Application note | 000748

Mass spectrometry

Sensitive and robust determination of genotoxic AZBT impurity in six sartan drug products

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Keywords

Azido, sartans, candesartan, irbesartan, losartan, olmesartan, telmisartan, valsartan, AZBT, drug products, TTC, Vanquish Flex UHPLC, TSQ Quantis MS, LC-MS/MS, liquid chromatography, triple stage quadrupole mass spectrometry

Key benefits

- A single method capable of detecting an azido impurity (AZBT) in six different sartan products—candesartan, irbesartan, losartan, olmesartan, telmisartan, and valsartan—saves time by testing multiple products in one sequence without separate method development.
- A robust method for quantification of AZBT was achieved by using a divert valve to prevent the mass spectrometer from the build-up of high concentrations of drug product samples. This helped maintain good sensitivity and reproducibility.
- This study is in line with regulatory information and requirements mentioned in EDQM[1], USFDA (Q2B)[2], EMEA[3], and ICH M7[4] guidelines.

Goal

To develop a sensitive and robust method for the determination of AZBT in six sartan drug products and validate according to European and ICH guidelines

Introduction

Azido impurity, (5-(4'-(azidomethyl)-[1,1'-biphenyl]-2-yl)-1H-tetrazole, also known as azidomethyl-biphenyl-tetrazole (AZBT), is a compound that can form during the manufacturing of the active ingredient in some sartan medications. It is known to damage DNA, and as a result, long-term exposure may increase an individual's risk of developing cancer.

Sartans, also known as angiotensin II receptor blockers (ARBs), belong to a pharmacological class that inhibits AT_1 (angiotensin II receptor type 1). Sartans are mainly used in the treatment of hypertension (high blood pressure), diabetic nephropathy (kidney damage due to diabetes), and congestive heart failure.

In April 2021, the European Directorate for the Quality of Medicines & HealthCare[1] (EDQM) reported that it had received information about the possible presence of potentially mutagenic azido impurities in certain sartan active substances. Investigations requested by the EDQM indicated that only a few sources were impacted. Several measures were taken to ensure that any active substance containing these impurities above the acceptable level would not be released onto the market. In addition, any impacted holders of a Certificate of Suitability of Monographs of the European Pharmacopoeia (CEP) were requested to take corrective action to ensure that such impurities do not exceed their acceptable limits in the future. The EDQM review of these actions in the impacted sources has been completed for some manufacturers and is well advanced for others.

In the absence of additional information from in vivo studies, it is necessary to ensure that this azido impurity is controlled at or below the Threshold of Toxicological Concern (TTC as per ICH M7),[4] which states acceptable intake of 1.5 µg per day for a mutagenic impurity. Therefore, it becomes important to determine the level of azido impurity present in sartan drug products.

The choice of analytical column chemistry becomes important to separate out the impurity peak from the peak of the drug substance. Thereafter, a suitable divert valve program is created with the purpose of sending the impurity into the mass spectrometer for detection while diverting the drug substance to waste. If these critical points are not considered during development of the method, it may lead to decreased analytical performance due to mass spectrometer contamination, and the robustness of the system may be compromised. Certain LC-MS/MS methods[5-8] are available for determination of AZBT in some of the sartans. In this study, we selected candesartan, irbesartan, losartan, olmesartan, telmisartan, and valsartan drug products and developed a single LC-MS/MS method for the determination of AZBT impurity in each of them. The separation of sartans and AZBT was achieved by reversed-phase chromatography using Thermo Scientific™ Accucore™ biphenyl chemistry; detection was achieved by a triple quadrupole mass spectrometer. High detection selectivity and sensitivity was achieved by taking advantage of selected reaction monitoring (SRM) of protonated AZBT ions. The suitable column chemistry and divert program in this method yield excellent sensitivity and robustness of the system.

Experimental

Sample preparation

Diluent solution and blank preparation

80 mL of methanol and 20 mL of water were mixed and sonicated as the diluent solution and blank.

Standard stock solution (100 µg/mL)

An appropriate amount of AZBT reference standard was weighed and dissolved in methanol to achieve a concentration of 100 µg/mL.

Preparation of standard (5 ng/mL)

10 µL of standard stock solution was transferred into a 20 mL volumetric flask and diluted to volume using diluent solution to prepare the intermediate dilution. Afterwards, 1 mL aliquot volume of the intermediate dilution was transferred into a 10 mL volumetric flask and diluted to volume with diluent solution. This standard solution was used for the system suitability experiment as well as the recovery evaluation at the mid concentration level.

