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Development and validation of a bioanalytical method based on LC–MS/MS analysis for the quantitation of CIGB-814 peptide in plasma from Rheumatoid Arthritis patients

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ABSTRACT

CIGB-814, originally named as E18-3 APL1 or APL1 in preclinical experiments, is a novel therapeutic peptide candidate for Rheumatoid Arthritis (RA). It is an altered peptide ligand containing a novel CD4+ T-cell epitope of human heat shock protein 60 (83–109, MW 2988.38 g/mol) with a mutation ($D^{100} \rightarrow L$) that increases its affinity for HLA-II type molecules associated to RA. A bioanalytical method, based on LC–MS/MS analysis, in the SRM mode was developed and fully validated to quantify this peptide in human plasma. An internal standard with the same amino acid sequence but labeled with three ($^{13}C_6{}^{15}N_2$)-Lys residues was used for quantitation. The method provides a linear range from 1.5 to 48 ng/mL (without matrix effect and carry over) and an accuracy and precision good enough for monitoring more than 80% of the AUC of the PK profile in a phase I clinical trial. The peptide was administered subcutaneously in three dose levels (1, 2.5 and 5 mg) not normalized to the body weight of patients with RA. The low doses imposed an analytical challenge; however, a LLOQ of 1.5 ng/mL enabled the PK analysis. The Cmax, reached at 0.5 h, showed a great variability, that was most likely due to the non-normalized doses; the proposed mechanism for this peptide; and the variability between patients. A rapid clearance of this peptide (4–6 h) is advantageous for an immunomodulatory drug, because the therapeutic schedule requires repeated dosages to restore peripheral tolerance.

1. Introduction

Rheumatoid Arthritis (RA) is a common, chronic, inflammatory, autoimmune disease of unknown etiology affecting approximately 1% of the world population [1]. In RA, both genetic and environmental factors produce a cascade of immune reactions leading to synovitis, joint and structural bone damages, pain and disability [1,2]. According to Smolen and Aletaha [3], RA has no cure despite the many new treatments developed over the last 30 years. Most of them decrease inflammation and pain; prevent joint damage and slow down the progression of the disease. In current medical practice, the drugs used for RA include non-steroidal anti-inflammatory drugs (NSAIDs), the disease modifying anti-

Abbreviations: RA, Rheumatoid Arthritis; NSAIDs, non-steroidal anti-inflammatory drugs; DMARDs, disease modifying anti-rheumatic drugs; Treg, T regulatory cells; LC–MS, liquid chromatography coupled to mass spectrometry; SRM, single reaction monitoring; IS, internal standard; QCs, quality control samples; CSs, calibration standard samples; LLOQ, lower limit of quantification; Cmax, maximum concentration; AUC, area under the curve; APL, altered peptide ligand.

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rheumatic drugs (DMARDs) and more recently, the biologics and their combinations [4,5].

These drugs do not induce sustained remission and may cause immunosuppression leading to severe complications. Some of the strategies proposed for a long-term remission are based on the induction of immunological tolerance [6,7]. Also antigen-specific therapies enable the elimination of pathogenic cells leaving the immune system's ability to respond to infections unaltered. Within this approach, known autoantigens such as heat shock proteins, and their derived peptides, seems to be very promising as therapeutic candidates.

The CIGB-814 is a novel 27–mer altered peptide ligand (APL) derived from a CD4+ T-cell epitope of the human heat shock protein 60 (amino acids: ^{83}SIDLKDKYKNIGAKLVQLVANNTNEEA^{109}) [8]. This APL has a mutation ($D^{100} \rightarrow L$) that increases its affinity for several HLA-II type molecules associated to RA. In all preclinical experiments as well as in our previous publications [8–11], the peptide studied here (CIGB-814) was named as E18-3 APL1, or APL-1. Immunotherapy with CIGB-814 significantly inhibits the course of adjuvant induced arthritis (AA) in Lewis rats and collagen induced arthritis (CIA) in DBA/1 mice in a similar way as methotrexate does [9]. Additionally, there was statistically significant reduction in TNF-α levels after treatment with CIGB-814. CIGB-814 significantly reduced auto-reactive T cell viability evaluated in *ex-vivo* assays with PBMC isolated from patients with active RA [10]. All these experimental results reinforced the therapeutic potential of CIGB-814 for RA [8].

On the other hand, preclinical research showed the safety of this peptide in acute and accumulative toxicity studies in rats. Consequently, the National Regulatory Agency for the Control of Drugs in Cuba (http://www.cecmed.cu/) authorized a phase I clinical trial (Code: RPCEC00000238) where CIGB-814 was used in RA patients. For safety studies and also for the pharmacokinetic profile of this novel candidate, three dose levels (1, 2.5 and 5 mg), that were not normalized to the patient's weight, were administered subcutaneously.

The administration of low doses of CIGB-814 is mandatory for ethical reasons because there are previous reports that indicate severe immune system deregulation [12,13] when immunomodulatory drugs (including APLs) have been administered at high doses. Additionally, this drug modulates the population of T-cells with regulatory phenotypes, which is practically constant among individuals, therefore a normalization by the body weight as a reason to increase the administered doses is not justified.

There are several factors that also increase the challenges for the quantitative determination of the intact CIGB-814 in the context of plasma: (1) peptides are efficiently eliminated through kidneys; (2) CIGB-814 should be presented in the context of HLA molecules, internalized within the cell and proteolytically processed by the proteasome and (3) the subcutaneous route of administration also has an impact in decreasing the Cmax. The efficient removal of highly abundant proteins is a mandatory issue for the quantitative purposes since the analyte concentration asymptotically decreases over the time course in the context of plasma: the biological matrix with the widest dynamic concentration range (g/L – 10^{-12} g/L).

The triple quadrupole tandem mass spectrometer operated in selective reaction monitoring mode is probably one of the most commonly used mass analyzer for quantitative measurements of large [14–17]; as well as short polypeptides [18,19]. Their usage overcomes the bias toward the identification the most abundant proteins due its high selectivity targeting only the analyte(s) of interest after defining its *m*/*z* and retention time. If the analyzed sample is spiked with an internal standard of a known concentration, the absolute quantification can be achieved [20,21]. This method is highly sensitive [22] since no scanning is required thus facilitating very low limit of quantitation which is desired for a PK modeling. The calibration curves constructed using this procedure has an excellent linearity over a wide dynamic concentration range (3–5 orders) [22].

Regulatory agencies demand a full validation when developing and implementing a bioanalytical method for the first time for a new drug entity [23,24]. For these reasons, we developed and fully validated a method to quantify the CIGB-814 in the plasma of RA patients enrolled in a phase I clinical trial. The plasmatic proteins were precipitated with acetonitrile prior to LC–MS/MS analysis and mass spectrometric analysis was performed in a Quattro Ultima Triple-Q mass spectrometer (Waters, USA) operated in Single Reaction Monitoring mode ($[M+4H]^{4+} \rightarrow b_{25}{}^{4+}$). The internal standard (IS) used was a peptide with the same amino acid sequence as CIGB-814 but with three $^{13}C_6{}^{15}N_2$- Lysine residues, located at positions 5, 7 and 14.

2. Material and methods

2.1. Reagents and chemicals

All chemicals were HPLC grade. Acetonitrile was purchased from Caledon (Canada), formic acid (FA) was from Sigma (USA) and 1,5 octyl-β-D-glucopyranoside was from Bachem (Switzerland). Water was ultrapure quality obtained from the production plant facility at the Center for Genetic Engineering and Biotechnology (Havana, Cuba). All reagents for peptide synthesis were from Iris Biotech (Germany). Blood was collected in 4 mL EDTA lavender vacutainer tubes from Greiner Bio-One (Germany). Plasma samples for method validation were obtained from healthy volunteers; and the pharmacokinetic study used samples taken from patients with moderate disease according to the DAS-28 index [25]. The patients enrolled in the phase I clinical trial signed their informed consent.

