

Method for purifying FSH

Field of Invention

The invention relates to the field of the purification of follicle stimulating hormone (FSH).

Background of the invention

Follicle-stimulating hormone (FSH) is an injectable protein falling into the class of gonadotrophins. FSH is used in the treatment of infertility and reproductive disorders in both female and male patients.

In nature, FSH is produced by the pituitary gland. For pharmaceutical use, FSH may be produced recombinantly (rFSH), or it may be isolated from the urine of postmenopausal females (uFSH).

FSH is used in female patients in ovulation induction (OI) and in controlled ovarian hyperstimulation (COH) for assisted reproductive technologies (ART). In a typical treatment regimen for ovulation induction, a patient is administered daily injections of FSH or a variant (about 75 to 300 IU FSH/day) for a period of from about 6 to about 12 days. In a typical treatment regimen for controlled ovarian hyperstimulation, a patient is administered daily injections of FSH or a variant (about 150-600 IU FSH/day) for a period of from about 6 to about 12 days.

FSH is also used to induce spermatogenesis in men suffering from oligospermia. A regimen using 150 IU FSH 3 times weekly in combination with 2'500 IU hCG twice weekly has been successful in achieving an improvement in sperm count in men suffering from hypogonadotrophic hypogonadism [Burgues et al.; *Subcutaneous self-administration of highly purified follicle stimulating hormone and human chorionic gonadotrophin for the treatment of male hypogonadotrophic hypogonadism. Spanish Collaborative Group on Male Hypogonadotrophic Hypogonadism; Hum. Reprod.; 1997, 12, 980-6*].

Because of the importance of FSH in the treatment of fertility disorders, the provision of FSH of high purity and high specific activity is desirable. FSH treatment requires repeated injections. Highly purified FSH preparations can be administered

subcutaneously, permitting self-administration by the patient, thus increasing patient convenience and compliance.

Lynch *et al.* [The extraction and purification of human pituitary follicle-stimulating hormone and luteinising hormone ; *Acta Endocrinologica*, **1988**, 288, 12-19] describe a method for purifying human pituitary FSH. The method involves anion and cation exchange chromatography, immunoaffinity extraction and size exclusion chromatography. The method is said to result in pituitary FSH having a specific activity of 4,990 IU (immunoassay)/mg, with 16 IU/mg of LH. Protein content was determined either by dry weight or in solution by absorption at 280 nm (assuming that $A_{1\text{cm}}^{280}$ for 1 g/l is equal to 1).

WO 98/20039 (IBSA Institut Biochimique SA) describes a process for the purification of human urinary FSH starting with urinary extracts called human menopausal gonadotrophins (hMG). The process uses ion-exchange chromatography on weakly basic anionic exchange resins of the DEAE type followed by affinity chromatography on resin having an anthraquinone derivative as a ligand. The process is said to yield urinary FSH free from LH and having a specific activity of 6,870 IU (immunoassay)/mg. Protein content was determined by assuming that a water solution of 1 mg/ml of protein has an optical density of 0.62 at 277 nm, in quartz cuvettes with a 1 cm path length.

WO 00/63248 (Istituto Massone SA) describes a process for the purification of gonadotrophins, including FSH, from human urine. The process involves the following steps: ion exchange chromatography with a strong cationic resin of the type sulphopropyl, ion exchange chromatography with a strong anionic resin, and hydrophobic interaction chromatography (HIC). A FSH preparation having a specific activity of 8,400 IU/mg (Steelman-Pohley method: *Assay of the follicle stimulating hormone based on the augmentation with human chorionic gonadotrophin*; *Endocrinology*; **1953**, 53, 604-616) and less than 1 IU LH (rat seminal vesicle weight gain method: Van Hell H, Matthijsen R & GA Overbeek; *Acta Endocrinol*, **1964**, 47, 409) biological activity per 75 IU FSH is reportedly obtained. Protein content was performed by the Lowry method [O.H. Lowry *et al.*, *J. Biol. Chem.*, **1951**, 193, 265].

US 5,990,288 (Musick *et al.*) describes a method for purifying FSH from biological samples, such as human pituitary glands or human post-menopausal urine. The process uses cation exchange chromatography on Fractogel EMD SO₃-650M, followed by dye affinity chromatography on Mimetic Orange 1 resin, followed by a step
5 of hydrophobic interaction chromatography on Bakerbond Wide Pore HI-Propyl resin. The process is said to result in human pituitary FSH having a specific activity of 7,066 IU (immunoassay)/mg and less than 1 IU (immunoassay)/mg of LH, and a urinary FSH having a specific activity of 6,298 IU (immunoassay)/mg and less than 3 IU (immunoassay)/mg of LH. Protein content was determined by absorption at 280 nm
10 (assuming that $A_{1\text{cm}}^{280}$ for 1 g/l is equal to 1).

Chiba *et al.* [Isolation and partial characterisation of LH, FSH and TSH from canine pituitary gland ; *Endocrinol. J.*, **1997**, *44*, 205-218] describe a technique for purifying canine pituitary gonadotrophins, including FSH, using Concanavalin (Con) A affinity
15 chromatography, hydrophobic interaction chromatography (HIC) and immobilized metal ion chromatography with Cu⁺⁺. The resulting FSH is reported to have a specific activity of 2.17 IU/g protein using a radioreceptor assay for FSH for measuring biological activity and the BioRad protein assay kit (BioRad Laboratories CA USA) for determining protein content.

WO 88/10270 (Istituto di Ricerca Cesare Serono SPA) describes a method for purifying human FSH from urine. The process involves immunochromatography with FSH-specific immobilized monoclonal antibodies bound to Sepharose 4B by divinyl
20 sulphone, followed by reverse phase HPLC. The resulting FSH is free of LH and other urinary proteins and has a specific activity of 6,200 IU/mg of lyophilised powder (Steelman-Pohley method). The preparation was the first FSH preparation to be suitable for subcutaneous administration, due to its purity.

An ongoing need remains for new methods for purifying FSH and FSH variants. In
30 particular, there is a need for purification methods that avoid the use of the cost-intensive immunoaffinity chromatography steps.

