



(51) International Patent Classification:

C07K 16/18 (2006.01) G01N 33/68 (2006.01)
C07K 16/40 (2006.01)

(21) International Application Number:

PCT/US2018/024769

(22) International Filing Date:

28 March 2018 (28.03.2018)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/480,048 31 March 2017 (31.03.2017) US

(71) Applicant: ALEXION PHARMACEUTICALS, INC.
[US/US]; 100 College St., New Haven, CT 06510 (US).

(72) Inventors: PELTO, Ryan; 100 College Street, New
Haven, CT 06510 (US). CHEN, Meng; 100 College St.,
New Haven, Connecticut 06510 (US).

(74) Agent: REMILLARD, Jane, E. et al; Nelson Mullins Ri-
ley & Scarborough LLP, One Post Office Square, Boston,
MA 02109-2127 (US).

(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ,
CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO,
DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN,
HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP,
KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME,
MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ,
OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA,
SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,
TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ,
UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ,
TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,
EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV,
MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,
TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

- as to the identity of the inventor (Rule 4.1 7(i))
- of inventorship (Rule 4.1 7(iv))

Published:

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: METHOD FOR SIMULTANEOUS QUANTIFICATION OF ALXN1210 AND ECULIZUMAB IN HUMAN SERUM OR URINE

(57) Abstract: Provided herein are methods for simultaneously detecting and quantifying antibodies which bind to the same target and have high sequence identity, such as eculizumab and ALXN1210, present together or alone in a biological sample.



METHOD FOR SIMULTANEOUS QUANTIFICATION OF ALXN1210 AND ECULIZUMAB IN HUMAN SERUM OR URINE

RELATED INFORMATION

This application claims the benefit of the priority date of U.S. Provisional Application No. 62/480048, filed on March 31, 2017, the content OF which is hereby incorporated by reference in its entirety.

BACKGROUND

The ability to accurately and reliably determine the pharmacokinetics of pharmaceutical compounds in a variety of preclinical models and clinical samples is essential to designing clinical trials and dosing regimens for determining optimal efficacy and minimal toxicity. This maximizes the chances for success in late-stage clinical trials and is a necessary component in the regulatory approval process. Due to their high likelihood for success and reduced development times and costs, therapeutic monoclonal antibodies (mAbs) are becoming increasingly important to the pharmaceutical industry. These proteins are based on naturally generated immunoglobulins (IgGs), but can be protein engineered to modify their natural properties and functions and to act by binding a target and inhibiting its activity or removing it from circulation. Monitoring mAb concentrations in serum is commonly performed by using enzyme-linked immunosorbent assays (ELISAs). This technique is sensitive and fast enough to efficiently analyze thousands of samples. However, it suffers from significant limitations when it comes to the quantification of antibodies which bind to the same target or different versions of the same antibody binding to the same target. In addition, immunoassays may be subject to matrix interferences and can have long development times. Furthermore, when moving between preclinical models, or from preclinical to clinical samples, or rehumanizing or otherwise re-engineering the antibody to reduce toxicological or immunological effects in the preclinical model and human clinical trials, the assay often needs to be redeveloped as drug development progresses. Thus, it would be useful to develop other methods to quantify mAbs that result in improved data quality and reduce development times and costs.

SUMMARY

Provided herein is a method for simultaneously detecting and quantifying antibodies which bind to the same target and have high sequence identity, such as eculizumab and

ALXN1210 (ravulizumab), present together or alone in a biological sample (*e.g.*, human serum or urine). This method is significant because while the two antibodies both bind the same target they differ only in four amino acids for the entire heavy and light chains.

In one aspect, the method comprises quantifying and detecting the respective amounts of eculizumab and ALXN1210 present together in a biological sample (*e.g.*, human serum or urine) by: (a) treating the biological sample containing the antibodies with a protease to form a proteolytic peptide mixture of peptides from the antibodies in the biological sample, (b) analyzing a sample of the proteolytic peptide mixture by high performance liquid chromatography (HPLC) tandem mass spectrometry to detect a signature peptide from each of the antibodies in the biological sample, and (c) quantifying each antibody in the biological sample based on the signal ratio of its signature peptide to an internal control, wherein the internal control comprises a labeled form of the same signature peptide. In one embodiment, the signature peptide for eculizumab comprises or consists of SEQ ID NO: 1 and the signature peptide for ALXN1210 comprises or consists of SEQ ID NO: 2.

In another aspect, the method comprises detecting and quantifying the amount of eculizumab present in a biological sample (*e.g.*, human serum or urine) by: (a) treating the biological sample containing the antibody with a protease to form a proteolytic peptide mixture of the antibody in the biological sample, (b) analyzing a sample of the proteolytic peptide mixture by high performance liquid chromatography (HPLC) tandem mass spectrometry to detect a signature peptide, and (c) quantifying the amount of eculizumab in the biological sample based on the signal ratio of the signature peptide to an internal control, wherein the internal control comprises a labeled form of the same signature peptide. In one embodiment, the signature peptide comprises or consists of SEQ ID NO: 1.

In another aspect, the method comprises detecting and quantifying the amount of ALXN1210 present in a biological sample (*e.g.*, human serum or urine) by: (a) treating the biological sample containing the antibody with a protease to form a proteolytic peptide mixture of the antibody in the biological sample, (b) analyzing a sample of the proteolytic peptide mixture by high performance liquid chromatography (HPLC) tandem mass spectrometry to detect a signature peptide, and (c) quantifying the amount of ALXN1210 in the biological sample based on the signal ratio of the signature peptide to an internal control, wherein the

internal control comprises a labeled form of the same signature peptide. In one embodiment, the signature peptide comprises or consists of SEQ ID NO: 2.

In a particular embodiment, a method of detecting and quantifying the respective amounts of two antibodies having high sequence identity present together in a biological sample (*e.g.*, human serum or urine) is provided, wherein the antibodies are eculizumab and ALXN1210, the method comprising: (a) treating the biological sample containing the antibodies with a protease to form a proteolytic peptide mixture of the antibodies in the biological sample, (b) analyzing a sample of the proteolytic peptide mixture by high performance liquid chromatography (HPLC) tandem mass spectrometry to detect a signature peptide from each of the antibodies in the biological sample, wherein the signature peptide for eculizumab is selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 19, and SEQ ID NO: 20 and the signature peptide for ALXN1210 is selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 21, and SEQ ID NO: 22, and (c) quantifying each antibody in the biological sample based on the signal ratio of its signature peptide to an internal control, wherein the internal control comprises a labeled form of the same signature peptide.

In another embodiment, a method of detecting and quantifying the amount of an antibody present in a biological sample (*e.g.*, human serum or urine) is provided, wherein the antibody is eculizumab, the method comprising: (a) treating the biological sample containing the antibody with a protease to form a proteolytic peptide mixture of the antibody in the biological sample, (b) analyzing a sample of the proteolytic peptide mixture by high performance liquid chromatography (HPLC) tandem mass spectrometry to detect a signature peptide, wherein the signature peptide for eculizumab is selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 19, and SEQ ID NO: 20, and (c) quantifying the amount of eculizumab in the biological sample based on the signal ratio of the signature peptide to an internal control, wherein the internal control comprises a labeled form of the same signature peptide.

In yet another embodiment, a method of detecting and quantifying the amount of an antibody present in a biological sample (*e.g.*, human serum or urine) is provided, wherein the antibody is ALXN1210, the method comprising: (a) treating the biological sample containing the antibody with a protease to form a proteolytic peptide mixture of the antibody in the biological sample, (b) analyzing a sample of the proteolytic peptide mixture by high performance liquid chromatography (HPLC) tandem mass spectrometry to detect a signature peptide, wherein the

signature peptide for ALXN1210 is selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 21, and SEQ ID NO: 22, and (c) quantifying the amount of ALXN1210 in the biological sample based on the signal ratio of the signature peptide to an internal control, wherein the internal control comprises a labeled form of the same signature peptide.

The methods described herein can include additional steps. For example, in one embodiment, the methods further comprise contacting the biological sample with an affinity capture reagent, such as bead-supported Protein A, prior to treating it with a protease (*e.g.*, trypsin). In another embodiment, the method further comprises washing the Protein A bound antibodies to remove unbound components prior to proteolysis. In another embodiment, the method further comprises denaturing the antibody sample. In another embodiment, the method further comprises reducing the antibody sample. In another embodiment, the method further comprises alkylating the antibody sample. In one embodiment, the denaturation, reduction, and alkylation steps unfold the antibody protein and facilitates proteolytic digestion.

Any suitable mass spectrometry-based assay can be used in the methods described herein. In another embodiment, the mass spectrometry-based assay is reverse-phase UPLC-MS/MS.

The signature peptides for use in the methods described herein are no more than 20 amino acids in length. For example, in one embodiment, the signature peptide is no more than 20, 19, 18, 17, 16, or 15 amino acids in length. In one embodiment, the signature peptide comprises or consists of SEQ ID NO: 1. In another embodiment, the signature peptide comprises or consists of SEQ ID NO: 2. In one embodiment, the signature peptide for eculizumab is SEQ ID NO: 1. In another embodiment, the signature peptide for eculizumab is SEQ ID NO: 19. In another embodiment, the signature peptide for eculizumab is SEQ ID NO: 20. In another embodiment, the signature peptide for ALXN1210 is SEQ ID NO: 2. In another embodiment, the signature peptide for ALXN1210 is SEQ ID NO: 21. In yet another embodiment, the signature peptide for ALXN1210 is SEQ ID NO: 22.

Further provided are isolated peptides consisting of particular sequences. In one embodiment, an isolated peptide consisting of SEQ ID NO: 1 is provided. In another embodiment, an isolated peptide consisting of SEQ ID NO: 2 is provided. In yet another embodiment, an isolated peptide consisting of SEQ ID NO: 19 is provided. In a further embodiment, an isolated peptide consisting of SEQ ID NO: 20 is provided. In another

embodiment, an isolated peptide consisting of SEQ ID NO: 21 is provided. In another embodiment an isolated peptide consisting of SEQ ID NO: 22 is provided.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram of a hybrid immunocapture liquid chromatography mass spectrometry assay.

Figure 2 shows the sequences of the heavy and light chains of eculizumab and ALXN1210. The four amino acid differences between eculizumab and ALXN1210 heavy chain sequences are underlined.

Figure 3 shows the effect of different surfactants used during protease digestion on the ultimate signature peptide signal in mass spectrometry.

Figure 4 shows the effect of surfactant concentration on the ratio of the analyte being measured compared to a labeled internal standard of the same peptide at a known concentration.

Figure 5 shows the effect of 1% DMSO added to the mobile phase of the chromatography step of the procedure on the ultimate signal of the signature peptides in mass spectroscopy.

Figure 6 shows a representative extracted ion chromatogram (XIC) of a blank sample.

Figure 7 shows a representative extracted ion chromatogram (XIC) of an LLOQ sample.

DETAILED DESCRIPTION

Eculizumab, also known as Soliris[®], is a humanized monoclonal antibody manufactured in a single unit dosage form comprising 300 mg in 30 ml (10 mg/ml) solution for infusion and having binding specificity for the human complement protein C5. Eculizumab V regions and their humanization were described in US Patent No: 6,355,245, the teachings of which are hereby expressly incorporated by reference. Eculizumab is comprised of 1324 amino acids with a molecular mass of approximately 148 kDa.

