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Research Article

Method Development and Validation for Determination of Febuxostat from Spiked Human Plasma Using RP-HPLC with UV Detection

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A rapid, simple, selective, and specific reverse phase high performance liquid chromatography (RP-HPLC) method with UV detection (315 nm) was developed and validated for estimation of febuxostat from spiked human plasma. The analyte and internal standard (diclofenac) were extracted using LLE with diethyl ether. The chromatographic separation was performed on Shodex C-18-4E (5 μm; 250 × 4.6 mm) with a mobile phase comprised of methanol : acetate buffer pH 4, 20 mM (90 : 10 v/v), at a flow rate of 1 mL/min. Febuxostat was well resolved from plasma constituents and internal standard. The calibration curve was linear in the range of 250–8000 ng/mL. The heteroscedasticity was minimized by using weighted least square regression with weighing factor of $1/x$. The intraday and interday %RSD was less than 15. Results of recovery studies prove the extraction efficiency. Stability data indicated that febuxostat was stable in plasma after three freeze thaw cycles and upon storage at −20°C for 30 days.

1. Introduction

The determination of drugs from biological fluids is essential to pharmacologic and pharmacokinetic studies, which include determinations of the absorption, distribution, metabolism, and elimination of drugs in animals and humans. Biologic fluid assays also support studies of correlations of blood tissue levels of drugs and pharmacologic effects (pharmacodynamics), which are sought during drug development [1–3]. Sample preparation for analysis of biological samples is one of the key factors that determine the extent of recovery of the analyte from the sample matrix. In this context, LLE is the most widely used method for extracting analytes from aqueous biological fluids and separating them from endogenous interferants. LLE also provides a simple means of concentrating the analyte by evaporation of the solvent. The analyte is isolated by portioning between the organic phase and the aqueous phase [4, 5].

Febuxostat is chemically 2-[3-cyano-4-(2-methylpropoxy)phenyl]-4-methylthiazole-5-carboxylic acid (Figure 1). It is a non-purine selective inhibitor of xanthine oxidase that is indicated for use in the treatment of hyperuricemia and gout. Febuxostat inhibits xanthine oxidase noncompetitively, therefore reducing production of uric acid [6]. Several methods are reported in literature for estimation of febuxostat from rat plasma using techniques like LC-MS/MS [7–10], LC-MS [11] and HPLC with fluorescence detection [12–15]. However the methods reported involved use of costly sample preparation tools like solid phase extraction and relied on less commonly available internal standard. Hence the purpose of the present work was to develop a simple economical LLE-RP-HPLC-UV method for the estimation of febuxostat from spiked human plasma.

2. Experimental

2.1. Equipment and Materials. The analysis was performed using Agilent 1200 series quaternary pump HPLC system equipped with variable wavelength programmable UV detector with precision loop injector (Rheodyne 20 μL). 50 μL Hamilton injection syringe was used for sample injection. The data was processed using Chemstation (B.02.01) software.

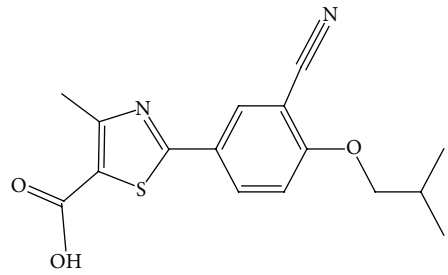

Figure 1: Chemical structure of febuxostat.

Pharmaceutical grade febuxostat was kindly provided by Ammi Life Sciences, Gujarat, India, and diclofenac by Aarti Drugs, Mumbai, India. Blank human plasma was procured as a gift sample from National Plasma Fractionation Centre, KEM Hospital, Mumbai, India. Plasma from six different sources was mixed thoroughly to get pooled blank plasma. Methanol and water used in the analysis were of HPLC grade and all other chemicals were of AR grade. All chemicals were purchased from SD Fine Chemicals, Mumbai, India. The 0.45 μm Nylon membrane filters were purchased from Pall India Pvt. Ltd. Mumbai, India.

2.2. Preparation of Calibration Curve (CC) Standards and Quality Control (QC) Samples. The stock solution (1 mg/mL) of febuxostat was prepared in methanol and this was then appropriately diluted with methanol to get working standard solutions having concentrations 10, 20, 40, 80, 160, and 320 μg/mL. 1 mL aliquots of blank human plasma were spiked with 25 μL of the working standard solutions to get CC standards containing 250, 500, 1000, 2000, 4000, and 8000 ng/mL of guaifenesin. The QC samples were similarly prepared to contain three concentrations [500 ng/mL lower quality control (LQC), 2000 ng/mL middle quality control (MQC), and 7500 ng/mL higher quality control (HQC)].

