IMPURITY ANALYSIS OF A PSYCHOACTIVE DRUG BY HPLC-DAD DETECTOR

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DISSERTATION

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ABSTRACT

IMPURITY ANALYSIS OF A PSYCHOACTIVE DRUG BY HPLC-DAD DETECTOR

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In this research, an HPLC-DAD technique was validated for the identification of impurities A and F in lamotrigine, a psychoactive drug. The study includes a comparative analysis of three pharmaceutical samples obtained from different countries. A C18 Phenomenex column with a dimension of 4.6 mm × 250 mm and a 4 μm particle size was utilized in the method. The gradient elution was used with mobile phase A consisting of monobasic potassium phosphate buffer (pH 3.5) and mobile phase B containing acetonitrile, at a flow rate of 1.0 mL/min and a column temperature of 40°C. Impurities were detected using diode array detection (DAD) at 215 nm. The injection volume for the HPLC-DAD analysis was 10 μL. The method was validated with precision, accuracy, linearity, limit of detection (LOD), limit of quantification (LOQ), specifity, robustness and stability. The method exhibited good linearities over the studied concentration ranges (r^2> 0.999). The limit of detection (LOD) and limit of quantification (LOQ) for Impurity-A were determined as 0.030 μg/mL and 0.09 μg/mL, respectively. For Impurity-F, these values were found to be 0.019 μg/mL and 0.058 μg/mL, respectively. The method demonstrated high selectivity and stability, making it ideal for determining impurities A and F in lamotrigine drug formulations. The results are reliable, thereby contributing to quality control in the pharmaceutical industry for determining related compounds in commercial lamotrigine tablets. Modern analytical methods, such as HPLC-DAD, are essential to safety, effectiveness, and regulatory compliance.

Keywords: Lamotrigine, Drug impurity, High-Performance Liquid Chromatography, Validation, Psychoactivedrug.

ÖZET

PSİKOAKTİF BİR İLACIN HPLC-DAD DEDEKTÖRÜ İLE SAFSIZLIK ANALİZİ

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Kimya Anabilim Dalı

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Bu araştırmada, psikoaktif bir ilaç olan lamotriginde bulunan A ve F safsızlıklarının tespiti için bir HPLC-DAD yöntemi valide edilmiştir. Çalışma, farklı ülkelerden elde edilen üç farmasötik örneğin karşılaştırmalı analizini içermektedir. Yöntemde 4,6 mm × 250 mm boyutlarında ve 4 µm parçacık boyutunda bir C18 Phenomenex kolonu kullanılmıştır. Monobazik potasyum fosfat tamponu (pH 3.5) içeren mobil faz A ve asetonitril içeren mobil faz B ile 1,0 mL/dk akış hızı ve 40°C kolon sıcaklığı ile gradiyent elüsyon kullanılmıştır. Safsızlıklar, 215 nm'de diyot dizi dedektörü (DAD) kullanılarak tespit edilmiştir. HPLC-DAD analizi için enjeksiyon hacmi 10 µL'dir. Yöntemin, hassasiyet, doğruluk, doğrusallık, gözlenebilme sınırı (LOD), tayin sınırı (LOQ), spesifiklik, sağlamlık ve kararlılık açısından doğrulandı. Yöntem, incelenen konsantrasyon aralıklarında iyi bir doğrusallık göstermiştir (r^2> 0.999). Gözlenebilme sınırı (LOD) ve tayin sınırı (LOQ) Impurity-A için sırasıyla 0.030 µg/mL ve 0.09 µg/mL olarak belirlenmiştir. Impurity-F için ise bu değerler sırasıyla 0.019 µg/mL ve 0.058 µg/mL olarak bulunmuştur. Yöntemin yüksek seçicilik, stabilite göstermesi lamotrijinin ilaç formülasyonlarındaki A ve F safsızlıklarının belirlenmesi için ideal kılmaktadır. Sonuçlar güvenilir olup, ticari lamotrigin tabletlerinde ilgili bileşenlerin belirlenmesi için farmasötik endüstrisinde kalite kontrolüne katkıda bulunmaktadır. HPLC-DAD gibi modern analitik yöntemler güvenlik, etkinlik ve mevzuata uygunluk açısından gereklidir.

Anahtar Sözcükler: Lamotrigin, İlaç safsızlığı, Yüksek Performanslı Kromatografisi, Validasyon, Psikoaktif ilaç.

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MOHAMMED ALI SALEH AHMAD ALSAEEDY

17/01/2024

STATEMENT OF COMPLIANCE WITH ETHICAL PRINCIPLES AND RULES

I hereby truthfully declare that this thesis is an original work prepared by me; that I have behaved in accordance with the scientific ethical principles and rules throughout the stages of preparation, data collection, analysis and presentation of my work; that I have cited the sources of all the data and information that could be obtained within the scope of this study, and included these sources in the references section; and that this study has been scanned for plagiarism with “scientific plagiarism detection program” used by Eskisehir Technical University, and that “it does not have any plagiarism” whatsoever. I also declare that, if a case contrary to my declaration is detected in my work at any time, I hereby express my consent to all the ethical and legal consequences that are involved.

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GLOSSARY OF SYMBOLS AND ABBREVIATIONS

AEDs:	:	Antiepileptic drugs
ADHD	:	Attention deficit hyperactivity disorder
α	:	Column Selectivity
CE	:	Capillary electrophoresis
CV	:	Coefficient of variation
DAD	:	Photodiode-array detection
EMA	:	European Medicines Agency
IMP	:	Impurity
FDA	:	Food and Drug Administration
GABA	:	Gamma-aminobutyric acid
GC	:	Gas chromatography
HPLC	:	High Performance Liquid Chromatography
ICH	:	International Council for Harmonisation
IR	:	Infrared spectroscopy
IMP	:	Impurity
IMP A	:	Impurity A
IMP F	:	Impurity F
K	:	Capacity Factor
LAM	:	Lamotrigine
LC	:	Liquid chromatography
LOD	:	Limit of detection
LOQ	:	Limit of Quantitation
LSD	:	Relative standard deviation
IMP F	:	Impurity F
IMP A	:	Impurity A
RSD	:	Relative standard deviation.
MS	:	Mass spectrometry
N	:	Efficiency Factor
SE	:	Standard error
SD	:	Standard deviation
R	:	Recovery
RSD		Relative standard deviation
SD		Standard deviation

1. INTRODUCTION

Impurity profiling and control is a highly regulated area in the pharmaceutical industry, as outlined in the ICH Q3A guidelines on "Impurities in the New Drug Substance." According to these guidelines, an impurity is any component of a medicinal product that is not the active ingredient, or an excipient listed in the product's formulation. Impurities are often chemical substances that are similar to the drug substance but lack pharmacological activity. They may be formed during the degradation of the drug substance and are therefore often referred to as "related substances," "related components," or "related impurities." It is important to identify and control impurities in order to ensure the quality and safety of the drug product (EMEA, 2006).

Impurities in drugs can have negative pharmacological or toxicological effects that may outweigh any potential benefits of the drug. As a result, it is important to fully characterize the impurities present in drugs intended for human use. However, there is limited information available in the literature about separation techniques such as high-performance liquid chromatography (HPLC) that can be used to analyze impurities in drugs (Nageswara Rao and Kumar Talluri, 2007). This project aims to use HPLC techniques to analyze impurities in a psychoactive drug, with the hope that the results will help to explore or open up new areas of research on analyzing drugs and the substances that contribute to the side effects of these drugs for remediation purposes (Venkatesan and Valliappan, 2014).

1.1. Importance of impurity evaluation in pharmaceutical industry

In the pharmaceutical industry, it is important to ensure that the active ingredients in a drug are pure and free from impurities. Impurities can be any substances that are present in the drug that are not the active ingredient. These impurities can be present in the raw materials used to make the drug, or they can be introduced during the manufacturing process. There are several reasons why impurity evaluation is important in the pharmaceutical industry:

Safety: Impurities can potentially be toxic or harmful to the patient. Evaluating and controlling the level of impurities in a drug helps to ensure that it is safe for use.

Efficacy: Impurities can interfere with the activity of the active ingredient and potentially reduce the effectiveness of the drug.

***Quality*:** Impurities can affect the physical and chemical properties of a drug, which can affect its stability and shelf life.

***Regulatory compliance*:** The level of impurities in a drug is regulated by government agencies such as the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA). Pharmaceutical companies are required to demonstrate that their drugs meet the specified impurity limits in order to obtain regulatory approval. To evaluate the level of impurities in a drug, pharmaceutical companies use various analytical techniques such as spectroscopy, chromatography, and mass spectrometry. These techniques allow them to identify and quantify the impurities present in the drug. By evaluating the impurities in a drug, pharmaceutical companies can ensure that their products meet the required quality standards and are safe and effective for use.

Chromatography is a widely used analytical technique in the pharmaceutical industry for the separation, identification, and quantification of impurities in a drug. Chromatography works by separating a mixture of compounds based on their physical and chemical properties, such as size, charge, and affinity for a stationary phase or mobile phase. There are several different types of chromatography, including liquid chromatography (LC), gas chromatography (GC), and thin-layer chromatography (TLC). Each type of chromatography is suited to different types of samples and impurities, and different techniques can be used in combination to fully characterize a sample.

In the pharmaceutical industry, chromatography is often used to:

Identify impurities: By separating the compounds in a sample, chromatography can help to identify the specific impurities present. This is done by comparing the retention time of the impurities to a reference standard or by analyzing the separated compounds using a detector such as a mass spectrometer.

Quantify impurities: Chromatography can also be used to determine the concentration of impurities in a sample. This is done by comparing the peak area or peak height of the impurities to a standard curve. Verify the purity of a drug: Chromatography can be used to confirm that a drug meets the specified purity limits. This is done by separating and quantifying all of the impurities present in the drug (Ramachandra, 2017).

1.2. Sources of impurities in pharmaceutical products

There are several sources of impurities that can be present in pharmaceutical substances. These impurities can come from a variety of sources, including raw materials, manufacturing processes, and storage and handling.

Raw materials: Impurities can be present in the raw materials used to make a pharmaceutical substance. These impurities can be intrinsic to the raw material, such as contaminants or impurities present in the natural source of the material. Impurities can also be introduced during the processing of the raw material, such as through the use of solvents or other chemicals.

Manufacturing processes: Impurities can also be introduced during the manufacturing process of a pharmaceutical substance. For example, impurities can be introduced through the use of solvents, catalysts, or other chemicals used in the synthesis or purification of the substance. Impurities can also be introduced through contamination by other substances, such as equipment or materials used in the manufacturing process.

Storage and handling: Impurities can be introduced during storage and handling of a pharmaceutical substance. For example, impurities can be introduced through contact with contaminants or through exposure to light or heat. Impurities can also be introduced through improper handling or storage, such as through contamination by other substances or through exposure to moisture or oxygen.

Packaging materials: Impurities can be introduced through the use of packaging materials that are not adequately cleaned or that contain contaminants.

***Environmental factors*:** Impurities can be introduced through exposure to environmental factors such as dust, humidity, and temperature fluctuations.

***Degradation: Impurities*:** can be formed through the degradation of the active ingredient or other components of the drug. Degradation can occur due to exposure to light, heat, or moisture, or due to the action of enzymes or other chemical reactions.

***Microbial contamination*:** Impurities can be introduced through microbial contamination, such as the growth of bacteria, fungi, or other microorganisms. This can occur during the manufacturing process or during storage and handling.

It is important to carefully control and monitor these sources of impurities in order to ensure the purity and quality of pharmaceutical substances. This may involve using clean and well-maintained equipment, properly storing and handling materials, and using

appropriate packaging materials. Regular testing and quality control measures can help to identify and eliminate impurities in pharmaceutical substances.

1.3. Types of impurities

There are several types of impurities that can be present in pharmaceutical substances. These impurities can be classified based on their source, chemical structure, or effect on the drug (Blessy et al., 2014).

- Process impurities: Process impurities are impurities that are introduced during the manufacturing process of a drug. These impurities can be byproducts of the synthesis or purification of the drug, or they can be contaminants introduced during the manufacturing process.
- Degradants: Degradants are impurities that are formed through the degradation of the active ingredient or other components of the drug. Degradants can be formed through exposure to light, heat, or moisture, or through the action of enzymes or other chemical reactions.
- Inactive excipients: Inactive excipients are non-active ingredients that are present in a drug formulation to improve its stability, appearance, or other properties. While these substances are not the active ingredient, they can still be considered impurities if they are present in excess or at levels that could potentially be harmful to the patient.
- Contaminants: Contaminants are impurities that are introduced during the manufacturing process or storage and handling of a drug. These impurities can come from a variety of sources, such as equipment, materials, or the environment.
- Residual solvents: Residual solvents are impurities that are present in a drug as a result of the use of solvents during the manufacturing process. These solvents can be toxic or harmful to the patient if present at high levels, and their presence must be carefully controlled.
- Heavy metals: Heavy metals are impurities that can be present in a drug as a result of contamination during the manufacturing process or storage and handling. Heavy metals such as mercury, lead, and arsenic can be toxic to the patient if present at high levels.
- Microbial contaminants: Microbial contaminants are impurities that result from the growth of microorganisms such as bacteria, fungi, or viruses. These impurities

can be introduced during the manufacturing process or during storage and handling of the drug.

- Pesticides and other environmental contaminants: Pesticides and other environmental contaminants can be present in a drug as a result of contamination during the manufacturing process or storage and handling. These impurities can potentially be harmful to the patient if present at high levels.
- Genetic impurities: Genetic impurities are impurities that result from the presence of genetic material, such as DNA or RNA, in a drug. These impurities can be introduced during the manufacturing process or during storage and handling of the drug.
- Radioactive impurities: Radioactive impurities are impurities that are present in a drug as a result of contamination with radioactive substances. These impurities can be introduced during the manufacturing process or during storage and handling of the drug.
- Allergens: Allergens are impurities that can cause an allergic reaction in some individuals. These impurities can be introduced during the manufacturing process or during storage and handling of the drug.
- Genotoxic impurities: Genotoxic impurities are impurities that have the potential to damage genetic material, such as DNA, and may cause cancer or other harmful effects.

These impurities can be introduced during the manufacturing process or during storage and handling of the drug.

It is important to carefully control and monitor all types of impurities in order to ensure the purity and quality of pharmaceutical substances. This may involve using clean and well-maintained equipment, properly storing and handling materials, and using appropriate packaging materials. Regular testing and quality control measures can help to identify and eliminate impurities in pharmaceutical substances.

1.4 Control of impurities in drugs

There are several steps that can be taken to control and reduce the level of impurities in drugs. These steps can be taken during the manufacturing process, storage, and handling of the drug. Here are a few examples (Ahuja, 2007) (Nath , Sharma, 2018):

Use of high-quality raw materials: One way to control impurities in drugs is to use high-quality raw materials that are free from contaminants and impurities. This can be achieved through careful sourcing and processing of the raw materials.

Use of clean equipment and facilities: Using clean and well-maintained equipment and facilities can help to reduce the risk of contamination and the introduction of impurities during the manufacturing process.

Proper handling and storage: Proper handling and storage of materials and finished products can help to reduce the risk of contamination and the introduction of impurities. This may involve using appropriate packaging materials and storing materials and products under controlled conditions such as temperature and humidity.

Use of appropriate manufacturing processes: Using appropriate manufacturing processes, such as purification techniques, can help to remove impurities from the drug.

Regular testing and quality control: Regular testing and quality control measures can help to identify and eliminate impurities in drugs. This may involve using analytical techniques such as chromatography or spectroscopy to identify and quantify impurities, and taking corrective action if impurities are found at unacceptable levels.

Overall, controlling and reducing the level of impurities in drugs is important in order to ensure their safety, efficacy, and quality. By carefully controlling the sources of impurities and implementing appropriate control measures, it is possible to produce high-quality pharmaceutical products that meet the required purity standards.

Table 1.1. *General table of criteria used to determine the proportion of impurities*

Level	Style
Impurity source	The amount of impurities present in the sample can be measured by various methods such as spectroscopy, chromatography, or mass spectrometry.
Impurity type	The type of impurities present in the sample can affect its quality and potential toxicity. Identification and characterization of impurities is important.
Impurity source	Impurities may come from various sources such as starting materials, reaction conditions, or contamination during purification. Understanding the source can help prevent future contamination.
Impurity concentration	The concentration of impurities can affect the potency and safety of the sample. Maximum acceptable levels of impurities may be set by regulatory agencies or industry standards.
Potential toxicity	Impurities may have toxic effects, particularly if present at high levels. Evaluating the potential toxicity of impurities is important for safety and risk assessment.

Note: The criteria listed above may vary depending on the specific substance, application, and regulatory requirements.

Table 1.2. *Criteria used to determine the proportion of impurities and degradation; based on the amount of dose taken (Strege, 1999) (EMEA, 2006)*

Maximum daily dose	The threshold for detection of IMP	Highest everyday dose	The threshold for of DPs in drugs
< 2 g	At 0.10%, or 0.9 mg daily, while one is less	Greater than 1 mg	At 1.0%, or 5 mg daily consumption,
> 2 g	0.05%	1 to 10 mg	At 0.5%, or 20 mg daily consumption,
		less 10 mg to 2 g	At 0.2%, or 2 mg daily consumption,
		At less 2 g	At 0.10%.

1.4. Control of impurities in drugs

The nomenclature, or system of naming, used for mentioning impurities in a drug sample depends on the type of impurity and the context in which it is being discussed (Strege, 1999) (Niazi, 2009). There are several different ways that impurities in drugs can be mentioned in chemical nomenclature, including:

By their common or trivial names: Impurities are often referred to by their common or trivial names, which are the names that are most commonly used to refer to the impurity. For example, iron oxide impurities in a sample of a drug might be referred to as "iron oxide impurities" or simply "iron oxide."

By their chemical formulas: Impurities can also be mentioned by their chemical formulas, which represent the types and numbers of atoms present in the impurity. For example, iron oxide impurities in a sample of a drug might be referred to as FeO impurities.

By their elemental symbols: Impurities can also be mentioned by their elemental symbols, which represent the types of atoms present in the impurity. For example, iron oxide impurities in a sample of a drug might be referred to as FeO impurities.

By their CAS number: The Chemical Abstracts Service (CAS) assigns a unique numerical identifier, known as a CAS number, to each chemical substance. Impurities in drugs can be mentioned by their CAS number in order to uniquely identify them.

Various pharmacopoeias utilize different nomenclature to describe the impurities or related compounds that are found in drug reference criteria(S Singh et al., 2012). The forms that were utilized are listed in Table 1.3. It's crucial to emphasize that the wording used in each case concludes with "(USP/Ph) (Int./IP)," "CRS (Eur./BP Ph.)," or just "CRS," which stands for "chemical reference substance." Additionally, in journals like as "Ph. Eur.," "BP," or "Ph. Int.," the final letter "RS" following a research article designates "reference standard." It is noteworthy that the "IMPs" referenced at the end of a modern monograph are typically ignored and have different numbers. As a result, the pharmacy corporation only offers "RS" and "CRS." (Nath , Sharma, 2018).

Table 1.3. *Different terminology is used to refer to impurities and related substances in the analysis of drug quality in various systems.*

Level	Style
USP	USP Based (A, B and C)
Ph. Eur.	This drug is categorized as impurity types A, B, and C.
BP	The type of drug is referred to as impurity (A, B, and C), CRS
Ph.	This drug is categorized as impurity types, (A, B, and C)
IP	The drug type is identified as impurity, labeled as (A, B, and C…) RS.

1.5. Determine the drug impurities

Determining the impurities present in a drug sample is an important step in the quality control and analysis of drugs. Impurities can be introduced into a drug sample during the manufacturing process or during storage and handling, and it is important to accurately identify and quantify these impurities in order to ensure the safety and effectiveness of the drug (Strege, 1999).

There are several methods that can be used to determine the impurities present in a drug sample, including (Niazi, 2009) (Singh et al., 2012):

- Chromatography is a technique that separates components in a mixture based on their physical and chemical properties. Chromatographic methods, such as gas chromatography and liquid chromatography, can be used to separate and identify the impurities present in a drug sample.
- Spectroscopy: Spectroscopy is a technique that involves the interaction of electromagnetic radiation with matter. Different spectroscopic methods, such as infrared spectroscopy, ultraviolet-visible spectroscopy, and nuclear magnetic resonance spectroscopy, can be used to identify the impurities present in a drug sample.
- Mass spectrometry: Mass spectrometry is a technique that measures the mass-to-charge ratio of ions. Mass spectrometry can be used to identify and quantify the impurities present in a drug sample by measuring the mass of the impurity ions and comparing them to known standards.
- Microscopy: Microscopy techniques, such as optical microscopy and electron microscopy, can be used to observe the physical characteristics of impurities present in a drug sample.

There are several other factors to consider when determining the impurities present in a drug sample (Jadhav, 2014) (Dong, 2007) (Holm, Elder, 2016):

Sampling: It is important to take a representative sample of the drug in order to accurately determine the impurities present. Sampling techniques, such as random sampling and stratified sampling, can be used to ensure that the sample accurately reflects the composition of the entire batch of drug.

Detection limits: The detection limits of the analytical method used to determine the impurities in the drug sample should be carefully considered. Detection limits refer to

the lowest concentration of an impurity that can be accurately detected and quantified using the analytical method.

Impurity limits: Impurity limits are established by regulatory agencies and specify the maximum allowable concentration of impurities in a drug sample. The impurity limits for a drug will depend on the specific drug and the intended use of the drug.

Documentation: It is important to accurately document the methods used to determine the impurities present in the drug sample, as well as the results obtained. This documentation is important for quality control purposes and for regulatory compliance.
In general, determining the impurities present in a drug sample is a complex process that requires careful consideration of the analytical methods used, the sampling and detection limits, and the regulatory requirements for impurity limits.

1.6. Steps involved in drug impurity identification.

Sample preparation: The first step in a drug impurity identification scheme is to prepare the sample for analysis. This may involve homogenizing the sample, extracting the active ingredient or desired compound, and separating the impurities from the active ingredient or desired compound (Venkatesan et al., 2014). Impurity separation: Once the sample has been prepared, the impurities must be separated from the active ingredient or desired compound. This may be done using chromatographic techniques, such as gas chromatography or liquid chromatography. *Impurity identification*: Once the impurities have been separated from the active ingredient or desired compound, the impurities must be identified (Ingale et al., 2011). This may be done using spectroscopic techniques, such as infrared spectroscopy or mass spectrometry, or by comparing the impurities to known standards. Impurity quantification: Once the impurities have been identified, they must be quantified to determine the concentration of each impurity in the sample. This may be done using analytical techniques, such as gravimetry or titration. Documentation: It is important to accurately document the steps taken in the drug impurity identification scheme, as well as the results obtained. This documentation is important for quality control purposes and for regulatory compliance, as shown in Fig 1.1.

1.7. Regulatory guidelines for controlling impurities of drugs

The ICH guidelines provide a globally harmonized approach to the development, registration, and post-approval of pharmaceuticals. These guidelines specify the

acceptable limits for impurities such as residual solvents, degradation products, and contaminants. The guidelines also provide guidance on the characterization and control of impurities in the drug substance and drug product (Sudhakar Babu et al., 2012) (Nageswara Rao, Kumar Talluri, 2007).

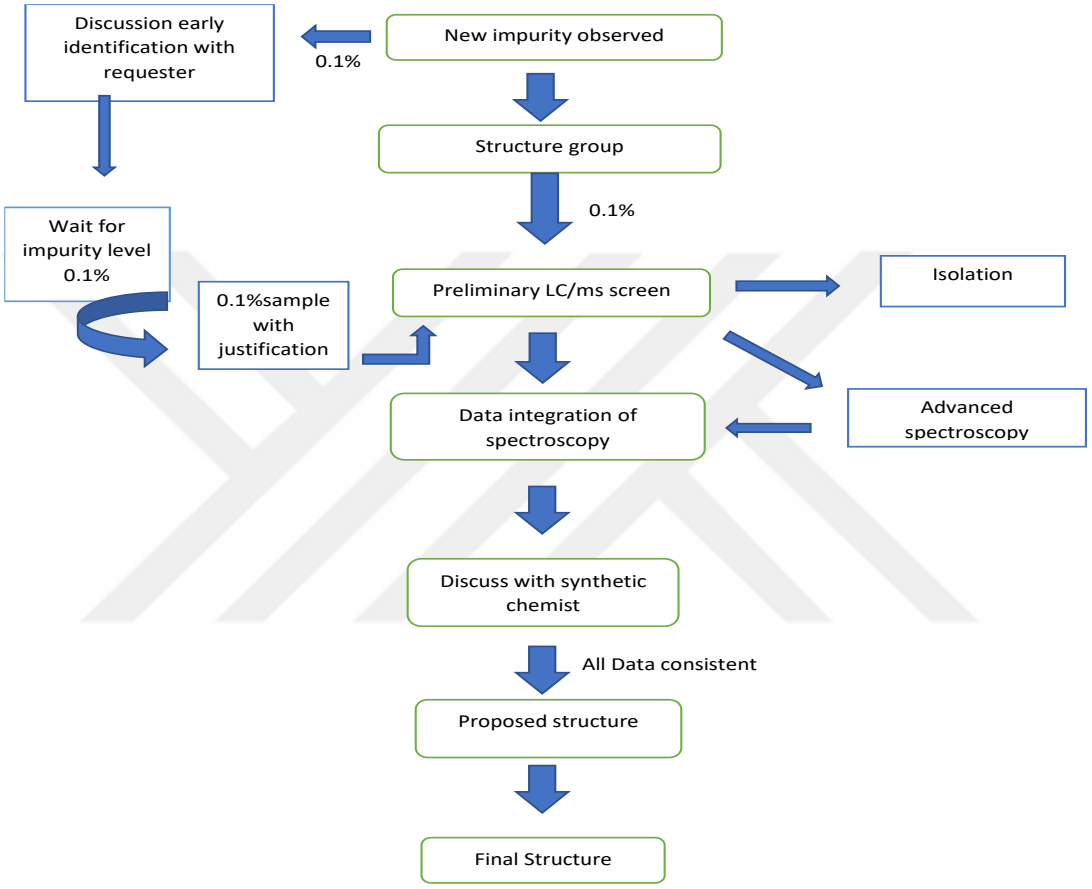

Figure 1.1. Compendial Terminology

The FDA's guidelines for impurities in drugs aim to protect public health by ensuring that pharmaceutical products are safe, effective, and of high quality. These guidelines establish acceptable levels of residual solvents, heavy metals, and other contaminants, and require characterization and control of impurities in the drug substance and product.

The EMA's guidelines for impurities in drugs also aim to ensure that pharmaceutical products are safe and of high quality for patient use. These guidelines provide guidance

on the acceptable levels of impurities, as well as on the characterization and control of impurities in drug substance and product (Eriksson, 2018).

U.S. Food and Drug Administration (FDA) guidelines: The FDA has established guidelines for impurities in drugs, including the acceptable levels of residual solvents, heavy metals, and other contaminants. The FDA also requires the characterization and control of impurities in the drug substance and drug product.

1.8. Challenges in impurity profiling

Impurity profiling can be a complex process due to several challenges. One of the main challenges is the complexity of impurities themselves. Impurities can originate from multiple sources and can be present in different forms, making it difficult to analyze and characterize them. Additionally, some impurities may be present at very low levels, making it challenging to detect and quantify them accurately using analytical methods. Interference from other substances or components can also affect the accuracy of impurity profiling results.

Another challenge in impurity profiling is the lack of standardization in methods and techniques used for detection, quantification, and characterization. This can result in variability in results between different laboratories or testing methods.

Sample preparation is also an important factor in impurity profiling, as errors during this step can lead to inaccurate results. Impurity profiling can also be time-consuming and expensive, particularly for complex samples, and there may be limited resources available for analysis (Vol et al., 2010) (Olsen, Baertschi, 2004).

Finally, compliance with regulatory requirements for impurities can be challenging, as standards and guidelines can change frequently. This requires continuous monitoring and updating of methods and procedures to ensure compliance.

