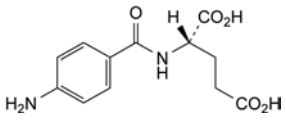
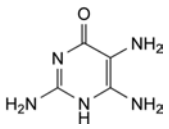
IMPURITIES

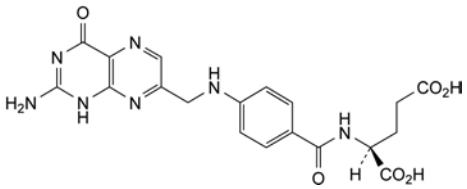
Specified impurities: A, B, C, D, E, F.



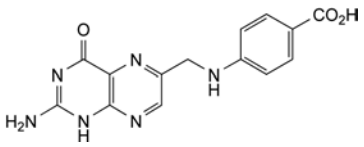
A. (2*S*)-2-[(4-aminobenzoyl)amino]pentanedioic acid (*N*-(4-aminobenzoyl)-L-glutamic acid),



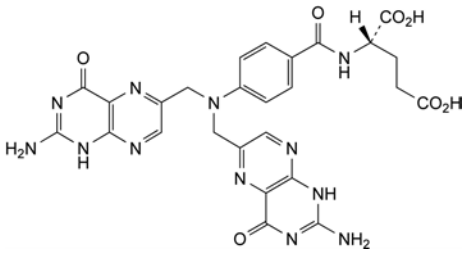
B. 2,5,6-triaminopyrimidin-4(1*H*)-one,



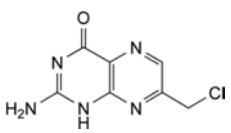
C. (2*S*)-2-[[4-[[(2-amino-4-oxo-1,4-dihydropteridin-7-yl)methyl]amino]benzoyl]amino]pentanedioic acid (isofolic acid),



D. 4-[[(2-amino-4-oxo-1,4-dihydropteridin-6-yl)methyl]amino]benzoic acid (pteroic acid),



E. (2*S*)-2-[[4-[bis[(2-amino-4-oxo-1,4-dihydropteridin-6-yl)methyl]amino]benzoyl]amino]pentanedioic acid (6-pterinylfolic acid),



F. 2-amino-7-(chloromethyl)pteridin-4(1*H*)-one.

01/2014:2285

FOLLITROPIN

Follitropinum

α-subunit

```
APDVQDCPEC TLQENPFFSQ PGAPILQCMG CCFSRAYPTP   40
LRSKKTMLVQ KNVTSESTCC VAKSYNRVTV MGGFKVENHT   80
ACHCSTCYYH KS                                 92
```

β-subunit

```
NSCELTNITI AIEKEECRFC ISINTTWCAG YCYTRDLVYK  40*
DPARPKIQKT CTFKELVYET VRVPGCAHHA DSLYTYPVAT  80*
QCHCGKCDSD STDCTVRGLG PSYCSFGEMK E          111*
```

glycosylation sites:
Asn-52, Asn-78, Asn-7*, Asn-24*

disulfide bridges:
7-31, 10-60, 28-82, 32-84, 59-87, 3*-51*, 17*-66*, 20*-104*, 28*-82*, 32*-84*, 87*-94*

M_r approx. 30 000 - 40 000

DEFINITION

Freeze-dried preparation of a heterodimeric glycoprotein having the structure of human follicle-stimulating hormone (FSH). It consists of 2 subunits: a 92-amino-acid α-chain common to other glycoprotein hormones and a specific 111-amino-acid β-chain.

Potency: 9000 IU to 17 000 IU per milligram of protein.

PRODUCTION

Follitropin is produced in mammalian cells by a method based on recombinant DNA (rDNA) technology.

Follitropin complies with the following requirements.

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: white or almost white powder.

