Journal of Pharmaceutical and Biomedical Analysis 159 (2018) 384–392

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Investigating the utility of minimized sample preparation and high-resolution mass spectrometry for quantification of monoclonal antibody drugs

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ARTICLE INFO

Article history:
Received 15 March 2018
Received in revised form 18 June 2018
Accepted 11 July 2018

Keywords:
Pharmacokinetic
Therapeutic monoclonal antibody
High resolution
Mass spectrometry
Bioanalysis

ABSTRACT

Determination of the pharmacokinetic (PK) properties of therapeutic monoclonal antibodies (mAbs) is essential for their successful development as drugs. For this purpose, besides the traditional ligand binding assay (LBA), LC–MS/MS method using low resolution mass spectrometers (*e.g.* triple quadrupole (QqQ)) has become routinely used, however, complicated and lengthy sample pre-treatment (employing immuno-affinity) is often necessary for obtaining sufficient sensitivity and selectivity. In this study, we investigate the capabilities of high-resolution MS instruments for circumventing the complex sample preparation currently needed for sensitive LC–MS/MS-based quantification of mAbs. Employing a simple one-step sample pre-treatment workflow, we compare the ability of three different LC–MS platforms for absolute quantification of a representative monoclonal antibody Rendomab-B1 in serum and plasma. The samples are subjected to protein precipitation with methanol, followed by pellet digestion with trypsin prior to LC–MS analysis. AQUA peptides based on two surrogate mAb peptides selected from an extensive *in-silico* and experimental screening are used as internal standards. MS/MS acquisitions are developed and systematically examined for 1) a low-resolution QqQ operated in selected reaction monitoring (SRM) acquisition mode, 2) a high-resolution hybrid Quadrupole-Orbitrap (Q-Orbitrap) operated in parallel reaction monitoring (PRM) acquisition mode and 3) a high-resolution hybrid Quadrupole-Time-of-flight (Q-TOF) operated in SRM acquisition mode with enhanced duty cycle (EDC) function. The sensitivity of the high-resolution Q-Orbitrap and Q-TOF methods was significantly higher (LOD of 80 ng/mL) in serum/plasma samples than the low-resolution QqQ method. Finally, the real-world utility of the developed high-resolution MS method with minimized sample handling was demonstrated and validated by determining the PK profile of Rendomab-B1 in mice by a 10-point *in vivo* study over 15 days.
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1. Introduction

Therapeutic monoclonal antibodies (mAbs) have facilitated considerable advances in the treatment of several major diseases including autoimmune, cardiovascular, infectious diseases, inflammation and cancer [1]. The therapeutic success of mAbs is due to an unusually long serum half-life combined with the ability to target and interfere with the function of a specific biological target. Moreover, humanized or chimeric mAbs have a similar sequence to human mAbs, in the whole sequence or in the Fc region, respectively, minimizing the risk of unwanted immunogenicity [2,3]. This similarity however poses several challenges in regards to the selective analysis of therapeutic mAbs in biological samples that also contain endogenous antibodies. Precise and sensitive quantification of active drug substances in biological samples is essential for the determination of drugs pharmacokinetic and pharmacodynamic properties, and a critical step for successful drug development.

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https://doi.org/10.1016/j.jpba.2018.07.012
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The current state-of-the-art technique for quantification of mAbs in biological samples; ligand-binding assays (LBA), has some limitations regarding selectivity, and a long development time (commonly 6–8 months) [4,5]. In recent years, liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) have shown great potential in obtaining specific and accurate measurements of mAbs in complex biological samples [5–9] LC–MS based methods can have shorter development times (weeks), wider linear dynamic ranges, higher specificity and provide options for monitoring degradation/modifications of the mAb [10]. LC–MS quantification of a mAb typically involves an enzymatic digestion step to produce a mixture of surrogate or signature peptides, following one or several appropriate sample clean-up strategies, *e.g.* immune-capture, protein depletion, solid phase extraction (SPE), 2D-SPE and protein co-precipitation [11]. The large majority of routine quantitative LC–MS assays for proteins and mAbs typically uses a LC system coupled to the low-resolution triple quadrupoles (QqQ) [8,12,13]. Such routine LC–MS assays often provide a low selectivity for clinical samples due to the complexity of the biological matrices, especially serum and plasma. A few recent studies have reported the use of quick sample-treatment procedures for LC–MS quantification of mAbs in plasma by utilizing affinity capture *via* streptavidin beads [14,15] or protein A [16,17] or G [18] beads followed by enzymatic digestion such as trypsin [14,16,18], papain and Lys-C [15] or immobilized trypsin and Lys-C on nanoparticles [17]. However, such procedures could still confront challenges concerning endogenous antibodies as they are just able to partly clean up the matrix. High resolution mass spectrometry (HRMS), for instance, hybrid quadrupole-Orbitrap or Time-of-flight mass (TOF) spectrometers, have recently emerged with the potential to perform selective and sensitive quantitative analyses of protein-based drugs [19,20]. The major advantage of HRMS over the conventional low-resolution MS is the ability to differentiate target protein-derived peptide analytes of very similar but not identical mass or structure due to a high mass resolution ranging from 25,000 to 140,000. These advances of HRMS appear ideally suitable for achieving sensitive and "direct" quantitative analysis of therapeutic mAbs in serum or blood samples from preclinical or clinical species without the need of intensive sample pre-treatment steps such as target specific immuno-affinity enrichment.

