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(54) **Title:** METHODS FOR DETECTING PROTEIN IN PLASMA

(57) **Abstract:** The present invention provides methods for detecting protein in plasma comprising contacting said plasma with a protease capable of digesting said protein into at least one detectable fragment and detecting said at least one detectable fragment using high performance liquid chromatography and mass spectroscopy.

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Methods for Detecting Protein in Plasma

Field of the Invention

The present invention provides methods for detecting protein in plasma comprising contacting said plasma with a protease capable of digesting said protein into at least one detectable fragment and detecting said at least one detectable fragment using high performance liquid chromatography and mass spectroscopy.

Background of the Invention

Therapeutic proteins are currently used to treat a wide variety of diseases. Therapeutic proteins can range in size from small synthetic polypeptides that are less than 50 amino acids in size to larger recombinant proteins, including fusion proteins, as well as monoclonal antibodies or fragments thereof. Typically, therapeutic proteins are larger than non-protein medications and, therefore, pose unique problems in determining their concentrations in tissue, serum and plasma. However, the pharmacokinetic aspects of a therapeutic protein must be determined during clinical testing and after in order to assess safety and efficacy. Usually, therapeutic protein concentration is detected using immunochemical techniques which employ such detection methods as colorimetry, flourimetry, or luminescence. However, immunochemical techniques merely provide non-selective results.

Additionally, mammals, including humans, naturally produce endogenous proteins that can be used as biomarkers for certain diseases or to predict response to certain therapeutics. For instance, natural production of certain cytokines by a mammal may be associated with diseases and disorders as well as inflammation. Additionally, certain tumour associated proteins can be produced by a mammal suffering from a particular type of cancer. Furthermore, mammals may have immunogenic responses to therapeutics, including small molecules, polypeptides and antibodies; therefore, producing endogenous antibodies against therapeutics administered to the mammal. Thus, a robust and sensitive method for determining therapeutic and/or endogenous protein from plasma is needed.

Summary of the invention

Methods are provided for detecting protein in plasma comprising contacting said plasma with a protease capable of digesting said therapeutic protein into at least one detectable fragment and detecting said at least one detectable fragment using high performance liquid chromatography and mass spectroscopy. The protein may be a therapeutic protein and/or an endogenous protein.

Brief Description of the Drawings

Figure 1: Amino Acid Sequence of SEQ ID NO.:1, wherein the Specific Peptide Fragments of SEQ ID NO:2 are underlined.

Figure 2: Amino acid [^{15}N $^{13}C_6$]-GLP1M (SEQ ID NO.:3) and GLP-1 peptide fragment (SEQ ID NO.:2) produced from the digestion of [^{15}N $^{13}C_6$]-GLP1M with endoproteinase Lys-C.

Figure 3: Structure of GLP-1 Specific Peptide Fragment (SEQ ID NO.:2) produced from the digestion of [^{15}N $^{13}C_6$]-GLP1M with endoproteinase Lys-C.

Figure 4: Method correlation plot for data generated using high performance liquid chromatography and mass spectroscopy methods and the ELISA methods.

Detailed Description of the Invention

Glossary

As used herein "therapeutic protein" means any polypeptide, protein, protein variant, fusion protein and/or fragment thereof which may be administered to a mammal as a medicament. By way of example, a therapeutic protein includes, but is not limited to, insulin, incretin mimetic, hormones, monoclonal antibodies which may be murine, human, humanized or chimeric, antibody fragments, domain antibodies, fusion proteins and/or fragments, variants and or conjugates of naturally occurring polypeptides.

As used herein "endogenous protein" means any polypeptide naturally produced by a mammal, including a human. By way of example, endogenous protein includes but is not limited to, antibodies, hormones, cytokines, tumor associated proteins, membrane associated proteins, including transmembrane proteins and ion channels, naturally

occurring peptide ligands, biomarkers and nucleic regulatory factors as well as, naturally occurring GLP-1, glucagon, and insulin.

"GLP-1 agonist composition" as used herein means any composition capable of stimulating the secretion of insulin, including, but not limited to an incretin hormone.

"Incretin hormone" as used herein means any hormone that potentiates insulin secretion. One example of an incretin hormone is GLP-1. GLP-1 is an incretin secreted by intestinal L cells in response to ingestion of food. In a healthy individual, GLP-1 plays an important role regulating post-prandial blood glucose levels by stimulating glucose-dependent insulin secretion by the pancreas resulting in increased glucose absorption in the periphery. GLP-1 also suppresses glucagon secretion, leading to reduced hepatic glucose output. In addition, GLP-1 delays gastric emptying time and slows small bowel motility delaying food absorption. GLP-1 promotes continued beta cell competence by stimulating transcription of genes involved in glucose dependent insulin secretion and by promoting beta-cell neogenesis (Meier, *et al. Biodrugs* 2003; 17 (2): 93-102).

"GLP-1 activity" as used herein means one or more of the activities of naturally occurring human GLP-1, including but not limited to, reducing blood or plasma glucose, stimulating glucose-dependent insulin secretion, suppressing glucagon secretion, delaying gastric emptying, and promoting beta cell competence and neogenesis.

An "incretin mimetic" as used herein is a compound capable of potentiating insulin secretion. An incretin mimetic may be capable of stimulating insulin secretion, increasing beta cell neogenesis, inhibiting beta cell apoptosis, inhibiting glucagon secretion, delaying gastric emptying and inducing satiety in a mammal. An incretin mimetic may include, but is not limited to, an incretin hormone, any polypeptide which has GLP-1 activity, including but not limited to, GLP-1, a GLP-1 fragment, variant and/or fusion, exendin 3 and exendin 4, including any fragments and/or variants and/or conjugates thereof.

"Hypoglycemic agent" as used herein means any compound or composition comprising a compound capable of reducing blood glucose. A hypoglycemic agent may include, but is not limited to, any GLP-1 agonist including incretin hormones or incretin mimetics, GLP-1 and/or fragment, variant and/or conjugate thereof. Other hypoglycemic agents include, but are not limited to, drugs that increase insulin secretion (*e.g.*, sulfonylureas (SU) and meglitinides), increase glucose utilization (*e.g.*, glitazones), reduce hepatic glucose production (*e.g.*, metformin), and delay glucose absorption (*e.g.*, α-glucosidase inhibitors).

"Polypeptide" refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds, *i.e.*, peptide isosteres. "Polypeptide" refers to both short chains, commonly referred to as peptides, oligopeptides or oligomers, and to longer chains, generally referred to as proteins. Polypeptides may contain amino acids other than the 20 gene-encoded amino acids. "Polypeptides" include amino acid sequences modified either by natural processes, such as posttranslational processing, or by chemical modification techniques that are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Polypeptides may be branched as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched and branched cyclic polypeptides may result from posttranslation natural processes or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. See, for instance, PROTEINS - STRUCTURE AND MOLECULAR PROPERTIES, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York, 1993 and Wold, F., Posttranslational Protein Modifications: Perspectives and Prospects, pgs. 1-12 in POSTTRANSLATIONAL COVALENT MODIFICATION OF PROTEINS, B. C. Johnson, Ed., Academic Press, New York, 1983; Seifter, *et al.*, "Analysis for protein modifications and nonprotein cofactors", *Meth. Enzymol.* (1990) 182:626-646 and Rattan, *et al.*, "Protein Synthesis: Posttranslational Modifications and Aging", *Ann NY Acad Sci* (1992) 663:48-62.

"Variant" as the term is used herein, is a polynucleotide or polypeptide that differs from a reference polynucleotide or polypeptide respectively, but retains essential properties. A typical variant of a polynucleotide differs in nucleotide sequence from another, reference polynucleotide. Changes in the nucleotide sequence of the variant may or may not alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Nucleotide changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence, as discussed below. A typical variant of a polypeptide differs in amino acid sequence from another, reference polypeptide. Generally, differences are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions in any combination. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. A variant of a polynucleotide or polypeptide may be a naturally occurring such as an allelic variant, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of polynucleotides and polypeptides may be made by mutagenesis techniques or by direct synthesis.

