ACADEMIA ROMÂNĂ
*Revue Roumaine de Chimie*
https://www.icf.ro/rrch/

*Rev. Roum. Chim.*,
**2023**, *68*(10–12), 527–532
DOI: 10.33224/rrch.2023.68.10-12.04

# PHARMACOKINETIC ANALYSIS OF FEBUXOSTAT IN HUMAN SERUM BY UFLC BASED ON FLUORIMETRIC DERIVATIZATION

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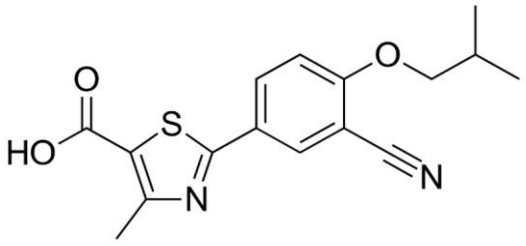
*Received September 20, 2022*

An ultra fast liquid chromatographic (UFLC) method using fluorimetric detection for the quantitation of Febuxostat (FEB) in human serum was developed by precolumn derivatization with 4-bromomethyl-7-methoxy coumarin (BrMmC). The derivatization was catalyzed with dibenzo-18-crown-6-ether to produce a fluorescent derivative which was determined through fluorescence measurement with excitation at 320 nm and emission at 395 nm wavelengths. A C18 column with 100 cm × 4.6 mm, 3 µm particule size was used for the UFLC separation procedure. A mixture of acetonitrile: methanol: 0.05 M aqueous ammonium acetate (pH 5.0) (40:40:20, v/v/v) was used as mobile phase. During the procedure, the flow rate was maintained at 0.5 mL/min.




A calibration curve was then plotted as 0.005–3.0 µg/mL with a detection limit of 0.0022 µg/mL and quantification limit of 0.0073 µg/mL. The mean recovery to reveal the accuracy and relative standard deviation (RSD) that indicates the precision of the method were found out to be 85.20 % and less than 3.52 %, respectively. The utility of the new method has been demonstrated by measuring the pharmacokinetics of FEB by administration of 80 mg tablets to a healthy female volunteer, aged 42.

## INTRODUCTION

Gout is a widely diagnosed rheumatic disease. Febuxostat (FEB), a novel non-purine selective inhibitor of xanthine oxidase, which is used for the treatment of hyperuricaemia in gout.[1]

In the literature, several methods of FEB analysis have been described in different matrices including spectrophotometric,[2-4] spectrofluorimetric,[5-7] high performance liquid chromatographic methods in combination with UV,[8,9] fluorimetric,[10] mass (MS)[11-13] and tandem mass MS/MS detections,[14] and a capillary chromatographic method.

In the current work, we introduce a novel sensitive and specific ultra-fast liquid chromatographic (UFLC) method combined with fluorescence detection. 4-bromomethyl-7-methoxycoumarin (BrMmC) is a fluorescent reagent and it is commonly used for the derivatization of analytes having carboxylic acid as functional group.[16-19] No methods have been described yet using the derivatization of FEB with BrMmC compound for biological matrices. UFLC is more favourable compared to conventional HPLC due to shorter duration and superior resolution features.[20-23]

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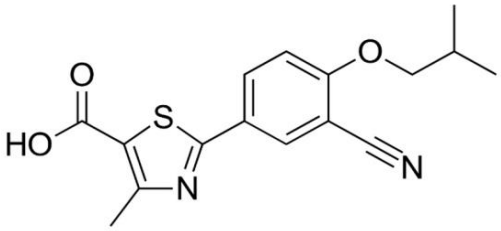


Fig. 1 – Chemical structure of FEB.

This study aimed to determine the FEB concentrations in serum by using UFLC method by derivatization with BrMmC. The new UFLC method was efficiently performed to determine the pharmacokinetic profile of FEB tablets.

## EXPERIMENTAL

### Chemicals and material

Febuxostat were provided by Dr Reddys Laboratories Limited (India). Adenuric film tablets® containing 80 mg FEB were taken local drug store. All reagents and chemicals procured were analytical grade. Sigma-Aldrich Chemie (Steinheim, GERMANY) is the supplier for the fluroscent derivatization reagent 4-bromomethyl-7-methoxy coumarin and the catalyst reagent dibenzo-18-crown-6-ether. Pure water for HPLC system was obtained using Elga Purelab Option Q (VWS DEUTSCHLAND) water purification system.

