Application Note

Pharmeceuticals

# Highly Sensitive Quantification of Mutagenic NDSRI N-Nitroso Propranolol in Propranolol API and 40 mg Tablets Using LC/MS/MS

## Detection of regulated genotoxic impurities from the drug manufacturing process


**Authors**

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## Abstract

Nitrosamine impurities are mutagenic impurities which come under the category of “cohorts of concern” as per ICH M7 guidelines and need to be controlled for the safe human consumption of medicines. Nitrosamine drug substance related impurities (NDSRI) are a class of nitrosamine impurities that share a structural similarity with APIs and can be generated during the manufacturing or storage period of the drug product. Since 2018, there have been multiple recalls of drug products due to the presence of nitrosamine impurities above the acceptable intake. Since July 2021, there have been multiple recalls specific to nitrosamine drug substance related impurities (NDSRI) in various drug products. One recent example is the recall of Propranolol tablets of various strengths by Pfizer Canada, due to the presence of N-Nitroso Propranolol above the acceptable intake. Consequently, there is a need to develop a highly sensitive LC/MS/MS method for the quantification of N-Nitroso Propranolol in Propranolol API and tablets. This application note describes a highly sensitive LC/MS/MS method with a limit of quantitation of 25 pg/mL for N-Nitroso Propranolol using the Agilent 6470 LC/TQ, and establishes all critical method performance-related parameters in both Propranolol API and tablets.

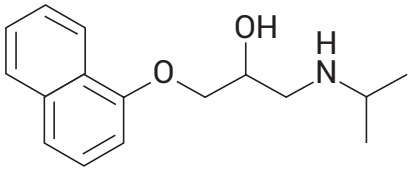
## Introduction

Nitrosamine impurity contamination in medicinal products has been a critical safety concern, falling under the category of "cohort of concern" under ICH M7 guidelines.[1] Several recalls of drug products like sartan, ranitidine, and metformin have occurred since 2018, due to the presence of nitrosamines above the acceptable intake.[2–4] Regulatory agencies like the USFDA and EMEA have published guidelines for the control of nitrosamine impurities in drug products which have the possibility to form nitrosamines.[5,6] Since July 2021, there have been multiple recalls of different categories of drug products due to the presence of nitrosamine drug substance related impurities (NDSRI) above acceptable intake limits.[7] One such example is the NDSRI-based recall by Pfizer Canada for Propranolol tablets at various strengths due to the presence of N-Nitroso Propranolol above such limits.[8]

Due to these complications, there is a need to develop highly sensitive analytical methods for the quantification of N-Nitroso Propranolol in Propranolol API and tablets. LC/MS/MS is an inherently selective and sensitive analytical technique that is well-suited to identify and quantify mutagenic impurities at very low levels, and has been widely adopted in the pharmaceutical industry. Multiple reaction monitoring (MRM) mode is used for quantification, as it 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.

Since there is little information available regarding the limits of N-Nitroso Propranolol, the method was developed with a widely accepted limit of 0.03 ppm in relation to Propranolol sample concentration, and achieved a much lower limit of quantification (LOQ) of 0.005 ppm in relation to the Propranolol sample concentration that corresponds to an absolute concentration of 25 pg/mL of N-Nitroso Propranolol. This study used the Agilent 6470 LC/TQ for the quantification of N-Nitroso Propranolol, and established all critical method performance-related parameters, including limit of detection (LOD), limit of quantification (LOQ), specificity, recovery, reproducibility, and linearity.

**A**

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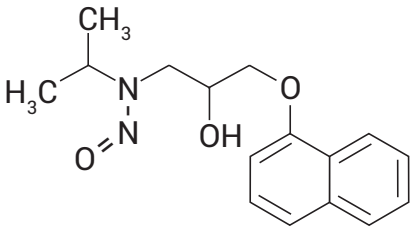


**Figure 1.** Chemical structures of (A) Propranolol and (B) N-Nitroso Propranolol impurity.

## Chemicals and reagents

An N-Nitroso Propranolol impurity standard and Propranolol API were obtained from a pharmaceutical company. Propranolol 40 mg tablets were procured from local pharmacies. Other LC/MS-grade solvents (methanol, water) were purchased from Honeywell (Charlotte, NC, USA). Ammonium acetate and formic acid were purchased from Fluka (now of Honeywell).

