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Determination of recombinant Interferon-α2 in *E. coli* periplasmic extracts by reversed-phase high-performance liquid chromatography

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ABSTRACT

Reversed-phase high-performance liquid chromatography (RP-HPLC) has been used to analyze Interferon α-2 (IFN-α2) as a pure protein or as a pharmaceutical preparation: a method for analyzing periplasmic IFN-α2 directly in osmotic shock extract has, however, never been reported. This work describes an RP-HPLC methodology for the qualitative and quantitative analysis of human IFN-α2a and IFN-α2b directly in bacterial periplasmic extracts or in purified preparations. The analytical method has been set up and validated for accuracy, precision, linearity, sensitivity and specificity. A recovery test indicated an average bias of ~1%, intra-day and inter-day quantitative determinations presented relative standard deviations always ≤ 5%, while the working sensitivity was of ~0.3 μg of IFN-α2 (RSD = 5%). The method proved to be suitable for detecting and quantifying also glycosylated and oxidized forms and N-methionylated IFN-α2 molecules, it was, however, not able to distinguish between IFN-α2a and IFN-α2b. This rapid methodology allows the application of RP-HPLC as a powerful tool to monitor the production yield and quality of IFN-α2 in osmotic shock fluids, right after, or even during the fermentation process.

1. Introduction

Interferons (IFNs) are cytokines with antiviral, antiproliferative and immune-modulatory properties [1]. At least 24 subtypes of human IFN–α have been identified, with a molecular mass range from 19 to 26 kDa. The production of IFN-α by DNA-recombinant technology has been focused on IFN-α2a and IFN-α2b, which differ in amino acid sequences by one residue (K23R) [2,3]. They are proteins with a molecular weight of approximately 19 kDa, structurally composed of 165 amino acid residues [4,5], mainly used for chronic viral hepatitis B and C, leukemia, multiple myeloma, hairy cell leukemia, melanoma, Kaposi sarcoma, follicular lymphoma and renal cell carcinoma therapy with or without complementary drugs [6–9]. They are currently produced in *Escherichia coli*, considering that their biological activity does not require glycosylation [10,11].

Recombinant IFN-α2, when expressed in genetically modified bacteria, can be directly stored as insoluble cytoplasmic inclusion bodies or secreted in the periplasmic space, thanks to the introduction of a suitable leader sequence in the constructed expression vector. While the first type of construction generates recombinant proteins with an extra N-terminal methionine, periplasmic secretion generates properly processed and folded proteins without the need of solubilization or renaturation. Besides this, the periplasmic product is free of the N-terminal methionine [12].

The detection and quantification of IFN-α2 are crucial for process control of IFN and IFN-related forms [13,14], especially during early downstream processing steps. Since the recombinant product has batch to batch differences, its composition needs to be estimated qualitatively and quantitatively before the purification downstream process steps [15,16].

Several analytical techniques for the quantitative and qualitative evaluation of IFN have been described, such as Enzyme-linked immunosorbent assay (ELISA), Surface Plasmon Resonance technique and densitometric analysis of the SDS-PAGE gel band [17–19].

Many authors have reported different chromatographic techniques to determine IFN-α2a or IFN-α2b concentration, stability and purity of pharmaceutical preparations or even their serum concentration. Zimmermann et al. [20] validated a Size-Exclusion HPLC method for recombinant IFN-α2a determination in pharmaceutical formulations. A Phenomenex™ Biosep-Sec-s2000® column (5 μm particles size and 150 Å pore size) was utilized and the IFN-α2a retention time was 17.2 min. Zarrin et al. [13] developed an RP-HPLC method for IFN-α2b quantitation from pharmaceuticals that was afterward applied to an Iranian post-marketing quality control program. A wide pore reversed-phase C_4 column

