Journal of Pharmaceutical and Biomedical Analysis 53 (2010) 221–227

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Combined use of immunoassay and two-dimensional liquid chromatography mass spectrometry for the detection and identification of metabolites from biotherapeutic pharmacokinetic samples

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ARTICLE INFO

Article history:
Received 11 December 2009
Received in revised form 22 April 2010
Accepted 23 April 2010
Available online 23 May 2010

Keywords:
Immunoassay
Two-dimensional liquid chromatography
Mass spectrometry
Metabolite identification
Monoclonal antibody
Anti-idiotype antibody

ABSTRACT

Peptides and monoclonal antibodies have both emerged as important therapeutic modalities, but each has challenges which limit their use. Non-recombinant chemical conjugation of peptides onto antibodies has the potential to minimize or eliminate altogether many of these limitations. Once such approach, pioneered by CovX has created the possibility for rapid stoichiometric fusion of pharmacophores to a single antibody platform. These molecules, called CovX-Bodies, maintain both the pharmacologic properties of a given peptide and the pharmacokinetic properties of a monoclonal antibody. The result is a new class of molecules wherein each component contributes desirable traits. In this paper, we demonstrate the use of immunoassay and two-dimensional liquid chromatography mass spectrometry (2DLC/MS) in combination to investigate the antibody conjugates of Glucagon-like peptide-1 (GLP-1) and analogs for intact protein metabolite identification directly from mouse serum. The information gained from combining these approaches has helped guide and expedite the optimization of our drug product development efforts.

1. Introduction

Targeted therapy using monoclonal antibodies has revolutionized medicine, with several already having demonstrated their clinical potential for a range of human diseases [1]. Peptides have advantages in terms of target affinity and specificity, and the ability to disrupt protein–protein interactions [2]. While monoclonal antibodies and peptides have emerged as important therapeutics, each has some important limitations. Each novel monoclonal antibody requires the development of a new cell line and manufacturing process. Peptides often suffer from rapid clearance and enzymatic degradation in the body. The development of highly specific chemical linkage technologies has allowed the coupling of pharmacologically active agents (specifically peptides) to an aldolase antibody in a stoichiometrically defined manner [3]. Furthermore, the platform nature of this aldolase antibody eliminates the need for new individual cell line development and optimization for each new biotherapeutic molecule [4]. This novel approach addresses the limitations of traditional peptide and antibody approaches by combining them into new bioconjugate molecules. These molecules, called CovX-Bodies, have both the pharmacologic properties of a given peptide and the pharmacokinetic properties of a monoclonal antibody. CovX-Bodies are created by covalently joining a pharmacophore via various linkers to two specific catalytic binding sites on a specially designed humanized monoclonal IgG1 antibody. The result is a well-characterized, bivalent antibody-drug conjugate that possesses the biologic actions of the peptide and the extended half-life of the antibody. We have successfully demonstrated the utility of this technology through the enhancement of both pharmacokinetic and pharmacodynamic profiles of peptides used in the treatment of several disease areas in recent human clinical trials [4]. Importantly, the use of the same antibody scaffold for multiple drug candidates allows for the use of a clone-specific anti-idiotype capture antibody which reduces drug development cycle time.

The antigenic specificity of a given antibody clone is dictated by the unique sequences of the (hypervariable) complementary determining regions (CDRs) located on the heavy and light chains of the antibody's variable domain. Collectively, these sequences form the "idiotype" of a given antibody clone. Thus, antibodies directed against the idiotypes (i.e. CDRs) of another antibody have become known as anti-idiotypic antibodies. Subsequently, anti-idiotype antibodies have been investigated as therapeutics for B-cell lymphomas and other cancers [5]. Additionally, anti-idiotypic antibodies have been used as highly specific reagents that

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doi:10.1016/j.jpba.2010.04.028

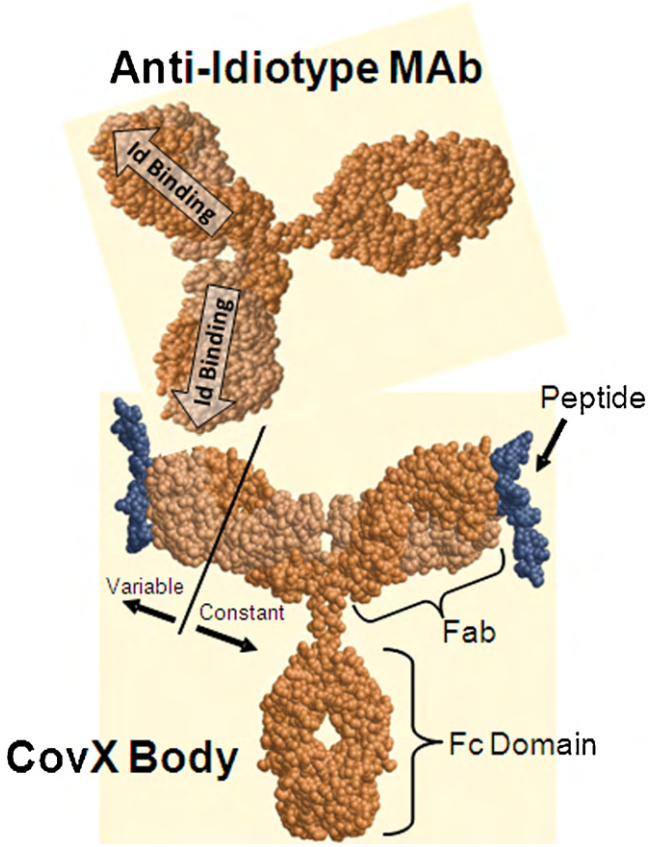

Fig. 1. Depiction of anti-idiotype antibody and CovX-Body interaction. CovX-Body composed of CovX antibody and peptide fused to Fab.

