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Comparative effects of stabilizing additives on the rates of heat inactivation of recombinant human interferon α-2b in solution

H.K. Šebėka *, B. Starkuvienė, O.V. Trepšienė, A.A. Pauliukonis, V.A. Bumelis

Institute of Biotechnology, Graičiūno 8, 2028 Vilnius, Lithuania

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Abstract

This study was aimed at the comparative evaluation of stabilizing additives used for the protection of the antiviral activity of interferon-α2b against thermal inactivation, at 60°C. The comparative effects of amino acids, polyhydric alcohols, saccharides and nonionic surfactants were studied. All were effective. Representing the thermal inactivation process with first order kinetics, a maximal prolongation of antiviral activity half-life of 39-fold was achieved with the most effective procedure. Inactivation rate constants varied from $(53.3 \pm 4.6) \times 10^{-3}$ to $(2.5 \pm 0.3) \times 10^{-3}$ min^{-1}. Human serum albumin, nonionic surfactants and monosaccharides increased half-life values by 5–39-, 5–23-, 4–20-fold, respectively. Amino acids, polyhydric alcohols and disaccharides increased $t_{1/2}$ values by 4–11-, 2–8- and 3–8-fold, respectively. These data provide useful information for the selection of stabilizing additives for IFN-α2b formulations.

Keywords: Interferon; Thermal stability; Additives; Antiviral activity

1. Introduction

The antiviral activity of interferon-α (IFN-α) and its maintenance in solution is affected by its molecular environment, as is the functional stability of a range of other proteins (Sedmak and Grossberg, 1981; Arakawa et al., 1991; Fagain, 1995; Chen and Arakawa, 1996). Inactivation can result from, for example, thermal stress, and/or chemical degradation. A range of additives is therefore widely used in protein drug formulations to improve their stability and to extend their respective shelf-lives (Cleland et al., 1993). The degree of protection provided by any one additive however is dependent on the nature of the specific protein on the one hand, and the nature of the additive, on the other hand (Ye et al., 1988; Gibson, 1996).

Due to the specific interest in the antiviral properties of IFN-α (Bonnem, 1991) a range of additives has been developed to protect the

* Corresponding author. Tel.: +370-260-2112; fax: +370-260-2116.

E-mail address: sereik@ibt.lt (H.K. Šebėka).

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molecule from loss of antiviral activity. These include sugars (Kohno et al., 1992), polyols (Hasegawa et al., 1986), inorganic salts (Sedmak and Grossberg, 1981), amino acids (Kwan, 1985; Patel, 1994), and albumins (Schepart et al., 1995; Appenheimer et al., 1998). The protective effects of different additives however are often not comparable because of differences in study design, assay methodologies and/or conditions under which experiments are carried out. Systematic investigations are therefore required for the evaluation of the relative protective effects of additives, to provide greater understanding of the chemical mechanisms involved in the stabilization of IFN-α, and to promote the preparation of stable IFN-α2b formulations.

The objective of this study was to compare the stabilizing effects of representatives of different classes of compounds against heat inactivation of IFN-α.

2. Materials and methods

2.1. Materials

Recombinant human α-2b interferon (IFN-α2b) was provided by Biofa AB (Lithuania). Leukocyte α interferon (LeIFN-α), derived from human leukocytes, was provided by Bakteriniai Preparatai AB (Lithuania). All monosaccharides, disaccharides, polyhydroxyl alcohols, Tween 20, Tween 80, Triton X-100, Triton X-405, Brij 35, Brij 58, were purchased from Fluka Chemie AG. Amino acids, human serum albumin (HSA), Pluronic F68, Myrj 52, Myrj 59, sodium chloride, sodium bicarbonate, gentamicin sulfate, trypsin, fetal bovine serum were purchased from Sigma Chemical Company. All amino acids (except for glycine) were of the L-configuration. Dulbecco's modified Eagle's medium (DMEM) and crystal violet were supplied by Serva Feinbiochemica. All reagents were of analytical grade quality.

2.2. Cells

Cultured L41 cells (transformed human bone marrow cells) were used for the determination of IFN-α2b activity. Cells were maintained as a monolayer culture in DMEM medium containing 3% fetal bovine serum, 2 mM L-glutamine, 0.16 mg ml^{-1} gentamicin sulfate.

