DOI: 10.22270/jmpas.2021.IC1I1.1940 ISSN NO. 2320–7418

International peer reviewed open access journal

Journal of Medical Pharmaceutical and Allied Sciences

Journal homepage: www.jmpas.com CODEN: JMPACO

Research article

Analytical method development and validation of combination of Anti-asthmatic drugs Montelukast and Doxofylline

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ABSTRACT

For qualitative and quantitative analysis, various analytical techniques are available such as Ultraviolet (UV) Spectrophotometry, High-performance liquid chromatography (HPLC), High-performance thin layer chromatography (HPTLC). As per literature survey, there are some UV, HPLC, Ultra-Performance Liquid Chromatography (UPLC) and HPTLC analytical methods are developed for Montelukast and Doxofylline individually and in a combination with other drugs too, since yet there are no significant stability studies indicating HPLC method reported for Montelukast and Doxofylline combinations. In the current study, the 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.

Keywords: Chromatography, Analytical method development, Anti-asthmatic drugs, HPLC, System suitability parameters, Validation parameters

Received - 20-09-2021, Reviewed - 02/10/2021, Revised/ Accepted- 30/10/2021

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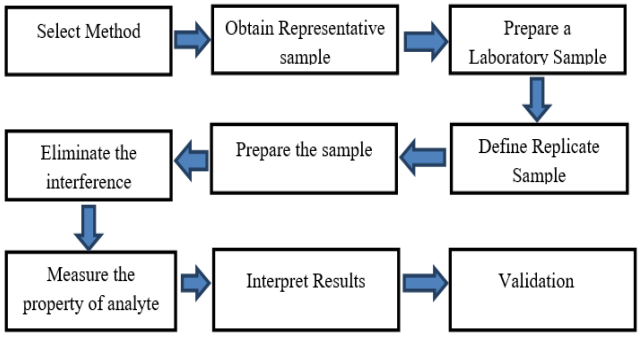
INTRODUCTION

In 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 and quantitative measurements of the substance present in bulk and pharmaceutical formulation [1-2]. The process of method development is often qualitative or quantitative. The number of drugs is introduced into the market has been increasing at an alarming rate. These drugs could be either new entities or partial structural modifications of the prevailing ones. Very often there is a time lag from the introduction of a drug into the market to the inclusion in pharmacopeias. This happens thanks to the possible ambivalence within the continual and wider usage of these drugs, reports of latest toxicities (resulting in their withdrawal from the market), development of patient resistance, and introduction of upper drugs by competitors. Under those conditions, standards and analytical procedures for these drugs would not be available within the pharmacopeias. Therefore, it is necessary to develop the latest analytical methods for such drugs [3-4].

The aim of the current work is to develop and validate quantitative analytical methods for anti-asthmatic agents in a 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 that are formed during the stability evaluation period. The extent of degradation of API under stress conditions will be studied.

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

Figure 1: Graphical representation of Steps in quantitative analysis

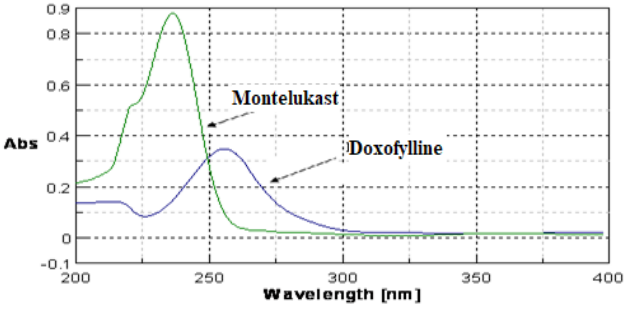


EXPERIMENTAL

Montelukast and Doxofylline both are soluble in Methanol. Twenty tablets were accurately weighed and average weight per tablet was calculated. Tablets were ground to fine powder and weighed tablet powder like 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.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. Setting the chromatographic conditions and stabilizing the instrument to gain a gentle 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. 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. Stock solutions (10µg/ml) of drugs were prepared in methanol and their isosbestic point is observed at 250 nm on UV- spectrophotometer. Overlain spectra shown in Fig.2

Figure 2: Overlain UV Spectra of Montelukast and Doxofylline



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.

Chromatographic separation study was carried out on the working standard solutions of Montelukast (10 µg/ml) and Doxofylline (10 µg/ml). Combination of Water: Acetonitrile (150:850 v/v) offered acceptable peak parameters. 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. Accurate 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.

