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Application News

Liquid Chromatography Mass Spectrometry

Streamlining Analytical Method Development for NDSRIs and Mutagenic Impurities in Pharmaceutical Formulations Using Single-Quadrupole LC-MS

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User Benefits

- LabSolutions™ MD enables simple and efficient development of LC conditions, such as mobile phases, columns, and gradient programs.
- Using a single-quadrupole LC-MS, NDSRIs in drug products can be quantified with high sensitivity and precision.
- Simultaneous screening of NDSRIs and mutagenic impurities can be performed.

■ Introduction

In September 2024, the FDA issued the second edition of "Control of Nitrosamine Impurities in Human Drugs: Guidance for Industry."[1] This guidance classifies nitrosamines into two categories: "small-molecule nitrosamines" and "nitrosamine drug substance-related impurities (NDSRIs)."

NDSRIs are formed through the reaction (nitrosation) of the active pharmaceutical ingredient (API) or API-related impurities with nitrites and related species. Unlike small-molecule nitrosamines, NDSRIs have structures similar to that of the API and exhibit a wide variety of API-derived structures. As a result, their toxicity varies depending on their structure. To address this issue, the FDA and EMA have developed the Carcinogenic Potency Categorization Approach (CPCA)[2], which estimates the carcinogenic risk of NDSRIs based on partial structural information and establishes acceptable intake limits (AI limits). Under the CPCA, NDSRIs are classified into Categories 1 to 5 according to their predicted toxicity, and different AI limits are assigned to each category. Furthermore, because NDSRIs differ in structure depending on the API, analytical methods need to be developed for each target NDSRI.

Atenolol (Fig. 1(a)) is a β-blocker used to treat mild to moderate hypertension, angina pectoris, and arrhythmia (premature beats), and its maximum daily dose is 100 mg/day. The NDSRI potentially formed from atenolol, N-nitrosoatenolol (Fig. 1(b)), is classified as Category 4 under the CPCA, with an AI limit of 1,500 ng/day.[3]

In this application, single-quadrupole LC-MS was used to analyze the API and NDSRI in an atenolol drug product, along with atenolol impurity D, a mutagenic impurity (Fig. 1(c)). LabSolutions MD (analytical method development support software) enabled efficient development of LC conditions, while the LCMS-2050 enabled simple, highly sensitive, and highly accurate analysis.

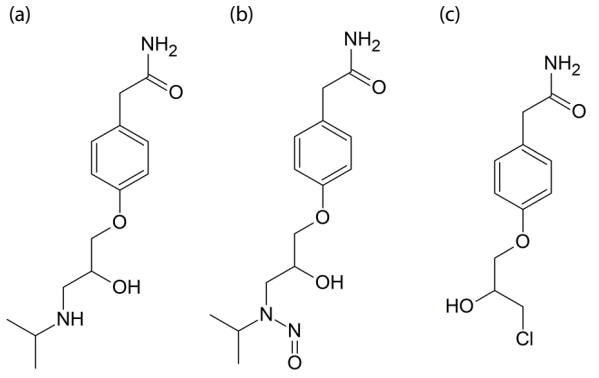

Fig. 1 Chemical structures
(a) Atenolol (API)
(b) N-nitrosoatenolol (NDSRI)
(c) Atenolol impurity D (mutagenic impurity)

■ Development of LC Conditions Using LabSolutions MD

Evaluation of Mobile Phases and Columns

In general, the early stage of LC method development involves evaluating several combinations of columns and mobile phases to identify conditions that provide good separation. LabSolutions MD enables efficient evaluation of the optimal mobile phase and column combination by using valve-switching systems for mobile phase switching and column selection.

Analytical conditions were evaluated for atenolol, N-nitrosoatenolol, and atenolol impurity D. As shown in Table 1, two columns ((1) and (2)), four mobile phases for solvent A (A1–A4), and two mobile phases for solvent B (B1–B2) were prepared, and a mixed standard solution containing the three analytes at 1 mg/L each was analyzed under all combinations. As shown in Fig. 2, LabSolutions MD allows users to select mobile phases and columns and generate an analysis batch. Conventionally, separate methods had to be created for each mobile phase and column combination, and the analyses had to be performed individually. By using this software, however, the evaluation can be carried out much more efficiently.

