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A New RP-HPLC Method Development and Validation for the Simultaneous Estimation of Febuxostat And Ketorolac in Bulk and Tablet Dosage Form

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Abstract

A simple, precise and accurate RP-HPLC technique was developed and the developed method was validated for the regular analysis of Febuxostat and Ketorolac. Chromatographic analysis was performed by selecting an Novapak RP-C18 column (150 x 3.9 µm i.d; particle size 4 µm), Phosphate buffer in water pH-5.8 adjusted with O-Phosphoric Acid: Methanol in the isocratic mode (40:60 v/v) as mobile phase, 1.2 ml/min as flow rate and 20 µl injection volume. The LC chromatographic peaks were eluted at 1.92 and 3.10 for Febuxostat and Ketorolac respectively at 321 nm as detection wavelength with PDA detector. The developed method was validated as per the ICH guidelines and the Validation parameters were specificity, accuracy, linearity, precision, LOD and LOQ.

Keywords: Febuxostat; Ketorolac; RP- HPLC; Validation.

Introduction

Analytical chemistry is often described as the area of chemistry responsible for characterizing the composition of matter, both qualitatively and quantitatively [1], for analyzing the drug sample in bulk, pharmaceutical formulations and biological fluids, many analytical techniques are used [2]. The instrumental method like High Performance Liquid Chromatography (HPLC) was derive from the classical column chromatography and, is widely used tool of analytical chemistry now a day [3].

Ketorolac is a pyrrolizine carboxylic acid derivative structurally related to indomethacin and chemically it is designated as 5-benzoyl-2,3-dihydro-1H-pyrrolizine-1-carboxylic acid. It is an NSAID and is used principally for its analgesic activity. Ketorolac is a Nonsteroidal anti-inflammatory drug (NSAID) chemically related to indomethacin and tolmetin. Ketorolac is a racemic mixture of [-]S- and [+]R-enantiomeric forms, with the S-form having analgesic activity. Its anti-inflammatory effects are believed to be due to inhibition of both cylooxygenase-1 (COX-1) and cylooxygenase-2 (COX-2) which leads to the inhibition of prostaglandin synthesis leading to decreased formation of precursors of prostaglandins and thromboxanes from arachidonic acid. The resultant reduction in prostaglandin synthesis and activity may be at least partially responsible for many of the adverse, as well as the therapeutic, effects of these medications.

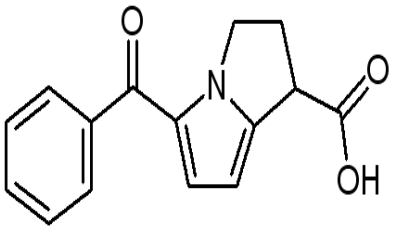

Figure 1: Structure of Ketorolac.

Febuxostat is a xanthine oxidase inhibitor. The active ingredient in Febuxostat is 2-[3-cyano-4-(2-

methylpropoxy) phenyl]-4-methylthiazole-5-carboxylic acid. It is approved by the European Medicines Agency 2008 and the FDA in management of hyper uricemia in patients with gout.

Febuxostat is a non-purine selective inhibitor of xanthine oxidase. It works by non-competitively blocking the molybdenum pterin center which is the active site on xanthine oxidase. Xanthine oxidase is needed to successively oxidize both hypoxanthine and xanthine to uric acid. Hence, Febuxostat inhibits xanthine oxidase, therefore reducing production of uric acid. Febuxostat inhibits both oxidized as well as reduced form of xanthine oxidase because of which Febuxostat cannot be easily displaced from the molybdenum pterin site [4-6].

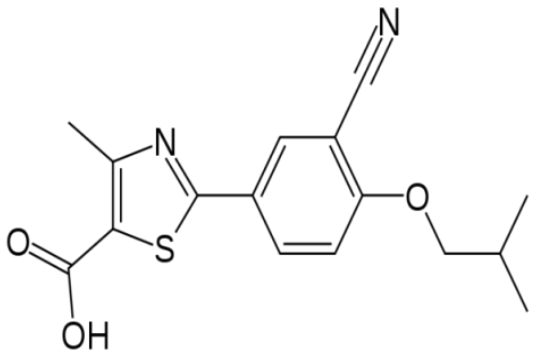

Figure 2: Structure of Febuxostat.

