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Physicochemical and biological assays for quality control of biopharmaceuticals: Interferon alfa-2 case study

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Abstract

A selection of physicochemical and biological assays were investigated for their utility in detecting changes in preparations of Interferon alfa-2a and Interferon alfa-2b (IFN-α2a, IFN-α2b), which had been subjected to stressed conditions, in order to create models of biopharmaceutical products containing product-related impurities. The stress treatments, which included oxidation of methionine residues and storage at elevated temperatures for different periods of time, were designed to induce various degrees of degradation, aggregation or oxidation of the interferon. Biological activity of the stressed preparations was assessed in three different in vitro cell-based bioassay systems: a late-stage anti-proliferative assay and early-stage assays measuring reporter gene activation or endogenous gene expression by quantitative real time Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). Relevant physicochemical methods such as SDS-PAGE, reverse phase (RP) chromatography, size-exclusion chromatography (SEC) and dynamic light scattering (DLS), proved their complementarity in detecting structural changes in the stressed preparations which were reflected by reductions in biological activity.

Keywords: Interferon; Bioassay; Dynamic light scattering; Biopharmaceutical; qRT-PCR

1. Introduction

Biopharmaceuticals are complex products which require rigorous quality control (QC) to ensure their safety and efficacy. During drug development, extensive characterisation of batches of the drug is conducted. No single analytical method is able to assess every relevant attribute, so a battery of physicochemical and biological assay techniques, providing complementary information, is required [1–3]. Some of the techniques commonly used for initial characterisation of products are costly, time-consuming or require a large amount of the test product. However, once the manufacturing process has been well established, a profile of the attributes of the drug, which show batch-to-batch variations, can be determined. It is then often possible to reduce the number of different types of assay necessary for QC for routine batch release. Subsequent changes in the manufacturing process may require a return to a wider range of analytical methods to demonstrate the comparability of batches pre- and post-change [1,3].

The purpose of QC for batch release is to demonstrate the similarity of a batch of drug to earlier batches, which were tested in clinical trials and shown to be safe and effective. For the majority of biopharmaceuticals, a single or a selection of physicochemical assays, which is practical to use for routine batch release, are not able to characterise the product sufficiently to predict its biological activity [4]. A biological assay, or bioassay, to measure biological activity, is therefore

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a regulatory requirement of the specifications for batch release for most biopharmaceuticals [2,5].

Selection of an appropriate set of assays and techniques for batch release has important consequences in terms of cost, efficiency and effectiveness. It requires an understanding of the complementarity of the various analytical techniques and of structure–function relationships for the particular product, which can be key to performing QC in a cost-effective way.

To demonstrate the benefits of such a selection of physicochemical and biological assays in assessing the quality of preparations of a model biopharmaceutical, we chose as a model interferon alfa-2 (IFN-α2).

IFN-α is currently one of the top selling biopharmaceutical drugs for therapeutic use against hairy-cell leukemia, AIDS-related Kaposi's sarcoma, hepatitis B and C [6]. As a result, there has been extensive research into its physicochemical properties and biological activity, which can be correlated to clinical experience. The amino acid sequences of IFN-α2a and IFN-α2b differ by only one residue (K23R) and comparison [7] of the three dimensional high resolution structure of human IFN-α2a determined by heteronuclear NMR spectroscopy in solution with the X-ray structure of IFN-α2b [8] concluded the overall structures are identical. However, a comparison by immuno-based methods [9] detected differences between IFN-α2a and IFN-α2b. Few published studies [10,11] have analysed simultaneously both physicochemical and biological properties of IFN-α2.

In this study we have investigated the impact of various physicochemical characteristics on the biological activity of IFN-α2a and IFN-α2b. In order for the results of such a study to be relevant to batch release testing, it is important that realistic samples are used; for example, mildly degraded samples reflecting possible product-related impurities in a batch of drug.

Therefore, in an attempt to mimic variations likely to be found between batches during pharmaceutical production, IFN-α2 degradation samples were generated from oxidation of methionine residues, as discussed by Drevenkar et al. [12], following a method recommended by the European Pharmacopoeia [13]. In addition, IFN-α2 samples with several degrees of aggregation were produced, as shown by Ruiz and co-workers [10], following storage at different conditions of time and temperature. For function and structure characterisation, we then applied a range of different biological assays, (early- and late-stage in vitro cell-based), including quantitative real time Reverse Transcription-Polymerase Chain Reaction (qRT-PCR); and a selection of physicochemical techniques, including SDS-PAGE, reverse phase (RP) chromatography, size-exclusion chromatography (SEC) and dynamic light scattering (DLS). Studies under accelerated stress conditions may also be useful in determining whether accidental exposures to unfavourable conditions (e.g. during transportation) are deleterious to the product, and to evaluate which specific test parameters may be the best indicators of product stability. We examined the utility of these various techniques in assessing the structural integrity and biological activity of IFN-α2a and IFN-α2b preparations, with particular interest in the potential utility of qRT-PCR and DLS, which are currently not so widely used in batch release testing.

2. Materials and methods

2.1. Samples

IFN-α2a and IFN-α2b chemical reference substances were purchased from European Directorate for the Quality of Medicines, EDQM CRS I03200300 and CRS I03200301, respectively, (Strasbourg, France), as a 1.46 mg/mL and a 7.19 mg/mL solution, at pH5 and pH7, respectively. The pH was measured using a pH PocketFET, Sentron 501 pH meter, with an electrode suitable for small volumes (20 μL), calibrated with pH buffer solutions for pH4, pH7 and pH10 (Thermo Fisher Scientific, UK) and indicator paper (VWR International, UK) for pH4–7 and pH5–10. Information on the formulation components (buffer salts, excipients) was not supplied and not available at the time of purchase. Although IFN-α2a and IFN-α2b supplied by EDQM are "*exclusively…. for use as standards or reference substance in the tests and assays indicated above, in accordance with the official methods of the Ph. Eur., and for no other purpose*" [14,15], we were able to show in a previous study that the EDQM CRS had similar biological activity to the respective International Standards (ISs) [16] in the bioassay systems used, and could thus be used as reference standards for biological activity. This enabled us to use the same preparation for both physicochemical and bioassay analyses. We also investigated whether the standards were appropriate for a wider range of physicochemical assays than specified by EDQM [14,15]. Table 1 summarises the various IFN-α2 preparations and codes used in this study.