2.3. Antiviral (cytopathic effect reduction) assay

The residual activities of sample solutions of IFN-α2b were determined by dye uptake assay using L41 cells and EMC (mouse encephalomyocarditis) virus. Initial dilution of samples was made with DMEM medium. Briefly, 0.1 ml of DMEM medium was dispensed to all wells of 96-well microplates; 0.1 ml sample and 0.1 ml appropriate dilution of the laboratory standard (calibrated in international units) were added to the inner six wells of the plate. A consecutive titration was then made using a 2-fold dilution through horizontal rows (outer rows of 96-well plates were used as controls for cells or virus). A further 0.1 ml L41 cell suspension was then added to serial diluted samples and cultured at 37°C in an atmosphere of 5% CO_2 for 24 h. Then, 0.05 ml EMC virus solution (appropriately diluted in DMEM) was placed in each well, except for the cell control row. Plates were returned to the CO_2 incubator for (20 ± 2) h. The medium was aspirated and 0.04 ml 0.05% crystal violet was added for 20 min. Plates were washed in a distilled water bath. The cell-bound dye was extracted using a mixed solution of ethanol and acetic acid (ethanol, 0.1% acetic acid in water 1:1). The absorbance of the dye was measured using an ELISA plate reader. The activity was expressed in international units (IU) according to the NIBSC international standard 82/576. The coefficient of variation in this assay was less than 15%. (Armstrong, 1981; Familletti et al., 1981; Finter, 1981; Imanishi et al., 1981).

2.4. IFN thermoinactivation

Thermal inactivation of IFN-α2b solutions was evaluated in test tubes in a constant-temperature (60°C) bath. Three millilitre of 0.01 M citrate buffer solution (pH 6.0), with or without additive, and 10 μl of IFN-α2b stock solution (0.18 mg ml^{-1}, activity, 3.2×10^8 IU mg^{-1}) were added to

each of three parallel tubes. At certain time intervals, 50–100 μl of the samples were withdrawn, cooled rapidly in an ice bath and residual activities were assayed according to the procedure given above.

The change in the potency (A) of IFN-α2b can be described as follows:

$$\text{if } \frac{dA}{dt} = -k_{in}A$$

$$\text{then } A_t = A_0 \exp(-k_{in}\, t) \quad (1)$$

where A_0 is the initial antiviral activity, A_t is the antiviral activity at the incubation time t, and k_{in} is the first order inactivation rate constant. This equation can also be transformed such that:

$$\ln\left(\frac{A_t}{A_0}\right) = -k_{in}t \quad (2)$$

Accordingly, to determine first order inactivation rate constants, residual activities were expressed as a percentage of the initial activity of the IFN solution and were plotted on a semilogarithmic axis, against incubation time. A linear regression was performed by the least squares method with the aid of the Sigma Plot (Jandel Co.) program. The calculations yielded mean values of inactivation rate constants and standard deviations around these values.

3. Results

The IFN-α2b stabilizing effects of amino acids, polyhydric alcohols, saccharides and nonionic surfactants were studied under conditions of thermal inactivation at a temperature of 60°C. This temperature was chosen for two reasons, (a) it provided experimentally convenient kinetic curves of antiviral activity decline, and (b) the acceleration of thermal inactivation provided greater scope for the comparison of additives as compared with studies at room temperature.

It was assumed that predictions of protein formulation stabilities could be based on higher temperature kinetic investigations (Yoshioka et al., 1994). Accelerated stability tests have earlier been reported for a range of proteins whose degradation occurred through a number of different pathways, including hydrolysis (Helm and Muller, 1990), and deamidation (Lee et al., 1992), and also for proteins whose degradation occurred through unidentified pathways (Yoshioka et al., 1991). Accelerated stability tests on β-interferon have also been reported (Geigert et al., 1987). Recently, an accelerated stability study of poliovirus indicated such studies to be good predictors of real-time stability (Burke et al., 1999).

Under the experimental conditions described in this paper thermal inactivation of IFN-α2b resulted in the exponential decay of antiviral activity in time, a process represented by first order kinetics. Kinetic constants were determined using linear regression analysis of the logarithm of residual activity as a function of time.

