Poster Reprint

ASMS 2025
Poster number MP 548

Highly Sensitive Quantitation of N-Nitroso Timolol Impurity in Timolol API using the Agilent 6495D LC/TQ

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Introduction

Nitrosamines are of concern as most of them have been reported to be potent mutagens in rodents and are potential carcinogens. Therefore, it is important to control these impurities at or below the stipulated specification limits. API-derived complex nitrosamines are known as nitrosamine drug substance-related impurities (NDSRIs). Multiple recalls of pharmaceutical drug products due to the presence of NDSRIs have drawn the attention of regulators and manufacturers. By considering the complexity of the global pharmaceutical supply chain, manufacturers must be more diligent to protect consumers by screening their APIs and drug products for the presence of NDSRIs. NDSRIs can be formed from both secondary and tertiary amines. Secondary amines can easily undergo nitrosation in the presence of trace amounts of acid. Timolol is beta-blocker class of drug used for hypertension. N-nitroso timolol is an API specific nitrosamine impurity. For most NDSRIs, analytical methods are not yet available, so manufacturers will need to develop, qualify, and validate a method that is suitable for the sample matrix and specific for the nitrosamines in question.

The analytical method developed must be sensitive enough to quantify the nitrosamines of interest down to a level that corresponds to 10% of specification limit.

In this poster we highlight a highly specific, sensitive, and reproducible method that was developed for the quantitation of N-nitroso Timolol impurity in Timolol drug substance using the 6495D triple quadrupole LC/MS (LC/TQ) coupled to the 1290 Infinity II LC.

Challenges in NDSRI analysis and Solutions

Sensitivity: ESI is the preferred ionization mode for NDSRIs. But there are exceptions.
Inclusion of additives i.e. Ammonium acetate, Ammonium formate, ATFA etc. to improve the sensitivity
Optimizing ratio of Organic modifier (combination of acetonitrile and methanol..)

Stability of solution: Impurity response may increase on storage
Hydrolytic and pH dependent degradation
Diluent (basified as per need), maintain Auto sampler temperature

Recovery: Not meeting at lower level (LOQ and/or Specification)
Higher recovery due to stability concern
Interfering of other peaks

Carry Over: Mostly associated with column stationary phase.
shall be minimized by optimizing the LC Gradient

Experimental

LC configurations and parameters:

Chromatography methods conditions, MRM and Source parameters are as describe in figure 1, 2 and 3, respectively.

Parameters	Value		
Instrument	Agilent 6495D LC/TQ coupled to 1290 Infinity II LC		
Needle Wash	S1: MeOH: Water (80/20); S2: Water: MeOH (80/20)		
Sample Diluent	MeOH: ACN (95:05, v/v)		
Multisampler Temperature	10 °C		
Injection Volume	7 µL		
Analytical Column	Agilent Infinity Lab Poroshell Phenyl-Hexyl, 150 × 3.0 mm, 2.7 µm, p/n 693975-312		
Column Temperature	40 °C		
Mobile Phase A	1 mM ammonium trifluoroacetate with 0.05% formic acid in water		
Mobile Phase B	0.1% of Formic acid in MeOH		
Flow Rate	0.4 mL/min		
Run Time	20 min		
Gradient	Time (min)	A (%)	B (%)
	0	55	45
	3	55	45
	6	25	75
	12	25	75
	14	10	90
	18	10	90
	18.1	55	45
	20	55	45

Figure 1. LC configurations and parameters

Precursor Ion (m/z)	Product Ion (m/z)	Dwell (ms)	CAV	CE	Polarity
346.2	128	200	4	10	Positive
346.2	144	200	4	21	Positive
346.2	130	200	4	25	Positive

Figure 2. Compound related parameters

Source/Gas Parameter (AJS-ESI)	Value
Gas Flow	12 L/min
Gas Temperature	250 °C
Sheath Gas Flow	12 L/min
Sheath Gas Temperature	350 °C
Polarity	Positive
Capillary Voltage	3000 V
Nebulizer Pressure	45 psi
Ion funnel mode	Standard

Figure 3. Source parameter optimized for selectivity and sensitivity

Results and Discussion

Linearity, Sensitivity at Limit of detection and Limit of Quantitation. Critical method development parameters like sensitivity, linearity, specificity, reproducibility, and recovery were established. Sensitivity parameters, LOD and LOQ were established, as defined by ICH guidelines, where the S/N values were 3.3 and 10 for the LOD and LOQ, respectively. Calibration curves constructed for N-nitroso timolol were found to be linear within the 5 to 1500 pg/mL (0.5 to 150 ppb with respect to 10 mg/mL API) concentration range. The value of R2 was 0.9990 for the equation y = mx + c, where m is the slope and c are the intercept with $1/x^2$ as weighting factor.

