



**DEVELOPMENT AND VALIDATION OF
NOVEL HPLC-DAD AND HPLC-ELSD
TECHNIQUES FOR THE ANALYSIS OF
LISINOPRIL DIHYDRATE AND ITS
IMPURITIES**

Hafsah Mohammed Hussein QABA

Eskişehir 2025

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Ph.D. DISSERTATION

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FINAL APPROVAL FOR THESIS

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ABSTRACT

DEVELOPMENT AND VALIDATION OF NOVEL HPLC-DAD AND HPLC-ELSD TECHNIQUES FOR THE ANALYSIS OF LISINOPRIL DIHYDRATE AND ITS IMPURITIES

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Department of Analytical Chemistry

Anadolu University, Graduate School, July 2025

Supervisor: Prof. Dr. Göksel ARLI

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This thesis provides the development and validation of robust analytical approaches for the determination of lisinopril dihydrate (LSDH) and its impurities (A and D) in both active pharmaceutical ingredients and tablet preparations. Two HPLC methods, HPLC-DAD and HPLC-ELSD, were developed and systematically validated. Their performance was evaluated using a 250 × 4.6 mm, 5 µm C18 column. The HPLC-DAD analysis was performed at a flow rate of 1.00 mL/min using a mobile phase consisting of a 60:40 (v/v) mixture of 23 mM phosphate buffer (pH 9.50) and methanol. Detection was performed at 210 nm using a DAD detector. For HPLC-ELSD analysis, a mobile phase consisting of water and methanol in a 60:40 (v/v) ratio was used at a flow rate of 1.00 mL/min. Detection was performed using an ELSD detector set at 60°C, with gain and filter settings of 7 and 2, respectively. The injection volume was set to 10 µL for both methods. - The findings obtained from the statistical evaluation demonstrated that both techniques used to measure LSDH yielded comparable results without significant differences. Both methods exhibited excellent selectivity, stability, and sensitivity, thereby confirming their effectiveness in accurately determining LSDH and related impurities (A and D) in pharmaceutical tablet formulations. In general, the use of modern chromatographic systems such as HPLC-DAD and HPLC-ELSD enhances analytical reliability, strengthens therapeutic value, and ensures compliance with pharmaceutical regulations.

Keywords: Lisinopril dihydrate, Impurity A, Impurity D, HPLC, UV/DAD, ELSD, Validation.

ÖZET

LİSİNOPRİL DİHİDRAT VE SAFSIZLIKLARININ EŞ ZAMANLI ANALİZİ İÇİN YENİ HPLC-DAD VE HPLC-ELSD YÖNTEMLERİNİN GELİŞTİRİLMESİ VE VALİDASYONU

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Danışman: Prof. Dr. Göksel ARLI

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Bu tez çalışmasının temel amacı, lisinopril dihidrat (LSDH) ve ilgili safsızlıkları (Safsızlık A ve D) içeren farmasötik hammaddeler ve tablet dozaj formlarında bu bileşiklerin eş zamanlı tayinine yönelik yeni ve güvenilir HPLC-DAD ve HPLC-ELSD yöntemlerinin geliştirilmesi ve validasyonudur. Bu kapsamda geliştirilen yöntemler, 250 mm × 4.6 mm boyutlarında ve 5 µm parçacık çapına sahip C18 kolonu kullanılarak değerlendirilmiştir. HPLC-DAD analizlerinde, fosfat tampon çözeltisi (pH 9,50; 23 mM) ile metanolün 60:40 (v/v) oranındaki karışımından oluşan mobil faz sayesinde, LSDH ve safsızlıkları etkili bir ayırma sağlanmıştır. Akış hızı 1,00 mL/dk olarak belirlenmiş ve analizler 210 nm dalga boyunda DAD dedektörü ile gerçekleştirilmiştir. HPLC-ELSD yöntemi ise LSDH ile safsızlık A ve D'nin kantitatif tayini için geliştirilmiş olup, su:metanol (60:40, v/v) karışımı mobil faz olarak kullanılmıştır. ELSD analizlerinde de sistem 1,00 mL/dk akış hızında çalıştırılmış ve enjeksiyon hacmi her iki yöntem için 10 µL olarak sabitlenmiştir. Her iki yöntemin istatistiksel değerlendirilmesi sonucunda, LSDH'nin kantitatif analizinde anlamlı bir fark bulunmamıştır. Geliştirilen yöntemlerin yüksek seçiciliğe, tekrarlanabilirliğe ve kararlılığa sahip olduğu doğrulanmış; böylece LSDH ve ilgili safsızlıklarının farmasötik formülasyonlarda doğru ve güvenilir biçimde tayini açısından uygunluğu ortaya konmuştur. Elde edilen sonuçlar, özellikle LSDH tabletlerinde kalite kontrol süreçlerinde etkin bir şekilde kullanılabilecek düzeyde güvenilir olup, ilaç endüstrisinde ürün güvenliği, etkinliği ve yasal düzenlemelere uyum açısından büyük önem taşımaktadır.

Anahtar Sözcükler: Lisinopril dihidrat, Safsızlık A, Safsızlık D, YPSK, UV/DAD, ELSD, Yöntem validasyonu.

STATEMENT OF COMPLIANCE WITH ETHICAL PRINCIPLES AND RULES

I hereby truthfully declare that this thesis/dissertation is an original work prepared by me; that I have behaved following 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 Anadolu 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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Hafsah Mohammed Hussein Qaba

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I have been ambitious since childhood; I have always dreamed of success with a meaningful societal role. Life is full of opportunities and experiences; everyone must strive and move forward, no matter the circumstances. From this perspective, I started achieving my goals one by one, and today, I have reached one of my greatest goals: completing my doctoral studies in my favorite field, analytical chemistry. The journey was never easy, but determination, persistence, and seeking help from God enabled me to continue this path and reap the fruits of its success.

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LIST OF ABBREVIATIONS

ACE	: Angiotensin-Converting Enzyme
ACN	: Acetonitrile
ANOVA	: Analysis of Variance
API	: Active Pharmaceutical Ingredients
CE	: Capillary Electrophoresis
DAD:	: Photodiod Array Dedector
ELSD	: Evaporative Light Scattering Dedector
GC	: Gas Chromatography
HPLC:	: High Performance-Liquid Chromatography
HPTLC	: High-Performance Thin Layer Chromatograph
ICH	: International Conference on Harmonization
LIS	: Lisinopril
LOD	: Limit of Detection
LOQ	: Limit of Quantitation
LSDH	: Lisinopril Dihydrate
MeOH	: Methanol
MS	: Mass spectrometry
NaH ₂ PO ₄	: Sodium Dihydrogen Phosphate
RSD	: Relative Standard Deviation
SD	: Standard Deviation
SEM	: Standard Error of the Mean
SFC	: Supercritical fluid chromatography
UV	: Ultraviolet

1 INTRODUCTION

Pharmaceutical analysis is widely regarded as one of the toughest areas in analytical chemistry. The primary aim of this field is to establish effective and validated techniques for the comprehensive analysis of APIs and pharmaceutical formulations, encompassing both qualitative identification and quantitative measurement. These methodologies are essential for providing analytical assistance in drug research, medical applications, quality assurance procedures, and many clinical studies. The majority of pharmaceutical analyses rely on instrumental analytical techniques. A key challenge for analysts is to develop methods that are very selective, accurate, precise, affordable, and efficient, while ensuring their accessibility and practicality for diverse users.

Each year, the Food and Drug Administration (FDA) approves a significant number of novel therapeutic agents, contributing substantially to advancements in public health and clinical practice. For therapeutic agents to exert their intended pharmacodynamic actions, they must be both effective and pose minimal risk. In today's drug design landscape, most active compounds are generated via synthetic chemistry. These substances must be free from process residues or by-products resulting from degradation, whether during synthesis or long-term storage. Even trace levels of such undesirable substances can lead to adverse pharmacological or toxicological outcomes. Therefore, in pharmaceutical manufacturing, which encompasses both bulk components and final formulations, impurity profiles must be kept within acceptable thresholds.

The drug needs to maintain its bioactive potency to show its intended clinical effect. The measurement of efficacy and detection of potential contaminants needs sensitive and reliable analytical platforms. Bioanalytical analyses help determine drug concentrations in physiological matrices and provide information about absorption, distribution, metabolism, and excretion (ADME) properties.

The development of an appropriate analytical protocol for evaluating a specific compound requires the selection of a detection strategy based on the physical and chemical properties of the analyte. Pharmaceutical industry analytical workflows need to show high specificity and robustness to deliver accurate results through efficient and cost-effective operation. The results need to fulfill strict quality standards, which can only be achieved through validation against established procedures or globally accepted scientific methods. The following goals serve as the framework for this study's

thorough assessment of lisinopril dihydrate (LSDH) and its related impurities (A and D): developing and analytical verification of a novel HPLC-PDA technique that uses photodiode array detection (PDA) to quantify LSDH and associated impurities (A and D) in both commercial tablet formulations and pure reference solutions, developing and identifying a different method for the same analytes in similar matrices that uses evaporative light scattering detection (HPLC-ELSD). Methodological comparison and explanation of the experimental results from both analytical systems, with a focus on sensitivity, accuracy, and how well the methods work. Using analysis of variance (ANOVA) to verify the consistency between methods and the sensitivity of the analysis. The methods that were developed underwent thorough validation processes and were then used to test LSDH and other impurities in a large number of pharmaceutical preparations. This made it possible to do a strong analytical assessment backed by statistical measurements.

2 LITERATURE REVIEW

2.1 Overview of Hypertension Management

The medical term hypertension describes the condition where blood pressure remains elevated at systolic (SBP) and/or diastolic (DBP) levels throughout an extended period. The condition raises the risk of cardiovascular problems, which eventually result in severe medical outcomes, including heart disease, stroke, organ failure and death (Carey et al., 2022). The biological pathways of hypertension include sympathetic nervous system activation and renin-angiotensin-aldosterone system (RAAS) stimulation and elevated oxidative stress levels (Basi and Turkoglu, 2019). According to the International Society of Hypertension and the World Health Organization (WHO), nearly two-thirds of adults 60 years of age and older have high blood pressure, and over one billion adults worldwide suffer from it. Both cardiovascular diseases and the deaths that result from them are significantly influenced by this condition. The World Health Organization (2023) estimates that 1.28 billion adults between the ages of 30 and 79 suffer from hypertension, which is the world's leading cause of premature death. Furthermore, an estimated 7.5 million deaths globally are linked to uncontrolled hypertension annually. The primary aim of antihypertensive treatment is to sustain a stable blood pressure reading of 140/90 mmHg, as recommended by the WHO in 2021. On the other hand, the issue of uncontrolled high blood pressure in clinical settings remains significant. When patients fail to achieve their desired blood pressure, healthcare providers may need to adjust their medications or encourage better treatment plan adherence. A lack of patient commitment, especially when lifelong therapy is necessary, can lead to insufficient blood pressure management. Additionally, fewer than half of adults with high blood pressure (42%) receive a diagnosis and appropriate treatment, and only 21% (one in five) successfully manage their condition. Global health initiatives have outlined a 33% reduction in hypertension prevalence from 2010 to 2030 as a primary goal for mitigating non-communicable diseases (Delacroix et al., 2014; Shyteyeva et al., 2024; Zhao et al., 2024). Existing blood pressure control strategies in hypertensive patients are often ineffective, with low adherence rates and the harmful impact of medication-related adverse events contributing to suboptimal outcomes (Shah et al., 2017).

High blood pressure, known as hypertension, can be categorized based on how severe it is. This classification depends on blood pressure readings and how other organs are

functioning. Clinically, hypertension can be categorized as either essential or secondary. Essential hypertension, which comprises most cases, often arises without a known underlying condition. Usually, high blood pressure is the first and only sign of their condition. Sometimes, hypertension is the result of other effects on the body and is called secondary hypertension. Blood pressure readings are given in millimeters of mercury (mmHg). Blood pressure is routinely measured in both systolic and diastolic phases of the cardiac cycle. Because of alterations in large arteries associated with aging, SBP rises aged 50 or younger, the increase in pressure is, on average, 2 mmHg per year, whereas the increase in diastolic pressure reaches 0.5–1 mmHg (Sukalo et al., 2016).

2.2 Hypertension Treatment

The start of antihypertensive drug treatment should be guided by a person's blood pressure and risk for atherosclerotic cardiovascular disease (ASCVD) (Carey et al., 2022). Choosing suitable antihypertensive treatment depends on various factors, including age, comorbidities, ethnic background, whether the patient is pregnant and other clinical factors for which an individualized approach is necessary. Based on distinct pharmacological and pharmacokinetic actions, antihypertensive drugs can be classified as follows:

- **Angiotensin-converting enzyme inhibitors (ACE):** Lisinopril (LIS), enalapril and captopril are the major first-line therapeutic agents that are prevalently used in the treatment of hypertension. These drugs are efficacious and tolerable. ACE inhibitors reduce the amount of the potent vasoconstrictor angiotensin II by stopping its conversion from angiotensin I, lowering high blood pressure.
 - **Angiotensin Receptor Blockers (ARBs):** deliver effects comparable to ACE inhibitors in lowering blood pressure, but are less likely to cause cough. Combination with ACE inhibitors may be considered for better Renin-Angiotensin-Aldosterone System (RAAS) blockade, though other drug classes (e.g., thiazide diuretics) are often preferred.
 - **Calcium Channel Blockers (CCBs):** such as amlodipine, inhibit calcium influx in vascular smooth muscle, reducing contraction.

Dihydropyridines primarily cause vasodilation and reduce systemic vascular resistance.

Non-dihydropyridines reduce heart rate and myocardial contractility.

- **Diuretics:** increase sodium and water excretion, improving cardiovascular outcomes and reducing stroke risk.

Thiazide diuretics, including hydrochlorothiazide (HCTZ) and chlorthalidone, are often recommended; however, they might raise the chances of developing type II diabetes.

Loop diuretics with potassium-sparing agents help prevent hypokalemia and hypomagnesemia.

Spironolactone is beneficial in heart failure but may cause hyperkalemia, gynecomastia, and renal impairment.

- **Beta-blockers:** are known to affect glucose metabolism and can mask the symptoms of hypoglycemia, requiring caution when prescribed to patients at risk for diabetes.
- **Aldosterone Antagonists:** often used in combination with ACE inhibitors, ARBs, CCBs, or diuretics, especially in heart failure, these drugs are effective antihypertensives in certain patients but may lead to electrolyte imbalances and renal dysfunction.
- **Alpha-blockers** are often prescribed as extra treatment for people with hard-to-control hypertension or for those who cannot tolerate first-line drugs. Additionally, they are particularly effective in older males with benign prostatic hyperplasia (BPH), helping to alleviate both hypertension and urinary symptoms associated with BPH. Combining an ACE inhibitor like lisinopril (LIS) with a diuretic such as hydrochlorothiazide (HCTZ) is regarded as an effective therapeutic strategy, owing to their complementary mechanisms that produce an additive effect in lowering blood pressure (Rizzoni et al., 2003; Delacroix et al., 2014; Shah et al., 2017).

2.3 LSDH

LSDH competes to block the ACE, which prevents angiotensin I from turning into angiotensin II, a strong substance that narrows blood vessels. It functions similarly to a version of enalapril and influences various components of the renin-angiotensin-aldosterone system. It is very important for managing high blood pressure and heart failure, and also helps prevent heart attacks and kidney problems associated with diabetes (Sukalo et al., 2016; Shulyak et al., 2021). LSDH increases renin activity in the blood by diminishing the normally occurring negative feedback via angiotensin II. The beneficial effects it produces in patients with high blood pressure are due to its ability to block the

renin-angiotensin-aldosterone system (RAAS). As a result, the activity of vasopressors and aldosterone declines, even in individuals exhibiting low renin concentrations. ACE inhibitors may also increase the risk of angioedema by blocking the breakdown of bradykinin (Min et al., 2023).

- Dosage form and administration

For managing hypertension, this medication is taken by mouth. Lisinopril is available in tablet form (2.5, 5, 10, 20, 30, and 40 mg) and as a liquid solution (1 mg/mL). It can be safely ingested with or without food. The typical daily dose for adults varies from 2.5 mg to 40 mg, based on what condition being treated. In patients with a glomerular filtration rate (GFR) ≤ 30 mL/min, clinicians often modify Lisinopril dosing to accommodate impaired renal function (Min et al., 2023). For those on diuretics, 5 mg once daily is recommended. A usual long-term dosage starts at 20 mg once a day, potentially increasing to 80 mg daily. For children aged six and older, an initial dose can be up to 0.07 mg/kg (or up to 5 mg daily). After taking the medication, it typically begins to work in about 1 to 2 hours and remains effective for around 24 hours. Lisinopril is absorbed from the gastrointestinal tract at a slow and steady rate when taken orally, reaching its highest concentration in the blood about 7 hours later. The distribution of the medication is around 25%. It is eliminated via the urine in its unchanged form and does not undergo metabolic transformation (Pund et al., 2017; Dode et al., 2023). Since LSDH is not metabolized at all, it is eliminated through urine (Zhao et al., 2024).

- Side effects

Common adverse effects linked to antihypertensive agents such as captopril, lisinopril, and enalapril include cough, dermatological reactions, taste disturbances, and angioedema. Due to these negative effects, there is an increasing focus on encouraging the creation of natural ACE inhibitors (Zhou et al., 2018). Additionally, LSDH medications can cause several side effects, such as:

- A persistent dry cough that doesn't improve
- Dizziness or feeling faint, particularly when standing or sitting up quickly
- Head pain
- Nausea or vomiting
- Loose stools

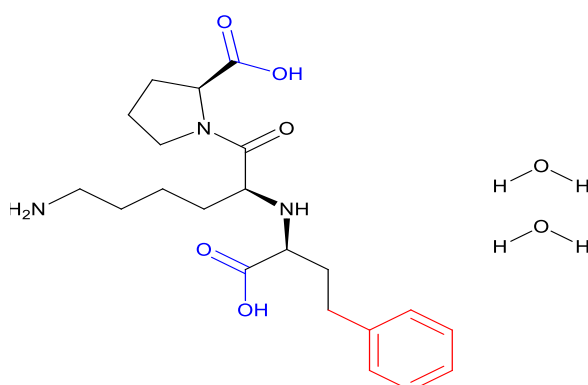
- Skin itching or a slight rash
- Seeing things in a blurry way (http-1).
- Cautions

A rare reaction known as angioedema can occur with LSDH. Common signs of this reaction are swelling in areas like the lips, face, tongue, or throat (http-2).

2.3.1 LSDH physical and chemical properties

The chemical identity of LSDH corresponds to (S)-1-[N²-(1-carboxy-3-phenylpropyl)-L-lysyl]-L-proline in its dihydrated form. Examining the chemical structure illustrated in Figure 2.1 reveals that LSDH contains a heteroatom, specifically nitrogen. This suggests that it is prone to oxidation. Additionally, the presence of an amide functional group increases the likelihood of undergoing hydrolysis (Vujanović et al., 2017). It differs from captopril and enalapril in several respects: it is hydrophilic, bypasses hepatic metabolism, and exhibits a prolonged half-life (Min et al., 2023). The physicochemical parameters of LSDH (water solubility, molecular weight, distribution coefficient) make it an ideal option for involvement in a transdermal delivery system (Shyteyeva et al., 2024). In addition, LSDH exhibits more distinct spectroscopic peaks in alkaline environments compared to acidic conditions (Özer and Senel, 1999). Due to its gastric irritant properties, LSDH is formulated with a cellulose acetate coating to mitigate gastrointestinal discomfort (Bhairav et al., 2017).

Figure 2.1
Chemical Structure of LSDH (Sbârcea et al., 2014)



- LSDH, possesses the chemical formula $C_{21}H_{31}N_3O_5 \cdot 2H_2O$ and is systematically named (2S)-1-[(2S)-6-amino-2-[[[(1S)-1-carboxy-3-phenylpropyl]amino}hexanoyl]pyrrolidine-2-carboxylic acid dihydrate. The compound features a modified peptide

chain with a phenyl-substituted alkyl segment and a proline derivative, alongside two integrated water molecules as part of its crystalline matrix.

- This white to off-white colored solid is an odorless powder that weighs nearly 441.525 g/mol, having a monoisotopic mass of 441.2475 g/mol. It melts at 160°C and is soluble in water and methanol but shows poor solubility in ethanol, acetone, acetonitrile, and chloroform. Its partition coefficient (Log P) was determined in a 0.1 M phosphate buffer at pH 10.2.
- LSDH remains chemically stable under typical ambient conditions (20–25°C) but may degrade if exposed to elevated thermal stress, specifically temperatures around 105°C. (Demarco and Brooks, 1992; Adam et al., 2012; Pund et al., 2017; Gul et al., 2017; Bozhanov et al., 2020; Dode et al., 2023).

2.3.2 Pharmacokinetics

LSDH is absorbed slowly and not completely by the gastrointestinal system, and eating does not affect how it gets absorbed. After taking it by mouth, the bioavailability of LSDH is low, between 10% and 30%. Following administration, the compound reaches peak levels within 6–8 hours and is primarily eliminated unchanged via urinary excretion. It has a half-life of around 12 hours, demonstrates minimal protein binding, and shows limited distribution in individuals with compromised cardiac function (Min et al., 2023).

2.3.3 Solution stability

Demarco and Brooks (1992) demonstrated that LSDH retained its stability at a concentration of 0.2 mg/mL across a pH range of 2.7 to 10.0, under constant ionic strength ($\mu = 0.3$). Their analysis included a graph depicting zero-order rate constants at 60°C and 80°C as a function of pH. Under acidic conditions, LSDH undergoes rapid degradation, predominantly producing diketopiperazine as its major decomposition product. The formation of diketopiperazine was shown to increase linearly over time. However, at neutral and higher pH levels above 7.0, the rate of decomposition is minimal.

2.4 Impurity

As per the International Council for Harmonisation (ICH) guidelines, any component in a pharmaceutical formulation that does not represent the pharmacologically active substance or a defined excipient with functional relevance is considered an impurity or foreign material (Ramachandra, 2017). Consequently, impurity profiling, which

encompasses identification, structural elucidation, and quantitative evaluation of these unwanted substances, remains a cornerstone of modern pharmaceutical analysis, especially in assessing formulations and bulk drug substances. This area has become increasingly important because unknown and possibly harmful impurities pose health risks. These impurities must be identified and investigated by certain methods to make drug treatment more safety (Bartos and Görög, 2008; Görög, 2018).

In recent years, many of the regulatory authorities, such as the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), the US Food and Drug Administration (FDA), the European Directorate for the Quality of Medicines (EDQM) and Health Canada, have accentuated the need of purity standards and impurity profiling of APIs. The determination of impurities is important in a rational-based approach to impurities in order to guarantee the safety of pharmaceuticals. This highlights the necessity of impurity profiling in pharmaceutical drug research (Bari et al., 2007).

Pharmaceutical impurities include any unwanted substances that can be formed in the drug active substances or drug products during the manufacturing process or with storage. The pharmacological potency and safety features of drug products are impaired even by trace quantities of foreign materials. Such chemicals may cancel the usefulness thereof by providing adverse pharmacological or toxicological effects. The sensitive and selective determination of relevant impurities is highly important in pharmaceutical quality control and clinical safety due to the strict regulation in the presence of impurities (Shah et al., 2012; Venkatesan and Valliappan, 2014; Ramachandra, 2017).

2.4.1 Impurity profiling

It involves the identification and characterization of known and unknown impurities in APIs manufactured with a controlled process. The identification, characterization and quantitative determination of impurities and degradation products in bulk drug substances and pharmaceutical preparations constitute an important part of contemporary industrial pharmaceutical research. This attention is guided by the general principle that all drugs must be free of toxic substances that threaten human health (Venkatesan and Valliappan, 2014; Görög, 2018). Pharmaceutical impurity profiling is accomplished through a variety of analytical methods, such as High-Performance Liquid Chromatography (HPLC) for pharmaceuticals, Gas Chromatography (GC) for related substances, High-Performance

Thin-Layer Chromatography (HPTLC)/Thin-Layer Chromatography (TLC) methods, and Capillary Electrophoresis (CE). Impurities are a major cause of poor quality drugs. The detection of these impurities is significant in the development of drugs. Hence, the application of sophisticated tools in pharmaceutical analysis, particularly impurity profiling, is essential. Spectroscopy techniques, namely NMR, IR and MS, are the most common practices employed to define the structural features of impurities and degradation products in drug substances. In the context of today's analytical methods, chromatographic methods such as HPLC, UPLC, and LC-MS are key technologies (Shah et al., 2012; Ramachandra, 2017).

2.4.2 Impurity type

According to ICH guidelines, impurities existing in chemically synthesized drug substances are usually classified in three principal classes:

2.4.2.1 Organic impurities

Such impurities may be derived from starting materials, intermediates, by-products, or decomposition products, and in general, are indicative of the production process and the drug per se.

2.4.2.2 Inorganic impurities

The production of pharmaceutical products may also lead to the presence of inorganic impurities, such as heavy metals, catalysts, ligands (suitable for metal and non-metal-based catalysts and intermediates), and reagents involved in processing, which are generally classified as inorganic salts or residual metals.

2.4.2.3 Residual solvents

Residual solvents, commonly referred to as volatile organic compounds, are contaminants originating from solvents utilized during drug synthesis. According to the United States Pharmacopeia (USP), impurities are classified into three major categories:

- Those present in an official pharmaceutical product
- General impurities
- Organic volatile impurities

“Genotoxic impurities (GTIs)” are chemical entities that possess mutagenic or carcinogenic potential, posing serious risks to human health even at trace concentrations.

Their detection and quantification at parts-per-million (ppm) or parts-per-billion (ppb) levels represent considerable analytical challenges in pharmaceutical development (Venkatesan and Valliappan, 2014; Ramachandra, 2017).

2.4.3 Impurity sources

- Pharmaceutical impurities may originate from diverse sources and can be present or develop at various stages of drug production. These include:

- Impurities originating from chemical reactions, equipment interactions, or procedural inconsistencies during the drug production
- Impurities that form while the product is stored
- Those that appear during the mixing of formulations and as drugs and products age.

On the other hand, most impurities result from the specific details of the synthetic manufacturing method (Ramachandra, 2017).

2.4.4 Formulation-related impurities

A drug product can contain impurities because of the inactive ingredients that are mixed with the active substance. The formulation process exposes the active compound to environmental factors and excipient interactions that can result in breakdown or deleterious reactions. Aqueous pharmaceutical preparations such as solutions and suspensions, are particularly prone to instability via hydrolysis. The water component contributes not only to the impurity profile but also facilitates hydrolytic and catalytic degradation pathways. Comparable reactions can take place in other solvents that could be utilized.

Impurities related to formulation can be categorized in several ways:

a. Environmental factors

Key environmental influences that can affect stability are listed below:

- **Temperature extremes:** substances that are sensitive to heat, especially in warm climates, can break down active ingredients and generate impurities. For example, vitamins lose their effectiveness when exposed to heat.
- **Light:** when photosensitive materials encounter light or UV rays, they can deteriorate, resulting in impurity formation.

- **Humidity:** it can harm both the raw materials of the drug and any formulation in solid dosage forms.
- Development of impurities over time:

The ingredients in a mixture can interact with each other, causing reactions that create impurities.

- Impurities Associated with Functional Groups
- **Ester breakdown:** aspirin, benzocaine, cefixime, cocaine, ethylparaben.
- **Hydrolysis process:** benzylpenicillin, barbitol, and chloramphenicol.
- **Degradation through oxidation:** hydrocortisone, methotrexate, compounds with heterocyclic aromatic rings, and nitroso/nitrile derivatives.
- **Photolytic degradation** can occur in pharmaceutical products exposed to light during manufacturing, hospital storage, or consumer handling prior to administration. Light-induced reactions may lead to chemical separation, compromising product stability and therapeutic integrity.
- **Certain carboxylic acids in solution**, notably p-amino salicylic acid, undergo decarboxylation when subjected to heat, resulting in the liberation of carbon dioxide (CO₂) (Venkatesan and Valliappan, 2014; Boob et al., 2018).

2.4.5 Breakdown compounds

These products arise when essential components in a formulation deteriorate under external influences, including elevated temperature, photolytic exposure, and humidity, potentially impacting drug quality and stability (Venkatesan and Valliappan, 2014; Boob et al., 2018)

Forced degradation studies, also known as stress testing, constitute a vital component of drug development, offering insights into the intrinsic stability of pharmaceutical compounds under extreme environmental conditions. Pharmaceutical research generally involves studies conducted on pure APIs and various formulated dosage forms such as solid preparations, aqueous solutions, and suspensions, which are often referred to as reference standards. This is done to investigate pathways, evaluate the stability of the drug in different forms, and identify potential degradation products, particularly considering interactions with specific excipients. Research activities are carried out under ICH Guidelines Q1A (R2), which specify different parameters, requirements, and

protocols for stress testing of pharmaceutical substances. Usually, the stress test conditions produce high temperatures of about 50 or 60 °C, with relative humidity greater than or equal to 75%, and hydrolytic, oxidative, and photolytic conditions within a wide-ranging pH spectrum. For hydrolytic degradation studies, the test substance is treated under acidic or basic conditions by either treating it with 0.1 mol.L⁻¹ hydrochloric acid (HCl) or sodium hydroxide (NaOH) for at least eight hours. For oxidative degradation, the active pharmaceutical ingredient is either dissolved directly in hydrogen peroxide (H₂O₂) or the solution is diluted with H₂O₂ from 3 to 30% concentration, depending on the severity required for the evaluation (Vujanović et al., 2017).

2.4.6 Limits for impurities

ICH According to ICH guidelines, impurities exist at 0.1% in new drugs, no detection is necessary for such impurities. This measure could be waived in exceptional circumstances if the impurities demonstrate extremely potent or harmful biological effects. The earlier guidelines also state the acceptability criteria for the presence of impurities are based on daily intake amounts: For drugs given in doses of less than 2 g per day, the assessment of acceptability must be carried out as impurities exist at more than 0.1% or impurities are present at too high a daily intake level of 1 mg per day, whichever is lower. For products administered above 2 g/day, the qualification threshold is reduced to 0.05%, reflecting the increased tolerance for lower relative impurity concentrations (Venkatesan and Valliappan, 2014).

2.4.7 Analytical methods for analyzing impurities

A range of analytical techniques are available for profiling impurities, using different Chromatographic and Spectroscopic methods. These can be utilized individually or in combination with other approaches. HPLC is the most widely utilized analytical technique for impurity profiling in pharmaceutical compounds. Its broad applicability is attributed to the diverse range of available detectors and stationary phases, high sensitivity, and favorable cost-efficiency, making it suitable for a wide array of analytical applications (Bari et al., 2007). During the last decade, a range of analytical protocols have emerged for assessing drug-related byproducts and molecular breakdown components. Nonetheless, chromatographic techniques such as liquid-phase separation coupled with ultraviolet absorption remain the predominant strategy for quantifying known residual compounds. In contrast, to decode the architecture of unidentified

chemical derivatives, approaches integrating mass spectrometric detection, magnetic resonance tools, and both coupled or direct NMR analyses without prior chromatographic resolution are preferentially employed. (Görög, 2018).

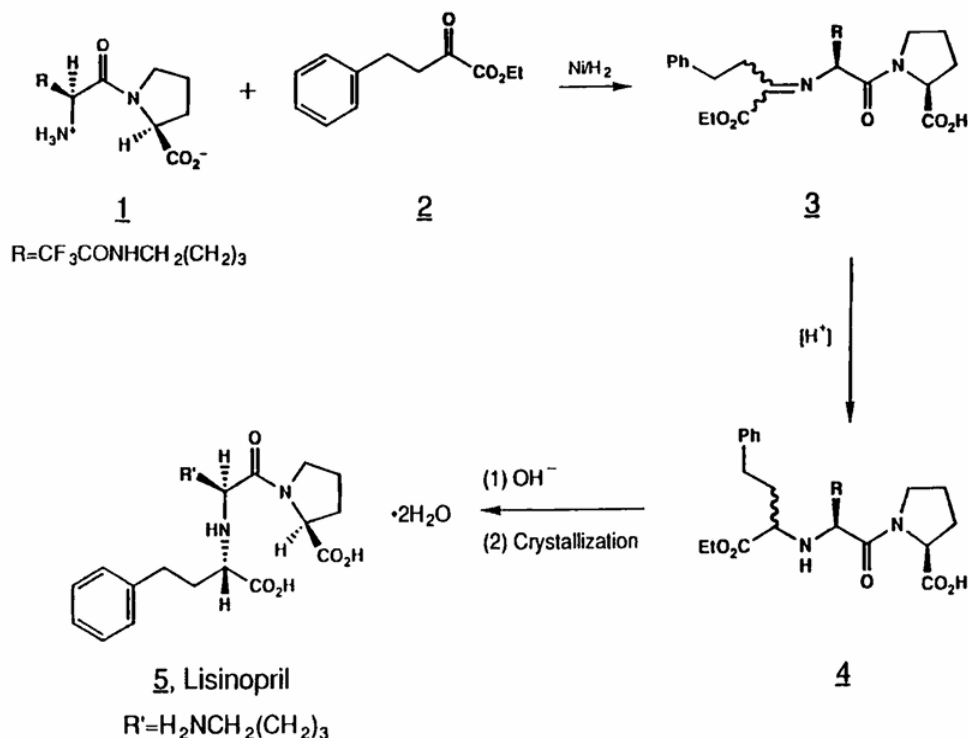
In the last two decades, considerable advancements have been made in modern analytical methodologies to overcome limitations associated with conventional techniques, particularly within the domain of pharmaceutical analysis. A range of hybrid and high-resolution approaches, such as UPLC, LC-MS, GC-MS, LC-NMR, and HPTLC, have been extensively utilized for the qualitative and quantitative evaluation of pharmaceutical substances and formulations (Ramachandra, 2017).

Impurities in pharmaceutical substances can be identified through several well-established analytical approaches. The utilization of reference standards represents a foundational approach in pharmaceutical analysis and manufacturing. These standards function as essential benchmarks for assessing the quality and performance of both production processes and final products. Their roles go beyond the proper identification and quantification of APIs; they are also critical in the detection and control of impurities, degradation products, starting materials, intermediates, and excipients, thereby ensuring consistency, regulatory compliance, and patient safety. Spectrometric techniques are widely used for impurity profiling as they deliver very detailed structural and compositional information. Ultraviolet spectrometry, infrared spectrometry, nuclear magnetic resonance, and mass spectrometry would elucidate changes present in molecular structure and impure compounds and give their chemical nature. Also, in pharmaceutical analysis, utmost importance is placed on separation techniques used for the isolation of impurities and proper measurement of their quantities. Different separation techniques are in use, such as thin layer chromatography, gas chromatography, high-performance liquid chromatography, capillary electrophoresis, and supercritical fluid chromatography. These techniques further increase analytical sensitivity by allowing accurate detection and analysis of trace impurities, hence by ensuring that the quality of the product does not suffer. Isolation techniques are ordinarily put to use before characterization, particularly when an in-depth analysis of impurities is required. Solid-phase extraction (SPE), liquid-liquid extraction (LLE), accelerated solvent extraction (ASE), column chromatography, and high-performance thin-layer chromatography (HPTLC) may all be used to separate impurities selectively from the complex

pharmaceutical matrices. These methods improve the subsequent structure elucidation step either by enriching or concentrating the analyte of interest. Such approaches give the possibility for impurity enrichment and purification for further study. Finally, combined separation and detection approaches are presently becoming more prevalent for the exhaustive characterization of impurities, such as gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), diode array detection-liquid chromatography-mass spectrometry (LC-DAD-MS), liquid chromatography-nuclear magnetic resonance (LC-NMR), tandem mass spectrometry (LC-MS/MS), and HPLC-DAD-NMR-MS. Impurity identification and characterization are efficiently done by the simultaneous applications of separation, detection, and structural elucidation (Shah et al., 2012).

2.4.8 LSDH impurities

LSDH was synthesized following the multi-step route summarized in Figure 2.2. The use of Raney nickel began with the reduction of 1, which is N α -trifluoroacetyl-L-lysyl-L-proline tyrosyl dipeptide, with 2, ethyl 2-oxo-4-phenylbutanoate, to obtain the Schiff base intermediate 3, which subsequently led to a diastereomeric mixture 4 of SSS and RSS stereoisomers. Then, hydrolysis removed the N α -trifluoroacetyl protective group, and saponification of the ethyl ester was induced to crystallize in the ethanolic/water system. Final recrystallization from water afforded lisinopril (SSS isomer, 5) with a chemical purity exceeding 98% and an overall yield of approximately 65%, calculated relative to intermediate (3) (Demarco and Brooks, 1992).