2.2. Modelling the isotopic ion distribution of CIGB-814 and the internal standard

The isotopic distributions of the $[M+4H]^{4+}$ ions corresponding to an equimolar mixture CIGB-814 and three different variants of IS were modeled by using the MassLynx software v4.1 (Waters, Manchester, UK) for determining their overlapping in the ESI–MS analysis. The three different variants of the IS were constructed by replacing one, two or three Lysine with $^{13}C_6{}^{15}N_2$-Lys residues. The m/Δm was fixed at 1 000 considering the resolution for precursor ion selection achieved in the triple quadrupole tandem mass spectrometer used in this PK study.

2.3. Synthesis of CIGB-814 and the ($^{13}C_6{}^{15}N_2$-Lys)$_3$ labeled IS

Both, CIGB-814 and its IS, were synthesized at the Peptide Synthesis Laboratory in the Center for Genetic Engineering and Biotechnology (Havana, Cuba) using Fmoc chemistry. CIGB-814 amino acid sequence corresponds to: H-Ser-Ile-Asp-Leu-Lys-Asp-Lys-Tyr-Lys-Asn-Ile-Gly-Ala-Lys-Leu-Val-Gln-Leu-Val-Ala-Asn-Asn-Thr-Asn-Glu-Glu-Ala-conh$_2$, ($MW_{average}$ = 2988.38 g/mol). Amino acid sequence of the IS was identical to CIGB-814, but lysine residues at positions 5, 7 and 14 were replaced by three residues of $^{13}C_6{}^{15}N_2$-Lys ($MW_{average}$ = 3012.47 g/mol) to avoid the overlapping between the isotopic ion distributions of precursor ions. Both peptides were purified to homogeneity (> 99% purity) by reversed-phase chromatography (C18). UV detection was monitored at 226 nm during the chromatographic separation.

2.4. Calibration standards and quality control samples

Stock solutions of CIGB-814 and its IS, were accurately prepared by determining the peptide content at 1 mg/mL in water using the specific extinction coefficient at 280 nm (ξ[1%, 280 nm]), determined experimentally by amino acid analysis (0.4937 $mL^{\bullet}mg^{-1}cm^{-1}$). The ξ[1%, 280 nm] used for the IS was identical to that determined for CIGB-814 since their amino acid sequence was the same. All dilutions from stock solutions were made in a 5 g/L of 1,5 octyl-β-D-glucopyranoside solution to avoid undesirable losses of the peptide associated to adsorption onto the plastic ware or the plate well of the auto-sampler tray [26]. To prepare Calibration Standards (CSs) and Quality Control Samples (QCs), 90 μL of plasma samples were spiked with 10 μL of an equivalent dilution. CIGB-814 concentrations for Calibrator Standard samples (CSs) were: 1.5, 3, 6, 12, 18, 24, 30, 36, 42 and 48 ng/mL, while for QCs, CIGB-814 concentrations were: 6, 18 and 36 ng/mL, covering the linear range of the calibration curve. IS concentration was 12 ng/mL for all samples.

2.5. Sample processing

Prior to the LC–MS/MS, 90 μL of plasma from RA patients were spiked with 10 μL of the IS stock solution to a final concentration of 12 ng/mL. CSs and QCs were prepared as explained above. In general, sample processing included an organic solvent precipitation step for the efficient removal of the plasma proteins. Two volumes of acetonitrile (200 μL) in relation to sample volume (100 μL), were added and vortexed for 1 min. This mixture was centrifuged at 15 366 RCF for 5 min at room temperature. The pellet was discarded and supernatant was evaporated to dryness in a Speed Vac (SAVANT, USA) without heating. Samples were stored at – 20 ± 5 °C until analysis. Prior to SRM analysis each sample was reconstituted by vortexing in 110 μL of 5 g/L of 1,5 octyl-β-D-glucopyranoside solution composed by 3.75% isopropanol/11.25% acetonitrile/0.2% formic acid (v/v/v) to minimize losses in this step (see discussions in Section 3.1.3). The samples were then centrifuged at 15 366 RCF for 5 min at room temperature. Supernatant was applied directly onto the auto-sampler well plate.

2.6. LC–MS/MS analysis of CIGB-814

An Alliance HPLC system model 2790 (Waters, USA) was used to perform chromatographic separation in a C18 column (Ø = 1 mm, H = 10 mm) packed with the Reprosil C18-AQ (5 μm) matrix (Ammerbuch-Entringen, Germany). Mobile phase consisted of A: 99.9% H_2O/0.1% FA (v/v) and B: 25% Isopropanol/74.8% Acetonitrile/0.2% Formic acid (v/v/v). A flow rate of 250 μL/min and a linear gradient from 15% to 95% of solution B in 1 min were used for the elution of the desired peptide. The entire cycle comprised 15 min, including also 10 min for column equilibration at 15% of solution B, and 4 min for desalting the sample using these conditions. MS analysis used a Quattro Ultima Triple-Q mass spectrometer (Waters, UK) in the Single-Reaction Monitoring (SRM) mode and an electrospray interface (ESI). The temperatures used for the source block and the desolvation were set to 95 °C and 350 °C, respectively. Nitrogen was used as nebulizing gas (170 L/h) and drying gas (440 L/h). The cone and capillary voltages were set to 35 V and 3 kV, respectively. The acquisition window was set to 0.5 min before and after CIGB-814 retention time. In the first quadrupole the $[M+4H]^{4+}$ ions of CIGB-814 (m/z = 747.60) and its IS (m/z = 753.60) were independently selected for fragmentation inside the collision cell using a resolution (m/Δm) of ~1000. The monitored transition for CIGB-814 and its IS, were their corresponding $b_{25}{}^{4+}$ fragments ions detected at m/z = 693.35 and m/z = 699.35, respectively.

To determine optimum collision energy, the peptide was dissolved at a concentration of approximately 1 pmol/mL in 50% of solutions A and B and sprayed at a flow of 5 μL/min using syringe pump (Harvard Apparatus, USA) into the ESI ion source operated with the same parameters described above. Under these experimental conditions the intensity ratio for the multiply-charged ions resembled the observed for CIGB814 peptide analyzed by LC–MS in the Quattro Ultima Triple-Q mass spectrometer (Waters, USA). To determine optimum collision energy, the $[M+4H]^{4+}$ ion of CIGB-814 was selected in the first quadrupole, fragmented in the collision chamber, and sixteen MS/MS spectra were obtained using different collision energies from 15 to 30 eV with gradual increases of 1 eV each.

In method validation as well as in the PK study, the precursor ions were fragmented by collision induced dissociation (CID) with collision energy of 21 eV and using argon as the collision gas at a pressure of 80 psi. Combinations of three transitions ($[M+4H]^{4+} \rightarrow b_n{}^{3+}$) corresponding to daughter ions with m/z higher than precursor ion ($b_{21}{}^{3+}$(m/z 771.9), $b_{22}{}^{3+}$(m/z 810.0), $b_{23}{}^{3+}$ (m/z 843.7), $b_{24}{}^{3+}$(m/z 881.8), $b_{25}{}^{3+}$(m/z 924.9), and $b_{26}{}^{3+}$(m/z 967.9)) were also evaluated to determine the selectivity and sensitivity of the method.

