FORMULATION AND ANALYSIS OF EGF FOR THE TREATMENT OF DIABETIC FOOT ULCERS

Ramesh. B¹*, K.Nagaiah¹, M.Laxminarasu²

2. Director-IST, JNTU, Hyderabad-500072, Andhra Pradesh, India.

*Corresponding Author E-mail: bhagam@gmail.com

ABSTRACT

Recombinant Human Epidermal Growth Factor (rH-EGF) enhances wound healing of diabetic foot ulcers. EGF in the form of gel is approved by regulatory bodies and is recommended by physicians for treatment of diabetic foot ulcers. There are some difficulties to physicians and patients during and after dressing the wound and applying EGF gel. In this study we present EGF liquid formulation for the treatment of diabetic foot ulcer. The main ingredients in liquid formulation are EGF at the concentration of 0.1 mg /mL, poloxamer, phosphate buffer, preservative, isotonic agent and water. Poloxamer 407 is used which is most important for this formulation, as this exists as fluid state at room temperature facilitating administration and becomes a gel at body temperature promoting sustained release of the formulation. The formulation proves its activity and needs further preclinical toxicology and clinical studies to prove its clinical efficacy.

Key words: Epidermal Growth Factor (EGF), Diabetic foot ulcers, poloxamer 407
INTRODUCTION:
Diabetes is the most common metabolic disease which is increasing worldwide. Diabetes is of two types; in type 1 (diabetes mellitus) body produces less or no insulin whereas in type 2 body tissues are resistant to insulin and high glucose levels damage the tissues. Diabetic foot ulcer is a major complication in diabetes mellitus with 15% lifetime risk in all diabetic patients. The main features are infection, ulceration or gangrene. Neuropathy, poor circulation and susceptibility to infection are the major contributors for development of a diabetic foot. Awareness of physicians about foot problems in diabetic patients, clinical examination, Para clinical assessment, patient education, hygienic practices, and provision of appropriate foot wear can reduce ulcer occurrence by 50%. Many treatments are available for treating diabetic foot ulcers. Besides regular therapy other treatments include use of EGF, vacuum compression therapy, peripheral stem cell injection, gene therapy, angiogenesis stimulance, immune stimulants and granulating agents.

Wound healing is a complex process and includes multiple events to repair the injured tissue. These events involve haemostasis, inflammation, proliferation and rebuilding. In haemostasis platelet aggregation and clot formation will occur and inflammatory phase involves accumulation of neutrophils and macrophages at the site of injury. Proliferation involves formation of new blood vessels generation of collagen, tissue formation and reepithelialisation.

Epidermal Growth Factor (EGF) is a single polypeptide chain of 53 amino acids residues with molecular weight of 6.2 kDa and contains three intramolecular disulfide bonds that are required for biological activity. EGF enhances wound healing of Diabetic Foot ulcers by initiating DNA synthesis, cell replication, activation of RNA and protein synthesis, stimulates keratinocyte differentiation, proliferation and reepithelialisation.

Though EGF was initially purified from urine, it is now produced by r-DNA technology using E.Coli as expression system. The expressed EGF is purified by using different chromatographic techniques and is well characterized before further use in non clinical or clinical use. EGF is approved by regulatory bodies for treatment of diabetic foot ulcer in the form of ointment. In this study we had developed a liquid formulation using poloxamer as gelling agent for treatment of diabetic foot ulcers.
Poloxamers are non ionic triblock polymers with central hydrophobic chain of polyoxypropylene flanked by two hydrophilic polyoxyethylene chains. Based on the length of polymer many Poloxamers exists with slightly different properties. Poloxamers are named PXXX, P indicates poloxamer, and first two digits multiplied by 100 gives the molecular mass of polyoxypropylene core and last digit multiplied by 10 gives the percentage of polyoxyethylene content. Poloxamers are used as surfactants due to their amphiphilic nature and also as gelling agent.

In this study poloxamer 407 is used which is most important for this formulation, as this exists as fluid state at room temperature facilitating administration and becomes a gel at body temperature promoting sustained release of the formulation$^1$.

