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Analyitical Assay Methods for Estimation of Drugs in Human Plasma by Liquid Chromatpgraphy Tandem Mass Spectrometry

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ABSTRACT

This study comprises the use of a liquid chromatography-tandem mass spectrometry (LCESI-MS/MS) assay with robust sensitivity, reliability, and selectivity for the precise quantification of febuxostat levels in human plasma. The internal standard (IS) employed in this testing is indomethacin. The analyte and IS were mined from a 200 µL sample of human plasma through the process of extraction (liquid-liquid), utilizing methyl tert-butyl ether as the solvent used for extraction. The experiment involved conducting chromatography using a Hypurity C18 column with dimensions of 100 mm × 4.6 mm and a particle having a size of 5 µm. The chromatography was carried out under isocratic circumstances. The analyte and internal standard were detected using tandem mass spectrometry, which was capable of running in a variety of negative ionization conditions and effectively monitor different modes of reaction. The observed product ion changes for indomethacin and febuxostat were m/z356.1 → 312.0 and 315.1 → 271.0, respectively, corresponding to the deprotonated precursor ions. The method exhibited a limit of detection (LOD) of 0.0025 µg/mL and a limit of quantitation (LOQ) of 0.05 µg/mL. The validated linear dynamic range for febuxostat was determined to be 0.05-6.00 µg/mL. The precision within batches and between batches, stated as the coefficient of variation (% CV), was found to be ≤7.1%. Additionally, the average extraction recovery of febuxostat across different quality control levels was determined to be >87%. The present study effectively employed the aforementioned method to investigate the bioequivalence of an 80 mg febuxostat tablet form in a cohort of 14 healthy male subjects of origin. Both fed and fasted circumstances were used for the experiment. Re-examining 110 incurred instances demonstrated the ability to duplicate the measurements of study results.

Introduction

Febuxostat, also known as FEB or 2-(3-cyano-4-isobutoxyphenyl)-4-methyl-1,3-thiazole-5-carboxylic acid, is a pharmacological agent that belongs to the class of non-purine urate-lowering drugs. It is commonly prescribed for the purpose of treating and managing gout and hyperuricemia [1-3]. Gout represents a prevalent condition that is marked by the presence of hyperuricemia and the manifestation of acute and chronic effects triggered by the buildup of urate crystals in the body's tissue and joints [4]. FEB is indeed a substance that inhibits each of the oxidized and reductive kinds of xanthine oxidase, an enzyme that catalyses the formation of uric acid from xanthine and hypoxanthine [4,5]. FEB exhibits a commendable oral bioavailability of 84% and exhibits prompt absorption by means of the gastrointestinal tract subsequent to being taken orally, necessitating approximately 60 minutes in individuals with sound health to achieve maximum concentrations in the plasma [2, 6]. The extent of dispersion for an oral dosage ranging from 10 to 300 mg of FEB exhibits a range of 29 to 751 L/kg during a state of

equilibrium, while the duration of half-life spans from 5 to 8 hours. It possesses a high protein binding level (99%), mostly to albumin, and it undergoes significant liver metabolism in phases I and II (glucuronidation and oxidation), with metabolites being excreted in the urine and faeces after [1,7,8]. Reverse-phase HPLC is being used to identify FEB in both bulk and medicinal dose forms [9–11]. Relatively few approaches [8,12–14], the majority of which are based on HPLC, have been published for the measurement of FEB in biological environments. The concentration in plasma of FEB was determined by Grabowski et al. [12] and Khosravan et al. [8] through a two-step process. Initially, they precipitated the proteins from 0.5 mL of human plasma using acetonitrile. Subsequently, they conducted an HPLC-fluorescence evaluation to determine the concentration. Both of these methods exhibited linear calibration curves ranging from 0.01-20 μg/mL. The aforementioned curves were subsequently utilized to investigate the impact of antacid or food [8] and hydrochlorothiazide [12] on the pharmacodynamics and pharmacokinetics of FEB in a cohort composed of fit volunteers. A comparable approach utilizing precipitation of proteins has been suggested, demonstrating 0.10 μg/mL of a sensitivity [13]. The study aimed to assess the bioequivalence and pharmacokinetics of FEB (80 mg) tablets in a cohort of healthy individuals from . In a recent study conducted by Lukram et al. (2014) a technique was developed for measuring the concentration of FEB (a chemical) in human blood plasma using ultra-performance liquid chromatography-tandem MS (LC-MS/MS). The technique was shown to have a quantifiable limit of 0.075 g/mL. The present study demonstrates an investigation into the carryover of impurities and the characterization of the impurity profile in the drug substance of FEB, utilizing LC-MS/MS [15]. Therefore, the current study proposes a validated LC-MS/MS method that is reliable, selective, and rapid for measuring FEB levels in human plasma. For the liquid-liquid mining process, just 200 L of human plasma is required by the approach. Each sample only takes about 5 minutes to run with this approach, demonstrating its exceptional resilience and efficiency. The empirical evidence has substantiated the presence of a dynamic linear correlation within the range of 0.05 to 6.00 g/mL. The methodology exhibits selectivity towards four drugs, explicitly aspirin, ibuprofen, naproxen, and diclofenac, as well as certain frequently prescribed medications among the study participants. The phenomenon of ion suppression/enhancement was investigated through the implementation of two experimental techniques: post-extraction spiking and post-column infusion of the analyte. The approach suggested in this study has been effectively utilized to conduct a bioequivalence investigation on a febuxostat (80 mg) tablet formulation. The study involved 14 healthy male participants from , who were subjected to both fed and fasting conditions. Through the use of incurred specimen reanalysis, the examination of reliability in measuring study samples is confirmed.

