# Review Article

# A Review about Better Analytical Technique for Determining Febuxostat in the Blood Plasma for Bioavailability and Bioequivalence Studies



**Abstract**

This comprehensive review provides a pioneering analysis of the instrumental role of Liquid Chromatography with Mass Spectrometry (LC-MS) in unraveling the bioavailability and bioequivalence of Febuxostat (FBS) in human plasma. Utilizing Febuxostat d9 and d7, along with other drugs as internal standards (IS), the review steers through the intricate view of pharmaceutical research, focusing on validation methods and the challenges presented by the complex pharmacokinetics of FBS. Unprecedented in its depth, the review aims to contribute valuable insights to the field by addressing the evolving paradigms in pharmaceutical analysis. FBS, a revolutionary advancement in the treatment of hyperuricemia and gout, takes center stage, highlighting its pivotal role in contemporary therapeutic approaches. LC-MS develops as a cornerstone analytical method for studying FBS, offering unparalleled sensitivity and selectivity. The review delves into the sample preparation techniques, emphasizing the significance of protein precipitation and liquid-liquid extraction in extracting FBS from human blood plasma. LC-MS/MS, chosen for its exceptional sensitivity and specificity, becomes a focal point in FBS analysis, with IS employed to enhance accuracy. The optimization of chromatographic conditions, encompassing the careful selection of stationary and mobile phases, is highlighted as crucial for establishing a robust and reliable LC method, ensuring accuracy in pharmacokinetic studies of FBS. This concept encapsulates the essence of the review, positioning LC-MS as a superior analytical technique for the precise

determination of Febuxostat in blood plasma, especially in the context of bioavailability and bioequivalence studies.

**Keywords**

Febuxostat, LC-MS, Pharmaceutical Analysis, Gout, Validation methods, Bioequivalence, Bioavailability study.



## 1. Introduction

Hyperuricemia and gouty arthritis, persistent conditions throughout human history, have witnessed a transformative evolution in mechanistic comprehension owing to recent research endeavors [1]. Despite these advancements, therapeutic interventions remain constrained. Gao *et al*. stated that a pivotal development in addressing this lacuna materialized with the FDA's endorsement of Febuxostat (FBS) in 2009 managed gouty arthritis or hyperuricemia [2]. FBS, a 2-(3-cyano-4-isobutoxyphenyl)-4-methyl-1,3-thiazole-5-carboxylic acid, has established itself as a secure and efficient treatment [3]. However, prolonged studies continue regarding its extended impact and its comparative efficacy over other anti-gout modalities.

Diverse methodologies have been devised for FBS determination, encompassing spectrophotometric and liquid chromatography methodologies [4] (**Table 1**). Our primary focus centers on a pioneering Liquid Chromatography-Mass Spectrometry (LC-MS) methodology for FBS quantification of human blood plasma. This methodology distinguishes itself through its inherent simplicity, discerning selectivity, and careful utilization of human plasma volume (a mere 100 ml) [5]. Yiwen *et al*. mentioned that the associated reduction in sample collection per progressive unit enhanced feasibility and extreme significance in augmenting data granularity, a critical consideration in bioavailability and future

pharmacokinetic investigations [6]. The singular employment of one-step solid-phase extraction ensures improved recoveries and obviates potential interference emanating from endogenous and exogenous sources [7]. The judicious inclusion of an isotope-labeled compound as an internal standard extends precision and accuracy. Ding *et al*. observed in their studies that with a shortened total run time of 2.0 minutes, LC-MS methodology emerged as an appealing bioanalytical mode for FBS in bioavailability and imminent pharmacokinetic inquiries [8].

**Table 1.** Differences between Spectrophotometry and Liquid Chromatography [9]

| **Parameter** | **Spectrophotometric** | **Liquid Chromatography** |
|---|---|---|
| Principle of Analysis | Measures the absorption or transmission of light by a substance at specific wavelengths | Separates the mixture and quantifies the components based on their interaction with the mobile and stationary phases, generating a chromatogram |
| Analytical Range | Suitable for analyzing a single analyte or a small group of closely related compounds | Extends broader versatility for analyzing complex mixtures and a wide range of compounds simultaneously |
| Sensitivity | High sensitivity, especially in specific wavelength ranges | Provides High sensitivity, and with the integration of mass spectrometry (LC-MS), it also determines the trace-level analysis |
| Selectivity | Relies on differences in absorbance spectra, creating it less selective for complex mixtures | Achieves high selectivity by using specific columns and mobile phases, allowing the separation of closely related compounds |
| Application | Quantitatively analyze specific compounds in solutions, such as determining concentrations of chromophores | Widely used for analyzing complex samples in various fields, including pharmaceuticals, environmental analysis, and biochemistry |
| Speed of Analysis | Faster and delivers quick results | The analysis will take time due to the separation process, but it offers superior resolution for complex mixtures |
| Detection Techniques | Primarily relies on absorbance or transmittance measurements | Coupled with various detectors, such as UV-Vis, fluorescence, or mass spectrometry, it enhances detection capabilities |

## 2. Overview of Febuxostat

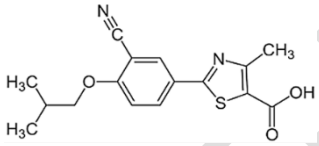
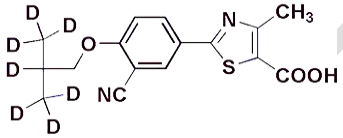
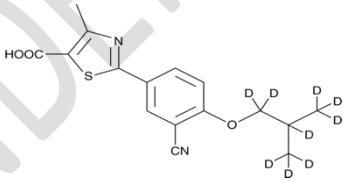
Febuxostat has emerged as an essential drug for hyperuricemia and gout treatment. This innovative medication, unlike traditional options like allopurinol, specifically targets xanthine oxidase (XO), which is liable for uric acid production [10]. This targeted approach offers several advantages. Cicero *et al*. stated that FBS boasts a unique ability to bind and inhibit both oxidized and reduced forms of XO [11]. This potent dual action significantly reduces uric acid production, leading to lower serum urate levels (sUA) and, consequently, improved management of gout symptoms. *In vitro* studies have convincingly demonstrated its efficacy, solidifying its potential as a promising alternative [12]. While its potential was evident, FBS faced heightened scrutiny due to increased focus on drug safety profiles. Despite this, its compelling clinical data convinced regulatory bodies, with the European Medicines Agency and the U.S. Food and Drug Administration approving it in 2008 and 2009, respectively [13]. This emphasizes its crucial role in modern therapeutic approaches for gout management.

