International Journal of ChemTech Research
CODEN (USA): IJCRGG, ISSN: 0974-4290, ISSN(Online):2455-9555
Vol.10 No.2, pp 870-880, 2017

A Validated Stability Indicating HPTLC method for analysis of Febuxostat and Characterization of degradation product

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Abstract : A simple, sensitive, precise, specific and stability indicating high-performance thin-layer chromatographic (HPTLC) method for the determination of febuxostat, both in bulk drug and pharmaceutical dosage form was developed and validated. The method employed aluminium plates precoated with silica gel G 60 F_{254} as the stationary phase. The solvent system consisted of ethylacetate : methanol : acetic acid (7.5:2.8:0.01, v/v/v). The *Rf* value of febuxostat was found to be 0.65.Densitometric analysis of febuxostat was carried out in the absorbance mode at 315 nm. Linear regression analysis showed good linearity ($r2$ = 0.9976) with respect to peak area in the concentration range of 30–180 ng $spot^{-1}$. The method was validated in accordance with ICH guidelines. Febuxostat was subjected to acid and alkali hydrolysis, oxidation, photodegradation and dry heat and wet heat conditions.The degraded product peak was well resolved from the pure drug with significantly different *Rf* values. Statistical analysis proved that the method is repeatable and specific for the estimation of febuxostat. As the method could effectively separate the drug from its degradation product, it can be regarded as stability indicating. The degraded product was characterized by IR, NMR and mass spectroscopic methods. Febuxostat was found to decompose in alkaline conditions to 2-(3-Carboxy-4-isobutoxyphenyl)-4-methylthiazole-5-carboxylic acid.
Keywords : Febuxostat, Stability indicating, HPTLC, Degradation, Characterisation.

Introduction

Febuxostat(FEB) is chemically known as 2-(3-Cyano-4 isobutoxyphenyl)-4-methyl 1,3-thiazole-5-carboxylic acid[1]. The chemical structure of febuxostat is shown in Figure 1. Febuxostat is a non-purine xanthine oxidase inhibitor used in the treatment of hyperuricaemia with chronic gout[2]. Quantification of febuxostat has been performed in the past using RP-HPLC [3–8], and LC/MS [9-13] either alone or in combination with other drugs and various stability indicating HPLC, UPLC and Capillary electrophoretic methods have been reported for assaying febuxostat [14-17]. Literature review revealed that no article related to the stability indicating high-performance thin-layer chromatographic (HPTLC) determination of febuxostat has been reported. The International Conference on Harmonization (ICH) guidelines entitled Stability Testing of New Drug Substances and Products requires the testing to be carried out to elucidate the inherent stability characteristics of the active substance [18]. Nowadays HPTLC is becoming a routine analysis technique due to advantages of low operating cost, high sample throughput, and need for minimum sample cleanup. The major advantage of HPTLC is that several samples can be run simultaneously using a small quantity of mobile phase unlike HPLC, thus lowering analysis time and cost per analysis.

The aim of the present work was to develop a precise, accurate, specific and stability-indicating HPTLC method using densitometric detection for the determination of febuxostat in the presence of its degradation products. The degradation product is characterised by IR,NMR and mass spectroscopic studies.

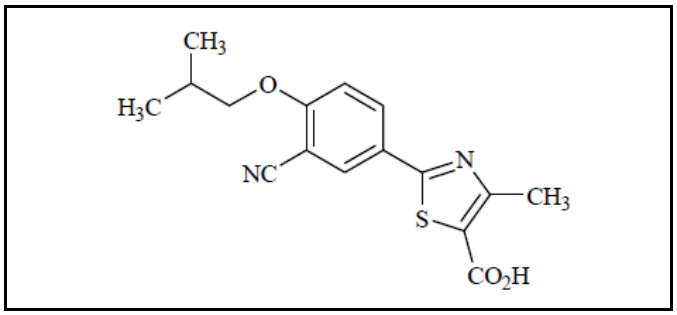


Figure 1. Structure of FEB

Experimental

Materials

Pharmaceutical grade of Febuxostat was kindly supplied as a gift sample by Centaur Pharmaceuticals Pvt Ltd, Pune, used without further purification, and certified to contain 99.5 % (w/w) on dried basis. Pharmaceutical dosage form Foxstat 40mg tablets, Batch no. ACI3004, Exp. 04/15) was procured from local market. All chemicals and reagents used were of analytical grade and were purchased from Merck Chemicals, Mumbai, India.

