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Simultaneous Low-level Quantitation of Four Impurities in Clozapine API using Triple Quadrupole LC-MS/MS

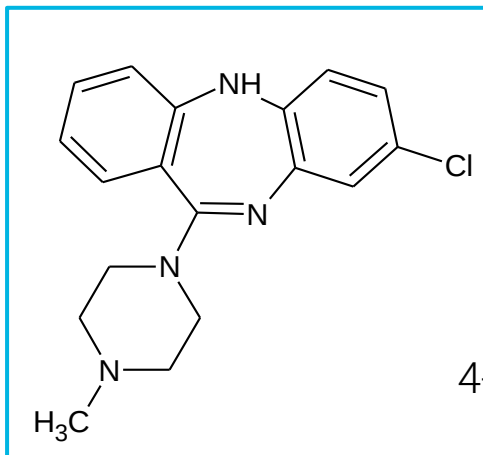
Prasanth Joseph1; Sivakumar S.1; Saikat Banerjee1; Kannan Balakrishnan1

1Agilent Technologies, Bangalore, India

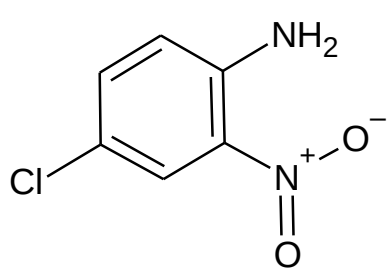
Introduction

Presence of potential toxic chemicals and impurities in drugs are one of the biggest challenges in the manufacturing of an Active Pharmaceutical Ingredient (API). Therefore, it is important to identify these impurities during the manufacturing process to avoid issues related to quality, efficacy, and safety of drugs.

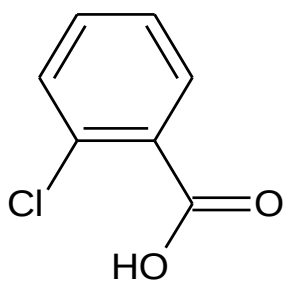
Screening and quantitation of these impurities in API is useful to identify potential problems when evaluating new suppliers, changing manufacturing sites, or during production scaleup. An LC-MS/MS method was developed for the simultaneous quantification of impurities namely, 4-Chloro 2-Nitrobenzene 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.



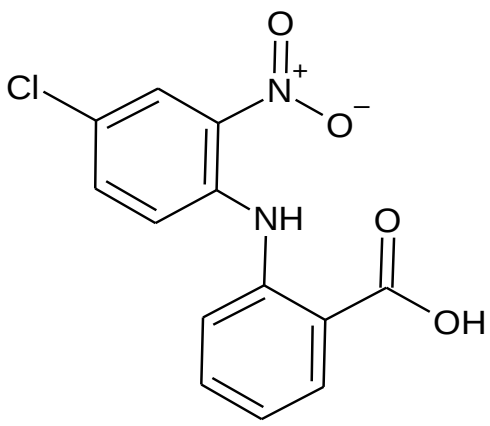
Clozapine



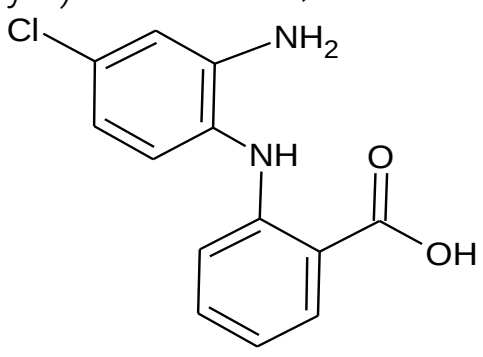
4-Chloro 2-nitro benzene amine (Impurity 2)



2- Chloro benzoic acid (Impurity 1)



2- (4-Chloro-2-nitrophenyl amino) benzoic acid (Impurity 3)



2- (2-amino-4-chloro phenyl amino) benzoic acid (Impurity 4)

Figure 1. Clozapine and 4 impurities.

Experimental

Method details.

