Research Article

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

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Abstract

The aim of the present study was the development and validation of a simple, precise, and specific RP-HPLC method for assay of Montelukast (MNT) and Rupatadine (RPT) in tablet dosage forms. The separation was achieved on Grace C-18 Column (4.6 × 250mm, 5μm) using Acetonitrile and 0.05% OPA (60:40, v/v) as mobile phase for assay and flow rate 1ml/ min & detection was carried out in U.V detector at 242.0nm. The retention time of RPT and MNT were found to be 3.86min and 7.60min respectively. The linearity of the RPT and MNT was found over the range of 5-25μg/ml. The system suitability test shows the response with retention time, theoretical plate, tailing factor and peak area for both the drugs. The validation of method carried out using ICH guidelines. The developed method was gave good resolution for drugs. The developed RP-HPLC method can be applied for routine quantitative and qualitative analysis of RPT and MNT in bulk and pharmaceutical formulations like tablets.

Keywords: Rupatadine; Montelukast; RP-HPLC; Validation; Actonitrile

Introduction

Rupatadine is a second generation antihistamine and PAF antagonist used to treat allergies. Rupatadine fumarate has been approved for the treatment of allergic rhinitis and chronic urticaria in adults and children over 12 years. The defined daily dose (DDD) is 10mg orally. It is soluble in methanol and ethanol slightly soluble in Chloroform and insoluble in water. It is off white to pinkish crystalline powder. Montelukast is a leukotriene receptor antagonist (LTRA) used for the maintenance treatment of asthma and to relieve symptoms of seasonal allergies. Montelukast is a CysLT1 antagonist; it blocks the action of leukotriene D4 (and secondary ligands LTC4 and LTE4) on the cysteinyl leukotriene receptor CysLT1 in the lungs and bronchial tubes by binding to it. It is off white hygroscopic powder. It is freely soluble in ethanol, methanol and water [1,2]. The development of analytical method for the determination of drugs in bulk, in dosage forms or in body fluids has received attention in recent years because of their importance in quality control, bioavailability and pharmacokinetic study. Literature review reveals that few analytical methods were evoked for the estimation of rupatadine fumarate and montelukast sodium, the present work is an attempt to estimate the same in combination by different method such as RP-HPLC method (Figure 1 and 2) [3,4].

Materials and Methods

Chemicals and reagent

Montelukast supplied as gift sample by Macleods Pvt. Ltd. Mumbai, India and its claimed purity was 99.3% and Rupatadine supplied as gift sample by Taj Pharmaceutical Limited Mumbai, India and have 99.5% purity. The RUPANEX M as a marketed formulation used which containing 10mg Rupatadine and 10mg
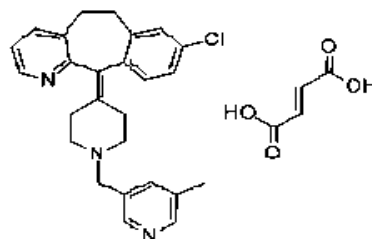

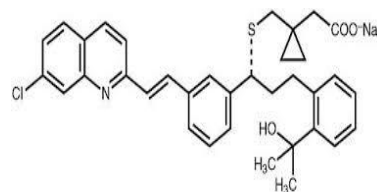**Figure 1:** Structure of Rupatadine fumarate [3].

Figure 2: Structure of Montelukast Sodium [4].

Montelukast, manufactured by Dr. Reddys, India Pvt. Ltd. HPLC grade Methanol, Water, Acetonitrile, Ortho Phosphoric Acid was procured. Hydrochloric acid (35% GR), Hydrogen peroxide, Sodium hydroxide was procured from Merck, India