As used herein "fragment," when used in reference to a polypeptide, is a polypeptide having an amino acid sequence that is the same as part but not all of the amino acid sequence of the entire naturally occurring polypeptide. Fragments may be "free-standing" or comprised within a larger polypeptide of which they form a part or region as a single continuous region in a single larger polypeptide. By way of example, a fragment of naturally occurring GLP-1 would include amino acids 7 to 36 of naturally occurring amino acids 1 to 36. Furthermore, fragments of a polypeptide may also be variants of the naturally occurring partial sequence. For instance, a fragment of GLP-1 comprising amino acids 7-30 of naturally occurring GLP-1 may also be a variant having amino acid substitutions within its partial sequence.

As used herein "conjugate" or "conjugated" refers to two molecules that are bound to each other. For example, a first polypeptide may be covalently or non-covalently bounded to a second polypeptide. The first polypeptide may be covalently bound by a chemical linker or may be genetically fused to the second polypeptide, wherein the first and second polypeptide share a common polypeptide backbone.

As used herein "tandemly oriented" refers to two or more polypeptides that are adjacent to one another as part of the same molecule. They may be linked either covalently or non-covalently. Two or more tandemly oriented polypeptides may form part of the same polypeptide backbone. Tandemly oriented polypeptides may have direct or inverted orientation and/or may be separated by other amino acid sequences.

As used herein, "reduce" or "reducing" blood glucose refers to a decrease in the amount of blood glucose observed in the blood of a patient after administration a hypoglycemic agent.

As used herein "diseases associated with elevated blood glucose" include, but are not limited to, Type I and Type II Diabetes and hyperglycemia.

As used herein "co-administration" or "co-administering" as used herein refers to administration of two or more compounds to the same patient. Co-administration of such compounds may be simultaneous or at about the same time (e.g., within the same hour) or it may be within several hours or days of one another. For example, a first compound may be administered once weekly while a second compound is co-administered daily.

"Recombinant expression system(s)" refers to expression systems or portions thereof or polynucleotides of the invention introduced or transformed into a host cell or host cell lysate for the production of the polynucleotides and polypeptides of the invention.

"Isolated" means altered "by the hand of man" from its natural state, i.e., if it occurs in nature, it has been changed or removed from its original environment, or both. For example, a polynucleotide or a polypeptide naturally present in a living organism is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated," as the term is employed herein. Moreover, a polynucleotide or polypeptide that is introduced into an organism by transformation, genetic manipulation or by any other recombinant method is "isolated" even if it is still present in said organism, which organism may be living or non-living.

Intact antibodies include heteromultimeric glycoproteins comprising at least two heavy and two light chains. Aside from IgM, intact antibodies are usually heterotetrameric glycoproteins of approximately 150Kda, composed of two identical light (L) chains and two identical heavy (H) chains. Typically, each light chain is linked to a heavy chain by one covalent disulfide bond while the number of disulfide linkages between the heavy chains of different immunoglobulin isotypes varies. Each heavy and light chain also has intrachain disulfide bridges. Each heavy chain has at one end a variable domain (VH)

followed by a number of constant regions. Each light chain has a variable domain (VL) and a constant region at its other end; the constant region of the light chain is aligned with the first constant region of the heavy chain and the light chain variable domain is aligned with the variable domain of the heavy chain. The light chains of antibodies from most vertebrate species can be assigned to one of two types called Kappa and Lambda based on the amino acid sequence of the constant region. Depending on the amino acid sequence of the constant region of their heavy chains, human antibodies can be assigned to five different classes, IgA, IgD, IgE, IgG and IgM. IgG and IgA can be further subdivided into subclasses, IgG1, IgG2, IgG3 and IgG4; and IgA1 and IgA2. Species variants exist with mouse and rat having at least IgG2a, IgG2b. The variable domain of the antibody confers binding specificity upon the antibody with certain regions displaying particular variability called complementarity determining regions (CDRs). The more conserved portions of the variable region are called Framework regions (FR). The variable domains of intact heavy and light chains each comprise four FR connected by three CDRs. The CDRs in each chain are held together in close proximity by the FR regions and with the CDRs from the other chain contribute to the formation of the antigen binding site of antibodies. The constant regions are not directly involved in the binding of the antibody to the antigen but exhibit various effector functions such as participation in antibody dependent cell-mediated cytotoxicity (ADCC), phagocytosis via binding to Fcγ receptor, half-life/clearance rate via neonatal Fc receptor (FcRn) and complement dependent cytotoxicity via the C1q component of the complement cascade.

A bispecific antibody is an antibody having binding specificities for at least two different epitopes. Methods of making such antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the coexpression of two immunoglobulin H chain-L chain pairs, where the two H chains have different binding specificities see Millstein *et al*, Nature 305 537-539 (1983), WO93/08829 and Traunecker *et al* EMBO, 10, 1991, 3655-3659. Because of the random assortment of H and L chains, a potential mixture of ten different antibody structures are produced of which only one has the desired binding specificity. An alternative approach involves fusing the variable domains with the desired binding specificities to heavy chain constant region comprising at least part of the hinge region, CH2 and CH3 regions. It is preferred to have the CH1 region containing the site necessary for light chain binding present in at least one of the fusions. DNA encoding these fusions, and if desired the L

chain are inserted into separate expression vectors and are then cotransfected into a suitable host organism. It is possible though to insert the coding sequences for two or all three chains into one expression vector. A bispecific antibody may be composed of a H chain with a first binding specificity in one arm and a H-L chain pair, providing a second binding specificity in the other arm, see WO94/04690. Also see Suresh *et al* Methods in Enzymology 121, 210, 1986.

Antibody fragments may be functional antigen binding fragments of intact and/or humanized and/or chimeric antibodies such as Fab, Fab', $F(ab')_2$, Fv, ScFv fragments of the antibodies. Traditionally such fragments are produced by the proteolytic digestion of intact antibodies by e.g. papain digestion (see for example, WO 94/29348) but may be produced directly from recombinantly transformed host cells. For the production of ScFv, see Bird *et al.* (1988) Science, 242, 423-426. In addition, antibody fragments may be produced using a variety of engineering techniques as described below.

Fv fragments appear to have lower interaction energy of their two chains than Fab fragments. To stablize the association of the VH and VL domains, they have been linked with peptides (Bird *et al*, (1988) Science 242, 423-426, Huston *et al*, PNAS, 85, 5879-5883), disulphide bridges (Glockshuber *et al*, (1990) Biochemistry, 29, 1362-1367) and "knob in hole" mutations (Zhu *et al* (1997), Protein Sci., 6, 781-788). ScFv fragments can be produced by methods well known to those skilled in the art see Whitlow *et al* (1991) Methods companion Methods Enzymol, 2, 97-105 and Huston *et al* (1993) Int.Rev.Immunol 10, 195-217. ScFv may be produced in bacterial cells such as *E. coli* but are more preferably produced in eukaryotic cells. One disadvantage of ScFv is the monovalency of the product, which precludes an increased avidity due to polyvalent binding, and their short half-life. Attempts to overcome these problems include bivalent $(ScFv')_2$ produced from ScFV containing an additional C terminal cysteine by chemical coupling (Adams *et al* (1993) Can.Res 53, 4026-4034 and McCartney *et al* (1995) Protein Eng. 8, 301-314) or by spontaneous site-specific dimerization of ScFv containing an unpaired C terminal cysteine residue (see Kipriyanov *et al* (1995) Cell. Biophys 26, 187-204). Alternatively, ScFv can be forced to form multimers by shortening the peptide linker to 3 to 12 residues to form "diabodies", see Holliger *et al* PNAS (1993), 90, 6444-6448. Reducing the linker still further can result in ScFV trimers ("triabodies", see Kortt *et al* (1997) Protein Eng, 10, 423-433) and tetramers ("tetrabodies", see Le Gall *et al* (1999) FEBS Lett, 453, 164-168). Construction of bivalent ScFV molecules can also be