IDENTIFICATION

A. It complies with the requirements described under Assay.

B. Isoelectric focusing (*2.2.54*).

Test solution. Dissolve the substance to be examined in *water R* to obtain a concentration of about 2 mg/mL, then desalt and concentrate using a suitably validated procedure. Dissolve the recovered material in *water R* to obtain a concentration of 5 mg/mL.

Reference solution. Dissolve the contents of a vial of *follitropin CRS* in *water R*. Desalt and concentrate using a suitably validated procedure. Dissolve the recovered material in *water R* to obtain a concentration of 5 mg/mL.

Focusing:

- *pH gradient*: a combination of ampholytes and electrode buffers giving a functional separation in the isoelectric point (pI) range of 3.5-5.5 is selected, as defined by the system suitability criteria; where pre-cast gels are employed, proprietary electrode solutions may be used in conjunction; otherwise, suitable dilute mineral or organic acids and bases are employed at pH levels respectively lower and higher than the functional range of the ampholytes;
- *catholyte*: 20.0 g/L solution of *glycine R*;
- *anolyte*: solution containing 3.4 g/L of *aspartic acid R* and 3.6 g/L of *glutamic acid R*, adjusted to pH 2.8-3.8;
- *application*: 10 µL.

Detection: as described in *2.2.54*.

System suitability:

– in the electropherogram obtained with the reference solution, the number of bands seen in the pI region 3.5-5.5 corresponds to that shown in the electropherogram supplied with *follitropin CRS*; the distribution of bands in the pI region 3.5-5.5 is qualitatively similar to that shown in the electropherogram supplied with *follitropin CRS*.

Results: examine the electropherogram obtained with the test solution; identify the bands observed by comparison with the electropherogram obtained with the reference solution; the pattern of bands is qualitatively similar to that seen with the reference solution.

C. Examine the chromatograms obtained in the test for follitropin oligomers.

Results: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution.

D. Peptide mapping (*2.2.55*).

SEPARATION OF THE α- AND β-SUBUNITS. Liquid chromatography (*2.2.29*).

Test solution. Dissolve the substance to be examined in mobile phase A to obtain a concentration of about 0.4 mg/mL.

Reference solution. Dissolve *follitropin CRS* in mobile phase A to obtain a concentration of about 0.4 mg/mL.

Precolumn:

– *size*: *l* = 0.02 m, Ø = 4.0 mm;
– *stationary phase*: *butylsilyl silica gel for chromatography R* (5 µm).

Column:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *butylsilyl silica gel for chromatography R* (5 µm) with a pore size of 30 nm.

Mobile phase:

– *mobile phase A*: dilute 1 mL of *trifluoroacetic acid R* to 1 L with *water R*;
– *mobile phase B*: *trifluoroacetic acid R*, *water R*, *acetonitrile R* (0.9:50:950 *V/V/V*);

Time (min)	Mobile phase A (per cent *V/V*)	Mobile phase B (per cent *V/V*)
0 - 2	100	0
2 - 8	100 → 76	0 → 24
8 - 17	76	24
17 - 36	76 → 70	24 → 30
36 - 41	70 → 25	30 → 75
41 - 46	25	75
46 - 47	25 → 100	75 → 0
47 - 57	100	0

Flow rate: 1.0 mL/min.

Detection: spectrophotometer at 226 nm.

Injection: 800 µL.

Retention time: β-subunit = about 14 min; α-subunit = about 30 min.

Collect the fractions containing the α- and β-subunits and freeze-dry them.

REDUCTION, MODIFICATION AND DESALTING OF THE PURIFIED SUBUNITS

Reduction and modification

Solution A. Dilute 10 µL of *tributylphosphine R* to 2 mL with *propanol R*. Saturate with nitrogen.

Solution B. Dilute 20 µL of *4-vinylpyridine R* to 200 µL with *propanol R*. Saturate with nitrogen.