Here we have developed a direct LC–MS/MS workflow using a simple one-step sample pre-treatment for absolute quantification of a model therapeutic monoclonal antibody (Rendomab-B1, a mouse monoclonal antibody that targets the human endothelin B receptor (hEtBR)) [21] in serum and plasma samples. To investigate the capabilities of HRMS for sensitive quantification of mAbs using this workflow, three different LC–MS-platforms including low-resolution triple-Quadrupole (QqQ), high resolution Q-Orbitrap (Q-OT) and Q-Time of-flight instruments (Q-TOF) are used and systematically compared in term of analytical performance. The real-world utility of the developed quantitative high-resolution MS workflow is subsequently utilized for an *in vivo* Pharmacokinetic (PK) study of 29 mice from 0 to 15 days after administration of Rendomab-B1.

2. Experimental

2.1. Chemical and reagents

Monoclonal antibody Rendomab-B1 and single chain variable fragment (scFv-B1) were generated in-house using protocols described in Allard et al. 2013 [21] and Ortega et al. 2013 [22], respectively. ^{15}N-scFv was produced using the same protocol with scFv-B1 [22] except that a minimum media with labeled ammonium chloride as exclusive nitrogen source (15 N, 99%, Cambridge Isotope Laboratories Inc., Andover MA, USA) was used as growth media. scFv functionality was tested using protocols published by Martin et al. 2016 [23].

Mouse plasma and *in vivo* samples from individual mice were also supplied in-house. HeavyPeptide (Absolute QUAntitation, stable isotope labeled (SIL)-peptides) AQUA Ultimate was from Thermo Fisher Scientific (Germany). Mouse CD1 Serum and plasma, pool of donor mice were obtained from Biopredic International (Saint-Gregoire, France). Trypsin (bovine pancreas -Type I and sequencing grade modified), bovine serum albumin (BSA), ammonium bicarbonate, formic acid (FA), perchloric acid, iodoacetamide (IAA), dithiothreitol (DTT), methanol (MeOH) and acetonitrile (ACN) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals and reagents were of at least analytical grade.

2.2. Instrumentation

The three following liquid chromatography coupled to mass spectrometry (LC–MS) setups were used in this study.

LC–MS 1 was an Agilent 1100 Series HPLC System (Agilent, Santa Clara, CA) coupled to a TSQ Quantum Ultra Triple Quadrupole MS (Thermo Scientific, San Jose, CA).

LC–MS 2 was a Dionex Ultimate 3000 UHPLC System coupled to a Thermo Q-Exactive hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA).

LC–MS 3 was a Waters NanoAQUITY UPLC coupled to a Synapt G2 Q-TOF High definition mass spectrometer (Waters, Wilmslow, UK).

LC–MS method parameters for all three setups used for quantification were optimized and summarized in Table 1.

2.3. Selection of signature peptides

2.3.1. Peptide mapping

5.0 μL of stock solution containing 920 μg/mL of Rendomab-B1 mAb was diluted 20 times by 50 mM ammonium bicarbonate (AB50). 40 μL of the solution was transferred to a new tube and pH was checked to be 8 with pH paper. The samples were reduced by adding 10 μL of 45 mM DTT (55 °C, 25 min) followed by alkylation with 10 μL of 100 mM IAA (RT, 35 min). 5 μL of 0.5 μg/μL trypsin was then added to 40 μL of the sample (37 °C, overnight). Finally, 5 μL of 10% FA (in 50:50 MeOH:H_2O) was added to quench the proteolytic activity of trypsin. The final concentration of mAb was 30 μg/mL.

Liquid chromatography was performed on the LC–MS2 system described above, with a 20 min linear gradient from 5% to 60% B solvent containing 0.1% formic acid in acetonitrile at 200 μL/min. The mass spectrometric analysis was performed in a two-part method, where each cycle had a run time of ~1000 ms. Part a) Full scan analysis with 100 ms acquisition at 70,000 resolution. Part b) The five most intense peaks in the full scan spectra were automatically selected for five subsequent MS/MS runs with a normalized collision energy (NCE) of 20% a dynamic exclusion of 30 s. An exclusion of peaks with unassigned charge states or single charges was chosen. The experiment was performed in duplicate, and only peptides observed in both experiments was included.

2.3.2. Data analysis

Identification of peptides was performed manually using the Xcalibur software (Thermo Scientific). ExPASy PeptideMass (SIB, Lausanne, CH) was used for *in silico* digestion of Rendomab-B1 mAb. The identified peptides were then confirmed by the presence of two or more b/y ions in the MS/MS acquisition with the use of MS-Product (a part of ProteinProspector) (University of California, San Francisco CA). The manual obtained data was compared to the results obtained using the Pinpoint software (Thermo Scien-

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