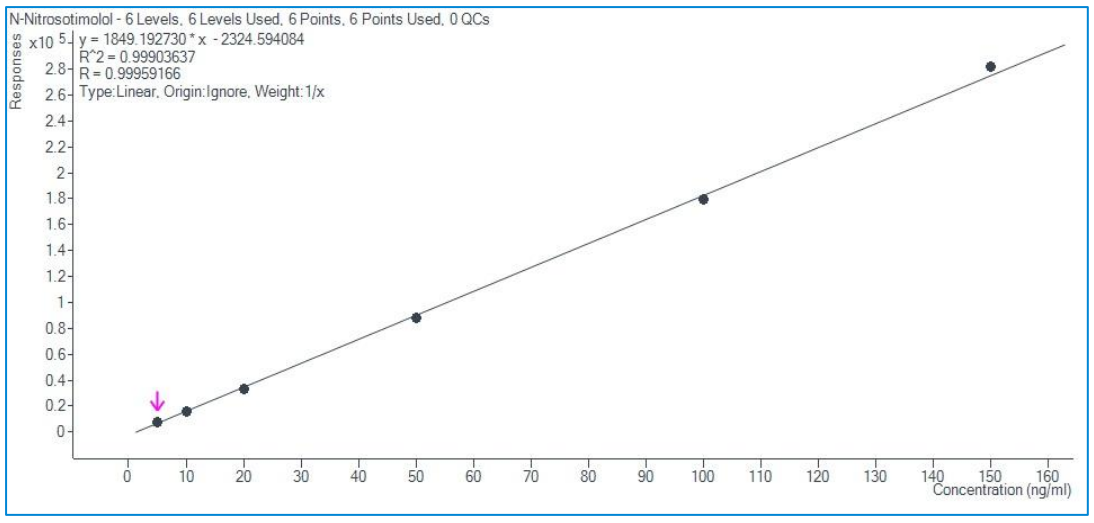

Figure 3. Linearity from 5 pg/mL to 1500 pg/mL

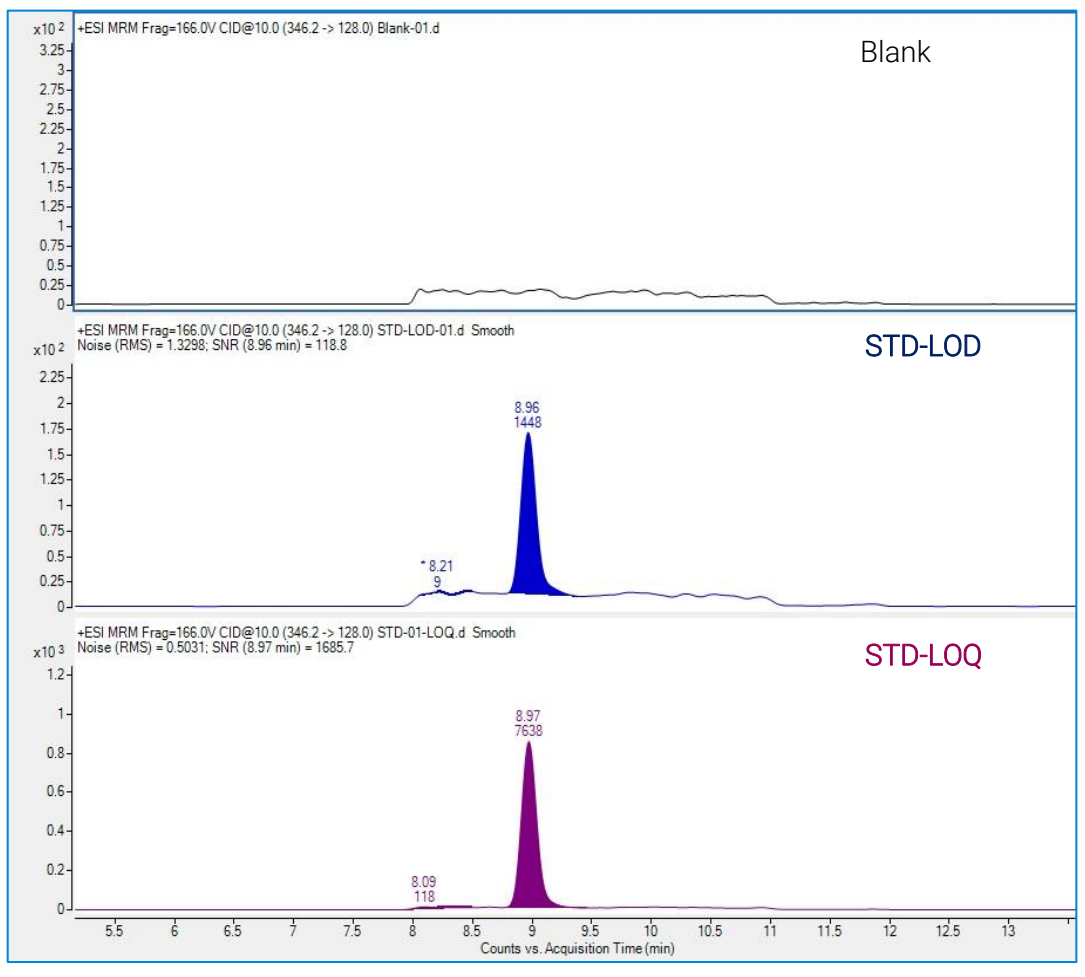

Figure 4. LOD 2 pg/mL (0.2 ppb) and LOQ 5 pg/mL (0.5 ppb) with S/N > 118:1 and S/N > 1680:1 respectively, calculated using RMS.

Method Chromatography separation, Selectivity and Specificity.

The method conditions were optimized to provide sufficient selectivity and sensitivity and achieve better resolution between the impurity and the API. Reversed-phase chromatography was performed using the Agilent Poroshell PhenylHexyl column (3 × 150 mm, 2.7 µm), with a mobile phase consisting of ammonium trifluoroacetate and formic acid in water as aqueous phase, and 0.1% formic acid in MeOH as organic phase.

