Detection: spectrophotometer at 254 nm.

Injection: 20 µl; inject the test solution and reference solutions (d) and (e).

System suitability: reference solution (d):
- resolution: minimum 4.0 between the peaks due to impurity A (1st peak) and oxytetracycline (2nd peak) and minimum 5.0 between the peaks and due to oxytetracycline and impurity B (3rd peak); adjust the 2-methyl-2-propanol content in the mobile phase if necessary,
- symmetry factor: maximum 1.25 for the peak due to oxytetracycline.

Limits:
- impurity A: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (e) (0.5 per cent),
- impurity B: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (e) (2.0 per cent),
- impurity C (eluting on the tail of the principal peak): not more than 4 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (e) (2.0 per cent),
- disregard limit: 0.02 times the area of the peak due to oxytetracycline in the chromatogram obtained with reference solution (d) (0.1 per cent).

Heavy metals (2.4.8): maximum 50 ppm.

0.5 g complies with limit test F. Prepare the standard using 2.5 ml of lead standard solution (10 ppm Pb) R.

Water (2.5.12): 6.0 per cent to 9.0 per cent, determined on 0.250 g.

Sulphated ash (2.4.14): maximum 0.5 per cent, determined on 1.0 g.

ASSAY
Liquid chromatography (2.2.27) as described in the test for related substances with the following modification.

Injection: test solution and reference solution (a).

Calculate the percentage content of C₂₂H₂₄N₂O₉.

STORAGE
In an airtight container, protected from light.

IMPURITIES

A. R₁ = NH₂, R₂ = N(CH₃)₂, R₃ = R₄ = H, R₅ = OH:
(4S,4aR,5,5aR,6S,12aS)-2-acetyl-4-(dimethylamino)-3,5,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (4-epoxytetracycline),
B. R₁ = NH₂, R₂ = R₄ = R₅ = H, R₃ = N(CH₃)₂:
- tetracycline,
- 12a-hexahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide hydrochloride.

Substance produced by the growth of certain strains of Streptomyces rimosus or obtained by any other means.

Content: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS
Appearance: yellow, crystalline powder, hygroscopic. Solubility: freely soluble in water, sparingly soluble in alcohol. Solutions in water become turbid on standing, owing to the precipitation of oxytetracycline.

IDENTIFICATION
A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 5 mg of the substance to be examined in methanol R and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 5 mg of oxytetracycline hydrochloride CRS in methanol R and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 5 mg of oxytetracycline hydrochloride CRS, 5 mg of tetracycline hydrochloride R and 5 mg of minocycline hydrochloride R in methanol R and dilute to 10 ml with the same solvent.

Plate: TLC octadecylsilica gel F₂₅₄ plate R.

Mobile phase: mix 20 volumes of acetonitrile R, 20 volumes of methanol R and 60 volumes of a 63 g/l solution of oxalic acid R previously adjusted to pH 2 with concentrated ammonia R.

Application: 1 µl.

Development: over 3/4 of the plate.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm.

System suitability: the chromatogram obtained with reference solution (b) shows 3 clearly separated spots.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

Oxytetracycline hydrochloride

Mobile phase: weigh 60.0 g of 2-methyl-2-propanol R and transfer to a 1000 ml volumetric flask with the aid of 200 ml of water R; add 60 ml of 0.33 M phosphate buffer solution pH 7.5 R, 50 ml of a 10 g/l solution of tetrabutylammonium hydrogen sulphate R adjusted to pH 7.5 with dilute sodium hydroxide solution R and 30 ml of a 0.4 g/l solution of sodium edetate R adjusted to pH 7.5 with dilute sodium hydroxide solution R; dilute to 1000 ml with water R.

Flow rate: 1.0 ml/min.

OXYTETRACYCLINE HYDROCHLORIDE

Oxytetracyclini hydrochloridum

\[ \text{C}_{22}\text{H}_{24}\text{N}_{2}\text{O}_{9} \]

M_r 496.9
B. To about 2 mg add 5 ml of sulphuric acid R. A deep red colour develops. Add the solution to 2.5 ml of water R. The colour becomes yellow.