achieved by genetic fusion with protein dimerizing motifs to form "miniantibodies" (see Pack *et al* (1992) Biochemistry 31, 1579-1584) and "minibodies" (see Hu *et al* (1996), Cancer Res. 56, 3055-3061). ScFv-Sc-Fv tandems ((ScFV)2) may also be produced by linking two ScFv units by a third peptide linker, see Kurucz *et al* (1995) J.Immol.154, 4576-4582. Bispecific diabodies can be produced through the noncovalent association of two single chain fusion products consisting of VH domain from one antibody connected by a short linker to the VL domain of another antibody, see Kipriyanov *et al* (1998), Int.J.Can 77,763-772. The stability of such bispecific diabodies can be enhanced by the introduction of disulphide bridges or "knob in hole" mutations as described *supra* or by the formation of single chain diabodies (ScDb) wherein two hybrid ScFv fragments are connected through a peptide linker see Kontermann *et al* (1999) J.Immunol.Methods 226 179-188. Tetravalent bispecific molecules are available by e.g. fusing a ScFv fragment to the CH3 domain of an IgG molecule or to a Fab fragment through the hinge region see Coloma *et al* (1997) Nature Biotechnol. 15, 159-163. Alternatively, tetravalent bispecific molecules have been created by the fusion of bispecific single chain diabodies (see Alt *et al*, (1999) FEBS Lett 454, 90-94. Smaller tetravalent bispecific molecules can also be formed by the dimerization of either ScFv-ScFv tandems with a linker containing a helix-loop-helix motif (DiBi miniantibodies, see Muller *et al* (1998) FEBS Lett 432, 45-49) or a single chain molecule comprising four antibody variable domains (VH and VL) in an orientation preventing intramolecular pairing (tandem diabody, see Kipriyanov *et al*, (1999) J.Mol.Biol. 293, 41-56). Bispecific $F(ab')_2$ fragments can be created by chemical coupling of Fab' fragments or by heterodimerization through leucine zippers (see Shalaby *et al*, (1992) J.Exp.Med. 175, 217-225 and Kostelny *et al* (1992), J.Immunol. 148, 1547-1553). Also available are isolated VH and VL domains (Domantis plc), see US 6, 248,516; US 6,291,158; US 6, 172,197.

Heteroconjugate antibodies are composed of two covalently joined antibodies formed using any convenient cross-linking methods. See, for example, U.S. Patent No. 4,676,980.

Hypoglycemic agents may be used in the treatment of both Type I and Type II Diabetes to lower glucose concentration in blood. Insulinotropic peptides have been implicated as possible therapeutic agents for the treatment of diabetes. Insulinotropic peptides include incretin hormones such as, but not limited to, gastric inhibitory peptide (GIP) and glucagon like peptide-1 (GLP-1) as well as fragments, variants, and/or conjugates thereof. Insulinotropic peptides also include exendin 3 and exendin 4. GLP-1 is a 36 amino acid long incretin hormone secreted by the L-cells in the intestine in response to ingestion of food. GLP-1 has been shown to stimulate insulin secretion in a physiological and glucose-dependent manner, decrease glucagon secretion, inhibit gastric emptying, decrease appetite, and stimulate proliferation of β-cells. In non-clinical experiments GLP-1 promotes continued beta cell competence by stimulating transcription of genes important for glucose dependent insulin secretion and by promoting beta-cell neogenesis (Meier, et al. *Biodrugs.* 2003; 17 (2): 93-102).

In a healthy individual, GLP-1 plays an important role regulating post-prandial blood glucose levels by stimulating glucose-dependent insulin secretion by the pancreas resulting in increased glucose absorption in the periphery. GLP-1 also suppresses glucagon secretion, leading to reduced hepatic glucose output. In addition, GLP-1 delays gastric emptying and slows small bowel motility delaying food absorption.

In people with Type II Diabetes Mellitus, the normal post-prandial rise in GLP-1 is absent or reduced (Vilsboll T, *et al.*, *Diabetes.* 2001. 50; 609-613). Accordingly, the rationale for administering exogenous GLP-1, an incretin hormone, or an incretin mimetic, is to replace or supplement endogenous GLP-1 in order to increase meal-related insulin secretion, reduce glucagon secretion, and slow gastrointestinal motility. Native GLP-1 has a very short serum half-life (<5 minutes). Thus, incretin mimetics used to simulate GLP-1 typically have a longer half-life than endogenous GLP-1.

The present invention provides methods for detecting at least one protein in plasma from a mammal comprising contacting said plasma with a first protease capable of digesting said at least one protein into at least one detectable fragment and detecting said at least one detectable fragment using high performance liquid chromatography and mass spectroscopy. In one aspect, said at least one protein is a therapeutic protein. In another aspect, said at least one protein is an endogenous protein to said mammal. In yet another aspect, the mammal is a human. Methods of the present invention further comprises

calculating pharmacokinetic parameters of at least one therapeutic and/or endogenous protein.

As is understood in the art various methods may be employed to collect, measure and assess pharmacokinetic data such as active compound concentration in blood, plasma and/or other tissue. As is also understood in the art, various methods may be employed to collect, measure and assess various pharmacodynamic data such as, but nor limited to, glucose, insulin, C peptide, glucagons and other biomarker levels in blood and/or plasma and/or other tissue. A skilled artisan will understand the various methods for measuring and calculating the pharmacokinetic (for example, but not limited to, Cmax, AUC, Tmax, serum half-life) and pharmacodynamic (for example, but not limited to, serum, plasma and blood glucose levels) parameters described herein. Furthermore, the skilled artisan will understand the various methods for making statistical comparisons (for example, but not limited to, comparisons of change from baseline to post-treatment and/or comparisons among treatment groups) and/or analysis of the pharmacokinetic and pharmacodynamic parameters described herein. Furthermore, the skilled artisan will understand and be able to employ various other methods for collecting and analyzing pharmacokinetic, pharmacodynamic and other clinical data.

A therapeutic protein of the present methods may be an incretin mimetic. An incretin mimetic can be selected from the group of: incretin hormone and/or fragment, variant and/or conjugate thereof and GLP-1 agonist and/or fragment, variant and/or conjugate thereof. Incretin mimetics may comprise at least one fragment and/or variant of human GLP-1 conjugated with a fusion partner. Fragments and variants of GLP-1 may comprise GLP-1(7-36(A8G)), wherein alanine 8 of wild type GLP-1, such alanine being mutated to a glycine (hereinafter designated as "A8G") (*See* for example, the mutants disclosed in U.S. Pat. No. 5,545,618, herein incorporated by reference in its entirety). Fragments and variants of GLP-1 may be genetically fused to human serum albumin. Fragments and variants of GLP-1 may comprise at least two GLP-1(7-36(A8G)) tandemly and genetically fused to the human serum albumin. GLP-1(7-36(A8G)) may be genetically fused at the N-terminus of the human serum albumin. Therapeutic proteins of the present invention may comprise SEQ ID No.: 1.

