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Molecular Characterization of Recombinant Human Interferon Alpha-2b Produced in Cuba

✍ Héctor Santana,[1] Eduardo Martínez,[1] Julio C Sánchez,[1] Galina Moya,[2] Raudel Sosa,[1] Eugenio Hardy,[1] Alejandro Beldarraín,[1] Vivian Huerta,[3] Luis J González,[3] Lázaro Betancourt,[3] Vladimir Besada,[3] Rudka Brito,[1] Tania Currás,[2] Joel Ferrero,[2] Vivian Pujols,[1] Miriela Gil,[1] Luis Herrera[1]

Divisions of [1]Production, [2]Quality Control, [3]Physical-Chemistry.
Center for Genetic Engineering and Biotechnology, PO Box 6162, Havana 10600, Cuba.
Fax: (53-7) 21 8070, 33 6008; E-mail: Santana@.cigb.edu.cu

ABSTRACT

The recombinant human interferon α 2b (IFN-α_{2b}) produced by Cuban technology is obtained from *Escherichia coli*. This is the active principle of the product registered in Cuba as Heberon alfa R® trademark (IFN-α_{2b}, CIGB, Havana), which has been successfully used worldwide for the therapy of several viral diseases and neoplasms. Here we describe the purity and identity tests used for its molecular characterization. The data show a product with a well-established identity, a high purity and a specific activity higher than 1.4 x 10^8 IU/mg of proteins. We also compared the final preparation with other IFN-α_2 products available in the international market. It behaved very similar to Intron A® and Roferon A®, and showed a higher homogeneity when compared with Bioferon® and Interimmun®.

Keywords: IFN, molecular characterization, recombinant human interferon alfa 2b

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RESUMEN

El interferón α 2b humano recombinante (IFN-α_{2b}) producido mediante la tecnología cubana es obtenido a partir de *Escherichia coli.* Éste constituye el principio activo del producto registrado en Cuba con la marca comercial Heberon alfa R® (IFN-α_{2b}, CIGB, La Habana), el cual ha sido usado exitosamente en muchos lugares del mundo para la terapia de varias enfermedades virales y neoplasias. En este trabajo se describen los ensayos de pureza e identidad para su caracterización molecular. Los datos muestran un producto de identidad bien establecida, de alta pureza y de actividad específica mayor que 1,4 x 10^8 UI/mg de proteínas. Además, se comparó la preparación final con otros productos IFN-α_2 similares distribuidos en el mercado internacional. El interferón cubano mostró características muy similares a Intron A® y Roferon A®, y mayor homogeneidad cuando se comparó con Bioferon® e Interimmun®.

Palabras claves: caracterización molecular, IFN, interferón alfa 2b humano recombinante

*I*ntroduction

Interferons (IFNs) were originally discovered because of their ability to protect cells against viral infections [1]. Several interferon types and subtypes have been identified and characterized to date. The main biological properties of IFNs are their antiviral, antiproliferative and immunomodulator effects [2-4]. These actions support the different applications of IFNs in the therapy of viral and neoplastic diseases, as well as in other conditions [5].

In Cuba, the production of natural IFN from Sendai virus-induced leukocytes from blood donors was achieved in 1981 [6]. This product was used for dengue hemorrhage fever the same year [7] and afterwards, for other applications [8]. An *Escherichia coli* strain that expresses the human IFN-α_{2b} gene was achieved by recombinant DNA techniques, and a process was designed to obtain a pure product [9-11] that has been widely used in general practice [8, 12]. This product is presented as a lyophilized powder, formulated with 1.5 mg of human serum albumin (HSA), at 1, 3, 5, 9 and 10 x 10^6 IU of IFN-α_{2b} per vial.

This work presents a detailed characterization of IFN-α_{2b} (CIGB, Havana, Cuba) using analytical methods with the sensitivity and resolution required to evidence its quality. Finally, the formulation was compared with other similar preparations available in the international market.

*M*aterials and Methods

IFN-α_{2b} was produced at the Center for Genetic Engineering and Biotechnology (CIGB) in Havana, Cuba. Commercial IFN preparations are: Intron A® (Schering-Plough Corp, NJ, USA), Roferon A® (F. Hoffmann-La Roche, Basel, Switzerland), Bioferon® (Instituto SIDUS S.A., Buenos Aires, Argentina), Interimmun® (INMUNO S.A., Buenos Aires, Argentina), and Heberon Alfa R® (Heber Biotec S.A., Havana, Cuba). All other chemicals were of analytical grade and from commercial sources.