In an effort to identify conditions which promoted the maintenance of the antiviral activity of IFN-α2b, the effects of manipulation of a number of experimental variables on these kinetic constants were evaluated. These variables included medium pH, amino acid concentration, and concentrations of such additives as polyhydric alcohols, monosaccharides, disaccharides, nonionic surfactants, HSA and some mixtures of additives.

3.1. Effects of pH and concentration of amino acids

The pH optimum for the promotion of IFN stability was determined in the presence of amino acids. Detailed pH-inactivation rate studies were performed in the presence of 0.05 M lysine, glutamic acid and glycine over the pH range from 4.0 to 8.0. The k_{in} values obtained are indicated in Fig. 1. The pH stability profiles observed were dependent on the nature of the amino acid used. Sodium glutamate and glycine provided greater protection at acidic pH values, in the region of pH 5.0–6.5. Outside this range, despite the presence of these amino acids, the thermal inactivation rate constant (k_{in}) increased approximately 2–3 times. Lysine was more effective at more basic pH values (from pH 5.5 to 7.5). The heat stability of IFN-α2b generally was maximal in the pH range 5.5–6.5. All subsequent experiments therefore were conducted at pH 6.0.

Optimal glycine, lysine and sodium glutamate concentrations were deduced from concentration curves over the concentration range 10–150 mM (Fig. 2). At low amino acid concentrations (10 mM) inactivation constants reached high levels, $(71 \pm 8) \times 10^{-3}$ (glycine), $(17.6 \pm 0.6) \times 10^{-3}$ (lysine), $(39.9 \pm 4.3) \times 10^{-3}$ min^{-1} (glutamate). Increasing concentrations, to levels of between 40 and 50 mM reduced the inactivation rate by 2–5-fold. Higher concentrations had no significant additional effect.

The effect of increasing KCl concentrations over the concentration range used for the evaluation of the effects of amino acids as described above, was also evaluated, as shown in Fig. 2. The inactivation rate was reduced by 2-fold at high concentrations of KCl (150 mM). At lower concentrations (50 mM), KCl was 2–5-fold less protective than were the amino acids tested.

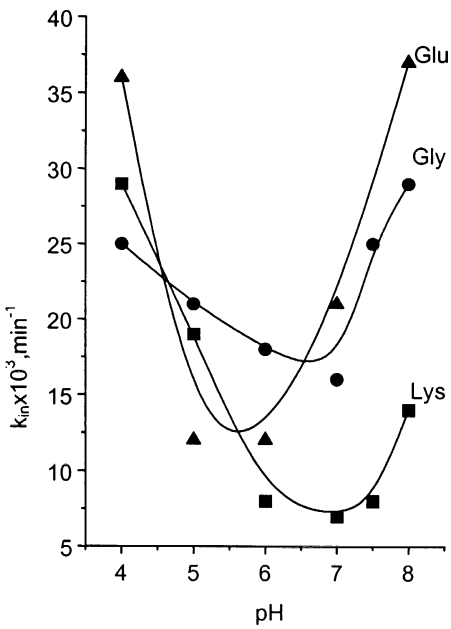

Fig. 1. pH effect on IFN-α2b thermal inactivation rate constants in the presence of amino acids. Thermal inactivation rate studies were performed in the 0.01 M citrate-phosphate buffer system. For conditions of k_{in} measurement, see Section 2. (▲), Glu; (●), Gly; (■), Lys. The data are means from three experiments, S.D. was less than ± 15% at each point.

