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The development of different analysis platforms with LC-MS for pharmacokinetic studies of protein drugs

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Summary

Three different analysis platforms using LC-MS were successfully developed for pharmacokinetic (PK) studies of an antibody drug in serum. These analysis platforms can be selectively used for different types of protein drugs, which ranged from a very specific for a particular drug (antibody enrichment), to a less specific for any antibody drugs with Fc domain (protein A enrichment), and to a very generic method that can be used for any protein drugs (albumin depletion method). In this manner the three platforms will be applicable to a wide range of antibody therapeutic studies for different species. The analysis using an albumin depletion method (with SDS-PAGE) achieved the detection of the drug (to 1 ng) in an aliquot of serum (30 μ L) with a 5-order magnitude of linearity. The analysis using protein A enrichment (with SDS-PAGE) achieved the detection of the drug at a 50 fold lower level (to 0.02 ng). Without using SDS-PAGE for separation, using protein A enrichment achieved the detection to 10 ng and using the anti-drug antibody enrichment achieved the detection to 0.1 ng, with a similar linear dynamic range. These three analysis platforms produced good agreement with a mimic PK study of the drug in monkey serum, as compared to ELISA approach. In addition, these analysis platforms can be selectively applied for PK studies of drugs with different requirements of development time and resources. Such as, the antibody enrichment method can be used in a high throughput manner but limited to a specific protein drug only. On the other hand, the albumin depletion method can be applied to many types of protein drugs, but with the laborious sample preparation steps (SDS-PAGE and the subsequent in-gel digestion). When anti-drug antibodies are not available for antibody drugs, or the sensitivity requirement is not stringent (e.g. > 10 ng), using protein A enrichment (without using SDS-PAGE) seems to be a good choice for PK studies which require fast throughput.

Introduction

Pharmacokinetic (PK) studies of drugs, which measure the clearance rate of drugs in animal and human serum, provide important guidance to dose drugs efficiently.^{1,2} PK studies of small molecule drugs have been using the mass spectrometry technology extensively,^{3–5} often because of the lack of proper antibodies against the small molecules. On the other hand, antibodies, which are often successful against protein drugs, are used largely in ELISA approaches for PK of protein drugs.^{6,7} ELISA approaches are often sensitive for low level drug detection with high throughput capacity. However, the drawbacks are relatively long

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development time and narrow dynamic range for antibody therapeutics. Additionally, ELISA approaches have specificity and detection problems against antibody drugs with the potential competition from patient's own antibodies, such as developed immunogenicity.^{8,9}

Recently, the use of on-line liquid chromatography coupled to mass spectrometry (LC-MS) has advanced greatly for protein quantitation.^{10–18} The LC-MS approaches usually quantitate intensities of the desired peptides, corresponding to the protein drugs, by multi-stage reaction monitoring (MRM) or selected reaction monitoring (SRM) approaches.^{10–18} Compared to ELISA, LC-MS usually has short development times with high specificity, and the same platform can be easily applied to PK studies of different protein drugs. Since the detection of the antibody drugs by LC-MS approach is targeting unique sequences of the drugs, potential competition from patient's own antibodies (usually with different protein sequences) against the drugs is not likely to interfere in the analysis, as for the ELISA approach.

However, the complexity of the serum proteome still presents challenges for efficient sample preparation and adequate sensitivity for LC-MS analysis of protein drugs, and enrichment procedures prior to the drug analysis are often needed. One such enrichment procedure is depletion of high-abundance serum proteins, which will reduce the complexity of serum proteome as well as improve the dynamic range for the analysis.^{19,20} However, the depletion procedures may unintentionally remove protein drugs, particularly, drugs at a low concentration.^{21,22} In addition to the depletion approach, several specific and non-specific enrichment strategies, such as using an idiotypic antibody (specific for a particular antibody drugs), protein A affinity (for antibody drugs containing the Fc region of IgG), gel or column separations can also be applied to reduce the serum sample complexity and enhance detection sensitivity.^{23–25}

In this study, we have evaluated several of these enrichment strategies, and achieved a rationale for the selection of specific analysis platforms to study PK of protein drugs at different stages of development.