A survey of literature [4-10] reveals that, other reported methods for Febuxostat include Spectrophotometry, HPLC and LC-MS in varied Matrices like formulations and biological fluids. As highlighted earlier, the use of the above drug has become very wide spread. It is however, surprising to note that, only few methods are only available at present for the estimation of the drug in pharmaceutical formulations. The present project seeks to bridge this gap by developing a novel method.

Materials and Methods

Instrumentation and analytical conditions

Quantitative HPLC was performed on a Liquid Chromatography of Waters separation 2996, PDA detector module equipped with automatic injector with injection volume 20 µl, and 2693 pump. A Novapak- RP-C18 column (150x3.9 mm i.d; particle size 4 µm) was used. The HPLC system was equipped with Empower Software. Chromatographic conditions were tabulated in the Table 1.

Table 1: Chromatographic conditions for Ketorolac and Febuxostat.

Parameter	Content
Column	An Novapak RP-C_{18} column (150x3.9 µm i.d; particle size 4 µm)
Mobile Phase	Phosphate buffer in water pH-5.8 adjusted with O-Phosphoric Acid: Methanol in the isocratic mode (40:60 v/v)
Flow Rate	1.2 ml/min
Run time	8 min
Temperature	Ambient
Injection Volume	20 µl
Detection and Wavelength	PDA Detector, 321 nm
Retention times	1.9302 min for Febuxostat and 3.104 min for Ketorolac

Chemicals and reagents used

1. Pure API (Active Pharmaceutical Ingredient) sample of Ketorolac, 99%, Molecular Weight=255.26 g/mole was acquired from Mylan Laboratories Pvt. Ltd, Hyderabad, India and Febuxostat, 99%, Molecular Weight=316.37 g/mole was acquired from Sun pharmaceuticals private limited, Chennai, India. Water HPLC grade (Millipore Corporation – Milli-Q).
2. Ortho-phosphoric acid HPLC Grade (S.D Fine-Chem Ltd).
3. Acetonitrile HPLC Grade (Qualigens Fine Chemicals Pvt Ltd).
4. Methanol HPLC Grade (Millipore Corporation– Milli-Q).
5. Sodium Di Hydrogen Orthophosphate AR grade (S.D Fine-Chem Ltd).

Chromatographic trials for simultaneous estimation of Ketorolac and Febuxostat by RP- HPLC

Trial 1: For Febuxostat and Ketorolac

Mobile Phase: Methanol and Water were mixed in the ratio of 45:55 v/v and sonicated to degas.

Chromatographic Conditions

Flow rate: 1.20 ml/min
Column: C18 BDS column, 5 µ
Detector wavelength: 321 nm
Column temperature: Ambient
Injection volume: 20 µl

Run time: 8 min
Retention time: 3.549 and 4.192 min for FEB and KET

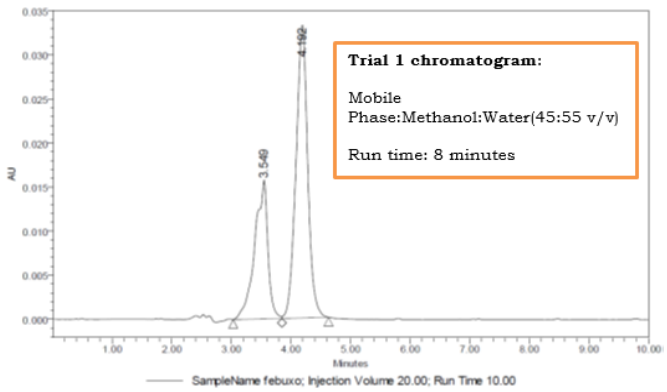

Figure 3: Chromatogram of Trial 1.

Observation

Theoretical plates are less, peak shape is not good, and fronting is there in graph and plate count is less than 2000.

