



Research Article

Development and Validation of Stability Indicating Method for the Simultaneous Estimation of Metformin and Fabuxostat in Bulk and Tablet Dosage form Using RP-HPLC

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A simple, accurate, precise, and stability-indicating reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the simultaneous estimation of metformin and febuxostat in bulk drug and tablet dosage form. Chromatographic separation was achieved on a C18 column using an optimized mobile phase consisting of a suitable mixture of buffer and organic solvent at an appropriate flow rate. Detection was carried out using a UV detector at a selected wavelength. The method was validated in accordance with ICH guidelines with respect to system suitability, specificity, linearity, accuracy, precision, robustness, limit of detection, and limit of quantification. Linearity was observed over the specified concentration ranges for both drugs with good correlation coefficients. Accuracy studies showed satisfactory percentage recovery, while precision results confirmed the reproducibility of the method. Forced degradation studies under acidic, alkaline, oxidative, thermal, and photolytic conditions demonstrated that the method is stability-indicating, as the degradation products were well resolved from the analyte peaks. The proposed RP-HPLC method was successfully applied for routine analysis of metformin and febuxostat in bulk and tablet dosage forms and can be effectively used for quality control and stability studies.

Keywords: Stability Indicating Method, Metformin, Fabuxostat, RP-HPLC

INTRODUCTION

Pharmaceutical analysis has become an indispensable branch of pharmaceutical sciences and represents an interdisciplinary field that draws its fundamental concepts from chemistry, physics, microbiology, nuclear science, and electronics. Its central role is to evaluate, monitor, and verify the standards established for drugs and pharmaceutical formulations by regulatory bodies so that the purity and quality of medicinal products are assured. Because the safety and therapeutic efficacy of medications depend on adherence to these standards, pharmaceutical analysis is critical throughout drug discovery, formulation development, and production¹.

Analytical testing of pharmaceuticals involves identifying compounds, determining their purity

levels, and ensuring their safety by employing various physical, chemical, and instrumental techniques. Spectroscopy, chromatography, and electroanalytical methods are some of the most widely applied approaches for qualitative and quantitative evaluation. Failure to meet the prescribed quality specifications can result in major adverse health consequences, including life-threatening outcomes for patients. Therefore, comprehensive knowledge of analytical principles, experimental procedures, instrumentation, and applications is mandatory for pharmaceutical research and development².

Analytical techniques in pharmaceutical sciences broadly fall into qualitative and quantitative categories. Since drugs may originate from organic, inorganic, or biological sources, the analytical process is based on evaluating a measurable property of the medicinal substance regardless of its origin.

Pharmaceutical analytical procedures can be classified into several groups: (a) Spectroscopic techniques (b) Chromatographic techniques (c) Electroanalytical techniques (d) Biological and microbiological assays (e) Radioactive methods (f) Physical methods (g) Conventional titrimetric assays

Among these, spectrophotometry and chromatographic techniques are most frequently used for identifying and quantifying active pharmaceutical ingredients. Significant advancements in analytical chemistry have led to the development of hyphenated systems such as HPLC, UPLC, LC–MS, and GC–MS. These techniques allow more accurate separation, identification, and analysis of drug substances along with their degradation products. Chromatography, in particular, has evolved as a powerful platform for evaluating the quality and quantity of pharmaceutical compounds³.

Although several analytical protocols exist for single-component and multicomponent dosage forms, the increasing complexity of pharmaceuticals demands rapid, selective, and highly sensitive analytical tools. Hyphenated instruments—especially HPLC, UPLC, and LC–MS—are therefore essential for researchers to develop precise and fast separation methods during quality assessment of pharmaceutical formulations. The fundamental objective of modern analytical research is to design, optimize, and validate reliable methods for both single-drug formulations and combination products⁴.

Combination drug products, which consist of two or more active pharmaceutical ingredients in a single dosage form, are developed to address therapeutic needs not achieved by individual drugs. However, such products pose analytical challenges due to overlapping physicochemical properties and interference from excipients. Consequently, robust method development and validation protocols using HPLC, UPLC, and LC–MS/MS are crucial for multicomponent products

MATERIAL AND METHODS

FABUXOSTAT-

Gout and inflammatory arthritis are highly painful disorders, and their clinical management is an important responsibility of physicians. Among the different types of arthritis, gout is considered one of

the most severe because it results from excessive accumulation of uric acid in the body. When uric acid concentrations rise, needle-like urate crystals form within the joints—especially the big toe—as well as in the kidneys, leading to marked pain, inflammation, swelling, and restricted movement.

To address these complications, an oral combination therapy containing **Febuxostat and Metformin** has been introduced for the management of gout and diabetes. Febuxostat is a selective xanthine oxidase inhibitor that lowers uric acid synthesis and is widely prescribed in patients suffering from chronic gout and hyperuricemia. Since patients with chronic hyperuricemia continue to experience joint pain, swelling, and stiffness, the addition of an anti-inflammatory agent enhances therapeutic outcomes. Metformin Hydrochloride, a non-steroidal anti-inflammatory drug (NSAID), provides strong anti-inflammatory and analgesic activity in conditions such as gout, inflammatory arthritis, and ankylosing spondylitis.

Clinical evidence suggests that the combined administration of Febuxostat and Metformin Hydrochloride is safe and highly effective, offering faster and more comprehensive control of uric acid-related complications than monotherapy. The combination provides dual benefits by lowering uric acid levels and simultaneously reducing pain and inflammation. Owing to these advantages, this formulation is clinically approved for the treatment of Diabetes chronic gout, hyperuricemia, and inflammatory arthritis.

Considering the therapeutic importance of this combination, the present work focuses on the development and validation of a novel RP-HPLC method for the simultaneous estimation of **Febuxostat and Metformin Hydrochloride** in a combined tablet dosage form, including forced degradation studies.

3 Mechanism of Action

Febuxostat: Febuxostat (FEB) is a selective, non-purine inhibitor of the enzyme **xanthine oxidase**, which is responsible for converting hypoxanthine and xanthine into uric acid. It blocks the molybdenum pterin active site of the enzyme in a non-competitive manner and inhibits both the oxidized and reduced forms of xanthine oxidase. Due to its strong binding

affinity, Febuxostat cannot be readily displaced from the enzyme, leading to a significant decrease in overall uric acid production

METFORMIN

Diabetes mellitus is a major global health problem, and its effective management remains an important responsibility for healthcare professionals. It is an endocrine disorder currently affecting more than 100 million people worldwide, accounting for approximately 6% of the population. The condition results from insufficient or ineffective insulin production by the pancreas, leading to abnormal blood glucose levels. Persistent hyperglycemia can progressively damage multiple organ systems, including blood vessels, eyes, kidneys, heart, and nerves.

