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Original Article

NEW METHOD DEVELOPMENT AND VALIDATION FOR THE DETERMINATION OF FEBUXOSTAT IN HUMAN PLASMA BY LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

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ABSTRACT

Objective: To develop a new method and validate the same for the determination of Febuxostat (FBS) in human plasma by liquid chromatography–mass spectrometry (LCMS).

Methods: The present method utilized reversed-phase high-performance liquid chromatography with tandem mass spectroscopy. Febuxostat D9 (FBS D9) was used as internal standard (IS). The analyte and internal standard were separated from human plasma by using solid phase extraction method. Zorbax Eclipse XDB, C_8, 100 mm x 4.6 mm, 3.5 µm column was used and HPLC grade acetonitrile, 5 millimolar (mM) ammonium format (80: 20, v/v) as mobile phase, detected by mass spectrometry operating in positive ion and multiple reaction monitoring modes.

Results: The parent and production transitions for FBS and internal standard were at m/z 317.1 →261.0 and 326.1→262.0 respectively. The method was validated for system suitability, specificity, carryover effect, linearity, precision, accuracy, matrix effect, sensitivity and stability. The linearity range was from 20.131 ng/ml to10015. 534 ng/ml with a correlation coefficient of 0.999. Precision results (%CV) across six quality control samples were within the limit. The percentage recovery of FBS and internal standard from matrix samples was found to be 76.57% and 75.03% respectively.

Conclusion: Present study describes new LC-MS method for the quantification of FBS in a pharmaceutical formulation. According to validation results, it was found to be a simple, sensitive, accurate and precise method and also free from any kind of interference. Therefore the proposed analytical method can be used for routine analysis for the estimation of FBS in its formulation.

Keywords: Febuxostat, Febuxostat D9, Liquid chromatography, Mass spectrometry

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INTRODUCTION

Chronic hyperuricemia and gouty arthritis [1-4] have ailed humans for centuries. Recent advancement of research in the understanding of the mechanism of their progress has changed our perception of the disease process. In spite of these developments, the treatment options are limited. The FDA approval of FBS for the treatment [5-8] of gouty arthritis or hyperuricemia has been a significant step forward. Since its approval during 2009, FBS has been proved to be a safe and efficacious treatment, although concerns remain about its long-term effects and superiority over other anti-gout agents, such as allopurinol.

Chemically the compound FBS is known as 2-(3-cyano-4-isobutoxyphenyl)-4-methyl-

1, 3-thiazole-5-carboxylic acid. Fig. 1 represents the chemical structure.

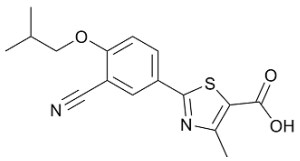

Fig. 1: Chemical structure of FBS

The literature survey indicates that there are certain methods available for the determination of FBS in bulk drug form, formulation, metabolites and in the spiked plasma sample. Spectrophotometric methods were developed [9, 10] for determination of FBS in bulk and formulation. Reverse phase liquid chromatography was developed [11-16] determination of FBS in bulk, formulation and plasma sample. One method was reported [17] for the determination of FBS by using ultra-pressure liquid chromatography/mass spectroscopy and also there were two methods [18, 19] which used liquid chromatography-mass spectroscopy. Our developed new method has got unique advantages over these existing methods which are as follows

The proposed LC-MS method is a simple and selective for the determination of FBS in human plasma. The method employs only 100 microliter of human plasma volume and achieved good sensitivity. Use of low plasma volume for the analysis, the sample to be collected per time point from an individual during the study is reduced significantly. This allows the inclusion of additional points. The analyte and the IS were extracted from plasma using one-step solid–phase extraction with no drying, evaporation and reconstitution steps. Solid–phase extraction allows higher recoveries and the elimination of possible interference from endogenous and exogenous components. Isotope-labeled compound used as an IS to get better precision and accuracy. The total run time (2.0 min) is short enough compared to existing methods and makes it an attractive bioanalytical procedure of FBS for bioavailability and also will be in pharmacokinetic studies which will be studied shortly.

MATERIALS AND METHODS

Instrumentation

HPLC: Shimadzu

Mass spectrophotometer: API 3000 LC-MS/MS system

Reagents

Reagents/materials-Manufacturer/supplier

Methanol-JT Baker

Acetonitrile (HPLC grade)-JT Baker

Ammonium formate (AR grade)-Merck

Formic acid (AR grade)-Merck

HPLC grade water-Rankem

Strata X polymeric sorbent cartridges (30 mg/1cc)-Phenomenex

Working standards

Drug: Febuxostat

Batch No: VL/S-FB-004/b

Purity: 99.59% (HPLC)

Supplier: Vivan life sciences private limited.

Drug: Febuxostat D9

Batch No: VL/D-FB-206/a

Purity: 99.96% (HPLC)

Supplier: Vivan life sciences private limited.

Mass spectrometry operating conditions

Compound	Febuxostat	Febuxostat D9
Detection	Positive	Positive
M/z	317.10 (parent) 261.00 (product)	326.10 (parent) 262.00 (product)
Ion spray voltage (ISV)	4000 V, 4000 V	
Temperature (TEM °C)	550 °C	550 °C
Curtain gas (CUR)	10 psi	10 psi
Collision gas (CAD)	10 psi	10 psi
NEB	6 psi	6 psi
Declustering potential (DP)	26 V	26 V
Collision energy (CE)	26 V	26 V
Collision cell exit potential (CXP)	12 V	12 V
Focusing potential (FP)	128 V	128 V
Entrance potential (EP)	10 V	10 V
Dwell time	200 ms	200 ms

Preparation of standard stock and plasma samples

FBS stock solution: Weighed about 10.00 mg of FBS working standard and transferred to a 5 ml clean glass volumetric flask, dissolved in HPLC grade methanol and made up the volume with the same to produce a solution of 2 mg/ml. Corrected the above concentration of FBS solution accounting for its potency and the actual amount weighed.