Dwell time was set to 0.35 s for all transitions, 10 and 20 ms were used as interscan and interchannel delays. At least eight data points per curve were used to monitoring transitions, according to Gallien et al. [22]. All samples analyzed in the PK study by SRM mode were measured by triplicate. The Area Under the Curve (AUC) for CIGB-814 and the IS, were continuously monitored by using the transition described above. Raw data processing to quantify CIGB-814 was performed by triplicate using MassLynx® v4.1 software (Waters, UK).

The MS/MS spectra of $[M+3H]^{3+}$ ions corresponding to CIGB-814 (m/z = 997.1) and its IS (m/z = 1005.1) were also acquired using higher collision energies from 30 to 46 eV with gradual increment of 1 eV each to determine the optimal collision energy and the selection of the most favorable transition for quantification purposes.

2.7. Method validation

The Matrix Factor (MF) from human plasma was assessed for 6 healthy volunteers by duplicate, to estimate the possible interferences of plasma in the quantitation of CIGB-814. An equimolar mixture of both peptides was added to the processed plasma samples and latter analyzed in SRM mode. The same equimolar mixture was analyzed in the absence of plasma directly under the same experimental conditions to determine the matrix factor (MF). The extraction efficiency was evaluated for both peptides at three different concentrations covering the entire analysis range: low (6 ng/mL), medium (18 ng/mL) and high (36 ng/mL). Plasma samples spiked with the known concentrations of CIGB-814 and the IS (four replicates for each one, in all evaluated concentration levels) were processed as described above. Finally, each sample was spiked with the same amount of IS or CIGB-814, to evaluate peptide recovery normalized to the IS and vice versa.

Five calibration curves (from 1.5 ng/mL to 48 ng/mL) were prepared using different lots of plasma and measured in five different days during method validation. Each calibration curve included ten concentration levels (see Section 2.4), a zero standard (containing only IS at 12 ng/mL) and a blank sample that contained only processed plasma. The calibration curves were constructed by using the MassLynx v4.1 graphical output. Method sensitivity was evaluated in terms of LLOQ. It was experimentally assessed by five replicates (at 1.5 ng/mL) to evaluate accuracy and precision. Concentration integrity was evaluated for low concentration samples, in a further effort to diminish a LLOQ.

On the other hand, accuracy and precision of the method were evaluated in three batches on three different days of method validation. Five replicates of QCs at three concentration levels (6, 18 and 36 ng/mL) were analyzed in one day for repeatability. Method variability between days was evaluated by triplicate per day, from each concentration level, in three different days of method validation.

CIGB-814 and IS stability was assayed under the same experimental conditions used for clinical samples in terms of storage and environmental stress. According to FDA Guidelines [24] and our own risk evaluation, we considered the evaluation of: a) stability of stock solutions, b) processed sample stability when stored at − 20 °C, c) processed sample stability when stored at 4 °C, d) bench top stability, e) freeze-thaw stability and f) post-validation long-term stability at − 80 °C. Except for the stock solution stability, the three concentration levels of QCs were evaluated (6, 18 and 36 ng/mL).

Stock solutions for both peptides were prepared by duplicate at 1 mg/mL. They were analyzed by RP-HPLC using UV detection (λ = 226 nm), immediately after thawing and later kept at room temperature for 6 h. During **processed sample stability stored at − 20 °C** processed plasma samples without reconstitution were stored at − 20 °C for 1, 3 and 7 days. Three replicates at each concentration were compared with freshly processed samples. Then for **processed sample stability stored at 4 °C**, processed samples reconstituted in the analysis solution were loaded onto the well plate of the auto-sampler and the temperature was kept at 4 °C for 0, 4 and 8 h. All samples were analyzed for peptide stability in terms of CV%, against fresh processed samples. **Bench top stability** measures the effect of keeping both peptides in unprocessed plasma samples for four hours at room temperature. The analysis was performed by triplicate. For the **freeze-thaw stability** study the same three concentrations levels were tested. A group of samples was submitted to three freeze-thaw cycles (−20 °C/25 °C) every 24 h. After the last cycle, the samples were processed and analyzed by LC–MS/MS in SRM mode. These samples were compared to those that were not treated, but processed immediately after thawing.

In the **long-term stability** study, sufficient samples were prepared at three concentrations to perform triplicate analyses at each time point. Samples were stored under the same conditions used for clinical samples (− 80 °C). The first group was analyzed at t = 0 (freshly prepared), and the remaining groups were analyzed at 2, 6, 9 and 12 months. All samples from method validation were evaluated in terms of CV% and RE% according to the FDA guidelines [24]. Additionally one way ANOVA was used in the stability evaluation of CIGB-814 and its IS.

2.8. Pharmacokinetic study

The phase I clinical trial in patients diagnosed with RA, complied with the *Declaration of Helsinki (World Medical* Association, 2013) [27]. A more detailed information on the phase I clinical trial of CIGB-814 is provided in the Public Cuban Repository Database of Clinical Trials (http://registroclinico.sld.cu/en/trials/RPCEC00000238-En). The pharmacokinetic study enrolled 20 patients with moderate RA activity distributed into three groups corresponding to the administration of three dose levels of CIGB-814: 1 mg (six patients), 2.5 mg (five patients) and 5 mg (nine patients). They received the peptide weekly as a single subcutaneous injection for the first month, and monthly injection until the sixth month. The pharmacokinetic study was performed after the first administration of the CIGB-814. The blood collection schedule was as follow: t = 0 (before peptide administration) and t = 0.5, 1, 1.5, 2, 4, 6, 8, 12, 18 and 24 h (after administration). All blood samples were collected in EDTA vacutainer tubes from Greiner BioOne (USA) to obtain plasma by centrifugation at 700 RCF for 15 min, immediately after collection. Plasma was aliquoted and stored at − 80 °C until the analysis by LC–MS/MS in SRM mode.

2.9. Analytical data processing

The response in terms of CIGB-814 concentration was calculated by MassLynx v4.1 as follow: IS concentration * (CIGB-814 area/IS area). Linear regression was used to calculate the equation best fitting the curve. No Axis transformation was needed. Statistical analysis based on the Coefficient of Variation (CV%), Relative Error (RE%) and Analysis of Variance (one way ANOVA) was performed using the Microsoft Excel from Windows 10 data analysis package and GraphPad Prism® v6.01 software (GraphPad Software Inc., USA). Pharmacokinetic data analysis and parameter estimations were carried out with WinNonLin v3.0 software (Pharsight Corp., USA).

3. Results and discussion

3.1. Method development

3.1.1. Design and synthesis of the internal standard for CIGB-814

Following the principle of isotopic dilution [28], the sequence proposed for the IS was identical to the sequence of CIGB-814 to ensure identical physicochemical properties for both peptides. The $[M+4H]^{4+}$ ion was selected as the precursor ion to be fragmented in the SRM measurements because it was the most abundant signal observed in the ESI–MS analysis (Fig. 1A) regardless the capillary and cone voltages assessed. Since the resolution ($m/\Delta m$) of the first quadrupole is around 1000, it was evident that several isotopically-labeled amino acids needed to be included in the IS sequence to separate both isotopic ion distributions. This mass shift avoids mutual interference between both peptides during precursor selection in the first quadrupole for their fragmentation by CID; and at the same time, it guarantees a reliable quantitation.

