ELSEVIER

International Journal of Pharmaceutics 187 (1999) 259–272

international journal of pharmaceutics

www.elsevier.com/locate/promis

Hybrid (BDBB) interferon-α: preformulation studies

John D. Allen, David Bentley, Rowan A. Stringer, Nicholas Lowther *

Drug Preformulation and Delivery Department, Ciba Pharmaceuticals (now Novartis Horsham Research Centre), Wimblehurst Road, Horsham, West Sussex RH12 5AB, UK

Received 28 June 1998; accepted 9 June 1999

Abstract

A number of techniques, including RP-HPLC, HP-SEC and SDS-PAGE have been used in the delineation of degradative mechanisms of recombinant hybrid (BDBB) interferon-α (IFN-α) in the solution phase. Different degradation profiles are found according to medium pH. At pH 4.0 the major routes of degradation are via chemical transformation of the monomeric protein to a species which retains antiviral activity, and by self-proteolytic hydrolysis. At pH 7.6, methionine-oxidation is the major chemical degradative process. Protein aggregation is also a significant route of degradation at the higher pH. The results have assisted in a targeted preformulation screen of potentially stabilising excipients and possible parenteral solution dosage forms have been identified. Preliminary 'real-time' storage data confirm excellent chemical and physical stability of IFN-α in vehicles formulated at pH 7.6 or, especially, pH 4.0 under the proposed shelf conditions.

Keywords: Interferon; Protein; Stability; Degradation; Preformulation; Analysis

1. Introduction

The interferons are families of inducible secretory proteins produced in response to viral and other stimuli. The α-interferons are currently being used in the clinic for the treatment of a number of different disease states including hairy-cell leukaemia, Karposi's sarcoma in AIDS, hepatitis-B and -C and basal cell carcinoma (Spiegel, 1987; Itri, 1992; Lucivero, 1992; Stuart-Harris et al., 1992; Rieger, 1995).

Recombinant human α-interferon B/D hybrid moieties have been constructed carrying various portions of the sequence of the parent α-interferon B and D species in order to create molecules with advantageous properties (Meister et al., 1986a). The parent molecules are divided in order to exchange the DNA segments coding for peptide sequences 1–60, 61–92, 93–150 and 151–166. The BDBB hybrid (hereafter referred to as IFN-α) has been found to exhibit particularly interesting preclinical anti-viral and anti-proliferative activity (Meister et al., 1986a; Gangemi et al., 1989; Hochkeppel et al., 1992).

Development of stable dosage forms of biopharmaceuticals such as proteins and peptides presents many technical challenges to the formulator. A thorough understanding of the physico-

* Corresponding author.

0378-5173/99/$ - see front matter
PII: S0378-5173(99)00124-6

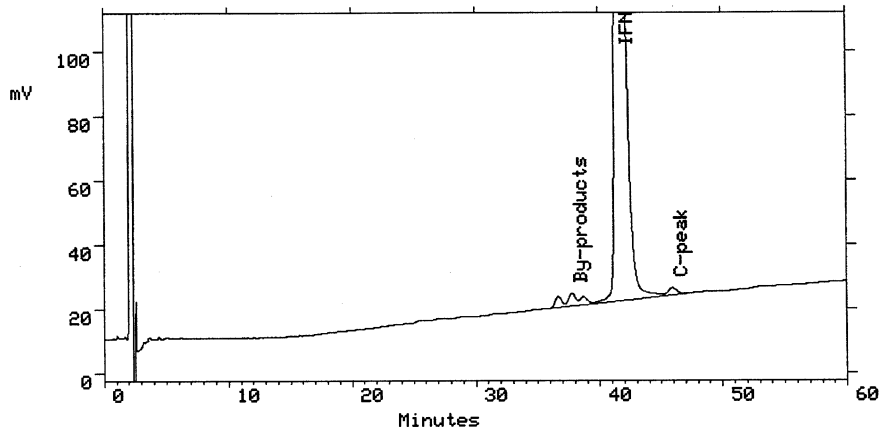


Fig. 1. Typical RP-HPLC of IFN-α bulk concentrate, as received.

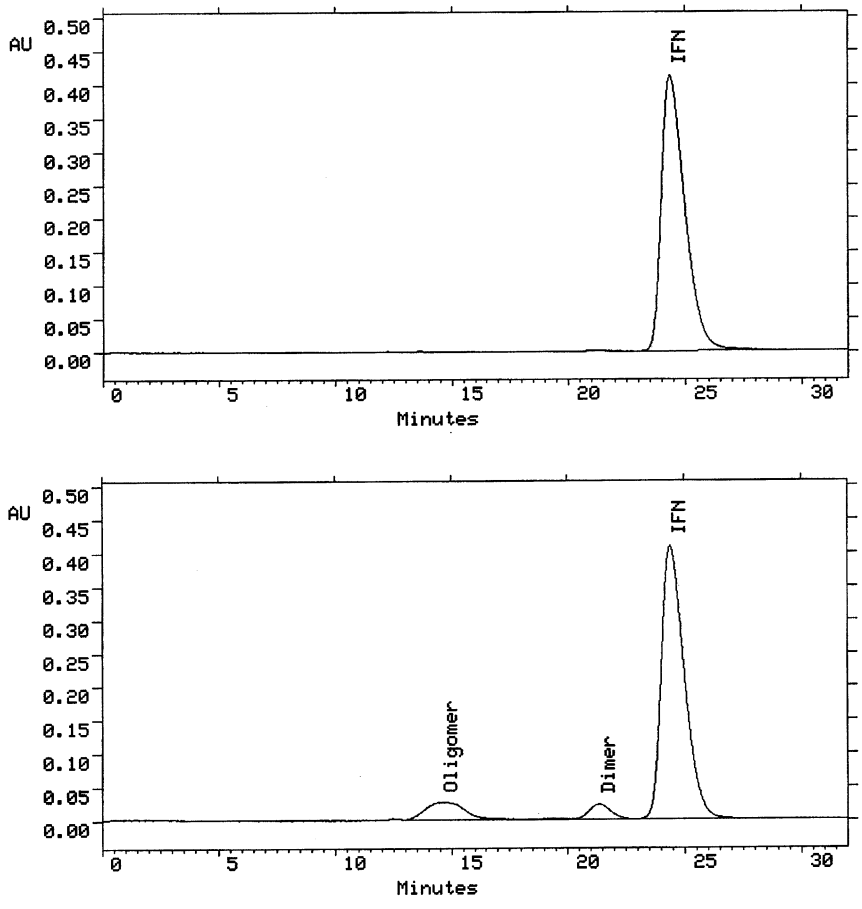
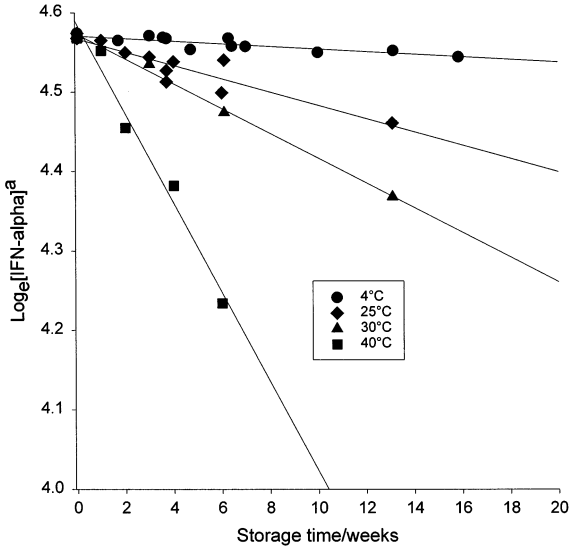


