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Development and validation of RP-UPLC method for quantification of Interferon in pharmaceutical preparation of Interferon

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ABSTRACT

A new specific, rapid, and precise reverse phase ultra performance liquid chromatography (RP-UPLC) method was developed and validated for quantification of Interferon in pharmaceutical preparation of Interferon (IFN). Chromatography was performed on a RP C-18 (2.1 mm ID × 50 mm L, 300 Å, 1.7 µm) using a mobile phase containing 0.1% Trifluoroacetic acid (TFA) in Milli-Q water and 0.1% TFA in Acetonitrile with gradient program at 0.6 mL/ min flow rate. Detection was carried out at 210 nm. Quantification was accomplished with internal reference standard (qualified using EP reference standard) The method was validated as per ICH guidelines for linearity (correlation coefficient > 0.99) (n=24), range, accuracy (n=12), precision (n=12) and robustness. Robustness was confirmed by different factors like wavelengths, column temperatures, and sample compartment temperatures and with different lot of columns during method development. The method was linear over the concentration range of 0.008mg/mL–0.028 mg/ mL. The precision of the method in terms of relative standard deviation was evaluated from intra- and inter-day replicate injections of different concentration of Interferon sample using different lot of columns. Components of within and between-batch variances were found to be below 2% (n = 18) and 5%, respectively, which constituted an acceptable level of variation. Retention time was found to be about 2.0 min for Interferon.The developed method is accurate, precise, linear, robust and rapid. The total analysis time (~5.0 min) allows the large numbers of samples in a short period of time, therefore would be cost effective in the pharmaceutical industry.

KEYWORDS: *Interferon alfa-2b; Protein concentration; Quantification; Ultra Performance Liquid Chromatography (UPLC); validation.*

1 INTRODUCTION

Interferons are a family of naturally-occurring proteins that are made and secreted by cells of the immune system. Three classes of interferons have been identified: alpha, beta, and gamma. Alpha interferon have been categarized in two sub classes: Interferon –alpha 2a [1] and Interferon alpha -2b [2].

Recombinant Human Interferon-alpha 2b, produced in E.Coli is a single, non-glycosylated, polypeptide chain containing 165 amino acids and having a formula of $C_{860}H_{1353}N_{229}O_{255}S_9$ and molecular mass of 19271 Dalton[2]. This protein is produced by recombinant DNA technology and resembles interferon secreted by leukocytes. IFN-alpha 2b is purified [3] by proprietary chromatographic techniques. It has been successfully used worldwide for the treatment of chronic hepatitis C, chronic hepatitis B, hairy cell leukemia, chronic myelogenous leukemia, multiple myeloma, follicular lymphoma, carcinoid tumor, and malignant melanoma therapy of several viral diseases and neoplasm. Interferons do not directly kill viral or cancerous cells; They boost the immune system response and reduce the growth of cancer cells by regulating the action of several genes that control the secretion of numerous cellular proteins that affect growth. Molecular Characterization of Recombinant Human Interferon Alpha-2b was reported by Héctor Santana.[2] Detailed structure of recombinant

interferon alpha-2b (rHu IFN alpha-2b) was evaluated by mass spectrometry, chromatography and CD spectroscopy.[3] Since recombinant protein molecule readily undergoes structural changes as a result of oxidation, deamination and aggregation (dimer and polymer formation), appropriate formulation of a medicinal product ensuring its stability throughout the reported shelf life, is important.[4-5] Pharmaceutical preparations of recombinant interferon are now available from several manufacturers around the world. These formulated products require specific analytical methods to control the quality of the product at different stage. Cueto-Rojas HF et. al. has developed a chromatographic method for quantitaion of IFN in inclusion bodies. [6] Where as Zarrin A et. al. have developed a HPLC method for quantitation of interferon-alpha 2b in dosage forms and delivery systems. [7] Collaborative study for establishment of an HPLC-method for batch consistency control of recombinant interferon-alfa-2 was done by Buchheit KH et. al.. [8] As per our knowledge there is no RP-UPLC method for quantification of IFN in intermediate and formulation of IFN. The objective of our work was hence to develop a rapid, simple and accurate RP-UPLC method with UV detection useful for routine quality control of IFN at intermediate and in final formulation. The method developed was validated as per ICH guidelines [9-11] for parameters such as specificity, linearity and range, accuracy, precision and robustness.