Mobile phase A	0.1% Acetic acid in water
Mobile phase B	Acetonitrile
Flow rate	0.5 mL/min
Injection volume	20 µL
Column temperature	50 °C
Sample diluent	Methanol: Water (70:30)
Column	Agilent Poroshell HPH C18 (4.6 x 150 mm, 2.7 µm) P/N693975-702 (T)

10 mg of Clozapine is accurately weighed and extracted with 1 ml of diluent by vortexing the mixture for 2 minutes. It is filtered through a PVDF filter.

A highly selective multiple reaction monitoring (MRM)-based LC/MS/MS method was developed using an Agilent 6470 triple quadrupole LC-MS/MS (G6470B). Four impurities were ionized in electrospray ionization technique operated in negative ionization mode. Well- resolved API, Clozapine is diverted to the waste line with the help of an integrated diverter valve during sample analysis.

Figure 2. 6470 Triple Quadrupole LC/MS System

ID	Precursor m/z	Product m/z	Frag.	CE
Clozapine	327	270.1	147	24
Impurity 1	155	111	71	4
Impurity 2	171	141	70	16
Impurity 3	291	216	92	16
Impurity 4	261	217	45	16

Figure 3. MRM parameters for Clozapine and 4 impurities.

Separation between API and 4 impurities

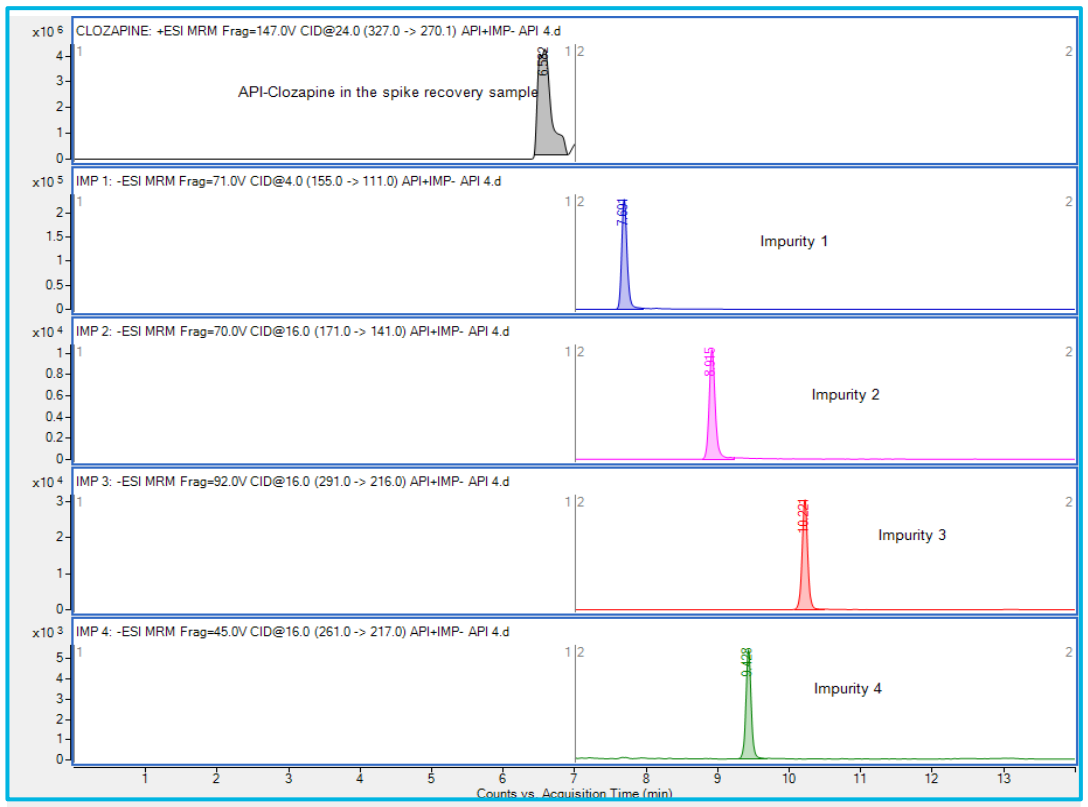


Figure 4. Chromatogram of API and 4 impurities

With the optimized chromatography, API was eluting at 6.58 minutes whereas retention time of impurities from 1-4 were found to be at 7.69 minutes, 8.91 minutes, 10.22 minutes, and 9.43 minutes respectively. The flow of LC effluent from 0 minutes to 7 minutes is diverted to waste to avoid contamination from API injected at a high concentration.