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12.5% or 15% polyacrylamide gels, essentially as described by Laemmli [13]. Samples were diluted 1:1 in 50 mM Tris-HCl buffer (pH 6.8) containing 2% SDS, 11.6% (v:v) glycerol and 0.001% bromophenol blue with (reduced) or without (non-reduced) 1% (v:v) β-mercaptoethanol. The samples were heated in a boiling waterbath for 3 min and loaded onto the gels. After electrophoresis, proteins were visualized using silver nitrate or Coomassie® brilliant blue staining.

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✍ Corresponding author

Western blot

Following SDS-PAGE, proteins were transferred to 0.45 µm nitrocellulose membranes (Schleicher and Schüll, Germany) using a semi-dry electroblotting device (Bio-Rad, California). Membranes were blocked with 0.2% Tween 20 and then incubated for 1 h with in-house raised rabbit anti-IFN-α_{2b} serum diluted 1:10 in phosphate-buffered saline solution containing 0.2% Tween 20. Following washing with the same solution, the membranes were incubated with a protein A-peroxidase conjugate (1:2000), and stained with diaminobenzidine reagent (Sigma, St. Louis, MO).

Isoelectric focusing

Isoelectric focusing pH 4.0-7.0 (Pharmacia Immobiline DryPlate, Uppsala, Sweden) was performed in a Multiphore II system (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions, and the temperature was set to 10 °C. The isoelectric point (Ip) was estimated using a broad Ip calibration kit supplied with the gel. Proteins were visualized by silver nitrate staining.

Reverse-phase high-pressure liquid chromatography (RP-HPLC)

RP-HPLC analysis was perfomed on a Bakerbond (Phillipsburg, NJ, USA) wide pore octyl (C8) column (5 µm; 100 x 4.6 mm). Solvents and gradient were: A, 0.1% aqueous trifluoroacetic acid (TFA); B, 0.1% TFA in acetonitrile (0% B to 60% B in 50 min). The flow rate was 0.8 mL/min. Detection was performed at 226 nm with automatic data processing using BioCrom version 2.0 software (CIGB, Havana, Cuba) for data acquisition and analysis.

Amino acid analysis

IFN-α_{2b} was hydrolyzed in a previously pyrolyzed, vacuum-sealed glass vial with 100 µL of 6 N HCl containing 0.1% β-mercaptoethanol and 0.1% phenol for 24 h at 110 °C. Amino acid analysis was carried out in an automatic analyzer Alpha Plus 4151 (Pharmacia-LKB, Sweden) using the standard sodium buffer system for the analysis of protein hydrolysates and fluorescence detection with *o*-phtalaldehyde.

Peptide mapping

IFN-α_{2b} was dried in a SVC 100 (SAVANT, France) evaporator centrifuge. Dry protein (ca. 100-200 µg), was dissolved in 1% ammonium bicarbonate buffer solution pH 8.0, and digested with trypsin at a 1:100 enzyme to substrate ratio for 4 h at 37 °C. The trypsin-digested IFN-α_{2b} samples were analyzed by RP-HPLC using a Vydac (Herperia) C-18 column (250 x 4.6 mm). Solvents and gradients were: A, 0.1% aqueous TFA; B, 0.1% TFA in acetonitrile (0% B to 60% B in 120 min). Detection was performed by monitoring UV light absorbance at 226 nm. The tryptic peptides were collected and the solvent evaporated in vacuum to determine their molecular masses by mass spectrometry. Mass spectra were obtained in a JEOL HX-110HF double sector mass spectrometer equipped with a FAB ion gas, a collision source and a JEOL JMS DA-5000 data processing system. Samples were bombarded with a beam of xenon atoms (4 kV), and the generated ions —$(M+H)^+$— were accelerated to 10 kV and detected in a post-acceleration detector operated at -20 kV. The magnetic field was calibrated by using cesium iodide signals.