However, research continues to optimize FBS effectiveness. Further studies into its bioavailability and bioequivalence are ongoing, aimed at ensuring consistent and reliable performance within different individuals. Dina *et al*. mentioned that FBSs' unique mechanism of action, coupled with its proven efficacy and safety profile, positions it as a crucial component in the evolving view of gout management [14]. Ongoing research promises further refinement, solidifying its role in offering patients a more effective and well-tolerated approach to managing this condition.

Beyond the primary compound, derivatives such as Febuxostat d9 and d7 have gained attention in pharmaceutical research. FBS d9 and FBS d7 are isotopically labeled compounds, providing valuable tools in analytical and pharmacokinetic studies (**Table 2**). Yichao Xu *et al*. detailed that introducing deuterium atoms at specific positions in these derivatives facilitated precise quantification and elucidation of metabolic pathways during pharmacological

investigations [15]. FBS d9 and d7 have been employed with advanced analytical techniques, including LC-MS, to enhance the sensitivity and accuracy of quantitative analyses in biological samples, contributing to a deeper understanding of the FBS pharmacokinetic profile [16]. The isotopic labeling of these derivatives delivers invaluable insights into the absorption, distribution, metabolism, and excretion of FBS, contributing to the refinement of therapeutic strategies and optimization of dosage regimens [14].

**Table 2.** Chemical Structure of Febuxostat and its derivatives, FBS- d7 and d9 [https://pubchem.ncbi.nlm.nih.gov/compound/134018]

| Drug | Chemical Structure | Chemical Name | M. Wt. (g/mol) |
|---|---|---|---|
| Febuxostat |  | 2-(3-cyano-4-isobutoxyphenyl)-4-methyl-5-thiazolecarboxylic acid | 316.4 |
| Febuxostat - d7 |  | 2-[3-Cyano-4-(2-methylpropoxyphenyl]-4-methyl-5-thiazolecarboxylic Acid-d7 | 323.4 |
| Febuxostat - d9 |  | 2-[3-cyano-4-[1,1,2,3,3,3-hexadeuterio-2-(trideuteriomethyl)propoxy]phenyl]-4-methyl-1,3-thiazole-5-carboxylic acid | 325.4 |



## 3. **Importance of** FBS **Bioavailability and Bioequivalence Studies**

In the realm of pharmaceuticals, bioavailability, and bioequivalence studies stand as pillars in ensuring drug efficiency and safety. For FBS, an essential drug in hyperuricemia management, unraveling its bioavailability and bioequivalence becomes imperative (**Figure 1**) [17]. This review explores the methodologies, especially focusing on the application of LC-

MS techniques in the determination of FBS in blood plasma [18]. With the keen emphasis on regulatory scrutiny for safety, understanding how LC-MS surpasses other techniques in specifying accurate drug measurement becomes crucial for advancing pharmacokinetic studies. The present review delves into the pivotal role of LC-MS as an analytical technique for elucidating the bioavailability and bioequivalence of FBS in blood plasma using FBS d9 and d7 and other drugs as internal standards (IS) [19]. By examining impurity profiles and the challenges posed by their complex pharmacokinetics, this review aims to contribute to the evolving view of pharmaceutical research and analysis.