are well suited for the detection of therapeutic antibodies *in vivo* [6], particularly in monkeys and humans, where the large amounts of endogenous antibodies often interfere with standard polyclonal anti-human IgG reagents. Fig. 1 displays a graphical representation of the anti-idiotype and CovX-Body interaction that is used to isolate the CovX-Body from serum.

Therapeutic drug monitoring is critical to achieving optimal patient care. Immunoassays and liquid chromatography have been used to assay small and large molecule therapeutics. The most common methods used in the clinical laboratories are immunoassays and liquid chromatography coupled with ultraviolet detection. Both methods are subject to interference by the metabolites formed *in vivo*, and affect the reliability of the assay performance because of the overestimation of drug concentrations due to nonspecific cross-reaction from their metabolites [7]. Liquid chromatography coupled with mass spectrometry (LC/MS) is a powerful analytical tool that provides high specificity and sensitivity. Because of the simplified sample preparation and the high sensitivity and specificity as compared to immunoassays, LC/MS has shown great potential to be the method of choice for the analysis of immunosuppressants *in vivo* [8]. In addition LC/MS can measure multiple ions to determine multiple drugs in a single analytical run. Like any other technique, however, LC/MS has its own limitations. The most widely used ionization source for MS (electrospray) is vulnerable to ion suppression [9], which can lead to significant sensitivity loss and erroneous results. Ion suppression occurs when co-eluting compounds suppress the ionization of the target compounds in the ionization source. The detailed mechanism of ion suppression is not clear, but the presence of complex matrices such as blood and serum often amplifies the problem. One effective way to eliminate ion suppression is to remove extraneous matrix components through sample cleanup procedures. Two-dimensional liquid chromatography reduces suppression by using two sample cleanup or separation steps prior to MS analysis. Two-dimensional liquid chromatography in combination with mass spectrometry has the ability to analyze biological samples with specificity and selectivity and is becoming the method of choice for biomarker discovery [10] and complex proteomic samples [11]. Proteolytic degradation and/or metabolite formation analysis is usually accomplished by peptide mapping or MS based sequencing techniques [12] and top-down or intact protein based methods are emerging techniques since they preserve post-translational modifications [13–14].

An anti-idiotypic antibody was created and used to detect and quantify specific intact and metabolized forms of CovX-Bodies directly from serum. By combining mass spectrometry and immunoassay approaches, a new way to detect intact large molecule therapeutics directly from the serum of animals or humans is demonstrated. In this paper, the analysis of GLP-1 peptide is investigated, a peptide which possesses several physiological properties making it the subject of intense investigation as a potential treatment for obesity and type 2 diabetes mellitus. GLP-1 has several know and important metabolites [15]. An aminopeptidase (dipeptidyl peptidase IV) which is found both in the endothelium of the local capillary bed within the intestinal wall and in the serum, readily degrades GLP-1 to generate N-terminal truncated metabolites. Exendin-4, a 39 amino acid peptide with 53% structural homology to GLP-1, has a longer half-life *in vivo* and similar biological properties to GLP-1 [16]. CovX-Bodies containing GLP-1 and Exendin-4 peptides were analyzed in serum samples over time by both immunoassay and 2DLC/MS to monitor the intact antibody, as well as, various metabolites. Both the immunoassay and the first dimension in 2DLC/MS used the same anti-idiotype antibody to capture the CovX-Body from serum. The immunoassays detect the CovX-Body using an antibody specifically reactive with the intact N-terminus of the GLP-1 peptide, the C-terminus of the Exendin-4 peptide, or human IgG. The 2DLC/MS detects the captured CovX-Body by switching the affinity column fraction into a reversed phased column with a time-of-flight mass spectrometer for the analysis of the intact antibody. A new method (2DLC/MS) was developed to directly analyze intact antibodies present in the serum and was used in conjunction with immunoassay to analyze time course serum samples for the qualitative analysis of metabolites.

2. Experimental

2.1. Reagents and chemicals

All chemicals purchased were of analytical grade unless otherwise specified. The solvents (acetonitrile and 2-propanol) were HPLC grade and purchased from J. T. Baker (Phillipsburg, NJ), except for the in-house water, which was purified by a Milli-Q system (Millipore Corp., Milford, MA). The acids, formic and trifluoroacetic, were purchased from Pierce (Rockford, IL, Part Nos. 28904 and 28905).

