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Development And Validation Of Rp-Hplc Method For Simultaneous Estimation Of Bepotastine Besilate And Montelukast Sodium In Synthetic Mixture

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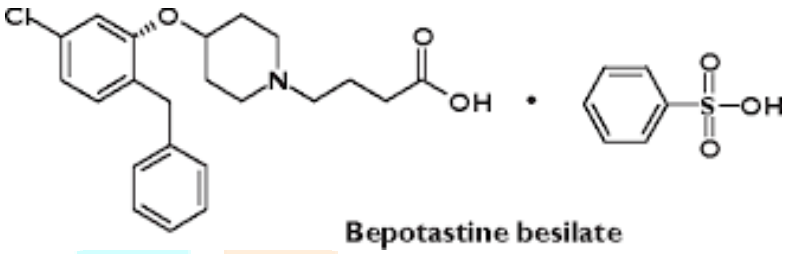
ABSTRACT : It is simple, sensitive and accurate RP-HPLC method was developed for simultaneous estimation for Montelukast Sodium and Bepotastine besilate A reversed–phase high-performance liquid chromatography method is developed and validated for the determination of both drugs. With the help of RP-HPLC it gives us to good resolution and better separation for the both drugs. The separation was conducted by using Cybersil C18 column (250 mm×4.6 mm×5 µm) with mobile phase consisting Phosphate buffer, Methanol Sodium and ACN (70:20:10)% v/v/v. The mobile phase was delivered at the flow rate of 1.0 ml/min. The eluent was monitored at wavelength 225 nm and found a sharp and symmetrical peak of Montelukast and Bepotastine besilate were found to be 4.36 min and 5.42 min respectively. The method was validated for linearity, accuracy, precision, system suitability. The method was found to be linear over the concentration range for both drugs 10-60µg/ml with coefficient R^2 for MON 0.9979 and BEP 0.998. Therefore, proposed method can be successfully used for routine analysis of Montelukast Sodium and Bepotastine besilate in bulk as well as synthetic mixture.

[**Keywords**: Montelukast Sodium(MON), Bepotastine besilate(BEP),reversed–phase high-performance liquid chromatography (RP-HPLC).]

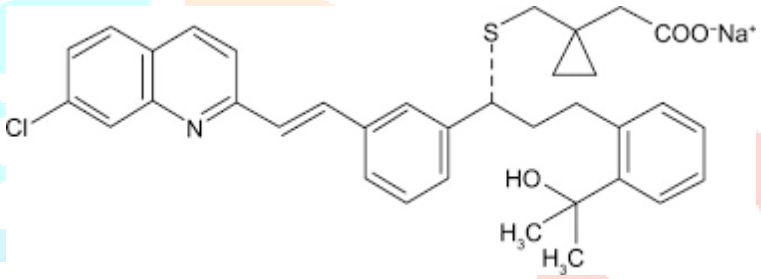
INTRODUCTION: Bepotastine is chemically described as (S)-4-{4-[(4-chlorophenyl) (pyridin-2-yl) methoxy] piperidin-1-yl} butanoic acid[22-23]. It is a non-sedating, selective antagonist of the histamine 1 (H1) receptor. Bepotastine was approved in Japan for use in the treatment of allergic rhinitis and uriticaria/pruritus[2]. Bepotastine works to relieve itchy eyes by three primary mechanisms of action. It is a non-sedating, selective antagonist of the histamine 1 (H1) receptor, a mast cell stabilizer, and it suppresses the migration of eosinophils into inflamed tissues to prevent tissue damage and worsening of allergic inflammation of the conjunctiva.Montelukast is chemically described as Sodium 2-[1-({[(1R)-1-{3-[(E)-2-(7-chloroquinolin-2-yl) ethenyl] phenyl}-3- [2-(2-hydroxypropan-2-yl) phenyl] propyl] sulfanyl} methyl)

cyclopropyl] acetate[24-25]. It is a selective, potent and orally active antagonist of the cysteinyl, CysTL1, leukotriene receptor used for the treatment of asthma in children and adults[3]. Montelukast blocks the action of leukotriene D4 on the cysteinyl leukotriene receptor CysLT1 in the lungs and bronchial tubes by binding to it. This reduces the bronchoconstriction otherwise caused by the leukotriene and results in less inflammation[47]. The need of validation of the analytical method development and validation emerged due to international competition, new development, maintaining the standard of products in high commercial & market value and ethical reasons[4-6]. High Performance Liquid Chromatography is the most common analytical separation tool, components by distributing between mobile phase and stationary phase[11-18].

Structural Formula :



Bepotastine besilate



Montelukast sodium

MATERIALS AND METHODS: Sample of Montelukast Sodium (MON) procured from APMP Lifescience, Gujarat. Bepotastine Besilate (BEP) as procured from Angle Bio Pharma, Gujarat.

Experimental condition:

Apparatus: HPLC manufactured by Cyber Lab having LC-100 model no. was used in these method development .Cyber-Sil, C18 column (250mm x 4.6mm, 5 µg) was used as an stationary phase. For Identification of api by using UV Visible Spectrophotometer and FT-IR UV Visible Spectrophotometer is manufactured by Shimadzu having UV 1700 model no and FT-IR is manufactured by Agilent Technologies having Cary 630 model no.

Chemical: HPLC Grade Water, Methanol, Acetonitrile which is manufactured by Ranchem Ltd. AR Grade Potassium Dihydrogen Phosphate, Orthophosphoric acid which is manufactured by Ranchem Ltd.

IDENTIFICATION OF API:

MELTING POINT: Melting point of Montelukast Sodium and Bepotastine Besilate was carried out by capillary tube method in paraffin bath. The melting point study was performed in Thieles tube that was filled with liquid paraffin. 50 mg of powdered drug was filled in capillary that was attached with the tip of thermometer with the help of thread.Then thermometer was placed in Thiele's tube and was heated.Temperature at which the drug powder melted was noted down. It was performed in triplicate.

SOLUBILITY STUDY: Solubility of Montelukast Sodium and Bepotastine Besilate was performed using various solvents like water, methanol, acetonitrile etc.

IR SPECTRA: Drug was placed in sample compartment of FT-IR instrument, where it was scanned in the range of 4000 - 650 cm^{-1}. Principle IR peaks were observed for drug are shown in table and from this data it was concluded that drugs were foundto be authentic.

UV ABSORPTION STUDY: Accurately weighed 10 mg of Montelukast Sodium and Bepotastine Besilate were transferred separately in 10 ml volumetric flasks, dissolved in small volume of methanol and then volume was adjusted to the mark with methanol to obtain concentration of 1000 µg/ml.These solutions were further diluted to obtain concentration of 10 µg/ml.These standard solutions of MON and BEP in methanol were scanned in UV range, 200-400 nm in 1 cm cell using methanol as blank and maximum absorbance was measured for selection of λmax of MON and BEP.

METHOD DEVELOPMENT AND VALIDATION:

SELECTION OF DILUENT: Based on solubility, Montelukast Sodium (MON) and Bepotastine Besilate (BEP) was sparingly soluble in methanol. Hence, methanol was selected as diluent.

PREPARATION OF STOCK SOLUTION: Accurately weighed and transferred about 10 mg of Montelukast Sodium (MON) and 10 mg of Bepotastine Besilate (BEP) in to 100 ml of volumetric flask, 50 ml of methanol was added and sonicated to dissolve. Volume was making up to the mark with diluent. Concentration of Montelukast Sodium (MON) and Bepotastine Besilate (BEP) 100 µg/ml. The optimum wavelength was selected for the estimation was 225 nm where gives maximum absorbance, which was obtained by scanning solution in the range of 200-400 nm in UV spectrophotometer.