Figure 2.2*Synthesis of Lisinopril (Demarco and Brooks, 1992)*

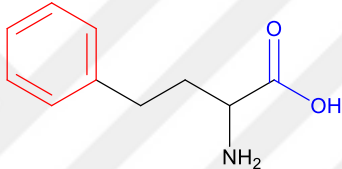
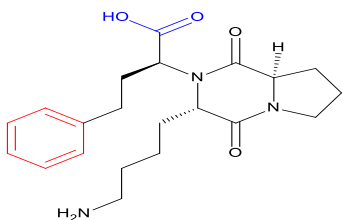
Compounds such as LSDH, which are known for their low absorption rates, require detailed examination of their stability. It is essential to determine the principal variables influencing their stability and to determine the specific conditions required to protect this drug. LSDH is highly sensitive to oxidative degradation and basic hydrolysis. Generally, higher temperatures accelerate the degradation process of LSDH. Degradation occurs at varying rates under various stress factors, depending on the specific degradation mechanism. The European Pharmacopoeia (Ph. Eur. 8.0, 2013 edition) specifies six defined impurities, referred to as impurities A through F, in the monograph for lisinopril (LSDH). The monograph provides comprehensive physicochemical data, including not only structural characterization but also fundamental parameters such as the acid dissociation constant (pK_a) and partition coefficient ($\log P$) for LSDH, as well as each impurity detected. These parameters as summarized in Table 2.1, are important for characterizing acid-base properties and lipophilicity of compounds and hence are among the salient parameters considered during method development and impurity profiling (Vujanović et al., 2017). In this thesis, two impurities, A and D, were selected for this study. Table 2.2 specifies the characterization of LSDH impurities.

Table 2.1*P* partition Coefficient and Value of Dissociation Constant of LIS Impurities (Vujanović et al., 2017)

Substance	log P	pKa ₁	pKa ₂
Lisinopril	1.61	3.17; 3.85	8.01; 10.21
Impurity-A	1.52	2.55	9.52
Impurity-B	1.67	-2.14	-
Impurity-C	1.44	3.79	10.21
Impurity-D	1.44	3.79	10.21
Impurity-E	1.61	3.17; 3.85	8.01; 10.21
Impurity-F	2.07	3.22; 3.88	7.99; 10.21

Table 2.2

Structural Information About Impurity A and D of LSDH

Impurity Systematic (IUPAC) Name	Chemical structure	Properties
Impurity A DL- Homophenylalanine		C ₁₀ H ₁₃ NO ₂ 179.22 g mol ⁻¹ Solid (White Powder) Storage: Store at 2-8°C. Solubility: Aqueous Acid (Slightly), Aqueous Base (Slightly) Melting point: 282 °C (dec.) (lit.) pKa ₁ : 2.55; pKa ₂ : 9.52 Partition coefficient: Log P 1.52 (http-4; http-5; http-6)
Impurity D Lisinopril (S,S,R)- Diketopiperazine		C ₂₁ H ₂₉ N ₃ O ₄ 387.47g mol ⁻¹ White to off-white solid Storage: Moisture-sensitive; store at -20°C Dissolution behavior: Slight in DMSO, methanol, and water Chemical integrity: Hygroscopic; avoid humidity exposure Melting point: >214°C PKa ₁ : 3.79; pKa ₂ : 10.21 Partition coefficient: Log P 1.44 (http-7; http-8).

2.5 Analytical Methods

This study presents the first documentation of the HPLC-ELSD technique for analyzing LSDH and its related impurities (A and D). This research aims to design and validate a fast, efficient, accurate, robust, and economically feasible analytical method for the determination of LSDH and its associated impurities (A and D) in both standard solutions and commercial tablet formulations. The thesis aims to develop fast, simple, precise,

robust, reliable, budget-friendly, and environmentally sustainable methods using HPLC-PDA and HPLC-ELSD. In this study, LSDH and its associated impurities were analyzed in both reference solutions and pharmaceutical tablet formulations using high-performance liquid chromatography combined with diode array detection (HPLC-DAD) and evaporative light scattering detection (HPLC-ELSD). The combined application of these complementary techniques enabled the creation of a comprehensive profile by leveraging the distinct analytical strengths of each detector.

2.5.1 History of chromatography and HPLC

Chromatography remains a fundamental technique for the resolution, identification, and purification of components in complex mixtures exhibiting diverse molecular sizes and weights.

In 1900, D.T. Day, an American chemist, was the pioneer of this method in chemical analysis. Then, in 1906, M.S. Tswett, a Polish botanist, investigated plant pigments with the use of adsorption columns. Chromatography is widely used to separate and purify both solid and liquid substances in a mixture. In column chromatography, the technique relies on a Stationary solid substrate with a flowing liquid phase (Touchstone, 1993; Akash and Rehman, 2020).

Over the next few decades, numerous chromatographic techniques—including paper chromatography, gas chromatography, and ion exchange chromatography—were introduced and refined by researchers. The advent of HPLC in the 1960s marked a transformative advancement, elevating chromatography to a central position in modern analytical science.

HPLC emerged in the 1970s, pioneered by Kirkland and Huber as an advancement of classical column chromatography. The method uses high-pressure liquid delivery through a packed column to achieve effective component separation. The technique of HPLC stands as a common method for separating different components within a mixture. When a sample enters a chromatographic column, its components travel at distinct speeds through the stationary phase. The separation of components occurs as a result of their distinct chemical properties, which interact with both stationary and mobile phases (Akash and Rehman, 2020; Siddique, 2021). The applications of HPLC extend throughout chemical and pharmaceutical analysis and drug development and treatment, as well as biomedical research and environmental science and food testing. High-

performance liquid chromatography serves as a crucial tool in pharmaceutical analysis because it facilitates drug development and regulatory compliance through precise measurements of active ingredients and their impurities and degradation products across various pharmaceutical dosage forms (Yabré et al., 2018; Zuvela et al., 2019; Siddique, 2021).

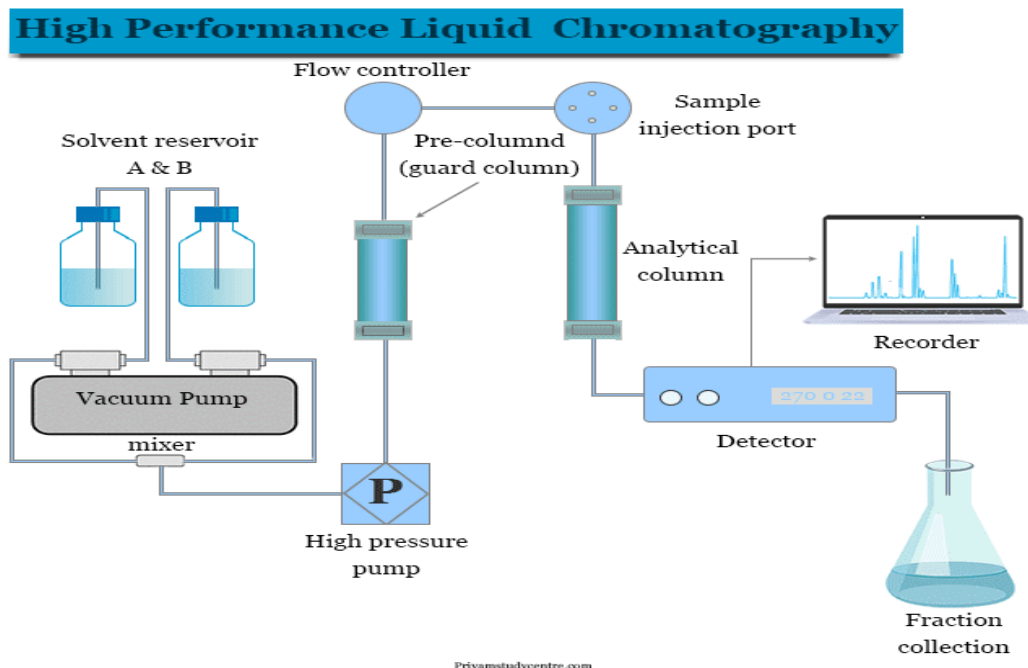
2.5.2 Principle of HPLC technique

The fundamental operation of HPLC relies on compounds experiencing different degrees of interaction with a fixed stationary phase and a flowing mobile phase. Chromatographic separation enables the separation of chemical compounds through their distinct interactions with two different phases. The immobilized medium, typically confined within a tubular structure, engages target compounds via mechanisms such as surface adherence, phase distribution, electrostatic interaction, or molecular filtration, contingent on the applied analytical strategy. The dynamic carrier, generally comprising a fluid or solvent mixture, transports analytes through this medium. As analytes encounter distinct affinities for the fixed phase, their migration velocities differ, resulting in temporal dispersion and thus enabling component-wise resolution of the sample (Siddique, 2021; Siddique, 2023).

In HPLC, columns with 2,000 to 7,000 theoretical plates are typically sufficient for most separations but are inadequate in certain cases:

- When the selectivity factor (α) for critical analyte pairs is too low (< 1.10 - 1.05).
- When the capacity factors (k') of the compounds cannot be sufficiently increased.
- In complex mixtures, where improving resolution for one analyte pair may decrease it for others. For these scenarios, separations requiring 20,000 to 70,000 theoretical plates can be achieved in less than one hour (Verzele and Dewaele, 1982).

Figure 2.3
Schematic Representation of a Typical HPLC System (http-9)



2.5.3 Types of HPLC

The classification of HPLC techniques is determined by the underlying separation principle, encompassing normal-phase, reversed-phase, ion-exchange, and size-exclusion chromatographic modes. Normal-phase (NP) chromatography achieves separation based on polarity by utilizing a nonpolar stationary phase and a polar mobile phase, facilitating the retention of polar analytes. Conversely, reversed-phase (RP) chromatography employs a polar stationary matrix in conjunction with a nonpolar mobile solvent, making it particularly well-suited for analyzing hydrophobic compounds due to their stronger retention in the nonpolar environment. The stationary phase of exchange chromatography contains charged functional groups that interact electrostatically with analytes to enable ionic property-based separation. The size exclusion chromatography method separates molecules according to their hydrodynamic volume because analytes travel through a porous stationary matrix based on their molecular size (Žuvela et al., 2019; Akash and Rehman, 2020; Siddique, 2021).

The chromatographic separation method of RP-HPLC serves as the dominant technique in more than ninety percent of all HPLC applications throughout scientific fields. The method finds widespread application throughout numerous scientific fields which include drug development together with environmental and wastewater monitoring and food

safety analysis and clinical diagnostics and biomedical research. The high adaptability of HPLC enables it to separate diverse chemical types such as nonpolar and polar compounds as well as ionizable and ionic compounds through a single analytical procedure. The mobile phase in RP-HPLC consists of a buffer solution and water-miscible organic modifiers including methanol or acetonitrile which are both widely available and safe to use. This chromatographic technique uses a non-polar stationary phase in conjunction with a polar hydro-organic mobile phase, which can be supported by buffer salts to enhance analytical stability. The retention time of solutes increases proportionally with the increasing polarity of the mobile phase due to (i) the high hydrophobic character of the analyte, (ii) the increased hydrophobicity of the stationary phase surface, and (iii) the decreased solubility of the solutes and increased interactions with the nonpolar matrix (Žuvela et al., 2019).

2.5.4 Key parts of the HPLC instrument

The principal components of the HPLC apparatus include:

- **Mobile Phase Reservoir:** Provides a customizable mixture of polar and nonpolar liquids, adapted to the physicochemical properties of the sample and the methodological requirements of the separation.
- **Pump:** the pump draws the mobile phase from the solvent container.
- **Sample Injector:** the sample injector is designed to add the liquid sample into the system, usually using volumes between 5 and 20 μl .
- **Column:** in HPLC, columns are usually constructed from either sturdy glass or shiny stainless steel. The selected materials must withstand the extreme pressures that develop during chromatographic procedures. The stationary phases of columns consist of porous silica (3–10 μm) because it provides a large surface area and adsorption capabilities. Pellicular materials consist of non-porous glass or polymeric spheres that measure 30 to 40 μm and provide fast separation with minimal interaction.
- **Detection unit:** The detector functions as an essential element in HPLC that detects and quantifies components that leave the chromatographic column. The detector translates physicochemical characteristics such as absorbance, fluorescence, refractive index and electrochemical responses into detectable signals that allow precise analysis of chromatographic components. The selection of HPLC detectors

depends on analyte nature and analytical needs among ultraviolet-visible (UV/Vis) detectors, photodiode array (PDA) detectors, refractive index (RI) detectors, fluorescence detectors, evaporative light scattering detectors (ELSD) and amperometric, potentiometric and other electrochemical detectors (Akash and Rehman, 2020).

2.5.5 Factors influencing HPLC performance

Several critical factors require constant observation to improve both resolution and separation efficiency. The chemical composition of the mobile phase, combined with its gradient system, determines the duration that analytes spend in the column and their elution times. Temperature control of the column impacts how analytes interact with the stationary phase, which results in changes to retention patterns and selectivity of chromatographic separation. The speed of mobile phase flow determines analyte movement speed through the column, yet increasing flow speed reduces analysis duration and resolution quality (Siddique, 2021; Siddique, 2023). The typical approach for HPLC method optimization involves changing a single parameter while maintaining all other variables constant. The approach leads to necessary separation but fails to ensure optimal conditions. The conventional approach for developing HPLC methods demands extensive time investment, together with high costs and labor-intensive procedures, while making it difficult to correct errors and obtain consistent results and peak analytical performance (Sahu et al., 2018).

2.5.6 Choosing the appropriate column

The selection of suitable RP-HPLC columns directly determines method development success when analyzing complex sample matrices. The characterization of chromatographic columns has received extensive research attention because scientists focus on assessing the stationary phase quality and separation performance. Research into RP-HPLC analyte retention and selectivity mechanisms has experienced substantial growth during recent years (Žuvela et al., 2019). Analytical columns dominate the market because they represent the industry standard for use with UV-Vis detectors, which provide sensitive and precise detection of eluting compounds (Akash and Rehman, 2020).

2.5.7 Mobile phase selection

The typical mobile phase compositions for RP-HPLC consist of aqueous solvents that include pH-adjusting reagents and ionic strength modifiers, along with organic solvents acetonitrile (ACN) and methanol (MeOH) to separate analytes. However, both solvents are labeled as hazardous because of their toxic properties, which raises significant concerns regarding their waste disposal. While MeOH is viewed as an eco-friendlier option compared to ACN, safety in handling and waste disposal remains a key priority. MeOH should be preferred over ACN whenever possible. The suitability of ACN and MeOH for RP-HPLC stems from their favorable physicochemical properties: they are fully miscible with water, exhibit low viscosities in solution (especially ACN), possess minimal UV absorbance at short wavelengths, and are commercially available in ultra-pure forms required for high-resolution separations. Additionally, their inertness concerning sample constituents and chromatographic hardware minimizes interference and degradation (Yabré et al., 2018).

2.5.8 Selection of buffer solution in HPLC

Accurate pH measurement in analytical protocols typically involves the use of a glass electrode paired with a reference electrode, commonly a silver/silver chloride type containing a concentrated potassium chloride (KCl) solution. In liquid chromatography, particularly RP-HPLC, buffered mobile phases are routinely employed to facilitate the separation of analytes with acid-base properties. The mobile phase's pH significantly affects analyte retention behavior, with additives such as acetic acid and triethylamine frequently utilized to modulate acidity or alkalinity. Variations in buffering agents, buffer concentration, and organic solvent proportion (e.g., ACN or MeOH) impact the pH of the hydro-organic system. Consideration of an analyte's pK_a about the mobile phase pH enables calculation of its degree of ionization, which is critical to understanding retention mechanisms. Furthermore, assessment of retention times for neutral and fully ionized forms allows prediction of chromatographic behavior of weak acids and bases across a range of pH conditions (Subirats et al., 2007; Li et al., 2010).

2.5.9 Isocratic and gradient separations

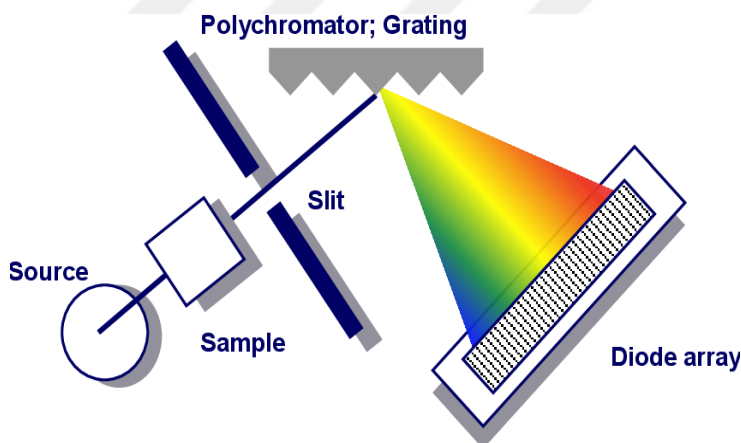
In general, isocratic separations are seen as more consistent compared to gradient separations. A stable eluent composition creates equilibrium in the column, leading to a constant speed for the compounds passing through it. Accordingly, analyte affinity

toward the stationary and mobile phases exhibits minimal fluctuation, preserving separation reproducibility. This reliability makes isocratic separations easier to predict, even though their separation power, the capability to distinguish many different compounds, is fairly limited. In isocratic separations, there is a limit to the maximum capacity, meaning that as a component stays on the column longer, the peak produced gets wider. Conversely, gradient separations greatly improve the system's ability to separate components, mostly because they greatly boost apparent efficiency, resulting in narrower peaks (Chandrul and Srivastava, 2010).

2.5.10 Photodiode Array Detector (PDA)

According to the diagram in Figure 2.4, PDA, a polychromator is an improved type of monochromator. While monochromators need to rotate a dispersing component around its vertical axis to scan wavelengths, a polychromator uses electronic scanning of the multichannel detector. This method removes the requirement for any mechanical movement. (Choi, 2003).

Figure 2.4
Schematic of Photodiode Array Spectrophotometer (<http-10>)



Diode arrays come in sizes from 128 to 1024 elements, and some can even have 4096. These multichannel detectors are excellent sensors for capturing the full spectrum in a UV-VIS dispersive spectrophotometer. They allow for the simultaneous collection of various wavelengths, which improves analytical performance. Considering that application, recent diode arrays have been developed featuring neighboring diodes that are each 25.6 mm in length and have a distance of 25 mm between them. Each diode includes a P-doped area that measures 13 mm by 2.5 mm, which serves as its cathode. The PDA, which functions as a UV-VIS spectrophotometric detector, fulfills these needs

while also allowing the collection of the full UV-VIS spectrum at once, a feature not found in traditional systems. Because it captures spectra in parallel, it greatly reduces, if not completely removes, several typical problems encountered by UV-VIS spectrophotometer users, including sample photodegradation, non-linear scans, and difficulties in detecting transient events.

The PDA UV-Vis spectrophotometer is equipped with a microprocessor-controlled multi-channel detector, allowing it to gather spectral data at various wavelengths all at once. On the other hand, a standard UV-Vis spectrophotometer relies on a single-channel detector that measures data for only one wavelength at a time. The PDA model provides increased speed and consistency in results because it does not include moving components such as gratings or prisms, which are common in traditional UV-Vis spectrophotometers. These moving parts, which are driven by a stepper motor to select specific wavelengths, can affect the accuracy of measurements (Jones, 1985).

- PDA detectors offer numerous advantages, including:
- Fast scanning capability
- High signal strength relative to background noise
- Accurate wavelength measurement
- Reduction of stray light effects (Choi, 2003).

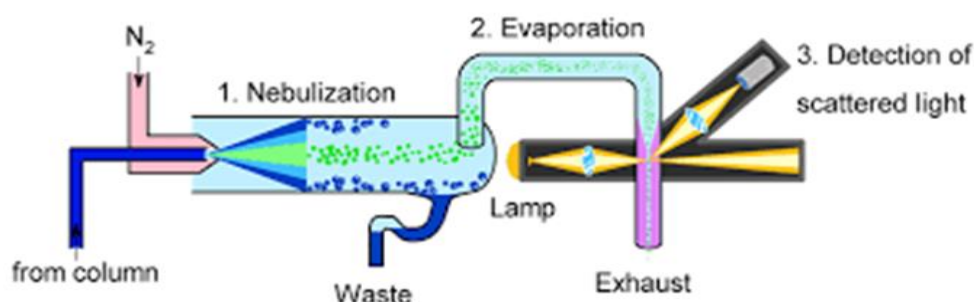
2.5.11 ELSD detector

Evaporative light scattering detection (ELS) is a new method that is increasingly used in HPLC and supercritical fluid chromatography (SFC) to analyze various compounds. J. M. Charlesworth conducted the first investigation about ELSD working mechanisms in 1978 which subsequently led to major progress in both analytical theory and method development for this detection method. The chromatography literature presents LC methods using ELSD which apply to various substances including lipids and oils and carbohydrates together with polymers and anions and cations and drugs. Additionally, ELSD has been recommended for measuring impurities in bulk drugs and controlling the purity of enantiomers to the 0.1% level as required by ICH. The ELSD response enables fast and reliable results in terms of both quantity and purity, particularly under certain conditions (Young and Dolan, 2004; Douville et al., 2006; Lucena et al., 2007; Mojsiewicz-Pieńkowska, 2009).

ELSD works by turning the liquid coming from the column into tiny droplets, which are then blown by a gas such as air or nitrogen through an evaporator tube. These droplets move toward a light beam. When light hits the leftover particles of non-volatile substances, it scatters, and this scattering is detected by either a photomultiplier or a photodiode oriented at an angle of either 90° or 120° concerning the incident light path is employed to detect scattered radiation. The strength of the signal indicates the concentration of the solute in the liquid and helps in its measurement. Figure 2.5 illustrates the three primary functions of the detector:

- **Nebulization:** involves using a nebulizer to combine a gas, usually air or nitrogen, with the liquid coming from a column. This creates a fine mist made up of small droplets. This method effectively converts the liquid sample into a format suitable for analysis in the detection system.
- **Volatilization of the mobile phase components:** as the aerosol traverses the drift tube, the mobile phase evaporates under elevated temperatures, enabling the conversion of dissolved analytes into particles that scatter light for quantification.
- **Detection:** when the dried particles leave the drift tube, they encounter light that makes them scatter. Photon detection is achieved through a photodiode or photomultiplier tube strategically placed at a specific angular orientation to the excitation source. This scattering is key to measuring the amount of analyte in the sample. For optimal consistency in the detector's response, it's crucial to keep the flow rates of both nebulization gas and eluent steady, maintain a uniform vaporization temperature, and avoid any partial vaporization of the solute (Dreux and Lafosse, 1995; Young and Dolan, 2004).

Figure 2.5
Schematic of the ELSD (<http-11>)



Critical parameters, such as particle morphology and population, established during the nebulization and evaporation phases, directly impact ELSD's analytical sensitivity and linearity. The quantitative relationship between scattering efficiency depends on particle size, together with dispersion characteristics (Mathews et al., 2004). ELSD provides practical benefits through its ability to operate with different eluents and additives as well as its stable baseline during gradient elution separations. The detector functions without dependence on analyte or solvent spectral characteristics, which produces its unique detection capability. The broad applicability of ELSD in chromatographic analysis results from its ability to deliver stable and dependable sensitivity to different chemical compounds, which spectroscopic detectors cannot match due to their analyte-dependent response variability (Young and Dolan, 2004).

The application of liquid chromatography with evaporative light scattering detection (LC-ELSD) has gained prominence in pharmaceutical quality assurance for compounds that do not possess UV-absorbing chromophores. The non-volatile analyte specificity of ELSD makes it a proper replacement for UV detection systems while also offering superior analytical capabilities when compared to amperometric or refractometric detectors (Douvillie et al., 2006).

2.5.12 HPLC validation

The ICH guidelines define HPLC method validation as a systematic review process that produces factual evidence demonstrating the method achieves defined criteria for its intended application. Validation of analytical methods demands a thorough assessment of performance metrics to verify adherence to established acceptance criteria. This evaluation facilitates a detailed understanding of the method's operational characteristics, particularly its accuracy, precision, and reliability, with special emphasis on its efficacy at low analyte concentrations.

The following are typical analytical performance characteristics that are commonly evaluated during method validation:

- **Accuracy:** In analytical terms, accuracy is defined as the closeness of an experimental measurement to the accepted or true value, typically expressed as the difference between the average observed result and the reference value. Generally, accuracy is

measured by how well-known quantities of an analyte are recovered during the test process.

- **Precision:** Precision indicates how consistently an analytical method can produce the same results. It has two main components: repeatability and intermediate precision. Repeatability explains the differences observed when an analyst uses the same device and conditions. Precision is usually measured using relative standard deviation (RSD), which is defined as the ratio of standard deviation to mean and is typically reported as a percentage.
- **Linearity:** The linearity of a method reflects its ability to produce results that are consistently proportional to the amount of analyte present in the sample and supports accurate quantification. This attribute allows the method to accurately determine various analyte concentrations. The confidence interval which surrounds the regression line slope represents this feature.
- **Limits of Detection and Quantitation in Method Validation:** The Method validation requires understanding the detection limits of methods as well as their quantitation limits. The detection limit (LOD) represents the minimum analyte concentration that generates an observable signal above background noise but lacks sufficient accuracy for quantification. The limit of quantification (LOQ) establishes the lowest concentration of analyte that can be measured with precise accuracy and exactness under defined method parameters. The standard deviation (SD) of response measurements near the LOD and the slope (S) of the calibration curve are used to calculate LOD and LOQ through the formulas $LOD = 3.3 \times (SD / S)$ and $LOQ = 10 \times (SD / S)$.
- **Robustness:** An analytical method demonstrates robustness when it maintains consistent performance after deliberate small changes to procedural parameters which proves its stability during routine operations (Vidushi et al., 2017).

2.6 Review of Analytical Methods Employed in LSDH Analysis

The ongoing development of analytical approaches for LSDH represents a substantial advancement within a field that continuously strives to improve methods while addressing their existing challenges. The technological advancements have enabled researchers to optimize LSDH analytical methods, which now deliver enhanced reliability

at lower costs and expanded applications throughout pharmaceutical quality control and biological research settings.

Analytical methods for pharmaceutical compounds are very important in drug discovery and quality control. These techniques are efficient, reliable, accurate and cost-effective, enabling the quantitation of drug components such as active pharmaceutical ingredients (APIs), finished dosage forms and biological matrices (blood, urine, plasma). Several methods have been found in literature for the analysis of LSDH by spectrophotometry, HPLC, LC-MS, atomic absorption spectroscopy and potentiometric titration. Of those, HPLC is generally recognized as the method of choice for LSDH quantification in LSD due to its excellent resolution and reproducibility. And new methods as microwave extraction, gas-liquid chromatography, fluorimmunoassay and capillary electrophoresis, have been studied for their capability to analyze these compounds. Nevertheless, spectrophotometry remains a widely used method as a result of its ease of use, low cost and relatively good selectivity and sensitivity. Yet it has some drawbacks, like limited fitness and being over-complex when some complicated conditions come up. The novel method has contributed substantially to the promotion of lisinopril analysis, effectively overcoming the limitations of the past and providing analytical convenience and reliability. In addition, the development of spectrofluorimetric methods, electrophoresis and capillary electrophoresis has broadened the range of drug analysis and allowed for more accuracy, precision and versatility in pharmaceutical determinations (Naveed, 2014; Gul et al., 2017; Shulyak et al., 2021a; Dode et al., 2023).

A literature review of known scientific references confirmed that few analytical studies in fact address the determination of LSDH and impurities in bulk materials and numerous drug formulations. Most of available analytical approaches focus on separation and detection of lisinopril rather than LSDH. Several methods exist for monitoring impurities in normal compounds and drug formulations. The majority of these techniques rely on gradient elution techniques, using organic solvent compositional changes and the modification of the flow rate to increase separation capacity. One of the major objectives of this study was the establishment of an inexpensive and rapid, easy-to-operate analytical routine, ultimately aimed at practical application in routine quality control or industrial laboratories.

A literature review of reliable scientific sources has shown that there are only a few analytical studies that focus on measuring LSDH and impurities in bulk materials and different types of drugs. Most of the current analytical methods focus primarily on the isolation and identification of lisinopril rather than LSDH. A variety of techniques are available for detecting impurities in both standard substances and pharmaceutical formulations. Most of these methods utilize gradient elution, involving variations in organic solvent concentrations and adjustments in flow rates to enhance separation efficiency. A central aim of this research was the development of a streamlined, economically viable, and time-efficient analytical method, optimized for implementation in routine quality control and industrial laboratory environments.

Tab.2.3 provides an overview of analytical research performed for the determination of LSDH/LIS and their impurities. Based on our survey of reports, no validated HPLC method with ELSD detection for the simultaneous determination of LSDH and its impurities of interest in bulk raw materials and different pharmaceutical dosage forms has been reported so far.

Quantitative analysis of LSDH and its impurities in drug products and biological specimens has been achieved through multiple instrumental approaches, such as NMR, FTIR, TLC, CE, UPLC-MS, and potentiometric methods. A brief overview of some of these methods is provided below.

Hillaert et al. (2001) reported a capillary electrophoresis (CE) protocol enabling the simultaneous quantification of hydrochlorothiazide in conjunction with various angiotensin-converting enzyme (ACE) inhibitors, specifically enalapril, lisinopril, quinapril, fosinopril, ramipril, and cilazapril. Among the evaluated methodological parameters, the pH of the running buffer emerged as the most influential factor affecting analyte resolution and quantification. Analytical separation was conducted using a 100 mM sodium phosphate buffer adjusted to pH 7.25 within a fused silica capillary (total length: 52 cm; internal diameter: 375 μ m). The validated method demonstrated efficacy in accurately determining the target compounds within their respective pharmaceutical formulations, highlighting its applicability in multi-component drug analysis.

Shinde et al. (2007) reported an LC–UV method for the determination of lisinopril using a phosphate buffer and acetonitrile dual gradient system. The analysis revealed an unknown impurity at the level of about 0.14% with a relative retention time of 3.26.

Subsequent LC-MS analysis with a dual gradient system of ammonia-ammonium acetate buffer (pH 5.0) and acetonitrile characterized the impurity as having a molecular structure of $C_{31}H_{41}N_3O_7$. The impurity was separated through preparative high-performance liquid chromatography (HPLC) [on column: X-Terra RP18e column (300 mm $\times$ 19mm, 7 μ m; Waters, USA)] with distilled water (E) and acetonitrile (F) as mobile phase. Structural analysis and confirmation with ^1H and ^{13}C NMR, mass spectrometry (MS), and Fourier transform infrared spectroscopy (FTIR) indicated the impurity to be a 4-phenylbutanoic acid derivative of lisinopril, designated as CL.

Digambar et al. (2014) established a simple, sensitive and cost-effective UV-visible spectrophotometric method based on the area under the curve (AUC) technique for the determination of LSDH in bulk drug substances and tablet dosage forms. Spectra were recorded on a Shimadzu 1800 UV/VIS double-beam spectrophotometer with a 1 cm path length quartz cell. Analytical measurements were performed at 202–216 nm. The method exhibited a great linearity in the concentration range of 10–30 μ g/mL (correlation coefficient [R^2] of 0.9993). The sensitivity (RSD) was also acceptable. The average recovery percentage was 99.905%, and the recovery studies demonstrated the accuracy of the method. Sensitivity parameters were well characterized; LOD and LOQ were found to be 0.814 and 2.468 μ g/mL, respectively.

Sbarcea et al. (2014) described an innovative spectrophotometric method which is selective, sensitive, simple, rapid and cost-effective for the determination of LSDH in three fixed-dose combination pharmaceutical formulations (Lisigamma®, Lisinopril Sandoz® and Ranolip®). The method has been developed and validated according to the International Council for Harmonisation (ICH) and the applied analytical criteria conformed with the well-known guidelines. Spectra were recorded in a 300UV (Spectronic Unicam) UV-Visible Spectrophotometer with 1 cm path length glass cuvettes. The established method had LOD and LOQ values of 1.16 and 3.52 μ g mL $^{-1}$ for LSDH, respectively, indicating that it could be applied to routine quality control analysis.

Quinn et al. (2015) offered, for the first time, the co-administration of a triple combination of aspirin, LSDH and atorvastatin calcium trihydrate simultaneously, using a soluble polymeric microneedle (MN) delivery system. The compounds were quantified by reversed-phase high-performance liquid chromatography (RP-HPLC) with an Agilent

1200 R Automatic Sampler (BK). Chromatographic separation was achieved on a Luna C18 (ODS1) column, 150 × 4.6 mm, 5 µm (Phenomenex, Macclesfield, UK) with gradient elution. UV detection was accomplished at 215 nm and 250 nm wavelengths. The retention time of LSDH was around 2.6 min. sensitivity parameter of the method,⁴³ limit of detection (LOD) and limit of quantification (LOQ) were calculated as 0.39 µg/mL and 1.19 µg/mL, respectively.

Through a detailed forced degradation study, Vujanović et al. (2017) showed that LSDH is stable under various stress conditions, including thermal exposure at 25 °C and 50 °C. They characterized the stability profile using high-performance liquid chromatography (HPLC) after treatment with different aqueous media. These included distilled water, 0.1 M hydrochloric acid, 0.01 M and 0.1 M sodium hydroxide solutions, and 3% hydrogen peroxide. Chromatographic separation took place on a Hypersil ODS C18 column (250 mm × 4.6 mm, 5 µm), with the mobile phase pH adjusted to 3.0 using concentrated phosphoric acid. They conducted a quantitative evaluation using UV absorbance at 225 nm. The results showed that LSDH is especially sensitive to hydrolytic degradation under alkaline conditions at 25 °C. Nearly complete degradation happened within one minute. Under neutral and acidic conditions, impurity D was regularly found as the main degradation product. Based on these results, it is advised that formulations with LSDH be protected from oxidative and hydrolytic stress and high temperatures. It is important to maintain the integrity of parenteral formulations during storage and use.

Shah et al. (2017) developed a highly sensitive and fast liquid chromatography-tandem mass spectrometry (LC-MS/MS) method using isotopically labeled internal standards (IS) to measure lisinopril (LIS) and hydrochlorothiazide (HCTZ) in human plasma at the same time. For sample preparation, they used solid-phase extraction (SPE) with 100 µL of plasma in Waters Oasis HLB cartridges. They performed chromatographic separation on a Thermo Scientific Hypersil Gold C18 column (50 mm × 3.0 mm, 5 µm) held at 30 °C, achieving analyte resolution in 2.0 minutes. The mobile phase included acetonitrile and 5.0 mM ammonium formate buffer (pH 4.5, adjusted with 0.1% formic acid) in an 85:15 (v/v) ratio, using isocratic elution. The analysis showed excellent linearity for both analytes in the concentration range of 0.50–250.0 ng/mL. This validated method was successfully used to measure drug levels in plasma samples from 18 healthy Indian

individuals after they took a fixed-dose combination tablet containing 10 mg of lisinopril and 12.5 mg of hydrochlorothiazide.

Gundersen et al. (2018) used ultra-high-performance liquid chromatography and tandem mass spectrometry (UHPLC-MS/MS) to assess twenty-one antihypertensive drugs, including lisinopril, in human serum. They optimized the method by testing various chromatographic columns. The BEH C18 column (2.1×50 mm) was linked to a matching guard column in a Waters I-Class system to achieve the best analytical results. They varied the mobile phase compositions by including formic acid or ammonium formate with methanol or acetonitrile under alkaline pH conditions. Detection was done with an Xevo TQ-S mass spectrometer, which had an electrospray ionization source and used argon gas as the collision-induced dissociation medium. The optimized method showed high selectivity and sensitivity for simultaneous multi-analyte quantification in serum samples.

Bozhanov et al. (2020) introduced a straightforward algorithm for evaluating acid-base systems. This approach allows for the quick and precise measurement of lisinopril dihydrate in 20 tablet samples using direct potentiometric titration. The titration took place at 25°C in a temperature-controlled vessel. It maintained a constant ionic strength of 0.2 mol/L KCl.

Min et al. (2023) presented and validated UV spectrophotometric and Fourier transform infrared (FTIR) spectroscopic methods for evaluating different commercial lisinopril tablet formulations. In the UV spectrophotometric analysis, tablet samples were diluted with distilled water, and their absorbances were measured to find the lisinopril concentration. At the same time, the FTIR spectroscopic method analyzes powder samples to assess their structural properties, confirm the presence of important functional groups, and check the purity of all brands. When used together, these validated methods offer useful analytical information about the quality control and consistency of lisinopril tablet formulations, ensuring they meet pharmacopeia regulations.