Experimental

Materials

The protein drug, CNTO736, was provided by Centocor R&D (Radnor, Pennsylvania) in a liquid formulation (20 mg/mL). CNTO736 is a homodimer, with the variable domain of the heavy chain replaced with a GLP-1 peptide which fused via a flexible linker sequence to a truncated heavy chain containing the hinge region and the CH2 and CH3 domains of an IgG4 antibody.²⁶ Thus, the homodimer is consistent with the two heavy chains linked together by the two interchain disulfide bonds (without the two light chains), with molecular weight of 60 kDa. The antibody (anti-CNTO736), an antibody against the GLP-1 portion of CNTO736, was called CNTO1626 and also provided by Centocor R&D in a liquid formulation (1 mg/mL). Human serum, dithiothreitol (DTT), iodoacetamide (IAA), guanidine hydrochloride, and ammonium bicarbonate, were obtained from Sigma-Aldrich (St. Louis, MO). The three albumin depletion kits were from (1) Albumin Depletion Kit (Pall Corporation, Ann Arbor, MI). (2) AlbuSorb (Biotech Support Group, North Brunswick, NJ), and (3) Vivapure Anti-HSA Kit (Vivascience, Hannover, Germany). Dynabeads Protein A were purchased from Invitrogen (Carlsbad, California). Achromobacter protease I (Lys-C) was obtained from Wako (Richmond, VA), and trypsin (sequencing grade) was purchased from Promega (Madison, WI). Formic acid, and acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ), and the HPLC-grade water, used in all experiments, was from J. T. Baker (Bedford, MA).

Preparation of serum with spike-in experiments of drug substance

The aliquots of CNTO736 (variable amounts as specified in the results section), spiked to 30 μ L or 1 mL human serum, were used for different enrichment studies, as described in the following.

Albumin depletion method

Three albumin depletion kits were analyzed in the following. Briefly, for use of the Albumin Depletion Kit (Pall Corporation), serum (i.e. 30 μ L) containing the protein drug was added to a binding buffer provided by the vendors (i.e. 120 μ L) and then loaded to an albumin depleting spin column. After incubating the sample for 2 min at room temperature, the albumin depleting spin column was centrifuged for 1 min and the filtrate was retained for further analysis. For AlbuSorb (Biotech Support Group), serum (i.e. 30 μ L) containing the protein drug was added to a binding buffer provided by the companies (i.e. 250 μ L) and then added 40 mg of Albusorb powder in a spin-tube. After mixing for 5–10 min on a rotating shaker at room temperature, the spin-tube was centrifuged for 2 min and the supernatant was collected for further analysis. For Vivapure Anti-HSA Kit (Vivascience), serum (i.e. 30 μ L) containing the protein drug was added to a binding buffer provided by the companies (i.e. 300 μ L) and then loaded to a spin column containing the HAS-affinity resin. After incubating for 30 min at room temperature on a rotating shaker, the spin column was centrifuged for 2 min and the flow through was collected for further analysis. One fifteenth of the above albumin depleted solution (i.e. 10 out of 150 μ L), which was the maximum capacity of a mini gel, was used for the subsequent SDS-PAGE separation.

Protein A enrichment method

Serum (i.e. 30 μ L) containing protein drug was added to 0.1M sodium (Na) phosphate buffer pH 8 (i.e. 70 μ L) with protein A magnetic beads (in 100 μ L buffer provided by the company). After incubating with slow tilt rotation mixing for 1 h at room temperature, protein A magnetic beads was washed three times with 0.5mL 0.1M Na-phosphate buffer, pH 8. The protein drug was eluted by adding 60 μ L 0.1M citrate pH 2–3 to protein A magnetic beads or boiling protein A magnetic beads with 60 μ L 2% SDS buffer for 10 min. The procedure was facilitated by use of a magnet. The eluent (or the supernatant without magnetic beads) was then concentrated to ~10 μ L for the subsequent SDS-PAGE separation. Alternatively, after incubation and wash, the protein drug was eluted with 60 μ L 6M guanidine hydrochloride containing 100mM ammonium bicarbonate, the eluent was then reduced with DTT and alkylated with IAA, and buffer exchanged for Lys-C digestion (without using SDS-PAGE for separation in this case).

