BENZATHINE BENZYLPPENICILLIN TETRAHYDRATE

(BENZATHINI BENZYLPPENICILLINUM TETRAHYDRICUM)

Draft proposal for revision in The International Pharmacopoeia

(October 2021)

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 (schmidt@who.int), with a copy to Ms Sinéad Jones (jonessi@who.int) by 6 December 2021.

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/21.879:

BENZATHINE BENZYL-PENCILLIN TETRAHYDRATE
(BENZATHINI BENZYL-PENCILLINUM TETRAHYDRICUM)

<table>
<thead>
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<th>Date</th>
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<tbody>
<tr>
<td>Revision drafted based on information found in the scientific literature,</td>
<td>March 2021</td>
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<tr>
<td>other pharmacopoeias, in particular the European Pharmacopoeia, submitted</td>
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<td>by manufacturers, and on laboratory investigations.</td>
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<td>Discussion at the Consultation on Screening Technologies, Laboratory Tools</td>
<td>May 2021</td>
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<td>and Pharmacopoeial Specifications.</td>
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<tr>
<td>Monograph sent out for public consultation.</td>
<td>October – December 2021</td>
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<td>Further follow-up action as required.</td>
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(Note from WHO Secretariat. It is proposed to revise the monograph on Benzathine benzylpenicillin. The revision is based on information found in the scientific literature, other pharmacopoeias - in particular the European Pharmacopoeia - submitted by manufacturers and on laboratory investigations.

The monograph is expected to play an important role in ensuring access to safe, effective and quality assured Benzathine Benzylpenicillin worldwide. Manufacturers of this product, regulatory authorities, procurement agencies and other stakeholders are therefore invited to provide their feedback on the draft revision.

If not already done, manufacturers are also invited to submit information and samples of their products. With their support, manufacturers will help ensure that the proposed monograph adequately controls the quality of the products they
manufacture. For further information, please contact Dr Herbert Schmidt at schmidt@who.int.

Changes from the original text are indicated by insert and delete.
**BENZATHINE BENZYLPIenicillin TETRAHYDRATE**

*BENZATHINI BENZYLPIenicillINUM TETRAHYDRICUM*

**Benzathine benzylpenicillin (non-injectable)**

**Benzathine benzylpenicillin, sterile**

**Molecular formula.** \((C_{16}H_{18}N_{2}O_{4}S)_2, C_{16}H_{20}N_{2} \cdot 4H_2O,\)

**Relative molecular weight.** 909.1 (anhydrous) 981.

**Graphic formula.**

![Graphic formula](image)

**Chemical name.** \(\text{N,N'-Dibenzylethylenediamine compound with (2S,5R,6R)-3,3-dimethyl-7-oxo-6-(2-phenylacetamido)4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (1:2); N,N'-bis(phenylmethyl)-1,2-ethanediamine compound with [2S-(2α,5α,6β)]-3,3-dimethyl-7-oxo-6-[(phenylacetyl)amino]-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (1:2); N,N'-dibenzylethylenediamine salt of benzylpenicillin; CAS Reg. No. 1538-09-6 (anhydrous). \(\text{N,N'-Dibenzylethylethane-1,2 diamine bis[(2S,5R,6R)-3,3-dimethyl-7-oxo-6-(2-phenylacetamido)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate]tetrahydrate; CAS Reg. No. 41372-02-5.}\)

**Other name.** Penicillin G benzathine.
Description. A white or almost white, slightly hygroscopic powder. A white powder; odourless or almost odourless.

Solubility. Very slightly soluble in water R; freely soluble in dimethylformamide R and in formamide R, slightly sparingly soluble in ethanol (~750 g/L) TS; practically insoluble in ether R.


Storage. Benzathine benzylpenicillin tetrahydrate should be kept in a tightly closed container or, if sterile, in a hermetically closed container, protected from light, and stored at a temperature not exceeding 30 °C.

Labelling. The designation sterile Benzathine benzylpenicillin tetrahydrate indicates that the substance complies with the additional requirements for sterile Benzathine benzylpenicillin tetrahydrate and may be used for parenteral administration or for other sterile applications.

Additional information. Benzathine benzylpenicillin contains a variable amount of water of crystallization. Benzathine benzylpenicillin tetrahydrate is a salt obtained from a fermentation product. Dispersion or suspending agents (e.g. lecithin and polysorbate 80) may be added.