The stock solutions were diluted to suitable concentrations using a mixture of acetonitrile and HPLC grade water in the ratio of (60:40 v/v) for spiking into the plasma to obtain calibration curve (CC) standards, quality control (QC) samples and DIQC samples. For the preparation of calibration curve standards and quality control samples, two separate stock solutions were prepared and used. All other final dilutions (system suitability dilutions, aqueous mixture, etc.) were prepared in the mobile phase.-FBS D9 Stock Solution (Internal standard): Weighed about 2.0000 mg of FBS D9 hydrochloride, transferred to a 2 ml volumetric flask, dissolved in HPLC grade methanol and made up the volume with the same to produce a solution of 1 mg/ml. Corrected the above concentration of FBS D9 accounting for its molecular weight, potency and the actual amount weighed. The stock solution was diluted to a suitable concentration using diluent for internal standard dilution.

Note: Stock solutions and further dilutions of FBS D9 were prepared under the yellow monochromatic light.

Biological matrix

Eight lots of K2-EDTA human plasma, including one lipemia and one hemolytic plasma, were screened for selectivity test. All eight human plasma lots, including hemolytic and lipemic plasma, were found free of any significant interference for FBS and IS.

Selectivity and matrix test was performed before bulk spiking. After bulk spiking, 300 µl aliquot of each of the spiked calibration standards and quality control samples were pipetted out into 5 ml RIA vial, and stored in a deep freezer at–70 °C, except twelve replicates each of LQC and HQC, which were stored in a deep freezer at–20 °C for generation of stability data at–20 °C.

Calibration curve standards and quality control samples

Calibration curve standard consisting of a set of ten non-zero concentrations ranging from 20.131 ng/ml to 10015.534 ng/ml of FBS were prepared. Prepared quality control samples consisted of concentrations of 20.799 ng/ml (LLOQ QC), 61.174 ng/ml (LQC), 1529.341 ng/ml (MQC1), 5097.802 ng/ml (MQC2) and 7608.659 ng/ml (HQC) for FBS as given in table 1 and table 2. These samples were stored at–70 °C until use. Twelve sets of LQC and HQC were stored at–20 °C deep freezer to check-20 °C stability. Twenty-four sets of quality control samples for dilution integrity were prepared by spiking about 1.60 times the higher standard concentration of FBS (16797.041 ng/ml). From these six sets each of two times dilutions and four times dilutions were performed.

Table 1: Calibration curve (standard)

Final concentration- CC (ng/ml)	Label
20.131	1
40.262	2
201.312	3
503.281	4
1006.561	5
2013.122	6
4026.245	7
6009.320	8
8012.427	9
10015.534	10

*CC- Calibration curve

Table 2: Quality control (sample)

LLOQ QC (ng/ml)	20.799
LQC	61.174
MQC1	1529.341
MQC2	5097.802
HQC	7608.659
D1QC (ULQC)	16797.041

*LLOQ- Lower limit of quantification, QC- Quality control, LQC- Low-quality control, MQC- Medium quality control, HQC-High quality control, DIQC- Dilution integrity quality controle. ULQC- Upper limit quality control.

Sample preparation

The samples were thawed at room temperature and vortexed to ensure complete mixing of the contents. 200 µl of the plasma sample was pipetted in 5 ml polypropylene RIA vial, 20 µl of internal standard dilution (30598.856 ng/ml of FBS D9) was added to it and vortexed, except in blank plasma samples where 20 µl diluents was added and vortexed. Then, 600 µl of 0.1% formic acid buffer was added and vortex. The analyte and the IS were extracted from plasma using one-step solid–phase extraction with no drying, evaporation and reconstitution steps. SPE allows higher recoveries and the elimination of possible interference from endogenous and exogenous components. The sample mixture was loaded into the strata X 33 µm polymeric sorbent (30 mg/1 ml) cartridges that were preconditioned with 1.0 ml of HPLC grade methanol followed by 1.0 ml Milli Q HPLC grade water (new cartridge for each sample). After applying the maximum pressure, the extraction cartridge was washed with 2 ml of Milli Q/HPLC grade water (1.0 ml of each time). Then the samples were eluted with 1 ml of mobile phase and transferred to autosampler loading vials (amber color) and loaded into the auto-sampler.

Method development

Chromatographic separation was achieved after several trials using various combinations of solvents like acetonitrile, methanol, buffer (ammonium acetate, ammonium formate, formic acid, acetic acid at different concentrations) with varying proportions of each component on different columns like C18 and C8 of different brands like Grace, Chromolith, Hypersil, Hypurity advance, Kromasil, Zorbax, Ace and Intertsil. Use of 5 mM ammonium formate buffer helped in achieving good response for the detection in the positive ionization mode.

Validation parameters

Chromatography: Chromatographic conditions were optimized to achieve good sensitivity and peak shapes for the compounds, as well as a runtime which could be as short as it is possible. The liquid chromatographic conditions were optimized after a number of trials. A mobile phase consisting of acetonitrile and ammonium formtate 5 mM buffer (80:20, v/v) was found the most suitable. Zorbax eclipse XDB, C8, 100 x 4.6 mm, 3.5 µm (make: Agilent technology) column was the most useful one for developing the method.