### Equipment and conditions for chromatography

Shimadzu LC 20A UFLC (Kyoto, Japan) Ultra Fast liquid chromatograph system was used for the chromatographic separation. The system composes of an autosampler system model SIL-AT-HT, a column oven model a SPD-20A HT and CTO 10 AC. The detector was adjusted to 320 nm for excitation of fluorescence and to 395 nm for fluorescence emission measurement using fluorimetric detector. Separation was performed on a C18 column (100 × 4.6 mm, 3 µm) (GL-Sciences,Tokyo-JAPAN) under isocratic elution using a mobile phase formed with a mixture of acetonitrile: methanol:0.05 M aqueous ammonium acetate (pH 5.0) (40:40:20, v/v/v) under flow rate of 0.5 mL/min where temperature for the column was tuned as 25ºC.

### Solution preparation

An adequate amounf of FEB was dissolved in methanol to yield 100 mg/mL concentration of FEB and diluted with DMSO to reach the final concentration of 1 mg/mL. Stock solution was diluted to prepare standard solutions of FEB by dilution with methanol. The stock solutions were stable for 30 days when kept at 4°C.

Reagent BrMmC stock solutions at 100 µg/mL concentration was formed in acetonitrile freshly every day. Acetonitrile was used to prepare 18-crown-6 solution at 1 µg/mL concentration.

### Preparation and derivatization of samples

The ethics committee approval to conduct the study was obtained from the Bezmialem Vakif University (BVU), Clinical Research Ethics Committee. The blood samples were collected from a 42-years-old female healthy volunteer. In order to prevent any coagulation, the samples were transferred in a tube with heparin as anticoagulant. Centrifugation of the samples was performed at 3000 rpm for 30 minutes to have a good separation of the aliquot. Then, serum samples of 0.2 mL were taken and transferred into 10 mL glass test tubes with stoppers. Volumes of 50 µL FEB solution at different concentrations were added into these glass test tubes. After mixing the serum and FEB solutions in the glass test tubes for 10s, a volume of 0.5 mL of acetonitrile was transferred on to the mixture. After the resulting mixtures were vortexed for 30s, centrifugation was carried out at 10000 rpm during 5 min. Until the dry residue was obtained, the 0.5 mL of supernatant layers were evaporated in a water bath kept at 50°C and under a slow flow of gaseous nitrogen.

The dried residue was derivatized with a 300 µL volume of BrMmC solution in acetonitrile, 50 µL solution of 18-crown-6-ether and 2 mg of $K_2CO_3$. To conclude the chemical reaction, this mixture was shaken for 70 min at 60°C in a water bath then, the mobile phase was added to complete the volume to 500 mL. Samples were filtrated using a 0.45 µm PTFE filter. Then, a volume of 20 µL filtrate was applied to the UPLC system.

### Validation of UFLC method

Method validation is performed according to ICH Guidelines in terms of selectivity, accuracy, precision, recovery, linearity, sensitivity, reproducibility, stability parameters of in spiked samples of the analyte.[24]

After the deproteinization process, standard solution series of 0.005, 0.1, 0.5, 1.0, 1.5, 2.0 and 3.0 µg/mL FEB were prepared from the 50.0 µL stock FEB solution and added to a 200.0 µL volume of blank serum extract. Finally, seven different solutions at different FEB concentration series were obtained and they were studied for the calibration curve.

The samples were trialed as previously described derivatization process. The calibration curve was constructed by plotting the known concentrations of FEB with the corresponding analyte peaks found in the UFLC analysis. The regression equation was used for calculation of the unknown FEB concentrations in the respective samples. The precision of the method was expressed by calculating the relative standard deviation (RSD) of “intra-day” and “inter-days” assays which were carried out with six repetitions of sample analysis (0.005, 1.0, and, 3.0 µg/mL) in a specific working day or on 5 different days.