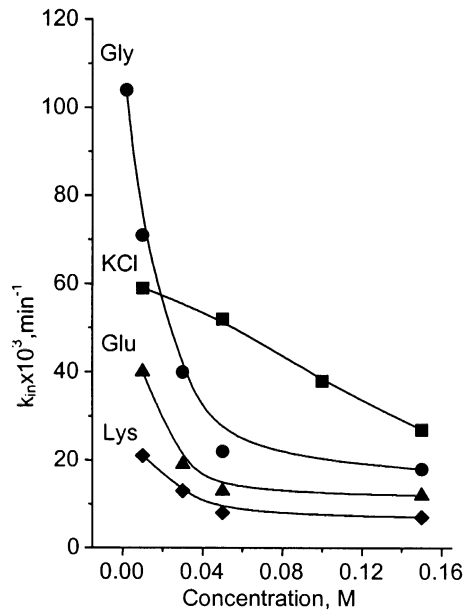

Fig. 2. Comparative effects of amino acids and KCl concentrations on IFN-α2b thermal inactivation rate constants (k_{in}). IFN-α2b solutions were incubated at 60°C and pH 6.0 in the presence of Lys (◆), Glu (▲), Gly (●) or KCl (■) at concentrations ranging from 0.01 to 0.15 M. The data are means from three experiments, S.D. was less than ± 15% at each point.

3.2. Screening of amino acids

The protection provided by 16 amino acids against antiviral activity loss was examined under the optimal conditions identified, as described above. The kinetic parameters observed are indicated in Table 1. All were effective, since antiviral activity half-life was prolonged by 4–11-fold compared with that in the absence of amino acids. Aromatic (Phe, Trp), basic or acidic amino acids (Lys, Glu) and Met were slightly more effective (about 2-fold) than the others tested.

3.3. Effects of polyhydric alcohols

The protective effects of a range of polyhydric alcohols, containing two to six hydroxy groups, were also evaluated over the concentration range of 0.03–0.40 M. The kinetics of antiviral activity loss are demonstrated in Fig. 3 on a semilogarith-

mic scale. The kinetic parameters are indicated in Table 2. Kinetic analysis revealed that the polyhydric alcohols tested reduced the inactivation rate of IFN-α2b and prolonged antiviral activity half-life approximately 2–8-times in comparison to control. Inactivation rate constants remained fairly constant for all tested compounds over the concentration range of 0.1–0.4 M.

3.4. Effects of monosaccharides

The effects of rhamnose, fructose and glucose on the maintenance of antiviral activity of IFN-α2b subjected to heat treatment were evaluated using concentrations of 0.1 and 0.3 M (Table 3). Kinetic analysis indicated a reduction in IFN-α2b inactivation rate and prolongation of the antiviral activity half-life by about 4–20-fold the control value. Glucose proved approximately 4-fold more protective against thermal inactivation at 60°C than the other monosaccharides tested.

Table 1
Kinetic parameters of IFN-α2b thermal inactivation in the presence of amino acids (60°C)[a]

L-amino acid	$k_{in} \times 10^3$ (min^{-1})	Antiviral activity half-life, $t_{1/2}$ (min)
Gly	18.4 ± 1.7	38 ± 4
Ala	18.9 ± 2.2	37 ± 4
Val	17.8 ± 1.4	39 ± 3
Leu	18.1 ± 1.8	38 ± 4
Ile	17.2 ± 1.1	40 ± 3
Phe	9.8 ± 0.5	71 ± 4
Trp	11.7 ± 0.8	59 ± 4
Met	9.5 ± 0.6	73 ± 5
His	19.1 ± 2.5	36 ± 5
Arg	13.9 ± 1.5	50 ± 5
Lys	7.9 ± 0.8	88 ± 9
Asp	11.1 ± 0.8	62 ± 5
Glu	11.6 ± 0.9	60 ± 5
Gln	10.4 ± 0.9	67 ± 6
Ser	17.7 ± 0.7	39 ± 2
Thr	20.6 ± 1.9	34 ± 3

[a] Control (without amino acids) $k_{in} = (91.5 \pm 8.1) \times 10^{-3}$ min^{-1}; $t_{1/2} = (8 \pm 1)$ min. Concentrations of amino acids were 0.05 M. The results are given as mean ± S.D.

