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Stability-indicating capillary zone electrophoresis method for the assessment of recombinant human interleukin-11 and its correlation with reversed-phase liquid chromatography and biossay

Ricardo Bizogne Souto, Fernanda Pavani Stamm, Jéssica Barbieri Schumacher, Clovis Dervil Appratto Cardoso Jr., Guilherme Weber de Freitas, Rafaela Ferreira Perobelli, Sérgio Luiz Dalmora *

Department of Industrial Pharmacy, Federal University of Santa Maria, 97105-900 Santa Maria, RS, Brazil

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ABSTRACT

A stability-indicating capillary zone electrophoresis (CZE) method was validated for the analysis of recombinant human interleukin-11(rhIL-11) using rupatadine fumarate, as internal standard (IS). A fused-silica capillary, (50 μm i.d.; effective length, 40 cm) was used at 25 °C; the applied voltage was 20 kV. The background electrolyte solution consisted of 50 mmol L^{-1} sodium dihydrogen phosphate solution at pH 3.0. Injections were performed using a pressure mode at 50 mbar for 45 s, with detection by photodiode array detector set at 196 nm. Specificity and stability-indicating capability were established in degradation studies, which also showed that there was no interference of the excipients. The method was linear over the concentration range of 1.0–300 μg mL^{-1} (r^2=0.9992) and the limit of detection (LOD) and limit of quantitation (LOQ) were 0.2 μg mL^{-1} and 1.0 μg mL^{-1}, respectively. The accuracy was 100.4% with bias lower than 1.1%. Moreover, the *in vitro* cytotoxicity test of the degraded products showed significant differences ($p < 0.05$). The method was applied for the content/potency assessment of rhIL-11 in biopharmaceutical formulations, and the results were correlated to those of a validated reversed-phase LC method (RP-LC) and an TF-1 cell culture assay, showing non-significant differences ($p > 0.05$). In addition the CZE and RP-LC methods were applied for the analysis of rhIL-11 in human plasma. Therefore, the proposed alternative method can be applied to monitor stability, to assure the batch-to-batch consistency and quality of the bulk and finished biotechnology-derived medicine.

1. Introduction

Thrombocytopenia occurs in patients with malignancies undergoing myelosuppressive chemotherapy, which may lead to hemorrhagic tendency and treatment delay. Recombinant human interleukin-11 (rhIL-11) is a kind of cytokine produced by DNA technology in *Escherichia coli*, now marketed worldwide as Oprelvekin, for clinical use in prevention of severe chemotherapy-induced thrombocytopenia and to reduce the need for platelet transfusions in patients with nonmyeloid malignancies [1–3].

The rhIL-11 biomolecule consists of 177 amino acids polypeptide chain, non-glycosylated with a molecular mass of 19 kDa and isoelectric point of 11.7. Differs from the naturally occurring human interleukin-11 (IL-11) only by the absence of an amino-terminal proline, and the presence of two residues of Met^{58} and Met^{122} [4].

The bioassays are useful to assess the efficacy and quality of those proteins, which cannot be adequately characterized only by physicochemical methods. Early signals triggered by IL-11 were assessed in a multifactor-dependent human erythroleukemic cell line TF1, which showed that this protein stimulated cell proliferation [5] and was applied to assess the bioactivity of biopharmaceuticals formulations [6]. However, a major concern of using a bioassay is its precision, which is generally inferior to the precision of physicochemical techniques [7–9].

Physicochemical techniques are used to monitor content/potency, purity, chemical stability of biopharmaceutical proteins obtained through recombinant DNA technology. No single technique can satisfactorily provide sufficient information about the protein and therefore a combination of physicochemical, immunological, and biological methods is recommended, and has been applied in correlation studies [10–13]. The reversed-phase liquid chromatography (RP-LC) method offers a high level of accuracy and sensitivity for the analysis of closely related protein variants or degradation products which may have reduced activity and altered immunogenicity [14,15]. A linear gradient RP-LC method using C_4

* Corresponding author. Tel./fax: +55 55 3220 8952.
E-mail address: sdalmora@terra.com.br (S.L. Dalmora).

