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Original Article

RP-HPLC METHOD DEVELOPMENT AND VALIDATION OF MACITENTAN WITH ITS KNOWN AND UNKNOWN DEGRADATION IMPURITIES IN ITS TABLET DOSAGE FORM

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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

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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].

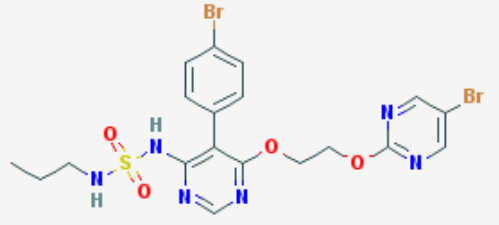

Fig. 1: Structure of macitentan

MATERIALS AND METHODS

In the present research work, an attempt was made to develop and validate macitentan tablet dosage form with its Known and unknown Degradation Impurities with RP-HPLC method. acetonitrile, methanol, ammonium acetate, potassium hydrogen Phosphate, hydrochloric acid, sodium hydroxide, hydrogen peroxide, glacial acetic acid and phosphoric Acid were produce from Merck. The sample of Macitentan API, Tablets and impurities were kindly gifted by ZYDUS CADILA HEALTH CARE, Moraiya, Ahmedabad [13].

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 µm 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.

Table 2: **Gradient program**

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

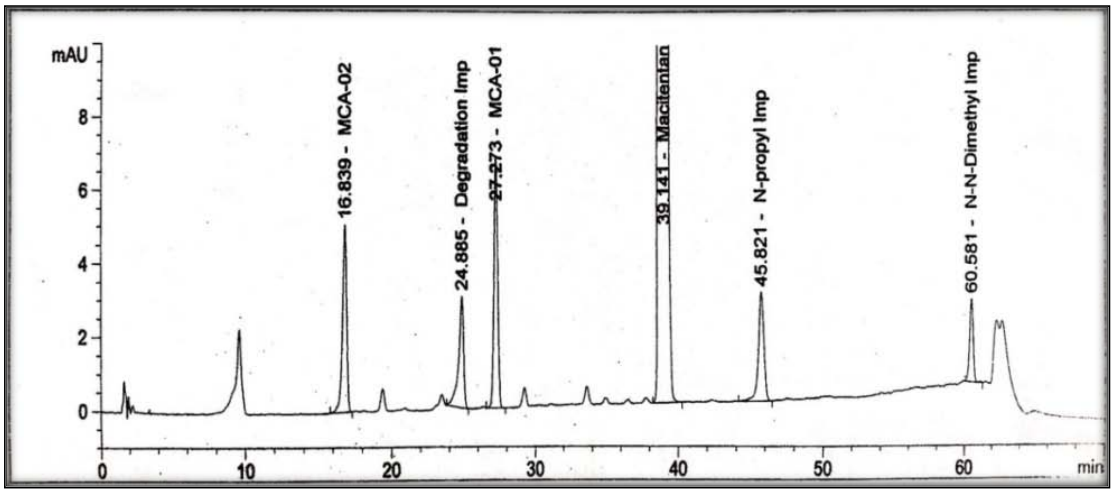

Fig. 2: Chromatogram of macitentan with its impurities

The developed Method was validated for linearity, precision, accuracy, robustness and is applied for forced degradation studies as per the ICH guidelines.

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.

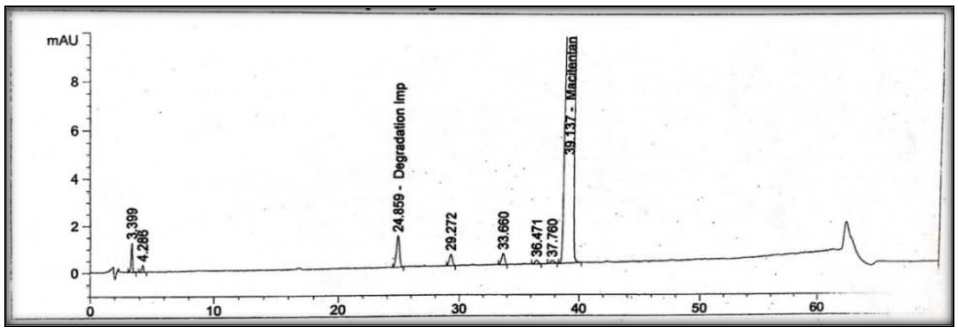

Fig. 3: Acid degradation for macitentan

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.