## Experimental

Sample preparation for Propranolol API (5 mg/mL):

1. Accurately weigh 25 mg of drug API into a 15 mL centrifuge tube.
2. Add 5 mL of sample diluent.
3. Vortex the solution for one minute, followed by 15 minutes of sonication so that the API is completely soluble.
4. Transfer the solution to HPLC vials and load onto LC/MS/MS for analysis.

Sample preparation for Propranolol 40 mg tablets (API final sample concentration 5.0 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 25 mg API.
3. Transfer to a 15 mL centrifuge tube and add 5 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, transfer into an HPLC sample vial, and load onto the LC/MS/MS for analysis.

## LC configuration and parameters

**Table 1.** UHPLC configuration and settings.

| Parameter | Value |
|---|---|
| Instruments | Agilent 1290 Infinity II high speed pump (G7120A)<br>Agilent 1290 Infinity II multisampler (G7167B)<br>Agilent 1290 Infinity II multicolumn thermostat (G7116B)<br>Agilent 1290 Infinity II variable wavelength detector (G7114B) |
| Needle Wash | methanol/water (80/20) |
| Sample Diluent | 100% methanol |
| Multisampler Temperature | 5 °C |
| Injection Volume | 10 µL |
| Analytical Column | Agilent InfinityLab Pursuit Diphenyl XRS, 3.0 × 150 mm, 2.7 µm (p/n A6021150X030) |
| Column Temperature | 40 °C |
| Mobile Phase A | 5 mM ammonium acetate |
| Mobile Phase B | 0.1% formic acid in methanol |
| Flow Rate | 0.4 mL/min |
| Gradient | Time (min) % A % B Flow (mL/min)<br>0 90 10 0.4<br>2.0 90 10 0.4<br>5.0 50 50 0.4<br>9.0 30 70 0.4<br>13.0 25 75 0.4<br>13.2 10 90 0.4<br>15.2 10 90 0.4<br>15.3 90 10 0.4<br>18.0 90 10 0.4 |
| Stop Time | 18 minutes |
| Wavelengths | 230 nm |

## Triple quadrupole mass spectrometer configuration and parameters

**Table 2.** Mass spectrometer configuration and source settings.

| Parameter | Value |
|---|---|
| Instrument | Agilent 6470 Triple Quadrupole mass spectrometer |
| Ion Source | Agilent Jet Stream Source (AJS) Electrospray Ionization |
| MS/MS Mode | MRM |
| Ion Mode | Postive |
| Gas Temperature | 200 °C |
| Gas Flow | 10 L/min |
| Nebulizer Pressure | 45 psi |
| Sheath Gas Heater | 350 °C |
| Sheath Gas Flow | 12 L/min |
| Capillary Voltage, Positive | 5,500 V |
| Nozzle Voltage | 1,000 V |
| MS1/MS2 Resolution | 0.7/0.7 (unit/unit) |

## MS/MS compound information for analytes

**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 |
|---|---|---|---|---|---|---|---|
| N-Nitroso Propranolol (Quantifier) | 289.1 | 259.2 | 200 | 60 | 2 | 5 | + |
| N-Nitroso Propranolol (Qualifier) | 289.1 | 259.2 | 200 | 60 | 4 | 5 | + |

## Data analysis

The data were acquired and analyzed using Agilent MassHunter software version 10.1 MS/MS. Transitions were obtained and optimized using 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.

## Results and discussion

Method development was performed in positive mode using an Agilent Jet Stream Source for the optimization of mass spectrometry parameters for N-Nitroso Propranolol, 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.

MRM transitions were carefully chosen to avoid the interference of the matrix. Chromatographic separation was achieved between N-Nitroso Propranolol and Propranolol using the Agilent InfinityLab Pursuit XRs Diphenyl column. Specificity was also established with Propranolol N-Formyl Impurity with the same optimized column and method conditions.

