Sensitive and selective analytical method for the quantification of two potential Genotoxic impurities in Azilsartan drug substance by using LC-MS/MS with Multiple Reaction Monitoring(MRM mode)

Abstract

Sensitive and selective LC-MS/MS MRM mode method was developed and validated for the quantification of two potential genotoxic impurities; Impurity-A and Impurity-B at trace levels in Azilsartan drug substance.The concentration limits of the both genotoxic impurities was calculated a limit of 37.5ppm based on the concept of TTC(threshold of toxicological concern) and MDD(maximum daily dosage which is 40mg/day for Azilsartan drug substance).The method was found to be Selective and Sensitive for the application.The limit of detection(LOD) was found to be 1.4ppm for both Impurity-A and Impurity-B.The limit of quantification(LOQ) for Impurity-A was 4.7ppm and Impurity-B was 4.5ppm respectively The method was found to be linear from 4.7ppm to 78.4ppm for Impurity-A and 4.5ppm to 75.7ppm for Impurity-B .The method was found to be specific,precise,linear and accurate.

Keywords: LC-MS/MS, MRM(Multiple reaction monitoring,TTC(Threshold of toxicological concern),MDD(Maximum daily dosage),Azilsartan,validation

1. Introduction

Azilsartan, Chemical name 2-ethoxy-1-((2'-(5-oxo-4,5-dihydro-1,2-4-oxadiazol-3-yl)-[1,1'-biphenyl]-4-yl)methyl)-1H-benzo[d]imidazole-7-carboxylic acid, which is used to treat high blood pressure(hypertension).Azilsartan belongs to a class of drugs called angiotensin receptor blockers(ARBs). Impurity-A used as a key starting material for synthesis of Azilsartan drug substance.Impurity-B will arise during synthesis of Azilsartan drug substance. ROS of Azilsartan shown in figure-1.Structue and chemical name of Impurity-A and Impurity-B shown in figure-2 and figure-3 respectively.Impurity-A and Impurity-B confirmed as a potential genotoxic impurities based on the available literature[1-7].The presence of trace level impurities present in the drug substance or drug product may potentially cause severe harmful effects on human health.The concentration limit of genotoxic impurities, Impurity-A and Impurity-B has been calculated based on TTC[1-7] and maximum daily dose[1-7].So Impurity-A and Impurity-B each must to be controlled at below 37.5ppm.

Literature survey show some work related to Azilsartan and Azilsartan medoxomil [8-15]related substances by using high performance liquid chromatographic methods. So the accurate quantification of Impurity-A and Impurity-B at ppm levels the above Literature methods are inadequate. Literature survey reveals that there was no sensitive and selective method available for the quantification of Impurity-A and Impurity-B by using LC-MS/MS Multiple reaction monitoring (MRM) mode. .So, the objective of this work is to develop and validate a highly Sensitive, accurate and selective LC-MS/MS MRM mode method developed and validated for the determination of trace level quantification of Impurity-A and Impurity-B in Azilsartan drug substance.

Quantitative structure-activity relationship (QSAR) analysis carried out for all the rawmaterials, reagents, intermediate, impurities and reagents used in the process of Azilsartan dru substance to identfy the Mutagenic impurities. We have found the Impurity A and Impurity B are mutagenic due to certain electrophilic moieties within a chemical structures. Both compounds are mutagenic as well as DNA-reactive. i.e. Impurity-A is intermediate of Azilsartan and Impurity-B is process impurity due to hydrolysis of intermediate. Following illustration below shows.