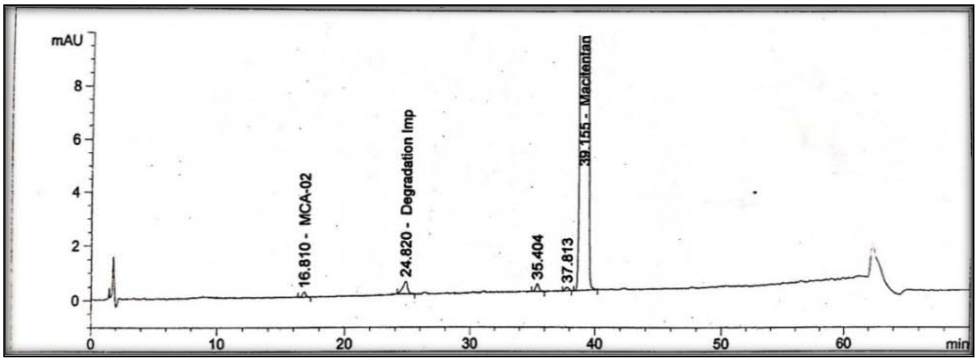

Fig. 4: Base degradation for macitentan

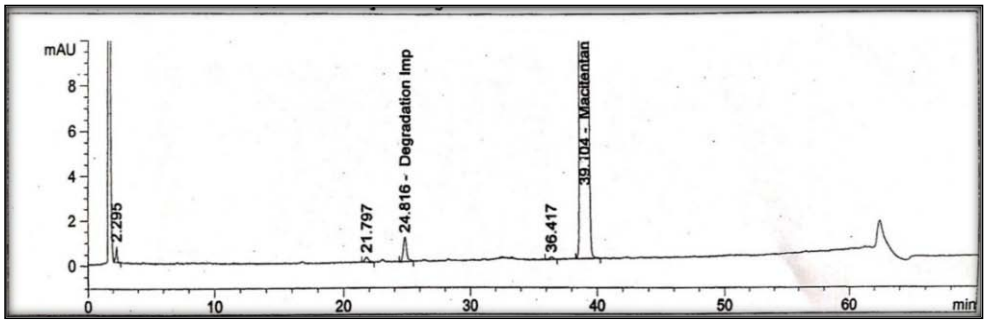

Fig. 5: Peroxide degradation for macitentan

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% H_2O_2 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.