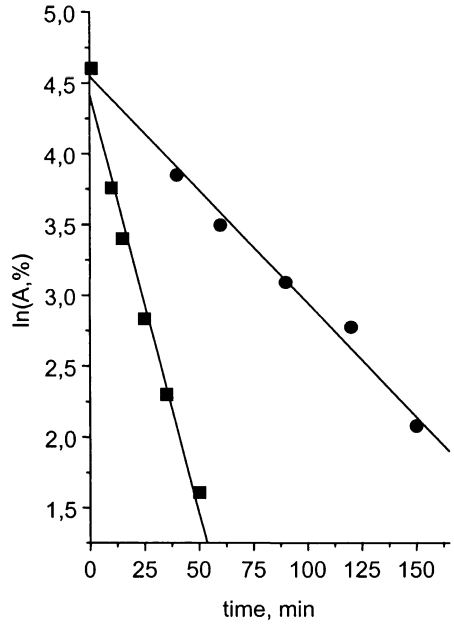

Fig. 3. Kinetics of antiviral activity decay under IFN-α2b heating in a semilogarithmic scale in the presence of polyhydric alcohols. IFN-α2b solutions were incubated at 60°C and pH 6.0 in the presence of 0.4 M sorbitol (●) or mannitol (■) for the indicated times. Thereafter, antiviral activities were determined in the solutions and regression lines were fitted as outlined under Section 2.

3.5. Effects of disaccharides

The protective effects of sucrose, lactose, trehalose, maltose, and cellobiose were also determined, the latter three over the concentration range from 0.1 to 0.3 M (Table 4). These disaccharides decreased inactivation rate and promoted the maintenance of antiviral activity half-life of IFN-α2b by a factor of 3–8-fold. This effect did not vary over the concentration ranges tested. Trehalose and sucrose were 2–3 times more effective than other disaccharides.

3.6. Effects of nonionic surfactants

The effects of a range of nonionic surfactants on the rate of inactivation of IFN-α2b were also evaluated. These included polyoxyethylene alkyl, alkylphenyl ethers (e.g. Brij and Triton derivatives) and esters (e.g. Myrj and Tween derivatives)

with hydrophobic groups containing dodecyl or hexadecyl carbon chains and hydrophilic groups in which the number of oxyethylene units varied between 9 and 95. Polymeric surfactants such as copolymers of polyethylene and polypropylene glycol (e.g. Pluronic) were also tested. The kinetic

Table 2
Kinetic parameters of IFN-α2b thermal inactivation in the presence of polyhydric alcohols[a]

Polyhydric alcohol	Concentration (M)	$k_{in} \times 10^3$ (min^{-1})	Antiviral activity half-life, $t_{1/2}$ (min)
1,2-Propanediol	0.1	11.6 ± 0.9	60 ± 5
	0.4	12.4 ± 0.7	56 ± 3
Glycerol	0.1	20.1 ± 2.3	34 ± 4
	0.2	21.2 ± 2.5	33 ± 4
	0.4	19.3 ± 1.8	36 ± 3
Mannitol	0.03	50.2 ± 5.3	14 ± 1
	0.1	53.3 ± 4.6	13 ± 1
	0.4	47.2 ± 3.7	15 ± 1
Inositol	0.1	15.9 ± 0.9	44 ± 2
	0.4	16.2 ± 1.3	43 ± 3
Dulcitol	0.4	17.1 ± 2.2	40 ± 5
Sorbitol	0.4	15.8 ± 1.1	44 ± 3
Adonitol	0.1	15.4 ± 1.9	45 ± 5

[a] Control (without polyhydric alcohols) $k_{in} = (93.1 \pm 7.3) \times 10^{-3}$ min^{-1}; $t_{1/2} = (7 \pm 1)$ min. The results are given as mean ± S.D.

Table 3
Kinetic parameters of IFN-α2b thermal inactivation in the presence of the monosaccharides[a]

Monosaccharide	Concentration (M)	$k_{in} \times 10^3$ (min^{-1})	Antiviral activity half-life, $t_{1/2}$ (min)
(L−)-Galactose	0.2	12.3 ± 1.1	56 ± 5
(D+)-Glucose	0.1	5.4 ± 0.8	128 ± 19
	0.3	4.9 ± 0.6	141 ± 17
(D−)-Fructose	0.1	21.2 ± 2.1	33 ± 3
	0.3	22.4 ± 3.2	31 ± 4
(L+)-Rhamnose	0.1	18.9 ± 2.1	37 ± 4
	0.3	20.7 ± 2.6	34 ± 4
(D+)-Mannose	0.2	23.5 ± 2.1	30 ± 3

[a] Control (without monosaccharides) $k_{in} = (97.2 \pm 9.2) \times 10^{-3}$ min^{-1}; $t_{1/2} = (7 \pm 1)$ min. The results are given as mean ± S.D.