Sample preparation was optimized in terms of extraction solvent and time, and achieved the desired recovery for N-Nitroso Propranolol in Propranolol API and tablets.

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

LOQ and LOD limits and S/N values are captured in Table 4. Reproducibility data, including bracketing standards at a limit level of 0.03 ppm and LOQ, are captured in Tables 5 and 6.

The calibration concentrations ranged from 0.025 ng/mL to 10 ng/mL, with specific details mentioned in Table 4.

$R^2$ values are greater than 0.99 for N-Nitroso Propranolol, displaying linear responses throughout the concentration range.

A summary of recovery experiments for N-Nitroso Propranolol at both the limit and LOQ level in Propranolol API and at the 0.12 ppm level in Propranolol 40 mg tablets are captured in Tables 7 and 8.

Figures 2 to 4 display the representative extracted ion MRM chromatograms from the 6470 LC/TQ, showing elution and separation of N-Nitroso Propranolol in standard, LOQ, and spiked formulations. DAD chromatograms were acquired to bracket the retention times of higher concentrations of Propranolol API, which need to be diverted to waste to avoid mass spectrometer contamination. This high concentration of Propranolol API is diverted to waste using the inbuilt diverter valve, by creating a diverter valve program as mentioned in Table 9.

**Table 4.** Representative S/N ratio data for LOQ, LOD, and linearity data for N-Nitroso Propranolol.

| S. No. | Compound | LOD | | LOD (S/N) | LOQ | | LOQ (S/N) | $R^2$ | Linearity Range | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | ng/mL | ppm | | ng/mL | ppm | | | ng/mL | ppm |
| 1 | N-Nitroso Propranolol | 0.0075 | 0.0015 | 34.7 | 0.025 | 0.005 | 107.3 | 0.99504 | 0.025 to 10 | 0.005 to 2 |

*S/N was calculated using the RMS algorithm, noise width (0.5 min), with reference selected as sample using Agilent MassHunter Quantitative software version 10.1.