Figure 1 Structures of mutagenic impurities

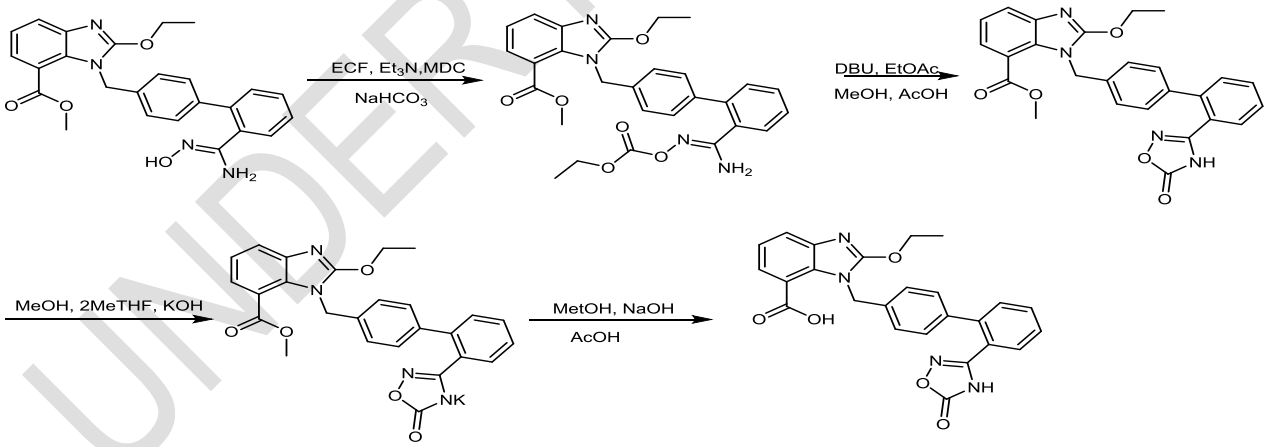

Figure 2 synthetic route of Azilsartan drug substance

2. Material and methods

2.1 Materials and methods

All chemicals were purchased from Sigma Aldrich and used directly. Reaction progress was monitored by thin-layer chromatography (TLC) using silica gelaluminium sheets (60F-254) and UV light. 1H nuclear magnetic resonance (NMR) spectra were recorded on Bruker Avance 400 MHz spectrometer with tetramethylsilane (TMS) as an internal standard. Splitting patterns were described as singlet (s), doublet (d), triplet (t), quartet (q), or doublet of doublet (dd) and multiplet (m). The broad (br) signals were also indicated. The value of chemical shifts (d) is given in ppm and coupling constants (J) in Hertz (Hz). Mass spectra were obtained using waters XEVO TQ LCMS instrument was used with an electrospray(ESI) positive and negative ionization modes.

2.2 Synthesis

Synthesis of Methyl(Z)-2-ethoxy-1-((2'-(N'-hydroxycarbamimidoyl)-[1,1'-biphenyl]-4-yl)methyl)-1H-benzo[d]imidazole-7-carboxylate (Impurity-A): To the stirred solution of Hydroxylamine hydrochloride (16.8g ,0.24mmol) and Sodium bicarbonate (30.6g ,0.36mmol) in Dimethyl sulfoxide (100 mL) heated to 50°C for 1-2h, Methyl 1-((2'-cyano-[1,1'-biphenyl]-4-yl)methyl)-2-ethoxy-1H-benzo[d]imidazole-7-carboxylate (10g, 0.024mmol) was added slowly at 50°C. The reaction mixture was stirred for 12h at 80°C and then further stirred at 40 °C. The reaction progress was monitored by TLC. The inorganic solids were filtered, and the obtained filtrate was poured into water (100 mL) and then stirred for 1 h. The obtained solid was filtered, and washed with water (50 mL) and dried under reduced pressure. The purified compound was then obtained by crystallization in Methanol to afford compound (A). White colour solid, yield:70%,1H NMR (400 MHz, DMSO-d6) d 9.16 (s, 1H, OH), 7.69 (dd J=8 Hz and 1Hz, 1H ArH), 7.38 (m, 6H, ArH), 7.29 (dd, J=8 Hz and 1Hz, 1H ArH), 7.19 (t, 1H, ArH), 6.94 (d, J=8 Hz ,2H, ArH), 5.52 (m, 2H, CH2), 5.51 (m, 2H, NH2), 4.62 (q, 2H, CH2), 3.72 (s, 3H, OCH3), 1.42 (t, 3H, CH3). MS (m/z): Calculated mass for C25H24N4O4 and measured mass for Impurity-A $[M+H]^+$:445.21.