2 MATERIALS AND METHODS

2.1 Material, reagent, and chemicals:

HPLC grade Acetonitrile was purchased from Merck; Tri-fluoro-acetic acid (TFA) was purchased from Sigma Aldrich. Ultra pure water was obtained using Milli-Q® UF-Plus (Millipore) system. Concentrated IFN solution [13-14] (Active Pharmaceutical Ingredient) having concentration of 2.3 mg/ mL was used as working standard, it was qualified using EP reference standard. Other API, Intermediate and formulated sample of IFN was used as test samples. Other chemicals such as Na_2HPO_4 $2H_2O$, NaH_2PO_4 H_2O, Disodium Ethylene Diamine Tetra Acetate (Di sodium EDTA) and Polysorabate 80 were used.

2.2 Preparation of standard, mobile phase and dilution buffer:

Diluted IFN standard was prepared by using 2.3 mg/mL of IFN IRS (Internal Reference standard) [12] and it was used for preparation of difference working standard using diluent. All dilutions were made using calibrated digital micro-pipettes. Mobile phase 'A' consisted of 0.1% V/V TFA in Milli Q water and mobile phase 'B' consisted of 0.1% V/V TFA in Acetonitrile. Formulation buffer used as diluent having the pH 7.0 contains 10mM Na_2HPO_4 $2H_2O$, 9.4mM NaH_2PO_4 H_2O and 0.01 % w/v of polysorabate 80 and Disodium EDTA.

2.3 Instrumentation:

A liquid chromatograph (Waters Acquity) system equipped with Binary pump, auto sampler, and an injection valve (Rheodyne), thermostatted column compartment and tunable UV detector. The UPLC system was well equipped with Empower 2 software for data processing.

2.4 Chromatographic condition:

A reversed-phase water BEH C_{18} column (2.1 mm* 50 mm L, porosity 300 Å, particle size1.7 µm) was used for separation. To get the optimum results, the mobile phase with a flow rate of 0.6 mL /min was used with gradient program, (time (% of mobile phase B), 0min (40%), 0.54 min (40%), 1.7 min (48%), 2.0 min(52%), 2.70 min(60%),3.40 min(70%), 3.6 min(40%) and 5.0 min (40%). The column temperature and sample compartment were maintained at 30°C and 10°C respectively and the detection was monitored at wavelength 210nm. The injection volume was 10 µL.

2.5 Validation of chromatographic method:

The developed and optimized chromatography method was validated according to the procedures described in ICH guidelines Q2 (R1) [9-11]. The following validation characteristics were addressed: specificity, linearity and range, accuracy, precision, and robustness. The guidance for the methodology and statistical tests and associated limits for the validation procedure is provided in the ICH Q2B.[10]