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column and UV detection at 214 nm was employed to determine the oxidative effect of plastic tubes used for storage of protein samples [16]. Optimal storage stability of lyophilized rhIL-11 was evaluated by quantifying the oxidation levels and cleavage products by the gradient RP-LC [17]. A stability-indicating RP-LC method was validated using a C_4 column with PDA detection at 214 nm, and was applied to the assessment of rhIL-11 in biopharmaceutical formulations [6]. Capillary electrophoresis (CE) has expanded its scope as a powerful analytical technique for pharmaceutical analysis, allowing the determination of biotechnology-derived medicines and their degraded forms, charged variants and isoforms [9,10,18–20]. At the moment, rhIL-11 is not included in any Pharmacopoeia and no CE method has been published for quality control analysis. However, validation of the method is essential to show that the procedure is suitable for its intended purpose [21].

The aim of this research was to develop and validate a specific, sensitive and stability-indicating capillary zone electrophoresis (CZE) method for the analysis of rhIL-11; to correlate the results with a validated RP-LC method and with an *in vitro* bioassay; and to evaluate the bioactivity and the cytotoxicity of the degraded forms, thus contributing to the development of an alternative method to monitor stability, improve quality control, and thereby assuring the therapeutic efficacy of the biotechnology-derived medicine.

2. Experimental

2.1. Chemicals and reagents

Reference reagent Interleukin-11, human rDNA derived, (*R-rhIL*-11 WHO 92/788), for bioassays was obtained from the National Institute for Biological Standards and Control-NIBSC (Herts, UK). Biological reference substance of rhIL-11, (*BRS-rhIL*-11), for physicochemical assays was supplied by Amoytop Biotech Co., Ltd. (Xiamen, Fujian, China). Rupatadine fumarate (IS) was purchased from by Sequoia Research Products (Oxford, UK). A total of ten batches of Plaquemax® Bergamo (São Paulo, Brazil), containing 5 mg/vial of rhIL-11 were identified by numbers from 1 to 10 and two batches of Neumega® Wyeth (São Paulo, Brazil), containing 5 mg/vial of rhIL-11 were identified by numbers from 11 to 12. Samples were obtained from commercial sources within their shelf life period. Acetonitrile, disodium hydrogen phosphate, glycine, methanol, sodium dihydrogen phosphate, sodium dodecyl sulfate and trifluoroacetic acid used as reagents or excipients were purchased from Merck (Darmstadt, Germany). Fetal bovine serum, RPMI-1640 medium and thiazolyl blue formazan (MTT) were acquired from Sigma-Aldrich (St. Louis, MO, USA). All chemicals used were of pharmaceutical or special analytical grade. For all of the analyses, ultrapure water was obtained using an Elix 3 coupled to a Milli-Q Gradient A10 system Millipore (Bedford, MA, USA).

2.2. Apparatus

CE experiments were performed on an Agilent ^{3D}CE apparatus Agilent Technologies (Waldbronn, Germany) consisting of a photodiode array (PDA) detector, a temperature-controlling system (4–60 °C) and a power supply able to deliver up to 30 kV. The CE ChemStation software was used for instrument control, data acquisition and analysis. The pH of the solutions was measured using a pH-meter, Thermo Orion Model 420 (Beverly, MA, USA).

The RP-LC method was carried out on a Shimadzu LC system (Kyoto, Japan) equipped with a SCL-10A_{VP} system controller, a LC-10 AD_{VP} pump, a DGU-14A degasser, a SIL-10AD_{VP} autosampler, and a SPD-M10A_{VP} PDA detector. Peak areas were integrated automatically by computer using a Shimadzu Class VP® V 6.14 software program.

2.3. Capillary zone electrophoresis method

2.3.1. Solutions preparation

Stock solutions were prepared by diluting the *BRS-rhIL*-11 reference solution and the sample of biopharmaceutical formulation in water, to a final concentration of 400 μg mL^{-1}. IS was diluted to a final concentration of 200 μg mL^{-1} in methanol. The stock solutions were stored at 2–8 °C protected from light and daily diluted with BGE to working concentrations of 40 μg mL^{-1} and 20 μg mL^{-1} respectively, for the rhIL-11 and IS, and filtered through a 0.22 μm membrane Millex Millipore (Bedford, MA, USA).