Preparation of Montelukast Sodium (MON) solution: Pipette out 1, 2, 3, 4, 5 and 6 ml of Montelukast Sodium (MON) 100 µg/ml in 10 ml of volumetric flask. Further diluted above solution to 10 ml volumetric flask and volume was make up to the mark with diluent to get 10, 20, 30, 40, 50 and 60 µg/ml concentration of Montelukast Sodium (MON).

Preparation of Bepotastine Besilate (BEP) solution: Pipette out 1, 2, 3, 4, 5 and 6 ml of Bepotastine Besilate (BEP) 100 µg/ml in 10 ml of volumetric flask.Further diluted above solution to 10 ml volumetric flask and volume was make up to the mark with diluent to get 10, 20, 30, 40, 50 and 60 µg/ml concentration of Bepotastine Besilate (BEP).

SELECTION OF MOBILE PHASE: The mobile phase selection process included choosing a buffer, pH of the buffer, solvent, and buffer-to-solvent ratio. The nature of the sample, its molecular weight, and solubility all have a role in the HPLC method selection. A buffer is necessary for pH regulation. The acidic component is kept at a low pH, whereas the base is kept at a high Ph.The mobile phase was chosen based on separation, peak purity, Tailing factor, theoretical plate, and other factors. To achieve a sharp peak of Montelukast Sodium (MON) and Bepotastine Besilate, various mobile phases were attempted in varied compositions and pH levels.

SELECTION OF WAVELENGTH: An ideal wavelength is the one that gives Maximum response for the drugs that was to be detected. For selection of wavelength U.V spectrophotometer is used or using HPLC assisted with UV detector, UV overlay spectra of Montelukast Sodium (MON) and Bepotastine Besilate were obtained. For High Performance Liquid Chromatography 225 nm was selected wavelength where both drug show good absorbance.

PREPARATION OF BUFFER: Dissolve 1.19 g of disodium hydrogen phosphate dihydrate R and 8.25 g of potassium dihydrogen phosphate R in water R and dilute to 1000.0 ml with the same solvent. Mix well and sonicate. Filter through 0.45 µm membrane filter paper.

PREPARATION OF MOBILE PHASE: Prepare a mixture of phosphate buffer, Methanol and ACN in the volume ratio 70:20:10 % v/v/v. Mix well and sonicate to degas the mixture. Adjust pH 5.8 with orthophosphoric acid.

SELECTION OF COLUMN: Montelukast Sodium (MON) and Bepotastine Besilate (BEP) are polar in nature. So, C18 analytical column were selected for HPLC method. The column was used Cybersil C18 column (250 mm × 4.6 mm, 5 µm) was used for the development of the method.

METHOD VALIDATION:

CALIBRATION CURVE: From the above prepared stock solution, pipette out appropriate volume of aliquot from standard stock solution of each of individual drug volumetric flask and transfer it to different volumetric flask of 10ml and volume adjusted upto mark with methanol, six different concentrations for MON prepared with ranges from 10 – 60 µg/ml and for BEP with ranges from 10 – 60 µg/ml were prepared from their individual respective stock solutions.

SYSTEM SUITABILITY TEST: The system-suitability tests are integral part of gas and liquid chromatography. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for analysis to be done. The tests are based on concept that the equipment, electronics, analytical operations, and sample to be analysed constitute an integral system that can be evaluated as such. The system suitability parameters like resolution, theoretical plates and asymmetric factor were calculated and compared with standard values.The system suitability test was carried out on freshly prepared working standard stock solution of MON (10 µg/ml) and BEP (10 µg/ml), respectively.

LINEARITY: The linearity of method was analysed by establishing calibration curve at six concentration levels over the range of 10 – 60 µg/ml for MON and 10 – 60 µg/ml for BEP, respectively.The calibration curve was established by plotting Peak area versus Concentration (n= 6) and straight-line regression equation was obtained. The calibration range was made in such a way that the ratio of combination was maintained throughout analysis.

PRECISION: In RP – LC method, repeatability has been carried out by injection repeatability.
Injection **Repeatability** was carried out by analysing the samples of Montelukast Sodium (MON) and Bepotastine Besilate (BEP) with the concentration of 30 µg/ml and 30 µg/ml respectively by injecting six times and peak retention time of each drug concentration was measured.Precision was estimated in terms of intraday and interday precisions.**Intraday precision** was determined by analysing sample solutions of Montelukast Sodium (MON) and Bepotastine Besilate (BEP) by three different levels MON (10 µg/ml, 30 µg/ml, 60 µg/ml) and BEP (10 µg/ml, 30 µg/ml, 60 µg/ml) which covers low, medium, and high concentrations of the calibration curve three times on the same day.**Interday precision** was determined by analysing sample solutions of Montelukast Sodium (MON) and Bepotastine Besilate (BEP) at three levels covering low, medium, and high concentrations over the 3 different successive days.

ACCURACY: The accuracy of method was determined by calculating recoveries of drug by standard addition method at three different level 50 %, 100 % and 150 % of standards to pre-quantified sample solution of synthetic mixture. For MON 10µg/ml, 20 µg/ml and 30µg/ml were spiked to pre-quantified sample solution of synthetic mixture for MON 20 µg/ml and For BEP 10µg/ml, 20 µg/ml and 30µg/ml were

spiked in pre-quantified sample solution of synthetic mixture for BEP 20 µg/ml, respectively.

LOD and LOQ: The LOD was estimated from the set of 3 calibration curves used to determine method linearity.

The LOD may be calculated as, **LOD = 3.3 × (SD/Slope)**

The LOQ may be calculated as, **LOQ = 10 × (SD/Slope)**

ROBUSTNESS: Small and deliberate changes were made in mobile phase, flow rate, temperature and analytical wavelength.The effect on the result were examined for MON (20 µg/ml) and BEP (20 µg/ml) combination solution. The effect on peak area and % RSD were calculated.

SPECIFICITY: The specificity of the method was ascertained by comparing retention time of MON and BEP in chromatogram obtained from standard drug and synthetic mixture.The standard concentration of MON and BEP taken was 20 and 20 µg/ml respectively.The synthetic mixture of MON and BEP was prepared in ratio of 10 mg: 10 mg, respectively.Common excipients like Microcrystalline cellulose (980 mg), Magnesium Stearate (20 mg), (HPMC) Hydroxypropyl methylcellulose K100 (200 mg), Cross Carmellose Sodium(100 mg) were weighed accurately and transfer into motor pestle along with 100 mg of MON and 100mg of BEP which is equivalent to 10 tablets.From the above stock solution 20 µL volume was injected and compare the chromatogram with standard sample without excipients.

ANALYSIS OF SYNTHETIC MIXTURE: The synthetic mixture of MON and BEP was prepared in ratio of 10 mg: 10 mg, respectively.Common excipients like Microcrystalline cellulose (980 mg), Magnesium Stearate (20 mg), (HPMC) Hydroxypropyl methylcellulose K100 (200 mg), Cross Carmellose Sodium (100 mg) were weighed accurately and transfer into motor pestle along with 100 mg of MON and 100mg of BEP which is equivalent to 10 tablets.Weight accurately equivalent to 10mg of Montelukast Sodium (MON) and 10mg of Bepotastine Besilate (BEP) and transfer it in 10 ml volumetric flask containing 5.0 ml of methanol and sonicated for 15 min.The solution was filtered using Whatman filter paper No.42 and collects the filtrate in another 10 ml volumetric flask and the residue was wash with 3.0 ml amount of methanol, the filtrate and residue was combined and volume was diluted to the mark with the methanol.Pipette out 1.0 ml aliquot from the above solution, transfer it in another 10 ml volumetric flask and volume made upto the mark with methanol, from above solution, Pipette out 1.0 ml aliquot and transfer it into another 10 ml volumetric flask and makeup volume upto the mark and from the above solution, Pipette out 3.0 ml aliquot from the above solution, transfer it in another 10 ml to obtain final concentration of 10 µg/ml for MON and 10 µg/ml for BEP respectively.From the above stock solution 20 µL volume was injected and compare the chromatogram with standard sample without excipients.The possibility of interference from other components of the synthetic mixture in the analysis was studied.It was analysed under proposed chromatographic conditions

and chromatogram recorded.The amount of Montelukast Sodium (MON) and Bepotastine Besilate (BEP) were computed using regression equation.