Variants of GLP-1(7-37) may be denoted for example as Glu^{22}-GLP-1(7-37)OH which designates a GLP-1 variant in which the glycine normally found at position 22 of

GLP-1(7-37)OH has been replaced with glutamic acid; Val^{8}-Glu^{22}-GLP-1(7-37)OH designates a GLP-1 compound in which alanine normally found at position 8 and glycine normally found at position 22 of GLP-1(7-37)OH have been replaced with valine and glutamic acid, respectively. Examples of variants of GLP-1 include, but are not limited to, those presented below:

Val^{8}-GLP-1(7-37)OH	Gly^{8}-GLP-1(7-37)OH	Glu^{22}-GLP-1(7-37)O- H
Asp^{22}-GLP-1(7-37)OH	Arg^{22}-GLP-1(7-37)OH	Lys^{22}-GLP-1(7-37)OH
Cys^{22}-GLP-1(7-37)OH	Val^{8}-Glu^{22}-GLP-1(7-37)OH	Val^{8}-Asp^{22}-GLP-1(7-37)OH
Val^{8}-Arg^{22}-GLP-1(7-37)OH	Val^{8}-Lys^{22}-GLP-1(7-37)OH	Val^{8}-Cys^{22}-GLP-1(7-37)OH
Gly^{8}-Glu^{22}-GLP-1(7-37)OH	Gly^{8}-Asp^{22}-GLP-1(7-37)OH	Gly^{8}-Arg^{22}-GLP-1(7-37)OH
Gly^{8}-Lys^{22}-GLP-1(7-37)OH	Gly^{8}-Cys^{22}-GLP-1(7-37)OH	Glu^{22}-GLP-1(7-36)OH
Asp^{22}-GLP-1(7-36)OH	Arg^{22}-GLP-1(7-36)OH	Lys^{22}-GLP-1(7-36)OH
Cys^{22}-GLP-1(7-36)OH	Val^{8}-Glu^{22}-GLP-1(7-36)OH	Val^{8}-Asp^{22}-GLP-1(7-36)OH
Val^{8}-Arg^{22}-GLP-1(7-36)OH	Val^{8}-Lys^{22}-GLP-1(7-36)OH	Val^{8}-Cys^{22}-GLP-1(7-36)OH
Gly^{8}-Glu^{22}-GLP-1(7-36)OH	Gly^{8}-Asp^{22}-GLP-1(7-36)OH	Gly^{8}-Arg^{22}-GLP-1(7-36)OH
Gly^{8}-Lys^{22}-GLP-1(7-36)OH	Gly^{8}-Cys^{22}-GLP-1(7-36)OH	Lys^{23}-GLP-1(7-37)OH
Val^{8}-Lys^{23}-GLP-1(7-37)OH	Gly^{8}-Lys^{23}-GLP-1(7-37)OH	His^{24}-GLP-1(7-37)OH
Val^{8}-His^{24}-GLP-1(7-37)OH	Gly^{8}-His^{24}-GLP-1(7-37)OH	Lys^{24}-GLP-1(7-37)OH
Val^{8}-Lys^{24}-GLP-1(7-37)OH	Gly^{8}-Lys^{23}-GLP-1(7-37)OH	Glu^{30}-GLP-1(7-37)OH
Val^{8}-Glu^{30}-GLP-1(7-37)OH	Gly^{8}-Glu^{30}-GLP-1(7-37)OH	Asp^{30}-GLP-1(7-37)OH
Val^{8}-Asp^{30}-GLP-1(7-37)OH	Gly^{8}-Asp^{30}-GLP-1(7-37)OH	Gln^{30}-GLP-1(7-37)OH
Val^{8}-Gln^{30}-GLP-1(7-37)OH	Gly^{8}-Gln^{30}-GLP-1(7-37)OH	Tyr^{30}-GLP-1(7-37)OH
Val^{8}-Tyr^{30}-GLP-1(7-37)OH	Gly^{8}-Tyr^{30}-GLP-1(7-37)OH	Ser^{30}-GLP-1(7-37)OH
Val^{8}-Ser^{30}-GLP-1(7-37)OH	Gly^{8}-Ser^{30}-GLP-1(7-37)OH	His^{30}-GLP-1(7-37)OH
Val^{8}-His^{30}-GLP-1(7-37)OH	Gly^{8}-His^{30}-GLP-1(7-37)OH	Glu^{34}-GLP-1(7-37)OH
Val^{8}-Glu^{34}-GLP-1(7-37)OH	Gly^{8}-Glu^{34}-GLP-1(7-37)OH	Ala^{34}-GLP-1(7-37)OH
Val^{8}-Ala^{34}-GLP-1(7-37)OH	Gly^{8}-Ala^{34}-GLP-1(7-37)OH	Gly^{34}-GLP-1(7-37)OH
Val^{8}-Gly^{34}-GLP-1(7-37)OH	Gly^{8}-Gly^{34}-GLP-1(7-37)OH	Ala^{35}-GLP-1(7-37)OH
Val^{8}-Ala^{35}-GLP-1(7-37)OH	Gly^{8}-Ala^{35}-GLP-1(7-37)OH	Lys^{35}-GLP-1(7-37)OH
Val^{8}-Lys^{35}-GLP-1(7-37)OH	Gly^{8}-Lys^{35}-GLP-1(7-37)OH	His^{35}-GLP-1(7-37)OH
Val^{8}-His^{35}-GLP-1(7-37)OH	Gly^{8}-His^{35}-GLP-1(7-37)OH	Pro^{35}-GLP-1(7-37)OH
Val^{8}-Pro^{35}-GLP-1(7-37)OH	Gly^{8}-Pro^{35}-GLP-1(7-37)OH	Glu^{35}-GLP-1(7-37)OH
Gly^{8}-Glu^{35}-GLP-1(7-37)OH	Val^{8}-Ala^{27}-GLP-1(7-37)OH	Val^{8}-His^{37}-GLP-1(7-37)OH
Val^{8}-Glu^{22}-Lys^{23}-GLP-1(7-37)OH	Val^{8}-Glu^{22}-Glu^{23}-GLP-1(7-37)OH	Val^{8}-Glu^{22}-Ala^{27}-GLP-1(7-37)OH
Val^{8}-Gly^{34}-Lys^{35}-GLP-1(7-37)OH	Val^{8}-His^{37}-GLP-1- (7-37)OH	Gly^{8}-His^{37}-GLP-1(7-37)OH
Val^{8}-Glu^{22}-Ala^{27}-GLP-1(7-37)OH	Gly^{8}-Glu^{22}-Ala^{27}-GLP-1(7-37)OH	Val^{8}-Lys^{22}-Glu^{23}-GLP-1(7-37)OH
Gly^{8}-Lys^{22}-Glu^{23}-GLP-1(7-37)OH.	Val^{8}-Glu^{35}-GLP-1(7-37)OH	

Variants of GLP-1 may also include, but are not limited to, GLP-1 or GLP-1 fragments having chemical modification of one or more of its amino acid side groups. A chemical modification includes, but is not limited to, adding chemical moieties, creating new bonds, and removing chemical moieties. Modifications at amino acid side groups

include, without limitation, acylation of lysine-ε-amino groups, N-alkylation of arginine, histidine, or lysine, alkylation of glutamic or aspartic carboxylic acid groups, and deamidation of glutamine or asparagine. Modifications of the terminal amino group include, without limitation, the des-amino, N-lower alkyl, N-di-lower alkyl, and N-acyl modifications. Modifications of the terminal carboxy group include, without limitation, the amide, lower alkyl amide, dialkyl amide, and lower alkyl ester modifications. Furthermore, one or more side groups, or terminal groups, may be protected by protective groups known to the ordinarily-skilled protein chemist.

GLP-1 fragments or variants may also include polypeptides in which one or more amino acids have been added to the N-terminus and/or C-terminus of GLP-1(7-37)OH of said fragment or variant. The amino acids in GLP-1 in which amino acids have been added to the N-terminus or C-terminus are denoted by the same number as the corresponding amino acid in GLP-1(7-37)OH. For example, the N-terminus amino acid of a GLP-1 compound obtained by adding two amino acids to the N-terminus of GLP-1(7-37)OH is at position 5; and the C-terminus amino acid of a GLP-1 compound obtained by adding one amino acid to the C-terminus of GLP-1(7-37)OH is at position 38. Thus, position 12 is occupied by phenylalanine and position 22 is occupied by glycine in both of these GLP-1 compounds, as in GLP-1(7-37)OH. Amino acids 1-6 of a GLP-1 with amino acids added to the N-terminus may be the same as or a conservative substitution of the amino acid at the corresponding position of GLP-1(1-37)OH. Amino acids 38-45 of a GLP-1 with amino acids added to the C-terminus may be the same as or a conservative substitution of the amino acid at the corresponding position of glucagon or exendin-4.

