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

Background:
Rifampin, also referred to as rifampicin, is a member of the rifamycin class of antibiotics. It is used in combination with other antibiotics for various infections, including tuberculosis. One of the starting materials in the synthesis of rifampin, 1-amino-4-methyl piperazine, could lead to the formation of 1-Methyl-4-Nitrosopiperazine (MNP) under the right conditions.

Rifapentine is also a member of the rifamycin class of antibiotics and is used with other agents to treat mycobacterium infections such as tuberculosis. In May 2020, FDA received a report from an applicant holder that a single lot of rifapentine drug substance contained 1-Cyclopentyl-4-Nitrosopiperazine (CPNP).

OTR had been assigned to develop and validate a method and test for MNP in rifampin and CPNP in rifapentine drug product and drug substance to determine the level of these two nitrosopiperazine impurities. MNP and CPNP belong to the nitrosamine class of compounds, some of which are classified as probable or possible human carcinogens.

Conclusions:
A combined LC-ESI-HRMS method was developed and validated following ICH Q2(R1) for the detection and quantitation of MNP in rifampin and CPNP in rifapentine drug substance and drug product. The limit of detection (LOD), limit of quantitation (LOQ) and range of the method are summarized below:

	MNP	**CPNP**
LOD (ng/mL)	0.3	0.1
(ppm)	0.010	0.003
LOQ (ng/mL)	0.5	0.5
(ppm)	0.017	0.017
Range (ng/mL)	0.5 – 200	0.5 – 200
(ppm)	0.017 – 6.67	0.017 – 6.67

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Purpose
This method will be used to quantitate 1-Methyl-4-Nitrosopiperazine (MNP) in rifampin and 1-Cyclopentyl-4-Nitrosopiperazine (CPNP) in rifapentine drug substance and drug product.

Principle
MNP and CPNP are nitrosamine impurities in rifampin and rifapentine, respectively. These impurities can be separated from their respective drug substances by reverse phase chromatography and then detected by a high-resolution and high-mass accuracy (HRMS) mass spectrometer. High sensitivity detection is achieved by monitoring the accurate *m/z* value of the fragment ions of the impurities. Quantitation is performed by comparing the peak area of the MNP or CPNP impurity in extracted ion chromatograms of the samples to the peak area of the MNP or CPNP reference standard in an external calibration standard.

Reagents
- MNP Reference Standard
- CPNP Reference Standard
- Ammonium Formate, LC/MS grade (Fisher A11550 or equivalent)
- Methanol, LC/MS grade (Fisher A456-4 or equivalent)
- Water, LC/MS grade or equivalent

Equipment/Instrument
- HPLC or UHPLC system equipped with temperature-controlled autosampler and column compartment
- Q Exactive™ hybrid quadrupole-orbitrap mass spectrometer (Thermo-Fisher Scientific)
- HPLC column: Ace Ultracore SuperPhenylHexyl, 2.5 µm 90 Å, 50 x 4.6 mm (Mac-Mod, Part No. CORE25B0546U), or equivalent
- Analytical Balance
- Vortex Mixer
- 15 mL glass centrifuge tubes
- Wrist action shaker
- 0.22 µm PVDF syringe filters
- Centrifuge
- HPLC vials

Mobile phase preparation
- 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.
- Mobile Phase B: Methanol

Diluent and Blank: Methanol

Mixed MNP and CPNP Intermediate Stock Standard preparation (100 ng/mL)
Prepare a 100 ng/mL MNP and CPNP mixed intermediate stock standard solution in methanol using commercially available MNP and CPNP reference standards.

Working Standard Preparation (3 ng/mL)
Transfer a 0.75 mL aliquot volume of the mixed intermediate stock standard into a 25 mL volumetric flask and dilute to volume with methanol. **Prepare fresh daily.**

Drug substance sample preparation
Accurately weigh 150 mg of drug substance into a 15 mL glass centrifuge tube. Add 5.0 mL of methanol and mix the solution using a vortex mixer until dissolved. Filter the solution using a 0.22 µm PVDF syringe filter, discard the first 1 mL and transfer the filtered sample into an HPLC vial for LC/MS analysis.