Fig. 2. Typical HP-SEC of IFN-α bulk concentrate as received (top) compared with an IFN-α 'standard' known to contain solubilised aggregates (bottom).



[a] estimated as the % of total RP-HPLC peak area.

Fig. 3. Degradation kinetics of IFN-α vs. storage temperature at pH 4.0 (vehicle comprising 40 mM citrate–200 mM mannitol). [a] Estimated as the percent of total RP-HPLC peak area.

chemical properties of the active ingredient is a prerequisite. Due to the plethora of chemical functionalities, proteins and peptides have many potential chemical and physical degradative pathways rendering careful assessment using an array of complementary techniques essential (Manning et al., 1989). Usually, proteins exhibit poor physico-chemical stability; their biological effectiveness can be compromised during isolation, purification, dosage form manufacture and storage (Evans and Grassam, 1986). The development of pharmaceutically acceptable dosage forms of protein medicinal agents is usually more complicated; the analytical challenges necessary to support the formulation effort invariably far outweigh those needed for conventional low molecular weight drugs (Nail, 1997). Since proteins have poor oral bioavailability, the most common approach is to present them as injectable products in order to achieve efficient drug delivery. Because of their instability in solution, interferons are, generally, formulated as lyophilised (freeze-dried) powders for reconstitution immediately prior to injection by subcutaneous, intramuscular or intravenous routes (Sedmak and Grossberg, 1981). However, the freeze-drying process is time consuming and expensive. Great care must be taken in design of the lyophilisation protocol to avoid protein degradation. Hence, a parenteral solution formulation of requisite stability would offer obvious advantages. Protein stability can be classified into two broad categories, namely chemical and physical. Chemical stability refers to all processes whereby the protein is modified via bond cleavage or formation. Examples of chemical degradative pathways include fragmentation, deamidation (Asp, Glu), oxidation (cysteine, methionine or tryptophan–SH), disulphide exchange, reduction (cystine–SS–), β-elimination, deglycation, racemisation (L-Asn ⇒ D-Asp), cross-linking (inter- and intra-molecular, mostly by –SS– bridging) and isomerisation (*cis*/*trans*-proline) (Geigert, 1989; Oliyai et al., 1991; van den Oetelaar et al., 1992; Nail, 1997). Physical stability does not involve covalent modification but rather changes in the higher order structure of the protein, e.g. via denaturation, aggregation or precipitation (Geigert, 1989; Oliyai et al., 1991; van den Oetelaar et al., 1992; Nail, 1997).

An array of analytical techniques is, invariably, necessary to characterise protein degradative processes adequately. The most commonly encountered techniques include sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (detection of fragmentation, crosslinking and aggregation), reverse phase high performance liquid chromatography (RP-HPLC) (deamidation, oxidation, disulphide scrambling, crosslinking), high performance size exclusion chromatography (HP-SEC) (fragmentation, aggregation), iso-electric focussing (deamidation) and peptide mapping (fragmentation) (Geigert, 1989; van den Oetelaar et al., 1992).

It is, obviously, necessary to maintain the chemical integrity of IFN-α during shelf storage of the chosen formulation. It is, also, highly desirable to maintain the protein in a disaggregated state in order to eliminate any possible adverse immunogenic effects and/or inconsistent dosing during therapy. At a desirable concentration for use as an injectable therapeutic agent, IFN-α is physically unstable in water; its solubility is compromised and it exists in an aggregated state.

Hence, in the first place, careful control of medium pH must be exercised via the use of appropriate buffered systems. Inclusion of stabilising excipients also needs investigation in order to optimise shelf-life of the finished formulation. In this study, the effectiveness of a series of known classes of 'protein stabilisers' is assessed for IFN-α.

