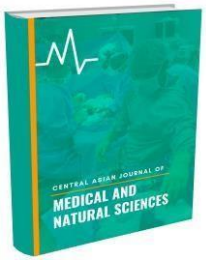

CENTRAL ASIAN JOURNAL OF MEDICAL AND NATURAL SCIENCES
https://cajmns.centralasianstudies.org/index.php/CAJMNS
*Volume: 05 Issue: 04 | October 2024 ISSN: 2660-4159*

*Article*

# Determination of Some Drugs in Blood Plasma Using Liquid Chromatography-MS

**Qasim Mezban Salih**[1]

1. College of Science, Wasit University, Wasit, Iraq
* Correspondence: kgamli@uowasit.edu.iq

**Abstract:** The accurate quantification regarding febuxostat levels in human plasma will be accomplished using a liquid chromatography - tandem mass spectrometry (LCESI-MS/MS) assay with a robust sensitivity, selectivity, and reliability. Indomethacin was used as the internal standard (IS) in the test. Using methyl tert-butyl ether as extraction solvent, the analyte as well as IS have been mined from 200µL human plasma sample using extraction procedure (liquid - liquid). A Hypurity C-18 column with particle size dimensions of 100mm x 4.6mm and a column with 5µm particle size were used in the experiment in order to perform the chromatography. Under isocratic circumstances, the chromatography has been performed. Tandem mass spectrometry, which could operate in a negative ionization reaction condition range and efficiently observe various reaction modes, has been used for the purpose of detecting the analyte and internal standard. The deprotonated precursor ions were represented by observed changes of product ions for the febuxostat and indomethacin, which have been m/z 356.10 → 312 and 315.10 → 271, respectively. This technique had demonstrated a 0.05µg/mL limit of quantitation and a 0.0025 µg/mL limit of detection. Validated linear dynamic range of febuxostat has been found to be 0.05g/mL–6g/mL. Precision, which is expressed as the coefficient of variation (% CV) within and across batches, has been determined to be ≤7.1%. It was revealed that average extraction recovery of the febuxostat at various levels of quality control has been >87%. In order to ascertain bio-equivalence of 80mg febuxostat tablet form in a group that includes 14 healthy males of origin, the current study successfully used the previously indicated methodology. The experiment was conducted under both fed and fasted circumstances. The potential for duplicating the measurements regarding study outcomes was shown by re-examining 110 incurred cases.

**Keywords:** spectrometry, febuxostat human plasma, bioequivalence, chromatography

**Citation:** Salih, Q. M. Determination of Some Drugs in Blood Plasma Using Liquid Chromatography-MS. Central Asian Journal of Medical and Natural Science 2024, 5(4), 78-92.

Received: 2nd Jun 2024
Revised: 9th Jun 2024
Accepted: 28rd Jun 2024
Published: 23rd Jul 2024



## 1. Introduction

Non-purine urate-lowering drugs, such as Febuxostat (sometimes referred to as 2-(3cyano-4iso-butoxyphenyl)-4methyl-1,3-thiazole-5-carboxylic acid), are a family of pharmaceuticals. It is often recommended to treat and control gout and hyperuricemia [1-3]. Gout can be defined as a common illness that is characterized by the hyperuricemia as well as the manifestation regarding chronic and acute consequences that are triggered through the accumulation of urate ion in body's joints and tissue [4]. The enzyme xanthine oxidase, which catalyses synthesis of uric acid from xanthine and hypoxanthine, is in fact inhibited by FEB, which is a substance [4,5].

FEB has an excellent oral bioavailability of 84% and is quickly absorbed through the gastrointestinal tract after oral administration; in healthy persons, this takes around 60 mins to reach peak plasma concentrations [2,6]. When the oral dosage of FEB is between

10 and 300 mg, the extent of dispersion shows a 29L/kg to 751L/kg range in an equilibrium state. The half-life is between 5 and 8 hours. It's highly protein-bound (99%), mainly to the albumin, and it is significantly metabolized by the liver in phase I and phase II (glucuronidation and oxidation), excreting metabolites in feces and urine after [1,7,8].

FEB is identified by HPLC in both bulk and pharmaceutical dosage forms [9–11]. For the measurement regarding FEB in biological contexts, very few methods [8,12–14] were reported, the bulk of which are based on HPLC. Khosravan *etal*. [8] and Grabovvski *etal*. [12] have used a 2-step procedure to assess the concentration of FEB in plasma. First, they used acetonitrile to precipitate proteins from 0.5mL of human plasma. They then have carried out HPLC-fluorescence analysis to ascertain concentration. The linear calibration curves for both of such techniques ranged from 0.01 to 20 μg/mL. The aforementioned curves have been then applied to a cohort of healthy individuals in order to examine the effects of meals [8], hydrochlorothiazide [12], and antacids on the pharmacokinetics and pharmacodynamics of the FEB.

A similar approach using protein precipitation was suggested with a 0.10μg/mL sensitivity [13]. Using a cohort of healthy adults, the research sought to assess FEB pharmacokinetics and bio-equivalence (80 mg) tablets. A recent research through Lukram et al. (2014) devised a method for using ultra-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) to measure content of a molecule called FEB in human blood plasma. There is a measurable limit of 0.075 g/mL with this approach. This work presents an analysis of the impurity carryover and characterization of the impurity profile using LC-MS/MS in drug substance of FEB [15].

Thus, the current work suggests a fast, accurate, and selective LC-MS/MS approach that was validated for determining amounts of FEB in the human plasma. The method only needs 200 L of human plasma for the process of liquid-liquid mining. This method's outstanding durability and efficiency are demonstrated by the fact that each sample runs in around 5 minutes. Within the range of 0.05g/mL to 6g/mL, empirical data has confirmed the existence of a dynamic linear association. The technique demonstrates selectivity toward 4 drugs, specifically aspirin, naproxen, ibuprofen, and diclofenac, and specific commonly prescribed medications amongst the participants of the study.

Two experimental procedures were used to study the phenomenon of ion suppression/enhancement: post-extraction spiking and post-column analyte infusion. Bioequivalence research on a formulation of febuxostat (80 mg) tablets was successfully carried out using the methodology proposed in this work. Fourteen male, healthy subjects from were put through both fed and fasted situations during the investigation. The investigation of measurement sample reliability is verified by means of incurred specimen reanalysis.