Test solutions. Dissolve each of the α- and β-subunit fractions obtained from the test solution in the previous step in 300 µL of *guanidine-tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.5 R* and incubate at 37 °C for 60 min in a thermostatically controlled water-bath. Add 100 µL of solution A, mix and saturate with nitrogen. Incubate at 37 °C for 90 min. Add 10 µL of solution B, mix and saturate with nitrogen. Incubate at 37 °C for 45 min. Add 100 µL of a 10 per cent *V/V* solution of *trifluoroacetic acid R* and mix.

Reference solutions. Prepare at the same time and in the same manner as for the test solutions but using the α- and β-subunit fractions obtained from the reference solution in the previous step.

Desalting

Dilute the α- and β-subunit test and reference solutions to 840 µL with mobile phase A.

Column:

– *size*: *l* = 0.02 m, Ø = 4.6 mm;
– *stationary phase*: *butylsilyl silica gel for chromatography R* (5 µm).

Mobile phase:

– *mobile phase A*: dilute 1 mL of *trifluoroacetic acid R* to 1 L with *water R*;
– *mobile phase B*: *trifluoroacetic acid R*, *water R*, *acetonitrile R* (1:300:700 *V/V/V*);

Time (min)	Mobile phase A (per cent *V/V*)	Mobile phase B (per cent *V/V*)
0 - 7	100	0
7 - 27	100 → 0	0 → 100
27 - 27.01	0 → 100	100 → 0
27.01 - 32	100	0

Flow rate: 1.0 mL/min.

Detection: spectrophotometer at 226 nm.

Injection: 800 µL.

For each solution the chromatogram shows a principal peak due to the monovinylpyridine-modified subunit and several minor peaks due to the di- and oligovinylpyridine-modified subunits. Only the fraction containing the monovinylpyridine-modified subunit is used for digestion in the following step.

Retention time: *α-subunit solution*: monovinylpyridine-modified α-subunit = about 15 min; *β-subunit solution*: monovinylpyridine-modified β-subunit = about 16 min.

Collect the fractions containing the monovinylpyridine-modified subunits and freeze-dry them.

SELECTIVE CLEAVAGE OF THE PEPTIDE BONDS

Solution C (8 M urea solution). Dissolve 480 g of *urea R* in 600 mL of *water R* and dilute to 1 L with the same solvent. Add about 3-5 g of mixed-bed resin and stir for about 1 h. Filter through a glass filter before use.

Solution D. Dissolve 15.8 g of *ammonium hydrogen carbonate R* and 8.3 g of *sodium edetate R* in 800 mL of *water R*. Adjust to pH 7.8 (*2.2.3*) with an 80 g/L solution of *sodium hydroxide R* and dilute to 1 L with *water R*.

Test solutions. Dissolve each of the modified α- and β-subunits obtained from the test solutions in the previous step in 42.5 µL of solution C and incubate at room temperature for 30 min. Add 42.5 µL of solution D and mix. To 42.5 µL of these solutions add 35 µL of a solution containing about 23 mU/µL of endoproteinase Lys-C and mix. Incubate at 37 °C for 4 h, then add 35 µL of the same endoproteinase Lys-C solution and mix. Incubate at 37 °C overnight, then dilute to 420 µL with mobile phase A.

Reference solutions. Prepare at the same time and in the same manner as for the test solutions but using the fractions obtained from the reference solutions in the previous step.

CHROMATOGRAPHIC SEPARATION. Liquid chromatography (*2.2.29*).

Precolumn:

– *size*: *l* = 0.02 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

Column:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 30 nm.

Mobile phase:

– *mobile phase A*: dilute 1 mL of *trifluoroacetic acid R* to 1 L with *water R*;

– *mobile phase B*: *trifluoroacetic acid R*, *water R*, *acetonitrile R* (1:300:700 *V/V/V*);

Time (min)	Mobile phase A (per cent *V/V*)	Mobile phase B (per cent *V/V*)
0 - 7	100	0
7 - 77	100 → 30	0 → 70
77 - 82	30 → 0	70 → 100
82 - 87	0	100
87 - 92	0 → 100	100 → 0
92 - 107	100	0

Flow rate: 1.0 mL/min.