Summary of the invention

It is an object of the invention to provide a new method for purifying recombinant FSH or a recombinant FSH variant.

5 In a first aspect, the invention provides a method for purifying recombinant human FSH or an FSH variant starting from a liquid containing the crude FSH, comprising the following steps :

- (1) dye-affinity chromatography;
- (2) hydrophobic interaction chromatography; and
- 10 (3) reverse phase chromatography ;

which may be carried out in any order.

Brief description of the drawings

Figure 1 illustrates the present invention, i.e. a process of purification comprising the
15 steps of :

- dye affinity chromatography,
- hydrophobic interaction chromatography,
- reverse phase chromatography,

Figure 2 shows a flow chart of a specific embodiment of the present invention, i.e. a
20 process of purification comprising the steps of :

- ultrafiltration/diafiltration,
- anion-exchange chromatography,
- dye affinity chromatography,
- hydrophobic interaction chromatography,
- 25 • reverse phase chromatography,
- anion-exchange chromatography,
- nanofiltration,
- ultrafiltration/diafiltration;

Abbreviations

The following abbreviations are used in the description of the invention:

DF: diafiltration

FSH: follicle stimulating hormone;

r-FSH: recombinant FSH;

hFSH: human FSH;

r-hFSH: recombinant human FSH

BV: Bed volume

5 DEAE: diethylaminoethyl

ELISA: enzyme linked immunoassay

DAC: dye affinity chromatography

IMAC: immobilised metal ion affinity chromatography

OD: optical density

10 HIC: Hydrophobic interaction chromatography

HPLC : high performance liquid chromatography

IRMA: immunoradiometric assay

KD or kD: kiloDalton

HCP: host cell protein, proteins arising from the host cell used for expression of FSH

15 IPC: In process controls

IEF: isoelectric focussing

PES: polyethersulphone

RP-HPLC: reverse phase high performance liquid chromatography

Q FF: anion exchange on Q Sepharose FF

20 RT : Room Temperature

UF: ultrafiltration

WFI: water for injection

Detailed description of the invention

25 The invention provides a method for purifying recombinant human FSH or a recombinant FSH variant starting from a liquid containing the crude FSH, comprising the steps :

(1) dye affinity chromatography;

(2) hydrophobic interaction chromatography; and

30 (3) reverse phase chromatography ;

which may be carried out in any order.

The purification method of the invention affords a recombinant FSH bulk of high purity which may then be formulated to the final medicament, e.g. Gonal-F (Serono). It has the advantage of affording a high degree of purity without using immunoaffinity

chromatography. The crude FSH which forms the starting material for the purification according to the present invention consists in cell culture harvests containing recombinant FSH.

In a preferred embodiment, an antioxidant or a free amino acid or dipeptide with
5 antioxidant and scavenging effect is included in some or all of the steps of the purification method according to the present invention. More precisely, the antioxidant is present in any of the buffers used to purify and/or concentrate and/or filter the r-hFSH. The antioxidant prevents oxidation of the FSH during processing. A preferred antioxidant is L-methionine. Preferably, L-methionine is used at a concentration of at
10 or about 10-100 mM. Further examples of an antioxidant include t-butyl-4-methoxyphenol, 2,6-bis(1,1-dimethylethyl)-4-methyl phenol; potassium or sodium bimeta-bisulfite, sodium bisulfite. Examples of free amino acid and dipeptide with antioxidant and scavenging effect are histidine, taurine, glycine, alanine, carnosine, anserine, 1-methylhistidine or combinations thereof.

15 Typically, the starting material is clarified first and then and optionally concentrated (e.g. by using ultrafiltration) and/or buffer exchanged (e.g. through a diafiltration step) prior to being captured on the first chromatographic step .

In the steps of chromatography, polymer-based and agarose-based resins may be used. It is also possible to use membrane chromatography, in which the resin is
20 replaced with a functionalised membrane.

The 3 purification steps of the present invention (i.e. dye affinity chromatography, hydrophobic interaction chromatography, reverse phase chromatography) are in the following outlined more in detail.

The dye affinity chromatography step (1)

25 The method of the invention involves a step of dye affinity chromatography (1). In a preferred embodiment, the step of dye affinity chromatography is carried out using a resin having as an immobilised ligand a dye compound which is well known to a person skilled in the art, i.e. Cibacron Blue F3G-A. The term "immobilized" is well understood by a person skilled in the art and means that the ligand is derivatised in
30 the sense that it is chemically linked to the resin. A particularly preferred resin is Blue

Sepharose FF (obtainable from Amersham Biosciences Inc.). The technical features of Blue Sepharose FF are as follows :

TECHNICAL SPECIFICATIONS	
Ligand	Cibacron Blue F3G-A
Ligand coupling method	Triazine coupling
Binding capacity	≈18 mg human serum albumin/ml drained gel
Matrix	Highly cross-linked agarose, 6%
Exclusion limit (M_r)	4×10^6
Particle size range	45–165 μm
Linear flow rate*	≈ 750 cm/h
Ligand density	≈ 7 μmol Cibacron Blue/ml medium
pH stability	4–12 (long term), 3–13 (short term)
Chemical stability	40 °C for 7 days in: 70% ethanol, 6 M guanidine hydrochloride, 8 M urea

It is understood that the method may be performed with alternate resins, having similar characteristics. Examples of alternative resins include : Toyopearl AF-blue-HC-650M (Tosoh Bioscience), Toyopearl SuperButyl 550, Toyopearl Phenyl 650, Blue Cellthru BigBead (Sterogene), SwellGel Blue (Pierce), Cibachrome blue 3GA-agarose 100 (Sigma), Affi-Gel Blue (BioRad), Econo-Pac blue cartridges (Bio-Rad), Blue sepharose HP (Amersham), Cibacron Blue 3GA (Sigma).