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was utilized and IFN-α2b retention time was about 9.2 min. Silva et al. [14] validated an RP-HPLC technique for the determination of IFN-α2a in pharmaceuticals. The method was also applied to the analysis of IFN-α2a and its related proteins in serum: a reversed-phase Jupiter C_4 column was utilized and the IFN-α2a retention time was 32.2 min. For Pegylated IFN-α2a determination, directly from pharmaceutical formulations, Ghazaly et al. [21] developed an RP-HPLC technique where a non-pegylated chemical reagent standard of IFN-α2a proved to suit as a primary (calibrant) standard. In this case a C_8 reversed-phase column was utilized and the IFN-α2a and pegylated IFN-α2a retention times were, respectively, 21 and 23 min. Cueto-Rojas et al. [10] reported a Reversed-Phase Ultra Performance Liquid Chromatography technique to quantify rhIFN-α2b in inclusion bodies. A reversed-phase C_{18} column was utilized and the rhIFN-α2b retention time was about 4 min. By this method, sample preparation includes its drying and solubilization.

With the exception of denaturing SDS-PAGE and immunoblotting, most of these techniques are applied to purified or semi-purified IFN lots in order to quantify the unmodified cytokine in the presence of host proteins and IFN-related isoforms.

The setting up of a method for the analysis of either Interferon-α2a or −α2b secreted in soluble form in bacterial periplasmic space, has not been described yet.

In the present work, therefore, an RP-HPLC methodology for the qualitative and quantitative analysis of recombinant human IFN-α2a and IFN-α2b, directly in bacterial periplasmic extracts without any previous purification step, is proposed. Once validated, it will allow the monitoring of their synthesis in terms of production yields, quality and isoform formation during the bioprocess.

2. Materials and methods

2.1. Chemicals, reagents and IFN preparations

Water was obtained from a Millipore Milli-Q plus water purification system (Bedford, MA, USA). Acetonitrile (HPLC-grade) (Mallinckrodt Baker, USA) and all other chemicals were analytical reagent-grade. *E. coli*-derived authentic IFN-α2a- and IFN-α2b-CRS (chemical reagent standard, European Pharmacopoeia, Strasbourg, France), glycosylated IFN-α2a and −α2b expressed in human cells (HEK-293) from Sigma-Aldrich (St. Louis, MO, USA), N-methionylated IFN-α2a expressed in *E. coli* cytoplasm (Sigma-Aldrich) and a pharmaceutical formulation of authentic *E. coli*-derived IFN-α2a (Roferon®- A, Hoffmann-La Roche, Switzerland) were used.

2.2. Bacterial strains, plasmids and cultivation

An *E. coli W3110* strain previously transformed with the vector λPL-DsbA-IFN-α2a developed in our laboratory was utilized. This plasmid construction used a cassette expression vector (λP_L-Amp®-3286pb) described for the first time by Soares et al. [12]. IFN-α2a synthesis in the periplasm of *E. coli* based on the λPL promoter is analogous to that described for the constitutive expression of other proteins [22–24]. The signal sequence utilized was that of DsbA (a bacterial disulfide oxidoreductase), encoding a peptide with 19 amino acids. The theoretical values of molecular mass for the IFN-α2a (165 aa) and of his precursor DsbA-IFN-α2a (184 aa) are 19237.2 Da and 21209.8 Da, respectively. The cDNA containing the restriction sites of *Nde*I and BamHI, DsbA signal peptide and IFN-α2a gene was introduced by GenScript Corporation (Piscataway, NJ, USA) in the pUC57 plasmid. The vector λPL–DsbA– IFN-α2a was thus obtained by replacing the gene of mouse prolactin with that of IFN-α2a in the plasmid λPL–DsbA–mPRL [24]. The cultivation was performed in 250 ml Luria-Bertani medium in 500 ml flasks, at 30 °C and 150 rpm. After 16 h, the culture was cooled and immediately submitted to osmotic shock. The same procedure was carried out with a non-transformed *E. coli* W3110 strain in order to set up a mock periplasmic extract.