HPTLC Instrumentation and Chromatographic Conditions

The HPTLC plates were prewashed with methanol and activated at 110°C for 5min prior to chromatography. The samples were spotted in the form of bands 6mm width with a Camag 100 µL sample syringe (Hamilton, Bonaduz, Switzerland) on silica gel precoated HPTLC aluminum plate G60 F_{254}, [(20 × 10 cm) with 250 μm thickness[E. Merck,Darmstadt, Germany, supplied by Anchrom Technologists, Mumbai] using a Camag Linomat IV applicator (Switzerland). A constant application rate of 0.1 μL s^{-1} was used and the space between two bands was 6 mm. Linear ascending development was carried out in 20 cm×10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The mobile phase consisted of ethylacetate : methanol : acetic acid (7.5:2.8:0.01, v/v/v) and 20mL were used per chromatography run. The optimized chamber saturation time for mobile phase was 30min using saturation pads at room temperature (25°C ± 2). The length of chromatogram run was 8 cm. Densitometric scanning was performed using a Camag TLC scanner III in the reflectance absorbance mode and operated by CATS software (V 3.15, Camag). The slit dimension was kept at 5mm×0.45mm and the scanning speed was 10mms^{-1}. The source of radiation used was a deuterium lamp emitting a continuous UV spectrum between 190 and 400 nm. All determinations were performed at ambient temperature with a detection wavelength of 315nm. Concentrations of the compound chromatographed were determined from the intensity of the diffused light. Evaluation was by peak areas with linear regression.

Preparation of Standard Solution

A stock solution of FEB (1000 μgmL^{-1}) was prepared in methanol. This solution was further diluted with methanol to obtain working standard solutions of FEB in the concentration range of 30–180 μgmL^{-1}.

Method Validation[19-23]

The HPTLC method was validated as per the ICH guidelines.

Linearity and Range

One µL from each working standard solution was spotted on the HPTLC plate to obtain final concentration range of 30–180 ng $spot^{-1}$. Each concentration was spotted six times on the HPTLC plate. The

plate was developed using the previously described mobile phase and scanned. The peak areas were plotted against the corresponding concentrations to obtain the calibration graph. Linear calibration curve was generated using least squares linear-regression analysis.

Precision

Precision of the method was verified by repeatability and intermediate precision studies. Repeatability studies were performed by analysis of concentration of 50 ng spot^{-1} of the drug in hexaplicate on the same day. Intermediate precision of the method was checked by repeating studies on different days.

Limit of Detection and Limit of Quantitation

In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), blank methanol was spotted following the same method. The signal-to-noise ratio was determined. LOD was considered as 3 : 1 and LOQ as 10 : 1. The LOD and LOQ were experimentally verified by diluting known concentrations of standard solution of febuxostst until the average responses were approximately 3 or 10 times the standard deviation of the responses for six replicate determinations.

Robustness of the Method

By introducing small changes in the mobile-phase composition (±0.1mL for each component), the effects on the results were examined. Mobile phase composition was varied by changing ±0.1 ml of ethyl acetate and chromatograms were run. Time from spotting to chromatography and from chromatography to scanning was varied by +10 min. Robustness of the method was done at 50 ng spot^{-1} of FEB for 6 times.

Accuracy

Accuracy of the method was determined by standard addition method in which the known amount of standard febuxostat solutions were added to preanalyzed tablet solution. These amounts corresponded to 80, 100, and 120 % (90, 100 and 110 ng spot^{-1}) of the amounts claimed on the label. The amounts of febuxostat were estimated by applying these values to the regression equation of the calibration curve. Accuracy study was performed for six times, and % recovery of febuxostat was calculated.