As shown in Fig. 3, the results can be displayed in a tabulated format. In addition, as shown in the matrix view in Fig. 4, chromatograms obtained under each set of conditions can be displayed simultaneously for comparison. In Fig. 4, chromatograms with higher minimum resolution are shown in darker colors, while those with lower minimum resolution are shown in lighter colors. Thus, large numbers of datasets can be analyzed simultaneously by sorting tables and chromatograms using various parameters. In this application, the data were sorted as shown in Fig. 3, focusing on the resolution and sensitivity of the NDSRI. As a result, the combination of mobile phase A (5 mM aqueous ammonium formate), mobile phase B (methanol), and the Shim-pack Velox™ SP-C18 column was found to be optimal in terms of both sensitivity and resolution. The chromatogram obtained under these conditions is shown in Fig. 5.

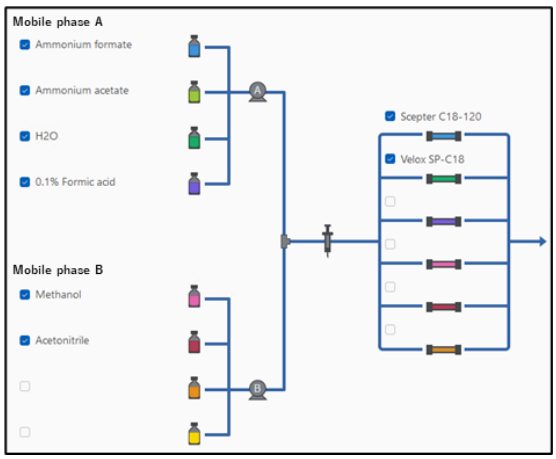

Fig. 2 Mobile phase and column setup screen in LabSolutions MD

			Compound (N-nitroso-atenolol)		
Mobile Phase A	Mobile Phase B	Column	Ret. Time	Area	Resolution
5mM AF aq.	MeOH	Velox SP C18	2.014	967319	2.883
5mM AA aq.	MeOH	Velox SP C18	2.056	962725	2.794
5mM AA aq.	MeOH	Scepter C18	2.291	939975	2.028
5mM AF aq.	MeOH	Scepter C18	2.289	933480	2.066

Fig. 3 Results sorted by resolution and sensitivity for N-nitrosoatenolol under different conditions

Fig. 4 Representative matrix view for different conditions

Table 1 Analytical Conditions for the LCMS-2050

System	: Nexera X3
Column	: (1) Shim-pack Velox SP-C18*1 (50 mm×2.1 mm I.D., 2.7 µm) (2) Shim-pack Scepter C18-120*2 (50 mm×2.1 mm I.D., 3 µm)
Temperature	: 40 ˚C
Injection volume	: 1 µL
Mobile phases	: A-1: 5 mM ammonium formate in H_2O A-2: 5 mM ammonium acetate in H_2O A-3: 0.1% Formic acid in H_2O A-4: H_2O B-1: Methanol B-2: Acetonitrile
Flow rate	: 0.4 mL/min
Time program (%B)	: 20% (0 min)→95% (5.00-6.00 min)→20% (6.01 -9.00 min)
System	: LCMS-2050 (DUIS Positive/Negative)
Nebulizing gas	: 3 L/min
Drying gas	: 3 L/min
Heating gas	: 7 L/min
DL temp.	: 250 ˚C
Desolvation temp.	: 500 ˚C
Interface voltage	: +0.5 kV/-2.0 kV
Scan range *m/z*	: (+/-) 100-1000
SIM *m/z*	: Atenolol(+) 267.17 N-nitrosoatenolol(+) 296.16 Atenolol Impurity D(+) 244.07

*1 P/N：227-32003-02
*2 P/N：227-31014-03

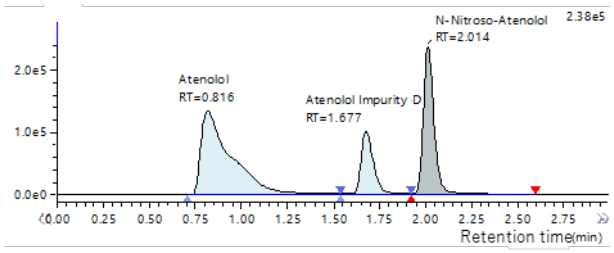

Fig. 5 Chromatogram obtained under the high-sensitivity and high-resolution conditions for the NDSRI determined using LabSolutions MD

Evaluation of Gradient Conditions

After the mobile phase and column combination had been selected, gradient conditions were evaluated, as is commonly done in LC method development. As shown in Fig. 5, the peaks of N-nitrosoatenolol and atenolol impurity D were closely eluting. To improve the separation between these compounds, gradient conditions were investigated using LabSolutions MD. Based on the LC conditions shown in Table 1, parameters such as the initial B concentration, hold time, and gradient slope were systematically varied for evaluation. In this software, various gradient programs can be generated automatically, as shown in Fig. 6, simply by entering the parameter ranges and the number of steps.

