Determination of blood concentration of linker cyanobacterial protein cyanovirin-N by LC-MS/MS

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Abstract

Cyanovirin-N(CV-N) is an antiviral agent that induced the inflammation of HSV-1.In our previous study, a (Gly4Ser)3 linker was fused to the N-terminus of CV-N and fabricated linker-CV-N(LCV-N) protein. A selective sensitive liquid chromatography tandem-mass spectrometry method was developed to determine the concentration of linker cyanobacterial protein cyanovirin-N in the plasma and to study its pharmacokinetics.

Keywords

LC/MS, standard curve, CV-N.

1. Introduction

A prokaryprotic protein treated as topical microbicide shows a latently antiviral activity resist many types of viruses, such as influenza virus and hepatitis C virus. There are one typical prokaryo protein named the cyanobacterial cyanovirus-N (CV-N)[1]. It separated from cyanobacteria (Blue-green algae) Candida and is 11 KD natural polypeptide with potent human immunodeficiency virus(HIV)-inactivity[2]. Cyanovirin-N can selectively recognize and integrate the membrane of HIV particles. According to this characteristic, Cyanovirin-N is able to prevent HIV combining with antigen of the host cells[3-5]. Researches have shown that the anti-HIV activity of CV-N is mainly manifested in many aspects. It can irreversibly inactivate T lymphocyte trophoblasts and inhibit the activity of type I, type II HIV and simian immunodeficiency virus. Furthermore, it also inhibits clinically macrophage trophoblasts (M-tropic) ,T cells and T cells which were acquired HIV-1.

In addition, there are existing nonnegligible drawbacks of cyanovirus-N which are shown in protein-relevant pharmaceutics including rapid renal clearance. To overcome these defects, we designed a mutant of cyanovirus-N. We fused a (Gly4Ser)3 molecule that is a linker peptide to the N-terminus of CV-N and composed the linker-CV-N. Our previous studies suggested that the toxicity and immunogenicity of linker-CV-N(LCV-N) were markedly lower than those of CV-N[6-9]. But LCV-N how metabolize in the body haven't been studied. Here we focused on the way how to examine the determination the concentration of LCV-N and its Pharmackinetics characters.

Pharmackinetics(PK) mainly studies the dynamic disposition changes of drugs which includes absorption, distribution biochemical conversion and the concentration changes of drug in body. Here plasma concentration changes is a one way to evaluate to the safety and effect of drugs. With the emergence of liquid chromatography tandem-mass spectrometry, as an accurate technology, it makes pharmacokinetics studies result more convinced[10-13]. In this study, we determine the concentration of linker cyanobacterial protein cyanovirin-N by LC-MS/MS and provide a systematic way to research PK characters of L-CVN.

2. Materials and Methods

2.1 Stock and working solution preparation

The standard curve and QC stock solutions were prepared by adding appropriate DMSO to original solution to generate the final concentration of 4.75mg/ml stock solution. And for IS stock solution, IS was dissolved in 50% ACN containing 0.1% formic acid (FA) to generate 1.0 mg/ml IS stock solution. The standard curve and QC working solutions were prepared by using 1×PBS:DMSO (v:v=9:1) solution diluted to desirable concentrations. The IS working solution was prepared by diluting the IS stock solution using 10% ACN at a final concentration at 50.0 ng/ml. The standard curve samples and QC samples were freshly prepared on each analysis day by diluting the working solution with blank SD rat plasma in polypropylene tube.

2.2 Pharmacokinetic study.

In order to assess the effect of (GGGGS)3 on pharmacokinetics characteristic of LCVN. Four rats (two female and two male) were injected with LCVN at administrated a single dose of 1.0 mg/kg LCVN body weight through by jugular vein injection. Blood samples were collected into vials containing K2-EDTA via tubes from the jugular vein at selected time points of 5, 15, 30, 60, 120, 240, 360, 480 and, 1440 min after injection administration. At appropriate times, the blood samples were centrifuged and the pRats plasma was placed in labeled vials and frozen samples were obtained by centrifugation and stored at (-80 ℃) until for further analysis. Plasma levels of LCVN were quantified using LC-MS/MS.