Table 2.3*Summary of Literature-Based Analytical Methods Applied to LSDH and Its Impurities*

Compound, Sample	Method	Column (stationary phase)	Mobile Phase composition, Elution type	Detector, Inj. Vol. (μL)	LOD, LOQ (μg.mL ⁻¹)	Reference
LIS, dosage forms and spiked human plasma	RP-HPLC	RP-18, 250 mm × 4 mm, 5 μm	0.02 M NaH ₂ PO ₄ (pH 3.0): MeOH (45:55, v/v), Isocratic Elution	F 540 nm (excitation at 470 nm), 20	0.008, -	El-Emam et al., 2004
LIS, human plasma and urine	HPLC	C18, 250 mm × 4.6 mm, 5 μm, with a guard column, 4 mm × 3 mm	A) 0.02 M PB (pH 3.2) B) MeOH, Gradient Elution	F 477 nm, with excitation at 383 nm, 20	0.002, 0.005	Sagirli and Ersoy, 2004
LIS, DKP, methyl paraben and propyl paraben, LIS extemporaneous formulation	RP-HPLC	Platinum EPS C8, 250 mm × 4.6 mm, 5 μm	30 mM PPB (pH 2.2): ACN (91:9, v/v), Gradient Elution	DAD 215 nm, 20	0.0075, -	Beasley et al., 2005
LIS and CL impurity of LIS	HPLC-MS, LC-MS/MS,	C-8, 250 mm × 4.6 mm, 5 μm	A: NaH ₂ PO ₄ , pH 5 in 970 ml H ₂ O and 30 ml ACN B: NaH ₂ PO ₄ , pH 5 in 400 ml H ₂ O and 600 ml ACN, Gradient Elution	MS (m/z 568)-, 20	-	Shinde et al., 2007
A novel impurity of LIS, bulk drug	HPLC NMR	C18, 150 mm × 4.6 mm, 5 μm	PH ₂ PO ₄ (pH 4.9): ACN (1:9, v/v), Gradient Elution	UV 210 nm, -	-	Zhu et al., 2008
Pure drug of LSDH	RP-HPLC	C18, 250 mm × 4.6 mm, 5 μm	TBAOH (0.03M aqueous) pH 6.5 adjusted with diluted OPA (10 % aqueous) and ACN (4:1), Isocratic Elution	DAD 210 nm, 50	0.61, 1.87	Adam et al., 2012
LSDH and HCTZ, combined dosage form	RP-HPLC	C18, 250 mm × 4.6 mm, 10 μm	NaH ₂ PO ₄ (pH 4.5): MeOH (70:30 v/v), Isocratic Elution	UV 225 nm, 20	(0.296 and 0.893 for LSDH) and (0.421 and 1.42 for HCTZ)	Wagh et al., 2012
LIS and NSAIDs, bulk materials, pharmaceutical dosage formulations and human serum	RP-HPLC	C18, 250 mm × 4.6 mm, 5 μm	MeOH: H ₂ O (pH 3.0): ACN (80:17.5:2.5 v/v), Isocratic Elution	UV-VIS 225 nm, 20	0.9, 2.8 for LIS	Arayne et al., 2013
Aspirin, LSDH, atorvastatin calcium trihydrate and Salicylic Acid	RP-HPLC	C18, 150 mm × 4.6 mm, 5 μm	25 mM NaH ₂ PO ₄ (pH 2.5): MeOH-ACN (50:50, v/v), Gradient Elution	UV 215 and 250 nm, -	0.39, 1.19	Quinn et al., 2015
LIS, LIS impurity A, LIS impurity B, LIS impurity C, amlodipine and amlodipine impurity D, tablets	HPLC	C18, 250 mm × 4.6 mm, 5 μm	A: ACN-H ₂ O-PA (10:90:0.1) B: ACN- H ₂ O-PA (90:10:0.1), Gradient Elution	DAD 215 nm, 20	-	Yu et al., 2016
LSDH and its impurity D, pharmaceutical form	RP-HPLC	C18, 250 mm × 4.6 mm, 5 μm	MeOH: H ₂ O: ACN (80:17.5:2.5 V/V/V), pH 3.0 adjusted with concentrated PA, Isocratic Elution	UV 225 nm, 20	-	Vujanović et al., 2017

Table 2.3 (Continued)

LIS and HCTZ, human plasma	LC-MS/MS	C18, 5 mm×3.0 mm, 5 µm	5.0 mM ammonium formate, (pH 4.5): ACN- (85:15, v/v), Isocratic Elution	MS, LIS-d5, 5.0	-	Shah et al., 2017
21 antihypertensive drugs (including LIS), serum	UHPLC-MS/MS	C18, 2.1x50 mm, 1.7 µm with UPLC C18, 2.1x5mm, 1.7 µm	Mix of MeOH/ACN/isopropanol/1 % (v/v) PA in H ₂ O (1:1:1:1, v/v, Gradient Elution	MS, -	0.00006, 0.00024 for LIS	Gundersen et al., 2018
Ca ²⁺ ion by taking advantage of the chelating nature of LIS molecule	HPLC-ELSD	-	-	nitrogen gas flow rate of ELSD 2.5 L/min	-	Abdallah, 2021
Amlodipine, Besylate and LSDH, Bulk and Pharmaceutical Dosage Form	RP-HPLC	C8, 250 mm x 4.6 mm, 5 µm	PB pH 6.5: MeOH ((25: 75 v/v), Isocratic Elution	UV 215nm, 5	-	Babar et al., 2021
77 commercial drugs including LIS and Captopril	HPLC-ESI-MS/MS	C18, 150 mm x 1 mm, 5 µm	A: H ₂ O (1% PA) B: ACN (1% PA), Gradient Elution	ESI-MS/MS, -	0.001866, 0.005664	Bhatti et al., 2021
12 tablets combined standard solution of atorvastatin and LIS	RP-HPLC	C18, 250 mm × 4 mm, 5 µm	10 Mm PH ₂ PO ₄ ACN and potassium hexafluorophosphate (55:45:0.4, v/v/v), Isocratic Elution	UV-VIS 215 nm, 10	0.025, 0.27	Shulyak et al., 2021b
LIS and carboxylic acids	RP-HPLC	A Kinetex Biphenyl 100 Å, 250 mm × 4 mm, 5 µm	CH ₃ CN and 0.1 % FA in Milli-Q H ₂ O, (15:85 v/v), Isocratic Elution	DAD 210 nm, -	-	Zhao et al., 2024
Degradation products of LIS	LC-MS/MS	C18, 100 mm × 2.1 mm, 1.7 µm	A: H ₂ O with 0.2 % ACA B: ACN with 0.1 %PA, Gradient Elution	MS, -		
8 Cardiovascular Drugs (including LIS), dosage form and spiked rat plasma	HPLC	C18, 4.6×250 mm, 5 µm	50 mM PB (pH 3) and MeOH, Isocratic Elution	DAD 220 nm, 10	-	Mourad et al., 2024
LSDH and ibuprofen, transdermal drug delivery	RP-HPLC	Knauer Eurosphere II 100–5 C18A	A: 0.8 g/L NaH ₂ PO ₄ , (pH 3.5) with PA 85 % B: ACN, Gradient Elution	UV 210 nm, 20		Lammerding et al., 2025

LIS: lisinopril, 7-chloro-4-nitrobenzol-2-oxa-1,3-diazole: (NBD-Cl), MeOH: methanol, ACN: acetonitrile, NaH₂PO₄: sodium dihydrogen phosphate, PH₂PO₄: potassium dihydrogen phosphate, hydrochlorothiazide: HCTZ, Chlorothiazide: CTZ, sulfonamide: DSA), diketopiperazine: DKP, phosphate buffer: PB, potassium phosphate buffer: PPB, KOH: potassium hydroxide, TBAOH: tetra butyl ammonium hydroxide, OPA: orthophosphoric acid, NSAIDs: Naproxen; Flurbiprofen; Diclofenac sodium; and Mefenamic acid, ACA: acetic acid,

3 MATERIALS AND METHODS

The chemical substances used in the analyses, their brands, and analytical purity levels are detailed in Table 3.1. All materials used were carefully selected to meet analytical grade purity standards.

Table 3.1
Chemical Substances used in the Study

Material Name	Brand	Quality Level
Methanol, ethanol, Acetonitrile, and water	Sigma - Aldrich (Germany)	HPLC grade $\geq 99,9$
Ortho-phosphoric acid	Sigma-Aldrich (Germany)	85-90%
Sodium phosphate monobasic	Sigma-Aldrich (Germany)	$\geq 99\%$
Sodium hydroxide	Sigma-Aldrich (Germany)	Analytical pure, %98-100,5, pellet
LSDH	Sigma-Aldrich (Germany)	≥ 97.00
Lisinopril Dihydrate		
Impurity A	Tokyo Chemical Industry, Japan	≥ 97.00
DL-Homophenylalanine	Gong Rd, Shanghai, China	
Impurity D	Toronto Research Chemicals Inc. (Toronto, Canada)	% 90
Lisinopril Diketopiperazine	Sigma-Aldrich (Germany)	≥ 97.00
Ethyl paraben	France	Milli-Q A10 Synthesis Water Purification System
Ultrapure water		

3.1 Pharmaceutical Dosage Forms (Tablet Samples)

- 5 mg Sinopryl ®
- 20/12.5 mg (every single tablet=21.78 mg LIS + 12.5 Hidroklorotiyazaid) Sinoretik ®
- 20/25 mg (every single tablet=21.78 mg LSDH + 25 mg Hidroklorotiyazaid) Sinoretik Fort ®, were obtained from a local (Turkey) pharmacy.

3.2 Devices and Instruments

Table 3.1 lists all of the chemicals used in the analyses along with their brands and levels of analytical purity.

Table 3.2*Instrumentation Overview: Brands and Models Utilized*

Instrument Name	Brand/Model
HPLC	Shimadzu Nexera-i LC2040C 3D-PDA, Liquid Chromatograph, (Tokyo, Japan)
SVEA C18 Gold, 150 × 4.6 mm, 3.5 µm	Nanologica, HPLC column
SVEA C18 Gold, 150 mm, 4.6 mm, 5 µm	Nanologica, HPLC column
SVEA C18 Gold, 250 mm, 4.6 mm, 5 µm	Nanologica, HPLC column
Supelcosil Analytical C18, 250 mm, 4.6 mm, 5 µm	Sigma-Aldrich (Germany)
Ultra PFPP C18 Column 200 mm, 4.6 mm, 5 µm	USA
PDA	Shimadzu, Nexera-i (LC-2040C 3D), (Japan)
ELSD	ELSD LT – II Low-Temperature Evaporate Light Scattering Detector (Tokyo, Japan) Shimadzu
Precision Balance	Ohaus PA214C
Ph Meter	Ohaus Starter 3100
Ultrasonic Bath	Lab companion (BW-20H-CS-10) ultrasonic cleaner (Korea)
Vortex Shaker	Heidolph (Germany)
Syringe	Part No: SN02522 – P
Filter paper (pore size 0.45µm)	Sartorius AG.37070, (Goetting, Germany)
Pipettes (100-1000 µL and 20-100 µL)	(Eppendorf, Germany)

4 METHODS

In this thesis, two distinct analytical approaches were utilized to quantify SDH along with Impurities A and D, in pure solutions and commercial tablets. The analyses were conducted using HPLC-DAD and HPLC-ELSD techniques.

4.1 HPLC-DAD Technique

4.1.1 HPLC-DAD solvent system

The mobile phase was prepared as a 40:60 (v/v) mixture of methanol and phosphate buffer, wherein the buffer component consisted of 23.1 mM sodium phosphate monobasic (NaH_2PO_4) solution adjusted to pH 9.50. Specifically, 2.770 g of NaH_2PO_4 was accurately weighed and dissolved in ultrapure water, and the final volume was brought to 1000 mL in a volumetric flask. The pH was adjusted using sodium hydroxide (NaOH) and verified with a calibrated pH meter. To ensure the removal of particulate matter, the buffer solution was vacuum-filtered through a 0.45 μm cellulose membrane filter. Degassing was performed via ultrasonic treatment for 15 minutes. The methanol and phosphate buffer were subsequently mixed in the specified volume ratio using the low-pressure gradient mixing system integrated within the HPLC instrument.

4.1.2 Preparing standard solutions

In order to prepare standard stock solutions of the substances being tested, exact amounts of reference standards, certified by the United States Pharmacopeia (USP), were carefully measured and dissolved in methanol and water mixed in a 40:60 ratio. The analytical method development utilized 10 mg each of LSDH and Impurity A, along with 1 mg of Impurity D, for stock solution preparation. These were subsequently combined and diluted in a consistent solvent medium to produce a final test solution containing 1000 $\mu\text{g/mL}$ of each analyte. Before storage at $-20\text{ }^\circ\text{C}$, all stock solutions were filtered using syringe filters with a pore size of 0.45 μm to ensure particulate-free samples.

Ethyl paraben (EP) served as the internal standard (IS) throughout the experimental protocol - stock IS solution (1000 $\mu\text{g mL}^{-1}$) was prepared by dissolving 10 mg of EP in 10 mL of methanol (MeOH). The working IS solution was obtained by dilution in 50% methanol (v/v), resulting in a final concentration of 100 $\mu\text{g mL}^{-1}$.

4.1.3 Chromatographic evaluation

The previously prepared solution was employed for preliminary trials, in which various combinations of methanol (MeOH) and buffer were examined in the mobile phase. Chromatographic performance was characterized through the analysis of essential system suitability indicators, namely, asymmetry factor, resolution, and column efficiency. Through systematic analysis, the most favorable conditions for achieving optimal chromatographic separation were determined.

4.1.4 Assessment of analytical findings

Integrated peak area served as the principal analytical parameter throughout the study. Calibration curves were generated by plotting peak area against analyte concentration to establish a linear quantitative relationship. Quantification was subsequently performed using the linear regression equation derived from the calibration data.

4.1.5 Analytical method validation

According to the validation protocols specified in ICH Q2(R1) and USP guidelines (USP Q2(R1), 2022; USP 37, 2014), the method was rigorously evaluated for accuracy, precision, sensitivity (LOD and LOQ), and overall analytical performance. System suitability parameters were concurrently examined to confirm consistent chromatographic behavior and instrument precision.

4.1.5.1 Evaluation of System Suitability (SST)

Instrument performance was verified as a critical aspect of analytical method development by evaluating key chromatographic parameters, including resolution (R_s), tailing factor (T), symmetry factor (A_s), theoretical plates (N), capacity factor (K'), and selectivity ratio (α). All calculations were conducted using Shimadzu LabSolutions software.

4.1.5.2 Assessment of linearity

Linearity assessment was conducted following USP guidelines, evaluating the correlation between analyte concentration and instrument response. Calibration curves for LSDH, Impurity A, and Impurity D were constructed across a concentration range of 1.00–400 $\mu\text{g mL}^{-1}$. Three independent batches were analyzed over three days to ensure reproducibility. Peak normalization ratios ($R = \text{PN}_{\text{Std}}/\text{PN}_{\text{IS}}$; PN = peak area/retention time) were used to

evaluate response consistency. Linear regression yielded slope and intercept values employed for the quantitative determination of pharmaceutical potency. All statistical evaluations were performed using Microsoft Excel.

4.1.5.3 Accuracy

Accuracy, as defined by ICH Q2(R1) (2022), measures the closeness between the experimentally obtained results and the true concentration of analytes. In this study, accuracy was assessed via percentage recovery across three concentration levels for LSDH (40, 75, 180 $\mu\text{g mL}^{-1}$), Impurity A (30, 65, 145 $\mu\text{g mL}^{-1}$), and Impurity D (6.5, 15, 25 $\mu\text{g mL}^{-1}$). Each concentration level was assessed via triplicate measurements across three distinct days to ensure methodological reproducibility.

4.1.5.4 Assessment of method precision

Precision is a critical parameter in analytical validation, representing the degree of agreement among repeated measurements under specified conditions. The method's repeatability (within-day) and intermediate precision (between-day) were determined and calculated using standard deviation (SD) and relative standard deviation (RSD) metrics.

For precision testing, a reduced calibration concentration was utilized as the reference solution and analyzed three times over three consecutive days. The tested concentrations for LSDH were 45, 95, and 145 $\mu\text{g mL}^{-1}$, while those for Impurity A were 35, 75, and 120 $\mu\text{g mL}^{-1}$, and for Impurity D, 8, 12, and 20 $\mu\text{g mL}^{-1}$. These findings confirmed the method's reproducibility and reliability.

4.1.5.5 LOD and LOQ

The detection limit (LOD) and quantification limit (LOQ), which remain for the lowest concentrations at which an analyte can be detected and quantified, respectively, were assessed in order to estimate the sensitivity of the analytical procedure. The following formulas were used to determine these parameters based on the calibration curve's slope (m) and the response's standard deviation (SD):

LOD is equal to $3.3 \times \text{SD}/m$.

LOQ is equal to $10 \times \text{SD}/m$.

These computations offer strong analytical sensitivity by making it easier to accurately assess analytes at low concentrations.

4.1.5.6 Method specificity and selectivity

They show how well the new technique can differentiate analyte responses from other potential interferences, including matrix elements, impurities, and degradation products. Potential interferences from the pharmaceutical dosage form's matrix were examined in order to assess specificity. Under carefully regulated stress conditions, the ability of three distinct tablet formulations to degrade due to bases was examined.

To assess degradation behavior, commercial tablet formulations containing 5 mg, 20/12.5 mg, and 20/25 mg of the active pharmaceutical ingredient were individually weighed, and the average mass of ten tablets was calculated. Tablets were homogenized via mortar and pestle, and a 10 mg aliquot of each powdered sample was dissolved in 10 mL of 0.01 M sodium hydroxide (NaOH) solution through ultrasonic treatment for 10 minutes. The resulting solutions were filtered using 0.45 μm nylon syringe filters to eliminate particulate matter. Final sample concentrations of 100 and 500 $\mu\text{g mL}^{-1}$ were analyzed at time intervals of 0, 48, and 72 hours to monitor degradation. Chromatographic purity of the target analyte was verified by examining the peak purity index of each degraded sample.

4.1.5.7 Method robustness evaluation

Robustness in analytical methods refers to their capacity to generate consistent and dependable results, even when subject to minor, deliberate alterations in experimental conditions. In this investigation, to determine method robustness, essential chromatographic conditions influencing retention, such as flow rate, column temperature, and mobile phase composition, were intentionally modified and monitored for consistent analytical performance. Controlled modifications were applied to evaluate the method's stability and ensure its applicability for routine analyses under varying laboratory conditions.

To examine the impact of these adjustments on analytical performance, standard solutions (n=3) of LSDH, Impurity A, and Impurity D were injected. By testing the method with various critical parameters, including buffer pH (7.52 and 7.55), column temperature (33 $^{\circ}\text{C}$ and 38 $^{\circ}\text{C}$), MeOH (%38 and %42), and DAD detection wavelengths (215 nm and 220 nm), the method's dependability was examined. Through these assessments, the method's resilience in preserving accuracy and precision in the face of slight variations in analytical conditions was confirmed.

4.1.5.8 Assessment of stability

In order to replicate actual analytical and processing environments, the stability of the developed method was investigated under a variety of conditions during the validation process. The following stability evaluations were part of the study:

- **Benchtop stability, also known as "short-term temperature stability,"** was evaluated by keeping standard solutions at room temperature for a full day in order to ascertain their integrity under normal handling.
- **"Freeze-Thaw Stability"** was examined by subjecting the analyte solutions to three freeze–thaw cycles at -24°C , simulating repetitive temperature stress conditions to assess compound resilience.
- **"Long-Term Stability":** to assess extended preservation conditions, stock solutions were maintained at -24°C for one month and subsequently analyzed for any degradation or variability.

4.1.6 Preparation of tablet samples

To demonstrate applicability, the developed method was implemented using LSDH dosage forms with active ingredient strengths of 5 mg, 20/12.5 mg, and 20/25 mg. Initially, each tablet was weighed separately, followed by determining the average weight of a set of ten tablets. Tablet samples were finely pulverized using a mortar and pestle to obtain a homogeneous powder. A portion corresponding to the average mass of a single tablet was accurately weighed and dissolved in 10 mL of a methanol: water (40:60, v/v) solvent system. The suspension was sonicated for 10–30 minutes to promote dissolution and subsequently filtered through nylon syringe filters ($0.45\ \mu\text{m}$) to eliminate particulates. Prepared aliquots of 5, 50, 250, and $450\ \mu\text{g mL}^{-1}$ from the 5 mg tablet solution and 20, 200, 1000, and $1800\ \mu\text{g mL}^{-1}$ from the 12.5/25 mg and 20/25 mg tablet formulations were injected into the HPLC system for detailed chromatographic profiling.

4.2 HPLC-ELSD Technique

4.2.1 Mobile phase preparation

The analysis of LSDH and its impurities A and D using the HPLC-ELSD technique was performed without the need for a buffer solution. Methanol and water, mixed at a 40:60 volume ratio, served as the mobile phase. To ensure the removal of insoluble particulates, the freshly prepared solution was vacuum-filtered through a $0.45\ \mu\text{m}$ cellulose membrane.

Dissolved gases were eliminated via sonication in an ultrasonic bath for approximately 15 minutes. Solvent mixing at the specified ratio was performed using the HPLC system's low-pressure gradient mixing module, thereby ensuring homogeneity and optimal chromatographic performance.

4.2.2 Preparation of stock and working standard solutions

A methanol–water mobile phase was used to dissolve USP-grade standards of LSDH, Impurity A, and Impurity D (10 mg, 10 mg, and 1 mg, respectively). The resulting stock solutions were combined and diluted to yield a test solution with analyte concentrations of 1000 $\mu\text{g mL}^{-1}$. Filtration through 0.45 μm syringe filters was conducted to remove particulates, and the solutions were stored at $-20\text{ }^{\circ}\text{C}$ until further experimental applications, maintaining their stability for analytical procedures.

4.2.3 Analytical conditions

In the HPLC-ELSD procedure, the column was eluted with a water–methanol mobile phase (60:40, v/v) at a constant flow rate of 1.0 mL/min. Before initiating sample analysis, the chromatographic system was stabilized by circulating the mobile phase mixture. To eliminate impurities and residual contaminants within the autosampler system during sample transitions, the HPLC system was operated for 10 minutes before each injection.

The analytes were injected at a volume of 10 μL , with the column temperature precisely regulated between $40\text{ }^{\circ}\text{C}$ and $50\text{ }^{\circ}\text{C}$ throughout the chromatographic procedure. Isocratic conditions were maintained using a fixed mobile phase composition, ensuring consistent elution profiles. A C18 column ($250 \times 4.6\text{ mm}$, 5 μm) was utilized to maximize resolution and peak symmetry.

4.2.4 Evaluation of results

Since ELSD is not a spectroscopic technique, it does not conform to the Lambert-Beer law. Quantification using ELSD requires nonlinear calibration models, as the detector response is dictated by the physical properties of aerosolized particles rather than concentration alone. This behavior is best described using a logarithmic function. Scattering principles are modeled using three key parameters that reflect particle size effects on light deflection and intensity. ELSD response is not directly proportional to analyte concentration, as it is modulated by mass, droplet size coefficients, and system-

dependent variables like gas flow and temperature (Guiochon et al., 1988). The ELSD response is governed by a nonlinear relationship expressed as:

$$A = am^b \quad (4.1)$$

where A denotes the peak area, m represents the analyte mass, a is the response factor, and b corresponds to the slope of the response curve. According to Lafosse et al. (1987) and Mengerink et al. (1991), this equation takes on a linear form after undergoing the logarithmic transformation:

$$b \log m + \log a = \log A \quad (4.2)$$

By creating a linear correlation between $\log_{10}(A)$ and $\log_{10}(m)$, this transformation makes calibration easier and provides a strong mathematical foundation for deciphering the behavior of ELSD signals.

4.2.5 Validation of the HPLC-ELSD analytical method

The ICH Q2(R1) guideline and USP standards were followed in the method validation. Aspects like accuracy, precision, LOD, and LOQ were checked as part of this validation. Additionally, the system's suitability was assessed to make sure the analytical performance of the instrument met expectations.

4.2.5.1 Evaluation of system performance

Evaluating the chromatographic system's operational efficiency and dependability is an essential part of method development. To this end, key system suitability parameters were evaluated, including resolution (R_s), tailing factor (T) or symmetry factor (A_s), theoretical plate number (N), capacity factor (K'), and selectivity coefficient (α). These metrics provide quantitative insight into column performance, peak shape, and separation quality. In line with USP 30 protocols, Shimadzu LabSolutions software was utilized to derive all relevant system suitability metrics. The specific equations applied in these calculations are detailed in Table 4.1, as referenced in section 4.1.5.1.

4.2.5.2 Assessment of linearity

To evaluate method linearity, a series of standard solutions were analyzed to establish the correlation between analyte concentration and detector response, as outlined by the United States Pharmacopeia. The linearity of the proposed analytical method was evaluated for LSDH and Impurity A across a concentration range of 9.00–800 $\mu\text{g mL}^{-1}$,

and for Impurity D within 9.00–600 $\mu\text{g mL}^{-1}$. Calibration curves were constructed for each analyte using standard solutions prepared at the specified concentration levels. To assess method reliability and reproducibility, three distinct mixtures containing all analytes were analyzed over three consecutive days. The calibration model was based on the logarithmic transformation of peak area ($\log_{10}A$) versus analyte concentration ($\log_{10}m$), enabling linear regression analysis to determine the slope and intercept parameters. These regression coefficients were subsequently applied to calculate the potency of the pharmaceutical dosage form. All statistical evaluations were performed using Microsoft Excel, ensuring consistency in data processing and interpretation.

4.2.5.3 Assessment of accuracy

In analytical method validation, accuracy is an essential factor that shows how close the measured value is to the actual value (ICH-Q2(R1), 2022). Various methods can be used to prove accuracy. For this research, we focused on the recovery percentage. Over a three-day validation period, triplicate analyses were performed for three concentration levels of each analyte: LSDH (170, 390, 610 $\mu\text{g mL}^{-1}$), Impurity A (110, 290, 530 $\mu\text{g mL}^{-1}$), and Impurity D (90, 270, 510 $\mu\text{g mL}^{-1}$).

4.2.5.4 Assessment of precision

Achieving reliable results relies heavily on precision, which refers to how closely repeated measurements align in a set of experiments. To assess analytical precision, both intra-day and inter-day evaluations were performed, and the resulting data were summarized using SD and RSD metrics.

For this analysis, selected low-concentration levels were analyzed in triplicate across three separate days, enabling assessment of both intra-day and inter-day variability. The concentrations tested were as follows: LSDH at 180, 420, and 700 $\mu\text{g mL}^{-1}$, Impurity A at 130, 310, and 630 $\mu\text{g mL}^{-1}$, and Impurity D at 100, 300, and 590 $\mu\text{g mL}^{-1}$.

4.2.5.5 Estimation of LOD and LOQ in method validation

The analytical sensitivity was assessed through the determination of LOD and LOQ, which represent the lowest detectable and quantifiable concentrations, respectively. These parameters were derived using the standard deviation of the response and the slope of the calibration curve, as follows:

$$\text{LOD} = 3.3 \times \text{standard deviation (SD)} / \text{slope (m)} \quad (4.3)$$

$$\text{LOQ} = 10 \times \text{standard deviation (SD)} / \text{slope (m)}$$

4.2.5.6 Characterization of method selectivity and target specificity

The ability of the chromatographic method to precisely measure the target analyte in the presence of matrix components and structurally related impurities is what defines its selectivity. A System Suitability Test (SST) was used to measure LSDH, Impurity A, and Impurity D in order to assess this parameter. The reference standard $\alpha > 1$, which denotes adequate resolution between neighboring peaks and little co-elution, was used to confirm selectivity. This made it possible to reliably separate and measure each analyte without being impacted by nearby compounds.

4.2.5.7 Robustness study

The ability of the method to yield precise and accurate results for LSDH, Impurity A, and Impurity D in spite of controlled changes in chromatographic parameters was the main focus of the robustness evaluation.

In order to confirm the method's dependability under common environmental and procedural variability, the robustness study included controlled degradation of ELSD and column temperatures in addition to the composition of the mobile phase.

4.2.5.8 Stability profile determination for analytical reliability

To verify the prepared reference solutions' suitability for routine analytical use, their chemical stability was assessed using the procedure outlined in Section 4.1.5.8.

4.2.6 Preparation of tablet samples

LSDH tablets with different strengths (5 mg, 20/12.5 mg, and 20/25 mg) were used to develop the analytical process. A representative sample of ten tablets was weighed, and the mean weight was determined. The LSDH tablet formulations were finely pulverized using a mortar and pestle to obtain a homogeneous powder. A portion of this powder, corresponding to the average weight of a single tablet, was accurately weighed and dispersed in 10 mL of mobile phase. The resulting suspension was subjected to sonication for 10–30 minutes to facilitate complete dissolution of the active pharmaceutical ingredients. Following sonication, the solution was filtered through 0.45 μm nylon syringe filters to remove particulate matter. From the clarified filtrate, aliquots were

precisely prepared at concentrations of 50, 150, and 250 $\mu\text{g mL}^{-1}$ for the 5 mg tablet, and 200, 600, and 1000 $\mu\text{g mL}^{-1}$ for the 20/12.5 mg and 20/25 mg tablet formulations, respectively. These samples were subsequently injected into the HPLC system for quantitative analysis.



5 RESULTS AND DISCUSSION

5.1 Optimization and Method Development of HPLC-DAD

Developing an HPLC analytical method requires careful consideration of several factors that influence chromatographic efficiency and accuracy. Important elements influencing chromatographic separation include the physicochemical characteristics of the analytes, mobile phase composition and pH, column selection, and stringent temperature regulation. Additionally, detector wavelength and other instrument settings must be optimized to ensure analytical precision and reproducibility. Adjusting these parameters helps establish a robust and reliable method for effective analysis.

5.1.1 Eluent phase optimization

Selection of the mobile phase was strategically tailored to the characteristics of the C18 Gold SVEA column (250×4.6 mm, $5 \mu\text{m}$), thereby maximizing chromatographic performance. The choice of solvent system was based on key physicochemical attributes of the analytes, including pKa values, partition coefficients, solubility, and polarity. The mobile phase in HPLC analysis commonly comprises water in combination with organic solvents possessing miscibility, including MeOH, ACN, EtOH, and 2-propanol, to facilitate optimal separation. A review of the literature regarding LSDH and its related impurities indicated that MeOH and ACN are widely used, either alone or in combination with buffer solutions, to enhance separation efficiency. Consequently, multiple mobile phase compositions were systematically tested during the method development stage. Among these, MeOH was identified as the most effective organic solvent, yielding superior peak shapes for LSDH and its impurities (A and D) compared to ACN and EtOH.

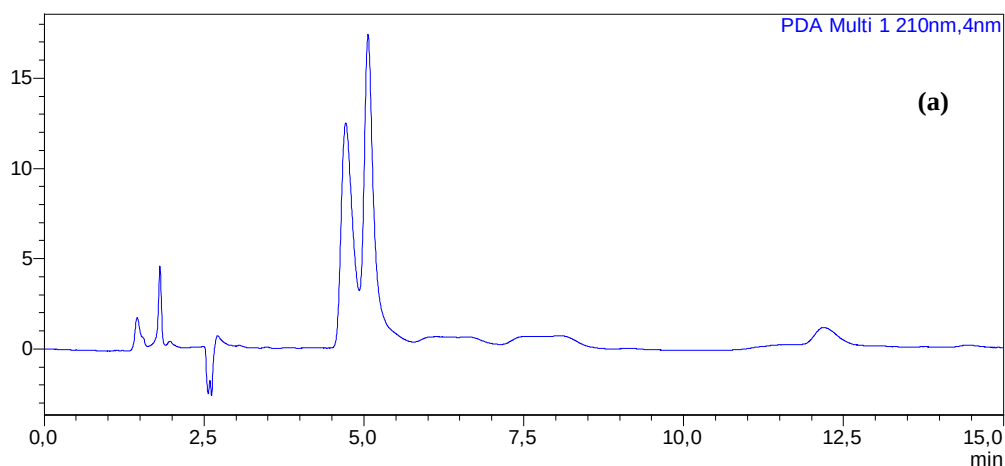
Among the drugs classified as ACE inhibitors, lisinopril stands out due to its fascinating and intricate chemistry regarding acid-base reactions. This compound has four sites that can bind protons, which consist of two carboxylate groups, one primary amine, and one secondary amine. The protonation of the carboxylate groups is likely to be significantly overlapping, which complicates their analysis. To explore how lisinopril ionizes and becomes protonated, researchers conducted potentiometric tests in water and mixtures of methanol and water. The pKa values for lisinopril are identified as 2.5, 4.0, 6.7, and 10.1. 3.17 is the most acidic pKa ever measured, and 10.21 is the most basic (Takács-Novák et al., 2013).

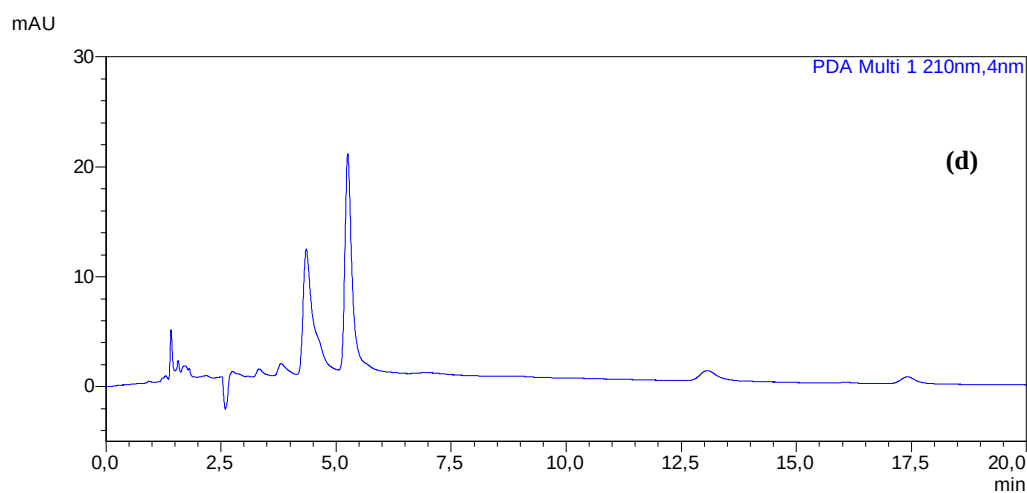
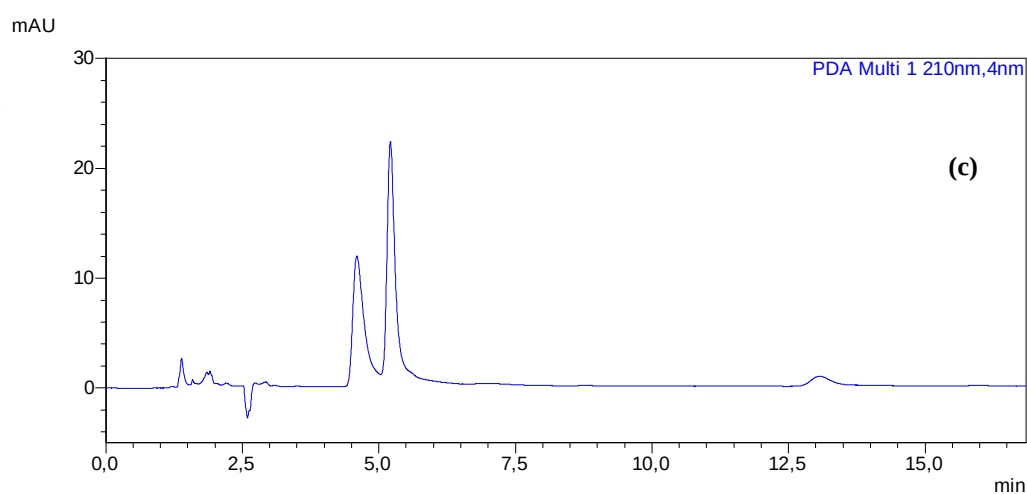
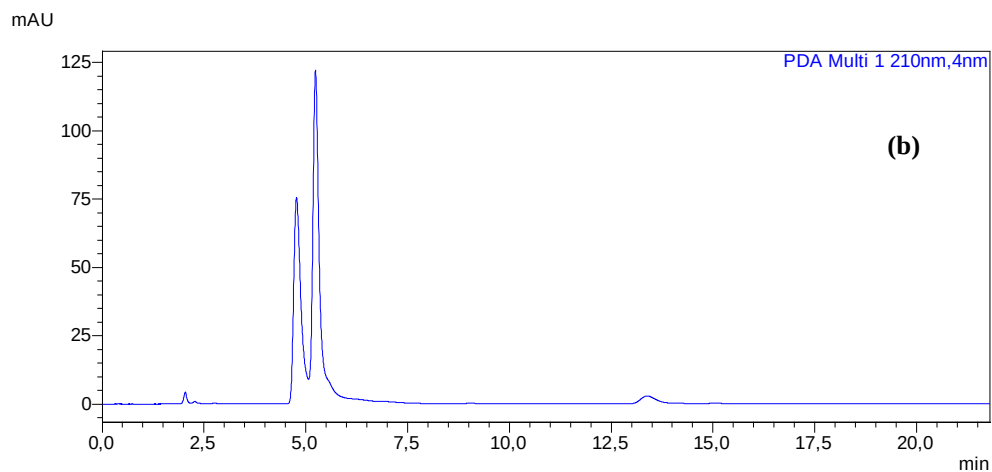
High-performance liquid chromatography relies heavily on buffer systems to control retention characteristics and boost repeatability, especially when analyzing ionizable analytes. Phosphate, acetate, formate, and citrate are some of the most often used buffers in HPLC because they offer pH control over a broad range (pH 2–12). Because of their low UV absorbance, phosphate buffers are excellent for detection at wavelengths shorter than 200 nm, making them a preferred choice for chromatographic applications requiring high sensitivity. The pH of the mobile phase was optimized through a series of experiments evaluating various buffer solutions at pH levels of 2.5, 3.0, 3.5, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, and 10.5. For the purpose to choose the best pH value for the procedure, these investigations concentrated on analyzing peak areas and peak shapes. Each compound underwent a System Suitability Test (SST) to guarantee consistent method performance and analytical reliability. This allowed for quick analysis without sacrificing sensitivity or separation quality. A mobile phase comprising sodium dihydrogen phosphate (NaH_2PO_4 , pH 9.50) and methanol (MeOH) in a 60:40 volume ratio was used to achieve the highest separation efficiency and high-resolution performance. Figure 5.1 shows the chromatograms generated during method optimization.