Trail 2: Febuxostat and Ketorolac

Mobile Phase

Buffer and Acetonitrile were mixed in the ratio of 50:50 v/v and sonicated to degas.

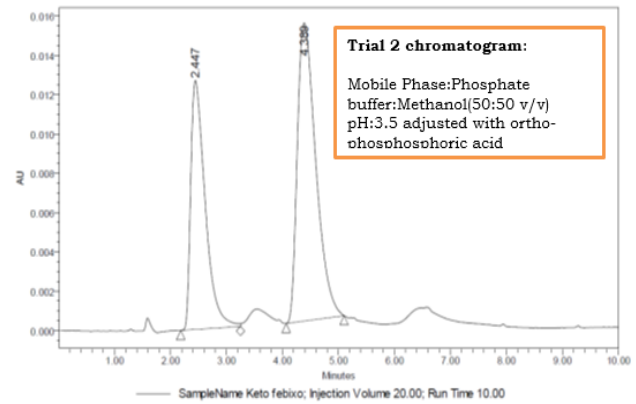

Figure 4: Chromatogram of Trial 2.

Chromatographic Conditions

Flow rate: 1.2 ml/min
Column: BDS C18 column (250x4.6x5µ)
Detector wavelength 321 nm
Column temperature: Ambient
Injection volume: 20 µl
Run time: 10 min

Retention time: 2.447 and 4.389 min for FEB and KET

Observation

The retention time is more, and asymmetry is high, asymmetry is more than limit sound noise ratio (S/N) is not meeting the limits.

Trail 3: Febuxostat and Ketorolac

Mobile Phase

Acetate Buffer and Water HPLC grade were mixed in the ratio of 50:50 v/v and sonicated to degas.

Chromatographic Conditions

Flow rate: 1.2 ml/min
Column: Xtterra C18 column (250x4.6x5µ)
Detector wavelength: 321 nm
Column temperature: Ambient
Injection volume: 20 µl
Run time: 10 min
Retention time: 2.382 and 4.096 min for FEB and KET

Observation

Retention time is high, and asymmetry is high

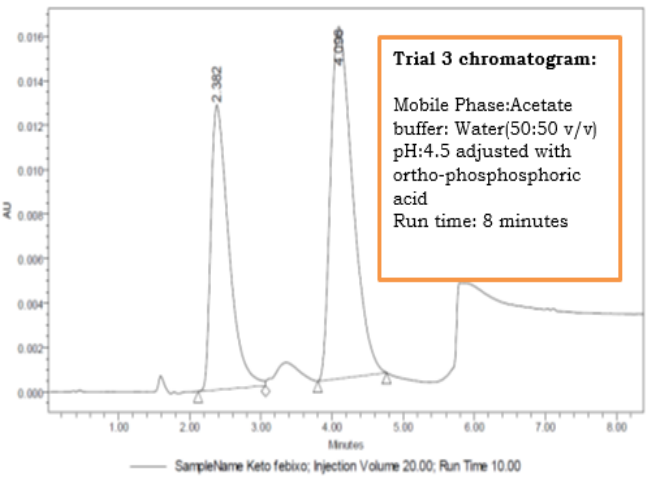

Figure 5: Chromatogram of Trial 3.

Trial 4: Chromatographic condition (Optimized Method)

Mobile Phase

Phosphate buffer in water pH-5.8 adjusted with O-Phosphoric Acid: Methanol in the isocratic mode (40:60 v/v)

Chromatographic Conditions

Flow rate: 1.2 ml/min
Column: An Novapak RP-C18 column (150x3.9 µm i.d; particle size 4 µm)
Detector wavelength: 321 nm
Column temperature: Ambient
Injection volume: 20 µl
Run time: 8min
Retention time: 1.9302 and 3.104 min for FEB and KET

Buffer Preparation

Accurately weighed 1.19 g of Di Sodium hydrogen phosphate Dihydrate and 8.25 g of Potassium Dihydrogen Phosphate in HPLC water and dilute to 1000 ml with the same solvent, pH (5.8) was adjusted to with Ortho phosphoric acid, filtered through 0.45 µm nylon membrane filter and degassed.