RP-HPLC method development and optimization

Instrumentation [4]: The HPLC analysis was performed using Younglin (S.K Gradient) equipped with UV 730D detector, column Grace C-18 (4.6 × 250 mm, 5μm) and the output signals were monitor and processed using data processor Autochro-3000. and UV Spectrophotometer (Shimadzu,Model-1700) was used. Ultrasonicator (RC-SYSTEMMU-17000) was used to sonicating the
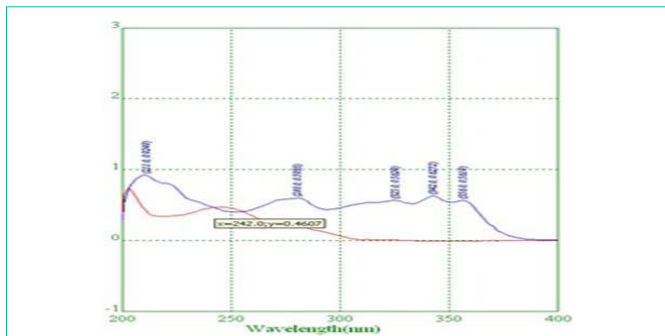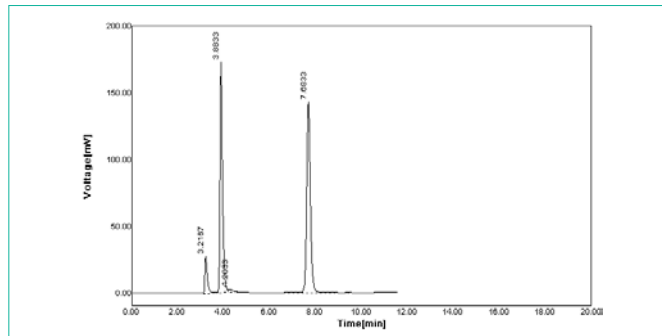
Figure 3: Determination of λ max.

Figure 4: Chromatogram of using Acetonitrile: KH$_2$PO$_4$ (60:40%, v/v).

Figure 5: Chromatogram of using Acetonitrile: KH$_2$PO$_4$ (70:30%, v/v).

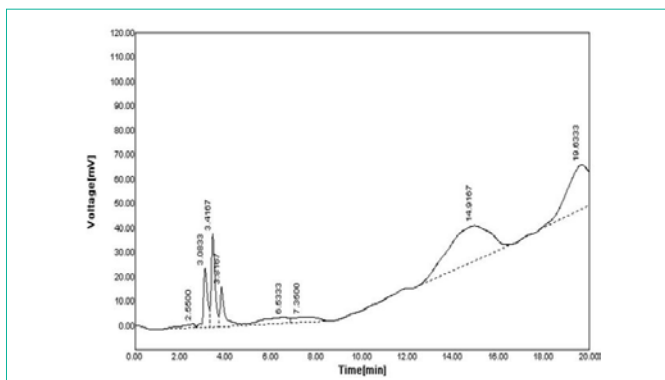**Figure 6:** Chromatogram using Acetonitrile: 0.05%OPA (90:10%, v/v).

Figure 7: Chromatogram of using Acetonitrile: water (90:10%, v/v).

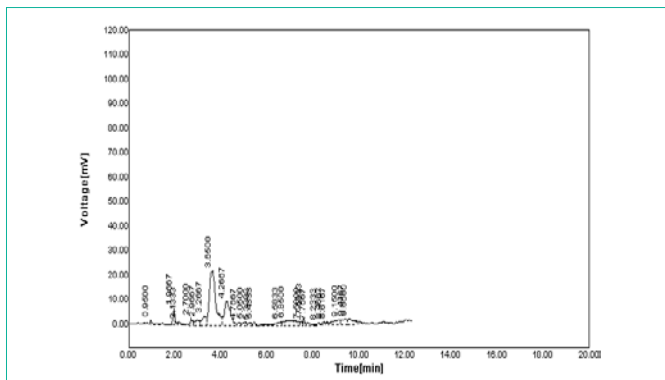**Figure 8:** Chromatogram of RPT and MNT using Acetonitrile: 0.05%OPA (60:40%, v/v).

