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Analytical Method Development And Validation Of Combination Of Anti-Asthmatic Drugs Montelukast And Doxofylline

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Abstract:

For qualitative and quantitative analysis there are different analytical techniques are available i.e. UV Spectrophotometry, HPLC and HPTLC chromatographic techniques. As per literature survey there are some UV, HPLC, UPLC and HPTLC analytical methods are available for Montelukast and Doxofylline individually and in a combination with other drugs but yet there is no stability indicating HPLC method reported for Montelukast and Doxofylline combinations. In current study analytical method develop and validate HPLC method is developed and validated for simultaneous quantitative estimations of Montelukast and Doxofylline. These present techniques are more efficient and sensitive as compared to other analytical techniques. The chromatographic separation achieved on Oyster C8 150 x4.6 mm 5 micron utilizing a mobile phase Water: Acetonitrile (150:850, v/v) and flow rate was 1 ml/min which shows good resolution and symmetric peak with retention time 4.507±0.04min and 9.561±0.1min for Montelukast and Doxofylline respectively. The detection wavelength selected was 250 nm. Linearity was observed in the range of 1-10 µg/ml for Montelukast and 4-24 µg/ml for Doxofylline. The degradation peaks developed under various stress condition for both Montelukast and Doxofylline were well separated from the peak of the intact drugs. The peaks of the Montelukast and Doxofylline were not remarkably shifted in the presence of the degradation peaks, which specify the stability-indicating character of the developed method.

Key word: Montelukast, Doxofylline, HPLC and stability indicating.

INTRODUCTION

Pharmaceutical analysis, discipline of chemistry involves isolation, characterization, quantification, separation, identification and determination of the relative amounts of components making up a sample of matter. It is mainly involved in the qualitative (identification or detection of compounds) and quantitative measurements of the substance present in bulk and pharmaceutical formulation. [1-5]

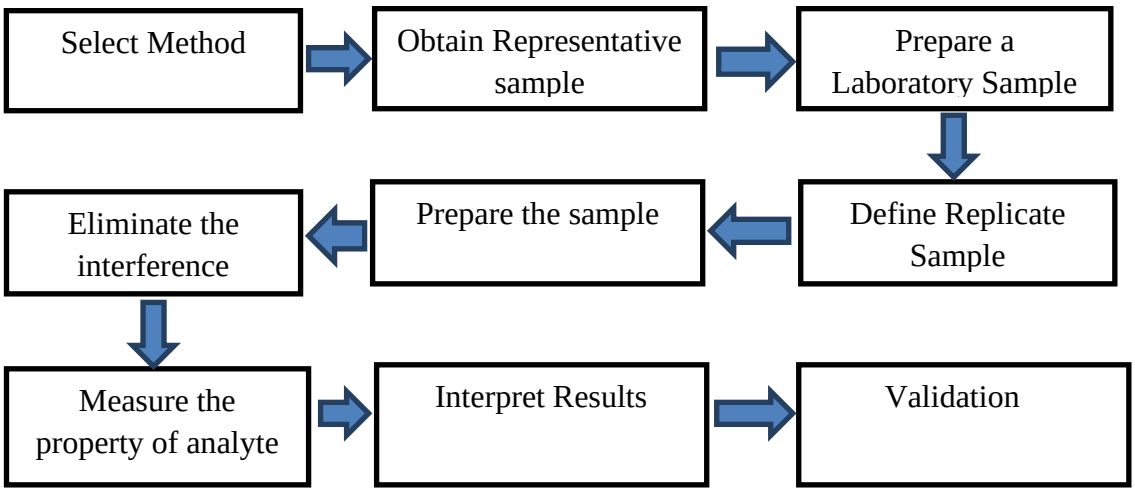

Fig. 1: Steps in quantitative analysis

Method development is the formalized process by which a set of experimental conditions designed to create a good analysis of a particular sample.