Considering that CIGB-814 has four lysine residues located at positions 5, 7, 9 and 14, we modeled the minimum number of doubly-labeled $^{13}C_6{}^{15}N_2$-Lys residues required to separate the isotopic ion distributions of $[M+4H]^{4+}$ ions for both peptides considering the resolution ($m/\Delta m \sim 1000$) achievable by the first quadrupole of our mass spectrometer (Fig. 1B–D). The introduction of a single residue of a doubly-labeled $^{13}C_6{}^{15}N_2$-Lys (Fig. 1B) is not enough to separate both isotopic ion distributions since they overlap at 30% of the maximum intensity (considering an equimolar mixture). The introduction of two residues of $^{13}C_6{}^{15}N_2$-Lys still shows evidence of partial overlapping (Fig. 1C). The inclusion of three $^{13}C_6{}^{15}N_2$-Lys residues in the IS produces a 24 Da mass shift from the CIGB-814 and permitted a base line separation of both isotopic ion distributions (Fig. 1D).

Analyzing the Lys content of the CIGB-814 sequence (Lys^5, Lys^7, Lys^9 and Lys^{14}), and also that Lys^9 was the most difficult coupling step in the solid phase peptide synthesis, the IS was synthesized with three $^{13}C_6{}^{15}N_2$-Lys residues located at positions 5, 7 and 14 of the sequence. Fig. 1E shows the ESI–MS spectrum of the synthetized IS having the same behavior as CIGB-814 by yielding the $[M+4H]^{4+}$ as the most intense signal. An expanded region in the ESI–MS spectrum (Fig. 1F) shows how the isotopic ion distributions of the $[M+4H]^{4+}$ ions corresponding to an artificial mixture of CIGB-814 and the IS, labeled with three $^{13}C_6{}^{15}N_2$-Lys residues, were separated down to the base line under our experimental conditions.

3.1.2. Mass spectrometric experimental conditions

The $[M+4H]^{4+}$ ion was selected as the precursor ion for the quantitation of CIGB-814 not only because it is most intense ion the in the ESI–MS spectrum (Fig. 1A), but also because it is fragmented

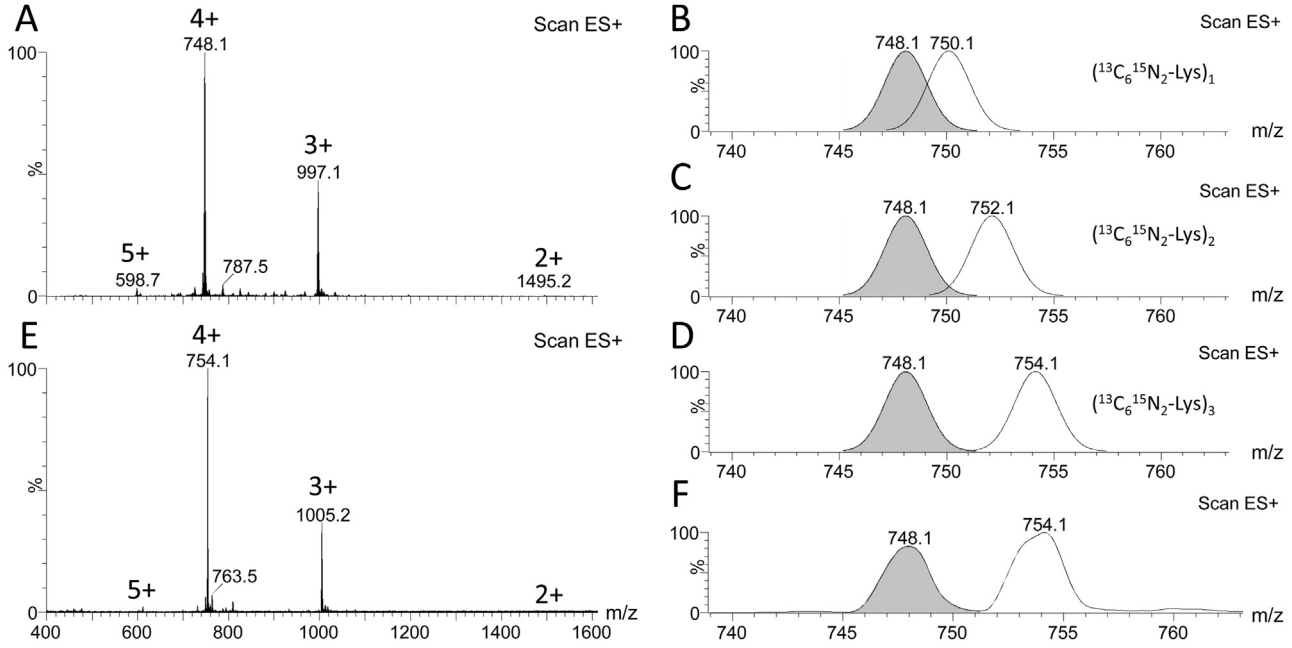

Fig. 1. **(A)** ESI–MS spectrum of the CIGB-814 peptide. The theoretical ESI–MS spectra shown in **(B)**, **(C)** and **(D)** contain the isotopic distributions of the $[M+4H]^{4+}$ ions of the CIGB-814 peptide (shaded in gray) and the proposed internal standard containing one, two and three $^{13}C_6{}^{15}N_2$-Lys, respectively. The isotopic distributions of CIGB-814 and its IS variants were modeled with the MassLynx software v4.1. The m/Δm was fixed at 1000 considering the resolution for the selection of precursor ion in the triple quadrupole tandem mass spectrometer used in this PK study. **(E)** ESI–MS spectrum of the IS containing three $^{13}C_6{}^{15}N_2$-Lys residues. **(F)** Expanded region of the experimental ESI–MS spectrum showing the $[M+4H]^{4+}$ ions of CIGB-814 and IS.

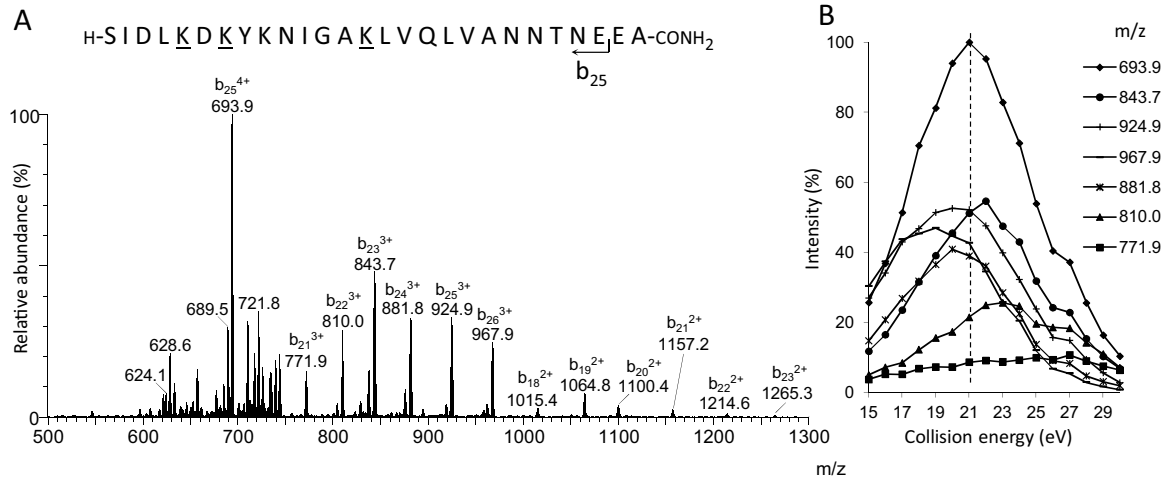

Fig. 2. **(A)** ESI–MS/MS spectrum of CIGB-814 using collision energy of 21 eV. The nomenclature agrees with that proposed by Roepstorff and Fohlman [39]. (B) Collision energy optimization to select among the fragment ions ($b_n{}^{2+}$ and $b_n{}^{3+}$), the best transition ($[M+4H]^{4+} \rightarrow b_{25}{}^{4+}$) guaranteeing the highest sensitivity, selectivity and the quantitation in the PK study. The dotted line indicates the collision energy used to induce CIGB-814 and IS fragmentation for method validation and PK study. The amino acid residues underlined in the CIGB-814 sequence indicate the position of the three $^{13}C_6{}^{15}N_2$-Lys residues in the IS sequence. The arrow indicates the $b_{25}{}^{4+}$ N-terminal fragment ion used as a transition for the quantitative analysis in the SRM mode.

