DETERMINATION OF 1-METHYL-4-NITROSOPIPERAZINE (MNP) IN RIFAMPICIN PRODUCTS BY LC-MS/MS

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Disclaimer: The testing method below provides option and guidance for the users to determine MNP in Rifampicin products. The method should be validated by users to ensure it is fit for its intended use.

1 Scope

This document outlines the test method for the determination of 1-methyl-4-nitrosopiperazine (MNP) in Rifampicin products by Liquid Chromatography Hybrid Tandem Mass Spectrometry (LC-MS/MS).

2 Determination of MNP by LC-MS/MS

2.1 Reagents and Chemicals

1-Methyl-4-nitrosopiperazine (MNP)
1-Methyl-4-nitrosopiperazine – d_4 (MNP-d_4, IS)
Methanol, HPLC grade (MeOH)
Ammonium formate, AR grade
Ammonium hydroxide, AR grade
Deionized water (DI water)
80% MeOH: MeOH / DI water (80/20, v/v)
Diluent: 10 ng/mL MNP-d_4 in 80% MeOH
Mobile Phase A: 10 mM ammonium formate in water, pH = 9.0;
Accurately weigh 630 mg of ammonium formate and transfer into a 1 L volumetric flask. Dilute to volume with water. Adjust the pH to 9.0 with ammonium hydroxide.

2.2 Instruments and Apparatus

Liquid Chromatography Tandem Mass Spectrometry (QTRAP 6500+ / 5500 MS/MS coupled with Agilent 1290 / 1260 Infinity LC)
Centrifuge
Ultrasonic bath
Volumetric flask (Class A, 5 and 10 mL)
Membrane syringe filter (PVDE 0.2 µm)
Micropipette
2 mL vials
1.5 mL Eppendorf tube
Conical bottom centrifuge tube, Polypropylene (PP)

2.3 LC-MS/MS Method

<u>HPLC</u>

Column:	Phenomenex® Luna Phenyl-Hexyl analytical column (4.6 mm x 150 mm, 3 µm), or equivalent
Column oven temperature:	30 °C
Autosampler Temperature	6 °C
Injection volume:	5 µL
Mobile phase A:	10 mM Ammonium formate in water, pH = 9.0
Mobile phase B:	Methanol
Flow rate:	0.60 mL/min

Gradient:	Time (min)	Mobile phase A (%)	Mobile phase B (%)
	0	60	40
	3	60	40
	7	0	100
	11	0	100
	11.1	60	40
	15	60	40

[Note: The flow rate or run time may be varied to obtain optimum separation.]

MS/MS

MS:	QTRAP 6500+ /5500					
Polarity:	Positive					
Ionization mode:	APCI (Atmospheric Pressure Chemical Ionization)					
MS parameter:	CUR: 30 psi; CAD: Medium; TEP: 400 ^{O}C; GSI: 40 psi; CXP: 11					
Valve switches*:	Time (min)			Position		Remark
	0.0-4.0			A		To waste
	4.0-6.2			B		To MS
	6.2-15			A		To waste
MRM:	ID	Q1	Q3	DP	EP	CE
	MNP 1	130.1	100.1	30	12	10
	MNP 2	130.1	58.1	30	12	22
	MNP IS	134.2	104.1	25	10	12

[*Note: Valve switches window may be adjusted depending on the different system to avoid excessive contamination of MS detector from API and excipients (subject to the RTs of the target analytes)]

2.4 Standard, Sample, Sample Blank and Spiked Sample Preparation

2.4.1 Standard Preparation

1. *Stock Standard Solution* (200 mg/L): Prepared from commercially available MNP standard in 10 mL volumetric flask, top up to volume with MeOH.
2. *Intermediate Stock Standard Solution* (1 mg/L): accurately transfer 50 µL of *Stock Standard Solution* to a 10 mL volumetric flask and top up to volume with 80% MeOH.
3. *Stock Internal Standard Solution* (200 mg/L): Prepared from commercially available MNP-d4 standard in 10 mL volumetric flask, top up to volume with MeOH.
4. *Intermediate Stock Internal Standard Solution* (1 mg/L): accurately transfer 50 µL of *Stock Internal Standard Solution* to a 10 mL volumetric flask and top up to volume with 80% MeOH.
5. *LOD Spiking Standard Solution* (0.5 ng/mL): accurately transfer 50 µL of *Intermediate Stock Standard Solution* and 1 mL of *Intermediate Stock Internal Standard Solution* to a 100 mL volumetric flask and top up to volume with Diluent.
6. *Working Standard Solutions* (prepared in 10 mL volumetric flask individually):

Working Standard Solution	Standards Conc.	Vol of *Mix Stock Standard solution* (1 mg/L)	Vol of *Mix Stock IS Solution* (1 mg/L)	
1	0	0	100 µL	Top up to volume (10 mL) using 80% methanol
2	0.5 µg/L	5 µL	100 µL	
3	1 µg/L	10 µL	100 µL	
4	5 µg/L	50 µL	100 µL	
5	10 µg/L	100 µL	100 µL	
6	20 µg/L	200 µL	100 µL	
7	50 µg/L	500 µL	100 µL	
8	100 µg/L	1000 µL	100 µL	

[Note: Protect all Standard Solutions from light. The working standard solutions are valid for two days if being kept at 4-8 °C.]