Fusion partners of therapeutic proteins of the present invention can comprise at least one polypeptide, variant and/or fragment thereof of a polypeptide selected from the group of: albumin, transferrin, and Fc portion of an IgG. Therapeutic proteins of the present invention can be selected from the group of: exendin 3, exendin 4, human GLP-1 and fragments, variants, and/or or conjugates thereof. Therapeutic proteins also include isolated polypeptide fragments, variants and/or conjugates thereof, monoclonal antibodies, which may be murine, human, humanized or chimeric, and fragments thereof or a domain antibodies.

Methods are also provided herein for detecting at least one endogenous protein in plasma from a mammal comprising contacting said plasma with a protease capable of

digesting said at least one protein into at least one detectable fragment and detecting said at least one detectable fragment using high performance liquid chromatography and mass spectroscopy. The endogenous protein may be selected from the group of antibody, cytokine, tumor associated protein, at least one biomarker, and transmembrane protein.

Additionally, methods are provided for detecting at least one protein in plasma from a mammal comprising contacting said plasma with a protease capable of digesting said at least one protein wherein the protease is selected from the group of: Lys-C, Trypsin, Asp-N, Arg-C, Asn-C, Post-Proline cleaving enzyme, and CNbr. In some aspects, the mammal is a human.

Methods are also provided further comprising preparing a stable detectable standard peptide. The stable detectable standard peptide can be prepared in a separate container from said at least one protein. In such an embodiment, the stable detectable standard peptide may be digested in a separate container or may not need to be digested. In another embodiment, the standard detectable marker may be a synthetic peptide. The stable detectable standard peptide would then be used to quantify the amount of at least one protein in plasma using the methods described herein. In another aspect methods are provided wherein said stable detectable standard peptide is contacted with said plasma. The stable detectable standard peptide may be digested using a second protease, if necessary. This second protease can be the same protease used to prepare said at least one protein or may be a different protease. The stable detectable standard peptide can be detected from the same sample as the protein of interest. In one aspect, the stable detectable standard peptide is isotopically labeled.

In another aspect, methods are provided further comprising generating a peptide library of said protein. Additional methods comprise selecting a fragment of said at least one protein that will be produced upon digesting with said protease. The fragment can be separated from all other fragments of said therapeutic protein produced by said protease based on one or more of the following characteristics: charge, size, secondary structure, and tertiary structure.

In yet another aspect, high throughput methods are provided for detecting at least one endogenous protein in plasma from a mammal. In another aspect, the methods are quantitative.

The methods of the present invention may be combined with several other technologies for rapid detection of at least one protein in plasma. For instance, the methods of the present invention can be combined with microarray technology and/or nanotechnology, including microfluidic analysis systems for detection and quantification of samples using mass spectroscopy. An example of a nanoliter microfluidic analysis system is described in Andersson, *et al., Anal. Chem.* 79:4022-4030 (2007).

The following examples illustrate various aspects of this invention. These examples do not limit the scope of this invention which is defined by the appended claims.

Examples

Example 1 – SEQ ID NO.:1 in Human Plasma

The following parameters were assessed:

- Selectivity, sensitivity and linearity
- Bias and precision
- Stability of SEQ ID NO.:1 in human plasma at room temperature
- The effect of freeze-thaw on the stability of SEQ ID NO.:1 in human plasma
- Stability of SEQ ID NO.:1 in processed samples
- The ability to dilute samples above the higher limit of quantification (HLQ)

Validation Procedure

SEQ ID NO.:1 is a 73,012 Dalton (645 amino acid) genetic fusion protein consisting of two modified 30 amino acid GLP-1 peptides linked in tandem to recombinant human serum albumin. This example describes HPLC-MS/MS methods for the determination of SEQ ID NO.:1 in human plasma. 200 μL human plasma sample from a human administered SEQ ID NO.:1 was combined with an isotopically labeled GLP-1 internal standard, [$^{15}N\,^{13}C_6$]-GLP1M (SEQ ID NO.:3) (Figure 3), and digested using the endoproteinase enzyme Lys-C.

Two GLP-1 specific peptide fragments (1005 and 1012Da) both from SEQ ID NO.:1 and [$^{15}N\,^{13}C_6$]-GLP1M were extracted from 200 μL of human plasma by solid

phase extraction. Extracts were analyzed by HPLC-MS/MS using a TurboIonSpray™ interface and multiple reaction monitoring.

Sample Preparation

Human plasma was transferred into 1.2 mL polypropylene 96-well tubes (Micronic Systems, Lelystad, Holland). [^{15}N $^{13}C_6$]-GLP1M was then added to each plasma sample. The plasma was enzymatically digested using endoproteinase lys-C to produce specific peptide fragments for both SEQ ID NO.:1 and [^{15}N $^{13}C_6$]-GLP1M (See Figures 3 and 2). The digestion was halted by adding acidified water. The prepared samples were loaded onto a mixed mode strong cat-ion exchange plate, washed, eluted, and evaporated under nitrogen before reconstituting and injection onto HPLC–MS/MS system for analysis. MS/MS detection was performed on an API4000 (Applied Biosystems/MDS Sciex) triple quadrupole instrument with thermally assisted electrospray ionization (TurboIonSpray™) and multiple reaction monitoring.

The following steps were used to prepare sample:

- 200 μL of plasma from a human patient, standard or QC were aliquoted into the polypropylene 96-well tubes;
- 50 uL of isotopically labeled GLP-1 internal standard ([^{15}N $^{13}C_6$]-GLP1M) was added to all plasma samples
- 50 uL of DI water were added to double blank samples
- 100 μL of endoproteinase lys-C (60 mIU) were added to all plasma samples and incubated at ambient room temperature for 24 hours;
- 300 μL of 0.1 N HCL in Milli-Q water was added to all plasma samples;
- 500 μL of methanol was added to a Strata X-C 10 mg/well (Phenomenex) SPE 96 well extraction plate followed by 500 μL 0.01N HCl for conditioning;
- Prepared samples were loaded onto the plate and allowed to sit for approximately five minutes before applying vacuum;
- 500 μL 0.01N HCl followed by 500 μL methanol was added to the plate for washing;
- 500 μL 5% NH_4OH in methanol was added to the plate to elute samples;
- Each sample was evaporated under nitrogen at 50°C to dryness;

- 200 µL 0.1% Formic Acid /Acetonitrile (70/30, v/v) was added to reconstitute each sample; and
- Each reconstituted sample was injected directly onto HPLC-MS/MS system for analysis.