Figure 9: Standard Chromatogram of RPT and MNT.
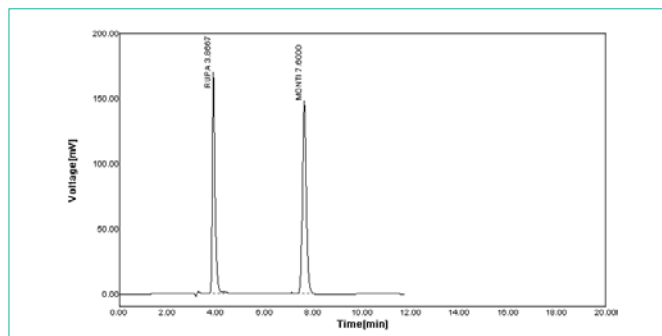
Figure 10: Sample chromatogram of RPT and MNT.

mobile phase and samples. Standard and sample drugs were weighed by using analytical balance model DS-852JSERIES. And pH of mobile phase was adjusted by using Digisun digital pH meter.

Chromatographic condition [5,6]: The separation was achieved on Younglin (S. K Gradient) System with Grace C18 (4.6 x 250mm, 5μm) column. The mobile phase consists of a mixture of Acetonitrile: 0.05% ortho phosphoric acid 60:40 v/v and the pH of mobile phase is adjusted to 2.5. The mobile phase was set at a flow rate of 1ml/
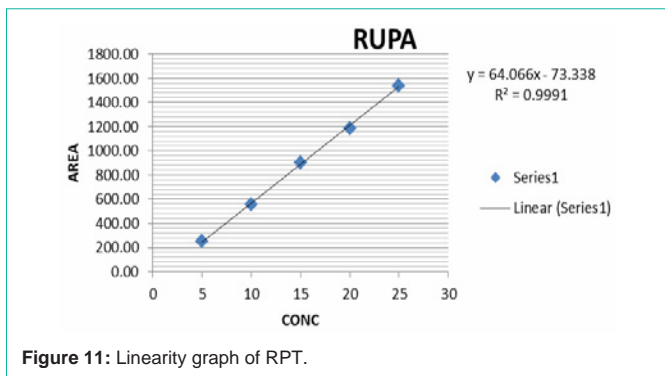
Figure 11: Linearity graph of RPT.

Figure 12: Linearity graph of MNT.

Figure 13: Linearity chromatogram in ratio of 5µg/ml RPT and 5µg/ml MNT.

Figure 14: Linearity chromatogram in ratio of 10µg/ml RPT and 10µg/ml MNT.

Figure 15: Linearity chromatogram in ratio of 15µg/ml RPT and 15µg/ml MNT.

Figure 16: RPT and MNT in ratio of 20µg/ml RPT and 20µg/ml MNT.

Figure 17: RPT and MNT in ratio of 25µg/ml RPT and 25µg/ml MNT.
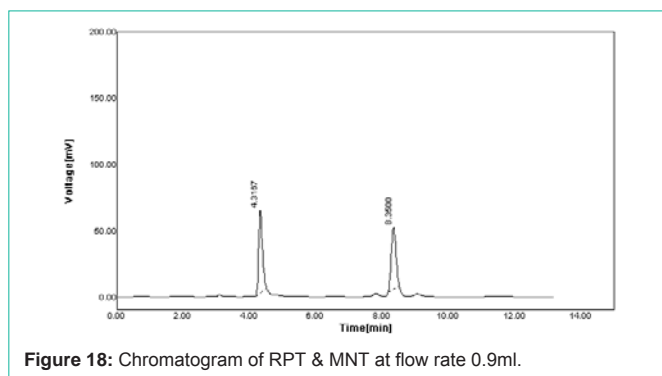
Figure 18: Chromatogram of RPT & MNT at flow rate 0.9ml.

min.wavelength selected for the determination of RPT and MNT was 242.0nm.

Preparation of mobile phase: The mobile phase was prepared by mixing Acetonitrile (HPLC Grade) and 0.05% Orthophosphoric acid in the ratio of 60:40 v/v. pH was made up to the 2.5. Then resulting solution was filtered through 0.45µ.Filtered and degassed using sonicator.

Preparation of stock and working solution for RPT & MNT:

Figure 19: Chromatogram of RPT & MNT at flow rate 1.1ml.

Figure 20: Chromatogram of RPT & MNT at wavelength 241nm.

Figure 21: Chromatogram of RPT & MNT at wavelength 243nm.

Figure 22: Chromatogram of RPT & MNT at Acetonitrile: 0.05%OPA (61:39%, v/v).

