

Analytical Scale Isolation and Purification of Propranolol Impurities Including Nitrosamine Drug Substance Related Impurity (NDSRI)

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Abstract

Nitrosamine drug substance-related impurities (NDSRIs) are a class of potentially carcinogenic compounds that can compromise drug safety. These impurities can form during manufacturing or storage and may lack carcinogenicity data, structural information, and reference standards. To enable structural characterization and identification, NDSRIs can be isolated using liquid chromatography (LC)-based purification with fraction collection. The work in this application note details an analytical scale isolation and purification workflow of an N-nitroso-propranolol, a nitrosated derivative of propranolol drug. Pure fractions of N-nitroso-propranolol and other low-level impurities were collected using a Waters™ Fraction Manager - Analytical (WFM-A) configured with an Arc™ Premier System, 2998 Photodiode Array (PDA) Detector, and an ACQUITY™ QDa™ II Mass Detector. The specificity of the mass-triggered fraction collection enabled efficient isolation of target impurities. Focused gradient elution enhanced resolution of closely eluting components, while time-based collection mode ensured precise

targeting of known retention windows. The MassLynx™ with the FractionLynx™ Application Manager Software facilitated the collection process.

Benefits

- An analytical scale purification of trace-level impurities using Waters Fraction Manager - Analytical (WFM-A) configured with an Arc Premier System, 2998 PDA Detector, and ACQUITY QDa II Mass Detector
- Detection and isolation of low-levels impurities using a low dispersion system configuration with sub-5 µm particle size analytical columns designed to deliver narrow peaks, fast chromatography with enhanced resolution
- Collection of pure fractions to achieve high-purity materials by optimizing chromatographic separation of coeluting peaks using a shallow focused gradient without increasing the total run time

Introduction

NDSRIs are a class of nitrosamines that share structural similarity to the active pharmaceutical ingredients (APIs) of drug products.¹⁻² These impurities can form through chemical reactions between the APIs and the nitrosating agents, such as residual nitrites, present in excipients used to formulate drug products during manufacturing and storage.¹⁻² NDSRIs pose a carcinogenic risk to drug safety and human health.

Propranolol hydrochloride, a widely used beta-blocker for the treatment of hypertension, angina, arrhythmias, and other cardiovascular conditions.³ Under acidic conditions, propranolol can react with nitrite, a common nitrosating agent, to form N-nitroso-propranolol, a nitrosated derivative structurally related to the parent API. Like other NDSRIs, N-nitroso-propranolol is considered a potential human carcinogen, raising concerns about its presence as a low-level impurity in drug formulations.

When new impurities and degradation products are observed during synthesis, manufacturing, stability studies, and storage of drug substances and drug products, they should be correctly characterized and identified.⁴⁻⁶ To accurately quantify new impurities, the use of appropriate reference standards is essential. When structural information is available, these standards can often be synthesized. However,

if synthesis is not feasible, an alternative approach involves isolating and purifying impurities directly from the drug product or formulation, allowing them to be used as reference materials for further analysis.⁷

Work in this application note illustrates a workflow for isolation and purification of N-nitroso-propranolol and other low-level impurities of the propranolol API through fraction collection using the WFM-A configured with an Arc Premier System. A focused gradient was applied to optimize the chromatographic separation between an impurity co-eluting with the propranolol API peak, allowing for isolation of pure fractions. Purity of the collected fractions was verified through analysis using an orthogonal column chemistry. The workflows and tools presented here provide analytical scientists with a reliable approach for isolating and purifying trace-level impurities in pharmaceutical drug substances and drug products at the analytical scale.

Experimental

Materials

Mass spectrometry (optima LC/MS) grade solvents and formic acids were obtained from Fisher Scientific. N-nitroso-propranolol purchased from Toronto Research Chemicals (Toronto, Ontario). Propranolol hydrochloride was purchased from Sigma Aldrich.