Requirements

Definition. Benzathine benzylpenicillin contains not less than 96.0% and not more than 100.5% of total penicillins calculated as \((C_{16}H_{18}N_{2}O_{4}S)_{2}C_{16}H_{20}N_{2}\) and not less than 24.0% and not more than 27.0% of \(C_{16}H_{20}N_{2}\), both calculated with reference to the anhydrous substance. Benzathine benzylpenicillin contains not less than 94.5% and not more than 102.0% of benzathine benzylpenicillin \(((C_{16}H_{18}N_{2}O_{4}S)_{2}C_{16}H_{20}N_{2})\) without correction for dispersion or suspending agents, and not less than 24.0% and not more than 27.0% of benzathine \((C_{16}H_{20}N_{2})\), both calculated with reference to the anhydrous substance and without correction for dispersion or suspending agents.
Identity tests

Either test A alone or any two of tests B, C or D may be applied.

A. Carry out the test as described under 1.7 Spectrophotometry in the infrared region. The infrared adsorption spectrum is concordant with the spectrum obtained from benzathine benzylpenicillin RS or with the reference spectrum of benzathine benzylpenicillin tetrahydrate.

B. Carry out the test as described under 1.14.4 High-performance liquid chromatography using the conditions given under “Assay”. The retention times of the two principal peaks in the chromatogram obtained with solution (1) correspond to the retention times of the peaks due to benzathine and benzylpenicillin obtained with solution (2).

C. Carry out the test as described under 1.14.1 Thin-layer chromatography using silica gel R5 as the coating substance and a freshly prepared mixture of acetone R and a solution of 154 g/L of ammonium acetate R, adjusted to pH 7.0 with ammonia R, (30:70 V/V) as the mobile phase. Apply separately to the plate 1 µL of each of the following two solutions in methanol R. For solution (A), use 5.0 mg of the test substance per mL. For solution (B), use 5.0 mg of benzathine benzylpenicillin RS per mL. Develop the plate for 2/3 of its length. After removing the plate from the chromatographic chamber, allow it to dry in air or in a current of air. Expose the plate to iodine vapour until the spots appear and examine in daylight. The test is not valid unless the chromatogram obtained with solution (B) shows two clearly separated spots. The two principal spots in the chromatogram obtained with solution (A) correspond in position, appearance and intensity to those in the chromatogram obtained with solution (B).

DA. To 2 mg in a test-tube, add 1 drop of water followed by 2 mL of sulfuric acid (~1760 g/L) TS and mix; the solution is almost colourless. Immerse the test-tube for 1 minute in a water-bath; the solution remains almost colourless. Place
2 mg in a second test-tube, add 1 drop of water and 2 mL of formaldehyde/sulfuric acid TS and mix; the solution is almost colourless and, after a few minutes, the colour changes to yellow-brown. Immerse the test-tube for 1 minute in a water-bath; a reddish brown colour is produced.

B. Shake 0.1 g with 2 mL of sodium hydroxide (1 mol/l) VS for 2 minutes, extract the mixture with 2 quantities, each of 3 mL of ether R, evaporate the combined extracts, and dissolve the residue in 1 mL of ethanol (~375 g/l) TS. Add 5 mL of trinitrophenol (7 g/l) TS, heat at 90 °C for 5 minutes, and allow to cool slowly. Collect the precipitate and recrystallize it from hot ethanol (~150 g/l) TS that contains 10 mg/mL of trinitrophenol R; melting temperature, about 214 °C (picrate).

Water. Determine as described under 2.8 Determination of water by the Karl Fischer method, Method A, using about 0.200 ± 0.05 g of the substance; the water content is not less than 50 mg/g and not more than 80 mg/g.

pH value. pH of a saturated solution containing about 0.05 g in 10 mL of carbon-dioxide-free water R, 5.0–7.5.

Acidity or alkalinity. To 0.50 g, add 100 mL of carbon dioxide-free water R, shake for 5 minutes and filter through a sintered-glass filter. To 20 mL of the filtrate, add 0.1 mL of bromothymol blue/ethanol TS. The solution is green or yellow. Not more than 0.2 mL of sodium hydroxide (0.02 mol/L) VS is required to change the colour of the indicator to blue.

Related substances. Prepare the solutions immediately before use and by diluting to volume immediately after dissolution of the test and reference substance. Carry out the test as described under 1.14.4 High-performance liquid chromatography, using a stainless steel column (4.6 mm x 15 cm) packed with end-capped particles of silica gel.
the surface of which has been modified with chemically-bonded octadecylsilyl groups (3 µm)\(^1\).

Use the following conditions for gradient elution:

- mobile phase A: 10 volumes phosphate buffer pH 3.3, 30 volumes of methanol R, and 60 volumes of water R;
- mobile phase B: 5 volumes phosphate buffer pH 3.3, 70 volumes of methanol R, and 25 volumes of water R.

