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RP-HPLC Method Development and Validation for Simultaneous Estimation of Doxofylline and Montelukast in Pharmaceutical Dosage Form

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ABSTRACT

A simple, precise, accurate, and robust reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the simultaneous estimation of Doxofylline and Montelukast in combined pharmaceutical dosage forms. The primary objective was to establish a stability-indicating method capable of separating the active pharmaceutical ingredients from their degradation products under various stress conditions. Chromatographic separation was achieved using a Discovery C18 column (250 mm × 4.6 mm, 5 µm) with a mobile phase of acetonitrile and 0.01 potassium dihydrogen phosphate (50:50, v/v) at a flow rate of 1.0 mL/min, and detection at 240 nm. The method was validated as per ICH Q2(R1) guidelines and demonstrated excellent linearity (R^2 = 0.999) in the concentration ranges of 40–240 µg/mL for Doxofylline and 1–6 µg/mL for Montelukast. The LOD and LOQ were found to be 1.26 and 3.81 µg/mL for Doxofylline and 0.01 and 0.02 µg/mL for Montelukast, respectively. Precision, accuracy, and robustness studies confirmed the method's reliability. Forced degradation studies under acid, alkali, oxidative, thermal, photolytic, and neutral conditions showed no interference at the analyte retention times, confirming the method's stability-indicating capability. The validated method is suitable for routine quality control and stability testing of combined Doxofylline and Montelukast formulations in the pharmaceutical industry.

Keywords: *Doxofylline, Montelukast, RP-HPLC, method validation, stability-indicating method, forced degradation, ICH Q2(R1), pharmaceutical analysis.*

1. INTRODUCTION

The quality and efficacy of pharmaceutical products are critically dependent on precise and reliable analytical methods, especially for combination therapies widely employed in respiratory disorders such as asthma and chronic obstructive pulmonary disease (COPD). Doxofylline, a bronchodilator, and Montelukast, a leukotriene receptor antagonist, are frequently co-administered in fixed-dose formulations due to their complementary mechanisms in managing airway inflammation and bronchoconstriction [1]. The simultaneous estimation of these drugs in pharmaceutical dosage forms is

essential for quality control, ensuring therapeutic efficacy, and regulatory compliance. Among the various analytical techniques, reverse-phase high-performance liquid chromatography (RP-HPLC) remains the method of choice due to its superior sensitivity, selectivity, accuracy, and reproducibility. Although several individual and combination assay methods have been reported, limitations such as longer run times, poor peak resolution, and inadequate stability-indicating capability necessitate the development of a novel, rapid, and validated RP-HPLC method [2]. Therefore, the present study aims to develop and validate a stability-indicating RP-HPLC method for the simultaneous estimation of Doxofylline and Montelukast in bulk and pharmaceutical formulations in accordance with ICH Q2(R1) guidelines, ensuring the method's suitability for routine quality control applications [3].

2. MATERIALS AND METHODS

Chemicals and Reagents

Doxofylline and Montelukast pure drug substances (API) were procured from Spectrum Labs. A marketed tablet formulation containing combination of drug was sourced from the local market. HPLC-grade solvents including acetonitrile, methanol, and water, along with A.R grade potassium dihydrogen phosphate and orthophosphoric acid, were obtained from Rankem. All other reagents used were of analytical grade.

Instrumentation

The chromatographic analysis was performed on a Waters Alliance 2695 HPLC system equipped with a PDA detector. Separation was achieved on a Discovery C18 column (250 mm × 4.6 mm, 5 µm). Other equipment included a UV-Visible spectrophotometer (Microprocessor UV-Visible Single Beam), analytical balance (Sortorious, Scaletec BSA224s-CW), ultrasonicator (Lab Man), pH meter (Lab Man), hot air oven (Sisco), and vortex mixer (Remi).

Chromatographic Conditions

The optimized mobile phase consisted of acetonitrile and 0.01N potassium dihydrogen phosphate buffer (50:50, v/v). The flow rate was set at 1.0 ml/min, with a detection wavelength of 240 nm. The injection volume was 10 µl, and the column temperature was maintained at 30°C. The total run time was 5 minutes. A diluent comprising acetonitrile and water in the ratio of 50:50 (v/v) was used throughout the analysis [4, 5].

Preparation of Standard and Sample Solutions

Standard Stock Solutions

Accurately weighed 2 mg of Montelukast and 80 mg of Doxofylline were transferred into a 50 mL volumetric flask, dissolved in 3/4th volume of diluent, sonicated for 10 minutes, and made up to volume with diluent. (Concentration: 40 µg/ml Montelukast, 1600 µg/ml Doxofylline) [6].