A. Chromatography

Chromatography represents a physical separation technique that involves the distribution of components or solutes to be parted across two phases. The stationary phase is the one that does not change, whereas the mobile phase moves in a predetermined direction. The aforementioned analytical tool is commonly utilized for the purpose of separating and identifying pharmaceutical and chemical components within intricate mixtures. In order to achieve retention and separation, it is imperative for the components to come into contact with the stationary phase. The mobile phase can consist of a liquid/supercritical fluid, gas, or that facilitates the movement of components by passing through or over the stationary phase. The mass spectrometer is commonly employed for compound-based the quantification within matrices and complex mixtures. The utilization of LC-MS or MS for the purpose of analysing of drugs is widely employed within the pharmaceutical sector.

B. Mass spectrometry

MS has developed into a potent analytical tool for utilization in both qualitative and quantitative fields. Over the past decade, considerable technological advances have been made in MS,

allowing it to be used with peptides, polysaccharides, proteins, DNA, medications, as well as numerous other biologically significant compounds. Due to ionization processes, MS has evolved into a vital instrument in the field of biology. The MS concept involves ionizing compounds in order to produce charged portions of molecules and then measuring the mass-to-charge ratio of those molecules.

Material and Methods

Chemicals and materials

Fabrica Italiana Sintetici and Zydus Cadila provided the corresponding standard elements for indomethacin (99.8%) and febuxostat (100.4%), respectively. From Mallinckrodt Baker Inc. and S.D. Fine Chemicals Ltd. HPLC-grade methanol, glacial acetic acid, ammonium acetate, and orthophosphoric acid were acquired. The Milli Q water purifying system from Millipore was used to process the deionized water for the LC-MS/MS analysis. K3EDTA-added human plasma was acquired from Cliantha Research Ltd. and kept at -20°C.

LC-MS/MS Equipment and Environment

The study utilized a Shimadzu liquid chromatography scheme, manufactured in Japan, along with a Hypurity C18 column (100 mm × 4.6 mm, 5.0 µm) sourced from Thermo Scientific, USA. The mobile phase involved a 70:30:0.01 volumetric ratio of methanol, ammonium acetate (10 millimolar), and glacial acetic acid, respectively. preserving isocratic circumstances, the auto sampler's operational temperature remained at 4 °C with 0.80 mL/min of the fluid flow rate. The eluent obtained from the column was divided in a ratio of 20:80, with 20% of the eluent directed towards the ISP interface. The flow rate produced by the ISP interface was 160 µL/min. The ionizing and identification of the analyte and internal standard (IS) were conducted using a triple quadrupole mass spectrometer, specifically the API-3000 model prepared with Turbo Ion spray® technology. The present analysis has been done utilizing the negative ion mode. compound FEB and 356.1 to 311.0 for the IS. The Analyst program, version 1.4.2, was used to set all MS and LC parameters. MS key parameters are summarized in Table 1. The source-dependent variables retained for FEB and IS were depicted as: ion spray voltage (ISV) was set to -4500 V, Gas 1 (Nebulizer gas) was set at 11 psi, turbo heater temperature (TEM) was kept at 500 °C, collision activation dissociation (CAD) was set to 5 psi, curtain gas (CUR) was set to 12 psi, and entrance potential (EP) was kept at -10V. The optimization of compound-dependent variables, such as focusing potential (FP), cell exit potential (CXP), collision energy (CE), and de-clustering potential (DP) was performed at specific voltage values. For the FEB instrument, the optimized values were -200 V for FP, -30 V for DP, -11 V for CXP, and -20 V for CE. Conversely, for the IS instrument, the optimized values were -180 V for FP, -11 V for DP, -10 V for CXP, and -11 V for CE. Quadrupoles 1 and 3 were operated at a resolution of one unit, with dwell times set at 800 milliseconds for FEB and 400 milliseconds for IS.

Plasma sample and stock preparation

The 1000 g/mL FEB common stock solution was made by dissolving the required amount in methanol. The initial solution was subsequently diluted using the identical diluent, resulting in the formation of an intermediate solution with a concentration of 120 µg/mL. Stock solutions of indomethacin, with a dosage of 100 µg/mL, were meticulously prepared through the dissolution of the requisite amount of indomethacin in methanol. A viable resolution, characterized by an amount of 3.00 µg/mL, was meticulously created through the process of dilution, wherein the original (stock) solution was mixed with deionized water. All of the aforementioned solutions remained at a temperature of 4 degrees Celsius until they were ready to be utilized. The calibration standards (CS) were prepared at concentrations of 0.05, 0.10, 0.20, 0.50, 1.00, 2.00, 4.00, 5.00, and 6.00 µg/mL. In addition, quality control (QC) specimens had been generated, which encompassed the low-quality control (LQC) at a level of 0.15 µg/mL, the lower limit of quantitation quality control (LLOQ QC) at a concentration of 0.05 µg/mL, the medium quality

control MQC-1 at a level of 1.80 µg/mL, the medium quality control MQC-2 at a level of 0.80 µg/mL, the upper limit of quantitation quality control (ULOQ QC) at a concentration of 6.00 µg/mL, and the high quality control (HQC) at a concentration of 4.50 µg/mL. These specimens were prepared by adding their respective employed solutions to blank plasma, with the working concentration solutions comprising 5% of the entire volume of plasma. The specific details of the standards used for calibration as well as quality controls control specimens can be found in Table 2 and Table 3. The plasma samples containing spiked substances at various concentrations were stored at a temperature of -20 degrees Celsius for the purpose of validation and subsequent analysis of subject samples.

The protocol pertaining to the preparation of samples

Prior to performing the test, the spiked plasma samples were subjected to a warming process utilizing a water bath, allowing them to attain thermal equilibrium with the surrounding ambient temperature. Before pouring, the samples were thoroughly blended utilizing a vortexer. Screw tubes were employed for the purpose of transferring aliquots measuring 200 µL of the plasma solution. This solution consisted of 10 µL of the solution used for work and 190 µL of blank plasma obtained from the FEB. For study samples, 10 µL of methanol was added while for QC and CS samples, 10 µL of deionized water was added. Then, 100 L of an effective solution of IS (3.00 g/mL) was put into every tube and mixed well with a vortexing method. The ortho-phosphoric acid solution (2.5% v/v) was introduced to the screw tubes in a volume of 50 µL and subsequently vortexed. Afterward, a volume of 4.0 mL of methyl tert-butyl ether (MTBE) was introduced into the tubes.

