01/2008:1316

ERYTHROPOIETIN CONCENTRATED SOLUTION

Erythropoietini solutio concentrata

APPRLICDSR VLERYLLEAK EAENITTGCA
EHCSLNENIT VPDTKVNFYA WKRMEVGQQA
VEVWQGLALL SEAVLRGQAL LVNSSQPWEP
LQLHVDKAVS GLRSLTTLLR ALGAQKEAIS
PPDAASAAPL RTITADTFRK LFRVYSNFLR
GKLKLYTGEA CRTGD

M_r approx. 30 600

DEFINITION

Erythropoietin concentrated solution is a solution containing a family of closely-related glycoproteins which are indistinguishable from the naturally occurring human erythropoietin (urinary erythropoietin) in terms of amino acid sequence (165 amino acids) and average glycosylation pattern, at a concentration of 0.5-10 mg/ml. It may also contain buffer salts and other excipients. It has a potency of not less than 100 000 IU/mg of active substance determined using the conditions described under Assay and in the test for protein.

PRODUCTION

Erythropoietin is produced in rodent cells *in vitro* by a method based on recombinant DNA technology.

Prior to batch release, the following tests are carried out on each batch of the final product, unless exemption has been granted by the competent authority.

Host cell-derived proteins: the limit is approved by the competent authority.

Host cell- and vector-derived DNA: the limit is approved by the competent authority.

CHARACTERS

Appearance: clear or slightly turbid, colourless solution.

IDENTIFICATION

A. It gives the appropriate response when examined using the conditions described under Assay.

B. Capillary zone electrophoresis (*2.2.47*).

Test solution. Dilute the preparation to be examined with *water R* to obtain a concentration of 1 mg/ml. Desalt 0.25 ml of the solution by passage through a micro-concentrator cartridge provided with a membrane with a molecular mass cut-off of not more than 10 000 Da. Add 0.2 ml of *water R* to the sample and desalt again. Repeat the desalting procedure once more. Dilute the sample with *water R*, determine its protein concentration as described under Tests and adjust to a concentration of approximately 1 mg/ml with *water R*.

Reference solution. Dissolve the contents of a vial of *erythropoietin BRP* in 0.25 ml of *water R*. Proceed with desalting as described for the test solution.

Capillary:

– *material*: uncoated fused silica,

– *size*: effective length = about 100 cm, Ø = 50 µm,

Temperature: 35 °C.

CZE buffer concentrate (0.1 M sodium chloride, 0.1 M tricine, 0.1 M sodium acetate). Dissolve 0.584 g of *sodium chloride R*, 1.792 g of *tricine R* and 0.820 g of *anhydrous sodium acetate R* in *water R* and dilute to 100.0 ml with the same solvent.

1 M putrescine solution. Dissolve 0.882 g of *putrescine R* in 10 ml of *water R*. Distribute in 0.5 ml aliquots.

CZE buffer (0.01 M tricine, 0.01 M sodium chloride, 0.01 M sodium acetate, 7 M urea, 2.5 mM putrescine). Dissolve 21.0 g of *urea R* in 25 ml of *water R* by warming in a water-bath at 30 °C. Add 5.0 ml of CZE buffer concentrate and 125 µl of 1 M putrescine solution. Dilute to 50.0 ml with *water R*. Using *dilute acetic acid R*, adjust to pH 5.55 at 30 °C and filter through a 0.45 µm membrane filter.

Detection: spectrophotometer at 214 nm.

Set the autosampler to store the samples at 4 °C during analysis.

Preconditioning of the capillary: rinse the capillary for 60 min with *0.1 M sodium hydroxide* filtered through a 0.45 µm membrane filter and for 60 min with CZE buffer. Apply voltage for 12 h (20 kV).

Between-run rinsing: rinse the capillary for 10 min with *water R*, for 5 min with *0.1 M sodium hydroxide* filtered through a 0.45 µm membrane filter and for 10 min with CZE buffer.

Injection: under pressure or vacuum.

Migration: apply a field strength of 143 V/cm (15.4 kV for capillaries of 107 cm total length) for 80 min, using CZE buffer as the electrolyte in both buffer reservoirs.