System suitability

System suitability study was performed by applying six consecutive applications of FBS and FBS D9 LLOQ concentration and thereby observing the results in terms of retention time and area response and calculating %CV.

Selectivity

Selectivity test of the developed new method was conducted by injecting blank human plasma, spiked six samples at concentrations of (ULOQ) for FBS and the internal standard in plasma, compared the responses FBS and internal standard in the blank with a mean response of applied (ULOQ). The peak area of FBS at the respective retention time in blank should not be more than 20% of the mean peak area of (ULOQ) of FBS. Similarly, the peak area of FBS D9 at the respective retention time in blank should not be more than 5% of the mean peak area of (ULOQ) of FBS D9. Observations were there, whether any interfering compounds appeared in the chromatogram.

Sensitivity

The lower limit of reliable quantification for FBS in human plasma was set in the concentration of the LLOQ 20.131 ng/ml. The precision and accuracy for the analyte should be calculated and results in terms of accuracy and precision should be verified whether they are within the limit.

Matrix effect

To predict the variability of matrix effects in samples from individual subjects, matrix effect was quantified by determining the matrix factor, which was calculated as follows:

Matrix Factor = Peak response ratio in the presence of extracting matrix (post extracted)/Peak response ratio in aqueous standards.

Six lots of blank biological matrices were extracted each in triplicates and post spiked with the aqueous standard at the low, high QC level, and compared with aqueous standards of the same concentration. The overall precision of the matrix factor is expressed as a coefficient of variation (CV %) and %CV should be<15%.

Recovery

The extraction recovery of FBS and FBS D9 from human plasma was determined by analyzing quality control samples. Recovery at three concentrations (LQC, MQC2 and HQC) was determined by comparing peak areas obtained from the plasma sample and the standard solution spiked with the blank plasma residue.

Linearity

A regression equation with a weighting factor of 1/(concentration ratio) 2 of FBS to FBS D9 concentration was judged to get the best fit for the concentration--detector response relationship for FBS in plasma. The correlation coefficient (r^2) value should be greater than 0.99 in the designed concentration range for FBS.

Precision and accuracy

The accuracy of the developed method is defined as the absolute value of the ratio of the calculated mean values of the LLOQ, low, middle and high-quality control samples to their respective nominal values, expressed in percentage. The precision of the developed method was measured by the percent coefficient of variation over the concentrations of LLOQ QC, LQC, MQC and HQC samples during the course of validation

Stability studies

Stability studies were performed by taking the sample passed through different physicochemical conditions like bench top, freeze-thaw, wet extract, dry extract, autosampler, dilution integrity, long term stability, etc.

RESULTS AND DISCUSSION

Method development

Optimization of liquid chromatography and mass spectrometry conditions

A detailed comparative study of the various published methods with the newly developed method is discussed in table 3. During method development while conducting trials, it was observed that increase in the proportion of buffer in mobile phase resulted in increased retention time, reduction in flow rate below 1 ml/minute also

increased the retention time, similarly reduction of column temperature below 40 °C also contributes in prolongation of retention time. However, there was no substantial amount of variation in the number of theoretical plates or tailing factor. With the optimized method condition, we could able to limit the moderate run time that is 2 min, which is short enough compared to the existing method [18, 19] which used liquid chromatography-mass spectroscopy.

Moreover, if we look at the peak obtained in the case of Febuxostat D7 in the method developed by Babu Rao Chandu, Kanchanamala Kanala, Nagiat T Hwisa, Prakash Katakam, Mukkanti Khagga [19] it seems there were certain impurities along with the compound as the main peak was closely accompanied with additional peak. It may be an interference effect also. However, in our proposed method no such issues are associated.

Table 3: Comparative table of FEB

Method	Solvent system	Con. range	Detection	Reference
UV spectroscopy	Methanol	0.2-15 µgm/ml	315 nm	[9]
UV spectroscopy	Zero order Methanol	2-30 µgm/ml	314 nm	[10]
	First order	1-30 µgm/ml	293-336 nm	
RP-HPLC	Sodium acetate, ACN	0.1-200 µgm/ml	254 nm	[11]
RP-HPLC	Methanol, OPA	45.42-2559.64 ngm/ml	310 nm	[12]
RP-HPLC	Ammonium acetate, ACN	50-400 µgm/ml	275 nm	[13]
RP-HPLC	Methanol, ACN	40-100 µgm/ml	218 nm	[14]
RP-HPLC	Pot. di. H. PO4, ACN	5-60 µgm/ml	320 nm	[15]
RP-HPLC	Methanol, Sodium acetate	250-8000 ng/ml	315 nm	[16]
UPLC-MS	Formic acid, ACN	2-10000 µgm/ml	MRM-MD	[17]
LC-MS	Formic acid, CAN, water	10-20000 ng/ml	MRM-MD	[18]
LC-MS	Ammonium formate, ACN	1-8000 ng/ml	MRM-MD	[19]

*CAN-Acetonitrile, OPA-Ortho phosphoric acid, Pot. di. H. PO4-Potassium dihydrogen phosphate, MRM-Multi reaction monitoring mode, MD-Mass detector.

In the present method, separation was achieved by using mobile phase as Acetonitrile and 5 mM ammonium formate (80:20, v/v) in isocratic elution technique at a flow rate of 1.00 ml/min. fig. 2 represents the mass spectra of FEBU and FEBU D9. Fig. 3 represents chromatogram of the blank plasma and blank plasma with internal standards.

