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RESEARCH ARTICLE

Analytical Method Development and Validation of Simultaneous Estimation of Pure and Tablet Dosage Form by RP-HPLC

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Abstract

The aim of this analytical research is to establish and validate the RP-HPLC (Reverse Phase High Performance Liquid Chromatography) method for concurrently quantifying Montelukast Sodium, Fexofenadine Hydrochloride, and Acebrophylline in a pharmaceutical formulation. The method utilized Rosagiline mesylate as an internal standard, employing a Shim-Pack Solar C18 Column (4.6 x 150 mm, 5 µm) as the stationary phase and a mobile phase composed of acetonitrile, methanol, and 10 mM Na_2HPO_4 buffer (50:30:20 % v/v/v, pH 5.5). The flow rate was maintained at 1.0 mL/min, and detection occurred at 210 nm. Validation followed ICH guidelines. The linear concentration ranges were 5-30 µg/ml, 10-60 µg/ml, and 10-100 µg/ml at 210 nm for Montelukast Sodium, Fexofenadine Hydrochloride, and Acebrophylline, respectively. Retention times were 7.643 min, 2.117 min, 3.863 min, and 3.050 min for Montelukast sodium, Fexofenadine Hydrochloride, Acebrophylline, and Rosagiline mesylate respectively. The RP-HPLC analysis of marketed formulations yielded concentrations within the ranges of 98.3–100.9%, 99.46–101.23%, and 99.82–101.74% for Montelukast sodium, Fexofenadine Hydrochloride, and Acebrophylline, respectively. Recovery fell within the range of 95.81-101.35% for both drugs. The proposed method has been validated as per ICH guidelines and successfully applied to the estimation of Montelukast, Fexofenadine and Acebrophylline in their combined tablet dosage form.

Keywords: Acebrophylline; Fexofenadine Hydrochloride; Internal Standard; Montelukast Sodium; RP-HPLC

Introduction

Montelukast (MON) is an oral dose drug that is FDA-approved for treating chronic asthma and prophylaxis and the prevention of exercise-induced bronchoconstriction. It is also approved for the relief of symptoms of both seasonal and perennial allergic rhinitis [1-4]. Fexofenadine (FEX) is a medication used in the management and treatment of allergic rhinitis and chronic urticaria. It is a second-generation antihistamine. Fexofenadine comes in multiple different forms. It may be administered orally as a tablet, oral suspension (syrup), or orally disintegrating tablets [5, 6]. Acebrophylline (ACE) has mucolytic properties and is used as bronchodilators. Ambroxol acefyllinate is given in an oral dose of 100 mg twice daily [7]. ACE is an airway mucus regulator with anti-inflammatory action. The molecule contains ambroxol, which facilitates various steps in the biosynthesis of pulmonary surfactant, theophylline-7 acetic acid whose carrier function raises blood levels of ambroxol, thus rapidly and intensely stimulating surfactant

production. The resulting reduction in the viscosity and adhesivity of the mucus greatly improves ciliary clearance. ACE also exerts an inflammatory effect [8]. All these drugs individually or in combination with other drugs are reported and have been estimated either in the combination of two or separately but do not involve simultaneous determination of MON, FEX, and ACE Review of literature revealed that RP- HPLC, mass spectroscopy, HPTLC (High Performance Thin Layer Chromatography) and UV-Visible (Ultraviolet Visible) spectroscopic methods have been done for simultaneous estimation of Montelukast and Fexofenadine in pharmaceutical dosage form [9-12]. Likewise, estimation of Montelukast and Acebrophylline simultaneously by UV spectroscopic method and RP- HPLC method has been reported by evaluating their different method validation parameters; Limit of Detection, Limit of Quantitation, Linearity, Range [13-16]. There is no analytical method for Montelukast, Fexofenadine and Acebrophylline of the combined dosage form. For this purpose, pure and tablet dosage form of Montelukast, Fexofenadine, and Acebrophylline were used. Hence, a successful attempt has been made to estimate these drugs simultaneously by RP-HPLC method in the present work. The proposed methods were optimized and validated as per ICH guidelines