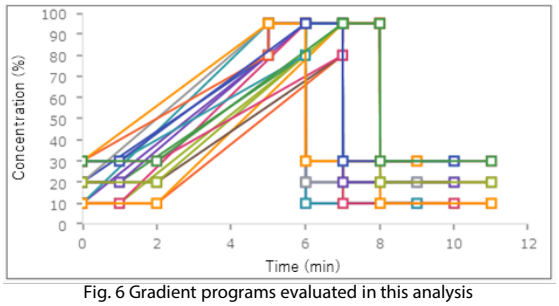

Fig. 6 Gradient programs evaluated in this analysis

As with the evaluation of mobile phases and columns, the tested conditions can also be sorted and evaluated to identify the gradient program that provides the highest resolution. Furthermore, as shown in Fig. 7, chromatograms (retention times) corresponding to changes in the gradient program can be predicted using artificial intelligence (AI). AI can also predict the gradient program that maximizes the resolution of the NDSRI. The predicted gradient program and chromatogram obtained through gradient optimization in this analysis are shown in Fig. 8.

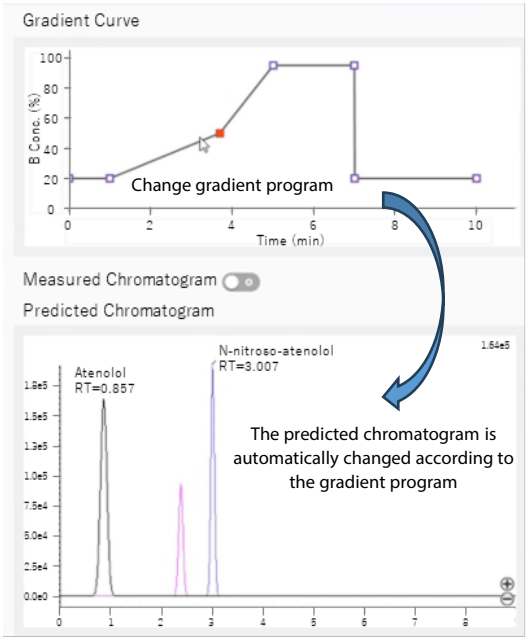

Fig. 7 Predicted chromatograms for different gradient programs

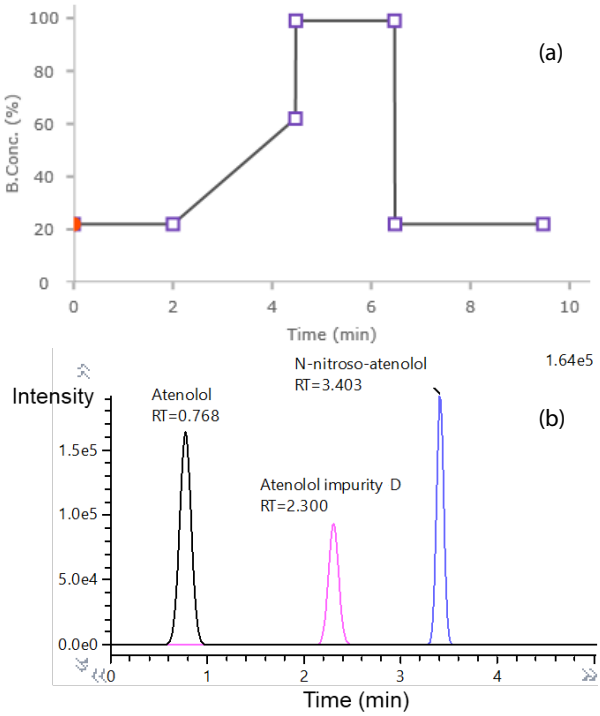

Fig. 8 AI-optimized gradient program (a) and predicted chromatogram (b)

Using the optimized gradient program shown in Fig. 8, a mixed standard solution containing the three components at 1 mg/L each was analyzed, and the resulting chromatogram is shown in Fig. 9. A chromatogram similar to that predicted in Fig. 8 was obtained, confirming that the separation between N-nitrosoatenolol and atenolol impurity D was improved relative to the chromatogram obtained under the initial conditions shown in Fig. 5. Based on this gradient program, further adjustments were made to improve sensitivity and other analytical parameters, and the LC conditions shown in Table 2 were ultimately adopted as the final analytical conditions. The resulting chromatogram is shown in Fig. 10.

