



Bioanalysis of Febuxostat in Human Plasma by Liquid Chromatography/Tandem Mass Spectrometry

**Surya Prakasarao Kovvasu^{3,4}, Steven Yeung⁴,
Priyanka Kunamaneni⁴, Balaji Kodali^{1,2*}**

^{*1}AxisClinicalslimited, Miyapur, Hyderabad-500049, Telangana, India.

²University College of Pharmaceutical Sciences, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur- 522510, Andhra Pradesh, India.

³University College of Pharmaceutical Sciences, Andhra University, Visakhapatnam- 530 003, Andhra Pradesh, India.

⁴College of Pharmacy, Western University of Health Sciences, Pomona, CA 91766, United States.

Abstract: The objective of this paper was to describe the development and validation of a novel liquid chromatography / tandem mass spectrometry (LC-MS/MS) method for the determination of Febuxostat in human plasma using febuxostat-d9 as internal standard (IS). An API-4000 triple quadrupole mass spectrometer operated in MRM mode was used for the selective quantification of Febuxostat in human plasma. Sample extraction utilizes protein precipitation (PP), followed by analyte and the IS were chromatographed on a C18 column using an isocratic mobile phase composed of 5mM ammonium formate and acetonitrile (20:80, v/v) pumped at a flow rate of 1.0 mL/min. Precision and accuracy of the method was determined using five analytical batches in the concentration range of 15.0–8000 ng/mL. All the validation experiments were carried out as per the US FDA guidelines and results met the acceptance criteria. The developed method was rapid with a total chromatographic run time of 2.0 min.

Keywords : Febuxostat; Human plasma; Method validation; LC–MS/MS.

Introduction

Febuxostat is used for the treatment of gout and hyperuricemia. It inhibits xanthine oxidase, thus reducing production of uric acid in the body ^[1,2]. Febuxostat was well tolerated at once-daily doses of 10-240 mg. Febuxostat on oral administration, rapidly absorbed and reach maximum plasma concentration around 0.5-1.3 h. Febuxostat was extensively metabolised and renal function did not seem to play an important role in its elimination from the body ^[3]. It was approved by US FDA in the year of 2009. The drug was also approved in China and EU recommended at 40 mg or 80 mg once daily ^[4].

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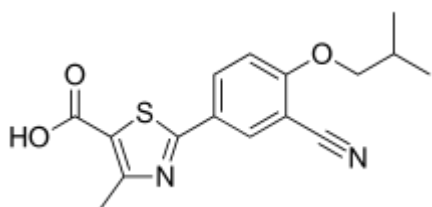
Few LC-MS/MS methods ^[5-8] have been reported for Febuxostat quantification in plasma. The author Vakaet *et al.*, 2013 ^[5] published a method for the determination of febuxostat in human plasma with a run time of >5 min. Similarly, Lukram *et al.*, 2012 ^[6] reported an UPLC-MS/MS for the Febuxostat in human plasma with an LLOQ of 0.075 µg/mL. Both the methods have utilized liquid-liquid extraction (LLE) technique for sample preparation. Moreover, Lukram *et al.*, 2012 employs a volume of 500 µL plasma for sample preparation, which is relatively high volume for a routine bioequivalence studies. The author's Wang *et al.*, 2013 ^[7] and Ding *et al.*, 2012 ^[8] described a LC-MS/MS method for the determination of febuxostat in plasma but both the methods employed protein precipitation (PP) technique for the sample preparation with chromatographic run time of >4 min. An effective bioanalytical method should be simple, sensitive, reproducible, rapid (short analytical run time) and it should require low sample volume for sample processing ^[9, 10].

The current paper described in detail about the LC-MS/MS method development and validation for the quantification of febuxostat in human plasma using febuxostat-d9 as internal standard (IS). The deuterated IS which mimics the behavior as like analyte (congener) in terms of ionization and extraction. The congener behavior nullifies the matrix effect. A simple protein precipitation (PP) was used to extract the analyte and IS from plasma. No significant interference was observed at the retention time of analyte and IS with proposed method conditions.