Antibody (anti-CNTO736) enrichment method

The preparation of the antibody column and the antibody enrichment procedure are described in the following. Briefly, 100 μ L of protein A magnetic beads was activated by washing three times with 0.5mL 0.1M Na-phosphate buffer pH 8, then 10 μ g CNTO 1626 (anti-CNTO736 antibody) was added to the activated protein A magnetic beads. After tumbling overnight at 4°C, the antibody-bead complex was washed twice with 1mL PBS-NP40 buffer, pH 7.4, then twice with 1mL 0.2M triethanolamine buffer, pH 8.2. After that, the antibody-bead complex was resuspended in 1mL of 20mM DMP (dimethyl pimelimidate dihydrochloride) in 0.2M triethanolamine buffer, pH 8.2 and incubated with rotational mixing for 1 h at room temperature. The cross-linking reaction (antibody chemically attached to the beads) was stopped by resuspending the antibody-bead complex in 1mL of 50mM tris buffer, pH7.5 and incubating for 15 min with rotational mixing. The cross-linked beads were washed three times with 1mL PBS-NP40 buffer, pH 7.4. The serum (i.e. 30 μ L) containing protein drug was then added to 0.1M Na-phosphate buffer pH 8 (i.e. 70 μ L) with the antibody cross-linked beads. After incubating (with tilting and rotation) for 1 h at 4°C, the antibody cross-linked beads were

washed three times with 1mL PBS-NP40 buffer, pH 7.4, then three times with 1mL 1M NaCl, pH 7.0. The protein drug was eluted by adding 60 μ L 0.1M citrate buffer, pH 2 to the antibody cross-linked beads or by boiling the antibody cross-linked beads with 60 μ L of 2% SDS buffer for 10 minutes. The eluent was then concentrated to ~10 μ L for the subsequent SDS-PAGE separation. Alternatively, after the incubation and wash step, the protein drug was eluted from the antibody cross-linked beads with 60 μ L 6M guanidine hydrochloride containing 100mM ammonium bicarbonate. The eluent was then reduced with DTT and alkylated with IAA, and buffer exchanged for Lys-C digestion (without using SDS-PAGE for separation in this case).

ELISA method

The method is a slightly different version from a traditional ELISA method, described as Electrochemiluminescence-based immunoassay (ECLIA) in the following. The spiked cynomolgus monkey serum samples were diluted in 0.1 M PBS, pH 7.4, containing 2 % (w/v) bovine serum albumin and 10 % (v/v) cynomolgus monkey serum. Standards were prepared separately in the same diluent as the serum spikes. Ten (10) μ L of each diluted sample or standard plus 100 μ L of a master mix containing streptavidin-coated paramagnetic beads and biotinylated anti-CNTO736 mouse monoclonal capture antibody were added per well to a 96-well round bottom polypropylene plate (Costar, Corning, NY). The plate was incubated for 1 h at room temperature on a plate rotator at a speed sufficient to keep the streptavidin beads in suspension. One hundred (100) μ L of a ruthenium-labeled anti-CNTO736 mouse monoclonal detection antibody was added to each well and the plate was incubated again for 1 h at room temperature on the rotator. The plate was read on a BioVeris M-8/384 electrochemiluminescence plate reader (BioVeris Corporation, Gaithersburg, MD). Electrochemiluminescence (ECL) units were proportional to CNTO736 concentration. Data was imported into SoftMax Pro version 4.6 software (Molecular Devices, Sunnyvale, CA). A standard curve of CNTO736 concentration versus ECL units was generated using a 4-parameter logistic fit without weighting. CNTO736 concentration of the spiked serum samples was obtained by interpolation from the standard curve.

SDS-PAGE and in-gel tryptic digestion

A mini gel (8 $\times$ 8 cm and 4 to 20% Tris-Glycine) with Coomassie blue staining was used to further separate the eluent isolated from either the albumin or the protein A depletion, or the antibody enrichment approach. The gel bands, contained the expected protein drug (expected molecular weight), were cut out. Each cut-out band was further minced into small pieces (approximately 0.5 mm²) and subjected to 2 to 3 cycles of gel dehydration with acetonitrile and rehydration with ammonium bicarbonate buffer (0.1 M, pH 8.0) in order to remove the Coomassie stain, as describing in the following. Briefly, the gel slices were further washed with 300–400 μ L of water for 15 min and centrifuged to remove the liquid. Acetonitrile was then added (300 μ L) to the gel slice, which was placed in a microcentrifuge tube (spin for ~15 min), the liquid (acetonitrile) was removed. The gel slice was further dried in a Speed-Vac, and then rehydrated with 300 μ L of 0.1 M NH₄HCO₃ for 10–15 min. An equal amount of acetonitrile was subsequently added, and the sample was vortexed for 15–20 min and then centrifuged to remove the liquid. This procedure was repeated up to 3 times, as necessary, or until no visible Coomassie stain remained. The remaining gel slice was then reduced with DTT by the addition of 250 μ L of 10 mM DTT in 0.1 M NH₄HCO₃ and incubated for 30 min at 56 °C. The sample (gel slice) was subsequently alkylated at room temperature and in the dark for 60 min with 250 μ L of 55 mM IAA in 0.1 M NH₄HCO₃. After removal of the liquid, an aliquot of 250 μ L of trypsin digestion solution (containing 10 ng/ μ L trypsin in 50 mM NH₄HCO₃, pH 8.0) was added to the gel slice, and the sample was then incubated for 30–35 min at 4 °C. The incubated solution was then replaced with sufficient 50 mM NH₄HCO₃ to cover the gel pieces (50–100 μ L) and then incubated overnight at 37 °C. The supernatant was removed and saved. The remaining gel pieces were further extracted with 5% formic acid (100 μ L) at 37 °C for 5

