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Recombinant human interferon analysis in pharmaceutical formulations

Análise de interferon humano recombinante presente em formulações farmacêuticas

Sinéa Mendes de Andrade[I,*]

Manuela da Silva[II]

Filipe Soares Quirino da Silva[I]

ABSTRACT

Introduction: Due to the interest in the treatment of hepatitis, the industrial production process of INF-α has been developed and perfected over the last few years. **Objective:** The present work aimed to develop a protocol to characterize the molecular structure of INF-α2b in pharmaceutical formulations by MALDI-TOF mass spectrometry. **Method:** Initially, a reversed-phase liquid chromatography method was developed to promote the separation of active and minor constituent INF-α2b and human serum albumin, also present in the pharmaceutical formulations, to obtain samples with protein homogeneity revealed by electrophoresis. Samples were hydrolyzed with trypsin and submitted to MALDI-TOF. In order to analyze the molecular structure, a procedure based on immunoaffinity and gel filtration chromatography was developed. **Results:** Prepared samples by these methods showed protein homogeneity by SDS-PAGE, and were analyzed by circular dichroism and fluorescence, which showed three - dimensional structure degradation. **Conclusions:** This work provides important data that support the establishment of a protocol for the analysis of INF-α2b in final product, which could replace the traditional peptide mapping by liquid chromatography, with the advantage of resulting in a larger amount of information about the structure of the biopharmaceutical.

KEYWORDS: Alpha Interferon; Protein; MALDI-TOF; Circular Dichroism; Fluorescence

RESUMO

Introdução: Em virtude do interesse para o tratamento da hepatite, o processo de produção industrial do INF-α foi desenvolvido e aperfeiçoado ao longo dos últimos anos. **Objetivo:** O presente trabalho teve como objetivo desenvolver um protocolo para caracterizar a estrutura molecular do INF-α2b em formulações farmacêuticas por espectrometria de massa do tipo MALDI-TOF. **Método:** Inicialmente foi desenvolvido um método de cromatografia líquida baseado em fase reversa para promover a separação entre o INF-α2b constituinte ativo e minoritário e a soro albumina humana, também componente presente nas formulações farmacêuticas, obtendo-se amostras com homogeneidade proteica, revelada por eletroforese. As amostras em solução foram submetidas à digestão com tripsina, levadas ao espectrômetro de massa MALDI-TOF. Para que fosse analisada a estrutura molecular, foi desenvolvido um procedimento baseado em imunoafinidade e cromatografia de gel filtração. **Resultados:** As amostras preparadas por estes métodos apresentaram homogeneidade proteica por eletroforese, sendo analisadas por dicroísmo circular e fluorescência, o que demonstrou ter degradação da estrutura tridimensional. **Conclusões:** Esse trabalho fornece dados importantes que subsidiam o estabelecimento de um protocolo para a análise de INF-α2b em produto final, que poderia substituir o mapa de peptídeos tradicional por cromatografia líquida, com a vantagem de resultar em um maior número de informações sobre a estrutura molecular do biofármaco.

PALAVRAS-CHAVE: Interferon-alfa; Proteínas; MALDI-TOF; Dicroísmo Circular; Fluorescência

[I] Instituto Nacional de Controle de Qualidade em Saúde, Fundação Oswaldo Cruz (INCQS/Fiocruz), Rio de Janeiro, RJ, Brazil

[II] Fundação Oswaldo Cruz (Fiocruz), Rio de Janeiro, RJ, Brazil

***E-mail:** sinea.mendes@incqs.fiocruz.br

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INTRODUCTION

Chronic infection caused by the hepatitis B virus affects about 240 million people worldwide. It is also the main cause of cirrhosis and hepatocellular carcinoma. It is estimated that 686,000 people die every year as a result of chronic or acute hepatitis[1]. Control of this disease involves preventive vaccination and the use of antivirals in infected individuals. The drugs recommended by the Health Surveillance's Department of Sexually Transmissible Infections (STI), HIV/AIDS and Viral Hepatitis (DIAVH) in this case are interferon-alpha (IFN-α), pegylated interferon-alpha, lamivudine, tenofovir, entecavir and adefovir[2].

Interferons are a group of proteins in the cytokine family produced by the body in response to viral infections. They are classified according to their sequences in type I, families α, ß, ε, κ, ω, and J, for example. In the case of viral hepatitis, the importance of IFN-α was identified as a factor that prevents viral replication, contributing to the treatment of this disease[3,4,5,6].

Because of the interest in the treatment of hepatitis, the industrial production process of IFN-α has been developed and improved over the last years. Overall, IFN-α is produced by overexpression in *Escherichia coli* cells. Following the overexpression, IFN-α is purified through a combination of various chromatographic methods[7].

Because of its protein structure, IFN-α is not orally active. Therefore, finished products in bulk should be converted into a suitable formula for administration by injection. In addition to the properties inherent in injectable products, such as pH, isotonicity, pyrogenicity and the presence of antioxidant agents, this formulation must ensure the stability of IFN during its shelf-life[8]. The most commonly used excipient is human serum albumin (HSA), whose main function is to stabilize the product, preventing the aggregation of the molecules, avoiding the loss of potency and the appearance of antibodies against the biopharmaceutical[9,10].

The basic formulation of IFN-α contains 1.5 mg of HSA per ml. As the ratio between mass and activity is 1.4 x 10^8 IU/mg[11], depending on the presentation, each ml of formulation contains a given mass of IFN-α. At the lowest dose, as described in the package insert, the formulation has about 20 µg of IFN-α and 1.5 mg of HSA. We can see that the lowest molar ratio is of 1 IFN-α to 24 in the final protein composition.