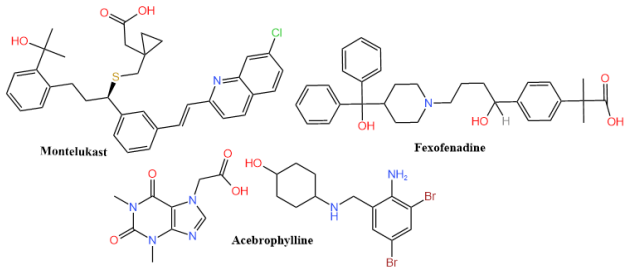

Figure 1: *Chemical structure of the drugs; Fexofenadine, Montelukast, and Acebrophylline*

Materials and Methods

Chemicals and Apparatus

Fexofenadine Hydrochloride, Montelukast Sodium, and Acebrophylline in % w/w were obtained from Triveni Pharmaceuticals Pvt. Ltd. Methanol and Acetonitrile of HPLC grade were purchased from Rankem (RFCL limited) New Delhi. Disodium hydrogen orthophosphate, orthophosphoric acid and Rosagiline mesylate (standard) were purchased from Central Drug House (CDH) Pvt. Ltd New Delhi (India). The nylon filters (25mm, 0.2 µm) were purchased from Advanced Micro Devices Pvt. Ltd. Chandigarh, India. Double distilled water was used throughout the experiment which was generated in-house. The sample drug formulation FEX, MON, ACE tablets was obtained from the Xieon Life Sciences Pvt. Ltd. The chromatographic analysis was performed on HPLC system of Shimadzu LC-20AT (Milford, USA) composed of 515 HPLC pump as a solvent delivery system equipped with Rheodyne injection valve with a 20 µL loop, Shimadzu SPD-20A UV-Visible detector and separation was performed on Shim-Pack Solar C18 column (4.6 mm × 150 mm, 5 µm i.d.) at 25℃ column temperature. Chromatographic data were recorded and processed using Spinchrome CFR software (Version 2.1.4.93).

Preliminary analysis of drugs

Montelukast sodium and Fexofenadine hydrochloride are official in Indian Pharmacopoeia (IP) 2010. Hence, preliminary analysis of each drug was performed according to IP 2010. Acebrophylline monograph is not officially in any pharmacopeia. Hence, preliminary analysis of ACE was performed by referring to Drug bank online DB13141. Preliminary analysis was done by studying description (colour and texture), solubility (in methanol, ethanol, and water), and

identification test was performed by scanning in FTIR (Fourier-Transform Infrared Spectroscopy).

Chromatographic conditions

The composition of Acetonitrile: Methanol: Disodium hydrogen orthophosphate (50:30:20 % v/v/v) has been found to be satisfactory for the complete separation of individual compounds. Before use, the mobile phase was prepared by mixing 50 mL of acetonitrile, 30 mL of methanol and 20 mL of 10mM Na_2HPO_4 buffer whose pH was adjusted to 5.5 using 1% orthophosphoric acid. The mobile phase was filtered through 0.2 µm super 200 membrane filter using vacuum pump and ultrasonicated for 10 mins. In the present study, Rosagiline mesylate (ROSA) was selected as internal standard based on the compatibility of internal standard with the drugs, resolution, and sharpness of the peak. The work was carried out in an air-conditioned room maintained at temperature 25±2 ℃ and total run time was 12 min.

Selection of wavelength

The standard stock solution of FEX (60 µg/mL), MON (5 µg/mL), ACE (100 µg/mL), and ROSA (10 µg/mL) were prepared in HPLC grade methanol. The resulting solutions were scanned over the UV range (200-400 nm), maximum absorbance was found at λ_{max} 210 nm for all drugs (overlaid spectra)

Preparation of mobile phase and stock solutions and test solutions

The mobile phase was prepared by mixing 50 ml of acetonitrile, 30 ml of methanol and 20 ml of 10mM Na_2HPO_4 buffer whose pH was adjusted to 5.5 using 1% orthophosphoric acids. The mobile phase was filtered through 0.2 µm super 200 membrane filter using vacuum pump and ultrasonicated for 10 mins.