Mobile Phase

Buffer and Methanol were mixed in the ratio of 40:60 v/v and sonicated to degas.

Preparation of working standard solution

Aliquots ranging from 0.5 ml to 1.5 ml of KET and 0.2 ml to 0.6 ml were taken from working stock solution (100 µg/ml-KET, 1000 µg/ml-FEB) and diluted to 10ml with mobile phase to give final concentration of 5-15 µg/ml of ketorolac and 20-60 µg/ml of Febuxostat.

Preparation of sample drug solution for pharmaceutical formulations

The marketed formulations containing 10 mg of Ketorolac and 40 mg of Febuxostat was taken. From the above solution subsequent concentrations of 5-15 µg/ml and 20 to 60 µg/ml were prepared with mobile phase, sonicated and filtered through 0.45 µ membrane filtered

Procedure

Initially the mobile phase was pumped for about 30 min to saturate the column thereby to set the baseline corrected. Then 20 µl of the standard and sample solutions were injected separately. A quantitative determination of the active ingredients was made by comparison of the peak area of the sample injection with the corresponding peak area of the standard injection. The amount of Febuxostat and Ketorolac present in the sample was calculated through the standard calibration curve.

Procedure for calibration curve

The contents of the mobile phase were filtered before use through 0.45 µ membrane and pumped from the respective solvent reservoirs to the column at a specified flow rate. Prior to injection of the drug solutions, the column was equilibrated for at least 30 min with the mobile phase flowing through the system. The chromatographic separation was achieved using a mobile phase consisting of Buffer and Methanol at 40:60 v/v the eluent was monitored using UV detector at a wavelength of 321 nm. The column was maintained an ambient temperature (250c) and an injection volume of 20 µl of each of standard and sample solutions were injected into the HPLC system to get the chromatograms. The retention time, peak area of drug was recorded graph was plotted by taking concentration of the drug on x-axis and peak area on y-axis.

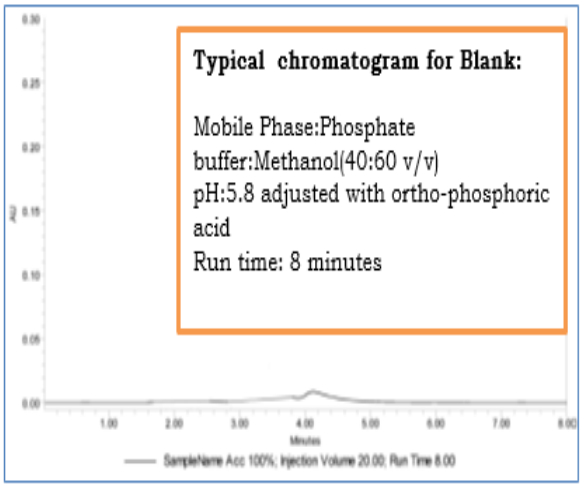

Figure 6: Chromatogram of Blank of Febuxostat (FEB) and Ketorolac (KET).

Results and Discussion

Method validation parameters

Specificity

The system suitability for specificity was carried out to determine whether there is any interference of any

impurities in retention time of analytical peak. The specificity was performed by Injecting blank, standard, sample and placebo solutions [11,12].

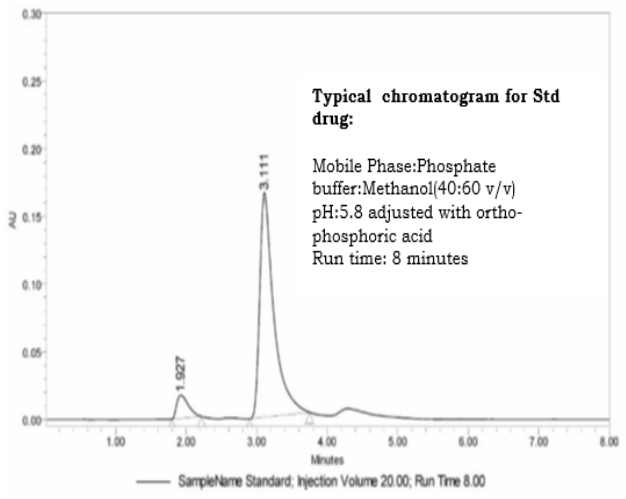

Figure 7: Typical chromatogram of Standard Febuxostat and Ketorolac.

