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Development and application of a reversed-phase high-performance liquid chromatographic method for quantitation and characterization of a Chikungunya virus-like particle vaccine

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ABSTRACT

To effectively support the development of a Chikungunya (CHIKV) virus-like particle (VLP) vaccine, a sensitive and robust high-performance liquid chromatography (HPLC) method that can quantitate CHIKV VLPs and monitor product purity throughout the manufacturing process is needed. We developed a sensitive reversed-phase HPLC (RP-HPLC) method that separates capsid, E1, and E2 proteins in CHIKV VLP vaccine with good resolution. Each protein component was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectrometry (MS). The post-translational modifications on the viral glycoproteins E1 and E2 were further identified by intact protein mass measurements with liquid chromatography–mass spectrometry (LC–MS). The RP-HPLC method has a linear range of 0.51–12 μg protein, an accuracy of 96–106% and a precision of 12% RSD, suitable for vaccine product release testing. In addition, we demonstrated that the RP-HPLC method is useful for characterizing viral glycoprotein post-translational modifications, monitoring product purity during process development and assessing product stability during formulation development.

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1. Introduction

Chikungunya virus is a mosquito-borne alphavirus. It is an enveloped positive-stranded RNA virus with a diameter of 60–75 nm that causes acute illness including fever, rash and severe arthralgia. Although rarely fatal, CHIKV causes incapacitating and prolonged joint pain that presents serious economic and social impact. Since the first isolation of CHIKV in Tanzania in 1952, sporadic outbreaks occurred in Central and Southern Africa and South East Asia between 1960s and 1990s. Outbreaks resurged between 2004 and 2007 in Reunion Islands, India and Europe with hundreds of thousands of reported cases [1–3]. Due to the disease severity, the high infection rate during outbreaks, and the extensive geographic distribution, there is an urgent need for an effective CHIKV vaccine. In 2011, Nabel et al. reported the expression of CHIKV virus-like particles (VLPs) in human embryonic kidney cells [4]. Vaccination using these CHIKV VLPs protected Rhesus macaques from challenge with wild-type virus. Additionally, serum antibodies from the vaccinated macaques provided protection from a lethal dose of CHIKV in a mouse model. These results established the proof-of-concept that CHIKV VLPs were sufficient to elicit a protective humoral response against CHIKV infection. The success of these experiments warranted further characterization of the CHIKV VLPs.

Traditionally, vaccines composed of VLPs are characterized by SDS-PAGE for purity and quantified by colorimetric protein assays such as Bradford, bicinchoninic acid (BCA) or Lowry assay. Both approaches have disadvantages. SDS-PAGE is labor and time intensive. Colorimetric protein assays can be sensitive to detergents, reducing agents or certain salts. In addition, the colorimetric protein assays measure total protein concentration, and are therefore not specific for the antigenic components of the vaccine product. To effectively support process and formulation development, it is highly desirable to have a sensitive and robust method available that can be automated to measure both vaccine purity and antigen-specific vaccine mass.

High-performance liquid chromatography (HPLC) has become an attractive analytical tool due to its high sensitivity and reproducibility. HPLC methods have been applied for the identification

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and quantitation of virus proteins and VLPs including influenza virus [5–8], lentivirus [9], Sendai virus [10], poliovirus [11,12], human papillomavirus VLP [13], adenovirus types 3 and 5 [14,15], and Hepatitis B VLP [16]. However, due to the hydrophobic nature of most viral glycoproteins and the presence of lipids with enveloped virus, it has been technically challenging to achieve good resolution and recovery for all the viral components [9,10,17].

The CHIKV VLP has three structural proteins and is organized as follows: an outer surface composed of 240 copies of glycoproteins E1 and E2 embedded in a lipid bilayer surrounding a nucleocapsid made of 240 copies of capsid protein [18]. Our goal was to develop a RP-HPLC assay that would separate E1, E2 and capsid proteins of CHIKV VLPs. This method would serve to evaluate and quantitate the mass and purity of the vaccine product. Additionally, this method would be a tool to assess both protein degradation and post-translational modifications for formulation and process development.

2. Materials and methods

2.1. Chemicals and reagents

HPLC grade acetonitrile (ACN), 2-propanol, trifluoroacetic acid (TFA), 0.1% TFA in acetonitrile, and 0.1% TFA in water were purchased from Fisher (Fair Lawn, NJ, USA). Zwittergent 3–12 detergent and C18 ZipTip were from Millipore (Billerica, MA, USA). Trypsin was from Promega (Madison, WI, USA). Formic acid, ammonium bicarbonate (NH_4HCO_3), α-cyano-4-hydrocinnamic acid (CHCA), iodoacetamide were from Sigma-Aldrich (St. Louis, MO, USA). Dithiothreitol (DTT) and GelCode Blue Stain Reagent were from Thermo Scientific (Pittsburgh, PA, USA). SilverQuest Staining Kit was from Invitrogen (Carlsbad, CA, USA). The expression and purification of CHIKV VLPs from the mammalian and the insect cell systems were described by Wagner et al. [19]. Briefly, HEK293 cells were transiently transfected with a plasmid DNA encoding the CHIKV structural genes. The cell culture supernatant was clarified, concentrated and purified with Q Sepharose XL anion exchange column. In the insect cell system, the high-pH adapted *Spodoptera frugiperda* insect cells (*Sf*Basic) were infected by baculovirus encoding the CHIKV structural genes. The culture supernatant was clarified, concentrated and purified with Sephacryl S-400 HR size exclusion column and Q-Sepharose XL anion exchange column.