Stock solution was prepared by dissolving FEX, MON and ACE (10 mg each) that were weighed accurately and separately transferred into 100 mL volumetric flasks. Similarly, the ternary mixture of FEX, MON and ACE was also prepared as that of stock solutions by dissolving 10 mg of all drugs in 50 mL of mobile phase, then sonicated for 10 min and diluted up to 100 mL. A series of solutions were prepared in the concentration range of 10-60, 5-30, 10-100 µg/mL of FEX, MON and ACE respectively. Standard stock solution of FEX, MON, ACE, and ROSA was prepared at concentration 10 µg/mL.

Calibration curve for FEX, MON, and ACE

The calibration curve was prepared by injecting concentration of 10-60, 5-30, 10-100 µg/mL of FEX, MON and ACE, and ternary mixture in triplicate to the HPLC system at detection wavelength of 210 nm. Mean of n = 6 determinations was plotted as the standard curve. The calibration curve was tested and validated with inter-day and intra-day measurements. Peak areas ratios between MON to ROSA, FEX to ROSA and ACE to ROSA were calculated.

Sample preparation of tablet formulation

Twenty tablets of MON, FEX and ACE (Airflow HD) were weighed and crushed to obtain fine powder. An accurately weighed tablet powder equivalent to about (10 mg of MON 120 mg of FEX, 200 mg of ACE) was transferred to 100 mL volumetric flask and sonicated for 30 min in 50 ml of HPLC grade methanol and made up the volume with same solvent to give a solution of 10 µg/mL of MON, 120 µg/mL of FEX and 200 µg/mL of ACE. The solution was filtered through Whatman filter paper and this solution was used as 'Sample Stock A'. from 'stock sample A' required concentration was prepared (5 µg/mL, 60 µg/mL, and 100 µg/mL).

RP-HPLC method

For marketed formulation, an assay was performed to check the purity of each drug in

formulation and percentage purity of the drugs was calculated. The 50 µL of standard and sample solution were injected by knowing the peak area of FEX, MON, and ACE and the number of drugs in sample was calculated.

RP- HPLC Method validation

Validation of the new simultaneous RP-HPLC methods was carried out as recommended by the International Conference on Harmonization[17] for all the validation parameters including accuracy, precision, linearity and range, limit of detection (LOD), limit of quantitation (LOQ), robustness, and system suitability.

Accuracy

The accuracy of the method was determined by recovery studies using the standard addition method. Pre analyzed samples were spiked with standard drugs (FEX, MON, ACE and ROSA) at three different concentration levels, i.e., 80%, 100% and 120 % and the mixtures were re-analyzed by the proposed method in triplicates. Data obtained was analyzed for percent recovery. For RP-HPLC method, known amount of the standard solution at concentration of 80%, 100% and 120 % individual drug were added to a pre-analyzed sample solution of FEX (120 µg/ mL) MON (10 µg/mL), ACE (200 µg/mL) and ROSA (10 µg/mL).

Precision

The precision of an analytical method was studied by performing intermediate precision (intra-day and inter day precision) and repeatability. Moreover, precision can be reported as standard deviation or relative standard deviation for a statistically significant number of replicate measurements.

Linearity and Range

The linearity of the method was determined by analyzing several aliquots of standard solution of FEX, MON, and ACE. For RP-HPLC method, linear correlations were obtained between peak area and concentration for FEX, MON, and ACE in the concentration ranges of 10-60, 5-30, 10-100 µg/mL, respectively.

