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Some factors affecting the stability of interferon alpha 2b in solution

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Abstract

In this paper we evaluated the influence of the protein concentration and a formulation vehicle on the stability of recombinant human Interferon alpha 2b (rhIFN-α2b) in solution. The effect of the protein content (from 1 to 100 MIU/ml) at 37 °C, showed that higher concentration of this cytokine protected against the loss of bioactivity (antiviral titration) better than the lower concentrations. In contrast, rhIFN-α2b at 50 and 100 MIU/ml decreased the SDS/PAGE- and RP-HPLC-determined purity faster than samples at 1 or 10 MIU/ml. According to these results, 10 MIU/ml rhIFN-α2b was the best choice to evaluate the influence of a formulation on the stability of this cytokine. Taking this into consideration, we studied the stability of a liquid and albumin-free formulation of this protein at the recommended storage temperature (5 ± 3 °C) and under accelerated conditions (28 ± 2 °C). Accelerated storage results showed an acceptable biochemical stability of the active ingredient throughout 2 months. Real-time storage data confirmed the good biochemical stability of this formulation for 30 months.

Keywords: Protein concentration; Interferon; Albumin-free formulation; Stability

1. Introduction

Interferons (IFNs) were originally discovered due to their ability to protect cells against viral infections [1]. However, IFNs have also potent immunomodulatory effects and antiproliferative activity against malignant cells [2].

According to the World Health Organization, potency, purity, identity and stability are the most important properties for the quality control of these cytokines [3]. Unfortunately, the characterization of naturally occurring IFNs was limited before 1980 due to the low quantities produced from natural sources [4]. This problem was solved using *Escherichia coli* strains that express the genes encoding IFNs, and processes designed to obtain a very pure active ingredient [5]. In these conditions, IFNs have been commercialized in a single vial containing minimal amounts (from 0.1 to 100 MIU/ml) of the active ingredient, lyophilized in the presence of an excess of human serum albumin (HSA) [6].

The role of HSA in these preparations has been to protect IFNs against potential chemical and physical degradations mechanisms. However, the use of this blood-derived has been problematic because of the danger for potential viral contamination and the formation of high molecular weight complexes which may induce neutralizing antibodies against the cytokine, and also due to the potential for viral contamination [7].

Recently, we developed a liquid and albumin-free formulation of recombinant human IFN alpha 2b

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(rhIFN-α2b). For this, we evaluated the influence of packing material and single and combined excipients on the stability of the cytokine in solution [8]. However, other factors such as protein concentration can also affect the stability of this cytokine.

In this work, we evaluated the influence of the concentration of rhIFN-α2b on the stability of this biomolecule. We also studied the accelerated and long-term stability of rhIFN-α2b in a new liquid and albumin-free vehicle.

2. Materials and methods

2.1. Materials

The Center for Genetic Engineering and Biotechnology (CIGB, Havana, Cuba) supplied rhIFN-α2b with the characteristics previously described [5].

All chemicals used were of analytical grade.

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. Influence of protein concentration on the stability of rhIFN-α2b

rhIFN-α2b was diluted to 1, 10, 50 and 100 MIU/ml) in 100 mM sodium phosphate buffer, pH 7.4 (81 mM sodium phosphate monobasic dihydrate, 19 mM sodium phosphate dibasic anhydrous), stored at 37 ± 3 °C and periodically analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC), SDS/PAGE under reduced conditions and determination of the biological activity (antiviral titration). The kinetics (k_{obs} and half-life) constants presented here were calculated from the linear relationships between the logarithms of residual relative purity or bioactivity, and time.

2.3. Stability assessment of rhIFN-α2b in a liquid and albumin-free vehicle

A new liquid and albumin-free formulation of this cytokine (0.1 mg/ml polysorbate 80, 1 mg/ml ethylenediaminetetraacetic acid disodium salt dihydrate, 4.89 mg/ml sodium chloride, 1.2 mg/ml methyl paraben, 0.12 mg/ml propyl paraben, 3.43 mg/ml sodium phosphate monobasic dihydrate, 12.68 mg/ml sodium phosphate dibasic anhydrous) was designed according to a preformulation study already published [8].

The formulation was dispensed into primary packs of 2R type I borosilicate glass vials, stored at 5 ± 3 or 28 ± 2 °C and periodically subjected to biological activity assay (antiviral titration), ELISA, RP-HPLC, pyrogens and sterility testing, abnormal toxicity screening, organoleptic evaluation, and measurement of pH.

