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No. LSD/CUD1629/053101/2012

31 MAY 2012
2012-05-31

Via E_mail
pbisht@gwalior
CONFIRMATION VIA COURIER

Sub: New Patent Application # 1655/DEL/2012 dated 31.5.2012 on "A Process for the Synthesis, Fractionation and Dry Formulation of Site Specific Mono-pegylated Interferon Alpha Molecule" - **Filing Intimation**

Dear Dr Bishen

This is to inform you that a patent application, along with the provisional application covering the subject matter of above-mentioned invention, has been filed at Patent Office, New Delhi on 31.5.2012 and the said application has been accorded application # **1655/DEL/2012.**

A copy of the Provisional Application, as filed, and Official Filing Receipt (OFR) is being mailed to you through courier today for your reference and records.

Please note that as per The Patents Act 1970, as amended till date, the complete specification has to be filed before the expiry of 12 months' time (**31.5.2013**) from the date of filing this patent application. Our next action, to draft and file the complete specification, will be initiated soon and the drafted claims will be mailed to you for your approval in due course of time.

In all your future correspondence with us, please quote our File Ref.# **UD1655.**

In case, you have any query in this connection, please do not hesitate to call us.

Thanking you

Sincerely yours
(C M Gaind)

Enclosed : As stated

Prof Prakash Singh Bishen
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Agent Number:

Serial Number	Reference Number/Application Type	Application Number	Title/Remarks	Amount Paid	Amount Computed	Fee Payment
1	ORDINARY APPLICATION Pages:-12 , Claims:-10	1652/DEL/2012	A CUTTING APPARATUS WITH ROTARY KNIFE FOR A FORM.	1000	1000	Full
2	ORDINARY APPLICATION Pages:-5 , Claims:-0	1653/DEL/2012	IMPROVED WHEEL OF VEHICLE.	4000	4000	Full
3	ORDINARY APPLICATION Pages:-6 , Claims:-0	1654/DEL/2012	AN IMPROVED WHEEL OF VEHICLE.	4000	4000	Full
4	ORDINARY APPLICATION Pages:-26 , Claims:-0	1655/DEL/2012	PROCESS FOR THE SYNTHESIS, FRACTIONATION AND DRY.	4000	4000	Full

Continue...

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Serial Number	Reference Number	Application Number	Title/Remarks	Amount Paid	Amount Computed	Fee Payment
Total Amount				13000	13000	

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PROVISIONAL

SPECIFICATION

SECTION 10

TITLE

A Process for the Synthesis, Fractionation and Dry Formulation of Site Specific Mono-pegylated Interferon Alpha Molecule

APPLICANT

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The following specification describes the nature of invention.

FIELD OF INVENTION

The present invention provides a novel process for the synthesis of mono-pegylated interferon alpha molecule specially IFN alpha 2b conjugate, separation of PEG-Interferon alpha conjugate by ammonium sulfate fractionation followed by vacuum drying.

BACKGROUND OF THE INVENTION

Novel therapeutic protein and peptide drugs have been developed in an attempt to improve pharmacokinetic and pharmacodynamic properties, and reduce the immonogenecity by their modifications. For example, PEG-Interferon alpha conjugate was prepared by conjugating PEG molecule (polymer) to the IFN alpha 2b (protein). Methods for creating protein – polymer conjugate are known in the art.

Conjugation of polymer to protein may result in a single polymer molecule conjugated to a protein or multiple such conjugation to a single protein or double protein conjugate to a single polymer resulting into the mixture of mono and oligo species of pegylated molecule. The multiple coupling of polymer to protein leads to the loss in the specific activity of the protein. The degree of conjugation depends on the reaction conditions and desired result. In the present invention, the reaction is controlled to get site specific mono-pegylated IFN alpha 2b conjugate. When single PEG molecule linked to interferon alpha molecule through hydrolytically stable carbamate linkage, the resulting PEG-Interferon alpha conjugate can comprise a mixture of positional isomers. In a preferred embodiment, one of the positional isomers is an interferon alpha molecule linked to a PEG molecule at a His_{34} residue having highest

specific activity. 77.3 % of His_{34} linked pegylated IFN alpha 2b is obtained in the reaction mixture.

Once, IFN alpha 2b is modified by polyethylene glycols (PEG), should be separated from uncoupled PEG, uncoupled IFN alpha 2b molecules and reaction enhancers. Multiple column chromatographies are in the practice to separate the PEG-Interferon alpha conjugates. Multiple column chromatography takes long process time which cause loss in the activity of the conjugate as proteins are highly unstable in aqueous solution and highly sensitive to the alteration in pH and conductivity of the chromatographic media. Moreover, Multiple column chromatographics are not very friendly to the large scale production as it involves expensive set up and maintenance. The need has been generated to overcome the issues associated with the purification.

In the present invention, a process has been developed for the separation of PEG-Interferon alpha conjugates. It is based upon the alteration in physiochemical properties of Interferon alpha by pegylation. It involves multiple step fractionation of the conjugate by ammonium sulfate. The fractionation of the conjugate is based upon the volume exclusion effects according to which the conjugates are sterically excluded from the solvent that are occupied by ammonium sulfate. Since, the aqueous solubility of the PEG-Interferon alpha conjugates and unconjugated interferon alpha varies, the PEG-Interferon alpha conjugates get precipitated first when its solubility is exceeded. In thermodynamic terms, the steric exclusion leads to an increase in the chemical potential of the protein until it exceeds that of the "pure solid state", leading to the precipitation of the conjugate. This happens mainly because of a large unfavorable free energy of interaction between ammonium sulfate and the PEG-

Interferon alpha conjugates leading to preferential hydration of the PEG-Interferon alpha conjugates attributed to the steric exclusion effects. The preferential hydration helps in maintaining the native structure of the conjugate.