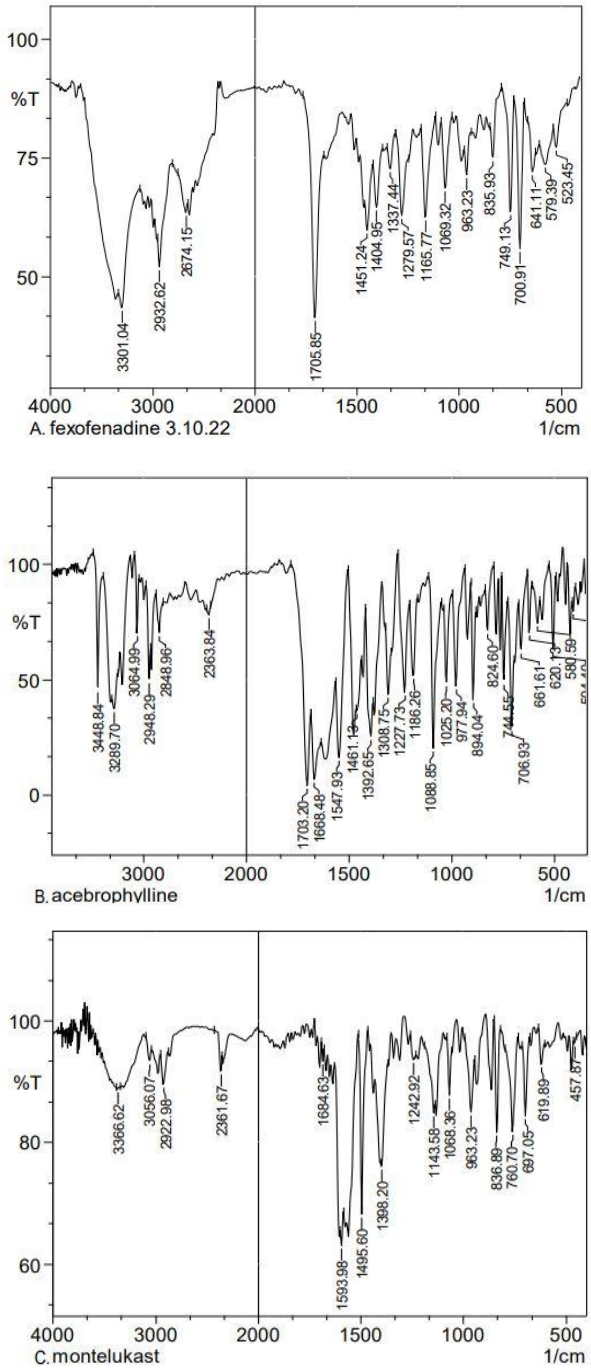

Figure 2: *FTIR Spectrum of Fexofenadine (A), Acebrophylline (B), and Montelukast (C)*

Limit of detection and Limit of Quantification

In analytical method development and validation, the Limit of detection (LOD) is the

lowest analyte concentration detectable but not precisely quantifiable, typically determined at a 3:1 signal-to-noise ratio. The Limit of quantification (LOQ) is the lowest analyte concentration measurable with acceptable accuracy and precision under defined conditions, often established at a 10:1 signal-to-noise ratio. Both LOD and LOQ are crucial parameters in assessing analytical procedure performance.

Hence, the LOD and LOQ were calculated as [18]:

LOD = 3.3×σ/S

LOQ =10×σ/S

Where, σ = Standard deviation of the lowest standard concentration; S = Slope of the standard curve.

Robustness

Combined standard solutions of MON (5 µg/mL), FEX (60 µg/ml) and ACE (100 µg/mL) with ROSA (10 µg/mL) were prepared and analyzed at different pH (5.39, 5.5, 5.61) and at different flow rate (0.98, 1.00, 1.02 mL/min) and different organic solvent content in mobile phase (51:30:19, 50:30:20, 49:30:21 %v/v/v), separately.

System Suitability

Combined standard solutions of MON (5 µg/mL), FEX (60 µg/ml) and ACE (100 µg/mL) with ROSA (10 µg/mL) were prepared and analyzed six times. Chromatograms were studied for different parameters such as tailing factor, resolution and theoretical plates to determine that whether they comply with the recommended limit or not.