2.2. CovX-body generation

The peptides were prepared in-house by solid phase synthesis utilizing Fmoc chemistry. The GLP-1 peptide was tethered at the C-terminus and contained a diketone linker, as shown in [18]. Exendin-4 and a modified version of Exendin-4 peptides were tethered at the C-terminus and mid-linked respectively, both with an azetidinone linker. Fig. 2 shows the amino acid sequence of the GLP-1 and Exendin-4 peptides. An aglycosylated version of the CovX antibody was used since it produced a stronger intensity signal

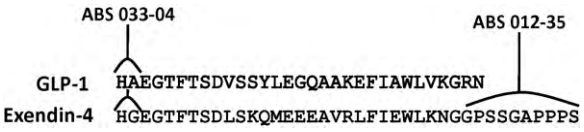

Fig. 2. GLP-1 and Exendin-4 peptide sequences and N-terminal and C-terminal specific reagents used in the immunoassays.

and simplified the identification of metabolites due to the lack of multiple glycoforms.

Stock solutions of the peptide (10 mM in water) were fused with the aglycosylated antibody (15.4 mg/mL, in 10 mM Histidine, 10 mM glycine, 2% sucrose, pH 6.5) at a 3:1 molar ratio at room temperature overnight. Protein fusion was verified by intact LC/MS analysis [17]. Peptide mapping has confirmed that the CovX-Body, as shown in Fig. 1, has two peptides fused to a lysine in the variable region of the Fab. Stock solutions were stored at 4 °C and diluted in PBS to a final working concentration of 3 mg/mL prior to dosing.

2.3. Anti-CovX antibody idiotype monoclonal antibody generation

The CovX antibody is a humanized IgG1, κ antibody selected to have aldolase activity [18]. An anti-idiotype antibody against the CovX antibody was produced by standard hybridoma technology. Balb/c mice were immunized and boosted with a Fab fragment of the CovX antibody and screened to select animals with positive responses. Spleens were removed and fused with myeloma cells to form hybridomas and plated. After an incubation period to allow hybridomas to stabilize, wells were screened for reactivity to CovX antibody and lack of reactivity to human IgG (Sigma, St. Louis, MO). Suitable wells were expanded, subcloned, and secondary screens were conducted with various IgG preparations by Biacore and ELISA methods to select a private, non-blocking anti-idiotype clone, i.e. a clone with specificity for CovX antibody, no reactivity to normal human IgG, and similar binding to CovX-Bodies with peptides of diverse size and chemical makeup. Fig. 1 shows a diagrammatic representation of the potential interaction of the anti-idiotype antibody with a CovX-body.

2.4. Total CovX-body concentration ELISA

A 96 well high binding plate (Costar®, Corning Inc.) was coated with the monoclonal anti-idiotype antibody (as described above) at 1 μg/mL in PBS (Cellgro®, Mediatech Inc., VA) by incubating the plate overnight (ON) at 4 °C. The next day, the plate was washed with 1X wash buffer (KPL Inc., MO; three cycles per wash) and was blocked with Super Block (ScyTek Laboratories, UT) for 1 h at room temperature (RT). CovX-Bodies (Standards/QCs/samples) prepared in 100% mouse serum (Rockland Inc., PA) were diluted 20 times using Super Block and were added to the washed blocked plate. The plate was incubated at RT on a horizontal orbital microplate shaker (VWR) at 400 rpm. The plate was washed and the captured CovX-Bodies were then detected by using Horseradish peroxidase (HRP-) conjugated Goat anti-human IgG (H + L) antibodies (Bethyl Laboratories Inc., TX; 20 ng/mL). The plate was further incubated for 1 h at RT at 400 rpm. After incubation, the plate was washed and the bound HRP was detected by the addition of SureBlue™ TMB Microwell Peroxidase substrate (1-component, KPL Inc.) for 6 min at RT. The reaction was stopped by addition of 2 M sulfuric acid. The absorbance was measured at 450 nm with wavelength correction set at 650 nm using a microplate reader (SpectraMax Plus®, Molecular Devices, CA).

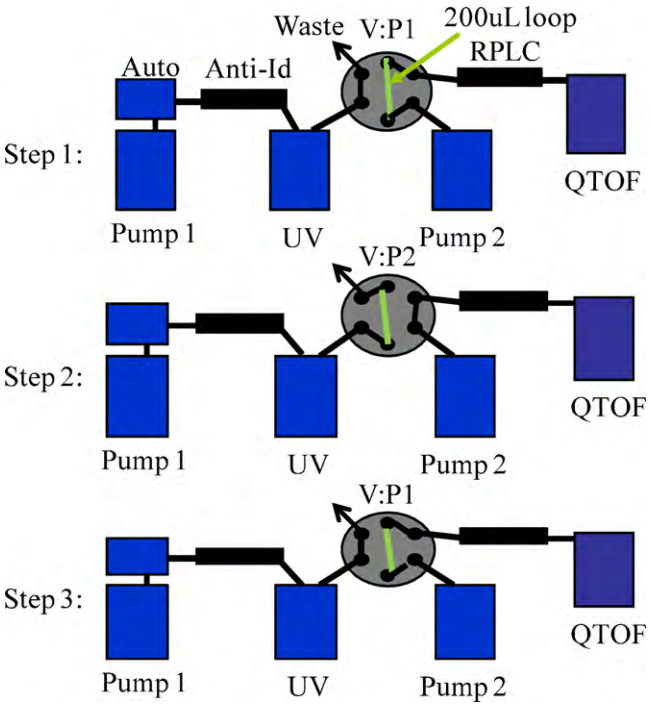

Fig. 3. 2DLC/UV/MS instrument diagram and analysis scheme.

