



Validated RP-HPLC and HPTLC methods for simultaneous estimation of febusostat and diclofenac sodium in pharmaceutical dosage form

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ABSTRACT

The present work reports a reverse phase high performance liquid chromatography (RP-HPLC) method and high performance thin layer chromatography (HPTLC) method for the simultaneous determination of febusostat and diclofenac sodium in pharmaceutical dosage form. HPLC was performed using a Thermo Hypersil C₁₈ column (250 × 4.6 mm i.d., 5 μm particle size) using a mixture of methanol: KH₂PO₄ (0.02 M) (70:30, v:v) as mobile phase. Ultraviolet detection was carried out at 280 nm. The retention time of febusostat and diclofenac sodium were found to be 6.725 and 8.892 min, respectively. The HPTLC separation was conducted on Merck HPTLC aluminum sheets of silica gel 60 F₂₅₄ as stationary phase using toluene:methanol in the ratio of 7:3 (v:v) as the mobile phase. The detection of febusostat and diclofenac sodium was carried out at 297 nm. The R_f values of febusostat and diclofenac sodium were found to be 0.48 and 0.60, respectively. Both the methods were validated as per ICH guidelines. The proposed methods were found to be suitable for the quantification of the selected combination of drugs in pharmaceutical dosage form.

1. 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 febusostat is shown in Figure 1. Febuxostat is a non-purine xanthine oxidase inhibitor used in the treatment of hyperuricaemia with chronic gout [2]. Diclofenac (DIC) is chemically 2-(2,6-dichloranilino) phenyl acetic acid [1]. The chemical structure of diclofenac is shown in Figure 2. It is a nonsteroidal anti-inflammatory drug (NSAID) taken or applied to reduce inflammation and as an analgesic for reducing pain in certain conditions [2]. Literature review revealed that spectrophotometric method [3], HPLC methods [4-7] and stability indicating studies [8-12] have been reported for estimation of febusostat and HPLC [13,14] and spectrophotometric [15-17] methods have been reported for diclofenac alone. Further a validated HPLC method [18] for simultaneous estimation of febusostat and diclofenac potassium in bulk and tablet dosage form on C₁₈, (250 mm × 4.6 mm × 5 μm) column using acetonitrile:methanol:water (30:30:40, v:v:v; pH adjusted to 5.0 with TEA and o-phosphoric acid) as mobile phase and validated simultaneous equation and absorbance ratio methods [19,20] for the simultaneous

estimation of febusostat and diclofenac in tablet dosage form have been reported.

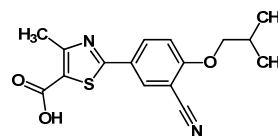


Figure 1. Structure of febusostat.

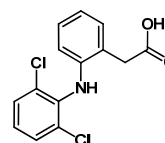


Figure 2. Structure of diclofenac.

In this work, a simple RP-HPLC method using methanol: KH₂PO₄ (0.02 M) (70:30, v:v) as mobile phase and HPTLC method using toluene:methanol in the ratio of 7:3 (v:v) as mobile phase have been reported.

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.

2. Experimental

2.1. Instrumentation

The HPLC system (Jasco Corporation, Tokyo, Japan) consisted of dual Pump (Model: Jasco PU-2080 Plus and Jasco PU-2087 Plus Intelligent Prep.) along with manual injector sampler programmed at 20 μ L capacity per injection. The UV/VIS (Model: Jasco UV 2075) detector was used. LC separations were performed on a Thermo Hypersil C₁₈ column (250 mm \times 4.6 mm i.d., 5 μ m particle size). Camag HPTLC system with Camag linomat IV automatic sample applicator, Camag TLC scanner III and Camag WinCATS software were used for HPTLC method. Saturation was done in Camag Twin-trough chamber (20 \times 10 cm). Merck plates coated with silicagel 60 F₂₅₄ (250 μ m thickness) on aluminium sheets was used as stationary phase.

2.2. Reagents and chemicals

Working standards of pharmaceutical grade FEB and DIC were obtained as gift samples from Centaur Pharmaceuticals Ltd., Pune. The tablet XANFEB DSR containing 40 mg FEB and 100 mg DIC was procured from the local pharmacy. All the chemicals and reagents used were of HPLC grade obtained from Merck Ltd., Mumbai, India.

