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(54) **Title**: A THERMOSTABLE LIQUID FORMULATION OF GONADOTROPINS

(57) **Abstract**: The present invention relates to an aqueous formulation of recombinant human gonadotropins which is stabilized to maintain the activity of gonadotropins for a prolonged period of time.

A THERMOSTABLE LIQUID FORMULATION OF GONADOTROPINS

RELATED APPLICATION

This application is related to Indian Provisional Application 584/MUM/2010 filed 5 March, 2010 and is incorporated herein in its entirety.

FIELD OF INVENTION

The present invention relates to a thermostable aqueous formulation of gonadotropins comprising acetic or lactic acid or its salts thereof and carbonate or bicarbonate buffer system to maintain the activity of gonadotropins for a prolonged period of time at ambient or body temperature to guarantee a reasonable shelf life. The said formulation further comprises an amino acid either alone or in combination thereof along with suitable anti-oxidant, non-ionic surfactant, and optionally with one or more preservative.

BACKGROUND OF THE INVENTION

Gonadotropins form a family of structurally related glycoprotein hormones. Typical members include human chorionic gonadotropin (hCG), follicle stimulating hormone (FSH), luteinizing hormone (LH) and thyroid stimulating hormone (TSH). These gonadotropins are synthesized and secreted by the pituitary gland in most vertebrate species. Purified FSH, alone or in combination with semi-purified human menopausal gonadotropins containing a mixture of FSH and LH, has been used to stimulate the development of ovarian follicles which is required for assisted reproduction techniques such as the IVF (*in vitro* fertilization) method.

Structurally, the gonadotropins are heterodimers composed of two dissimilar subunits namely alpha and beta, which are associated by non-covalent bonds. The alpha subunit is essentially identical for each member of the gonadotropin family. The beta subunits are different for each protein, i.e. hCG, FSH, TSH and LH, but show ample homology in structure. In humans, the alpha subunit consists of 92 amino acid residues, while the beta subunit varies in size for each member: 111 residues in FSH, 121 residues in LH, 118 residues in TSH and 145 residues in hCG (*Combamous, Y. 1992, Endocrine Reviews, 13, 670-691; Lustbader, J. W. et al. 1993, Endocrine Reviews, 14, 291-311*).

In the biopharmaceutical industry, the long term storage of gonadotropins, prepared using recombinant DNA technology in aqueous formulations, is generally a difficult task. This is particularly true for pure gonadotropins in relatively dilute solutions. To overcome the stability problem of proteins in aqueous formulations, gonadotropin products are stabilized via lyophilization (freeze-drying). For example, US5270057 discloses a lyophilized formulation comprising gonadotropin stabilized with polycarboxylic acid or a salt thereof, preferably citric acid and a non-reducing disaccharide, sucrose. US5650390 discloses a lyophilized formulation comprising FSH, LH or hCG stabilized by means of a combination of sucrose and glycine. Commercially, human menopausal gonadotropin (hMG) a mixture of FSH and LH in an approximate ratio of 1:1 and human chorionic gonadotropin (hCG) are available in lyophilized form, under the trade names Humegon® and Pregnyl® respectively, from Schering Plough. However, lyophilized products are inconvenient because they need to be reconstituted in Water for Injection (WFI) prior to use. Further, the production process of the lyophilized products involves a freeze-drying step which could result in freeze-injury to the protein lyophilized.

US5929028 discloses a liquid formulation of gonadotropin, which is prepared by dissolving gonadotropin using a diluent composed of a stabilizing amount of polycarboxylic acid or a salt thereof, a stabilizing amount of a thioether compound, a non-reducing disaccharide like sucrose and a non-ionic surfactant. According to this art, sodium citrate is described as the most preferred form of polycarboxylic acid or a salt thereof and the formulation contains 1.47% sodium citrate. This composition should be stored refrigerated at 2-8 °C until dispensed. Upon dispensing, the product may be stored at 2-8°C until the expiration date, or at or below 25°C (77°F) for three months, whichever occurs first.

EP1610822 A2 describes the pharmaceutical formulations of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and mixtures of FSH and LH, and methods of producing such formulations. The invention describes a liquid or freeze-dried formulation of FSH or LH or FSH and LH comprising a surfactant selected from Pluronic®F77, Pluronic®F87, Pluronic®F88, and Pluronic®F68. The composition as described in this patent application should be stored refrigerated at 2-8°C until dispensed. Upon reconstitution with the diluent, the product may be stored by the patient at 2-8°C until the expiration date, or at room temperature (20-25°C) for up to three months or until the expiration date, whichever occurs first.

WO1996029095 discloses a gonadotropin containing liquid pharmaceutical compositions. More precisely, it concerns liquid formulations of hCG stabilized with a polyalcohol or a non-reducing sugar. The composition is stabilized with mannitol in phosphate buffer at pH 7. Such compositions are ready to be injected and, therefore, the step of reconstitution of the lyophilized powder is avoided, thus simplifying the way of use.

WO2000004913 discloses a formulation comprising FSH or an FSH variant, containing an alpha subunit and a beta subunit, and a preservative selected from the group consisting of phenol, m-cresol, p-cresol, o-cresol, chlorocresol, benzyl alcohol, alkylparaben, benzalkonium chloride, benzethonium chloride, sodium dehydroacetate and thimerosal, or mixtures thereof in an aqueous diluents. The formulation further comprises isotonic agent and a physiologically acceptable buffer such as phosphate buffer saline.

WO2007037607 discloses an aqueous formulation of human Follicle stimulating hormone (FSH) which is stabilized to maintain the activity of FSH for a prolonged period of time. The patent application claims an aqueous formulation comprising a therapeutically effective amount of FSH stabilized in a phosphate buffer containing glycine, methionine and a non-ionic surfactant, preferably polysorbate 20, which is capable of maintaining the activity of FSH for an extended period of time.

WO2009098318 discloses a liquid pharmaceutical composition comprising a FSH polypeptide and benzalkonium chloride and benzyl alcohol as preservatives. The composition further comprises optionally one or more additional pharmaceutically acceptable excipients. In one embodiment, the composition contains methionine as an antioxidant. The composition shows good storage stability when stored at 2-8°C. This FSH aqueous formulation comprising of polysorbate 20 as a surfactant, mannitol as a tonicity modifier, phosphate as a buffer, methionine as a stabilizing agent, and benzyl alcohol and benzalkonium chloride as preservatives prevents protein loss also and dissociation of the protein into constituent monomers, thereby, stabilizing FSH for a prolonged period of time.

