CHARACTERS

A white or almost white, crystalline powder, slightly hygroscopic, freely soluble in water, soluble in alcohol.

It melts at about 148 °C or it may occur in either of 2 other crystalline forms which melt respectively at about 134 °C and 139 °C. Mixtures of these forms melt within the range 134 °C to 147 °C.

IDENTIFICATION

First identification: A, B, D.

Second identification: B, C, D.

A. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *tetracaine hydrochloride CRS*.

B. To 10 ml of solution S (see Tests) add 1 ml of *ammonium thiocyanate solution R*. A white, crystalline precipitate is formed which, after recrystallisation from *water R* and drying at 80 °C for 2 h, melts (*2.2.14*) at about 131 °C.

C. To about 5 mg add 0.5 ml of *fuming nitric acid R*. Evaporate to dryness on a water-bath, allow to cool and dissolve the residue in 5 ml of *acetone R*. Add 1 ml of *0.1 M alcoholic potassium hydroxide*. A violet colour develops.

D. Solution S gives reaction (a) of chlorides (*2.3.1*).

TESTS

Solution S. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Dilute 2 ml of solution S to 10 ml with *water R*. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

pH (*2.2.3*). Dilute 1 ml of solution S to 10 ml with *carbon dioxide-free water R*. The pH of the solution is 4.5 to 6.5.

Related substances. Examine by thin-layer chromatography (*2.2.27*), using a *TLC silica gel GF_{254} plate R*. Carry out a preliminary development over a path of 12 cm using a mixture of 4 volumes of *glacial acetic acid R*, 16 volumes of *hexane R* and 80 volumes of *dibutyl ether R*. Remove the plate and dry it in a current of warm air for a few minutes. Allow the plate to cool before use.

Test solution. Dissolve 1.0 g of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 50 mg of *4-aminobenzoic acid R* in *water R* and dilute to 100 ml with the same solvent. Dilute 1 ml of the solution to 10 ml with *water R*.

Apply to the plate 5 µl of each solution. Develop over a path of 10 cm using a mixture of 4 volumes of *glacial acetic acid R*, 16 volumes of *hexane R* and 80 volumes of *dibutyl ether R*. Dry the plate at 100 °C to 105 °C for 10 min and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.05 per cent). The principal spot in the chromatogram obtained with the test solution remains at the starting point.

Heavy metals (*2.4.8*). 12 ml of solution S complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (*2.2.32*). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

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

ASSAY

Dissolve 0.250 g in 50 ml of *alcohol R* and add 5.0 ml of *0.01 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 ml of *0.1 M sodium hydroxide* is equivalent to 30.08 mg of $C_{15}H_{25}ClN_2O_2$.

STORAGE

Store protected from light.

01/2008:0644

TETRACOSACTIDE

Tetracosactidum

H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-OH

$C_{136}H_{210}N_{40}O_{31}S$ M_r 2933

[16960-16-0]

DEFINITION

Tetracosactide is a synthetic tetracosapeptide in which the sequence of amino acids is the same as that of the first twenty-four residues of human corticotropin. It is available as an acetate and contains water. It increases the rate at which corticoid hormones are secreted by the adrenal glands. The potency is not less than 800 International Units per milligram, calculated with reference to the anhydrous, acetic acid-free substance.

CHARACTERS

A white or yellow, amorphous powder, sparingly soluble in water.

IDENTIFICATION

A. It increases the amount of corticosterone produced by isolated rat adrenal cells in the conditions of the assay.

B. Examine by electrophoresis (*2.2.31*) and thin-layer chromatography (*2.2.27*) to obtain a two-dimensional separation using two plates with *cellulose for chromatography R1* as the coating substance.