Drug product sample preparation
Empty the contents of the drug product capsule(s) or crush the appropriate number of tablet(s) to obtain a target concentration of 30 mg/mL of drug substance in methanol, and transfer into a 15 mL glass centrifuge tube. Add the appropriate volume of methanol and mix for 1 minute using a vortex mixer. Shake the sample for 40 minutes using a mechanical wrist action shaker.

After extraction, centrifuge the sample for 10 minutes at 4000 rpm. Filter the supernate using a 0.22 µm PVDF syringe filter, discard the first 1 mL and transfer the filtered sample into an HPLC vial for LC/MS analysis.

Chromatographic Conditions

HPLC Column	HPLC column: Ace Ultracore SuperPhenylHexyl, 2.5 µm 90 Å, 50 x 4.6 mm (Mac-Mod, Part No. CORE25B0546U)		
Column Temp.	30 °C		
Flow Rate	0.5 mL/min		
Mobile Phase A	10 mM Ammonium Formate in water, pH = 9.0		
Mobile Phase B	Methanol		
Gradient	Time (min)	A%	B%
	0	60	40
	2.0	60	40
	6.0	0	100
	9.0	0	100
	9.1	60	40
	13.0	60	40
Injection Volume	3 µL		
Autosampler Temp.	4 – 8 °C		
Needle Wash	Methanol		

Mass spectrometer conditions

- Instrument
  Q Exactive™ mass spectrometer (Thermo-Fisher)
- Ion Source Settings
  Note: Ion source parameters can be adjusted to achieve the desired sensitivity.

Sheath Gas Flow Rate	55 arbitrary units
Aux Gas Flow Rate	15 arbitrary units
Sweep Gas Flow Rate	0 units
Spray Voltage	3.5 kV
Capillary Temp.	300 °C
Aux Gas Heater Temp.	300

- Scan Settings
  Note:
  1. *The scan start-end time should be adjusted for the user's HPLC system since the retention time of the MNP or CPNP impurity may vary between different hplc systems.*
  2. *The divert valve can be used to divert the eluent to waste when a scan is not performed.*

Scan Settings	MNP	CPNP
Scan Type	PRM	PRM
Polarity	Positive	Positive
Scan Start -End (min)	1.0 – 2.5	4.8 – 5.5
m/z Isolated for PRM	130.0975	184.1444
NCE	30	30
Isolation Window	1.0 m/z	1.0 m/z
Microscans	3	1
Resolution	70,000	70,000
AGC target	1e6	1e6
Maximum IT	300 ms	100 ms

Injection Sequence

- Inject Blank (use diluent) at least once at the beginning of a sequence or in between sample injections to reduce carry over.
- Inject Standard solution for six consecutive times before the injection of the first sample
- Inject Standard solution once every six injections of samples and at the end of a sequence. Example sequence:

Order	Solution	No. of Injections
1	Blank	2
2	Standard	6
3	Blank	1
4	Sample 1	1
5	Sample 2	1
6	Sample 3	1
7	Sample 4	1
8	Sample 5	1
9	Sample 6	1
10	Blank	1
11	Standard	1
12	Blank	1
…	…	…

System Suitability

- The % RSD (n = 6) of the MNP or CPNP peak areas for the first six injections of the standard solution should be no more than 10%.
- The cumulative % RSD of the MNP or CPNP peak areas should be no more than 15%. (cumulative % RSD of the peak area is calculated by combining the initial six replicate injections of the standard solution and each subsequent bracketing standard).

Data Processing

- MNP or CPNP peak areas from the extracted ion chromatograms (EIC) with a *m/z* extraction window of ***15 ppm*** are used for quantitation. The *m/z* value to be extracted for MNP or CPNP is listed below:

Nitrosamine	**MNP**	**CPNP**
m/z to be extracted	100.0999	98.0969

- The retention time difference of the MNP or CPNP impurity in the analyzed samples should be no more than 2% of the retention time of the corresponding MNP or CPNP peak in the reference standard solution.