Currently, in the market, there are two commercial recombinant human FSH liquid products; Gonal-F RFF® from Merck Serono and Follistim AQ® from Schering Plough, in phosphate and citrate buffer respectively at pH 7.0. Ovidrel® (250 mcg hCG /0.5 ml) single dose Pre-filled

Syringe is also available from Sereno. Pergoveris® from Sereno containing follitropin alfa and lutropin alfa is also formulated in phosphate buffer.

The nature of additives and stabilizers in commercial protein formulations can vary. However, the common feature of the commercial formulations of proteins both in powder and in aqueous form is the presence of a buffer. A buffer is required to maintain the pH of the formulation where protein achieves its maximum stability. In some cases, different buffers or buffer combinations at different pH can be used to enhance the stability of formulation for a prolonged period of time. This may be achieved at pH away from the isoelectric point of the protein.

All the commercial products and prior art available as of now for gonadotropin formulations contemplate either on phosphate buffer or citrate buffer at pH 6.5 – 7.5 with storage conditions at 2-8°C. Products with storage conditions at 2-8°C, usually, require maintenance of cold chain during transportation and delivery also. This is not only costly but also challenging and difficult to maintain for resource constrained countries such as India. Consequently, there remains a need in the art not only to decrease the product cost but also to increase the patient compliance by developing a thermostable formulation of gonadotropins which may be stored at room temperature, thereby avoiding the need of cold-storage. These thermostable preparations are especially needed where extended treatments are required. Also, there is a need to provide the products that can be used and approved for multi-use administration over a period of time and can be stored at room temperature for prolonged period of time.

SUMMARY OF THE INVENTION

The present invention provides a thermostable aqueous formulation of gonadotropin in a novel buffer system capable of maintaining the activity of the protein for a prolonged period of time at ambient or body temperature to guarantee a reasonable shelf life.

In one aspect, the formulation of gonadotropin or its variant comprises a buffer system selected from acetate, lactate, carbonate and bicarbonate or their combination.

In another aspect, the formulation of gonadotropin or its variant comprises a buffer system selected from the group consisting of acetate, lactate, carbonate and bicarbonate or their

combination and an ampholyte selected from the group consisting of glycine, lysine and arginine or a combination thereof.

In yet another aspect, the formulation of gonadotropin or its variant comprises a buffer system selected from the group consisting of acetate, lactate, carbonate and bicarbonate or a combination thereof, and a ampholyte selected from the group consisting of glycine, lysine and arginine or a combination thereof, one or more polyols, a non-ionic surfactant, an antioxidant and optionally one or more preservatives.

The invention provides a formulation of gonadotropin wherein the polyol is selected from the group consisting of sucrose, trehalose, maltose, mannitol, xylitol, maltitol and sorbitol or a combination thereof.

In another aspect, the non-ionic surfactant of the gonadotropin formulation is selected from the group consisting of a polysorbate-based non-ionic surfactant and a poloxamer-based non-ionic surfactant or a combination thereof.

The gonadotropin formulation further consists of antioxidant selected from the group consisting of L-methionine, sodium bisulfite, salts of ethylenediamine tetraacetic acid (EDTA), butylated hydroxytoluene (BHT) and butylated hydroxyl anisole (BAH).

In a further aspect, the formuation optionally consists of preservative selected from group consisting of phenol, m-cresol, p-cresol, o-cresol, chlorocresol, alkylparaben, benzethonium chloride, sodium dehydroacetate and thimerosal or a combination thereof.

The gonadotropin of the invention is naturally occurring or recombinantly prepared and the pH of the formulation is maintained in the range of 6.5 to 9.0.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the comparison of dissociation of alpha and beta subunits of recombinant FSH formulations at different pH after 4 Hours at 55 °C

Lane 1: 3% intact and 5% dissociated

Lane 2: F-1

Lane 3: F-2

Lane 4: F-3

Lane 5: F-4

Lane 6: F-5

Figure 2 shows the comparison of dissociation of alpha and beta subunits of recombinant FSH formulations at different molar concentrations after 1 hour at 55 °C.

Lane 1: 3% intact and 5% dissociated

Lane 2: F-6

Lane 3: F-7

Lane 4: F-8

Lane 5: Initial (0 time)

Lane 6: RMP

Figure 3 shows the comparison of dissociation of alpha and beta subunits of recombinant FSH formulations with different buffers after 2 Hours at 55 °C.

Lane 1: 3% intact

Lane 2: 5% dissociated

Lane 3: F-9

Lane 4: F-10

Lane 5: F-11

Lane 6: F-12

Lane 7: F-13

Lane 8: F-14

Figure 4 shows the typical RP-HPLC Chromatograms of rHu-FSH compositions showing the well separated peaks of buffer, m-cresol, β- and α- subunits along with oxidized α- subunit.

Figure 5 shows the comparison of dissociation of alpha and beta subunits of recombinant FSH formulation at 40°C (**6A** – 4 days, **6B** – 7 days & **6C** – 14 days)

Lane 1: 5% intact and 5% dissociated

Lane 2: Control (In-house Gonal F-RFF)

Lane 3: F-15

Lane 4: F-16

Lane 5: F-17

Lane 6: F-18

Figure 6A & 6B shows the DSC profile of Reference Medicinal Product and the formulation numbered F-15

Figure – 7: SDS-PAGE gel picture of hCG formulations at different pH after 4 Hours at 55 °C

Lane 1: Control
Lane 2: H-1
Lane 3: H-2
Lane 4: H-3

DESCRIPTION OF THE INVENTION

The present invention provides a novel and thermostable aqueous formulation, stabilized to maintain the activity of gonadotropins for a prolonged period of time to guarantee a reasonable shelf-life at room temperature.

In one embodiment, the aqueous formulations comprises recombinant gonadotropins along with novel buffers, suitable anti-oxidants, non-ionic surfactants, stabilizers, polyols, optionally with one or more preservative.

The present invention envisages Gonadotropins produced by recombinant DNA technology. Gonadotropins being a complex heterodimeric protein, a eukaryotic cell line has been selected for expression work (Chinese hamster ovary cells). The pharmaceutical preparation of recombinant gonadotropins differ from that of human menopausal gonadotropin (hMG) and the first generation of urinary gonadotropins in terms of source of protein, purity, specific activity, and batch to batch consistency. So, recombinant gonadotropin compositions are the best one for stimulating ovarian follicular development.