Quality control of IFN can be done by two groups of assays. The first group, which apply assays that are common to liquid pharmaceutical forms for internal use (injectable), consists of tests of medium volume, pH, safety, sterility and bacterial endotoxins. When the final product is freeze-dried, the residual moisture – important for the stability of the product – should be evaluated[12].

The second group of assays relates to the particularities of IFN-α as to the protein nature of this biopharmaceutical. These assays vary according to the monographs for IFN-α in each pharmacopoeia. In Brazil, the 5th edition of the Brazilian Pharmacopoeia (BP) does not include a specific monograph for IFN-α. The BP does not present general recommendations for products produced by recombinant DNA either, unlike other international pharmacopoeias[13].

The United States Pharmacopoeia 39 (USP-39) does not present a specific monograph for IFN-α. On the other hand, the USP-39 recommends a series of requirements that should be assessed for the characterization of articles derived from biotechnology and suggests methods for this evaluation, like amino acid analysis, capillary electrophoresis, polyacrylamide gel electrophoresis and total protein assay. It is worth noting that there are specific indications for the tests applicable to intermediate stages and the final product, and it is up to the manufacturer to carry out evaluations during or at the end of the process[14].

Like the USP-39, the European Pharmacopoeia 8.0. ed. (EP)[11] also prescribes general requirements for recombinant DNA biotechnology products. The EP determines that the product must be characterized as to its identity, purity, potency and stability, using chemical, physical, immunochemical and biological methods. No specific assay is recommended. Some methodologies are suggested to determine production consistency. These methodologies involve characterizing amino acid composition, N-terminal region sequencing, peptide map by Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC), total protein content and contaminant proteins from the expression system.

At the end of the general recommendations, the EP determines that the specific monographs list the assays that are applicable to each product. The EP has a specific monograph to evaluate the raw material of the IFN-α before the biomedicine is formulated. This monograph recommends the following tests:

- Map of RP-HPLC peptides – protein identification method by comparison between standard and sample IFN-α chromatograms. Both standard and sample are to be previously submitted to trypsin hydrolysis;
- Isoelectric focusing – identifies degradation that changes the isoelectric point (pI) of the IFN-α protein;
- Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) – compliance with expected molecular mass in relation to protein standard and homogeneity;
- Protein content;
- Related proteins – these are IFN degradation products. These impurities are intrinsically related to the protein's active principle. These are aggregates, oxidized forms and hydrolysis products;
- Potency – determined by the measurement of its cellular protection effect against viral cytopathic effect in MDBK cells induced by vesicular stomatitis virus. The potency is compared to an international standard preparation, the result of which relates the product to the specification, expressed in terms of international units per milligram of protein.

The related methodologies provide information on the purity, identity and potency of the IFN-α and are classically placed as parameters for the evaluation of changes that may imply lack of efficacy and/or immunogenicity of the product[11].

The peptide map by RP-HPLC is an identity technique that essentially requires a comparison between hydrolysates of an IFN-α standard and the sample. It is also a time-consuming technique, since it requires the use of a gradient, which leads to analyses of up to two hours for each sample[11].

The absence of monographs for finished products in pharmacopoeias makes it difficult the work of regulatory authorities in relation to the minimum quality requirement for this type of product[11,12,13].

The pharmacological effect of proteins is related to their complex molecular structure. Failure in protein expression, with modifications of the primary structure and denaturation of the three-dimensional structure, for example, may lead to a reduction of receptor affinity, decreasing or even eliminating the pharmacological effect. Physical degradation, such as loss of secondary and tertiary structures, compromises the ability of IFN-α to bind to the receptor, thereby compromising its effect[15,16,17]. For this reason, developing methods for the evaluation of the final product is relevant, mainly due to the possibility of incorporating new methodologies that evaluate the molecular structure of the biopharmaceutical product in detail.

Spectroscopic methods can be used to evaluate the protein content in the preparation and provide information on the molecular structure. The identity can be determined by mass spectrometry and the stability of the three-dimensional structure can be determined by circular dichroism and fluorescence[18].

The present work was developed with the objective of searching for more modern and efficient analytical methodologies for the quality control of IFN-α in pharmaceutical formulations, in order to obtain information about the safety, quality and efficacy of the biopharmaceutical and support the work of sanitary surveillance bodies.

METHOD

Samples

We used samples of IFN-α_{2b} from a Brazilian producer, purchased by the Ministry of Health's Department of Science, Technology and Strategic Supplies for the National Viral Hepatitis Control Program. We analyzed formulation batches with potency of 10 x 10^6 IU/mL/vial.

Reagents

All solutions in this study were prepared with ultrapure water using the Milli-Q® system (Millipore). The reagents were supplied by Sigma, GE, Bio-Rad and Merck, among others. The reagents specific to certain techniques were related as described in the procedures. The standard of IFN-α_{2b} Batch # 3 we used was provided by the European Pharmacopoeia (EP).

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

The RP-HPLC was used to separate IFN-α_{2b} from the HSA and the other components of the formulation. The experiment was carried out in an LC-10 (Shimadzu) high performance liquid chromatograph, equipped with SCL-$10A_{vp}$ controller system, LC-10 AD_{vp} pump, Shimadzu FCV-10AL solvent selector valve, SIL- $10AD_{vp}$, sample cooler, FRC-10A fraction collector, and UV/VIS SPD 10A detector. The column we used was ACE 3 C18-300 (250 mm x 4.6 mm), maintained at room temperature. Mobile phase A consisted of 0.1% trifluoroacetic acid (0.1% TFA) in water, mobile phase B consisted of acetonitrile (ACN): 0.1% TFA, gradient described in the EP[19]; flow rate of 1.0 mL/min and detection wavelength of 214 nm. Data was processed using Class - VP 6.13 SP2. The injection volume was 100 µL.