2.3.2. Electrophoretic procedure

All experiments were carried out on a fused-silica capillary with 50 μm i.d. and 48.5 cm of total length (effective length 40 cm), thermostatized at 25 °C, and using a PDA detector set at 196 nm. At the beginning of each working day, the capillary was conditioned by rinsing with 1 mol L^{-1} sodium hydroxide for 5 min, followed by water for 2 min and 1 mol L^{-1} phosphoric acid for 5 min, and then by water for 2 min and with a running BGE solution for 5 min. Samples were injected using the pressure mode at 50 mbar for 45 s with a constant voltage of 20 kV (current about 55.2 μA) applied during the analysis. Since electrolysis can change the electroosmotic flow (EOF) and affect the migration time, efficiency and selectivity, the running electrolyte was replaced by a fresh solution after each three injections. The Background electrolyte solution (BGE) consisted of 50 mmol L^{-1} sodium dihydrogen phosphate at pH 3.0, adjusted by adding 8.5% phosphoric acid.

2.3.3. Validation of the capillary zone electrophoresis method

The method was validated using samples of a biopharmaceutical formulation of rhIL-11 with a label claim of 5 mg/vial, by determinations of the following parameters: specificity, linearity, range, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ), robustness, stability, and system suitability test, following the ICH guidelines [21,22]. Rupatadine fumarate (IS) was selected as internal standard to compensate for any injection errors and minor fluctuations of migration time, thus improving the reproducibility and performance of the CZE method.

2.3.4. Forced degradation studies

The stability-indicating capability of the CZE method was determined by subjecting a *BRS-rhIL*-11 reference solution (400 μg mL^{-1}) and a biopharmaceutical formulation (400 μg mL^{-1}) to accelerated degradation by different acidic, basic, oxidative, photolytic and temperature conditions [23,24]. Working solutions prepared in 1 mmol L^{-1} hydrochloric acid were used for acidic hydrolysis and working solutions in 1 mmol L^{-1} sodium hydroxide for the basis hydrolysis evaluation. Both solutions were maintained at room temperature for 10 min and 1 h, respectively, and neutralized with base or acid, as necessary. Oxidative degradation was induced by maintaining the solutions in 3% hydrogen peroxide, at ambient temperature for 3 min, protected from light. Photodegradation was induced by exposing the sample in a photostability chamber to 200 W h m^{-2} of near ultraviolet light from 1 to 24 h. For a study under neutral condition, sample solutions were diluted in water and heated at 80 °C for 3 h. Then, the solutions were diluted with the BGE solution to final concentrations of 40 μg mL^{-1}. The interference of the excipients of the biopharmaceutical formulation was determined by the injection of a sample containing only a placebo (in-house mixture of all the formulation excipients), and by the standard addition method, where a calibration curve

was constructed by the addition of known amounts of the reference substance to the placebo [21]. Then, the specificity of the method was established by determining the peak purity of rhIL-11 in the samples using a PDA detector.

2.4. Reversed-phase LC method

The validated gradient RP-LC method was performed as described elsewhere [6]. Briefly, the elution was carried out 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 25 °C. A security guard holder was used to protect the analytical column. The elution was performed using a linear gradient at a constant flow rate of 1 mL min^{-1} and using photodiode array (PDA) detection at 214 nm. Mobile phase A consisted of water with 0.1% trifluoroacetic acid (TFA) and mobile phase B was acetonitrile with 0.1% TFA, run as follows: time 0–0.1 min 40% of B; from 0.1 to 30 min linear up to 65% of B; from 30.01 to 31 min linear down to 40% of B, maintained up to 40 min. The injection volume was 50 μL of a solution containing 50 μg mL^{-1} for both standard *BRS-rhIL*-11 and samples.

2.5. In vitro TF-1 cell proliferation bioassay

The assay was performed as described elsewhere [6], and the growth-promoting activity of rhIL-11 assessed on TF-1 cell line (ATCC number CRL-2003), and the absorbance was assessed at 595 nm, using microplate reader Thermo Scientific Multiskan FC (Vantaa, Finland). The biological potencies were calculated against the *R-rhIL*-11 (WHO 92/788) with the biological potency of 1000 IU μg^{-1} by the parallel line statistical method using the CombiStats® software (European Directorate for the Quality of Medicines & HealthCare, EDQM Council of Europe).

2.6. In vitro cytotoxicity test

The *in vitro* cytotoxicity assay was performed as described elsewhere [13] based on a neutral red uptake (NRU) assay, with the exposure of NCTC clone 929 cell line (mammalian fibroblasts, ATCC number CCL-1) to the degraded samples of rhIL-11, and the absorbance was measured at 540 nm.