Prepare the phosphate buffer pH 3.3 by dissolving 34 g of potassium dihydrogen phosphate R in 900 mL of water R. Adjust to pH 3.3 with phosphoric acid (~1440 g/L) TS and dilute 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>0–2</td>
<td>85</td>
<td>15</td>
<td>Isocratic</td>
</tr>
<tr>
<td>2–16</td>
<td>85 to 0</td>
<td>15 to 100</td>
<td>Linear gradient</td>
</tr>
<tr>
<td>16–26</td>
<td>0</td>
<td>100</td>
<td>Isocratic</td>
</tr>
<tr>
<td>26–27</td>
<td>0 to 85</td>
<td>100 to 15</td>
<td>Return to initial composition</td>
</tr>
<tr>
<td>27–40</td>
<td>85</td>
<td>15</td>
<td>Re-equilibration</td>
</tr>
</tbody>
</table>

Operate with a flow rate of 1.5 mL per minute. As a detector, use an ultraviolet spectrophotometer set at a wavelength of 220 nm. Maintain the column temperature at 50 °C.

Prepare as a diluent a solution containing 1.3 g/L of disodium hydrogen phosphate R and 6.8 g/L of potassium dihydrogen phosphate R in water R.

\(^{1}\) A YMC-Pack ODS-A and a Waters Atlantis T3 column were found suitable.
Prepare the following solutions:

For solution (1), dissolve 70.0 mg of the test substance in 25 mL of methanol R and dilute to 50.0 mL with the diluent. For solution (2), dilute 1.0 mL of solution (1) to 100.0 mL with a mixture of equal volumes of methanol R and the diluent. For solution (3), dilute 1.0 mL of solution (2) to 10.0 mL with a mixture of equal volumes of methanol R and the diluent. For solution (4), dissolve 3 mg of benzathine benzylpenicillin for peak identification RS (containing benzathine benzylpenicillin and the impurities A, B, C, D, E, F, G, H, I, J and K) in 1 mL of methanol R and dilute to 2 mL with the diluent.

Inject 20 µL each of solutions (1), (2), (3) and (4).

Use the chromatogram supplied with benzathine benzylpenicillin for peak identification RS and the chromatogram obtained with solution (4) to identify the peaks due to benzylpenicillin, benzathine and the impurities A, B, C, D, E, F, G, H, I, J and K in the chromatogram obtained with solution (1). Benzathine and the impurities, if present, are eluted at the following relative retentions with reference to benzylpenicillin (retention time about 7 minutes): impurity A about 0.18; benzathine about 0.30; impurity D about 0.36; impurity G about 0.38; impurity J about 0.44; impurity E about 0.51 and 0.60; impurity B about 0.69; impurity F about 0.84 and 0.88; impurity H about 1.22; impurity I about 1.42; impurity C about 1.75; and impurity K about 2.90.

The test is not valid unless, in the chromatogram obtained with solution (4), the resolution between the peaks due to the epimers of impurity F is at least 1.0 and between the peaks due to the impurities D and G is at least 1.5. Also, the test is not valid unless, in the chromatogram obtained with solution (3), the peak due to benzylpenicillin is obtained with a signal-to-noise ratio of at least 20.

In the chromatogram obtained with solution (1):
the sum of the areas of any peaks corresponding to impurity E (the sum of the isomers), when multiplied with a correction factor of 1.9, is not greater than 3 times the sum of the areas of the peaks due to benzathine and benzylpenicillin in the chromatogram obtained with solution (3) (0.3 %);

- the sum of the areas of any peaks corresponding to impurity F (the sum of the epimers), when multiplied with a correction factor of 1.5, is not greater than 3 times the sum of the areas of the peaks due to benzathine and benzylpenicillin in the chromatogram obtained with solution (3) (0.3 %);

- the area of any peak corresponding to impurity C is not greater than 2 times the sum of the areas of the peaks due to benzathine and benzylpenicillin in the chromatogram obtained with solution (2) (2.0 %);

- the area of any peak corresponding to impurity K is not greater than 0.5 times the sum of the areas of the peaks due to benzathine and benzylpenicillin in the chromatogram obtained with solution (2) (1.0 %);

- the area of any peak corresponding to impurity J is not greater than 0.5 times the sum of the areas of the peaks due to benzathine and benzylpenicillin in the chromatogram obtained with solution (2) (0.5 %);

- the area of any peaks corresponding to impurities A, B, D, G, H or I is not greater than twice the sum of the areas of the peaks due to benzathine and benzylpenicillin in the chromatogram obtained with solution (3) (0.2 %);

- the area of any other impurity peak is not greater than twice the sum of the areas of the peaks due to benzathine and benzylpenicillin in the chromatogram obtained with solution (3) (0.2 %).

