Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Pharmaceutics 264 (2003) 57–72
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Long-term stabilization of recombinant human interferon α 2b in aqueous solution without serum albumin

Llamil Ruiz, Nuria Reyes, Ladys Duany, Abrisleyda Franco, Kethia Aroche, Eugenio Hardy Rando*

Formulation Development Department, Center for Genetic Engineering and Biotechnology (CIGB), P.O. Box 6162, Havana, Cuba

Received 6 January 2003; received in revised form 18 April 2003; accepted 10 July 2003

Abstract

The development of parenteral solution dosage forms of interferon α 2 (rhIFN-α2) without human albumin may significantly diminish the problem of forming highly immunogenic rhIFN-α2b aggregates and the potential risk of blood-transmitted diseases caused by infectious viruses and often living pathogens that may be present in the plasma. With this purpose, we evaluated the compatibility of type I borosilicate glass vials and chlorobutyl stoppers with rhIFN-α2b in an aqueous solution. At the same time, we carried out a targeted formulation screen at 37 °C of single or combined (e.g. polysorbate 80, EDTA Na_2, PEG 400) potentially stabilizing excipients. Quantified biochemical results from 12 independent batches of rhIFN-α2b in a polysorbate/benzyl alcohol-based vehicle formulated at pH 7.4 were all found within the limits established by the World Health Organization for this cytokine. Real-time storage data confirmed the excellent biochemical long-term (30 months) stability of rhIFN-α2b in this aqueous solution formulation. Analyses were performed at intervals throughout the time period using reverse-phase high-performance liquid chromatography, a sandwich-type enzyme-linked immunosorbent assay, and antiviral activity as stability-indicating assays. Furthermore, both the physical stability (color, odor, appearance, pH, and absence of particulate material) and the sterility of this formulation were maintained under the proposed shelf conditions.

Keywords: Interferon; Stability; Excipient; Formulation; Degradation pathway

1. Introduction

Interferons have proven to be highly active against a variety of RNA and DNA viruses, exhibiting potent immunomodulatory effects (e.g. the regulation of NK cell activity and the modulation of the expression of MHC-encoded proteins), and having antiproliferative activity against malignant cells (Zoon et al., 1986; Bordens et al., 1997). Due to this broad spectrum of biological activities, many potential therapeutic uses for interferons have been investigated. In particular, genetically engineered interferon α 2 (rhIFN-α2) produced in recombinant strains of microorganisms (e.g. *Escherichia coli*) is currently used worldwide for the therapy of various cancer and chronic viral diseases. Some of the most rhIFN-α2-treated diseases are leukemia (Mahon et al., 1998), multiple myeloma (Kirkwood, 2002), carcinoma (Marshall et al., 1996), and hepatitis B (Bayraktar et al., 1996) and hepatitis C (Solinas et al., 1993; Davis et al., 1998), with or without other complementary drugs.

* Corresponding author. Fax: +537-2714764/336008.
E-mail address: ehardy@cigb.edu.cu (E.H. Rando).

0378-5173/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0378-5173(03)00388-0

One of the most widely used pharmaceutical dosage forms of rhIFN-α2 consists of a glass vial containing minimal amounts (from 0.1×10^6 to 100×10^6 IU/ml) of the active ingredient lyophilized in the presence of an excess of human serum albumin (HSA) (Kato et al., 1987; Kwan, 1985). The role of HSA in this pharmaceutical preparation has been to protect rhIFN-α2 against known potential chemical degradation mechanisms (e.g. proteolysis, oxidation, or deamidation) and physical influences (aggregation, precipitation, or adsorption). Pharmaceutical preparations of HSA-stabilized rhIFN-α2 in its lyophilized form have retained the physico-chemical and biological properties of rhIFN-α2 when stored at 2–8 °C, for at least 2 years (Gross et al., 1998; Yuen and Kline, 1998, 1999).

Unfortunately, the use of HSA to stabilize rhIFN-α2 has drawbacks: (i) rhIFN-α2 and HSA can form high molecular weight complexes which in turn may cause neutralizing antibody formations against rhIFN-α2 and (ii) blood-derived HSA is problematic in view of the potential contamination by viruses or other pathogens (Gross et al., 1998; Yuen and Kline, 1998, 1999).

Recently, new HSA-free pharmaceutically acceptable preparations of rhIFN-α2 have been described which display the bioactivity and physico-chemical stability of the active ingredient in an aqueous solution for extended storage periods (Gross et al., 1998; Yuen and Kline, 1998, 1999). In addition to rhIFN-α2, these formulations comprise other auxiliary excipients which act as stabilizers (e.g. non-ionic detergents), isotonizing agents (sodium chloride, mannitol, glycerol), preserving agents (benzyl alcohol, phenol, *m*-cresol, and parabens), and buffers providing a pH of 4.5–7.2. The excipients of these liquid formulations have been carefully selected from a variety of potentially suitable agents and have been adequately combined. In our opinion, these previous works have significantly contributed to the foundation of subsequent developments of HSA-free liquid dosage forms of rhIFN-α2, to be registered and approved by national regulatory agencies, for their use in humans.

In this paper, a preformulation study followed by the design, manufacture, and consistency assessment of a new HSA-free rhIFN-α2b liquid formulation was undertaken. Only controlled stability trials could unequivocally support a long-term expiration date of at least 30 months for this aqueous pharmaceutical dosage form of rhIFN-α2b under the appropriate storage conditions.

2. Materials and methods

2.1. Materials

The Center for Genetic Engineering and Biotechnology (CIGB, Havana, Cuba) supplied rhIFN-α2b with the following characteristics: (i) a specific activity higher than 1.4×10^8 IU/mg, (ii) a molecular weight of 19,500 Da, and (iii) an isoelectric point of 6.06. This cytokine has been demonstrated to contain 165 amino acids with a secondary structure characterized by two intramolecular disulfide bonds, Cys 1–Cys 98 and Cys 29–Cys 138 (Padrón et al., 1989). When analyzed by mass spectrometry, the peptide mapping of trypsin-digested rhIFN-α2b has shown the following mass/charge ratio (*m*/*z*) profile (Santana et al., 1999b): peak 1, 613; peak 2, 618; peak 3, 740.9; peak 4, 902.8; peak 5, 750.1; peak 6, 1450; peak 7, 2225.8; peak 8, 1955.3; peak 9, 1481.6; peak 10, 1076.6; peak 11, 910.4, 1337.4, 2245.5, 1337.4, 2245.5; peak 12, 910.3, 1209.2, 2117.1; peak 13, 1313.3, 3303.6, 4617.1; peak 14, 1313.4, 4737, 6049.4; peak 15, 2459.7. Circular dichroism (CD) spectroscopy in the near- and far-UV regions has indicated that 55% of the secondary structure of this interferon corresponds to α-helix, 20% to not organized structures, and 25% to others; no β structures have been detected (Beldarraín et al., 2001).

All chemicals used were of analytical grade. In particular, the following chemicals were purchased from Merck (Darmstadt, Germany): polysorbate 80, EDTA Na_2, polyethylene glycol 400 (PEG 400), benzyl alcohol, benzaldehyde, hydrogen peroxide, glycerol, ammonium acetate, sodium chloride, methyl paraben, propyl paraben, sodium phosphate monobasic dihydrate, and sodium phosphate dibasic anhydrous. Acetonitrile was purchased from Caledon (Georgetown, Ont., Canada) and trifluoroacetic acid was acquired from Pierce (Rockford, IL, USA).

Type I borosilicate glass vials were acquired from Nuova OMPI (Piombino Dese, Italy) and rubber stoppers plus flip-off seals were from Helvoet Pharma (Alken, Belgium).

2.2. Preformulation of rhIFN-α2b

2.2.1. Compatibility of type I borosilicate glass vials with rhIFN-α2b in an aqueous solution

rhIFN-α2b was diluted at 1.5, 3, 5, 10, 12, 18, or 24 million international units per milliter (MIU/ml) in sodium phosphate buffer (19 mM/l NaH_2PO_4 and 81 mM/l $Na_2HPO_4 \times 2H_2O$), pH 7.4, and 1 ml was dispensed in borosilicate glass vials. Each vial was sealed with a chlorobutyl stopper and a 13-mm flip-off aluminum seal. Sealed vials were stored at 4 °C for 24 h. The compatibility of glass vials with rhIFN-α2b was estimated by determining the concentration of enzyme-linked immunosorbent assay (ELISA)-quantified interferon present at 24 h of storage, compared to the initial concentration.

2.2.2. Compatibility of chlorobutyl stoppers with rhIFN-α2b in an aqueous solution

rhIFN-α2b was diluted to 3 MIU/ml, in sodium phosphate buffer, pH 7.4, or formulated as described in Table 1 (A). The interferon-containing samples of 1 ml were dispensed into borosilicate glass vials and stored at 37 °C with or without contact with the chlorobutyl stoppers. They were then analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC) on the initial day and after 3, 6, 9, 15, 21, and 30 days of storage.