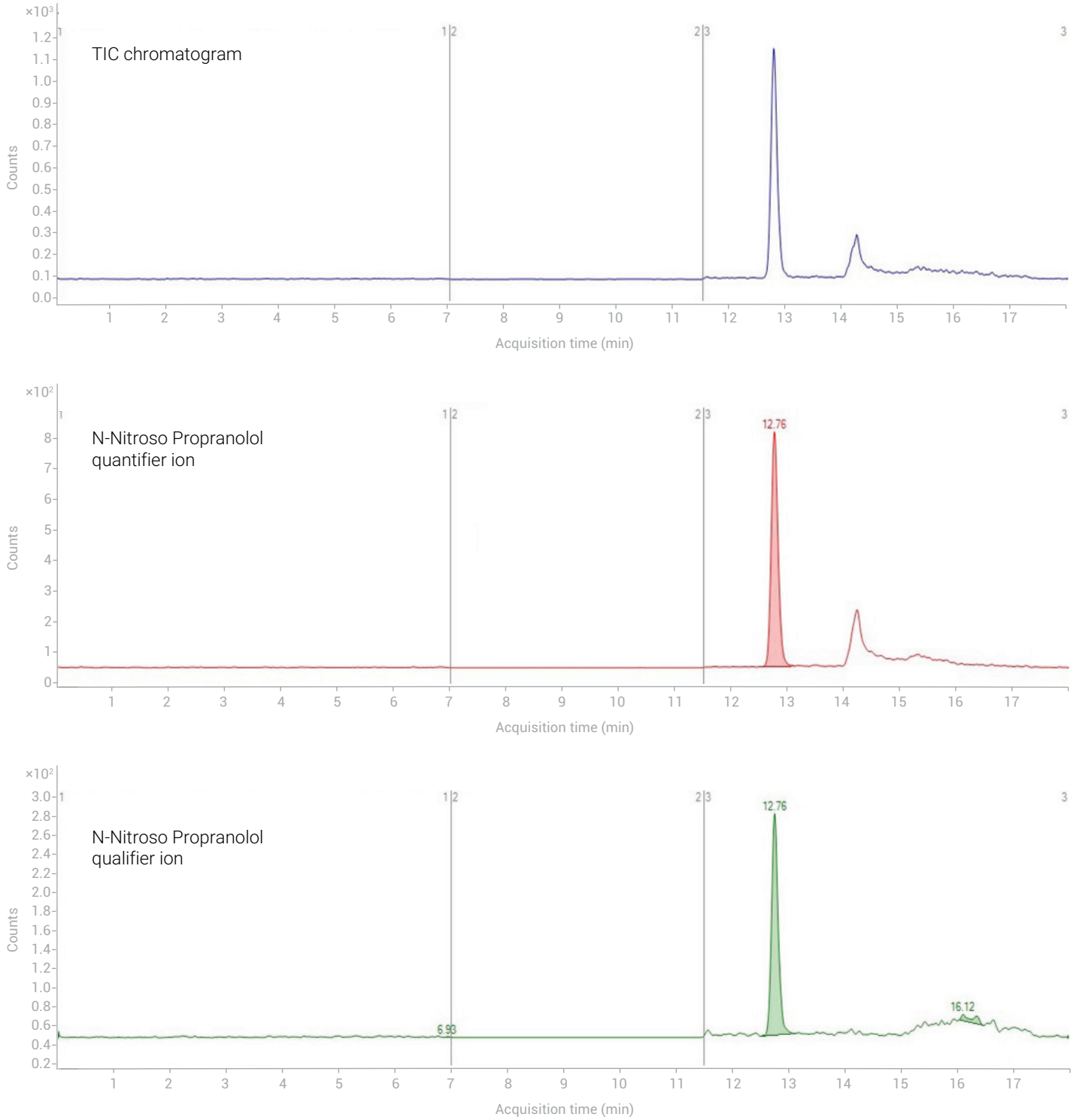


**Figure 2.** Representative standard chromatogram of N-Nitroso Propranolol at 0.15 ng/mL (0.03 ppm in relation to an API concentration of 5.0 mg/mL).