RESULTS AND DISCUSSION:

IDENTIFICATION OF DRUG:

MELTING POINT STUDY: The observed melting point of each mentioned drugs were similar to the standard melting point reported for respective drugs as evident from Table 1.

Table 1 Melting Point Study:

Drugs	Reported Melting Point(°C)	Observed Melting Point(°C)
Montelukast Sodium (MON)	145 °C -148 °C [25]	146 °C -148 °C
Bepotastine Besilate (BEP)	159◦C -163◦C [27]	160◦C -163◦C

N = 3, Mean of 3 replicates

SOLUBILITY STUDY: The solubility of substance fundamentally depends on the physical and chemical properties of the solute and solvent as well as temperature, pressure and the pH of the solution. The solubility profile is used for solvent selection in method development.The solubility of each drug in different solvent in shown in Table 2.

Table 2 Solubility Study:

Drugs	Montelukast Sodium (MON)	Bepotastine Besilate (BEP)
Water	Freely soluble	Very Slightly Soluble
Methanol	Very Soluble	Slightly Soluble
Acetonitrile	Insoluble	Insoluble

UV ABSORPTION STUDY:UV spectra of drugs in methanol depicted that the wavelength maxima of MON and BEP were at 224 nm and 225 nm respectively as shown in Figure 1. For High Performance Liquid Chromatography 225 nm was selected wavelength.

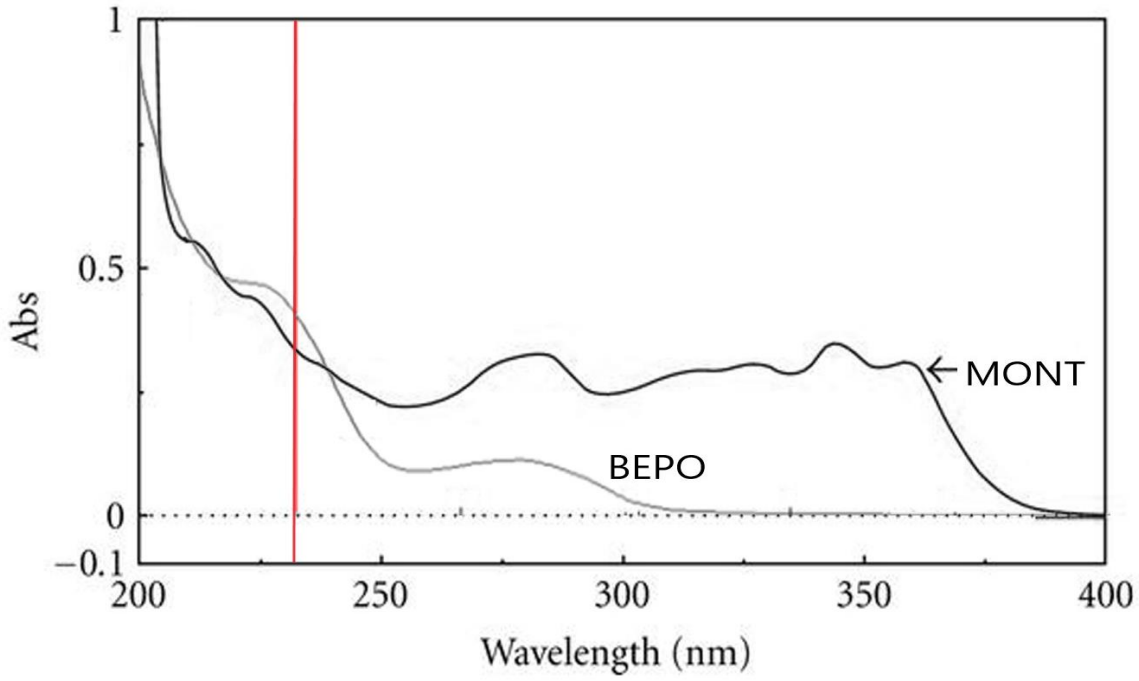


Figure 1: Overlain UV Spectrum in methanol

IR SPECTRA: An IR spectrum of reference sample shown in figure 2 and figure 3.

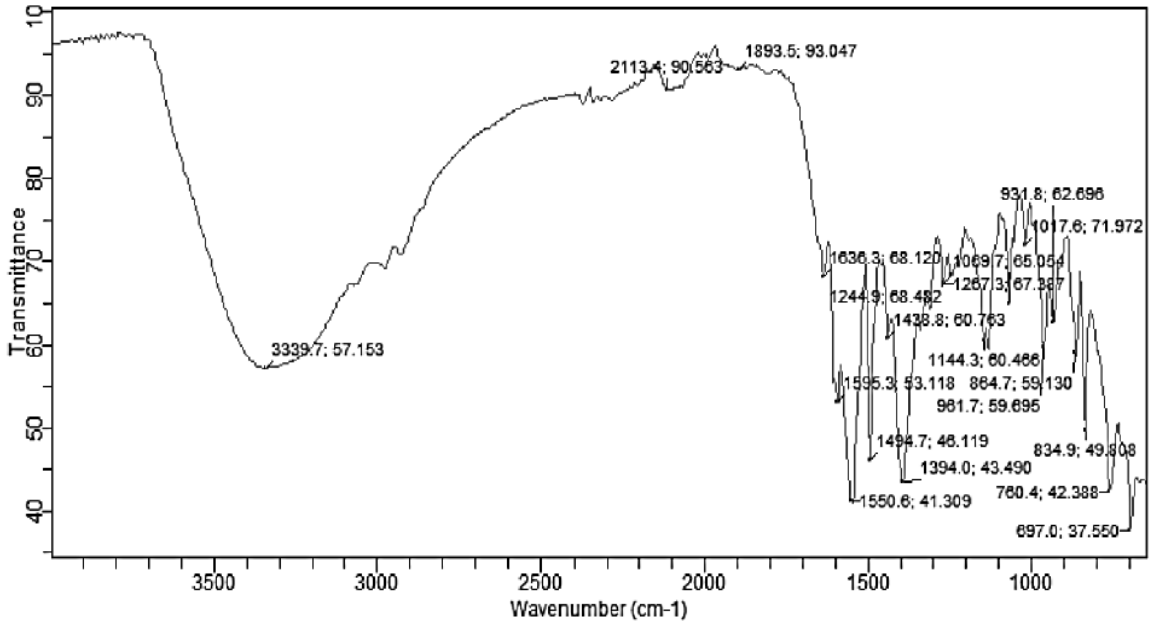


Figure 1 IR Spectrum of Montelukast Sodium (MON)