- The sum of the areas of all impurity peaks, including the corrected areas of any peaks corresponding to impurities E and F, is not greater than 3.5 times the sum of the areas of the peaks due to benzathine and benzylpenicillin in the chromatogram obtained with solution (2) (3.5%). Disregard all peaks with an area of less than 0.5 times the sum of the areas of the peaks due to benzathine and benzylpenicillin in the chromatogram obtained with solution (3) (0.05%). Disregard also the peak due to benzathine.
**Assay.** Prepare the solutions immediately before use and by diluting to volume immediately after dissolution of the test and reference substance. Carry out the test as described under *1.14.4 High-performance liquid chromatography*, using the conditions given above under “Related substances” with the following modifications:

As the mobile phase, use a mixture of 15 volumes of mobile phase B and 85 volumes of mobile phase A.

Prepare the following solutions: for solution (1), dissolve 40.0 mg of the test substance in 50 mL of methanol R and dilute to 100.0 mL with the diluent. For solution (2), dissolve 40.0 mg of benzathine benzylpenicillin RS in 50 mL of methanol R, dilute to 100.0 mL with the diluent.

Inject 20 µL each of solutions (1) and (2) and record the chromatogram for 30 minutes. The substances are eluted in the order: benzathine, benzylpenicillin.

Measure the areas of the peaks corresponding to benzathine \((C_{16}H_{20}N_2)\) and to benzylpenicillin \((C_{16}H_{18}N_2O_4S)\) obtained in the chromatograms of solutions (1) and (2) and calculate the percentage content of benzathine \((C_{16}H_{20}N_2)\) and benzathine benzylpenicillin \((C_{16}H_{18}N_2O_4S)_2(C_{16}H_{20}N_2)\) in the sample using the declared contents of benzathine \((C_{16}H_{20}N_2)\) and benzathine benzylpenicillin \((C_{16}H_{18}N_2O_4S)_2(C_{16}H_{18}N_2O_4S)\) in benzathine benzylpenicillin RS.

A. For total penicillins. Dissolve about 0.065 g, accurately weighed, in 10 mL of dimethylformamide R and dilute with sufficient water to produce 1000 mL. Transfer two 2.0 mL aliquots of this solution into separate stoppered tubes. To one tube add 10.0 mL of imidazole/mercuric chloride TS, mix, stopper the tube and place it in a water-bath at 60 °C for exactly 25 minutes. Cool the tube rapidly to 20 °C (solution A).

To the second tube add 10.0 mL of water and mix (solution B).
Without delay measure the absorbance of a 1-cm layer at the maximum at about 325 nm against a solvent cell containing a mixture of 2.0 mL of water and 10.0 mL of imidazole/mercuric chloride TS for solution A and water for solution B.

From the difference between the absorbance of solution A and that of solution B, calculate the amount of \((\text{C}_{16}\text{H}_{18}\text{N}_2\text{O}_4\text{S})_2\text{C}_{16}\text{H}_{20}\text{N}_2\) in the substance being tested by comparison with 0.050 g of benzylpenicillin sodium RS similarly and concurrently examined, taking into account that each mg of benzylpenicillin sodium RS \((\text{C}_{16}\text{H}_{17}\text{N}_2\text{O}_4\text{S})\) is equivalent to 1.275 mg of \((\text{C}_{16}\text{H}_{18}\text{N}_2\text{O}_4\text{S})_2\text{C}_{16}\text{H}_{20}\text{N}_2\).

In an adequately calibrated spectrophotometer, the absorbance of the reference solution should be 0.62 ± 0.03.

B. For \(\text{C}_{16}\text{H}_{20}\text{N}_2\). To about 1 g, accurately weighed, add 30 mL of sodium chloride \((400 \text{ g/l})\) TS and 10 mL of sodium hydroxide \((~150 \text{ g/l})\) TS, shake well, and extract with four quantities, each of 50 mL of ether R. Wash the combined extracts with three quantities, each of 10 mL of water, extract the combined washings with 25 mL of ether R, and add the extract to the main ether solution. Evaporate the ether solution to a low bulk, add 2 mL of dehydrated ethanol R and evaporate to dryness. To the residue add 50 mL of glacial acetic acid R and titrate with perchloric acid \((0.1 \text{ mol/l})\) VS, using 1 mL of 1-naphtholbenzein/acetic acid TS as indicator. Repeat the operation without the substance being examined; the difference between the titrations represents the amount of perchloric acid required to neutralize the liberated base. Each mL of perchloric acid \((0.1 \text{ mol/l})\) VS is equivalent to 12.02 mg of \(\text{C}_{16}\text{H}_{20}\text{N}_2\).