Detection: spectrophotometer at 210 nm.

Injection: 400 µL.

System suitability:

α-subunit:

– the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram of follitropin α-subunit digest supplied with *follitropin CRS*; both chromatograms show peaks due to the L4, L6, L3, L5 and L1-2/L1 fragments;

– retention times obtained with the test and reference solutions differ by not more than 5 per cent for fragments L4, L6 and L3, not more than 3 per cent for fragment L5 and not more than 2 per cent for fragments L1-2/L1;

β-subunit:

– the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram of follitropin β-subunit digest supplied with *follitropin CRS*; both chromatograms show peaks due to the L5, L7, L6, and L1-4 fragments;

– retention times obtained with the test and reference solutions differ by not more than 5 per cent for fragment L5, not more than 2 per cent for fragments L7 and L6 and not more than 1 per cent for fragments L1-4.

Results: for each subunit, the profile of the chromatogram obtained with the test solution is similar to that of the chromatogram obtained with the corresponding reference solution.

E. Glycan analysis (*2.2.59*). Carry out either method A or method B.

METHOD A

PROTEIN DENATURATION

Test solution. Dissolve 500 µg of the substance to be examined in 60 µL of *0.05 M phosphate buffer solution pH 7.5 R*. Add 6 µL of a 10 mg/mL solution of *sodium dodecyl sulfate R* and 35 µL of a 1 per cent *V/V* solution of *2-mercaptoethanol R*. Mix using a vortex mixer, centrifuge and incubate at 37 °C for 15 min.

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

SELECTIVE RELEASE OF THE GLYCANS

Test solution. To the test solution obtained in the previous step add 0.75 µL of octylphenyl-polyethylene glycol and mix using a vortex mixer. Add 25 mU of *peptide N-glycosidase F R*, mix using a vortex mixer and centrifuge. Incubate at 37 °C for 24 h. Remove the protein fraction using a suitable, validated procedure. The following method has been found to be appropriate. Add 600 µL of *anhydrous ethanol R*, previously cooled at – 20 °C for 45 min. Mix using a vortex mixer and centrifuge. Precipitate the proteins at – 20 °C for 15 min, then centrifuge at 10 600 *g* at 4 °C for 5 min. Transfer the supernatant to a separate tube and evaporate the ethanol for 15 min. Add 1 L of *particle-free water R* and resume evaporating until the remaining volume is about 500-800 µL, then freeze-dry.

Label the liberated glycans contained in the sample with 2-aminobenzamide. The procedure employs a combination of reagents optimised and validated for the efficient labelling of glycans, and for the subsequent extraction and recovery of the labelled glycans from the reaction. Recover the sample in 1.5 mL of *particle-free water R*.

Reference solution. Prepare at the same time and in the same manner as for the test solution but using the reference solution obtained in the previous step.

CHROMATOGRAPHIC SEPARATION. Liquid chromatography (*2.2.29*).

Column:

– *size*: *l* = 0.075 m, Ø = 7.5 mm;

– *stationary phase*: *weak anion-exchange resin R* (10 µm);

– *temperature*: 30 °C.

Mobile phase:

– *mobile phase A*: *acetonitrile R*;

– *mobile phase B*: *0.5 M ammonium acetate buffer solution pH 4.5 R*; filter through a membrane filter (nominal pore size 0.22 µm);

– *mobile phase C*: *particle-free water R*;

Time (min)	Mobile phase A (per cent *V/V*)	Mobile phase B (per cent *V/V*)	Mobile phase C (per cent *V/V*)
0 - 5	20	0	80
5 - 21	20	0 → 4	80 → 76
21 - 61	20	4 → 25	76 → 55
61 - 62	20	25 → 50	55 → 30
62 - 71	20	50	30
71 - 72	20	50 → 0	30 → 80
72 - 117	20	0	80

Flow rate: 0.4 mL/min.