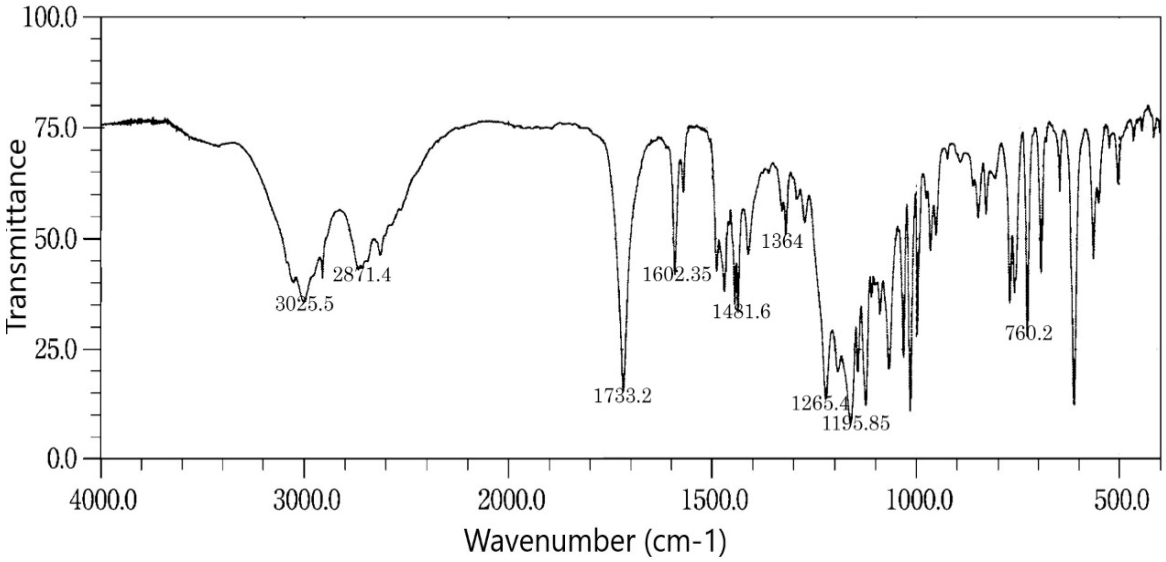


Figure 2 IR Spectrum of Bepotastine Besilate (BEP)

OPTIMIZATION OF RP-HPLC CHROMATOGRAPHIC CONDITION:

Table 3 Method Development Trial

Trial No.	Condition	*Observation*
1	Column: C 18 (250 mm x 4.6 mm),5 µm Mobile Phase: Methanol: Water (20:80 % V/V) Flow Rate: 1 ml/min Wavelength: 225 nm Injection Volume: 20 µl	Lower retention time of Drug and peak shape was not proper only.
2	Column: C 18 (250 mm x 4.6 mm),5 µm Mobile Phase: Acetonitrile:Water (20:80 V/V) Flow Rate: 1 ml/min Wavelength: 225 nm Injection Volume: 20 µl	Lower retention time of Drug and peak shape was not proper only
3	Column: C 18 (250 mm x 4.6 mm),5 µm Mobile Phase: Water: Acetonitrile: Methanol (30:30: 40 %V/V) Flow Rate: 1 ml/min Wavelength: 225 nm	Higher retention time of Drug and peak shape was not proper.

	Injection Volume: 20 µl	
4	Column: C 18 (250 mm x 4.6 mm),5 µm Mobile Phase: Water: Methanol: Acetonitrile (50:30: 20 %V/V) Flow Rate: 1 ml/min Wavelength: 225 nm Injection Volume: 20 µl	Higher retention time of Drug and peak shape was not proper.

5	Column: C 18 (250 mm x 4.6 mm),5 µm Mobile Phase: Phosphate buffer: Methanol: Acetonitrile (60:30: 10 %V/V) 5 pH of buffer Flow Rate: 1 ml/min , Wavelength: 225 nm Injection Volume: 20 µl	Shorter retention time of Drug and peak shape was proper but resolution not good
6	Column: C 18 (250 mm x 4.6 mm),5 µm Mobile Phase: Phosphate buffer: Methanol: Acetonitrile (60:30: 10 %V/V) 5.8 pH of buffer Flow Rate: 1 ml/min Wavelength: 225 nm Injection Volume: 20 µl	Shorter retention time of Drug and peak shape was proper but resolution not good
7	Column: C 18 (250 mm x 4.6 mm),5 µm Mobile Phase: Phosphate buffer, Methanol and ACN in the volume ratio 70:20:10 % v/v/v, 5.8 pH of buffer Flow Rate: 1 ml/min ,Wavelength: 225 nm Injection Volume: 20 µl	Shorter retention time of Drug and peak shape was proper, resolution good

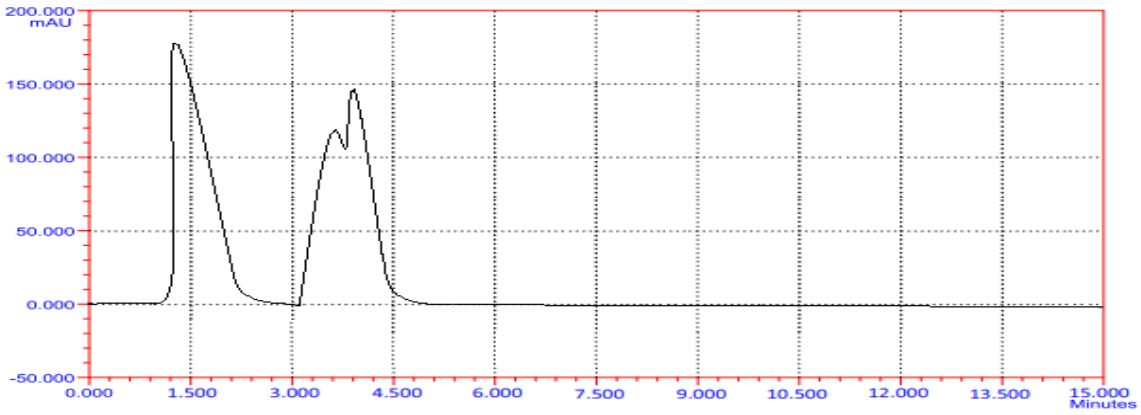


Figure 3 Trail 1: HPLC Chromatogram: Mobile Phase: Methanol: Water (20:80 % V/V)

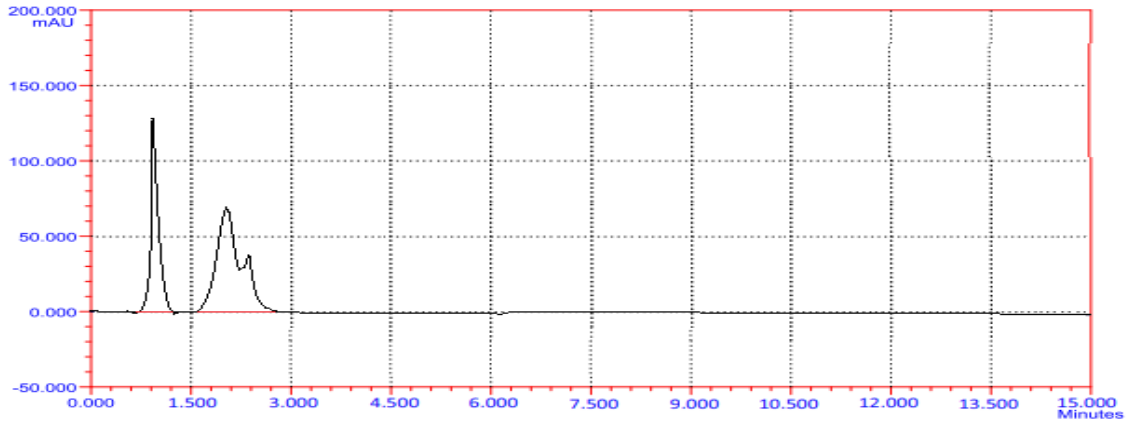


Figure 4 Trail 2: HPLC Chromatogram: Mobile Phase: Acetonitrile: Water (20:80% V/V)