Method Validation

System suitability

Consecutive six injections were applied for FBS and IS LLOQ concentration in the chromatographic system. The mean retention time was found to be 0.9733 min and 0.9616 min respectively. If it is compared with any other existing method [18, 19] it is easy to understand the rapid nature of the present method. Mean area, standard deviation, and %CV were found to, 18528.0 and 2508497.3, 1805.26 and 223043.3, 9.74 and 8.89 for FBS and IS respectively which are well within the acceptable limit.

Selectivity and specificity

The selectivity and specificity of the present analytical method were established by examining any interfering compounds which elute along with FBS. The response of both analyte and IS in blanks was compared with the mean response of injected LLOQ.

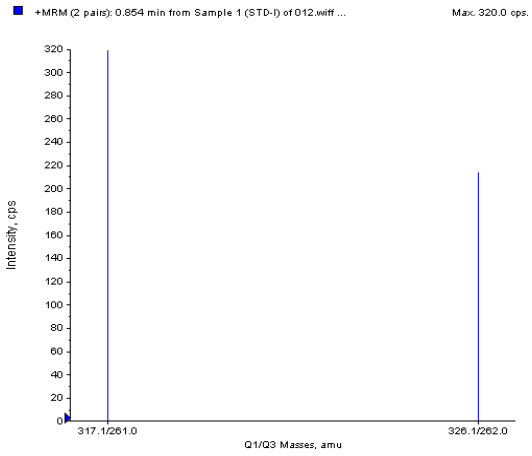

Fig. 2: Q1/Q3 mass spectrum of FBS along with the IS

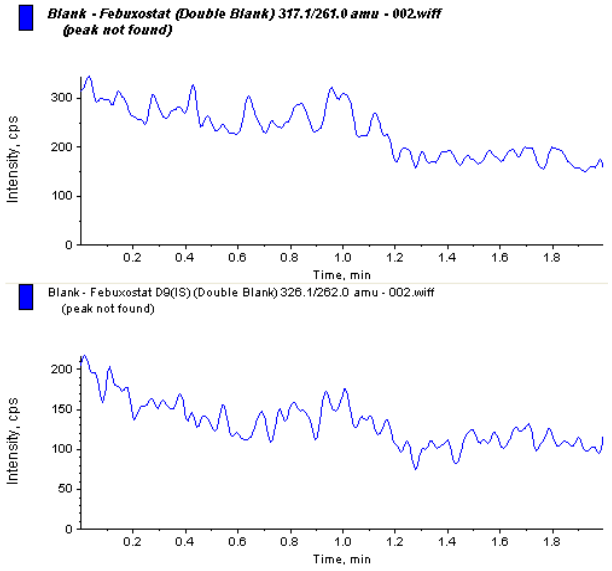

Fig. 3: Chromatogram of blank plasma sample of FBS and FBS D9

There were no interfering peaks formed at FBS retention time and IS retention time in the plasma blanks. Fig. 4 shows chromatograms of blank human plasma samples. The results were shown in table 4 and table 5. The analytical study of FBS and FBS D9 using the multiple reaction monitoring functions was highly selective and no interfering compounds were observed.

Recovery

As the sample and internal standard FBS D9 were extracted from human plasma, and the test for recovery was determined by analyzing quality control samples (LQC, MQC2 and HQC) by means of comparison of peak areas obtained from the plasma sample and the standard solution spiked with the blank plasma residue, the average percentage recoveries were found to be 76.56% and 75.03% respectively. This yield is up to the mark and comparable with any other mass spectrophotometric method. Detailed results are narrated in table 6 and table 7.

Table 4: Result of system suitability

S. No.	t_R in Minutes		Peak response (area) LLOQ	
	Analyte	Internal standard	Analyte	Internal standard
1	0.97	0.96	15844	2213163
2	0.97	0.96	17086	2300690
3	0.97	0.96	20729	2748657
4	0.97	0.96	18409	2461078
5	0.98	0.97	19729	2735237
6	0.98	0.96	19371	2592159
Mean (±)	0.973	0.961	18528.0	2508497.3
SD	0.00516	0.00408	1805.26	223043.34
CV%	0.530	0.424	9.74	8.89

*tR-Retention time, LLOQ-Lower limit of quantification

Table 5: Specificity of FBS and internal standard

Sample Id	FBS Peak area	IS peak area	% interference at-t_R of FBS	% interference at-t_R of IS
ULOQ FBS 1	6125002	0	NA	0
ULOQ FBS 2	5948204	0	NA	0
ULOQ FBS 3	5658120	0	NA	0
ULOQ FBS 4	5450620	0	NA	0
ULOQ FBS 5	5408965	0	NA	0
ULOQ FBS 6	6844515	0	NA	0
The mean response of FBS in presence of IS	5905904			
Mean response of FBS D9 in presence of FBS		0		0
Blank+IS (FBS D9) 1	0	1996826	0	NA
Blank+IS (FBS D9) 2	0	2123014	0	NA
Blank+IS (FBS D9) 3	0	1858814	0	NA
Blank+IS (FBS D9) 4	0	2087960	0	NA
Blank+IS (FBS D9) 5	0	2133141	0	NA
Blank+IS (FBS D9) 6	0	1999478	0	NA
Mean response of FBS D9 in presence of FBS		2033210		
Mean response of FBS			0	