Due to high physiochemical instability of proteins in aqueous solutions,[1-4] it is a common practice to formulate proteins as dry powder to retain the desired storage shelf life of the product. Lyophilization has been the traditional process of the choice for the formulation of the protein as dry powder. Lyophilization involves long process time, expensive set up and maintenance of the lyophilization system, and above all, the instabilities incurred upon proteins because of the steps involved in lyophilization. Due to these key issues, a need has been realized to develop alternative technologies to overcome the limitations of lyophilization.[5-6]

The alternative technologies to lyophilization reported in the literature include spray drying,[7] spray freeze drying,[8] bulk crystallization,[9] supercritical fluid technology,[10] vacuum drying,[11] and foam drying.[12]. Although, these processes have been shown to overcome some of the problems associated with lyophilization, several limitations still exist in each of these processes. For example, the presence of large air / water interface has been associated with protein aggregation in spray drying.[13] Similarly, the use of supercritical fluids involves organic solvents that may not be favorable for the existence of protein conformation[14] and simple vacuum drying may not result in acceptable powder form of the product.[15]

The present invention contemplates a process of drying the pure PEG-Interferon alpha conjugate by vacuum drying after

ammonium sulfate fractionation. The finally fractionated pure PEG-Interferon alpha conjugate was dried under vacuum with sugars at 3°-5°C to achieve the powder state.

Novel biodrugs have been developed in attempts to improve pharmacokinetic and pharmacodynamic properties, and reduce the immunogenicity of therapeutic proteins and peptides by their modifications. They are modified by polyethylene glycols (PEG) leading into mono-pegylated and oligo-pegylated protein molecules, uncoupled protein and PEG molecules and reaction enhancers. The mono-pegylated protein molecules are separated from oligo-pegylated protein molecules, uncoupled PEG and protein molecules and reaction enhancers by ion exchange followed by gel exclusion column chromatographies.

Conjugation of polymers to protein leads to a single polymer molecule conjugated to a protein or multiple such conjugation to a single protein or double protein conjugate to a single polymer through hydrolytically stable carbamate linkage. Multiple coupling of polymer to protein leads to loss in the specific activity of the protein.

Multiple column chromatographies are in the practice to separate the PEG-Interferon alpha conjugates. Multiple column chromatography takes long process time which cause loss in the activity of the conjugate as proteins are highly unstable in aqueous solution and highly sensitive to the alteration in pH and conductivity of the chromatographic media. Moreover,

Multiple column chromatographies are not very friendly to the large scale production as it involves expensive set up and

maintenance. The need has been generated to overcome the issues associated with the purification.

In the present invention, a process has been developed for the synthesis of His_{34} site specific mono-pegylated IFN alpha conjugate that is having highest specific activity.

The multiple column chromatography is replaced by the fractionation of the conjugate that is purely based upon the volume exclusion effects according to which the conjugates are sterically excluded from the solvent that are occupied by ammonium sulfate and separated from uncoupled IFN alpha and PEG molecules. The separation of PEG-Interferon alpha conjugate is based upon the alteration in physiochemical properties of interferon alpha by pegylation.

OBJECTS OF THE INVENTION

The main objective of the present invention is to develop a controlled process to achieve single PEG molecule conjugated to Interferon alpha.

Another objective of the present invention to provide a controlled process wherein equimolar of PEG and interferon is used at pH 6.5 at 4°C resulting into mono-pegylated interferon alpha 2b conjugate.

Yet another objective of the present invention is to devise a process for fractionation of PEG-Interferon alpha conjugates from the reaction mixture of uncoupled interferon alpha and PEG molecules wherein the inclusory or exclusory molecule is

ammonium sulfate and the concentration of exclusory reagent is 5 % to saturated aqueous solution of ammonium sulfate.

Still another objective of the present invention is that number of fractionations employed for isolation of pure PEG-Interferon alpha is 2 to 12.

Yet another objective of the present invention is to develop a method of fractionation segregating into PEG-Interferon alpha conjugate, PEG and uncoupled interferon alpha molecules.

A further objection of the present invention is that the fractionation of PEG-Interferon alpha conjugates comprise single PEG molecule conjugated to single interferon alpha molecule and having PEG of mass 5,000 Da to 40,000Da.

An additional object of the present invention to devise a process wherein the drying of PEG- Interferon alpha conjugates involves the precipitation with ammonium sulphate followed by vacuum drying .

Yet another additional object of the invention is develop a process where the operation parameters of pressure, temperature and duration of vacuum drying operates in the range of 0.5 mm to 50 mm.; 2° - 45°C and 10 minutes to 48 hours respectively.

The foregoing has outlined some of the pertinent objectives of the invention. These objectives should be construed to be merely illustrative of some of the more prominent features and applications

of the intended invention. Many other beneficial results can be obtained by applying the disclosed invention in a different manner or modifying the invention within the scope of disclosure. Accordingly, other objectives and a full understanding of the invention and the detailed description of the preferred embodiment in addition to the scope of invention are to be defined by the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Further objects and advantages of this invention will be more apparent from the ensuing description when read in conjunction with the accompanying drawings and wherein:

Fig. 1 : Synthesis of mPEGSC, electrophillic derivative of mPEG and coupling with IFN alpha 2b.

Fig. 2 : Synthesis of mPEGSC, electrophillic derivative of mPEG and coupling with IFN alpha 2b.

Fig. 3 : SDS PAGE gels pictures showing the PEG : IFN; 4:1 and 8:1 molar ratio at different reaction time in the PEGylation of IFN alpha 2b.

Fig. 4 : A flow chart showing the fractionation steps of PEG-IFN conjugate. In step one of ammonium sulfate fractionation, purity of IFN-PEG conjugate was improved to 26.3% that improved further to 37.6% in step-2, 54.1% in step -3, 66.7% in step - 4, 89.5% in step - 5 and > 95 % in step – 6

Fig. 5 : SDS PAGE showing the step- wise fractionation of PEG-IFN alpha 2b conjugate.