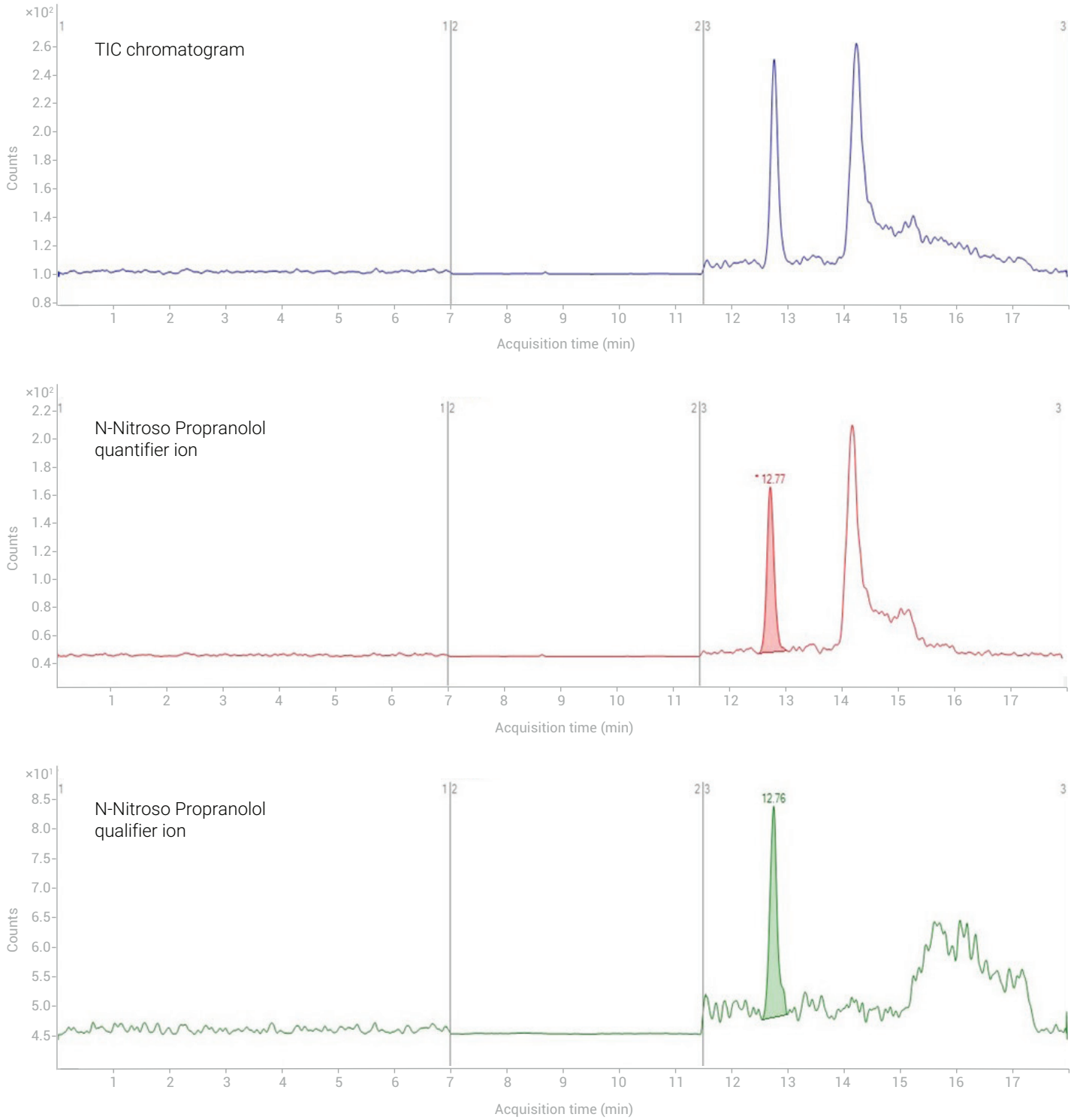


**Figure 3.** Representative LOQ chromatogram of N-Nitroso Propranolol at 0.025 ng/mL (0.005 ppm in relation to an API concentration of 5.0 mg/mL).