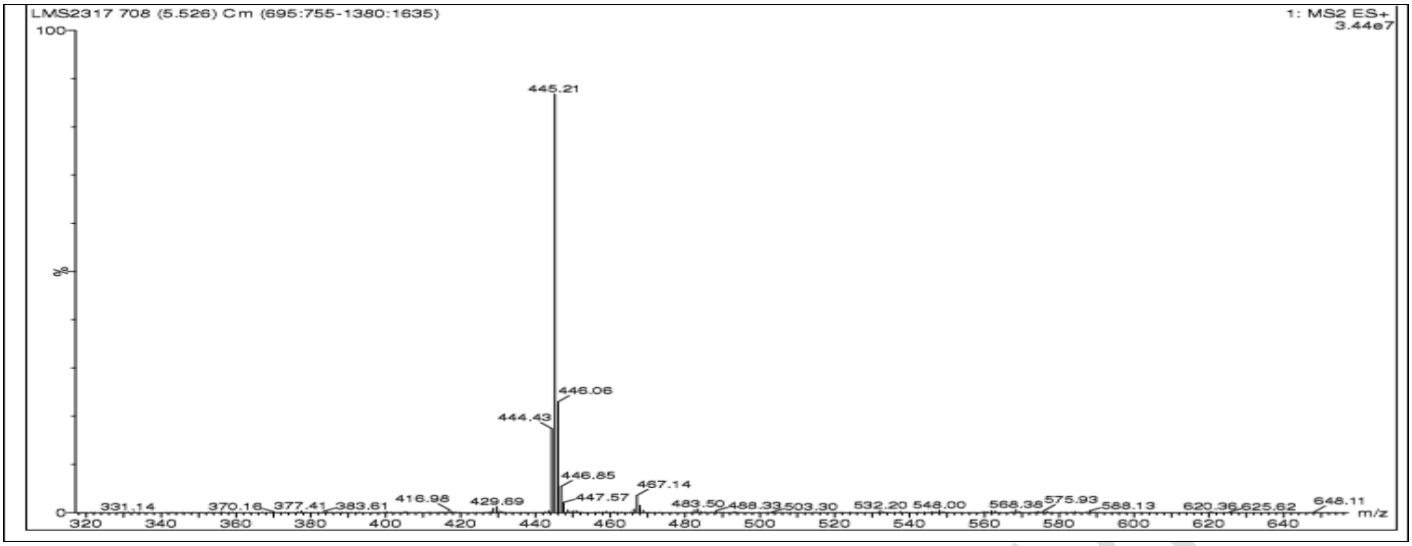

Figure 3 Mass spectra of Impurity-A: [M+H]=445.21

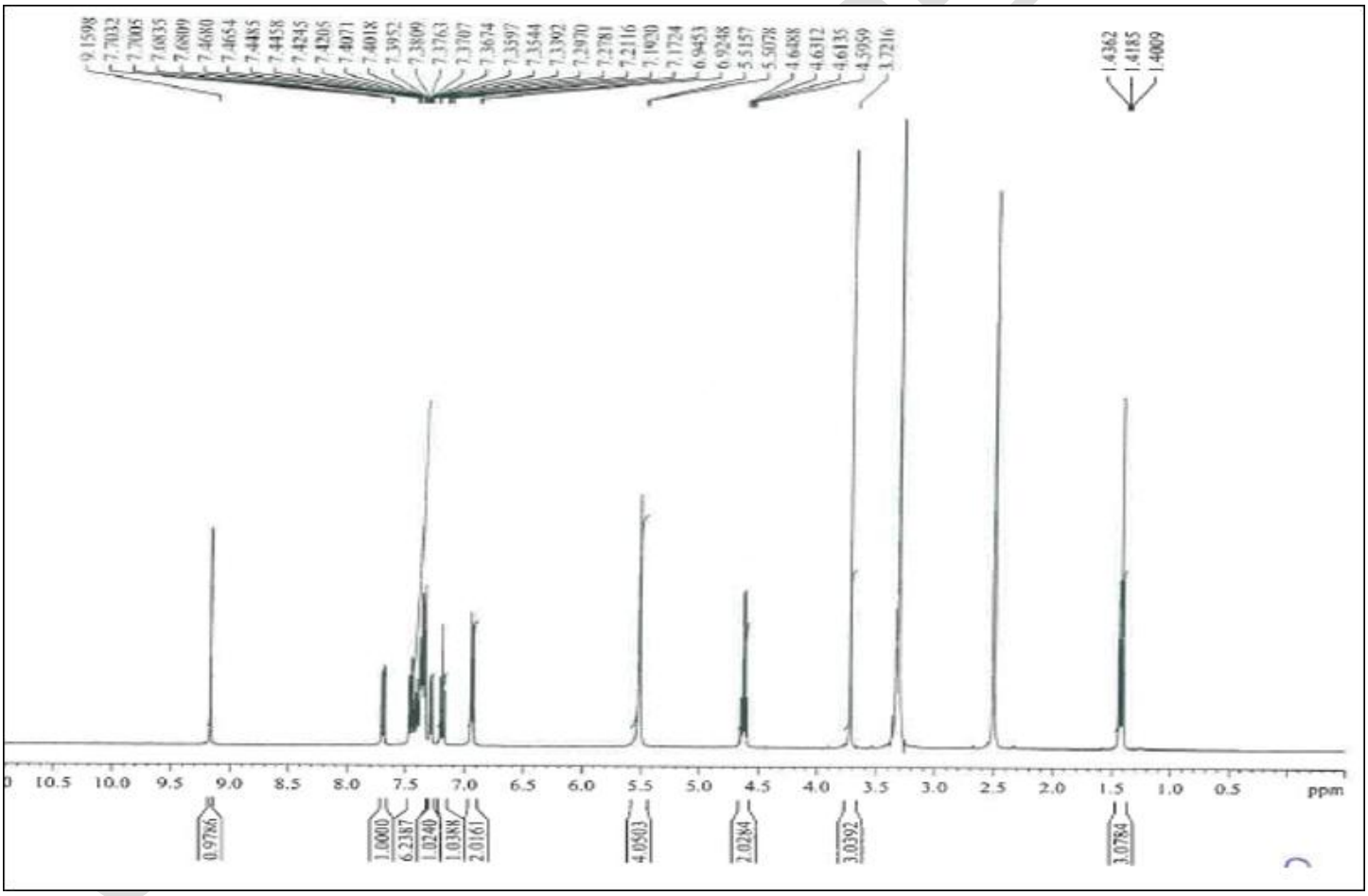

Figure 4: ^{1}H NMR Spectrum of Impurity-A

Synthesis of 2-ethoxy-1-((2'-(N'-hydroxycarbamimidoyl)-[1,1'-biphenyl]-4-yl)methyl)-1H-benzo[d]imidazole-7-carboxylic acid (Impurity-B) : To the stirred solution of Impurity-A (10g ,0.22mmol) and Sodium hydroxide (2.7g ,0.067 mmol) in Methanol (50 mL) and water (50 mL) mixture , The reaction mixture was stirred for 5h at 60°C. The reaction progress was monitored by TLC then cooled to 30 °C. The obtained reaction mass was poured into water (100 mL) and Hydrochloric acid (15mL) then stirred for 1 h , the obtained solids were filtered, and dried under reduced pressure to to afford compound (B). White colour solid, yield:85%, 1H NMR (400 MHz, DMSO-d6) d 9.17 (s, 1H, COOH), 7.65 (d, 1H, ArH), 7.53(d, 1H, ArH), 7.38 (m, 6H, ArH), 7.17 (t, 1H ArH), 7.01 (d, 2H, ArH), 5.66 (s, 2H, CH2), 5.55 (s, 2H, NH2) 4.61 (q, 2H, CH2), 1.42 (t, 3H, CH3). MS (m/z): Calculated mass for C24H22N4O4 and measured mass for Impurtiy-B $[M+H]^+$:431.07.