SUMMARY OF THE INVENTION:

The interferon alpha family consists of small proteins that have clinically important anti – infective and anti-tumor activity. Interferon alpha -2b combination therapy with ribavirin is the current standard of care for the treatment of chronic hepatitis C virus infection. A drawback of this therapy however, is the short serum half life and rapid renal clearance. Interferon alpha -2b is pegylated by attaching a mono-methoxy polyethylene glycol to enhance its serum half life, reduce renal clearance and immunogenicity.

PEG – interferon alpha conjugates are prepared by site specific conjugation of PEG molecule on the backbone of interferon alpha resulting into carbamate linkage between polymer and the protein. In the preferred embodiments, the PEG – interferon alpha conjugates of the present invention comprise interferon alpha -2 a (Roferon, Hoffman La- Roche, Nutley, NJ), interferon alpha -2 b (Intron, Schering – Plough, Madison, NJ), interferon alpha-2 c (Berofor Alpha, Bochringer Ingelheim, Ingelheim, Germany).

Methoxy Polyethylene Glycol is a polymer having covalently attached repeating chemical units (O CH_2 CH_2)n. Hydroxyl group (-OH) at one end of the polymer is converted into methoxy group (-CH_3O) to protect the polymer coupling with protein at both the ends and resulting into undesirable monopegylated di protein conjugates. The mass of the polymer is designated with a number following the name of the repeated chemical unit. For example, "PEG" or

polyethylene glycol (12,000)" refers to a polymer of polyethylene glycol having an average mass of approximately 12,000. In a PEG polymer, the number of repeated polyethylene glycol units in the polymer is approximately 273. Since, it is often impossible to manufacture the polymer of precise and uniform mass or number of repeated units, it is always designated as average mass in the art.

Methods for preparation of protein – polymer conjugate are well known in the art. For example, U.S. Pat. No. 5,612,460 to Zalipsky , U.S. Pat. No. 5,711,944 to Gilbert, et. al., U.S. Pat. No. 5,691,154 to Callstrom et al, U.S. Pat. No. 5,686,071 to Subramanian et Al, U.S. Pat. No.5,492,821 to to Callstrom et al, U.S. Pat. No. 5,447,722 to Lang et al and U.S. Pat. No.5,091,176 to Braatz et al. reveals methods for producing protein –polymer conjugate.

The synthesis of mPEGSC, electrophillic derivatives of mPEG and coupling with IFN alpha 2b has been depicted in Figure 1.

Conjugation of polymers to protein may result in a single polymer molecule conjugated to a protein or multiple such conjugation to a single protein or double protein conjugate to a single polymer through hydrolytically stable carbamate linkage. Since, multiple coupling of polymer to protein leads to loss in the specific activity of the protein, a controlled method is developed to achieve mono pegylated protein. The degree of conjugation depends on the reaction conditions. In the present invention the reaction was controlled to get single polymer conjugated to protein. When single PEG molecule linked to interferon alpha molecule through hydrolytically stable carbamate linkage, the resulting PEG-Interferon alpha conjugate can comprise a mixture of positional isomers. In a

preferred embodiment, one of the positional isomers is an interferon alpha molecule linked to a PEG molecule at a His_{34} residue having highest specific activity. 77.3 % of His_{34} linked pegylated IFN alpha 2b is obtained in the reaction mixture.

The present invention of fractionation is not limited to the specific concentration of excludor. Examples include but not limited to excludor such as ammonium sulfate. Likewise, the present invention is not limited to any particular amount of excludor. In one embodiment, excludor is present sufficient enough to fractionate the PEG-Interferon alpha conjugate from free Interferon alpha and PEG. In such an embodiment, excludor can be present from 1 % to 60 %, preferably 5 % to 40% and most preferably 10 % to 25 %.

PEG- interferon alpha conjugates, however, are subject to damage during and after lyophilization. Damage to PEG- interferon alpha conjugates can be characterized by the loss of protein, loss of biological activity, alteration in the conformation of the protein, alteration of degree and / or nature of conjugation of the interferon alpha, aggregation of PEG- interferon alpha conjugates. For example, a PEG- interferon alpha conjugate may degrade into free PEG and interferon alpha, reduction in the degree of conjugation. Likewise, the resulting free PEG may react with another interferon alpha of the PEG- interferon alpha conjugates, potentially resulting in the increase of the degree of conjugation in that target molecule. Similarly, a PEG- interferon alpha conjugate may undergo an intramolecular shift of the PEG from one site of conjugation to another within the same molecule, thereby changing the nature of conjugation of the interferon alpha.

The present invention protects PEG- interferon alpha conjugates from damage during lyophilization. In the preferred embodiment, the method involves the precipitation of PEG-interferon alpha conjugates and drying them under vacuum at 3° - 5° C in presence of sugar.

While present invention is not limited to a specific sugar, example includes, but are not limited to sugars. A preferred sugar is a sugar alcohol. A preferred sugar alcohol is mannitol. The invention is not limited to any particular amount of sugar used. In such an embodiment, sugar can be present in a concentration of about 0.1 % to 80 %, preferably 0.5 % to 60 % and most preferably 1 % to 10 %.

The present invention is not limited to a specific duration, extent of vacuum and temperature for vacuum drying. In one embodiment, vacuum time is continued till the complete drying to the powder form. The duration of vacuum may be from 10 minutes to 48 hours, preferably 1 hour to 24 hours and most preferably 2 hours to 10 hours. Likewise, temperatures of vacuum drying are 2° -45° C, preferably 4 ° -25° C and most preferably 2 ° - 10° C. Similarly, vacuum for drying may be from 0.1 mm to 100 mm, preferably 0.5 mm to 50 mm and most preferably 1 mm to 10 mm.