1. Also highlight the bioavailability and Bioequivalence

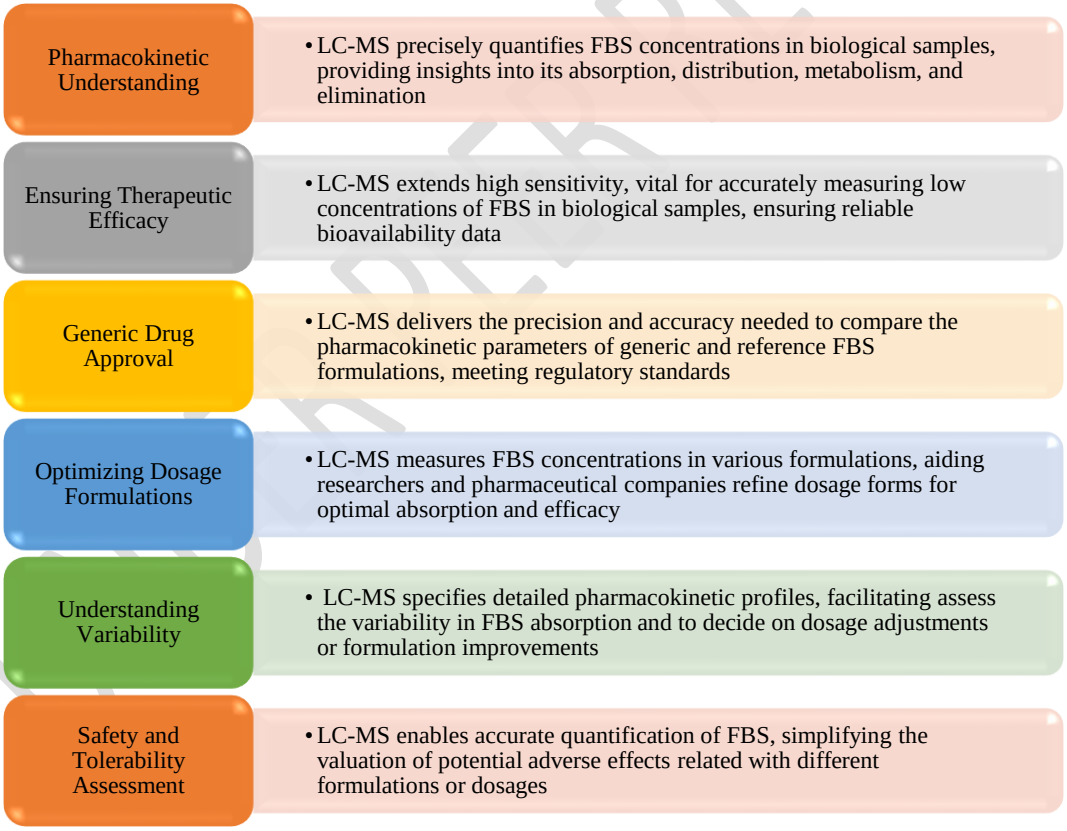



**Figure 1.** Requirements of Bioequivalence and Bioavailability Studies of Febuxostat by LC-MS

## 4. Analytical Techniques in Pharmacokinetic Studies

Analytical techniques in pharmacokinetic studies encompass a spectrum of methodologies designed to elucidate a drug's behavior within the body. These are essential for comprehending how drugs interact with the body over time. Some of the commonly used techniques include Gas Chromatography (GC), Mass Spectrometry (MS), High-Performance Liquid Chromatography (HPLC), and more recently, advanced hyphened methods such as LC-MS (**Table 3**) [20]. HPLC is widely utilized for its capacity to separate components in liquid samples with high sensitivity. Kamel B *et al*. specified that LC-MS combines separation with mass analysis, offering specificity and the ability to identify compounds in complex matrices [21]. GC-MS is ideal for analyzing volatile compounds in environmental and forensics fields. Hybrid techniques, such as LC-MS/MS, integrate multiple methods for enhanced capabilities [22]. Each method has its strengths, addressing specific analytical challenges in drug quantification, impurity profiling, and metabolite identification. Researchers choose techniques based on drug characteristics, often combining methods for a comprehensive pharmacokinetic understanding.

**Table 3.** Different Analytical Techniques Used in Pharmaceutical Drug Analysis References missing

| Parameter | GC | MS | HPLC | LC-MS |
|---|---|---|---|---|
| Compound Compatibility | Less commonly applied | Not much applied | Deals with liquid samples and compounds requiring high-resolution separation | Analyze a broad range of compounds, like polar and non-volatile, found in drug formulations |

| | | | | |
|---|---|---|---|---|
| Sample Types | Volatile Drugs | Used only when coupled with LC | Drug formulations | Pharmaceutical drugs, metabolites, and biomarkers with diverse chemical properties |
| Sensitivity & Selectivity | Less sensitive due to its limited use in volatile compounds | Exceptionally sensitive to delivering structural information, and valuable for pharmaceutical R&D | Provides high sensitivity & selectivity to meet pharmaceutical quality control and impurity profiling | Delivers high sensitivity & selectivity vital for detecting low concentrations of drugs and their metabolites in complex matrices |
| Versatility | Less versatile in pharmaceuticals with specific analysis | Beneficial for in-depth structural characterization of pharmaceutical compounds | Commonly employed for routine pharmaceutical analysis, particularly in QC | Adaptable for analyzing pharmaceuticals, handling a wide variety of drug compounds |
| Quantitative Analysis | Limited | Potent device for accurate quantitative analysis | Routinely used for the precise determination of drug concentrations | Extensively used for drugs and their metabolites analysis, providing accurate and reliable results |

### 4.1 **Significance of Accurate Drug Measurement**

Accurate drug measurement is paramount in pharmacokinetic studies, serving as a cornerstone for reliable data vital in drug development and clinical applications. The precision of these measurements holds substantial influence over the assessment of crucial pharmacokinetic parameters, with absorption, distribution, metabolism, and excretion (ADME) of pharmaceutical compounds [23]. Any inaccuracies in drug measurement can introduce biases, leading to distorted pharmacokinetic profiles that may compromise the safety and efficacy of the drug. Sharma S *et al*. observed that analytical techniques played a pivotal role in confirming the accuracy of drug measurements by providing high sensitivity, selectivity, and the capacity to separate complex mixtures of drugs and metabolites [24]. This precision is essential in establishing a robust foundation for pharmacokinetic studies, contributing significantly to the reliability of data that informs clinical decision-making. As regulatory

standards become more stringent, the emphasis on accurate drug measurement grows, reinforcing its pivotal role in shaping the pharmaceutical background.

## 5. Liquid Chromatography: An Overview

Liquid Chromatography is a fundamental analytical technique crucial for separating and quantifying components within mixtures. The technique relies on the differential partitioning of a sample between a mobile liquid phase and a stationary phase, leading to the separation of individual components based on their chemical properties [25]. As per a study by Faraz Rahimi *et al*., LC comprehends various modes, including HPLC and Ultra-High-Performance Liquid Chromatography (UHPLC), allowing customization of column type, the composition of mobile phase, and detection methods, which is a requisite tool in the pharmaceutical analysis [26].

At the core of LC lies a dynamic process that reveals the secrets of mixtures. The technique involves sample injection into the mobile phase stream, followed by column chromatography. The mobile phase transports the sample over a column packed with the stationary phase [27]. Different molecules interact uniquely with the stationary phase, leading to varied retention times. As the separated components emerge, a detector measures their presence and amount, translating the information into a chromatogram (**Figure 2**). This discloses the identity and quantity of each component in the mixture [28]. LCs' versatility is further highlighted by its applications, ranging from pharmaceuticals, food and beverage analysis, environmental analysis, and biotechnology, to forensic science [29].