Thermostability is the quality of a substance to resist irreversible changes in its chemical or physical structure at a relatively high temperature. Thermostability with respect to the present invention refers to the storage stability of protein, i.e. stability at body temperature (37 °C) for more than two weeks or until the expiration date at 2-8 °C, whichever occurs first.

The concentration of gonadotropin used in the present invention is dependent on the solubility of the gonadotropin and on the therapeutic amount for a given dose and the gonadotropin is selected from the group consisting of FSH, LH or hCG either alone or combination thereof. A suitable concentration of gonadotropin may range from 2 - 2000 µg/ml. The gonadotropins of the current invention are naturally occurring or recombinantly prepared.

Buffers are suitable for maintaining pH of the formulation. In one embodiment, the buffer system of the present FSH formulation comprises acetate, lactate and bicarbonate buffers either alone or in suitable combination, with an amino acid selected from the group of glycine, lysine and arginine either alone or in combination. Buffer in the invention is used at a concentration of 0.1mM to 500mM. The preferred pH range is 4.0 – 10.0 and more preferably the pH is range is 6.5 to 8.5.

The aqueous formulation of the present invention includes amino acids such as glycine, lysine or arginine as ampholyte(s). Amino acids such as glycine, lysine or arginine in solution state enable gathering of greater numbers of water molecules around gonadotropins, thereby, further stabilizing the outermost hydrophilic amino acids among numerous amino acids constituting the protein and consequently stabilizing it. These amino acids further stabilize the formulation by maintaining the pH of the solution at a desired value.

The preferred concentration of the amino acids in the present invention is between 0.5mM – 500mM and most preferably between 1- 300 mM. The pH of the liquid formulation of the present formulation is in between 4.0 – 10.0, preferably between pH 6.0 to 9.0 and most preferably between pH 6.5 to 8.5. The suitable molar concentration of acetate, lactate and bicarbonate buffers is between 0.001- 1000mM, preferably between 0.01mM- 500mM and most preferably between 0.1mM- 100mM.

Antioxidant as used herein refers to a chemical that reduces the quantity of oxidizing gonadotropins within a solution or delivery device. The antioxidant of the present invention is selected from the group consisting of methionine, sodium bisulfite, salts of ethylenediamine tetraacetic acid (EDTA), butylated hydroxytoluene (BHT), and butylated hydroxyl anisole (BHA); the most preferred one among them is methionine. Preferred concentration of anti-

oxidant is in the present formulation between 0.001 – 10 mg/ml and most preferably, 0.05- 5 mg/ml.

Non-ionic surfactant is used in the present invention in order to prevent adsorption of gonadotropins on the surface of the vial, ampoule, carpoule, cartridge or syringe. Non-ionic surfactants lower surface tension of a protein solution, thereby, preventing its adsorption or aggregation on the hydrophobic surface. Preferred examples of the non-ionic surfactant that can be used in the present invention may include a polysorbate-based non-ionic surfactant and a poloxamer-based non-ionic surfactant, either alone or in combination. The most preferred concentration of non-ionic surfactants used in this invention is in the range of 0.01 – 10 mg/ml and most preferably, 0.05- 5 mg/ml.

Stabilizers used in the present invention are selected from the saccharide group. Monosaccharides, such as glucose, fructose and mannose, and the like, polyols such as sucrose, trehalose, lactose, cellobiose, maltose, and the like, and polysaccharides such as dextran, chitosan, cellulose, pullulan and the like, either alone or in combination thereof may be used. Sugar polyalcohols such as glycerol, erythritol, ribitol, arabitol, sorbitol, galactitols, mannitol and xylitol, and the like, either alone or in combination thereof may be used. According to the present invention, the concentration of stabilizer is in the range of 5-500 mM and most preferred concentration is between 20-350 mM.

Inorganic salt such as sodium chloride, potassium chloride, calcium chloride, etc. may also be used to adjust the osmolality in the range of 250-350 mOsm. However, the use of salts in the present invention is optional.

Preservative refers to a composition or substance added to a formulation to act as a bacteriostatic agent. A preserved gonadotropin containing formulation of the present invention preferably meets statutory or regulatory guidelines for preservative effectiveness to be a commercially viable multi-use product, preferably in humans. Preservatives used in the present invention are selected from the group consisting of phenol, m-cresol, p-cresol, o-cresol, chlorocresol, alkylparaben (methyl, ethyl, propyl, butyl and the like), benzethonium chloride, sodium dehydroacetate or thimerosal, either alone or in combination thereof. However, in the present invention, the use of preservative is optional and is preferred while designing multi-dose formulations. The preferred

concentration of preservative used in the formulation of present invention is in the range of 0.5-10 mg/ml and most preferably in between 1.0-5.0mg/ml.

Gonadotropin used in the present invention is in a quantity sufficient to form a therapeutically useful concentration of the protein for parenteral (e.g. subcutaneous, intramuscular or intravenous) administration. These formulations may also be suitable as alternative delivery systems, e.g., but not limited to, nasal, pulmonary, transmucosal, transdermal, oral, subcutaneous, intramuscular or parenteral sustained release, dry or liquid formulation.

The liquid gonadotropin containing formulations of the invention may be stored in the liquid state at various temperatures for prolonged periods while retaining the biological activity and physical stability of the gonadotropin. Preferably the storage temperature is below 30 °C and above the freezing temperature. The preferred storage temperature range is between approximately 2 °C and 8 °C.

In a further aspect of the invention there is provided a cartridge containing a sterile liquid formulation according to the invention. As used herein a cartridge means a closed container, such as an ampoule, a vial, a bottle or a bag.

A cartridge may contain an amount of the liquid gonadotropin formulation corresponding to one or more therapeutic doses of the gonadotropin. In a further aspect of the invention there is provided a device for administration comprising a cartridge containing a sterile liquid formulation according to the invention.

The formulation of the present invention may be administered in the form of liquid, lyophilized powder, gels, emulsions, liposomes, vesicles, nanoparticles, creams, ointments etc.

The present invention is not just limited to gonadotropins. It may be applicable to other proteins and peptides such as abatacept, etanercept, erythropoietin (EPO), Darbepoetin, enzymes, human growth hormone (hGH), insulin, growth factors, interferon α2a (IFN α 2a) , interferon α2b (IFN α 2b), interleukin-2 (IL-2), tumor necrosis factor alpha (TNF- α), immunoglobulins, tissue plasminogen activator (tPA), monoclonal antibodies (mAbs), albumin, antithrombin III, Clotting factor VIII, vaccines, hirudin, etc.

