ECRONICON OPEN ACCESS

EC PHARMACOLOGY AND TOXICOLOGY
Research Article

Determination of Novel Phase I Metabolite of Febuxostat Followed by *In Vitro*, *In Vivo* Pharmacokinetic and *In Silico* Studies

Kapendra Sahu[1,2], Anees A Siddiqui[1]* and Mohammad Shaharyar[1]

[1]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Jamia Hamdard (Hamdard University), Hamdard Nagar, New Delhi, India
[2]National Dope Testing Laboratory (MYAS), New Delhi, India

***Corresponding Author**: Anees A Siddiqui, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Jamia Hamdard (Hamdard University), Hamdard Nagar, New Delhi, India.

Received: July 21, 2017; **Published:** September 07, 2017

Abstract

A selective and fast technique was developed for the determination of phase I metabolites in human liver microsomal preparation using ultra performance liquid chromatography–tandem mass spectrometry. The metabolism of febuxostat was examined using human liver Microsomes (HLMs). The resulting four metabolites (M1-M4) were detected; out of those solely M3 was not reported. The kinetic of M3 formation were calculated at zero to fifty minutes in HLMs. Moreover, a validated method of M3 in rat plasma was developed and with success applied on pharmacokinetic studies. Additionally, *in silico* metabolic and toxicity studies were carried out to establish the M3 metabolite and toxicity respectively. Furthermore, the *in silico* toxicity studies showed that M3 is not probably to exhibit toxicity.

Keywords: *Febuxostat Metabolism; Human Liver Microsomes; UPLC-MS/MS; In Vitro; In Vivo; Pharmacokinetics*

Introduction

The development of a replacement bioactive molecule is complicated, time intense and extremely expensive and additionally the developed medication could suffer from unwanted facet effects, resistance, toxicity etc. It is the first thought of someone within the pharmaceutical chemistry field to develop potent and safe drugs. Fashionable drug discovery may be a multidisciplinary enterprise consisting of disease-based target recognition and validation in colligation with high throughput screening (HTS) of natural and chemical product libraries. This is often followed by the careful improvement of elite lead compounds, *in vitro* and *in vivo* pharmacokinetics, toxicokinetic studies and bioavailability testing leading, finally, to diagnosis and clinical studies [1-3].

In last decades, screening for toxicity of the drug discovery method, if effective, would presumptively comb out such failures and alter medicative chemists to focus their lead improvement programs on drug candidates with acceptable levels of each bioactivity and toxicity. Unluckily, there has been a scarcity of *in vitro* technologies that have the requisite output to deal with early-stage pharmacological medicine which will adequately mimic human metabolism to predict the chance of drug candidate toxicity [3]. Absorption distribution metabolism excretion-Toxicity (ADME-Tox) property of molecules is a very important parameter for the success of some compounds in preclinical and clinical studies. Furthermore, *In silico* ways to predict toxicity and Absorption Distribution Metabolism Excretion (ADME) properties even before a drug candidate was synthesized is wide employed in drug discovery to know the properties that square measure necessary to convert leads into smart medicines, that increase the success rate associated with the preceding, the synthesized compounds were subjected to *in silico* metabolism and toxicity studies so as to predict their metabolic and toxicity profile [1,4]. A preliminary plan of the metabolic and toxic profile of a compound may be of nice facilitate in assessing the suitableness of the planned compounds as a probable medicament candidate and still on avoid the presence functionalities which may cause toxic effects [5].

Gout is a common illness among old individuals and its incidence will elevate yearly. It is an illness characterized by acute joint inflammation ensuring from the deposition of salt crystals. Uric acid is the outcome of hypoxanthine metabolism to xanthine, catalyzed by the enzyme named xanthine oxidase [4,6,7]. Febuxostat, 2-[3-cyano-4-(2-methylpropoxy)phenyl]-4-methylthiazole- 5-carboxylic acid (Figure 1a), is a novel selective non-purine inhibitor of Xanthine Oxidase and was approved by the United States of America, Food and Drug Administration (FDA) in 2009 for the management of hyperuricemia in older-age. In 1964, It is the primary agent approved within the USA for the treatment of gout since allopurinol was first approved [8]. For scouting for alternative metabolites of febuxostat, MetabolExpert of the CompuDrug International, Inc. (South San Francisco, USA) were used. Many metabolites (Figures 1b-1l) were foreseen once febuxostat was subjected to *in silico* metabolism. However, solely the existence of M2-M3 (Figure 1c and 1f) was proven by computer modelling on MetabolExpert.

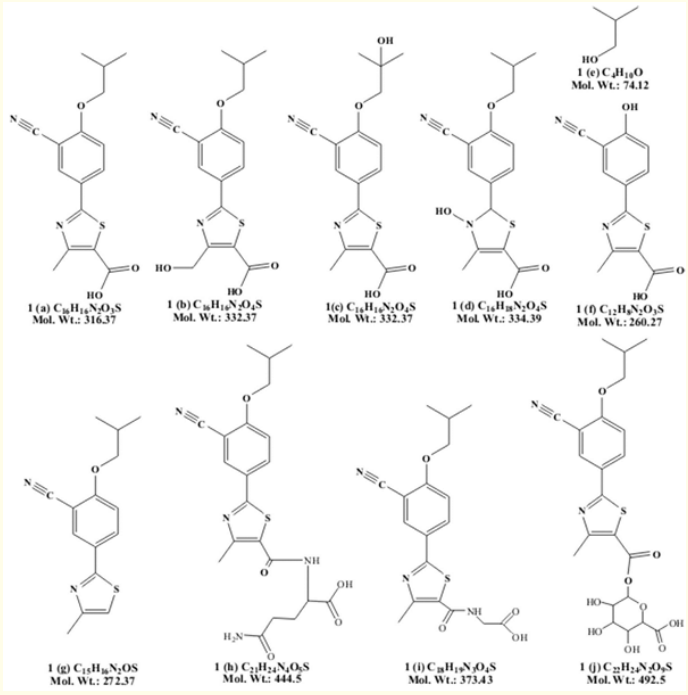

Figure 1: *The chemical structure of febuxostat (1a) and the tentative metabolites (1b-1j).*

