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Research Article

Validation of a Statistically Optimized Stability Indicating Method for the Estimation of Febuxostat in a Solid Dosage Form

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Abstract

In this study stability indicated liquid chromatographic method was used to determine the stability of Febuxostat (FBX) in solid dosage form being optimized by 2^3 factorial designs. The absorption maxima (λ max) were detected in methanol (70% v/v), using UV spectrophotometer. Various mobile phases were studied to select the optimized chromatographic conditions (mobile phase, pH and flow rate) using 2^3 factorial design. Total 8 methods (A1 to A8) were studied to select the optimized one and to explore for further studies. The statistical analysis was carried out using design Expert software version 8.0.5.2 (Stat-Ease Inc., Minneapolis, USA). The optimized solvent system comprised of methanol (70% v/v), pH-3 (UV detection at 315 nm) was used to separate over C-18 column with the flow rate of 0.8 mL/min (A5). The optimized method had asymmetry and thus triethylamine (TEA) was added to it, as peak modifier. The selected chromatographic method comprised of mobile phase with methanol:water (70:30 v/v), pH = 3, flow rate = 0.8 mL/min and TEA (0.3 mL/L) was further utilized. The present method generated a linear calibration curve with $r^2 = 0.9999$ over the range of 2 µg/mL to 50 µg/mL. The present method was also found to be reproducible for slope, intercept, correlation coefficient (r) and coefficient of analysis (r^2) values. The technique was found accurate and precised for three quality levels of low quality control (LQC-8 µg/mL), middle quality control (MQC-30 µg/mL) and high quality control (HQC-45 µg/mL) with percent relative standard deviation (% RSD) value of 0.004 to 0.018 for intraday precision and 0.002 to 0.011 for interday precision parameters. The resulting limit of detection (LOD) and limit of quantification (LOQ) values were found out to be 0.006 µg/mL and 0.002 µg/mL respectively. Furthermore method was found to be more rugged and robust on account of analytical parameters. Finally to justify the objectives the assay and forced degradation studies of FBX were also quantified. In this way the present method was successfully employed for routine quality control analysis of FBX in bulk samples and its pharmaceutical formulations.

Keywords: *Liquid chromatography; Febuxostat; Factorial design; Validation*

Introduction

Febuxostat (FBX) is [2-{3-cyano-4-(2-methylpropoxy)-phenyl}-4-methylthiazole]-5-carboxylic acid [1], white crystalline powder pharmaceutical and relatively insoluble in water. FBX is amongst the latest drug approved by the European medicine agency and US [2] for the treatment of hyperuricemia and gout. The present state of arts of FBX provides an alternative to the patients not tolerating or having inadequate reduction in serum uric acid level with allopurinol [3]. It is orally administered nonpurine selective xanthine oxidase inhibitor and the pharmaceutical (FBX) indicated for the chronic management of hyperuricemia in gout patients. It is an uricostatic [4] which inhibits both oxidized and reduced forms of xanthine oxidase [5] and also the conversion of hypoxanthine to xanthine and of xanthine to uric acid leading to a lower uric acid serum level in hyperuricemic patients. Hence, it is not recommended for the treatment of

asymptomatic hyperuricemia [6]. Similar to allopurinol, FBX does not structurally resemble a purine or pyrimidine pharmaceutical. It is thus more selective because it does not affect other enzymes involved in purine or pyrimidine synthesis. About 1-6% of FBX is excreted in the urine as unchanged drug and has minimal effects on other enzymes of purine and pyrimidine metabolism [7]. FBX is not yet official in any of the pharmacopeia. Regulatory agencies have heightened their scrutiny of the safety profile. Currently the emphasis has been placed on the development of analytical technique for the estimation of the drug release and content from the dosage form.

The determination of FBX in bulk drug and pharmaceutical formulations has been the subject of intense analytical research which leads to the development of sensitive and reproducible analytical techniques including UV [8] HPLC [9] LC-MS [10] and UPLC-MS [11]. Current status revealed that very few efforts have been made to determine the content of FBX through a solid dosage form by means of liquid chromatographic analytical procedures based on high performance liquid chromatography (HPLC) [12].

Statistical optimizing techniques like Factorial design enables to vary all the factors simultaneously and allowing quantification of the effects caused by independent variables and interactions between them. To study the significance of the independent factors over the dependent factors is also possible [13]. Many researchers have optimized pharmaceutical formulations using factorial design [14] but this is the first attempt to optimize any analytical method using factorial design.

In this present work author attempt to develop simple, sensitive and accurate method to analyze the drug stability profile of FBX. Simultaneously, the studies were designed to optimize the chromatographic method for independent variables (organic content, pH and flow rate) in order to achieve desired retention time, asymmetry and number of theoretical plates with precision and accuracy.

Materials and Methods

Chemicals and reagents

FBX was provided as a generous gift by Lupin Pharma Ltd, India. Acetonitrile, Methanol, Triethylamine and Ortho Phosphoric acid were purchased from Qualigens, Mumbai, India. HPLC grade water was purchased from Rankem, India.