2.3. Preparation of Internal Standard Stock Solution and Working Standard Solution. The stock solution (1 mg/mL) of diclofenac was prepared in methanol and then appropriately diluted with methanol to get working standard solution of 240 μg/mL.

2.4. Sample Preparation. 1 mL of human plasma spiked sample was taken in a glass tube of 15 mL capacity. To this 5 mL of diethyl ether was added and the contents of the tube were mixed on a vortex mixer for 2 min. The tubes were kept in an inclined position on a reciprocating shaker at 100 strokes per min for 45 min. After extraction, the tubes were centrifuged at 3000 rpm for 15 min at 4°C. 3 mL of the organic layer was pipetted out into a separate tube and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 250 μL of the mobile phase and subjected to chromatographic analysis using the optimized chromatographic conditions.

2.5. Chromatographic Conditions. The chromatographic separation was performed in a Shodex C 18-4E column (5 μm; 250 × 4.6 mm, Showa Denko America Inc., USA). The mobile phase consisted of a mixture of methanol : acetate buffer pH 4, 20 mM (90 : 10 v/v). The injection volume was 20 μL and the run time was 10 min. Detection was carried out at 315 nm.

2.6. Calibration Studies. All CC standards were analyzed in six replicates and the data of concentrations and the corresponding area ratios of febuxostat to internal standard were subjected to unweighted and weighted least square linear regression. The equations so generated were used to calculate the interpolated concentrations of the CC standards and the % relative error (%RE) was calculated for each CC standard as

$$\%\text{RE} = \frac{\text{Interpolated concentration} - \text{nominal concentration}}{\text{Nominal concentration}} \times 100. \quad (1)$$

The calibration model that resulted in minimum total %RE for the interpolated concentrations of CC standards was selected as the one that gave least error.

2.7. Validation. The developed method was validated as per the recommendations of US FDA Guidance for Industry: Bioanalytical Method Validation [16]. Selectivity was studied at the lower limit of quantification (LLOQ) of 250 ng/mL by comparing the blank responses of plasma from six different sources with the peak areas afforded by the LLOQ samples. Accuracy and precision were studied by analyzing five replicates of the lower quality control (LQC), middle quality control (MQC), and higher quality control (HQC) samples over five days. The concentration of febuxostat in the QC samples was determined by referring to the area ratio of the drug to internal standard, obtained from the QC samples, to the calibration equation generated on the same day. The accuracy was estimated as the mean %RE while the precision was measured in terms of %RSD.

The recovery of the extraction procedure was calculated by comparing the peak areas of the processed QC samples to those of corresponding standard dilutions.

Stability of febuxostat was evaluated under various conditions, namely, three freeze thaw cycles, stability at room temperature for 6 h, and short term stability at −20°C. For the determination of freeze thaw stability, five replicates of LQC and HQC samples were frozen at −20°C for a minimum of 24 h and then allowed to thaw unassisted at room temperature. For short term room temperature stability, five replicates of LQC and HQC samples were kept at room temperature for 6 h, short term stability at −20°C was determined by using five replicates of LQC, and HQC was kept at −20°C for one month. The amount of the drug in stability samples was found and the % nominal and %RSD were calculated.

Table 1: Area ratio from calibration experiments on febuxostat.

CC number	Amount of drug (ng/mL)	Mean area ratio (±SD*)
1	250	0.197 ± 0.009
2	500	0.321 ± 0.013
3	1000	0.569 ± 0.011
4	2000	1.325 ± 0.033
5	4000	2.570 ± 0.065
6	8000	5.061 ± 0.034

*Standard deviation.

Table 2: Blank responses and the peak areas at LLOQ.

Serial number	Blank response (mAU·sec)	Peak areas at LLOQ (mAU·sec)
1	3.46	38.32
2	6.64	38.67
3	3.47	38.02
4	3.62	37.97
5	3.86	38/16
6	3.63	38.95

3. Results and Discussion

Chromatographic conditions were selected after several trials on different columns using mobile phases comprised of methanol and acetate buffer at varying pH and in different proportions. The Shodex C-18-4E column (250 × 4.6 mm, 5 μm) and mobile phase of methanol : acetate buffer pH 4, 20 mM (90 : 10 v/v), gave adequate resolution and satisfactory peak shapes for febuxostat and internal standard. The detection wavelength of 315 nm was chosen for maximum sensitivity.