2.2. RP-HPLC

Samples containing CHIKV VLPs were analyzed on XBridge BEH300 C4 column (3.5 μm, 4.6 × 150 mm, 300 Å, Part # 186004504, from Waters) held at 60 °C with a linear AB gradient elution. Mobile phase A contained 0.1% TFA in water. Mobile phase B contained 30% ACN, 70% 2-propanol and 0.1% TFA. The separation was carried out with a 60-min gradient ranging from 0% to 100% mobile phase B followed by a 7-min re-equilibration with mobile phase A at a flow rate of 1 mL/min. Eluted proteins were detected by fluorescence at excitation at 280 nm and emission at 350 nm. Samples were incubated with 5% Zwittergent 3-12 detergent and injected at a volume of 100 μL. In the linearity study, the reference standard was diluted at 5.1–120 μg/mL in the presence of 5% Zwittergent 3-12 detergent. It was noted that in the early applications, for example studies that monitored the degradation and post-translational modifications, sample pre-treatment was not yet implemented. However, since Zwittergent was used to improve recovery, the lack of Zwittergent should not impact the characteristics of eluted E1 and E2.

2.3. Protein identification: RP-HPLC peak collection, SDS-PAGE, in-gel digestion, and MALDI-ToF MS

CHIKV VLP fractions were collected from RP-HPLC run, dried in a Savant SpeedVac equipped with a cold acetone vapor trap. The fractions were then reconstituted in SDS-PAGE sample loading buffer and analyzed using a NOVEX gel apparatus in NOVEX 4–20% tris-glycine 1.5 mm gels. The gels were stained using either SilverQuest Staining Kit or GelCode Blue Stain Reagent.

For protein identification by MALDI-ToF MS, gels were first stained using GelCode Blue Stain. Protein bands were then excised along with a blank portion of the gel used as a negative control. Gel bands were placed into individual eppendorf tubes and crushed. They were destained by three cycles of dehydration with 25 mM ammonium bicarbonate (NH_4HCO_3)/50% ACN (v/v) and rehydration with 25 mM NH_4HCO_3. Destained gel pieces were dried in a Savant SpeedVac, reduced in 10 mM DTT/25 mM NH_4HCO_3 at 55 °C for 1 h, and alkylated with 55 mM iodoacetamide/25 mM NH_4HCO_3 at room temperature for 45 min in the dark. Gel pieces were washed with 25 mH NH_4HCO_3 and dried in a Savant SpeedVac. The pieces were incubated in a solution of high purity trypsin (5 ng/μL in 25 mM NH_4HCO_3) for 16 h at 37 °C. The resulting tryptic peptides were eluted from the gel pieces by vortexing with a solution of 5% formic acid/50% ACN (v/v). The extracted peptides were prepared for analyses by MALDI-ToF MS with C18 ZipTips per the manufacturer's instructions. Peptides were eluted from the ZipTips with 1 μL of matrix consisting of 8 mg/mL of CHCA in 0.05% TFA/50% ACN (v/v) and spotted onto a MALDI target. Samples were analyzed on a Bruker Autoflex III operated in reflector mode and spectra were acquired in positive ion mode in an *m/z* range of 700–4000 using a laser power of 76%. Proteins were identified by comparing observed masses to theoretical tryptic masses of the E1, E2, and capsid proteins.

2.4. Characterization of post-translational modifications: Intact protein mass analysis by LC–MS

The proteins in the VLP samples were separated and analyzed by LC–MS using a Waters Acquity LC system coupled to a Synapt G2 mass spectrometer (Waters, Milford, MA). The mobile phases were slightly modified as stated below. Mobile phase A was 0.1% formic acid (v/v) in water and mobile phase B was 0.1% formic acid (v/v) in 30% ACN/70% isopropanol. Because TFA caused ion suppression, formic acid was used as the mobile phase modifier. In order to increase the signal intensity for intact protein accurate mass measurement, we used 10 consecutive injections of 25 μL of the sample. These injections were made with a short 2 min isocratic flow of 5% mobile B. Following the last injection, the proteins were eluted using a linear gradient of 5–80% of mobile phase B in 18 min at a flow rate of 0.2 mL/min. Mass spectra were obtained in positive mode by spraying the eluent into the mass spectrometer using an ESI source. The capillary, source cone, and extraction cone voltages were set at 3 kV, 20 V, and 4 V, respectively. Nitrogen was used as a desolvation gas at a flow rate of 800 L/h. The source and desolvation temperatures were set at 110 and 450 °C, respectively. The instrument was operated in Sensitivity mode and spectra were acquired in an *m/z* range of 1000–2500. Data acquisition and analysis (deconvolution) were performed with Waters MassLynx 4.1 software. Protein spectra were deconvoluted to obtain the observed intact protein masses. MaxEnt deconvolution parameters were set with output mass range of 40,000–60,000 and resolution of 0.1 Da/channel. Minimum intensity ratios were 33% for both the left and right parameters. A uniform Gaussian model was used with width at half height of either 1 or 0.8 Da. For spectra with width at half height of 1 Da, a maximum of 10 iterations were used. For spectra with width at half height of 0.8 Da, a maximum of