System suitability: in the electropherogram obtained with the reference solution, a pattern of well separated peaks corresponding to the peaks in the electropherogram of erythropoietin supplied with *erythropoietin BRP* is seen, and the largest peak is at least 50 times greater than the baseline noise. If necessary, adjust the sample load to give peaks of sufficient height. Identify the peaks due to isoforms 1 to 8. Isoform 1 may not be visible. The peak due to isoform 8 is detected and the resolution between the peaks due to isoforms 5 and 6 is not less than 1. Repeat the separation at least 3 times. The baseline is stable, showing little drift, and the distribution of peaks is qualitatively and quantitatively similar to the distribution of peaks in the electropherogram of erythropoietin supplied with *erythropoietin BRP*. The relative standard deviation of the migration time of the peak due to isoform 2 is less than 2 per cent.

Limits: identify the peaks due to isoforms 1 to 8 in the electropherogram obtained with the test solution by comparison with the electropherogram obtained with the reference solution. Calculate the percentage content of each isoform from the corresponding peak area. The percentages are within the following ranges:

Isoform	Content (per cent)
1	0 - 15
2	0 - 15
3	1 - 20
4	10 - 35
5	15 - 40
6	10 - 35
7	5 - 25
8	0 - 15

C. Polyacrylamide gel electrophoresis and immunoblotting.

(a) Polyacrylamide gel electrophoresis (*2.2.31*)

Gel dimensions: 0.75 mm thick, about 16 cm square.

Resolving gel: 12 per cent acrylamide.

Sample buffer: *SDS-PAGE sample buffer (concentrated) R*.

Test solution (a). Dilute the preparation to be examined in *water R* to obtain a concentration of 1.0 mg/ml. To 1 volume of this solution add 1 volume of sample buffer.

Test solution (b). Dilute the preparation to be examined in *water R* to obtain a concentration of 0.1 mg/ml. To 1 volume of this solution add 1 volume of sample buffer.

Reference solution (a). Dissolve the contents of a vial of *erythropoietin BRP* in 0.25 ml of *water R*. To 1 volume of this solution add 1 volume of sample buffer.

Reference solution (b). Dissolve the contents of a vial of *erythropoietin BRP* in *water R* and dilute with the same solvent to obtain a concentration of 0.1 mg/ml. To 1 volume of this solution add 1 volume of sample buffer.

Reference solution (c). A solution of molecular mass markers suitable for calibrating SDS-polyacrylamide gels in the range of 10-70 kDa.

Reference solution (d). A solution of pre-stained molecular mass markers suitable for calibrating SDS-polyacrylamide gels in the range of 10-70 kDa and suitable for the electrotransfer to an appropriate membrane.

Sample treatment: boil for 2 min.

Application: 20 µl, in the following order: reference solution (c), reference solution (a), test solution (a), empty well, reference solution (b), test solution (b), reference solution (d).

At the end of the separation, remove the gel-cassette from the apparatus and cut the gel into 2 parts: the first part containing reference solution (c), reference solution (a) and test solution (a); the second part containing reference solution (b), test solution (b) and reference solution (d).

Detection: by Coomassie staining on the first part of the gel.

System suitability: reference solution (c):

– the validation criteria are met.

Results: the electropherogram obtained with test solution (a) shows a single diffuse band corresponding in position and intensity to the single band seen in the electropherogram obtained with reference solution (a).

(b) Immunoblotting

Transfer the second part of the gel onto a membrane suitable for the immobilisation of proteins, using commercially available electrotransfer equipment and following the manufacturer's instructions. After electrotransfer, incubate the membrane in a neutral isotonic buffer containing a suitable blocking agent (for example, 50 g/l of dried milk or 10 per cent *V/V* foetal calf serum), for 1-2 h, followed by incubation for 1-14 h in the same blocking solution with a suitable dilution of either a polyclonal or monoclonal anti-erythropoietin antibody. Detect erythropoietin-bound antibody using a suitable enzyme- or radiolabelled antibody (for example, an alkaline phosphatase-conjugated second antibody). The precise details of blocking agents, concentrations and incubation times should be optimised using the principles set out in *Immunochemical methods* (*2.7.1*).

System suitability: in the electropherogram obtained with reference solution (d), the molecular mass markers are resolved on the membrane into discrete bands, with a linear relationship between distance migrated and logarithm_{10} of the molecular mass.

Results: the electropherogram obtained with test solution (b) shows a single broad band corresponding in position and intensity to the single band seen in the electropherogram obtained with reference solution (b).