2.3 Sample Preparation

Each plasma sample (30 μL), calibration standards samples and QC samples were mixed with 90μL of isopropanol and vortexed for 10 min. The mixture was centrifuged at 1500 rpm for 10 min at 4℃. After removal of the supernatant, the protein pellets were reconstituted in 120 μL of 200 mM ammonium bicarbonate in 10 % MeoH with addition of 10μL of internal standard (IS) solution (concentration: 50.0ng/mL) and 10μL of trypsin which was used to digest the protein pellets. The digested mixture was then loaded onto Waters Oasis® MCX SPE plate. The plate was washed by 1 ml 2 %FA in 5 % MeOH, followed by 1 ml MeOH. The surrogate peptide was eluted by 500 μl 5 % NH4OH in 40 % ACN. The eluted solution was dried under nitrogen. The dry residue was reconstituted with 200 μl of 10 % CAN. The same procedure was applied to the calibration standards and the internal control samples. 10μL aliquot of the completed samples was injected to the LC-MS/MS system for analysis. Pharmacokinetic calculations were performed on each individual set of data using the pharmacokinetic software WinNonlin™ Version 6.3 (Pharsight, Mountain View, CA) by non-compartmental model.

2.4 Liquid chromatography tandem-mass spectrometry (LC-MS/MS)

The chromatography was performed on a Waters Acquity UPLC® BEH300 C18 (2.1×50 mm, 1.7 μm,(Waters Corporation, USA) analytical column and maintained at 65 ℃.The mobile phase a consisted of 0.05 % in water and the mobile phase B consisted of 0.1 % FA in MeOH. Samples were separated by gradient elution using the following program: 0-1 min 98 % A; 1-1.5 min 98-90 % A; 1.5-6 min 90-86 % A; 6-7 min 86-15 % A; 7-8 min 15 % A; 8-8.01 min 15-98 % A and 8.5 min stop. The flow rate was set at 0.45 ml/min from 0-6 min and 0.5 ml/min from 6.01-8.5 min. The surrogate peptide's retention time of analyte was 2.65 min.

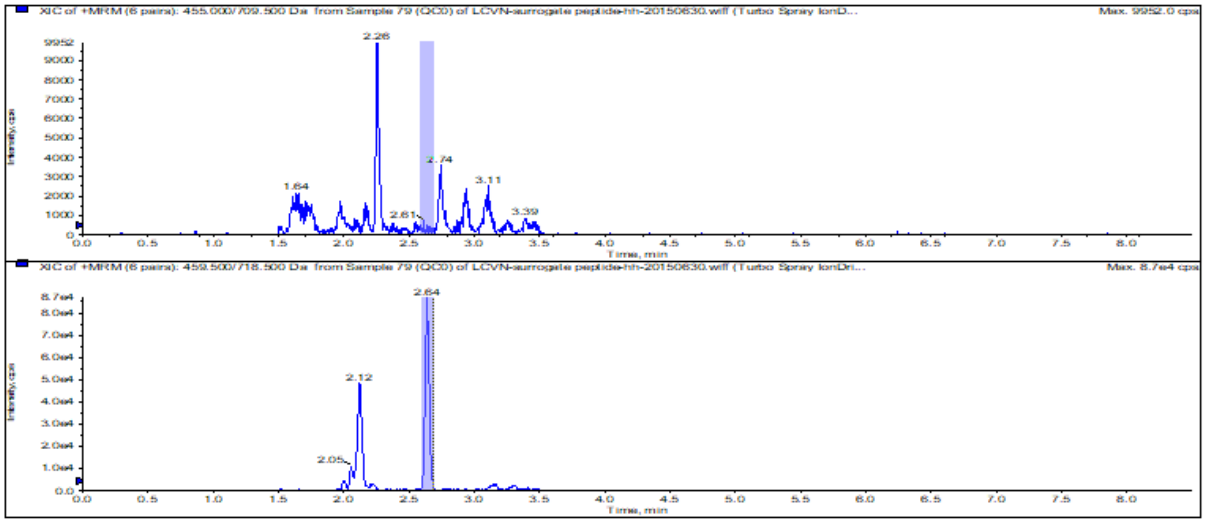
The API 6500 MS system (Applied Biosystems/Sciex, USA), equipped with a heated ESI source, was operated in the positive ion mode with a multiple reaction monitor (MRM) was used for LC-MS/MS analysis. The instrument parameters were optimized as follows: IonSpray Voltage, +5500 V, vaporized temperature and heated capillary temperatures, 550 ℃. CAD 10 unit, and collision energy, 20 eV. The surrogate peptide of analyte is AQQFVSTK, and the internal standard is AQQFVSTK(C13-Phe lable, provided by WuXi Apptec). The following precursor-to-product ion