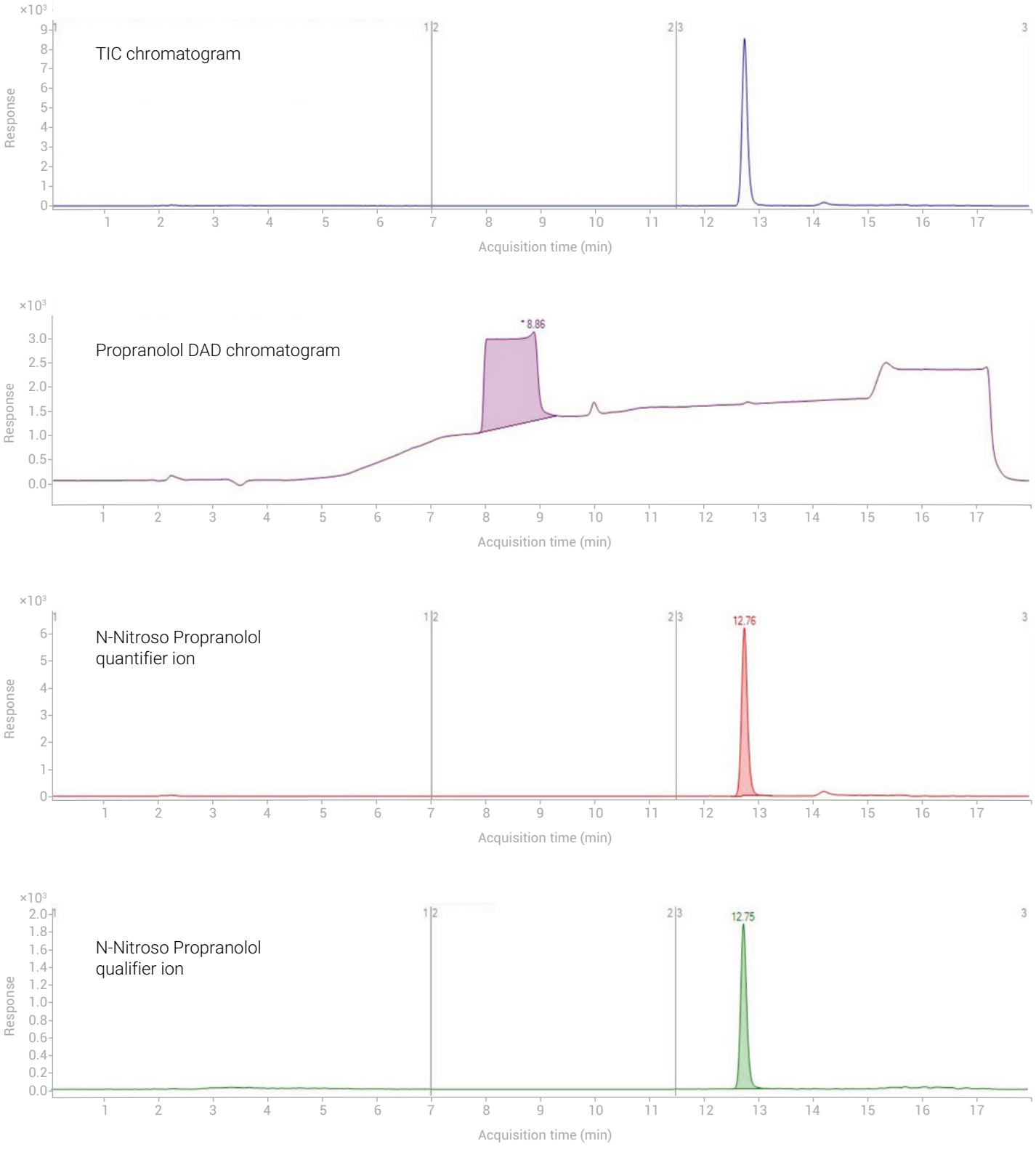


**Figure 4.** Representative chromatograms of standard N-Nitroso Propranolol at 0.6 ng/mL (0.12 ppm in relation to an API concentration of 5.0 mg/mL) spiked into Propranolol 40 mg tablets.

## Accuracy and reproducibility

The calibration curve for N-Nitroso Propranolol demonstrated an accuracy rate within 15% of the expected concentration. Calibration levels were as shown in Table 4, and reproducibility across all levels exhibited CVs less than 15%. Below is the calibration curve generated from the 6470 LC/TQ system.

## Specificity of N-Nitroso Propranolol impurity with respect to Propranolol N-Formyl impurity

### Preparation of Propranolol N-Formyl impurity

1. Accurately weigh 25 mg of Propranolol and transfer into a 15 mL centrifugal tube.
2. Add 5 mL of 2 N formic acid.
3. Place the centrifugal tube in a hot water bath and allow the reaction to be carried out at 70 °C for 90 minutes.
4. Transfer mixture into an HPLC vial for LC/MS analysis.

The data were acquired in full scan mode, and the chromatogram was extracted at 288 *m/z* in positive mode (Propranolol Formyl Impurity molecular weight: 287).

Figure 6. Representative chromatograms showing the separation of Propranolol formyl Impurity (EIC) and N-Nitroso Propranolol at 0.6 ng/mL (0.12 ppm in relation to an API concentration of 5.0 mg/mL) to demonstrate the specificity.