Circular dichroism (CD) spectroscopy

CD spectra of IFN-α_{2b} in the near- and far-UV regions were obtained in 10 mM phosphate buffer pH 8.0 with a Jasco spectropolarimeter J-710 equipped with a personal computer. Temperature was maintained using a circulating-water bath connected to a water-jacketed cell holder. The sample was scanned five times using a protein concentration of 0.1 mg/mL in a 1 mm rectangular cell. The results were expressed as residual ellipticity [θ] (deg cm^2 $dmol^{-1}$) using 116.9 as the mean residue weight, after subtracting the buffer scan, which is defined as:

where:
θ_{obs}: measured ellipticity in degrees
c: concentration in mg/mL
l: light path length in cm
MRW: mean residue weight

The estimation of secondary structure composition was made by deconvolution of the CD data with the software CCA [14].

Biological activity

IFN antiviral activity was assayed by the inhibition of the cytopathic effect (CPE) produced by Mengo virus on HEp-2 cells (ATCC No. CCL23), as previously reported [15, 16]. Cell monolayers in 96-well microtiter plates were incubated for 24 h at 37 °C, under 3% CO_2 and 95% humidity, with IFN samples (serially diluted 1:2 in minimum essential medium with 2% fetal calf serum, 40 µg/mL gentamicin). Virus (10^7 TCID) was then added to each well and incubation proceeded under the same conditions until CPE (90% cell lysis) was evident (approx. 18-20 h) in the control virus wells (incubation without IFN). The degree of cell destruction was measured by fixing and staining the remaining cells with crystal violet. Cytopathic effect was determined using the plate photometer of an ultramicroanalytical system device (Tecnosuma, Havana, Cuba). A validated software was used to convert the raw non-linear sigmoid data to linear regression through a probit transformation. The unit of antiviral activity is defined as the reciprocal of the sample dilution that yields a 50% protection of cells from the virus CPE. The potency of each sample was expressed in IU by comparison to a secondary reference material calibrated against the 69/19 international WHO IFN standard.

*R*esults and Discussion

Molecular characteristics of IFN-α_{2b} raw active material

IFN-α_{2b} is constituted by 165 amino acids with a secondary structure characterized by two intramolecular disulfide bonds, Cys1-Cys98 and Cys29-Cys138 [17]. This protein is produced in transformed *E. coli* as intracellular insoluble aggregates, which protect it against proteolytic degradation and make purification easier.

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During extraction and immunopurification, different forms of IFN-α_{2b} can be obtained: acetylated, with N-terminal methionine, oxidized amino acids, scrambled disulfide bond species, with partially or completely reduced cysteines and molecular aggregates. Some of these molecular species have a lower specific activity [18] and could be responsible for adverse reactions and immunogenicity. The purification procedure that we have developed removes all the abnormal forms of IFN-α_{2b}, as shown below.

SDS-PAGE analysis. A typical SDS-PAGE of IFN-α_{2b} is shown in Figures 1A and B, under reducing and non-reducing conditions, respectively. Since as much as 25 µg/lane were loaded, even low levels of impurities could be readily detected. The product IFN-α_{2b} demonstrated to have less than 1% impurities in non-reduced samples. In fact, the impurity content of the product is often less than 0.2% including IFN-α_{2b} dimers. These results agree with the international requirements established for IFN-α_{2b} [19]:

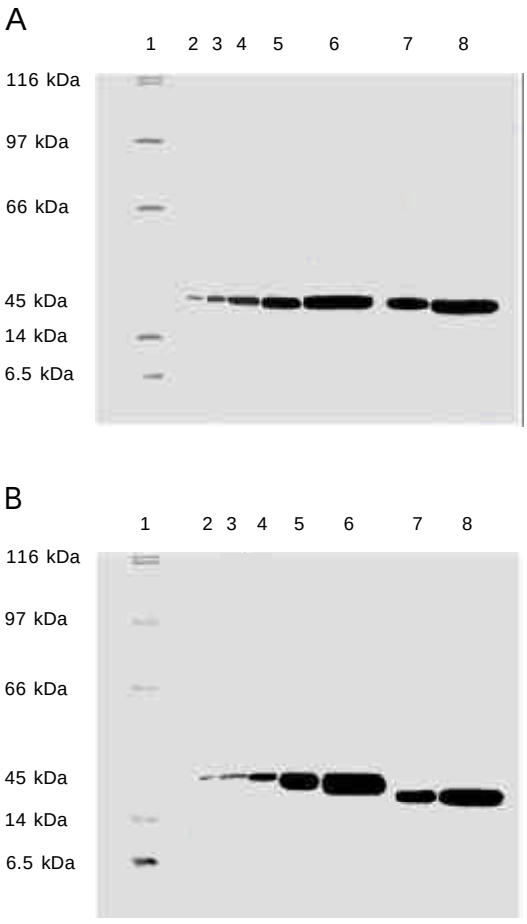

Figure 1. 15 % SDS-PAGE, silver stained. Lane 1, molecular weight markers (MW): 116 kDa (β-galactosidase), 97 kDa (phosphorilase B), 66 kDa (bovine serum albumin), 45 kDa (ovalbumin), 14 kDa (lysozyme) and 6.5 kDa (aprotinin); lanes 2-6, sensitivity curve with reference IFN-α_{2b} 0.05, 0.25, 1.25, 6.125 and 31.25 µg, respectively, all of them under reducing conditions (A and B); lanes 7 and 8, 5 and 25 µg of IFN-α_{2b}, respectively. (A) Under reducing conditions. (B) Under non-reducing conditions.

- The test is not valid unless the molecular weight marker is distributed along 80% of the gel length.
- The mobility of IFN-α_{2b} must be within 300 Da of the reference standard.
- A band should be seen in the electrophoretogram obtained with 0.05 µg of the reference preparation.
- A gradation of staining intensity should be seen in the electrophoretograms obtained with the sample (5 and 25 µg) and with the reference preparation (from 0.05 to 31.25 µg).

Stringent specifications have been established for the purity of IFN-α_{2b} on SDS-PAGE [19]. These specifications apply to reduced as well as non-reduced samples:

- There should be no more than 2% of the total impurities by SDS-PAGE.
- No impurity band should be 1% more intense than the principal band and no more than three such bands should be 0.2% more intense than the principal band.
- The amount of IFN-α_{2b} dimer should not be more than 1% in the non-reduced sample.

Isoelectric focusing analysis. An IFN-α_{2b} isoform with correct disulfide bonds (Cys1-Cys98 and Cys29-Cys138, called IFM1) and identical conformation as the native molecule, has been reported. They are indistinguishable in 15% SDS-PAGE, both under reducing and non-reducing conditions. However, they have been distinguished by isoelectric focusing with isoelectric points of 5.85 and 6.06 for IFM1 and the homogeneous native IFN-α_{2b} (IFM2), respectively [20].

Figure 2 shows the analysis performed on IFN-α_{2b}. Only one band corresponding to IFM2 isoelectric form appeared, discarding the presence of IFM1, deamidation of asparagine or glutamine residues or other modifications.

RP-HPLC analysis. A typical RP-HPLC analysis of IFN-α_{2b} is shown in Figure 3. The chromatogram shows a single and symmetric peak indicating more than 99% purity. The use of this method allows a rigorous evaluation of protein purity and homogeneity. This technique has been used for the analysis of different IFN-α_{2b} molecular forms. It has resolved

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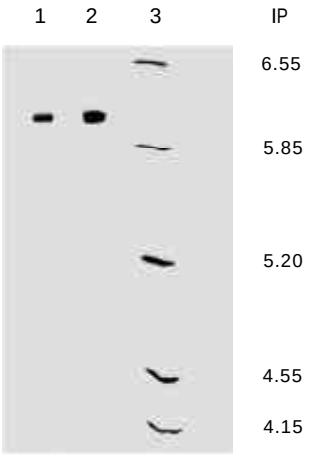

Figure 2. Isoelectric focusing of IFN-α_{2b} (CIGB) as described in Materials and Methods. Lanes 1 and 2, 5 and 10 µg of IFN-α_{2b} (CIGB), respectively; lane 3, isoelectric point marker (IP) for the valves, 6.55, 5.85, 5.20, 4.55, 4.15 (the most alkaline is near the application point).

