the data from the initial calibration curve.

Acceptance criteria:

At each QC sample level, the mean nominal concentration should fall between the range of 85% to 115%, and the precision should be less than or equal to 15% of the CV%.

Note:

Keep in mind that ±15% of each level's nominal value should be occupied by a minimum of 67% (4 out of 6) of the QC's injected.

7.4.1.13 Wet Extract Stability

The purpose of testing the wet extract stability is to determine how well processed samples hold up when left at room temperature for an extended length of time.

- To ensure injection is possible within the suggested stability periods, process a sufficient number of low- and high-quality control samples (6 repetitions).
- Auto sampler samples should be kept at room temperature or the temperature you specify.
- Make a new analyte standard stock solution on the stability day.

- Inject six samples of QC together with the newly spiked calibration standards in each replication. The concentration range of the standards should match that used to calculate precision and accuracy.
- After the sample preparation time is subtracted from the auto sampler stability durations, determine the injection time for the first QC.
- Use the data from the calibration curves of the recently spiked calibration standards to recalculate quality control concentrations.

Calculations:

$$\text{Mean \% nominal concentration} = \frac{\text{Mean concentration of LQC or HQC samples}}{\text{Respective nominal conc. of LQC or HQC}} \times 100$$

Acceptance criteria:

At each QC sample level, the mean nominal concentration should fall between the range of 85% to 115%, and the precision should be less than or equal to 15% of the CV%.

Note:

Keep in mind that ±15% of each level's nominal value should be occupied by a minimum of 67% (4 out of 6) of the QC's injected.

7.4.1.14 Carryover

In order to see whether the instrument is carrying over any data from one sample to the next, a carryover test will be conducted.

We ensure that carryover is less than 20% of the LLOQ on a peak area basis by injecting the sample without an internal standard right after the highest standard in each batch.

Calculations:

$$\text{\% Of drug carry over} = \frac{\text{Drug response in COT BLANK}}{\text{Average drug response in COT LLOQ}} \times 100$$

Acceptance criteria:

Carry over is not regarded significant if the blank sample response is less than or equal to 20% of the LOQ sample.

7.4.1.15 Recovery

- The six sets of quality control samples (LQC, MQC, or HQC) have been eliminated from the deep freezer.
- The quality control samples, which are extracted samples, should be processed and injected.
- Twenty-four samples of blank matrices should be processed.
- For the processed blank matrix samples, which stand for 100% analyte extraction (non-extracted samples), spike six sets of each QC dilution at low, middle, and high concentrations without an internal standard.
- To represent 100% extraction of the internal standard (non-extracted sample), spike the six processed blank matrix samples with the working concentration of the standard's dilution.
- Then, inject six separate samples that have not been removed.
- Record the peak area responses of both the extracted and non-extracted samples for each of the three QC levels: low, middle, and high.
- Compare the extracted (medium QC level) with non-extracted internal standard samples by tabulating their peak area responses.
- Determine the mean response, standard deviation, and %CV.
- Determine the average absolute percentage of recovery for the analyte and internal standard at each QC concentration using the following formula:

Mean absolute % recovery of drug =

$$\frac{\text{Mean peak area response of extracted samples at LQC, MQC or HQC}}{\text{Mean peak area response of non-extracted samples at LQC, MQC or HQC}} \times 100$$

Mean absolute % recovery of internal standard =

$$\frac{\text{Mean peak area response of extracted samples at medium QC level}}{\text{Mean peak area response of non-extracted samples}} \times 100$$

Finally, calculate the overall recovery of analyte.

Overall absolute % recovery = Average of mean of absolute % recovery at LQC, MQC and HQC

Acceptance criteria:

- Every quality control concentration level must have a C.V% of at least 15% for the mean analyte(s) and internal standard recovery.
- Any discrepancy in recovery rates, from lowest to highest, should not exceed 25%.

7.4.1.16 Dilution Integrity

- Get twelve sets of quality control (QC) samples that are 1.5-1.8 times the concentration of the highest standard (ULOQ).
- Dilute six sets of these samples twice, and then dilute another six sets four times before adding the screened blank matrix for extraction.
- The QC concentrations can be determined by injecting these samples with the undiluted calibration curve standards and multiplying the results by 2 for samples that were diluted twice and 4 for those that have been diluted four times.

Acceptance criteria:

The QC precision within a batch with the same dilution factor would be less than or equal to 15%. The ±15% tolerance for QC within a batch with the identical dilution factor is required.

Note :

Please be informed that a minimum of 4 out of 6 QC's injected at every level should fall within 15% of their corresponding nominal value.

7.5: Results and Discussion

7.5.1 Chromatography

Included here are chromatograms that serve as examples of the following: aqueous standard analyte and internal standard mixture; blank plasma with internal standard; Duloxetine LLOQ QC sample; LQC sample; MQC sample; and HQC sample.

7.5.2. SELECTIVITY

Figure 2 shows a representative chromatogram of the plasma (blank) sample that was extracted. In all batches that were examined, there was no discernible interference from endogenous components at the masses transitions of Duloxetine and the internal standard.

7.5.3. SENSITIVITY

The value of 0.5105 ng/mL was chosen as the Lowest Limit of Quantification (LLOQ) for duloxetine.

Table 7.1: Concentration-response Linearity Data for Sensitivity (Duloxetine)

	Conc. (ng/mL)									
	STD-A	**STD-B**	**STD-C**	**STD-D**	**STD-E**	**STD-F**	**STD-G**	**STD-H**	**STD-I**	**STD-J**
CC#	0.5105	1.0209	2.5524	5.1047	10.2094	30.0278	60.0556	120.1112	160.1482	200.1853
Lower Limit	0.4084	0.8678	2.1695	4.3390	8.6780	25.5236	51.0473	102.0945	136.1260	170.1575
Upper Limit	0.6126	1.1740	2.9353	5.8704	11.7408	34.5320	69.0639	138.1279	184.1704	230.2131
1	0.5326	0.9425	2.5166	4.9146	10.4636	30.3919	59.9395	116.3263	178.3631	193.6547
% Nominal	104.33	92.32	98.60	96.28	102.49	101.21	99.81	96.85	111.37	96.74

Table 7.2: Regression Data

	Slope	**Intercept**	**r^2**
CC# 1	0.0066	0.0002	0.9966

Table 7.3: Precision and Accuracy (within Batch) forSensitivity of Duloxetine

	Nominal Conc. (ng/mL)	
SEN- LLOQ	**0.5105**	**% Accuracy**
Lower Limit	**0.4084**	**80.00**
Upper Limit	**0.6126**	**120.00**
1	0.5422	106.21
2	0.5111	100.12
3	0.5145	100.78
4	0.5215	102.16
5	0.5194	101.74
6	0.5060	99.11
Mean	**0.51912**	
S.D	**0.012619**	
C.V %	**2.43**	
%Nominal	**101.69**	
N	**6**	

Observation:

At this concentration, duloxetine showed a precision of 2.43% and an accuracy of 101.69%.

7.5.4. Matrix Effect

Across all eight plasma batches tested, there was no discernible matrix impact for both low (LQC) and high (HQC) amounts of duloxetine.

Observation:

The IS normalized matrix factor had a precision of 2.09% at the LQC level and 1.14% at the HQC level. The IS normalized factor for LQC was 1.000 and for HQC it was 0.983.

Table 7.4: Matrix Effect Assessment of Duloxetine without Matrix Ions

S. No.	LQC			HQC		
	Area of Analyte	Area of Internal Standard	Area Ratio	Area of Analyte	Area of Internal Standard	Area Ratio
1	22182	2053669	**0.01**	2347287	1863487	**1.26**
2	20963	2017490	**0.01**	2192102	1791328	**1.22**
3	21097	1977709	**0.01**	2201552	1792786	**1.23**
4	21091	2002633	**0.01**	2301659	1851408	**1.24**
5	22149	2041272	**0.01**	2199544	1767441	**1.24**
6	21388	1979359	**0.01**	2192505	1809132	**1.21**
		Mean	**0.011**		**Mean**	**1.235**

$$\text{IS-Normalized Matrix Factor} = \frac{\text{Peak response area ratio in the presence of matrix ions}}{\text{Mean peak response area ratio in the absence of matrix ions}}$$

IS normalized Matrix Factor = 1.0± 0.15

Table 7.5: Matrix Effect of Duloxetine (Matrix Ions Presence)

Matrix Lot. No.	LQC				HQC			
	Area of Analyte	Internal Standard Area	Area Ratio	IS Normalized Matrix Factor	Area of Analyte	Internal Standard Area	Area Ratio	IS Normalized Matrix Factor
Blank-P040212-613-B	20246	1934768	**0.01**	**0.98**	2175126	1825317	**1.19**	**0.96**
Blank-P040212-614-B	21006	1956000	**0.01**	**1.01**	2235957	1820722	**1.23**	**0.99**
Blank-P040212-616-B	21889	1993074	**0.01**	**1.03**	2262208	1856468	**1.22**	**0.99**
Blank-P040212-	20949	1940484	**0.01**	**1.01**	2289365	1853112	**1.24**	**1.00**

617-B								
Blank-P040212-618-B	20117	1955434	**0.01**	**0.96**	2221553	1845484	**1.20**	**0.97**
Blank-P040212-619-B	20807	1935574	**0.01**	**1.01**	2179282	1802119	**1.21**	**0.98**
Blank-P060911-527-L-Lipemic	20599	1901889	**0.01**	**1.01**	2246829	1844191	**1.22**	**0.99**
Blank-P060911-528-P-Hemolysed	20547	1942970	**0.01**	**0.99**	2205164	1823749	**1.21**	**0.98**
	Mean			**1.000**	**Mean**			**0.983**
	SD			**0.0209**	**SD**			**0.0112**
	% CV			**2.09**	**% CV**			**1.14**
	N			**8**	**N**			**8**

7.5.5. LINEARITY

Table 7.6: Concentration-response Linearity Data (Duloxetine)

	Nominal Conc.(ng/mL)									
	STD-A	**STD-B**	**STD-C**	**STD-D**	**STD-E**	**STD-F**	**STD-G**	**STD-H**	**STD-I**	**STD-J**
CC#	0.5105	1.0209	2.5524	5.1047	10.2094	30.0278	60.0556	120.1112	160.1482	200.1853
Lower Limit	0.4084	0.8678	2.1695	4.3390	8.6780	25.5236	51.0473	102.0945	136.1260	170.1575
Upper	0.6126	1.1740	2.9353	5.8704	11.7408	34.5320	69.0639	138.1279	184.1704	230.2131

Limit										
1	0.5326	0.9425	2.5166	4.9146	10.4636	30.3919	59.9395	116.3263	178.3631	193.6547
2	0.5123	1.0283	2.5231	4.8586	10.2039	29.8088	59.0164	117.1380	181.0673	193.8122
3	0.5163	1.0072	2.4795	5.1043	10.3958	30.6194	59.5037	113.2920	177.2949	190.5224
Mean	0.52040	0.99267	2.50640	4.95917	10.35443	30.27337	59.48653	115.58543	178.90843	192.66310
S.D.	0.010753	0.044708	0.023522	0.128770	0.134701	0.418098	0.461789	2.027213	1.944426	1.855572
C.V. %	2.07	4.50	0.94	2.60	1.30	1.38	0.78	1.75	1.09	0.96
% Nominal	101.94	97.23	98.20	97.15	101.42	100.82	99.05	96.23	111.71	96.24
N	3	3	3	3	3	3	3	3	3	3

Table7.7: Regression Data

CC#	Slope	Intercept	r^2
1	0.0066	0.0002	0.9966
2	0.0067	0.0001	0.9970
3	0.0066	0.0001	0.9974

Observation:

While the concentration of duloxetine ranged from 0.5105 ng/mL to 200.185 ng/mL, the correlation coefficient (r) remained above 0.99.

7.5.6. Precision and Accuracy

During validation, we examined the assay's precision by looking at the % coefficient of variation over the different concentrations of LLOQQC, LQC, MQC1, MQC2, and HQC samples, in that order. To determine the assay's accuracy, we took the percentage of the computed mean values of the LLOQ, low, middle, and high-quality control samples and compared them to their respective nominal values [32].

Within-batch Precision and Accuracy:

Table 7.8: Precision and Accuracy (within Batch) for Duloxetine

	Nominal Conc. (ng/mL)									
	LLOQ		**LQC**		**MQC1**		**MQC2**		**HQC**	
QC#	**0.5121**	**% Accuracy**	**1.5062**	**% Accuracy**	**40.1657**	**% Accuracy**	**100.4143**	**% Accuracy**	**170.1937**	**% Accuracy**
Lower Limit	**0.4097**	**80.00**	**1.2803**	**85.00**	**34.1408**	**85.00**	**85.3522**	**85.00**	**144.6646**	**85.00**
Upper Limit	**0.6145**	**120.00**	**1.7321**	**115.00**	**46.1906**	**115.00**	**115.4764**	**115.00**	**195.7228**	**115.00**
1	0.4889	95.47	1.4945	99.22	40.5414	100.94	105.3542	104.92	168.1813	98.82
2	0.5171	100.98	1.5124	100.41	38.9043	96.86	102.0409	101.62	164.9271	96.91
3	0.5139	100.36	1.5653	103.93	40.5762	101.02	104.8529	104.42	169.5194	99.60
4	0.5078	99.16	1.4636	97.17	40.3468	100.45	104.4938	104.06	169.0611	99.33
5	0.4711	92.00	1.5195	100.88	40.1643	100.00	102.9754	102.55	166.7102	97.95
6	0.4917	96.03	1.5851	105.24	38.9558	96.99	105.2406	104.81	168.2177	98.84
Mean	**0.49842**		**1.52340**		**39.91480**		**104.15963**		**167.76947**	
S.D.	**0.017656**		**0.044967**		**0.777141**		**1.347531**		**1.691283**	
C.V.%	**3.54**		**2.95**		**1.95**		**1.29**		**1.01**	
% Nominal	**97.33**		**101.14**		**99.38**		**103.73**		**98.58**	
N	**6**		**6**		**6**		**6**		**6**	
7	0.5159	100.75	1.4501	96.28	39.6914	98.82	101.0756	100.66	161.7545	95.04

8	0.5164	100.83	1.5013	99.68	38.9242	96.91	101.9241	101.50	162.0066	95.19
9	0.4960	96.86	1.5201	100.92	40.4943	100.82	105.3358	104.90	167.9778	98.70
10	0.5321	103.90	1.4828	98.45	39.8756	99.28	101.3008	100.88	165.5318	97.26
11	0.5178	101.12	1.5136	100.49	39.4788	98.29	102.5322	102.11	161.2483	94.74
12	0.4925	96.18	1.5176	100.76	40.2247	100.15	102.3848	101.96	165.7732	97.40
Mean	**0.51178**		**1.49758**		**39.78150**		**102.42555**		**164.04870**	
S.D.	**0.014888**		**0.027023**		**0.556430**		**1.537626**		**2.752711**	
C.V.%	**2.91**		**1.80**		**1.40**		**1.50**		**1.68**	
% Nominal	**99.94**		**99.43**		**99.04**		**102.00**		**96.39**	
N	**6**		**6**		**6**		**6**		**6**	
13	0.5313	103.75	1.6517	109.66	39.7626	99.00	103.8061	103.38	165.0294	96.97
14	0.4979	97.22	1.6161	107.29	40.2708	100.26	104.7309	104.30	167.2083	98.25
15	0.5052	98.66	1.6980	112.73	41.1154	102.36	105.5570	105.12	164.9553	96.92
16	0.5098	99.55	1.6904	112.23	41.0231	102.13	103.5863	103.16	167.4120	98.37
17	0.5148	100.54	1.6803	111.56	40.8792	101.78	105.7529	105.32	171.7419	100.91
18	0.5251	102.54	1.7021	113.00	40.6307	101.16	105.0833	104.65	166.3935	97.77
Mean	**0.51402**		**1.67310**		**40.61363**		**104.75275**		**167.12340**	
S.D.	**0.012469**		**0.033224**		**0.516148**		**0.895948**		**2.491557**	
C.V.%	**2.43**		**1.99**		**1.27**		**0.86**		**1.49**	
% Nominal	**100.37**		**111.08**		**101.12**		**104.32**		**98.20**	
N	**6**		**6**		**6**		**6**		**6**	

Observation:

The following ranges were recorded for the within-batch precision: 2.43–3.54% for LLOQ QC, 1.80%–2.95% for LQC, 1.27%–1.95% for MQC1, 0.86–1.50% for HQC, and 1.01%–1.68% for HQC. From 97.33% to 100.37%, 99.43% to 111.08%, 99.04% to 101.12%, 102.00% to 104.32%, and 96.39% to 98.58% were the within-batch accuracy ranges for LLOQ QC, LQC, MQC1, MQC2, and HQC, respectively.

Table 7.9: Intraday Precision and Accuracy (Duloxetine)

	Nominal Conc.(ng/mL)									
	LLOQ QC		LQC		MQC1		MQC2		HQC	
QC#	0.5121	% Accuracy	1.5062	% Accuracy	40.1657	% Accuracy	100.4143	% Accuracy	170.1937	% Accuracy
Lower Limit	0.4097	80.00	1.2803	85.00	34.1408	85.00	85.3522	85.00	144.6646	85.00
Upper Limit	0.6145	120.00	1.7321	115.00	46.1906	115.00	115.4764	115.00	195.7228	115.00
1	0.4889	95.47	1.4945	99.22	40.5414	100.94	105.3542	104.92	168.1813	98.82
2	0.5171	100.98	1.5124	100.41	38.9043	96.86	102.0409	101.62	164.9271	96.91
3	0.5139	100.36	1.5653	103.93	40.5762	101.02	104.8529	104.42	169.5194	99.60
4	0.5078	99.16	1.4636	97.17	40.3468	100.45	104.4938	104.06	169.0611	99.33
5	0.4711	92.00	1.5195	100.88	40.1643	100.00	102.9754	102.55	166.7102	97.95
6	0.4917	96.03	1.5851	105.24	38.9558	96.99	105.2406	104.81	168.2177	98.84
7	0.5159	100.75	1.4501	96.28	39.6914	98.82	101.0756	100.66	161.7545	95.04
8	0.5164	100.83	1.5013	99.68	38.9242	96.91	101.9241	101.50	162.0066	95.19
9	0.4960	96.86	1.5201	100.92	40.4943	100.82	105.3358	104.90	167.9778	98.70
10	0.5321	103.90	1.4828	98.45	39.8756	99.28	101.3008	100.88	165.5318	97.26
11	0.5178	101.12	1.5136	100.49	39.4788	98.29	102.5322	102.11	161.2483	94.74
12	0.492	96.18	1.517	100.7		100.1	102.38	101.9	165.77	97.40

	5		6	6	40.2247	5	48	6	32	
Mean	**0.50510**		**1.51049**		**39.84815**		**103.29259**		**165.90908**	
S.D.	**0.017064**		**0.037852**		**0.648153**		**1.649292**		**2.918929**	
C.V. %	**3.38**		**2.51**		**1.63**		**1.60**		**1.76**	
% Nominal	**98.63**		**100.28**		**99.21**		**102.87**		**97.48**	
N	**12**		**12**		**12**		**12**		**12**	

Observation:

LLOQ QC, 2.51%, 1.63%, 1.60%, and 1.76% were the intra-day precision values for LQC, MQC1, MQC2, and HQC, respectively.

The following intra-day accuracy rates were recorded: 98.63% for LLOQ QC, 100.28% for LQC, 99.21% for MQC1, 102.87% for HQC, and 97.48% for HQC.

Table 7.10: Nominal Concentration (ng/mL)

	Nominal Conc.(ng/mL)									
	LLOQ QC		**LQC**		**MQC 1**		**MQC 2**		**HQC**	
QC #	**0.5121**	**% Accuracy**	**1.5062**	**% Accuracy**	**40.1657**	**% Accuracy**	**100.4143**	**% Accuracy**	**170.1937**	**% Accuracy**
Lower Limit	**0.4097**	**80.00**	**1.2803**	**85.00**	**34.1408**	**85.00**	**85.3522**	**85.00**	**144.6646**	**85.00**
Upper	**0.6145**	**120.00**	**1.7321**	**115.00**	**46.1906**	**115.00**	**115.4764**	**115.00**	**195.7228**	**115.00**

Limit										
1	0.4889	95.47	1.4945	99.22	40.5414	100.94	105.3542	104.92	168.1813	98.82
2	0.5171	100.98	1.5124	100.41	38.9043	96.86	102.0409	101.62	164.9271	96.91
3	0.5139	100.36	1.5653	103.93	40.5762	101.02	104.8529	104.42	169.5194	99.60
4	0.5078	99.16	1.4636	97.17	40.3468	100.45	104.4938	104.06	169.0611	99.33
5	0.4711	92.00	1.5195	100.88	40.1643	100.00	102.9754	102.55	166.7102	97.95
6	0.4917	96.03	1.5851	105.24	38.9558	96.99	105.2406	104.81	168.2177	98.84
7	0.5159	100.75	1.4501	96.28	39.6914	98.82	101.0756	100.66	161.7545	95.04
8	0.5164	100.83	1.5013	99.68	38.9242	96.91	101.9241	101.50	162.0066	95.19
9	0.4960	96.86	1.5201	100.92	40.4943	100.82	105.3358	104.90	167.9778	98.70
10	0.5321	103.90	1.4828	98.45	39.8756	99.28	101.3008	100.88	165.5318	97.26
11	0.5178	101.12	1.5136	100.49	39.4788	98.29	102.5322	102.11	161.2483	94.74
12	0.4925	96.18	1.5176	100.76	40.2247	100.15	102.3848	101.96	165.7732	97.40
13	0.5313	103.75	1.6517	109.66	39.7626	99.00	103.8061	103.38	165.0294	96.97
14	0.4979	97.22	1.6161	107.29	40.2708	100.26	104.7309	104.30	167.2083	98.25
15	0.5052	98.66	1.6980	112.73	41.1154	102.36	105.5570	105.12	164.9553	96.92
16	0.509	99.55	1.690	112.2	41.02	102.1	103.5	103.1	167.4	98.37

	8		4	3	31	3	863	6	120	
17	0.5148	100.54	1.6803	111.56	40.8792	101.78	105.7529	105.32	171.7419	100.91
18	0.5251	102.54	1.7021	113.00	40.6307	101.16	105.0833	104.65	166.3935	97.77
Mean	**0.50807**		**1.56469**		**40.10331**		**103.77931**		**166.31386**	
S.D.	**0.015901**		**0.086448**		**0.698613**		**1.580463**		**2.772333**	
C.V. %	**3.13**		**5.52**		**1.74**		**1.52**		**1.67**	
% Nominal	**99.21**		**103.88**		**99.84**		**103.35**		**97.72**	
N	**18**		**18**		**18**		**18**		**18**	

Observation:

Between-batch precision:

LLOQ QC (3.13%), LQC (5.52%), MQC1 (1.74%), MQC2 (1.52%) and HQC (1.67%).

Between-batch accuracy:

LLOQ QC (99.21%), LQC (103.88%), MQC1 (99.84%), MQC2 (103.35%) and HQC (97.72%).

7.5.7. Stabilities

Room temperature (20 ± 5 °C) spiking solution stability testing was achieved for Duloxetine, Duloxetine D5 (Internal Standard) at 9 hours. This was achieved by spiking 6 replicates of made stock dilutions of Duloxetine equal to the final MQC2 QC concentration and Duloxetine D5 at the final working concentration from the stock solution. Stability samples for Duloxetine and Duloxetine D5 were evaluated against newly prepared comparison samples for mean area response at 9 h [33].

Table 7.11: Stock Solution Stability (Room Temperature) of Duloxetine (0, 9 hours)

Stock Solution ID	Hours	%Stability
DST-01	0	100.00
DST-03	9	98.75

	Stability standard stock	Comparison standard stock
S. No.	**Area**	**Area**
1	1343508	1360081
2	1360589	1364533
3	1352245	1382509
4	1351136	1370394
5	1341898	1372839
6	1343905	1343671
Mean	**1348880.2**	**1365671.2**
SD	**7153.61**	**13219.29**
%CV	**0.53**	**0.97**
N	**6**	**6**
% Stability	**98.75**	

Table 7.12: Stock Solution Stability (Room Temperature) of Duloxetine D5 (0, 9 hours)

Stock Solution ID	Hours	%Stability
DDST-01	0	100.00
DDST-02	9	98.20

	Stability standard stock	Comparison standard stock
S. No.	**Area**	**Area**
1	1732166	1717852
2	1723276	1732342
3	1722340	1747973
4	1686176	1771997
5	1728476	1781882
6	1686155	1733584
Mean	**1713098.2**	**1747605.0**
SD	**21165.05**	**24838.71**
%CV	**1.24**	**1.42**
N	**6**	**6**
% Stability	**98.20**	

Calculations:

$$\% \text{ Stability} = \frac{\text{Average response of stability sample} \times \text{Concentration of reference stock} \times 100}{\text{Average response of reference sample} \times \text{Concentration of stability stock}}$$

Observation:

The room-temperature stock solution stability of Duloxetine was evaluated at 20 ± 5°C. The stability value at 0 hour was observed as 98.75%, while the precision after 9 hours ranged from 0.53% to 0.97%.

Similarly, Duloxetine-D5 stock solution showed 98.20% stability at 0 hour under the same room-temperature condition. After 9 hours, the precision values were recorded between 1.24% and 1.42%.

Room Temperature Spiking Solution Stability of Duloxetine and Duloxetine D5:

Stability of Duloxetine and Duloxetine D5 (Internal Standard) was tested in room temperature (20 ± 5 °C) spiking solution at 9 hours. Six replicates of stock dilutions of Duloxetine equal to the final MQC2 quality control concentration and Duloxetine D5 at the final working concentration, respectively were injected from the spiking solution. Duloxetine and Duloxetine D5 stability samples were compared to freshly generated comparison samples at 9 hours for mean area response.

Table 7.13: Room Temperature Spiking Solution Stability of Duloxetine (0, 9 Hours)

Spiking solution ID	Hours	%Stability
DSD-03	0	100.00
DSD-10	9	99.80

	Stability standard stock Dilution	Comparison standard stock Dilution
Sr. No.	**Area**	**Area**
1	1388075	1360081
2	1375303	1364533
3	1367061	1382509
4	1333700	1370394
5	1363926	1372839
6	1351145	1343671
Mean	**1363201.7**	**1365671.2**
SD	**18953.92**	**13219.29**
%CV	**1.39**	**0.97**
N	**6**	**6**
% Stability	**99.80**	

Table7.14: Room Temperature Spiking Solution Stability of Duloxetine D5 (0, 9 Hours)

Spiking solution ID	Hours	%Stability
DDSD-01	0	100.00
DDSD-03	9	99.80

	Stability standard stock Dilution	Comparison of standard stock Dilution
S.No	**Area**	**Area**
1	1741695	1717852
2	1743520	1732342
3	1762489	1747973
4	1712857	1771997
5	1739921	1781882
6	1745714	1733584
Mean	**1741032.7**	**1747605.0**
SD	**16025.10**	**24838.71**
%CV	**0.92**	**1.42**
N	**6**	**6**
% Stability	**99.80**	

$$\% \text{ Stability} = \frac{\text{Mean response of stability samples} \times \text{Comparison stock concentration} \times 100}{\text{Mean response of comparison stock samples} \times \text{Stability stock concentration}}$$

Observation:

Stability of Duloxetine in spiking solution at room temperature (20 ± 5 □C) was reported to be 1.39% to 0.97% at 0 hour and 99.80% stable at 9 hour.

The stability of duloxetine D5 in a spiking solution at room temperature (20 ± 5 □C) was found to be 99.80% at 0 hours and 1.42% at 9 hours respectively.

Refrigerated Stock Solution Stability of Duloxetine and Duloxetine D5 at (2 - 8 °C):

To verify the stability of the cooled stock solution six different injections of the stock dilution were conducted at the final MQC2 level. The stock solutions were kept for four days without any modification.

Table 7.15: Refrigerated Stock Solution Stability of Duloxetine at 2 - 8 °C (4 Days)

Stock solution ID	Days	%Stability
DST-01	0	100.00
DST-05	4	100.57

S. No.	Stability standard stock	Comparison standard stock
	Area	Area
1	1774107	1756112
2	1732656	1747626
3	1729879	1767605
4	1768509	1741419
5	1759226	1705120
6	1729503	1721597
Mean	**1748980.0**	**1739913.2**
SD	**20632.03**	**22965.37**
%CV	**1.18**	**1.32**
N	**6**	**6**
% Stability	**100.57**	

Table 7.16: Refrigerated Stock Solution Stability of Duloxetine D5 at 2 - 8 °C (4 Days)

Stock solution ID	Days	%Stability
DDST-01	0	100.00
DDST-03	4	97.70

	Stability standard stock	Comparison standard stock
S.No.	**Area**	**Area**
1	2316337	2436408
2	2297576	2395827
3	2335573	2398046
4	2322345	2386261
5	2313333	2298379
6	2312299	2360068
Mean	**2316243.8**	**2379164.8**
SD	**12516.97**	**46588.20**
%CV	**0.54**	**1.96**
N	**6**	**6**
% Stability	**97.70**	

$$\% \text{ Stability} = \frac{\text{Average response of stability samples} \times \text{Comparison stock concentration} \times 100}{\text{Average response of comparison stock samples} \times \text{Stability stock concentration}}$$

Observation:

Duloxetine has a percentage of stability of 100.57% and a precision ranging from 1.18% to 1.32%.

Duloxetine D5 has a stability percentage of 97.70% and an accuracy ranging from 0.54% to 1.96%.

Refrigerated Spiking Solution Stability of Duloxetine and Duloxetine D5 at (2 - 8 °C):

They injected six different spiking dilutions at the final MQC2 concentration level to ensure the stability of the spiked solution in refrigeration. Over the course of four days, the spike solution remained stable.

Table 7.17: Refrigerated Spiking Solution Stability of Duloxetine at 2 - 8 °C (4 Days)

Spiking solution ID	Days	%Stability
-DSD-03	0	100.00
DSD-23	4	100.28

	Standard stock Dilution (Stability)	Standard stock Dilution (Comparison)
Sr. No.	**Area**	**Area**
1	1772766	1756112
2	1716669	1747626
3	1745732	1767605
4	1774469	1741419
5	1746555	1705120
6	1707613	1721597
Mean	**1743967.3**	**1739913.2**
SD	**27699.68**	**22965.37**
%CV	**1.59**	**1.32**
N	**6**	**6**
% Stability	**100.28**	

Table 7.18: Refrigerated Spiking Solution Stability of Duloxetine D5 at 2 - 8 °C (4 Days)

Spiking solution ID	Days	%Stability
DDSD-01	0	100.00
DDSD-08	4	97.22

	Standard stock Dilution (Stability)	Standard stock Dilution (Comparison)
Sr. No.	**Area**	**Area**
1	2338012	2436408
2	2293910	2395827
3	2252498	2398046
4	2299180	2386261
5	2348480	2298379
6	2297433	2360068
Mean	**2304918.8**	**2379164.8**
SD	**34498.82**	**46588.20**
%CV	**1.50**	**1.96**
N	**6**	**6**
% Stability	**97.22**	

Calculations:

$$\% \text{ Stability} = \frac{\text{Average response of stability samples} \times \text{Comparison stock concentration} \times 100}{\text{Average response of comparison stock samples} \times \text{Stability stock concentration}}$$

Observation:

A stability percentage of 100.28% was determined, while the precision varied between 1.32 and 1.59%.

The percentage of stability was determined to be 97.22%, while the precision varied between 1.50% and 1.96%.

Freeze-thaw Stability

Four freeze-thaw cycles were used to test the stability of Duloxetinein human plasma. Following three cycles of freezing and thawing, 6 replicates of LQC and HQC were examined. The accuracy and precision were determined by comparing the freeze-thaw quality control samples to standards from a recently spiked calibration curve, whose concentration range was the same as that utilized for the calculations.

Table 7.19: Freeze Thaw Stability Data of Duloxetine (IV Cycles)

	Nominal Conc. (ng/mL)			
	LQC		HQC	
QC#	**1.5062**	**% Accuracy**	**170.1937**	**% Accuracy**
Lower Limit	**1.2803**	**85.00**	**144.6646**	**85.00**
Upper Limit	**1.7321**	**115.00**	**195.7228**	**115.00**
83	1.4840	98.52	165.8722	97.46
84	1.5724	104.40	166.9185	98.08
85	1.4827	98.44	168.1446	98.80
86	1.5737	104.48	170.9211	100.43
87	1.5420	102.38	166.3796	97.76
88	1.5976	106.07	166.7745	97.99
Mean	**1.54207**		**167.50175**	
S.D.	**0.048787**		**1.837876**	
C.V.(%)	**3.16**		**1.10**	
% Nominal	**102.38**		**98.42**	
N	**6**		**6**	

Observation:

Over the course of four freeze-thaw cycles, the nominal percent varied between 98.42% and 102.38%, while the precision varied between 1.10% and 3.16%.

Bench Top Stability

The stability of duloxetine at the top was tested after 11 h for six sets of LQC and HQC, respectively. Quality control samples were measured against standards from a freshly spiked calibration curve for accuracy and precision. These standards had a concentration range similar to that used for precision and accuracy calculations. The half life of duloxetine was discovered to be eleven hours.

Table 7.20: Bench Top Stability Data of Duloxetine for 11 hours

	Nominal Conc.(ng/mL)			
	LQC		HQC	
QC#	**1.5062**	**% Accuracy**	**170.1937**	**% Accuracy**
Lower Limit	**1.2803**	**85.00**	**144.6646**	**85.00**
Upper Limit	**1.7321**	**115.00**	**195.7228**	**115.00**
155	1.4944	99.22	166.4495	97.80
156	1.5521	103.05	159.5742	93.76
157	1.5223	101.07	168.5407	99.03
158	1.5540	103.17	167.0918	98.18
159	1.5502	102.92	164.9309	96.91
160	1.5993	106.18	164.3492	96.57
Mean	**1.54538**		**165.15605**	
S.D.	**0.035162**		**3.122010**	
C.V.(%)	**2.28**		**1.89**	
% Nominal	**102.60**		**97.04**	
N	**6**		**6**	

Observation:

For eleven hours, the nominal percent fluctuated between 97.04% and 102.60%. The accuracy was between 1.89 and 2.28% for a duration of 11 hours.

Short-term Plasma Samples Stability at -20 °C:

The stability of Duloxetine in the case of plasma samples was temporarily stored in a -20°C deep freezer for five days was determined by quantification of six sets of LQC and HQC against the freshly spiked calibration curve standard. The stability % was obtained by comparing the newly spiked samples with the quality control samples.

Table 7.21: Short Term Stability Data of Duloxetine for Freshly Spiked QC's

	Nominal Concentration (ng/mL)			
	LQC		HQC	
QC#	**1.5063**	**% Accuracy**	**170.2027**	**% Accuracy**
Lower Limit	**1.2804**	**85.00**	**144.6723**	**85.00**
Upper Limit	**1.7322**	**115.00**	**195.7331**	**115.00**
1	1.5846	105.20	165.7090	97.36
2	1.5610	103.63	165.1669	97.04
3	1.5134	100.47	170.4253	100.13
4	1.5360	101.97	170.1785	99.99
5	1.4979	99.44	165.2254	97.08
6	1.5272	101.39	165.1536	97.03
Mean	**1.53668**		**166.97645**	
S.D.	**0.031698**		**2.585249**	
C.V.(%)	**2.06**		**1.55**	
% Nominal	**102.02**		**98.10**	
N	**6**		**6**	

Table 7.22: Short Term Stability Data of Duloxetine at –20 ºC(5 Days)

	Nominal Concentration (ng/mL)			
	LQC		HQC	
QC#	**1.5062**	**% Accuracy**	**170.1937**	**% Accuracy**
Lower Limit	**1.2803**	**85.00**	**144.6646**	**85.00**
Upper Limit	**1.7321**	**115.00**	**195.7228**	**115.00**
119	1.5706	104.28	165.7248	97.37
120	1.5327	101.76	168.5556	99.04
121	1.5197	100.89	168.8028	99.18
122	1.5212	101.00	165.3567	97.16
123	1.4938	99.18	164.1428	96.44
124	1.6029	106.42	165.8205	97.43
Mean	**1.54015**		**166.40053**	
S.D.	**0.039613**		**1.865218**	
C.V.(%)	**2.57**		**1.12**	
% Nominal	**102.25**		**97.77**	
N	**6**		**6**	
% Stability	**100.23**		**99.66**	

$$\% \text{ Stability} = \frac{\text{Average concentration of stability (LQC/HQC) samples} \times 100}{\text{Average concentration of freshly prepared (LQC/HQC) samples}}$$

Observation:

The precision varied from 1.12% to 2.57% and the percent stability from 99.66% to 100.23%.

Wet Extract Stability at (2 - 8 °C):

The wet extract stability was evaluated by processing and chilling six sets of QC samples, including both LQC and HQC, at temperatures ranging from 2 to 8 °C. After 40 hours, the samples were injected and measured against newly spiked standards of calibration curves with concentration ranges similar to those used for accuracy and precision calculations. After 40 hours in the fridge, the treated samples showed no signs of degradation, according to the results (2-4 °C).