Preparation of linearity standards, limits of detection (LOD) and limits of quantitation (LOQ)

An intermediate dilution of 1,000 ng/mL was prepared by transferring 0.1 mL of standard stock solution (100 µg/mL) into a 10 mL volumetric flask and the volume made up to the mark with diluent solution. From this intermediate dilution, a suitable volume was serially diluted to achieve six linearity standards of concentrations 0.5 (LOQ), 1.25, 5, 10, 20, and 40 ng/mL. LOQ was further diluted appropriately to prepare LOD of 0.025 ng/mL.

Sample preparation procedure

Approximately 5 to 10 tablets of the sartan drug product were ground into powder and mixed properly. An appropriate weight corresponding to 5 mg of active content was weighed and transferred into a 15 mL centrifuge tube. The sample was taken up with 5 mL of diluent solution, vortexed briefly, and then sonicated for 30 minutes in an ultrasonic bath. The samples were vortexed for 1 minute and centrifuged at 4,500 rpm and 5 °C for 15 minutes. The clear supernatant was transferred into HPLC vials for analysis.

Instrumentation

- Thermo Scientific™ Vanquish™ Flex Binary UHPLC system equipped with a temperature-controlled autosampler and column compartment
- Thermo Scientific™ Vanquish™ Diode Array Detector (P/N VF-D11-A-01) for UV detection
- Thermo Scientific™ TSQ Quantis™ triple quadrupole mass spectrometer (P/N TSQ02-10001) with heated electrospray ionization (H-ESI) source

Consumables/reagents

- Reference standards procured from Cleanchem Laboratories
  - 5-(4'-(azidomethyl)-[1,1'-biphenyl]-2-yl)-1H-tetrazole (AZBT)
- Fisher Scientific™ Formic acid, Optima™ LC/MS grade (Fisher Scientific P/N A117-50 or equivalent)
- Fisher Scientific™ Methanol, Optima™ LC/MS grade (Fisher Scientific P/N A456-4 or equivalent)
- Fisher Scientific™ Water, Optima™ LC/MS grade (Fisher Scientific P/N AAB-W6-4 or equivalent)
- Thermo Scientific™ Nunc™ 15 mL extraction/ conical sterile polypropylene centrifuge tubes (P/N 339652)
- Thermo Scientific™ Chromacol™ GOLD HPLC vials (2-SVG)
- Thermo Scientific™ Accucore™ Biphenyl 100 × 2.1 mm, 2.6 µm (P/N 17826-102130)
- Thermo Scientific™ Vanquish™ Diode Array Detector, Standard Flow Cell, path length 10 mm (13 µL, SST) (P/N 6083.0510)

Chromatographic conditions

Table 1. LC conditions

Parameter	Value
HPLC column	Accucore Biphenyl 100 mm × 2.1 mm, 2.6 µm
Column temperature	35 °C
Flow rate	0.400 mL/min
Solvent A	0.1% formic acid in water
Solvent B	0.1% formic acid in methanol
Injection volume	5 µL
Autosampler temp.	10 °C
Needle wash	80:20, methanol:water
Pump mixing volume	200 µL
UV wavelength (sartans)	224 nm

Table 2. Gradient program

Time (min)	Solvent A %	Solvent B %
0	45	55
3	45	55
4	40	60
6	10	90
14	10	90
14.2	45	55
17	45	55

Mass spectrometer parameter settings

Table 3. Ion source settings

Parameter	Value
Ion source type	H-ESI
Polarity	Positive
Positive voltage	3,000 V
Sheath gas flow rate	50 arbitrary units
Aux gas flow rate	10 arbitrary units
Sweep gas flow rate	0 arbitrary units
Ion transfer tube temperature	275 °C
Vaporizer temperature	350 °C

Table 4. Divert valve settings

Time (min)	Position	Remarks
0	1-6	Diverted to UV
2	1-2	MS
5.2	1-6	Diverted to UV

Table 5. SRM properties

Parameter	Value
RF Lens (V)	105
Q1 resolution (FWHM)	0.7
Q3 resolution (FWHM)	0.7
CID gas (mTorr)	1.5
Source fragmentation	10
Chromatographic peak width (s)	6
Use Chromatographic Filter	TRUE