Test solution. Dissolve 1 mg of the substance to be examined in 0.2 ml of a 15.4 g/l solution of *ammonium acetate R* adjusted to pH 8.2 with *dilute ammonia R2*. Add 10 µl of a 2 g/l solution of *trypsin R*, maintain the mixture at 37 °C to 38 °C for 40 min, heat on a water-bath for 3 min and add 5 µl of *glacial acetic acid R*. Evaporate to dryness at 40 °C at a pressure not exceeding 3 kPa, dry the glassy residue at 40 °C for 1 h and dissolve in 0.1 ml of *glacial acetic acid R*. Dry the solution from the frozen state, dissolve the residue in 0.1 ml of *water R* and dry again from the frozen state. Dry the final residue at 45 °C for 1 h at a pressure not exceeding 3 kPa and dissolve in 50 µl of *water R*.

Reference solution. Prepare at the same time and in the same manner as the test solution, using *tetracosactide CRS* instead of the substance to be examined.

Spray the plates with the electrolyte solution which consists of a solution containing 0.2 per cent *V/V* of *glacial acetic acid R* and 0.2 per cent *V/V* of *pyridine R*. Place the filter paper tongues to connect the plates with the appropriate compartment of each trough so that each

tongue covers an area 1.5 cm wide at one end of the plate. Close the tank and allow to stand for 30 min. Apply the solutions on the anodic side. Apply to the first plate, at a point about 2.5 cm from each of two adjacent edges, 4 µl of the test solution. Apply to the second plate, at a similar position, 4 µl of the reference solution. Apply to both plates a potential of 280 V for a plate 200 mm long and allow electrophoresis to proceed for 90 min. Allow the plates to dry in air for 30 min and then dry in a current of air at 30 °C for 30 min. Carry out a second separation on each plate by thin-layer chromatography. Develop at right angles to the direction of electrophoresis over a path of 15 cm using a mixture of 8 volumes of *glacial acetic acid R*, 24 volumes of *pyridine R*, 30 volumes of *water R* and 38 volumes of *butanol R*. Dry the plates in a current of air and spray with *ninhydrin solution R1*. The principal spots in the chromatogram obtained with the test solution are similar in position to those in the chromatogram obtained with the reference solution, but their intensity may differ.

TESTS

Specific optical rotation (*2.2.7*). Dissolve 10.0 mg in 1.0 ml of a mixture of 1 volume of *glacial acetic acid R* and 99 volumes of *water R*. The specific optical rotation is – 99 to – 109, calculated with reference to the anhydrous, acetic acid-free substance.

Absorbance (*2.2.25*). Dissolve 1.0 mg in *0.1 M hydrochloric acid* and dilute to 5.0 ml with the same acid. Examined between 240 nm and 280 nm, the solution shows an absorption maximum at 276 nm. The absorbance at the maximum is 0.51 to 0.61, calculated with reference to the anhydrous, acetic acid-free substance. The ratio of the absorbance at the maximum at 276 nm to the absorbance at 248 nm is 2.4 to 2.9.

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 L-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 °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.

Express the content of each amino acid in moles. Calculate the relative proportions of the amino acids, taking that for valine to be equivalent to three. The values fall within the following limits: lysine 3.5 to 4.7; histidine 0.9 to 1.1; arginine 2.7 to 3.3; serine 1.1 to 2.2; glutamic acid 0.9 to 1.1; proline 2.5 to 3.5; glycine 1.8 to 2.2; methionine 0.9 to 1.1; tyrosine 1.7 to 2.2; phenylalanine 0.9 to 1.1. Not more than traces of other amino acids are present, with the exception of tryptophan.

Related peptides

A. Examine by liquid chromatography (*2.2.29*). Use degassed solvents.

Test solution. Dissolve 1.0 mg of the substance to be examined in 1 ml of *water R*.

Reference solution (a). Dissolve 1.0 mg of the substance to be examined in 1 ml of a 1 per cent *V/V* solution of *glacial acetic acid R* and add 50 µl of a mixture of 1 volume of *strong hydrogen peroxide solution R* and 999 volumes of *water R*. Allow to stand for 2 h.