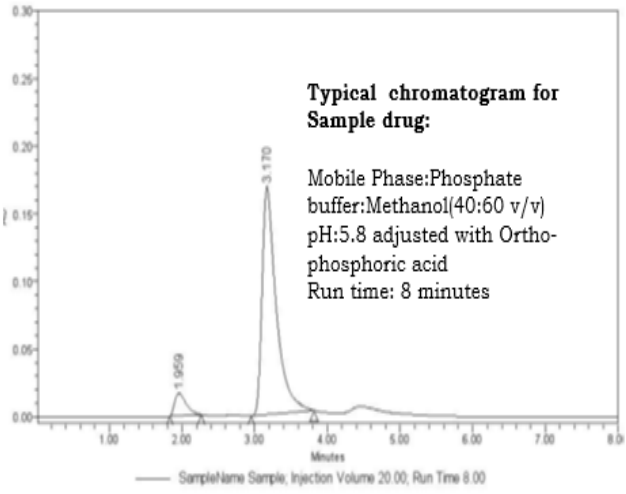

Figure 8: Chromatogram of Working Sample of Febuxostat and Ketorolac.

Linearity

The linearity of an analytical method is its ability to elicit test results that are directly or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. Serial dilutions of Febuxostat and Ketorolac (5- 15 µg/ml and 20-60 µg/ml) were injected into the column and detected at a wavelength set at 321 nm. The calibration curve was obtained by plotting the concentration vs. peak area [10].

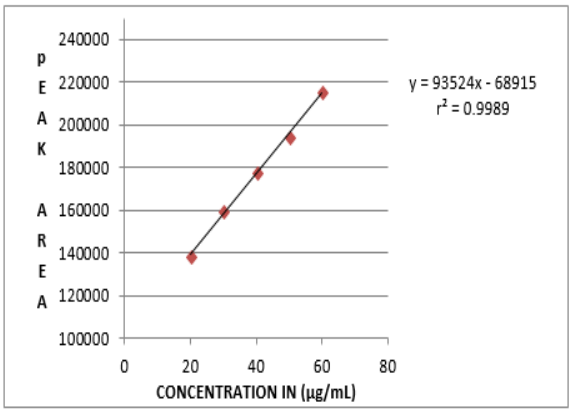

Figure 9: Standard Calibration Curve of Febuxostat.

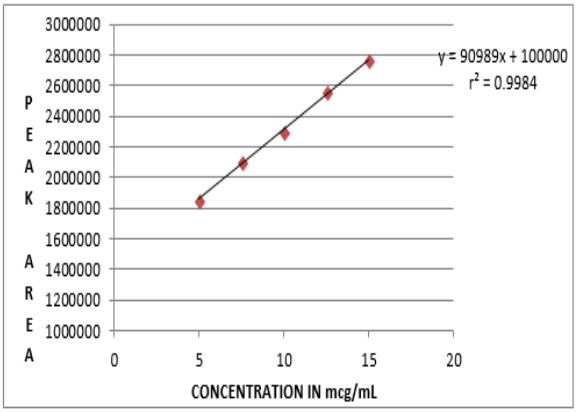

Figure 10: Standard Calibration Curve of Ketorolac.

Acceptance criteria

Correlation Coefficient should be not less than 0.999. 8% of RSD for level 1 and level 6 should be not more than 2.0%.

Recovery Studies

Recovery studies were conducted by analyzing the formulations in the first instance for the active ingredients in the concentration of 50% of the working standard solution, 100% of the working standard solution (40 µg/ml of Febuxostat and 10 µg/ml of Ketorolac) and 150% of the working standard solution by the proposed method. Each concentration was injected three times and the peak areas were recorded. The known amount of the pure drug of the working standard solution contains was

added to each three previously analyzed formulations and the total amount of the drug was again determined by the proposed method (each concentration was injected three times) by keeping the active ingredient concentration within the linearity limits.