2. Also highlight about Mass Spectrometry

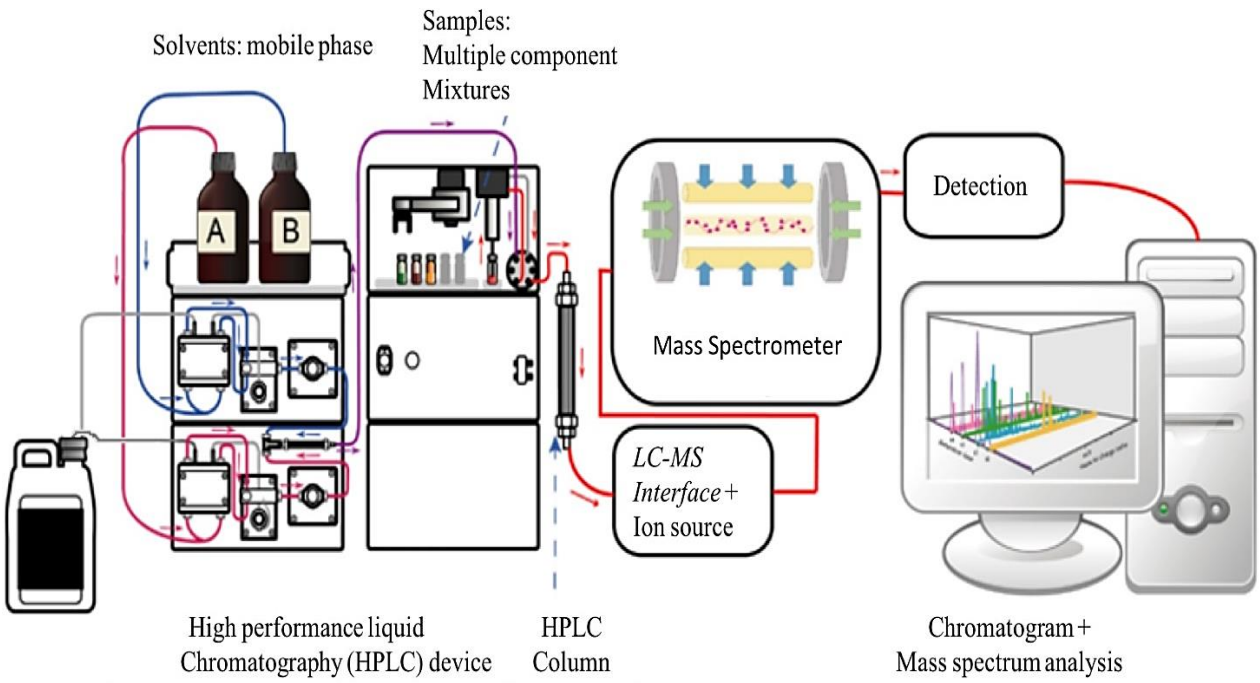



**Figure 2.** Schematic representation of a Liquid Chromatography Mass Spectrometer. [Credit: Cwszot, Dagui1929, CasJu, and YassineMrabet, reproduced under the Creative Commons CC0 1.0 Universal Public Domain Dedication license.]



### 5.1 **Advantages of LC in Pharmaceutical Analysis**

LC occupies a central role in pharmaceutical analysis and its strength lies in its exceptional sensitivity, allowing the detection of minute drug quantities and impurities within complex mixtures. Srinivas B *et al*. stated that LC is a vital method for ensuring safety and efficacy, as even trace amounts can have a substantial impact [30]. Furthermore, LC stands out in separating individual components with remarkable precision (**Figure 3**). This meticulous separation enables accurate identification and quantification of each element, regardless of their similarities [31]. Additionally, LC demonstrates remarkable flexibility, analyzing a wide range of molecules, from small drugs to large proteins. This adaptability makes it an indispensable tool for both research and quality control.

Beyond its core strengths, LC offers several other advantages [32].

- *Precise measurements*: LC delivers accurate quantitative results, critical for ensuring consistent drug dosages and safety profiles.
- *Broad applicability*: It effectively analyzes diverse analytes, including hydrophobic and hydrophilic compounds, covering the varied chemical properties of drug molecules.
- *High-throughput analysis*: Modern automated systems process large batches of samples efficiently, crucial for high-volume pharmaceutical testing.
- *Diverse detection methods*: Compatibility with UV-Vis, fluorescence, and mass spectrometry detectors enhances analysis and characterization of pharmaceutical compounds.
- *Regulatory compliance*: Widely accepted methods ensure results comply with pharmaceutical standards, facilitating new drug approval processes.

LCs' unique combination of sensitivity, precision, versatility, and efficiency firmly establishes it as the go-to technique for pharmaceutical analysis. Its ability to provide accurate and reliable data across diverse sample types makes it an invaluable asset in validating the safety, efficiency, and superiority of pharmaceutical products.

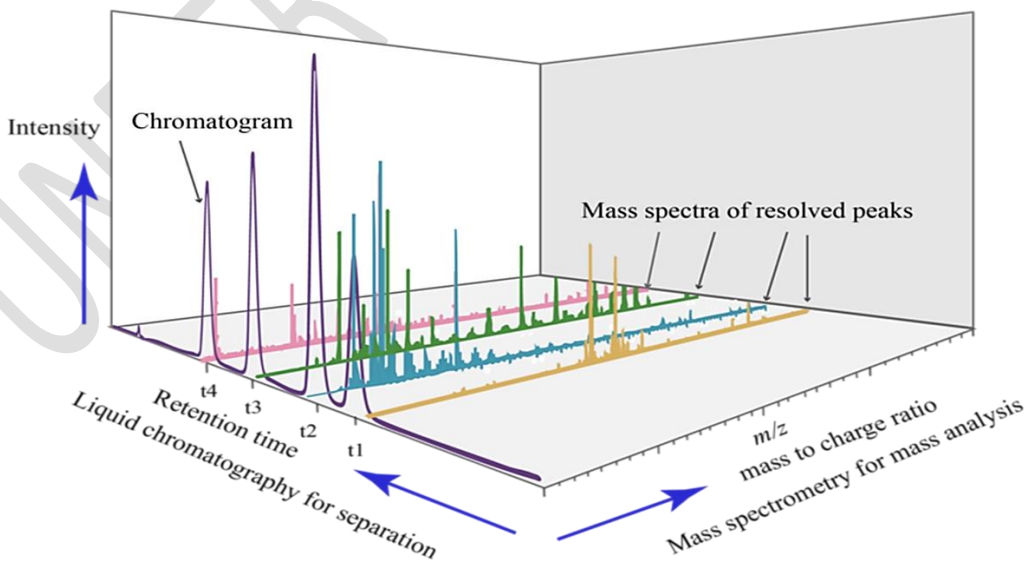


**Figure 3.** Example of the outline of LC-MS analysis. [Credit: Daniel Norena-Caro, reproduced under the Creative Commons CC0 1.0 Universal Public Domain Dedication license.]

## 6. Febuxostat: Properties and Importance in Clinical Studies of Gout

FBS claims a distinct molecular structure critical to its pharmacological actions. Bin Fan *et al*. mentioned that the nonpurine selective inhibitor, FBS, helps to manage hyperuricemia associated with gout [33]. Excess uric acid in joints causes Gout, an inflammatory condition. FBS molecule's core structure includes a 1,3-thiazole monocarboxylic acid core, a 3-cyano-4-(2-methylpropoxy) phenyl substituent, and nitrogen and sulphur atoms. These attributes contribute to its stability, binding affinity to XO, and overall pharmacological profile. Arrigo *et al*. stated that FBSs' unique properties, such as potent XO inhibition, oral availability, selectivity, and prolonged action, make them a valued therapeutic option [34]. Clinical studies focus on the efficacy and safety of FBS in gout management. Ruixia Sun specified that FBS effectively reduces the frequency and severity of flares, diminishes urate calculi, and maintains serum uric acid levels below recommended levels [35]. FBS demonstrates superior tolerability and safety, with fewer side effects compared to allopurinol [33]. Despite these, FBSs' impact on gout treatment positions it as a key player in clinical practice, for improved joint health and a better quality of life for patients.

