Short communication

Interferon-α 2b quantification in inclusion bodies using Reversed Phase-Ultra Performance Liquid Chromatography (RP-UPLC)

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ABSTRACT

Interferon-α 2b (IFN-α 2b) is a recombinant therapeutic cytokine produced as inclusion bodies using a strain of *Escherichia coli* as expression system. After fermentation and recovery, it is necessary to know the amount of recombinant IFN-α 2b, in order to determine the yield and the load for solubilization, and chromatographic protein purification steps. The present work details the validation of a new short run-time and fast sample-preparation method to quantify IFN-α 2b in inclusion bodies using Reversed Phase-Ultra Performance Liquid Chromatography (RP-UPLC). The developed method demonstrated an accuracy of 100.28%; the relative standard deviations for method precision, repeatability and inter-day precision tests were found to be 0.57%, 1.54% and 1.83%, respectively. Linearity of the method was assessed in the range of concentrations from 0.05 mg/mL to 0.5 mg/mL, the curve obtained had a determination coefficient (r^2) of 0.9989. Detection and quantification limits were found to be 0.008 mg/mL and 0.025 mg/mL, respectively. The method also demonstrated robustness for changes in column temperature, and specificity against host proteins and other recombinant protein expressed in the same *E. coli* strain.

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1. Introduction

Interferons are a family of cytokines with antiviral, antiproliferative and immunomodulatory properties [9,10,18]. Particularly, Interferon-α 2b (IFN-α 2b) is a protein with a molecular weight of approximately 19 kDa, structurally composed of 166 aminoacids [11]. IFN-α 2b does not require glycosylation for its biological activity [12], thus it is large-scale produced using a recombinant strain of *Escherichia coli* [13,14].

In spite of the benefits of expressing the IFN-α 2b gene in *E. coli*, it is well known that a high-level expression of recombinant human proteins in prokaryotic systems often results in their deposition in the cytoplasm as inclusion bodies [4,15]. The recombinant protein contained inside the inclusion bodies is neither biologically active nor soluble [3,4], thus the production process of IFN-α 2b involves fermentation, cellular disruption and inclusion bodies' recovery (known as upstream process), followed by solubilization, folding and chromatographic purification steps (known as downstream process) in order to obtain a high-purity and biologically active protein [3,6].

The detection and quantification of IFN-α 2b is crucial for in-process control of intermediates containing this product [5,7], especially during early downstream processing steps, because the amount of recombinant product inside inclusion bodies has batch to batch differences; its content needs to be estimated before the downstream process steps of solubilization and folding begin. Due to the nature of the sample, content of host proteins, residual DNA and other non-protein contaminants [3,4,6], the quantification of recombinant proteins in inclusion bodies is often difficult. There are reports of quantification and content estimation of proteins in complex matrixes using electrophoretic methods, like SDS-PAGE gels densitometry [16] and HPLC [5,7,17]. An alternative to these methods is UPLC, a chromatographic technique that uses sub-2 μm particles, mobile phases at high linear velocities and instrumentation that operates at high pressure [8,19]. These characteristics increase the resolution, sensitivity and speed of the chromatographic analysis, which are desirable for a chromatographic method to quantify IFN-α 2b in-process control.

A complete validation is essential to demonstrate that a procedure is suitable for the intended purpose [5,20], this is the main reason why the aim of the present work was to develop and validate a Reversed Phase-Ultra Performance Liquid Chromatography (RP-UPLC) method to be used to determine the amount of IFN-α 2b in inclusion bodies' samples.