2. Materials and methods

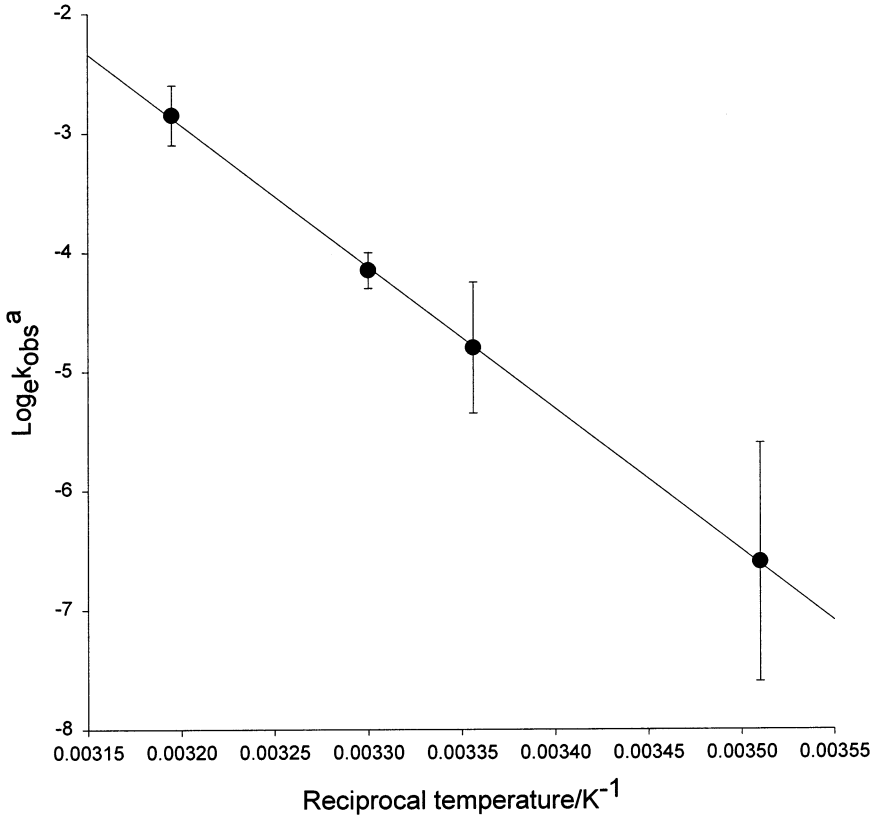
2.1. Materials

IFN-α was supplied by Dr A. Meister, Biopharmaceutical Development, Ciba-Geigy AG, Basle, Switzerland. IFN-α was isolated and purified via a multistage procedure (Meister et al., 1986b). Material was supplied in the form of frozen (− 78°C) bulk concentrate consisting of IFN-α (ca. 3 mg/ml) in 2 M ammonium chloride–25 mM Tris–(hydroxymethyl)aminomethane hydrochloride.

Acetonitrile (HPLC grade), trifluoroacetic acid (TFA, sequenal quality), and nanopure water were used to prepare mobile phases. All other materials were of reagent grade or better and used without further purification.

2.2. RP-HPLC

A 15 cm × 4.6 mm i.d. stainless steel Vydac 218TP54, 5 μm, 300 Å, C_{18} protein and peptide



[a] Pseudo first-order degradation rate constants as computed from the slopes of log-linear [IFN-α] *versus* time plots

Fig. 4. Arrhenius degradation kinetics of IFN-α at pH 4.0 (vehicle comprising 40 mM citrate–200 mM mannitol).[a] Pseudo first-order degradation rate constants as computed from the slopes of log-linear [IFN-α] vs. time plots.

Table 1
Relative stability of IFN-α versus medium pH and buffer composition

Buffer system	Medium pH	Relative IFN-α degradation rate at 4°C[a]
Citrate	2.0	4.9
Citrate	3.0	5.0
Citrate	4.0	2.9
Citrate	4.5	4.2
Acetate	3.4	>10
Acetate	4.0	>10
Acetate	5.0	>10
Glycine–HCl	1.0	>10
Glycine–HCl	2.0	>10
Glycine–HCl	3.0	1.5
Glycine–HCl	4.0	1
Glycine–HCl	4.5	1.1
Glycine–HCl	5.0	ND[b]
Glycine–HCl	6.0	ND[b]
Phosphate	7.6	1.4

[a] As assessed by RP-HPLC.
[b] ND, not determined, IFN-α insufficiently soluble.

column was used with UV detection at 214 nm. The mobile phases comprised TFA (0.22%) in H_2O (A) and TFA (0.22%) in MeCN (B). A linear gradient was applied: time/min (% A) 0 (54%), 65 (38%). The flow rate was 1 ml/min. A typical column loading was 20 μg protein.

2.3. HP-SEC

Protein aggregation in the solution phase was assessed using a 30 cm × 15 mm i.d. glass Superdex 75 (Pharmacia) agarose based size exclusion column and a mobile phase comprising 200 mM NaH_2PO_4–Na_2HPO_4 at pH 7.6. The flow rate was 0.5 ml/min and UV detection was at 214 nm. A typical column loading was 20 μg protein. The molecular weight was calibrated using standard protein markers (ribonuclease A, chymotrypsin A, ovalbumin and BSA).

2.4. Turbidity by light scattering

Insoluble aggregation was assessed by light scattering at 700 nm.

2.5. SDS-PAGE

SDS-PAGE was conducted using a Phastsystem (Pharmacia, Uppsala, Sweden) with precast 20% Phastgels under non-reducing and reducing (+β-mercaptoethanol) conditions. The molecular weight was calibrated using silver stain (PhastGel silver kit, Pharmacia, Uppsala, Sweden).

2.6. Preparative RP-HPLC

Selected samples were fractionated by preparative RP-HPLC using the analytical RP-HPLC

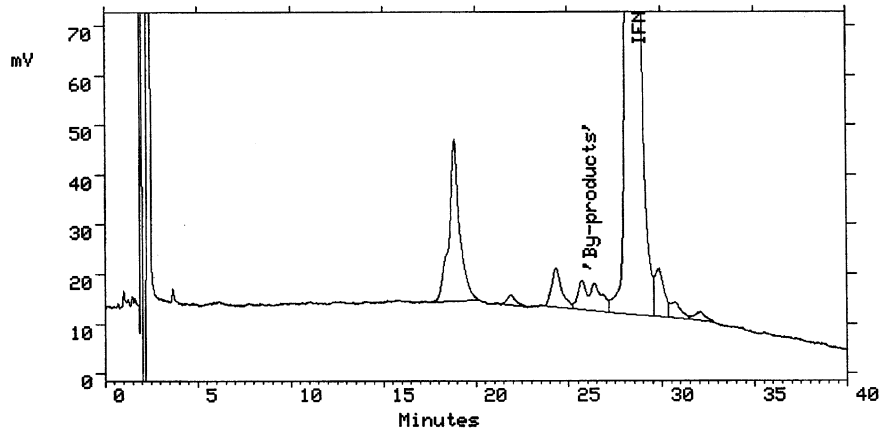


Fig. 5. RP-HPLC of IFN-α after storage at 40°C for 6 weeks at pH 4.0.

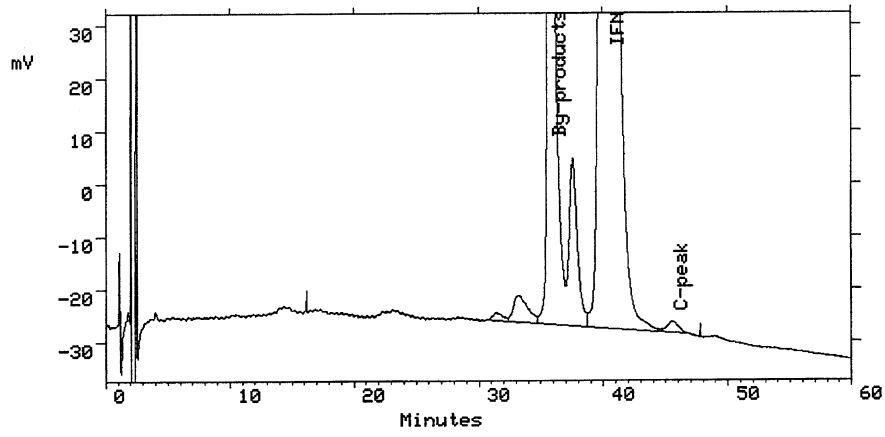


Fig. 6. RP-HPLC of IFN-α after storage at 40°C for 6 weeks at pH 7.6.

system described above. The column was loaded with ca. 250 μg of analyte and fractions corresponding to peaks of interest were collected. The samples were then evaporated and reconstituted with 80 mM glycine HCl–200 mM mannitol (pH 4.0) or 30 mM NaH_2PO_4–Na_2HPO_4–200 mM mannitol (pH 7.6) to a nominal protein concentration of 0.3 mg/ml.