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 (λ 1 nm). The method sensitivity was determined with reference to detection and quantitation limit. They were determined from respective regression equations obtained for Montelukast and Doxofylline. 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. 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 hold 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 keep for 30 min in dark room. 0.4 ml of resulting solution was diluted with mobile phase upto10 ml (16 µg/ml) and then injected in stabilized chromatographic conditions. 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. Before prepared solutions for chromatographic conditions were tested for system suitability testing.

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. 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 keep for 30

min in dark place. 0.6 ml of resulting solution was withdrawal 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 keep 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. 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 keep 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 keep 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. 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 keep 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, Dry heat studies were performed by keeping drug sample as individual in oven (1000 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. 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 [5-6].

RESULTS AND DISCUSSION

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.

Table 1: Precision: Results are depicted

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

Table 2: Accuracy: Results are shown

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

Table 3: Sensitivity: Results are shown

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

Specificity: Fig.3 Chromatogram of Montelukast (2 µg/ml) and Doxofylline (8 µg/ml) in tablet

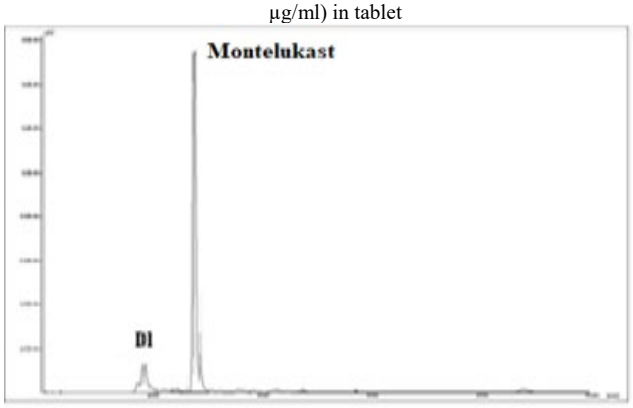


Table 4 Robustness: Results are shown

Parameters	Drug	% R.S.D.
Flow rate		
+ 1 min.	Montelukast	0.89
	Doxofylline	0.94
-1 min	Montelukast	0.84
	Doxofylline	0.91
Wavelength		
+ 1nm	Montelukast	0.41
	Doxofylline	0.54
-1nm	Montelukast	0.68

Table.5 System Suitability parameters: Results are shown

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 6 Forced Degradation Study: The results are shown

Agent	Exposure time (hr.)	Number of Degradation products (Retention time in minute)		% of drug remaining after degradation	
		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

Figure 4: Chromatogram of Montelukast after acid degradation with degradation product

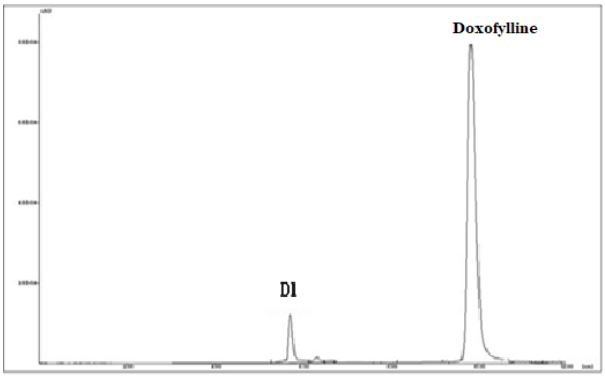
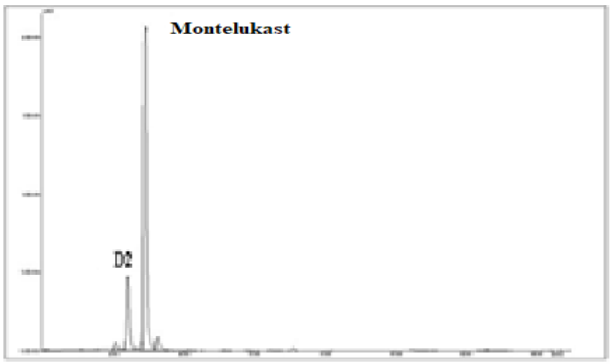


Figure 5: Chromatogram of Doxofylline after acid degradation with degradation product



Acid degradation

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 shown in above Figure. 4 & 5

Alkaline degradation

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 shown in Fig. 6 & 7.