Figure 5.1

Influence of Mobile Phase pH on LSDH and Impurities: (a) Unbuffered, (b) pH 7.5, (c) pH 8.0, (d) pH 8.5

mAU





5.1.2 Column selection and optimization

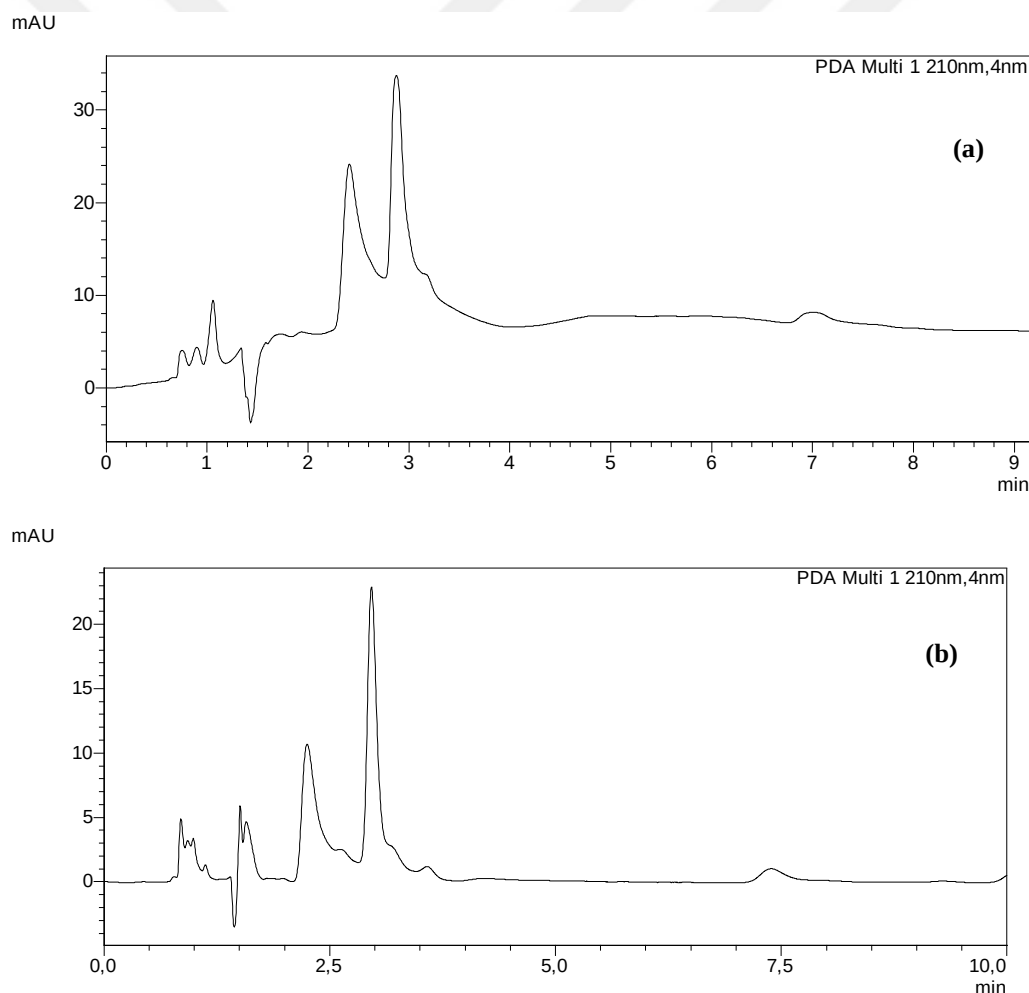
In HPLC, the column serves as the cornerstone of the separation process. Its selection is dictated by the physicochemical attributes of both the analytes and the stationary phase, which collectively determine separation efficiency. Among the widely employed stationary phases in reversed-phase HPLC are C18 and C8, based on octadecyl and octyl silica bonding, respectively. This study investigated three C18 columns with variations

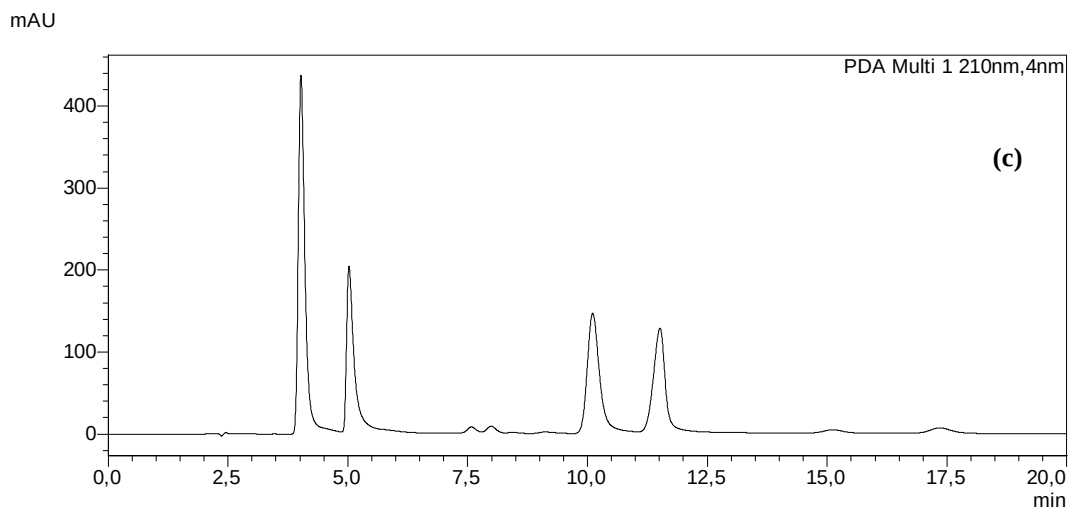
in length and particle size, 150 mm and 250 mm $\times$ 4.6 mm, with packing materials of 3.5 μ m and 5 μ m, each selected to assess their impact on separation efficiency and resolution.

Several practical trials were conducted to assess column performance, resulting in the selection of a C18 column (250 mm $\times$ 4.6 mm, 5 μ m) due to its high separation efficiency. This column was then utilized under refined chromatographic conditions to ensure reliable analytical outcomes. The chromatograms for each column are presented in Figure 5.2.

Figure 5.2

Chromatograms Illustrating Method Development Using C18 Columns of Varying Specifications: (a) 150 mm $\times$ 4.6 mm, 3.5 μ m; (b) 150 mm $\times$ 4.6 mm, 5 μ m; (c) 250 mm $\times$ 4.6 mm, 5 μ m



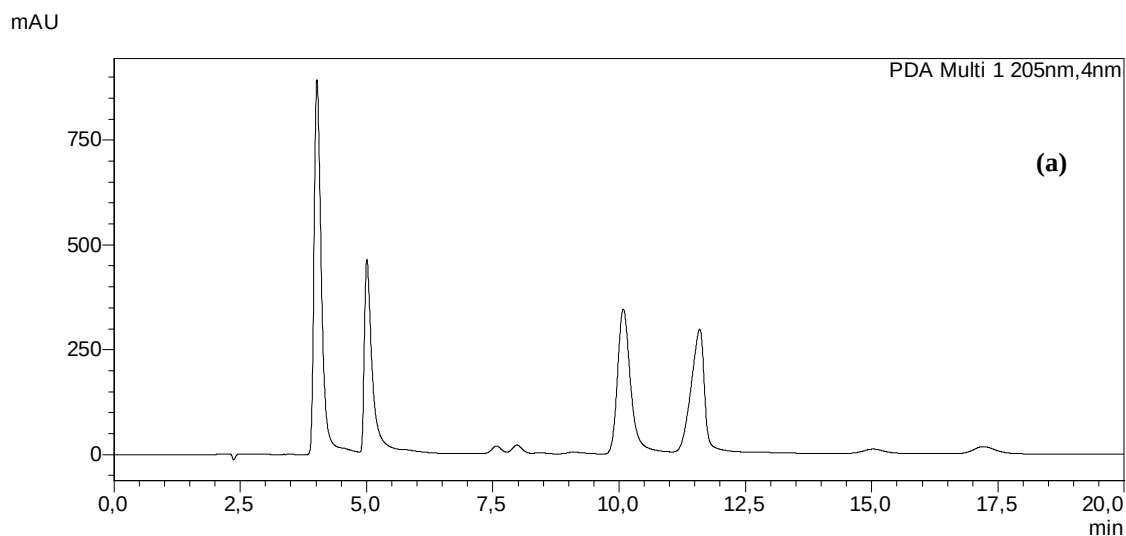


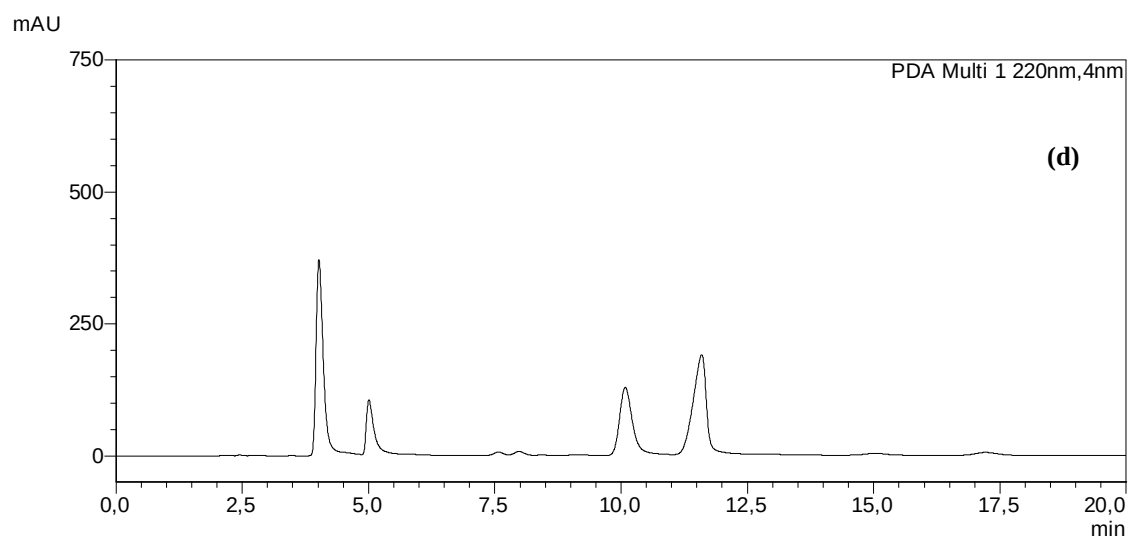
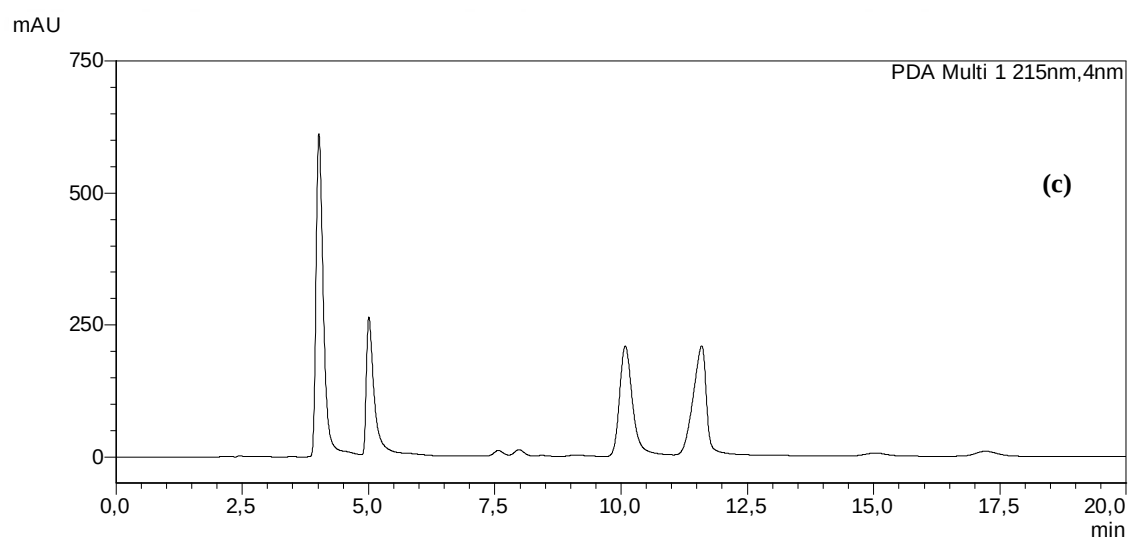
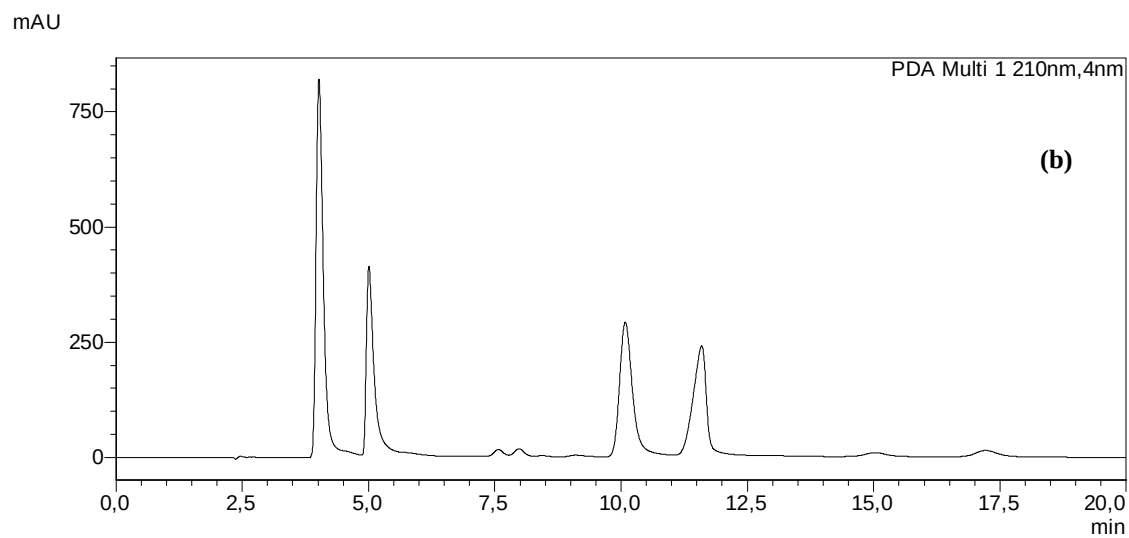
5.1.3 Optimization of wavelength

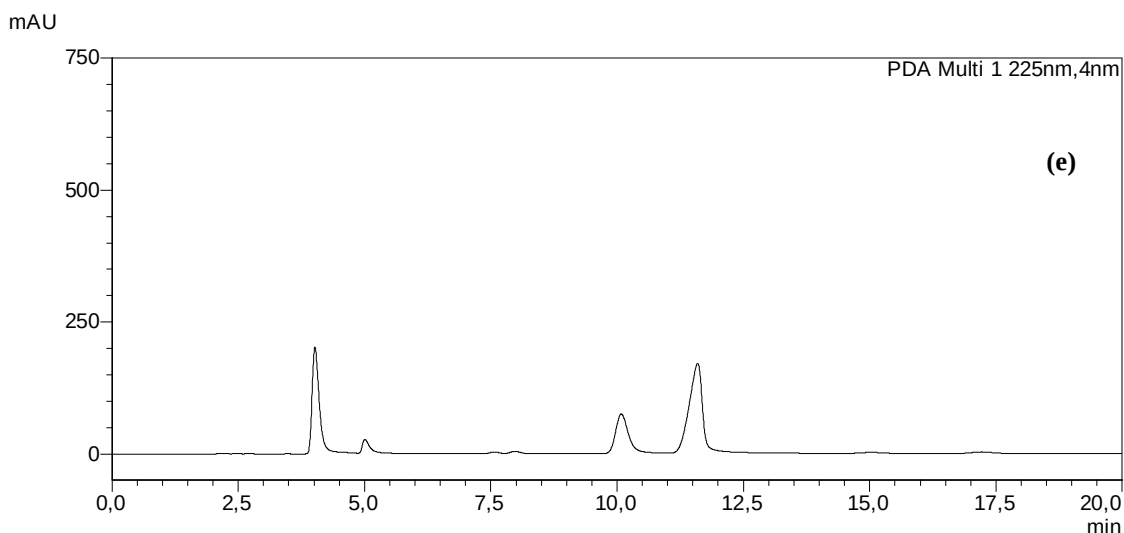
To identify the active substances, an evaluation of previously reported wavelengths was conducted. Spectral data acquisition was performed using a UV-DAD detector configured to monitor wavelengths at 205, 210, 215, 220, and 225 nm. Among these, 210 nm was determined to be the most suitable wavelength for detection. Corresponding chromatograms for each wavelength are presented in Figure 5.3.

Figure 5.3

Chromatograms Associated with the Method Development for LSDH and its impurities at various wavelengths: (a) 205 nm, (b) 210 nm, (c) 215 nm, (d) 220 nm, (e) 225 nm



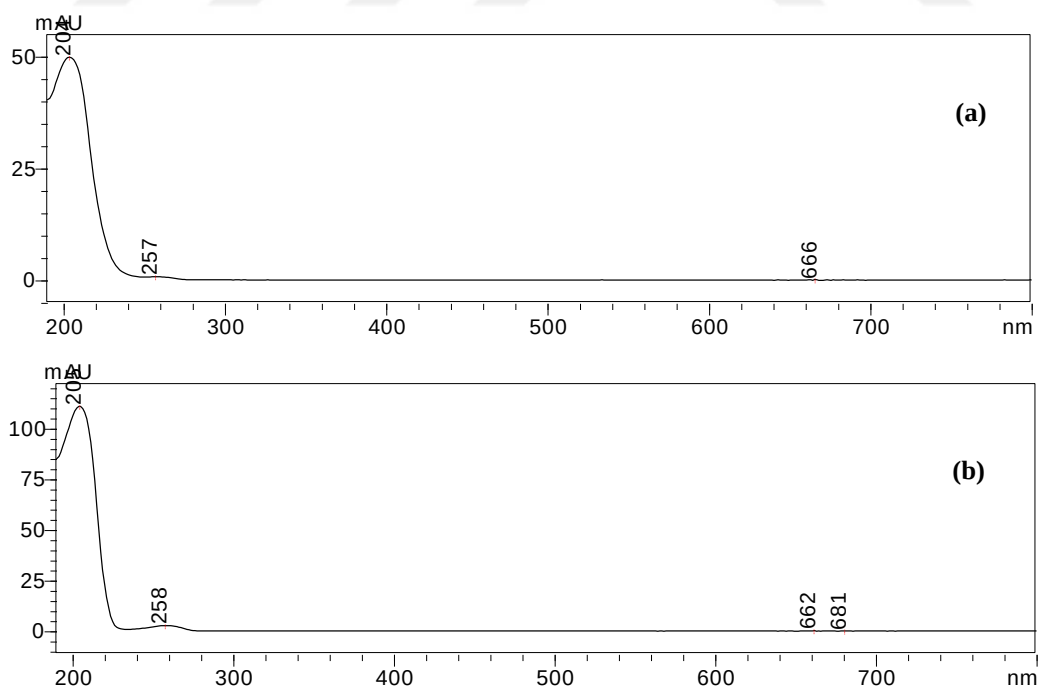




Preliminary UV spectroscopic analysis revealed that the target compounds exhibited prominent absorbance within the lower wavelength region, particularly between 200 and 220 nm. Based on these findings, a detection wavelength of 210 nm was deemed optimal for subsequent HPLC-UV analyses. The relevant absorption spectra are illustrated in Figure 5.4, and the related spectrums of LSDH for tablet samples are given in Figure 5.5.

Figure 5.4

Comparative UV–Vis Spectra of LSDH and Selected Impurities: (a) LSDH, (b) Impurity A, (c) Impurity D



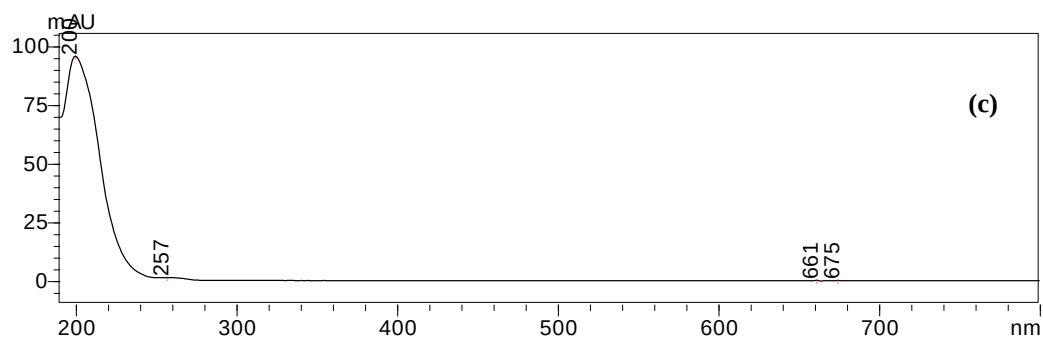
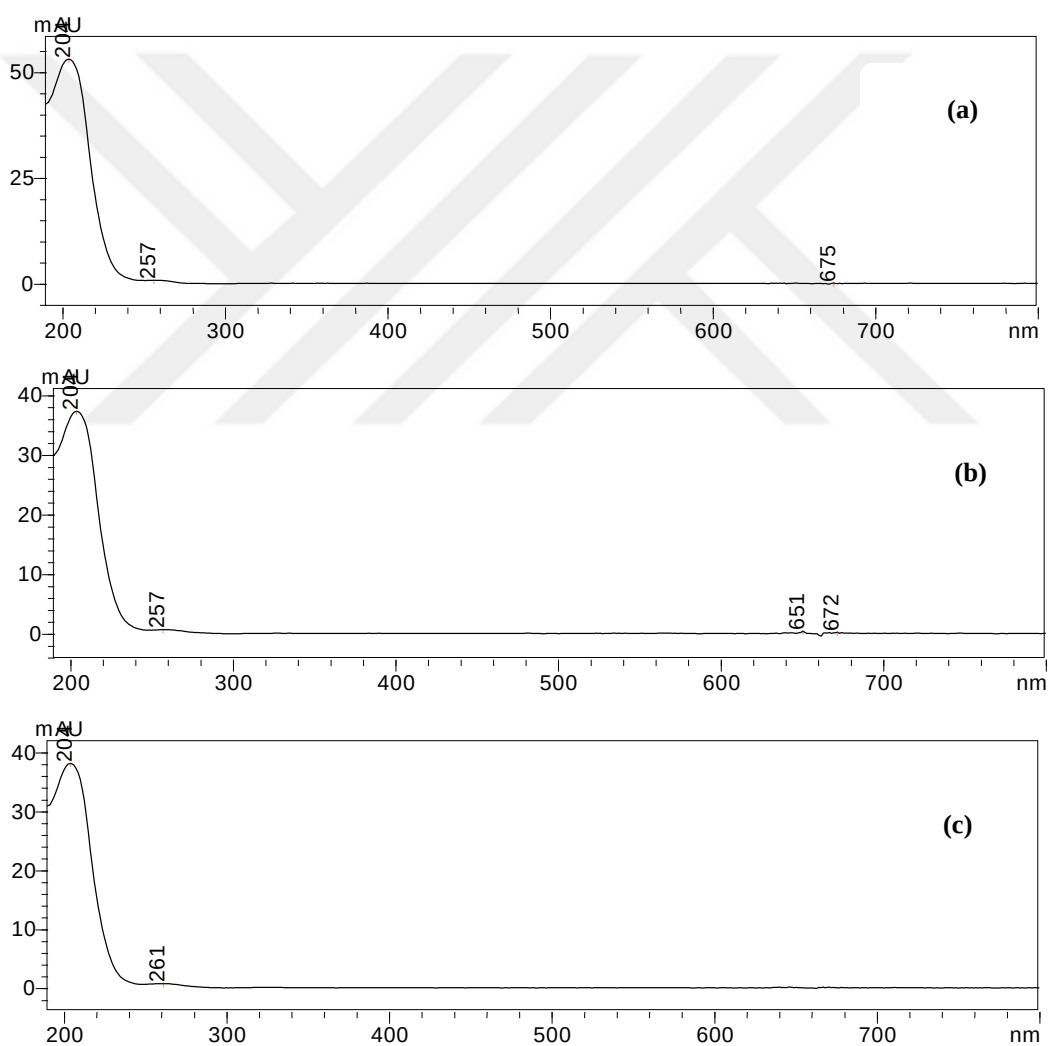


Figure 5.5

UV-Vis Spectral Profiles of LSDH In Various Tablet Samples: (A) 5 mg Dose, (B) 20/12.5 mg Formulation, and (C) 20/25 mg Formulation



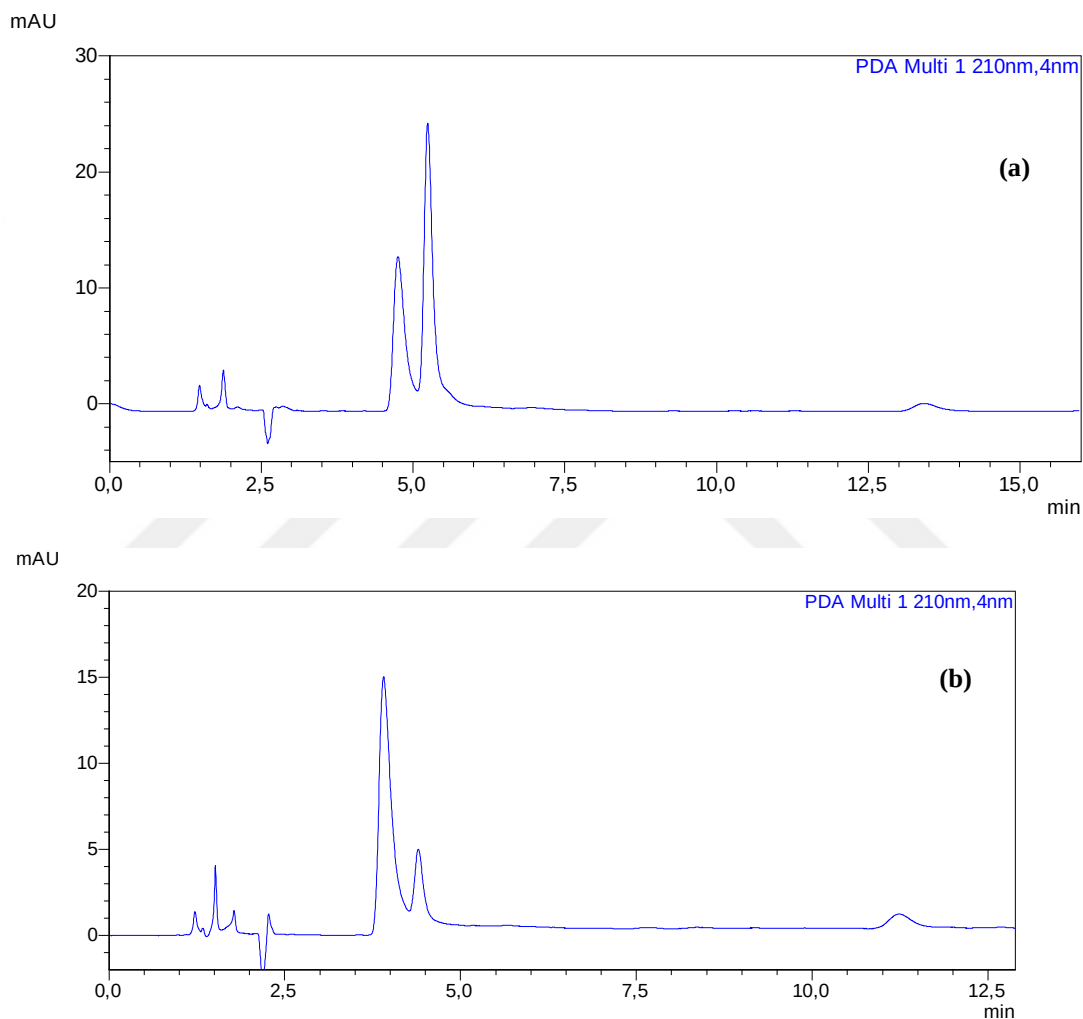
5.1.4 Column temperature and flow rate optimization

Maintaining consistent temperature control during analysis is crucial because it affects chromatographic selectivity. To enhance the resolution of LSDH from impurities A and

D, a series of experiments were conducted varying column temperature (25 °C, 30 °C, 35 °C, and 40 °C) and flow rate (0.7–1.2 mL/min). The optimal conditions identified were a flow rate of 1.0 mL/min and a column temperature in the range of 35–40 °C. Details of parameter adjustments and the corresponding chromatograms are shown in Figure 5.6.

Figure 5.6

Chromatograms from the Method Development for LSDH and its Impurities: (A) Column Temperature (25–40 °C) and (B) Constant Flow Rate (1.20 mL/min)



5.1.5 Optimization of injection volume

To assess the impact of different injection volumes, a systematic investigation was conducted using injection volumes of 10.0 μL and 20.0 μL . Standard analyte combinations ($100 \mu\text{g mL}^{-1}$) were analyzed using a flow rate of 1.0 mL/min, a consistent injection volume of 10.0 μL , and UV detection at 210 nm. During the entire investigation, the column temperature remained steady at 40 °C.

5.1.6 Optimum method conditions

As a result of systematic optimization studies, the chromatographic conditions for the developed method have been succinctly summarized in Table 5.1. To illustrate the outcomes obtained under the most favorable conditions, Figure 5.7 presents the chromatogram acquired at the optimized parameters.

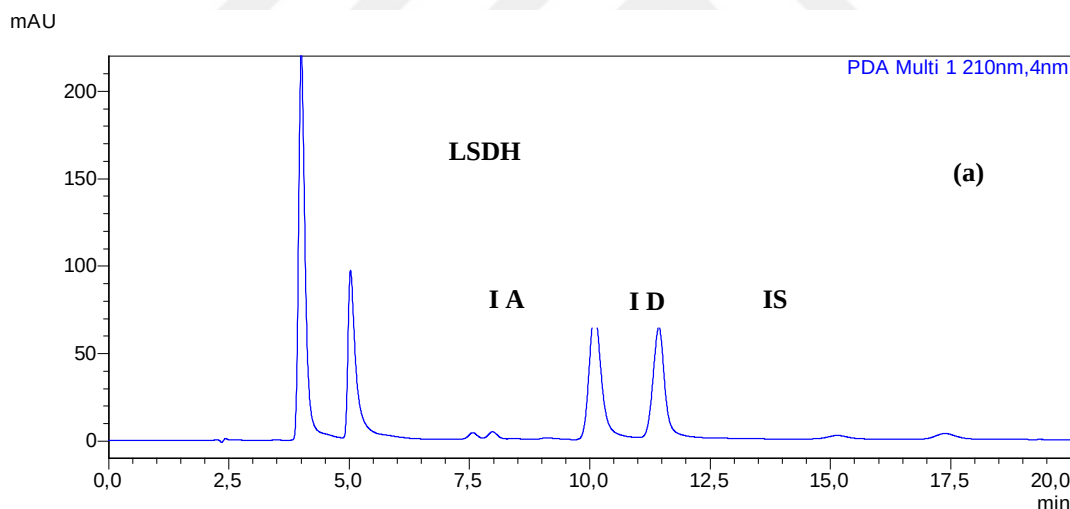
Table 5.1

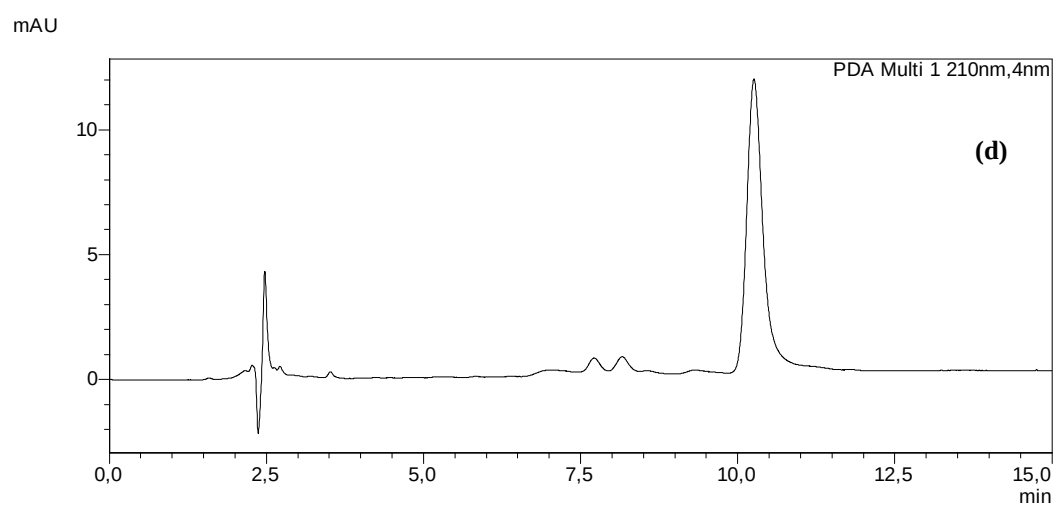
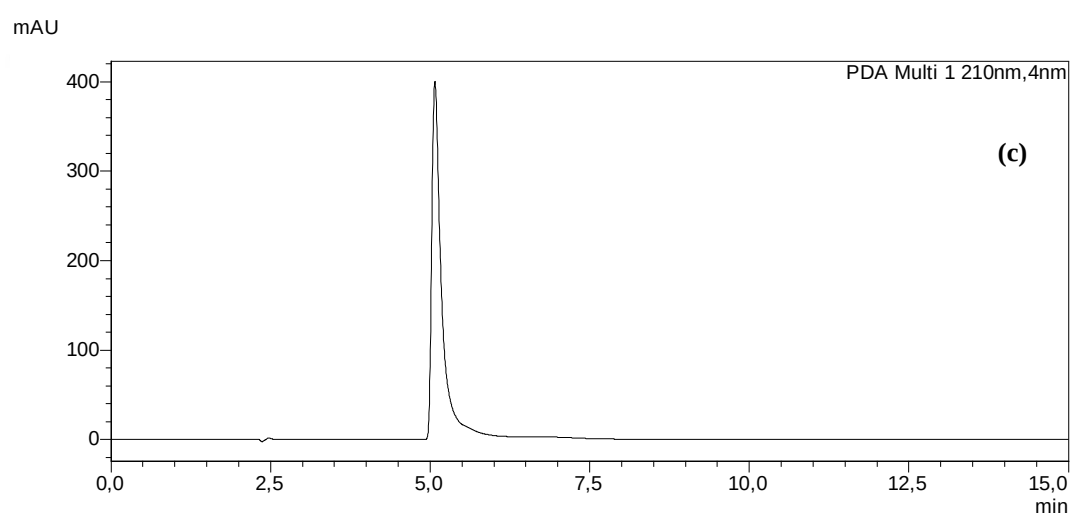
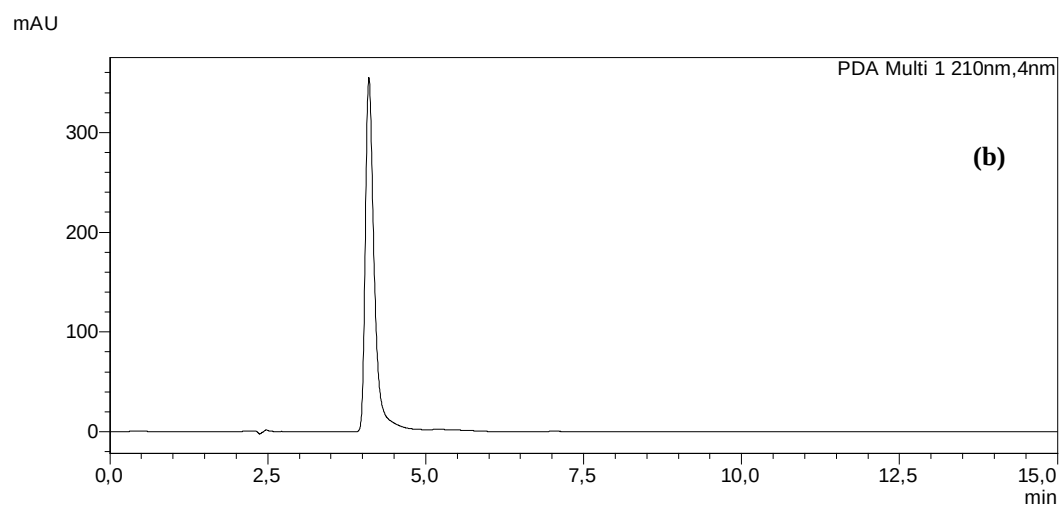
Optimized Chromatographic Parameters for the Determination of LSDH and Its Related Impurities

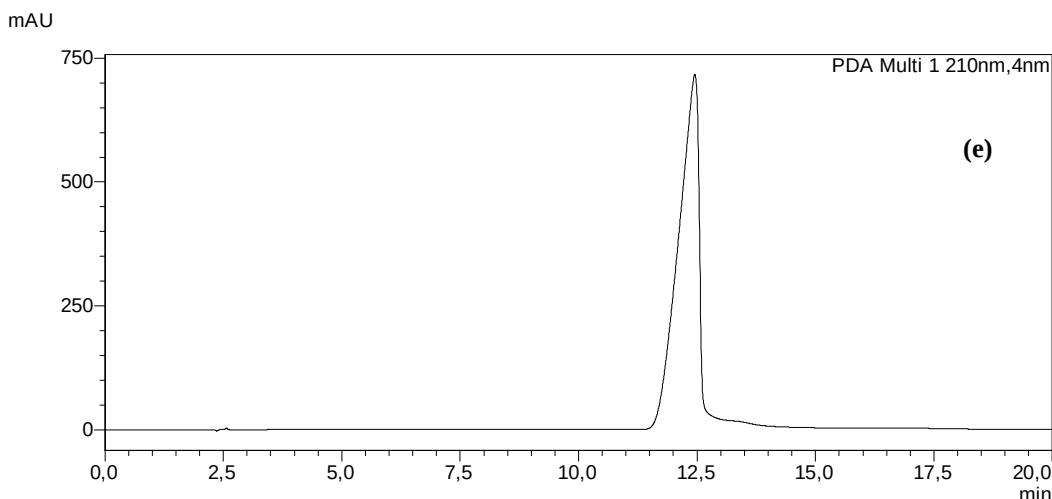
Parameter	Value
Column	C18 250 mm, 4.6 mm, 5 μ m
Buffer solution	23 Mm NaH ₂ PO ₄ , pH 9.50
Mobile phase	Buffer: MeOH (60:40) v/v
Column temperature	(35- 40) °C
Flow rate	1.0 mL min ⁻¹
Injection volume	10 μ L
Wavelength	DAD: 210 nm

Figure 5.7

Typical Chromatograms were Obtained at Optimum Conditions for (a) Mix Standards and Internal Standards, (b) LSDH, (C) Impurity A, (d) Impurity D and (e) Internal Standard (Ethyl Paraben)







5.1.7 System Suitability Assessment (SST)

System suitability test plays a critical role in method validation, ensuring the chromatographic system consistently delivers reliable results of LSDH, impurity A and impurity D. Under the established optimal parameters shown in Table 5.2, effective separation of LSDH and its impurities (A and D) was achieved. To confirm the method's applicability in quality control laboratories, key system suitability parameters were evaluated following ICH guidelines.

