Application Note

Pharmaceuticals

Highly Sensitive LC/MS/MS Method for the Simultaneous Quantification of Mutagenic Azido Impurity Analogues in Five Different Sartan APIs and Formulations

Detection of regulated genotoxic impurities from the drug manufacturing process

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Abstract

In 2018, the first incident of contamination of a potential genotoxic nitrosamine impurity N-nitrosodimethylamine (NDMA) was reported in the angiotensin II receptor blocker (ARB) drug Valsartan. This later expanded to the addition of more nitrosamine impurities in various ARB category drug substances and products, which lead to multiple product recalls by the US-FDA and EMA agencies. Another known mutagenic azido impurity 5-(4'-(azidomethyl)-[1,1'-biphenyl]-2yl)-1H-tetrazole (AZBT) triggered multiple recalls by various regulatory agencies such as EMEA, UK MHRA, Health Canada, and TGA for various sartan tetrazole ring therapeutics, which contain drugs such as Irbesartan, Losartan, and Valsartan. The European Directorate for the Quality of Medicines and Health Care (EDQM) also alerted its CEP holders to perform the required confirmatory tests. Azido impurities can form as a byproduct during the synthesis of sartan active pharmaceutical ingredients (APIs) with a tetrazole ring.

This application note describes a highly sensitive and selective method using an Agilent 6470 triple quadrupole LC/MS for the simultaneous determination of two azido impurities 5-(4'-(azidomethyl)-[1,1'-biphenyl]-2yl)-1H-tetrazole (AZBT) and 4'-(azidomethyl)-[1,1'-biphenyl]-2-carbonitrile (AZBC) in five different sartan APIs and formulations, including Losartan, Valsartan, Irbesartan, Candesartan, and Olmesartan.

Introduction

Azido impurities are known mutagenic impurities, which need to be controlled as per the ICH M7 guidelines regarding safe human consumption. Formation of azido impurities is possible during the synthesis of sartan active pharmaceutical ingredients (APIs) containing a tetrazole ring. The formation of the tetrazole ring in sartans is achieved by the reaction between appropriate nitrile and azido groups, which leads to the formation of azide impurity byproducts at trace levels. Several recalls of sartan therapeutics, including Irbesartan, Valsartan, and Losartan were triggered by UK MHRA and Health Canada due to the presence of AZBT. EDQM also alerted its CEP holders to perform the required confirmatory tests. Regulatory agencies such as EDQM, Swissmedic, and the Taiwan FDA have published LC/MS/MS methods for the determination of AZBT in different sartan APIs and formulations. LC/MS/MS is an inherently selective and sensitive analytical technique that is well-suited to detect and quantify trace-level potential genotoxic impurities, and is widely used in the pharmaceutical industry. Multiple reaction monitoring (MRM) is the mode of acquisition used for quantitative analysis using triple quadrupole instruments. This mode selectively filters the precursor ion and product ions of the compound of interest, thereby increasing the sensitivity and selectivity of the analysis. The Agilent Jet Stream (AJS) ionization source used in this application note works by using thermal gradient focusing technology, which helps to increase the sensitivity of the instrument to reach lower detection limits. While regulatory limits for AZBT impurities are defined as 4.0 ppm with a LOQ of 0.4 ppm, this study concluded that even lower LOQs of 0.2 ppm for AZBT impurities could be obtained using the Agilent 6470 triple quadrupole LC/MS. AZBT is the main impurity of regulatory concern, to be quantified in sartans, while 4'-(azidomethyl)-[1,1'-biphenyl]-2-carbonitrile (AZBC) quantification is for information purposes only, as mentioned in the regulatory agencies published methods.

The LC/MS/MS methods described in this application note were developed using the highly sensitive 6470 triple quadrupole LC/MS instrument. This method was developed for the simultaneous determination of both azido impurity analogues AZBT and AZBC in five different sartan APIs and formulations, including Losartan, Valsartan, Irbesartan, Candesartan, and Olmesartan which are captured in Figure1. Of the two methods published in this application note, method 1 refers to the simultaneous quantification of AZBT and AZBC in five sartan API and drug formulations, and method 2 refers to the quantification of AZBT only, in five sartan APIs. Both methods differed in terms of sample preparation with respect to the API sample concentration. Liquid chromatography and mass spectrometry conditions remained the same for both methods.

