ABSTRACT

Objective: To develop and validate macitentan with its known and unknown degradation impurities in its tablet dosage form.

Methods: The RP-HPLC method for macitentan and its impurities was developed and three potential degradation impurities MCA-02, MCA-01 and degradation impurity and N-propyl derivative and N-N dimethyl derivative process impurities were separated. Chromatographic separation was achieved within 70 min on Inertsil C8 (250*4.6 mm, 5 µm) column, Using mobile phase A [Ammonium acetate (ph 4.5 adjusted with glacial acetic acid)] and mobile phase B acetonitrile in gradient elution. Other hplc parameter which was optimized flow rate 1.5 ml/min, detection wavelength 266 nm, column oven temperature 30 °C and injection volume 20μl. macitentan was subjected to forced degradation also known as stress testing. It was validated as per ICH guidelines.

Results: The drug showed extensive degradation in acidic and basic conditions, a slight degradation in oxidative condition. The developed method was statistically validated for linearity (0.45-2.25 ppm). The result of precision (%RSD<5), robustness, LOD(0.15 ppm) and LOQ(0.45 ppm) are well within limits/% Recovery at LOQ, 50%, 100% and 150% was found to be within limit 80-120%.

Conclusion: RP-HPLC method was successfully developed with satisfactory separation of macitentan and its impurities. The proposed method was found to be specific, accurate, precise and robust can be used for estimation of macitentan and its impurities and can be successfully employed in the routine analysis of macitentan.

Keywords: RP-HPLC, Macitentan, Forced Degradation

INTRODUCTION

Macitentan is chemically a (5-{4-bromophenyl}-6-{2-[(5-bromo-pyrimidin-2-yl)oxy]ethoxy}pyrimidin-4-yl)sulfamoyl}(propyl) amine with molecular weight of 588.273g/mol [1].

Macitentan blocks the ET1-dependent rise in intracellular calcium by inhibiting the binding of ET-1 to ET receptors. Blocking of the ETA receptor subtype seems to be of more importance in the treatment of PAH than blocking of ETB, likely because there are higher numbers of ETA receptors than ETB receptors in pulmonary arterial smooth muscle cells [2-4].

A survey of literature revealed that RP-HPLC, first order Derivative UV Spectroscopy, and stability indicating analytical methods have been reported for macitentan. On literature survey, it was found that there are few RP-HPLC analytical methods available, but in my work impurities to be estimated are other than the reported one. Hence it was thought worthwhile to develop a method for estimation of impurities and related substance in macitentan using HPLC [5-9].

Therefore, it was of thought interest to develop precise, accurate, sensitive, selective chromatographic method for estimation of macitentan in Tablet dosage form which will provide valuable information that can be used to assess the inherent stability of the drug under various stressed conditions, eventually to improve formulation and manufacturing process. The aim of work was to carry out RP-HPLC method development and validation for macitentan tablet dosage form [10-12].

Table 1: List of Impurities with their specification

| S. No. | Impurity                  | Acceptance criteria |
|--------|---------------------------|---------------------|
| 1      | (MCA-01)                  | Not more than 0.15% |
| 2      | (MCA-02)                  | Not more than 0.15% |
| 3      | (Degradation)             | Not more than 0.15% |
| 4      | (N-propyl derivative)     | Not more than 0.10% |
| 5      | (N-N Dimethyl derivative) | Not more than 0.15% |
Equipment

The analysis was performed on HPLC Agilent technologies 1200 series, fitted with a gradient pump photodiode array detector and rheodyne injector with 20μl loop volume. Inertsil C8 (250 mm *4.6 mm)5 µm) column which is maintained at 30 ° C temperature. Chem-station software was applied for data collecting and processing.

Preparation of mobile phase

Prepare a Mobile phase A [Ammonium acetate (ph 4.5 adjusted with glacial acetic acid)] and Mobile phase B Acetonitrile in gradient elution. A buffer was sonicated for 5 min (minute) for degassing and filtered through 0.45 µ Millipore filter.