3. Elaborate the mechanism of inflammation in gout

### 6.1 Role of FBS in Gout Management

Gout, inflammatory arthritis formed in joints, has found a powerful connection with FBS (**Figure 4**). Unlike traditional medications that manage symptoms, Kraev KI *et al*. in their research found that FBS challenges the root cause by targeting and inhibiting XO, the enzyme responsible for uric acid production [36]. This dual action against the oxidized and reduced

forms of XO effectively lowers serum urate (sUA) levels, significantly reducing the risk of gout flares and promoting joint health [37]. Clinical studies have validated its efficacy in this regard, showcasing its ability to not only decrease the frequency and severity of flares but also maintain healthy uric acid levels for extended periods. Gout, being a crystal-induced disease, requires addressing the source of the crystals: uric acid [38]. Santwona Dash *et al*. also detailed that by inhibiting XO and suppressing uric acid production, FBS directly targets the root cause, offering a more comprehensive solution than simply managing symptoms [39]. Additionally, its safety profile and tolerability hold significant value in regulatory scrutiny. Understanding FBSs' unique properties not only sheds light on their effectiveness in managing gout but also paves the way for further targeted research and advancements in gout treatment [40].

**Figure 4.** Foot affected by Gout exposing swelling, joint rigidity, and pain around the joint. [Credit- Creative Commons Attribution-Share Alike 4.0 International http://www.scientificanimations.com/wiki-images/]



7. **Need for High-Performance Analytical Techniques**

Analyzing FBS presents a set of challenges that necessitate the application of high-performance analytical techniques. The compound's intricate molecular structure, as an XO inhibitor, requires careful study [41]. Traditional analytical methods may struggle to provide the necessary resolution and sensitivity due to the complexity of Febuxostats' pharmacokinetics. The structural nuances, coupled with potential interference from

endogenous compounds, demand advanced techniques that can direct these challenges. A recent study by Tingting Wang *et al*. specified that high-performance analytical methods, particularly LC-MS, emerge as an essential tool to overcome these hurdles, ensuring accurate quantification and reliable pharmacokinetic data (**Table 4**) [42].

**Table 4.** Difference and prominence between LC-MS and Traditional methods references missing

| Parameter | Traditional Method | LC-MS |
|---|---|---|
| Sensitivity & Specificity | Often lack the sensitivity and specificity leading to potential challenges in detecting low-concentration analytes | Delivers higher sensitivity and specificity due to the combination of LC for separation and MS for precise detection |
| Structural Information | Don't provide detailed structural information, causing compound identification challenges | Extends structural information through mass analysis, aiding in the compound identification based on their mass-to-charge ratios |
| Multiple Analyte Detection | Often involve separate assays for each analyte, leading to longer analysis times and increased resources | Enables concurrent detection of multiple analytes in a single run, improving efficiency |
| Interference & Matrix Effects | Have challenges with interference and matrix effects, especially in complex sample matrices | Mitigate interference from matrix components through selective detection with the use of internal standards |
| Quantitative Accuracy | Relies on external calibration, potentially leading to variations in accuracy | Allows the use of internal standards, enhancing accuracy in quantification |
| Lower Detection Limits | Have higher LOD and LOQ, limiting their utility for trace-level analysis | Attains lower LOD and Quantitation LOQ, enabling the detection of compounds at very low concentrations |
| Regulatory Compliance | Require additional validation steps to meet regulatory standards | Mostly preferred in pharmaceutical and clinical research for its ability to meet stringent regulatory requirements |



### 7.1 **Importance of Accuracy in Bioavailability Studies**

The accuracy of bioavailability studies is paramount in understanding the therapeutic profile of FBS. Bioavailability is the portion of a directed dose that reaches the systemic circulation and is a crucial parameter influencing a drug's effectiveness [43]. For FBS, precision in bioavailability measurement becomes imperative. Greco V *et al*. observed that the

inaccuracies in quantifying FBS levels in blood plasma can lead to misguided assessments of its absorption, distribution, and elimination [44]. High-performance analytical techniques, particularly those offering sensitivity, specificity, and automated exact mass measurement like LC-MS, play a pivotal role in ensuring the accuracy required for robust bioavailability studies [45]. The reliability of these studies is paramount in shaping dosage recommendations, optimizing therapeutic outcomes, and meeting regulatory standards in the development of FBS as a key player in gout management.

## 8. **Liquid Chromatography in Pharmaceutical Analysis**

LC is a vital technique in pharmaceutical analysis, rooted in principles that support its widespread application. The method relies on the differential partitioning of a sample between a mobile liquid phase and a stationary phase [46]. The sample is injected into the mobile phase, typically a liquid solvent, and passes through a column containing the stationary phase. Components in the sample interact differently with the stationary phase, leading to their separation based on factors such as polarity, size, and affinity. Leonardo Perez *et al*. indicated that the key variations, including HPLC and UHPLC, enhance speed and resolution, focusing on LC as a versatile and powerful tool in pharmaceutical analysis [47].

### 8.1 **Application in Drug Quantification**

LCs' application in drug quantification is integral to pharmaceutical analysis, particularly in pharmacokinetic studies. Hector *et al*. indicated that LCs' ability to precisely separate and quantify individual components within a complex mixture shows it is well-suited for determining drug concentrations in biological samples [48]. In drug quantification, a sample containing the drug of interest is introduced into the LC, and the detector records the signals as separated components elute from the column [32]. By comparing these signals to those of

known standards, the drug concentration in the sample can be accurately verified. The techniques' sensitivity, selectivity, and adaptability to various detection methods, such as UV or MS, enhance their utility in drug quantification [49]. Deschamps E *et al*. cited that LC played a crucial role in ensuring the precision and reliability required for accurate drug quantification, contributing extensively to the advancement of pharmaceutical research and analysis [50].

### 8.2 **Methodology: Liquid Chromatography for Febuxostat Analysis**

Choosing the appropriate LC technique is a critical step in ensuring the accurate and reliable analysis of febuxostat. HPLC and UHPLC are common choices. The decision is often guided by factors such as the required resolution, sensitivity, and speed of analysis. UHPLC, with its higher pressure and smaller particle sizes, allows for faster separations and improved resolution compared to traditional HPLC [51]. The selection depends on the specific demands of the analysis, emphasizing the need for optimal separation and quantification of FBS from complex matrices.

#### 8.2.1 **Sample Preparation**

For analyzing FBS, sample preparation is a crucial step in LC, notably impacting the accuracy and sensitivity of the analytical method. In the context of biological samples, the Shimaa *et al*. study detected that the blood plasma required meticulous preparation to eliminate potential interferences and enhance the purity of the analyte before chromatographic analysis [52]. Commonly employed techniques for extracting FBS from the blood plasma include protein precipitation or liquid-liquid extraction [53].