2.1.1. Methionine oxidation

Oxidation of methionine residues in IFN-α2a material was carried out as described in the method recommended by the European Pharmacopoeia [13]. Some modifications were

Table 1
Summary of the various degradation samples of IFN-α2a and IFN-α2b used in this study, following deliberate oxidation of methionine residues and storage at different conditions of time and temperature

Preparation	Study code	Treatment	Study code
EDQM CRS IFN alfa-2a (I0320300)	IFN-α2a	Methionine oxidation	IFN-α2a, OX
		Oxidation control	IFN-α2a, OXcontrol
		Storage −70 °C	IFN-α2a, −70 °C
		Storage +37 °C	IFN-α2a, +37 °C
EDQM CRS IFN alfa-2b (I0320301)	IFN-α2b	Storage −70 °C	IFN-α2b, −70 °C
		Storage +37 °C	IFN-α2b, +37 °C
2nd International standard IFN alfa-2a (code 95/650)	IS IFN-α2a	Storage −70 °C	IS IFN-α2a, −70 °C
2nd International standard IFN alfa-2b (code 95/566)	IS IFN-α2b	Storage −70 °C	IS IFN-α2b, −70 °C

made to obtain a larger amount of oxidised product, by extending the oxidation reaction to 3 h. A control sample of IFN-α2a was exposed to the same conditions as the modified material, without the oxidising reagent (hydrogen peroxide). All samples were stored at −70 °C prior to assay.

2.1.2. Storage conditions

Preparations of IFN-α2a and IFN-α2b material were diluted to 0.4 mg/mL in phosphate-buffered saline (PBS, pH7; Invitrogen Ltd., Paisley, UK) and stored at −70 °C, +4 °C, +20 °C and +37 °C, for periods up to 25 weeks. The pH of the diluted solutions of IFN-α2 was measured before storage as pH6.4 and pH7, respectively, for IFN-α2a and IFN-α2b. The 2nd ISs for IFN-α2a (code 95/650) and IFN-α2b (code 95/566), used as reference standards for the bioassay analysis, were reconstituted as described on the instructions for use [17,18] and stored at −70 °C prior to assay.

2.2. In vitro cell-based assay systems

In this study, three different in vitro cell-based assay systems were developed or adapted to measure the relative activity of the preparations of IFN-α2a and IFN-α2b. These bioassays comprised an anti-proliferative assay, i.e. one measuring a late-stage cellular response, a reporter gene activation assay, i.e. one measuring an early-stage cellular response and a qRT-PCR based assay [19].

Bioassay systems are affected by a wide variety of factors, some of which are difficult or impossible to control. As a result, responses measured can vary between assays, so the activity of the test sample is measured relative to that of a reference standard of a similar material. Any variation in the assay system should affect the response of the test sample and of the reference standard to the same extent, and hence the potency of the test sample relative to that of the reference standard, i.e. the relative potency, should remain constant. In the bioassays reported here, biological activities were measured relative to appropriate reference standards as detailed for each case.

2.2.1. Daudi cell anti-proliferative assay

Cells (CCL-213™, LGC Promochem, UK) were maintained in culture medium (RPMI-1640 modified medium supplemented with 10% (v/v) Foetal Bovine Serum (FBS); LGC Promochem, UK) in 75 cm^2 flasks (Corning®, 430641; Thermo Fisher Scientific, UK) and passaged three times a week, seeding at approximately $1-3 \times 10^5$ cells/mL ($2.2-6.6 \times 10^6$ cells/flask). Under this maintenance regime, the Daudi cells were shown to maintain their responsiveness to IFN-α2 over many passages (from P2 to greater than P60). For assay, cells were seeded in 96-well culture plates (Nunc™, 167008; Thermo Fisher Scientific, UK) at a density of 5×10^4 cells/mL (1×10^4 cells/well) in culture medium and dosed on seeding with a 2-fold dilution series range of stock solution of IFN-α2a or IFN-α2b in culture medium. After incubation (37 °C, 5% CO_2 in air) for 72 h, 40 μL/well of alamarBlue™ (BUF012B; Serotec Ltd., Oxford, UK) was added and the plates were incubated for further 16 h. The response was measured as the absorbance ($A_{570nm} - A_{600nm}$), using a spectrophotometer, SpectraMax® 340PC (Molecular Devices Ltd., UK). Development and optimization of this protocol is described in Refs. [16,20].

2.2.2. Luciferase reporter gene assay

A549/93D7 cells, which are transfected with the promoter of the *M×A* gene fused to a luciferase reporter gene (kindly donated by Dr G. Adolf, Boehringer Ingelheim), were maintained in culture medium (DMEM without phenol red supplemented with 10% (v/v) heat-inactivated FBS, 50 U/mL penicillin, 50 μg/mL streptomycin, 500 μg/mL antibiotic G418 geneticin and 2 mM L-glutamine; Sigma–Aldrich Co Ltd., UK) in 75 cm^2 flasks (Falcon®, 353024; Thermo Fisher Scientific, UK) and passaged twice a week. Plates were seeded at approximately 3.25×10^4 cells/mL (6.5×10^5 cells/flask). For assay, cells were seeded in white 96-well culture plates with shielded wells (6005688; Perkin Elmer LAS, Beaconsfield, UK) at a density of 5×10^5 cells/mL (5×10^4 cells/well) in culture medium and incubated (37 °C, 5% CO_2 in air) for 16 h. After this period, supernatant was removed and cells were dosed with a 2-fold dilution series range of stock solution of IFN-α2a or IFN-α2b in assay medium, (culture medium with 0.2% (v/v) FBS instead of 10%), and incubated (37 °C, 5% CO_2 in air) for 6 h. The 2-fold dilution series was first prepared on polypropylene 96-well plates (ScreenMates™, Matrix products; Thermo Fisher Scientific, UK) and then 100 μL/well transferred into the assay plate. After the 6 h incubation with IFN-α2, 100 μL/well of LucLite® (Perkin Elmer LAS Ltd., UK) was added and the plates were incubated at room temperature for 10–20 min, before measuring the luminescence, using a liquid scintillation and luminescence counter, Perkin Elmer 1450 Wallac Microbeta® Trilux. The response was measured as the number of counts per well in a 5-s counting period. Note that LucLite® has recently been replaced by SteadyLite plus™ (Perkin Elmer LAS Ltd., UK). Development and optimization of this protocol is described in Refs. [16,20].

