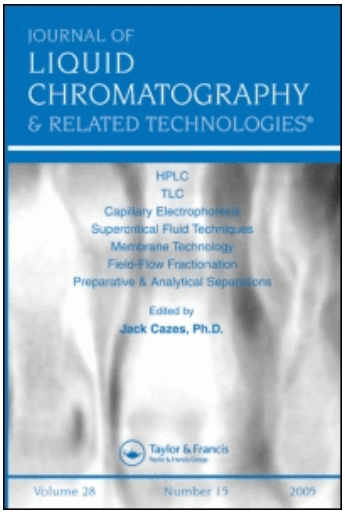

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Abstract: A reversed phase liquid chromatography (RP-LC) method was validated for the determination of interferon-α2a in pharmaceutical formulations. The RP-LC method was carried out on a Jupiter C_4 column (250 mm × 4.6 mm I.D.), maintained at room temperature. The mobile phase A consisted of 0.1% TFA and the mobile phase B was acetonitrile with 0.1% TFA, run at a flow rate of 1 mL/min, and using photodiode array (PDA) detection at 214 nm. The chromatographic separation was obtained with the retention time of 32.6 min, and was linear in the concentration range of 0.5–50 MIU/mL ($r^2 = 0.9999$). The specificity was proven through degradation studies, which also showed that there was no interference of the excipients. The accuracy was 100.84% with bias lower than 1.87%. The limits of detection and quantitation were 0.19 and 0.5 MIU/mL, respectively. Moreover, method validation demonstrated acceptable results for precision and robustness. The proposed method was applied for the analysis of the interferon-α2a and their related proteins in parenteral dosage forms, contributing to establishing alternatives to improve the quality control assuring the therapeutic efficacy of the biological medicine.

Keywords: Formulations, Interferon-α2a, Pharmaceutical, Reversed phase liquid chromatography, Validation

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INTRODUCTION

Interferons are cytokines with antiviral, antiproliferative, and immunomodulatory properties.[1–4] Due to this broad spectrum of biological activities, the recombinant human interferon-α2a (rhIFN-α2a) produced by recombinant DNA technology, it is currently being used worldwide for the therapy of various neoplastic disorders and chronic viral diseases, which includes leukemia, multiple myeloma, and hepatitis B and C, with or without other complementary drugs.[5–7] The polypeptidic structure of rhIFN-α2a consists of 165 amino acids with two disulphide bonds between Cys_1 and Cys_{98} and Cys_{29} and Cys_{138}, and molecular mass of 19.2 kDa.[4,8]

The antiviral assays, the majority based on cell cultures, were the first type of biological assays to measure the relative activity of interferons. The assessment of the biological potency of rhIFN-α2a has been performed by the *in vitro* cytophatic assay based on its protective effect of a variety of cell/virus systems and assay designs. Mainly, the rhIFN-α2a sensitive human amniotic cell line (WISH) has been used against the vesicular stomatitis virus (VSV), evaluating the responses as viable protected cells stained with vital dyes, such as tetrazolium salts and alamar blue, followed by spectrophotometric measurements of dye absorbances in the microplates reader.[9,10] However, subsequent discoveries of several other biological activities of interferon has opened the way to the development of assays based on one or other of these activities. The latter include inhibition of cell proliferation, regulation of functional cellular activities, regulation of cellular differentiation, and immunomodulation.[1]

The availability of robust, precise, and low time consuming assay methods for detection and quantitation of rhIFN-α2a in pharmaceutical matrices, is crucial in the development, process monitoring, and quality control of pharmaceuticals.[11,12] Liquid chromatography (LC) has been successfully used to monitor the purity, identity, chemical stability, and potency of biologicals obtained through recombinant DNA technology, being also capable of monitoring minor structural as well as conformational variations occurring in the protein structure, which could lead to significant changes in biological activity of the drug.[13–16] Size exclusion liquid chromatography (SE-LC) is performed under separation conditions, which are expected not to affect the conformational structure of the protein, and was used to quantify native protein of biopharmaceuticals and to determine dimers and oligomers.[17,18] LC, with UV circular dichroism and intrinsic fluorescence detection, was applied to monitor conformational properties of rhIFN-α2b during reversed phase liquid chromatography (RP-LC) analysis, and to discriminate conformational properties of aggregates from the corresponding native protein during SE-LC.[17] Besides, the RP-LC was applied for quantification of pharmaceutical proteins, including interferons, and for the analysis of closely related protein