Diluent

The drug was dissolved in acetonitrile.

Preparation of standard stock solution (200 ppm)

Transfer an accurately weighed quantity of about 20 mg of Macitentan working standard into 100 ml of volumetric flask. Add about 50 ml of diluent and sonicate to dissolve. Make the volume up to mark with diluent and mix.

Preparation of standard solution (10 ppm)

Take 5 ml from std. A stock solution was transferred into the 100 ml volumetric flask and then diluted with the diluents.

Preparation of impurities solution: (10 ppm)

MCA-01: Weigh 1.012 mg of MCA-01 dissolve in 10 ml of diluent 2. Take 1 ml of it and dissolve in 10 ml diluents and mix well.

MCA-02: Weigh 1.005 mg of MCA-02 dissolve in 10 ml of diluent 2. Take 1 ml of it and dissolve in 10 ml diluents and mix well.

Degradation impurity: Weigh 1.003 mg of degradation impurity dissolve in 10 ml of diluent 2. Take 1 ml of it and dissolve in 10 ml diluents and mix well.

N-N Dimethyl derivative impurity: Weigh 1.042 mg of N-N Dimethyl derivative impurity dissolve in 10 ml of diluent 2. Take 1 ml of it and dissolve in 10 ml diluents and mix well.

N-propyl derivative: Weigh 1.023 mg of N-propyl derivative impurity dissolve in 10 ml of diluent 2. Take 1 ml of it and dissolve in 10 ml diluents and mix well.

(Diluent 2: 0.05% v/v HCL in ACN)

Spiked impurity mixture: (Specification limit of impurities =0.15 %)

Take 1 ml of the stock solution of standard, 1 ml of MCA-01 Stock solution, 1 ml of MCA-02 solution, 1 ml of Degradation impurity solution, 1 ml of N-N Dimethyl derivative impurity solution, 1 ml of N Propyl derivative impurity solution dilute up to 20 ml with ACN. Filter solution with 0.45 μ PVDF Filter.

As such sample preparation: (1000 ppm)

[Label claim: 10 mg]

The average of 10 Tablet was determined and grounded in a mortar. Weigh and transfer crush tablet equivalent to 50 mg (182.3 mg) into 50 ml of volumetric flask. Add 30 ml diluent (ACN) and sonicate for 45 min and makeup to 50 ml with diluents Mix well. Filter with 0.45 µm PVDF Filter.

Chromatographic conditions

Inertsil C8 (250*4.6 mm, 5 µm column was used as the stationary phase. Using mobile phase A [Ammonium acetate (ph 4.5 adjusted with glacial acetic acid)] and mobile phase B Acetonitrile in gradient elution. It was filtered through 0.45μ (micron) membrane filter and degassed. The mobile phase was pumped at 1.5 ml/min. The eluents were monitored at 266 nm. The injection volumes of sample and standard were 20μl (microliter). Total run time is 70 min.

| Time | MP A | MP B |
|------|------|------|
| 0    | 66   | 34   |
| 5    | 66   | 34   |
| 15   | 60   | 40   |
| 30   | 50   | 50   |
| 50   | 40   | 60   |
| 60   | 25   | 75   |
| 62   | 66   | 34   |
| 70   | 66   | 34   |

Table 2: Gradient program

Fig. 2: Chromatogram of macitentan with its impurities
RESULTS AND DISCUSSION

Method development
ICH prescribed stress conditions such as acidic, basic and oxidative stresses were carried out.

Acid degradation
Sample preparation
The average of 10 Tablet was determined and grounded in a mortar. An accurately weighed the amount of powder equivalent to 10 mg of macitentan (152.5 mg) sample dissolve in 10 ml of diluent (ACN) sonicate for 30 min then add 1 ml of 5 N HCL and heat at 80 ° C in water bath for 1 h. Then cool it at RT and neutralize it with 1 ml of 5 M NaOH. Makeup to volume 25 ml with Diluent. Filter it.