The CDR1, CDR2 and CDR3 heavy chain sequences of eculizumab are shown in SEQ ID NOs: 3, 4, and 5, respectively. The CDR1, CDR2 and CDR3 light chain sequences of eculizumab are shown in SEQ ID NOs: 6, 7, and 8, respectively. The heavy chain variable region sequence of eculizumab is shown in SEQ ID NO: 9 and the light chain variable region sequence is shown in SEQ ID NO: 10. The heavy and light chain sequences of eculizumab are shown in SEQ ID NOs: 12 and 13, respectively.

ALXN1210 is an anti-C5 antibody described in PCT/US20 15/0 19225 and US Patent No. 9,079,949, the teachings of which are hereby expressly incorporated by reference. ALXN1210 is a humanized monoclonal antibody that is highly structurally related to eculizumab. ALXN1210 was recently given the generic name ravulizumab and was derived through minimal targeted engineering of eculizumab by introducing four unique amino acid substitutions into the heavy chain, with the objective of enhancing the duration of terminal complement inhibition, while maintaining key eculizumab attributes such as epitope and low immunogenicity. Therefore, ravulizumab (ALXN1210) and eculizumab share over 99% primary sequence identity and have highly similar pharmacology, except ravulizumab has a Tl/2 in humans of greater than 40 days as compared to eculizumab' s Tl/2 in human of approximately 12 days. Ravulizumab and ALXN1210 are used interchangeably herein. ALXN1210 selectively binds to human complement protein C5, inhibiting its cleavage to C5a and C5b during complement activation.

The CDR1, CDR2 and CDR3 heavy chain sequences of ALXN1210 are shown in SEQ ID NOs: 17, 18, and 5, respectively. The CDR1, CDR2 and CDR3 light chain sequences of ALXN1210 are shown in SEQ ID NOs: 6, 7, and 8, respectively. The heavy chain variable region sequence of ALXN1210 is shown in SEQ ID NO: 14. The light chain variable region sequence of ALXN1210 is shown in SEQ ID NO: 10. The entire heavy chain sequence of ALXN1210 is shown in SEQ ID NO: 16. The entire light chain sequence of ALXN1210 is shown in SEQ ID NO: 13.

The present disclosure addresses the important need for accurately quantifying eculizumab and ALXN1210 simultaneously in a biological sample from a patient (e.g., human serum or urine), particularly in a clinical setting where the patient is being switched from one antibody to the other during the course of treatment. The methods described herein can be adapted to switching from eculizumab to other anti-C-antibodies or other complement inhibitors. Such quantification is critical to determine accurate dosing regimens. Concurrent quantification of eculizumab and ALXN1210 has been extremely challenging due to the highly similar sequences of the two antibodies, which, as described, differ by only four amino acids, and because the antibodies bind to the same target. The methods described herein would be more easily adapted to switching from eculizumab to a an alternative complement inhibitor having more structural differences from eculizumab than does ALXN1210. Ligand binding assays, such as ELISA, quantify antibodies by their epitopes, and thus are insufficient to accurately quantify

eculizumab and ALXN1210, because of the similarity in their sequence and the fact that these antibodies both bind to human C5 at the same epitope. Mass spectrometry relies on unique amino acid (peptide) sequences to distinguish among proteins. Accordingly, in view of the greater than 99% sequence identity between eculizumab and ALXN1210, prior mass spectrometry assays have lacked the necessary sensitivity or selectivity to discriminate between these antibodies. Moreover, this problem is exacerbated by the fact that human serum contains a multitude of endogenous antibodies, many of which also share regions of sequence identity and/or homology with eculizumab and ALXN1210, thus creating further noise and hindering accurate quantification of the antibodies.

The assay described in the present disclosure solves the above-discussed problem and provides experimental data demonstrating that the methods described herein can be used to reliably detect and quantify eculizumab and ALXN1210 in human serum and urine samples.

Digestion

In order to produce signature peptides for analysis by mass spectrometry, a biological sample such as serum or urine containing eculizumab and/or ALXN1210 is treated with a protease, either with or without an initial capture step, to form a digested antibody sample containing one or more signature peptides from the respective antibodies. The term "biological sample", as used herein, refers to any component derived or separated from an animal or human patient and includes urine, blood, plasma, and serum.

The term "signature peptide", as used herein, refers to a peptide that shows experimentally advantageous chromatographic and mass spectrometric properties and is unique (i.e., specific to eculizumab or ALXN1210). "Advantageous chromatographic performance", as used herein, can be defined as narrow peaks, low background noise with high peptide recovery. "Good mass spectrometric performance", as used herein, can be indicated by relatively high parent ion and fragment ion intensities with a high degree of selectivity for the sequence of the signature peptide. In one embodiment, the signature peptide is no more than 20, 19, 18, 17, 16, or 15 amino acids in length. In another embodiment, the signature peptide for eculizumab is selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 19, and SEQ ID NO: 20. In another embodiment, the signature peptide for ALXN1210 is selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 21, and SEQ ID NO: 22.

The term "protease", as used herein, refers to an enzyme capable of cleaving or hydrolyzing peptides or proteins into fragments in either a specific or generic, random manner. Example proteases include trypsin, papain, endoproteinase LysC, endoproteinase ArgC, *staph aureus* V8, chymotrypsin, Asp-N, Asn-C, pepsin, and endoproteinase GluC.

In one embodiment, prior to digestion, the biological sample is first contacted with an affinity capture reagent. After washing with an appropriate biological buffering system, an enriched biological sample is then eluted and treated with the protease. The term "affinity capture reagent", as used herein, refers to an antibody capture reagent or an antigen immobilized to a solid substrate. In one embodiment, the affinity capture reagent is Protein A or G. In another embodiment, the affinity capture reagent is the target antigen of the antibody or complement protein C5 or complement protein C5 attached to a solid substrate. In another embodiment, the affinity capture reagent is biotinylated. In another embodiment, the protein A or G is immobilized or conjugated onto a matrix and placed in a chromatography column format. In another embodiment, the protein A or G is immobilized on magnetic beads and the unbound material may be washed away and the bound antibodies may be digested by proteases while still attached to the beads.

After the biological sample is contacted and bound with the affinity reagent, it is then washed to remove non-specifically bound host proteins or other biomolecules. In a particular embodiment, the antibody bound to the affinity capture reagent is denatured to facilitate efficient elution from the affinity reagent and complete protease digestion. In one embodiment, the denaturation is performed using a surfactant. In one particular embodiment, the denaturation is performed using RAPIGEST™. In another particular embodiment, the denaturation is performed using PROTEASEMAX™. In certain embodiments, the antibody bound to the affinity reagent is also reduced to break any disulfide bonds and to further facilitate protease digestion. In a particular embodiment, reduction is performed using dithiothreitol (DTT). In another embodiment the reduced antibody is alkylated to prevent reformation of disulfide bonds. In one embodiment, alkylation is performed using iodoacetic acid.

High Performance Liquid Chromatography and Mass Spectrometry

Following digestion, the processed sample is analyzed using high performance liquid chromatography in tandem with mass spectrometry to quantitate the amount of each respective antibody present in the biological sample.

Mass spectrometry techniques are well known in the art (e.g., reviewed in Yates, et al., *Annu Rev Biomed Eng.* 2009; 11:49-79). In one embodiment, the mass spectrometry is performed by liquid chromatography/mass spectrometry (LC/MS). Other forms of mass spectrometry which can be employed include, for example, ultra performance liquid chromatography (UPLC) or tandem mass spectrometry (MS/MS). In a particular embodiment, reverse phase liquid chromatography tandem mass spectrometry (RPLC/MS/MS) is used. Ionization techniques which can be used include electron impact ionization (EI), chemical ionization (CI), desorption chemical ionization (DCI), fast atom bombardment (FAB), atmospheric pressure chemical ionization (APCI), electrospray ionization (ESI), matrix-assisted laser desorption/ionization (MALDI). Mass analyzers which can be employed include Quadrupole, Time-of-Flight (TOF), Orbitrap, and Linear ion trap. In some embodiments, the analyzers are used for tandem MS. In one embodiment, a triple quadrupole mass analyzer is used.

As used herein, "chromatography" refers to a process in which a chemical mixture carried by a liquid or gas is separated into components as a result of differential distribution and separation of the chemical entities as they flow around or over a stationary liquid or solid phase.

As used herein, "liquid chromatography" (LC) means a process of separating the components of a fluid solution as the fluid uniformly flows through a column of a finely defined substance, particle or through capillary passageways. The separation results from the distribution of the components according to size and/or chemical characteristics of the mixture as differential amounts of time is spent associated between one or more stationary phases and the bulk fluid, (i.e., mobile phase), as this fluid moves relative to the stationary phase(s). Liquid chromatography includes high performance liquid chromatography (HPLC), reverse phase liquid chromatography (RPLC), and high turbulence liquid chromatography (HTLC), hydrophilic interaction liquid chromatography (HILIC), and normal phase liquid chromatography (NPLC).

The chromatographic column typically includes a medium (i.e., a packing material) to facilitate separation of chemical moieties (i.e., fractionation). The medium may include minute particles. The particles may include a bonded surface that covalently binds different chemical

functional groups that interact with the various chemical moieties within the sample mixture to facilitate separation of the chemical moieties such as the signature peptides quantified in the experiments herein. Separation is often dependent on both size and chemical characteristic. One suitable bonded surface is a hydrophobic bonded surface such as an alkyl bonded surface. Alkyl bonded surfaces may include C4, C8, or C18 bonded alkyl groups to separate increasingly hydrophobic entities. The chromatographic column includes an inlet for receiving a sample and an outlet for discharging an effluent that includes the fractionated and separated sample components. The digested antibody sample is applied to the column at the inlet, eluted with a solvent or solvent mixture or solvent gradient, and discharged at the outlet. Different solvent modes may be selected for eluting different peptides of interest. For example, liquid chromatography may be performed using a gradient mode, an isocratic mode, or a polytypic (i.e., mixed) mode. In one embodiment, the starting or sample loading solvent is an aqueous solvent with 5% organic modifier such as acetonitrile and elution occurs as the concentration of acetonitrile is increased to 50% over 50 minutes. In some embodiments, an additional mobile phase additive is used. In other embodiments, the mobile phase additive is DMSO.

The chromatographically separated components can be coupled with a mass spectrometer to measure the molecular weight of a particular fractionated species present in each peak eluting from the chromatography column. Data from mass spectrometry can then be used to detect and quantify the amount of signature peptide as it elutes from the column, and thus the respective amount of antibody present in the biological sample may be calculated and compared, using art recognized techniques as discussed below.

Quantification

Quantification of the signature peptides normalized to analogous internal standard peptides can be achieved using calculations from a standard curve.

In one embodiment, absolute quantifications of the signature peptides and the corresponding source antibodies are determined. Quantitative measurements of the signature peptides can be performed using internal standard peptides, such as the signature peptides in labeled form (i.e., so they are structurally and chemically similar to the signature peptides, but the mass is detectably different due to an isotopic label). Thus, the eluent from the separation process comprises an amount of the signature peptides that is in proportion to the known amount

of the labeled internal standard peptides in the sample. It is then possible to relate the amount of the signature peptides in the sample to the amounts of the antibody from which it originated via the standard curve. In one embodiment, the internal standard peptides are isotopically labeled and incubated at a defined concentration with the antibody fragments. As used herein, "isotopically labeled" refers to a peptide that has been enriched synthetically with one or more heavy atom isotopes (e.g., stable isotopes such as Deuterium, ^{13}C , ^{15}N , ^{18}O , ^{37}Cl or ^{81}Br).

Methods

Provided herein are methods for simultaneously detecting and quantifying antibodies which bind to the same target and have high sequence identity, such as eculizumab and ALXN1210 (ravulizumab), present together or alone in a biological sample (e.g., human serum or urine).