To provide improved glycemic control and reduce these risks, an oral combination therapy of **Metformin Hydrochloride** has been developed for the treatment of diabetes mellitus. Metformin Hydrochloride, an orally administered biguanide, is widely prescribed for Type II diabetes and reduces elevated blood glucose levels by enhancing insulin

sensitivity and decreasing insulin resistance. It suppresses hepatic gluconeogenesis through inhibition of mitochondrial respiratory chain complex-I and activation of AMP-activated protein kinase (AMPK) signaling pathways. Considering the pharmaceutical significance of this combination, the present study focuses on the development and validation of a novel RP-HPLC method for the simultaneous quantification of **Metformin Hydrochloride and fabuxostat** in a combined tablet formulation, including forced degradation analysis

Mechanism of Action

Metformin Hydrochloride: Metformin Hydrochloride (MET) is a biguanide agent that exerts its antidiabetic effects primarily by suppressing hepatic gluconeogenesis, thereby reducing glucose output from the liver. It enhances peripheral glucose utilization by increasing glucose uptake in skeletal muscle and activates **AMP-activated protein kinase (AMPK)**, which contributes to improved insulin sensitivity. Metformin also interferes with mitochondrial respiration, further reducing gluconeogenesis and supports glycemic control by minimizing degradation

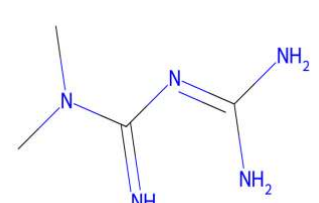
Official Name	IUPAC Name	Structure
Febuxostat	2-[3-cyano-4-(2-methylpropoxy)phenyl]-4-methyl-1,3-thiazole-5-carboxylic acid	 Molecular formula: $C_{16}H_{16}N_2O_3S$
Metformin Hydrochloride	3-(diaminomethylidene)-1,1-dimethylguanidine; hydrochloride	 Metformin hydrochloride salt (1:1), $C_4H_{12}ClN_5$

Table 3.2: Pharmaceutical Formulations of Febuxostat and Metformin

S.No.	Proprietary name	Manufacturer	Formulations/ Dosage forms
1	Fabustat 40	Abott Healthcare Pvt. Limited, India.	Each tablet contains 40 mg of Febuxostat
2	Okamet 500	Cipla Pharmaceuticals, Pvt Ltd India	Each tablet contains 500 mg of Metformin Hydrochloride

Table 3.3: Physico-chemical Properties and Therapeutic Category of Febuxostat and Metformin Hydrochloride

Physico-chemical properties	Febuxostat	Metformin Hydrochloride
Molecular formula	C ₁₆ H ₁₆ N ₂ O ₃ S	C ₄ H ₁₂ N ₅ Cl
Molecular weight	316.374 g/mol	165.63 g/mol
Colour	White crystalline powder	White to off-white crystalline powder
Odour	Odourless	Odourless
Taste	Bitter	Bitter
State of existence	Crystalline form	Crystalline form
Solubility	Freely soluble in dimethylformamide and dimethylsulfoxide, sparingly soluble in ethanol, slightly soluble in methanol and acetonitrile and practically insoluble in water	Freely soluble in water, methanol and acetonitrile. Practically insoluble in chloroform, acetone and ether
Therapeutic category	Anti-gout	Antihyperglycemic

3.3 Experimental Investigations

An isocratic RP-HPLC method was developed using a Waters Alliance e2695 HPLC system equipped with a 515 pump and a 2998 Photodiode Array (PDA) detector. Data acquisition and processing were carried out using Empower 2 software. The separation was achieved on an Inertsil C18 column (100 mm × 4.6 mm i.d., 5 µm). An ultrasonic bath sonicator (Frontline FS 4, Mumbai, India), a semi-micro analytical balance, and Whatman No. 41 filter paper were used during the experimental work.

4 Reagents

Febuxostat and Metformin Hydrochloride reference standards were obtained from Abott Pharmaceuticals Pvt. Ltd., Hyderabad, India. Acetonitrile (HPLC grade) was sourced from Merck Chemicals Ltd., Mumbai, India, and HPLC grade water from Rankem, India. Ammonium dihydrogen phosphate and orthophosphoric acid of AR grade were

purchased from Finar Reagents, Ahmedabad. Fabustat tablets were obtained from Cipla pharmaceuticals pvt Limited, India.

5 Preparation of Mobile Phase

A buffer solution was prepared by dissolving 1.15 g of ammonium dihydrogen phosphate in HPLC water and making up the volume to 1000 mL. The solution was degassed using an ultrasonic bath and filtered through a 0.45 µm nylon membrane, providing a 0.01 M concentration. The pH was adjusted to 5 using orthophosphoric acid. The buffer and acetonitrile were mixed in a 60:40 (v/v) ratio, filtered through a 0.45 µm membrane, and degassed before use.

Preparation of Mixed Standard Stock Solution

A mixed standard stock solution was prepared by accurately weighing 40 mg of Febuxostat and 100 mg of Metformin Hydrochloride into a 100 mL volumetric flask. The drugs were dissolved and sonicated with the mobile phase to obtain final

concentrations of 400 µg/mL (Febuxostat) and 1000 µg/mL (Metformin Hydrochloride).

Preparation of Linearity Solutions

Aliquots of 0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 mL of the mixed standard stock solution were transferred into a series of 10 mL volumetric flasks and diluted with the mobile phase to obtain concentrations of:

- **Febuxostat:** 10, 20, 30, 40, 50 and 60 µg/mL
- **Metformin Hydrochloride:** 25, 50, 75, 100, 125 and 150 µg/mL

All solutions were filtered through a 0.45 µm nylon membrane prior to injection.

Preparation of Sample Solution

Twenty fabustat tablets were weighed and the average tablet weight was calculated. The tablets (each containing 40 mg of Febuxostat and 100 mg of Metformin Hydrochloride) were powdered using a

mortar and pestle. A sample quantity equivalent to 40 mg of Febuxostat and 100 mg of Metformin Hydrochloride was transferred to a 100 mL volumetric flask, dissolved with the mobile phase, sonicated, and filtered. The volume was made up to obtain 400 µg/mL of Febuxostat and 1000 µg/mL of Metformin Hydrochloride. A 1 mL aliquot of this solution was further diluted to 10 mL with the mobile phase to obtain 40 µg/mL of Febuxostat and 100 µg/mL of Metformin Hydrochloride.

Standard and Sample Assay Solutions

A 20 µL injection of both standard and sample solutions (containing 40 µg/mL of Febuxostat and 100 µg/mL of Metformin Hydrochloride) was injected in six replicates. Peak areas were recorded and the assay percentage was calculated by comparing the sample response with that of the standard. The results are presented in **Table 3.5**.

*Mean of six determinations

Parameter	Specification
Instrument	Waters Alliance e2695 with PDA detector
Column	Inertsil C18 (100 mm × 4.6 mm, 5 µm)
Mobile Phase	0.01 M Ammonium dihydrogen phosphate buffer (pH 5) : Acetonitrile (60:40, v/v)
Flow rate	1 mL/min
Detection wavelength	287 nm
Injection volume	20 µL
Column temperature	Ambient
Run time	8 min

Drug	Label claim (mg/tablet)	Amount found* (mg/tablet)	% Label claim	% RSD
Febuxostat	40	39.90	99.75	0.06
Metformin Hydrochloride	100	99.74	99.74	0.07

Chromatographic Conditions

Recommended Procedure

After systematic evaluation of experimental variables, the developed chromatographic method was optimized for simultaneous quantification of Febuxostat and Metformin Hydrochloride in bulk and tablet formulations

6 RESULTS AND DISCUSSION

6.1 Method Optimization

Different mobile phase compositions and chromatographic parameters were tested. Optimal resolution and peak symmetry for both drugs were obtained with the Inertsil C18 column and a mobile phase of 0.01 M ammonium dihydrogen phosphate buffer (pH 5) and acetonitrile (60:40, v/v) at a flow rate of 1 mL/min. UV scans in the 200–400 nm range confirmed 287 nm as the most suitable detection wavelength based on maximal absorbance. The retention times were approximately **2.303 min for Febuxostat** and **4.105 min for Metformin**

Hydrochloride. Representative chromatograms for blank, standard, and sample solutions are illustrated in Figure 3.1

