Application Note

Pharmaceuticals

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Simultaneous Quantitation of Four Impurities in Clozapine API Using the Agilent 6470 Triple Quadrupole LC/MS

Quantitation of impurities 4-chloro 2-nitrobenzene amine, 2-chlorobenzoic acid, 2-(4-chloro 2-nitro phenyl amino) benzoic acid and 2-(2-amino 4-chloro phenyl amino benzoic acid) in API (Clozapine)

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Abstract

The presence of potential toxic chemicals and impurities in drugs are one of the biggest challenges in the manufacturing of active pharmaceutical ingredients (APIs). Therefore, it is important to identify these impurities during the manufacturing process to avoid issues related to the quality, efficacy, and safety of drugs. This application note describes an LC/MS/MS-based MRM method for the simultaneous quantitation of the four impurities 4-chloro 2-nitrobenzene amine, 2-chlorobenzoic acid, 2-(4-chloro 2-nitro phenyl amino) benzoic acid, and 2-(2-amino 4-chloro phenyl amino) benzoic acid in API (Clozapine). This application note demonstrates LOD-LOQ determination of impurities based on the calibration curve method. The detection limit is calculated as $3.3 \times \sigma/S$, whereas the quantification limit is calculated by $10 \times \sigma/S$, where σ is the standard deviation of the response, and S is the slope of the curve.

Quantitation of these impurities in clozapine API is extremely challenging due to the high matrix effect caused by the API, resulting in inaccurate quantitation. Therefore, chromatographic separation of the impurity from the API is necessary. The analysis is made further complex, as the sensitivities of these four impurities are not similar.

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Figure 1. Agilent 1290 Infinity II LC coupled to an Agilent 6470 triple quadrupole LC/MS.

Introduction

The presence of potential toxic chemicals and impurities in drugs is one of the biggest challenges in the manufacturing of API. Therefore, it is important to identify these impurities during the manufacturing process to avoid issues related to the quality, efficacy, and safety of drugs. Screening and quantitation of these impurities in APIs is useful to identify potential problems when evaluating new suppliers, changing manufacturing sites, or during production scale-up. An LC/MS/MS method was developed for the simultaneous quantification of the impurities 4-chloro 2-nitrobenzene amine, 2-chlorobenzoic acid, 2-(4-chloro 2-nitro phenyl amino) benzoic acid, and 2-(2-amino 4-chloro phenyl amino) benzoic acid in API for Clozapine.

In this application note, a highly selective multiple reaction monitoring (MRM)-based LC/MS/MS method was developed using an Agilent 6470 triple quadrupole LC/MS (LC/TQ). The sensitivity of the 6470 LC/TQ can easily detect compounds at the required limits of detection. The special design of the ion optics and stable electronics of the system provide consistent results across multiple batches.

Experimental

Chemicals and reagents

The four impurities: 4-chloro 2-nitrobenzene amine, 2-chlorobenzoic acid, 2-(4-chloro 2-nitro phenyl amino) benzoic acid, and 2-(2-amino 4-chloro phenyl amino) benzoic acid were provided by a potential customer. LC/MS-grade solvents such as methanol, acetonitrile, and water were purchased from Honeywell (Charlotte, NC, USA). Formic acid, MS grade was purchased from Fluka (now of Honeywell).

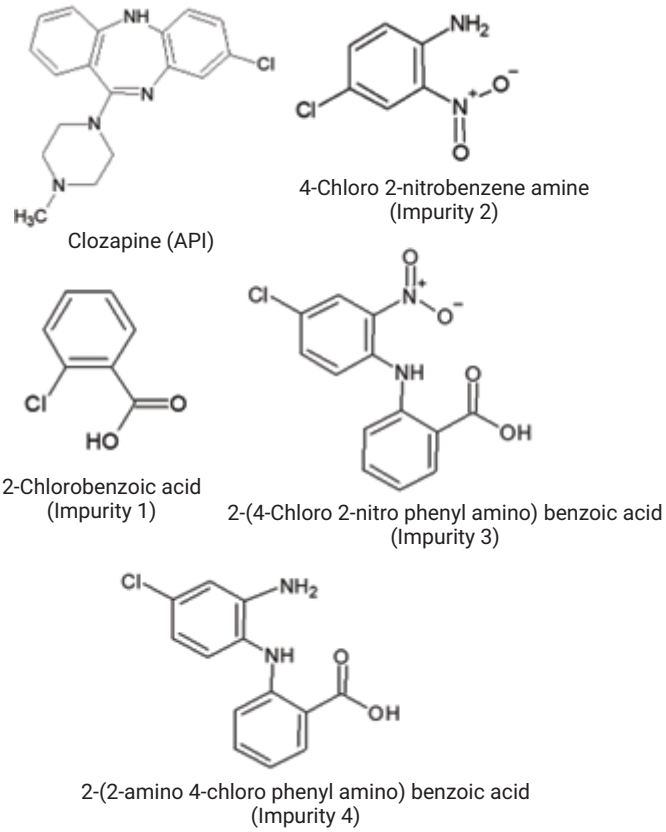

Figure 2. Structures of Clozapine and four impurities.

Instrument configuration

- 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 diode array detector (G7117A)
- Agilent 6470 triple quadrupole LC/MS (G6470B)

Table 1. Chromatography conditions.

Parameter	Value
Mobile Phase A	0.1% Acetic acid and 1 mM ammonium fluoride in water
Mobile Phase B	Methanol (100%)
Flow Rate	0.5 mL/min
Injection Volume	20 µL
Column Temperature	50 °C
Needle Wash	Methanol/water (70/30)
Column	Agilent Poroshell HPH C18, 4.6 × 150 mm, 2.7 µm (p/n 693975-702) (T)

Table 2. Gradient.

Time (min)	%A	%B
0	95	5
2	95	5
5	40	60
8	25	75
10	25	75
10.2	0	100
13	0	100
13.1	95	5
Post run	2 minutes	

Preparation of working standards for LOD-LOQ calculation

Table 3.1. Preparation of working standards, set 1.