Table 4: Linearity data for MCA-02, MCA-01 and degradation impurity

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

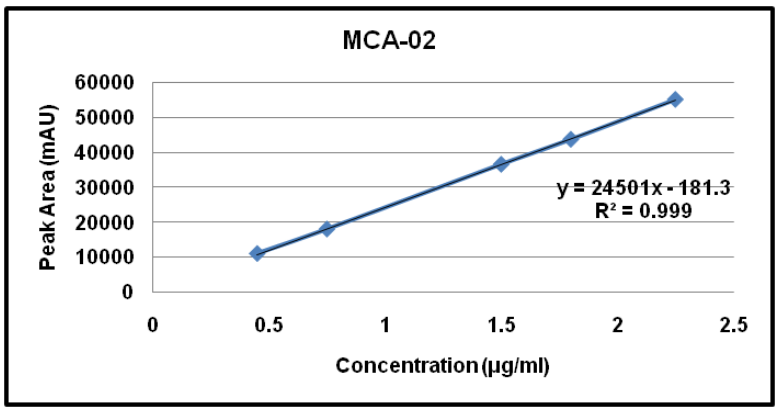

Fig. 6: Calibration curve of MCA-02

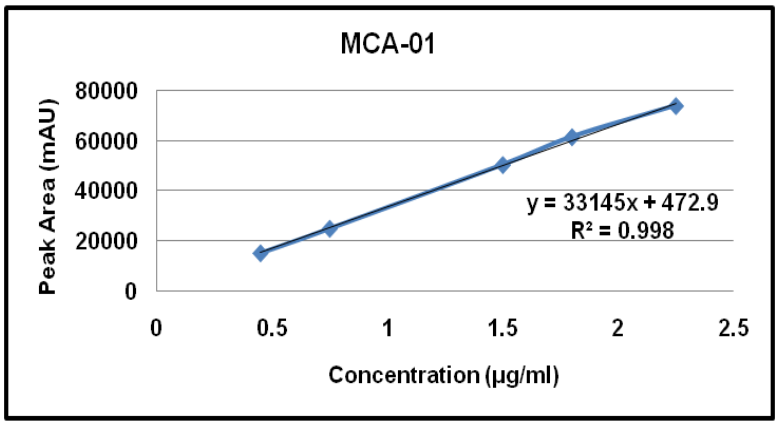

Fig. 7: Calibration curve of MCA-01

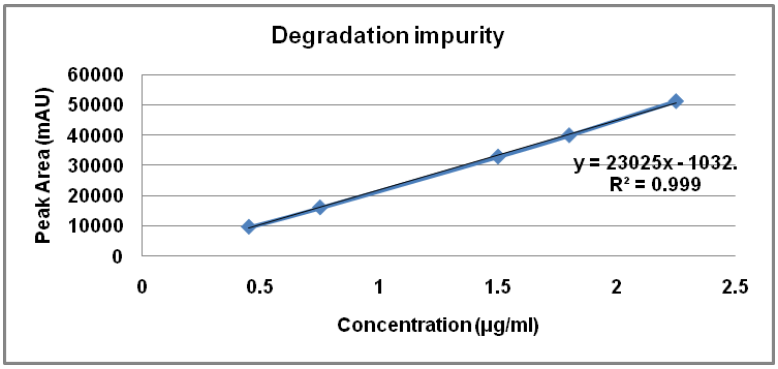

Fig. 8: Calibration curve of degradation impurity

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		
30%	0.45	11345	0.466	103.55	102.51±1.00	0.97
	0.45	11234	0.461	102.44		
	0.75	18138	0.745	99.33	97.37±0.33	
50 %	0.75	18098	0.743	99.06		0.34
	0.75	18212	0.748	99.73		
	1.5	35735	1.46	97.33	97.77±0.381	
100 %	1.5	35918	1.47	98.00		0.38
	1.5	35824	1.47	98.00		
	2.25	54554	2.24	99.55	99.40±0.254	
150 %	2.25	54312	2.23	99.11		0.25
	2.25	54624	2.24	99.55		

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
	0.45	16212	0.472	104.8	105.46±1.15	
LOQ	0.45	16524	0.481	106.8		1.09
30%	0.45	16224	0.472	104.8		
	0.75	25233	0.735	98	98.00±0.230	
50 %	0.75	25148	0.732	97.6		0.24
	0.75	25255	0.735	98		
	1.5	50021	1.457	97.13	97.00±0.231	
100 %	1.5	49812	1.451	96.73		0.24
	1.5	50013	1.457	97.13		
	2.25	74334	2.165	96.22	96.01±0.045	
150 %	2.25	74331	2.164	96.17		0.05
	2.25	74282	2.163	96.13		

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
	0.45	9118	0.411	91.33	91.34±1.110	
LOQ	0.45	9013	0.406	90.22		1.22
30%	0.45	9228	0.416	92.44		
	0.75	15830	0.714	95.20	95.82±0.669	
50 %	0.75	15911	0.718	95.73		0.70
	0.75	16045	0.724	96.53		
	1.5	33586	1.514	100.9	101.00±0.655	
100 %	1.5	33816	1.526	101.7		0.64
	1.5	33404	1.507	100.4		
	2.25	51151	2.309	102.6	102.56±0.251	
150 %	2.25	50998	2.302	102.3		0.24
	2.25	51258	2.313	102.8		

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
1	1.5	35740	50381	32378
2	1.5	35948	50581	31318
3	1.5	34998	49380	32484
4	1.5	36141	51008	32980
5	1.5	35889	50451	32035
6	1.5	36030	50661	32123
% Mean recovery±SD*		35791±411.17	50410±550.13	32220±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	50%	20432	20124	20278±217.98	1.07
2	50%	20213	20598	20406±272.24	1.33
3	50%	20513	20188	20351±229.80	1.13
100 % level					
Set	Level	Morning	Evening	mean±SD*	RSD
1	100%	36981	35991	36486±700.03	1.92
2	100%	36607	36033	36320±405.87	1.12
3	100%	36108	35997	36053±78.48	0.22
150 % level					
Set	Level	Morning	Evening	mean±SD*	RSD
1	150%	55814	55125	55470±487.19	0.88
2	150%	56124	55899	56012±159.09	0.28
3	150%	55754	55160	55457±420.02	0.76

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

Table 11: Intraday precision of MCA-01

50 % level					
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					
Set	Level	Morning	Evening	mean±SD*	RSD
1	100%	52254	51789	52022±328.80	0.63
2	100%	52312	52013	52163±211.42	0.41
3	100%	52159	51936	52048±157.68	0.30
150 % level					
Set	Level	Morning	Evening	mean±SD*	RSD
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