2.3. Osmotic shock

Osmotic shock was utilized to extract all proteins from the bacterial periplasm without cell disruption [25], as previously described [12,22]. Briefly, the product from fermentation broth is harvested and the bacteria are precipitated by centrifugation at 3000*g* for 5 min. All subsequent steps were carried out at 4 °C in an ice bath. Pellets were resuspended in ice-cold 10 mM Tris-HCl, pH 7.5 adding 1 ml of 20% sucrose (w/v) for each 100 A_{600} units; then, 33 ml 0.5 M EDTA, pH 8.0, were added for each ml of solution and incubation on ice continued for 10 min. The suspension was centrifuged again at 3000*g* for 10 min, the pellet being resuspended by adding 1 ml of ice-cold 1 mM Tris-HCl, pH 7.5 per each 100 A_{600} units, incubating 10 min and centrifuging again at 3000 g for 10 min. The collected supernatant (periplasmic fluid) was stored at −80 °C.

2.4. Reversed-phase high-performance liquid chromatography (RP-HPLC)

A Shimatzu Model SPD-20A HPLC apparatus (Kyoto, Japan) was used, employing the LC Solution software, also from Shimatzu. The column was a C_4 Grace (Hesperia, CA, USA) 214TP54 (25 cm x 4.6 mm I.D., pore diameter of 300 Å and particle diameter of 5 μm). The set-up method was based on a previously reported reversed-phase HPLC technique that had been applied to IFN determination from pure samples [14,26], but was unable to resolve the IFN peak from other periplasmic proteins.

The elution procedure was, thus, modified as follows. The mobile phase consisted of 0.1% TFA (Solution A) and of an aqueous solution of 90% acetonitrile plus 10% (v/v) solution A (Solution B). The mobile phase gradient was programmed for a total run-time of 45 min divided into three stages: a step with 47% of Solution B for 10 min, a first gradient from 47% to 58% of solution B for 25 min, and a second gradient from 58% to 47% of solution B for 10 min. The flow rate was 1 ml/min, UV detection at 220 nm, column temperature maintained at 30 °C and sample volume from 10 to 100 μl.

2.5. High-performance size-exclusion chromatography (HPSEC)

This classical methodology was utilized to quantify samples containing purified IFN in comparison with RP-HPLC. HPSEC was carried out in the same Shimatzu equipment. A Tosoh Bioscience TSK Gel G2000 SW column (60 cm x 7.5 mm I.D., particle size of 10 μm and pore size of 125 Å), purchased from Tosohaas (Montgomeryville, PA, USA), was coupled to a 7.5 cm x 7.5 mm I.D. A TSK Gel guard column SW was used. The mobile phase was 0.15 M NaCl, 0.02 M sodium phosphate, pH 7.0, with a flow-rate of 1.0 ml/min and UV detection at 220 nm, as described by Oliveira et al. [27].

2.6. Gel electrophoresis and western-blot analysis

These techniques were utilized to confirm the identity and purity of the pooled osmotic shock fractions, collected from RP-HPLC. SDS-PAGE based on 12% poly-acrylamide gels was carried out under non-reducing conditions as previously described [28]. Western blot analysis was based on mouse anti-IFN-α2a first antibody (1:5000) and Goat Anti-Mouse IgG HRP conjugate, second antibody (1:10,000) (Millipore, Darmstadt, Germany) using a nitrocellulose membrane. Blots were visualized with Luminata Forte (Millipore, Darmstadt, Germany) and revealed in an X-ray film.

2.7. Sensitivity determination

To determine the theoretical sensitivity of the RP-HPLC methodology, the Rodbard's formulation was used, as previously described by Oliveira *et al.* [27].

The minimal detectable dose of the RP-HPLC methodology was calculated based on the standard deviation (n = 3) of the lowest dose