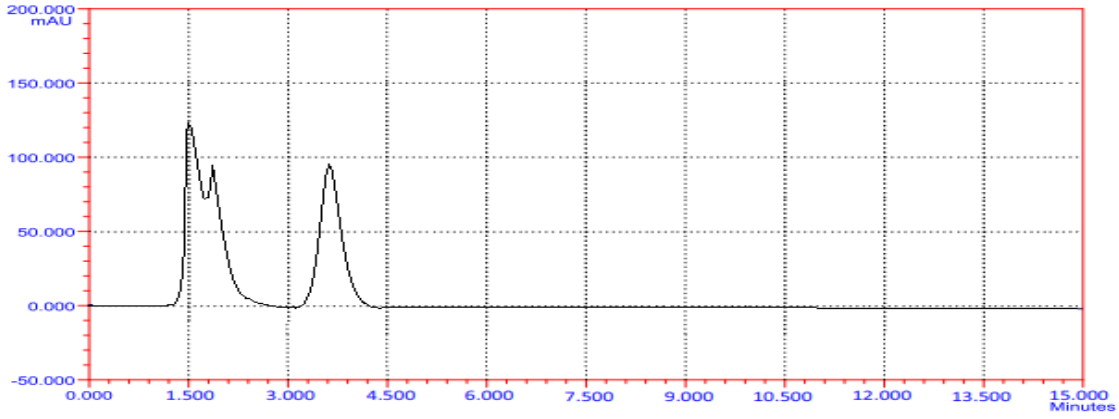


Figure 5 Trail 3:HPLC Chromatogram :Mobile Phase: Water: Acetonitrile: Methanol (30:30: 40 % V/V)

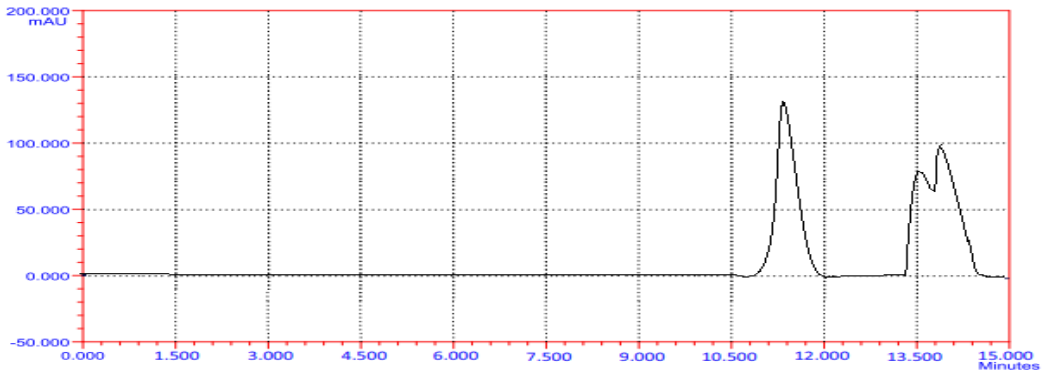


Figure 6 Trail 4: HPLC Chromatogram: Mobile Phase: Water: Methanol: Acetonitrile (50:30: 20 % V/V)

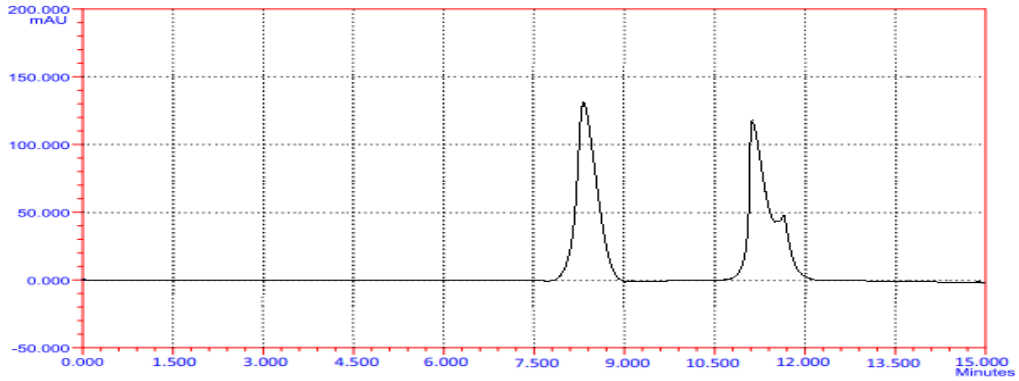


Figure 7 Trail 5: HPLC Chromatogram: Mobile Phase: Phosphate buffer: Methanol: Acetonitrile (60:30: 10 %V/V) 5 pH of buffer

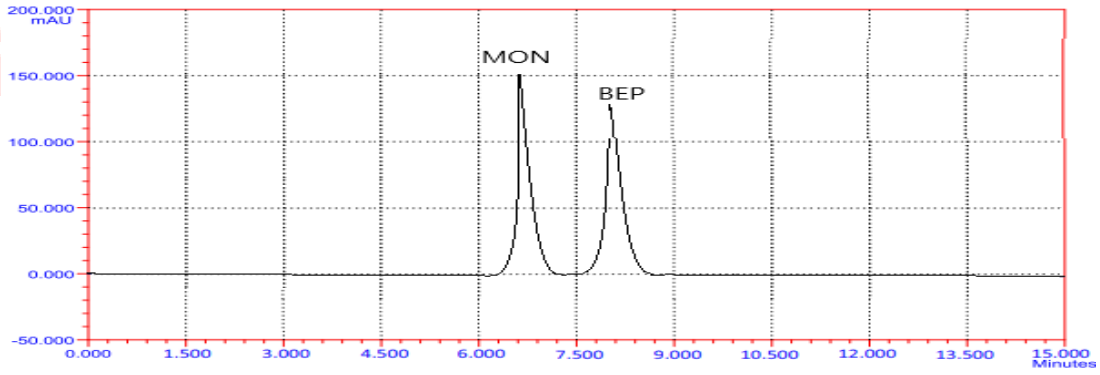


Figure 8 Trail 6: HPLC Chromatogram: Mobile Phase: Phosphate buffer: Methanol: Acetonitrile (60:30: 10 %V/V) 5. 8 pH of buffer

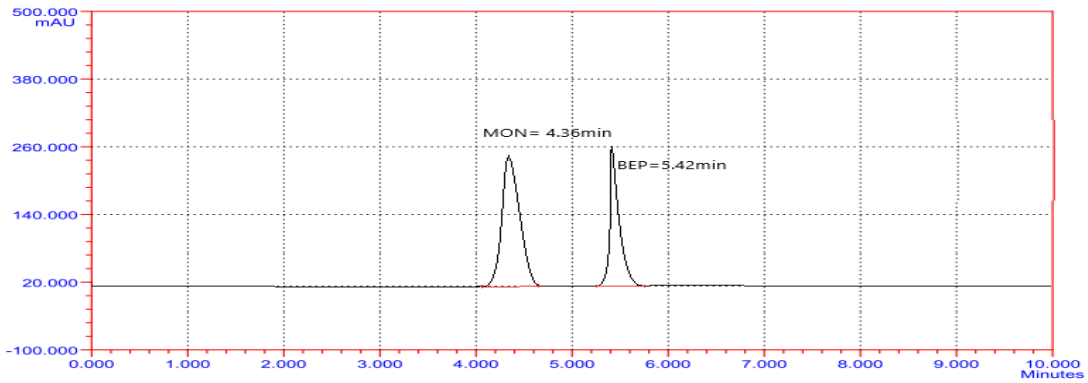


Figure 9 Trail 7: HPLC Chromatogram: Phosphate buffer, Methanol and ACN in the volume ratio 70:20:10 % v/v/v, 5.8 pH of buffer

Table 4 Optimization of RP-HPLC chromatographic condition

Sr. No.	**Chromatographic parameter**	**Optimize Condition**	
1	Flow Rate	1 ml/min	
2	Detection Wavelength	225nm	
3	Mobile Phase composition	Phosphate buffer, Methanol and ACN in the volume ratio 70:20:10 % v/v/v	
4	Column	C18 (250 mm×4.6 mm×5 µm)	
5	Injection Volume	20 µl	
6	pH of buffer	5.8 ± 0.02	
7	Retention time (min)	MON	BEP
		4.36 min	5.42 min

Individual drug peak was confirmed by injecting the MON and BEP in optimized chromatographic condition shown in **figure 8** and **figure 9**.Blank chromatogram shown in **figure 10**.