Table 6. SRM table

Compound	Start time (min)	End time (min)	Precursor (*m/z*)	Product (*m/z*)	Collision energy (V)
AZBT	0	17	278.1	235.0	8.4
			278.1	207.0*	14.9
			278.1	192.0#	27.1

Qualifiers: *Confirming ion 1, #Confirming ion 2

Injection order

- One injection of diluent blank
- Six replicate injections of standard solution (5 ng/mL) for system suitability
- Three replicate injections of LOD (0.025 ng/mL)
- One injection each of linearity standards from low to high concentration level
- All as such (unspiked) samples in triplicate
- For spiked sample recovery evaluation: Six replicate injections of neat standards at low, mid, and high concentrations along with three replicates of spiked samples at three levels, i.e., low, mid, and high concentrations
- Suitable number of bracketing standards and diluent blank injections throughout the sequence.

Note: Samples of each sartan drug product (i.e., candesartan, irbesartan, losartan, olmesartan, telmisartan, and valsartan) were prepared separately.

System suitability requirements

- % RSD of peak area for AZBT impurity for the first six replicate injections of standard solution should not be more than 5%.
- The cumulative % RSD of the peak area should be no more than 10%. (Cumulative % RSD of the peak area is calculated by combining the initial six replicate injections of the standard solution and all subsequent bracketing standard.)

Calculation of AZBT content in drug products[5]

$$\text{Amount of AZBT in the sample } (\mu g/g) = \frac{C \times V}{M \times 1{,}000}$$

Where,

C: the concentration of AZBT in the sample solution calculated by the standard calibration curve (ng/mL)

V: the final make-up volume of the sample (mL)

M: the weight of the sample (g)

Calculation of ion ratio and acceptance limits[5]

The ion ratio is calculated on the relative product ion intensities by dividing the peak area response of the confirming ion (Qualifier) with that of the quantitation ion (Quantifier). Acceptance of the ion ratio is defined by the criteria in Table 7 as per the European Commission Decision (2002/657/EC),[9] where different values of the ion ratio have different acceptable windows or relative tolerance.

Table 7. Ion ratio acceptance criteria

Ion ratio	Ion ratio in percentage	Relative tolerance (2002/657/EC)
>0.5	>50%	±20%
0.20–0.50	20%–50%	±25%
0.10–0.20	10%–20%	±30%
<0.10	<10%	±50%

Calculation of recovery percentage[3]

Recovery is calculated by comparing peak area responses of spiked and unspiked samples against neat standards of AZBT. The following formula is used to calculate recovery:

$$\text{\% Recovery of AZBT} = \frac{\text{Peak area response in spiked sample - Peak area response in unspiked sample}}{\text{Average peak area response of 6 replicates of neat standard}} \times 100$$

% Recovery observed at low, mid, and high concentration levels should be within 70–130%.[3]

Data analysis

Data analysis was performed using Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software. Chromeleon CDS features allow all major activities such as acquisition, data processing, reporting of interactive tables, interactive charts, and ion ratio calculations. This study has been performed using Chromeleon CDS version 7.3.

Results and discussion

The following validation parameters were evaluated in this study:

- System suitability
- Reproducibility of system suitability standards, including the bracketing standards
- Linearity from 0.5 ng/mL to 40 ng/mL
- Relative ion ratio between quantifying and qualifying ions
- LOD and LOQ based on peak area responses
- Signal-to-noise ratio
- Recovery at low (0.5 ng/mL), mid (5 ng/mL), and high (40 ng/mL) concentration levels, determined by comparing peak area responses of AZBT observed in spiked samples against neat standards

The data quality was found to be excellent with additional confirmation of acceptable ion ratio for qualifier ions. Analysis was done in SRM mode using the TSQ Quantis triple quadrupole mass spectrometer with H-ESI ionization mode.

The robustness of the system and optimized method is reflected by the excellent reproducibility in system suitability exercise and cumulative reproducibility including the bracketing standards of the sequence that was run for approximately 117 injections lasting 36 hours.

System suitability

System suitability was performed by six replicate injections of standard solution at 5 ng/mL to evaluate the performance of the LC-MS/MS instrument. Table 8 shows the results of %RSD (reproducibility). Table 9 shows the results of cumulative % RSD (reproducibility) with bracketing standards.