Enzyme digestion

The trypsin solution (Promega V5228A) was prepared by dissolving 100 µg in 100 µl of 50 mM acetic acid (stock solution 1.0 µg/µl). From this stock solution, 14 µl were taken and diluted in 1 ml of 50 mM ammonium bicarbonate (NH_4HCO_3) to prepare a working trypsin solution. We prepared a blank with the buffer and other reagents and a standard concentration of 7 µg/mL of IFN-α_{2b} in NH_4HCO_3 buffer, pH 8.4. This concentration is what is expected for the sample. The fractions collected from the RP-HPLC-FR freeze-dried and then 100 µl of NH_4HCO_3 buffer, pH 8.4, were added, mixed in vortex, plus 10 µL of trypsin working solution. The samples remained in Thermomixer®, with stirring at 37° C for 18 h. In the second digestion step, we added another 10 µl of the trypsin working solution to the tubes with a 4 h period in Thermomixer®, with stirring and temperature of 37° C. The following sample, standard and blank were subjected to the digestion procedure[20,21].

The tubes with the peptides were stored at -20° C for further analysis by mass spectrometry.

Mass spectrometry (MS)

For the identification of the samples collected in RP-HPLC, the Peptide Mass Fingerprint (PMF) technique was used with the Matrix Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-TOF)[22].

The matrix used in the preparation of the samples was a-cyano-p-hydroxycinnamic acid (CHCA). A saturated solution of this matrix was prepared in water, ACN (1: 2) and TFA (0.2%). This solution was mixed with peptide solution in equal parts so as to have the ratio of 1,000 to 10,000 times more matrix molecules than peptide molecules. To the fraction of 5 µL of the sample previously submitted to digestion, we added 5 µL of the matrix; 0.5 µl of this mixture was applied to a sample holder which crystallized after drying[22].

For the acquisition of the spectra, the spectrometer was operated in the reflected mode, with detection for positive ions. The calibration of the equipment was done with Brucker Daltonics standard peptide kit. The spectra were processed in the XACQ program[23].

Gel filtration high-performance liquid chromatography (GF-HPLC)

This experiment was carried out in the same chromatograph used in the RP-HPLC. Specific conditions were: the Superdex® 75 GL (GE Healthcare) column with the mobile phase buffer, 50 mM PB pH 7.0, 150 mM NaCl, 5% propanol, flow rate: 0.4 mL/min, wavelength of 214 nm, volume of 100 µL. The fraction corresponding to IFN-α_{2b} was manually collected and concentrated in Speed-Vac®[24,25].

Quantitative protein determination

The quantitative determination of proteins was used to monitor the fractions collected from the chromatographic methods, which were determined by the Bradford method using the protocol for determination in microplates[26].

Protein electrophoresis with sodium dodecyl sulfate on polyacrylamide gel (SDS-PAGE)

SDS-PAGE was used to evaluate the protein homogeneity of fractions collected from the procedures using RP and GF-HPLC. Aliquots of 10 mL of the GF-HPLC fractions collected, added with 10 mL of ß-mercaptoethanol solution, were applied in 5% concentrator gel and 15% acrylamide gel, according to Laemmli[27], and placed in the Mini-Protean III (Bio-Rad) electrophoresis system, under the race conditions set out in the manufacturer's protocol. Silver staining was applied to evidence the IFN-α_{2b}[28] bands[28]. The obtained gels were digitized in the GS-800 Bio-Rad densitometer, with the aid of the Quantity One (Bio-Rad) program. The molecular weights of each band were estimated comparatively to low molecular mass standards.

Use of immunoaffinity membrane to remove HSA from samples

An immunoaffinity membrane procedure was used to decrease the amount of HSA and facilitate the chromatographic separation of IFN-α_{2b}. The manufacturer's protocol was followed[29].

Circular dichroism spectroscopy

The circular dichroism spectroscopy methodology was used to evaluate the secondary structure of IFN-α_{2b} from the purification by immunoaffinity/gel filtration[30]. The fractions collected from GF-HPLC, concentrated on Speed-Vac®, were subjected to the dialysis procedure using a 10 kDa cut-off membrane against 10 mM phosphate buffer, pH 7.4[31]. An IFN-α2a standard from the EP was diluted with 10 mM phosphate buffer, pH 7.4, so as to obtain a concentration similar to that of the collected fraction. The spectra were obtained in the Jasco spectrometer in 1 cm quartz cells.

Fluorescence Spectroscopy

Fluorescence spectroscopy was used to evaluate the tertiary structure of fractions collected from GF-HPLC. These collected fractions were concentrated in Speed-Vac®, subjected to the dialysis procedure, using a 10 kDa cut-off membrane against 10 mM phosphate buffer, pH 7.4. The spectra were obtained by Shimadzu RF-5301 PC spectrofluorimeter in 1.0 cm optical path quartz cells, using wavelengths for excitation of 280 nm and 295 nm, with a slit size of 5.0 nm. The emission was read from 200 nm to 400 nm. The reading of the phosphate buffer was subtracted as blank[32].