Table 1: Parameters for MS optimization

Precursor ion to product ion transitions used for quantitation						
Analyte Name	**Precursor ion (m/z)**				**Product ion (m/z)**	**Dwell time (msec)**
Febuxostat	315.1				271.0	800
Indomethacin (IS)	356.1				312.0	400
Source dependent parameters for Febuxostat and Indomethacin (IS)					**MS detection parameters for Febuxostat and Indomethacin (IS)**	
Collision activation dissociation (CAD)	5 psi				**Period**	1
Curtain gas (CUR)	12 psi				**Experiment**	1
Gas 1 (GS1)	11 psi				**Scan type**	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 ON				**Resolution Q1** **Resolution Q3**	Unit Unit
Entrance potential (EP)	-10 V				**Intensity threshold**	0.00 cps
Compound dependent parameters					**Settling time**	0.0 msec
					MR pause	5.0070 msec
Parameters	**DP (V)**	**FP (V)**	**CE (V)**	**CXP (V)**	**Multiple channel analysis (MCA)**	No
Febuxostat	-30	-200	-20	-11	**Step size**	0.00 amu
Indomethacin (IS)	-11	-180	-11	-10	**Synchronization mode**	LC Sync

The tubes were then sealed and subjected to vortex mixing for a duration of 3 minutes. Subsequently, the samples underwent centrifugation at a force of 1811 times the acceleration due to gravity for a duration of 5 minutes. Organic materials have been identified after the aqueous layer became frozen in a dry ice bath. The organic component layer was then gently evaporated at an atmosphere pressure of 15 psi and at a temperature of 40 °C until it was completely dry. The remaining substance was dissolved in 400 µL of reconstitution solution, and a volume of 5.0 µL was subsequently inserted into LC-MS/MS framework, utilizing a partial loop mode when necessary.

Validation Methodology

The method's validation was conducted using a selectivity test performed on twelve distinct batches of blank human plasma, which encompassed both lipemic and haemolysed plasma samples. The samples were taken utilizing K3EDTA as an anticoagulant agent. Two replicas, each consisting of 190 µL, were subjected to the addition of methanol (10 µL) from each of the 12 distinct lots. In the initial group, the plasma sample devoid of both the analyte and IS was subjected to direct injection subsequent to the extraction process. Conversely, the second group involved the addition of solely the IS prior to the extraction step. Furthermore, it is worth noting that a solitary sample for the system's suitability was meticulously prepared, with an average proportion of 0.10 µg/mL at CS-2. Additionally, two separate copies of the LLOQ proportion were likewise diligently prepared at CS-1. The aforementioned procedure was executed by introducing 10 µL of the corresponding practical solution of FEB into 190 µL of human plasma devoid of any substances of interest. The identification of potential drug interactions was conducted. The substances utilized by human participants in this study encompassed caffeine, acetaminophen, chlorpheniramine maleate, pseudoephedrine, and cetirizine all of which are frequently employed. Furthermore, this study examined the chromatographic interference, ionization properties, and analytical recuperation of four drugs: ibuprofen, naproxen, diclofenac, and aspirin. Dissolving the appropriate amount in methanol yielded 100 µg/mL stock solutions. Moreover, solutions with a concentration of 20.0 µg/mL were equipped using methanol as the solvent. These solutions were then added to plasma samples and subjected to analysis using identical parameters. The LQC and HQC levels were assessed in triplicate. The sets were processed with recently created calibration curve standards (CS) and a pair (8 samples) of QC samples, HQC, LQC, MQC-1, MQC-2. According to the specified acceptance standards, the percentage accuracy must fall within the range of 85 to 115 percent. The current study aimed to examine the alterations observed in the Multiple Reaction Monitoring (MRM) techniques when applied in the negative ionization process mode for four frequently utilized pharmaceutical substances: naproxen (229/185), ibuprofen (205/161), aspirin (179/137), and diclofenac (294/250).

In order to evaluate any potential interference brought on by carryover, the setup of the study included several types of injections, comprising plasma samples such as LLOQ samples, LLOQ samples, and ULOQ samples. The method's linearity was assessed using non-zero levels and calibration curves. The area ratio responses for FEB/IS from manifold reactions being tracked were taken into account for regression analysis. The LLOQ was chosen by selecting the smallest standard from the calibration curve, under the condition that the analyte reaction exhibited a reaction in the retrieved plasma that was a minimum of ten times greater than the reaction that was observed in the drug-free (blank) sample.The evaluation of the accuracy and precision of FEB encompassed a comprehensive assessment across numerous batches, encompassing both intra-batch and inter-batch analyses, conducted over a span of three successive validation days. Six quality control levels were used, including LLOQ QC, ULOQ QC, MQC-2, MQC-1, LQC, and HQC. The average precision was expected to be consistent within ±15%, with the exception of the LLOQ, which might deviate by ±20%. It is imperative that no less than two-thirds of the quality control specimens exhibit adherence to the rigorous criterion of falling within a range of

±15% in relation to the nominal concentration. The process of ensuring the reproducibility of reinjection was accomplished by reintegrating a complete validation batch back into the system.

The investigation evaluated recuperation, matrix impact, and procedural proficiency at HQC, LQC MQC-1, MQC-2 levels in six repetitions [17]. The assessment of relative recovery was conducted through the comparison of pre-extraction spiked specimens with post-extraction spiked specimens at various levels of quality control. Here, PE (ME × RE)/100 determined the overall process efficiency. The evaluation of the influence of the relative matrix on the quantification of analytes was conducted over a span of eight groups of K3EDTA plasma, encompassing samples that exhibited both lipemia and hemolysis. A total of four samples were meticulously prepared from every batch, with a particular focus on both the LQC and HQC levels. It is imperative that the discrepancies observed between developed norms and quality controls remain within the confines of a tolerance range not surpassing ±15%.