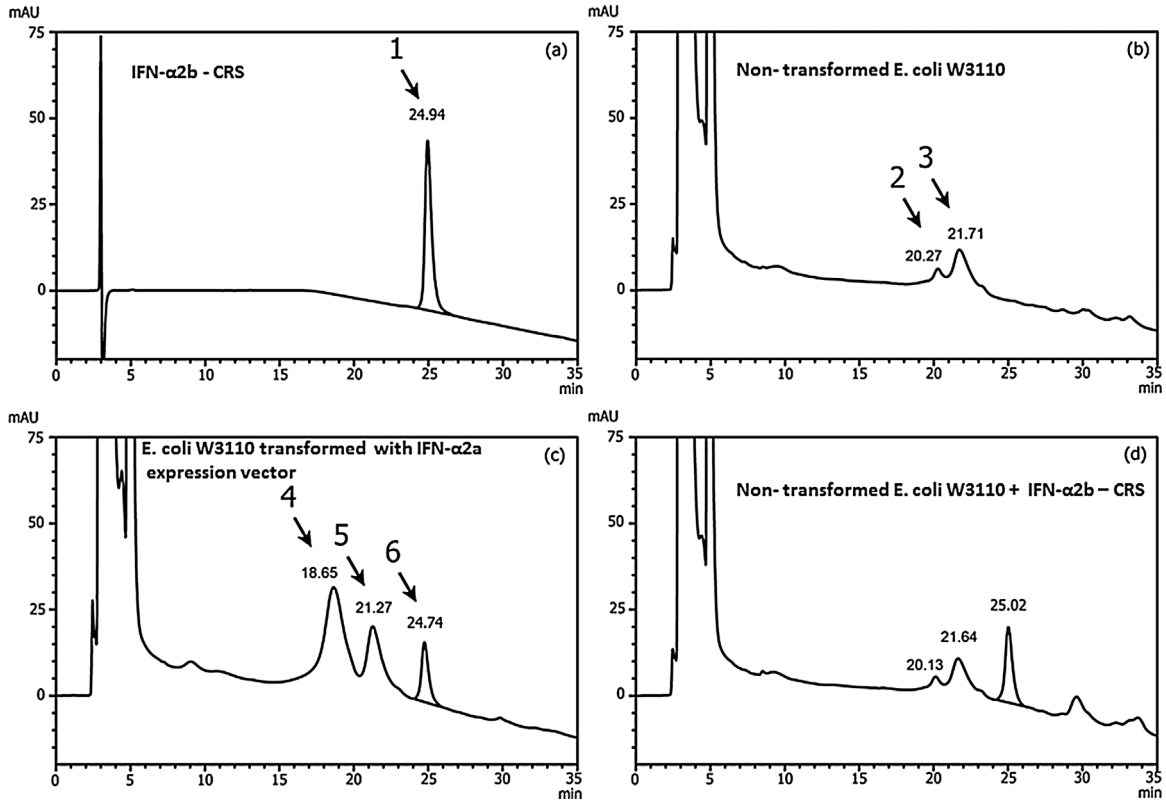

Fig. 1. RP-HPLC chromatograms on a C4 Vydac 214TP54 column of IFN-α2b-CRS and of different periplasmic fluids obtained from *E. coli* W3110. The arrows indicate the peaks collected for SDS-PAGE and Western Blot analyses (see Fig. 2). Injected volume: 100 µl.

used (0.625 µg) and of the zero dose (only blank addition), using a *t*-test (one-sided, $P = 0.05$) [29], according to the following equation:

$$y_{\min} = y_0 + ts_p \sqrt{\frac{1}{n_0} + \frac{1}{n_1}}$$

$$s_p = \sqrt{\frac{s_0^2(n_0 - 1) + s_1^2(n_1 - 1)}{n_0 + n_1 - 2}}$$

where $Y_{\min}$ = minimal detectable response (area units); Y_0 = response of the zero dose; n_0 = number of replicates of the zero dose; s_0 = standard deviation of the zero dose; n_1 = number of replicates of the lowest dose; s_1 = standard deviation of the lowest dose; t = Student's *t*-test value.

The lowest dose used in the calibration curve was 0.625 µg, and the zero dose response was calculated by simulated sample application of elution buffer. The minimal detectable dose ($X_{\min}$) was then calculated via the calibration curve.

Working sensitivity was considered the experimental dose, added to a mock periplasmic extract, that provided an intra-day relative standard deviation (RSD) of ~ 5% (n = 3)

2.8. Preparation of an oxidized form

This preparation was used to prove the capacity of the RP-HPLC technique to identify oxidized isoforms [14]. Thirty percent H_2O_2 was thus added to a 1 mg/mL solution of IFN-α2b in a proportion of 2:3. The reaction was incubated for 1 h at room temperature and then applied to RP-HPLC.