Reference solution (b). Dissolve 1.0 mg of *tetracosactide CRS* in 1 ml of *water R*.

The chromatographic procedure may be carried out using:

– a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (10 µm),
– as mobile phase at a flow rate of 2.0 ml/min, a mixture of 365 ml of *acetonitrile R*, 10.0 ml of *glacial acetic acid R* and 10.0 g of *ammonium sulphate R*, diluted to 2000 ml with *water R*,
– as detector a spectrophotometer set at 280 nm.

Inject 20 µl of each solution ensuring that the syringe used to inject the test solution is not contaminated with peroxide. The chromatogram obtained with reference solution (a) shows a peak due to tetracosactide corresponding to the principal peak in the chromatogram obtained with the test solution and a peak with a lower retention time, due to tetracosactide sulphoxide, of significantly greater area than any corresponding peak in the chromatogram obtained with the test solution. The test is not valid unless in the chromatogram obtained with reference solution (a), the resolution between the peaks corresponding to tetracosactide and tetracosactide sulphoxide is at least 7. In the chromatogram obtained with the test solution, the area of the peak corresponding to tetracosactide sulphoxide is not greater than 4 per cent of the sum of the areas of all the peaks, disregarding any peaks due to the solvent and the mobile phase.

B. Examine by thin-layer chromatography (*2.2.27*), using *cellulose for chromatography R* as the coating substance.

Test solution. Dissolve 3.0 mg of the substance to be examined in 1.5 ml of a mixture of equal volumes of *dilute acetic acid R* and *water R*.

Reference solution (a). Dilute 0.5 ml of the test solution to 10 ml with a mixture of equal volumes of *dilute acetic acid R* and *water R*.

Reference solution (b). Dilute 5 ml of reference solution (a) to 10 ml with a mixture of equal volumes of *dilute acetic acid R* and *water R*.

Reference solution (c). To 0.5 ml of the test solution add 50 µl of a mixture of 1 volume of *strong hydrogen peroxide solution R* and 999 volumes of *water R*. Allow to stand for 2 h.

Apply to the plate as 1 cm bands 10 µl of each solution. Develop over a path of 15 cm using a mixture of 4 volumes of *glacial acetic acid R*, 24 volumes of *pyridine R*, 30 volumes of *water R* and 42 volumes of *butanol R*. Dry the plate in a current of air and spray with *ninhydrin solution R1*. In the chromatogram obtained with reference solution (c), the band corresponding in position

to the principal band in the chromatogram obtained with the test solution is reduced in intensity, and a prominent band, due to tetracosactide sulphoxide, of lower R_F is present. In the chromatogram obtained with the test solution, any band, apart from the principal band and any band due to tetracosactide sulphoxide, is not more intense than the band in the chromatogram obtained with reference solution (a) (5.0 per cent) and at most one such band is more intense than the band in the chromatogram obtained with reference solution (b) (2.5 per cent).

Peptide. Not less than 85.0 per cent of peptide, expressed as $C_{136}H_{210}N_{40}O_{31}S$, calculated with reference to the anhydrous, acetic acid-free substance.

Examine the chromatograms obtained in test A for related peptides. Calculate the content of $C_{136}H_{210}N_{40}O_{31}S$ from the peak heights or areas in the chromatograms obtained with the test solution and reference solution (b), and the declared content of $C_{136}H_{210}N_{40}O_{31}S$ in *tetracosactide CRS*.

Acetic acid (*2.5.34*): 8.0 per cent to 13.0 per cent.

Test solution. Dissolve 10.0 mg of the substance to be examined in a mixture of 5 volumes of mobile phase B and 95 volumes of mobile phase A and dilute to 10.0 ml with the same mixture of solvents.

Water (*2.5.12*): 5.0 per cent to 16.0 per cent, determined on 80.0 mg by the semi-micro determination of water.