Method development

Selection of suitable wavelength

Appropriate wavelength for detection of the drug was calculated using UV-visible spectrophotometer, by dissolving the drug in methanol and acquiring the range of 200-400 nm by means of Shimadzu UV-visible 1700 (E) spectrophotometer.

Development of analytical method

Development of the analytical method was made through a 2^3 factorial design with volume fraction of organic solvent in mobile phase (X^1), pH of the solution (X^2) and flow rate (X^3) as independent variables while considering the retention time (Y^1), asymmetry (Y^2) and number of theoretical plates (Y^3) as dependent variables. Total-Eight different conditions (A1-A8) were attempted by varying the conditions like different fraction of Methanol like 70% and 90%, pH (3.0 and 3.5) and flow rate (0.8 mL/min and 1.2 mL/min) to get a suitable analytical method (Table 1) with the optimized parameters such as symmetry, retention time and number of theoretical plates. The condition of mobile phase was optimized so that there was no interference from solvents for this FBX was injected into various mobile phases and their resolution was noted.

Statistical analysis of data

The effect of independent variables on the responses was modeled by Design Expert software version 8.0.5.2 (Stat-Ease, Inc., Minneapolis, USA). Polynomial equations were generated for the dependent variables that were reduced by removing non-significant coefficients by applying one way ANOVA. Level of significance was set at $p < 0.05$.

Trial Name	X1	X2	X3	Y1	Y2	Y3
A1	1	-1	-1	12.89	2.570	4125
A2	1	1	-1	11.79	2.722	3865
A3	1	-1	1	12.60	2.715	4012
A4	1	1	1	11.11	2.872	3814
A5	-1	-1	-1	15.26	1.655	6109
A6	-1	1	-1	14.36	2.105	4712
A7	-1	1	1	13.21	2.118	4562
A8	-1	-1	1	14.03	1.954	4672
A9	0	0	0	13.16	2.334	4484
A10	-1 + TEA	-1	-1	15.34	1.124	6105
A*	-1	-0.46	+0.1	14.352	1.899	5132.13

Table 1: *Pathogens of concern and control methods for various product categories (FDA, 2014).*

Validation of experimental design

The experimental design was validated by an extra design check point analytical condition (A9) and software tool by comparing the predicted and the observed value. The predicted values for retention time and asymmetry, generated by their respective polynomial equations were compared with experimental values and tested for statistical significance using pooled t-test at 95% confidence interval, and degree of freedom = 4 ($p < 0.05$).

Selection of optimized analytical condition

The effect of independent variables on the responses was modeled by using Design Expert software version 8.0.5.2 (Stat-Ease, Inc., Minneapolis, USA). The polynomial equations were generated for the dependent variables that were reduced by removing non-significant coefficients by applying one way ANOVA. Level of significance was set at $p < 0.05$.

Modification of the peak

Triethylamine (0.5 mL/l) was added to the selected optimized mobile phase as peak modifier (A10).

Chromatography

Preparation of mobile phase

The mobile phase was prepared by taking Methanol (HPLC grade) and water in the ratio of 70:30 (v/v). Triethylamine (0.5 mL/l) was added in mobile phase as a peak modifier. The final pH of buffer solution was maintained to 3.0 using orthophosphoric acid. The mobile phase was filtered through 0.45 µm filter and sonicated for 10 min prior to its use in HPLC.

Preparation of stock solution

Stock solution was prepared by accurately weighing 25 mg of FBX and dissolving in 50 mL volumetric flask with mobile phase to prepare a stock solution with concentration of 500 µg/mL. Working solutions for HPLC injections were prepared freshly from the stock solution and filtered through a 0.45 µm whatman membrane filter prior to injection.

Dilutions

Dilutions of concentrations 2.0, 5.0, 10, 15, 25, 40 & 50 µg/mL were prepared from the stock solution. All the dilutions were prepared in mobile phase. Three quality samples were prepared of concentration 20, 30 and 45 µg/mL from the stock solution. These samples were used for precision study.

Chromatography

Analysis was performed with a Shimadzu chromatograph equipped with LC-20AT solvent delivery system, a universal loop injector Rheodyne 7725i of injection capacity 20 µL, and an SPD-20A UV-visible detector (Shimadzu) set at 315 nm. The equipment was controlled by a PC work station. Compounds were separated on a 250 mmX 4.6 mm internal diameter; 5 µm particle size, Phenomenex Luna C-18 column under reversed phase partition chromatographic condition with flow rate of 0.8 mL/min. The selected mobile phase with TEA and pH of 3.0 was used for the analysis and the run time was selected as 20 min. Before analysis both the mobile phase and sample solution were degassed by the use of a bath sonicator and filtered through a 0.45 µm filter. Chromatography was performed in an air-conditioned room maintained at 25 ± 2°C.

System suitability tests

The system suitability test was performed to ensure that the complete testing system was suitable for the intended application. Peak area, retention time, tailing factor and theoretical plates were measured. The working standard stock (500 µg/mL) solution (0.3 mL) was diluted to 10mL with the mobile phase to final concentration of 15 µg/mL. Six replicate injection of this solution were made and % RSD values of all the parameter were noted.