1.9. Psychoactive drugs

A psychoactive drug is a substance that alters brain function and results in changes in perception, mood, consciousness, cognition, or behavior. These drugs can be used medically, recreationally, or for spiritual reasons. They may be prescribed by physicians for therapeutic purposes, such as treating neuropsychiatric disorders, pain, or Parkinson's disease. Some psychoactive substances may be used in detoxification and rehabilitation

programs for individuals who have become dependent or addicted to other mind-altering substances (Bialer, 2012) (Di Trana et al., 2020).

They have been used for centuries for both medicinal and recreational purposes. Psychoactive drugs can be classified into several groups based on their chemical structure and mechanism of action.

While psychoactive drugs can bring about changes in consciousness and mood that the user finds rewarding, prolonged use can lead to addiction and physical or psychological dependence. This can result in negative consequences, such as compulsive drug use, withdrawal symptoms, and adverse effects on physical and mental health.

1.9.1. Classes of psychoactive drug

Antiepileptic drugs AEDs, antidepressants, antipsychotics, and mood stabilizers are classes of psychoactive drugs used to treat specific neuropsychiatric conditions, such as epilepsy, depression, psychosis, and bipolar disorder, respectively.

Stimulants, which include drugs like Ritalin (Methylphenidate) and Adderall (Amphetamine), can also be used to treat neuropsychiatric disorders, such as attention deficit hyperactivity disorder (ADHD). However, these drugs can also cause serious side effects, such as heart problems, anxiety, and addiction, and require close monitoring by healthcare providers (Graziano et al., 2019) (Taschwer et al., 2017).

It's important to note that each psychoactive drug class can have different effects, risks, and benefits.

1.9.2. Antiepileptic drugs

Antiepileptic drugs are a group of drugs that are used to treat epilepsy and other seizure disorders. They work by reducing the frequency of seizures by modulating the activity of neurons in the brain. Seizures are caused by excessive and synchronized electrical activity in the brain, and AEDs work by altering the balance between inhibitory and excitatory neurotransmitters in the brain to prevent seizures (Khoschsorur et al., 2001).

AEDs can work by enhancing inhibitory neurotransmission, for example, by potentiating the effect of the neurotransmitter GABA *Glutamate modulators* at GABA receptors. GABA is an inhibitory neurotransmitter that helps to regulate the excitability of neurons in the brain. By enhancing GABA's effect, AEDs can help to reduce seizure

activity. Alternatively, AEDs can also work by inhibiting excitatory processes in the brain, for example, by inhibiting ligand-gated glutamate receptors or blocking voltage-gated sodium channels. Glutamate is an excitatory neurotransmitter that helps to regulate the excitability of neurons in the brain. By blocking glutamate receptors or inhibiting the flow of sodium ions into neurons, AEDs can reduce the excitability of neurons and prevent seizures (Budakova et al., 2008).

AEDs are a structurally diverse group of drugs, meaning that they come in different chemical structures. However, they all share the common goal of reducing seizure frequency, and each AED can have unique mechanisms of action and side effects. The choice of AED depends on the individual's seizure type, severity, and other factors. It's important to work with a healthcare provider to determine the best AED for an individual's specific needs and health history. It's important to note that not all AEDs work the same way for all individuals, and the choice of AED depends on many factors, such as the type and severity of seizures, age, medical history, and other medications being taken. In addition, while AEDs can effectively control seizures in many individuals, they may not cure epilepsy. AEDs are usually taken on a daily basis and may need to be taken for an extended period of time, sometimes for the rest of an individual's life (Khoschsorur et al., 2001).

1.9.2.1. Classification antiepileptic drugs (AEDs)

Antiepileptic drugs (AEDs) can be classified into several categories based on their mechanism of action (Di Trana et al., 2020):

Sodium channel blockers: These drugs block the activity of sodium channels, which are involved in the initiation and spread of seizures. Examples include carbamazepine and lamotrigine.

Gamma-aminobutyric acid (GABA) modulators: These drugs enhance the activity of GABA, an inhibitory neurotransmitter, to reduce seizure activity. Examples include valproate and gabapentin.

Glutamate modulators: These drugs target the activity of glutamate, an excitatory neurotransmitter, to reduce seizure activity. Examples include topiramate and felbamate.

Calcium channel blockers: These drugs block the activity of calcium channels, which are involved in the generation and spread of seizures. Examples include ethosuximide and valproate.

Other mechanisms: Some AEDs work by other mechanisms, such as inhibiting the metabolism of neurotransmitters or reducing oxidative stress. Examples include levetiracetam and zonisamide. It's worth noting that some AEDs may belong to more than one category, and some AEDs may have multiple mechanisms of action. Additionally, some AEDs may be used off-label for the treatment of other conditions, such as migraine headaches or bipolar disorder.

1.10. History of chromatography and HPLC

Chromatography is a technique for separating and analyzing mixtures of chemical compounds. The history of chromatography can be traced back to the early 20th century, when scientists first began experimenting with various methods for separating and analyzing mixtures of chemical compounds.

One of the earliest forms of chromatography was developed in 1906 by a Russian botanist named Mikhail Tswett. Tswett used a technique called column chromatography, which involved passing a mixture of chemical compounds through a column filled with a solid adsorbent material. The different components of the mixture would interact differently with the adsorbent material, causing them to separate and travel at different rates through the column (Touchstone, 1993).

In the following decades, scientists developed a variety of different chromatography techniques, such as paper chromatography, gas chromatography, and ion exchange chromatography. However, it was not until the development of high-performance liquid chromatography (HPLC) in the 1960s that chromatography truly came into its own as a powerful and versatile analytical technique.

HPLC is a form of liquid chromatography that uses high pressure to pump a liquid mobile phase through a column packed with a solid stationary phase. The components of the mixture being analyzed interact differently with the stationary phase, causing them to separate and travel at different rates through the column. HPLC is now widely used in a wide range of fields, including biomedical research, environmental science, and the pharmaceutical industry (Lapeyre-Mestre, 2016).

1.10.1. Characteristics of the chromatographic process

The chromatographic process has several characteristics that determine its efficiency and effectiveness in separating and purifying compounds. These characteristics

include the optimization of a chromatogram often involves adjusting parameters such as Resolution, capacity factor, efficiency factor, and column selectivity (Ovliv et al., 2013).

A. Resolution

Resolution in chromatography refers to the ability of the chromatographic process to separate two or more closely related compounds in a mixture. It is a measure of the separation between two peaks of different compounds, and is an important characteristic of the chromatographic process, as it determines the ability of the method to distinguish between similar compounds in a mixture.

The resolution of a chromatographic process is determined by a combination of factors, including the selectivity of the separation, the efficiency of the column, and the peak shape of the compounds. The selectivity of the separation is determined by the properties of the stationary phase and mobile phase, as well as the conditions of the separation. The efficiency of the column is determined by the number of theoretical plates that the column can generate per unit length. The peak shape of the compounds is determined by the properties of the compounds and the conditions of the separation.

The resolution of a chromatographic process can be improved by using a more selective stationary phase or mobile phase, by using a column with a higher efficiency, or by optimizing the conditions of the separation to improve the peak shape of the compounds. In general, the resolution of a chromatographic process can be improved by adjusting the conditions of the separation to suit the properties of the compounds being separated.

$$R = 2\,(t_2 - t_1) / (w_1 + w_2) \qquad (1.1)$$

Where

R = Resolution between a peak of interest (peak 2) and the peak preceding it (peak 1),

W_1 = Width of the base of component peak 1,

W_2 = Width of the base of component peak 2.

There are several ways to quantify resolution, but the most widely used method is the resolution equation, which is defined as the ratio of the distance between two peaks of different compounds to the width of the peak at its half-height. A resolution value of 1 or higher indicates that the peaks are well separated and distinct from each other, while a

resolution value of less than 1 indicates that the peaks are not well separated and may overlap.

B. Capacity factor

The capacity factor, also known as the retention factor or k value, is a measure of how strongly a compound interacts with the stationary phase in a chromatographic system. It is a dimensionless number that compares the retention time of a compound to that of a reference compound, typically an unretained compound such as the mobile phase solvent. A higher capacity factor indicates that a compound has a stronger interaction with the stationary phase and will therefore be retained for a longer period of time (Philosophy, 2011).

Capacity factor is used to compare the relative retention of different compounds in a chromatographic system.

$$K_1 = (t_2 / t_0) - 1 \qquad (1.2)$$

Where, K_1 = Capacity factor,

t_2 = Retention time of a compound,

t_0 = Retention time of the reference.

In normal phase chromatography, compounds with higher polarities will have a lower capacity factor and elute earlier, while in reverse-phase chromatography, compounds with lower polarities will have a lower capacity factor and elute earlier.

The capacity factor is also used to determine the column loading capacity. It is the amount of solute that can be retained by the stationary phase before the column becomes saturated. The column loading capacity is inversely proportional to the capacity factor, which means that as the capacity factor increases, the column loading capacity decreases.

In short, the capacity factor is a measure of how much a compound interacts with the stationary phase and how long it takes to elute, this parameter is useful to understand the retention of a compound and how much of it can be retained in a column. It can also provide some information about the polarity of the compound.

C. Efficiency

The efficiency factor, also known as the plate count or n value, is a measure of the resolving power or performance of a chromatographic system. It is defined as the number of theoretical plates per unit length of the column. The theoretical plate is a hypothetical unit of measurement that represents the separation of two peaks in a chromatogram. The higher the number of theoretical plates, the better the resolution of the system.

Efficiency factor is calculated as the ratio of the column length to the number of theoretical plates (n):

$$N = 16 (t / w)^2 \quad (1.3)$$

Where, N = Theoretical plates,

t = Retention time of the component,

W = Width of the base of the component peak using tangent method.

The efficiency factor can be affected by many factors such as the type of column, column temperature, mobile phase composition, and flow rate. A high efficiency factor indicates that the system is able to separate closely eluted peaks and a low efficiency factor means that the system is not able to separate closely eluted peaks.

Higher efficiency factor results in smaller peaks with sharper peaks, which is beneficial in trace analysis. In contrast, a lower efficiency factor results in broader peaks, which may be beneficial for sample loading capacity and reducing analysis time.

D. Column selectivity

Column selectivity is the ability of a chromatographic column to separate different compounds based on their chemical properties. The selectivity of a column depends on the type of stationary phase and the interaction between the stationary phase and the solute (Kalyan Chakravarthy and Gowri Sankar, 2012).

There are different types of stationary phases that can be used in chromatography, each with different selectivity. Column selectivity can be described by the relative retention of two peaks. The ratio of the retention times of two peaks is known as the selectivity factor (alpha) and is calculated as:

$$\alpha = (t_2 - t_a) / (t_1 - t_a) \quad (1.4)$$

Where, α = Relative retention,

t_1 = Retention time of the first peak measured from point of injection,

ta = Retention time of an inert peak not retained by the column,

measured from point of injection.

A high selectivity factor (alpha) indicates that the column is able to separate the two peaks well, while a low selectivity factor indicates that the column is not able to separate the two peaks.

Column selectivity is also affected by the column temperature, mobile phase composition and flow rate. For example, increasing the temperature of the column will increase the selectivity of the column for certain types of stationary phases, while increasing the mobile phase flow rate will decrease the selectivity of the column. As well as important parameter to consider when selecting a column for a specific analysis. It is also important to consider the selectivity of the column when optimizing the chromatographic conditions to obtain the best separation of the compounds of interest.

Table 1.4. *Ranges of chromatography parameters (Ambadekar et al., 2018)*

Parameter	Typical Range
Resolution (R)	1.5 - 2.5
Capacity Factor (K)	0.1 - 10
Efficiency Factor (N)	1000 - 10000 plates/m
Column Selectivity(α)	0.1 - 10

1.10.2. High-performance liquid chromatography (HPLC)

Basics of the method HPLC has become a versatile and widely used analytical method. It allows for the physical separation, detection, and quantification of individual analytes in biological, organic, and inorganic samples. The separation is achieved through various interactions between analytes in the liquid mobile phase and the stationary phase.

Depending on the strength and type of chemical and physical interactions, the retention of analytes in the stationary phase is different and characteristic for each analyte,

so they can be detected separately without mutual overlap, but optimal conditi ons must be established (Phadke et al., 2018) (Ambadekar et al., 2018).

The polarity plays an important role in the retention of analytes in the stationary phase. In a more polar stationary phase, polar analytes will be better retained due to the non-polarity of the mobile phase. This is reminded by the Latin saying "similia similibus solvuntur" (translated as "like dissolves like"), which means that polar analytes dissolve in polar solvents, the same applies to non-polar solvents and non-polar analytes. Reverse-phase HPLC is the most commonly used, in which hydrophobic interactions predominate among the analytes. The stationary phase is non-polar, and the mobile phase is polar. Therefore, the polar analytes will be eluted first and will reach the detector dissolved in the polar mobile phase. Normal phase HPLC works on a similar but reversed principle.

In this case, the mobile phases are non-polar (hexane, heptane) with a small addition of a polar modifier (ethanol, methanol), which can be used to regulate the retention of the analyte in the column. Because polar interactions predominate - the particles of the stationary phase have many polar -OH groups-even 1 volumetric percent can significantly affect retention. By far the most common implementation is reverse-phase HPLC, which is used in almost 90% of all chromatographic analyses of low molecular mass samples. One of the reasons for such dominance is the ability to separate very similar substances. This is enabled by weak forces that allow separation. Small changes in weak (hydrophobic, van der Waals) interactions are more sensitive than small changes in stronger (polar) interactions (See Fig. 1.2).

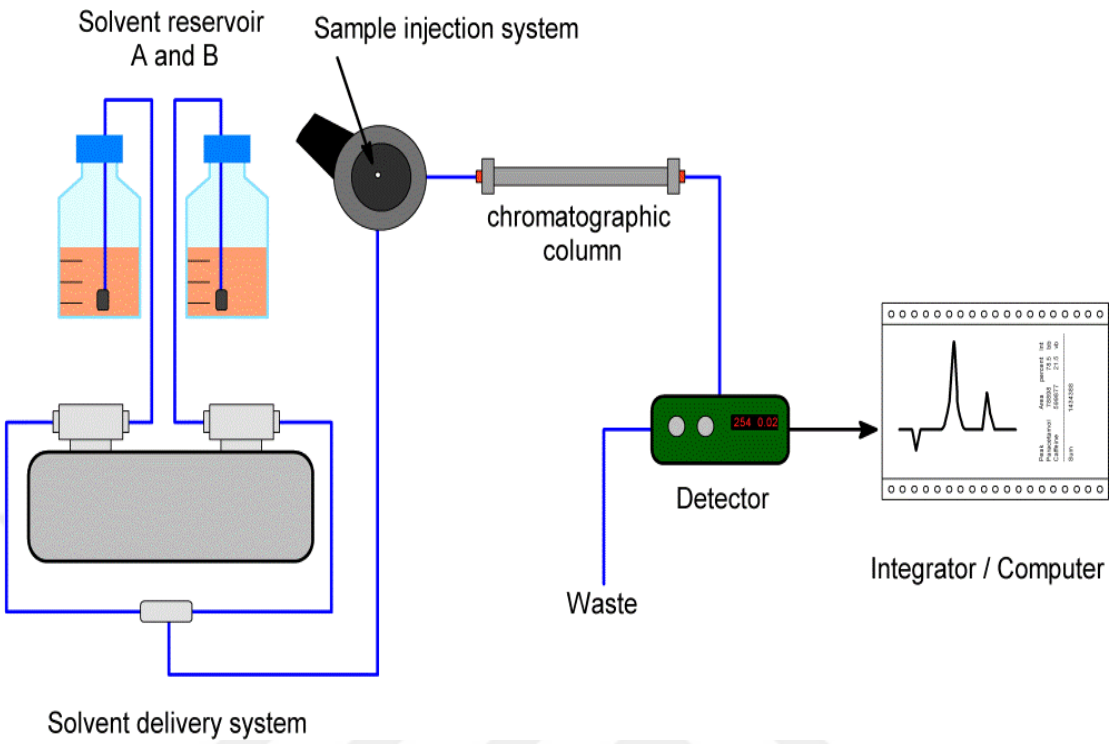

Figure 1.2. *Schematic layout of a HPLC system*

1.10.2.1. Selection of acids and bases in HPLC

Selection of acids and bases used in HPLC is based on several important properties, including the strength of the acid or base (measured by the pKa value), volatility, UV cutoff, and polarity (Arar et al., 2018).

pKa: The pKa value indicates the strength of an acid or base. A lower pKa value indicates a stronger acid or base. Volatility: Refers to whether the acid or base can evaporate at room temperature. Non-volatile acids and bases are preferred in HPLC because they do not affect the mobile phase.

UV Cutoff: Refers to the highest wavelength at which the compound absorbs ultraviolet light. UV detection is a common detection method in HPLC, so it is important to select acids and bases that do not interfere with this method.

Polarity: Refers to the degree of attraction that the compound has for ions. Polarity affects the solubility of compounds in the mobile phase and can impact the separation of compounds in HPLC.

The choice of acid or base to use in HPLC will depend on the nature of the sample being analyzed and the desired selectivity for separation. For example, Trifluoroacetic

acid (TFA) is a strong acid that is commonly used to control the moving phase's pH. Sodium hydroxide (NaOH) and potassium hydroxide (KOH) are strong bases that are commonly used to form ionizable species.

It is important to consider these properties when selecting acids and bases for use in HPLC to ensure optimal performance of the system and accurate results, as shown in Table 1.5

Table 1.5. *Selection of acids and bases used in HPLC*

Acid/Base	pKa (25°C)	Compound	Volatile	UV Cutoff
Acetic Acid	4.75	CH_3COOH	Yes	190 nm
Formic Acid	3.75	HCOOH	Yes	210 nm
Trifluoroacetic Acid (TFA)	0.25	CF_3COOH	No	190 nm
Sodium Hydroxide (NaOH)	14.0	NaOH	No	N/A
Potassium Hydroxide (KOH)	14.0	KOH	No	N/A

Note: The choice of acid or base to use in HPLC will depend on the nature of the sample being analyzed and the desired selectivity for separation. The pKa value indicates the strength of an acid or base. A lower pKa value indicates a stronger acid. The volatile property refers to whether the acid or base can evaporate at room temperature. The UV cutoff refers to the highest wavelength at which the compound absorbs ultraviolet light.

1.10.2.2. Selection of buffer salts in HPLC

Selection of buffer salts in HPLC is important in maintaining a consistent pH in the mobile phase during the separation process (Pandya and Rajput, 2018).

Buffer salts work by providing a source of ions that can react with hydrogen ions (H^+) to regulate the pH of the mobile phase. This helps to stabilize the pH during the separation process, which is important for accurate and reproducible results.

Common buffer salts used in HPLC include sodium acetate, potassium phosphate, and sodium borate. The choice of buffer salt will depend on the desired pH range, buffering capacity, and stability of the salt, as shown in table 1.6.

Table 1.6. *Summarizing the selection of buffer salts in HPLC*

Buffer Salt	pH Range	Buffering Capacity	Stability
Dihydrogen Phosphate	6 to 8	Neutral	Stable in solution
Hydrogen Phosphate	7 to 8	Weakly acidic	Stable in solution
Phosphate	6 to 8	Neutral	Stable in solution
Dihydrogen Citrate	4 to 5	Weakly acidic	Stable in solution
Hydrogen Citrate	4 to 5	Weakly acidic	Stable in solution
Citrate	3 to 4	Weakly acidic	Stable in solution
Acetate	4 to 6	Weakly acidic	Stable in solution
Formate	3 to 4	Weakly acidic	Stable in solution

Here is a table summarizing the selection of mobile phase components used in HPLC and their properties:

Table 1.7. *Summarizing the selection of mobile phase components in HPLC*

Solvent	Separation Mode	Polarity Index	Dielectric Constant
Water	RP-HPLC	10.2	80
Acetonitrile	RP-HPLC	5.8	37.5
Methanol	RP-HPLC	5.1	32.7
THF	RP or NP-HPLC	4.0	7.6
Acetone	RP or NP-HPLC	5.1	20.7
Ethanol	RP or NP-HPLC	4.3	24.6
Isopropanol	RP or NP-HPLC	3.9	19.9
Hexane	NP-HPLC	0.1	1.9

Note: The Polarity Index is a measure of solvent polarity and is used to determine the suitability of a solvent for a particular separation. The Dielectric Constant is a measure of the solvent's ability to dissolve polar compounds. RP-HPLC stands for reverse-phase high-performance liquid chromatography and NP-HPLC stands for normal-phase high-performance liquid chromatography. The selection of mobile phase components will depend on the nature of the sample being analyzed and the desired selectivity for separation.

1.10.2.3. Composition of HPLC system

The composition of an HPLC system includes the various components that work together to perform the separation, detection, and quantification of individual compounds in a sample. The system can be configured in different ways depending on the application

and the type of sample, and additional components or accessories can be included to enhance the performance of the system (Subirats et al., 2007). The main components of an HPLC system are:

***A pump**:* The pump is one of the main components of an HPLC system. It is responsible for generating a high-pressure flow of the mobile phase (the liquid that carries the sample through the column) through the system. The pump is a critical component of the system as it helps to ensure that the mobile phase is delivered to the column at the correct flow rate and pressure, which is essential for achieving optimal separation of the analytes.

There are several types of pumps that can be used in an HPLC system, including:

- Reciprocating pump: This type of pump uses a piston to generate the flow of the mobile phase. It is a relatively simple design, but it is not as precise as other types of pumps.
- Syringe pump: This type of pump uses a syringe to generate the flow of the mobile phase. It is a more precise and accurate pump than a reciprocating pump, but it can be less reliable.
- Gradient pump: This type of pump allows for a gradual change in the composition of the mobile phase during the analysis. It is typically used in gradient elution chromatography, where a change in the mobile phase composition is used to separate different classes of compounds.
- Dual-piston pump: This type of pump uses two pistons that work in opposite directions to generate the flow of the mobile phase. It is more precise and accurate than the reciprocating pump and it is used in high-pressure applications.
- Membrane pump: This type of pump uses a flexible diaphragm or membrane to generate the flow of the mobile phase. It is a precise and reliable pump that is well-suited for use with small sample volumes.

All these types of pumps have their own benefits and limitations, and the choice of pump depends on the application, the sample, and the desired level of precision and accuracy (S. Eldin et al., 2017).

A sample injector: The sample injector is a key component of an HPLC system that allows the sample to be introduced into the system. The sample injector is responsible for introducing a precise amount of sample into the mobile phase, which then flows through the column for separation and detection (Luo et al., 2018).

A sample injector for HPLC typically consists of a sample vial or container and a needle or loop. The sample is loaded into the vial or container and then the needle or loop is inserted into the sample. The sample is then injected into the mobile phase by either manually pulling the plunger of the syringe or by using a pneumatic or electronic drive to push the sample through the needle or loop. The sample injector is typically located at the front of the HPLC instrument, it is connected to the pump, and the sample is introduced into the mobile phase at a specific flow rate and pressure.

The sample injectors can vary in design, some are manual, some are semi-automatic, and some are fully automated. A manual syringe injector is simple and inexpensive, it allows the user to manually inject a defined volume of the sample into the mobile phase. A semi-automatic injector uses a fixed volume loop to introduce the sample into the mobile phase, the loop is loaded with the sample, and it is automatically introduced into the mobile phase. A fully automated injector can hold multiple vials or containers with samples, it automatically takes the sample, injects it into the mobile phase and can also change between samples and vials.

A column: The column is the heart of an HPLC system. It is where the separation of the sample takes place. The column is a long, thin tube that is packed with a material called the stationary phase. This material can be a solid or a liquid, and it is chosen based on the characteristics of the sample and the type of separation required. The sample is introduced into the column, and as it flows through the column, the different components of the sample interact with the stationary phase in different ways. This interaction causes the components to separate from each other, with each component exiting the column at a different time, this is known as the retention time (Lei et al., 2017).

There are several types of columns that can be used in an HPLC system, including:

Normal phase columns: These columns use a non-polar stationary phase and a polar mobile phase. They are typically used to separate polar compounds.

Reversed phase columns: These columns use a polar stationary phase and a non-polar mobile phase. They are typically used to separate non-polar compounds.

Ion exchange columns: These columns use an ion exchange resin as the stationary phase. They are typically used to separate ions or ionizable compounds.

Size exclusion columns: These columns use beads with different pore sizes as the stationary phase. They are typically used to separate macromolecules based on their size.

***Affinity columns*:** These columns are based on the specific binding of a ligand to a receptor. They are typically used to purify specific biomolecules.

The choice of column depends on the application, the sample, and the desired level of separation. Each column has its own advantages and disadvantages, and the best column for a particular application will depend on the characteristics of the sample and the type of separation required.

A detector: The detector is an essential component of an HPLC system that is used to detect and quantify the separated components of a sample. The detector measures the presence of the analyte in the mobile phase as it exits the column. There are several types of detectors that can be used in an HPLC system, each with its own advantages and disadvantages (Kalyan Chakravarthy and Gowri Sankar, 2012).

UV-Visible detector: This type of detector measures the absorbance of the analytes at specific wavelengths. It is widely used in HPLC, particularly for compounds that have chromophores (compounds that absorb ultraviolet or visible light).

Fluorescence detector*:* This type of detector measures the fluorescence emitted by the analytes. It is particularly useful for detecting low levels of compounds and for compounds that do not absorb UV or visible light.

Refractive index detector*:* This type of detector measures the change in refractive index of the mobile phase caused by the presence of the analytes. It is useful for detecting non-chromophore compounds.

Mass spectrometer detector*:* This type of detector is used to identify and quantify the analytes. It works by ionizing the analyte molecules, separating them by their mass-to-charge ratio, and measuring the resulting ions. It is used in conjunction with other detectors, and it is particularly useful for identifying unknown compounds.

Electrochemical detector: This type of detector measures the current or potential difference between a sample and a reference electrode, it is useful for detecting electroactive compounds.

The choice of detector depends on the application, the sample, and the desired level of sensitivity and selectivity. In most cases, more than one detector is used in an HPLC system to provide a complete analytical picture of the sample (Kalyan Chakravarthy and Gowri Sankar, 2012).

A diode array detector (DAD) is a type of detector commonly used in High-Performance Liquid Chromatography (HPLC) to detect and quantify the components of a mixture.

The spectrometric detector with a diode array allows for the measurement of the entire absorption spectrum as a function of time, resulting in a 3D record (dependence of absorbance on wavelength and time). The detector consists of a silicon wafer containing 1024 photodiodes, the measured spectral range is in the range of 190-800 nm. The resulting chromatogram of a given wavelength is extracted from the three-dimensional image. The 3D record from the DAD detector is mainly used to assess the purity of the peak being monitored. The sensitivity is about one order of magnitude lower than that of UV/VIS detectors, that is 10^{-7}-10^{-8} g mL^{-1}.

The DAD uses multiple photodiodes, typically in the ultraviolet-visible (UV-Vis) range, to detect the absorbance of the components in the mixture as they exit the column as well as capable of simultaneous detection of multiple wavelengths, which allows for the identification and quantification of multiple components in the mixture. And also connected to a computer and data handling software, which processes the data and displays it in a chromatogram. The chromatogram shows the retention time and absorbance of each component in the mixture. This information can be used to identify and quantify the components in the mixture, and it is widely used in HPLC because they are sensitive, specific, and able to detect a wide range of compounds. They are commonly used in applications such as pharmaceutical analysis, environmental analysis, and food analysis (Al Bratty et al., 2020).

It is important to note that DAD is not suitable for all types of compounds, as it requires the compound to be UV-Vis absorbent, if the compound is not UV-Vis absorbent other types of detector such as Fluorescence or mass spectrometer detector should be used. In addition, consists of multiple photodiodes that are sensitive to ultraviolet-visible (UV-Vis) radiation. As the components of the mixture pass through the detector, they are exposed to the photodiodes, which measure the absorbance of the components at different wavelengths. This allows for the simultaneous detection of multiple components in the mixture. Additionally, DADs are known for their high sensitivity and wide dynamic range, which allows for the detection of a wide range of concentrations of the components in the mixture. This is especially useful in applications where the concentration of the analytes can vary greatly, such as in environmental samples. Another advantage is their

ability to provide full-spectral data, which can be used for advanced data analysis techniques such as multivariate analysis. This can help to identify and quantify complex mixtures and uncover hidden information that might be missed using traditional single-wavelength detection methods. also have the ability to detect the fluorescence of compounds, when DADs are equipped with fluorescence detection capability, they can provide additional information about the compounds in the mixture, allowing for even more accurate identification and quantification. also, highly versatile in terms of their compatibility with different types of HPLC systems. They can be used with various types of HPLC columns, including reverse-phase, normal-phase, and ion-exchange columns, and can work with different types of mobile phases. This allows the user to tailor the analysis to the specific sample and the type of information required (Mohan et al., 2020).