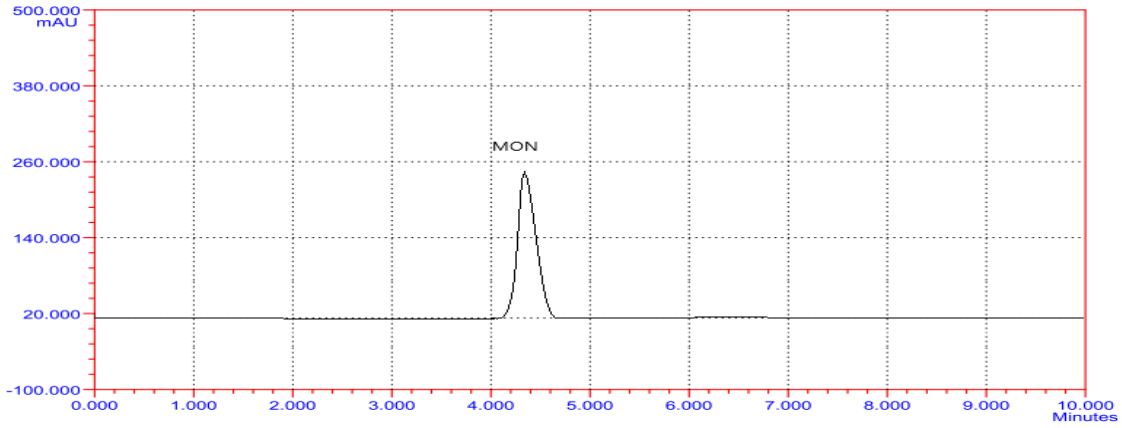


Figure 8 HPLC Chromatogram of Montelukast sodium: Phosphate buffer, Methanol and ACN in the volume ratio 70:20:10 % v/v/v, 5.8 pH of buffer

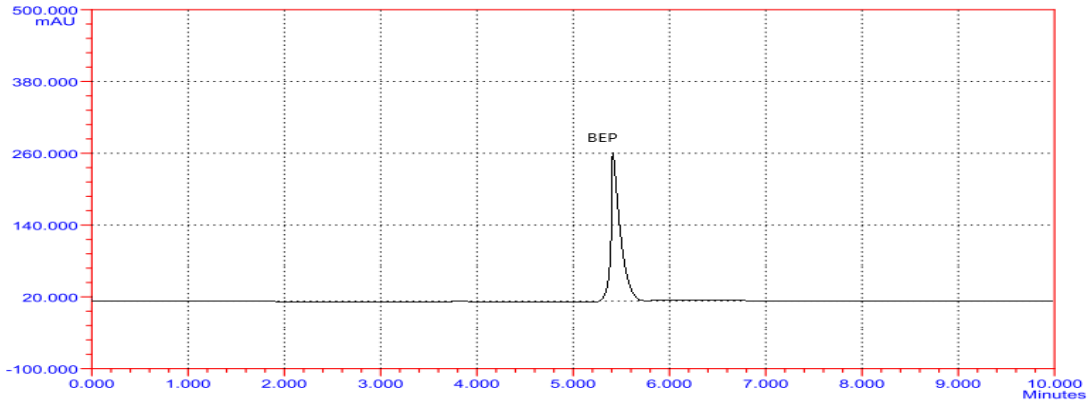


Figure 9 HPLC Chromatogram of Bepostatine: Phosphate buffer, Methanol and ACN in the volume ratio 70:20:10 % v/v/v, 5.8 pH of buffer

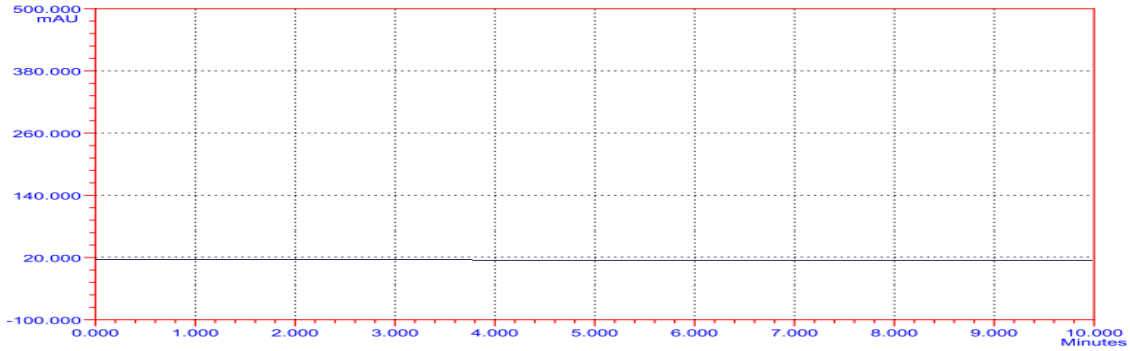


Figure 10 Blank HPLC Chromatogram

SYSTEM SUITABILITY TEST: The system suitability parameters were calculated and all system suitability parameter are within the acceptable range**.**

Table 5 System Suitability Parameter:

Parameter	**MON**	**BEP**
Retention Time(min)	4.36	5.42
Resolution	0.00	4.24
Theoretical plate	2925.31	4945.55
Symmetric Factor	1.10	0.98
Peak Area	24444	33533

METHOD VALIDATION

LINEARITY AND CALIBRATION CURVE: Calibration curve for MON was found to be 10-60 µg/ml with regression coefficient of 0.9979 showed in (Figure 11) and For BEP it was 10-60 µg/ml with correlation coefficient of 0.998 (Figure 12).Figure 3 shows the overlay chromatogram of MON (10-60 µg/ml) and BEP (10-60 µg/ml) at 225 nm.The calibration range was prepared in such a way that the ratio of combination was maintained throughout simultaneous estimation of both drugs in bulk and synthetic mixture.The result of calibration curve and regression analysis of calibration curve are shown in Table 6,7 and 8.

Table 6 Result of calibration curve for Montelukast Sodium (MON)

Concentration (µg/ml)	**Area ^{n}**	**SD ^{n}**	**%RSD**
10	28560.55	468.60	1.64
20	40371.53	787.01	1.95
30	52651.82	902.10	1.71
40	63231.32	876.40	1.39
50	75143.23	1078.48	1.44
60	83927.33	1342.52	1.60

n=Average of 6 replicate

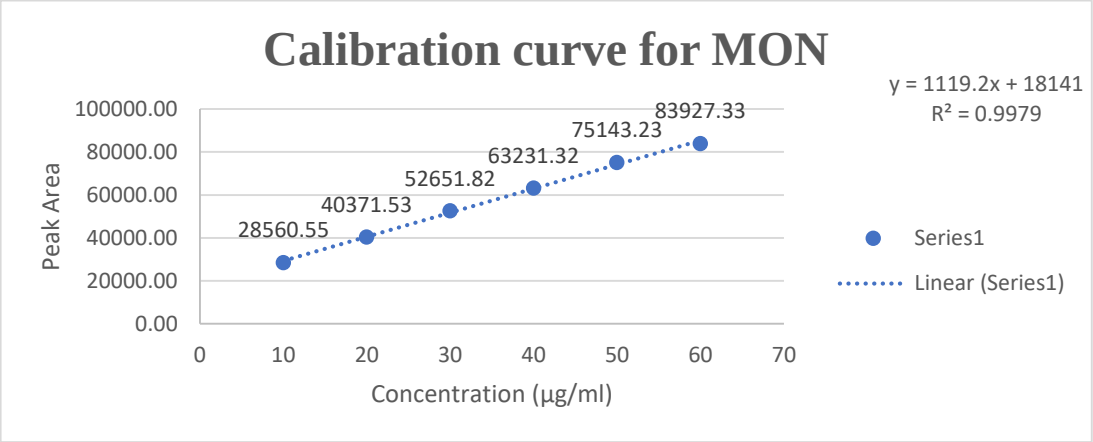


Figure 11 Calibration curve for MON **at** 225 **nm**

Table 7 Result of calibration curve for BEP **at 225 nm**

Concentration (µg/ml)	Area	SD	%RSD
10	33463.65	339.74	0.53
20	54683.38	611.53	0.47
30	80543.28	426.05	0.93
40	102481.00	896.58	0.64
50	127887.77	2095.94	0.76
60	156548.57	2555.42	1.78