Construction of calibration curve

Solutions of the pure drug of different concentration were prepared from the working stock of the standard solution. Final dilutions were prepared using mobile phase. These dilutions were chromatographed by injecting 20 µL and the peak areas were noted. The peak area of drug was then plotted against the respective concentration of the drug to plot the calibration curve. The unknown samples used for analysis were quantified with reference to these calibration plots.

Validation

The method was validated for linearity, sensitivity, accuracy, precision, robustness & ruggedness [15]. Selectivity and specificity of the method was accessed by injecting solution containing drug, which gave a sharp peak.

Linearity, range and reproducibility

Linearity of the method was determined by injecting the eight different concentrations and constructing the calibration curve by plotting peak area of the drug and against the respective concentration. The calibration range was determined for the linear curve with coefficient of correlation (r) 0.9999 and coefficient of determination (r^2) 0.9999 (n = 8). Reproducibility was determined by performing the calibration for five times (n = 5). Linear regression analysis was performed to plot the linear regression equation by determining the slope and intercept values.

Accuracy and Precision

The accuracy and precision of the developed method was confirmed by conducting recovery studies. The recovery studies were made by determining the concentration of a sample with known concentration of API. For this purpose three quality sample solutions of LQC (8 µg/mL), MQC (30 µg/mL) and HQC (45 µg/mL) of FBX were prepared from the stock solution with mobile phase. These samples were filtered through 0.45 µm whatman filter and three determination were made (n = 3). Accuracy is expressed as % Relative Standard deviation. Interday and intraday precision analysis was done and studied using the above three quality control samples 8 µg/mL, 30 µg/mL and 45 µg/mL. For study of intra-day precision the observation was made at three different intervals on the same day, while for inter-day precision the concentration of drug was calculated on three different days. Each concentration level was prepared from the stock solution freshly at three different times. Precision was expressed by % Relative Standard Deviation (% RSD). This study was performed in six replicates.

LOD and LOQ

LOD and LOQ were measured to evaluate the detection and quantization limits of the method to determine whether these were affected by the presence of the impurities. These were calculated by the use of equations:

LOD = 3.3 σ/S

LOQ = 10 σ/S

Where- σ is representing the standard deviation (S.D.) of the intercept and S is the slope of the calibration curve.

Robustness

The robustness of the assay method was established by introducing small changes in the HPLC conditions which included wavelength (310 and 320 nm), methanol: water in mobile phase (72:28 and 78:22) and flow rate (0.65 mL/min and 0.95 mL/min). Robustness of the method was studied using six replicates at a concentration level of 25 µg/mL of FBX. For this study 0.5 mL of working standard stock solution (500 µg/mL) was diluted to 10 mL to achieve a concentration of 25 µg/mL.

Ruggedness

Ruggedness of the method was established by changing the analyst. To prepare working dilution 0.3 mL of working standard stock solution (500 µg/mL) was diluted to 10mL to achieve a concentration of 15 µg/mL. All the solution was prepared freshly on other day followed by the routine analysis and the injections were done by different analyst. Six replicate injections of this solution were made and % RSD values of all the parameters were noted.

Assay of tablet formulation

Twenty Tablets, each containing 40 mg of FBX were weighed and finely powdered. An amount of FBX powder equivalent to 10 mg was weighed and transferred to 10 mL of volumetric flask. The drug (FBX) was dissolved in small amount of standard solvent (Methanol) and vortexed for 10-15 min and final volume was made up to 10 mL with the mobile phase. The solution was filtered using 0.45 µ Nylon syringe filter. The filtrate is used to prepare appropriate dilutions of different concentration were prepared in mobile phase. From the tablet stock solution six replicates of the required dilution were prepared and sonicated for 10 min. The prepared solutions were used for quantitative analysis.

Forced degradation studies

Stress studies were performed to evaluate the stability indicating properties and specificity of the method [16]. All samples were diluted with mobile phase to give a final concentration of 10 µg/mL and filtered before injection.

Acidic degradation

Acidic degradation was performed by preparing the drug solution (1 mg/mL) in 0.1 N Hydrochloric acid. The resulting solution was refluxed for 30 min at 60°C in thermostat, cooled and then the stressed sample was neutralized and diluted with mobile phase as per the requirement before injected in to the HPLC system.

Alkaline degradation

Alkaline degradation was performed by preparing the drug solution (1 mg/mL) in 0.1 N sodium hydroxide. The resulting solution was refluxed for 30 min at 60°C in thermostat, cooled and then the stressed sample was neutralized and diluted with mobile phase as per the requirement before injected in to the HPLC system.

Oxidation degradation

Oxidation degradation was performed by preparing the drug solution (1 mg/mL) in 3% H_2O_2. The resulting solution was refluxed for 30 min at 60°C in thermostat, cooled and then the stressed sample was diluted with mobile phase as per the requirement before injected in to the HPLC system.

Thermal degradation

For thermal stress testing, the drug solution (1 mg/mL) was heated in thermostat at 60°C for 30 min, cooled and then the stressed sample was diluted with mobile phase as per the requirement before injected in to the HPLC system.

Results and Discussion

Selection of wavelength

From the UV-spectra the wavelength selected for the method was 315 nm (Figure 1). Solutions of substance in mobile phase were injected in HPLC and peak areas of the drug and internal standard were recorded at 315 nm. It was observed that at this wavelength there was no interference from mobile phase or baseline disturbance, thus 315 nm was the most appropriate wavelength for analysis of the drug.