Table 8. System suitability results

Inj #	Injection name	RT (min)	Area (counts · s)	Calculated conc. (ng/mL)
3	Standard-1	4.1	188079	5.019
4	Standard-2	4.1	183377	4.894
5	Standard-3	4.1	184983	4.936
6	Standard-4	4.1	186634	4.981
7	Standard-5	4.1	186418	4.975
8	Standard-6	4.1	184438	4.922
Average		**4.1**	**185655**	
SD		**0.00**	**1705.2**	
%RSD		**0.0**	**0.9**	

Table 9. System suitability results – reproducibility with bracketing standards

Inj #	Injection name	RT (min)	Area (counts · s)	Calculated conc. (ng/mL)
3	Standard-1	4.1	188079	5.019
4	Standard-2	4.1	183377	4.894
5	Standard-3	4.1	184983	4.936
6	Standard-4	4.1	186634	4.981
7	Standard-5	4.1	186418	4.975
8	Standard-6	4.1	184438	4.922
9	Bracket_Standard-1	4.1	191682	5.115
10	Bracket_Standard-2	4.1	187648	5.008
11	Bracket_Standard-3	4.1	158178	4.222
12	Bracket_Standard-4	4.1	200838	5.359
Average		**4.1**	**185228**	
SD		**0.01**	**10742.0**	
%RSD		**0.3**	**5.8**	

Linearity

Linearity was determined by injection of low to high calibration standards of the desired concentration range i.e., 0.5 ng/mL to 40 ng/mL. Figure 1 shows the linearity of the AZBT standard.

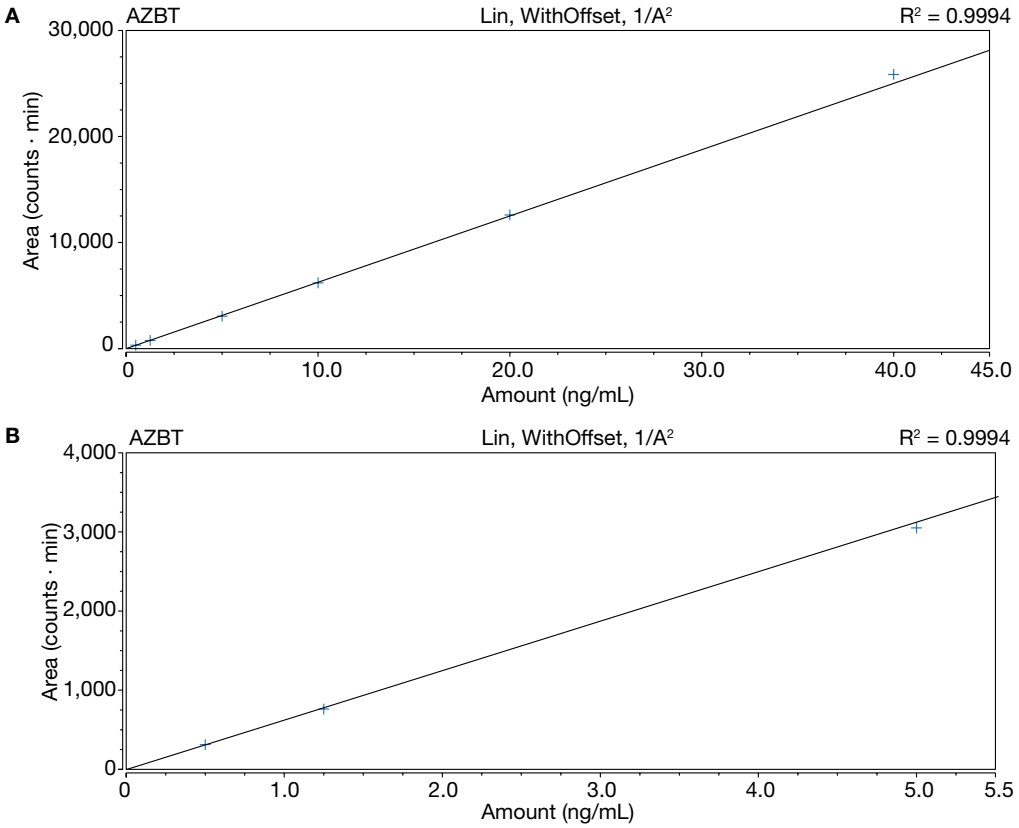

Figure 1. (A) Calibration plot and (B) zoomed view for bottom 3 points

Relative ion ratio was determined by comparing responses of confirming ions with that of quantitation ion. Peak area response of Confirming ion 1 (Conf 1) was found to be 44% and Confirming ion 2 (Conf 2) was found to be 8% of that of Quantitation ion (Quan) by averaging the ion ratio of all calibration curve points as shown in Table 10.