Table 5.2

Evaluation of System Suitability Parameters for LSDH and Related Impurities

Parameters	LSDH	Impurity A	Impurity D	IS (EP)	Recommended value
t_R (min)	4.07	5.13	10.28	11.99	-
Capacity factor (k')	0.63	1.05	3.12	3.80	$1 < K' < 10$
Tailing Factor (T)	0.89	1.50	1.57	1.38	< 2
symmetry factor (A_s)	1.04	1.15	1.16	1.19	$0.95 < A_s < 1.2$
Selectivity Factor (α)	1.67	2.94	3.59	-	> 1
Resolution (R_s)	7.78	3.16	11.16	3.24	> 2
Theoretical Plates (N)	4698	3847	8291	11707	> 2000

5.1.8 HPLC-DAD validation

In compliance with ICH guidelines on method validation, the developed approach for LSDH and its impurities (A and D) underwent systematic evaluation. Accuracy, precision, linearity, and robustness were rigorously tested to ensure dependable analytical performance. Furthermore, statistical analyses were conducted using Microsoft Excel software, enabling comprehensive data evaluation and verification of the method's suitability for precise quantification.

5.1.8.1 Linearity

For LSDH and impurities in the 2–400 $\mu\text{g mL}^{-1}$ range, the calibration curve's linearity was examined. Both internal standard (IS) and peak normalization (PN) ratios ($R = \text{PNStd}/\text{PNIS}$, where $\text{PN} = \text{peak area}/\text{peak retention time}$) were employed in the computations. Linearity was assessed using the least squares regression equation. The standard calibration curves for LSDH, Impurity A, and Impurity D are displayed in Figures 5.8, 5.9, and 5.10, respectively. To ensure precise quantification and system stability, the calibration curve range was chosen to preserve ideal peak morphology and avoid column overload.

The linear properties of the technique created for the analysis of LSDH and related impurities are clarified by the data and associated figures displayed in Tables 5.3, 5.4, and 5.5. Standard solutions with LSDH and impurities (A and D) at varying concentrations were tested at 2, 4, 8, 16, 20, 40, 80, 100, 200, 300, and 400 $\mu\text{g mL}^{-1}$ to evaluate linearity. The least squares regression formula was used to graph and analyze the results. High correlation coefficients ($r^2 \geq 0.9993$) confirmed the linearity of the calibration models for LSDH and its impurities. The analytical sensitivity and baseline offsets were defined by slope and intercept values of 0.0283/0.0198 (LSDH), 0.013/0.0351 (Impurity A), and 0.0071/0.0187 (Impurity D). A strong linear dependency between peak area and analyte concentration was observed for LSDH and its impurities (A and D). The linear equation parameters, slope and intercept, define this relationship.

Choosing the calibration curve focused on enhancing the peak shape and avoiding overload in the column. The related figures also back the method's linearity, which shows it is both reliable and precise. The findings validate the linear nature of the method developed, confirming that it is appropriate for quantitatively analyzing LSDH and its impurities.

Table 5.3
Linearity of LSDH

Sr. No.	Concentration (ug mL ⁻¹)	Mean PNStd/PNIS (n=3)
1	2.00	0.05
2	4.00	0.10
3	8.00	0.21
4	16.00	0.44
5	20.00	0.58
6	40.00	1.17
7	80.00	2.35
8	100.0	2.86
9	200.0	5.75
10	300.0	8.47
11	400.0	11.33
Correlation coefficient (r ²)		0.9999
Slope		0.0287
Intercept		0.0198
SD		0.0014
LOD		0.166 ug mL ⁻¹
LOQ		0.503 ug mL ⁻¹

Figure 5.8
Linearity of LSDH

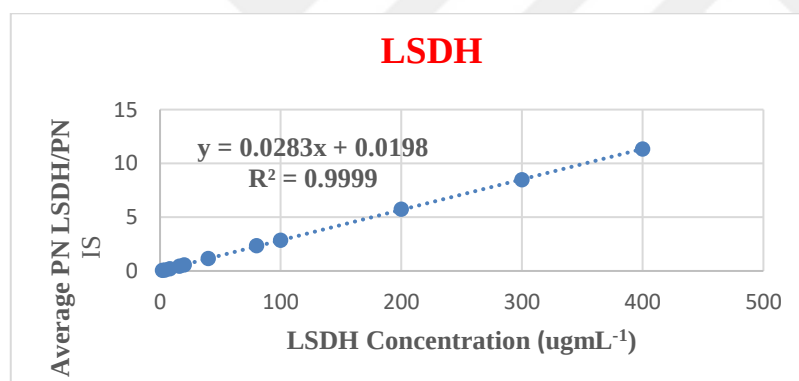
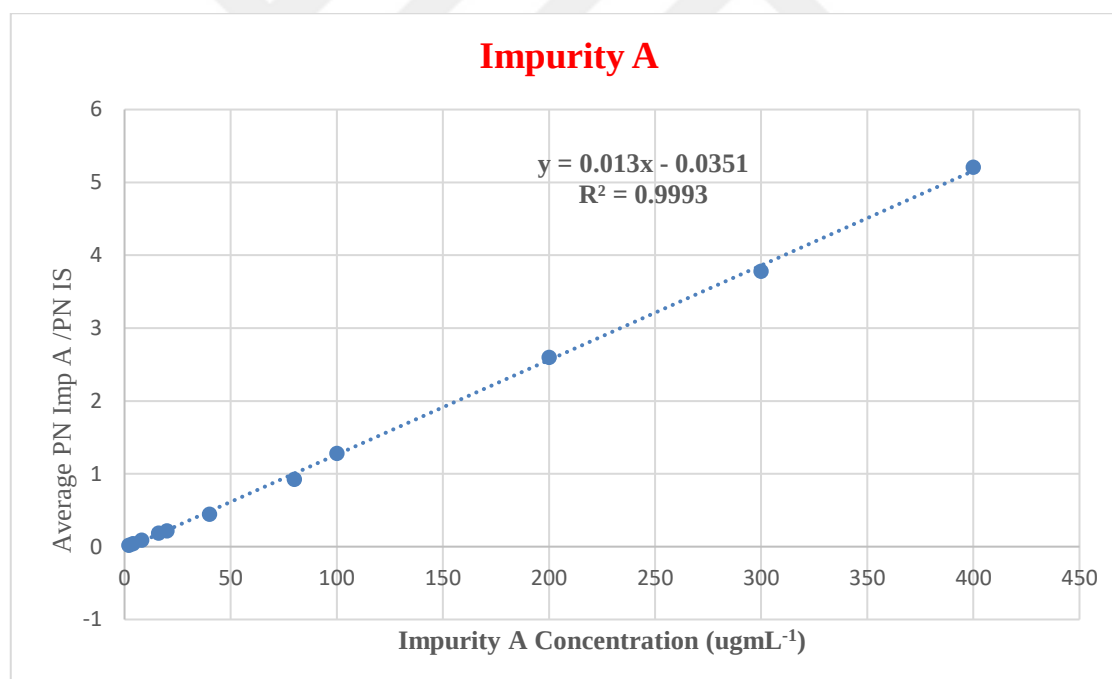


Table 5.4 reports the calibration results for Impurity A, listing concentration levels and average chromatographic responses across triplicate injections. The strong correlation ($r^2 = 0.9993$) and associated regression parameters (slope = 0.013; intercept = 0.0351) demonstrate consistent linear behavior throughout the studied range. This means that as the concentration of impurity A goes up, the peak area also rises proportionally. The high r^2 value of 0.9993 reflects an excellent fit between the experimental data and the regression line, indicating a strong linear relationship. As depicted in Figure 5.9, the slope and intercept define the calibration model linking peak area to the concentration of Impurity A. Data presented in Table 5.4 confirm the linearity of the developed method across the tested concentration range.

Table 5.4
Linearity of Impurity A

Sr. No.	Concentration ($\mu\text{g mL}^{-1}$)	Mean PNStd/PNIS (n=3)
1	2.00	0.020
2	4.00	0.042
3	8.00	0.089
4	16.00	0.18
5	20.00	0.21
6	40.00	0.44
7	80.00	0.92
8	100.0	1.28
9	200.0	2.59
10	300.0	3.78
11	400.0	5.21
Correlation coefficient (r^2)		0.9993
Slope		0.01325
Intercept		0.035
SD		0.0011
LOD		0.282 $\mu\text{g mL}^{-1}$
LOQ		0.856 $\mu\text{g mL}^{-1}$

Figure 5.9
Linearity for Impurity A.



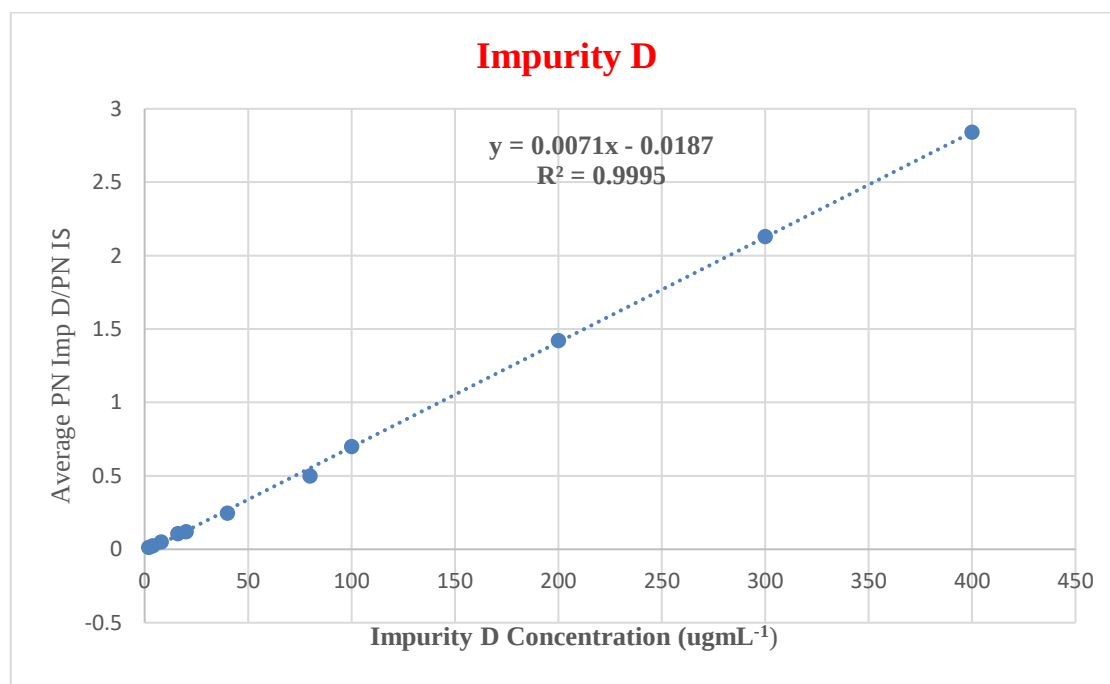
The method used to measure Impurity D's linearity was evaluated by creating a graph that shows the concentration of Impurity D against the average PN Impurity D/PN IS, which is illustrated in Figure 5.10. In Table 5.5, information about the linearity of Impurity D is provided. This table includes 11 varying concentrations of Impurity D and the average area (n=3) for each one. Moreover, the correlation coefficient (r^2) is mentioned, reflecting how strong the linear connection is between concentration and average area. In this

instance, the r^2 value stands at 0.9995, indicating a robust linear relationship. Furthermore, the regression parameters for Impurity D (slope = 0.0071; intercept = 0.0187) confirm a strong calibration curve linearity, validating the method's suitability for quantifying this impurity.

Table 5.5
Linearity of Impurity D

Sr. No.	Concentration ($\mu\text{g mL}^{-1}$)	Mean PNStd/PNIS (n=3)
1	2.00	0.012
2	4.00	0.024
3	8.00	0.050
4	16.00	0.10
5	20.00	0.12
6	40.00	0.24
7	80.00	0.49
8	100.0	0.70
9	200.0	1.42
10	300.0	2.13
11	400.0	2.84
Correlation coefficient (r^2)		0.9995
Slope		0.00709
Intercept		0.0194
SD		0.000608
LOD		0.283 $\mu\text{g mL}^{-1}$
LOQ		0.857 $\mu\text{g mL}^{-1}$

Figure 05.10
Linearity for Impurity D

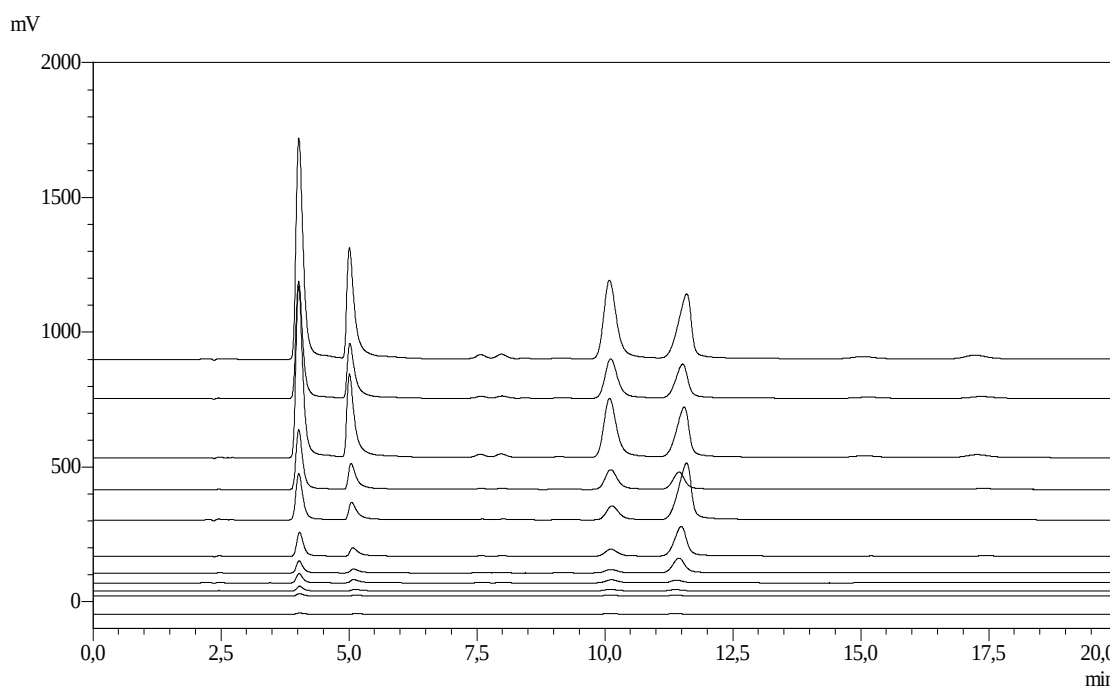


Linear regression results presented in Figures 5.8 through 5.10 demonstrate strong correlations between concentration and peak area for LSDH and its related impurities. LSDH yielded a correlation coefficient (r^2) of 0.9999 with a regression equation of $Y = 0.0283x + 0.0198$. Impurity A and Impurity D exhibited similarly high correlation coefficients of 0.9993 and 0.9995, described by the equations $Y = 0.013x - 0.0351$ and $Y = 0.0071x - 0.0187$, respectively, supporting the method's linearity across the tested range.

In conclusion, as shown in Fig. 5.11, the developed method exhibits outstanding linearity for the drug substance and its related impurities across the entire working concentration range. This fundamental attribute highlights the method's strength, dependability, and appropriateness for accurate measurement in drug analysis, reinforcing its applicability for routine quality control and regulatory compliance.

Figure 5.11

The Chromatograms of Working Concentration Range for LSDH, Impurity A, Impurity D, and IS



5.1.8.2 Accuracy assessment

The method's accuracy was confirmed through recovery studies conducted at three concentration levels for LSDH (40, 75, and 180 $\mu\text{g mL}^{-1}$), Impurity A (30, 65, and 145 $\mu\text{g mL}^{-1}$), and Impurity D (6.5, 15, and 25 $\mu\text{g mL}^{-1}$). Analyses were tested using standard solutions, and recovery percentages were calculated using calibration curve equations.

Each experiment was replicated three times over three consecutive days. Table 5.6 lists %Recovery, SD, and RSD% values obtained for all analytes.

The recovery experiments showed that the average recovery rates were 100.02% for LSDH, 99.97% for Impurity A, and 101.83% for Impurity D. Overall, the data validate the method's suitability for the accurate determination of LSDH and its impurities, confirming its applicability for routine analysis. The experiments provide detailed data on recovery rates at various standard concentrations. In addition, these findings are essential for creating analytical techniques used in the quality control of pharmaceuticals.

Table 5.6
Accuracy Study Results of HPLC-DAD Method for LSDH, Impurity A, and Impurity D in Standard Solutions

Compound	Concentration ($\mu\text{g mL}^{-1}$)	Recovery% (n=3)	Average Recovery%	SD	RSD%
LSDH (IS)	40	99.10	100.02	0.0014	0.12
	75	99.95		0.00057	0.03
	180	101.01		0.00083	0.02
Impurity A (IS)	30	96.77	99.97	0.0015	0.44
	65	103.84		0.0021	0.25
	145	99.30		0.00098	0.05
Impurity D (IS)	6.5	102.55	101.82	0.00022	0.78
	15	102.14		0.00079	0.88
	25	100.77		0.00050	0.31

5.1.8.3 Precision assessment

Precision parameters, including repeatability and intermediate precision, were assessed to confirm the reliability of the developed method under routine analytical conditions. Precision assessments were conducted at three lower calibration levels for LSDH (45, 95, and 145) $\mu\text{g mL}^{-1}$, Impurity A (35, 75, and 120) $\mu\text{g mL}^{-1}$, and Impurity D (8, 12, and 20) $\mu\text{g mL}^{-1}$. Method precision was examined through SD, RSD%, and SEM calculations. Table 5.7 presents the results, with RSD% values remaining below the accepted threshold of 2.0%, demonstrating the method's adequacy for quantitative analysis.

Table 5.7

Statistical Analysis of Precision Studies for the HPLC-DAD Method for LSDH, Impurity A, and Impurity D Standard Solutions

Compound	Concentration ($\mu\text{g mL}^{-1}$)	Statistical Parameter	Intra-day			Inter-day (n=9)
			Day-1 (n=3)	Day-2 (n=3)	Day33 (n=3)	
LSDH (IS)	45	Average	1.33	1.34	1.35	1.34
		SD	0.0011	0.0019	0.0017	0.00042
		SEM	0.00062	0.0011	0.00097	0.00014
		RSD%	0.08	0.14	0.12	0.03
	95	Average	2.72	2.73	2.73	2.73
		SD	0.012	0.0028	0.0039	0.0047
		SEM	0.0067	0.0017	0.0023	0.0016
		RSD%	0.43	0.10	0.15	0.17
	145	Average	4.08	4.09	4.08	4.09
		SD	0.018	0.014	0.018	0.0028
		SEM	0.010	0.0078	0.011	0.00092
		RSD%	0.44	0.30	0.45	0.068
Impurity A (IS)	Concentration ($\mu\text{g mL}^{-1}$)	Statistical Parameter	Intra-day			Inter-day (n=9)
			Day-1 (n=3)	Day-2 (n=3)	Day-3 (n=3)	
	35	Average	0.43	0.45	0.44	0.43
		SD	0.0014	0.0015	0.0082	0.0039
		SEM	0.00082	0.00089	0.0047	0.0013
		RSD%	0.331	0.34	1.87	0.89
	75	Average	0.93	0.95	0.94	0.93
		SD	0.0047	0.0025	0.012	0.0050
		SEM	0.0027	0.0014	0.0070	0.0016
		RSD%	0.51	0.26	1.29	0.53
	120	Average	1.48	1.51	1.53	1.50
		SD	0.0048	0.0023	0.016	0.0072
		SEM	0.0028	0.0013	0.0092	0.0024
		RSD%	0.33	0.15	1.05	0.48
Impurity D (IS)	Concentration ($\mu\text{g mL}^{-1}$)	Statistical Parameter	Intra-day			Inter-day (n=9)
			Day-1 (n=3)	Day-2 (n=3)	Day-3 (n=3)	
	8	Average	0.043	0.044	0.044	0.044
		SD	0.00023	7.89E-05	0.00010	7.88E-05
		SEM	0.00013	4.55E-05	5.85E-05	2.62E-05
		RSD%	0.513	0.18	0.23	0.18
	12	Average	0.072	0.070	0.071	0.071
		SD	0.00031	0.00015	0.00081	0.00034
		SEM	0.00018	8.64E-05	0.00045	0.00011
		RSD%	0.43	0.21	1.12	0.476
	20	Average	0.124	0.126	0.125	0.13
		SD	0.00059	0.00070	0.00077	8.87E-05
		SEM	0.00034	0.00041	0.00045	2.95E-05
		RSD%	0.48	0.56	0.62	0.070

he highest RSD% values observed in both intra-day and inter-day assessments further confirm the method's precision and overall analytical accuracy. In Table 5.7, a detailed statistical assessment of the precision studies for LSDH, Impurity A, and Impurity D in standard solutions is shown. The table summarizes the analyte and impurity concentration

levels along with corresponding mean PN S/PN IS ratios, SD, RSD%, and SEM for both intra-day and inter-day precision assessments.

The consistent observation of RSD% values below 2.0% for all analytes and concentration levels demonstrates the precision and reliability of the developed analytical method. The information in Table 5.6 offers a thorough assessment of the method's accuracy in LSDH, Impurity A, and Impurity D analysis, demonstrating its applicability for precise and repeatable quantification.

5.1.8.4 Specificity and selectivity

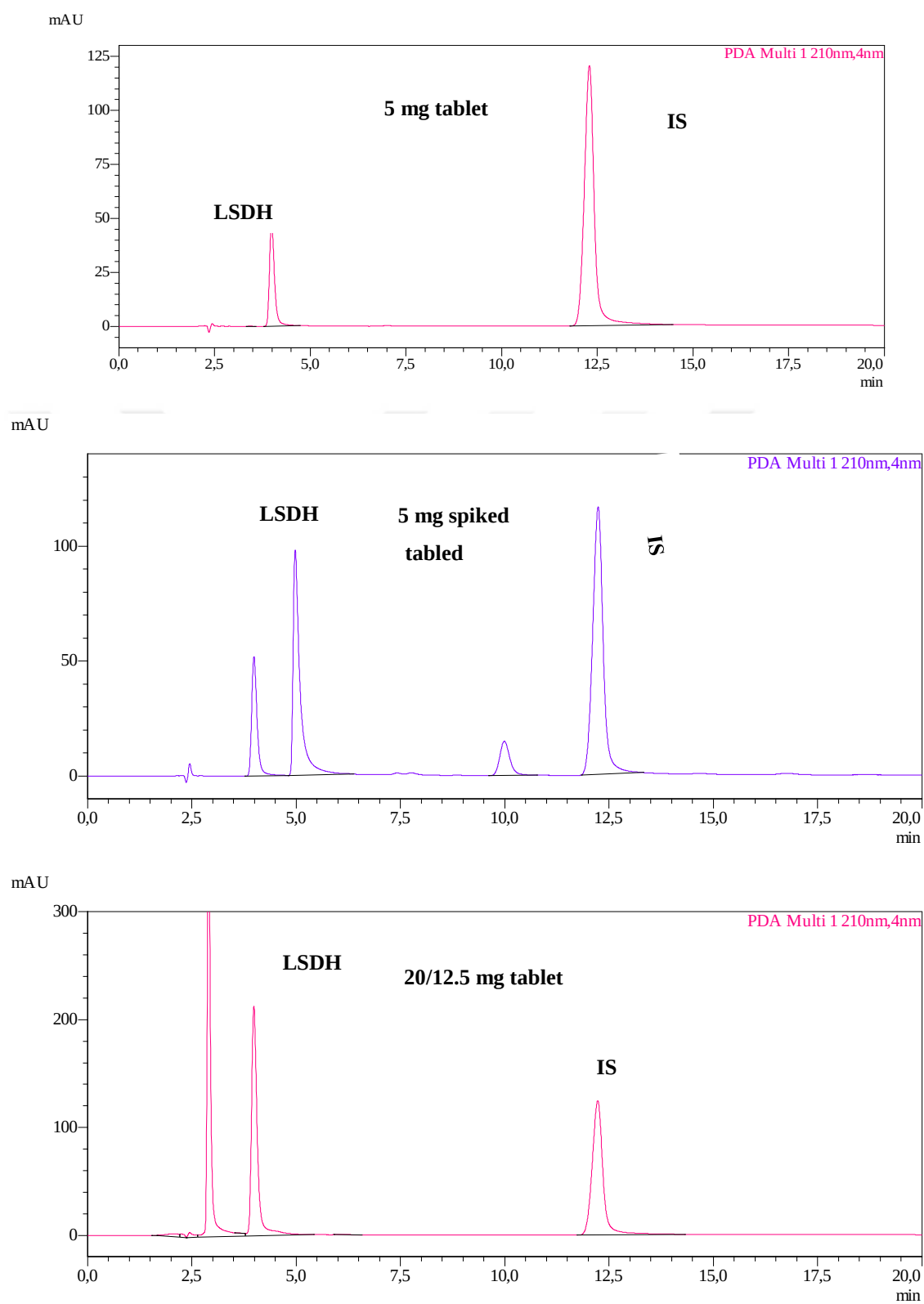
In order to assess the capacity to differentiate and precisely analyze target analytes in the presence of possible interferences, the specificity method is a crucial investigation created to generate degradation products and associated impurities.

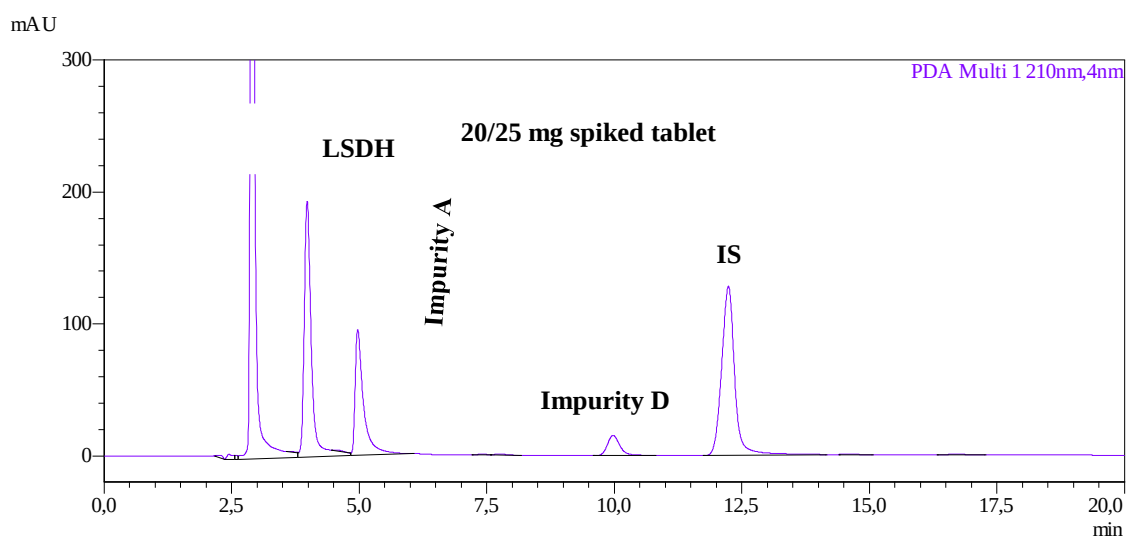
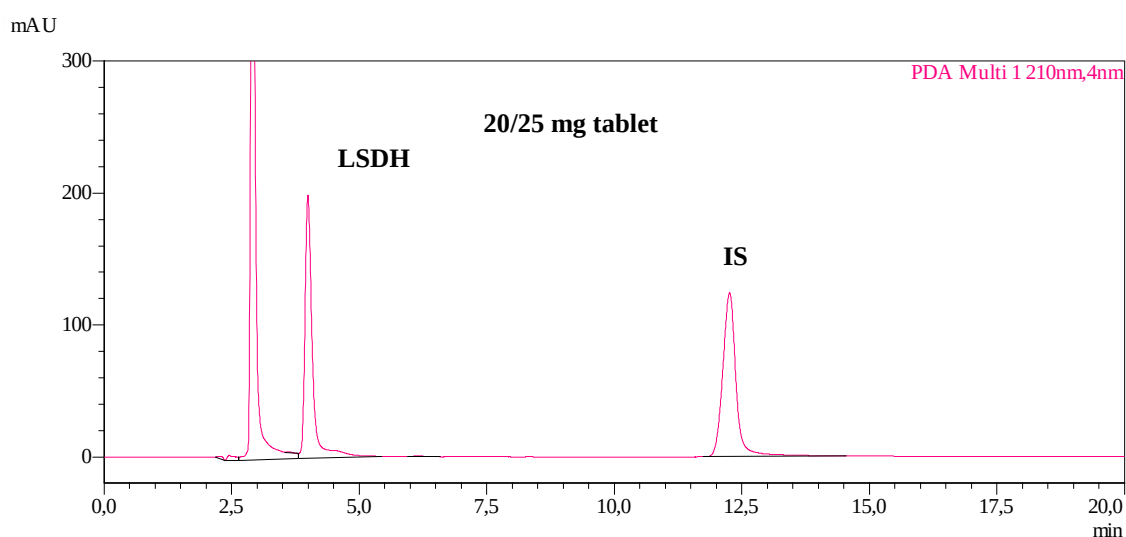
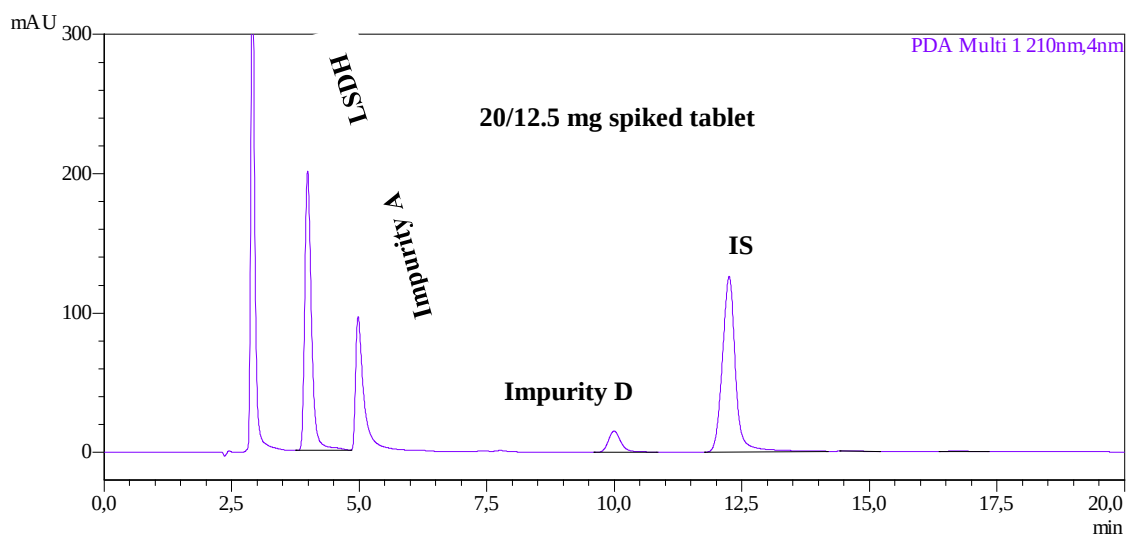
Three different tablet formulations were examined for degradation caused by base exposure under strict stress conditions. Tablets containing 5 mg, 20/12.5 mg, and 20/25 mg of the active pharmaceutical component were weighed individually. The average weight was calculated based on measurements from ten units per dosage strength. To create a uniform powder, the tablets were finely crushed using a mortar and pestle. A 10 mg sample from each tablet was precisely measured and dissolved in 10 mL of a 0.01 M NaOH solution and subjected to sonication for 10 minutes to improve dissolution. The filtered solution was then passed through a 0.45 μm nylon syringe filter. The concentration changes of the tablet samples (100 and 500 $\mu\text{g mL}^{-1}$) were tracked at specific time points of 0, 48, and 72 hours.

The resulting chromatogram, shown in Figure 5.12, exhibited no discernible peaks, emphasizing the method's specificity. The HPLC-DAD method's capability to distinguish analytes from each other is known as selectivity. The peaks did not interfere with each other, and the resolution values of LSDH indicated that effective separation was achieved (>1.5), as shown in Figure 5.12.

Figure 5.12

The Chromatograms for Specificity Show Tablets and Spiked Tablets Chromatograms





Significant degradation was observed under basic stress conditions for LSDH (n=3). The percent degradation for the tablets was found to be 43.6%, 52.9%, and 51.5% for the 5

mg tablet; 54.2%, 56.7%, and 51.3% for the 20/12.5 mg tablet; and 52.0%, 49.0%, and 48.2% for the 20/25 mg tablet, respectively.

To quantify the extent of degradation under basic conditions, the following equation was applied:

Degradation (%) = [(Average peak area of the untreated sample – Average peak area of the degraded sample) / Average peak area of the untreated sample] × 100.

This formula enables the comparative assessment of active ingredient loss due to basic hydrolytic stress.

