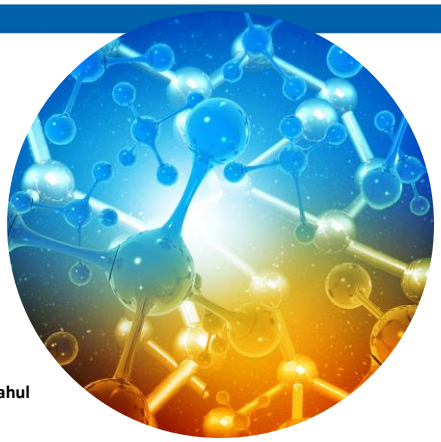

TN-1334

Sensitive and Reproducible Quantification of N-Nitroso Propranolol in a Propranolol Drug Substance and Product

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Introduction

Propranolol, a synthetic amino alcohol, is a competitive nonselective β-adrenoreceptor antagonist extensively used to treat hypertension, angina pectoris, and other cardiac diseases. β-adrenergic blocking drugs such as Propranolol, Atenolol, Metoprolol, Nadolol, Oxprenolol, and Sotalol contain amine groups. As a result, these drugs react with Sodium Nitrite in a Hydrochloric Acid solution to produce N-Nitrosamines.

Per regulatory guidance, Nitrosamine impurity limits are defined and must be controlled in drugs due to their carcinogenicity. Previous studies have revealed that the N-Nitroso Propranolol impurity is approximately 16-fold more carcinogenic than the N-Nitroso Dimethylamine (NDMA) impurity. Therefore, there is an important need to develop assays for the sensitive detection and quantification of the N-Nitroso Propranolol impurity in drug substance and drug products.

This technical note describes the quantification of the N-Nitroso Propranolol impurity in a Propranolol drug substance and product using a Kinetex 2.6 µm Biphenyl HPLC column and a SCIEX® QTRAP® 6500+ system. A lower limit of quantification (LLOQ) of 0.01 ng/mL was achieved for N-Nitroso Propanol with high reproducibility and accuracy (**Figure 1**). In addition, linearity was achieved between 0.01 ng/mL and 10.00 ng/mL, providing a linear dynamic range (LDR) of 3 orders of magnitude.

Sample Preparation

Standard preparation: A 1 mg/mL N-Nitroso Propranolol solution was prepared in Methanol. The stock solution was diluted with Acetonitrile / Water (80:20, v/v) to prepare calibration standards at concentrations ranging from 0.005 to 10 ng/mL that were stored under refrigerated conditions.

Drug substance: Propranolol was weighed and diluted to a final concentration of 1 mg/mL in Acetonitrile / Water (80:20, v/v). The sample was shaken for 20 minutes using a mechanical wrist action shaker. After extraction, the sample was centrifuged for 10 minutes at 4000 rpm. The supernatant was collected and filtered using a 0.22 µm PVDF syringe filter. The filtered solution was transferred into an HPLC vial for analysis.

Drug product: The drug product was crushed and weighed. A 1 mg/mL solution of the drug product with respect to the active pharmaceutical ingredient (API) was prepared using Acetonitrile / Water (80:20, v/v). The solution was vortexed for 2 minutes and shaken for 30 minutes using a mechanical wrist action shaker. After extraction, the sample was centrifuged for 10 minutes at 4000 rpm. The supernatant was collected and filtered using a 0.22 µm PVDF syringe filter. The filtered solution was transferred into an HPLC vial for analysis.

LC Conditions

Column: Kinetex™ 2.6 µm Biphenyl
Dimensions: 150 x 3.0 mm
Part No.: 00F-4622-Y0
Mobile Phase: A: 1 mM Ammonium Formate with 0.1 % Formic Acid in Water
B: 0.1 % Formic Acid in Acetonitrile

Gradient:

Time (min)	%B
0.00	35
3.00	35
5.00	55
6.00	75
8.00	75
8.10	95
10.0	95
10.1	35
14.0	35

Flow Rate: 0.4 mL/min
Injection Volume: 15 µL
Temperature: 40 °C
LC System: SCIEX ExionLC™
Detection: MRM
Detector: SCIEX QTRAP 6500+

MRM Conditions

Polarity: Positive
Source Temperature: 450 °C
GS1: 60 psi
GS2: 60 psi
CUR: 40 psi
CAD: 10
IS: 5500 V

MRM Transitions and Parameters

Analyte	Q1 (m/z)	Q3 (m/z)	Dwell (sec)	DP (V)	EP (V)	CE (V)	CXP (V)
N-Nitroso Propanolol-1	289.1	259.1	150	50	10	8	14
N-Nitroso Propanolol-2	289.1	72.1	150	50	10	15	15

Note: N-Nitroso Propanolol-1 was used as a quantifier transition and N-Nitroso Propanolol-2 was used as a qualifier transition.