2.3. Mobile phase

The mobile phase used in HPLC was methanol: KH₂PO₄ (0.02 M) (70:30, v:v). The flow rate was set to 1.0 mL/min. In HPTLC, toluene:methanol in the ratio of 7:3 (v:v) was used as mobile phase.

2.4. Preparation of standard stock solution

Standard stock solution of FEB and DIC containing 100 μ g/mL and 1000 ppm were prepared for HPLC and HPTLC methods, respectively. From the above stock solution, concentrations in the range of 2-12 μ g/mL of FEB and 5-30 μ g/mL of DIC for HPLC and 40-280 ng/band of FEB and 100-600 ng/band of DIC for HPTLC were prepared. The peak area for the different concentrations of FEB and DIC were recorded. The chromatogram in Figure 3 shows the retention time of FEB and DIC as 6.725 and 8.892 min, respectively, by HPLC method. The calibration curves were constructed between concentration against their respective peak area for FEB and DIC, respectively. Figure 4 shows the UV overlay spectrum of FEB and DIC and Figure 5 represents 3-D Chromatogram obtained in HPTLC study showing peaks of FEB and DIC in different concentrations. The densitogram of FEB and DIC is shown in Figure 6.

2.5. Sample preparation

For analysis of the tablet dosage form, twenty tablets (XANFEB DSR, Indoco Remedies Ltd.) containing 40 mg of FEB and 100 mg of DIC were weighed individually and their average weight was determined.

For HPLC method, the tablets were crushed to a fine powder and powder equivalent to the weight of 40 mg of FEB and 100 mg of DIC was transferred to a 100 mL volumetric flask and dissolved in about 30 mL of mobile phase. The solution was shaken for 5 min and then ultrasonicated for 15-20 min and filtered through 0.45 μ m Whatman filter paper. The

residue was washed with mobile phase and the combined filtrate was made up to the mark with the same solvent. 1 mL of this solution was diluted to 100 mL solvent to get the final solution that consists of concentration of 4 μ g/mL of FEB and 10 μ g/mL of DIC. The solution (20 μ L) was then injected for quantitative analysis. The identities of both the compounds were established by comparing retention time of the sample solution with those of standard mixed solution.

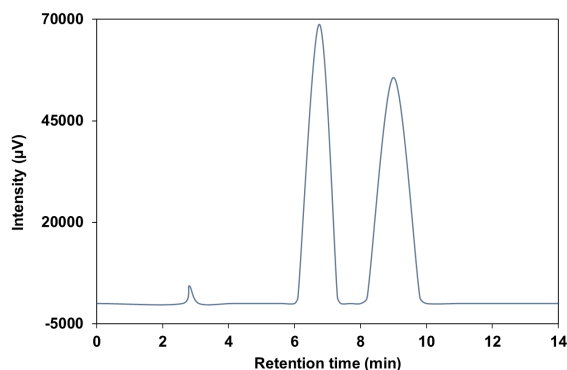


Figure 3. Chromatogram showing R_t of FEB and DIC.

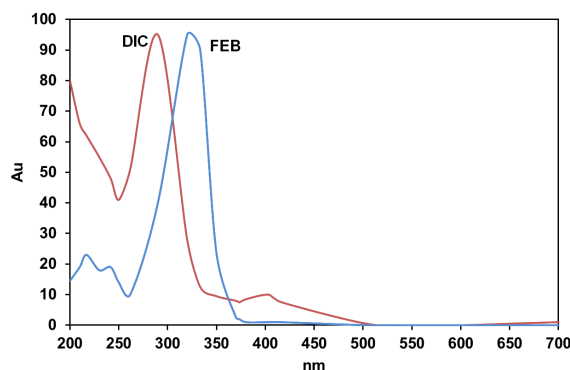


Figure 4. UV overlay of FEB and DIC.

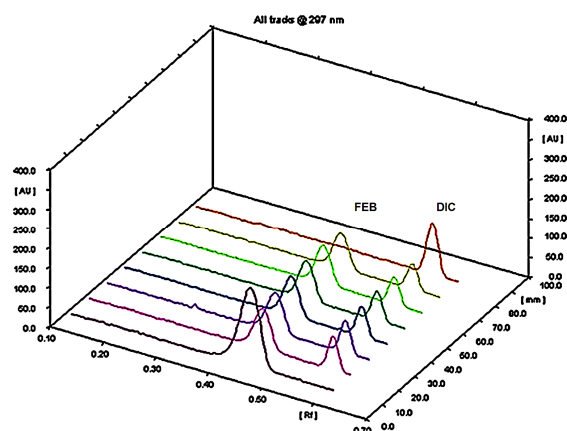


Figure 5. 3-D Chromatogram showing peaks of FEB and DIC in different concentrations at 297 nm.