2.5. N-terminal peptide ELISA

The assay was performed as described above with following changes. For determination of the intact N-terminus of the GLP-1/Exendin-4 peptides (Fig. 2), a biotinylated anti-GLP-1 monoclonal antibody (custom biotinylation of ABS 033-04 by Assay designs, MI) was used. The above monoclonal antibody was added (75 ng/mL for GLP-1 CovX-Body assays; 750 ng/mL for Exendin-4 CovX-Body assays) and the plate was incubated for 1 h at RT at 400 rpm. After washing, the bound biotinylated antibody was detected using Streptavidin HRP (BD Pharmingen™, CA; 1:6000).

2.6. C-terminal peptide ELISA

The assay was perfomed as described above with following changes. For determination of the intact C-terminus of the Exendin-4 peptide (Fig. 2), a biotinylated anti-Exendin-4 monoclonal antibody (Antibody Shop®, BioPorto® Diagnostics, Grusbakken, Denmark; ABS 012-35) was added to the plate at 200 ng/mL.

2.7. Two-dimensional liquid chromatography

The equipment used for the 2DLC experiments utilizes affinity chromatography in the first dimension and reversed phase liquid chromatography (RPLC) in the second dimension. The 2DLC mode of operation was a heart-cut or partial sampling of the first dimension into the second dimension [19] and shown in Fig. 3. A Waters nanoAcquity (Milford, MA) HPLC pump and autosampler was used for the affinity separation. An Agilent 1100 binary pump and ultraviolet detector (Santa Clara, CA) were used in the second dimension which was connected to the Waters quadrupole time-of-flight mass spectrometer (QTOF micro). The anti-idiotype affinity column was made in-house by coupling the anti-idiotype antibody to Ultralink support (Pierce, Rockford, IL) as per the instruction sheet provided. The resulting resin was packed into a 2.0 mm × 50 mm stainless steel column. The reversed phase column was a 1.0 mm × 100 mm Proto 300 C4 with 5 μm particles (The Nest Group, Southborough,

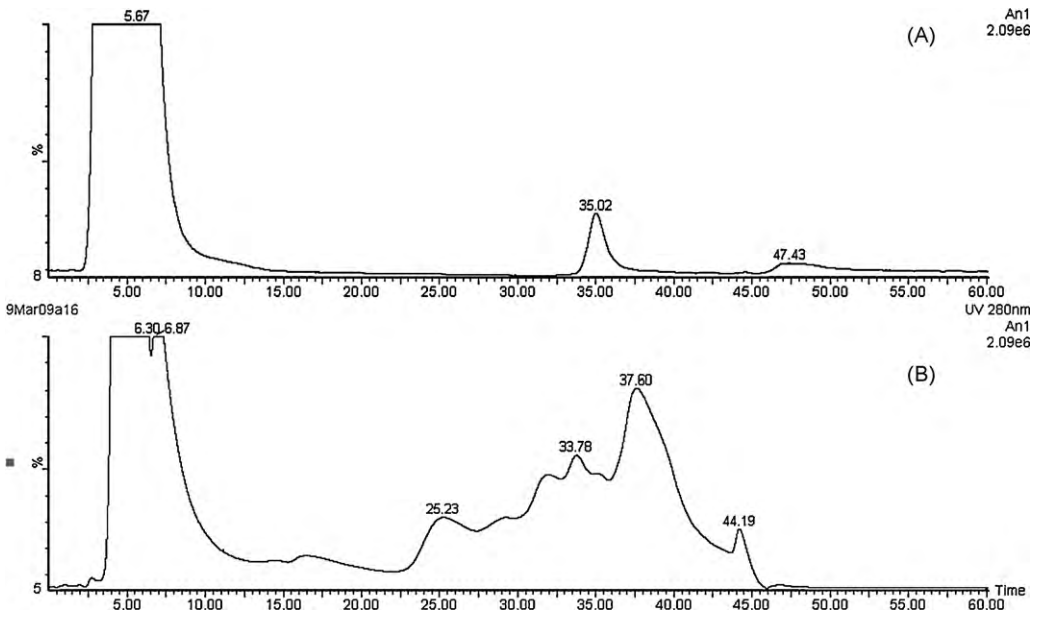

Fig. 4. Affinity chromatography of fusion antibodies in mouse serum with anti-idiotype resin (A) or protein A resin (B) with UV detection at 280 nm.

