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A simple and rapid HPLC method for quantitation of interferon-α2b in dosage forms and delivery systems

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Abstract

Development of reliable assay methods for quantitation of interferons in dosage forms has encountered serious limitations because of the physicochemical nature of these proteins as well as the sensitivity/selectivity issues. A rapid, available, and easy-to-use reversed-phase HPLC method has been developed for quantitative analysis of interferon-α2b in pharmaceuticals. The reversed- phase method was based on a gradient system using a wide-pore C4 column and produced linear response in drug concentration range of 0.25–5 MIU ($r = 0.9997$). The average within-run and between-run variables of the method were 4.19 and 9.40%, respectively, with corresponding average accuracies of 99.48 ± 4.11 and $102.83 \pm 9.51\%$. The limits of detection (LOD) and quantitation (LOQ) of the method were 0.125 and 0.25 MIU/ml, respectively. The practical applicability of the method was proven throughout a post-marketing quality control program on interferon-α2b products (PD-feron®) produced and marketed in Iran.

Keywords: Interferon-α2b; HPLC; Gradient HPLC; protein assay

1. Introduction

Interferons (IFNs), first discovered by Isaacs and Lindenmann in late 1950s [1], are bioactive agents now well established as highly pleiotropic cytokines exhibiting antiviral activity against a variety of RNA and DNA viruses, potent immunomodulatory effects (e.g. the regulation of NK cell activity and modulation of the expression of MHC-encoded proteins), and antiproliferative activity against malignant cells [2–4]. Due to this broad spectrum of biological activities, a variety of potential therapeutic uses have been established for interferons. In particular, genetically engineered interferon-α2 (rh IFN-α2) produced by recombinant DNA technology, is currently being used worldwide in management of various neoplastic disorders and chronic viral diseases including hairy-cell leukemia, multiple myeloma, chronic myelogenous leukemia (CML) [5–7], renal cell carcinoma [8], malignant melanoma [9], and hepatitis [10]. Interferon-α2b (IFN-α2b), available in market as both lyophilized and soluble injectable preparations, is the most widely used subclass in clinical settings.

The availability of simple and reliable assay methods for detection and quantitation of IFN-α2b in pharmaceutical matrices, is of crucial role in development, process monitoring, and quality control of pharmaceuticals containing this protein drug. Unfortunately, the majority of commonly used analysis methods for IFN-α2b, including bioassay [3,11], immunoassay [3,12], isoelectric focusing (IEF), and gel electrophoresis [13] methods are, while being highly sensitive, sophisticated in nature and, accordingly, suffer more or less from the limited popularity problem mainly because of their high cost, limited availability, long analysis time, lack of specificity, low sensitivity, and external interferences [12,14]. Chromatographic methods, on the other hand, may potentially circumvent the majority of the above-mentioned problems owing to their simple, well-defined, robust and relatively reproducible physicochemical nature. As a result, these methods have long been used in pharmaceutical biotechnology as powerful tools in separation, purification, and qualitative as well as quantitative analysis of proteins/peptides. In addition, these methods are capable of being used during the production/formulation processes for monitoring of minor structural as well as conformational variations occurred in protein structure which can lead to significant changes in biological activity of the drug [15]. Most of the chromatographic methods presented for IFN-α2b analysis have gain limited atten-

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tion in practical use because of a series of inherent problems including very long run-time [13,16–19], the need for special columns and/or other equipments, high variations in responses and, finally, the high expertise needed for use and interpretation of the results obtained from these methods [13,18–22]. Therefore, in this article we have developed and validated a simple and available gradient reversed-phase HPLC method with UV detection for quantitation of IFN-α2b in parenteral dosage forms and delivery systems.

2. Experimental

2.1. Chemicals

IFN-α2b standard was kindly donated by Pooyesh Darou Co. (Tehran, Iran). IFN-α2b ampoules (PD-feron® 3 and 5 MIU/ml, manufactured by Pooyesh Darou Co., Tehran, Iran) were purchased from a local hospital pharmacy. Other chemicals and solvents were from chemical lab or HPLC purity grades, whenever needed, and purchased locally.