For HPTLC method, quantity equivalent to 40 mg of FEB and 100 mg of DIC was taken in 100 mL volumetric flask and dissolved in 40 mL methanol and the final volume was made up with the same solvent. The solution was filtered through 0.45 μ m nylon syringe filter.

Table 1. Analysis of the marketed formulation by HPLC and HPTLC methods *.

Drug	HPLC			HPTLC		
	Amount taken, µg/mL	Drug content (%)±S.D.	%RSD	Amount taken, ng/band	Drug content (%)±S.D.	%RSD
FEB	4	99.821±0.081	0.019	120	99.68±0.018	0.158
DIC	10	100.190±0.154	0.055	300	99.21±0.134	0.183

* n=6.

Table 2. Linear regression data for the calibration curves by HPLC and HPTLC methods ^a.

Parameter	HPLC		HPTLC	
	FEB	DIC	FEB	DIC
Linearity range	2-12 µg/mL	5-30 µg/mL	40-280 ng/band	100-600 ng/band
r ²	0.9997	0.9997	0.9989	0.9999
Slope ± standard error	33170±268.3	44490±385.2	26.76±0.4507	14.01±0.0663
Intercept ± standard error	3093±20.89	1098±75.00	209.47±70.22	46.289±25.84
Confidence limit of slope ^b	32430 to 33920	43420 to 45550	25.50±28.01	13.82±14.19
Confidence limit of intercept ^b	-2708 to 8893	-19720 to 21920	14.55±40.4	-25.43±118.0
Sy.x ^c	2244	8057	75.42	27.75
p value ^d	<0.0001	<0.0001	<0.0001	<0.0001

^a n=6.^b 95% confidence intervals.^c Standard deviation of residuals from line.^d p value is < 0.0001, considered highly significant.**Table 3.** Accuracy studies for the determination of FEB and DIC by HPLC and HPTLC methods *.

Drug	HPLC				
	Amount added (%)	Theoretical content (µg/mL)	Measured conc.±SD	Recovery (%)	% R.S.D
FEB	80	4.8	4.85± 0.06	96.29	1.212
	100	6	6.05±0.02	100.8	0.399
	120	7.2	7.25 ± 0.04	100.75	0.687
DIC	80	12	11.94 ± 0.01	99.5	0.153
	100	15	14.94±0.08	99.6	0.084
	120	18	18.17±0.09	100.94	0.094
Drug	HPTLC				
	Amount added (ng)	Total amount (ng)	Amount recovered (ng)	Recovery (%)	% R.S.D
FEB	20	60	58.58	97.64	1.01
	40	80	81.52	101.9	0.98
	60	100	97.73	97.73	1.15
DIC	50	150	150.68	100.45	1.16
	100	200	197.86	98.93	0.48
	150	250	247.45	98.98	1.37

* n=6.

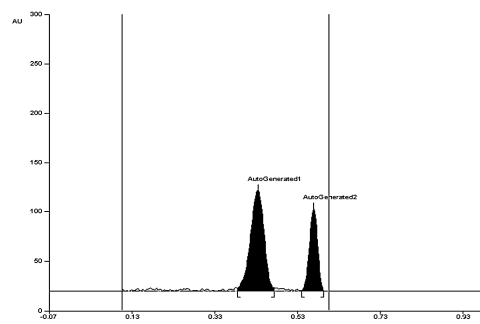
From the resultant solution 1 mL was diluted to 10 mL which contains 40 and 100 ppm of FEB and DIC respectively. 3 µL volume was spotted for six times to achieve a final concentration of 120 ng/band for FEB and 300 ng/band for DIC. The plate was developed in the previously described chromatographic conditions and the peak area of the spots was measured at 297 nm. The results of analysis of marketed formulation by HPLC and HPTLC methods are reported in Table 1.