In one aspect, the method comprises quantifying and detecting the respective amounts of eculizumab and ALXN1210 present together in a biological sample (e.g., human serum or urine) by: (a) treating the biological sample containing the antibodies with a protease to form a proteolytic peptide mixture of peptides from the antibodies in the biological sample, (b) analyzing a sample of the proteolytic peptide mixture by high performance liquid chromatography (HPLC) tandem mass spectrometry to detect a signature peptide from each of the antibodies in the biological sample, and (c) quantifying each antibody in the biological sample based on the signal ratio of its signature peptide to an internal control, wherein the internal control comprises a labeled form of the same signature peptide. In one embodiment, the signature peptide for eculizumab comprises or consists of SEQ ID NO: 1 and the signature peptide for ALXN1210 comprises or consists of SEQ ID NO: 2.

In another aspect, the method comprises detecting and quantifying the amount of eculizumab present in a biological sample (e.g., human serum or urine) by: (a) treating the biological sample containing the antibody with a protease to form a proteolytic peptide mixture of the antibody in the biological sample, (b) analyzing a sample of the proteolytic peptide mixture by high performance liquid chromatography (HPLC) tandem mass spectrometry to detect a signature peptide, and (c) quantifying the amount of eculizumab in the biological sample based on the signal ratio of the signature peptide to an internal control, wherein the internal

control comprises a labeled form of the same signature peptide. In one embodiment, the signature peptide comprises or consists of SEQ ID NO: 1.

In another aspect, the method comprises detecting and quantifying the amount of ALXN1210 present in a biological sample (*e.g.*, human serum or urine) by: (a) treating the biological sample containing the antibody with a protease to form a proteolytic peptide mixture of the antibody in the biological sample, (b) analyzing a sample of the proteolytic peptide mixture by high performance liquid chromatography (HPLC) tandem mass spectrometry to detect a signature peptide, and (c) quantifying the amount of ALXN1210 in the biological sample based on the signal ratio of the signature peptide to an internal control, wherein the internal control comprises a labeled form of the same signature peptide. In one embodiment, the signature peptide comprises or consists of SEQ ID NO: 2.

In one embodiment, a method of detecting and quantifying the respective amounts of two antibodies having high sequence identity present together in a biological sample (*e.g.*, human serum or urine) is provided, wherein the antibodies are eculizumab and ALXN1210, the method comprising: (a) treating the biological sample containing the antibodies with a protease to form a proteolytic peptide mixture of the antibodies in the biological sample, (b) analyzing a sample of the proteolytic peptide mixture by high performance liquid chromatography (HPLC) tandem mass spectrometry to detect a signature peptide from each of the antibodies in the biological sample, wherein the signature peptide for eculizumab is selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 19, and SEQ ID NO: 20 and the signature peptide for ALXN1210 is selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 21, and SEQ ID NO: 22, and (c) quantifying each antibody in the biological sample based on the signal ratio of its signature peptide to an internal control, wherein the internal control comprises a labeled form of the same signature peptide.

In another embodiment, a method of detecting and quantifying the amount of an antibody present in a biological sample (*e.g.*, human serum or urine) is provided, wherein the antibody is eculizumab, the method comprising: (a) treating the biological sample containing the antibody with a protease to form a proteolytic peptide mixture of the antibody in the biological sample, (b) analyzing a sample of the proteolytic peptide mixture by high performance liquid chromatography (HPLC) tandem mass spectrometry to detect a signature peptide, wherein the signature peptide for eculizumab is selected from the group consisting of SEQ ID NO: 1, SEQ

ID NO: 19, and SEQ ID NO: 20, and (c) quantifying the amount of eculizumab in the biological sample based on the signal ratio of the signature peptide to an internal control, wherein the internal control comprises a labeled form of the same signature peptide.

In yet another embodiment, a method of detecting and quantifying the amount of an antibody present in a biological sample (*e.g.*, human serum or urine) is provided, wherein the antibody is ALXN1210, the method comprising: (a) treating the biological sample containing the antibody with a protease to form a proteolytic peptide mixture of the antibody in the biological sample, (b) analyzing a sample of the proteolytic peptide mixture by high performance liquid chromatography (HPLC) tandem mass spectrometry to detect a signature peptide, wherein the signature peptide for ALXN1210 is selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 21, and SEQ ID NO: 22, and (c) quantifying the amount of ALXN1210 in the biological sample based on the signal ratio of the signature peptide to an internal control, wherein the internal control comprises a labeled form of the same signature peptide.

The methods described herein can include additional steps. For example, in one embodiment, the methods further comprise contacting the biological sample with an affinity capture reagent, such as bead-supported Protein A, prior to treating it with a protease (*e.g.*, trypsin). In another embodiment, the method further comprises washing the Protein A bound antibodies to remove unbound components prior to proteolysis. In another embodiment, the method further comprises denaturing the antibody sample. In another embodiment, the method further comprises reducing the antibody sample. In another embodiment, the method further comprises alkylating the antibody sample. In one embodiment, the denaturation, reduction, and alkylation steps unfold the antibody protein and facilitates proteolytic digestion.

Isolated Peptides

Further provided are isolated peptides consisting of particular sequences. In one embodiment, an isolated peptide consisting of SEQ ID NO: 1 is provided. In another embodiment, an isolated peptide consisting of SEQ ID NO: 2 is provided. In yet another embodiment, an isolated peptide consisting of SEQ ID NO: 19 is provided. In a further embodiment, an isolated peptide consisting of SEQ ID NO: 20 is provided. In another embodiment, an isolated peptide consisting of SEQ ID NO: 21 is provided. In another embodiment an isolated peptide consisting of SEQ ID NO: 22 is provided.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments described herein. Such equivalents are intended to be encompassed by the following claims. In addition, all references cited throughout this application, are hereby incorporated by reference herein in their entirety, as though individually incorporated by reference. All sequence listing information also is part of the present disclosure.

EXAMPLES

Example 1: Hybrid immunocapture UPLC-MS/MS approach for concurrent detection and quantification of ALXN1210 and eculizumab in human serum

To concurrently detect and quantify ALXN1210 and eculizumab in a human serum sample, the following hybrid immunocapture UPLC-MS/MS approach was developed (shown schematically in **Figure 1**). Due to the high molecular weight of ALXN1210 and eculizumab, practical direct quantitative analysis using LC/triple quadrupole mass spectrometric technology was not feasible. An immunoaffinity capture approach was developed to enrich ALXN1210 and eculizumab from human serum using magnetic beads coated with Protein A. The extracted natural antibodies and drug antibody proteins were subjected to on-bead proteolysis: denaturation with low concentration of organic solvents, reduction with dithiothreitol (DTT) at 60°C, alkylation with iodoacetic acid, and simultaneous or subsequent digestion with the protease trypsin. Next, characteristic "signature" peptides generated from each antibody after tryptic proteolysis were then used as surrogates for the detection and quantification of ALXN1210 and eculizumab in human serum using UPLC-MS/MS. Two signature tryptic peptides were selected for monitoring in the assay, as described below.

Example 2: Identification of signature peptide sequences for ALXN1210 and eculizumab

ALXN1210 and eculizumab share significant sequence identity (i.e., only four amino acid differences exist between the two antibody drugs, as shown in **Figure 2**). Accordingly, for specific detection of each antibody, signature peptides unique to each of ALXN 12 10 and eculizumab were generated using in silico tryptic digestion. Six peptides were selected which

contained amino acid differences between the sequences of the two antibodies, as shown in **Table 1**.

Table 1. Eculizumab and ALXN1210 Signature Peptides

SEQ ID NO	Amino Acid	Purpose
SEQ ID NO: 21	QAPGQGLEWMGEILPGSG H TEYTENFK	Quantification of ALXN1210
SEQ ID NO: 22	WQEGNVFSCSV L HEALH S HYTQK	Quantification of ALXN1210
SEQ ID NO: 2	ASG H IFSNIYWIQWVR	Quantification of ALXN1210
SEQ ID NO: 19	QAPGQGLEWMGEILPGSG S TEYTENFK	Quantification of eculizumab
SEQ ID NO: 20	WQEGNVFSCSV M HEALH N HYTQK	Quantification of eculizumab
SEQ ID NO: 1	ASG I IFSNIYWIQWVR	Quantification of eculizumab

Example 3: Immunocapture of ALXN1210 and eculizumab in human serum

An immunocapture assay was developed for the enrichment of ALXN1210 and eculizumab from human serum samples, as described below. Sensitivity was one of the major challenges for the development of the assay. The existence of endogenous proteins of high abundance from the other captured antibodies and other components in biological matrices leads to high background, significant ion suppression, and interferences of quantification. An immunocapture approach was examined using Protein A. Whole serum direct digestion was used as the control.

Protein A is a protein of microbial origin with the ability to bind to human IgG1, IgG2, and IgG4 with high affinity to the antibody Fc region. Even through both eculizumab and ALXN1210 contain a non-naturally occurring protein engineered heavy chain constructed with elements of both IgG2 and IgG4, both retain the ability to bind protein A. Protein A magnetic beads were incubated with human serum samples, during the period of incubation, IgGs from the sample were bound to the beads via their Fc regions (PUREPROTEOME™ Protein A Magnetic Bead, EMD Millipore, Product No. LSKMAGA10). With the assistance of an external magnet (PUREPROTEOME™ Magnetic Stand, Millipore, Product No. LSKMAGS15), the IgG-Protein A bead complexes were separated and washed from other unbound serum components. Protease digestion was performed directly on bead-bound proteins using MS Grade Trypsin Protease, (Pierce Product No. 90305B).

For whole serum digestion, direct tryptic digestion of the sample was performed without any pre-treatment for the enrichment of target proteins or to reduce background. It was found that signature peptides were recovered using the whole serum digestion approach. The background was higher than was observed using other immunocapture approaches. However, the analyte responses (detection) from Protein A capture was high, resulting in excellent sensitivity and quantification. This technique using Protein A capture also showed high accuracy and precision when spiked QCs were examined.

For trypsin digestion, certain detergents were often used to denature proteins and maintain their solubility for better digestion and recovery. During method development, RAPIGEST™, a surfactant that unfolds antibodies preferentially before proteases, was initially assessed to assist protein digestion. Alternatively, another surfactant PROTEASEMAX™ was tested for comparison, as the signature peptide recovery was less than optimal. It was found that PROTEASEMAX™ was much more effective than RAPIGEST™, as shown in **Figure 3**. Surprisingly, further investigation indicated that digestion efficiency was best where there was no addition of PROTEASEMAX™, as shown in **Figure 4**. Importantly, these results suggested that denaturation was not necessary for complete digestion of the antibodies and recovery and that detergents may negatively affect enzymatic digestion for certain antibodies.

Example 4: Concurrent quantification of ALXN1210 and eculizumab using UPLC-MS/MS approach

Following immunocapture and protease digestion, samples of proteolytic peptide mixtures were subjected to reversed-phase UPLC-MS/MS. ALXN1210 and eculizumab were used as anti-C5 antibodies with high affinity towards complement protein C5. The assay was desirable because clinical trials were ongoing where one antibody was being switched to the other for treatment, thus quantification of their relative amounts at any given time during the switch study was necessary. The assay was further complicated due to the various levels of endogenous C5 bound to the administered antibodies, therefore the impact of the C5 concentration on signature peptide recovery from ALXN1210 and eculizumab was assessed. In addition to normal human C5 levels serum, two levels of quality control, low C5 and high C5 concentrations, were prepared to have three different levels of C5 concentrations: C5 depleted, endogenous C5 level in normal human serum, and 100 µg/mL fortified in normal human serum.