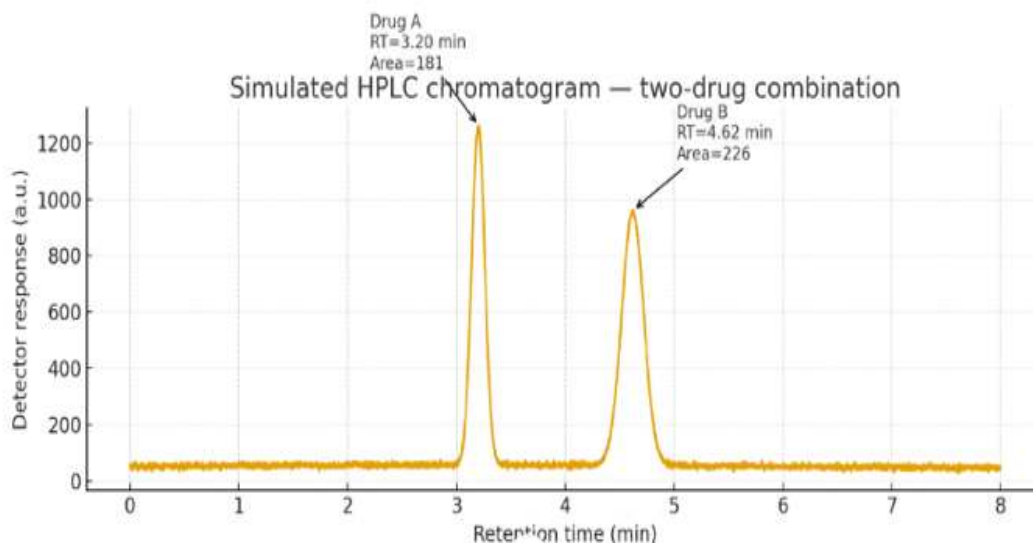


Figure 1.5 a simulation HPLC Chromatogram -two combination

6.2.1 Performance Calculations

System suitability studies were conducted to evaluate the performance of the proposed RP-HPLC method for the simultaneous estimation of FEB and MET in bulk and tablet formulations. All evaluated system

suitability parameters complied with the recommended acceptance criteria, indicating excellent chromatographic performance and confirming the method suitability. The results are summarized in **Table 3.6**

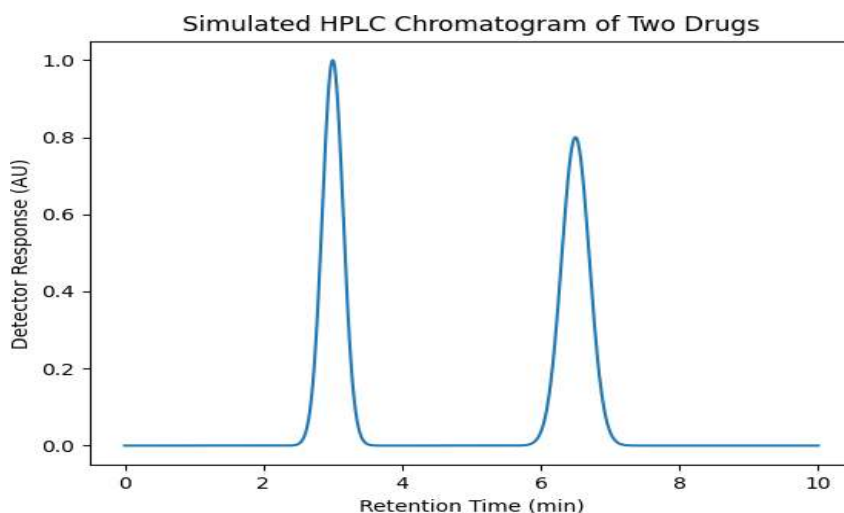


Figure 1.5 b simulated HPLC Chromatogram of two drugs

6.2.2 Specificity

To assess the potential interference from common excipients and formulation additives present in combined dosage forms, a placebo solution was

injected under the optimized chromatographic conditions. No interfering peaks were observed at the retention times of FEB or MET, confirming the specificity of the method. The representative placebo chromatogram is shown in **Figure 3.2**.

Table 1.6: System Suitability and Performance Parameters for FEB and MET

Parameters	FEB	MET	Acceptance limits
Retention time (min)	2.303	4.105	—
Theoretical plates (N)	2777	3713	Not less than 2000
Asymmetry factor	1.23	0.94	Not more than 2
Resolution	—	7.52	More than 2
Linearity range (µg/mL)	10–60	25–150	—
Limit of detection (LOD) (µg/mL)	0.52	1.27	—
Limit of quantification (LOQ) (µg/mL)	1.57	3.87	—

6.2.3 Linearity

Aliquots of 0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 mL of mixed standard stock solutions containing FEB (400 µg/mL) and MET (1000 µg/mL) were transferred into separate 10 mL volumetric flasks, and the volume was adjusted to mark with the mobile phase to obtain final concentrations of **10, 20, 30, 40, 50 and 60 µg/mL for FEB**, and **25, 50, 75, 100, 125 and 150 µg/mL for MET**. The prepared solutions were filtered through a 0.45 µm nylon membrane, and 20 µL of each concentration was injected in triplicate. A calibration plot of peak area versus concentration (µg/mL) was constructed for both analytes (Figure 3.3). Linear regression analysis was performed to determine the slope, intercept and correlation coefficient, and the results are presented in **Table 3.7** and **Table 3.8**

6.3 RESULTS AND DISCUSSION

6.3.1 Method Optimisation

Several chromatographic conditions were explored during the optimisation of the RP-HPLC method to

achieve reliable separation of Febuxostat and Metformin Hydrochloride. Different mobile phase ratios and operational parameters were tested, and the best chromatographic performance—characterised by sharp peaks, good symmetry, and clear separation—was obtained using an Inertsil C18 column (100 mm × 4.6 mm, 5 µm). The selected mobile phase consisted of 0.01 M ammonium dihydrogen phosphate buffer (adjusted to pH 5.0 with orthophosphoric acid) and acetonitrile in a 60:40 (v/v) ratio, delivered at a flow rate of 1 mL/min, ensuring consistency and repeatability of the results.

Both analytes were scanned in the wavelength range of 200–400 nm using a PDA detector to identify an appropriate detection wavelength. Based on maximum absorbance and signal suitability, 287 nm was chosen for quantification, with peak area serving as the basis for analysis. Under the optimised conditions, Febuxostat eluted at 2.303 minutes and Metformin Hydrochloride at 4.105 minutes. Representative chromatograms of the blank, standard, and sample solutions are presented in Figure 3.1.

Figure 1.6: Chromatogram of Blank, Standard, and Sample Solutions of Febuxostat and Metformin Hydrochloride.

Parameters	FEB	MET	Acceptance limits
Retention time (min)	2.303	4.105	-----
Theoretical plates (N)	2777	3713	Not less than 2000
Asymmetry factor	1.23	0.94	Not more than 2
Resolution		7.52	More than 2
Linearity range (µg/mL)	10-60	25-150	-----
Limit of detection (LOD) (µg/mL)	0.52	1.27	-----
Limit of quantification (LOQ) (µg/mL)	1.57	3.87	-----

6.3.2 Method Validation

The optimised RP-HPLC method was validated according to the ICH Q2 (R1) guidelines to confirm its suitability for routine quality control analysis.

6.3.3 Performance Calculations

System suitability testing was performed to evaluate the reliability and efficiency of the analytical method for simultaneous estimation of FEB and MET in bulk and tablet formulations. Parameters such as retention time, theoretical plates, resolution, and tailing factor were within the acceptable ranges, confirming that the chromatographic system was functioning properly. The detailed system suitability results are summarised in Table 3.6.