Analysis of Marketed Pharmaceutical Dosage Form

To determine the content of febuxostat in marketed pharmaceutical dosage form (Foxstat 40mg tablets, Batch no. ACI3004, Exp. 04/15) twenty tablets were weighed and powdered. An accurate weight of the powder equivalent to 32.52 mg febuxostat was weighed and transferred into a 100mL volumetric flask containing 50mL methanol, sonicated for 30min and di luted to 100mL with methanol. The resulting solution was centrifuged at 3000 rpm for 5min and supernatant was analyzed for drug content. 1mL of the above supernatant solution was transferred into 20mL volumetric flask and diluted to volume with methanol. The concentration achieved after the above dilution was 100 μgmL^{-1}. 1 μL volume was spotted for six times to achieve a final concentration of 100 ng spot^{-1}. The plate was developed in the previously described chromatographic conditions. The possibility of excipient interference in the analysis was studied. The peak areas of the spots were measured at 315 nm and concentrations in the samples were determined using multilevel calibration curve developed on the same plate under the same conditions using linear regression equation.

Forced Degradation Studies

Decomposition studies were performed in solutions containing febuxostat at a concentration of 1000 μgmL^{-1}. Samples were withdrawn at suitable time intervals and subjected to HPTLC analysis. The drug was subjected under different stress conditions as follows.

Acid-Induced Degradation

To 10mL of methanolic stock solution 10mL of 0.1N HCl was added. This mixture was refluxed at 80°C.

Base-Induced Degradation

To 10mL of methanolic stock solution 10mL of 0.1N NaOH was added, and the solution was refluxed at 80˚C.

Oxidative Degradation

To 10mL of methanolic stock solution 10mL each of hydrogen peroxide 3% (v/v) and 35% (v/v) was added separately. The solution was kept at room temperature and then heated in boiling water bath for 10min to completely remove the excess of hydrogen peroxide.

Wet Heat Degradation

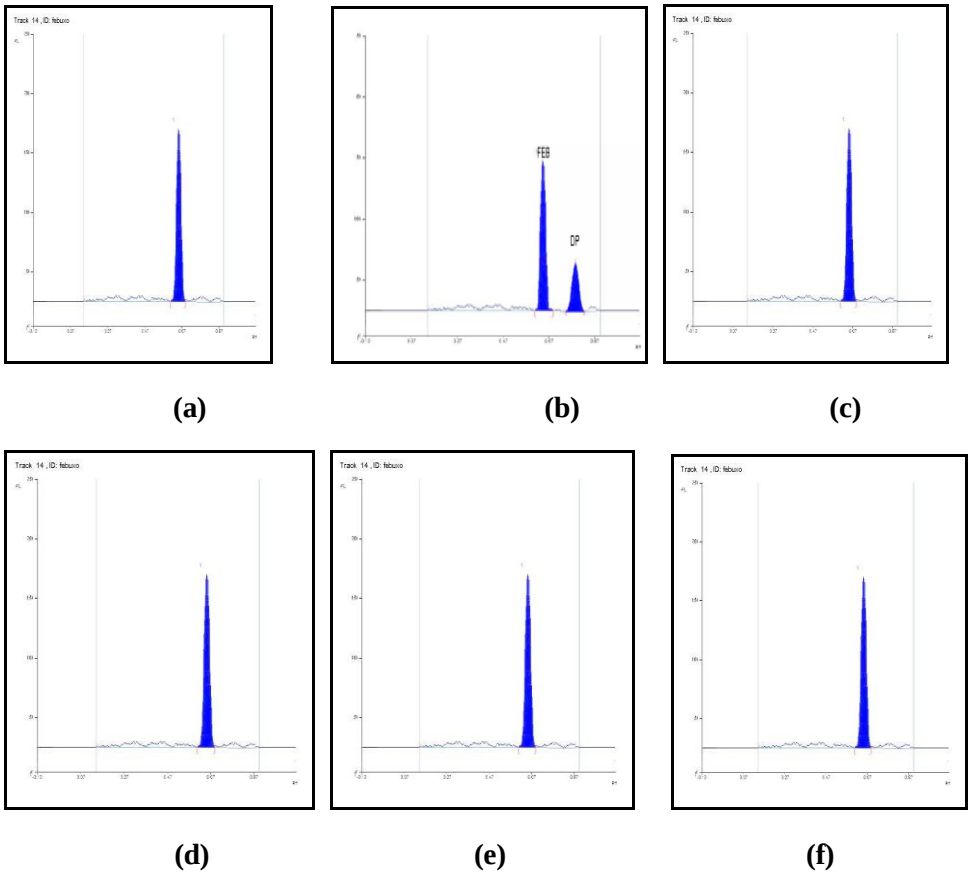
Studies under neutral conditions were performed by dissolving the drug substance in distilled water and solution was refluxed at 80˚C for 2 days.