As indicated below in **Tables 2 and 3**, C5 concentration had no impact on the detection and quantification of ALXN1210 and eculizumab, respectively.

Table 2. Quantification of ALXN1210 in Human Serum with Different C5 Concentrations

	C5 Depleted Human Serum		Normal Human Serum		Normal Human Serum Fortified C5 at 100 µg/mL	
	QC 31 (µg/mL)	QC 35 (µg/mL)	QC 41 (µg/mL)	QC 45 (µg/mL)	QC 51 (µg/mL)	QC 55 (µg/mL)
	2.68	397	2.7	337	2.58	322
	2.34	403	2.64	335	2.43	326
	2.52	396	2.73	344	2.41	310
	2.87	370	2.5	349	2.51	354
	2.72	393	2.61	365	2.36	326
	2.62	387	2.75	360	2.23	336
N	6	6	6	6	6	6
Theoretical						
Theoretical Concentration	2.5	375	2.5	375	2.5	375
Mean	2.62	391	2.66	348	2.42	329
S.D.	0.179	11.3	0.092	12.1	0.124	15
%C.V.	6.83	2.89	3.46	3.47	5.14	4.55
% Difference from Theoretical	4.94	4.27	6.25	-7.15	-3.16	-12.3

Table 3. Quantification of Eculizumab in Human Serum with Different C5 Concentrations

	C5 Depleted Human Serum		Normal Human Serum		Normal Human Serum Fortified C5 at 100 µg/mL	
	QC 31 (µg/mL)	QC 35 (µg/mL)	QC 41 (µg/mL)	QC 45 (µg/mL)	QC 51 (µg/mL)	QC 55 (µg/mL)
	16	391	15.1	356	14.1	336
	15.3	402	15.2	363	14	316
	15.6	398	16	347	15.1	322
	16.2	387	14.2	359	14.5	367
	15.3	389	14.9	395	14.5	350
	15.5	393	15.1	380	13.5	342
N	6	6	6	6	6	6
Theoretical						
Theoretical Concentration	15	375	15	375	15	375
Mean	15.6	393	15.1	367	14.3	339
S.D.	0.35	5.73	0.566	17.7	0.525	18.7
%C.V.	2.23	1.46	3.75	4.84	3.67	5.51
% Difference from Theoretical	4.31	4.92	0.487	-2.23	-4.78	-9.6

The effect of DMSO as a mobile phase additive was then investigated. The following chromatography conditions were used (with and without 1% DMSO):

LC Pump: One Agilent 1100 Series pump and Agilent 1200 SL series pump
 Pre-column Frit: 2-µm stainless steel inline solvent filter, Upchurch Scientific - Rheodyne, Product No. A-103x

Analytical Column:	Acquity BEH C8, 2.1 mm x 50 mm, 1.7 μ m, Waters, Product No. 186002877
Column Temperature:	50 °C
Pump Program:	See method
Mobile Phase A:	100:0.1, Water/Formic Acid
Mobile Phase B:	100:0.1:1, Acetonitrile/Formic Acid/DMSO
Flow Rate:	0.4-0.5 mL/min
Injection Volume:	25 μ L
LC Pressure:	-280 bar
Autosampler Wash 1:	5:25:30:40:0.1 TFE/IPA/Ethanol/Acetonitrile/Formic Acid, v/v/v/v/v
Autosampler Wash 2:	80:20:0.1, Water/MeOh/Formic Acid
Approximate Run Time:	7.5 min

The peptide elution gradient pump program, makeup pump program, ACH valve program, MS conditions, and other parameters are shown in **Tables 4, 5, 6, 7, and 8**, respectively.

As shown in **Figure 5**, when 1% DMSO was added to mobile phase B, the response of ALXN1210 signature peptide was doubled (DMSO, Thermo Scientific, Product No. TS20684). For eculizumab, the original precursor ion ([M+3H]) was completely converted to precursor ion [M+2H], which was selected for quantification. Sensitivity for eculizumab was increased by about 15%. The peptide elution and column washing gradient used are shown below in **Table 4**.

Table 4. Eluting Pump Program

Step	Total Time(min)	Flow Rate(μ l/min)	A (%)	B (%)
0	0.00	400	86	14
1	1.00	400	86	14
2	4.00	400	54	46
3	4.20	400	54	46
4	4.30	500	10	90
5	5.00	500	10	90
6	5.01	500	90	10
7	5.30	500	90	10
8	5.31	500	10	90
9	6.50	500	10	90
10	6.60	500	86	14
11	7.40	500	86	14
12	7.50	400	86	14

Table 5. Makeup Pump Program

Step	Total Time (min)	Flow Rate (μ L/min)	Composition	
			A (%)	B (%)
0	0.00	200	50	50
1	7.50	200	50	50

Table 6. ACH Valve Program

Total Time (min)	Position	Comments
Initial	Left	Flow from Makeup pump to MS
3.00 *	Right	Flow from column to MS
4.00	Left	Flow from Makeup pump to MS
4.5	Right	Flow from column to MS
4.7	Left	Flow from Makeup pump to MS

Table 7. MS Conditions

Compound ID	Q1 Mass (Da)	Q3 Mass (Da)	Dwell (msec)	DP	CE
Peptide A	622.0	701.3	50	45	28
Peptide A IS	625.3	711.3	50	45	28
Peptide B	945.5	588.3	50	80	41
Peptide B IS	950.5	598.3	50	80	41

Table 8. Other Parameters

Other Parameters	
CUR	35
IS	5500
TEM	550
GS1	60
GS2	50
CAD	8
EP	10
CXP	5

Next, the carryovers from different phase columns were evaluated. A C18 reversed phase column was initially chosen for the method. Due to the relative hydrophobic nature of the two signature peptides, substantial carryovers were observed (-103% for ALXN1210 and -118% for eculizumab peptides, comparing to LLOQ level in the blank immediately following ULOQ). A C8 column was then evaluated and the carryover for ALXN1210 and eculizumab peptides was significantly reduced to less than 50% and 30%, respectively, which was acceptable with injection order protection.

Overall, the hybrid immunocapture UPLC-MS/MS method was successfully validated for eculizumab and ALXN1210 detection and quantification using the signature tryptic peptides from each (SEQ ID NOs: 1 and 2, respectively). The standard curve range was 1.00-500 $\mu\text{g/mL}$ for ALXN1210 and 5.00-500 $\mu\text{g/mL}$ for eculizumab using a diluted serum sample volume of 25 μL and an MRD at 10. Linear regression was used and correlation coefficient >0.996 was observed for both mAbs during validation. Excellent intra- and inter-day assay precisions and accuracies from all quality control (QC) concentration levels were demonstrated for both ALXN1210 and eculizumab, as shown in **Tables 9** and **10**, respectively. The method was also successfully cross-validated with individual ligand-binding assays for ALXN1210 and eculizumab.

Table 9. Typical Precision and Accuracy Performance for ALXN1210

Run ID	IA 0	IA 1	IA 2	IA 3	IA 4	IA 5
	($\mu\text{g/mL}$)					
IREIV2-A-1	0.880	2.34	4.73	12.6	74.4	344
	1.00	2.46	4.77	15.5	76.7	375
	0.943	2.04	5.02	15.0	77.6	411
	0.961	2.23	5.08	15.1	78.6	396
	0.900	2.68	4.79	14.9	78.9	400
	0.926	2.47	4.45	14.7	78.7	395
N	6	6	6	6	6	6
Theoretical						
Concentration	1.00	2.50	5.00	15.0	75.0	375
Mean	0.936	2.37	4.81	14.6	77.5	387
S.D.	0.0439	0.222	0.228	1.04	1.71	24.0
%C.V.	4.69	9.36	4.73	7.10	2.21	6.19
% Difference						
from Theoretical	-6.44	-5.14	-3.84	-2.57	3.31	3.23

Table 10. Typical Precision and Accuracy Performance for Eculizumab

Run ID	IA 2	IA 3	IA 4	IA 5
	($\mu\text{g/mL}$)	($\mu\text{g/mL}$)	($\mu\text{g/mL}$)	($\mu\text{g/mL}$)
IREIV2-A-2	5.27	13.3	75.4	350
	5.98	15.9	81.3	386
	5.85	15.3	80.5	408
	5.69	15.4	79.5	395
	5.40	16.0	83.6	415
	5.20	16.5	77.3	395
N	6	6	6	6
Theoretical				
Concentration	5.00	15.0	75.0	375
Mean	5.56	15.4	79.6	392
S.D.	0.321	1.13	2.95	23.1
%C.V.	5.77	7.35	3.70	5.89
% Difference				
from Theoretical	11.3	2.57	6.15	4.43

Example 5: Quantification of ALXN1210 in human urine

The matrix effects present in urine samples can hinder the quantification of mAbs using traditional ligand-binding assays. To overcome these challenges, the UPLC-MS/MS method described above was optimized to quantify ALXN1210 in human urine. Briefly, ALXN1210

was subject to denaturation, reduction with dithiothreitol (DTT) at 65°C, alkylation with iodoacetamide, and trypsin digestion. The signature peptide generated from ALXN1210 (SEQ ID NO: 2), was then used for the quantification of ALXN1210 using UPLC-MS/MS. **Tables 11 and 12** list the equipment and reagents used, respectively.

Table 11. Equipment

Equipment	Vendor/Model
SPE System	Agela, SPE-M96 Positive Pressure SPE Device
1.4 mL Non-coded Push Cap Tubes U-bottom*	Micronic, MP 32022
96-Well Square Well Plate, 2.0 mL per well	MicroLiter, Product# 07-7400
Mass Spectrometer	SCIEX6500
Micro- Analytical Balance	Capable of weighing 0.001 mg
Analytical Balance	Capable of weighing 0.01 mg
Water System	Sys#. 56-2
Vortex Mixer	VWR, VX-2500
Advantage Plug Cap Strips	Analytical sales and service: Cat#96128-2
Disposable sterile pipet tips with extended length and ultrafine point	VWR, VWR#37001-524
Eppendorf MixMate	Eppendorf, Product No. 5353
96 well silicone mat	Agela, Cat#96GP2036-M
Protein LoBind tube, 2 mL	Eppendorf, Product No. 022431102
Cleanert PEP 96-well SPE Microplate, 5mg/1mL/well	Agela, Cat#PE00501-MW
Analytical Column	Acquity BEH C8, 2.1mm X 50 mm, 1.7µm, Waters, Product No. 186002877

Table 12. Reagents

Item Description	Vendor
HPLC Grade Acetonitrile (ACN)	Fisher
Water	Purified
GR Grade Formic Acid (FA)	ACROS
GR Grade Ammonium Bicarbonate	VWR
HPLC Grade Methanol (MeOH)	Fisher
HPLC Grade Isopropanol (IPA)	Fisher
DL-Dithiothreitol (DTT)	Sigma, Product No. D0632-10G
Iodoacetamide (IAA)	Sigma, Product No. I1149-5G
MS Grade Trypsin Protease	Pierce, Product No. 90059
RAPIGEST™ Box of (5) 1mg vials	Waters Product# 186001861
Human Urine	BioreclamationIVT
HPLC Grade Dimethylsulfoxide (DMSO)	Thermo Scientific

Carryover was one of the major technical challenges during development of the assay for detection of ALXN1210 and eculizumab in human serum. The HPLC gradient of the assay for human urine was improved to include a higher starting mobile phase B percentage (from 14% to 25%). This change resulted in no detectable carryover and the elimination of an additional column wash cycle, which led to shorter sample run time. The carryover performance during validation is shown in **Table 13**.