2.2. Instrument and HPLC method

The reversed-phase HPLC method consisted, as mobile phase, of a gradient of 0.1% trifluoroacetic acid (TFA) in water (A) and 0.08% TFA in acetonitril (B) with initial A/B ratio of 60/40 which changed linearly to the final A/B of 40/60 within 12 min. The reversal to the initial condition was occurred within 2 min and, finally, the system was re-equilibrated over 6 min (total run-time of 20 min). The analyte separation was carried out by a wide-pore protein analysis column (Symmetry® C_4; 4.6 mm × 50 mm, pore size 300 Å; Waters, MA, USA) operated at ambient temperature and equipped by a guard column of the same packing (Waters, MA, USA). The solvent delivery system used was a double-reciprocating pump (Waters, model 600, MA, USA) with a flow rate of 0.75 ml/min. A UV-detector (Waters, model 486, MA, USA) with a wavelength of 220 nm was used for detection with the outputs processed and recorded by a compatible integrator (Waters, model 746, MA, USA). Sample injection to system (50 μl) was made by a loop injector (Rheodyne® 7725i, Cotati, CA, USA).

2.3. Standard preparation

A stock solution of 5 MIU/ml was prepared from IFN-α2b standard in phosphate buffer (KH_2PO_4, 0.05 M; pH 7.4) and the concentrations of 0.25, 0.5, 1, and 2.5 MIU/ml were prepared by serially diluting this solution with the proper amount of the same buffer.

2.4. System suitability tests

The following parameters were calculated as system suitability indices of the developed method:

$$\text{number of theoretical plates } (N) = 16\left(\frac{t_R}{w}\right)^2,$$

$$\text{peak symmetry} = \frac{w}{2f},$$

$$\text{retainability } (K') = \frac{t_R}{t_a} - 1,$$

where t_R is the retention time of the analyte, w the width of the analyte peak at its 0.05 height, f the front half-width of the analyte peak at its 0.05 height and t_a the retention time of non-retained analyte (solvent front).

2.5. Analysis validation tests

2.5.1. Standard curve (linear range)

Serial dilution samples, prepared as described, were injected directly to chromatograph in three separate runs and, in each case, the linear regression analysis was carried out on known concentrations of IFN-α2b against the corresponding peak heights and, then, the regression coefficient (r), slope, and intercept of the resulting calibration curves were determined.

2.5.2. Within-run variations

In one run, three samples with concentrations of 0.25, 1, and 5 MIU/ml (from high, middle, and low regions of the standard curve) were prepared in triplicate and analyzed by developed HPLC method. Then, the coefficient of variations (CV%) of the corresponding determined concentrations were calculated in each case.

2.5.3. Between-run variations

On three different runs, samples from upper, intermediate, and lower concentration regions used for construction of standard curve (the same as within-run variation test) were prepared and analyzed by HPLC method. Then, the corresponding CV% values were calculated.

2.5.4. Absolute recovery (accuracy)

For each sample tested for within- and between-run variations, the absolute recovery of the method was determined as the percent ratio of the measured concentration (determined using standard curve) to the corresponding nominal added concentration.

2.5.5. Limits of detection and quantitation

Limit of detection (LOD) of the method was determined as the lowest IFN-α2b concentration producing a signal-to-noise (S/N) ratio of about 3. Limit of quantitation (LOQ) was determined as the lowest IFN-α2b concentration capable of being quantitated with enough accuracy (≥90%) and precision (≤20%).

2.6. Applicability test

The applicability of the developed method was tested by analyzing 10 units from each of two IFN-α2b preparations manufactured and marketed in Iran (PD-feron® 5 and 3 MIU/ml) as a part of a post-marketing quality control (PMQC) study.