The information in Table 5.8 indicates the level of degradation over specific time periods. It shows that degradation happens quickly, even with a weaker base like 0.01M NaOH. At a temperature of 25°C, LSDH degraded by 43.6% right away, and after 48 hours, the total degradation reached about 56.7%. The chromatograms illustrating the base degradation study are provided in Figure 5.13.

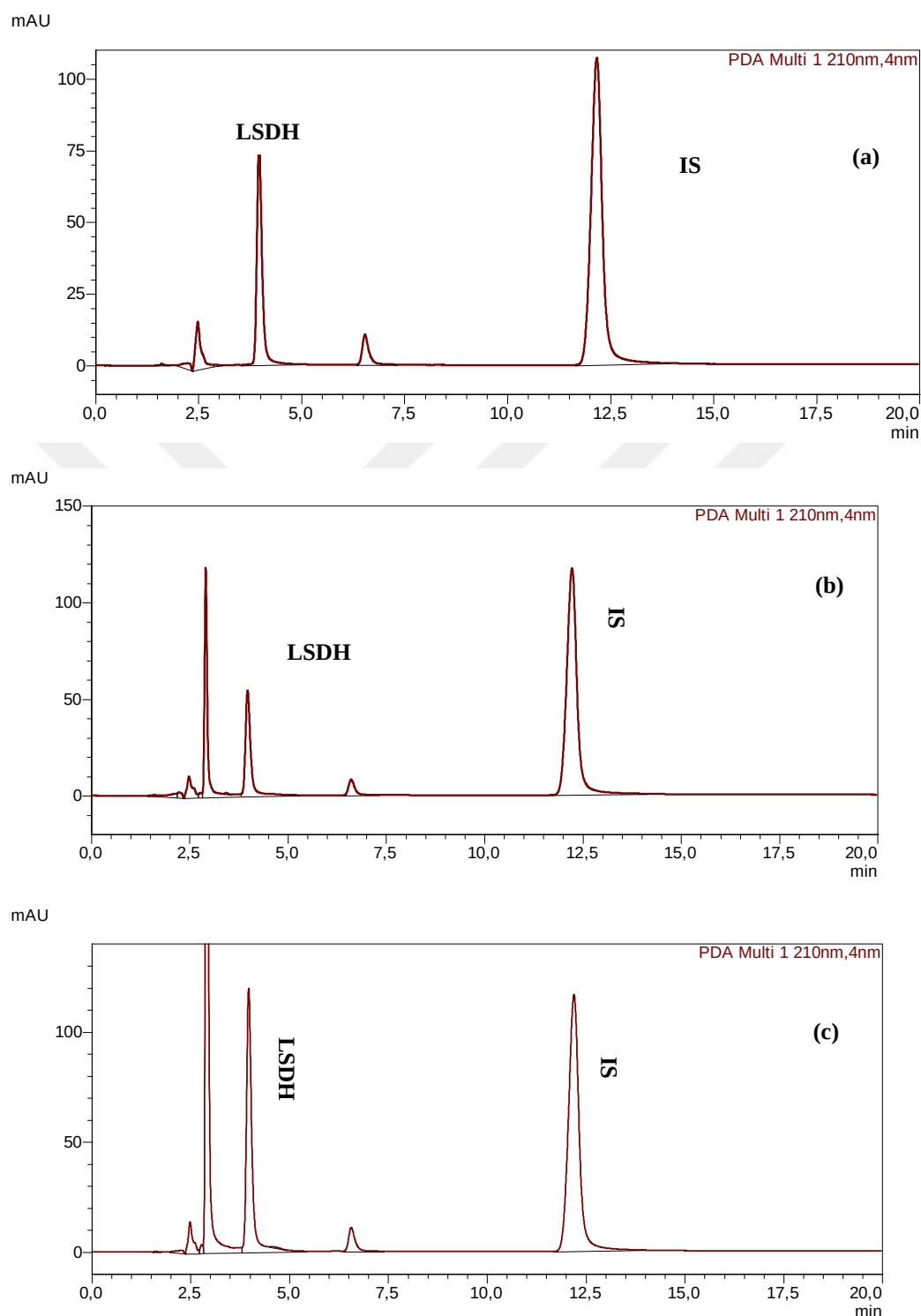
Table 5.8

Comparative Presentation of the Percent Degradation of LSDH in Tablet Solutions After Base Degradation (0.01 M NaOH) Study at Defined Time Intervals

Tablet Formulation	Time (Hours)	Average peak area (Untreated Tablet)	Average peak area (Degraded Tablet)	Percent Degradation (%)
5 mg	0	0.61	0.34	43.64
	48	3.21	1.51	52.89
	72	0.61	0.29	51.48
20/12.5 mg	0	2.83	1.29	54.19
	48	13.26	5.74	56.67
	72	2.83	1.38	51.29
20/25 mg	0	2.75	1.32	52.03
	48	12.93	6.59	49.01
	72	2.80	1.45	48.24

Figure 5.13

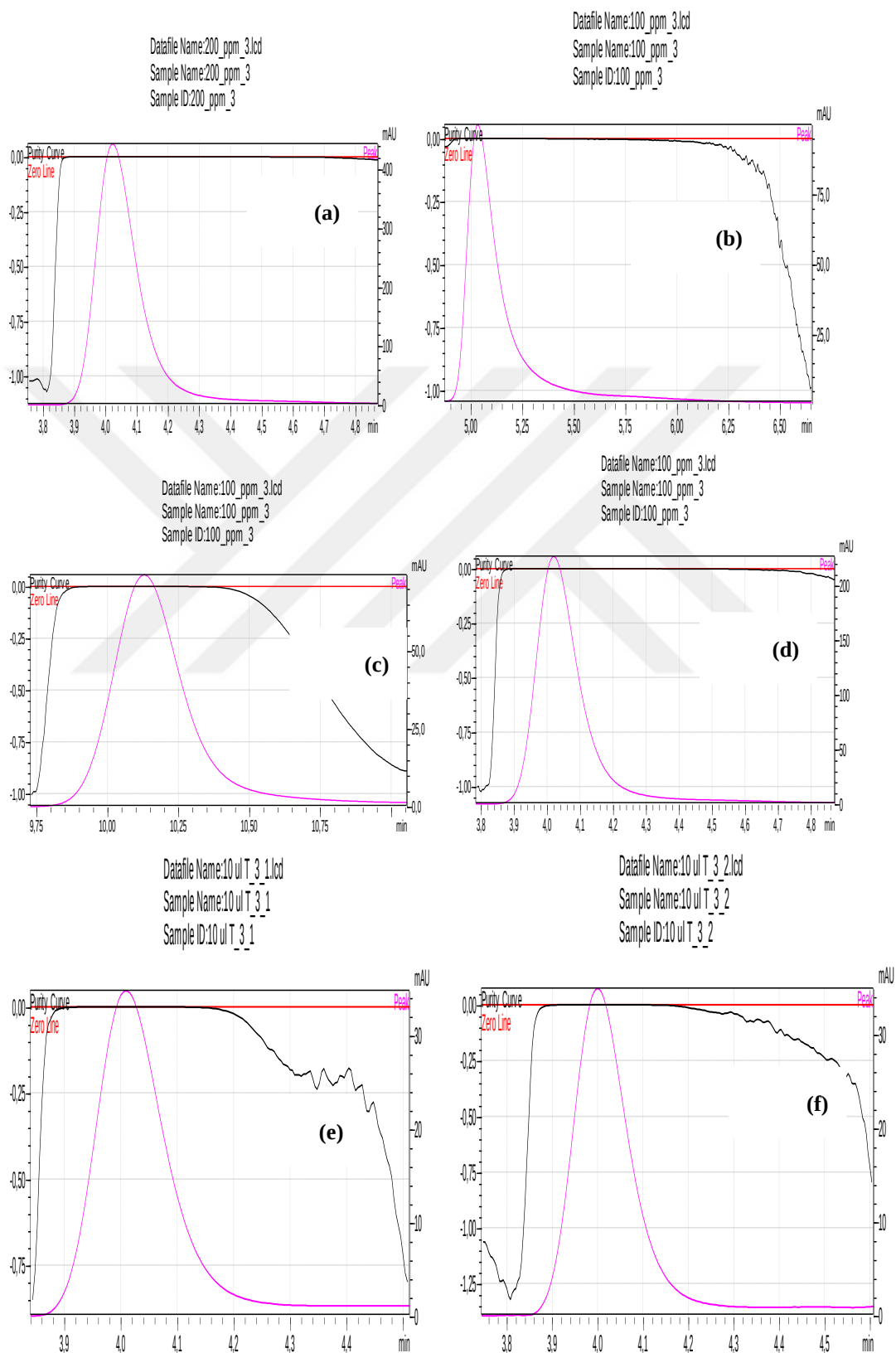
Chromatograms Demonstrating the Effect of Basic Stress Conditions on Tablets Containing: (A) 5 mg, (B) 20/12.5 mg, and (C) 20/25 mg of LSDH

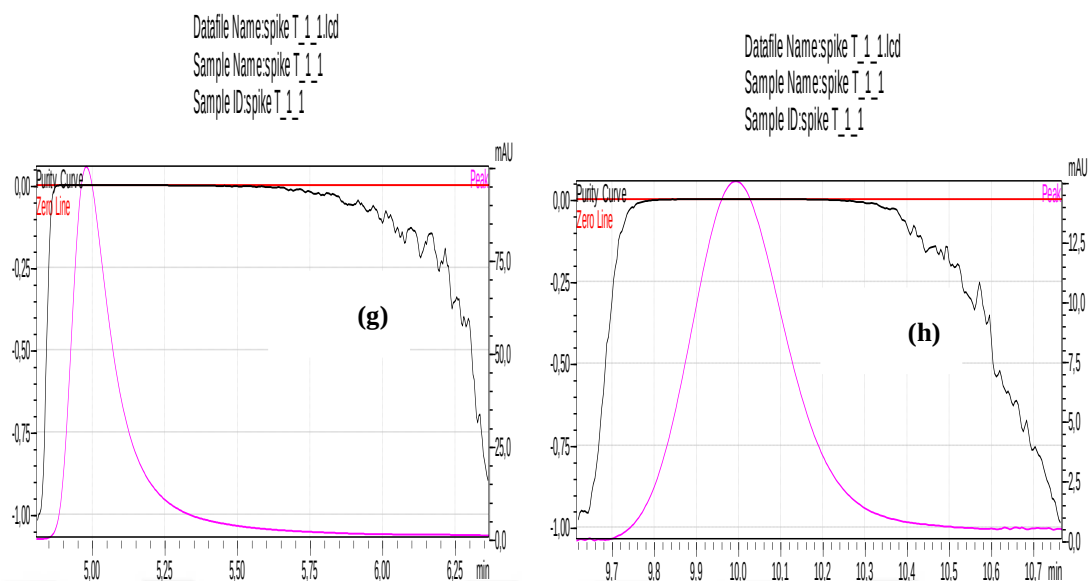


To verify method selectivity, analyte peaks were analyzed using a DAD detector to assess spectral homogeneity. The obtained spectra indicated that all analytes exhibited high peak purity, with no co-eluting interferences. Figure 5.14 displays the corresponding peak purity profiles of standards and tablet samples.

Figure 5.14

Peak Purity Spectra of (a) LSDH, (b) Impurity A, (c) Impurity D, (d) LSDH in 5 mg Tablet Formulation, (e) LSDH in 20/12.5 mg Tablet, (f) LSDH in 20/25 mg Tablet, (g) Impurity A in 5 mg Spiked Tablet and (h) Impurity D in 5 mg Spiked Tablet





5.1.8.5 Detection (LOD) and quantitation (LOQ) limits

LOD and LOQ values for LSDH and its impurities (A and D) were determined to assess the method's sensitivity. The LOD was calculated using the formula $3.3 \times (SD/m)$, while the LOQ was derived from $10 \times (SD/m)$.

A linearity assessment was performed to check the sensitivity of the new analytical method designed for identifying impurities, accompanied by calculated LOD and LOQ. The time required for analysis is aligned with a retention period of 20 minutes. The developed method exhibited strong sensitivity, achieving LOD and LOQ values of 0.17 and 0.50 $\mu\text{g mL}^{-1}$ for LSDH; 0.282 and 0.857 $\mu\text{g mL}^{-1}$ for Impurity A; and 0.283 and 0.857 $\mu\text{g mL}^{-1}$ for Impurity D. These figures demonstrate the method's reliability in detecting and quantifying all analytes at trace levels.

5.1.8.6 Robustness

The robustness of the developed HPLC-DAD method for the quantification of LSDH and its impurities (A and D) was evaluated by analyzing its stability under controlled variations in key analytical parameters. Robustness of the method was assessed by analyzing LSDH (10 $\mu\text{g mL}^{-1}$), Impurity A (20 $\mu\text{g mL}^{-1}$), and Impurity D (5 $\mu\text{g mL}^{-1}$) in triplicate while varying pH, column temperature, and mobile phase composition. As shown in Table 5.9, all RSD% values remained below 2%, confirming the method's consistency and reliability for routine application.

Table 5.9
Results of Robustness Study

Compound	Parameter	Condition	Average Area	SD	%RSD
LSDH (IS)	Buffer pH	7.52	0.23	0.00064	0.27
		7.55	0.23	0.00091	0.38
	MeOH %	38 %	0.21	2.08E-06	0.00095
		42 %	0.27	7.18E-06	0.0026
	Temperatures	33 °C	0.28	0.0029	1.042
		38 °C	0.21	0.00058	0.27
	Wavelengths	215 nm	0.24	0.0031	1.25
		220 nm	0.21	2.72E-06	0.0012
	Buffer pH	7.52	0.23	0.00064	0.27
		7.55	0.26	0.00071	0.27
		38 %	0.22	1.65E-06	0.00074
Impurity A (IS)	MeOH %	42 %	0.21	4.69E-06	0.0021
		33 °C	0.22	0.0033	1.50
	Temperatures	38 °C	0.28	0.00050	0.17
		215 nm	0.21	0.00084	0.40
	Wavelengths	220 nm	0.22	1.083E-06	0.00048
	Buffer pH	7.52	0.011	0.00024	1.13
		7.55	0.011	0.00023	1.032
		38 %	0.015	5.39E-08	0.00034
	MeOH %	42 %	0.012	2.15E-07	0.0016
		33 °C	0.014	1.29E-05	0.087
Impurity D (IS)	Temperatures	38 °C	0.011	3.06E-06	0.027
		215 nm	0.014	6.57E-05	0.46
	Wavelengths	220 nm	0.015	8.24E-08	0.00053

5.1.8.7 Stability assessment

The stability of LSDH, Impurity A, and Impurity D standard solutions was assessed to ensure their reliability over time. The prepared solutions were analyzed under controlled storage conditions, with standard solutions kept at +4°C for one month and at room temperature for 24 hours. In this study, LSDH (10 µg mL⁻¹), Impurity A (20 µg mL⁻¹), and Impurity D (5 µg mL⁻¹) were each analyzed through triplicate injections. The corresponding results are presented in Table 5.10. The data confirm that all standard compounds remained chemically stable under all tested conditions.

Table 5.10
Results of Stability Study

Compound	Parameter	Average Area	SD	RSD %
LSDH (IS)	First Day	0.23	3.82E-06	0.0016
	Short Term			
	Stability	0.24	0.0037	1.51
	Freeze and			
	Thaw Stability	0.23	0.0036	1.59
Impurity A (IS)	Long Term			
	Stability	0.23	0.00063	0.26
	First Day	0.23	2.095E-06	0.00087
	Short Term			
	Stability	0.23	1.57E-05	0.0066
Impurity D (IS)	Freeze and			
	Thaw Stability	0.24	2.63E-06	0.0011
	Long Term			
	Stability	0.23	4.23E-06	0.0018
	First Day	0.011	3.05E-07	0.0025
	Short Term			
	Stability	0.012	1.33E-06	0.011
	Freeze and			
	Thaw Stability	0.012	2.13E-06	0.017
	Long Term			
	Stability	0.0085	2.42E-06	0.028

5.1.9 Analytical evaluation of LSDH content across tablet formulations

To assess the effectiveness of the created method, it was used to measure LSDH and any impurities present in tablet forms. The findings from these tests can be found in Tables 5.11-5.15, while the data for spiked tablets is included in Table 5.13. Additionally, Figure 5.15 illustrates the chromatograms for tablet samples.

Table 05.11
LSDH Assay Results for 5 mg Film Tablet (n=3)

Parameter	5 µgm L ⁻¹	50 µg mL ⁻¹	250 µg mL ⁻¹	450 µg mL ⁻¹
Average (µg mL ⁻¹)	4.51	49.41	249.77	449.52
Minimum (µg mL ⁻¹)	4.79	49.23	249.63	449.33
Maximum (µg mL ⁻¹)	4.23	49.59	249.91	449.72
SD (µg mL ⁻¹)	0.22	0.19	0.18	0.097
RSD %	0.62	0.36	0.06	0.021
SEM (µg mL ⁻¹)	0.11	0.073	0.057	0.069
Bias%	-9.8	-1.18	-0.092	-0.106
% Content Uniformity	95.8	99.18	99.96	99.94

Table 5.12
LSDH Assay Results for 20/12.5 mg Film Tablet (n=3)

Parameter	20 µg mL ⁻¹	200 µg mL ⁻¹	1000 µg mL ⁻¹	1800 µg mL ⁻¹
Average (µg mL ⁻¹)	19.68	198.90	998.18	1795.84
Minimum (µg mL ⁻¹)	19.58	198.58	996.87	1793.66
Maximum (µg mL ⁻¹)	19.78	199.22	999.49	1798.01
SD (µg mL ⁻¹)	0.05	0.16	0.65	1.087
RSD %	0.25	0.08	0.066	0.061
SEM (µg mL ⁻¹)	0.035	0.13	0.46	0.76
Bias%	-1.6	-0.55	-0.18	-0.23
% Content Uniformity	98.9	99.6	99.95	99.89

Table 5.13*LSDH Assay Results for 20/25 mg Film Tablet (n=3)*

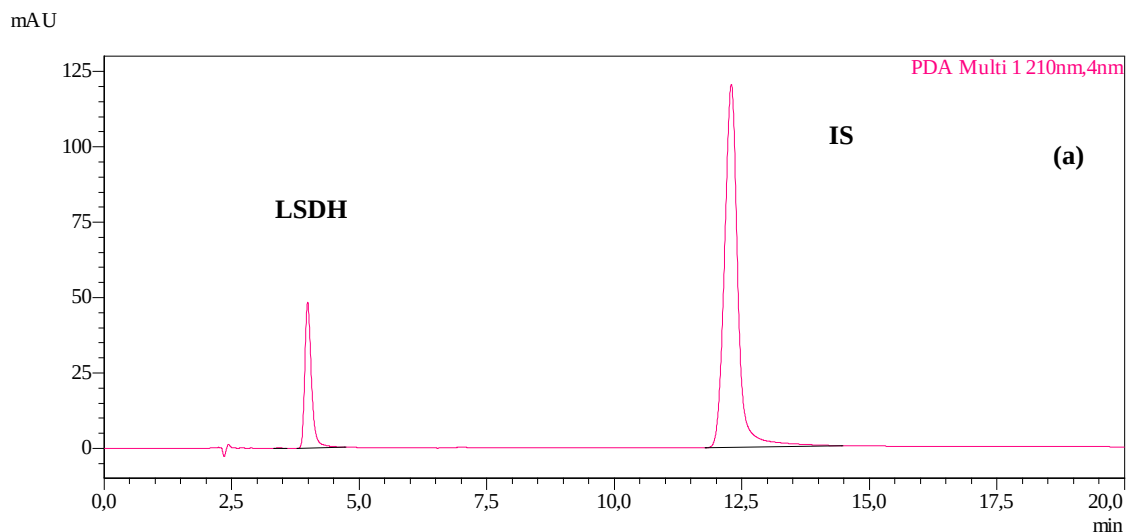
Parameter	20 µg mL ⁻¹	200 µg mL ⁻¹	1000 µg mL ⁻¹	1800 µg mL ⁻¹
Average (µg mL ⁻¹)	19.81	193.39	912.86	1787.19
Minimum (µg mL ⁻¹)	19.45	191.72	906.42	1784.64
Maximum (µg mL ⁻¹)	19.99	196.48	919.61	1788.81
SD (µg mL ⁻¹)	0.31	2.67	6.60045	2.24
RSD %	1.56	1.38	0.72	0.12
SEM (µg mL ⁻¹)	0.028	0.064	0.031	0.73
Bias%	-1.8	-0.69	-10.056	-0.73
% Content	99.07	96.69	91.28	91.29
Uniformity				

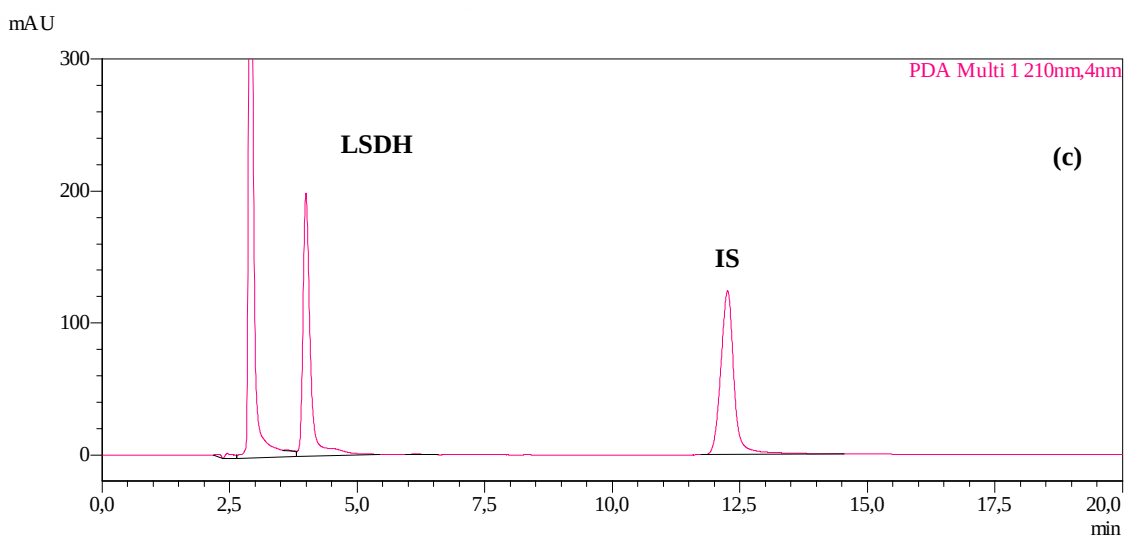
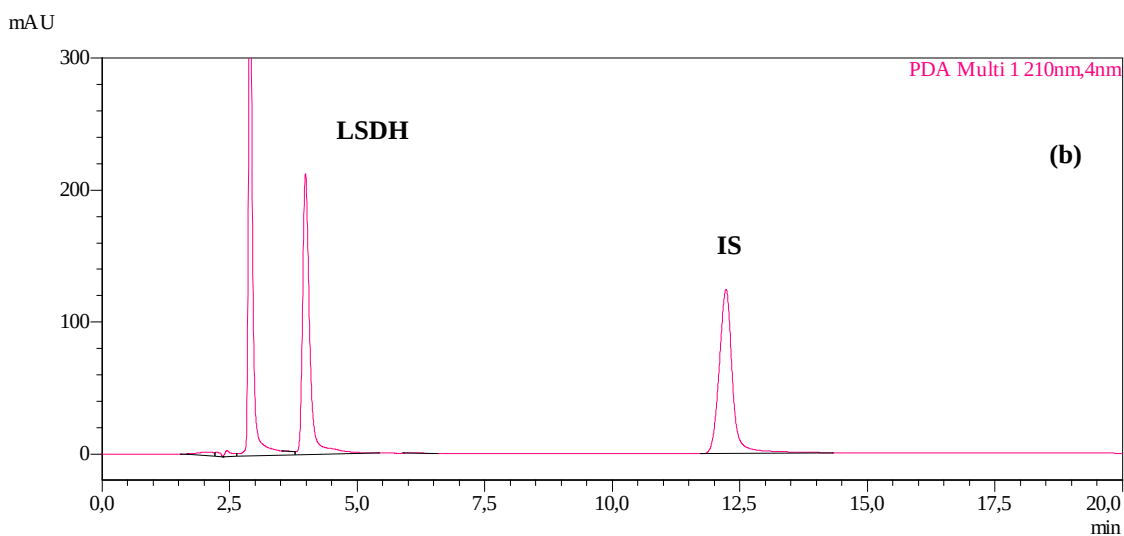
Table 5.14*Assay Data for Impurity A in Spiked Tablet Samples (n = 3)*

Tablet	5 mg	20/12.5 mg	20/25 mg
Amount Added (µg mL ⁻¹)	100	100	100
Amount Found (µg mL ⁻¹)	100.25	100.51	100.90
SD (µg mL ⁻¹)	0.0083	0.0045	0.0048
RSD %	0.32	0.39	0.42
% Recovery	100.25	100.51	100.9

Table 5.15*Assay Data for Impurity D in Spiked Tablet Samples (n = 3)*

Tablet	5 mg	20/12.5 mg	20/25 mg
Amount Added (µg mL ⁻¹)	20	20	20
Amount Found (µg mL ⁻¹)	20.25	20.95	20.89
SD (µg mL ⁻¹)	0.0024	0.00081	0.00084
RSD %	1.61	0.58	0.60
% Recovery	101.25	104.75	104.45

Figure 5.15*Chromatograms of (a) 5 mg Tablet, (b) 20/12.5 mg Tablet and (c) 20/25 mg Tablet*



Across the three tablet types, the 5 mg version displayed the smallest peak areas and concentrations, revealing that it contains less drug content. In contrast, both the 20/12.5 mg and 20/25 mg tablets exhibited larger peak areas and concentrations due to their higher nominal contents. The analysis verified that all three tablet types comply with pharmaceutical requirements for homogeneous content and accuracy. The HPLC-DAD method has shown accuracy, sensitivity, and repeatability for a range of sample sizes, making it a good option for routine LSDH tablet quality control. Furthermore, the consistency of the tablet production process and the dependability of the analytical technique are reinforced by the lack of any notable irregularities or inconsistencies.

There are variations in recovery and sensitivity between dosage forms, according to the analysis results for Impurity A and Impurity D in the additional tablet samples. The 5 mg tablet yielded a 99.96% recovery for Impurity A, suggesting a slight overestimation. The

accuracy of the impurity measurement was demonstrated by the 20/12.5 mg and 20/25 mg tablets, which yielded recovery rates of 99.98% and 99.07%, respectively. With RSD values for each of the three formulations ranging from 0.32% to 0.42%, the analytical method showed a high degree of precision. This demonstrates outstanding reproducibility and validates the accuracy of the Impurity A measurement method. The experimental results, however, consistently overestimated the recoveries for Impurity D in all formulations, with the 5 mg, 20/12.5 mg, and 20/25 mg tablets yielding recoveries of 99.96%, 104.75%, and 104.45%, respectively. These percentages fall within acceptable bounds for measuring impurities, despite being marginally higher than the optimal 100% threshold. With RSD values ranging from 0.58% to 1.61%, the method also showed respectable precision and reproducibility across formulations; however, the 5 mg tablet displayed somewhat greater variability. In summary, the analytical method designed for quantifying Impurity A and Impurity D in LSDH tablets achieves satisfactory accuracy and precision, offering consistent and dependable results across various tablet formulations, thus making it suitable for regular impurity analysis in pharmaceutical products.

5.1.10 Summary evaluation of the HPLC-DAD analytical approach

Most pharmaceutical organizations now require that the purity of drug formulations is free from impurities. Impurities in medications can pose serious health risks to people. As a result, it's essential to create an analytical method that meets the standards set for quality control labs regularly.

This thesis presents the development of a straightforward isocratic RP-HPLC-DAD technique that runs for 20 minutes, aimed at measuring LSDH and its impurities (A and D) in drugs. For this method, a C18 column (SVEA Gold), 250 × 4.6 mm, 5 μm, was optimized, resulting in successful separation along with good peak shape, resolution, and a shorter analysis duration.

The basic compounds LSDH and its impurities (A and D) were effectively separated by using a phosphate buffer at pH 9.5 with a concentration of 23 mM. The mobile phase consisted of methanol and buffer solution in a ratio of 40 to 60 (v/v). This newly developed method satisfied all the required acceptance criteria for system suitability testing. It has been thoroughly validated, demonstrating robustness and precision with a low limit of detection, making it a favorable option compared to the official USP method.

This successful method development achieved two goals of the thesis: producing promising results.

5.11 Optimization and Method Development of HPLC-ELSD

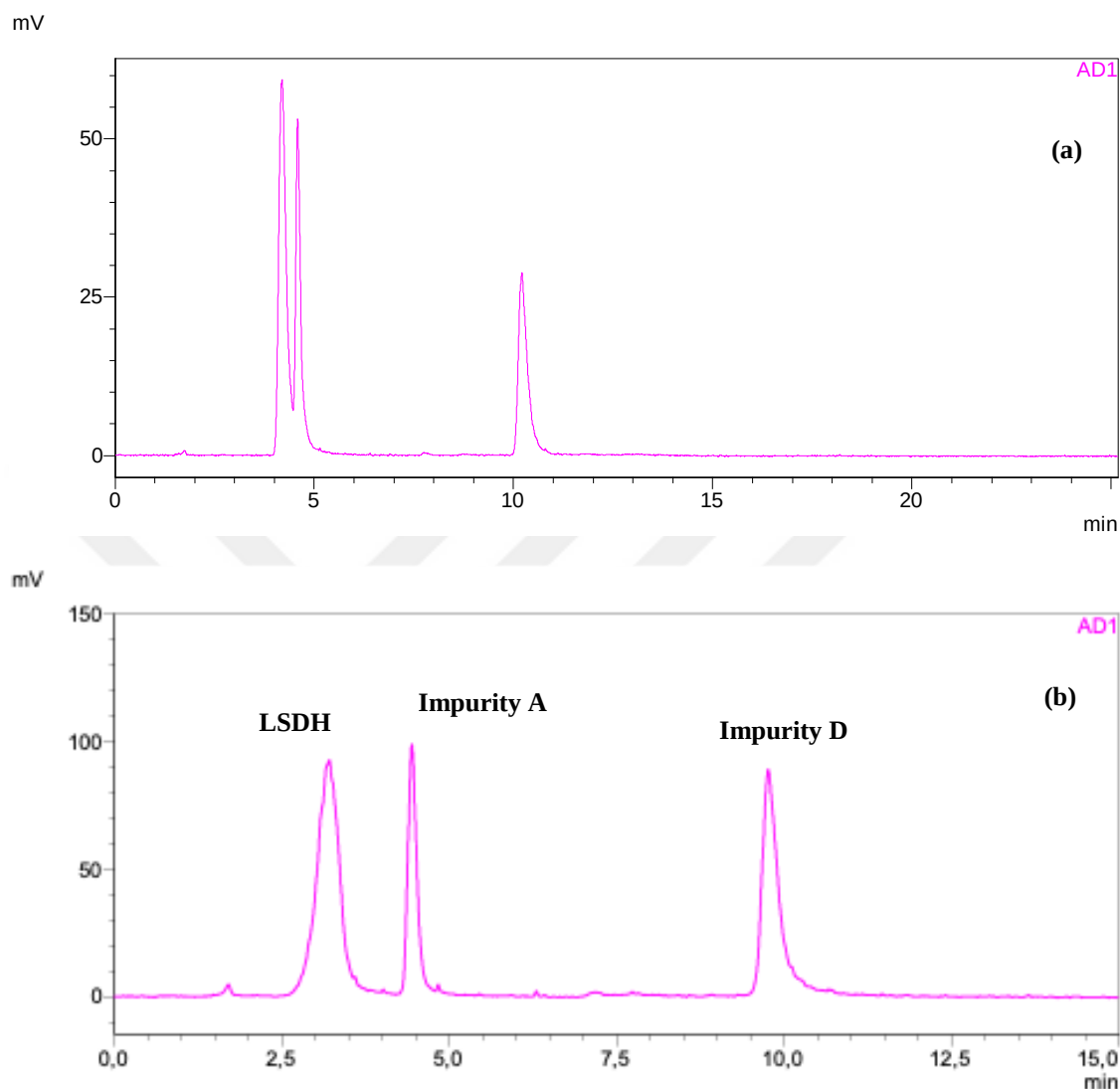
The process of developing a method for quantifying LSDH and its impurities (A and D) using an ELSD Detector was initiated. Unlike the analyses done with the HPLC-UV/DAD system, the HPLC-ELSD technique was used to find LSDH in different tablet forms in the pharmaceutical industry. There is not much research available on using ELSD for this purpose. ELSD is influenced by a broader range of factors compared to other detectors. Thus, to ensure the best separation, various factors such as eluent composition, column and nebulizer temperatures, nitrogen gas pressure, gain, and filter settings were carefully studied and optimized through detailed experiments.

5.11.1 Mobile phase optimization

The A Gold SVEA C18 column (250×4.6 mm, $5\ \mu\text{m}$) was employed for mobile phase optimization. The choice of solvents was informed by analyte-specific parameters such as pKa, solubility, partition coefficient, and polarity. Water and organic solvents compatible with reversed-phase HPLC, namely MeOH, ACN, EtOH, and 2-propanol, were evaluated for their suitability. A review of existing literature regarding the analysis of LSDH and its impurities reveals that MeOH and ACN, often used with or without buffers, are the solvents of choice, typically in different proportions. Drawing from these insights, various mobile phases were assessed during the method development phase. MeOH emerged as the favored organic solvent for the separate evaluations of LSDH and its impurities (A and D), offering better peak shapes than ACN and EtOH. For improved resolution, a 40:60 (v/v) methanol–water mixture was employed as eluent, applying this mixture in all following analyses. Figure 5.16 shows three distinctly separated peaks that displayed greater resolution than the overlapping peak observed with other mobile phase combinations.

Figure 5.16

Effect of Methanol Concentration in the Mobile Phase on the Retention Behavior of LSDH and its Impurities: (a) 50% MeOH, (b) 40% MeOH

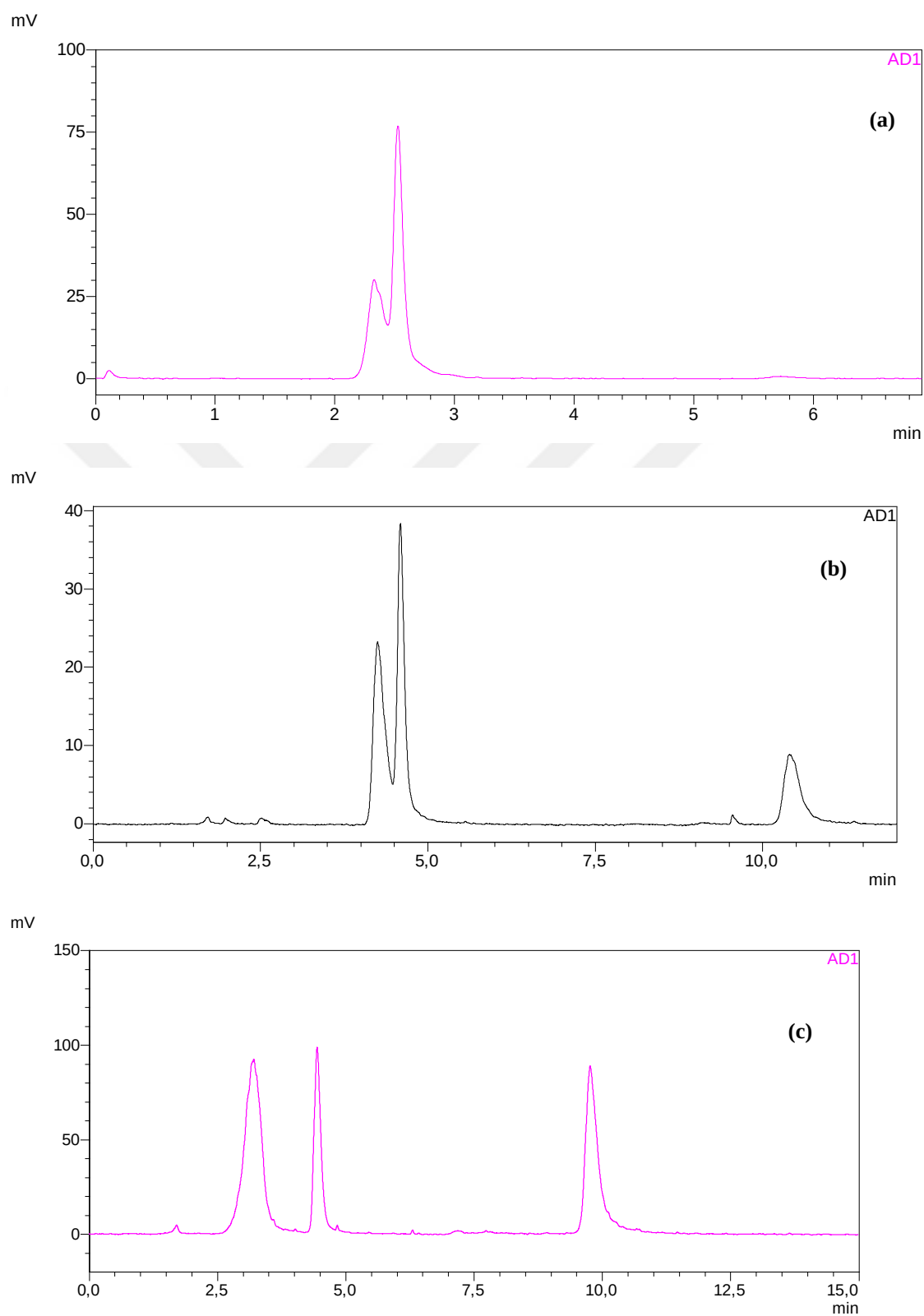


5.11.2 Column selection

Multiple reversed-phase C18 columns were systematically assessed to optimize the chromatographic separation of LSDH and its impurities (A and D). Columns tested included 150×4.6 mm formats with $3.5 \mu\text{m}$ and $5 \mu\text{m}$ particles, as well as a 250×4.6 mm column with $5 \mu\text{m}$ particles. Based on comparative performance, the 250 mm column with $5 \mu\text{m}$ particles was selected for its superior resolution. Consequently, this chosen C18 column was utilized under optimized conditions for chromatography. The chromatograms for the various columns can be seen in Figure 5.17.