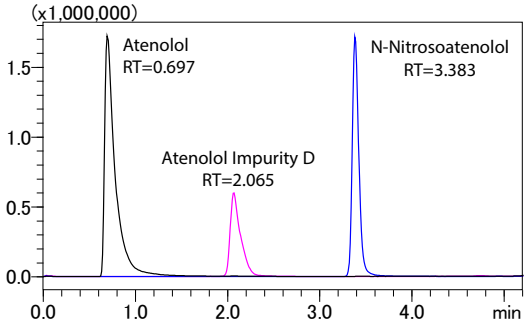

Fig. 9 Chromatogram obtained using the optimized gradient program

Table 2 Final Optimized LC Conditions

System	: Nexera X3
Column	: Shim-pack Velox SP-C18 (50 mm×2.1 mm I.D., 2.7 µm)
Temperature	: 40 °C
Injection volume	: 1 µL
Mobile phases	: A: 5 mM ammonium formate in H_2O B: Methanol
Flow rate	: 0.4 mL/min
Time program (%B)*	: 20% (0-2.00 min)→99% (5.00-7.00 min)→20% (7.01-10.00 min)

* The flow was diverted to the drain from 0 to 1.5 min during the analysis.

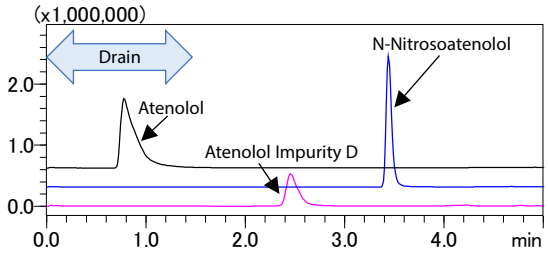

Fig. 10 Chromatogram obtained under the optimized LC conditions

■ Analysis of Standard Solutions

Using the optimized analytical conditions, mixed standard solutions of N-nitrosoatenolol and atenolol impurity D were prepared by serial dilution with methanol and analyzed by LC-MS. The resulting calibration curves are shown in Fig. 11. For N-nitrosoatenolol, the accuracy criterion of 80–120% was met over the concentration range of 0.2–200 ng/mL, while for atenolol impurity D, it was met over the range of 2–200 ng/mL. In addition, the coefficients of determination (r^2) for the calibration curves were all 0.999 or higher, indicating excellent linearity. The SIM chromatograms at the lowest concentration level of each calibration curve are shown in Fig. 12. Good peak shapes were obtained even at low concentrations.

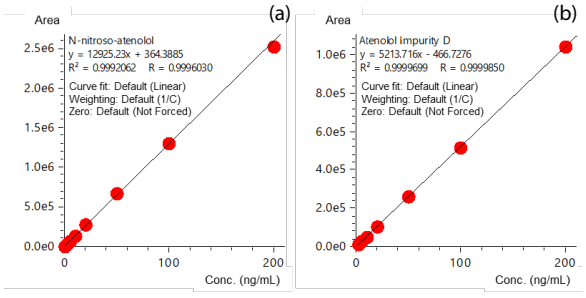

Fig. 11 Calibration curves for
(a) N-nitrosoatenolol and (b) Atenolol impurity D

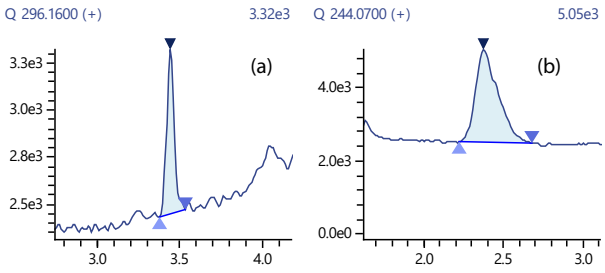

Fig. 12 SIM chromatograms at the lowest calibration level
(a) N-nitrosoatenolol, (b) Atenolol impurity D

■ API Analysis

Because the maximum daily dose of atenolol is 100 mg/day and the AI limits for N-nitrosoatenolol and atenolol impurity D are both 1,500 ng/day[3,4], the allowable concentration of each impurity is calculated to be 15 ppm relative to the API. In this application, a methanolic solution of atenolol (50 mg/75 mL) was prepared as the API sample. N-nitrosoatenolol was spiked into the sample at 1.5 ppm and 15 ppm relative to the API, while atenolol impurity D was spiked at 15 ppm, and spike recovery tests were performed. The results are summarized in Table 3. Good recovery and repeatability were obtained for both compounds.