The process of method development can be qualitative or quantitative. The number of drugs are introduced into the market has been increasing at an alarming rate. These drugs may be either new entities or partial structural modification of the existing one. Very often there is a time lag from introduction of a drug into the market to the inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (resulting in their withdrawal from the market), development of patient resistance and introduction of better drugs by competitors. Under these conditions standards and analytical procedures for these drugs may not be available in the pharmacopoeias. Therefore it becomes necessary to develop newer analytical methods for such drugs.[6,7]

Reasons for the development of newer methods of drug analysis are: [8, 9]

- The drug or drug combination may not be official in any pharmacopoeias.
- A literature search may not reveal any proper analytical procedure for the drug due to patent regulations.
- Analytical methods for a drug in combination with other drugs may not be available.
- Analytical methods may not be available for the drug combination due to interference caused by excipients.
- Analytical methods for the quantitation of the drug in biological fluids may not be available.

Tswett defined chromatography as the technique in which the components of a mixture are separated on an adsorbent column in a flowing system. [9]

Aim of the current work is to develop and validate quantitative analytical methods antiasthamatic agents in combined dosage form that are competent to meet up the requirements to be entitled as 'stability indicating method'. The developed method must be proficient for resolving potential interferents specifically degradation products which are formed during stability evaluation period. The extent of degradation of API under stress conditions will be studied.

Extensive literature survey with respect to 'Stability-indicating analytical methods' revealed that stability indicating methods for antiasthamatic agents in combined dosage form as bulk and/or pharmaceutical formulations are not reported. Based on these observations, objectives of the study are framed i.e. Montelukast and Doxofylline.

Material and Methods

Pharmaceutically pure sample or working standard / drug sample of Montelukast was obtained as a gift sample from Unnati Pharmaceuticals Pvt Ltd and Doxofylline was obtained as a gift sample from Pure and Cure Healthcare Pvt.Ltd. Haridawar. The marketed formulation Spirodin M (Koye Pharmaceutical) is available in market purchased and used for work. All other chemicals used in the analysis were HPLC grade purchase from local market.

Instrumentation

Chromatographic separation was performed on revers phase Preparative HPLC with Flash System (RP-HPLC) system model Shimadzu, Oyster C8 150 x4.6 mm 5 micron, UV Detector, equipped with a LC-P 3000-M pump, sample injector and column thermostats. LC solution software was applied for data collecting and processing.

Chromatographic condition

Mobile phase : Water: Acetonitrile: Methanol (150:850 V/V)
Column : Oyster C8 150 x4.6 mm 5 micron
Detector wavelength : 250nm
Injection volume : 20 µl
Flow rate : 1.2 mL/min
Run time : 10 min

Solvent

Montelukast and Doxofylline both are soluble in Methanol. So methanol was used as a solvent.

Selection of Detection Wavelength:

Stock solutions (10µg/ml) of drugs were prepared in methanol and their isobestic point is observed at 250 nm on UV- spectrophotometer. Overlain spectra shown in **Fig. 2**

Preparation of Standard stock solution

Standard stock solution of Montelukast was prepared by dissolving 10 mg of drug in 10 ml methanol to achieve concentration of 1000 µg/ml which was diluted further with same solvent to obtain final concentration 10 µg/ml.

Standard stock solution of Doxofylline was prepared by dissolving 40 mg of drug in 10 ml methanol to get concentration 4000 µg/ml. The resulting solution was diluted to get final concentration 40 µg /ml.

Selection of mobile phase and chromatographic conditions:

Chromatographic separation study was carried out on the working standard solutions of Montelukast (10 µg/ml) and Doxofylline (10 µg/ml). Initially different solvents like methanol, acetonitrile, buffers in different proportions were tried. Finally the combination of **Water: Acetonitrile (150:850 v/v)** offered acceptable peak parameters. This mobile phase system observed to give good resolution with sharp peaks and the retention time as **4.507 ± 0.04 min** and **9.561 ± 0.15 min** for Montelukast and Doxofylline respectively. Results are shown in **Table 1** and **Fig. 3.**

Analysis of tablet formulation:

Twenty tablets were accurately weighed and average weight per tablet was calculated. Tablets were ground to fine powder and weighed tablet powder equivalent to 10 mg of Montelukast and 40 mg of Doxofylline and was transferred to 100 ml volumetric flask and dissolved in methanol. It was sonicated for 10 min and filtered through whatman filter paper no.41. Then the volume was made up to mark with methanol to obtain the concentration of 1000 µg/ml for Montelukast (4000 µg/ml for Doxofylline). Aliquot of this solution was diluted with mobile phase to get a final concentration 2 µg/ml of Montelukast and 8 µg/ml of Doxofylline. After setting the chromatographic conditions and stabilizing the instrument to obtain a steady baseline, the tablet sample solution was injected, and chromatogram was obtained. The injections were repeated six times. The peak areas were determined. The amount of each drug present in sample was calculated from the respective calibration. Results are shown in **Table 2** and **Fig. 4.**

Validation of Analytical Method [78]

Linearity

From standard stock solutions Montelukast, aliquots of 1, 2, 4, 6, 8, 10 ml were transferred into 10 ml volumetric flasks and diluted up to mark with mobile phase such that the final concentration in the range of 1-10 µg/ml.