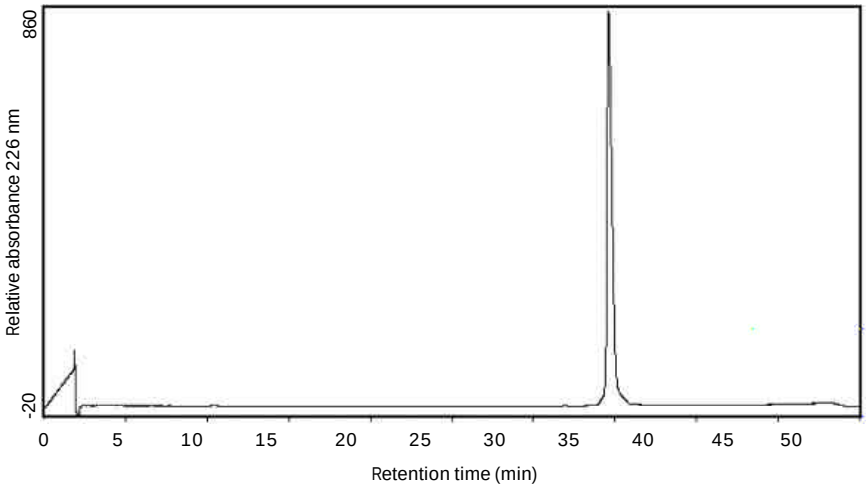

Figure 3. Figure 3. RP-HPLC analysis of recombinant IFN-α_{2b} preparations was performed using a Bakerbond wide pore octyl (C8) column equilibrated with solution A (0.1% TFA). Elution was carried out with solution B (0.1% TFA in acetonitrile) from 0% to 6% (v:v) in 50 min.

acetylated, oxidized, and scrambled disulfide bond species [21].

Amino acid analysis. It is important to stress that the amino acid recoveries for a given protein depend not only on the performance of the analysis, but also on the nature of the linkages in each particular protein. Table 1 shows the typical results obtained for IFN-α_{2b}. The observed deviations for each amino acid from the theoretical values predicted from cDNA sequence remain within the range for this procedure. Also, these data are in agreement with the results previously discussed to a great extent for this molecule. The lower recovery values obtained for Ser, Ile and Met can be explained on the basis of their chemical behavior during the acid hydrolysis. Ser is partially dehydrated, Ile is one of the amino acids that can form chemically resistant bonds and Met is oxidized by the remaining oxygen present in the ampule, with a recovery usually about 50% under this procedure. The 125% of recovery obtained for Gly is in accordance with the well known abundance of background signals for this amino acid. The low recovery value of Lys has been obtained consistently for IFN-α_{2b} in our laboratory [22].

Peptide mapping. Tryptic digestion and RP-HPLC analysis of IFN-α_{2b} are shown in Figure 4. The chromatogram obtained is the same as previously reported [21]. Nevertheless, sequence verification by mass spectrometry was performed on every peak observed in the map. Mass values were determined, and assignment to each region of the molecule was done [21]. The peptides identified by m/z 1313.6 (peaks 13 and 14), corresponding to the N-terminal region, confirmed the protein integrity at this end. These values also indicate the total absence of IFN-α_{2b} species with modified *N*-terminus. Automatic sequencing was done to confirm this result and the expected amino acids in each cycle were observed, with a very good yield. It was possible to verify 98% of the amino acid sequence. Additionally, the correct formation of the two disulfide bonds (between Cys1 and Cys98 by m/z 4617.4 and 6049.4 [peaks 13 and 14, respectively], and Cys29 and Cys138 by m/z 2246.0 and 2118.0 [peaks 11 and 12, respectively]), was verified [21].

CD spectroscopy. The crystal structure of human IFN-α_{2b} has been determined recently at 2.9-Å resolu-

Table 1. Amino acid composition of IFN-α_{2b}.

Amino acid	Number of residues per molecule ± standard deviation*	Values predicted from cDNA sequence	Recovery (%)
Asp + Asn	11.82 ± 0.18	12	98.5
Thr	9.37 ± 0.27	10	93.7
Ser	11.93 ± 0.76	14	85.2
Glu + Gln	28.86 ± 0.48	26	111.0
Gly	6.24 ± 1.28	5	124.8
Ala	8.62 ± 0.37	8	109.8
Val	6.53 ± 0.21	7	93.3
Met	3.46 ± 0.24	6	57.7
Ile	6.65 ± 0.16	8	83.1
Leu	20.15 ± 0.58	21	96.0
Tyr	4.78 ± 0.44	5	95.6
Phe	9.22 ± 0.38	10	92.2
His	2.92 ± 0.24	3	97.3
Lys	6.97 ± 0.18	10	69.7
Arg	9.80 ± 0.39	10	98.0

*Average of three independent determinations.