In the field of drug development, also commonly used to monitor the purity and stability of drug compounds during the development process. They are also used to identify and quantify impurities that may be present in drug samples.

A data system: A data system in HPLC is an essential component of the system that is used to collect, process, and store data generated by the detector. The data system typically consists of a computer and software that are used to control the HPLC instrument, process the data, and store the data for analysis and reporting. The data system can be used to control the flow rate, pressure, and temperature of the mobile phase, as well as the detector settings. It can also be used to collect data from the detector in real-time and store it for later analysis (Chen et al., 2020).

The data system software is used to process the data and perform calculations such as peak integration, quantification, and identification of the analytes. It can also be used to generate reports and graphical representations of the data. The data system can be connected to other devices such as mass spectrometers, chromatographic fraction collectors, and other analytical instruments to provide a comprehensive analytical picture of the sample.

A fraction collector: A fraction collector is a device that is used to collect specific fractions of the eluted sample from the column in HPLC. It works by collecting a certain volume of the eluted sample at specific time intervals or based on specific conditions such as a specific peak eluting from the column. The fractions can then be collected into individual vials or containers for further analysis or purification.

There are several types of fraction collectors that can be used in an HPLC system, including (Palakurthi et al., 2020):

Time-based fraction collectors: These collectors collect a specific volume of the eluted sample at specific time intervals. *Peak-based fraction collectors*: These collectors collect a specific volume of the eluted sample based on specific conditions such as a specific peak eluting from the column. *Multi-port fraction collectors*: These collectors have multiple collection ports that allow for the collection of multiple fractions simultaneously.

The choice of fraction collector depends on the application, the sample, and the desired level of separation. A fraction collector can be used in conjunction with other analytical instruments such as mass spectrometers to provide a more detailed analysis of the collected fractions. It is particularly useful for purifying specific compounds or isolating specific fractions for further analysis.

1.11. Analytical method validation

Analytical method validation is the process of demonstrating that an analytical method is suitable for its intended use. It involves conducting a series of experiments to evaluate the performance of the method and to establish its limitations. The main objective of analytical method validation is to ensure that the method is reliable, accurate, and reproducible (Prajapati et al., 2020).

The process of analytical method validation typically includes the following steps:

- Defining the scope and objectives of the validation.
- Selecting appropriate test samples and standards.
- Establishing the performance characteristics of the method, including specificity, linearity, precision, accuracy, and robustness.
- Evaluating the performance of the method using the test samples and standards.
- Documenting the results of the validation, including any limitations or special considerations.
- Implementing a plan for ongoing monitoring and maintenance of the method.

The specific requirements for analytical method validation may vary depending on the type of analysis, the regulatory agency, and the industry guidelines. For example, the

International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines provide a framework for the validation of analytical methods used in the pharmaceutical industry (EMEA, 2006).

It's worth mentioning that the analytical method validation should be periodically reviewed and updated as necessary to ensure that the method continues to meet the needs of the analysis and to reflect any changes in the regulatory requirements or industry standards.

1.11.1. Validation protocol

The validation process must follow the validation protocol. It is written by a suitably qualified person and approved by the quality management department. It should include the entire process of preparing reagents, samples, instruments, performing analyzes and final calculations. It must also contain all the data necessary to carry out the validation: type of method, number of repetitions, acceptance criteria of individual parameters, details of the required apparatus, and more (Tome et al., 2020).

1.11.1.1. Validation parameters

Validation parameters are the characteristics that are evaluated during the process of analytical method validation to ensure that the method is reliable, accurate, and reproducible.

These validation parameters are essential in order to determine the performance of the analytical method and its suitability for the intended use. It is important to follow the regulatory agency's guidelines and industry standards while performing the validation and to document the results in a clear and comprehensive validation report. listed below is typical validation factors to consider (Ambadekar et al., 2018).

(a) Accuracy

Accuracy is a measure of how closely the results of an analytical method agree with the true or accepted value of the sample. It is one of the most important validation parameters in analytical method validation.

A common way to assess accuracy is to compare the technique's output with a standard or reference method that is regarded as the "true value". This can be achieved by applying the test technique and the reference method to analyze a sample with a known

concentration of the analyte and comparing the outcomes. The difference between the two values is known as the bias (Arous et al., 2018).

The accuracy can be expressed in different ways depending on the context, but in general, the most common ways are:

- Recovery: the percentage of the analyte that is recovered by the method compared to the added amount. This parameter is calculated by comparing the results of the method with the amount of the analyte that was added to the sample.
- Absolute error: the absolute difference between the results obtained by the method and the reference value.
- Relative error: the relative difference between the results obtained by the method and the reference value.

Acceptance criteria for accuracy are usually established based on the specific requirements of the analysis and the regulatory guidelines. In general, a bias of less than 20% and a relative error of less than 15% are considered to be acceptable levels of accuracy.

It's worth noting that accuracy is affected by various factors such as the quality of the reagents and standards, the performance of the instrument, and the skill of the analyst. Therefore, it is important to perform regular calibration and maintenance of the instrument and to ensure that the reagents and standards are of high quality.

(b) Precision

Precision is a measure of how reproducible the results of an analytical method are when the same sample is analyzed multiple times under the same conditions. It is one of the most important validation parameters in analytical method validation (Hegazy et al., 2018).

Precision is usually evaluated by repeating the analysis of the same sample multiple times and calculating the standard deviation or coefficient of variation of the results. The standard deviation is a measure of the spread of the values around the mean, while the coefficient of variation (CV) is the standard deviation expressed as a percentage of the mean. A smaller standard deviation or CV indicates that the results are more precise.

There are two types of precision:

- Repeatability: The precision that can be obtained when the analysis is performed by the same operator using the same equipment, under the same conditions, over a short period of time.
- Reproducibility: The precision that can be obtained when the analysis is performed by different operators, using different equipment, in different laboratories, over an extended period of time.

Acceptance criteria for precision are usually established based on the specific requirements of the analysis and the regulatory guidelines. In general, a repeatability CV of less than 2% and a reproducibility CV of less than 5% are considered to be acceptable levels of precision.

(c) Specificity

Specificity is the ability of an analytical method to unambiguously identify and quantify the analyte of interest in the presence of other components that may be present in the sample matrix. The sample matrix consists of all other components such as impurities, degradation products, and matrix interferences that may be present in the sample (Sowmithri et al., 2018).

The specificity of a method is demonstrated by the fact that the analyte of interest elutes independently at a precisely determined time without interactions with the matrix. In other words, the analyte should be separated from other components in the sample and should not be affected by the presence of other compounds.

This separation is typically achieved through the use of chromatographic technique such as HPLC. The specificity of a method can be influenced by various factors such as the type of column and mobile phase used, the pH and temperature of the system, and the nature of the analyte and sample matrix. To demonstrate specificity, a series of specificity tests are usually performed.

These include:

- Blank matrix: The method is applied to a blank matrix, i.e., a matrix that is similar to the sample matrix but doesn't contain the analyte, to verify that no interferences are present.

- Spiking known interferences: The method is applied to a sample that has been spiked with known interferences to evaluate the effect of these interferences on the results.
- Comparison with alternative method: The results obtained using the method of interest are compared with those obtained using an alternative method. A method with high specificity will give the same results as alternative method, where as a method with low specificity will give different results.

Overall, Specificity is a critical parameter in analytical chemistry, as it helps to ensure that the results obtained are specific to the analyte of interest and not affected by the presence of other compounds. This allows for accurate and reliable results, and it is a key aspect of method validation and regulatory compliance.

(d) Linearity

Linearity is an important parameter in analytical chemistry that describes the relationship between the concentration of an analyte in a sample and the response of the analytical method. It refers to the ability of an analytical method to produce results that are directly proportional to the amount of analyte present within a specified range (S. Eldin et al., 2017).

Linearity is typically determined by measuring various known concentrations of the analyte, usually by diluting a standard solution of the analyte. This is done by preparing a series of solutions with different concentrations of the analyte, and then measuring the response of the analytical method for each solution. Linearity can be determined graphically by plotting the response of the analytical method against the concentration of the analyte. The resulting graph should be a straight line with a slope that is directly proportional to the concentration of the analyte.

The intercept of this line should be zero, indicating that there is no response in the absence of the analyte.

Linearity can also be determined mathematically by fitting the data to a linear equation of the form $y = mx + b$, where y is the response of the analytical method, x is the concentration of the analyte, m is the slope of the line and b is the y-intercept.

The linearity range is the range of analyte concentrations over which the method is linear. Generally, it is recommended that a linearity range should be at least five-fold or more above and below the expected concentration of the analyte in the sample. It is

important to note that non-linearity can occur due to various factors such as changes in the chemical or physical properties of the analyte, changes in the sample matrix, or the presence of interfering compounds. Therefore, linearity should be evaluated and confirmed for each analytical method and sample matrix.

(e) Limit of detection and limit of quantitation

1) Limit of detection

Detection limit, also known as the limit of detection (LOD), is a measure of the sensitivity of an analytical method. It is defined as the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated as an exact value.

The detection limit is usually expressed as the concentration of the analyte in the sample or as the amount of analyte per unit of sample. The unit of measurement is often in parts per million (μg/mL)) or parts per billion (ppb) for trace level analysis.

There are different ways to calculate LOD. Based on Signal-to-Noise ratio (S/N). A signal-to-noise ratio of 3 or 2:1 is generally deemed appropriate for evaluating the detection limit. This method is only applicable to analytical methods with baseline noise. The most widely used methods is based on the standard deviation of the response and the slope, The LOD may be expressed as:

$$LOD = 3.3\ \sigma / S \qquad (1.5)$$

σ standard deviation of the response, S slope of the calibration curve.

It's important to note that the LOD can vary depending on the sample matrix, the nature of the analyte, and the analytical method used. Factors such as the instrumentation, the type of column, the mobile phase, and the sample preparation techniques can also affect the LOD (Sangeetha and Vadlamudi, 2017).

2) Limit of quantitation

The Quantitation Limit (QL) is a parameter in analytical chemistry that refers to the minimum amount of a substance that can be accurately and precisely measured.

The Quantitation Limit (QL) based on Signal-to-Noise (S/N) ratio is a commonly used method to determine the minimum amount of a substance that can be accurately and

precisely measured. This method is based on the measurement of the signal (peak height or area) generated by the analyte in the presence of the background noise in the sample.

The QL is defined as the concentration at which the signal generated by the analyte is equal to or greater than a specified minimum signal-to-noise ratio, typically S/N=10. This means that the signal generated by the analyte should be at least ten times greater than the background noise in order to be considered a reliable and accurate measurement.

The QL based on S/N ratio is useful for determining the limit of detection (LOD) and limit of quantitation (LOQ) of an analytical method. The Quantitation Limit (QL) based on the Standard Deviation of the Response (SD) and the Slope is a method used in analytical chemistry to determine the minimum amount of a substance that can be accurately and precisely measured. This method takes into account both the variability in the response and the slope of the calibration curve. The QL is defined as the concentration at which the standard deviation of the response is equal to or less than a specified fraction of the slope. For example, a commonly used value is the

$$QL = 10 * SD / slope \qquad (1.6)$$

where σ standard deviation of the response ,S slope of the calibration curve.

This means that the standard deviation of the response should be less than 10% of the slope in order to be considered a reliable and accurate measurement. The QL based on SD and slope is useful for determining the limit of detection (LOD) and limit of quantitation (LOQ) of an analytical method.

(f) Robustness

Robustness is a measure of the capacity of an analytical method to remain unaffected by small, but deliberate variations in method parameters. It provides an indication of the reliability of the method during normal usage and is a measure of its ability to perform consistently under different conditions, and also typically evaluated by deliberately varying one or more method parameters, such as the pH of the mobile phase, the column temperature, the flow rate, or the sample injection volume, and then measuring the effect of these variations on the performance of the method. This is usually done by analyzing a sample containing a known amount of the analyte and comparing the results obtained under the different conditions. For instance, if a method is resilient to pH variations, then minor variations in the mobile phase's pH shouldn't significantly affect

the method's performance. However, if a method is not robust, then small changes in the pH of the mobile phase may cause significant changes in the results (Ramachandra, 2017).

Robustness is often evaluated by performing a series of replicate measurements on a sample containing a known amount of the analyte under different conditions. The precision and accuracy of the method are typically determined by comparing the results obtained under the different conditions to a reference value. It's important to note that the robustness of a method can be affected by several factors such as the type of analyte, the sample matrix, the instrumentation, and the method conditions. Thus, it is important to evaluate the robustness of an analytical method prior to its routine use to ensure that the method can be used in practice with minimal variations.

(g) Stability

In a stability study, the standard solutions are the solutions that were examined for short-term (1 day), freeze-thaw, and long-term (30 days) stability. These solutions may contain one or more active ingredients, excipients, and other ingredients that are typically used in the final product. The stability study is conducted to ensure that the product remains within its predetermined specifications over a specific period of time under specified storage conditions (Chandorkar et al., 2008).

During the stability study, the solutions are stored under different conditions such as different temperatures and lighting conditions to mimic real-world storage conditions. The solutions are then analyzed using HPLC at specific intervals to check for any changes in the composition of the solution. The HPLC analysis helps to determine if the active ingredients, excipients, and other ingredients in the solution have degraded or if there are any impurities present.

The stability study solution HPLC is an important step in the development and quality control of a product as it ensures that the product will remain stable and effective throughout its shelf life. The results of the stability study are used to determine the shelf life of the product and to establish appropriate storage conditions.

(h) A system suitability test

A system suitability test (SST) is a set of procedures that are used to verify the performance of a chromatographic method. These tests are performed to ensure that the equipment, analytical operations, and samples are functioning as intended, and that the data obtained will be accurate and precise. The SST is an essential part of the

chromatographic method, as it helps to confirm that the system is suitable for the analysis to be performed.

The SST is based on the principle that the equipment, analytical operations, and samples constitute an integral system that can be evaluated as a whole. The test is used to evaluate the performance of the system, including the resolution and reproducibility of the system. The SST is performed by investigating several parameters that are critical to the performance of the chromatographic method.

The parameters that are typically evaluated during an SST include the capacity factor, tailing factor, theoretical plates number, resolution, and the relative standard deviation (RSD) of the peak areas. The capacity factor is a measure of the efficiency of the separation, and it is calculated by comparing the retention time of a compound to the retention time of a reference compound. The tailing factor is a measure of the shape of the peaks, and it is calculated by comparing the width of the peak at the base to the width of the peak at the top. The theoretical plates number is a measure of the efficiency of the column, and it is calculated by counting the number of theoretical plates present in the column. The resolution is a measure of the separation between two peaks, and it is calculated by comparing the distance between the two peaks to the width of the peaks.

The RSD of the peak areas is a measure of the reproducibility of the data, and it is calculated by comparing the variation in the peak areas to the average peak area.

1.12.2. Aims and objectives

Aims: The aim of the work undertaken was to identify the possible impurities products present in lamotrigine, which we obtained from three different countries as (Turkey, Yemen, and India), and the comparison between them.

Objectives:

The objectives of the present work include:

- To identify process-related or inherent impurity in an active pharmaceutical ingredient (API).
- Development of new, simple, accurate, and economical analytical method for the estimation of lamotrigine impurities.
- The present work objective is to develop a stability-indicating analytical method for major impurity profile in a pharmaceutical dosage form which covers all impurities.

- Validation of developed analytical method in accordance with the ICH guideline Q3A (R2).

2. DRUG PROFILE

LAMOTRIGINE

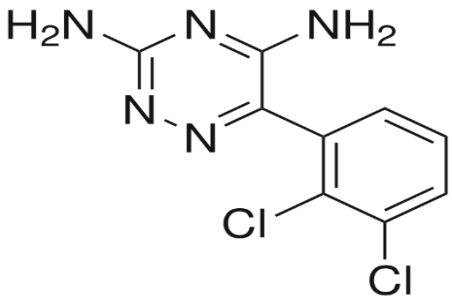

Figure 2.1. *Chemical Structure of Lamotrigine*

Molecular Formula : $C_9H_7C_{l2}N_5$

Molecular Weight : 256.091

Chemical Name : 6-(2, 3-dichlorophenyl)-1, 2, 4-triazine-3, 5-diamine

Appearance : White to off-white, crystalline powder

Solubility : Soluble DMSO, methanol, very slightly soluble in water

Melting point range : 171– 181^0C

Therapeutic Category **:** Broad-spectrum antiepileptic

Storage : Should be stored at room temperature, between 15°C and 30°C (59°F and 86°F). It should be kept in its original container and protected from moisture, light, and heat.

Dose : Stored in high-density polyethylene bottle (HDPE), with silica gel desiccant canisters or sachets, and polyester fiber packing material

Lamotrigine is a chemical compound with the molecular formula $C_9H_7C_{l2}N_5$. It is a white to pale cream-colored powder that is soluble in water and other polar solvents.

Lamotrigine is an antiepileptic and mood-stabilizing drug that works by modulating the release of certain neurotransmitters in the brain, particularly glutamate, which is involved in the regulation of excitability in the nervous system. It is classified as a triazine anticonvulsant and is used to treat a variety of neurological and psychiatric conditions, including epilepsy and bipolar disorder (Carrier et al., 2008) (Ventura et al., 2017).

Mechanism of action

The mechanism of action of Lamotrigine refers to the way in which it works in the body to produce its effects. One theory is that Lamotrigine affects sodium channels in the brain. Sodium channels play a role in transmitting electrical signals in the brain and are involved in regulating the release of certain neurotransmitters.

In vitro studies, which are studies done in a laboratory setting using cells or tissues, suggest that Lamotrigine may inhibit the activity of these sodium channels and stabilize the neuronal membranes. This stabilization effect may then lead to a modulation, or change, in the release of certain neurotransmitters, such as glutamate and aspartate, which are known as excitatory amino acids. However, it is important to note that this proposed mechanism of action for Lamotrigine has not yet been fully established in human studies. Further research is needed to confirm and understand the exact way in which Lamotrigine works in the bod (Martins et al., 2010).

Pharmacokinetics

Lamotrigine has complex pharmacokinetics, meaning how the drug is absorbed, distributed, metabolized, and excreted in the body. This complexity leads to highly variable half-life (the time it takes for half of the drug to be eliminated from the body) and blood plasma levels of the drug.

Despite this complexity, Lamotrigine has fewer drug interactions compared to many other anticonvulsant drugs. However, there is a specific interaction with Sodium Valproate that requires monitoring of blood levels of Lamotrigine. This interaction highlights the importance of careful monitoring and management of drug interactions and their impact on the pharmacokinetics of Lamotrigine (Ruiz et al., 2012).

Precautions

Patients should also avoid alcohol and CNS depressants, as these can worsen the side effects of Lamotrigine. Additionally, it is important for patients to inform their physician of any pre-existing medical conditions, such as liver or kidney disease, mental illness, anemia, high blood pressure, angina, or heart problems, as Lamotrigine may not be suitable for some individuals with these conditions. Finally, patients should also inform their physician if they have a history of alcohol or drug use, if they are nursing or pregnant, or if they plan to become pregnant, as these factors may also impact the safety and efficacy of Lamotrigine (Li et al., 2010).

Side effects and toxicity

Some common side effects may include headache, dizziness, drowsiness, blurred vision, double vision, nausea, vomiting, abdominal pain, loss of coordination, tremors, sleep problems, and skin rash. Less common side effects may include anxiety, depression, irritability, confusion, memory problems, speech difficulties, muscle pain or weakness, joint pain, decreased appetite, weight loss, menstrual problems, and hair loss.

In rare cases, severe skin rashes or hypersensitivity reactions, including Stevens-Johnson syndrome, toxic epidermal necrolysis, and exfoliative dermatitis, have been reported with the use of Lamotrigine. If a severe skin rash or hypersensitivity reaction occurs, it is important to discontinue Lamotrigine and seek medical attention immediately (Committee et al., 2020).

The toxic effects of lamotrigine impurities can vary depending on the specific impurity present and its concentration. Some potential toxic effects associated with impurities in lamotrigine may include:

Genotoxicity: Certain impurities in lamotrigine may exhibit genotoxic properties, which may cause damage to genetic material and increase the risk of mutations or cancer development.

Carcinogenicity: Impurities with carcinogenic properties can pose a significant health risk if present in lamotrigine formulations, which may lead to the development of cancer upon prolonged exposure.

Neurotoxicity: Impurities with neurotoxic effects may affect the central nervous system, resulting in adverse neurological symptoms or disabilities.

Hepatotoxicity: Some impurities in lamotrigine may have hepatotoxic properties, causing liver damage and affecting its normal function.

Reproductive and Developmental Toxicity: Certain impurities can affect reproductive health and fetal development if present in lamotrigine formulations, posing risks to pregnant individuals and their offspring.

Allergic Reactions: Impurities may trigger allergic reactions in individuals who are sensitive to certain compounds, resulting in hypersensitivity reactions or other allergic manifestations.

Therapeutic uses

Lamotrigine is used for the treatment of several neurological conditions, including:

- Epilepsy: Lamotrigine is commonly used as an antiepileptic drug to treat various types of seizures, including partial seizures and tonic-clonic seizures.
- Bipolar disorder: Lamotrigine is used as a mood stabilizer in the treatment of bipolar disorder to help prevent mood swings and manic or depressive episodes.
- Schizophrenia: Lamotrigine has been investigated as a potential adjunctive therapy in the treatment of schizophrenia, particularly for negative symptoms such as apathy and depression.
- Neuropathic pain: Lamotrigine has been shown to be effective in treating neuropathic pain conditions, such as post-herpetic neuralgia and painful diabetic neuropathy.
- Depression: Lamotrigine has been used as an antidepressant for the treatment of major depressive disorder and has been found to be effective for some patients.

It is important to note that Lamotrigine is not approved for the treatment of all these conditions by regulatory agencies and the use of Lamotrigine for a particular condition should be guided by a healthcare provider based on individual patient's needs and medical history (Jacob and Nair, 2016).

Impurity A

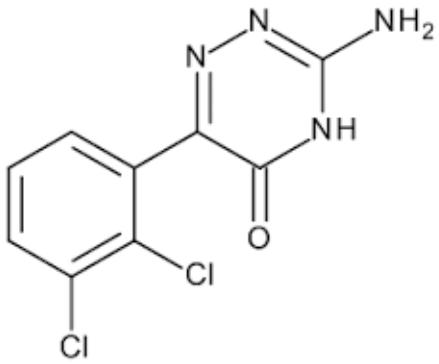

Figure 2.2. 3-Amino-6-(2,3-dichlorophenyl)-1,2,4-triazin-5(2H)-one

Molecular Formula	: $C_9H_6C_{l2}N_4O$
Molecular Weight	: 257.08
Chemical Name	: 3-Amino-6-(2,3-dichlorophenyl)-1,2,4-triazin-5(2H)-one
Appearance	: White, powder.
Solubility	: Soluble DMSO, methanol, very slightly soluble in water
Melting point range	: 161– 121°C
Storage	: 2- 8°C

In the case of 3-Amino-6-(2,3-dichlorophenyl)-1,2,4-triazin-5(2H)-one (Lamotrigine), the term "Impurity A" refers to a specific substance that is present in the drug but is not part of its intended structure. The presence of Impurity A in Lamotrigine can impact the quality and safety of the drug and its efficacy in treating neurological conditions. Further information about Impurity A in Lamotrigine, including its chemical structure, potential health effects, and regulatory standards (Ravisankar and Babu, 2012).

Impurity F

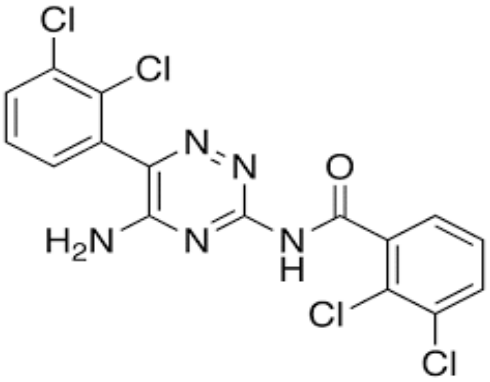

Figure 2.3. N-[5-Amino-6-(2,3-dichlorophenyl)-1,2,4-triazin-3-yl]-2,3-dichlorobenzamide

Molecular Formula	: $C_{16}H_9Cl_4N_5O$
Molecular Weight	: 429.09
Chemical Name	: 3-Amino-6-(2,3-dichlorophenyl)-1,2,4-triazin-5(2H)-one
Appearance	: White to Off-White Solid
Solubility	: DMSO (Slightly), Methanol (Slightly, Heate)
Melting point range	: >235°C
Storage	: -20°C, Inert atmosphere, Light sensitive

Impurity F N-[5-Amino-6-(2,3-dichlorophenyl)-1,2,4-triazin-3-yl]-2,3-dichlorobenzamide is a chemical contaminant found in the drug substance lamotrigine. It is composed of a triazine ring with an amino group and a benzamide group, both of which are substituted with chlorine atoms. This impurity can affect the quality and safety of the lamotrigine drug and its presence is monitored and controlled during the drug's production process to meet regulatory requirements. The presence of impurities in pharmaceuticals is a common issue and the industry closely monitors the levels of these contaminants to ensure the safety and efficacy of the final product (Ashton et al., 1999).

3. LITERATURE REVIEW

Analytical viewpoints refer to the approach or perspective used to analyze data or information. In the context of impurities in the drug substance lamotrigine, the analytical viewpoint would refer to the methods and techniques used to identify, quantify, and characterize these impurities. These methods could include chromatographic techniques, such as HPLC, as well as other analytical methods, such as spectroscopy or mass spectrometry. The analytical viewpoint would also consider the theoretical perspectives of the analysis, such as the underlying mechanisms of degradation or the chemical properties of impurities that are relevant to the analysis. The analytical viewpoint in the context of impurities in lamotrigine is crucial for ensuring the quality and safety of the drug substance and for understanding how impurities can impact the efficacy and stability of the drug.

There are few analytical methods in the literature for determining Lamotrigine impurities in tablets and pharmaceutical formulations.

Despite this, various analytical methods have been reported for the determination of lamotrigine and its impurities in pharmaceutical dosage forms and biological matrices, providing valuable information about the molecule's behavior under different chromatographic conditions

Youssef and Taha (2007) have developed and validated three methods for the determination of lamotrigine in the presence of its impurity, 2,3-dichlorobenzoic acid. These methods included a spectrophotometric method, a TLC separation method, and an HPLC method. The spectrophotometric method used p-chloranilic acid to form a colored product with a maximum wavelength of 519 nm. The TLC method used ethyl acetate, methanol, and ammonia 35% as a mobile phase, and the HPLC method used acetonitrile, methanol, and potassium orthophosphate. The methods were successfully applied to bulk powder, dosage form, and the presence of impurities, and were validated according to USP guidelines. The results showed that there was no significant difference between the three methods and a reported one, and that all three methods had high accuracy and sensitivity.

Narender Rao et al., (2012) have reported on the process development of Lamotrigine and the identification of five unknown peaks observed in HPLC analysis. These peaks were identified as impurities and were individually synthesized and characterized based on their spectral data. The study describes the formation, synthesis,

and characterization of these impurities. The authors used infrared spectroscopy (IR), mass spectroscopy, and nuclear magnetic resonance spectroscopy (NMR) to determine the structures of these impurities and confirm their identity. The results of this study provide a deeper understanding of the impurities found in Lamotrigine and the methods used to identify and characterize them.

Carrier et al., (2008) have developed an analytical method for the trace analysis of a synthetic impurity, 14W80, in lamotrigine. They used LC-MS/MS method with APCI and MRM for adequate sensitivity and specificity in the analysis. The authors used an Agilent 1100 HPLC and an API4000 triple quadrupole mass spectrometer for the analysis. The separation of lamotrigine and 14W80 was achieved using a gradient profile of acetonitrile and ammonium acetate, The HPLC gradient was set to 15/85 A/B volume fraction for 10min with a linear gradient up to 95/5 A/B volume fraction over 10 min. A Luna C18 (2) 50 mm×2 mm, 3.5 μm (Phenomenex, with a flow rate of 0.4 mL/min was used. The detection limit of 14W80 was 25 ppb mass fraction relative to lamotrigine, which was improved to 2 ppb using SPE.