2.7. Analysis of rhIL-11 in biopharmaceutical formulations

For the quantitation of rhIL-11 in biopharmaceutical formulations, the stock solutions were diluted to appropriate concentrations of 40 μg mL^{-1} and 50 μg mL^{-1}, respectively, with a BGE solution, or a mobile phase, for the electrophoretic and chromatographic methods, injected in triplicate and the percentage recoveries calculated against the *BRS-rhIL*-11, that was calibrated against the *R-rhIL*-11 (WHO 92/788).

2.8. Analysis of rhIL-11 in human plasma

Plasma samples were spiked with 40 μg mL^{-1} of rhIL-11 and 20 μg mL^{-1} of IS and separated using a Waters Oasis® HLB extraction cartridges (Milford, MA, USA). The biomolecule was eluted with the mixture of water and acetonitrile (20:80,v/v), and 0.1% TFA. After evaporation under a nitrogen stream to obtain 1 ml, precipitation with 5 mL of methanol (−20 °C) for at least 1.5 h eliminated the remaining impurities, and the supernatant was used for the analysis by the CZE and RP-LC methods.

3. Results and discussion

3.1. Optimization of the electrophoretic conditions

To develop the CZE method, it was considered the isoeletric point of 11.7 as at this pH the rhIL-11, although charged, behaves as if it is neutral and has no tendency to migrate in the electrical field [25,26]. Then some electrolyte solutions were tested with variations in the composition, ionic strength and pH as shown in (Table 1), selecting sodium dihydrogen phosphate. The optimum pH of a BGE solution containing 50 mmol L^{-1} sodium dihydrogen phosphate was investigated in the range of 2–4, which enabled the separation (Fig. 1). Higher pHs resulted in peak tailing and increased migration time, therefore pH 3.0 was selected since it showed better peak symmetry (about 1.08). The sodium dihydrogen phosphate was evaluated at concentrations of 10–90 mmol L^{-1} at pH 3.0 (Fig. 2), which demonstrated a significant effect on the separation performance through its influence on the EOF and the current produced in the capillary. A 50 mmol L^{-1} solution was selected due to its low effect on current and non-significant increase on the migration time. The temperature effect on the separation was investigated in the range of 20–35 °C, and a temperature of 25 °C was chosen due to short run time and acceptable current. The effect of the voltage was studied through changes from 10 to 30 kV, showing that a potential of 20 kV yielded a short analysis time with an acceptable current (about 55.2 μA). Sample solutions were injected using a pressure mode at 50 mbar for 45 s, equivalent to a injection volume of 86 nL. The electrophoretic buffers usually have a minimal background in the whole UV region. This allows the use of capillary electrophoresis techniques with UV detector operating in 190–215 nm, where many organic analytes have a significant higher adsorption. On the contrary, application of chromatographic methods which use organic solvents in the liquid phases is limited, as within a short UV wavelength range most of the commonly used organic solvents absorb UV light strongly [27]. Then, wavelength detection was

Table 1
BGE conditions tested and current observed for the CZE method.

BGE	Ionic strength (mmol L^{-1})	pH	Current (μA)
Boric acid	20–30	4.0–7.0	20–35
Bis–tris propane	10–70	3.0–7.0	15–60
Di-sodium tetraborate	25–40	3.5–7.0	20–40
Potassium phosphate	10–60	3.0–6.0	15–100
MES [2-(N-morpholino) ethanesulfonic acid]	15–50	4.0–7.0	20–60
Sodium dihydrogen phosphate	10–90	2.0–4.0	15–110
Tris(hydroxymethyl)aminomethane	10–30	2.0–5.0	20–40

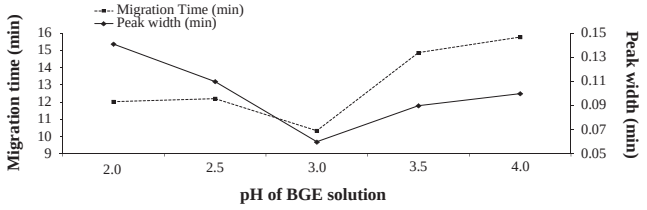

Fig. 1. Effect of pH on migration time and peak width of rhIL-11 40 μg mL^{-1}. Running buffer 50 mmol L^{-1} sodium dihydrogen phosphate. Conditions: capillary, 50 μm i.d. × 40 cm; applied voltage, 20 kV; UV detection, 196 nm; column temperature, 25 °C. Pressure injection at 50 mbar for 45 s.

evaluated in the range of 190–400 nm, and a wavelength of 196 nm was chosen due to better sensitivity and signal-to-noise ratio.