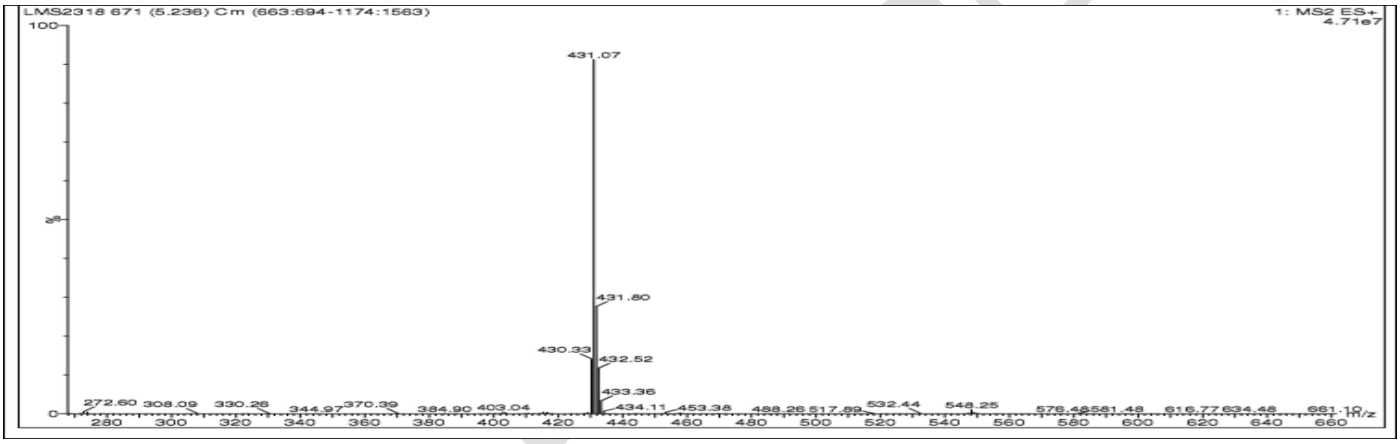

Figure 5 Mass spectra of Impurity-B: [M+H]=431.07

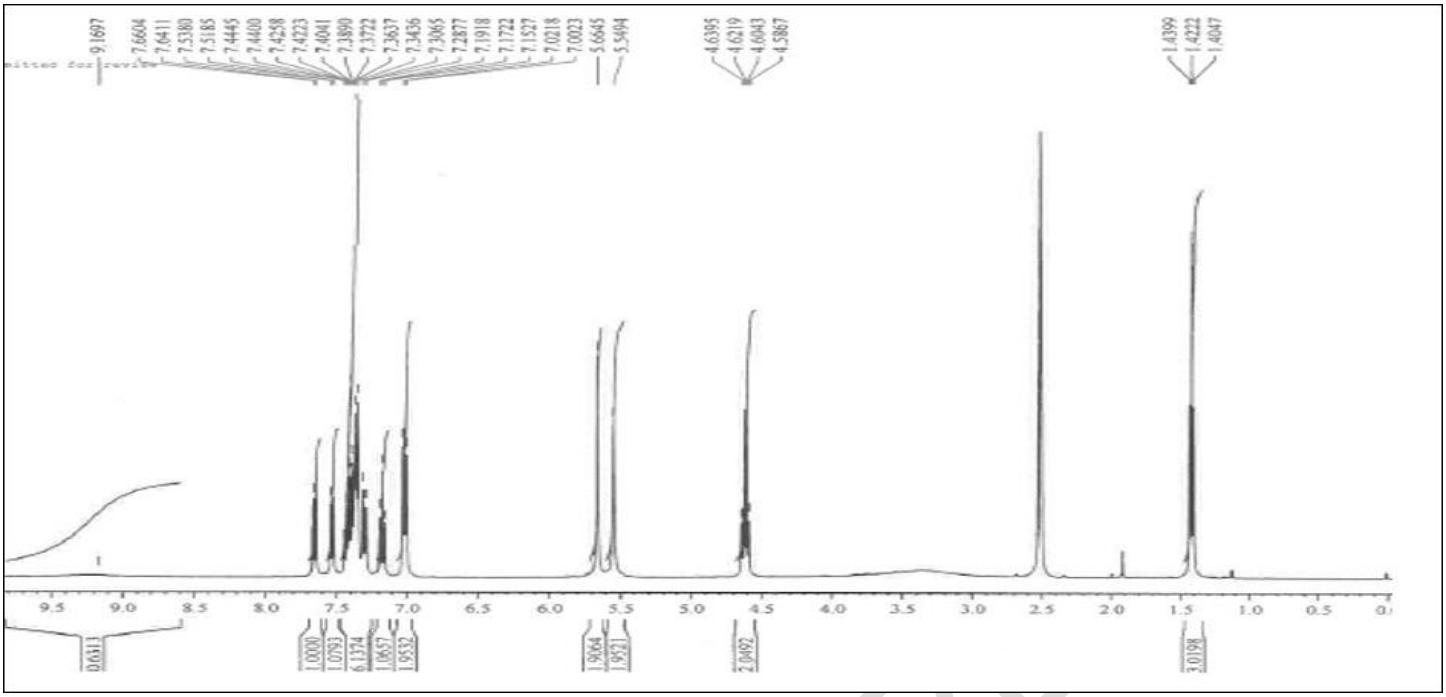

Figure 6 1H NMR Spectrum of Impurity-B

2.3 Methodology

LC-MS grade of Ammonium formate and formic acid from sigma-Aldrich.LC-MS grade Acetonitrile from Fisher chemicals. Purified water collected from Mill-Q plus water purification system. The method development and method validation was performed in Water's Acquity UPLC H-Class connected to Xevo TQ MS/MS detector. The data were collected and processed using Mass lynx software.