Standard Working Solutions 1 mL of each stock solution was diluted to 10 ml with diluent to obtain final concentrations of 4 µg/ml Montelukast and 160 µg/ml Doxofylline [7].

Sample Stock Solutions

Ten tablets were weighed, powdered, and a quantity equivalent to one tablet was transferred to a 100 ml volumetric flask. About 25 ml of diluent was added, sonicated for 25 minutes, diluted to volume with diluent, and filtered through HPLC filter [8].

Sample Working Solutions

0.4 ml of the filtered sample stock solution was transferred to a 10 ml volumetric flask and diluted to volume with diluent.

Method Validation

System Suitability: Six replicate injections of standard solution were assessed for retention time, theoretical plates, tailing factor, and resolution.

Specificity: Blank, placebo, and sample solutions were analysed to check for interference at the retention times of Doxofylline and Montelukast.

Linearity: Calibration curves were constructed at six concentration levels for both drugs in the range of 40–240 µg/ml for Doxofylline and 1–6 µg/ml for Montelukast.

Precision: System precision, method precision (intra-day), and intermediate precision (inter-day) were evaluated by calculating %RSD for replicate injections.

Accuracy: Recovery studies were performed at 50%, 100%, and 150% levels by standard addition method.

Limit of Detection (LOD) and Limit of Quantification (LOQ): Determined based on the standard deviation of the response and the slope of the calibration curve.

Robustness: The effect of small deliberate changes in flow rate, mobile phase composition, and column temperature on system suitability parameters was evaluated.

Specificity/Selectivity: Confirmed by analysing degradation samples and checking for separation of degradation products from drug peaks [9, 10, 11].

3. RESULTS AND DISCUSSION

Method Development and Optimization

Multiple chromatographic conditions were optimized by varying the mobile phase composition and column parameters. Trials using different ratios of methanol, acetonitrile, and buffer resulted in suboptimal peak shapes and retention times. The final optimized method utilized a mobile phase of acetonitrile and 0.01N potassium dihydrogen phosphate (50:50, v/v), achieving sharp, well-resolved peaks at retention times of 2.241 min for Doxofylline and 2.955 min for Montelukast with satisfactory theoretical plate counts and tailing factors. The optimized chromatogram illustrating this separation is shown in Figure 1.

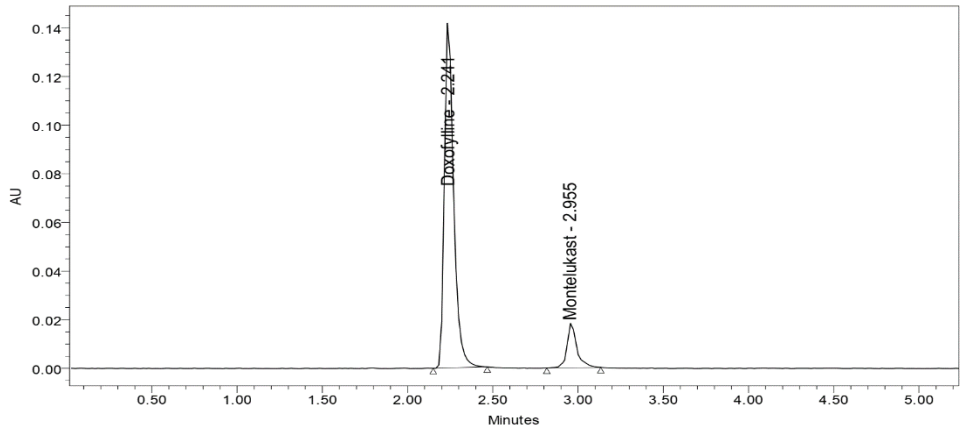

Figure 1. Optimized chromatogram showing separation of Doxofylline and Montelukast

System Suitability

The system suitability parameters such as retention time, USP plate count, tailing factor, and resolution were evaluated by six replicate injections of the standard solution. The results are shown in **Table 1**, confirming all parameters were within acceptable limits as per ICH Q2(R1) guidelines. Representative system suitability chromatograms are presented in Figure 2.