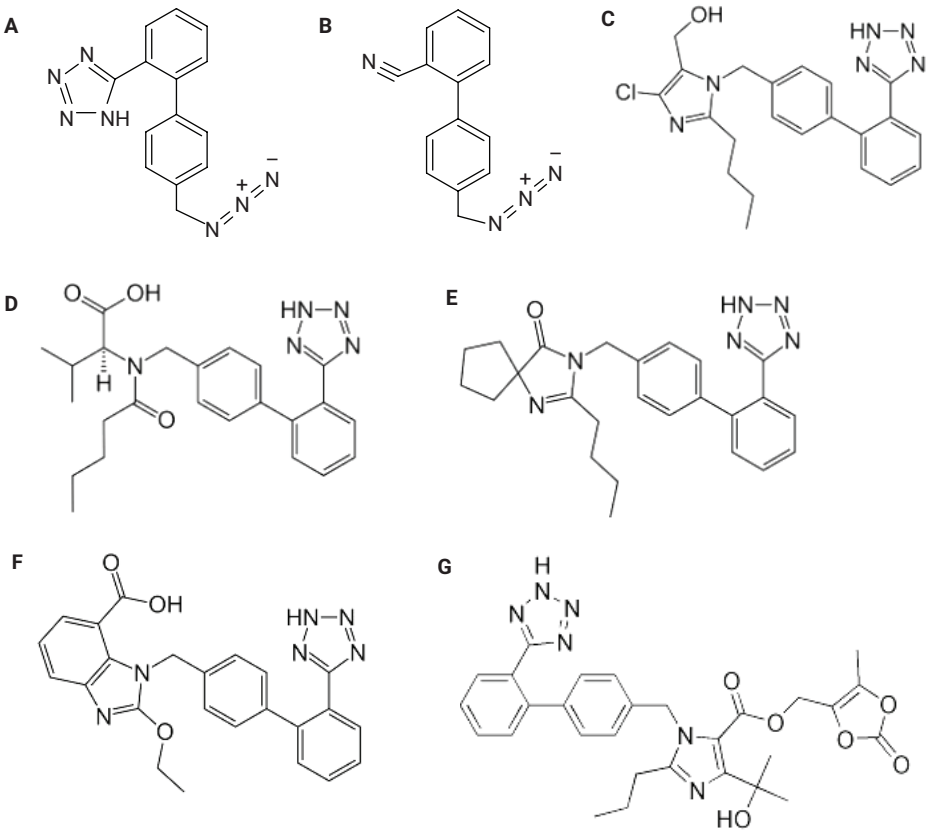

Figure 1. Chemical structures of (a) azido biphenyl tetrazole impurity, (b) azido biphenyl Carbonitrile impurity, (c) Losartan API, (d) Valsartan API, (e) Irbesartan API, (f) Candesartan API, (g) Olmesartan API.

Chemicals and reagents

Sartan APIs and azido impurity standards AZBT and AZBC were locally procured from pharmaceutical company. Drug formulations were purchased from local pharmacies. Other LC/MS-grade solvents (methanol, water) were purchased from Honeywell (Charlotte, NC, USA). Formic acid was purchased from Fluka (now of Honeywell).

Experimental

Method 1

Sample preparation for Sartan drug APIs (API final sample concentration 2.5 mg/mL)

Losartan, Valsartan, and Olmesartan drug API:

1. Accurately weigh 250 mg of drug API into a 15 mL centrifuge tube.
2. Add 10 mL of sample diluent.
3. Vortex the solution for 1 minute, followed by 10 minutes of sonication so that the API is completely soluble.
4. Transfer 100 µL of the prepared solution into an HPLC sample vial and add 900 µL of diluent.
5. Vortex the mixture a final time before loading onto the LC/MS/MS for analysis.

Irbesartan and Candesartan drug API:

1. Accurately weigh 250 mg of drug API into a 15 mL centrifuge tube.
2. Add 10 mL of sample diluent.
3. Vortex the solution for one minute, followed by 10 minutes of sonication.
4. Centrifuge for 15 minutes at 4,500 rpm.
5. Transfer 100 µL of the supernatant solution into an HPLC sample vial and add 900 µL of diluent.
6. Vortex the mixture a final time before loading onto the LC/MS/MS for analysis.

Sample preparation for Losartan 100 mg, Valsartan 160 mg, and Irbesartan 300 mg drug products (API final sample concentration 2.5 mg/mL)

1. Using the labeled concentration, crush enough tablets to obtain at least three times the API target weight.
2. Find the average weight per tablet and accurately weigh the equivalent of 250 mg API.
3. Transfer to a 15 mL centrifuge tube and add 10 mL of diluent.
4. Vortex for one minute, followed by 40 minutes of shaking using a shaker.
5. After extraction, centrifuge the samples at 4,500 rpm for 15 minutes.
6. Filter the supernatant solution using a 0.22 µm PVDF membrane, and transfer 100 µL into an HPLC sample vial, followed by the addition of 900 µL of diluent.
7. Vortex the mix a final time before loading onto the LC/MS/MS for analysis.

Sample preparation for Candesartan 32 mg and Olmesartan 40 mg drug products (API final sample concentration 1.25 mg/mL)

1. Using the labeled concentration, crush enough tablets to obtain at least three times the API target weight.
2. Find the average weight per tablet and accurately weigh the equivalent of 125 mg API.
3. Transfer to a 15 mL centrifuge tube and add 10 mL of diluent.
4. Vortex for one minute, followed by 40 minutes of shaking using a shaker.
5. After extraction, centrifuge the samples at 4,500 rpm for 15 minutes.
6. Filter the supernatant solution using a 0.22 µm PVDF membrane, and transfer 100 µL into an HPLC sample vial, followed by the addition of 900 µL of diluent.
7. Vortex the mix a final time before loading onto the LC/MS/MS for analysis.

Method 2

Sample preparation for Losartan, Valsartan, Irbesartan, Candesartan, and Olmesartan drug APIs (API final sample concentration 0.3 mg/mL)

1. Accurately weigh 30 mg of drug substance into a 15 mL centrifuge tube and add 10 mL of diluent.
2. Vortex for one minute, followed by 10 minutes of sonication to ensure complete solubility.
3. Transfer 100 µL into an HPLC sample vial and add 900 µL of diluent.
4. Vortex the mix a final time before loading onto the LC/MS/MS for analysis.

AZBT and AZBC preparation: standard and calibration level

1. Accurately weigh 10 mg of each of AZBT and AZBC.
2. Transfer each mixture into 10 mL volumetric flasks and add 100% methanol to obtain final concentrations of stock 1 at 1,000 µg/mL.
3. Prepare a 10 µg/mL sample of stock 2 for AZBT and AZBC by adding 100 µL of stock 1 to 9.9 mL of 100% methanol.
4. Further dilute stock 3 to 1 µg/mL by adding 1 mL of stock 2 to 9 mL of 100% methanol.
5. Prepare a series of calibration standards from 1 µg/mL using 80% acetonitrile in water to obtain the final concentrations of 50, 40, 20, 10, 5, 1, 0.25, and 0.1 ng/mL for AZBT, and 50, 40, 20, 10, 5, and 1 ng/mL for AZBC.

Instrument parameters and setpoints

Table 1. UHPLC configuration and settings.