Detection: fluorimeter at 330 nm for excitation and at 420 nm for emission.

Injection: 50 µL.

System suitability: reference solution:

- the chromatogram obtained is qualitatively similar to the chromatogram supplied with *follitropin CRS*;
- by comparison with the chromatogram supplied with *follitropin CRS*, identify the peaks due to neutral, mono-, di-, tri- and tetra-sialylated forms; determine the area of each peak and express it as a percentage of the total; calculate the *Z* number using the following expression:

$$(A_0 \times 0) + (A_1 \times 1) + (A_2 \times 2) + (A_3 \times 3) + (A_4 \times 4)$$

A_0 = peak area percentage due to the neutral form;

A_1 = peak area percentage due to the mono-sialylated form;

A_2 = peak area percentage due to the di-sialylated form;

A_3 = peak area percentage due to the tri-sialylated form;

A_4 = peak area percentage due to the tetra-sialylated form.

The *Z* number obtained for the reference solution is in the range 177-233.

Examine the chromatogram obtained with the test solution and calculate the *Z* number as described above.

Result: *Z* = 177-233.

METHOD B

PROTEIN DENATURATION

Solution A. To 1.952 g of *2-[N-morpholino]ethanesulfonic acid R* and 57.32 g of *guanidine hydrochloride R*, add 1 mL of a 15.4 g/L solution of *dithiothreitol R*, 10 mL of an 18.61 g/L solution of *sodium edetate R* and 20 mL of *water R*. Maintain in a water-bath at about 37 °C for 1 min to dissolve the components. Adjust to pH 8.1 (*2.2.3*) with an 80 g/L solution of *sodium hydroxide R* and dilute to 100.0 mL with *water R*. Mix.

Solution B. Dissolve 37 mg of *iodoacetamide R* in 1 mL of *water R* and mix. Protect from light.

Solution C. Dissolve 26.7 g of *disodium hydrogen phosphate dihydrate R* and 11.2 g of *sodium edetate R* in 3 L of *water R* and mix. Adjust to pH 7.5 (*2.2.3*) with a 40 g/L solution of *sodium hydroxide R*.

Test solution. Dissolve 1 mg of the substance to be examined in 0.2 mL of solution A and incubate in a water-bath at 37 ± 1 °C for 2 h. Add 20 µL of freshly prepared solution B, mix and incubate at 37 ± 1 °C for a further 2 h, protected from light. Add 10 µL of *2-mercaptoethanol R* and mix. Dialyse against 1 L of solution C. Add 200 µL of solution C and mix. Determine the protein content of the solution.

Reference solution (a). Prepare in the same manner as for the test solution but using *follitropin CRS* instead of the substance to be examined. Determine the protein content of the solution.

Reference solution (b). Prepare in the same manner as for the test solution but using fetuin instead of the substance to be examined. Determine the protein content of the solution.

SELECTIVE RELEASE OF THE GLYCANS

Test solution. Dilute the test solution obtained in the previous step with solution C to obtain a concentration of 1.1 g/L. Add 1 U of *peptide N-glycosidase F R* to 500 µg of the solution, mix and incubate at 37 ± 1 °C for 24 h. Place the solution in ice. Precipitate the protein and salts with 3 volumes of ice-cold *anhydrous ethanol R* and allow to stand in ice for 10 min. Centrifuge at 16 000 *g* for about 5 min and transfer the supernatant to a separate tube. Add 3 µL of a 1 µg/µL solution of *maltotriose R*, then freeze-dry. Dissolve in 100 µL of *water R*.

Reference solution (a). Prepare in the same manner as for the test solution but using the reference solution obtained with *follitropin CRS* in the previous step.

Reference solution (b). Prepare in the same manner as for the test solution but using the reference solution obtained with fetuin in the previous step.

CHROMATOGRAPHIC SEPARATION. Liquid chromatography (*2.2.29*).

