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

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, C8, 100 mm x 4.6 mm, 3.5 µm column was used and HPLC grade acetonitrile, 5 millimolar (mM) ammonium formate (90:20, 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 to 10015.534 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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DOJ: http://dx.doi.org/10.22159/ijpps.2016v8i9.11968

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](image-url)
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) | 326.10 (parent) |
| 261.00 (product)                      | 262.00 (product) |
| Ion spray voltage (ISV)               | 4000 V, 4000 V | 550 °C |
| 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 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
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. 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 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 0.1% formic acid 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.

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.

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 formate 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.

Validation parameters

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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.

Sample preparation

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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 spectrometry.

Moreover, if we look at the peak obtained in the case of Febuxostat D7 in the method developed by Babu Rao Chandu, Kanchanamala Kanala, Naglat T Hwis, 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                  | Concentration 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]      |
| RP-HPLC                       | Sodium acetate, ACN             | 0.1-200 µgm/ml      | 293-336 nm         | [11]      |
| RP-HPLC                       | Methanol, OPA                   | 45.2-2559.64 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         | 10-80000 ng/ml      | MRM-MD             | [18]      |
| LC-MS                         | Ammonium formate, ACN           | 1-80000 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.

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. | tR in Minutes | Analyte Peak response (area) LLOQ | Analyte Internal standard Peak response (area) LLOQ |
|--------|---------------|-----------------------------------|---------------------------------------------------|
| 1      | 0.97          | 0.96                              | 15944                                             |
| 2      | 0.97          | 0.96                              | 17086                                             |
| 3      | 0.97          | 0.96                              | 20729                                             |
| 4      | 0.97          | 0.96                              | 18409                                             |
| 5      | 0.98          | 0.97                              | 19729                                             |
| 6      | 0.98          | 0.96                              | 19371                                             |
| Mean (x) | 0.973      | 0.961                             | 18528.0                                           |
| SD     | 0.00516       | 0.00408                           | 1805.26                                           |
| CV%    | 0.530         | 0.424                             | 9.74                                              |

*9.7% 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 tR of FBS | % interference at tR 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 | 6844515       | 0            | NA                         | 0                         |
| ULOQ FBS 5 | 5408965       | 0            | NA                         | 0                         |
| ULOQ FBS 6 | 5450620       | 0            | NA                         | 0                         |

The mean response of FBS in presence of IS 5905904
Mean response of FBS D9 in presence of IS 2033210

Mean response of FBS D9 in presence of FBS 6215002
Mean response of FBS D9 in presence of IS 5905904

*ULOQ-Upper limit of quantification, FBS-Febuxostat, IS-Internal standard, tR-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 area |
|----------|------------------|--------------------------------|------------------|-----------------------------------|---------------|
| LQC      | AQS-LQC-1        | 51867                          | EXT-LQC-1        | 39587                             | 72%           |
|          | AQS-LQC-2        | 53188                          | EXT-LQC-2        | 39724                             | 72%           |
|          | AQS-LQC-3        | 54426                          | EXT-LQC-3        | 39027                             | 72%           |
|          | AQS-LQC-4        | 52778                          | EXT-LQC-4        | 38041                             | 72%           |
|          | AQS-LQC-5        | 53780                          | EXT-LQC-5        | 37193                             | 72%           |
|          | AQS-LQC-6        | 54577                          | EXT-LQC-6        | 37261                             | 72%           |
| Mean (x) | 534360.0         | 38472.2                        | 1131.98          | 2.94                              | 72%           |
| SD       | 1035.04          | 1411.1                        | 1131.98          | 2.94                              | 72%           |
| CV%      | 1.94             | 3.46                          | 1131.98          | 2.94                              | 72%           |
| MQC      | AQS-MQC-1        | 4120459                        | EXT-MQC-1        | 3214184                           | 74.23%        |
|          | AQS-MQC-2        | 3997420                        | EXT-MQC-2        | 3067145                           | 74.23%        |
|          | AQS-MQC-3        | 4263493                        | EXT-MQC-3        | 316738                            | 74.23%        |
|          | AQS-MQC-4        | 4340363                        | EXT-MQC-4        | 3051183                           | 74.23%        |
|          | AQS-MQC-5        | 4040216                        | EXT-MQC-5        | 3068832                           | 74.23%        |
|          | AQS-MQC-6        | 4280130                        | EXT-MQC-6        | 3026803                           | 74.23%        |
| Mean (x) | 4173680.2        | 3098134.2                     | 72971.65         | 2.36                              | 74.23%        |
| SD       | 140637.05        | 1131.98                       | 72971.65         | 2.36                              | 74.23%        |
| CV%      | 3.37             | 2.36                          | 72971.65         | 2.36                              | 74.23%        |
| HQC      | AQS-HQC-1        | 5731755                        | 4859028          | 83.47%                            |
|          | AQS-HQC-2        | 5649408                        | 47931.10         | 83.47%                            |
|          | AQS-HQC-3        | 5618990                        | 4777081          | 83.47%                            |
|          | AQS-HQC-4        | 5728449                        | 4717922          | 83.47%                            |
|          | AQS-HQC-5        | 5696802                        | 4654670          | 83.47%                            |
|          | AQS-HQC-6        | 5765760                        | 4736922          | 83.47%                            |
| Mean (x) | 5698527.3        | 4756435.5                     | 70023.94         | 1.47                              | 83.47%        |
| SD       | 5524.76          | 70023.94                      | 70023.94         | 1.47                              | 83.47%        |
| CV%      | 0.97             | 1.47                          | 70023.94         | 1.47                              | 83.47%        |

