Journal of Chromatography A, 1334 (2014) 64–71

Contents lists available at ScienceDirect

Journal of Chromatography A

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

A versatile method for protein-based antigen bioanalysis in non-clinical pharmacokinetics studies of a human monoclonal antibody drug by an immunoaffinity liquid chromatography–tandem mass spectrometry

Ichio Onami*, Miho Ayabe, Naoaki Murao, Masaki Ishigai

Fuji-Gotemba Research Labs. Chugai Pharmaceutical Company, Ltd., 1-135 Komakado, Gotemba, Shizuoka 412-8513, Japan

ARTICLE INFO

Article history:
Received 9 December 2013
Received in revised form 23 January 2014
Accepted 31 January 2014
Available online 7 February 2014

Keywords:
Receptor activator of nuclear factor-κB ligand (RANKL)
LC–MS/MS
Immunoaffinity
Immune complex

ABSTRACT

A versatile immunoaffinity liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed to quantify the total concentration of a protein-based antigen in non-clinical pharmacokinetics (PK) studies of a human monoclonal antibody drug. The method combines using magnetic beads that have been coated with a commercial anti-human Fc region antibody to capture an immune complex of the antigen and antibody drug, with subsequent digestion and quantification of the antigen-derived tryptic peptide via LC–MS/MS. Although a typical immunoassay or an immunoaffinity LC–MS/MS assay requires an antigen-specific antibody that uses a different epitope from the antibody drug, this method requires only a commercial anti-human Fc region antibody. The method was applied to quantify total receptor activator of nuclear factor-κB ligand (RANKL) in the presence of denosumab, a humanized monoclonal antibody (mAb) specific to RANKL. The assay was validated as fit-for-purpose and found to be accurate (<115% interbatch accuracies) and precise (<15%, interbatch coefficient of variation) across a range of 3.13–200 ng/mL RANKL. Commercial enzyme-linked immunosorbent assay (ELISA) kit was not able to determine the total RANKL because interference by denosumab decreased recovery. In contrast, the antibody drug had less effect on the LC–MS/MS method. The method now provides a bioanalytical platform for developing other protein-based antigen assays in the early drug stage.

1. Introduction

Monoclonal antibody (mAb)-based therapeutic agents play a central role in the treatment of many human diseases [1,2]. The increasing number of mAbs in the non-clinical development stage represents a tough challenge of rapidly establishing a suitable bioanalytical method. A reliable method of determining mAb and its target molecule in plasma or serum is crucial for assessing the pharmacokinetic (PK)/pharmacodynamic (PD) relationship, especially in the non-clinical development stage, when mAb concentrations are used to understand PK in non-clinical species. In addition, interpreting the target molecule concentration is important for understanding the dynamics of the mAb to the target molecule, which leads to the model-based determination of dose and regimen [3]. PK/PD models can be built on measurement of free and/or total antigen levels as well as free and/or total mAb levels. However, in many cases, because free antigen concentration is so low and may vary with assay conditions, quantification may be unreliable. For that reason, free antigen concentrations used in PK/PD models are often calculated using total antigen concentrations and in vitro data such as equilibrium dissociation constant (K_D) [4]. Therefore, reliable total antigen concentrations are necessary. In addition, recent antibody engineering has created mAbs that have improved PK, longer target molecule neutralization, and target molecule sweeping activity [5–7]. This has led to a growing further need to quantify the total antigen concentrations after an administration of mAb. These target molecules include those such as TNFα and RANKL that are multivalent and form an oligomeric structure that presents multiple antigenic sites for interaction with more than one mAb at the same time. These multivalent antigens and mAbs form immune complexes with different degrees of size and configuration at varying antigen–antibody molar ratios or temperatures [8,9]. Therefore, quantification of the total multivalent antigens is a tough challenge.

The immunoassay, such as the enzyme-linked immunosorbent assay (ELISA), is a well-established platform for measuring protein-based antigens because of their ease of use, high sensitivity, and high throughput. However, using an immunoassay to find the total target molecule after administration of a drug candidate typically

* Corresponding author. Tel.: +81 550 87 6377; fax: +81 550 87 5326.
E-mail address: ohnami.ichio39@chugai-pharm.co.jp (I. Onami).

0021-9673/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.chroma.2014.01.082

requires two reagent antibodies that bind to epitopes other than the therapeutic antibody's epitope and that are different from each other. These reagents can be difficult to generate, especially for protein-based antigens in the early non-clinical stage that may possess fewer discrete epitopes [4].

In recent years, liquid chromatography–tandem mass spectrometry (LC–MS/MS) has evolved as a promising and complementary technique to quantify mAb therapeutics, biomarkers, and antigens in complex biological matrices, such as serum and plasma [10–13]. Multiple reaction monitoring (MRM), which quantifies protein by monitoring the appropriate surrogate peptides that originate from the protein, is gaining in popularity [14–19]. MRM experiments are characterized by high specificity, high sensitivity, and a wide dynamic range, and they offer the potential for multiplexed detection and quantification of various proteins. To improve assay sensitivity, the sample complexity can be reduced by protein-based immunoaffinity enrichment of a target molecule using target-specific antibodies [20–22], and peptide-based immunoaffinity enrichment of a surrogate peptide after digestion using surrogate peptide-specific antibodies [21,23–25]. This type of hybrid assay, combining MRM detection with immunoaffinity enrichment, provides high sensitivity and specificity for protein quantification. However, these immunoaffinity enrichment techniques require many resources and much time to prepare the target molecule-specific capture antibodies that bind to epitopes different from those in the therapeutic mAb. Preparing capture antibodies against multivalent antigens is rendered even more difficult by steric hindrance and epitope overlapping. Although the many reports noted above discuss protein quantification, there are only a few reports about using LC–MS/MS to measure antigen concentration profiles after administering a drug candidate. Recently, Neubert et al. [25] developed a method of quantifying total nerve growth factor (NGF) in the presence of the humanized mAb tanezumab, which is specific to NGF, by an immunoaffinity LC–MS/MS. Although this method employed a polyclonal antibody specific to NGF to prevent interference from tanezumab, the data showed a decrease in the recovery of NGF as tanezumab concentration was raised, a result of the antibody drug effect.