2.7. Preparation of solution formulations.

Aqueous solution formulations of desired IFN-α loading (ca. 0.02–0.30 mg/ml) were prepared from the bulk concentrate using an ultrafiltration technique. The donor solution was diluted, by a minimum of 40 fold, with the buffered medium of choice in a laboratory AMICON cell fitted with a YM10 10 k MW cut-off membrane. The cell was pressurised to 50–60 psig using oxygen-free nitrogen or, in some cases, air or oxygen. The volume of solution was reduced by a factor of 8–10, with continuous stirring, and rediluted with buffered medium to the original volume. This cycle was repeated 3–4 times. The final protein concentration was confirmed using a Bradford assay (Bio-Rad, Munich, Germany).

All formulations prepared for stability testing were physiologically isotonic, as assessed using a Roebling automatic micro-osmometer (Camlab, Cambridge, UK).

2.8. Formulation stability studies

All solution formulations were sterile filtered (0.2 μm nitro-cellulose) and filled into primary packs comprising 2 ml Type I glass vials with teflon-coated rubber septa and aluminium crimp seals. Vials, seals and stoppers were autoclaved prior to filling. The vials were stored, upright, in the dark, in isothermally controlled (4, 25, 30 and 40°C) storage cabinets. The samples were pulled

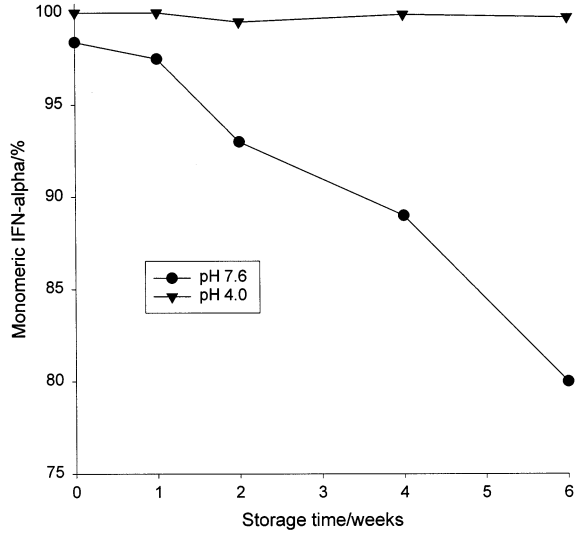


Fig. 7. Aggregation of IFN-α (as indexed by remaining monomer as the percent of total HP-SEC peak area) vs. time after storage at pH 4.0 and 7.6, respectively, at 40°C.

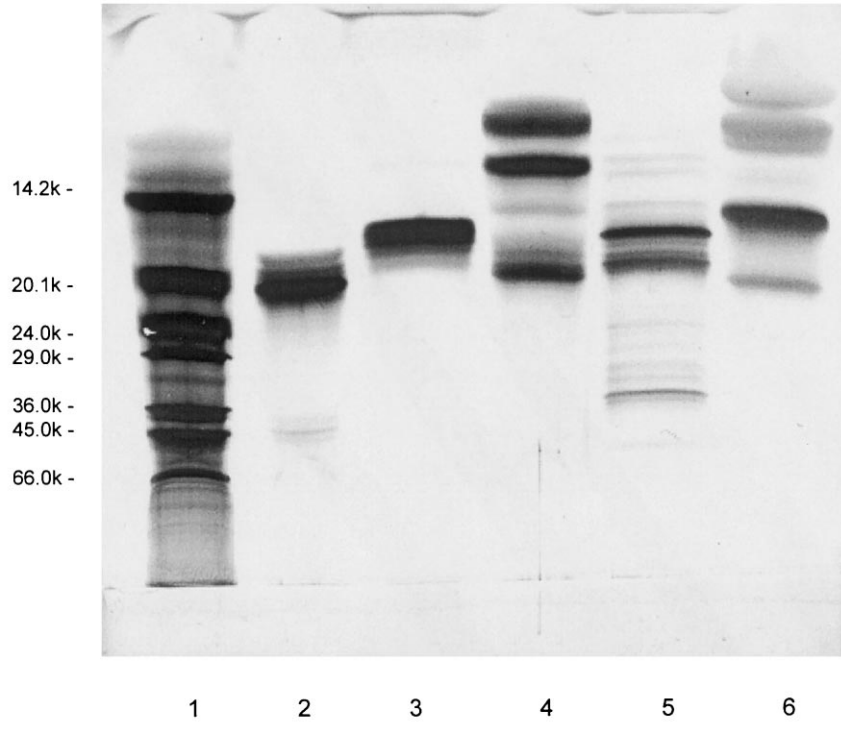


Fig. 8. SDS-PAGE of IFN-α after storage at 40°C for 6 weeks at pH 4.0 and 7.6, respectively.

at given intervals, and analysed using the techniques described above. The antiviral activity of selected samples was assessed using a cytopathic effect reduction assay (Meager, 1987). Maintenance of the sterility of vial contents was also checked periodically.

3. Results and discussion

3.1. Characterisation of hybrid (BDBB) interferon-α

A typical RP-HPLC chromatogram of IFN-α bulk concentrate (starting material 'as received') is shown in Fig. 1. The chromatogram shows three early eluting 'by-product' moieties, the main peak and a later eluting 'C peak'. The latter has been identified as an *N*-methyl derivative of IFN-α and represents a trace impurity (typically $< 1\%$ of the total peak area) remaining from the isolation protocol. The three early eluting species represent, respectively, met-101 and 112 sulphoxides of IFN-α. The latter two peaks exhibit identical primary structure, their resolution into two chromatographically distinct species being explained by chirality of the met-112 sulphoxide moiety. Typically, bulk concentrate, as received, contained $< 2\%$ by peak area of 'by-products'.

A typical HP-SEC chromatogram of IFN-α bulk concentrate is shown in Fig. 2 (for comparison, the profile of a sample known to contain

aggregated protein is appended). Typically, extremely low levels of soluble aggregates are detected in the bulk concentrate, as received.