Working Standard	Volume Taken	Volume of Diluent	Total Volume	Resultant Concentration
1 ppm	0.75 mL	4.25 mL	5.0 mL	150 ppb
1 ppm	0.60 mL	4.4 mL	5.0 mL	120 ppb
150 ppb	1.0 mL	1.0 mL	2.0 mL	75 ppb
120 ppb	1.875 mL	3.125 mL	5.0 mL	45 ppb
150 ppb	1.0 mL	4.0 mL	5.0 mL	30 ppb
30 ppb	1.0 mL	1.0 mL	2.0 mL	15 ppb
15 ppb	1.0 mL	1.0 mL	2.0 mL	7.5 ppb
15 ppb	0.5 mL	4.5 mL	5.0 mL	1.5 ppb

100 µL of each of the diluters were pipetted and filled up to 1 mL with diluent.

Table 3.2. Preparation of working standards, set 2.

Working Standard	Volume Taken	Volume of Diluent	Total Volume	Resultant Concentration
150 ppb	0.1 mL	0.9 mL	1.0 mL	15 ppb
120 ppb	0.1 mL	0.9 mL	1.0 mL	12 ppb
75 ppb	0.1 mL	0.9 mL	1.0 mL	7.5 ppb
45 ppb	0.1 mL	0.9 mL	1.0 mL	4.5 ppb
30 ppb	0.1 mL	0.9 mL	1.0 mL	3.0 ppb
15 ppb	0.1 mL	0.9 mL	1.0 mL	1.5 ppb
7.5 ppb	0.1 mL	0.9 mL	1.0 mL	0.75 ppb
1.5 ppb	0.1 mL	0.9 mL	1.0 mL	0.15 ppb

Table 4. MRM parameters.

ID	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Dwell Time (ms)	Fragmentor (V)	Collision Energy (V)	Cell Accelerator Voltage (V)	Polarity
Clozapine	327	270.1	50	147	24	4	Positive
Impurity 1	155	111	50	74	4	4	Negative
Inpurity 2	171	141	50	70	16	4	Negative
Inpurity 3	291	216	50	92	16	4	Negative
Impurity 4	261	217	50	45	16	4	Negative

Concentration levels ranging from 0.15 to 15 ppb were prepared as explained in Tables 3.1 and 3.2. Calibration curves were plotted to establish the LOD and LOQ levels. Limit of detection and limit of quantitation were calculated from the slope and standard error of the predicted y-value for each x in the regression of the calibration curve.

Instrument MRM parameters (Table 4) and source parameters (Table 5) were optimized to maximize sensitivity, while maintaining consistency in the method performance for large batches.

Table 5. MS source parameters.

Parameter	Value
Ionization Souce	AJS ESI
Gas Temperature	300 °C
Gas Flow	10 L/min
Nebulizer	30 psi
Sheath Gas	250 °C
Sheath Gas Flow	10 L/min
Capillary Voltage	3,500 V
Nozzle Voltage	500 V

Once the chromatographic separation between the API and impurities was established, the time program was set to divert the API to waste with the help of an integrated diverter valve (Table 6).

Table 6. Diverter valve program.

Start Time (min)	Scan Type	Diverter Valve
0	MRM	To waste
8	MRM	To MS

Sample preparation

API preparation

20 mg of API was weighed in a centrifuge tube. One milliliter of 60/40 methanol/water was added and vortexed for almost two minutes. The mixture was kept in an ultrasonic bath for five minutes, and the API was partially dissolved. However, the impurities have good solubility in the solvent system used (60/40 methanol/water). The partial solubility of the API is helpful in reducing the matrix effect. The contents were then filtered through a PVDF filter into a 2 mL HPLC vial for injection.

Data acquisition and data analysis

All samples were acquired using Agilent MassHunter Data Acquisition software, version 10.1. MRM transitions were obtained and optimized using the Agilent MassHunter Acquisition optimizer software. This tool automatically optimized Fragmentor voltages for the Q1 precursor ions, and collision energies for the Q3 product ions.

A standard solution of concentration, 500 ng/mL, was introduced to the MS by Flow Injection Analysis with an injection volume of 5 µL. Through the automated workflow, 10 product ions from each impurity were selected for creation to MRM transitions.

Chromatograms were viewed through MassHunter qualitative analysis software, version 10.0. Quantitation of each batch was carried out using MassHunter quantitative analysis software, version 10.1.

Validation parameters such as linearity, reproducibility, recovery, specificity, and sensitivity, in terms of limit of quantification (LOQ) and limit of detection (LOD), were characterized to ensure good method performance. Accuracies for calibration points were within ±20%. No manual integration was needed.

Results and discussion

The LOD and LOQ were calculated using the calibration curve method. Calibration curves from 0.15 to 15 ppb were used to establish the LOD and LOQ.

The detection limit is expressed as DL = 3.3 × σ/S

The quantification limit is expressed as QL = 10 × σ/S

Where σ = the standard deviation of the response and
S = slope of the curve.

Considering all four impurities, the LOD and LOQ are fixed as 1.0 and 3.0 ppb, respectively.

Calibration curves generated from 0.15 ng/mL (LOQ) to 15 ng/mL for LOD-LOQ calculation

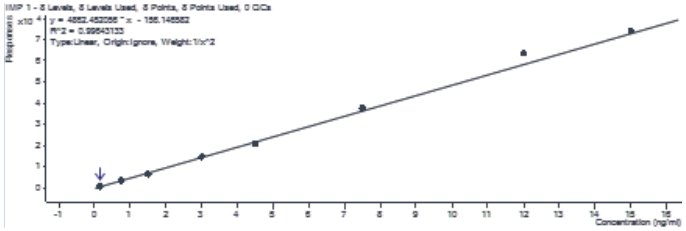

Figure 3.1. Calibration curve generated for Impurity 1 from 0.15 ng/mL (LOQ) to 15 ng/mL for LOD-LOQ calculation.

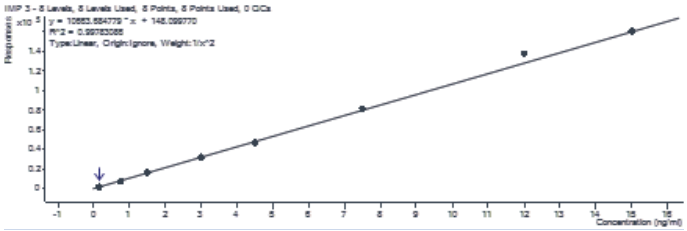

Figure 3.2. Calibration curve generated for Impurity 2 from 0.15 ng/mL (LOQ) to 15 ng/mL for LOD-LOQ calculation.