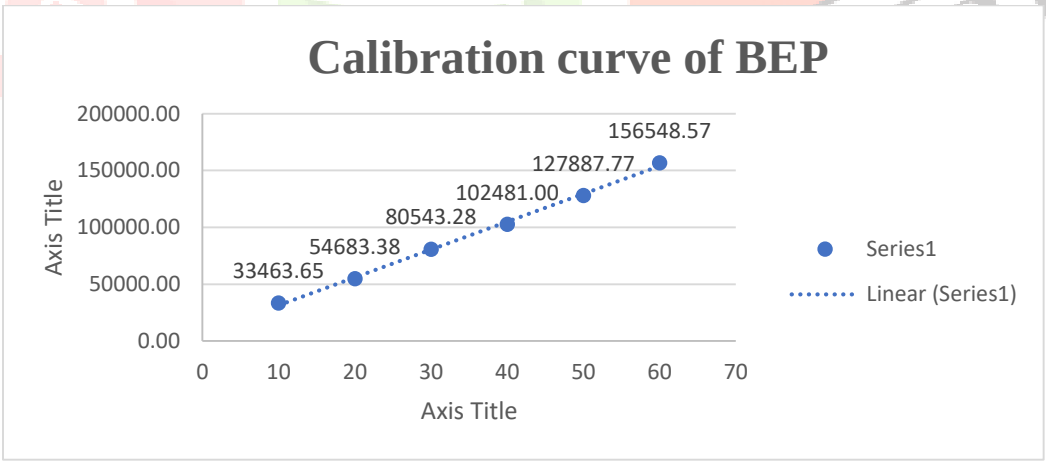


Figure 12 Calibration curve for BEP at 225 nm

Table 8 Linear regression parameters for estimation of MON and BEP

Parameters	MON	BEP
Calibration range (µg/ml)[a]	10-60	10-60
Regression equation	y = 1119.2x + 18141	y = 2448.5x + 6903.7
Standard deviation of slope	25.880	54.10
Standard deviation of intercept	603.32	1400.15
Correlation coefficient (r^2)	0.9979	0.998

[a] = 6 replicates

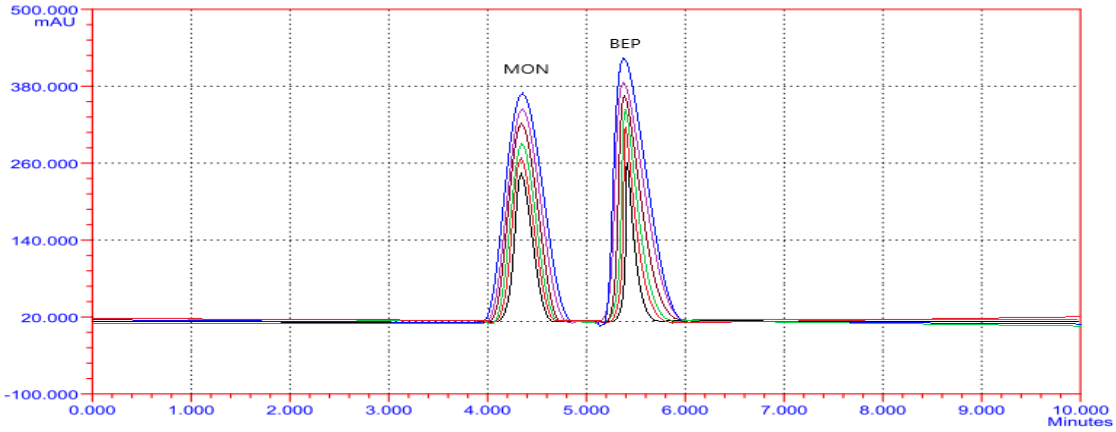


Figure 13 Overlay chromatogram of MON (10-60 µg/ml) and BEP (10-60 µg/ml) at 225 nm

PRECISION:The peak areas obtained were used to calculate mean and % RSD values.In RP-HPLC method, repeatability has been carried out by injection repeatability. Injection repeatability was carried out by analysing the samples of MON and BEP samples in which MON (30 µg/ml) and BEP (30µg/ml) six times and peak area was measured which was shown in **Table 9**.

Table 9 Repeatability data

Sr. No.	MON (30µg/ml) Peak Area (n=6)	BEP (30µg/ml) Peak Area (n=6)
1	80585.2	53552.25
2	81135.8	51552.35
3	80747.5	52871.81
4	80563.2	53514.75
5	80382.4	51551.18

6	79845.6	52865.76
Average	80543.28333	52651.24
SD	426.05	902.10
% RSD	0.53	1.71

The precision of method was determined by carring out intraday and interday precision.

Intraday precision was determined by analysing sample solution of MON (10 µg/ml, 30 µg/ml and 60 µg/ml) and for BEP (10 µg/ml, 30 µg/ml and 60 µg/ml) which covers lower, medium and high concentrations of the calibration curve three times on the same day.Intraday precision was determined by analysing sample solution of MON and BEP with same concentration as intraday at three levels covering lower, middle and high concentration over the 3 different successive days.The peak areas obtained were used to calculate mean and % RSD values shown in **Table 10**. The percentage (%) RSD was found to be less than 2 % which indicate method is precise.

Table 10 Precision data for MON and BEP

Drug	Conc. (µg/ml)	Intraday (n=3) ± SD	% RSD Intraday	Interday (n=3) ± SD	% RSD Interday
MON	10	28247.53 ± 292.88	1.04	28514.20 ± 501.96	1.76
	30	52979.48 ± 490.45	0.93	52658.48 ± 1016.92	1.93
	60	82927.33 ± 1000	1.21	83179.89 ± 1396.07	1.68
BEP	10	33338.57 ± 262.46	0.79	33488.73 ± 342.18	1.02
	30	80822.83 ± 282.92	0.35	80697.07 ± 781.75	0.97
	60	156688.04 ± 1402.24	0.89	158006.84 ± 2177.93	1.38

ACCURACY: The accuracy of method was determined by calculating % recovery of drug by standard spiking of 50 %, 100 % and 150 % of standard in pre quantified sample solution from synthetic mixture. For MON (10µg/ml, 20 µg/ml, 30 µg/ml) were spiked, and for BEP (10µg/ml, 20 µg/ml, 30 µg/ml) were spiked in pre quantified sample solution which were prepared from synthetic mixture. Method was accurate with % recovery of 98.19-102.37 % for MON and 98.22-100.65 % for BEP shown in **Table 11**.

Table 11 Accuracy study

Drug	% Level of spike	Amount of drug taken (µg/ml)	Amount of drug spiked	Total Amount of drug	Mean Area^n ± SD	Amount of drug found (µg/ml)	%Recovery
MON	0	20	0	20	40119.90 ± 409.75	19.64	98.19
	50	20	10	30	52512.82 ± 380.00	30.71	102.37
	100	20	20	40	63893.43 ± 695.48	40.88	102.20
	150	20	30	50	74818.90 ± 1512.80	50.64	101.28
BEP	0	20	0	20	54999.77 ± 437.31	19.64	98.22
	50	20	10	30	80822.83 ± 282.92	30.19	100.63
	100	20	20	40	103781.87 ± 1279.26	39.57	98.92
	150	20	30	50	130125.07 ± 2081.67	50.33	100.65

n= Replicate of 3

LOD and LOQ:LOD and LOQ of MON and BEP were determined by equation according to ICH guideline. LOD for MON and BEP was found to be 1.62 and 1.71 µg/ml respectively. LOQ for MON and BEP was found to be 5.39 and 5.72 µg/ml respectively as shown in **Table 12** indicating sensitivity of the method.