***** ULOQ-Upper limit of quantification, FBS-Febuxostat, IS-Internal standard, t_R-Retention time, NA-Not applicable.

Table 6: Recovery of FBS from matrix

Standard	Identifying code	Unextracted standard peak area	Identifying code	Extracted matrix standard peak area	%recovery
LQC	AQS-LQC-1	51867	EXT-LQC-1	39587	
	AQS-LQC-2	53188	EXT-LQC-2	39724	
	AQS-LQC-3	54426	EXT-LQC-3	39027	
	AQS-LQC-4	52778	EXT-LQC-4	38041	
	AQS-LQC-5	53780	EXT-LQC-5	37193	
	AQS-LQC-6	54577	EXT-LQC-6	37261	
Mean (±)		534360.0		38472.2	72%
SD		1035.04		1131.98	
CV%		1.94		2.94	
MQC	AQS-MQC-1	4120459	EXT-MQC-1 EXT-MQC-	3214184	
	AQS-MQC-2	3997420	2	3067145	
	AQS-MQC-3	4263493	EXT-MQC-3	3161738	
	AQS-MQC-4	4340363	EXT-MQC-4	3051183	
	AQS-MQC-5	4040216	EXT-MQC-5	3068832	
	AQS-MQC-6	4280130	EXT-MQC-6	3026803	
Mean (±)		4173680.2		3098314.2	74.23%
SD		140637.05		72971.65	
CV%		3.37		2.36	
HQC	AQS-HQC-1	5731755		4859028	
	AQS-HQC-2	5649408		4793110	
	AQS-HQC-3	5618990		4777081	
	AQS-HQC-4	5728449		4717922	
	AQS-HQC-5	5696802		4654670	
	AQS-HQC-6	5765760		4736922	
Mean (±)		5698527.3		4756455.5	83.47%
SD		55247.60		70023.94	
CV%		0.97		1.47	

*LQC-Low quality control, AQS-Aqueous, EXT-Extracted, SD-Standard deviation, CV-Co-efficient of variance, MQC-Medium quality control, HQC-High Quality control.

Table 7: Recovery of internal standard from matrix

Standard	Identifying code	Unextracted peak area	Identifying code	Extracted peak area	%recovery
Internal standard	AQS-LQC-1	2541331	SPIKED-LQC-1	1942265	
	AQS-LQC-2	2615551	SPIKED-LQC-2	1948802	
	AQS-LQC-3	2662715	SPIKED-LQC-3	1955042	
	AQS-LQC-4	2613782	SPIKED-LQC-4	1886018	
	AQS-LQC-5	2643611	SPIKED-LQC-5	1844533	
	AQS-LQC-6	2689044	SPIKED-LQC-6	1812939	
	AQS-MQC-1	2376143	SPIKED-MQC-1	1882419	
	AQS-MQC-2	2315155	SPIKED-MQC-2	1791023	
	AQS-MQC-3	2406262	SPIKED-MQC-3	1866519	
	AQS-MQC-4	2544363	SPIKED-MQC-4	1789813	
	AQS-MQC-5	2336335	SPIKED-MQC-5	1778645	
	AQS-MQC-6	2449825	SPIKED-MQC-6	1770304	
	AQS-HQC-1	2291491	SPIKED-HQC-1	1831302	
	AQS-HQC-2	2257716	SPIKED-HQC-2	1806206	
	AQS-HQC-3	2264027	SPIKED-HQC-3	1814292	
	AQS-HQC-4	2310835	SPIKED-HQC-4	1762670	
	AQS-HQC-5	2307111	SPIKED-HQC-5	1724347	
	AQS-HQC-6	2311513	SPIKED-HQC-6	1757634	
Mean (±)		2440933		1831376.3	
SD		154156.27		69079	
%CV		6.32		3.77	

*FBS-Febuxostat, LQC-Low quality control, AQS-Aqueous, SD-Standard deviation, CV-Co-efficient of variance, MQC-Medium quality control, HQC-High Quality control.

Linearity, accuracy, and precision

Linearity, accuracy, and precision: The calibration curve was constructed using 8 calibration standards ranging from 20.131ng/ml to 10015.534ng/ml. a straight line fit was made through the data points. The correlation coefficient was found to be ≥0.999. The lower limit of quantification (LLOQ) was found to be 20.799ng/ml. Accuracy was calculated in terms of percentage recovery and precision in terms of percentage coefficient variation. For the concentration of LLOQ the accuracy result and precision value were found to be 100.10% and CV% 5.69 respectively. The test result for inter-batch accuracy was 97.91%; precision for LLOQ, LQC, MQC and HQC was 4.96, 3.77, 3.47 and 2.18 respectively. The test result for inter-batch accuracy was 99.77% (first batch), 97.24% (second batch); precision for LLOQ, LQC, MQC and HQC for the first batch was 3.04, 3.62, 4.44 and 0.64; for second batch 4.49, 1.13, 2.90 and 0.69 respectively. The test result for intraday accuracy was 98.51%; precision for LLOQ, LQC, MQC1, MQC2 and HQC was 3.85, 2.72, 4.36, 4.46 and 1.49 respectively. All the results of linearity, accuracy, and precision were within the limit. Fig. 4 represents the calibration curve, fig. 5 to fig. 9 represent the chromatograms of FBS and FBS D9 for the concentrations of LLOQ, LQC, MQC1 and HQC. Table 8 to table11 contains the results of accuracy and precision.