2.2.3. qRT-PCR

A549/93D7 or A549 (CCL-185; LGC Promochem Ltd., Teddington, UK) cells were prepared and dosed with IFN-α2 as described in Section 2.2.2. After 90 min incubation with IFN-α2a or IFN-α2b, total RNA was extracted from control (unstimulated) and IFN-stimulated A549/93D7 and A549 cells, using RNAeasy spin-column technology (RNAeasy Protect Mini Kit; Qiagen Ltd., Crawley, UK), according to the manufacturer's instructions. As an alternative procedure, RNA was extracted from samples that had been incubated with IFN-α2 and then treated with lysis buffer (Qiagen Ltd., Crawley, UK) and stored at −70 °C for later processing. Complementary DNA (cDNA) was synthesised simultaneously from all RNA samples using MMLV-RT Superscript II (Promega Ltd., Southampton, UK) as previously described [19]. Quantitative RT-PCR amplification reactions were performed on a Rotor-Gene™ 6000 series real time rotary system

(Corbett Research Ltd., Cambridge, UK) containing 2× Sensimix (Quantace Ltd., London, UK), 50× SYBR green, forward and reverse primers (500 nM final concentration), sample cDNA (1/3 dilution in water) and standard DNA (generated from a purified PCR product of the gene of interest). Selected forward and reverse PCR primers were used to detect the expression of the genes of interest. Human *M×A* primer sequences were forward; 5′-CTCCCACTCCCTGAAATCTG-3′, reverse; 5′-CTGTTCTCCTGCACCTCCTT-3′. The housekeeping gene *GAPDH* was amplified in the same samples to allow for normalisation of the initial amount of cDNA. Primer sequences for *GAPDH* were as previously published [21]. PCR reactions included 40 cycles consisting of 95 °C denaturation for 10 s, annealing for 10 s at 55 °C, a 72 °C extension phase for 10 s followed by a 77 °C extension phase for 1 s. Fluorescence measurements were taken at the end of the 77 °C extension phase to avoid primer–dimer interference.

2.2.4. Statistical analysis of bioassay data

All raw data were expressed as means ± SEM. For the antiproliferative and reporter gene assays, relative potencies were calculated from dose-response curves using an in-house program, WRANL [22]. Comparisons among estimates of activity were made using analysis of variance of the logarithms of the estimates. Where estimates were combined, they were calculated as geometric means, and the variances among the means were summarised as geometric coefficients of variation (GCV). Confidence intervals have been based on the variability of the logarithms of the estimates combined, typically for the variation among estimates from separate plates in independent assays where between-assay variation has been shown to be similar to between-plate variation. Where a single estimate from a single plate was available, the confidence intervals are based on the between-replicate variation within the assay plate [23]. The qRT-PCR data were analysed as parallel-line assays, after selecting a linear portion of the dose-response curve (log transformed response against log dose). An in-house program was used.

2.3. Physicochemical characterisation

IFN-α2 preparations were analysed by SDS-PAGE, RP- and SEC- high performance liquid chromatography techniques (HPLC), and DLS.

SDS-PAGE

Samples were treated with a solution of Laemmli buffer and run on 4–12% SDS homogeneous gels, in reducing conditions, 2 μg or 4 μg of material was loaded into each lane of the gel and detection of resolved protein bands was by Silver staining (PlusOne™, 17-1150-01; Amersham Biosciences, NJ, USA) or Coomassie Blue staining (Colloidal Coomassie; Invitrogen Ltd., Paisley, UK). Stained gels were scanned using a BioRad GS800 densitometer and percentage areas for the product bands were calculated.

RP-HPLC

Chromatographic analyses were carried out on a JASCO HPLC with PU-2080 Plus pumps equipped with a UV PDA detector (MD-2010 Plus; Spectrum Max Plot), autosampler and column oven. Injections consisted of 50 μL of sample solutions at 0.2 mg/mL. The protocol for the oxidation analysis was based on the method recommended by the European Pharmacopoeia [13] modifying the percentages of TFA (trifluoroacetic acid) used in the two elution buffers to improve resolution. In particular, 0.1% TFA in chromatographic buffer A and 0.085% TFA in buffer B were used, as suggested by the column application notes.

SEC-HPLC

Analyses were carried out in PBS on a Phenomenex BioSep S2000 column, at 1 mL/min flow rate. Injections consisted of 10 μL of sample solutions at 1 mg/mL were interspersed with blank injections. Detection at 280 nm. The column was calibrated for molecular weight determination by injecting a 1 mg/mL sample of myoglobin (17,000 Da, RT = 8.45 min). The molecular weight (MW) of monomeric IFN-α2a predicted by the amino acid sequence is 19,241 Da and 19,269 Da for IFN-α2b.

DLS

Analyses were carried out on a Nano-ZS (Malvern Instruments) in a low volume disposable cuvette at 25 °C. The sample solutions, 0.2 mg/mL in PBS, were analysed using the manufacturer's dispersion technology software. The hydrodynamic radius (R_H) of the major species present in solution (evaluated by the volume distribution) was estimated by multimodal analysis of the intensity size distribution.