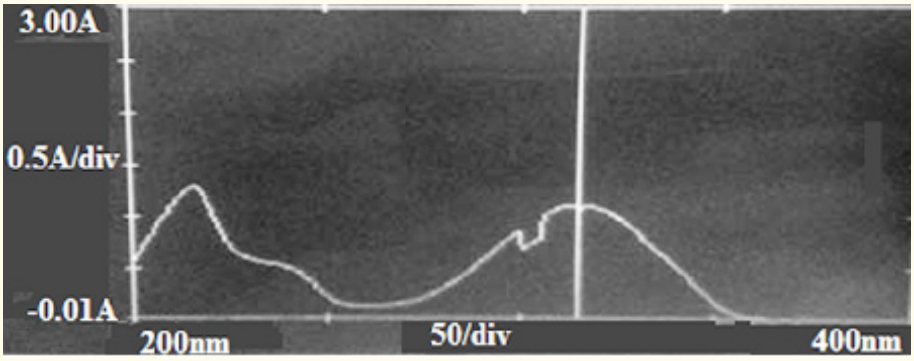

Figure 1: *UV curve of FBX solution in methanol.*

Development of analytical condition

A total of eight analytical trials were made through 2^3 factorial designs. Methanol was selected as the organic part for chromatography because of the solubility of the FBX in it. Water was selected as the retarding agent so that the FBX get partitioned towards the organic column. The detail of various mobile phases and conditions which were used for developing the method and the results regarding retention time and symmetry are given in table 1. To adjust the tailing factor chromatographic conditions were optimized by selecting the suitable mobile phase composition, pH of the medium and flow rate.

Retention time

Retention time for the analysis ranged from 11.11 min (A4) to 15.26 min for A5. On applying one way ANOVA it was observed that the design had significant influence over the retention time. Any change in retention time was influenced by the organic content of the mobile phase, working pH and flow rate. During this study it was observed that decreasing the pH of the mobile phase increases the retention time of the FBX due to the presence of carboxylic group in the drug leading to the partition of FBX towards the column. The statistical analysis showed that there was the significant role of flow rate over the retention time but increasing the flow rate decreased the retention time that might happen due to the quicker movement of the drug molecules with the solvent towards elute. Influence of increasing the organic content of the organic solvent in the mobile phase decreased the retention time due to the higher solubility of the FBX in the mobile phase leading to easy partition of FBX toward it.

Asymmetry

Asymmetry for the analytical method ranged from 1.655 (A5) to 2.872 (A4). Design had a significant influence over the asymmetry. All the dependent factors, except the factor of pH, had the significant influence over the asymmetry. During this study it was observed that decreasing the organic content of the mobile phase decreased the asymmetry of the peak. While decreasing pH of the mobile phase decreased the asymmetry. Flow rate had a significant role over the asymmetry as evident that A4 (+1) with high flow rate had higher asymmetry (2.872) while A2 (-1) with low flow rate had lower symmetry (2.570).

Number of theoretical plates

Number of theoretical plates for all the model studies ranged from 6109 (A5) to 3814 (A4). The statistical analysis showed that all the independent factors had the significant influence on the number of theoretical plates. It is evident from the A5 with lower organic content; pH and flow rate had higher number of theoretical plates while A4 with higher organic content, pH and flow rate had lower number of theoretical plates. Thus it can be said that number of theoretical plates decreased with increase in organic content, pH and flow rate. The higher numbers of theoretical plates gives a better, symmetrical and sharp separation of the content thus it is desired for the method to have higher number of theoretical plates. But from the study it is evident that higher number of theoretical plates resulted from the slower flow of mobile phase that resulted in higher retention time but with lower asymmetry.

Statistical analysis

Statistical analysis was done by Design expert software version 8.0.5.2 (Stat-Ease, Inc., Minneapolis, USA) and the third order polynomial equations were derived. The transformed equations are,

$Y_1 = 13.156 - 1.059X_1 - 0.539X_2 - 0.109X_1X_2 + 0.176X_1X_3 - 0.0387X_2X_3 - 0.0587X_1X_2X_3$

$Y_2 = 2.339 + 0.381X_1 + 0.0759X_3 - 0.0381X_1X_2 - 2.125X_1X_3 - 0.0351X_2X_3 + 0.0364X_1X_2X_3$

$Y_3 = 4483.875 - 529.875X_1 - 245.625X_2 - 218.875X_3 + 131.125X_1X_2 + 177.875X_1X_3 + 168.625X_2X_3 - 153.125X_1X_2X_3$

Where X_1, X_2 and X_3 are the independent variables.

The significance, interaction and the effects of various independent variables on the dependent variables as observed from the statistical analysis are illustrated in Figure 2, Figure 3 and Figure 4. Figures provided the evidences for the theory that the design had the significance for the three dependent factors. Figure 2 showed that X_1, X_2 and X_3 had the significance for the variation in the value of independent factors by giving zero deviation from the reference points. Figure 3 signified the interaction of independent factors but not within the design limit. Figure 4 showed the influence of the independent factors over the dependent factors.