2.4. Protein analysis

The biological activity determination of rhIFN-α2b was performed as previously described [9], and was based on the inhibition of the cytopathic effect produced by Mengo virus on Hep-2 cells (ATCC No. CCL23). RP-HPLC was undertaken on a Vydac (Hesperia, Calif., USA) wide-pore octyl (C8) column (5 μm; 125 × 4.6 mm), as previously described [8]. Purity was calculated as percentage of the main peak divided by the total area. The samples were also analyzed by SDS/PAGE as described by Laemmli [10]. Sterility, pyrogens and pH were determined in agreement with USP 26 [11]. Abnormal toxicity was qualitatively evaluated according to the British Pharmacopoeia [12]. ELISA was performed as described by Santana et al. [13], and the organoleptic characteristics were verified by checking the transparency and absence of suspended solids against the light, as usual.

3. Results and discussion

3.1. Influence of protein concentration on the stability of rhIFN-α2b

The biological activity decreased throughout the study, probably due to the storage condition (37 ± 3 °C) (Table 1). However, higher (50 and 100 MIU/ml)

Table 1
Kinetic parameters of the thermal stability of rhIFN-α2b, at four concentrations

Concentration (MIU/ml)	$k \times 10^{-3}$ (day^{-1})	$t_{1/2}$ (day)
	Biological activity	
1	16.54 ± 1.44	42.15 ± 81.85
10	14.55 ± 1.15	47.79 ± 37.55
50	11.61 ± 0.98	59.74 ± 48.19
100	9.23 ± 1.25	75.32 ± 29.09
	Purity[a] by RP-HPLC	
1	3.21 ± 0.15	216.56 ± 75.64
10	3.35 ± 0.19	210.11 ± 48.08
50	4.16 ± 0.22	169.02 ± 64.22
100	4.63 ± 0.15	150.65 ± 22.18
	SDS/PAGE[b]	
10	0.71 ± 0.02	990.15 ± 48.07
50	1.13 ± 0.01	630.15 ± 81.11
100	1.64 ± 0.02	433.12 ± 16.35

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

[a] Results corresponded to the analysis of the purity of the rhIFN-α2b native peak, as determined by RP-HPLC.

[b] Results corresponded to the analysis of the purity of rhIFN-α2b main band, as determined by SDS/PAGE under reduced conditions.

concentrations of rhIFN-α2b protected better than lower (1 and 10 MIU/ml) concentrations of this cytokine against the loss of bioactivity (Table 1). Kinetic analysis showed that 10, 50 and 100 MIU/ml increased the bioactivity and half-life of rhIFN-α2b by about 1.14-, 1.42- and 1.79-fold, respectively, on the basis of the results obtained from samples at 1 MIU/ml (Table 1).

The effect of protein concentration on the biological activity of different active ingredients has been previously studied [14]. Lecker and Khan enhanced the enzymatic activity of alpha amylase throughout the increment of the concentration of the enzyme [14]. They speculated that this behavior could be explained due to the formation of non-covalent aggregates which might protect proteins against denaturation. It was based on: (i) the inhibition of denaturation because of steric repulsion of neighboring molecules and (ii) changes on the monomeric conformation of protein to active and more stable dimers and multimers [15].

Taking into account these data, it seems that high concentrations of rhIFN-α2b are very convenient to efficiently maintain the bioactivity of this cytokine.

On the other hand, the RP-HPLC analysis showed an accelerated formation of a pre- and post-main rhIFN-α2b native peak by-products in samples at all four evaluated concentrations (Fig. 1). The first of such degradation products could correspond to a Met sulfoxide by-product as Bordens and coworkers previously reported [2]. The exact chemical identity of the later fraction remains under investigation.

Electrophoretic profile was characterized by a main band plus a less intensive band with a higher molecular weight, which may correspond to dimers of the cytokine according to the molecular weight standard (Fig. 2).

The purity of rhIFN-α2b also decreased throughout the study when the samples were stored at 37 ± 3 °C. However, in contrast with data from the biological activity determination, higher (50 and 100 MIU/ml) concentrations reduced the purity of the active ingredient faster than the lower (1 and 10 MIU/ml) concentrations (Table 1).

