ASSAY

The activity of aprotinin is determined by measuring its inhibitory action on a solution of trypsin of known activity. The inhibiting activity of the aprotinin is calculated from the difference between the initial activity and the residual activity of the trypsin.

The inhibiting activity of aprotinin is expressed in European Pharmacopoeia Units. 1 Ph. Eur. U. inhibits 50 per cent of the enzymatic activity of 2 microkatals of trypsin.

Use a reaction vessel with a capacity of about 30 ml and provided with:

- a device that will maintain a temperature of 25 ± 0.1 °C;
- a stirring device, such as a magnetic stirrer;
- a lid with 5 holes for accommodating the electrodes, the tip of a burette, a tube for the admission of nitrogen and the introduction of the reagents.

An automatic or manual titration apparatus may be used. In the latter case the burette is graduated in 0.05 ml and the pH-meter is provided with a wide reading scale and glass and calomel electrodes.

Test solution. Prepare a solution of the substance to be examined in *0.0015 M borate buffer solution pH 8.0 R* expected to contain 1.67 Ph. Eur. U./ml (about 0.6 mg (*m* mg) per millilitre).

Trypsin solution. Prepare a solution of *trypsin BRP* containing about 0.8 microkatals per millilitre (about 1 mg/ml), using *0.001 M hydrochloric acid* as the solvent. Use a freshly prepared solution and keep in iced water.

Trypsin and aprotinin solution. To 4.0 ml of the trypsin solution add 1.0 ml of the test solution. Dilute immediately to 40.0 ml with *0.0015 M borate buffer solution pH 8.0 R*. Allow to stand at room temperature for 10 min and then keep in iced water. Use within 6 h of preparation.

Dilute trypsin solution. Dilute 0.5 ml of the trypsin solution to 10.0 ml with *0.0015 M borate buffer solution pH 8.0 R*. Allow to stand at room temperature for 10 min and then keep in iced water.

Maintain an atmosphere of nitrogen in the reaction flask and stir continuously; introduce 9.0 ml of *0.0015 M borate buffer solution pH 8.0 R* and 1.0 ml of a freshly prepared 6.9 g/l solution of *benzoylarginine ethyl ester hydrochloride R*. Adjust to pH 8.0 with *0.1 M sodium hydroxide*. When the temperature has reached equilibrium at 25 ± 0.1 °C, add 1.0 ml of the trypsin and aprotinin solution and start a timer. Maintain at pH 8.0 by the addition of *0.1 M sodium hydroxide* and note the volume added every 30 s. Continue the reaction for 6 min. Determine the number of millilitres of *0.1 M sodium hydroxide* used per second (n_1 ml). Carry out, under the same conditions, a titration using 1.0 ml of the dilute trypsin solution. Determine the number of millilitres of *0.1 M sodium hydroxide* used per second (n_2 ml).

Calculate the aprotinin activity in European Pharmacopoeia Units per milligram from the expression:

$$\frac{4000\,(2n_2 - n_1)}{m}$$

The estimated activity is not less than 90 per cent and not more than 110 per cent of the activity stated on the label.

STORAGE

In an airtight, tamper-proof container, protected from light.

LABELLING

The label states the number of European Pharmacopoeia Units of aprotinin activity per milligram.

01/2008:0579

APROTININ CONCENTRATED SOLUTION

Aprotinini solutio concentrata

[9087-70-1]

DEFINITION

Aprotinin concentrated solution is a solution of aprotinin, a polypeptide consisting of a chain of 58 amino acids, which inhibits stoichiometrically the activity of several proteolytic enzymes such as chymotrypsin, kallikrein, plasmin and trypsin. It contains not less than 15.0 Ph. Eur. U. of aprotinin activity per millilitre.

PRODUCTION

The animals from which aprotinin is derived must fulfil the requirements for the health of animals suitable for human consumption to the satisfaction of the competent authority.

The manufacturing process is validated to demonstrate suitable inactivation or removal of any contamination by viruses or other infectious agents.

The method of manufacture is validated to demonstrate that the product, if tested, would comply with the following tests.

Abnormal toxicity (*2.6.9*). Inject into each mouse a quantity of the preparation to be examined containing 2 Ph. Eur. U. diluted with a sufficient quantity of *water for injections R* to give a volume of 0.5 ml.

Histamine (*2.6.10*): maximum 0.2 µg of histamine base per 3 Ph. Eur. U.

CHARACTERS

Appearance: clear and colourless liquid.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

Test solution. Solution S (see Tests).

Reference solution. Aprotinin solution BRP.

Plate: *TLC silica gel G plate R.*

Mobile phase: *water R, glacial acetic acid R* (80:100 *V/V*) containing 100 g/l of *sodium acetate R*).

Application: 10 µl.

Development: over a path of 12 cm.

Drying: in air.

Detection: spray with a solution of 0.1 g of *ninhydrin R* in a mixture of 6 ml of a 10 g/l solution of *cupric chloride R*, 21 ml of *glacial acetic acid R* and 70 ml of *ethanol R*. Dry the plate at 60 °C.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

B. Determine the ability of the preparation to be examined to inhibit trypsin activity using the method described below.

Test solution. Dilute 1 ml of solution S to 50 ml with *buffer solution pH 7.2 R*.

Trypsin solution. Dissolve 10 mg of *trypsin BRP* in *0.002 M hydrochloric acid* and dilute to 100 ml with the same acid.

Casein solution. Dissolve 0.2 g of *casein R* in *buffer solution pH 7.2 R* and dilute to 100 ml with the same buffer solution.