Elution in the step of immobilised dye affinity chromatography should preferably be carried out using a buffer of phosphate, particularly preferably sodium phosphate. The pH of the eluent should preferably be at or about 6.0 to at or about 11.5, more preferably at or about 6.5 to at or about 8, particularly preferably at or about 7.0. Alternate buffers appropriate for maintaining a pH of 7.0 include the following: MES, Bis-Tris, ADA, PIPES, ACES, BES, MOPS, TES, HEPES. The elution buffer for the step of dye affinity chromatography should preferably contain a salt to increase the conductivity, preferably NaCl,.

In a particularly preferred embodiment, the product-contacting buffers for the step of dye affinity chromatography (equilibration, wash and elution) contain an antioxidant, such as L-methionine. Further examples of an antioxidant include t-butyl-4-

methoxyphenol, 2,6-bis(1,1-dimethylethyl)-4-methyl phenol; potassium or sodium bimetabisulfite, sodium bisulfite.

The hydrophobic interaction chromatography step (2)

5 The method also involves a step of hydrophobic interaction chromatography (2). In a preferred embodiment, the hydrophobic interaction chromatography is carried out with a resin such as Toyopearl Butyl 650M (obtainable from Tosoh Biosep Inc.).

It is understood that step (2) may be performed using alternate resins, having similar characteristics. Alternative resins that may be used are as follows : Phenyl Sepharose
10 6 Fast Flow (low sub) ; Phenyl Sepharose 6 Fast Flow (high sub) ; Butyl Sepharose 4 Fast Flow ; Octyl Sepharose 4 Fast Flow ; Phenyl Sepharose High Performance; SOURCE 15ETH; SOURCE 15ISO; SOURCE 15PHE all from Amersham Biosciences (800) 526-3593; (see www.amershambiosciences.com). Still further resins are :
Hydrocell C3 or C4; Hydrocell Phenyl from BioChrom Labs Inc. (812) 234-2558; (see
15 www.biochrom.com)

Binding on the HIC resin is achieved in a buffer with a high conductivity, obtained through the addition of salt (NaCl, $(\text{NH}_4)_2\text{SO}_4$ or Na_2SO_4 for example). Elution in the step of hydrophobic interaction chromatography is preferably carried out by reducing
20 the conductivity of the mobile phase (reducing salt concentration), using a buffer having a pH at or about 6 to at or about 8, more preferably at or about 6.5 to at or about 7.5, most preferably at or about 7). A particularly preferred system contains sodium phosphate for buffering preferably at a pH of at or about 7, and ammonium sulfate. Alternative buffers are mentioned above.

25 In a particularly preferred embodiment, the product-contacting buffers for the step (2) of HIC (equilibration, wash, elution) contain an antioxidant, such as L-methionine. Alternative antioxidants are mentioned above.

The reverse phase chromatography step (3)

30 The method of the invention also comprises a step of reverse phase chromatography (RPC) (3). The RPC is preferably carried out using a resin such as SOURCE 30 RPC (obtainable from Amersham Biosciences). It is understood that step (3) may be

performed using alternative resins which are well known to a person skilled in the art and which have similar characteristics.

The chromatography is preferably carried out using a mobile phase buffering at mildly alkaline pH, for example at or about pH 7-8.5, more preferably at or about 7.5 or 7.6.

- 5 In a preferred embodiment, the buffering species is ammonium acetate. Alternate buffers adequate for a pH at or around 7.6 include: BES, MOPS, Phosphate, TES, HEPES. The buffer solutions used for this step may also contain an organic modifier, the concentration of which is modulated for different phases of the chromatography step (load, wash, elution and regeneration). In a preferred embodiment, the organic
- 10 modifier is a water miscible organic solvent, preferably an alcohol (such as methanol, ethanol, etc.), most preferably 2-propanol (*iso*-propanol).

In a particularly preferred embodiment, the product-contacting buffers for the step of RPC (equilibration, wash, elution) contain an antioxidant, such as L-methionine. Alternate antioxidants are mentioned above.

15 Optional further purification step 0 - ion exchange chromatography

Further to the 3 main purification steps – outlined above – the present invention may include additional purification steps.

- In one embodiment, the purification method of the invention involves a preliminary step of ion exchange chromatography (0) carried out, preferably with a strong anion
- 20 exchange resin, particularly preferably a quaternary ammonium resin, such as Q Sepharose FF (obtainable from Amersham Biosciences), having the following characteristics :

Type of ion exchanger:	Strong anion
Total capacity (mmol/ml):	0.18-0.25
Exclusion limit (globular proteins) :	4×10^6
Bead form :	Spherical, diameter 45-165 μm
Bead structure:	Cross-linked agarose, 6%
Operational pH stability:	2-12
Cleaning pH stability:	1-14
Linear flow rate at 25°C 1 bar 15 cm bed height, XK 50/30 column:	400-700 cm/h

Alternatively, the ion-exchange chromatography step (0) may be carried out using a resin such as Fractogel EMD TMAE HICAP (obtainable from Merck KGaA, Darmstadt Germany), or a resin having similar characteristics, see below:

Support	Fractogel® EMD TMAE
Cat.No.	1.16887
Particle size S-type	20-40µm
Type of chromatography	ion-exchange chromatography
Functional group	trimethylaminoethyl group (Q-type)
Monomer structure	$\text{CH}_2=\text{CH}-\text{CONH}-(\text{CH}_2)_2\text{N}^+(\text{CH}_3)_3$
Protein binding capacity	120 mg BSA/ml of gel
pH stability range	pH 2 up to pH 12
pK value	> 13
Elution conditions	high salt concentrations
Pressure limit (bed: 150x10 mm)	20 bar (pressure drop along the column)
Working temperature	4°C to room temperature
Preservative	20 % ethanol
Ready to use cartridge	50-10 mm
Bulk material S-types	100 ml; 500 ml
Linear flow rate	1.27 - 6.35 cm/min

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The step of ion-exchange chromatography is preferably carried out using a buffer having a mildly alkaline pH (e.g. at or about 7.2 to at or about 9.0, or at or about 8.0 to at or about 9.0, most preferably at or about 8.5). Suitable buffers include, for example borate buffer, triethanolamine/iminodiacetic acid Tris, ammonium acetate, tricine, bicine, TES, HEPES, TAPS. Most preferred is borate buffer, at a pH of at or about 8.5. Elution from the ion-exchange resin is achieved by increasing the conductivity of the mobile phase through the addition of salt, preferably NaCl. In a particularly preferred embodiment the product-contacting buffers for the ion-exchange chromatography (equilibration, wash, elution) contain an antioxidant, preferably L-methionine. Alternative antioxidants are mentioned above.