3 RESULTS AND DISCUSSION

3.1 Method development:

To acquire the best chromatographic conditions, the stationary phase, mobile phase composition, UV detection by wavelength, column temperature, sample compartment temperature and flow rate were adequately selected. The stationary phase C_{18} was chosen for optimum profile and result. C_{18} types of column was considered suitable for quantification of IFN as it is also suggested for impurity quantification of interferon alpha concentrated solution in official monograph of European Pharmacopoeia[13] and Indian Pharmacopoeia[14]. Four different wavelengths (207nm, 210nm, 213nm and 220 nm) tried for maximum and specific detection. As narrow bore column used, flow rate was set based on the column pressure and run time, it was varied from 0.5 mL/min to 0.7 mL/min. The column temperature was varied between 25°C and 35°C and the analysis at 30°C was preferred to better result however there was no major change was observed during the study. As part of gradient chromatographic to get the optimum, specific and efficient detection, mobile phase composition was varied and set chromatographic conditions described in "Chromatographic condition" section 2.4. No interference of other excipients was observed as shown in figure 1.The capacity factor (k') of the IFN peak was 9.0. The asymmetry of the peak was found to be 1.53 while the tailing factor parameter IFN was found to be 1.33. USP plate count for the IFN peak was to be 22343 for replicate injections of IFN standard; the % RSD of the main peak area was found to be below 2.0%, and there was no significant variation in the retention time (<0.1 min). Available IFN formulation in the market contains 0.012 mg/mL and 0.015 mg/mL of IFN and the range was selected as 0.008 mg/mL to 0.028 mg / mL. As this quantification method (Assay), detection limit (LOD) and quantification limit (LOQ) was not required for the study. Different lots of reverse phase C_{18} column used and compared in terms of percentage variation of recovery of IFN component, it was not more than 5% in all samples. Retention time of the principal peak of IFN was found to be around 2.0 min (**figure 2**).

3.2 Method validation:

3.2.1 Specificity:

Specificity is the ability of method to measure the analyte, IFN, response in the presence of its potential impurities, and excipients such as Na_2HPO_4 $2H_2O$, NaH_2PO_4 H_2O, Polysorabate 80 and Disodium EDTA was studied in terms of resolution of peaks observed and peak area of IFN. To prove this formulation buffer containing excipients, mobile phase A, and IFN standard were injected onto UPLC separately. Five different concentrations of IFN (0.008, 0.012, 0.015, 0.018 and 0.025 mg/mL) were prepared in the mobile phase A as well as in the formulation buffer and were injected in triplicate onto UPLC. From **figure 1** it is evident that there is no interference and the method developed is specific for IFN. The percent RSD for recovery of each concentration of each diluent was found to be less than 3.0 % with respective calibration curve.

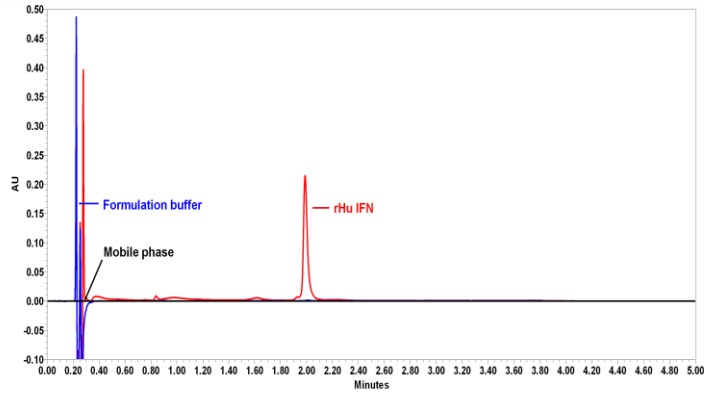

Figure 1: Overlap chromatogram of (A) Mobile phase (0.1%TFA in Milli Q water) as blank, (B) Formulation buffer (as diluent) and (C) Interferon alpha 2b drug product. Mobile phase, formulation buffer and Interferon alpha 2b drug product were injected into HPLC separately. (A) Mobile phase containing 0.1 % TFA in Milli Q water and 0.1% TFA in Acetonitrile. (B) Formulation buffer having the pH 7.0 --containing 10 mM Na_2HPO_4 $2H_2O$, 9.4mM NaH_2PO_4 H_2O and 0.01 % w/v of polysorabate 80 and Disodium EDTA and (C) Interferon alpha 2b drug product –containing 0.018 mg/mL of interferon alpha 2b