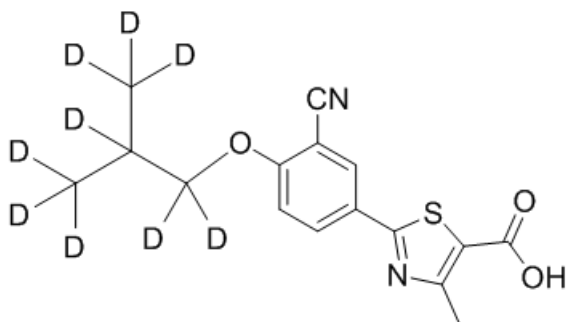
Materials and Methods

Standards and reagents

The working standards of febuxostat (99.6%) and febuxostat-d9 (Fig.1) (99.4%) were obtained from Vivan Lifesciences Limited (Mumbai, India). HPLC grade acetonitrile and methanol were purchased from J.T. Baker (Phillipsburg, NJ, USA), while analytical grade ammonium formate was obtained from Merck Ltd (Mumbai, India). LC-MS grade water was collected by using Milli Q water purification system procured from Millipore (Bangalore, India). The drug free K₂ human plasma was obtained from Deccan's Pathological Lab's (Hyderabad, India).



Febuxostat



Febuxostat-d9 (IS)

Figure 1: Chemical structure of Febuxostat and febuxostat-d9 (IS).

LC–MS/MS instrument conditions

An API-4000 (MS/MS) (AB Sciex, Foster City, CA, USA) triple quadrupole mass spectrometer equipped with a Turbo ion spray™ in positive ion mode was used for the quantification. The mass spectrometer was coupled with HPLC system (Shimadzu, Kyoto, Japan). An aliquot of 10 µL of the processed sample was injected into the column (Kromasil 100-5C18, 100 mm × 4.6 mm, 5 µm), maintained at ambient (25±2°C) temperature. A mixture of 5mM ammonium formate and acetonitrile (20:80, v/v) was used to separate the analyte from interferences. The mobile phase was pumped at a flow rate of 0.8 mL/min. Mass spectrometer was maintained with interface temperature 500 °C and ion spray voltage was set at 5000 V. Detection of the ions was carried out in the multiple–reaction monitoring (MRM) mode, by monitoring the transition pairs of m/z 317.1 precursor ion to the m/z 261.0 product ion for febuxostat and m/z 326.1 precursor ion to the m/z 262.0 product ion for the IS. Quadrupoles (Q1 and Q3) were operated in unit resolution. The source parameters viz. the nebulizer gas (GS1), auxiliary gas (GS2), curtain gas and collision gas were set at 40, 40, 35, and 10 psi, respectively. The voltage values for declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) were set at 50, 26, 10, 11 V for febuxostat and for the IS. The data acquisition was performed by using Analyst software™ (version 1.4.2).

Preparation of calibration standards and quality control samples

All stock solutions were prepared in methanol at a concentration of 1 mg/mL and respective working solutions were prepared using diluent (water and methanol 70:30, v/v). The spiked samples were prepared in the linear range of 15-8000 ng/mL in plasma. The five QC concentrations were prepared at 15.1 ng/mL (lower limit of quantitation quality control, LLOQ QC), 50.0 ng/mL (low quality control, LQC), 1200 ng/mL (medium quality control, MQC1), 4000 ng/mL (MQC2) and 6000 ng/mL (high quality control, HQC).

Extraction procedure

A 250 µL aliquot of human plasma sample was mixed with 25 µL of the internal standard working solution (10.0 µg/mL of febuxostat-d9). To this, 100 µL of 0.1% formic acid (5% v/v) was added after vortex mixing for 10 s. Then the samples were precipitated with 1 mL of acetonitrile. After vortex–mixing for 30 s and centrifugation at 4000 rpm for 20 min, the supernatant was transferred to glass test tubes and evaporated under gentle stream of nitrogen (20 psi) at 45°C and samples were reconstituted with 500µL of mobile phase. The reconstituted solution was transferred into 1 mL vials.

Method validation

The proposed method was validated for selectivity, sensitivity, matrix effect, linearity, precision and accuracy, dilution integrity, recovery, and stability as per the current US FDA guidelines^[11].

Results and Discussion

Method development

API-4000 mass spectrometry conditions (source dependent and compound dependent) were optimized using 100 ng/mL working solution of analyte and the IS. We found more intense peaks in positive mode when compare to the negative mode using ESI source. MRM technique was used to monitor the ions for better selectivity and specificity^[12-15]. Mass spectrometry source and compound dependent parameters were suitably tuned to get better intense signals and reproducible response. From the MSMS spectrum of analyte and IS in positive ion mode, the transition pairs of m/z 317.1 precursor ion to the m/z 261.0 product ion for febuxostat and m/z 326.1 precursor ion to the m/z 262.0 product ion for the IS were selected to monitor in MRM. The chemical structures of febuxostat and febuxostat-d9 were shown in Fig. 1.