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Thus in a preferred embodiment, the method of the invention comprises the following steps:

- (0) anion-exchange chromatography, preferably on a strong anion exchange resin, [preferably a quaternary ammonium resin, such as Q Sepharose FF or Fractogel EMD TMAE];
- (1) dye affinity chromatography [preferably on Blue Sepharose FF];
- (2) hydrophobic interaction chromatography [preferably on Toyopearl Butyl 650M];
- (3) reverse phase chromatography [preferably on Source 30 RPC].

Optional further purification step (-1) – ultrafiltration/diafiltration

- Prior to the step of ion exchange chromatography (0), it may be desirable to carry out a step of ultrafiltration, in order to concentrate the crude FSH. The ultrafiltration (or diafiltration) is preferably carried out using a membrane having a cut-off of at or about 3 –10 kD, most preferably at or about 8 kD.

Optional further purification step (4) – anion-exchange chromatography

- In a further preferred embodiment, the method of the invention also comprises a second step of anion-exchange chromatography (4). A preferred resin is Fractogel EMD TMAE HICAP (obtainable from Merck KGaA, Darmstadt Germany), or a resin having similar characteristics, as mentioned above. Alternatively the second step of anion-exchange chromatography may be carried out on Q Sepharose FF, or other resin having similar characteristics, as mentioned above.

The steps of anion exchange chromatography, dye affinity chromatography, hydrophobic interaction chromatography (HIC), reverse phase chromatography and second step of anion-exchange chromatography may be carried out in any order, although it is preferred to carry out a step of anion exchange chromatography first. The remaining steps of dye affinity chromatography, hydrophobic interaction chromatography (HIC), RPC and optional second anion-exchange chromatography may be carried out in any order, although it is preferred to follow the order shown below:

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(0) anion-exchange chromatography, (1) dye affinity chromatography, (2) hydrophobic interaction chromatography (HIC), (3) reverse phase chromatography RPC; and (4) second anion-exchange chromatography.

Optional further purification step (5) – ultrafiltration/diafiltration

In a further preferred embodiment, after any of the steps of chromatography (particularly after a step of reverse phase chromatography), the FSH sample is subjected to a concentration step. Preferably the step is performed using ultrafiltration combined by
5 diafiltration in order to obtain a bulk having the desired composition. The ultrafiltration (or diafiltration) is preferably carried out using a membrane having a cut-off of at or about 3 –10 kD, most preferably at or about 5 kD.

In a particularly preferred embodiment, the following steps are carried out in the order
10 shown below:

- (-1) Ultrafiltration (preferably with a membrane having a cut-off of at or about 8 kD),
- (0) anion-exchange chromatography (preferably using a Q Sepharose FF column);
15
- (1) dye affinity chromatography (preferably using a Blue Sepharose FF column);
- (2) hydrophobic interaction chromatography (HIC) (preferably using a Butyl 650Mcolumn);
- (3) reverse phase chromatography (RPC) (preferably using a Source 30 RPC column);
20
- (4) anion-exchange chromatography on a strongly basic anion exchange resin (preferably using a TMAE hicap resin); and
- (5) ultrafiltration (preferably with a membrane having a cut-off of 5 kD).

25 It may be desirable to subject the FSH sample to a step of nanofiltration, in particular as a virus clearance step; i.e. to reduce the risk of contamination of the FSH preparation with viruses or virus-like particles originating from the cell culture.

Nanofiltration may be done at any stage of the purification process, however, it is
30 particularly preferred to carry out nanofiltration after the 2nd step of ion exchange chromatography, or after reverse phase chromatography or after hydrophobic interaction chromatography. Nanofiltration may be performed more than one time, for example it may be performed twice.

In a particularly preferred embodiment, the method of the invention comprises the following steps:

- (-1) Ultrafiltration (preferably with a membrane having a cut-off of at or about 8 kD),
- (0) anion-exchange chromatography (preferably with Q Sepharose FF),
- (1) dye affinity chromatography (preferably with Blue Sepharose FF),
- (2) hydrophobic interaction chromatography (HIC) (preferably with Butyl 650M),
- (3) reverse phase chromatography (RPC) (preferably with Source 30 RPC),
- (4) anion-exchange chromatography on a strongly basic anion- exchange resin (preferably TMAE hicap resin);
- (4') nanofiltration,
- (5) ultrafiltration (preferably with a membrane having a cut-off of 5 kD).

The advantage of the present invention is that the purification method is devoid of a cost intensive immuno-affinity chromatography step and provides anyhow a high degree of FSH purity and specific bioactivity. Also, the purified FSH of the present invention does not contain undesired impurities added by the immuno affinity chromatography (e.g. immunoglobulins leached from the resin)

Storage/Lyophilisation

The liquid composition resulting from the purification process as described above and containing purified FSH may be frozen for storage as is, or after purification, the eluate may be subjected to lyophilisation ("freeze-drying") to remove solvent. The resulting liquid or lyophilised product is termed "FSH Bulk".

FSH formulations

FSH or an FSH variant of the invention or purified according to the method of the invention may be formulated for injection, either intramuscular or subcutaneous, preferably subcutaneous. The FSH formulation may be freeze-dried, in which case it is dissolved in water for injection just prior to injection. The FSH formulation may also be a liquid formulation, in which case it can be injected directly, without prior dissolution.

The FSH formulation may be single dose or multiple dose. If it is multiple dose, it should preferably contain a bacteriostatic agent, such as, for example, benzyl alcohol, meta-cresol, thymol or phenol, preferably benzyl alcohol or meta-cresol. Single dose formulations may also comprise a bacteriostatic agent.