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2. Materials and methods

2.1. Chemicals

IFN-α 2b standard was supplied in-house as Active Pharmaceutical Ingredient (API) obtained by the standard production bioprocess; this API was lyophilized, salt-free and had a purity of 97%. IFN-α 2b inclusion bodies were supplied in-house, these inclusion bodies had a 34% dry-mass. Milli Q water was produced by a Biocel® Millipore® system, acetonitrile (ACN) and 2-propanol (IPA) were HPLC grade and purchased from J.T. Baker; Guanidine Hydrochloride (GdnHCl), Tris Hydrochloride (TrisHCl), 2-Mercaptoethanol 98% (2-ME) and Iodoacetamide (IA) and Methanol HPLC grade were purchased from Sigma–Aldrich.

For the specificity test inclusion bodies of a recombinant Leucin aminopeptidase (rLAP) supplied in-house with 23% of dry-mass were used. This rLAP was expressed in the same *E. coli* strain and used the same vector as IFN-α 2b.

2.2. Solutions

2.2.1. Solubilization buffer

A buffer solution of GdnHCl 6 M, TrisHCl 50 mM, 2-ME 100 mM and IA 10 mM, at pH 8 was used to dissolve inclusion bodies' samples and standard as well.

2.2.2. Instrument and UPLC method

An Acquity® UPLC system (Waters™) was used for the development and validation of the chromatographic method, Empower® software (Waters™) was used to integrate and process the data obtained.

A 2.1 × 50 mm BEH C$_{18}$ column, with particle and pore diameters of 1.7 μm and 146 Å, respectively was used to carry out the experiments. The UV detector was programmed at a wavelength of 210 nm; column and sample temperatures were set at 50 °C and 10 °C, respectively. Flow rate was set at 0.208 mL/min and the injection volume was 2 μL in all cases.

0.2% TFA in Milli Q water (A) and 0.2% TFA in ACN (B) were used as mobile phases for the UPLC method. The mobile phase gradient was programmed for a total run-time of 8 min as follows: the initial condition was set at 56% A and 44% B, which was maintained during 1 min, then from minute 1.00 until minute 3.00 the % B was raised from 44% to 54.5%, this last condition was maintained for 0.5 min, and from minute 3.50 until minute 4.00 the % B was raised from 54.5% to 70%; the ratio 30% A and 70% B was maintained during 1.40 min and from minute 5.40 until minute 6.00 the % A was raised from 30% to 56% using a linear gradient; this condition was maintained 2 min to equilibrate the column for the next injection.

2.2.3. Standard preparation

API standard solution was prepared by dissolving 50 mg of IFN-α 2b in 5 mL of solubilization buffer to obtain a final concentration of 10 mg/mL. The concentrations used in the validation tests were prepared by dissolving the proper volume of API standard solution with solubilization buffer. The solution was treated in a Thermomixer during 10 min, at 37 °C and 500 rpm; finally was filtered using a 0.45 μm membrane.

2.2.4. Sample preparation

Stock solutions of inclusion bodies both, IFN-α 2b and rLAP were prepared respectively by dissolving 50 mg of inclusion bodies' dry-mass in 5 mL of solubilization buffer to obtain a final concentration of 10 mg/mL. The concentrations used in the validation tests were prepared by dissolving the proper volume of stock solution with solubilization buffer. The solutions were treated in a Thermomixer

during 10 min, at 37 °C and 500 rpm; and finally were filtered using a 0.45 μm membrane.

2.3. Validation tests

2.3.1. System suitability tests

API solution of 0.3 mg/mL was injected six times, the theoretical plate number, retention time of the standard, tailing factor, and capacity factor were calculated and analyzed.

2.3.2. Standard curve

API solutions of 0.05 mg/mL, 0.1 mg/mL, 0.2 mg/mL, 0.3 mg/mL, 0.4 mg/mL and 0.5 mg/mL were prepared and injected in three separated runs using the chromatographic method described in Section 2.2.2. The linear regression analysis was carried out with the known API concentrations against corresponding peak areas, and the determination coefficient, slope and intercept of the resulting calibration curve were calculated.