A representative chromatogram of diluent blank, LOD, and LOQ injections that contains the peak area responses of the Quan ion and Conf 1 and 2, as shown in Figure 2.

Similarly, chromatograms of the calibration standard at the lowest level (Calibration_Std_1) and at the highest level (Calibration_Std_6) containing peak area responses of the Quan and Conf 1 and Conf 2 ions are shown in Figure 3.

Ion ratio evaluation results are compiled in Table 11.

Chromatographic separation of sartans (peak identified in UV) and AZBT showing the benefit of using a divert valve is shown in Figure 4. Mobile phase coming from the LC was sent to the MS only for a defined range of 2 to 5.2 minutes and the rest of the time the flow was sent to the UV, thereby protecting the MS from contamination of heavy sample load and maintaining system robustness.

Table 10. Relative ion ratio

Injection name	Theoretical conc. (ng/mL)	Observed conc. (ng/mL)	Quan area (counts·s)	Conf 1 area (counts·s)	Conf 1 ion ratio %	Conf 2 area (counts · s)	Conf 2 ion ratio %
Calibration_STD_1	0.500	0.505	18751	7970	42.50	1593	8.50
Calibration_STD_2	1.250	1.224	45719	20122	44.01	4026	8.81
Calibration_STD_3	5.000	4.885	183043	80542	44.00	15073	8.23
Calibration_STD_4	10.000	9.914	371701	163458	43.98	29609	7.97
Calibration_STD_5	20.000	20.163	756168	332047	43.91	60852	8.05
Calibration_STD_6	40.000	41.354	1551127	665936	42.93	119958	7.73
				Average	**44**		**8**