Mobile phase preparation:

Preparation of Buffer solution: Weighed about 0.63g of Ammonium formate salt and dissolved in 1000mL of water and adjusted pH 3.00±0.05 with formic acid.

Preparation of Mobile phase-A: Buffer:Acetonitrile(90:10)%v/v

Preparation of Mobile phase-B: Buffer:Acetonitrile(20:80)%v/v

Diluent for samples and standard preparation:Acetonitrile:Water(50:50)%v/v

Preparation of standard solution:Weighed about each 20mg of Impurity-A and Imprity-B and transferred in 100mLvolumetric flask and dissolved with diluent(Stock-1). Pippet out 1.0mL from Stock-1 into

100mLvolumetric flask and make upto the mark with diluent(Stock-2).Further transferred 1.0mL of stock-2 solution into 100mL volumetric flask and makeup to the mark with diluent(Standard solution).

Preparation of sample solution: Weighed about 50mg of Azilsartan drug substance sample transferred into 100mL volumetric flask , dissolved and make upto the mark with diluent.

LC-MS/MS Operating Conditions:

Expermentation performed using Water's Acquity UPLC H-Class connected to Xevo-TQ MS/MS detector with ESI Source(Electron spray ionization).Inertsil ODS-3V (150x4.6mm),5µm column used to separate the Impurity-A,Impurity-B and Azilsartan. Chromatographic method developed using isocratic mode of elution with Mobile phase-A and Mobile phase-B(60:40)%v/v with a flow rate of 0.8mL/min and a runtime of 10minutes for standards solution and 25mins runtime for samples solution. Column oven temperature maintained at 30°C and auto sampler temperature maintained at 10°C with an with a injection volume of 5µL.

A triple quardrupole MS equipped with a positive electron spray ionization (ESI) source was used in the MRM mode. The equipment was set with a Capillary voltage3.2kV, Cone voltage20V, Source temperature 150°C, Desolvation temperature 600°C, Desolvation Gas flow 850L/hr.

Table 1.Impurity-A and Impurity-B MRM transitions

S.No	Analyte	Parent(m/z)	Daughter (m/z)	Dwell(s)	Collision Energy(eV)
1	Impurity-A	445.21	207.12	0.078	30
			225.14	0.078	30
2	Impurity-B	431.07	207.12	0.078	20
			225.10	0.078	20

2.4 Method validation:

Method validation study was successfully completed for the developed method in terms of specificity, precision, limit of detection (LOD),Limit of quantification(LOQ),Linearity, Accuracy and Solution stability.The linearity study was evaluated by preparing and analyzing six different levels of concentration in the range of 4.7ppm to 78.4 ppm for Impurity-A and 4.5ppm to 75.7ppm for Impurity-B. Slope, Y-intercept, Correlation

coefficient and residual sum of squares reported from linearity study.Limit of detection(LOD)and Limit of quantification were established for Impurity-A(4.7ppm) and Impurity-B(4.5ppm) was established by using signal to noise ratios of 3:1(LOD) and 10:1(LOQ) respectively. Performed Precision at LOQ and Accuracy at LOQ to prove LOQ concentration was Accurate and precisice.Method precision was performed by preparing six preparations of the spiked sample solution of Azilsartan drug substance containing concentration 37.5ppm each Impurity-A and Impurity-B and evaluated the %relative standard deviation(%RSD) of content of Impurity-A and Impurity-B.Accuracy of the method was proved by adding known amount of Impurity-A and Impurity-B to the solution of Azilsartan drug substance at 50%,100% and 150% level and calculated % recovery.

3. Results and discussion

Method development and optimization:

The objective of LC-MS/MS in this study to develop a sensitive, selective and Accurate method for quantification of Impurity-A and Impurity-B in Azilsartan drug substance. Different acidic mobile phases such as formic acid, trifluoroacetic acid, diflouoroacetic acid mix with organic modifiers such as Acetonitrile and methanol isocratic mode elution have been tested. Different stationary phases like C18,C8 and Phenyl HPLC columns has been tested and found Inertsil ODS-3V(150x4.6mm),5µm has been(Make:GL Sciences,Japan) separation of Impurity- A,Impurity-B and Azilsartan drug substance.Gaussian curve peak shapes observed in Ammonium formate mobile phase pH=3.00, pre-mix with Acetonitrile in the isocratic mode elution with flow rate 0.8mL/min.Finalised mobile phase conditions pre mix of 10Mm Ammonium formate buffer pH=3.00:Acetonitrile(90:10)%v/v as Mobilephase-A and pre mix of 10Mm Ammonium formate buffer pH=3.00:Acetonitrile(20:80)%v/v as Mobilephase-B. Isocratic elution of Mobilephase-A:Moblie phase-B(60:40)%v/v found good sensitivity and separation of Analytes.