D. Peptide mapping (*2.2.55*). Liquid chromatography (*2.2.29*).

Test solution. Dilute the preparation to be examined in *tris-acetate buffer solution pH 8.5 R* to a concentration of 1.0 mg/ml. Equilibrate the solution in *tris-acetate buffer solution pH 8.5 R* using a suitable procedure (dialysis against *tris-acetate buffer solution pH 8.5 R*, or membrane filtration using the procedure described under Identification B, but reconstituting the desalted sample with *tris-acetate buffer solution pH 8.5 R*, are suitable). Transfer the dialysed solution to a polypropylene centrifuge tube. Freshly prepare a solution of *trypsin for peptide mapping R* at a concentration of 1 mg/ml in *water R*, and add 5 µl to 0.25 ml of the dialysed solution. Cap the tube and place in a water-bath at 37 °C for 18 h. Remove the sample from the water-bath and stop the reaction immediately by freezing.

Reference solution. Dissolve the contents of a vial of *erythropoietin BRP* in 0.25 ml of *water R*. Prepare as for the test solution, ensuring that all procedures are carried out simultaneously, and under identical conditions.

Column:

– *size*: *l* = 0.25 m, Ø = 4.6 mm,

– *stationary phase*: *butylsilyl silica gel for chromatography R* (5-10 µm).

Mobile phase:

– *mobile phase A*: 0.06 per cent *V/V* solution of *trifluoroacetic acid R*,

– *mobile phase B*: to 100 ml of *water R* add 0.6 ml of *trifluoroacetic acid R* and dilute to 1000 ml with *acetonitrile for chromatography R*,

Time (min)	Flow rate (ml/min)	Mobile phase A (per cent *V/V*)	Mobile phase B (per cent *V/V*)
0 - 10	0.75	100	0
10 - 125	0.75	100 → 39	0 → 61
125 - 135	1.25	39 → 17	61 → 83
135 - 145	1.25	17 → 0	83 → 100
145 - 150	1.25	100	0

Detection: spectrophotometer at 214 nm.

Equilibration: at initial conditions for at least 15 min. Carry out a blank run using the above-mentioned gradient.

Injection: 50 µl.

System suitability: the chromatograms obtained with the test solution and the reference solution are qualitatively similar to the chromatogram of erythropoietin digest supplied with *erythropoietin BRP*.

Results: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

E. *N*-terminal sequence analysis.

The first 15 amino acids are: Ala - Pro - Pro - Arg - Leu - Ile - (no recovered peak) - Asp - Ser - Arg - Val - Leu - Glu - Arg - Tyr.

Perform the Edman degradation using an automated solid-phase sequencer, operated in accordance with the manufacturer's instructions.

Desalt the equivalent of 50 µg of erythropoietin. For example, dilute a volume of the preparation to be examined equivalent to 50 µg of the active substance in 1 ml of a 0.1 per cent *V/V* solution of *trifluoroacetic acid R*. Pre-wash a C18 reverse-phase sample preparation cartridge according to the instructions supplied and equilibrate the cartridge in a 0.1 per cent *V/V* solution of *trifluoroacetic acid R*. Apply the sample to the cartridge, and wash successively with a 0.1 per cent *V/V* solution of *trifluoroacetic acid R* containing 0 per cent, 10 per cent and 50 per cent *V/V* of *acetonitrile R* according to the manufacturer's instructions. Lyophilise the 50 per cent *V/V acetonitrile R* eluate.

Redissolve the desalted sample in 50 µl of a 0.1 per cent *V/V* solution of *trifluoroacetic acid R* and couple to a sequencing cartridge using the protocol provided by the manufacturer. Run 15 sequencing cycles, using the reaction conditions for proline when running the 2nd and 3rd cycles.

Identify the phenylthiohydantoin (PTH)-amino acids released at each sequencing cycle by reverse-phase liquid chromatography. The procedure may be carried out using the column and reagents recommended by the manufacturer of the sequencing equipment for the separation of PTH-amino-acids.

The separation procedure is calibrated using:

- the mixture of PTH-amino acids provided by the manufacturer of the sequencer, with the gradient conditions adjusted as indicated to achieve optimum resolution of all amino acids,
- a sample obtained from a blank sequencing cycle obtained as recommended by the equipment manufacturer.

TESTS

Protein (*2.5.33, Method I*): 80 per cent to 120 per cent of the stated concentration.

Test solution. Dilute the preparation to be examined in a 4 g/l solution of *ammonium hydrogen carbonate R*.

Record the absorbance spectrum between 250 nm and 400 nm. Measure the value at the absorbance maximum (276-280 nm), after correction for any light scattering, measured up to 400 nm. Calculate the concentration of erythropoietin taking the specific absorbance to be 7.43.