Figure 6 Chromatogram of Montelukast after alkaline degradation with degradation product

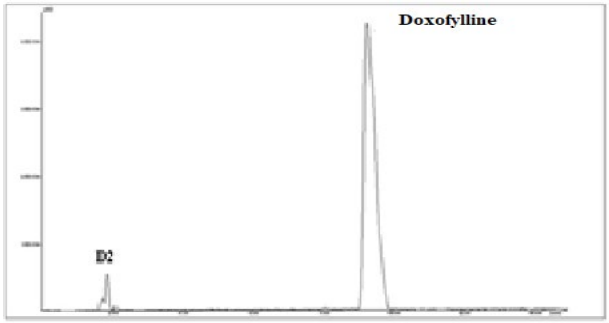
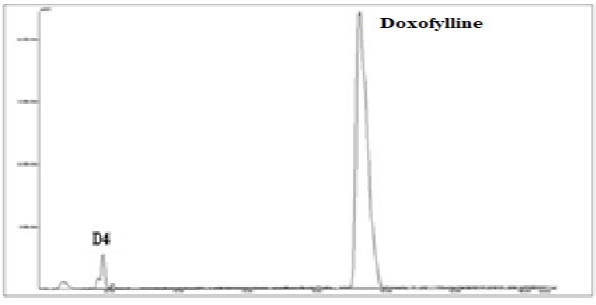


Figure 7: Chromatogram of Doxofylline after alkaline degradation with degradation product



Neutral Hydrolytic Degradation

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% H2O2 and 8 ml of methanol. The solution was keep for 30 min in dark place. 0.6 ml of resulting solution was diluted with mobile phase upto10 ml (6 µg/ml). 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

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

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 7: 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 8: Forced degradation studies of Montelukast and Doxofylline

Agent	Exposure time (hr)	Number of Degradation products (Retention time in minute)		% of drug remaining after degradation
		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 3 88.98

Figure 8: Chromatogram of Montelukast (1µg/ml)

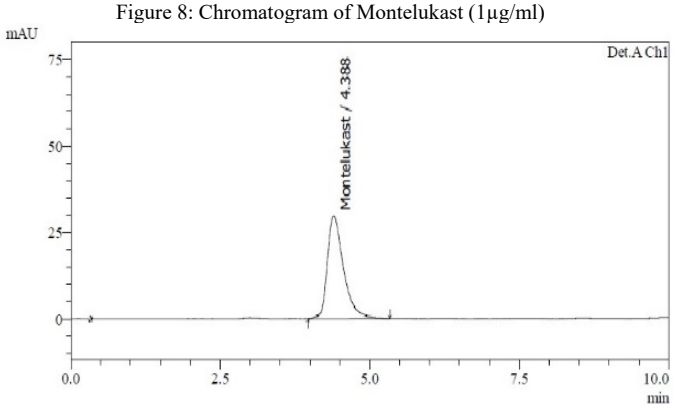


Figure 9: Chromatogram of Montelukast (2µg/ml)

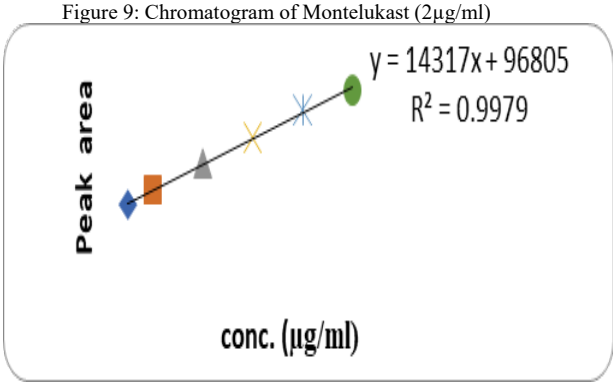


Figure 10: Calibration curve of Montelukast

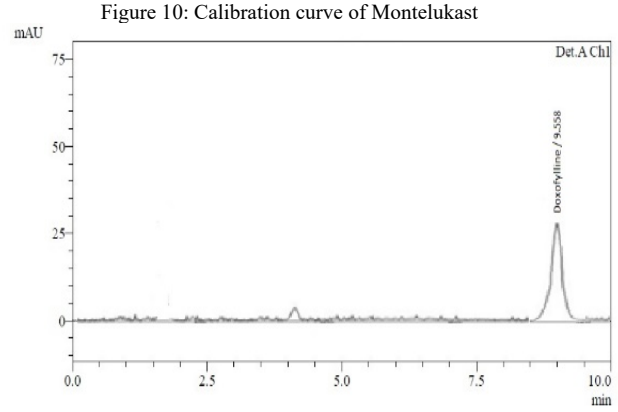


Figure 11: Chromatogram of Doxofylline (4µg/ml)

