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Article

Evaluation of recombinant human interferon beta 1b by liquid chromatography methods and bioassay

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Recombinant human interferon beta 1b (rhIFNβ–1b) is clinically used to treat multiple sclerosis. A reversed–phase liquid chromatography (RP–LC) method was carried out on a Jupiter C_4 column (250 mm × 4.6 mm i.d.). The mobile phase A consisted of 0.1% trifluoroacetic acid (TFA) in water, and the mobile phase B was acetonitrile with 0.1% TFA run at a flow rate of 1.0 mL/min. A size exclusion liquid chromatography (SE–LC) method was carried out on a BioSep–SEC–S 2000 column (300 mm × 7.8 mm i.d.). The mobile phase consisted of 1 mM monobasic potassium phosphate, 8 mM sodium phosphate dibasic and 200 mM sodium chloride buffer pH 7.4, run isocratically at a flow rate of 0.8 mL/min. Retention times were 31.87 and 17.78 min, and calibration curves were linear over the concentration range of 1−200 µg/mL (r^2 = 0.9998) and 0.50−200 µg/mL (r^2 = 0.9999), respectively, for RP–LC and SE–LC, with detection at 214 nm. Liquid chromatography (LC) methods were validated and employed in conjunction with the *in vitro* bioassay to assess the content/potency of rhIFNβ–1b, contributing to improve the quality control and to ensure the efficacy of the biotherapeutic.

Keywords: Recombinant human Interferon beta 1b. Biotechnology-derived medicine. Bioassay. Reversed-phase liquid chromatography. Size-exclusion liquid chromatography.

INTRODUCTION

Interferons (IFNs) are natural proteins produced by immune system cells that have antiviral, antiproliferative and immunomodulatory properties. Interferon betas represent the first class of disease modifying therapies (DMTs) for multiple sclerosis (MS) and have contributed considerably to the understanding of the immunomodulatory mechanisms. Human interferon beta (hIFN-β) is a hydrophobic glycoprotein that contains 166 amino acids produced by fibroblasts. Advances in the recombinant DNA technology facilitate the expression of hIFN–β resulting in a large-scale production of biopharmaceutical formulations. Recombinant human interferon beta 1b (rhIFNβ-1b) is engineered as a non-glycosylated protein in *Escherichia coli* (*E. coli*) with a serine residue instead of a cysteine at amino acid position 17 and lacks the methionine at the N-terminus. The substitution at position 17 was made to eliminate the free sulfhydryl of cysteine to obtain a product that is more stable upon storage. The polypeptide structure is composed of 165 amino acids with a molecular mass of 18.5 kDa. It is currently being used worldwide to treat MS in a large number of patients (Mark *et al*., 1984; Dendrou, Fugger, Friese, 2015; EMEA, 2017).

The biological potency of rhIFNβ–1b has been assessed by *in vitro* bioassays using a variety of cells/virus systems and has also been used in a collaborative study that established the 3rd international standard. An *in vitro* cytopathic bioassay based on the effect of the VSV virus cell line (ATCC® No. VR-158™) against the sensitive WISH cell line (ATCC® No. CCL-25™) has been widely used, evaluating the responses as viable protected cells stained with vital dyes such as AlamarBlue or tetrazolium salts. The antiproliferative assays using the Daudi cell line (ATCC® No. CCL-213™) or A–549 cell line (ATCC® No. CCL-185™) were applied to evaluate the potency of the biomolecule, measuring the responses with MTT. A genetically modified cell line with promoter activation

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and expression of enzyme markers such as alkaline phosphatase was also developed (Borden, Hogan, Voelkel, 1982; Meager, Das, 2005; Basu *et al*., 2006).