Recently, some methods on LC-MS/MS were developed and validated [9,10]. However, in these methods, a new formed metabolite (M3) of phase I metabolism is not reported. It may be due to lack of sensitivity. In the present study, a sensitive and selective method for febuxostat metabolites including M3, which is not reported yet were developed and validated as per USFDA guidelines. Moreover, Triple quadrupole mass spectrometers coupled with UPLC have tried to be extraordinarily powerful in quantitative drug metabolism studies owing to their selectivity, sensitivity and simple of use [11,12]. Frequently introduced quadruple-time of flight (ESI-Q-TOF) mass spectrometers has clear benefits of MS/MS analysis [12-16]. Further, these instruments permit correct mass measurements within the MS and MS/MS modes that facilitate information for interpretation [17,18]. Based on the supported facts, the present study describes the development of speedy separations for the identification of metabolites in microsomal (*in vitro*)/ pharmacokinetics (*in vivo*) samples by LC-MS/MS. Additionally, the pharmacokinetics of new metabolite M3 was performed. Moreover, the proposed metabolic pathways were developed with the assistance of MS/MS analysis.

Experimental

Chemicals and Reagents

Febuxostat was obtained as a gratis sample from Micro Labs Ltd (Banglore, India). The di hydroxyl metabolite (M3) used in the study was synthesized in our laboratory. Pooled human liver microsomes from 30 donors were purchased from BD Gentest (Woburn, MA, USA). Midazolam and 6-Hydroxy midazolam were procured from Sigma-Aldrich Co. (St Louis, MO, USA). HPLC-grade methanol and acetonitrile were purchased from the Merck Co. (Darmstadt, Germany). Methanol, acetonitrile and Glacial acetic acid (GAA) were purchased from Merck limited (Mumbai, India). Tris-HCl, KCl, ammonium acetate, magnesium chloride and ethyl acetate were all purchased from Merck (Germany). NADPH reduced tetrasodium salt was obtained from Sisco Research Laboratories, (Mumbai, India). All other chemicals for this study were of analytical grade and utilized without further purification. Flash column chromatography (CombiFlash Rf 200i, Teledyne, USA) was performed on silica gel (200 - 300 mesh), using chloroform:methanol (40:60) mixtures at a flow rate of 10 mL/min. The ^{1}H NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer with DMSO-d_6 as the solvent and TMS as the internal standard. Prior approval from the Institutional Animal Ethics Committee (IAEC), Jamia Hamdard (Approval No. 899) was sought for care and experimental studies with animals. All experiments, euthanasia, and disposal of carcasses were performed in accordance with the rules arranged by IAEC for animal experimentation.

Instrumentation and chromatographic conditions

Ultra-performance liquid chromatography (UPLC)

UPLC was equipped with a binary solvent delivery pump, an auto sampler with photodiode array (PDA) detector of acquity UPLC system manufactured by Waters Corporation (Milford, MA, USA); data were acquired and processed using Empower software. The chromatographic separation of the analyte and metabolite was carried out using a Waters Acquity BEH 150 x 2.1 mm, 1.7 µm, C18 column. The column was heated to 30ºC and 5 µL of the sample were injected. The composition of mobile phases was acetonitrile-10 mM ammonium acetate buffer (pH 5.0) within the ratio of 60:40. The optimum flow rate was 0.2 ml/min. The eluent from the column was additionally monitored by PDA detector at 310 nm. The retention times (RT), relative retention times (RRT) for febuxostat and its metabolite are given in table 1.

Quadrupole-Time of Flight-Mass Spectrometry (ESI-Q-TOF-MS)

Mass spectrometry (UPLC-ESI-Q-TOF) was carried out on a Waters Q-TOF Premier (Micromass MS Technologies, Manchester, UK) mass spectrometer, in which LC part consist of UPLC (Waters). The nebulization gas was set to 550 L/h, the cone gas was set to 55 L h^{-1} and the source temperature was set to 105ºC. The Q-TOF Premier was operated in reflectron mode with a resolution of 8600 mass with 1.0 min scan time and 0.02s inter-scan delay. The accurate mass and composition for the precursor ions and the fragment ions were determined using the MassLynx V 4.1 software in build in the system. Argon gas was used as the collision gas at a pressure of 5.3 x 10^{-5} torr. The capillary voltage and sample cone voltage were set to 3.0 KV and 40 V, respectively. Quantitation of analytes was performed utilizing Synapt mass spectrometer (Q-TOF) of the transitions of febuxostat and its metabolite is given in table 2.

Working solutions and samples preparation

Microsomal studies

The stock solution of 1 mg/ml concentration for febuxostat was ready by dissolving 1 mg of the analyte, made up to a final volume of 1 ml in methyl alcohol (methanol). Working standard solutions of febuxostat was prepared by combining the aliquots of every primary stock solution and diluting with methanol. Calibration standards of febuxostat (2, 5, 10, 25, 50, 100 and 200 ng/mL) were prepared by spiking the working standard solutions into incubation mixture. Calibration standards were prepared by spiking of pooled human liver microsomes with the appropriate working solution of febuxostat on the day of analysis.

All the stock solutions were stored at -4˚C until analysis. Quality control (QC) samples were prepared by individually spiking control rat plasma at four concentration levels [0.8 ng/mL (lower limit of quantitation, LLOQ), 3 ng/mL (QC low), 80 ng/mL (QC medium) and 180 ng/mL (QC high)] and stored at −75 ± 10˚C until analysis.

Pharmacokinetics studies for metabolite (M3)

The primary stock solutions of metabolite (M3, 1 mg/ml) were prepared by dissolving one milligram of analyte in water. Working standard solutions of M3 were prepared by adding the aliquots of every primary stock solution and diluting with water. Calibration standards of M3 (2, 5, 10, 25, 50, 75, 100 and 200 ng/ml) were prepared by spiking the working standard solutions into pooled blank rat plasma. All the stock solutions were stored at 4˚C until analysis. Quality control (QC) samples were developed by individually spiking control rat plasma at three concentration levels [3 ng/ml (QC low), 80 ng/ml (QC medium) and 180 ng/ml (QC high)] and stored at −75 ± 10˚C until analysis.