very easily in the collision chamber and the ESI–MS/MS spectrum contains several fragment ions that can be studied to select the best transition(s) (Fig. 2A). Several ESI–MS/MS spectra for the $[M+4H]^{4+}$ using different collision energies from 15 to 30 eV were acquired and monitored the relative intensities of the main backbone ions detected (Fig. 2B). This result evidenced that $b_{25}{}^{4+}$ ($m/z = 693.9$) is the most favorable fragmentation. Fig. 2A shows the ESI–MS/MS spectrum of CIGB-814 fragmented at 21 eV, which was the optimal collision energy.

Although several $b_n{}^{3+}$ and $b_n{}^{2+}$ fragments ions were also observed in the ESI–MS/MS (Fig. 2B) and in principle they may be also useful for quantitation, the $b_{25}{}^{4+}$ ion at least duplicated its intensity compared to the remaining fragment ions. We also assessed the use of several transitions other than $[M+4H]^{4+} \rightarrow b_{25}{}^{4+}$including the combinations of three transitions ($[M+4H]^{4+} \rightarrow b_{21}{}^{3+}$-$b_{26}{}^{3+}$), for a better selectivity of the method, particularly those daughter ions with m/z higher than the precursor [22]. However, the overall sensitivity of the method was compromised by one order of magnitude due to the considerably lower intensities of other fragment ions (Fig. 2B). This has an unfavorable impact on lowering the LLOQ (data not shown) thus impairing the quantification of samples in the elimination phase of this PK study.

Additionally, considering other transitions besides the $b_{25}{}^{4+}$, significantly decreased the number of data points per curve, which

is critical for the reliability in the quantitation [22], especially for the concentration values close to the LLOQ.

In contrast, if we follow only one transition (SRM mode) for both peptides ($[M+4H]^{4+} \rightarrow b_{25}{}^{4+}$, CIGB-814: 748.1→693.9, and IS: 754.1→699.9), with the previously fixed dwell time, the number of data points per peak increased up to 20 points (Fig. 3A), which is also important for reliable quantitation results. Moreover, the increased intensity of $b_{25}{}^{4+}$ compared to the other fragment ions led us to set a longer dwell time for registering this fragmentation ($b_{25}{}^{4+}$), thereby improving sensitivity. On the other hand, cycle time was also optimized to increase signal quality and intensity, especially for lower concentration samples. According to Gallien et al. [22], cycle time directly affects sensitivity and precision of quantitation. With a chromatographic peak width of 20 s, a cycle time of 1 s, accurately reproduces the elution profile.

The above mentioned aspects were relevant in decreasing the LLOQ of this bioanalytical method down to 1.5 ng/mL (see discussion in Section 3.2.2) and in ensuring better sensitivity, which is essential for plasmatic detection/quantitation of CIGB-814 administered subcutaneously at very low doses.

Therefore, instead of following several transitions for this peptide, we decided to evaluate the selectivity of the method by analyzing only one transition and the values obtained for the absolute concentration values of the quality control samples measured during the entire validation process and the matrix factor (see discussions in Section 3.2.1).

No mutual interference was detected between CIGB-814 and the IS when both peptides dissolved in PBS were separately injected and both transitions (748.1 → 693.9 and 754.1→699.9) were monitored by SRM analysis (Fig. 3A and B). The transitions detected in both cases corresponded only to the injected peptide. Additionally, blank processed human plasma, was analyzed under the same experimental conditions, and showed no traces of transitions for both peptides (Fig. 3C). This result demonstrates that plasma has no interference in the quantitation of either peptides, CIGB-814 or its IS. Also a human plasma sample spiked with IS, was analyzed (Fig. 3D) and no interference at the transition corresponding to CIGB-814 was detected.

Similar experiments to those described above were also conducted for $[M+3H]^{3+}$ (m/z 997.1, Fig. 1A) considering that its intensity is approximately the half of the most abundant multiply-charged ion ($[M+4H]^{4+}$ (see Fig. 1A). The $[M+3H]^{3+}$ was not fragmented efficiently, and it required higher collision energies than the selected for ($[M+4H]^{4+}$ to observe an appreciable fragmentation, probably due to the less availability of mobile protons [29]. The $b_{26}{}^{3+}$ ion at m/z 967.27 was the most favorable fragmentation for $[M+3H]^{3+}$ in all of the assayed conditions but this transition implied an overall drop of 1/5 the sensitivity of the method which is unacceptable for PK modeling considering the low dosage evaluated in this study.

3.1.3. Sample processing

To eliminate most of the proteins in the plasma several precipitation approaches were assessed using organic acids and solvents. The best peptide recovery (≅ 60%) and reproducibility, as evaluated by reverse phase liquid chromatography, was by adding two volumes of pure acetonitrile to the plasma sample. Similar procedures have been successful in the enrichment of peptides [30] or low molecular weight serum protein prior to mass spectrometric analysis [31]. According to the authors, acetonitrile precipitation produces a reproducible depletion of abundant and high molecular weight proteins from the serum.

The supernatant was vacuum-dried and before LC–MS/MS analysis and reconstituted in 15% of solution B used as the mobile phase in the chromatographic separation but containing 0,5 g/L of 1,5 octyl-β-D-glucopyranoside. These conditions maximize the recovery of the dried CIGB-814 and did not affect its retention in the column. At the same time, this solution assured the non-retention of several impurities that were more hydrophilic than CIGB-814 and that remained after the initial protein precipitation step.

Although solid phase extraction is very frequently used at the initial steps of sample processing to eliminate interference substances, proteins and/or metabolites that affect the results of LC–MS/MS experiments [26], it was finally discarded due to the irreproducible results that compromised sensitivity, precision and accuracy of the method.

3.1.4. Chromatographic conditions

The PK study as well as the implementation and the entire validation process comprised the analysis of a considerable number of samples: 350 and ~500, respectively. Considering that the major contributor to the PK analysis time remains the chromatographic step [32], the length of the gradient was optimized as much as possible (1 min from 15 to 95% of solution B) without compromising the selectivity of the method while enabling the analysis of multiple samples in less time. In spite several attempts were addressed to shorten the desalting and equilibrium steps, they were failed and compromised the overall sensitivity of the method as well as the retention of the peptide by the column. The usage of UPLC [33] and/or monolithic columns [34] would be a source for further optimization of the chromatographic analysis.

The co-injection of both peptides under the chromatographic conditions rendered a single peak that reproducibly eluted at 8.58 min with a FWMH of 0.13 min (Fig. 3A, B). This demonstrates that there no isotopic effects and both peptides have the same behavior in the LC–MS/MS analysis as was expected for the ^{13}C and ^{15}N-labeling of the IS [28]. These issues are very important to obtain reliable results in terms of quantitation.