Table 1
Composition of the formulations used to compare the solution stability of rhIFNα-2b at 37 °C

Components	A[a]	B[b]	C[c]	D[d]
rhIFN-α2b (MUI/ml)	3	3	3	3
Ammonium acetate (mg/ml)	–	–	0.77	–
Glycerol (mg/ml)	–	–	20	–
Benzyl alcohol (mg/ml)	10	–	10	–
Polysorbate 80 (mg/ml)	0.2	0.1	0.2	–
$NaH_2PO_4 \times 2H_2O$ (mg/ml)	2.96	–	–	2.96
$Na_2HPO_4 \times 2H_2O$ (mg/ml)	14.4	–	–	14.4
NaCl (mg/ml)	4.67	7.5	–	–
$NaH_2PO_4 \times H_2O$ (mg/ml)	–	1.3	–	–
Na_2HPO_4 (mg/ml)	–	1.8	–	–
EDTA Na_2 (mg/ml)	–	0.1	–	–
Methyl paraben (mg/ml)	–	1.2	–	–
Propyl paraben (mg/ml)	–	0.12	–	–

Formulation B was prepared as described by Yuen and Kline (1998, 1999) and formulation C as described by Gross et al. (1998).
[a] rhIFNα-2b formulation developed here.
[b] rhIFNα-2b formulation described by Yuen and Kline (1998).
[c] rhIFNα-2b formulation described by Gross et al. (1998).
[d] Control formulation.

2.2.3. Influence of additives on the stability of the rhIFN-α2b solution

2.2.3.1. The effect of selected single excipients. rhIFN-α2b was diluted to 3 MIU/ml in sodium phosphate buffer, pH 7.4, and then the following chemicals were added to the solution: polysorbate 80 (0.01, 0.02, 0.05%), EDTA Na_2 (0.05, 0.1, 0.5%), PEG 400 (0.5, 1, 2%), benzyl alcohol (0.5, 1, 2%), benzaldehyde (0.001, 0.01, 0.05%), and hydrogen peroxide (0.01, 0.1, 0.5 mM/ml). The interferon solutions containing highly pure benzyl alcohol were bubbled with gaseous nitrogen, dispensing 1 ml into borosilicate vials. They were then sealed with chlorobutyl stoppers and 13-mm flip-off aluminum seals, and stored at 37 °C. Analysis with RP-HPLC was performed at time intervals of 0, 3, 7, 14, 21, and 30 days of storage.

2.2.3.2. Influence of combined excipients on the stability of rhIFN-α2b solution. The additives which previously provided the best stability results were used to design a new albumin-free liquid formulation of rhIFN-α2b (Table 1, A). This aqueous solution formulation was then compared with other formulations established by Schering Plough (Table 1, B) and Hoffman La Roche (Table 1, C). Each bulk formulation was sterile filtered using minisart filters (0.2 μm) (Sartorius, Goettingen, Germany) before dispensing in primary packs of 3 ml type I glass vials closed with chlorobutyl stoppers/13-mm flip-off aluminum seals. Vials, stoppers, and seals were autoclaved before filling. When benzyl alcohol was used as a preservative, the formulation was bubbled with nitrogen gas during the preparation and vial filling. The vials containing the formulations with or without full contact with the stoppers were stored (in the dark) at 37 °C. The samples were taken at the given intervals, and multianalyzed by RP-HPLC, ELISA plus the antiviral assay.

2.3. Manufacturing a new albumin-free liquid formulation of rhIFN-α2b

2.3.1. Consistency of the formulation

Twelve independent, consecutive lots of the new formulation (Table 1, A) were produced at 3, 5, 6, and 10 MIU/vial of rhIFN-α2b and the consistency of the

formulation process was evaluated. To assure that the correct dose was present in each vial, we prepared every batch with a constant concentration (10 MIU/ml) and we only varied the volume dispensed in each container, which depended on the required dose. In addition, the required overfilling, determined as described in the United States Pharmacopoeia 24 (USP 24, 2000), was taken into account. Thus, batches of 3, 5, 6, and 10 MIU/vial were filled up to 350, 600, 700, and 1100 μl/vial, respectively. The manufacturing process was carried out under GMP conditions. An automatic line for vial filling and a sealing machine (Bausch Ströbel, Ilshofen, Germany) were used for the formulation-containing vials. Both the bulk formulation and the filled vials were carefully bubbled with nitrogen gas.

2.3.2. Long-term stability studies of the present formulation

Three consecutive, independent batches of this liquid formulation were produced to investigate its long-term stability. Vials were stored at 4 °C and periodically analyzed by antiviral assay (Section 2.4.1), RP-HPLC (Section 2.4.2), ELISA (Section 2.4.3), and other Section 2.4.4 described assays (sterility of vials contents, pyrogens, abnormal toxicity, organoleptic characteristics, pH) on the day they were released and after 3, 6, 9, 12, 18, 24, and 30 months of storage.

2.4. Analyses of the formulated rhIFN-α2b

2.4.1. Biological activity determination

rhIFN-α2b antiviral activity was assayed by inhibition of the cytopathic effect produced by the Mengo virus on Hep-2 cells (ATCC No. CCL23) as previously described (Ferrero et al., 1994). Briefly, cells monolayers in 96-well microtiter plates were incubated for 24 h at 37 °C, under 3% CO_2 and 95% humidity, with rhIFN-α2b samples serially diluted 1:2 (v/v) in a minimum essential medium containing 2% fetal calf serum and 40 μg/ml gentamicin. The virus (10^7 TCID) was then added to each well and the plates were incubated under the same conditions until the cytopathic effect (90% cell lysis) was evident (approximately 18–20 h) in the virus control (incubation without rhIFN-α2b) wells. The degree of cell destruction was measured by fixing and staining the remaining cells with crystal violet. A plate photometer of an ultramicroanalytical system device (Tecnosuma, Havana, Cuba) was used to accurately determine this cytophatic effect. 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 was defined as the reciprocal of the sample dilution that yields 50% protection of cells against the virus cytopathic effect. The potency of each sample was expressed in IU compared to a secondary reference material calibrated against the 69/19 International World Health Organization (WHO) rhIFN-α2b Standard.

2.4.2. Reverse-phase high-performance liquid chromatography

RP-HPLC analysis was performed on a Vydac (Hesperia, CA, USA) wide pore octyl (C8) column (5 μm; 125 mm × 4.6 mm). Solvents and gradients were, A: 0.1% aqueous trifluoroacetic acid (TFA) and B: 0.1% TFA in acetonitrile (15–60% B in 40 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) for data acquisition and analysis.

2.4.3. Enzyme-linked immunosorbent assay

This procedure was performed as previously described (Cruz et al., 1990; Santana et al., 1999a). Briefly, each well of the microtiter plate was coated with 1 μg anti-recombinant rhIFN-α2b CBIFNA 2.3 MAb (CIGB) in 0.1 ml coating buffer (0.05 M Na_2CO_3, 0.05 M Na_2HCO_3, pH 9.6) and incubated for 3 h at 37 °C. The coated plate was briefly washed twice with a washing solution (10 mM sodium phosphate and 145 mM NaCl (PBS), pH 7.2, containing 0.05% (v/v) polysorbate 20). Then, 100 μl samples were added per well at the appropriate dilution in assay buffer (PBS containing 0.5% (w/v) skim milk). After incubating for 30 min at 37 °C, the plate was washed five times with PBS/polysorbate 20. Then, 70 μl of the conjugated second antibody, anti-rhIFN-α2b CBIFNA 2.4 MAb (CIGB) labeled with horseradish peroxidase, diluted 1:6000 (v/v) in the assay buffer were added, followed by incubation at 23 °C for 1 h. The plate was washed eight times with PBS/polysorbate 20, and then incubated for 15 min at 23 °C with 100 μl of the substrate solution. The substrate solution contained 0.1 M Na_2HPO_4, 0.048 M monohydrated citric acid, pH 5.5, containing 0.45 g *o*-phenylenediamine/l

and 0.3 g H_2O_2/l. The reaction was stopped by adding 50 μl of 2.5 M H_2SO_4. Absorbance at 492 nm was measured by using a conventional plate reader. As a reference for quantification, the samples were assayed in parallel with serially diluted amounts (from 0.625 to 12.5 ng/ml) of highly pure (98% or more) RP-HPLC-purified rhIFN-α2b.

2.4.4. Other analyses

The concentration of benzyl alcohol in the final pharmaceutical dosage form of rhIFN-α2b was evaluated with the aid of a Pye Unicam PU 4550 gas chromatographer (Cambridge, UK) coupled to a Shimadzu CR 3A Chromatopac integrator (Kyoto, Japan). The working temperature was 230 °C for the column and 250 °C for both the injector and flame ionization detector. This assay was done using a Porapak Q capillary column (1.5 m × 4 mm). The concentration of benzyl alcohol in the problem formulation samples was mathematically determined against a calibration curve of benzyl alcohol prepared at 0.25, 0.5, 0.75, and 1% in the formulation buffer.