The following examples illustrate the described pharmaceutical compositions of the present invention and the means of carrying out the invention to obtain a stable pharmaceutical formulation of gonadotropins. These examples should not, however, be construed as limiting the scope of the invention.

Example 1: Optimization of pH for maximum protein stability

Recombinant Hu-FSH is a dimeric protein containing alpha and beta subunits which on exposure to stress (e.g. elevated temperatures) dissociates into its individual monomeric units. Also it is prone to form high molecular weight species under stress conditions. Different aqueous formulations of rHu-FSH were prepared and pH of the solutions was adjusted from pH 6.5 to 8.5 by using HCl or NaOH to determine the pH where the protein is most stable. These formulations also contained other excipients as per details given in Table 1. Samples were incubated at 55°C for 4 hours and then analyzed by SDS- PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) (12% homogeneous resolving, non-reducing) to determine the optimum pH where the protein is maximally stable. SDS-PAGE was used as an analytical technique which can separate the dissociated and high molecular weight aggregates from native protein based on molecular weight. SDS-PAGE gel (Figure-1) of different formulations (F-1 to F-5) shows the dissociation of protein into alpha and beta subunits after 4 hours at 55°C. It was observed from the gel picture that FSH is more stable in the range of 8.0-8.5 where the dissociation was found to be minimal (not more than 5% dissociation). At other pH, it was found that protein is highly dissociated (more than 5% dissociation). However, there was no significant difference in stability of different formulation having different pH in terms of aggregation.

Table 1: Composition of rHu-FSH aqueous solution

Formulation code	Formulation pH	Details of the formulation
F-1	6.5	rHu-FSH (0.3 mg/ml)
F-2	7.0	Glycine (50mM)
F-3	7.5	lactic acid (50mM)
F-4	8.0	Sucrose (60mg/ml)
F-5	8.5	Polysorbate20 (0.2mg/ml)
		Methionine (0.1mg/ml)

Example 2: Optimization of molar concentrations of buffer and ampholytes

Different aqueous formulations of rHu-FSH were prepared and effect of molar strength of lactic acid and glycine on formulation stability was studied. These formulations also contained other excipients as per details given in Table 2. Samples were incubated at 55°C for 1 hour and then analyzed by SDS-

PAGE as discussed in Example-1 to determine the optimum molar strengths of excipients where the protein is most stable. SDS-PAGE gel (Figure-2) of different formulations (F-6 to F-8) shows the dissociation of protein into alpha and beta subunits. Reference Medicinal Product (RMP) from Merck Sereno composition was also prepared in house and used as positive control to compare with different formulations of FSH containing different concentrations of lactic acid or glycine. Formulation F-6 with 5 mM concentration of lactic acid and glycine was found to be most stable as there was no dissociation or aggregation after 1 hour of incubation at 55°C (Lane-2 in Figure-2) and the profile is comparable to that of initial non-incubated sample (Lane-5 in Figure-2). The formulation was found to be significantly stable than RMP (Lane-6 in Figure-2). Formulation containing excipients with strength at 10mM was comparable to RMP. However, other formulation containing higher molar strength (i.e. 20 mM) of glycine and lactate was not so stable at elevated temperatures.

Example 3: Screening of different formulations

Table 2: Compositions of rHu-FSH aqueous formulations

Formulation code	Molar strength of lactic acid (mM)	Molar strength of Glycine (mM)	Details of the formulation
Initial	5	5	rHu-FSH (0.15 mg/ml) Sucrose (60mg/ml), Polysorbate 20 (0.2 mg/ml), Methionine (0.1 mg/ml), Phenol (3.0 mg/ml)
F-6	5	5	
F-7	10	10	
F-8	20	20	
Control (In-house Gonal F-RFF)	rHu-FSH (0.15 mg/ml), Sodium dihydrogen phosphate -0.45mg/ml Disodium hydrogen phosphate -1.1mg/ml, (Sucrose (60mg/ml), Polaxamer188 (0.1mg/ml), Methionine (0.1mg/ml), m-Cresol (3.0mg/ml)),		

Different aqueous formulations of rHu-FSH were prepared and effect of buffers viz. tris, sodiumbicarbonate and histidine at pH 8.0 on formulation stability was studied. These formulations also contained other excipients as per details given in Table 3. Samples and RMP were incubated at 55°C for 2 hours and then analyzed by SDS- PAGE as discussed in Example-1 to identify the buffer in which the protein is most stable. Different molar concentrations of glycine were also tried viz, 100 and 200mM. SDS-PAGE gel (Figure-3) of different formulations (F-9 to F-14) shows the dissociation of protein into alpha and beta subunits. The gel picture showed that tris and histidine buffers were not appropriate for as there was almost complete dissociation of FSH in 2 hours at 55°C. Bicarbonate buffer was found to protect the protein in efficient manner from dissociation among the three buffers tried.

Table 3: Composition of rHu-FSH aqueous solution

Formulation Code	Formulation Details	Formulation Code	Formulation Details
F-9	Glycine – 100 mM	F-12	Glycine – 200 mM
	Acetic acid - 5 mM		Acetic acid - 5 mM
	Tris - 10 mM		Sodium Bicarbonate - 10 mM
	Methionine - 0.5 mg/mL		Methionine - 0.5 mg/mL
	Phenol - 3.0 mg/mL		Phenol - 3.0 mg/mL
	Polysorbate 20 - 0.2 mg/mL		Polysorbate 20 - 0.2 mg/mL
	Acetic acid / NaOH to adjust the pH		Acetic acid / NaOH to adjust the pH
F-10	Glycine – 200 mM	F-13	Glycine – 100 mM
	Acetic acid - 5 mM		Acetic acid - 5 mM
	Tris - 10 mM		Histidine - 10 mM
	Methionine - 0.5 mg/mL		Methionine - 0.5 mg/mL
	Phenol - 3.0 mg/mL		Phenol - 3.0 mg/mL
	Polysorbate 20 - 0.2 mg/mL		Polysorbate 20 - 0.2 mg/mL
	Acetic acid / NaOH to adjust the pH		Acetic acid / NaOH to adjust the pH
F-11	Glycine – 100 mM	F-14	Glycine – 200 mM
	Acetic acid - 5 mM		Acetic acid - 5 mM
	Sodium Bicarbonate - 10 mM		Histidine - 10 mM
	Methionine - 0.5 mg/mL		Methionine - 0.5 mg/mL
	Phenol - 3.0 mg/mL		Phenol - 3.0 mg/mL
	Polysorbate 20 - 0.2 mg/mL		Polysorbate 20 - 0.2 mg/mL
	Acetic acid / NaOH to adjust the pH		Acetic acid / NaOH to adjust the pH

Example 4: Stability studies of FSH formulations at 40°C.

