TEST FOR 1-NITROSO-4-METHYL PIPERAZINE (MeNP) IN
RIFAMPICIN ACTIVE PHARMACEUTICAL INGREDIENT OR
RIFAMPICIN TABLETS BY HPLC-MS/MS

Draft proposal for inclusion in The International Pharmacopoeia
(June 2022)

DRAFT FOR COMMENTS

Please send any comments you may have on this draft working document to Dr Herbert Schmidt, Technical Officer, Norms and Standards for Pharmaceuticals, Technical Standards and Specifications (email: schmidth@who.int), with a copy to Ms Sinéad Jones (email: jonessi@who.int) by 2 September 2022.

Our working documents are sent out electronically and they will be placed on the WHO Medicines website (https://www.who.int/teams/health-product-and-policy-standards/standards-and-specifications/pharmaceuticals/current-projects) for comments under the “Working documents in public consultation” link. If you wish to receive our draft guidelines, please send your e-mail address to jonessi@who.int and your name will be added to our electronic mailing list.

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Schedule for the Adoption Process of Document QAS/21.899:

Test for 1-nitroso-4-methyl piperazine (MeNP) in Rifampicin Active Pharmaceutical Ingredient or Rifampicin Tablets by HPLC-MS/MS

<table>
<thead>
<tr>
<th>Description</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drafting of the test based on a method developed and validated by the Health Science Authority, Singapore.</td>
<td>August 2021</td>
</tr>
<tr>
<td>Discussion of the draft method with a small group of experts</td>
<td>September 2021</td>
</tr>
<tr>
<td>Draft method sent out for public consultation</td>
<td>June – September 2022</td>
</tr>
<tr>
<td>Presentation to the 57th WHO Expert Committee on Specifications for Pharmaceutical Preparations.</td>
<td>October 2023</td>
</tr>
<tr>
<td>Further follow-up action as required.</td>
<td></td>
</tr>
</tbody>
</table>

[Note from the Secretariat. It is intended to publish a method to test for MeNP in the Supplementary information section of The International Pharmacopoeia (under Test methods used during development or manufacture).]
Test for 1-Nitroso-4-methyl piperazine (MeNP) in Rifampicin active pharmaceutical ingredient or Rifampicin tablets by HPLC-MS/MS

Molecular formula. C$_5$H$_{11}$N$_3$O

Relative molecular mass. 129.16.

Graphic formula

\[
\begin{array}{c}
\text{CH}_3 \\
\text{N} \\
\text{N} \\
\text{N} \\
\text{N} \\
\text{O}
\end{array}
\]

Chemical name. 1-methyl-4-nitrosopiperazine; CAS Reg. No. 16339-07-4.

Carry out the test as described under 1.14.1 Chromatography. High-performance liquid chromatography using a stainless steel column (15 cm x 4.6 mm) packed with porous particles of silica gel, the surface of which has been modified with chemically-bonded phenyl groups (3 µm).\(^1\)

Use the following conditions for gradient elution:

- **Mobile phase A**: 10 mM ammonium formate solution, pH = 9.0;
- **Mobile phase B**: methanol R.

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\(^1\) A Phenomenex Luna Phenyl-Hexyl column was found suitable.
Prepare the 10 mM ammonium formate solution, pH 9.0, by dissolving 630 mg of ammonium formate R in 900 mL of water R, adjust the pH to 9.0 by adding ammonia (~260 g/L) TS and diluting to 1000 mL with water R.

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Mobile phase A (% v/v)</th>
<th>Mobile phase B (% v/v)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–3</td>
<td>60</td>
<td>40</td>
<td>Isocratic</td>
</tr>
<tr>
<td>3–7</td>
<td>60 to 0</td>
<td>40 to 100</td>
<td>Linear gradient</td>
</tr>
<tr>
<td>7–11</td>
<td>0</td>
<td>100</td>
<td>Isocratic</td>
</tr>
<tr>
<td>11–11.1</td>
<td>0 to 60</td>
<td>100 to 40</td>
<td>Return to initial composition</td>
</tr>
<tr>
<td>11.1–15</td>
<td>60</td>
<td>40</td>
<td>Re-equilibration</td>
</tr>
</tbody>
</table>

Operate with a flow rate of 0.6 mL per minute. Maintain the column at 30 °C. As a detector, use a triple-quadrupole mass spectrometer equipped with an Atmospheric Pressure Chemical Ionization (APCI) interface.

Set the ion source and scan settings of the MS spectrometer as follows and optimize the settings for ion source temperature and auxiliary gases. Use the additional MRM transition of 130.1→ 58.1 to identify the MeNP response.

