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Notes: New monograph	

Follitropin Injection

Follitropin Injection

Action and use
Recombinant human follicle-stimulating hormone; treatment of female infertility.

DEFINITION

Follitropin Injection is a sterile solution of Follitropin or Follitropin Concentrated Solution in a suitable liquid. It is supplied as a ready-to-use solution. Either the alpha or beta follitropin glycoform can be used.

The injection complies with the requirements stated under Parenteral Preparations and with the following requirements

Content of follitropin
90.0 to 110.0% of the stated amount

Potency
The estimated potency is not less than 80% and not more than 125% of the stated potency.

IDENTIFICATION

A. In the test for oxidised follitropin, the principal peak in the chromatogram obtained with solution (1) has the same retention time as the principal peak in the chromatogram obtained with solution (3).

B. In the protein test of the Assay, the principal peak in the chromatogram obtained with solution (1) has the same retention time as the principal peak in the chromatogram obtained with solution (2).

TESTS

Follitropin oligomers
Use method A or method B.

A. Carry out the method for *size-exclusion chromatography*, Appendix III C, using the following solutions.

Solution A A solution of 1.65% w/v *disodium hydrogen orthophosphate dihydrate*, 0.12% w/v *sodium dihydrogen orthophosphate* and 30% w/v *sucrose* in *water*.

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

(1) *For injections less than 0.025% w/v concentrate the injection using a centrifugal filter unit to achieve the concentration required (Merck Amicon Ultra-0.5 is suitable).* Dilute the injection or the concentrated injection in solution A to give a protein concentration of 0.025% w/v. A lower protein concentration may be used if validated.

(2) Dissolve the contents of a vial of *follitropin EPCRS* 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 protein concentration of 0.025% w/v.

CHROMATOGRAPHIC CONDITIONS

(a) Use a column (30 cm × 7.8 mm) packed with *hydrophilic silica gel for chromatography* of a grade suitable for the fractionation of globular proteins in the molecular mass range of 10,000 to 500,000 (Biosep-SEC-S is suitable).

(b) Use isocratic elution and the mobile phase described below.

(c) Use a flow rate of 0.5 mL per minute.

(d) Use an ambient column temperature.

(e) Use a detection wavelength of 215 nm.

(f) Inject 100 µL of each solution.

MOBILE PHASE

1.42% w/v solution of *anhydrous sodium sulfate* in *0.1 M phosphate buffer solution pH 6.7*, filtered through a membrane filter (nominal pore size 0.45 µm).

When the chromatograms are recorded under the prescribed conditions, the peak due to follitropin has a retention time of 14 to 16 minutes.

SYSTEM SUITABILITY

The test is not valid unless, in the chromatogram obtained with solution (2) the resolution between the peaks due to bovine albumin and follitropin is at least 1.5.

LIMITS

In the chromatogram obtained with solution (1) the sum of the areas of any peaks eluting before the principal peak is not greater than 2% by *normalisation*.

B. Carry out the method for *size-exclusion chromatography*, Appendix III C, using the following solutions.

(1) Dilute a quantity of injection if necessary, freeze dried if necessary, in sufficient mobile phase to produce a solution expected to contain the equivalent of 0.0004% of protein. A lower protein concentration may be used if validated.

(2) Dissolve the contents of a vial of *follitropin EPCRS* in in sufficient mobile phase or water to produce a solution containing 0.004% w/v of protein.

(3) 0.00144% w/v of freeze-dried follitropin, 0.00004% w/v of free subunits and 0.00003% w/v of follitropin oligomers in the mobile phase. Free subunits are prepared by heating dissolved freeze-dried follitropin dissolved in water at 100° for at least 5 minutes. Follitropin oliogmers are prepared by heating freeze-dried follitropin at 120° for at least 30 minutes. Combine freeze-dried follitropin, prepared oligomers and prepared free subunits in mobile phase.

CHROMATOGRAPHIC CONDITIONS

(a) Use a column (30 cm x 10 mm) packed with a composite of cross-linked agarose and dextran of a grade

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suitable for the fractionation of globular proteins in the molecular mass range of 3,000 to 70,000 (Superdex 75 HR 10/30, Superdex HR 75 10/300 GL and Superdex 75 10/300 GL are suitable).

(b) Use isocratic elution and the mobile phase described below.

(c) Use a flow rate of 0.4 mL per minute.

(d) Use an ambient column temperature.

(e) Use a detection wavelength of 210 nm.

(f) Inject 100 µL of each solution.

MOBILE PHASE

20 volumes of *acetonitrile R1* and 80 volumes of a pH 7.0 buffer prepared as follows. Dissolve 35.6 g of disodium hydrogen orthophosphate dihydrate in 900 mL of water, adjust the pH to 7.0 with orthophosphoric acid and add sufficient water to produce 1000 mL.