Currently, analytical methodologies based on biological and physicochemical properties have proven to be particularly useful for the analysis of therapeutic proteins and have also been applied to correlation studies (Qian *et al*., 2008; Skrlin *et al*., 2010; Cardoso *et al*., 2017). Reversed-phase liquid chromatography (RP-LC) and size-exclusion liquid chromatography (SE-LC) methods offer a high level of accuracy and sensitivity for the analysis of closely related protein variants, degradation products, and high-molecular-weight (HMW) substances with reduced or absent activity and altered immunogenicity (Fekete *et al*., 2014; Moussa *et al*., 2016). A gradient RP-LC method was used to evaluate the long-term stability of hIFN-β with 214TP C_4 column with detection at 214 nm (Geigert *et al*., 1988). A RP-LC method was performed on a Jupiter C_4 column, together with a SE–LC method on an SEC UPLC column, with UV and fluorescence detection, to evaluate the effect of the excipients on the stability of the formulation and to characterize and quantitate aggregates in preparations of rhIFNβ-1b, thus correlating the biophysical characteristics with immunogenicity (Abdolvahab *et al*., 2016a; Abdolvahab *et al*., 2016b). An RP-LC method using a C_4 column and an SE-LC using an SEC UPLC column were applied to monitor the mechanism of aggregation of IFNβ-1b by heating, oxidizing, or seeding of an unformulated monomeric solution (Fazeli *et al*., 2014). SE-LC using a TSK G2000S column was applied to characterize and quantitate aggregates, evaluating potential links to any immune response (Barnard, Babcock, Carpenter, 2013). A literature search shows the necessity of validated methods for the analysis of rhIFNβ-1b to meet the acceptance criteria suggested for biotechnology-derived proteins (FDA, 2015; EP, 2017).

This research aimed to develop and validate specific, stability-indicating RP-LC and SE-LC methods to assess the content/potency of rhIFNβ-1b in biopharmaceutical formulations; to correlate the results with the *in vitro* bioassay and to evaluate the bioactivity and the cytotoxicity of related proteins and HMW substances. This work thus will contribute to the development of methods to improve the quality control of biotechnology-derived medicine.

MATERIAL AND METHODS

Reagents and chemicals

The standard Interferon Beta Ser17 Mutein, human rDNA derived (BRS-IFNβ-1b), WHO 00/574, for bioassay, was obtained from the National Institute for Biological Standards and Control (NIBSC, Hertz, UK) with 64,000 IU per vial. The recombinant human biological reference substance of Interferon beta 1b (Rec–IFNβ–1b), for physicochemical assays, was supplied by United States Biological (Swampscott, Massachusetts, USA) with 2 µg/mL. A total of six batches of Betaferon® Bayer HealthCare (São Paulo, Brazil), containing 300 µg/vial (9.600.000 IU/vial = 9.6 MIU/vial) of rhIFNβ-1b, were labeled from 1 to 6. The samples were acquired from commercial sources within their shelf–life period. Monobasic potassium phosphate, sodium phosphate dibasic, sodium chloride, acetonitrile, trifluoroacetic acid (TFA), human serum albumin (HSA) and mannitol were supplied by Merck (Darmstadt, Germany). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and AlamarBlue™ cell viability reagent were acquired from Sigma-Aldrich (St. Louis, MO, USA). All chemicals used were of HPLC grade or special analytical grade. For all analyses, ultrapure water was purified using an Elix 3 coupled to a Milli–Q Gradient A10 system from Millipore (Bedford, MA, USA).

Apparatus

RP-LC and SE-LC analyses were performed on a Shimadzu LC system (Kyoto, Japan) equipped with a CBM–20A system controller, a LC-20 AD pump, a DGU-20A_S degasser, a SIL-20AC_{HT} autosampler, a CTO column oven and a SPD-M20A photodiode array (PDA) detector. Peak areas were automatically integrated by the computer by using LC Solution version 1.22 SP1 software. The absorbance of the *in vitro* cell culture bioassay was measured on a Thermo Scientific Varioskan® Flash microplate reader (Vantaa, Finland).