From standard stock solutions of Doxofylline, aliquots of 1, 2, 3, 4, 5, 6 ml were transferred into 10 ml volumetric flasks and diluted up to mark with mobile phase such that the final concentration of in the range 4-24 µg/ml. Volume of 20µl of each sample was injected with help of syringe. All measurements were repeated six times for each concentration and calibration curve was constructed by plotting peak area versus concentration. The observation table shown in **Table 3** and calibration curve of Montelukast and Doxofylline shown in **Fig. 5 and 6** respectively**.**

Range

Montelukast = 1-10µg/ml

Doxofylline = 4-24 µg/ml

Precision:

The precision study was performed by Intra-day and Inter-day variation study. In the intra-day study, three replicates of three different concentrations of Montelukast (4, 6, 8 µg/ml) and of Doxofylline (12, 16, 20 µg/ml) were analyzed in a day and percentage RSD was calculated. For the inter-day variation study, three replicates of three different concentrations of Montelukast (4, 6, 8 µg/ml) and of Doxofylline (12, 16, 20 µg/ml) were analyzed on three consecutive days and percentage RSD was calculated. Results are depicted in **Table 4.**

Accuracy: Accuracy of the method was studied by % recovery. To the sample solution (2 µg/ml Montelukast and 8 µg/ml Doxofylline) a known amount of standard drug was added at 80, 100, and 120 % and re-analyzed by the proposed method. Results are shown in **Table 5**

Sensitivity: The method sensitivity was determined with reference to detection and quantitation limit. They were determined from respective regression equations obtained for Montelukast and Doxofylline. Results are shown in

Table 6.

Specificity : Specificity of the developed method was confirmed by injecting standard and tablet formulation solution containing Montelukast and Doxofylline into HPLC system to check the interference of excipients. Peaks for both drugs were confirmed by comparing the spectra and retention times of Montelukast and Doxofylline with that of standard drugs. Spectra shown in **Fig.7.**

Robustness: As per the ICH, method robustness expresses its capacity to remain unaltered through small, deliberate variations in parameters of method. The parameters altered were change in flow rate of mobile phase (± 0.1 ml min^{-1}) and wavelength (± 1 nm). Results are shown in **Table 7.**

System Suitability parameters: System suitability testing is essential for the quality performance of the chromatographic system. It was performed to ensure that the complete testing system was suitable for the intended applications. Earlier prepared solutions for chromatographic conditions were tested for system suitability testing. Results are shown in **Table.8.**

Forced Degradation Study: Forced degradation studies were carried under condition of acid, base, neutral hydrolysis, oxidation, dry heat and photolysis to access the stability of both the drugs. Dry heat and photolytic degradation were conceded out in solid state. The results are shown in **Table. 9.**

Acid degradation : From the standard stock solution of Montelukast (1000 µg/ml) 1 ml solution was mixed with 1ml of 0.1N HCl and 8 ml of methanol. The solution was kept for 30 min in dark place. 0.6 ml of resulting solution was withdrawl and diluted upto10 ml with mobile phase (6 µg/ml). Similarly from the standard stock solution of Doxofylline (4000 µg/ml) 1ml solution was mixed with 1ml of 0.1 N HCl and 8 ml of methanol. The solution was

kept for 30 min in dark place. 0.4 ml of resulting solution was diluted with mobile phase upto10 ml (16µg/ml) and then injected in stabilized chromatographic conditions. After acid treatment, Montelukast showed one additional peaks of degradation at Rt 1.8 min with 85.16 % recovery and Doxofylline showed peak of degradation at Rt 5.82 min with 91.81 % recovery.