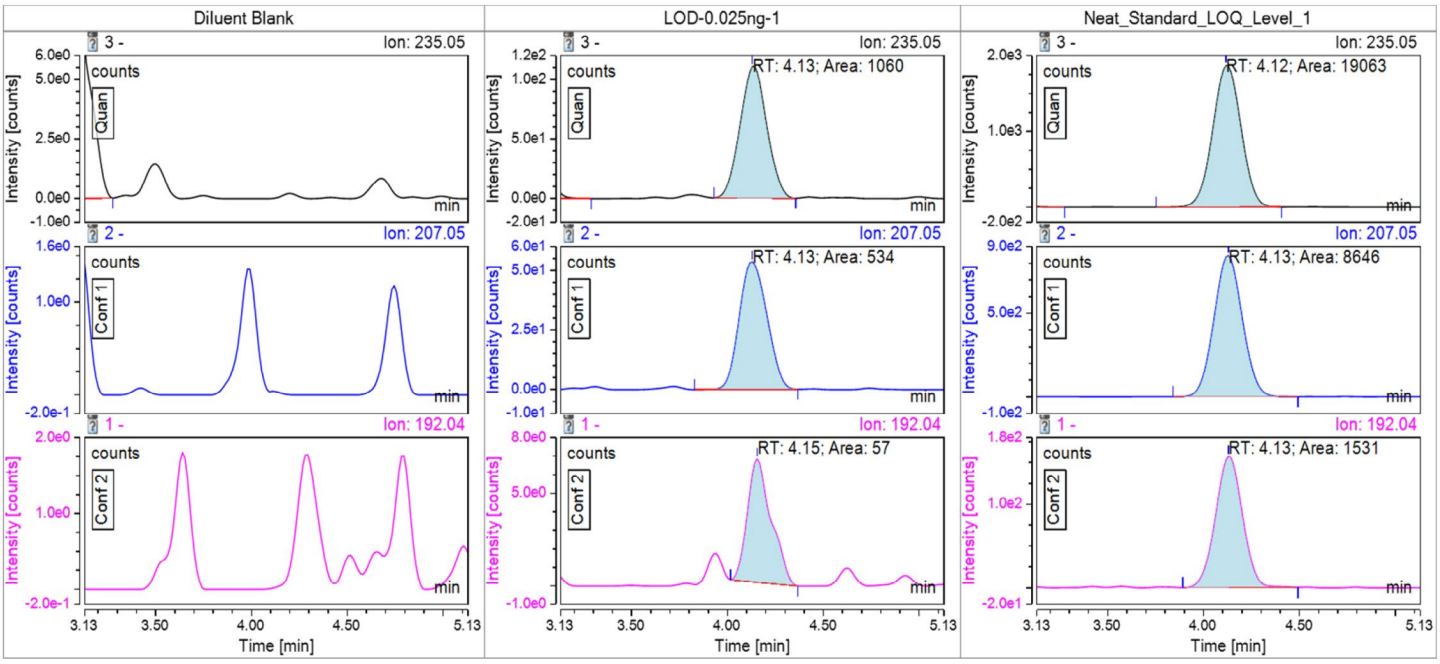

Figure 2. Relative ion intensities for ion ratio (Blank-Left, LOD-Middle, LOQ-Right)

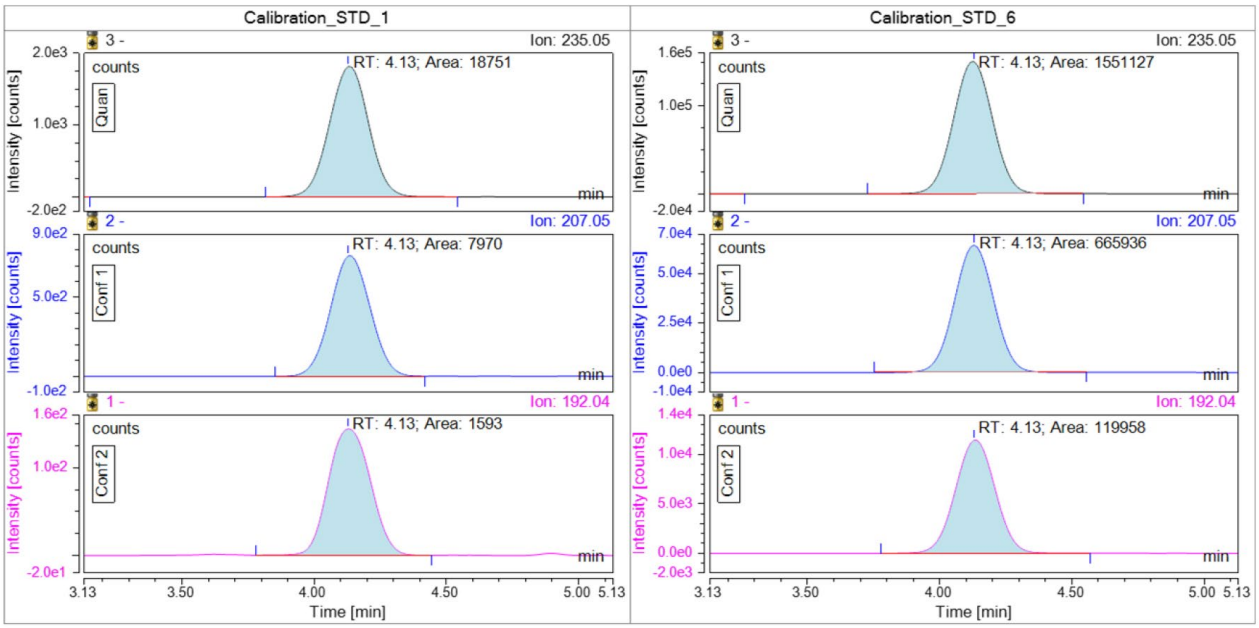

Figure 3. Chromatograms of calibration standards at lowest and highest concentration levels.

Table 11. Ion ratio results calculated for all relevant samples of the sequence

Confirming ion #	*m/z*	Expected ion ratio (Average ion ratio of all standards of calibration curve)	Ion ratio tolerance	Acceptable range	Observed range	Status
Conf 1	207.054	44%	±25%	33% to 55%	41% to 53%	Ok
Conf 2	192.042	8%	±50%	4% to 12%	5% to 10%	Ok