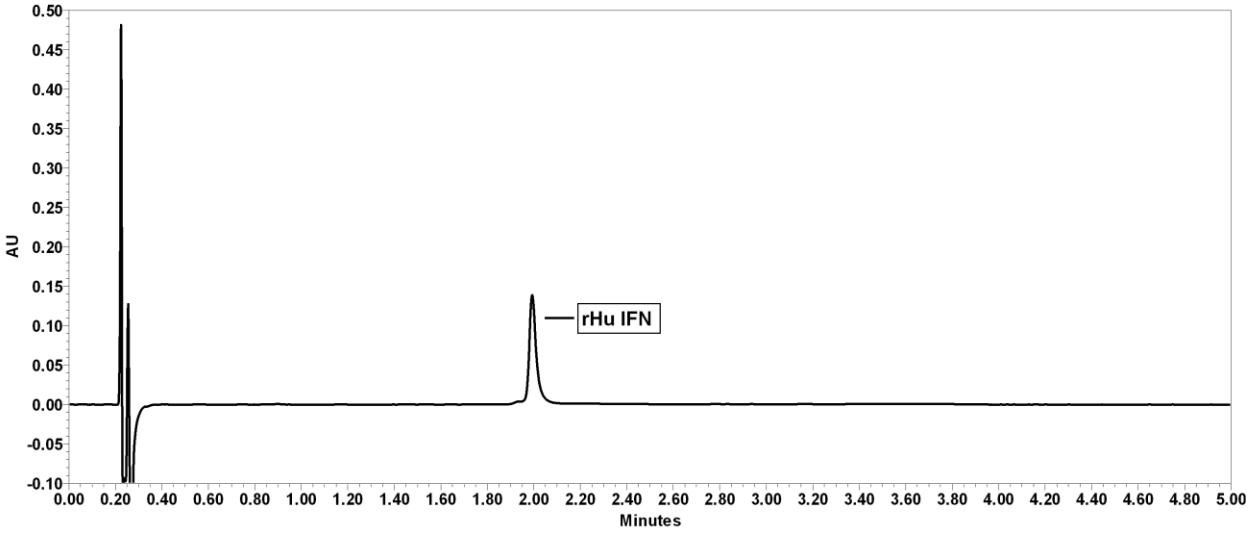

Figure 2: Chromatogram of Interferon alpha 2b drug product- containing 0.012 mg/mL of interferon alpha 2b