Table 1 System suitability parameters for Doxofylline and Montelukast

S. No.	Doxofylline RT (min)	USP Plate Count	Tailing	Montelukast RT (min)	USP Plate Count	Tailing	Resolution (Rs)
1	2.255	7583	1.21	2.863	10998	1.19	6.1
2	2.258	7573	1.25	2.865	10221	1.20	6.1
3	2.258	7601	1.22	2.867	10250	1.21	6.0
4	2.266	7526	1.21	2.871	10767	1.20	6.1
5	2.270	7772	1.23	2.872	9393	1.21	6.1
6	2.271	7768	1.24	2.874	10201	1.20	6.0

Specificity The method's specificity was assessed by comparing chromatograms of blank, placebo, and sample solutions. No interfering peaks were observed at the retention times of Doxofylline and Montelukast, confirming the method's selectivity. The chromatograms for blank, placebo, and typical sample are presented in Figures 2-4.

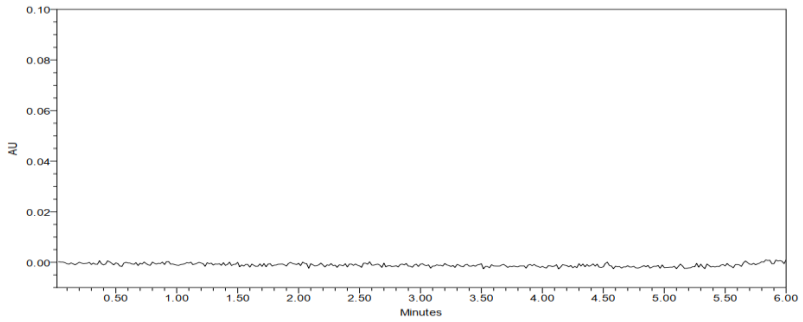

Figure 2. Chromatogram of Blank.

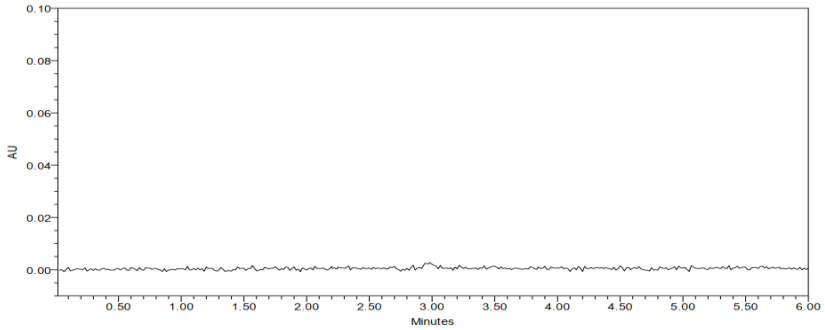

Figure 3. Chromatogram of Placebo

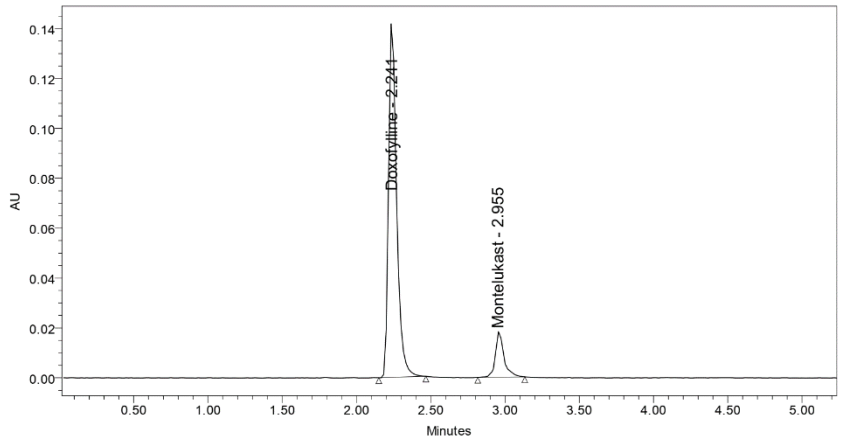

Figure 4. Typical sample chromatogram showing peaks of Doxofylline and Montelukast.

Linearity

The method demonstrated excellent linearity in the concentration ranges of 40–240 µg/ml for Doxofylline and 1–6 µg/ml for Montelukast. The calibration curves for both drugs showed a strong correlation between peak area and concentration. The regression equation for Doxofylline was found to be $y = 3085.1x + 2556.6$ and for Montelukast, $y = 36630x + 263.61$. The correlation coefficient (R^2) for both drugs was 0.999, indicating excellent linearity across the specified concentration ranges. The linearity data obtained for both Doxofylline and Montelukast are summarized in Table 2, and the corresponding calibration curves are depicted in Figure 5.