Optimization of MS/MS Conditions:

MS/MS Conditions optimization started with electron spray ionization (ESI) source in positive mode.MRM(multiple reaction monitoring) mode showed higher sensitivity than SIR(Single ion recording)mode. MRM(multiple reaction monitoring)mode had greater advantage to improve sensitivity due to both parent ion and daughter ions are monitored at a time when compared to SIR(single ion recording),here only parent ion only studied.Injected standard solution of Impurity-A,observed its [M+H]+ at m/z 445.35 and further MS/MS fragmentation with collision energy 30eV found stable daughter ions with higher sensitivity at m/z 207.15 and 225.18 respectively .Similarly injected standard solution of Impurity-B observed its [M+H]+ at

m/z of 431.33 and further MS/MS fragmentation with collision energy 20eV found stable daughter ions with higher sensitivity at m/z values 207.24 and 225.16 respectively.

Validation results of the method:

The developed method for the quantification of trace level determination of Impurity-A and Impurity-B in Azilsartan drug substance was validated as per ICH guidelines. The method was evaluated for its specificity, Sensitivity, LOD (limit of detection), LOQ(Limit of quantification),Linearity, Accuracy, Precision and solution stability.

Specificity:

The specificity of the method was verified by injecting the individual impurity standards Impurity-A and Impurity-B each at about 37.5ppm level with respect to 0.5mg/mL analyte concentration, azilsartan drug substance at 0.5mg/mL ,Spiked sample solution of Azilsartan drug substance containing Impurity-A and Impurity-B.(Table:2) .

Table 2. TIC: Total ion Chromatogram, PDA: Photodiode array detector

S.No	Component name	Retention time(min)	
		Data from Individual standards	Data from Spiked sample standards
1	Impurity-A	6.74(From TIC)	6.75(From TIC)
2	Impurity-B	3.22(From TIC)	3.22(From TIC)
3	Azilsartan	21.18(From PDA detector)	21.19(From PDA detector)

Sensitivity:

The Limit of detection (LOD) (Figure.4) and Limit of quantification(LOQ) was determined from Signal to noise ratio(S/N) method. Prepared and injected a series of diluted solutions from individual standard solutions.Based on S/N ratios of diluted solutions reported LOD and LOQ concentrations of Impurity-A and Impurity-B reported. LOD and LOQ concentrations of Impurity-A and Impurity-B reported in Table-3 and Table-4 respectively.

Table 3.Limit of detection (LOD) results

S.No	Component name	S/N Ratio	LOD Concentration
1	Impurity-A	3.2	1.4ppm
2	Impurity-B	3.4	1.4ppm

Table 4.Limit of quantification (LOQ) results

S.No	Component name	S/N Ratio	LOQ Concentration
1	Impurity-A	10.4	4.7ppm
2	Impurity-B	10.2	4.5ppm

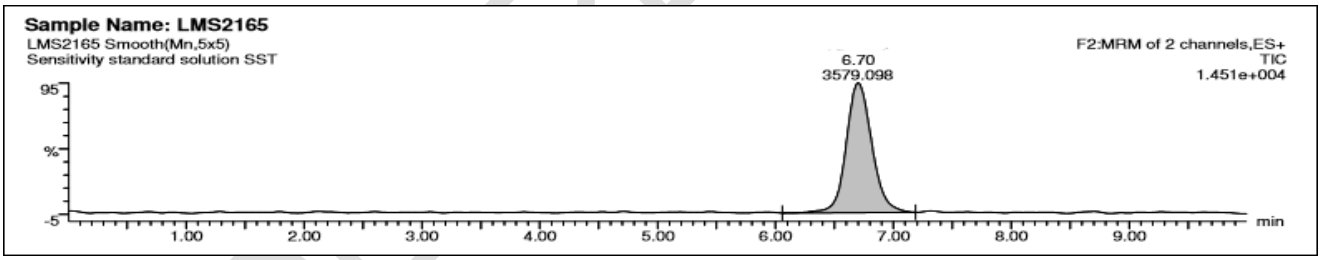

Figure 7: LOQ(Limit of quantification)MRM Chromatogram of Impurity-A

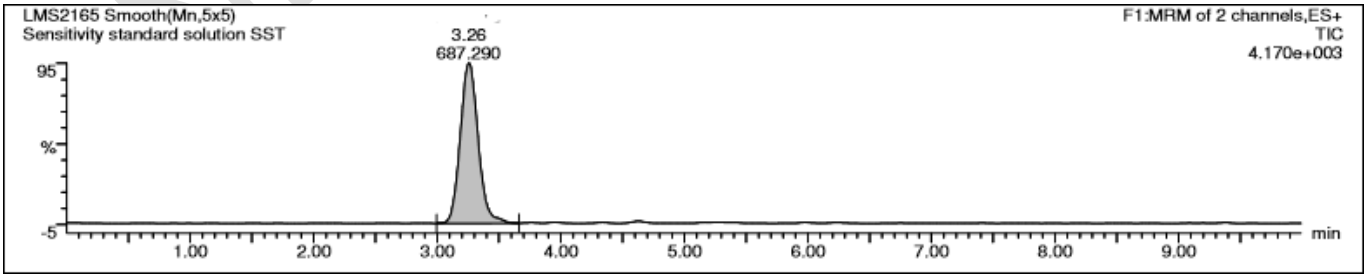

Figure 8 : LOQ (Limit of quantification)MRM Chromatogram of Impurity-B

Precision at LOQ:

Prepared and injected the LOQ standard solution (each Impurity-A and Impurity-B at concentration of 4.7ppm and 4.5ppm respectively. Recorded the MRM peak areas of each Impurity-A and Impurity-B and calculated the %RSD from six replicate injections Results are tabulated in Table-5.