Sample Description

Propranolol stock solution prepared in methanol at 5 mg/mL was diluted with 20:80 acetonitrile/water diluent to the working concentration of 1 mg/mL. N-nitroso-propranolol stock was prepared at 10 mg/mL in 10:90 DMSO/Acetonitrile and spiked to propranolol working solution at 10 µg/mL level.

Method for Fraction Collection

LC system:

Arc Premier System with Binary Solvent
Manager (BSM-R) and Flow-Through Needle
(FTN-R)

2998 PDA and ACQUITY QDa II Mass Detector

	Waters Fraction Manager-Analytical (WFM-A)
Vials:	LCMS Maximum Recovery 2 mL (p/n: 600000749CV)
Column:	XSelect™ Premier™ CSH C ₁₈ Column, 4.6 mm internal diameter (ID) x 100 mm, 2.5 μm (p/n: 186009873)
Column temperature:	30 °C
Sample temperature:	15 °C
Injection volume:	45 μL
Mobile phase:	A: Water with 0.1% formic acid B: Methanol with 0.1% formic acid
Wash solvents:	Purge/Sample Wash: water/acetonitrile 80:20 Seal Wash: water/acetonitrile 80:20
UV detection:	210–450 nm, derived at 230 nm, sampling rate: 10 pts/sec

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	1.00	90.0	10.0	6
10.00	1.00	10.0	90.0	6
10.50	1.00	10.0	90.0	6
10.60	1.00	90.0	10.0	6
13.50	1.00	90.0	10.0	6

MS Conditions

MS system: ACQUITY QDa II Mass Detector

Ionization mode: ESI+

Acquisition range: 85-350 *m/z*

Capillary voltage: 0.8 kV (+)

Cone voltage: 10 V

Probe temperature 450 °C

Data: Centroid

Isocratic Solvent Manager (ISM) Conditions

Makeup solvent: 50:50 water/acetonitrile with 0.1% formic acid

Flow rate: 0.5 mL/min, with 5:1 split and dilute ratio

Data Management

Data acquisition and control software: MassLynx™ version 4.2 SCN1049

MassLynx application manager: FractionLynx™

Method for Analysis of Collected Fractions

LC system: Arc Premier System with Quaternary Solvent Manager (QSM-R) and Flow-Through Needle (FTN-R)
2998 PDA Detector and ACQUITY QDa II Mass Detector

Column: XSelect Premier HSS T3 Column, 4.6 mm ID x 100 mm, 2.5 µm (p/n: 186009851)

Column temperature: 30 °C

Mobile phase: A: Water with 0.1% formic acid
B: Methanol with 0.1% formic acid

Flow rate: 1.2 mL/min

Separation: Gradient elution

UV detection: 210–450 nm, derived at 230 nm, sampling rate: 10 pts/sec

MS detection: Same as in method for fraction collection

Chromatography data software (CDS): Empower™ Chromatography Data System (CDS)

Results and Discussion

In a purification workflow, the goal is to achieve high purity materials through efficient fraction collection. This starts with the development of a robust chromatographic method capable of separating the target compounds from other sample components. The purity of the collected fractions is generally verified using an orthogonal column chemistry to reduce the risk of missing co-eluting peaks.

For the analysis of propranolol and related impurities, chromatographic separation was carried out using a 100 mm XSelect Premier CSH C₁₈ Column with 4.6 mm ID (Figure 1). The analysis was run on an Arc Premier System connected to a WFM-A Fraction Collector and two detectors: a PDA Detector and an ACQUITY QDa II Mass Detector. Both the PDA and mass data were acquired simultaneously for each injection. With the WFM-A in injection mode, the system was instructed to make sample injections only, without collecting fractions. This data acquisition facilitated an assessment of the chromatographic resolution and accurate identification of the target sample components. Subsequently, with the fraction collection enabled, the WFM-A was used to isolate N-nitroso-propranolol and other low-level impurities of propranolol. The mass spectral data facilitated identification of the unknown components and spectral peak purity verification of the collected fractions.