3. Results

3.1. Oxidised IFN-α2a

IFN-α2a exposed to oxidising conditions (IFN-α2a, OX) was compared with untreated material (IFN-α2a, OxControl). Oxidation was confirmed by RP-HPLC with the appearance of an earlier-eluting peak, as shown in Fig. 1. The percentage of IFN-α2a oxidised was estimated as approximately 60% by comparison with a calibration curve as described in the European Pharmacopoeia [13]. The biological activity of the oxidised preparation was compared with that of the control preparation by the anti-proliferative and the reporter gene bioassays. By both assay systems, the oxidised IFN-α2a showed significantly reduced specific biological activity. By the anti-proliferative assay, the relative activity of the oxidised IFN-α2a compared with the control material was 0.55 (95% confidence interval, 0.45–0.68, geometric mean of 7 estimates from 2 independent assays, 3 or 4 plates per assay). By the reporter gene assay, the relative activity of the oxidised IFN-α2a was 0.35 (95% confidence interval, 0.31–0.39, geometric mean of 4 estimates from 1 assay). Selected dose-response curves for the 2 assay systems are shown in Fig. 2.

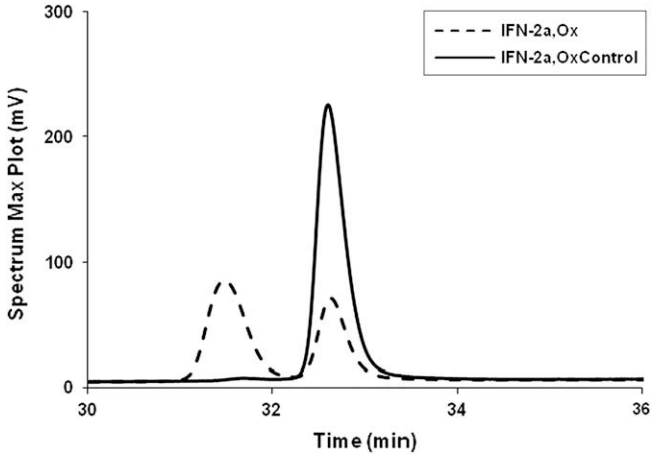

Fig. 1. Representative RP-HPLC trace of methionine oxidised EDQM CRS IFN-α2 (IFN-α2a, Ox, dotted line), compared to a control (IFN-α2a, OxControl, solid line), $n = 2$.

3.2. Storage at elevated temperature

Screening studies to determine storage conditions required to generate IFN-α2 samples with partial loss of biological activity for physicochemical analyses were carried out for IFN-α2a and IFN-α2b. Assessment by the anti-proliferative and reporter gene assays of the biological activity of these samples stored at the various temperatures (−70 °C, +4 °C, +20 °C and +37 °C, for periods up to 25 weeks) in 3 separate stress studies showed a time- and temperature-dependent loss of activity. Selected time points are shown in Table 2. In addition, samples from all time points at the different temperatures were also subject to analyses by SDS-PAGE (data not shown), to demonstrate the sensitivity of the various methods in detecting changes in the preparations. For logistical reasons, and to avoid freeze-thaw cycles, samples were analysed immediately by bioassay at the different storage time points and, as a consequence, could not all be analysed in the same assay. At each time point, the biological activities of the samples stored at the various temperatures were measured relative to the equivalent IFN-α2a and IFN-α2b samples stored at −70 °C, as summarised in Table 2. To permit inter-assay comparisons, the ISs were included in each assay. At +4 °C (data not shown), estimates of relative activity did not differ significantly between the different time points and showed no trend across the different time points. At 9 weeks, samples IFN-α2b stored at +37 °C showed around 30% of the activity of samples stored at −70 °C and this was judged to be an appropriate degree of degradation for testing the utility of the analytical techniques. This time point was not sampled for IFN-α2a in the initial

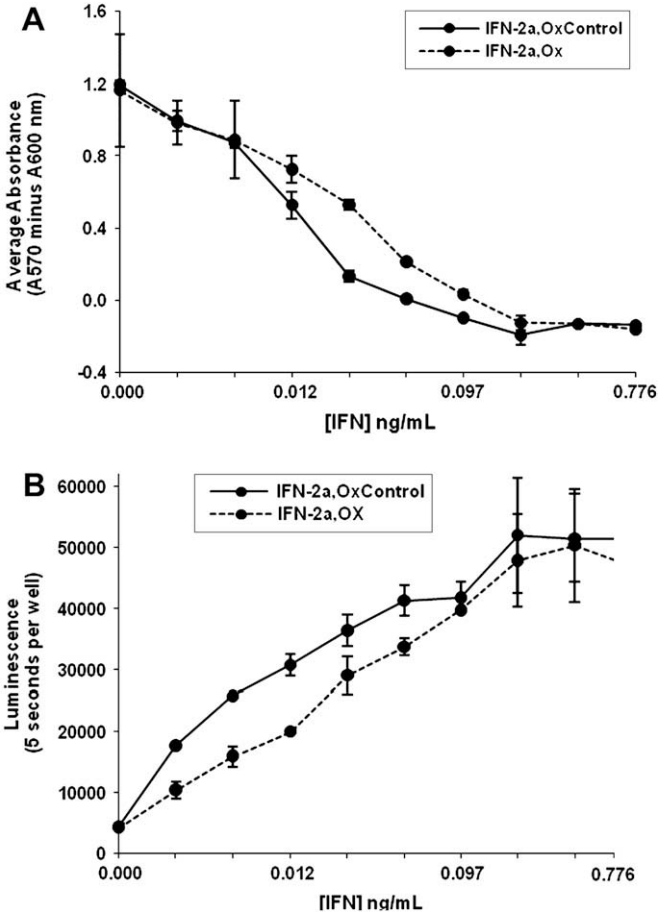

Fig. 2. Selected IFN-α2 dose-response curves for methionine oxidised EDQM CRS IFN-α2a (IFN-α2a, Ox, dotted lines) compared to a control (IFN-α2a, OxControl, solid lines). (A), Daudi anti-proliferative assay; (B), luciferase reporter gene assay. Data are the means ± SEM ($n = 2$).