**Chromatography**

Chromatography can be defined as a physical separation method which includes distributing solutes or components to be split across 2 phases. While mobile phase travels in a predetermined direction, stationary phase remains constant. For isolating and identifying pharmacological and chemical constituents in complex mixes, the aforementioned analytical instrument is frequently used. It is essential that the components make contact with stationary phase for accomplishing separation and retention. The mobile phase is made up of a liquid, gas, or supercritical fluid which makes it easier for components to move through or over stationary phase. Mass spectrometer is frequently used for the compound-based quantification in the complicated mixtures and matrices. Pharmaceutical industry makes extensive use of the ion of MS or LC-MS for drug analysis.

**Mass spectrometry**

With applications in both quantitative and qualitative domains, MS has grown into a powerful analytical tool. Thanks to substantial technological advancements over the last

ten years, MS may now be utilized to analyze peptides, proteins, polysaccharides, medications, DNA, and a wide range of other biologically relevant compounds. MS has developed into a crucial tool in the study of biology as a result of ionization processes. For the measurement of mass-to-charge ratio of the molecules, MS idea entails ionizing compounds to form charged regions of molecules.

## 2. Materials and Methods

### Chemicals and materials

The equivalent standard febuxostat (100.40%) and indomethacin (99.80%) elements were supplied by Fabrica Italiana Sintetici and Zydus Cadila, respectively. We purchased ammonium acetate, HPLC-grade methanol, glacial acetic acid, and orthophosphoric acid from Mallinckrodt Baker Inc. and SD Fine Chemicals Ltd. For LC-MS/MS analysis, deionized water has been processed using Milli Q ion purifying equipment from Millipore. Human plasma with K3EDTA added was purchased from Cliantha Research Ltd. and stored at -20°C.

### Equipment and Environment of LC-MS/MS

The research employed a Japanese-manufactured Shimadzu liquid chromatography system and a Hypurity C-18 column (100mm x 4.6mm, 5μm) from Thermo Scientific, U.S. The volumetric ratio of methanol, glacial acetic acetic acid, and ammonium acetate was 70:30:0.01, respectively, in the mobile phase. Auto sampler's operating temperature has been kept at 4°C with a sample flow rate of 0.80mL/min to maintain isocratic circumstances. About 20% of the column's produced eluent has been sent toward the ISP interface in an 80:20 ratio. The ISP interface generated a flow rate of 160.0μL/min. A triple quadrupole mass spectrometer—more precisely, API3000 model made with Turbo Ion spray® technology—was used for identification and ionizing of analyte and internal standard (IS).

The negative ion mode was used to do the current analysis. Compound FEB and the IS, 356.1 to 311.0. The MS and LC parameters were all established using the Analyst program, version 1.4.2. Table 1 provides a summary of the MS key parameters. The following source-dependent variables have been maintained for IS and FEB: ISV (i.e., ion spray voltage) has been set to -4500V, gas 1 (nebulizer gas) has been set to 11psi, 500 °C was maintained for turbo heater temperature (TEM), 5 psi was set for CAD (i.e., collision activation dissociation), 12 psi has been set for the curtain gas (CUR), and -10V has been maintained for entrance potential (EP). Collision energy (CE), Focusing potential (FP), cell exit potential (CXP), and de-clustering potential (DP) are examples of compound-dependent variables that were optimized at particular voltage levels. The ideal values for FEB instrument have been -11V for CXP, -20V for CE, -200V for FP, and -30 V for DP. On the other hand, optimum values for IS instrument have been -11V for DP, -180 V for FP, -11V for CE, and -10V for CXP. Operated at a single unit resolution, quadrupoles 1 and 3 have dwell periods of 400 milliseconds for IS and 800 milliseconds for FEB.

### Plasma sample and stock preparation

Through dissolving the necessary amount in methanol, a 1000g/mL FEB common stock solution has been created. The initial solution was then diluted with the same diluent to produce an intermediate solution with a 120g/mL concentration. Indomethacin stock solutions with a 100μg/mL dosage have been accurately created by dissolution ion of necessary quantity of the indomethacin in the methanol. After careful dilution of the original (stock) solution with deionized water, a workable resolution with a concentration of 3.00 μg/mL was eventually produced. Until they were ready to be used, all of the previously described solutions were kept at a temperature of 4 degrees Celsius. At concentration levels of 0.05, 0.1, 0.2, 0.5, 1, 2, 4, 5, and 6μg/mL, the calibration standards (CS) were made. The lower limit of specimen quality quantitation (LLOQ QC) at concentrator rate of 0.05μg/mL, medium quality control MQC1 at the level of 1.8g/mL, medium quality control

MQC2 at the 0.80μg/mL level, and upper limit of specimen quality quantitation (ULOQ QC) at a concentrator rate of 0.80μg/mL were also included.

The working concentration solutions made up 5% of the total plasma volume, and those specimens have been created by the addition of corresponding utilized solutions into the blank plasma. Tables 2 and 3 include the specifics of standards that have been utilized for calibration together with the quality control samples. For purposes of validation and subsequent analyses of subject substances, plasma samples that contain spiked substances at varying concentration levels have been kept at a -20°C temperature.

**The protocol of sample preparation**

The spiked plasma samples have been warmed using a water bath in order for them to reach thermal equilibrium with surrounding ambient temperature prior to when the test was conducted. The samples were properly mixed using a vortexer prior to pouring. A screw tube system was utilized to transport 200 μL aliquots of the plasma solution. This mixture was made up of 190μL of blank plasma from FEB and 10μL of the work solution. 10 μL of methanol were added to the research samples, and 10 μL of deionized water were added to the QC and CS samples. Subsequently, 100 L of an IS effective solution (3g/mL) has been added to each one of the tubes and well mixed by using the vortexing technique. After adding 50 μL of ortho-phosphoric acid solution (2.50% v/v) to each screw tube, the tubes were vortexed. Next, 4.0 milliliters of MTBE (i.e., methyl tert-butyl ether) were added to the tubes.