Example- 1

This example provides the description of the synthesis of mono pegylated IFN alpha 2b. PEG-Interferon alpha 2b conjugate was synthesized by the electrophilic and nucleophilic interactions of mPEG-SC and IFN alpha 2b in 10 mM Phosphate buffer, pH 6.5 at 4°C for 1 hour where His_{34} becomes the predominant site of coupling. As the molar ratio of PEG increases, amount of mono-

pegylated IFN alpha increases. Oligo-pegylated species start appearing from molar ratio of PEG : IFN; 2:1 and go on increasing with increase in molar ratio of PEG. Mono-pegylated interferon alpha was synthesized with equimolar ratio of PEG and Interferon alpha, resulting into the mixture of mono-pegylated interferon alpha 2b and uncoupled interferon alpha 2b but no oligo-pegylated species.

Figure 2 shows SDS PAGE gels diagrams indicating the PEG and IFN alpha 2b ration inn the PEGylation of IFN alpha 2b. As the molar ratio of PEG increases, amount of mono-pegylated IFN increases but oligo pegylated species also start appearing from the molar ratio of PEG:IFN::2:1 and increase with the increase in molar ratio of PEG to IFN alpha 2b

GEL -1

LANE	SAMPLE
1	PEG-IFN , MOLAR RATIO 1:1
2	PEG-IFN , MOLAR RATIO 2:1
3	PEG-IFN , MOLAR RATIO 4:1
4	IFN ALPHA 2b
5	MOL. WT. MARKER

GEL -2

LANE	SAMPLE
1	PEG-IFN , MOLAR RATIO 8:1
2	PEG-IFN , MOLAR RATIO 16:1
3	PEG-IFN (PEG INTRON) (SCHERING PLOUGH)
4	IFN ALPHA 2b (INTRON A) (SCHERING PLOUGH)

Example- II

This example provides the description of the studies on the synthesis of pegylated IFN alpha 2b using PEG : IFN; 4:1 and 8:1 molar ratio for different reaction time periods.

PEG-Interferon alpha 2b conjugate was synthesized by the electrophilic and nucleophilic interactions of mPEG-SC and IFN alpha 2b in 10 mM phosphate buffer, pH 6.5 at 4°C for different time periods ranging from 1 hour to 18 hours with PEG : IFN; 4:1 as well as 8:1 molar ratios. As the reaction time increases, no substantial increase in mono PEG-IFN was observed whereas oligo-pegylated species start appearing and increase with the reaction time.

Figure 3 shows SDS PAGE gels diagrams indicating the PEG:IFN : 4:1 and 8:1 molar ratio at different reaction time in the PEGylation of IFN alpha 2b. As the reaction time increases, no substantial increase in mono PEG-IFN was observed whereas oligo-pegylated species start appearing and increase with the reaction time.

Gel : 1

Lane	Sample
1	Mol. Wt. Marker
2	PEG-IFN (4:1) 1 hour reaction
3	PEG-IFN (4:1) 2 hour reaction
4	PEG-IFN (4:1) 3 hour reaction
5	PEG-IFN (4:1) 4 hour reaction

6	PEG-IFN (4:1) 5 hour reaction
7	PEG-IFN (4:1) 6 hour reaction
8	PEG-IFN (4:1) 7 hours reaction
9	PEG-IFN (4:1) 8 hours reaction
10	PEG-IFN (4:1) 18 hours reaction

Gel : 2

Lane	Sample
1	Mol. Wt. Marker
2	PEG-IFN (8:1) 1 hour reaction
3	PEG-IFN (8:1) 2 hour reaction
4	PEG-IFN (8:1) 3 hour reaction
5	PEG-IFN (8:1) 4 hour reaction
6	PEG-IFN (8:1) 5 hour reaction
7	PEG-IFN (8:1) 6 hour reaction
8	PEG-IFN (8:1) 7 hours reaction
9	PEG-IFN (8:1) 8 hours reaction
10	PEG-IFN (8:1) 18 hours reaction

Example- III

This example provides the description of a fractionation method for isolating PEG-Interferon alpha conjugate. Monopegylated Interferon alpha is fractionated from uncoulpled interferon alpha by excludor ammonium sulfate in different concentrations at multiple steps.

Figure 4 depicts a flow chart showing the fractionation stepas of PEG-IFN conjugate. In step one of ammonium sulfate

fractionation, purity of IFN-PEG Congujate was improved to 26.3% that improved further to 37.6% in step 2, 54.1% in step -3, 66.7% in step-4, 89.5% in step-5 and > 95% in step -6.

Further in step-I, the conjugation reaction mixture is treated with 50% saturated ammonium sulfate (v/v), resulting into the precipitation of PEG-Interferon alpha conjugate which is centrifuged at 10,000 g. The pellet is dissolved in 10 mM Phosphate buffer, pH 6.5 to the protein concentration of 1.0 mg /ml. This is further (Step-II) treated with 25% saturated ammonium sulfate (v/v), resulting into the precipitation of PEG-Interferon alpha conjugate which is centrifuged at 10,000 g. The pellet is dissolved in 10 mM Phosphate buffer, pH 6.5 to the protein concentration of 1.0 mg /ml. The step-II is repeated for four times to achieve pure PEG-Interferon alpha conjugate. The fractionation details are given in the following table. The pure PEG-Interferon alpha conjugate is characterized for its specific activity and compared with that of the conventionally column purified conjugate.