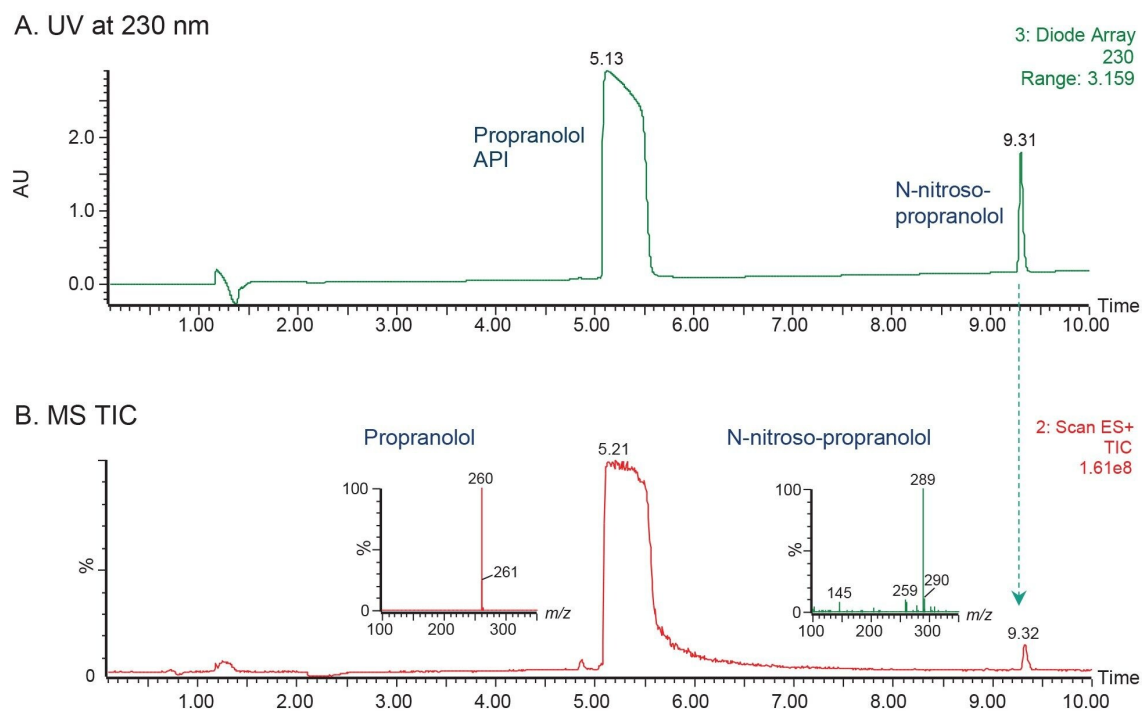


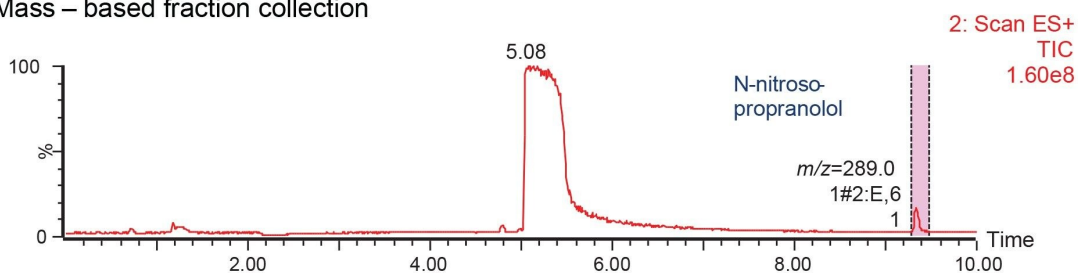
Figure 1. Chromatographic separation of propranolol and N-nitroso-propranolol. UV at 230 nm (A) and MS TIC with mass spectral data (B).

N-Nitroso-Propranolol: Isolation and Fraction Analysis

The chromatographic data acquired using the WFM-A System showed excellent resolution between propranolol API and N-nitroso-propranolol peaks (Figure 1). The detected mass for N-nitroso-propranolol impurity was found to be $[M+H]^+$ with an m/z of 289. The MS-triggered and time-based fraction collection were applied to isolate the impurity peak (Figure 2).