Table 7.23: Wet Extract Stability Data of Duloxetine for 40 hours

	Nominal Conc. (ng/mL)			
	LQC		**HQC**	
QC#	**1.5062**	**% Accuracy**	**170.1937**	**% Accuracy**
Lower Limit	**1.2803**	**85.00**	**144.6646**	**85.00**
Upper Limit	**1.7321**	**115.00**	**195.7228**	**115.00**
149	1.5629	103.76	171.7728	100.93
150	1.5227	101.10	168.2410	98.85
151	1.4421	95.75	164.8167	96.84
152	1.5348	101.90	166.1765	97.64
153	1.5130	100.45	166.6719	97.93
154	1.5701	104.24	167.4493	98.39
Mean	**1.52427**		**167.52137**	
S.D.	**0.046011**		**2.385088**	
C.V.(%)	**3.02**		**1.42**	
% Nominal	**101.20**		**98.43**	
N	**6**		**6**	

Observation:

The percentage nominal at 40 hours ranged from 98.43% to 101.20% and precision range from 1.42% to 3.02%.

7.9 Auto sampler Stability:

To evaluate the stability of the auto sampler for Duloxetine, six sets of QC samples, including LQC and HQC, were processed and loaded into the device. Following 45 hours of infusion, the samples were measured using newly spiked calibration curve standards within a concentration range similar to that utilized for accuracy and precision calculations.

Table 7.24: Auto sampler Stability Data of Duloxetine for 45 hours

	Nominal Concentration (ng/mL)			
	LQC		HQC	
QC#	**1.5062**	**% Accuracy**	**170.1937**	**% Accuracy**
Lower Limit	**1.2803**	**85.00**	**144.6646**	**85.00**
Upper Limit	**1.7321**	**115.00**	**195.7228**	**115.00**
143	1.5228	101.10	167.2250	98.26
144	1.5599	103.56	166.2271	97.67
145	1.5919	105.69	169.9140	99.84
146	1.5370	102.04	164.5049	96.66
147	1.5855	105.27	164.2154	96.49
148	1.5522	103.05	172.5750	101.40
Mean	**1.55822**		**167.44357**	
S.D.	**0.026916**		**3.256349**	
C.V.(%)	**1.73**		**1.94**	
% Nominal	**103.45**		**98.38**	
N	**6**		**6**	

Observation:

The results showed that the samples treated were stable for 45 hours. Percent nominal at 45 hours was between 98.38% and 103.45% with precision between 1.73% and 1.94%.

Re Injection Stability:

Reinjection stability was performed by processing and analyzing six sets of QC samples including LQC and HQC utilizing a calibration curve reference. The QC samples were re-injected in the auto sampler after 24 h and quantified using the results of the initial calibration curve. The mean concentration of the original injection QCs was compared with that of the reinjected QCs.

Table 7.25: Concentration-response Linearity Data of Duloxetine for Reinjection Stability

	Concentration (ng/mL)									
	STD-A	**STD-B**	**STD-C**	**STD-D**	**STD-E**	**STD-F**	**STD-G**	**STD-H**	**STD-I**	**STD-J**
CC#	0.5105	1.0209	2.5524	5.1047	10.2094	30.0278	60.0556	120.1112	160.1482	200.1853
Lower Limit	0.4084	0.8678	2.1695	4.3390	8.6780	25.5236	51.0473	102.0945	136.1260	170.1575
Upper Limit	0.6126	1.1740	2.9353	5.8704	11.7408	34.5320	69.0639	138.1279	184.1704	230.2131
1	0.5326	0.9425	2.5166	4.9146	10.4636	30.3919	59.9395	116.3263	178.3631	193.6547
% Nominal	104.33	92.32	98.60	96.28	102.49	101.21	99.81	96.85	111.37	96.74

	Slope	Intercept	r^2
CC# 1	0.0066	0.0002	0.9966

Table 7.26: Reinjection Stability Data for Duloxetine (o hour)

	Nominal Concentration (ng/mL)			
	LQC		HQC	
QC#	**1.5062**	**% Accuracy**	**170.1937**	**% Accuracy**
Lower Limit	**1.2803**	**85.00**	**144.6646**	**85.00**
Upper Limit	**1.7321**	**115.00**	**195.7228**	**115.00**
1	1.4945	99.22	168.1813	98.82
2	1.5124	100.41	164.9271	96.91
3	1.5653	103.93	169.5194	99.60
4	1.4636	97.17	169.0611	99.33
5	1.5195	100.88	166.7102	97.95
6	1.5851	105.24	168.2177	98.84
Mean	**1.52340**		**167.76947**	
S.D.	**0.044967**		**1.691283**	
C.V.(%)	**2.95**		**1.01**	
% Nominal	**101.14**		**98.58**	
N	**6**		**6**	

Table 7.27: Reinjection Stability Data for Duloxetine (24 hour)

	Nominal Concentration (ng/mL)			
	LQC		HQC	
QC#	**1.5062**	**% Accuracy**	**170.1937**	**% Accuracy**
Lower Limit	**1.2803**	**85.00**	**144.6646**	**85.00**
Upper Limit	**1.7321**	**115.00**	**195.7228**	**115.00**
1	1.5711	104.31	169.1871	99.41
2	1.5696	104.21	168.6116	99.07
3	1.5773	104.72	167.0405	98.15
4	1.5941	105.84	167.4273	98.37
5	1.5602	103.58	166.3733	97.76
6	1.5482	102.79	166.7048	97.95
Mean	**1.57008**		**167.55743**	

S.D.	**0.015535**		**1.111791**	
C.V.(%)	**0.99**		**0.66**	
% Nominal	**104.24**		**98.45**	
N	**6**		**6**	
% Stability	**103.06**		**99.87**	

Observation:

The stability of the reinjected samples for 24 hours was demonstrated by the results. There was a wide range of precision (0.66% to0.999%) and percent stability (99.87% to 103.16%) throughout the course of 24 hours.

7.5.8. Carryover:

They extracted matrix blanks that were suitable for the experiment (COT BLANK), as well as high and low concentrations of the analytes that were within the calibration curve range (COT ULOQ and COT LLOQ, respectively).

Table 7.28: Carry Over Test Data for Duloxetine

COT LLOQ	Area of Analyte	Area of Internal Standard
1	7824	2264137
2	7641	2246263
3	7618	2240951
4	8168	2173396
5	8124	2259290
6	7477	2106306
Mean	**7808.7**	**2215057.2**
COT ULOQ	**Area of Analyte**	**Area of Internal Standard**
1	2739612	2066387
2	2663267	2081300
3	2703124	2087336

4	2645021	1997907
5	2655166	1946183
6	2576415	1961663
Mean	**2663767.5**	**2023462.7**

	Analyte		**Internal Standard**	
Blank	**Area response**	**% Carry over**	**Area response**	**% Carry over**
COT Blank-1	0	0.00	0	0.00
COT Blank-2	0	0.00	0	0.00
COT Blank-3	0	0.00	0	0.00
COT Blank-4	0	0.00	0	0.00
COT Blank-5	0	0.00	449	0.02
COT Blank-6	0	0.00	0	0.00

Observation:

The results show that the analyte and internal standard did not have a substantial carryover impact.

7.9. Recovery:

They extracted the analyte to 100%. Eighteen blank plasma samples were amalgamated with six sets of final dilutions of LQC, MQC2 and HQC with 500µL of

sample as well as an internal standard (non-extracted samples). Samples were handled and injected from three sets, LQC (161–166), MQC2 (71–76) and HQC (161–166). The extracted Duloxetine samples were compared to the non-extracted LQC, MQC2 and HQC samples. The internal standard response in all 18 non-extracted quality control samples was associated to the extracted samples (1-18).

Table 7.29: Assessment of Duloxetine Recovery from Human Plasma Samples

S. No	LQC Response		MQC2 Response		HQC Response	
	Extracted QC	Non-Extracted QC	Extracted QC	Non-Extracted QC	Extracted QC	Non-Extracted QC
	LQC (161-166)	LQC (1-6)	MQC2 (71-76)	MQC2 (1-6)	HQC (161-166)	HQC (1-6)
1	25446	30808	1562233	1764696	2494588	2787532
2	24877	28666	1543987	1795532	2429640	2851110
3	25222	28527	1558429	1736046	2393105	2825310
4	24247	28357	1551968	1797394	2395748	2849467
5	25945	28469	1585770	1752982	2352653	2861670
6	24531	28181	1531176	1794822	2399689	2763534
Mean	**25044.7**	**28834.7**	**1555593.8**	**1773578.7**	**2410903.8**	**2823103.8**
S.D.	**621.51**	**980.44**	**18476.00**	**26123.64**	**47790.19**	**39460.10**
C.V.(%)	**2.48**	**3.40**	**1.19**	**1.47**	**1.98**	**1.40**
N	**6**	**6**	**6**	**6**	**6**	**6**
% Recovery	**86.86**		**87.71**		**85.40**	

Table 7.30: Overall Determination of Duloxetine Recovery in Human Plasma Matrix

Mean	86.65
S.D.	1.168
N	3
% Difference	2.31

Observation:

The verage overall recovery of Duloxetine was 86.65% with a precision from 1.19% to 3.40%.

Table 7.31: Recovery of Duloxetine D5 from Human Plasma

Extracted QC ID	Extracted QC area	Non-Extracted QC ID	Non-Extracted QC area
EXTRACTED-LQC-161	2228961	NON-EXTRACTED-LQC-1	2710087
EXTRACTED-LQC-162	2176410	NON-EXTRACTED-LQC-2	2677540
EXTRACTED-LQC-163	2206763	NON-EXTRACTED-LQC-3	2783647
EXTRACTED-LQC-164	2222539	NON-EXTRACTED-LQC-4	2639051
EXTRACTED-LQC-165	2236961	NON-EXTRACTED-LQC-5	2678522
EXTRACTED-LQC-166	2216968	NON-EXTRACTED-LQC-6	2692160
EXTRACTED-MQC2-71	2137465	NON-EXTRACTED-MQC2-1	2479504
EXTRACTED-MQC2-72	2102264	NON-EXTRACTED-MQC2-2	2510473
EXTRACTED-MQC2-73	2071762	NON-EXTRACTED-MQC2-3	2502544
EXTRACTED-MQC2-74	2109287	NON-EXTRACTED-MQC2-4	2562515
EXTRACTED-MQC2-75	2121626	NON-EXTRACTED-MQC2-5	2526196
EXTRACTED-MQC2-76	2076543	NON-EXTRACTED-MQC2-6	2521740
EXTRACTED-HQC-161	2095692	NON-EXTRACTED-HQC-1	2393534
EXTRACTED-HQC-162	1998886	NON-EXTRACTED-HQC-2	2411029
EXTRACTED-HQC-163	2019654	NON-EXTRACTED-HQC-3	2372185
EXTRACTED-HQC-164	2038488	NON-EXTRACTED-HQC-4	2414462
EXTRACTED-HQC-165	2009435	NON-EXTRACTED-HQC-5	2430501
EXTRACTED-HQC-166	2043184	NON-EXTRACTED-HQC-6	2336563
Mean	**2117382.7**	**Mean**	**2535680.7**
SD	**80843.62**	**SD**	**133146.66**
CV%	**3.82**	**CV%**	**5.25**

N	**18**	**N**	**18**
% Recovery	**83.50**		

Observation:

The mean recovery of internal standard Duloxetine D5 was 83.50% with a precision ranging from 3.82% to 5.25%.

7.10. Dilution Integrity:

Dilution integrity of Duloxetine was assessed by preparing 24 sample sets at a concentration level 1.75 times higher than the upper calibration standard, equivalent to 350.3987 ng/mL. From these samples, twelve sets were subjected to 2-fold dilution, while another twelve sets were diluted 4-fold before processing.

The diluted QC samples were analyzed together with undiluted calibration curve standards from PA-2 for precision and accuracy evaluation. The final QC concentrations were calculated by applying the respective dilution factors. The results confirmed that Duloxetine maintained acceptable dilution integrity at both 2-fold and 4-fold dilution levels.

Table 7.32: Concentration Response Linearity Data of Duloxetine for Dilution Integrity

	Nominal Conc. (ng/mL)									
	STD-A	**STD-B**	**STD-C**	**STD-D**	**STD-E**	**STD-F**	**STD-G**	**STD-H**	**STD-I**	**STD-J**
CC#	0.5105	1.0209	2.5524	5.1047	10.2094	30.0278	60.0556	120.1112	160.1482	200.1853
Lower Limit	0.4084	0.8678	2.1695	4.3390	8.6780	25.5236	51.0473	102.0945	136.1260	170.1575
Upper Limit	0.6126	1.1740	2.9353	5.8704	11.7408	34.5320	69.0639	138.1279	184.1704	230.2131
2	0.5123	1.0283	2.5231	4.8586	10.2039	29.8088	59.0164	117.1380	181.0673	193.8122
% Nominal	100.36	100.72	98.85	95.18	99.95	99.27	98.27	97.52	113.06	96.82

	Slope	Intercept	r^2
CC# 2	0.0067	0.0001	0.9970

Table 7.33: Dilution Integrity Data for Duloxetine (2 Times and 4 Times)

	NOMINAL CONC.(ng/mL)				
	Four times dilution			Two times dilution	
QC#	**DIQC (1-6)**			**DIQC (7-12)**	
	350.3987	**% Accuracy**		**350.3987**	**% Accuracy**
Lower Limit	297.8389	85.00	**Lower Limit**	297.8389	85.00
Upper Limit	402.9585	115.00	**Upper Limit**	402.9585	115.00
1	348.8465	99.56	7	340.3172	97.12
2	355.3539	101.41	8	331.7812	94.69
3	353.7157	100.95	9	331.0072	94.47
4	362.7736	103.53	10	343.5826	98.05
5	350.2342	99.95	11	333.7681	95.25
6	349.4372	99.73	12	338.5307	96.61
Mean	353.39352		**Mean**	336.49783	
S. D	5.258174		**S. D**	5.074626	
C.V %	1.49		**C.V %**	1.51	
%Nominal	100.85		**%Nominal**	96.03	
N	6		**N**	6	

Observation:

At a 2-dilution factor, the accuracy and precision of duloxetine were 96.03% and 1.51%, respectively. Likewise, with a dilution factor of 4, the accuracy and precision for duloxetine were 100.85% and 1.49%, respectively.

7.11. Ruggedness:

Separate columns of the same manufacturer and various solutions were employed for processing and analysis of 1 precision and accuracy batch (PA-3).

Table 7.34: Precision and Accuracy (within Batch) of Duloxetine for Ruggedness

	NOMINAL CONC. (ng/ml)									
	LLOQ QC		LQC		MQC-1		MQC-2		HQC	
QC#	0.5121	% Accuracy	1.5062	% Accuracy	40.1657	% Accuracy	100.4143	% Accuracy	170.1937	% Accuracy
Lower Limit	0.4097	80.00	1.2803	85.00	34.1408	85.00	85.3522	85.00	144.6646	85.00
Upper Limit	0.6145	120.00	1.7321	115.00	46.1906	115.00	115.4764	115.00	195.7228	115.00
13	0.5313	103.75	1.6517	109.66	39.7626	99.00	103.8061	103.38	165.0294	96.97
14	0.4979	97.22	1.6161	107.29	40.2708	100.26	104.7309	104.30	167.2083	98.25
15	0.5052	98.66	1.6980	112.73	41.1154	102.36	105.5570	105.12	164.9553	96.92

16	0.5098	99.55	1.6904	112.23	41.0231	102.13	103.5863	103.16	167.4120	98.37
17	0.5148	100.54	1.6803	111.56	40.8792	101.78	105.7529	105.32	171.7419	100.91
18	0.5251	102.54	1.7021	113.00	40.6307	101.16	105.0833	104.65	166.3935	97.77
Mean	0.51402		1.67310		40.61363		104.75275		167.12340	
S.D.	0.012469		0.033224		0.516148		0.895948		2.491557	
C.V.%	2.43		1.99		1.27		0.86		1.49	
% Nominal	100.37		111.08		101.12		104.32		98.20	
N	6		6		6		6		6	

Observation:

Within-batch precision:

LLOQ QC (2.43%), LQC (1.99%), MQC1 (1.27%), MQC2 (0.86%) and HQC (1.49%).

Within-batch accuracy:

LLOQ QC (100.37%), LQC (111.08%), MQC1 (101.12%), MQC2 (104.32%) and HQC (98.20%).

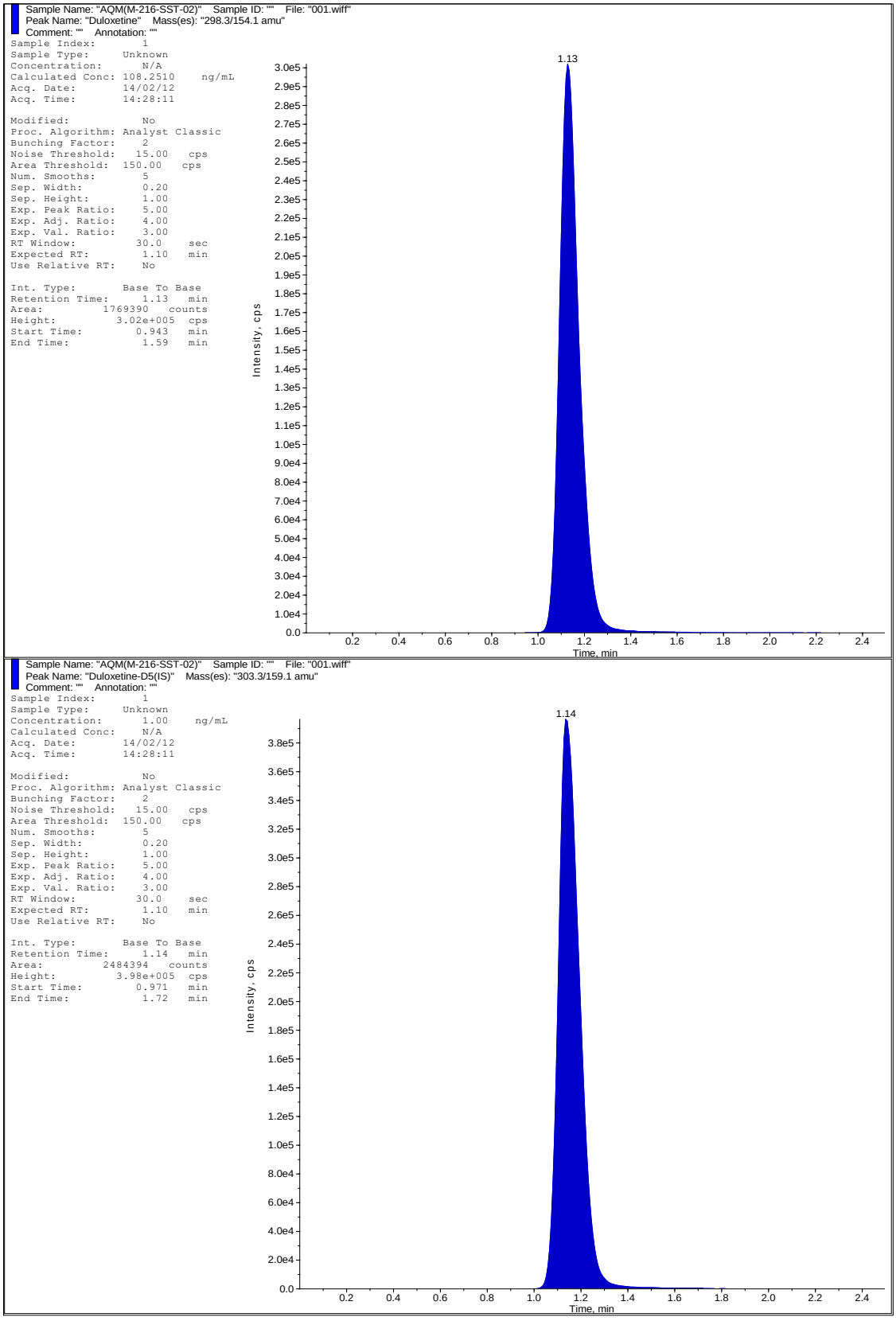


Fig 7.4: A Representative Chromatogram of an Aqueous Standard and Internal Standard Mixture of Duloxetine