**MATERIALS AND METHODS:**

**Materials:**

All the equipments and / instruments required for analysis or manufacturing are procured from reputed manufacturers. All the chemicals used were of analytical/reagent grade obtained from merck or SRL. All the biochemicals, enzymes and other required were obtained from Sigma / Biorad / Invitrogen. Poloxamer 407 (lutrol ®S 127) was obtained from BASF. All the glassware used was procured from Borosil /Schott Duran.

**Cloning and transformation:**

The EGF gene was cloned into pET vector; presence of gene was verified by sequencing. The plasmid containing EGF gene was transformed into E.Coli BL21 (DE3). The transformed culture was plated on to LB agar media plate with ampicillin (100µg/ml) and incubated for 3-4 hours. A colony was picked and inoculated into 10 mL of LB medium containing ampicillin.

**Shake flask studies:**

10mL of the overnight grown culture at OD 1.2-1.4 was sub cultured into 100mL terrific broth and incubated at 37$^\circ$C for 5-6hrs.20mL of the grown culture is again subcultured to 200mL of the Terrific broth media with 2% glucose and ampicillin (100µg/mL) and incubated at 37$^\circ$C for 3-4hrs.

**Fermentation studies:**

The grown culture with OD of about 3.5-4.0 is inoculated in the fermentor with 9L of media (LB broth, DIFCO) Fermentation was continued for 6hrs and the culture was induced with 1mM isopropyl β-D-1 isopropyl thiogalactopyranoside (IPTG) and continued for 4hrs. The culture was harvested, centrifuged at 6000rpm and the supernatant was discarded. The pellet is
resuspended in lysis buffer and lysed by passing through High pressure homogenizer at 800 bar. The resultant lysate is centrifuged at 8000 rpm for 30 min and the pellet is discarded to collect the supernatant.

**Purification process:**

The lysed supernatant was clarified using 750Kd hollow fiber. The clarified and concentrated sample was purified using ion-exchange chromatography, sample concentration with 1kD TFF cassette, and followed by preparative HPLC to get a purity of ≥99.0%. Sample from preparative HPLC was buffer exchanged with water and stored at -20°C.

**Analytical methods:**

There are many established analytical techniques to check the purity and characterize the purified therapeutic proteins.

**Protein estimation:**

Protein estimation was done by Bradford’s method using Coomassie brilliant G-250.

**SDS-PAGE:**

Since the invention of electrophoresis technique by Laemmli has become important separating tool for Biochemist to check purity of the product and to determine the molecular weight of the unknown protein. Sample was tested with both reducing and non reducing conditions. Samples for reducing gel were boiled for 3 minutes at 90 °C and both reducing and non reducing samples were loaded onto the gel and constant voltage of 150 V for 60 minutes was applied. After completion of run, gel was stained with coomassie blue and destained with destaining solution.

**Immunoblotting:**

Identification of EGF was done by using immunoblotting technique by using procedure used for running the non reducing SDS gel as mentioned above. Following the electrophoresis run, the gel was rinsed with transfer buffer to remove electrophoresis buffer salts and was transferred over to PVDF membrane and was placed in blocking buffer, washed with TBST buffer. Primary antibodies are added and incubated for 2 hours and followed by secondary antibodies are incubated for 2 hours. The membrane was finally washed and membrane was developed.

**RP-HPLC:**

Purity of EGF was tested by using RP-HPLC which is carried out by using a zorbax SB C₈ column on Shimadzu chromatography system with UV detector, mobile phase A is 0.1% TFA in water and mobile phase B is 0.1% TFA in 900 mL ACN and 100 mL water and the flow is
gradient. Analysis was carried at 214 nm and the run time is 60 minutes.

**Dimers and related substances:**
Formation of dimers and aggregates is a major issue in all biopharmaceutical products, which are identified and quantified by using size exclusion chromatography. TSKgel G2000SWXL was used UV detector at 220 nm, run time is 60 minutes, and mobile phase is sodium phosphate buffer.