Table -1

% fractionation of PEG-Interferon alpha conjugate

(Densitometric analysis)

Sr. No.	Fractionation			
	Centrifugate (% Recovery of Interferon alpha)		Pellet (% Purity of uncoupled Interferon alpha and PEG-Interferon alpha conjugate)	
	Interferon alpha	PEG-Interferon alpha conjugate	Interferon alpha	PEG-Interferon alpha conjugate
01	24 %	0 %	73.7 %	26.3 %
02	10.6 %	0 %	62.4 %	37.6 %
03	13.6 %	0 %	45.9 %	54.1 %

04	14.2 %	0 %	33.3 %	66.7 %
05	2.8 %	0 %	10.5 %	89.5 %
06	0.8 %	0 %	0 %	> 95 %

Table -2

Net yield of PEG-IFN conjugate

PEG-IFN conjugate (Chromatographic method i.e. ion exchange followed by gel filtration)	PEG-IFN conjugate (Fractionation method)
7.3% of IFN used in PEGylation	10.8% of IFN used in PEGylation

Figure 5 depicts SDS PAGE showing the step-wise fractionation of PEG-IFN alpha 2b conjugate. The free IFN alpha 2b is removed from the PEG-IFN alpha 2b conjugate in 6 steps of ammonium sulfate fractionation.

C	:	Crude PEG-IFN
MW	:	Molecular Weight Marker
F 1 - F6	:	Centrifuate at different steps 1-6 showing the fractionation of IFN
R1- R6	:	Residue at different steps 1-6 showing the fractionation of PEG-IFN Conjugate
Pure IFN	:	Pure IFN alpha 2b for reference.

Example- IV

The PEG-Interferon alpha conjugate is mixed with mannitol in the concentration of 10 mg of mannitol per mg of protein. Dried under desiccated vacuum (< 5 mm) at 3°C to 5°C till the precipitate gets dried to powder state. The dried PEG-Interferon alpha

conjugate is characterized for its specific activity and compared with that of the conventionally dried by lyophilization.

Table –3

Specific activity of PEG-Interferon alpha conjugates in different conditions

Interferon alpha used : 2.9 X 10^8 IU/mg

Specific activity is determined using NIBSC standards by Cytopathy Effect employing EMC virus infected WISH cells

Specific Activity of Column purified PEG-Interferon alpha 2b		**Specific Activity of Fractionated PEG-Interferon alpha 2b**	
1.24 X 10^8 IU/mg (42.8 % Retention)		1.62 X 10^8 IU/mg (55.8 % Retention) (12.8 % Gain)	
Lyophilized Powder	Vacuum dried Powder	Lyophilized Powder	Vacuum dried Powder
0.99 X 10^8 IU/mg (79.8 % Retention)	1.12 X 10^8 IU/mg (90.3 % Retention) (10.5 % Gain)	1.31 X 10^8 IU/mg (80.8 % Retention)	1.50 X 10^8 IU/mg (92.6% Retention) (11.8 % Gain)

Table shows the specific activity of PEG-Interferon alpha conjugates obtained through column chromatography (ion exchange followed by gel filtration) and fractionation; lyophilized and vacuum dried. The 12.8 % gain in the specific activity of PEG-Interferon alpha conjugate obtained when it is fractionated which was further enhanced by 11.8 % when vacuum dried instead of freeze drying.

References

1. Chen T. Formulation concerns of protein drug. Drug Dev Ind Pharm. 1992; 18:1311-1354.

2. Manning MC, Patel K, Borchardt RT. Stability of protein pharmaceuticals Pharm Res. 1989; 6:903 – 918.

3. Arakawa T, Prestrelski S, Kenney WC, Carpenter JF, Factors affecting short term and long term stabilities of proteins. Adv. Drug Deliv Rev. 2001; 46:307 – 326.

4. Ahern TJ, Manning MC. Stability of Protein Pharmaceuticals, Part A : Chemical and Physical Pathways of protein degradation. New York, NY : Plenum Press; 1990.

5. Gomez G, Pikal MJ, Rodriguez – Homedo N. Effect of initial buffer composition on pH changes on far-from-equilibrium freezing of sodium phosphate buffer solution. Pharm Res. 2001; 18:90-97.

6. Franks F. Protein destabilization at low temperatures, In: Anfinsen CB, EDsall JT, Richards FM, Eisenberg DS, eds. Advances in Protein Chemistry : Protein Stability. New York, NY : Academic Press; 1995 : 105 -137.

7. Mumenthaler M, Hsu CC, Pearlman R. Feasibility study on spray-drying protein pharmaceuticals: Recombinant human growth hormone and tissue –type plasminogen activator. Pharm Res. 1994; 11:12-20.

8. Mumenthaler M, Leuenberger H. Atmospheric spray-freeze drying: A suitable alternative in freeze drying technology. Int J Pharm. 1991; 72:97 -110.

9. Shenoy B, Wang Y, Shan W, Margolin AL. Stability of crystalline proteins. Biotechnol Bioeng. 2001 ; 73 : 358-369.

10. Winters MA, Knutson BL, Debebedetti PG, et. al. Precipitation of proteins in super critical carbon dioxide. J Pharm Sci. 1996; 85; 586 – 594.

11. Mattern M, Winter G, Rudolph R, Lee G. Formulation of proteins in vacuum dried glasses. Part I. Improved vacuum drying of sugars using crystallizing amino acids. Eur J Pharm Biopharm. 1997; 44:177-185.

12. Brohnstein V. Scalable long term shelf preservation of sensitive biological solutions and suspensions. US patent 6 509 146, January 21, 2003.

13. Maa YF, Nguyen PA, Andya JD, et. al.. Effect of spray drying and subsequent processing conditions on residual moisture content and physical / biochemical stability of protein inhalation powders. Pharm Res. 1998; 15 : 768 -775.

14. Winters MA, Knutson BL, Debebedetti PG, et. al. Precipitation of proteins in super critical carbon dioxide. J Pharm Sci. 1996; 85; 586 – 594.

15. Mattern M, Winter G, Rudolph R, Lee G. Formulation of proteins in vacuum dried glasses. Part I. Improved vacuum drying of sugars using crystallizing amino acids. Eur J Pharm Biopharm. 1997; 44:177-185.