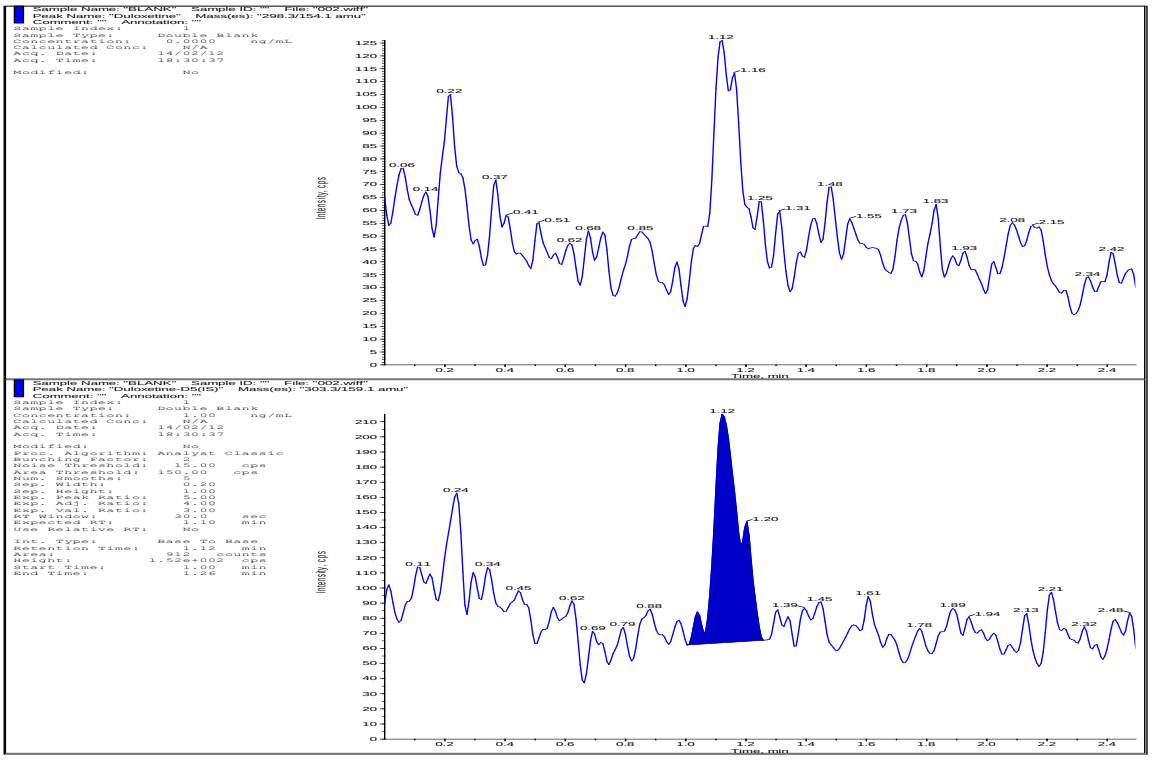


Fig 7.5: Chromatogram of Blank Plasma Sample of Duloxetine

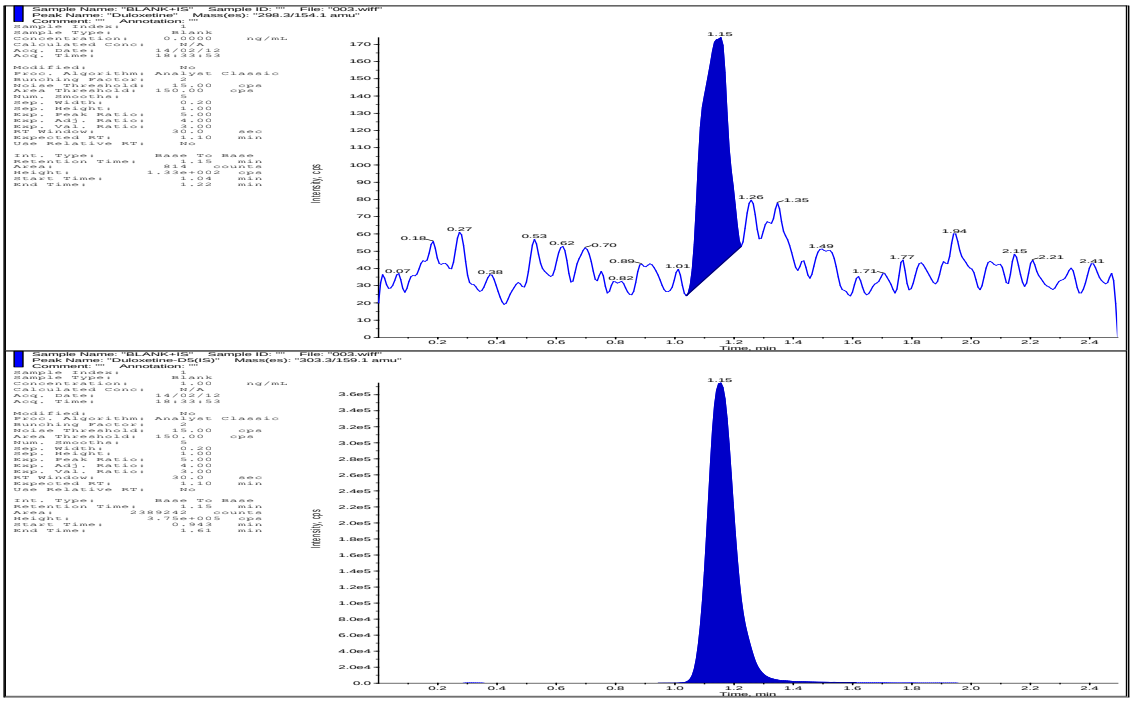


Fig 7.6: Chromatogram of Blank Plasma with IS Sample of Duloxetine

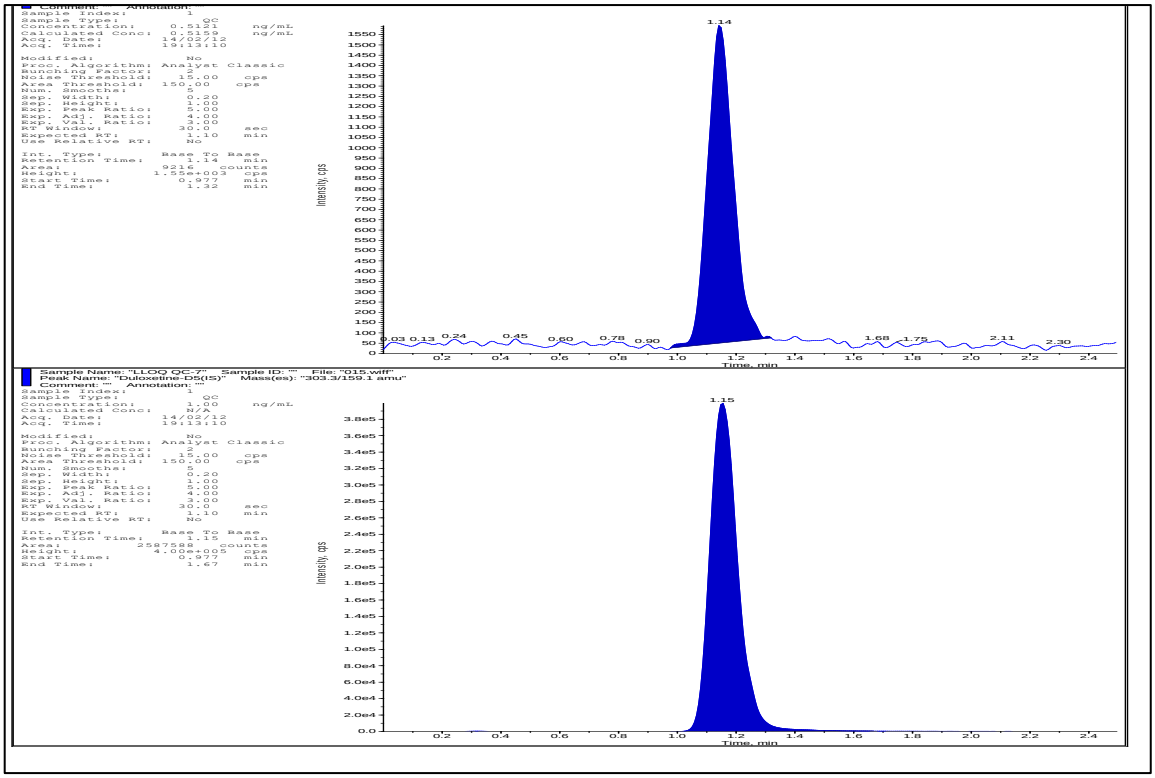


Fig 7.7: Chromatogram of LLOQ QC of Duloxetine

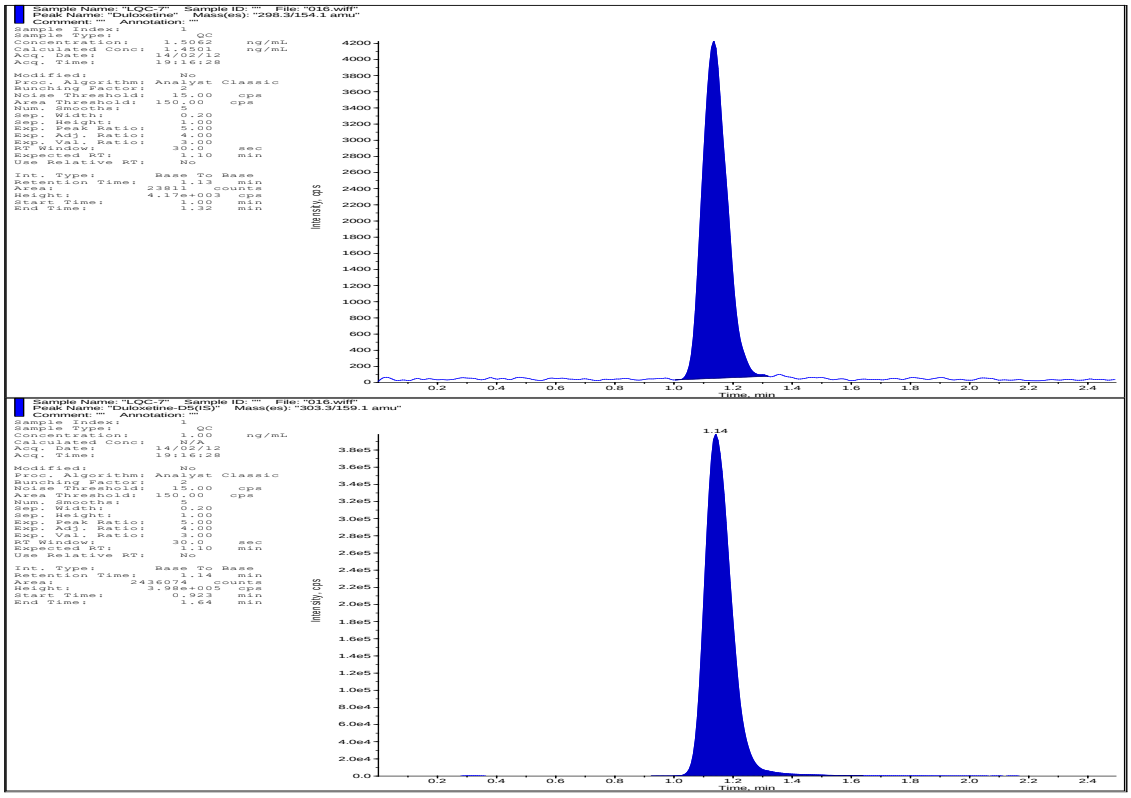


Figure 7.8: A Chromatogram of LQC of Duloxetine

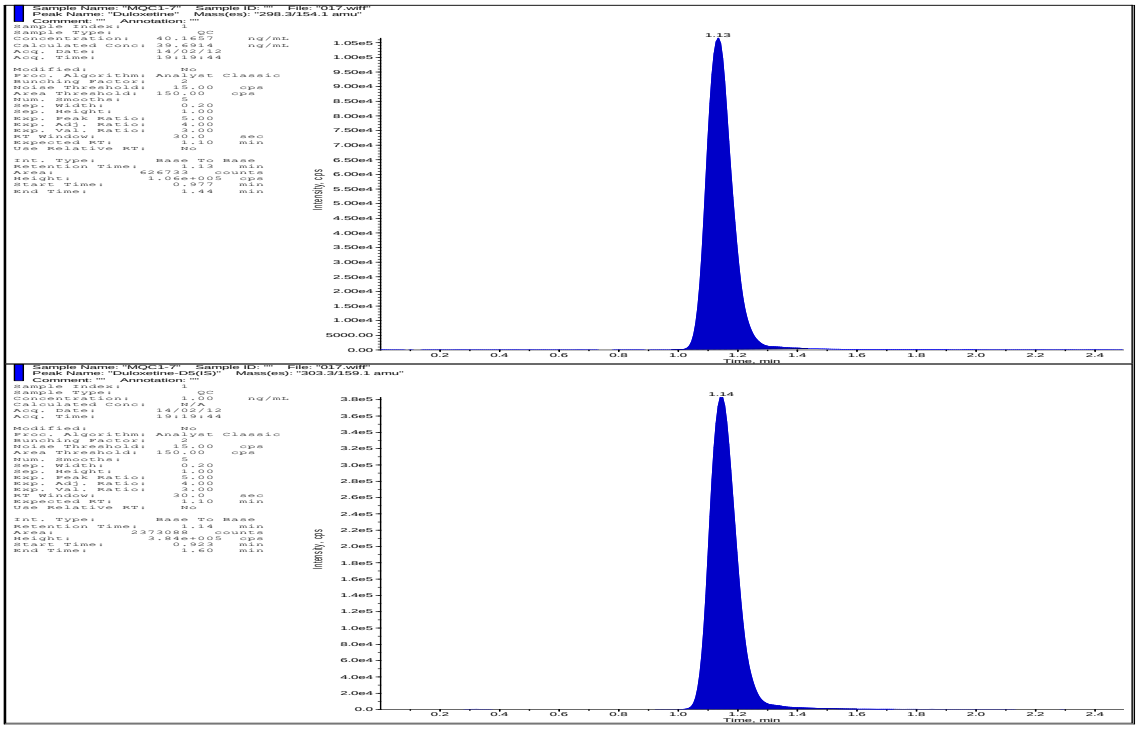


Fig 7.9: Chromatogram of MQC1of Duloxetine

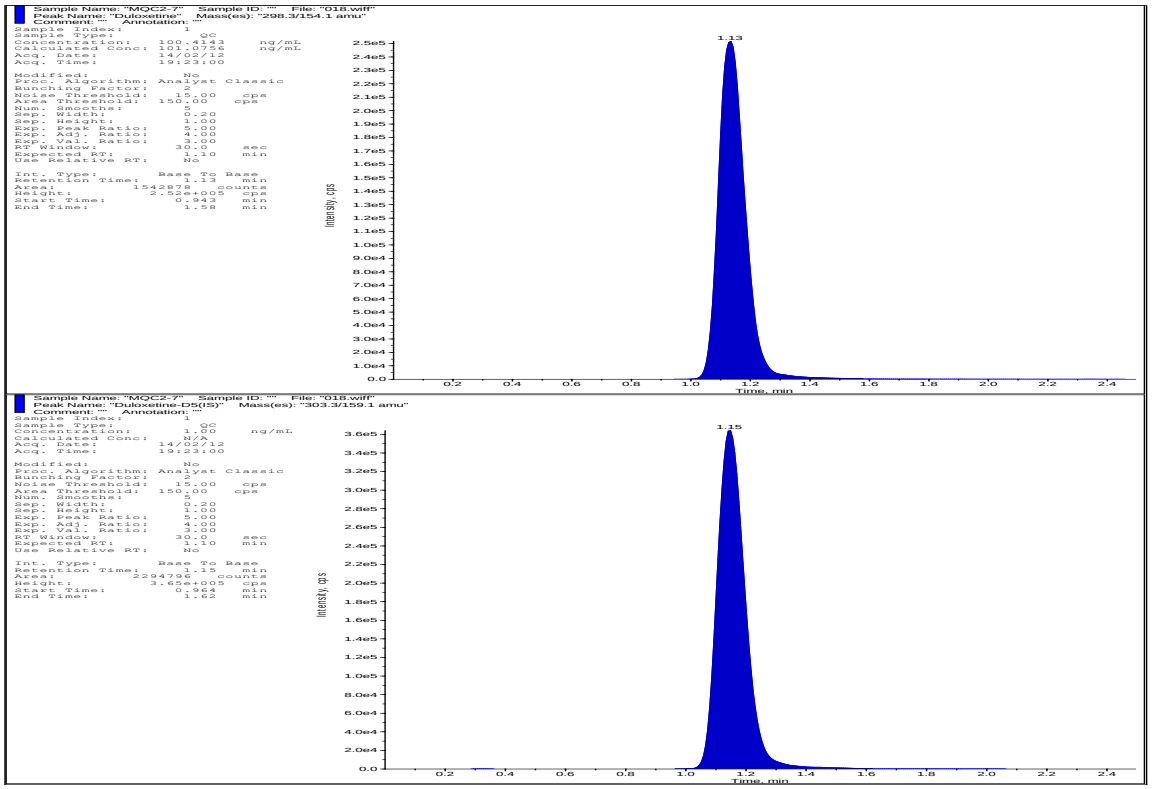


Fig 7.10: Chromatogram of MQC2 of Duloxetine

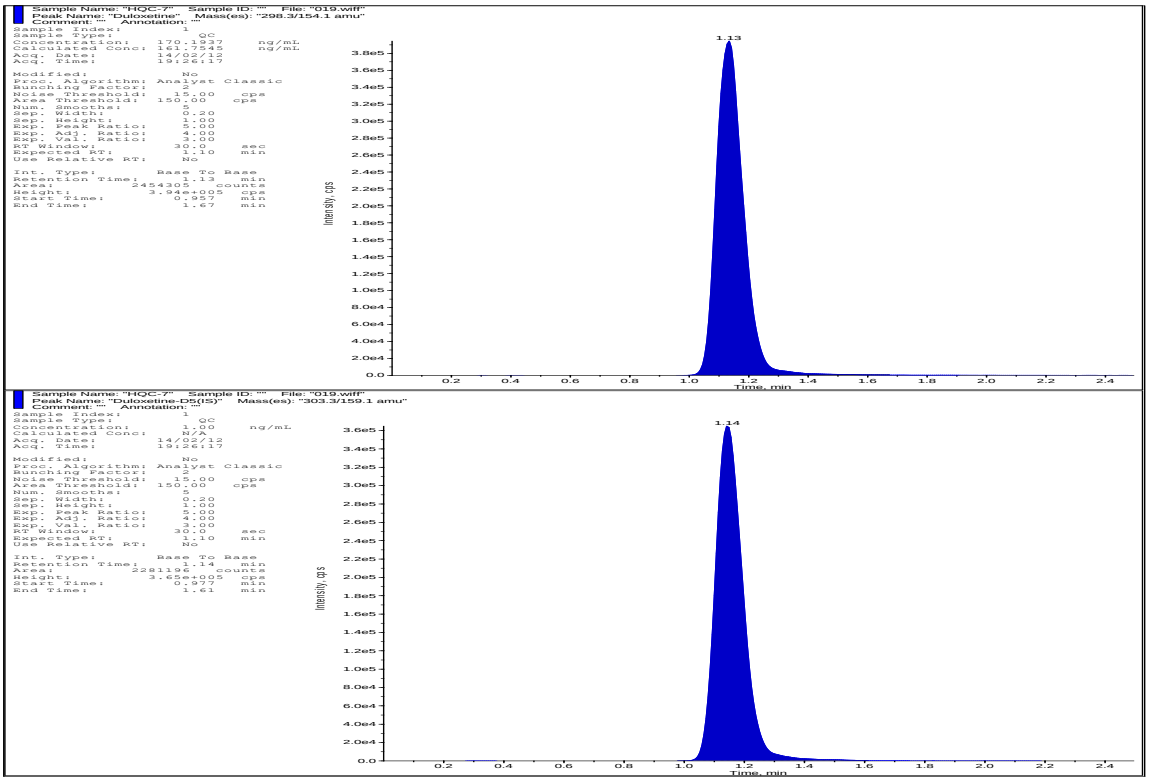


Fig 7.11: A Chromatogram of HQC of Duloxetine

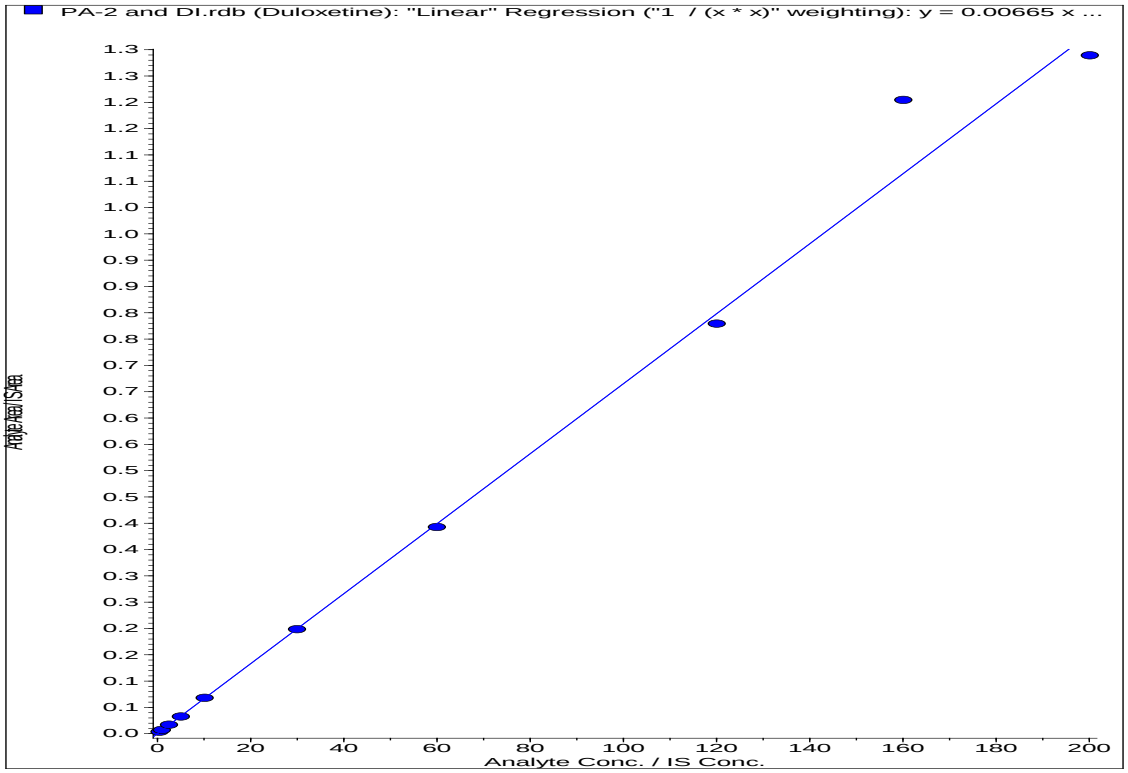


Fig 7.12: A Calibration Curve for Regression Analysis of Duloxetine

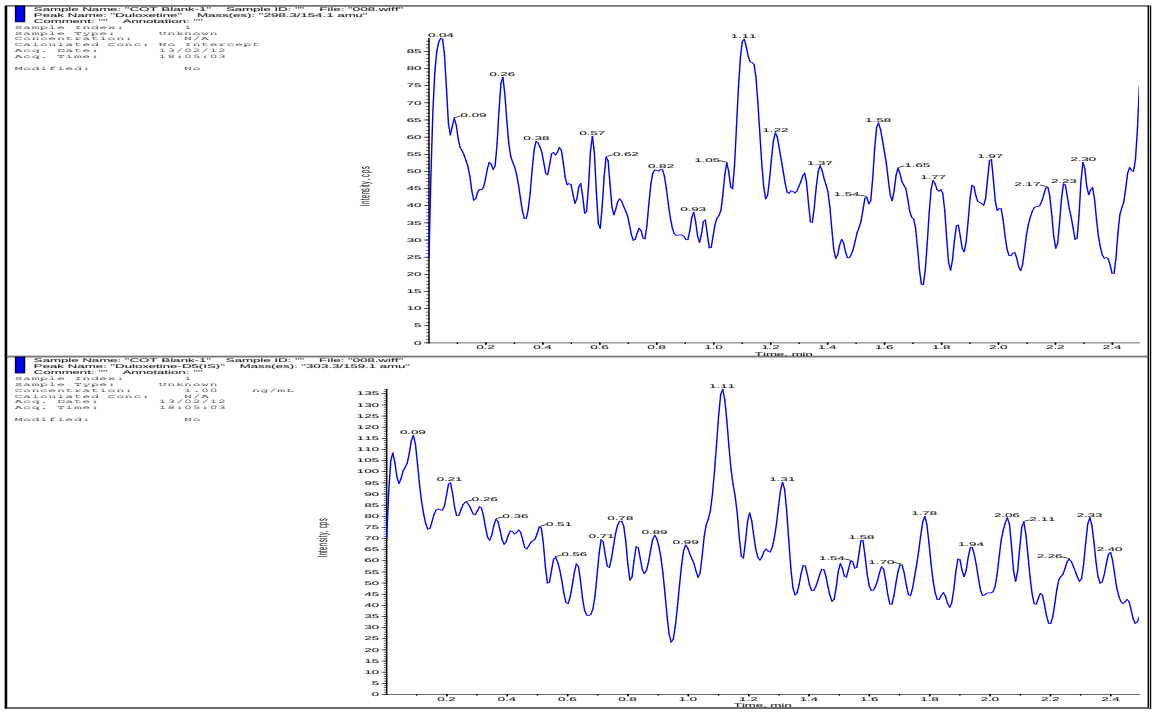


Fig 7.13: A Chromatogram of Extracted plasma (blank) sample analyzed for Carryover Test

7.11: Discussion

The quantitative determination of Anti-depressant drugs was required for the scheduled pharmacokinetic and pharmacodynamic study of plasma samples. Available methods by means of LC-MS/MS had been published for the simultaneous estimation of these drugs in human plasma in the literature. Among published methods, only individual or combination of these drugs were analyzed in biological samples in support to clinical trials. These reported methods require lengthy steps and extensive labor for sample preparation. As a result, undesirable variability of precision and accuracy in quantitative measurements of all analyte at trace levels (ng/ml) can be introduced. I have successfully developed and validated this LC-MS/MS method to meet the sensitivity levels required. As solid phase extraction and protein precipitation were relatively time consuming and cost affecting when associated with other method called, liquid-liquid extraction which was simple, suitable for estimation of analyte within less time and also economical.

Due to the varying polarities of Duloxetine, developing a chromatographic method encompassing the single analyte proved to be challenging. During method

development, it was observed that analyte yielded unacceptable tailing and peak splitting with Intersil ODS, 4.6× 50mm,5µm, thus methods utilizing these columns might not be feasible for method validation. After having evaluated several columns, ZORBAX SB-C18 2.1× 50mm,5µmwas chosen for acceptable chromatography and quantitation.

During MS/MS experiments "multiple reactions monitoring mode (MRM)" was chosen for needed selectivity and sensitivity. Among potential transitions from precursor ion to product/daughter ion, it was noted that compound (Duloxetine hydrochloride), plus internal standards (Duloxetine-D5 oxalate) were observed during MS/MS scans. For instance, transitions from 298.90 to 154.10 were evident, which may be used in MRM detections. For internal standards the MRM transitions were from 303.30 (parent) and 159.10 (product) for Duloxetine-D5 oxalate. Selection of the above ion transitions for detection and quantitation was made upon adequate selectivity and sensitivity in the actual K_2EDTA human plasma matrix.

The method validation batches met the acceptance criteria for linearity (at least 75% of non-zero standards at 85-115% accuracy (80-120% at the LLOQ) and of the regression statistics > 0.990) for all analytes. The above method validation batches met the acceptance criteria for precision and accuracy of quality control samples at the LLOQ (at least 2/3 QCs at 80-120%, inter- and intra-batch average accuracy of 80-120%, inter and intra-batch %CV < 20%), inter- and intra-batch at the low mid and high QC levels (at least 2/3 QCs at 85-115% accuracy, inter and intra-batch average accuracy between 85-115%, inter and intra-batch %CV < 15%). The analysis showed no interfering peaks at the retention time of the analytes or internal standards in the specificity from the six individual lots of K_2EDTA human plasma used.

The recovery of the liquid-liquid extraction procedure was also evaluated. Extracts of the "low, mid and high QC levels" were compared with injection of neat standards at the same final concentration. The recoveries were typically within 73-100% for all analytes (86.65% for Duloxetine hydrochloride). Freeze-thaw stability bench-top and auto-sampler "stability of low, mid and high-quality control samples" was validated for freezing and thawing over three cycles and storing samples at auto-sampler respectively. The analytes showed no instability due to freezing and thawing and storage at auto-sampler temperature conditions.

7.12: Summary

A simple, specific, sensitive, economical, and validated method is developed using LC-MS/MS with Atmospheric pressure ionization (API) in human plasma for estimation of drug (Duloxetine hydrochloride) used for major depressivedisorder. Separation of analyte and internal standard (Duloxetine-D5 oxalate) was performed on ZORBAX SB-C18 2.1× 50mm,5µmcolumn with a runtime of 2.50 min. Duloxetine hydrochloride were run in positive mode using Duloxetine-D5 oxalate as IS with mobile phase composition ofHPLCgrade Acetonitrile: water: ammonium acetate (5 mM) in an 85:15 (v/v) ratio In order to facilitate rapid analysis, the samples were treated using liquid-liquid extraction (LLE). Over a dynamic concentration range of 0.5-200 ng/ml for Duloxetine, respectively, the approach was validated. In line with the US FDA Bio analytical technique validation criteria, the method has been validated to cover precision, accuracy, selectivity, linearity/range, recoveries, freeze-thaw cycles, bench-top, and auto-sampler stabilities. The method's precision, accuracy, and linearity were all satisfactory. There was a wide range of recoveries, from 73% to 100%.

7.13: Conclusion:

For the purpose of estimating duloxetine hydrochloride in human plasma, a rapid, easy, sensitive, specific, cost-effective, and LC-MS/MS approach has been designed and validated. The use of a liquid-liquid extraction (LLE) clean-up stage gives simplicity of application and is capable of processing no. of samples with in short time. Intra-assay and inter-assay variability has also shown the capability of the method described here to provide reproducible determination of analytes in a matrix.

Moreover, significant decrease in analysis time would lead to increased throughput of samples providing a more viable approach for estimation of analyte than is currently available. The method showed good applicability for measuring these drugs in plasma samples.

Chapter - 8

METHOD DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF EMETREXED AND CISPLATIN

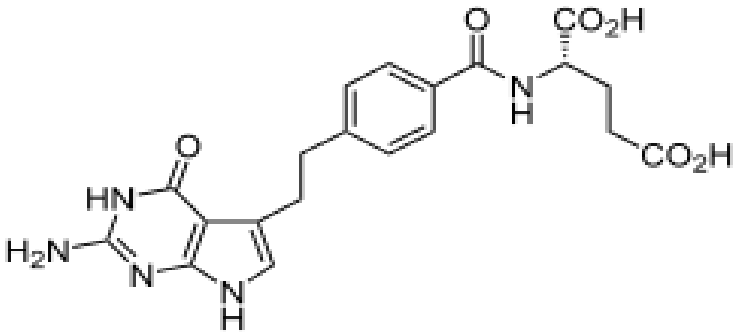
8.1. DRUG PROFILE

Drug: Pemetrexed

Synonym: LY-231514, MTA (Multi-targeted antifolate)

Drug category: Antineoplastic, antifolate,antimetabolite [1]

Structure:



Folate analog antimetabolite (pyridopyrimidine nucleus)

Chemical name/ Nomenclature / IUPAC Name:

~N-[4-[2-(2-amino-4,7-dihydro-4-oxo-1H-pyrrolo[2,3-d]pyrimidin-5-yl)ethyl]benzoyl] -L-glutamic acid disodium[2-5]

Molecular Formula: $C_{20}H_{21}N_5O_6$

Molecular Weight: 427.41 g/mol

Official Pharmacopoeia: Usually listed in regional pharmacopoeias (e.g., British Pharmacopoeia, Indian Pharmacopoeia; check country specific sources)[6-10]

8.1.1. Physicochemical Properties [11-15]

Description (Physical State): Powder for concentrate for infusion (white to off-white)

Solubility: Soluble in water for injection (formulated for IV use)

Stability: Stable when lyophilized; dilute and use per label instructions

Storage Conditions: Store unopened vials at controlled room temperature (20–25 °C)

Dosage: Tablet

8.1.2. Pharmacokinetic Properties [16-21]

Bioavailability: Not applicable — IV administration

Half-life: ~3.5 hours

Absorption: IV – complete systemic availability

Volume of Distribution: ~9 L/m² (~16.1 L total)

Protein binding: ~81%

Metabolism: Minimal hepatic metabolism

Metabolites: None significant

Time of peak action: (Not applicable to cytotoxic chemo)

Excretion: ~70–90% unchanged in urine within 24 hr

Adverse effects/Side effects:

Common: fatigue, nausea, myelosuppression (neutropenia, anaemia), stomatitis, vomiting, constipation. [22-26]

8.1.3. Pharmacodynamics

Mechanism of action: Multi targeted antifolate — inhibits thymidylate synthase (TS), dihydrofolate reductase (DHFR), and GARFT, blocking DNA and RNA synthesis. [27-32]

Therapeutic efficacy/Indications:

- Malignant pleural mesothelioma (with cisplatin)
- Non-small cell lung cancer (non-squamous), first-line with cisplatin
- Maintenance or single agent therapy in selected settings

Adverse reactions: Myelosuppression, mucositis, rash, GI symptoms

Contraindications: Hypersensitivity, severe renal impairment (Creatinine clearance <45 mL/min)[33-39]

Interactions: NSAIDs (e.g., ibuprofen) may increase toxicity at impaired renal function

Drug interactions: Cisplatin does not change pemetrexed PK

Food interactions: Not applicable (IV drug)

8.1.4. Drug Formulation: Pemetrexed

S. No	Drug name	Label Claim	Brand name	Company
1	Pemetrexed	500 mg/vial	Alimta	Eli Lilly

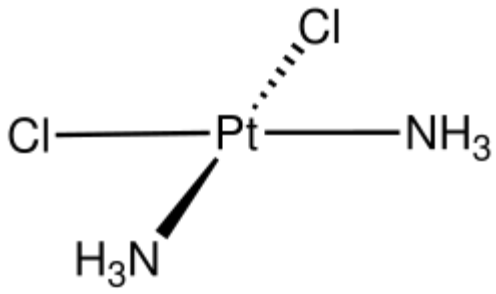
8.2. Drug Profile

Drug: Cisplatin

Synonym: Cis-diammine-dichloro-platinum (II), CDDP

Drug category: Platinum based antineoplastic/alkylating agent [40]

Structure:



$[Pt(NH_3)_2Cl_2]$ square planar coordination complex

Chemical name/ Nomenclature / IUPAC Name: Cis-diammine-dichloro-platinum(II)

Molecular Formula: $[Pt(NH_3)_2Cl_2]$

Molecular Weight: 300.05 g/mol

Official Pharmacopoeia: Listed widely in major pharmacopoeias (e.g., BP, IP, USP)[41-44]

8.2.1. Physicochemical Properties

Description (Physical State): Yellow orange crystalline powder

Solubility: Soluble in water/saline; ~1 mg/mL

Stability: Stable under normal conditions; protection from light recommended in solution.[45-49]

Storage Conditions: As per product label (typically controlled room temp)

Dosage: Tablet

Melting point: ~207 °C

pKa (strongest acidic): Not applicable (coordination complex) [50]

Log P: ~-2.19 (water soluble)

8.2.2. Pharmacokinetic Properties

Bioavailability: 100% (IV)

Half-life: ~30–100 hrs (terminal)

Absorption: Not applicable (IV)

Volume of Distribution: ~11-12 L/m² steady state

Protein binding:>95%

Metabolism: Not metabolized significantly (reacts with proteins/DNA)

Metabolites: Platinum protein adducts (not active metabolites)

Time of peak action: (Not defined for cytotoxic drugs)

Excretion: Renal excretion of platinum species (varies by protein binding)

Adverse effects/Side effects:

Nephrotoxicity,nausea/vomiting, myelosuppression, ototoxicity, neurotoxicity, electrolyte disturbances. [51-53]

8.2.3. Pharmacodynamics

Mechanism of action: Binds DNA at guanine bases forming cross links → prevents replication & transcription → apoptosis.

Therapeutic efficacy/Indications:

- Advanced testicular cancer
- Ovarian cancer
- Bladder cancer
- Lung, head/neck carcinomas (often in combination)

Adverse reactions: Nephrotoxicity, severe emesis, neurotoxicity, ototoxicity, myelosuppression

Contraindications: Hypersensitivity to platinum agents, severe renal impairment,

pregnancy/breastfeeding

Interactions: Nephrotoxic agents ↑ toxicity; hydration & antiemetics required

Drug interactions: Cisplatin may interact with other cytotoxins & nephrotoxins

Food interactions: None specific (IV drug)

8.3. Drug Formulation: Cisplatin

S. No	Drug name	Label Claim	Brand name	Company
1	Cisplatin	50 mg/vial	Platinol	Various

8.4. Materials

8.4.1 List of Instruments

S. No	Instruments And Glass wares	Model
1	HPLC	WATERS Alliance 2695 separation module, software: Empower 2, 996 PDA detector.
2	pH meter	Lab India
3	Weighing machine	Sartorius
4	Volumetric flasks	Borosil
5	Pipettes and Burettes	Borosil
6	Beakers	Borosil

8.4.2. List of chemicals used:

S. No	Chemical	Brand names
1	Pemetrexed	Alimta
2	Cisplatin	Platinol
3	Water and Methanol for HPLC	LICHROSOLV (MERCK)
4	Acetonitrile for HPLC	Merck

8.5 Method Development:

8.5.1. Preparation of standard solution:

Accurately weigh 10 mg of Pemetrexed and Cisplatin working standards and transfer them into 10ml of clean dry volumetric flasks add about 7ml of Methanol sonicate to dissolve and elimination of air entirely and bring volume up to the mark with the same Methanol.

Pipette 0.1ml of the above Pemetrexed and 0.3ml of the Cisplatin stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

8.5.2. Procedure:

Inject the samples by varying the chromatographic conditions and record the chromatograms. Note the conditions for proper peak elution to perform the validation parameters as per ICH guidelines.

8.5.3. Mobile Phase Optimization:

The original mobile phases were Methanol: Water, Water: Acetonitrile and Methanol: Phosphate Buffer: ACN with different ratios. Finally, the mobile phase was adjusted to Acetonitrile: Phosphate Buffer in ratio 45 : 55 v/v respectively.

8.5.4. Optimization of Column:

The procedure was run on different columns such as C18 column, Symmetry column and Zodiac column. The optimal particle size was discovered to be Phenomenex Luna C18 (4.6×250mm, 5µm) as it produced good peak shape and resolution at 1ml/min flow rate.

8.5.5. Chromatographic conditions:

Instrument used : Waters HPLC with auto sampler and PDA Detector 996 model.

Temperature : 35ºC

Column : Phenomenex Luna C18 (4.6×250mm, 5µm) particle size

Buffer	:	Dissolve 6.8043 of potassium dihydrogen phosphate in 1000 ml HPLC water and adjust the pH 4.6 with diluted ortho-phosphoric acid. Filter and sonicate the solution by vacuum filtration and ultrasonication.
pH	:	4.6
Mobile phase	:	Acetonitrile: Phosphate Buffer (45:55 v/v)
Flow rate	:	1ml/min
Wave length	:	230 nm
Injection volume	:	10 µl
Run time	:	7 min

8.5.6. Preparation of Potassium dihydrogen Phosphate (KH_2PO_4) buffer (pH-4.6):

Dissolve 6.8043 g of potassium dihydrogen phosphate in 1000 ml of HPLC water and adjust the pH to 4.6 using diluted orthophosphoric acid. Vacuum filter and sonicate the solution.

8.5.7. Preparation of mobile phase:

The exact volume of 450 ml (45%) of Methanol and 550 ml of Phosphate buffer (55%) were combined and degassed in digital ultra sonicator for 15 minutes and then filtered through 0.45 µ filter using vacuum filtration.

8.6. Method Validation

8.6.1. System Suitability

Take 10mg of Pemetrexed and 10mg of Cisplatin working standard accurately weigh and transfer into a 10ml of clean dry volumetric flasks add around 7mL of Diluents and sonicate for dissolving it entirely and make volume up to the mark with the same solvent. (Stock solution) Pipette 0.1ml of the aforesaid Pemetrexed and 0.3ml

of the Cisplatin stock solutions in to a 10ml volumetric flask and dilute up to the mark with Diluent.

Procedure:

The standard solution was injected five times and area was determined for all five injections in HPLC. The %RSD of the area of five duplicate injections was observed within the limits.

8.6.2. Specificity:

Preparation of Standard Solution:

Accurately weigh and transfer 10mg of Pemetrexed and 10mg of Cisplatin working standard into a 10ml of clean dry volumetric flasks add around 7mL of Diluents and sonicate to dissolve it entirely and make volume up to the mark using the same solvent. Further Pipette 0.1ml of the aforesaid Pemetrexed and 0.3ml of the Cisplatin stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.(Stock solution)

Preparation of Sample Solution:

Average weight of one Tablet and crush in mortar using pestle and weigh 10 mg equivalent weight of Pemetrexed and Cisplatin sample into 10mL clean dry volumetric flask and add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with same solvent.

Pipette out 0.1ml of the above Pemetrexed and 0.3ml of the Cisplatinstock solutions into a 10ml volumetric flask and dilute to volume with Diluent.

Procedure:

Inject the three replicate injections of standard and sample solutions. Calculate the assay using formula:

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

8.6.3. Linearity:

Weigh and shift with care Take Pemetrexed 10 mg and Cisplatin 10mg working standard into 10ml clean dry volumetric flasks add around 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark using the same solvent. (Stock solution)

Preparation of Level – I (6 ppm of Pemetrexed& 18ppm of Cisplatin):

Pipette out 0.06ml of Pemetrexed and 0.18ml of Cisplatin stock solutions were taken in a 10ml of volumetric flask and diluted up to the mark with diluent.

Preparation of Level – II (8 ppm of Pemetrexed & 24ppm of Cisplatin):

Take 0.08ml Pemetrexed and 0.24ml Cisplatin stock solutions in a 10ml of volumetric flask and dilute up to the mark with diluents.

Preparation of Level – III (10 ppm of Pemetrexed & 30ppm of Cisplatin):

0.1 ml of Pemetrexed and 0.3ml of Cisplatinstock solutions was pipette out in a 10ml of volumetric flask diluted up to the mark with diluents.

Preparation of Level – IV (12 ppm of Pemetrexed & 36ppm of Cisplatin):

Take 0.12ml of Pemetrexed and 0.36ml of Cisplatin stock solutions in a 10ml of volumetric flask dilute up to the mark with diluent. Pipette out

Preparation of Level – V (14 ppm of Pemetrexed & 42ppm of Cisplatin):

0.14ml Pemetrexed and 0.42ml Cisplatinstock solutions in a 10ml volumetric flask and make up the volume to the mark with diluent.

Procedure:

Inject each level into the chromatographic apparatus and record the peak area. Plot graph of Peak area Vs Concentration (Concentration on X-axis and Peak area on Y-axis) and calculate the correlation coefficient.

8.6.4. Precision

8.6.4.1. Repeatability

Preparation of Pemetrexed and Cisplatin Product Solution Accuracy

Weigh accurately and transfer 10 mg of Pemetrexed and 10mg of Cisplatin working standard into a 10ml clean dry volumetric flask add around 7mL of Diluents and sonicate to dissolve it entirely and make volume up to the mark with same solution. (Stock-Solution)

Add 0.1ml of Pemetrexed and 0.3ml of Cisplatin stock solutions to 10ml volumetric flask and dilute up to the mark with Diluent.

The standard solution was injected five times and the area was determined for all five injections by HPLC. The %RSD of area for five duplicate injections was within the prescribed limits.

8.6.4.2. Intermediate Precision:

Precision was done on multiple days with same conditions to evaluate intermediate precision (also called Ruggedness) of the approach.

Procedure:

DAY 1:

The standard solution was injected for Six times and area was determined for all Six injections in HPLC. The %RSD for area of Six duplicate injections was confirmed to be within the prescribed ranges.

DAY 2:

The reference solution was injected for Six times and the area for all Six injections was measured in HPLC. The %RSD for the area of Six replicate injections was confirmed to be within the specified limits.

8.6.5. Correctness:

50% Standard stock solution:

Weigh accurately and transfer 10 mg of Pemetrexed working standard and 10mg of Cisplatin working standard into a 10ml of clean dry volumetric flasks add

around 7mL of Diluents sonicate to dissolve it entirely and make volume up to the mark with the same solvent. (solution stock)

Further Pipette 0.05ml of above Pemetrexed and 0.15ml of the Cisplatinstock solutions into 10ml volumetric flask and dilute up to the mark with Diluent.

Preparation of 100% Standard stock solution:

Accurately weigh and transfer 10 mg of Pemetrexed and 10mg of Cisplatin working standard into a 10ml of clean dry volumetric flasks add around 7mL of Diluents and sonicate to dissolve it entirely and make volume up to the mark with same solvent. (Stock-Solution)

• Pipette 0.1ml of the aforesaid Pemetrexed and 0.3ml of the Cisplatin stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Standard stock solution (150% preparation):

Accurately weigh and transfer 10 mg of Pemetrexed and 10mg of Cisplatin working standard into a 10ml of clean dry volumetric flasks add around 7mL of Diluents and sonicate to dissolve it entirely and make volume up to the mark with same solvent. (Stock solution)

Pipette 0.15ml Pemetrexed and 0.45ml Cisplatin from the above stock solutions into a 10ml volumetric flask and dilute to the mark with diluents.

Method:

Inject the Three injections of individual concentrations (50%, 100%, 150%) were made under the optimal conditions. Chromatogram was recorded and peak response was found Calculate the Amount found and Amount added for Pemetrexed and Cisplatin Calculate the individual and average recovery results.

8.6.6. Robustness:

The study was performed under different conditions in order to detect variability of the findings of testing. The following conditions are evaluated for variance of result.

Standard Solution Preparation:

Weigh accurately and transfer 10 mg of Pemetrexed and 10mg of Cisplatin working standard into a 10ml of clean dry volumetric flasks add around 7mL of Diluents and sonicate to dissolve it entirely and make volume up to the mark using the same solvent. (Stock-Solution)

Further pipette 0.1ml of the above Pemetrexed and 0.3ml of the Cisplatin stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Effect of flow conditions variation:

The sample was run at 0.9 ml/min and 1.1 ml/min instead of 1ml/min with all other parameters staying the same. 10µl of aforesaid sample was injected and chromatograms were recorded.

Effect of Variation of Organic Composition of Mobile Phase:

The sample was analyzed by change of mobile phase i.e. Acetonitrile: Phosphate Buffer was taken in the ratio of 50:50, 40:60 instead (45:55) remaining conditions are equal. The identical material was injected (10 µ l) and the chromatograms recorded.6.7. Results and Discussion

Optimized Chromatogram (Standard)

Mobile phase : Acetonitrile: Phosphate Buffer (pH-4.6) (45:55 v/v)

Column : Phenomenex Luna C18 (4.6×250mm, 5µm) particle size

Flow rate : 1 ml/min

Wavelength : 230 nm

Column temp : 35ºC

Injection Volume : 10 µl

Run time : 7 minutes

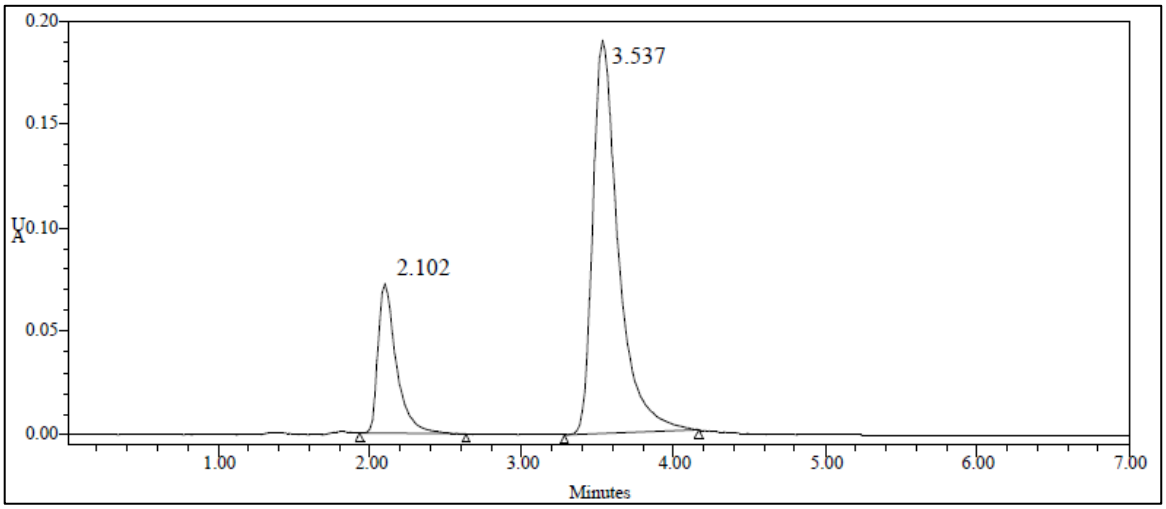


Fig 8.1 -: Optimized Chromatogram (Standard)

Table 8.1: - Peak results for Optimized Chromatogram

S. No	Peak name	R_t	Area	Height	USP Tailing	USP plate count
1	Cisplatin	2.102	765789	69584	0.97	5587.0
2	Pemetrexed	3.537	2532158	190049	1.26	5398.0

Observation: From the aforesaid chromatogram it was seen that peaks of Pemetrexed and Cisplatin are properly separated and they show proper retention period, peak tail and plate count.

Optimized Chromatogram (Sample)

Mobile phase : Acetonitrile: Phosphate Buffer (pH-4.6) (45:55 v/v)

Column : Phenomenex Luna C18 (4.6×250mm, 5µm) particle size

Flow rate : 1 ml/min

Wavelength : 230 nm

Column temp : 35ºC

Injection Volume : 10 µl

Run time : 7 minutes

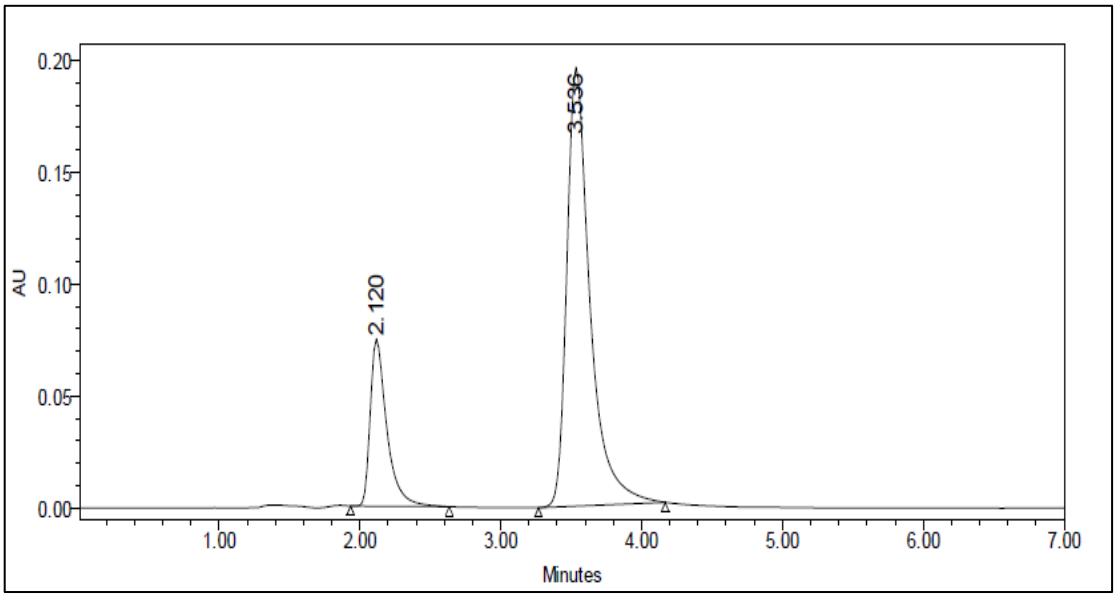


Fig: 8.2 Optimized Chromatogram (Sample)

Table 8.2: Optimized Chromatogram (Sample)

S. No.	Peak name	R_t	Area	Height	USP Tailing	USP plate count
1	Cisplatin	2.120	775684	13124	0.99	6365.0
2	Pemetrexed	3.536	2658478	937405	1.23	7458.0

Acceptance criteria:

- Resolution between two medicines should be 2 or more.
- 2000 theoretical plates minimum
- Tailing factor should be not less than 0.9 and not more than 2.
- From above data it was observed that all the system suitable parameters for created approach lies inside limit.