Four different formulations were selected as given in Table 4 for stability studies. The effect of different ampholytes such as glycine or lysine was assessed on the stability of the protein at the elevated temperature. Similarly the effect of sucrose or trehalose was also studied. In these formulations, polysorbate 20 was used as a surface active agent, methionine was used as an anti-oxidant and phenol was used as a preservative. Acetic acid and bicarbonate buffer was used at a molar concentration of 5 mM each and the pH of the formulations was adjusted to 8.0 by using HCl or NaOH. Formulated solutions were sterilized by using 0.2 µm filter under laminar air flow and stored at 40 °C for 14 days. The samples were withdrawn at predetermined time points at 0, 4, 7 and 14 days and analyzed by RP-HPLC and SDS-PAGE.

Reverse phase high performance liquid chromatography (RP-HPLC) is a method which resolves the product related impurities i.e. oxidized impurities, based on their hydrophobicity. RP-HPLC

was done by using Reverse phase Grace Vydac C4 column (4.6 X 2.50 mm, 5µm, 300Å) in Agilent 1200 series HPLC. Mobile phase used was (A): 70% ACN and (B): 100mM Na_2SO_4; pH 2.5 ± 0.1.

Table 4: Composition of rHu-FSH aqueous formulations

Formulation Code	Formulation Details	Formulation Code	Formulation Details
F-15	Glycine –100mM	F-17	Lysine –50mM
	Acetic acid-5mM		Acetic acid-5mM
	Sodium Bicarbonate - 5 mM		Sodium Bicarbonate - 5 mM
	Sucrose – 100 mM (34.28mg/ml)		Sucrose - 75mM (26mg/ml)
	Methionine - 0.5 mg/mL		Methionine - 0.5 mg/mL
	Polysorbate 20 - 0.2 mg/mL		Polysorbate 20 - 0.2 mg/mL
	Phenol - 3.0 mg/mL		Phenol - 3.0 mg/mL
	HCl / NaOH to adjust the pH 8.0		HCl / NaOH to adjust the pH to 8.0
F-16	Glycine –100mM	F-18	Lysine –50mM
	Acetic acid-5mM		Acetic acid-5mM
	Sodium Bicarbonate - 5 mM		Sodium Bicarbonate - 5 mM
	Trehalose - 75mM (28.35mg/ml)		Trehalose - 75mM (28.35mg/ml)
	Methionine - 0.5 mg/mL		Methionine - 0.5 mg/mL
	Polysorbate 20 - 0.2 mg/mL		Polysorbate 20 - 0.2 mg/mL
	Phenol - 3.0 mg/mL		Phenol - 3.0 mg/mL
	HCl / NaOH to adjust the pH 8.0		HCl / NaOH to adjust the pH 8.0

Recombinant Hu-FSH is a dimeric protein containing alpha and beta subunits which on exposure to stress (e.g. elevated temperatures) not only dissociates into its individual monomeric units but also gets oxidized. Beta and Alpha subunits of rHu FSH have 1 and 3 methionine residue respectively. The major product related impurity is oxidized variants of beta and alpha subunits. Oxidation decreases the hydrophobicity of proteins with respect to native proteins. Oxidized impurities elute before the native proteins when analyzed by RP-HPLC and quantify these impurities accurately. This method is able to resolve such modified forms and thus can be used for determination of oxidized impurities. Typical chromatogram of FSH showing the well separated peaks of buffer, m-cresol, β- and α- subunits along with oxidized α- subunit has been shown in Figure 4. rHu-FSH profile gives two major peaks: one corresponding to beta subunit and another to alpha subunit. Two major oxidized impurities of rHu-FSH alpha elute before main rHu-FSH alpha peak. Percentage oxidation data of different formulations of rHu-FSH is shown in Table 5. The data showed that the oxidation values of different formulations are comparable to that of RMP.

Table 5: Percentage oxidation of different FSH formulations at 40 °C

Formulation Code	% Oxidation			
	0 Day	4 Day	7 Day	14 Day
Control	1.43	2.81	2.94	3.24
F-15	2.12	2.22	2.41	3.05
F-16	2.25	2.61	3.04	3.73
F-17	2.17	2.91	3.63	5.15
F-18	2.21	2.88	4.14	5.39

SDS-PAGE data of rHu-FSH compositions incubated at 40°C for 4, 7 and 14 days are shown in Figure 5(A), 5(B) and 5(C) respectively. The picture shows that the RMP is highly dissociated and the rate of dissociation increases with the duration of incubation. On the other hand, compositions F15 to F17 show very little dissociation when compared with innovator's composition on respective days. Interestingly, it was observed that there is no significant increase in the amount of dissociated protein with increased duration of incubation i.e. from day 4 to day 14. This clearly indicates the thermal stability of our formulations.

Example 5: DSC profile of rHu- FSH formulation vs innovator's product

Differential Scanning Calorimetry (DSC) was used to measure the stability in terms of unfolding of protein in different formulations by heating the protein at a constant rate. The transition midpoint T_m is the temperature where 50% of the protein is in its native confirmation, and the other 50% is denatured. In general, the higher the T_m, the more stable the protein. Proteins which are more stable are less susceptible to unfolding and precipitation. Using T_m data from DSC, one can design and select the most stable engineered protein/variant, optimize process development, and screen for the most stable liquid formulations. DSC (Microcal, USA) was used to determine the T_m value of innovator's product and FSH formulation (F-15). Figure 6A and 6B shows the DSC profile and T_m value of RMP and FSH formulation (F-15) respectively. From DSC analysis, T_m value of RMP was found to be 70.73°C whereas T_m value of F-15 formulation was 72.45°C which clearly indicates enhanced thermostability of latter formulation.

Example 6: Optimization of pH of HCG with acetate-bicarbonate buffer

Different aqueous formulations of HCG were prepared in acetate-bicarbonate buffer and and pH of the solutions was adjusted from pH 7.0 to 8.0 by using HCl or NaOH to determine the pH where HCG is most stable. These formulations also contained other excipients as per details given in table 6.