min, and an equal amount of acetonitrile was subsequently added, and the sample was shaken for 15 min. The formic acid and acetonitrile solution, containing tryptic peptides, was combined with the previous supernatant and concentrated to ~10 μ L. The concentrated solution (trypsin-digested peptides) was subjected to LC-MS analysis.

In-solution Lys-C Digestion

The eluent (~60 μ L) either from the protein A isolation or from the antibody enrichment was reduced with 5mM DTT for 60 min at 75 °C and alkylated with 20mM IAA in the dark for 60 min at room temperature. The eluent (after the reduction and alkylation) was then transferred to a Microcon centrifugal filter with a 10 kDa molecular weight cut-off (MWCO) to remove guanidine hydrochloride and excess DTT and IAA, and buffer exchanged to Lys-C digestion buffer (100mM ammonium bicarbonate, pH 8). Lys-C (1:50 w/w) was added to the eluent and incubated for 4 h at 37 °C. The Lys-C digested solution was concentrated to ~10 μ L in a speed-vac for subsequent LC-MS analysis.

LC-MS analysis

The tryptic (in-gel) or the Lys-C (in-solution) peptides were analyzed by an LTQ XL mass spectrometer (Thermo Fisher, San Jose, CA) equipped with New Objective (Waltham, MA) nanospray source coupled to a Dionex nano U3000 LC instrument (Sunnyvale, CA) with a 75 μ m i.d. $\times$ 15 cm C-18 capillary column packed with Magic C18 (3 μ m, 200 Å pore size) (Michrom Bioresources, Auburn, CA). The mass spectrometer was operated in Selected Reaction Monitor (SRM) mode for the desired peptides. The LC gradient was from 5% B to 65% B in 60 min (A: water with 0.1% formic acid; B: acetonitrile with 0.1% formic acid), then from 65% B to 80% B in 10 min, and hold at 80% B for 10 min. The column flow rate was maintained at 200 nL/min after splitting.

Selection of desired peptides for SRM

The desired peptides were selected from the variable region (the active peptide) of the CNTO736, as described in the following text. Different enzymatic fragments with different charge states were evaluated by the LC-MS analysis first using the data-dependent mode. Based on the mass spectrometry sensitivity (intensities of the peptide peaks) and the LC-MS separation (minimum interference from the background), the peptide candidates were further evaluated by LC-MS using the SRM mode for quantitation. Trypsin was chosen for the in-gel digestion because Lys-C digestion resulted in lower peptide recovery in that protocol. On the other hand, Lys-C was used for the in-solution digestion because less numbers of peptides were generated, which produced less potential interference for SRM quantitation. Based on the linearity and reproducibility of the peak areas, three peptide fragments with the desired performance either from the tryptic peptides or the Lys-C peptides were selected. We looked for potential modifications of the three selected peptides in the corresponding extracted ion chromatogram and no measurable levels of such modifications were observed. In these two sets of the three desired peptides (one from the in-gel tryptic peptides and the other from the Lys-C peptides), two of the three peptides were identical (with the same trypsin and Lys-C cleavages), and the other had a slightly longer peptide length (two more amino acids) for the Lys-C than the corresponding tryptic peptide. The three peptides, eluted at least 5 min apart from each other in the LC-MS chromatogram, named as peptide A (from the N-terminus of the protein drug sequence), peptide B (from the middle of the sequence), and peptide C (from the hinge region of the sequence).