Sample preparation for pharmacokinetic studies

A simple protein precipitation extraction method using methanol as a precipitating solvent was followed by the extraction of febuxostat from rat plasma. To 100 µl of plasma in a tube, next a 400 µl aliquot of extraction solvent, methanol was added. The mixture was then vortexes for 5min, accompanied by centrifugation for 5 min at 2000g at 20°C on Sigma 3-16 K (Frankfurt, Germany). The supernatant organic layer (300 µl) was separated and evaporated to dryness under vacuum in speedvac concentrator (Savant Instrument, Farmingdale, New York, USA). The residue was reconstituted in 100 µL of the mobile phase and 5 µL was injected into the UPLC-MS/MS system for analysis.

***In vitro* study**

The metabolite formation of febuxostat was carried out employing human liver microsomes (HLMs). The study was performed in triplicate at a final reaction volume of 500 µL. The incubation temperature of the microsomal mixture containing human liver microsomes was 37˚C followed by bench-top Lab-Line shaker. The incubation mixture consist of 0.5 mg protein/ml microsomes, 5 mM $MgCl_2$, 2 mM NADPH 50 mM Tris-HCl buffer (pH 7.4) and 50 µM febuxostat in a final volume of 500 µl. The enzyme reaction was initiated by spiking NADPH followed by pre-incubation of 10 minutes. The reaction was terminated by spiking 2 ml of cold ethyl acetate at different time intervals viz., 10, 20, 30, 40 and 50 min. The solution was vortex-mixed and centrifuged at 4˚C for 10 min at 3500 rpm. The supernatant was transferred to a test tube and evaporated to dryness under vacuum in the speedvac concentrator. The residue was reconstituted in 100 µL of the mobile phase, and 5µl of this solution was injected to MS analysis. Further, the controls groups were checked viz., vehicle control (Microsomes incubated without the febuxostat but with vehicle), negative control (Microsomes combined with the febuxostat, and then ethyl acetate had added before the NADPH) and metabolic positive control (midazolam metabolism to 6-hydroxy midazolam effective in the presence of cyp3A4) [19-21].

***In vivo* study**

The pharmacokinetic (PK) studies of M3 were performed in healthy male Wistar rats (n = 5 per time point) utilizing weight range of 210–230 g. Overnight fasted rats, febuxostat were administered orally at a dose of 5 mg/kg in 0.25% sodium carboxy methyl cellulose (CMC) and 5% tween 80 suspensions. Blood samples (Approx. 200 µL) were collected from the retro-orbital plexus of rats under light ether anesthesia into microfuge tubes containing heparin as an anti-coagulant at 0.5, 1, 2, 3, 4, 5, 8, 10, 12 and 24 h post-dosing. Rat plasma was harvested by centrifuging the blood at 12000 rpm for 10 min and stored at -20°C until analysis. The data was accepted based on the performance of QCs prepared to use rat blank plasma (three QCs each at three concentration levels). The criteria for acceptance of the analytical runs comprehended given by USFDA guidance viz., (1) not higher than 33% of the QC samples were greater than 15% of the nominal concentration; (2) not less than 50% at each QC concentration level must meet the acceptance criteria. Plasma concentration-time data of febuxostat metabolite M3 were analyzed by the non-compartmental method employing WinNonlin Version 5.1 (Pharsight Corporation, Mountain View, California, USA) [17,22,23].

Application to *In vitro* metabolic stability

The *in vitro* metabolic stability of febuxostat was executed using HLMs. The triplicate glass tubes contain reaction volume of 500 µL. Incubation of Febuxostat with human liver Microsomes was carried out at 37°C in a bench-top Lab-Line shaker. The incubation solution contained 100 mM potassium phosphate buffer (pH 7.4), 0.5 mg protein/mL microsomes, 5 mM $MgCl_2$, 2 mM NADPH and 50 µM Febuxostat in a final volume of 500 µL. The enzyme reaction was initiated by adding NADPH after an initial 10-min pre-incubation. The reaction was terminated by adding 2 mL of ice-cold ethyl acetate (contain 200 ng/mL I.S) at different interval 0, 15, 30, 45, 60, 90 and 120 minutes. The solution was vortex-mixed and centrifuged at 4°C for 10 min at 3500 rpm. The supernatant was transferred to a test tube and evaporated to dryness under vacuum in speedvac concentrator. The residue was reconstituted in 100 µL of mobile phase and 5 µl of this solution was injected to UPLC. Percent drug remaining versus time graph were plotted and resulting parameters like rate constant (K) and half-life ($t_{1/2}$) were determined using graph pad prism version 5.1 [11,14,20,21].

***In silico* metabolites and toxicity prediction**

The metabolites and also the toxicity profile of the molecules were foreseen by the computational method using MetabolExpert (Pallas 3.8.1.2). A well-established practice of prediction of drug metabolism is that the use of *in silico* methods for the expected potential metabolic pathway. The likely MetabolExpert program employ for metabolite identification usually screens for potential metabolites on the premise of notable metabolic alterations including steric prospects of any drug candidate [1,2,24,25].

Synthesis of M3 by dealkylation of febuxostat

To a solution of BBr_3 (1 ml, 1.0 M) and febuxostat (315 mg, 1.0 M) in CH_2Cl_2 (1 mL) was added at 40ºC (Figure S2). The mixture was stirred for 1.5 - 2 Hrs and monitored by TLC. After the starting material (febuxostat) had been completely consumed, add water (4ml) then basify at pH 8.0 by sodium bicarbonate. The mixture was extracted with 5ml Dichloromethane (DCM). The combined organic layers were washed with aq. Na_2CO_3 and then with saturated NaCl. The obtained organic layer was dried over anhydrous Na_2SO_4. The solvent had been evaporated under vacuum, the residue was purified by flash column chromatography on silica gel to give the desired product (M3). The successful dealkylation of the febuxostat with the boron tribromide was verified using 1H NMR and ESI-MS (Figure S3).