Results and Discussion

The Kinetex™ 2.6 µm Biphenyl LC column was selected for separation, as it provided high separation power. Under the optimal LC-MS/MS conditions, the N-Nitroso Propranolol impurity eluted at a retention time of 7.30 minutes. Matrix interferences were not observed in the diluent or control samples, demonstrating the selectivity and specificity of the assay. A limit of detection (LOD) of 0.005 ng/mL and LLOQ of 0.010 ng/mL for the N-Nitroso Propranolol impurity were achieved. The representative chromatograms for the matrix blank and sample at the LOD, LLOQ, and specification limit of the sample are shown in **Figure 2**. The signal-to-noise (S/N) ratios observed at the LOD and LLOQ levels were >10 and >20, respectively.

The upper limit of quantification (ULOQ) was selected based on the highest levels of analytes expected to be observed and is not a reflection of system capability. The calibration curve generated for the concentrations tested is shown in **Figure 3** with an overlay of the linearities for both the quantifier and qualifier transition responses. Linearity was demonstrated over a concentration range of 0.01 ng/mL to 10.00 ng/mL. A correlation coefficient (r^2) of >0.99 was achieved for responses from both transitions.

Accuracy was expressed as percentage deviation from the nominal value at each respective concentration level. The precision of the assay was measured by the percent coefficient of variation (%CV) at each of the concentrations. Accuracy and %CV were determined by analyzing 6 replicates of the samples at the LLOQ and specification limit in a single analytical run. As shown in **Table 1**, the %CV values at the LLOQ and specification limit were 6.74 % and 3.59 %, respectively. Accuracy ranged from 96 % to 115 % at the LLOQ and 107 % to 116 % at the specification limit, averaging 105 % and 110 %, respectively. Overall, these data confirm the accuracy and high reproducibility of the method for quantifying N-Nitroso Propranolol (**Table 1**).

Two MRM transitions were used to provide an additional level of specificity for N-Nitroso Propranolol quantification (**Table 2**). The ion ratios for the N-Nitroso Propranolol spike samples are similar to the ratios observed in the standard solution, indicating that under the optimized LC-MS/MS conditions, the interferences at the retention time of N-Nitroso Propranolol are negligible. Representative chromatograms shown in **Figure 4** highlight the ion ratio lines with a tolerance of ±30 %. Recovery was evaluated in 3 matrices, including the placebo, API, and drug product. Recovery was assessed at 0.01 ng/mL, 0.03 ng/mL, 1 ng/mL, and 5 ng/mL for the placebo and API samples. Since N-Nitroso Propranolol was detected in the drug product at 0.185 ng/mL, the recovery analysis was performed at 1 ng/mL and 5 ng/mL. In the case of the placebo sample, no area response was observed at the retention time of N-Nitroso propranolol. **Table 2** summarizes the recoveries observed for all evaluated matrix samples. Recoveries between 85 % and 111 % were achieved, indicating that the extraction workflow achieved good recovery for N-Nitroso Propranolol in both the drug substance and drug product.

Figure 1. Representative Chromatograms of the Quantifier and Qualifier Transitions for N-Nitroso Propranolol in the Solvent Blank and at the LLOQ.

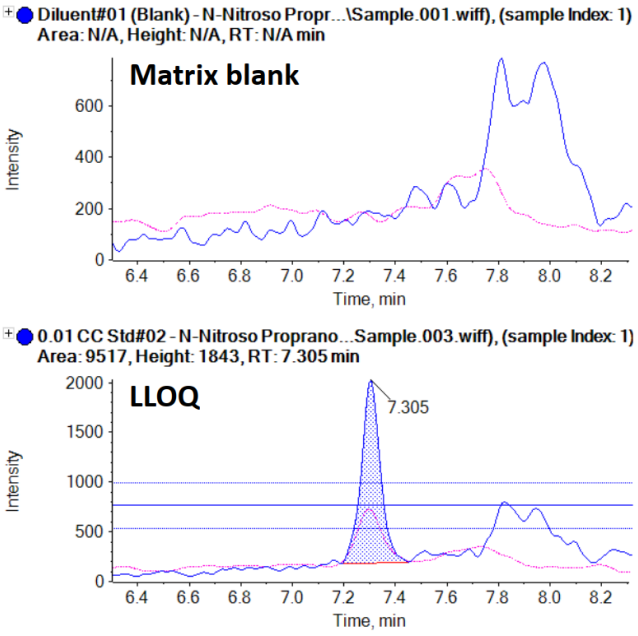


Figure 2. Chromatograms of the Matrix Blank and Sample at the LOD, LLOQ, and Specification Limit.