2.6. Validation

The validation of an analytical method verifies that the characteristics of the method satisfy the requirements of the application domain. The proposed method was validated in the light of ICH Guidelines [21]. The developed method was validated for linearity, accuracy, precision, repeatability, selectivity and specificity study as per ICH guidelines. All the validation studies were carried out by replicate injection of the sample and standard solutions.

2.6.1. Linearity

Linear relationship was observed in concentrations ranging from 2-12 µg/mL for FEB and 5-30 µg/mL for DIC in HPLC and 40-280 ng/band for FEB and 100-600 ng/band for DIC in HPTLC. From the data obtained correlation coefficient, y-intercept and slope were calculated to provide mathematical estimates of the degree of linearity (Table 2).

**Figure 6.** Densitogram of FEB (0.48) and DIC (0.60) standard.

2.6.2. Accuracy

Accuracy of the developed methods was carried out by adding known amount of each drug corresponding to three concentration levels; 80, 100 and 120% of the label claim in HPLC and 50, 100 and 150% of the label claim in HPTLC. The accuracy was expressed as the percentage of analytes recovered by the assay methods. The results of percentage recovery are shown in Table 3.

2.6.3. Precision

Precision was studied by repeatability and intermediate precision studies.

Table 4. Results of Precision study by HPLC and HPTLC methods *.

Drug	HPLC			HPTLC				
	Drug conc. ($\mu\text{g/mL}$)	Repeatability Found conc. \pm S.D.	% RSD	Intermediate precision Found conc. \pm S.D.	% RSD	Drug conc. (ng/band)	Intraday precision % RSD	Interday precision % RSD
FEB	2	1.9394 \pm 0.0012	0.065	1.9312 \pm 0.0122	0.632	40	1.42	1.55
	6	6.0677 \pm 0.0008	0.013	6.0816 \pm 0.0501	0.824	120	1.11	1.48
	12	11.917 \pm 0.0043	0.036	11.8529 \pm 0.1297	1.094	240	1.71	1.71
DIC	5	5.019 \pm 0.0307	0.612	5.1356 \pm 0.0985	1.919	100	1.53	1.002
	15	14.898 \pm 0.0192	0.128	14.826 \pm 0.1184	0.798	300	0.121	1.53
	30	30.7689 \pm 0.1954	0.650	29.9605 \pm 0.4040	1.348	600	0.434	0.625

* n=6.

Table 5. Limit of detection(LOD) and limit of quantitation(LOQ) in HPLC and HPTLC methods.

Parameter	HPLC		HPTLC	
	FEB	DIC	FEB	DIC
LOD	0.32 $\mu\text{g/mL}$	1.13 $\mu\text{g/mL}$	0.24 ng/band	0.13 ng/band
LOQ	0.98 $\mu\text{g/mL}$	2.87 $\mu\text{g/mL}$	0.70 ng/band	0.37 ng/band

Table 6. Robustness evaluation ^a of the method by HPLC (n = 6).

Factor	Level	Retention time (t_R)		Asymmetry (T)	
		FEB	DIC	FEB	DIC
<i>A: Flow Rate (mL/min)</i>					
0.9	-1	6.712	6.687	1.02	1.15
1.0	0	6.725	8.892	1.01	1.17
1.1	+1	6.613	8.788	1.01	1.16
<i>B: Percentage of methanol in the mobile phase (v:v)</i>					
71	-1	6.712	8.824	1.33	1.23
70	0	6.725	8.892	1.31	1.22
72	+1	6.798	8.878	1.29	1.21
<i>C: Columns from different manufacturers</i>					
Hypersil C18 column		6.725	8.892	1.31	1.22
HiQ-Sil™ HS C18 column		6.798	8.811	1.32	1.23

^a Average of three concentrations 2, 6 and 12 $\mu\text{g/mL}$ for FEB, 5, 15 and 30 $\mu\text{g/mL}$ for DIC.**Table 7.** Robustness evaluation of the method by HPTLC (n = 6).

Parameter	SD of peak area for FEB	% RSD	SD of peak area for DIC	% RSD
Mobile phase composition (\pm 0.1 mL)	15.26	0.46	22.77	0.620
Amount of mobile phase (\pm 5%)	11.51	0.31	16.53	0.254
Time from application to development (+10 min)	7.74	0.25	9.81	0.240
Time from development to scanning (+10 min)	14.63	0.45	7.38	0.154

The results are reported in terms of relative percentage standard deviation (% RSD) as in Table 4.