Figure 23: Chromatogram of RPT & MNT at Acetonitrile: 0.05%OPA (59:41%, v/v).

temperature and made up to the mark with methanol (HPLC Grade) which gave the final concentrations of 1000μg/ml and 1000μg/ml for RPT and MNT respectively. Take 0.05ml from stock solution of RPT and 0.05ml from stock solution of MNT respectively in a 10.0ml volumetric flask and make up the volume up to the mark with mobile phase to get 5μg/ml RPT & 5μg/ml MNT.

Preparation of sample solution: Take the powder weight of tablet equivalent to 544mg in 50.0 ml of volumetric flask and add sufficient mobile phase and sonicate it for 15min. Make up the volume up to the mark with mobile phase and filtered it with 0.24μ to get 1000μg/ml of RPT and MNT respectively. Take 0.05ml of RPT and 0.05ml of MNT from above solution of RPT and MNT respectively in a 10.0ml volumetric flask and make up the volume up to the mark with mobile phase to get 5μg/ml RPT & 5μg/ml MNT.

Validation of method

Linearity [7]: To establish the linearity of the analytical method, a series of dilutions with mobile phase were prepared in order to obtain the mixture of MNT & RPT ranging from 1-5μg/ml for MNT and 1-5μg/ for RPT. A constant volume of 20.0μL of each sample was injected and calibration curve was constructed by plotting the peak area versus the drug concentration.

System Suitability [7]: The system suitability parameter with respect to tailing factor, theoretical plates, relative standard deviation and resolution between MNT & RPT peaks was defined.

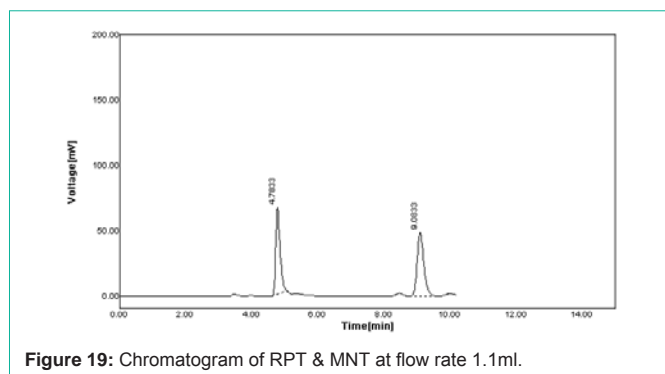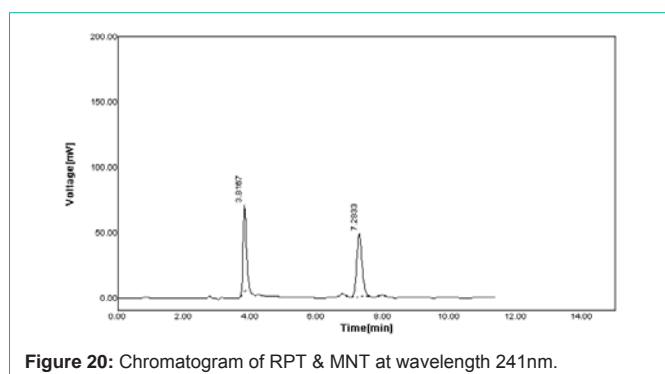**Accuracy [8]:** Recovery studies were carried out by standard addition method by adding the known amount of MNT & RPT separately to the reanalyzed sample at three different concentration levels i.e. 80%, 100% and 120% of assay concentration and percent recoveries were calculated.

Precision [9]: The method precision was evaluated by preparing 6 samples (sample preparation) as per the test method representing a single batch were applied in triplicate and injected this sample

An accurately weighed quantity of RPT working standard about 10.0 mg and MNT working standard about 10mg were transferred separately into 100.0 ml volumetric flask. About 10.0ml of methanol (HPLC Grade) was added to each of the volumetric flask and sonicated to dissolve the drug. The solution was cooled to the room

Table 1: Results for estimation of RPT in marketed formulation.