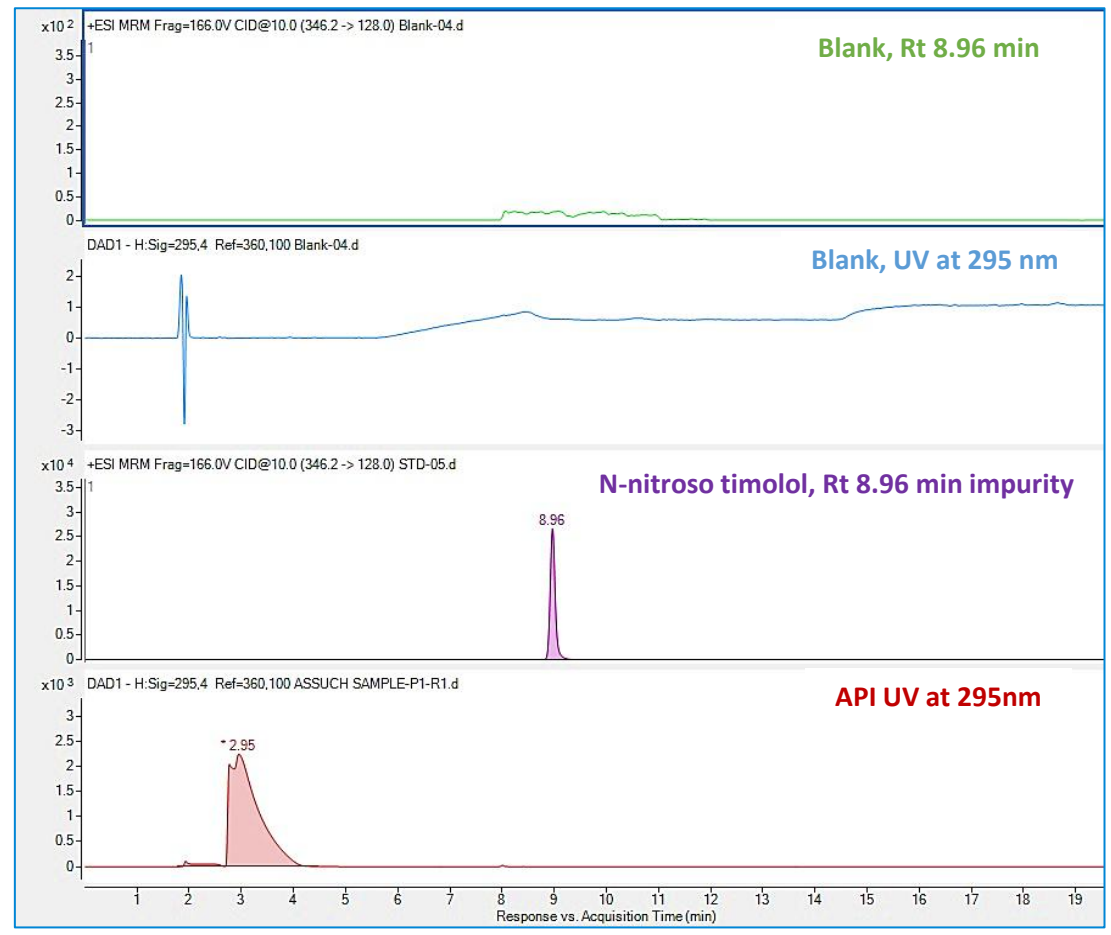

Figure 4. Separation between impurity and API

Specificity in sample matrix is studied and evaluated with the precise selection of Quantifier and Qualifier ion

MRM chromatogram is acquired for standard at 100 pg/mL to select the quantifier ion and qualifier ion to ensure selectivity of the analyte into sample matrix.

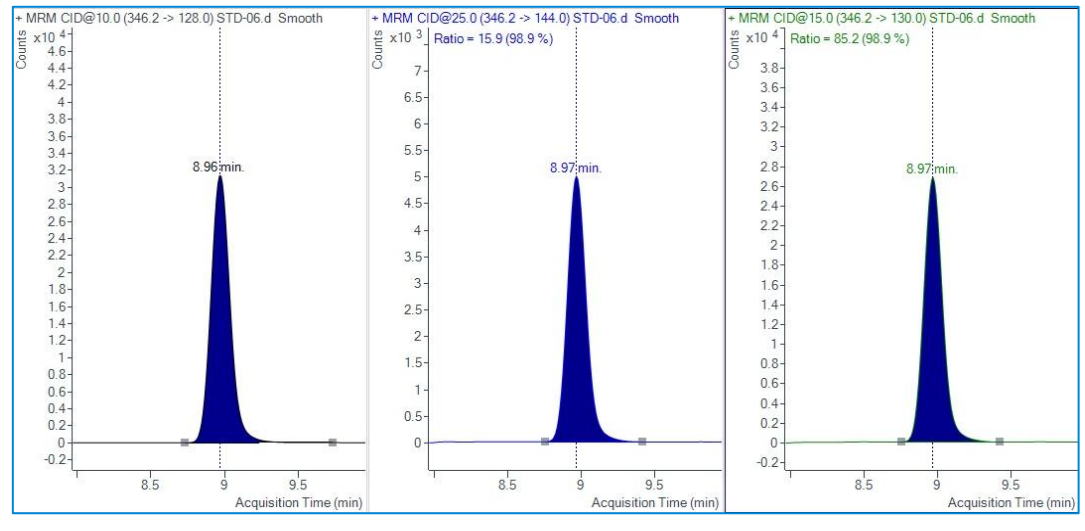

Figure 5. Chromatogram displays Quantifier ion and two Qualifier ion in standard at 100 pg/mL

Results and Discussion

Method Precision and Reproducibility:

Method parameters such as method precision was evaluated at three concentration levels. Including LOD, LOQ concentration. Each level was injected 6 times to calculate the % RSD

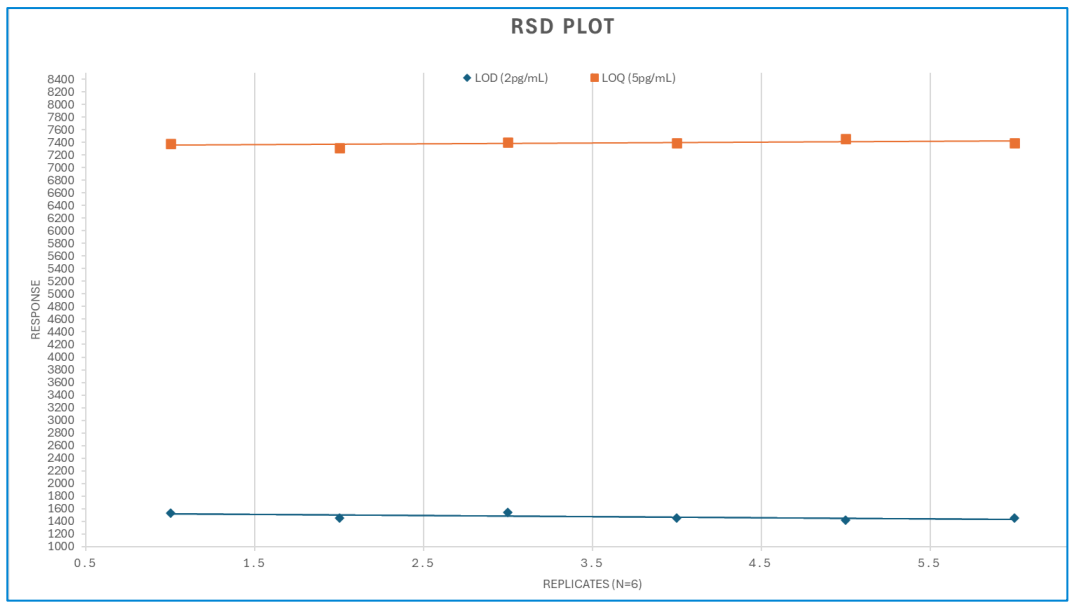

Figure 6. Plot represent the precision at LOD, LOQ at each levels injected (N=6).

Method Recovery and Accuracy:

Recovery is performed for ensuring the accuracy and reliability of analytical results. If the recovery is low, it could lead to underestimation of the analyte concentration, while high recovery may indicate contamination or interference.