8.7: Result & Discussion

8.7.1. Validation Parameters

Blank:

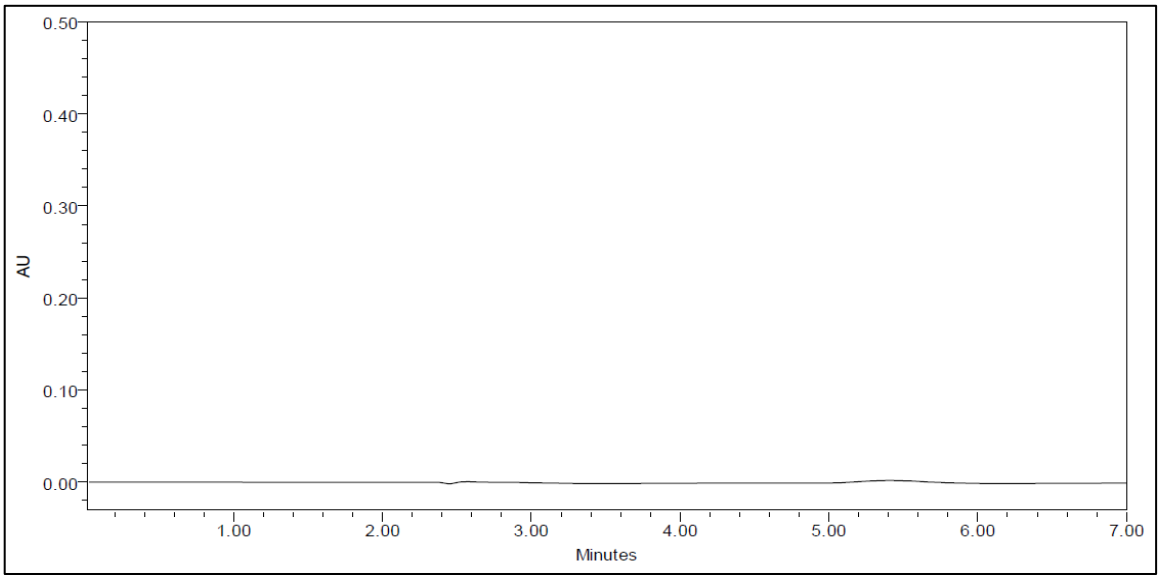


Fig 8.3: Chromatogram showing blank (mobile phase preparation)

System suitability:

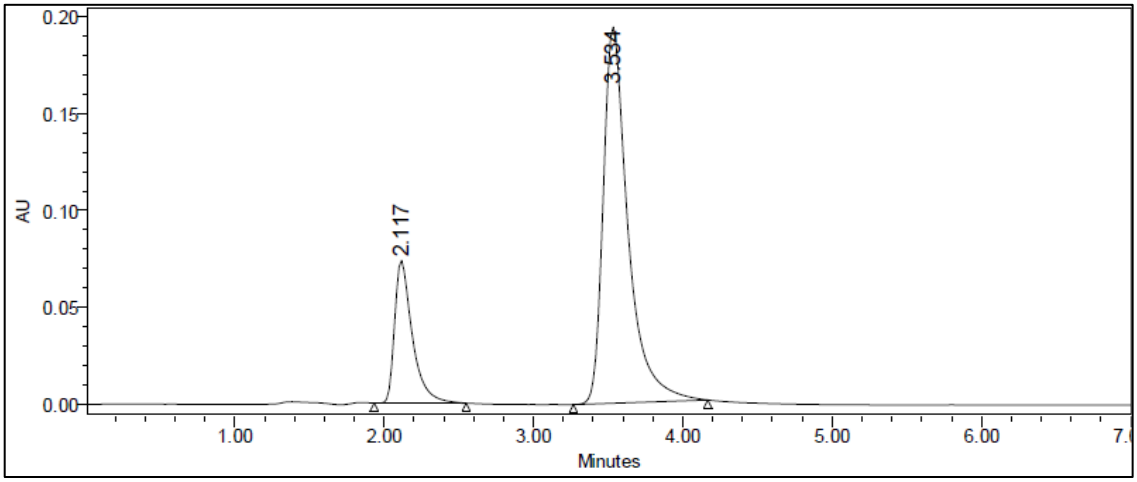


Fig: 8.4 Chromatogram showing injection -1

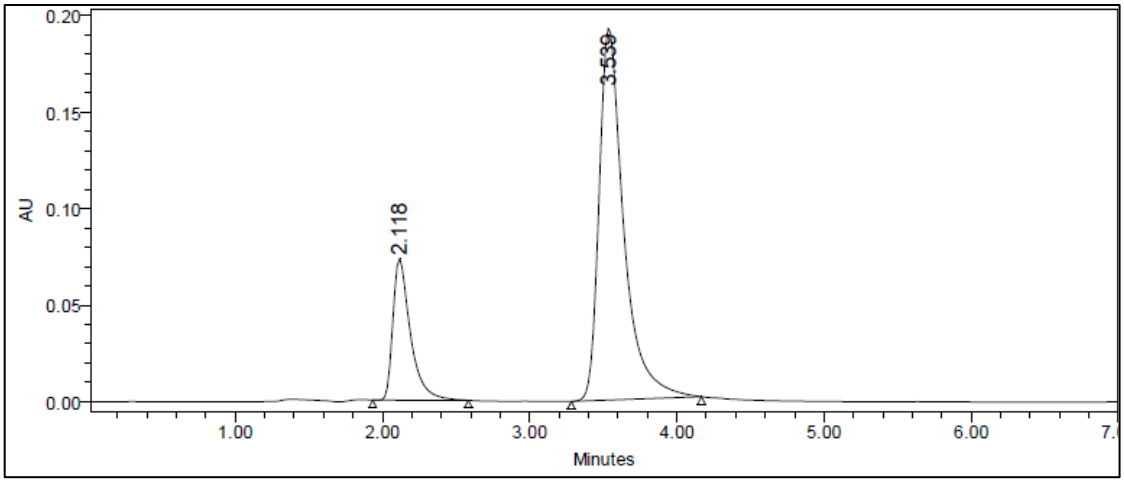


Fig 8.5: Chromatogram showing injection -2

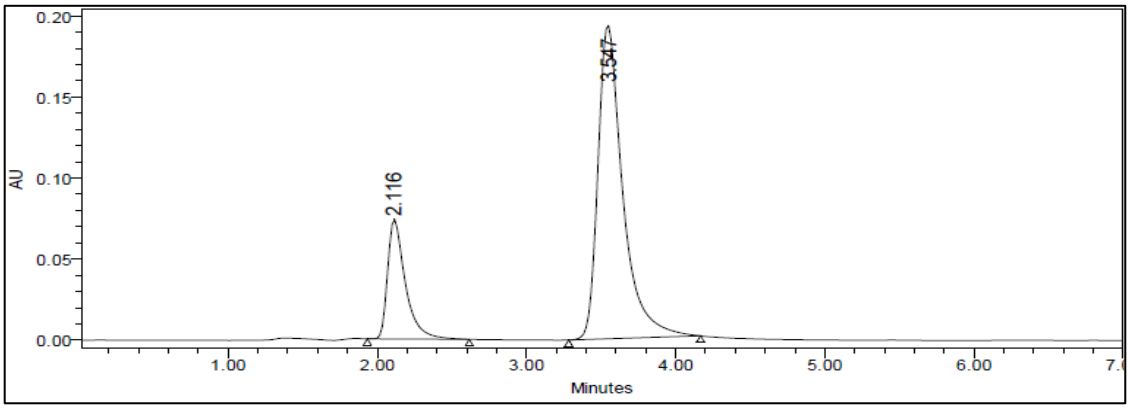


Fig 8.6: Chromatogram showing injection -3

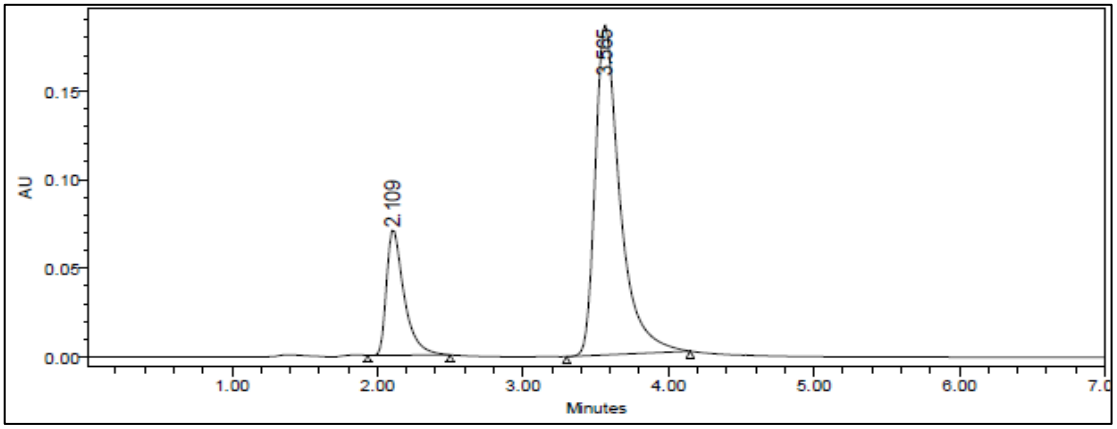


Fig8.7: Chromatogram showing injection -4

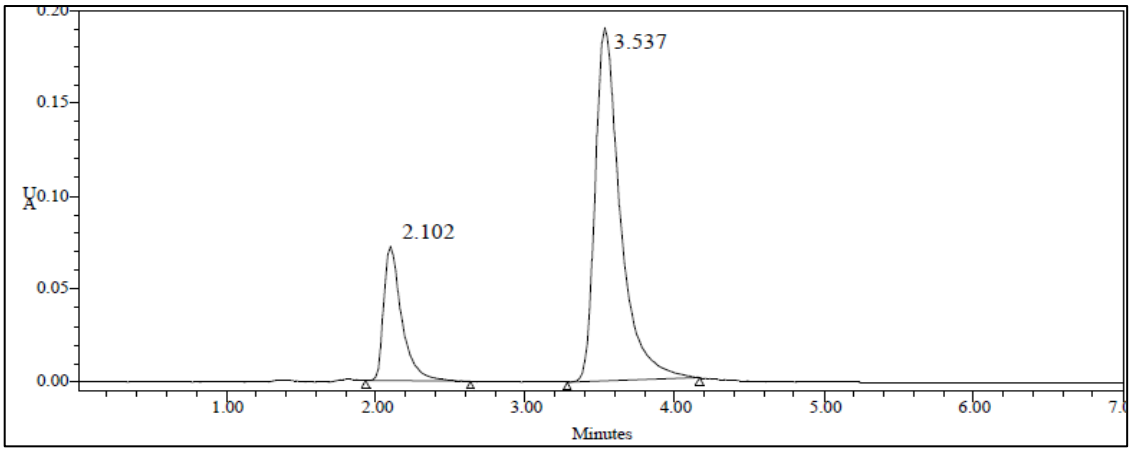


Fig 8.8: Chromatogram showing injection -5

Table 8.3: Results of system suitability for Cisplatin

S.no	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Cisplatin	2.117	765843	69587	5589	1.9

2	Cisplatin	2.118	766594	69854	5576	1.6
3	Cisplatin	2.116	765487	70211	5658	1.6
4	Cisplatin	2.109	765928	69213	5642	1.7
5	Cisplatin	2.102	765426	69558	5685	1.6
Mean			765855.6			
Std. Dev			466.6522			
% RSD			0.060932			

Acceptance criteria:

- %RSD of five distinct sample solutions shall not be more than 2
- %RSD obtained is within the limit hence the procedure is suitable.

Table 8.4: Results of system suitability for Pemetrexed

S.no	Name	R_t	Area	Height	USP plate count	USP Tailing
1	Pemetrexed	3.547	2534658	190058	5365	1.2
2	Pemetrexed	3.539	2536854	190052	5348	1.4
3	Pemetrexed	3.547	2535879	190078	5389	1.5
4	Pemetrexed	3.565	2533564	190035	5347	1.6

5	Pemetrexed	3.537	2534214	190085	5364	1.6
Mean			2535034			
Std. Dev			1183.309			
% RSD			0.046678			

Acceptance criteria:

- %RSD for sample should be <= 2.
- The %RSD of standard answer is less than 1 which is within limits, ergo approach is precise.

Specificity

Specificity is the capacity to measure the analyte clearly in the presence of components that may be expected to be present in the sample, e.g. contaminants, degradants and matrix components, according to the ICH criteria. The method was validated for specificity for accurate quantification of Pemetrexed and Cisplatin in drug products.

Assay (Standard):

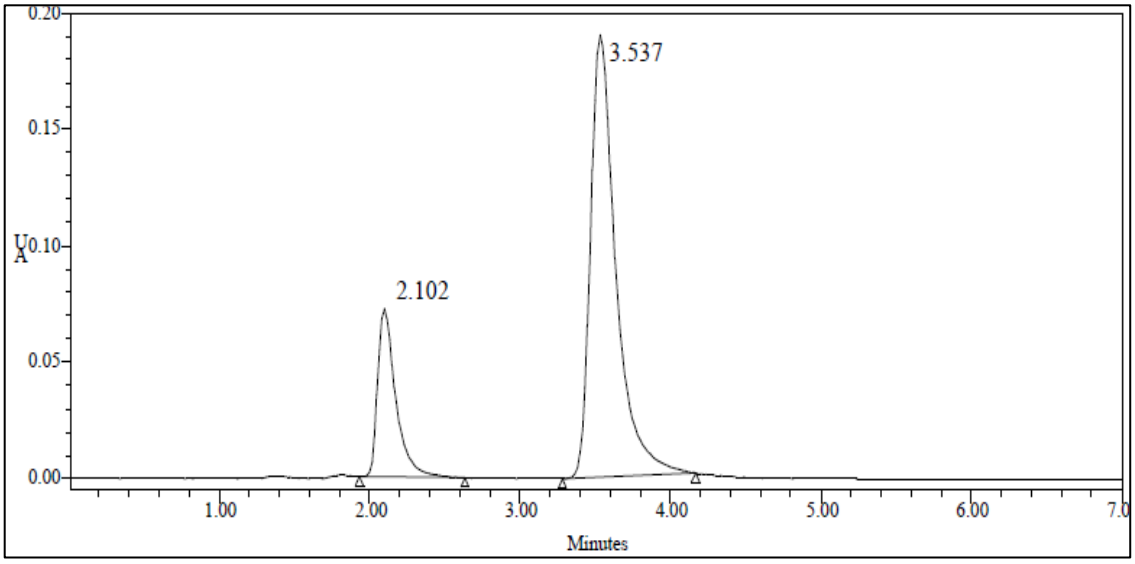


Fig 8.9-: Chromatogram showing assay of standard injection -1

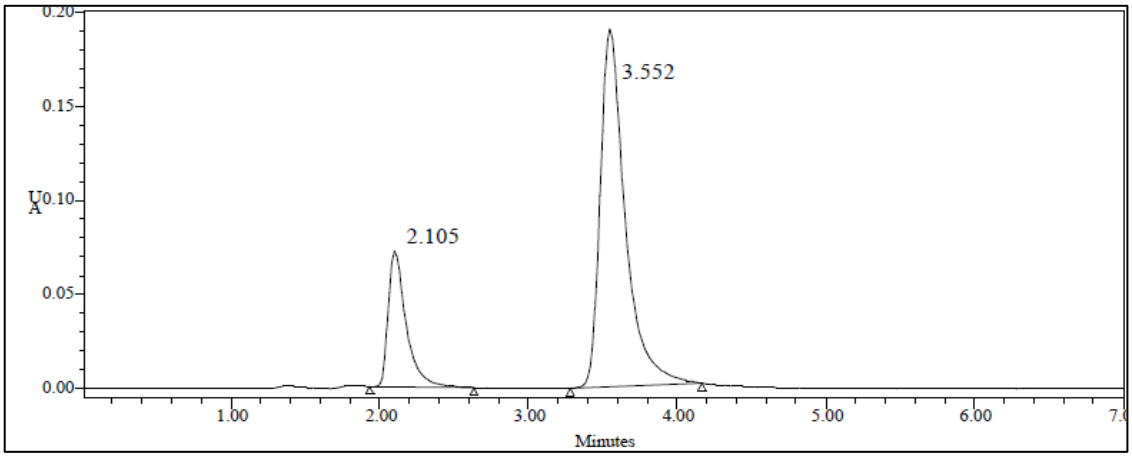


Fig8.10-: Chromatogram showing assay of standard injection -2

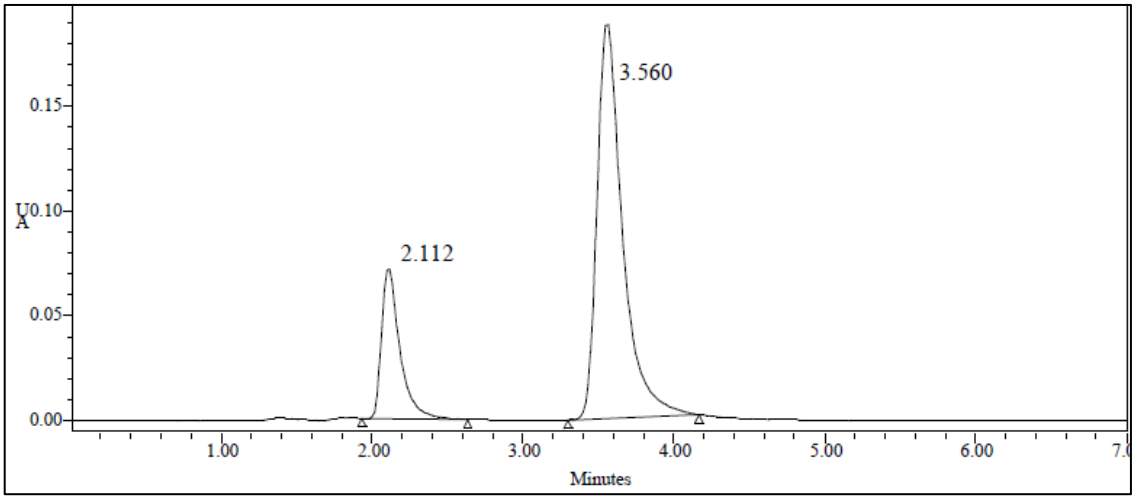


Fig 8.11-: Chromatogram showing assay of standard injection -3

Table-8.5: Peak results for assay standard

S no	Name	Rt	Area	Height	USP Tailing	USP plate count
1	Cisplatin	2.102	759868	71255	1.7	5689
2	Pemetrexed	3.537	2458754	215654	1.6	5362
3	Cisplatin	2.105	759458	72541	1.7	5748
4	Pemetrexed	3.552	2465885	226565	1.6	5452
5	Cisplatin	2.112	759245	72584	1.7	5584
6	Pemetrexed	3.560	2489578	221542	1.6	5456

Assay (Sample):

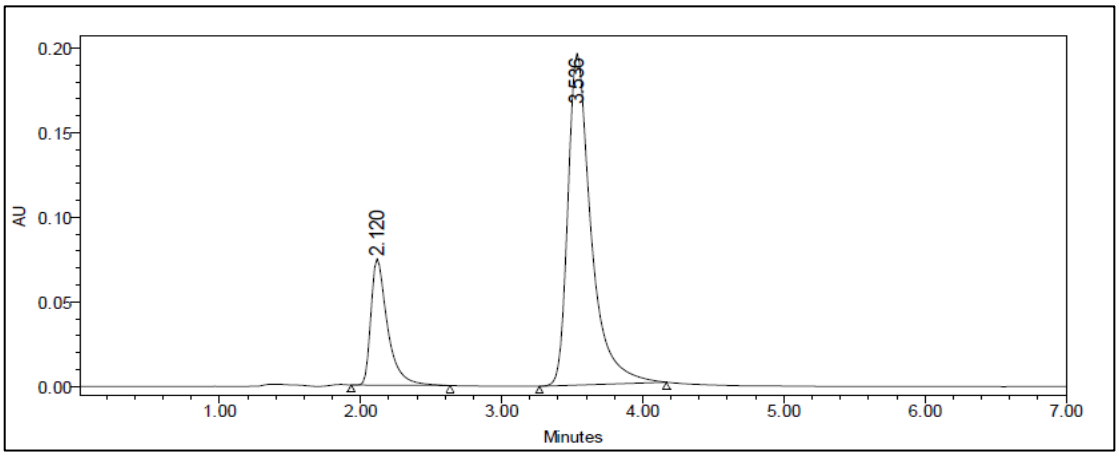


Fig-8.12: Chromatogram showing assay of sample injection-1

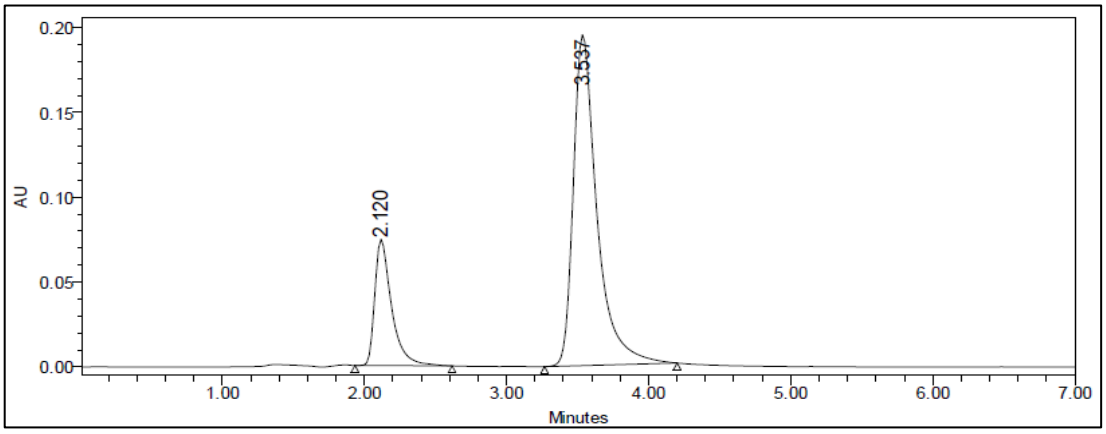


Fig-: 8.13 Chromatogram showing assay of sample injection-2

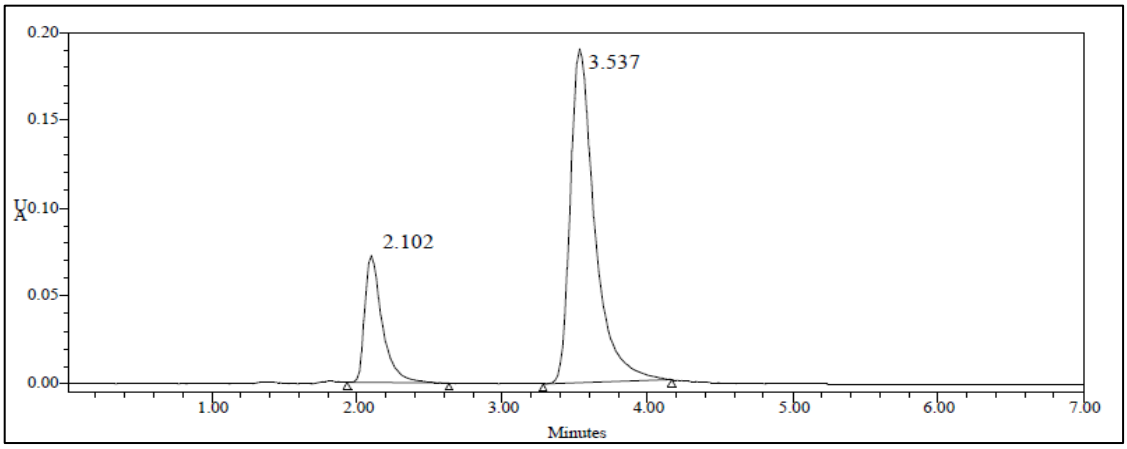


Fig 8.14: Chromatogram showing assay of sample injection-3

Table 8.6: Peak results for Assay sample

S no	Name	R_t	Area	Height	USP Tailing	USP plate count
1	Cisplatin	2.120	756985	68958	0.98	7253
2	Pemetrexed	3.536	2569856	198564	1.23	8836
3	Cisplatin	2.120	758745	69857	1.05	6530
4	Pemetrexed	3.537	2598654	195682	0.99	7270
5	Cisplatin	2.102	756848	69588	1.7	7586
6	Pemetrexed	3.537	2587454	192541	1.6	8371

%ASSAY =

$$\frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

The % purity of Pemetrexed and Cisplatin in pharmaceutical dosage form was found to be 99.8 %.

Linearity

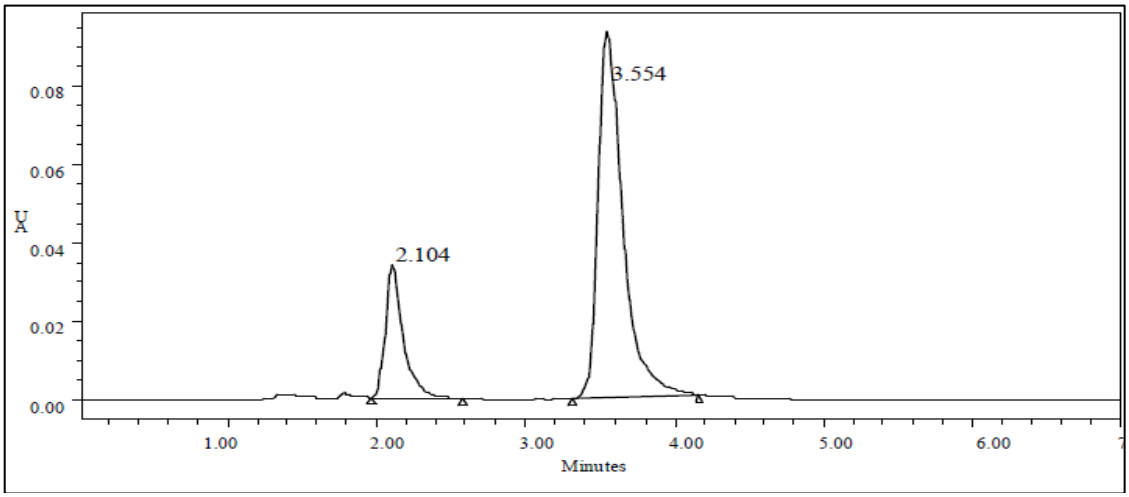


Figure 8.15-: Chromatogram for linearity concentration-6µg/ml of Cisplatin& 18 µg/ml of Pemetrexed

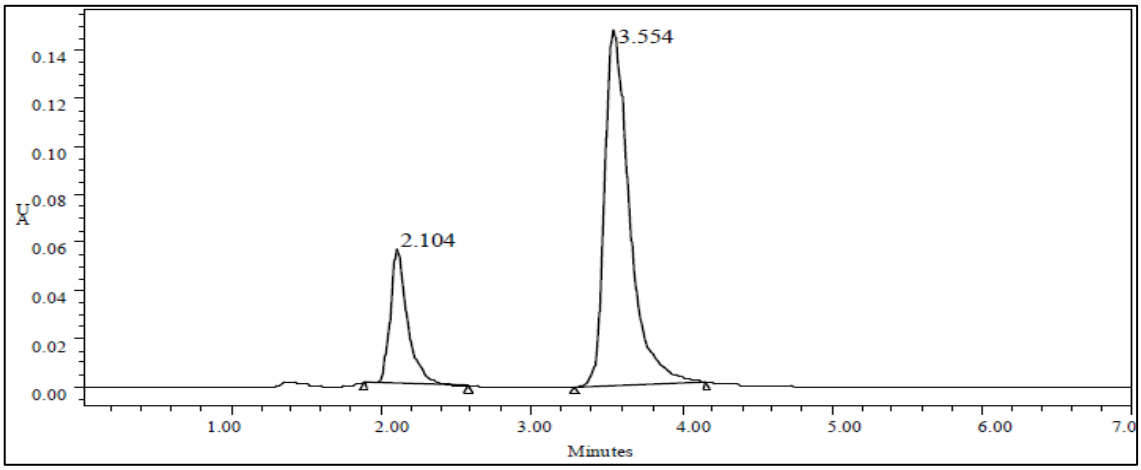


Figure 8.16-: Chromatogram for linearity concentration-8 µg/ml of Cisplatin& 24µg/ml of Pemetrexed

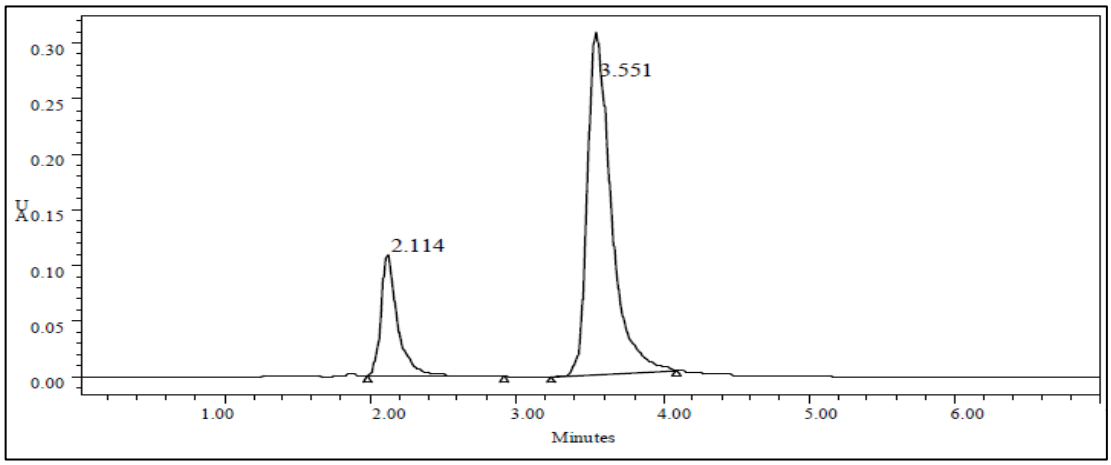


Figure 8.17-: chromatogram for linearity concentration-10 µg/ml of Cisplatin& 30µg/ml of Pemetrexed

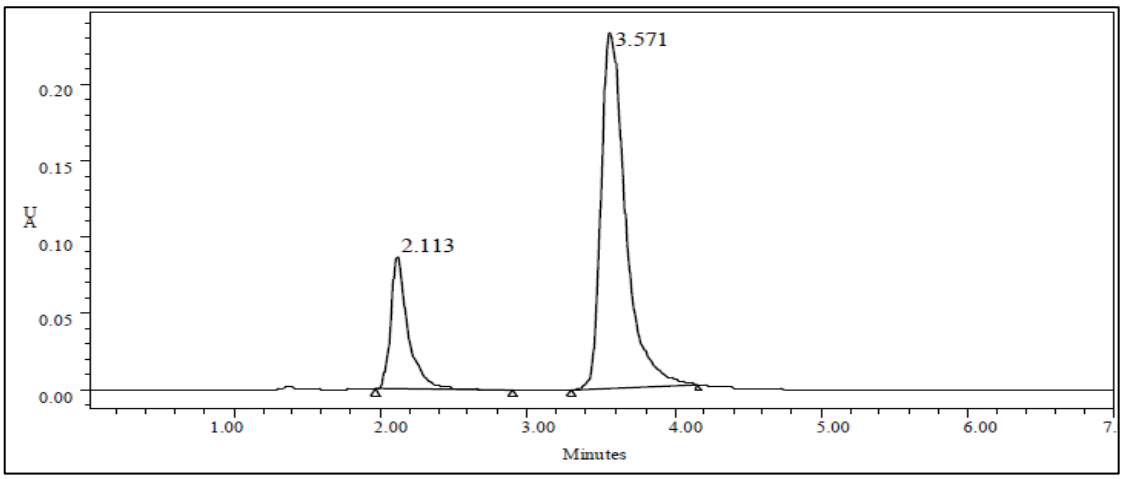


Figure 8.18-: chromatogram for linearity concentration-12 µg/ml of Cisplatin& 36 µg/ml of Pemetrexed

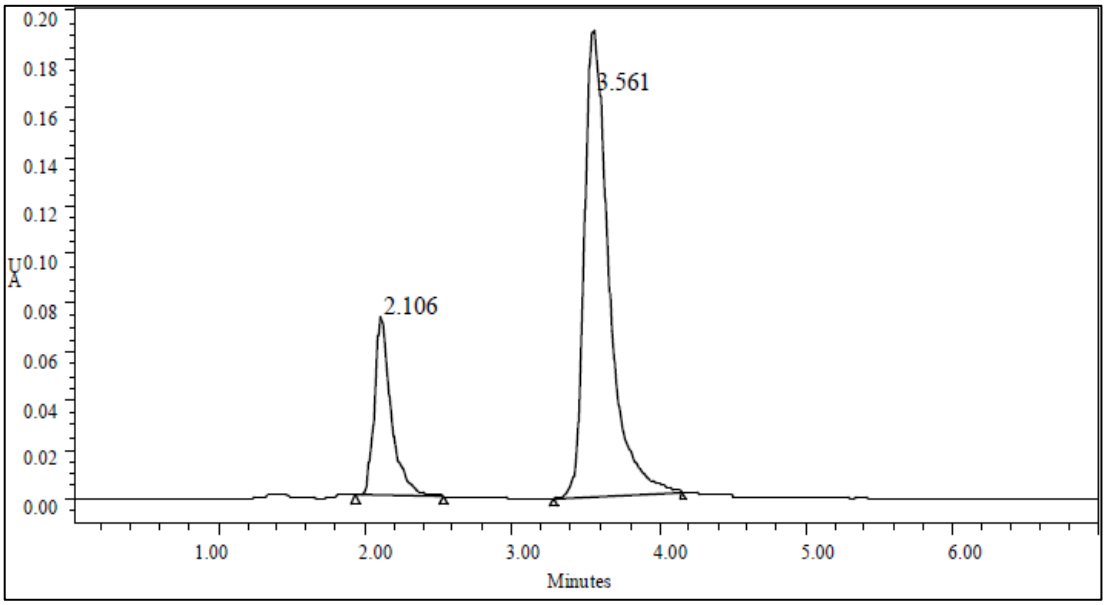


Figure 8.19-: Chromatogram for linearity concentration-14 µg/ml of Cisplatin& 42 µg/ml of Pemetrexed

Linearity Data:

Pemetrexed:

Concentration (µg/ml)	Average Peak Area
10	349877
20	688574
30	999895
40	1326522
50	1673877

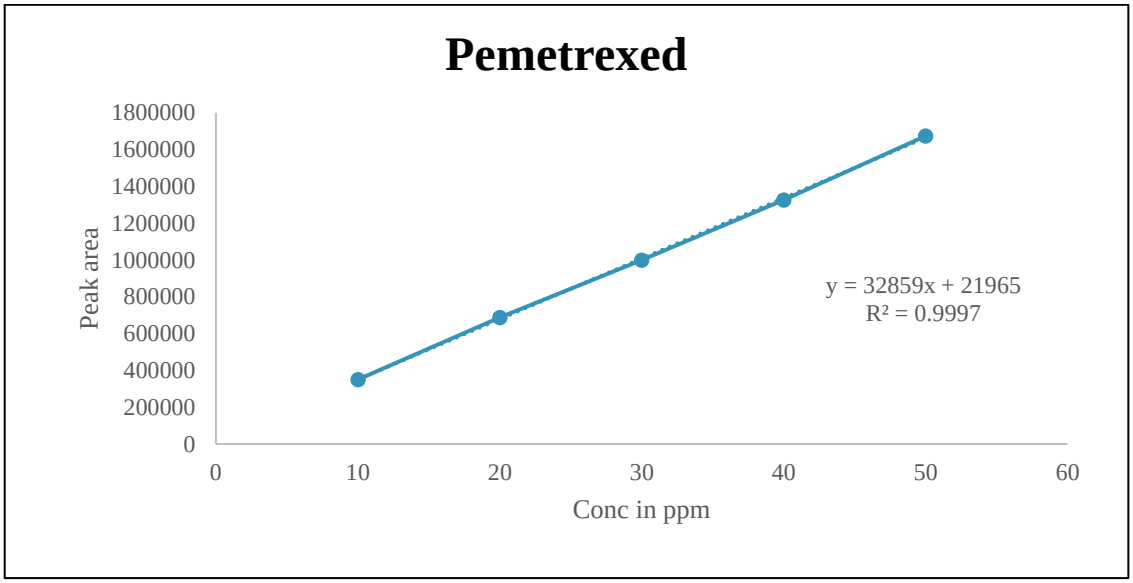


Figure 8.20: Calibration Graph for Pemetrexed

Plot of Concentration (x) vs Average Peak Area (y) of Pemetrexed is straight line.

$$Y = mx + c$$

Slope (m) = 33173

Intercept (c) = 10459

Correlation Coefficient (r) = 0.999

Validation Criteria: The response linearity is confirmed if the Correlation Coefficient is greater than or equal to 0.99. The correlation coefficient (r) is 0.99 with intercept of 10459. These values satisfy the validation requirements.

Cisplatin:

Concentration (µg/ml)	Average Peak Area
10	1896545
20	3685798
30	5389557
40	7096443
50	8878478

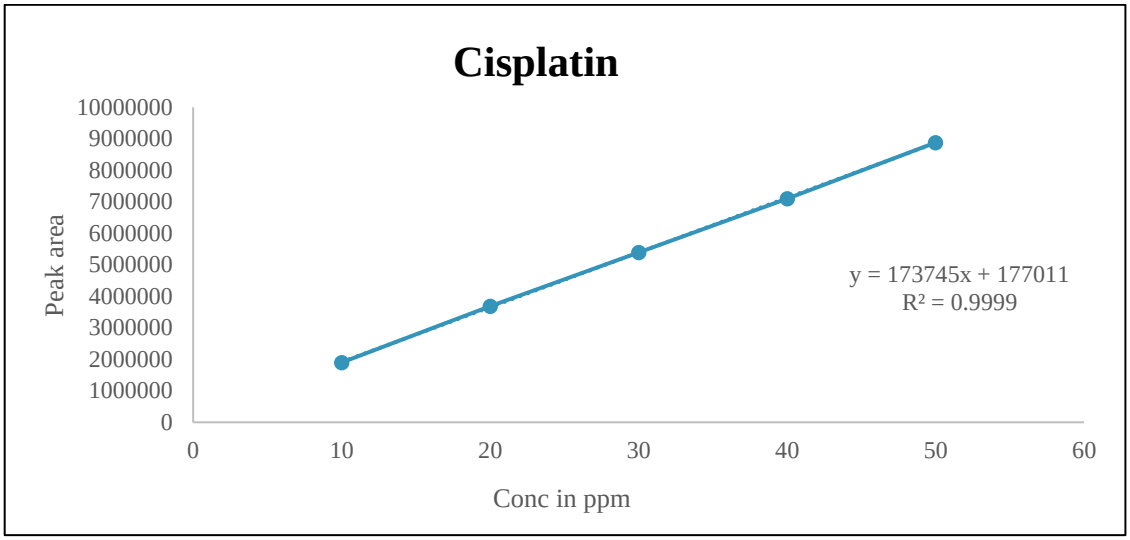


Figure 8.21: Calibration Graph for Cisplatin

The plot of Average Peak Area (y) vs Concentration (x) data of Cisplatin is a straight line.

$$Y = mx + c$$

Slope (m) =176274

Intercept (c) = 84291

Correlation Coefficient (r) = 0.999

Validation Criteria: If the Correlation Coefficient is 0.99 and above, the answer is proven to be linear. Correlation Coefficient (r) = 0.99 Intercept = 84291 These values satisfy the validation criteria.