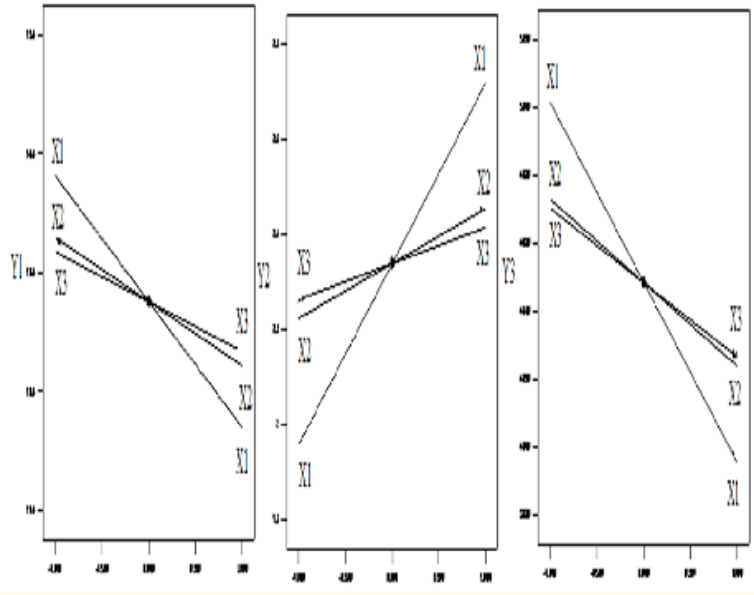

Figure 2: *Perturbation curves derived by the software analysis showing significance of the independent factors on the dependent factors.*

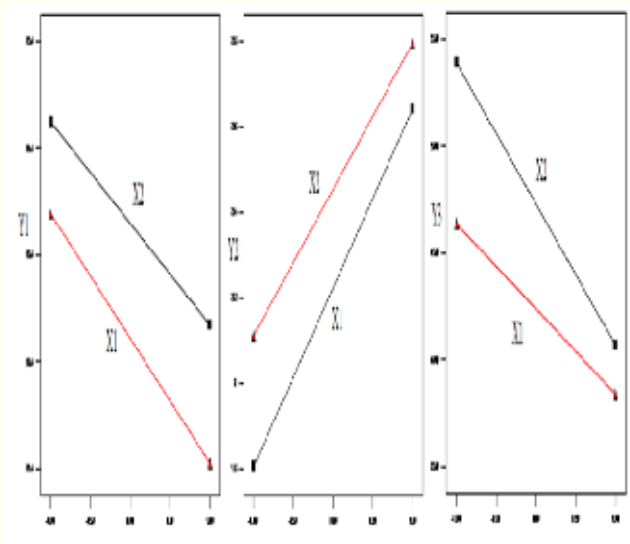

Figure 3: *Interaction curves for the independent factors for dependent factors.*

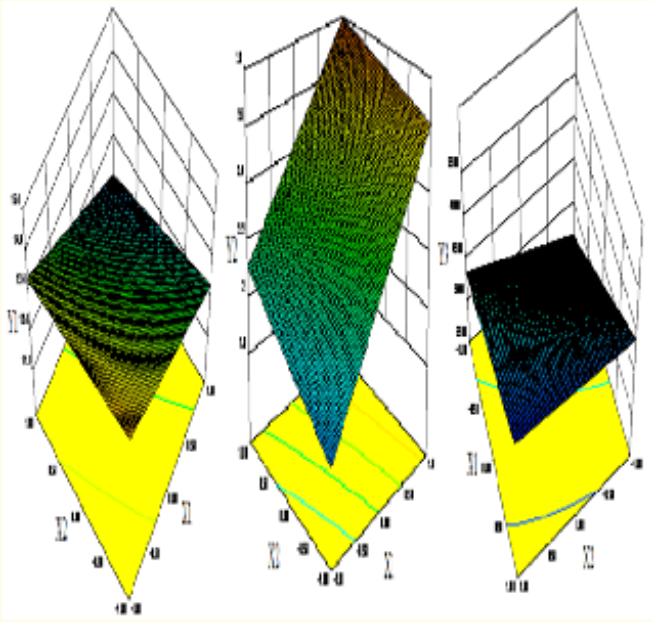

Figure 4: *Contour plots elaborating the effect of independent factors over dependent factors.*

Validation of the experimental design

An extra design check point analysis (A9) was made and the predicted value and experimental values of dependent variables were compared using pooled t-test at 95% confidence interval, degree of freedom 4 and $p < 0.05$. No significant difference was recorded between the two values thereby establishing validity of the generated model. On comparison of the predicted and the observed value generated by the software (Figure 5) also gave a straight line giving a proof for the validity of the design.