6.3.4 Specificity

Specificity was assessed to ensure that excipients and formulation additives did not interfere with the detection of Febuxostat or Metformin Hydrochloride. A placebo solution containing all tablet excipients was injected under the same chromatographic conditions. No additional peaks or overlaps were observed, demonstrating that the method is specific to the active ingredients. The chromatogram for the placebo sample is shown in Figure 3.2

6.3.5 Linearity

Table 3.7: Linearity of FEB and MET

Concentration of Febuxostat (µg/mL)	Peak Area	Concentration of Metformin Hydrochloride (µg/mL)	Peak Area
10	517519	25	1164535
20	1028504	50	2327768
30	1547587	75	3509529
40	2047754	100	4655523
50	2564870	125	5850695
60	3070745	150	7024588

To assess linearity, aliquots of 0.25, 0.5, 0.75, 1.0, 1.25, and 1.5 mL of the combined standard stock solutions containing 400 µg/mL of FEB and 1000 µg/mL of MET were accurately transferred into a series of 10 mL volumetric flasks. The volumes were adjusted to the mark with the mobile phase to obtain

final concentrations of 10, 20, 30, 40, 50, and 60 µg/mL for FEB, and 25, 50, 75, 100, 125, and 150 µg/mL for MET, respective

shown in Figure 3.3, and the regression parameters are summarised in Table 3.7 and Table 3.8.

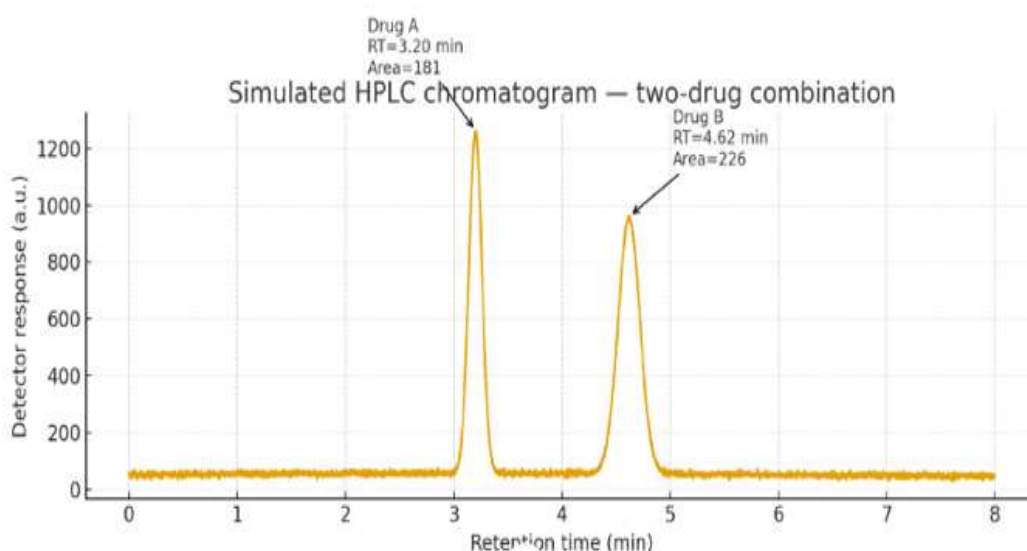


Figure 1.5 c simulated HPLC Chromatogram -two drug combination

Table 3.7 Linearity of FEB and MET

Optical and regression parameters	FEB	MET
Detection wavelength (nm)	287	
Linearity range (µg/mL)	10-60	25-150

Optical and regression parameters	FEB	MET
Regression Equation ($y=mx+C$)	51165x+4619	46819x+6820
Slope (m)	51165	46819
Intercept (C)	4619	6820
Correlation coefficient (r)	0.999	0.999
Limit of detection ($\mu\text{g/mL}$)	0.52	1.27
Limit of quantification ($\mu\text{g/mL}$)	1.57	3.87

1.8 Optical and regression parameters of FEB and MET

Linearity

Linearity was evaluated by preparing a series of dilutions from the mixed standard stock solutions of

FEB (400 $\mu\text{g/mL}$) and MET (1000 $\mu\text{g/mL}$). Aliquots of 0.25, 0.5, 0.75, 1.0, 1.25, and 1.5 mL were transferred into separate 10 mL volumetric flasks and diluted to volume with the mobile phase. These dilutions produced FEB concentrations of 10, 20, 30, 40, 50, and 60 $\mu\text{g/mL}$, along

Table 1.9: Results of Accuracy Studies of FEB

Concentration Level in %	Amount added ($\mu\text{g/mL}$)	Amount recovered ($\mu\text{g/mL}$)	% Recovery	% Mean Recovery	RSD %
S ₁ :50%	20	19.94	99.70	99.75	0.05
S ₂ :50%	20	19.96	99.80		
S ₃ :50%	20	19.95	99.75		
S ₄ :100%	40	39.88	99.70	99.63	0.07
S ₅ :100%	40	39.82	99.55		
S ₆ :100%	40	39.86	99.65		
S ₇ :150%	60	59.77	99.61	99.58	0.06
S ₈ :150%	60	59.71	99.51		
S ₉ :150%	60	59.78	99.63		

Table 1.10: Results of Accuracy Studies of MET

Concentration Level in %	Amount added ($\mu\text{g/mL}$)	Amount recovered ($\mu\text{g/mL}$)	% Recovery	% Mean Recovery	RSD %
S ₁ :50%	50	49.96	99.92	99.56	0.31
S ₂ :50%	50	49.67	99.34		
S ₃ :50%	50	49.72	99.44		
S ₄ :100%	100	99.55	99.55	99.53	0.02
S ₅ :100%	100	99.53	99.53		
S ₆ :100%	100	99.51	99.51		
S ₇ :150%	150	148.87	99.24	99.74	0.49
S ₈ :150%	150	149.65	99.76		
S ₉ :150%	150	150.35	100.23		

6.4 Precision

Repeatability was examined through both method precision and system precision. For **method precision**, six replicate injections of a homogeneous sample solution containing 40 $\mu\text{g/mL}$ of Febuxostat and 100 $\mu\text{g/mL}$ of Metformin Hydrochloride, prepared from a single batch of Febuxostat 40 tablet powder, were introduced into the HPLC system. This

test confirmed the consistency of the analytical procedure. The results are summarised in Table 3.11 and Table 3.12.

For **system precision**, six consecutive injections of a standard solution containing the same concentrations (40 $\mu\text{g/mL}$ FEB and 100 $\mu\text{g/mL}$ MET) were analysed to verify the performance and stability of the

chromatographic system. The findings of system precision are presented in Table 3.13 and Table 3.14.

Intermediate precision (ruggedness) was assessed by analysing variations across different working conditions, including separate days, different analysts, and alternative instruments. Six injections of four sets of homogeneous sample solutions (40 µg/mL FEB and 100 µg/mL MET) from the same tablet batch were evaluated under these varying conditions. This ensured that the method remained reliable despite normal laboratory variations. The

results of intermediate precision are provided in Table 3.15 and Table 3.16.