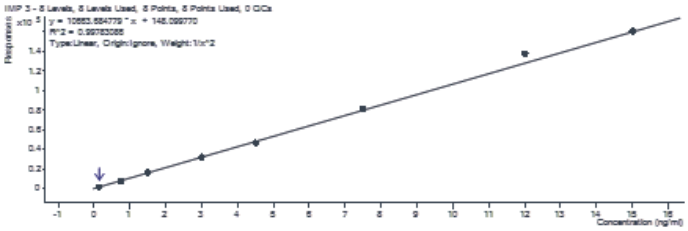

Figure 3.3. Calibration curve generated for Impurity 3 from 0.15 ng/mL (LOQ) to 15 ng/mL for LOD-LOQ calculation.

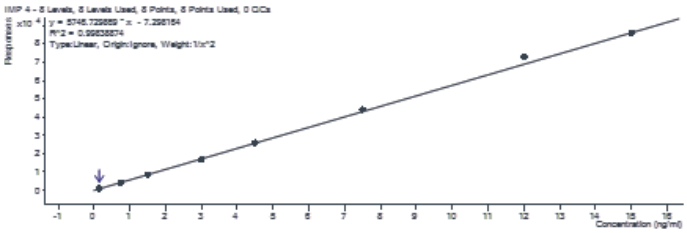

Figure 3.4. Calibration curve generated for Impurity 4 from 0.15 ng/mL (LOQ) to 15 ng/mL for LOD-LOQ calculation.

Chromatographic conditions were developed to achieve maximum separation between the impurities and API. The API was diverted to waste to avoid severe contamination of the MS using the integrated diverter valve. As per the diverter valve time program, only eluent with retention times between 8 and 16 minutes proceeded to the MS.

Table 7. LOD and LOQ calculation.

Concentration in (ng/mL) Used in Calibration Curve	Response/Area Impurity 1	Response/Area Impurity 2	Response/Area Impurity 3	Response/Area Impurity 4
0.15	585	727	1773	867
0.75	3322	4497	7527	4006
1.50	6515	9098	16017	8500
3.01	14678	18586	32129	16928
4.51	20709	27898	46782	25811
7.52	37606	48433	81777	44177
12.02	63404	80680	137752	73156
15.03	74222	97088	160420	86234
Correlation	0.99876	0.9996	0.9988	0.9992
STDEV (σ)	1513.7	1159.5	3195.0	1383.3
Slope (S)	5105.7	6592.6	10988.7	5887.8
LOD	0.98	0.58	0.96	0.78
LOQ	2.96	1.76	2.91	2.35

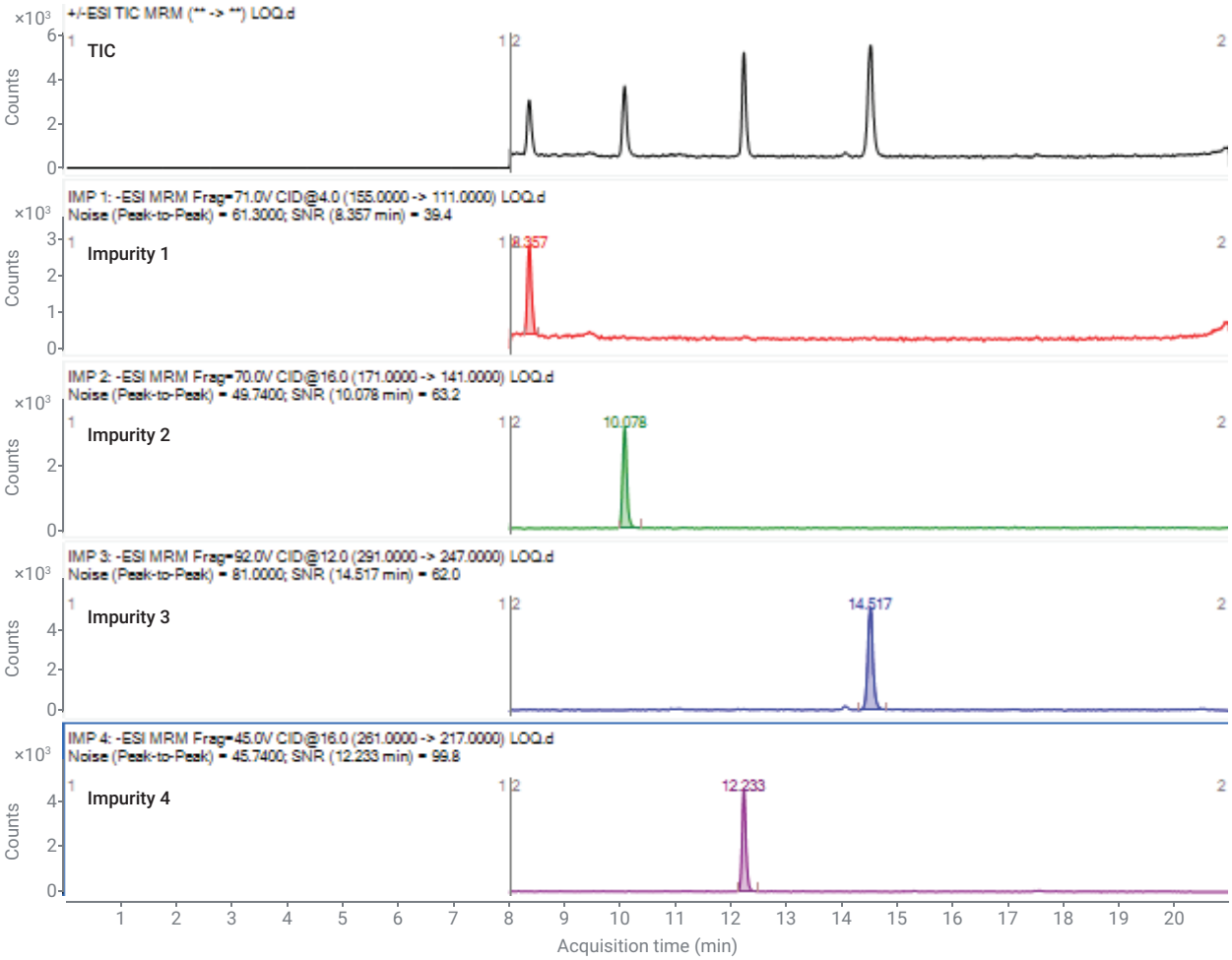

Figure 4. Chromatographic separation between the API and the impurity. The API is diverted to waste.