Alkaline degradation: From the standard solution of Montelukast (1000 µg/ml) 1 ml solution was mixed with 1ml of 0.1N NaOH and 8 ml of methanol. The solution was kept for 30 min in dark place. 0.6 ml of resulting solution was diluted with mobile phase upto10 ml (6µg/ml).

Similarly from the standard stock solution of Doxofylline (4000 µg/ml) 1ml solution was mixed with 1ml of 0.1 N NaOH and 8 ml of methanol. The solution was kept for 30 min in dark place. 0.4 ml of resulting solution was diluted with mobile phase upto10 ml (16 µg/ml) and then injected in stabilized chromatographic conditions. After alkaline treatment, Montelukast showed one additional peaks of degradation at Rt 2.51 min with 89.83 % recovery and Doxofylline showed peak of degradation at Rt 1.91 min with 88.43 % recovery.

Neutral Hydrolytic Degradation: From the standard solution of Montelukast (1000 µg/ml) 1 ml solution was mixed with 1ml of water and 8 ml of methanol. The solution was kept for 30 min in dark place. 0.6ml of resulting solution was diluted with mobile phase upto10 ml (6 µg/ml). Similarly from the standard stock solution of Doxofylline (4000 µg/ml) 1ml solution was mixed with 1ml of water and 8 ml of methanol. The solution was kept for 30 min in dark place. 0.4 ml of resulting solution was diluted with mobile phase upto10 ml (16 µg/ml) and then injected in stabilized chromatographic conditions. After neutral treatment, Montelukast shows 90.50 % recovery without extra peak of degradation and Doxofylline showed peak of degradation at Rt 7.30 min with 87.26 % recovery.

Oxidative degradation: From the standard solution of Montelukast (1000 µg/ml) 1 ml solution was mixed with 1ml of 30% H_2O_2 and 8 ml of methanol. The solution was kept for 30 min in dark place. 0.6 ml of resulting solution was diluted with mobile phase upto10 ml (6 µg/ml).

Similarly from the standard stock solution of Doxofylline (4000 µg/ml) 1ml solution was mixed with 1ml 30% H_2O_2 and 8 ml of methanol. The solution was kept for 30 min in dark place. 0.4 ml of resulting solution was diluted with mobile phase upto10 ml (16 µg/ml) and then injected in stabilized chromatographic conditions. After oxidative treatment, Montelukast shows 88.66 % recovery without extra peak of degradation while Doxofylline showed peak of degradation at Rt 1.92 min with 91.62 % recovery.

Dry heat degradation: Dry heat studies were performed by keeping drug sample as individual in oven (100^0 C) for a period of 1 hour. Samples were withdrawn after 1hr, dissolved in methanol and diluted appropriately to get concentration of 6 µg/ml for Montelukast and 16 µg/ml for Doxofylline. The chromatogram obtained for

Montelukast and Doxofylline after dry heat treatment showed no extra peak and there was no considerable change in peak area which denoted the drug stability in dry heat condition.

Photo-degradation studies

Photolytic study was carried out by exposure of drug individually to UV light up to 200 watt hours/square meter for period of 4 hrs. Sample was weighed, dissolved and diluted to get 6µg/ml for and 16µg/ml for resp. After photo degradation study Montelukast shows 94.55% recovery without extra peak of degradation while Doxofylline showed peak of degradation at Rt 1.93 min with 88.98 % recovery.

Table 1 Summary of Chromatographic parameters

Sr. no.	Parameter	Conditions used for Analysis
1.	Mobile phase	Water : Acetonitrile (150: 850 v/v)
2.	Flow rate	1 ml/min
3.	Detection Wavelength	250 nm
4.	Sample injector	20 µl loop
5.	Column	Oyster C8 150 x4.6 mm 5 micron
6.	Column temperature	Ambient

Table 2 Analysis of Montelukast and Doxofylline Tablet formulation

Brand	Label claim (mg)	Amount taken (µg)	Amount found (µg)	% Drug content	S.D.*	% R.S.D.*
Spirodin M (Koye Pharmaceutical)	10 Montelukast	2	1.99	99.50	1.96	1.96
	400 Doxofylline	8	7.94	99.25	0.89	0.90

*Average of six determinations

Table 3 Linearity studies of Montelukast and Doxofylline

Sr. no	Conc. of Montelukast (µg)	Mean Peak area	Conc. of Doxofylline ((µg)	Mean Peak area
1	1	109235.8	4	151352.3