transitions were used 455.0, 709.5 for the surrogate peptide of analyte, 459.5, 718.5 is for internal standard.

3. Results

3.1 Detection peak of L-CV-N

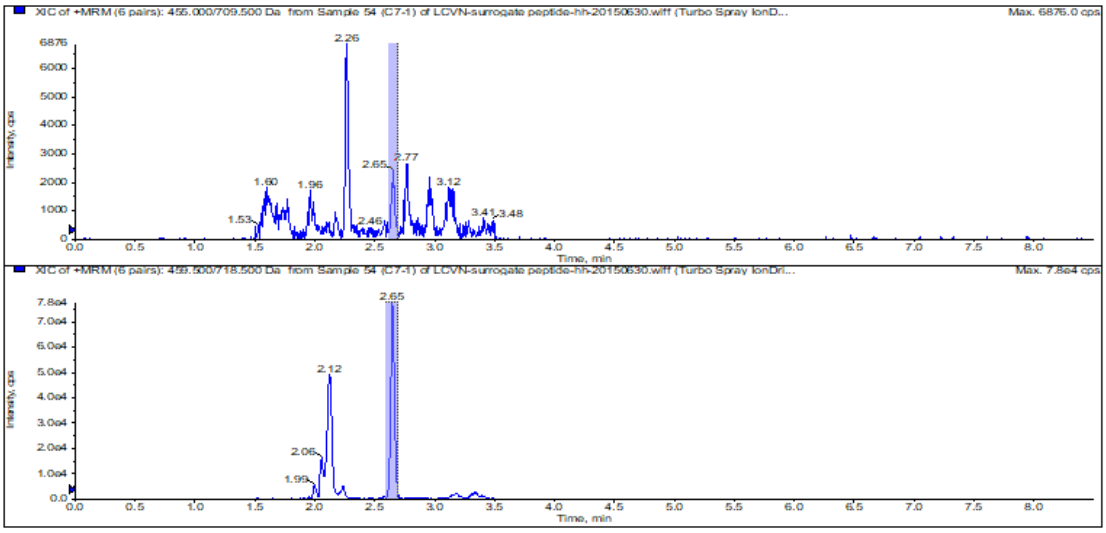
In order to detect the plasma concentration of L-CVN, we use AQQFVSTK as surrogate peptide to examine L-CV-N and AQQFVSTK（C13-Phe label）as the internal standard. As it shows in Fig.1. It was found there was no significant interfering peak at the retention time of analyte and IS (approximately 2.65 min).



Time(min)

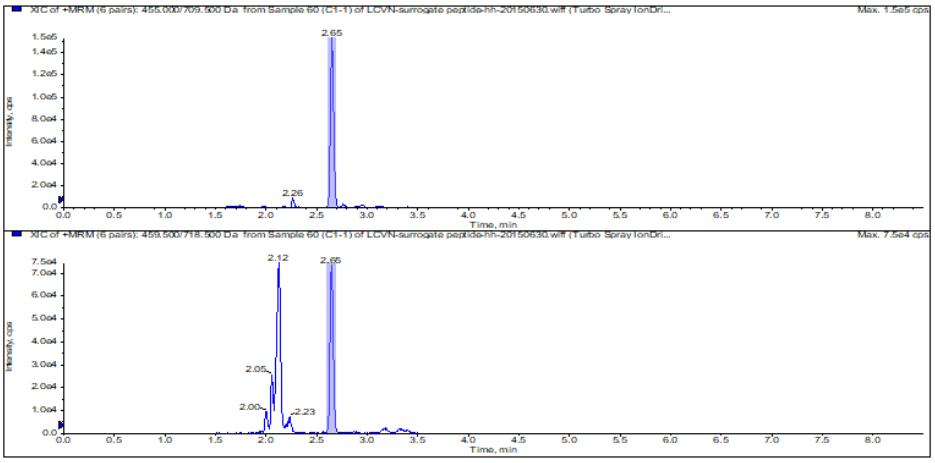
Figure 1. MRM chromatograms of LCVN and IS in blank plasma. The picture above is the MRM of L-CVN and the other is IS.