Parameter	Value
Instruments	Agilent 1290 Infinity II high speed pump (G7120A) Agilent 1290 Infinity II multisampler (G7167B) Agilent 1290 Infinity II multicolumn thermostat (G7116B) Agilent 1290 Infinity II variable wavelength detector (G7114B)
Needle Wash	methanol/water (80/20)
Sample Diluent	acetonitrile/water (80/20)
Multisampler Temperature	15 °C
Injection Volume	5 µL
Analytical Column	Agilent InfinityLab Poroshell 120 PFP, 3.0 × 100 mm, 2.7 µm (p/n 695975-308)
Column Temperature	40 °C
Mobile Phase A	0.1% formic acid in water
Mobile Phase B	0.1% formic acid in acetonitrile/water (95/5)
Flow Rate	0.4 mL/min
Gradient	Time (min) % A % B Flow (mL/min) 0 60 40 0.4 0.1 60 40 0.4 5.5 60 40 0.4 12 0 100 0.4 14 0 100 0.4 14.1 60 40 0.4 18 60 40 0.4
Stop Time	18 minutes
Wavelengths	260 nm

Data analysis

Data were acquired and analyzed using Agilent MassHunter software version 10. MS/MS transitions were obtained and optimized using the Agilent MassHunter Acquisition optimizer software to determine optimal precursor and product ions, fragmentor voltages, and collision energies upon injection of a neat solution at a concentration level of 1,000 ng/mL, with 1 µL injection volume in flow injection mode.

Table 2. Mass spectrometer configuration and source settings.

Instrument	Agilent 6470 Triple Quadrupole Mass Spectrometer (G6470B)
Ion Source	Agilent Jet Stream (AJS)
MS/MS Mode	MRM
Ion Mode	Positive
Gas Temperature	300 °C
Gas Flow	6 L/min
Nebulizer Pressure	30 psi
Sheath Gas Heater	300 °C
Sheath Gas Flow	10 L/min
Capillary Voltage, Positive	4,000 V
Nozzle Voltage	1,000 V
MS1/MS2 Resolution	0.7/0.7 (unit/unit)
Dwell Time	50 ms

Table 3. Detailed MRM settings in MRM mode on the Agilent 6470 LC/TQ.

Compound	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Dwell Time (ms)	Fragmentor (V)	Collision Energy (V)	CAV (V)	Polarity
AZBT (Quantifier)	278.2	235.1	50	50	4	5	+
AZBT (Qualifier)	278.2	207.1	50	50	14	5	+
AZBC (Quantifier)	207.0	179.1	50	80	30	5	+
AZBC (Qualifier)	207.0	151.0	50	80	40	5	+

Table 4. Diverter Valve program used to divert all sartan APIs to waste.

No.	Start Time (min)	Scan Type	Diverter Valve
1	0	MRM	MS
2	1.0	MRM	Waste
3	3.2	MRM	MS
4	4.1	MRM	Waste
5	6.2	MRM	MS
6	8.2	MRM	Waste
7	16	MRM	MS

Results and discussion

Method 1

Method development was performed in positive mode using an Agilent Jet Stream Source for the optimization of mass spectrometry parameters for AZBT and AZBC, including precursor and product ions, capillary voltage, fragmentor voltages, and collision energies to obtain the desired sensitivity. Gas temperatures, gas flows, and dwell times were optimized to establish the response reproducibility. Chromatographic separation was achieved between AZBT, AZBC, and all five sartans using the Poroshell PFP column.

Sample preparation was optimized in terms of extraction time, and achieved the desired recovery for AZBT in all five sartan APIs and formulations Losartan, Valsartan, Irbesartan, Candesartan, and Olmesartan, and AZBC in Losartan, Valsartan, Irbesartan, and Olmesartan formulations only.

Critical parameters such as specificity, reproducibility, linearity, recovery, LOQ, and LOD are established.

LOQ and LOD limits and S/N values are captured in Table 5. Reproducibility data, including bracketing standards at a limit level of 4.0 ppm and LOQ are captured in Tables 6 and 7. The calibration concentrations ranged from 0.1 to 50 ng/mL, with specific details mentioned in Table 5. R^2 values are greater than 0.99 for both AZBT and AZBC impurities, displaying linear responses throughout the concentration range.

A summary of recovery experiments for AZBT and AZBC at the limit level in five Sartan APIs and at the limit level and LOQ level in sartan formulations are captured in Tables 8, 9, and 10.

Table 5. Representative S/N ratio data* for LOQ, LOD, and linearity data for both azido impurities AZBT and AZBC.

No.	Compound	LOD		LOD (S/N)	LOQ		LOQ (S/N)	R^2	Linearity Range	
		ng/mL	ppm		ng/mL	ppm			ng/mL	ppm
1	AZBT	0.125	0.05	29.1	0.5	0.2	132.8	0.9955	0.1 to 50	0.04 to 20
2	AZBC	0.625	0.25	9.2	2.5	1	20.3	0.9993	1.0 to 50	0.4 to 20

* S/N was calculated using the RMS algorithm, noise width (0.6 min), with reference selected as sample using Agilent MassHunter Quantitative software version 10.

Table 6. Representative data for reproducibility of the method at 10.0 ng/mL (4 ppm in relation to an API concentration of 2.5 mg/mL), including bracketing standards.

	Number	AZBT	AZBC
Initial Replicates	1	69,035	2,703
	2	68,732	2,609
	3	70,275	2,391
	4	71,332	2,526
	5	69,552	2,408
	6	69,222	2,632
Bracketing Standard	7	69,094	2,692
	Average	69,606.0	2,565.9
	STD DEV	906.4	127.8
	%RSD	1.3	5.0

Table 7. Representative data for reproducibility of the method at LOQ 0.5 ng/mL (0.2 ppm) of AZBT and 2.5 ng/mL (1.0 ppm) of AZBC in relation to an API concentration of 2.5 mg/mL.