Purity verification of the fractions collected via MS-trigger was performed on an Arc Premier System controlled by the Empower Software (Figure 3). Both the chromatographic and spectral purity of the N-nitroso-propranolol were evaluated. A chromatographic purity of 96.1% was observed (Figure 3A). Additionally, the spectral analysis of the mass and UV data confirmed that the N-nitroso-propranolol peak is spectrally homogeneous, showing no evidence of coeluting impurities.

A. Mass – based fraction collection



B. Timed – based fraction collection

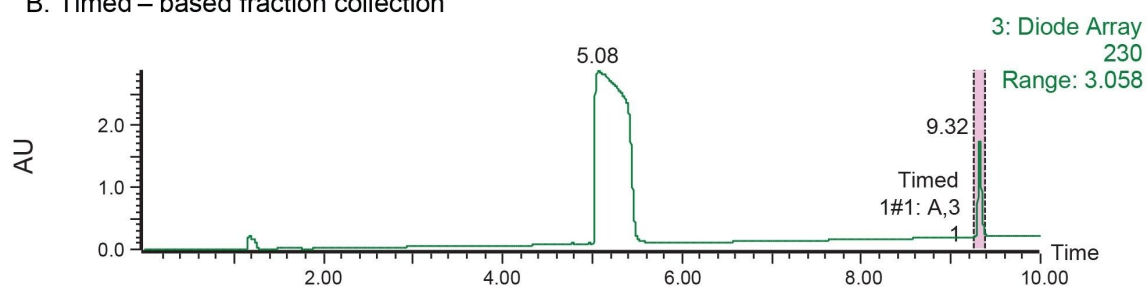


Figure 2. Fraction collection of N-nitroso-propranolol impurity using MS-based trigger (A) and time-based trigger (B).