Working standard.	Volume of working std., ml	Volume of diluent, ml	Resultant Conc.	ID
Imp1, 2 (10 ppm) &Imp 3,4 (100 ppb)	0.1	0.9	Imp1, 2 (1000 ppb) &Imp 3,4 (10 ppb)	Calibration level 7
Imp1, 2 (1000 ppb) &Imp 3,4 (10 ppb)	0.5	0.5	Imp1, 2 (500 ppb) &Imp 3,4 (5 ppb)	Calibration level 6
Imp1, 2 (500 ppb) &Imp 3,4 (5 ppb)	0.2	0.8	Imp1, 2 (100 ppb) &Imp 3,4 (1 ppb)	Calibration level 5
Imp1, 2 (100 ppb) &Imp 3,4 (1 ppb)	0.5	0.5	Imp1, 2 (50 ppb) &Imp 3,4 (0.5 ppb)	Calibration level 4
Imp1, 2 (50 ppb) &Imp 3,4 (0.5 ppb)	0.2	0.8	Imp1, 2 (10 ppb) &Imp 3,4 (0.1 ppb)	Calibration level 3
Imp1, 2 (50 ppb) &Imp 3,4 (0.5 ppb)	0.5	0.5	Imp1, 2 (5 ppb) &Imp 3,4 (0.05 ppb)	Calibration level 2
Imp1, 2 (5 ppb) &Imp 3,4 (0.05 ppb)	0.5	0.05	Imp1, 2 (2.5 ppb) &Imp 3,4 (0.025 ppb)	Calibration level 1

Figure 5. Standard mix dilutions for generating the calibration curve.

Working standards from 0.025 ng/ml to 10 ng/ml were prepared for impurity 3 and 4 , working standards from 2.5 ng/ml to 1000 ng/ml were prepared for impurity 1 and dilutions from 5 ng/ml to 1000 ng/ml were prepared for impurity 2 in order to plot the calibration curves.

Calibration curves

The calibration curves were found linear using linear regression and 1/X weighing with regression coefficient above 0.995 for all the four impurities. With respect to the sample concentration of 10 mg/ml, the LOQ values of impurities 1-4 in ppm were 0.25 ppm, 0.5 ppm, 0.0025 ppm and 0.0025 ppm respectively.