Table 3 Recovery rates and concentration repeatability (%RSD) for the API sample spiked at (a) 1.5 ppm and (b) 15 ppm sample (n = 5)

(a) 1.5 ppm spiked	Recovery	%RSD, n=5
N-nitrosoatenolol	106.6%	3.0
(b) 15 ppm spiked	**Recovery**	**%RSD, n=5**
N-nitrosoatenolol	100.4%	1.6
Atenolol Impurity D	96.3%	3.3

The chromatogram of a sample spiked with N-nitrosoatenolol and atenolol impurity D at 15 ppm each relative to the API is shown in Fig. 13. Good peak shapes were obtained, suggesting the validity of the spike recovery test described above. However, for N-nitrosoatenolol, an interference peak was observed near the target peak (R.T. ≈ 3.25 min). The positive-mode mass spectrum at this retention time is shown in Fig. 14. A strong peak was detected at *m/z* 295, and the peak at *m/z* 296 is considered to be its isotope peak. This detected *m/z* is thought to correspond to the ^{13}C isotope peak of the protonated molecule of N-formylatenolol ($C_{15}H_{22}N_2O_4$, monoisotopic mass = 294.16).

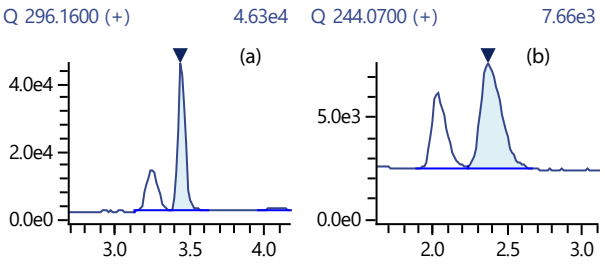

Fig. 13 Chromatograms obtained from the sample spiked at 15 ppm relative to the API
(a) N-nitrosoatenolol, (b) Atenolol impurity D

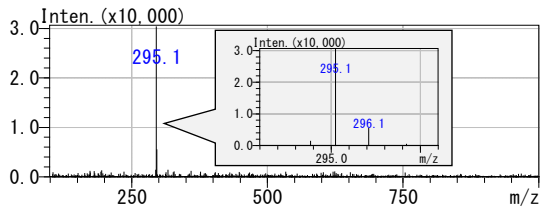

Fig. 14 Positive-mode mass spectrum near R.T. = 3.25 min

■ Drug Product Analysis

As with the API sample, spike recovery tests were performed for the drug product sample. As a preliminary step, atenolol tablets containing 25 mg/tablet were purchased, and the weights of 10 tablets were measured to determine an average tablet weight of 107.5 mg. The 10 tablets were pretreated according to the workflow shown in Fig. 15. After pulverization, a 215 mg portion of the powdered sample, corresponding to 50 mg of atenolol, was weighed and extracted with methanol. The extract was then centrifuged, and the supernatant was analyzed by LC-MS. During extraction, N-nitrosoatenolol was spiked at 1.5 ppm and 15 ppm, whereas atenolol impurity D was spiked at 15 ppm, and spike recovery tests were performed.