variants.[19] Different analytical techniques such as peptide mapping, RP-LC on a C_4 column and mass spectrometry, were used to characterize the primary structure of rhIFN-α2b produced by *Escherichia coli*.[4] The RP-LC method was developed and partially validated on a C_4 column with UV detection and retention time of 9.67 min, for the quantification of rhIFN-α2a in pharmaceutical dosage forms and delivery systems. However, the robustness of the method was not demonstrated, the resolution is not appropriate, and the related proteins were not considered in the method development. The complete validation of the method is essential to show that the procedure is suitable for the intended purpose.[11,20,21]

The aim of the present study was to optimize and fully validate a specific and accurate gradient RP-LC method with UV detection that could be used for the quantitative analysis of rhIFN-α2a in pharmaceutical formulations, contributing to establishing alternatives to improve the quality control and to assure the therapeutic efficacy of the biological.

EXPERIMENTAL

Chemicals and Reagents

European Pharmacopoeia Certificated Reference Standard (Ph. Eur. CRS) for interferon-α2a, a total of eight batches of rhIFN-α2a from three manufacturers, containing 6 MIU/mL of rhIFN-α2a was identified by Arabic numbers from 1 to 6, and two batches containing 9 MIU/mL were identified by Arabic number 7 and 8. The samples were obtained from commercial sources within their shelf life period. Hydrogen peroxide 30% in aqueous solution, potassium phosphate monobasic, polyssorbate 80, acetonitrile, and trifluoroacetic acid were purchased from Merck (Darmstadt, Germany). All chemicals used were of pharmaceutical or special analytical grade. For all the analyses, ultrapure water was purified using an Elix 3 coupled to a Milli-Q Gradient A10 system (Millipore, Bedford, MA, USA).

Apparatus and Analytical Conditions

The LC method was carried out on a Shimadzu LC system (Shimadzu, Kyoto, Japan) equipped with a SCL-10A_{VP} system controller, LC-10 AD_{VP} pump, DGU-14A degasser, SIL-10AD_{VP} autosampler, and a SPD-M10A_{VP} PDA detector. The peak areas were integrated automatically by computer using a Shimadzu Class VP® V 6.14 software program. The experiments were performed on a reversed phase Phenomenex (Torrance, USA) Jupiter C_4 column (250 mm × 4.6 mm I.D., with a pore size of 300Å). A security guard holder was used to protect the analytical column. The Shimadzu

LC system was operated at ambient controlled temperature (25°C). The elution was performed by a gradient at a constant flow rate of 1 mL/min. Mobile phase A consisted of 0.1% trifluoroacetic acid (TFA) and mobile phase B consisted of 0.1% TFA in acetonitrile. The applied gradient was as follows: 0.1 min 38% of B, from 1–5 min linear to 43% of B, from 5.01–20 min linear to 45% of B, from 20.01–30 min linear to 48% of B, from 30.01–40 min linear back to 38% and 40–42 min 38% of B. The mobile phases were filtered through a 0.22 μm membrane filter (Millipore, Bedford, MA, USA). The injection volume was 50 μL for both standard and samples.

Procedure

Samples and Standard Solutions

Working standard and sample solutions of rhIFN-α2a were prepared daily by diluting the Ph. Eur. CRS for rhIFN-α2a and the samples of pharmaceutical formulations in phosphate buffer (KH_2PO_4, 0.05 M; pH 7.4) containing 0.5 mg/mL polyssorbate 80, to a final concentration of 6 MIU/mL, respectively.

Validation of the Method

Once the chromatographic and the experimental conditions were optimized, the method was validated by the determination of the following parameters: specificity, linearity, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ), robustness, and system suitability test, following the International Conference on Harmonisation (ICH) guidelines.[21]

Specificity

Specificity of the method towards the drug was established through the determination of the peak purity of the reference sample solution of rhIFN-α2a (6 MIU/mL) subjected to degradation by oxidative conditions, by adding 30 μL of hydrogen peroxide 30% during 1 and 2 hours. Besides, the in-house mixture of the pharmaceutical formulations excipients were also analyzed by the RP-LC method using a PDA detector.