The evaluation of matrix ion suppression impacts on the sensitivity of MRM. LC-MS/MS was conducted through the utilization of the post-column analyte infusion test [18]. A classical remedy, comprising FEB (1.80 µg/mL) and IS (3.00 µg/mL) in the mobile phase, was added into the mobile stage 5.0 µL/min flow rate utilizing a Harvard infusion pump, with a 'T' connector situated post the column. Subsequent to the extraction process, aliquots measuring 5.0 µL were obtained from the sample with a concentration of 6.00 µg/mL, as well as from the plasma sample serving as the double blank. These aliquots were next introduced into the LC-MS/MS system, specifically utilizing the MRM technique. Chromatograms were subsequently acquired for the analyte of interest, FEB, as well as for the IS. The stability of stable samples was assessed using area ratio response (FEB/IS) against reference standards at HQC and LQC levels. The stability of the stock solutions of IS and FEB was assessed through rigorous examination of their long-term and short-term behavior under ambient temperature conditions as well as at a controlled temperature of 4°C. The stability of solutions was deemed satisfactory when their deviation compared to the nominal value did not exceed or equal to 10.0%. Six duplicates were used for achieving top stability, processing specimen stability at ambient and chilling temperatures, freeze-thaw stability, and stability over -20°C. The accuracy and CV must be within 15% of the acceptance criterion. An experiment was carried out to assess the integrity of dilution using FEB at an HQC level, along with a concentration that was five times higher than the ULOQ. The quantification of six replicated specimens was conducted utilizing a dilution factor of 10, in accordance with the newly established curve of calibration for FEB.

Study design and ISR in bioequivalence research

The conducted bioequivalence examination was executed in an open-label manner, employing a two-sequence, two-period, two-treatment, randomized, and balanced crossover design. The objective of the study was to assess the comparative oral bioequivalence between a generic company's test manufacturing comprising febuxostat (80 mg) tablets and a reference formulation commonly referred to as ULORIC®. The reference formulation, produced by Takeda Pharmaceuticals America, USA, also consisted of 80 mg febuxostat tablets. The research encompassed the active involvement of 14 individuals of optimal health hailing from the population. The main focus of this investigation was to evaluate the state of bioequivalence in the context of both fed and unfed conditions. The study involved participants aged 18 to 45 years, with a desirable body mass index (range:18.5-24.9 kg per height squared). The selection criteria included age, physical examination, electrocardiogram assessment, and laboratory tests. Participants underwent a 10-hour fast before drug administration and were offered a high-fat breakfast 30 minutes before the drug. The samples of blood were obtained at different time intervals, encompassing both pre- and post-drug management. The plasma sample was isolated and stored at a temperature of -20°C until it was ready for utilization. The estimation of the pharmacokinetic variables for FEB was conducted employing WinNonlin software with version

5.2.1. A computerized random selection method was used for sample reanalysis (ISR) on 110 subject samples, focusing on samples taken during the near Cmax and eliminating phase. The outcomes obtained have been compared to previously collected data for samples that were identical, allowing for an acceptable deviation of ±20%.

Results and Discussion

Procedure for method development

The carboxylic acid group of FEB and IS necessitated the use of negative ionization for the optimal selection of MS factors for IS and FEB. The predominant deprotonated precursor ions of the analyte and IS had masses of 315.1 and 356.1, respectively. The ion with the highest abundance and remarkable consistency observed in FEB's product ion MS corresponded to m/z 271.0. The description of this specific ion is elaborated through the process of CO2 dissociation from the precursor ion, as shown in Figure 1(a). Due to CO2 being eliminated from the precursor ion, the main production for IS was at m/z 312.0 (Figure 1b). The optimal potential of 4500 V was kept in order to obtain a perfect Taylor cone and spectral response. For a constant and stable reaction, the CAD gas and nebulizer gas were also fine-tuned. Three different analytical columns from Thermo Scientific were used in a series of studies to help build a dependable and successful chromatography process, specifically the Hypurity C8 (4.6 mm×100 mm, 5.0 µm), Hypurity C18 (4.6mm×100 mm ×, 5.0 µm), and BDS Hypersil C18 (4.6mm×100 mm, 5.0 µm). Two previously employed methodologies [8, 12] have utilized the Phenomenex Capcell Pak C18 column; though, the chromatographic analysis was not visually presented. Diligent endeavors were undertaken to achieve heightened sensitivity accompanied by commendable peak shapes, reduced duration of analysis, enhanced throughput, and the attainment of desired selectivity while minimizing the impact of extraneous matrix interference. Several volume proportions of the mobile phase of water compositions (methanol and acetonitrile) were used for instance 50:50, 60:40, 70:30, and 80:20, v/v, etc. Furthermore, ammonium acetate and glacial acetic acid at values ranging from 2 to 20 mM were used when combined with these solution columns.

Moreover, the influence of flow rate was also examined within the range of 0.3 to 1.0 mL/min, a parameter that played a pivotal role in ensuring the attainment of chromatographic peaks with satisfactory shapes. The attainment of efficient chromatography, characterized by satisfactory response, peak shape, and retention was made feasible through the utilization of a mobile phase comprising methanol, glacial acetic acid in a volumetric ratio of 70:30:0.01, and 10 millimolar ammonium acetate. On a Hypurity C18 column, the mobile phase was implemented at a rate of flow of 0.8 ml per minute. The outcomes observed across all three columns exhibited a similar degree of comparability. Nevertheless, it is worth noting that the elapsed time exceeded five minutes specifically when employing the Hypurity C8 column. The utilization of BDS Hypersil C18 yielded consistent and satisfactory outcomes. However, it is worth noting that the retention and peak shapes were notably superior when employing Hypurity C18 for all quality control samples. Consequently, Hypurity C18 was chosen for implementation in the current study.

The duration of retention for FEB and IS was observed to be 2.48 and 3.73, respectively, during a 5.0-minute run. The reproducibility based on retention times was determined to be ≤ 0.7% for 100 injections performed on the similar column. The utilization of indomethacin as an internal norm was employed in order to mitigate potential fluctuations in the analytical process resulting from solvent evaporation and ionization efficiency. The process of extracting FEB from human plasma was conducted quantitatively through the utilization of protein precipitate with acetonitrile. Nevertheless, the response derived was found to be incompatible, demonstrating ion suppression at the levels of the LQC and LLOQ.