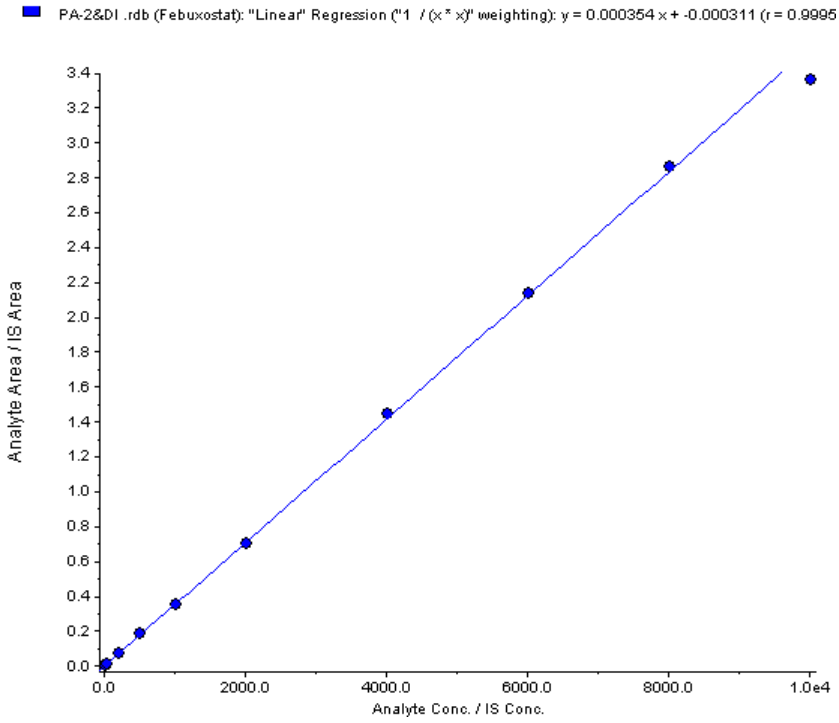

Fig. 4: Calibration curve for regression analysis of FBS

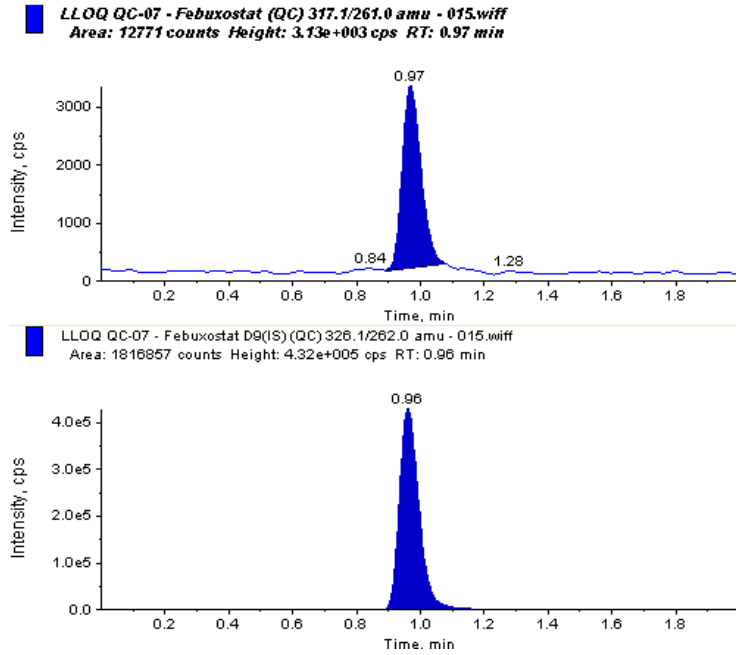

Fig. 5: Chromatogram of LLOQ QC sample of FBS and FBS D9

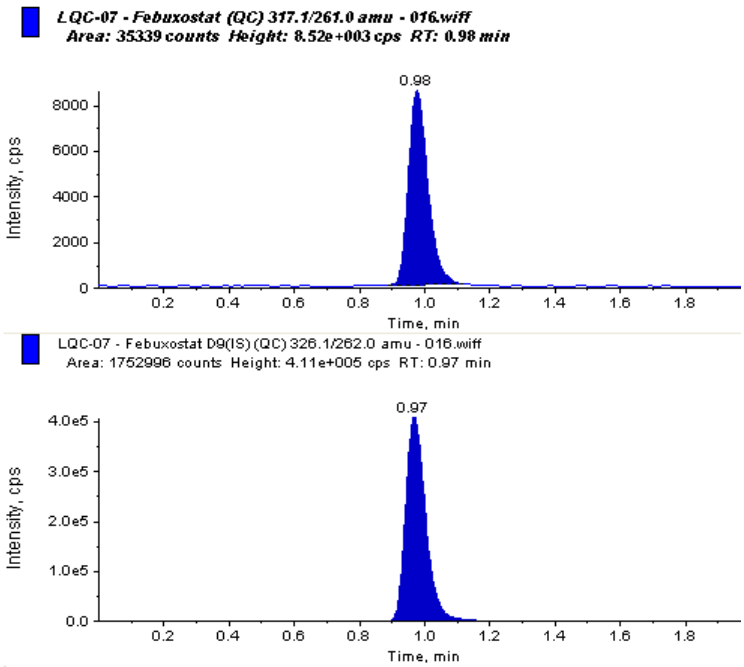

Fig. 6: Chromatogram of LQC sample of FBS and FBS D9

Table 8: Precision and accuracy for LLOQ (sensitivity)

	FBS	
Identifying code	**Nominal (ng/ml)**	**% Accuracy**
LLOQ-1	19.892	98.81
LLOQ-2	20.982	104.23
LLOQ-3	18.861	93.69
LLOQ-4	19.183	95.29
LLOQ-5	21.931	108.98
LLOQ-6	20.054	99.62
Mean	20.1518	
SD	1.14587	
CV%	5.69	
% nominal	100.10	

*Number of replicates = 6, FBS-Febuxostat, LLOQ-Lower limit of quantification, SD-Standard deviation, CV-Co-efficient of variance.