ADVANTAGES OF THE INVENTION

This invention will be useful to

i) synthesize only site specific mono pegylated interferon molecules and there would not be need for the removal of oligo pegylated interferon species. The His_{34} site specific mono pegylated interferon molecules usually have maximum specific activity.

ii) separate mono pegylated interferon molecules from uncoupled interferon and PEG molecules without column chromatographic methods where there is possibility of loosing the specific activity of interferon.

iii) formulate the product without lyophilization that is not only very critical process but also having the possibilities of denaturing the protein and loosing the specific activity of the conjugate.

Dated : 30th day of May, 2012

(C M Gaind)
of L S Davar & Co.,
APPLICANTS' AGENT

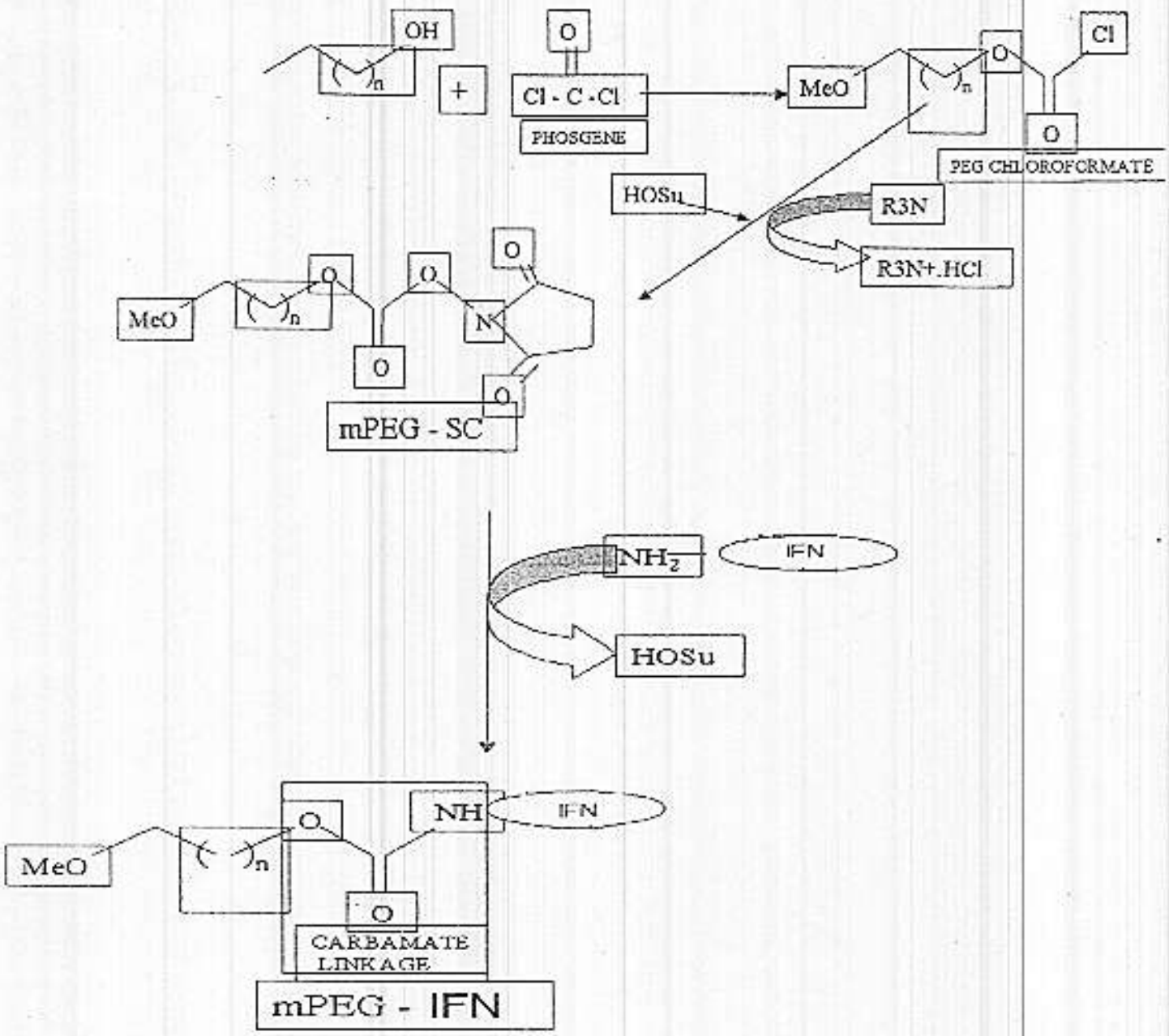

Fig. 1

(C. M. Gaind)
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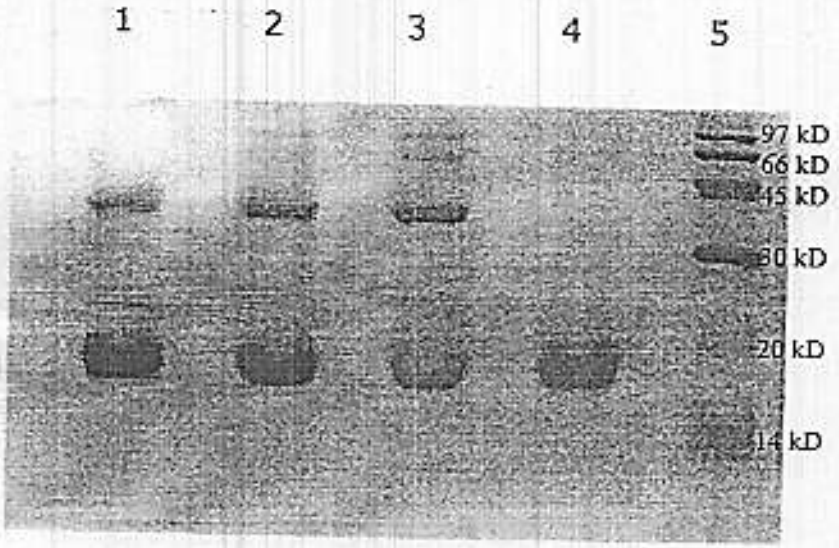
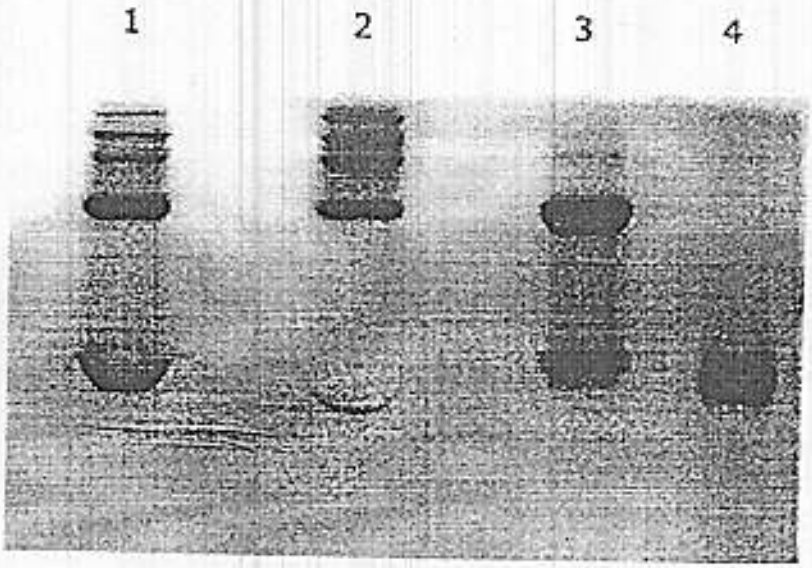
APPLICANT'S AGENT



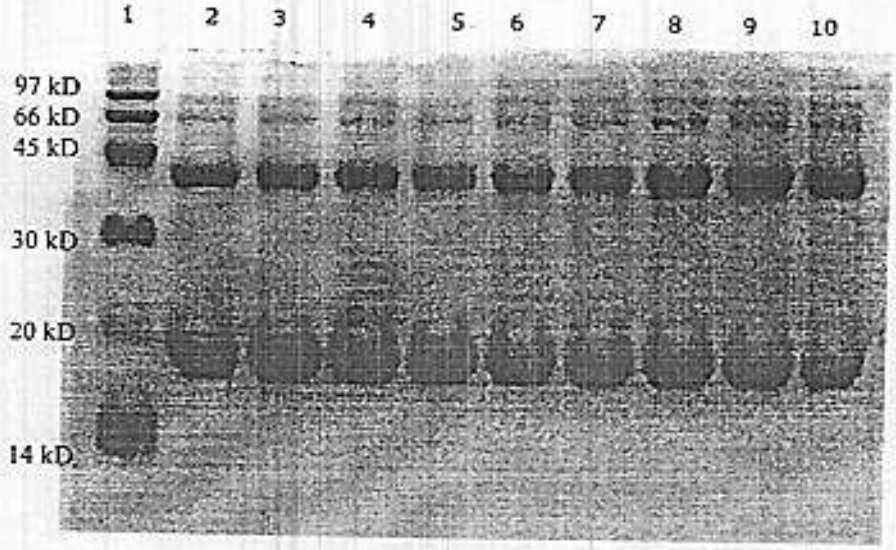
Gel : 1



Gel : 2

Fig. 2

(C. M. Gaind)
of. L.S. Davar & Co.
APPLICANT'S AGENT



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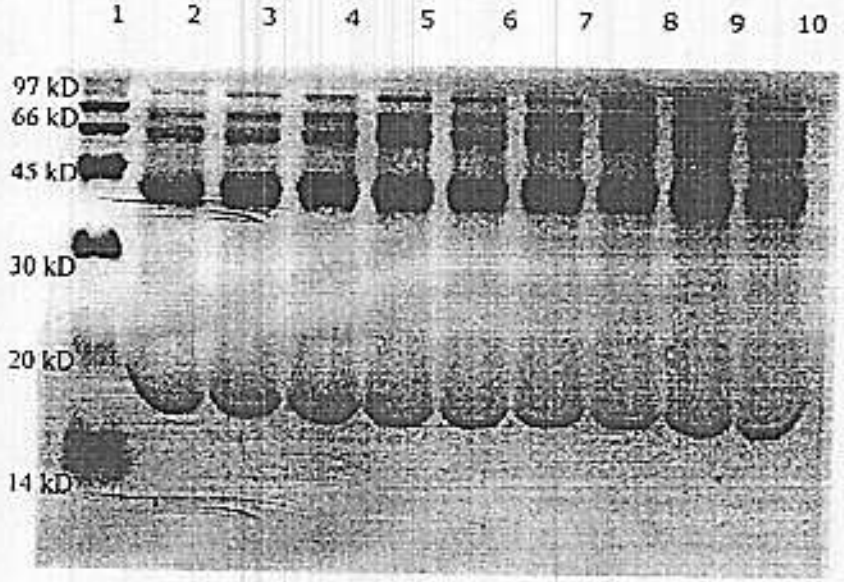

