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# Analytical Method Development and Validation for "MONTELUKAST Sodium" And "DOXOFYLINE" Using "RP-HPLC"

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**Abstract:**
Developed by using a novel rapid HPLC method was developed for simultaneous determination of montelukast and doxofylline in bulk and pharmaceutical dosage forms. Development of an analytical method for simultaneous estimation of drugs requires a lot of efforts.it is a challenging task. The method was Agilent C18 column (250 x 4.6mm) 5 $\mu$m particle size. Mobile phase consisting of methanol and acidic water (0.1% OPA) at pH 2.9; the flow rate of 1.0 mL/min and ultraviolet detection at 240 nm. Both drugs were sufficiently resolved having retention time of 4.7 min and 2.2min for montelukast and doxofylline, respectively. This method will give good result or sharp peaks. The method was validated as per ICH Guidelines for various parameters like precision, linearity, accuracy, ruggedness, and robustness. The validated method was applied to the commercially available pharmaceutical dosage form and obtained the desired result

**Keywords:** montelukast sodium ,doxofyline ,

## I. Introduction:

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 is 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.

Reasons for the development of newer methods of drug analysis are:

- 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.

Method development or Analytical validation should be carried out by different method that are enlisted below.

1. UV-Visible Spectrophotometer
2. High performance liquid chromatography
3. High performance Thin layer chromatography
4. Ultra performace liquid chromatography
5. RP-HPLC [Reverse phase liquid chromatography]

But we have to carried out the work on UV and HPLC method for development and analytical validation.

Ultraviolet Visible [UV-visible spectrophotometry] is a technique that Used to measure light absorbance across the ultraviolet and visible ranges of electromagnetic spectrum. When incident light

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strikes the matter, it should be absorbed, reflected or transmitted UV visible Ranges from 200 to 400 NM. The atomic excitation which carries out the transition from low energy to high energy i.e., (ground state to excited state).

The main principle of UV-visible spectrophotometer is can quantify the analytes in a sample based on their absorption characteristics.

Using the UV spectrophotometer we can determine the analytical evaluation parameter like precision, Linearity, accuracy, Roughdness, Robustness or another different evolutionary parameter should be examined by using UV-spectrophotometer.

HPLC is the (High performance liquid chromatography) is a technique that should be used for separation purpose. It is used to conduct the chromatography.

Chromatography is used to separate protein, lipids, nucleic acid and many different small mixtures.

HPLC technique that should be easily determine analytical or Preparatory sample by easy way. This also helps to evaluation and validation of different analytical parameter.

## II. Materials and Methods:

**Selection and Procurement of Drug**

Drug sample supplier

**Table 1: Drug and Drug Supplier**

| Name of Drug | Drug Supplier |
|---|---|
| Doxofylline and Montelukast | Swapnroop drug and pharmaceutical |

List of reagents & chemicals used

**Table 2: List of Reagents and Chemicals used**

| Sr. No. | Name of chemicals | Manufacturer. |
|---|---|---|
| 1. | Methanol (HPLC grade) | Merck Ltd., India |
| 2. | Acetonitrile (HPLC grade) | Merck Ltd., India |
| 3. | Ortho-phosphoric Acid buffer (HPLC grade) | Merck Ltd., India |

**Selection of formulation:**

From the literature survey and market survey we selected Maxide formulation for work.

Marketed Preparation**:**

**Brand Name :Singulair**

**Content :** Doxofylline and Montelukast

**Marketed by : unichem laboratories pvt .ltd**

The marketed preparation was obtained from local market and is referred here after in this thesis by the name as such.

**Selection of Analytical Technique**

HPLC was selected as analytical technique for estimation of Doxofylline and Montelukast.

**Instruments:**

The analysis of the drug was carried out on Agilent Tech. Gradient System with Auto injector, (DAD) & Gradient Detector. Equipped with Reverse Phase (Agilent) $C_{18}$ column (4.6mm x 250mm; 5µm), and UV730D Absorbance detector and running chemstation 10.1 software.

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**Instruments and Equipment**

**Table. 3: Instrument (HPLC) Details used during Method Development**

| | Name of Instrument | Company Name |
|---|---|---|
| 1 | HPLC Instrument | Agilent Tech. Gradient System with Auto injector (Chemstation software). |
| 2 | UV-Spectrophotometer | Analytical Technology. |
| 3 | Column($C_{18}$) | Agilent $C_{18}$ (250mmX 4.6mm,5µm). |
| 4 | pH meter | VSI pH meter (VSI 1-B). |
| 5 | Balance | WENSAR™ High Resolution Balance. |
| 6 | Sonicator | Ultrasonics' electronic instrument. |

**a) Chromatographic conditions:**

The following chromatographic conditions were established by trial and error and were kept constant throughout the experimentation.