3. Results

The transformed *E. coli W3110* strain was cultivate in flasks, reaching an optical density of ~4 A_{600} units, a specific yield of 0.5-1.5 µg IFN-α2a/(mL A_{600}) and a concentration higher than 50 µg/mL in the periplasmic fluid extract.

An adequate resolution between the osmotic shock fluid components and IFN-α2, after previous experimental studies (data not shown), was achieved using an RP-HPLC C_4 column with one isocratic and two gradient steps based on acetonitrile, at 30 °C. Fig. 1 shows examples of chromatograms obtained for pure IFN and for periplasmic fluid extracts obtained by osmotic shock. It is evident that the non-transformed strain does not present any interfering peak while the IFN peak is eluted at a t_R of ~25 min and it is well resolved from the bulk of periplasmic proteins.

This methodology allows, therefore, an initial rapid evaluation of the quality and quantity of IFN-α2 secreted in the periplasmic space right after, or even during, the fermentation process. It was not possible, however, to distinguish between IFN-α2a and IFN-α2b.

In order to confirm the identity and purity of IFN-α2 eluted from RP-HPLC, the product-related peaks 4, 5 and 6, showed on Fig. 1C, were analyzed by SDS-PAGE. Key peaks were thus compared based on their molecular weight and the presence of IFN-α2 was confirmed by Western blot analysis (Fig. 2). The SDS-PAGE and WB analysis confirmed that the periplasmic fluid fraction (peak 6 from Fig. 1) corresponds to pure and immunologically active IFN-α2. The IFN-α2b reference preparation (IFN-α2b – CRS) and peak 1 did not show alterations, suggesting that this methodology can be useful for quickly obtaining pure IFN directly from periplasmic fluid.

Peaks 4 and 5 correspond to proteins with a higher molecular mass and without immunological activity against anti-IFN-α2 antibody.

The present RP-HPLC methodology for the quantification of IFN-α2 in periplasmic fluid was validated by analyzing the following parameters: accuracy, linearity of the calibration curve, precision, sensitivity and specificity. A recovery test was carried out by adding known amounts of pure recombinant interferon (IFN-α2b-CRS) to a periplasmic fluid from non-transformed *E. coli* W3110 strain (blank). The mean recovery of IFN-α2a was 99%, therefore with an average bias of

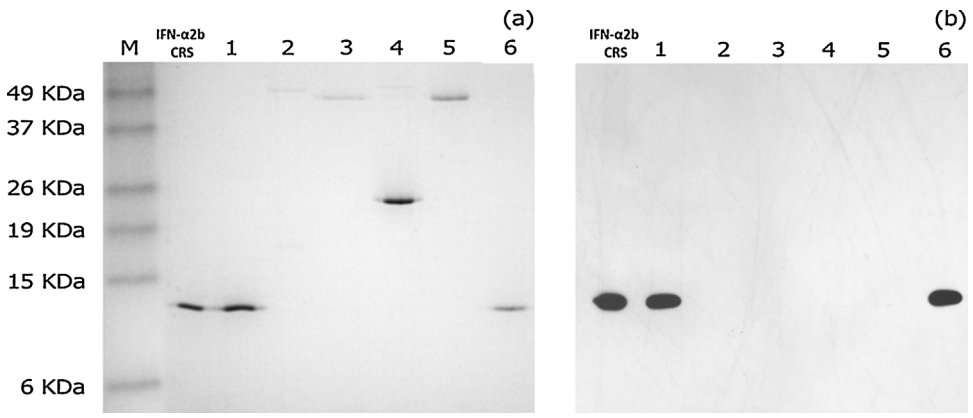

Fig. 2. SDS-PAGE under non-reducing conditions (a) and Western-blot analysis (b) of relevant peaks present in RP-HPLC chromatograms. Lane: M, Protein Molecular Mass Marker; Standard preparation, IFN-α2b-CRS; 1–6, eluted peaks from RP-HPLC (see Fig. 1).

Table 1
Recovery test of known amounts of pure recombinant IFN-α2b added to mock periplasmic fluids. Each point was determined in triplicate.