Dimers and related substances of higher molecular mass. Size-exclusion chromatography (*2.2.30*).

Test solution. Dilute the preparation to be examined in the mobile phase to obtain a concentration of 0.2 mg/ml.

Reference solution. To 0.02 ml of the test solution add 0.98 ml of the mobile phase.

Column:

- *size*: *l* = 0.6 m, Ø = 7.5 mm,
- *stationary phase*: *hydrophilic silica gel for chromatography R*, of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 20 000 to 200 000.

Mobile phase: dissolve 1.15 g of *anhydrous disodium hydrogen phosphate R*, 0.2 g of *potassium dihydrogen phosphate R* and 23.4 g of *sodium chloride R* in 1 litre of *water R* (1.5 mM potassium dihydrogen phosphate, 8.1 mM disodium hydrogen phosphate, 0.4 M sodium chloride, pH 7.4); adjust to pH 7.4 if necessary.

Flow rate: 0.5 ml/min.

Detection: spectrophotometer at 214 nm.

Injection: 100 µl.

Run time: minimum 1 h.

System suitability: the area of the principal peak in the chromatogram obtained with the reference solution is 1.5 per cent to 2.5 per cent of the area of the principal peak in the chromatogram obtained with the test solution.

Limits:

- *total of any peaks eluted before the principal peak*: not more than the area of the principal peak in the chromatogram obtained with the reference solution (2 per cent).

Sialic acids: minimum 10 mol of sialic acids (calculated as *N*-acetylneuraminic acid) per mole of erythropoietin.

Test solution (a). Dilute the preparation to be examined in the mobile phase used in the test for dimers and related substances of higher molecular mass to obtain a concentration of 0.3 mg/ml.

Test solution (b). To 0.5 ml of test solution (a) add 0.5 ml of the mobile phase used in the test for dimers and related substances of higher molecular mass.

Reference solution (a). Dissolve a suitable amount of *N-acetylneuraminic acid R* in *water R* to obtain a concentration of 0.1 mg/ml.

Reference solution (b). To 0.8 ml of reference solution (a) add 0.2 ml of *water R*.

Reference solution (c). To 0.6 ml of reference solution (a) add 0.4 ml of *water R*.

Reference solution (d). To 0.4 ml of reference solution (a) add 0.6 ml of *water R*.

Reference solution (e). To 0.2 ml of reference solution (a) add 0.8 ml of *water R*.

Reference solution (f). Use *water R*.

Carry out the test in triplicate. Transfer 100 µl of each of the test and reference solutions to 10 ml glass test tubes. To each tube add 1.0 ml of *resorcinol reagent R*. Stopper the tubes and incubate at 100 °C for 30 min. Cool on ice. To each tube, add 2.0 ml of a mixture of 12 volumes of *butanol R* and 48 volumes of *butyl acetate R*. Mix vigorously, and allow the 2 phases to separate. Ensuring that the upper phase is completely clear, remove the upper phase, taking care to exclude completely any of the lower phase. Measure the absorbance (*2.2.25*) of all samples at 580 nm. Using the calibration curve generated by the reference solutions, determine the content of sialic acids in test solutions (a)

and (b) and calculate the mean. Calculate the number of moles of sialic acids per mole of erythropoietin assuming that the relative molecular mass of erythropoietin is 30 600 and that the relative molecular mass of *N*-acetylneuraminic acid is 309.

System suitability:

- the individual replicates agree to within ± 10 per cent of each other,
- the value obtained from reference solution (a) is between 1.5 and 3.3 times that obtained with test solution (a).

Bacterial endotoxins (*2.6.14*): less than 20 IU in the volume that contains 100 000 IU of erythropoietin.

ASSAY

The activity of the preparation is compared with that of *erythropoietin BRP* and expressed in International Units (IU).

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

Carry out the determination of potency by Method A or B.

A. In polycythaemic mice

The activity of the preparation is estimated by examining, under given conditions, its effect in stimulating the incorporation of ^{59}Fe into circulating red blood cells of mice made polycythaemic by exposure to reduced atmospheric pressure.

The following schedule, using treatment in a hypobaric chamber, has been found to be suitable.

Induce polycythaemia in female mice of the same strain, weighing 16-18 g. Place the mice in a hypoxic chamber and reduce the pressure to 0.6 atmospheres. After 3 days at 0.6 atmospheres, further reduce the pressure to 0.4-0.5 atmospheres and maintain the animals at this pressure for a further 11 days (the partial vacuum is interrupted daily for a maximum of 1 h at about 11:00 a.m., in order to clean the cages and feed the animals). At the end of the specified period, return the mice to normal atmospheric conditions. Randomly distribute the mice into cages, each containing 6 animals, and mark them.