	Number	AZBT	AZBC
Initial Replicates	1	3,814	744
	2	4,171	884
	3	4,061	801
	4	3,687	756
	5	3,974	818
	6	4,111	721
	Average	3,969.7	787.3
	STD DEV	186.0	59.6
	%RSD	4.7	7.6

Table 8. Summary of the recovery experiment in sartan drug APIs at 10 ng/mL (4.0 ppm in relation to an API concentration of 2.5 mg/mL).

API Sample Concentration 2.5 mg/mL				Recovery %	
Number	API Name	ng/mL	ppm	AZBT	AZBC
1	Losartan	10	4.0	95.3	80
2	Valsartan	10	4.0	106.9	101.4
3	Irbesartan	10	4.0	106.4	90
4	Candesartan	10	4.0	91.5	89.9
5	Olmesartan	10	4.0	97.4	95.3

Table 9. Summary of recovery experiment in sartan drug products at 10 ng/mL (4.0 ppm in relation to an API concentration of 2.5 mg/mL).

				Recovery %	
Number	Formulation Name	ng/mL	ppm	AZBT	AZBC
1	Losartan 100 mg	10	4.0	100.5	99.7
2	Valsartan 160 mg	10	4.0	100.2	103.7
3	Irbesartan 300 mg	10	4.0	96.3	102.7
4	Candesartan 32 mg	10	4.0	88.8	NA*
5	Olmesartan 40 mg	10	4.0	99.2	108

* NA: Satisfactory recovery was not observed in Candesartan 32 mg tablets for AZBC.

Table 10. Summary of recovery experiment in sartan drug products at LOQ (0.2 ppm of AZBT and 1.0 ppm of AZBC in relation to an API concentration of 2.5 mg/mL).

Number	API Name	LOQ		Recovery %*	LOQ		Recovery %
		ng/mL	ppm	AZBT	ng/mL	ppm	AZBC
1	Losartan	0.5	0.2	107.7	2.5	1	102.4
2	Valsartan	0.5	0.2	97.4	2.5	1	106
3	Irbesartan	0.5	0.2	99	2.5	1	97.9
4	Candesartan	0.5	0.2	87	2.5	1	NA**
5	Olmesartan	0.5	0.2	98.8	2.5	1	113.5

* Recovery experiment performed in duplicates in five different sartan APIs at 4.0 ppm, sartan drug products at 4.0 ppm, and LOQ levels using 2.5 mg/mL API test concentration. Chromatographic separation can be further optimized if any interference is observed in formulation samples due to matrix components with impurities retention times.

** NA: Satisfactory recovery not observed in Candesartan 32 mg tablets for AZBC.

Figures 2, 3, and 4 show the representative extracted ion MRM chromatograms from the 6470 LC/TQ, showing elution and separation of azido impurities in standard and spiked sartan APIs and formulations. DAD chromatograms were acquired to bracket the retention times of higher concentrations of all sartan APIs, which need to be diverted to waste to avoid mass spectrometer contamination. These high concentration of sartan APIs are diverted to waste using the inbuilt diverter valve of the Agilent 6470 LC/MS by creating a diverter valve program as mentioned in Table 4.