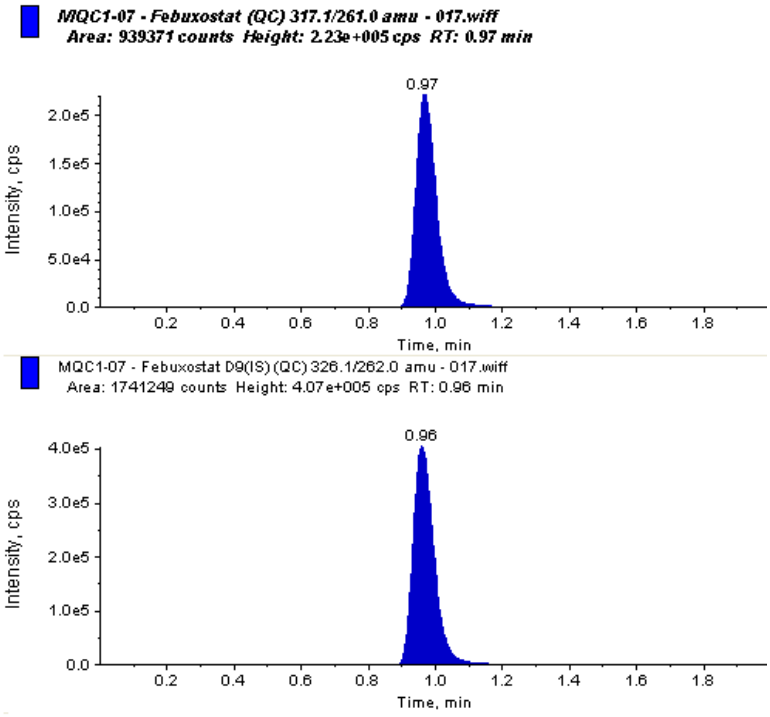

Fig. 7: Chromatogram of MQC1 sample of FBS and FBS D9

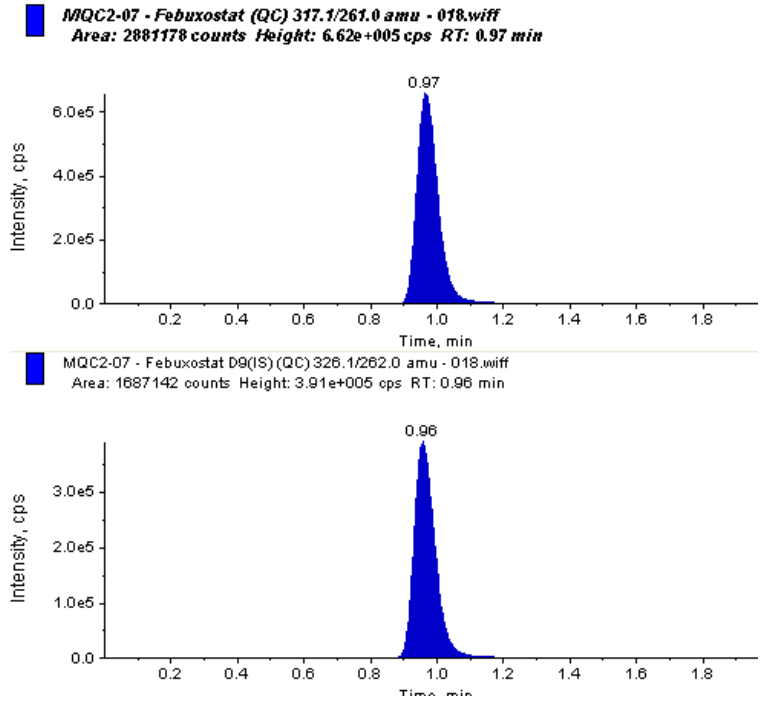

Fig. 8: Chromatogram of MQC2 sample of FBS and FBS D9

Table 9: Inter batch precision and accuracy

Batch	LLOQ (ng/ml)		LQC (ng/ml)		MQC1 (ng/ml)		MQC2 (ng/ml)		HQC (ng/ml)	
QC	20.779	%Accuracy	61.174	%Accuracy	1529.341	%Accuracy	5097.802	%Accuracy	7608.659	%Accuracy
Mean	20.3139	97.6	58.1238	95.01	1529.5868	99.49	4887.0549	95.87	7449.3205	97.91
SD	1.00710		2.18880		3.79		169.56247		162.41094	
CV%	4.96		3.77		99.49		3.47		2.18	

*Number of replicates = 30, LQC-Low quality control, MQC-Medium quality control, HQC-High Quality, SD-Standard deviation, CV-Co-efficient of variance control.