2.3.3. Quantification and detection limits

The detection and quantification limits were calculated using the data from the linearity test with the following equations: LOD = 3.3 SD/m and LOQ = 10 SD/m; where SD is the standard deviation of the intercept and m is the slope of the calibration curve.

2.3.4. System and method precision

A standard API solution of 0.3 mg/mL was prepared and injected six times, the area mean value and Relative Standard Deviation (RSD) for the set of injections were calculated.

An inclusion bodies' solution of 0.5 mg/mL was prepared and injected six times, on two different days. The area mean value and Relative Standard Deviation for the set of injections were calculated.

2.3.5. Total recovery (accuracy)

Mixtures of API and inclusion bodies' samples with concentration of IFN-α 2b of 0.15 mg/mL, 0.2 mg/mL and 0.3 mg/mL were prepared and injected three times. The obtained IFN-α 2b peak areas were used to calculate the experimental concentration; this data was compared to the theoretical IFN-α 2b concentration to obtain the total recovery percentage.

2.3.6. Method linearity

The method linearity was assessed with a set of IFN-α 2b inclusion bodies' solutions of 0.2 mg/mL, 0.3 mg/mL, 0.4 mg/mL, 0.5 mg/mL and 0.6 mg/mL, this sample set covers a range of concentrations from 0.08 mg/mL to 0.24 mg/mL of IFN-α 2b pure drug substance, the average amount of each sample was measured with the standard calibration curve and a linear regression analysis was done, both curves were compared in order to determine if there was statistical difference using Student's *t*-test at 95%.

2.3.7. Robustness

IFN-α 2b inclusion bodies' solution of 0.5 mg/mL was injected three times, using different mobile phase flow rates (0.198 mL/min, 0.208 mL/min and 0.218 mL/min) and column temperatures (49 °C, 50 °C and 51 °C), the mean value and relative standard deviation of each condition were calculated; Student's *t*-test at 95% was made in order to determine if there were differences between the conditions tested.

2.3.8. Specificity

Solubilized inclusion bodies of rLAP and IFN-α 2b were prepared and injected three times, separately and mixed; the separation factor and the comparative analysis of the chromatograms were made.
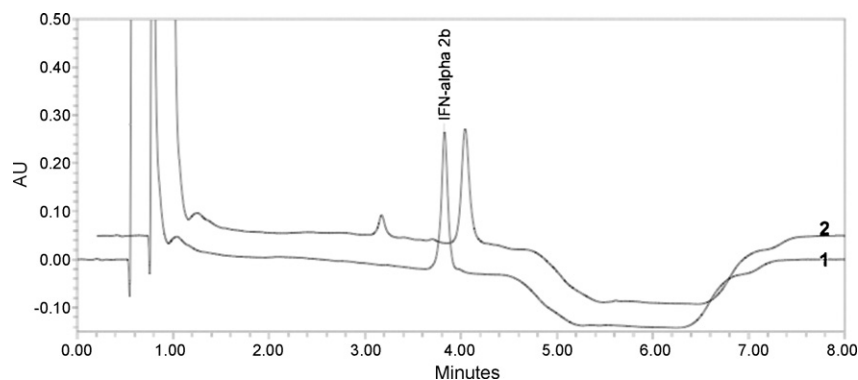
Fig. 1. Typical chromatographic profiles of standard and inclusion bodies' samples are presented as follows: (1) IFN-α 2b standard and (2) IFN-α 2b inclusion bodies.

3. Results and discussion

3.1. Method development

Due to the lack of an accurate, fast and reproducible method for IFN-α 2b quantification in inclusion bodies' samples, our research team developed a UPLC method to this purpose.