The concentration of polysorbate 80 on the final liquid formulation of rhIFN-α2b was determined on the basis of its ability to form a blue complex with a cobalt thiocyanate solution (Nonionic Surfactants as CTAS, 1989). For this, 0.5 ml of the problem samples in Eppendorf tubes were centrifuged in a vacuum drier until water was completely eliminated. Then, 100 μl of formic acid and 1 ml of ethylene dichloride were added to each sample. The mixture-containing samples were centrifuged at 600 × *g* in a Hettich Mikroliter centrifuge (Tuttlingen, Germany) for 30 min after 1 ml of cobalt thiocyanate was added to each Eppendorf tube. The aqueous phase was then discarded, and the absorbance of the organic phase was spectrophotometrically measured. Parallelly assayed samples containing serially diluted amounts (from 100 to 500 μg/ml) of polysorbate 80 were used as a reference for quantification.

Sterility, pyrogens, and pH tests were developed in agreement with USP 24 (2000). Briefly, the sterility test was based on membrane (0.45 μm of nominal porosity) filtration of the test formulation. Remaining traces of benzyl alcohol that might remain after sample filtration were removed from the membrane by washing with bacteriological peptone solution. The membranes, which were contained in two separate sterile filter vessels, were independently incubated in fluid thioglycollate medium at 30–35 °C or in tryptone soya broth at 20–25 °C for not less than 14 days. The contents of the two vessels were examined on a regular basis for signs of microbial growth (development of turbidity or surface growth) over the specified period. The requirements of the test for sterility were met only when no growth was observed. The test for pyrogens used rabbits and measured their temperature after an injection of 150,000 IU of formulated rhIFN-α2b per kg of body weight. The pH was measured on the basis of the potentiometric titration of the hydrogen ion concentration in the sample, by using an adequate electrode and a digital pH meter.

Abnormal toxicity was determined under the requirements of the British Pharmacopoeia (1998). Toxicity was qualitatively evaluated for a single formulated rhIFN-α2b dose of 600,000 IU/animal (OF1 mice and Hartley guinea pigs).

The organoleptic characteristics of the liquid formulation were verified by checking its transparency and absence of suspended solids, as usual.

2.4.5. Statistical analysis

The statistical significance of the experimental data was determined by the unpaired Student's *t*- or ANOVA tests, after a comparison of the homogeneity of variance (Bartlett test) (Sigarroa, 1985).

When $P \leq 0.05$, ANOVA test was followed by a Duncan Multiple Range test to determine the specific groups showing significant differences.

3. Results and discussion

3.1. Compatibility of the bottling material with the stability of the rhIFN-α2b solution

3.1.1. Type I borosilicate glass vials

Proteins are known to adsorb to many surfaces, such as glass containers and air/water interfaces (Wang, 1999). Surface adsorption is usually a concentration-dependent phenomenon, reaching a maximum at certain protein concentrations that may result in the loss or destabilization of proteins. Here, we did not find statistical differences between the recovery of rhIFN-α2b in the solution-containing vials at the initial time and after 24 h of storage (Table 2). This fact

Table 2
Compatibility of borosilicate glass vials with rhIFNα-2b in solution

Concentration ($\times 10^6$ IU/ml)	Time (h)	ELISA concentration (μg/ml)	Recovery (%)	α (0.05)
1	0	13.67	–	
	24	13.43	98.27	0.80
3	0	30.57	–	
	24	31.31	102.44	0.78
6	0	47.57	–	
	24	45.02	94.65	0.40
12	0	118.53	–	
	24	118.92	100.33	0.96
18	0	168.20	–	
	24	172.08	102.31	0.09
24	0	236.83	–	
	24	236.28	99.77	0.98

Samples were dispensed into borosilicate vials, stored at 4 °C for 24 h, and analyzed by ELISA.
Differences between 24 h and time 0 results were considered significant at $P < 0.05$. α (0.05) was determined for primary data. Experiments were done in triplicate.

clearly indicated the absence of rapid adsorption on the glass wall or any other degradation route generally induced at interfaces. The ELISA technique used in this experiment has proven its ability to quantify the correctly folded interferon and recognizes when degraded species of rhIFN-α2b coexist in the analyzed sample as well (Santana et al., 1999a), enhancing the reliability of this observation. Therefore, the use of additives to avoid vial–rhIFN-α2b interactions and prevent the early loss of the active principle might not be necessary, instead, it would only be needed to guarantee its stability during storage or against protein dilution as discussed later.

3.1.2. Chlorobutyl stoppers

Before evaluating the compatibility of stoppers with rhIFN-α2b and the effect of various additives (see the following description) on the stability of this cytokine solution, the main degradation products formed during storage were determined. After analyzing cytokine damage, a suitable formulation could be designed and optimized for rhIFN-α2b stability by rationally choosing excipients. The protein degradation at 37 °C was followed with the aid of analytical RP-HPLC, a technique capable of separating modified forms of interferon involving oxidation, crosslinking, disulfide scrambling, or deamidation. Consequently, any changes in retention time or the appearance of the additional peaks as compared to controls (samples without contact, Table 1, D) were used to monitor any occurrence of chemical instability.

The major degradation products detected under our experimental conditions (3 MIU/ml interferon in 100 mM sodium phosphate buffer at pH 7.4, incubated at 37 °C) were an early eluting species (retention time (RT) of 32.6 min) and a moiety eluting just after the rhIFN-α2b main peak (RT of 34.7 min). The exact chemical identity of the early and later fractions remains under investigation and will be published elsewhere.

The effect of stoppers or stabilizing additives (see the following description) combined with storage temperature (37 °C) and length of storage (up to 30 days) was estimated by (i) calculating the area under the curve of the main native rhIFN-α2b peak (RT of 34.1 min) and (ii) measuring the relative area of the main native interferon peak. Hereinafter, the former parameter will be referred as the concentration of native rhIFN-α2b. These two parameters offer an idea of the true concentration of unmodified interferon in the sample and its purity, respectively. The acceleration of the thermal inactivation of rhIFN-α2b resulted in an experimentally convenient decline of both parameters with time to provide a wider scope for the comparison of excipients as compared with similar studies at a lower temperature (e.g. 4 °C). It may be expected that the decomposition kinetics of rhIFN-α2b like those of a variety of proteins does not follow a unimolecular rate-determining step, given the multitude of interdependent protein decomposition pathways, as well as the general complexity of decomposition kinetics in aqueous solution. Nevertheless, to quantitatively compare the difference in stability of the various rhIFN-α2b preparations, we speculatively adjusted the thermal inactivation of rhIFN-α2b under the experimental conditions described in this work, to a first order kinetic model. Thus, the kinetics (k_{obs} and half-life) constants presented later were calculated from linear relationships (r^2 between 98.24 and 99.99) between the logarithms of residual relative purity or concentration of native rhIFN-α2b by RP-HPLC and time.

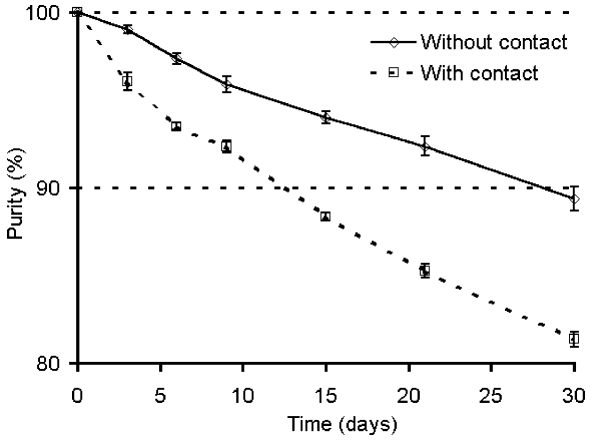

Fig. 1. Effect of rubber chlorobutyl stoppers on the solution stability of rhIFNα-2b. Samples were dispensed into borosilicate glass vials, stored at 37 °C with or without contact with gum stoppers, and the purity was determined by RP-HPLC. Details as described in Section 2.

As shown in Fig. 1, the stability by RP-HPLC of the samples in contact with the stoppers notably decreased as compared to that of rubber stopper-free samples. The accelerated formations of pre-shoulder- and post-main-rhIFN-α2b native peak by-products were observed. The corresponding kinetic parameters are shown in Table 3. Although the decomposition effect was the same for both purity and area of the native rhIFN-α2b-related chromatographic curve (Table 3), it was remarkably greater with time for this latter parameter. This trend, which was seen throughout all the kinetic analysis, might reflect the additional occurrence of degradation routes affecting the concentration of native rhIFN-α2b other than those affecting its purity. The contact with stoppers increased the apparent degradation rate constant and decreased the half-life of interferon by about 1.8-fold for purity and 1.4-fold for the concentration of native rhIFN-α2b in comparison to control on Table 1 (D).