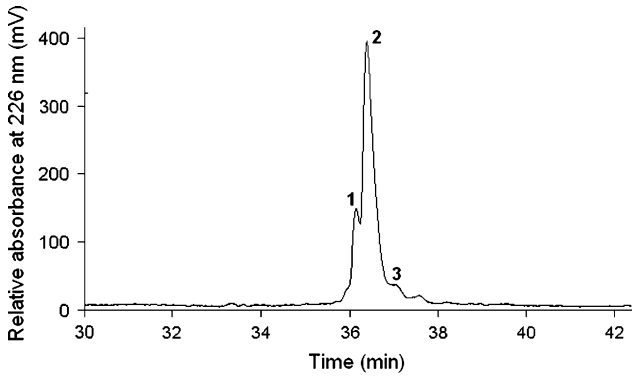

Fig. 1. Effect of accelerated storage conditions on the RP-HPLC profile of rhIFN-α2b, after 15 days at 37 ± 3 °C. Peak 1: early eluting species; peak 2: native rhIFN-α2b, peak 3: post-main rhIFN-α2b native peak by-product.

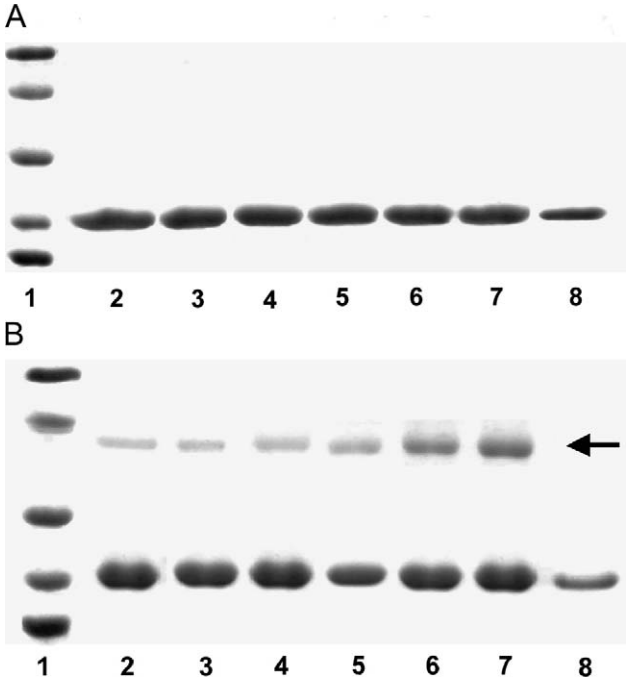

Fig. 2. Effect of the rhIFN-α2b concentration on the SDS/PAGE profile of this cytokine in solution, at the beginning of the study (A) and after 15 days of storage, at 37 ± 3 °C (B). Lane 1 corresponds to the molecular weight standard markers: lysozyme (14.3 kDa), β-lactoglobulin (18.4 kDa), tripsinogen (24 kDa), ovalbumin (43.5 kDa) and bovine serum albumin (67 kDa); lanes 2 and 3 correspond to 10 MIU/ml rhIFN-α2b; lanes 4 and 5 correspond to 50 MIU/ml rhIFN-α2b; lanes 6 and 7 correspond to 100 MIU/ml rhIFN-α2b and lane 8 correspond to the control.

According to kinetic analyses, samples at 10, 50 and 100 MIU/ml reduced the RP-HPLC-determined purity and the half-life of the cytokine by about 1.03-, 1.28- and 1.44-fold, respectively, as compared with samples at 1 MIU/ml (Table 1). Additionally, samples at 50 and 100 MIU/ml reduced the SDS/PAGE-determined purity and half-life by about 1.57- and 2.28-fold, respectively, as compared to samples at 10 MIU/ml (Table 1). Due to the low concentration of the protein, rhIFN-α2b at 1 MIU/ml was not analyzed by this technique.

The influence of protein concentration on the purity of several of these macromolecules has also been studied [16]. It has been suggested that concentration higher than 0.02 mg/ml may facilitate the aggregation of proteins [16]. This influence can be explained due to the increment on the intermolecular collisions which may increase the rate of degradation reactions on these molecules.

From these results, lower concentrations seem preferable to maintain the purity of this active ingredient in solution.