HPLC conditions were optimized by using a variety of volatile buffers ammonium formate, ammonium acetate, as well as acid additives like formic acid and acetic acid at different strength in mobile phase to achieve highest sensitivity. Also, a variety of columns like C₁₈ and C₈ of different makes were checked for good peak shape and for better selectivity. Finally, the mobile phase composed of 5mM ammonium formate and acetonitrile (20:80, v/v) gives better peak shape with a Kromasil 100-5C18 (100 × 4.6, 5 µm) column. The

mobile phase was pumped at a flow rate of 0.8 mL/min and the analyte and the IS were retained at about 1.25 min with total analytical run time of 2 min.

Initially we tested a variety of extraction techniques like PP, SPE and LLE. SPE was tested with Starata X polymeric sorbent, Oasis HLB, Bond ElutPlexa and Orpheus C₁₈, and LLE was tried with hexane, MTBE, ethyl acetate and diethyl ether. Both SPE and LLE methods gave good recovery (>80%) for the analyte and the IS. But the SPE cartridges are expensive and require special SPE equipment to process the samples. Similarly, LLE requires more non-polar solvents, which involves drying and evaporation. As a purpose to develop a simple and inexpensive extraction method PP was tested with acetonitrile and methanol. Acetonitrile gives clear precipitation and more recovery than the methanol, hence we used for the present purpose. Addition of formic acid helped to obtain high and reproducible recovery for the analyte. Deuterated compounds as internal standard limit the possible matrix suppression or enhancement effect and increases the bioanalytical assay precision and accuracy. Hence, in the present work febuxostat stable labeled isotope febuxostat-d₉ was used as an internal standard.

Linearity, precision and accuracy

The proposed method was validated in the concentration range of 15–8000 ng/mL. A total of five calibration curves were analyzed. Each curve contains blank, blank sample with the IS, 8 non-zero calibration standards and 6 sets of each QCs (LLLOQ QC, LQC, MQC1, MQC2 and HQC). The correlation coefficient value was >0.99 during entire course of validation.

Intra-day and inter-day precision and accuracy results of febuxostat for five analytical runs in spiked quality control samples are summarized in Table 1. Accuracy values were ±15% of their nominal values and the precision was <15%. The chromatograms of blank plasma, blank plasma along with IS and extracted LLOQ samples were shown in Fig:2-4.

Table 1: Precision and accuracy data for febuxostat.

Quality control	Run	Concentration found Mean±SD (ng/mL)	Precision (%)	Accuracy(%)
Intra-day variations (n=12 at each concentration)				
LLOQ		16.1 ± 0.71	4.42	107.0
LQC		44.8 ± 1.22	2.72	89.6
MQC1		1149 ± 50.0	4.36	95.7
MQC2		3729 ± 166.0	4.46	93.2
HQC		5765 ± 85.7	1.49	96.1
Inter-day variations (n=30 at each concentration)				
LLOQ		15.8 ± 0.74	4.71	105.0
LQC		44.9 ± 1.93	4.29	89.9
MQC1		1171 ± 44.6	3.81	97.6
MQC2		3776 ± 153.0	4.04	94.4
HQC		5751 ± 117.0	2.03	95.8
Nominal concentrations of LLOQ, LQC, MQC1, MQC2 and HQC are 15.1, 50.0, 1200, 4000 and 6000 ng/mL, respectively.				