Table 13. Injection Carryover Results for ALXN1210

Run Date	Watson Run ID	Sequence No.	Double Blank Sample		STD 1 Sample	Mean IS Peak Area from Accepted Stds and QC	Carryover %	
			Analyte Peak Area	IS Peak Area	Analyte Peak Area		Analyte	IS
16Jun17	1	14	0	0	4294	509613	0.0	0.0
		15	0	0			0.0	0.0
		16	0	0			0.0	0.0
16Jun17	2	14	0	0	5289	633759	0.0	0.0
		15	0	0			0.0	0.0
		16	0	0			0.0	0.0
19Jun17	3	14	0	0	5585	586840	0.0	0.0
		15	0	0			0.0	0.0
		16	0	0			0.0	0.0
20Jun17	4	14	0	0	5592	680500	0.0	0.0
		15	0	0			0.0	0.0
		16	0	0			0.0	0.0

Additional testing was conducted to evaluate the performance of the assay under conditions of elevated protein and red blood cell (hemolyzed) levels, as they are often present in patients' urine. Urine Interference Quality Control (QC) samples were prepared containing 15% serum or 2% hemolyzed whole blood (volume %). Standard samples ("std-") did not contain any serum or hemolyzed blood. The recovery of ALXN1210 signature peptide at low QC level with 15% serum was substantially reduced in comparison to the calibration curve and QC samples prepared in a 100% urine matrix, as shown in **Table 14**.

Table 14. Recovery of the QC samples with serum and hemolyzed whole blood

Sample Name	Accuracy (%)	Analyte Concentration (µg/mL)	Calculated Concentration (µg/mL)	Analyte Peak Area (counts)	IS Peak Area (counts)
Std-1	103.46	0.08	0.083	612	182644
Std-2	94.86	0.16	0.152	1396	198555
Std-3	89.79	1	0.898	10427	222809
Std-4	91.73	5	4.587	57254	235224
Std-5	99.53	10	9.953	129894	245341
Std-6	106.95	20	21.391	306661	269217
Std-7	107.31	32	34.341	522564	285664
Std-8	106.35	40	42.541	678037	299169
15%Srum-Q2-1	17.08	5	0.854	3137	70549
15%Srum-Q2-2	15.39	5	0.77	2453	61381
15%Srum-Q2-3	17.86	5	0.893	865	18582
15%Srum-Q2-4	13.55	5	0.678	636	18155
15%Srum-Q2-5	20.82	5	1.041	807	14836
15%Srum-Q2-6	14.12	5	0.706	504	13794

Human serum was added to all of the samples (5% volume of the sample) during sample processing to normalize calibration curve QCs and the QC samples containing serum or hemolyzed whole blood. Standard samples ("std-") and plate acceptance QC samples ("Low-", "Mid-", and "High-") did not contain any serum or hemolyzed blood. Digestion duration was then prolonged to enhance the recovery of ALXN1210 signature peptide in urine samples containing human serum. The conditions had good recovery at both 15% serum and 2% hemolyzed whole blood at LLOQ QC level, as shown in **Table 15**. The assay also had good sensitivity with a 20 µl sample volume. Representative extracted ion chromatograms (XIC) of blank and LLOQ are shown in **Figures 6 and 7**.

Table 15. Recovery of the QC samples containing serum or hemolyzed whole blood with the addition of human serum and overnight tryptic digestion

Sample Name	Accuracy (%)	Analyte Concentration (ng/mL)	Calculated Concentration (ng/mL)	Area Ratio	Analyte Peak Area (counts)	IS Peak Area (counts)
Std1	97.36	0.08	0.078	0.00734	236	32120
Std2	91.71	0.16	0.147	0.01432	407	28409
Std3	101.44	1	1.014	0.1023	3358	32826
Std4	103.27	5	5.164	0.52304	15635	29892
Std5	95.66	10	9.566	0.9695	30695	31660
Std6	106.72	20	21.345	2.16388	67566	31224
Std7	107	32	34.241	3.4716	107142	30862
Std8	102.23	40	40.891	4.14592	130912	31576
15%Serum-LLOQ-1	119.11	0.08	0.095	0.0091	269	29549
15%Serum-LLOQ-2	104.53	0.08	0.084	0.00792	261	32897
15%Serum-LLOQ-3	101.92	0.08	0.082	0.00771	228	29620
15%Serum-LLOQ-4	112.57	0.08	0.09	0.00857	256	29818
15%Serum-LLOQ-5	98.29	0.08	0.079	0.00741	204	27577
15%Serum-LLOQ-6	93.5	0.08	0.075	0.00702	213	30352
2%Hem-LLOQ-1	105.4	0.08	0.084	0.00799	293	36720
2%Hem-LLOQ-2	103.03	0.08	0.082	0.0078	298	38204
2%Hem-LLOQ-3	84.7	0.08	0.068	0.00631	245	38790
2%Hem-LLOQ-4	93.49	0.08	0.075	0.00702	172	24496
2%Hem-LLOQ-5	81.42	0.08	0.065	0.00604	160	26390
2%Hem-LLOQ-6	96.11	0.08	0.077	0.00724	209	28868
Low-1	86.67	0.24	0.208	0.02053	600	29217
Mid-1	93.94	5	4.697	0.47574	12131	25500
High-1	110.27	30	33.082	3.35413	99122	29552
Low-2	89	0.24	0.214	0.0211	704	33350
Mid-2	98.26	5	4.913	0.49766	17907	35982
High-2	107.49	30	32.248	3.26953	95121	29093

In summary, the UPLC-MS/MS method was successfully developed and validated for the quantitation of ALXN1210 in human urine using a signature tryptic peptide. With a sample volume of 20 μ L, the calibration curve range of the assay was 0.0800 - 40.0 μ g/mL for ALXN1210. Samples above the ULOQ could be diluted up to 10 fold and 2 fold using pooled human urine (data not shown). Excellent intra- and inter-day assay precisions and accuracies from all quality control (QC) concentration levels were demonstrated in addition to linearity,

recovery, dilution integrity, processed sample stability, QC bench-top stability, matrix effect, batch-size, reinjection stability, QC freeze/thaw stability, carryover, and interference from analyte on IS (data not shown). This method demonstrates the capability of the UPLC-MS/MS method to overcome the matrix effect in urine and quantify ALXN1210 with excellent sensitivity.

SEQUENCE LISTING

<p>SEQ ID NO: 1 amino acid sequence for eculizumab signature peptide</p> <p>ASGY<u>I</u>FSNYWIQWVR</p>
<p>SEQ ID NO: 2 amino acid sequence for ALXN1210 signature peptide</p> <p>ASGH<u>I</u>FSNYWIQWVR</p>
<p>SEQ ID NO: 3 amino acid sequence of heavy chain CDR1 of eculizumab (as defined under combined Kabat-Chothia definition)</p> <p>GYIFSNYWIQ</p>
<p>SEQ ID NO: 4 amino acid sequence of heavy chain CDR2 of eculizumab (as defined under Kabat definition)</p> <p>EILPGSG<u>S</u>TEYTFNKD</p>
<p>SEQ ID NO: 5 amino acid sequence of the heavy chain CDR3 of eculizumab and ALXN1210 (as defined under combined Kabat definition).</p> <p>YFFGSSPNWYFDV</p>
<p>SEQ ID NO: 6 amino acid sequence of the light chain CDR1 of eculizumab and ALXN1210 (as defined</p>

under Kabat definition)

GASENIYGALN

SEQ ID NO: 7

amino acid sequence of light chain CDR2 of eculizumab and ALXN1210 (as defined under Kabat definition)

GATNLAD

SEQ ID NO: 8

amino acid sequence of light chain CDR3 of eculizumab and ALXN1210 (as defined under Kabat definition)

QNVLNTPLT

SEQ ID NO: 9

amino acid sequence of heavy chain variable region of eculizumab

QVQLVQSGAEVKKPGASVKVSCKASGYIFSNYWIQWVRQAPGQGLEWM
 GEILPGSGSTEYTENFKDRVMTTRDTSSTVYMELSLRSEDTAVYYCARY
 FFGSPNWFYFDVWGQGLVTVSS

SEQ ID NO: 10

amino acid sequence of light chain variable region of eculizumab and ALXN1210

DIQMTQSPSSLSASVGDRVTITCGASENIYGALNHWYQQKPGKAPKLLIYG A
 TNLADGVPSRFS GSGSGTDFTLTISLQPEDFATYYCQNVLNTPLTFGQGTK
 VEIK

SEQ ID NO: 11

amino acid sequence of heavy chain constant region of eculizumab

ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVH
 TFP AVLQSSGLYSLSSVTVPSNFGTQTYTCNVDHKPSNTKVDKTVKRC
 CVECPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSDPEVQF

<p>NWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKV SNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPS DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCS VMHEALHNHYTQKSLSLGLGK</p>
<p>SEQ ID NO: 12 amino acid sequence of entire heavy chain of eculizumab</p> <p>QVQLVQSGAEVKKPGASVKVSCASGYIFS^UNYWIQWVRQAPGQGLEWM GEILPGSG^USTEYTENFKDRVTMTRDTS^TSTVYMELSLRSEDTAVYYCAR YFFGSSPNWYFDVWGQGLVTVSSASTKGPSVFPLAPCSRSTSESTAALGCL VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSNFGTQTYT CNVDHKPSNTKVDKTKVERKCCVECPAPPVAGPSVFLFPPKPKDTLMISR TPEVTCVVVDVSDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLT VLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMT KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTV DKSRWQEGNVFSCSVMHEALHNHYTQKSLSLGLGK</p>
<p>SEQ ID NO: 13 amino acid sequence of entire light chain of eculizumab and ALXN1210</p> <p>DIQMTQSPSSLSASVGDRVTITCGASENIYGALNWYQQKPGKAPKLLIYG ATNLADGVPSRFSGSGGTDFLTISLQPEDFATYYCQNVLNTPLTFGQ GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDN ALQSGNSQESVTEQDSKDS^TYSLSS^TLTLSKADYEKHKVYACEVTHQGLSPV TKSFN^RRGEC</p>
<p>SEQ ID NO: 14 amino acid sequence of heavy chain variable region of ALXN1210</p> <p>QVQLVQSGAEVKKPGASVKVSCASGHIFS^UNYWIQWVRQAPGQGLEW MGEILPGSGH^UTEYTENFKDRVTMTRDTS^TSTVYMELSLRSEDTAVYYC ARYFFGSSPNWYFDVWGQGLVTVSS</p>
<p>SEQ ID NO: 15</p>

amino acid sequence of heavy chain constant region of ALXN1210

ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGV
HTFPAVLQSSGLYSLSSVVTVPSNFGTQTYTCNV^DHKPSNTKVDK^TVER
KCCVECPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSDQEDPE
VQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYK
CKVSNKGLPSIEKTISKAKGQPREPQVYTLPPS QEEMTKNQVSLTCLVKG
FYPSDIAVEWESNGQPENNYK^TTPPVLDSDGSFFLYSRLTVDKSRWQEGN
VFSCSVLHEALHSHYTQKSLSLSLGK