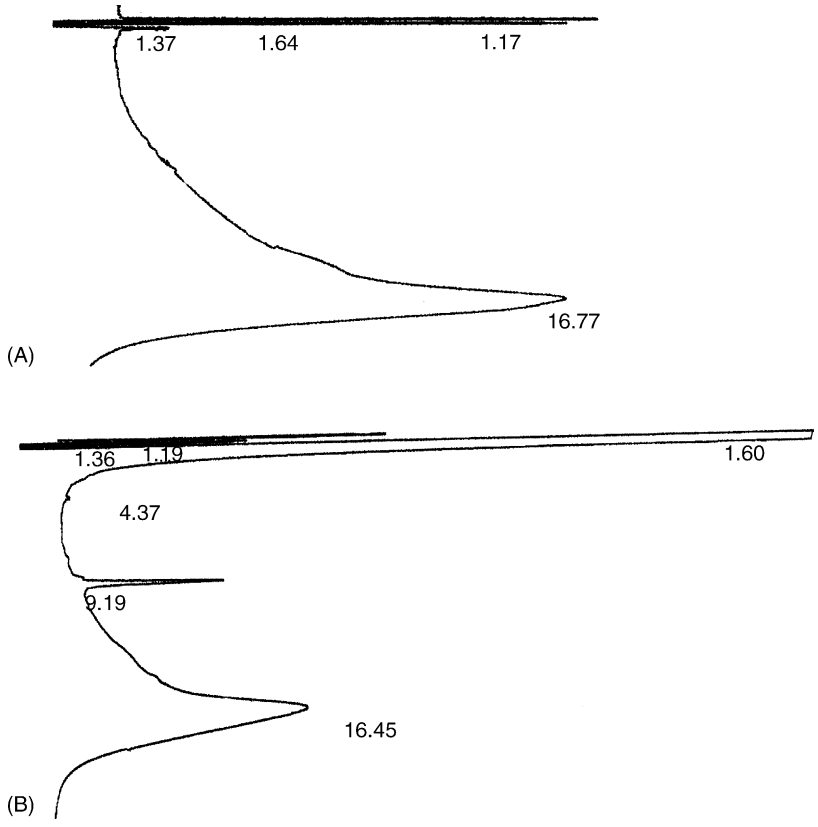

Fig. 1. Typical chromatograms of the developed HPLC method for quantitation of IFNα2b: (A) blank sample and (B) sample containing 2.5 MIU/ml of IFNα2b (t_R = 9.19 min).

3. Results and discussion

3.1. Method development

In response to lack of an available, reliable, and easy-to-use analysis method as a part of production process monitoring, in process (IP) and finished product quality control (QC), post-marketing quality control (PMQC) and formulation development studies on IFN-α2b as well as in vitro characterization tests on novel delivery systems containing this protein drug, a gradient reversed-phase HPLC method was developed for quantitation of the drug in pharmaceutical matrices. To this end, initially a series of isocratic conditions using different usual protein assay mobile phase compositions and polarities were tested which did not resulted in acceptable outputs in all cases. As a result, gradient systems consisting of two mobile phase components regularly used in protein analysis (i.e., solutions of trifuoroacetic acid (TFA) in water and acetonitril) with different concentrations, initial and final ratios, and total gradient and run cycles were tested extensively. The other parameters tested in this factorial design were mobile phase flow rate, column temperature, detection wavelength (UV detection was used in all cases regarding to its popularity), and volume of injection. Considering the whole body of data obtained from this extensive study, the set of conditions indicated earlier in this article, was selected for further validation. A typical

Table 1
Within-run variations of the HPLC method for quantitation of IFNα2b (n = 3)

Nominal added concentration (MIU/ml)	Run number	Measured concentration	Mean ± S.D.	CV%	Accuracy (%)	Mean ± S.D.
	1	5.03			100.60	
5	2	5.12	5.09 ± 0.05	1.02	102.40	101.80 ± 1.04
	3	5.12			102.40	
	1	1.05			104.53	
1	2	1.02	1.01 ± 0.05	4.61	102.46	100.88 ± 4.65
	3	0.96			95.64	
	1	0.24			97.24	
0.25	2	0.22	0.24 ± 0.02	6.93	88.52	95.77 ± 6.64
	3	0.25			101.56	

Table 2
Between-run variations of the HPLC method for quantitation of IFNα2b ($n = 3$)

Nominal added concentration (MIU/ml)	Run number	Measured concentration	Mean ± S.D.	CV%	Accuracy (%)	Mean ± S.D.
	1	5.16			103.20	
5	2	5.03	5.08 ± 0.07	1.38	100.60	101.53 ± 1.45
	3	5.04			100.80	
	1	2.08			83.20	
2.5	2	2.44	2.31 ± 0.20	8.66	97.60	92.27 ± 7.89
	3	2.40			96.00	
	1	1.29			129.00	
1	2	0.97	1.07 ± 0.19	17.7	97.00	107.00 ± 19.08
	3	0.95			95.00	
	1	0.42			84.00	
0.5	2	0.54	0.51 ± 0.08	15.69	108.00	101.33 ± 15.14
	3	0.56			112.00	
	1	0.29			116.00	
0.25	2	0.27	0.28 ± 0.01	3.57	108.00	112.00 ± 4.00
	3	0.28			112.00	

chromatogram of the developed HPLC method is shown in Fig. 1.

3.2. System suitability tests

The number of theoretical plates (N), peak symmetry, and retainability (K') of the method for IFN-α2b were 2111, 1.125, and 6.92, respectively. These data shows that the developed method is of appropriate separation efficiency and peak shape, both of which are important factors in evaluation of the chromatographic method outputs.

3.3. Method validation tests

3.3.1. Linearity

The method produced linear responses throughout the IFN-α2b concentration range of 0.25–5 MIU/ml, which is suitable for intended purposes.