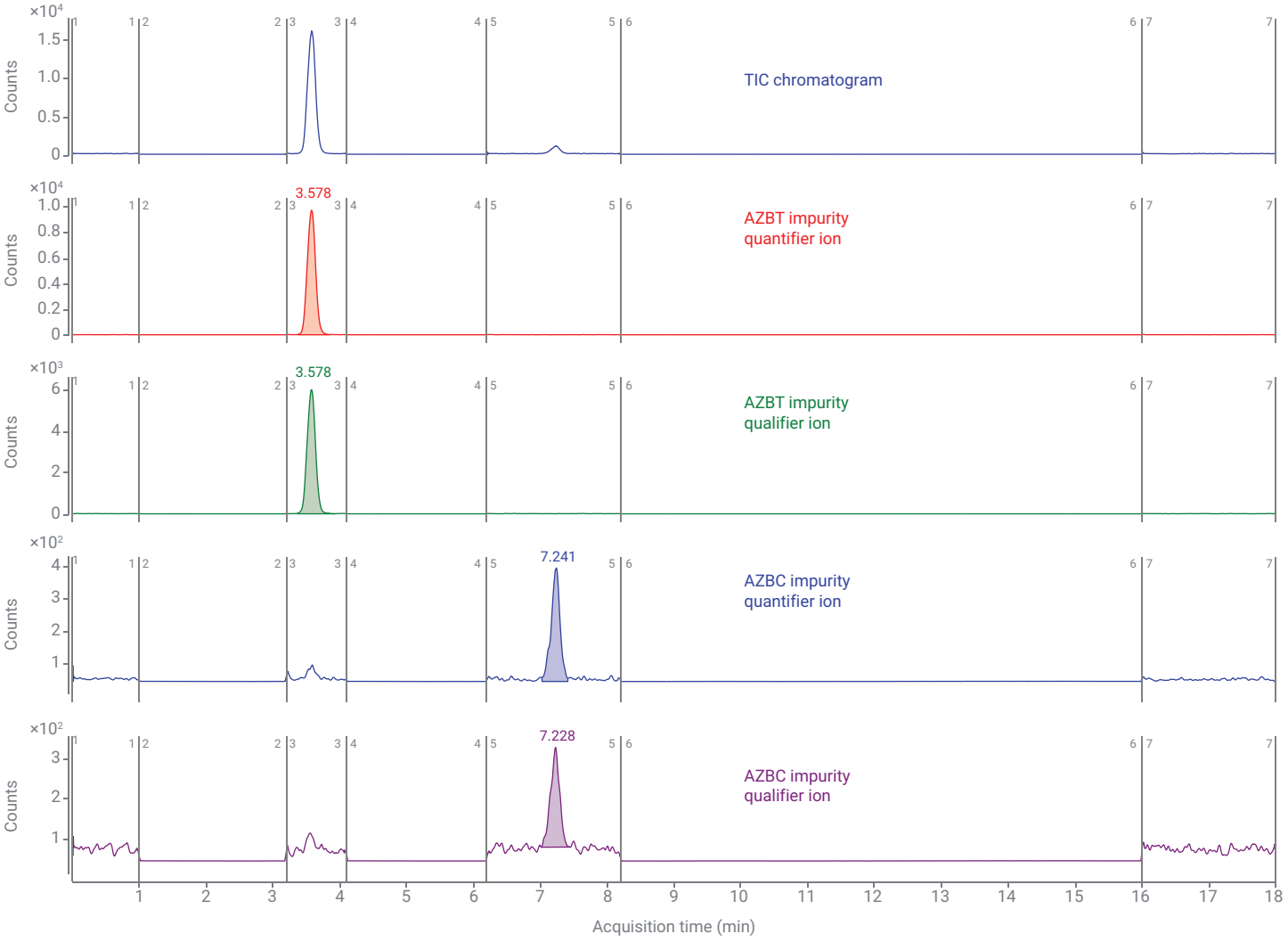

Figure 2. Representative standard chromatogram of AZBT and AZBC at 10 ng/mL (4.0 ppm in relation to an API concentration of 2.5 mg/mL).

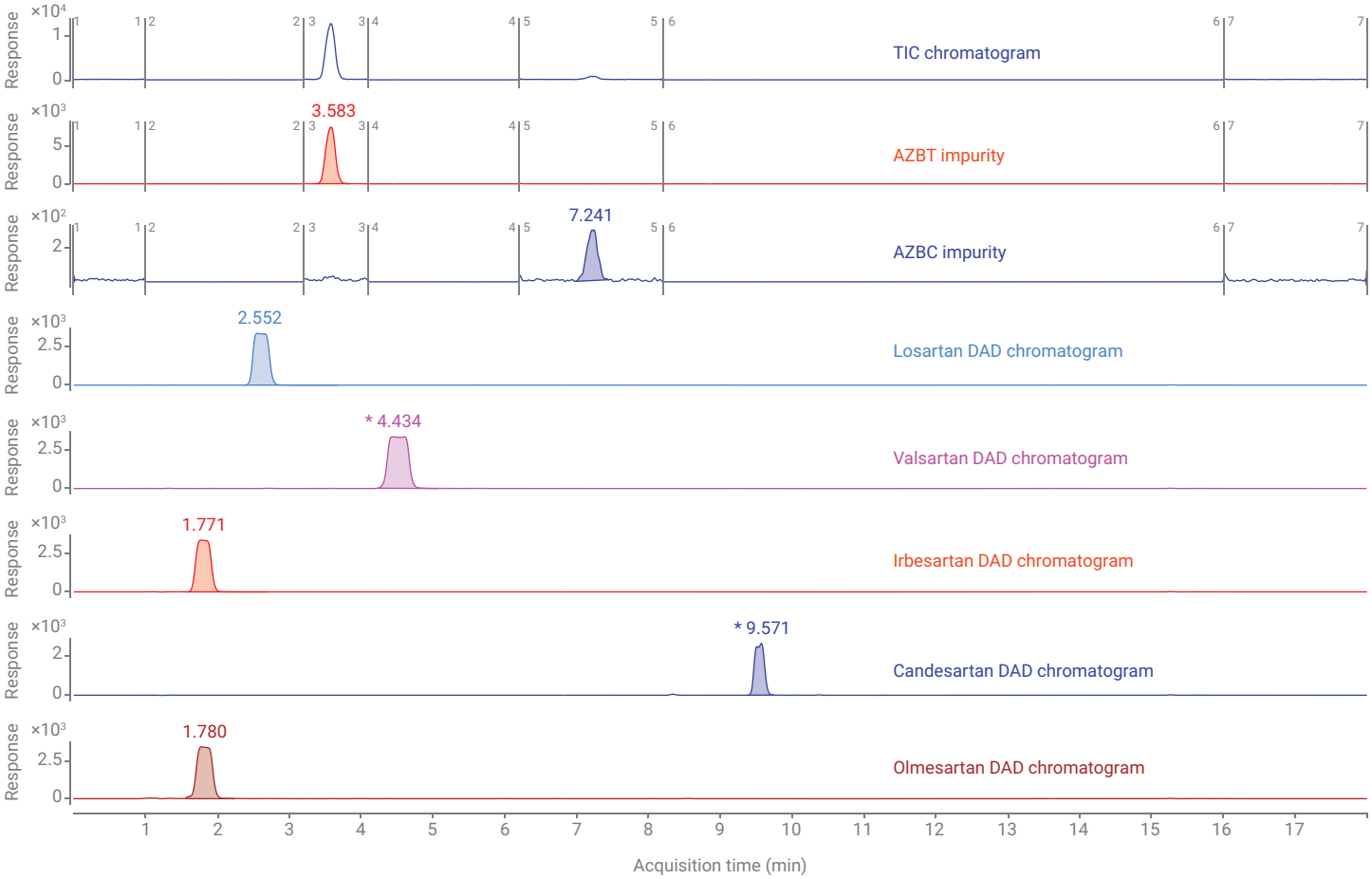

Figure 3. Representative chromatograms of the chromatographic separation between AZBT and AZBC impurity standards, with all five sartans: Losartan, Valsartan, Irbesartan, Candesartan, and Olmesartan.