RESULTS AND DISCUSSION

Separation of IFN-α_{2b} from the other components of the formulation by RP-HPLC

The RP-HPLC is a technique widely used in the separation of proteins due to its high resolution. In this study we could separate IFN-α_{2b} from HSA with good resolution (7.3), as shown in Figure 1 (a), of the chromatographic profile obtained from the IFN-α_{2b} formulation. The chromatographic peak for HSA, present in excess in the sample, showed retention time of 58.1 min. The second peak, identified as IFN-α_{2b}, shows retention time of 66.8 min, which was confirmed by injection of the EP standard as shown in Figure 1 (b). RP-HPLC proved efficient for the separation of IFN-α_{2b}/HSA. The hydrophobicity of IFN-α_{2b} is higher than that of HSA, as can be seen in the chromatograms of Figure 1 (a) and (b), which greatly facilitated the separation.

The protein homogeneity of the peak of 66.8 min was evaluated by SDS-PAGE of fractions collected from the RP-HPLC, shown in Figure 1 (c). A 19 kDa band was observed, which corresponds to the molecular weight of IFN-α_{2b}. Furthermore, there are higher molecular weight bands than IFN-α_{2b}, with 57 kDa. In the literature, such bands are related to IFN-α_{2b}[33,34] trimmers. This result proved that IFN-α_{2b} was in a purity state suitable for characterization by mass spectroscopy.

IFN-α_{2b} analysis by MS

Initially, to establish the methodology, we used IFN-α_{2b} and HSA (Sigma) standards hydrolyzed by trypsin. Although the peaks collected in the RP-HPLC presented adequate purity, an HSA standard was also prepared for MS (results not shown). The objective of this experiment was to determine whether there was contamination by HSA that had not been detected in the SDS-PAGE.

The table shows the expected amino acid sequences of the peptides of this digestion and the corresponding theoretical and observed $[M + H]^+$ masses of each peptide, for both the EP standard and the fraction collected from RP-HPLC.

Under these conditions, the observed sequence coverage for the IFN-α_{2b} standard was 72.7%. Peptides corresponding to 120 of the total of 165 amino acids in IFN-α_{2b} were observed. Considering

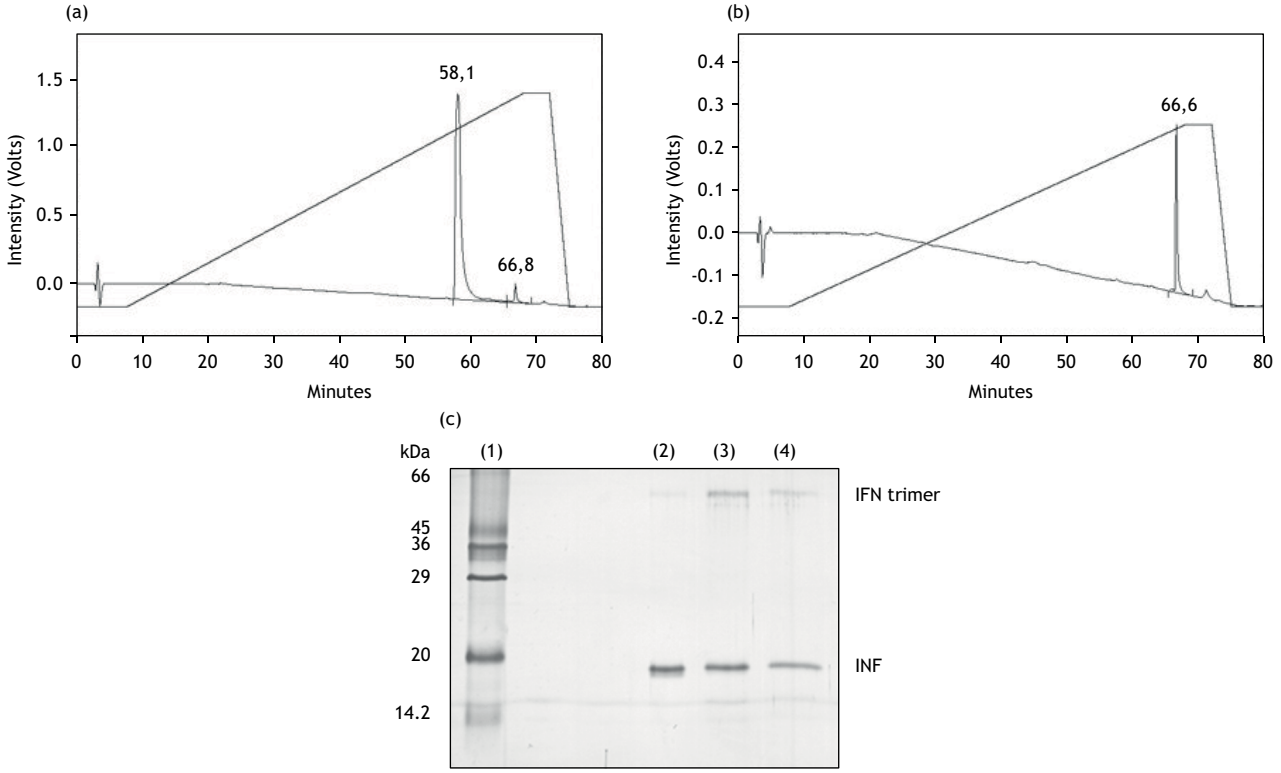

Figure 1. (a) Chromatogram of the formulation of IFN-α_{2b}; (b) Chromatogram of the IFN-α-$_{2b}$ EP standard. Experiments performed under the same conditions: 100 µL solution, 35 µg/100 µL, ACE 3 C18-300 column, 250 mm C x 4.6 mm diameter, mobile phase A: 0.1% TFA in water, mobile phase B: acetonitrile: TFA 0.1%, gradient, flow rate 1.0 mL/min, λ 214 nm. The overlapped line shows the concentration of phase B; (c) SDS-PAGE analysis of the fractions collected from the RP-HPLC in (a): (1) molecular weight standard; lines (2), (3), and (4) fractions collected from RP-HPLC, with retention time of

Table. Expected amino acid sequences of the peptides for tryptic hydrolysis of IFN-α_{2b} and the corresponding monoisotopic masses [M + H]$^+$. The column of the experimental masses refers to the values observed in the spectra of the IFN-α_{2b} standard of the EF and the fraction collected.