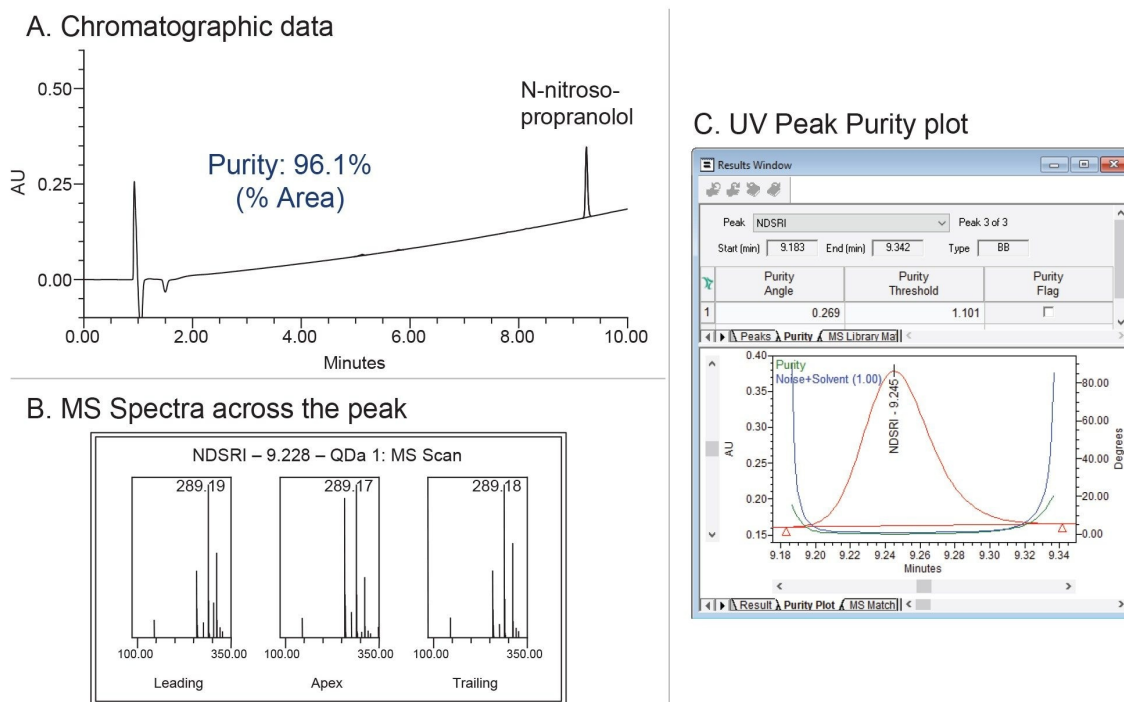


Figure 3. Analysis of the collected fraction containing the N-nitroso-propranolol impurity using an orthogonal column chemistry. Chromatographic data (A), mass spectral data across the N-nitroso-propranolol peak (B), and UV peak purity plot (C) confirming spectral homogeneity.

Focused Gradient: Isolation of Low-Level Impurities

Focused gradients can be used to decrease the gradient slope for a targeted portion of the chromatographic region, in order to increase the resolution for closely eluting peaks without increasing the total run time.⁸⁻⁹ A focused gradient calculator assists in determining the focus gradient conditions for preparative analysis.¹⁰ These gradients are created based on the relationship between the percent organic composition and analyte retention times, while accounting for system dwell volume.

In this study of propranolol impurities, the chromatographic data showed presence of additional low-level impurities eluting before the propranolol API peak (Figure 4). While the method successfully separated the first impurity, the second impurity coeluted with the API. A focused gradient was applied to optimize the resolution between these two peaks. Using a calculator, the focused gradient conditions were determined to separate the impurity coeluting with the API (Figure 5). The method conditions for

the start gradient were entered into the calculator, including column dimensions, focusing limits, flow rate, start and end of the separation. The retention of the target compound specified in the calculator was used to calculate the mobile phase composition in a focused gradient in order to improve the separation. When the shallow slope ended, a high percentage of organic was applied as a column washing step.

Performing the analysis using a focused gradient with a shallow slope provided a base-line separation of the impurity from the API peak (Figure 6). The first impurity peak also eluted under the focused gradient conditions. Optimizing the resolution for the target sample components by applying a focused gradient enabled effective collection of the low-level impurities (Figure 6B).

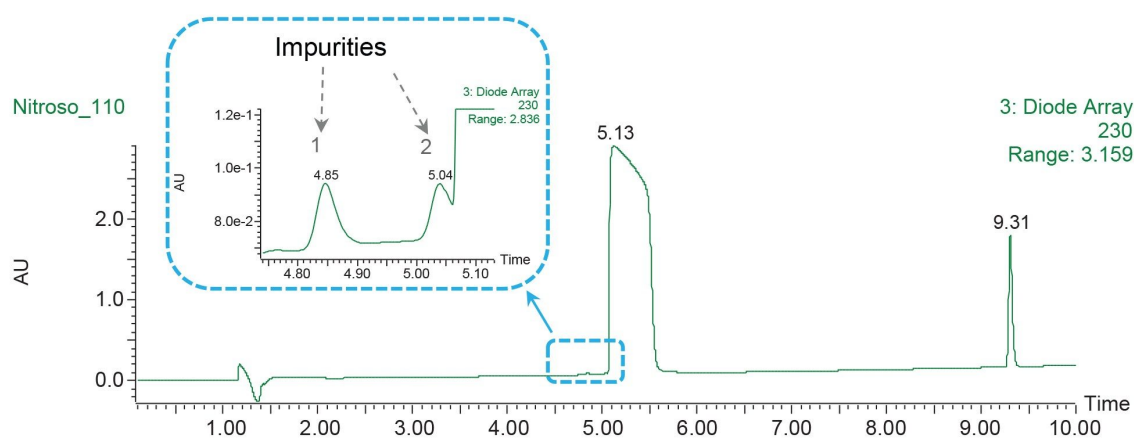


Figure 4. Chromatographic separation of impurities eluting before the propranolol API peak. UV at 230 nm, without use of a focused gradient.