Test solution (a). Dilute the substance to be examined in *phosphate-albumin buffered saline pH 7.2 R1* to obtain a concentration of 0.2 IU/ml.

Test solution (b). Mix equal volumes of test solution (a) and *phosphate-albumin buffered saline pH 7.2 R1*.

Test solution (c). Mix equal volumes of test solution (b) and *phosphate-albumin buffered saline pH 7.2 R1*.

Reference solution (a). Dissolve *erythropoietin BRP* in *phosphate-albumin buffered saline pH 7.2 R1* to obtain a concentration of 0.2 IU/ml.

Reference solution (b). Mix equal volumes of reference solution (a) and *phosphate-albumin buffered saline pH 7.2 R1*.

Reference solution (c). Mix equal volumes of reference solution (b) and *phosphate-albumin buffered saline pH 7.2 R1*.

Radiolabelled ferric [^{59}Fe] chloride solution, concentrated. Use a commercially available solution of [^{59}Fe]ferric chloride (approximate specific activity: 100-1000 MBq/mg of Fe).

Radiolabelled [^{59}Fe]ferric chloride solution. Dilute the concentrated radiolabelled [^{59}Fe]ferric chloride solution in *sodium citrate buffer solution pH 7.8 R* to obtain a solution with an activity of 3.7×10^4 Bq/ml.

The concentrations of the test solutions and reference solutions may need to be modified, based on the response range of the animals used.

3 days after returning the animals to atmospheric pressure, inject each animal subcutaneously with 0.2 ml of one of the solutions. The 6 animals in each cage must each receive one of the 6 different treatments (3 test solutions and 3 reference solutions), and the order of injection must be separately randomised for each cage. A minimum of 8 cages is recommended. 2 days after injection of the test or reference solution, inject each animal intraperitoneally with 0.2 ml of radiolabelled [^{59}Fe]ferric chloride solution. The order of the injections must be the same as that of the erythropoietin injections, and the time interval between administration of the erythropoietin and the radiolabelled ferric chloride solution must be the same for each animal. After a further 48 h, anaesthetise each animal by injection of a suitable anaesthetic, record body weights and withdraw blood samples (0.65 ml) into haematocrit capillaries from the bifurcation of the aorta. After determining the packed cell volume for each sample, measure the radioactivity.

Calculate the response (percentage of iron-59 in total circulating blood) for each mouse using the expression:

$$\frac{A_s \times M \times 7.5}{A_t \times V_s}$$

A_s = radioactivity in the sample,

A_t = total radioactivity injected,

7.5 = total blood volume as per cent body weight,

M = body weight, in grams,

V_s = sample volume.

Calculate the potency by the usual statistical methods for a parallel line assay. Eliminate from the calculation any animal where the packed cell volume is less than 54 per cent, or where the body weight is more than 24 g.

B. In normocythaemic mice

The assay is based on the measurement of stimulation of reticulocyte production in normocythaemic mice.

The assay may be carried out using the following procedure:

Test solution (a). Dilute the preparation to be examined in *phosphate-albumin buffered saline pH 7.2 R1* to obtain a concentration of 80 IU/ml.

Test solution (b). Mix equal volumes of test solution (a) and *phosphate-albumin buffered saline pH 7.2 R1*.

Test solution (c). Mix equal volumes of test solution (b) and *phosphate-albumin buffered saline pH 7.2 R1*.

Reference solution (a). Dissolve *erythropoietin BRP* in *phosphate-albumin buffered saline pH 7.2 R1* to obtain a concentration of 80 IU/ml.

Reference solution (b). Mix equal volumes of reference solution (a) and *phosphate-albumin buffered saline pH 7.2 R1*.

Reference solution (c). Mix equal volumes of reference solution (b) and *phosphate-albumin buffered saline pH 7.2 R1*.

The exact concentrations of the test solutions and reference solutions may need to be modified, based on the response range of the animals used.

At the beginning of the assay procedure, randomly distribute mice of a suitable age and strain (8-week old B6D2F1 mice are suitable) into 6 cages. A minimum of 8 mice per cage is recommended. Inject each animal subcutaneously with 0.5 ml of the appropriate treatment (one solution per cage)

and put the animal in a new cage. Combine the mice in such a way that each cage housing the treated mice contains one mouse out of the 6 different treatments (3 test solutions and 3 reference solutions, 6 mice per cage). 4 days after the injections, collect blood samples from the animals and determine the number of reticulocytes using a suitable procedure.