**Additional Requirements for Benzathine Benzylpenicillin for sterile use**

**Storage.** Sterile Benzathine benzylpenicillin should be kept in a hermetically closed container, protected from light.

**Bacterial endotoxins.** If intended for use in the manufacture of a parenteral dosage form without a further appropriate procedure for the removal of bacterial endotoxins,
carry out the test as described under 3.4 Test for bacterial endotoxins; contains not more than 0.01 IU of endotoxin RS per mg of benzathine benzylpenicillin.

**Sterility.** If intended for use in the manufacture of a parenteral and/or sterile dosage form without a further appropriate sterilization procedure, complies with 3.2 Test for sterility.

Additional requirements for Benzathine benzylpenicillin for parenteral use.

Complies with the monograph for "Parenteral preparations".

**Impurities**

A. \( N^1 \)-benzylethane-1,2-diamine.

B. Phenylactic acid.

C. \((2,5,4S)-2-\{(1\Xi)-2-\text{benzyloxy}-2-(\text{benzylamino})\text{ethyl}\}\text{amino}-2-\text{oxo}-1-(2\text{-phenylacetamido})\text{ethyl}-5,5\text{-dimethyl}-1,3\text{-thiazolidine-4-carboxylic acid (benzylpenicilloic acids benzathide).}
D. \((3S,7R,7aR)-5\text{benzyl-}2,2\text{-dimethyl-}2,3,7,7a\text{-tetrahydroimidazo}[5,1-}
\[b][1,3]\text{thiazole-3,7-dicarboxylic acid (penillic acid of benzylpenicillin).}

E. \((2\Xi,4S)-2\text{-(\Xi)-carboxy(2-phenylacetamido)methyl}-5,5\text{-dimethyl-1,3-}
\text{thiazolidine-4-carboxylic acid (penilloic acids of benzylpenicillin).}

F. \((2RS,4S)-5,5\text{-dimethyl-2-[(2-phenylacetamido)methyl]-1,3-thiazolidine-4-}
\text{carboxylic acid (penilloic acids of benzylpenicillin).}

G. \((2S,5R,6R)-6\text{-[2-(4-hydroxyphenyl)acetamido]-3,3-dimethyl-7-oxo-4-thia-1-}
\text{azabicyclo[3.2.0]heptane-2-carboxylic acid.}
H. \((2S,5R,6R)-6-[(3Z)-\text{hex-3-enamido}]-3,3\text{-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (isopenicillin F).}\)

I. \((2S,5R,6R)-6\text{-hexanamido-3,3\text{-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (dihydropenicillin F).}\)

J. Unknown structure.

K. \((2R,2'R,4S,4'S)-2,2'\text{-[(4R,11R)-6,9\text{-dibenzyl-2,5,10,13-tetraoxo-1,14-diphenyl-3,6,9,12-tetraazatetradecane-4,11-diyl]bis(5,5\text{-dimethyl-1,3-thiazolidine-4-carboxylic acid).}\)
Reference substances evoked in the monograph

Benzathine benzylpenicillin for peak identification RS

It is intended to refer to the corresponding reference substance established by the European Pharmacopoeia.

Benzathine benzylpenicillin RS

ICRS to be established.

Reagents to be established or revised

Sodium hydroxide (~0.8 g/L) TS

A solution of sodium hydroxide R containing about 0.8 g/L of NaOH (approximately 0.02 mol/L).

Bromothymol blue/ethanol TS

Procedure. Warm 0.1 g of bromophenol blue R with 3.2 mL of sodium hydroxide (0.05 mol/L) VS and 5 mL of ethanol (~710 g/L) TS; after solution has been effected add a sufficient quantity of ethanol (~150 g/L) TS to produce 250 mL.

Procedure. Dissolve 50 mg of bromothymol blue R in a mixture of 4 mL of sodium hydroxide (~0.8 g/L) TS and 20 mL of ethanol (~710 g/L) TS R and dilute to 100 mL with water R.

Test for sensitivity. To 0.3 mL of bromothymol blue solution R1, add 100 mL of carbon dioxide-free water R. The solution is yellow. Not more than 0.1 mL of 0.02 M sodium hydroxide is required to change the colour to blue.

Colour change: pH 5.8 (yellow) to pH 7.4 (blue).