**Table 1**. MS optimization Parameters

| Precursor ion to the product ion transitions that are utilized for the quantitation | | | |
|---|---|---|---|
| Analytes | Precursor ions (m/z) | Product ion (m/z) | Dwell time (msec) |
| Febuxostat | 315.10 | 271.00 | 800 |
| Indomethacin (IS) | 356.10 | 312.00 | 400 |
| Source dependent parameters for the Febuxostat and Indomethacin (IS) | | Parameters of MS detection for Febuxostat and Indomethacin (IS) | |
| Collision activation dissociation (CAD) | 5psi | Period | 1 |
| Curtain gas (CUR) | 12psi | Experiment | 1 |
| Gas1 (GS1) | 11psi | Type of the Scan | MRM |
| Gas 2 (GS2) | - | Polarity | Negative |
| Ion spray voltage (IS) | -4500 V | Ion source | Turbo Ion Spray |
| Turbo heater temperature (TEM) Interface heater (Ihe) | 500 °C<br>ON | Resolution Q-1<br><br>Resolution Q-3 | Unit<br>Unit |

| | | | | | | |
|---|---|---|---|---|---|---|
| Entrance potential (EP) | -10 V | | | | Threshold of Intensity | 0.00cps |
| Compound dependent parameters | | | | | Settling time | 0msec |
| | | | | | MR pause | 5.0070msec |
| Parameters | DP (V) | FP (V) | CE (V) | CXP (V) | Multiple channel analysis (MCA) | No |
| Febuxostat | -30 | -200 | -20 | -11 | Size of the Step | 0.00amu |
| Indomethacin (IS) | -11 | -180 | -11 | -10 | Synchronization mode | LC Sync |

After that, the tubes were shut and vortexed for a duration of three minutes. The samples then went through five minutes of centrifugation at force equal to 1,811 times the acceleration caused by gravity. After aqueous layer had solidified in a dry ice bath, organic compounds were detected. After that, the organic component layer has been gradually evaporated until it was entirely dry, at a temperature of 40 °C and an atmospheric pressure of 15 psi. A volume of 5.0 μL was then added to the LC-MS/MS model, using partial loop mode where necessary, after remaining substance had been dissolved in 400μL of the reconstitution solution.

**Validation Methodology**

A test of selectivity on 12 different blank human plasma sample batches, including both lipemic as well as haemolysed plasma samples, has been used to validate the approach. The anticoagulant agent K3EDTA was used to take the samples. The addition of 10 μL of methanol from each of the 12 different lots was applied to two replicas, each with 190 μL. Following the extraction procedure, a plasma sample free of IS and analyte was used in the initial group. The second group, on the other hand, only included the IS before the extraction process. A single sample for system's suitability has been meticulously prepared, with an average 0.1g/mL proportion at CS2. This is noteworthy. Furthermore, two different copies of LLOQ proportion have been diligently prepared at CS-1. Through adding 10 L of appropriate practical FEB solution to 190L of human plasma that was free of any substances of interest, the previously described process was executed.

Identification of potential medication interactions was carried out. Human participants in this study used a variety of medications, including acetaminophen, caffeine, pseudoephedrine, chlorpheniramine maleate, and chlorpheniramine. The ionization characteristics, ionization characteristics, and chromatographic recuperation of four drugs—aspirin, ibuprofen, diclofenac, and naproxen—were also studied in this study. The right amount of methanol was dissolved to produce stock solutions that contained 100 μg/mL. Furthermore, solutions have been provided with a solvent of methanol at a concentration of 20.0 μg/mL.

Identical solutions were then combined with plasma samples and analyzed using plasma parameters. Three separate assessments of the HQC and LQC levels were made. The sets were processed using two (eight samples) of QC samples, LQC, HQC, MQC-2, and MQC-1, as well as newly developed calibration curve standards (CS). Within the range of 85 to 115%, the percentage accuracy should meet the stipulated acceptance standards. The purpose of the current research has been investigating the changes that were seen in Multiple Reaction Monitoring (MRM) methods when they were used for four commonly used pharmaceutical substances: ibuprofen (205/161), aspirin (229/185), diclofenac (294/250), and naproxen (179/137).

The setting of the research includes a variety of injection types, including plasma samples, like LLOQ, LLOQ, and ULOQ samples, for assessing any possible interference that is caused by carryover. The approach's linearity has been evaluated with the use of

calibration curves and non-zero values. Regression ion analysis was performed using area ratio responses for the FEB / IS from the various reactions that were being watched. The LLOQ was determined by choosing the calibration curve's smallest standard, provided that analyte reaction in the recovered plasma showed a reaction at least 10 times larger than the reaction that has been seen in drug-free (i.e., the blank) sample.

The assessment of FEB's precision and accuracy involved a thorough analysis conducted over three consecutive validation days, spanning multiple batches and including both intra- and inter-batch assessments. There were six quality control levels in use: MQC-1, MQC-2, HQC, LQC, LLOQ QC, and ULOQ QC. With the possible exception of the LLOQ, which might vary by ±20%, the average precision was predicted to remain constant within ±15%. The quality control criteria must be met by at least two-thirds of the specimens, with a rigorous range of 15% with respect to the nominal concentration. Re-integrating a whole validation batch back into the system allowed for the process of verifying the reproducibility of reinject ion.

Matrix impact, recuperation, and procedural proficiency were assessed in six iterations at the LQC, HQC, MQC-2, and MQC-1 levels of the study [17]. Comparing pre-extraction spiked samples with post-extraction spiked samples at different quality control levels allowed for the evaluation of relative recovery. The entire process efficiency was determined in this case by PE (ME RE)/100. The assessment of the impact of relative matrix on analyte quantification has been carried out on eight K3EDTA plasma groups, which included samples that demonstrated both hemolysis and lipemia. With special attention to the LQC and HQC levels, a total of 4 samples have been meticulously prepared from each batch. The differences that are found between developed norms and quality controls must always stay inside a tolerance range of no more than ±15%.

The post-column analyte infusion test has been employed in order to assess effects of matrix ion suppression on sensitivity regarding MRM. LC-MS/MS [18]. A Harvard infusion pump was used to infuse a classical remedy, consisting of IS (3.00 μg/mL) and FEB (1.80 μg/mL) in mobile phase, to the mobile stage at flow rate of 5.0 μL/min. A 'T' connector was positioned after the column. Following the extraction procedure, 5.0 L aliquots were taken from plasma sample used as the double blank and the sample with a 6g/mL concentration. The LC-MS/MS equipment was then used to add such aliquots, specifically using the MRM approach. The analyte of interest, FEB, and the IS were then each given chromatograms.

The stable sample stability has been evaluated with the use of the FEB ratio response (FEB/IS) against the reference standards at the HQC and IS levels. We rigorously examined long- and short-term behavior of stock solutions of FEB and IS at both room temperature and at a controlled temperature of 4°C in order to evaluate their stability. In the case when the variation of the solutions from the nominal value was less than or equal to 10.0%, the stability of the solutions was considered good. To achieve top stability, freeze-thaw stability, stability above -20°C, and processing specimen stability at chilling and ambient temperatures, six duplicates were employed. Within 15% of the acceptance threshold is where the accuracy and CV should be. With the use of FEB at HQC level and a concentration that is 5 times higher than ULOQ, an experimentation has been conducted in order to evaluate dilution integrity. In accordance with the recently developed curve for FEB calibration, the quantification of 6 duplicated specimens has been performed by using a dilution factor of 10.