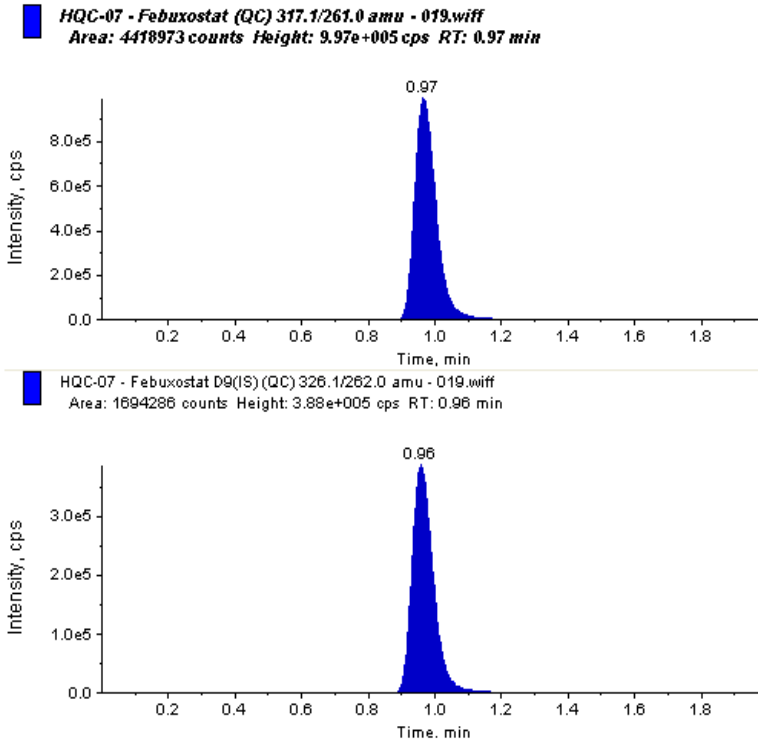

Fig. 9: Chromatogram of HQC sample of FBS and FBS D9

Table 10: Intraday precision and accuracy

	LLOQ (ng/ml)		LQC (ng/ml)		MQC1 (ng/ml)		MQC2 (ng/ml)		HQC (ng/ml)	
QC	20.799	**%Accuracy**	61.174	**%Accuracy**	1529.341	**%Accuracy**	5097.802	**%Accuracy**	7608.659	**%Accuracy**
Mean	20.4502	98.32	58.2138	95.16	1493.5383	97.66	4847.3108	95.09	7495.0603	98.51
SD	0.78632		1.58526		65.06109		215.9665		111.369	
CV%	3.85		2.72		4.36		4.46		1.49	

*Number of replicates = 12, QC-Quality control, LLOQ-Lower limit of quantification, LQC-Low quality control, MQC-Medium quality control, HQC-High Quality, SD-Standard deviation, CV-Co-efficient of variance control.

Table 11: Intra batch precision and accuracy

Batch	**LLOQ (ng/ml)**		**LQC (ng/ml)**		**MQC1 (ng/ml)**		**MQC2 (ng/ml)**		**HQC (ng/ml)**	
QC	**20.799**	**%Accuracy**	**61.174**	**%Accuracy**	**1529.34**	**%Accuracy**	**5097.802**	**%Accuracy**		**%Accuracy**
1st batch										
Mean	20.6940	99.50	58.7045	95.96	1457.2373	95.29	4806.0205	94.28	7591.3512	99.77
SD	0.62943		2.12693		64.67799		262.02031		48.95283	
CV%	3.04		3.62		4.44		5.45		0.64	
N	6		6		6		6		6	
2nd batch										
Mean	20.2063	97.15	57.7230	94.36	1529.839	100.03	4888.60	95.90	7398.769	97.24
SD	0.90631		0.65334		44.34646		172.81499		51.36160	
CV%	4.49		1.13		2.90		3.54		0.69	

*N-Number of replicates = 6, QC-Quality control, LLOQ-Lower limit of quantification, LQC-Low quality control, MQC-Medium quality control, HQC-High Quality control, SD-Standard deviation, CV-Co-efficient of variance.

Carry over effect

The sequence of injections consisting two blank samples and two samples of ULOQ concentration were analyzed alternately to find out if there is any carry over affect on the blank sample. It was found that there was no carryover effect observed in the present method.

Stability results

As stability studies were performed by taking FBS passed through different physicochemical conditions like bench top, freeze-thaw, wet extract, dry extract, autosampler, dilution integrity, long-term stability, the mean response, percentage recovery and percentage coefficient variation were found within the limit. Table 12 represents the results of stability studies in details.

Dilution integrity

Dilution integrity was performed by taking two times and four times dilution of ULOQ concentration (16797.041 ng/ml). The percentage accuracy and %CV were found to be within the acceptance criteria (table 13).

Table 12: Stability data of QC samples in human plasma

Nominal Conc	Stability	Mean (ng/ml)	% Accuracy	Precision (CV %)
	Bench top 15 h	59.1428	96.68	5.90
LQC	Freeze-thaw	59.7635	97.69	3.64
(61.174 ng/ml)	Wet extract	62.6802	102.46	5.75
	Autosampler	60.0737	98.20	4.16
	Freshly spiked QC	58.4463	95.56	1.77
	60 d	59.4030	95.56	0.72
	Bench top 15 h	7608.659	100.95	1.20
HQC	Freeze-thaw	7737.6602	101.70	1.61
(7608.946 ng/ml)	Wet extract	7803.0808	102.56	1.19
	Autosampler	7714.5567	101.39	1.22
	Freshly spiked QC	7756.0290	101.96	0.72
	60 d	7656.8233	100.63	0.80

*Number of replicates, QC-Quality control, LQC-Low quality control, HQC-High quality control, CV-Co-efficient of variance.

Table 13: Data acquired on dilution integrity

Nominal concentration (NC) ng/ml				
DIQC	**Two times dilution**		**Four times dilution**	
	NC = 16797.041	**% Accuracy**	**NC = 16797.041**	**% Accuracy**
Mean	16355.7257	97.37	16542.7382	98.49
SD	444.99782		436.31186	
CV%	2.72		2.64	

*DIQC-Dilution integrity quality control, NC-Nominal concentration, SD-Standard deviation, CV-Co-efficient of variation.

CONCLUSION

The new developed method is a rapid, simple, specific and accurate liquid chromatography mass spectrophotometry for the determination of FBS as the results of all the validation parameters were found within the limit. The sophisticated solid phase extraction technique has yielded in high precision values. As per C_{max} of FBS the range was designed and the method was very sensitive with a low value of LLOQ. The newly developed method can be used for regular determination of Febuxostat in the laboratory.

CONFLICT OF INTERESTS

Declared none

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