Samples and standard solutions

A commercial batch of Betaferon® labeled as 300 µg/vial, equal to 9.6 MIU/vial, was calibrated against the BRS-IFNβ-1b and Rec-IFNβ-1b and used as an in–house biological reference substance of rhIFNβ-1b (BS-IFNβ-1b) for the LC methods. Stock solutions were prepared by diluting the BS-IFNβ-1b and samples of biopharmaceutical formulations in ultrapure water to a final concentration of 50 µg/mL (1.6 MIU/mL) and 25 µg/mL (0.8 MIU/mL), respectively, for RP-LC and SE-LC; and to a range of concentrations starting with 1 IU/mL of BRS-IFNβ-1b and of BS-IFNβ-1b in culture medium DMEM containing 2% (v/v), fetal bovine serum, for the cell culture bioassay.

Procedures

Reversed-phase liquid chromatography (RP-LC)

The experiments were performed on a reversed-phase Phenomenex (Torrance, USA) Jupiter C_4 column (250 mm × 4.6 mm i.d., with a particle size of 5 µm and pore size of 300 Å) maintained at 30 °C. A security guard holder was used to protect the analytical column. The mobile phase A consisted of 0.1% TFA in water and the mobile phase B was 0.1% TFA in acetonitrile and was run as follows: time 0 to 0.01 min 38% of B; from 0.01 to 35 min linear up to 60% of B; from 35.1 to 38 min linear down to 38% of B maintained up to 42 min. The flow rate was 1 mL/min, with PDA detection at 214 nm. The injection volume was 50 µL.

Size-exclusion liquid chromatography (SE-LC)

The experiments were accomplished using a size-exclusion Phenomenex (Torrance, USA) BioSep-SEC-S 2000 column (300 mm × 7.8 mm i.d., with a particle size of 5 µm and pore size of 145 Å) maintained at 25 °C. A security guard holder was used to protect the analytical column. The mobile phase consisted of 1 mM monobasic potassium phosphate, 8 mM sodium phosphate dibasic; 200 mM sodium chloride buffer, pH 7.4. The flow rate was 0.8 mL/min with PDA detection at 214 nm. The injection volume was 30 µL.

Antiproliferative assay

The bioassay was performed as described elsewhere (Borden, Hogan, Voelkel, 1982) with some adjustments. The A-549 cell line of human alveolar adenocarcinoma (ATCC® No. CCL-185™) was maintained in DMEM culture medium supplemented with 10% (v/v) FBS in 75-cm^2 flasks. The cells were seeded in 96-well Costar® microplates (Corning, NY, USA) at a density of 1×10^4 cells/mL and dosed upon seeding with four concentrations (nine–fold dilution series) starting with 1 IU/mL of rhIFNβ-1b. BRS-IFNβ-1b was used as standard and to calibrate the BS-IFNβ-1b, and the control was DMEM. Briefly, the plates were incubated at 37 °C, 5% CO_2 for 120 h. Then, 20 µL of AlamarBlue™ was added per well, and the plates were incubated for a further 4 h. The response was calculated as the difference between the absorbances measured at 570 and 600 nm. The biological activity was calculated with the parallel line statistical method by using CombiStats™ software (EDQM, Council of Europe, Strasbourg, France).

In vitro cytotoxicity test

The assay was performed as described elsewhere (Maldaner *et al*., 2017) based on the neutral red uptake (NRU) assay, with the NCTC clone 929 cell line (mammalian fibroblasts, ATCC® No. CCL-1™) exposed to altered samples of rhIFNβ-1b. The absorbance was measured at 540 nm.

Validation of LC methods

Validation of the RP–LC and SE-LC methods was performed by using samples of rhIFNβ-1b with a label claim of 300 µg/vial (9.6 MIU/vial), and the parameters were assessed according to the guidelines (FDA, 2015; ICH, 2005).