**Isoelectric focusing (IEF):**
Isoelectric focusing (IEF) is an electrophoretic technique by which amphoteric compound are fractionated according to their pI values along a continuous pH gradient. The process of IEF in carrier ampholytes (CA) and in immobilized pH gradients (IPG) provides an additional force which counteracts diffusion of CA and so maximizes the ratio of separative to dissipative transports, this substantially increases the resolution of the fractionation method. Isoelectric point is very important in any purification process. IEF gel was prepared by mixing 1.5 grams of acrylamide and bisacrylamide (29:1), 2.2 g urea, ampholyte pH range 3-5 (0.1 mL), ampholyte pH range 3-19 (0.04 mL ), 0.07µL of 10% APS,0.08 µL of TEMED. Cathode buffer used is 1M sodium hydroxide and anode buffer is 1 M phosphoric acid. Sample is loaded onto the gel and applied constant voltage of 100 for 1 hour followed by 200 V for 1 hour and 500 V for 2 hours. After completion of run the gel is placed in fixing solution followed by staining and destaining process.

**Host cell derived proteins:**
HCPs are contaminants generated during fermentation and cell lysis step and may cause immunogenic reactions if present in larger amount. Acceptable limit of HCPs are less than 100 ppm in final purified sample. HCPs contamination is checked by ELISA method by using kit manufactured by alpha diagnostic international, USA.

**Host cell DNA contamination:**
Host cell contamination was tested by PCR method. Host (E.coli BL21 (DE3) cell were grown to OD of 2.0 at 600 nm , culture was centrifuged and pellet was taken , DNA was isolated by standard procedure as given by kit manufacturer and quantified DNA by measuring absorbance at 260 and 280 nm. Pure DNA has an A260/A280 ratio of 1.8~1.9, lower ratios indicate protein contamination and higher indicates RNA contamination. An A_{260} is equal to 1.0 indicates a [DNA] =50 µg/ µL. Standard DNA are diluted to 10,1,0.1 and 0.01 ng , all the standards and the samples were used for amplification. After completion of PCR run, 10 µL of the PCR product was loaded.
on a 1% agarose gel with DNA marker and the gel is visualized using gel documentation system. Acceptable limit of residual DNA contamination is less than 10 ng.

**Bacterial endotoxin test:**

Endotoxins are fever causing agents which are generated from cell wall disruption of E.coli which are detected by gel clot. Gel clot method is simple and well used method which LAL reagent is added to the sample and incubated at 37°C for one hour in water bath or dry heat block. Presence of endotoxins is indicated by formation of gel clot and vice versa.

**ELISA:**

*In-vitro* activity of EGF was tested by quantikine human EGF immunoassay kit (R&D systems). A monoclonal antibody specific for EGF has been precoated onto a microplate. Standards and samples are pipetted into the wells and any EGF present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme linked polyclonal antibody specific for EGF is added to the wells. Following a wash to remove any unbound antibody enzyme reagent, a 1:1 ratio mix of hydrogen peroxide and TMB substrate solution is added to the wells and color develops in proportion to the amount of EGF bound in the initial step. The color development is stopped by adding 2N sulfuric acid and the intensity of the color is measured within 30 minutes at 450 nm.

**Formulation development:**

EGF drug substance which was fully characterized as described above was used for formulation development which includes stress studies, selection of buffer system, compatibility studies with manufacturing components like filters, tubing, optimization of manufacturing conditions and release profile of drug.

We had developed different formulations and the final formulation selected for further study contains EGF as active ingredient 0.1 mg/mL, dibasic sodium phosphate as buffering agent 28.4 mg/mL, sodium dihydrogen phosphate as buffering agent 24 mg/mL, Benzyl alcohol as preservative 0.01 mL/mL, poloxamer 407 as gelling agent 150 mg/mL, mannitol as isotonic agent 50 mg/mL.