3.2. Method validation

3.2.1. Matrix factor and extraction efficiency

The estimated MF of the analyzed samples was 1.16 with a CV% of 7.29%. According to this, the analysis of CIGB-814 and its IS with or without processed plasma did not show a matrix effect. The recoveries of the method were similar in the three concentration levels: 6, 18 and 36 ng/mL, with an extraction efficiency of 44.9% for CIGB-814 (CV% ≤ 11.1%) and 53.1% for IS (CV% ≤ 8.4%). Even with the differences between both peptides a consistent, precise and reproducible recovery was obtained throughout the analysis range.

3.3. Sensitivity

A linear response ($R^2 \geq 0.99$) from 1.5 ng/mL to 48 ng/mL (Fig. 4A and B) was found. The relative error and precision were determined in five replicates for the LLOQ (1.5 ng/mL) and it ranged from −12.5% to 12.5%, with a coefficient of variation CV% of 9.9%. These results met the acceptance criteria of FDA for methods based on LC–MS analysis [24].

3.4. Calibration range and response

The R^2 coefficient was higher than 0.99 for all assessed curves. Fig. 4A shows the 5th curve in the MassLynx v4.1 graphical output. The replicates for each QCs (low, medium and high concentration) had a coefficient of variation lower than 4.34%. The differences between all concentration levels in the curves showed a CV% lower than 12.9%. The upper limit of quantitation (ULOQ) was set at 48 ng/mL for two reasons: (1) higher concentrations were not needed in the calibration curves because the Cmax for CIGB-814 after a subcutaneous injection were not very high due to the low doses and (2) when more concentrated samples were injected a

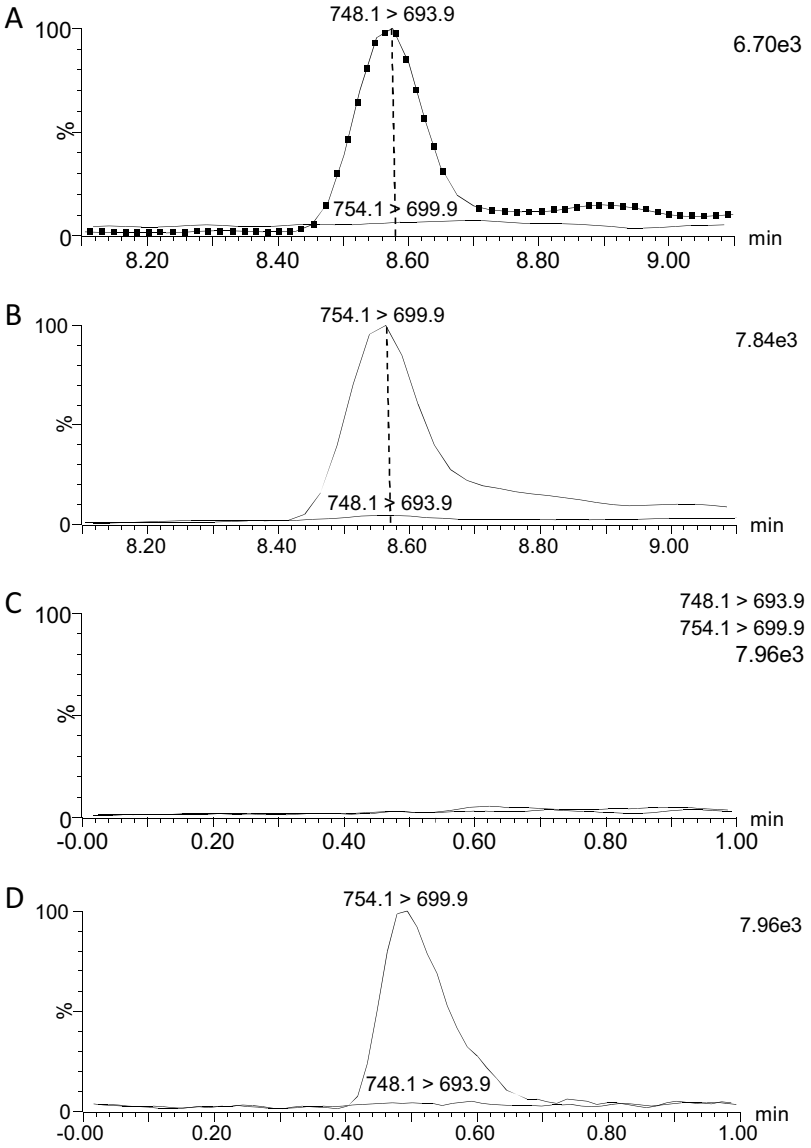

Fig. 3. Evaluation of the mutual interference between CIGB-814 and IS by monitoring their corresponding transitions, 748.1 → 693.9 and 754.1 → 699.9, in the SRM mode. PBS solutions containing only CIGB-814 or its IS were injected alone in **(A)** and **(B),** respectively. In the experiments shown in **(A)** and **(B)** both transitions were monitored simultaneously. The black squares in **(A)** indicate the data points used to monitor the transition of IS according to the dwell time and the inter-scan delay. In **(C)**, the blank plasma (without CIGB-814 and its IS) was injected and the transitions were monitored. In **(D)** a processed plasma sample was spiked with the IS and the transitions for both peptides were monitored simultaneously.

carry-over was observed, in some cases higher that 20% respect to the LLOQ, and it would makes more complicated the validation procedure unnecessarily by including this aspect in the full validation process [35]. Therefore, in this bioanalytical method it was not necessary to assess dilution integrity. In a further effort to obtain even lower LLOQ. Concentration integrity was evaluated for low concentration samples, but it had a negative impact on CIGB-814 peptide quantitation (data not shown).

3.5. Accuracy and precision

Regarding repeatability, a CV% ≤ 4.3% and −4.7 ≤ RE% ≤ 5.1% were obtained. Variability between days was also evaluated in terms of CV% and RE%. All samples met the FDA acceptance criteria for bioanalytical methods based on LC–MS (CV% ≤ 15%, RE% ± 15%) [24], with CV% ≤ 6.7% and −9.8% ≤ RE% ≤ 8.2%.

3.6. Sample stability

Sample stability studies are summarized in Table 1. Regarding **Stock solutions**, there were not important variation in peptide concentration (≤7.8%) for the different conditions assayed. Total impurities expressed as peak area% were less than 5% of the total peak area detected by RP-HPLC, and none of the peaks corresponding to impurities individually exceeded 1%. When processed samples corresponding to each concentration (6, 18 and 36 ng/mL) were stored at −20 °C for 1, 3 and 7 days and compared with freshly processed samples, the results showed that both peptides were stable under the experimental conditions ($p \geq 0.2$). (Table 1). Additionally, the processed sample reconstituted in the analysis solution and stored at 4 °C for 0–8 h in the well plate of the auto-sampler, in the three evaluated concentrations (6, 18 and 36 ng/mL) showed no significant differences in the CV% compared to samples analyzed immediately after processing ($p \geq 0.2$).