Table 2
Summary of screening studies to determine storage conditions required to generate IFN-α2 samples with partial loss of biological activity for physicochemical analyses

Sample	Storage temp.	Study	Storage time (weeks)						
			2	6	9	9.5	12	24	25
Specific activity measured by Luciferase reporter gene assay									
IFN-α2a	+20 °C	S1	1.03	1.06	–	–	–	0.92	–
		S2	1.10	1.11	–	–	1.09	1.01	–
	+37 °C	S1	1.06	0.61	–	–	–	0.05 (3)	–
		S2	0.91	0.43	–	–	0.14 (3)	0.07	–
IFN-α2b	+20 °C	S3	1.08	1.40	1.22	–	1.28	–	1.12
	+37 °C	S3	0.84	0.29	0.11 (3)	–	0.10 (3)	–	0.02 (3)
Specific activity measured by Daudi cell anti-proliferative assay									
IFN-α2a	+20 °C	S1	0.90	1.14	–	0.85	–	1.23	–
		S2	1.09	–	–	–	1.13	1.18	–
	+37 °C	S1	0.77	0.80	–	0.47 (3)	–	0.12 (3)	–
		S2	0.77	–	–	–	0.19 (3)	0.07	–
IFN-α2b	+20 °C	S3	1.14	–	1.19	–	1.54	–	0.86
	+37 °C	S3	0.97	–	0.30	–	0.19 (3)	–	0.02 (3)

Comparison by early-stage luciferase reporter gene assay or late-stage Daudi cell anti-proliferative assay of specific activities of EDQM CRS IFN-α2a and EDQM CRS IFN-α2b preparations, stored at +20 °C or +37 °C for periods up to 25 weeks, relative to a sample of the same IFN-α2 preparation stored at −70 °C, for the same periods. At the end of each storage period, activity was measured immediately, relative to that of a sample stored at −70 °C for the same period. Three separate storage studies, coded S1, S2 and S3, were conducted. Numbers represent the mean activity of two estimates from 2 plates, except where noted (3)For three plates. IFN-α2 samples stored at +37 °C for 6 or 9 weeks showed a loss of activity judged suitable for testing the performance of analytical techniques.

stress studies, but was judged to produce a degree of change in IFN-α2b, which would be suitable for study by other analytical techniques. To produce similarly stressed samples of IFN-α2a, aliquots were subjected to 9 weeks at +37 °C and a similar loss of biological activity was demonstrated as shown in the dose-response curves of Fig. 3A,B. To determine whether changes causing such a loss in biological activity could be detected by other techniques, samples of IFN-α2a and IFN-α2b from 9 weeks storage at + 37 °C or −70 °C were subjected to analyses by a range of physicochemical techniques, and a further bioassay system, qRT-PCR.

Quantitative RT-PCR measurements, using the A549/93D7 reporter gene cells, also showed a loss of activity in the IFN-α2a and IFN-α2b samples stored at +37 °C for 9 weeks, compared with samples stored at −70 °C. For IFN-α2a, the estimate of potency of the +37 °C sample was 0.389 (95% confidence interval, 0.213−0.669, geometric mean of 1 independent assay, 1 estimate) relative to the −70 °C sample (Fig. 3C). For IFN-α2b, the estimate of potency of the +37 °C sample was 0.234 (95%, confidence interval, 0.162−0.328, 1 estimate) relative to the −70 °C sample (Fig. 3D). The use of the A549/93D7 cell line enabled direct comparison by qRT-PCR and reporter gene activation assays of the same cell stock, dosed with the same IFN-α2 preparations in the same experiment to facilitate comparison of the two assay systems. Similar results were obtained for the non-transfected A549 cells, (data not shown), for the qRT-PCR. No differences were observed between results obtained from samples processed immediately following incubation of the cells and those obtained from samples lysed and stored at −70 °C for later processing, as reported in previous studies [19].

SDS-PAGE analysis of the samples of IFN-α2a and IFN-α2b stored at elevated temperatures showed time- and temperature-dependent changes. Fig. 4 shows the profiles for the samples stored for 9 weeks at −70 °C and +37 °C. High molecular weight (HMW) bands were detected at +37 °C for both preparations. The SEC results for the control samples stored at −70 °C reveal >95% monomer content, with little or no HMW species (Fig. 5 and Table 3). DLS is considered as a complementary technique to SEC; while SEC is invaluable for detecting low molecular weight oligomers (LMW; i.e. dimers, trimers, etc.), the strength of DLS lies in its ability to detect very small amounts of HMW aggregates. However, DLS does not resolve the LMW species. The DLS results for the IFN-α2 samples (Fig. 6 and Table 3) reveal that the major species are particles in the size range 0−10 nm, which can be attributed to the monomer, with some HMW aggregates in the 100−1000 nm size range. When the DLS results are normalised by the volume of the scattering particle (data not shown), the contribution from the HMW species is very low: 0% for IFN-α2b and 0.1−0.2% for IFN-α2a. After 9 weeks storage at +37 °C, the IFN-α2b material showed protein aggregation amounting to 39% of HMW species, when analysed by SEC (Table 3). In addition, DLS showed an increase in the R_H of the main peak (Fig. 7 and Table 3), indicative of a shift in the population towards HMW species. All the samples exhibit a large polydispersity index that is indicative of a sample with a range of species of different MW, (within the instrument resolution capability − 3 times the size or 12 times the MW). The higher R_H measured for IFN-α2b sample after stressing conditions (Fig. 7 and Table 3) corresponds to the SEC results (Fig. 5), indicating the presence in solution of