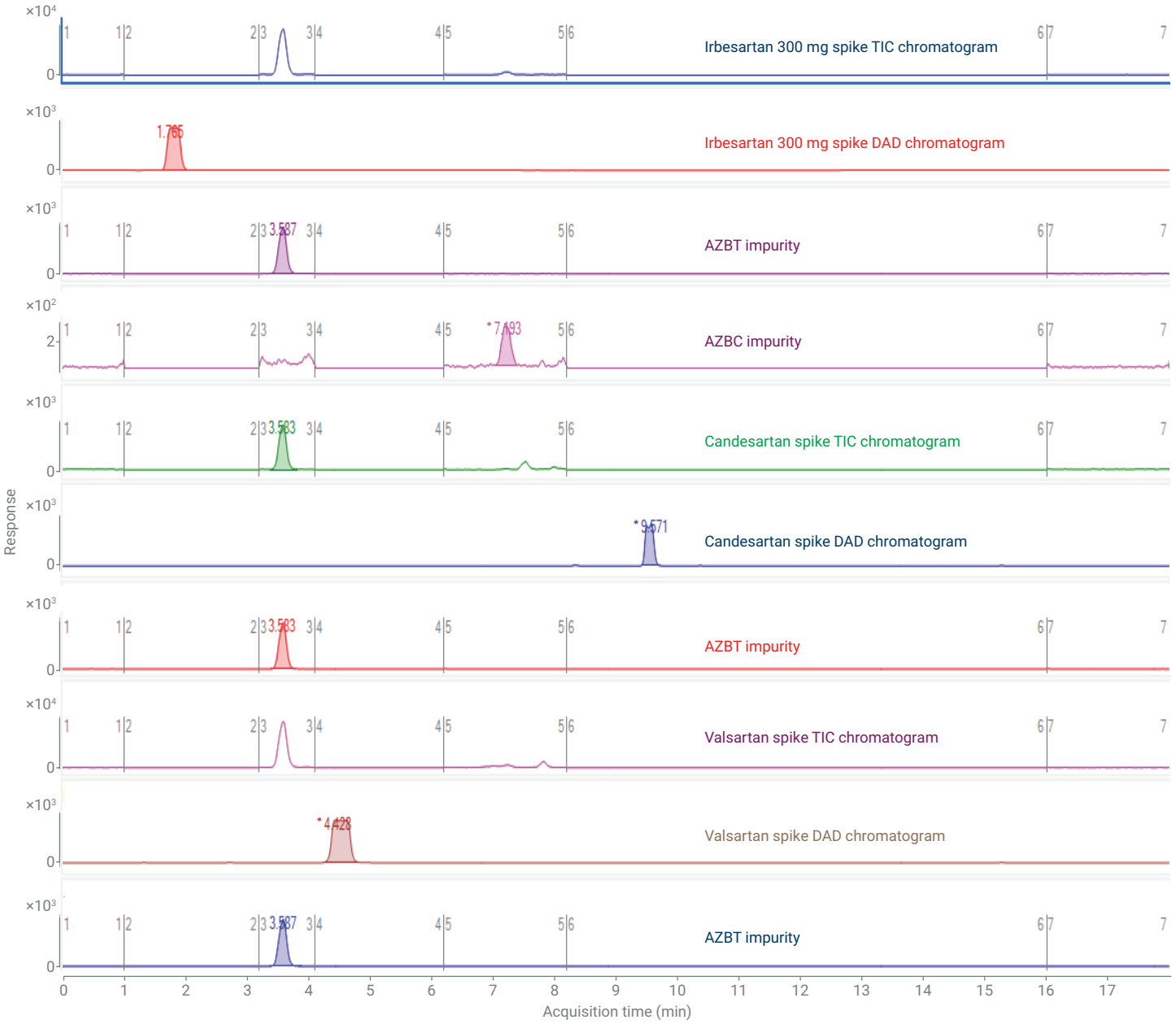

Figure 4A. Representative chromatograms of standards AZBT and AZBC at 10 ng/mL (4.0 ppm in relation to an API concentration of 2.5 mg/mL) spiked into sartan formulations Irbesartan 300 mg, Candesartan 32 mg, and Valsartan 160 mg.