SRM Quantitation

SRM, which only selected the precursor ions of the three desired peptides for fragmentation in the MS/MS mode, was used for quantitation. In this SRM procedure, the precursor ions were

acquired with the average of two microscans, and the MS/MS acquisitions for each precursor ion were acquired with the average of ten microscans in a given retention window. The ion target value was set at 30,000 and a maximum of 100 ms scan time for the precursor ion scan (MS mode) and the ion target value at 10,000 and a maximum of 200 ms scan time for the fragment ion scan (MS/MS mode). Using SRM in a linear ion trap mass spectrometer, all fragment ions per peptide are simultaneously observed, not just the few transition ions per peptide often used in a triple quadrupole mass spectrometer measurement. Thus, all fragment ions of the precursors observed in this study (fragmentation pattern) are used to compare to the fragmentation pattern of the same precursor ions from different samples to assure the same peptide (protein) identity. The extracted ions from the fragment ions per peptide, often only the highest intensity ion or the ion with little interference from background ions, were used for peak area comparison. Thus, the quantitation analysis for peptide A was applied using the transition of m/z 705.80 (triply charged precursor ion) $\rightarrow$ m/z 984.44 (b_{19}^{2+} product ion), for peptide B using the transition of m/z 503.66 (doubly charged precursor ion) $\rightarrow$ m/z 359.23 (y_3^{1+} product ion), and for peptide C using the transition of m/z 812.98 (doubly charged precursor ion) $\rightarrow$ m/z 737.29 (y_7^{1+} product ion). It should be noted that the peptide length is slightly longer for the corresponding Lys-C peptide. Thus, the quantitation for peptide C (Lys-C digestion) was applied using the transition of m/z 919.64 (doubly charged precursor ion) $\rightarrow$ m/z 1100.20 (b_{14}^{1+} product ion). The extracted ion chromatograms (XIC) of these specified product ions were used for quantitation. Often, only the best peptide measurement per targeted protein (i.e. Peptide C in this case) was selected for quantitation based on the robustness in isolation from the in-gel/solution digestion and efficient/robust ionization in the mass spectrometry. The other peptides (i.e. Peptide A and Peptide B) can then be used for identification purpose.

Analysis of CNTO736 in monkey Serum

Pooled cynomolgus monkey serum was purchased from Bioreclamation, Inc. (Liverpool, NY, USA). Cynomolgus monkey blood was collected into serum collection tubes (Vacutainer, Becton-Dickinson, VWR, Bridgeport, NJ) and allowed to clot overnight at 2–8 °C. The blood was centrifuged at $3,000 \times g$ for 30 minutes in a refrigerated centrifuge. Serum was aspirated and stored at –20 °C. CNTO736 was added to the serum to give a working stock concentration of 1 mg/mL. Final serum concentrations for analysis were prepared by diluting the working stock to 250, 200, 100, 50, 10, 1 and 0.1 µg/mL. The monkey sample analysis was similar to CNTO736 spiked to human serum samples. However, the amounts of CNTO736 spiked into the samples were unknown initially, so that the analyses were performed as a blind study. In brief, 30 µL of each monkey serum sample went through either the albumin depletion method or protein A enrichment, and then SDS-PAGE was used for the subsequent separation. Alternatively, each monkey serum sample was purified using anti-drug antibody enrichment (without using SDS-PAGE) or protein A enrichment (without using SDS-PAGE).

Results and Discussion

We evaluate several different enrichment strategies by spiking the protein drug into human serum and then using a LC-MS (SRM) approach for detection and quantitation of the drug. The detection sensitivity and sample preparation procedures with different enrichment strategies are compared. These enrichment strategies are further evaluated by a mimic PK study of the drug in a monkey serum. The pros and cons, and the rationale of using these analysis platforms for different types of protein drugs are discussed in the following.

Using albumin depletion

Albumin (~ 30 to 50 mg/mL) is the highest abundant protein in serum and often needs to be removed to improve the detection of low abundant proteins, such as the protein drugs (~ low

to sub $\mu\text{g/mL}$) in this study. The work flow for the albumin depletion strategy is shown as route (1) in Figure 1. Different amounts of the protein drug from 0, 1, 10, 100, 1000, to 10,000 ng were spiked into human serum (30 μL). The spike-in samples were loaded onto an albumin depletion kit and one aliquot of the depleted solution (1/15) was loaded onto a SDS-PAGE for further separation (Figure 2). The expected drug band (based on molecular weight) from the SDS-PAGE separation was cut out for in-gel tryptic digestion and the resulting peptides were analyzed by LC-MS analysis using SRM of the desired peptide ions (Figure 3). As shown in Figure 3, the peak areas of the three desired peptides were measured. The peptides were chosen by the criteria of the uniqueness of the peptide sequences to the protein (vs. the human protein database), and their sensitivity in the mass spectrometric analysis. The peak areas from different spike-in samples were displayed as a linear curve (Figure 4A, 4B, and 4C). As shown, all three desired peptides were linear from 0 to 10,000 ng with r^2 greater than 0.99 (0.9992 for peptide A, 0.998 for peptide B, and 0.9999 for peptide C). Even in the low concentration range (0, 1, and 10 ng), the linearity was still good (r^2 was 0.9849 for peptide A, 0.9909 for peptide B, and 1.000 for peptide C), as shown in the insert of figures 4A, 4B, and 4C. Although the slope at low concentrations was slight different from the slope using for the whole range, the concentration determination (at low concentration) was quite similar using either of the slopes.