**Table No.4: chromatographic conditions (HPLC) details used during method Development**

| | | |
|---|---|---|
| 1. | HPLC | Agilent Tech. Gradient System with Auto injector |
| 2. | Software | Chemstation |
| 3. | Column | (Agilent) C18 column (4.6mm x 250mm) |
| 4. | Particle size packing | 5 µm |
| 5. | Stationary phase | C-18 (Agilent) |
| 6. | Mobile Phase | Methanol: Water (0.1% OPA) 40: 60 |
| 7. | Detection Wavelength | 240 nm |
| 8. | Flow rate | 1 ml/min |
| 9. | Temperature | Ambient |
| 10. | Sample size | 20 µl |
| 11. | pH | 3 |
| 12. | Run Time | 10 min |
| 13. | Filter paper | 0.45 µm |

**Study of Doxofylline and Montelukast on the chromatographic conditions used in method development of HPLC for the Following Mobile phase were tried:**

**METHOD DEVELOPMENT OF HPLC:**

**List of Mobile Phase:**

**Table No.5: Selection of mobile Phase.**

| Sr.No. | Mobile Phase |
|---|---|
| 1. | Methanol: water (0.1%OPa PH-3) (70:30 % v/v) |
| 2 | Methanol: Water 0.1% OPA (50: 50%v/v) PH3 |
| 3 | Methanol: Water 0.1% OPA (40: 60%v/v) PH3 flow 0.8 ml/min |
| 4 | Methanol: Water 0.1%OPA (40: 60%v/v) PH3Flow 1 ml/min |

**7.3.** Analysis of standard drugs was done by following parameters:

- Melting point
- Solubility
- UV spectra and $\lambda_{max}$
- HPLC chromatogram and retention time

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**7.4. Selection of wavelength by UV-Visible Spectrophotometry:-**

**7.5.1. Preparation of standard stock solution:-**

- **Doxofylline standard stock solution : ( Stock I )**

An accurately weighed quantity, 200 mg of Doxofylline (DOXO) was dissolved in Methanol in a 10 ml volumetric flask and volume made up to 10.0 ml to produce a solution of 20,000 µg/ml. **(Fig No: 13)**

- **Montelukast standard stock solution : ( Stock II )**

An accurately weighed quantity,5 mg of Montelukast (MONTE)was dissolved in Methanol in 10 ml volumetric flask and volume made up to 10.0 ml to produce a solution of 500 µg/ml. **(Fig No: 13)**

- **Preparation of Stock Standard Combination Solution :( Stock III) [MET+DAPA]**

Accurately weight and transfer 200 mg Doxofylline and Montelukast 5 mg working standard into 10 ml volumetric flask as about diluent Methanol completely and make volume up to the mark with the same solvent to get 20,000µg/ml standard Met and 500 µg/ml for Dapa (stock solution) and 15 min sonicate to dissolve it and remove the unwanted gas,further an aliquots portion of Doxofylline and Montelukast stock solution in ratio of 40:60 were mixed in volumetric flask in 10 ml and volume was adjusted up to mark with mobile phase from the resulting solution 0.2 ml was transferred to 10 ml volumetric flask and the volume was made up to the mark with Methanol: Acidic water(0.1% OPA) , prepared in (7.5ml Methanol: 2.ml Acidic water)solvent .Result as shown as; **(Fig No:14)**

**7.5.2. HPLC used for chromatographic condition apply on the Preparation of standard solution:-**

- **Preparation of std. Doxofylline solution: ( Stock I )**

From the freshly prepared standard stock solution (20,000 µg/ml), 0.2 ml stock solution was pipeted out in 10 ml of volumetric flask and volume was made up to 10 ml with mobile phase to get final concentration of 400 µg/ml. **(Fig No:14)**

- **Preparation of std. Montelukast solution: ( Stock II )**

From the freshly prepared standard stock solution (500 µg/ml), 0.2 ml stock solution was pipeted out in 10 ml of volumetric flask and volume was made up to 10 ml with mobile phase to get final concentration 10 µg/ml. **(Fig No:14)**