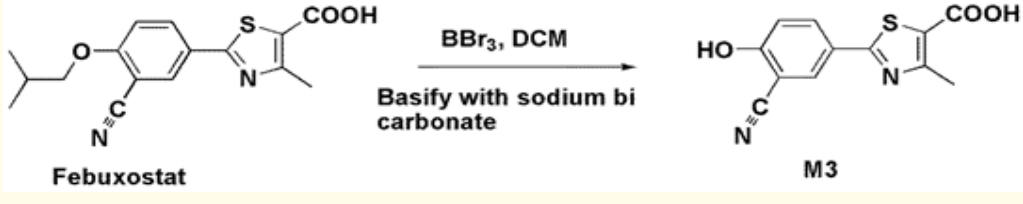

Figure S2: *Synthesis of M3 by dealkylation of febuxostat.*

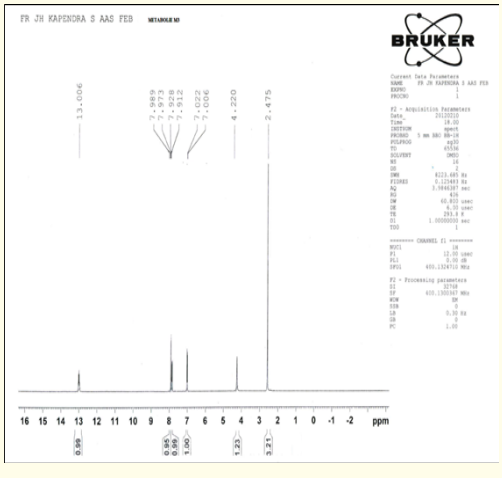

Figure S3: *NMR Spectra of M3.*

Validation procedures in plasma

As per the FDA requirements, a complete validation was executed for the assay in rat plasma [11,12].

Specificity and selectivity

The specificity of the method was assessed by analyzing rat plasma samples collected from six rats to analyze the potential interferences at the UPLC peak region for an analyte using the planned extraction procedure for LC conditions [26].

Matrix impact

The impact of rat plasma constituents over the separation of febuxostat and its metabolite were determined by comparing the responses of the post extracted plasma standard QC samples (n = 6) with the response of analytes from neat standard samples at corresponding concentrations. The matrix effect for febuxostat was determined at QC low, QC medium, and QC high concentrations, viz., 3, 80 and 180 ng/ml.

Calibration curve

The calibration curve was acquired by plotting the ratio of the peak area versus the nominal concentration of calibration standards. The final concentrations of calibration standards obtained for plotting the calibration curve were 2, 5, 10, 20, 50, 75, 100, 200 ng/ml. The results were fitted to linear regression analysis using $1/X^2$ as a weighting factor. The calibration curve had to have a correlation coefficient (r) of 0.995 or better. The acceptance criteria for back-calculated standard concentration were ± 15% deviation from the nominal value except at LLOQ, which was set at ± 20% [16,17].

Precision and accuracy

The intra-day assay precision and accuracy were estimated by analyzing six replicates at four different QC levels, i.e., 3, 80 and 180 ng/ml. The inter-day assay precision was determined by analyzing the four levels QC samples on three different runs. The criteria for acceptability of the data included accuracy within ± 15% standard deviation (S.D.) from the nominal values and precision of within ± 15% relative standard deviation (R.S.D.), except for LLOQ, where it should not exceed ± 20% of accuracy as well as precision.

Stability experiments

All stability studies were conducted at two concentration levels, i.e. QC low and QC high, using six replicates at each concentration levels. Replicate injections of processed samples were analyzed up to 18 h to establish the auto-sampler stability of analyte at 4°C. The peak area of analyte obtained at initial cycle was used for the reference to determine the stability at subsequent points. The stability of febuxostat in the biomatrix during 4h exposure at room temperature in rat plasma (bench top) was determined at ambient temperature (25 ± 2°C). Freeze/thaw stability was evaluated up to three cycles. In each cycle, samples were frozen for at least 12h at -75 ± 10°C. Freezer stability of febuxostat in rat plasma was assessed by analyzing the QC samples stored at -75 ± 10°C for at least 15 days. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (i.e., 15% SD) and precision (i.e. 15% RSD).

Recovery

The extraction recovery of analytes, through protein precipitation extraction procedure, was determined by equating the peak areas of extracted plasma (pre-spiked) standard QC samples (n = 6) to those of the post-spiked standards at equivalent concentrations. Recoveries of M3 were determined at three concentration levels QC low, QC medium and QC high concentrations viz., 3, 80, and 180 ng/ml.

Statistical analysis

The experimental data are expressed as mean ± SD. The pharmacokinetic parameters were calculated followed by the non-compartmental method employing WinNonlin Version 5.1 (Pharsight Corporation, Mountain View, California, USA).

Results

Optimization of the UPLC conditions

During the initial method development, the analyte was analyzed on a BEH C18 column employing water: acetonitrile (50:50) as a mobile phase at a flow rate of 0.15 ml/min. and a column temperature of 25ºC. Under these conditions, the shape of analyte peak was not found acceptable. Consequently, various trials were made on microsomal samples by varying the composition of acetonitrile, pH, and temperature. The best response was attained in the same column at 30˚C. Finally, a mobile phase consisting of acetonitrile and 10 mM ammonium acetate buffer (85:15 v/v, pH 5) at a flow rate of 0.2 ml/min was ascertained to be appropriate during UPLC optimization and additionally established the determination of the electrospray ionization response for febuxostat and its metabolite (M3). The detection wavelength was 310 nm at constant injection volume of 5µL for UPLC. The retention times (R_T) and relative retention times (RR_T) for febuxostat and its metabolite (M3) are given in table 1. The UPLC chromatogram contains the peak of febuxostat and its metabolite obtained from the analysis of microsomal samples is depicted in figure 2. In addition, the developed method is also follows the principles of green chemistry by reducing solvents use and time.

Metabolite / Parameters	M4	M3	M2	M1	FEB.
R_T	2.72	3.68	4.17	4.92	5.23
RR_T	0.52	0.70	0.80	0.94	1.00
Mol. Wt	346	260	332	332	316
% found	2.79	0.31	5.44	4.60	86.23
Oncogenicity	NC	0	NC	NC	NC
Mutagenicity	NC	29	NC	NC	NC
Teratogenicity	NC	17	NC	NC	NC
Sensitivity	NC	0	NC	NC	NC
Immunotoxicity	NC	0	NC	NC	NC
Neurotoxicity	NC	29	NC	NC	NC

Table 1: *Formation of different metabolites (M1-M4) in UPLC chromatogram and In silico predicted toxicities of the M3.*

R_T = Retention Time; RR_T = Relative Retention Time; NC = Not Calculated

Mass spectrometry (ESI-Q-TOF)

For optimization of electron spray ionization (ESI) conditions for febuxostat and its metabolite (M3), ESI-Q-TOF full scans were assessed in positive ion mode. In LC-MS experiment, the mass spectra for febuxostat and its metabolite (M1-M4) revealed peaks at m/z 317 amu and 333 (M1, M2 at different R_T), 261 (M3), 347 (M4) amu respectively as protonated molecular ions $[M+H]^+$. The predominant fragment ion mass spectrum for febuxostat and its metabolite (M1-M4) are given in table 2. Supported to UPLC coupled with MS detection, a small amount of polar hydroxylated metabolite (M3) at R_T 3.68 minute was detected. The M3 responded better to positive ESI mode, and the full-scan mass spectrum presented $[M+H]^+$ at m/z 261.2 amu as the main ion and a major fragment ions at m/z 217.2 amu. Therefore, the transition m/z 261 → 217 was selected for M3 and the m/z 317 → 261 for febuxostat for further quantitative analysis in multiple reactions monitoring (MRM) mode followed by MassLynx software. The separation of M3 would be BEH column and positive mode ionization.