Specificity

The specificity of the RP-LC method was assessed by subjecting a BS–IFNβ–1b solution and a sample of the biopharmaceutical formulation (300 µg/mL) to photodegradation by exposing the sample to 200 Wh/m^2 near-UV light in a photostability chamber for 24 h. The oxidative condition induced by hydrogen peroxide 3% for 3 h was also tested. The solutions were diluted with ultrapure water to a final concentration of 50 µg/mL. For the SE-LC, a BS-IFNβ-1b solution and a sample of biopharmaceutical formulation (300 µg/mL) were subjected to neutral hydrolysis (60 °C for 2 h) and shaken for 30 min. Solutions were diluted with ultrapure water to a final concentration of 25 µg/mL. In addition, possible interference from excipients of the biopharmaceutical formulation was determined by analyzing a sample that contained only placebo (in–house mixture of formulation excipients). The specificity of the LC methods was also established by determining the peak purity of rhIFNβ-1b and degraded forms by overlaying the spectra captured at the apex, upslope, and downslope using a PDA detector.

Linearity

The linearity of the methods was determined by constructing three independent analytical curves, each with eight concentrations of the BS-IFNβ-1b solution. Three replicates of 50 µL and 30 µL injections of the reference solutions were prepared to verify the repeatability of the detector response. The peak areas were plotted against the respective concentrations of BS-IFNβ-1b solution to obtain the analytical curve. The results were subjected to regression analysis by the least squares method to calculate the calibration equation and the determination coefficient (r^2).

Precision and accuracy

Precision was determined by means of repeatability

(intra-day) and intermediate precision (inter–days and between–analysts). Repeatability was examined by six analyses of a sample of rhIFNβ-1b, at concentrations of 50 and 25 µg/mL for the RP-LC and SE-LC methods, respectively, on the same day and under the same experimental conditions. The inter-days precision was assessed by analysis of two samples of the biopharmaceutical formulations on three different days. The between-analysts precision was assessed by submitting the samples to analysis by different analysts in the same laboratory. The accuracy was assessed by analysis of the in–house mixture of excipients with known amounts of the biomolecules to obtain solutions at concentrations of 40, 50 and 60 µg/mL for the RP-LC, and 20, 25 and 30 µg/mL for the SE-LC methods, equivalent to 80, 100 and 120%, respectively, of the working concentration solutions. The accuracy was calculated as the percentage of drug recovered from the formulation and expressed as the percentage relative error (bias %).

Detection and quantitation limits

The detection limit (DL) and the quantitation limit (QL) were calculated as defined by (ICH, 2005), using the mean values of the three independent analytical curves determined by a linear-regression model, where the factors 3.3 and 10 for the DL and QL, respectively, were multiplied by the ratio from the standard deviation of the intercept and the slope. The QL was also evaluated in an experimental assay.

Robustness

The robustness of an analytical procedure provides an indication of its reliability for routine analysis. The RP–LC and SE-LC methods were tested analyzing the same samples, containing 50 µg/mL and 25 µg/mL, respectively, under one-variable-at-a-time (OVAT) conditions, to evaluate the described parameters (Tables I and III). The stability of sample solutions was tested after storage, and any changes in the chromatographic pattern were compared with the freshly prepared samples.

System suitability test

The system suitability test was performed to analyze five replicate injections of 50 µg/mL and 25 µg/mL BS-rhIFNβ-1b. The peak area, retention time, theoretical plates, and tailing factor (peak symmetry) were measured.

Analysis of rhIFNβ-1b in biopharmaceutical formulations

Biopharmaceutical samples available for clinical use, with potency expressed in µg/mL and MIU/mL, were identified compared to Rec-IFNβ-1b. For the RP-LC and SE-LC methods, the solutions were diluted to appropriate concentrations of 50 and 25 µg/mL in ultrapure water, respectively, and injected in triplicate, and the percentage recoveries were calculated against the BS-IFNβ-1b.

RESULTS AND DISCUSSION

Biotherapeutics manufactured by the recombinant DNA technology are complex and heterogeneous and possess characteristics that are highly dependent on the processes used for their manufacture. This includes the expression system, raw materials, protein production, isolation and purification processes, as well as the formulation and storage of the final product. As a consequence, even if the proteins are produced using the same gene sequence, it is highly likely that the quality of the products differs considerably, with potential impact on clinical efficacy and safety. Then, an important issue for biotechnology–derived medicines is the research of biological and LC methods necessary to assure the content/potency of the biotherapeutics, selected due to their capabilities and validated following the international guidelines for the qualitative and quantitative analysis.