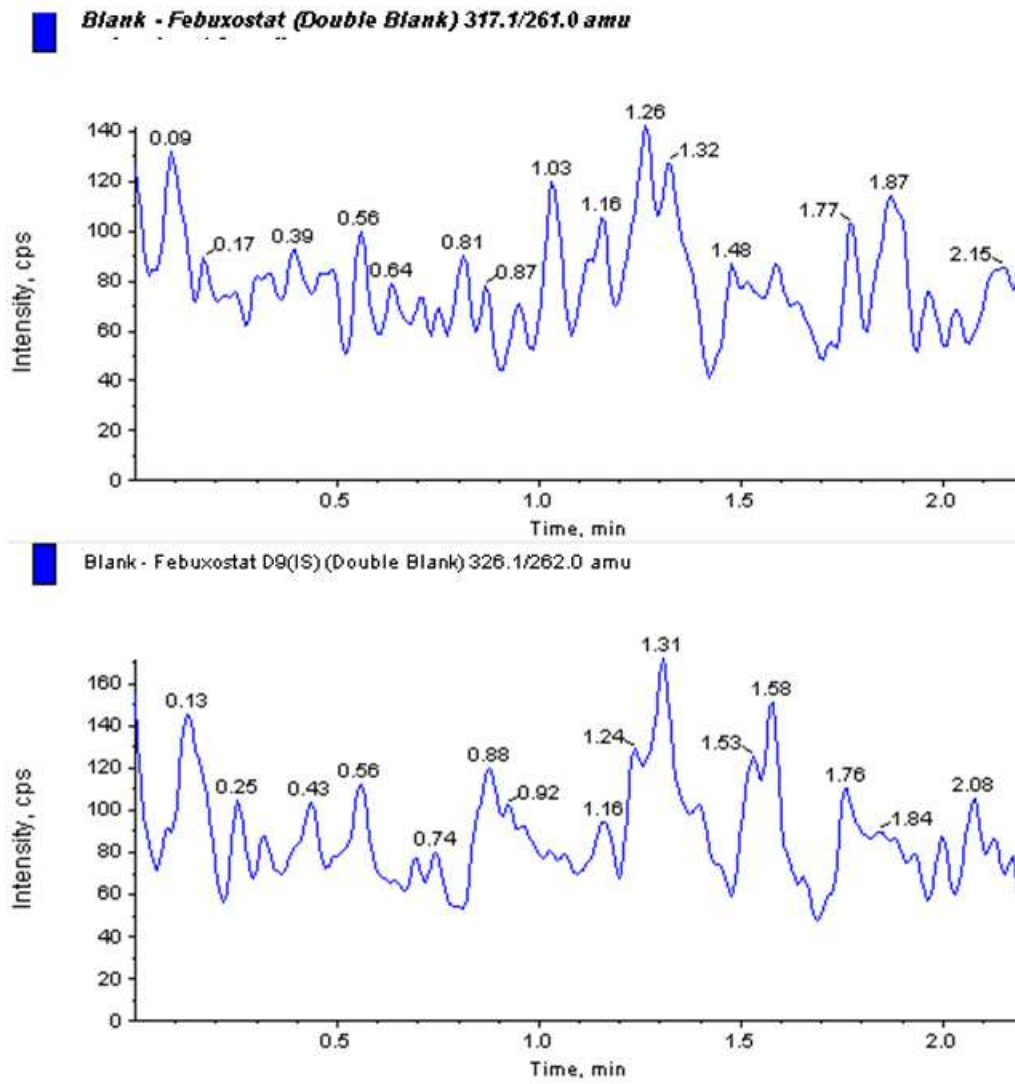


Figure 2: Typical MRM chromatogram of febuxostat and IS in human blank plasma.