- **Preparation of std. Doxofylline and Montelukast solution :( Stock III)**

From the freshly prepared standard stock solution (20,000 µg/ml:500 µg/ml), 0.1 ml stock solution was pipette out in 10 ml of volumetric flask and volume was made up to 10 ml with mobile phase to get final concentration 200 µg/ml of Doxofylline and 5 µg/ml was Montelukast. **(Fig No:)**

**7.5.3. Selection of mobile phase:**

Each mobile phase was vacuum degassed and filtered through 0.45µ membrane filter. The mobile phase was allowed to equilibrate until steady baseline was obtained. The standard solution containing mixture of Doxofylline and Montelukast was run with different individual solvents as well as combinations of solvents were tried to get a good separation and stable peak. From the various mobile phases tried, mobile phase containing Methanol & Acidic water with pH adjust (3)OPA was selected since it gave sharp, well resolved peaks with symmetry within the limits and significant reproducible retention time for Doxofylline and Montelukast. Chromatograms of Doxofylline and Montelukast are shown in **(Table No:)** respectively.

**7.6. Studies of Calibration plot :-**

**7.6.1. Optimization of Chromatographic condition :**

The following chromatographic conditions were established by trial and error and were kept constant throughout the analysis.

| | |
|---|---|
| Column | : Agilent C18 ( 250 mm× 4.6mm) |
| Particle size packing | : 5µm |
| Detection wavelength | : 240 nm |
| Flow rate | : 1 ml/min |
| Temperature | : Ambient |
| Sample size | : 20 µl |
| Mobile phase | : Methanol: water (0.1%OPA) ( 40 : 60 ) |

**7.7. Procedure for calibration curve of Doxofylline and Montelukast:**

The mobile phase was allowed to equilibrate with stationary phase until steady baseline was obtained. From the freshly prepared standard stock solution, pipette out 200 mg Doxofylline and 5 mg Montelukast in 10 ml of volumetric flask and diluted with mobile phase. From it 0.1, 0.2, 0.3, 0.4 and 0.5ml of solution were pipette out in 10 ml volumetric flask and volume was made up to 10 ml with mobile phase to get final concentration 200,400,600,800,1000 µg/ml of Doxofylline and

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5,10,15,20,25 µg/ml of Montelukast. sample were injected and peaks were recorded at 240 nm as the graph plotted as concentration of drug verses peak area is depicted in **(fig. no. 15, 16 )** respectively.

**7.8. Study of system suitability parameters:**
The system suitability is used to verify, whether the resolution and reproducibility of the chromatographic system are adequate for analysis to be done. The test was performed by collecting data from five replicate injections of standard solution.

**7.9.1. Calibration Experiment:**
➢ **RP-HPLC Method :**
**a) Preparation of Calibration curve standard:**
The above standard stock solution (20000:500µg/ml) of Doxofylline and Montelukast was diluted with mobile phase to yield Five calibration curve (cc) standards with concentrations of 200,400,600,800,1000 µg/ml of Doxofylline and 5,10,15,20,25 µg/ml of Montelukast **(Table No.19) & (Table No.21)**
➢ **UV Spectrophotometric method:**
**a) Selection of detection Wavelength :**
Standard solutions were scanned in the range of 200-400nm, against 10 ml Methanol and volume make with Methanol solvent system as reference Doxofylline **(Figure No:6)** and Montelukast **(Figure No:7)** were showed absorbance maxima (lamda max) at 273 nm and 238nm respectively **(Figure No:8)**.
If Two Doxofylline and Montelukast sample Interact with this point is called Isobestic point Then detection of wavelength in isobestic point in 240 nm were selection wavelength is HPLC Method can be used.
**c) Calibration standard drug and regression equation data :**
From the standard stock solution of Doxofylline and Montelukast, different concentration were prepared respectively in the range of 200-1000 µg/ml for Doxofylline **(Figure No:28)** and 5-25µg/ml for Montelukast and measured at 273 nm and 238nm. The calibration curves were plotted **(Figure No:29)** and Regression equation data presented in **(Table No: 19 and Table No: 21)**
**d) Calibration runs and regression analysis :**
These calibration standard solutions were analyzed in three replicates using the under mentioned chromatographic conditions.

- Analytical column: Agilent C18 Column (250mm x 4.6mm, 5µm partical size).
- Injection volume : 20µl.
- Flow rate : 1 ml/min.
- Mobile phase : Methanol: Acidic water(0.1%0PA) (40: 60 % V/V).
- Detection : 240 nm.