Development and optimization of chromatographic conditions

Tests were performed to determine which mobile phase would lead to satisfactory selectivity and sensitivity in a short separation time. For the RP–LC, TFA in water and TFA in acetonitrile resulted in higher sensitivity related to phosphate buffer, and lower retention time compared to sodium phosphate buffer. Resolution was improved, using mobile phases containing 0.1% TFA in water and 0.1% TFA in acetonitrile. For the SE-LC method, a mobile phase composed by 1 mM monobasic potassium phosphate, 8 mM sodium phosphate dibasic buffer and 200 mM sodium chloride resulted in higher sensitivity than phosphate buffer and phosphate buffered saline. The optimal wavelength was selected using a PDA detector. Typical chromatograms demonstrating the resolution of the symmetrical peaks corresponding to rhIFNβ-1b are shown in Figure 1 (a, b) and Figure 2 (a, b).

Validation of LC methods

The stability-indicating capacity of the RP-LC method was evaluated under oxidative and photodegradation conditions, which showed decreases

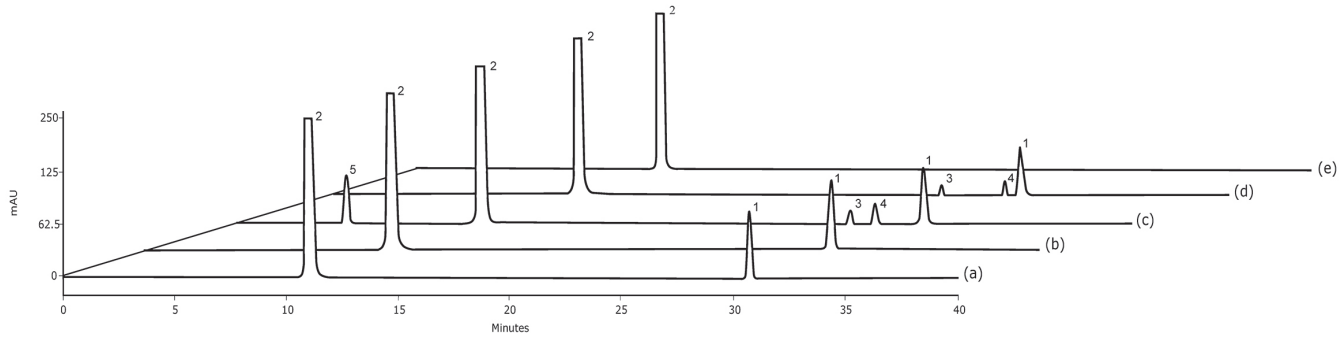

FIGURE 1 – Representative RP–LC chromatograms showing peak 1 = rhIFNβ–1b; peak 2 = human serum albumin; peaks 3 and 4 = related proteins; peak 5 = hydrogen peroxide. (a) Biological reference substance of rhIFNβ–1b. (b) Sample of biopharmaceutical formulation, untreated and after: (c) degradation by hydrogen peroxide; (d) photodegradation; (e) placebo.