Precipitating solution. Mix 1 volume of *glacial acetic acid R*, 49 volumes of *water R* and 50 volumes of *ethanol R*.

Mix 1 ml of the test solution with 1 ml of the trypsin solution. Allow to stand for 10 min and add 1 ml of the casein solution. Incubate at 35 °C for 30 min. Cool in iced water and add 0.5 ml of the precipitating solution. Shake and allow to stand at room temperature for 15 min. The solution is cloudy. Carry out a blank test under the same conditions using *buffer solution pH 7.2 R* instead of the test solution. The solution is not cloudy.

TESTS

Solution S. Prepare a solution containing 15 Ph. Eur. U./ml, if necessary by dilution on the basis of the activity stated on the label.

Appearance of solution. Solution S is clear (*2.2.1*).

Absorbance (*2.2.25*): maximum 0.80 by measuring at the absorption maximum at 277 nm.

Prepare from the concentrated solution a dilution containing 3.0 Ph. Eur. U./ml.

Protein impurities of higher molecular mass. Size-exclusion chromatography (*2.2.30*).

Freeze-dry the preparation to be examined using a pressure of 2.7 Pa and a temperature of – 30 °C; the operation, including freeze-drying and a period of drying at 15-25 °C, takes 6-12 h.

Use *cross-linked dextran for chromatography R2*. Use a 180 g/l solution of *anhydrous acetic acid R* to swell the gel and as the eluent. Prepare a column of gel 0.8 m to 1.0 m long and 25 mm in diameter, taking care to avoid the introduction of air bubbles. Place at the top of the column a quantity of the preparation to be examined containing 300 Ph. Eur. U. dissolved in 1 ml of a 180 g/l solution of *anhydrous acetic acid R* and allow to elute. Collect the eluate in fractions of 2 ml. Measure the absorbance (*2.2.25*) of each fraction at the absorption maximum at 277 nm and plot the values on a graph. The chromatogram obtained does not present an absorption maximum before the elution of the aprotinin.

Specific activity of the dry residue: minimum 3.0 Ph. Eur. U. of aprotinin activity per milligram of dry residue.

Evaporate 25.0 ml to dryness in a water-bath, dry the residue at 110 °C for 15 h and weigh. From the mass of the residue and the activity determined as described below, calculate the number of European Pharmacopoeia Units per milligram of dry residue.

Bacterial endotoxins (*2.6.14*): less than 0.14 IU per European Pharmacopoeia Unit of aprotinin, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

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The inhibiting activity of aprotinin is expressed in European Pharmacopoeia Units. 1 Ph. Eur. U. inhibits 50 per cent of the enzymatic activity of 2 microkatals of trypsin.

Use a reaction vessel with a capacity of about 30 ml and provided with:

– a device that will maintain a temperature of 25 ± 0.1 °C;

– a stirring device, such as a magnetic stirrer;

– a lid with 5 holes for accommodating the electrodes, the tip of a burette, a tube for the admission of nitrogen and the introduction of the reagents.

An automatic or manual titration apparatus may be used. In the latter case the burette is graduated in 0.05 ml and the pH-meter is provided with a wide reading scale and glass and calomel electrodes.

Test solution. With *0.0015 M borate buffer solution pH 8.0 R* prepare an appropriate dilution (*D*) of the concentrated solution expected on the basis of the stated potency to contain 1.67 Ph. Eur. U./ml.

Trypsin solution. Prepare a solution of *trypsin BRP* containing about 0.8 microkatals per millilitre (about 1 mg/ml), using *0.001 M hydrochloric acid* as the solvent. Use a freshly prepared solution and keep in iced water.

Trypsin and aprotinin solution. To 4.0 ml of the trypsin solution add 1.0 ml of the test solution. Dilute immediately to 40.0 ml with *0.0015 M borate buffer solution pH 8.0 R*. Allow to stand at room temperature for 10 min and then keep in iced water. Use within 6 h of preparation.

Dilute trypsin solution. Dilute 0.5 ml of the trypsin solution to 10.0 ml with *0.0015 M borate buffer solution pH 8.0 R*. Allow to stand at room temperature for 10 min and then keep in iced water.

Maintain an atmosphere of nitrogen in the reaction flask and stir continuously; introduce 9.0 ml of *0.0015 M borate buffer solution pH 8.0 R* and 1.0 ml of a freshly prepared 6.9 g/l solution of *benzoylarginine ethyl ester hydrochloride R*. Adjust to pH 8.0 with *0.1 M sodium hydroxide*. When the temperature has reached equilibrium at 25 ± 0.1 °C, add 1.0 ml of the trypsin and aprotinin solution and start a timer. Maintain at pH 8.0 by the addition of *0.1 M sodium hydroxide* and note the volume added every 30 s. Continue the reaction for 6 min. Determine the number of millilitres of *0.1 M sodium hydroxide* used per second (n_1 ml). Carry out, under the same conditions, a titration using 1.0 ml of the dilute trypsin solution. Determine the number of millilitres of *0.1 M sodium hydroxide* used per second (n_2 ml).

Calculate the aprotinin activity in European Pharmacopoeia Units per millilitre from the expression:

$$4000\,(2n_2 - n_1) \times D$$

D = dilution factor of the aprotinin concentrated solution to be examined in order to obtain a solution containing 1.67 Ph. Eur. U./ml.

The estimated activity is not less than 90 per cent and not more than 110 per cent of the activity stated on the label.

STORAGE

In an airtight, tamper-proof container, protected from light.

LABELLING

The label states the number of European Pharmacopoeia Units of aprotinin activity per millilitre.