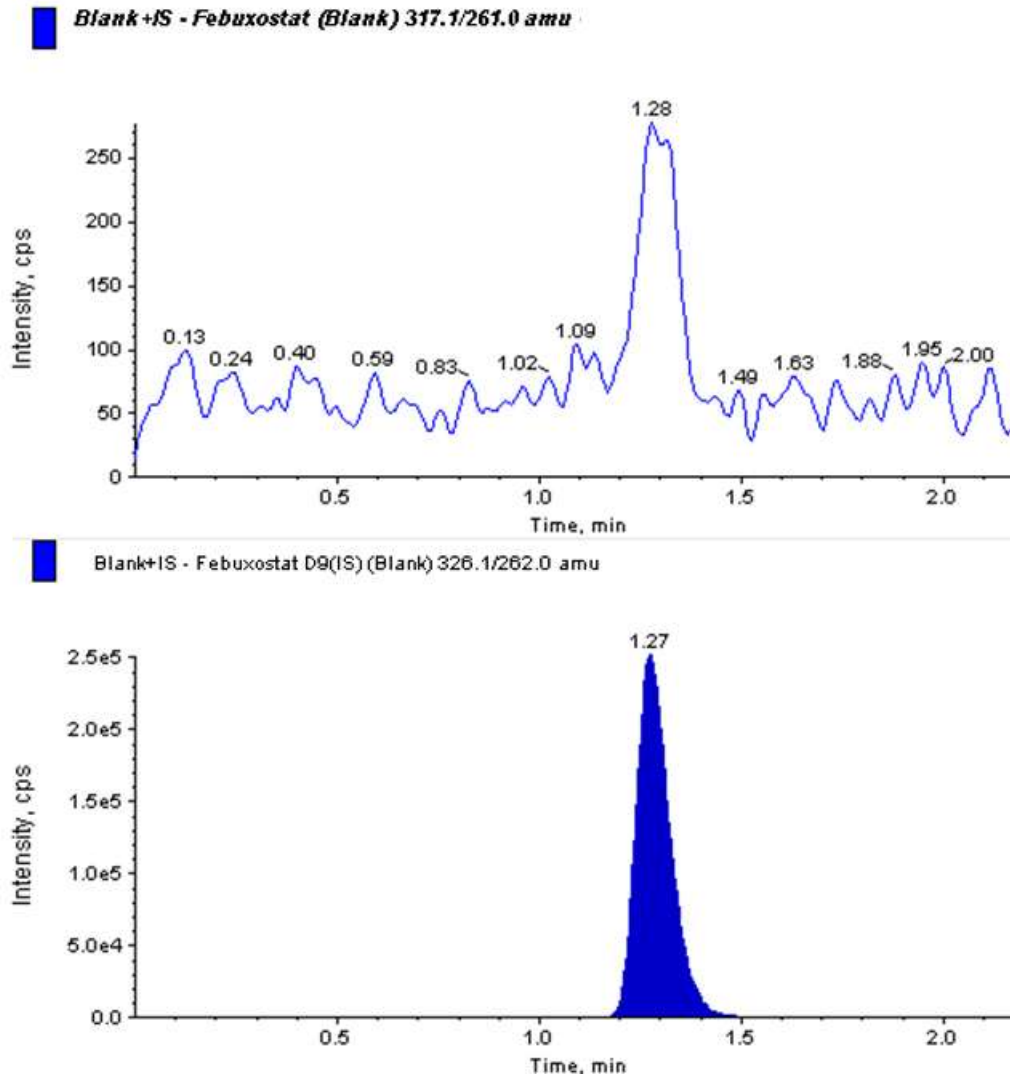


Figure 3: Typical MRM chromatogram of febxostat and IS in human plasma spiked with IS.