Inclusion bodies are complex in nature [3,4], and therefore their recombinant protein content is difficult to quantify for the presence of host proteins and other cell components like DNA, lipids and carbohydrates. Part of our work was to test different solubilization protocols to find the best for our purpose. The conditions mentioned in Section 2.2.4 showed the best performance for IFN-α 2b inclusion bodies' solubilization; these conditions are a modification of those reported by Valente et al. [3]. The rationale for using these conditions was that (i) inclusion bodies are soluble under extreme conditions using chaotropic and reducing agents [3]; (ii) for quantitative purposes it is necessary to dissolve the maximum amount of inclusion bodies, thus the presence of a chaotropic agent and a highly reducing environment is needed; (iii) this condition permits the existence of a single conformational form of the protein (reduced and unfolded form), which avoids the presence of diverse peaks of the same protein. In other studies carried out in our laboratory this phenomenon was observed, especially in chromatograms of downstream process samples from folding and dialysis steps (data not shown).

After the solubilization barrier was overcome, the next step in the development of the analytical method was to find the optimal chromatographic conditions. The conditions indicated in Section 2.2.2 were selected.

Immediately after sample injection there was a load wash to get rid of unbound material for 1 min at 44% of B, then a first gradient to eliminate contaminants was performed for 2 min until 54.5% of B was reached. In order to get a better resolution between contaminants and the main product a 30 s step hold was introduced. Then a second gradient for IFN-α 2b elution was performed until 70% of B was reached by 0.5 min. After Interferon elution and additional wash at 70% of B was applied to clean the column for 1.4 min. Finally the column was re-equilibrated at 44% of B for 2 min to prepare next injection. The whole time run is 8 min between injections. This represents a major advantage over most of densitometric protocols, which tend to be time-consuming because of their inherent run-time, staining, gel and sample preparation. Conventional chromatographic methods are simple, robust, reproducible and reliable procedures that detect small structural changes in a protein [7]. However these HPLC methods are time-consuming and were designed to quantify Interferon in final formulations [5,7,17]. In contrast the UPLC method we report here is a powerful technique that saves solvents, time, sample and is able to resolve Interferon

in inclusion bodies' samples. Thus, this method can be used for in-process control to estimate the productivity and yield during the upstream and downstream processes, respectively.

The chromatographic profiles of inclusion bodies' sample and IFN-α 2b as API were compared in order to find the IFN-α 2b peak in the inclusion bodies. Typical chromatograms of both samples are showed in Fig. 1.

3.2. System suitability tests

The chromatographic analysis of IFN-α 2b API peaks from six injections was used to determine the system suitability parameters. The number of theoretical plates, the retention time, capacity factor and tailing factor were 11092, 3.825 (SD 0.009), 6.650 (SD 0.017) and 0.999 (SD 0.016), respectively. These results suggest that this method is suitable for the intended purpose in accordance with the literature [2].

3.3. Standard curve and method linearity

A calibration curve was built with API solutions in the range of concentrations from 0.05 mg/mL to 0.5 mg/mL. The method was lineal in the range of concentrations used. It was found that the behavior of the method is described by the equation $y = 7472142x - 57417$, where y is the area of the peak and x is the concentration of the API solution; a determination coefficient (r^2) of 0.9989 was found.

On the other hand, the behavior of the data obtained for method linearity tests is described by the equation $y = 7487214.4x - 56665.8$; in this case the determination coefficient was found to be 0.9985, and no statistical difference between this model and standard curve was found.

According to Épshtein [1] a value of $r^2 > 0.99$, suggests that the method is linear throughout the assessed range of concentrations.

3.4. Quantification and detection limits

According to the data obtained, the standard deviation of the intercepts was found to be 18623.7, and the values of LOQ and LOD calculated were 0.025 mg/mL and 0.008 mg/mL, respectively.