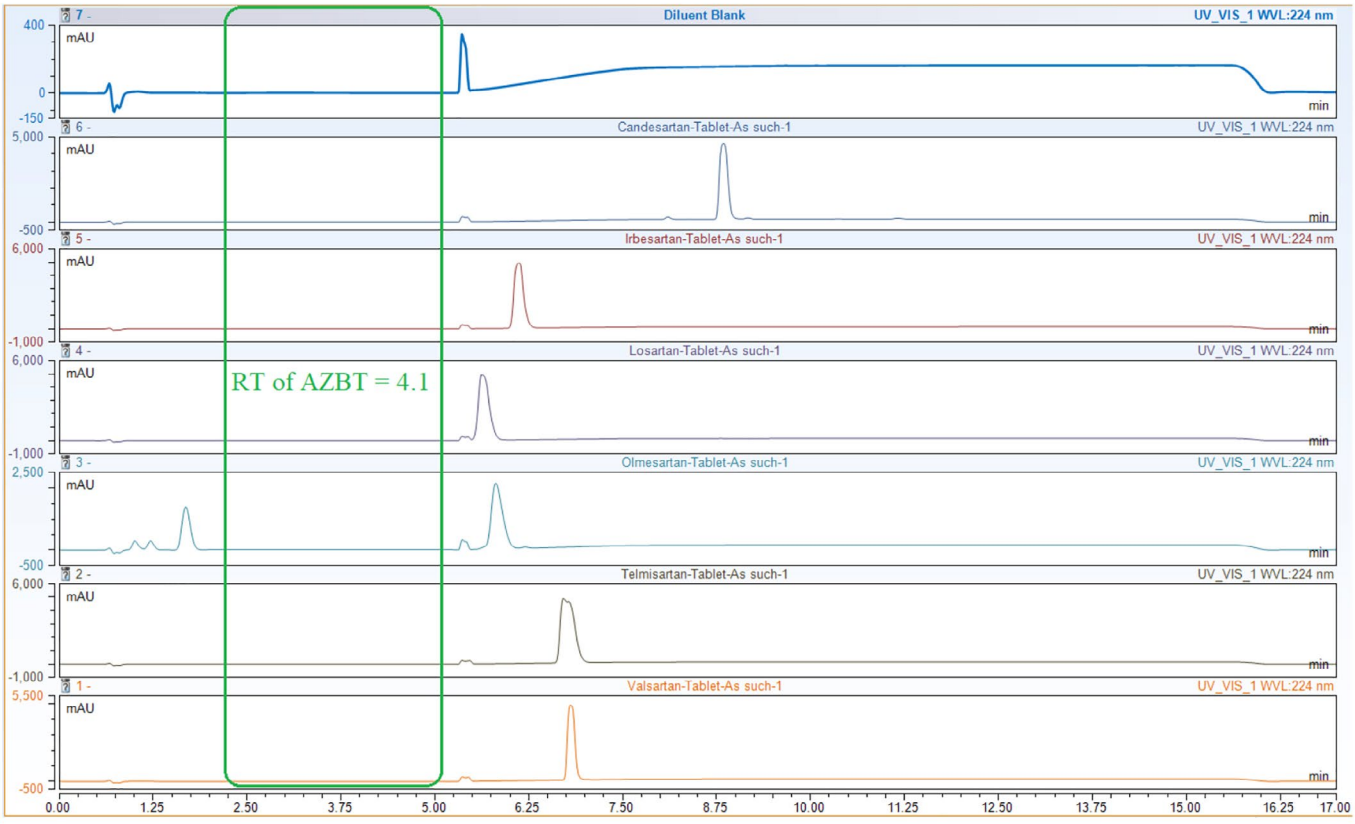

Figure 4. Chromatographic separation of AZBT and sartans

The data corresponding to LOD and LOQ has been summarized in Table 12. LOD was determined based on peak area response and S/N ratio to be >3. LOQ was kept 10 times lower than the standard limit of 5 ppm with respect to the drug substance and was determined based on peak area response to be sufficient to carry out the required validation experiments, e.g., recovery where peak area response should be sufficiently reproducible and S/N to be >10. The signal-to-noise ratio was determined through Chromeleon CDS by using the following formula:

$$S/N = 2\times \frac{\text{Peak height}}{\text{Noise}}$$

Depending upon the acceptable intake of 1.5 µg/day for genotoxic impurity as per the ICH M7 guideline[4] and 300 mg as the maximum daily dose of sartans (for the six sartans evaluated here), the standard limit is calculated to be 5 ppm. Based on this calculation as well as the sample preparation of 1 mg/mL equivalent of drug substance, the LOD concentration is 0.025 ppm and the LOQ concentration is 0.5 ppm. This method far exceeds these regulatory sensitivity requirements.