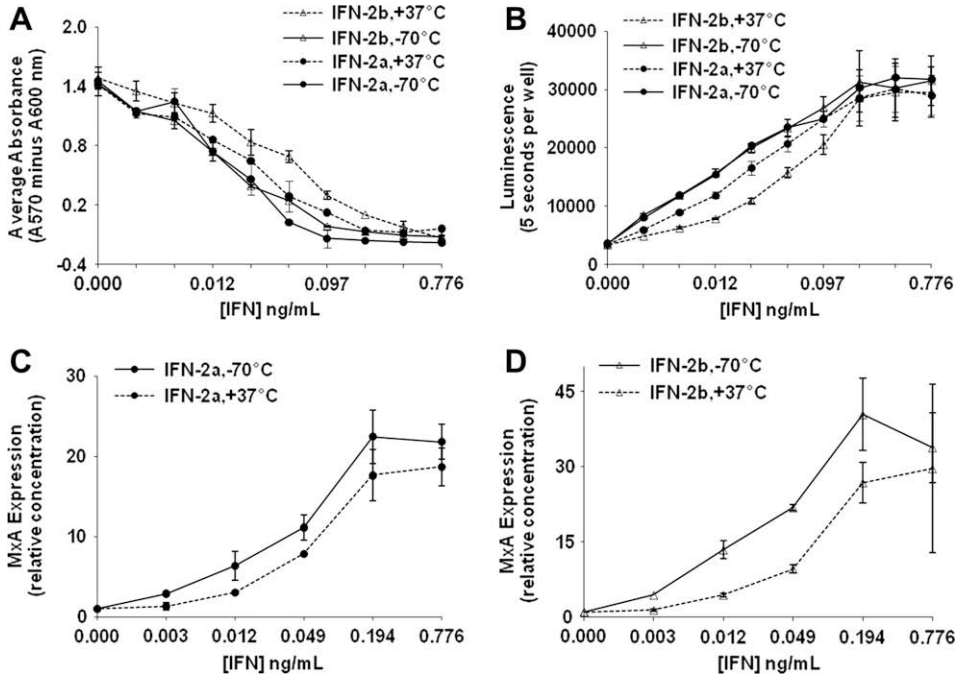

Fig. 3. Selected IFN-α2 dose-response curves for EDQM CRS IFN-α2a and EDQM CRS IFN-α2b preparations, after 9 weeks at −70 °C (IFN-α2a,−70 °C, IFN-α2b,−70 °C) and +37 °C (IFN-α2a,+37 °C, IFN-α2b, +37 °C). (A), Daudi anti-proliferative assay; (B), luciferase reporter gene assay, and (C) and (D) qRT-PCR. Data are the means ± SEM ($n = 2$).

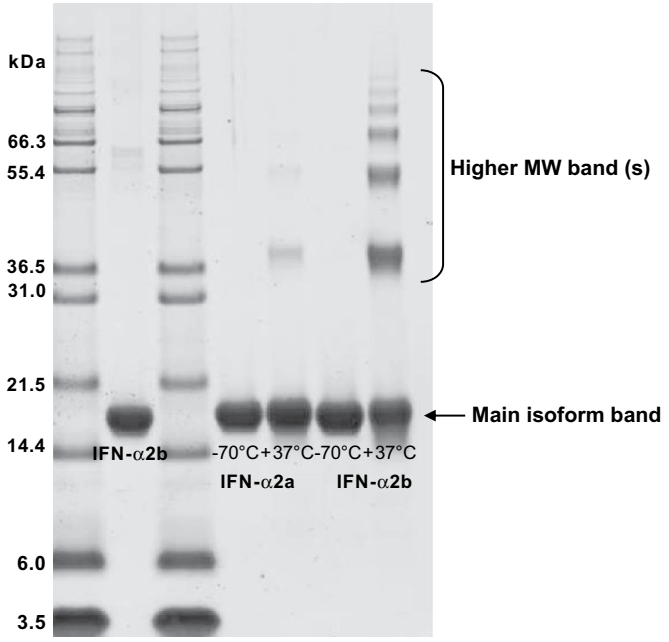

Fig. 4. Selected SDS-PAGE gel in 4–12% reducing conditions for EDQM CRS IFN-α2a and EDQM CRS IFN-α2b preparations, after storage at −70 °C and +37 °C for 9 weeks (4 μg loading). Molecular weight (MW) ladder (kDa) is Marker 12 (Invitrogen Ltd.).

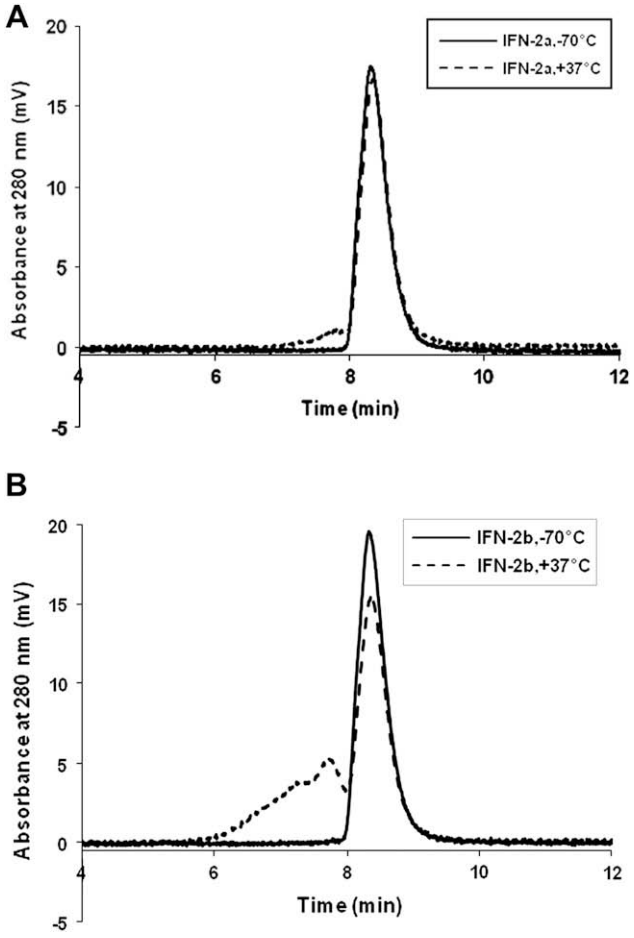

Fig. 5. Representative SEC-HPLC chromatograms of (A), EDQM CRS IFN-α2a; and (B), EDQM CRS IFN-α2b preparations, after 9 weeks storage at −70 °C (IFN-α2a,−70 °C, IFN-α2b,−70 °C, solid lines) and +37 °C (IFN-α2a, +37 °C, IFN-α2b, +37 °C, dotted lines). Absorbance measured at 280 nm ($n = 2$).

aggregate species of HMW. The IFN-α2 preparations were then examined by RP-HPLC, previously demonstrated to detect oxidised IFN-α2a (Fig. 1). IFN-α2, +37 °C showed a chromatographic profile characteristic of oxidised material (data not shown); a peak eluting before the main isoform with a similar shift in retention time to material that had been exposed to accelerated oxidative conditions.