Table 2: Standard Calibration Values of Febuxostat

Concentration of Febuxostat (µg/ml).

Concentration of Febuxostat (µg/ml)	Peak Area
20	138292
30	159718
40	177651
50	194564
60	215947

Range

Based on precision, linearity and accuracy data it can be concluded that the assay method is precise, linear and accurate in the range of 5-55 µg/ml and 20-60 µg/ml for Febuxostat and Ketorolac respectively.

Table 3: Standard Calibration Values of Ketorolac.

Concentration of Febuxostat (µg/ml)	Peak Area	
5.0	1857080	
7.5	2098367	
10.0	2294605	
12.5	2558229	
15.0	2764507	

Accuracy

To determine the accuracy of the proposed method, different amounts of bulk sample of Febuxostat and Ketorolac within linearity limits was taken and analyzed by the proposed method.

Calculations

$$\% \text{ Assay} = \frac{At \times Ws \times Avg.Wt \times P}{As \times Wt \times ClaimWt}$$

Where,
At=Average area due to Formulation peak in sample preparation
As=Average area due to peak in the Standard preparation
Ws=Weight of the working standard
Wt Weight of the sample Formulation
P=Potency of the working standard
Avg.Wt=Average Weight.

Precision

The precision was repeated with the formulated sample for the same concentrations by injecting the working sample solutions containing 40 µg/ml of Febuxostat and 10 µg/ml of Ketorolac. The sample Febuxostat and Ketorolac was processed six times for the response of peak area. The % Relative Standard Deviation (RSD), were calculated and presented in Tables: 6 & 7 respectively.

Acceptance criteria

The individual assays of Febuxostat and Ketorolac should be not less than 98% and not more than 102% and %RSD of assay should be NMT 2.0% by both analysts.

Table 4: Recovery data for Febuxostat.

%Conc (specification Level)	Area	Amount Added (mg)	Amount recovered (mg)	% Recovery	Mean Recovery
50%	1164128	5.0	5.07	101.34%	101.00%
100%	2331828	10.0	10.15	101.49%	
150%	3451918	15.0	15.02	100.16%	

Table 5: Recovery data for Keterolac.

%Conc (at specification Level)	Area	Amount Added (mg)	Amount recovered (mg)	% Recovery	Mean Recovery
50%	93780	5.0	5.07	101.42 %	100.84%

100%	188160	10.0	9.99	101.75 %	
150%	275594	15.0	14.90	99.35%	

Table 6: Precision of Recommended Procedure Using Standard Drugs (Febuxostat & Ketorolac).

Injection Number	Name of the Drug & Concentration (40 µg/ml)	Retention time in min	Peak Area	Name of the Drug & Concentration (10 µg/ml)	Retention time in min	Peak Area
1	Febuxostat	1.932	202686	Ketorolac	3.107	2341653
2	Febuxostat	1.923	201719	Ketorolac	3.104	2330075
3	Febuxostat	1.936	202770	Ketorolac	3.114	2327554
4	Febuxostat	1.934	205665	Ketorolac	3.114	2334942
5	Febuxostat	1.926	208243	Ketorolac	3.114	2335520
Mean		1.9302	204216.6		3.1106	2333949
Standard Deviation			2691.8			5447.697
% RSD			1.32			0.23

Assay Results: (Febuxostat)

207762 3.0 10 10 99.9 111.23

---------- x---------x --------- x--------- x ----------x----------x100 =100.56%

206453 100 10 27.8 1.2 100

Assay Results: (Ketorolac)

2286541 10 1.2 10 10 111.23 99.9

---------- x ----------x --------- x ------ x --------x 100 x ---------x---------- = 99.24%

2302475 10 10 27.8 1.2 40 100

Accuracy (Ketorolac)

To determine the accuracy of the proposed method, different amounts of bulk sample of Ketorolac within linearity limits was taken and analyzed by the proposed method.