**Study design and ISR in the bio-equivalence research**

The performed bio-equivalence test has been carried out in an open-label manner by using a 2-period, 2-sequence, 2-treatment, ion, and balanced crossover design. Study goal: evaluate the oral bioequivalence regarding a reference formulation known as ULORIC® vs the test manufacturing of a generic firm that included febuxostat (80 mg) tablets. The reference formulation, made in the United States by Takeda Pharmaceuticals America, included tablets containing 80 mg of febuxostat as well. A total of 14 people in good health who were members of the population participated actively in the study. Evaluation of the

state of bioequivalence under both unfed and fed conditions has been the primary goal of this work.

Participants in the research ranged in age from 18 to 45 and had an acceptable body mass index (18.50-24.90 kg per height squared). Physical examination, age, ECG evaluation, and blood testing were among the selection criteria. Prior to the drug's delivery, participants have been asked to fast for ten hours and were given the option of a high-fat breakfast 30min. beforehand. Blood samples have been taken at various points in time, including pre- as well as post-medication periods. The plasma sample has been separated then kept at a -20$^{o}$C plasma temperature until it has been suitable for use. Version 5.2.1 of the WinNonlin software was used to estimate the pharmacokinetic variables for FEB. Sample reanalysis (ISR) was performed on 110 subject samples with the use of a computerized random selection procedure. The samples that have been acquired during the near Cmax and elimination phase were the focus of the ISR. The results acquired for identical samples were compared to data gathered before, with an acceptable ±20% deviation.

## 3. Results

### Procedure for method development

For the best choice of MS factors for IS and FEB, negative ionization was required due to the carboxylic acid group of both compounds. The masses of analyte and IS's major deprotonated precursor ions were 315.10 and 356.10, respectively. In product ion MS of FEB, the ion with highest abundance and most consistent pattern was m/z 271.0. Fig 1(a) illustrates how the dissociation of CO2 from the precursor ion elaborates the description of this particular ion. The primary synthesis for IS occurred at m/z 312.0 as a result of CO2 being removed from precursor ion (Fig1-b). To get a optimal Taylor cone and spectral response, the ideal potential of 4500 V has been maintained. Both the CAD gas and the nebulizer gas were adjusted to ensure a steady and consistent reaction. A number of investigations used three different analytical columns from Thermo Scientific—Hypurity C-8 (4.6 mm×100mm, 5μm), the Hypurity C-18 (4.6 mm×100mm ×, 5.0μm), and the BDS Hypersil C-18 (4.6 mm×100mm, 5μm)—to assist develop a dependable and successful chromatography procedure.

The Phenomenex Capcell Pak C18 column has been used in two previously used techniques [8, 12]; however, the chromatographic analysis was not graphically presented. Increased sensitivity, excellent peak shapes, reduced durat ion interference analysis, increased throughput, and the achievement of the targeted selectivity with the least amount of unwanted matrix interference have been all achieved through diligent efforts. Several volume ratios, such as 50:50, 60:40, 70:30, and 80:20, v/v, were utilized in the mobile phase of water compositions (methanol and acetonitrile). Also, such solution columns have been mixed with ammonium acetate and glacial acetic acid at concentrations varying between 2mM and 20mM.

Additionally, the flow rate effect has been investigated in a range of 0.30 to 1mL/min, a component that was crucial in guaranteeing the achievement of chromatographic peaks with appropriate forms. It was possible to achieve effective chromatography with acceptable response, peak form, and retention by using a mobile phase that included 10 millimolar ammonium acetate, 70:30:0.01 volumetric ratio of glacial acetic acid, and methanol. The mobile phase has been introduced onto a Hypurity C18 column at a flow rate of 0.8 milliliters per minute. The results that have been seen in each of the three columns showed a comparable level of comparability. However, it is noteworthy that total time surpassed 5min., particularly in the case of utilizing Hypurity C-8 column. BDS Hypersil C-18 application had produced reliable and acceptable results. It is noteworthy, nonetheless, that all quality control samples showed noticeably better retention and peak forms when Hypurity C-18 has been used. Hypurity C18 was thus selected for use in this investigation. The findings indicate that throughout a 5.0-minute run, the duration of retention for IS and FEB were 3.73 and 2.48 minutes, respectively.

For 100 injections on a comparable column, the reproducibility depending upon the retention times has been determined to be 0.70%. To reduce potential variations in the analytical procedure caused by solvent evaporation and ionization efficiency, indomethacin has been utilized as internal norm. Quantitative extraction of FEB from human plasma has been achieved by the use of the acetonitrile and protein precipitate. The response obtained, however, was discovered to be incompatible and showed evidence of ion suppression at the LQC and LLOQ levels.

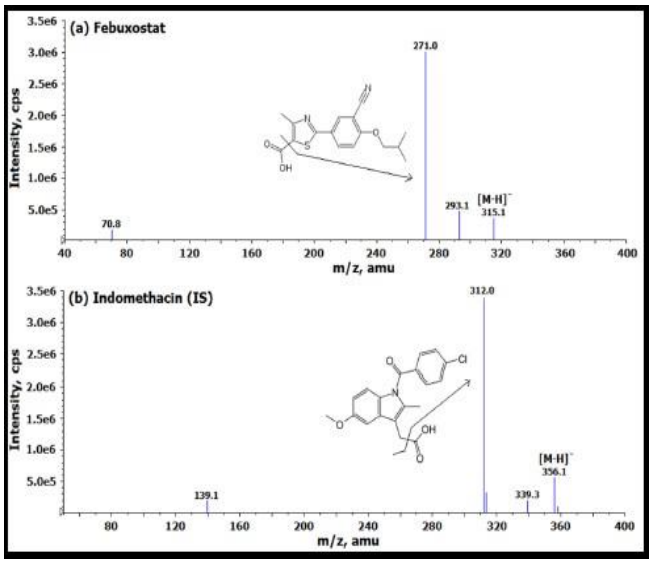


**Figure 1**. Ion MS of in negative mode of ionization (a) febuxostat (315.10 ⌾271*m*/*z*, scanning range 40amu-400amu) and (b) indomethacin (IS, 356.10⌾312*m*/*z*, scanning range 50amu-400amu)

The liquid-liquid extraction (LLE) process has been carried out with the use of several solvent systems; yet, the recovery rates that were observed were inconsistent and only ranged from roughly 50% to 60%. The incorporation of ortho-phosphoric acid has been used in order to impede drug-plasma binding, consequently guaranteeing that analyte and internal standard (IS) persisted in their non-ionized state. The recovery rates of FEB as well as IS compounds with MTBE consistently exceeded 85% across all levels of quality control, indicating a high degree of precision and reliability in contrast to alternative solvents. During the liquid-liquid extraction procedure, 200 μL of human plasma was used, which led to an adequate recovery of febuxostat. According to the research that is currently available, rate of febuxostat recovery in the UPLC-MS/MS is almost 100%. It is important to note, nonetheless, that the plasma volume used in this study's febuxostat extraction was significantly less than that of other procedures reported for the biological samples.