Table 5.LOQ precision results

S.No	Name of the solution	Impurity-A area	Impurity-B area
1	LOQ Standard solution injection-1	3712.226	732.009
2	LOQ Standard solution injection-2	3909.835	721.173
3	LOQ Standard solution injection-3	3821.25	731.111
4	LOQ Standard solution injection-4	3636.271	722.679
5	LOQ Standard solution injection-5	3666.535	768.314
6	LOQ Standard solution injection-6	3649.165	663.937
7	Mean	3732.5	723.2
8	Standard deviation	109.8102	33.7400
9	%RSD	2.9	4.7

Linearity:

Linearity studies were performed for Impurity-A and Impurity-B at different concentrations from QL to 200%(QL, 25, 50,100,150 and 200%) of the specification level with respect to analyte concentration. Plotted a linear graph by taking the MRM peak areas on Y-axis and corresponding concentration on X-axis. Reported the values of correlation co-efficient, slope, y-intercept and residual sum of squares from linearity study. For linearity results of Impurity-A refer Table-6 and for Impurity-B refer Table-7 respectively

Table 6.Impurity-A Linearity results

Level	Concentration(ppm)	MRM Peak Area
LOQ	4.7066688	3717.891
25%	9.80556	7535.895
50%	19.61112	15223.849
100%	39.22224	30506.014
150%	58.83336	45692.574
200%	78.44448	60327.133
Correlation co-efficient		1.0000
Slope		770.6755555
Y-Intercept		113.5046674
Residual sum of square		160669.0451

Table 7.Impurity-B Linearity results

Level	Concentration(ppm)	MRM Peak Area
LOQ	4.54477272	675.027
25%	9.4682765	1510.123
50%	18.936553	3205.101
100%	37.873106	6368.086
150%	56.809659	9875.648
Correlation co-efficient		**0.9997**
Slope		171.0879622
Y-Intercept		-70.94994526
Residual sum of square		80280.73968

Precision:

Method precision:

Prepared the spiked sample solution in six times containing each Impurity-A and Impurity-B at specification level at each preparation and injected each once. Calculated the content of each Impurity-A and Impurity-B and

reported % RSD for Impurity-A, Impurity-B content from six spiked sample preparations. Results are tabulated n Table-8.

Table 8. Method precision results

Level	Impurity-A(ppm)	Impurity-B(ppm)
Method Precision Preparation-1	39.227	42.528
Method Precision Preparation-2	38.73	43.066
Method Precision Preparation-3	40.229	43.884
Method Precision Preparation-4	38.987	43.044
Method Precision Preparation-5	39.789	42.806
Method Precision Preparation-6	39.339	42.608
Mean	39.4	43.0
Standard deviation	0.5459	0.4901
%RSD	1.39	1.14

Accuracy:

The accuracy of the test method was demonstrated by prepared the un spiked sample solutions and spiked sample solution with known concentration of Impurity-A, Impurity-B at LOQ level,50%,100% and 150% of the specification limit. Calculated the %recovery of Impurity-A and Impurity-B at each level. The mean %recovery of Impurity-A at LOQ,50%,100% and 150% level were 99%,99%100% and 102% respectively. The mean %recovery of Impurity-B at LOQ,50%,100% and 150% was93%, 101%,103% and 103% respectively. Results were tabulated in table-9.

Table 9. %Recovery of Impurity-A and Impurity-B

	LOQ	50%(18.8ppm)	100%(37.5ppm)	150%(56.3ppm)

Mean % recovery for Impurity-A (n=3)	99	99	100	102
Mean % recovery for Impurity-B(n=3)	93	101	103	103