4. Discussion

QC for batch release of a biopharmaceutical is based on a limited set of complementary techniques that should permit detection and assessment of any likely, clinically important, differences between the batch and those used in clinical trials. A bioassay for potency measurement is usually a required part of the specifications for batch release. Potency measurements may be sensitive to a number of structural features if these affect the biological activity in the assay system. Bioassays can present a variety of logistical problems, and, in this study, three different bioassay systems were used, revealing a number of advantages and disadvantages to each system.

Since no single analytical method is able to compare every aspect of protein structure or function, a number of physicochemical techniques are also commonly used to provide complementary information to assess the properties of the product. This study examined the utility of a range of physicochemical and bioassays techniques in testing a model biopharmaceutical, IFN-α2, which had been subjected to stress conditions, in order to create models of biopharmaceutical products containing product-related impurities, that might be encountered in production and in stability studies, to represent the type of variation that would need to be detected if it occurred in batches of the product. Oxidation and aggregation were found to provide a suitable set of samples with a range of degrees of degradation. Alternative degradation techniques such as deamidation or fragmentation could probably have generated a similarly suitable set of degraded preparations.

A wide range of bioassay systems is used in release testing. Here we have compared assays of two commonly used types: a cell-based late-stage bioassay, measuring change in cell

Table 3
Summary of the results for SEC-HPLC and DLS for the various degradation samples of EDQM CRS IFN-α2a and EDQM CRS IFN-α2b, following deliberate storage at different temperatures for 9 weeks

Sample	SEC		DLS	
	% monomer	% HMW species	R_H (nm)	Width (nm)
IFN-α2a, −70 °C	100	0	4.58 (± 0.36)	0.862
IFN-α2a, +37 °C	95	5	4.87 (± 0.27)	0.817
IFN-α2b, −70 °C	100	0	5.20 (± 0.31)	1.150
IFN-α2b, +37 °C	61	39	5.93 (± 0.12)	1.560

HMW, high molecular weight; and R_H, hydrodynamic radius.

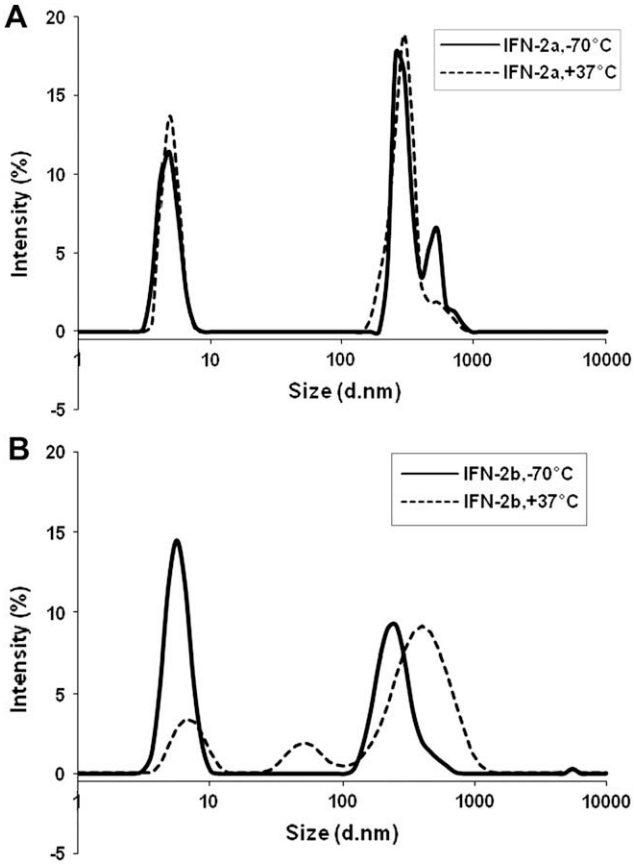

Fig. 6. Representative DLS data of (A), EDQM CRS IFN-α2a; and (B), EDQM CRS IFN-α2b preparations, after 9 weeks storage at −70 °C (IFN-α2a,−70 °C, IFN-α2b,−70 °C, solid lines) and +37 °C (IFN-α2a,+37 °C, IFN-α2b, +37 °C, dotted lines). Size distribution by intensity ($n = 9$).