**Evaluation of system selectivity, carryover, suitability, and interference in the analytical approaches**

As part of validation procedure, system's suitability testing precision has been assessed depending on the employed strategy. For the time of retention and area reaction of IS and FEB components, the coefficient of variation (%CV), a precision measure, ranged from 0.14 to 0.45% and 1.2 to 2.1%, respectively. The chromatograms for double blank plasma (which lacks IS), blank plasma (which contains IS), and peak rate of FEB at LQC are shown in Fig2(a-c), which highlights results of selectivity. No indication of interference with commonly utilized drugs, including caffeine, cetrizine, pseudoephedrine, acetaminophen, and chlorpheniramine maleate, was found. Chromatograms from the study, showing effects of oral administration of an 80mg tablet of FEB after a duration of 0.75hrs, demonstrate the clarity of the situation (Figure 2d).

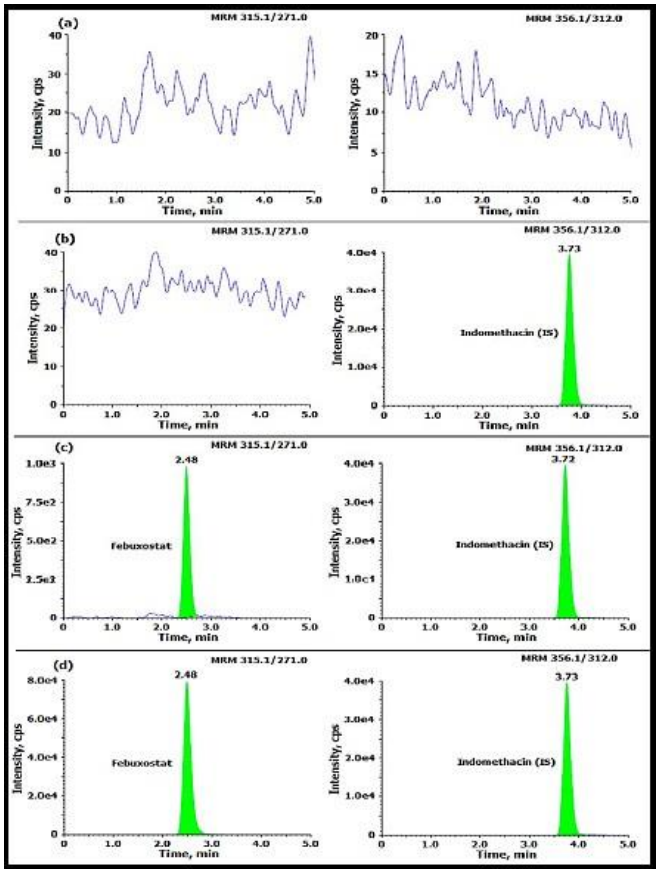


**Figure 2**. In (a) double blank plasma, (b) IS with blank plasma, (c) LLOQ-based febuxostat, (d) a tangible example after administrating 80mg dosage, corresponding ions for the indomethacin (IS, m/z356.1312.0) and febuxostat (m/z315.1271.0) can be observed in the MRM ion-chromatograms

Gout control often requires the use of anti-inflammatory drugs along with the urate-lowering medications [21]. According to results of the current investigation, none of the NSAID drugs exa mined affected the measurement of the FEB. Under ideal experimental conditions, durations of retention for the diclofenac, aspirin, naproxen, and ibuprofen were recorded as 2.81, 2.65, 3.13, and 2.20 minutes, respectively. However, the discrete MRM transitions had no effect on the quantification of FEB. For both quality control levels, the percentage accuracy metrics for FEB ranged from 97.4% to 104.3%. To ascertain whether it has any bearing on precision and accuracy of suggested method, a carry-over assessment was carried out. After ULOQ, a plasma assay has been run, and it was found that the analyte was only slightly present. This suggests that there was no residual carry-over in the future rounds. There were no noticeable peaks in the statistical analysis of a blank sample.

**Recovery, Matrix Factor, Matrix Effect, and Ion Suppression**

The information about absolute matrix effect, process-based efficiency, and relative recovery of FEB is shown in Table 2. The real recovery of an analyte is measured by measuring the reaction regarding the analyte's area ratio to sample in extracted (spiked following extraction procedure) as well as unextracted (spiked prior to extraction) samples. This recovery measure is independent of the matrix. Over 85% of the time, FEB and IS recovery rates have been seen at all quality assurance levels. Additionally, as Table 5 illustrates, matrix effect, which assesses variation in values of precision (% CV) between several batches (i.e., origins) of the plasma specimens following extraction, showed a range of 0.40-3.60 for FEB (LQC & HQC levels).

**Table 2**. Febuxostat (n=4): LQC and HQC plasma specimens that show differential matrix effects

| Plasma lots | LQC (0.15µg/mL) | HQC (4.50µg/mL) |
|---|---|---|
| | Avg. calculated conc. (%CV) | Avg, calculated conc. (%CV) |
| Lot1 | 0.15 (1.8) | 4.35 (1.3) |
| Lot2 | 0.15 (0.6) | 4.32 (1.6) |
| Lot3 | 0.15 (1.1) | 4.16 (2.4) |
| Lot4 | 0.16 (1.1) | 4.15 (0.7) |
| Lot5 | 0.15 (1.3) | 4.22(1.4) |
| Lot6 | 0.14 (0.4) | 4.25(1.5) |
| Lot7 (haemolysed) | 0.14 (1.2) | 4.09 (1.6) |
| Lot8 (lipemic) | 0.14 (3.6) | 4.19 (1.4) |
| CV represents the coefficient of variation | | |
| N represents the No. of replicates at every one of the levels | | |

The results of post-column analyte infusion testing, which are shown in Figure 3, show that there has not been any ion suppression or IS generation throughout the retention rate of FEB and IS. At 1.6 minutes, there was a small drop in the response rate, but it had no effect on the procedure of the quantification. Mean matrix factor value, which was derived through dividing rate of response of post-spiked samples by reaction from neat solution (in reconstituted solution) at a level of LQC, has been found to be 0.97, which implies a small impact of suppression of about 3%.