2.6.4. Limit of detection (LOD), and limit of quantification (LOQ)

The lowest amount of the analyte in the sample which can be detected and the lowest amount of analyte which can be quantitatively determined were studied and, LOD and LOQ values are reported in Table 5.

2.6.5. Robustness

The robustness of the methods was determined by subjecting the methods to slight changes in the chromatographic conditions. It was observed that there was no marked change in the chromatogram which demonstrated that the methods developed are robust (Table 6 and 7).

2.6.6 Selectivity

The selectivity was checked by injecting the solution of both the drugs into the HPLC system and it was observed that two sharp peaks of FEB and DIC having resolution of 2.511 were obtained at retention time of 6.725 and 8.892 min, respectively. In HPTLC, the R_f values of FEB and DIC were found to be 0.48 and 0.60, respectively. It was observed that the excipients did not interfere with the retention time and R_f values of the drugs. So, the methods developed for this combined dosage form are said to be selective.

2.6.7. Specificity

Specificity of the method was assessed by comparing the chromatograms obtained for standard drugs with the chromatogram obtained for tablet solution. The retention time and R_f values of standard drugs and the drugs in sample solution were same, so the methods are specific. The results of system suitability parameters in HPLC are shown in Table 8.

2.6.8. Stability of analytical solution in HPLC

Stability of sample solution was established by storage of sample solution at ambient temperature for 48 hours. FEB and DIC sample solution was re-analyzed after 24 and 48 hr time intervals and assay value was determined and compared against fresh sample. Sample solution did not show any appreciable change in assay value when stored at ambient temperature up to 48 hr. It was found that the percentage labeled amounts of FEB at 0, 24 and 48 hr were 99.8, 100.1 and 100.5, respectively and the percentage labeled amounts of DIC were 100.1, 99.9 and 100.3, respectively.

3. Results and discussion

Column chemistry, solvent type, solvent strength, detection wavelength and flow rate were varied to determine the chromatographic conditions giving the best separation. The mobile phase conditions were optimized so that the components were free from the interference of solvent and excipients.

Table 8. System suitability parameters in HPLC.

Parameter	FEB	DIC	Reference values
Theoretical plates (N)	2216.17	2074	N > 2000
Peak asymmetry (T)	1.01	1.17	T ≤ 2
Capacity factor (K')	1.06	1.61	1 < K' < 10
HETP (H) ^a	0.11	0.12	
Selectivity (α) ^b	1.39		A > 1
Resolution (Rs) ^b	2.511		Rs ≥ 2

^aHETP (Height Equivalent to Theoretical Plate).^bWith respect to previous peak.

Mobile phase and flow rate selection was based on peak parameters like height, area, tailing, theoretical plates, capacity factor, resolution and run time. In HPTLC, the effect of chamber saturation time on peak shapes, development pattern and R_f value were studied. The chamber saturation time was fixed as 30 min as the R_f value and resolution were satisfactory for FEB and DIC. Plate equilibration time was fixed as 20 min as the plates developed after 20 min of saturation showed good precision and reproducibility of peak area of drugs. The composition of solvent constituting the mobile phase was varied in order to study its effect on resolution of drugs. The analytes should have distinct retention time and R_f values for good resolution. The best result was obtained by use of 70:30 (v:v) ratio of methanol and 0.02 M KH_2PO_4 in HPLC and toluene:methanol in the ratio (7:3, v:v) in HPTLC. Under the optimum chromatographic conditions, the retention time obtained for FEB and DIC were 6.725 and 8.892 min, respectively, in HPLC method and R_f values for FEB and DIC were found to be 0.48 and 0.60, respectively, in HPTLC method. The values obtained for the validation parameters show that, the chromatographic conditions are appropriate for separation and determination of the compounds.

4. Conclusion

A new RP HPLC method and HPTLC method have been developed for simultaneous analysis of Febuxostat and Diclofenac in combined dosage form. The developed methods were found to be linear, accurate, reproducible, repeatable, precise, selective and specific proving the reliability of the methods. The recovery studies indicate that there was no interference of excipients. The proposed methods may be successfully applied to routine analysis of samples containing Febuxostat and Diclofenac.

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