IFN added Mass (μg)	Determined IFN mass (μg) [a]	% Recovery
1.25	1.16 ± 3.8%	92.8
2.50	2.55 ± 2.3%	102.0
5.00	5.19 ± 2.0%	103.8
10.0	9.74 ± 2.3%	97.4
M ± SD		99.05 ± 4.97

[a] M ± RSD, n = 3.

~1% (n = 12, Table 1). A good accuracy for IFN determination was thus demonstrated. The highly significant correlation between added and recovered IFN is represented by the equation: $Y_{recov} = 0.98\ X_{added} + 0.09$ (r = 0.9981; P < 0.001).

The calibration curve (added IFN mass vs peak area) in the range 0.625–10 μg is shown in Fig. 3 and Table 2. The equation of the calibration curve is:

Yau = 754.7 Xμg + 72.6 where "au" are measured area units of the peak. The highly significant correlation coefficient (r = 0.998; P < 0.001, for n = 18) confirmed the linearity of the response obtained in this range.

The sensitivity of the method obtained according to Rodbard's formulation [29] was of 7.2 ng. The value of 0.30 μg IFN, experimentally providing an intra-day relative standard deviation (RSD) ~ 5.0% in the presence of a mock periplasmic extract, was considered the "working sensitivity" of the method. The intra- and inter-day precision of the RP-HPLC system was evaluated via triplicate determinations, on a single day and on three different days, using three IFN-α2a samples, run under the same conditions. For both intra-day and inter-day assays (Table 3),

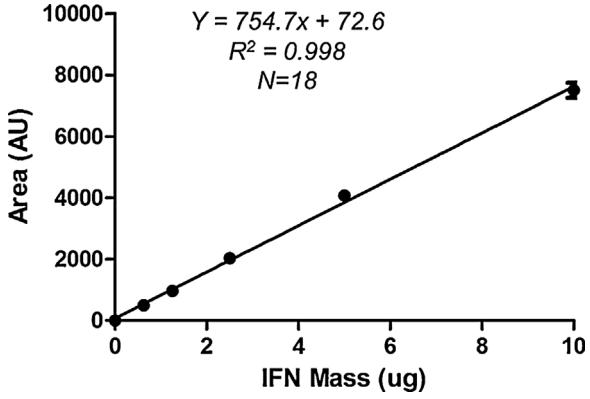

Fig. 3. Standard curve for IFN-α2 determination by RP-HPLC, relating peak area (au = area units) to known added amounts of pure protein.

Table 2
IFN mass and relative peak areas utilized to plot the Standard curve.

IFN Mass (μg)	Peak area (AU)[a]	RSD%[b]
0.000	1.16e0 ± 0.08	6.97
0.625	4.94e2 ± 4.56	0.92
1.250	9.62e2 ± 3.08	0.32
2.500	2.03e3 ± 7.80	0.12
5.000	4.07e3 ± 5.05	0.12
10.00	7.50e3 ± 245	3.27

[a] M ± SD, n = 18.
[b] Expressed as percentage of the mean for 3 replicates.

Table 3
Intra-day determinations of IFN-α2a by RP-HPLC in different periplasmic shock fluids.

Sample	Intraday[a] (μg)	Interday[a](μg)
1	0.30 ± 5.20%	0.31 ± 4.33%
2	1.07 ± 0.54%	08 ± 0.92%
3	4.50 ± 2.09%	4.70 ± 3.32%

[a] M ± RSD, n = 3.

the sample utilized were periplasmic extracts, covering the range 0.3–5 μg of IFN.

In Fig. 4, IFN-α2 from different biological sources was evaluated utilizing the proposed methodology. Among the samples analyzed is a pharmaceutical formulation of IFN-α2a produced in *E. coli* (Roferon®)-A, with a retention time (t_R) identical to that of the IFN-α2b-CRS. Glycosylated IFN-α2a, oxidized and the N-methionylated IFN-α2a seem to be less hydrophobic than IFN-α2b-CRS. Three main peaks are identified in the glycosylated isoform, none of which with a t_R similar to that of CRS. A difference of approximately 5 min between the t_R of the N-methionylated IFN-α2a and that of the IFN-α2b-CRS was also observed. The oxidized isoform shows a t_R of 23.32 min against 24.86 min for non-oxidized IFN-α2b.