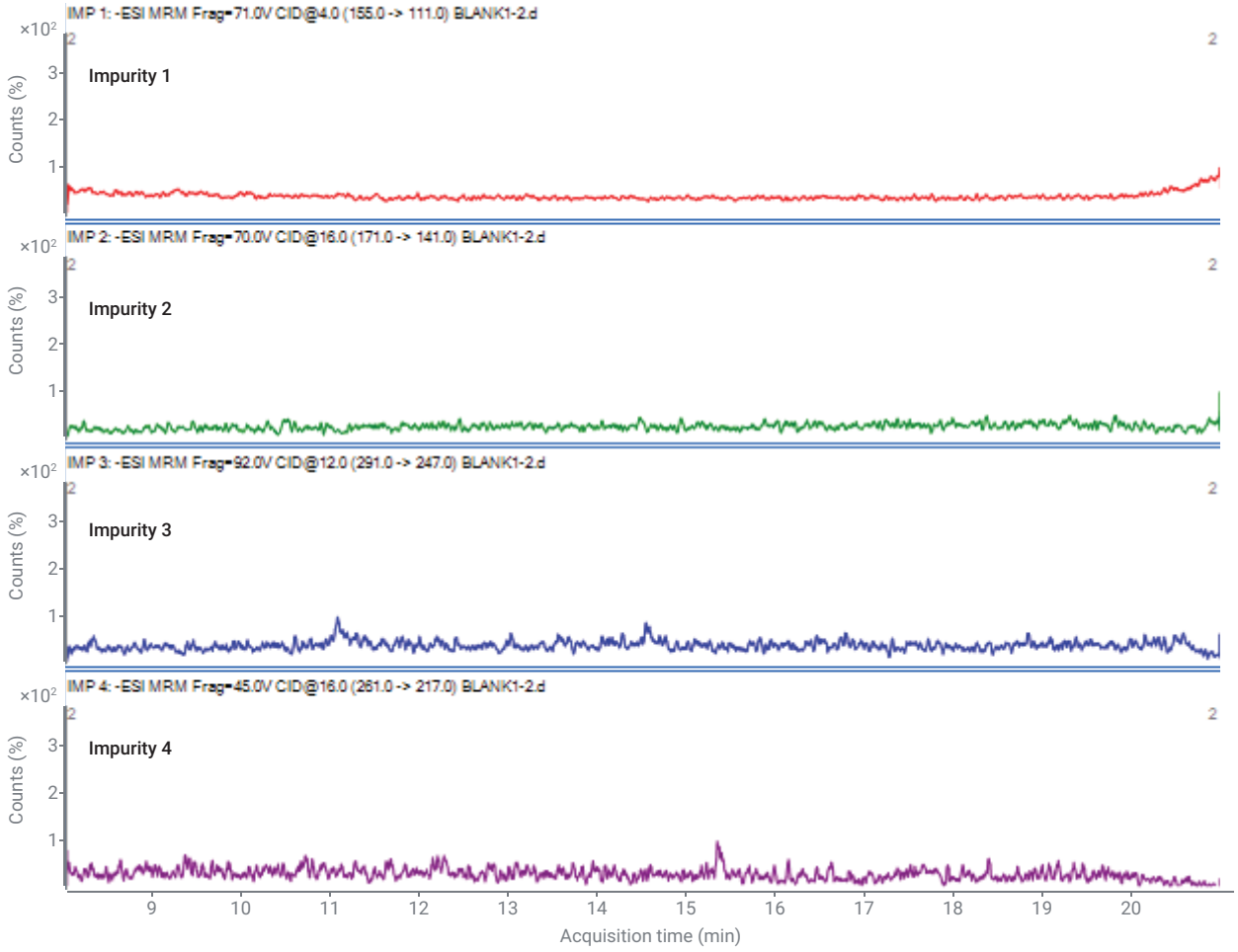

Figure 5. Blank chromatogram.

The reproducibility of the response is evaluated at the limit of quantitation level by giving 10 injections of the mixture of four impurities at a fixed LOQ level of 3 ng/mL.

Table 8. Percent RSD of 10 injections at LOQ level.

Compound Name	Count	Avg. RT	Avg. Area	RSD Area
IMP 1	10	8.4	14,544.9	2.3%
IMP 2	10	10.1	18,431.6	1.4%
IMP 3	10	14.5	31,377.4	1.8%
IMP 4	10	12.3	17,824.3	1.9%

Ten injections at the LOQ level were performed, and the %CV of area response (impurities 1 to 4) were found to be 2.3%, 1.37%, 1.8%, and 1.9% respectively.