Gel : 2

Fig. 3

(C. M. Gaind)
of. L.S. Davar & Co.
APPLICANT'S AGENT

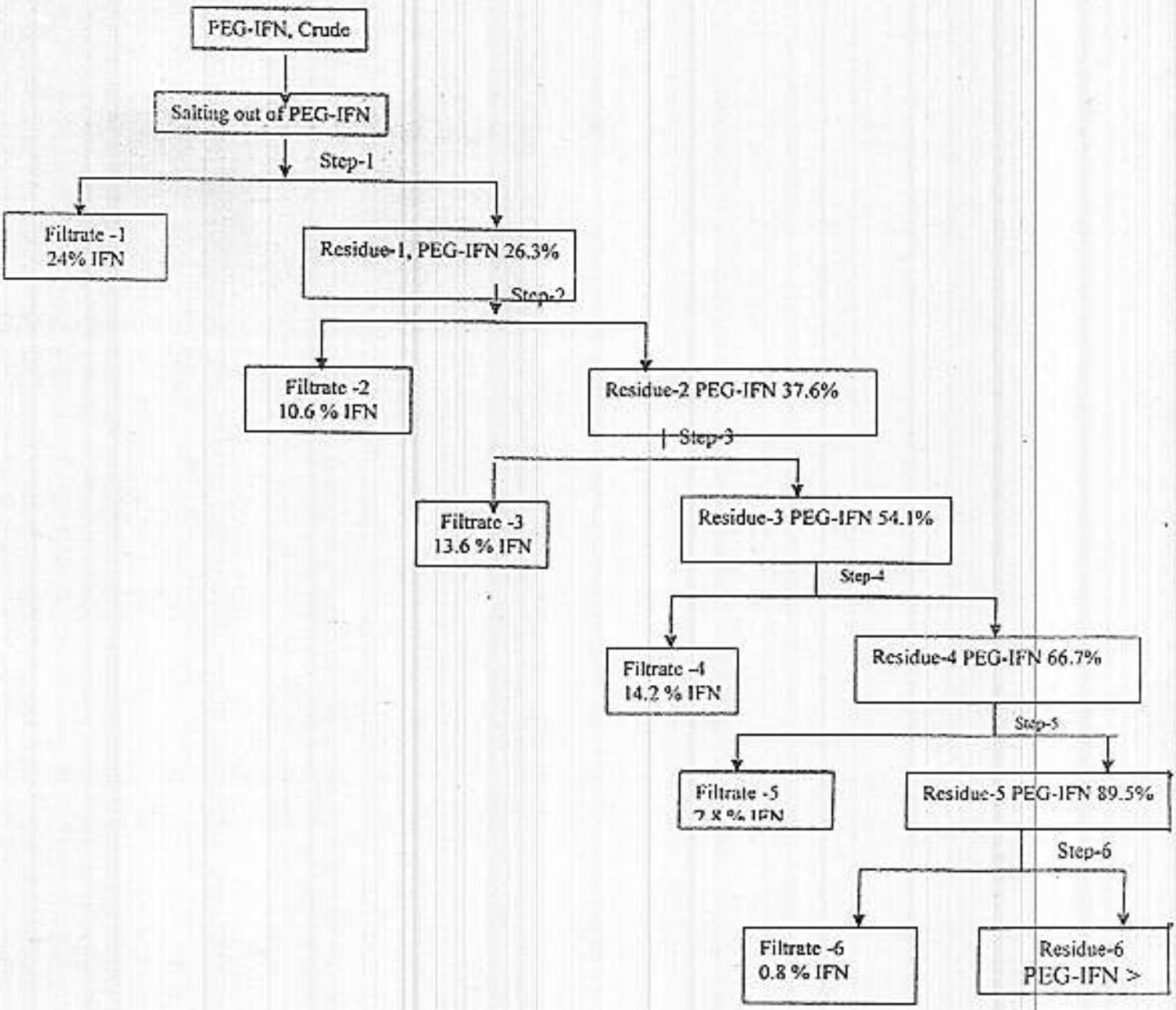

Fig 4

(C. M. Gaind)
of. L.S. Davar & Co.
APPLICANT'S AGENT

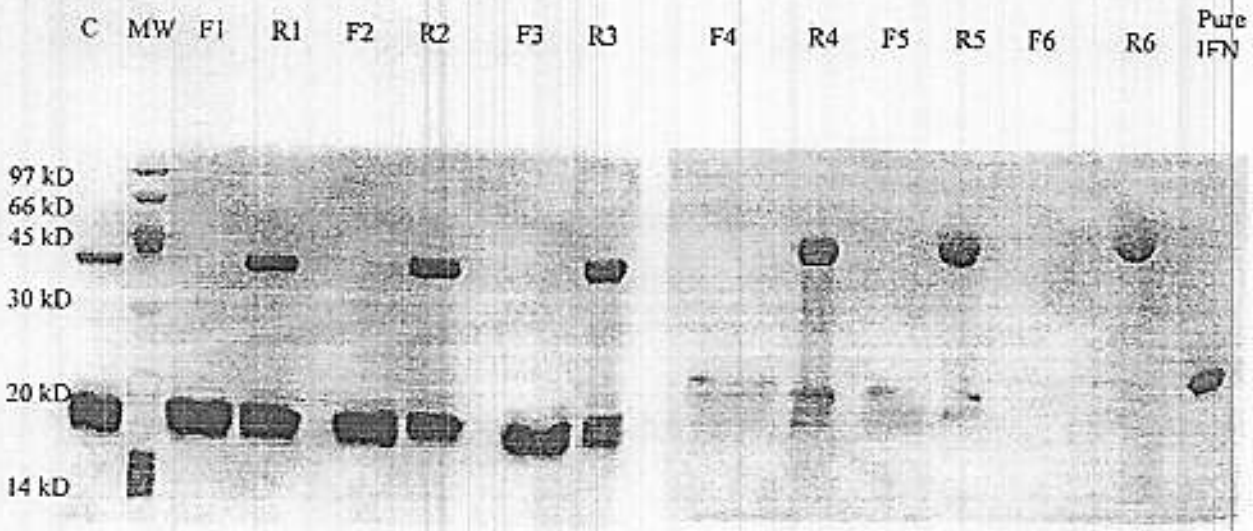

Fig 5

(C. M. Gaind)
of. L.S. Davar & Co.
APPLICANT'S AGENT