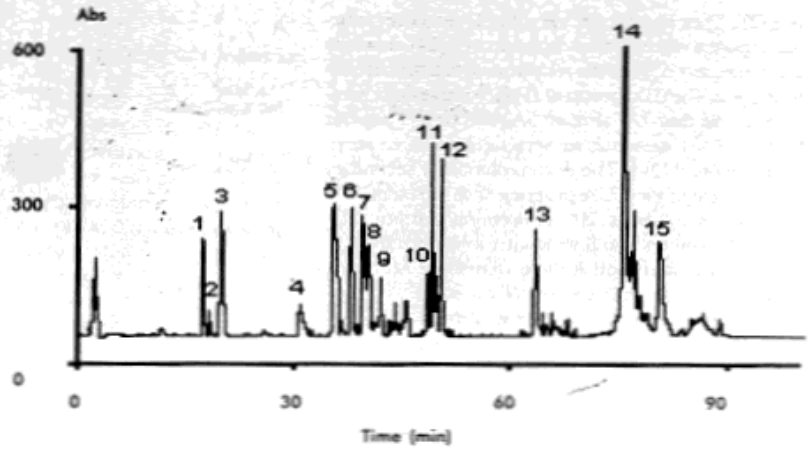

Figure 4. Tryptic digestion and RP-HPLC of IFN-α_{2b}. For details, see Materials and Methods. Mass/charge ratio (m/z) of different peaks are: 1, 613.0; 2, 618.0; 3, 740.9; 4, 902.8; 5, 750.1; 6, 1450.0; 7, 2225.8; 8, 1955.3; 9, 1481.6; 10, 1076.6; 11, 910.4, 1337.4, 2245.5, 1337.4, 2245.5; 12, 910.3, 1209.2, 2117.1; 13, 1313.3, 3303.6, 4617.1; 14, 1313.4, 4737.0, 6049.4; 15, 2459.7 [21].

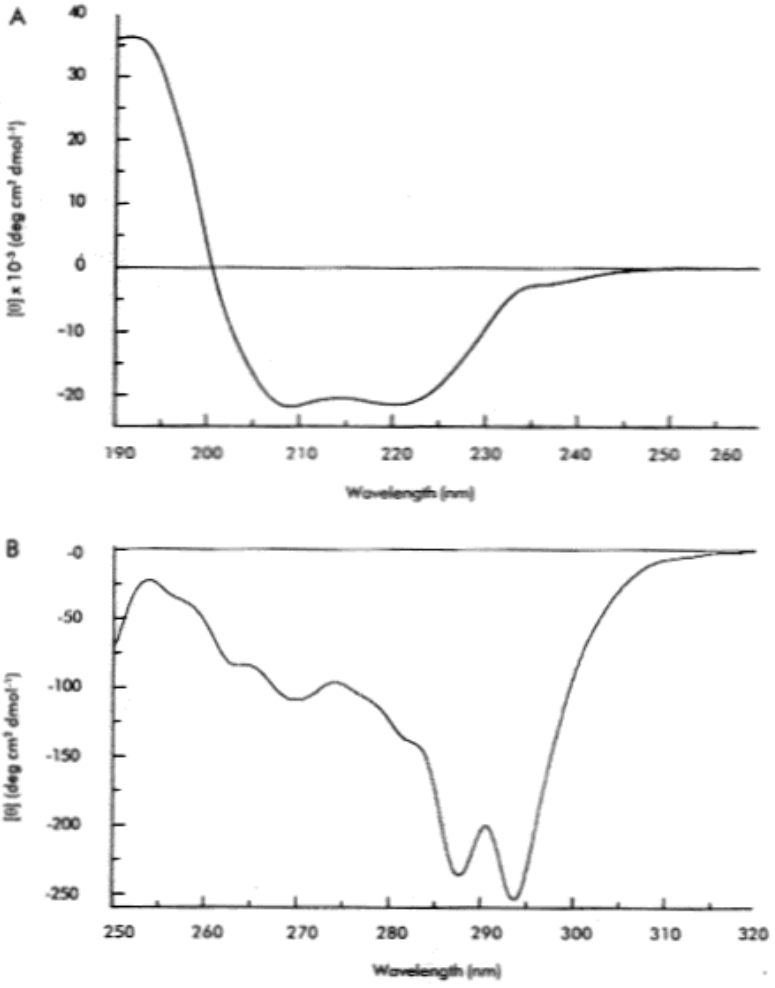

Figure 5. Circular dichroism spectra of IFN-α_{2b}. (A) Near UV region. (B) Far UV region.