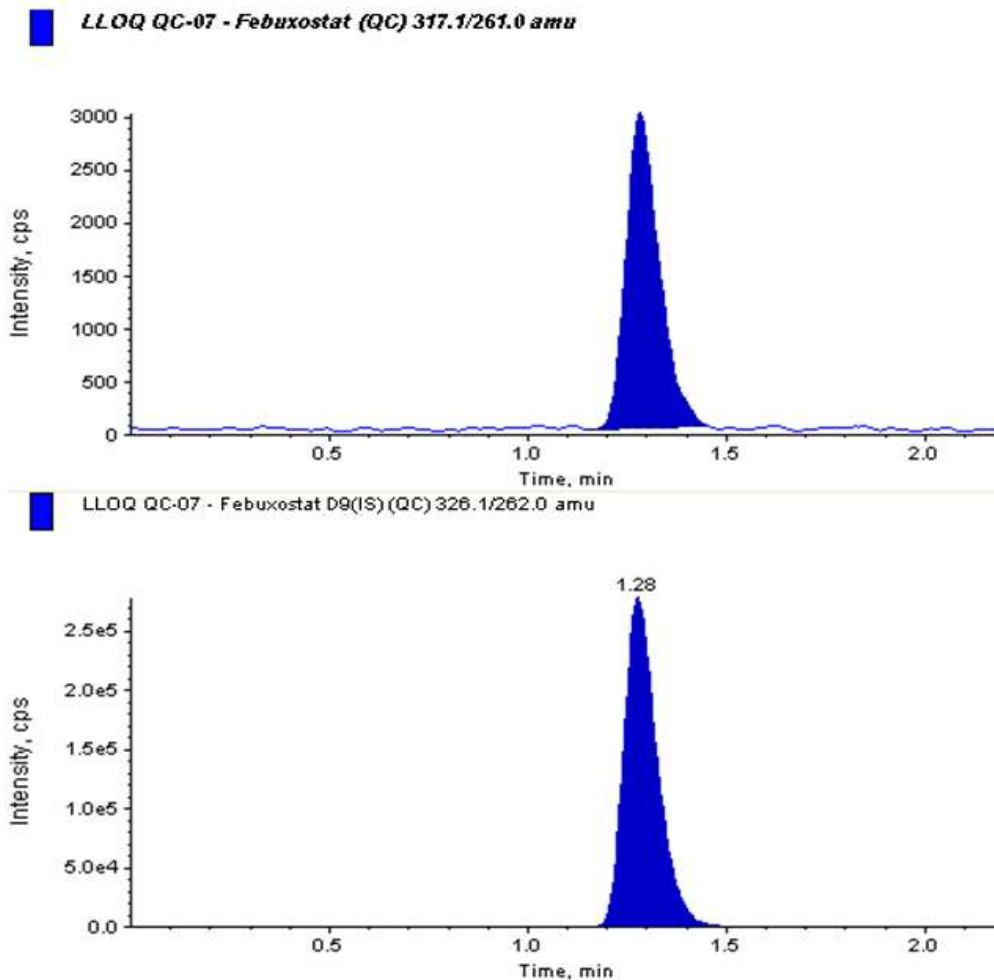


Figure 4: Typical MRM chromatogram of febuxostat and IS in human plasma at LLOQ level.

Chromatography, selectivity and sensitivity

Analyzed six blank plasma samples obtained from different sources and all are found to be free from significant interference from endogenous components at the RT of analyte and the IS. Also, no cross talk was found from the IS at the RT of analyte. Similarly, no interference was found from OTC drugs like paracetamol, ibuprofen, caffeine, pantoprazole, dicyclomine etc.

The signal-to-noise ratio (S/N) was measured at a concentration of 15 ng/mL, which is set as a lowest limit of reliable quantification (LLOQ) for the analyte. The S/N ratio at this concentration was found to be ≥ 5 . The precision and accuracy at LLOQ concentration were found to be 6.23% and 101%, respectively. An LLOQ sample of analyte along with the IS was presented.

Dilution integrity

The ULOQ value for the present method is 8000 ng/mL, and this can be extended (16000 ng/mL) by sample dilution with blank plasma. Half (1:2) or quarter (1:4) dilution was performed and quantified under undiluted calibration curve. The precision (%CV) for dilution integrity of half and quarter dilution was found to be 4.74% and 2.38% and the accuracy results were found to be 98.5% and 98.9%, respectively.

Extraction efficiency

Simple precipitation with acetonitrile gives highest and reproducible recovery for the analyte and the IS. The mean overall recovery obtained for febuxostat was $80.1 \pm 0.85\%$ with the precision (%CV) range of 1.47%–12.3% and the recovery of the IS was 75.4%.

Matrix effect

The calculated IS normalised matrix factor values were in the range of 0.9-1.2 at LQC, HQC level indicates there was no significant matrix effect. The % CV was found to be < 6%.

Stability studies

The mean % nominal values were found to be within $\pm 15\%$ of the predicted concentrations for the analyte at their LQC and HQC levels and the precision (% CV) values were within 15% (Table 2) for all the stability tests carried out during the entire course of method validation. All the above stability results were found to be within the acceptable limits during the entire validation.

Table 2: Stability data for febuxostat in plasma (n=6).

Stability test	QC (spiked concentration, ng/mL)	Mean \pm SD (ng/mL)	Precision (%)	Accuracy/Stability (%)
Autosampler stability (at 10°C for 35 h)	50.0	47.4 \pm 3.87	8.17	94.8
	6000	6042 \pm 206	3.41	101
Wet extract stability (at 2–8°C for 32 h)	50.0	46.9 \pm 1.55	3.31	93.8
	6000	6133 \pm 204	3.32	102
Bench top stability (16 h at room temperature)	50.0	46.8 \pm 3.38	7.22	93.6
	6000	5983 \pm 166	2.77	99.7
Freeze–thaw stability (3 cycles)	50.0	47.3 \pm 2.39	5.05	94.6
	6000	5871 \pm 315	5.37	97.9
Reinjection stability (32 h)	50.0	45.8 \pm 2.96	6.46	91.5
	6000	5652 \pm 327	5.78	94.2
Long–term Stability (at –80°C for 40 days)	50.0	49.2 \pm 2.33	4.74	98.3
	6000	6131 \pm 303	4.94	102

Conclusion

The proposed LC–MS/MS assay is simple, rapid and sensitive for the determination of febuxostat in human plasma. The method describes the complete method development and validation process as per US FDA guidelines and is well suitable for pharmacokinetic or bioavailability/bioequivalence application. Simple PP extraction gives high and reproducible recovery febuxostat and the IS. Also, the method was rapid with run time of 2 min and alternative for existing methods for routine drug analysis. Thus, the advantage of this method is that a relatively large number of samples can be analyzed in short time, thus increasing the output.

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