RP-HPLC and HP-SEC chromatograms of solution formulations derived from the bulk concentrate are, typically, indistinguishable from the starting material.

3.2. Degradation mechanism of IFN-α in solution

Preliminary accelerated degradation studies were carried out at 25, 30 and 40°C, in addition to the proposed storage condition (4°C). Across the range of pH studied, degradation of IFN-α was found to approximate to pseudo first-order (log linear concentration versus time) kinetics (see, for example, Fig. 3). Adherence of Arrhenius degradation kinetics (for example, Fig. 4) suggested that rate-limiting degradative mechanisms remain unaltered over the temperature range studied, thus validating these as true accelerated conditions.

The stability of IFN-α varies significantly according to both medium pH and buffer composition (Table 1). Clearly, a glycine–HCl based buffer is superior to either a citrate or, especially, acetate buffer at a particular pH. Initial indications suggest that a pH either above, or below, the iso-electric point (pH 5.5–5.6) might prove advantageous; the pH 4.0 (glycine–HCl) and 7.6 (phosphate) systems being identified as potential formulation vehicles. The low pH medium has the advantage of maximising the solubility of IFN-α up to ca. 1.5 mg/ml; at pH 7.6 some insolubles are detected at an IFN-α loading of >0.3–0.4 mg/ml.

Accelerated stability studies of IFN-α in solution at pH 4.0 and pH 7.6 yield different degradation profiles by RP-HPLC (for example, Figs. 5 and 6). The major degradation products detected at low pH are an early eluting species (relative retention time (RRT) 0.67) and a moiety eluting just after the IFN-α main peak (RRT 1.05); the levels of Met sulphoxide by-products remain largely unaltered. However, at pH 7.6, the material balance is largely accounted for by oxidation (Met sulphoxide) by-products (RRT 0.88 and 0.93), consistent with a well-known mode of protein degradation (Borchardt et al., 1993). Light scattering at 700 nm indicates the absence of insoluble aggregate formation in both pH 4.0 and pH 7.6 formulations (up to 0.3 mg/ml protein loading — at concentrations above this insolubles are detected at pH 7.6) after accelerated storage (7 months at 25°C). HP-SEC analysis shows a clear propensity for pH 7.6 samples to form soluble aggregates under accelerated storage conditions. However, at pH 4.0, this does not appear to be a major degradative route (Fig. 7). SDS-PAGE after stress storage at pH 4.0 and 7.6 again shows

Table 2
SDS-PAGE (reducing and non-reducing conditions) of IFN-α after storage in pH 4.0 and 7.6 solutions for 6 weeks at 40°C

Medium pH	Fraction	Non-reducing	Reducing[a]
4.0	Unfractionated	Major species monomeric	Monomer band loses intensity. Two bands appear at lower MW
	RRT 0.67	Major species monomeric. Some dimeric protein[b]	Monomer band loses intensity. Two bands appear at lower MW
	RRT 1.05	Major species monomeric. Some dimeric protein[b]	Major species monomeric
7.6	Unfractionated	Major species monomeric + several bands at both lower and higher MW	Bands at higher MW disappear, replaced with bands at MW < monomeric protein
	RRT 0.88	Major species monomeric + band showing aggregated protein	Aggregate band remains, monomer band becomes a multiplet
	RRT 0.93	Major monomer and aggregate bands	Aggregate bands lose intensity

[a] After addition of β-mercaptoethanol.
[b] Probably methodologically induced.

Table 3
Influence of excipients upon the solution phase stability of IFN-α

Vehicle	pH	Relative IFN-α degradation rate at 25°C[a]
60 mM phosphate	7.6	11
60 mM phosphate–200 mM mannitol	7.6	1.5
60 mM phosphate–0.3% polygeline	7.6	18
60 mM phosphate–5% HSA	7.6	30
60 mM phosphate–5% lactose	7.6	12
60 mM phosphate–0.5% glycocholate	7.6	60
30 mM phosphate–200 mM mannitol	7.6	1.4
30 mM phosphate–200 mM lactose	7.6	8
30 mM phosphate–200 mM mannitol–0.1% Tween 20	7.6	41
30 mM phosphate–200 mM mannitol–0.1% EDTA	7.6	0.8
30 mM phosphate–200 mM mannitol–0.1% ascorbic acid	7.6	78
30 mM phosphate–200 mM mannitol–0.01% ferric citrate	7.6	6.5
80 mM glycine HCl–200 mM mannitol	4.0	1
80 mM glycine HCl–200 mM lactose	4.0	1
80 mM glycine HCl–200 mM mannitol–0.1% BZK	4.0	1.3
80 mM glycine HCl–200 mM mannitol–0.1% benzyl alcohol	4.0	2.0
80 mM glycine HCl–200 mM mannitol–0.1% propyl paraben	4.0	1.1
80 mM glycine HCl–200 mM mannitol–0.1% *m*-cresol	4.0	1.1
80 mM glycine HCl–200 mM mannitol–0.1% Tween 20	4.0	>100
80 mM glycine HCl/200 mM mannitol/0.1 % EDTA	4.0	0.6
80 mM glycine HCl–200 mM mannitol–0.1% ascorbic acid	4.0	>100
80 mM glycine HCl–200 mM mannitol–0.01% ferric citrate	4.0	1.6

[a] As assessed by RP-HPLC.

differences in the degradation profile. At pH 4.0 lower molecular weight degradation products are detected. At pH 7.6, significant levels of aggregated protein are found (Fig. 8).

Accelerated degradation samples have been further characterised by fractionating the eluted peaks by preparative RP-HPLC. Fractions corresponding to peaks eluting at RRT 0.67 and 1.05 (pH 4.0), and 0.88 and 0.93 (pH 7.6), were analysed and characterised by SDS-PAGE. The results are summarised in Table 2. The pH 4.0 results suggest that the early eluting species (RRT 0.67) by RP-HPLC contains fragmented protein, and the later eluting moiety (RRT 1.05) is monomeric IFN-α. It is interesting that bands associated with low MW species in pH 4.0 samples only appear under reducing conditions. It is likely that the absence of low MW bands under non-reducing conditions is due to the attachment of cleaved protein fragments by disulphide bridges, reduction of which results in the generation of lower molecular species. The early eluting fraction has been further characterised using N-terminal sequence analysis. The sample contains three different clipped species with cleavage sites at G103/V104 (approximately 50%), L111/M112 (approximately 40%) and V106/I107 ($\leq 10\%$). The identity of the later eluting moiety remains to be determined, but it is closely related to the parent IFN-α as samples containing this degradation product show no significant losses in antiviral activity.