50 % level					
Set	Level	Morning	Evening	mean±SD*	RSD
1	50%	16381	15989	16185±277.18	1.71
2	50%	16261	15994	16128±188.79	1.17
3	50%	16221	15931	16076±205.06	1.28
100 % level					
Set	Level	Morning	Evening	mean±SD*	RSD
1	100%	32132	31818	31975±222.03	0.69
2	100%	32331	32121	32226±148.49	0.46
3	100%	32880	32590	32735±205.06	0.63
150 % level					
Set	Level	Morning	Evening	mean±SD*	RSD
1	150%	51121	51159	51140±26.87	0.05
2	150%	52310	51817	52064±348.60	0.62
3	150%	51731	51234	51483±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 2 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

50 % level					
Set	Level	Day-1	Day-2	mean±SD*	RSD
1	50%	20432	20812	20622±268.70	1.30
2	50%	20213	20787	20500±405.87	1.98
3	50%	20513	20013	20263±353.55	1.74
100% level					
Set	Level	Day-1	Day-2	mean±SD*	RSD
1	100%	36981	37130	37056±105.35	0.28
2	100%	36607	36917	36762±219.20	0.60
3	100%	36108	36718	36413±431.33	1.18
150 % level					
Set	Level	Day-1	Day-2	mean±SD*	RSD
1	150%	55814	56132	55973±224.8	0.40
2	150%	56124	56338	56231±151.32	0.27
3	150%	55754	56124	55939±261.62	0.47

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

Table 14: Interday precision of MCA-01

50 % Level					
Set	Level	Day-1	Day-2	mean±SD*	RSD
1	50%	26013	26454	26234±311.83	1.19
2	50%	26312	26818	26565±375.79	1.35
3	50%	26567	26121	26344±315.36	1.20
100 % level					
Set	Level	Day-1	Day-2	mean±SD*	RSD
1	100%	52254	52535	52395±198.69	0.38
2	100%	52312	52117	52215±137.88	0.26
3	100%	52159	52652	52406±348.60	0.67
150% level					
Set	Level	Day-1	Day-2	mean±SD*	RSD
1	150%	74718	74968	74843±176.77	0.24
2	150%	74812	74528	74873±106.77	0.27
3	150%	74520	74912	74716±277.18	0.37

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

Table 15: Interday precision of degradation impurity

50 % Level					
Set	Level	Day-1	Day-2	mean±SD*	RSD
1	50%	16381	16525	16453±101.82	0.62
2	50%	16261	16434	16348±122.32	0.75
3	50%	16221	16623	16372±213.54	1.30
100 % level					
Set	Level	Day-1	Day-2	mean±SD*	RSD
1	100%	32132	32722	32427±417.19	1.29
2	100%	32331	32918	32625±415.07	1.27
3	100%	32880	32581	32731±211.42	0.65
150% level					
Set	Level	Day-1	Day-2	mean±SD*	RSD
1	150%	51121	51438	51280±224.15	0.44
2	150%	52310	51912	52111±281.42	0.54
3	150%	51731	51934	51833±143.54	0.28

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

Table 16: Robustness result of MCA-02

Parameter	**Change**	**Area**			**%Mean recovery±SD***	**RSD**
		1	2	3		
Flow rate	1.3 ml	35312	35138	35381	35842.33±431.91	
(ml/min)	1.5 ml	36013	36133	36131		1.20
	1.7 ml	36142	36108	36223		
Coloumn	25 °C	36131	36150	36300	35936.22±352.13	
temp.	30 °C	35648	35830	36101		0.97
	35 °C	36130	36138	35998		

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

Table 17: Robustness result of MCA-01

Parameter	**Change**	**Area**			**% Mean recovery±SD***	**RSD**
Flow Rate		**1**	**2**	**3**		
(ml/min)	1.3 ml	50324	50128	50155	50907**±**546.83	
	1.5 ml	51312	50998	51212		1.07
	1.7 ml	51502	51304	51228		
Coloumn	25 °C	51034	50938	50868	51267.67**±**258.33	
Temp.	30 °C	51341	51554	51344		0.50
	35 °C	51334	51558	51438		

SD*-Standard deviation, RSD*-relative standard deviation, number of experiments (n)-3

Table 18: Robustness result of degradation impurity

Parameter	**Change**	**Area**			**%Mean recovery±SD***	**RSD**
Flow rate		1	2	3		
(ml/min)	1.3 ml	33133	32734	33187	35278.67±357.68	1.01
	1.5 ml	33077	33412	33132		
	1.7 ml	33814	33581	33781		
Column	25 °C	32812	33018	32918	33072.44±176.45	0.53
temp.	30 °C	33118	33418	33216		
	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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