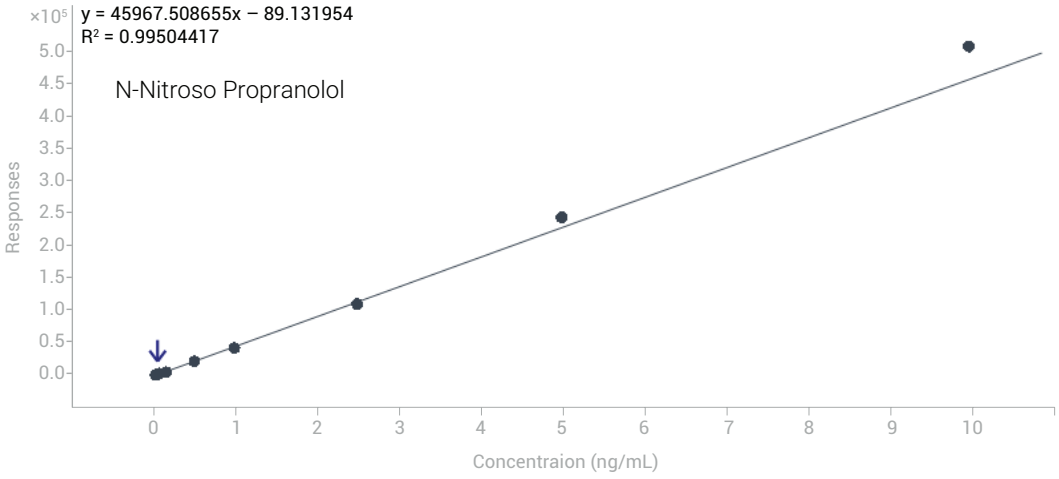


**Figure 5.** Representative calibration curve from the Agilent 6470 LC/TQ for N-Nitroso Propranolol dispersed throughout the chromatogram. The calibration curve used a $1/x^2$ weighting factor.