A different pattern emerges from the pH 7.6 fractions. In addition to monomeric protein (Met sulphoxide by-products), significant levels of aggregated IFN-α are clearly detected in both fractions. Under reducing conditions the RRT 0.93 aggregate bands lose intensity slightly implying that this fraction probably contains non-covalently linked and covalently linked aggregated protein.

Hence, in pH 4.0 and pH 7.6 solution, IFN-α degrades via different mechanisms to yield different degradation products. At pH 4.0, the major degradation species are both monomeric (but chemically modified) and cleaved IFN-α, the latter probably being formed via self-proteolytic hydrolysis which has been reported in acidic

solutions of proteins (Manning et al., 1989). At pH 7.6, the major degradation products appear to be monomeric protein, in the form of the methionine sulphoxide moieties, together with aggregated IFN-α.

3.3. Preformulation studies

The influence of medium pH and buffer composition of the medium upon the stability of IFN-α has already been addressed (Table 1). Vehicles of pH 4.0 (glycine–HCl based) and pH 7.6 (phosphate based) were selected for further optimisation studies with stabilising excipients.

Carbohydrates are known to stabilise proteins via an exclusion mechanism whereby, in solution, the protein is preferentially hydrated (Arawaka and Timasheff, 1982). Mannitol is commonly used in this role. In this study, incorporation of either mannitol or lactose improves the solution stability of IFN-α at pH 4.0. However, at pH 7.6 although mannitol appears to be a useful stabiliser, lactose causes accelerated degradation via the formation of both Met sulphoxide by-products plus additional unidentified pre-and post-main peak eluting moieties by RP-HPLC.

Non-ionic surfactants, such as polysorbates (Tweens) have been used in order to minimise protein denaturation at solution–air and/or solution–container interfaces (Wang and Hanson, 1988). However, Tween 20 has a deleterious effect upon the solution stability of IFN-α at either pH 4.0 or 7.6, causing accelerated formation of Met sulphoxide by-products together with several, unidentified, early eluting species by RP-HPLC. An additional, late eluting, moiety is also observed at pH 7.6.

Table 4
Influence of medium pH and headspace (during processing and storage) upon the rate of chemical degradation of IFN-α in solution

IFN-α batch no.	Medium pH (atmosphere)	IFN-α degradation rate at 4°C (%/week by RP-HPLC ± S.D.)
019	7.6 (N_2)	0.073 ± 0.028
019	7.6 (O_2)	0.095 ± 0.006
021	7.6 (N_2)	0.070 ± 0.045
028	7.6 (N_2)	0.074 ± 0.057
028	7.6 (O_2)	0.127 ± 0.031
019	4.0 (N_2)	0.026 ± 0.018
019	4.0 (O_2)	0.025 ± 0.025
021	4.0 (N_2)	0.030 ± 0.074
028	4.0 (N_2)	0.002 ± 0.023
028	4.0 (O_2)	0.003 ± 0.027

Metal ions, such as copper and iron, have been proposed as catalysts for oxidation of proteins and peptides in solution. We have found that introduction of metal ions (in the form of ferric citrate) promotes degradation of IFN-α via the formation Met sulphoxide by-products at pH 7.6. However, little change is found at pH 4.0. These results are consistent with the major degradative mechanisms elucidated earlier — the protein is much less susceptible to oxidation at low pH. Chelating agents, such as EDTA, have been used in parenteral formulations primarily for inhibition of oxidation via removal of such metal ions (Wang and Hanson, 1988). Inclusion of EDTA as an antioxidant for IFN- has been shown in this study to offer protection against the formation of Met sulphoxide by-products.

Ascorbic acid has also been investigated as a protein antioxidant (Vemuri et al., 1993). However, this excipient has a deleterious effect upon the solution stability of IFN-α at either pH 4.0 or 7.6, causing a similar degradation profile to that induced by the inclusion of Tween 20.

Although human serum albumin (HSA) has been commonly used as a stabiliser in protein formulations, including interferons (Nail, 1997), exploratory studies with IFN-α showed no advantage of inclusion of this material. The same preliminary screen also identified glycocholate and polygeline (Haemaccel®) as ineffective stabilisers of IFN-α. In each case, multiple degradation products are found by RP-HPLC.

Inclusion of pharmaceutically acceptable preservatives would be necessary for a multi-dose IFN-α product. Examples of the major classes of known preservatives (Evans and Grassam, 1986) were investigated in this study. Benzyl alcohol has an adverse effect upon the solution stability of IFN-α. However, propyl paraben, benzalkonium chloride (BZK) or *m*-cresol appear to be viable preservatives. A summary of the results generated

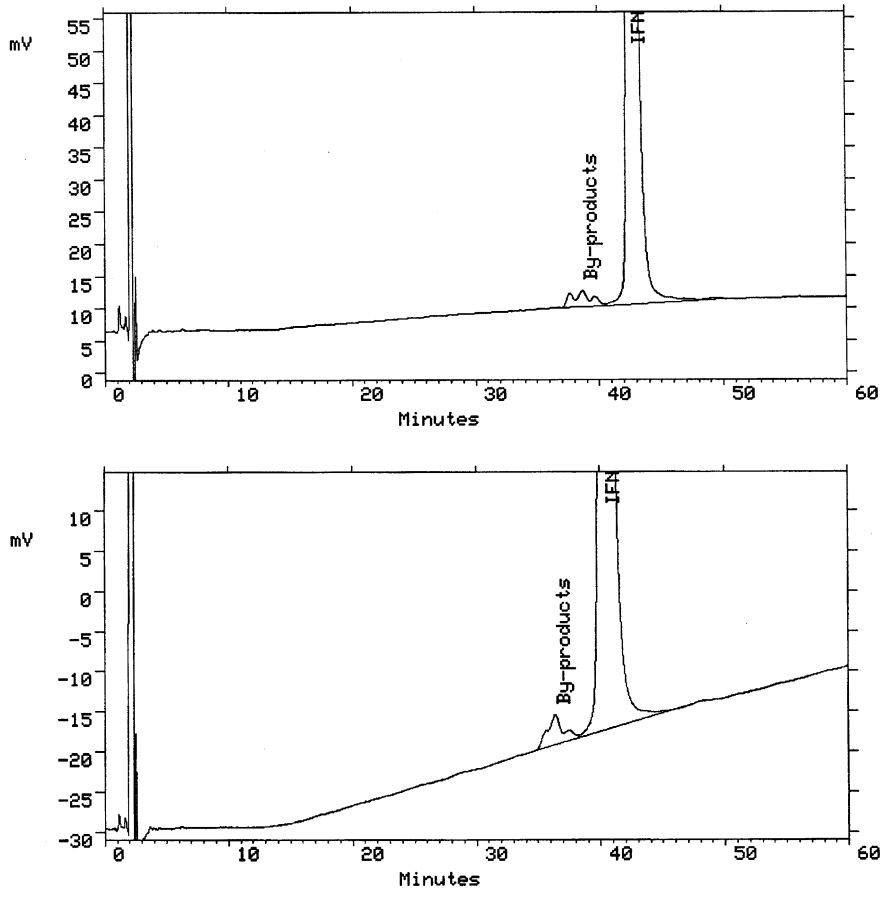


Fig. 9. RP-HPLC of IFN-α at t = 0 (top) and after storage at 4°C for 30 weeks at pH 7.6 (bottom).

using potential 'stabilising' excipients and preservatives is shown in Table 3.