MA). The 10-port valve (Rheodyne/Idex, Oak Harbor, WA) was used between the first and second dimension separation systems. A 200 μL loop was used to collect a 4 min fraction from the affinity column which was injected onto the reversed phase column. The first dimension affinity separation flowed at 50 μL/min with a linear gradient elution from 0% to 100% B in 30 min then equilibrated for a total run of 90 min. Mobile phase A is 50 mM citric acid, pH 7.2, and mobile phase B is 0.1 M acetic acid, pH 2.2. The second dimension RPLC separation flowed at 50 μL/min with 20% B from 0 to 39 min, then a linear gradient to 90% B in 40 min. Mobile phase A was water with 0.2% formic acid, 0.05% trifluoro acetic acid, and 10% 2-propanol. Mobile phase B was acetonitrile with 0.2% formic acid, 0.05% trifluoroacetic acid, and 10% 2-propanol. The QTOF MS is operated in the scam mode from 1000 to 4000 *m*/*z* with 3 s/scan, a cone voltage of 70 V, capillary voltage of 3000 V, source at 120 °C, desolvation heater at 300 °C, and desolvation gas 400 L/h of house nitrogen. To process the mass spectra approximately 15 min of the protein chromatographic profile was averaged. The spectra was expanded from 2400 to 3400 *m*/*z* then deconvoluted using a mass range of 140,000 to 180,000, and iterated until convergence with a 10 Da/channel resolution.

The 2DLC/MS analysis takes place in 3 steps which are shown in Fig. 3. The first step is the injection of 50 μL of serum directly onto the anti-idiotype affinity column, and equilibration of the RPLC column. As the CovX-body starts to elute off the anti-idiotype column the valve switches to position 2 and collects the antibody in the loop for the second step. In the third step, the valve was switched back to position 1 and the collected fraction in the loop is injected onto the RPLC column and detected with the QTOF MS.

2.8. Animals and serum preparation

Twenty-seven male Swiss Webster mice (CFW, Charles Rivers, Hollister, CA) weighing approximately 18–20 g at the start of dosing were used in this study. Upon receipt, mice were housed in a room controlled environment (temperature 72–74 °F and humidity 46–59%RH) and provided with rodent chow and tap water (ad libitum). Mice were acclimated to laboratory conditions for at least 48 h before the start of dosing. On the day of the experiment, mice were subdivided into groups of three mice per sampling time points. A single intravenous dose (30 mg/kg) of the CovX-Body test articles were administered to mice via tail vein injection. Following dose administration, blood was collected at 6 designated time points over the course of a 2-day period (0.08, 1, 6, 24, 32 and 48 h post-dose). Mice were anesthetized with isoflurane (USP grade, Baxter Healthcare Corp., Deerfield IL) and 100 μL of blood volume were taken from three mice via retro orbital sinus bleed at 0.08–6 h. For the remaining time points up to 48 h, mice were sacrificed by CO2 inhalation and terminal cardiac samples were drawn. Samples were collected in serum separator tubes and placed on ice (∼30–60 min). A protease inhibitor cocktail (Sigma) was added to all blood collection tubes prior to sampling. Blood samples were then centrifuged at 12,000 rpm for 5–10 min. Following centrifugation, serum samples were transferred to individual micro centrifuge tubes and stored at −80 °C until analysis.

3. Results and discussion

3.1. Anti-idiotype chromatography

Chromatographic methods remain the most widely used tools in protein analysis. The separation or fractionation of a biological sample in order to reduce its complexity is often a prerequisite to qualitative or quantitative proteomics. Affinity chromatography is an efficient protein separation method based upon the interaction between target proteins and specific immobilized ligands. There is a large range of available ligands to separate complex biological matrices into different protein classes or for the isolation of low abundance proteins. Due to its high specificity, affinity chromatography reduces the complexity of a protein mixture for subsequent analysis by electrophoresis or gel-free based approaches like LC/MS. Affinity chromatography using protein A as the ligand has been widely used for the isolation of IgG from various species. In the specific case of CovX-Body isolation, anti-idiotype affinity chromatography results in greater specificity than traditional protein A separations. Fig. 4 displays the UV chromatograms of a CovX-Body at 100 μg/mL in mouse serum using both protein A and anti-idiotype ligands. The anti-idiotype column retains only the CovX-Body, whereas, the protein A column retains many IgG type antibodies. The improved specificity of the anti-idiotype affinity column reduces the background interference from other IgG antibodies and improves overall 2DLC/MS sensitivity due to reduced