Figure 05.17

Chromatograms Demonstrating Method Development Using: (a) C18 Column (150 mm × 4.6 mm, 3.5 μ m), (b) C18 Column (150 mm × 4.6 mm, 5 μ m), and (c) C18 Column (250 mm × 4.6 mm, 5 μ m)

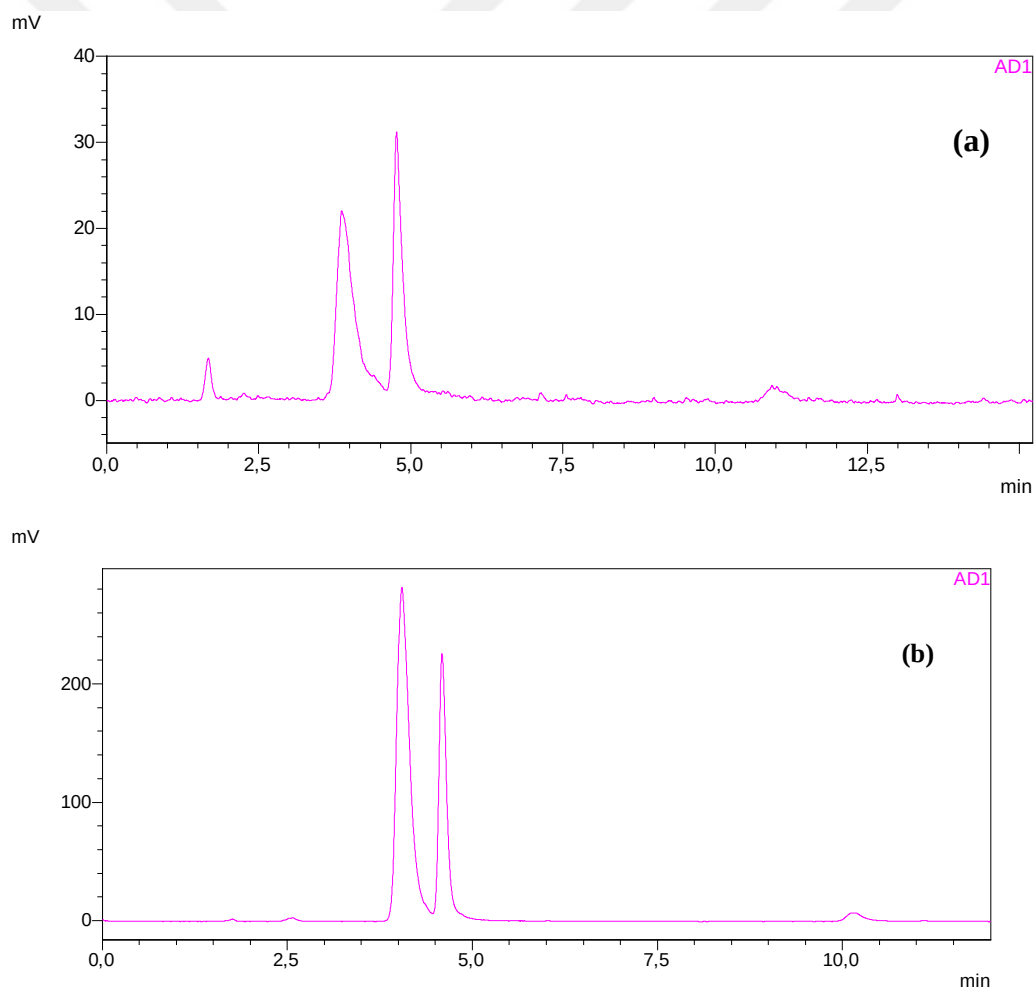


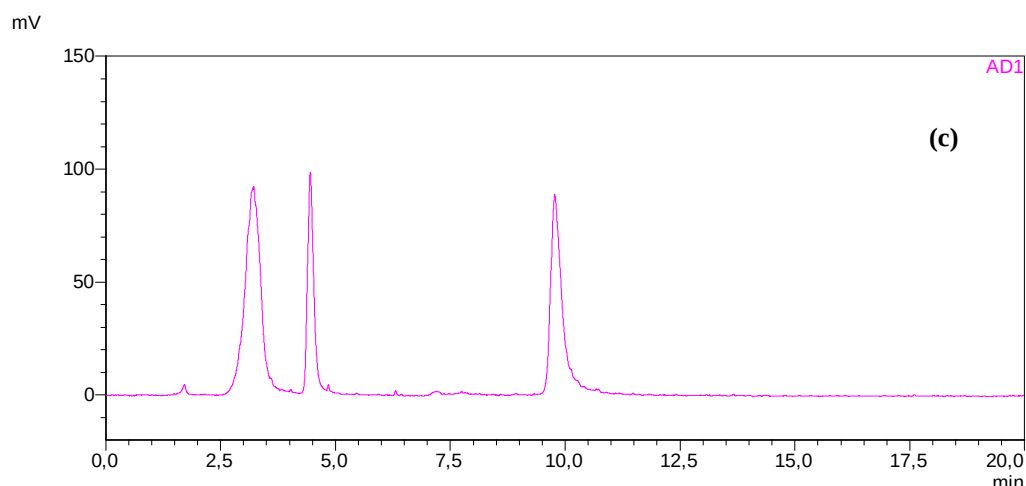
5.11.3 Optimization of ELSD temperature

An important parameter for ELSD is the nebulizer temperature, which is one of the most critical factors affecting the analysis. A series of experiments were conducted to assess the influence of ELSD temperature (30–60 °C) on the chromatographic separation of LSDH and its impurities. Increasing the detector temperature led to improved peak definition and resolution, with notable enhancement observed for Impurity D. Based on these data, the nebulizer temperature was set to 60 °C. The detector signals corresponding to the increase in temperature are presented in Figure 5.18.

Figure 50.18

Comparative Chromatograms Showing the effect of ELSD Temperature Adjustments, (a) 40 °C, (b) 50 °C, and (c) 60 °C, on the Separation Efficiency of LSDH and its Related Impurities





5.11.4 Optimization of ELSD gain and filter

The influence of gain and filter parameter variations on ELSD performance was systematically investigated. These parameters directly affect the symmetry, sharpness, and area of the chromatograms. The analysis results indicated that the optimal gain value is 7, while the optimal filter value is 2.

5.11.5 Optimization of ELSD gas pressure

The effects of nitrogen gas pressure on the peak area and morphology for the determination of LSDH and its impurities (A and D) were also investigated. Nitrogen gas pressure was adjusted to 200, 250, 300, 350, and 400 kPa, respectively, and applied to the HPLC-ELSD system. Based on the results, 350 kPa was determined as the optimal pressure.

5.11.6 Effects of additional experimental parameters on analytical outcomes

The impact of supplementary chromatographic parameters was evaluated by varying flow rate and column temperature. Flow rates of 1.0, 1.1, and 1.2 mL/min were tested, with 1.0 mL/min providing optimal peak symmetry and a balanced analysis time. To ensure consistent selectivity, column oven temperatures of 35 °C, 40 °C, 45 °C, and 50 °C were evaluated, with a constant temperature of 60 °C ultimately chosen for its ability to produce sharper peaks, particularly for Impurity D. The results, including chromatographic profiles and quantitative data, are presented in Figure 5.19. The optimized conditions produced symmetrical and well-resolved peaks within a suitable analysis window, as detailed in Table 5.16. Representative chromatograms for LSDH and its related impurities (A and D) under these conditions are shown in Figure 5.20.

Figure 05.19

Separation Profiles of LSDH and its Impurities Obtained Under Column Temperature Ranges of (a) 35–40 °C, (b) 40–50 °C, and (c) 45–50 °C

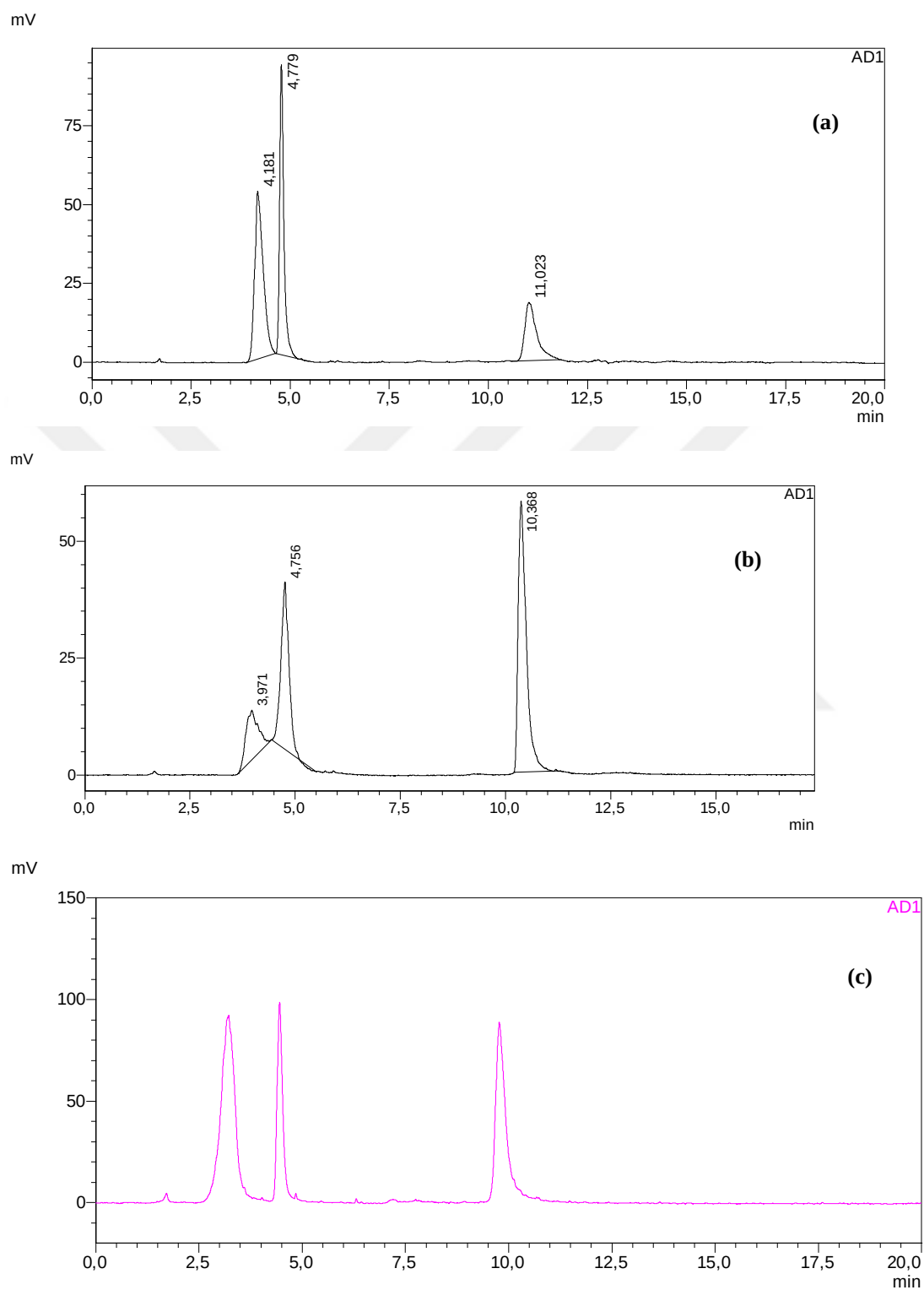


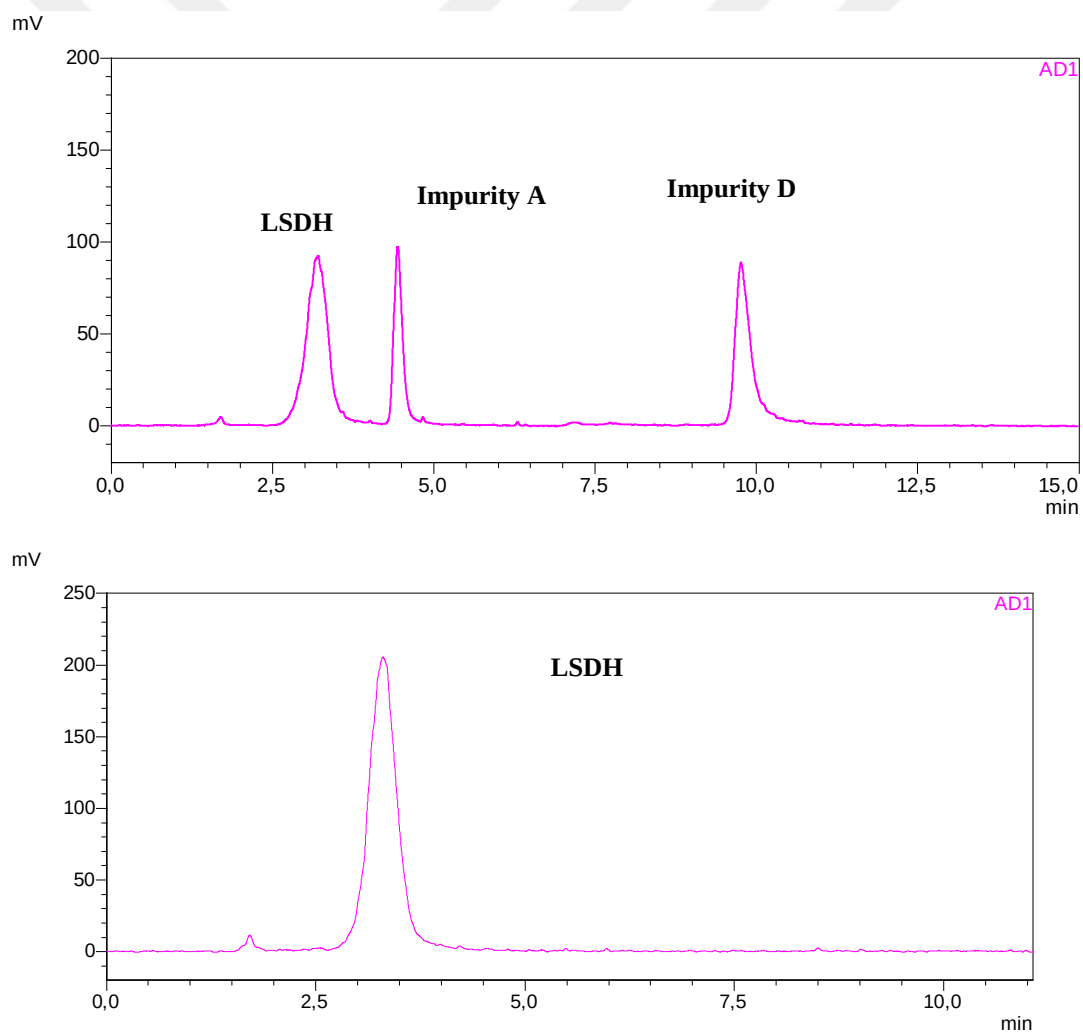
Table 5.16

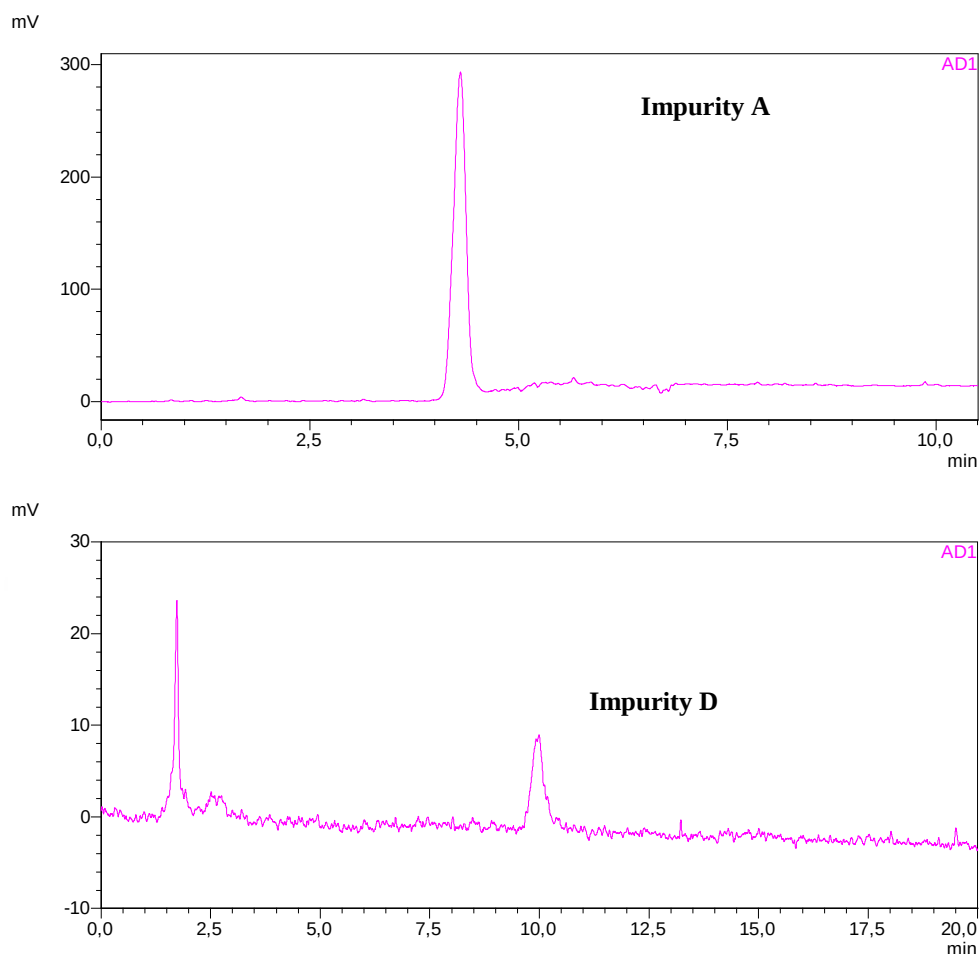
Finalized Chromatographic Conditions for the Quantitative Analysis of LSDH and Associated Impurities A and D)

Instrument	Shimadzu, Nexera – i (LC-2040C 3D)
Column	SVEA gold C18 250 mm, 4.6 mm, 5 µm
Mobile phase	Water: MeOH (60:40) v/v/
Detector	ELSD
ELSD Temperature	60 °C
ELSD pressure	350 kPa
Gain	7
Filter	2
Column temperature	(45- 50) °C
Flow rate	1.0 mL min ⁻¹
Injection volume	10 µL

Figure 05.20

Typical Chromatograms were Obtained at Optimum Conditions for the Mix Standards, LSDH, Impurity A and Impurity D





5.11.7 SST

The system suitability test (SST) was conducted to validate the reliability and consistency of the chromatographic system used for LSDH and its impurities (A and D). As shown in Table 5.17, effective separation was achieved under optimized conditions. The method's suitability for quality control applications was confirmed by evaluating key SST parameters by ICH guidelines.

Table 5.17
Summary of System Suitability Parameters Evaluated for LSDH and Associated Impurities A and D

Parameters	LSDH	Impurity A	Impurity D	Recommended value
t_R (min)	3.21	4.45	9.77	-
Capacity factor (K')	0.87	1.59	4.70	1 < K' < 10
Tailing Factor (T)	0.95	1.32	1.83	< 2
Symmetry factor (A_s)	0.95	0.99	1.11	0.95 < A _s < 1.2
Selectivity Factor (α)	1.82	2.94	-	> 1
Resolution (R_s)	2.39	2.03	9.83	> 2
Theoretical Plates (N)	3801	3803	3848	> 2000

5.11.8 Validation of HPLC-ELSD method

The validation parameters for both LSDH and its impurities, A and D, were assessed, and statistical analysis was conducted using Microsoft Excel. In each analytical computation, logarithmic transformations were applied to both the peak area ($\log_{10}A$) and analyte concentration ($\log_{10}m$), facilitating linearization and improved regression modeling over direct use of untransformed data.

5.11.8.1 Linearity assessment

The linearity of the proposed method was evaluated for LSDH and Impurity A across concentrations from 9.00 to 800 $\mu\text{g mL}^{-1}$, while Impurity D was tested within a range of 9.00 to 600 $\mu\text{g mL}^{-1}$. For the calibration curve, LSDH and its impurities (A and D) were assessed in the same concentration range, but LSDH was specifically examined from 60 to 800 $\mu\text{g mL}^{-1}$. This selection aimed to achieve clear peak shapes and avoid overloading the column.

Intra-day and inter-day linearity were assessed, and corresponding regression parameters, slope, intercept, and r^2 , were derived using Microsoft Excel. In the ELSD system, the amount of analyte injected into the column does not conform to the Beer–Lambert law; consequently, the calibration curves did not display linearity. The resulting curves for LSDH, Impurity A, and Impurity D are shown in Figures 5.21–5.23.

Figure 5.21
Calibration Curve of LSDH (Concentration-Peak Area)

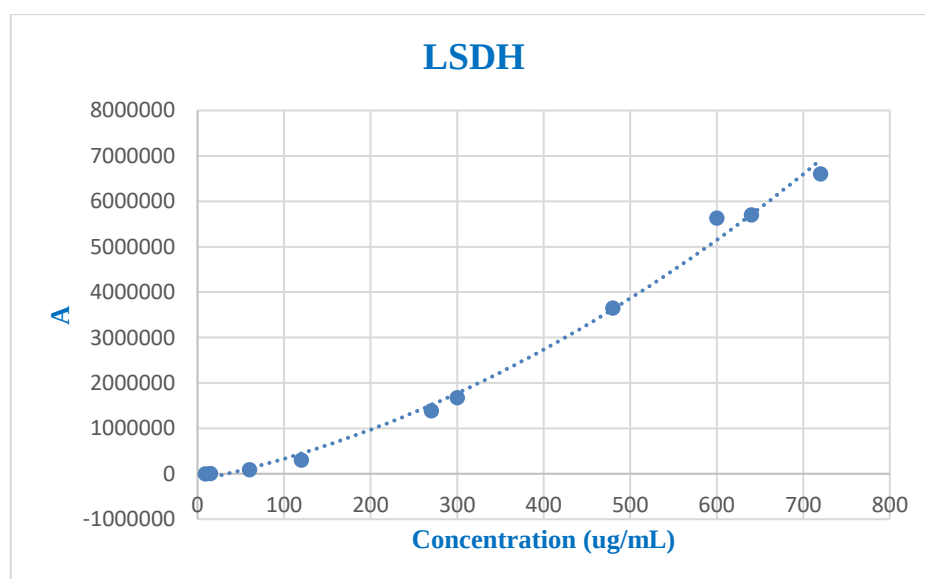


Figure 5.22
Calibration Curve of Impurity A (Concentration-Peak Area)

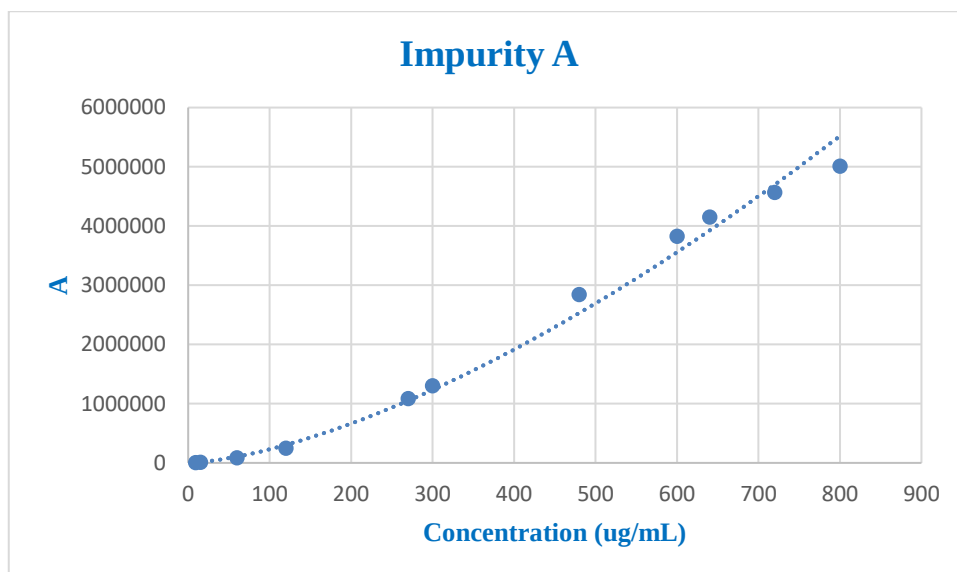
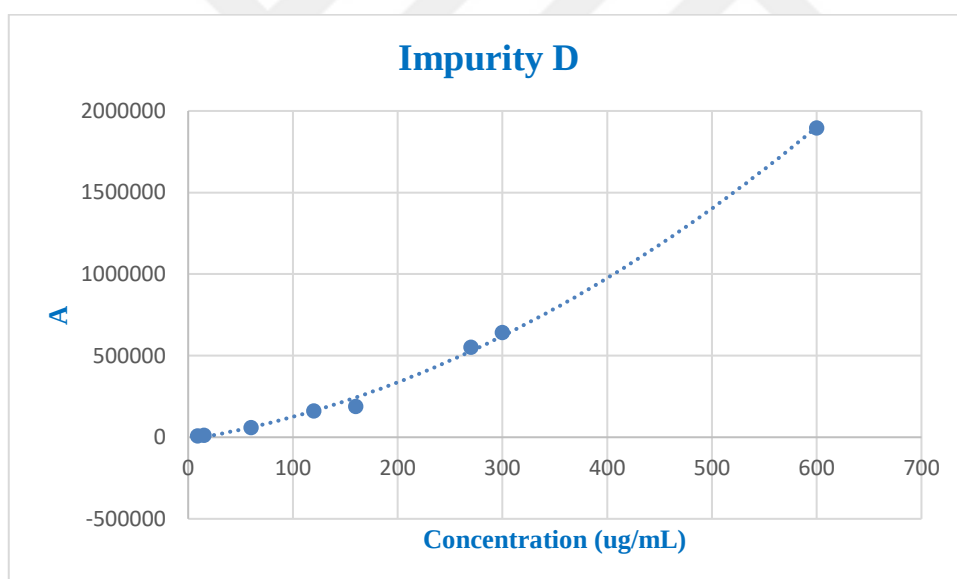


Figure 5.23
Calibration Curve of Impurity D (Concentration-Peak Area)



Logarithmic values of peak area and concentration ($\log_{10}A$ and $\log_{10}m$) were utilized in all calculations to enhance calibration curve linearity. Table 5.18 provides intra-day and inter-day statistical data, and Figures 5.24–5.26 display the calibration plots for LSDH, Impurity A, and Impurity D.

Table 5.18*The Results of the Linearity Analysis of LSDH, Impurity A and Impurity D*

	Inter-days (n=9)		
	LSDH	Impurity A	Impurity D
Range ($\mu\text{g mL}^{-1}$)	9.0-800	9.0-800	9.0-600
Slope $\pm$ SD	1.7272 ± 0.0059	1.5275 ± 0.0029	1.33 ± 0.00092
Intercept $\pm$ SD	1.9076 ± 0.0148	2.3075 ± 0.00407	2.5049 ± 0.0017
r^2	0.9984	0.9982	0.9944
LOD ($\mu\text{g mL}^{-1}$)	0.075	0.10	0.19
LOQ ($\mu\text{g mL}^{-1}$)	0.23	0.31	0.57

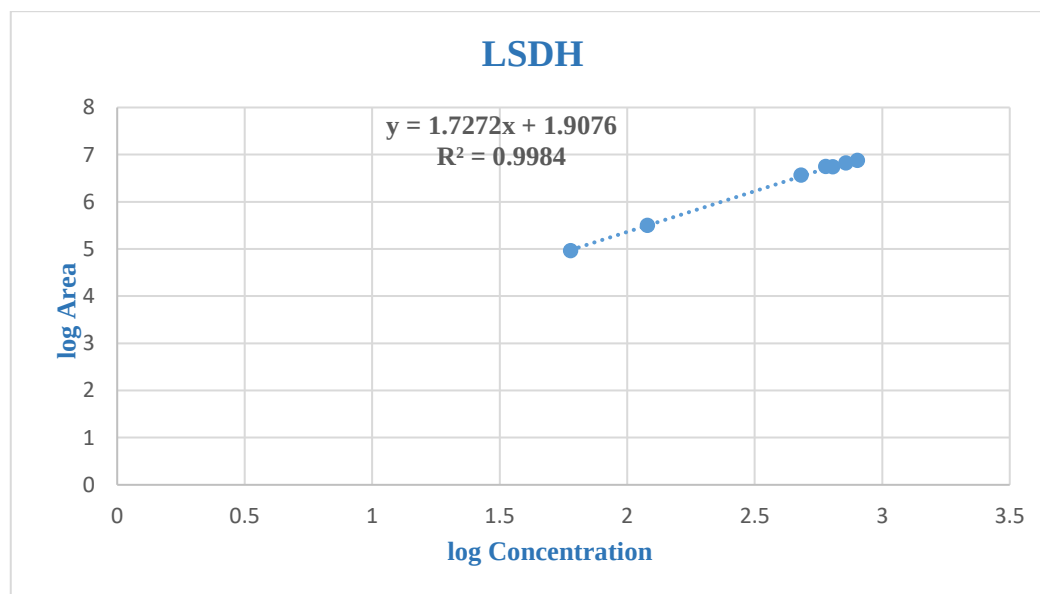
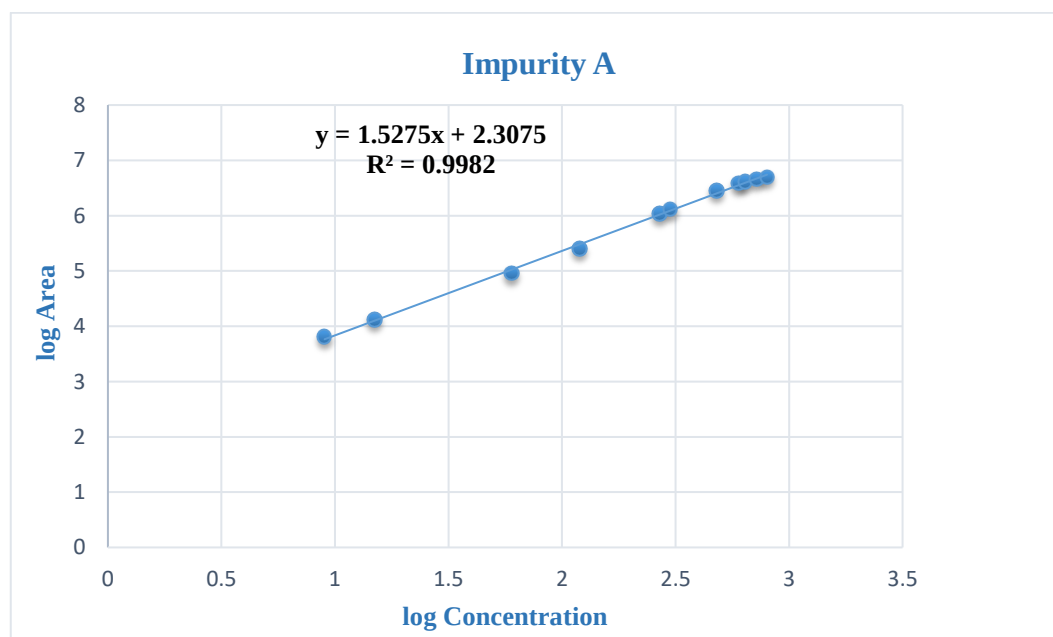
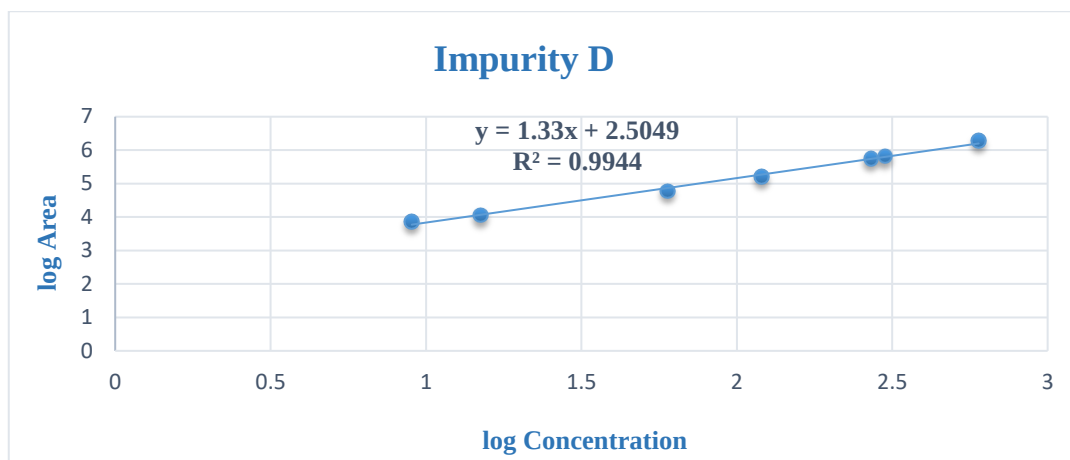
Figure 05.24*Calibration Curve of LSDH***Figure 05.25***Calibration Curve of Impurity A*

Figure 5.26
Calibration Curve of Impurity D



5.11.8.2 Accuracy

Recovery experiments were conducted to verify method accuracy across a range of concentrations: LSDH (170, 390, 610 $\mu\text{g mL}^{-1}$), Impurity A (110, 290, 530 $\mu\text{g mL}^{-1}$), and Impurity D (90, 270, 510 $\mu\text{g mL}^{-1}$). Analytical measurements were performed using standard solutions, and recovery values were computed to confirm the method's precision and reliability. The analyses were repeated three times over three days. The accuracy values were derived from the calibration curve equations. In Table 5.19, you can find the % Recovery, SD, and RSD% for LSDH, Impurity A, and Impurity D.

The recovery results showed that the mean values were 99.71% for LSDH, 101.08% for Impurity A, and 99.91% for Impurity D. The results confirm that the method is accurate and reliable for quantifying LSDH and its impurities across various concentration levels. Recovery experiments provided detailed insights into performance consistency, supporting its application in pharmaceutical quality control.

Table 5.19
Summary of Accuracy Data for LSDH, Impurity A, and Impurity D in Standard Solutions Using HPLC-ELSD

Compound	Concentration ($\mu\text{g mL}^{-1}$)	Average Log Area	Average Recovery% (n=3)	Average Recovery% (n=9)	Average SD	Average RSD%
LSDH	170	5.31	98.98	99.70	0.0014	0.0098
	390	6.147	99.47		0.00014	0.0023
	610	6.60	100.66		0.00026	0.0040
Impurity A	110	5.44	102.92	101.09	0.0016	0.0302
	290	6.06	99.817		0.0036	0.060
	530	6.47	100.50		0.0024	0.038
Impurity D	90	5.10	100.31	99.90	0.00087	0.017
	270	5.73	98.65		0.00079	0.013
	510	6.11	100.74		0.00081	0.013

5.11.8.3 Precision study

Repeatability and intermediate precision were examined to ensure the method's reproducibility. Intra-day precision was determined by repeated measurements within the same day, whereas inter-day precision involved analyses conducted over several days. The assessment was conducted using three distinct concentration levels: low, moderate, and high, derived from the calibration curves of LSDH, Impurity A, and Impurity D. The tested concentrations were as follows:

- LSDH: 180, 420, and 700 $\mu\text{g mL}^{-1}$
- Impurity A: 130, 310, and 630 $\mu\text{g mL}^{-1}$
- Impurity D: 100, 300, and 590 $\mu\text{g mL}^{-1}$

To assess precision, standard statistical methods were used, including calculating the SD and RSD%. RSD% values reported in Table 5.20 were consistently below 2.0, indicating high precision and confirming the method's reliability for the determination of LSDH and its impurities.