Precolumn:

- *size*: *l* = 0.05 m, Ø = 4.0 mm;
- *stationary phase*: *strongly basic anion-exchange resin for chromatography R*;

Column:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *strongly basic anion-exchange resin for chromatography R*.

Mobile phase:

- *mobile phase A*: 20 g/L solution of *sodium hydroxide R*; maintain under helium;
- *mobile phase B*: *water R*; maintain under helium;
- *mobile phase C*: dissolve 41 g of *anhydrous sodium acetate R* in 800 mL of *water R*, dilute to 1 L with the same solvent, then mix; filter through a membrane filter (nominal pore size 0.45 µm); maintain under helium.

Time (min)	Mobile phase A (per cent *V/V*)	Mobile phase B (per cent *V/V*)	Mobile phase C (per cent *V/V*)
0 - 0.2	20	80	0
0.2 - 94.0	20	80 → 34	0 → 46
94.0 - 97.0	20	34	46
97.0 - 97.1	20	34 → 80	46 → 0
97.1 - 115.0	20	80	0

Flow rate: 1.0 mL/min.

Detection: pulsed amperometric detector.

Injection: 45 µL.

System suitability:

- the chromatogram obtained with reference solution (b) is qualitatively similar to the chromatogram for fetuin supplied with *follitropin CRS*;
- the chromatograms obtained with the test solution and reference solution (a) are qualitatively similar to the chromatogram supplied with *follitropin CRS*;
- by comparison with the chromatogram supplied with *follitropin CRS*, identify the peaks due to neutral, mono-, di-, tri- and tetra-sialylated forms in the chromatogram obtained with reference solution (b); determine the area of each peak and express it as a percentage of the total; calculate the *Z* number using the following expression:

$$(A_0 \times 0) + (A_1 \times 1) + (A_2 \times 2) + (A_3 \times 3) + (A_4 \times 4)$$

A_0 = peak area percentage due to the neutral form;

A_1 = peak area percentage due to the mono-sialylated form;

A_2 = peak area percentage due to the di-sialylated form;

A_3 = peak area percentage due to the tri-sialylated form;

A_4 = peak area percentage due to the tetra-sialylated form.

The *Z* number obtained for reference solution (b) is in the range 290-325.

Examine the chromatogram obtained with the test solution and calculate the *Z* number as described above.

Result: *Z* = 178-274.

TESTS

Follitropin oligomers. Size-exclusion chromatography (*2.2.30*). Use the normalisation procedure.

Solution A. Dissolve 118 mg of *sodium dihydrogen phosphate R*, 1.65 g of *disodium hydrogen phosphate dihydrate R* and 30.0 g of *sucrose R* in 40 mL of *water R* and dilute to 100.0 mL with the same solvent.

Solution B. Dissolve 1.0 mg of *bovine albumin R* in 30 mL of solution A.

Test solution. Dissolve the substance to be examined in solution A to obtain a concentration of 0.25 mg/mL.

Reference solution. Dissolve the contents of a vial of *follitropin CRS* in 200 µL of solution A and mix with the same volume of solution B. If necessary, dilute further with solution A to obtain a concentration of 0.25 mg/mL.

Column:

- *size*: *l* = 0.3 m, Ø = 7.8 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 10 000 to 500 000 (5 µm).

Mobile phase: dissolve 28.4 g of *anhydrous sodium sulfate R* in 2 L of *0.1 M phosphate buffer solution pH 6.7 R* and filter through a membrane filter (nominal pore size 0.45 µm).

Flow rate: 0.5 mL/min.

Detection: spectrophotometer at 215 nm.

Injection: 100 µL.

Retention time: follitropin = 14-16 min.

System suitability: reference solution:

- *resolution*: minimum 1.5 between the peaks due to bovine albumin and follitropin;
- no peak is detected between 5 min and 16 min in blank injections.

Limit:

- *sum of the peaks with a retention time less than that of the principal peak*: maximum 0.5 per cent.