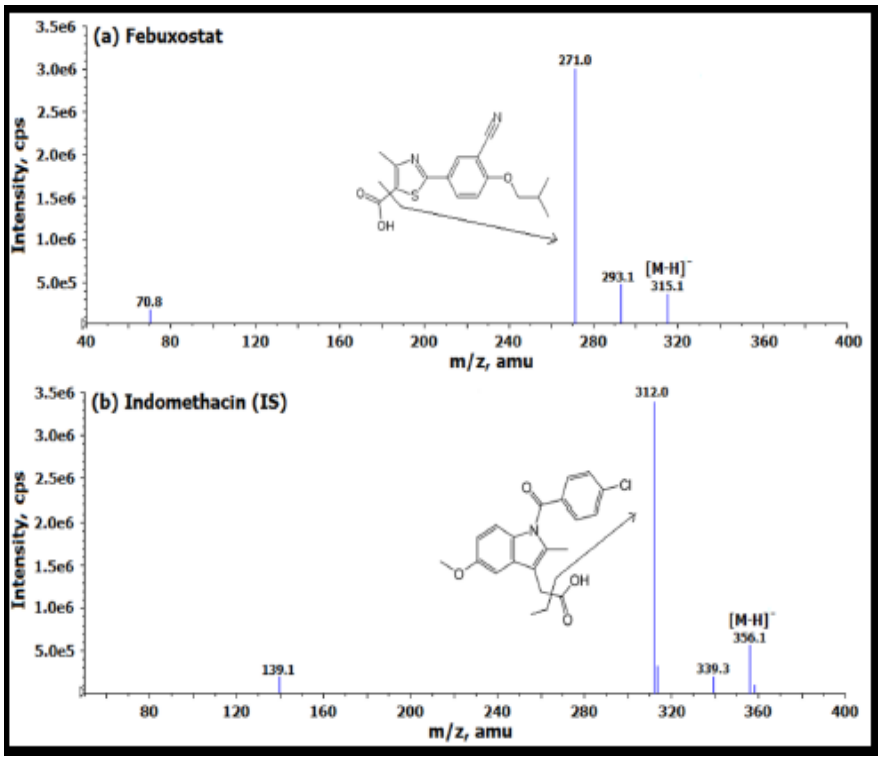

Figure1. Ion MS of in negative ionization mode (a) febuxostat (315.1 →271.0 *m/z*, scanning range 40-400 amu) and (b) indomethacin (IS, 356.1→312.0 *m/z*, scanning range 50-400 amu)

The liquid-liquid extraction (LLE) procedure was conducted using various solvent systems; however, the observed recovery rates were relatively low, ranging from approximately 50% to 60%, and exhibited inconsistency. The addition of ortho-phosphoric acid was employed to disrupt the binding between the drug and plasma, thereby ensuring that both the analyte and internal standard (IS) remained in their non-ionized form. In comparison to alternative solvents, MTBE demonstrated a high level of accuracy and reliability in recovering both FEB and IS compounds, with recovery rates consistently exceeding 85% across all quality control levels. A volume of 200 µL of human plasma was utilized in the liquid-liquid extraction process, resulting in sufficient recovery of febuxostat. In the existing research, it was observed that the febuxostat recovery rate in UPLC-MS/MS was nearly 100%. However, it is worth noting that the plasma volume utilized for febuxostat extraction in this particular investigation was comparatively smaller in comparison to other methodologies documented for biological specimens.

The assessment of system selectivity, suitability, carryover, and interference in analytical methods.

The evaluation of the system's suitability test precision was conducted as part of the validation process based on the strategy that was utilized. The coefficient of variation (%CV), which is a measure of precision, was between 0.14 and 0.45% for the duration of retention and between 1.2 and 2.1% for the area reaction of the FEB and IS components. Figure 2(a-c) illustrates the selectivity outcomes, presenting the chromatograms corresponding to the double blank plasma (lacking IS), the blank plasma (containing IS), and the peak responses of FEB at the LLQC. There was no evidence of any interference with commonly utilized medications such as caffeine, chlorpheniramine maleate, acetaminophen, pseudoephedrine, and cetrizine. The clarity of the matter becomes evident upon examination of the subject under scrutiny, namely the chromatograms obtained from the study, which depict the effects of the ingestion of an orally administered 80 mg tablet of FEB after a duration of 0.75 hours. (Figure 2d).