Reddy et al., (2008) have reported on the impurity profile of Lamotrigine, a medication. In the preparation process, five potential impurities were detected in intermediate 2 through HPLC and LC-MS analysis. The molecular weight of these impurities is the same and they were characterized as isomeric impurities of intermediate 2. During the conversion of intermediate 2 to Lamotrigine, the same impurities were converted and reflected in the final product, characterized as different compounds. A high-performance liquid chromatography method was developed to separate these impurities and to quantify them. The method used a C18 column with a mobile phase consisting of ammonium acetate and methanol with a pH of 4.5, and UV detection at 210 nm. The method was successful in separating the impurities, which ranged from 0.05 to 0.10% in the presence of the parent compound.

Ashton et al., (1999) have developed an analytical method for the detection of trace amounts of the principal synthetic route indicative impurity in lamotrigine (3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine). The method involves the preconcentration of a sample extract by normal-phase high-performance liquid chromatography (HPLC) and subsequent analysis by reversed-phase HPLC-thermospray mass spectrometry (TSP-MS). The sample extraction and concentration process include a preliminary separation of the impurity from the lamotrigine using semipreparative normal-phase

chromatography and the use of an organic solvent. The collected fraction is then subjected to reversed-phase HPLC-TSP-MS. The study also looked at the impact of ultrasonic extraction on the method. When the method was applied to lamotrigine tablets, a shake flask partitioning step using 1 mg/ml EDTA in water-dichloroethane was used instead of ultrasonic extraction. The results showed that the route indicative impurity could be detected in the 50-100 ppb range with detection limit and recovery measurements.

Ravisankar Babu, (2012) the authors investigated lamotrigine, a benzotriazine derivative used to treat disorders of the central nervous system. During the process development of lamotrigine, five unknown peaks were observed in HPLC analysis, which were identified and characterized as impurities based on their spectral data (IR, Mass, and NMR). The authors synthesized each of these impurities individually and assigned their structures as 2-(2,3-dichlorophenyl)-2-(guanidiny limino) acetonitrile, N-guanidinyl-2,3-dichlorobenzamide, 3-amino-6-(2,3-dichlorophenyl)-4H-1,2,4-triazin-5-one, N-[5-amino-6-(2,3-dichloro phenyl)-1,2,4-triazin-3-yl]-2,3-dichloro benzamide, and 3,5-bis-(2,3-dichloro benzamido)-6-(2,3-dichloro phenyl)-1,2,4-triazine, respectively. The authors described the formation, synthesis, and characterization of these impurities.

Michail et al., (2013) have reported a method of Stress Degradation Assessment of Lamotrigine. In this study, a sensitive and stability-indicating HPTLC method was developed to determine lamotrigine. Lamotrigine was exposed to various stress conditions including heating in acidic, basic, and neutral media, as well as oxidative stress, humidity, high temperature, and direct sunlight. The separation of lamotrigine from degradation impurities was achieved using TLC silica gel plates and a mobile phase composed of ethyl acetate, methanol, and ammonia. The linear regression analysis showed good linearity over the concentration range of 10-300 ng/spot and the forced degradation studies revealed that lamotrigine is susceptible to degradation under acidic, basic, neutral, and oxidative conditions, with the highest degradative potential being alkaline-induced hydrolysis. On the other hand, the drug was found to be stable under heat, humidity, and daylight stress factors.

The developed HPTLC method was applied to analyze commercial tablet formulations and the results showed no degradation of the drug in the analyzed marketed products. The HPTLC instrument used in the study was a Camag Linomat IV sample applicator and a Camag TLC scanner III. The TLC plates were made of silica gel and aluminum and the mobile phase consisted of a 17:2:1 ratio of ethyl acetate-methanol-ammonia. The chromatogram was developed and scanned densitometrically at 310 nm using a deuterium lamp.

El-Enany et al., (2012) have developed a sensitive, simple, and selective spectrofluorimetric method for the determination of LAM in pharmaceutical formulations and biological fluids. The method is based on the reaction of Lamotrigine with o-phthalaldehyde in the presence of 2-mercaptoethanol in a borate buffer of pH 9.8, resulting in the formation of a highly fluorescent derivative that can be measured at 448 nm after excitation at 337 nm. The fluorescence-concentration plot was found to be rectilinear over the range of 0.1–1.0 μg mL^{-1} with a lower limit of detection of 0.02 μg mL^{-1} and a limit of quantification of 0.06 μg mL^{-1}. The proposed method was applied to the analysis of commercial tablets and found to be effective, with results comparable to a reference method in terms of accuracy and precision. Additionally, the method was applied to in-vitro and in-vivo determination of LAM in spiked and real human plasma, with mean percentage recoveries of 95.78±1.37 and 90.93±2.34, respectively. Interference from co-administered drugs was also investigated and a reaction pathway with o-phthalaldehyde was proposed.

Szulfer et al., (2013) have reported Evaluating the performance of different stationary phases in the liquid chromatography analysis of LAM and related compounds. The study was conducted using 28 brands of stationary phases and a system suitability test was performed to determine their suitability for the lamotrigine separation. The liquid chromatography was carried out using a Waters system and the results were analyzed using factor analysis as a chemometric technique. The selected stationary phase was Hypersil BDS 100-C18 150×4.6 mm. The study found that the system suitability test was effective in determining the suitability of the columns for the lamotrigine separation.

Srinivasulu et al., (2009) have published a study that describes the development of a simple and time-efficient liquid chromatography method for evaluating the quality and stability of LAM and its impurities. The method uses a Symmetry C18 column and a mobile phase of aqueous trifluoroacetic acid and acetonitrile. It was validated for linearity, accuracy, precision, specificity, and robustness, and was found to be linear in the range of 5-60 μg mL^{-1} with a correlation coefficient of 0.9999. The method can be used to evaluate the quality and stability of regular production and stability samples of lamotrigine and separates all impurities. This is claimed to be the first validated stability-indicating liquid chromatography method for LAM.

Čolović et al., (2019) have developed an analytical method for LAM, and its impurities A and G in tablets using chaotropic chromatography.

The authors used a robust optimization approach and Box-Behnken design to evaluate the effect of acetonitrile content, chaotropic agent concentration, and pH on

critical method attributes. Monte Carlo simulations were used to estimate the predictive distribution of responses. A design space with 95% minimum reliability was defined and the method was validated using a Finnigan Surveyor chromatographic system and Zorbax Extend C18 columns with a particle size of 5 μm, a column dimension of 150 mm × 4.6 mm, and UV detection at 270 nm. The flow rate was set at 1 mL min^-1 and the column temperature was maintained at 30°C The authors reported active main effects and interactions in the obtained models and confirmed the reliability of the method for routine pharmaceutical analysis.

Sripalakit et al., (2008) have developed a high-performance liquid chromatography method for determining lamotrigine in a dissolution medium and tablet formulation. The chromatographic separation was performed using a Gemini C18 column (250 mm x 4.6 mm i.d., 5 mm) with a mobile phase of 0.05 M $(NH_4)H_2PO_4$-acetonitrile (68:32, v/v, pH 2.68) delivered at a flow rate of 1.2 mL/min, and detected by ultraviolet at 265 nm and was validated for specificity, linearity, accuracy, and precision. The conditions for the dissolution test of lamotrigine tablets were established as a paddle stirring speed of 50 rpm, a medium volume of 900 mL, a temperature of 37±0.5 °C, and using three different pH solutions as dissolution media. The proposed method was successfully applied for the quality control of commercial lamotrigine tablets and for comparison of their in vitro performance.

4. EXPERIMENT AND METHOD

This section reports the various aspects relating to HPLC method for the determination of impurities in the fixed-dose tablet.

4.1. Materials

4.1.1. Chemicals

Individual standards of lamotrigine were Purchased from Sigma-Aldrich (St. Louis, MO, USA) with a minimum purity level of 99.0%, Impurities A and F are Purchased from Biosynth Carbosynth market with purity 99.5%. The lamotrigine tablets containing 50 mg were obtained from three countries: Turkey, Yemen, and Indian markets. The inactive ingredient uses as the drug–matrix including microcrystalline cellulose, sodium starch glycolate, and lactose were supplied from faculty of Pharmacy. Analytical grade monobasic potassium phosphate with a purity exceeding 99.99% purchased from Sigma-Aldrich, methanol with a purity range of 98-100% was purchased from Sigma-Aldrich, and hydrochloric acid with a minimum purity level of 99.0%, obtained from Fluka and HPLC grade acetonitrile with purity 99.0% were purchased from Merck (Germany), Phosphoric acid, approximately 98% pure, obtained from Fluka Analytical in Switzerland and double distilled water.

4.2. Instruments

- HPLC detector (Agilent, 1260 diode-array detector detector, Germany)
- HPLC (Agilent, 1260 Infinity Binary (consisting of dual pump, deaerator, autosampler and thermostatic column sections) Liquid Chromatography System, Germany)
- Sonication in Ultrasonic bath (Ultrasonics Selec, Vetra, Italy)
- Centrifuge (Centrifuge, R 5810, Germany)
- Filter paper (4 mm 0.22 μm)
- Filter paper (pore size 0.45 μm)
- pH meter (Multi-model pH meter, Seven brand, Mettler Toledo, Switzerland)
- Pipettes (20-100 μL and 100-1000 mL; Eppendorf, Germany)
- Column (Phenomenex C18 column, 4.6 mm, 250 mm, 4 μm, United States)
- Electronic Balance (Mettler Toledo, XP205, Switzerland)
- Vortex mixer (Velp Scientifica, ZX3, Italy)
- Ultrapure water device (Millipore, Ultrapure Water System, France)

- Refrigerator (Arçelik, No Frost, Electronic, Türkiye)

4.3. Chromatographic system and conditions

Using an Agilent 1260 and a Diode-Array detector, the HPLC technique was applied. Utilizing Agilent ChemStation software (Rev. B. 04.03-SP2 (105)), the chromatographic data were collected. The separation process required using a C18 Phenomenex column in gradient mode (4.6 mm, 250 mm, 4 μm) to help separate impurity. The development of perfect conditions depended on the specific drugs under evaluation. The mobile phase consisted the following two components:

A) KH_2PO_4 with pH 3.5.

B) ACN (acetonitrile).

The flow rate was adjusted to 1.0 mL/min, and the injection volume was 10 μm.

An appropriate wavelength is critical for the concurrent quantification of the drug and two impurity substances. The identity of the most efficient wavelength for detecting the drug and two impurities in the mobile phase with includes wavelength scanning spanning the 200–400 nm range using Diode Array Detection, as well as was carried out detection at 215 nm.

4.4. Preparation of solutions

4.4.1. Preparation of mobile phase

For this experiment, the mobile phase was developed as follows: Mobile phase A), 1400 mg of KH_2PO_4 were dissolved in 1 L of double-distilled water to create a buffer solution of monobasic potassium phosphate at a concentration of 0.02 M. The pH of the mobile phase was subsequently adjusted to 3.5 by adding orthophosphoric acid. Mobile phase B) ACN. The resulting solution was then degassed in a sonicator for 15 minutes before being filtered through a 0.45 m membrane filter.

This prepared buffer solution was subsequently introduced into the HPLC system using a gradient elution method. The specific gradient profile employed for solvent B was as follows: At 5% at 0 min, 15% at 5 min, 80% at 14 min, and 90% at 22 min.

4.4.2. Preparation of of standard solutions

1.0 mg of lamotrigine was weighed accurately and taken into a sample tube. 1.0 mL of methanol was added and dissolved, and properly mixed in an ultrasonic bath for 15 min. Thus, a solution containing 1000 μg/mL lamotrigine was obtained.

4.4.3. Preparation of impurity standards

The stock solution of impurities was prepared by weighing 0.5 mg of Impurity A and Impurity F on a precision balance. These were then transferred into a 1.5 mL sample tube, followed by the addition of methanol. The mixture was sonicated for 15 minutes and made up to 1.0 mL with methanol. As a result, a solution containing 500 μg/mL of Impurity A and Impurity F. Appropriate dilutions of this stock solution was prepared using methanol to obtain solutions of known concentrations to be used for linearity studies and assay purposes.

4.4.4. Standard solutions of drug and related substances in the drug–matrix

Substances in the drug–matrix samples of 1 mg LAM or 0.5 mg related substances (weighing by aliquot method) mixing with appropriate proportions of the drug–matrix components. The mixture was dispersed and diluted to 1.0 mL using methanol. The solution was sonicated for 15 min, centrifuged at 4000 rpm for 10 min and the supernatant was used to prepare methanolic solutions containing various quantities of drug or related substances.

4.4.5. Standard solution

Considering investigating pharmaceutical items' qualitative and quantitative compose was 25 μg/mL of pharmaceuticals, 10 μg/mL for each impurity, and a specific amount of adjunct compounds, according to the inquiry into the qualitative and quantitative content of drugs.

4.4.6. Assay sample preparation

The pharmaceutical product, namely LAM tablets, was systematically evaluated using the proposed and validated approach. In this analytical process, each tablet underwent a precise weighing procedure, followed by the calculation of the mean weight derived from ten tablets. Subsequently, the tablets were meticulously pulverized into a

fine powder using a mortar and pestle. An equivalent amount of powder, corresponding to the calculated mean weight of a single tablet, was dissolved and homogenized in a 50 mL solution of methanol. The mixture was agitated thoroughly to facilitate dissolution and then brought to its predetermined volume, ensuring a uniform distribution. Following this, the solution was subjected to centrifugation, and one milliliter of the resulting supernatant was methodically diluted to achieve a concentration of 25 µg/mL. To further prepare the sample for HPLC analysis, 5.0 mL of this diluted solution were meticulously filtered through a Millipore filter with a pore size of 0.45 µg/mL. Subsequently, 10 µL of the filtered solution was accurately injected into the HPLC column for further analysis.

4.5. Development of the method

To develop an HPLC technology capable of simultaneously determining the presence of impurities A and F, optimization studies were carried out. This comprehensive inquiry encompassed an extensive array of factors, spanning wavelength, selection of column, pH levels, concentration of buffer solution, temperature variations, mobile phase gradient profile, flow rate, and the volume of injection. A thorough evaluation of peak shapes and peak area values served as the foundation to selecting the most suitable settings.

4.5.1. Optimization of wavelength

The wavelengths that have been investigated in the literature for active substances were examined. To study the spectra of the analytes, the UV detector was adjusted to the following wavelengths: 210 nm, 215 nm, 226 nm, 270 nm, and 305 nm. The optimal wavelength was determined to be 215 nm.

4.5.2. Determination of mobile phase

Several mobile phases have been evaluated throughout the technique development phase. ACN developed as the preferred organic solvent for the individual evaluations of impurities A and F, according to the literature currently in use. Furthermore, it was discovered that ACN has a better ability than methanol to produce superior peak shapes. As a result, ACN was used as the preferred organic solvent, and several phosphate buffer solutions were used to achieve pH changes.

- Phosphate buffer solutions at pH 2.0 were prepared using a combination of orthophosphoric acid (H_3PO_4) and monpotassium phosphate (KH_2PO_4)
- To prepared phosphate buffer solutions with a pH of 3.5, orthophosphoric acid (H_3PO_4) was utilized in combination with monopotassium phosphate (KH_2PO_4).
- To prepared phosphate buffer solutions with a pH of 6.0, monosodium phosphate (NaH_2PO_4) and sodium hydroxide (NaOH) were employed in the formulation process.

The optimal mobile phase composition was established through a rigorous evaluation of pH and buffer solution concentration in optimization studies, as well as the analysis of gradient profiles at pH 3.5.

4.5.3. Select of column

After a series of practical experiments on the quality of the columns, the column (C18, 250 mm, 4.6 mm, 4 μm) was selected. Based on the preferences of this column, column C18 (L1) 25 cm in length and 4.6 mm in diameter were used with all the optimal conditions installed.

4.5.4. Optimization of mobile phase pH

The pH of the mobile phase was then adjusted by the use of experiments to evaluate several buffer solutions at pH levels of 2.5, 3.0, 3.5, 4.5, and 6.0. These investigations focused on evaluating peak areas and peak shapes, which finally helped choose the ideal pH level.

4.5.5. Optimization of buffer concentration

After optimizing the pH of the mobile phase, the assessment of buffer concentration involved analyzing solutions at concentrations of 20.0 mM, 100.0 mM, 150 mM, and 150.0 mM. The selection of the resulted from a careful analysis of peak area values and peak shape characteristics, the ideal concentration level was selected.

4.5.6. Optimization of temperature

A range of temperatures, including 25°C, 30°C, 35°C, and 40°C, were methodically investigated to determine the right temperature. This assessment included a thorough examination of peak area and peak shape data, which eventually resulted in the choice of the ideal temperature.

4.5.7. Optimization of gradient profile

Different gradient profiles were evaluated in specifics, and peak shapes and peak area values were carefully examined. Subsequently, the optimal gradient profile was selected based on these evaluations.

4.5.8. Optimization of injection volume

A thorough analysis of peak forms and peak area values for injection volumes ranging from 5.0 to 20 µL were done in order to optimize the injection volume. Based on the findings of this experiment, the best injection volume was chosen.

4.5.9. Optimization of flow rate

A thorough investigation was done to determine how to best use the injection volume. This investigation looked at peak areas and peak shapes for flow rate ranging from 0.5 to 1.5 mL/min. The results of this experiment were used to determine the ideal flow rate.

4.6. Validation of the method

The analytical method was validated through a series of laboratory studies, with the main objective to verify that the method's performance characteristics suitable with the prerequisites for its intended analytical application. In order to achieve this objective, a carefully designed experimental design was used to show through evidence the test method's ability to consistently deliver dependable, reliable, and reproducible results within the set acceptability levels. It is important to note that the specific acceptance criteria for the aforementioned validation parameters were carefully defined within the different experimental designs. Concurrently, detailed records of observations and findings were carefully kept on dedicated technique validation data sheets. Following that, the outcomes from the method's validation efforts were clearly presented, and

analytical inferences were formed from these results. Finally, sound conclusions were reached by carefully interpreting the results of the method validation process.

4.6.1. Validation parameters

The parameters have been provided include the system suitability test, and validation parameters such as accuracy, precision, specificity, linearity, stability, and robustness (EMEA, 2006).

4.6.1.1. System suitability test

The standard solution was prepared using lamotrigine and an impurity standard mixture solution, following the test method. To determine these parameters, a study was conducted using a standard mixture solution with a concentration of 10 µg/mL, and the results were obtained from six separate injections and was injected into the HPLC system. Standard chromatograms were used to calculate the capacity factor, tailing factor, theoretical plate number, resolution, and RSD of the peak areas in order to assess the system suitability parameters.

4.6.1.2. Accuracy

The accuracy of an analytical method is the measure of the closeness of the obtained value to the true value or the reference value. Accuracy can be demonstrated by several approaches. In our study, accuracy was determined by the recovery method. Solutions were obtained as described below, corresponding to 1.0, 5.0 and 50 µg/mL of the active ingredients and, 0.1, 1.0, and 50 µg/mL levels of each impurity.

4.6.1.3. Precision

Precision assessments were performed using intraday and interday for analysis six replicate sample preparations as per method (n=6) evaluations. The Relative Standard Deviation (RSD) of the recovery values acquired from the Quality Control (QC) standard solutions was used to evaluate the precision of the approach. Analyses of these solutions were conducted with six replications.

4.6.1.4. Specificity

Evaluate the presence of any interferences stemming from the compelled degradation analysis Procedure.

LAM was put through a comprehensive suite of tests that included thermal degradation, photo-degradation, oxidative degradation, base-induced degradation, acid-induced degradation, and base-induced degradation. Following careful preparation of blank and stressed samples for each degradation condition, injections were carried out. For the primary peak in each of the prepared degraded samples, the peak purity index was evaluated.

- No additive interference:

  By injecting a blank, a placebo, a sample, and a standard composed of the same substance, this was proved. Lamotrigine retention time does not reach a peak. This demonstrates that there was no interference from the excipients or the blank.

  A chromatogram for the placebo is shown in Figure 2 and one for the blank - diluents is shown in Figure 1. The system precision chromatogram is displayed in Figure 3. The sample's chromatogram is displayed in Figure 4.

- The degradation products do not cause any interference:

  Demonstrated this carrying out forced degradation of sample with 5 N HC1, 1 N NaOH, 30% H_2O_2, heating in water bath at 80°C for 15min and keeping under room temperature for 24 hrs. Prepare samples as per test preparation and inject into HPLC with photodiode array detector.

- Acid degradation

  Set up 100 mg LAM tablets, unaltered, into a 100 mL volumetric flask. Incorporate 50 mL of a suitable diluent and sonicate the mixture for 15 minutes. After that, add 5 mL of 5 M HCl and immerse the flask in an 80°C water bath for 15 minutes. After cooling, adjust the volume to the mark with the same diluent and thoroughly homogenize the solution after neutralization with 5 mL of 5 M NaOH.

- Alkali degradation

  A 50-mL volumetric flask was filled with 5.0 mL of the stock solution sample, 10 mL of a diluent, and it was thoroughly mixed after that. Then, 2.0 mL of 1 M NaOH were added, and the mixture was heated for 30 minutes in

a water bath with a temperature of 80°C. 2 mL of 1 N hydrochloric acid (HCl) were added to the resulting solution to neutralize it. The solution was then allowed to cool before being thoroughly mixed and brought to the desired volume with more diluent. A subsequent injection of this prepared solution into the High-Performance Liquid Chromatography (HPLC) system was made.

- Peroxide degradation

  A 50 mL volumetric flask was filled with a 5 mL sample stock solution. After that, 10 mL of the proper diluent was added to the flask. A accurate addition of 0.5 mL of a 3.0% hydrogen peroxide (H_2O_2) solution to the mixture was carried out to start the desired chemical reaction. The resulting mixture was subsequently put through a carefully controlled thermal treatment in a water bath that was thermostatically adjusted at an elevated temperature of 80°C and kept there for five minutes. Following the prescribed thermal exposure, the solution was allowed to naturally equilibrate to ambient temperature. Subsequently, the solution was expanded to the designated calibration mark on the volumetric flask using the aforementioned diluent, with meticulous attention to volumetric precision. Post volumetric adjustment, thorough mixing was employed to ensure complete homogenization of the solution. The sample was injected, in accordance with this procedural, was subsequently introduced into the HPLC.

- Thermal degradation

  Transfer of a 5 mL portion of a stock solution into a 50 mL volumetric flask. Subsequently, 10 mL of an appropriate diluent was added and thoroughly mixed. The resulting mixture was then subjected to controlled heating at 80°C for 30 minutes in a water bath. After cooling to room temperature, the solution was diluted to the volumetric mark using the same diluent and mixed thoroughly. Finally, this carefully prepared solution was injected into the HPLC system for subsequent analytical assessment.

4.6.2. Linearity

The linearity curve was created by using a series of eight concentration levels (from 1.0 to 100 µg/mL) and (0.1-10 µg/mL), which included both LAM and any associated impurities. These concentration levels were detected concentration in the standard

solution. The use of linear regression analysis was then used to evaluate the association between peak areas and concentrations. Under the chromatographic operating conditions outlined above, 10 µL of each solution was injected. Peak areas vs LAM concentrations were plotted to create calibration curves.

4.6.3. Stability studies

Stability studies were carried out using solutions containing 1.0 and 5.0 µg/mL of LAM standard solution and 1.0, 5.0 µg/mL of IMP A and F standard solutions. In order to examine the stability of LAM solutions in the short term (24 hrs, at room temperature), long term (2 weeks, at -20°C) and after the thawing-freezing cycle (3 cycles), three solutions containing 1.0 and 5.0 µg/mL of standard solution were prepared. While, one of these three solutions was kept at room temperature for 24 hours, the second was kept at -20°C for 2 weeks. The third solution was subjected to a triple freeze-thaw cycle. LAM solution was added to each solution that completed the waiting conditions and three analyzes were performed for each. With the analysis data under each condition, recovery% values were calculated and the stability of standard drug solution under the specified conditions was evaluated.

4.6.4. Robustness

According to the ICH, an analytical procedure's robustness refers to its ability to be unaffected by minor and intended changes to the method parameters. Adjustments to some instrumental parameters that have a major impact on retention were performed in this investigation to evaluate the robustness of the newly proposed approach. By injecting standard solutions (n=5) of LAM, IMP A and IMP F, the impact of these parameter changes on the final results was studied. Adjustments in the pH, column temperature, and flow rate were performed to assess the robustness of the established approach. The buffer pH values of 3.4 and 3.6 were changed by 0.1 pH units, the column temperature was tested at 39°C and 41°C, and the flow rate was measured at 1.1 and 0.9 mL/min, respectively.

4.6.5. Calibration solutions

Six different concentrations of the standard drug solution, ranging from 1.0 to 100 µg/ml, and 0.1-10 µg/ml for IMP were prepared in order to create the calibration curve.

Chromatographic analysis was conducted six times for each of these dilutions. The assessment of linearity was conducted through the utilization of linear regression analysis.

To ensure consistency and accuracy, the chromatography column underwent a pre-injection equilibration period of no less than 30 minutes, during which the mobile phase circulated through the system. Six independent determinations were executed for each solution, and each solution was filtered through a 0.45 μm PTFE filter and transferred to vials. These prepared solutions were then injected into the chromatographic system. Peak area values were obtained for each analyte. Calibration curves for each analyte were constructed by plotting peak area values (y-axis) against concentration (x-axis) using the standard addition method. Subsequently, a correlation plot was constructed by graphing the peak areas obtained at the optimal detection wavelength against the quantities of the respective concentrations that were injected.

5. RESULTS

5.1. Development of the method

5.1.1. Optimization of wavelength

The investigation involved studying the wavelengths of 210 nm, 215 nm, 226 nm, and 270 nm. Methanol was used to dilute stock solutions to obtain the 100.0 µg/mL standard mixing solution used in this investigation. Phosphate buffer solution (A) with a pH of 3.5 and ACN (B) flowing at a rate of 1 mL/min consisting of the mobile phase. The gradient profile, in terms of time and %B, was set as follows: at 0 minutes, 5% B; at 5 minutes, 15% B; at 14 minutes, 80% B; and at 22 minutes, 90% B. The temperature of the column was kept at 40ºC, and the injection volume was 10.0 µL. In light of a comprehensive review of the existing academic literature and in consideration of the spectral attributes of the analytes, the wavelength of 215 nm was designated as the optimal choice. Subsequently, this particular wavelength was employed in the subsequent research endeavors.

5.1.2 Select of column

The study encompassed an evaluation of chromatographic columns identified as L1, L2, and L3. This analysis encompassed the utilization of a standard mixture solution, with a concentration of 100 µg/mL, prepared through dilution of stock solutions with methanol. The mobile phase consisted of a solution with a pH of 3.5 and a concentration of 0.02 M phosphate buffer (referred to as A), in conjunction with acetonitrile (referred to as B). The flow rate of 1 mL/min. The gradient profile, established with consideration to time and the proportion of solvent B (%B), followed this pattern: at 0 minutes, 5% B; at 5 minutes, 15% B; at 14 minutes, 80% B; and at 22 minutes, 90% B. The volume of the sample injected into the column was precisely 10.0 µL, while the column temperature was rigorously maintained at 40ºC. The results, meticulously detailed in Table 5.1, provided empirical validation for the appropriateness of the chosen experimental parameters.

Table 5.1 *Column selection*

No	Type of column	IMPA Peak height	IMPF Peak height
1	**(L1)** Phenomenex (C18, 250 mm, 4.6 mm, 4 µm)	**729**	**691**
2	(**L2)** Zorbax C18 (3 mm, 150 mm, 3.5 µm)	**524**	**455**
3	**(L3)** Inertsil 3ODS (150 x 4.6 mm, 5 µm)	**412**	**478**

5.1.3. Optimization of mobile phase pH

In order to optimize the mobile phase pH, we conducted experiments utilizing buffer solutions with distinct pH values, specifically 2.5, 3.0, 3.5, and 4.5. These buffer solutions were meticulously prepared as outlined in Section 4.5.1. Using appropriately diluted stock solutions with methanol, we prepared 100 µg/mL standard mixing solutions for this study. We used a mobile phase that consisted of 0.02 M buffer solutions (A) and ACN (B), flowing at a steady 1 mL/min. The gradient profile has the following characteristics, indicated by (time, %B): 5% B at 0 min, 15% B at 5 min, 80% B at 14 min, and 90% B at 22 min. In each injection, we used a fixed volume of 10.0 µm. The wavelength selected for analysis was 215 nm, and the column temperature was precisely maintained at 40ºC. The chromatograms acquired during these experiments are visually depicted in Figure 5.1.