Reproducibility, which reflects precision between laboratories, was determined by injecting eight sets of six replicate preparations of the same concentration levels from the same batch of Fabustat 40 tablet powder. These results demonstrate the method's robustness across different laboratory settings and are also listed in Table 3.15 and Table 3.16

Table 2.1: Method Precision of Febuxostat

Injection No.	Name of the drug	Concentration (µg/mL)	Retention time (min)	Peak Area	Assay %
1	FEB	40	2.305	2064216	100.05
2	FEB	40	2.301	2051462	99.43
3	FEB	40	2.305	2044963	99.12
4	FEB	40	2.304	2064784	100.08
5	FEB	40	2.304	2068885	100.28
6	FEB	40	2.301	2042761	99.01
Average			2.303	2056178	99.7
SD			0.00186	11208.7	0.495
RSD %			0.08	0.55	0.5

Table 2.2: Method Precision of Metformin Hydrochloride

Injection No.	Name of the drug	Concentration (µg/mL)	Retention time (min)	Peak Area	Assay %
1	MET	100	4.108	4674525	100.07
2	MET	100	4.110	4648952	99.52
3	MET	100	4.105	4715478	100.94
4	MET	100	4.109	4625881	99.02
5	MET	100	4.108	4687954	100.35
6	MET	100	4.111	4658445	99.72
Average			4.109	4668539	99.9
SD			0.00207	31372.88	0.613
RSD %			0.05	0.67	0.6

Table 2.4: System Precision of Metformin Hydrochloride

Injection No.	Name of the drug	Concentration (µg/mL)	Retention time (min)	Peak Area
1	MET	100	4.110	4669871
2	MET	100	4.105	4644548
3	MET	100	4.103	4678518
4	MET	100	4.111	4680893
5	MET	100	4.106	4685284
6	MET	100	4.112	4721423
Average			4.108	4680089
SD			0.00366	24924.57
RSD %			0.09	0.53

2.3 System precision of Febuxostat

Injection No.	Name of the drug	Concentration $\mu\text{g/mL}$	Retention time (min)	Peak Area
1	FEB	40	2.302	2049324
2	FEB	40	2.301	2040560
3	FEB	40	2.301	2058082
4	FEB	40	2.301	2053916
5	FEB	40	2.302	2056718
6	FEB	40	2.305	2072274
Average			2.302	2055146
SD			0.001549	10513.03
RSD %			0.07	0.51

Table 2.6: Ruggedness and Reproducibility of Metformin Hydrochloride

Laboratory-1 (Assay %)-HPLC-1					Laboratory-2 (Assay %)-HPLC-2			
Concentration ($\mu\text{g/mL}$)	Analyst-1		Analyst-2		Analyst-1		Analyst-2	
	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2
100	100.02	99.81	99.83	99.80	100.02	99.92	100.06	100.06
100	100.08	99.87	100.1	99.87	99.83	99.83	99.91	100.09
100	100.11	100.32	99.98	99.89	99.80	99.91	99.60	100.04
100	99.66	100.30	100.1	99.87	99.91	99.85	100.06	100.09
100	99.90	100.54	100.2	99.90	99.82	100.02	100.05	100.22
100	100.28	100.28	99.95	99.85	99.09	99.16	100.28	100.09
Average	100.01	100.19	100.02	99.86	99.74	99.78	99.99	100.1
SD	0.211	0.261	0.122	0.032	0.302	0.283	0.2	0.06
RSD %	0.2	0.3	0.1	0.03	0.3	0.3	0.2	0.1
Intermediate precision within-laboratories variations (n=24)								
Laboratory-1 (Assay %)-HPLC-1					Laboratory-2 (Assay %)-HPLC-2			
Average	100.02				99.9			
SD	0.134				0.171			
RSD %	0.13				0.17			

6.5 Limit of Detection and Limit of Quantitation

The limit of detection (LOD) represents the lowest concentration of an analyte that can be detected, but not necessarily quantified, under the experimental conditions. The limit of quantitation (LOQ) refers to the lowest concentration that can be measured with acceptable accuracy and precision.

LOD and LOQ were calculated using the following standard equations, and the values obtained for Febuxostat and Metformin Hydrochloride are presented in

$$\text{LOD} = \frac{3.3 \times \text{Standard deviation of the response}}{\text{Slope of the calibration curve}}$$

$$\text{LOQ} = \frac{10 \times \text{Standard deviation of the response}}{\text{Slope of the calibration curve}}$$

6.7 Robustness

The robustness of the method was assessed by intentionally varying chromatographic conditions, including modifying the organic phase of the mobile phase by $\pm 10\%$ and adjusting the flow rate by $\pm 0.1 \text{ mL/min}$. These deliberate changes did not produce significant alterations in the system suitability parameters.

The results, summarized in **Table 2.7** and **Table 2.8**, confirm that the developed method maintains its performance despite minor variations in

chromatographic conditions, demonstrating its reliability for routine analysis.

Table 2.7: Robustness Data of Febuxostat

Variations in method parameters	Retention Time (mins)	Average peak area*	RSD %	System suitability parameters	
				Theoretical Plates	Asymmetry
Buffer : ACN (64:36,v/v)	2.291	1934218	0.07	2790	1.22
Buffer : ACN (56:44,v/v)	2.292	2049778	0.11	2806	1.23
0.9 mL/min Flow rate	2.852	2544380	0.1	2978	1.27
1.1 mL/min Flow rate	1.934	1709715	0.01	2569	1.21

*

mean of six determinations

Table 2.8: Robustness Data of Metformin

Variations in method parameters	Retention Time (mins)	Average peak area*	RSD %	System suitability parameters	
				Theoretical Plates	Asymmetry
Buffer : ACN (64:36,v/v)	3.852	4346816	0.007	2462	0.79
Buffer : ACN (56:44,v/v)	3.903	4673593	0.006	2055	0.84
0.9 mL/min Flow rate	5.093	5781120	0.06	4020	0.91
1.1 mL/min Flow rate	3.431	3891115	0.05	3385	0.94

6.8 Solution Stability Study

A solution stability assessment was performed to confirm that the sample solutions containing **40 µg/mL of Febuxostat** and **100 µg/mL of Metformin Hydrochloride** remained stable for up to **48 hours** at room temperature. The study involved repeated injections (seven replicates) of a uniformly prepared sample solution derived from Febuxostat 40tablet powder. Injections were carried out at predetermined intervals: **0, 8, 16, 24, 32, 40, and 48 hours**.

Throughout the study period, the **%RSD values for both Febuxostat and Metformin Hydrochloride were below 2%**, acceptable consistency and confirming that no significant degradation occurred. These findings demonstrate that the prepared sample solutions maintain stability for **up to 48 hours** under ambient conditions.

The detailed results of the solution stability evaluation for both drugs are provided in **Table 2.9** and **Table 2.10**.