When liquid-liquid extraction was performed using different immiscible solvents like diethyl ether, ethyl acetate, and dichloromethane, it was found that both the drug and internal standard were appreciably extracted with diethyl ether. The extraction recovery for febuxostat was 72–75%, while that of diclofenac was 73–78%.

During calibration experiments it was revealed that the standard deviation of area ratio of CC standards increased with concentration indicating a need for weighted linear regression. The area ratios from calibration experiments are depicted in Table 1.

The use of the unweighted regression calibration model with linear regression equation $Y = 0.000633129X + 0.01185$ resulted in heteroscedasticity and a large total %RE. When weighting factor of $1/x$ was used, the variances of area ratios of CC standards were homogenized over the calibration range with a decrease in the %RE. Thus, weighted linear regression with a weighting factor of $1/X$ was selected as a calibration model which resulted in the equation $Y = 0.0006X + 0.0198$. During the validation studies it was found that the peak area for the LLOQ samples was more than five times the blank responses obtained using six different plasma sources, as can be seen in Table 2. This proved that the method was selective at the LLOQ of 250 ng/mL. The representative chromatogram of blank plasma shows no interference at the retention times of febuxostat and diclofenac (Figure 2).

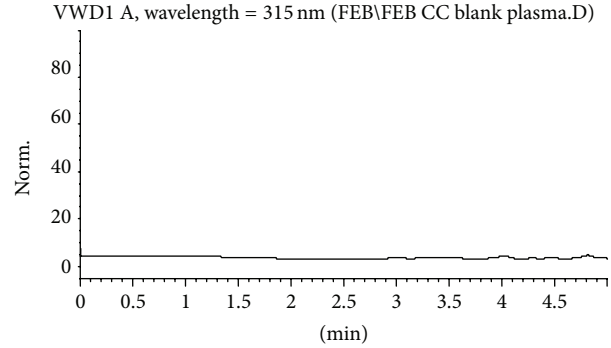

Figure 2: Representative chromatogram of blank plasma extract showing a lack of significant interference at the retention times of febuxostat and the internal standard, diclofenac.

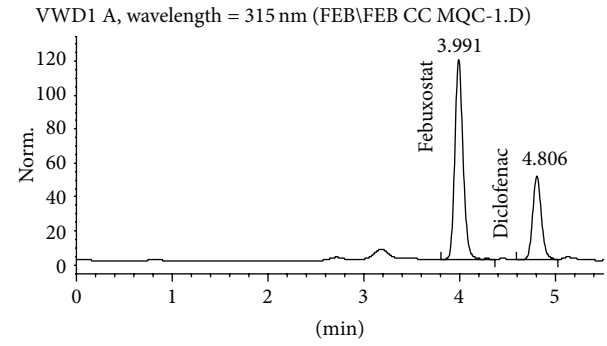

Figure 3: Representative chromatogram of MQC sample of febuxostat showing febuxostat (RT = 3.991 min) and internal standard, diclofenac (RT = 4.806 min).

The representative chromatogram of a MQC sample of febuxostat (Figure 3) shows both the drug and an internal standard extracted from plasma. The evaluation of accuracy and precision showed that the intraday %RE was between +15% and −15%, while the %RSD was less than 15%. The USFDA Guidance requires that the %RE be between +15% and −15%, while the %RSD should be less than 15%. The results of assay precision and accuracy as well as extraction recovery for febuxostat at LQC, MQC, and HQC and for internal standard are represented in Table 3.

From the stability studies, it can be concluded, that for each stability study, % nominal concentration was between 85 and 115 and %RSD was less than 15, as shown in Table 4, which falls within the acceptable limits. This indicates that the deviation of results from nominal concentration may be attributed to the variability of the assay and imply that the drug had remained stable after stability cycles.

4. Conclusion

In this report a rapid, simple, sensitive, and accurate HPLC-UV method was described for the determination of febuxostat in spiked human plasma using liquid-liquid extraction.

Table 3: Results of assessment of accuracy, precision, and %recovery of febuxostat.