Precision:

Precision: The closeness of agreement (degree of scatter) between a series of measurements obtained from replicate sampling of the same homogenous sample under prescribed conditions.

Repeatability

Five (5) replicates of 100% accuracy solution obtained according to experimental circumstances. Peak regions were recorded and % RSD was determined.

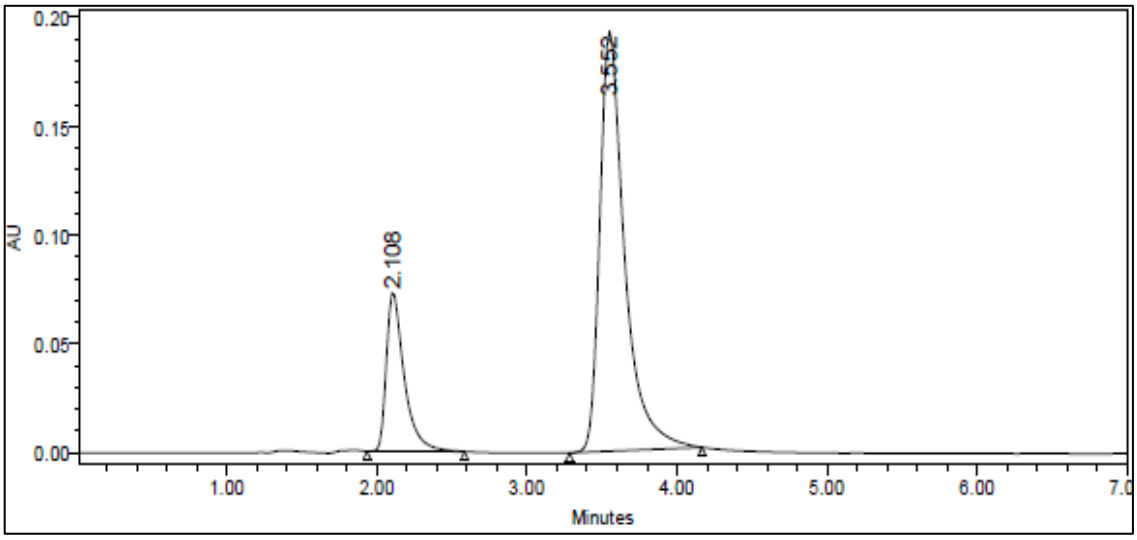


Fig 8.22-: Chromatogram showing precision injection -1

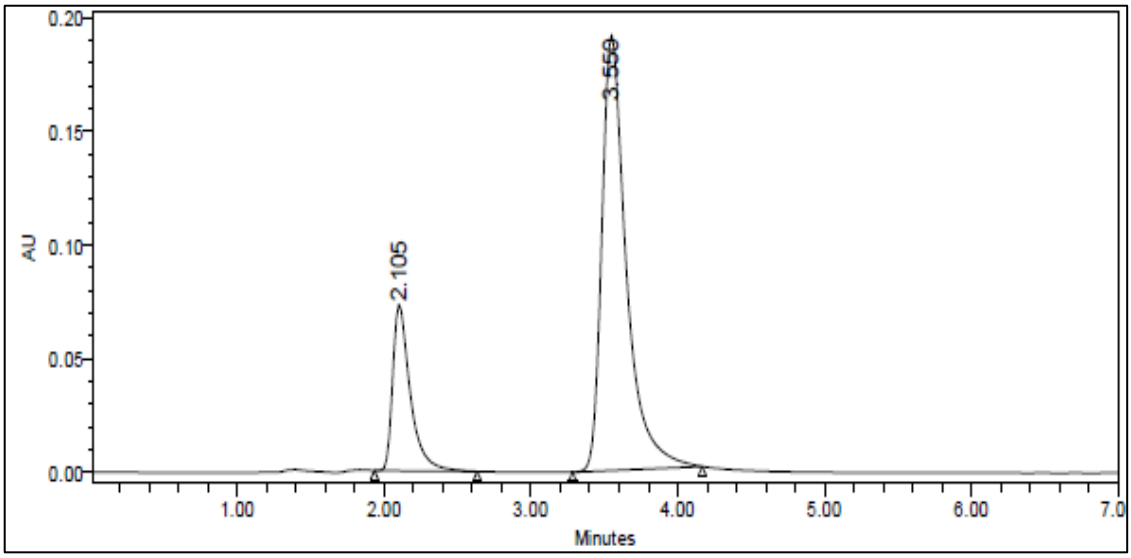


Fig 8.23-: Chromatogram showing precision injection -2

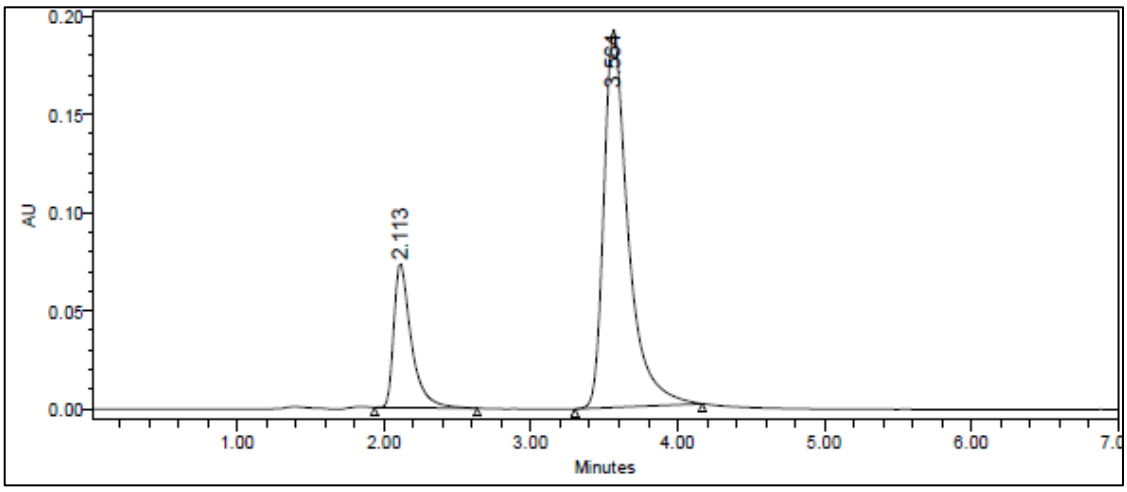


Fig 8.24-: Chromatogram showing precision injection -3

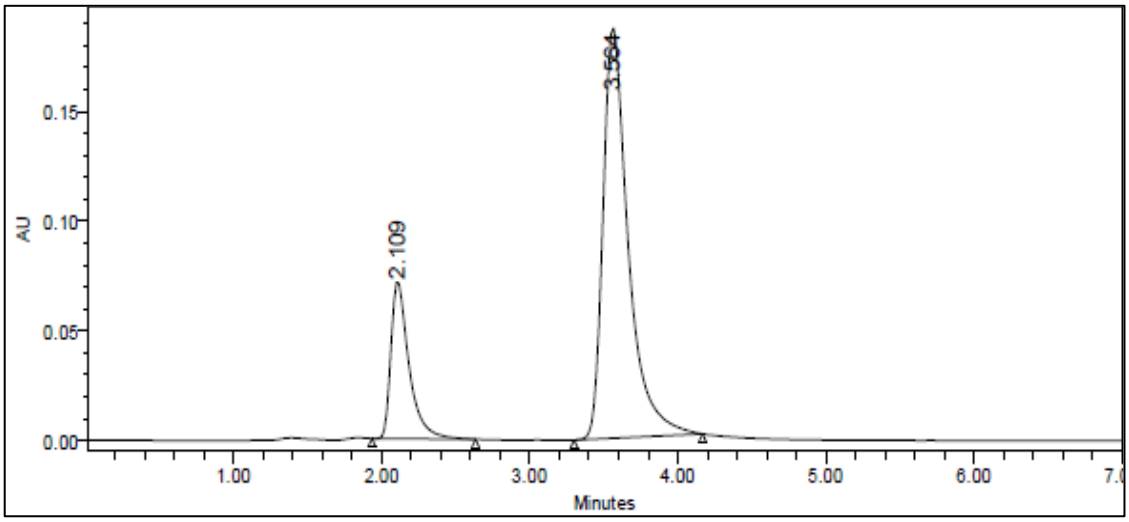


Fig8.25-: Chromatogram showing precision injection -4

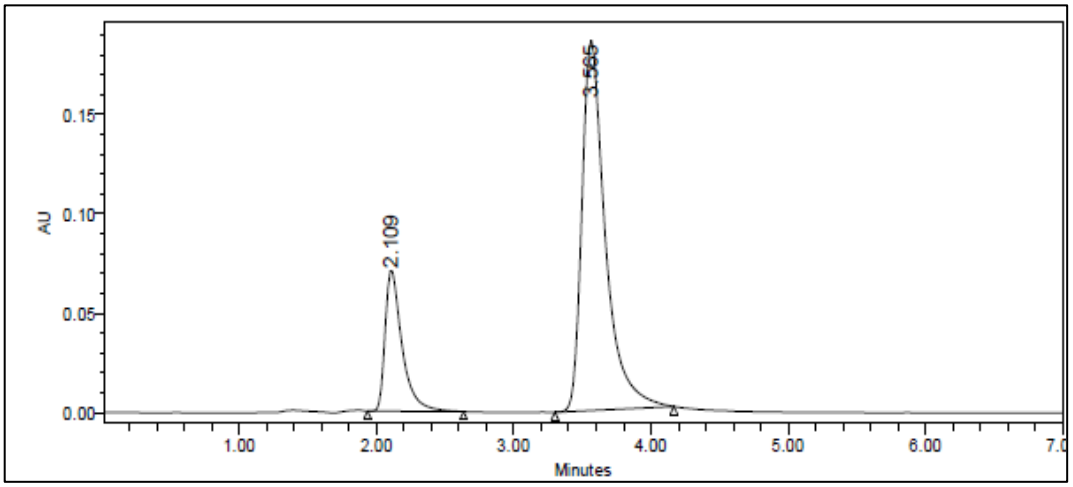


Fig 8.26-: Chromatogram showing precision injection -5

Table 8.7: Results of Repeatability for Cisplatin:

Sno	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Cisplatin	2.108	766854	702564	5685	1.6
2	Cisplatin	2.105	765884	698789	5584	1.4
3	Cisplatin	2.113	765842	701235	5521	1.6
4	Cisplatin	2.109	768985	700124	5525	1.9
5	Cisplatin	2.109	765845	698986	5578	1.7
Mean			766682			
Std. Dev			1357.973			
% RSD			0.177123			

Acceptance criteria:

- NMT %RSD for sample 2
- RSD for standard solution is less than 1 which is in limits consequently approach is precise.

Table 8.8-: Results of method precision for Pemetrexed:

Sno	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Pemetrexed	3.552	2569865	2231111	5365	1.6
2	Pemetrexed	3.550	2578474	2674210	5425	1.6
3	Pemetrexed	3.564	2568985	2231261	5368	1.5
4	Pemetrexed	3.564	2586845	2421301	5359	1.5
5	Pemetrexed	3.565	2545898	2324710	5498	1.6
Mean			2570013			
Std. Dev			15309.45			
% RSD			0.595695			

Acceptance criteria:

- NMT %RSD for sample 2
- RSD for standard solution is less than 1 which is in limits consequently approach is precise.

Intermediate precision:

Day 1:

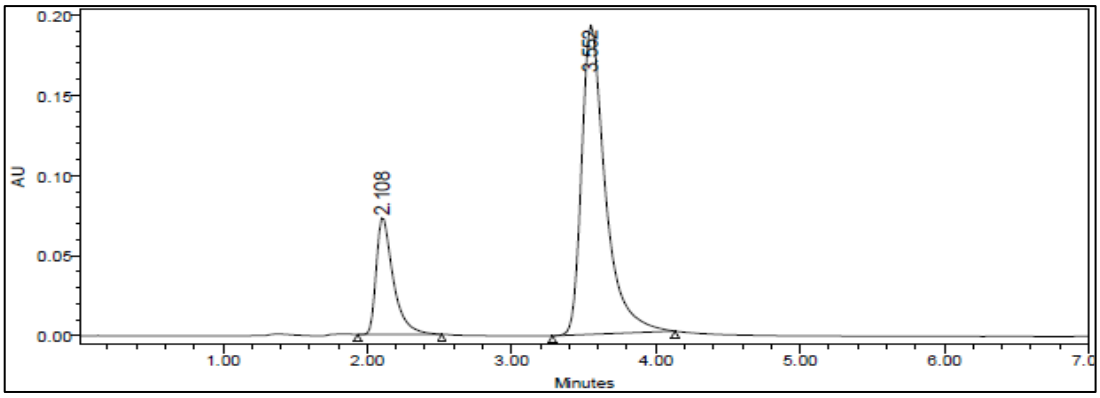


Fig 8.27: Chromatogram showing Day1 injection -1

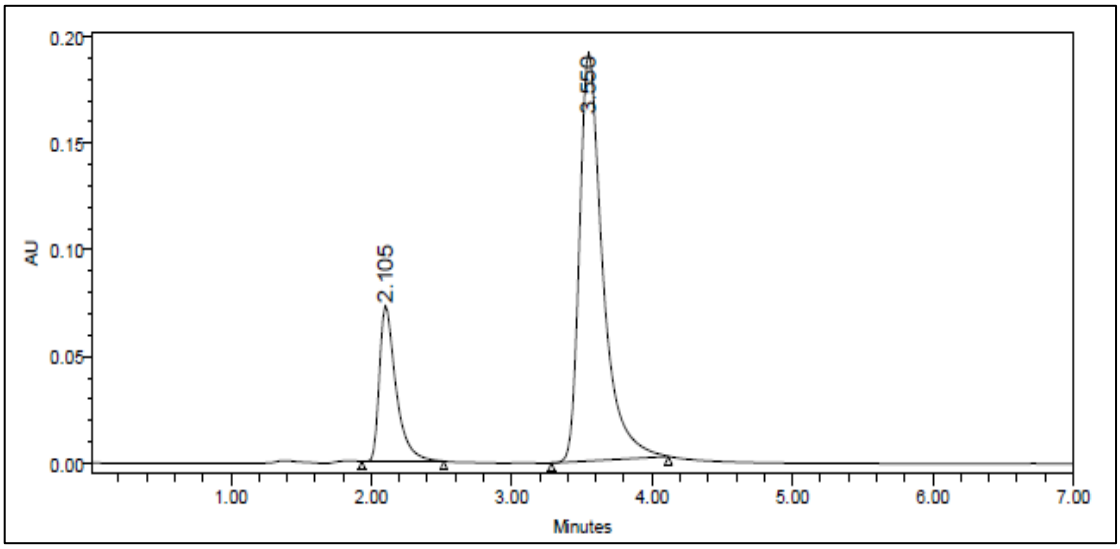


Fig8.28 -: Chromatogram showing Day1 injection -2

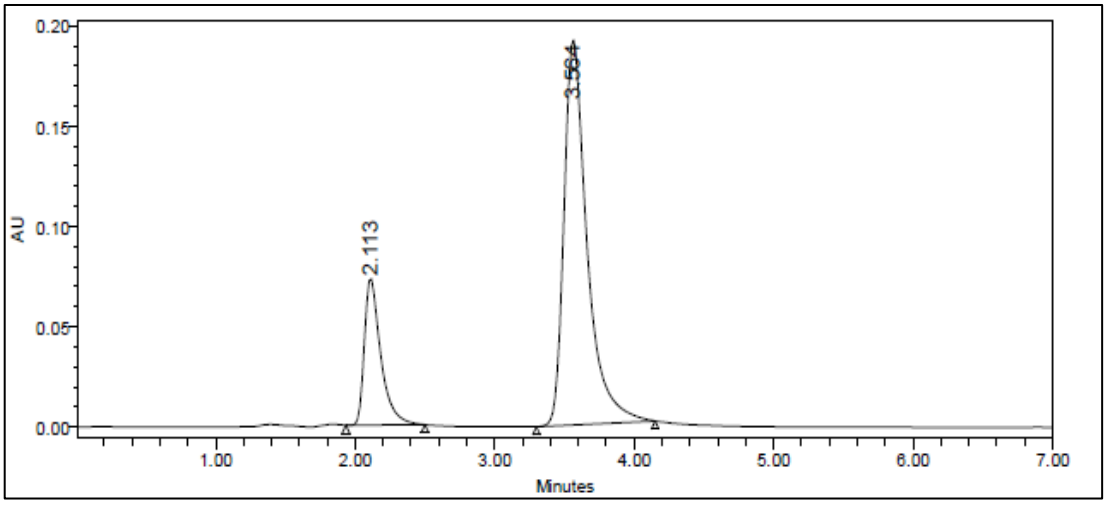


Fig 8.29-: Chromatogram showing Day1 injection -3

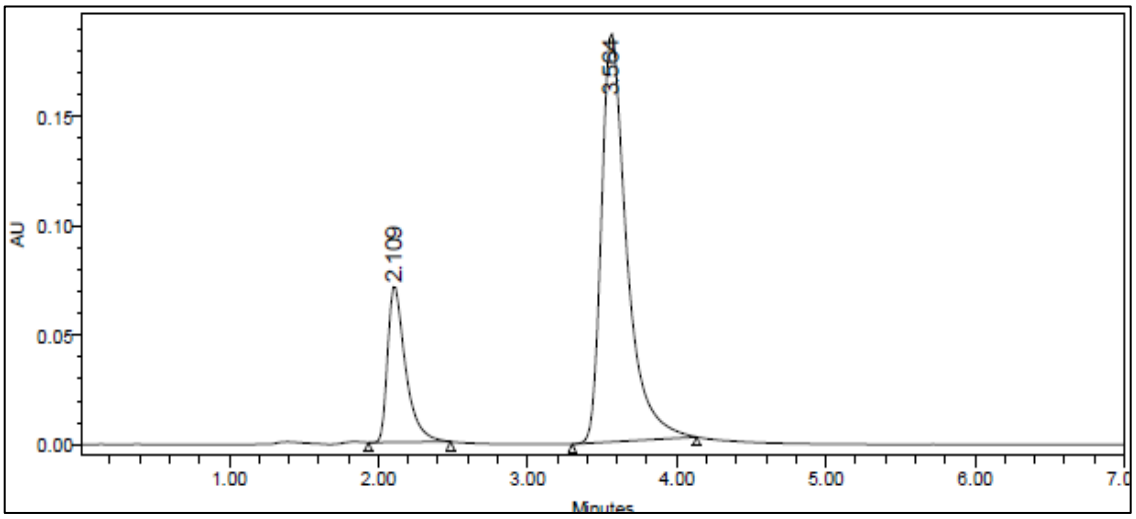


Fig8.30: Chromatogram showing Day1 injection -4

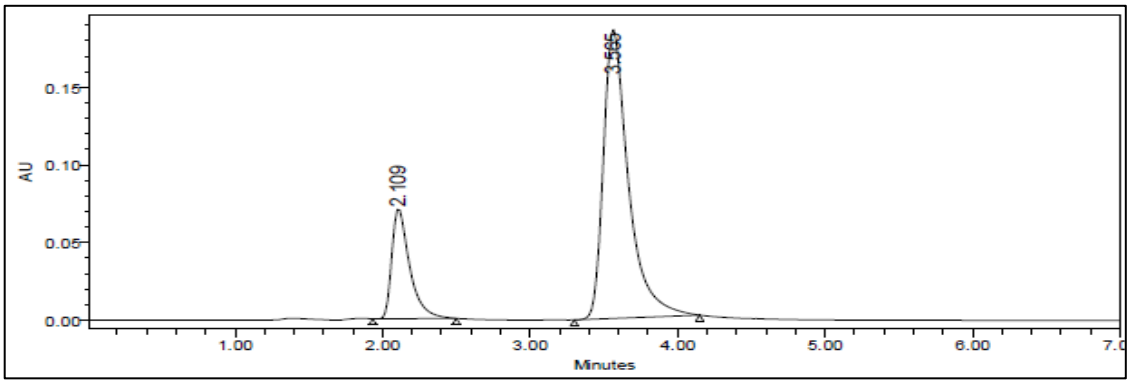


Fig 8.31-: Chromatogram showing Day1 injection -5

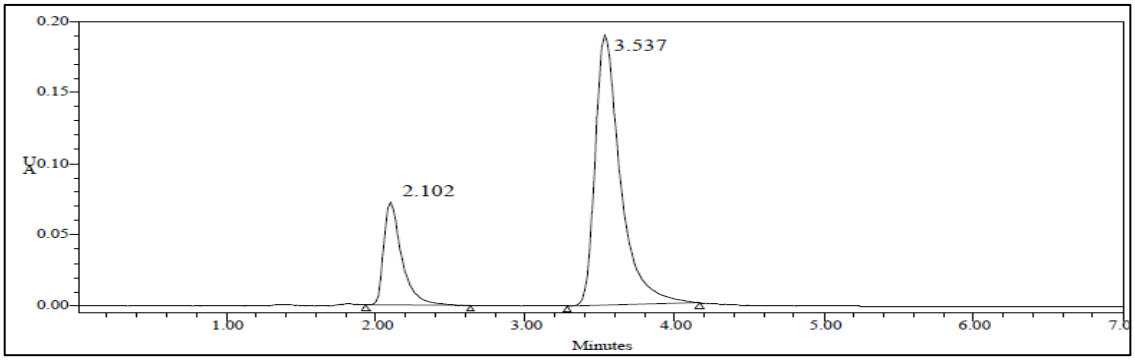


Fig 8.32-: Chromatogram showing Day1 injection -6

Table 8.9-: Results of Intermediate precision for Cisplatin

Sno	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Cisplatin	2.108	758955	68986	5785	1.6
2	Cisplatin	2.105	759869	68957	5698	1.4
3	Cisplatin	2.113	758985	68545	5689	1.6
4	Cisplatin	2.109	756894	68952	5781	1.9
5	Cisplatin	2.109	759854	68595	5785	1.7
6	Cisplatin	2.102	756985	68952	5693	1.6
Mean			758590.3			
Std. Dev			1339.793			
% RSD			0.176616			

Acceptance criteria:

- %RSD of Six different sample solutions should not more than 2.

Table 8.10-: Results of Intermediate precision for Pemetrexed

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Pemetrexed	3.552	2659852	190025	5485	1.5	2.04
2	Pemetrexed	3.550	2648574	190048	5421	1.6	2.03
3	Pemetrexed	3.564	2659865	190054	5468	1.6	2.01
4	Pemetrexed	3.564	2658547	190078	5487	1.6	2.05
5	Pemetrexed	3.565	2648981	190016	5492	1.6	2.02
6	Pemetrexed	3.537	2654652	190057	5463	1.6	2.03
Mean			2655079				
Std. Dev			5242.086				
% RSD			0.197436				

Acceptance criteria:

- %RSD of Six different sample solutions should not be greater than 2.
- %RSD obtained is within the limit hence the procedure is tough.

Day 2:

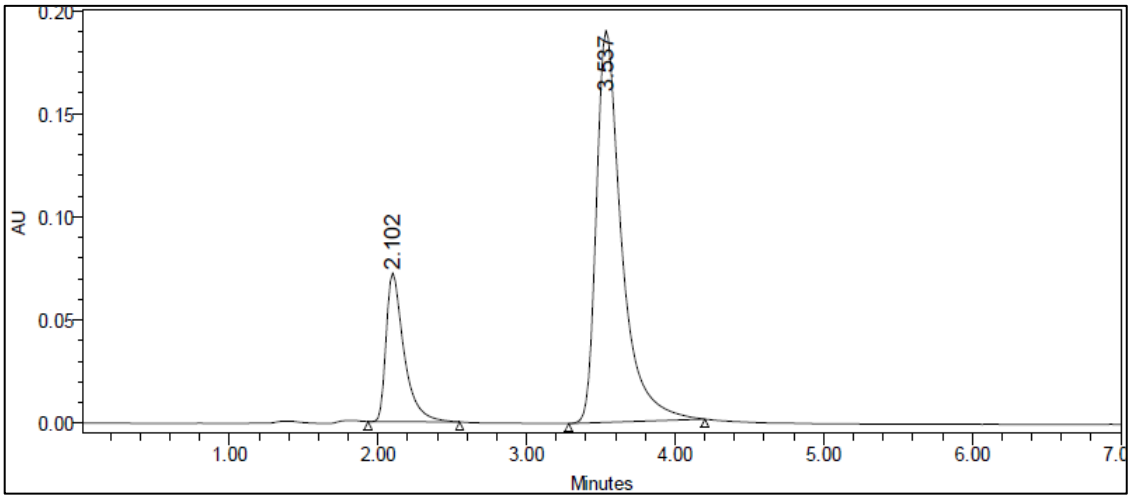


Fig 8.33-: Chromatogram showing Day 2 injection -1

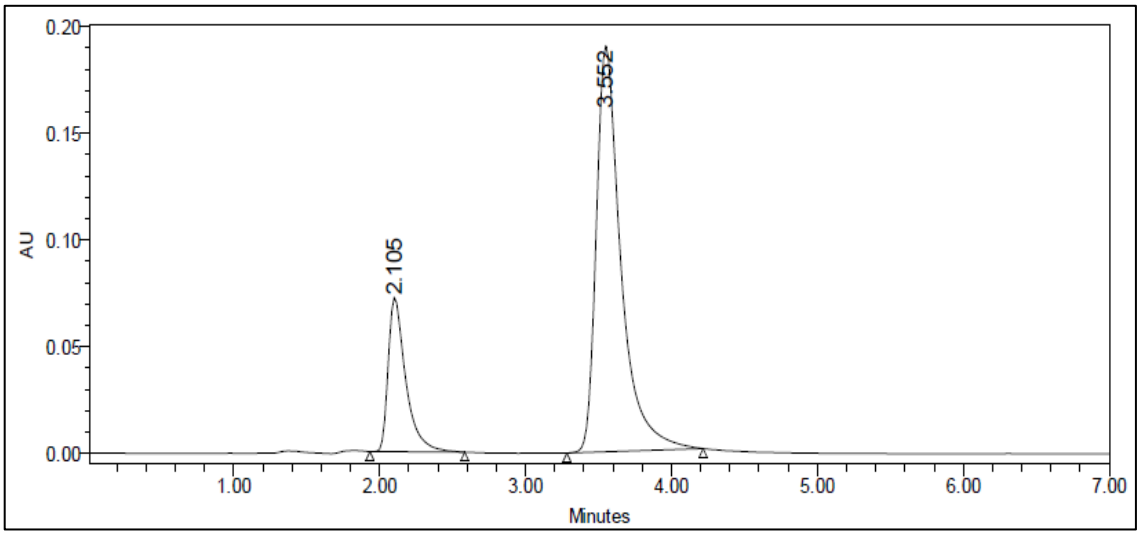


Fig 8.34-: Chromatogram showing Day 2 injection -2

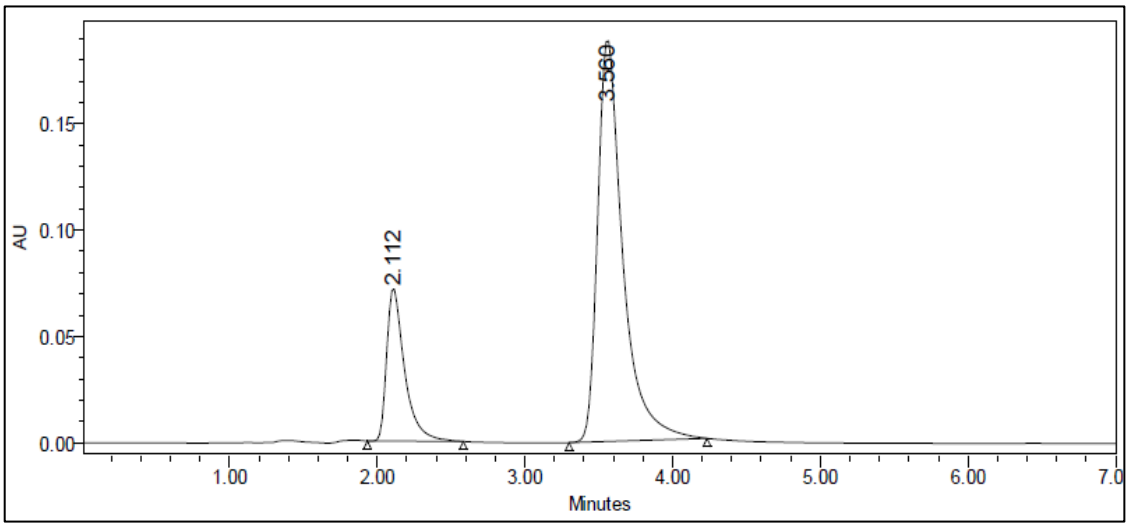


Fig-8.35: Chromatogram showing Day 2 injection -3

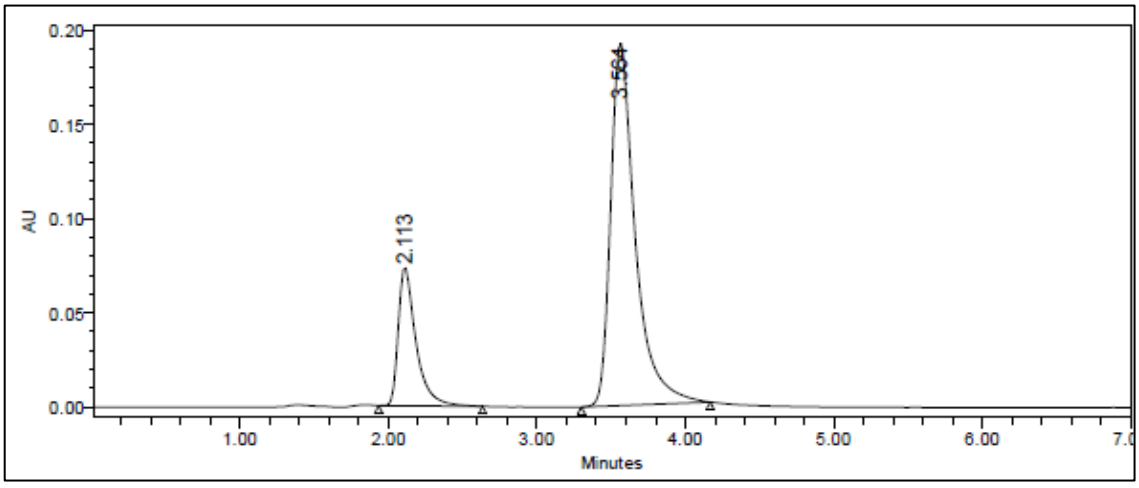


Fig 8.36-: Chromatogram showing Day 2 injection -4

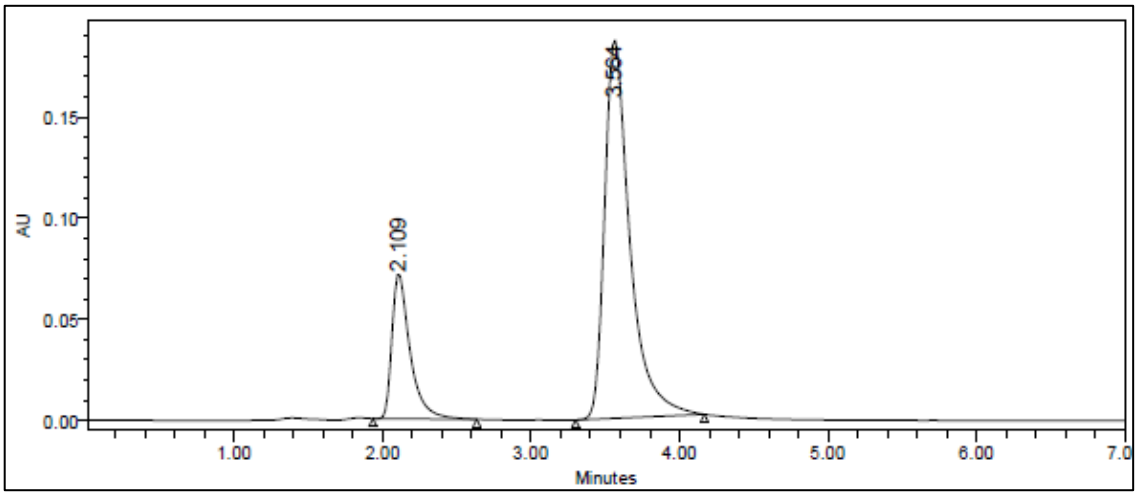


Fig 8.37-: Chromatogram showing Day 2 injection -5

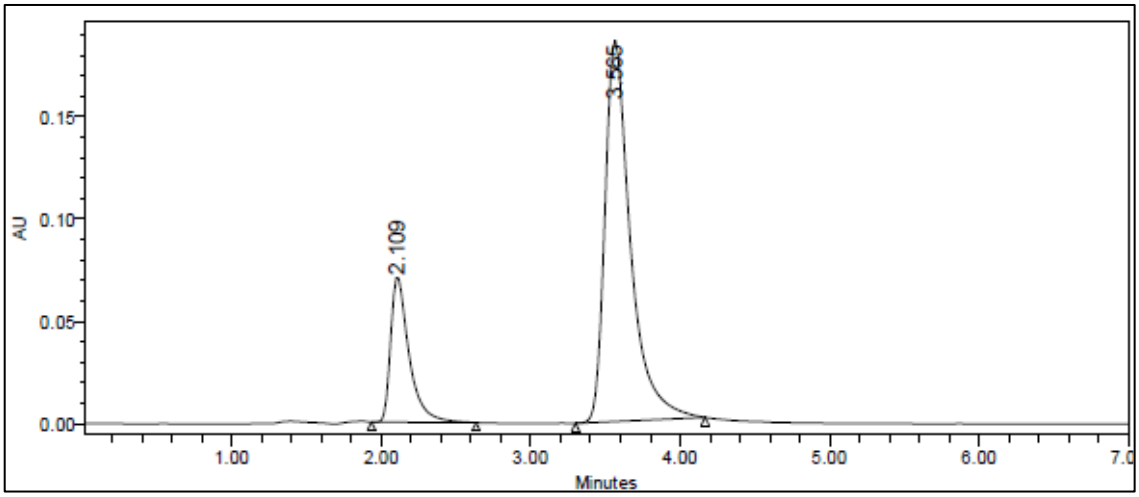


Fig 8.38: Chromatogram showing Day 2 injection -6

Table 8.11: Results of Intermediate precision Day 2 for Cisplatin

Sno	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Cisplatin	2.102	766895	69858	5586	1.5
2	Cisplatin	2.105	765988	69854	5636	1.6
3	Cisplatin	2.112	766532	69824	5432	1.6
4	Cisplatin	2.113	766214	69875	5468	1.6
5	Cisplatin	2.109	765897	69854	5546	1.9
6	Cisplatin	2.109	765245	69848	5507	1.7
Mean			766128.5			
Std. Dev			567.7234			
% RSD			0.074103			

Acceptance criteria:

- %RSD of Six distinct sample solutions should be not greater than 2.

Table 8.12-: Results of Intermediate precision for Pemetrexed

Sno	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Pemetrexed	3.537	2653254	190110	5428	1.6	7.98
2	Pemetrexed	3.552	2648985	190058	5452	1.6	6.4
3	Pemetrexed	3.560	2658213	190142	5498	1.6	8.9
4	Pemetrexed	3.564	2653652	190031	5442	1.5	8.3
5	Pemetrexed	3.564	2648978	190058	5489	1.5	7.5
6	Pemetrexed	3.565	2658985	190047	5463	1.6	5.3
Mean			2653678				
Std. Dev			4313.355				
% RSD			0.162543				

Acceptance criteria:

- %RSD of Six distinct sample solutions should not greater than 2%
- The %RSD obtained is within limit and hence the procedure is rugged.

Accuracy:

Accuracy at different concentrations (50%, 100% and 150%) was prepared and % recovery was calculated.