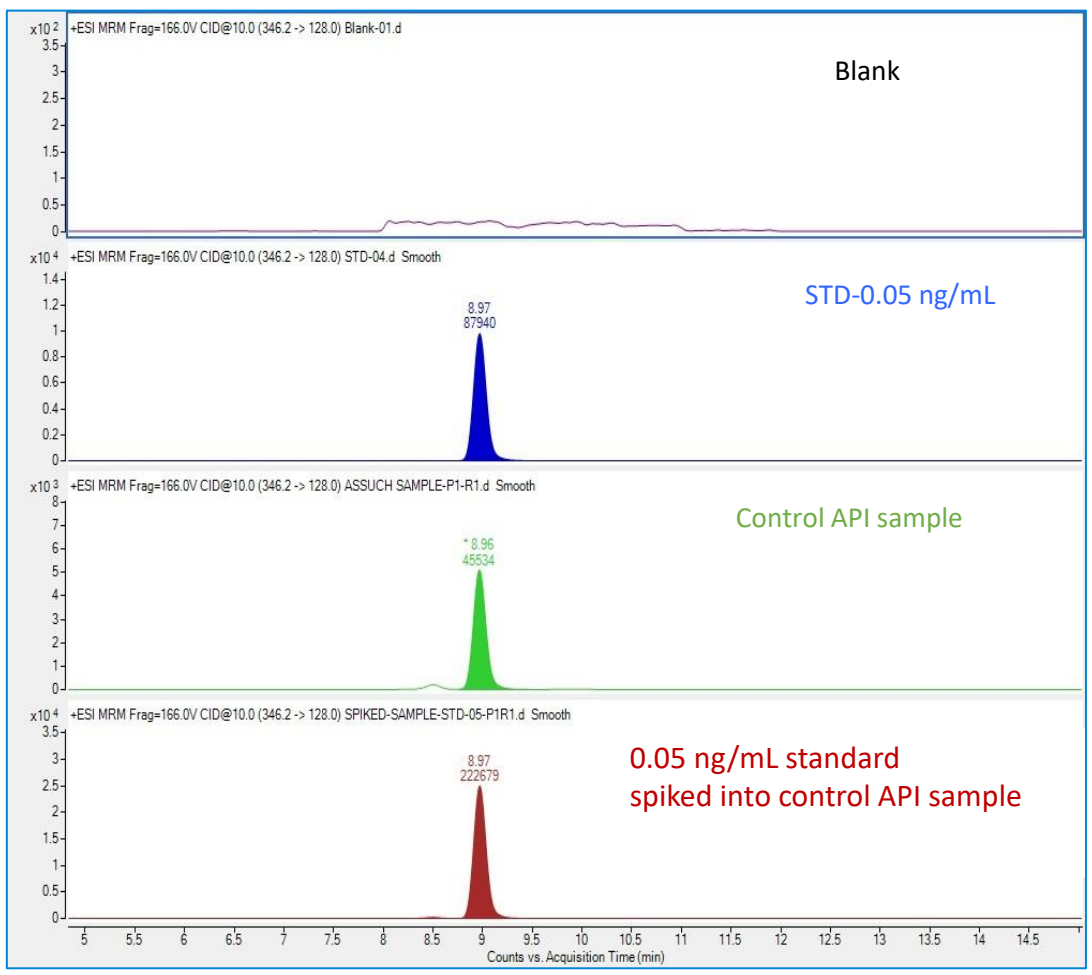

Figure 7. MRM of 346.2>128.0 m/z of standard specification limit (50 pg/mL; 50 ppb), control sample and spiked sample (API load 10mg/mL)

Recovery is typically expressed as a percentage and calculated using the formula:

Recovery (%)=(Amount of analyte recovered/Amount of analyte spiked)×100%

Preparation/Replicates	Response of STD-50 pg/mL	Response of control sample	Response of spiked-sample-STD-50 pg/mL
P1-R1	179280	45502	222679
P1-R2	179452	45413	222497
P1-R3	178988	45237	221951
P2-R1	178095	46732	221802
P2-R2	178967	46556	221784
P2-R3	178630	45759	221823
Average	178902	45866	222089
Recovery (%)= (Average response in spiked sample-Average response in as such sample)/ Average response in standard			**98.5**

Figure 8. Recovery is established in API sample at 50 pg/mL(5 ppb with respect to API load 10 mg/mL) after spiking the know standard concentration 50 pg/mL into the control sample.

Recovery found to be within acceptance window of 90 to 110%

Conclusions

A highly sensitive and robust multiple reaction monitoring (MRM) method was developed utilizing the Agilent 6495D LC/TQ to quantify N-nitroso timolol impurity in timolol API.

- The chromatographic method provided desirable separation between the impurity and the API to avoid interference.
- The developed method demonstrated excellent linearity over the range of 5 to 1500 pg/mL (0.5 to 150 ppb with 100 mg/mL API load) with an R^2 value greater than 0.9990
- The LOD and LOQ values achieved were 2 and 5 pg/mL respectively for targeted NDSRI
- The method provided recovery between 90 and 110%, which is within the acceptance criteria

References

1. Recommended Acceptable Intake Limits for Nitrosamine Drug Substance-Related Impurities (NDSRIs) Guidance for Industry. U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER) August 2023 Pharmacology/Toxicologyhttps://www.fda.gov/drugs/guidance-compliance-regulatory-information/guidances-drugs.
2. 5994-7066E; App note: Low-Level Quantitation of N-Nitroso Dabigatran Etexilate Impurity in Dabigatran Etexilate Mesylate API Using the Agilent 6495C LC/TQ.

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DE-006717

Published in USA, May 15, 2025

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