Results and Discussion

Preliminary analysis of drug

Preliminary analysis of MON, FEX and ACE such as description, solubility and identification test by using FTIR analysis studied and the results were found to be satisfactory as shown in (**Fig. 2**). The FTIR spectra were obtained over a scan range of 500-4000 cm^{-1} frequencies and each functional group was analysed. The FTIR spectra of FEX includes; -OH stretching (3301 cm^{-1}), -CH aromatic stretching (2932 cm^{-1}), -N-H stretching (3301 cm^{-1}), $-CH_2$ aliphatic stretching (2932 cm^{-1}), $-CH_3$ stretching (2674 cm^{-1}), and -C=O carboxylic acid stretching (1705 cm^{-1}) (**Fig. 2A**). The prominent FTIR spectra for AEC were reported as; $-NH_2$ stretching (3289 cm^{-1}), -OH stretching (3448 cm^{-1}), -Br stretching (744 cm^{-1}), -CH aromatic stretching (2948 cm^{-1}), $-CH_2$ aliphatic stretching (3064 cm^{-1}), -C=O stretching (1668 cm^{-1}), $-CH_3$ stretching (3064 cm^{-1}), and -C=O carboxylic acid stretching (1703 cm^{-1}) (**Fig. 2B**). For MON, the important functional groups and their FTIR spectrum include; -OH stretching (3400 cm^{-1}), H-Cl stretching (760 cm^{-1}), -COOH stretching (3366 cm^{-1}), $-CH_2$ aromatic stretching (2922 cm^{-1}), $-CH_2$ aliphatic stretching (3056 cm^{-1}), and S-H stretching (1398 cm^{-1}) (**Fig. 2C**).

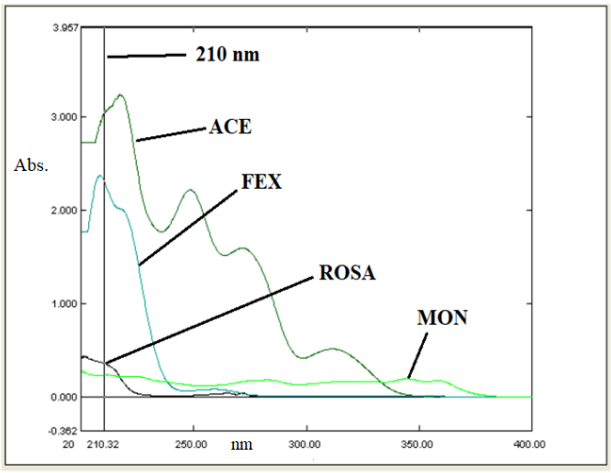

Figure 3: *UV-Visible overlain Spectra of MON (5 µg/mL), FEX (60 µg/mL), ACE (100 µg/mL) and ROSA (10 µg/mL) in Methanol to determine the analytical wavelength*

Selection of analytical wavelength

To select analytical wavelength, all these drugs i.e., MON, FEX, ACE and ROSA were prepared in mobile phase separately in concentration of 5, 60, 100 and 10 µg/mL.

These solutions were scanned in the UV region of 200-400 nm and the overlain spectra were observed for selection of analytical wavelength. It was found that all the three drugs showed good absorbance at 210 nm. So, 210 nm was selected as an analytical wavelength (**Fig. 3**).

Table1: Linear regression analysis of calibration curves for MON, FEX, and ACE

Parameters	MON	FEX	ACE
Linearity range (µg/mL)	5-30	10-50	10-60
Slope	0.0831	0.0859	0.1065
Intercept	0.1089	0.5085	0.1790
Correlation coefficient (r^2)	0.9997	0.9991	0.9995
LOD	0.9865	1.254	1.7624
LOQ	3.2554	4.1388	5.8159

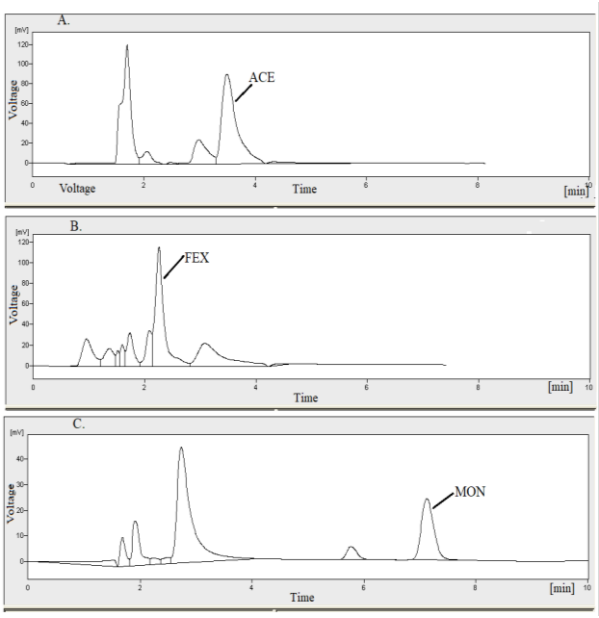

Figure 4: *Chromatography of optimized condition for A. ACE, B. FEX, and C. MON at a selected mobile phase of ACN, methanol and buffer (50:30:20) at a pH of 5.5 with a flow rate of 1 mL/min*