N[1]	Position	Peptide aa sequence	[M + H]+ (Da)[2]				
			Theoretical	P[4]	Ir[3] (%)	FC5	Ir[3] (%)
1	84-112	FYTELYQQLNDLEACVIQGVGVTETPLMK	3902.6	n.o.[6]	-	n.o.	-
2	50-70	AETIPVLHEMIQQIFNLFSTK	2459.3	2459.2	4.5	n.o	-
3	34-51	HDFGFPQEEFGNQFQKDR	-	2225.9	98.3	2225.9	100.0
4	34-49	HDFGFPQEEFGNQFQK	1954.8	1954.8	87.4	1954.8	33.7
5	150-162	SFSLSTNLQESLR	1481.7	1481.7	100	1481.7	71.4
6	71-83	DSSAAWDETLLDK	1450.6	1450.6	6.2	n.o	-
7	1-12	CDLPQTHSLGSR	1313.6	1313.6	38.3	n.o	-
8	135-144	YSPCAWEVVR	1209.5	1209.1 + 2116.9	51.4	1209 + 2116.3	44.9
9	14-22	TLMLLAQMR	1076.5	1076.5	81.5	1076.7	26.8
10	24-31	ISLFSCLK	910.5	910.6 + 2116.9	26.5	910.6 + 2116.3	14.3
11	113-120	EDSILAVR	902.4	902.6	9.5	901.9	19.5
12	126-131	ITLYLK	750.4	750.7	4.4	n.o	-
13	145-149	AEIMR	619.3	n.o.	-	n.o	-
14	122-125	YFQR	613.3	n.o.	-	n.o	-

1N: Peptide number; 2[M + H]$^+$Theoretical (MH^+): obtained by subjecting the IFN-α_{2b} sequence to the *PeptideMass* tool (www.expasy.org); 3Ir: relative intensity; 4 P: standard EP; 5 FC: mass observed in the hydrolysis of the collected fraction; 6 n.o.: not observed.

that due to the hydrolytic specificity of trypsin, with the formation of free amino acids and di/tripeptides that are not detectable under these conditions, the total amino acids that could be detected with this method is 154, a coverage of 78% has been achieved, which is high coverage[20].

Important observations can be made from the results of the EP standard. According to the Table, peptides 3 and 5 have characteristics in their sequences that are correlated to the increase in the ionization[35]. Both have arginine at the C-terminus, and peptide 3 also has histidine at the N-terminus. It is important to note that peptide 5 is the one with the most intense signal in published mass spectra of IFN-α_{2b}[20,36].

On the other hand, the less intense peptides have characteristics in their sequences that reduce ionization[35]. As for C-terminus,

peptide 6 has lysine, peptide 7 has arginine, in addition to cysteine in the N-terminus. Both have aspartates as internal residues, and 6 also has a tryptophan.

The disulfide bridges, important bonds for the stability of the three-dimensional structure of proteins, are presented correctly, according to the results. Another point that was observed in the spectra of the samples was the conservation of the disulfide bridge between cysteines 29 and 138 ($[M + H]^+$ 2117.5), demonstrating that this important feature of the IFN-α_{2b} structure is preserved in the commercial product analyzed after the procedures were performed. An intense peak was observed with MH^+ of 2116.9. This value corresponds to the association, via disulfide bridge, of peptides 8 and 10. Since no reducer was used in the sample preparation, part of the sulfide bridge was maintained and part was reduced, forming peptides 8 and 10.

An intense peak with $[M + H]^+$ equal to 2225.9 was observed (peptide 3). This value corresponds to the mass of a peptide where no hydrolysis occurs by trypsin in the KDR sequence. The loss of this trypsin hydrolysis site had already been observed previously in the analysis of IFN-α_{2b}[20].

There was a coincidence of unobserved peptides with preliminary results from the literature for the analysis of $IFN_{\alpha 2b}$ raw material[20] and in the EP[36] standard analysis.

The next step was the analysis of the IFN-α_{2b} present in the formulations. The material collected in the RP-HPLC was then subjected to trypsin hydrolysis and the resulting peptides were analyzed by MS.

Peptides 2, 6 and 12, less intense in the standard spectrum, were not observed in the sample spectra. Likewise, peptide 7 was not observed in the samples.

The sequence coverage achieved with this analysis for the samples of the pharmaceutical formulations was 34.5%, with 57 amino acids being identified in a total of 165. Starting from the possibility of detection of 154 amino acids, we achieved 37% of sequence coverage[20].

The most intense peptides observed in the standard, 3, 4, 5, 8, 9 and 10 were also observed in the sample prepared from the hydrolysis of the reverse phase collected. The sequence coverage achieved 45.5%, and we identified 75 amino acids in 165. Considering the possibility of detection of 154 amino acids, we achieve 48.7% coverage.