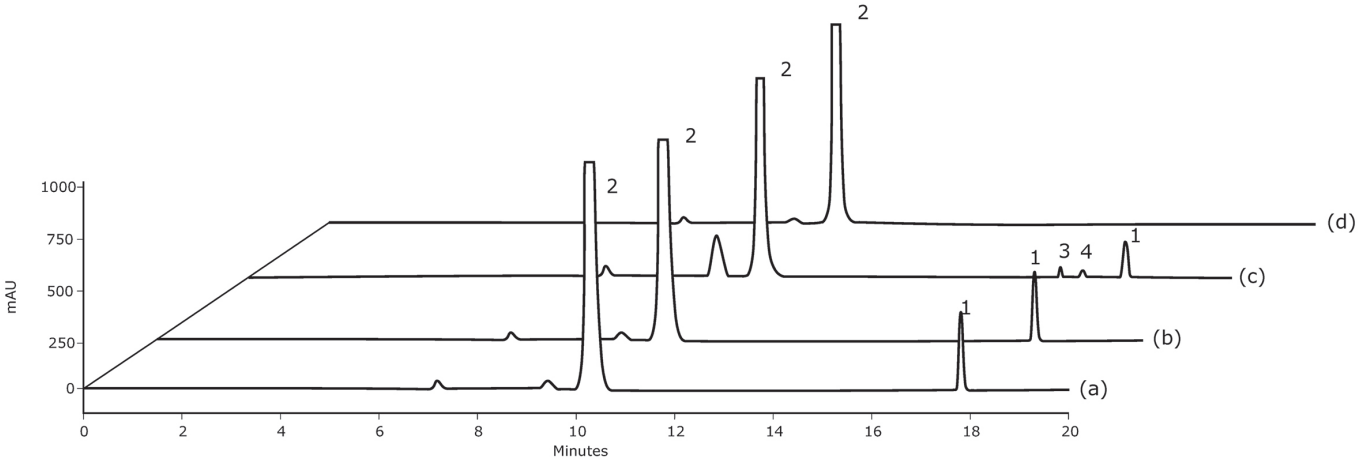

FIGURE 2 – Representative SE–LC chromatograms showing peak 1 = rhIFNβ–1b; peak 2 = human serum albumin; peaks 3 and 4 = high–molecular–weight substances. (a) Biological reference substance of rhIFNβ–1b. (b) Sample of biopharmaceutical formulation, untreated and after: (c) neutral hydrolysis; (d) placebo.

in the area of the main peak (at 31.87 min) and additional peaks attributed to deamidated/sulfoxides, with retention times at 27.74 and 28.62 min (Figure 1c) and at 27.44 and 30.07 min (Figure 1d), respectively. The specificity of the SE–LC method, evaluated by neutral hydrolysis, showed the peak related to the monomer detected at 17.78 min and two additional peaks of HMW substances, with retention times at 16.58 and 17.69 min (Figure 2c). Moreover, the injection of a sample containing only the placebo showed one peak at 9.78 min, related to the HSA. Together with the peak purity index of 0.9999-1, the data showed that the peaks were free of any co–eluting peak and that the excipients, mainly HSA, did not interfere in the analysis, which confirmed that the LC methods were specific for the analysis of rhIFNβ-1b.

For the RP–LC method, analytical curves were found to be linear over a concentration range of 1-200 µg/mL (0.032 - 6.4 MIU/mL). The determination coefficient was calculated as $r^2 = 0.9998$, $y = (31936 \pm 919.76)\,x - (11051 \pm 8647.04)$, where x is concentration and y is the peak absolute area. For the SE–LC method, the analytical curves were found to be linear over a concentration range of 0.50-200 µg/mL (0.016 – 6.4 MIU/mL). The determination coefficient was calculated as $r^2 = 0.9999$, $y = (231645 \pm 165037.74)\,x - (2182 \pm 1001.77)$.

The precision of the LC methods was studied by calculating the mean values and relative standard deviation (RSD %). For repeatability, the obtained RSD values were 0.10 and 0.04%. The inter-days precision was tested, giving RSD values of 0.40 and 0.71% for the RP-LC, and 0.83 and 0.94% for the SE-LC. The between-analysts precision was also determined; the RSD values were found to be 0.57 and 1.19% for the RP-LC and 0.25 and 0.35% for the SE-LC method.

The accuracy was assessed, and the absolute means were 100.42 and 100.45% with bias lower than 0.69 and 0.82% (Table I), respectively, for the RP-LC and SE-LC

methods; these values showed the accuracy under the experimental conditions.