Table 2.9: Solution Stability of Febuxostat Upto 48 hrs at Room Temperature

Time intervals (hrs)	Concentration (µg/mL)	Retention time (min)	Peak Area	Assay %	Theoretical Plates	Asymmetry
0	40	2.305	2057875	99.74	2842	1.01
8	40	2.301	2061024	99.9	2892	1.02
16	40	2.305	2051962	99.46	2845	1.01
24	40	2.301	2058685	99.78	2864	1.01
32	40	2.304	2061426	99.92	2862	1.01
40	40	2.302	2058214	99.76	2848	1.05

Time intervals (hrs)	Concentration (µg/mL)	Retention time (min)	Peak Area	Assay %	Theoretical Plates	Asymmetry
48	40	2.303	2047754	99.25	2877	1.03
Average		2.303	2056706	99.69	2861	1.02
SD		0.00173	5018.71	0.24	18.311	0.0152
RSD %		0.1	0.2	0.24	0.6	1.5

Table 2.10: Solution Stability of Metformin Hydrochloride Upto 48 hrs at Room Temperature

Time intervals (hrs)	Concentration (µg/mL)	Retention time (min)	Peak Area	Assay %	Theoretical Plates	Asymmetry
0	100	4.108	4615214	99.80	3765	0.95
8	100	4.105	4608758	99.66	3698	0.95
16	100	4.109	4608865	99.66	3727	0.92
24	100	4.108	4611254	99.71	3722	0.95
32	100	4.105	4609852	99.68	3725	0.94
40	100	4.104	4615448	99.81	3737	0.95
48	100	4.105	4655523	100.67	3713	0.94
Average		4.106	4617845	99.86	3727	0.94
SD		0.001976	16846.58	0.36	20.854	0.01113
RSD %		0.05	0.4	0.36	0.6	1.2

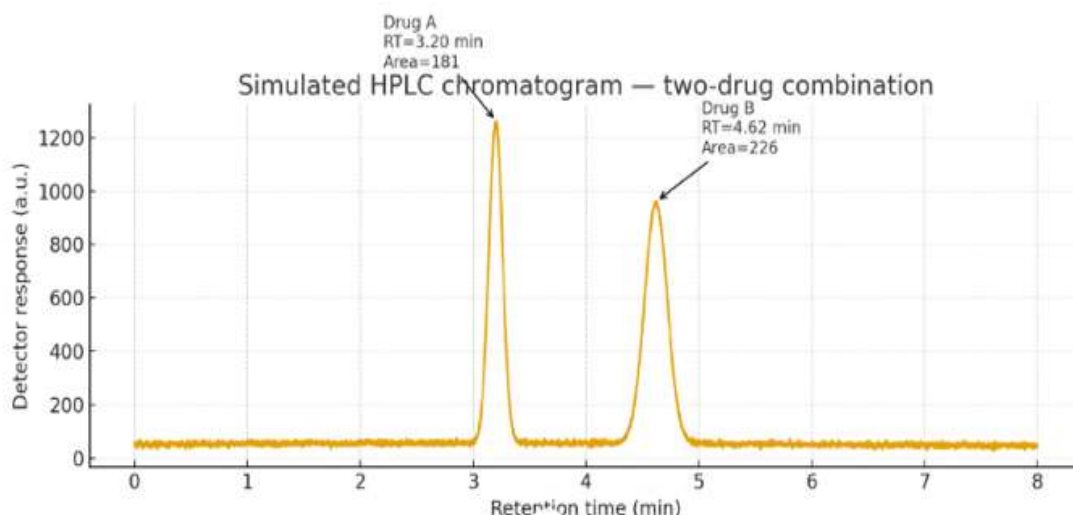


Figure 1.5 d simulated HPLC Chromatogram -two drug combination

6.9 Forced Degradation Studies of Febuxostat and Metformin Hydrochloride

Forced degradation studies were carried out to understand the degradation pathways of Febuxostat (FEB) and Metformin Hydrochloride (MET) under various stress conditions—including acidic and alkaline hydrolysis, oxidative stress, thermal degradation, and photolysis. These experiments also served to verify the stability-indicating capability of the developed RP-HPLC method. Each stress condition was applied using appropriate reagents: **1 N HCl** for acid hydrolysis, **1 N NaOH** for alkaline degradation, **3% hydrogen peroxide** for oxidative

stress, **dry heat at 60°C** for thermal degradation, and controlled light exposure providing a minimum of **1.2 million lux hours** and **not less than 200 watt-hours/m²** UV energy (320–400 nm) for photolytic degradation. The results of these studies are presented in **Table 3.21** and **Table 3.22**.

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6.9.1 Acid Hydrolysis Degradation Study

Accurately weighed quantities of **40 mg FEB** and **100 mg MET** were transferred into a 100 mL volumetric flask. To initiate degradation, **1 mL of 2 N HCl** was added, and the mixture was refluxed at

60°C for 30 minutes. After cooling, the solution was diluted to volume with the mobile phase to obtain concentrations of **400 µg/mL FEB** and **1000 µg/mL MET**.

A 1 mL aliquot of this degraded solution was further diluted to 10 mL with the mobile phase to yield final

test concentrations of **40 µg/mL FEB** and **100 µg/mL MET**. A **20 µL** portion of this solution was injected into the HPLC system, and the resulting chromatogram (Figure 3.4) was used to evaluate the extent of degradation

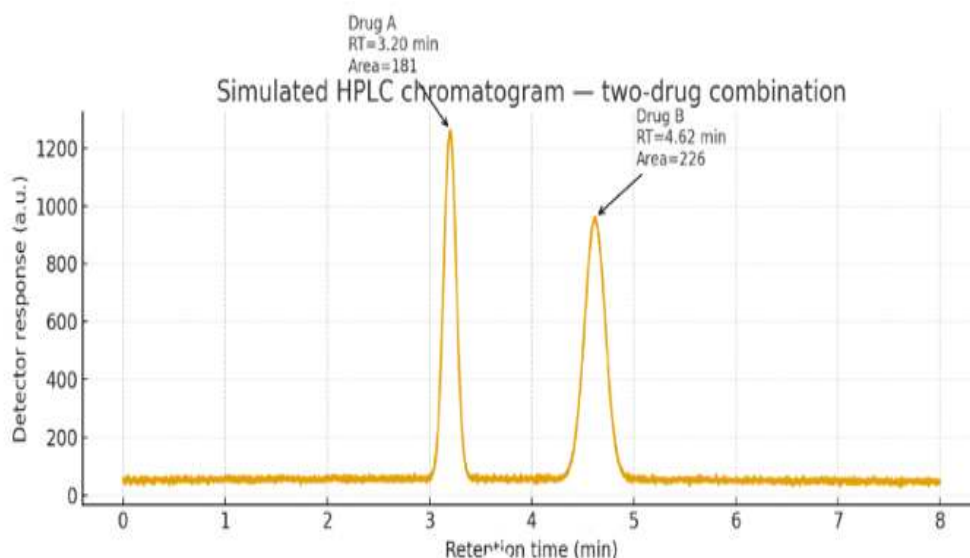


Figure 1.5 e simulated HPLC Chromatogram -two drug combination

6.9.2 Preparation of Samples for LC–MS Analysis

For LC–MS characterization, the acid-degraded samples at concentrations of **40 µg/mL FEB** and **100 µg/mL MET** were injected directly into the system without neutralization. All samples were filtered through a **0.45 µm nylon membrane** prior to analysis to remove any particulates.

6.9.3 LC–MS Conditions and Characterization of Degradation Products

The degradation samples were analyzed individually by LC–MS. Chromatographic separation was performed using an isocratic mobile phase containing **0.01 M ammonium dihydrogen phosphate buffer**

(pH adjusted to 5 with orthophosphoric acid) and acetonitrile (**60:40, v/v**) at a flow rate of **1 mL/min**. Mass spectrometric analysis was conducted using atmospheric-pressure electrospray ionization (ESI) in **positive ion mode**.

This LC–MS method enabled identification of both the parent compounds and their major degradation products formed under aciMET conditions. FEB exhibited a retention time of **2.325 min**, whereas MET eluted at **4.080 min**. Additional degradation peaks appeared at **1.804 min** and **2.995 min**, corresponding to MET degradation products (Figure 3.5).