Optimization of chromatographic condition

For optimizing chromatographic condition, a number of trials have been tried on Shim-Pack Solar C18 Column (4.6× 150 mm, 5 µm). For selecting the mobile phase composition MON, FEX, and ACE were prepared separately of 100 µg/mL concentration in HPLC grade methanol. Trials were started with different composition of ACN: Water like 10:90, 20:80, 30:70, 40:60, 50:50, 60:40, 70:30, 80:20, 90:10 %v/v, with 1 mL/min flow rate. It was observed that in 10:90 and 90:10 ratio of ACN and water, the three drugs have shown the peaks but did not show good theoretical plate.

Trials have continued with buffer (Na_2HPO_4) and ACN at a pH of 6.5 with the ratio of 30:70. Even though there were some disturbances peaks, ACE and FEX shows good peaks. Then, 0.1% TEA has been added to the mobile phase with a composition of ACN, methanol and TEA (50:30:20) at a pH of 6, 5 and 5.5. At pH 6, FEX shows splitting of peaks and MON shows two separate peaks. So, trials were continued at pH 5 which shows the merging of FEX peaks better. It was noted that at acidic pH, FEX shows better peaks. TEA was replaced by Disodium buffer which is adjusted with 1% o-phosphoric acid. Different pH like 6, 6.2, 7, 7.1 and 7.5 have been tried but, in the basic mobile phase FEX did not show response. So, a mobile phase of ACN, methanol and buffer (50:30:20) at a pH of 5.5 with a flow rate of 1 mL/min was selected because all the three drugs showed good theoretical plates (above 2000) and asymmetry of less than 2. In the selected mobile phase, ACE peak was eluted at 3.923 min, MON at 7.692 min, and FEX at 2.178 min, respectively (**Fig. 4**).

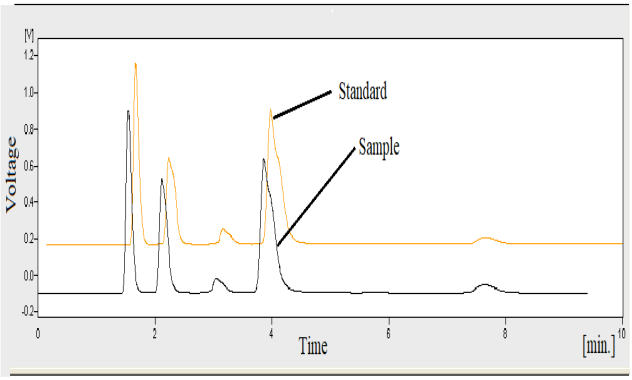

Figure 5: *Overlain (3D view) chromatograms of sample and standard solution*

Linearity Study

MON, FEX and ACE were found to be linear in the concentration range of 5-30 µg/mL, 10-60 µg/mL and 10-100 µg/mL, respectively. The r^2 values obtained for the three drugs were 0.9997, 0.9991 and 0.9995 respectively (**Table 1, Fig. 5**).

Assay of Marketed Formulation

The amounts of drugs present in the marketed formulation. (Airflow HD) was calculated using single point equation (**Table 2**). The mean % of MON, FEX and ACE were found in the range from 98.3-100.9%, 99.46-101.23% and 99.73-101.74%, respectively. Marketed formulation was analyzed by the proposed method and assay result of marketed formulation was shown in **Table 3**.