The DL and QL of the LC methods were calculated as 0.47 and 1.57 µg/mL, for the RP–LC and 0.10 and 0. 34 µg/mL, for the SE-LC. The evaluated experimental QL with a precision with error below 5% and accuracy within ± 5% (Shabir *et al*., 2007), was found to be 1 µg/mL (0.032 MIU/mL) and 0.50 µg/mL (0.016 MIU/mL), respectively, which are suitable for quality control analysis.

The results and experimental range of the selected variables evaluated for robustness using an OVAT approach are given in Tables II and III, together with the optimized values, demonstrating that they were within the acceptable deviation (RSD ≤ 2%), with non-significant differences ($p > 0.05$), as calculated by analysis of variance. The stability tests also showed non-significant changes ($p > 0.05$).

The suitability of the system was tested and the RSD values calculated for the retention time, peak symmetry and peak area were 0.01, 0.40 and 1.96%, respectively, for the RP-LC, and 0.04, 0.28 and 0.22%, respectively, for the SE-LC method. The number of theoretical plates was 107554, with an RSD of 0.81%, and 9579 with an RSD of 0.88%. These results were considered acceptable (RSD < 2%).

Application of the LC methods and bioassay

The validated LC methods were applied to determine rhIFNβ-1b in biopharmaceutical formulations, giving the content/potencies shown in Table IV, with mean

TABLE I - Accuracy of RP–LC and SE–LC for rhIFNβ–1b in biopharmaceutical formulations

Method	Nominal concentration (µg/mL)	Mean concentration measured[a] (µg/mL)	RSD[b] (%)	Accuracy (%)	Bias[c] (%)
RP–LC	40	40.00	0.03	99.99	–0.01
	50	50.30	0.85	100.59	0.59
	60	60.41	0.50	100.69	0.69
SE–LC	20	20.16	0.08	100.80	0.82
	25	25.04	0.06	100.16	0.16
	30	30.12	0.19	100.40	0.40

[a]Mean of three replicates. [b]RSD = Relative standard deviation. [c]Bias = [(Measured concentration - Nominal concentration)/Nominal concentration] × 100.

TABLE II - Chromatographic conditions and range investigated during robustness test for the RP-LC method

Variable	Range investigated	rhIFNβ–1b[a] (%)	RSD[b] (%)	Optimized value
TFA (%)	0.05	98.97	1.08	0.10
	0.10	100.16	0.94	
	0.15	99.13	1.24	
Temperature (ºC)	28	99.35	0.23	30
	30	100.16	0.04	
	32	99.35	0.36	
Flow rate (mL/min)	0.9	99.79	0.19	1.0
	1.0	99.39	0.14	
	1.1	99.31	0.28	
Solution stability	Autosampler 24 h	100.47	0.56	–
	2 – 8 ºC 24 h	100.05	0.16	–
	2 – 8 ºC 48 h	100.68	0.83	–
Wavelength (nm)	190 – 320	–	–	214

Mean of three replicates. [b]RSD = Relative standard deviation.

TABLE III - Chromatographic conditions and range investigated during robustness test for the SE-LC method

Variable	Range investigated	rhIFNβ–1b[a] (%)	RSD[b] (%)	Optimized value
Mobile phase pH	7.0	99.27	1.33	7.4
	7.4	99.30	0.60	
	7.8	100.55	1.21	
Monobasic potassium phosphate (mM)	0.9	99.25	1.20	1.0
	1.0	99.59	0.78	
	1.1	100.03	0.85	
Sodium phosphate dibasic (mM)	7.5	99.56	0.89	8.0
	8.0	100.33	0.78	
	8.5	98.24	0.84	
Sodium chloride (mM)	180	99.86	0.94	200
	200	100.10	0.54	
	220	100.22	1.09	
Flow rate (mL/min)	0.7	99.95	0.90	0.8
	0.8	99.99	0.78	
	0.9	99.94	0.89	
Solution stability	Autosampler 24 h	99.46	1.06	–
	2 – 8 ºC 24 h	99.35	0.59	–
	2 – 8 ºC 48 h	99.45	0.91	–
Wavelength (nm)	190 – 320	–	–	214

