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Method of Test for 1-Methyl-4-Nitrosopiperazine in Rifampin Drug Substance

1. Scope

This method is applicable to the determination of 1-methyl-4-nitrosopiperazine (MNP) in rifampin drug substances.

2. Method

After extraction, 1-methyl-4-nitrosopiperazine (MNP) is determined by liquid chromatography/tandem mass spectrometry (LC-MS/MS).

2.1. Equipment

2.1.1. Liquid chromatograph/tandem mass spectrometer

2.1.1.1. Ion source: electrospray ionization, ESI.

2.1.1.2. Column: Poroshell 120 Phenyl Hexyl, 2.7 μm, 4.6 mm i.d. × 10 cm, or an equivalent product.

2.1.2. Vortex mixer.

2.1.3. Ultrasonicator.

2.1.4. Centrifuge: centrifugal force ≥ 3000 ×g.

2.2. Chemicals

Methanol, HPLC grade;
Ammonium formate, AR grade;
Ammonia water (25%), reagent grade;
Deionized water, resistivity ≥ 18 MΩ · cm at (25°C);
1-methyl-4-nitrosopiperazine (MNP), reference standard;
1-methyl-4-nitrosopiperazine-d_4 (MNP-d_4), isotope-labeled internal standard.

2.3. Apparatus

2.3.1. Volumetric flask: 10 mL, amber flask.

2.3.2. Centrifuge tube: 15 mL, PP.

2.3.3. Membrane filter: 0.22 μm, PVDF.

2.4. Mobile phase

2.4.1. Solvent A

Accurately weigh 0.63 g of ammonium formate and transfer into a 1 L volumetric flask. Dilute to volume with deionized water. Adjust the pH to 9.0 with ammonium water. Filter with a membrane filter.

2.4.2. Solvent B

Methanol.

2.5. Internal standard solution preparation

Transfer about 10 mg of MNP-d_4 internal reference standard accurately weighed into a 10 mL volumetric flask, dissolve and dilute to volume with methanol as the internal standard stock solution. Store under freezing and protect from light. Upon use, dilute the internal standard stock solution with methanol to 200 ng/mL as the internal standard solution.

2.6. Standard solution preparation

Transfer about 10 mg of MNP reference standard accurately weighed into a 10 mL volumetric flask, dissolve and dilute to volume with methanol to as the standard stock solution. Store under freezing and protect from light. Upon use, mix appropriate volume of the standard stock solution and the internal standard solution, and dilute with methanol to 0.5-100 ng/mL (containing 20 ng/mL internal standard) as the standard solutions.

2.7. Standard calibration curve establishment

Accurately inject 5 μL of the standard solution into LC-MS/MS separately, and operate according to the following conditions. Establish the standard calibration curve of MNP by the ratios of the peak area of MNP to that of the internal standard vs. the concentrations of MNP.

LC-MS/MS operating conditions(note):

Column: Poroshell 120 Phenyl Hexyl, 2.7 μm, 4.6 mm i.d. × 10 cm.

Column temperature: 35°C.

Mobile phase: a gradient program of solvent A and solvent B is as follows.

Time (min)	A (%)	B (%)
0.0 → 5.0	60 → 60	40 → 40
5.0 → 6.0	60 → 0	40 → 100
6.0 → 10.5	0 → 0	100 → 100
10.5 → 11.0	0 → 60	100 → 40
11.0 → 15.0	60 → 60	40 → 40

Flow rate: 0.5 mL/min.

Inject volume: 5 μL.

Ion spray voltage: 4.5 kV.

Ionization mode: ESI^+.

Ion source temperature: 500°C.

Nebulizer gas, Gas 1: 60 psi.

Heated gas, Gas 2: 40 psi.

Curtain gas: 25 psi.

Collision gas: Low.

Detection mode: multiple reaction monitoring (MRM). Selected ion pair, declustering potential and collision energy are as follows.

Analyte	Ion pair Precursor ion (*m/z*) > Product ion (*m/z*)	Declustering potential (V)	Collision energy (eV)
MNP	130 > 100*	50	11
	130 > 58	40	23
MNP-d_4	134 > 104	100	10

* Quantitative ion pair

Note: 1. If a divert valve is available, the direction of the mobile phase can be diverted as follows.

Time (min)	Position
0.0 → 2.0	Waste
2.0 → 4.0	MS
4.0 → 15.0	Waste

2. All the parameters can be adjusted depending on the instruments used if the above conditions are not applicable.

2.8. Sample solution preparation

Transfer about 0.1 g of sample accurately weighed to a 10 mL volumetric flask, and add 1 mL of the internal standard solution and 7 mL of methanol. Mix well, sonicate for 10 min, and dilute with methanol to volume. Transfer to a 15 mL centrifugal tube, and centrifuge at 3000 ×g for 5 min. Filter the supernatant with a membrane filter, and take the filtrate as the sample solution.