The stability of the serum samples was determined by analyzing 3 various concentrations of FEB (0.005, 1.0, and 3.0 µg/mL). The serum samples were tested after storage process at ambient temperatures on a benchtop. For this purpose, 3 replicate analyses were performed in the first and third days. Stability of FEB in serum was assayed after 3 freeze–thaw cycles and the stability of the serum sapmles at -20°C were also trialed. The recovery at 3 various concentrations of FEB (0.005, 1.0, and 3.0 µg/mL) was monitored by comparison of the analyte peaks gained from serum samples.

### Applicability

A pharmacokinetic study was conducted on a healthy female volunteer. After administering a 80 mg FEB single oral dose to the volunteering individual, the venous blood samples were gathered into heparinized glass test tubes according to the following blood sampling schedule expressed as hours: 0, 0.25,

0.5, 0.75, 1, 1.25, 1.6, 1.8, 1.9,2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0, 12, 16, 18 and 24 hours. The serum samples were prepared from the collected venous blood samples. The method used for the preparation of the serum samples were previously defined in the "Sample Preparation" section of this article. Till analysis, the samples were kept at –20°C. Pharmacokinetic parameters were measured according to the data using the SAS system version 9.4 (SAS Institute, Cary, NC).

## RESULTS AND DISCUSSION

### Conditions for chromatography

The optimal separation conditions were achieved by using a C18 column (100 × 4.6mm, 3µm) and a mobile phase which was composed of acetonitrile: methanol: 0.05 M aqueous ammonium acetate (pH 5.0) (40:40:20, v/v/v). The procedure was carried out under the 0.5 mL/min flow rate. The fluorescent derivatization procedure was performed with BrMmC as the reagent using dibenzo-18-crown-6-ether as the reaction catalyzer. The fluorescence of FEB derivative was measured at $\lambda_{ext.}$= 320 nm and $\lambda_{em.}$= 395 nm using a fluorimetric detector. The FEB derivative retention time was calculated under previously described chromatographic conditions and it was found out to be approximately 3.0 min (Fig. 2).