tion [23]. Their results coincide with CD secondary structure reported more than fifteen years ago [24], in which approximately 65% of the residues adopt a helical conformation. The CD spectra of IFN-α_{2b} have been measured in the near-UV (Figure 5A) and far-UV regions (Figure 5B). Both signals are very similar to those previously reported [24]. The deconvolution of secondary structure using the CCA program indicates that 55% corresponds to α-helix, 20% to not organized structures and 25% to others. No β structures were detected.

Bioassay. It is well known that there are many factors that can influence IFN potency, as determined in bioassays for antiviral activity. The specific biological activity of IFN-α_{2b} was higher than 1.4×10^8 IU/mg of proteins, which fulfills the international requirements [19]. A polyclonal antibody raised against IFN-α_{2b} was used to demonstrate that the antiviral activity was indeed due to IFN-α_{2b}.

Comparison of IFN-α_{2b} formulated with other preparations

Tested IFN-α_2 products were for clinical use and were all analyzed before expiration date.

SDS-PAGE and Western blot analyses. As seen in Figure 6A, all IFN-α_2 preparations show the band corresponding to this molecule. Besides, a more intense band corresponding to HSA appears, as well as other minor bands coming from the HSA preparations. IFN-α_{2b} is formulated at 1.5 mg/mL of HSA, a concentration slightly higher than that used for Intron A®, Bioferon® and Interimmun® (1 mg/mL). Roferon A® is presented as an albumin-free formulation.

The electrophoresis did not show any difference between the products. However, a band migrating at the size of an interferon dimer could be appreciated in the case of Interimmun® by Western blot (Figure 6B). The rest of the products showed a single IFN-α_2 band at the monomer size.

RP-HPLC analysis. Bioferon® and Interimmun® yielded a small peak before the main one (Figure 7), indicating a lower homogeneity of these preparations compared to Intron A®, Roferon A® and IFN-α_{2b}, for which only one symmetrical peak was obtained.

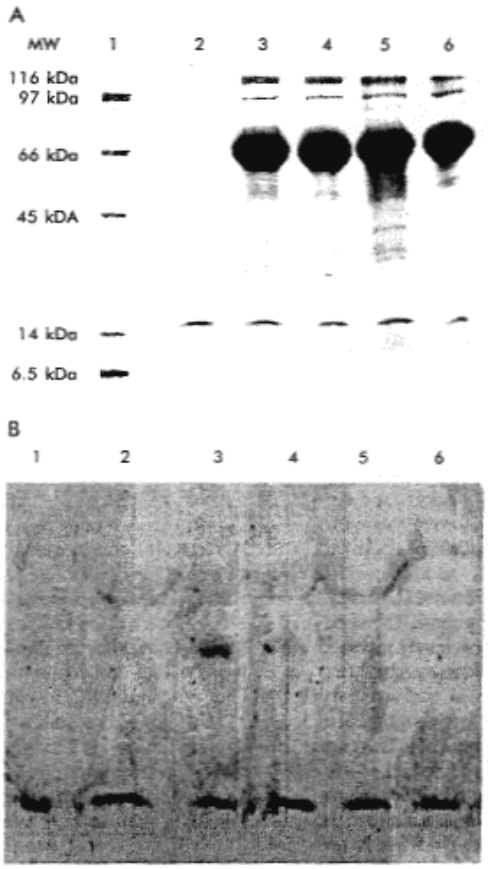

Figure 6. Comparative analysis of IFN-α_{2b} from different commercial brands. (A) 12.5% SDS-PAGE under reducing conditions, stained with Coomassie blue. Lane 1, molecular weight markers (MW): 116 kDa (β-galactosidase), 97 kDa (phosphorilase B), 66 kDa (bovine serum albumin), 45 kDa (ovalbumin), 21.5 kDa (soybean trypsin inhibitor), 14 kDa (lysozyme) and 6.5 kDa (aprotinin); lane 2, Roferon A®; lane 3, IFN-α_{2b}; lane 4, Bioferon®; lane 5, Interimmun® and lane 6, Intron A®. (B) Comparative analysis by Western blot. Lane 1, IFN-α_{2b}, active raw material; lane 2, IFN-α_{2b}; lane 3, Interimmun®; lane 4, Bioferon®; lane 5, Intron A® and lane 6, Roferon A®. Equivalent amounts of IFN-α_{2b} were applied (6×10^5 IU).