Calculations

Drug Substance:

MNP or CPNP impurity (ppm) = $\frac{A_{spl}}{As} \times C_s \times \frac{1\,mg}{1\times 10^6\,ng} \times \frac{V}{W} \times 10^6$

Where: A_{spl} = Area of the MNP or CPNP impurity peak in the sample solution
As = Average area (n = 6) of the MNP or CPNP impurity peak from the first six consecutive injections of the standard solution
C_s = Concentration of the nitrosamine impurity in the standard solution (ng/mL)
W = Weight of drug substance (mg)
V = Volume of the diluent in the sample solution (mL)

Drug Product:

MNP or CPNP impurity (ppm) = $\frac{A_{spl}}{As} \times C_s \times \frac{1\ mg}{1\times10^6\ ng} \times \frac{1}{30\ mg/mL} \times 10^6$

Where: A_{spl} = Area of the MNP or CPNP impurity peak in the sample solution
As = Average area (n = 6) of the MNP or CPNP impurity peak from the first six consecutive injections of the standard solution
C_s = Concentration of the nitrosamine impurity in the standard solution (ng/mL)

Report

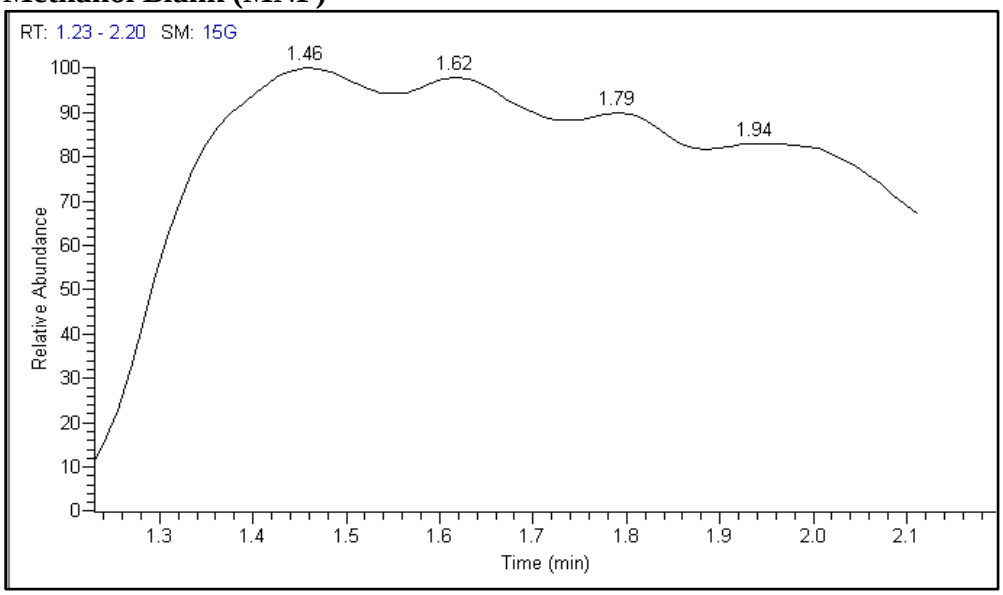
- Report the nitrosamine impurity content in ppm with three significant figures if the value is ≥ LOD
- Report 'not detected' if the nitrosamine impurity is not detected, or the value is < LOD