The stopper-induced degradation mechanism is not clear as yet, though two factors may account for this: the possible release of heavy metal ions which are used during the vulcanization of the rubber for the chlorobutyl stoppers, and other less clear degradation routes induced at the rubber–liquid interface. Speculatively, in line with this we have observed a reduction of interferon degradation after the use of either EDTA Na_2 (discussed as follows), a very well-known chelator agent, or polysorbate 80 (see the following description).

Although the influence of chlorobutyl stoppers on the stability of proteins in solution has been less studied than the stability of proteins in a freeze-dried state (Earle et al., 1992), it seems that this degradation pathway must be carefully taken into account during the development of any type of interferon-based liquid formulation.

3.2. Role of single excipients on rhIFN-α2b stabilization

The influence of pH and buffer composition of the medium on the stability of rhIFN-α2b has been previously studied (Gross et al., 1998; Yuen and Kline, 1998). Accordingly, we theoretically selected an appropriate vehicle of pH around 7.4 which is based on phosphate, for further screening studies with stabilizing additives.

3.2.1. Polysorbate 80

Two mechanisms that explain the effect of non-ionic surfactants, including polysorbate 80, on the

Table 3
Kinetic parameters of rhIFNα-2b thermal stability with or without contact with gum stoppers

Condition	Purity		Area[a]	
	$k \times 10^3$ (day^{-1})	$t_{1/2}$ (day)	$k \times 10^3$ (day^{-1})	$t_{1/2}$ (day)
With contact	6.6 ± 0.1	105 ± 2.9	41.4 ± 0.4	17 ± 1.2
Without contact	3.7 ± 0.7	187 ± 3.5	30.0 ± 0.7	23 ± 1.2

Samples were dispensed into borosilicate glass vials, stored at 37 °C with or without contact with gum stoppers, and analyzed by RP-HPLC. Details as described in Section 2.

The results are expressed as mean ($n = 3$) ± S.D.

[a] Determination of the recovery area under the native rhIFNα-2b main peak as determined by RP-HPLC.

preservation of proteins against physical and chemical destabilization have been described (Bam et al., 1998; Wang, 1999). The first one is based on the competition with protein for adsorption on various interfaces, such as air–solution or vial–solution, protecting against denaturation and aggregation at these interfaces. The second one involves specific interactions with the surface of the protein, covering hydrophobic sites where aggregation could occur, or acting as "chaperonins" to catalyze refolding of partially unfolded proteins.

Here, incorporation of polysorbate 80 improved solution stability of rhIFN-α2b at 37 °C (Fig. 2). Its impact on the degradation rate was significantly higher when 0.01/0.02% of the detergent was used. The half-life kinetic parameters in Table 4 (rows 1–3) revealed that 0.05% polysorbate 80 had the lowest impact in stabilizing rhIFN-α2b against degradation. Nonetheless, even under this condition, polysorbate 80 influenced a decrease in the degradation rate of about 1.3-fold for purity and 2-fold for the concentration of native rhIFN-α2b, as compared to the control on Table 1 (D).

On the other hand, although polysorbate 80 appears to be a useful stabilizer, it seems clear that the use of this detergent in high concentrations may restrain the remaining peroxides which can accelerate degradation and protein damage (Hora et al., 1992). Both, this previous information and our results have led us to choose 0.02% polysorbate 80 for further formulation studies with rhIFN-α2b.

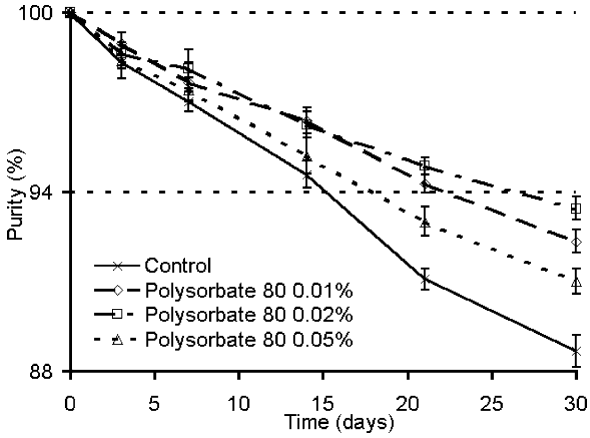

Fig. 2. Effect of polysorbate 80 at three concentrations on the stability of rhIFNα-2b. Samples were dispensed into borosilicate glass vials, stored at 37 °C, and the purity was determined by RP-HPLC. Details as described in Section 2.

3.2.2. EDTA Na_2

This chelating agent has sometimes been used as a stabilizer in protein formulations, including interferons (Tsai et al., 1993), basically for the inhibition of oxidation during the removal of metal ions, such as copper, iron, and others. In certain cases, however, the effect of EDTA Na_2 is more complex and may destabilize a protein by binding to its critical metal ions, increasing degradation or significantly changing the distribution of oxidation of protein-derived products (Wang, 1999). In this study, the inclusion of EDTA

Table 4
Kinetic parameters of rhIFNα-2b thermal stability in the presence of different additives

Condition	Concentration (%)	Purity		Area[a]	
		$k \times 10^3$ (day^{-1})	$t_{1/2}$ (day)	$k \times 10^3$ (day^{-1})	$t_{1/2}$ (day)
Polysorbate 80	0.01	2.6 ± 0.6	267 ± 4.0	11.8 ± 0.7	59 ± 1.8
Polysorbate 80	0.02	2.2 ± 0.1	315 ± 3.9	8.2 ± 1.4	85 ± 4.0
Polysorbate 80	0.05	3.1 ± 0.1	224 ± 2.7	14.4 ± 2.1	48 ± 3.0
EDTA Na_2	0.05	2.5 ± 0.2	277 ± 5.6	22.8 ± 2.8	30 ± 2.3
EDTA Na_2	0.1	2.0 ± 0.3	347 ± 4.5	18.7 ± 0.7	37 ± 2.0
EDTA Na_2	0.5	1.8 ± 0.2	385 ± 3.3	17.2 ± 3.5	40 ± 1.9
PEG 400	0.5	18.3 ± 0.9	39 ± 3.2	42.6 ± 2.1	16 ± 2.4
PEG 400	1.0	28.1 ± 2.0	25 ± 4.0	55.5 ± 2.8	12 ± 1.4
PEG 400	2.0	60.8 ± 2.7	11 ± 2.1	84.9 ± 3.5	8 ± 1.5
Control	–	4.0 ± 0.3	173 ± 4.3	28.8 ± 2.1	24 ± 2.4

Samples were dispensed into borosilicate glass vials, stored at 37 °C, and analyzed by RP-HPLC. Details as described in Section 2. The results are expressed as mean ($n = 3$) ± S.D.

[a] Determination of the recovery area under the native rhIFNα-2b main peak as determined by RP-HPLC.

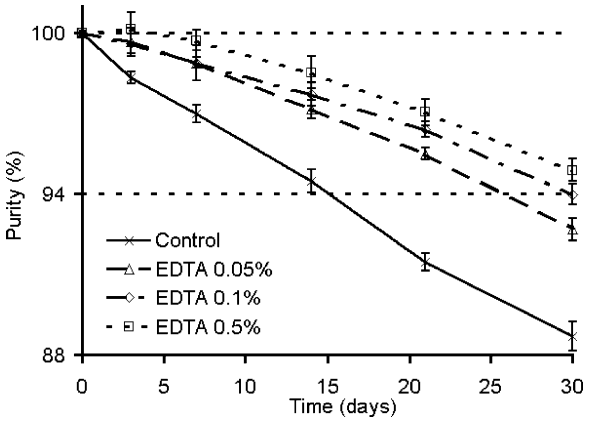

Fig. 3. Effect of EDTA Na_2 at three concentrations on the solution stability of rhIFNα-2b. Samples were dispensed into borosilicate glass vials, stored at 37 °C, and the purity was determined by RP-HPLC. Details as described in Section 2.

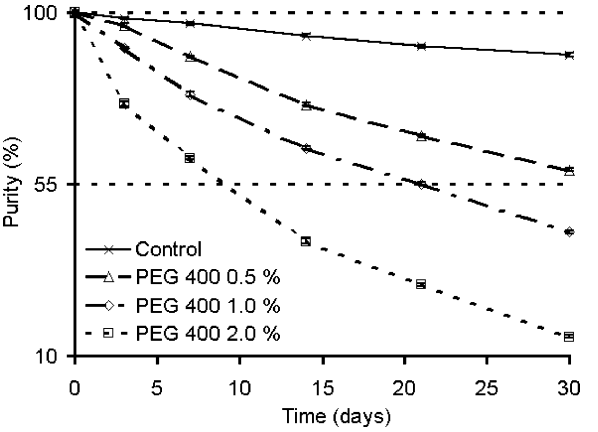

Fig. 4. Effect of PEG 400 at three concentrations on the stability of rhIFNα-2b. Samples were dispensed into borosilicate glass vials, stored at 37 °C, and the purity was determined by RP-HPLC. Details as described in Section 2.