'Real time' stability studies in the 'preferred' vehicles formulated at pH 4.0 and 7.6, at the proposed storage condition (4°C), have been carried out. Degradation approximates to zero-order kinetics over the time periods studied which correspond to small ($< 3\%$) absolute levels of chemical degradation of the protein. This enables a simple 'percent degradation per week' index of protein stability. The results are shown in Table 4. Chemical degradation rates are slightly higher at pH 7.6 than at pH 4.0. At pH 7.6, independent of the batch of material used, degradation of IFN-α at 4°C is indexed by a slow but definite increase in the level of Met sulphoxide by-products (see, for example, Fig. 9). At pH 4.0, the Met sulphoxide by-products remain largely unaltered. With some batches of IFN-α, trace levels (typically $< 0.5\%$) of the late eluting moiety (RRT 1.05), as identified during accelerated screening, are detectable after prolonged storage at 4°C (Fig. 10). However, no additional degradation products are detectable after 30 weeks storage at 4°C with other batches of IFN-α (Fig. 11). No fragmented protein has, so far, been detected under these storage conditions.

IFN-α in pH 4.0 solution is not significantly affected by oxygen. However, oxidation appears to be accelerated in pH 7.6 solution prepared and stored under oxygen or, to a lesser extent, air (data not shown), so maintenance of an inert atmosphere during formulation and storage of a solution of IFN-α at this pH may well be necessary.

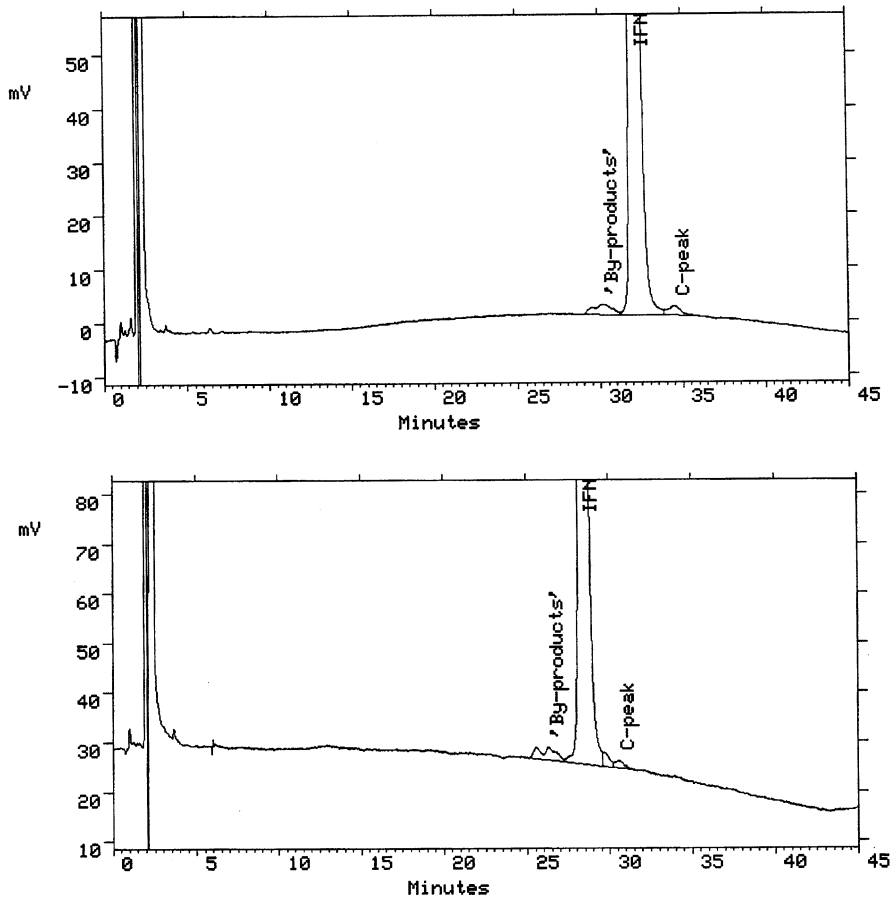


Fig. 10. RP-HPLC of IFN-α (batch 021) at $t = 0$ and after storage at 4°C for 30 weeks at pH 4.0 (bottom).

Table 5
Influence of medium pH upon the solution phase aggregation of IFN-α

Medium pH	IFN-α batch	Storage condition	IFN-α by HP-SEC (%)		
			Monomer	Dimer	Oligomer
7.6	021	4°C 10 week	99.7	0.3	ND[a]
	028	4°C 20 week	98.2	1.4	0.4
	019	4°C 20 week	98.2	0.7	1.1
4.0	021	4°C 10 week	100	ND[a]	ND[a]
	028	4°C 20 week	100	ND[a]	ND[a]
	019	4°C 20 week	99.0	ND[a]	1.0

[a] ND, not detected.