Validation procedures

Calibration curve

The regressions analysis of metabolite (M3) was extrapolated between concentrations versus peak area over the concentration range 2 - 200 ng/ml for M3 in rat plasma. The linearity of M3 was found an optimum.

A best-fitted equation of the calibration curve was: y = 1.231x + 42.662, r2 = 0.985, where "y" represents M3 peak area and "x" represents the plasma concentration using a weighing factor of $1/X^2$.

Specificity, recovery and matrix effect

The selectivity and specificity were studied by using individual plasma samples from six different rats. As depicted in figure 2, there is no important interference from plasma found at retention time of either the febuxostat or its metabolite. The retention time (R_T)and relative retention time (RR_T) of febuxostat and its metabolite are given in table 1.

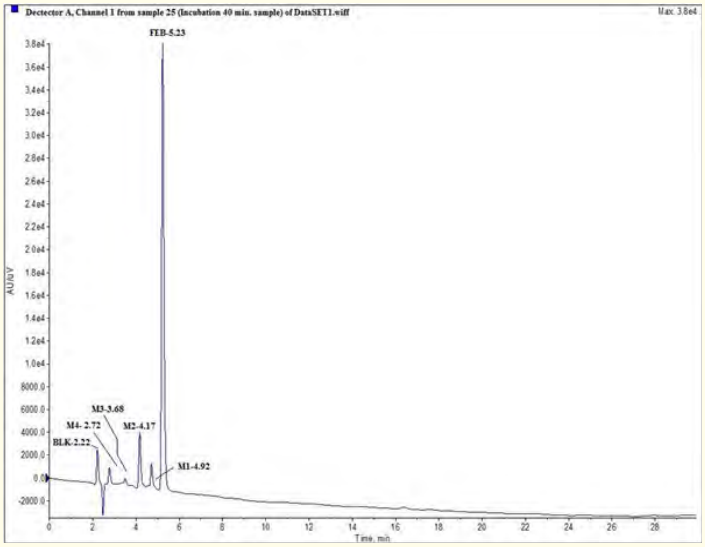

Figure 2: *UPLC Chromatogram shows separation of febuxostat and its metabolite in a mixture of microsomal preparations.*

The extraction recovery was calculated in comparison with the peak areas of pre-spiked standards at three QC levels (3, 80 and 180 ng/ml) with those of post-extraction blank plasma standards levels spiked with their respective concentrations. The mean recoveries of M3 metabolite were 92.2, 96.3 and 97.6% (n = 5) at concentrations of 3, 80 and 180 ng/ml, respectively.

The matrix impact was evaluated by analyzing QC low (3 ng/ml), QC medium (80 ng/ml) and QC high samples (180 ng/ml). The average matrix effect values were 97.21%, 95.49%, and 97.22%.

Precision and accuracy

The precision of the method was evaluated by calculating RSD for QCs at three concentration levels over a period of two days. The intra-day and inter-day precision was found in the range of 0.054 - 2.533 and 0.422 - 4.762 respectively at every QC level (3, 80, and 180 ng/ml). The accuracy of the method ranged from 93.167 to 99.819% at each QC level. Assay performance information is given in table 3. The above finding shows that the values are within the acceptable limits, and also, the method is correct and precise.

Stability

The stability studies showed that febuxostat spiked into rat plasma was stable for 2h at room temperature, for 30 days at -20°C, and through three freeze–thaw cycles. Stability of febuxostat extracts in the sample solvent on an autosampler was also observed over a period of twenty-four hours. The results of stability experiments are shown in table 4.

Nominal Concentration (ng/ml)	Observed Concentration		[a]Precision (%)	[b]Accuracy(%)
	Mean	±SD		
Inter-day				
3	2.893	0.055	1.901	96.433
80	79.855	0.823	1.031	99.819
180	178.94	0.857	0.479	99.411
Intra-day				
3	2.795	0.073	2.6118068	93.167
80	79.593	0.496	0.62317038	99.491
180	179.563	0.76	0.42324978	99.757

Table 3: *Precision and accuracy for M3 of quality control sample in rat plasma (n = 5).*

[a]*Expressed as % R.S.D. = (S.D./mean) × 100.*

[b]*Calculated as (mean determined concentration/nominal concentration) × 100.*

Condition	Spiked concentration (ng/ml)	Found concentration (ng/ml)	[a]Precision (%)	[b]Accuracy (%)	[c]RE (%)
Ambient, 2h	3 180	2.933 179.442	2.147 1.514	97.767 99.690	-2.233 -0.310
-20°C, 30 days	3 180	2.730 178.873	2.057 1.195	91.000 99.374	-9.000 -0.626
Three freeze–thaw	3 180	2.757 178.981	2.339 2.048	91.900 99.434	-8.100 -0.566
Autosampler ambient 24h	3 180	2.860 178.350	1.227 1.499	95.333 99.083	-4.667 -0.917
10 days -80˚C	3 180	2.453 17.493	2.124 1.936	95.100 99.718	-4.900 -0.282

Table 4: *Summary of stability of M3 under various storage conditions (n = 5).*

[a]*Expressed as % R.S.D. = (S.D./mean)×100.*

[b]*Calculated as (mean determined concentration/nominal concentration)×100.*

[c]*Calculated as (observed concentration – nominal concentration/nominal concentration) ×100.*

Discussion

In silico studies

The key benefit of *in silico* metabolism studies is to allow prediction of metabolites that are probably to be formed *in vitro* and *in vivo*. Recent trends in metabolic analysis seem to favour the employment of *in vitro* microsomal metabolism studies. Usually, microsomal metabolism methods do not ascertain an entire image of *in vivo* metabolic alterations viz., valuable information concerning metabolic stability and essential metabolites of the drug. Particularly, the oxidative and the hydrolytic metabolites seem within the sample inferred from microsomal studies. Many metabolites (Figures 1b-1e) were prefigured when the febuxostat was subjected to *in silico* studies. But, solely the existence of M3 was proven in *in vivo* and *in vitro* studies as shown in Figure 1b.