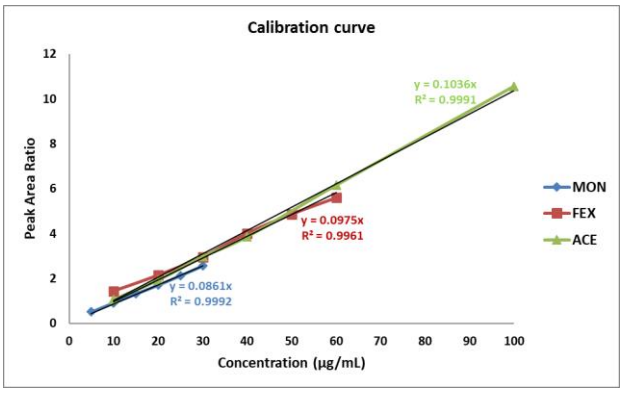

Figure 6: *Linear calibration curve of MON, FEX, and ACE for accuracy study*

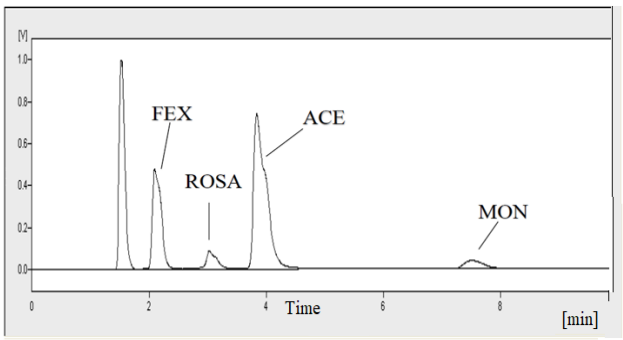

Figure 7: *Chromatogram of mixed standard solution of MON (5 µg/mL), FEX (60 µg/mL), ACE (100 µg/mL) and ROSA (10 µg/mL)*

Table 2: Results of chromatogram of sample solution (Airflow HD)

Analyte	RT	Area (mV.s)	TF	TP	Resolution
MON	7.643	1074.196	1.379	2882	2.678
FEX	2.117	6029.016	1.517	3133	4.785
ACE	3.863	11272.181	1.881	2341	2.145
ROSA	3.050	1157.573	1.289	4070	-

RT = Retention Time (min), TF = Tailing Factor, TP = Theoretical Plates

Table 3: Assay results of tablet formulation by RP-HPLC Method

Statistics	Amount found			% Assay		
	MON (100 µg/mL)	FEX (120 µg/mL)	ACE (200 µg/mL)	MON (100 µg/mL)	FEX (120 µg/mL)	ACE (20 µg/mL)
Mean	99.8	120.38	201.34	99.93	100.31	100.71
±SD	0.0983	0.8679	1.7899	0.9121	0.7235	0.8337
%RSD	0.9853	0.7209	0.8890	0.9127	0.7212	0.8279

Table 4: Statistical validation data for accuracy study

Level of % Recovery	Mean (% recovery) ±Sd			%RSD		
	MON	FEX	ACE	MON	FEX	ACE
80%	100.08±1.282	100.57±1.442	100.57±0.0513	1.2756	1.4379	0.0512
100%	99.73±0.736	100.34±0.736	100.34±0.444	0.66984	0.7345	0.4442
120%	99.72±1.343	100.01±0.578	100.01±0.427	1.3359	0.5765	0.4275

Table 5: Summary of validation parameters

Parameters	MON	FEX	ACE
Recovery %	99.73-100.08	100.29-101.12	100.01-100.57
Repeatability (C.V. n = 6)	0.921	3.941	6.151
Precision (% RSD)			
Intra-day (n = 3)	0.8301-1.4052	0.4684-0.0615	1.3174-0.1352
Inter-day (n = 3)	1.6281-1.7730	0.9361-1.2433	1.7665-0.0162
LOD	0.9865	1.254	1.7624
LOQ	3.2554	4.1388	5.8159
Robustness	Robust	Robust	Robust
Solvent stability	Suitable	Suitable	Suitable

Validation parameters

This method was validated in accordance to ICH guidelines. Accuracy was determined by calculating the recovery. The different level percentage recovery along with their % RSD is given in **Table 4** and **Fig. 6**.