In the protein precipitation method, blood samples are collected, and plasma is separated using centrifugation. Internal standards were added to ensure accuracy. Jiawen Lyu *et al*. cited that protein precipitation was induced by introducing a protein-denaturing solvent,

followed by centrifugation to separate proteins from the supernatant [54]. The resulting supernatant, containing the target analytes, was then transferred to a new vial. After undergoing evaporation and reconstitution, the prepared sample is ready for injection into the LC-MS system for analysis [55].

On the other hand, the liquid-liquid extraction method involves mixing plasma with an organic solvent to facilitate extraction. After the organic phase containing analytes was separated, it was collected, subjected to evaporation, modified, and then infused into the LC-MS system. The choice between these sample preparation methods depends on various factors, including the specific analytical method, the nature of the sample matrix, and the desired sensitivity [56]. Optimization of the sample preparation process is crucial to ensure accurate and reliable results in FBS analysis from blood plasma.

### 8.2.2 **Chromatographic Conditions**

For chromatographic analysis of FBS, the selection of chromatographic conditions is pivotal for the success of the technique. Key factors, such as the choice of stationary and mobile phases, significantly influence the separation and detection of the target compound. Typically, a reversed-phase column is preferred for FBS analysis due to its compatibility with polar compounds [57]. The optimization of the mobile phase composition, flow rate, and detection wavelength is crucial to achieving maximum sensitivity and resolution in the chromatographic separation. Thorough consideration and fine-tuning of these parameters contribute to the progress of a robust and consistent LC technique for FBS analysis (**Table 5**). This optimized method ensures accurate and precise data, which is essential for conducting pharmacokinetic studies. Patel B *et al*. specified that by providing a well-defined separation of FBS and other components in the sample matrix, these chromatographic conditions contributed to the overall success of the analytical method in pharmaceutical research and development [58].

**Table 5.** Chromatographic conditions for the determination of FBS [53, 59-62]

| Drugs | Matrix | Stationary Phase | Mobile Phase | Analytical Wavelength (nm) |
|---|---|---|---|---|
| FBS | Human Plasma | Phenomenex Luna C18 | Acetonitrile: water, 60:40, v:v, 0.032% glacial acetic acid | 380 |
| FBS | Human Plasma | Inertsil Symmetry C18 column | Methanol/1% formic acid (80:20; v/v) | 210 |
| FBS | Human Plasma | Bondapack C18 | Acetonitrile and 0.5% aqueous phosphoric acid (52: 48, v/v) | 315 |
| FBS | Human Plasma | Nucleosil C18 | 10 mM ammonium acetate buffer (buffer of pH 4.0 adjusted with 0.2% triethyl amine) and acetonitrile in the ratio (15: 85, v/v) | 275 |
| FBS | Human Plasma | Purospher® RP-18 | Trifluoroacetic acid (0.1%) in water and acetonitrile in a ratio of 42:58 v/v | 230 |

## 9. **Validation of the Analytical Method**

Method validation is a crucial step in establishing the reliability and robustness of an analytical procedure, ensuring that the results generated are accurate, reproducible, and appropriate for the intended purpose [63]. In the context of LC for FBS analysis, method validation is paramount for confirming the appropriateness of the technique for its proposed claim in pharmacokinetic studies [64]. Validated methods introduce confidence in the accuracy of the data, contributing to the overall quality and credibility of the analytical results.

### 9.1 **Parameters and Criteria for Validation**

During the validation of an analytical method for FBS analysis, several key parameters and criteria play a crucial role in ensuring the methods' accuracy and reliability. Ramanl al *et al*. stated that one fundamental parameter was specificity, which evaluated the methods' ability to accurately and selectively measure FBS in the presence of potential impurities or matrix components [65]. Ensuring specificity is essential to guarantee that the detected signals solely

originate from the analyte of interest. Precision assesses the degree of repeatability and reproducibility of the method. Precision is evaluated through measures such as intra-day and inter-day precision, offering insights into consistent results under varying conditions [66].

Accuracy measures the closeness of calculated values to the true values and is often determined through recovery studies. Kamel B *et al*. cited that these studies involved comparing the measured concentration to the known concentration of FBS in spiked samples, establishing the accuracy of the method [21]. The linearity of the method is assessed to determine its ability to provide results that are directly proportional to the concentration of FBS in the sample. Calibration curves constructed over a range of concentrations help evaluate and establish the linearity of the analytical method. Parameters such as Limit of Detection (LOD) and Limit of Quantitation (LOQ) signify the lowest concentrations at which FBS can reliably detect and quantify, respectively. These parameters define the sensitivity of the method, crucial for pharmacokinetic studies [17]. Stability studies are conducted to assess the robustness of the method over time, including the stability of FBS in solution and the stability of analytical solutions under various storage conditions [67]. Diligently addressing these parameters during validation provides a comprehensive understanding of the analytical methods' competencies, establishing their aptness for pharmacokinetic studies involving FBS.

### 9.2 **Clinical Validation Method in Healthy Subjects**

This review was focused on studying to assess the bioavailability of Febuxostat in human blood plasma following oral administration. Aaron *et al*. developed a highly sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method, featuring a 10-fold higher sensitivity, and employed liquid-liquid extraction for sample preparation [68]. FBS d7 & FBS d9 and a few other drugs were utilized as internal standards to enhance accuracy and mitigate matrix effects. Babu *et al*. stated that the validated method demonstrated exceptional

sensitivity with an LLOQ with a minimum of 1 ng/mL, successfully quantifying FBS concentrations [69]. The validation of FBS on precision, accuracy, recovery, and stability study results supported a comprehensive clinical pharmacokinetic study, contributing valuable data for dosage recommendations and therapeutic optimization (**Table 6, 7**). The chromatograms of the studied Blank and the FBS standard are displayed in **Figures 5 & 6**, respectively [78].