Accuracy50%:

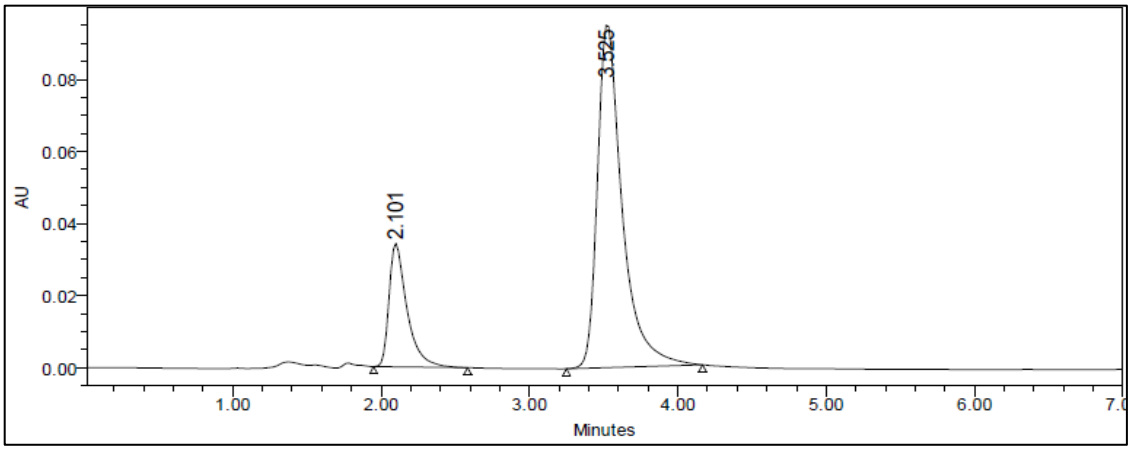


Fig 8.39-: Chromatogram showing accuracy-50% injection-1

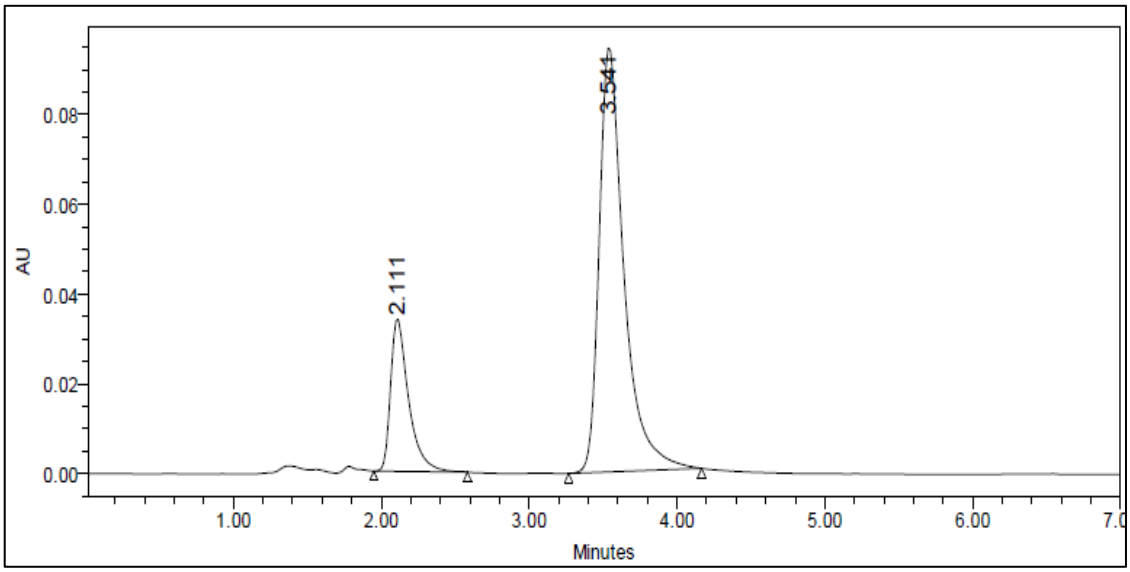


Fig 8.40-: Chromatogram showing accuracy-50% injection-2

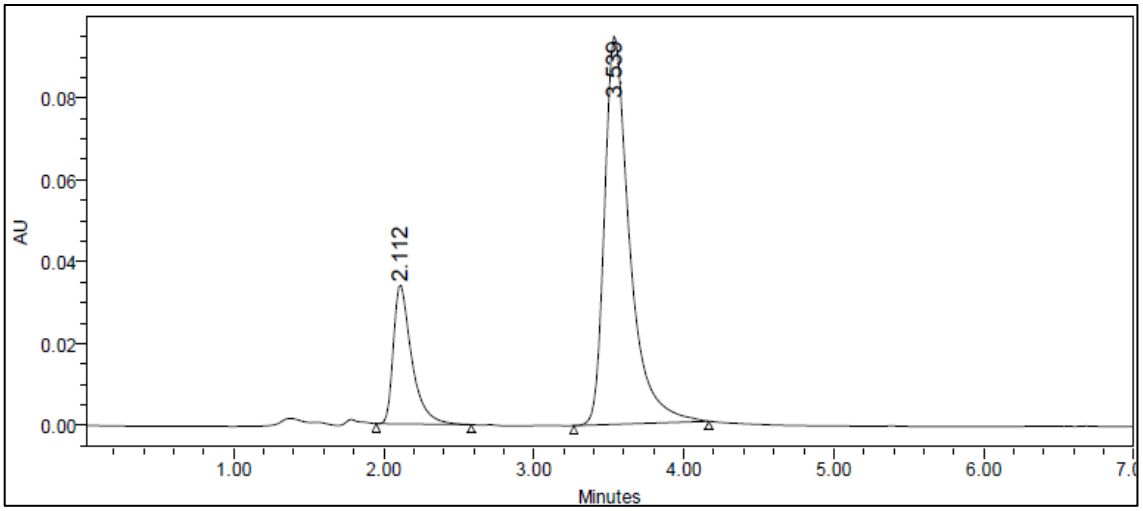


Fig 8.41 -: Chromatogram showing accuracy-50% injection-3

Table 8.13-: Results of Accuracy for concentration-50%

Sr. no.	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Cisplatin	2.101	392985	48525		1.6	4658	1
2	Pemetrexed	3.525	205245	168547	6.00	1.6	4256	1
3	Cisplatin	2.111	393104	47981		1.5	4652	2
4	Pemetrexed	3.541	204652	167898	5.04	1.5	4281	2
5	Cisplatin	2.112	392586	48954		1.6	4668	3
6	Pemetrexed	3.539	204989	168747	8.02	1.6	4265	3

Accuracy100%:

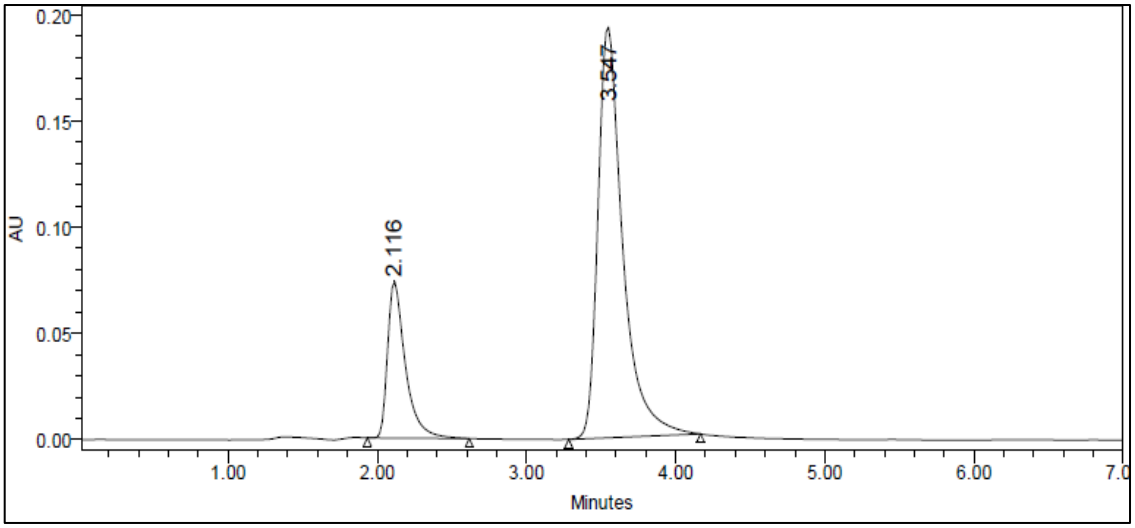


Fig 8.42-: Chromatogram showing accuracy-100% injection-1

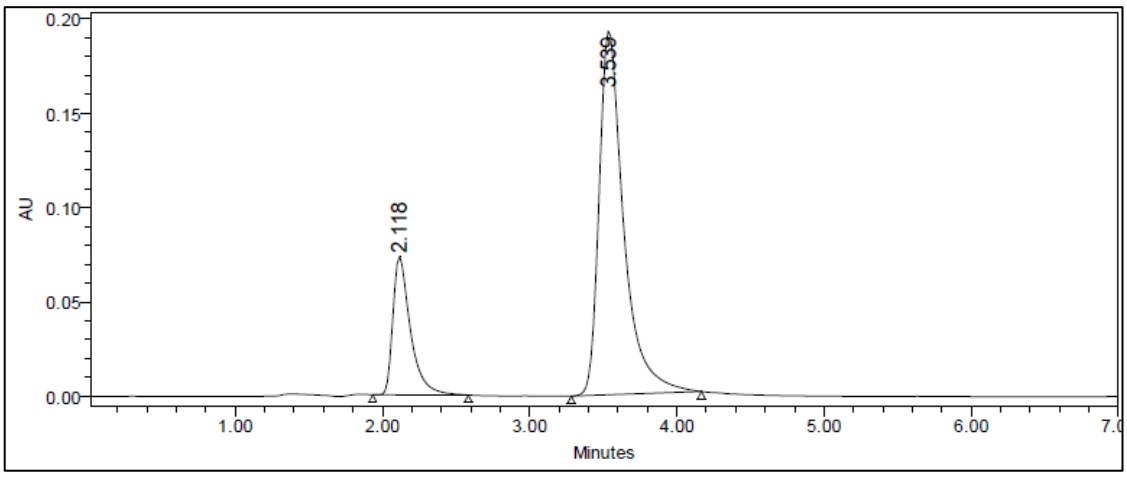


Fig 8.43: Chromatogram showing accuracy-100% injection-2

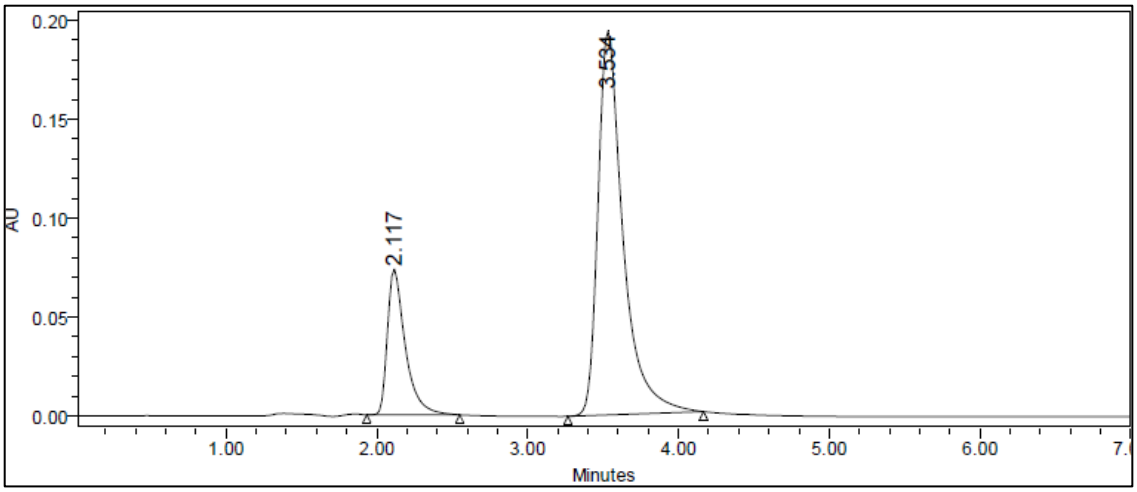


Fig8.44-: Chromatogram showing accuracy-100% injection-3

Table 8.14-: Results of Accuracy for concentration-100%

Sno	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Cisplatin	2.116	781898	69854		1.6	5685	1
2	Pemetrexed	3.547	365254	190014	7.5	1.6	5428	1
3	Cisplatin	2.118	782101	69898		1.5	5624	2
4	Pemetrexed	3.539	364587	190087	6.04	1.5	5426	2
5	Cisplatin	2.117	781989	69985		1.6	5698	3
6	Pemetrexed	3.534	365213	190068	9.03	1.6	5463	3

Accuracy150%:

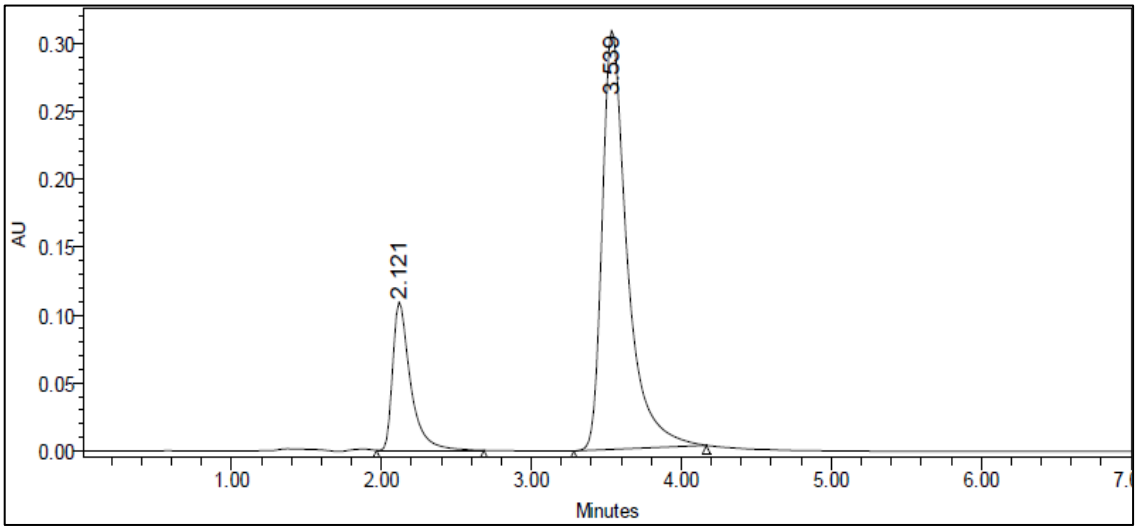


Fig 8.45-: Chromatogram showing accuracy-150% injection-1

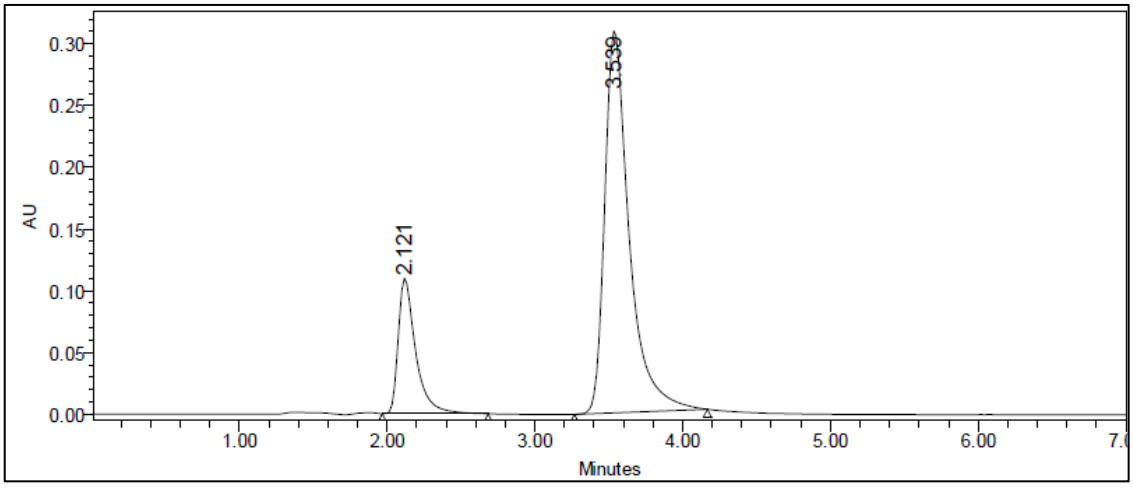


Fig-8.46: Chromatogram showing accuracy-150% injection-2

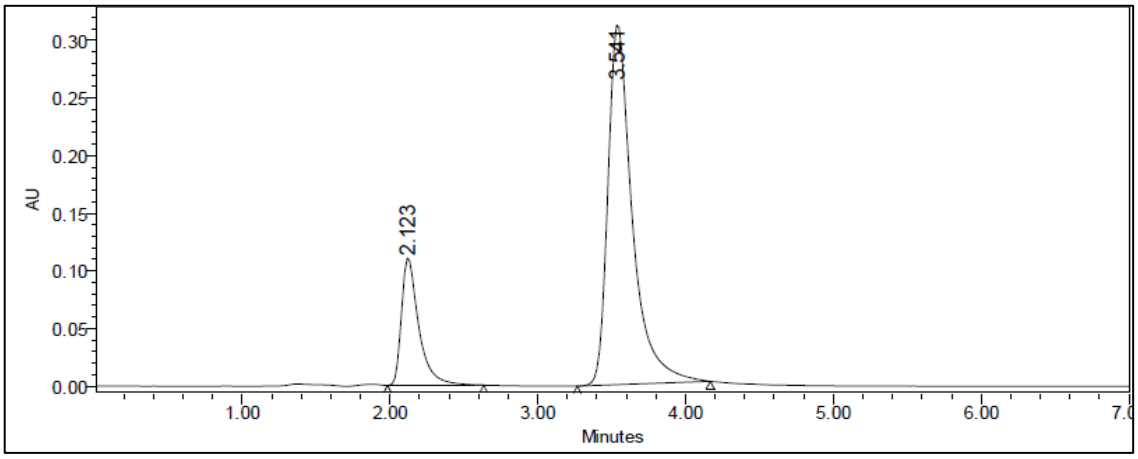


Fig8.47-: Chromatogram showing accuracy-150% injection-3

Table 8.15 -: Results of Accuracy for concentration-150%

Sr. no.	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Cisplatin	2.121	1165248	73625		1.6	5895	1
2	Pemetrexed	3.539	521582	236524	6.98	1.6	5723	1
3	Cisplatin	2.121	1178582	73745		1.5	5874	2
4	Pemetrexed	3.539	520368	236458	5.00	1.5	5798	2
5	Cisplatin	2.123	1172134	74585		1.6	5898	3
6	Pemetrexed	3.541	521243	234589	9.03	1.6	5784	3

Table 8.16 -: The accuracy results for Cisplatin

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	392891.7	5	5.027	100.540%	100.351%
100%	781996	10	10.026	100.260%	
150%	1171988	15	15.038	100.253%	

Table-8.17: The accuracy results for Pemetrexed

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	*204962*	*15*	*15.156*	*101.040%*	100.93%
100%	*365018*	*30*	*30.378*	*101.260%*	
150%	*521064.3*	*45*	*45.218*	*100.484%*	

Acceptance Criteria:

- The recovery percentage was found to be within limit (98-102%).

The given results for recovery in 50%, 100% and 150% are within limitations. The process is hence accurate.

Detection Limit

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

$$\mathbf{LOD = 3.3 \times \sigma / s}$$

Were

σ = Standard deviation of the response

S = Slope of the calibration curve

Result:

Pemetrexed:

0.6µg/ml

Cisplatin:

0.8µg/ml

Limit of Quantitation

The limit of quantitation of a single analytical method is the lowest amount of analyte in a sample that can be quantitatively measured.

$$\mathbf{LOQ=10\times\sigma/S}$$

Were

σ = Standard deviation of the response

S = Slope of the calibration curve

Result:

Pemetrexed:

1.8µg/ml

Cisplatin:

2.4µg/ml

Robustness

Robustness was checked for flow rate variations from 0.9 ml/min to 1.1ml/min and mobile phase ratio variations from more organic phase to less organic phase ratio for Pemetrexed and Cisplatin. The approach is rugged under the decreased flow condition and the procedure is robust even with Mobile phase change of ± 5 %. Standard and samples of Pemetrexed and Cisplatin were injected and the chromatography settings were altered. Parameters such as resolution, tailing factor, asymmetry factor and plate count did not change significantly.

Variation in flow

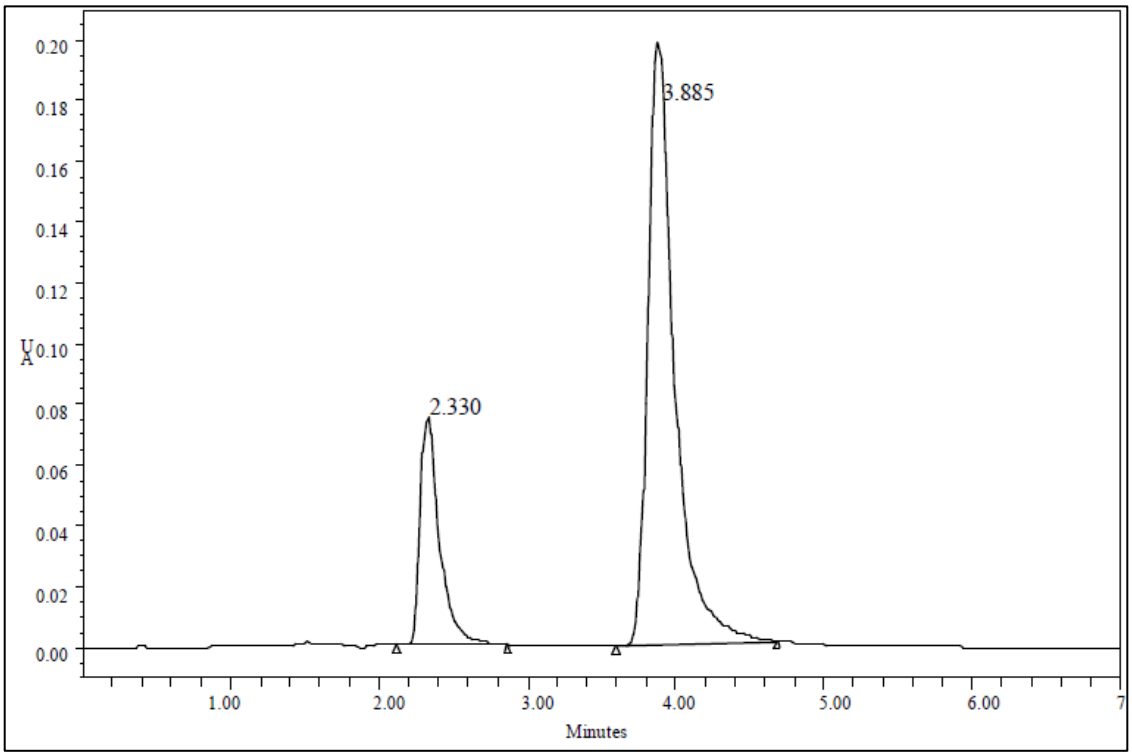


Figure-8.48: chromatogram showing less flow of 0.9ml/min

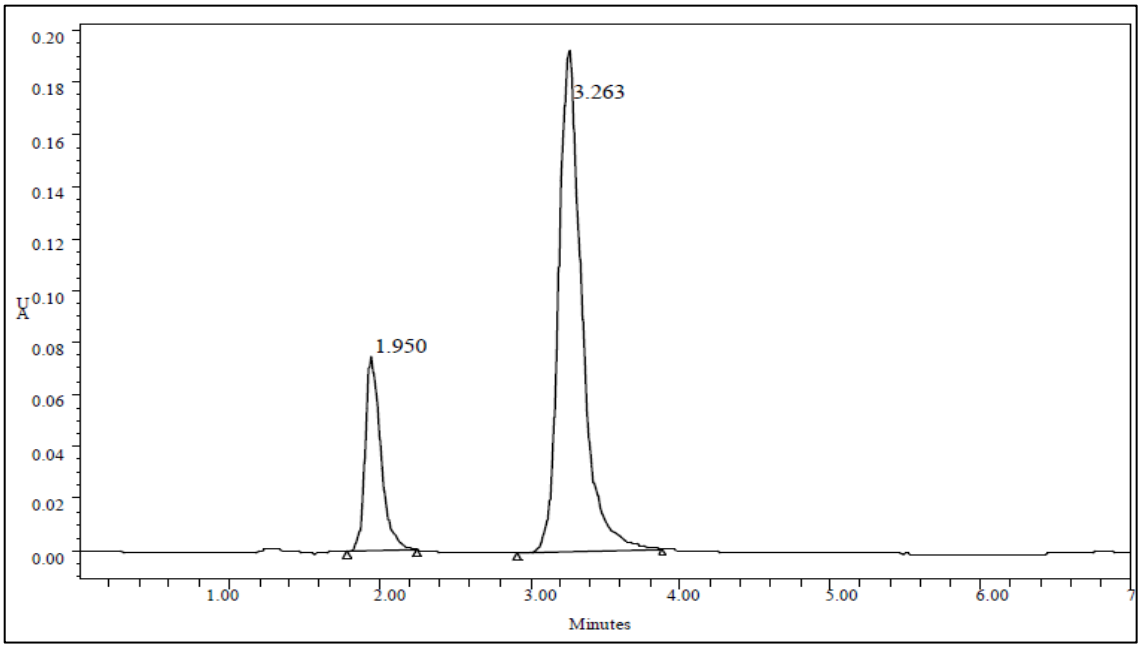


Fig: 8.49 Chromatogram showing more flow of 1.1 ml/min

Variation of mobile phase organic composition

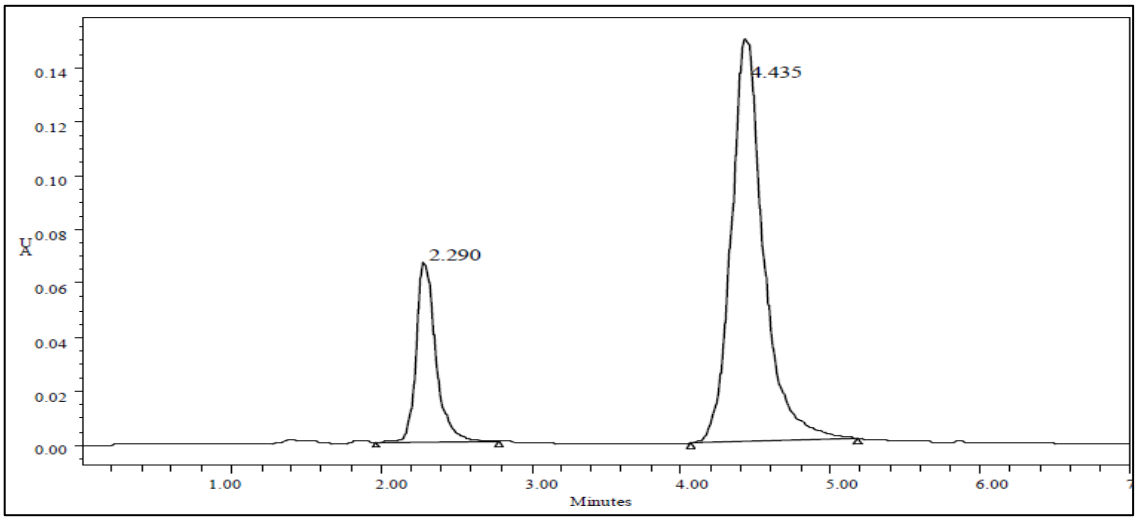


Fig 8.50: Chromatogram showing less organic composition

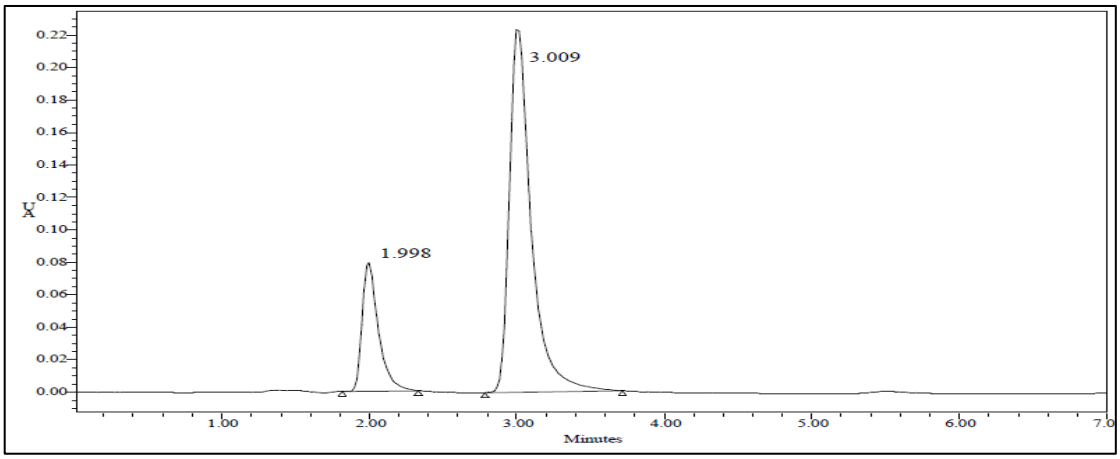


Figure 8.51: Chromatogram showing more organic composition

Table 8.18-: Results for Robustness

Pemetrexed:

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	765789	2.102	5587	1.7
Less Flow rate of 0.9 mL/min	758698	2.330	5458	1.7
More Flow rate of 1.1 mL/min	7689584	1.950	5696	1.7
Less organic phase	758412	2.290	5586	1.4
More organic phase	769852	1.998	5355	1.5

Acceptance criteria:

The tailing factor should be < 2.0 and the number of theoretical plates (N) should be > 2000.

Cisplatin:

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	2532158	3.537	5398	1.6
Less Flow rate of 0.9 mL/min	2458692	3.885	5329	1.7
More Flow rate of 1.1	2658642	3.263	5256	1.7
Less organic phase	2452148	4.435	5214	1.2
More organic phase	2653894	3.009	5524	1.0

Acceptance criteria:

The tailing factor should be < 2.0 and the number of theoretical plates (N) should be > 2000.

8.8. Summary and conclusion

8.8.1 Summary

A Reverse Phase High-Performance Liquid Chromatographic (RP-HPLC) method was developed and validated for the simultaneous quantification of Pemetrexed and Cisplatin in bulk and pharmaceutical formulations. The method used was C18 column with customized composition of mobile phase for the clean separation of both the medications. The procedure settings were carefully optimized to get crisp peaks, good resolution and consistency. The method was validated according to the guidelines of ICH Q2 (R1) for parameters including linearity, accuracy, precision, specificity, limit of detection (LOD) and limit of quantitation (LOQ). The results showed that the method is accurate, precise and resilient and acceptable for routine analysis of these drugs in combined dose forms.

8.8.2 Conclusion

The devised RP-HPLC method is simple, quick, reliable and cost-effective analytical procedure for simultaneous measurement of Pemetrexed and Cisplatin. It demonstrates good sensitivity and specificity, as well as acceptable linearity, precision and accuracy. This technology can be successfully utilized for the quality control and routine analysis of pharmaceutical formulations including these medications to ensure consistent therapeutic efficacy and safety.

Chapter - 9

SUMMARY

The number of pharmaceutical products entering the global market has increased at an unprecedented rate over the past few decades. Many of these drugs are either entirely new chemical entities or structural modifications of existing molecules and are often listed in only a limited number of pharmacopoeias or not included at all. As a consequence, officially recognized standards and validated analytical procedures for such drugs are frequently unavailable, inaccessible, or inadequate. This situation necessitates the development of new, reliable, and validated analytical methods to ensure the quality, safety, and efficacy of pharmaceutical substances and dosage forms. In this context, pharmaceutical analysis plays a crucial role in quality assurance and quality control of both bulk drug substances and finished pharmaceutical products. Analytical techniques are essential for confirming identity, purity, potency, stability, and performance characteristics of drugs throughout their lifecycle, from development to post-marketing surveillance. The rapid evolution of drug design, formulation strategies, and regulatory expectations further emphasizes the need for robust analytical methodologies that are simple, cost-effective, sensitive, selective, and compliant with regulatory guidelines.

Modern pharmacists and pharmaceutical analysts are therefore required to possess strong expertise in analytical method development and validation. The ability to design, optimize, and validate analytical procedures enables pharmaceutical industries, research laboratories, and regulatory bodies to maintain high standards of drug quality and to respond efficiently to the challenges posed by novel drug molecules. In addition, validated bioanalytical methods are indispensable for pharmacokinetic, bioavailability, and bioequivalence studies, which form the backbone of clinical drug development and regulatory approval. With this background, the present thesis is aimed at the development and validation of new analytical and bioanalytical methods using UV spectrophotometry, high-performance liquid chromatography (HPLC), liquid chromatography–mass spectrometry (LC-MS/MS) and RP-HPLC method for selected pharmaceutical drugs. The methods developed were systematically optimized and rigorously validated by evaluating relevant validation

parameters in accordance with established regulatory guidelines, particularly those of the United States Food and Drug Administration (USFDA) and the International Council for Harmonisation (ICH).

Six drugs of therapeutic importance—Lamivudine (LAM), Sitagliptin (SITA), Naproxen (NAP), Duloxetine (DUL) ,Pemetrexed And Cisplatin—were selected for this investigation. These drugs represent different pharmacological classes and dosage requirements, thereby providing a comprehensive framework to demonstrate the applicability of different analytical techniques. Lamivudine, an antiviral agent, was selected for UV spectrophotometric method development due to its suitability for simple and economic analysis. Sitagliptin, an antidiabetic drug, was chosen for chromatographic method development using HPLC, reflecting the need for higher specificity and precision in formulation analysis. Naproxen, a non-steroidal anti-inflammatory drug, and Duloxetine, a centrally acting antidepressant, were selected for bioanalytical method development using LC-MS/MS owing to the requirement for highly sensitive and selective quantification in biological matrices. Pemetrexed and Cisplatin, are antineoplastic drug, were chosen for chromatographic method development using RP-HPLC.

The thesis is organized into multiple chapters that systematically present the theoretical background, experimental work, results, discussion, and conclusions. The introductory chapter provides a comprehensive overview of analytical chemistry principles with emphasis on UV spectrophotometry, HPLC, and bioanalytical method development. Fundamental concepts such as Beer–Lambert's law, electronic transitions, solvent effects, and factors influencing absorption spectra are discussed to establish the theoretical basis for spectroscopic analysis. The chapter also highlights the principles of chromatographic separation, method optimization strategies, and the importance of method validation. Method development is presented as a critical process driven by the absence of official analytical procedures, interference from formulation excipients, lack of bio-analytical methods, and the need for economical and reproducible techniques. The selection of analytical methods is guided by criteria such as simplicity, specificity, accuracy, precision, robustness, and suitability for routine application. The validation requirements outlined by ICH, including specificity, linearity, accuracy, precision, limits of detection and quantitation, robustness, and

reproducibility, are discussed in detail. In addition, bioanalytical method development principles are elaborated with emphasis on pharmacokinetics, bioavailability, bioequivalence, sample preparation techniques, and mass spectrometric detection.

The importance of bioanalytical method validation in drug development is emphasized, particularly in relation to regulatory expectations for investigational and marketed products. Sample preparation techniques such as protein precipitation, liquid–liquid extraction, and solid-phase extraction are discussed, with liquid–liquid extraction highlighted for its simplicity, cost-effectiveness, and suitability for high-throughput analysis. The principles of mass spectrometry, ionization techniques, multiple reaction monitoring, and validation parameters specific to bioanalytical studies—including matrix effect, recovery, stability, dilution integrity, and ruggedness—are also addressed. The experimental chapters describe the development and validation of analytical methods for each selected drug. For Lamivudine, a UV spectrophotometric method was developed based on its oxidation reaction and absorption characteristics. The method demonstrated good linearity within the studied concentration range and complied with Beer–Lambert's law. The developed method was found to be simple, accurate, precise, and economical, with no interference from formulation excipients, making it suitable for routine analysis of bulk and tablet dosage forms.

For Sitagliptin, a robust HPLC method was developed and optimized by selecting appropriate chromatographic conditions, including mobile phase composition, pH, detection wavelength, and flow rate. The method exhibited good linearity, precision, accuracy, and robustness over a wide concentration range. The absence of interference from excipients and the simplicity of the procedure without requiring extensive sample preparation highlighted its suitability for routine quality control analysis in pharmaceutical laboratories. Bioanalytical method development for Naproxen involved the establishment of a sensitive and selective LC-MS/MS method using negative ionization mode and an appropriate internal standard. Liquid–liquid extraction was employed for sample preparation to minimize matrix effects and improve recovery. The validated method demonstrated acceptable performance in terms of selectivity, linearity, precision, accuracy, recovery, stability, and dilution integrity over a broad concentration range in human plasma. The method complied with USFDA bioanalytical validation guidelines and was suitable for pharmacokinetic and

bioequivalence studies. Similarly, a rapid and sensitive LC-MS/MS method was developed and validated for the quantification of Duloxetine in human plasma using positive ionization mode and a deuterated internal standard. The method addressed challenges related to chromatographic behavior and analyte polarity by selecting an appropriate column and mobile phase composition. Liquid–liquid extraction enabled efficient sample clean-up and high recovery. The validated method demonstrated excellent sensitivity, precision, accuracy, and stability across the required concentration range, fulfilling regulatory requirements for bioanalytical applications.

All the developed methods were validated as per USFDA and ICH guidelines and demonstrated consistency, reliability, and reproducibility within predefined acceptance criteria. The results obtained confirm that the analytical and bioanalytical approaches developed in this thesis are suitable for their intended purposes and can be successfully applied in pharmaceutical quality control laboratories, research settings, and clinical studies.