[a]Mean of three replicates. [b]RSD = Relative standard deviation.

values 1.27 and 1.05% higher for RP-LC and SE-LC, respectively, compared to the *in vitro* bioassay. The Pearson's correlation coefficient was calculated, showing significant correlation for the RP-LC ($r = 0.9626$), and the SE–LC ($r = 0.9367$), related to the bioassay. Furthermore, biopharmaceutical samples were artificially degraded, as described in the section on specificity, were analyzed by the LC methods, and subjected to the *in vitro* bioassay. These samples showed bioactivities reduced by 6.50% ± 3.40 ($n = 3$) and 44.30% ± 2.15 ($n = 3$) for the deamidated/sulfoxides and HMW substances, respectively, except for the oxidative condition, which was not tested due to the possible interference of H_2O_2. The results showed the capability of each method, which can be apply also to biosimilarity studies of rhIFNβ–1b and the recently used innovative formulation of beta1a PEGylated interferon (Kálmán-Szekeres, Olajos, Ganzler, 2012; Madsen, 2017). The noninnovator "copy" versions are likely to vary in their quality, eg., physicochemical characteristics and biological activity, with important implications for clinical efficacy and safety (Meager *et al.,* 2011). Then the studies showed the potential of each method, and that using them in combination represents advancement in terms of analytical techniques, contributing also to the characterization of this therapeutic biosimilar.

Cytotoxicity evaluation

The cytotoxicity test was performed on degraded forms giving mean IC_{50} = 8.68 ± 0.71 MIU/mL, IC_{50} = 12.17 ± 0.20 MIU/mL, for photolytic and neutral hydrolysis conditions, respectively. Differences calculated by Student's t-test were significant ($p < 0.05$) compared to the intact molecule, which had an IC_{50} of 6.89 ± 0.13 MIU/mL. Such evaluations are now necessary, mainly due to recent concerns about possible undesirable effects in humans resulting from the instability of samples during storage (Pineda *et al*., 2015).

CONCLUSIONS

The results of the validation studies show that the LC methods are specific, sensitive and accurate, and can be initially applied in combination with the *in vitro* bioassay for content/potency assessment of biopharmaceutical formulations. Due to the agreement between the results, LC methods can be used as alternative for evaluating biotechnology processes and through subsequent purification steps, to monitor the stability, and to ensure the batch-to-batch consistency of the bulk and finished biotechnology-derived medicines.

TABLE IV - Comparative content/potency evaluation of rhIFNβ–1b in biopharmaceutical formulations by bioassay and LC methods

Sample	Theoretical Amount	Bioassay[a]		RP–LC[a]		SE–LC[a]	
		Potency	Confidence intervals	Main peak	Deamidated/ sulfoxides	Monomer	HMW
	(μg)	(%)	(P = 0.95)	(%)	(%)	(%)	(%)
1	300	99.70	(94.20 – 105.60)	100.83	0.81	100.58	0.68
2	300	100.20	(94.20 – 106.70)	100.41	0.59	100.12	0.49
3	300	98.80	(92.90 – 105.00)	100.31	0.46	100.98	0.39
4	300	102.40	(96.90 – 108.10)	104.05	0.36	103.90	0.47
5	300	98.70	(93.30 – 104.50)	99.87	0.78	99.16	0.89
6	300	95.60	(90.20 – 101.30)	97.57	0.99	96.96	0.84
Mean	–	99.23	–	100.50	0.73	100.28	0.68
SD[b]	–	2.22	–	2.08	–	2.27	–
2[c]	–	93.70	(85.60 – 102.50)	89.34	7.36	–	–
2[d]	–	55.90	(50.80 – 61.60)	–	–	65.40	11.36
2[e]	–	Nt[f]	–	72.08	25.47	–	–

[a]Mean of three replicates. [b]SD = Standard deviation. [c]UVdegraded. [d]Neutral hydrolysis. [e]Oxidative condition. [f]Nt = Not tested.

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