3.2. Validation of the method

The CZE method was validated for the analysis of rhIL-11 in biopharmaceutical formulations with a migration time of about 10.31 min, as shown in typical electropherograms (Fig. 3a, b). The stability-indicating capability of the method was tested under basic condition which showed decrease of the area, and only one additional peak was detected at 9.73 (Fig. 3c). The acidic condition resulted in a decrease of the area with two additional peaks at 9.62 and 14.10 min. (Fig. 3d). The forced photolytic condition showed decrease of the area with one additional peak at 12.31 (Fig. 3e). The forced oxidative degradation studies exhibited one peak related to the hydrogen peroxide at 7.43 min and one additional peak at 9.91 (Fig. 3f). Under the neutral hydrolysis condition described, decrease of the area was observed, without any additional peak. The specificity of the method was established by determining the peak purity of the analyte and the IS in the working BRS-rhIL-11 reference solution, by overlaying the spectra captured at the apex, upslope and downslope using a PDA detector. Additionally, the standard addition method was applied to evaluate the interference from formulation excipients. Non-significant difference ($p > 0.05$) was found between the slopes calculated for the calibration curve and the standard addition method. The data, together with the peak purity index in the range of 0.9999–1, showed that the peak was free from any co-migrating peak, with no interference of excipients, thus confirming that the proposed method is specific for the analysis of rhIL-11. Additionally the *BRS-rhIL*-11 and the degraded samples were subjected to the *in vitro* cytotoxicity test.

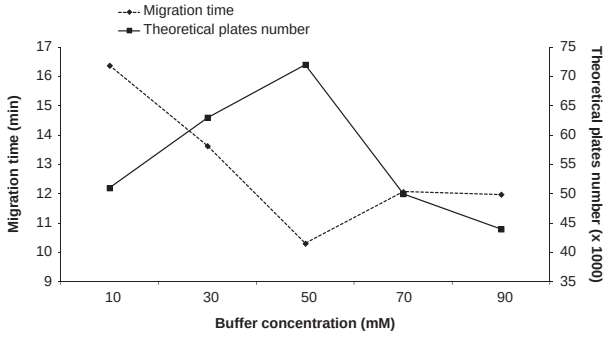

Fig. 2. Effect of buffer concentration on migration time and theoretical plate number of rhIL-11. Running buffer sodium dihydrogen phosphate pH 3.0, and conditions as described in the caption of Fig. 1.

The linearity determined by constructing three calibration curves, each one with ten concentrations of *BRS-rhIL*-11 solution in the 1.0–300 μg mL^{-1} range, spiked with IS at 20 μg mL^{-1}. The value of the determination coefficient calculated by a least-squares regression analysis ($r^2 = 0.9992$, $n = 10$, $y = (0.0349 \pm 0.0012)x + (0.0473 \pm 0.0097)$, where, x is concentration in μg mL^{-1} and, y is the peak-area ratio of *BRS-rhIL*-11 to IS, indicated linearity of the calibration curve for the method.

The precision of the method was evaluated by calculating the relative standard deviation (RSD%) of the migration time and the peak-area ratio, for eight determinations at a concentration of 40 μg mL^{-1}, performed on the same day and under the same experimental conditions. The obtained RSD values were 0.8 and 1.1% for the migration time and the peak-area ratio, respectively. The intermediate precision was assessed by analyzing two samples of the biopharmaceutical formulation on three different days (inter-days) giving RSD values of 1.4 and 0.8%, respectively. The between-analysts precision was determined by calculating the RSD for the analysis of two samples by three analysts; the values were calculated as 1.1 and 0.6%, respectively.

The accuracy was assessed from three replicate determinations of three solutions of in-house mixtures of the excipients with known amounts of the biomolecule, containing 30, 40, and 50 μg mL^{-1}. The absolute means obtained with a mean value of 100.4% and a bias lower than 1.1% as given in Table 2, shows that the method is accurate within the desired range [28].