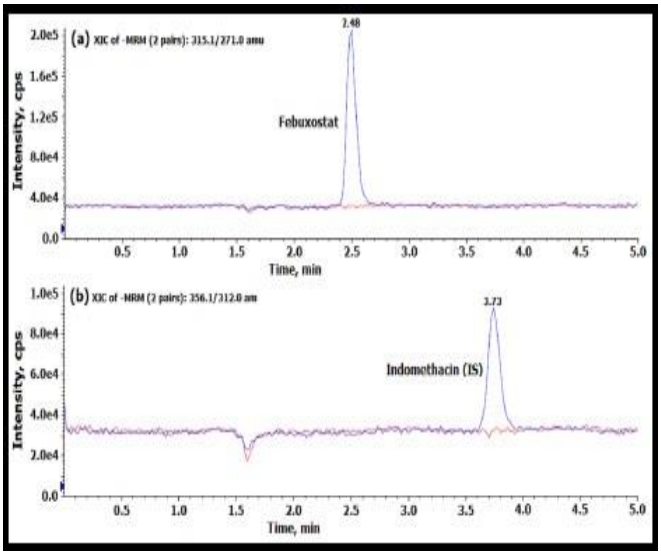


**Figure 3**. Indomethacin Chromatograms (IS, m/z356.1312.0) and febuxostat (m/z 315.1271.0) after the typical post-column analyte injection MRM LC-MS/MS overlay chromatography (a) Febuxostat's XIC chromatogram

The observed precision values for ULOQ (3.00 µg/mL) as well as HQC (0.45 µg/mL) levels of dilution integrity were found to be 2.0% and 1.5%, respectively. The accuracy results for such dilutions have been comparably determined to be 101.6 and 98.2%, respectively. It is important to note that these results demonstrate the reliability and validity of measurements because they are within the permitted range of 15% for precision and 85 to 115% for accuracy (% CV).

**Integrity with respect to Stability and Dilution**

To evaluate FEB stability in solution stock and plasma samples under a variety of the circumstances, stability studies have been executed. In the case when held at a 4°C temperature, stock solution stability has been reported to be maintained for 7 hours at room temperature. After 12 days at a 4°C temperature, the intermediate stock solution in the methanol had exhibited very little change in percentage. For a duration of 24 hours and 6 cycles of freezing and thawing, the stability of FEB has been preserved in a control-led plasma environment. The analyte's stability in extracted plasma samples has been seen to last for 96 hours at ambient temperatures and 96 hours in refrigerators at 4°C. Samples of plasma were spiked and then preserved to assure long-term viability. The samples showed stability for a duration of 75 days. The percentage fluctuations seen in each stability trial are shown in Table 3.

**Table 3**. Stability examination of the febuxostat under a variety of the conditions. (n=6)

| Benchtop Stability | Storage conditions | Level | Mean stability sample ±SD (µg/mL) | % change |
|---|---|---|---|---|
| | Room temperature (24h) | LQC | 0.150 ± 0.00190 | 0.00 |
| | | HQC | 4.690 ± 0.04260 | 4.20 |
| Constancy of extracted samples after processing | Auto sampler ($4^0$C, 96h) | LQC | 0.150 ± 0.00260 | 0.00 |
| | | HQC | 4.100 ± 0.07870 | -8.90 |
| | Room temperature (96h) | LQC | 0.150 ± 0.00130 | 0.00 |
| | | HQC | 4.120 ± 0.13890 | -8.40 |
| Constancy of extracted samples after processing | After 6th cycle at -$20^0$C | LQC | 0.150 ± 0.00060 | 0.00 |
| | | HQC | 4.260 ± 0.03800 | -5.30 |
| | 75 days at $20^0$C | LQC | 0.140 ± 0.00220 | -6.70 |
| | | HQC | 4.230 ± 0.04500 | -6.00 |

$$\%\text{Change}=\frac{Mean\ stability\ samples - Mean\ comparison\ samples}{Mean\ comparison\ samples}\times 100$$

LQC represents low quality control, SD represents standard deviation, and HQC represents high quality control.

**Approach has been tested on the healthy human subjects**

In a group that included 14 physically sound male persons of descent, a bioequivalence analysis was carried out using the validated approach. The test as well as reference formulations regarding FEB, each having an 80 mg dosage, were given to these subjects, who were also placed under both fasting and fed conditions. Figure 4 shows a visual picture of statistical representation of pharmacokinetic research investigation descriptions, 1682 samples, including calibration, volunteer samples, and quality control, were efficiently executed and thoroughly examined. Table 3 shows the mean pharmacokinetic variables that have been obtained for experimental and control formulations in the two studies. Research has indicated that the ingestion of meal causes a significant reduction in rate and volume of FEB absorption [8].

When evaluated under fasting settings as opposed to fed states, results for t 1/2 and Cmax showed a significant rise. On the other hand, the value of Tmax that was seen below the fed condition has been around 3 times higher than the value that was seen during fasting. There was a notable degree of resemblance between the pharmacokinetic variables

during the fasting state and the results published by Menon *etal*. [13] in their investigation with volunteers that have been in good health. Table 4 displays average logarithmic ratios of parameters and their related 90% CI values. The results indicate that the parameters fall within the designated range of bioequivalence. Figure 5 shows observed percentage variation in the specimens of subject that have been chosen randomly for incurred sample reanalyses. This variation was within a range of 8 to -9.7%. The assertion above supports verifiability and robustness of the suggested method.