Table 2. Linearity data for Doxofylline and Montelukast

Doxofylline Conc. (µg/ml)	Peak Area	Montelukast Conc. (µg/ml)	Peak Area
0	0	0	0
40	126403	1	36808
80	252357	2	73630
120	374175	3	111488
160	493928	4	146069
200	621002	5	182421
240	741513	6	220656

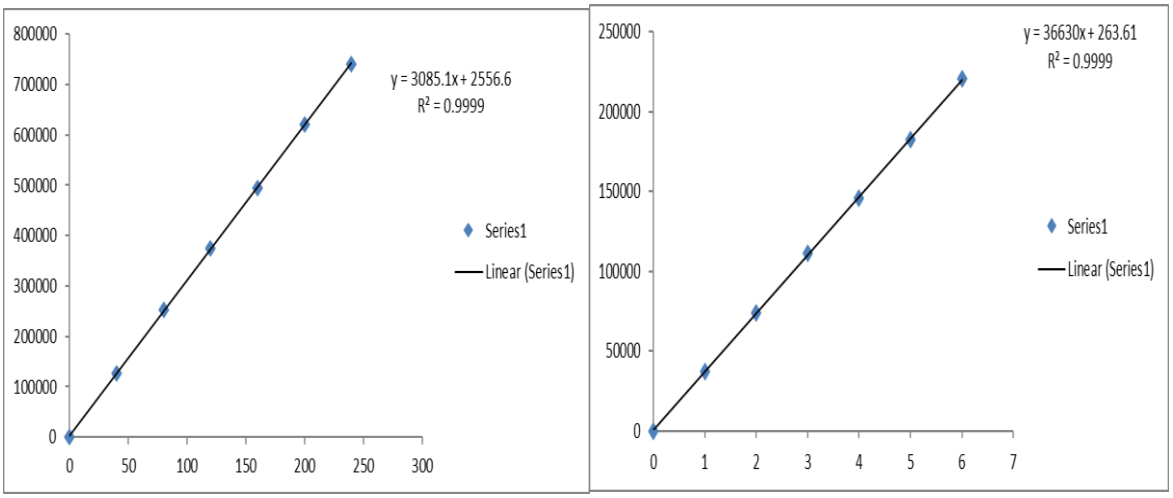

Figure 5. Calibration curves of Doxofylline and Montelukast.

Precision

For system precision, six replicate injections of the standard solution were analysed, and the %RSD was found to be 1.0% for Doxofylline and 0.7% for Montelukast, indicating excellent instrument repeatability (Table 3). Method precision was assessed by preparing and analysing six individual sample solutions under the same conditions on the same day. The %RSD values obtained were 0.4% for Doxofylline and 0.3% for Montelukast (Table 3), confirming good repeatability of the method. Intermediate precision was determined on a different day by a different analyst using the same method. The %RSD values were 0.7% for Doxofylline and 1.0% for Montelukast, demonstrating good reproducibility of the method (Table 3).

Table 3. System precision, method precision, and intermediate precision data

Type	S. No	Doxofylline Area	Montelukast Area
System Precision	**1**	505454	145518
	2	504132	146654
	3	495445	148276
	4	494780	147595
	5	503366	145789

	6	504350	146519
	%RSD	1	0.7
Method Precision	**1**	498403	146894
	2	503896	146196
	3	501089	145912
	4	499167	147136
	5	499692	146758
	6	501951	146240
	%RSD	0.4	0.3
Intermediate Precision	**1**	499209	146473
	2	493839	144651
	3	492329	144423
	4	491436	147863
	5	497408	147294
	6	498471	146916
	%RSD	0.7	1

Accuracy

The accuracy of the developed method was assessed by the recovery study using the standard addition method at three concentration levels: 50%, 100%, and 150% of the test concentration. At each level, three determinations were performed. The mean percentage recoveries were found to be within acceptable limits, demonstrating the method's accuracy for both analytes. The detailed accuracy data are provided in Table 4. The overall mean percentage recoveries were 99.80% for Doxofylline and 99.91% for Montelukast, confirming the accuracy of the method.