Samples were incubated at 55°C for 4 hours and then analyzed by SDS- PAGE (12% homogeneous resolving, non-reducing) to determine the optimum pH where the protein is maximally stable. SDS-PAGE gel (Figure-7) of different formulations (H-1 to H-3) shows the dissociation of protein into alpha and beta subunits after 4 hours at 55°C. RMP was also prepared in house and used as positive control to compare with different formulations of HCG at different pH. It was observed from the gel picture (Figure 7) that HCG is more stable at pH 7.0 where the aggregation was found to be less than other pH (7.5 and 8.0) after 4hrs at 55°C. However, there was no significant difference in stability of different formulation having different pH in terms of dissociation. The formulation was found to be stable than the innovator's formulation (Lane-1 in Figure-7). Acetate- bicarbonate buffer was found to protect HCG in efficient manner from aggregation.

Table 6: Composition of rHu-hCG aqueous solution

Formulation code	Formulation pH	Details of the formulation
Control	**7.0**	RMP
H-1	**6.5**	Glycine –100mM, Acetic acid-5mM, Sodium Bicarbonate - 5 mM, Sucrose – 100 mM (34.28mg/ml), Methionine - 0.5 mg/mL, Polysorbate 20 - 0.2 mg/mL, Phenol - 3.0 mg/mL, HCl / NaOH to adjust the pH
H-2	**7.0**	
H-3	**7.5**	

The formulations prepared by the said invention, comprising an effective amount of biologically active gonadotropins, are useful in treating infertility. They are used preferably as injectable aqueous solutions.

A novel and thermostable formulation of gonadotropins described in the present invention offers the following advantages:

1. Involve operational simplicity
2. Involve use of buffers which prevent the dissociation of alpha and beta subunits of human Follicle stimulating hormone
3. Provide better stability to the aqueous formulation to maintain its activity for a prolonged period of time to guarantee a reasonable shelf-life
4. Provide better stability to the aqueous formulation to maintain its activity even at a higher temperature
5. Avoid cold-chain shipment
6. Cost effective and enhanced patient convenience

Claims

We claim

1. A formulation comprising human gonadotropin or its variant and a buffer system selected from the group consisting of acetate, lactate, carbonate and bicarbonate or their combination at a pH in the range of 6.5 to 9.0.
2. A formulation comprising human gonadotropin or its variant, a buffer system selected from the group consisting of acetate, lactate, carbonate and bicarbonate or their combination and a ampholyte selected from the group consisting of glycine, lysine and arginine or a combination thereof at a pH in the range of 6.5 to 9.0.
3. A formulation comprising human gonadotropin or its variant, a buffer system selected from the group consisting of acetate, lactate, carbonate and bicarbonate or a combination thereof, and a ampholyte selected from the group consisting of of glycine, lysine and arginine or a combination thereof, one or more polyols, a non-ionic surfactant, an antioxidant and optionally one or more preservatives at a pH in the range of 6.5 to 9.0.
4. The formulation of claim 3 wherein the said polyol is selected from the group consisting of sucrose, trehalose, maltose, mannitol, xylitol, maltitol and sorbitol or a combination thereof.
5. The formulation of claim 3 wherein the non-ionic surfactant is selected from the group consisting of a polysorbate-based non-ionic surfactant and a poloxamer-based non-ionic surfactant or a combination thereof.
6. The formulation of claim 3 wherein the antioxidant is selected from the group consisting of L-methionine, sodium bisulfite, salts of ethylenediamine tetraacetic acid (EDTA), butylated hydroxytoluene (BHT) and butylated hydroxyl anisole (BAH).
7. The formulation of claim 3 wherein the preservative is selected from the group consisting of phenol, m-cresol, p-cresol, o-cresol, chlorocresol, alkylparaben, benzethonium chloride, sodium dehydroacetate and thimerosal or a combination thereof.
8. The formulation of claim 1 to 3 wherein the human gonadotropin is naturally occurring or recombinantly prepared.
9. A formulation comprising human recombinant gonadotropin, glycine, acetate and bicarbonate buffer, sucrose, polysorbate, methionine and phenol at a pH of about 6.5 – 8.5.
10. A formulation comprising human recombinant gonadotropin in a range of 2 – 2000µg/ml, glycine in the range of 1 – 300mM, acetate and bicarbonate buffer in the range of 0.1mM to 500mM, sucrose in the range of 20-350mM, polysorbate in the range of 0.05 – 5 mg/ml,

methionine at a concentration of 0.05-5mg/ml and phenol in the range of 1-5 mg/ml at a pH of about 6.5 -8.5.