Method of manufacturing: About 80% of the batch size of water for injection at 2-8 °C was taken, poloxamer 407 was added and dissolved until a clear solution is formed. To it mannitol, dibasic sodium phosphate and sodium dihydrogen phosphate were added and dissolved, rEGF was added and mixed and then benzyl alcohol was added and mixed well. The formulation was made up to the required
volume with cooled water for injection. The formulation was filtered through 0.2 um filter membrane and filled into containers. The recommended storage temperature of the product is 2-8°C. The final formulation was analyzed for physical appearance, pH and identification by immunoblotting, purity by SDS PAGE and RP-HPLC, SEC-HPLC, potency (invitro), endotoxins and benzyl alcohol.

RESULTS AND DISCUSSION:

Physical appearance:
The solution is clear and colorless solution, there is no turbidity or opalescence produced during the entire process of formulation which indicates that there are no solubility issues of the excipients, preservatives and drug substances and there is no change during storage and stability studies which indicates suitability and its compatibility with excipients of formulation.

pH:
pH is an important parameter to be considered during the formulation development and limit of pH is 6.2 ~6.7 and the pH of the formulation remains unchanged as the formulation contains buffer system.

Immunoblotting:
The protein of interest is identified by using immunoblotting technique. After SDS-PAGE, the protein is transferred onto PVDF membrane by using semidry western blotting apparatus by using transfer buffer, membrane was removed and washed with TBS and then incubated with 5% skim milk powder in tris buffered saline (TBS) (blocking agent), then membrane was incubated with monoclonal anti EGF antibody as primary antibody followed by anti mouse IgG alkaline phosphatase antibody as second antibody. Membrane was washed in between every step i.e before and after block, primary, secondary body addition TBST (0.05% tween 20 in TBS) and membrane was developed by using 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP) / nitro-blue tetrazolium chloride (NBT) solution which was prepared by dissolving 1 tablet each of (BCIP)/ (NBT) in 10 mL of water for injection until clear bands are visible on the membrane.
**Figure-1**: Western blot analysis of EGF sample (Lane 1 EGF drug substance and 2 formulated sample)

**SDS PAGE:**

15% gel was prepared and standard conditions (150 V, 60 minutes) are used for running the gel. After completion of the run, gel was stained with Coomassie blue and destained with destaining solution. The gel picture conforms the drug substance and drug product migration and shape of the band are similar, which means there is no effect of formulation excipients and preservatives on EGF drug substance. SDS PAGE is used as stability indicating factor in both drug substance and drug product.

**Figure-1**: SDS-PAGE analysis EGF sample.

(Lane 1 EGF drug substance and 2 formulated sample)
RP-HPLC:

The purity of EGF was checked by RP-HPLC. The zorbax SB C<sub>8</sub>, UV detector at 214 nm, run time is 60 minutes, and mobile phase A is 0.1% TFA in water and mobile phase B is 0.1% TFA in 900 mL ACN and 100 mL water and the flow is gradient.

![Figure-3: RP-HPLC analysis of EGF sample. The purity of the sample is >99.4 %](image)

TABLE-1:

SEC-HPLC:

The dimers and other related substances of EGF was checked by SEC-HPLC. The TSKgel G2000SWXL (7.8 x 300) UV detector at 220 nm, run time is 60 minutes, and mobile phase is sodium phosphate buffer.

![Figure-4: SEC-HPLC analysis of EGF sample. The purity of the sample is >99 %](image)
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286
Potency:

Potency or activity of the product was evaluated as per the procedure described in methods and materials.

Bacterial endotoxins test:

BET test was performed as per procedure described in methods and the endotoxins content was less than 30 EU/mL.

CONCLUSION:

The data shows the release of EGF indicating a better formulation for treatment of diabetic foot ulcer. The main advantages of this formulation are convenient to apply i.e no need to apply by using hands or other means and dressing will be easy. Stability, Preclinical and clinical evaluation is to be carried out before human use and various factors which effect the activity of EGF have to be extensively studied.

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3. Matti laato, Juha Niinikoski, Bengt Gerdin, Lena lebel; Stimulation of wound healing by Epidermal Growth Factor.