Base degradation
Preparation of sample
The average of 10 Tablet was determined and grounded in a mortar. An accurately weighed the amount of powder equivalent to 10 mg of macitentan (152.6 mg) sample dissolve in 10 ml of diluent (ACN) sonicate for 30 min then add 1 ml of 5 M NaOH and heat at 80 °C in a water bath for 1 h. Then cool it at RT and neutralize it with 1 ml of 5 N HCL. Make up to volume 25 ml with Diluent. Filter it.

Table 3: Degradation summary

| Type                       | Solution | Area   | % Degradation |
|----------------------------|----------|--------|---------------|
| As Such                    | macitentan | 159223 | -             |
| Acid Degradation           | macitentan | 136124 | 14.50%        |
| Base Degradation           | macitentan | 141223 | 11.30%        |
| Peroxide Degradation       | macitentan | 150013 | 5.78%         |
Peroxide degradation

Preparation of sample

152.5 mg sample dissolve in 10 ml with diluents sonicate for 30 min then add 1 ml of 10% H₂O₂ and heat at 80 °C in a water bath for 1 h then cool the sample at RT and make up a sample with Diluent. Filter it.

Method validation

The described method has been validated which include parameters like linearity, accuracy, precision, robustness, LOD (limit of detection) and LOQ (limit of quantification).

Linearity

The linearity of this method was evaluated by linear regression analysis and calculated by a least square method and studied by preparing stock solutions of MCA-01, MCA-02 and Degradation impurities at different concentration levels.

The calibration curve showed good linearity in the range of 0.45-2.25µg/ml. Generate linearity plot of area versus percentage of concentration. Linearity curve it should be more than 0.998 that shows linear detector response. The results are given in table 4.

| Drug            | Conc* (µg/ml) | Area    |
|-----------------|--------------|---------|
| MCA-02          |              |         |
| 0.45            | 11030        |         |
| 0.75            | 18032        |         |
| 1.5             | 36568        |         |
| 1.8             | 43723        |         |
| 2.25            | 55123        |         |
| MCA-01          |              |         |
| 0.45            | 15218        |         |
| 0.75            | 25003        |         |
| 1.5             | 50423        |         |
| 1.8             | 61517        |         |
| 2.25            | 73930        |         |
| Degradation Impurity |          |         |
| 0.45            | 9718         |         |
| 0.75            | 16131        |         |
| 1.5             | 33001        |         |
| 1.8             | 40051        |         |
| 2.25            | 51358        |         |

Conc*-concentration

![Fig. 6: Calibration curve of MCA-02](image)

![Fig. 7: Calibration curve of MCA-01](image)
Table 5: Recovery data of MCA-02

| Conc level | Amount added | Area observed | Amount recovered | % recovery | % Mean recovery ± SD | %RSD |
|------------|--------------|---------------|-----------------|------------|---------------------|------|
| LOQ        | 0.45         | 11123         | 0.457           | 101.55     | 102.51 ± 1.00       | 0.97 |
| 30%        | 0.45         | 11345         | 0.466           | 103.55     | 97.37 ± 0.33        | 0.34 |
|            | 0.75         | 18138         | 0.745           | 99.33      | 99.77 ± 0.381       | 0.38 |
| 50%        | 0.75         | 18098         | 0.743           | 99.06      | 99.11 ± 0.254       | 0.25 |
|            | 1.5          | 35735         | 1.47            | 98.00      | 98.00 ± 0.230       | 0.24 |
| 100%       | 1.5          | 35918         | 1.47            | 98.00      | 97.13 ± 0.231       | 0.24 |
|            | 2.25         | 54554         | 2.24            | 96.22      | 100.90 ± 0.110      | 1.22 |
| 150%       | 2.25         | 54624         | 2.24            | 96.13      | 102.6 ± 0.251       | 0.24 |