A comparison between the RP-HPLC determinations validated in this study and a classical physico-chemical method (HPSEC) is presented in Table 4. There was a good agreement between the two methods, presenting values that are quite close to the nominal, declared amounts.

4. Discussion

An RP-HPLC methodology for qualitative and quantitative analysis of recombinant human IFN-α2 in purified preparations and in *E. coli* crude periplasmic extracts has been set up. The performance of the method was defined concerning accuracy, precision, linearity, sensitivity and specificity, and the described technique showed to be useful

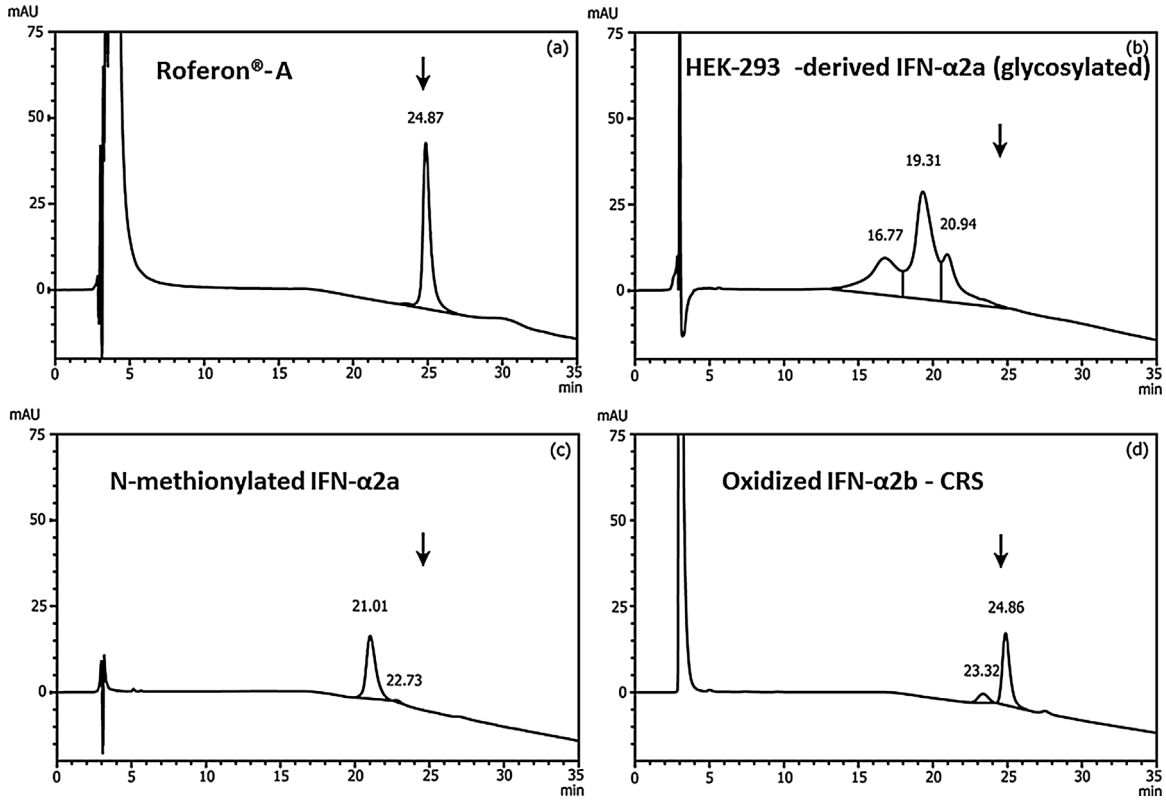

Fig. 4. RP-HPLC chromatograms of different IFN preparations and related isoforms. The amount of applied protein (2.5 μg) was based on the declared nominal vial content. (a) Roferon® A, (b) glycosylated HEK-293-derived IFN-α2a, (c) N-methionylated IFN-α2a expressed in *E. coli* cytoplasm (Sigma-Aldrich) and (d) oxidized IFN-α2b-CRS. The arrow indicates IFN-α2b-CRS retention time (24.74 min).

