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Highly selective and sensitive LC-MS/MS quantification of a therapeutic protein in human serum using immunoaffinity capture enrichment

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ABSTRACT

We report the development, validation and application of a liquid chromatography-tandem mass spectrometry (LC-MS/MS) bioanalytical method for the determination of a recombinant protein drug candidate, NVS001, in human serum. A unique surrogate peptide, IPAETTIYNR (IPA), was identified to distinguish NVS001 from its endogenous counterpart, i.e., the full length and the C-terminal of protein X in LC-MS/MS. The selection of IPA for the LC-MS/MS determination of NVS001 was supported by the absence of peak responses due to endogenous components in the LC-MS/MS chromatograms of the extracted blank human serum samples. The optimal chromatographic separation of IPA from the extracted matrix components was achieved on a Waters Cortecs C_{18} (100 × 2.1 mm, 2.7 µm) column using gradient elution with a run cycle time of approximately 7.5 min. The mobile phases were water containing 0.1% formic acid (mobile phase A) and acetonitrile containing 0.1% formic acid (mobile phase B). The method was validated for specificity, sensitivity, matrix effect, recovery, linearity, accuracy and precision, dilution integrity, and stability. The validated assay dynamic range was 10.0 to 1000 ng/mL using a 50 µL sample volume. The accuracy and precision for the LLOQ (10.0 ng/mL) sample results were within ± 9.2% bias and ≤6.0% CV, respectively. From the intra-day and inter-day assay performance evaluations, the precision of the QC sample (30, 500 and 750 ng/mL) results were ≤3.5% CV and the accuracy within ± 3.3% bias, respectively. An additional assessment of incurred sample reanalysis (ISR) was conducted to demonstrate the ruggedness and robustness of the assay method. The validated method was successfully implemented in support of a first-in-human study.

1. Introduction

Protein therapeutics has been expanding exponentially in the past a few decades for the treatment of various diseases such as cancers, immune disorders and infections. The success of protein therapeutics is largely due to the high specificity and minimal adverse effects of the therapies [1,2]. Currently, > 130 therapeutic proteins have been approved for clinical use by the US FDA [3]. The continuous growth of protein therapeutics in the pharmaceutical industry creates a strong demand for a robust bioanalytical platform to support the development of the therapeutics, particularly in the clinical stage of the drug development programs.

Ligand binding assays (LBA) have been considered the gold standard for the quantification of various therapeutic proteins in biological fluids for many years [4,5]. However, the performance of LBA is largely limited by the availability of specific reagent(s) and assay sensitivity is often subjected to experimental conditions such as sample dilution, incubation time and soluble target levels [6,7]. Therefore, developing a reliable LBA method is often difficult and time consuming. In contrast, liquid chromatography with tandem mass spectrometry features high specificity, wide dynamic range, short method development time and multiplexing capabilities [8–10]. Lately, the technique has been increasingly utilized as an important alternative to LBA for quantitative bioanalysis of therapeutic proteins in various biological matrices [2,3]. Differing from LBA whose specificity relies on the specific binding of the ligand (target molecules) to the receptors (capture reagents), LC-MS/MS quantification of proteins in biological samples commonly involves enzymatic digestion of the extracted proteins, followed by the measurement of one or more surrogate peptide(s) from the digested samples. The assay specificity is achieved by (1) chromatographic separation of the selected surrogate peptide(s) from unwanted matrix components and (2) tandem mass spectrometric detection of the selected surrogate peptides in selected reaction monitoring (SRM) mode.

Numerous peptides are produced from enzymatic digestion of the extracted proteins; therefore, selection of suitable surrogate peptide becomes a critical step in LC-MS/MS bioanalysis of proteins [9]. In

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practice, the selected peptide(s) need to be (1) unique and specific to the target proteins to avoid interference from endogenous background; (2) formed rapidly and reproducibly by the enzymatic digestion procedure, (3) stable throughout the entire sample preparation and analysis procedure; (4) retainable by LC column and (5) easily ionized during MS/MS analysis [3]. In addition to selection of surrogate peptide, selection of suitable sample preparation workflow is another important aspect as different workflows are often associated with different achievable assay sensitivity. Direct digestion of plasma/serum samples after reduction and alkylation [11] and pellet digestion [12,13] are often employed in the LC-MS/MS of proteins with LLOQ at μg/mL level. However, this sensitivity is not sufficient for many protein analytes with systemic concentration in ng/mL range. In practice, in order to quantify a therapeutic protein by LC-MS/MS with a LLOQ at ng/mL level or lower in biological samples, a pre-concentration or enrichment step has to be employed. A common approach to enriching the protein analytes of interest is immunoaffinity capture [9]. Immunoaffinity capture utilizes a specific antibody that is immobilized to a solid support, such as magnetic particles, to exclusively extract the desired protein analytes while unwanted matrix components are washed away. Upon denaturation, reduction, alkylation, and digestion on the magnetic particles, the procedure leads to generation of much cleaner sample extracts as compared to those obtained from direct digestion or pellet digestion. Immunoaffinity capture has become the most widely used method for quantitative LC-MS bioanalysis of therapeutic proteins at low concentration levels.

NVS001 is a protein drug candidate currently being investigated by Novartis. This recombinant protein is engineered from an endogenous protein, protein X, which is found in human serum at approximately 200 ng/mL and can be naturally cleaved in vivo into N-terminal and C-terminal portions with molecular weight of ~25 kDa for each. NVS001 shares a high similarity to the C-terminal of protein X with an exception of the following: (1) the lysine at positon 423 of the amino acid sequence is substituted by glutamine and (2) there are 10 amino acids less than the C-terminal of protein X in the amino acid sequence. Due to such a high similarity, various LBA approaches that had been tested were found not specific enough, as the capture antibody failed to differentiate NVS001 from the C-terminal or the full length protein (protein X) in human serum. The many failed attempts (details not shown) led the focus of bioanalytical method development on LC-MS/MS.