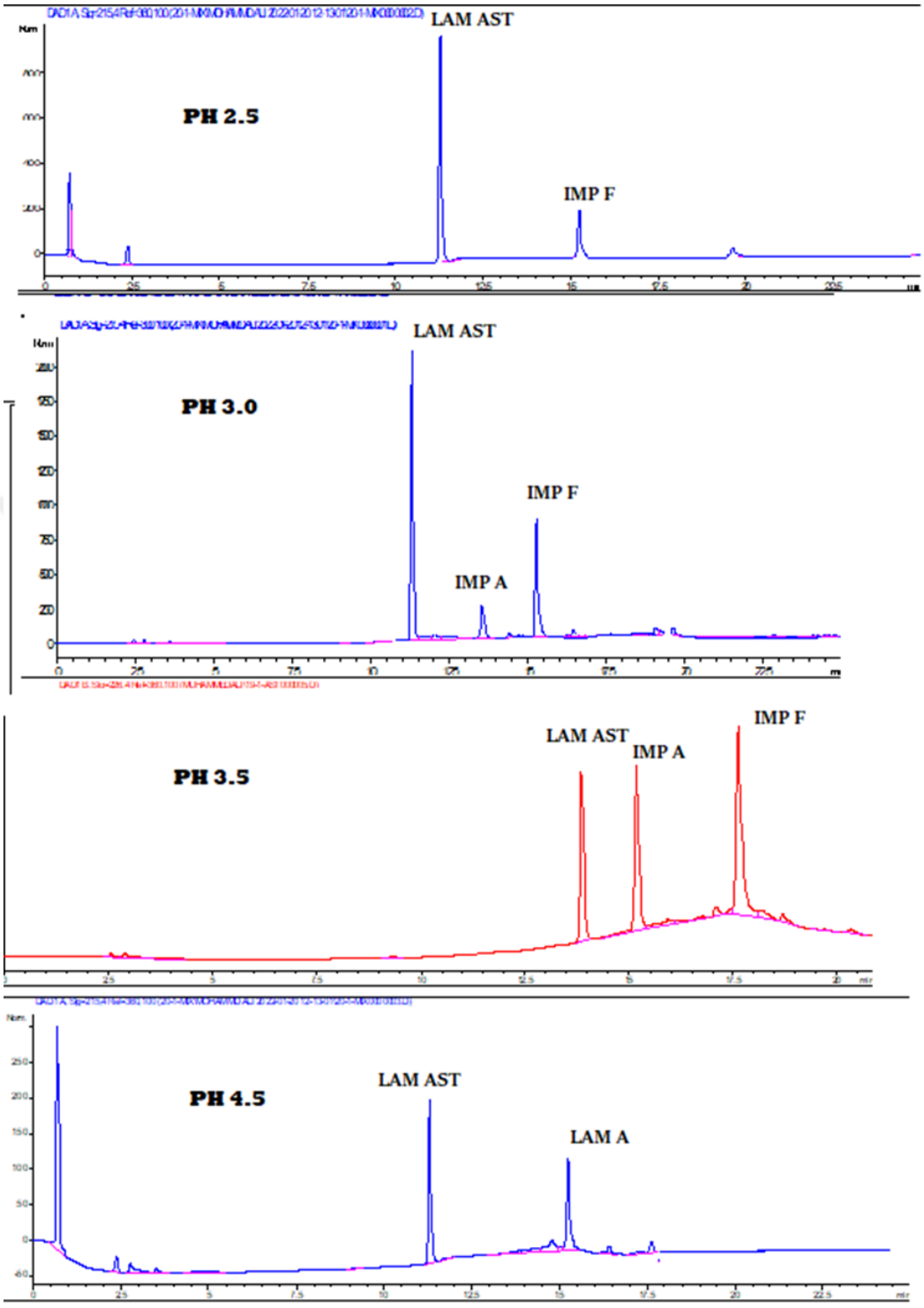

Figure 5.1. *Chromatograms were produced using 0.02 mM buffer solutions and ACN at 1 mL/min flow rate, with a 10.0 µL injection volume, 215 nm wavelength, and 40°C column temperature. This aimed to optimize the mobile phase pH.*

Table 5.2 illustrates the impact of mobile phase pH on the resulting peak area values.

The data highlights the influence of pH variation on peak areas for the compounds IMPA and IMPF. Understanding how pH impacts peak areas is crucial for method optimization, indicating the potential for pH 3.5 to be favorable for IMPA and IMP F and demonstrating varying behaviors of different compounds under different pH conditions. Further exploration and complete data collection at all pH values can lead to a more comprehensive method development for HPLC analysis.

Table 5.2. *The effect of varying the pH of the mobile phase between 2.5 and 4.5 on the resulting peak area values was investigated*

pH	IMPA Peak Area	IMP F Peak Area
2.5	1788.3	4283.5
3.0	2321.5	4159.2
3.5	2450.8	4236.5
4.5	1867.3	-

5.1.4 Optimization of buffer concentration

After optimizing the mobile phase pH, the study proceeded to evaluate buffer concentration. Specifically, phosphate buffer solutions at a pH of 3.5 were tested, varying concentrations at 20.0 mM, 100.0 mM, and 150.0 mM. Our experimental approach involved employing 100 µg/mL standard mixture solutions, which were meticulously prepared by diluting stock solutions with a mixture of water and ACN in a 50:50 ratio. The mobile phase consisted of a pH 3.5 phosphate buffer solution (designated as A) and ACN (B), flowing at a rate of 1 mL/min. The gradient profile, defined in terms of time and B%, was as follows: at 0 min, 5% B; 5 min, 15% B; 14 min, 80% B; and finally, at 22 min, 90% B.

The injection volume was precisely set at 10.0 µL. A wavelength of 215 nm was chosen for detection, and the column temperature was rigorously maintained at 40ºC. The resulting chromatograms have been thoughtfully illustrated in Figure 5.2.

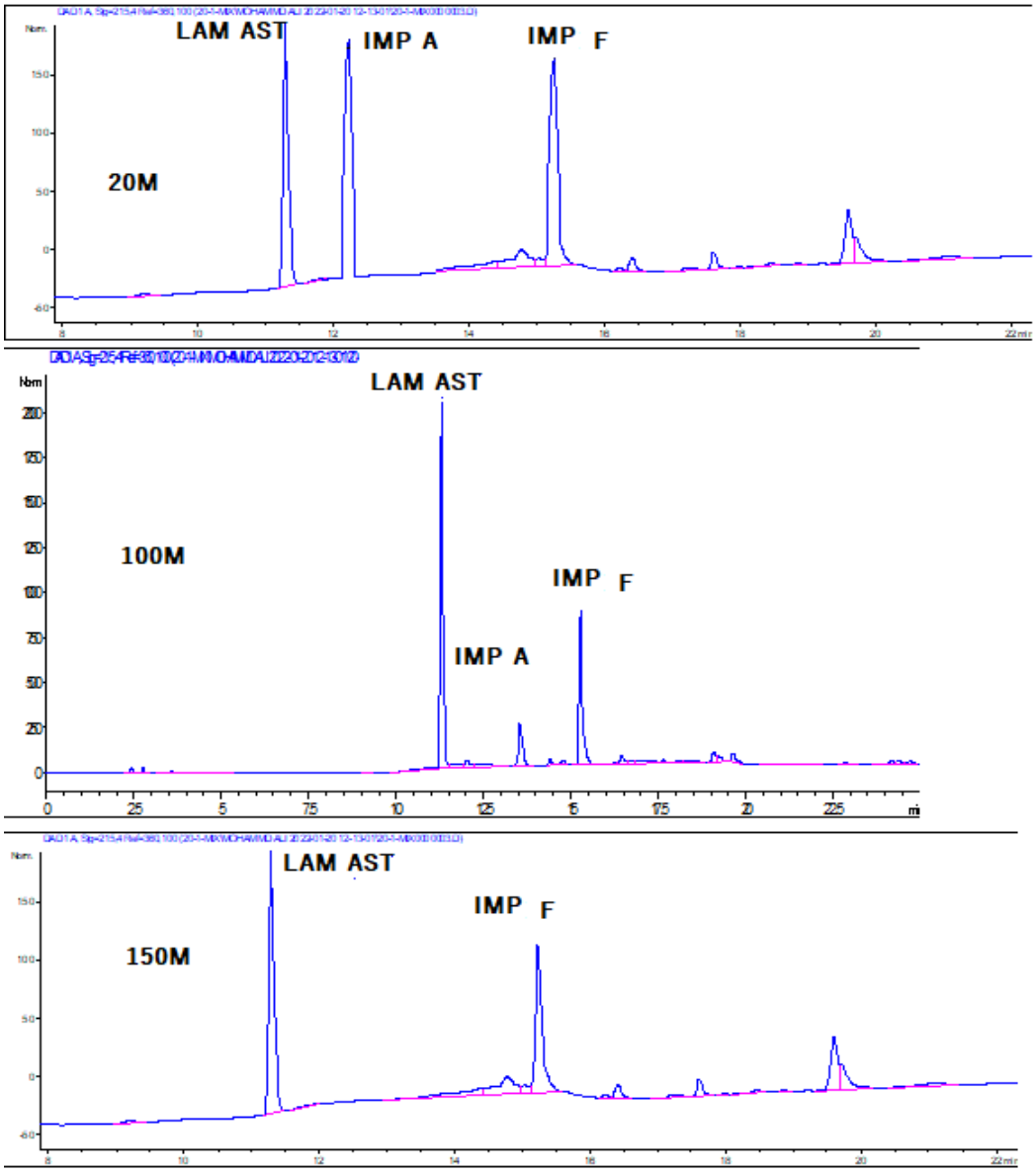

Figure 5.*2* *Effect of buffer concentration ranging from 20.0 to 150.0 mM on the recorded peak area values.*

The impact of buffer concentration on the measured peak area values is presented in the associated table (Table 5.3).

Table 5.3. *Effect of buffer concentrations to the peak area values.*

Buffer Concentration (µg/mL)	IMPA Peak Area	IMPF Peak Area
20.0	1339	1213
100.0	7025	6065
150.0	-	9097

The highest recorded peak area values and symmetrically were achieved through the utilization of a 20.0 µg/mL phosphate buffer solution. As a result of this observation, 20.0 µg/mL was identified as the optimal buffer concentration. Subsequently, buffer solutions with this concentration were employed in the subsequent phases of the study.

5.1.5 Optimization of temperature

In the optimization temperature, a range of temperatures, including 25℃, 30℃, 35℃, and 40℃, was systematically examined. The research utilized 10 µg/mL standard mixture solutions, prepared by diluting stock solutions with methanol. Acetonitrile (B) and a 20.0 mM pH 3.5 phosphate buffer solution (A) were used as the mobile phase, with a flow rate of 1 mL/min. The gradient profile, defined by time and %B, followed this sequence: beginning at 0 min with 5% B, rising to 15% B at 5 min, increasing to 80% B by 14 min, and reaching 90% B at 22 min. During every trial, a consistent 10.0 µL injection volume was employed, with detection carried out at a 215 nm wavelength. The resultant chromatograms have been visually represented in Figure 5.3.

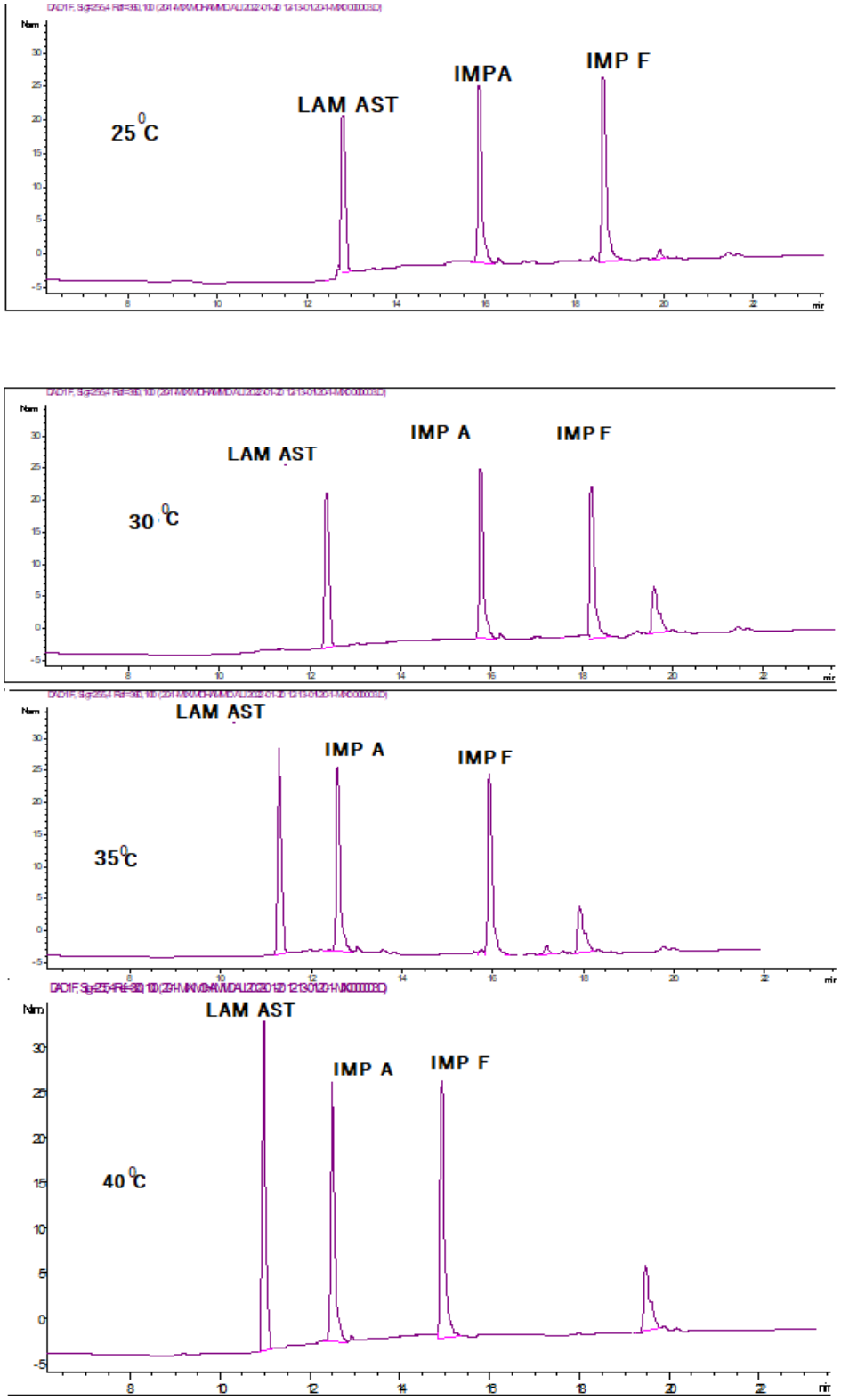

Figure 5.3. *The chromotograms produced by optimizing temperature at 25℃, 30℃, 35℃, and 40℃.*

The impact of temperature on the measured peak area values is presented in Table Table 5.4.

Table 5.4. *Temperature Impact on Peak Area Values (25 – 45ºC Range)*

Temperature (ºC)	IMPA Peak Area	IMPF Peak Area
25	3749.2	7986.7
30	3953.6	8046.5
35	4089.8	8249.4
40	4153.6	8546.5
45	2560.3	5076.7

Observations indicated that as the temperature decreased, the peak area values also decreased, and at temperatures exceeding 40ºC, peak splitting was observed.
Conversely, elevated temperatures resulted in shorter retention times. Based on these findings, the optimal temperature was determined to be 40ºC, attributed to its association with the highest peak area values. This selected temperature was subsequently employed for the ensuing phases of the study.

5.1.6 Optimization of gradient profile

To evaluate the influence of gradient profiles, multiple profiles named A, B, C, D, and E were examined, outlined in detail in Table 5.5. 100.0 µg/mL standard mixing solutions were used in these studies, which were created by diluting stock solutions with methanol. The mobile phase, running at a rate of 1 mL/min, comprised a 20.0 mM pH 3.5 phosphate buffer solution (referred to as A) combined with acetonitrile (B). Consistently, an injection volume of 10.0 µL was used for each run, with detection conducted at a wavelength of 215 nm. The temperature was maintained at 40ºC throughout the study.

The chromatograms obtained have been visually depicted in Figure 5.4. Additionally, the characteristics of the applied gradient profiles are outlined in Table 5.6, while the influence of these profiles on the acquired peak area values is detailed in Table 5.5.

Table 5.5. *Characteristics of Employed Gradient Profiles*

Gradient Profile	Characteristics of the System				
A	Time (min)	0	10	15	20
	ACN%	20	60	60	20
B	Time (min)	0	5	10	15
	ACN%	30	60	60	30
C	Time (min)	0	5	10	15
	ACN%	30	70	70	30
D	Time (min)	0	5	14	22
	ACN%	5	15	80	90
E	Time (min)	0	5	10	15
	ACN%	30	90	90	30

Table 5.6. *Shows how the gradient system affects the values of the measured peak areas*.

ACN Ratio (%)	IMP.A Peak Area	IMP.F Peak Area
20 (Gradient A)	3858.7	8696.7
30 (Gradient B)	4151.4	9061.3
30 (Gradient C)	3821.9	7017.1
30 (Gradient D)	3491.9	6282.6
30 (Gradient E)	3171.8	5141.2

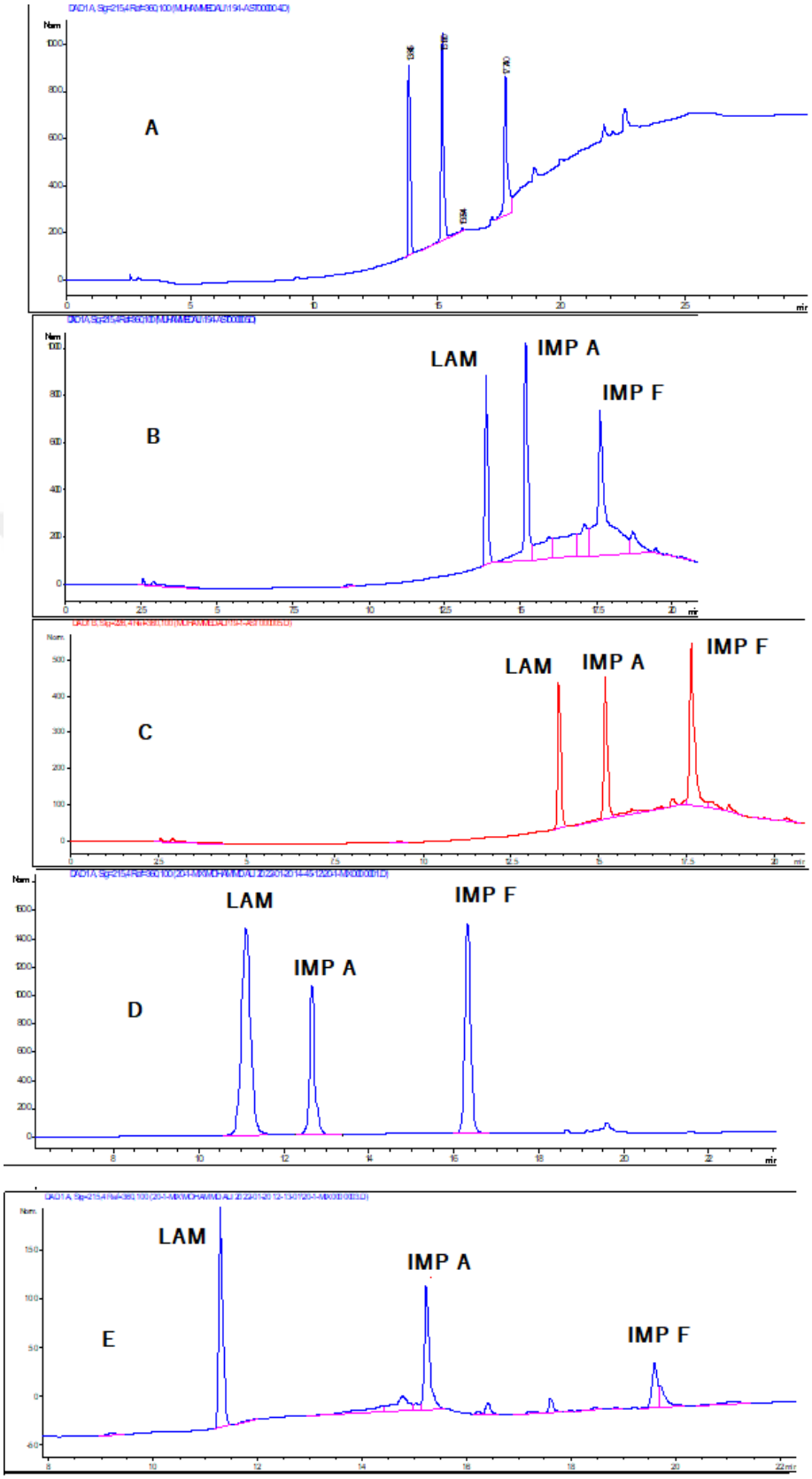

Figure 5.4. Displays the chromatograms obtained through the utilization of gradient systems A, B, C, D.

The objective of the gradient profile optimization was to get symmetrical, well-defined peaks with no tailing. Of particular, gradient profiles D was employed, which produced no peak tailing and shorter retention durations. Following a thorough analysis, profile D was found to be the ideal gradient profile, mainly because it had higher peak area values than gradient systems E. Gradient profile D was then chosen to be used in the latter stages of the investigation.

5.1.7 Optimization of injection volume

To evaluate the influence of various injection volumes, a systematic investigation was conducted, encompassing volumes of 5.0 µL, 10.0 µL, 15.0 µL, and 20.0 µL. These experiments were conducted using 100 µg/mL standard mixture solutions, which were meticulously prepared through dilution from stock solutions using methanol. The mobile phase, flowing at a rate of 1 mL/min, comprised a 20.0 mM pH 3.5 phosphate buffer solution (referred to as A) and acetonitrile (B). For every run, a steady injection volume of 10.0 µL was used, and detection occurred at a wavelength of 215 nm. The column temperature was maintained at 40ºC throughout the study. The chromatograms that were produced are shown in Figure 5.5.

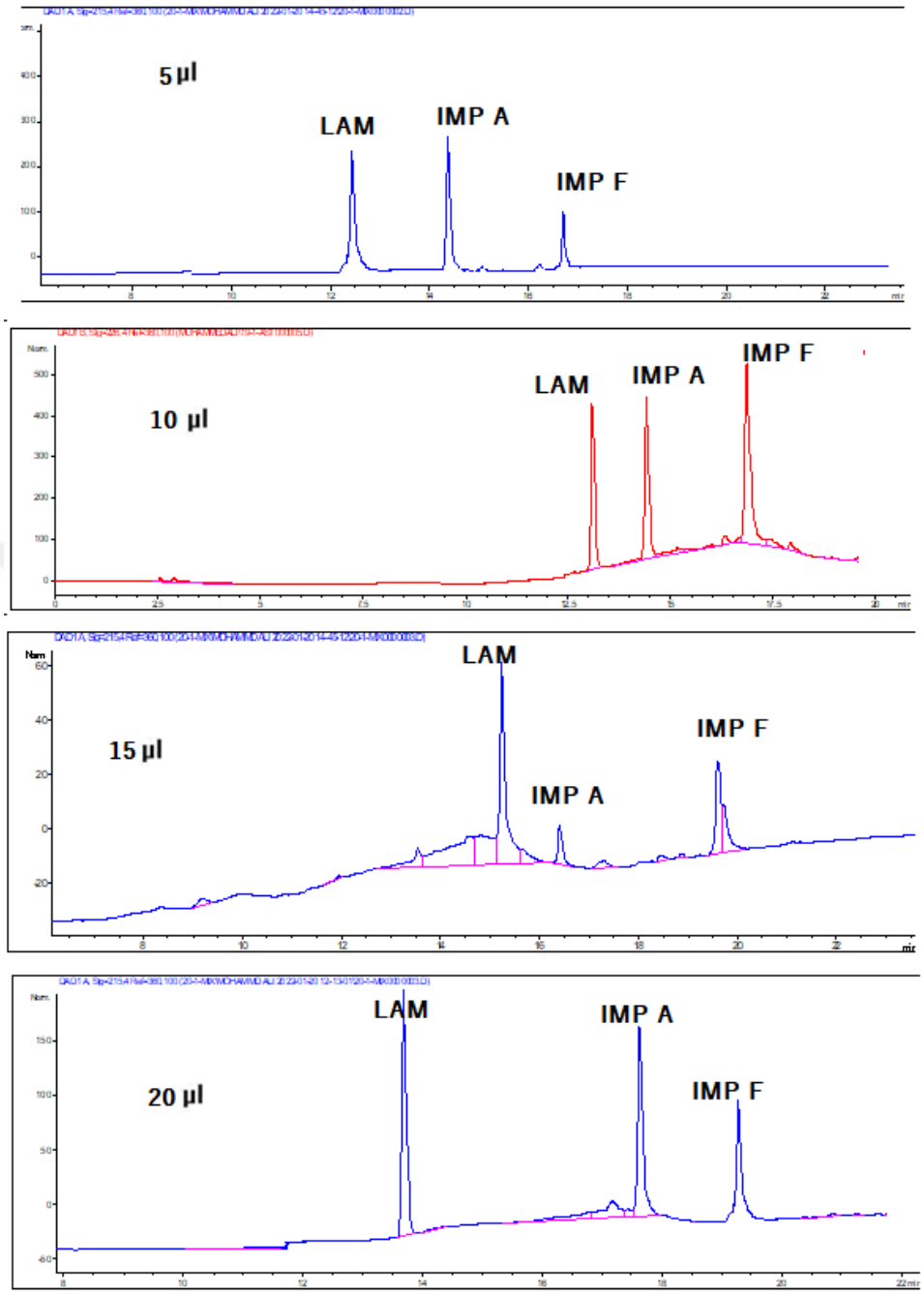

Figure 5.5. *Exhibits chromatograms obtained using different injection volumes: 5.0 µL, 10.0 µL, 15.0 µL, and 20.0 µL, while keeping a consistent flow rate.*

The impact of the injection volume on the measured peak area values is presented in Table 5.7.

Table 5.7. *Provides an overview of the influence of injection volumes ranging from 5.0 µL to 20.0 µL on the measured peak area values.*

Injection volumes (µL)	IMP.A Peak Area	IMP.F Peak Area
5.0	1708.0	3172.1
10.0	3432.3	6132.3
15.0	5405.0	9771.1
20.0	7345.3	12302.3

There was a positive correlation observed between the peak area values and the increasing injection volume. It's worth highlighting that the peak shapes obtained using a 10.0 µL injection volume were notably favorable. Consequently, an injection volume of 10.0 µL was determined to be the optimal choice and was subsequently utilized in the subsequent phases of the study.

5.1.8 Optimum method conditions

As a result of the systematic optimization studies conducted, we have succinctly compiled the chromatographic conditions relevant to our developed method, as delineated in Table 5.8. For a visual of the outcomes achieved under the most favorable circumstances, provide Figure 5.6, depicting the chromatogram acquired at the optimal conditions.