2.4.2 Sample Preparation(capsule/tablet)

1. Weigh 10 units together and calculate the average mass of one unit.
2. Accurately weigh an amount of powdered sample, corresponding to 250 mg of the API into a 15 mL PP conical centrifuge tube. (Powder sample should be prepared freshly.)
3. Add 5 mL of *Diluent*, vortex to mix well and sonicate for 10 min [Note: Scale down the sample amount if necessary].
4. After extraction, centrifuge the sample for 10 minutes at 4000 rpm.
5. Filter the supernatant into a HPLC vial through a 0.2 µm PVDF Membrane filter.
6. Dilute sample with *Diluent* accordingly when the concentration of the Sample Solution exceeds the calibration range.
   [Note: Protect sample solutions from light. In the situation when the amount of powder is too much for effective sample extraction, please reduce the powder amount or increase the extraction solvent volume. In this case, the LOD of the method will be affected and needed to be recalculated accordingly.]

2.4.3 Spiked Sample Preparation (Negative sample)

1. Accurately weigh an amount of powdered sample, corresponding to 250 mg of API into a 15 mL PP Conical bottom centrifuge tube.
2. Using 5 mL *LOD Spiking Standard Solution* as extraction solvent and repeat step 3-5 as in Section 2.4.2 to obtain *Spike Sample Solution* [0.5 ng/mL in *Spiked Sample Solution*, corresponding to 10 ng/g in sample, LOD of MNP)].

2.4.4 Sample Blank Preparation

Sample Blank Solution: Prepare the Sample Blank as described for the Sample Preparation in Section 2.4.2 but without the sample addition.

2.5 Test Procedure

1. Select method: *MNP_ MRM_APCI*;
2. Inject solvent blank (80% MeOH).
3. Inject *Standard Solution*s (Duplicate).
4. Inject solvent blank.
5. Inject *Sample Blank.*
6. Inject *Sample Solutions* (Duplicate).
7. Inject *Spiked Sample Solutions* (for negative samples).
8. Inject solvent blank.
9. Flush LC-MS/MS system immediately after the analysis.

2.6 Interpretation of Results

1. For negative identification, the result is valid only if:

   i. No peaks corresponding to MNP was observed in the chromatogram obtained from the *Sample Solution*. Positive results are obtained in *Spiked Sample Solution*;
   ii. Report as 'Not Detected' and indicate the LOD of the MNP.
   iii. The LOD for the MNP is listed as below:

	MNP
Instrument LOD	0.5 ng/mL
Method LOD	10 ng/g

[Note: The LOD was calculated with reference to API used in the drug substance].

2. For positive identification, the result is valid only if:

   i. The peaks corresponding to MNP in the chromatogram of all the ion pairs from the *Sample Solution* have close retention time (±0.3 min) to the corresponding peaks from the Standard Solutions chromatogram;
   ii. The deviation of the ion ratios of MNP obtained from the *Standard Solution* and *Sample Solution* for the two MRM transitions are not more than 20%.

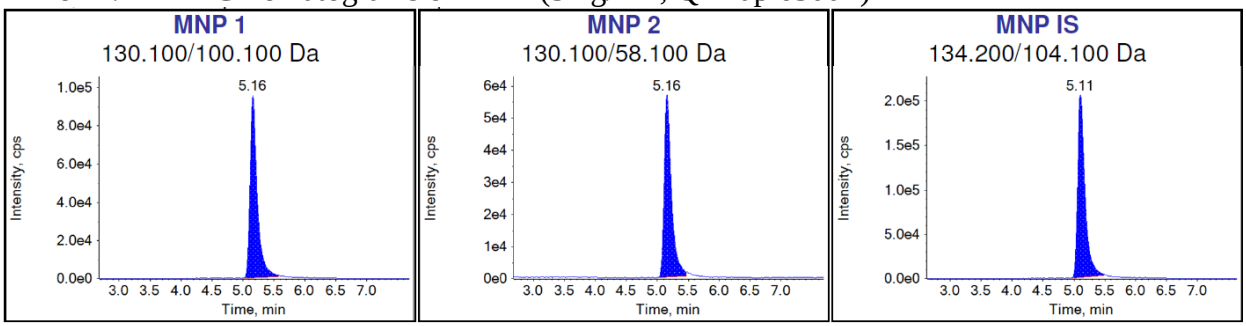
3. The quantification is performed using the peak area ratio of MNP (130.1/100.1) to that of MNP-d_4 (134.2/104.1) obtained in the chromatograms through linearity plot from *Working Standard Solutions* 1, 3-8. The quantification result is valid only if:
   i. The deviation of the peak area ratios for MNP obtained from duplicated *Sample Solution* are not more than 20%;
   ii. The linearity coefficient of the calibration plot is greater than 0.99;
   iii. Report as 'Less than 20 ng/g' if the analysis result of MNP is above 10 ng/g but less than 20 ng/g.

   The LOQ for the MNP is listed as below:

	MNP
Instrument LOQ	1.0 ng/mL
Method LOQ	20 ng/g

[Note: The LOQ was calculated with reference to API used in the drug substance].

Annex 1. MRM Chromatograms of MNP (5 ng/mL, Q-Trap 6500+)



Annex 2. MRM Chromatograms of MNP (5 ng/mL, Q-Trap 5500)