An analytical method using Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) was developed and validated for the simultaneous estimation of Pemetrexed and Cisplatin in bulk and pharmaceutical formulations. The method utilized a C18 column with an optimized mobile phase composition to achieve clear separation of both drugs. The method parameters were systematically optimized to ensure sharp peaks, good resolution, and reproducibility. Validation was carried out according to ICH Q2(R1) guidelines, evaluating parameters such as linearity, accuracy, precision, specificity, limit of detection (LOD), and limit of quantitation (LOQ). The results demonstrated that the method is accurate, precise, and robust, suitable for routine analysis of these drugs in combined dosage forms.

Chapter - 10

CONCLUSION AND FUTURE SCOPE

10.1 Conclusion

The results show that the new UV Spectrophotometric approach is easy to use, cost-effective, and provides accurate estimations of Lamivudine in both bulk and tablet dose forms. For the identification of organic molecules, cerium IV ammonium sulphates are widely used since they are strong oxidizing agents. Very low absorbance within the lamivudine detection ranges All concentrations between 2 and 18 µg ml-1 adhere to Beer's law. Lamivudine on oxidation with (IV) in acidic medium had shown a maximum absorption at 280 nm. The λmax lamivudine was found to be 280 nm. Hence it can be easily estimated using UV Visible Spectrophotometry and can be concluded as new accurate, precise and highly economical method and there was no interference from excipients in tablets. Also, it can be utilized for the routine analysis in massive and tablet dosage form.

The results of this investigation demonstrate the practicality of the suggested HPLC approach for SITA detection. After eight minutes of running time, this dependable HPLC method successfully determines SITA with a high degree of precision and sufficient sensitivity. The proposed method is quite simple and do not require any pre-treatment of the drug and tedious extraction procedure. The method has wider linear range with good accuracy. In addition, typical formulation additives and excipients do not interfere with the process. Laboratory generated combinations using the described approach showed good agreement in the assay findings. Since the chromatographic parameters did not undergo any significant changes, it may be concluded that the established method was resilient. In pharmaceutical quality control laboratories, the suggested method was determined to be appropriate for routine SITA measurement.

In accordance with the parameters specified in the "Guidance for Industry—Bioanalytical Method Validation (May 2001)" document by the USFDA and CDER, this report details the outcomes for "selectivity, matrix effect, sensitivity, linearity, precision and accuracy, stabilities, recovery, dilution integrity, and concurrent drug effect" that are considered acceptable for bioanalytical batches.

Within the concentration range of 500.1 ng/mL to 100028.5 ng/mL, the afore-mentioned analytical method, when used in negative ion mode, may detect "Naproxen m/z - 229.00 (parent) and 185.00 (product), in addition to the internal standard Zidovudine m/z - 267.00 (parent) and 222.90 (product)" in human plasma.

A simple, specific, sensitive, economical, and validated method is developed using LC-MS/MS with Atmospheric pressure ionization (API) in human plasma for estimation of drug (Duloxetine hydrochloride) used for major depressive disorder. Separation of analyte and internal standard (Duloxetine-D5 oxalate) was performed on ZORBAX SB-C18 2.1× 50mm,5µmcolumn with a runtime of 2.50 min. Duloxetine hydrochloride were run in positive mode using Duloxetine-D5 oxalate as IS with mobile phase composition of HPLC grade Acetonitrile: water: ammonium acetate (5 mM) in an 85:15 (v/v) ratio In order to facilitate rapid analysis, the samples were treated using liquid-liquid extraction (LLE). Over a dynamic concentration range of 0.5-200 ng/ml for Duloxetine, respectively, the approach was validated. In line with the US FDA Bio analytical technique validation criteria, the method has been validated to cover precision, accuracy, selectivity, linearity/range, recoveries, freeze-thaw cycles, bench-top, and auto-sampler stabilities. The method's precision, accuracy, and linearity were all satisfactory. There was a wide range of recoveries, from 73% to 100%.

The developed RP-HPLC method is a simple, rapid, reliable, and cost-effective analytical procedure for the simultaneous estimation of Pemetrexed and Cisplatin. It exhibits high sensitivity and specificity, with acceptable linearity, precision, and accuracy. This method can be effectively applied for quality control and routine analysis of pharmaceutical formulations containing these drugs, ensuring consistent therapeutic efficacy and safety.

10.2: Future Scope

The present thesis successfully developed and validated UV spectroscopic, HPLC, LC–MS/MS bioanalytical methods and RP-HPLC method for Lamivudine, Sitagliptin, Naproxen, Duloxetine, Pemetrexed and Cisplatin in accordance with USFDA guidelines. While these methods are suitable for routine quality control and bioanalytical applications, several avenues exist for future research to further strengthen and expand the scope of pharmaceutical analysis. Future work may focus on the development of stability-indicating methods capable of resolving drugs from their

degradation products under forced degradation conditions, in line with ICH Q1A (R2). Such studies would enhance understanding of degradation pathways and support formulation development and shelf-life determination. Additionally, extending the validated methods to simultaneous estimation of drug combinations commonly used in therapy would be valuable, particularly for fixed-dose combinations and poly-pharmacy scenarios.

Advanced green analytical chemistry approaches should be explored to minimize solvent consumption, toxicity, and environmental impact. The use of eco-friendly solvents, miniaturized techniques, and reduced analysis time can improve sustainability without compromising method performance. Further, hyphenated techniques such as UPLC–MS/MS and high-resolution mass spectrometry may be investigated to achieve higher sensitivity, selectivity, and throughput, especially for trace-level bioanalysis. In the bioanalytical domain, future studies may include pharmacokinetic, bioavailability, and bioequivalence evaluations of the selected drugs in special populations, as well as method adaptation for alternative biological matrices such as saliva, urine, or dried blood spots. The application of automation and chemometric tools for method optimization and data interpretation can also improve robustness and efficiency. Continued research in analytical method development, aligned with evolving regulatory expectations and emerging technologies, will further support quality assurance, regulatory compliance, and rational drug therapy. The present work thus provides a strong foundation for future advancements in pharmaceutical and bioanalytical research.

Chapter - 11

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ANALYTICAL METHOD DEVELOPMENT FOR SITAGLIPTIN USING REVERSE PHASE HPLC: A COMPREHENSIVE STUDY

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Abstract

The investigators in this work hope to one day be able to measure Sitagliptin in pharmaceutical formulations using a Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) method. An essential medication for managing type 2 diabetes, sitagliptin acts as a selective inhibitor of the enzyme dipeptidyl peptidase-4 (DPP-4), which controls blood glucose levels. The need of precise quantification in providing consistent therapeutic effects and safety is highlighted by the research. Hypersil ODS C18 column and methanol/triethylamine mobile phase were optimized for RP-HPLC using high sensitivity UV detection at 237 nm. The method is suitable for routine pharmaceutical analyses since it is linear, exact, accurate, and resilient. The method's suitability for establishing a consistent Sitagliptin quality control is confirmed by its low limits of detection (LOD: 0.6773 µg/mL and LOQ: 2.1881/µg/mL), indicating a high level of sensitivity.

Keywords: Sitagliptin, RP-HPLC, DPP-4 Inhibitor, Pharmaceutical Formulations, Method Validation.

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1.INTRODUCTION

Indeed, one of the most often recommended medications for type 2 diabetes is sitagliptin. An key enzyme in glucose metabolism, dipeptidyl peptidase 4 (DPP-4) is inhibited, causing it to act. The incretin hormones, which regulate insulin secretion and blood glucose levels, are broken down by DPP-4. These hormones include glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP). Sitagliptin reduces blood glucose levels by boosting the release of insulin and elevating these hormones by blocking DPP-4. As a result, sitagliptin has become an essential medication for the management of blood glucose in individuals with diabetes.
It is critical to have accurate and reliable quantification of Sitagliptin in pharmaceuticals because of its importance in diabetes treatment. To avoid potentially harmful side effects from overdosing or insufficient therapeutic results from underdosing, proper dosage is essential. In order to ensure that pharmaceutical goods containing Sitagliptin are of high quality and safe for patients, this work seeks to build a Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) method for accurately measuring Sitagliptin concentrations.

1.1. Importance of Accurate Quantification in Pharmaceutical Products

Several factors make accurate pharmacological dosage determination of Sitagliptin essential. A change in the drug's composition could lead to unsuccessful therapy or unwanted side effects; the precise dosage determines the drug's therapeutic efficacy. The pharmaceutical preparations containing sitagliptin should be of excellent quality and homogeneity in order to ensure that each dosage unit or tablet contains the necessary amount of active ingredient. Treatment efficacy and patient safety might be compromised by improper formulation or contamination. To ensure that each batch of pharmaceuticals meets the specified standard, it is crucial to have reliable quality control methods of analysis for everyday quality control in the production process.

1.2. Reverse Phase HPLC Method for Sitagliptin Determination

The exceptional sensitivity, selectivity, and adaptability of Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) to complex pharmaceutical matrices have earned it the status of a gold standard analytical technology. Non-volatile, hydrophobic substances like Sitagliptin can

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be analyzed using RP-HPLC since it depends on the individual analytes' affinities in the hydrophobic stationary phase. This method is a solid choice for pharmaceutical quality control because of how exact and reliable it is. This study aims to develop and optimize a Sitagliptin RP-HPLC system that may be used as a routine analytical tool in pharmaceutical labs; this system will be fast, accurate, and dependable.

1.3. Research Objectives

The research aimed to design and validate an accurate RP-HPLC method for quantifying Sitagliptin in pharmaceutical preparations. The method's performance was evaluated in terms of sensitivity, linearity, precision, and accuracy.

- The goal is to develop a reliable RP-HPLC assay that can accurately and reliably measure the concentration of Sitagliptin in pharmaceutical formulations.
- In order to ensure that the RP-HPLC method is suitable for routine usage, it must be tested for linearity, precision, accuracy, and durability.
- Identifying the Sitagliptin Limit of Detection (LOD) and Limit of Quantification (LOQ) will allow us to gauge the method's sensitivity.

2. DRUG PROFILE OF SITAGLIPTIN

Molecular structure, action mechanism, solubility property, and adverse effects are all detailed in this section of the Sitagliptin pharmacological profile. With a generally positive safety profile, sitagliptin is a selective DPP-4 inhibitor used to improve blood sugar levels in type 2 diabetic patients.

2.1. Molecular Structure

One key enzyme in glucose metabolism is dipeptidyl peptide-4 (DPP-4), and sitagliptin is a specific and powerful inhibitor of this enzyme. The chemical formula is C16H15F6N5O and the molecular weight is 407.314 g/mol; it is a triazolopyridine derivative. The binding capacity and effectiveness in inhibiting the DPP-4 enzyme and improving the insulin secretion mechanism are both enhanced by the structure's many functional entities. Patients with diabetes may benefit from

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its long-term use because of its unique composition, which allows it to dodge rapid metabolic breakdown.

2.2. Mechanism of Action

One mechanism by which sitagliptin exerts its effects is by inhibiting Dipeptidyl Peptidase-4 (DPP-4), an enzyme that is responsible for the degradation of incretin hormones like GLP-1 and GIP. Important for controlling blood sugar levels, incretin hormones are secreted naturally all across the GI system in response to eating. Pancreatic beta cells secrete more insulin and alpha cells secrete less glucagon when sitagliptin inhibits DPP-4, which in turn increases GLP-1 and GIP levels. Both effects work together to improve glycemic control in type 2 diabetes by lowering blood glucose levels, particularly after eating. One benefit of this method is that it has no hypoglycemia risk, unlike other anti-diabetic medications, because its action is glucose-dependent. This means that insulin production is increased in response to an increase in blood glucose levels.

2.3. Solubility

For the most part, sitagliptin is water- or alcohol-soluble. To dissolve it, you need a polar organic solvent like methanol or N,N-dimethyl formamide (DMF). But its poor solubility in water might reduce its absorption when taken orally. The formulation of Sitagliptin makes it more acceptable to boost absorption and guarantee that this drug can be effective in its therapeutic use, which is a frequent difficulty for most oral drugs due to their low solubility. Tablet formulations that include excipients to increase the rate of dissolving are primarily responsible for addressing the low water solubility.

2.4. Adverse Effects

Compared to other oral anti-diabetic medications, sitagliptin is thought to have a superior adverse effects profile, and the drug's side effects are usually acceptable. However, some people may experience negative effects because it is a medicine. Common adverse effects include stuffy nose, headaches, and infections in the upper respiratory tract. These are just minor side effects that, in most cases, go away after a few days of use. Pancreatitis, or inflammation of the pancreas, is another rare but serious side effect of Sitagliptin that has been documented in a small number of

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post-marketing monitoring studies. Patients with a history of pancreatitis are less likely to be prescribed Sitagliptin due to concerns about the potential danger, even though the exact cause-and-effect relationship between the two has not been completely determined. A low blood sugar, or hypoglycemia, is not a typical side effect of sitagliptin when used alone but may occur when combined with insulin, sulfonylureas, or other anti-diabetic medications. Less common but still possible are gastrointestinal problems including nausea and diarrhea. Sitagliptin has a reduced risk of weight gain, which is good news for many people who have type 2 diabetes.

3. REVIEW OF LITREATURE

In order to determine the Sitagliptin phosphate content in tablets, Tang et al. (2012) developed and validated a stability-indicating RP-HPLC method. The goal of this process was to remove any potential contaminants from the analytical data by isolating Sitagliptin and its breakdown products. We utilized a UV detector operating at 237 nm in conjunction with a methanol and water mobile phase and a Symmetry C18 column. The method is suitable for regular stability testing of Sitagliptin in pharmaceutical preparations because of its effectiveness, linearity, precision, and strength.

Ravanello et al. (2013) created an additional LC method for Sitagliptin phosphate tablets that indicates stability. At 267 nm, they found the product using an isocratic mobile phase consisting of acetonitrile and phosphate buffer. It was determined that the method was accurate, exact, linear, and selective. Based on the results of the experiment, the method might be used to test the stability of Sitagliptin in commercial pharmaceutical formulations since it could distinguish between Sitagliptin and its degradation products.

To estimate Sitagliptin phosphate in pharmaceutical dose forms, Monila et al. (2014) established visible spectrophotometric techniques. Sitagliptin was complexed with two dyes, Bromo Thymol Blue (BTB) and Bromo Cresol Green (BCG), to form colored complexes that could be measured at 412 nm and 419 nm, respectively, according to these procedures. These procedures have been proven to be accurate, sensitive, and reproducible; they provide a cheap, easy way to check the quality of your pharmaceutical analyses.

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Sitagliptin phosphate may be easily and accurately measured in pharmaceutical and bulk forms using a new spectrophotometric technique developed by Balasekaran and Indumathy (2012). The procedure yielded a yellow substance by means of a condensation reaction involving Sitagliptin phosphate, acetyl acetone, and formaldehyde. We tested the method for linearity, accuracy, and precision, and then we conducted the spectrophotometric measurement at 430 nm. Its low price and high reliability make it an excellent choice for regular use in pharmaceutical quality control analyses.

A spectrofluorimetric technique for the determination of Sitagliptin in both tablet form and spiking human serum was devised by Caglar et al. (2013). Aside from assessing Sitagliptin's inherent fluorescence, this study also used a method to measure its reaction with fluorescein, which yielded a derivative with enhanced fluorescence. The linear range of the first approach was 0.5-10.4 µg/mL, whereas the second method covered 0.4-1.8 µg/mL. Both approaches were shown to be very effective in detecting Sitagliptin in complicated pharmaceutical and biological matrices, and they were verified for linearity, precision, sensitivity, and accuracy.

4. METHODOLOGY

This study aimed to design and establish a dependable Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) method of the quantification of Sitagliptin in pharmaceutical preparations.

4.1. RP-HPLC Method Development

The RP-HPLC technique involved the use of Hypersil ODS C18 column (250 mm x 4.6 mm, 5 uM) where the mobile phase consisted of methanol and 0.5% triethylamine in a 66:34 v/v mixture. Optimum flow rate was set at 1.0 mL/min with UV recording at 237 nm that is sensitive to the quantification of Sitagliptin. Optimization was made to these conditions to obtain good separation and peak resolution.

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4.2. Sample Preparation

Sitagliptin tablets were powdered and triturated then dissolved in methanol. The vacuum filter was used to filter the solution and proper dilution of the solution was made in methanol to be within the linear range of the calibration curve.

4.3. Instruments Used

- HPLC-UV detector: It is necessary to quantify Sitagliptin due to its absorbance of 237 nm.
- UV-Visible Spectrophotometer: This instrument is applicable in the determination of the λmax of Sitagliptin.
- pH Meter: The meter was used to titrate the pH of the mobile phase to 3.0 ± 0.1.
- Vacuum Filter: S ensured sample clarity by eliminating the particulates prior to analysis.

4.4. Method Validation

The technique was tested as linear, precise, accurate, and robust in accordance to ICH requirements. Calibration curves were plotted and reliability of the method was tested using study of repeatability and accuracy.

5. RESULT

This part includes the findings of the RP-HPLC method development of Sitagliptin quantification, determination of 1 / 1max, method optimization, linearity, precision, accuracy, and sensitivity. The results confirm the strength of the technique, sensitivity, and the applicability of the technique to normal pharmaceutical studies.

5.1. Determination of λmax

In the methanol absorption spectrum, the absorption spectra of Sitagliptin were acquired and the drug showed a typical peak at 237 nm which was chosen to be used in HPLC analysis in U.V. detection. The absorption spectrum of Sitagliptin in methanol in figure 1 has a typical peak at 237 nm which was chosen as the UV absorbing wavelength in the HPLC analysis.

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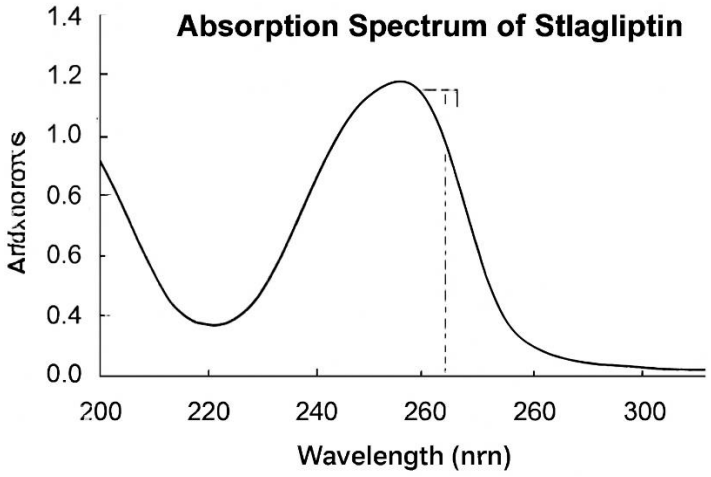


Figure 1: Absorption spectrum of Sitagliptin at 237 nm.

The highest point at 237 nm indicates the maximum absorption wavelength (maximum absorption) (6) of Sitagliptin meaning the wavelength which the drug absorbs UV light best. The selected wavelength (UV) was used in the HPLC analysis to detect the UV in order to achieve a high level of sensitivity and precise determination of Sitagliptin in pharmaceutical preparations. The steepness of the peak indicates that the technique is very selective to Sitagliptin and other compounds will not interfere with the technique.

5.2. Method Optimization

The optimum mobile phase (Methanol:0.5% Triethylamine, 66:34 v/v) gave a retention time (Rt) of 3.6 minutes which provided good peak resolution. The chromatogram presented below shows that Sitagliptin is separated significantly in the tablet matrix in comparison with other excipients. The figure shows the optimized chromatogram of Sitagliptin, which was obtained in the application of a mobile phase, made up of a mixture of methanol:0.5% triethylamine (66:34 v/v). A retention time (Rt) of 3.6 minutes is observed in the chromatogram, which indicates that Sitagliptin is effectively separated with other excipients in the tablet matrix.

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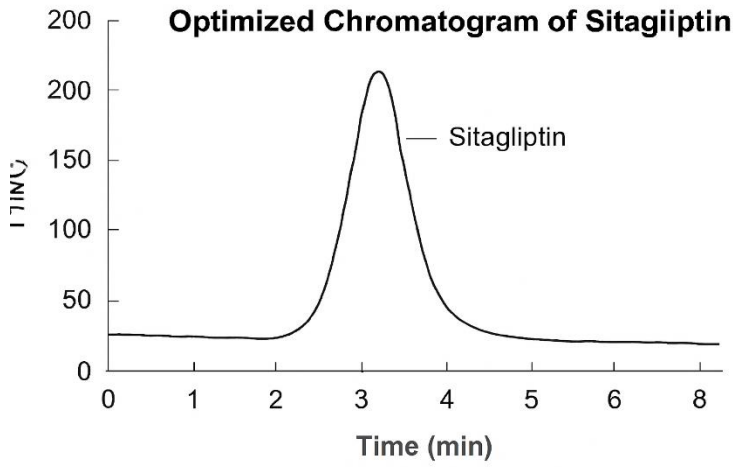


Figure 2: Optimized Chromatogram of Sitagliptin.

It can be seen that the chromatogram shows that the optimized mobile phase is capable of separating Sitagliptin and excipients well with a sharp and distinct peak at 3.6 minutes. This confirms the approach ability to give the straight answer thus creating the right quantification of Sitagliptin in the pharmaceutical formulations. The clear distinction underlines the applicability of the method in the regular quality control in pharmaceutical industry.

5.3. Linearity and Calibration

The figure represents the calibration curve of Sitagliptin that indicates the dependence of the peak area on the concentration (20-200 µg/mL). The high linear relationship with a correlation coefficient of 0.9999 assures the strength and quality of method to measure Sitagliptin.

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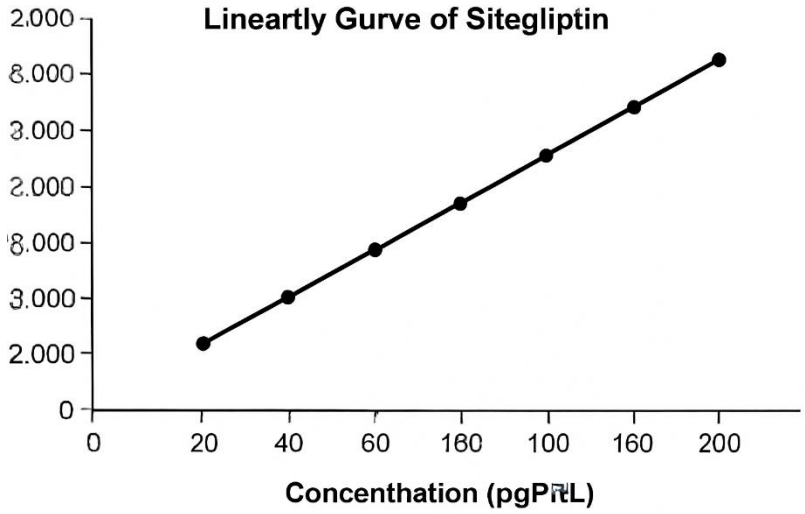


Figure 3: Linearity curve of Sitagliptin.

The linearity curve shows that the concentration of Sitagliptin is directly proportional to the area of the peak with high correlation coefficient (0.9999) indicating that the technique is effective and precise in measuring the concentration of Sitagliptin in pharmaceutical preparations.

Calibration was developed with a concentration of 20-200 ug/ ml. The data on the linearity (Table 1) showed a correlation of 0.9999 which validated the strength of the method in the determination of the correct quantification.

Table 1: Linearity data for Sitagliptin.

Conc. (µg/mL)	Peak Area
20	1.973
40	3.767
60	5.452
80	7.331

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120	10.843
160	14.365

The results demonstrate a steady rise in the peak area with the Sitagliptin concentration, which proves the existence of a strong linear correlation between them and validity of the approach to precise quantification.

5.4. Precision

Repeatability and intermediate precision tests were run to ensure the technique was accurate. The approach is reproducible across and within trials, as shown by the low values of the percent RSD in Tables 2 and 3.

In terms of concentration (mg/ml), peak area with standard deviation (SD), and percentage relative standard deviation (percent RSD), the findings of the precision (repeatability) of the Sitagliptin quantification method are displayed in the table below.

Table 2: Precision (Repeatability) study results.

Conc. (µg/mL)	Area ± SD	%RSD
20	1.836 ± 0.01	0.54
40	3.684 ± 0.04	1.08
80	7.192 ± 0.07	0.97
120	10.943 ± 0.16	1.46
200	17.434 ± 0.28	1.60

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Because the percent RSD numbers are modest, we can say that the approach is highly consistent and repeatable. The method is applicable for routine analysis since it accurately quantifies Sitagliptin and the percentage relative standard deviation values fall within the range of 0.54 to 1.60.

The results of the different trials for Sitagliptin at an intermediate level of accuracy are shown in this table together with their concentration (µg/mL), peak area with standard deviation (± SD), and percentage relative standard deviation (% RSD).

Table 3: Precision (Intermediate Precision) study results.

Conc. (µg/mL)	Area ± SD	%RSD
20	1.820 ± 0.02	1.09
40	3.678 ± 0.04	1.08
80	7.256 ± 0.10	1.37
120	10.988 ± 0.16	1.45
200	17.492 ± 0.28	1.60

Results from the repeatability study and the intermediate precision study for the percentage of RSD are comparable, demonstrating that the approach maintains its accuracy and reproducibility when tested by different operators. The soundness of the methodology and its capacity to give consistent results in varied situations are demonstrated by the estimated logarithms of RSD, which range from 1.09 percent to 1.60.

5.5. Accuracy

Spiking standard solutions with 50, 100, and 150 percent of the correct concentration allowed for the determination of accuracy. The method's accuracy was demonstrated by the recovery values, which ranged from 100.97 to -101.3. Table 4 shows the area responses and percentages of recovery

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from a Sitagliptin recovery study conducted at different concentration levels (50, 100, and 150 percent).

Table 4: Results of Recovery.

Level (%)	Area Response SITA	Recovery (%)
50	5.617	101.86 ± 0.10
100	7.211	100.97 ± 1.5
150	9.125	101.09 ± 0.8

Proof of the method's accuracy and reliability are recovery percentages near 100, 100.97-101.86. Low standard deviation values indicate that the approach is suitable for quantitative analysis of pharmaceutical preparations since it produces consistent and trustworthy results across a range of concentrations.

5.6. LOD and LOQ

The verification of the method's sensitivity led to the determination of the limit of detection (LOD) as 0.6773 µg/mL and the limit of quantitation (LOQ) as 2.188 1µg/mL. Table 1 displays the Limit of Detection (LOD) and Limit of Quantification (LOQ) for Sitagliptin, which indicate the lowest values at which the method can be used with any degree of certainty.

Table 5: Result of LOD and LOQ.

Parameter	Sitagliptin
LOD (µg/mL)	0.6773
LOQ (µg/mL)	2.1881

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A LOD value of 0.6773 0g/ml and LOQ of 2.1881 0g/ml prove the high sensitivity of the method. The above values affirm that the technique can identify and quantify Sitagliptin accurately even at a low concentration and thus it is used in the accurate analysis of pharmaceutical products.

6.CONCLUSION AND RECOMMENDATIONS

In order to quantify sitagliptin in pharmaceutical preparations, this research was able to develop and demonstrate an effective Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) method. Across a wide concentration range of 20200 µg/mL, the method demonstrated exceptional sensitivity, accuracy, and precision. A correlation coefficient of 0.9999, an optimum retention time of 3.6 minutes, and effective separation of Sitagliptin from other excipients proved that the approach was robust enough for routine usage. Validation results, such as small values of the percent reproducible s.d. and high recovery levels, show that the approach is suitable for quantifying Sitagliptin and quality controlling it in commercial preparations. Furthermore, the method's great sensitivity is supported by its low detection and quantification limits (LOD: 0.6773 ug/mL, LOQ: 2.1881/mL).

- Routine Quality Control Testing: The designed RP-HPLC technique can be implemented in routine to measure the concentrations of Sitagliptin in the pharmaceutical products to provide control over the product quality and safety of the patients throughout the production batches.
- Additional Method Optimization: Additional optimization of the mobile phase composition and column configurations can be considered in future applications to improve the efficiency and throughput of the method, which might decrease the time of analysis of large-scale testing.
- Application to Biological Samples: It is suggested that the method should be adapted to analyze Sitagliptin in biological samples including serum or plasma to add value to its application in pharmacokinetic research and in therapeutic drug monitoring.

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BIOANALYTICAL METHOD DEVELOPMENT FOR NAPROXEN USING LC-MS/MS: VALIDATION AND APPLICATION

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Abstract

To measure naproxen in human plasma, a sensitive LC-MS/MS method must be developed and validated in order to acquire the appropriate pharmacokinetic and bioequivalence study. This paper describes the process of creating an LC-MS/MS method that analyzes naproxen using Zidovudine as an internal standard. In various chromatographic settings, the method was reproducible, sensitive, and selective. According to FDA guidelines, the validation was carried out using tests for linearity, accuracy, precision, recovery, and stability. Excellent chromatographic isolation, crisp peak resolution, and minimal plasma interference were demonstrated by the method. A linear response with a correlation coefficient (r2) of 0.9962 was discovered between 500.1 ng/mL and 100028.5 ng/mL. There was little variation in the recovery rate with Naproxen and internal standards, which was shown to be constant (>80%). Tests of the method's stability under various circumstances, including room temperature, freeze-thaw cycles, and prolonged storage, were conducted. This work presents a bioanalytical methodology that is suitable for bioanalytical research and may be used to measure the amounts of naproxen in blood plasma in both preclinical and clinical studies.

Keywords: LC-MS/MS, Naproxen, Bioanalytical Method, Validation, Sensitivity, Stability, Internal Standard

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1.INTRODUCTION

The process of creating and approving reliable bioanalytical instruments is essential to pharmaceutical research in order to precisely measure the medications and their metabolites in a biological setting. These methods support research in pharmacokinetics, bioavailability, and bioequivalence, which forms the basis of clinical trials and regulatory filing. High sensitivity, selectivity, and reproducibility have made liquid chromatography-tandem mass spectrometry (LC-MS/MS) the gold standard among analysis techniques. In order to evaluate Naproxen's clinical and pharmacological effects, the current work aims to establish a valid LC-MS/MS technique for quantitatively detecting this medication in human plasma.

1.1 Background and Significance

Chemically, multi(S)-2-naphthalen-2-yl propanoic acid(+)-(S)-2-naphthalen-2-yl propanoic acid (C 14 H 14 O 3 MW = 230.26 g / mol) is the powerful analgesic and antipyretic ingredient of Naproxen, a popular non-steroidal anti-inflammatory medicine (NSAID). As one of the aryl acetic acid derivatives, it is often prescribed to treat a variety of conditions, including primary dysmenorrhea, osteoarthritis, spondylitis, tendinitis, bursitis, and rheumatoid arthritis. Naproxen's ability to block the activity of cyclooxygenase enzymes, specifically COX-1 and COX-2, which promote the synthesis of prostaglandins that induce pain, inflammation, and fever, is thought to be the basis for its therapeutic action. The medicine's pharmacokinetic unpredictability and limited therapeutic index need the creation of a precise and reliable analytical approach for drug quantification in biological samples, despite its effectiveness.

1.2 Rationale for Method Development

Pharmacokinetic profiling, bioequivalence, and therapeutic medication analysis all benefit greatly from naproxen plasma analysis. Common problems with traditional chromatographic techniques include low recovery, low sensitivity, and matrix interference. As a result, the development of the liquid chromatography-tandem mass spectrometry (LC-MS/MS) technique offers special

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advantages such high detection limits, low interference, and good selectivity. Using Zidovudine as an internal standard, the study aimed to develop an efficient, sensitive, and repeatable LC-MS/MS method for quantifying Naproxen in human plasma. Following the US-FDA's Bioanalytical Method validation guidelines, the method was validated, with particular attention paid to essential parameters such as linearity, sensitivity, accuracy, precision, recovery, matrix effect, carry-over, and stability under different analysis settings.

1.3. Research Objectives

The subsequent research objectives are the summarised objectives of the development and validation of a robust LC-MS/MS technique in the quantification of Naproxen in human plasma platform, with consideration to the performance of the method, selectivity and reliability of the technique towards bioanalytical use.

- To develop and validate an LC-MS/MS method for accurate Naproxen quantification in human plasma, ensuring high sensitivity, selectivity, and reproducibility across various analytical parameters.
- To assess chromatographic performance and selectivity, ensuring clear peak resolution and minimal matrix interference for reliable bioanalytical applications.
- To evaluate method robustness through stability, dilution integrity, and ruggedness testing, confirming its reliability for pharmacokinetic and bioequivalence studies.

2. REVIEW OF LITREATURE

Adye et al. (2023) used 3D-printed sorbents as extraction media and developed an LCMS/MS method for analyzing non-steroidal anti-inflammatory medications (NSAIDs) in rat plasma. According to their analysis, the new 3D-printed sorbents exhibited a higher analyte recovery yield and fewer matrix effects because of their improved surface interaction. Pre-clinical pharmacokinetic assessment could be performed using the validated technique, which was proven to be very linear, exact, and accurate. The study showed how advanced materials can change the way samples are prepared for bioanalytical procedures.

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Bhatt et al. (2018) created a Quality by Design (QbD)-based LC–MS/MS bioanalysis method to assess diclofenac and paracetamol in human plasma at the same time. They employed statistical design-of-experiment (DoE) techniques to identify the critical variables affecting the methods' performance. With a linear calibration, high recovery, and minimal interference, the validation revealed that the result was consistent with US-FDA bioanalytical principles. According to their findings, a QbD strategy could improve the method's reproducibility and robustness, giving bioanalytical research a strong foundation for regulatory compliance.

A straightforward and sensitive LC-MS/MS method for measuring moxidectin in plasma was developed and tested by Chhonker et al. (2019) and then applied to in vitro metabolism studies. The process showed a satisfactory degree of accuracy, precision, and stability between the validation batches, and the sample was prepared using protein precipitation. The technique's validity in pharmacokinetic research was confirmed by their work, which also highlighted the importance of LC-MS/MS in toxicokinetic and drug metabolism studies.

Elkady and colleagues (2018) created an LC-MS/MS bioassay for four proton pump inhibitors (PPIs) in human plasma. Additionally, the authors have adjusted the chromatographic settings to provide good sensitivity and selectivity by achieving the clear separation of the analytes in a very short run time. With good intra and inter-day precision and recovery, the method has been shown to be valid according to EMA guidelines. The study confirmed the usefulness of LC-MS/MS in simultaneously identifying several medications in intricate biological materials.

Eure et al. (2021) developed an LCMS/MS assay for riluzole and etoricoxib in rat brain tissue and plasma, which they applied to a pre-clinical rat model of traumatic brain injury. Both medications may be monitored simultaneously in different biological media because to the technique's high sensitivity and strength. LC-MS/MS/MS is a versatile analytical technique in neuropharmacological and toxicological research, as demonstrated by the confirmed assay's information on distribution in tissues and pharmacokinetics.

3. MATERIALS AND METHODS

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The development of a reliable and reproducible LC-MS/MS bioanalytical method for estimating naproxen in human plasma required careful material selection, chromatographic condition optimization, and rigorous validation procedures. To maintain the integrity of the analysis and lower variability, the experiment was carried out in a controlled environment in the lab. All validation parameters, including selectivity, accuracy, precision, linearity, recovery, matrix effect, and stability, were carefully considered because the method was developed using the guidelines provided in the US-FDA (2001) document on Bioanalytical Method Validation, which also includes industry guidance.

3.1 Chemicals and Reagents

The internal standard (IS), pure naproxen and zidovudine, was acquired from approved pharmaceutical producers. All of the solvents displayed, including acetone, methanol, and ammonium acetate, were of HPLC quality and were acquired from reputable vendors of analytical supplies. Ultra-pure water was created using a Milli-Q purification system to prevent any contaminants that would obstruct chromatographic detection. Accredited biorepositories were used to get the human plasma samples, ensuring ethical consideration and tracking. 0.22 0 membrane filters were used to produce and filter all of the reagents and solutions.

3.2 Instrumentation and Chromatographic Conditions

The Agilent Technologies Zorbax Eclipse XDB phenyl column (4.6 75 mm, 3.5 µm) was used for the chromatography to separate naproxen and the internal standard. This column was selected due to its high efficiency and dependability in the separation of aromatic chemicals. The optimal peak symmetry and signal response were obtained with a 90:10 (v/v) mixture of acetonitrile and 20 mM ammonium acetate as the mobile phase. With an injection volume of 10 µL and a flow rate of 0.8 mL/min, each sample was run for less than five minutes on average.

A triple quadrupole mass spectrometer (API system) using an electrospray ionization (ESI) source in negative ion mode performed the measurement. Several Reaction Monitoring (MRM) transitions were detected at m/z 267/222.9 Zidovudine and m/z 229/185 Naproxen. To increase

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ionization efficiency and signal intensity, source parameters such ion spray voltage, collision energy, and declustering potential were optimized.

3.3 Sample Preparation

To achieve high separation of the analyte, internal standard, and plasma components, the sample was prepared using the liquid-liquid extraction (LLE) method. Typically, a 500 00L plasma sample was vortexed and spiked with an appropriate quantity of Zidovudine (IS) in a working solution. Organic solvents such as methyl tert-butyl ether or ethyl acetate were used in the extraction process to increase the analytes' recovery. After centrifuging the samples for 10 minutes at 4000 rpm, the organic layer was carefully removed and evaporated using a low pressure nitrogen flow at 40 degrees Celsius. Prior to being transferred to LC-MS/MS autosampler vials, the residue was vortexed, dried, and reconstituted in the mobile phase.