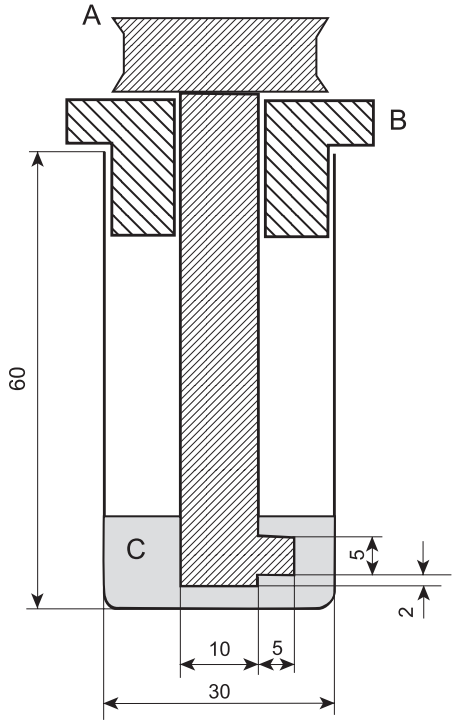
ASSAY

The potency of tetracosactide is estimated by comparing in given conditions its activity in increasing the amount of corticosterone produced by isolated rat adrenal cells with that of the International Reference Preparation of tetracosactide or a reference preparation calibrated in International Units.

The International Unit is the activity contained in a stated amount of the International Reference Preparation which consists of a quantity of synthetic tetracosactide with mannitol. The equivalence in International Units of the International Reference Preparation is stated by the World Health Organisation.

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits (P = 0.95) of the estimated potency are not less than 64 per cent and not more than 156 per cent of the stated potency.

Use siliconised glassware and rinse well with *water R* before use. Euthanise four male rats, each weighing between 200 g and 400 g, by exsanguination. Remove the adrenal glands, carefully free them of adhering fat and immerse in solution B maintained at 4 °C. Cut each gland into four equal pieces and transfer to a suitable plastic stirring apparatus (see Figure 0644.-1) containing 5 ml of solution C maintained at 37 °C. Disperse the adrenal cells by stirring the mixture at 500 r/min. After 20 min, remove the supernatant liquid, cool to 4 °C, add 5 ml of solution C and repeat the dispersal procedure. Repeat the operation a further three times. Combine the five supernatant liquids, centrifuge at 4 °C in a polyethylene test-tube for 30 min after slow acceleration to 100 *g*. Suspend the resulting pellet in 8 ml of solution D and centrifuge for 30 min at 100 *g*. Again suspend the residue in 8 ml of solution D and filter the mixture through nylon gauze with 100 µm pores into a polyethylene beaker and add a suitable volume of solution D to the filtrate (a total volume of 65 ml to 105 ml has been found suitable). Maintain the resulting suspension at 4 °C.



A - pulley and stirring paddle,

B - bearing,

C - incubation medium.

Figure 0644.-1. – *Stirring Apparatus*

Dimensions in millimetres

Prepare four independent dilutions, with two-fold dose intervals, from solutions of suitable concentrations of the substance to be examined and of the reference preparation using solution E as diluent. Pipette 0.1 ml of each dilution into each of four polystyrene test-tubes and add 1.0 ml of the cell suspension prepared above to each tube. Incubate at 37 °C for 2 h and then cool to 4 °C. Transfer 1 ml of the contents of each tube to a glass tube containing 1.4 ml of *methylene chloride R*, mix in a vortex mixer for 10 s and centrifuge at 3000 *g* for 5 min. Transfer 1 ml of each of the methylene chloride layers, avoiding taking up aqueous phase, to a glass tube containing 0.6 ml of a mixture of 15 volumes of *alcohol R* and 35 volumes of *sulphuric acid R*. Mix in a vortex mixer for 10 s and centrifuge at 1500 *g* for 5 min. Allow each tube to stand for 30 min. Examine by fluorimetry (*2.2.21*), irradiating the lower layer with an excitant beam of suitable wavelength such as 436 nm or 470 nm and measuring the fluorescence at the maximum between 530 nm and 545 nm. If the solutions are not transferred to spectrophotometric measurement cells, select glass tubes that give fluorescence values for a standard corticosterone which do not differ from each other by more than 5 per cent. Calculate the result of the assay by the usual statistical methods using the linear portion of the log dose-response curve.