Dry Heat Degradation

For dry heat degradation, the standard drug was placed in an oven at 80˚C for 2 days.

Photochemical Degradation

The photochemical stability of the drug was studied by exposing the solution to direct sunlight for 12 h kept on a terrace. The chromatograms obtained under different stress conditions are shown in Figure 2.



(a) **(b)** **(c)**

(d) **(e)** **(f)**

Figure 2. Degradation of Febuxostat by Acid(a), Base(b), Peroxide(c), Wet heat(d), Dry heat(e) and Light(f)

Characterization of degradation product

Febuxostat showed degradation only by alkaline hydrolysis. Peak of the degraded product was well resolved from febuxostat with reasonably different R_f value. The degradation product was characterized by IR(Figure 3), ^{1}HNMR(Figure 4), ^{13}C NMR(Figure 5) and Mass spectroscopic techniques(Figure 6).

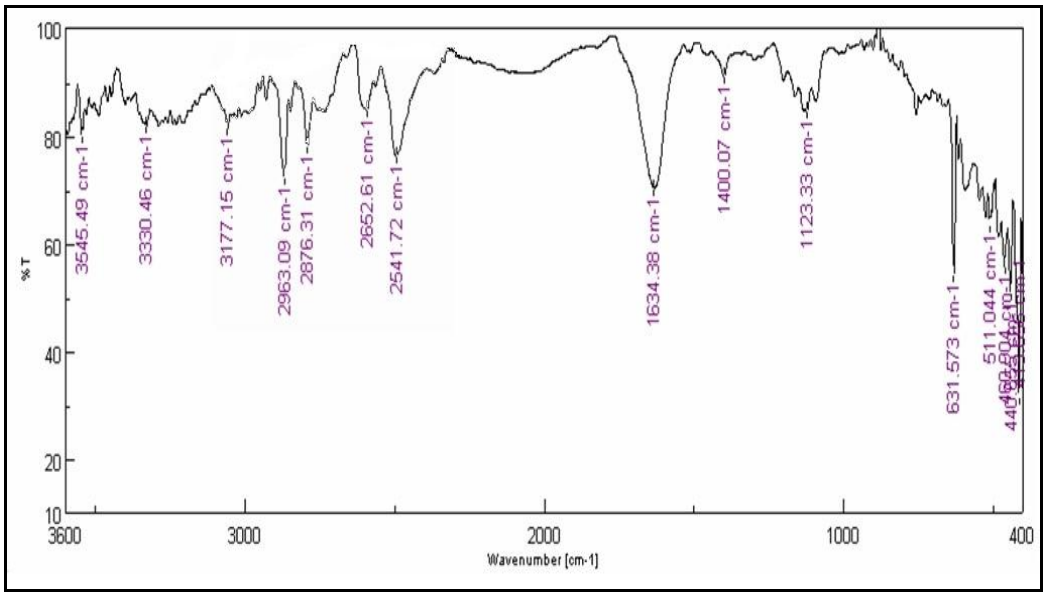


Figure 3. IR spectrum of FEB Degradation Product

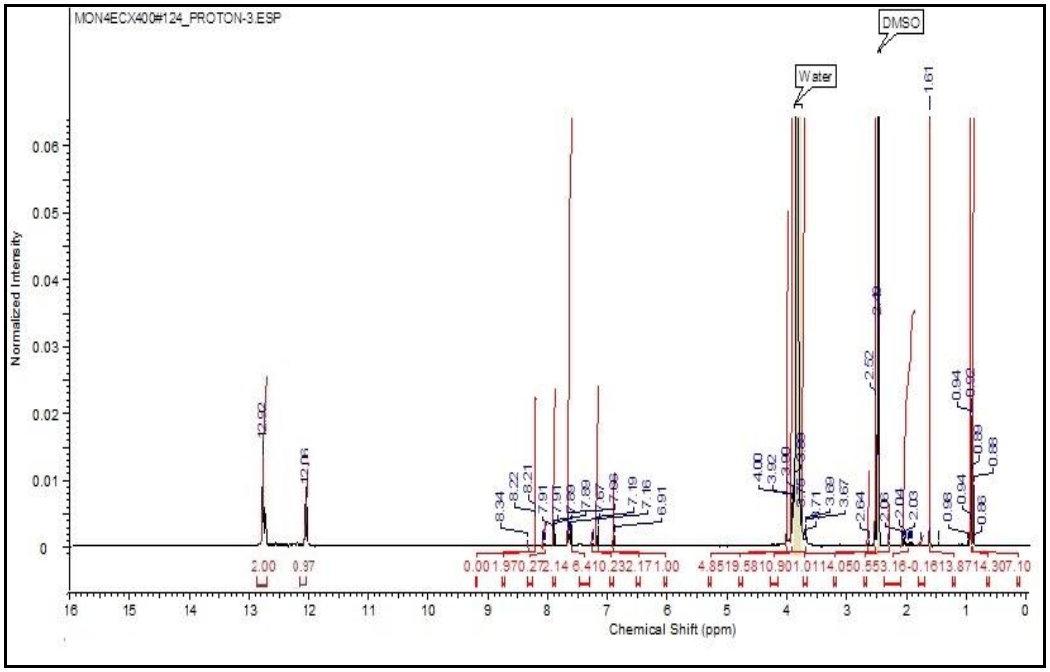


Figure 4. ^{1}H NMR spectrum of FEB Degradation Product

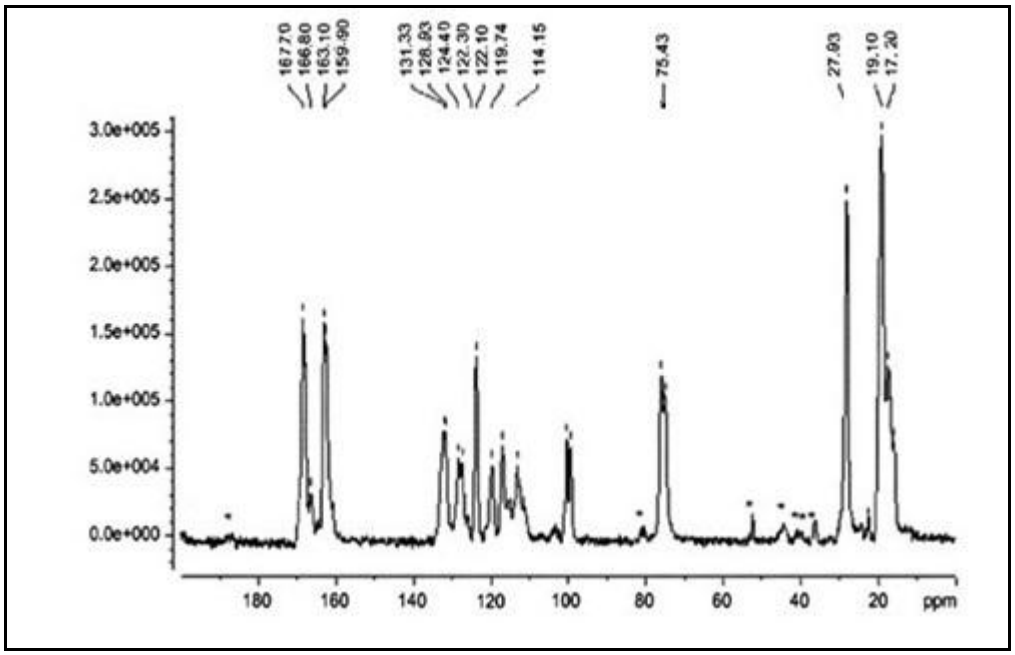


Figure 5.^{13}C NMR spectrum of FEB Degradation Product

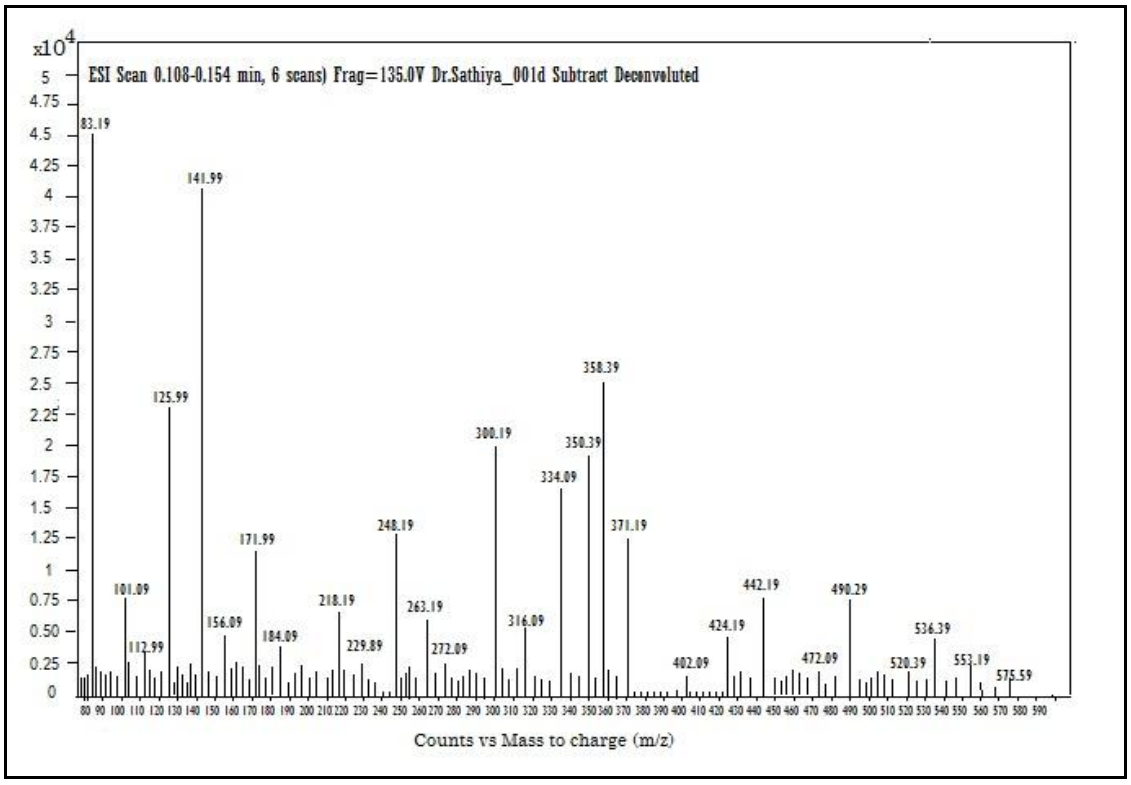


Figure 6.Mass spectrum of FEB Degradation Product

Results and Discussion

Optimization of the Chromatographic Conditions

The HPTLC procedure was optimized with a view to develop stability-indicating assay method. Both the pure drug and degraded drug solutions were spotted on HPTLC plates and run in different solvent systems. From the literature survey initially Ethyl acetate : methanol and Ethyl acetate: Tolune in different proportions were tried as mobile phases. Toluene was found to reduce the movement of febuxostat significantly. Ethyl acetate:Methanol at the ratio of 7:3 resulted in good desired movement of the spot. However the peak shape was