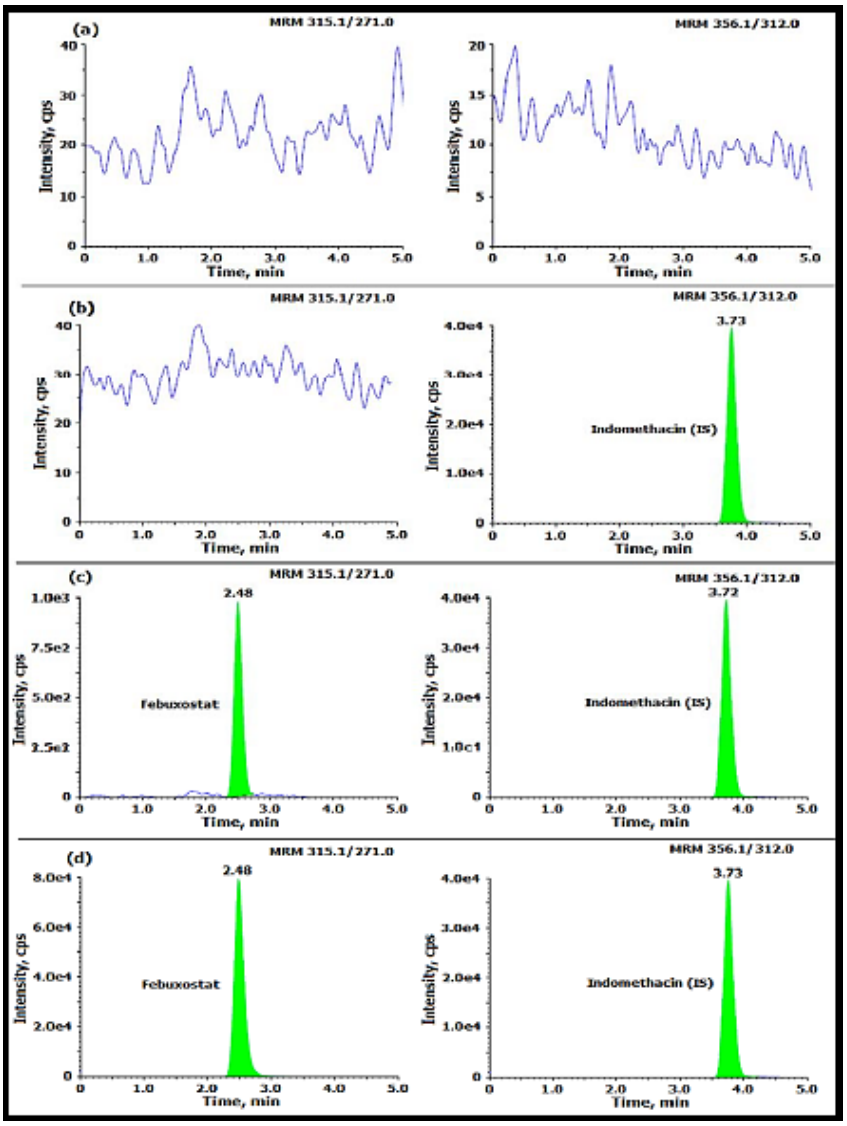

Figure 2. In (a) double blank plasma, (b) IS with blank plasma, (c) LLOQ-based febuxostat, and (d) a tangible example after administration of an 80 mg dose, the corresponding ions for indomethacin (IS, m/z 356.1312.0) and febuxostat (m/z 315.1271.0) can be seen in MRM ion-chromatograms.

The control of gout typically necessitates the utilization of anti-inflammatory medications in conjunction with urate-lowering agents [21]. The current study found that not any of the NSAIDs drugs examined had any impact on the measurement of FEB. The retention times for naproxen, ibuprofen, aspirin, and diclofenac were monitored to be 2.65, 2.81, 2.20, and 3.13 minutes, respectively, under the optimized conditions of experimentation. Nonetheless, the quantification of FEB was not affected by the distinct MRM transitions. The percentage accuracy metrics for FEB both at the quality control levels ranged from 97.4% to 104.3%. A carry-over assessment was performed to determine whether it has any effect on the accuracy and precision of the proposed approach. A subsequent plasma analysis was performed subsequent to the ULOQ, wherein it was observed that the analyte exhibited a minimal presence, thereby indicating the lack of any residual carry-over in the following iterations. The statistical evaluation of a blank sample did not yield any discernible peaks.

Recovery, Matrix Effect, Matrix Factor, and Ion Suppression

Table 2 displays the data pertaining to the relative recovery, process-based efficiency, and absolute matrix effect of FEB. The true recovery of an analyte is determined through assessing

the area ratio reaction of an analyte to the IS in both unextracted (spiked after the extraction process) and extracted (spiked before extraction) samples. This measure of recovery remains unaffected by the matrix. The recovery rates for FEB and IS were found to surpass 85% across all levels of quality assurance. Furthermore, the matrix effect, which evaluates the fluctuation of precision values (% CV) among diverse batches (origins) of plasma specimens after extraction, demonstrated an interval of 0.4-3.6 for FEB (LQC and HQC levels), as illustrated in Table 5.

Table 2: Febuxostat (n=4): LQC and HQC plasma samples showing differential matrix effect.

Plasma lots	**LQC (0.15 µg/mL)**	**HQC (4.50 µg/mL)**
	Mean calculated conc. (%CV)	**Mean calculated conc. (%CV)**
Lot-1	0.15 (1.8)	4.35 (1.3)
Lot-2	0.15 (0.6)	4.32 (1.6)
Lot-3	0.15 (1.1)	4.16 (2.4)
Lot-4	0.16 (1.1)	4.15 (0.7)
Lot-5	0.15 (1.3)	4.22(1.4)
Lot-6	0.14 (0.4)	4.25(1.5)
Lot-7 (haemolysed)	0.14 (1.2)	4.09 (1.6)
Lot-8 (lipemic)	0.14 (3.6)	4.19 (1.4)
CV: coefficient of variation n: number of replicates at each level		

The findings from the post-column analyte infusion test depicted in Figure 3 demonstrate that there is no occurrence of development or ion suppression at a time of retention of the IS and FEB. A slight decrease in the response rate was noticed at 1.6 minutes; yet, it didn't have any impact on the quantification process. The mean matrix factor value, determined by dividing the response rate of the post-spiked specimens by the reaction from the neat solution (in the reconstituted solution) at an LQC level, was found to be 0.97. This suggests a slight suppression effect of approximately 3%.