C. It gives reaction (a) of chlorides (2.3.J).

TESTS

**pH (2.2.3):** 2.3 to 2.9.

Dissolve 0.1 g in 10 ml of carbon dioxide-free water R.

**Specific optical rotation (2.2.7):** –188 to –200 (anhydrous substance).

Dissolve 0.250 g in 0.1 M hydrochloric acid and dilute to 25.0 ml with the same acid.

**Specific absorbance (2.2.25):** 270 to 290 determined at 353 nm (anhydrous substance).

Dissolve 20.0 mg in buffer solution pH 2.0 R and dilute to 100.0 ml with the same buffer solution. Dilute 10.0 ml of the solution to 100.0 ml with buffer solution pH 2.0 R.

**Light-absorbing impurities.** Carry out the measurements within 1 h of preparing the solutions.

Dissolve 20.0 mg in a mixture of 1 volume of 1 M hydrochloric acid and 99 volumes of methanol R and dilute to 10.0 ml with the same mixture of solvents. The absorbance (2.2.25) determined at 430 nm has a maximum of 0.50 (anhydrous substance).

Dissolve 0.100 g in a mixture of 1 volume of 1 M hydrochloric acid and 99 volumes of methanol R and dilute to 10.0 ml with the same mixture of solvents. The absorbance (2.2.25) determined at 490 nm has a maximum of 0.20 (anhydrous substance).

**Related substances.** Liquid chromatography (2.2.29).

**Test solution.** Dissolve 20.0 mg of the substance to be examined in 0.01 M hydrochloric acid and dilute to 25.0 ml with the same acid.

**Reference solution (a).** Dissolve 20.0 mg of oxytetracycline CRS in 0.01 M hydrochloric acid and dilute to 25.0 ml with the same acid.

**Reference solution (b).** Dissolve 20.0 mg of 4-epi oxytetracycline CRS in 0.01 M hydrochloric acid and dilute to 25.0 ml with the same acid.

**Reference solution (c).** Dissolve 20.0 mg of tetracycline hydrochloride CRS in 0.01 M hydrochloric acid and dilute to 25.0 ml with the same acid.

**Reference solution (d).** Dissolve 8.0 mg of α-apo oxytetracycline CRS in 5 ml of 0.01 M sodium hydroxide and dilute to 100.0 ml with 0.01 M hydrochloric acid.

**Reference solution (e).** Dissolve 8.0 mg of β-apo oxytetracycline CRS in 5 ml of 0.01 M sodium hydroxide and dilute to 100.0 ml with 0.01 M hydrochloric acid.

**Reference solution (f).** Mix 1.5 ml of reference solution (a), 1.0 ml of reference solution (b), 3.0 ml of reference solution (c), 3.0 ml of reference solution (d) and 3.0 ml of reference solution (e) and dilute to 25.0 ml with 0.01 M hydrochloric acid.

**Reference solution (g).** Mix 1.0 ml of reference solution (b), 4.0 ml of reference solution (c) and 40.0 ml of reference solution (e) and dilute to 200.0 ml with 0.01 M hydrochloric acid.

**Column:**

- **size:** l = 0.25 m, Ø = 4.6 mm,
- **stationary phase:** styrene-divinylbenzene copolymer R (8 µm),
- **temperature:** 60 °C.

**Mobile phase:** weigh 30.0 g (for mobile phase A) and 100.0 g (for mobile phase B) of 2-methyl-2-propanol R and transfer separately to 1000 ml volumetric flasks with the aid of 200 ml of water R; to each flask add 60 ml of 0.33 M phosphate buffer solution pH 7.5 R, 50 ml of a 10 g/l solution of tetrabutylammonium hydrogen sulphate R adjusted to pH 7.5 with dilute sodium hydroxide solution R and 10 ml of a 0.4 g/l solution of sodium edetate R adjusted to pH 7.5 with dilute sodium hydroxide solution R; dilute each solution to 1000 ml with water R.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Mobile phase A (per cent V/V)</th>
<th>Mobile phase B (per cent V/V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 15</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>15 - 30</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>30 - 45</td>
<td>70</td>
<td>30</td>
</tr>
</tbody>
</table>