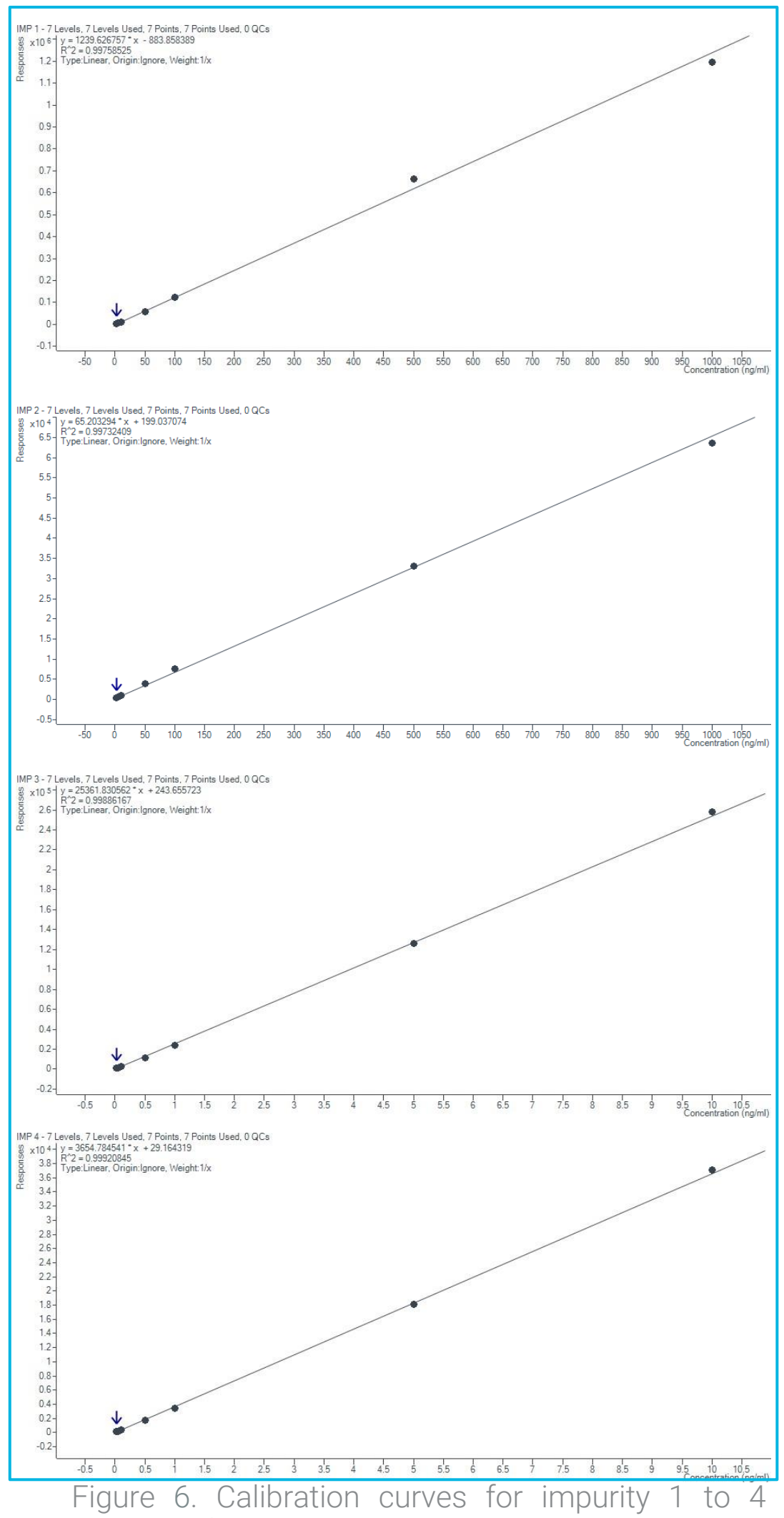


Figure 6. Calibration curves for impurity 1 to 4 respectively

Results and Discussion

Reproducibility in result

Method showed good reproducibility with a % CV of less than 5 % for 6 repeated injections of impurity standard mix at 5 ppb concentration of impurity 1 and 2 and at 0.5 ppb concentration of impurity 3 and 4.