When the chromatograms are recorded under the prescribed conditions the retention times relative to follitropin (retention time, about 24 minutes) are: oligomers, about 0.83 and free subunits, about 1.17.

SYSTEM SUITABILITY

The test is not valid unless, in the chromatogram obtained with solution (3) the resolution between the peaks due to follitropin and free subunits is at least 0.8.

Inject solution (2) six times. The test is not valid unless the relative standard deviation of the area of the principal peaks is not greater than 2.5 per cent.

LIMITS

In the chromatogram obtained with solution (1) the sum of the areas of any peaks eluting before the principal peak is not greater than 2% by *normalisation*.

Free subunits

Use method A or method B. The choice of method A or method B should be based on compliance with the system suitability requirements.

A. Carry out the method for *polyacrylamide gel electrophoresis*, Appendix III F, under non-reducing conditions, using slab gels 1.5 mm thick and 12% *acrylamide* as the resolving gel and the following solutions.

(1) *For injections less than 0.02% w/v concentrate the injection using a centrifugal filter unit to achieve the concentration required (Merck Amicon Ultra-0.5 is suitable).* Dilute the injection or the concentrated injection in *water* to give a protein concentration of 0.2% w/v. A lower protein concentration may be used if validated. To 55 µL of this solution add 55 µL of *SDS-PAGE sample buffer (concentrated).* Allow to stand for 4 hours at room temperature.

(2) Dissolve the contents of a vial of *follitropin EPCRS* in water to obtain a protein concentration of 0.2% w/v. To 25 µl of the solution add 25 µL *SDS-PAGE sample buffer (concentrated).* To 40 µL of this solution add 180 µL of *SDS-PAGE sample buffer (concentrated)* and 180 µL of water. Allow to stand for 4 hours at room temperature, then boil for 5 minutes.

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

In well 1 apply 40 µL of solution (2); In well 2 apply 30 µL of solution (2); In well 3 apply 20 µL of solution (2); In well 4 apply 15 µL of solution (2); In well 5 apply 10 µL of solution (2); In well 6 apply 5 µL of solution (2); In well 7 apply 50 µL of solution (1); In well 8 apply 50 µL of solution (1) and 25 µL of solution (2); In well 9 apply 10 µL of solution (3).

Detect using Coomassie staining.

SYSTEM SUITABILITY

The test is not valid unless:

The result obtained from well 9 meets the validation criteria of Appendix III F;

The bands corresponding to the follitropin heterodimer and subunits are clearly separated in the electrophoretogram obtained from well 8;

No bands corresponding to the follitropin heterodimer are observed in any of the electrophoretograms due to solution (2);

Recovery is between 75% and 125%.

LIMITS

Not more than 5%.

B. Carry out the method for *size-exclusion chromatography*, Appendix III C, using the following solutions.

(1) Dilute a quantity of injection if necessary, freeze dried if necessary, in sufficient mobile phase to produce a solution expected to contain the equivalent of 0.0004% of protein. A lower protein concentration may be used if validated.

(2) Dissolve the contents of a vial of *follitropin EPCRS* in in sufficient mobile phase or water to produce a solution containing 0.004% w/v of protein.

(3) 0.00144% w/v of freeze-dried follitropin, 0.00004% w/v of free subunits and 0.00003% w/v of follitropin oligomers in the mobile phase. Free subunits are prepared by heating dissolved freeze-dried follitropin dissolved in water at 100° for at least 5 minutes. Follitropin oliogmers are prepared by heating freeze-dried follitropin at 120° for at least 30 minutes. Combine freeze-dried follitropin, prepared oligomers and prepared free subunits in mobile phase.

CHROMATOGRAPHIC CONDITIONS

(a) Use a column (30 cm x 10 mm) packed with a composite of cross-linked agarose and dextran of a grade suitable for the fractionation of globular proteins in the molecular mass range of 3,000 to 70,000 (Superdex 75 HR 10/30, Superdex HR 75 10/300 GL and Superdex 75 10/300 GL are suitable).

(b) Use isocratic elution and the mobile phase described below.

(c) Use a flow rate of 0.4 mL per minute.

(d) Use an ambient column temperature.

(e) Use a detection wavelength of 210 nm.

(f) Inject 100 µL of each solution.

MOBILE PHASE

20 volumes of *acetonitrile R1* and 80 volumes of a pH 7.0 buffer prepared as follows. Dissolve 35.6 g of disodium hydrogen orthophosphate dihydrate in 900 mL of water, adjust the pH to 7.0 with orthophosphoric acid and add sufficient water to produce 1000 mL.

When the chromatograms are recorded under the prescribed conditions the retention times relative to

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follitropin (retention time, about 24 minutes) are: oligomers, about 0.83 and free subunits, about 1.17.

SYSTEM SUITABILITY

The test is not valid unless, in the chromatogram obtained with solution (3) the resolution between the peaks due to follitropin and free subunits is at least 0.8.