We present here the technical development of an analytical method of quantifying antigen using LC–MS/MS coupled with an immunoaffinity method in which magnetic beads (MB) are coated with a commercial anti-human Fc region antibody to capture antigen–antibody immune complex in mouse plasma. As model proteins of the target molecule and the antibody drug, we chose receptor activator of nuclear factor-κB ligand (RANKL) and denosumab: RANKL, a TNF family member, assembles into a homotrimer in either membrane-bound or soluble form [26–28]; denosumab, a fully human mAb that inhibits osteoclastic-mediated bone resorption by binding to osteoblast-produced RANKL, has been clinically used [29]. This quantification method requires only a mAb therapeutic and a commercial antibody, making it unnecessary to prepare target molecule-specific antibodies that recognize different epitopes from the mAb therapeutic. Furthermore, the method developed in this study has the potential to quantify any antigen for which immunoassays are not readily available. The method has been validated as fit-for-purpose and the limit of quantification of RANKL is low ng/mL in mouse plasma. Results from an in vivo study are compared with those obtained by ELISA.

2. Experimental

2.1. Materials

Recombinant human RANKL was purchased from Cell Signaling Technology (Boston, MA, USA; >98% by SDS-PAGE; concentration verified by manufacturer). The RANKL was confirmed to be a trimer by size exclusion chromatography (Please see supporting material in Appendix A for details). Recombinant human osteoprotegerin (OPG) was purchased from Sino Biological Inc. (Beijing, China; >97% by SDS-PAGE; concentration verified by manufacturer). Denosumab was purchased from Daiichi Sankyo (Tokyo, Japan). MS300 low carboxyl MB were purchased from JSR Life Science (Tokyo, Japan). Lysozyme (chicken egg white), anti-human IgG (γ-chain specific), $F(ab')^2$ fragment antibody produced in goat, iodoacetamide (IAA), phosphate buffered saline (PBS), PBS with Tween 20, and bovine serum albumin (BSA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dithiothreitol (DTT), ammonium bicarbonate, and urea were purchased from Wako Pure Chemical Industries, Ltd (Tokyo, Japan). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Sequencing-grade trypsin was obtained from Promega (Madison, WI, USA). Mouse plasma was purchased from Charles River Laboratories (Kanagawa, Japan). Human RANKL ELISA kit was obtained from BioVendor Laboratory Medicine, Inc. (Brno, Czech Republic) and used according to the manufacturer's instructions. All other chemicals and solvents were of analytical reagent grade.

2.2. Cross-linking of anti-human Fc region antibody to MB

A 1-mL aliquot of MS300 low carboxyl MB was washed once with 1 mL of 0.1 M MES buffer (pH 5.0). Anti-human IgG (γ-chain-specific), $F(ab')^2$ fragment antibody (100 μg) in 900 μL of 0.1 M MES buffer (pH 5.0) and 100 μL of 10 mg/mL EDC in MES buffer (pH 5.0) were incubated with MB for 3 h at room temperature (RT). Subsequently, MB coupled with antibody were washed four times with 800 μL of 10 mM PBS/0.05% Tween 20. The antibody-linked beads were stored at 4 °C in 2 mL of 10 mM PBS containing 0.05% Tween 20.

2.3. Sample processing and preparation of RANKL digests for LC–MS/MS

The sample processing and MB separation were performed in a 96-well plate format. MB separation was achieved using a DynaMag 96 side magnet (Invitrogen, Oslo, Norway). For washing and incubation steps, the plate was removed from the magnet.

Calibrants were prepared by diluting the recombinant human RANKL in mouse plasma, resulting in concentrations of 3.13, 6.25, 12.5, 25.0, 50.0, 100, and 200 ng/mL. Quality control (QC) samples were prepared in mouse plasma in a similar manner, resulting in concentrations of 3.13 ng/mL (lower limit of quantitation [LLOQ]), 6.25 ng/mL (low), 50.0 ng/mL (medium), and 200 ng/mL (high). An aliquot of 10 μL of plasma, calibrants, and QC samples was added to a polypropylene 96-well plate (MBBio, Tokyo, Japan), followed by adding 40 μL of 50 μg/mL denosumab in 1% BSA in PBS. The plate was sealed and shaken for 2 h at RT to form antigen–antibody immune complexes. A 50-μL aliquot of the functionalized MB was added to each well of the sample plate and incubated for 2 h at RT to capture antigen–antibody immune complexes. The beads were washed three times with 200 μL of 10 mM PBS containing 0.05% Tween 20 and once with 200 μL of PBS. After 25 μL of 100 ng/mL lysozyme as internal standard (IS) in 50 mM ammonium bicarbonate/7.5 M urea/8 mM DTT was added to each well, the plate was sealed and incubated for 45 min at 56 °C. Then 2 μL of 500 mM IAA in 50 mM ammonium bicarbonate was added, and the plate was sealed and incubated for 30 min at 37 °C in darkness. Subsequently, 160 μL of 625 ng/mL trypsin in 50 mM ammonium bicarbonate was added and the plate was sealed and incubated at 37 °C overnight. Finally, the supernatant in each well was transferred to a deep well plate (Waters Corp., Milford, MA, USA) followed by adding 10%