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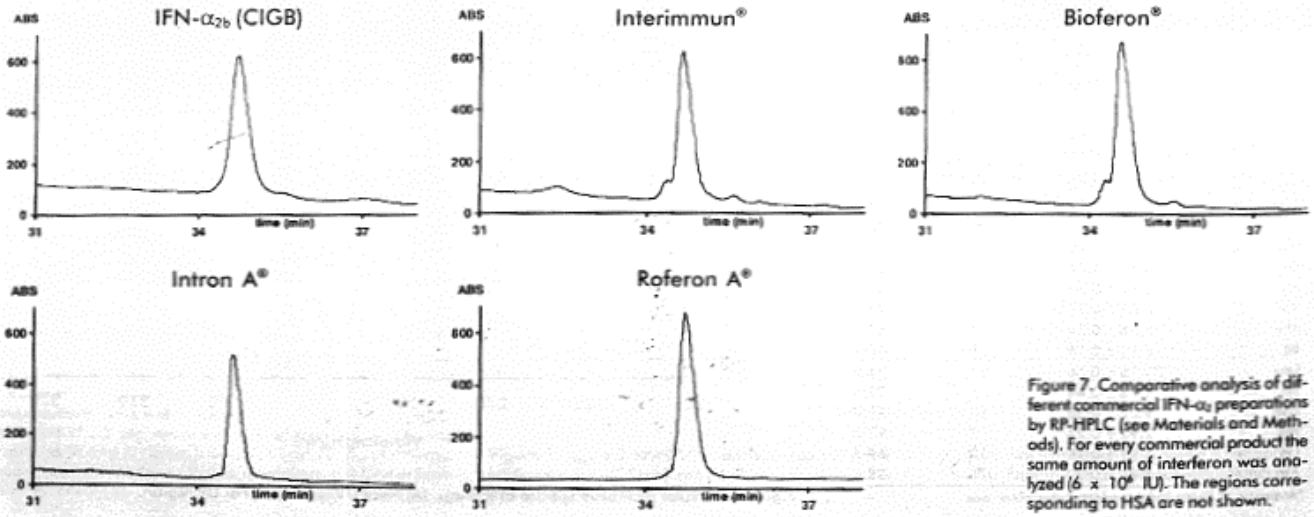

Figure 7. Comparative analysis of different commercial IFN-α_2 preparations by RP-HPLC (see Materials and Methods). For every commercial product the same amount of interferon was analyzed (6×10^5 IU). The regions corresponding to HSA are not shown.

Table 2. Bioassay comparison of different recombinant interferon-α marketed products.

Product name	Biological activity[a] (x 10^8 IU)
Intron A®	1.489-3.563
Roferon A®	2.406-4.804
Bioferon®	2.060-4.957
Interimmun®	2.298-4.182
IFN α_{2b}	2.224-3.525

[a]The 95% confidence intervals for six independent determinations.

Bioassay. The potency of the different commercial preparations was also compared through the viral CPE inhibition assay. As shown in Table 2, the specified value (3 x 10^8 IU) was included in the confidence intervals for each commercial product.

The detection of protein aggregates of IFN-α in formulations (IFN α:IFN-α and IFN-α:HSA) and their possible contribution to immunological reactions during therapy, have been recently published [25]. In Cuba, a study on the antigenicity of IFN-α_{2b} was conducted and antibody induction—all of neutralizing type—in 6.4% (19/296) of treated patients was obtained [26]. These results are similar to those previously reported for IFN-α_{2b}.

In conclusion, the results of the this study demonstrate the well characterized identity, high purity and molecular homogeneity of IFN-α_{2b} fulfilling the highest standard requirements for product quality. Comparative studies with similar products already in the market showed very similar standards in the analysis with respect to Intron A® and Roferon A®, and higher homogeneity when compared with Bioferon® and Interimmun®.

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