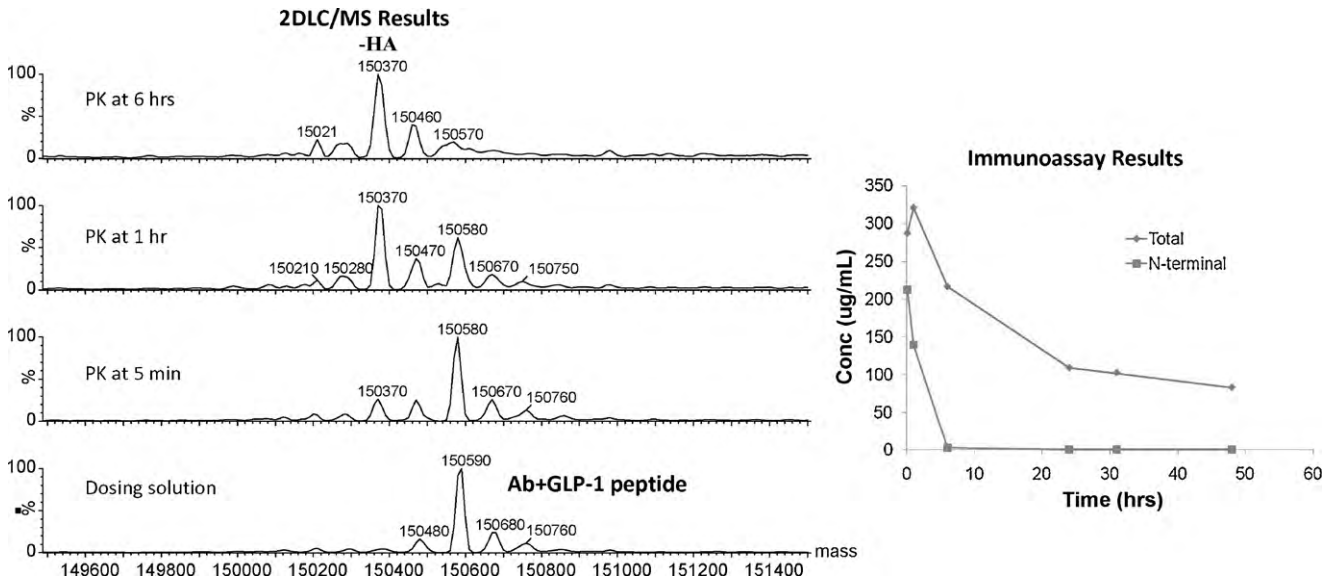

Fig. 5. Immunoassay and 2DLC/MS results for CovX-Body GLP-1 peptide conjugate.

complexity and potential suppression in the LC/MS, the second dimension of the 2DLC/MS.

3.2. CovX-body GLP-1 peptide conjugate

The immunoassay and 2DLC/MS results for the murine serum samples containing the CovX-Body GLP-1 peptide conjugate are shown in Fig. 5. This particular CovX-Body contains only one peptide when using the diketone linker confirmed by the 2DLC/MS results of the dosing solution. The aglycosylated antibody has a mass of 146,630 Da and the mass of the GLP-1 peptide plus linker contributes 3957 Da. Thus the mass at 150,590 ± 10 Da is attributed to the CovX-Body GLP-1 peptide conjugate containing one peptide. As stated in the introduction, GLP-1 is known to be degraded by dipeptidyl peptidase IV to generate N-terminally truncated metabolites. For the murine serum samples containing the CovX-Body GLP-1 peptide conjugate, the N-terminal immunoassay shows a quick decline in concentration with the conjugate virtually eliminated by 6 h. The total immunoassay shows that the CovX-Body is still present out to 48 h, but has decreased in concentration by one third of the original concentration. The 2DLC/MS results in Fig. 5 correlate well with the immunoassay in that the intact CovX-Body GLP-1 conjugate is decreasing rapidly and non-detectable after 6 h. As the intact CovX-Body GLP-1 conjugate decreases over time, the metabolite or proteolytic cleavage product at 150,370 ± 10 Da is shown to be increasing. The metabolite at 150,370 Da corresponds to a mass loss of 210 Da from the intact CovX-Body GLP-1 conjugate and corresponds to the loss of 2 amino acids from the N-terminus. The loss of the His-Ala dipeptide from the N-terminus was predicted [14], and is the reason for the drop in concentration detected by the N-terminal immunoassay since the detection antibody is specific for the N-terminus dipeptide (Fig. 2), and the C-terminus is a linkage site. The accurate mass loss of the His-Ala dipeptide is theoretically 208 Da and within the experimental error of measuring the intact antibody mass of ±10 Da.

3.3. CovX-body Exendin-4 peptide conjugate

The immunoassay and 2DLC/MS results for the analyses of the CovX-Body Exendin-4 peptide conjugate in the mouse serum samples are shown in Fig. 6. This molecule contains two peptides per antibody and was confirmed by 2DLC/MS analysis of the dosing solution. The aglycoslyted antibody has a mass of 146,630 Da and the mass of the Exendin-4 peptide plus linker contributes 4932 Da. Thus the mass at 156,500 ± 10 Da is attributed to the CovX-Body Exendin-4 peptide conjugate containing two peptides. The total CovX-Body Exendin-4 peptide conjugate concentration over time as detected by the immunoassay is similar to the GLP-1 containing CovX-body in that there is approximately one third left remaining after 48 h. The N-terminal immunoassay in Fig. 6 shows an improved concentration profile as compared to the GLP-1 CovX-body in Fig. 5. There is a divergence in the N-terminal peptide concentration in Fig. 6 after 6 h as compared to the total CovX-body concentration. A similar trend was seen in the 2DLC/MS data where a metabolite was detected after 6 h at a mass 200 Da less than the intact CovX-Body Exendin-4 peptide conjugate. It was attributed to the proteolytic cleavage of the His-Gly dipeptide from the N-terminus and due to antibody linking at the C-terminus of the peptide. The cleavage at a mass of 156,300 ± 10 Da (and first detected in the 24 h time point) increases in intensity over time. The predicted loss of the dipeptide is theoretically 194 Da and within the experimental error of measuring the intact antibody of ±10 Da. The loss of the second dipeptide (−400 Da from intact CovX-Body Exendin-4 peptide conjugate) from the opposite Fab of the antibody is also detected in the 48 h sample at a mass of 156,140 ± 10 Da.