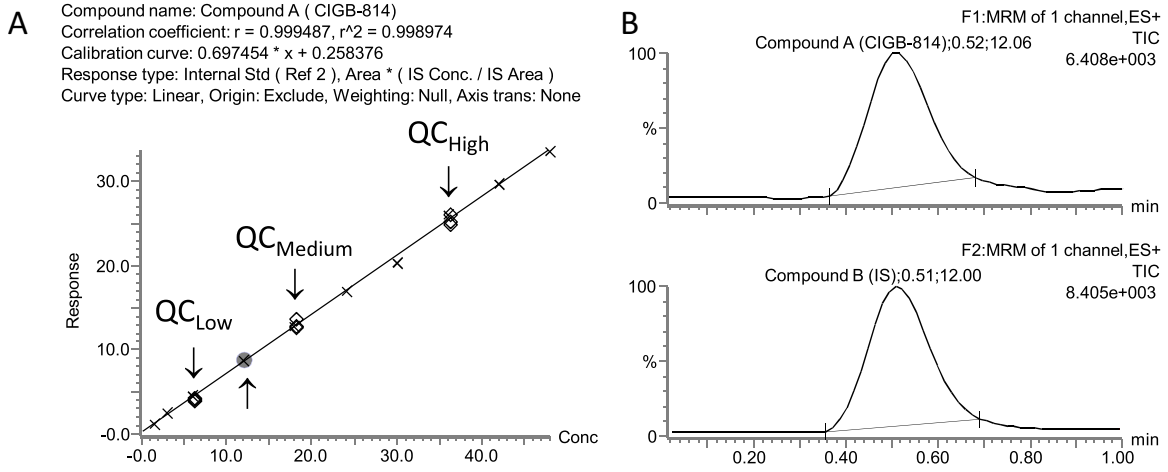

Fig. 4. (A) Graphical output from MassLynx v4.1 software for the 5th calibration curve obtained at method validation. Head down arrows indicate the QCs quantified with this curve covering the entire linear range (CV% ≤ 4.34%). The crosses indicate data points used to construct the calibration curve (1.5; 3.0; 6.0; 12; 18; 24, 30; 36; 42 and 48 ng/mL). **(B)** Transition peaks of the CIGB-814 (upper panel) and its IS (bottom panel) used to perform peptide quantitation in terms of response. They correspond to an equimolar mixture at a concentration of 12 ng/mL in the plasma (indicated by the head up arrow in panel A). Retention time obtained for CIGB-814 and its IS were 0.52 and 0.51 min, respectively. The calculated response enabled the estimation of CIGB-814 concentration which was 12.06 ng/mL.

Table 1
Stability evaluation for CIGB-814 and its IS in human plasma. Results are expressed in terms of CV%[a] and ANOVA[b].

	QCs nominal concentrations		
	6 ng/mL	18 ng/mL	36 ng/mL
Processed sample stability stored at − 20 °C			
Mean (ng/mL)	5.7	17.3	33.4
CV%	5.0	4.9	3.5
p	0.6	0.2	0.4
Processed sample stability stored at 4 °C			
Mean (ng/mL)	6.2	19.1	38.1
CV%	7.1	6.3	4.2
p	0.4	0.6	0.2
Bench top stability			
Mean (ng/mL)	6.5	18.5	38.0
CV%	3.3	2.2	2.3
p	0.3	1.0	0.6
Freeze-thaw stability			
Mean (ng/mL)	5.8	19.0	37.5
CV%	7.6	6.4	2.7
p	0.1	0.3	0.8
Long-term stability			
Mean (ng/mL)	6.6	18.3	36.8
CV%	6.7	6.0	2.3
p	0.1	1.0	0.7

[a] CV% < 15% meets the FDA acceptance criteria [24].
[b] Significance level of α = 0.05 to establish that peptides were stable under the assayed condition.

In the **bench top stability**, the analysis revealed no differences between unprocessed plasma samples incubated for 4 h and those analyzed immediately ($p \geq 0.3$). The **freeze-thaw stability** study after the last cycle, revealed that both peptides were unstable ($p < 0.05$, data not shown). Additionally, the experiment was repeated by freeze-thawing the samples only two times every 24 h, and both peptides were found to be stable ($p \geq 0.1$). The **long-term stability** study showed that samples prepared at three concentrations were stable for 2, 6, 9 and 12 months stored at − 80 °C ($p \geq 0.1$).

In accordance with the above results, the bioanalytical method developed here for the absolute quantitation of CIGB-814 in human plasma was fully validated according to the FDA acceptance criteria [24]. Overall validation results are summarized in Table 2.

3.7. Application of the bioanalytical method to pharmacokinetic studies in RA patients

The validated method was applied to study PK profile of CIGB-814 in plasma obtained from 20 patients with moderate RA enrolled in the phase I clinical trial. The CIGB-814 concentrations plotted against time for the three dose levels are shown in Fig. 5A. The measured AUC for 1 mg, 2.5 mg and 5 mg dose levels represent 92.9%, 89.34% and 95.83% of the total AUC extrapolated to infinity, respectively (see Table 3). According to the Note for Guidance on the Investigation of Bioavailability and Bioequivalence released by EMA, 80% would be considered as enough to appropriate PK parameter determination [36]. The main PK parameters were calculated by Non Compartmental Analysis and are shown in Table 3.

A Tmax of 0.5 h for all dose levels (Fig. 5A) agrees with those obtained in Lewis rats after administering the ^{125}I-labeled CIGB-

Table 2
Overall validation results included in the FDA acceptance criteria [24].

Validation parameters	FDA Acceptance criteria	Results
Matrix factor MF/(CV%)	MF = 1	1.16/7.3%
Variability of LLOQ (CV%)	CV% ≤ 20%	15.8%
Variability of calibrator standards (CV%)[a]	CV% ≤ 15%	≤12.9%
LLOQ/(CV%)	CV% ≤ 20%	1.5 ng/mL/9.9%
Repeatability in one day (CV%)[a]	CV% ≤ 15%	≤4.3%
Variability between days (CV%)[a]	CV% ≤ 15%	≤6.7%
Extraction efficiency for **CIGB-814** (%) [CV%]	CV% ≤ 15%	44.9% [≤ 11.1%]
Extraction efficiency for **EI** (%) [CV%]	CV% ≤ 15%	53.1% [≤ 8.4%]
Stability of the stock solution for **CIGB-814** (Δc%)	Δc% ≤ 10%	7.2%
Stability of the stock solution for **EI** (Δc%)	Δc% ≤ 10%	7.8%
Long-term stability at −80 °C (CV%) [a] [p] [b]	CV% ≤ 15%	≤6.7% [$p \geq 0.1$]
Sample stability after three freeze-thaw cycles every 24 h (CV%) [a] [p] [b]	CV% ≤ 15%	≤7.6% [$p \geq 0.1$]
Bench-top stability (CV%)[a] [p][b]	CV% ≤ 15%	≤3.3% [$p \geq 0.3$]
Processed sample stability store at −20 °C (CV%)[a] [p][b]	CV% ≤ 15%	≤5.0% [$p \geq 0.2$]
Processed sample stability store at 4 °C (CV%)[a] [p][b]	CV% ≤ 15%	≤7.1% [$p \geq 0.2$]

[a] It was reported the nearest value to the acceptance criterion among all results obtained from the evaluation at three concentration levels: QD_L, QC_M and QC_H.
[b] High probability value obtained from the one way ANOVA regarding those parameters ($\alpha = 0.05$).