Conc. (μg/ml)	Area	Amount found	% Label claim
20	166. 73	19.35	96.78
20	185.38	19.64	98.24
Mean	1176.06	19.44	97.51
SD	13.19	0.21	0.35
% RSD	1.12	0.83	0.37

Table 2: Statistical data for estimation of RPT and MNT in marketed Formulation.

Conc. (µg/ml)	Area	Amount found	% Label claim
20	1353.84	19.92	99.6
20	1356.6	19.96	99.8
Mean	1355.22	19.94	99.7
SD	1.95	0.03	0.64
% RSD	0.14	0.14	0.61

Table 3: Statistical data for estimation of RPT and MNT in marketed Formulation.

Sr. No.	RPT		MNT	
	Assay (mg)	Assay (%)	Assay (mg)	Assay (%)
1	120.85	99.83	4.47	99.69
2	119.24	99.83	4.49	99.7
3	119.02	99.8	4.46	99.71
Mean	119.7	99.84	4.47	99.71
SD	0.1138	0.024119	0.351	0.0452
% RSD	0.061	0.648763	0.35	0.417598

preparation, but before diluent, placebo, and standard solution in six replicates injected in HPLC system. Determine the assay of these samples and evaluate the precision of the method by computing the % RSD of the assay results.

Robustness [10]: The Robustness of the method was evaluating the effect of small variation in the chromatographic conditions, such as changing the flow rate by ± 10%, and wavelength by ± 2nm, system suitability was done for each condition.

Ruggedness [11]: The ruggedness of the method was performed by analyzed the drug in the intra and inter day variation.

Result and Discussion

Determination of λ max (selection of wavelength)

Determination of λ max and selection of Analytical Wavelength: From the overlain spectra the two drugs RPT and MNT having the intersection at 242nm so for further studies wavelength selected is 242nm (Figure 3).

Optimization of chromatographic condition

- The following chromatographic conditions were established by trial and error and were kept constant throughout the method. Trial -1 Acetonitrile : KH_2PO_4 (60:40%, v/v).

- Trial -2 Acetonitrile: KH_2PO_4 (70:30%, v/v).

Table 5: Statistical Validation Data for Accuracy.

Level of % Recovery	RPT			MNT		
	Mean	SD	% RSD	Mean	SD	% RSD
80%	100.23	1.3	1.3	98.42	0.33	0.33
100%	95.97	1.07	1.11	102.33	1.08	1.06
120%	101.26	0.42	0.41	98.61	0.92	0.93

Table 6: Results of precision study.

Sr.No.	RPT		MNT	
	Peak Area	% Assay	Peak Area	% Assay
1	550.4	98.39	621.32	98.1
2	899.65	101.57	981.63	97.74
3	1159.85	97.47	1369.13	100.73
4	552.2	98.59	631.32	98.94
5	909.11	101.89	992.3	99.46
6	1169.85	97.47	1364.46	100.5
	Mean	**99.23**	**Mean**	**99.24**
	SD	**1.96**	**SD**	**1.22**
	%RSD	**1.97**	**%RSD**	**1.22**

Table 7: Result of Linearity study.

Sr. No.	Conc. (µg/ml)		Peak Area	
	RPT	MNT	RPT	MNT
1	5	5	250.42	292.39
2	10	10	558.99	631.07
3	15	15	904.09	996.16
4	20	20	1186.35	1377.17
5	25	25	1538.38	1709.8
Slope	64.006	71.618		
Intercept	73.338	72.958		
(r²)	0.9991	0.9995		

- Trial -3 Acetonitrile: 0.05%OPA (90:10%, v/v).

- Trial -4 Acetonitrile: water (90:10%, v/v).

- Trial -5 Acetonitrile: 0.05% OPA (60:40%, v/v).

Figure 4 shows resolution of peaks were poor, in this chromatogram many noisy peaks were observed hence this method was not suitable. Similarly chromatogram obtained using Acetonitrile: KH_2PO_4 (70:30%, v/v) separation of peaks were not proper, the two peaks of drugs were not identified so this method was not suitable

Table 4: Results of Accuracy.