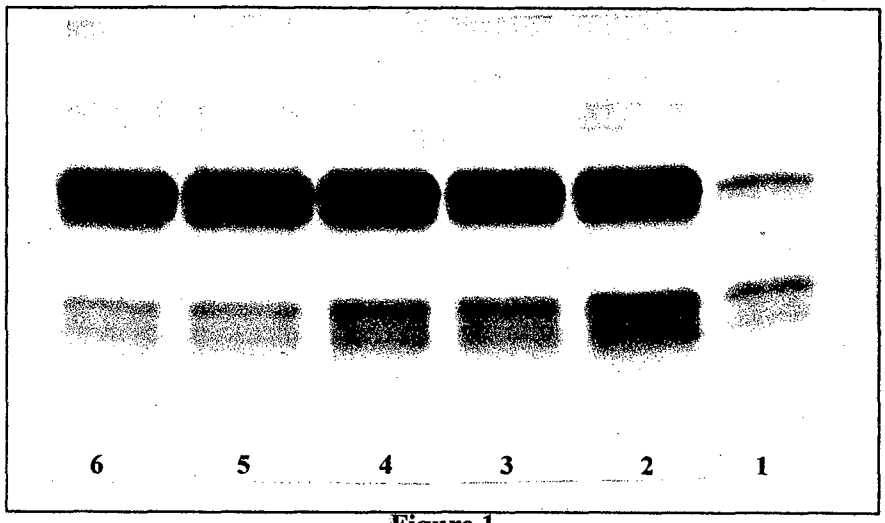


Figure 1

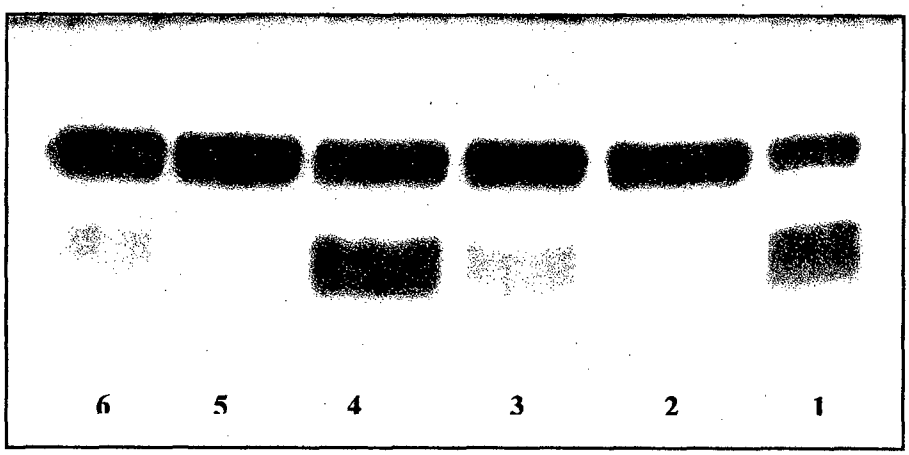


Figure 2

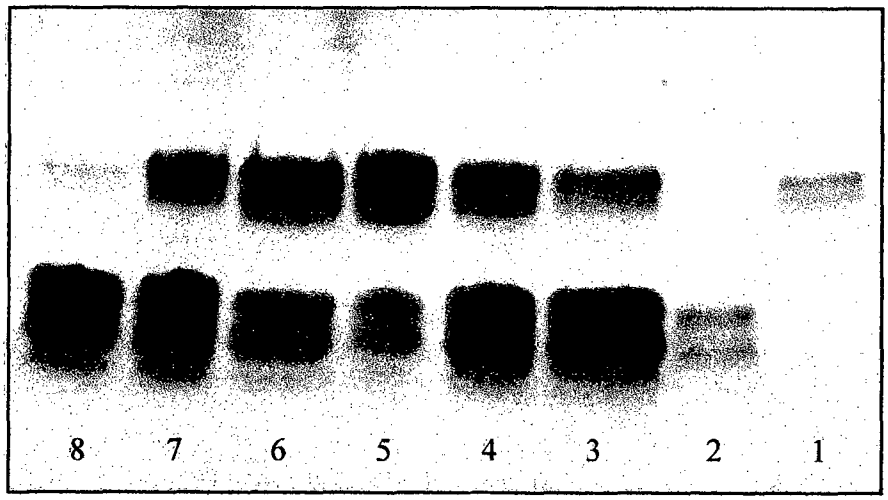


Figure 3

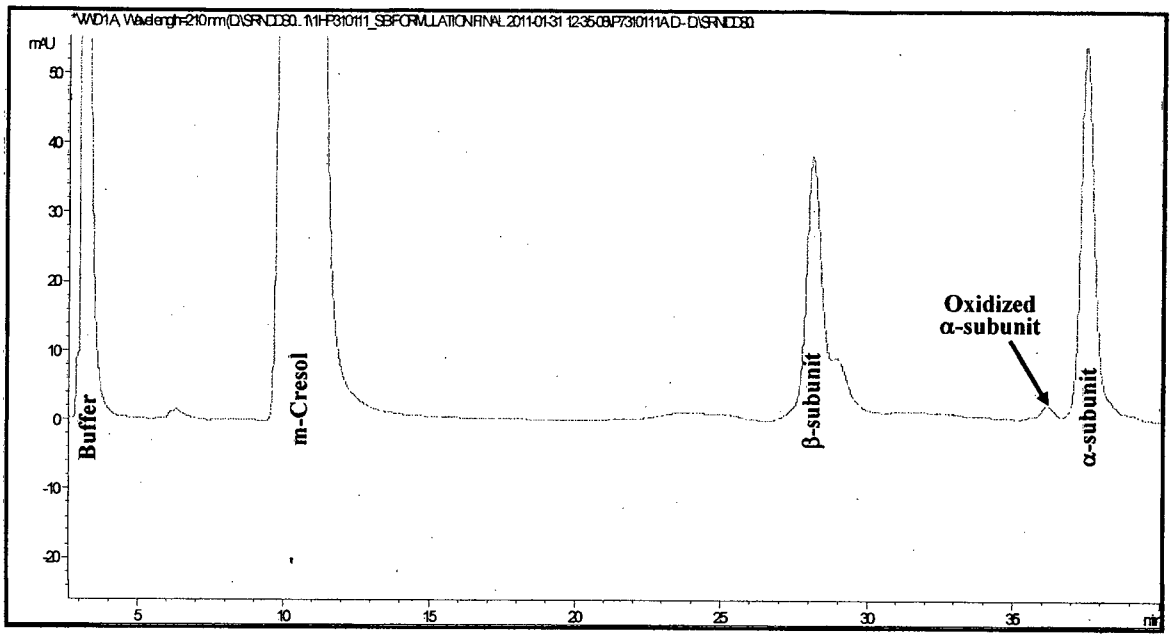


Figure 4

(A) 4 Days

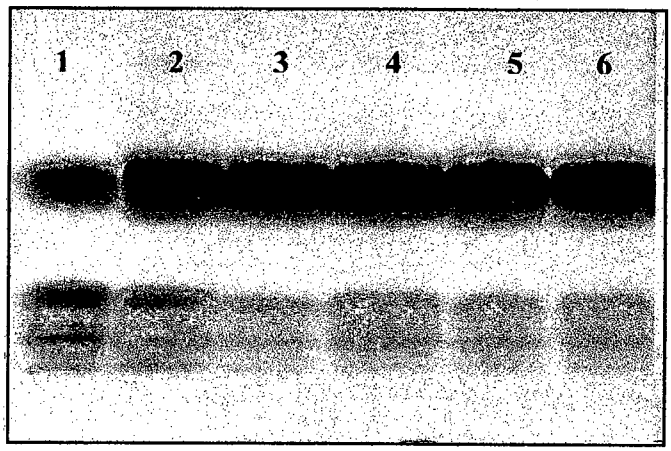


Figure 5A

(B) 7 Days