Linearity

Linearity was determined by constructing three analytical curves, each one with eight reference standard substance concentrations of rhIFN-α2a, in the range of 0.5–50 MIU/mL prepared in phosphate

buffer containing 0.5 mg/mL polyssorbate 80. Before injection of the solutions, the column was equilibrated for at least 20 min with the mobile phase flowing through the system. Three replicates of 50 μL injections of the reference solutions were made to verify the repeatability of the detector response. The peak areas of the chromatograms were plotted against the respective concentrations of rhIFN-α2a to obtain the analytical curve. The results were subjected to regression analysis by the least squares method to calculate calibration equation and determination coefficient.

Precision and Accuracy

The precision of the method was determined by repeatability and intermediate precision. Repeatability was examined by six evaluations of the same concentration sample of rhIFN-α2a, on the same day, under the same experimental conditions. The intermediate precision of the method was assessed by carrying out the analysis on three different days (inter-days) and also by other analysts performing the analysis in the same laboratory (between-analysts). The accuracy was evaluated applying the proposed method to the analysis of the in-house mixture of the excipients with known amounts of the drug, to obtain solutions at concentrations of 4.8, 6, and 7.2 MIU/mL, equivalent to 80, 100, and 120% of the nominal analytical concentration, respectively. The accuracy was calculated as the percentage of the drug recovered from the formulation and also expressed as the percentage relative error (bias %) between the measured mean concentrations and added concentrations.

Limits of Detection and Quantitation

The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated, as defined by ICH,[21] using the mean values of three independent analytical curves, determined by a linear regression model, where the factors 3.3 and 10 for the detection and quantitation limits, respectively, were multiplied by the ratio from the standard deviation of the intercept and the slope. The LOQ was also evaluated in an experimental assay.

Robustness

The robustness of an analytical procedure refers to its ability to remain unaffected by small and deliberate variations in method parameters and provides an indication of its reliability for the routine analysis. The robustness was determined by analyzing the same samples (6 MIU/mL) under a variety of conditions of the method parameters, such as: injection volume, percent of acetonitrile, flow rate, and the wavelength. To assess the stability of sample solutions of rhIFN-α2a, the samples were tested

being maintained at 2–8°C for 48 h and also placed into the autosampler, at room temperature, for 24 h. The stability of these solutions was studied by performing the experiment and observing any change in the chromatographic pattern, compared with freshly prepared solutions.

System Suitability Test

To ensure the validity of the analytical procedure, data from five injections of 50 μL of the working standard solution containing 6 MIU/mL were used for evaluation of the system suitability parameters, such as asymmetry, number of theoretical plates, retention time, and area, through the CLASS-VP® V 6.14 software.

Analysis of rhIFN-α2a in Pharmaceutical Formulations

For the quantitation of rhIFN-α2a in the pharmaceutical formulations, the respective solutions were diluted to appropriate concentration with phosphate buffer containing 0.5 mg/mL polyssorbate 80, injected in triplicate, and the percentage recoveries of the drug calculated against the reference standard substance.

RESULTS AND DISCUSSION

Optimization of Chromatographic Conditions

To obtain the best chromatographic conditions, the mobile phases were optimized to provide appropriate selectivity and sensitivity. The use of gradient elution resulted in better sensitivity, improving the peak symmetry (about 1.04) with the retention time suitable for the separation also of deamidates and sulphoxides, as usually suggested.[9,19] The PDA detector was used for the selection of the best wavelength detection, and the LC method was validated due to the capability and application for the analysis of rhIFN-α2a in bulk and pharmaceutical formulations.

Method Validation

Specificity

The specificity of the analytical method was indicated by the oxidative conditions that generated the main peak at 32.6 min and additional peaks of the related proteins with the retention times at 30.5 min and 31.8 min,

as shown in the typical chromatograms in Figure 1. No interference from formulation excipients was found, showing that the peak was free from any coeluting peak, with values of peak purity index higher than 0.9999, thus demonstrating that the proposed method is specific for the analysis of rhIFN-α2a.