SEQ ID NO: 16

amino acid sequence of entire heavy chain of ALXN1210

QVQLVQSGAEVKKPGASVKVSCKASGHIFS^NYWIQWVRQAPGQGLEWM
GEILPGSGHTEYTENFKDRVTMTRDTS TSTVYMELSLRSEDTAVYYCAR
YFFGSSPNWYFDVWGQGLVTVSS ASTKGPSVFPLAPCSRSTSESTAALGCL
VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSNFGTQTYT
CNVDHKPSNTKVDK^TVERKCCVECPCPAPPVAGPSVFLFPPKPKDTLMISR
TPEVTCVVVDVSDQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLT
VLHQDWLNGKEYKCKVSNKGLPSIEKTISKAKGQPREPQVYTLPPS QEEMT
KNQVSLTCLVKG^FYPSDIAVEWESNGQPENNYK^TTPPVLDSDGSFFLYSRLTV
DKSRWQEGNVFSCSVLHEALHSHYTQKSLSLSLGK

SEQ ID NO: 17

amino acid sequence of heavy chain CDR1 of ALXN1210

GHIFS^NYWIQ

SEQ ID NO: 18

amino acid sequence of heavy chain CDR2 of ALXN1210

EILPGSGHTEYTENFKD

SEQ ID NO: 19

amino acid sequence for eculizumab candidate signature peptide

QAPGQGLEWMGEILPGS GSTEYTENFK

SEQ ID NO: 20

amino acid sequence for eculizumab candidate signature peptide

WQEGNVFSCSVMHEALHNHYTQK

SEQ ID NO: 21

amino acid sequence for ALXN1210 candidate signature peptide

QAPGQGLEWMGEILPGS GHTEYTENFK

SEQ ID NO: 22

amino acid sequence for ALXN1210 candidate signature peptide

WQEGNVFSCSVLHEALHSHYTQK

CLAIMS

We claim:

1. A method of detecting and quantifying the respective amounts of two antibodies having high sequence identity present together in a biological sample, wherein the antibodies are eculizumab and ALXN1210, the method comprising:
 - (a) treating the biological sample containing the antibodies with a protease to form a proteolytic peptide mixture of the antibodies in the biological sample;
 - (b) analyzing a sample of the proteolytic peptide mixture by high performance liquid chromatography (HPLC) tandem mass spectrometry to detect a signature peptide from each of the antibodies in the biological sample, wherein the signature peptide for eculizumab comprises SEQ ID NO: 1 and the signature peptide for ALXN1210 comprises SEQ ID NO: 2; and
 - (c) quantifying each antibody in the biological sample based on the signal ratio of its signature peptide to an internal control, wherein the internal control comprises a labeled form of the same signature peptide.

2. A method of detecting and quantifying the amount of an antibody present in a biological sample, wherein the antibody is eculizumab, the method comprising:
 - (a) treating the biological sample containing the antibody with a protease to form a proteolytic peptide mixture of the antibody in the biological sample;
 - (b) analyzing a sample of the proteolytic peptide mixture by high performance liquid chromatography (HPLC) tandem mass spectrometry to detect a signature peptide, wherein the signature peptide comprises SEQ ID NO: 1; and
 - (c) quantifying the amount of eculizumab in the biological sample based on the signal ratio of the signature peptide to an internal control, wherein the internal control comprises a labeled form of the same signature peptide.

3. A method of detecting and quantifying the amount of an antibody present in a biological sample, wherein the antibody is ALXN1210, the method comprising:

- (a) treating the biological sample containing the antibody with a protease to form a proteolytic peptide mixture of the antibody in the biological sample;
 - (b) analyzing a sample of the proteolytic peptide mixture by high performance liquid chromatography (HPLC) tandem mass spectrometry to detect a signature peptide, wherein the signature peptide comprises SEQ ID NO: 2; and
 - (c) quantifying the amount of ALXN1210 in the biological sample based on the signal ratio of the signature peptide to an internal control, wherein the internal control comprises a labeled form of the same signature peptide.
4. The method of any of the preceding claims, wherein the protease is trypsin.
 5. The method of any of the preceding claims, further comprising contacting the biological sample with an affinity capture reagent prior to treating the biological sample with a protease.
 6. The method of claim 5, wherein the affinity capture reagent is bead-supported Protein A.
 7. The method of claims 5 or 6, further comprising washing the Protein A bound antibodies to remove unbound components prior to proteolysis.
 8. The method of any of the preceding claims, further comprising denaturing the antibody sample.
 9. The method of any of the preceding claims, further comprising reducing the antibody sample.
 10. The method of any of the preceding claims, further comprising alkylating the antibody sample.
 11. The method of any of the preceding claims, wherein the denaturation, reduction, and alkylation steps unfold the antibody protein and facilitates proteolytic digestion.

12. The method of any of the preceding claims, wherein the mass spectrometry is reverse-phase UPLC-MS/MS.
13. The method of any of the preceding claims, wherein the biological sample is serum or urine.
14. The method of any of the preceding claims, wherein the signature peptide comprises no more than 20 amino acids.
15. The method of any of the preceding claims, wherein the signature peptide comprises no more than 15 amino acids.
16. An isolated peptide consisting of SEQ ID NO: 1.
17. An isolated peptide consisting of SEQ ID NO: 2.
18. A method of detecting and quantifying the respective amounts of two antibodies having high sequence identity present together in a biological sample, wherein the antibodies are eculizumab and ALXN1210, the method comprising:
 - (a) treating the biological sample containing the antibodies with a protease to form a proteolytic peptide mixture of the antibodies in the biological sample;
 - (b) analyzing a sample of the proteolytic peptide mixture by high performance liquid chromatography (HPLC) tandem mass spectrometry to detect a signature peptide from each of the antibodies in the biological sample, wherein the signature peptide for eculizumab is selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 19, and SEQ ID NO: 20 and the signature peptide for ALXN1210 is selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 21, and SEQ ID NO: 22; and
 - (c) quantifying each antibody in the biological sample based on the signal ratio of its signature peptide to an internal control, wherein the internal control comprises a labeled form of the same signature peptide.

19. A method of detecting and quantifying the amount of an antibody present in a biological sample, wherein the antibody is eculizumab, the method comprising:

- (a) treating the biological sample containing the antibody with a protease to form a proteolytic peptide mixture of the antibody in the biological sample;
- (b) analyzing a sample of the proteolytic peptide mixture by high performance liquid chromatography (HPLC) tandem mass spectrometry to detect a signature peptide, wherein the signature peptide for eculizumab is selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 19, and SEQ ID NO: 20; and
- (c) quantifying the amount of eculizumab in the biological sample based on the signal ratio of the signature peptide to an internal control, wherein the internal control comprises a labeled form of the same signature peptide.

20. A method of detecting and quantifying the amount of an antibody present in a biological sample, wherein the antibody is ALXN1210, the method comprising:

- (a) treating the biological sample containing the antibody with a protease to form a proteolytic peptide mixture of the antibody in the biological sample;
- (b) analyzing a sample of the proteolytic peptide mixture by high performance liquid chromatography (HPLC) tandem mass spectrometry to detect a signature peptide, wherein the signature peptide for ALXN1210 is selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 21, and SEQ ID NO: 22; and
- (c) quantifying the amount of ALXN1210 in the biological sample based on the signal ratio of the signature peptide to an internal control, wherein the internal control comprises a labeled form of the same signature peptide.

21. The method of any of the preceding claims, wherein the protease is trypsin.

22. The method of any of the preceding claims, further comprising contacting the biological sample with an affinity capture reagent prior to treating the biological sample with a protease.

23. The method of claim 22, wherein the affinity capture reagent is bead-supported Protein A.

24. The method of claims 22 or 23, further comprising washing the Protein A bound antibodies to remove unbound components prior to proteolysis.
25. The method of any of the preceding claims, further comprising denaturing the antibody sample.
26. The method of any of the preceding claims, further comprising reducing the antibody sample.
27. The method of any of the preceding claims, further comprising alkylating the antibody sample.
28. The method of any of the preceding claims, wherein the denaturation, reduction, and alkylation steps unfold the antibody protein and facilitate proteolytic digestion.
29. The method of any of the preceding claims, wherein the mass spectrometry is reverse-phase UPLC-MS/MS.
30. The method of any of the preceding claims, wherein the biological sample is serum or urine.
31. The method of any of the preceding claims, wherein the signature peptide comprises no more than 20 amino acids.
32. The method of any of the preceding claims, wherein the signature peptide comprises no more than 15 amino acids.
33. The method of claim 18 or 19, wherein the signature peptide for eculizumab is SEQ ID NO: 19.

34. The method of claim 18 or 19, wherein the signature peptide for eculizumab is SEQ ID NO: 20.
35. The method of claim 18 or 20, wherein the signature peptide for ALXN1210 is SEQ ID NO: 21.
36. The method of claim 18 or 20, wherein the signature peptide for ALXN1210 is SEQ ID NO: 22.
37. The method of any of the above claims, wherein the affinity capture reagent is magnetic bead-supported Protein A.
38. An isolated peptide consisting of SEQ ID NO: 19.
39. An isolated peptide consisting of SEQ ID NO: 20.
40. An isolated peptide consisting of SEQ ID NO: 21.
41. An isolated peptide consisting of SEQ ID NO: 22.

Schematic diagram for hybrid immunocapture UPLC-MS/MS workflow

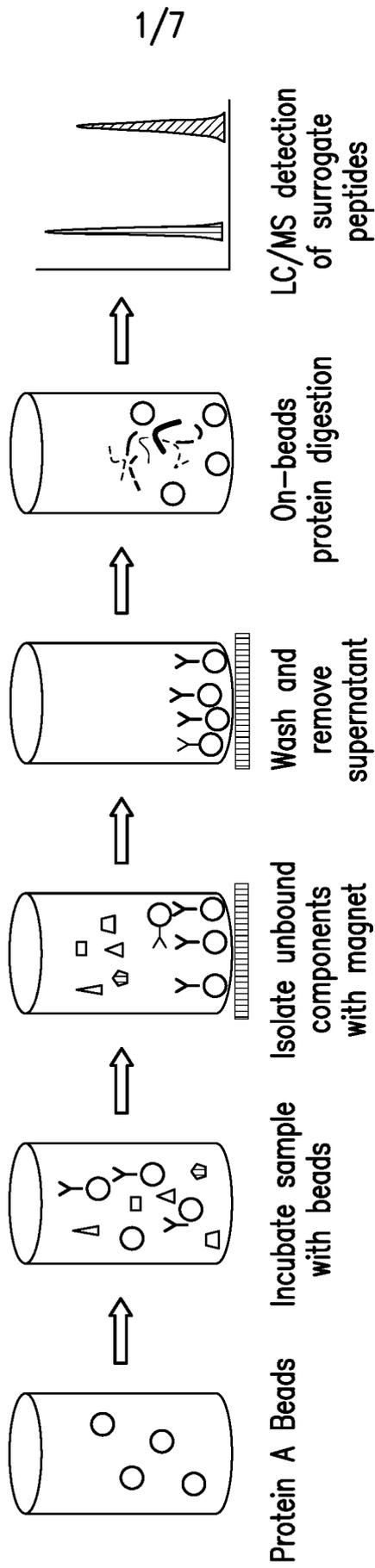


FIG.1

Sequences of eculizumab (EC) and ALXN1210

Light chain for both

DIQMTQSPSSLSASVGDRVTITCGASENIYGALN^{WY}QKPGKAPKLLIYGATNLADGVPSRFSGS
 GSGTDFTLTISSLQPEDFATYYCQNVLNTPLTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTA
 SVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSYSTLSSTLTLSKADYEKHKVYA
 CEVTHQGLSSPVTKSFNRGEC