Acceptance criteria

The mean % recovery of the Febuxostat and Ketorolac at each level should be not less than 95.0% and not more than 105.0%.

Table 7: Precision of Recommended Procedure Using Sample (Febuxostat & Ketorolac).

Injection Number	Name of the Drug & Concentration (40µg/mL)	Retention time in min	Peak Area	Name of the Drug & Concentration (10 µg/mL)	Retention time in min	Peak Area
1	Febuxostat	1.997	217118	Ketorolac	3.210	2350782
2	Febuxostat	2.009	216128	Ketorolac	30228	2304212
3	Febuxostat	2.002	214423	Ketorolac	3.221	2312475
4	Febuxostat	2.001	217780	Ketorolac	3.215	2305900
5	Febuxostat	2.008	217780	Ketorolac	3.214	2311894
Mean		2.0034	215547		3.2176	2317053
Standard Deviation			2218.636			19199.61
% RSD			1.03			0.83

Robustness

Effect of variation of flow rate

A study was conducted to determine the effect of variation in flow rate. Standard solution prepared as per the test method was injected into the HPLC system using flow rates, 1.0 ml/min and 1.2 ml/min. The system suitability parameters were evaluated and found to be

within the limits for 1.0 ml/min and 1.2 ml/min flow.

Febuxostat (FEB) and ketorolac (KET) was resolved from all other peaks and the retention times were comparable with those obtained for mobile phase having flow rates 1.0 ml/min.

Acceptance criteria

The Tailing Factor of FEB & KET standards should be NMT 2.0 for Variation in Flow.

Table 8: Summary of Validation data of Febuxostat and Ketorolac.

Parameters	Results	
	Febuxostat	**Ketorolac**
Linearity range (µg/ml)	20-60	5-15
Wave Length (λ max)	321 nm	321 nm
Coefficient of determination	0.9991 ± 0.01	0.9991 ± 0.01
Limit of detection (µg/ml)	0.04	0.12
Limit of quantification (µg/ml)	0.16	0.42
% Recovery (n=3)	101.00	100.84
Precision (%RSD)	1.32	0.23

Limit of Detection (LOD) and Limit of Quantification

The detection limit of the method was investigated by injecting standard solutions into the HPLC column. By using the signal-to-noise (S/N) method, the peak–to-peak noise around the analyte retention time is measured. Subsequently, the concentration of the analyte which would yield a signal equal to certain value of noise to signal ratio was also estimated. A signal-to-noise ratio (S/N) of 3 was generally accepted for estimating LOD and signal-to-noise (S/N) ratio of 10 was used for estimating LOQ.

The LOD was found to be 0.04 µg/ml for Febuxostat and 0.12 µg/ml for Ketorolac. The LOQ was found to be 0.16 µg/ml for Febuxostat and 0.42 µg/ml for Ketorolac.

Summary and Conclusion

There are only few reported methods on the RP-HPLC determination of Febuxostat and Ketorolac in Tablet dosage form in the literature prior to the commencement of this work. The author has developed a sensitive, accurate and precise RP-HPLC procedure for the estimation of Febuxostat and Ketorolac in bulk drug and also in pharmaceutical formulations.

From the typical chromatogram of Febuxostat and Ketorolac, it was found that the retention times were 1.923 min for Febuxostat and 3.104 min for Ketorolac. A mixture of Disodium hydrogen phosphate dehydrate and Potassium Dihydrogen Phosphate in Water HPLC grade (pH adjusted to 5.8 with 1% Ortho-Phosphoric Acid: Methanol (40:60 v/v) was found to be the most suitable solvent for elution. A good linear relationship (r=0.9998) was observed between the concentration range of linearity in the range of 20-60 µg/ml for Febuxostat and 5-15 µg/ml for Ketorolac respectively.

Thus, the present procedures constitute the RP-HPLC method with good precision, accuracy and sensitivity for the simultaneous estimation of Febuxostat and Ketorolac in pure stage and in pharmaceutical formulations.

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