Table 4
Kinetic parameters of IFN-α2b thermal inactivation in the presence of disaccharides[a]

Disaccharide	Concentration (M)	$k_{in} \times 10^3$ (min^{-1})	Antiviral activity half-life, $t_{1/2}$ (min)
Sucrose	0.2	12.9 ± 1.2	54 ± 5
Trehalose	0.1	11.7 ± 1.8	59 ± 9
	0.3	13.3 ± 1.6	52 ± 6
Maltose	0.1	27.5 ± 2.9	25 ± 3
	0.3	26.1 ± 2.5	27 ± 3
Cellobiose	0.1	22.4 ± 2.6	31 ± 4
	0.3	21.6 ± 2.8	32 ± 4
Lactose	0.2	33.2 ± 2.6	21 ± 2

[a] Control (without disaccharides) $k_{in} = (94.8 \pm 11) \times 10^{-3}$ min^{-1}; $t_{1/2} = (7 \pm 1)$ min. The results are given as mean ± S.D.

Table 5
Kinetic parameters of IFN-α2b thermal inactivation in the presence of surfactants[a]

Surfactants	$k_{in} \times 10^3$ (min^{-1})	Antiviral activity half-life, $t_{1/2}$ (min)
Brij 35	5.3 ± 0.6	131 ± 15
Brij 58	3.7 ± 0.5	187 ± 25
Myrj 52	5.5 ± 0.4	120 ± 8
Myrj 59	6.7 ± 0.5	103 ± 8
Pluronic F-68	4.3 ± 0.4	161 ± 6
Triton X-100	17.6 ± 1.2	39 ± 3
Triton X-405	13.9 ± 0.5	50 ± 2
Tween 20	4.4 ± 0.5	158 ± 18
Tween 80	4.8 ± 0.6	144 ± 17

[a] Control (without detergents) $k_{in} = (84.3 \pm 10.1) \times 10^{-3}$ min^{-1}; $t_{1/2} = (8 \pm 1)$ min. Concentrations of nonionic surfactants were 1 mM, except Pluronic F-68 — 0.1% (w/v). The results are given as mean ± S.D.

data are summarized in Table 5. All surfactants tested effectively promoted the maintenance of half-life activity. Under the experimental conditions used, half-life values were 5–23-fold higher than control values.

3.7. Effect of human serum albumin (HSA)

HSA was tested for its efficacy to prevent IFN-α2b from thermoinactivation in the concentration range of 0.3–10 mg ml^{-1}, which is commonly used in protein formulations. The addition of HSA effectively reduced inactivation rate, so that antiviral activity half-life was approximately 5–39-fold the control value (Table 6). Interestingly, the inactivation rate was increased, and the antiviral activity half-life decreased at the higher HSA concentrations used.

3.8. Combined effects of mixtures of additives

Protective effects of several simultaneously used additives, chosen from different classes of compounds studied, were also determined. The kinetic parameters of antiviral activity reduction measured under these conditions are given in Table 7. Evidently, the mixture methionine/HSA or trehalose/HSA exhibited the same protective effect as HSA alone at concentrations of 1 and 5 mg ml^{-1}, respectively. No difference in stabilizing effect was observed with methionine (0.05 M)/trehalose (0.1 M) addition, as compared with methionine (0.05 M) or trehalose (0.1 M) alone (Tables 1 and 4). However, with the addition of the HSA/Tween 20 combination the inactivation observed was considerably more rapid and the half-life about 2-fold shorter than was observed with the addition of the single ingredients.

It would be useful to learn whether the results could be extended to IFN-αs or others than IFN-α2b. For this purpose tests with human interferon α from human leukocytes (LeIFN-α) were carried out under the same conditions as for IFN-α2b. The results are shown in Table 7. It can be seen that the additional inclusion of methionine or trehalose reduced half-life of LeIFN-α in comparison with HSA alone by 4- and 5-fold, respectively. In contrast, IFN-α2b inactivation parameters were not modified by additional inclusion of trehalose or methionine. This means that the results obtained with IFN-α2b could not be extended to LeIFN-α.