### 9.3 **Impurity Profile Determination using LC-MS Quadrupole Time-of-Flight (Q-TOF)**

The impurity study of FBS Active Pharmaceutical Ingredient (API) involves a thorough examination of impurities within the drug substance, including an assessment of carryover impurity from intermediate stages and raw materials [79]. To achieve this, an LC-MS Q-TOF instrument was employed for impurity profiling, providing automated exact mass measurement and structural confirmation through infrared (IR), nuclear magnetic resonance (NMR), and mass analysis following isolation [80, 81]. The Q-TOF instrument delivers high-quality data, offering insights into the elemental composition and structural characteristics of impurities. Mustakhusen *et al*. cited that this detailed impurity profile is of paramount importance for regulatory compliance, ensuring adherence to safety standards, and maintaining the efficacy of the FBS API [82]. The overall impurity study involves a meticulous process starting with method development, utilizing techniques like HPLC or LC-MS for sensitive impurity detection [83, 84]. Sample preparation ensures representative drug substance samples, and chromatographic analysis, often with LC-MS, is conducted for the separation, identification, and quantification of impurities. Techniques like mass spectrometry, NMR spectroscopy, and IR spectroscopy contribute to structural characterization. Margaret *et al*. mentioned that validation of the method is crucial for regulatory compliance, culminating in the identification,

quantification, and reporting of impurities, ensuring the pharmaceutical product's safety, efficacy, and adherence to quality standards [85].

## 10. **Future Perspectives and Innovations in LC**

Ongoing research is eagerly exploring potential breakthroughs in LC, primarily targeting advancements in column technology. Novel stationary phases and column designs, such as monolithic columns and superficially porous particles, are under study [86]. These innovations aim to elevate separation efficiency, increase analysis speed, and enhance selectivity, thereby pushing the boundaries of what LC can achieve in drug analysis. Concurrently, a study by Deyber *et al*. quoted an outstanding trend gaining momentum involving the miniaturization and incorporation of microfluidics into LC systems [87]. This shift towards micro-scale LC systems offers compelling advantages, including reduced solvent consumption, heightened sensitivity, and the prospect of conducting high-throughput analyses.

Also, the future view of LC holds exciting prospects in terms of hyphenation with advanced detection techniques [88]. Integrating LC with cutting-edge methods such as capillary electrophoresis, ion mobility spectrometry, and high-resolution MS presents a promising avenue for achieving unparalleled sensitivity, specificity, and comprehensive characterization of complex drug samples [89]. Besides, automation is anticipated for the potential emergence of high-throughput platforms in LC. Met Han *et al*. specified that the automated processes encompassing sample preparation, injection, and data analysis were balanced to contribute significantly to the efficiency, reproducibility, and overall advancement of drug analysis methodologies [90].

## 11. **Emerging Technologies in Pharmaceutical Research**

Pharmaceutical research is undergoing a profound transformation, marked by several key trends and innovations. Artificial Intelligence (AI) is at the forefront, leveraging machine learning and deep learning for transformative impacts on drug discovery processes [91]. A study by Sarkar *et al*. mentioned that predictive modeling, virtual screening, and the analysis of intricate biological data are domains witnessing significant advancements, showcasing the potential of AI to revolutionize the entire drug development pipeline [92]. Further, the pharmaceutical industry is experiencing a shift towards continuous manufacturing processes, gaining momentum due to their potential for improved efficiency, reduced costs, and heightened control over production. Innovations in drug delivery systems are also unfolding, with nanotechnology taking center stage. Nano-sized carriers are demonstrating the ability to enhance drug solubility, improve bioavailability, and enable targeted delivery, offering the potential for more effective and less toxic treatments [93]. In the field of clinical trials, the application of blockchain technology is emerging as a disruptive force. Aithal *et al*. specified that blockchain brings transparency, security, and data integrity to clinical trials, with smart contracts and decentralized platforms updating trial processes and data management [94]. These advancements, linked with the continuous evolution of LC and the integration of emerging technologies, are collectively shaping a dynamic future for pharmaceutical research and analysis.

4. Reference are not appropriate.

## 12. Conclusion

In the realm of pharmaceutical analysis, LC-MS reigns supreme, especially when delving into the intricate pharmacokinetics of drugs like Febuxostat. Its versatility offers a unique collection of advantages. This comprehensive review of LC-MSs' high sensitivity, selectivity, and customizable conditions makes them indispensable for quantifying Febuxostat and other pharmaceutical drugs with precision, generating reliable pharmacokinetic data

crucial for understanding drug behavior. FBSs' adaptability to various detection methods, robust sample preparation techniques, and advancements in column technologies further solidify its lead. Beyond separation, LC-MS significantly contributes to the accuracy and specificity demanded in pharmacokinetic studies, with reliable validation protocols ensuring data integrity and regulatory compliance. LC-MSs' proven track record in providing impurity profiles, simultaneous metabolite determination, and successful bioavailability studies position it as a cornerstone for future endeavors. Thus, LC-MS's superiority in Febuxostat analysis set the stage for a future where pharmacokinetic studies become insightful, efficient, and transformative, shaping the future of pharmaceutical research.

**Table 6.** HPLC method for the determination of FBS & FBS- d7 &d9 in Human Blood Plasma [69-74]

| Drug | Matrix | Stationary Phase | Mobile Phase at Flow Rate (FR) | Internal Standard | Detection | Sensitivity Parameters | | Precision (%) | Accuracy (%) | Recovery (%) | Stability (ng/ml) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | LOD (µg/ml) | LOQ (ng/ml) | | | | |
| FBS | Human Plasma | Capcell Pak C18 | Acetonitrile–5mM ammonium acetate–formic acid (85:15:0.015, v/v/v) at 0.6 mL/min | FBS-d7 | LC-MS/MS | 0.01-5 | 10 | 4.2 | 105 | 95.9 | 18.9-21.8 |
| FBS | Human Plasma | Zorbax SB-C18 | 5mM ammonium formate and acetonitrile (40:60, v/v) at 0.5mL/min | FBS-d7 | LC-MS/MS | 0.01-6 | 1.1 | 7.69 | 110 | 82.7 | 3.13-3.24 |
| FBS | Human Plasma | Ascentis Express C18 | 10 mM Ammonium formate: Acetonitrile (20:80 v/v) at 0.8 mL/min | FBS-d7 | LC-MS/MS | 0.01-8 | 1 | 8.44 | 103.19 | 81.59 | 3.03-3.11 |
| FBS | Human Plasma | Hypersil Gold-C18 | Mobile phase A [Water (0.1% formic acid)], and mobile phase B [acetonitrile (0.1% formic acid)] at 0.3 mL/min | FBS-d9 | LC-MS | 0.01-8 | 10 | 8.68 | 104.86 | 112.06 | 29.80-31.80 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| FBS | Human Plasma | Zorbax Eclipse XDB, C8 | Acetonitrile and HPLC grade water in the ratio of (60:40 v/v) | FBS-d9 | LC-MS | 0.02-10 | 20.13 | 5.69 | 100.1 | 76.56 | 58.44-62.68 |
| FBS | Human Plasma | Kromasil 100-5C18 | 5mM ammonium formate and acetonitrile (20:80, v/v) at 1.0 mL/min | FBS-d9 | LC-MS/MS | 0.015-8 | 15.1 | 4.71 | 105 | 80.1 | 45.8-49.2 |