Table 5.8. *Optimal method parameters*

Column	Phenomenex, C18 (250 mm, 4.6 mm, 4 µm)				
Mobile Phase	ACN with a 0.02 mM phosphate buffer at pH 3.5				
Gradient Profile	Time (min)	0	5	14	22
	ACN (%)	5	15	80	90
Injection	10.0 µL				
Volume flow rate	1 mL/min				
Column Temperature	40ºC				
Wavelength	DAD: 215 nm				

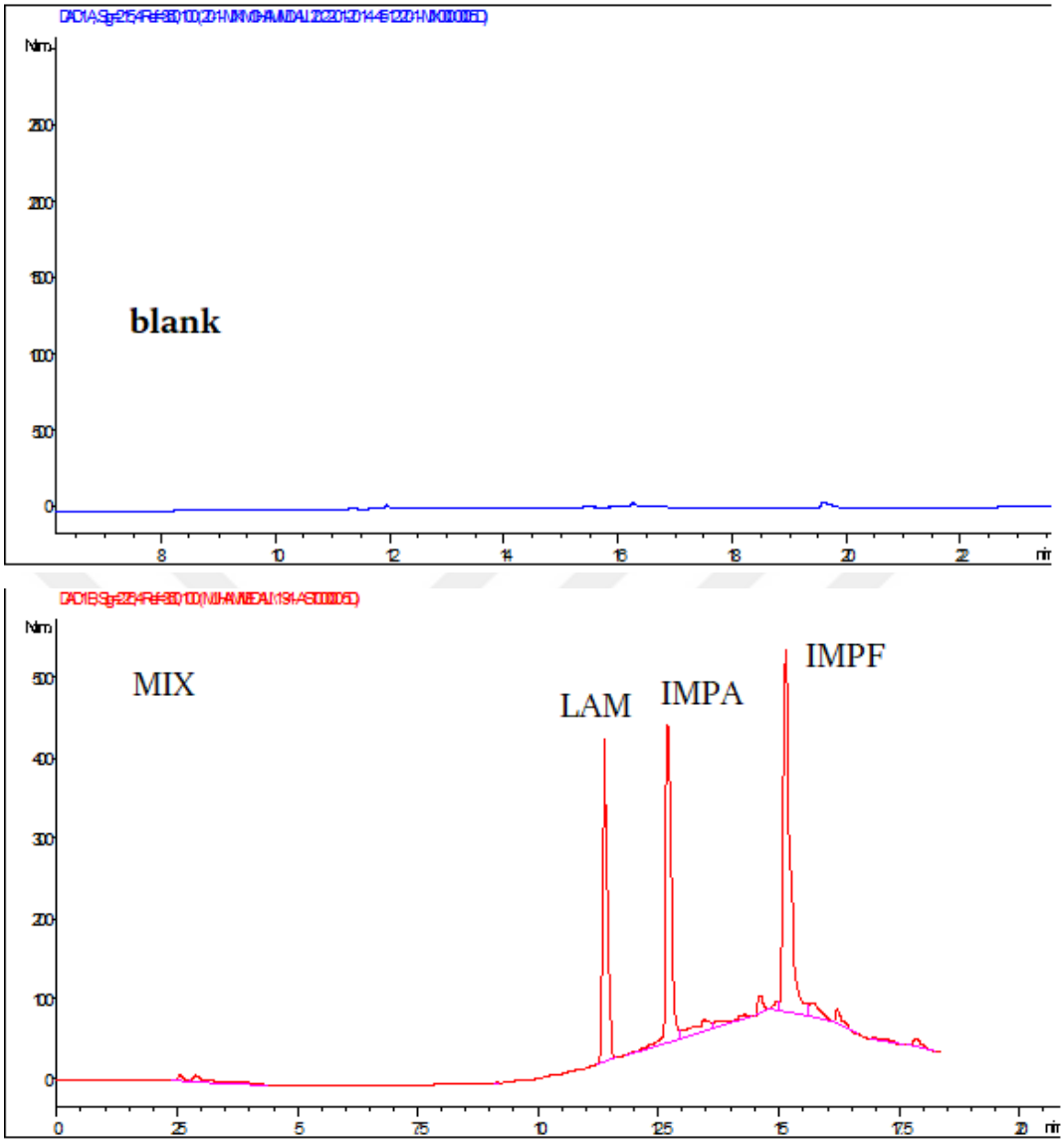

Figure 5.6. *Typical chromatogram obtained at optimum conditions*

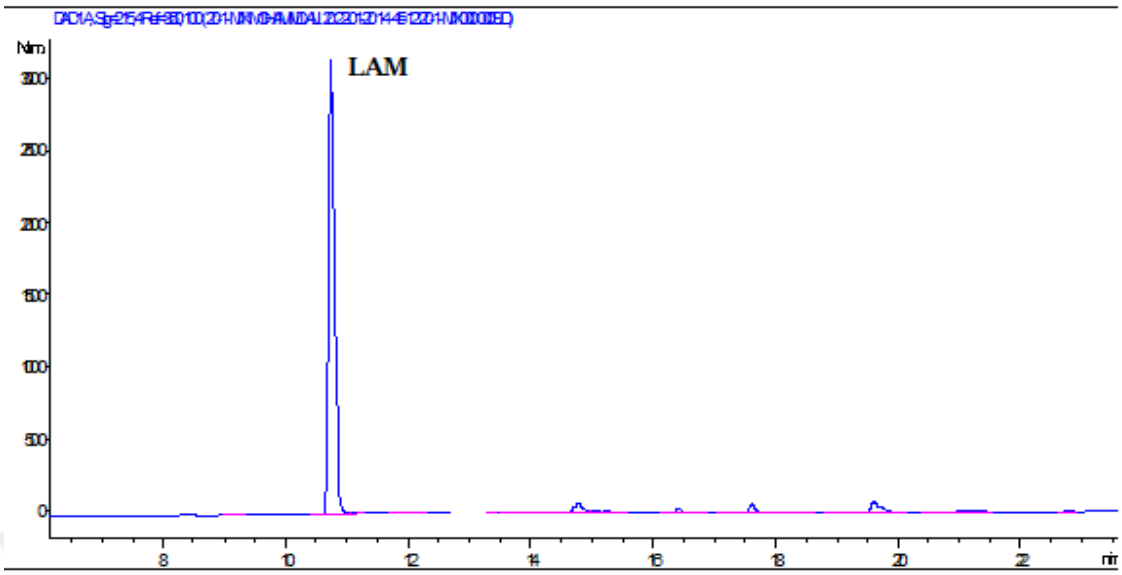

Figure 5.7. *Typical chromatogram obtained at optimum conditions of Lamotrigine standard*

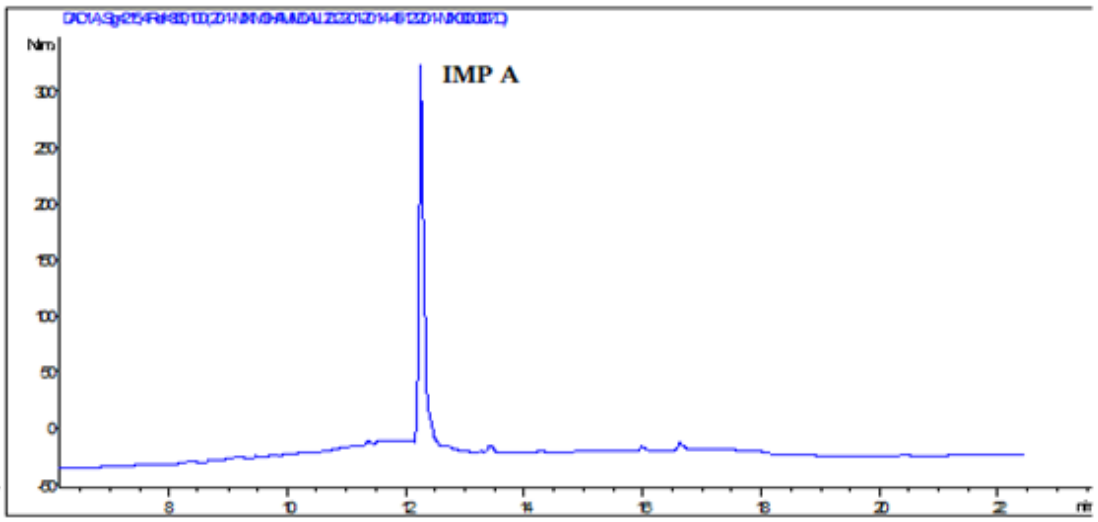

Figure 5.8. *Typical chromatogram obtained at optimum conditions of impurity A*

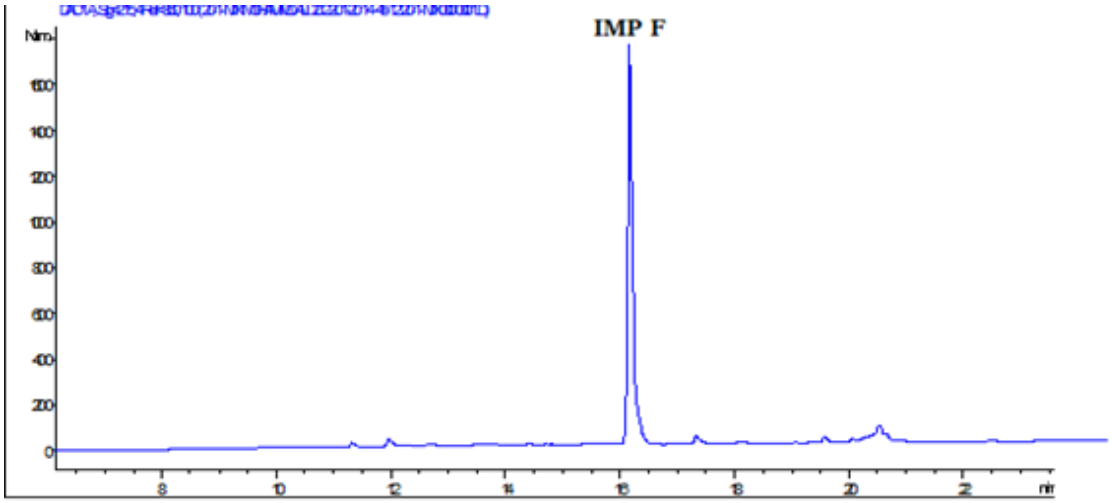

Figure 5.9. *Typical chromatogram obtained at optimum conditions of impurity F*

Validation of HPLC method

Validation parameters were assessed for impurity and statistical analyses were conducted using GraphPad Prism 6 and Microsoft Excel software.

5.2.1 System suitability test

Statistical calculations were made using the area ratios obtained from the analyzes performed with solutions containing 10 µg/mL LAM, from which results were generated through six injections. The findings from the system suitability test have been methodically documented in Table 5.9, offering valuable insights for reference and evaluation. The system suitability test is an essential aspect of method validation, ensuring the reliability and consistency of the chromatographic system for the analysis of the target substances. In this study, the parameters recommended by ICH guidelines were applied, and the results obtained for the system suitability test are summarized in the table below.

Table 5.9. *Results of system suitability test (n=6)*

Parameter	IMP A	IMP F	Recommendation
Capacity factor (k)	7.62	9.92	k' > 2
Tailing Factor (T)	1.12	0.992	$T \leq 2$
Theoretical Plates (N)	99575	183530	N > 2000
Resolution (R_S)	4.66	21.85	$R_S > 2$
RSD (Peak Area)	0.49	0.45	RSD ≤ 1

The capacity factor (k) represents the extent of separation between the analyte peaks and the void volume. As per ICH guidelines, k value greater than 2 is indicative of adequate separation. In our analysis, both IMP A and IMP F demonstrated favorable k values of 7.62 and 9.92, respectively, signifying effective separation.

The tailing factor (T) is a critical parameter indicating peak symmetry, and a value equal to or less than 2 is considered acceptable. Our results show that both IMP A and IMP F met this criterion with T values of 1.12 and 0.992, suggesting well-symmetrical peaks.

The theoretical plates (N) represent the efficiency of the chromatographic system, and a value exceeding 2000 is typically desired. Notably, both IMP A and IMP F displayed remarkably high theoretical plate counts, far exceeding the recommended threshold, with values of 99575 and 183530, respectively.

Resolution (Rs) is a measure of the separation between adjacent peaks. A value greater than 2 indicates effective separation. Our analysis revealed that both IMP A and IMP F surpassed this benchmark, with Rs values of 4.66and 21.85, emphasizing their distinctive separation.

The relative standard deviation (RSD) of peak area signifies the precision and reproducibility of the method. An RSD less than or equal to 1 is indicative of favorable precision. Both IMP A and IMP F demonstrated low RSD values of 0.493 and 0.457, reinforcing the method's reliability.

Linearity		
Sr. No.	**Concentration (µg/mL)**	**Mean A/RT (n=6)**
		Lamotrigine
1	1.0	68.099
2	2.5	130.89
3	5.0	231.20
4	10	486.01
5	25	1046.65
6	50	2105.73
7	100	4050.97
Correlation coefficient (R^2)		**0.9997**
Slope		**40.314**
Intercept		**28.3**

Table 5.10. *Linearity of lamotrigine*

5.2.2 Linearity

The calibration curve's linearity was established by plotting the concentrations of lamotrigine (n=6) against their respective peak areas. We determined the linearity by analysis of combined standard solution in the range of (1.0, 2.5, 5.0, 10, 25, 50, 100 µg/mL) for lamotrigine, (0.1, 0.5, 1.0, 2.5, 5.0, 10.0 µg/mL for IMP A and IMP F. The linearity was checked by using the least square regression equation. The parameters of the curve for lamotrigine standard solution obtained in Table 5.10 lists the data for regression analysis. Fig. 5.10, 5.11 and 5.12 represent standard plot curve for LAM, IMP A and IMP F. The selection of calibration curve was depending on getting good peak shape and avoiding column overloading.

The data presented in Table 5.11 and the accompanying figures provide information about the linearity of the method developed for analyzing lamotrigine and its impurities. The linearity was determined by analyzing combined standard solutions at a range of concentrations for lamotrigine (1.0, 2.5, 5.0, 10, 25, 50, 100 µg /mL). The data was plotted and analyzed using the least square regression equation. The correlation coefficient (R^2) value of 0.9997, slope of 40.314, and intercept of 28.3 indicate a strong linear relationship

between the peak area and the concentrations of lamotrigine and its impurities. The slope and the intercept values are the coefficients of the linear equation that relates the peak area to the lamotrigine concentration.

The selection of the calibration curve was made based on achieving good peak shape and avoiding column overloading. The figures provided also support the linearity of the method, demonstrating the consistency and accuracy of the results. The data confirms the linearity of the developed method.

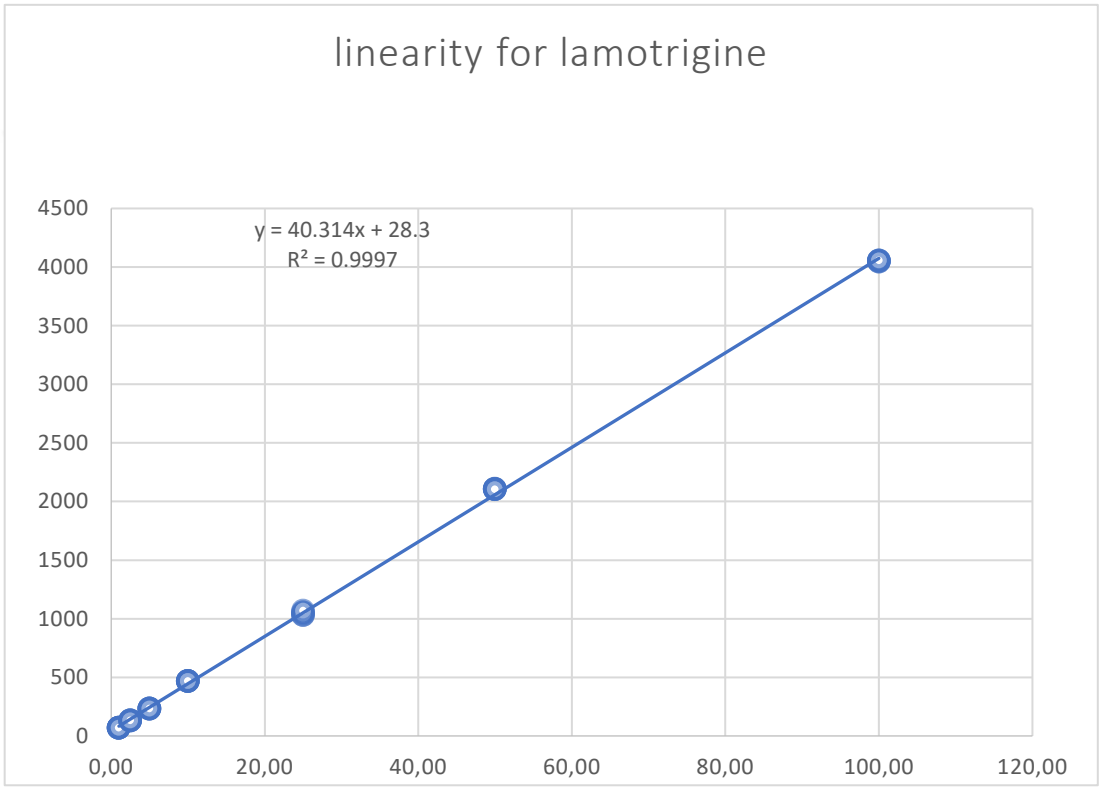

Figure 5.10. *Linearity for lamotrigine*

Table 5.11 presents the results of the linearity study for impurity A. The table lists the concentrations of impurity A used in the study and the corresponding mean area (n=6) for each concentration. The correlation coefficient (R^2) of 0.9995, slope of 32.972, and intercept of 1.886 indicate a strong linear relationship between the peak area and the concentrations of impurity A. This suggests that as the concentration of impurity A increases, so does the peak area. The R^2 value of 0.9995 is a measure of how well the data points fit to the regression line, R^2 value closer to 1 represents a better fit as showed Fig 5.11. The slope and the intercept values are the coefficients of the linear equation that

relates the peak area to the impurity A concentration. The slope represents the change in the y-variable (peak area) per unit change in the x-variable (concentration) and the intercept is the point where the line crosses the y-axis when x is zero, as well, the data in Table 5.11 confirms that the method developed for impurity A is linear over the concentration range studied.

Table 5.11. *Linearity of impurity A*

Linearity IMP A		
Sr. No.	**Concentration (µg/mL)**	**Mean A/RT (n=6)**
		IMP A
1	0.1	4.90
2	0.5	18.88
3	1.0	37.10
4	2.5	83.29
5	5.0	163.81
6	10	334.17
Correlation co-efficient (R^2)		**0.9995**
Slope		**32.972**
Intercept		**1.886**

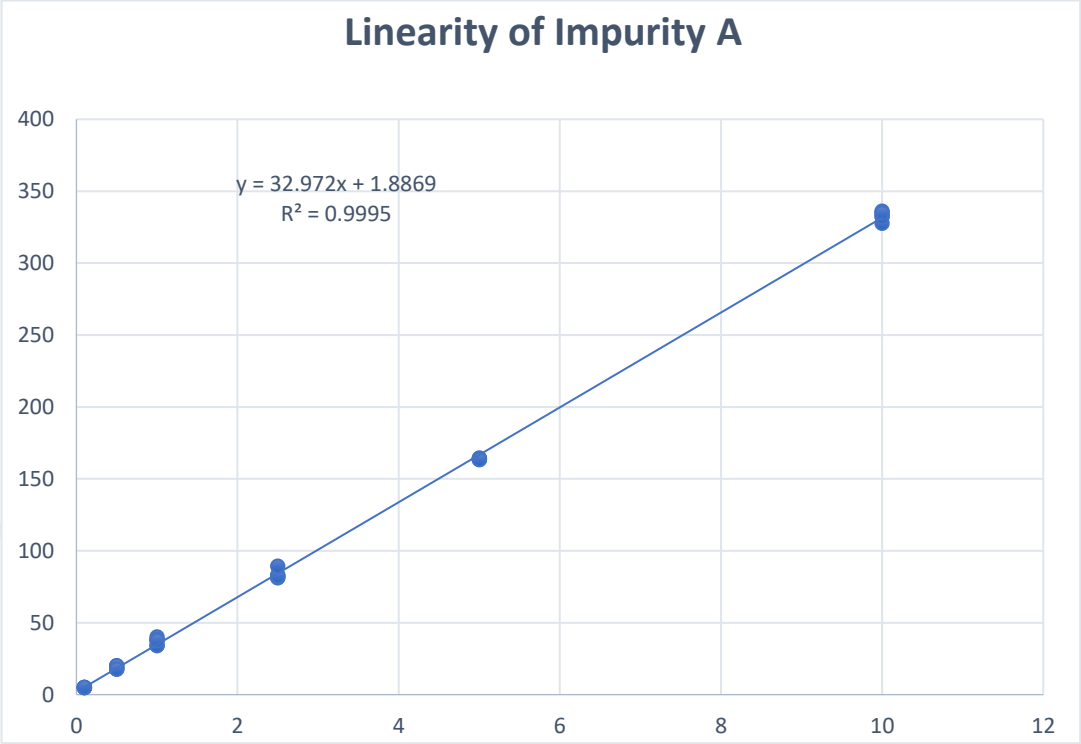

Figure 5.11. *Linearity for impurity A*

The linearity of the method for quantifying IMP F was assessed by plotting the concentration of IMP F against the corresponding mean A/RT as showed Fig 5.12. Table 5.12 presents data on the linearity of a substance called IMP F. The table lists six different concentrations of IMP F, along with the mean area (n=6) for each concentration. The correlation coefficient (R^2) is also given, which indicates the strength of the linear relationship between concentration and mean area. In this case, the R^2 value is 0.9995, which suggests a very strong linear relationship. Additionally, the slope and intercept of the linear regression line are given as 15.654 and 1.6776, respectively, the data in this table indicate that there is a strong linear relationship between the concentration of IMP.

Table 5.12. *Linearity of impurity F*

Linearity IMP F		
Sr. No.	**Concentration (µg/mL)**	**Mean A/RT (n=6)**
		IMP F
1	0.1	3.39
2	0.5	8.09
3	1.0	18.95
4	2.5	40.62
5	5.0	79.89
6	10	158.20
Correlation co-efficient (R^2)		**0.9995**
Slope		**15.654**
Intercept		**1.6776**

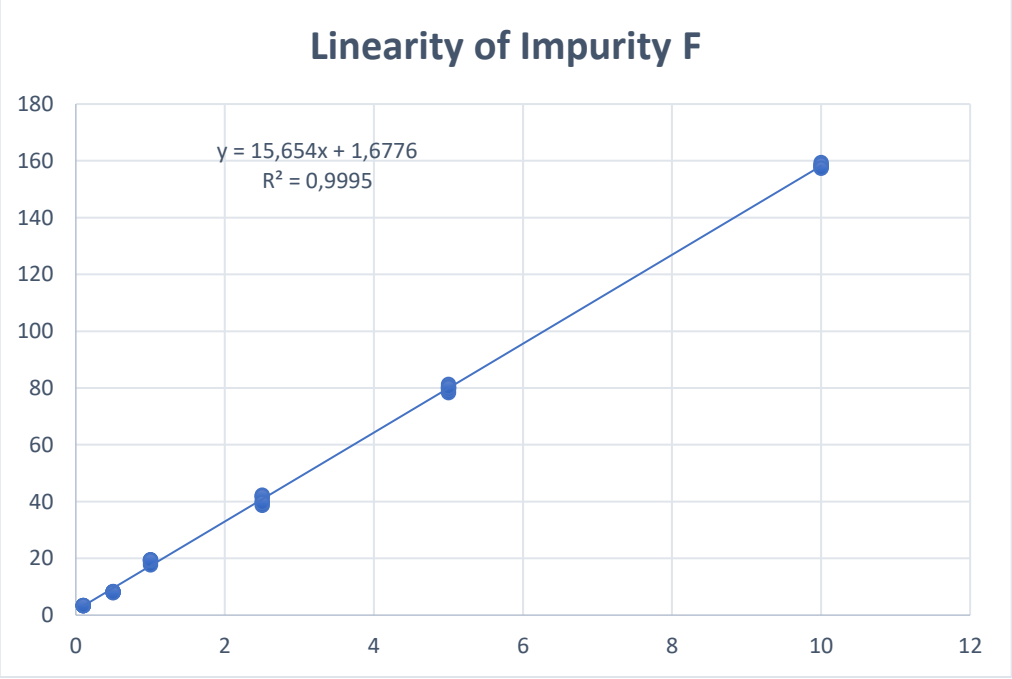

Figure 5.12. *Linearity for impurity F*

The graphs (Fig 5.10, Fig 5.11 and Fig 5.12) show that correlation coefficient of the lamotrigine was R^2 =0.9994 with linear regression equation Y = 40.314x +28.3 8; for IMP-A R^2 = 0.9997 with Y =32.972x +1.8869, and for IMP-F R^2 = 0.9995 with Y = 15.654x + 1.6776.

In conclusion, it is evident that the method exhibits exceptional linearity for both the drug substance and related impurities over the entire working concentration range. This fundamental characteristic underscores the method's reliability and suitability for accurate quantification in pharmaceutical analysis.

5.2.3 Accuracy

In the present study, the accuracy of the method was estimated using recovery studies. Three impurity concentrations (0.1, 1.0, and 5.0 µg/mL) and (1.00, 5.00, and 50 µg/ml) for standard solution were analysed six times a day for three days. The results of the recovery experiments revealed that the mean recovery of impurity A and impurity F were 100.2%, and 100.8%, respectively. Importantly, all recoveries were within the acceptable range ±5%. These results demonstrate that the approach is reliable and precise for analysing impurities in the sample. The recovery experiments are presented in Table 5.13, providing detailed information on the recovery percentages for different impurity concentrations. Additionally, the chromatograms for 0.1, 1.0, and 5.0 µg/mL of impurities spiking are depicted in the figure, an observable representation of the method's accuracy. These results provide valuable information for the development of analytical methods for the quality control of drug products.

Table 5.13. *The accuracy of the HPLC method for determining impurity compounds in standard solutions*

Compound	Standard concentrations (µg/mL)	Standard solutions	
		Observed concentrations (µg/mL)	Recovery (%)
LAM	1.0	0.98	98.78
	5.0	4.99	99.97
	50	50.41	100.83
Average recovery			**99.83**
R.S.D.%			**1.38**
Impurity A	0.1	0.10	100.7
	1.0	0.98	100.2
	5.0	5.00	101.0
Average recovery			**100.2**
R.S.D.%			**1.99**
Impurity F	0.1	0.099	99.08
	1.0	0.98	99.24
	5.0	5.20	104.2
Average recovery			**100.80**
R.S.D.%			**0.042**

5.2.4 Precision

The precision of a newly developed analytical method was evaluated through an investigation of its Intraday and Interday precision. This study utilized three concentrations of the calibration curve for lamotrigine standard and impurities A and F. Standard statistical techniques were used to evaluate the precision of the method, which included computing the standard deviation (SD), relative standard deviation (RSD), and standard error of mean (SE). Table 5.14 displays the precision outcomes of the investigation, which demonstrated that the method was analytically suitable with an RSD% below 2.0.

Table 5.14. *Statistical evaluation of precision studies for lamotrigine and its impurities standard solutions*

Analyte	Concentration (µg/mL)	Intra-day			Inter-day		
		Mean	SD	RSD	Mean	SD	RSD
LAM	1.0	37.519	0.086	0.23	37.517	0.22	0.60
	5.0	246.39	0.527	0.112	246.348	2.43	0.98
	50	2148.25	1.247	0.058	2119.01	2.325	0.41
IMP A	0.1	2.280	0.001	0.043	2.275	0.016	0.744
	1.0	32.302	0.026	0.081	32.366	0.573	1.772
	5.0	161.86	0.036	0.022	160.94	0.497	0.309
IMP F	0.1	2.682	0.0557	0.349	0.122	0.003	2.86
	1.0	15.94	0.055	0.349	15.913	0.119	0.748
	5.0	92.53	0.082	0.089	92.624	0.257	0.278

The highest RSD value for intraday and interday studies were calculated, respectively. The results confirmed the precision of the developed method.

The table results show the statistical evaluation of precision studies for the analyte lamotrigine and its impurities standard solutions. The table includes the concentration levels of the analyte and impurities, as well as their mean, SD, and RSD values for both Interday and Intra-day studies. The data indicates that the precision of the developed method is relatively high, with the highest RSD values being less than 1% for both intraday and interday studies. The results confirm the precision of the developed method.