Level of % Recovery	Amount of Std. Drug Added (µg/m l)		Total Amount Recovered (µg/ml)		%Recovery	
	RPT	MNT	RPT	MNT	RPT	MNT
80%	8	8	8.09	7.67	101.5	98.19
	8	8	7.87	9.75	99.31	98.65
100%	10	10	9.52	10.15	98.21	101.5
	10	10	9.67	10300	99.72	101
120%	12	12	12.18	11.75	101.5	98.96
	12	12	12.01	11.91	100.9	99.26

Table 8: Results of Linearity study of Rupatadine.

Sr. no	Conc. (µg/ml)	Mean Peak Area	SD	%RSD
1	5	250.42	2.22	0.76
2	10	558.99	2.3	0.36
3	15	904.09	1.92	0.19
4	20	1186.35	17.85	1.3
5	25	1538.38	13.87	0.81

Table 9: Results of Linearity study of Montelukast.

Sr.no	Conc. (µg/ml)	Mean Peak Area	SD	%RSD
1	5	292.39	0.78	0.31
2	10	631.07	1.12	0.2
3	15	996.16	5.43	0.6
4	20	1377.17	0.15	0.01
5	25	1709.8	13.48	0.88

Table 10: System suitability RPT and MNT.

Parameters	RPT	MNT
Retention time	3.86 min	7.60 min
Theoretical plate	6283.7	12113.1
Tailing factor	1.4	1.2
Capacity factor	0.64	4.31

(Figure 5).

By using Acetonitrile: 0.05% OPA (90:10%, v/v), RPT & MNT were separated with good peaks, minimum tailing having retention time 5.66 for RPT and 11.11 for MNT (Figure 6) but resolution between peaks were not sufficient. Hence this method was not suitable.

Good resolution with minimized tailing also proper peak shape and system suitability was observed within the limits. Retention time for RPT was 3.86 and retention time for MNT was 7.50 hence the above chromatographic parameters were finalized (Figure 7,8).

Estimation of Rupatadine and Montelukast from marketed formulation [12]

After establishing the chromatographic conditions, Mix standard and marketed preparation were prepared and analyzed by following procedure described under experimental work. It gave accurate, reliable results and was extended for estimation of drugs in marketed tablet formulation.

Formula for determination of drug concentration and % Label claim as shown below

$$\% \text{Label claim} = \frac{At}{As} \times \frac{Ds}{Dt} \times \frac{A}{Lc} \times 100$$

whereas,

At: Area count for sample solution; As: Area count for standard solution; Ds: Dilution factor for standard;

Dt: Dilution factor for sample; Lc: Label claim; A: Average weight.

Amount of drugs was calculated using formula

$$E_w = \frac{A_u}{A_s} \times C_s \times d$$

Table 11: Results of ruggedness study for MNT.

Sr. No	Observations	% Drug estimation		
		Intra-day	Inter-day	Different Analyst
1	I	98.1	98.94	98.876
2	II	97.74	99.46	99.624
3	III	100.73	100.5	99.922
Mean		976.96	995.46	99.472
±S.D.		6.6	4.48	0.474
%R.S.D.		0.68	0.45	0.476

Table 12: Results of ruggedness study for RPT.

Sr. No	Observation	% Drug estimation		
		Intra-day	Inter-day	Different Analyst
1	I	98.39	98.59	98.932
2	II	101.57	101.89	99.53
3	III	97.47	97.4	99.676
Mean		902.68	905.82	99.573
±S.D.		4.29	4.65	0.493
%R.S.D.		0.47	0.51	0.495

Table 13: Result of Robustness study at flow rate 0.9ml.

Flow rate 0.9ml		
Conc. (µg/ml)	Area for (RPT)	Area for (MNT)
10	490.6	460.46
10	495.62	463.32
Mean	493.11	461.89
±SD	3.55	2.02
% RSD	0.72	0.44

Table 14: Result of Robustness study at flow rate 1.1ml.

Flow rate 1.1ml		
Conc. (µg/ml)	Area for (RPT)	Area for (MNT)
10	601.08	677.98
10	610.36	610.03
Mean	605.72	644.01
±SD	6.56	8.05
% RSD	1.08	1.24

EW: Drug estimated in sample weight, mg; CS: Concentration of standard, µg/ml; Au: Area of unknown; As: Area of standard; D: Dilution factor.