SD*: Standard deviation, RSD*: Relative standard deviation, Number of experiments (n)-3

Table 6: Recovery data of MCA-01

| Conc level | Amount added | Area observed | Amount recovered | % recovery | % Mean recovery ± SD | %RSD |
|------------|--------------|---------------|-----------------|------------|---------------------|------|
| LOQ        | 0.45         | 16212         | 0.472           | 104.8      | 105.46 ± 1.15       | 1.09 |
| 30%        | 0.45         | 16524         | 0.481           | 106.8      | 98.00 ± 0.230       | 0.24 |
|            | 0.75         | 25233         | 0.735           | 98.06      | 96.53 ± 0.045       | 0.05 |
| 50%        | 0.75         | 25148         | 0.732           | 97.6       | 96.22 ± 0.231       | 0.24 |
|            | 1.5          | 50021         | 1.457           | 97.13      | 97.13 ± 0.231       | 0.24 |
| 100%       | 1.5          | 49812         | 1.451           | 96.73      | 96.17 ± 0.045       | 0.05 |
|            | 1.5          | 50013         | 1.457           | 97.13      | 96.17 ± 0.045       | 0.05 |
|            | 2.25         | 74334         | 2.165           | 96.22      | 96.22 ± 0.045       | 0.05 |
| 150%       | 2.25         | 74331         | 2.164           | 96.17      | 96.17 ± 0.045       | 0.05 |
|            | 2.25         | 74282         | 2.163           | 96.13      | 96.13 ± 0.045       | 0.05 |

Number of experiments (n)-3, SD*: Standard deviation, RSD*: Relative Standard deviation

Table 7: Recovery data of degradation impurity

| Conc level | Amount added | Area observed | Amount recovered | % recovery | % Mean recovery ± SD* | %RSD |
|------------|--------------|---------------|-----------------|------------|-----------------------|------|
| LOQ        | 0.45         | 9118          | 0.411           | 91.33      | 91.34 ± 1.110         | 1.22 |
| 30%        | 0.45         | 9013          | 0.406           | 90.22      | 95.20 ± 0.669         | 0.70 |
|            | 0.75         | 9228          | 0.416           | 92.44      | 95.73 ± 0.669         | 0.70 |
| 50%        | 0.75         | 15830         | 0.714           | 95.20      | 95.20 ± 0.669         | 0.70 |
|            | 1.5          | 15911         | 0.718           | 95.73      | 95.73 ± 0.669         | 0.70 |
| 100%       | 1.5          | 33056         | 1.514           | 100.9      | 101.00 ± 0.655        | 0.64 |
|            | 1.5          | 33404         | 1.507           | 100.4      | 102.6 ± 0.251         | 0.24 |
|            | 2.25         | 33816         | 1.526           | 101.7      | 102.56 ± 0.251        | 0.24 |
| 150%       | 2.25         | 33404         | 1.507           | 100.4      | 102.6 ± 0.251         | 0.24 |
|            | 2.25         | 51151         | 2.309           | 102.6      | 102.6 ± 0.251         | 0.24 |
|            | 2.25         | 50999         | 2.302           | 102.3      | 102.3 ± 0.251         | 0.24 |
|            | 2.25         | 51258         | 2.313           | 102.8      | 102.8 ± 0.251         | 0.24 |

SD*: Standard deviation, Conc*: concentration, RSD*: Relative Standard deviation, Number of experiments (n)-3
Accuracy

The accuracy of the method was determined at LOQ (30%), 50%, 100% and 150% by calculating recovery of Impurities in the solution. Each solution was injected in triplicate and the % recovery was calculated. Recovery (individually) at each level is between 91–106%. RSD of % recovery is not more than 5. The results are given in table 5-7.