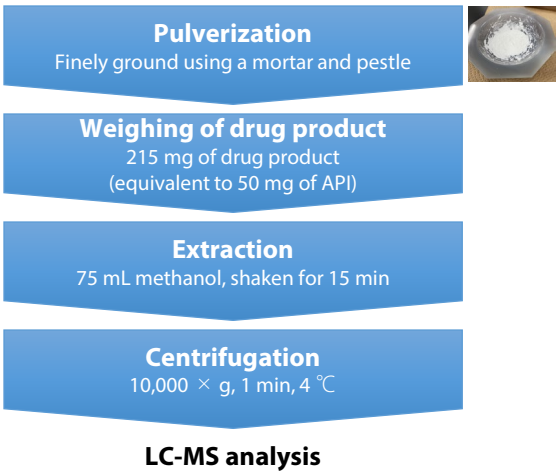

Fig. 15 Sample preparation workflow for the drug product sample

The results of the spike recovery tests are summarized in Table 4. Good recovery and repeatability were obtained for both compounds. Similarly, good recovery and repeatability were achieved in the analysis of the drug product sample. In addition, the chromatograms of a drug product sample spiked with N-nitrosoatenolol and atenolol impurity D at 15 ppm relative to the API are shown in Fig. 16. As with the API sample, good peak shapes were observed. In the drug product sample as well, a peak at *m/z* 296 was observed near R.T. = 3.25 min and is considered to be derived from N-formylatenolol. Because good recovery and repeatability were obtained in all cases, the developed analytical method is considered to enable quantification without interference from these impurities.

Table 4 Recovery and concentration repeatability (%RSD) for the drug product sample spiked at (a) 1.5 ppm and (b) 15 ppm sample (n = 5)

(a) 1.5 ppm spiked	Recovery	%RSD, n=5
N-nitrosoatenolol	106.4%	1.8

(b) 15 ppm spiked	Recovery	%RSD, n=5
N-nitrosoatenolol	110.0%	1.5
Atenolol Impurity D	103.0%	1.9

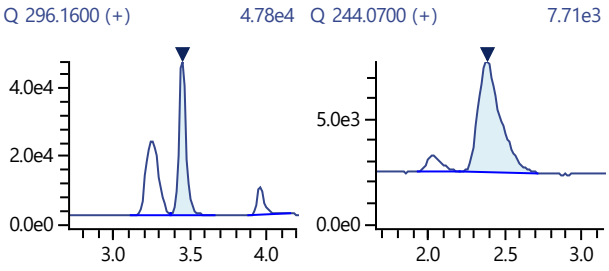

Fig. 16 Chromatograms of N-nitrosoatenolol and atenolol impurity D obtained from the drug product sample spiked at 15 ppm relative to the API
(a) N-nitrosoatenolol, (b) Atenolol impurity D

■ Conclusion

- LabSolutions MD enabled simple and efficient development of LC conditions (mobile phases, columns, and gradient programs) capable of separating the API, NDSRI, and impurities.
- Using the optimized LC conditions, the LCMS-2050 enabled highly sensitive detection of the NDSRI and impurities.
- In spike recovery tests using the API sample, good recovery and repeatability were confirmed for the NDSRI at one-tenth of the AI limit concentration and for the mutagenic impurity at a concentration equivalent to the AI limit.
- In the drug product sample, good recovery and repeatability were also confirmed for the NDSRI at one-tenth of the AI limit concentration and for the mutagenic impurity at a concentration equivalent to the AI limit, even with simple pretreatment consisting only of methanol extraction.

These results confirmed that the combination of LC conditions optimized using LabSolutions MD and the LCMS-2050 enables highly sensitive and highly precise quantification of NDSRIs in drug products. In addition, mutagenic impurities could also be quantified simultaneously at concentrations equivalent to the AI limit, demonstrating the applicability of single-quadrupole LC-MS to screening analysis of impurities in drug products.

References

1) US Food and Drug Administration: "Control of Nitrosamine Impurities in Human Drugs Guidance for Industry", Revision2, September 2024.
2) US Food and Drug Administration: "Recommended Acceptable Intake Limits for Nitrosamine Drug Substance-Related Impurities (NDSRIs) Guidance for Industry", August 2023.
3) US Food and Drug Administration: "FDA Recommended AI Limits for Certain Hypothetical NDSRIs and Other Identified Nitrosamine Impurities", Updated: 3/19/2026
4) ICH M7(R2) Guideline: ASSESSMENT AND CONTROL OF DNA REACTIVE (MUTAGENIC) IMPURITIES IN PHARMACEUTICALS TO LIMIT POTENTIAL CARCINOGENIC RISK, Adopted on 24 May 2022.

Related Applications

1. Determination of N-Nitroso-Nebivolol in Nebivolol Drug Substance by LCMS-2050, Application News No. 03-LCMS-055-EN

01-01236-EN First Edition: Aug. 2026

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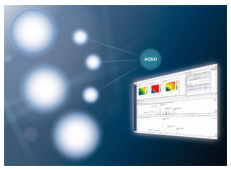

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