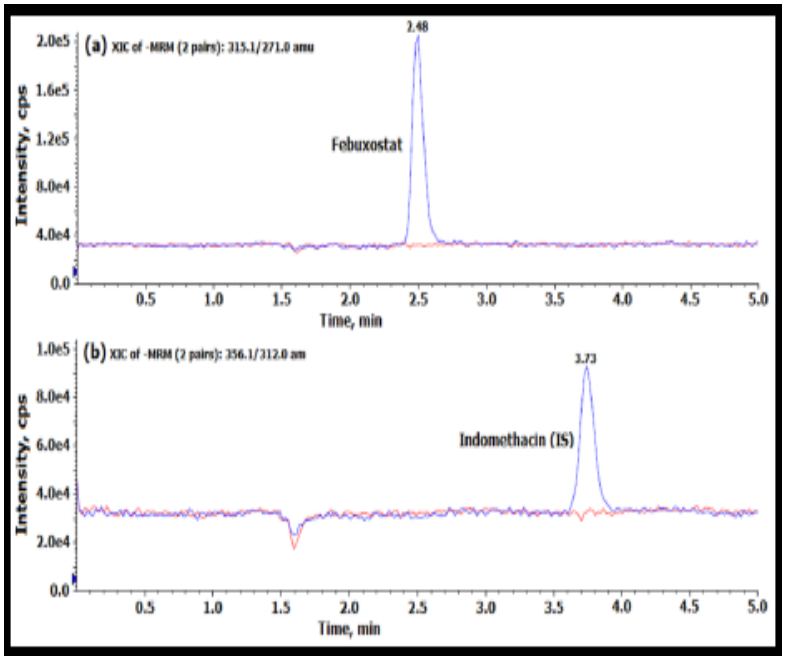

Figure 3: Chromatograms of indomethacin (IS, m/z 356.1312.0) and febuxostat (m/z315.1271.0) after typical post-column analyte injection MRM LC-MS/MS overlay chromatography (a) Febuxostat's XIC chromatogram.

The precision values pertaining to the dilution integrity of HQC (0.45 µg/mL) and ULOQ (3.00 µg/mL) level were observed to be 1.5 and 2.0%, respectively. Similarly, the accuracy outcomes for these dilutions were determined to be 98.2 and 101.6%, respectively. It is worth noting that these values fall within the acceptable range of 85 to 115 % for accuracy and 15 % for precision (% CV), thereby indicating the reliability and validity of the measurements.

Integrity with respect to Stability and Dilution

Stability studies were executed to assess the steadiness of FEB in both solution stock and samples of plasma across various circumstances. The stability of the stock solution was observed to be maintained for a duration of 7 hours at the ambient temperature and for a significantly longer period of 182 days when stored at a temperature of 4°C. The intermediate stock solution in methanol showed minimal percentage change at 4°C for 12 days. FEB's stability was maintained in a controlled plasma environment for 24 hours and six cycles of freezing and thawing. The analyte's stability in extracted plasma samples lasted 96 hours when subjected to refrigerated conditions at 4°C and 96 hours when exposed to room temperature. In order to ensure long-term viability, samples of plasma were spiked and subsequently stored, demonstrating stability for a duration of 75 days. Table 6 presents the percentage fluctuations observed in all stability experiments.

Table 3: An examination of the stability of febuxostat under diverse conditions. (n=6)

Stability of the Benchtop	**Storage condition**	**Level**	**Mean stability sample±SD (µg/mL)**	**% change**
	Room temperature (24h)	LQC	0.15 ± 0.0019	0.0
		HQC	4.69 ± 0.0426	4.2
Constancy of extracted samples after processing	Auto sampler (4^0C, 96h)	LQC	0.15 ± 0.0026	0.0
		HQC	4.10 ± 0.0787	-8.9
	Room temperature (96h)	LQC	0.15 ± 0.0013	0.0
		HQC	4.12 ± 0.1389	-8.4
Constancy of extracted samples after processing	After 6^{th} cycle at -20^0C	LQC	0.15 ± 0.0006	0.0
		HQC	4.26 ± 0.0380	-5.3
	75 days at 20^0C	LQC	0.14 ± 0.0022	-6.7
		HQC	4.23 ± 0.0450	-6.0

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

LQC: low quality control, HQC: high quality control, SD: standard deviation

Approach was tested on healthy human participants.

The method that underwent validation was employed to conduct a bioequivalence investigation of FEB in a cohort of 14 physically sound male individuals of descent. These subjects were administered both the test and reference formulations of FEB, each containing a dosage of 80 mg while being subjected to both fed and fasting conditions. The statistical representation of the pharmacokinetic descriptions for the research investigations is visually depicted in Figure 4. A total of 1682 samples, encompassing calibration, quality control, and volunteer samples, were effectively executed and subjected to comprehensive analysis. The average pharmacokinetic variables acquired for the experimental and control formulations in both investigations are displayed in Table 3.Studies have demonstrated that the consumption of food leads to a notable reduction in both the speed and magnitude of the absorption process of FEB [8]. The results obtained for t1/2 and Cmax exhibited a notable increase when assessed under fasting conditions

in comparison to fed states. Conversely, the value of Tmax observed below fed state was approximately threefold greater than its fasting counterpart. The pharmacokinetic variables observed during the fasting state exhibited a remarkable degree of similarity to the findings reported by Menon et al. [13] in their study involving volunteers who were in good health. The calculated average logarithmic ratios of the parameters, along with their corresponding 90% confidence intervals, were found to fall within the specified range of bioequivalence, as represented in Table 8. The observed percentage variation in the subject specimens selected at random for the purpose of incurred sample reanalysis fell within the range of 8.0 to -9.7%, as visually depicted in Figure 5. The aforementioned statement substantiates the verifiability and resilience of the proposed methodology.

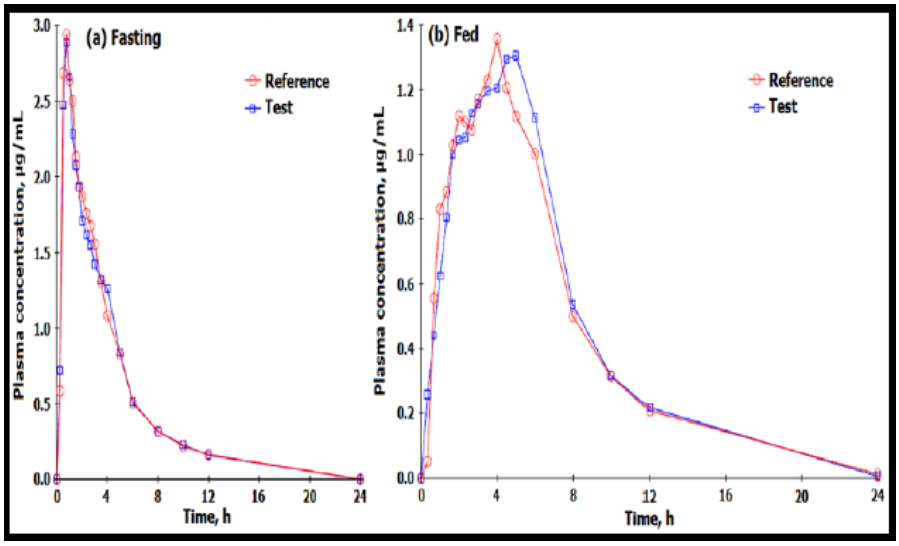

Figure 4 The average level of febuxostat in the blood over time after it was given by mouth to 14 healthy people who were either fasting or eating. ULORIC® and Takeda Pharmaceuticals America, Inc. were the test and standard formulations.