Limit of detection (LOD) and limit of quantification (LOQ)

According to the ICH recommendation, the approach based on the standard deviation (SD) of the response and slope was a use of the determining the LOD and LOQ values. The LOD and LOQ were found to be 0.15µg/ml and 0.45µg/ml for MCA-01, MCA-02 and Degradation impurity estimated by using the S/N ratio. The low values of LOD and LOQ illustrate that the developed method was sensitive, accurate and precise as it can be detected and quantify with very low concentration.

Acceptance criteria: LOQ

It is estimated the progressive lower concentration of impurity until a signal to noise (S/N) ratio remains greater than 10.

LOD

It is estimated by injecting the diluted concentration until the peak of impurity is able to detect. The results are given in table 8.

Table 8: S/N Ratio for LOD and LOQ of impurity

| Name of impurity       | LOD (S/N Ratio) | LOQ (S/N Ratio) |
|------------------------|----------------|-----------------|
| MCA-02                 | 8.17           | 58.1            |
| MCA-01                 | 5.92           | 48.5            |
| Degradation Impurity   | 6.17           | 65.4            |

LOD-Limit of detection, LOQ-Limit of quantification

Precision

Repeatability

For Repeatability sample containing all impurities at 100% level injected for six times and for the intermediate precision sample containing all impurities at 50%, 100%, 150% level injected for Intraday precision and Interday precision it is injected in 3 sets. Sample spiked with all known impurities at 100% level injected six times. All impurity peak area calculated for RSD. % RSD is not more than 5. The results are given in table 9.

Table 9: Repeatability data of MCA-02, MCA-01, degradation impurity

| S. No. | Concentration PPM (100% level) | Peak area | MCA-02 | MCA-01 | Degradation impurity |
|--------|--------------------------------|-----------|--------|--------|---------------------|
|        |                                |           | Mean±SD* |        |                     |
|        | % Mean recovery±SD*            |           | 3579±141.17 | 5041±550.13 | 3222±553.08 |
|        | %RSD                           |           | 1.15    | 1.09   | 1.72                |

SD*-Standard deviation, RSD*-Relative standard deviation, Number of experiments (n)=6, Conc*-concentration

Intraday precision

Intraday precision was performed by injecting stock impurities preparations two times (Morning and Evening) on the day by maintaining the optimized chromatographic conditions and calculate % relative standard deviation of retention time and peak areas for macitentan. All impurity area calculated for RSD for morning and evening. % RSD is not more than 5. so method is precise. The results are given in table 10, 11, and 12.

Table 10: Intraday precision of MCA-02

| 50 % level | Set | Level | Morning | Evening | mean±SD* | RSD |
|------------|-----|-------|---------|---------|----------|-----|
| 1          | 1   | 50%   | 20432   | 20124   | 2027±21.79 | 1.07 |
| 2          | 2   | 50%   | 20213   | 20598   | 2040±272.24 | 1.33 |
| 3          | 3   | 50%   | 20513   | 20188   | 2035±229.80 | 1.13 |
| 100 % level| Set | Level | Morning | Evening | mean±SD* | RSD |
| 1          | 1   | 100%  | 36981   | 35991   | 3648±700.03 | 1.92 |
| 2          | 2   | 100%  | 36607   | 36033   | 3632±405.87 | 1.12 |
| 3          | 3   | 100%  | 36108   | 35997   | 3605±70.48  | 0.22 |
| 150 % level| Set | Level | Morning | Evening | mean±SD* | RSD |
| 1          | 1   | 150%  | 55814   | 55125   | 5544±487.18 | 0.88 |
| 2          | 2   | 150%  | 56124   | 55899   | 5601±159.09 | 0.28 |
| 3          | 3   | 150%  | 55754   | 55160   | 5545±420.02 | 0.76 |