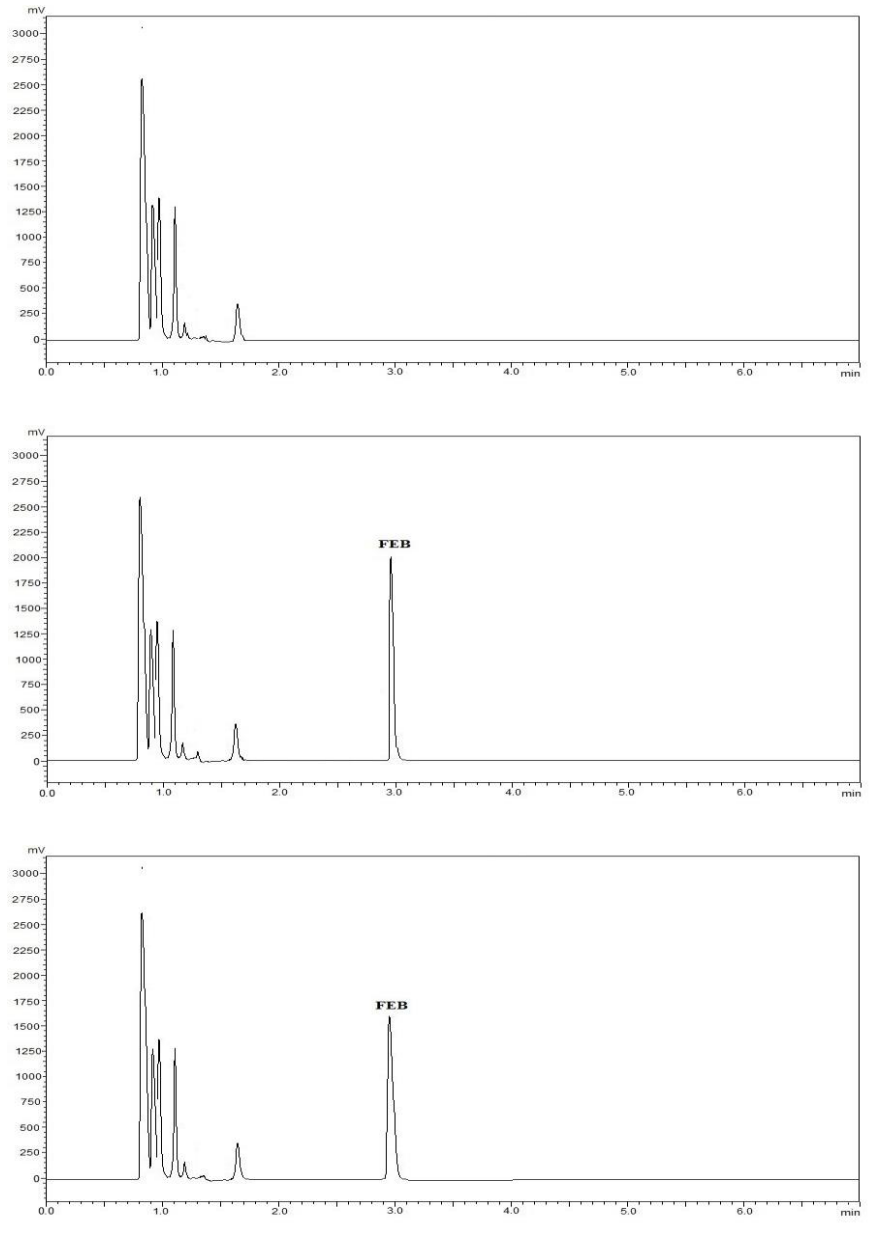


Fig. 2 – Chromatograms of: A) Blank serum sample; B) Serum sample spiked with FEB (3 µg/mL); C) Serum sample obtained from the healty volunteer 2.5 h after oral administration of 80 mg FEB.

**Optimum reaction parameters for derivatization**

According to a previous study, FEB was derivatized in pharmaceutical preparations with BrMmC reagent and dibenzo-18-crown-6-ether was used to catalyze the reaction.[15] Reaction conditions were given in Table 1.

*Table 1*

Optimum derivatization conditions

| Parameter | Optimum value |
|---|---|
| Derivatization time (min) | 70 |
| Temperature (°C) | 60 |
| Volume of BrMmC (µL) | 300 |
| Volume of 18-crown-6 solution (µL) | 50 |
| Amount of $K_2CO_3$ (mg) | 2 |

**Method validation**

Calibration curves were constructed for the serum concentrations of FEB within the range of 0.005 - 3.0 µg/mL. To construct the curve, the serum concentrations of FEB (expressed in µg/mL) were established with the corresponding peak responses obtained in the assay. The mean linear regression equation of the calibration curve was calculated with the formula as follows $y = 0.227x + 0.0067$ ($r^2 = 0.9979$), where $y$ indicates the area of FEB and $x$ indicates the serum concentrations of FEB.

The formula below was used in order to calculate the limit of quantitation (LOQ) and the limit of detection (LOD):

$$\text{LOD or LOQ} = \kappa SDa/b;$$

where κ was taken as 3 for LOD or 10 for LOQ, "SD" was the intercept's standard deviation, and $b$ indicated the slope. Using this formula, the LOQ value was calculated to be 0.0022 µg/mL and the LOD value was determined to be 0.0073 µg/mL.

To investigate the mean recovery following the extraction procedure, spiked serum samples at three various concentration levels of FEB were selected. The FEB concentrations of 0.005, 1.0, and 3.0 µg/mL were selected and analyzed. The estimated recovery was calculated to be 86.30% (Table 2).

*Table 2*

Extraction recovery values of FEB from serum samples

| Added concentration (µg/mL) | Found concentration (µg/mL)± SD | Recovery% | RSD% * |
|---|---|---|---|
| 0.005 | 0.00426 ± 0.00015 | 85.20 | 3.52 |
| 1.0 | 0.853 ± 0.0272 | 85.30 | 3.15 |
| 3.0 | 2.652± 0.0725 | 88.40 | 2.73 |

*RSD: Relative Standart Deviation

The "intra-day" and "inter-days" RDS values were analyzed within the same day and in the following consecutive days with 5 replicated tests. In this method; the calculated RSD values were determined to be 1.69–3.20% and 1.82–3.84% for the "intra-day" and "inter-days" that indicates high precision of the method (Table 3), respectively. These figures indicated a good level of precision. The results were presented in Table 3.