- 5 The FSH of the invention may be formulated with known excipients and stabilizers, for example, sucrose and mannitol. It may also comprise an antioxidant, such as methionine. It may further comprise a surfactant, such as TWEEN (preferably TWEEN 20), or Pluronic (preferably Pluronic F68).
- 10 In a particularly preferred multidose formulation, FSH produced by the method of the invention is formulated by dissolving it in water for injection with sucrose, phosphate buffer (pH 7), Pluronic F68, methionine and meta-cresol or benzyl alcohol.

- A particular preferred liquid multi-dose formulation of recombinant FSH for
- 15 subcutaneous or intramuscular injection is the following :

Components of FSH multi-dose liquid formulations				
Component #	Description	300 IU FSH	450 IU FSH	900 IU FSH
1	rhFSH (µg/cartridge)	22.2 (305 IU)	33.3 (458 IU)	66.7 (916 IU)
2	Sucrose (mg/cartridge)	30.0	45.0	90.0
3	NaH ₂ PO ₄ ·H ₂ O (mg/cartridge)	0.225	0.337	0.675
4	Na ₂ HPO ₄ ·2H ₂ O (mg/cartridge)	0.555	0.832	1.665
5	Pluronic F68 (mg/vial)	0.050	0.075	0.150
6	Methionine (mg/vial)	0.050	0.075	0.150
7	<i>m</i> -cresol (mg/vial)	1.50	2.25	4.50
8	pH	7.0	7.0	7.0
9	WFI	q.s. to 0.5 ml	q.s. to 0.75 ml	q.s. to 1.5 ml

Indications

The FSH of the invention is suitable for use in all treatments where FSH is indicated. It is particularly suited for subcutaneous administration in ovulation induction, controlled ovarian hyperstimulation for assisted reproductive technologies, and in the treatment of oligospermia. It may be used in conjunction with other gonadotrophins, such as LH and hCG. It may also be used with further compounds which augment the response to FSH, such as clomiphene citrate, aromatase inhibitors, such as Anastrozole, Letrozole, Fadrozole and YM-511.

Sequences:

SEQ ID NO. 1: human glycoprotein α -subunit;

SEQ ID NO. 2: hFSH β -subunit

SEQ ID NO. 3: hFSH β -subunit variant 1

SEQ ID NO. 4: hFSH β -subunit variant 2

SEQ ID NO. 5: hFSH β -subunit variant 3

Follicle stimulating hormone, or FSH, as used herein refers to human FSH (hFSH) produced as a full-length mature protein. FSH is a dimer composed of the human glycoprotein alpha-subunit and the human FSH beta-subunit. The protein sequence of the human glycoprotein alpha subunit is provided in SEQ ID NO: 1, and the protein sequence of the human FSH beta subunit is given in SEQ ID NO: 2.

The use of the term "recombinant" refers to preparations of FSH that are produced through the use of recombinant DNA technology (see for example WO 85/01958).

One example of a method of expressing FSH using recombinant technology is by transfection of eukaryotic cells with DNA sequences encoding an alpha and beta subunit of FSH, whether provided on one vector or on two vectors with each subunit having a separate promoter, as described in European patent nos. EP 0 211 894 and EP 0 487 512. The DNA encoding FSH may be a cDNA or it may contain introns.

Another example of the use of recombinant technology to produce FSH is by the use of homologous recombination to insert a heterologous regulatory segment in operative connection to endogenous sequences encoding one or both of the subunits of FSH, as described in European patent no. EP 0 505 500 (Applied Research Systems ARS Holding NV). Also contemplated are methods such as those disclosed in WO 99/57263 (Transkaryotic Therapies), wherein one of the subunits is inserted heterologously into a cell, and the other subunit is expressed by activation of genomic

sequences by insertion of a heterologous regulatory segment by homologous recombination. The method of the invention may be used to purify FSH expressed using any of these methods and other methods.

The expression "recombinant cell" refers to a cell produced by inserting heterologous DNA, including any of the above-mentioned methods of genetic manipulation.

Preferably the FSH is produced recombinantly in Chinese hamster ovary (CHO) cells transfected with a vector or vectors comprising DNA coding for the human glycoprotein alpha-subunit and the beta-subunit of FSH. DNA encoding the alpha and beta-subunits may be present on the same or different vectors.

The expression "FSH variant" is meant to encompass those molecules differing in amino acid sequence, glycosylation pattern or in inter-subunit linkage from human FSH but exhibiting FSH-activity. Examples include CTP-FSH, a long-acting modified recombinant FSH, consisting of the wild type α -subunit and a hybrid β -subunit in which the carboxy terminal peptide of hCG has been fused to the C-terminal of the β -subunit of FSH, as described in LaPolt *et al.*; *Endocrinology*; **1992**, *131*, 2514-2520; or Klein *et al.*; *Development and characterization of a long-acting recombinant hFSH agonist*; *Human Reprod.* 2003, *18*, 50-56]. Also included is single chain CTP-FSH, a single chain molecule, consisting of the following sequences (from N-terminal to C-terminal):

β FSH	β hCG-CTP(113-145)	α FSH
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wherein β FSH signifies the β -subunit of FSH, β hCG CTP (113-145) signifies the carboxy terminal peptide of hCG and α FSH signifies the α -subunit of FSH, as described by Klein *et al.* [*Pharmacokinetics and pharmacodynamics of single-chain recombinant human follicle-stimulating hormone containing the human chorionic gonadotrophin carboxyterminal peptide in the rhesus monkey*; *Fertility & Sterility*; **2002**, *77*, 1248-1255]. Other examples of FSH variants include FSH molecules having additional glycosylation sites incorporated in the α - and/or β -subunit, as disclosed in WO 01/58493 (Maxygen), and FSH molecules with intersubunit S-S bonds, as disclosed in WO 98/58957. Further examples of FSH variants include

chimeric molecules comprising sequences from FSH and sequences from hCG or LH, such as those described in WO 91/16922 and WO 92/22568.