Reagents for Sample preparation are described in Table 1 below:

Table 1 Reagents for Sample Preparation

Reagent	Function	Preparation
[^{15}N $^{13}C_6$]-GLP1M	Internal Standard	H-His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-**Glu-Phe-Ile-Ala-Trp-[$^{15}N^{13}C_6$]Leu-Val-Lys**-Gly-Arg-OH (SEQ ID NO.:3) Bachem, King of Prussia, PA
Lys C	Digestion Reagent	Endoproteinase Lys-C from *Lysobacter enzymogenes,* 3IU Sigma, St. Louis, MO
Lys C Solution	Digestion Reagent	Add 5 mL 100mM Bicarbonate Buffer, pH 8.5 to 3IU Lys C. Prepare fresh daily
100mM Sodium Bicarbonate Buffer, pH 8.5	Digestion Buffer	Add 8.401g of sodium bicarbonate to 1 L DI water. Adjust pH to 8.5 using NaOH. Filter buffer using 0.2 micron filter.
Water containing 0.1% (v/v) formic acid	Mobile Phase A	Add 1 mL of formic acid to water and make up to 1000 mL with water.
5% NH_4OH in MeOH	Elution Solution	Add 50 mL NH_4OH to 950 mL MeOH
70:30 0.1% Formic Acid:Acetonitrile (v/v)	Reconstitution Solution	Add 70 mL of 0.1% Formic acid to 30 mL acetonitrile.
40:40:20 Acetonitrile:Isopropanol:0.1% Formic acid in water	Injector and syringe Solvent Wash	Add 40 mL Acetonitrile to 40 mL Isopropanol, and 20 mL 0.1% Formic acid in water
75:25 0.1% Formic acid in water:Acetonitrile	Injector and syringe Solvent Wash	Add 75 mL 0.1% Formic acid in water to 25 mL Acetonitrile

Unless specified, all chemicals and solvents are at least AR and HPLC grade, respectively.

High Performance Liquid Chromatography

HPLC-MS/MS data were acquired and processed (integrated) using the proprietary software application Analyst (Version 1.4.1, Applied Biosystems/MDS Sciex, Canada). Calibration plots of analyte/internal standard peak area ratio versus SEQ ID NO.:1 concentration were constructed and a weighted ($1/x^2$) linear regression (sigma (m * x + c – y) ^ 2) applied to the data. Concentrations of SEQ ID NO.:1 in validation samples were determined from the constructed calibration line, and used to calculate the bias and precision of the method.

HPLC conditions are described in Table 2 and MS/MS conditions are described in Table 3.

Table 2 – HPLC Conditions

HPLC conditions Autosampler	CTC HTS PAL
Injector Wash Solvent 1	40:40:20 Acetonitrile:Isopropanol:0.1% Formic acid in water; 2 washes
Injector Wash Solvent 2	75:25 0.1% Formic Acid in water:Acetonitrile; 2 washes
Syringe Wash Solvent 1	40:40:20 Acetonitrile:Isopropanol:0.1% Formic acid in water; 1 wash
Syringe Wash Solvent 2	75:25 0.1% Formic Acid in water:Acetonitrile; 1 wash
Typical Injection Volume	5 µL, loop injection
Chromatography System	Flux Instruments Rheos 2000 HPLC
Flow Rate	350 µL/min
Analytical Column	Waters Atlantis dC18 2.1 x 50 mm, 3µm
Column Temperature	30°C
Run Time/Data Acquisition Time	3.0 minutes
Autosampler Blocking Time	30 seconds
Mobile Phase B	ACN
Mobile Phase D	0.1% (v/v) formic acid in water
Gradient Composition	

Time (min)	%B	%D
0.0	15	85
0.15	15	85
1.1	75	25
2.0	75	25
2.1	15	85

Alternative, equivalent HPLC equipment may be used as appropriate. Minor changes to chromatographic conditions may be made

Table 3 - MS/MS Conditions

Mass Spectrometer	Applied Biosystems/MDS Sciex API-4000
Ionisation Interface and Temperature	TurboIonSpray® at 750°C
Pause Time	5 msec
Gas 1 Setting (Zero Air)	60 psi
Gas 2 Setting (Zero Air)	60 psi
Curtain Gas Setting (Nitrogen)	30
Collision Gas Setting (Nitrogen)	8

Analytical method parameters are typical but may vary from instrument to instrument in order to achieve an equivalent response

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Test Compounds and Biological Matrix

The amino acid sequence of SEQ ID NO.:1 is presented in Figure 1 and its GLP-1 specific peptide fragment (SEQ ID NO.:2) is presented in Figure 3. The amino acid sequence of isotopically labeled GLP-1 internal standard [^{15}N $^{13}C_6$]-GLP1M (SEQ ID NO.:3) and its GLP-1 specific peptide fragment (SEQ ID NO.:4) is presented in Figure 2. Sufficient volumes of both pooled control human plasma (EDTA) for the preparation of validation samples and control human plasma from 6 individual volunteers to establish the selectivity of the method were obtained from Bioreclamation Inc. (Hicksville, NY) and stored frozen at -80°C.

Preparation of Calibration Standards and Validation Samples

Validation samples to establish the bias and precision of the method were prepared from an independent set of analytical standard solutions to give nominal concentrations of SEQ ID NO.:1 in human plasma at 50, 200, 2000, 40000 and 50000 ng/mL according to the dilution schedule specified in Table 4.

Table 4: Calibration standards are prepared fresh as follows and thoroughly mixed.

Standard Concentration (ng/mL)	Volume of Working Solution (µL)				Total Volume in Human (EDTA) Plasma (µL)
	A4 1 µg/mL	A3 10 µg/mL	A2 100 µg/mL	A1 1000 µg/mL	
50	50	-	-	-	1000
100	100	-	-	-	1000
250	-	25	-	-	1000
500	-	50	-	-	1000
1000	-	100	-	-	1000
2500	-	-	25	-	1000
5000	-	-	50	-	1000
10000	-	-	100	-	1000
25000	-	-	-	25	1000
50000	-	-	-	50	1000

The total volumes prepared may be scaled up or down as required

In addition to blanks and a duplicate set of calibration standards, 6 replicates at each validation sample concentration were analyzed on 3 separate occasions by the

HPLC-MS/MS method. The lyophilized powder of [^{15}N $^{13}C_6$]-GLP1M was reconstituted in the appropriate amount of water to give a 1.0 mg/mL stock solution.

Quality Control/Validation Sample Preparation

Quality controls (QC) and validation samples (VS) were prepared according to the dilutions of Table 5 and thoroughly mixed. Replicate 1.4 mL aliquots were transferred to appropriate assay tubes for storage at - 80°C.

Table 5 – Quality Control and Validation Samples

QC/VS Concentration (ng/mL)	Volume of Spiking Solution (µL)				Total Volume in Human (EDTA) Plasma (mL)
	B4 1 µg/mL	B3 10 µg/mL	B2 100 µg/mL	B1 1000 µg/mL	
50 [1]	500	-	-	-	10
200	-	200	-	-	10
2000	-	-	200	-	10
40000	-	-	-	400	10
50000 [1]	-	-	-	500	10
250000 [1]	-	-	-	500	2

1. Prepared for validation only
The total volumes prepared may be scaled up or down as required

Digestion Procedure:

Samples were subject to digestion according to the following steps:

- Aliquot 200 µL of sample, standard or QC into Micronic tube;
- Add 50 uL of I.S. (5000 ng/mL) and vortex mix.
- Add 100 µL of Lys C Solution in 100mM Sodium Bicarbonate buffer, pH 8.5 to all tubes; and
- Cap all tubes, vortex for 2 min., Centrifuge for 5 min. and place on shaker for 24 hours at room temperature.

Regression Model:

Peak area ratios with $1/x^2$ weighted linear regression.

Selection of the SEQ ID NO.:1 Fragment

SEQ ID NO.: 2, a 1005 Da fragment of SEQ ID NO.:1, was selected for monitoring based on the analysis of the peptide map generated using the endoproteinase

enzyme Lys-C. This fragment contains the amino acid sequence that is specific for the GLP-1 moiety of the fusion protein and there are no other similar amino acid sequences contained within this protein that can be formed when digested with Lys-C. Since two tandem copies of GLP-1 are linked to one molecule of human serum albumin SEQ ID NO.:2 is released from SEQ ID NO.:1 at a molar ratio of 2/1. The selectivity of the 1005 fragment in human plasma was confirmed during the selectivity phase of the validation as described below.