SD*-Standard deviation, RSD*-Relative Standard deviation, Number of experiments (n)=3
Table 11: Intraday precision of MCA-01

| Set  | Level | Morning | Evening | mean±SD | RSD    |
|------|-------|---------|---------|---------|--------|
| 1    | 50%   | 26013   | 25981   | 25997±22.62 | 0.09   |
| 2    | 50%   | 26312   | 26121   | 26217±135.05 | 0.52   |
| 3    | 50%   | 26567   | 26056   | 26312±361.33 | 1.37   |
| 100% | Level |         |         |         |        |
| 1    | 100%  | 52254   | 51789   | 5202±328.80  | 0.63   |
| 2    | 100%  | 52312   | 52013   | 5216±211.42  | 0.41   |
| 3    | 100%  | 52159   | 51936   | 52048±157.68 | 0.30   |
| 150% | Level |         |         |         |        |
| 1    | 150%  | 74718   | 74135   | 74427±412.24 | 0.55   |
| 2    | 150%  | 74812   | 73556   | 73684±181.01 | 0.25   |
| 3    | 150%  | 74520   | 74132   | 74326±274.35 | 0.37   |

SD*-Standard deviation, RSD*-Relative Standard deviation, Number of experiments (n)-3

Table 12: Intraday precision of degradation impurity

| Set  | Level | Morning | Evening | mean±SD | RSD   |
|------|-------|---------|---------|---------|-------|
| 1    | 50%   | 16381   | 15989   | 1618±277.18 | 1.71  |
| 2    | 50%   | 16261   | 15994   | 16128±188.79 | 1.17  |
| 3    | 50%   | 16221   | 15931   | 1607±205.06 | 1.28  |
| 100% | Level |         |         |         |       |
| 1    | 100%  | 32132   | 31818   | 3252±180.23 | 0.69  |
| 2    | 100%  | 32331   | 32121   | 3222±148.49 | 0.46  |
| 3    | 100%  | 32880   | 32590   | 3273±205.06 | 0.63  |
| 150% | Level |         |         |         |       |
| 1    | 150%  | 51121   | 51159   | 51140±268.79 | 0.05  |
| 2    | 150%  | 52310   | 51817   | 5206±348.60 | 0.62  |
| 3    | 150%  | 51731   | 51234   | 5148±287.79 | 0.56  |

SD*-Standard deviation, RSD*-Relative standard deviation, Conc*-concentration, Number of experiments (n)-3

Interday precision

Inter-day precision was performed by injecting stock impurity preparations three times into chromatographic system on two different days by maintaining the optimized chromatographic conditions and calculate % relative standard deviation of retention time and peak areas for macitentan. All impurity area calculated for RSD for Day-1 and Day-2 %RSD is not more than 5 so method is precise. The results are given in table 13-15.

Robustness

According to robustness, there is the minor deliberate change made such as in chromatograph parameter with reference of flow rate and column temperature. To observe robustness, 100 % level solution used. Robustness was checked by changing the flow rate and column temperature in the optimized chromatographic condition. This method said to be robust as % RSD for each studied factor was found to be less than 5. The results are given in table 16, 17, and 18.

Table 13: Interday precision of MCA-02

| Set  | Level | Day-1 | Day-2 | mean±SD | RSD   |
|------|-------|-------|-------|---------|-------|
| 1    | 50%   | 20432 | 20812 | 2062±268.70 | 1.30  |
| 2    | 50%   | 20213 | 20787 | 2050±405.87 | 1.98  |
| 3    | 50%   | 20513 | 20013 | 2026±353.55 | 1.74  |
| 100% | Level |       |       |         |       |
| 1    | 100%  | 36981 | 37130 | 3706±105.35 | 0.28  |
| 2    | 100%  | 36607 | 36917 | 3676±219.20 | 0.60  |
| 3    | 100%  | 36108 | 36718 | 3641±341.33 | 1.18  |
| 150% | Level |       |       |         |       |
| 1    | 150%  | 55814 | 56132 | 5597±224.8 | 0.40  |
| 2    | 150%  | 56124 | 56338 | 5623±351.32 | 0.27  |
| 3    | 150%  | 55754 | 56124 | 5599±261.62 | 0.47  |