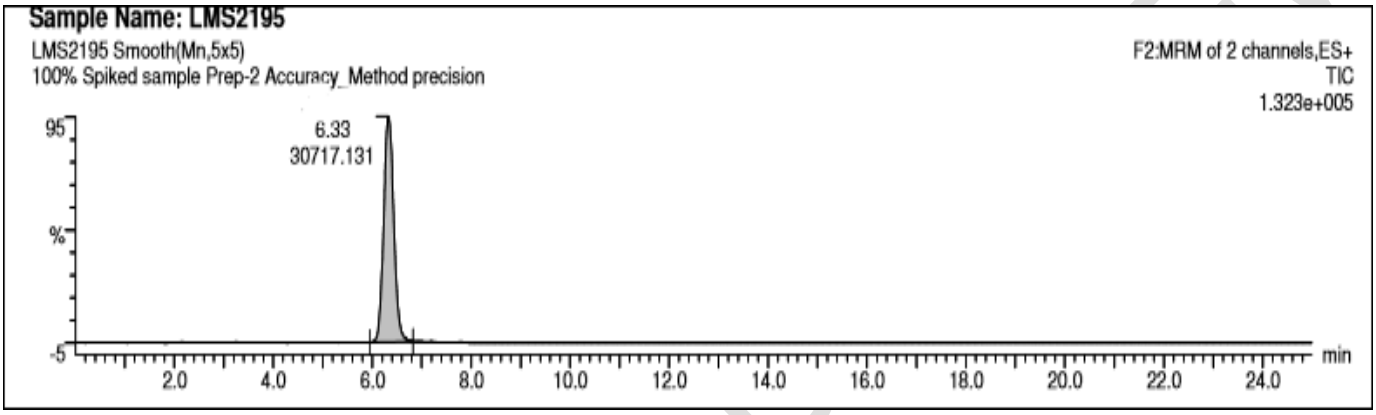

Figure 9: 100% Spiked sample MRM Chromatogram of Impurity-A

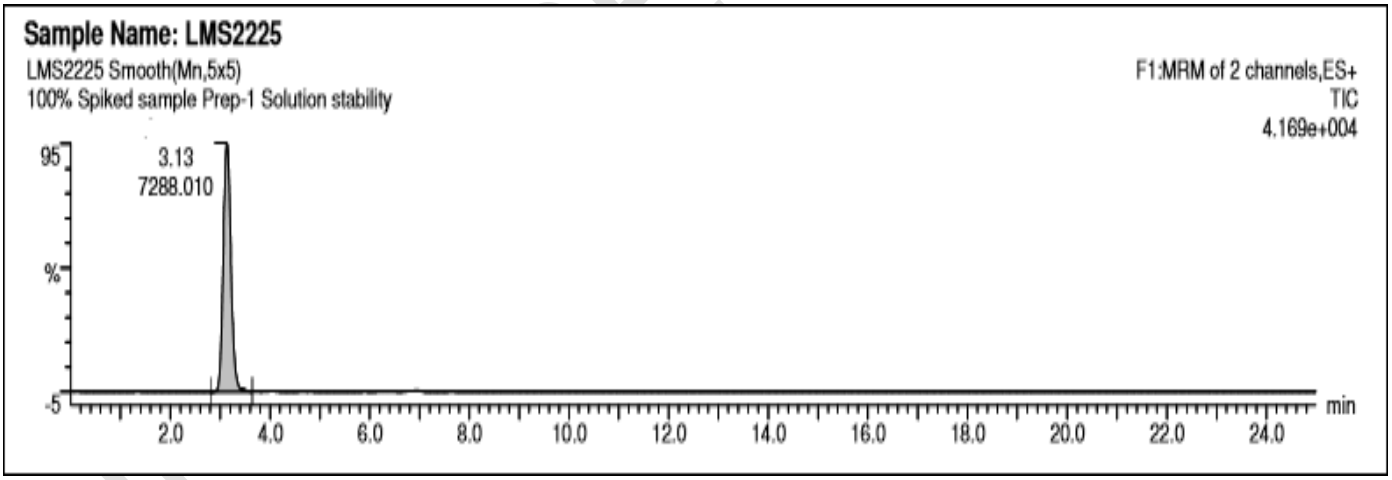

Figure 10: 100% Spiked sample MRM Chromatogram of Impurity-B

Robustness:

Robustness of the method was proved by making small deliberate changes in experimental conditions like flow rate and column oven temperature. The actual flow rate of the mobile phase was 0.8mL/min and changed flow rate from 0.8mL±0.1mL i.e,0.7mL and 0.9mL.The actual column oven temperature was 30°C and changed column oven temperature from 30°C±5°C i.e, 25°C and 35°C.No significant change in chromatographic performance was observed due to this small deliberate changes in experimental conditions it indicates robustness of the method.

4. Conclusion

A sensitive and selective LC-MS/MS MRM quantification method for genotoxic impurities i.e; Methyl(Z)-2-ethoxy-1-((2'-(N'-hydroxycarbamimidoyl)-[1,1'-biphenyl]-4-yl)methyl)-1H-benzo[d]imidazole-7-carboxylate (Impurity-A) and 2-ethoxy-1-((2'-(N'-hydroxycarbamimidoyl)-[1,1'-biphenyl]-4-yl)methyl)-1H-benzo[d]imidazole-7-carboxylic acid (Impurity-B) in Azilsartan drug substance developed and validated as per ICH method validation guidelines. The method can be suitable for trace level quantification of these two potential genotoxic impurities in Azilsartan drug substance.

COMPETING INTERESTS DISCLAIMER:

AUTHORS HAVE DECLARED THAT NO COMPETING INTERESTS EXIST. THE PRODUCTS USED FOR THIS RESEARCH ARE COMMONLY AND PREDOMINANTLY USE PRODUCTS IN OUR AREA OF RESEARCH AND COUNTRY. THERE IS ABSOLUTELY NO CONFLICT OF INTEREST BETWEEN THE AUTHORS AND PRODUCERS OF THE PRODUCTS BECAUSE WE DO NOT INTEND TO USE THESE PRODUCTS AS AN AVENUE FOR ANY LITIGATION BUT FOR THE ADVANCEMENT OF KNOWLEDGE. ALSO, THE RESEARCH WAS NOT FUNDED BY THE PRODUCING COMPANY RATHER IT WAS FUNDED BY PERSONAL EFFORTS OF THE AUTHORS.

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