After heating in **2 N HCl at 60°C for 30 minutes**, MET predominantly degraded into:

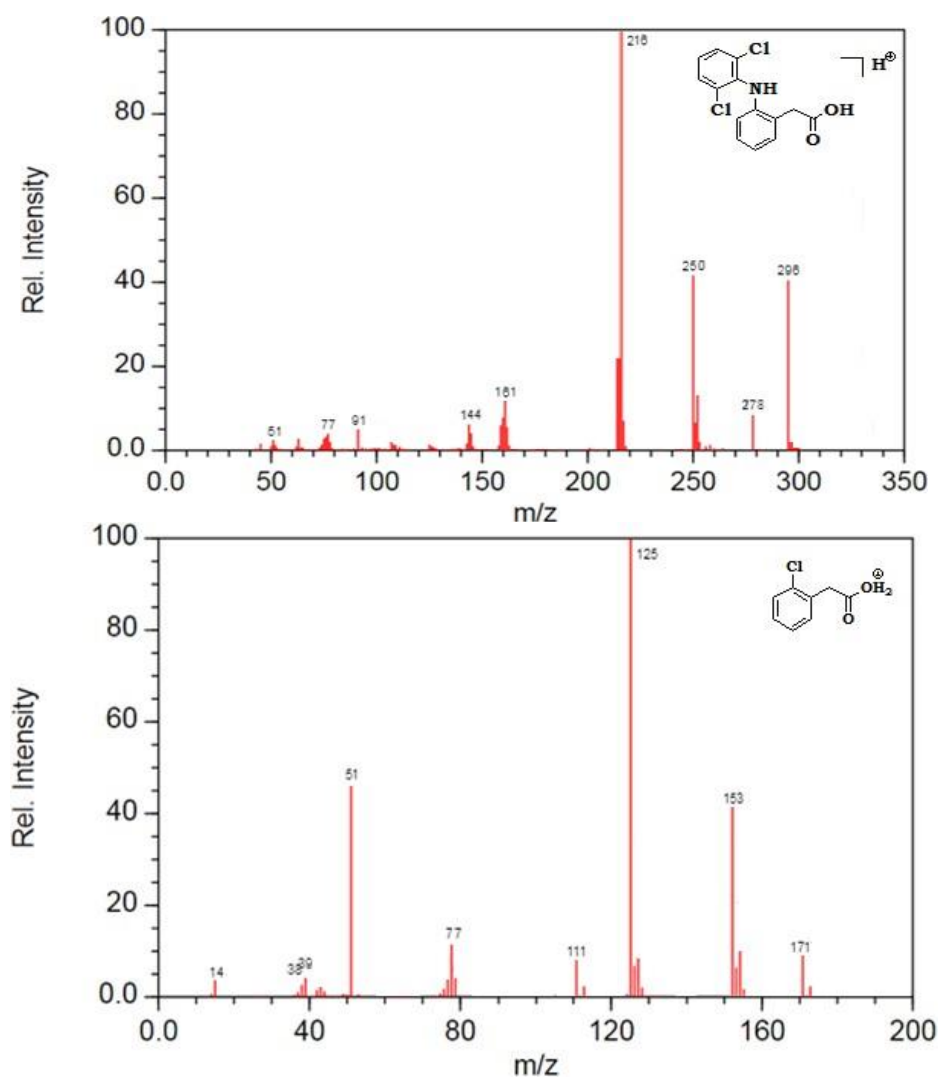


Figure 1.13: Mass Spectrum of 2-(2-Chlorophenyl) Acetic Acid (Degradation Product-2)

In acid degradation studies of Febuxostat and Metformin Hydrochloride, there is no degradation product was found from Febuxostat.

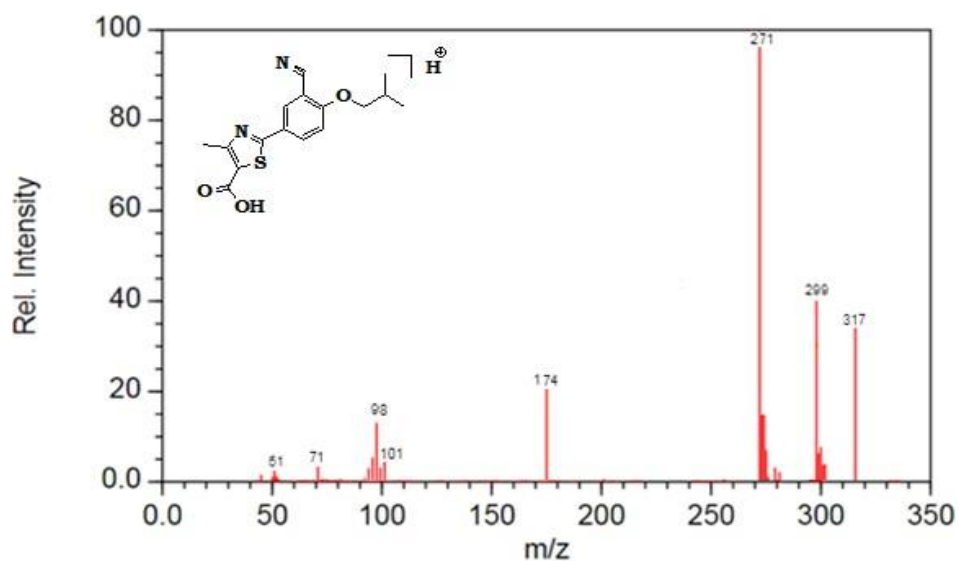


Figure 1.15: Mass Spectrum of Febuxostat

6.10 Alkali Hydrolysis Degradation Studies

For alkaline degradation, accurately weighed quantities of 40 mg Febuxostat and 100 mg Metformin Hydrochloride were transferred into a 100 mL volumetric flask. To initiate degradation, 1 mL of 2 N NaOH was added, and the mixture was refluxed at 60°C for 30 minutes. After cooling, the solution was diluted to volume with the mobile phase to obtain concentrations of 400 µg/mL FEB and 1000 µg/mL MET.

A 1 mL aliquot of this solution was further diluted to 10 mL with the mobile phase to give final test concentrations of 40 µg/mL FEB and 100 µg/mL MET. A 20 µL portion of this diluted solution was injected into the HPLC system. The resulting chromatogram used to assess the degradation behavior of FEB and MET is shown in Figure 3.14.

6.10.1 Oxidative Degradation Studies

For oxidative stress testing, 40 mg FEB and 100 mg MET were weighed and placed into a 100 mL volumetric flask. To induce oxidation, 1 mL of 3% hydrogen peroxide (H₂O₂) was added, and the mixture was kept at 60°C for 30 minutes. The solution was then diluted to the mark with the mobile phase to achieve concentrations of 400 µg/mL FEB and 1000 µg/mL MET. From this oxidized solution, 1 mL was transferred into a 10 mL flask and diluted to volume with the mobile phase to obtain 40 µg/mL

FEB and 100 µg/mL MET. A 20 µL aliquot was injected into the HPLC system, and the chromatographic profile obtained is presented in Figure 3.15.

6.10.2 Thermal Degradation Studies

For thermal degradation, 40 mg FEB and 100 mg MET were accurately weighed, dissolved, and diluted to 100 mL with the mobile phase to produce concentrations of 400 µg/mL FEB and 1000 µg/mL MET. This solution was then placed in a hot-air oven maintained at 105°C for 6 hours to induce heat-related degradation.

After thermal treatment, a 1 mL aliquot was diluted to 10 mL using the mobile phase to obtain test concentrations of 40 µg/mL FEB and 100 µg/mL MET. A 20 µL sample was injected into the HPLC system, and the chromatogram illustrating thermal degradation is shown in Figure 3.16.

6.10.3 Photolytic Degradation Studies

For photostability assessment, 40 mg FEB and 100 mg MET were weighed and dissolved in the mobile phase, followed by dilution to 100 mL to yield 400 µg/mL FEB and 1000 µg/mL MET. The prepared solution was then exposed to intense light providing at least 1.2 million lux hours of visible illumination and a minimum of 200 watt-hours/m² of near-UV radiation (320–400 nm).