Free subunits. Polyacrylamide gel electrophoresis (*2.2.31*) under non-reducing conditions.

Gel dimensions: 1.5 mm thick.

Resolving gel: 12 per cent acrylamide.

Sample buffer. Concentrated SDS-PAGE sample buffer R.

Test solution. Dissolve the substance to be examined in *water R* to obtain a concentration of 2 µg/µL. To 55 µL of the solution add 55 µL of the sample buffer. Allow to stand for 4 h at room temperature.

Reference solution (a). Dissolve the contents of a vial of *follitropin CRS* in *water R* to obtain a concentration of 2 µg/µL. To 25 µL of the solution add 25 µL of the sample buffer. To 40 µL of this solution add 180 µL of the sample buffer and 180 µL of *water R*. Allow to stand for 4 h at room temperature, then boil for 5 min.

Reference solution (b). A solution of molecular mass markers suitable for calibrating SDS-polyacrylamide gels in the range of 14.4-94 kDa.

Application:

Well	Solution(s)	Volume (µL)
1	Reference solution (a)	40
2	Reference solution (a)	30
3	Reference solution (a)	20
4	Reference solution (a)	15
5	Reference solution (a)	10
6	Reference solution (a)	5
7	Test solution	50
8	Test solution + reference solution (a)	50 + 25
9	Reference solution (b)	10

Detection: by Coomassie staining.

System suitability:

- reference solution (b): the validation criteria are met (*2.2.31*);
- test solution + reference solution (a): the bands corresponding to the follitropin heterodimer and subunits are clearly separated;
- reference solution (a): no bands corresponding to the follitropin heterodimer are seen;
- recovery is between 75 per cent and 125 per cent.

Limit:

- *free subunits*: maximum 3 per cent.

Oxidised follitropin. Liquid chromatography (*2.2.29*).

Solution A. Dissolve about 3.3 mg of *2,4-dichlorobenzoic acid R* in 10.0 mL of *ethanol (96 per cent) R*.

Test solution. Dissolve the substance to be examined in *water R* to obtain a concentration of 300 µg/mL.

Reference solution (a). Dissolve the contents of a vial of *follitropin CRS* in *water R* to obtain a concentration of 300 µg/mL.

Reference solution (b). Dissolve the contents of a vial of *follitropin CRS* in *dilute hydrogen peroxide solution R* to obtain a concentration of 300 µg/mL. Incubate for 30-45 min. Add 10 µL of solution A and inject immediately.

Column:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *butylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 30 °C.

Mobile phase:
– *mobile phase A*: *0.2 M phosphate buffer solution pH 2.5 R*;
– *mobile phase B*: *water R*, *acetonitrile R* (40:60 *V/V*);
– *mobile phase C*: *water R*;

Time (min)	Mobile phase A (per cent *V/V*)	Mobile phase B (per cent *V/V*)	Mobile phase C (per cent *V/V*)
0 → 8.4	50	25 → 39	25 → 11
8.4 → 8.5	50	39 → 45	11 → 5
8.5 → 15	50	45	5
15 → 15.1	50	45 → 25	5 → 25
15.1 → 25	50	25	25

Flow rate: 1.0 mL/min.

Detection: spectrophotometer at 210 nm.

Injection: 25 µL.

System suitability: reference solution (b):
– the peaks due to the oxidised follitropin α- and β-subunits are separated from the peaks due to the non-oxidised follitropin subunits and from the peak due to 2,4-dichlorobenzoic acid;
– the chromatogram obtained is similar to the chromatogram supplied with *follitropin CRS*.

Calculate the percentage of oxidation of the follitropin subunits using the following expression:

$$\frac{(A_2 + A_4) \times 100}{A_1 + A_2 + A_3 + A_4}$$

A_1 = area of the peak due to the follitropin α-subunit;

A_2 = area of the peaks due to the oxidised follitropin α-subunit;

A_3 = area of the peak due to the follitropin β-subunit;

A_4 = area of the peak due to the oxidised follitropin β-subunit.