Another point that we observed in the sample spectra was the conservation of the disulfide bridge between cysteines 29 and 138 ($[M + H]^+$ 2117.5), demonstrating that this important feature of the structure of IFN-α_{2b} is preserved in the commercial product analyzed after the procedures performed.

Evaluation of the molecular structure of IFN-α_{2b}

To be able to evaluate the molecular structure of IFN-α_{2b} we had to develop a non-denaturing HSA/IFN-α_{2b} separation method. The RP-HPLC is considered a denaturing technique with acid pH and high concentration of organic solvent, which changes the three-dimensional structure during the analysis[37]. The technique initially tested for separation was GF-HPLC. This type of chromatography proved adequate due to the difference in hydrodynamic volume between IFN and HSA, which, in principle, would enable the separation. In addition, the analysis takes place in low denaturing conditions, with low organic solvent concentration and pH close to neutral, which minimizes the possibility of degradation of the biopharmaceutical agent during the process. The collected material would be in the right conditions for the experiments of circular dichroism and fluorescence[33].

GF-HPLC

The GF-HPLC is a widely used method for analysis of aggregates in proteins, and various parameters such as the chromatographic column, mobile phase saline concentration, use of surfactant and organic modifiers can be optimized to improve the separation[38,39]. We tested several parameters to obtain a better separation of IFN-α_{2b} from HSA (results are not shown). The best condition was achieved with the addition of 5% n-propanol to the mobile phase in order to decrease the hydrophobic interactions of the formulation with the matrix of the stationary phase[38,39].

Under these conditions we observed the biggest difference in retention time obtained between the peak of HSA, present in excess, and the peak of IFN-α_{2b}. It was of about six minutes, as can be seen in Figure 2(a). For this reason, the second peak was collected, concentrated and analyzed by SDS-PAGE, to evaluate its protein homogeneity.

The result of SDS-PAGE is shown in Figure 2(b). The fractions we collected presented bands corresponding to the molecular masses of HSA and IFN-α_{2b}, which shows that effective separation was not completed.

Despite the separation obtained in the GF-HPLC, the SDS-PAGE result of the fraction corresponding to IFN-α_{2b} still showed HSA contamination. The difficulty in improving the separation between the two proteins could be associated with the small resolution achieved by the GF-HPLC and by the great difference in concentration between both. Therefore, we chose a process that reduces the concentration of HSA in the samples. With less HSA, the saturation of the column sites would be lower and the separation would probably be better.

Use of immunoaffinity membrane to remove HSA from the samples

The formulation was submitted to the immunoaffinity procedure and accompanied by SDS-PAGE, as shown in Figure 3. It can be seen that the resin treatment greatly reduced the presence of HSA in the samples, but did not completely eliminate this protein. Probably the large molar excess of HSA completely saturated the resin, with part of the protein eluted together with IFN-α_{2b}.

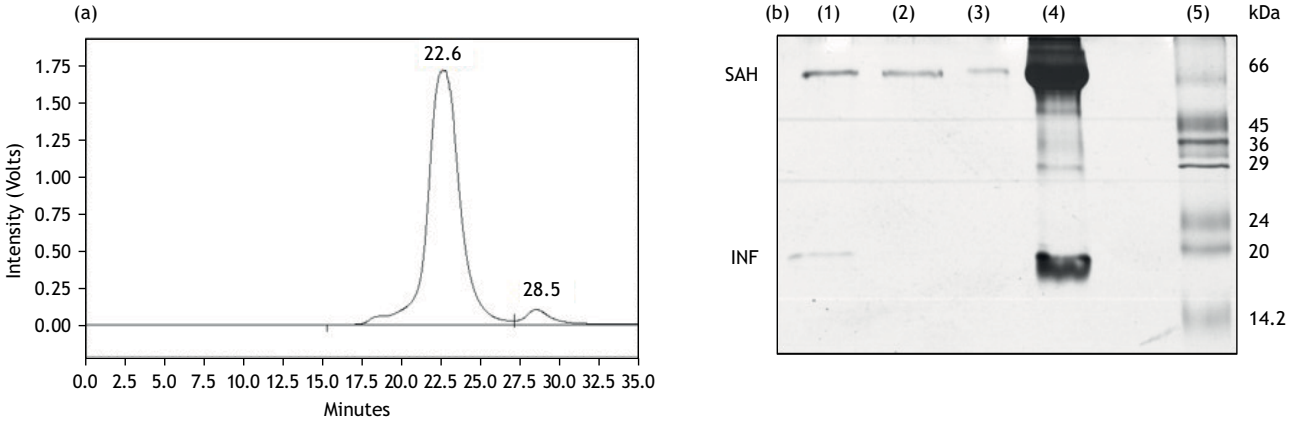

Figure 2. (a) Chromatogram of the obtained IFN-α_{2b} formulation. Conditions: Superdex® 75 HR 10/30 column, 30 cm C × 10.0 mm diameter, mobile phase: buffer, 50 mM PB pH 7.0, 150 mM NaCl, 5% propanol, flow rate: 0.4 mL/min , λ 214 nm. (1) Fraction of 28 min, mobile phase: buffer, 50 mM PB pH 7.0, 150 mM NaCl, 5% nPrOH, (2) SDS-PAGE analysis of fractions collected, from the three systems tested, Fraction of 28 min, mobile phase: buffer, 50 mM PB pH 7.0, 150 mM NaCl, (3) mobile phase: buffer, 50 mM PB pH 7.0, (4) formulation, (5) standard marker of weight molecular.