Table 6
Kinetic parameters of IFN-α2b thermal inactivation in the presence of HSA[a]

Kinetic parameters	HSA concentration (mg ml^{-1})			
	0.3	1.0	5.0	10.0
$k_{in} \times 10^3$ (min^{-1})	2.5 ± 0.3	4.1 ± 0.5	17.1 ± 1.8	21.1 ± 2.4
Half-life (min)	277 ± 33	169 ± 21	41 ± 4	33 ± 4

[a] Control (without additions) $k_{in} = (98.1 \pm 10.2) \times 10^{-3}$ min^{-1}; $t_{1/2} = (7 \pm 1)$ min. The results are given as mean ± S.D.

Table 7
Kinetic parameters of IFN-α2b and LeIFN-α thermal inactivation in the presence of the mixtures of additives[a]

Additives and their concentrations	$k_{in} \times 10^3$ (min^{-1})	Antiviral activity half-life, $t_{1/2}$ (min)
IFN-α2b		
HSA 1 mg ml^{-1} + Met 0.05 M	4.3 ± 0.4	161 ± 15
HSA 5 mg ml^{-1} + Met 0.05 M	17.3 ± 2.2	40 ± 5
HSA 1 mg ml^{-1} + Trehalose 0.1 M	4.6 ± 0.4	151 ± 13
HSA 5 mg ml^{-1} + Trehalose 0.1 M	24.6 ± 2.7	28 ± 3
HSA 1 mg ml^{-1} + Tween 20 1mM	8.7 ± 0.8	80 ± 7
Met 0.05 M + Trehalose 0.1 M	7.1 ± 0.9	98 ± 12
Met 0.05 M + Glycerol 0.4 M	21.6 + 2.9	32 + 4
LeIFN-α		
HSA 1 mg ml^{-1} + Met 0.05 M	34.8 ± 4.1	20 ± 3
HSA 1 mg ml^{-1} + Trehalose 0.1 M	41.5 ± 4.3	17 ± 2

[a] Control for IFN-α2b (in the presence of 1 or 5 mg ml^{-1} HSA) is given in the Table 6. Control for LeIFN-α (in the presence of 1 mg ml^{-1} HSA) $k_{in} = (8.6 \pm 1.3) \times 10^{-3}$ min^{-1}; $t_{1/2} = (81 \pm 12)$. Results are given as mean ± S.D.

4. Discussion

All the additives tested over the course of this study promoted the stability of the antiviral activity of IFN-α2b, under conditions of thermal inactivation at 60°C. These observations are consistent with earlier reports. Our observations on the protective effects of the addition of glycine and alanine are consistent with a report by Kwan (1985) who reported the protection of activity of freeze-dried IFN-α2b, stored over a period of 6 months at 20°C and reconstituted in solution. In addition, our observations on the effects of the addition of methionine and histidine are consistent with those earlier reported by Patel (1994) who reported an extension of shelf-life with these products at 40°C. Polyhydric alcohols such as glycerol, erythritol, arabitol, xylitol, sorbitol and mannitol, at concentrations of from 25 to 60% were also earlier reported to be effective stabilizers at 45°C over 24 h intervals and over a period of 1 month, at 37°C (Hasegawa et al., 1986). In addition, Yuen and Kline (1996) reported that nonionic surfactants such as Tween 20 and Tween 80 were effective in preventing losses of activity over periods of more than 2 years at 4–8°C.

Our results indicate that the most effective additives in the protection of the antiviral activity of IFN-α2b are serum albumin, nonionic surfactants, monosaccharides and amino acids, at concentrations suitable for injectable IFN-α2b preparations. The extension of antiviral activity half-life in the presence of these products was 5–39-, 5–23-, 4–20-, and 4–11-fold, respectively.

The monosaccharides galactose and glucose provided significantly greater protection in comparison with other hexoses (for example, rhamnose and mannose). An increase in the number of glucose residues per molecule however, as in the case of the disaccharides cellobiose and maltose, resulted in the loss of some of the protective effect. Inactivation rates increased by a factor of 4–5-fold, as compared with those observed after addition of the monomer. Recently, trehalose was described as a highly effective stabilizer of enzyme structure and function (Sola-Penna and Meyer-Fernandes, 1998). Indeed, in our own study, trehalose was 2–3-fold more effective that lactose, maltose, and cellobiose, but not more effective than sucrose in the protection of the antiviral activity of IFN-α2b.