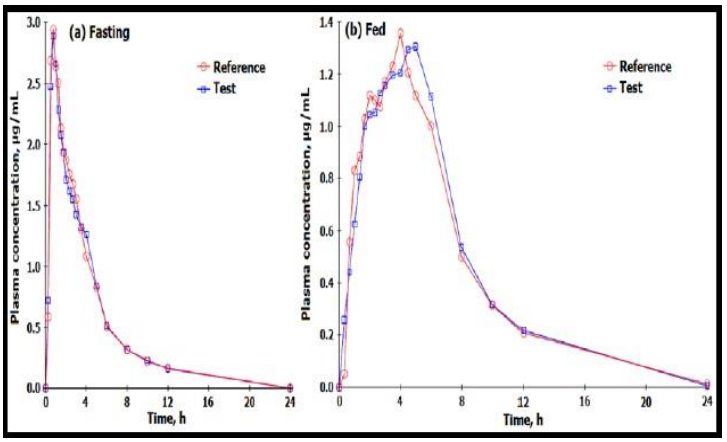


**Figure 4**. Mean febuxostat level in blood with time after it has been given orally to 14 healthy subjects that have either been eating or fasting. ULORIC® and Takeda Pharmaceuticals America, Inc. have been tested and the standard formulations

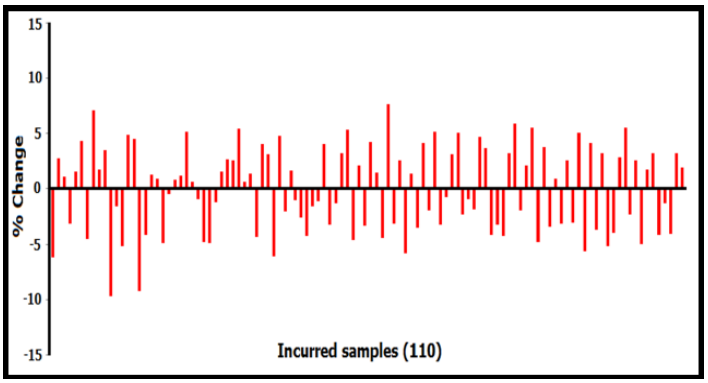


**Figure 5**. Findings for 110 incurred febuxostat samples

## 4. Accuracy and Precision

A high coefficient of correlation of r2 ≥0.9989 indicated that there was a substantial linear association between a concentration that ranges between 0.05 and 6µg/mL in all 6 calibration curves. A least squares regression analysis was performed on the data points, leading to the development of linear equation, which appropriately depicts relationship between analyte's measured concentration (x) and analyte/IS peak area ratio (y). This study yielded the mean linear equation y = (0.7626 ± 0.0378) x + (0.0040 ± 0.0035). The precision and accuracy (% CV) of calibration curve standards ranged between 1.7 and 5.3% and 95.3 to 105%, respectively. Minimum concentration (LLOQ) for accurately and precisely measuring a substance has been determined to be 0.05g/mL, ensuring an SNR of a minimum 50 and 0.0025g/Ml limit of detection (LOD). While a factor of five could potentially lower LLOQ, the examination of the subject samples may not be the primary cause. The obtained sensitivity was discovered to be 0.050µg/mL, exceeding the threshold of 0.075µg/mL (Lukram *etal*. [14]).

**Table 4**. Inter- and Intra-batch precision and accuracy for the febuxostat

| QC ID | Concentration added (µg/mL) | Intra-batch | | | | Inter-batch | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | n | Mean conc. found (µg/mL)[a] | Accuracy (%) | CV (%) | n | Mean conc. found (µg/mL)[b] | Accuracy (%) | CV (%) |
| LLO1 | .01 | 5 | 0.04 | 101.0 | 1.2 | 17 | 0.03 | 98 | 1.2 |
| LQC1 | 0.12 | 5 | 0.2 | 101.0 | 1.5 | 17 | 0.12 | 99.12 | 5.2 |
| MQC-3 | 0.78 | 5 | 0.12 | 103.5 | 0.6 | 17 | 0.54 | 95.32 | 2.1 |
| MQC-4 | 1.12 | 5 | 1.43 | 102.7 | 1.3 | 17 | 1.83 | 96.22 | 6.4 |
| HQC-1 | 3.98 | 5 | 3.3 | 103.9 | 2.3 | 17 | 3.12 | 93.22 | 7.12 |
| ULOQ-1 | 5.98 | 5 | 4.12 | 96.0 | 2.4 | 17 | 4.18 | 95.22 | 4.1 |

Summation of the observations = n

Mean of 6 duplicate measurements that have been taken at every concentration and their related variation coefficient average of 18 independent measurements that have been taken throughout three different analytical runs

Through the runs of validation at different levels of quality control, accuracy and precision within and between batches have been determined. The accuracy ranged from 97.0 to 102.9 percent, while precision within batch showed a range of 0.90-2.80 percent. The precision values showed a range of 1.6-7.1 throughout the inter-batch tests, indicating a significant degree of variability. On the other hand, the accuracy results indicated a consistent level of measurement accuracy, falling between 100.0 and 105.6% within an acceptable range.

## 5. Conclusion

There are very few methods of quantification of FEB in biological matrices described in academic literature, and there are also very few detailed descriptions of the method's creation and validation. From here on, the principal goal of this project has been to develop a sensitive, robust, and rapid method for the LC-MS/MS quantification of FEB in human plasma. For FEB, the extraction (liquid-liquid) technique used in this research produced comprehensive and dependable retrievals across all quality control levels. In comparison to all previously published methods used in the examination of human plasma, the current methodology demonstrates an increased level of sensitivity. A 5.0µL injection volume resulted in a maximum on-column loading of 15ng for ULOQ. Maintaining the durability and effectiveness of a column greatly depends on its loading capacity. Moreover, it is significant because the quantification limit is low enough to allow for careful observation of FEB concentration over a minimum of five half-lives. Excellent levels of intra- and inter-assay reproducibility are also present, as shown by the quality controls' percentage CV.

The adopted methodology demonstrated selectivity in identifying the presence of four nonsteroidal anti-inflammatory drugs (NSAIDs) and numerous commonly used medications among the human cohort pharmaceutical participants. A broad range of studies on pharmacokinetics and bioequivalence are made possible by the suggested methodology's level of ion sensitivity, which is considered adequate. The study's findings on rapid and nourished subjects show that, while food consumption slows down the rate and extent of febuxostat absorption, it has no discernible effects on the pharmacokinetics of febuxostat in a healthy cohort. Therefore, it is reasonable to use febuxostat in conjunction with or

instead of nutrition during anti-hyperuricemic therapy to treat gout symptoms. The successful use of incurred sample-based reanalysis serves as an efficient illustration of the reproducibility of the evaluation of subject specimens.

**REFERENCES**


[1] C. Han, C. B. Davis, and B. Wang, Eds., *Evaluation of Drug Candidates for Preclinical Development: Pharmacokinetics, Metabolism, Pharmaceutics, and Toxicology*, vol. 12. John Wiley & Sons, 2015.