**7.10. Validation of method for analysis of Doxofylline and Montelukast:[41]**
➢ The developed method was validated as per ICH guidelines.

**7.10.1 Linearity:**
Linearity of an analytical method is its ability to elicit test results that are directly or by a well defined mathematical transformation, proportional to the concentration of analyte in samples within a given range, The Result are shown in; **(Table No and Table No )**

- **Determination:**

The linearity of the analytical method is determined by mathematical treatment of test results obtained by analysis of samples with analyte concentrations across the claimed range. Area is plotted graphically as a function of analyte concentration. **(Fig No. )** Percentage curve fittings are calculated**.** The Result are shown in; **(Table No.);( Fig No. and Fig No. )**

- **Acceptance Criteria:**

The plot should be linear passing through the origin. Correlation Coefficient should not be less than 0.999. The Result are shown in;

- **Preparation of standard stock solution for linearity:**

Average weight of tablet sample (equivalent to 200 mg of Doxofylline and 5mg of Montelukast) were weighed and transfered to 10 mL volumetric flask & diluent was added to make up the volume. Sonicated for 10 min with occasional swirling. 0.1 ml of this solution diluted upto 10 ml volumetric flask with diluents was added to make up the volume.

- **Preparation of linearity solution:**

A series of standard preparations of working standard of were prepared.

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**Table No.13:Table of linearity for Rp-HPLC Method**

| Concentration (µg/mL) | |
|---|---|
| Doxofylline | Montelukast |
| 200 | 5 |
| 400 | 10 |
| 600 | 15 |
| 800 | 20 |
| 1000 | 25 |

**7.10.2 Accuracy (recovery):**

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. Accuracy may often the expressed as percent recovery by the assay of known added amounts of analyte.The accuracy of an analytical method is determined by applying the method to analyzed samples, to which known amounts of analyte have been added. The accuracy is calculated from the test results as the percentage of analyte recovered by the assay, The RP-HPLC Method Result are shown in; **(Table No:)**

- **Acceptance Criteria:**

Mean recovery should be in the range of 98-102%.
The Relative Standard Deviation should not be more than 2.0%.

- **Preparation of standard stock solution:**

200mg of Doxofylline and 5 mg of Montelukast working standards were weighed and transfered to 10 mL volumetric flask & diluent was added to make up the volume 0.1 ml of this solution diluted upto 10 ml with diluent.

- **Application of proposed method for analysis of tablet formulation:**
- **Accuracy**

The accuracy was determined by Doxofylline and Montelukast (equivalent to 200 mg of Doxofylline and 5 mg of Montelukast (80 %, 100 % and 120 % of the label claimed, respectively) to quantity equivalent to average weight of marketed tablets. This powder mixture containing 200 mg of Doxofylline and 5 mg of Montelukast were triturated and then subjected to chromatographic analysis using the described method. The resulting mixtures were analyzed in triplicates over three days. The % recovery of added drug was taken as a measure of accuracy**.**
The Result are shown in; **( Fig No :)**

**Table No. 14 :Table of Accuracy Rp-HPLC Method**

| Sample | Amount Added (mg) | |
|---|---|---|
| | Doxofylline | Montelukast |
| Accuracy 80% | 160 | 4 |
| Accuracy 100% | 200 | 5 |
| Accuracy 120% | 240 | 6 |

**7.10.3 Repeatability:**

Precision of the system was determined with the sample of RP-HPLC Method for . Six replicates of sample solution containing 200 mg of Doxofylline and 5 mg Montelukast were injected and peak areas were measured and %RSD was calculated. is was repeated for five times :result are shown in; **(Table No : )& (Fig No :)**

- **Application of proposed method for analysis of tablet formulation:**

Average weight of tablet sample (equivalent to 200 mg of Doxofylline and 5 mg Montelukast) were weighed and transfered to 10 mL volumetric flask & diluent was added to make up the volume. Sonicated for 10 min with occasional swirling. The above solution was filtered through 0.45µm membrane filter 0.1 ml of this solution diluted upto 10 ml with diluent.