Limit:
– *total oxidised forms*: maximum 6 per cent.

Bacterial endotoxins (*2.6.14*): less than 0.1 IU per International Unit of follitropin activity, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Protein. Size-exclusion chromatography (*2.2.30*).

Solution A. Dissolve 100 mg of *poloxamer 188 R* in 900 mL of *water R* and dilute to 1.0 L with the same solvent.

Test solution. Dissolve the substance to be examined in solution A to obtain a concentration of about 0.03 mg/mL.

Reference solution. Dissolve the contents of a vial of *follitropin CRS* in solution A to obtain a concentration of about 0.03 mg/mL.

Column:
– *size*: *l* = 0.3 m, Ø = 7.8 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 10 000 to 500 000 (5 µm).

Mobile phase: mix 6.74 mL of *phosphoric acid R*, 14.2 g of *anhydrous sodium sulfate R* and 900 mL of *water R*, adjust to pH 6.7 (*2.2.3*) with a 0.5 g/mL solution of *sodium hydroxide R* and dilute to 1.0 L with *water R*; filter through a membrane filter (nominal pore size 0.45 µm).

Flow rate: 1 mL/min.

Detection: spectrophotometer at 214 nm.

Injection: 100 µL.

System suitability: reference solution:
– *number of theoretical plates*: minimum 1300, calculated for the peak due to follitropin.

Calculate the content of follitropin taking into account the assigned content of *follitropin CRS*.

Potency

The follicle-stimulating activity of follitropin is estimated by comparing under given conditions its effect in enlarging the ovaries of immature rats treated with chorionic gonadotrophin with the same effect of the International Standard preparation of human recombinant follicle-stimulating hormone or of a reference preparation calibrated in International Units. The International Unit of FSH is the activity contained in stated amounts of the International Standard of human recombinant follicle-stimulating hormone. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Use immature female rats of the same strain, 19-28 days old, differing in age by not more than 3 days and having masses such that the difference between the heaviest and the lightest rat is not more than 10 g. Assign the rats at random to 6 equal groups of at least 5 rats. If sets of 6 litter mates are available, assign 1 litter mate from each set to each group and mark according to litter.

Choose 3 doses of the reference preparation and 3 doses of the preparation to be examined such that the smallest dose produces a positive response in some of the rats and the largest dose does not produce a maximal response in all of the rats. Use doses in geometric progression and as an initial approximation total doses of 1.5 IU, 3.0 IU and 6.0 IU may be tried, although the dose will depend on the sensitivity of the rats used, which may vary widely.

Dissolve separately the total quantities of the preparation to be examined and of the reference preparation corresponding to the daily doses to be used in sufficient *phosphate-albumin buffered saline pH 7.2 R* such that the daily dose is administered in a volume of about 0.5 mL. The buffer solution shall contain in the daily dose not less than 14 IU of chorionic gonadotrophin to ensure complete luteinisation. Add a suitable antimicrobial preservative such as 4 g/L of phenol or 0.02 g/L of thiomersal. Store the solutions at 5 ± 3 °C.

Inject subcutaneously into each rat the daily dose allocated to its group. Repeat the injection of each dose 24 h and 48 h after the 1st injection. About 24 h after the last injection, euthanise the rats and remove the ovaries from each rat. Remove any extraneous fluid and tissue from the ovaries and weigh the 2 combined ovaries of each rat immediately. Calculate the results by the usual statistical methods (for example, *5.3*), using the mass of the 2 combined ovaries as the response. (The precision of the assay may be improved by a suitable correction of the organ mass with reference to the mass of the rat from which it was taken; an analysis of covariance may be used.)

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.

STORAGE

In an airtight container, at a temperature not exceeding – 20 °C.

LABELLING

The label states:
– the potency in International Units per milligram of protein;
– where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.