Solution A

Sodium chloride R	6.60 g
Potassium chloride R	0.353 g
Sodium hydrogen carbonate R	0.840 g
Potassium dihydrogen phosphate R	0.161 g

Magnesium sulphate R	0.291 g
Calcium chloride R	0.373 g
2-[4-(2-Hydroxyethyl)piperazin-1-yl]ethane sulphonic acid R	4.77 g

Dissolve the above ingredients in about 950 ml of *water R*, adjust to pH 7.4 with *1 M sodium hydroxide* and add 60 mg of *benzylpenicillin sodium R* and a quantity of *streptomycin sulphate R* equivalent to 100 mg of streptomycin. Dilute to 1000 ml with *water R*.

Solution B

Add 2 g/l of *glucose R* to solution A.

Solution C

To solution B add 1 g/l of a preparation of collagenase obtained from *Clostridium histolyticum* and of a grade suitable for the preparation of dispersed cells.

Solution D

Add 5 g/l of *bovine albumin R* to solution B.

Solution E

A 9 g/l sterile solution of *sodium chloride R* containing 1 g/l of *bovine albumin R* and adjusted to pH 2.0 with *1 M hydrochloric acid*.

STORAGE

Store under nitrogen, protected from light, at a temperature between 2 °C and 8 °C.

LABELLING

The label states:

– the potency in International Units per milligram,

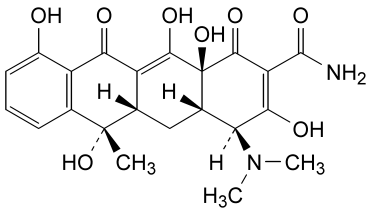
– the peptide content per container,

– the storage conditions.

01/2008:0211
corrected 6.0

TETRACYCLINE

Tetracyclinum



$C_{22}H_{24}N_2O_8$ M_r 444.4
[60-54-8]

DEFINITION

(4*S*,4a*S*,5a*S*,6*S*,12a*S*)-4-(Dimethylamino)-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide.

Substance produced by certain strains of *Streptomyces aerofaciens* or obtained by any other means.

Content: 88.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

Appearance: yellow, crystalline powder.

Solubility: very slightly soluble in water, soluble in ethanol (96 per cent) and in methanol, sparingly soluble in acetone. It dissolves in dilute acid and alkaline solutions.

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 *tetracycline hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

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

Plate: *TLC octadecylsilyl silica gel F_{254} 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).

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

C. Dissolve about 10 mg in a mixture of 1 ml of *dilute nitric acid R* and 5 ml of *water R*. Shake and add 1 ml of *silver nitrate solution R2*. Any opalescence in the solution is not more intense than that in a mixture of 1 ml of *dilute nitric acid R*, 5 ml of *water R* and 1 ml of *silver nitrate solution R2*.

TESTS

pH (*2.2.3*): 3.5 to 6.0.

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

Specific optical rotation (*2.2.7*): – 260 to – 280 (dried substance).

Dissolve 0.250 g in *0.1 M hydrochloric acid* and dilute to 50.0 ml with the same acid.

Related substances. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

Test solution. Dissolve 25.0 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 25.0 mg of *tetracycline hydrochloride CRS* in *0.01 M hydrochloric acid* and dilute to 25.0 ml with the same acid.

Reference solution (b). Dissolve 12.5 mg of *4-epitetracycline hydrochloride CRS* in *0.01 M hydrochloric acid* and dilute to 50.0 ml with the same acid.