Selectivity, Sensitivity and Linearity

The precursor ion at m/z 503 is the $[M+2H]^{2+}$ ion of the GLP-1 parent ion fragment. The characteristic precursor $[M+2H]^{2+}$ to product ions transitions, m/z 503 to 616 and $[M+H]^{1+}$ 507 to 623 are consistent with the structures of SEQ ID NO.:1 and the internal standard, respectively, and are used as multiple reaction monitoring transitions to ensure high selectivity. The selectivity of the method was established by the analysis of samples of control human plasma from 6 individual volunteers. The selectivity of the method was also assessed by the inclusion of blank and double blank samples prepared from pooled control human plasma in validation assays. HPLC-MS/MS chromatograms of the blanks and validation samples were visually examined and compared for chromatographic integrity and potential interferences. No unacceptable interferences at the retention times of SEQ ID NO.:1 and its internal standard SEQ ID NOs.:2 and 3 were observed.

Linear responses in the analyte/internal standard peak area ratios were observed over the range 50 to 50000 ng/mL. The correlation coefficients obtained using $1/x^2$ weighted linear regression were better than 0.9964.

Bias and Precision

Concentrations of SEQ ID NO.:1 in validation samples were determined from the calibration line on each occasion and are presented in Table 6 along with accuracy and precision values. At all validation sample concentrations examined, the bias was less than 15%, and is therefore acceptable. The maximum bias observed was -12.8%. At all validation sample concentrations examined, the within- and between-run precision values were less than or equal to 15%, and are therefore acceptable. The maximum within- and between-run precision values observed were 4.3% and 8.6%, respectively. As defined by

the lower and upper validation sample concentrations possessing acceptable accuracy and precision, the validated range of this method for SEQ ID NO.:1 based on 200 μL of human plasma is 50 to 50000 ng/mL. Bias, precision and individual sample concentrations for SEQ ID NO.:1 in human plasma are presented in Table 6.

Table 6. Bias, Precision and Individual Validation Sample Concentrations for SEQ ID NO.:1 in Human Plasma

	Nominal Concentrations (ng/mL)				
	50000.0	**40000.0**	**2000.0**	**200.0**	**50.0**
RUN 1	42484.2	36745.2	1973.5	190.2	52.0
	43269.5	34922.2	1858.4	191.7	53.8
	43978.6	35295.2	1939.2	198.3	51.9
	44625.2	37083.3	2051.7	191.7	48.1
	45750.7	37995.6	2019.3	185.7	52.4
	45348.3	37548.6	2022.9	191.1	49.5
Mean (ng/mL)	44242.7	36598.4	1977.5	191.4	51.3
S.D.	1245.2	1234.5	70.7	4.0	2.1
Precision (%CV)	2.8	3.4	3.6	2.1	4.1
Bias %	-11.5	-8.5	-1.1	-4.3	2.5
RUN 2	44794.1	36115.3	1969.9	183.9	49.9
	45633.8	36255.7	1942.6	193.7	49.2
	47046.1	36546.3	1905.1	191.0	51.6
	45970.4	38586.5	1993.3	194.8	47.7
	47692.8	38139.1	2017.4	192.9	53.7
	47105.3	38066.7	1936.7	194.2	51.8
Mean (ng/mL)	46373.7	37284.9	1960.8	191.8	50.6
S.D.	1089.3	1096.1	40.9	4.1	2.2
Precision (%CV)	2.3	2.9	2.1	2.1	4.3
Bias %	-7.3	-6.8	-2.0	-4.1	1.3
RUN 3	44778.2	36456.2	1932.0	205.2	47.0
	46028.4	37233.5	1976.7	200.9	43.8
	43876.0	37764.2	2004.3	193.5	42.9
	48575.9	38645.0	1895.1	199.8	43.5
	46740.1	37869.4	2073.3	191.1	41.9
	48170.0	39850.3	2051.7	194.8	42.6
Mean (ng/mL)	46361.4	37969.8	1988.9	197.5	43.6
S.D.	1850.0	1172.8	68.5	5.3	1.8
Precision (%CV)	4.0	3.1	3.4	2.7	4.1
Bias %	-7.3	-5.1	-0.6	-1.2	-12.8
	Overall Statistics				
Mean (ng/mL)	45659.3	37284.4	1975.7	193.6	48.5
S.D.	1695.3	1240.2	59.0	5.1	4.0
Precision (%)	3.7	3.3	3.0	2.6	8.3
Bias(%)	-8.7	-6.8	-1.2	-3.2	-3.0
Average Within-run precision (%)	3.1	3.1	3.1	2.3	4.2
Between-run precision (%)	2.4	1.3	Negligible	1.5	8.6

The ability to dilute samples containing SEQ ID NO.:1 at concentrations above the HLQ was demonstrated by performing 6 replicate 10-fold dilutions of human plasma

samples spiked at 250000 ng/mL. Concentrations of SEQ ID NO.:1 in these matrix dilution samples were determined and corrected for the dilution factor. The bias and within-run precision values are less than 15% (actually -3.4% and 4.5%, respectively) indicating that a 10-fold dilution of human plasma samples containing SEQ ID NO.:1 above the HLQ is valid.

Conclusion

This method was successfully validated over the range of 50.0-50000 ng/mL for SEQ ID NO.:1 in human plasma and proved to be highly selective, precise, and accurate. The use of specific proteolytic digestion followed by solid-phase extraction and quantitative tandem mass spectrometry proved to be a very successful approach, offering a substantial increase in robustness over conventional immunochemical methods. Based on this method, clinical SEQ ID NO.:1 samples can be processed in approximately the same period of time as many small molecule quantitative tandem mass spectrometry methods.

Example 2

An additional assessment of method performance was determined by comparing the results obtained from human clinical study samples with results obtained using sandwich ELISA technique.

In the validated ELISA procedure, clinical samples were diluted 100-fold with sample buffer prior to analysis. The fusion protein was captured using a rabbit anti-human GLP-1 (7-36) amide and detected using a rabbit anti-HSA conjugated to biotin. Streptavidin HRP conjugate was used as the detection enzyme in conjunction with a chemiluminescent substrate. Sample concentrations were determined by interpolation from the standard curve, which was fitted using a weighted (1/x). four parameter logistic equation. The validated range of this assay, based on 10 uL of human plasma is 50 to 15000 ng/mL.

A method correlation plot for data generated using high performance liquid chromatography and mass spectroscopy methods of Example 1 and the ELISA method of this example was generated using sample results obtained from 612 clinical samples. The method correlation plot is presented in Figure 4. The linear regression analysis yielded a correlation coefficient (r) of 0.9692, with an intercept at -83.4 ng/mL ($p<0.001$), and a slope of 0.725 ($p<0.001$) between the methods. The statistical results from the linear

regression analysis indicate that the intercept and the slope are significantly different from 0 ($p<0.001$) and 1 ($p<0.001$); however, the negative slope bias observed is well within the range of the combined method accuracies. It should be noted that although differences in sample concentration results exist between the two methods, the overall pattern of the clinical PK data remains the same.

Example 3

Incurred sample reanalysis of clinical samples was performed in order to confirm both assay robustness and reproducibility. Clinical samples from both healthy and type 2 diabetic patients were reanalyzed on three separate occasions. Incurred sample reproducibility is confirmed when the difference between run CV for the three runs is ≤15%. Results from the incurred sample reanalysis test are presented in Table 7. Incurred sample reproducibility was confirmed in both healthy and type 2 diabetic patients.