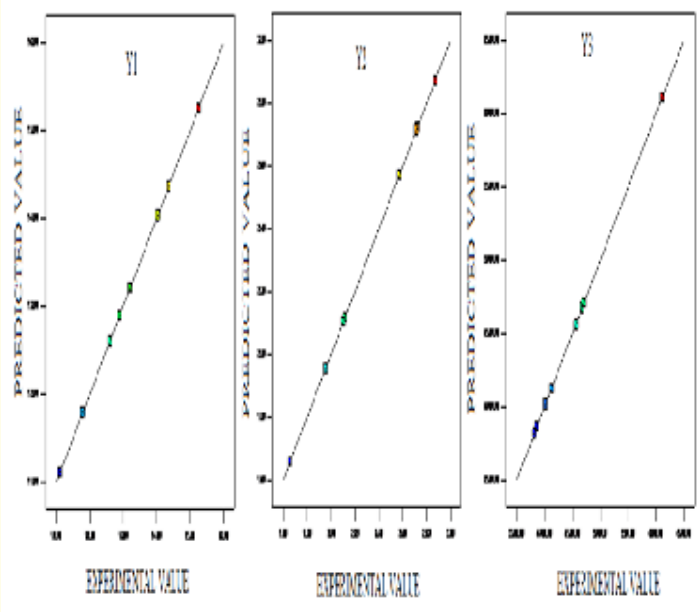

Figure 5: *Predicted and experimental value showing the validity of the design.*

Selection of optimized method

Eventually the method A5 with retention time, asymmetry, desirability factor and number of theoretical plates of 15.26 min, 1.655, 0.703 and 6109 respectively and identified as the optimized method from the design. Software analysis also gave desirability factor of 0.981 for the method not given in the design (A*) with the value of X_1 (-1), X_2 (-0.46) and X_3 (+0.10) giving the value of Y_1 (14.352), Y_2 (1.899) and Y_3 (5132.13).

Modification of the peak

Without adding triethylamine (TEA) the peak showed the asymmetry of 1.655 thus TEA (0.5 mL/l) was added as peak modifier (Figure 6) and the resulted method was termed as A10. The addition of the TEA resulted in the decreased asymmetry to 1.124 without influencing other factors. It is found that TEA is an ion-pairing reagent that alters selectivity in reverse-phase HPLC separations [17]. Addition of triethylamine improves the separation by masking polar silanol groups on the stationary phase by competing for them. Thus, the competition reduced the availability of the free silanols group for interaction, thus enabling analyte molecules to move through the column without interference from the stationary phase [18].

Dilution

Calibration curve was drawn with different concentration of FBX. Calibration curve is shown in the Figure 7. Calibration curve showed the coefficient of correlation (r value) of 0.9999 for the range of 2 µg/mL to 50 µg/mL showing linear curve with intercept value of 9.893 and slope value of 82.994 and coefficient of determination 0.9999. Linear regression equation generated for the calibration curve is Peak area (A) = 9.893 + 82.994C, Where C = concentration of FBX

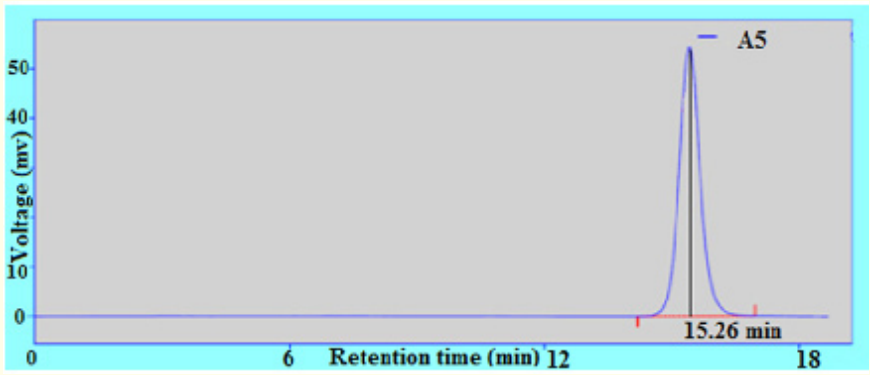

Figure 6: *Chromatogram of Febuxostat in Selected Mobile Phase Methanol: water (70% v/v) pH = 3.0, triethylamine.*

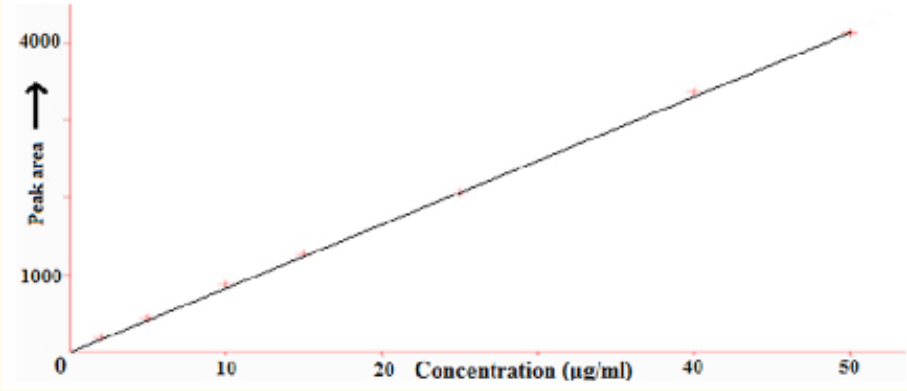

Figure 7: *Chromatogram showing System Suitability.*

System suitability test

The % RSD for the system suitability parameters (Table 2) like retention time (% RSD = 0), area (% RSD = 1.444), height (% RSD = 1.792), amount for recovery (% RSD = 1.443), width (% RSD = 1.475), asymmetry (% RSD = 0.840), symmetry (% RSD = 1.620), efficiency (% RSD = 0.623), efficiency/length (% RSD = 0.623) and HETP (% RSD = 0.000) were analyzed and found to be within the % RSD limit (2%).