The addition of polyhydric alcohols was less effective. A cyclic polyol inositol has earlier been reported to act as a strong stabilizer of protein hydrophobic interactions (Gekko, 1981), although it may also destroy hydrogen bonds. In our trials the effects of inositol did not differ markedly from those of other polyhydric alcohols. In the range of dihydric to hexahydric alcohols, mannitol was less effective and propanediol more effective than others by a factor of 2–5-fold.

All α-amino acids tested appeared to be effective. Hitherto a few of them, particularly methionine (Patel, 1994), have been used to stabilize liquid IFN-α2b formulations. Our data indicated that phenylalanine, glutamine and lysine were as effective as methionine.

Various nonionic surfactants were used at concentrations above their critical micelle concentrations. Surfactants are known to prevent protein aggregation by blocking hydrophobic surfaces (Katakam et al., 1995; Bam et al., 1996; Rozema and Gellman, 1996). However, no correlation was detectable between, for example, hydrophilic–lipophilic ratios, and/or molecular masses, and the protective effects observed. Only Triton X-100 and Triton X-405 were less effective than the other surfactants used. The solubilities of these two products, however, are known to be limited at 60°C. The nature of the surfactant used therefore may not be of significance with respect to the protection of the antiviral activity of IFN-α2b.

HSA is known to stabilize proteins (Tarelli et al., 1998) and is used as a carrier protein in the solution and freeze-dried commercial IFN-α formulations (Braun and Alsenz, 1997). However, considering the risk of viral contamination associated with the use of HSA, HSA-free IFN-α2b formulations are desirable. Appenheimer et al. (1998) compared the stability of antiproliferative activity of IFN-α2b solution stored for 42 days at 4°C in the presence and absence of albumin. Their results indicated that stability was not dependent on the presence of HSA.

Our results indicate that HSA did enhance the thermal stability of IFN-α2b more than did the other additives. The stabilizing effect was highly dependent upon the HSA concentration and decreased with increasing concentration over the concentration range of 0.3–10.0 mg ml^{-1}. This could be attributed to the increased formation of HSA-IFN-α2b dimers at higher HSA concentrations and higher temperatures. Their presence in IFN-α formulations has earlier been reported by Braun and Alsenz (1997). The results obtained indicate it is important to avoid excess HSA in the preparation of IFN-α2b formulations and standard solutions.

We also evaluated the stabilizing effects of combinations of additives. The addition of the second constituent either had no significant effect (within experimental error) on the half-life values observed after addition of the first or resulted in marked decreases in the half-life. The kinetic parameters observed after the addition of HSA were not modified with the further addition of methionine or trehalose. Similar results were obtained with the mixture of methionine and trehalose. These observations could be explained thermodynamically on the basis of the theory of protein stability developed by Timasheff and others (Lee, 2000). Accordingly, the addition of a second ingredient may not change the difference between the free energy (ΔG) of the native and denatured states of IFN-α2b, such that the half-life values would remain essentially the same.

The monophasic kinetics of the IFN-α2b inactivation process (Fig. 3) suggest that one thermally unstable domain is responsible for the decay of the antiviral activity of IFN-α2b, under conditions of thermal inactivation at 60°C. This observation is consistent with the report that the N-terminal domain, which is known to be involved in the high affinity receptor binding of IFN-α, is of lower conformational stability than the C-terminal domain (Skamlova et al., 1997). Therefore, the effects of the additives described above may well be mediated through protective effects exerted at the level of the IFN-α2b cell-binding domain.

Finally, an attempt was made to determine whether the observations on stabilization of IFN-α2b might also apply to other IFN-α subspecies. Leukocyte IFN-α, derived from human leukocytes, was chosen as a natural mixture of IFN-α moieties and tested in parallel with IFN-α2b. However, in three cases stability parameters (Table 7) were altered in a disparate manner by the same additives, an observation which might reflect differences in the molecular properties of IFN-α subspecies (Knight, 1984).

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