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Bioanalysis of therapeutic peptides: Differentiating between total and anti-drug antibody bound drug using liquid chromatography–tandem mass spectrometry quantitation

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ABSTRACT

An acylated peptide with MW ~4.5 kDa was measured in samples from pharmacokinetic, toxicology and clinical studies using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Lower limits of quantitation of 2 ng/mL and 50 pg/mL were achieved for animal and human plasma, respectively. Repeated drug administration may lead to anti-drug antibodies (ADA) which can inactivate the drug by formation of drug-ADA complexes. Hence, the LC–MS/MS assay incorporated cleavage of potential drug-ADA complexes to quantify the total plasma concentration. To obtain information on active drug levels, an assay that measures the free concentration or alternatively the ADA-unbound concentration would be needed. Ultrafiltration experiments through 100 kD cutoff membranes to remove Ig-bound peptide were not successful due to nonspecific binding. Extraction of Ig-bound drug using Protein A or G (bacterial cell wall proteins with high affinity to the Fc region of IgG) was suitable to distinguish between ADA-bound drug and [free + protein bound (not ADA-bound)] drug and correlated with findings from ELISA ADA measurement.

1. Introduction

Development of therapeutic biomolecules requires characterization of their pharmacokinetics and safety properties; hence bioanalytical methods are needed for drug concentration measurements. Large biomolecules are usually analyzed by ligand binding assays (ELISA), while peptides of molecular weights below 10 kDa can be measured in biological matrices by ESI-LC–MS(/MS) in their native form, employing similar procedures and regulatory standards as for small molecules [1–4]. However, peptides share with large molecules their ability to generate an immune response and trigger formation of anti-drug antibodies (ADAs) [5,6]. Similarly to small molecules, circulating peptide drug in the body is comprised of free and protein- (e.g., albumin, AGP (alpha-1-acid-glycoprotein) but not ADA) bound; in addition, similarly to large molecules, also ADA-bound drug may be present. There is the possibility of drug inactivation by formation of these drug-ADA complexes (neutralizing ADAs). For PK data interpretation it is essential to consider whether the free (or partially free) or the total drug is measured [7–10]. Bioanalysis of peptides by LC–MS/MS, which incorporates sample preparation that in most cases cleaves drug-ADA bonds and thus gives the total drug concentration, has been addressed already in numerous publications [11–16]. Nevertheless, properties and behavior vary significantly among peptides even with similar amino acid sequences, and each new method development bears challenges. Variable sensitivity requirements and the wide range of molecular properties usually ask for specific methods and prevent the use of generic assays. Special precautions for sample collection and handling may be required to address potential enzymatic instability and non-specific binding. To overcome chromatographic problems associated with certain peptides (peak broadening and tailing) and carryover issues, HPLC conditions need to be carefully optimized. Proper selection of the precursor ion and MRM transition(s) is critical for good selectivity and sensitivity. Sample enrichment/cleanup (on-line or off-line) usually disrupts non-ADA plasma protein binding and (partially) also ADA binding, but special procedures may be considered to effectively dissociate peptide-ADA complexes, for example acid treatment [17], to ensure total peptide measurement.

The drug addressed in this manuscript was a fatty-acid substituted peptide (38 natural AA, 2 aminoisobutyric acid molecules, palmitoyl chain bound to terminal lysine via acyl bond, MW 4.5 kDa). The fatty acid moiety leads to strong plasma protein binding and therefore, prolonged half-life [18]. An LC–MS/MS assay that

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quantifies the total plasma concentration of the drug (after dissociation of potential drug-ADA complexes) had to be developed and validated, which is discussed in detail. An ADA assay (sandwich ELISA, [19]) that determines the presence of ADAs was available, and ADA's were detected in samples from a cynomolgus monkey study after repeated drug administration. The question was raised whether it was possible to develop an assay that measures the free concentration of the drug: neither protein-bound nor Ig-bound (immunoglobulin-, ADA). A better solution was to develop an assay capable of differentiating between [free + bound to albumin, AGP] and Ig-bound peptide, that after correction for protein binding could provide information on presence of ADA-drug complexes and their amount. The assay should be robust and straightforward enough to be applicable to large sample numbers, i.e. from toxicity and possibly also clinical studies. Development of a specific ELISA for the quantification of free drug was not attempted due to complications such as generation of analyte-specific antibodies within required timelines [4]. Therefore, a selective sample preparation procedure followed by LC–MS/MS as for the total drug assay had to be developed. Several techniques (e.g., ultrafiltration, ultracentrifugation and dialysis) are available for the determination of free concentrations of small drug molecules [20]. In this work we refrained from attempts to measure the free peptide, because our previous experiences with ultrafiltration, equilibrium dialysis, TRANSIL^{XL} High Sensitivity Binding Assay and plasma immunodepletion revealed problems for this class of highly protein-bound peptides, due to adsorption on surfaces (unpublished data on file). Instead we focused on the possibility to distinguish between ADA-bound and ADA-unbound drug. Several biomolecule separation techniques were reviewed, considering that the equilibrium ADA-bound/unbound drug should remain undisturbed during sample preparation. Ultrafiltration (UF) through large molecular weight cutoff (MWCO) membranes appeared as a promising approach. This membrane separation technique is pressure-driven and primarily based on MW. Other factors, such as molecular shape, electrical charge, sample concentration, sample composition, and operating conditions also determine retention/passage of molecules through the UF membrane. Application of UF for biomolecule separation, e.g. albumin from IgG, was described before [21–23]. We tested 100 kD MWCO devices to separate drug bound to ADA (MW > 150 kDa) from [free drug + drug bound to plasma proteins; i.e. albumin, AGP] (MW < 100 kD). Affinity extraction was selected as second option. Protein A and G are bacterial cell wall proteins produced by *staphylococcus aureus* and various *streptococcus* species, respectively, that bind immunoglobulin, and that can be used for the purification of antibodies (mainly IgGs) through binding to the Fc region [24]. The albumin-binding site and the cell wall and cell membrane binding domains have been removed in recombinant Protein G to ensure maximum specific IgG binding capacity [25]. The proteins are commercially available immobilized onto agarose resins in a variety of formats, such as columns, beads and pipette tips [26]. Assessment of immunogenicity of protein therapeutics using Protein G beads was reported [27]. We expected the co-extraction of IgG- (ADA-) bound peptide drug together with IgGs on Protein A or G, whereas drug not bound to ADA (i.e. free and plasma protein bound peptide) will remain in the sample. The experiments and results obtained with both approaches, UF and affinity extraction, using spiked and study samples are described and discussed in this manuscript. To simulate drug-ADA binding in spiked samples, a positive control (purified rabbit polyclonal antibody [28]) was used. In this manuscript, we used the term "positive control (or PC)" when describing samples spiked with artificial rabbit antibody, whereas "ADA" referred to antibodies generated in vivo after drug administration to animals. "Protein-bound" refers to drug bound to plasma proteins other than ADA or Ig.