2	2	125614.5	8	277560.7
3	4	155091.7	12	431326.8
4	6	183491.2	16	581328.7
5	8	214484.5	20	746439.5
6	10	236727.7	24	893327.5

Table 4 Precision studies of Montelukast and Doxofylline

Parameter	Montelukast			Doxofylline		
	Amount taken(µg)	Amount found(%)	% RSD	Amount taken(µg)	Amount found(%)	% RSD
Intra-day [n= 3]	4	99.41	0.87	12	98.94	0.75
	6	99.85	1.33	16	99.85	0.50
	8	100.58	0.69	20	99.78	0.66
Inter-day [n= 3]	4	99.83	0.76	12	99.13	0.47
	6	99.77	0.78	16	99.39	1.09
	8	100.24	0.57	20	99.35	1.43

Table 5 % Recovery Studies of Montelukast and Doxofylline

Drug	Amount taken (µg)	Amount of standard drug added (µg)	Amount Recovered (µg)	% Amount Recovered	% R.S.D.*
Montelukast	2	1.6	3.59	99.74	0.70
	2	2	4.0034	100.07	1.74
	2	2.4	4.38	99.61	1.01
Doxofylline	8	6.4	14.36	100.01	0.59
	8	8	15.95	99.71	0.81
	8	9.6	17.44	99.13	1.00

*Average of three determination

Table 6 LOD& LOQ Studies of Montelukast and Doxofylline

Name of the drug	LOD(µg/ml)	LOQ (µg/ml)
Montelukast	0.56µg/ml	1.70 µg/ml
Doxofylline	0.44 µg/ml	1.33µg/ml

Table 7 Robustness studies of Montelukast and Doxofylline

Sr. No.	Parameters	Drug	% R.S.D.
1.	Flow rate		
	+ 1 min.	Montelukast	0.89
		Doxofylline	0.94
	-1 min	Montelukast	0.84
		Doxofylline	0.91
2.	Wavelength		
	+ 1nm	Montelukast	0.41
		Doxofylline	0.54
	-1nm	Montelukast	0.68
		Doxofylline	0.57

Table 8 System suitability parameters

Name of Drug	RT (Min)	Tailing factor (T)	Theoretical Plates (N)	Asymmetry Factor
Montelukast	5.507±0.04	0.94	6871	1.101
Doxofylline	9.561±0.15	1.12	7354	1.154

Table 9 Forced degradation studies of Montelukast and Doxofylline

Agent	Exposure time (hr)	Number of Degradation products (Retention time in minute)		% of drug remaining after degradatio	
		Montelukast	Doxofylline	Montelukast	Doxofylline
HCl (0.1N)	0.5	1 (1.8)	1 (5.82)	85.16	91.81
NaOH (0.1 N)	0.5	1 (2.51)	1 (1.91)	89.83	88.43
Water	0.5	No degradation	1 (7.30)	90.50	87.26
H_2O_2 (30%)	0.5	No degradation	1 (1.92)	88.66	91.62
Dry Heat	1	No degradation	No degradation	100.16	90.43
Photo degradation	4	No degradation	1 (1.93)	94.55	88.98