Table 4. Accuracy data for Doxofylline and Montelukast

% Level	Doxofylline Amount Spiked (µg/ml)	Amount Recovered	% Recovery	Montelukast Amount Spiked (µg/ml)	Amount Recovered	% Recovery
50%	80	80.0	100.0	2	1.99	99.75
	80	79.4	99.2	2	2.00	99.88
	80	79.7	99.6	2	1.98	99.05
100%	160	160.5	100.3	4	3.99	99.86
	160	159.6	99.8	4	3.97	99.15
	160	160.7	100.5	4	4.02	100.52
150%	240	240.4	100.2	6	5.97	99.56
	240	237.7	99.0	6	6.01	100.22

	240	239.1	99.6	6	6.07	101.19
Mean% Recovery	99.80			99.91		

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The sensitivity of the method was determined in terms of LOD and LOQ for both Doxofylline and Montelukast, based on the standard deviation of the response and the slope of the calibration curve. The calculated LOD and LOQ values are shown in Table 5. The low values confirm the method's high sensitivity for both analytes.

Table 5. LOD and LOQ values for Doxofylline and Montelukast

Molecule	LOD (µg/ml)	LOQ (µg/ml)
Doxofylline	1.26	3.81
Montelukast	0.01	0.02

Robustness

The robustness of the method was evaluated by deliberately varying chromatographic parameters, including flow rate (±0.1 ml/min), mobile phase composition (±5%), and column temperature (±3-°C). The %RSD values for both Doxofylline and Montelukast remained within acceptable limits under all altered conditions, indicating method robustness. The detailed results are presented in Table 6.

Table 6. Robustness data for Doxofylline and Montelukast

Condition	%RSD of Doxofylline	%RSD of Montelukast
Flow rate (-) 0.9 mL/min	0.6	0.2
Flow rate (+) 1.1 mL/min	0.5	1.9
Mobile phase (-) 45B:55A	0.7	1.5
Mobile phase (+) 55B:45A	0.3	0.2
Temperature (-) 27°C	0.5	0.8
Temperature (+) 33°C	0.7	1.3

Assay studies- Bearing the label claims Montelukast 10mg, Doxofylline 400mg. Assay was performed with the above formulation. Average % Assay for Doxofylline and Montelukast obtained was 99.69%and 99.66% respectively

Table 6. Assay study for Doxofylline and Montelukast

	Doxofylline			**Montelukast**		
S.no	Std Area	Sample area	% Assay	Std Area	Sample area	% Assay
1	505454	498403	99.23	145518	146894	99.91
2	504132	503896	100.33	146654	146196	99.44
3	495445	501089	99.77	148276	145912	99.25
4	494780	499167	99.38	147595	147136	100.08
5	503366	499692	99.49	145789	146758	99.82

6	504350	501951	99.94	146519	146240	99.47
Avg	501255	500700	99.69	146725	146523	99.66
Std Dev.	4808.9	2028.5	0.40	1052.9	475.2	0.32
%RSD	1.0	0.4	0.4	0.7	0.3	0.32

Degradation studies-Regarding the pH adjustment in mobile phase for the acid and base degradation studies have movement in retention time of drugs. But due to neutralized acid sample with 2N Base solution and base sample with 2N Acid solution there will be no change in retention time

Table 7. Degradation studies for Doxofylline and Montelukast

Type of degradation	**Doxofylline**			**Montelukast**		
	Area	% Recovered	% Degraded	Area	% Recovered	% Degraded
Acid	481419	95.85	4.15	140626	95.58	4.42
Base	468348	93.25	6.75	137866	96.38	3.62
Peroxide	468884	93.36	6.64	136747	96.81	3.19
Thermal	487790	97.12	2.88	144446	98.22	1.78
UV	495347	98.62	1.38	144572	99.06	0.94
Water	498240	99.20	0.80	145781	99.71	0.29

4. CONCLUSION

A simple, accurate, precise, and robust RP-HPLC method was successfully developed and validated for the simultaneous estimation of Doxofylline and Montelukast in pharmaceutical dosage forms. The method demonstrated excellent linearity, precision, accuracy, sensitivity, specificity, and robustness in accordance with ICH Q2(R1) guidelines. Forced degradation studies confirmed the stability-indicating capability of the method, as all degradation products were well-resolved and showed no interference at the retention times of the analytes under acid, alkali, oxidative, thermal, photolytic, and neutral conditions. The method exhibited satisfactory system suitability and recovery values within acceptable limits, with low LOD and LOQ values indicating high sensitivity. Given its reliability, rapid analysis time, and reproducibility, the validated method is suitable for routine quality control and stability testing of combined Doxofylline and Montelukast formulations in the pharmaceutical industry.

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Conflict of Interest

The authors declare no conflict of interest.

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