Linearity

The analytical curves constructed for rhIFN-α2a were found to be linear in the 0.5–50 MIU/mL range. The value of the determination coefficient calculated ($r^2 = 0.9999$, $y = (179698.24 \pm 626.08)\ x + (4895.37 \pm 11501.32)$, where, x is concentration and y is the peak absolute area) indicated the linearity of the analytical curve for the method. Moreover, the relative

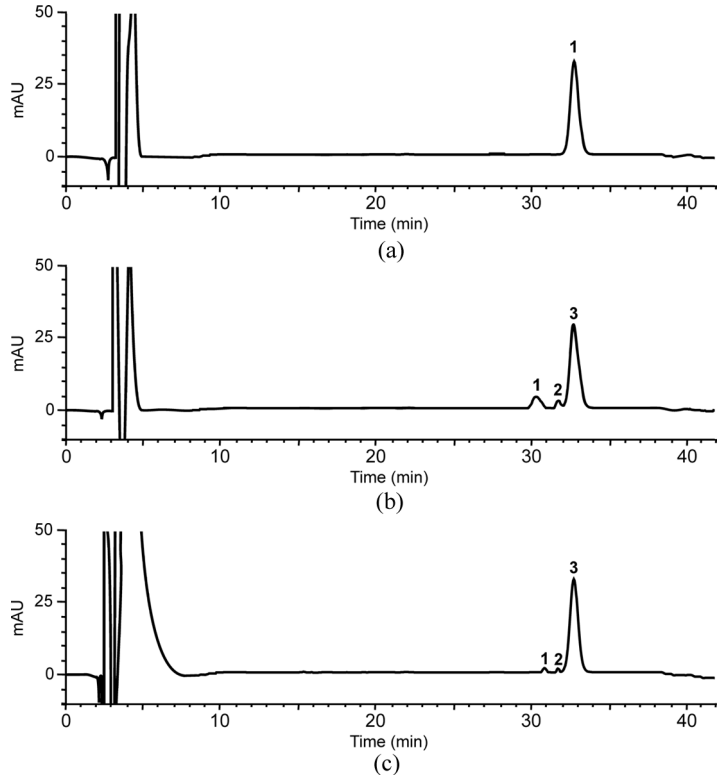

Figure 1. LC chromatograms of interferon-α2a (6 MIU/mL). (a) Interferon-α2a reference standard substance solution: peak 1 = rhIFN-α2a. (b) After degradation by oxidative conditions: peak 1 = deamidates, 2 = sulphoxides, 3 = rhIFN-α2a. (c) Interferon-α2a in pharmaceutical formulations: peak 1 = deamidates, 2 = sulphoxides, 3 = rhIFN-α2a.

standard error of slope can be used as a parameter with respect to the precision of the regression, as a general acceptance criterion for the linearity performance of the analytical procedure.[22] This parameter should be comparable to the relative standard deviation obtained in the evaluation of the precision. The result obtained for the relative standard deviation of the slope is 0.35%, which is lower than the mean value 0.60%, of the RSD of the precision.

Precision

The precision evaluated as the repeatability of the method was studied by calculating the relative standard deviation (RSD) for six determinations of the 6 MIU/mL performed on the same day and under the same experimental conditions. The RSD value obtained was 0.77%.

The intermediate precision was assessed by analyzing two samples of the pharmaceutical formulations on three different days (inter-day); the mean values obtained were 94.38 and 97.73% with RSD 0.55 and 0.41%, respectively. Between analysts precision was determined by calculating the mean values and the RSD for the analysis of two samples of the pharmaceutical formulations by three analysts; the values were found to be 95.04 and 98.01% with RSD 0.77 and 0.52%, respectively. The results are shown in Table 1.

Accuracy

The accuracy was assessed from three replicate determinations of three different solutions containing 4.8, 6, and 7.2 MIU/mL. The absolute means obtained for rhIFN-α2a are shown in Table 2 with a mean value

Table 1. Inter-day and between-analysts precision data of RP-LC for interferon-α2a in pharmaceutical formulations

	Inter-day			Between-analysts		
Sample	Day	Recovery[a] (%)	RSD[b] (%)	Analysts	Recovery[a] (%)	RSD[b] (%)
1	1	94.02		A	95.88	
	2	94.98	0.55	B	94.64	0.77
	3	94.15		C	94.59	
2	1	98.14		A	98.56	
	2	97.72	0.41	B	97.92	0.52
	3	97.33		C	97.56	

[a]Mean of three replicates.
[b]RSD = Relative Standard Deviation.

Table 2. Accuracy of RP-LC for interferon-α2a in pharmaceutical formulations

Nominal concentration (MIU/mL)	Mean concentration found[a] (MIU/mL)	RSD[b] (%)	Accuracy (%)	Bias[c] (%)
4.8	4.89	0.29	101.92	1.87
6	5.95	0.44	99.22	−0.83
7.2	7.30	0.22	101.38	1.25

[a]Mean of three replicates.
[b]RSD = Relative Standard Deviation.
[c]Bias = [(Measured concentration−Nominal concentration)/Nominal concentration] × 100.

of 100.84% and bias lower than 1.87%, demonstrating that the method is accurate within the desired range.