Table 5.20

Intra- and Inter-Day Precision Outcomes for LSDH and Its Impurities (A and D) Determined by HPLC-ELSD

Compound	Concentration ($\mu\text{g mL}^{-1}$)	Statistical Parameter	Intra-day			Inter-day (n=9)
			Day 1 (n=3)	Day 2 (n=3)	Day 3 (n=3)	
LSDH	180	Average Log Area	5.357	5.370	5.391	5.373
		SD	0.00229	0.00982	0.001006	0.017
		RSD%	0.0429	0.183	0.0187	0.319
	420	Average Log Area	6.2239	6.224	6.227	6.2249
		SD	0.00694	0.0030	0.0057	0.00176
		RSD%	0.11	0.049	0.0927	0.0282
	700	Average Log Area	6.750	6.746	6.742	6.746
		SD	0.00590	0.000535	0.000971	0.004
		RSD%	0.08750	0.00793	0.01440	0.0592
	Concentration ($\mu\text{g mL}^{-1}$)	Statistical Parameter	Intra-day			Inter-day (n=9)
			Day 1 (n=3)	Day 2 (n=3)	Day 3 (n=3)	
Impurity A	130	Average Log Area	5.5236	5.5394	5.5390	5.534
		SD	0.0068	0.00128	0.00332	0.00735
		RSD%	0.1247	0.0231	0.0601	0.13291
	310	Average Log Area	6.1147	6.1037	6.1053	6.1079
		SD	0.0141	0.00243	0.0001247	0.00485
		RSD%	0.2308	0.0399	0.00204	0.0794
	630	Average Log Area	6.5832	6.5888	6.5948	6.5889
		SD	0.0071	0.00504	0.00225	0.00473
		RSD%	0.1086	0.0765	0.0341	0.0718

Table 5.20 (continued)

Impurity D	Concentration ($\mu\text{g mL}^{-1}$)	Statistical Parameter	Intra-day			Inter-day (n=9)
			Day 1 (n=3)	Day 2 (n=3)	Day 3 (n=3)	
Impurity D	100	Average Log				
		Area	5.1623	5.1650	5.16716	5.1648
		SD	0.00463	0.00258	0.00116	0.00198
		RSD%	0.0897	0.0499	0.0224	0.0384
		Average Log				
		Area	5.8067	5.7996	5.7486	5.7849
	300	SD	0.00963	0.00078	0.0494	0.0258
		RSD%	0.1658	0.01348	0.8604	0.4473
		Average Log				
	590	Area	6.2776	6.2821	6.2899	6.2832
		SD	0.00691	0.00116	0.00180	0.00508
		RSD%	0.11017	0.0185	0.0286	0.08087

5.11.8.4 Specificity and selectivity

As in previously established methods, a selectivity factor (α) greater than 1 was taken as a reference value. As shown in Table 5.17, the results obtained from the SUT evaluation are consistent with the reference value.

5.11.8.5 Detection and quantitation limits (LOD and LOQ)

LOD and LOQ values were derived for LSDH and its impurities (A and D) using the formulas $3.3 \times (\text{SD}/m)$ and $10 \times (\text{SD}/m)$, respectively. These calculations, based on the standard deviation of the lowest concentration and the calibration curve slope, confirmed the method's sensitivity and suitability for low-level quantification.

To assess the sensitivity of the newly created analytical method for identifying impurities, we carried out a linearity analysis alongside the calculated LOD and LOQ. This analysis was performed over a retention time of 15 minutes. According to the data in Table 5.18, the LOD and LOQ results were $0.075 \mu\text{g mL}^{-1}$ and $0.23 \mu\text{g mL}^{-1}$ for LSDH, $0.10 \mu\text{g mL}^{-1}$ and $0.31 \mu\text{g mL}^{-1}$ for Impurity A, and $0.19 \mu\text{g mL}^{-1}$ and $0.57 \mu\text{g mL}^{-1}$ for Impurity D. These findings show that the analytical method is both sensitive and dependable for detecting and quantifying LSDH and its impurities, even at low concentration levels.

5.11.8.6 Robustness evaluation

A systematic robustness assessment was conducted on the HPLC-ELSD technique designed for the analysis of LSDH and its related impurities (A and D) were tested under intentional variations of critical analytical conditions. Specifically, the influence of ELSD detector temperature, column temperature, and mobile phase composition was systematically investigated, employing a strategy consistent with that used for the HPLC-

DAD method. Triplicate analyses were conducted for LSDH (250 $\mu\text{g mL}^{-1}$), Impurity A (240 $\mu\text{g mL}^{-1}$), and Impurity D (200 $\mu\text{g mL}^{-1}$) to determine the method's resilience. As shown in Table 5.21, all RSD% values were below 2.0%, confirming the method's robustness, stability, and suitability for routine application under minor chromatographic variations.

Table 5.21
Results of Robustness Study

Compound	Parameter	Condition	Mean	SD	% RSD
LSDH	Column temperature	42 °C	1132119	1.52	0.00013
		48 °C	1130724	31.72	0.0028
	ELSD Temperature	50 °C	1133112	4.04	0.00035
		55 °C	1133293	11.53	0.0010
	MeOH %	38 %	1129836	72.85	0.0064
		42 %	1132492	17.009	0.0015
Impurity A	Column Temperature	42 °C	606335	250.29	0.041
		48 °C	608312	30.44	0.0050
	ELSD Temperature	50 °C	625641	252.80	0.040
		55 °C	611464	81.07	0.013
	MeOH %	38 %	608654	172.53	0.02
		42 %	615580	254.24	0.041
Impurity D	Column Temperature	42 °C	72083	10.59	0.014
		48 °C	74644	154.79	0.20
	ELSD Temperature	50 °C	78132	100.87	0.129
		55 °C	81303	267.87	0.32
	MeOH %	38 %	75173	140.82	0.18
		42 %	73205	255.91	0.34

5.11.8.7 Stability assessment

The stability of the standard solutions for LSDH, Impurity A, and Impurity D was assessed by monitoring their chemical integrity over time under defined storage conditions. Storage conditions included +4 °C for one month and ambient temperature for 24 hours. Analytical testing was performed thereafter to detect any notable deviations. Triplicate injections were performed for LSDH 248 ($\mu\text{g mL}^{-1}$), Impurity A (240 $\mu\text{g mL}^{-1}$), and Impurity D (239 $\mu\text{g mL}^{-1}$). The corresponding results are presented in Table 5.22, demonstrating no substantial changes in the working standard solutions throughout the storage duration, confirming their stability and suitability for analytical applications under these conditions.

Table 5.22
Results of Stability Study

Compound	Parameter	Average Area	SD	RSD %
LSDH	First Day	792782	14.18	0.0017
	Short Term Stability	801258	17.89	0.0022
	Freeze and Thaw			
	Stability	784262	33.77	0.0043
	Long Term Stability	810269	21.36	0.0026
Impurity A	First Day	534064	21.57	0.0040
	Short Term Stability	570185	9.50	0.0016
	Freeze and Thaw			
	Stability	596344	29.70	0.0049
	Long Term Stability	570452	23.89	0.0041
Impurity D	First Day	497336	10.59	0.0021
	Short Term Stability	503172	25.63	0.0051
	Freeze and Thaw			
	Stability	515242	27.15	0.0052
	Long Term Stability	480604	15.71	0.0032

5.11.9 Application of LSDH in pharmaceutical tablet forms

The developed method was evaluated for its capability to quantify LSDH and its associated impurities in tablet formulations. Tables 5.23–5.27 provide a summary of the related test results, and Figure 5.24 displays representative chromatograms of the tablet samples.

When the three LSDH tablet formulations are compared, it can be seen that the 20/12.5 mg and 20/25 mg versions have slightly better precision, as evidenced by their lower SD and RSD% values. Since all three formulations met the accuracy and content uniformity requirements for LSDH and its impurities in tablet form, the method demonstrated adequate robustness and reliability.

In summary, the analytical process exhibits good precision, accuracy, and consistency across different concentration levels and LSDH tablet formulations. While the results on content uniformity demonstrate that the medication is dispersed uniformly throughout the tablets, low RSD values indicate high repeatability.

Table 5.23
Assay Results for LSDH 5 mg Film Tablet (n=3)

Parameter	50 ug mL ⁻¹	150 ug mL ⁻¹	250 ug mL ⁻¹
Average (ug mL ⁻¹)	48.59	147.16	250.4
Minimum (ug mL ⁻¹)	48.24	147.08	250.02
Maximum (ug mL ⁻¹)	48.94	147.25	250.78
SD	0.49	0.12	0.53
RSD %	1.01	0.081	0.21
SEM (ug mL ⁻¹)	0.28	0.069	0.31
Bias%	-2.82	-1.89	0.16
% Content Uniformity	97.18	98.11	100.16

Table 5.24*Assay Results for LSDH 20/12.5 mg Film Tablet (n=3)*

Parameter	200 ug mL ⁻¹	600 ug mL ⁻¹	1000 ug mL ⁻¹
Average(ug mL ⁻¹)	201.635	601.8	1001.07
Minimum (ug mL ⁻¹)	201.52	601.64	1000.91
Maximum (ug mL ⁻¹)	201.75	601.96	1001.23
SD	0.06	0.092	0.092
RSD %	0.032	0.015	0.0092
SEM (ug mL ⁻¹)	0.038	0.053	0.053
Bias%	0.82	0.3	0.11
% Content Uniformity	100.82	100.3	100.11

Table 5.25*Assay Results for LSDH 20/25 mg Film Tablet (n=3)*

Parameter	200 ug mL ⁻¹	600 ug mL ⁻¹	1000 ug mL ⁻¹
Average (ug mL ⁻¹)	187.51	587.51	994.90
Minimum (ug mL ⁻¹)	185.78	583.57	992.57
Maximum (ug mL ⁻¹)	189.24	591.46	997.24
SD	0.99	2.27	1.34
RSD %	0.53	0.38	0.13
SEM (ug mL ⁻¹)	0.57	1.31	0.77
Bias%	-6.25	-2.08	-0.51
% Content Uniformity	93.76	97.92	99.49

The developed technique for identifying Impurities A and D in formulations of spiked LSDH tablets satisfies established requirements for precision and accuracy. All of the tested formulations' recovery percentages stayed within reasonable bounds, demonstrating the method's ability to precisely and minimally deviate from the measured impurity levels. Its consistency and dependability are further demonstrated by the low RSD values. Impurity D in the 20/12.5 mg spiked tablet showed some variability, but this did not affect the method's overall suitability. These findings validate its suitability for regular LSDH tablet quality control, guaranteeing thorough tracking and measurement of important contaminants in pharmaceutical goods.

Table 5.26*Assay Results for Impurity A in Spiked Tablet Samples (n = 3)*

Tablet	5 mg	20/12.5mg	20/25 mg
Amount Added (ug mL ⁻¹)	100	100	100
Amount Found (ug mL ⁻¹)	100.71	100.63	100.49
SD	0.23	0.21	0.24
RSD %	0.232	0.21	0.24
% Recovery	100.71	100.63	100.49

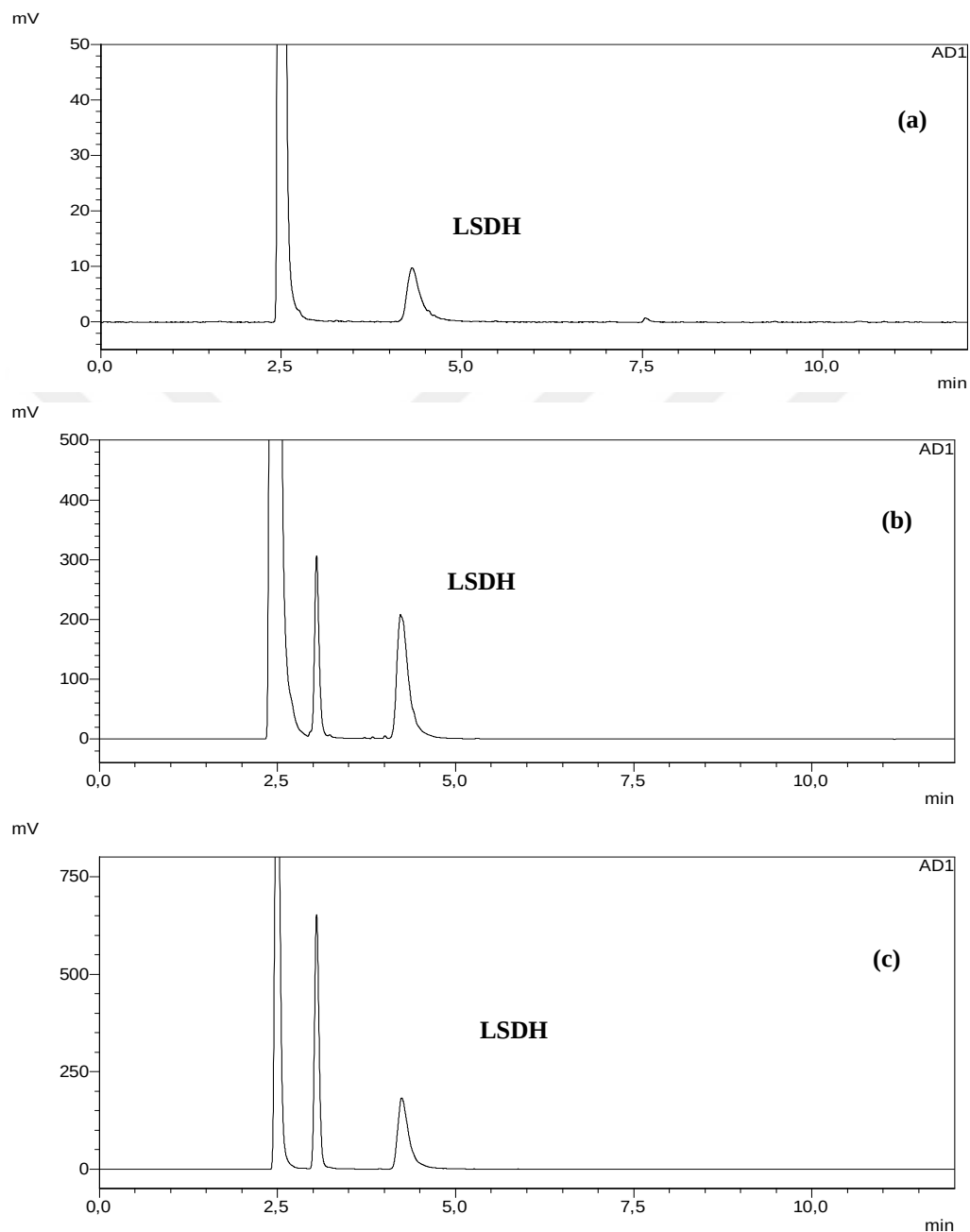
Table 5.27*Assay Results for Impurity D in Spiked Tablet Samples (n = 3)*

Tablet	5 mg	20/25 mg	20/25 mg
Amount Added (ug mL ⁻¹)	20	20	20
Amount Found (ug mL ⁻¹)	20.19	20.61	20.34
SD	0.14	0.38	0.180

RSD %	0.70	1.87	0.88
% Recovery	100.96	103.09	101.71

Figure 5.27

Chromatograms of (a) 5 mg Tablet, (b) 20/12.5 mg Tablet and (c) 20/25 mg Tablet



5.11.10 Overall analytical assessment of the HPLC-ELSD technique

A sensitive method using HPLC-ELSD was created and validated to detect LSDH, Impurity A, and Impurity D in various pharmaceutical products. To achieve effective

separation, a Gold SVEA C18 column was selected and optimized, resulting in improved peak symmetry, good resolution, and shorter analysis times.

Since LSDH and its impurities (A and D) are basic compounds, an effective quantification was accomplished with a mobile phase mixture of MeOH and Water (40:60 v/v). This newly developed method adhered to all recommended acceptance criteria for system suitability testing (SST). The method was validated to be robust and precise, with a notably low LOD, indicating improved performance over the USP standard. Its development facilitated the attainment of two key thesis objectives, resulting in a highly satisfactory outcome.

5.12 Analytical Comparison of Photodiode Array and Evaporative Light Scattering Detection in HPLC-Based Quantification

The developed methods were subjected to one-way ANOVA to compare their performance across three dosage forms. No significant differences were observed, suggesting comparable effectiveness in LSDH quantification. Thus, either technique can be successfully used for LSDH analysis. The statistical results are given in Tables 5.28-5.30.

Table 5.28

5 mg Film Tablet Assay Results by Using HPLC-DAD and HPLC-ELSD (n=3)

Parameters	HPLC-DAD	HPLC-ELSD
Mean ($\mu\text{g mL}^{-1}$)	249.77	250.4
RSD %	0.06	0.21
Bias%	-0.092	0.16
ANOVA (p-value)	> 0.05	> 0.05

Table 5.29

20/12.5 mg Film Tablet Assay Results by Using HPLC-DAD and HPLC-ELSD (n=3)

Parameters	HPLC-DAD	HPLC-ELSD
Mean ($\mu\text{g mL}^{-1}$)	998.18	1001.07
RSD %	0.06	0.009
Bias%	-0.18	0.11
ANOVA (p-value)	> 0.05	> 0.05

Table 5.30

20/25 mg Film Tablet Assay Results by Using HPLC-DAD and HPLC-ELSD (n=3)

Parameters	HPLC-DAD	HPLC-ELSD
Mean ($\mu\text{g mL}^{-1}$)	912.86	994.90
RSD %	0.72	0.13
Bias%	-10.05	-0.51
ANOVA (p-value)	> 0.05	> 0.05

The effectiveness of the two techniques for analyzing LSDH and its impurities (A and D) was evaluated through a comparative analysis of verification parameters. Assessing each method's effectiveness, sensitivity, precision, and dependability in precisely identifying and measuring the analytes was the aim.

The most crucial analysis parameters, such as accuracy, precision, linearity, robustness, and stability, are shown in comparison in Tables 5.31–5.33. Specifically, Table 5.31 offers comprehensive verification data for the standard LSDH solution, demonstrating dependability and consistency across various methodologies.

Table 5.31

Comparative Validation Data for LSDH Using Developed Analytical Methods

Parameters	HPLC-DAD	HPLC-ELSD
Time of Analysis (min)	20	15
Study Range ($\mu\text{g mL}^{-1}$)	2-400	9.0-800
r^2	0.9999	0.9984
LOD ($\mu\text{g mL}^{-1}$)	0.166	0.075
LOQ ($\mu\text{g mL}^{-1}$)	0.50	0.23
Inter-day Precision (RDS%) at Low Concentration	0.0310	0.319
Selectivity	Selective	Selective

Table 5.32

Validation Data Comparison for the Impurity A Standard Solution Using Developed Methods

Parameters	HPLC-DAD	HPLC-ELSD
Time of Analysis (min)	20	15
Study Range ($\mu\text{g mL}^{-1}$)	2-400	9.0-800
r^2	0.9993	0.9982
LOD ($\mu\text{g mL}^{-1}$)	0.282	0.10
LOQ ($\mu\text{g mL}^{-1}$)	0.856	0.31
Inter-day Precision (RDS%) at Low Concentration	0.88	0.13
Selectivity	Selective	Selective

Table 5.33

Validation Data Comparison for the Impurity D Standard Solution Using Developed Methods

Parameters	HPLC-DAD	HPLC-ELSD
Time of Analysis (min)	20	15
Study Range ($\mu\text{g mL}^{-1}$)	2-400	9.0-600
r^2	0.9995	0.9944
LOD ($\mu\text{g mL}^{-1}$)	0.283	0.186
LOQ ($\mu\text{g mL}^{-1}$)	0.857	0.565
Inter-day Precision (RDS%) at Low Concentration	0.18	0.038
Selectivity	Selective	Selective

Both HPLC-DAD and HPLC-ELSD are performing well for assay determination, with their respective advantages in various situations, according to the validation results of LSDH, Impurity A, and Impurity D. Naturally, the HPLC-ELSD conducted analyses more quickly (15 minutes as opposed to 20 minutes) and produced better LOD and LOQ for every compound, suggesting that it was more sensitive than the HPLC-DAD. Both

methods generally indicated excellent linearity ($r^2 > 0.994$), although the HPLC-DAD usually showed a slightly better correlation coefficient. On relative precision, considering the inter-day RSD% in low concentrations, both methods demonstrated good precision; however, HPLC-ELSD presented better precision, mainly for impurities. Both methods also demonstrated good selectivity. These results suggest that while HPLC-DAD is dependable and highly linear, HPLC-ELSD offers advantages in speed, sensitivity, and precision, making it especially useful for detecting trace impurities.

The findings indicate that both HPLC-DAD and HPLC-ELSD methods present high precision and accuracy in the quantification of LSDH and its impurities. The decision on which method to employ relies on the specific analytical objectives, including sensitivity requirements, matrix composition, and the instruments available within the laboratories.

6 CONCLUSION AND RECOMMENDATION

Pharmaceutical applications facilitate the enhancement of public health by reducing the spread of diseases and their associated sequelae. To obtain the desired therapeutic effect, the pharmaceutical must exert its pharmacological action with devoid of any toxic substances or harmful impurities unsafe to human health. As a result, pharmaceutical analysis is an ever-advancing field, focused on the development and refinement of analytical methodologies tailored to meet evolving laboratory and regulatory demands. The principal aim of this thesis is to generate detailed and reliable analytical data concerning LSDH, impurity A and impurity D. Numerous analytical techniques have been documented in the literature for measuring lisinopril (LSDH) in drug formulations, mainly using HPLC and other chromatography methods. Nevertheless, up until now, there have been no studies detailing the separation and measurement of LSDH, impurity A, and impurity D through the HPLC-ELSD technique.

The primary objective of this thesis is to establish and validate robust analytical procedures utilizing HPLC-DAD and HPLC-ELSD techniques for the simultaneous determination of LSDH and its related impurities (A and D) in both reference standards and commercial tablet formulations. Due to limitations in the availability of certified reference standards, the analysis was restricted to a subset of known impurities. A significant analytical challenge arises from the fact that many impurities found in pharmaceutical formulations are degradation products of the active pharmaceutical ingredient. These impurities often possess structural similarities to the parent compound, complicating their isolation, identification, and chromatographic separation. As a result, method development for such closely related compounds is particularly demanding. In this study, considerable effort was directed toward optimizing chromatographic parameters to achieve efficient resolution and accurate quantification of LSDH, impurity A and impurity D.

In this study, an isocratic elution system was developed for two analytical methods. The HPLC-DAD technique used a mobile phase made of a 23 mM NaH_2PO_4 solution mixed with methanol, while the HPLC-ELSD method employed a blend of water and methanol for its mobile phase. In both methods, a SVEA Gold 5 μm C18 column was chosen as the stationary phase. The DAD detector functioned at a wavelength of 210 nm. The temperature of the column during HPLC-DAD varied from 35 to 40 °C, while for HPLC-

ELSD, it was adjusted between 45 and 50 °C. The temperature for the ELSD detector was kept at 60 °C. A volume of 10.0 µL was injected for each solution. To find the amounts of the separated analytes, calibration curves were established using pure standards, allowing for the quantification of each analyte. The results are reported as recovery percentages.

The methods that were developed have been checked for accuracy, precision, selectivity, sensitivity, and linearity, with the outcomes displayed in tables. These findings indicated that the recovery rates fell within acceptable ranges, and the precision results, highlighted by an RSD% of under 2.0, validated the analytical effectiveness of the methods. Furthermore, tests on the repeatability of injections demonstrated that the relative standard deviations for peak areas and retention times of each substance met the established acceptance criteria. Moreover, suitability tests for the proposed chromatographic methods were carried out. The findings demonstrated that all substances met the requirements for theoretical plate count, peak asymmetry, resolution, selectivity, and tailing factor. In conclusion, the developed method showed high suitability regarding precision and repeatability, as reflected in the low standard deviation values; accuracy, corroborated by satisfactory recovery rates; and sensitivity, indicated by low LOD values and steep calibration curve slopes. Furthermore, the method displayed excellent linearity, with correlation coefficients (r^2) nearing 1.0, confirming its reliability for standard analytical processes.

In conclusion, the developed analytical methods exhibit excellent specificity, linearity, accuracy, and precision. The analysis of the validation parameters shows that they are strong and suitable for assessing both the quality and quantity of LSDH, impurity A, and impurity D. One key advantage of these methods is that they can identify and measure impurities at the same time, which has not been documented before in the literature for the HPLC-ELSD method used to determine LSDH and its associated compounds. Furthermore, the methodologies are practical, reliable, and adaptable, making them highly suitable for routine impurity profiling in pharmaceutical formulations. Accordingly, these methods serve as essential tools for quality control laboratories, supporting the assurance of pharmaceutical product safety and efficacy.

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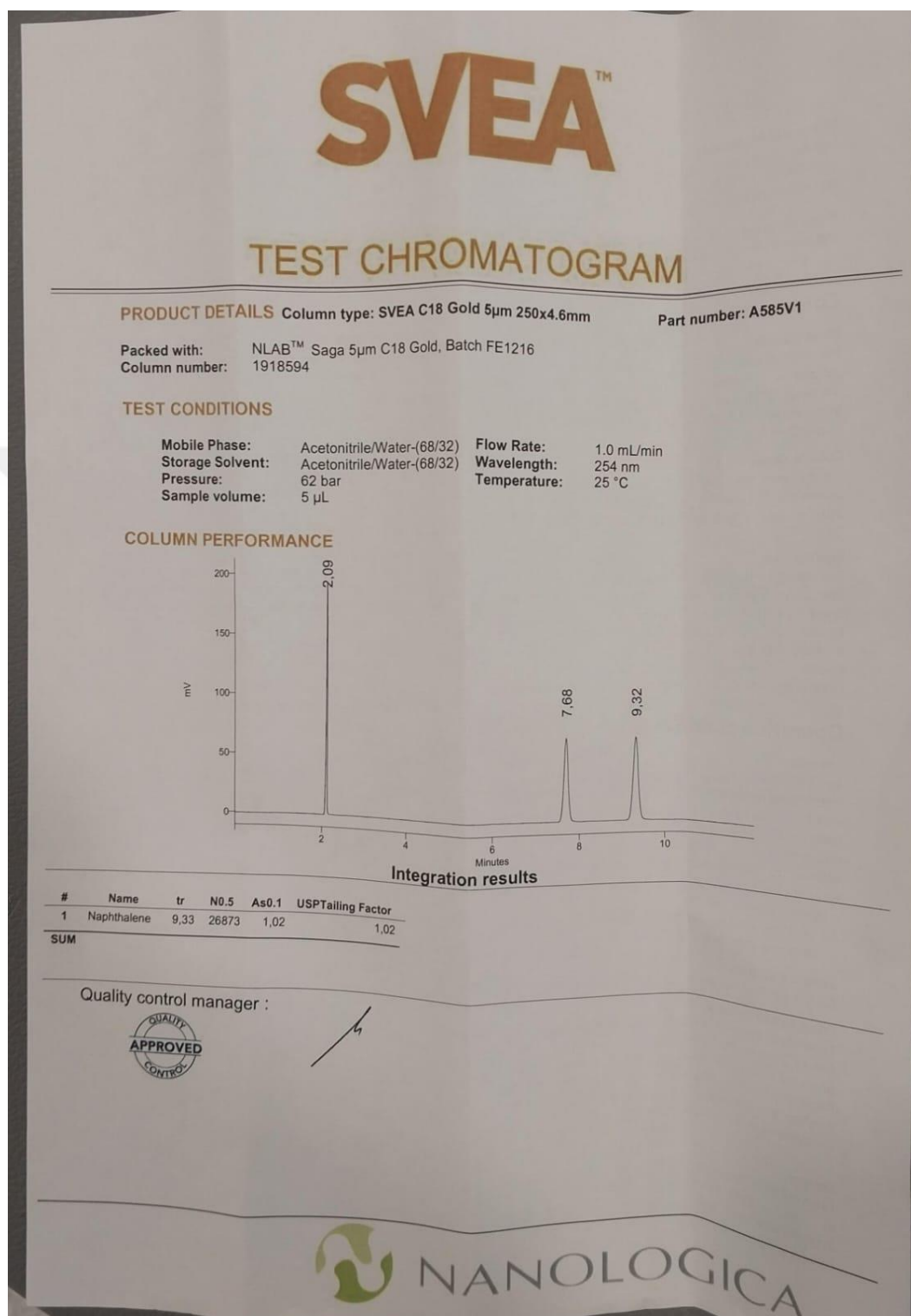
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APPENDICES



SVEATM

TEST CHROMATOGRAM

PRODUCT DETAILS Column type: SVEA C18 Gold 5µm 150x4.6mm

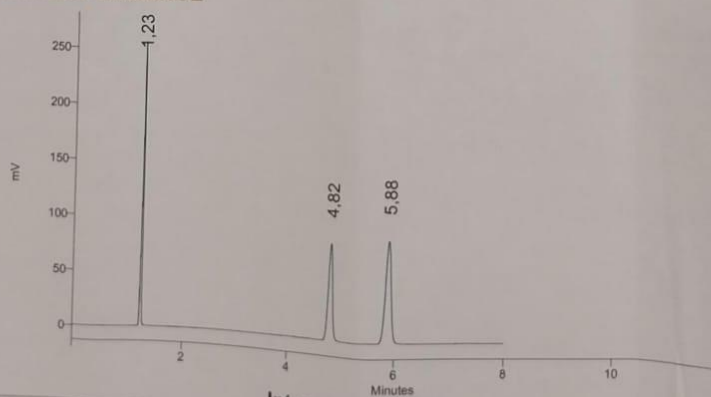
Part number: A565V1

Packed with: NLABTM Saga 5µm C18 Gold, Batch FE020
Column number: 19099261

TEST CONDITIONS

Mobile Phase:	Acetonitrile/Water-(68/32)	Flow Rate:	1.0 mL/min
Storage Solvent:	Acetonitrile/Water-(68/32)	Wavelength:	254 nm
Pressure:	50 bar	Temperature:	25 °C
Sample volume:	5 µL		

COLUMN PERFORMANCE



Integration results

#	Name	tr	N0.5	As0.1	USPTailing Factor
1	Naphthalene	5.88	16724	1.04	1.02
SUM					

Quality control manager :



NANOLOGICA



TEST CHROMATOGRAM

PRODUCT DETAILS Column type: SVEA C18 Gold 3.5µm 150x4.6mm

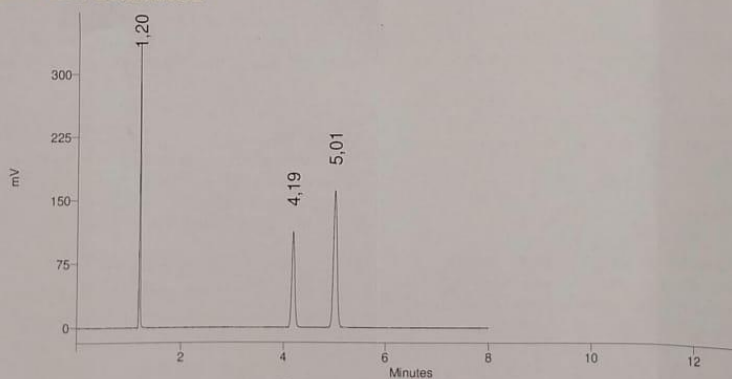
Part number: A365V1

Packed with: NLAB™ Saga 3.5µm C18 Gold, Batch FE1229
Column number: 2323015

TEST CONDITIONS

Mobile Phase:	Acetonitrile/Water-(70/30)	Flow Rate:	1.0 mL/min
Storage Solvent:	Acetonitrile/Water-(70/30)	Wavelength:	254 nm
Pressure:	112 bar	Temperature:	22 °C
Sample volume:	5 µL		

COLUMN PERFORMANCE



Integration results

#	Name	tr	N0.5	As0.1	USPTailing Factor
1	Naphthalene	5.01	24728	0.98	0.99

SUM

Quality control manager : Virginie KASZYCA
Product Manager



NANOLOGICA

CURRICULUM VITAE

HAFSAH MOHAMMED

HUSEIN QABA

● ABOUT ME

PhD Research Student

A research-oriented individual with excellent communication and analytical skills, mental strength, brainstorming, and dedication going to extra mile to complete assigned tasks using my knowledge and experience.

● EDUCATION AND TRAINING

15/10/2019 – CURRENT Eskisehir, Türkiye

PH.D Analytical Chemistry, Anadolu University

Computer Skills include:

- Ability to use spectroscopy and chromatography equipments as (HPLC-DAD, HPLC-ELSD)
- Prism statistical software, Origin Data Analysis Software
- Chemical Drawing software: Such as Chemdraw, Marvin...etc.
- Microsoft office (Word, Excel & Power point)

Website <https://www.anadolu.edu.tr/> |

Field of study Natural sciences, mathematics and statistics not further defined | **Final grade** 4/4 |

Thesis Determination of Lisinopril Dihydrate by Using Different Analytical Methods.

15/10/2017 – 30/07/2019 Eskisehir, Türkiye

MASTER Analytical Chemistry, Anadolu University

Website <https://www.anadolu.edu.tr/> | **Field of study** Analytical Chemistry | **Final grade** 3.71/4 |

Thesis Determination Of Polyphenolic Compounds in Plant Product Matrices by High-Performance Liquid Chromatographic Method.

30/09/2010 – 30/06/2014 Sana'a, Yemen

BS.C Chemistry, Sana'a University

Website <https://su.edu.ye/en/> | **Field of study** Chemistry | **Final grade** 93.78/100

● LANGUAGE SKILLS

Mother tongue(s): **ARABIC**

Other language(s):

	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken production	Spoken interaction	
TURKISH	C2	C2	C2	C2	C2
ENGLISH	C1	C1	C1	C1	C1

Levels: A1 and A2: Basic user; B1 and B2: Independent user; C1 and C2: Proficient user

● WORK EXPERIENCE

15/09/2015 – 30/06/2016 Sana'a, Yemen

CHEMISTRY TEACHER SECONDARY SCHOOL AL-FURSAN

● ORGANISATIONAL SKILLS

supervisor

Work as a supervisor in the first volunteer team to help Yemeni students applying for Turkish scholarships.

● HOBBIES AND INTERESTS

Reading Articles And Books

Writing

Traveling

Cooking

Swimming

Research interests

- Chromatography: HPLC-DAD, HPLC-ELSD, HPLC-MS/MS
- Green chemistry
- Medicinal chemistry

● CONFERENCES AND SEMINARS

19/10/2023 – 22/10/2023 Istanbul/Turkey

The 12th Aegean Analytical Chemistry Days (AACD 2023)

I have participated with two posters which are:

1. Determination of Lisinopril Dihydrate by Using Different Analytical Methods.
2. Quantification of Ellagic Acid and Rutin in Sambucol and Sambucus Black Elderberry Supplements and Certain Foods by HPLC Using A Phenyl Column"

Link <https://aacd2023.yildiz.edu.tr/>

24/10/2021 – 27/10/2021 Nur-Sultan, Kazakhstan/ Agrotechnical University

ASTANA INTERNATIONAL CONFERENCE ON SCIENTIFIC RESEARCH

Poster: THE ANTIVIRAL PROPERTIES OF THE PLANT ESSENTIAL OILS AS A POSSIBLE ADJUVANT SUPPORT THE HUMAN IMMUNE SYSTEM AGAINST RESPIRATORY INFECTIONS.

Link https://www.researchgate.net/publication/355889315_THE_ANTIVIRAL_PROPERTIES_OF_THE_PLANT_ESSENTIAL_O

● PUBLICATIONS

2022

[An overview of liquid chromatographic methods for analyzing new generation anti-epileptic drugs.](#)

Mohammed ALSaeedy, Arwa Al-Adhre, Hafsah Qaba, Abdulrahman Mashrah & Elif Mine Öncü-Kaya

Link <https://doi.org/10.1080/10826076.2022.2134146>

● PROJECTS

05/11/2021 – 05/11/2023

Determination of Lisinopril Dihydrate by Using Different Analytical Methods.

The aim of this project was to develop a cheap, simple and sensitive method for analysis of lisinopril dihydrate in pharmaceutical dosage form in Turkey and biological fluids. In the study, new analytical methods were developed using the HPLC-DAD system and HPLC-ELSD system. The results were evaluated statistically within methods and validation studies were applied.

● DIGITAL SKILLS

Microsoft Office | Microsoft Excel | Zoom | Microsoft Powerpoint | Google Drive

● HONOURS AND AWARDS

30/06/2014

The Second Position In BS.c – Sanaa University

30/07/2019

The first Position In Master – Anadolu University

30/06/2021

I got 4/4 score in Ph.D courses

● RECOMMENDATIONS

Prof. GÖKSEL ARLI Professor

Department of Analytical Chemistry, Faculty of Pharmacy, Anadolu University, Turkey