**7.10.4 Precision:**

Precision of an analytical method is the degree of agreement among Individual test results when the

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procedure is applied repeatedly to multiple Samplings of a homogenous sample. Precision of an analytical method is usually expressed as standard deviation or relative standard deviation. Also, the results obtained were subjected to one way ANOVA and within-day mean square and between-day mean square was determined and compared using F-test. **(Fig No :35)**

➢ **Result of Intra day and Inter day Precision studies on RP-HPLC method for Doxofylline and Montelukast**

**7.10.4.1 Intra-day precision:**

Sample solutions containing 200mg of Doxofylline and 5 mg three different concentration(200µg/ml,400µg/ml,600µg/ml)Monte lukast and (5µg/ml,10µg/ml,15µg/ml) Doxofylline and Montelukast were analyzed three times on the same day and %R.S.D was calculated. The Result are shown in; **(Table No.26)& (Fig No :36-41)**

**7.10.4.2 Inter-day precision:**

Sample solutions containing 200mg of Doxofylline and 5 mg three different concentration (200µg/ml,400µg/ml,600µg/ml)Montelukast and (5µg/ml,10µg/ml,15µg/ml) Doxofylline and Montelukast different days and % R.S.D was calculated. It is usually expressed as standard deviation or relative standard deviation. The Result are shown in; **(Table No.26)& (Fig No :42-44)**

- **Acceptance criteria:**

The Relative Standard Deviation should not be more than 2% for test

- **Preparation of standard stock solution:**

200 mg of Doxofylline and 5 mg Montelukast working standards were weighed and transfered to 10 mL volumetric flask & diluent was added to make up the volume. 0.1 ml of this solution diluted upto 10 ml with diluent.

**5. Robustness:**

The mobile phase composition was changed in (±1 ml/ $min^{-1}$) proportion and the flow rate was **(Fig No:)** of methanol: acidic water(40:60) in the mobile phase composition (±1ml/ $min^{-1}$) and the change in detection wavelength (±1 ml/ $min^{-1}$) and the effect of the results were examined**.(Fig No: )**and **(Fig No:)** it was performed using 600µg/ml and 15 µg/ml solution of Doxofylline and Montelukast in triplicate. The Result are shown in; **(Table No: ,Table No.)**

**7.10.5 Detection Limit**

Based on the S.D. of the response and the slope of calibration curve, the detection limit (DL) was calculated as,

$$DL = \frac{3.3\sigma}{S}$$

Where,

σ = the S.D. of the y-intercepts of regression lines.

S = the slope of the calibration curve.

The slope S may be estimated from the calibration curve and S.D. was used should be calculated from the y-intercepts of regression line in calibration curve.

The result are shown in: **(chapter:8)**

**7.10.6 Quantitation Limit**

Based on the S.D. of the response and the slope of calibration curve, the quantitation limit (QL) was calculated as,

$$QL = \frac{10\sigma}{S}$$

Where,

σ = the S.D. of the y-intercepts of regression lines.

S = the slope of the calibration curve.

The slope S may be estimated from the calibration curve and S.D. was used should be calculated from the y-intercepts of regression line in calibration curve.

The results are shown in **(chapter: 8)**

**7.11 Analysis of marketed formulation**

To determine the content of Doxofylline and Montelukast in marketed tablets (label claim 200mg of Doxofylline and 5 mg Montelukast), 20 tablets powder weighed in 20000 gms and average weight of powder was calculated in 500 gms.Tablets were triturated and powder equivalent to weighed in 305 mg The drug was extracted from the tablet powder with 10 mL Methanol. To ensure complete extraction it was sonicated for 15 min. 0.4mL of supernatant was then diluted up to 10 mL with mobile phase. The resulting solution was injected in HPLC and drug peak area was noted**.(Fig No: )** .

Regression equation was generated using peak areas of standard solutions. Using the regression equation and peak area of the sample the amount of Doxofylline and Montelukast in the sample was calculated. The amount of Doxofylline and Montelukast per tablet was obtained from the regression equation of the calibration curve as described in analysis of Tablet formulation are shown in **(Table No.).**

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**RESULT AND DISCUSSION-**

**8.1. Preliminary studies on Doxofylline and Montelukast**

**8.1.1. Melting point**

The procured reference standard of Doxofylline and Montelukast were found to melt in the range of 144-145.5$^0$C and 84-90$^0$C respectively.

**8.1.2. Solubility**

The drug was found to be

- Freely soluble in, methanol.
- Practically *insoluble* in water, but freely *soluble in organic solvents*.