The following method may be employed:

The volume of blood, dilution procedure and fluorescent reagent may need to be modified to ensure maximum development and stability of fluorescence.

Colorant solution, concentrated. Use a solution of thiazole orange suitable for the determination of reticulocytes. Prepare at a concentration twice that necessary for the analysis.

Proceed with the following dilution steps. Dilute whole blood 500-fold in the buffer used to prepare the colorant solution. Dilute this solution 2-fold in the concentrated colorant solution. After staining for 3-10 min, determine the reticulocyte count microfluorometrically in a flow cytometer. The percentage of reticulocytes is determined using a biparametric histogram: number of cells/red fluorescence (620 nm).

Calculate the potency by the usual statistical methods for a parallel line assay.

STORAGE

In an airtight container at a temperature below – 20 °C. Avoid repeated freezing and thawing.

LABELLING

The label states:

– the erythropoietin content in milligrams per millilitre,
– the activity in International Units per millilitre,
– the name and the concentration of any other excipients.

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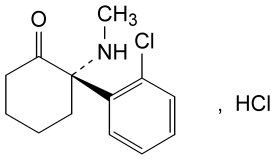
corrected 6.0

ESKETAMINE HYDROCHLORIDE

Esketamini hydrochloridum



, HCl

$C_{13}H_{17}Cl_2NO$ M_r 274.2
[33795-24-3]

DEFINITION

(2*S*)-2-(2-Chlorophenyl)-2-(methylamino)cyclohexanone hydrochloride.

Content: 99.0 per cent to 101.0 per cent.

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: freely soluble in water and in methanol, soluble in alcohol.

IDENTIFICATION

A. Specific optical rotation (*2.2.7*): + 85.0 to + 95.0.
Dilute 12.5 ml of solution S (see Tests) to 40.0 ml with *water R*.

B. Infrared absorption spectrophotometry (*2.2.24*).
Comparison: *Ph. Eur. reference spectrum of esketamine hydrochloride.*

C. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

Solution S. Dissolve 8.0 g in *carbon dioxide-free water R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

pH (*2.2.3*): 3.5 to 4.5.

Dilute 12.5 ml of solution S to 20 ml with *carbon dioxide-free water R*.

Impurity D. Liquid chromatography (*2.2.29*).

Test solution. Dissolve 25.0 mg of the substance to be examined in *water R* and dilute to 100.0 ml with the same solvent.

Reference solution (a). Dissolve 5 mg of *esketamine impurity D CRS* in *water R*, add 20 ml of the test solution and dilute to 50 ml with *water R*. Dilute 10 ml of this solution to 100 ml with *water R*.

Reference solution (b). Dilute 5.0 ml of the test solution to 25.0 ml with *water R*. Dilute 5.0 ml of this solution to 50.0 ml with *water R*.

Reference solution (c). Dilute 2.5 ml of reference solution (b) to 10.0 ml with *water R*. Dilute 1.0 ml of this solution to 10.0 ml with *water R*.

Precolumn:

– *size*: *l* = 0.01 m, Ø = 3.0 mm,
– *stationary phase*: *silica gel AGP for chiral chromatography R* (5 µm),
– *temperature*: 30 °C.

Column:

– *size*: *l* = 0.125 m, Ø = 4.6 mm,
– *stationary phase*: *silica gel AGP for chiral chromatography R* (5 µm),
– *temperature*: 30 °C.

Mobile phase: mix 16 volumes of *methanol R* and 84 volumes of a 6.8 g/l solution of *potassium dihydrogen phosphate R* previously adjusted to pH 7.0 with *potassium hydroxide R*.

Flow rate: 0.8 ml/min.

Detection: spectrophotometer at 215 nm.

Injection: 20 µl.

Run time: 20 min.

Relative retention with reference to esketamine (retention time = about 10 min): impurity D = about 1.3.

System suitability:

– *resolution*: minimum 2.0 between the peaks due to esketamine and impurity D in the chromatogram obtained with reference solution (a),
– *signal-to-noise ratio*: minimum 3 for the principal peak in the chromatogram obtained with reference solution (c).

Limit:

– *impurity D*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent).

Related substances. Liquid chromatography (*2.2.29*).

Test solution. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.