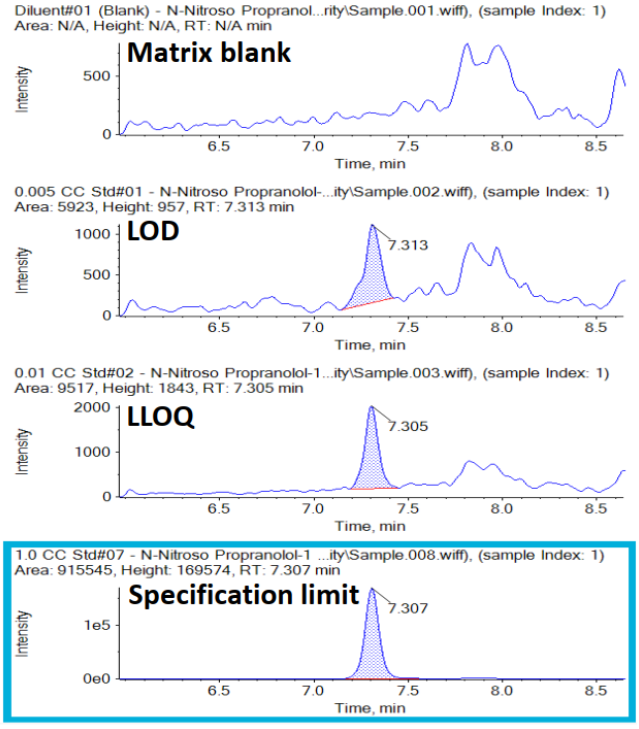


Figure 3. Calibration Curves were Generated using the Quantifier (Blue) and the Qualifier (Pink) Transition Responses.

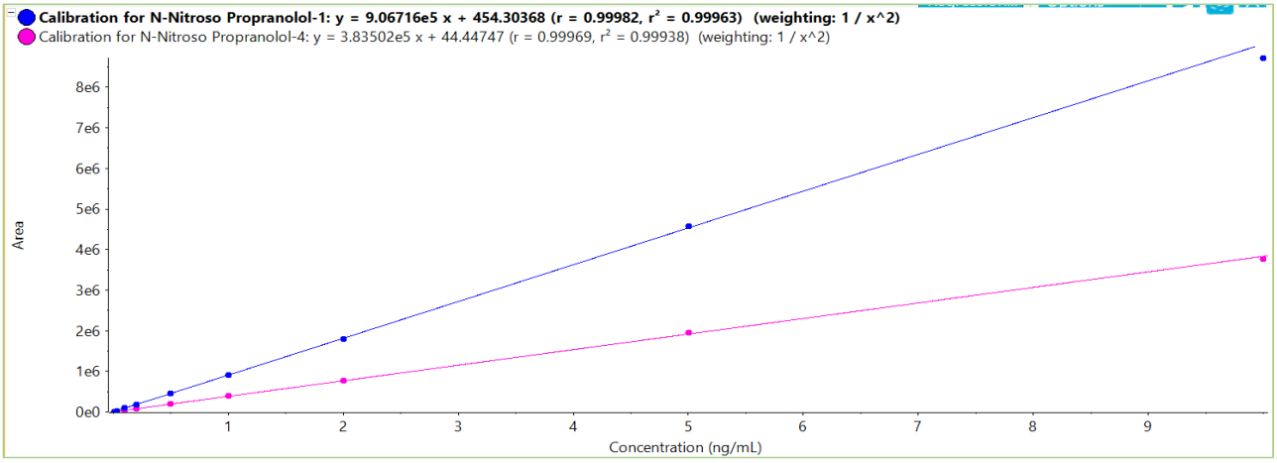


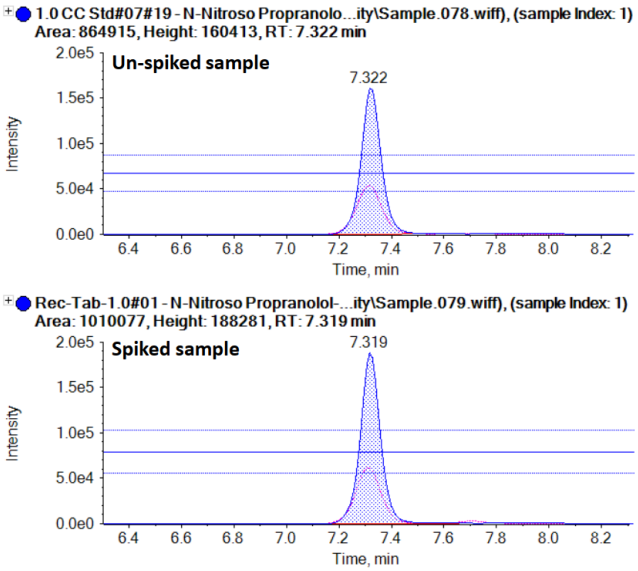
Table 1. Accuracy and %CV at 3 Concentration Levels.