The LOD and the LOQ were calculated from the slope and the standard deviation of the intercept determined by a linear-regression model, by using the mean values of the three independent calibration curves. The obtained values were 0.2 and 1.1 μg mL^{-1}, respectively. The evaluated experimental LOQ with a precision lower than 5% and an accuracy within ± 5%, [29,30] was determined as 1.0 μg mL^{-1}, which is suitable as an alternative comparable to the RP-LC, for quality-control analysis [6].

The robustness of the analytical procedure [31] was determined by analyzing samples of the *BRS-rhIL*-11 reference solution

Table 2
Accuracy of CZE for rhIL-11 in the formulations.

Nominal concentration (μg mL^{-1})	Mean concentration found[a] (μg mL^{-1})	RSD[b] (%)	Accuracy (%)	Bias[c] (%)
30	30.2	1.1	100.6	−0.7
40	40.1	0.3	100.1	−0.2
50	50.2	0.9	100.4	−1.1

[a] Mean of three replicates.
[b] RSD = relative standard deviation.
[c] Bias = [(measured concentration - nominal concentration)/nominal concentration] × 100.

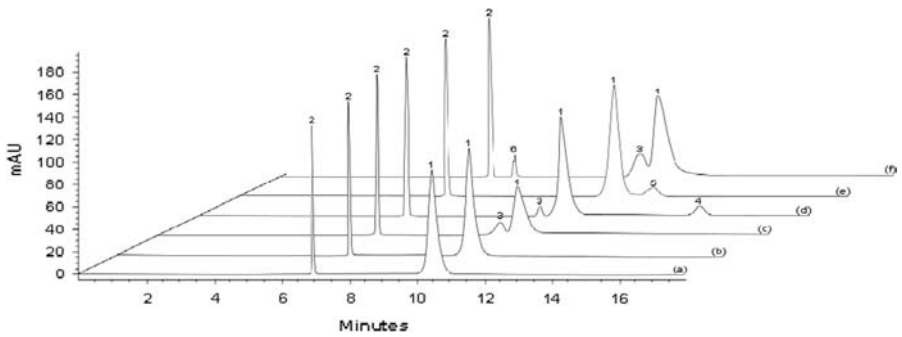

Fig. 3. Representative CZE electropherograms showing peak 1 = rhIL-11; peak 2 = internal standard (IS); peak 3, 4, 5 = degraded forms; peak 6 = hydrogen peroxide; peak 7 = glycine. (a) *BRS-rhIL*-11; (b) Sample of biopharmaceutical formulation. *BRS-rhIL*-11 following degradation under conditions: (c) basic hydrolysis, (d) acid hydrolysis, (e) photolytic, and (f) oxidative.

containing 40 μg mL^{-1} in triplicate by the one-variable-at-a-time (OVAT) approach. The results and the experimental range of the selected variables evaluated are given in Table 3, together with the optimized values. Additionally, the robustness was also evaluated and compared by the multi-variable-at-a-time (MVAT) approach [32] at three levels (one unit per parameter up or down around optimized values). This procedure gives results for minimum changing of the maximum number of parameters at a time, and is a very useful, rapid and efficient approach for a robustness determination. The results for the OVAT and MVAT procedures were within the acceptable deviation (RSD < 2%), and an analysis of the variance showed non-significant differences ($p > 0.05$). The analysis performed with a wider level of variations of the solution pH, temperature and voltage, showed changes of the migration time related to the optimized conditions. Moreover, the peak symmetry values were also evaluated, showing non-significant differences ($p > 0.05$). The electropherogram pattern was not altered and different capillary batches also indicated robustness under the conditions tested.

The stability of rhIL-11 in BGE was assessed after storage of the samples for 48 h at 2–8 °C, and also placed in an auto-sampler for 24 h at room temperature, showing non-significant changes (< 2%) relative to freshly prepared samples, as suggested [33].

A 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 *BRS-rhIL-11* reference solution containing 40 μg mL^{-1} of rhIL-11. The obtained RSD values for the migration time, peak area, peak symmetry and peak width were 0.1%, 1.3%, 1.2%, and 1.3%, respectively, as calculated by the standard deviation of the Gaussian function. The number of theoretical plates was approximately 56,238, with RSD of 0.9%. The parameters tested were within the acceptable range (RSD < 2%).

