3 min each time. To the combined organic layers add 10 ml of water R and shake for 3 min. The aqueous layer complies with the limit test for iron (10 ppm).

**Heavy metals** (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of lead standard solution (10 ppm Pb) R.

**Loss on drying** (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

**Sulphated ash** (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

**ASSAY**

Dissolve 0.150 g in 5 ml of anhydrous formic acid R. Add 30 ml of anhydrous acetic acid R. Titrate with 0.1 M perchloric acid, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M perchloric acid is equivalent to 18.12 mg of C₉H₁₁NO₃.

**STORAGE**

Store protected from light.

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**Definition**

Mixture of antimicrobial linear and cyclic polypeptides, isolated from the fermentation broth of *Brevibacillus brevis* Dubos. It consists mainly of gramicidins and tyrocidins as described above; other related compounds may be present in smaller amounts.

**Potency**: 180 IU/mg to 280 IU/mg (dried substance).

**Characters**

**Appearance**: white or almost white powder.

**Solubility**: practically insoluble in water, soluble in alcohol and in methanol.

**Identification**

First identification: B.

Second identification: A.

A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 5 mg of the substance to be examined in 4.0 ml of alcohol R.

Reference solution. Dissolve 5 mg of tyrothricin CRS in 4.0 ml of alcohol R.

Plate: TLC silica gel F₅₄ plate R.


Application: 1 µl.

Development: over 2/3 of the plate.

Drying: in a current of warm air.

Detection A: examine in ultraviolet light at 254 nm.

Results A: the principal spots or groups of principal spots in the chromatogram obtained with the test solution are similar in position and size to the principal spots or groups of principal spots in the chromatogram obtained with the reference solution. The upper group corresponds to gramicidins, the lower group to tyrocidins.

Detection B: spray with dimethylaminobenzaldehyde solution R2. Heat the plate in a current of warm air until the spots appear.

System suitability: reference solution:

– the chromatogram shows 2 clearly separated spots or groups of spots.

Results B: the principal spots or groups of principal spots in the chromatogram obtained with the test solution are similar in position, colour and size to the principal spots or groups of principal spots in the chromatogram obtained with the reference solution. The upper group corresponds to gramicidins, the lower group to tyrocidins.

B. It complies with the test for composition (see Tests).

**Tests**

**Composition**. Liquid chromatography (2.2.29): use the normalisation procedure. Prepare the solutions immediately before use.

**Test solution**. Dissolve 25 mg of the substance to be examined in 10 ml of methanol R and dilute to 25.0 ml with the mobile phase.

**Reference solution (a)**. Dissolve 25 mg of tyrothricin CRS in 10 ml of methanol R and dilute to 25.0 ml with the mobile phase.

**Reference solution (b)**. Dilute 1.0 ml of reference solution (a) to 50.0 ml with the mobile phase.

**Column**:

– size: l = 0.25 m, Ø = 4.6 mm,
– stationary phase: octadecylsilica gel for chromatography R (5 µm),
– temperature: 60 °C.

**Mobile phase**: 0.79 g/l solution of ammonium sulphate R, methanol R (25.75 V/V).

**Flow rate**: 1.2 ml/min.

**Detection**: spectrophotometer at 280 nm.

**Injection**: 25 µl.
Run time: 6 times the retention time of gramicidin A1. Use the chromatogram obtained with reference solution (a) and the chromatogram supplied with tyrothricin CRS to identify the peaks due to gramicidin A1, gramicidin A2 and the tyrocidins.

Relative retention with reference to gramicidin A1 (retention time = about 10 min): gramicidin C1 = about 0.8; gramicidin C2 = about 0.9; gramicidin A2 = about 1.1; tyrocidins = about 1.5 to 6.

System suitability: reference solution (a):
– resolution: minimum 1.5 between the peaks due to gramicidin A1 and gramicidin A2.

Composition:
– sum of gramicidins: 25 per cent to 50 per cent,
– sum of tyrocidins: 50 per cent to 70 per cent,
– total: minimum 85 per cent,

– disregard limit: the sum of the areas of the peaks due to gramicidins in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32): maximum 4.0 per cent, determined on 1.000 g by drying under high vacuum at 60 °C for 3 h.

Sulphated ash (2.4.14): maximum 1.5 per cent, determined on 1.0 g.

ASSAY
Carry out the microbiological assay of antibiotics (2.7.2) using the turbidimetric method. Use gramicidin CRS as the reference substance.

Test solution. Prepare a solution of tyrothricin containing about the same amount of gramicidin as the corresponding solution of gramicidin CRS i.e. 5 times more concentrated.

STORAGE
In an airtight container, protected from light.