Table 12. LOQ reproducibility and S/N at LOD and LOQ levels

Inj #	Injection name	RT (min)	Area (counts · s)	S/N
10	LOD-0.025ng-1	4.1	1060	89
11	LOD-0.025ng-2	4.1	1130	70
12	LOD-0.025ng-3	4.1	1047	78
40	Neat_Standard_LOQ_Level_1	4.1	19063	2280
41	Neat_Standard_LOQ_Level_2	4.1	18339	1137
42	Neat_Standard_LOQ_Level_3	4.1	18766	2243
43	Neat_Standard_LOQ_Level_4	4.1	18667	2761
44	Neat_Standard_LOQ_Level_5	4.1	18928	1477
45	Neat_Standard_LOQ_Level_6	4.1	18813	1898
Average		**4.1**	**18763**	
SD		**0.00**	**248.5**	
%RSD		**0.0**	**1.3**	

Recovery results

The recovery experiment was performed at three levels, LOQ level (Low), standard limit level (Mid), and highest-level concentration (High) in triplicate (Table 13).

Table 13. Recovery at low, mid, and high levels

Recovery level	Observed recoveries
Low (0.5 ng/mL)	79–120%
Mid (5 ng/mL)	99–121%
High (40 ng/mL)	101–113%

Conclusion

The results of the experiments described here demonstrate:

- An LC-MS/MS method for the analysis of AZBT impurity in six sartan drug products (candesartan, irbesartan, losartan, olmesartan, telmisartan & valsartan)
- The method met expectations even lower than the currently required concentrations. The coefficient of correlation R^2 was found to be 0.9994.
- The LOQ for AZBT was established on par with current regulatory expectations. The %RSD at the desired standard limit concentration as well as the LOQ concentration (5 ppm and 0.5 ppm, respectively, with respect to the drug substance) was less than 0.92% and 1.32%, respectively.
- Cumulative %RSD (i.e., including system suitability standard and bracketing standards) was found to be 5.8% for 117 injections (36 hours) in one go, which indicates the robustness of the system and the optimized method.
- %Recovery was performed as per ICH topic Q2 (R1), determined at the 5 ppm standard level, and found to be within the permissible limit (70–130%). Recovery was determined at two additional concentration levels, the LOQ level and the highest concentration level of the method, and the results were found to be within 70–130%.
- The TSQ Quantis LC-MS/MS system is capable of successfully achieving the desired results for AZBT within acceptable limits for all the experiments performed. The sensitivity of the instrument as well as the reproducibility of the method have been found to be suitable for the analysis of AZBT in six sartans.

References

1. Risk of the presence of mutagenic azido impurities in losartan active substance. [Online] https://www.edqm.eu/en/news/risk-presence-mutagenic-azido-impurities-losartan-active-substance
2. Guidance for Industry Q2B Validation of Analytical Procedures: Methodology November 1996 ICH.
3. 3ICH Topic Q2 (R1) Note for guidance on validation of analytical procedures: text and methodology, (cpmp/ich/381/95) June 1995.
4. International Council For Harmonisation Of Technical Requirements For Pharmaceuticals For Human Use (ICH), Assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals to limit potential carcinogenic risk, M7(R1), Current Step 4 version, dated 31 March 2017.
5. Taiwan FDA method - AZBT in sartan drug substances including candesartan, irbesartan, losartan, olmesartan, and valsartan. Dated: 2021-07-21. [Online] https://www.fda.gov.tw/tc/includes/GetFile.ashx?mid=189&id=37286&t=s
6. LGL method - LC-MS/MS method for the determination of AZBT in valsartan, irbesartan, candesartan and losartan drug substances. [Online] https://www.edqm.eu/sites/default/files/general-methodparameters-azbt-lcms.pdf
7. Test method 31_PV_185 for genotoxic substances in sartans (01.07.2021). [Online] https://www.edqm.eu/sites/default/files/medias/fichiers/OMCL_Network/31-pv-185-genotoxic-substances-sartans.pdf
8. Jireš, J. et al. The determination of two analogues of 4-(azidomethyl)-1,1'-biphenyl as potential genotoxic impurities in the active pharmaceutical ingredient of several Sartans containing a tetrazole group. *Journal of Pharmaceutical and Biomedical Analysis* **2021,** *205,* 114300.
9. European Commission Decision (2002/657/EC) – Publications Office of the European Union.

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