Linearity, Range and reproducibility

The calibration curve for the analytical method was found to be linear over a concentration range from 2 µg/mL to 50 µg/mL, which is indicated by coefficient of determination value of $r^2 = 0.9999$ as indicated in table 3.

Chromatogram. No.	Area	Height	Amount	Width	Asymmetry	Symmetry	Efficiency	EFF/ Length	HETP
MY LC- 4	1170.227	42.578	14.757	0.432	1.642	1.315	5326.551	53265.514	0.019
MY LC- 5	1214.622	42.793	15.316	0.437	1.625	1.344	5305.602	53056.019	0.019
MY LC- 6	1179.600	41.843	14.875	0.433	1.632	1.355	5395.517	53955.171	0.019
MY LC- 7	1188.355	41.176	14.985	0.443	1.649	1.362	5339.620	53396.195	0.019
MY LC- 8	1198.607	41.112	15.115	0.447	1.660	1.372	5340.983	53409.830	0.019
MEAN	1190.282	41.920	15.010	0.438	1.642	1.350	5341.655	53416.546	0.019
STDV	17.188	0.751	0.217	0.006	0.014	0.022	33.293	332.929	0.000
%RSD	1.444	1.792	1.443	1.475	0.840	1.620	0.623	0.623	0.000
RESULT	PASS	PASS	PASS	PASS	PASS	PASS	PASS	PASS	PASS
%RSD LIMIT	2.000	2.000	2.000	2.000	2.000	2.000	2.000	2.000	2.000

Table 2: *System suitability test results.*

S. No.	Slope	Intercept	r	r^2
CC 1	82.994	9.893	0.9999	0.9999
CC 2	82.519	9.789	0.9999	0.9999
CC 3	82.966	9.914	0.9999	0.9999
CC 4	82.818	9.853	0.9999	0.9999
CC 5	82.837	9.881	0.9999	0.9999
Mean	82.787	9.866	0.9999	0.9999
SD	0.206	0.048	0.0000	0.0000
%RSD	0.249	0.490	0.0000	0.0000

Table 3: *Various parameters for the calibration curve.*

Coefficient of determination, r^2 is a statistical data that will give the information regarding goodness of fit. The value of coefficient of determination, r^2 ranges from 0 to 1 [19]. If the value of r^2 is closer to 1 then it shows that the curve is straight i.e. curve is linear. Linear curve depicts that the analysis can be made with high accuracy. The value of r^2 was determined to be 0.9999. Thus the curve is found to be linear.

Reproducibility is determined by calculation the mean, standard deviation (SD), and % relative standard deviation (% RSD) for the five calibration curves for the values of slope, intercept, r and r^2 values. The method was found to be reproducible. All the curves were linear with % RSD values within the limit of 2.0 %.

Mean values for slope, intercept, r and r^2 were found to be 82.787, 9.866, 0.9999 and 0.9999 respectively. SD for slope, intercept, r and r^2 0.206, 0.048, 0.0000, 0.0000 and 0.0000. % RSD value of slope, intercept, r and r^2 were calculated to be 0.249, 0.490, 0.0000 and 0.0000. As the value of % RSD is within limit showing that the method is reproducible.

Value of coefficient of determination (r^2 = 0.9999) shows that the curve is straight. As the value of %RSD for r^2 is found 0.0000, showing that curve is reproducible.

Accuracy and Precision

Observations for the accuracy and precision study were given in Table 4. The results of accuracy were determined by Mean %recovery. Accuracy ranged from 99.912% to 100.180%. The observations depicted that the method is accurate as the %recovery was within limit of ± 2%.

Actual Concentration (µg/mL)	Mean % Recovery	S.D	% RSD for Accuracy	% RSD for Interday	% RSD for Intraday
8	99.817	0.446	0.447	0.002	0.018
				0.008	
				0.008	
30	99.912	0.163	0.163	0.008	0.016
				0.002	
				0.011	
45	100.180	0.100	0.100	0.002	0.004
				0.001	
				0.001	

Table 4: *Recovery, accuracy and precision of the method.*

Three different quality levels were marked as low quality control (LQC-8 µg/mL), middle quality control (MQC-30 µg/mL) and high quality control (HQC-45 µg/mL) to determine the precision of the developed method. The intraday and interday precision for FBX was analyzed on these three quality levels i.e. LQC, MQC and HQC for % RSD. The value of % RSD ranged from 0.004 to 0.018 for the three levels for the intraday precision. The observations showed that the method is precised for intraday study. Similarly the value of % RSD for the inter-day precision was found to be 0.002 to 0.011. The % RSD value found to be within the acceptable range of 2% in both Intraday and Inter-day analysis, thus it can be said that the method is precised.