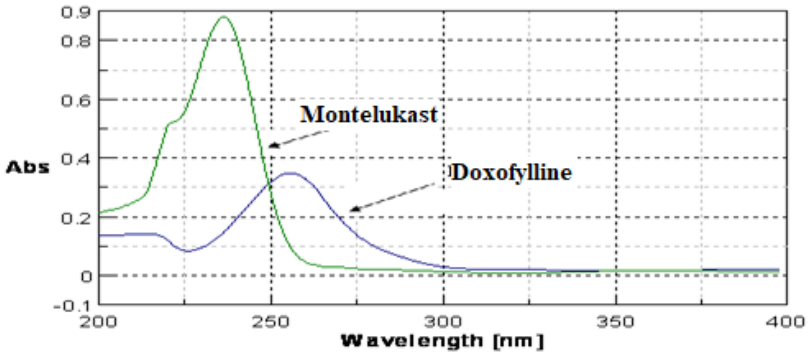

Fig 2: Overlain UV Spectra of Montelukast and Doxofylline

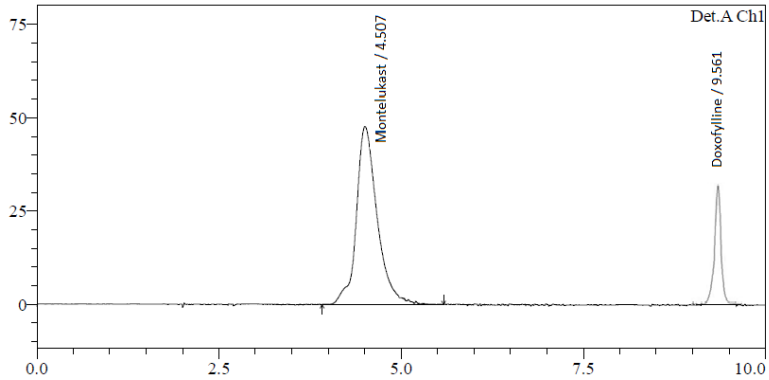

Fig 3: Chromatogram of Montelukast (10 µg/ml, Rt = 4.507 ± 0.04 min) and Doxofylline (10 µg/ml, Rt = 9.561 ± 0.15 min)