In the present work, we report the development and validation of an LC-MS/MS method for the quantitative bioanalysis of NVS001 in human serum. To achieve the needed assay specificity which was also associated with assay sensitivity, a unique surrogate peptide IPAETTIYNR (abbreviated as IPA) was identified and incorporated into the assay. To ensure the needed assay sensitivity, an immunoaffinity capture procedure using an anti C-terminal antibody as capture reagent was employed for the enrichment of NVS001 from the study samples. Upon enzymatic digestion, LC-MS/MS with multiple-reaction monitoring (MRM) was carried out with a stable isotope labeled IPA as the internal standard. The current method was not only specific with almost no background noise observed in the extracted blank human serum samples, but also sensitive with a validated LLOQ of 10 ng/mL using a sample volume of 50 μL. The assay specificity was further supported by the simultaneous monitoring of some additional surrogate peptides that corresponded to the endogenous full length, N-terminal and C-terminal of protein X. The method was fully validated for a dynamic range of 10 to 1000 ng/mL along with dilution integrity and stability and successfully implemented in support of the First-in-Human (FIH) study.

2. Materials and methods

2.1. Materials

The drug candidate, NVS001 (purity 100%), was obtained from Novartis Pharma AG (Basel, Switzerland). The mouse anti C-terminal antibody was obtained from Novartis Biologics Center. The stable isotope-labeled surrogate peptide internal standard, IPAE(Cam)TTIYNR ($^{13}C_6{}^{15}N_4$) (purity > 95%), and non-labeled surrogate peptide, IPAE (Cam)TTIYNR, were synthesized by New England Peptide (MA, USA). Sequencing grade modified trypsin was purchased from Promega Corporation (WI, USA). HPLC grade acetonitrile, isopropanol and bovine serum albumin (BSA) were purchased from Sigma (MO, USA). Formic acid was purchased from Fisher Scientific (NH, USA). Phosphate buffered saline (PBS) was purchased from Coring (VA, USA). Ammonium bicarbonate and urea were purchased from JT Baker (PA, USA). Dithiothreito (DTT), iodoacetamide (IAA) and streptavidin magnetic beads were purchased from Pierce (TX, USA). Tween-20 and Sulfo-NHS-LC-Biotinylation kit were purchased from Thermo Fisher (MA, USA). Human serum was obtained from Bioreclamation, Inc. (NY, USA). Deionized water was generated in-house using an ELGA PureLab Ultra Water System (MA, USA).

2.2. Matrix blanks, calibration standards and quality controls

NVS001 stock solutions were prepared at a final concentration of 10.0 mg/mL with deionized water. The solution was stored at ≤ −60 °C in small aliquots for long term use. The pooled human serum was pre-spiked with the drug candidate at a final concentration of 10 μg/mL as a starting stock solution (intermediate). Thereafter, calibration standards and QC samples were prepared by serial dilution of the 10 μg/mL intermediate into blank human serum. Eight non-zero calibration standards were prepared at the concentrations of 10.0, 20.0, 50.0, 100, 200, 400, 800 and 1000 ng/mL, respectively. QCs were prepared at concentrations of 10.0, 30.0, 500, and 750 ng/mL. Dilution QCs were prepared at 5000 ng/mL. After preparation, the standards and QCs were stored at ≤ −60 °C before use.

2.3. Biotinylation

Biotinylation of the anti C-terminal antibody was performed using EZ-Link™ Sulfo-NHS-LC-Biotin with approximately 20:1 molar ratio of biotin to the capture antibody. A 25 μL aliquot of 10 mM Sulfo-NHS-LC-Biotin solution was added into 2 mL of 0.9 mg/mL capture antibody. After incubation at room temperature for approximately 1 h and buffer exchange on wet ice, the biotinylated antibody was then purified using Zeba™ spin desalting columns via centrifugation at 1000 × *g* for 2 min.

2.4. Immunoaffinity capture and trypsin digestion

An aliquot of 5 mL of streptavidin magnetic beads (10 mg/mL) were washed once with PBST buffer (PBS with 0.1% Tween-20) and re-suspended into 25 mL of PBST. A 0.200 mL aliquot of biotinylated anti C-terminal antibody (0.9 mg/mL) was immobilized to the washed bead suspension. The mixture was incubated on a rotator overnight at 4 °C. The beads were then washed twice with 25 mL of PBST buffer to remove unbound antibody and re-suspended into 25 mL of PBST with 1% BSA.

A 50 μL aliquot of calibration standards, QCs, and unknown samples was transferred to assigned well of a Protein Lo Bind 1-mL 96-well plate (Eppendorf, NY, USA). A 150 μL aliquot of the magnetic bead suspension was added to each well of the plate. The mixture was then incubated for 2 h at ambient temperature in a Thermo-Mixer (Eppendorf, Hamburg, Germany) at 950 RPM. The beads were subsequently washed with PBST three times and PBS twice using Tomtec Quadra 4 automated liquid handler (Tomtec, Hamden, CT, USA) with a magnetic plate stand. After the last washing step, the residual PBS buffer was removed. A 50 μL aliquot of freshly prepared reducing-denaturing buffer (8 mM DTT, 10 M urea in 50 mM ammonium bicarbonate) was added to each well of the plate, followed by incubation for 45 min at 37 °C in a Thermo-Mixer at 950 RPM. A 10 μL aliquot of freshly prepared alkylation buffer (135 mM IAA in 50 mM ammonium bicarbonate) was added