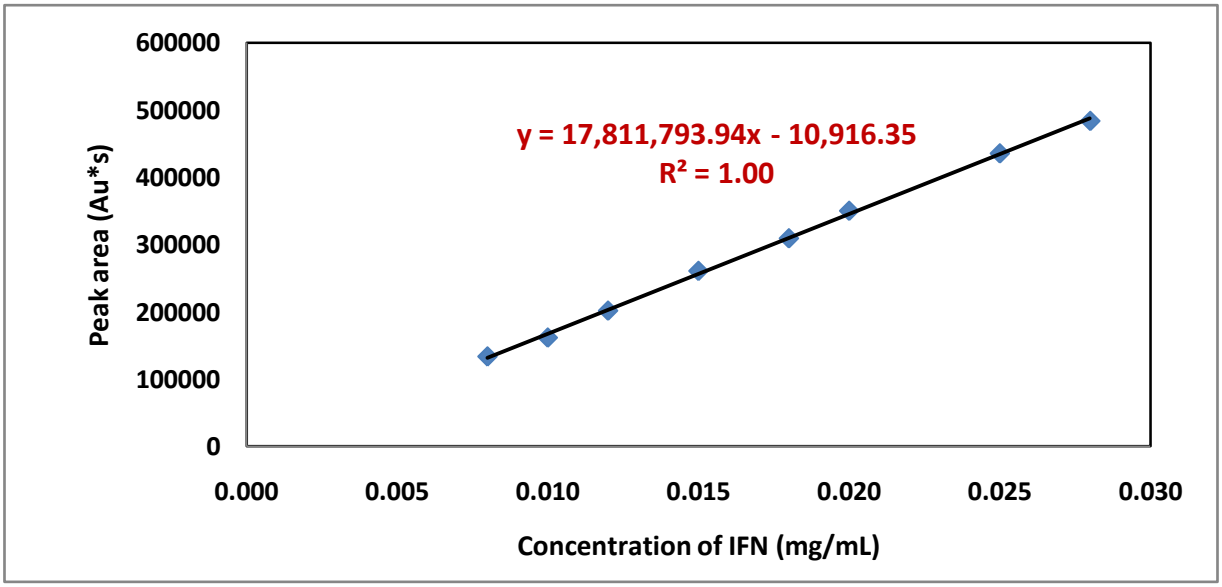

Figure 3: Linearity curve for interferon alpha 2b drug substance (2.3 mg/mL) diluted with formulation buffer for preparation of different concentration range from 0.008 mg/mL to 0.028 mg/mL and injected separately.

3.2.2 Linearity and Range:

The linearity of measurement was evaluated by analyzing different concentrations of the IFN internal reference standard solutions, ranging from 0.008 mg/mL to 0.028 mg/mL. Linearity curve was constructed by plotting average peak area against concentration and regression equation was computed (**Figure 3**). The correlation coefficient, slope, Y-intercept, regression equation of the calibration curve was determined and is shown in **Table 1**. The percent RSD for each standard dilution was found to be less than 2.0% while the percent recovery of each standard dilution was found to be in the range of 97 % to 102%.

3.2.3 Accuracy:

Accuracy was studied using four different solutions, containing 0.012, 0.015, 0.018 and 0.025 mg/ mL of IFN. Each solution was spiked in the diluent and injected onto HPLC (n = 12). The % recovery, assessed by comparing the analyte amount determined versus known amount

spiked, was found to be between 98% and 101% and the % RSD was found to be <2.0% as shown in **Table 3**.

3.2.4 Precision:

Precision was evaluated based on intra-day (repeatability) and inter-day (intermediate precision) variation. The repeatability was assessed with six independent samples of each system suitability solution, 0.012 mg/mL and 0.018 mg/mL of IFN. Single injection from each preparation was injected and the results are shown in **Table 4**. The % RSD of the main peak area was found to be < 2.0 %.

Intermediate precision was evaluated on different days with different lot of columns of same brand (waters, BEH C-18, 2.1 mm ID × 50 mm L, porosity 300 Å, particle size 1.7 µm) and equipment (UPLC system Waters Acquity) in the same laboratory. Five different concentration of IFN (0.008, 0.012, 0.015, 0.018 and 0.025 mg/ mL) sample were considered for study.

The % RSD for the main peak area of IFN standard within each set and between different sets was found to be less than 2.0 % and 5.0 % respectively. The % recovery of IFN standards was found to be between 95.0% and 105.0% within each set. The results are shown in **Tables 1, 3, and 4.**

3.2.5 Robustness:

To determined robustness of developed method, experimental condition was deliberately altered and four different concentrated IFN samples were injected. Evaluated altered conditions were concentration of TFA in mobile phase (± 20 %), flow of mobile phase (± 0.1 mL /min), different wavelength (±3 nm), column temperature (± 5°C) and sample compartment temperature (set temp. 10°C, altered temp. 30°C). Retention time of IFN peak, RSD for main peak area of IFN were evaluated and results are found satisfactory in altered condition of column temperature, sample compartment temperature and concentration of TFA in mobile phase while compared with the unaltered condition. Whereas, result for recovery was found higher than the expected in altered condition of flow and wavelength while compared with unaltered condition and hence these parameters (flow and wavelength) shall be controlled during the routine analysis (**Table 5**).