Inject solution (2) six times. The test is not valid unless the relative standard deviation of the area of the principal peaks is not greater than 2.5 per cent.

LIMITS

In the chromatogram obtained with solution (1) the area of the peak due to free subunits is not greater than 5% by normalisation.

Oxidised follitropin

Carry out the method for *liquid chromatography*, Appendix III D, using the following solutions.

(1) *For injections less than 0.025% w/v concentrate the injection using a centrifugal filter unit to achieve the concentration required (Merck Amicon Ultra-0.5 is suitable).* Dilute the injection or the concentrated injection in *water* to give a protein concentration of 0.03% w/v. A lower protein concentration may be used if validated.

(2) 0.033 % w/v solution of *2,4-dichlorobenzoic acid* in *ethanol (96%)*.

(3) Dissolve the contents of a vial of *follitropin EPCRS* in water to give a protein concentration of 0.03% w/v.

(4) Dissolve the contents of a vial of *follitropin EPCRS* in a 0.1% w/v solution of *hydrogen peroxide* to produce a solution containing 0.03% w/v. Incubate for 30-45 minutes. Add 10µL of solution (2) and inject immediately after preparation.

CHROMATOGRAPHIC CONDITIONS

(a) Use a column (25 cm x 4.6 mm) packed with *butylsilyl silica gel for chromatography* (5 µm) (Vydac 214TP54 is suitable).

(b) Use gradient elution and the mobile phase described below.

(c) Use a flow rate of 1 mL per minute.

(d) Use a column temperature of 30°.

(e) Use a detection wavelength of 210 nm.

(f) Inject 25 µL of each solution (1), (3) and (4).

MOBILE PHASE

Mobile phase A

0.2 M phosphate buffer solution pH 2.5

Mobile phase B

40 volumes of *water* and 60 volumes of *acetonitrile R1*

Mobile phase C

Water

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

SYSTEM SUITABILITY

The test is not valid unless, in the chromatogram obtained with solution (4), the peaks due to the oxidised follitropin alfa- and beta-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 with solution (4) is similar to the chromatogram supplied with *follitropin EPCRS*.

LIMITS

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

$$\frac{(A_{1-ox} + A_{2-ox}) \times 100}{A_1 + A_{1-ox} + A_2 + A_{2-ox}}$$

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

$A_{1\text{-ox}}$ = area of the peaks corresponding to the oxidised α-subunit of follitropin;

A_2 = area of the peak corresponding to the β-subunit of follitropin;

$A_{2\text{-ox}}$ = area of the peak corresponding to the oxidised β-subunit of follitropin.

In the chromatogram obtained with solution (1):

the total oxidised forms are not more than 15 %.

ASSAY

Protein

Carry out the method for size-exclusion chromatography, Appendix III C, using the following solutions.

Solution A 0.01% w/v *poloxamer 188* in *water*.

(1) Dilute the preparation to be examined in solution A to produce a solution containing 0.003% w/v.

(2) Dissolve the contents of a vial of *follitropin EPCRS* in solution A to obtain a concentration of about 0.003% w/v.

CHROMATOGRAPHIC CONDITIONS

(a) Use a stainless steel column (30 cm × 7.8 mm) packed with *hydrophilic gel for chromatography*, of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 10000 to 500000 (5 µm) (Biosep-SEC-S 2000 is suitable).

(b) Use isocratic elution and the mobile phase described below.

(c) Use a flow rate of 1 mL per minute.

(d) Use an ambient column temperature.

(e) Use a detection wavelength of 214 nm.

(f) Inject 100 µL of each solution.

MOBILE PHASE

Mix 6.74 mL of *orthophosphoric acid*, 14.2 g of *anhydrous sodium sulfate* and 900 mL of *water*, adjust to pH 6.7 with a 0.5 g/mL solution of *sodium hydroxide* and dilute to 1 L with *water*; filter through a membrane filter.

SYSTEM SUITABILITY

The test is not valid unless, in the chromatogram obtained with solution (2), the column efficiency is at least 1300 theoretical plates per metre.

DETERMINATION OF CONTENT

Calculate the content of follitropin in Follitropin Injection from the declared content of *follitropin EPCRS*.

Potency

In accordance with the General Notices, alternative test methods for biological activity may be used to demonstrate compliance with the monograph and the use of such tests is particularly encouraged when this leads to replacement or reduction of animal use or reduction of suffering.

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

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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 follicle-stimulating human recombinant hormone. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

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.

Dilute and dissolve respectively 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* 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 0.4% w/v of phenol or 0.002% w/v of *thiomersal*. Store the solutions at 5 ± 3°.

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 *supplementary chapter IV G*), 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 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

Follitropin Injection should be protected from light and stored at a temperature of 2° to 8°. It should not be allowed to freeze.

LABELLING

The label of the sealed container states the number of IU contained in it.

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