[2] S. Zhou, Q. Song, Y. Tang, and W. Naidong, "Critical Review of Development, Validation, and Transfer for High Throughput Bioanalytical LCMS/MS Methods," *Current Pharmaceutical Analysis*, vol. 1, no. 1, pp. 3-14, 2015.

[3] N. R. Srinivas, "Applicability of Bioanalysis of Multiple Analytes in Drug Discovery and Development: Review of Select Case Studies Including Assay Development Considerations," *Biomedical Chromatography*, vol. 20, no. 5, pp. 383-414, 2016.

[4] H. C. Liu, R. H. Liu, D. L. Lin, and H. O. Ho, "Rapid Screening and Confirmation of Drugs and Toxic Compounds in Biological Specimens Using Liquid Chromatography/Ion Trap Tandem Mass Spectrometry and Automated Library Search," *Rapid Communications in Mass Spectrometry*, vol. 24, no. 1, pp. 75-84, 2017.

[5] R. Oertel, K. Richter, T. Gramatte, and W. Kirch, "Determination of Drugs in Biological Fluids by High-Performance Liquid Chromatography with On-Line Sample Processing," *Journal of Chromatography A*, vol. 797, no. 1, pp. 203-209, 2018.

[6] B. K. Matuszewski, M. L. Constanzer, and C. M. Chavez-Eng, "Strategies for the Assessment of Matrix Effect in Quantitative Bioanalytical Methods Based on HPLC-MS/MS," *Analytical Chemistry*, vol. 75, no. 13, pp. 3019-3030, 2019.

[7] M. Dole, L. L. Mack, R. L. Hines, R. C. Mobley, L. D. Ferguson, and M. D. Alice, "Molecular Beams of Macroions," *The Journal of Chemical Physics*, vol. 49, no. 5, pp. 2240-2249, 2015.

[8] M. Yamashita and J. B. Fenn, "Electrospray Ion Source: Another Variation on the Free-Jet Theme," *The Journal of Physical Chemistry*, vol. 88, no. 20, pp. 4451-4459, 2016.

[9] M. L. Aleksandrov, L. N. Gall, N. V. Krasnov, V. I. Nikolayev, V. A. Pavlenko, and V. A. Shkurov, "Extraction of Ions from Solutions at Atmospheric Pressure," *Patel C.D. References K.B.I.P.E.R. Kadi Sarva Vishwavidyalaya, Gandhinagar*, p. 433 of 450, 2017.

[10] S. Zhou, Q. Song, Y. Tang, and W. Naidong, "Critical Review of Development, Validation, and Transfer for High Throughput Bioanalytical LCMS/MS Methods," *Current Pharmaceutical Analysis*, vol. 1, no. 1, pp. 3-14, 2015.

[11] B. L. Ackermann, M. J. Berna, J. A. Eckstein, L. W. Ott, and A. K. Chaudhary, "Current Applications of Liquid Chromatography/Mass Spectrometry in Pharmaceutical Discovery After a Decade of Innovation," *Annual Review of Analytical Chemistry*, vol. 1, pp. 357-396, 2018.

[12] D. B. Robb, T. R. Covey, and A. P. Bruins, "Atmospheric Pressure Photoionization: An Ionization Method for Liquid Chromatography-Mass Spectrometry," *Analytical Chemistry*, vol. 72, no. 15, pp. 3653-3659, 2020.

[13] J. A. Syage and M. D. Evans, "Photoionization Mass Spectrometry: A Powerful New Tool for Drug Discovery," *Spectroscopy-Eugene*, vol. 16, no. 11, pp. 14-21, 2021.

[14] I. N. Papadoyannis, *HPLC in Clinical Chemistry*, Dekker, 2020.

[15] S. Quaranta, C. Woloch, A. Paccou, M. Giocanti, C. Solas, and B. Lacarelle, "Microdose Pharmacokinetics of IDX899 and IDX989, Candidate HIV1 Non-Nucleoside Reverse Transcriptase Inhibitors, Following Oral and Intravenous Administration in Healthy Male Subjects," *The Journal of Clinical Pharmacology*, vol. 49, no. 12, pp. 1408-1416, 2019.

[16] H. C. Liu, R. H. Liu, D. L. Lin, and H. O. Ho, "Rapid Screening and Confirmation of Drugs and Toxic Compounds in Biological Specimens Using Liquid Chromatography/Ion Trap Tandem Mass Spectrometry and Automated Library Search," *Rapid Communications in Mass Spectrometry*, vol. 24, no. 1, pp. 75-84, 2020.

[17] R. Oertel, K. Richter, T. Gramatte, and W. Kirch, "Determination of Drugs in Biological Fluids by High-Performance Liquid Chromatography with On-Line Sample Processing," *Journal of Chromatography A*, vol. 797, no. 1, pp. 203-209, 2021.

[18] B. K. Matuszewski, M. L. Constanzer, and C. M. Chavez-Eng, "Strategies for the Assessment of Matrix Effect in Quantitative Bioanalytical Methods Based on HPLC-MS/MS," *Analytical Chemistry*, vol. 75, no. 13, pp. 3019-3030, 2019.

[19] D. A. Wells, *High Throughput Bioanalytical Sample Preparation: Methods and Automation Strategies*, vol. 5, Elsevier Science, 2003.

[20] J. M. Dethy, B. L. Ackermann, C. Delatour, J. D. Henion, and G. A. Schultz, "Demonstration of Direct Bioanalysis of Drugs in Plasma Using Nanoelectrospray Infusion from a Silicon Chip Coupled with Tandem Mass Spectrometry," *Analytical Chemistry*, vol. 75, no. 4, pp. 805-811, 2019.

[21] L. Li, F. Liu, X. Kong, S. Su, and K. A. Li, "Investigation of a Liquid–Liquid Extraction System Based on Non-Ionic Surfactant–Salt–H2O and Mechanism of Drug Extraction," *Analytica Chimica Acta*, vol. 452, no. 2, pp. 321-328, 2022.

[22] S. S. Sagdullaev, A. Z. Sadikov, T. T. Shakirov, and R. A. Rafikov, "Liquid-Liquid Extraction Technology for Production of the Antiarrhythmic Drug Aclezin from the Above-Ground Part of Aconitum Leucostomum," 2020.

[23] M. E. Ernst and M. A. Fravel, "Febuxostat: A Selective Xanthine Oxidase/Xanthine Dehydrogenase Inhibitor for the Management of Hyperuricemia in Patients with Gout," *Clinical Therapeutics*, vol. 31, pp. 2503-2518, 2019.