1.LNEARITY- From Doxofylline standard stock solution, different working standard solution (50-250µg/ml) were prepared in mobile phase Likewise from Montelukast standard stock solution different working standard solution (200-1000µg/ml) were prepared in mobile phase 20 µl of sample solution was injected into the chromatographic system using fixed volume loop injector. Chromatograms w ere recorded

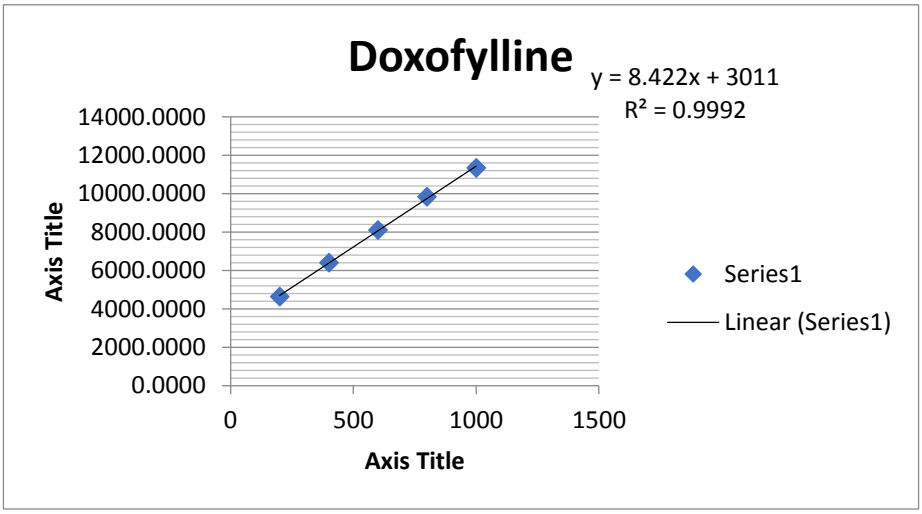


FIG NO.15 CALIBRATION CURVE FOR DOXOFYLINE

**Table No 19. Regression equation data for Doxofylline**

| **Regression Equation Data Y=mx+c** | |
|---|---|
| **Slope(m)** | 8.422 |
| **Intercept(c)** | 3011 |
| **Correlation Coefficient** | 0.999 |

**Table No 18. Linearity of Doxofylline**

| **Concentration µg/ml** | **Area Doxofylline** |
|---|---|
| 200 | 4635.5390 |
| 400 | 6401.6956 |
| 600 | 8105.3864 |
| 800 | 9840.4367 |
| 1000 | 11338.2000 |

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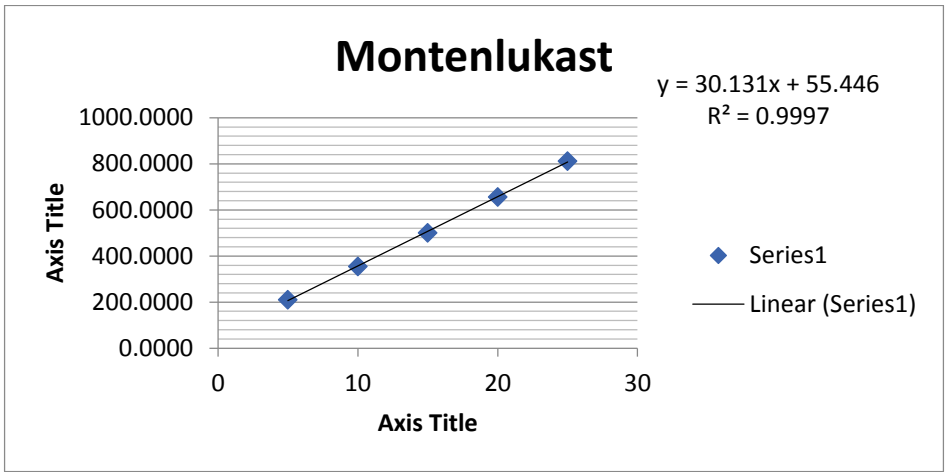


**Table.21. Regression equation data for Montelukast**

| Regression Equation Data Y=mx+c | |
|---|---|
| **Slope(m)** | 30.13 |
| **Intercept(c)** | 55.44 |
| **Correlation Coefficient** | 0.999 |

**Table No 20. Linearity of Montelukast**

| Concentration µg/ml | Area Montelukast |
|---|---|
| 5 | 210.4150 |
| 10 | 355.2100 |
| 15 | 501.6700 |
| 20 | 656.8900 |
| 25 | 812.8400 |