3.3. Cytotoxicity evaluation

The cytotoxicity test was performed on degraded forms related to the intact molecule, in order to detect possible effects resulting from the instability of the samples during storage, giving mean IC_{50} = 18.7 μg mL^{-1}, IC_{50} = 22.2 μg mL^{-1}, respectively, for acidic and photolytic conditions with significant differences as calculated by the Student's *t* test ($p < 0.05$) compared to the intact molecule that showed IC_{50} = 67.1 μg mL^{-1}. Such evaluations are now necessary, mainly due to the recent concerns related to possible human undesirable effects of the degraded forms [15,34].

3.4. Method application

The CZE method was applied to the determination of rhIL-11 in biopharmaceutical formulations and the results compared to those obtained using a validated RP-LC method and an *in vitro* bioassay, giving mean differences of the estimated content/potencies of 0.4% and 1.4% higher, respectively, as shown in Table 4. The experimental values were compared statistically by analysis of the variance (ANOVA), which showed non-significant differences ($p > 0.05$). Both of the methods showed similar results for degraded forms and related proteins, and the CZE method demonstrated better sensitivity to detect the degraded forms from forced degradation studies. The oxidation levels and cleavage products were formerly determined only by RP-LC [16,17]. CE methods were previously used to evaluate different therapeutic peptides and

Table 3
CZE conditions and range investigated during robustness testing with one-variable-at-a-time (OVAT) procedure.

Variable	Values	rhIL-11[a] (mg)	RSD[b] (%)	Migration time (min)	RSD[b] (%)	Symmetry	RSD[b](%)	Optimized condition
BGE solution pH	2.6	4.96	1.0	10.23	1.1	1.46	1.0	3.0
	2.8	4.96	0.6	10.40	1.0	1.57	1.1	
	3.0	4.99	0.3	10.27	0.4	1.08	0.5	
	3.2	4.94	0.9	10.52	0.9	1.16	0.9	
	3.4	4.94	0.7	10.34	1.0	1.32	1.2	
BGE (mM)	46	5.05	1.3	11.49	1.1	1.74	1.0	50
	48	5.07	0.8	11.08	0.6	1.62	0.4	
	50	5.03	0.2	10.32	0.2	1.31	0.3	
	52	5.04	0.7	10.26	0.8	1.48	0.6	
	54	4.96	0.6	10.21	1.2	1.52	0.8	
Temperature (°C)	21	4.84	0.3	10.44	0.7	1.42	0.7	25
	23	4.95	0.3	10.41	0.7	1.26	1.1	
	25	4.95	0.2	10.34	0.2	1.07	0.4	
	27	5.06	0.4	10.53	0.5	1.14	1.2	
	29	5.08	0.5	10.31	0.8	1.33	1.3	
Voltage (kV)	16	5.11	1.2	11.36	1.0	1.66	0.8	20
	18	5.12	0.8	11.01	0.8	1.89	0.4	
	20	5.03	0.3	10.36	0.5	1.24	0.2	
	22	4.96	0.7	10.31	1.0	1.57	0.4	
	24	4.90	0.9	10.28	1.2	1.92	0.6	
Time injection (s)	41	4.81	1.7	10.53	0.5	1.23	1.6	45
	43	4.89	1.3	10.42	0.3	1.49	1.3	
	45	4.95	0.7	10.30	0.1	1.16	0.7	
	47	4.93	0.8	10.37	0.3	1.37	1.0	
	49	4.93	1.3	10.41	0.4	1.88	1.1	
Wavelength (nm)	192	5.03	1.0	10.48	0.4	1.63	0.9	196
	194	5.07	1.1	10.29	0.5	1.54	1.0	
	196	5.00	0.6	10.27	0.3	1.14	0.7	
	198	5.01	0.8	10.36	0.8	1.49	1.0	
	200	5.08	0.7	10.42	0.8	1.32	0.9	

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

Table 4
Comparative content/potency evaluation of rhIL-11 in biopharmaceutical formulations by CZE and RP-LC methods, and bioassay.