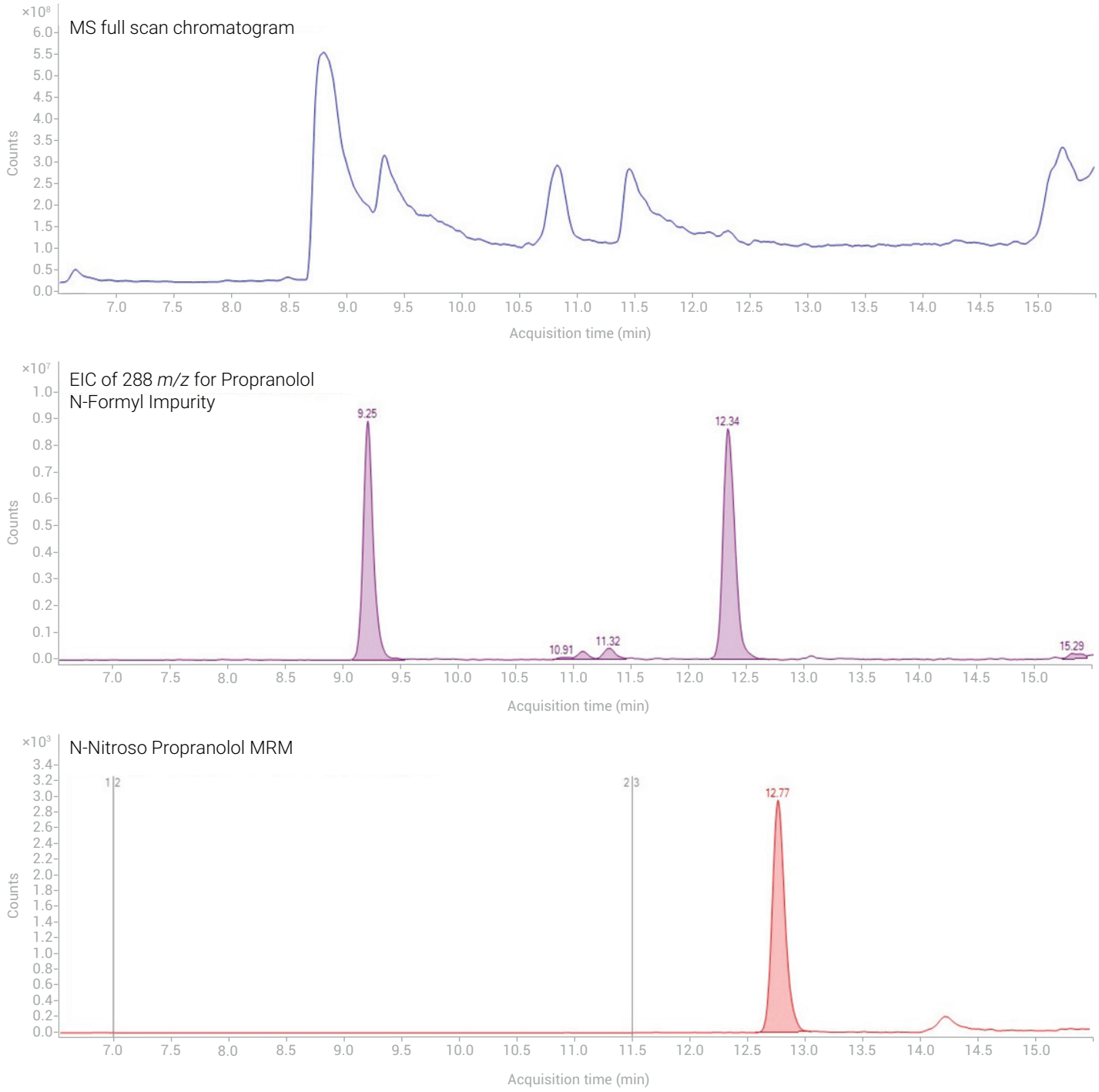


**Figure 6.** Representative chromatograms showing the separation of Propranolol formyl Impurity (EIC) and N-Nitroso Propranolol at 0.6 ng/mL (0.12 ppm in relation to an API concentration of 5.0 mg/mL) to demonstrate the specificity.

**Table 5.** Representative data for reproducibility of the method at 0.15 ng/mL (0.03 ppm in relation to an API concentration of 5 mg/mL), including bracketing standards.

| | S. No. | N-Nitroso Propranolol |
|---|---|---|
| Intial Replicates | 1 | 6,450 |
| | 2 | 6,582 |
| | 3 | 6,424 |
| | 4 | 6,461 |
| | 5 | 6,679 |
| | 6 | 6,139 |
| Bracketing Standard | 7 | 6,492 |
| | **Average** | **6,461.0** |
| | **Standard Deviation** | **167.5** |
| | **%RSD** | **2.6** |

**Table 6.** Representative data for reproducibility of the method at LOQ 0.025 ng/mL (0.005 ppm) in relation to an API concentration of 5.0 mg/mL.

| | S. No. | N-Nitroso Propranolol |
|---|---|---|
| Intial Replicates | 1 | 1,041 |
| | 2 | 1,088 |
| | 3 | 1,063 |
| | 4 | 1,101 |
| | 5 | 1,109 |
| | 6 | 1,155 |
| | **Average** | **1,092.8** |
| | **Standard Deviation** | **39.5** |
| | **%RSD** | **3.6** |

**Table 7.** Summary of the recovery experiment in Propranolol API at 0.15 ng/mL (0.03 ppm in relation to an API concentration of 5.0 mg/mL).

| S. No. | Name of Impurity | Recovery at 0.15 ng/mL (0.03 ppm) in Propranolol API |
|---|---|---|
| 1 | N-Nitroso Propranalol | 100.6% |

**Table 8.** Summary of the recovery experiment in Propranolol API at 0.6 ng/mL (0.12 ppm in relation to an API concentration of 5.0 mg/mL).

| S. No. | Name of Impurity | Recovery at 0.6 ng/mL (0.12 ppm) in Propranolol 40 mg Tablets |
|---|---|---|
| 1 | N-Nitroso Propranalol | 99.4% |

The recovery experiment was performed in duplicate for both Propranolol API (Table 7) and 40 mg tablets (Table 8). In tablets, the recovery experiment performed at a higher concentration due to the presence of N-Nitroso Propranolol already in the samples.

**Table 9.** Diverter Valve program used to divert all sartan APIs to waste.

| Number | Start Time (min) | Scan Type | Diverter Valve |
|---|---|---|---|
| 1 | 0 | MRM | MS |
| 2 | 7 | MRM | Waste |
| 3 | 11.5 | MRM | MS |

## Conclusion

In summary, a highly sensitive and selective method was developed for the quantification of N-Nitroso Propranolol in Propranolol API and tablets, and could establish all critical parameters of method performance using an Agilent 6470 LC/TQ. This application note is intended to demonstrate the reproducibility and sensitivity of the 6470 LC/TQ for Nitroso Propranolol quantification.

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