LOD and LOQ

LOD is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated under the stated experimental condition. The method showed LOD value of 0.002 µg/mL which means that the presence of FBX in as low concentration of 0.002 µg/mL can be qualitatively detected by the mentioned method. LOQ is the lowest amount of analyte in a sample that can be determined quantitatively with acceptable precision and accuracy under the stated experimental condition. The method showed the LOQ value of 0.006 µg/mL thus quantitative analysis can be made for as low concentration of 0.006 µg/mL. This low value of LOD and LOQ shows that the method is very sensitive.

Robustness

The results obtained from assay of the test solutions were not affected by varying the conditions and were in accordance with the results for original conditions and the percentage recovery was found to be 98.91-100.65%. The method was found to be robust with the variation in flow rate, wavelength and mobile phase. The parameters were within the acceptance limit of 2% RSD. When the flow rate of the mobile phase was varied from 0.8 mL/min to 0.95 mL/min the retention time changed from 15.34 min to 14.83 min for FBX while if the flow rate was changed to 0.65 mL/min, the retention time was changed from 15.34 min to 15.97 min for FBX. When the ratio of mobile phase (methanol:water) is changed from 70:30 to 65:35 the retention time changed from 15.34 min to 15.51 min while when ratio changed to 75:25 the retention time changed to 15.23 min. When the wavelength was changed no change in % recovery was observed. The results are given in table 5.

Parameters	Flow Rate			Mobile Phase			Wavelength		
Alterations	0.65	0.80	0.95	72:28	75:25	78:22	310	315	320
Change	15.97	15.34	14.83	15.51	15.34	15.23	99.84	99.88	99.86

Table 5: *Robustness of the method.*

Ruggedness

The method was found to be rugged when operated by another analyst, hence the method was found rugged and the parameters were within the acceptance limit of 2% RSD.

Assay

The method can be satisfactorily applied in the routine analysis for the assay of tablet dosage forms which is indicated by % Recovery values which ensures minimum interaction by the matrix (excipients) (Figure 8) and low % RSD values which ensures reproducibility. The results were found to be 99.713 ± 0.233. This study also showed that the method is specific for the FBX as no interference was observed in the peak by the excipients.

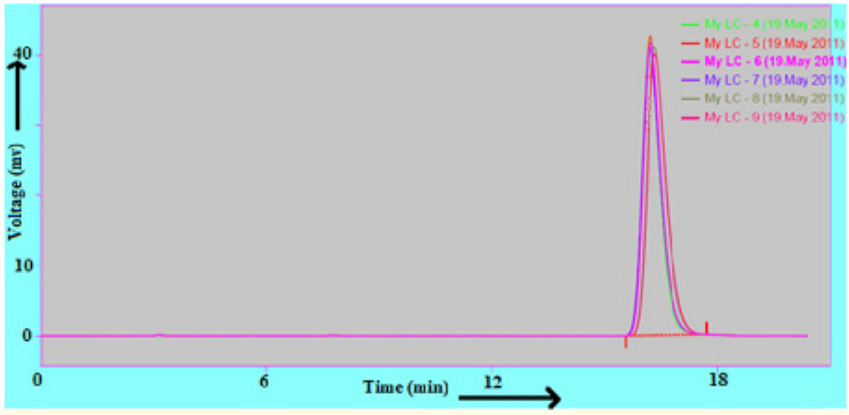

Figure 8: *Calibration curve for system suitability test.*

Forced degradation studies

The specificity of the developed method was determined by injecting FBX sample solutions of (10 µg/mL) which were prepared by forcibly degrading under stress conditions such as acid, base, oxidative agent and heat under the proposed chromatographic conditions. Result of the forced degradation study is given in table 6.

Condition	% Drug Recovery
Initial state	99.88 ± 0.12
Standard condition	99.86 ± 0.09
Acidic degradation	99.52 ± 0.06
Alkaline degradation	99.21 ± 0.14
Thermal degradation	99.81 ± 0.17
Oxidative degradation	98.73 ± 0.03

Table 6: *Drug recovery on stressed degradation study.*

The stability indicating capability of the method was established from the separation of FBX peak from the degraded samples derived from the inbuilt software. The degradation of FBX was found to be very similar for both the tablets and standard.

FBX standard and tablet powder was found to be quite stable under dry heat conditions and no decomposition was seen on exposure of FBX drug solution to heat. On the other hand the drug decomposition under acidic, alkaline and oxidation degradation was found to be less than 1.5% indicating that the drug is stable towards acidic, alkaline and oxidation conditions It can be concluded that FBX is more resistant towards acidic, alkaline, oxidative and thermal conditions.

Conclusion

The results showed that the developed method is simple, rapid, precise, accurate, rugged and robust, which have been successfully applied for the analysis of several formulations containing FBX. The above experimental data was suitable for the estimation of drug in formulation and bulk drugs. The simplicity, rapidity, reproducibility and economy of the proposed method completely fulfill the objective of the research. A stability indicating assay based on the HPLC method for FBX in pharmaceutical formulations was developed and validated for a concentration range of 2 - 50 µg/mL. Method was found to be accurate, sensitive, specific, rugged and robust and capable of producing reproducible result.

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Conflict of Interest

There is no conflict of interest of authors.

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