In the CovX-Body Exendin-4 peptide conjugate, the use of the azetidinone linker resulted in two peptides being fused to the antibody, but the proteolytic cleavage at the same position as seen in the CovX-Body GLP-1 peptide conjugate resulted in a degradation of the Exendin-4 CovX-body after 6 h.

3.4. Modified CovX-body Exendin-4 peptide conjugate

To reduce the N-terminal degradation of the Exendin-4 peptide portion of the conjugate, a modified version of the peptide was made and fused to the aglycosylated antibody. The immunoassay and 2DLC/MS results for the analyses of the modified CovX-Body Exendin-4 peptide conjugate in the murine serum samples are shown in Fig. 7. The modified CovX-Body Exendin-4 peptide conjugate contains two peptides per antibody and was confirmed by 2DLC/MS analysis. The aglycoslyted antibody has a mass of 146,630 Da and the mass of the modified Exendin-4 peptide plus linker contributes 4830 Da. Thus the mass at 156,310 ± 10 Da is attributed to the modified CovX-Body Exendin-4 peptide conjugate

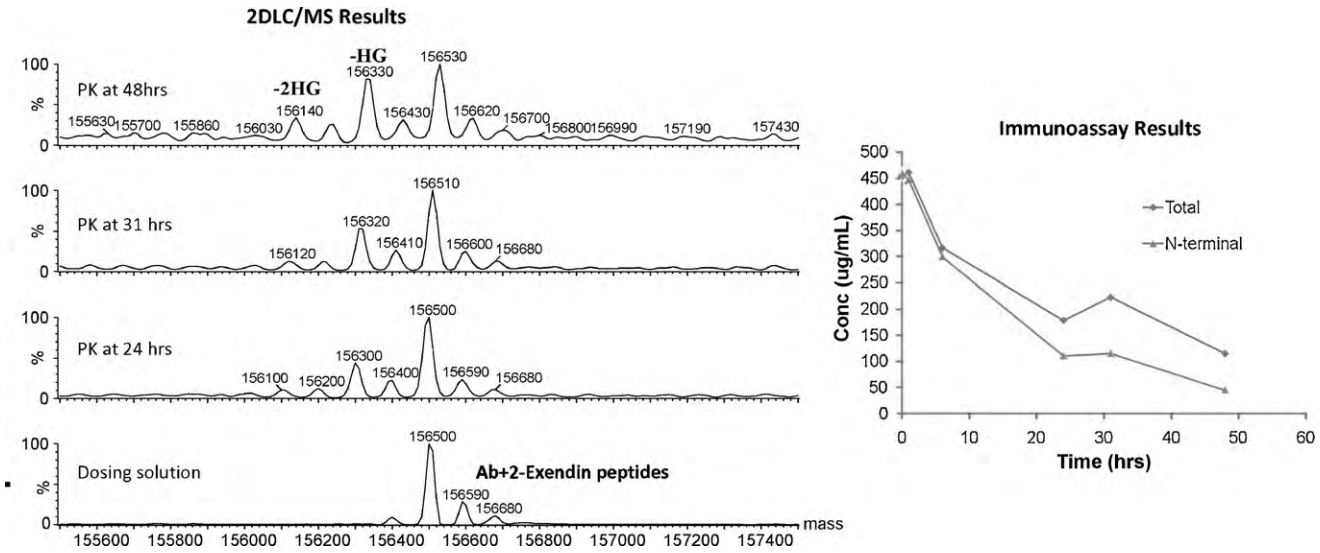

Fig. 6. Immunoassay and 2DLC/MS results for CovX-Body Exendin-4 peptide conjugate.