Na_2 offered protection against the formation of degraded interferon by-products at 37 °C (Fig. 3). In contrast to the trend shown by polysorbate 80, the solution stability of rhIFN-α2b regularly augmented by increasing the concentration of EDTA Na_2. This was clearer for the recovery of the native interferon-related main peak (Table 4, rows 4–6), which expressed a minor effect when using the lowest EDTA Na_2 concentration. Kinetic analyses showed that 0.5% EDTA Na_2 reduced the degradation rate and promoted the half-life of rhIFN-α2b in approximately 2.2-fold for purity and 1.7-fold for the concentration of the cytokine, as compared to the control on Table 1 (D). Under these experimental conditions, the role of EDTA Na_2 may have been to capture trace amounts of metal ions in solution, which would otherwise accelerate several protein degradation reactions (e.g. oxidation) and thus decrease stability (Cholewinski et al., 1996).

3.2.3. *Polyethylene glycol 400*

This polymer has been used to stabilize certain proteins by a preferential exclusion mechanism. According to this, PEG 400 should be preferentially excluded from the protein surface, affecting the free energy of the system and therefore leading from denatured forms of the protein to the native one (Carpenter et al., 1990, 1993; Arakawa et al., 1991). In this study, in contrast with polysorbate 80 or EDTA Na_2, the incorporation of PEG 400 did not improve the solution stability of rhIFN-α2b at 37 °C (Fig. 4). Instead, the rhIFN-α2b contained in this polymer was rapidly degraded and destabilized through the formation of both the pre-shoulder peak (probably Met sulfoxide by-products) plus an additional unidentified, more hydrophobic form of rhIFN-α2b by RP-HPLC (Fig. 5). According to the parameters evaluated (purity and concentration of the native rhIFN-α2b), degradation rate constant values (Table 4, rows 7 and 8) were about 4.4- to 14.8-fold and 1.5- to 2.9-fold higher, respectively.

It seems that factors other than the presence of PEG were present. Considering the high excipient to interferon ratio used in our experiment, it may be possible that traces of contaminant peroxides in the mixture

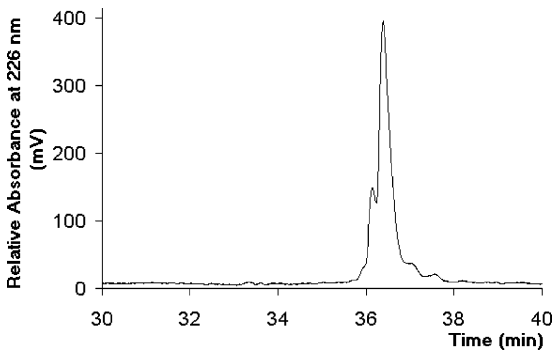

Fig. 5. Effect of PEG 400 at 1% on the RP-HPLC profile of rhIFNα-2b after 7 days of storage at 37 °C.

of PEG 400 and interferon in solution could have affected the solution stability of rhIFN-α2b. Johnson and Taylor (1984) studied the effect of PEG 400 on the oxidation of fenprostalene. They found that the degradation of this drug is closely related to the autoxidation of PEG 400 in the presence of oxygen, and determined that the use of antioxidants is very effective in retarding the rate of degradation. Nevertheless, in view of the present results we have absolutely discarded PEG 400 from the development of a long-term stable liquid formulation of rhIFN-α2b.

3.2.4. Benzyl alcohol

Because it arrests the growth of bacteria, benzyl alcohol is pharmaceutically useful in preserving a variety of drug-based medications. Its metabolism to benzoic acid in the human body is well determined (LeBel et al., 1988). Also, the toxicity (e.g. carcinogenicity, genotoxicity, acute, short-term and long-term toxicity), general biology, and clinical assessment of safety of this alcohol have been studied extensively (Nair, 2001, and citations therein). In particular, it has been found that when intravenously administered in excessive amounts (100–400 mg/kg/day), benzyl alcohol may be associated with severe toxicity (e.g. cardiovascular collapse, gasping respirations, metabolic acidosis, hematological abnormalities) in newborns which is sometimes fatal (Brown et al., 1982; Gershanik et al., 1982; Jardine and Rogers, 1989).

However, most drugs that have low doses of this alcohol are not suspected of causing complications in older infants, children, or adults (US Food and Drug Administration (US FDA), 1989; American Academy of Pediatrics Committee on Drugs, 1997). Even more, benzyl alcohol has been increasingly described as an effective local anesthetic when it has been intramuscularly injected to patients (Wilson and Martin, 1999; Bartfield et al., 2001). Given this, this agent at a concentration generally ranging from 0.5 to 3% (w/v) remains to be used as a bacteriostatic preservative in drug formulations for parenteral therapy (Powell et al., 1998), though it is not recommended for use in newborns.

Benzyl alcohol can adversely interact with proteins (e.g. rhIFN-γ) in a formulation-dependent manner (Lam et al., 1997). Here, we found that certain batches of this bacteriostatic agent have the ability to produce a deleterious effect on the solution stability of rhIFN-α2b, causing the rapid formation of a more hydrophilic form of the protein (Fig. 6A). The presence of aldehydes (e.g. benzaldehyde) contaminating the solution of benzyl alcohol and interferon might account for this fact, since the same hydrophilic form of rhIFN-α2b was also induced by its influence (Fig. 6B). The nature of this early eluting degradation form was further verified by the RP-HPLC analysis of rhIFN-α2b incubated at 37 °C with hydrogen peroxide. This oxidizer induced the same highly hydrophilic form of rhIFN-α2b, indicating the oxidized nature of the pre-main peak eluting moiety of interferon (Fig. 6C). In addition, this degraded form had a similar retention time to the one we previously detected using PEG 400. Thus, this may be a confirmation of the oxidized nature of the more hydrophilic species obtained with this polymer.

It should be noted that the British Pharmacopoeia has established that benzyl alcohol intended for the production of parenteral dosage forms must contain no more than 0.05% of benzaldehyde (British Pharmacopoeia, 1998). In our case, however, rapid oxidation-related degradations appeared in the rhIFN-α2b solution prepared and stored for 7 days at 37 °C with even lower concentrations of benzaldehyde (0.01%) (Fig. 6C). Thus, the need for using an ultrapure benzyl alcohol to formulate rhIFN-α2b in solution was experimentally identified as a critical point.

3.3. Stabilization of rhIFN-α2b by combined excipients

Based on the abovementioned results, we designed a liquid formulation which was finally composed of rhIFN-α2b diluted to 10 MIU/ml, sodium phosphate buffer to render a physiological pH (7.2–2.6), polysorbate 80 at 0.2 mg/ml, highly pure benzyl alcohol at 10 mg/ml, and sodium chloride at 4.9 mg/ml. In spite of the displayed ability of EDTA Na_2 to protect rhIFN from degradation, we did not believe it necessary to replace polysorbate 80 with this chelating agent in the design of our long-term stable formulation. Indeed, polysorbate 80 did show a better RP-HPLC-generated chromatogram peak recovery for native interferon, which could be related to its outstanding detergent properties, which was not shown by EDTA Na_2.

Gross et al. (1998) claim to use nitrogen during the formulation and filling process of albumin-free liquid