3.5. Precision

The relative standard deviation for the system precision test using API samples was found to be 1.54% (data not shown).
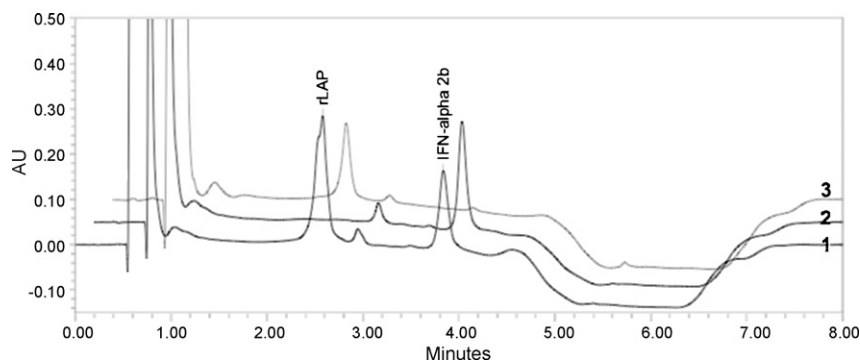
Table 1 summarizes the results obtained for the method precision and the inter-day precision tests using inclusion bodies' samples. In all cases the RSD values were less than 2%,

Table 1
Results obtained for method precision tests using IFN-α 2b inclusion bodies.

Day	Repetition	Concentration of IFN-α 2b (mg/mL)	Mean (mg/mL)	RSD (%)
1	1	0.204		
	2	0.205		
	3	0.202	0.204	0.57
	4	0.205		
	5	0.203		
	6	0.204		
2	1	0.207		
	2	0.210		
	3	0.210	0.210	1.07
	4	0.211		
	5	0.210		
	6	0214		
	Total mean	0.207	Total RSD	1.83

Table 2
Results obtained for accuracy tests.

Mixed amount of IFN-α 2b API and inclusion bodies (mg/mL)	Recovered amount of IFN-α 2b (mg/mL)	Recovery (%)	Mean (%)	RSD (%)
0.15	0.146	97.33		
	0.147	98.00	98.44	1.41
	0.150	100.00		
0.20	0.201	100.50		
	0.206	103.00	101.5	1.30
	0.202	101.00		
0.30	0.301	100.33		
	0.304	101.33	100.89	0.50
	0.303	101.00		

Fig. 2. Typical chromatographic profiles of rLAP, IFN-α 2b inclusion bodies and a mixture of those. The profiles are presented as follows: (1) rLAP + IFN-α 2b inclusion bodies, (2) IFN-α 2b inclusion bodies and (3) rLAP inclusion bodies.

which is considered appropriate for pure drug substances [1], demonstrating an adequate method precision for this complex sample.

3.6. Total recovery (accuracy)

According to the data obtained from the accuracy tests, an average total recovery of 100.28% was detected with a RSD less than 1.5% (Table 2). It is well known that according to Épshtein [1] an average recovery between 99.00–101.00% and an RSD value less than 2% indicates that the method is accurate.

3.7. Robustness

RSD and mean values for each test are summarized in Table 2. According to the data obtained, no statistical differences were found for the different column temperatures tested with a flow rate at the level of 0.198 mL/min with respect to the method conditions. However, precision of this method seems to be lower in the last case. The results suggest that the method is sensitive to mobile phase flow rate variations.

3.8. Specificity

The resolution factor (Rs) of the main peak with respect to the nearest peak, host proteins, was found to be 3.152 and Rs with respect to rLAP peak was found to be 6.156. A value of Rs > 2 is desirable [2]. The data obtained from specificity tests suggests that this method is capable of avoiding interferences from host proteins (Fig. 2).

4. Conclusions

The method presented and validated in the present article demonstrated the compliance with the acceptance criteria of the different desirable characteristics in a chromatographic method: these encompass linearity, precision, accuracy and specificity. The described method was shown to be robust with the different tem-

perature changes, but however was sensitive to changes in the mobile phase.

The described method compares well with other electrophoretic or chromatographic methods. The UPLC method which is simple, accurate, precise and fast allowed to analyze several samples with a high degree of confidence. This means that our developed method was suitable to quantify the total content of IFN-α 2b in inclusion bodies, and could be used in samples from different purification steps.

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