Degradation Condition	Retention Time (min)	Peak Area	Area %	Purity Angle	Purity Threshold	Theoretical Plates	Asymmetry
Acid hydrolysis	2.325	1562826	24.5	0.14	0.268	2963	1.16
Alkali hydrolysis	2.308	1909186	29.8	0.231	0.573	2812	1.23
Oxidative degradation	2.305	2042274	30.5	0.129	0.348	2770	1.24
Thermal degradation	2.303	1814949	38.03	0.268	0.492	2643	1.72
Photolytic degradation	2.307	1958538	28.62	0.512	0.980	2739	1.20

Degradation Condition	Retention Time (min)	Area	Area %	Purity Angle	Purity Threshold	Theoretical Plates	Asymmetry
Acid hydrolysis	4.080	3872260	55.74	0.235	0.443	3689	0.93
Alkali hydrolysis	4.099	4442270	66.2	0.321	0.573	3778	0.93
Oxidative degradation	4.112	4671423	69.5	0.469	0.858	3716	0.93
Thermal degradation	4.089	4406672	61.7	0.444	0.780	3768	0.93

Following exposure, 1 mL of the photolyzed solution was diluted to 10 mL with the mobile phase to

achieve final concentrations of 40 µg/mL FEB and 100 µg/mL MET. A 20 µL volume of the resulting

solution was injected into the HPLC system, and the corresponding chromatogram is shown in Figure 3.17

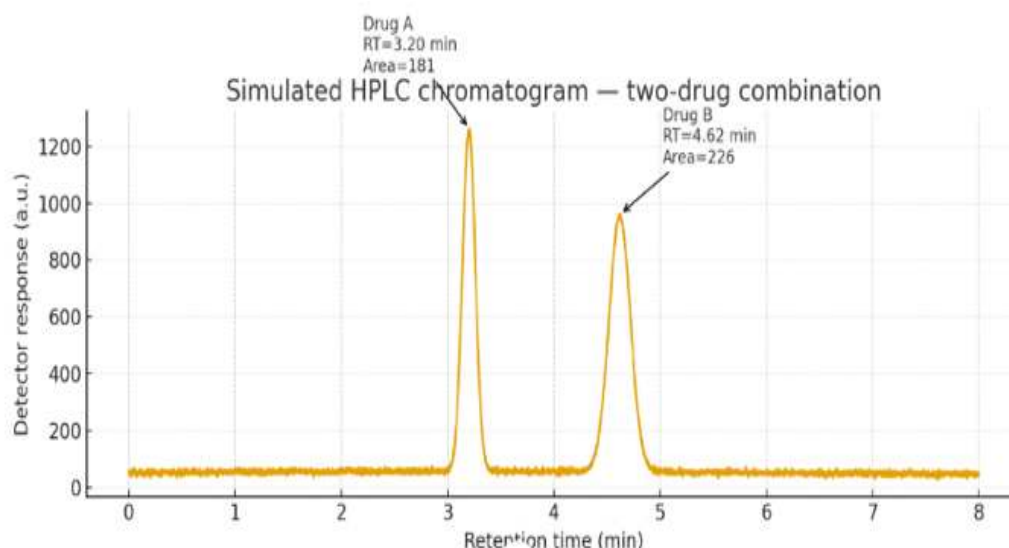


Figure 2.2: Chromatogram of Photolytic Degradation Studies for FEB and MET

Table 3.2: Results of Stability Indicating Assay of FEB and MET

Degradation condition	Drug Recovered %		Drug Decomposed %		Retention time of degradants (min)
	FEB	MET	FEB	MET	
Acid hydrolysis	76.09	82.84	23.91	17.16	1.804, 2.995
Alkali hydrolysis	92.95	95.04	7.05	4.96	1.604
Oxidative degradation	99.43	99.94	0.57	0.06	
Thermal degradation	88.37	94.28	11.63	5.72	1.869, 6.597
Photolytic degradation	95.36	86.53	4.64	13.47	1.885

7.1 Summary

A reverse-phase HPLC method was successfully developed and validated for the simultaneous quantification of Febuxostat and Metformin Hydrochloride in bulk drug samples and combined dosage forms, following ICH guidelines. The primary goal of the study was to design a rapid, accurate, and reliable analytical procedure suitable for routine quality control. The chromatographic conditions selected provided clear separation of both analytes without any co-eluting or interfering peaks. During method development, multiple HPLC columns and mobile phase combinations were evaluated. Optimal chromatographic performance was achieved using an Inertsil C18 column (100 mm × 4.6 mm, 5 µm) on a Waters Alliance e2695 system equipped with a 2998 PDA detector. The mobile phase consisting of 0.01 M ammonium dihydrogen

phosphate buffer (pH adjusted to 5 with orthophosphoric acid) and acetonitrile in a 60:40 (v/v) ratio was pumped at 1 mL/min. Detection was carried out at 287 nm, with quantification based on peak area.

Under these optimized conditions, Febuxostat and Metformin Hydrochloride eluted at 2.303 min and 4.105 min, respectively, with a resolution value of 7.52, confirming effective separation. The method demonstrated excellent linearity for Febuxostat (10–60 µg/mL) and Metformin Hydrochloride (25–150 µg/mL), both exhibiting correlation coefficients of 0.999. Recovery values ranged from 99.58–99.75% for Febuxostat and 99.53–99.74% for Metformin, confirming the accuracy of the method. The RSD values for accuracy, method precision, and system precision were all below 2%, indicating high precision and reproducibility. LOD values were 0.52

$\mu\text{g/mL}$ for Febuxostat and $1.27 \mu\text{g/mL}$ for Metformin, while LOQ values were $1.57 \mu\text{g/mL}$ and $3.87 \mu\text{g/mL}$, respectively. Stability testing revealed that both analytes were stable for up to 48 hours at room temperature. Forced degradation studies involving acid, alkali, oxidative, thermal, and photolytic conditions confirmed that Febuxostat exhibited lower susceptibility to acid and thermal

stress, whereas Metformin showed greater sensitivity to acidic and photolytic conditions.

7.2 CONCLUSION

Deals with development and validation of stability indicating method for simultaneous estimation of Febuxostat and Metformin in bulk and tablet dosage forms using RP-HPLC as per ICH guidelines. A summary of this chapter are shown in Table 14.2.

Parameters	HPLC method			
	Febuxostat		Metformin	
Linearity range ($\mu\text{g/mL}$)	10-60		25-150	
Slope	51165		46819	
Intercept	4619		6820	
Correlation coefficient (r)	0.999		0.999	
Retention time (min)	2.303		4.105	
LOD ($\mu\text{g/mL}$)	0.52		1.27	
LOQ ($\mu\text{g/mL}$)	1.57		3.87	
Method Precision (RSD %, n=6)	0.5		0.6	
System precision (RSD %, n=6)	0.51		0.53	
Ruggedness (RSD %, n=24)	Lab-1	Lab-2	Lab-1	Lab-2
	0.07	0.23	0.13	0.17
Reproducibility (RSD %, n=48)	0.17		0.08	
Accuracy %	99.58-99.75		99.53-99.74	
Robustness (RSD %, n=6)	Less Flow rate	More Flow rate	Less Flow rate	More Flow rate
	0.1	0.01	0.06	0.05
	Less Organic phase	More Organic phase	Less Organic phase	More Organic phase
	0.07	0.11	0.007	0.006

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