Table 4
Determination of IFN-α2 preparations by RP-HPLC in comparison with HPSEC determination.

Mass (μg)			
	Nominal amount	RP-HPLC[a]	HPSEC[a]
IFN-α2b CRS	2.50	2.53 ± 2.2%	2.54 ± 4.7%
Glycosylated IFN-α2b[b]	2.50	2.68	2.53
IFN-α2a CRS	2.50	2.43 ± 0.3%	2.53 ± 3.6%

[a] M ± RSD, n = 2.
[b] n = 1.

for the qualitative and quantitative analysis of IFN-α2 and of different IFN-related forms. The correlation coefficient of the dose-response curve relating the amount of an IFN-α2 International Standard to arbitrary area units was highly significant ($p < 0.001$).

The present approach was based on previous work from our group [27,30,31] exploring the hydrophobic differences existing between the host-cell proteins and the heterologous protein of interest. This study was possible thanks to the utilization of an IFN-α2a- secreting *E. coli* strain that provides high expression levels and whose periplasmic extracts contain relatively high concentrations of IFN (> 50 μg/mL).

From the point of view of "specificity", the described methodology permits to detect important structural differences related to the presence of carbohydrate, of oxidized isoforms and in particular to identify the N-methionylated isoform. This methodology was, however, not able to distinguish between IFN-α2a and IFN-α2b: the substitution of histidine (IFN-α2a) by arginine (IFN-α2b), at amino acid position 23, according to our RP-HPLC data did not significantly modify the hydrophobicity of the molecule. The described method should be very efficient in identifying N-methionylated interferon variant, which is an important advantage in relation to the method described in the European Pharmacopeia that could not identify N-methionylated interferon in the final product [1]. The possibility of determining the content of this isoform will be useful in investigating its possible presence in recombinant IFN-α2 preparations and in controlling its contents in commercial products, mainly considering the use of methionine aminopeptidase to produce the N-terminal methionine-free mature protein [1].

It was also observed that glycosylated IFN obtained in HEK-293 cells presents different forms, all less hydrophobic than the non-glycosylated protein, indicating that this methodology can also be useful to detect and study glycosylated IFN of different origins, possibly identifying different types of IFN-α2 isomers.

Another widely used physico-chemical method (HPSEC) was used for a comparative analysis of different samples of interferon (Table 4). Considering the characteristics of each technique a good inter-method correlation is shown, which indicates a good accuracy for the set-up methodology. As known, HPSEC cannot be applied to the analysis of periplasmic extracts, since it is usually unable to resolve the protein of interest from the bulk of bacterial proteins [31].

The applicability of this RP-HPLC methodology to recombinant IFN determination in crude osmotic shock fluids or in partially purified products makes it ideal for the development of a production process, from the choice of the best culture conditions to the monitoring of each purification steps. The use of RP-HPLC can thus greatly improve the quality, reproducibility and efficiency of the costly and delicate production process of recombinant IFN-α2, being useful for in-process control, in order to estimate the productivity and yield during upstream and downstream processes.

5. Conclusions

Due to lack of an accurate and reproducible method for the qualitative and quantitative analysis of IFN-α2 in *E. coli* periplasmic extracts,

obtained by osmotic shock, for the first time a suitable RP-HPLC technique was set-up, which can be also applied to purified preparations. This methodology was validated with regard to accuracy, precision and sensitivity and its specificity can be usefully applied to detect the presence of different IFN isoforms. The applicability of this RP-HPLC technique allows the production monitoring in terms of yields, quality and isoform formation and makes it ideal for planning and following each step of the fermentation and purification bioprocess. We are aware of the limitation of the method which, being unable to differentiate IFN-α2a from IFN-α2b, could not be applied in this specific identity test. It can, however, be applied in the synthesis, purification and quality control of both IFN-α2 isoforms.

Acknowledgements

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