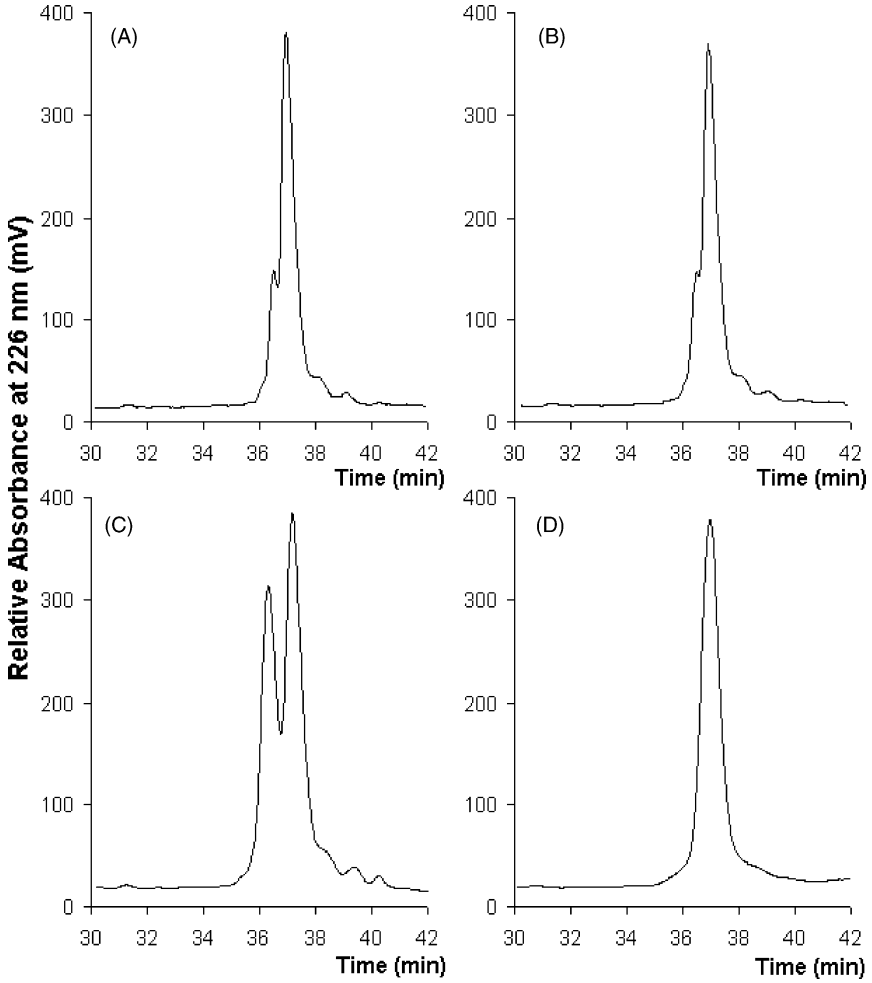

Fig. 6. Effect of stressing agents on the stability of rhIFNα-2b after 3 days of storage at 37 °C. (A) The effect of oxidized benzyl alcohol; (B) the effect of benzaldehyde; (C) the effect of hydrogen peroxide; (D) the compatibility of using highly pure benzyl alcohol plus gas nitrogen during the filling of vials.

formulations of interferon to prevent the possible oxidation effect of benzyl alcohol at 10 mg/ml, when it is used as a preservative. Taking this and our results into account, we decided to use benzyl alcohol at 10 mg/ml of the highest possible purity and fill both the bulk formulation and vials containing the rhIFN-α2b with nitrogen gas. As a consequence, the use of highly pure benzyl alcohol under these conditions did not affect the solution stability of rhIFN-α2b (Fig. 6D).

With a new formulation vehicle to be tested, our next logical step was to compare its protective effect with other widely marketed products involving similar or different types of additive mixtures. For this, we prepared our rhIFN-α2b in two other formulation vehicles described by Schering Plough (Table 1, B) and Hoffman La Roche (Table 1, C).

3.3.1. Short-term stability at high temperature

The preliminary accelerated stability analysis, carried out at 37 °C, yielded similar degradation profiles according to RP-HPLC. Although there were differences in intensity among the three formulations, they generally showed the same main degradation products eluting just after the rhIFN-α2b main peak. It might

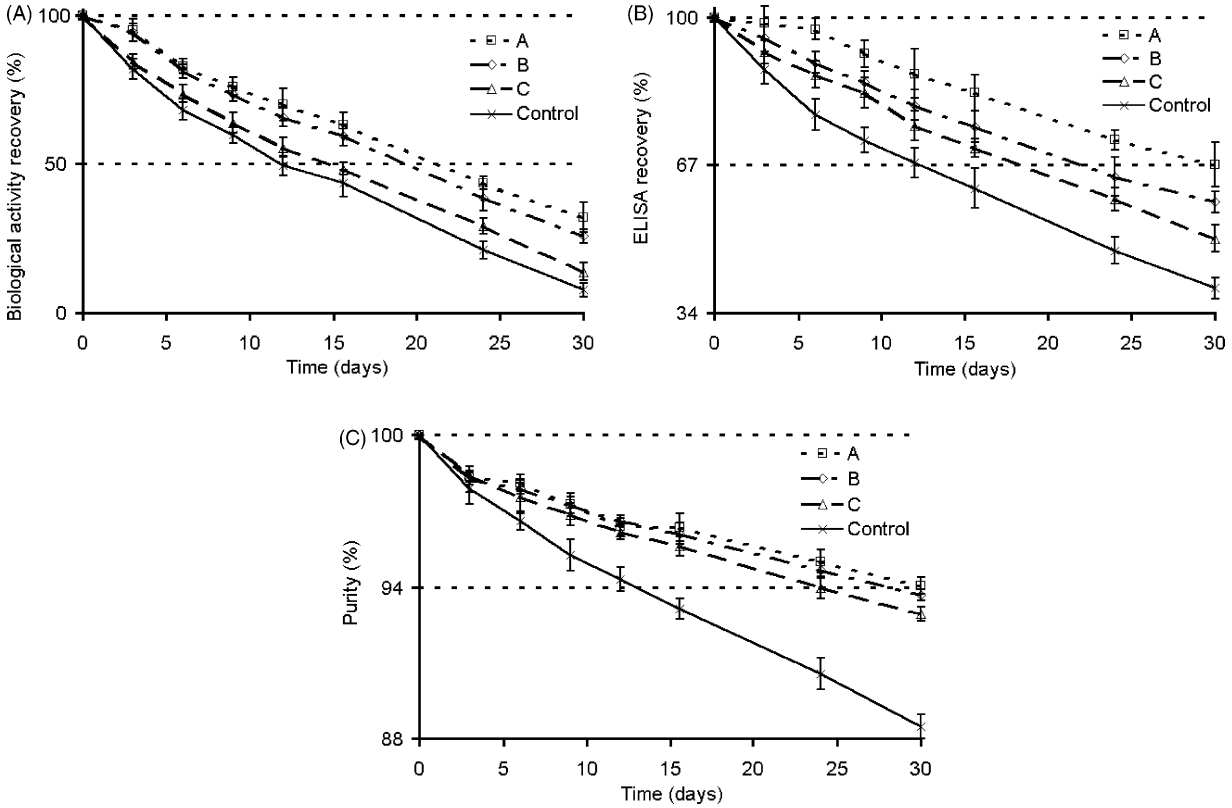

Fig. 7. Short-term stability of three different rhIFNα-2b formulations. Samples were stored at 37 °C and systematically analyzed by antiviral titration (A), ELISA (B), and RP-HPLC involving native rhIFNα-2b main peak purity (C) determination. Formulation A was developed in this work, whereas formulations B and C were prepared as described by Yuen and Kline (1998) and Gross et al. (1998), respectively.

be possible that the material balance was largely accounted for, by aggregation which is consistent with a well-known mode of interferon degradation (Wang, 1999); this requires, however, further research.

Antiviral biological assay analyses showed a clear trend for rhIFN-α2b samples to lose their activity under these accelerated storage conditions (Fig. 7A). The final concentration of rhIFN-α2b varied significantly from the original 16.12 ± 2.59 μg/ml concentration in the presence of vehicle A (Table 1), as shown with the aid of ELISA analysis (Fig. 7B). Similar results were noted with the other two aqueous formulation vehicles tested (B and C in Table 1). In general, however, these three aqueous formulations effectively protected rhIFN-α2b against degradation compared to the control on Table 1 (D) (Fig. 7A–C). This overall protective effect was clearly expressed in the absence or presence of rubber stoppers (Fig. 8), which were

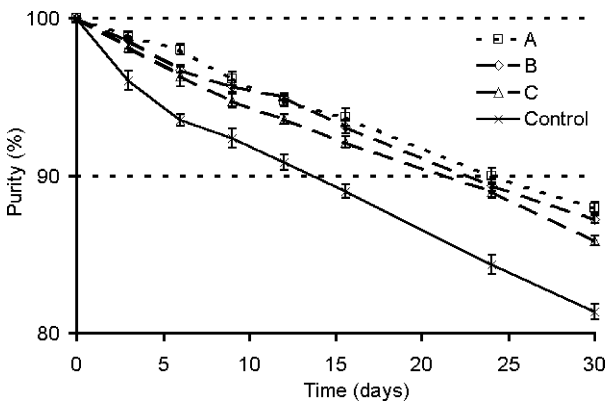

Fig. 8. Effect of three formulations on the protection of rhIFNα-2b against the influence of chlorobutyl rubber stoppers. Samples were dispensed into borosilicate glass vials, stored at 37 °C, and the purity was determined by RP-HPLC. Details as described in Section 2.

previously shown to destabilize rhIFN-α2b under our experimental conditions (Fig. 1; Table 3). In particular, the RP-HPLC- (Fig. 7C), bioactivity- (Fig. 7A), and ELISA- (Fig. 7B) generated results from ANOVA statistical analysis with $\alpha < 0.05$ suggested that our formulation preserved the integrity of rhIFN-α2b somewhat better than formulation C (Table 1, C) or the control (Table 1, D) and it was as good as formulation B (Table 1, B).

Quantitatively, the decrease of degradation rate constants (Table 5) in the presence of these three liquid vehicles were between 1.3- and 2.1-fold for ELISA analysis, between 1.3- and 2.1-fold for biological activity analysis, and between 1.7- and 2.1-fold for purity RP-HPLC-based analysis, in comparison to control on Table 1 (D). Taking into account these results, it can be expected that not only vehicles B and C but also vehicle A would be able to guarantee a long-term stability of rhIFN-α2b when stored under real refrigeration conditions (2–8 °C), as verified in the next section.