The detection sensitivity (1 ng) (see Figure 5A) and linearity (5-order magnitude) is as good as or even better than a typical ELISA approach (typically in the range of ~ 1 to 100 ng for detection sensitivity with 2 to 3 orders of linearity for antibody drugs). However, the sample preparation procedure (i.e. SDS-PAGE and in-gel digestion) prior to the LC-MS detection is not a high throughput approach.

It should be noted that the albumin depletion kits could remove some of the protein drug as well, and therefore we evaluated three commercial albumin depletion kits (Pall, Biotech Support Group, and Vivascience). In our evaluation, the Pall depletion kit could efficiently eliminate the majority of albumin, however, with the expense of significant drug loss. On the other hand, Vivascience depletion kit depleted a lesser amount of albumin but also with less loss of the drug (data not shown). Thus, instead of using the albumin depletion kits, we evaluated the use of protein A for the enrichment of the antibody drug.

Using protein A enrichment

Antibody drugs usually contain the constant region (Fc) of IgG. So, we can take advantage of using protein A to capture the Fc region of antibody drugs in serum. The work flow for the protein A enrichment is shown in as route (2) in Figure 1, which is similar to the albumin depletion method but without the need to deplete albumin. The protein drugs (from 0, 0.02, 0.1, 1, 10, and 100 ng) were spiked into an aliquot of human serum (i.e. 30 μL). The spike-in samples containing the drug were first captured by protein A beads. The bound drug (on the protein A beads) were then eluted with a strong elution buffer (i.e. either a low pH citric or a SDS buffer), and followed with the SDS-PAGE and in-gel digestion procedures for LC-MS analysis, similar to the albumin depletion method for the drug detection. As shown in Figure 5B and Figure 6, the lowest detection was achieved at 0.02 ng with a good linearity ($r^2 = 0.9818$). Based on the peak intensity at the lowest detection level (S/N ratio), using the protein A enrichment was approximately 50 fold more sensitive than using the albumin depletion method. It should be noted that the flow through (the unbound fraction), not the eluent, contained the drug in the albumin depletion method. Thus, the use of a strong wash was limited (to avoid albumin containment) in the albumin depletion method. On the other hand, a strong elution buffer could be used for the protein A enrichment to improve the detection limit. However, many serum proteins, particular immunoglobulin proteins (IgG) which contain the Fc domain, still co-eluted with the protein drug after the protein A enrichment step. To achieve high detection sensitivity (to 0.02 ng), a further separation procedure (i.e. SDS-PAGE) was

still needed in the protein A enrichment. Without using the SDS-PAGE step (route (2) b in Figure 1), the direct analysis of the eluent from the protein A enrichment would result in a 500-fold decrease in sensitivity (to 10 ng). It should be noted that the detection sensitivity (10 ng) might not be improved further for many classic antibody drugs (with two light and heavy chains of ~150 kDa molecular weight) even using SDS-PAGE for further separation since the serum IgGs would migrate to a similar location as for classic antibody drugs. Thus, fusion proteins, such as CNTO736 (with a molecular weight of 60 kDa in intact or 30 kDa in reduced form), may have a unique advantage to enhance the detection sensitivity to 0.02 ng using SDS-PAGE.

Nevertheless, to avoid the laborious steps of SDS-PAGE and the subsequent in-gel digestion, an enrichment method using the anti-drug antibody was evaluated.

Using antibody (anti-CNTO736) enrichment

An antibody against the idiotypic domain (specific for the GLP-1 portion) of the protein drug (CNTO736) was developed for the enrichment step. The work flow of using the antibody enrichment is shown as route (3) in Figure 1. The serum samples with the spike-in drug were captured by the immobilized antibody (see the experimental section for details). After several washing steps, the eluent containing the drug was collected and subjected to enzymatic digestion (Lys-C digestion). The Lys-C digested peptides were analyzed by the LC-MS method using SRM. It should be noted that we used Lys-C instead of trypsin to reduce the total numbers of peptides in the sample (approximately by one half) since less peptides (less possible with similar precursor ions) improved the sampling process in the LC-MS analysis. As shown in Figure 7, six human serum samples, which contained different amounts of the protein drug (0, 0.1, 1, 10, 100, and 1000 ng), were detected linearly with this enrichment procedure, as the r^2 of 0.9963 for the whole range and r^2 of 1.000 for the low concentration range (see insert of Figure 7).