**Flow rate:** 1 ml/min.

**Detection:** spectrophotometer at 254 nm.

**Injection:** 20 µl; inject the test solution and reference solutions (f) and (g).

**System suitability: reference solution (f):**

- **resolution:** minimum 4.0 between the peaks due to impurity A (1st peak) and oxytetracycline (2nd peak), minimum 5.0 between the peaks due to oxytetracycline and impurity B (3rd peak) and minimum 3.5 between the peaks due to impurity D (4th peak) and impurity E (5th peak); if necessary, adapt the ratio mobile phase A: mobile phase B and/or adjust the time programme used to produce the one-step gradient elution,

- **symmetry factor:** maximum 1.25 for the peak due to oxytetracycline.

**Limits:**

- **impurity A:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (g) (0.5 per cent),

- **impurity B:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (g) (2.0 per cent),

- **impurity C (eluting on the tail of the main peak):** not more than 4 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (g) (2.0 per cent),

- **total of impurities D, E and F (eluting between the latter two):** not more than the area of the peak due to impurity E in the chromatogram obtained with reference solution (g) (2.0 per cent),

- **disregard limit:** 0.02 times the area of the peak due to oxytetracycline in the chromatogram obtained with reference solution (f) (0.1 per cent).  

**Heavy metals (2.4.8):** maximum 50 ppm.

0.5 g complies with limit test F. Prepare the standard using 2.5 ml of lead standard solution (10 ppm Pb) R.

**Water (2.5.12):** maximum 2.0 per cent, determined on 0.500 g.

**Sulphated ash (2.4.14):** maximum 0.5 per cent, determined on 1.0 g.

**Bacterial endotoxins (2.6.14):** less than 0.4 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

**ASSAY**

Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.
Injection: test solution and reference solution (a).
Calculate the percentage content of $C_{22}H_{25}ClN_2O_9$ taking 1 mg of oxytetracycline as equivalent to 1.079 mg of oxytetracycline hydrochloride.

STORAGE
In an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING
The label states, where applicable, that the substance is sterile, store in a sterile, airtight, tamper-proof container.

IMPURITIES
from bacterial endotoxins.

**CHARACTERS**
A white or almost white powder, hygroscopic, very soluble in water and in dilute solutions of acetic acid and of ethanol.

**IDENTIFICATION**
Examine the chromatograms obtained in the assay. The retention time of the principal peak in the chromatogram obtained with the test solution is approximately the same as that of the principal peak in the chromatogram obtained with the reference solution.

**TESTS**

**pH** (2.2.3). Dissolve 0.200 g in carbon dioxide-free water R and dilute to 10.0 ml with the same solvent. The pH of the solution is 3.0 to 6.0.

**Amino acids.** Examine by means of an amino-acid analyser. Standardise the apparatus with a mixture containing equimolar amounts of ammonia, glycine and the L-form of the following amino acids:

- lysine
- threonine
- alanine
- leucine
- histidine
- serine
- valine
- tyrosine
- arginine
- glutamic acid
- methionine
- phenylalanine
- aspartic acid
- proline
- isoleucine

together with half the equimolar amount of t-cystine. For the validation of the method, an appropriate internal standard, such as DL-norleucine R, is used.

**Test solution.** Place 1.0 mg of the substance to be examined in a rigorously cleaned hard-glass tube 100 mm long and 6 mm in internal diameter. Add a suitable amount of a 50 per cent $V/V$ solution of hydrochloric acid R. Immerse the tube in a freezing mixture at $-5\,^\circ C$, reduce the pressure to below 133 Pa and seal. Heat at 110 °C to 115 °C for 16 h. Cool, open the tube, transfer the contents to a 10 ml flask with the aid of five quantities, each of 0.2 ml, of water R and evaporate to dryness over potassium hydroxide R under reduced pressure. Take up the residue in water R and evaporate to dryness over potassium hydroxide R under reduced pressure; repeat these operations once. Take up the residue in a buffer solution suitable for the amino-acid analyser used and dilute to a suitable volume with the same buffer solution. Apply a suitable volume to the amino-acid analyser.

See the information section on general monographs (cover pages)