not good. Hence acetic acid was added which resulted in compact spot with good peak shape. But the degradation peak eluted was moving towards upper side. Hence the solvent system was further adjusted and the optimum mobile phase was found to be ethylacetate : methanol : acetic acid (7.5:2.8:0.01, v/v/v) was found to resolve the degraded peak from the standard peak. The drug in presence of their degradation products was satisfactorily resolved with *Rf* value at 0.65. In order to reduce the neckless effect, the TLC chamber was saturated for 30min using saturation pads. The mobile phase was run upto distance of 8 cm, which takes approximately 30min for development of HPTLC plate. Under various stressed conditions, febuxostat showed degradation only by alkaline hydrolysis. Peak of the degraded product was well resolved from febuxostat with reasonably different R_f value and identified as 2-(3-Carboxy-4-isobutoxyphenyl)-4-methylthiazole-5-carboxylic acid through IR, NMR and Mass spectroscopic techniques.

Validation of the Method

Linearity and Range

Linear relationship was observed by plotting drug concentration against peak areas. Febuxostat showed linear response in the concentration range of 30–180 ng spot^{-1}. The corresponding linear regression equation was y = 17.72x – 17.33 with square of correlation coefficient (*r* 2) of 0.9976 for febuxostat. The linear regression data is shown in Table 1.

Table No.1: **Linear regression data**

Parameters	FEB
Linearity range	30-180 ng spot^{-1}
r2	0.9976
Slope ± Standard error	17.724 ±368.8
Intercept ± Standard error	17.333± 35.50
Confidence limit of slope[a]	25.50 to 28.01
Confidence limit of intercept[a]	14.55 to 404.4
Sy.x[b]	75.42
P value[c]	<0.0001

*n=6

[a] 95% confidence intervals.

[b] Standard deviation of residuals from line.

[c] *P* value is < 0.0001, considered highly significant.

Precision

The results of the repeatability and intermediate precision experiments are shown in Table 2. The developed method was found to be precise as the RSD% values for repeatability and intermediate precision studies were <2%, respectively.

Table No.2: Results of Precision study

Drug Conc (ng/band)	Intraday precision*		Interday precision *	
FEB	Area ± S.D ng/spot	% RSD	Area ± S.D ng/spot	% RSD
50	2367.83 ± 0.007	0.52	2358 ± 0.034	0.33

*n=6

Limit of Detection and Limit of Quantitation

The signal : noise ratios of 3 : 1 and 10 : 1 were considered as LOD and LOQ respectively. The LOD and LOQ were found to be 20 ng spot^{-1} and 25 ng spot^{-1} respectively.

Robustness of the Method

The standard deviation of peak areas was calculated for each parameter, and RSD% was found to be less than 2%. The low values of RSD% as shown in Table 3. indicated robustness of the method.

Table No.3: Robustness evaluation of the method (n=6)

Parameter	SD of peak area for PHE	% RSD
Mobile phase composition (±0.1ml)	18.21	0.62
Amount of mobile phase (± 5%)	16.80	0.5
Time from application to development(+10 min)	13.71	0.51
Time from development to scanning (+10 min)	19.22	0.72

Accuracy

As shown from the data in Table 4, good recovery % of the drug in the range from 98.45 to 99.97% was obtained at various added concentrations.

Table No.4: Accuracy studies

Amount added(ng)	Total amount(ng)	Amount recovered(ng) *	Recovery(%)	% R.S.D
40(80%)	90	87.99	98.44	0.63
50(100%)	100	98.45	99.40	0.12
60(120%)	110	97.99	98.81	0.46

*n=6

Analysis of Marketed Pharmaceutical Dosage Form

A single spot at *Rf* value of 0.65 was observed in the chromatogram of the drug samples extracted from tablets. There was no interference from the excipients that are commonly present in the formulations. The drug content was found to be 98.97% with a RSD% of 0.71 for six replicate determinations. It may, therefore, be inferred that degradation of febuxostat had not occurred in the marketed formulations that were analyzed by this method. The good performance of the method indicated the suitability of this method for routine analysis of febuxostat in pharmaceutical dosage form.The results of market sample analysis is shown in Table 5.

Table No.5: **Analysis of the marketed formulation**

Drug	Label Claim Mg	Amount found mg*	Drug content(%)±S.D	%RSD
FEB	40	39.2	99.53±0.28	0.71

* n=6.