3.3.2. Manufacture and long-term stability at low temperature

Table 6 shows the Quality Control release of 12 independent batches of our liquid formulation for rhIFN-α2b. Quantified biochemical results were within the limits established by WHO for rhIFN-α2b (WHO, 1988). Moreover, these batches of an aqueous solution formulation were physiologically isotonic, according to a KNAUER Semimicro osmometer DL (Berlin, Germany) and were released as non-pyrogenic, innocuous, sterile, colorless, transparent, and absolutely free from mechanical impurities; its pH was about 7.4 during the study. In addition, polysorbate 80 and benzyl alcohol content remained between 0.01–0.03 and 0.8–1.2%, respectively, and no sign of benzaldehyde was identified during the study. These data verified that the new liquid formulation is reproducible and consistent, which is one of the most indispensable features to guarantee its large-scale production.

To be useful as a therapeutic product, this formulation must also maintain the chemical integrity of rhIFN-α2b while being in a disaggregated state during shelf storage in order to eliminate any possible adverse immunogenic effects and/or inconsistent dosing during therapy. Given this and the fact that the long-term stability of the two formulations described earlier (B and C) has been successfully demonstrated, our next logical step should be to evaluate the prolonged stability of the present liquid formulation at

Table 5
Kinetic parameters of rhIFNα-2b thermal stability in the presence of different formulations

Formulation	$k \times 10^3$ (day^{-1})	$t_{1/2}$ (day)
ELISA		
A	14.3 ± 3.54	49 ± 2.20
B	18.1 ± 2.17	38 ± 2.55
C	22.3 ± 1.40	31 ± 1.83
Control	29.9 ± 2.82	23 ± 2.51
Biological activity		
A	37.8 ± 2.12	18 ± 1.08
B	44.7 ± 4.95	16 ± 0.59
C	61.7 ± 2.83	11 ± 0.69
Control	79.4 ± 1.41	9 ± 0.65
Purity[a]		
A	1.9 ± 0.31	365 ± 3.48
B	2.0 ± 0.11	347 ± 4.07
C	2.3 ± 0.06	301 ± 3.89
Control	3.9 ± 0.15	178 ± 5.70
Area[a]		
A	16.7 ± 1.44	42 ± 1.99
B	17.7 ± 2.89	39 ± 2.70
C	19.9 ± 2.15	35 ± 0.57
Control	27.9 ± 4.24	25 ± 1.21
Purity[b]		
A	4.4 ± 0.08	158 ± 3.63
B	4.6 ± 0.83	151 ± 6.13
C	4.9 ± 0.67	142 ± 3.45
Control	6.5 ± 1.59	107 ± 4.23
Area[b]		
A	23.9 ± 2.35	29 ± 1.81
B	25.3 ± 3.07	27 ± 0.82
C	28.2 ± 3.28	25 ± 0.77
Control	40.6 ± 3.52	17 ± 1.84

Samples were dispensed into borosilicate glass vials, stored at 37 °C without contact with gum stoppers, and systematically analyzed by ELISA, antiviral titration, and RP-HPLC involving native rhIFNα-2b main peak area recovery or purity determinations. Details as described Section 2.

The results are expressed as mean ($n = 3$) ± S.D.

[a] Determinations were accomplished with samples without contact with gum stoppers and correspond to the purity and recovery area under the native rhIFNα-2b main peak, respectively, as determined by RP-HPLC.

[b] Determinations were accomplished with samples in contact with gum stoppers and correspond to the purity and recovery area under the native rhIFNα-2b main peak, respectively, as determined by RP-HPLC.

Table 6
Batch release of 12 independent liquid interferon formulation batches

Batches	Doses (MIU/vial)	Biological assay (MIU/vial)	RP-HPLC (%)	ELISA
1	3	3.41	98.25	Identified
2		3.11	97.89	Identified
3		3.24	98.38	Identified
1	5	4.68	97.91	Identified
2		5.22	98.02	Identified
3		5.61	97.35	Identified
1	6	6.88	98.01	Identified
2		7.28	98.11	Identified
3		6.28	97.38	Identified
1	10	11.68	97.88	Identified
2		10.66	97.33	Identified
3		10.59	98.31	Identified

Each batch was analyzed according to the WHO specification requirements as described in Section 2.

low temperature. It is worth noting that the aforementioned results obtained during targeted preformulation screening of the candidate stabilizing excipients allowed us to expect a long shelf life for this formulation. However, it has certainly been recognized that these type of preformulations and short-term stability studies can only generally provide the basis for starting a long-term stability evaluation, but they may fail to reflect or completely predict the desired behavior of proteins (e.g. rhIFN-α2b) under real-time storage conditions. This is due to the possible multiple protein degradation pathways at different temperatures which may limit the extrapolation of stability data from a higher to a lower temperature.

For this, three independent and consecutive batches were manufactured; using rhIFN-α2b at 10 MIU/ml. Real-time stability studies at the proposed storage condition (4 °C) was undertaken. Prior to the specific analysis of this solution formulation, the glass vial contents were examined visually at each time interval to detect any physical evidence of incompatibility. The initial examination of the formulation indicated no physical change, such as color, precipitate formation, or cloudiness, over the 30-month period. Degradation is nearly of a zero kinetic order during 30 months, which corresponds to small (<5%) levels of chemical degradation of rhIFN-α2b by RP-HPLC. No additional fragmented interferon products have, so far, been detected under these storage conditions which would have indicated the occurrence of some specific chemical instability within the formulation. Consequently, the purity of rhIFN-α2b remained above 95% throughout this study. No loss of antiviral activity was detected after this prolonged storage, remaining between 64 and 156% of its nominal value, or were then differences detected between the rhIFN-α2b contents in the glass vials. Material balances were totally accounted for, suggesting no significant adsorption of rhIFN-α2b to the surfaces of the storage containers during this study. This liquid formulation of rhIFN-α2b proved to be non-pyrogenic, non-toxic, and sterile during the present study. The results are shown in Table 7. Note

Table 7
Long-term stability of rhIFNα-2b in the present formulation vehicle

Months	Antiviral activity ($\times 10^6$ IU/ml)			RP-HPLC (purity in %)		
	Batch 1	Batch 2	Batch 3	Batch 1	Batch 2	Batch 3
0	11.8	9.5	9.8	96.0	97.3	95.5
3	12.6	11.9	12.6	94.9	96.3	95.3
6	10.5	7.4	6.5	96.3	96.1	95.9
9	12.3	10.4	7.9	94.3	95.9	96.6
12	11.1	8.80	7.7	94.5	95.9	94.5
15	11.8	11.1	9.6	96.6	96.6	96.1
18	12.1	11.0	9.8	96.6	97.2	97.2
21	11.8	9.50	10.2	95.3	95.4	95.8
24	10.3	10.9	11.2	96.5	97.8	97.4
27	12.6	11.1	10.1	96.8	96.7	95.3
30	12.0	8.30	10.2	95.0	95.6	95.1

Samples were stored at 4 °C and systematically analyzed as described in Section 2.

that all evaluated parameters were found within the limits established by numerous regulatory agencies (WHO, 1988) and chemical degradation rates were remarkably lower at this low temperature than at 37 °C. Thus, at the concentration of 10 MIU/ml for use as an injectable therapeutic agent, rhIFN-α2b was chemically and physically stable in the aqueous solution, its solubility was not compromised and it was not in an aggregated state. At the same time, these data heavily argue in favor of the good quality of the product during transportation or prolonged storage.

This formulation has additionally been evaluated in bioequivalence clinical trial studies, from which it proved to be pharmacokinetic and pharmacodynamically equivalent to the commercial INTRON A; the detailed results will be described elsewhere.

Acknowledgements

We thank the colleagues from the Interferon Production, In-process Control, and Quality Control Departments of the CIGB (Havana, Cuba) for their invaluable technical assistance and contribution to the development of a new liquid formulation of rhIFN-α2b.

References

American Academy of Pediatrics Committee on Drugs, 1997. "Inactive" ingredients in pharmaceutical products: update (subject review). Pediatrics 99, 268–278.

Arakawa, T., Kita, Y., Carpenter, J.F., 1991. Protein–solvent interactions in pharmaceutical formulations. Pharm. Res. 8, 285–291.

Bam, N.B., Cleland, J.L., Yang, J., Manning, M.C., Carpenter, J.F., Kelley, R.F., Randolph, T.W., 1998. Polysorbate protects recombinant human growth hormone against agitation-induced damage via hydrophobic interactions. J. Pharm. Sci. 87, 1554–1559.

Bartfield, J.M., May-Wheeling, H.E., Raccio-Robak, N., Lai, S.Y., 2001. Benzyl alcohol with epinephrine as an alternative to lidocaine with epinephrine. J. Emerg. Med. 21, 375–379.