The FSH variants referred to herein also include the carboxy terminal deletions of the beta subunit that are shorter than the full length mature protein of SEQ ID NO:2.

Carboxy terminal deletions of the human beta subunit are provided in SEQ IDS NOS: 3, 4, and 5. It is understood that the carboxy terminal variants of the beta chain form complex with a known alpha subunit to form an FSH variant heterodimer.

In a preferred embodiment, the FSH is produced recombinantly in CHO cells, either in a serum or in a serum-free medium.

In a preferred embodiment, the purified FSH produced according to the method of the invention is suitable for subcutaneous administration, permitting self-administration by the patient.

The expression "crude recombinant FSH" refers to the cell culture supernatant from recombinant cells expressing FSH, before it has undergone any chromatographic step. The expression encompasses the raw form of the supernatant (as isolated from cells) as well as concentrated and/or filtered and/or ultrafiltered supernatant.

The term "biological activity" in relation to FSH activity, refers to the ability of an FSH formulation to elicit biological responses associated with FSH, such as ovarian weight gain in the Steelman-Pohley assay [Assay of the follicle stimulating hormone based on the augmentation with human chorionic gonadotrophin; Endocrinology; 1953, 53, 604-616], or follicular growth in a female patient. Follicular growth in a female patient can be evaluated by ultrasound, for example, in terms of the number of follicles having a mean diameter of at or about 16 mm on day 8 of stimulation. Biological activity is evaluated with respect to an accepted standard for FSH.

The LH content in an FSH preparation may be measured, for example, using an LH-specific immunoassay, such as the Delfia hLH Spec (Wallac Oy, Turku, Finland).

The term "specific activity", in reference to FSH, means the biological activity in IU of the preparation in a recognised biological assay for FSH, such as the Steelman

Pohley bioassay [divided by the amount of protein, as determined by an assay for total protein content, such as the Lowry assay [O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall (1951) *J. Biol. Chem.* **193**: 265; Hartree E. E. (1972). *Anal. Biochem.* **48**: 422; J.R. Dulley and P.A. Grieve (1975) *Anal. Biochem.* **64**: 136], the Bradford
5 assay [Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248], or by absorbance at 280 nm.

Preferably, the FSH obtained by the invention has a specific activity of greater than at or about 8000 IU/mg, more preferably greater than at or about 9000 IU/mg, even more preferably greater than at or about 10000 IU/mg, even more preferably at or about 14000 IU/mg wherein biological activity is measured by the Steelman-Pohley bioassay
10 and protein content is measured by SE-HPLC

The FSH samples may be analysed in respect of their purity at various stages of the procedure using, for example, techniques such as those listed below:

r-hFSH quantification/free alpha subunit/purity/ oxidised forms: RP-HPLC

As mentioned above, FSH is a heterodimeric glycoprotein, composed of an α -
15 and a β -subunit. Some dissociation of the subunits can occur, and this can be monitored by looking at the amount of free α -subunit present in a sample. In addition, the FSH subunits may become oxidised. The oxidised contaminants can be quantified using RP-HPLC, while the free subunits may be assessed using SDS-PAGE.

r-hFSH quantification: Immunoassay

FSH content in a sample can be determined using an immunoassay specific for FSH, such as the DELFIA FSH immunoassay.

Total protein: Bradford assay, Lowry Assay, Absorbance at 280 nm

As with any protein preparation, total protein content can be determined using
25 techniques such as a Bradford assay, a Lowry Assay or by absorbance at 280 nm.

Isoforms pattern: IEF

As mentioned above, FSH is a glycoprotein, having multiple oligosaccharide
30 residues attached at various places on both subunits. The oligosaccharide residues may have different degrees of branching and may be capped with sialic acid residues. Sialic acid residues are negatively charged (at neutral

pH). Differences in capping leads to heterogeneity, with a mixture of species having different isoelectric points (pI). This can be assessed using a technique that separates based on charge, such as isoelectric focussing (IEF)

Host cell protein (HCP)

- 5 Host cell protein can be analysed using an ELISA assay. For example, antibodies can be raised to a “mock culture”, which is a culture of host cells without FSH gene.

EXAMPLES

- 10 The present invention will now be illustrated by means of 2 examples.

Corresponding flow charts illustrating said 2 examples are presented in Figure 1 & 2. The resulting purified r-hFSH is termed “r-hFSH bulk”.

EXAMPLE 1 (cf Figure 1)

Step (1): Dye affinity chromatography on Blue Sepharose

- 15 The FSH starting material for the purification is prepared from cell culture harvests containing recombinant FSH, i.e. FSH which was produced recombinantly in CHO cells, either in a serum or in a serum-free medium. The dye affinity chromatography column (Blue Sepharose FF resin) is first equilibrated with a low conductivity buffer at a pH of 8.5 containing L-methionine. The liquid containing the FSH is then applied
20 directly to the resin. After the load, the unbound material is washed out using equilibration buffer. The FSH is finally eluted by flushing the column with Sodium phosphate buffer at pH 7.0, containing NaCl and L-methionine. The elution pool is directly processed to the next step. The step is performed at 2-8°C.

Step (2): Hydrophobic Interaction Chromatography (HIC) on Toyopearl Butyl 650M

- 25 The Blue sepharose FF eluate from step (1) is loaded onto a Toyopearl Butyl 650M column equilibrated against a Sodium phosphate buffer, pH 7.0, containing Ammonium Sulfate and L-methionine. The unbound material is flushed out with equilibration buffer. The FSH is eluted with the same buffer, but with a reduced concentration of Ammonium Sulfate. The eluate is processed to the next step. The
30 step is performed at RT

Step (3): Reverse Phase on Source 30 RPC

The HIC eluate (from step (2)) is first conditioned by addition of IPA (isopropanol). A Source 30RPC column is equilibrated against an ammonium acetate buffer, pH 7.6, containing L-methionine, and 2-propanol at a concentration equivalent to that of the conditioned load material. After flushing out the unbound material with equilibration
5 buffer, the resin is washed with ammonium acetate buffer, pH 7.6, containing L-methionine, and an increased concentration of 2-propanol. The FSH is finally eluted by increasing further the concentration of 2-propanol. The elution pool is finally diluted under stirring, with water containing L-methionine. The diluted pool is processed to
10 the next step. The step is performed at RT.