NADPH-dependent metabolism Studies in human liver Microsomes

The metabolism of febuxostat was performed in incubations of 50 µM in human liver Microsomes (HLMs) at the different time interval of 10, 20, 30, 40 and 50 minutes. The experimental results show that a human liver microsomal preparation in the presence of NADPH, metabolizes a vital portion of the parent compound. Consequently, the more polar, hydroxylated metabolite has appeared at RR_T 0.70 minute. The standard chromatogram of UPLC shown in figure 2 also shows an additional peak of the metabolite (M3) at R_T 3.68 (RR_T 0.70) in conjugation with a peak of the febuxostat at 5.23 (RR_T 1.000) that confirms the formation of the M3. Moreover, it absolutely was confirmed by utilizing MS/MS analysis shown in figure 3. In addition, the peaks at R_T 3.68 and R_T 5.23 were additionally cross-checked in MS detection for the relative molecular mass of metabolite (M3) and febuxostat respectively. On the idea of prevailed molecular mass, the mechanism of formation of M3 could be proposed. The concurrent application of isocratic elution and multiple reaction monitoring (MRM) can be created possibly to the discriminate febuxostat and its metabolite. The proposed metabolite was additional confirmed by employing standard metabolite. The fast isocratic elution UPLC with MS-MRM monitoring allows detecting the febuxostat and M3 in samples metabolized using human liver microsomal preparations. The mechanism of formation of the metabolite is depicted in figure 4. The experimental findings indicate that an HLMs preparation metabolizes an alkyl portion of febuxostat to create a hydroxyl, polar metabolite in higher concentration at 50 minutes. The detectable metabolite (M3) is 2-(3-cyano-4-hydroxyphenyl)-4-methylthiazole-5-carboxylic acid, the M3 were formed by the dealkylation of the febuxostat.

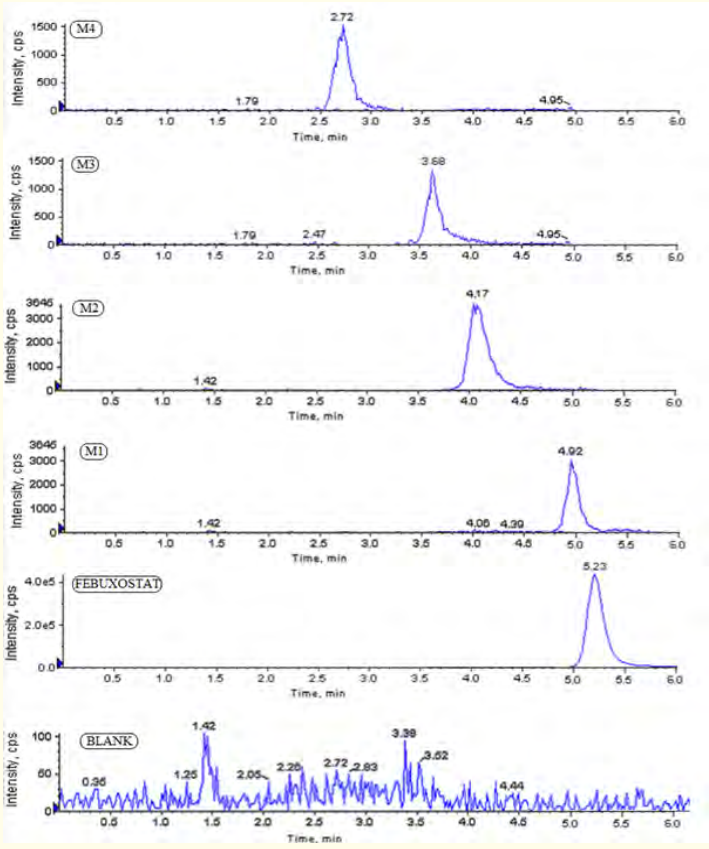

Figure 3: *MS/MS spectra of febuxostat (a) and its metabolites M1(b), M2(c), M3(d) and M4(e).*

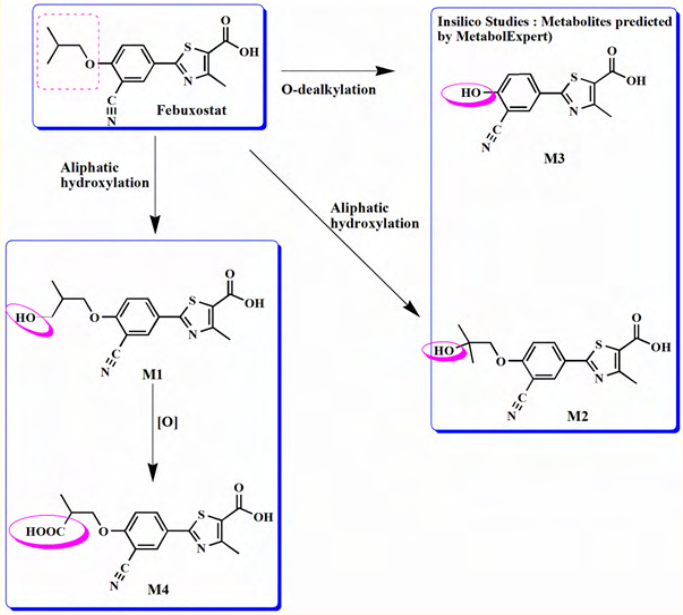

Figure 4: *A proposed pathway for the formation of metabolites M1-M4 of febuxostat.*