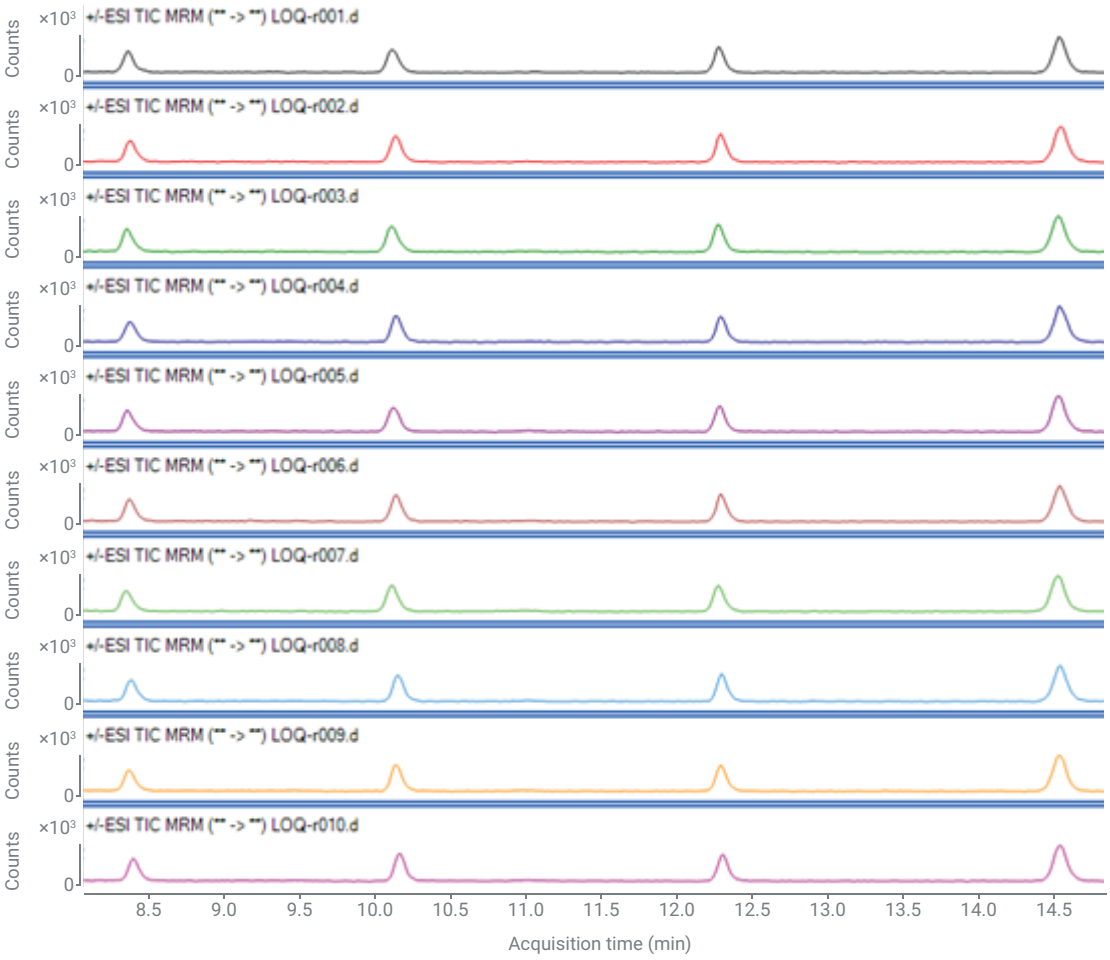

Figure 6. Chromatogram of 10 replicate injections at the LOQ level, showing the consistency in result.

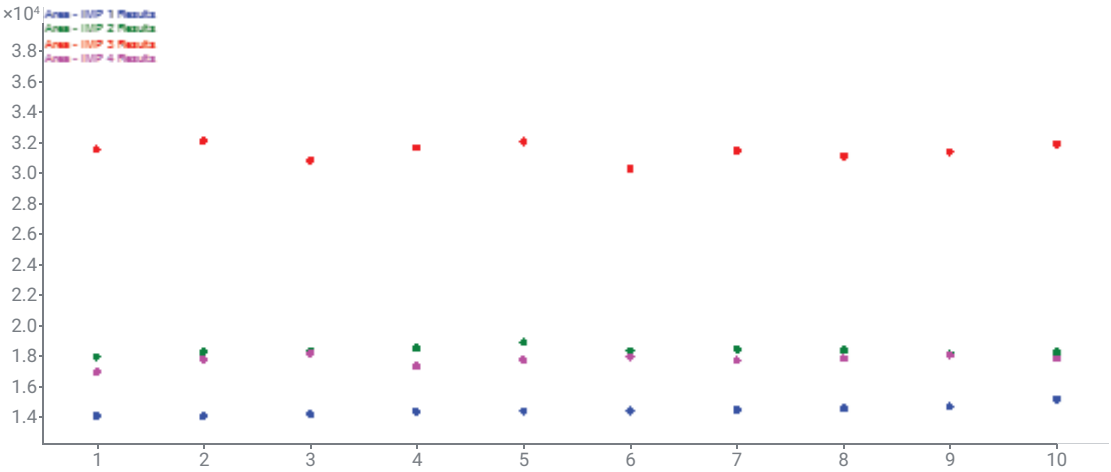

Figure 7. Matrix plot of responses of impurities 1 to 4 from 10 injections.

A linear concentration curve spanning three orders of magnitude was produced from 3.0 to 150 ng/mL (R^2 value of 0.9957 from linear regression and $1/x^2$ weighing). The lowest concentration of 3 ppb ng/mL demonstrated a S/N >39:1, 63:1, 62:1, and 99:1 for impurities 1 to 4, respectively, using the peak-to-peak algorithm for noise calculation. This demonstrates the sensitivity of the 6470 LC/TQ, and the possibility to analyze lower concentrations.

Table 9. Working standard preparation for plotting the calibration curve.

Working Standard	Volume Taken	Volume of Diluent	Total Volume	Resultant Concentration	Calibration Level
1 ppm	0.30 mL	1.7 mL	2.0 mL	150 ppb	Level 8
1 ppm	0.20 mL	1.8 mL	2.0 mL	100 ppb	Level 7
150 ppb	1.0 mL	1.0 mL	2.0 mL	75 ppb	Level 6
100 ppb	1.0 mL	1.0 mL	2.0 mL	50 ppb	Level 5
50 ppb	1.0 mL	1.0 mL	2.0 mL	25 ppb	Level 4
150 ppb	0.2 mL	1.8 mL	2.0 mL	15 ppb	Level 3
100 ppb	0.2 mL	1.8 mL	2.0 mL	10 ppb	Level 2
150 ppb	1.0 mL	4.0 mL	5.0 mL	30 ppb	
30 ppb	0.2 mL	1.8 mL	2.0 mL	3.0 ppb (LOQ)	Level 1

Preparation of spike samples

Spiking was performed at different concentration levels, namely LOQ, the 25 ppb level, 50 ppb level, and 75 ppb level. 20 mg of the API was weighed in a centrifuge tube. Appropriate volumes of working standards were spiked to achieve the desired concentration of impurities in the API. 1 mL of 60/40 methanol/water was added, and vortexed for almost two minutes. The API was partially dissolved. However, the impurities have good solubility in the solvent system used (60/40 methanol/water). The contents were then filtered through PVDF filter in to a 2 mL HPLC vial for injection. The concentrations obtained from spiked samples were compared with the generated calibration curve.