Limits of Detection and Quantitation

For the calculation of the LOD and LOQ, a calibration equation, $y = 179698.24x + 4895.37$, was generated by using the mean values of the three independent analytical curves. The LOD and LOQ were obtained by using the mean of the slope, 179698.24±626.08, and the standard deviation of the intercept of the independent curves, determined by a linear regression line as 11501.32. The LOD and LOQ calculated were 0.19 and 0.64 MIU/mL, respectively. The LOQ evaluated in an experimental assay, with the precision ($\leq 5\%$) and accuracy within ±5%, was found to be 0.5 MIU/mL, which is significant, also considering that the previously published value of 0.25 MIU/mL for the LOQ was found with the precision ($\leq 20\%$).[11]

Robustness

The results and the experimental range of the selected variables evaluated in the robustness assessment are given in Table 3, together with the optimized values. There were no significant changes in the chromatographic pattern when the modifications were made in the experimental conditions, thus showing the method to be robust. The stability of the sample solutions was studied and the data obtained showed the stability during 24 h into the autosampler and during 48 h when maintained at 2–8°C.

System Suitability

The system suitability test was carried out to evaluate the resolution and reproducibility of the system for the analysis to be performed, using five replicate injections of a reference standard substance solution containing

Table 3. Chromatographic conditions and range investigated during robustness testing

Variable	Range investigated	Interferon-α2a[a] (%)	RSD[b] (%)	Optimized value
Flow rate (mL/min)	0.9	98.54	1.69	
	1	97.81	1.45	1
	1.1	98.25	1.35	
Injection volume (μL)	30	96.28	0.38	
	40	96.86	1.89	50
	50	97.55	0.34	
Acetonitrile (%)	46	95.37	0.66	
	48	97.01	0.28	48
	50	97.74	0.35	
Solution stability	Auto sampler 24 h	97.90	0.36	–
	2–8°C 24 h	97.33	1.22	–
	2–8°C 48 h	96.95	0.68	–
Wavelength (nm)	210–320	–	–	214

[a]Mean of three replicates.
[b]RSD = Relative Standard Deviation.

6 MIU/mL of rhIFN-α2a. The RSD values calculated for the retention time, peak symmetry, and peak area were 0.39, 0.66, and 0.90%, respectively. The number of theoretical plates was about 220250.86, with RSD of 1.29%. The experimental results show that the parameters tested were within the acceptable range (RSD <2.0%), indicating that the system is suitable for the analysis intended.

Method Application

The proposed method was applied for the determination of rhINF-α2a in parenteral formulations giving content/potencies within 91.53 and 100.75% of the stated potency, with calculated RSD lower than 1.93%, and related proteins lower than 0.78%, as shown in Table 4, meeting the specifications existing only for concentrated solutions (<5%),[9] and demonstrating also the quality of the pharmaceutical preparations.

CONCLUSION

The results of the validation studies show that the gradient RP-LC method is specific, accurate with mean value of 100.84%, and possesses

Table 4. Determination of interferon-α2a (rhIFN-α2a) in pharmaceutical formulations by the RP-LC method

Theoretical amount		Experimental amount		
		Content/potencies		
Sample	MIU/mL[a]	MIU/mL	rhIFN-α2a[a] (%)	Deamidates/ Sulphoxides (%)
1	6	5.99	99.81	n.d.[b]
2	6	5.52	91.99	0.42
3	6	5.56	92.61	0.34
4	6	5.49	91.53	0.56
5	6	5.58	92.95	0.78
6	6	5.87	97.84	n.d.[b]
7	9	9.07	100.75	n.d.[b]
8	9	8.61	95.62	0.22

[a]Mean of three replicates.
[b]n.d. = not detected.

significant linearity and precision characteristics without any interference from the excipients. The separation was achieved with the retention time of 32.6 min, and the method has been successfully used for the analysis of commercial pharmaceutical formulations, with advantages, also, of lower time consumption related to the biological assay. Moreover, the RP-LC method can represent an important alternative, improving the existing procedures for the potency evaluation of rhIFN-α2a, which can be applied to the purification process and to the batch consistency assessment in bulk and finished biological products.

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