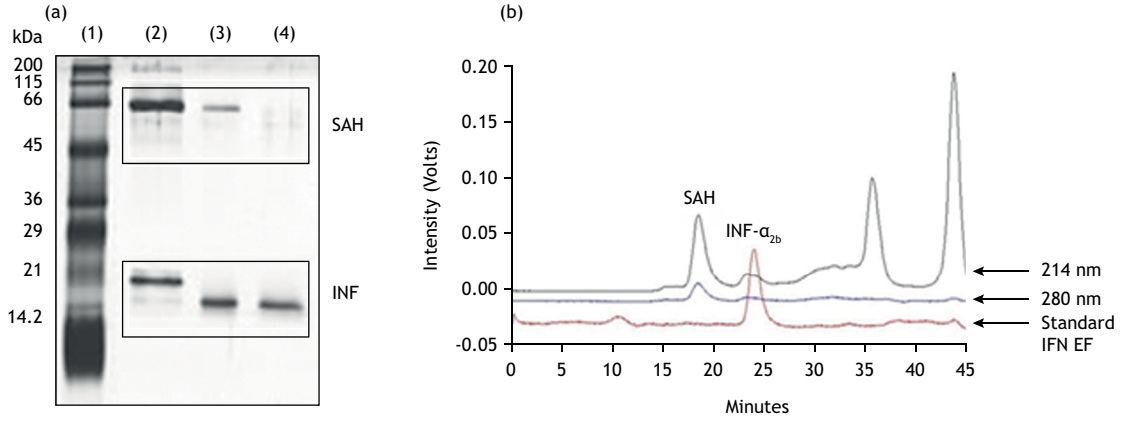

Figure 3. (a) SDS-PAGE analysis of fractions of the IFN-α_{2b} (1) molecular mass standard; (2) Formulation submitted to immunoaffinity (3) Fraction corresponding to the 28 min peak collected from Superdex 75 column (4) Fraction of Superdex 75 column collection, from sample injection obtained by immunoaffinity; (b) Chromatographic profile of the collected fraction of immunoaffinity membrane, and IFN EP standard (red), conditions: Superdex® 75 HR 10/30 column, 30 cm C x 10.0 mm diameter, mobile phase: buffer, PB, 50 mM NaCl, 150 mM NaCl, 5% propanol pH 6.9, flow rate: 0.4 mL/min, UV detector λ_1 214 nm.

From this result, the immunoaffinity and GF-HPLC procedures were combined, which enabled us to obtain pure INF-α_{2b} from the formulations. The samples prepared like that showed protein homogeneity by SDS-PAGE, as can be seen in Figure 3(a).

Circular dichroism

The pharmacological effect of biopharmaceutical agents depends on the integrity of their molecular structure. Physical degradation, such as loss of secondary and tertiary structures, compromises the ability of IFN-α_{2b} to bind to the receptor, impairing its effect[15,16,17]. Therefore, we had to evaluate this integrity, and the techniques of circular dichroism and fluorescence can be used for this purpose[40].

The fractions collected from the immunoaffinity/gel filtration purification scheme were then analyzed by circular dichroism. The results are shown in Figure 4. The standard profile was similar to that described by other authors for IFN-α_{2b} at pH 7.4[31,32,41]. Bands appear at 209 nm (-12.81) and 219.4 nm (-11.56), with lower intensity. This profile is characteristic of proteins with alpha helices[30], as is the case of IFN-α_{2b}[41].

The fraction collected showed a profile that was distinct from the standard. The intensity of the light deviation decreased, there was a deviation from negative bands to longer wavelengths: 209.8 nm and 225.6 nm, respectively. Furthermore, there was a reversal of the intensity of the bands, and the second band (-11,11) was more intense than the first band (-7.87) (Figure 4).

These results indicate that there is a loss of secondary structure of the IFN-α_{2b} present in the formulations. To complement this data, the samples were also evaluated by fluorescence.

Fluorescence

The spectra of the collected fraction and the EP standard were compared, as shown in Figure 5. The standard showed a spectrum with emission maxima at 336 nm (λ_{ex} 280 nm) and 333 nm (λ_{ex} 295 nm). The fractions collected showed emission maxima at 338 nm (λ_{ex} 280 nm) and 334 nm (λ_{ex} 295 nm). These results demonstrated that the IFN-α_{2b} present in the formulations has a side chain exposure higher than that of the EP standard, indicating the possibility of denaturation of the tertiary structure[31].

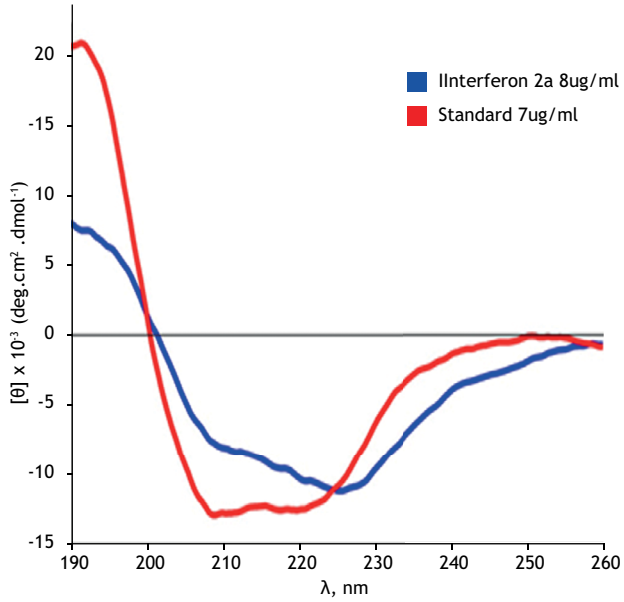

Figure 4. Circular dichroism spectra of the IFN-α_{2b} standard of EP (7 µg/mL) in red, and the fraction collected from the Immunoaffinity/GF-HPLC (8 µg/mL) in blue. Samples in 10 mM PB buffer, pH 7.4.