Table 6: Result of robustness study: variation in organic solvent ratio in mobile phase

MP	Analyte	RT	TF	TP	Resolution
51:30:19	MON	7.612	1.352	2922	2.615
	FEX	2.096	1.493	3206	4.189
	ACE	3.859	1.874	2219	2.173
	ROSA	3.032	1.275	4086	-
50:30:20	MON	7.643	1.379	2882	2.678
	FEX	2.117	1.517	3133	4.785
	ACE	3.863	1.881	2241	2.145
	ROSA	3.050	1.289	4070	-
49:30:21	MON	7.7033	1.386	2809	2.234
	FEX	2.205	1.564	2977	4.817
	ACE	3.869	1.890	3107	2.176
	ROSA	3.061	1.282	4064	-

MP = Mobile phase (ACN: MeOH: Na_2HPO_4), RF = Retention factor, TP = Theoretical plates, TF = Tailing factor. The % RSD was found to be less than 4 % for each drug; Mean of 3 estimations.

Moreover, percentage of recovery of MON, FEX and ACE were found in the range from 99.73-100.08%, 100.29-101.12% and 100.01-100.57%, respectively (**Table 5**). Precision of the method was determined by % RSD found among intra-day precision (n = 3), inter-day precision (n = 3), and repeatability (n = 6). It was found to be less than 2 (Table 5). The repeatability of MON, FEX, and ACE was found to be 0.921, 3.941, and 6.51, respectively. The LOD and LOQ of MON were found to be 0.9865 and 3.2554, respectively. LOD and LOQ of FEX were found to be 1.2542 and 4.1388, respectively. LOD and LOQ of ACE were found to be 1.7624 and 5.8159, respectively (**Table 5**).

The method was specific as no interference observed when the drugs were estimated in presence of excipients. For robustness study, the effect of change in pH (2%) of mobile phase, organic phase ratio (2%) and flow rate (2%) on the retention time, asymmetry factor, theoretical plates and resolution were studied. Combined standard solutions of MON (5 µg/mL), FEX (60 µg/mL), ACE (100 µg/mL) with ROSA (10 µg/mL) were prepared and analyzed at different pH (5.39, 5.5, 5.61) of the mobile phase, at different organic phase ratio (51:30:19, 50:30:20, 49:30:21 v/v/v) and at different flow rate (0.98, 1, 1.02 mL/min) (**Fig. 7**). Percentage RSD of retention time of each peak in all three variables was found to be less than 4% (**Table 6**). The % RSD value for system suitability was found to be less than 2% (**Table 7**).

Table 7: System suitability results of the proposed method (n=6)

Analyte	RF	TP	TF	%RSD	
				Rt	Peak area ratio
MON	2.778	3419	1.348	1.542	0.627
FEX	4.583	4362	1.261	0.298	0.584
ACE	2.245	2517	1.590	0.964	0.817
ROSA	-	4298	1.152	1.160	-
Required limits	R>2	N>2000	T<2	RSD < 2%	

RF =Retention factor, TP = Theoretical plates, TF = Tailing factor

Conclusions

RP- HPLC method for estimation of MON, FEX and ACE was developed with ROSA as internal standard. The method was validated according to ICH guidelines. Results of assay and validation study were found to be satisfactory. The proposed HPLC method provide simple, specific, precise, accurate, and reproducible quantitative analysis for simultaneous analysis of MON, FEX and ACE in combined dosage form. So, the methods can be applied for the routine analysis of MON, FEX and ACE from marketed formulation in

pharmaceutical industry. the medicines, healthcare, biology and chemistry.

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Author's Contribution Statement

Shankar Thapa: Conceptualization, Methodology, Experiment, Analysis, Writing-original manuscript **Bipindra Pandey:** Analysis, Writing-Review & Editing; **Mahalakshami Suresha Biradar**: Writing-Review & Editing

Conflict of Interest

All author declared no conflict of interest.

Data Availability Statement

The data that support the findings of this study can be made available from the corresponding author, upon reasonable request.

Abbreviations

ACE: Acebrophylline

FEX: Fexofenadine Hydrochloride

HPTLC: High Performance Thin Layer Chromatography

ICH: International Council for Harmonization of Technical Requirements of Pharmaceuticals for Human Use

IP: Indian Pharmacopoeia

MON: Montelukast Sodium

ROSA: Rosagiline mesylate

RP-HPLC: Reverse Phase High Performance Liquid Chromatography

UV-Visible Spectroscopy: Ultraviolet Visible Spectroscopy

Reference:

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