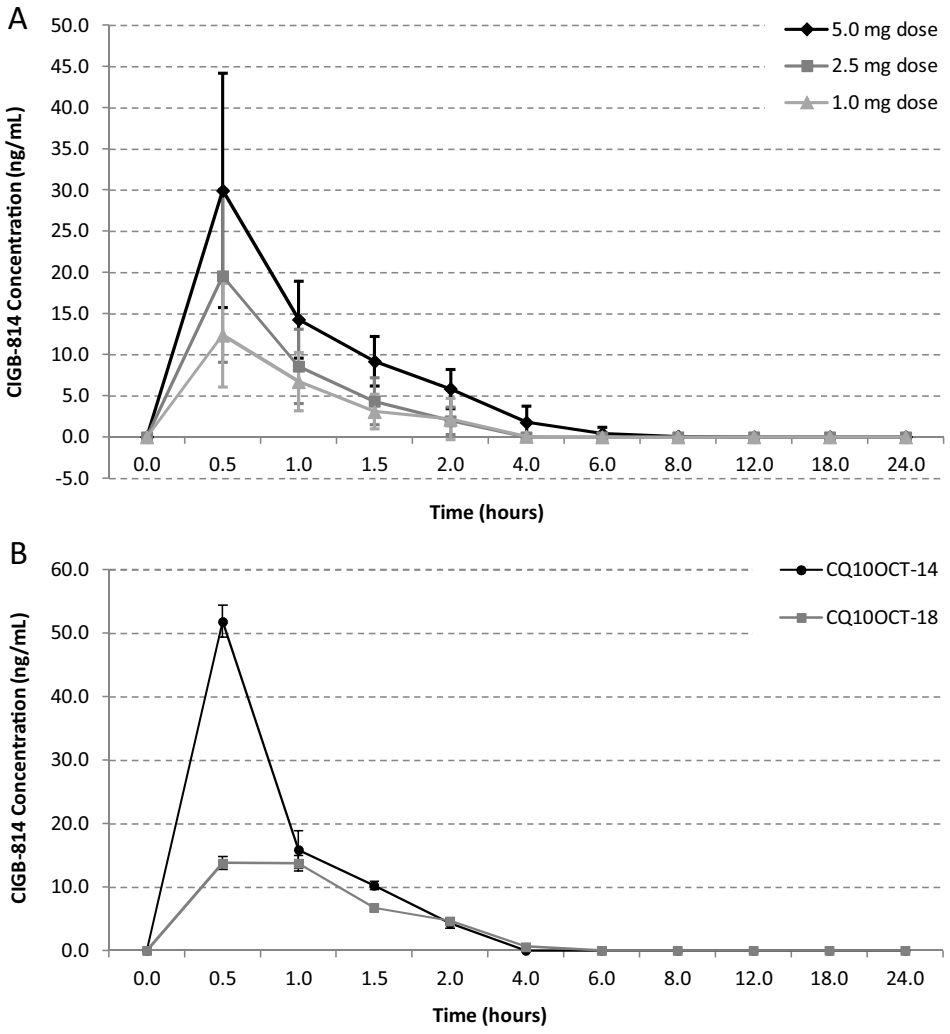

Fig. 5. (A) Plasma concentration profile (ng/mL) of CIGB-814 for 24 h post-administration in the three dose levels of the PK study (see the legend inset). Each time point in the graph represents an average of the absolute concentration of CIGB-814 with their corresponding STD bars for all patients included in each dose level. **(B)** Plasma concentration profile of two patients reaching the highest (CQ10OCT-14) and the lowest (CQ10OCT-18) Cmax values in the 5 mg dose level.

814 (manuscript in preparation). The average of Cmax values showed a wide dispersion (Fig. 5A) and it is associated with the biological variability between individuals and the non-normalized doses respect to the body weight. Plasma concentration plotted against time in the two patients displaying the highest and lowest Cmax within the 5 mg dose level is shown in Fig. 5B. The very small standard deviation bars in these two graphs (Fig. 5B) reinforced the idea that large dispersion found for all data in the three dose levels is not associated with this analytical method.

Table 3
The main PK parameters calculated by NCA using WinNonLin v2.1 software.

PK parameters[a]	Dose levels		
	1.0 mg (*N* = 5)	2.5 mg (*N* = 5)	5.0 mg (*N* = 8)
Tmax (h)	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00
Cmax (ng/mL)	12.38 ± 6.29	19.59 ± 10.48	29.93 ± 14.23
λ_z (h^{-1})	1.32 ± 0.68	2.05 ± 1.24	0.76 ± 0.33
$t_{1/2}\lambda$ (h)	0.69 ± 0.42	0.42 ± 0.19	1.10 ± 0.48
MRT (h)	0.92 ± 0.21	0.80 ± 0.06	1.30 ± 0.40
CL (L/h)	0.10 ± 0.06	0.21 ± 0.16	0.15 ± 0.06
V_z (L)	0.10 ± 0.09	0.12 ± 0.10	0.23 ± 0.12
AUC (ng/mL h)[b]	13.95 ± 8.54	18.77 ± 10.57	38.62 ± 15.30
AUC_{last} (ng/mL h)[c]	12.96 ± 7.98	16.77 ± 9.02	37.01 ± 14.94
AUC Covered (%)[d]	92.90 ± 5.57	89.34 ± 4.73	95.83 ± 2.66

[a] All values are expressed as Mean ± STD.
[b] AUC represents the total area under the curve extrapolated to infinity.
[c] AUC last represents the area under the curve until the last point measured in the PK profile.
[d] AUC Covered shows the fraction represented by the AUC last respect to the AUC expressed in% [(AUClast/AUC)*100%].

On the other hand, there was no correlation between the Cmax and body weight (r = – 0.033). CIGB-814 is an APL that acts as an immunomodulator inducing peripheral tolerance through the proliferation of the T-cells with regulatory phenotypes (Treg). This specific immune cell population of Treg cells is basically constant among all individuals; therefore the administration of CIGB-814 does not necessarily need to be normalized respect to the patient's weight. In contrast with this, it was reported that the administration of high doses of immunomodulatory drugs can lead to severe immune system deregulation and produce no therapeutic effect [37,38]. The LLOQ of the bioanalytical method was reached at about 6 h post-administration for dose levels of 2.5 and 5 mg, but it was attained just after 4 h post-administration in the 1 mg dose level. This is typical for the systemic administration of therapeutic peptides with low-molecular weight and it also demonstrates the rapid clearance of CIGB-814 following this administration schedule. This favors CIGB-814 as a therapeutic drug candidate for RA because many immunomodulatory therapies report adverse events due to the persistence of the drug in the body for long periods [12] and at the same time, it may enables the use of the repeated dose schedules required for immunomodulatory drugs.

Based on the estimation of the 95% confidence intervals (95% CI), it was impossible to establish a dose dependent study of CIGB-814 PK parameters. Although the AUC and the Mean Residence Time (MRT) showed a slightly increases with the dose levels, all 95% CI for these PK parameters overlapped. Due to the low doses, is very probable that CIGB-814 plasma concentration did not reach the distributive equilibrium which would not allow us to establish dose dependency. This is, however, irrelevant when considering that the expected effect for this immunomodulatory peptide could be reached with a minimal availability of the drug.

4. Conclusions

The bioanalytical method developed for the absolute quantitation of CIGB-814 in human plasma was fully validated according to FDA guidelines. All parameters met the acceptance criteria for reliable peptide quantitation. The application of the validated method to the analysis of clinical samples during the phase I clinical trial in RA patients, enabled the estimation of the main PK parameters, that included more than 85% of the total AUC. Although dose dependence was not established for the main PK parameters, there was a trend of the Cmax and consequently the AUC, to increase when the dosages augmented. When the plasma concentration was plotted against time, it was found that CIGB-814 was rapidly cleared from plasma, for all doses, with an average clearance half-life of 0.74 ± 0.34 h. This result is very promising for an immunomodulatory drug candidate in terms of safety because long-lasting self-peripheral tolerance would probably require repeated doses. In general, the PK study in the phase I clinical trial reinforce the therapeutic potential of CIGB-814 as immunomodulatory drug candidate.

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