A



Time(min)

B



Time(min)

Figure 2: MRM chromatograms of LLOQ (A) and ULOQ(B)

3.2 Establishment of a standard curve for radioisotope labeling

The effect of $(GGGGS)_3$ on pharmacokinetics characteristic of LCV-N were measured by LC-MS/MS followed intravenous injection. A standard curve ranging from 10.0-1000.0 ng/ml were firstly established to be y= 0.00363x+0.00244, R2= 0.994. The representative mass chromatograms of blank sample, lower limit of quantification (LLOQ) sample and upper limit of quantification (ULOQ) sample are presented in Fig.2

3.3 Determination of blood concentration

The plasma concentration-time curve after administration of 1.0 mg/kg LCV-N is shown in Fig 3. The area under curve (AUC0-last) was 3925±360 h· ng/ml. The half-life was calculated to be 12.4±3.05 h. Mean values of apparent volume of distribution (Vdss) and clearance (CL) were 2.80±0.608 L/kg and 3.42± 0.455 ml/min/kg, respectively. More importantly, even 24 h after injection, LCV-N still can be detected in plasma.

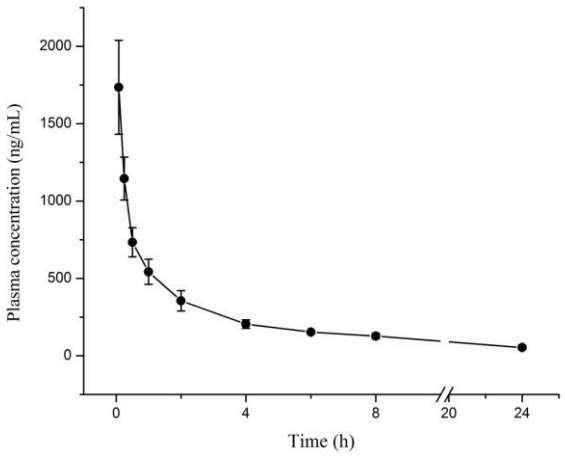


Fig 3. Mean plasma concentration versus time curves of LCVN after intravenous injection in rat for 24 h. Data were (Mean ± SD for four rats). Each rat given a single 1.0 mg/kg dose. Plasma levels of LCVN were measured by LC-MS/MS.

4. Discussion

Immunoassays are the common used methods to study the PK characters of protein drugs like antibodies and recombinant proteins with high sensitivity and accuracy[14]. Based on this theory and other novel technologies, many methods like immunosorbent [14, 15]and radioimmunoassay[16] have been developed. Protein drugs(antigen) specifically combine with their antibodies is the basic requirement of immunoassays. However, these will lead to non-specific binding[17] and it's time-consuming to produce corresponding antibodies[17], and the radioimmunoassay cannot be carry out in clinic. With the development of chromatographic technology, LC-MS/MS has been used to depict the PK characters of protein drug because it's high sensitivity, selectivity and linearity[18, 19]. In this study, after injection with 1.0 mg/kg LCVN in rat through jugular vein, we used LC-MS/MS method to analysis LCVN PK characters by quantifying the surrogate peptide AQQFVSTK with AQQFVSTK (C13-Phe lable) as the internal standard. Although it not carried out on large scale animal experiments, we can also expect it's a good method to analyze LCVN in the complex biological matrix with high sensitivity (10 ng/ml) and without interference by serum and other possible proteins. Our results found that after single dose of 1.0 mg/kg LCVN, it still can be detected in serum (mean concentration is 53.8 ng/ml). The effective anti-viral concentration of LCVN is 2.24nM, with 1.0 mg/kg administration, it can maintain almost 24 h effective anti-viral concentration. It indicated that LCVN can be a potential anti-viral candidate to be used in clinic.

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