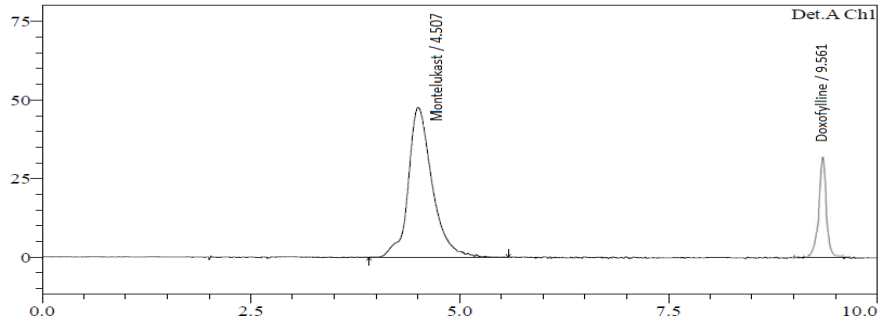

Fig 4: Chromatogram of Montelukast (2 µg/ml) and Doxofylline (8 µg/ml) in tablet

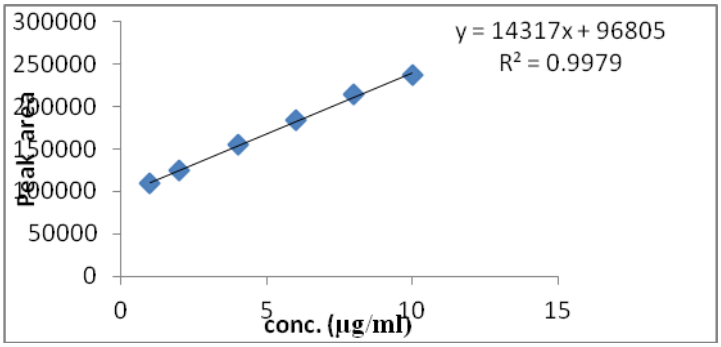

Fig 5: Calibration curve of Montelukast

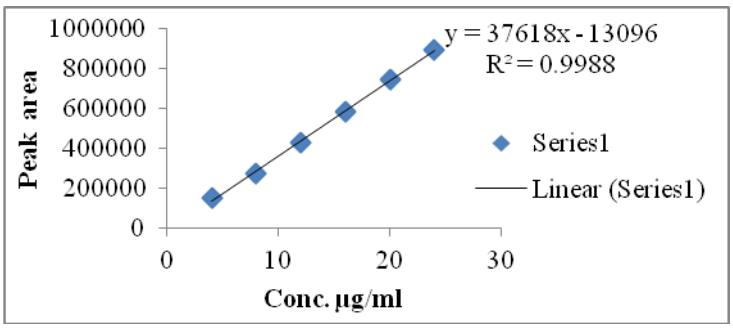

Fig 6: Calibration curve of Doxofylline

DISCUSSION

The chromatographic separation achieved on Oyster C8 150 x4.6 mm 5 micron utilizing a mobile phase **Water: Acetonitrile (150:850, v/v**) and flow rate was 1 ml/min which shows good resolution and symmetric peak with retention time **4.507±0.04min and 9.561±0.1min** for Montelukast and Doxofylline respectively. The detection wavelength selected was 250 nm. Linearity was observed in the range of 1-10 µg/ml for Montelukast and 4-24 µg/ml for Doxofylline. The percentage recoveries of Montelukast and Doxofylline in the marketed dosage form were found to be **99.50% and 99.25%** respectively. The correlation coefficients for Montelukast and Doxofylline were 0.997 and 0.998 respectively. The method was applied to marketed tablet formulation and the % amount of drug estimated was in good relationship with label claim. The method was validated as per ICH guidelines for Linearity, accuracy, precision and robustness. The accuracy of the method was studied by recovery at three different concentration levels and found to be 99.61**% to 100.07 % for Montelukast and 99.13% to 100.01% for Doxofylline**. The results of precision study in part of Intra-day and inter-day showed % RSD less than 2 indicate method is precise. The low value of LOD and LOQ indicates sensitivity of the method. The % RSD less than 2 for robustness study confirmed method is robust as per ICH guideline. The system suitability test parameters were checked as per USP. Method summary given in **Table 10**

Montelukast and Doxofylline were exposed to various stress degradation conditions i.e. acid, base, neutral, oxidative, dry heat and photolytic. Montelukast and Doxofylline were exposed to various stress degradation conditions. Peaks obtained from the samples degraded by acid, alkali, neutral, hydrogen peroxide, dry heat and photo treatment showed well separated peak of the pure drugs and few degradation peaks at various Retention time. Montelukast showed degradation product peak under acid (**1.8**) and alkali (**2.51**) conditions but did not show any observable peak in neutral, oxidation, dry heat and photo condition. Doxofylline showed degradants peaks for acid (**5.82**), alkali (**1.91**), neutral (**7.30**), oxidation (**1.92**) and photo (**1.93**) condition but did not show any observable peak in dry heat stress condition. The degradation peaks developed under various stress condition for both Montelukast and Doxofylline were well separated from the peak of the intact drugs. The peaks of the Montelukast and Doxofylline were not remarkably shifted in the presence of the degradation peaks, which specify the stability-indicating character of the developed method.

Table 10: Summary of Stability Indicating HPLC method for Montelukast and Doxofylline

Parameter	Montelukast	Doxofylline
Stationary Phase	Oyster C8 150 x4.6 mm 5 micron	
Mobile Phase	Water : Acetonitrile (150: 850 v/v)	
Flow Rate (ml/min)	1 ml/min	
Detection Wavelength	250 nm	
System sitability parameter		
RetentionTime (Rt) (minute)	4.507± 0.04	9.561± 0.15
Theoretical plate (N)	6871	7354
Tailing Factor (T)	0.94	1.12
Assmetry factor	1.101	1.154
Regression coefficient	0.997	0.998
Range (µg/ml)	1-10	4-24
Method validation		
Precision (Intra-day) (% RSD)	0.69-1.33	0.50-0.75
Precision (Inter-day) (% RSD)	0.57-0.78	0.47-1.43
Accuracy (% recovery)	99.61-100.07	99.13-100..01
LOD (µg/ml)	0.56	0.44
LOQ (µg/ml)	1.70	1.33
Robustness	Robust	Robust
Stability Study	Executed	Executed

CONCLUSION

For qualitative and quantitative analysis there are different analytical techniques are available i.e. UV Spectrophotometry, HPLC and HPTLC chromatographic techniques.

According to literature survey there are some UV, HPLC, UPLC and HPTLC analytical methods are available for Montelukast and Doxofylline individually and in a combination with other drugs but yet there is no no stability indicating HPLC method reported for Montelukast and Doxofylline combinations.

In present study analytical method develop and validate HPLC method is developed and validated for simultaneous quantitative estimations of Montelukast and Doxofylline. These present techniques are more efficient and sensitive as compared to other analytical techniques.

In general, HPLC is versatile and extremely precise when it comes to identifying and quantifying chemical components. With many steps involved, the precision of HPLC is largely down to the process being automated and therefore highly reproducible.

Stability indicating analytical method developed and validated for estimation of Montelukast and Doxofylline in bulk and tablet dosage form has been developed. Developed methods are found to be accurate, precise and robust as per ICH guidelines. The methods can be used in industry for simultaneous quantitative estimation of drugs.

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