Upon following the above procedure, the factor of purification – i.e. the ratio of FSH purity in the purified sample versus the FSH purity in the starting material (crude FSH) - is at about 40.000.

EXAMPLE 2 (cf Figure 2)

Step (-1): Ultrafiltration/Diafiltration of concentrated r-hFSH

All the operations were performed in refrigerated conditions (2-8°C). The crude FSH forming the starting material for the purification is derived from cell culture harvests containing recombinant FSH.

Clarification

Crude r-hFSH was filtered through a 0.5 µm depth filter (such as Pall Profile II filters or equivalent).

Ultrafiltration

The clarified crude was first concentrated by ultrafiltration using a 10KD polyether sulfone membrane. The concentrated retentate was then diafiltered against at least 5 diavolumes of borate buffer, pH 8.5 containing L-methionine as antioxidant. The conductivity and pH of the retentate were measured to monitor the progress of the
30 diafiltration. The retentate was then concentrated further before draining the system. The ultra-filtration unit was finally flushed with diafiltration buffer and the rinsate mixed with the recovered retentate. The pool was progressed to the next step.

Filtration

The concentrated product was filtered through a 0.2 µm polyether sulphone filter (or equivalent).

Step (0): Anion Exchange on Q Sepharose FF

- 5 The filtered material was then applied to a strong anion exchange (Q-sepharose FF) resin equilibrated against Sodium Borate buffer, pH 8.5, containing L-methionine. After the load, the column was rinsed with equilibration buffer in order to flush all unbound material. The column was then eluted with Sodium Borate buffer pH 8.5, containing NaCl (to increase conductivity) and L-methionine (as an antioxidant). The
10 elution pool collected was processed to the dye affinity chromatography.

Step (1): Dye affinity chromatography on Blue Sepharose

- The dye affinity chromatography column (Blue Sepharose FF resin) was first equilibrated with the elution buffer from the Q-Sepharose FF step. The capture eluate
15 was then applied directly to the resin. After the load, the unbound material was washed out using equilibration buffer. The FSH was finally eluted by flushing the column with Sodium phosphate buffer at pH 7.0, containing NaCl and L-methionine. The elution pool was directly processed to the next step. The step was performed at 2-8°C.

20 **Step (2): Hydrophobic Interaction Chromatography on Toyopearl Butyl 650M**

- The Blue sepharose FF eluate was loaded onto a Toyopearl Butyl 650M column equilibrated against a Sodium phosphate buffer, pH 7.0, containing Ammonium Sulfate and L-methionine. The unbound material was flushed out with equilibration buffer. The FSH was eluted with the same buffer, but with a reduced concentration of
25 Ammonium Sulfate. The eluate was processed to the next step. The step was performed at RT.

Step (3): Reverse Phase on Source 30 RPC

- The HIC eluate (from step (2)) was first conditioned by addition of IPA
30 (isopropanol). The Source 30RPC column was equilibrated against an ammonium acetate buffer, pH 7.6, containing L-methionine, and 2-propanol at a concentration equivalent to that of the conditioned load material. After flushing out the unbound material with equilibration buffer, the resin is washed with ammonium acetate buffer,

pH 7.6, containing L-methionine, and an increased concentration of 2-propanol. The FSH is finally eluted by increasing further the concentration of 2-propanol. The elution pool is finally diluted under stirring, with water containing L-methionine. The diluted pool is processed to the next step. The step was performed at RT.

5

Step (4) anion-exchange chromatography on Fractogel EMD TMAE hicap resin

A Fractogel EMD TMAE hicap column was first equilibrated with Sodium Borate buffer, pH 8.5, containing L-methionine. The diluted post-RPC material (from step (3)) was loaded onto the column. The unbound material was flushed out using equilibration buffer. The FSH is eluted from the column increasing the salt concentration in a linear fashion. The step was performed at 2-8°C.

10

Step (4') Nanofiltration

The eluate from the Fractogel EMD-TMAE step (4) was applied directly to a 20nm nanofiltration device at a pressure of 3 bar under nitrogen. The filtrate is processed to the next step. The operation was performed at 2-8°C.

15

Step (5) Bulk Ultrafiltration

The nanofiltered FSH material was concentrated by tangential flow filtration on a 5KD polyether sulphone membrane. When the retentate reached about half of the initial volume, the material was buffer-exchanged by diafiltration against WFI.

20

The Purity of the Samples

The purity of the FSH samples after the purification steps was determined :

25

Purification Step	Purity
Step (-1): Ultrafiltration/Diafiltration	19 % FSH - FSH determined by RP-HPLC - Total protein content determined by Bradford Assay
Step (0): Anion Exchange on Q Sepharose FF	44% FSH FSH determined by RP-HPLC

	• Total protein content determined by Bradford
Step (1): Dye affinity chromatography on Blue Sepharose	68 % FSH • FSH determined by RP-HPLC; • Total protein content determined by Absorbance at 280 nm or about 420'000 ppm HCP • FSH content determined by RP-HPLC; • host cell protein content determined by ELISA
Step (2): Hydrophobic Interaction Chromatography on Toyopearl Butyl 650M	Amount of impurity : 3400 ppm • FSH content determined by RP-HPLC; • host cell protein content determined by ELISA
Step (3): Reverse Phase on Source 30 RPC	Amount of impurity : 170 ppm • FSH content determined by RP-HPLC; • host cell protein content determined by ELISA
Step (4) Anion-exchange chromatography on Fractogel EMD TMAE hicap resin	Amount of impurity : < 80 ppm • FSH content determined by RP-HPLC; • host cell protein content determined by ELISA

Biological activity of samples

The biological activity of the purified r-hFSH was measured using the Steelman-Pohley ovarian weight gain method. Specific activity was calculated using the biological activity divided by the FSH content as determined by an SE-HPLC method, as described below.