Replicate No.	LLOQ (0.01 ng/mL)	0.03 ng/mL	Specification Limit (1 ng/mL)
1	0.011	0.029	1.156
2	0.010	0.031	1.096
3	0.010	0.030	1.084
4	0.011	0.031	1.070
5	0.011	0.032	1.082
6	0.010	0.031	1.160
Mean	*0.011*	*0.031*	*1.108*
SD (%)	*0.001*	*0.001*	*0.040*
%CV	*6.74*	*3.30*	*3.59*
% Accuracy	*105*	*103*	*111*

Table 2. Recovery Results from the Standard Solutions and Spiked Samples.

Sample Type	Concentration (ng/mL)	Mean Area Response in Control Samples (Mean ± SD)	Mean Area Response in Standard Solutions (Mean ± SD)	Mean Area Response in Spiked Samples (Mean ± SD)	Recovery (%)
Placebo	0.01	Not Detected	9979 ± 642	10622 ± 1155	106
	0.03		28446 ± 924	29886 ± 886	105
	1		1005374 ± 36034	934359 ± 6170	92.9
	5		4878501 ± 221584	4858043 ± 37729	99.6
API	0.01	11248 ± 698.7	9979 ± 642	19525 ± 863	91.9
	0.03		28446 ± 924	36689 ± 420	92.4
	1		1005374 ± 36034	945876 ± 5629	93.0
	5		4878501 ± 221584	4651926 ± 28735	95.1
Drug Product	1	168938 ± 8961.1	1005374 ± 36034	1008797 ± 8748	85.9
	5		4878501 ± 221584	4669906 ± 45067	92.5

Figure 4. Chromatograms of N-Nitroso Propranolol for Standard Solutions and Spiked Samples.



Conclusions

Low-level quantification of the N-Nitroso Propranolol impurity in Propranolol drug product was achieved using a Kinetex™ 2.6 µm Biphenyl HPLC column in conjunction with the SCIEX® QTRAP® 6500+ system. Accurate and highly reproducible quantification of N-Nitroso Propranolol was demonstrated. Linearity was achieved between 0.01 to 10 ng/mL, generating an LDR of 3 orders of magnitude. The method demonstrated a simple sample preparation workflow with high recovery. The recovery was evaluated in placebo, API, and drug product matrices.

Kinetex Ordering Information

2.6 µm Midbore™ Columns (mm)						SecurityGuard™ ULTRA Cartridges (mm)‡
Phases	**30 x 3.0**	**50 x 3.0**	**75 x 3.0**	**100 x 3.0**	**150 x 3.0**	**3/pk**
EVO C18	00A-4725-Y0	00B-4725-Y0	—	00D-4725-Y0	00F-4725-Y0	AJ0-9297
PS C18	00A-4780-Y0	00B-4780-Y0	—	00D-4780-Y0	00F-4780-Y0	AJ0-8950
Polar C18	—	00B-4759-Y0	—	00D-4759-Y0	00F-4759-Y0	AJ0-9531
Biphenyl	—	00B-4622-Y0	—	00D-4622-Y0	00F-4622-Y0	AJ0-9208
XB-C18	00A-4496-Y0	00B-4496-Y0	00C-4496-Y0	00D-4496-Y0	00F-4496-Y0	AJ0-8775
C18	00A-4462-Y0	00B-4462-Y0	00C-4462-Y0	00D-4462-Y0	00F-4462-Y0	AJ0-8775
C8	00A-4497-Y0	00B-4497-Y0	00C-4497-Y0	00D-4497-Y0	00F-4497-Y0	AJ0-8777
HILIC	00A-4461-Y0	—	—	00D-4461-Y0	00F-4461-Y0	AJ0-8779
Phenyl-Hexyl	—	00B-4495-Y0	—	00D-4495-Y0	00F-4495-Y0	AJ0-8781
F5	—	00B-4723-Y0	—	00D-4723-Y0	00F-4723-Y0	AJ0-9321

for 3.0 mm ID

‡SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-9000

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