References

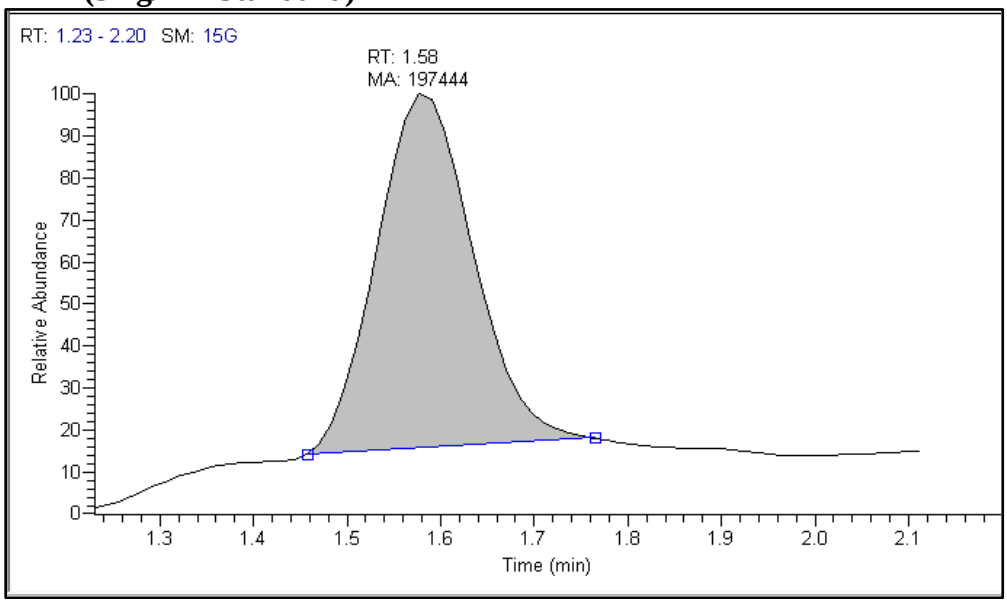
1. Rifampicin | C43H58N4O12 - PubChem
2. Rifapentine | C47H64N4O12 - PubChem
3. Q2 (R1) Validation of Analytical Procedures: Text and Methodology
4. Guidance for Industry: Analytical Procedures and Methods Validation for Drugs and Biologics
5. Control of Nitrosamine Impurities in Human Drugs

Example Chromatograms

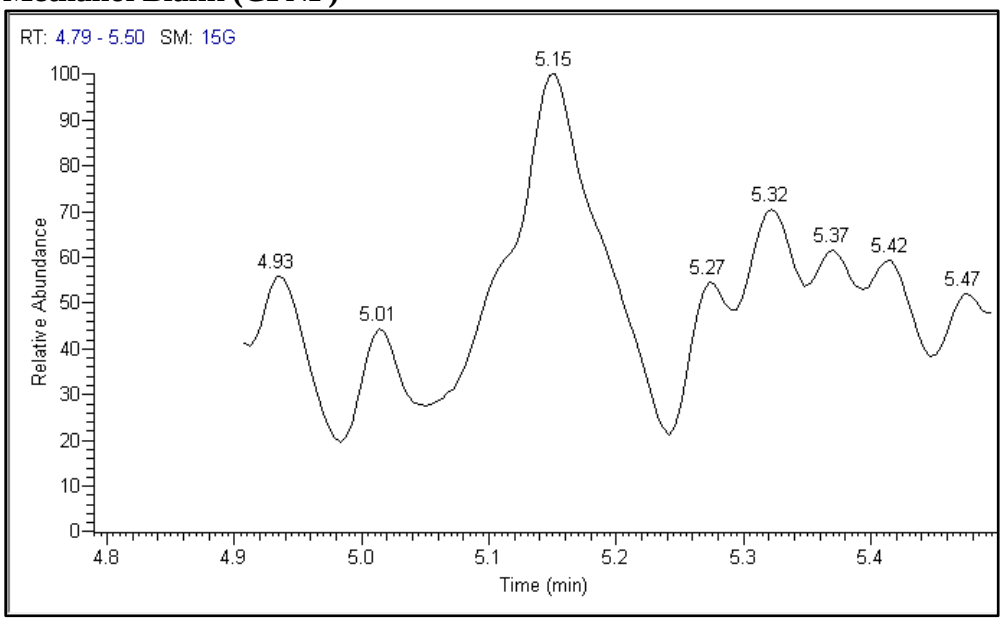
Methanol Blank (MNP)



MNP (3 ng/mL Standard)



Methanol Blank (CPNP)



CPNP (3 ng/mL Standard)