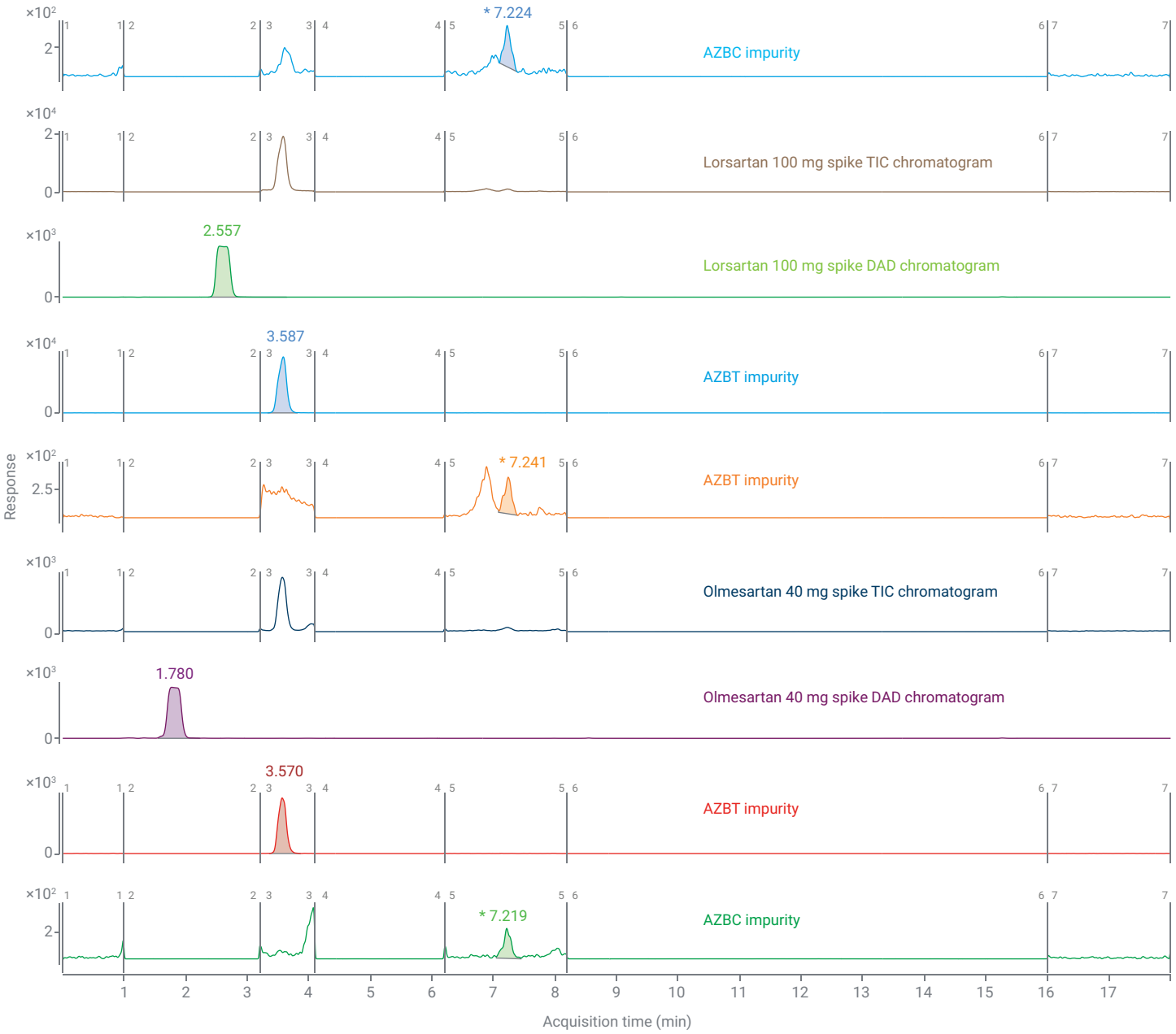

Figure 4B. Representative chromatograms of standards AZBT and AZBC at 10 ng/mL (4.0 ppm in relation to an API concentration of 2.5 mg/mL) spiked into sartan formulations Losartan 100 mg and Olmesartan 40 mg.

Accuracy and reproducibility

The calibration curve for both azido impurities demonstrated an accuracy rate within 15% of the expected concentration. Calibration levels were as shown in Table 5, and reproducibility across all levels exhibited CVs less than 15%. Figure 5 shows the calibration curves generated from the 6470 LC/TQ system.

Method 2

Method 1 was developed for simultaneous quantification of both AZBT and AZBC impurities at 2.5 mg/mL as an API test concentration, whereas method 2 was developed for the quantification of AZBT only, using 0.3 mg/mL API concentration for all five sartans: Losartan, Valsartan, Irbesartan, Candesartan, and Olmesartan, with a limit of 4.0 ppm and LOQ of 0.4 ppm.

All sartan APIs are completely soluble using the sample preparation method mentioned earlier for method 2, and chromatographic separation performed using the same conditions as in method 1.

All critical parameters (reproducibility, recovery, linearity, and LOQ) are established for AZBT in all five sartan APIs.

Table 11. Representative data for reproducibility of the method at 1.25 ng/mL (4 ppm in relation to an API concentration of 0.3 mg/mL) including bracketing standards.

	Number	AZBT
Initial Replicates	1	8,437
	2	8,763
	3	8,937
	4	9,172
	5	9,104
	6	8,899
Bracketing Standard	7	8,877
	Average	8,884.1
	STD DEV	241.1
	%RSD	2.7

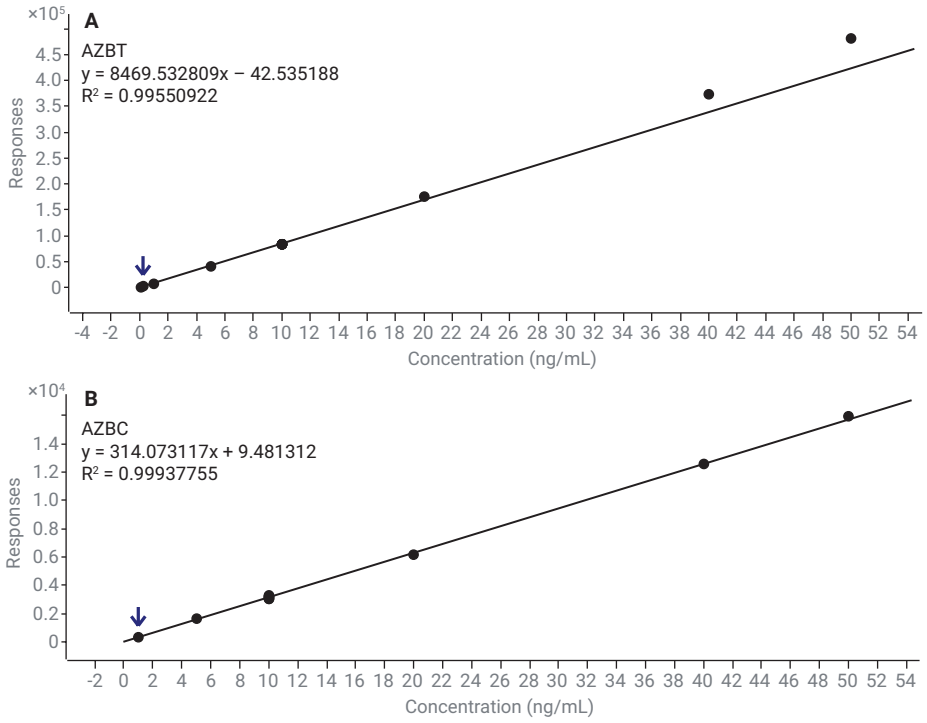

Figure 5. Representative calibration curves for AZBT (A) and AZBC (B) from the Agilent 6470 LC/TQ for all azido impurities dispersed throughout the chromatogram. The calibration curves used a $1/x^2$ weighting factor.