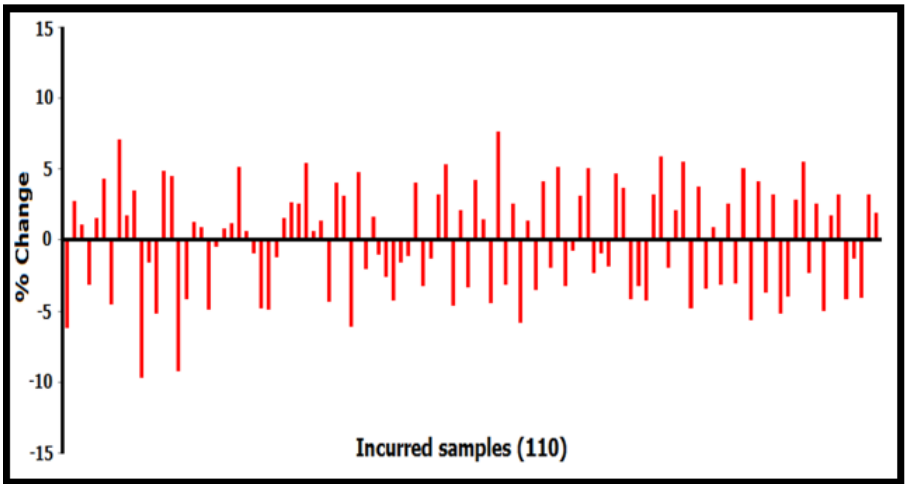

Figure 5: Findings for 110 incurred specimens of febuxostat

Accuracy and Precsion

A concentration ranging from 0.05 – 6.00 µg/mL was found to have a significant linear relationship in all six curves of calibration, as evidenced by a high correlation coefficient of r2 ≥0.9989. The data points were subjected to a least square regression study, resulting in the establishment of a linear equation that accurately represents the relation between the measured concentration of the analyte (denoted as x) and the peak area ratio for the analyte/IS (denoted as y). The mean linear equation obtained from this analysis is y = (0.7626 ± 0.0378) x + (0.0040 ±

0.0035). The calibration curve standards exhibited a range of precision and accuracy (% CV) spanning from 1.7 to 5.3% and 95.3 to 105.0 %, respectively. The minimum concentration (LLOQ) for reliably quantifying a substance with satisfactory accuracy and precision was determined to be 0.05 µg/mL, ensuring a SNR of at least 50 and 0.0025 µg/Ml limit of detection (LOD). Although there was potential to decrease the LLOQ by a factor of five, it was not necessarily due to the subject samples' analysis. The sensitivity attained was found to be 0.05µg/mL, surpassing that of 0.075 µg/mL (Lukram et al. [14]).

Table 4: Inter-batch and Intra-batch precision and accuracy for febuxostat

QC ID	Conc. 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

sum of observations = n

Mean of six duplicate measurements taken at each concentration and their associated coefficient of variation baverage of 18 independent measurements taken throughout 3 separate analytical runs

The precision and accuracy within and between batches were determined through validation runs at various quality control levels. The precision within the batch exhibited a range of 0.9-2.8, whereas the accuracy displayed a range of 97.0-102.9%. During the inter-batch examinations, the precision values exhibited a range of 1.6-7.1, suggesting a notable degree of variability. Conversely, the accuracy values fell within an acceptable range of 100.0 to 105.6%, suggesting a uniform degree of accuracy in measurement.

Conclusion

There exists a paucity of techniques documented in scholarly literature pertaining to the quantification of FEB in biological matrices, accompanied by a dearth of comprehensive details regarding the process of method establishment and validation. Henceforth, the primary aim of this endeavor was to devise a discerning, resilient, and expeditious technique for the quantification of FEB in human-based plasma via LC-MS/MS. The extraction (liquid-liquid) method utilized in the current study yielded reliable and complete retrievals for FEB across all quality control levels. The present methodology exhibits a heightened level of sensitivity in relation to all previously documented methodologies employed in the analysis of human plasma. The ULOQ exhibited a maximum on-column loading of 15 ng when utilizing a 5.0 µL injection volume. The loading capacity of a column is of utmost importance in maintaining both its efficiency and durability. Furthermore, it is noteworthy that the limit of quantification is sufficiently low, enabling the vigilant monitoring of the concentration of FEB for a minimum of five half-lives. This is accompanied by commendable levels of inter and intra-assay reproducibility, as indicated by the % CV for the quality controls. The methodology employed exhibited selectivity in detecting the presence of four NSAIDs as well as several frequently utilized pharmaceuticals within the cohort of human participants. The proposed methodology exhibits a level of sensitivity that is deemed sufficient to facilitate a diverse array of investigations pertaining to pharmacokinetics and bioequivalence. The conducted investigation pertaining to rapid and nourished circumstances

demonstrates that while the consumption of sustenance induces a reduction in the pace and scope of febuxostat assimilation, it does not engender any noteworthy alterations in the pharmacokinetics of febuxostat among a cohort of healthy participants. Hence, it is plausible to administer febuxostat with or without sustenance in the course of anti-hyperuricemic therapy aimed at ameliorating the symptoms of gout. The efficient demonstration of reproducibility in the evaluation of subject specimens is effectively exemplified through the utilization of incurred sample-based reanalysis.

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