2. Experimental

2.1. Chemicals, materials, solutions and standards

Ethanol and methanol (Lichrosolv for HPLC) were obtained from Merck (Darmstadt, Germany) and acetonitrile (supra gradient) from Biosolve (Valkenswaard, Holland). Ammonium formate (p.a.), formic acid 98–100% (Suprapur grade), and acetic acid were purchased from Fluka (Buchs, Switzerland). Water used for preparation of all solutions was from a Milli-Q apparatus (Millipore, Billerica, MA, USA). Ammonium hydroxide solution ca. 25% NH3 (p.a.), 2-propanol (p.a.) Tween® 80 and aprotinin (saline solution, 3–7 TIU/mg protein from bovine lung) were purchased from Sigma–Aldrich (St. Gallen, CH). Bovine serum albumin (BSA) and phosphate buffered saline (PBS) were obtained from Eurobio (Les Ulis, France).

Blank animal plasma was prepared from EDTA blood (rat, cynomolgus monkey) in the laboratories at F. Hoffmann-La Roche Ltd. (Basel, Switzerland). Human EDTA plasma was purchased from Bioreclamation (Hicksville, NY, USA). Addition of 100 KIU aprotinin/mL of plasma was performed to stabilize the analyte.

Deep-well-plates (DWP 96) were purchased from Milian (Geneva, Switzerland), low bind Nunc Minisorp PP tubes 4.0 mL from Thermo Scientific (Waltham, MA, USA) and Oasis MCX 96-well SPE Plates 30 μm (30 mg) from Waters (Milford, MA, USA). The following ultrafiltration devices with 100 kD MWCO and 500 μL volume were used: Amicon Ultra® (Millipore), Vivaspin (Sartorius, Goettingen, Germany), Spin-X UF (Corning, Tewskbury, MA, USA), Nanosep Omega (Pall Schweiz AG, Basel, CH). Thermo Scientific Aspire Protein A or G Tips with corresponding buffer kits were used; the 1000 μL pipette tips contained 150 μL of Thermo Scientific Pierce Immobilized Protein A or G Plus resin slurry (50 μL resin bed) with the capacity to purify ≥1.0 mg of human IgG.

Peptide Y (MW = 4473 g/mole) and an exact analog containing one stable-labeled amino acid, $^{13}C_9{}^{15}N$-phenylalanine, used as internal standard (IS) were manufactured by F. Hoffmann-La Roche Ltd. Stock solutions of analyte (0.5 mg/mL) were prepared in 0.1 M acetic acid/ethanol 50:50 (v/v), and small aliquots were spiked to blank plasma for the preparation of the highest calibration standard and quality control (QC) samples. Other calibration standards and QCs were prepared by serial dilution of the highest concentration with blank plasma. Calibration ranges for peptide Y were 2.00–500 ng/mL and 0.0500–10.0 ng/mL. IS stock solution (1 mg/mL) was prepared in 0.1 M acetic acid/ethanol 50:50 (v/v) and further diluted in ethanol–water–formic acid 90:10:0.1 (v/v/v) to working solutions at 500 ng/mL for the high calibration range and 100 ng/mL for the low range. Rabbit polyclonal anti-peptide antibody used as positive control (PC) for the ADA ELISA assay and to mimic drug-ADA binding in our experiments was produced by Roche Diagnostics, Penzberg, Germany. PC QCs were prepared by spiking variable levels of PC (1.00–20.0 μg/mL) to peptide plasma QCs. Selected QCs (with and without PC) were also prepared in BSA, diluted BSA or diluted plasma up to a dilution factor of 100 (using PBS).

2.2. Liquid chromatography–mass spectrometry assay

2.2.1. Sample preparation

2.2.1.1. Protein precipitation. Aliquots of 50 μL plasma were pipetted into a 1.2 mL DWP, 20 μL of 500 ng/mL IS working solution (or ethanol/water/formic acid 90:10:0.1 (v/v/v) for double blank samples) was added followed by 20 μL of 15% formic acid in acetonitrile. After mixing the sample, the solution was incubated for two hours at room temperature to cleave drug-ADA bonds. For protein precipitation 600 μL of ethanol/acetonitrile/formic acid 33:65:2 (v/v/v) were added and, after heat sealing, the sample solution was mixed