Heavy chain for EC

QVQLVQSGAEVKKPGASVKVSCKASGYIFSNYWIQWVRQAPGQGLEWMGEILPGSGSTEYTEN
 FKDRVTMTRDTSTSTVYME^{LS}SLRSEDTAVYYCARYFFGSSPNWYFDVWGQGLTVTVSSASTK
 GPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVT
 VPSSNFGTQYTCNV^{DH}KPSNTKVDKTKVERKCCVECPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVDVSDQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTIISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPPVLDSDGSFFLYSRLTVDKSRWQEGN^VFCSSVMHEALHNHYTQKSLSL^{SL}GLGK

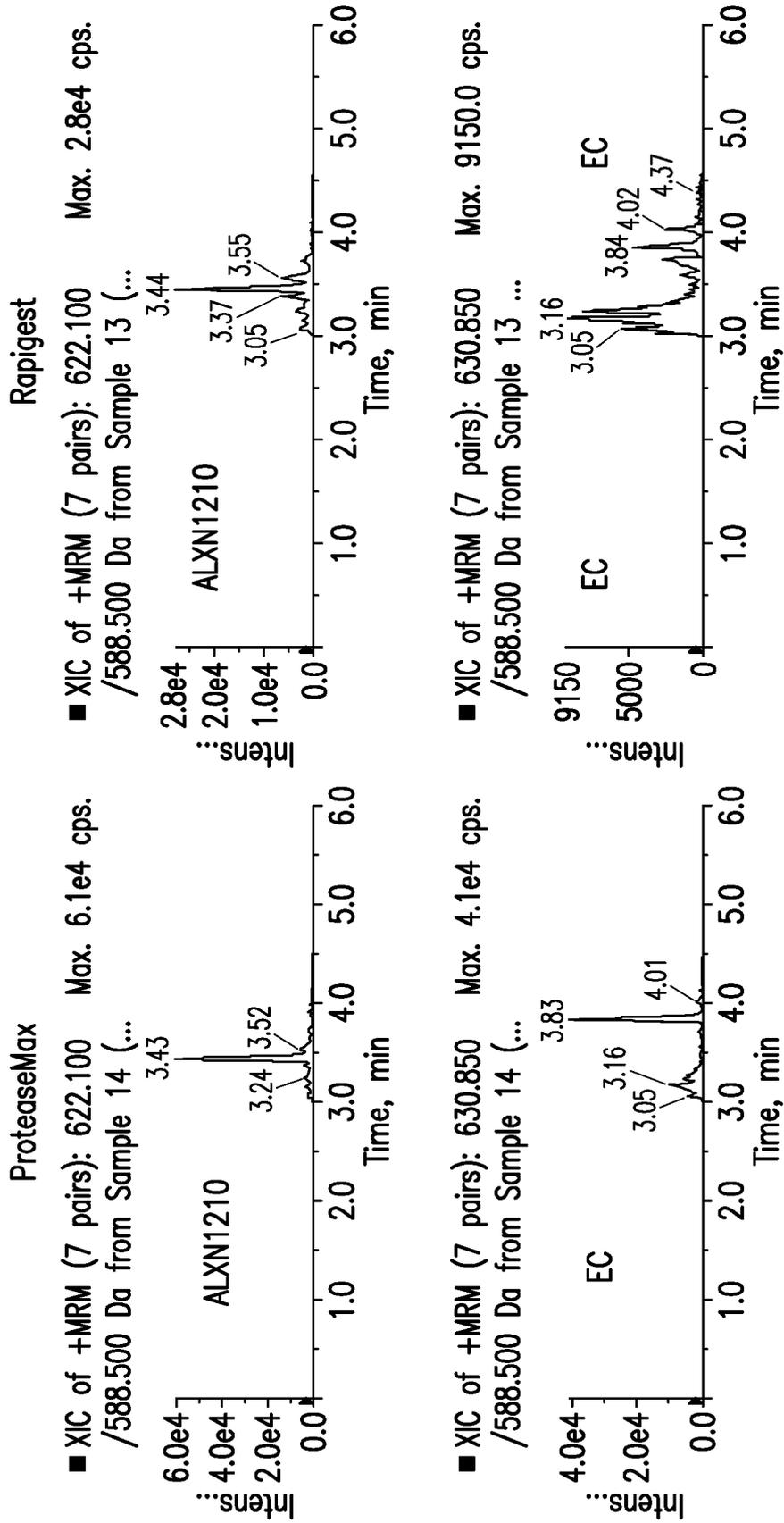
Heavy chain for ALXN1210

QVQLVQSGAEVKKPGASVKVSCKASGHIFSNYWIQWVRQAPGQGLEWMGEILPGSGHTEYTEN
 FKDRVTMTRDTSTSTVYME^{LS}SLRSEDTAVYYCARYFFGSSPNWYFDVWGQGLTVTVSSASTK
 GPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVT
 VPSSNFGTQYTCNV^{DH}KPSNTKVDKTKVERKCCVECPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVDVSDQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTIISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPPVLDSDGSFFLYSRLTVDKSRWQEGN^VFCSSV^LHEALHSHYTQKSLSL^{SL}GLGK