The enrichment of using antibody capture simplified the sample preparation procedures (no SDS-PAGE and in-gel digestion) with added advantage of high sensitivity (0.1 ng) (see Figure 5C), wide linear dynamic range (5 order), and with a throughput capability comparable to a typical ELISA assay. However, the development of an suitable antibody takes time and resources, and the analysis platform is also quite specific for a particular drug. By comparison, the previous two methods, the protein A enrichment seems to be generic to antibody drugs which contain the Fc region, and the albumin depletion method is potentially generic to many protein drugs. In the initial stage of drug development, when the antibody is not available or the throughput is not an issue, protein A enrichment or albumin depletion method (depending on protein types) can be used with little development time and resources. In the later stage of drug development, when the antibody becomes available or the throughput is important, the antibody enrichment procedure or ELISA assay can then be applied.

Analysis of CNTO736 in Monkey Serum

To further evaluate the above strategies (albumin depletion, protein A, and antibody enrichment), a mimic PK study of the drug in a monkey serum was used. In this study, the known amounts of CNTO736 spiked in monkey serum at levels that mimic the time course of the drug's clearance in a monkey serum. However, the amounts of spike-in CNTO736 were unknown to us initially. Seven different monkey serum samples (represent the entire time course of the drug clearance) were analyzed using the albumin depletion approach (followed by SDS-PAGE and in-gel digestion), and the analysis results were shown in Table 1 (second row). In addition, since the concentration range of the sample set was determined to be relatively high, a different sample preparation procedure using protein A enrichment (without using SDS-PAGE, and using Lys-C digestion) was also applied and gave comparable results

(see Table 1, the third row). Moreover, the seven monkey serum samples were also analyzed using the antibody enrichment approach (without using SDS-PAGE, and using Lys-C digestion), which produced very similar results as using either the albumin depletion or the protein A enrichment (see Table 1, the fourth row). After the samples were un-blinded, we found that these three analysis platforms were indeed in a good agreement with ELISA measurement (Table 1, the fifth row). The next step in the development of a LC-MS method for a PK study would be the preparation of a suitable labeled antibody drug for use as internal standard.

It should be noted that the antibody or protein A enrichment procedure (without SDS-PAGE) generated results with a much faster analysis time, with a capacity of 7 to 10 samples per day per LC-MS instrument. These approaches will be valuable when a high throughput is important and multiple LC-MS instruments are available. The albumin depletion or protein A enrichment method (with SDS-PAGE) would need two additional days for the sample preparation prior to the LC-MS analysis (or takes 7 to 10 samples per 3 days for one LC-MS instrument). Nevertheless, the protein A or albumin depletion method are preferred for one can proceed without the anti-idiotypic antibody. In addition, if the sensitivity requirement is not stringent (e.g. > 10 ng), such as the case for the monkey serum samples, using protein A enrichment (without SDS-PAGE) should be a good choice for PK studies with fast development and good analysis throughput.

Conclusion

Other approaches, such as using chromatography (ion exchange or reversed phase columns) instead of using SDS-PAGE for the protein separation step produced low recovery for the protein drug and thus resulted to much poor detection sensitivity (~ 1000 fold less or to 1 μ g detection level) for the protein drug. We also tried to use albumin depletion method first, and then digested the depleted albumin solution (flow through fraction) for the subsequent LC-MS analysis of peptides, either 1-dimensional LC - or 2-dimensional LC (ion exchange and reversed phase) with MS analysis. However, the complexity of serum proteomes prevented the efficient digestion of the protein drug, particularly, when the drug concentration becomes low (lower than 1 μ g) in an aliquot serum sample (30 μ L). We also used the already digested standard (neat drug) to spike in the depleted albumin solution (after digestion), and observed the peptides of the drug at 10 ng and 1 ng for 1D and 2D LC-MS, respectively. These results again indicated that inefficient digestion of the protein drug was due to the complexity of serum proteomes. Thus, we believe that the three analysis platforms, with reasonable detection as presented in this paper, are appropriate for PK study assessments of protein drugs. These analysis platforms can be selectively used for different types of protein drugs, and applied for drugs with different requirements of development time and resources. Although this work focused on serum, based on the nature of the methods, we believe that the approaches (i.e. albumin depletion and protein A enrichment using antibody drug Fc region) are quite generic with less interference by typical anticoagulants such as EDTA or citrate, and should be applicable to plasma samples. The antibody enrichment approach, similar to ELISA, may not directly apply to plasma samples without further study. Thus the result described here provide insightful information for the pharmaceutical industry to study a wide range of antibody therapies across different species. In addition, the detection of the antibody drug by the LC-MS approach is targeting to specific amino acid sequences of the drug. Thus, patients own antibodies (usually with different protein sequences) against the drug are not likely to interfere with the analysis (when using albumin depletion or protein A enrichment). Thus, the LC-MS approach can also be used to check if there is a competition from patient's own antibodies against the drug which may not be observed in ELISA approach. In general, the antibody enrichment method can be used in a high throughput manner but limited to a specific protein drug only. Alternatively, the albumin depletion method can be applied for many types of protein