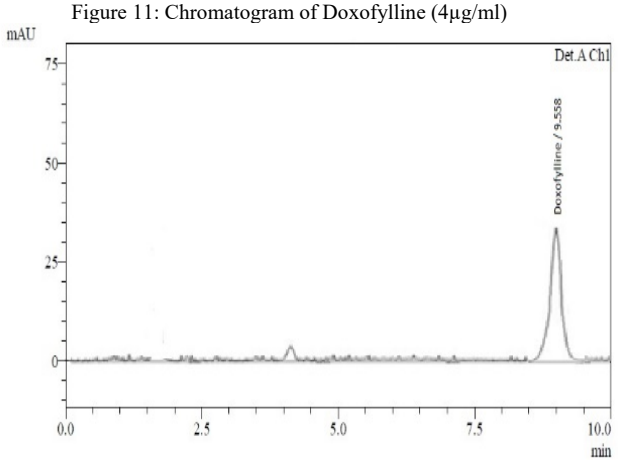


Figure 12: Chromatogram of Doxofylline (8µg/ml)

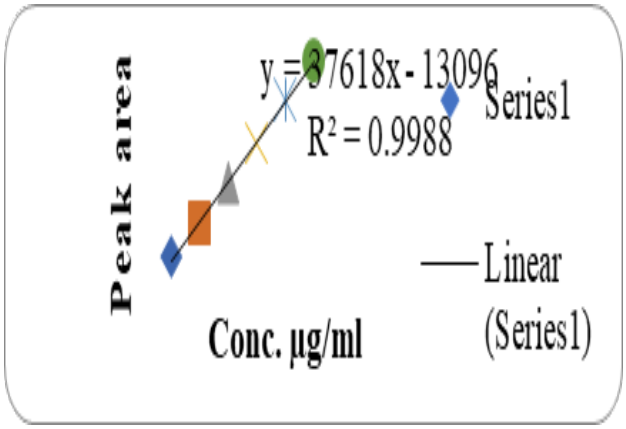


Figure 13: Calibration curve of Doxofylline

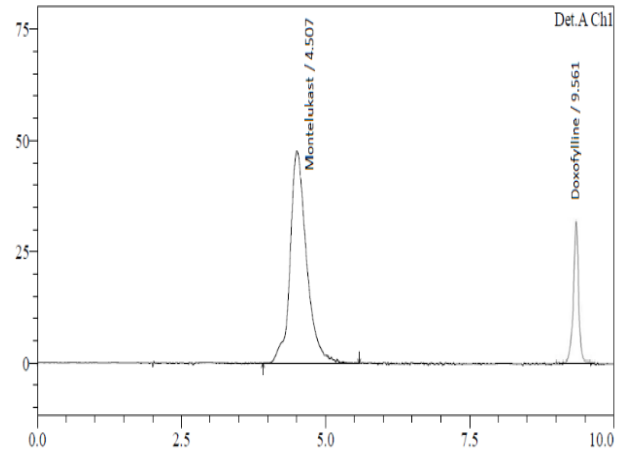
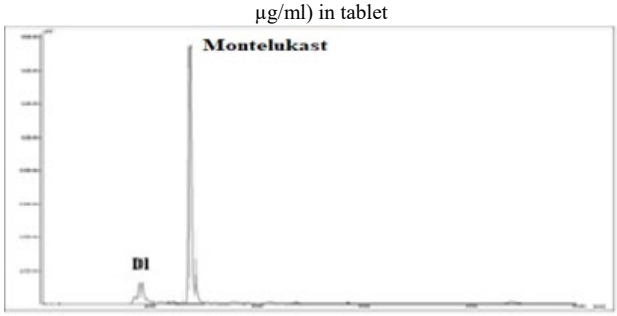


Figure 14: Chromatogram of Montelukast (2 µg/ml) and Doxofylline (8 µg/ml) in tablet



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 approve 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 1.7 Montelukast and Doxofylline were exposed to various stress degradation conditions i.e. acid, base, neutral, oxidative, dry heat and desorption. 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 9: Summary of Stability Indicating HPLC methods 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 development 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.

Normally, HPLC is adjustable and very precise when it involves identifying and quantifying chemical components. With more steps involved, the precision of HPLC is essentially right down to the method being automated and thus 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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How to cite this article

Sachin N. Kapase, Dr. M. S. Ranawat, 2021. "Analytical method development and validation of combination of Anti-asthmatic drugs Montelukast and Doxofylline". Jour. of Med. P'ceutical &Allied. Sci. V 10 - I 6, 1940, P-3494-3399. doi: 10.22270/jmpas.V10I6.1940