Bayraktar, Y., Koseoglu, T., Somner, C., Kayhan, B., Ternizer, A., Uzunalimoglu, B., 1996. The use of deferoxamine infusions to enhance the response to interferon-α treatment of chronic viral hepatitis B. J. Viral Hepat. 3, 129–135.

Beldarraín, A., Cruz, C., Cruz, O., Navarro, M., Gil, M., 2001. Purification and conformational properties of a human interferon alpha 2b produced in *Escherichia coli*. Biotechnol. Appl. Biochem. 33, 173–182.

Bordens, R., Grossberg, S.E., Trotta, P.P., Nagabhushan, T.L., 1997. Molecular and biologic characterization of recombinant interferon-alpha2b. Semin. Oncol. 24, S9-41–S9-51.

British Pharmacopoeia, 1998. London, UK.

Brown, W.J., Buist, N.R., Gipson, H.T., Huston, R.K., Kennaway, N.G., 1982. Fatal benzyl alcohol poisoning in a neonatal intensive care unit. Lancet 29, 1250.

Carpenter, J.F., Arakawa, T., Crowe, J.H., 1990. Interactions of stabilizing additives with proteins during freeze-thawing and freeze-drying. In: International Symposium on Biological Product Freeze-drying and Formulation. Bethesda, USA. Dev. Biol. Stand. 74, 225–239 (Karger, Basel, 1991).

Carpenter, J.F., Prestrelski, S.J., Arakawa, T., 1993. Separation of freezing- and drying-induced denaturation of lyophilized proteins using stress-specific stabilization. I. Enzyme activity and calorimetric studies. Arch. Biochem. Biophys. 303, 456–464.

Cholewinski, M., Luckel, B., Horn, H., 1996. Degradation pathways, analytical characterization and formulation strategies of a peptide and a protein. Calcitonine and human growth hormone in comparison. Pharm. Acta Helv. 71, 405–419.

Cruz, S., Duarte, C., Ferra, E., Fonterroche, G., Vázquez, J., Martínez, L., Arteaga, N., Pérez, E., Gavilondo, J., 1990. Cuantificación de interferón alfa 2b humano recombinante mediante anticuerpos monoclonales. Biotecnol. Apl. 7, 132–141.

Davis, G.L., Esteban-Mur, R., Rustgi, V., Hoefs, J., Gordon, S.C., Trepo, C., Shiffman, M.L., Zeuzem, S., Craxi, A., Ling, M.H., Albrecht, J., 1998. Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. International Hepatitis Interventional Therapy Group. New Engl. J. Med. 339, 1493–1499.

Earle, J.P., Bennett, P.S., Larson, K.A., Shaw, R., 1992. The effects of stopper drying on moisture levels of *Haemophilus influenzae* conjugate vaccine. Dev. Biol. Stand. 74, 203–210.

Ferrero, J., Ochagavía, M.E., Aguilera, A., López-Saura, P., 1994. Interferon antiviral activity titration using the "SUMA" device system. Biotecnol. Apl. 11, 34–42.

Gershanik, J., Boecler, B., Ensley, H., McCloskey, S., George, W., 1982. The gasping syndrome and benzyl alcohol poisoning. New Engl. J. Med. 25, 1384–1388.

Gross, G., Del Terzo, S., Kumar, S., 1998. Stabilizer interferon alpha solutions. United States Patent 5762923.

Hora, M.S., Rana, R.K., Wilcox, C.L., Katre, N.V., Hirtzer, P., Wolfe, S.N., Thomson, J.W., 1992. Development of a lyophilized formulation of interleukin-2. Dev. Biol. Stand. 74, 295–303.

Jardine, D.S., Rogers, K., 1989. Relationship of benzyl alcohol to kernicterus, intraventricular hemorrhage and mortality in preterm infants. Pediatrics 83, 153–160.

Johnson, D.M., Taylor, W.F., 1984. Degradation of fenprostalene in polyethylene glycol 400 solution. J. Pharm. Sci. 73, 1414–1417.

Kato, Y., Hayakawa, E., Furuya, K., Kondo, A., 1987. Method for solubilization of interferon. United States Patent 4675183.

Kirkwood, J., 2002. Cancer immunotherapy: the interferon-alpha experience. Semin. Oncol. 29, 18–26.

Kwan, H.K., 1985. Biologically stable alpha-interferon formulations. United States Patent 4496537.

Lam, X.M., Patapoff, T.W., Nguyen, T.H., 1997. The effect of benzyl alcohol on recombinant human interferon-gamma. Pharm. Res. 14, 725–729.

LeBel, M., Ferron, L., Masson, M., Pichette, J., Carrier, C., 1988. Benzyl alcohol metabolism and elimination in neonates. Dev. Pharmacol. Ther. 11, 347–356.

Mahon, F.X., Faberes, C., Pueyo, S., Cony-Makhoul, P., Salmi, R., Boiron, J.M., Marit, G., Bilhou-Nabera, C., Carrere, A., Montastruc, M., Pigneux, A., Bernard, P., Reiffers, J., 1998. Response at three months is a good predictive factor for newly diagnosed chronic myeloid leukemia patients treated by recombinant interferon-alpha. Blood 92, 4059–4065.

Marshall, M.E., Wolf, M., O'Rourke, M., Barnett, T.C., Crawford, E.D., 1996. Treatment of stage D2 hormone refractory carcinoma of the prostate with 5-fluorouracil and Roferon-A: a Southwest Oncology Group Study. Cancer Biother. Radiopharm. 11, 119–123.

Nair, B., 2001. Final report on the safety assessment of benzyl alcohol, benzoic acid, and sodium benzoate. Int. J. Toxicol. 20, 23–50.

Nonionic Surfactants as CTAS, 1989. Standard Methods for Examination of Water and Wastewater, 17th ed. pp. 5.64–5.67.

Padrón, G., Besada, V., Agraz, A., Quiñonez, Y., Herrera, L., Shimonishi, Y., 1989. Spectrometric analysis of recombinant human α-2 interferon. Anal. Chim. Acta 223, 361–369.

Powell, M.F., Nguyen, T., Baloian, L., 1998. Compendium of excipients for parenteral formulations. PDA J. Pharm. Sci. Technol. 52, 238–311.

Santana, H., Espino, Y., Franco, A., Furrazola, G., Hardy, E., 1999a. A sandwich-type enzyme-linked immunosorbent assay for the analysis of recombinant human interferon α-2b. Biotech. Tech. 5, 341–346.

Santana, H., Martínez, E., Sánchez, J.C., Moya, G., Sosa, R., Hardy, E., Beldarraín, A., Huerta, V., Ferrero, J., Pujols, V., González, L.J., Betancourt, L., Besada, V., Brito, R., Currás, T., Gil, M., Herrera, L., 1999b. Molecular characterization of recombinant human interferon alpha-2b produced in Cuba. Biotecnol. Apl. 16, 154–159.

Sigarroa, A., 1985. Biometry and Experimental Design. Pueblo y Educación, La Habana, pp. 383–395.

Solinas, A., Cossu, P., Poddighe, P., Tocco, A., Deplano, A., Garrucciu, G., Diana, M.S.A., 1993. Changes of serum 2′,5′-oligoadenylate synthase activity during interferon treatment of chronic hepatitis C. Liver 13, 253–258.

Tsai, P.K., Volkin, D.B., Dabora, J.M., Thompson, K.C., Bruner, M.W., Gress, J.O., Matuszewska, B., Keogan, M., Bondi, J.V., Middaugh, C.R., 1993. Formulation design of acidic fibroblast growth factor. Pharm. Res. 10, 649–659.

US Food and Drug Administration, 1989. Parenteral drug products containing benzyl or other antimicrobial preservatives: withdrawal of notice of intent. Fed. Regul. 54, 49772.

USP 24, 2000. United States Pharmacopoeia Convention, Inc., Rockville, MD.

Wang, W., 1999. Instability, stabilization, and formulation of liquid protein pharmaceuticals. Int. J. Pharm. 185, 129–188.

World Health Organization, 1988. Requirements for Human Interferons Made by Recombinant DNA Techniques. Technical Series, No. 771.

Wilson, L., Martin, S., 1999. Benzyl alcohol as an alternative local anesthetic. Ann. Emerg. Med. 33, 495–499.

Yuen, P., Kline, D., 1998. Stable, aqueous alpha interferon solution formulations. United States Patent 5766582.

Yuen, P., Kline, D., 1999. Stable aqueous alpha interferon solution formulations. United States Patent 5935566.

Zoon, K., zur Nedden, D., Enterline, J., Manischewitz, J., Dyer, D., Boykins, R., Gerrard, T., 1986. Chemical and biological characterization of natural human lymphoblastoid interferon alphas. In: Biology of the Interferon System. Martenus Nijkoff Publishers, Dertecht, p. 567.