Specific activity of the final bulk obtained are typically between 10'000 to 17'000 IU/mg. Exemplary values for 2 samples of a final bulk FSH obtained following the method of Example 2 are given in Table 1.

Table 2. Specific activity of bulk purified rhFSH of the invention

5

Analysis	Sample 1	Sample 2
Protein concentration by SE-HPLC (mg/ml)	0.61	0.54
Specific activity (Biological activity/SE-HPLC)	12'600 IU/mg	14'600 IU/mg

Claims

1. A method for purifying recombinant FSH or a FSH variant comprising subjecting a liquid containing FSH to :
 - (1) a dye affinity chromatography;
 - (2) a hydrophobic interaction chromatography; and
 - (3) a reverse phase chromatography ;which may be carried out in any order.
2. The method of claim 1, wherein the dye affinity chromatography of Step (1) is carried out with a resin having immobilised Cibacron Blue F3G-A.
3. The method of claim 1 or 2, wherein the resin used for dye affinity chromatography of Step (1) is Blue Sepharose FF.
4. The method of any one of claims 1 to 3, wherein the dye affinity chromatography of Step (1) is carried out using sodium phosphate buffer at a pH of at or about 6.5 to 11.5 as eluent.
5. The method of any one of claims 1 to 4, wherein the hydrophobic interaction chromatography (HIC) of Step (2) is carried out using Toyapearl Butyl 650M, or a resin having similar characteristics.
6. The method of any one of claims 1 to 5, wherein the hydrophobic interaction chromatography of Step (2) is carried out using sodium phosphate / ammonium sulphate as eluent.
7. The method of any one of claims 1 to 6, wherein the step of reverse phase chromatography of Step (3) is carried out using Source 30 RPC as resin.
8. The method of any one of claims 1 to 7, wherein the step of reverse phase chromatography of Step (3) is carried out using ammonium acetate with 2-propanol as eluent.

9. The method of any one of claims 1 to 8, wherein the steps are carried out in the following order:

- (1) dye affinity chromatography;
- (2) hydrophobic interaction chromatography; and then
- (3) reverse phase chromatography.

10. The method of any one of claims 1 to 9, further comprising a step of anion-exchange chromatography step (0).

11. The method of claim 10, wherein the step of anion-exchange chromatography is carried out before steps (1), (2) and (3).

12. The method of claim 11, wherein the steps are carried out in the order:

- (0) anion-exchange chromatography;
- (1) dye affinity chromatography;
- (2) hydrophobic interaction chromatography; and
- (3) reverse phase chromatography.

13. The method of claim 10, 11 or 12, wherein the anion-exchange chromatography of Step (0) is carried out with Q Sepharose FF resin or Fractogel EMD TMAE HiCap resin.

14. The method of any one of claims 10 to 13, wherein the ion exchange chromatography of Step (0) is carried out using borate buffer as eluent.

15. The method of claim 14, wherein the borate buffer is at a pH of at or about 8.5.

16. The method of any one of claims 10 to 15, further comprising a second step of anion-exchange chromatography step (4).

17. The method of claim 16, wherein the second step of anion-exchange chromatography of Step (4) is carried out after steps (1), (2) and (3).

18. The method of claim 17, wherein the steps are carried out in the order:

- (0) Anion-exchange chromatography;

- (1) dye affinity chromatography;
- (2) hydrophobic interaction chromatography;
- (3) reverse phase chromatography; and
- (4) anion-exchange chromatography.

5

19. The method of any one of claims 16 to 18, wherein the second step of anion-exchange chromatography of Step (4) is carried out using Fractogel EMD TMAE HiCap resin or Q Sepharose FF resin.

10

20. The method of claim 16 to 19, wherein the second step of anion-exchange chromatography of Step (4) is carried out using borate buffer and a gradient of increasing NaCl concentration.

15

21. The method of any one of claims 16 to 20, comprising a step of nanofiltration step (4'), carried out the second step of anion-exchange chromatography step (4).

22. The method of claim 21, comprising a step of ultrafiltration (5), after the step of nanofiltration (4').

20

23. The method of any one of claims 1 to 22, wherein any of the eluents and/or buffers may contain an anti-oxidant, in particular L-methionine.

25

24. A method for purifying human recombinant FSH comprising the steps of subjecting FSH to:

- (-1) ultrafiltration;
- (0) anion exchange chromatography on Q Sepharose FF with borate / NaCl, L-methionine pH at or about 8.5 as eluent;
- (1) subjecting the eluate of step (0) to a step of dye affinity chromatography on Blue Sepharose FF, and phosphate, NaCl, L-methionine, at a pH of at or about 7 as eluent;
- (2) subjecting the eluate of step (1) to a step of hydrophobic interaction chromatography on Toyopearl Butyl 650M, with phosphate, ammonium sulphate, L-methionine, at a pH of at or about 7 as eluent;

30

- (3) subjecting the eluate of step (2) to a step of reverse phase chromatography on Source 30 RPC with ammonium acetate, L-methionine, with 2-propanol at a pH of at or about 7.6 as eluent;
 - (4) subjecting the eluate of step (3) to a step of anion-exchange chromatography on Fractogel EMD TMAE hicap resin, with borate, L-methionine, at a pH of at or about 8.5, and NaCl;
 - (4') subjecting the eluate of step (4) to a step of nanofiltration; and
 - (5) subjecting the permeate of step (4') to a step of ultrafiltration.
- 10 25. A recombinant human FSH or FSH variant, obtainable by the process according to any of claims 1 to 24.
- 15 26. A pharmaceutical composition comprising human FSH or a FSH variant according to claim 25 as well as a pharmaceutically acceptable excipient.
- 20 27. A pharmaceutical composition according to claim 26, comprising the FSH according to claim 25, Pluronic F68, sucrose, methionine, *m*-cresol, and an aqueous phosphate buffer at a pH of at or about 7.0.
- 20 28. Use of a recombinant human FSH or FSH variant according to claim 25 for the preparation of a medicament for the treatment of fertility disorders.