Journal of Pharmaceutical and Biomedical Analysis 105 (2015) 107–114

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Bio-analytical method based on MALDI-MS analysis for the quantification of CIGB-300 anti-tumor peptide in human plasma

Ania Cabrales-Rico[a], Beatriz G. de la Torre[b], Hilda E. Garay[a], Yoan J. Machado[c], Jose A. Gómez[c], Enrique Audain[c], Orlando Morales[c], Vladimir Besada[d], Jose Luis Marcelo[e], Vilcy Reyes[f], Yasser Perera[f], Silvio E. Perea[f], Osvaldo Reyes[a], Luis Javier González[d,*]

[a] Chemical Synthesis Department, Center for Genetic Engineering and Biotechnology, Havana, Cuba
[b] Departament de Ciències Experimentals i de la Salut, UPF/PRBB, Barcelona, Spain
[c] Mass Spectrometry Department, Center for Molecular Immunology, Havana, Cuba
[d] Mass Spectrometry Laboratory, Proteomics Department, CIGB, Havana, Cuba
[e] Quality Assurance Unit, CIGB, Havana, Cuba
[f] Pharmaceutical Research Division, CIGB, Havana, Cuba

ARTICLE INFO

Article history:
Received 12 September 2014
Received in revised form
24 November 2014
Accepted 25 November 2014
Available online 3 December 2014

Keywords:
Anti-tumor peptide
MALDI-TOF
Human plasma
Bio-analytical method
Full validation

ABSTRACT

A fully validated bio-analytical method based on Matrix-Assisted-Laser-Desorption/Ionization-Time of Flight Mass Spectrometry was developed for quantitation in human plasma of the anti-tumor peptide CIGB-300. An analog of this peptide acetylated at the N-terminal, was used as internal standard for absolute quantitation. Acid treatment allowed efficient precipitation of plasma proteins as well as high recovery (approximately 80%) of the intact peptide. No other chromatographic step was required for sample processing before MALDI-MS analysis. Spectra were acquired in linear positive ion mode to ensure maximum sensitivity. The lower limit of quantitation was established at 0.5 μg/mL, which is equivalent to 160 fmol peptide. The calibration curve was linear from 0.5 to 7.5 μg/mL, with $R^2 > 0.98$, and permitted quantitation of highly concentrated samples evaluated by dilution integrity testing. All parameters assessed for five validation batches met the FDA guidelines for industry. The method was successfully applied to analysis of clinical samples obtained in a phase I clinical trial following intravenous administration of CIGB-300 at a dose of 1.6 mg/kg body weight. With the exception of C_{max} and AUC, pharmacokinetic parameters were similar for ELISA and MALDI-MS methods.

1. Introduction

The peptide CWMSPRHLGTC was identified by screening a 9-mer random cyclic peptide phage display library using the human papilloma virus (HPV-16) E7 acidic domain as target [1]. To ensure optimum penetrability of this cyclic peptide into the cell nucleus it was fused to the cell penetrating peptide (GRKKRRQRRRPPQ) of the TAT protein from HIV using a beta Alanine residue as a linker (-βA-). This pro-apoptotic cyclic peptide, named CIGB-300 (GRKKRRQRRRPPQ-(βA)-CWMSPRHLGTC), impairs CK2 mediated phosphorylation *in vitro* and *in vivo*, and leads to a dose-dependent anti-proliferative effect in a variety of tumor cell lines. Prominently, CIGB-300 elicits significant anti-tumor effect both by local and systemic administration in murine syngenic tumors and human tumors xenografted in nude mice. In a First-in-Human trial with CIGB-300 in patients with cervical malignancies this peptide was found to be safe and well tolerated in the dose range studied. Likewise, signs of clinical benefit were clearly identified after the CIGB-300 treatment as evidenced by significant decrease of the tumor lesion area and histological examination [2]. At molecular level, the systemic administration of CIGB-300 induced apoptosis in the tumor as evidenced by *in situ* DNA fragmentation analysis. In addition, there are evidences that ^{99m}Tc-labeled CIGB-300

Abbreviations: MALDI-TOF MS, Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry; QCs, quality control samples; CSs, calibration standard samples; TFA, trifluoroacetic acid; IS, internal standard; LLOQ, lower limit of quantification; AUC, area under the curve.

* Corresponding author at: Physics and Chemistry Division, Center for Genetic Engineering and Biotechnology, PO Box 6162, Havana, Cuba.
E-mail addresses: luis.javier@cigb.edu.cu, ania.cabrales@cigb.edu.cu (L.J. González).

http://dx.doi.org/10.1016/j.jpba.2014.11.043
0731-7085/

was certainly accumulated on the tumors after administration by systemic and/or intra-tumor routes [3]. Other reports reinforce and support the use of CIGB-300 for targeted cancer therapy [4–6].