Table 12. Summary of the recovery experiment in sartan drug substances at 1.25 ng/mL (4.0 ppm in relation to an API concentration of 0.3 mg/mL).

API Sample Concentration 0.3 mg/mL				Recovery %
Number	API Name	ng/mL	ppm	AZBT
1	Losartan	1.25	4.0	99.7
2	Valsartan	1.25	4.0	115.9
3	Irbesartan	1.25	4.0	96.6
4	Candesartan	1.25	4.0	95.2
5	Olmesartan	1.25	4.0	95.1

Table 13. Representative S/N ratio data for LOQ and LOD for AZBT.

		LOD			LOQ		
Number	Compound	ng/mL	ppm	LOD (S/N)	ng/mL	ppm	LOQ (S/N) *
1	AZBT	0.033	0.11	3.5	0.1	0.33	13.9

* S/N was calculated using the RMS algorithm, noise width (0.6 min) with reference selected as sample using MassHunter Quantitative software, version 10.

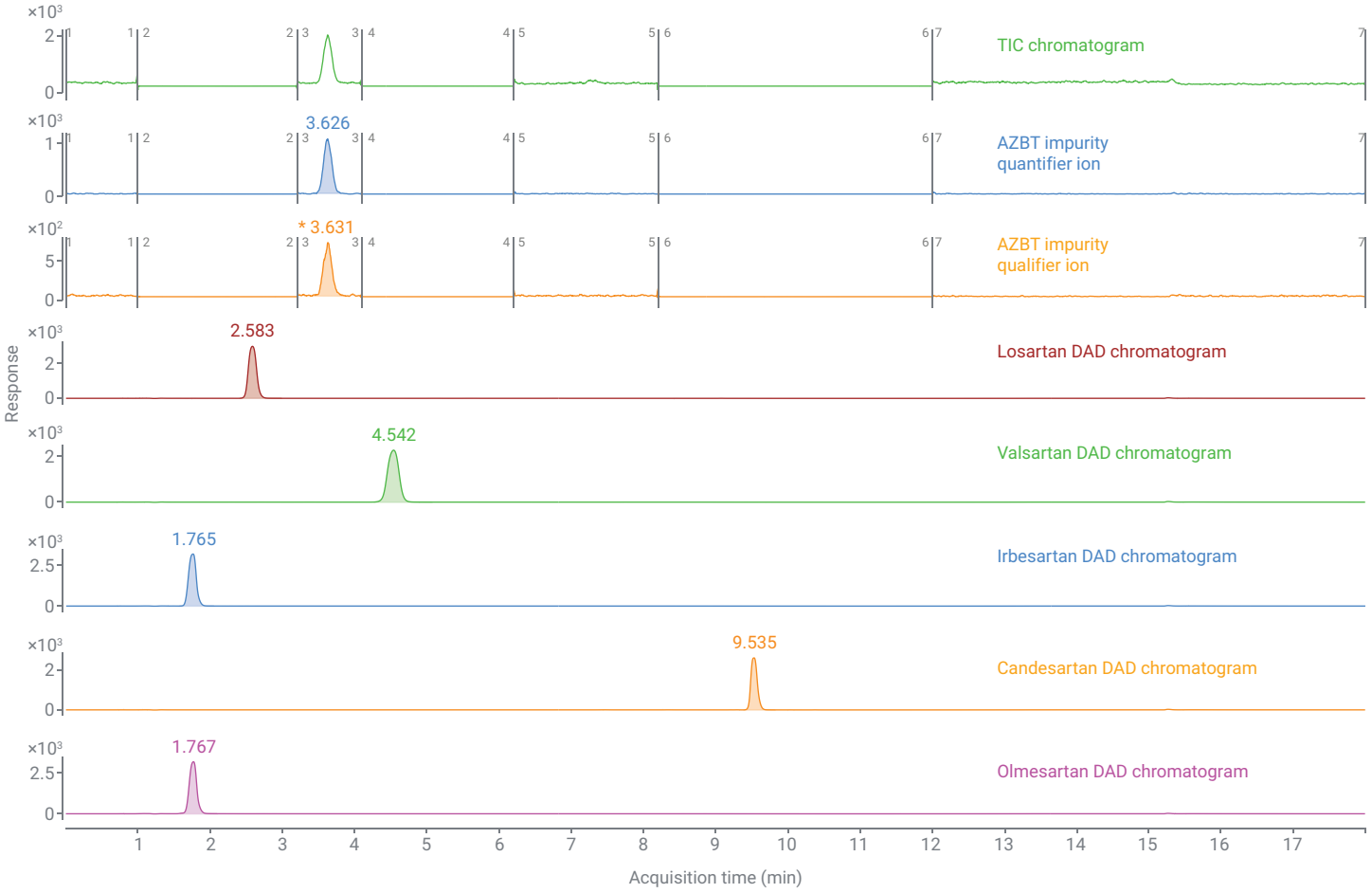

Figure 6. Representative chromatogram of the chromatographic separation between AZBT at 1.25 ng/mL, and the five sartans at 0.3 mg/mL sample concentration (Losartan, Valsartan, Irbesartan, Candesartan, and Olmesartan).

Conclusion

Two different methods have been developed in terms of differing API sample concentrations, and could establish all critical parameters of method performance for azido impurities using an Agilent 6470 LC/TQ. This study analyzed azido impurities at very low concentration levels, demanded by regulatory requirements. This study also detected the presence of AZBT impurities below the limit level in the analyzed Losartan formulations, using the developed method conditions. This application note is intended to demonstrate the reproducibility and sensitivity of the 6470 LC/TQ in the detection of azido impurities in the five different sartan APIs and formulations Losartan, Valsartan, Irbesartan, Candesartan, and Olmesartan.

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