Table 12 LOD and LOQ for HPLC method

Parameters	MON (µg/ml)	BEP (µg/ml)
LOD	1.62	1.71
LOQ	5.39	5.72

ROBUSTNESS: The small deliberate variations in liquid chromatography conditions were used to evaluate the robustness of the assay method.

In this study, the chromatographic parameters monitored were retention time, area, capacity factor, tailing factor and theoretical plates.The results of analysis of robustness study are as shown in **Table 13**, where %RSD less than 2 indicate that the method is robust, and is not affected by small changes in routine use.

Table 13 Robustness data

EFFECT OF CHANGE IN VOLUME OF PHOSPHATE BUFFER									
	65 ml			**70 ml**			**75 ml**		
	Peak Area	**% RSD**	**Rt (min)**	**Peak Area**	**% RSD**	**Rt (min)**	**Peak Area**	**% RSD**	**Rt (min)**
MON	39916.85	0.25	4.68	39975.65	0.444	4.32	41371.53	0.864	4.01
BEP	54738.6	1.236	5.97	54738.6	0.906	5.46	54298.7	0.122	4.89
EFFECT OF CHANGE IN FLOWRATE									
	0.9 ml/min			**1 ml/min**			**1.1 ml/min**		
	Peak Area	**% RSD**	**Rt (min)**	**Peak**	**% RSD**	**Rt (min)**	**Peak Area**	**% RSD**	**Rt (min)**
MON	41391.53	0.25	4.93	39975.65	0.444	4.32	39721.58	0.864	3.8
BEP	133345.9	1.236	5.86	54738.6	0.906	5.46	55863.7	0.122	4.12
EFFECT OF CHANGE IN DETECTION									
	220 nm			**225 nm**			**230 nm**		
	Peak Area	**% RSD**	**Rt (min)**	**Peak Area**	**% RSD**	**Rt (min)**	**Peak Area**	**% RSD**	**Rt (min)**

MON	39521.58	0.25	4.33	975.65	0.444	4.32	39115.51	0.864	4.33
BEP	4174.2	1.236	5.45	54738.6	0.906	5.46	54174.2	0.122	5.46

SPECIFICITY: There was no interfering peak at Rt of MON and BEP from the excipient added in preparation of synthetic mixture, thereby confirming the specificity of method.**Figure 14** shows the chromatogram of optimized chromatographic condition.

SUMARY OF VALIDATION

Table 14 Summary of validation parameter for MON and BEP by RP-HPLC method

Sr. No.	**Parameters**	**Result**	
		Montelukast Sodium	**Bepotastine Besilate**
1.	Concentration range (µg/ml)	10-60	10-60
2.	Retention time (min.)	4.36	5.42
3.	Detection limit (µg/ml)	1.62	1.71
4.	Quantification limit (µg/ml)	5.39	5.72
5.	Accuracy (% Recovery) (n=3)	98.19-102.37%	98.22-100.65%
6.	Precision (% RSD)		
	Repeatability (n=6)	0.53	1.71
	Intraday Precision (n=3)	0.93-1.21	0.35-0.89
	Interday Precision (n=3)	1.68-1.93	0.97-1.38
7.	Specificity	Specific	Specific
8.	Robustness	Robust	Robust

ANALYSIS OF SYNTHETIC MIXTURE: The proposed method was applied on determination of MON and BEP in its synthetic mixture and % amount of drug found to be more than 98%. The results were shown in **Table 15**, **Figure 14** shown shows overlay chromatogram of API and synthetic mixture of MON (20 µg/ml) and BEP (20 µg/ml) in form of overlay using optimized chromatographic condition.Synthetic mixture contains equivalent amount of 10 mg Montelukast sodium and 10 mg of bepostatine.

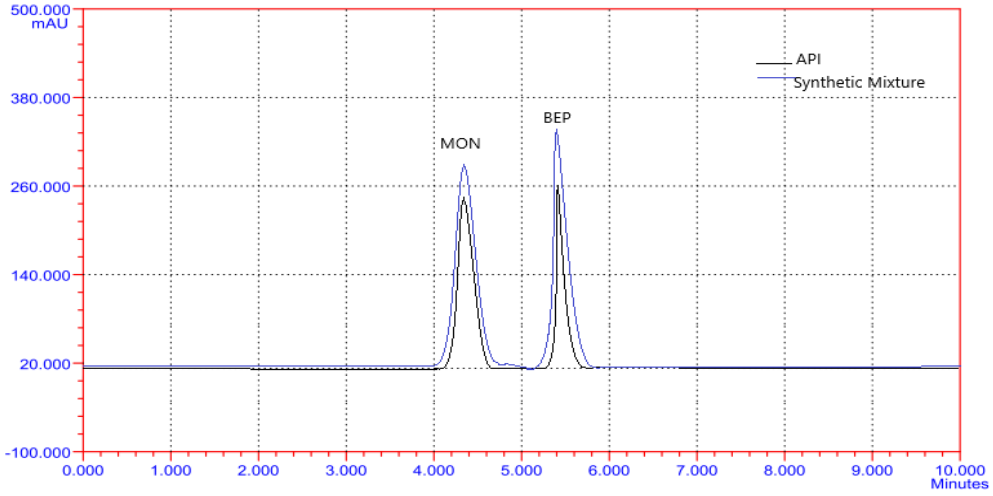


Figure 14 overlay chromatogram of API and synthetic mixture of MON and BEP

Table 15 Analysis of synthetic mixture

Sr. no.	Drug	Labeled amount(µg/ml)	Mean amount found[a]	% Assay Mean ± SD (n=3)	% RSD
1	MON	20	19.66 ± 0.37	98.32 ± 1.84	1.87
2	BEP	20	19.65 ± 0.28	98.19 ± 1.42	1.45

[a] **mean of three replicates**

CONCLUSION:

➢ A simple, sensitive and accurate RP-HPLC method was developed for simultaneous estimation Montelukast and Bepotastine besilate.In RP-HPLC method, good resolution and separation of two drugs was achieved. The C18 (250 mm×4.6 mm×5 µm) used as stationary phase, Phosphate buffer, Methanol and ACN in the volume ratio 70:20:10 % v/v/v was used as mobile phase and detection wavelength was 225nm. Retention time of Montelukast and Bepotastine besilate were found to be 4.36 min and 5.42 min respectively with a flow rate of 1 ml/min. The proposed RP-HPLC method was specific, accurate, precise and robust. Therefore, proposed method can be used for routine analysis of Montelukast and Bepotastine besilate in bulk as well as synthetic mixture.Validation parameters like Linearity, Accuracy, Precision, Robustness, System suitability, Specificity was tested. Observation of all these parameters leads to the point that developed RPHPLC method is linear, accurate, precise, specific and robust.LOD for MON and BEP was found to be 1.62 and 1.71 µg/ml respectively. LOQ for MON and BEP was found to be 5.39 and 5.72 µg/ml respectively. The accuracy of method was determined by calculating % recovery of drug by standard spiking of 50 %, 100 % and 150 % of standard in pre quantified sample solution was found to be % recovery of 98.19-102.37 % for MON and 98.22-100.65 % for BEP. It can be successfully adopted for routine quality control analysis of

Montelukast and Bepotastine besilate in synthetic mixture form without any interference from common excipients.

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