A substantial number of peptide-based drugs and vaccines are in clinical trials [7,8]. One of the major obstacles in developing novel peptide drugs is the full validation and implementation of tailored analytical methods for detection and quantification of these molecules in the complex context of biological fluids. Because of the short half-life of peptides, their rapid clearance, proteolysis and/or degradation by exo-peptidases, analytical methods must be reliable, accurate and selective. According to this, liquid chromatography coupled to mass spectrometry in combination with isotopic labeling has expanded their application to the absolute quantification of peptides in biological samples [9–14]. A sample cleanup step prior to LC–MS analysis is an absolute requirement to avoid potential interferences from substances present in complex biological matrices. Mass spectrometric detection has been mainly used in selective ion monitoring (SIM) [15,16] or multiple reaction monitoring (MRM) modes [17,18]. Triple quadrupole and ion traps are the mass analyzers most commonly used to quantify peptides in biological samples due to their high selectivity, sensitivity; fast scanning speeds and their versatility using different scan modes. However, the resolving power of the MS and the labeling method used, are important aspects to be considered for obtaining an acceptable mass shift between analyte and its internal standard, during absolute quantification. It is particularly critical, when peptides to be quantified are large or multiply-charged ions originated in the ionization process are potentiated by the presence of several basic amino acids within the sequence [19]. In these cases to ensure a reliable quantification, it is necessary to label isotopically a considerable number of amino acid residues in order to avoid the overlap between the isotopic distribution of the target peptide and its internal standard. Thus, MALDI-TOF mass spectrometry emerges as an alternative approach to analyze large peptide molecules. A great benefit of the MALDI-TOF MS-based methods is the absence of limit for the molecular weight either for protein or peptide which is required to achieve a quantitative determination. It opens the possibility of a direct quantification of the sample of interest without previous fractionation mainly if the molecular mass of the target peptide is quite separated from the matrix signals raised during the ionization process. Besides, MALDI-TOF MS has some advantages when compared to ESI-MS, such as the temporal detachment between chromatography and mass spectrometry. This offline coupling is beneficial for MALDI, because the time required for analysis does not depend on the sample LC elution. Another MALDI benefit is the higher salt and buffer tolerance during sample preparation. This allows the running of alternative LC separation methods that are incompatible with ESI-MS due to the presence of salts or even to omit the LC separation and perform a direct analysis saving time and labor [20]. Using this strategy, the angiotensinogen peptide and two proteins (RNase and its protein inhibitor) were quantified upon tryptic digestions [21]. Other reports describing similar methods to determine hepcidine hormone peptide in human urine [22], acetylcholine and choline in micro-dialysis samples, have been reported previously [23].

The aim of this work was the development and full validation of a bio-analytical method to quantify CIGB-300 anti-tumor peptide in human plasma by MALDI-TOF mass spectrometry. Sample cleanup previous to the analysis was carried out by acidic precipitation of major plasma proteins with trifluoroacetic acid (TFA). No further chromatography step was necessary prior to MALDI-TOF MS analysis. In order to achieve the absolute quantification of CIGB-300 an internal standard (IS), its analog acetylated at the N-terminal end, was synthesized and purified (>99% of purity estimated by RP-HPLC). The acetylation shifts the molecular mass of the IS + 42 Da respect to intact CIGB-300. It avoids the overlapping of both isotopic ion distributions and permits a reliable quantification. The ionization efficiency of the intact CIGB-300 as well as its IS are similar. The MALDI MS spectrum of an equimolar mixture of both peptides showed that CIGB-300 ionizes more efficiently than it IS. However, this fact did not affect analyte quantitation because it was corrected using the equation: C [CIGB-300] = ((A [CIGB-300]/A [IS]) − n)/m, where C [CIGB-300] was the analyte concentration, A [CIGB-300]/A [IS] was the ratio of the peaks areas from both species, and n and m were the intercept and the slope of the calibration curve, respectively. The method was applied to the analysis of clinical samples from a CIGB-300 pharmacokinetic study carried out in patients with solid tumors included in the phase I clinical trial (WHO primary registry number: RPCEC00000112). Most of the PK parameters obtained by applying the MALDI-MS-based method were comparable to those obtained by the ELISA-based method developed for similar purposes (in press).

2. Materials and methods

2.1. Reagents and chemicals

All chemicals were HPLC and Spectral grade. Acetonitrile (ACN) and acetone were from Caledon (Canada), trifluoroacetic acid (TFA) was from Thermo Scientific (USA) and α-cyano-4-hydroxylcinnamic acid (CHCA) matrix was obtained from LaserBioLabs (France). Water was ultrapure quality from CIGB, Cuba. Blood samples were collected in 4 mL EDTA vacutainers from Greiner Bio-One (Germany). All blood samples were obtained under informed consent of the donors, either from healthy volunteers during method validation or patients during the clinical trial where CIGB-300 was evaluated (RPCEC00000112).

2.2. Equipment

Mass spectrometric analysis was achieved in an AXIMA Performance^{TM} MALDI-TOF/TOF mass spectrometer (Kratos Analytical Ltd., UK), equipped with a 50 Hz N_2 laser (337 nm) and using a DE1583TA stainless steel sample plate. Mass spectrometry data acquisition and processing were accomplished with Shimadzu Biotech MALDI-MS software (Japan). RP-HPLC analysis was carried out in an YL9100 YOUNG LIN HPLC system (South Korea) equipped with a ZORBAX RP-C18 (5 μm) analytical column (4.6 × 150 mm) from Agilent (USA). RP-HPLC data acquisition and processing were through YL-Clarity chromatograph data system (South Korea). For sample preparation, previous to MS analysis, a vortex-genie 2 from Scientific Industries Inc. (USA) and a micro-centrifuge from Eppendorf (Germany) were used.

2.3. Peptide synthesis

The CIGB-300 peptide and its IS were synthesized in solid phase by using the Fmoc/tBu synthesis strategy [24], at the Peptide Synthesis Laboratory from the Center for Genetic Engineering and Biotechnology (Havana, Cuba). N-terminus acetylation of the IS was also carried out in solid phase, before cleaving the peptide from the resin. Both peptides were purified by RP-HPLC to over 95% purity by monitoring the absorbance at 226 nm. Identity of both peptides was confirmed by ESI-MS based on good agreement between the expected and experimental molecular mass with an error lower than 0.01%. The specific extinction coefficient at 280 nm for CIGB-300 was experimentally determined by amino acid analysis [$\xi_{1\%,\,280\,nm}$ = 17.38] as reported previously by McEntire [25]. Since N-terminal acetylation does not interfere with peptide absorbance at 280 nm, the extinction coefficient was considered same for both peptides.