Table 2
Accelerated stability of rhIFN-α2b at 10 MIU/ml, in the present formulation vehicle

Months	Biological activity (MIU/ml)			ELISA (μg/ml)			RP-HPLC (%)		
	Batch 1	Batch 2	Batch 3	Batch 1	Batch 2	Batch 3	Batch 1	Batch 2	Batch 3
0	8.54	9.17	10.75	59.48	56.47	57.49	96.59	98.42	98.13
1	9.18	9.66	9.49	57.51	55.39	54.94	95.75	97.18	97.55
2	10.77	9.14	10.28	56.97	56.24	56.48	95.18	96.29	95.03
3	9.79	9.21	9.37	58.64	57.48	55.79	93.61	92.42	94.15
6	9.94	9.18	9.89	56.74	55.68	54.94	71.84	83.85	71.93

Samples were stored at 28 ± 2 °C and systematically analyzed as described in Section 2.

3.2. Stability assessment of rhIFN-α2b in a liquid and albumin-free formulation

Although ICH guidance typically requires accelerated stability studies to be performed at 25 ± 2 °C, ambient temperature is globally increasing and usually remains between 28 and 30 °C, or even higher. Therefore, we considered 28 ± 2 °C as an appropriated temperature to study the stability of this new formulation in order to know what would happen if any failure of the cold chain occur during storage or transportation of this product. At this condition, the formulation was stable for 2 months (Table 2). During this storage period, the biological activity of the active ingredient varied from 8.54 to 10.77 MIU/ml, the RP-HPLC-determined purity remained above 95% and the concentration, as determined by ELISA, was also stable as shown in Table 2. In addition, the formulation was non-pyrogenic, non-toxic, sterile, colorless and without suspended solids. Furthermore, the pH was maintained between 7.2 and 7.6.

According to these data, this formulation would be robust against potential loss of stability due to short failures of the cold chain.

The 5 ± 3 °C data showed that the formulation retained its biochemical properties for 30 months (Table 3). In this case, the biological activity varied from 8.59 to 11.54×10^6 IU/ml, the RP-HPLC-determined purity remained at higher than 95% and the concentration, as determined by ELISA varied between 55.42 and 62.35 μg/ml (Table 3).

The effect of the components of this formulation has been studied by other authors [15]. Polysorbate 80 is a non-ionic detergent that stabilizes proteins at low concentrations [15]. This surfactant competes with proteins for adsorption to various interfaces where physical instabilities can be induced [15]. This additive can also bind weakly to proteins, covering hydrophobic sites and thus inhibiting their aggregation [15]. Results indicated that polysorbate 80 efficiently contributed to the stabilization of rhIFN-α2b at both, shelf-life and accelerated storage conditions.

The other stabilizer, ethylenediaminetetraacetic acid disodium salt dihydrate stabilizes proteins due to the inhibition of the oxidation reactions by the removal of metal ions [15]. On the other hand, sodium chloride is an isotonizing agent to guarantee an isotonic formulation avoiding the risks of tissue damages due to the effect of hypotonic or hypertonic solutions. Finally, methyl and propyl parabens are two preservatives that have been used in different formulations due to their capacity to inhibit the growth of microorganisms such as gram-positive and gram-negative bacteria, yeasts and moulds [17]. The activity of these two preservatives is currently enhanced by the presence of EDTA [17]. Consequently, the use of these additives in parenteral liquid preparations becomes very favourable to prevent any microbial

Table 3
Long-term stability of rhIFN-α2b at 10 MIU/ml, in the present formulation vehicle

Months	Biological activity (MIU/ml)			ELISA (μg/ml)			RP-HPLC (%)		
	Batch 1	Batch 2	Batch 3	Batch 1	Batch 2	Batch 3	Batch 1	Batch 2	Batch 3
0	8.84	9.19	10.15	57.84	61.08	62.18	96.59	98.42	98.13
3	9.15	10.28	8.59	59.54	59.87	59.84	96.43	98.39	98.73
6	8.59	10.48	11.54	55.42	59.81	60.48	95.77	98.13	97.52
9	9.48	9.38	8.69	58.36	62.14	61.55	95.31	97.98	96.23
12	11.15	11.28	10.27	55.91	60.44	62.35	96.83	97.92	96.19
18	10.28	10.87	10.58	57.39	59.87	59.88	95.21	95.27	95.97
24	9.58	9.79	9.87	61.09	60.18	61.09	95.39	95.71	95.42
30	8.97	8.97	9.08	58.75	62.01	62.17	95.61	96.97	96.31

Samples were stored at 5 ± 3 °C and systematically analyzed as described in Section 2.

contamination during the preparation or the filling of the formulation.

The combination of these excipients stabilized the active ingredient and therefore, this new formulation can be used as a therapeutic product for the human use.

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