*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        | 2626715               | SPIKED-LQC-3     | 1955042             |           |
|             | AQS-LQC-4        | 2616782               | 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     | 1802419             |           |
|             | 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        | 2499825               | 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 6.32 1831376.3
%CV 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 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 table 11 contains the results of accuracy and precision.
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)

| 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        | 100.10     |

*Number of replicates = 6, FBS-Feboxostat, LLOQ-Lower limit of quantification, SD-Standard deviation, CV-Co-efficient of variance.*
Table 9: Inter batch precision and accuracy

| Batch | LLOQ (ng/ml) | %Accuracy | LCQ (ng/ml) | %Accuracy | MQC1 (ng/ml) | %Accuracy | MQC2 (ng/ml) | %Accuracy | HQC (ng/ml) | %Accuracy |
|-------|--------------|-----------|-------------|-----------|--------------|-----------|--------------|-----------|-------------|-----------|
| QC    | 20.779       | 97.6      | 61.174      | 99.49     | 1529.341     | 99.49     | 5097.802     | 95.87     | 7608.659    | 97.91     |
| Mean  | 20.3139      | 95.01     | 58.1238     | 99.49     | 1529.5868    | 95.87     | 4887.0549    | 97.91     | 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. 7: Chromatogram of MQC1 sample of FBS and FBS D9

Fig. 8: Chromatogram of MQC2 sample of FBS and FBS D9
Table 10: Intraday precision and accuracy

| QC        | LLOQ (ng/ml) | LQC (ng/ml) | MQC1 (ng/ml) | MQC2 (ng/ml) | HQC (ng/ml) |
|-----------|--------------|-------------|--------------|--------------|-------------|
| QA        | 20.799       | 61.174      | 1529.341     | 5097.802     | 7608.659    |
| Mean      | 20.5402      | 58.2313     | 1493.5383    | 4847.3108    | 7495.0603   |
| 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 control, SD-Standard deviation, CV-Co-efficient of variance.

Table 11: Intra batch precision and accuracy

| Batch     | LLOQ (ng/ml) | LQC (ng/ml) | MQC1 (ng/ml) | MQC2 (ng/ml) | HQC (ng/ml) |
|-----------|--------------|-------------|--------------|--------------|-------------|
| 1st batch | 20.799       | 61.174      | 1529.341     | 5097.802     | 7608.659    |
| Mean      | 20.6940      | 58.7045     | 1457.2373    | 4806.0205    | 7591.3512   |
| SD        | 0.62943      | 64.67799    | 262.02031    | 48.95283     | 3.04        |
| CV%       | 3.04         | 4.44        | 5.45         | 6.14         | 4.49        |
| N         | 6            | 6           | 6            | 6            | 6           |
| 2nd batch | 20.2063      | 57.7230     | 1529.839     | 4888.60      | 7398.769    |
| Mean      | 20.0631      | 60.65334    | 44.34646     | 172.81499    | 51.36160    |
| SD        | 0.90631      | 6           | 6            | 6            | 6           |
| CV%       | 3.44         | 3.54        | 6.69         | 6.69         | 6.69        |

*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 effect 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).
The new developed method is a rapid, simple, specific and accurate liquid chromatography mass spectrophotometry for the determination of FBS in human plasma. As per Cmax were found within the limit. The sophisticated solid phase extraction determination of FBS as the results of all the validation parameters indicating RP-HPLC method for the determination of Febuxostat (a xanthine oxidase inhibitor). J Chromatogr Sci 2013;51:931-8.

CONFLICT OF INTERESTS
Declared none

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How to cite this article
• Narottam Pal, Avanapu Srinivasa Rao, Piggili Rayakumar. New method development and validation for the determination of febuxostat in human plasma by liquid chromatography–mass spectrometry. Int J Pharm Pharm Sci 2016;8(9):61-70.

Table 12: Stability data of QC samples in human plasma

| Nominal Conc | Stability | Mean (ng/ml) | % Accuracy | Precision (CV %) |
|-------------|-----------|-------------|------------|-----------------|
| LQC (61.174 ng/ml) | Bench top 15 h | 59.1428 | 96.68 | 5.90 |
| | Wet extract | 62.6802 | 97.69 | 3.64 |
| | 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 | 76.08.659 | 100.95 | 1.20 |
| HQC (7608.946 ng/ml) | Freeze-thaw | 77.37.6602 | 101.70 | 1.61 |
| | Wet extract | 78.03.0808 | 102.56 | 1.19 |
| | Autosampler | 77.14.5567 | 101.39 | 1.22 |
| | Freshly spiked QC | 77.56.0290 | 101.96 | 0.72 |
| | 60 d | 76.56.8233 | 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 |
|-------------------------------|------|-------------------|-------------------|
|                               | Mean | % Accuracy | NC = 16797.041 | % Accuracy | NC = 16797.041 | % Accuracy |
| Mean                           | 16355.7257 | 97.37 | 16542.7382 | 98.49 |
| SD                             | 444.99782 | 2.72 |
| CV%                            | 2.72 | 2.64 |

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