The data presented in Table 5.14 provides a thorough evaluation of the precision of the method developed for analyzing lamotrigine and its impurities. The precision of the method was evaluated through repeatability (intraday precision) and intermediate precision (interday precision) studies at three different concentrations (1.0, 5.0, 50 µg/mL) for lamotrigine and (0.1, 1.0, 5.0 µg/mL) for impurities A and F. SD, RSD, and SE were calculated to evaluate the precision of the method. The results indicate that the method is analytically sufficient, with RSD values consistently below 2.0% for all analytes and concentrations. Generaly, the data confirms the precision of the developed method.

5.2.5 Specificity

The specificity method a investigations aimed to generate degradation products and related impurities to challenge the method's ability to discriminate and accurately quantify the target analyte in the presence of such interfering substances. The analytes were exposed to stress conditions, and the chromatograms obtained from the solutions under stress conditions are shown in the respective figures.

The study was conducted on the tablet sample. In this investigation, the tablet's excipients were meticulously examined, and a matrix medium was formulated using materials accessible within the university's resources. The precise proportions of the tablet's excipients were provided by experienced pharmaceutical technologists. The excipients were accurately weighed and subsequently transferred to a mortar for homogenization. The resulting mixture was initially diluted to the required volume with methanol using a 10.0 mL volumetric flask and further diluted with the mobile phase. Following proper filtration, the solution was introduced into the analytical system. The resulting chromatogram is presented in Figure 5.13. Remarkably, no discernible peaks were observed, underscoring the method's specificity. The HPLC method's capability to distinguish analytes from each other is referred to as selectivity. It was found that peaks did not interfere with each other, and the resolution values of LAM indicated that effective separation was achieved (>1.5) as shown Fig 5.13.

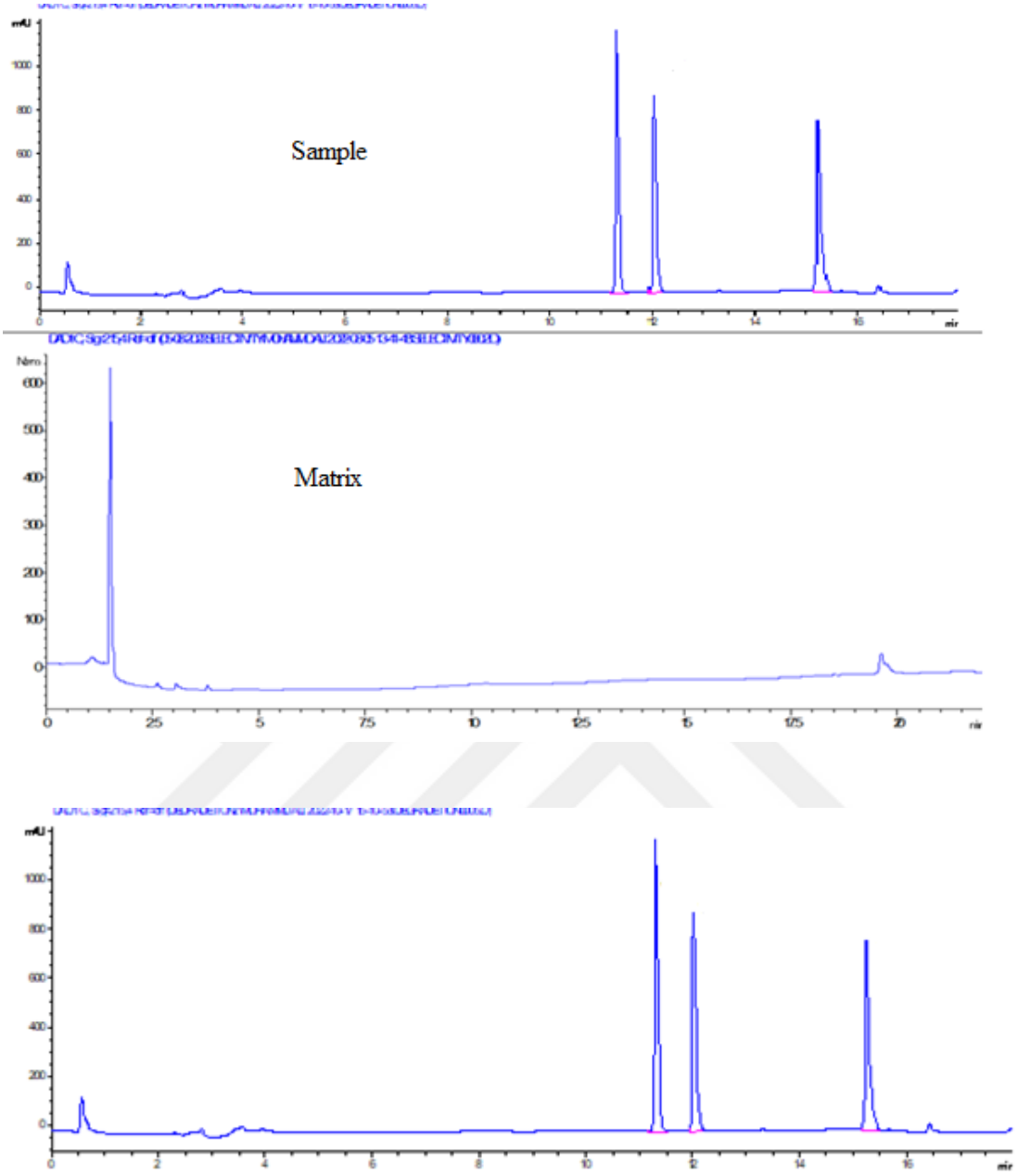

Figure 5.13. The chromatograms for specificity showing sample, matrix and spiked sample chromatograms

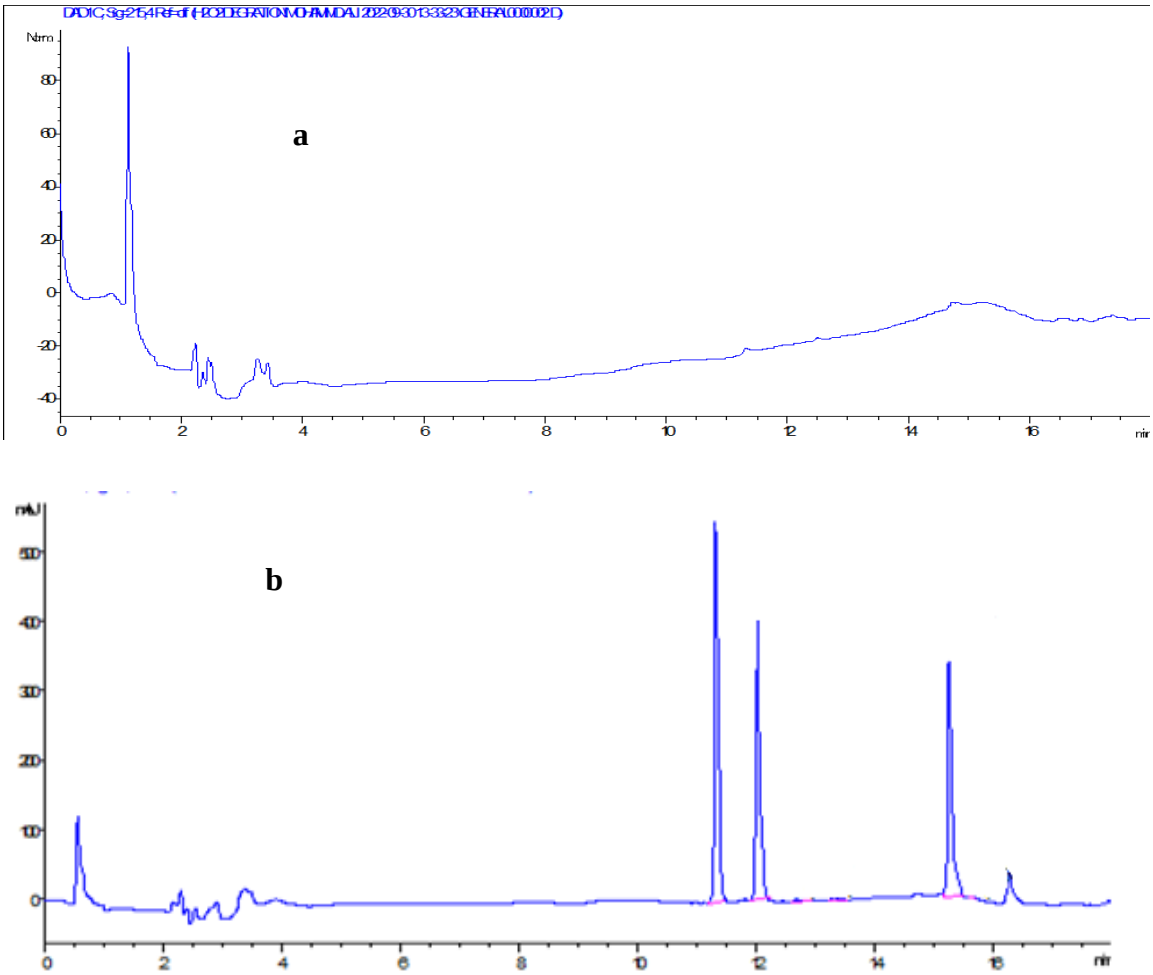

Figure 5.14. a) *The chromatogram of the blank prepared with HCl, b) The chromatogram of standard solution with HCl*

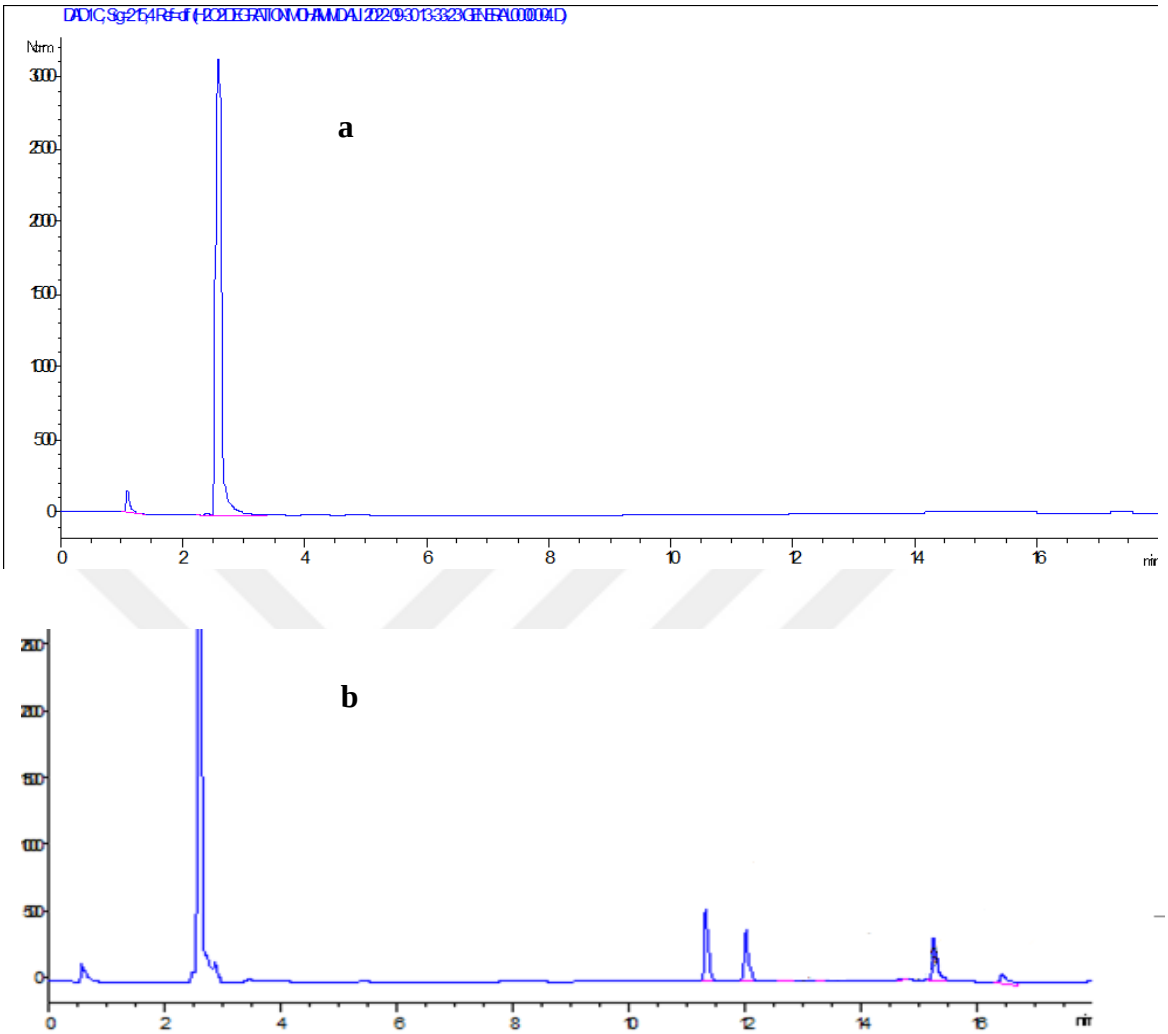

Figure 5.15. a) *The chromatogram of the blank prepared with H_2O_2 , b) The chromatogram of standard solution with H_2O_2*

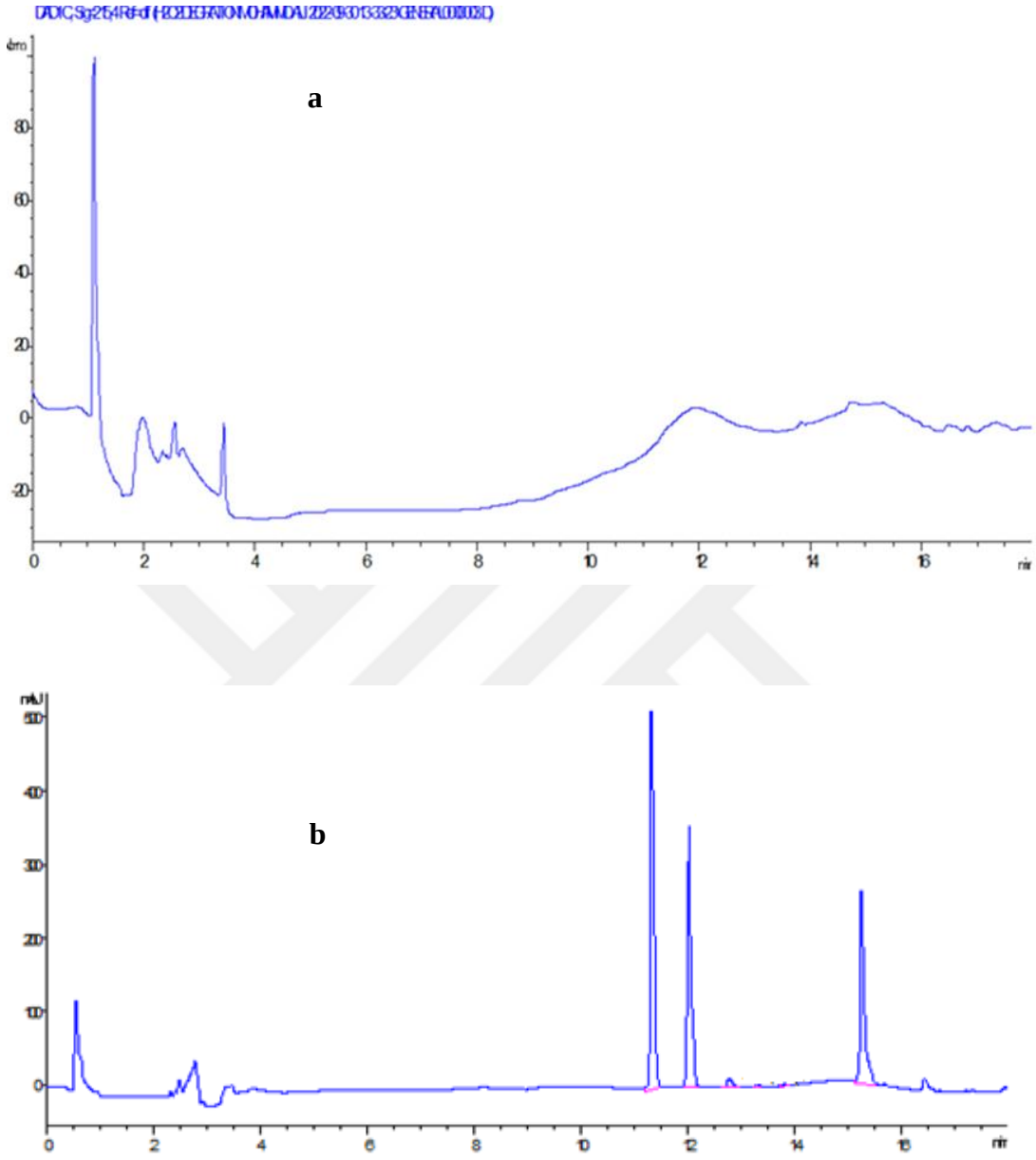

Figure 5.16. a) *The chromatogram of the blank prepared with NaOH, b) The chromatogram of standard solution with NaOH*

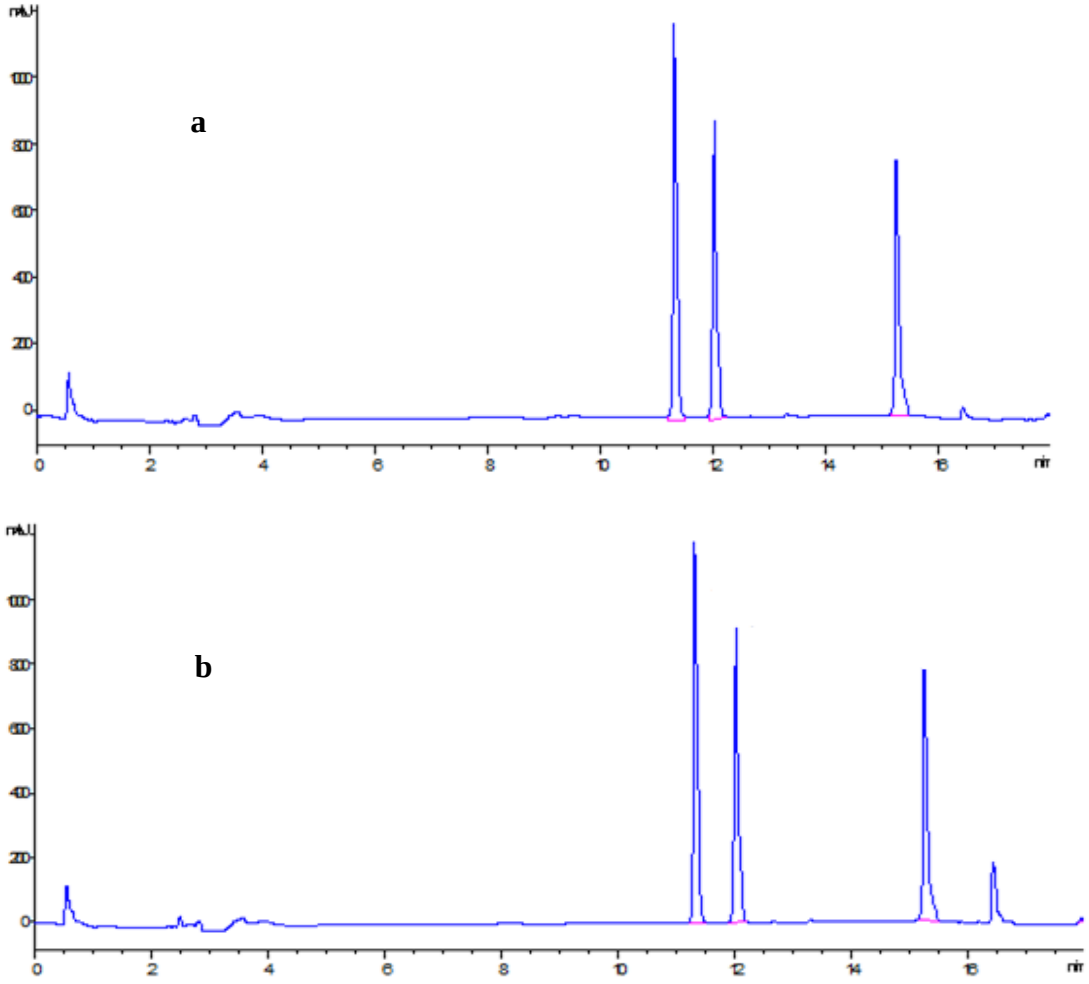

Figure 5.17. a) *Chromatogram of the sample without thermal degradation, b) The chromatogram of the sample prepared with thermal degradation.*

Table 5.15. *Percent degradation of lamotrigine*

Conditions	Degradation% of Lamotrigine (n=5)	Peak purity match	Peak purity result
Untreated stock solution (10 µg/mL)	-	-	-
Acid hydrolysis 0.1 N HCl	54.8	55.8	Pass
Alkali hydrolysis 0.1 N NaOH	57.5	60.6	Pass
Oxidative degradation of 3% H_2O_2	57.9	56.99	Pass
Thermal degradation	-	-	-

Table 5.16. *Percent Degradation of lamotrigine*

Conditions	Degradation% of Lamotrigine (n=5)	Peak purity match	Peak purity result
Untreated stock solution (10 µg/mL)	-	-	-
Acid hydrolysis 0.1 N HCl	54.8	999	Pass
Alkali hydrolysis 0.1 N NaOH	57.5	1000	Pass
Oxidative degradation of 3% H_2O_2	57.9	999	Pass
Thermal degradation	-	-	-

Table 5.16. *Percent Degradation of Impurity A*

Conditions	Degradation% of Imp A (n=5)	Peak purity match	Peak purity result
Untreated stock solution (10 µg/mL)	-	-	
Acid hydrolysis 0.1 N HCl	54.8	1000	Pass
Alkali hydrolysis 0.1 N NaOH	57.5	1000	Pass
Oxidative degradation of 3% H_2O_2	57.9	999	Pass
Thermal degradation	-	-	-

Table 5.17. *Percent Degradation of Impurity F*

Conditions	Degradation% of Imp F (n=5)	Peak purity match	Peak purity result
Untreated stock solution (10µg/mL)	-	-	-
Acid hydrolysis 0.1 N HCl	54.8	999	Pass
Alkali hydrolysis 0.1 N NaOH	57.5	1000	Pass
Oxidative degradation of 3% H_2O_2	57.9	999	Pass
Thermal degradation	-	-	-

Significant degradation was observed in the presence of acidic, basic, oxidative and thermal stress conditions for lamotrigine, respectively (n=5). Percent degradation for the standard drug was found as 54.8%, 57.5%, and 57% for lamotrigine, and 55.8%, 60.6%, and 56% for IMP A in the presence of acidic, basic, oxidative, degradation, respectively. While it was not notice that any degradation in the thermal degradation.

The percent degradation was calculated by the formula:

Degradation% = (Average peak area of untreated stock solution – average peak area of stock solution under specific degradation condition) / (average peak area of untreated stock solution) x 100).

The High-Performance Liquid Chromatography (HPLC) method described in this study demonstrates selectivity towards lamotrigine and two closely related compounds that may be present as impurities. An examination of the absence of excipients and impurities revealed that none of the chromatographic peaks align with the retention time of lamotrigine. This observation leads to the conclusion that the developed method exhibits selectivity with respect to the excipients and impurities within the final preparation.

5.2.6 Limit of detection (LOD) and quantitation (LOQ)

The limit of detection (LOD) and limit of quantitative determination (LOQ) values for impurity analysis were determined. To calculate the limit of detection (LOD) values, the formula 3.3 x (SD/S) was applied, whereas the limit of quantification (LOQ) values were obtained by applying the formula 10 x (SD/S). Here, SD refers to deviation of low concentration of the standard solution, while S represents the slope of the calibration curve.

The outcomes of the linearity analysis and calculations of LOD and LOQ values, which were performed to evaluate the sensitivity of the newly developed analytical method for detecting impurities at retention time 22 min as analysis time. The outcomes of the linearity analysis and calculations of LOD and LOQ values for lamotrigine 0.019 µg/mL, 0.058 µg/mL; for Impurity-A 0.047 µg/mL, 0.015 µg/mL, respectively, while those for Impurity-F were 0.030 µg/mL and 0.090 µg/mL, respectively. These findings indicate that the analytical method employed in this study had the ability to detect and measure these impurities at relatively low concentrations.

5.2.7 Robustness

Adjustments to some instrumental parameters that have a major impact on retention time were performed in this investigation to evaluate the robustness of the newly proposed approach. By injecting standard solutions (n=5) of LAM, IMP A, and IMP F, the impact of these parameter changes on the final results were studied (Table 5.18). Adjustments in the pH, column temperature, and flow rate were performed to assess the robustness of the established approach. The buffer pH values of 3.4 and 3.6 were changed by 0.1 pH units, the column temperature was tested at 39°C and 41°C, and the flow rate was measured at 1.1 and 0.9 mL/min, respectively. The robustness analysis results showed that the RSD % value was less than 2%, indicating that the developed approach was robust. Table 5.19 displays the outcomes of the robustness analysis, which indicated that modifications in the flow rate and mobile phase pH had no significant impact on the analyte response. The temperature variations, however, had a minor effect on the final outcomes.

Table 5.18. *Robustness parameters*

Parameter	The Lower Value	The Higher Value
Temperature	39ºC	41ºC
Flow rate	1.1 mL/min	0.9 mL/min
Mobile phase pH	3.4	3.5

Table 5.19. *Data of the robustness study of developed HPLC method (n=5)*

Parameter	Concentration (µg/mL)	IMP A		IMP F	
		SD	RSD	SD	RSD
Temperature: 39ºC	1.0	0.47	0.47	0.061	0.3
	5.0	0.33	0.33	0.095	0.10
Temperature:41ºC	5.0	0.17	0.17	0.09	0.66
	5.0	0.35	0.35	0.41	0.44
Flow rate: 0.9 mL/min	1.0	0.18	0.18	0.14	1.00
	5.0	1.22	1.22	0.36	0.44
Flow rate: 1.1 mL/min	1.0	0.094	0.094	0.24	1.56
	5.0	0.56	0.56	0.24	0.29
Mobile phase pH: 3.4	1.0	0.16	0.16	0.19	1.20
	5.0	1.02	1.02	0.30	0.32
Mobile phase pH: 3.6	1.0	0.07	0.07	0.19	1.20
	5.0	0.78	0.78	0.77	0.90

5.2.8 Stability

In this study, the stability of standard solutions of IMP A and IMP F was evaluated under various conditions. The stability of all compounds was monitored, and the results indicated that they were stable under all conditions studied. The recovery and RSD% values were calculated using the data obtained from the solution analysis.

The stability study includes three types of stability tests: 1) short term stability (24 hours at room temperature), 2) freeze and thaw stability, and 3) long term stability (4 weeks at -20˚C). The table 5.20 and table 5.21 display the results of the stability analysis of IMP A and IMPF and SD, RSD%, and the percentage of recovery for each of the three types of stability tests at different concentrations (1.0 µg/mL and 5.0 µg/mL) were given in the mentioned tables.

The short term stability test shows that the concentration of IMP A and IMP F remained stable within 24 hours at room temperature. The percentage of recovery for IMP A and IMPF were 100.31% and 98.90% respectively at 1.0 µg/mL, and 99.09% and 101.72% respectively at 5.0 µg/mL. The freeze and thaw stability test shows that the concentration of IMP A and IMPF remained stable after being frozen and thawed three times. The percentage of recovery for IMP A and IMPF were 100.33% and 98.18% respectively at 1.0 µg/mL and 99.41% and 102.7% respectively at 5.0 µg/mL. The long-term stability test shows that the concentration of IMP A and IMPF remained stable after being stored for 4 weeks at -20˚C. The percentage of recovery for IMP A and IMPF were 100.15% and 97.93% respectively at 1.0 µg/mL and 99.53% and 98.7% respectively at 5.0 µg/mL.

Generally, the stability study shows that the developed HPLC method is stable for lamotrigine impurities under different storage conditions. The percentage of recovery for IMP A and IMPF were in the range of 98% -102%, indicating that the developed method is suitable for stability testing of lamotrigine impurities.