Pharmacokinetic

This validated LC–MS/MS method is employed to determine the M3 concentrations in rat plasma samples after the administration of a single oral dose of febuxostat. The isocratic elution UPLC with MS-MRM monitoring allows reliable detection of metabolite (M3) in rat plasma samples. The m/z 317 amu precursor ion to the m/z 261 was used for quantification for febuxostat. Similarly, for M3 m/z 261 amu precursor ion to the m/z 217 amu was employed for the quantification purpose. The plot of parent drug remaining versus time curve after oral administration of a single 5 mg/kg dose of febuxostat for its metabolite (M3) is depicted in figure 5. Subsequently, the primary pharmacokinetic parameters from the non-compartment model analysis are given in table S1. The data of plasma samples were subjected to non-compartmental pharmacokinetics analysis using WinNonlin (version 5.1, Pharsight Corporation, Mountain View, USA). Consequently, the ascertained maximum plasma concentration (C_{max}) is 155.390 ng/mL and the time to reach the maximum plasma concentration (T_{max}) is 1.000 h were obtained from the experimental data. The area under the plasma concentration-time graph (AUC_{0-t} = 928.445 ng h/ mL) was calculated employing a linear trapezoidal technique. The clearance (CL) of M3 was 53.238 L/h/Kg, whereas the mean residual time (MRT_t) was 5.542h. The apparent elimination half-life ($t_{1/2}$ = 3.652h) was calculated as 0.693/k_{el} and also the k_{el} was estimated by linear regression of the log of plasma concentrations in the terminal phase.

Parameters	Observations
C_{max} (ng/ml)	142.737
T_{max} (h)	001.000
AUC_{0-t} (µg h/ml)	1208.708
$AUC_{0-\infty}$ (µg h/ml)	1291.930
CL (L/h/kg)	003.868
AUC%Extrap	000.713
$t_{1/2}$ (h)	003.029
MRT_t	006.660

Table S1: *Pharmacokinetic parameters of M3 after oral administration of single dosage 5 mg/kg febuxostat to rats (n = 5).*

In vitro metabolic stability

Metabolic stability is outlined as the percent of parent molecule disappear over time within the presence of the biological test system. As a result of a majority of drug metabolism appears within the liver, the *in vitro* human liver microsomal preparations have preferred to evaluate metabolic stability. Microsomes could also be additional applicable due to the low volumes utilized in these analytical assays and to attain the goal is to screen febuxostat in a high-throughput (HTP) way. The metabolic stability of febuxostat has designed and carried out using human liver Microsomes. The percentage of the intact febuxostat remained within the incubation mixture over the time of 120 minutes is about 60% as shown in figure 5. This shows that the febuxostat drug is unstable in human liver Microsomes up to 120 minutes at 37 ± 1˚C with a half-life of 54.51 hours. The information of non-linear regression showed sensible fitting with mono-exponential decay (degree of freedom = 18) were calculated by exploitation graph using graph pad prism software system. The ascertained parameters are half-life ($t_{1/2}$ mins), rate constant (K min^{-1}), regression co-efficient (r^2) and degree of freedom (df) shown in table 5. The developed technique in UPLC is fast, economical, reduces consumption of solvents and time for analysis and reduced time for studies using *in vitro* human Microsomes.

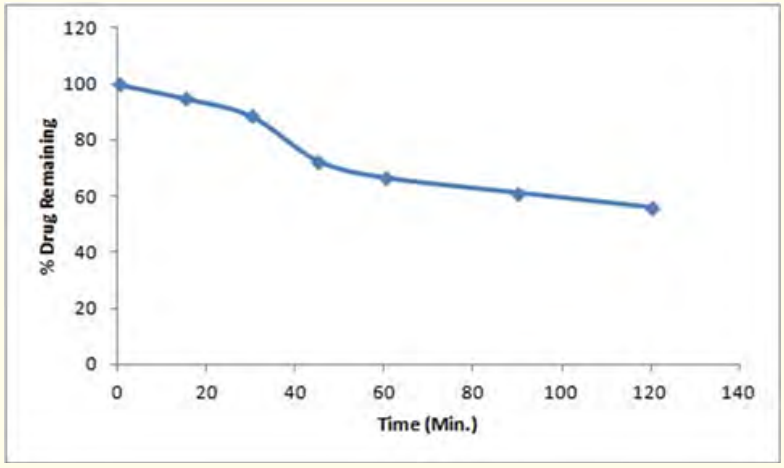

Figure 5: *Plot of percent of parent drug remaining in the incubation mixture versus time for febuxostat.*

Apart from the aforementioned deserves, the results of the current study additionally emphasize the importance of the hepatic metabolism which will be helpful to establish *in vivo-in vitro* correlation, phenotyping and Cyp inhibition studies of the febuxostat.

Conclusion

In this study, an economical bio-analytical method for the coincident quantitation of febuxostat and its phase I metabolite i.e., 2-(3-cyano-4-hydroxyphenyl)-4-methylthiazole-5-carboxylic acid (M3) followed by protein precipitation method in human hepatic microsomal preparations were studied for the first time. Furthermore, a validated method for M3 in rat plasma samples in UPLC-MS/MS was developed and the strategy was with success applied to a pharmacokinetic study of the M3 after oral administration of febuxostat (5 mg/kg). The fashioned metabolites of febuxostat were quantified that are generated after the enzymatic hydroxylation. In supported with *in silico* studies, the expected metabolite is 2-(3-cyano-4-hydroxyphenyl)-4-methylthiazole-5-carboxylic acid (M3). The plasma concentration versus time profile of metabolite of febuxostat in rats has been extrapolated following orally administration of febuxostat, and it has been depicted that febuxostat metabolizes to 2-(3-cyano-4-hydroxyphenyl)-4-methylthiazole-5-carboxylic acid *in vivo*. The developed bioanalytical method has extended relevance viz., various *in vivo* and *in vitro* metabolisms and would be suitable for clinical medical study. *In silico* metabolite and toxicity studies were also carried out for the synthesized molecule to assess their suitability in terms of their sus-

ceptibility to metabolism and toxicity profile. The present study correlates the significance of *in vivo*, *in vitro* and *in silico* studies followed by the confirmation of M3 metabolite.

Future Prospects

The *in silico* studies for metabolite prediction supports the *in vivo* and *in vitro* studies for the assessment of metabolite. These *in silico* and *in vitro* studies would be useful for researchers to assist with the identification of potential drug candidates to be additional evaluated using *in vivo* studies. The coordination of *in silico*, *in vitro* studies accompanied with UPLC-MS/MS can be a dramatic impact on the ability to perform high-throughput screening of compounds. By understanding the metabolic pathway of compounds prior in drug discovery, the potential for a drug candidate to fail within the development as results of pharmacokinetic reason would be also reduced.