*Table 3*

Intra-day, Inter-day precision and accuracy results of FEB from serum samples

| Added concentration (µg/mL) | Found concentration (µg/mL)± SD | RSD% * |
|---|---|---|
| **Intra-day** | | |
| 0.005 | 0. 00437 ± 0. 00014 | 3.20 |
| 1.0 | 0. 98 ± 0. 0183 | 1.86 |
| 3.0 | 2. 97 ± 0. 0504 | 1.69 |
| **Inter-day** | | |
| 0.005 | 0. 00416 ± 0. 00016 | 3.84 |
| 1.0 | 0. 96 ± 0. 0194 | 2.02 |
| 3.0 | 2.79 ± 0. 0305 | 1.82 |

*RSD: Relative Standart Deviation

Standard drug solutions were tested for stability to investigate the applicability of different storage conditions. The tested conditions for optimum storage conditions such as, ambient temperature at dark for 24 hours, keeping in auto-sampler for 24 hours, and a temperature of 4°C for one-month storage. The stability studies have provided evidence that the drug samples can be kept stable under these storage conditions described above.

### Analysis of Human Serum Samples

Human serum samples, obtained from a healthy female volunteer at 42 years of age, were analyzed using the developed method. For the analysis, 80 mg FEB single oral dose was administered to the fasting participant and venous blood samples were collected. $C_{max}$ was found to be 2.81 µg/mL at the hour 1.90 ($t_{max}$) following the administration of the drug (Table 4). The serum concentrations of FEB were monitored for up to 24 hours (Fig. 3).

*Table 4*

Pharmacokinetic parameters of Febuxostat after administration of 80 mg orally

| Parameter | Found value |
|---|---|
| $t_{max}$ [a](h) | 1.90 |
| $C_{max}$ [b](µg/mL) | 2.81 |
| $t_{1/2}$[c] (h) | 4.92 |
| AUC[d]$_{0\text{-}24}$ (µg h mL$^{-1}$) | 11.18 |

[a]Time to maximum concentration
[b]Maximum concentration,
[c]Elimination half life,
[d]Area under the concentration-time curve

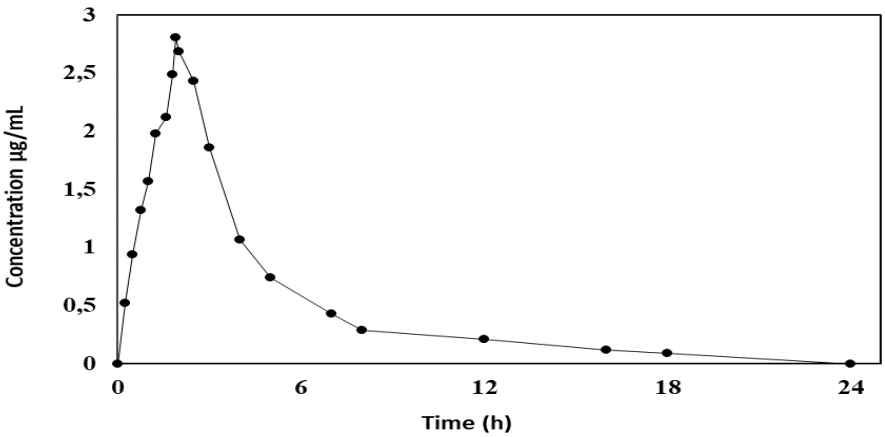


Fig. 3 – Serum concentration–time profile of FEB in a healthy volunteer after oral administration of 80 mg/dose.

## CONCLUSIONS

In this study, a new, simple, fast, and specific ultra fast liquid chromatography method with fluorimetric detection to quantify FEB in human serum was developed. The results prove that the proposed fluorimetric method provides pharmacokinetic investigation of the drug substance and gives the opportunity to monitorize the drug concentration levels in human serum. The main advantages of this method compare to listed references is short analysis time (about 3.0 minutes), rapid and simple analytical procedures, higher sensitivity with low LOD value (0.0022 µg/mL) and having not a requirement for sophisticated analytical instruments. The results of the presented study proves us it is possible to use this developed method for routine, toxicological and clinical assays of FEB in serum.

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