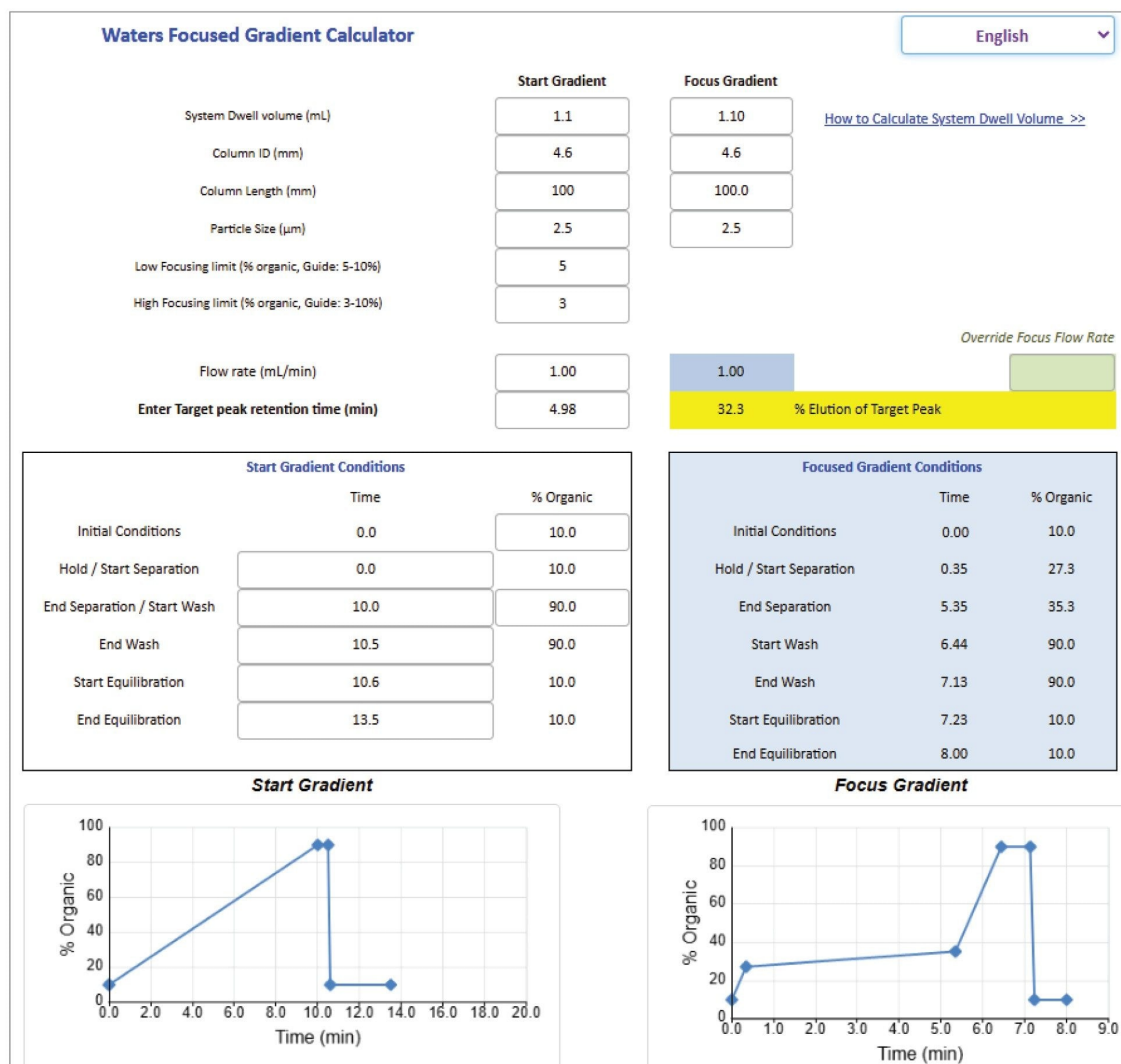
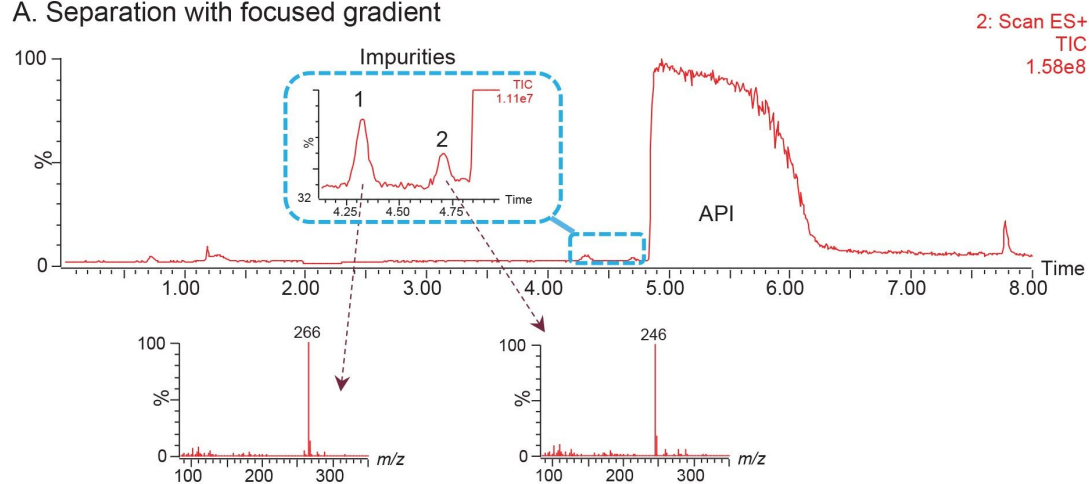