drugs but with limited throughput. When the sensitivity requirement is not stringent (e.g. > 10 ng), using protein A enrichment (without using SDS-PAGE) seems to be a good choice for PK studies.

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Figure 1.

Work flow for the three different strategies. (1) The albumin depletion strategy; (2) the protein A enrichment strategy, with (a) and without (b) SDS-PAGE separation; (3) the antibody (anti-CNT0736) enrichment strategy.

Figure 2.

Protein separation by SDS-PAGE (after albumin depletion) and the specified gel bands (with red squares) are cut out for in-gel tryptic digestion. Different amounts of protein drug (CNTO736) spiked in human serum are indicated at each gel lane, along with the neat CNTO736 drug (1 μ g) at the two sides. As noted in the insert, only 1/15 of the original spike-in drugs (or 10 out of 150 μ L) are loaded to the gel.

Figure 3. LC-MS analysis of the in-gel tryptic peptides. Three desired peptides are detected by SRM approach. The right panels are the fragmentation pattern (MS/MS spectra) of the three peptides (labeled as Peptide A, Peptide B, and Peptide C in the insert). The left panels are the extracted ion chromatogram (XIC) of the three peptides. The precursor and fragment ions used for XIC are indicated in the insert.

Figure 4A

Figure 4B

Figure 4C

Figure 4.

Quantitation of protein drug (CNTO736) in human serum samples using the albumin depletion strategy with SRM approach. Linearity of peptide A (**Figure 4A**), peptide B (**Figure 4B**), and peptide C (**Figure 4C**) of CNTO736 at different concentrations are shown, with the linearity of low concentration spike indicated in the insert.

Figure 5.

Limit of detection (LOD) for the three different strategies. (A) the albumin depletion strategy with S/N of 4 indicated in the insert; (B) the protein A enrichment strategy with SDS-PAGE separation with S/N of 5; (C) the antibody enrichment strategy (without SDS-PAGE separation) with S/N of 3.

Figure 6.

Quantitation of protein drug (CNTO736) in human serum samples using the protein A enrichment strategy (with SDS-PAGE separation). Linearity of peptide C of CNTO736 at different concentration is shown, with the linearity of low concentration spike indicated in the insert.

Figure 7.

Quantitation of protein drug (CNTO736) in human serum samples using the antibody enrichment strategy (without SDS-PAGE separation). Linearity of peptide C of CNTO736 at different concentration is shown, with the linearity of low concentration spike indicated in the insert. In this case, the peptide C is from Lys-C cleavage of CNTO736.

Table 1

Determination of protein drug concentration in seven monkey serum samples (mimic the entire drug clearance course) using the albumin depletion, protein A enrichment without using SDS-PAGE, antibody enrichment, and ELISA. The reported values were the average concentration of 3 measurements together with the % CV.

sample #	# 4	# 3	# 1	# 6	# 5	# 7	# 2
Albumin depletion (ug/mL)	254 ±2%	186 ±2%	71 ±2%	40 ±5%	9 ±8%	0.4 ±5%	0.2 ±13%
Protein A enrichment (ug/mL)	264 ±3%	162 ±4%	68 ±5%	36 ±4%	9 ±2%	1.1 ±12%	0.1 ±4%
Antibody enrichment (ug/mL)	257 ±2%	177 ±3%	70 ±5%	41 ±4%	12 ±9%	0.9 ±1%	0.1 ±2%
ELISA (ug/mL)	241 ±6%	178 ±0.3%	93 ±2%	46 ±4%	10 ±2%	0.9 ±5%	0.1 ±1%