SD*-Standard deviation, RSD*-Relative Standard deviation, Number of experiments (n)-3
### Table 14: Interday precision of MCA-01

| Set     | Level     | Day-1     | Day-2     | mean±SD* | RSD     |
|---------|-----------|-----------|-----------|----------|---------|
| 100 % level | 50%       | 26013     | 26454     | 2623±311.83 | 1.19    |
| 100%     | 50%       | 26312     | 26818     | 2656±375.79  | 1.35    |
| 100%     | 50%       | 26567     | 26121     | 2634±15.36   | 1.20    |

### Table 15: Interday precision of degradation impurity

| Set     | Level     | Day-1     | Day-2     | mean±SD* | RSD     |
|---------|-----------|-----------|-----------|----------|---------|
| 100% level | 50%       | 16381     | 16525     | 1645±101.82 | 0.62    |
| 100%     | 50%       | 16261     | 16434     | 1634±122.32 | 0.75    |
| 100%     | 50%       | 16221     | 16621     | 1637±13.54  | 1.30    |

### Table 16: Robustness result of MCA-02

| Parameter | Change | Area        | %Mean recovery±SD* | RSD     |
|-----------|--------|-------------|---------------------|---------|
| Flow rate | 1.3 ml | 35312       | 35138               | 35381   | 3584±343.91 | 1.20    |
| (ml/min)  | 1.5 ml | 36142       | 36106               | 36131   | 3622±123.54 | 0.75    |
| Coloumn   | 25 °C  | 36131       | 36150               | 36300   | 3536±253.12 | 0.50    |
| temp.     | 30 °C  | 35648       | 35830               | 36101   | 3590±258.33 | 0.50    |
| 35 °C     | 36130  | 36138       | 35998               |         |          |         |

### Table 17: Robustness result of MCA-01

| Parameter | Change | Area        | %Mean recovery±SD* | RSD     |
|-----------|--------|-------------|---------------------|---------|
| Flow Rate | 1.3 ml | 50324       | 50128               | 50155   | 5097±54.83  | 1.07    |
| (ml/min)  | 1.5 ml | 51312       | 50998               | 51212   | 5122±123.54 | 0.75    |
| Coloumn   | 25 °C  | 51034       | 50938               | 50668   | 5126±258.33 | 0.50    |
| Temp.     | 30 °C  | 51341       | 51554               | 51344   | 5143±258.33 | 0.50    |
| 35 °C     | 51334  | 51558       | 51438               |         |          |         |

### Table 18: Robustness result of degradation impurity

| Parameter | Change | Area        | %Mean recovery±SD* | RSD     |
|-----------|--------|-------------|---------------------|---------|
| Flow rate | 1.3 ml | 33133       | 32734               | 33187   | 3527±357.68 | 1.01    |
| (ml/min)  | 1.5 ml | 33077       | 33412               | 33132   | 3321±143.54 | 0.54    |
| Column    | 25 °C  | 32812       | 33018               | 32918   | 3307±176.45 | 0.53    |
| temp.     | 30 °C  | 33118       | 33418               | 33216   | 3303±176.45 | 0.53    |
| 35 °C     | 33141  | 32998       | 33013               |         |          |         |

SD*-Standard deviation, RSD*-Relative Standard deviation, Number of experiments (n)-3
CONCLUSION

All the parameters and results were found within the acceptance limit as given in the validation protocol. So we can conclude that the developed RP-HPLC Method was selective, specific, sensitive, linear, accurate, precise, and robust. Therefore the method is found to be specific for macitentan’s related substances with good resolution. It can be applied to the forced degradation study. So the proposed method can be used in the pharmaceutical analysis for Forced degradation study and routine quality control samples of macitentan Tablets.

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AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICT OF INTERESTS

Declared none

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