**2. Accuracy:-**

Recovery studies were performed to validate the accuracy of developed method. To pre analyzed tablet solution, a definite concentration of standard drug (80%, 100%, and 120%) was added and then its recovery was analysed

| METHOD | Drug | Level (%) | Amt. taken (µg/ml | Amt. Added (µg/ml | Area Mean* ± S.D. | Amt. recovered Mean *±S.D. | %Recovery Mean *± S.D. |
|---|---|---|---|---|---|---|---|
| RP-HPLC Method | Doxo | 80% | 200 | 160 | 361.31±0.26 | 161.3±0.26 | 100.94±0.17 |
| | | 100% | 200 | 200 | 403.88±0.17 | 203.0±0.175 | 101.50±0.09 |
| | | 120% | 200 | 240 | 437.24±0.42 | 237.54±0.42 | 98.97±0.17 |
| | Monte | 80% | 5 | 4 | 9.0±0.02 | 4±0.02 | 99.90±0.55 |
| | | 100% | 5 | 5 | 9.86±0.117 | 4.86±0.117 | 99.97±1.46 |
| | | 120% | 5 | 6 | 10.90±0.016 | 5.90±0.016 | 98.33±0.27 |

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| METHOD | Level of Recovery (%) | Drug | Mean % Recovery | Standard Deviation* | % RSD |
|---|---|---|---|---|---|
| Rp-HPLC Method | 80% | DOXO | 100.94 | 0.17 | 0.17 |
| | | MONTE | 98.90 | 0.022 | 0.55 |
| | 100% | DOXO | 101.50 | 0.09 | 0.09 |
| | | MONTE | 99.97 | 1.46 | 1.46 |
| | 120% | DOXO | 98.97 | 0.17 | 0.18 |
| | | MONTE | 98.33 | 0.016 | 0.27 |

***Denotes average of three determinations for RP-HPLC**

Accuracy of RP-HPLC method is ascertained by recovery studies performed at different levels of concentrations (80%, 100% and 120%). The % recovery was found to be within 98-102%.

**3. System suitability parameters :( Repeatability**

Repeatability studies on RP-HPLC for Doxofylline and Montelukast was found to be ,The %RSD was less than 2%, which shows high percentage amount found in between 98% to 102% indicates the analytical method that concluded.

| Method | Concentration of Doxofylline and Montelukast (mg/ml) | Peak area | Amount found (mg) | % Amount found |
|---|---|---|---|---|
| RP-HPLC Method for DOXO | 600 | 8149.77 | 101.65 | 101.65 |
| | 600 | 8150.282 | 101.72 | 101.72 |
| | | **Mean** | **101.69** | **101.69** |
| | | **SD** | **0.73** | **0.73** |
| | | **%RSD** | **0.01** | **0.01** |
| RP-HPLC Method for MONTE | 15 | 517.420 | 15.52 | 102.52 |
| | 15 | 510.770 | 15.54 | 102.54 |
| | | **Mean** | **15.53** | **102.53** |
| | | **SD** | **4.70** | **4.70** |
| | | **%RSD** | **0.91** | **0.91** |

**4. Precision:-**

The method was established by analyzing various replicates standards of Doxofylline and Montelukast. All the solution was analyzed thrice in order to record any intra-day & inter-day variation in the result that concluded. The result obtained for intraday is shown in**( Table No**

**Table No .25: Result of Intra day and Inter day Precision studies on RP-HPLC for Doxofylline and Montelukast**

| METHOD | Drug | Conc$^{n}$ (µg/ml) | Intraday Precision | | Interday Precision | |
|---|---|---|---|---|---|---|
| | | | Mean± SD | %Amt Found | Mean± SD | %Amt Found |
| Rp- | DOXO | 200 | 4692.29±1.78 | 99.82 | 200.40±0.58 | 100.20 |
| | | 400 | 6426.86±25.15 | 101.40 | 6444.90±6.72 | 101.93 |

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| | | | | | | |
|---|---|---|---|---|---|---|
| **HPLC METHOD** | | 600 | 8161.11±12.47 | 101.92 | 8150.74±0.82 | 101.71 |
| | **MONTE** | 4 | 421.33±0.82 | 100.22 | 418.54±2.39 | 99.53 |
| | | 6 | 625.33±0.05 | 100.78 | 625.20±0.31 | 100.76 |
| | | 8 | 815.96±3.03 | 99.39 | 815.79±0.48 | 99.37 |

Intraday and Inter day Precision studies on RP-HPLC for Doxofylline and Montelukast which shows the high precision %amount in between 98% to 102% indicates to analytical method that concluded

**5. Robustness:**

The Robustness of a method is its ability to remain unaffected by small deliberate changes in parameters. To evaluate the robustness of the proposed method, small but deliberate variations in the optimized method parameters were done. The effect of changes in mobile phase composition and flow rate, wavelength on retention time and tailing factor of drug peak was studied.