Level	Concentration added (ng/mL)	Intraday ($n = 5$)			Interday ($n = 5$)			%Recovery ($n = 5$)
		Mean concentration found (ng/mL)	RE*1, %	RSD*2, %	Mean concentration found (ng/mL)	RE, %	RSD, %	
LQC	500	502.41	0.48	3.17	509.46	1.88	3.49	74.34
MQC	2000	2070.65	0.82	3.49	1902.32	−4.88	3.34	72.13
HQC	7500	7640.86	1.88	4.98	7752.68	3.37	3.17	73.98
IS	—	—	—	—	—	—	—	76.13

*1Relative error; *2relative standard deviation.

Table 4: Results of stability studies for febuxostat ($n = 5$).

QC level	Stability at room temperature		Stability at −20°C		Freeze thaw stability	
	%nominal	%RSD	%nominal	%RSD	%nominal	%RSD
LQC	105.53	6.9	91.43	8.7	86.21	4.8
HQC	97.36	8.1	102.89	11.1	88.64	7.2

The method described does not require expensive chemicals and solvents and does not involve complex instrumentation or complicated sample preparation.

The developed method performs well with selectivity, precision, accuracy, stability, and linearity for the concentration range 250 to 8000 ng of febuxostat. The proposed method can be used for routine bioanalysis of febuxostat from plasma to support bioavailability bioequivalence studies.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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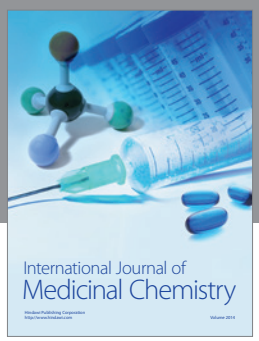

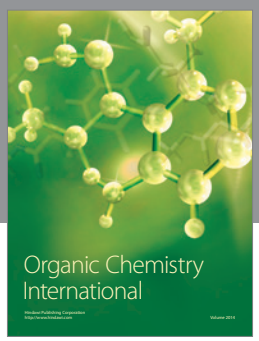

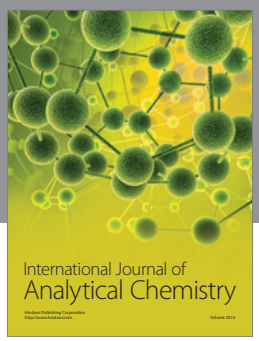

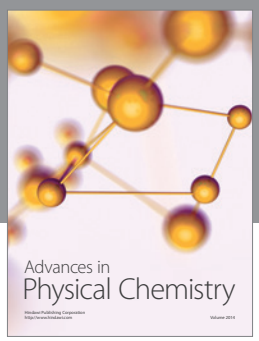

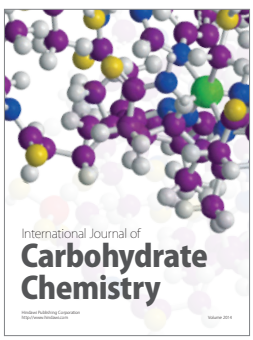

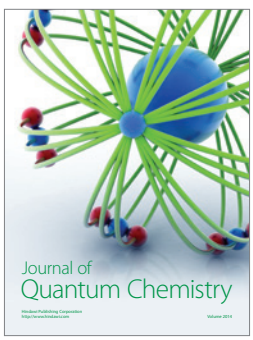

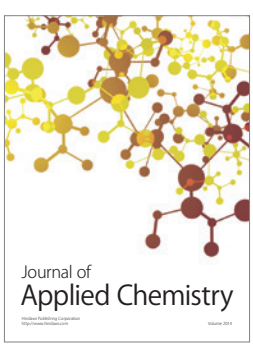

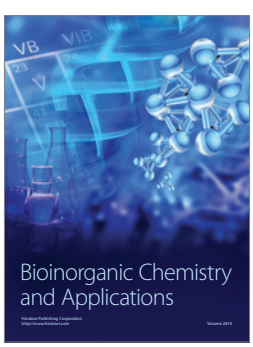

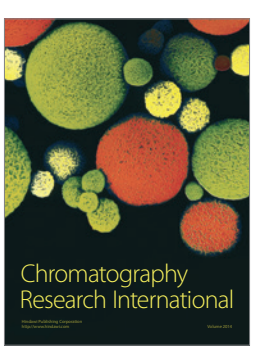

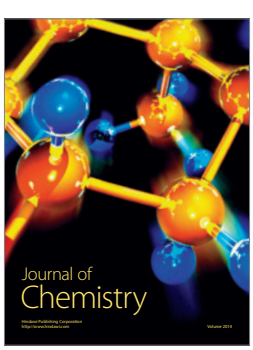

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