The estimation of drug from marketed formulation were carried out by using 20µg/ml concentration of both drug and amount of drug recovered as 19.44 µg/ml for RPT with % label claim as 97.51 & 19.94µg/ml for MNT with % label claim as 99.70 respectively (Figure 9,10).

The proposed method was applied to the determination of RPT & MNT in marketed formulation. The mean % amount found was 99.84 (RPT) & 99.71 (MNT) with %RSD values was NMT 2.0% indicates the developed method was successfully applied for analysis of marketed formulation. All the results found were in good agreement with the

Table 15: Result of Robustness study at wavelength 214nm.

Wavelength 241nm		
Conc. (µg/ml)	Area for (RPT)	Area for (MNT)
10	450.41	545.07
10	455.39	552.3
Mean	452.9	548.69
±SD	3.52	5.11
% RSD	0.78	0.93

Table 16: Result of Robustness study at wavelength 243nm.

Wavelength 243nm		
Conc. (µg/ml)	Area for (RPT)	Area for (MNT)
10	531.17	505.1
10	540.2	517.87
Mean	535.69	511.49
±SD	6.39	9.03
% RSD	1.19	1.77

Table 17: Result of Robustness study at Acetonitrile: 0.05% OPA (61:39%, v/v).

Conc. (µg/ml)	Area for (RPT)	Area for (MNT)
10	490.66	497.44
10	498.54	485.69
Mean	494.6	482.57
±SD	5.57	4.42
% RSD	1.13	0.92

Table 18: Result of Robustness study at Acetonitrile: 0.05% OPA (59:41%, v/v).

Conc. (µg/ml)	Area for (RPT)	Area for (MNT)
10	557.65	530.11
10	550.27	542.31
Mean	553.96	536.21
±SD	5.22	8.63
% RSD	0.94	1.61

label content of marketed formulation (Table 1-3).

Method validation

The method was validated for the parameters Accuracy, Precision, LOD & LOQ, Linearity, Standard Deviation, Specificity, Ruggedness, and Robustness.

Accuracy: The accuracy of the method was determined by recovery experiment using the standard addition method by adding the known amount of RPT and MNT separately to the reanalyzed sample at three different concentration levels i.e. 80%, 100% and 120% of assay concentration and percent recoveries were reported in Table 4.

For the level of 80% the mean of RPT recovered as 100.23% with RSD as 1.30% for 100% the mean RPT was recovered as 95.97 with RSD as 1.11 & for 120% total amount of RPT was found to be 101.26 with RSD value less than 2% indicate method was to be accurate for analysis. Similarly % recovery for MNT was found to be 98.42% to 102.33% with RSD values less than 2% respectively (Table 5).

Table 19: Summary of Validation parameters for RPT & MNT.

Parameters	RPT	MNT
Linearity (range) Y= mx + C	5-25 µg/ml Y = 64.066 X -73.338	5-25 µg/ml Y= 71.618 X -72.958
Correlation coefficient	0.9991	0.9995
% Recovery	99.84	99.71
Precision (% RSD)	1.97	1.22
Accuracy (% RSD)		
For 80%	0.33	1.3
For 100%	1.06	1.11
For 120%	0.93	0.42
Ruggedness (% RSD)		
Intra-Day	1.47	0.68
Inter-Day	0.51	0.45
Repeatability	0.79	0.97
Robustness (%RSD)		
Flow change 0.9ml	0.72	0.44
Flow change 1.1ml	1.08	1.24
Composition change 61:39	1.13	0.94
Composition change 59:41	0.92	1.61
Wavelength change 241nm	0.78	0.93
Wavelength change 243nm	1.19	1.77

Precision: It is a measure of degree of repeatability of an analytical method under normal operation and it is normally expressed as % of relative standard deviation (%RSD). The standard solution was injected for six times and measured area for all six replicates in HPLC system. The %RSD for the area of six replicate injections was found to be within the specified limits as shown in Table 6.