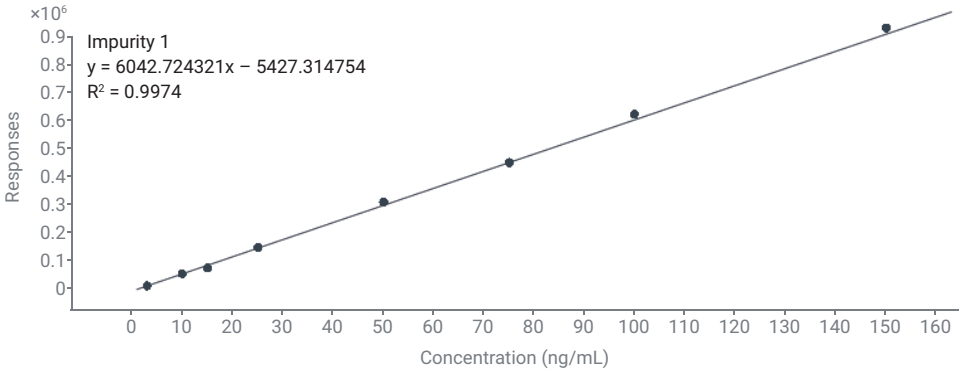

Figure 8. Calibration curve generated from 3 ng/mL (LOQ) to 150 ng/mL for Impurity 1.

Batch Table

Sample: SAMPLE CLZP-USP-2007012 SPIK Sample Type: <All> Compound: IMP 1

Sample				IMP 1 Me...	IMP 1 Results				
	Data File	Type	Level	Exp. Conc.	RT	Resp.	MI	Calc. Conc.	Accuracy
	BLANK1-2.d	Sample					☐		
	LOQ.d	Cal	1	3.00	8.355	13121	☐	3.0695	102.3
	LEVEL 2.d	Cal	2	10.00	8.341	53272	☐	9.7140	97.1
	LEVEL 3.d	Cal	3	15.00	8.327	76588	☐	13.5725	90.5
	LEVEL 4.d	Cal	4	25.00	8.351	146317	☐	25.1120	100.4
	LEVEL 5.d	Cal	5	50.00	8.320	308942	☐	52.0244	104.0
	LEVEL 6.d	Cal	6	75.00	8.316	448961	☐	75.1960	100.3
	LEVEL 7.d	Cal	7	100.00	8.327	617724	☐	103.1243	103.1
	LEVEL 8.d	Cal	8	150.00	8.313	920719	☐	153.2664	102.2
	BLANK2-1.d	Blank					☐		
	API SAMPLE-1_1-20...	Sample			8.196	7338	☐	2.1126	
	API SPIKE SAMPLE...	QC	1	3.00	8.242	23072	☐	4.7163	157.2
▸	API SPIKE SAMPLE...	QC	4	25.00	8.243	163116	☐	27.8919	111.6
	API SPIKE SAMPLE...	QC	5	50.00	8.259	312531	☐	52.6184	105.2
	API SPIKE SAMPLE...	QC	6	75.00	8.278	455964	☐	76.3549	101.8

Figure 9. Quantitative table for Impurity 1.

Table 10.1. Recovery study result for Impurity 1.

Added Impurity Concentration	Spike ID – Impurity 1	Sample Weight	Total Dilution	Obtained Concentration	Actual Concentration	Recovery
3 ppb	Spike 1 (LOQ)	20.0 mg	1.0 mL	2.60 ppb	3.0 ppb	86.7%
25 ppb	Spike 2	20.0 mg	1.0 mL	25.78 ppb	25.0 ppb	103.1%
50 ppb	Spike 3	20.0 mg	1.0 mL	50.51 ppb	50.0 ppb	101.0%
75 ppb	Spike 4	20.0 mg	1.0 mL	74.24 ppb	75.0 ppb	98.99%

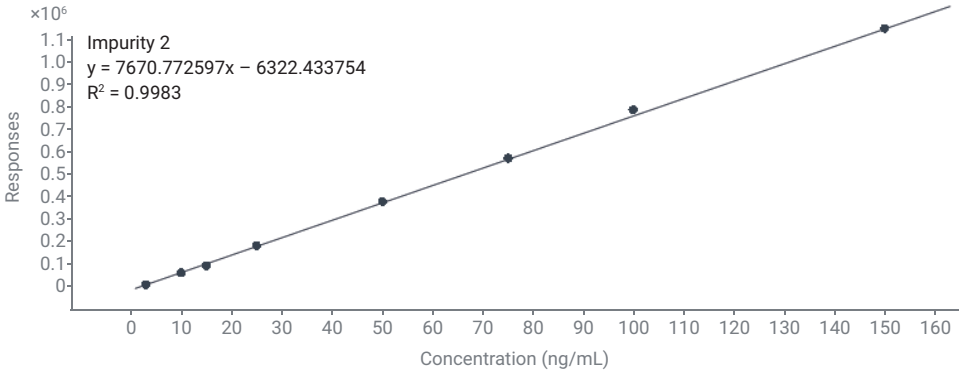

Figure 10. Calibration curve generated from 3 ng/mL (LOQ) to 150 ng/mL for Impurity 2.

Batch Table

Sample: SAMPLE CLZP-USP-2007012 SPIK Sample Type: <All> Compound: IMP 2

Sample				IMP 2 Me...	IMP 2 Results				
	Data File	Type	Level	Exp. Conc.	RT	Resp.	MI	Calc. Conc.	Accuracy
	BLANK1-2.d	Sample			10.389	1	☐		
	LOQ.d	Cal	1	3.00	10.078	17105	☐	3.0542	101.8
	LEVEL 2.d	Cal	2	10.00	10.064	68457	☐	9.7486	97.5
	LEVEL 3.d	Cal	3	15.00	10.050	99940	☐	13.8529	92.4
	LEVEL 4.d	Cal	4	25.00	10.088	188659	☐	25.4187	101.7
	LEVEL 5.d	Cal	5	50.00	10.054	385428	☐	51.0706	102.1
	LEVEL 6.d	Cal	6	75.00	10.050	576365	☐	75.9620	101.3
	LEVEL 7.d	Cal	7	100.00	10.071	785279	☐	103.1971	103.2
	LEVEL 8.d	Cal	8	150.00	10.050	11449...	☐	150.0903	100.1
	BLANK2-1.d	Blank			9.922	3	☐		
	API SAMPLE-1_1-20...	Sample			10.109	56433	☐	8.1811	
	API SPIKE SAMPLE...	QC	1	3.00	10.076	77762	☐	10.9617	365.4
▸	API SPIKE SAMPLE...	QC	4	25.00	10.078	237424	☐	31.7760	127.1
	API SPIKE SAMPLE...	QC	5	50.00	10.068	425500	☐	56.2945	112.6
	API SPIKE SAMPLE...	QC	6	75.00	10.088	602149	☐	79.3234	105.8

Figure 11. Quantitative table for Impurity 2.