A typical linear regression equation of the method was $y = 12713x - 322.63$ with x and y representing IFN-α2b concentration (in MIU/ml) and peak height (in arabitiary units), respectively, and the regression coefficient® of 0.9997.

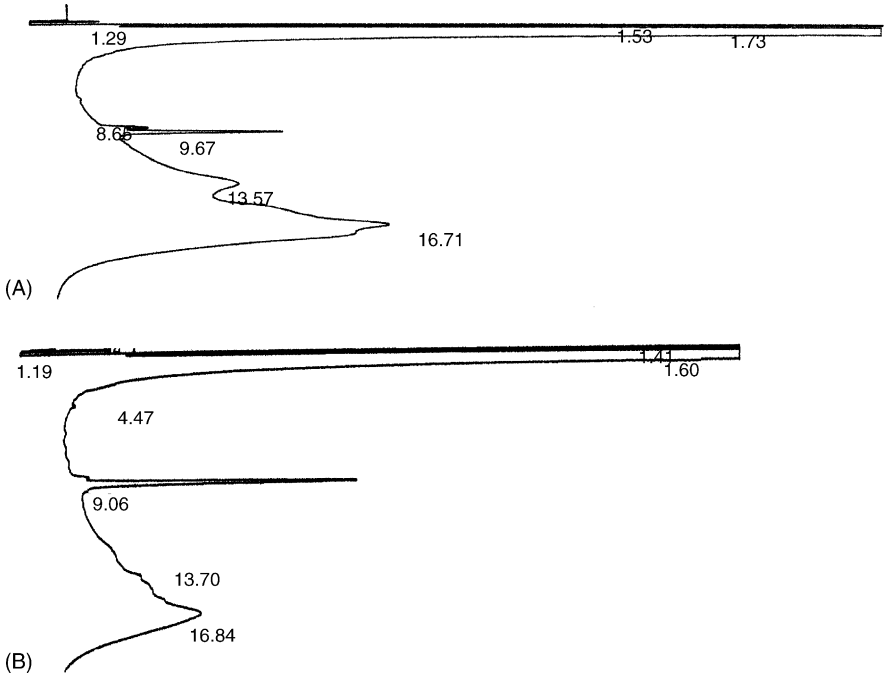

Fig. 2. Typical chromatograms of the assay test on two IFN-α2b preparations produced in Iran. (A) 3 MIU/ml and (B) 5 MIU/ml ($t_R = 9.67$ and 9.06 min, respectively, correspond to IFN-α2b in these chromatograms).

Table 3
Applicability of the developed method for quantitation of marketed products ($n = 10$)

	Product number										Mean ± S.D.	CV%
	1	2	3	4	5	6	7	8	9	10		
5 MIU/ml	5.03	5.12	4.99	5.12	4.92	5.04	5.03	5.08	5.11	4.97	5.04 ± 0.07	1.39
3 MIU/ml	3.08	3.12	3.03	2.99	3.02	3.11	2.98	2.98	3.00	3.06	3.04 ± 0.05	1.64

3.3.2. Within-run variations and accuracy

The within-run variations of the developed HPLC method as well as the corresponding absolute recoveries are shown in Table 1.

3.3.3. Between-run variations and accuracy

The between-run variations of the developed HPLC method as well as the corresponding absolute recoveries are shown in Table 2.

3.3.4. Limit tests

The limits of detection (LOD) and quantitation (LOQ) of the method were 0.125 and 0.25 MIU/ml, respectively.

In general, the results of the validation tests indicated that the developed method has a remarkable degree of accuracy, repeatability and reproducibility with application limits being in the desirable range for routine applications.

3.4. Applicability test

Typical chromatograms of IFN-α2b assay in two tested products of Iranian market are shown in Fig. 2. Listed in Table 3 are the assay results of 10 products of each dosage strengths. These data showed that the methods can be applied throughout the quality control programs on IFN-α2b marketed products.

4. Conclusion

The results of validation tests were, collectively, indicative for a method with a practically wide enough linear range, acceptable precision and accuracy both within- and between- analytical runs, and practically reliable sensitivity. System suitability tests showed that the developed method is of appropriate separation efficiency and peak shape. A notable advantage of this method over the previously reported HPLC methods for IFN-α2b [13,17–19] was its considerably shorter (at least two times) run-time, which is of a great importance in practice, particularly when a high number of samples are to be analyzed (e.g., in regulatory and industrial settings). The results of assay tests on IFN-α2b products marketed in Iran were representative for an applicable and easy-to-use method. In addition, the method is currently being used successfully in a novel delivery system development project in our lab.

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