survival and proliferation; and an early-stage response assay, measuring the induction of a reporter gene. The measured response in both assay systems depends on binding of the product to the same cell surface receptor. The two systems proved similar in their measurements of differences in potency between the various IFN-α2 samples, but offered different logistical advantages. The late-stage anti-proliferative assay required a total time of 88 h between dosing the sample and obtaining a relative potency value, while the early-stage reporter gene assay required only 6 h. In some situations, obtaining a potency value rapidly may be a significant advantage, possibly permitting adjustments in a production process, for example. As a consequence of shortened incubation times, sterile conditions may not be required for dosing in such an assay and there is a reduction in the time for which incubator facilities are required. However, the early-stage reporter gene assay did require the provision and maintenance of a genetically modified cell line and the consumables − reagents and assay plates − were more expensive than those required for the late-stage assay. The choice between two such systems would probably depend largely on the specific requirements of a biopharmaceutical company and its existing assay platforms. The study also briefly examined the induction of specific mRNA species and their quantification by qRT-PCR as a bioassay for IFN-α2. The relative potencies of different IFN-α2 preparations were similar to the results obtained for the late-stage assay and the reporter gene assay and the total assay time of 6 h was similar to that of the reporter gene assay. The results from this study indicate that qRT-PCR can serve as an early-stage bioassay to measure the biological activity of IFN-α2 preparations as recently reported for another biologically active molecule [19]. Its potential utility in release testing depends on a number of factors. It does require specialised equipment and reagents are relatively expensive but it can be performed in non-genetically modified cell lines. Furthermore, the very short incubation time for the product with the cells could allow considerable flexibility with sub-optimal cell culture conditions. Current equipment permits relatively low sample throughput, but on-going developments in the field and wider adoption of this type of assay platform for a variety of purposes may result in the availability of higher throughput, lower cost, equipment, which could make feasible the use of this technique for QC purposes.

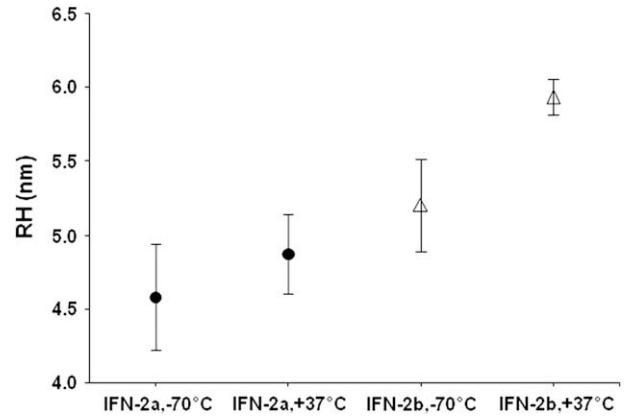

Fig. 7. Hydrodynamic radius (R_H) of EDQM CRS IFN-α2a and EDQM CRS IFN-α2b preparations, after 9 weeks storage at −70 °C (IFN-α2a,−70 °C, IFN-α2b,−70 °C) and +37 °C (IFN-α2a, +37 °C, IFN-α2b, +37 °C). Data are the means ± SD ($n = 9$).

The various degrees of degradation, aggregation or oxidation induced in the IFN-α2 preparations were analysed by SDS-PAGE, RP-HPLC, SEC-HPLC and DLS, which detected changes to the molecules that were reflected by the changes in biological activity. RP-HPLC analysis assessed the oxidation of the stressed preparations. IFN-α2b, +37 °C material showed the largest proportion of oxidative degradation, possibly reflecting differences in formulation and/or storage conditions such as the differences in pH described in Section 2. On SDS-PAGE, the band profile of the IFN-α2 preparations suggests association of molecules corresponding to dimers, trimers and other oligomers of IFN-α2a and IFN-α2b. SEC-HPLC analysis confirmed the presence of HMW material, with IFN-α2b forming a higher percentage of aggregated material than IFN-α2a, with some resolution of peaks at the elution times predicted for dimeric and trimeric species.

The data from this study indicate that the IFN-α2b is less stable than the IFN-α2a. However, it would be necessary to compare more than one preparation of each form of the

molecule in order to attribute observed differences to the different forms of the molecule rather than batch-to batch variation of the active pharmaceutical ingredient or formulation differences, as discussed by Robinson and co-workers [24]. It was not the purpose of this study to attribute observed differences in behaviour to differences in the molecular structure of the two interferons but rather to investigate the utility of selected analytical techniques in detecting differences between preparations of biologicals.

In this study, SEC-HPLC and SDS-PAGE showed no HMW species in the samples stored at −70 °C. However, DLS analysis exhibited a size distribution profile that contained larger particles (HMW species) in addition to smaller species which can be attributed to protein monomers.

SDS-PAGE, RP-HPLC, SEC-HPLC and DLS, as used in this study, thus differed in their detection of the various oxidised and aggregated impurities. The denaturing conditions of SDS-PAGE, for example, can dissociate non-covalent bonding aggregates which, as a result, are usually not detectable by this technique [25]. RP-HPLC may detect both covalent and non-covalent aggregates and different isoforms of protein aggregates such as dimers based on hydrophobicity differences [25]. In contrast, SEC is a commonly used technique for quantitative assessment of aggregate size and content but is limited for the determination of aggregates that may have been removed during filtration prior to injection [25]. In addition, SEC may underestimate aggregation due to nonspecific aggregate binding to the column matrix, or reversible or weakly associated aggregates breaking up in the column [1] or blocked on the pre-column filter.

DLS is less commonly used than SDS-PAGE, RP-HPLC or SEC-HPLC in batch release testing, but this study demonstrated some of its advantages. The technique measures the diffusion speed of particles, which is affected by conformational changes and aggregation [26]. As the intensity of light scattering is dependent on particle radius to the power of 6, DLS is a particularly sensitive technique for detecting larger particles even when these are present at very low concentration. The detection of even small concentrations of aggregates can be important as they can serve as nuclei for an accelerating rate of aggregation on storage. Detection of large aggregates is particularly important due to their potential in inducing immunogenic responses [27]. An additional advantage of DLS is the possibility of conducting the measurement in a variety of solution environments, without the need for denaturing conditions, particular solvents or prior filtration. If DLS is used in product characterisation, it may, in some cases, prove useful to extend its use to batch release testing.

This study illustrates the importance of selecting an appropriate range of analytical techniques for monitoring product quality, such that clinically important attributes, which cannot be assessed by one technique, are monitored by a complementary assay. The portfolio of analytical techniques appropriate for a given product and a given production method must be based on knowledge of the structural characteristics of the product and its biological properties, and needs to be determined empirically, on a case-by-case basis. This study indicated that two of the assay systems investigated, qRT-PCR, as a bioassay for potency measurement, and DLS for monitoring molecular size and aggregation, could prove more widely useful than is currently the case, in QC and batch release of biopharmaceutical products.

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