Table 5.20. *Data of the stability study of developed HPLC method for IMP A (n=5)*

Parameter	Concentration (µg/mL)	SD	RSD%	Recovery%
Short term stability (24 hours at room temperature)	1.0	0.91	0.91	100.3
	5.0	0.41	0.41	99.0
Freeze and thaw stability	1.0	1.0	1.0	100.3
	5.0	0.37	0.37	99.4
Long term stability (4 weeks, -20˚C)	1.0	1.60	1.60	100.1
	5.0	0.06	0.06	99.5

Table 5.21. *Data of the stability study of developed HPLC method for IMP F (n=5)*

Parameter	Concentration (µg/mL)	SD	RSD%	Recovery%
Short term stability (24 hours at room temperature)	1.0	1.195	1.217	98.90
	5.0	0.422	0.412	101.7
Freeze and thaw stability	1.0	0.414	0.422	98.19
	5.0	0.314	0.306	102.7
Long term stability (4 weeks, -20˚C)	1.0	0.445	0.456	97.93
	5.0	0.516	0.523	98.76

5.3. Analysis of lamotrigine in the pharmaceutical drugs

The results obtained from lamotrigine drug indicates the presence of A and F impurity in all tablets or drug samples, which were collected of lamotrigine from different countries such as India, Turkey and Yemen. The applicability of the developed method was assessed by using it for determination of lamotrigine tablet and its impurities to determine the presence of impurities. The results for assay of tablets form are evaluated in Table 5.22, 5.23 and 5.24. This impurity is considered one of the critical impurities that can arise during cyclization step, and it may be remained after conventional purification steps. Fig. 5.18, 5.19, and 5.20 show the HPLC chromatogram of lamotrigine and related impurities in a pharmaceutical formulation tablet.

Table 5.22. *Assay results of the Turkish lamotrigine tablet at a concentration of 25 µg/mL (n=5)*

Compound	Found Concentration (µg/mL)	Standard solutions	
		Theoretical concentration (µg/mL)	**Average recovery (%)**
Impurity A		-	-
SD			-
Mean			-
Assay%			-
Impurity F	0.50	-	-
SD			0.11
Mean			9.00
Assay%			1.14

Table 5.23. *Assay results lamotrigine Tablet 25 µg/ml (n=5) by using Yemeni tablets*

Compound	Found Concentration (µg/mL)	Standard solutions	
		Theoretical concentration (µg/mL)	**Average recovery (%)**
Impurity A	0.301	-	-
SD			1.02
Mean			11.11
Assay%			1.43
Impurity F	0.706	-	-
SD			0.2
Mean			12.09
Assay%			1.71

Table 5.24. *Assay Results lamotrigine Tablet 25 µg/mL (n=5) by using Indian tablets*

Compound	Found Concentration (µg/mL)	Standard solutions	
		Theoretical concentration (µg/mL)	Average recovery (%)
Impurity A	0.4822		-
SD			0.22
Mean			17.15
Assay%			1.84
Impurity F	0.706		-
SD			0.59
Mean			15.50
Assay%			2.26

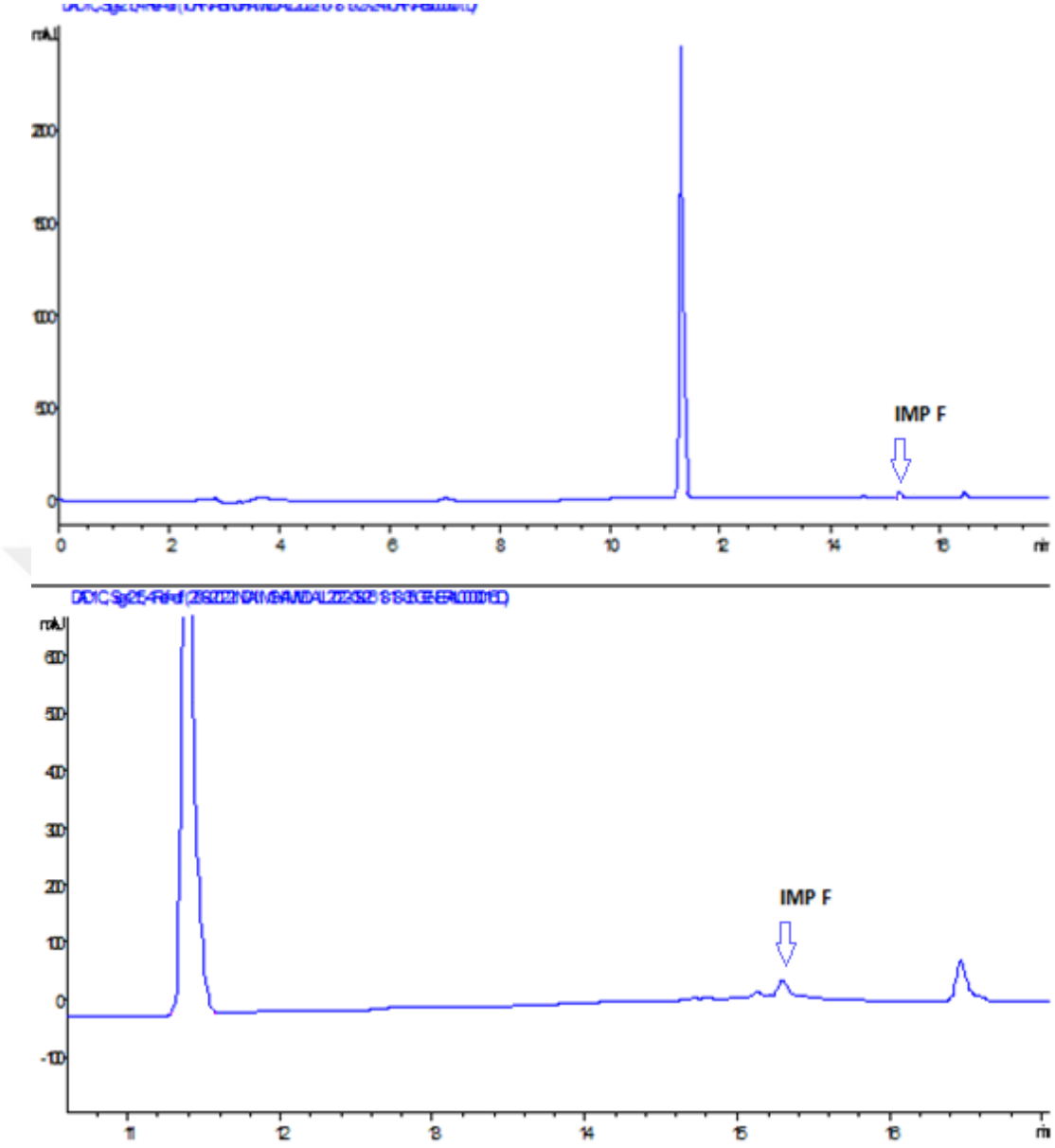

Figure 5.18. Chromatograms represent impurities in Turkish tablets

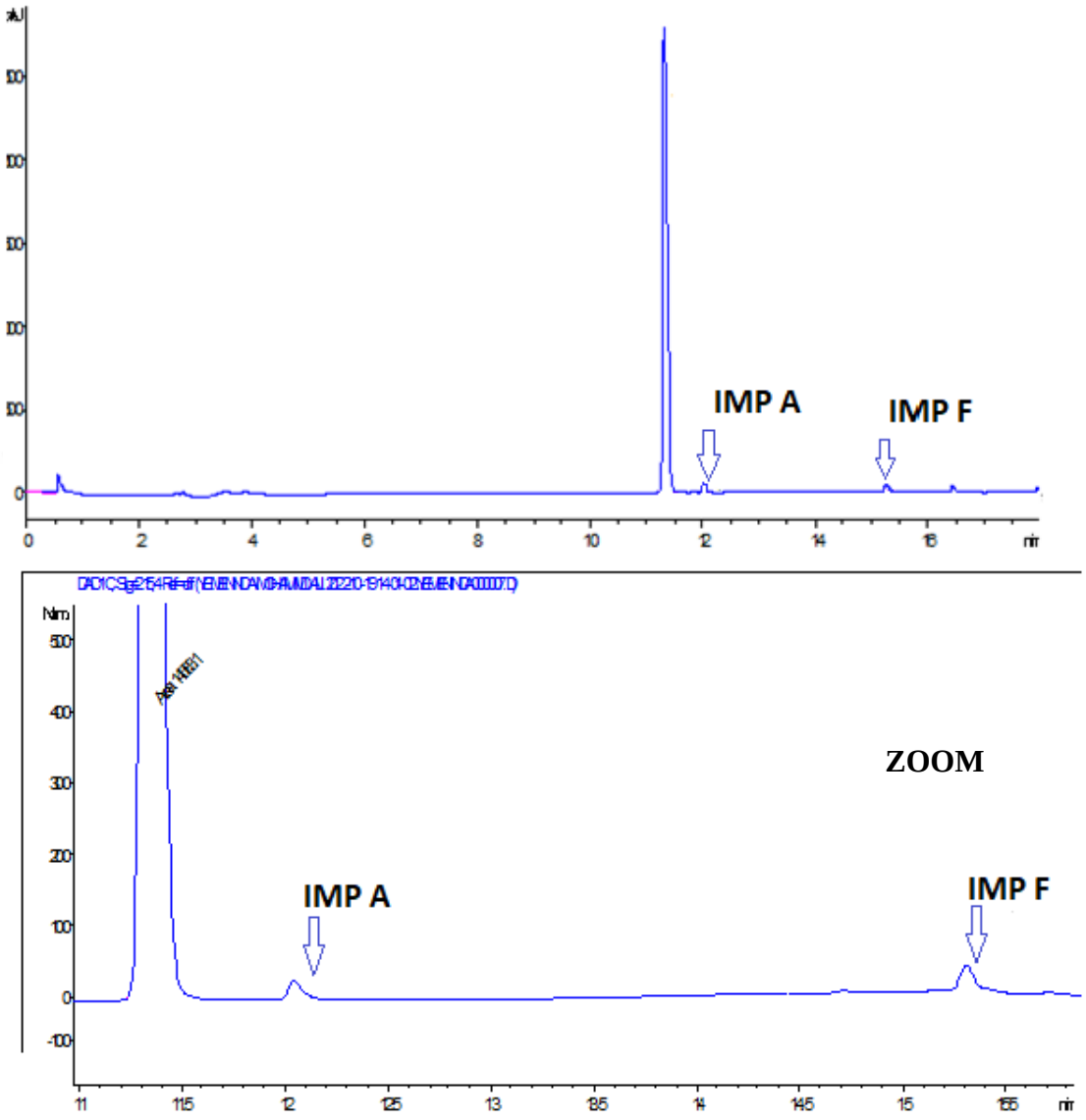

Figure 5.19. *Chromatograms represent impurities in Yemeni tablets*

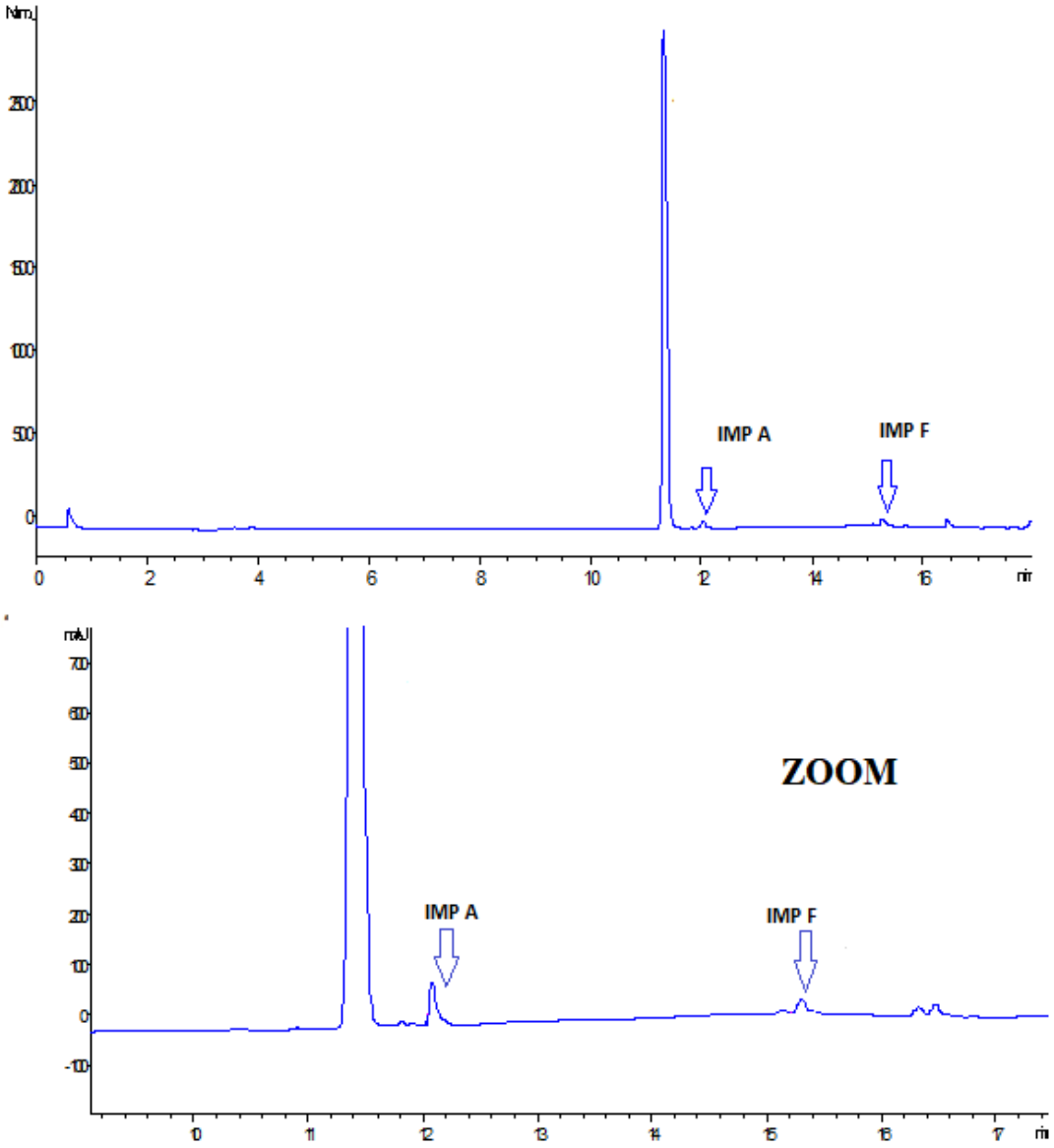

Figure 5.20. *Chromatograms represent impurities in Indian tablets*

For Turkish drug, one of the lamotrigine related compounds (IMP F) appeared clearly on the chromatogram, whereas Impurity A was not detected in any significant amount of lamotrigine drug of Turkish tablet. Indicating that the proposed method could differentiate between the active part and its associated impurities.

For Yemen drug, two of the lamotrigine related compounds (IMP F) and impurity A, appeared clearly on the chromatogram, indicating that the proposed method could differentiate between the active part and its associated impurities as well for the Indian drug, two impurities A and F appeared also in the chromatogram.

6. DISCUSSION AND CONCLUSIONS

In our study, a High-Pressure Liquid Chromatography method was developed and validated for the separation and quantification of the active ingredients of the antiepileptic drug lamotrigine (LAM), as well as certain impurities labeled as A and F. In our study, three samples of LAM were collected from three countries: Turkey, Yemen, and India.

In the literature, methods have been developed to determine the amount of active ingredients of LAM in dosage forms by using HPLC and other chromatography. However, we have not found a method in which the separation and determination of these active substances and their impurities are carried out simultaneously in our literature review. The aim of our study is to identify impurities A and F of LAM in pharmaceutical tablets. Only some of these impurities could be analyzed in our study. The reason for this is the difficulties in obtaining impurities. Since the impurities that pharmaceutical forms may contain consist of some degradation products of the active ingredients, the chemical structures of the impurities are similar to the active ingredients. This situation causes difficulties in developing a separation method. In our study, chromatographic conditions were optimized for the separation of LAM Impurity A, and Impurity F.

In the study, a gradient elution system was created with 0.020 M NaH_2PO_4 solution and acetonitrile as mobile phases. Phenomenex (C18, 4.6 mm, 250 mm, 4 μm) column was chosen as the stationary phase. DAD detector wavelength was 215 nm. The column temperature was 40°C and the autosampler temperature was 25°C. The injection volume for each solution was 10.0 μL. Then, in order to determine the quantities of the separated analytes, calibration curves were created based on pure standards, and the quantities of each analyte were determined. In our study, the pharmaceutical tablet solution containing the active ingredients was used as the matrix, and impurities that should not normally be present in a tablet and standard active ingredient solutions were added to this solution. Thus, both the recovery and the amount of active ingredient contained in the tablet were determined by using the standard addition method, which also eliminates the possible matrix effect. Results are expressed as recovery results in %. In addition, the solution prepared from pure standard active ingredients was also added to the standard active ingredient solutions and impurities were added and analyzed. A comparison of both studies shows no interference from the pharmaceutical tablet matrix.

The proposed High-Pressure Liquid Chromatography method has been validated in terms of accuracy, precision, selectivity, sensitivity, and linearity, and the results were

presented in tables. In accuracy and precision tests, three impurity concentrations (0.1, 1.0, and 5.0 µg/mL) and (1.0, 5.0, and 50 µg/mL) for standard solutions were analyzed six times a day for three days. The results of the recovery experiments revealed that the mean recoveries of impurity A and impurity F were 100.2%, and 100.8%, respectively. It was observed that the recovery % obtained was within the limits as well as displayed the precision demonstrated that the method was analytically suitable with an RSD% below 2.0, and (injection repeatability) test for the precision, the relative standard deviation of the peak areas and retention times obtained for each analyte complies with the specified criteria. Additionally, degradation studies were carried out for specificity and the solutions were kept in acidic, basic, H_2O_2, and thermal degradation environments noticed in this method that LAM when exposed to these conditions, degraded at different rates under all conditions, while it was not affected by thermal degradation.

In the regression lines obtained from the linearity study, the correlation coefficients (R^2) found for each active ingredient and impurities were greater than 0.995, the method exhibited good linearity for both the drug substance and related impurities over the entire working concentration range. This fundamental characteristic underscores the method's reliability and suitability for accurate quantification in pharmaceutical analysis.

In addition, system suitability sests were performed for the proposed chromatographic method, and it was observed that the appropriate theoretical plate number, peak asymmetry, resolution, selectivity, and tailing factors were provided for all analytes. Retention times for LAM drug, impurity A, and impurity F were 11.34; 12.07, and 15.30 minutes, respectively. LOD and LOQ values were found for LAM 0.019 µg/mL, 0.058 µg/mL, and for Impurity-A 0.015 µg/mL and 0.047 µg/mL, respectively, while for Impurity-F were 0.030 µg/mL and 0.090 µg/mL. As a result, the developed method is suitable in terms of precision and repeatability with low standard deviation values, accuracy with the results of recovery% studies, sensitivity with low LOD values and slope values of the calibration lines, linearity with correlation coefficient (r) values close to 1 and is routinely used.

In summary, the developed HPLC methodology exhibits good specificity, linearity, accuracy, and precision. The statistical analysis of validation parameters attests to their acceptability, thereby validating the method's appropriateness for both qualitative and quantitative assessments of LAM and impurities. A notable advantage of this technique is its capability to concurrently analyze impurities, a unique feature absents in literature

concerning HPLC-based determination of these specific LAM impurity drugs. Additionally, the approach is easy to use and dependable for routine impurity analysis of pharmaceutical products can be recommended for analysis.

It is worth mentioning that certain companies might choose to sell their products at lower prices by sacrificing quality, resulting in the presence of impurities surpassing acceptable limits. This was evidenced in lamotrigine tablets sourced from manufacturers in Yemen and India, where higher levels of impurities were detected compared to LAM tablets from a Turkish manufacturing company. Such elevated impurities could potentially induce toxic effects and contribute to various health complications.

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Foreign Language : English, Arabic, Urdu, Turkish

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Education and Professional Background:

- **2011-2013**, Master in Organic Chemistry, Maulana Azad of Arts, Science and Commerce, Aurangabad-Indai.
- **2004-2007**, Bachelor' Chemistry, Thamar University Faculty of Applied Sciences, Department, chemistry.

Publications and/or Scientific/Artistic Activities:

- ALSaeedy, Mohammed, Ahmed Hasan, Arwa Al-Adhreai, Ali Alrabie, Hafsah Qaba, Abdulrahman Mashrah & Elif Mine Öncü-Kaya "An overview of liquid chromatographic methods for analyzing new generation anti-epileptic drugs." Journal of Liquid Chromatography & Related Technologies 45.5-8 (2022): 66-83.
- ALSaeedy, Mohammed, Arwa Al-Adhreai, Elif Mine Öncü-Kaya & Erol Şener "An Overview of Advances in the Chromatography of Drugs Impurity Profiling" Critical Reviews in Analytical Chemistry 53:7 (2023): 1455-1471.
- Alrabie, Ali, Nabeel Abdullah Alrabie, and Mohammed AlSaeedy. "ICP-MS analysis, GC-MS identification of antimicrobial, Antioxidant, Antidiabetic volatile bioactive constituents from Tribulus terrestris fruits." (2022).
- Alrabie, A., Alrabie, N. A., AlSaeedy, M., Al-Adhreai, A., Al-Qadsy, I., Al-Horaibi, S. A., ... & Farooqui, M. (2023). An integrative GC–MS and LC–MS metabolomics platform determination of the metabolite profile of Bombax ceiba L. root, and in silico & in vitro evaluation of its antibacterial & antidiabetic activities. Natural Product Research, 37(13), 2263-2268.
- ALSaeedy, Mohammed et al. "Novel Hybrid Triazoline-Triazole Glycosides: Synthesis, Characterization ALSaeedy, Mohammed, Antimicrobial Activity study via In Vitro, and In Silico Means." Carbohydrate Research 532 (2023): 108877.
- Al-Madhagib, Haitham, and ALSaeedy, M. "Dual Docking of Some Synthesized Isoxazolidine Derivatives Against Cathepsin L and Main Protease as a Novel Treatment Strategy for COVID-19." Journal of Molecular Structure (2023): 137253.
- Al-horaibi, S. A., Al-Odayni, A. B., Alezzy, A., ALSaeedy, M., Saeed, W., Hasan, A., & El-Shishtawy, R. M. (2023). Development of new co-sensitizer based squaraine dyes for enhancing the performance of DSSC. Journal of Molecular Structure, 1292, 136130.

- Al-Adhreai, A., ALSaeedy, M., Alrabie, A., Al-horaibi, S. A., Al-Qadsy, I., Alezzy, A. A., ... & Farooqui, M. (2023). Enhanced synthesis of novel multisubstituted isoxazolidines as potential antimicrobial and antioxidant agents using zinc (II) catalyst, and in silico studies. Journal of Molecular Structure, 1292, 136146.
- Arwa, A. A., ALSaeedy, M., Alrabie, A., Al-Qadsy, I., Dawbaa, S., Alaizeri, Z. M., .. & Farooqui, M. (2022). Design and synthesis of novel enantiopure Bis (5-Isoxazolidine) derivatives: insights into their antioxidant and antimicrobial potential via in silico drug-likeness, pharmacokinetic, medicinal chemistry properties, and molecular docking studies. Heliyon, 8(6).
- Adhreai, A. A., ALSaeedy, M., Farooqui, M., Alrabie, A., Al-Qadsy, I., & Al-Timari, U. (2022). Stereoselective synthesis of novel chiral open-chain d-ribose and d-glucose-derived nitrones through 1, 3-dipolar cycloaddition of maleimide and maleic acid and investigation of their antimicrobial activity via molecular docking and ADMET studies. Journal of Molecular Structure, 1256, 132481.
- Al-Horaibi, Sultan A., Abdel-Basit Al-Odayni, Mohammed ALSaeedy, Fares Hezam Al-Ostoot, Adel Al-Salihy, Abdulmajeed Alezzy, Arwa Al-Adhreai, Faizaa A. Saif, Salama A. Yaseen, and Waseem Sharaf Saeed. "Exploring DSSC Efficiency Enhancement: SQI-F and SQI-Cl Dyes with Iodolyte Electrolytes and CDCA Optimization." Molecules 28, no. 20 (2023): 7129.
- Alezzy, A. A., Al-horaibi, S. A., Alnahari, H. A., Al-Odayni, A. B., ALSaeedy, M., AL-Adhreai, A., ... & Arif, P. M. (2023). Synthesis, Characterization, DFT Calculations, and Biological Studies of Novel N-(5-methylisoxazol-3-yl)-4-(((1E, 2E)-3-phenylallylidene) amino) benzenesulfonamide Schiff base (SMZ-CAL) and its Cu (II) and Ni (II) Metal Complexes. Journal of Molecular Structure, 137069.
- Al-horaibi, S. A., Al-Odayni, A. B., Alezzy, A., ALSaeedy, M., Al-Adhreai, A., Saeed, W. S., & Hasan, A. (2023). Novel Squaraine Dyes for High-Performance in Dye-Sensitized Solar Cells: Photophysical Properties and Adsorption Behavior on TiO2 with Different Anchoring Groups. Journal of Molecular Structure, 135943.
- Al Adhreai, A., ALSaeedy, M., Farooqui, M., Alrabie, A., Al-Qadsy, I., & Al-Timari, U. (2022). Stereoselective Synthesis of Novel Chiral Open-Chain D-Ribose and D-Glucose-Derived Nitrones through 1, 3-Dipolar Cycloaddition of Maleimide and Maleic Acid and Investigation of Their Antimicrobial Activity Via Molecular Docking and ADMET Studies. Journal of Molecular Structure, 1256.
- Al-horaibi, Sultan A., Abdel-Basit M. Al-Odayni, Abdulmajeed A. Alezzy, Mohammed A. ALSaeedy, Waseem S. Saeed, and Reda M. El-Shishtawy. "Novel D-Π-A Co-Sensitizers-Based Squaraine Dyes for Achieving High-Performance of Dye-Sensitized Solar Cells." Available at SSRN 4380095.
- Alrabie, Ali, Mohammed AlSaeedy, Arwa Al Adhreai, and Mazahar Farooqui. "In Silico, Molecular Docking And *in-vitro* Antibacterial Activity of Bioactive

- Compounds of Aqueous Extract of Caesalpinia Bonducella (L.) Roxb Seed Kernel." (2022).
- Alrabie, A., Al-Rabie, N. A., Al Saeedy, M., Al Adhreai, A., Al-Qadsy, I., & Farooqui, M. (2023). Martynia annua safety and efficacy: heavy metal profile, in silico and in vitro approaches on antibacterial and antidiabetic activities. Natural product research, 37(6), 1016-1022.
- Abod, Daiekh AA, S. M. AL-Bader, and Mohammed A. AL-Saeedy. "Synthesis of New D-xylose Derivatives and Study Their Antimicrobial Effect."2016
- Synthesis and Characterization of Some New Glycosides Derived from The Sugar D-ribose of Expected Biological (Journal of Purity, Utility Reaction and Environment Vol.5 No.1, February 2016, 31-42)
- Synthesis of New D-Glucose Derivatives and Study Their Antimicrobial EffectJournal of Purity, Utility Reaction and Environment, 2014-08-14 | journal-article.
- Synthesis, purification and identification of some D-xylosides (6^{th} International Pharmaceutical Science Conference, Faculty of Pharmacy, UPM-University-Malaysia 12, 13, March, 2015.

Authorships

❖ Book Title (Khat and chemical drug) Issued in 2016, with co-authorship Dr Arwa Al-Adhrea, Member at Dhamar University of Yemen.

Academic awards

- 2008, Scientific Achievement Certificate for obtaining the first highest score of BA degree in the academic year 2007/2008, awarded by the president of Yemen Republic.
- 2011, a scholarship in India at Department of Chemistry, Maulana Azad College of Arts, Science and Commerce, Aurangabad, India via ICCR and Al Bayda University program to prepare for Master degree.
- 2017, obtained a scholarship to prepare for a doctorate degree in analytical chemistry from Al Bayada University, Yemen.

Internship

- I got an internship in Italy at the Department of Pharmacology, Faculty of Pharmacy, University of Messina, 98122 Messina, for the academic year 2010-2011.

Professional Union/Association/Organization Memberships:

- A researcher of Organic Chemistry and analytical chemistry, Faculty of Science, Al Bayada University, Yemen