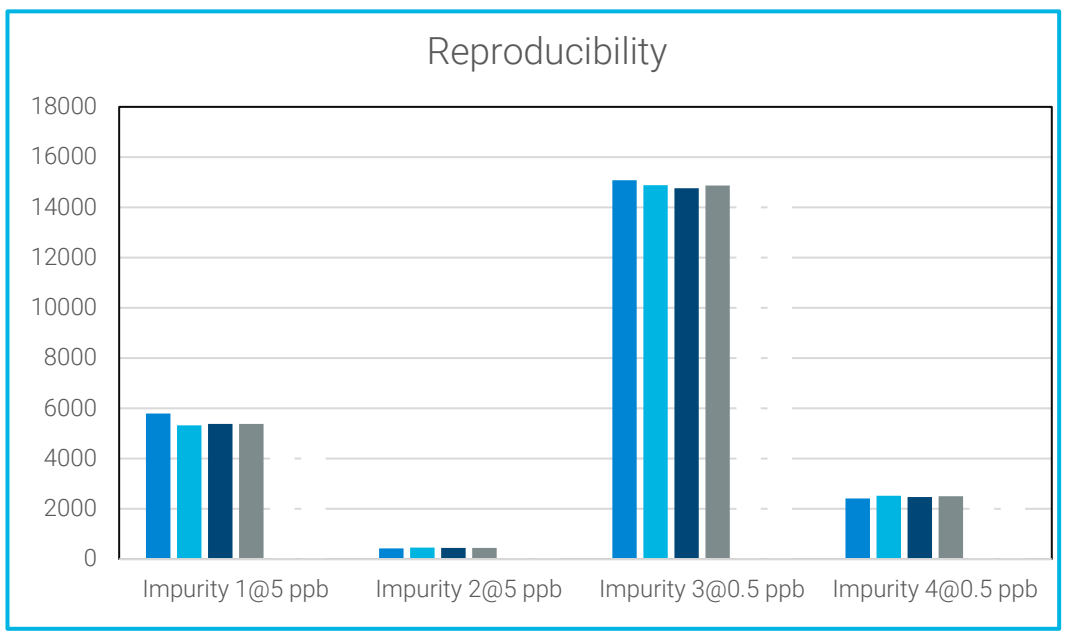


Figure 7. Bar diagram showing the reproducibility of 6 replicate injections

Recovery experiment was conducted by spiking 100 ul of highest concentration in calibration graph (Impurity 1, 2 (1 ppm), Impurity 3, 4 (10 ppb) spiked to 3 batches of 10mg/ml of API. It was observed that API was partially soluble in the diluent used. However, the recovery values of all the four impurities were between 85-117%. Three batches of API, Clozapine were tested for the presence of these impurities.