2/7

FIG.2

Effect of different detergents on digestion



3/7

FIG.3

4/7

Effect of different amounts of ProteaseMax on digestion efficiency

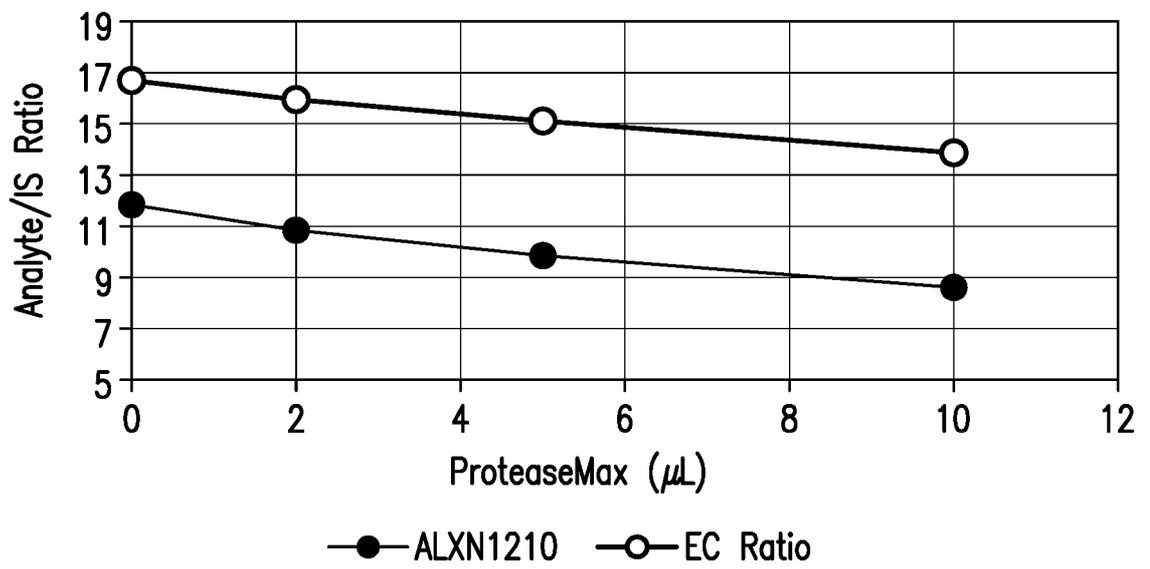


FIG.4

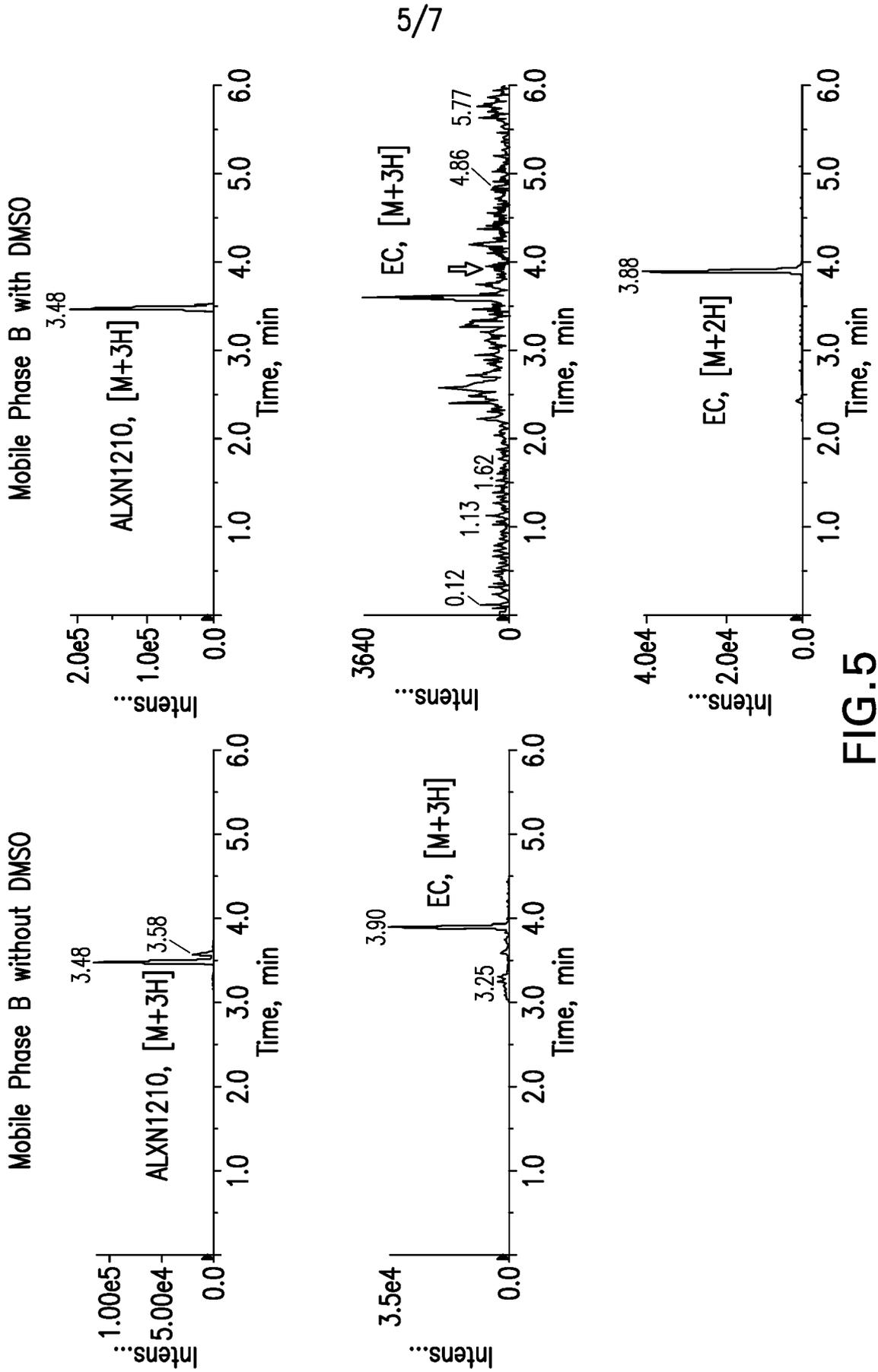


FIG.5

6/7

Representative XIC of Blank Sample

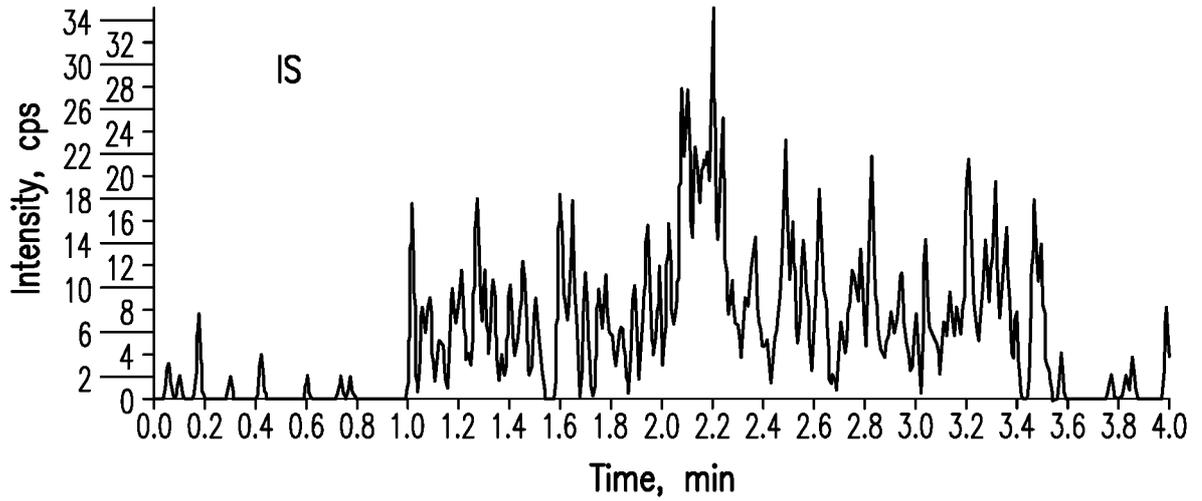
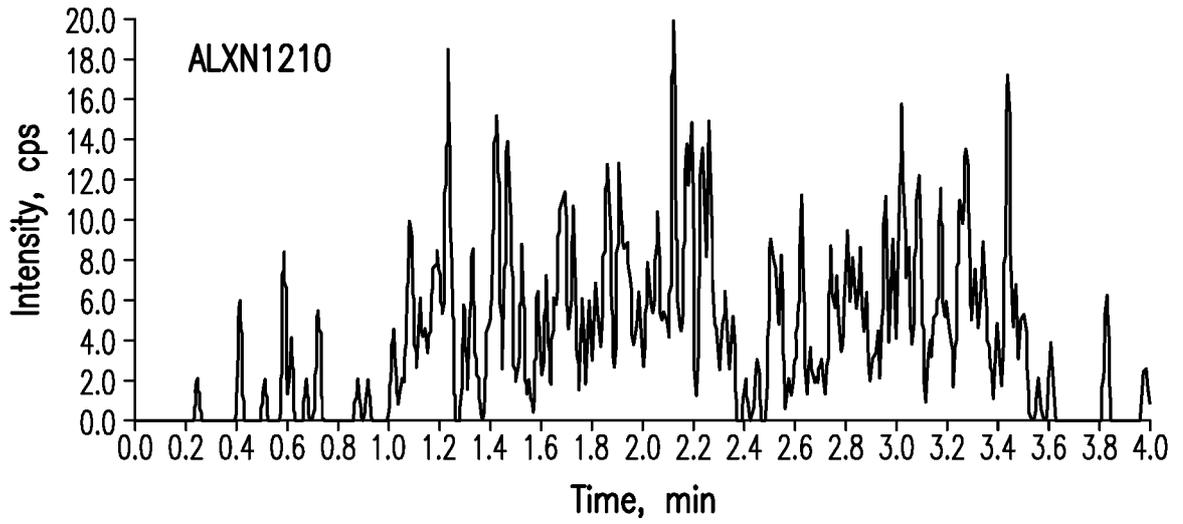


FIG. 6

7/7

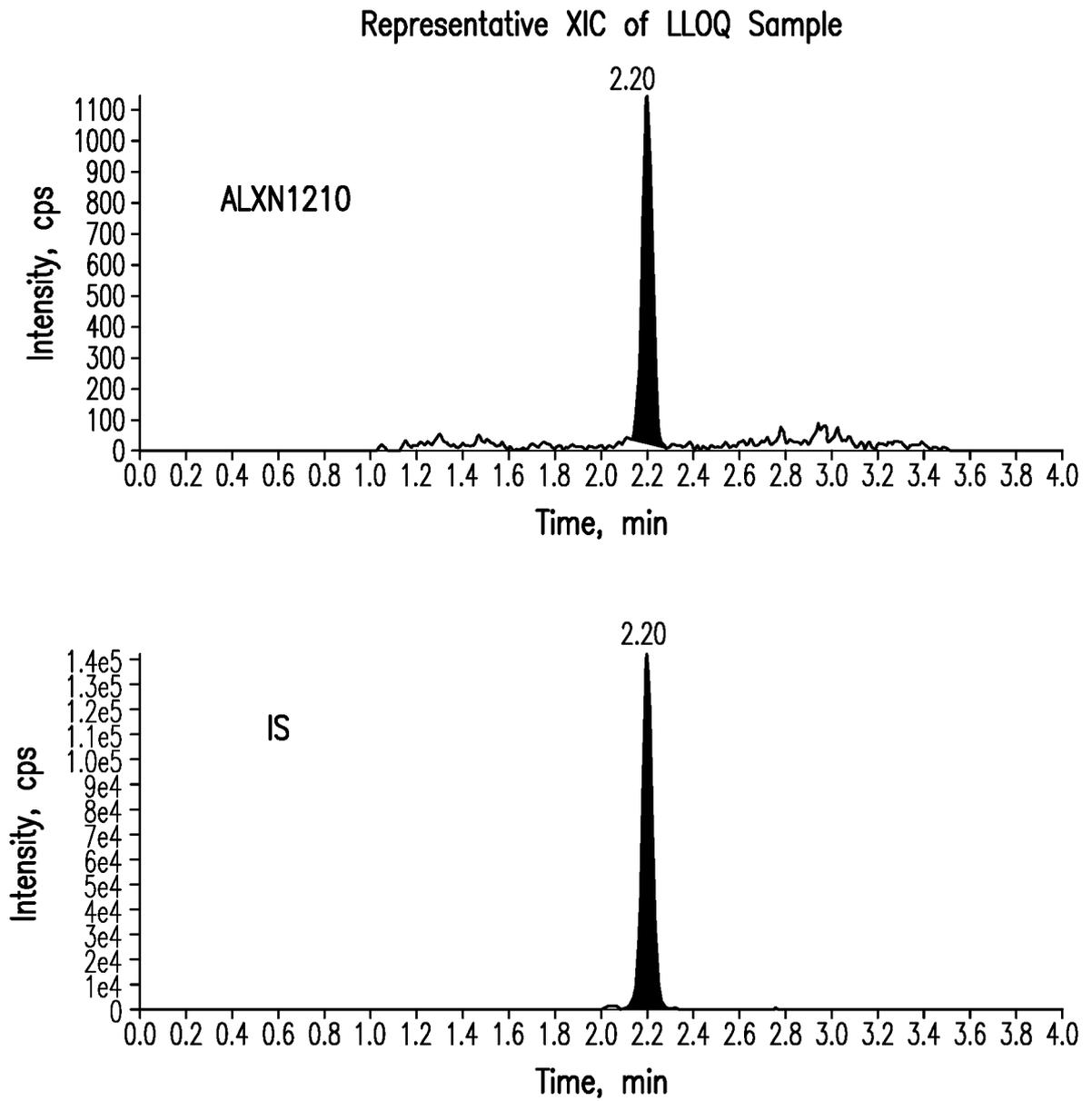


FIG. 7

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2018/024769

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/18 C07K16/40 G01N33/68
 ADD.
 According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
 Minimum documentation searched (classification system followed by classification symbols)
C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal , WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	wo 2016/018978 AI (MAYO FOUNDATION [US]) 4 February 2016 (2016-02-04) Abstract; claims; examples. -----	1-41
X	US 2012/087862 AI (HOOD LEROY [US] ET AL) 12 April 2012 (2012-04-12) Sequence 50986 -----	39
X	US 9 079 949 B1 (ANDRIEN JR BRUCE A [US] ET AL) 14 July 2015 (2015-07-14) cited in the application Abstract; examples; claims; sequences. -----	1-41

Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 15 May 2018	Date of mailing of the international search report 24/05/2018
---	---

Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Lopez Garcia, F
--	--

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/024769

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No PCT/US2018/024769

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2016018978	AI	04-02-2016	EP 3175242 AI 07-06-2017
			US 2018106815 AI 19-04-2018
			WO 2016018978 AI 04-02-2016

US 2012087862	AI	12-04-2012	AU 2007284651 AI 21-02-2008
			CA 2660286 AI 21-02-2008
			EP 2057465 A2 13-05-2009
			EP 2520935 A2 07-11-2012
			JP 5244103 B2 24-07-2013
			JP 2010500568 A 07-01-2010
			US 2012087862 AI 12-04-2012
			US 2014106981 AI 17-04-2014
			WO 2008021290 A2 21-02-2008

US 9079949	BI	14-07-2015	AR 099698 AI 10-08-2016
			AU 2015226977 AI 08-09-2016
			CA 2942165 AI 11-09-2015
			CN 106459189 A 22-02-2017
			CY 1118018 T1 17-05-2017
			DK 2970455 T3 28-11-2016
			EA 201691764 AI 30-12-2016
			EP 2970455 AI 20-01-2016
			EP 3095795 AI 23-11-2016
			ES 2589230 T3 11-11-2016
			HK 1217207 AI 30-12-2016
			HR P20161146 T1 18-11-2016
			HU E030944 T2 28-06-2017
			JP 6112469 B1 12-04-2017
			JP 2017113028 A 29-06-2017
			JP 2017512463 A 25-05-2017
			KR 20160126080 A 01-11-2016
			KR 20170125420 A 14-11-2017
			LT 2970455 T 25-10-2016
			MA 38859 B1 31-01-2017
			NZ 631007 A 30-10-2015
			NZ 711451 A 27-05-2016
			PL 2970455 T3 31-07-2017
			PT 2970455 T 25-10-2016
			SM T201600366 B 10-11-2016
			TW 201542590 A 16-11-2015
			US 9079949 B1 14-07-2015
			US 9107861 B1 18-08-2015
US 2015299305 AI 22-10-2015			
US 2016108115 AI 21-04-2016			
US 2016251433 AI 01-09-2016			
US 2017298123 AI 19-10-2017			
US 2018009885 AI 11-01-2018			
WO 2015134894 AI 11-09-2015			

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-41

Signature peptides and methods for detecting and quantifying eculizumab and ALXN1210 either simultaneously or individually measuring the said signature peptides.

1.1. claims: 1-17 (completely) ; 18-32 , 37(partially)

Methods of detecting and quantifying eculizumab and ALXN1210 simultaneously or individually characterized by measuring SEQ. ID. Nos.: 1 and 2 and the signature peptides of SEQ. ID. Nos.: 1-2

1.2. claims: 18, 21-32 , 37(also partially)

Methods of detecting and quantifying eculizumab and ALXN1210 simultaneously characterized by the difference mass spectra obtained for SEQ. ID. Nos.: 1, 19, 20 and SEQ. ID. Nos.: 2, 21, 22

1.3. claims: 33, 34, 38, 39(completely) ; 19-32 , 37(partially)

Methods of detecting and quantifying eculizumab and ALXN1210 individually and the signature peptides of SEQ. ID. Nos.: 19, 20

1.4. claims: 35, 36, 40, 41(completely) ; 19-32 , 37(partially)

Methods of detecting and quantifying eculizumab and ALXN1210 individually and the signature peptides of SEQ. ID. Nos.: 21, 22