Another important piece of information that fluorescence spectra provide is the measurement of the light intensity in λ_{ex}. This measure is proportional to light scattering, a phenomenon that occurs due to the presence of aggregates in solution. The standard had an intensity of 674.8 (λ_{ex} 280 nm) and 280.6 (λ_{ex} 295 nm). In both lengths the collected fractions presented an intensity of 1015.6, which is in the saturation range of the fluorimeter detector. This result shows that the aggregation of samples is high, which is not observed in the standard.

The results of the evaluation of the three-dimensional structure demonstrated that in the samples there was denaturation of this structure.

According to studies presented by Qian[37], the complexity of the protein structure and the various pathways of aggregation and degradation may be induced by some factors. The present study found the physical degradation probably caused by the procedures used to obtain the purified IFN-α_{2b}, such as temperature change, increase in salt concentration in the buffer composition, and the various steps necessary to obtain sufficient volume for analysis[16,38].

CONCLUSIONS

In this study, the separation of IFN-α_{2b} from the HSA present in the formulations was performed with the RP-HPLC method, resulting in an excellent separation of HSA and enabling the analysis by MALDI-TOF. The MALDI-TOF analysis of IFN-α_{2b} allowed 45.5% coverage of the biopharmaceutical sequence in pharmaceutical formulations.

In order to evaluate the integrity of the three-dimensional structure of the biopharmaceutical agent, we had to develop a protocol for the separation of IFN-α_{2b} from the other components of the commercial formulation, initially reducing the HSA with an immunoaffinity procedure and completing the purification with GF-HPLC and SDS -PAGE. The obtained fractions were analyzed

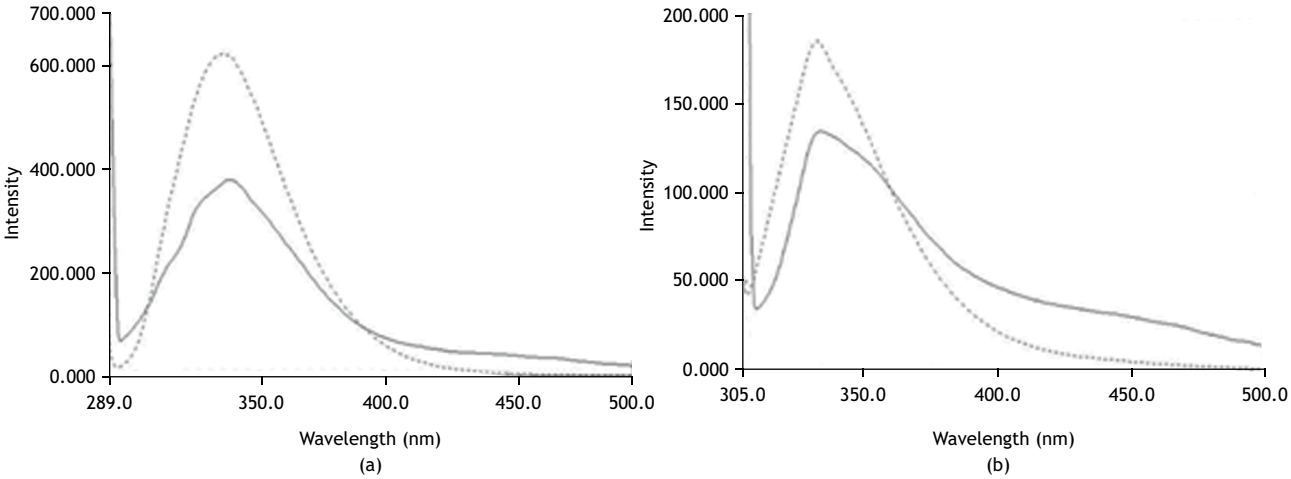

Figure 5. (a) Fluorescence profile of IFN-α_{2b} standard of the EP (dotted line) and the fraction collected from the GF-HPLC (regular line). Excitation at 280 nm. (b) Fluorescence profile of the IFN-α_{2b} of the EP (dotted line) and the fraction collected from the GF-HPLC (regular line). Excitation at 295 nm. Samples in 10 mM PB buffer, pH 7.4.

by circular dichroism and fluorescence, which allowed the verification of the degradation of the three-dimensional structure.

This study provides important data that support the establishment of a protocol for the analysis of IFN-α_{2b} in final products. This could replace the traditional peptide map by liquid chromatography, with the advantage of providing more information on the molecular structure of the biopharmaceutical agent.

The implementation of the MALDI-TOF analysis in the routine is expensive, mainly due to the cost of acquisition of the equipment. This could be a limitation to the adoption of this method in official compendia. On the other hand, from the sanitary standpoint, deeper knowledge about the product is important, because it helps to guarantee its effectiveness and reduce the risk in its use. Moreover, the high added value of IFN-α_{2b} and its chronic use justify the adoption of techniques such as MALDI-TOF.

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Conflict of Interest

Authors have no potential conflict of interest to declare, related to this study's political or financial peers and institutions.