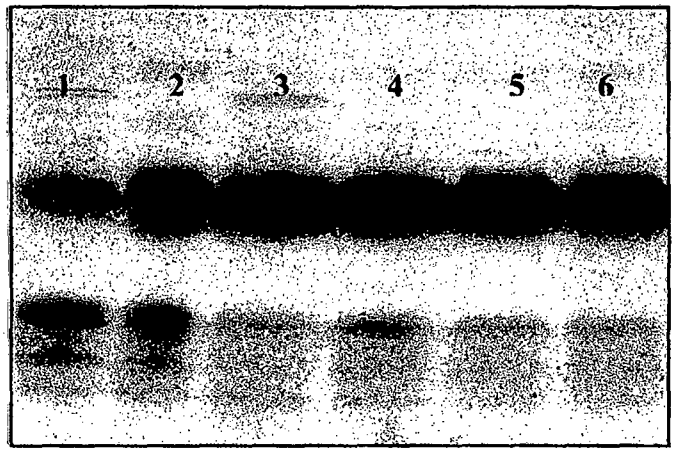


Figure 5B

(C) 14 Days

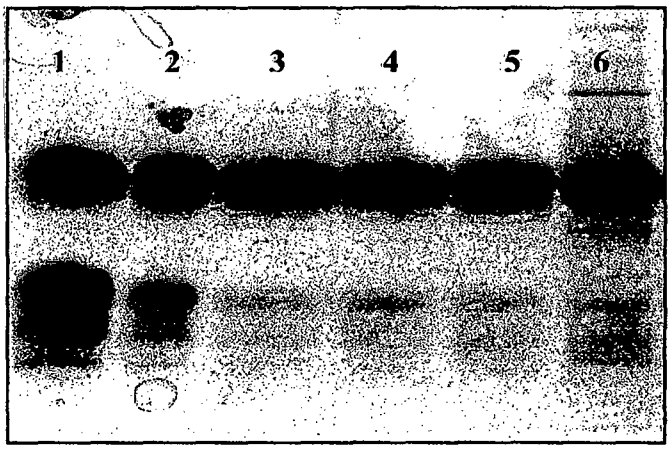


Figure 5C

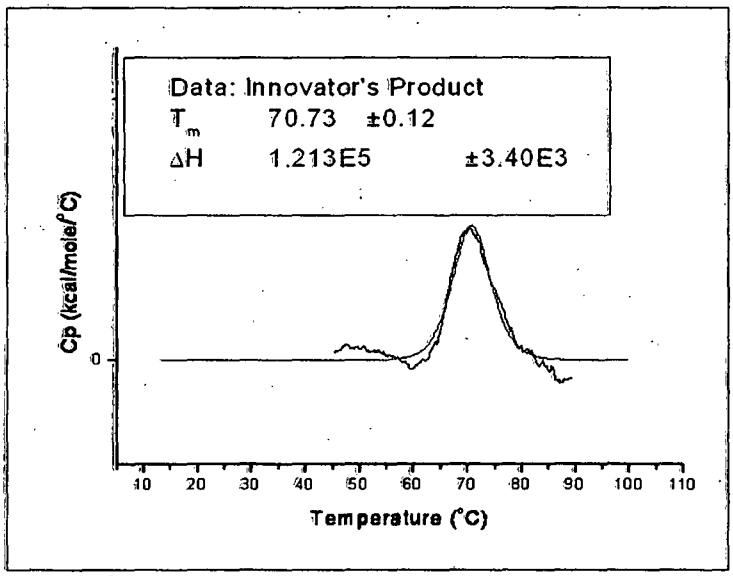


Figure 6A

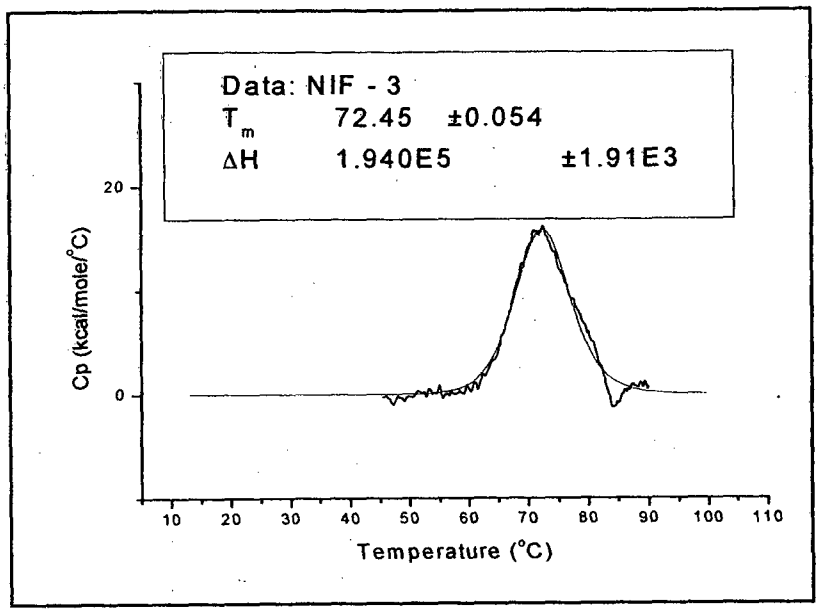


Figure 6B

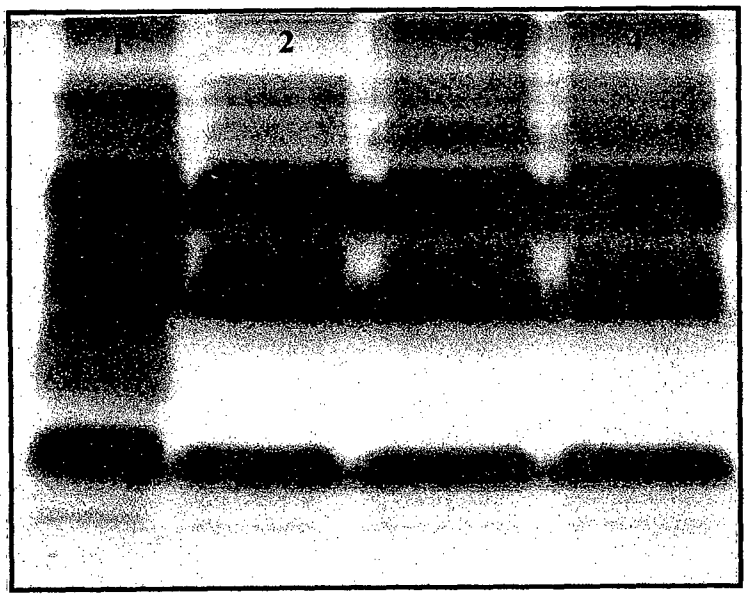


Figure 7