The mean% recovered as 99.23% for RPT while as 99.24% was recovered as MNT which means that developed method is highly precise for analysis of above drugs.

Linearity: The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration of analyte in the sample. Linearity was carried out for five levels. A graph was plotted with concentration on X axis and mean peak areas on Y axis. The r^2 value was found to be 0.999 for rupatadine and montelukast r^2 value should be 0.999. The result show that excellent correlation exists between concentration and mean peak areas within concentration range. From the studies carried out and result obtained the proposed method compared in terms of statistical data. The data were representing in Table 7 and the linearity curve was represented in Figure 11,12.

The linearity chromatogram was constructed with different concentration of RPT & MNT (5 to 25 µg/ml) are shown in Figure 13-17 & identical with standard supported by r^2 & retention time (Rt), % RSD value obtained from linearity study. Hence developed method of RP-HPLC for RPT & MNT is highly linear with respect to concentration vs. peak area obtained in chromatogram (Table 8,9).

System suitability: System suitability tests are an integral part of Method development and are used to verify whether the resolution and reproducibility of the chromatographic system are adequate for

analysis. Retention time (Rt), Theoretical plate (N), Tailing factor (T), Capacity factor were evaluated for five replicates for standard drug solution & result was expressed in Table 10.

Ruggedness: When the drugs RPT and MNT were analyzed by proposed method in different conditions like inter-day (during two days) intra-day (in single day at different time) and different analyst using similar operational procedure but by different analyst Peak area was measured for same concentration solutions. The % amount of drugs was found with %RSD (NMT than 2%) which was in agreement with system suitability. Therefore, the proposed HPLC method for the determination of RPT and MNT in a tablet was found to be sufficiently rugged (Table 11,12).

Robustness: The robustness of assay method was studied by incorporating small but deliberate changes in analytical method as,

1. Effect of variation in flow rate (±1) : If injection volume changed as 0.9ml for RPT using RP-HPLC method resulting peak area for RPT as 493.11 & %RSD value as 0.72 & 461.89 and %RSD as 0.44 for MNT as within acceptable limit (Table 13, Figure 18).

If flow rate changed as 1.1ml for both drugs mean peak area for RPT was found to be 605.72 & %RSD as 1.08 and 644.01 with % RSD as 1.24 for MNT as satisfactory (Table 14, Figure 19).

The % RSD was not more than 2% for both (RPT & MNT) which was in agreement with system suitability. Hence the proposed HPLC method for the determination of RPT and MNT in a tablet was found to be robust.

2. Change in wave length (±1): When analysis was carried out by changing wavelength for measurement as 241nm mean peak area as 452.90 & %RSD as 0.78 was obtained for RPT and 548.69 & %RSD 0.93 for MNT was obtained (Table 15, Figure 20).

It was observed that there is no effect on retention time, peak area of both drug if they are measured at 243nm the mean peak area for RPT was 535.69 & %RSD value as 1.19 & 511.49 along with %RSD as 1.77 for MNT indicate that change in wavelength slight changes in peak area of both drug supported by %RSD less than 2%. Hence above method was validated as per robustness parameters (Table 16, Figure 21).

3. Change in Mobile phase ratio (±1): When Mobile phase compositions consider as Acetonitrile: 0.05% OPA (61:39% V/V) and resulting chromatogram obtained from this study shown in Figure 22 The %RSD value for both RPT & MNT as 1.13 and 0.92 respectively (Table 17).

If mobile phase composition as 59:41 v/v for Acetonitrile: 0.05% OPA were selected for study Peak area, SD & %RSD value obtained reported in Table 18 was acceptable.

From the above study was concludes that change in wavelength (±1) does not affect on retention time of both RPT & MNT (Figure 23).

Summary of validation parameters: The validation parameters applied for study RPT & MNT from marketed formulation & resulting parameters along with %RSD values was found within limit prescribed by standard (Table 19).

Conclusion

The developed RP-HPLC method was found to be linear over wider concentration range. Therefore the developed RP-HPLC method can be applied for routine quantitative and qualitative analysis of RPT and MNT in bulk and pharmaceutical formulations like tablets. The developed RP-HPLC method was validated as per the ICH guidelines.

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