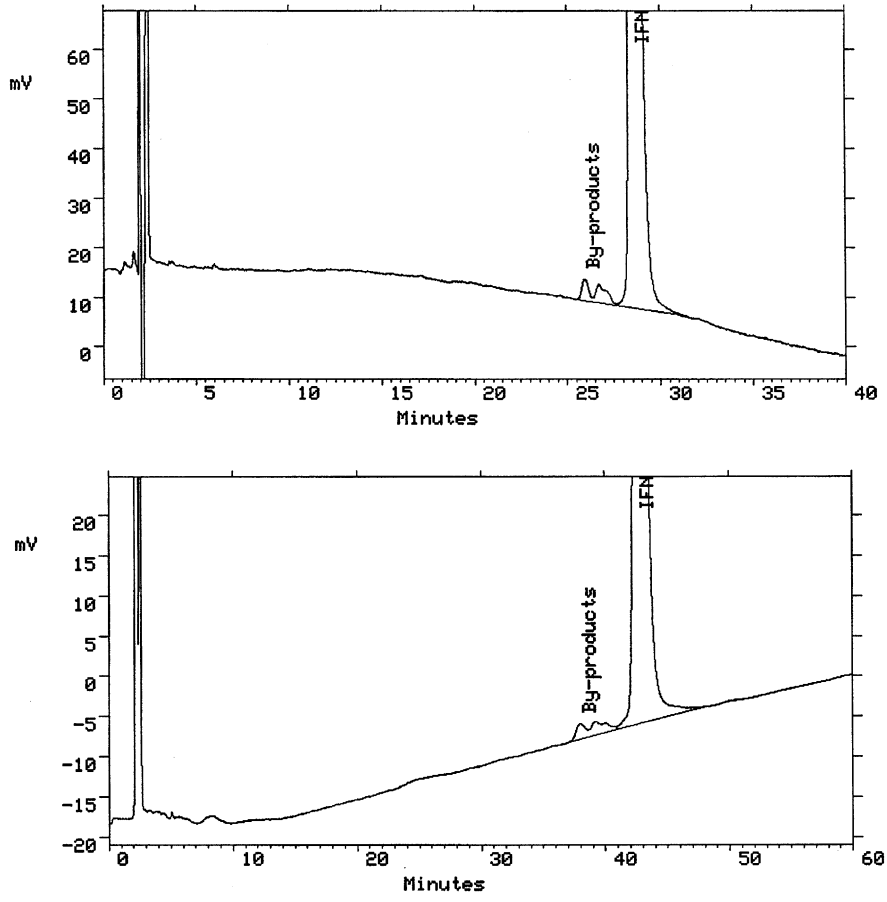


Fig. 11. RP-HPLC of IFN-α (batch 028) at $t = 0$ and after storage at 4°C for 30 weeks at pH 4.0 (bottom).

Overall, the results suggest that a pH 4.0 medium preserves the chemical integrity of IFN-α somewhat better than at pH 7.6.

Physical stability (colour, odour, appearance, pH, absence of insoluble aggregates) of both pH 4.0 and 7.6 formulations was maintained over 30 weeks storage at 4°C. Material balances were fully accounted for, suggesting no significant adsorption of protein to the surfaces of storage containers during this study. No detectable loss of antiviral activity was detected after prolonged storage at either pH at 4°C. However, the low pH medium holds the advantage in terms of preservation of the protein in the monomeric state, as evidenced by levels of soluble aggregates as detected by HP-SEC (Table 5).

On the basis of these results, a product shelf-life of several years appears to be a distinct possibility.

Acknowledgements

The authors acknowledge Dr Gabriele Fendrich's expert contribution to the elucidation of IFN-α by-products and thank her for carrying out N-terminal protein sequencing studies.

References

Arawaka, T., Timasheff, S.N., 1982. Stabilization of protein structure by sugars. Biochemistry 21, 6536–6544.

Borchardt, R.T., Li, S., Schoeneich, C., Wilson, G.S., 1993. Chemical pathways of peptide egradation. V. Ascorbic acid promotes rather than inhibits the oxidation of methionine to methionine sulphoxide in small model peptides. Pharm. Res. 10, 1572–1579.

Evans, S., Grassam, P, 1986. Considerations for parenteral dosage form development of natural alpha interferon. J. Parent. Sci. Technol. 40, 83–87.

Gangemi, J.D., Lazdins, J., Dietrich, F.M., Matter, A., Poncioni, B., Hochkeppel, H.-K., 1989. Antiviral activity of a novel recombinant human interferon-α B/D hybrid. J. Interferon Res. 9, 227–237.

Geigert, J., 1989. Overview of the stability and handling of recombinant protein drugs. J. Parent. Sci. Technol. 43, 220–224.

Hochkeppel, H.K., Grütter, M., Horisberger, M.A., Lazdins, J.K., 1992. Human IFN-alpha hybrids. Drugs Future 17, 899–914.

Itri, L.M., 1992. The interferons. Cancer 70 (Suppl. 4), 940–945.

Lucivero, G., 1992. The interferons in clinical practice. Int. J. Immunopathol. Pharmacol. 5, 83–92.

Manning, M.C., Patel, K., Borchardt, R.T., 1989. Stability of protein pharmaceuticals. Pharm. Res. 6, 903–918.

Meager, A. 1987, In: Clemens, M.J., Morris, A.G., Gearing, A.J.H. (Eds.) Lymphokines and Interferons: a Practical Approach, IRL Press, pp. 129–147.

Meister, A., Uzé, G., Morgensen, K., et al., 1986a. Biological activities and receptor binding of two recombinant interferons and their hybrids. J. Gen. Virol. 67, 1633–1643.

Meister, A., Meyer, F., Hinnen, A., Gruetter, M.G., Alkan, S, 1986b. Hybrid interferons, EP-0205404 A/B.

Nail, S.L., 1997. Formulation of proteins and peptides, Control. Drug Deliv., 185-203.

Oliyai, C., Schöneich, C., Wilson, G.S., Borchardt, R.T., 1991. Chemical and physical stability of protein pharmaceuticals. In: Crommelin, D.J.A, Midha, K.K. (Eds.), Top. Pharm. Sci., Proc. Int. Congr. Pharm. Sci. F.I.P. 51st, Stuttgart, pp. 23–46.

van den Oetelaar, P.J.M., Jansen, P.S.L., Melgers, P.A.T.A., Wagenaars, G.N., ten Kortenaar, P.B.W., 1992. Stability assessment of peptide and protein drugs. J. Control. Release 21, 11–21.

Rieger, P.T., 1995. Interferon-alpha: a clinical update. Cancer Pract. 3, 356–365.

Sedmak, J.J., Grossberg, S.E., 1981. Approaches for the stabilization of interferons. Tex. Rep. Biol. Med. 41, 274–279.

Spiegel, R.J., 1987. Clinical overview of alpha interferon. Studies and future directions. Cancer 59 (Suppl. 3), 626–631.

Stuart-Harris, R.C., Lauchlan, R., Day, R., 1992. The clinical applications of the interferons: a review. Med. J. Aust. 156 (12), 869–872.

Vemuri, S., Yu, C.T. and Roosdorp, N., 1993. In: Wang, Y.J. and Pearlman, R (Eds.), Stability and Characterisation of Protein and Peptide Drugs: Case Histories, New York, pp. 263–287.

Wang, J.W., Hanson, A.M., 1988. Parenteral formulations of proteins and peptides: stability and stabilizers. J. Parent. Sci. Technol. 42 (Suppl.), S4–S26.