**Table 7.** HPLC method for the determination of FBS & a few drugs as IS in Human Blood Plasma [60, 75-77]

| Drug | Matrix | Stationary Phase | Mobile Phase at Flow Rate (FR) | Internal Standard | Detection | Sensitivity Parameters | | Precision (%) | Accuracy (%) | Recovery (%) | Stability (ng/ml) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | LOD (µg/ml) | LOQ (ng/ml) | | | | |
| FBS | Human Plasma | ZORBAX SB-C18 | 0.2% formic acid (A) and acetonitrile (B) [0–8.6 min, 28%B; 8.6–8.8 min, 28–100% B; 8.8–10.7 min, 100% B; 10.7–11 min,100–28% B; 11–12.5 min, 28% B}at 0.08mL/min | Losartan Potassium | LC-MS/MS | 0.1-200 | 10 | 12.2 | 5.1 | 87.1-98.6 | - |
| FBS | Human Plasma | Hypurity C18 | Methanol: 10 mM ammonium acetate: glacial acetic acid (70:30:0.01, v/v/v) at 0.8 mL/min | Indomethacin | LC-MS/MS | 0.05-6 | 50 | 7.1 | 105.6 | 85.1 | 41.0-46.9 |
| FBS | Human Plasma | Bondapack C18 | Acetonitrile and 0.5% aqueous phosphoric acid (pH 3) (52: 48, v/v) | Ketoprofen | LC-MS/MS | 0.05-5 | 50 | 5.28 | 103.78 | 77.56 | 144-151 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| FBS | Human Plasma | BEH C18 | 0.1% formic acid and acetonitrile (25:75; v/v) at 0.5mL/min | Trandolapril | UPLC | 0.075-12 | 75 | 6.96 | 5.5 | 106.5 | 85.5-137.87 |

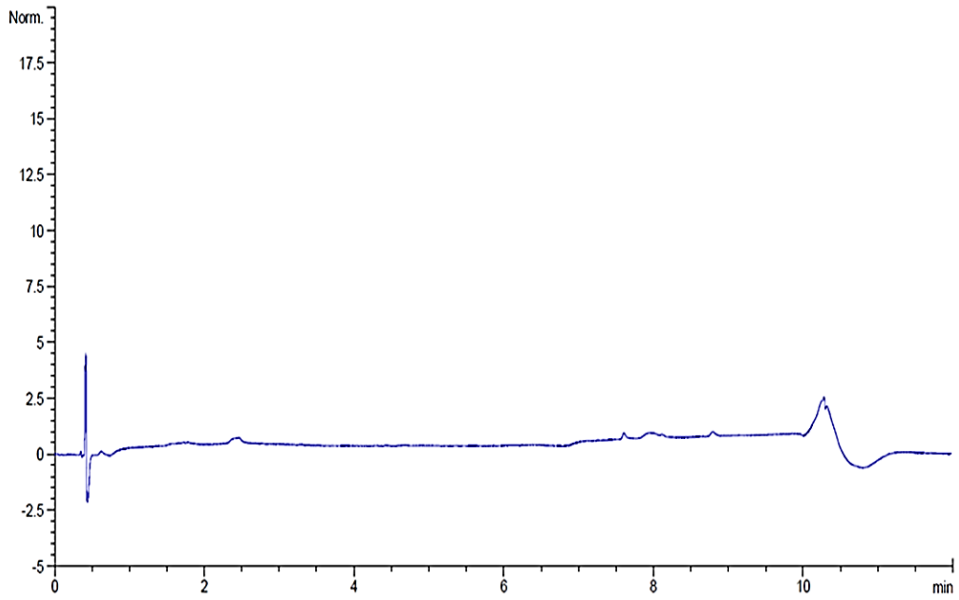



**Figure 5.** Chromatograms of the Blank Sample [78]

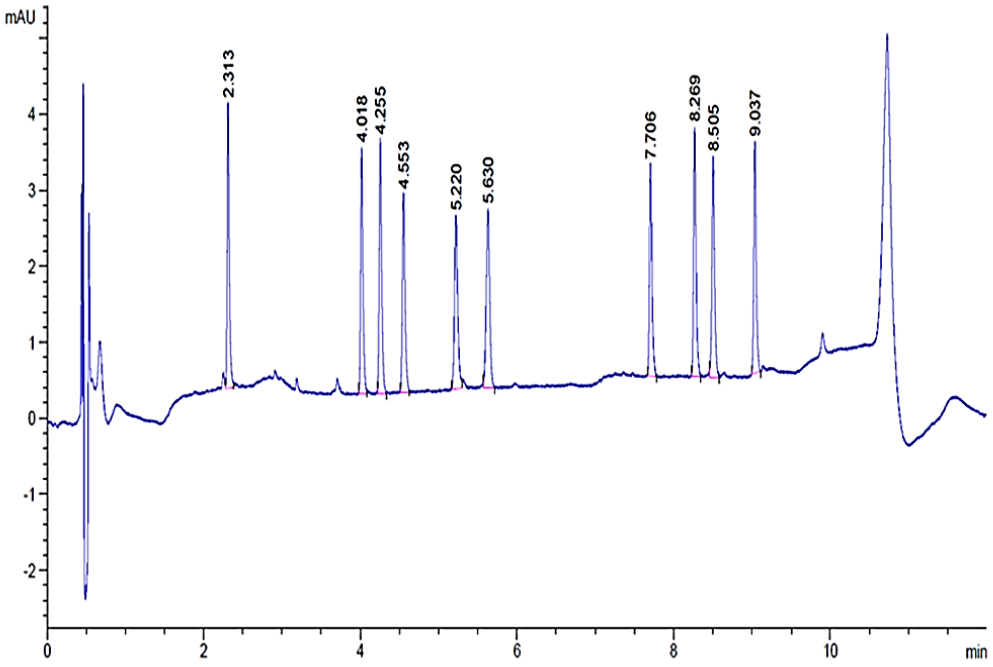



**Figure 6.** Chromatograms of the Febuxostat Standard [78]

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