Table 10.2. Recovery study result for Impurity 2.

Added Impurity Concentration	Spike ID – Impurity 2	Sample Weight	Total Dilution	Obtained Concentration	Actual Concentration	Recovery
3 ppb	Spike 1 (LOQ)	20.0 mg	1.0 mL	2.78 ppb	3.0 ppb	92.7%
25 ppb	Spike 2	20.0 mg	1.0 mL	23.22 ppb	25.0 ppb	92.9%
50 ppb	Spike 3	20.0 mg	1.0 mL	47.74 ppb	50.0 ppb	95.5%
75 ppb	Spike 4	20.0 mg	1.0 mL	70.77 ppb	75.0 ppb	94.4%

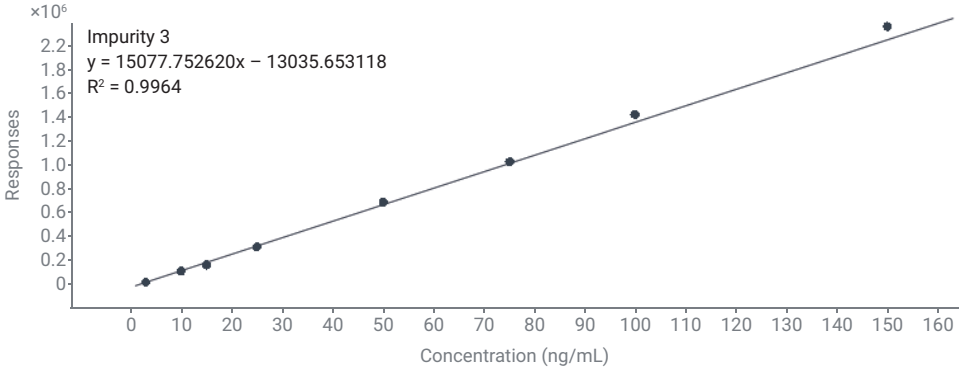

Figure 12. Calibration curve generated from 3 ng/mL (LOQ) to 150 ng/mL for Impurity 3.

Batch Table

Sample: SAMPLE CLZP-USP-2007012 SPIK Sample Type: <All> Compound: IMP 3

	Sample			IMP 3 Me...	IMP 3 Results				
	Data File	Type	Level	Exp. Conc.	RT	Resp.	MI	Calc. Conc.	Accuracy
	BLANK1-2.d	Sample			13.748	14	☐	0.8655	
	LOQ.d	Cal	1	3.00	14.551	33658	☐	3.0969	103.2
	LEVEL 2.d	Cal	2	10.00	14.488	130467	☐	9.5175	95.2
	LEVEL 3.d	Cal	3	15.00	14.464	190292	☐	13.4853	89.9
	LEVEL 4.d	Cal	4	25.00	14.471	358105	☐	24.6151	98.5
	LEVEL 5.d	Cal	5	50.00	14.450	757732	☐	51.1195	102.2
	LEVEL 6.d	Cal	6	75.00	14.443	11303...	☐	75.8341	101.1
	LEVEL 7.d	Cal	7	100.00	14.447	15642...	☐	104.6088	104.6
	LEVEL 8.d	Cal	8	150.00	14.440	23678...	☐	157.9095	105.3
	BLANK2-1.d	Blank			14.440	21	☐	0.8660	
	API SAMPLE-1_1-20...	Sample			14.450	41670	☐	3.6282	
	API SPIKE SAMPLE...	QC	1	3.00	14.436	82894	☐	6.3623	212.1
▸	API SPIKE SAMPLE...	QC	4	25.00	14.422	426580	☐	29.1566	116.6
	API SPIKE SAMPLE...	QC	5	50.00	14.401	832750	☐	56.0949	112.2
	API SPIKE SAMPLE...	QC	6	75.00	14.422	12515...	☐	83.8737	111.8

Figure 13. Quantitative table for Impurity 3.

Table 10.3. Recovery study result for Impurity 3.

Added Impurity Concentration	Spike ID – Impurity 3	Sample Weight	Total Dilution	Obtained Concentration	Actual Concentration	Recovery
3 ppb	Spike 1 (LOQ)	20.0 mg	1.0 mL	2.73 ppb	3.0 ppb	91.0%
25 ppb	Spike 2	20.0 mg	1.0 mL	25.53 ppb	25.0 ppb	102.1%
50 ppb	Spike 3	20.0 mg	1.0 mL	52.47 ppb	50.0 ppb	104.9%
75 ppb	Spike 4	20.0 mg	1.0 mL	80.25 ppb	75.0 ppb	107.0%

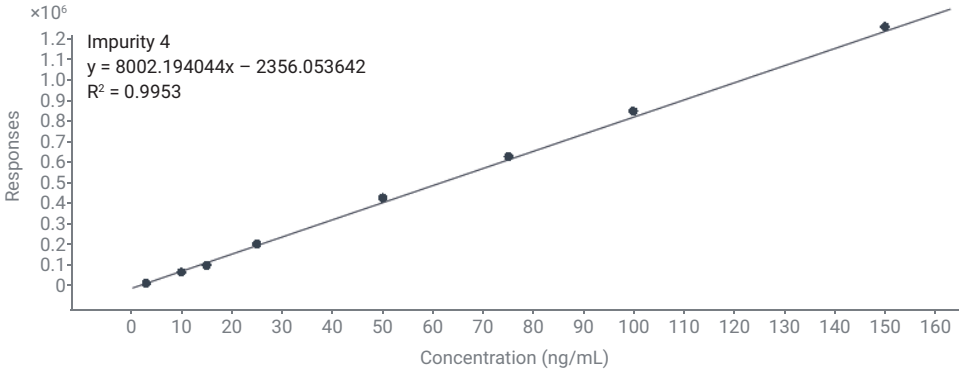

Figure 14. Calibration curve generated from 3 ng/mL (LOQ) to 150 ng/mL for Impurity 4.