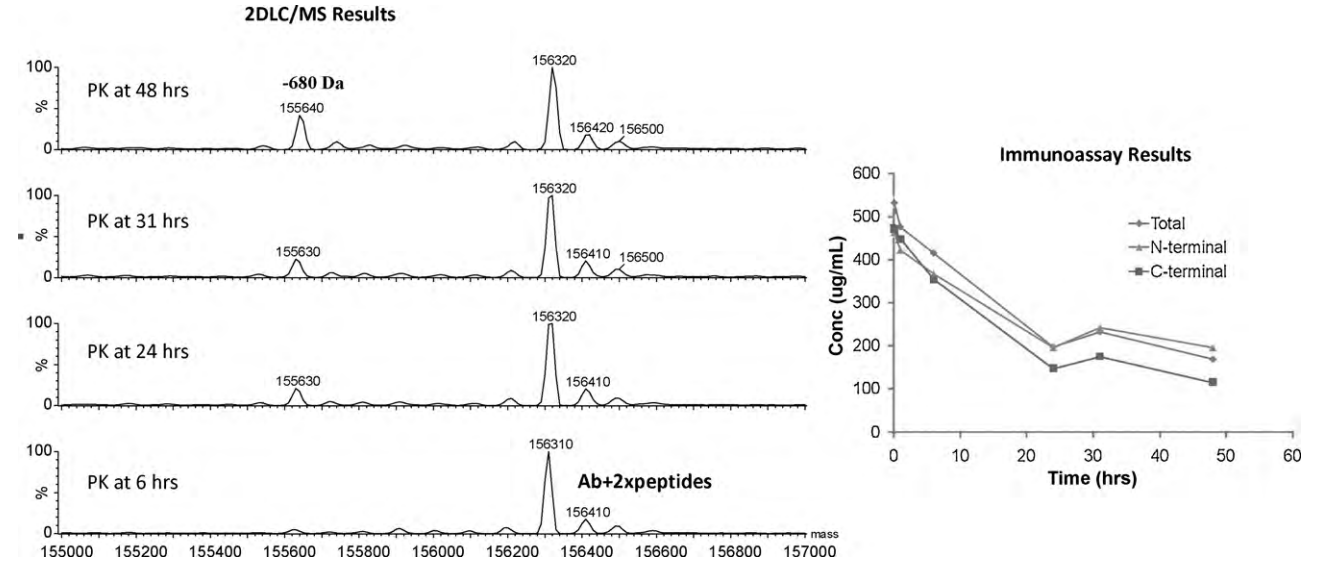

Fig. 7. Immunoassay and 2DLC/MS results for modified CovX-Body Exendin-4 conjugate.

containing two peptides. The total CovX-body concentration over time as detected by the immunoassay is similar to the GLP-1 and Exendin-4 containing CovX-bodies in that there is approximately one third left remaining after 48 h. However, the data from the N-terminal immunoassay in Fig. 7 shows an improved concentration profile as compared to the CovX-Body GLP-1 peptide conjugate in Fig. 5, and the CovX-Body Exendin-4 peptide conjugate in Fig. 6 due to modification of the N-terminal amino acid sequence. Since the modified CovX-Body Exendin-4 peptide conjugate was linked in the middle of the peptide there is a possibility for proteolytic cleavage to occur on both the N and C-terminus, so antibodies specific to these peptide regions were used (Fig. 2). Two separate immunoassays were used to determine if the N- and C-terminal ends of the peptide were intact and present in the CovX-bodies. There was a divergence in concentration observed in Fig. 7 after 6 h, but only for the data from the C-terminal immunoassay. There was a similar trend in the 2DLC/MS data where a metabolite at 680 Da less than the intact modified CovX-Body Exendin-4 peptide conjugate was detected after 6 h, and was attributed to the proteolytic cleavage of the eight C-terminal amino acids (C-terminal of Pro in Fig. 2). This cleavage is observed in the 2DLC/MS results in Fig. 7 (mass of 155,630 ± 10 Da), first detected in the 24 h time point and is shown to increase in intensity over time. The loss of this octapeptide corresponds to a theoretical mass of 680 Da and is within the experimental error of intact antibody measurement of ±10 Da.

4. Conclusions

The combination of immunoassay and 2DLC/MS for the successful investigation of intact antibody metabolites has been demonstrated. To overcome the limitations of these individual techniques, the specificity of an anti-idotype monoclonal antibody for capture of the CovX-Bodies from serum samples has been exploited. The anti-idiotype antibody has minimized interference from common serum components in the immunoassay, as well as, reduced the suppression of interfering proteins in the 2DLC/MS spectra making these two techniques ideal for investigation of intact antibody metabolites. While the immunoassay was able to quantitate intact antibody metabolites, the 2DLC/MS analysis greatly enhanced the overall quality of the analysis. The

2DLC/MS method described does not require N- and C-terminal specific reagents to identify individual residues where degradation occurs, but the immunoassays are helpful in narrowing the investigation to the areas where degradation does occur (either N- or C-terminus). The next level in this specific type of mass spectrometry identification would allow for MS/MS sequencing of the intact antibody metabolites [14]. The identification of antibody metabolites by combining these techniques will enable better understanding of biotherapeutic metabolism at the molecular level and possibly reduce drug development cycle times.

Acknowledgements

The authors thank Jeanette Dilley, Amisha Kamal Kizhakkedathu, Yasmina Addiche, Jessica Yu of Rinat, Marla Matin and Trina Osothprarop of CovX for the preparation of the anti-idiotype antibody. Colleen Brown, Charles Appah, Amber Pham, Mathias Rickert, Michael Chin of Rinat and Julie Hoye of CovX for the preparation of the aglycoslyated CovX antibody. James Kerr and Rodney Lappe of CovX for support and review of the manuscript.

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