Figure 5. Waters Focused Gradient Calculator used to determine focused gradient conditions to separate target peaks based on the specified retention time.

A. Separation with focused gradient



B. Fraction collection

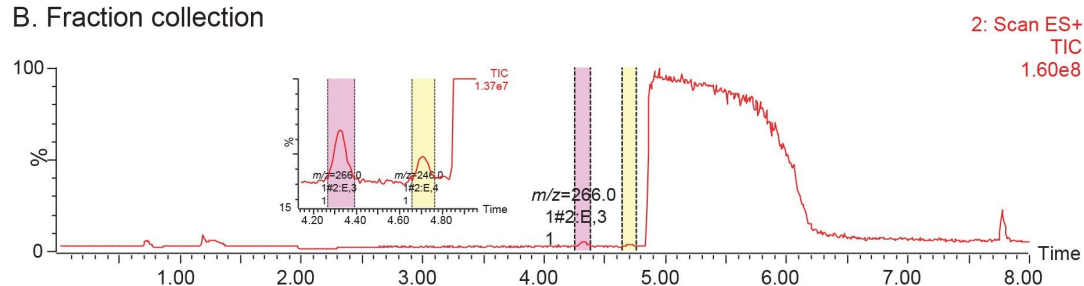


Figure 6. Chromatographic separation of impurities under focused gradient conditions (A) and fraction collection based on mass trigger (B). MS TIC data.

Conclusion

High purity fraction collection of N-nitroso-propranolol and the low-level impurities of propranolol API was successfully achieved using the WFM-A connected to an Arc Premier System. The specificity of the mass-directed trigger performed using the ACQUITY QDa II Mass Detector enabled efficient fraction collection of the target impurities. Applying a focused gradient with shallow slope improved the resolution between coeluting peaks without extending the overall run time, allowing for effective isolation of low-level impurities. The purity of the collection was successfully verified using orthogonal column chemistry, verifying the success of the purification strategy.

The workflow and tools described in this application note can be applied for the isolation and purification of pharmaceutical impurities and degradation products that may arise during development, manufacturing, or storage of drug products. The collection of pure fractions yields high purity materials that can be used for further characterization, identification, and as reference materials.

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