Repeatable recoveries (>80) with negligible matrix interaction and stability of naproxen throughout the assay were provided by this extraction method.

3.4 Calibration and Validation Protocol

A calibration curve was drawn between Naproxen and the concentration of the stock solutions at the concentration of 500.1 to 100028.5 ng/mL by serial dilution of the stock solutions in the blank plasma. Every calibration level and quality control (QC) sample containing low (LQC), medium (MQC), and high (HQC) concentrations was analyzed in 6 replicates.

The testing validation trial was conducted according to the FDA and EMA regulation and comprised of the following parameters:

- Selectivity and Specificity: blank plasma of six different sources was used to assess selectivity and specificity to ensure that no interference was occurring at the retention time of Naproxen and the internal standard.

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- Linearity: Decided because least-squares regression analysis produced a correlation coefficient (r^2) ≥ 0.99.
- Precision and Accuracy: Evaluated over (intra-day) and between (inter-day) batches, with a CV% of 15% (or less) at each level of QC.
- Recovery and Matrix Effect: Determined by the comparison of extracted samples and post-extraction spiked standards, and gives reproducibility and low ion suppression.
- Stability Studies: Involved bench-top, autosampler, freeze-thaw, short-term and long-term stability measures in order to determine the strength of Naproxen at varying storage and analytical conditions.

All validation parameters were found to be within regulatory acceptance and this ensured that the method was appropriate to be used in regular pharmacokinetic and bioequivalence investigations of Naproxen.

4. RESULTS AND DISCUSSION

The design and verification of the LC-MS/MS methodology of Naproxen determination in plasma entailed a thorough analysis of the chromatographic performance and selectivity. The procedure proved to be effective in the separation and clear resolution of Naproxen compared to the plasma matrix components and its internal standard (Zidovudine), which guaranteed the reliability and reproducibility of the results. The next section reveals the specifics of the chromatographic data, which is robust, sensitive, and specific.

4.1 Chromatography and Selectivity

Naproxen and Zidovudine could be separated well without any interference with plasma components. Below are representative chromatograms. The chromatogram of Naproxen and Zidovudine (internal standard) aqueous standard mixture is presented in Figure 1. The individual peaks of Naproxen and internal standard are indicated in the chromatogram and the Naproxen peak has a retention time of about 3.5 minutes and the internal standard peak has a retention time around 6 minutes. The clear peptides, as well as lack of major interference by the matrix components, guarantee the sensitivity of the LC-MS/MS method, and its potential to be used in quantification.

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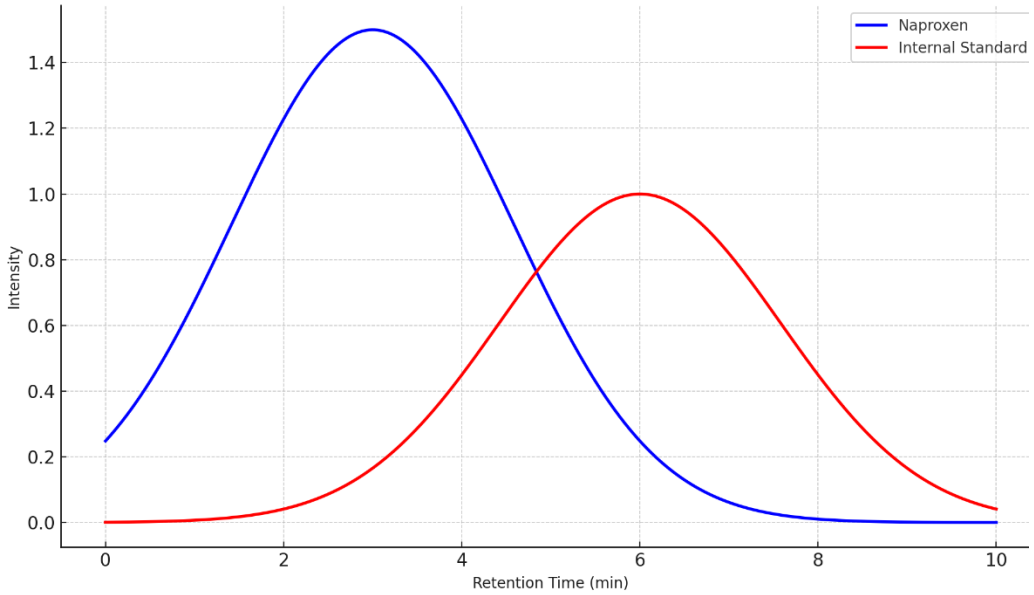


Figure 1: Analytical chromatogram showing Naproxen's aqueous and internal standard mixtures

The blue peak in this chromatogram is the Naproxen analyte and the red peak is the Zidovudine (internal standard). The acuity and definition of the peaks prove that the technique gives explicit separation between the analyte and internal standard. There is no baseline drift and no interference can be measured at the retention times of both the compounds, which indicates that the method is selective in the detection of Naproxen in plasma. This sharp chromatographic resolution is important in order to obtain precise and reliable results in further bioanalytical experiments, e.g. pharmacokinetic experiments.

The chromatogram of blank plasma and Zidovudine as internal standard is represented in figure 2. No major peaks of endogenous plasma components could be identified in the chromatogram, other than the internal standard peak which appeared at the retention time of about 6 minutes. This proves that there is no interference at the retention time of Naproxen and thus specificity of the method.

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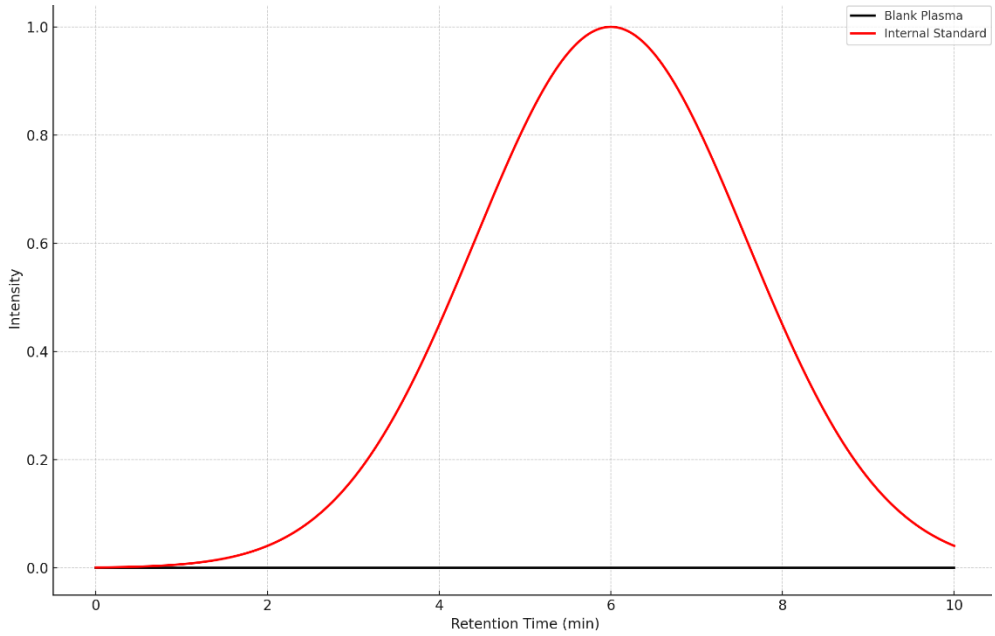


Figure 2. Typical Chromatogram of Undiluted Plasma Using an Internal Standard

The black trace in this chromatogram is the blank plasma that had only the internal standard (red peak) in it. The fact that no peaks of the analyte were detected in the blank sample is an indicator of the specificity of the method because the endogenous plasma substances would not be where Naproxen is retained. The evident observation of the internal standard peak is also in favour of the reliability of the chromatographic system, which suggests that the method is applicable in the quantification of Naproxen in plasma devoid of matrix interferences. This guarantees that further quantitative analyses done in the presence of the plasma matrix effects are accurate.

The chromatogram of LLOQ (Lower Limit of Quantification) QC of Naproxen is described in figure 3. It is evident that the peak of Naproxen is apparent at the retention time with a very small amount of background noise. It shows that it is a reliable method to measure the lowest concentration of Naproxen (500.1 ng/mL) at which a pharmacokinetic analysis can be done correctly.

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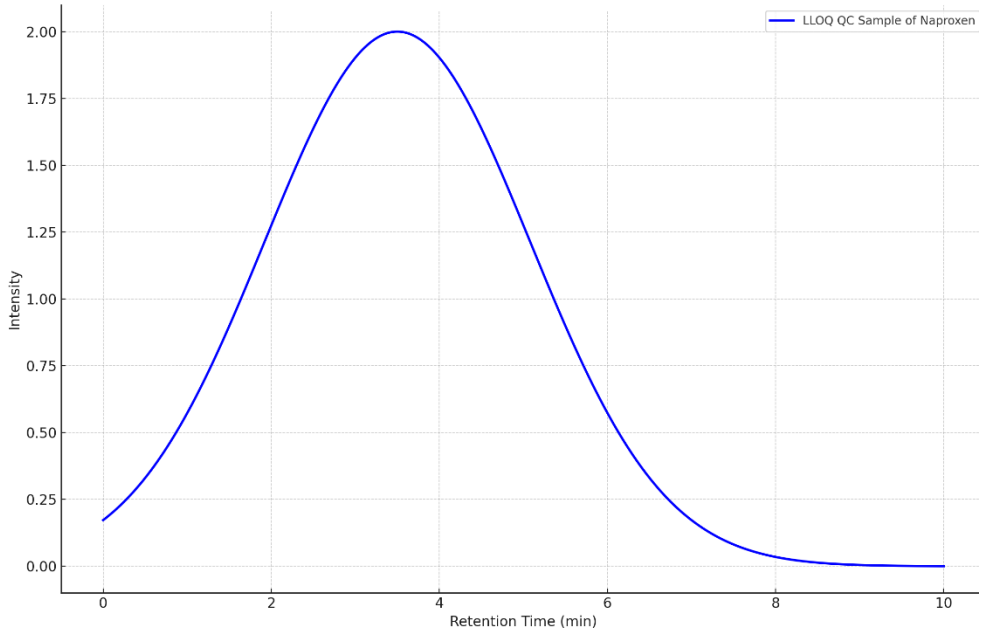


Figure 3. Analytical chromatogram of Naproxen LLOQ QC Sample

The blue peak in this chromatogram corresponds to Naproxen, and the method's great sensitivity even at low doses is demonstrated by its crispness and strong peak at the specified retention time. At the lower level of quantification, the approach demonstrates accuracy and precision with LLOQ QC samples showing a robust and well-resoluted peak free of matrix component interference. This is crucial for early-stage pharmacokinetic studies and other samples containing low levels of Naproxen, since it will ensure that the process is appropriate for detection and quantification of these compounds.

4.2 Linearity and Sensitivity

Data on the concentration-response linearity of Naproxen from 500.1 to 100028.5 ng/mL are presented in Table 1. There is a continuous and reproducible link between the concentration and the reaction, as shown by the good linearity of the approach and the r 2 of 0.9962.

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Table 1. Concentration-Response Linearity Data for Naproxen

Concentration (ng/mL)	Mean Response	SD	CV (%)	% Nominal	r^2
500.1 – 100028.5	Linear	—	<3.0	91.9–108.5	0.9962

With a very high value of r 2 of 0.9962 and excellent linearity over the complete concentration range tested, the approach is clearly demonstrated in Table 1. As a result, we know for sure that the method is bioanalytical and will provide accurate results within the given concentration range when used to measure Naproxen at low doses (500.1 ng/mL). With a low CV and a satisfactory percentage Additional proof of the method's accuracy and precision can be found in nominal values.

4.3 Accuracy and Precision

Table 2 displays the data for Naproxen's within-batch precision and accuracy for each of the five QC levels: LLOQ, LQC, MQC1, MQC2, and HQC. The results show that the approach is accurate and precise in a single batch, with CV values ranging from 2.08% to 9.00% and percent nominal values ranging from 107.9% to 112.8%.

Table 2. Within-Batch Precision and Accuracy of Naproxen

QC Level	Mean (ng/mL)	SD	CV (%)	% Nominal
LLOQ	564.4	22.2	3.93	112.8
LQC	1603.4	144.3	9.00	107.9
MQC1	16542.6	678.9	4.10	110.3
MQC2	56370.4	1170.0	2.08	112.7
HQC	95680.9	2417.9	2.53	112.6

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Table 2 demonstrates that the approach is far below the FDA-approved level and has a good degree of accuracy (91113) and a high degree of precision (CV < 10%) across all QC levels, which is considered quite acceptable. This approach has been validated for the measurement of Naproxen in human plasma and may be used for routine bioanalytical detection because the average values at every QC level are similar to the nominal values.

4.4 Recovery and Matrix Effect

Table 3 shows the recovery of Naproxen and Zidovudine (IS) internal standard. Naproxen showed 80.63% on average recovery, and Zidovudine at 81.93 that the two different compounds experienced low CV values (8.74% and 7.20%, respectively), which is an indication of an efficient extraction procedure.

Table 3. Recovery of Naproxen and Internal Standard (Zidovudine)

Compound	Mean Recovery (%)	CV (%)
Naproxen	80.63	8.74
Zidovudine (IS)	81.93	7.20

Table 3 shows that both Naproxen and Zidovudine have consistent recoveries, which have low variability (CV < 10%). This establishes that the process of sample extraction was very efficient which makes the method reliable in the determination of Naproxen in plasma samples with insignificant matrix effects. The recovery of both the analyte and internal standard is within acceptable range, which is evidence of the strength of the method.

4.5 Stability Studies

The summary of the stability data, of Naproxen, in different conditions of storage and handling is described in Table 4. These findings indicate that Naproxen is stable under all experimental conditions and thus, it is observed that the stability is between 99.38 and 110.17 and the CV values are not more than 10, which validates the strength of the method.

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Table 4. Summary of Stability Data for Naproxen

Stability Condition	Duration	% Stability	CV (%)	Result
Room Temperature (9 h)	99.38	2.13	Stable	
Refrigerated (7 days)	102.13	2.27	Stable	
Freeze-Thaw (4 cycles)	108.03–109.72	7.4–8.9	Stable	
Autosampler (49 h)	99.87–110.17	<9.8	Stable	
Short-Term (–20 °C, 6 days)	108.63–110.02	<5.8	Stable	

The stability information in Table 4 affirm that Naproxen is stable at a range of conditions, including room temperature, refrigeration and freeze-thaw, autosampler storage and short term deep freeze storage. The stability percentage in such conditions is in the acceptable limits (99.38% to 110.17%), and the values of CV are always low (less than 10%), which means low variability. Such outcomes justify that the approach is reliable and robust to be used in bioanalytical applications over the long term.

4.6 Dilution Integrity and Ruggedness

In Table 5, the results of the dilution integrity of Naproxen at two dilution factors (2× and 4×) are provided. The dilutions showed good values of the aspects of dilution (percent of the sample) (dilution) (97.81 percent and 90.69 percent) and dilution (value of the dilution) (0.92 percent and 0.86 percent), indicating that the method was reliable in the dilution of a sample.

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Table 5. Dilution Integrity Results

Dilution Factor	Mean Conc (ng/mL)	% Nominal	CV (%)	Conclusion
2× Dilution	156070.4	97.81	0.92	Acceptable
4× Dilution	146201.8	90.69	0.86	Acceptable

Table 5 demonstrates that the results obtained by the method are accurate and precise even in the case when the samples are diluted (2x and 4x). The nominal values and the values of the CV are very near to 100 and the value of CV is low and this means that the method is good to work with diluted samples. Moreover, ruggedness experiments also indicated that the procedure gives similar results when using different analysts, columns, and different reagents, indicating that the method can be reliable in different conditions.

5.CONCLUSION AND RECOMMENDATIONS

An accomplishment in determining the levels of Naproxen in human plasma, the design and establishment of the LC-MS/MS technology demonstrate the system's great sensitivity, selectivity, and repeatability. Zidovudine, the internal standard, and Naproxen were effectively separated chromatographically using this method. Zidovudine features low matrix interference and allows for reliable sample measurement even at low concentrations. Multiple metrics, including linearity, accuracy, precision, recovery, stability, and matrix effects, were used to confirm the technique, and they all met the stringent standards imposed by the FDA and EMA. Stability, dilution integrity, and ruggedness tests have also proven that the procedure is robust, which opens up some bioanalytical possibilities, like bioequivalence testing and pharmacokinetic research.

- It is suggestive that this LC-MS/MS/method be extended to other NSAIDs and Pharmacokinetic research, particularly in clinical trials to comply with drug levels and treatment efficacy.

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- The Stability Testing: Additional stability tests must be done under extreme conditions of the environment (e.g., long exposure to light, change in temperature cycles) to verify stability of the method in other environments.
- Sample Preparation: With high-throughput analysis being required, in future, the automation of the sample extraction process may be considered, with solid-phase microextraction (SPME) being the method to automate the bioanalytical process, minimize the number of errors in manual work, and enhance the throughput.

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Development and ICH Compliant Validation of a Spectrometric Method for the Determination of Lamivudine in Pharmaceutical Formulations

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Abstract: *The present study aimed at enhancing a straightforward UV-visible spectrophotometric technique to determine lamivudine in pharmaceutical preparations based on the guidance of the International Conference on harmonisation (ICH). Lamivudine is an antiretroviral drug, which is a nucleoside analogue used in a large number in treating HIV infection and therefore needs quality control analysis methods that are reliable. This research paper measured the analytical wavelength (λmax) of lamivudine with a UV-Visible spectrophotometer at 280 nm. It was performed by the BeerLambert law within the range of concentration of 218ug/mL, having a regression equation of A = 0.048C + 0.068 and correlation coefficient at 0.999 which indicated that it was quite linear. The accuracy, precision, detection limit, quantification limit and robustness was the parameter used to prove the developed method as per the ICH guidelines. The detection and quantification limit were also found within the range of 0.0033 ug/mL and 0.10043 ug/mL respectively. Recovery studies proved to be accurate between 99.70% to 100.34%,, whereas assay of a commercial tablet formulation (Lamivir-HBV) gave a recovery of 99.98% which showed that excipients did not interfere. The findings validated that the developed spectrophotometric technique is easy, correct, exact, cost-effective, and fit to the habitual quality control investigation of lamivudine in drug preparation.*

Keywords: *Lamivudine; UV–Visible spectrophotometry; Method validation; Pharmaceutical analysis; ICH guidelines.*

I. Introduction

Pharmaceutical analysis is important in quality, safety, and efficacy of the drug products. Quantitative analysis of active pharmaceutical ingredients in bulk drugs, as well as in finished pharmaceutical preparations, requires reliable analysis procedures. Spectrophotometric methods are one of the most common methods of analysis due to their simplicity, speed of analysis, low cost, and acceptable accuracy among other available methods of analysis. The validation and analytical techniques can be developed and verified by following internationally recognised guidelines (including those of the International Conference on Harmonisation (ICH)). This is to ensure that the method is appropriate to the purpose it is intended and yields reproducible and reliable results in normal quality control of pharmaceuticals.

1.1 Overview of Lamivudine

Lamivudine is an antiretroviral drug which is mainly used in the treatment of Human Immunodeficiency Virus (HIV) that is a nucleoside analogue drug. It is a nucleoside reverse transcriptase inhibitor (NRTIs), which is an essential companion of antiretroviral therapy. Chemically, lamivudine is known as 4-amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one, with a molecular formula $C_8\ H_{1\ 1}\ N_3\ O_3\ S$ and molecular weight 229.3 g/mol.

Lamivudine is a crystalline solid, white to off-white in color and it is well soluble in water. Its absorption through the gastrointestinal tract is efficient with an estimated bioavailability of about 86%, and it is mostly absorbed orally. Lamivudine has a plasma half-life of 5-7 hours; pharmacokinetically, this drug can be used to maintain good levels of the therapeutic drug in the body. These pharmacophysiological and pharmacokinetic activities render lamivudine as a significant part of combination antiretroviral therapy.

1.2 Mechanism of Action and Therapeutic Importance

Lamivudine can be administered and phosphorylated intracellularly to lamivudine triphosphate, the active metabolite. This is an active metabolite that works by inhibition of this enzyme HIV reverse transcriptase, which is involved in the conversion of viral RNA into DNA during the viral replication process. Lamivudine triphosphate competes with natural nucleosides and inserts into the viral DNA chain leading to the premature end of DNA elongation, thus preventing viral replication.

As a result of this process, lamivudine is one of the most common ingredients of HAART, in the treatment of HIV infection. The correct assessment of the lamivudine concentration in the pharmaceutical preparations is thus required to ascertain the quality of drugs, the effectiveness of therapy, and the regulations.

1.3 Need for Analytical Method Development

Several methods of analysis have been reported to determine lamivudine in drug dosage form such as HPLC, HPTLC, and UV spectrophotometry. The chromatographic methods are usually highly sensitive and specific but need costly instrumentation, multifaceted sample preparation methods, and laborious analysis.

Conversely, UV spectrophotometrics methods have a number of advantages including simplicity, low cost, speed of analysis and low volumes of solvents. Such attributes render spectrophotometric methods especially appropriate in quality control analysis regularly in pharmaceutical laboratories.

1.4. Research Objectives

The present study paper will design and establish a UV spectrophotometric procedure to determine lamivudine in pharmaceutical preparations in line with International Conference on Harmonisation (ICH) regulations. The aim of the study is as follows:

1. To come up with a bare and straightforward UV spectrophotometric technique of the quantification of lamivudine in pharmaceutical preparations.
2. To determine the analysis parameters of the developed method such as the wavelength to be used, calibration curve preparation, and the linearity range.
3. To test the developed analytical method against ICH guidelines by assessing the parameters, accuracy, precision, detecting limit, and quantifying limit, and robust to routine pharmaceutical analysis.

II. Review Of Literature

A variety of analytical methods had been established to the determination of lamivudine either as a separate or conjugated agent with other antiretroviral agents in pharmaceutical preparations and in biological samples. The main approach of these studies was to use chromatographic and spectrophotometric methods in order to obtain precise quantification and credible quality control.

Koppisetty et al. (2023) developed a reverse phase high-performance liquid chromatography (RP-HPLC) system with a Quality by Design (QbD) strategy in quantification of lamivudine and efavirenz in combined pharmaceutical preparations. The focus of their study was on systematic method development with the focus on the experimental design and optimization of the chromatographic conditions to have the reliable separation and accurate quantification of the drugs. The created method showed acceptable levels of analytical performance and emphasized the practicality of the QbD methodology in enhancing the high levels of robustness and reliability in the chromatographic methods.

Kumar et al. (2025) described an HPLC-based stability-indicating Analytical Quality by Design (AQbD) analysis of the three drugs lamivudine, tenofovir disoproxil fumarate, and efavirenz in plasma. The technique was created to assess the steadiness and quantitative estimation of these antiretroviral medications under different circumstances. Their work showed that the principles of AQbD could be applicable to the optimization of the method and provide guaranteed detection and quantification of the drugs in the complex biological samples**.**

Mohammed et al. (2023) developed a UV spectrophotometric assay of the dialysis of abacavir and lamivudine in pharmaceutical dosage forms. The research was aimed at developing an easy and affordable analytical method of regular pharmaceutical examination. The proposed spectrophotometric technique demonstrated good accuracy and precision and was deemed appropriate in the determination of these drugs in mixed formulations.

Raj et al. (2023) designed and tested a stability-indicating analytical procedure based on UV spectrophotometric techniques to determine ritonavir in bulk and pharmaceutical dosage forms. It was aimed at creating a simple and dependable analysis process that would help to trace the drug as well in the context of degradation products. The devised technique proved to have good validation parameters including linearity, precision, accuracy, and robustness which showed that UV spectroscopic methods would be effective to use in regular analysis of pharmaceutical quality control.

Somkuwar et al. (2024) carried out the comparative analysis of UV spectroscopy, reverse phase high-performance liquid chromatography (RP-HPLC), and high-performance thin layer chromatography (HPTLC) in quantifying the antiviral drug lamivudine in tablet formulations. They evaluated the performance of these three methods in their study in terms of sensitivity, accuracy and reliability. The study found that despite the increased sensitivity and selectivity of chromatographic methods, the UV spectrophotometric methods offered a more convenient, fast and cheaper method of pharmaceutical analysis of lamivudine routinely.

III. Materials And Methods

The present study project entailed the construction and verification of a UV-Visible spectrophotometric assay of lamivudine in pharmaceutical preparations. In the analysis process, standard and sample solutions were prepared, analytical wavelength had to be determined, the calibration curve had to be prepared and the developed method had to be validated, in accordance with the ICH, recommendations. All the experiments were conducted under reliable and controlled laboratory conditions using properly calibrated instruments and analytical grade reagents to guarantee reliability and accuracy of the results.

3.1 Instruments

The spectrophotometric study was conducted by a double beam UV- Visible spectrophotometer (UV-3092) using UV Win software to measure absorbance. The determination of pH in the course of solution preparation was done using a digital pH meter. The weigh of the sample powder of the drug and tablets was done using an analytical balance.

3.2 Chemicals and Reagents

Chemicals and reagents in the study were of analytical grade. The analysis was done using the following reagents:

- Cerium (IV) ammonium sulphate (0.1%)
- Perchloric acid (4 M)
- Methanol
- Distilled water

An oxidizing agent was cerium (IV) ammonium sulphate solution and perchloric acid served as the necessary acidic medium in the reaction. Drug dissolution and extraction was done using methanol and dilution of solutions was carried out using distilled water.

3.3 Preparation of Standard Solution

Lamivudine was accurately measured as 10mg in a 100 mL volumetric flask. In order to prepare the standard stock solution a quantity of the drug was dissolved in methanol and the volume was brought to the mark with a distilled water. Based on this stock solution, pertinent dilution of the stock solution was done to develop working standard solutions within the range of 2-18 ug/mL

3.4 Sample Preparation

To prepare the sample solution, twenty lamivudine tablets were weighed correctly and crushed to fine powder using mortar of pestle. One-tenth of the powdered tablet with lamivudine equal to 10 mg was moved into a volumetric flask. Methanol was added to the drug and thoroughly mixed with it before the solution was filtered to give a solution to take off the insoluble excipients. Distilled water was added to the filtrate to have a final solution of 10 µg/mL, which was analyzed.

3.5 Determination of Analytical Wavelength

The lamivudine solution was prepared, and the solution was scanned with the help of UVvis spectrophotometer with the purpose to identify the longest wavelength of maximum absorption. Lamivudine had its maximum absorbance (λmax) at 280 nm, and that was used as the wavelength of the quantitative analysis.

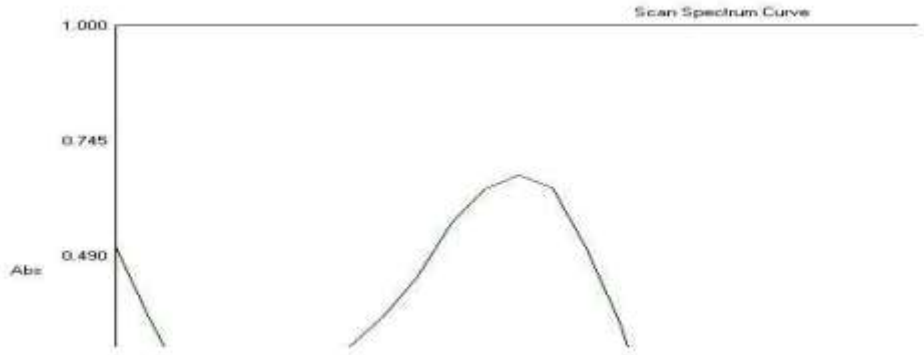


Figure 1: UV–Visible absorption spectrum of lamivudine showing maximum absorbance (λmax) at 280 nm

The UV absorption spectrum revealed that the lamivudine had a clear maximum absorbance peak with a value of 280 nm. This wavelength was subsequently chosen as the wavelength of analysis to be used to further determine lamivudine quantitatively because it gives optimum sensitivity and accuracy in the spectrophotometric analysis.

3.6 Construction of Calibration Curve

Volumetric aliquots of the standard lamivudine solution with the insoluble concentration in the range of 2-18 ug/mL were transferred into a series of volumetric flasks. Each flask was added 1 mL of solution of perchloric acid of 4 M and cerium (IV) ammonium sulphate. The final volume was put in using distilled water and the solutions were left to react in 5 minutes.

Absorbance of individual solutions at 280 nm using the UV -Visible spectrophotometer. This was followed by a calibration curve that was plotted as absorbance vs. lamivudine concentration.

3.7 Method Validation

The spectrophotometric procedure that has resulted has been proven to be valid according to the International Conference on harmonisation (ICH) guidelines. The parameters of validation reflected included the linearity, accuracy, precision, detection limit, quantifying and the robustness in order to establish the reliability and the appropriateness of the method to complete the routine pharmaceutical analysis.

IV. Result And Discussion

The generated UV -Visible spectrophotometric procedure to determine lamivudine in pharmaceutical formulations was assessed with respect to a number of parameters of analysis in line with the ICH guidelines. The results of the determination of wavelength, the construction of a calibration curve and the validation studies including linearity, accuracy, precision, detection limit, quantification limit, robustness, and assay of tablet formulation were critically reviewed. These parameters were evaluated to identify the reliability, sensitivity, and applicability of the devised analytical method in monitoring the analytical quality of the pharmaceuticals under regular quality supervision of lamivudine.

4.1 Optical Characteristics of Lamivudine

In acidic medium lamivudine had the maximum absorbance at 280 nm. The devised spectrophotometric procedure followed the law of BeerLambert at the range of 2-18 μg/mL, implying that the relationship between concentration and absorbance was linear.

The tables below (Table 1) show the optical properties and regression coefficients of lamivudine after the UV spectrophotometric analysis. The table will give a summary of the analysis wavelength, the range of law concentration Beer, and regression parameters obtained by using the calibration curve. Moreover, the key validation parameters to be used, which include the LOD, LOQ, correlation coefficient, slope, and intercept values are also provided.

Table 1: Optical Characteristics and Regression Parameters

Parameter	Value
Color	Colorless
λmax (nm)	280
Beer's law range (μg/mL)	2–18
Limit of detection (μg/mL)	0.0033
Limit of quantitation (μg/mL)	0.10043
Correlation coefficient	0.999
Slope	0.048
Intercept	0.068

Table 1 has indicated that lamivudine had optimum absorbance at 280 nm that was chosen as an analytical wavelength during its quantitative analysis. The procedure adhered to the law of Beer in the concentration range of 2-18 ug/mL which means that there is a high degree of linearity between the concentration and the absorbance. The value of the correlation coefficient was high (0.999) to assure high linearity of the calibration curve. The sensitivity of the developed spectrophotometric method was observed in the LOD (0.0033 μg/mL) and LOQ (0.10043 μg/mL). Moreover, the slope and intercept values showed that there was a good proportionality of the concentration and absorbance.

4.2 Calibration Curve of Lamivudine

Figure 2 shows the calibration curve that has been developed on lamivudine in the developed UV spectrophotometric method. The plot of absorbance versus corresponding concentrations of lamivudine in the range of 218 2 ug/mL at an analytical wavelength of 280 nm was used to construct the curve. The regression

equation obtained off the calibration data is A = 0.048C + 0.068 where A is the absorbance and C is the concentration of lamivudine.

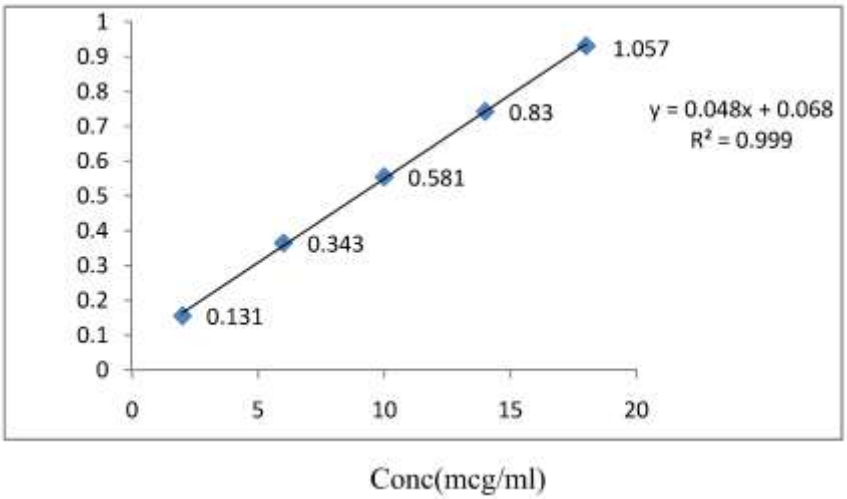


Figure 2: Calibration Curve of Lamivudine

The calibration curve showed the existence of a linear relationship between the absorbance and the concentration of lamivudine at the range studied. Regression equation A = 0.048C + 0.068 found that the proportionality between concentration and absorbance was good, which proved suitability of the Beer law. The linear nature of the calibration curve indicates the accuracy of the elaborated UV spectrophotometric technique of quantitative determination of lamivudine in pharmaceutical preparations.

4.3 Linearity

Table 2 indicates the absorbance values of the lamivudine through various concentrations applied to determine the linearity of the developed spectrophotometric method. The concentrations were 2-18 ug/mL and the values of the absorbance were obtained at 280nm.

Table 2: Linearity Data

Concentration (µg/mL)	Absorbance
2	0.131
6	0.343
10	0.581
14	0.830
18	1.057

Table 2 shows that the absorbance value rose in direct proportion to the concentration of lamivudine. This confirms the developed process obeys the law of BeerLambert in the range of 2-18 ug/mL of the concentration. The linearity and reliability of the suggested spectrophotometric procedure are also supported by the stable growth in the absorbance values.

4.4 Accuracy

Table 3 shows the outcomes of the recovery experiment that was conducted to test the validity of the designed spectrophotometric method. The recovery studies run with three different concentration levels namely low concentration, intermediate concentration and high concentration.

Table 3: Accuracy Data

Level	Concentration (µg/mL)	%Recovery
LC	2	99.70
IC	10	100.34
HC	18	100.09

The recovery values given in Table 3 were between 99.70% and 100.34% and this shows that the developed method has high accuracy. The findings can affirm that the technique can be used to reliably measure lamivudine with a minimal error in analysis**.**

4.5 Precision

Table 4 shows the results of precision study that was conducted to verify repeatability of the developed spectrophotometric method. Three days were used to determine the accuracy of the method by measuring the absorbance of different concentrations.

Table 4: Precision Data

Concentration (µg/mL)	Day 1	Day 2	Day 3
4	0.242	0.245	0.247
8	0.477	0.471	0.479
16	1.002	1.005	1.010

Table 4 shows that the obtained absorbance values on the various days were consistent which shows that there was good precision of the method of analysis. The fact that the standard deviation of the values of absorbance is low proves the repeatability and reliability of the designed spectrophotometric procedure.

4.6 Detection Limit and Quantitation Limit

Table 5 summarizes the summary of the limits of detection and quantification (DL and QL) of lamivudine using the developed method of UV spectrophotometry.

Table 5: DL and QL

Parameter	Value
Detection Limit (DL)	0.0033 µg/mL
Quantitation Limit (QL)	0.10043 µg/mL

Table 5 shows that the developed analytical method is sensitive enough to detect and quantify lamivudine at low concentrations because of the low values of DL and QL.

4.7 Robustness

Table 6 shows the findings of the robustness experiment carried out to test the integrity of the devised spectrophotometric method in the face of minimal changes in the conditions of analysis.

Table 6: Robustness Study

Concentration (µg/mL)	Mean Absorbance	%Recovery
4	0.245	101.24
8	0.476	99.79
16	1.006	100.40

Table 6 shows that minor changes in the experimental conditions did not have a significant impact on the analytical findings. The values of percentage recovery were very near to 100, which is a sign to suggest that the developed method is strong and stable.

4.8 Assay of Tablet Formulation

Table 7 indicates the assay outcomes of the lamivudine in a commercial tablet preparation through the developed UV spectrophotometric method.

Table 7: Analysis of Lamivudine Tablet

Brand	Drug Added (µg/mL)	Drug Found (µg/mL)	%Recovery
Lamivir-HBV	10	9.998	99.98

Table 7 indicates that the assay outcomes indicate that the designed spectrophotometric assay was effective in the quantification of lamivudine in the tablet formulation. The recovery of 99.98% means that it is very accurate and can be able to state that the method is not interfered with by tablet excipients.

V. Conclusion And Recommendations

The present study was able to design and establish an UV-Visible spectrophotometric technique to determine lamivudine in pharmaceutical preparations in the framework of ICH guidelines. The procedure exhibited peak absorbance at 280 nm and obeyed BeerLamberts law at 2-18 ug/mL concentration with 0.999 correlation coefficient. The validation parameters that were used to prove the reliability of the method include accuracy, precision, detection limit, quantification limit and robustness. There were also high recovery values (between 99.70% and 100.34%), and the commercial tablet formulation assay produced a recovery of 99.98%.

The results reveal that the method formulated is convenient, correct, and precise and suitable in the standard analysis of quality control of lamivudine in drug formulations.

- The constructed UV spectrophotometric method is applicable in the routine quality-control analysis of lamivudine in pharmaceutical preparations.
- Further studies can develop the technique of simultaneous determination of lamivudine and other antiretroviral medications in combination preparations.
- The procedure can also be used in the upcoming research to study the stability and degradation of lamivudine.

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