<table>
<thead>
<tr>
<th>Acquisition mode:</th>
<th>Multiple Reaction Monitoring (MRM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polarity:</td>
<td>Positive</td>
</tr>
<tr>
<td>MRM transitions (m/z):</td>
<td>130.1→ 100.1 (for MeNP)</td>
</tr>
<tr>
<td></td>
<td>134.2→ 104.1 (for the Internal Standard MeNP-d4)</td>
</tr>
</tbody>
</table>

Use valve switches to channel API and excipients to waste, as appropriate, to avoid excessive contamination of MS detector by API and excipients.
Prepare the following solutions freshly. As a diluent, use a mixture of 80 volumes of methanol R and 20 volumes of water R. After preparation, keep the solutions protected from light at about 4-8 °C or use an autosampler with cooling (4-8 °C).

For the MeNP stock solution (1), prepare a solution containing 1 µg of 1-nitroso-4-methyl piperazine R per mL.

For the Internal Standard (IS) stock solution (1), prepare a solution containing 1 µg of 1-nitroso-4-methyl piperazine-d4 R (MeNP-d4) per mL.

For the Internal Standard (IS) stock solution (2), dilute 1.0 mL of the Internal Standard (IS) stock solution (1) to 100.0 mL.

Prepare a set of 5 standard solutions (solutions (1) to (5)) and a sensitivity solution (solution (6)) by diluting the MeNP stock solution according the following table.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume of MeNP stock solution (mL)</th>
<th>Volume of IS stock solution (1) (mL)</th>
<th>Final volume (mL)</th>
<th>MeNP concentration (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.500</td>
<td>1.0</td>
<td>100.0</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>1.0</td>
<td>100.0</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>2.0</td>
<td>1.0</td>
<td>100.0</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>5.0</td>
<td>1.0</td>
<td>100.0</td>
<td>50</td>
</tr>
<tr>
<td>5</td>
<td>10.0</td>
<td>1.0</td>
<td>100.0</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>0.100</td>
<td>1.0</td>
<td>100.0</td>
<td>1</td>
</tr>
</tbody>
</table>

Prepare the sample solutions in duplicate (solutions (7) and (8)):

For the analysis of rifampicin active pharmaceutical ingredient, transfer 250.0 mg of the test substance each into a 15 mL conical centrifuge tube. Add 5.0 mL of Internal
Standard (IS) stock solution (2), mix and sonicate for 10 minutes. Centrifuge the dispersion for 10 minutes at 4,000 rpm, filter\(^2\) the supernatant and use the filtrate.

For the analysis of rifampicin tablets, weigh and powder 20 tablets. Transfer a quantity of the powdered tablets, nominally containing 250.0 mg of Rifampicin, each into a 15 mL conical centrifuge tube and proceed as described above.

For solution (9), transfer 5.0 mL of Internal Standard (IS) stock solution (2) into a 15 mL conical centrifuge tube, mix and sonicate for 10 minutes. Centrifuge the solution for 10 minutes at 4,000 rpm, filter the supernatant and use the filtrate.

Inject 5 µL each of solutions (1) to (9).

Monitor the 130.1 to 100.1 and the 134.2 to 104.1 (m/z) transitions and measure the signals due to MeNP and MeNP-d4 (retention times of about 5 minutes).

The test is not valid unless, in the chromatogram obtained with solution (6), the signal due to MeNP is detected with a signal-to-noise ratio of at least 10.

Plot the ratios of the signals due to MeNP and to MeNP-d4 versus the concentration of MeNP in each of the standard solutions (1) to (5) (in ng/mL) and determine the regression line using the least-squares method. From the graph so obtained, determine the concentration (C) of MeNP in the sample solutions (7) and (8) (in ng/mL).

The test is not valid unless the correlation coefficient of the calibration curve is at least 0.99. The test is also not valid unless the concentrations of MeNP in sample solutions (7) and (8) deviate by more than 15% from each other.

If the concentrations of MeNP in solutions (7) to (8) exceed the calibration range, prepare new sample solutions by either scaling down the sample amount, or diluting the sample solution using Internal Standard (IS) stock solution (2), and repeat the analysis.

\(^2\) A 0.2 µm PVDF membrane syringe filter was found suitable.
Calculate the concentration of MeNP in µg per g rifampicin

\[ R_f = \frac{C \times 5}{1000} / W \]

W = amount of rifampicin taken (in g) or labelled amount of rifampicin in the amount of rifampicin tablets taken (in g).

The result is the mean of the values \( R_f \) obtained for the two samples.

Reagents to be established

1-Nitroso-4-methyl piperazine R
A commercially available substance of suitable grade.

1-Nitroso-4-methyl piperazine-d4 RS
A commercially available substance of suitable grade..