The mobile phase composition was changed in (±1 ml/min$^{-1}$**)** proportion and the flow rate was varied by (±1ml/min$^{-1}$**)**, and wavelength change (±1 ml/min$^{-1}$**)** of optimized chromatographic condition. The results of robustness studies are shown in **(Table No.26, 27**).Robustness parameters were also found satisfactory; hence the analytical method would be concluded.

| **Parameters** | **Conc.(µg/ml)** | **Amount of detected(mean ±SD)** | **%RSD** |
|---|---|---|---|
| Chromatogram of flow change 0.9ml | 600 | 8105.75±0.03 | 0.0 |
| Chromatogram of flow change 1.1 ml | 600 | 8113.14±2.86 | 0.04 |
| Chromatogram of comp change 39ml Meoh+61ml OPA Water | 600 | 8161.637±0.59 | 0.01 |
| Chromatogram of comp change 61ml Methanol+39 ml OPA Water | 600 | 8137.41±46.73 | 0.57 |
| Chromatogram of comp change wavelength change 239nm | 600 | 8152.5±4.43 | 0.05 |
| Chromatogram of comp change wavelength change 241 nm | 600 | 8189.42±1.12 | 0.01 |

The changes were did flow rate (±1 ml/ min$^{-1}$),PH of mobile phase composition (±1 ml/ min$^{-1}$),and Wavelength (±1 ml/ min$^{-1}$) .%RSD for peak area was calculated which should be less than 2%.the result shown in analytical method that concluded

| **Parameters** | **Conc.(µg/ml)** | **Amount of detected(mean ±SD)** | **%RSD** |
|---|---|---|---|
| Chromatogram of flow change 0.9ml | 15 | 618.88±0.80 | 0.13 |
| Chromatogram of flow change 1.1 ml | 15 | 506.44±6.82 | 1.35 |
| Chromatogram of comp change 39ml Meoh+61ml OPA Water | 15 | 591.672±0.96 | 0.16 |
| Chromatogram of comp change 41ml Methanol+39 ml OPA Water | 15 | 565.47±1.62 | 0.29 |
| Chromatogram of comp change wavelength change 239nm | 15 | 581.2±1.40 | 0.24 |

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**Robustness Study of** Montelukast**:**
The changes were did flow rate (±1 ml/ $min^{-1}$),PH of mobile phase composition (±1 ml/ $min^{-1}$),and Wavelength (±1 ml/ $min^{-1}$) .%RSD for peak area was calculated which should be less than 2%.the result shown in analytical method that concluded

**6. Limit Detection**
The LOD is the lowest limit that can be detected. Based on the S.D. deviation of the response and the slope The limit of detection (LOD) may be expressed as:

**LOD = 3.3 (SD)/S**

*where*, SD = Standard deviation of Y intercept
S = Slope
**Limit of detection = 11.39** (µg/mL) of Doxofylline
**Limit of detection = 0.2145** (µg/mL)of Monte

The LOD of Doxofylline and Montelukast was found to be 11.39(µg/mL) and 0.214(µg/mL), analytical method that concluded.

**7. Limit Quantification**
The LOQ is the lowest concentration that can be quantitatively measured. Based on the S.D. deviation of the response and the slope,
The quantitation limit (LOQ) may be expressed as:

**LOQ** = 10 (SD)/ S

*where*, SD = Standard deviation Y intercept
S = Slope
**Limit of Quantitation = 34.54** (µg/mL)
**Limit of Quantitation = 0.6500** (µg/mL)

The LOQ of Doxofylline and Montelukast was found to be 34.54 µg/mL) and 0.6500 (µg/mL), analytical method that concluded.

**CONCLUSION**- the effective hplc method developed is sensitive, unique, precise,rapid and reproducible for montelukast and doxofyline pharmaceutical dosage form.the method was validated as per ICH guidelines.it is conclude that this method can be used by the industries or academician for their combination of drug estimation which is fast as well as effective

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