2.9. Identification and quantification

Accurately inject 5 μL of sample solution and standard solution into LC-MS/MS separately, and operate according to the conditions in section 2.7. Identify MNP based on the retention time and the relative ion

intensities(note). Calculate the amount of MNP in the sample by the following formula:

$$\text{The amount of MNP in the sample (μg/g)} = \frac{C \times V}{M} \times 10^{-3}$$

Where,

C: the concentration of MNP in the sample solution calculated by the standard calibration curve (ng/mL)

V: the final make-up volume of the sample (mL)

M: the weight of the sample (g)

Note: Relative ion intensities are calculated by peak areas of quantitative ions divided by peak areas of qualitative ions (≤ 100%). Maximum permitted tolerances of relative ion intensities by LC-MS/MS are as follows.

Relative ion intensity (%)	Tolerance (%)
> 50	± 20
> 20-50	± 25
> 10-20	± 30
≤ 10	± 50

Remark

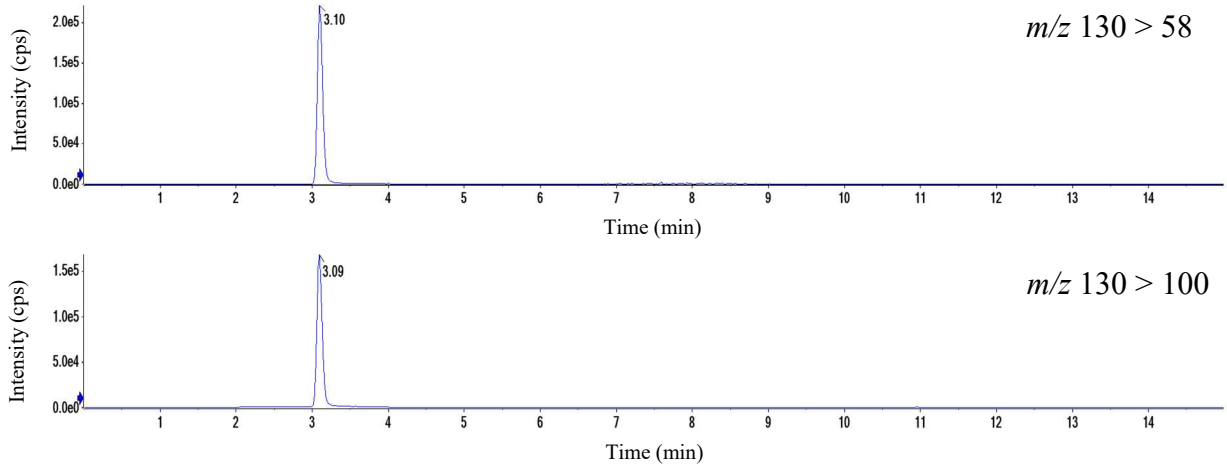
1. Limit of quantification (LOQ) for MNP is 0.05 μg/g.
2. Further validation should be performed when interference compounds appear in samples.

Reference

1. Tao, X., Tian, Y., Liu, W-H., Yao, S. and Yin, L. 2022. Trace level quantification of 4-methyl-1-nitrosopiperazin in rifampicin capsules by LC-MS/MS. Front. Chem. 10: 834124.
2. Wohlfart, J., Scherf-Clavel, O., Kinzig, M., Sörgel, F. and Holzgrabe, U. 2021. The nitrosamine contamination of drugs, part 3: Quantification of 4-methyl-1-nitrosopiperazine in rifampicin capsules by LC-MS/HRMS. J. Pharm. Biomed. Anal. 203: 114205.
3. U.S. Food and Drug Administration. 2020. Liquid chromatography-high resolution mass spectrometry (LC-ESI-HRMS) method for the determination of MNP in rifampin and CPNP in rifapentine drug substance and drug product. [https://www.fda.gov/media/142092/download]

Reference chromatogram

(A) MNP



(B) MNP-d_4 (I.S.)

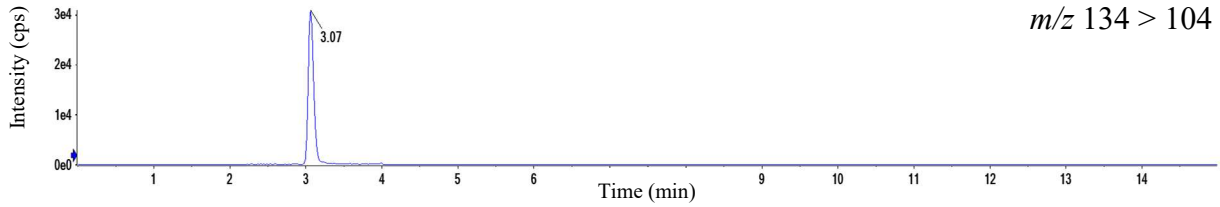


Figure. The MRM chromatograms of MNP standard (A) and MNP-d_4 internal standard (B) analyzed by LC-MS/MS.