Forced Degradation Studies

Stress testing of febuxostat under different conditions using ethylacetate : methanol : acetic acid (7.5:2.8:0.01, v/v/v) as the mobile solvent system suggested the following degradation behavior(Table 6)

Acid-Induced Degradation

Initially 0.1N HCl was used at 80˚C for 8 h, but no degradation was observed. The process was tried with 1N and 2N HCl. No degradation was observed with 1N HCl, with 2N HCl, the drug was completely degraded.

Base-Induced Degradation

The drug was found to be highly labile to alkaline degradation. The reaction was carried out by refluxing with 0.1N NaOH at 80˚C for 4h. One degradation peak at *Rf* 0.79 was observed.

Oxidative Degradation

Reaction in 3% peroxide at room temperature showed no degradation. Hence drug was exposed to 35% hydrogen peroxide at room temperature. No degradation was observed.

Wet Heat Degradation

The drug was found to be stable when refluxed with water at 80˚C for 48 h. No significant degradation was observed, reflux time was then increased for 5 days. No degradation peaks were observed.

Dry Heat Degradation

Drug was found to be stable when subjected to thermal degradation at 80˚C for 12 days. No degradation peaks were observed.

Photochemical Degradation

Febuxostat was found to be stable to photochemical degradation as no degradation peaks were observed after exposing drug to sunlight for 5 days. Degradation products obtained under different stress conditions are summarized in Table 6.

Table No.6: Degradation of FEB under various stress conditions

Stress Condition	Time (hr)	% Recovery	% Degradation	R_f of degradants
Acid 1N HCl (reflux	12	97.72	----	----
80ºC)	10	78.51	19.20	0.79
Base 1N NaOH (reflux	12	97.15	----	-----
80ºC)	12	96.53	-----	-----
H_2O_2 35% (room temp)	48	98.12	-----	-----
Photolytic	48	94.23	-----	-----
Dry Heat(80ºC)				
Wet Heat(80ºC)				

Characterisation of degradation product

The degradation product was identified as 2-(3-Carboxy-4-isobutoxyphenyl)-4-methylthiazole-5-carboxylic acid through IR, 1HNMR, C13 NMR and Mass spectroscopic techniques.

IR(incm^{-1}):

3537.57,2536.83,1689.70,1602.97,1282.35,1049.31,1022.31,918.15,756.12,678.97; **^{1}H NMR(300 MHz,DMSO,δ_H in ppm):**

0.86-0.98 (d,6H(C<u>H</u>$_3$)$_2$>CH),1.91- 2.06(m,1H((CH$_3$)$_2$>C<u>H</u>), 3.67-3.69(d,2H,>CH-C<u>H</u>$_2$),7.25-8.22(d,dd,3H,Ar-<u>H</u>),2.64(s,3H,-C<u>H</u>$_3$),12.06,12.92(s,1H,COO<u>H</u>);

^{13}C NMR (300 MHz, DMSO, δ_C in ppm)

C_1-166.8, C_2-159.9, C_3-124.4, C_4-17.2, C_5-163.1, C_6-122.3, C_7-131.3, C_8-119.7, C_9-122.1, C_{10}-114.1, C_{11}-128.9, C_{12}-167.7, C_{13}-75.4, C_{14}-27.9, $C_{15,}$ C_{16}-19.1.

MS m/z:334.9,358.39,371.19,442.19,472.09

Conclusion

The developed HPTLC technique is precise, specific, accurate and stability indicating. Statistical analysis proves that the method is repeatable and selective for the analysis of febuxostat as bulk drug and in pharmaceutical dosage form. As the method separates the drug from its degradation products, it can be employed as a stability-indicating one. The proposed HPTLC method reduces the duration of analysis and is suitable for routine determination of febuxostat in pharmaceutical formulation in quality control laboratories, where economy and time are essential. This study is a typical example of development of a stability indicating assay following the recommendations of ICH guidelines.

Acknowledgments

The authors thank Centaur Pharmaceuticals Pvt Ltd, Pune for providing gift sample of standard febuxostat.

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