Batch Table

Sample: SAMPLE CLZP-USP-2007012 SPI Sample Type: <All> Compound: IMP 4

	Sample			IMP 4 Me...	IMP 4 Results				
	Data File	Type	Level	Exp. Conc.	RT	Resp.	MI	Calc. Conc.	Accuracy
	BLANK1-2.d	Sample			12.528	11	☐	0.2958	
	LOQ.d	Cal	1	3.00	12.233	22580	☐	3.1161	103.9
	LEVEL 2.d	Cal	2	10.00	12.219	70613	☐	9.1187	91.2
	LEVEL 3.d	Cal	3	15.00	12.205	105393	☐	13.4650	89.8
	LEVEL 4.d	Cal	4	25.00	12.229	202032	☐	25.5415	102.2
	LEVEL 5.d	Cal	5	50.00	12.208	418726	☐	52.6209	105.2
	LEVEL 6.d	Cal	6	75.00	12.205	615061	☐	77.1560	102.9
	LEVEL 7.d	Cal	7	100.00	12.215	823857	☐	103.2483	103.2
	LEVEL 8.d	Cal	8	150.00	12.205	12177...	☐	152.4680	101.6
	BLANK2-1.d	Blank			12.194	43	☐	0.2998	
	API SAMPLE-1_1-20...	Sample			12.222	55226	☐	7.1958	
	API SPIKE SAMPLE...	QC	1	3.00	12.215	80754	☐	10.3859	346.2
▸	API SPIKE SAMPLE...	QC	4	25.00	12.208	281992	☐	35.5338	142.1
	API SPIKE SAMPLE...	QC	5	50.00	12.198	517219	☐	64.9290	129.9
	API SPIKE SAMPLE...	QC	6	75.00	12.212	741817	☐	92.9961	124.0

Figure 15. Quantitative table for Impurity 4.

Table 10.4. Recovery study result for Impurity 4.

Added Impurity Concentration	Spike ID – Impurity 4	Sample Weight	Total Dilution	Obtained Concentration	Actual Concentration	Recovery
3 ppb	Spike 1 (LOQ)	20.0 mg	1.0 mL	3.19 ppb	3.0 ppb	106.3%
25 ppb	Spike 2	20.0 mg	1.0 mL	28.34 ppb	25.0 ppb	113.4%
50 ppb	Spike 3	20.0 mg	1.0 mL	57.73 ppb	50.0 ppb	115.5%
75 ppb	Spike 4	20.0 mg	1.0 mL	85.80 ppb	75.0 ppb	114.4%

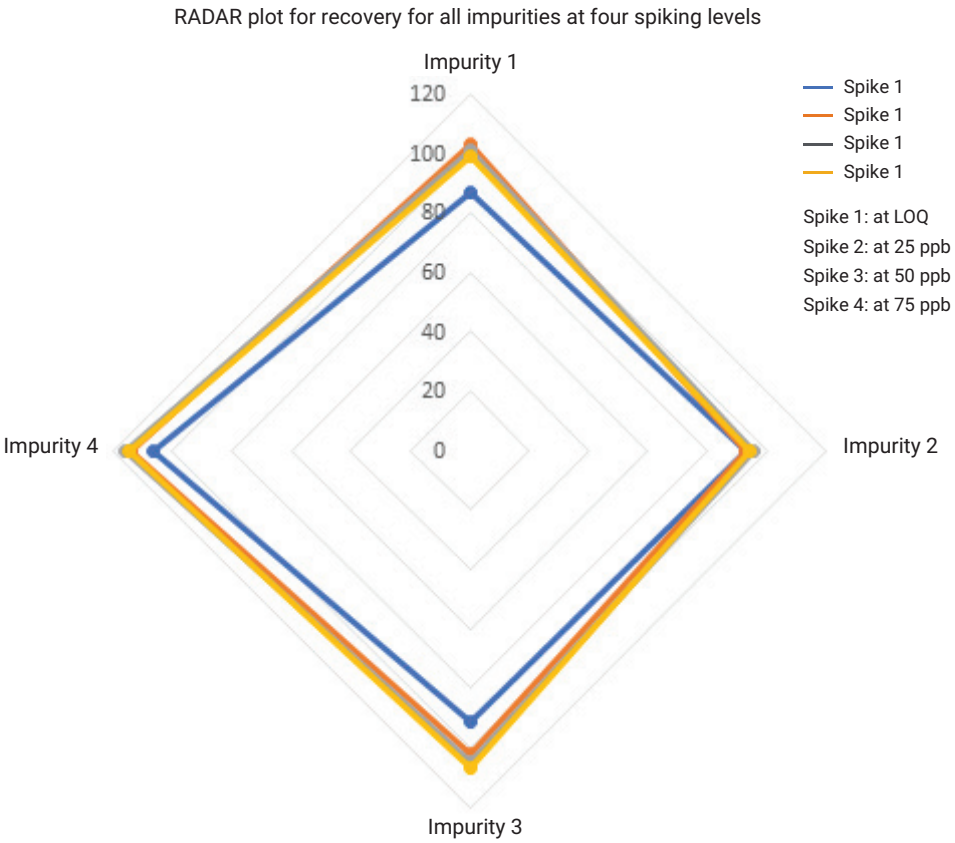

Figure 16. RADAR Plot showing the recovery percentage obtained for four different spiking levels.

Conclusion

A highly sensitive and robust MRM method was developed for quantitation of impurities 4-chloro 2-nitrobenzene amine, 2-chlorobenzoic acid, 2-(4-chloro 2-nitro phenyl amino) benzoic acid, and 2-(2-amino 4-chloro phenyl amino) benzoic acid in API, Clozapine. The chromatographic method developed provided good separation between the impurity and the API to avoid interference. To avoid contamination of the MS, an integrated diverter valve program was included to divert high concentration API as it elutes. The method showed an LOD of 1 ppb and LOQ of 3 ppb, calculated by the calibration curve method. Calibration curves were made between LOQ (3 ppb) level to 150 ppb for all four impurities, and were found to be linear with $1/x^2$ weighing.

The minimum signal to noise ratios for impurities 1 to 4 at the LOQ level were found to be 39:1, 63:1, 62:1, and 99:1 respectively. R^2 values were above 0.9950. Spike recovery analysis showed the efficiency of sample extraction with recovery percentage between 85 to 116%, at a test concentration of 20 mg/mL. The method developed was found to be highly reproducible at the LOQ level, with %RSD value for the area response of 10 replicate injections not more than 2.5%. The method can be used for quality control of the clozapine API.

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