Table 1: Linearity and range results

Sample name	conc. (mg/mL)	% RSD for peak area of replicate injections	Back calculated conc. (mg/mL)	% Recovery
Standard 1	0.008	1.68	0.0081	101
Standard 2	0.010	0.39	0.0097	97
Standard 3	0.012	0.26	0.0119	99
Standard 4	0.015	0.65	0.0152	102
Standard 5	0.018	0.57	0.0180	100
Standard 6	0.020	0.32	0.0203	101
Standard 7	0.025	1.59	0.0251	100
Standard 8	0.028	0.10	0.0278	99

Table 2: Result of different test parameters

Statistical parameter	Details / Results
Linearity & Range (Conc. in mg/mL)	0.008 - 0.028
Regression equation	y = 17811793.94x – 10916.35
Correlation coefficient	1.00
Total analysis time (minute)	5 minute
Retention time (minute)	2.0 minute
Flow rate	0.6 mL/min
Column	BEH C_{18} (2.1x50 mm), 1.7µm,300°A
Mobile phase	0.1%v/v TFA in Milli Q water , 0.1%v/v TFA in Acetonitrile
Method	Gradient
Validation parameter	
Specificity	No interference
Accuracy	Recovery 95 % - 105 %
Precision	RSD less than 2 %
Intermediate precision	RSD less than 5 %
Robustness	Except flow and wavelength, method is robust for studied parameter

Table 3: Accuracy results

Sample name	conc. (mg/mL)	% RSD for peak area of replicates	Back calculated conc. (mg/mL)	% Recovery
Standard 1	0.012	1.46	0.0121	101
Standard 2	0.015	0.12	0.0147	98
Standard 3	0.018	0.61	0.0178	99
Standard 4	0.025	1.59	0.0251	100

Table 4: Repeatability results

Preparation of IFN	For 0.012 mg/mL of IFN				For 0.018 mg/mL of IFN			
	Peak area (Au*s)	Av. Area (Au*s)	% RSD	Rt of IFN peak (min)	Peak area (Au*s)	Av. Area (Au*s)	% RSD	Rt of IFN peak (min)
1	198148	202318	1.6	1.9	310126	307961	0.7	1.9
2	203389			1.9	310106			1.9
3	207750			1.9	307084			1.9
4	201079			1.9	304385			1.9
5	201223			1.9	308102			1.9
6	202065			1.9	306582			1.9

Table 5: Robustness results

Altered parameter	Altered condition	Parameter verified	Results
Column Temperature			
	25°C	RT of IFN peak	Difference < 0.1 min
		% recovery	%RSD < 1 %
	35°C	RT of IFN peak	Difference < 0.1 min
		% recovery	%RSD < 1 %
Sample compartment			
	30°C	RT of IFN peak	Difference < 0.1 min
		% recovery	%RSD < 1.5 %
Concentration of TFA			
	0.08% V/V	RT of IFN peak	Difference < 0.1 min
		% recovery	%RSD < 5 %
	0.12% V/V	RT of IFN peak	Difference < 0.1 min
		% recovery	%RSD < 5 %
Flow			
	0.5 mL /min	RT of IFN peak	Difference < 0.1 min
		% recovery	%RSD > 5 %
	0.7 mL /min	RT of IFN peak	Difference < 0.1 min
		% recovery	%RSD > 5 %
Wavelength			
	207 nm	RT of IFN peak	Difference < 0.1 min
		% recovery	%RSD >10%
	210 nm	RT of IFN peak	Difference < 0.1 min
		% recovery	%RSD >10%
	220 nm	RT of IFN peak	Difference < 0.1 min
		% recovery	Sensitivity very less, %RSD > 30%

4 CONCLUSION

A convenient and rapid RP-UPLC method was demonstrated for quantifying of Interferon in the presence of other excipients. Validation study proved that the developed method was specific, linear, accurate and precise. RP- UPLC method described here was found to appropriate for quantification of IFN in interferon formulation. Since method is simple and rapid it may be successfully applied to quality control analysis of IFN in interferon formulation.

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