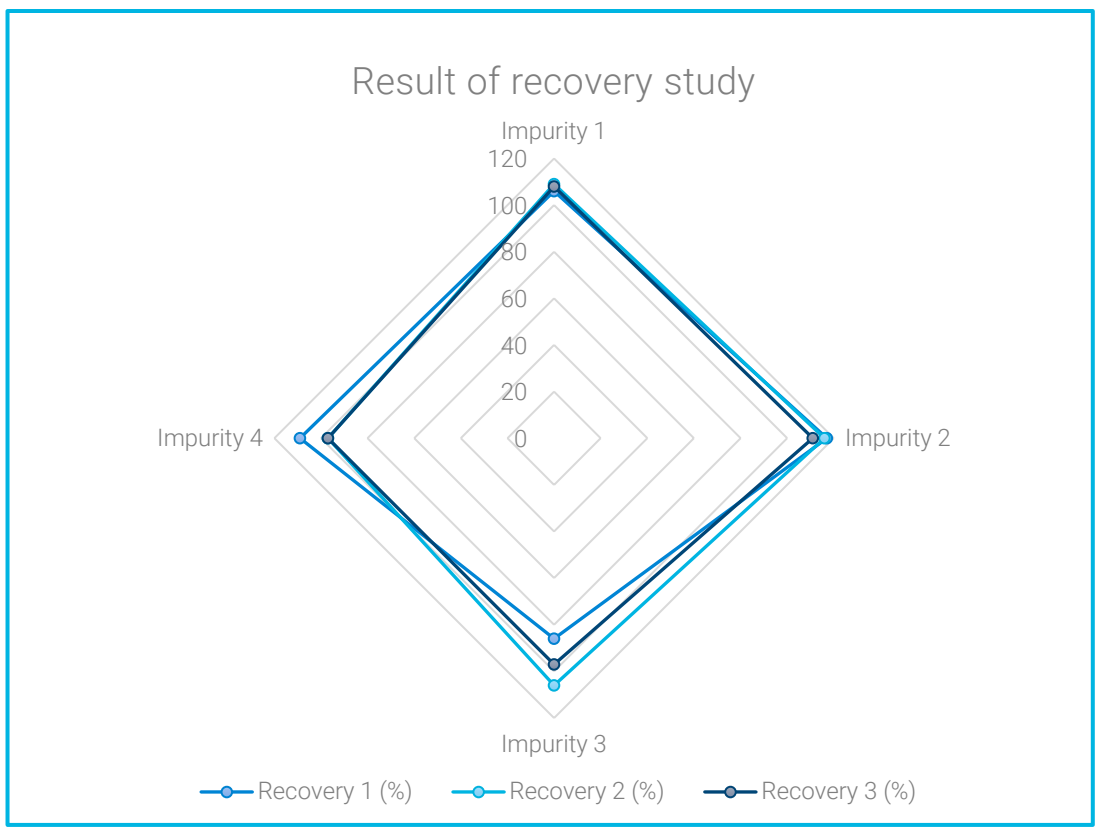


Figure 8. RADAR plot showing the Recovery (%) for impurity 1-4 studied in 3 different batches of API.

Carryover study

Carryover was also evaluated for all the four impurities. Initially carryover was observed for impurity 3 and 4 in a blank after injecting the highest concentration level of standard mix in the calibration curve. However, modifying the diluent composition and utilizing multiwash option to wash needle and needle seat helped in eliminating carryover.

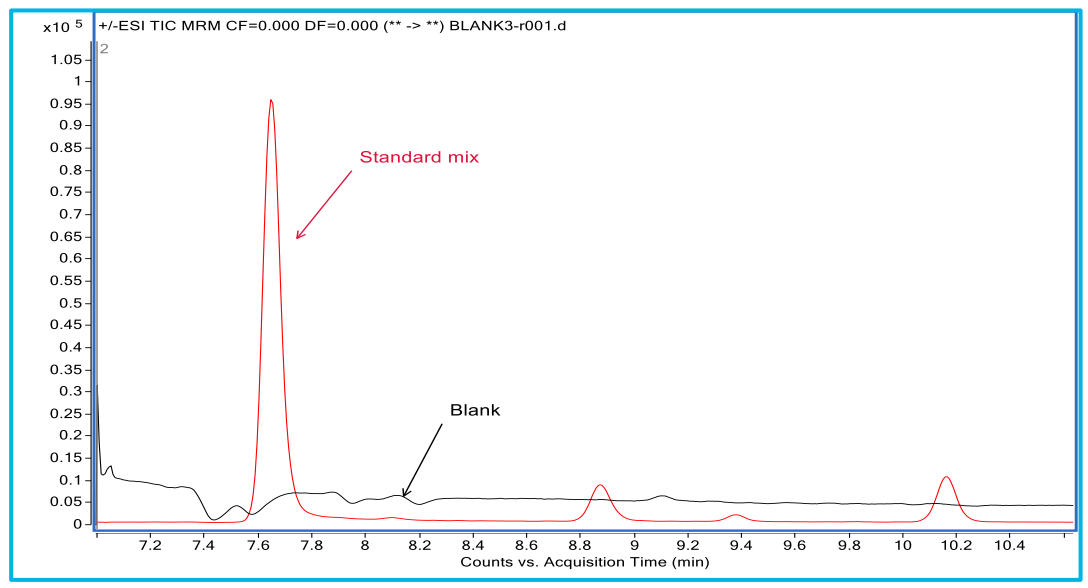


Figure 9. No significant carryover observed from the merged chromatogram of standard mix and blank.

Conclusions

- MRM based LC-MS/MS method is developed for the quantitation of 4 impurities in Clozapine API.
- Chromatographic separation is achieved between API and the 4 impurities.
- Calibration curves were made for all the 4 impurities and response found to be linear within the concentration range.
- Method developed found to be suitable for the routine quality control of API, clozapine.

References

[1]Determination of NDMA Impurity in Ranitidine Using the Agilent 6470 Triple Quadrupole LC/MS., Agilent Application note 5994-1668EN

[2]Determination of Nitrosamine Impurities Using the Ultivo Triple Quadrupole LC/MS, Agilent Application note 5994-1383EN

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