Acknowledgments

The authors are grateful to the Vice Chancellor, Jamia Hamdard, New Delhi for providing the experimental facilities for this research study. Dr. Kapendra Sahu wishes to thank the Department of Science and Technology (DST), New Delhi, for providing him Senior Research Fellowship.

Conflict of Interest

The authors declare that they have no conflict of interest.

Bibliography

1. AP L. "Screening for human ADME/Tox drug properties in drug discovery". *Drug Discovery Today* 6.7 (2001): 357-366.

2. Ekins S and Rose J. "In silico ADME/Tox: the state of the art". *Journal of Molecular Graphics and Modelling* 20.4 (2002): 305-309.

3. Kassel DB. "Applications of high-throughput ADME in drug discovery". *Current Opinion in Chemical Biology* 8.3 (2004): 339-345.

4. Mukoyoshi M., *et al*. "In vitro drug-drug interaction studies with febuxostat, a novel non-purine selective inhibitor of xanthine oxidase: plasma protein binding, identification of metabolic enzymes and cytochrome P450 inhibition". *Xenobiotica* 38.5 (2008): 496-510.

5. Kim MJ., *et al*. "High-throughput screening of inhibitory potential of nine cytochrome P450 enzymes in vitro using liquid chromatography/tandem mass spectrometry". *Rapid Communications in Mass Spectrometry* 19.18 (2005): 2651-2658.

6. Sahu KSM and Siddiqui Anees A. "Establishment of inherent stability of febuxostat and development of a validated stability-indicating method by UPLC according to ICH requirement". *Medicinal Chemistry Research* 22.4 (2013): 1641-1647.

7. Schumacher HR Jr. "Febuxostat: a non-purine, selective inhibitor of xanthine oxidase for the management of hyperuricaemia in patients with gout". *Expert Opinion on Investigational Drugs* 14.7 (2005): 893-903.

8. Yu KH. "Febuxostat: a novel non-purine selective inhibitor of xanthine oxidase for the treatment of hyperuricemia in gout". *Recent Patents on Inflammation and Allergy Drug Discovery* 1.1 (2007): 69-75.

9. Wu Y., *et al*. "Simultaneous determination of febuxostat and its three active metabolites in human plasma by liquid chromatography-tandem mass spectrometry and its application to a pharmacokinetic study in Chinese healthy volunteers". *Journal of Pharmaceutical and Biomedical Analysis* 114 (2015): 216-221.

10. Xie H., *et al*. "An HPLC-MS/MS method for simultaneous determination of the active metabolites of febuxostat (67M-1, 67M-2 and 67M-4) in human plasma". *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Science* 970 (2014): 24-30.

11. Rama Raju KS., *et al*. "Simultaneous determination of centchroman and tamoxifen along with their metabolites in rat plasma using LC-MS/MS". *Bioanalysis* 7.8 (2015): 967-979.

12. Wang H., *et al*. "Development and validation of a liquid chromatography-tandem mass spectrometry method for the determination of febuxostat in human plasma". *Biomedical Chromatography* 27.1 (2012): 34-38.

13. Furge LL and Guengerich FP. "Cytochrome P450 enzymes in drug metabolism and chemical toxicology: An introduction". *Biochemistry and Molecular Biology Education* 34.2 (2006): 66-74.

14. Guengerich FP. "Cytochrome P450s and other enzymes in drug metabolism and toxicity". *AAPS Journal* 8.1 (2006): E101-E111.

15. Mayer MD., *et al*. "Pharmacokinetics and pharmacodynamics of febuxostat, a new non-purine selective inhibitor of xanthine oxidase in subjects with renal impairment". *American Journal of Therapeutics* 12.1 (2005): 22-34.

16. Taneja I., *et al*. "Assessment of pharmacokinetic compatibility of short acting CDRI candidate trioxane derivative, 99-411, with long acting prescription antimalarials, lumefantrine and piperaquine". *Scientific Reports* 5 (2015): 17264.

17. Sahu K., *et al*. "Effect of Morin on pharmacokinetics of Piracetam in rats, in vitro enzyme kinetics and metabolic stability assay using rapid UPLC method". *Drug Testing and Analysis* 5.7 (2012): 581-588.

18. Taneja I., *et al*. "LC-ESI-MS/MS method for bioanalytical determination of osteogenic phytoalexin, medicarpin, and its application to preliminary pharmacokinetic studies in rats". *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Science* 1001 (2015): 9-16.

19. Eagling VA., *et al*. "Inhibition of the CYP3A4-mediated metabolism and P-glycoprotein-mediated transport of the HIV-1 protease inhibitor saquinavir by grapefruit juice components". *British Journal of Clinical Pharmacology* 48.4 (1999): 543-552.

20. Hariparsad N., *et al*. "In vitro methods in human drug biotransformation research: implications for cancer chemotherapy". *Toxicology In Vitro* 20.2 (2006): 135-153.

21. Hop CE., *et al*. "In vitro metabolite identification using fast gradient high performance liquid chromatography combined with tandem mass spectrometry". *Rapid Communications in Mass Spectrometry* 16.3 (2002): 212-219.

22. Singh SP., *et al*. "Reduced bioavailability of tamoxifen and its metabolite 4-hydroxytamoxifen after oral administration with biochanin A (an isoflavone) in rats". *Phytotherapy Research* 26.2 (2011): 303-307.

23. Singh SS. "Preclinical pharmacokinetics: an approach towards safer and efficacious drugs". *Current Drug Metabolism* 7.2 (2006): 165-182.

24. 3.8.1.2., P., ADME-Tox software. CompDrug International Inc, USA (2010).

25. Dearden JC. "In silico prediction of drug toxicity". *Journal of Computer-Aided Molecular Design* 17.2-4 (2003): 119-127.

26. Center for Drug Evaluation and Research (U.S.); Center for Veterinary Medicine (U.S.), Guidance for industry bioanalytical method validation. U.S. Dept. of Health and Human Services, Food and Drug Administration Center for Veterinary Medicine: Rockville, MD, (2001): 1.

Volume 4 Issue 3 September 2017