Sample	Theoretical amount (mg)	CZE[a]		RP-LC[a]		*In vitro* bioassay[a]	
		Found (mg)	Degraded Forms (mg)	Main peak (mg)	Deamidated/Sulfoxides (mg)	Potency (mg)	Confidence intervals ($p=0.95$)
1	5	5.01	0.02	5.00	0.01	4.88	4.69–5.42
2	5	4.96	0.07	4.93	0.05	4.84	4.32–4.87
3	5	4.84	0.10	4.77	0.07	4.72	4.17–4.93
4	5	4.59	0.31	4.65	0.24	4.65	4.43–4.78
5	5	5.28	0.12	5.19	0.08	5.13	4.85–5.21
6	5	5.17	0.03	5.12	0.04	5.25	4.72–5.60
7	5	5.36	0.02	5.21	0.02	5.11	4.73–5.41
8	5	5.22	0.00	5.07	0.00	5.06	4.67–5.32
9	5	4.81	0.00	4.98	0.00	4.77	4.35–5.56
10	5	4.97	0.01	4.92	0.02	4.90	4.37–5.48
11	5	4.66	0.05	4.61	0.09	4.56	4.09–4.84
12	5	5.00	0.06	5.22	0.04	5.17	4.53–5.47
Mean	–	4.99	0.07	4.97	0.05	4.92	–
SD [b]	–	0.24	0.09	0.21	0.07	0.22	–
					ANOVA Between-methods		*F* calculated [c] 1.31

[a] Mean of three replicates.
[b] SD=Standard deviation of the distribution.
[c] *F* critic for $p=0.05$.

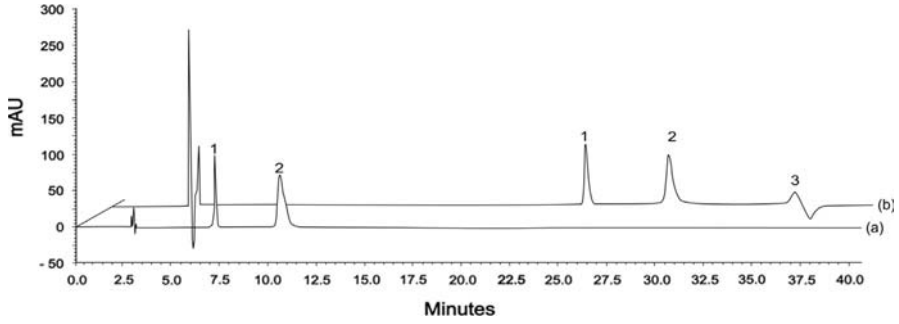

Fig. 4. Representative blank plasma spiked with rhIL-11 and internal standard, showing peak 1=internal standard; peak 2=rhIL-11; peak 3=excipient. *BRS-rhIL-11*: (a) CZE electropherogram, and (b) RP-LC chromatogram.

proteins, but the potential demonstrated by the validated method can be useful for the determination of rhIL-11, and to support biosimilarity studies [35], without prior separation of the excipients of the formulation, with the added advantages of small sample volumes without consumption of organic solvents, and a short analysis time.

The validated CZE and RP-LC methods were also applied for the analysis of rhIL-11 in human plasma after SPE extraction procedure, as shown in Fig. 4, giving mean recoveries of 97.0% and 98.3% by the CZE, and of 96.1% and 97.6%, by the RP-LC, respectively, for rhIL-11 and IS. The LLOQs evaluated in experimental assays, were found to be 10 $\mu g\ mL^{-1}$ and 5 $\mu g\ mL^{-1}$, respectively, for the CZE and RP-LC methods, with the precision of 8.5% and 9.0%, and accuracy of 107.5% and 102.3%, showing lower sensitivity for the CZE method, probably due to the low sample injection volume and the short optical path-length.

4. Conclusions

The results of the validation studies show that the CZE method is sensitive with a LOQ of 1.0 $\mu g\ mL^{-1}$ accurate with a mean value of 100.4%, possesses significant linearity ($r^2=0.9992$) and precision characteristics without any interference from the excipients. The proposed method was applied to the assessment of rhIL-11, showing a higher mean difference of the estimated content/potencies of 0.4% and 1.4% compared to the RP-LC and to the *in vitro* bioassay, respectively, but with significant correlation, as calculated by ANOVA ($p>0.05$). Therefore, represents an alternative to current methods which can be applied for quantitative analysis during the biotechnology process and through subsequent purification steps, to monitor its stability and to assure the quality of the bulk and finished biotechnology-derived medicine.

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