Table 7: Incurred Sample Reproducibility

	Run 1	**Run 2**	**Run 3**
	ng/mL	**ng/mL**	**ng/mL**
Healthy	3912.5	3669.2	3701.8
	3946.5	3868.8	3953.6
	3973.9	3826.6	4087.6
Mean	3944.3	3788.2	3914.3
%CV	0.8	2.8	5.0
Mean (Between run)		3882.3	
%CV (Between run)		**2.1**	
	Run 1	**Run 2**	**Run 3**
	ng/mL	**ng/mL**	**ng/mL**
T2DM	304.9	301.6	302.6
	319.8	316.6	317.3
	332.1	307.6	327.3
Mean	318.9	308.6	315.7
%CV	4.3	2.4	3.9
Mean (Between run)		314.4	
%CV (Between run)		**1.7**	

Claims

1. A method for detecting at least one protein in plasma from a mammal comprising contacting said plasma with a first protease capable of digesting said at least one protein into at least one detectable fragment and detecting said at least one detectable fragment using high performance liquid chromatography and mass spectroscopy.
2. The method of claim 1, wherein said at least one protein is a therapeutic protein.
3. The method of claim 1, wherein said at least one protein is an endogenous protein to said mammal.
4. The method of claim 2, wherein said therapeutic protein is an incretin mimetic.
5. The method of claim 4, wherein said incretin mimetic is selected from the group of: incretin hormone and/or fragment, variant and/or conjugate thereof and GLP-1 agonist and/or fragment, variant and/or conjugate thereof.
6. The method of claim 4, wherein said incretin mimetic comprises at least one fragment and/or variant of human GLP-1 conjugated with at least one fusion partner.
7. The method of claim 6, wherein said at least one fragment and variant of GLP-1 comprises GLP-1(7-36(A8G)).
8. The method of claim 7, wherein the at least one fragment and variant of GLP-1 is genetically fused to human serum albumin.
9. The method of claim 8, wherein at least one fragment and variant of GLP-1 comprises at least two GLP-1(7-36(A8G)) tandemly and genetically fused to the human serum albumin.
10. The method of claim 9, wherein the at least two GLP-1(7-36(A8G)) are genetically fused at the N-terminus of the human serum albumin.
11. The method of claim 4, wherein said therapeutic protein comprises SEQ ID NO: 1.
12. The method of claim 6, wherein said at least one fusion partner comprises at least one polypeptide, variant and/or fragment thereof of a polypeptide selected from the group of: albumin, transferrin, and Fc portion of an IgG.

13. The method of claim 2 wherein said therapeutic protein is an isolated polypeptide fragments, variants and/or conjugates thereof, monoclonal antibody and/or fragment thereof or a domain antibody.

14. The method of claim 2, wherein said therapeutic protein is selected from the group of: exendin 3, exendin 4, human GLP-1 and fragments, variants, and/or or conjugates thereof.

15. The method of claim 3, wherein said at least one endogenous protein is selected from the group of: antibody, cytokine, tumor associated protein, naturally occurring receptor ligand, hormone, GLP-1, glucagon, insulin, and transmembrane protein.

16. The method of claim 1, wherein said first protease is selected from the group of: Lys-C, Trypsin, Asp-N, Arg-C, Asn-C, Post-Proline cleaving enzyme, and CNbr.

17. The method of claim 1, wherein said mammal is a human.

18. The method of claim 1, further comprising preparing a stable detectable standard peptide.

19. The method of claim 18, wherein said stable detectable standard peptide is prepared in a separate container from said at least one protein.

20. The method of claim 18, wherein said stable detectable standard peptide is contacted with said plasma.

21. The method of claim 20, wherein said stable detectable standard peptide is further digested with a second protease.

22. The method of claim 21, wherein said second protease is selected from the group of: Lys-C, Trypsin, Asp-N, Arg-C, Asn-C, Post-Proline cleaving enzyme, and CNbr.

23. The method of claim 22, wherein said first and second protease are the same.

24. The method of claim 18, wherein said stable detectable standard peptide is isotopically labeled.

25. The method of claim 1, further comprising generating a peptide library of said therapeutic protein.

26. The method of claim 25, further comprising selecting a fragment of said at least one protein that will be produced upon digesting with said protease.

27. The method of claim 26, wherein said fragment can be separated from all other fragments of said therapeutic protein produced by said protease based on one or more of the following characteristics: mass, charge, size, secondary structure, and tertiary structure.

28. The method of claim 1, wherein said method is a high throughput method.

29. The method of claim 1, wherein said method is quantitative.

30. The method of claim 1, further comprising calculating at least one pharmacokinetic parameter of said at least one protein in plasma.

31. The method of claim 1, further comprising calculating at least one pharmacodynamic parameter of said at least one protein in plasma.

32. The method of claim 1, wherein sample reproducibility is confirmed by ELISA.

Figure 1. Amino Acid Sequence of SEQ ID NO.:1

His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Asp-Ala-His-Lys-Ser-Glu-Val-Ala-His-Arg-Phe-Lys-Asp-Leu-Gly-Glu-Glu-Asn-Phe-Lys-Ala-Leu-Val-Leu-Ile-Ala-Phe-Ala-Gln-Tyr-Leu-Gln-Gln-Cys-Pro-Phe-Glu-Asp-His-Val-Lys-Leu-Val-Asn-Glu-Val-Thr-Glu-Phe-Ala-Lys-Thr-Cys-Val-Ala-Asp-Glu-Ser-Ala-Glu-Asn-Cys-Asp-Lys-Ser-Leu-His-Thr-Leu-Phe-Gly-Asp-Lys-Leu-Cys-Thr-Val-Ala-Thr-Leu-Arg-Glu-Thr-Tyr-Gly-Glu-Met-Ala-Asp-Cys-111-111-Cys-Ala-Lys-Gln-Glu-Pro-Glu-Arg-Asn-Glu-Cys-Phe-Leu-Gln-His-Lys-Asp-Asp-Asn-Pro-Asn-Leu-Pro-Arg-Leu-Val-Arg-Pro-Glu-Val-Asp-Val-Met-Cys-Thr-Ala-Phe-His-Asp-Asn-Glu-Glu-Thr-Phe-Leu-Lys-Lys-Tyr-Leu-Tyr-Glu-Ile-Ala-Arg-Arg-His-Pro-Tyr-Phe-Tyr-Ala-Pro-Glu-Leu-Leu-Phe-Phe-Ala-Lys-Arg-Tyr-Lys-Ala-Ala-Phe-Thr-Glu-Cys-Cys-Gln-Ala-Ala-Asp-Lys-Ala-Ala-Cys-Leu-Leu-Pro-Lys-Leu-Asp-Glu-Leu-Arg-Asp-Glu-Gly-Lys-Ala-Ser-Ser-Ala-Lys-Gln-Arg-Leu-Lys-Cys-Ala-Ser-Leu-Gln-Lys-Phe-Gly-Glu-Arg-Ala-Phe-Lys-Ala-Trp-Ala-Val-Ala-Arg-Leu-Ser-Gln-Arg-Phe-Pro-Lys-Ala-Glu-Phe-Ala-Glu-Val-Ser-Lys-Leu-Val-Thr-Asp-Leu-Thr-Lys-111-Val-His-Thr-Glu-Cys-Cys-His-Gly-Asp-Leu-Leu-Glu-Cys-Ala-Asp-Asp-Arg-Ala-Asp-Leu-Ala-Lys-Tyr-Ile-Cys-Glu-Asn-Gln-Asp-Ser-Ile-Ser-Ser-Lys-Leu-Lys-Glu-Cys-Cys-Glu-Lys-Pro-Leu-Leu-Glu-Lys-Ser-His-Cys-Ile-Ala-Glu-Val-Glu-Asn-Asp-Glu-Met-Pro-Ala-Asp-Leu-Pro-Ser-Leu-Ala-Ala-Asp-Phe-Val-Glu-Ser-Lys-Asp-Val-Cys-Lys-Asn-Tyr-Ala-Glu-Ala-Lys-Asp-Val-Phe-Leu-Gly-Met-Phe-Leu-Tyr-Glu-Tyr-Ala-Arg-Arg-His-Pro-Asp-Tyr-Ser-Val-Val-Leu-Leu-Leu-Arg-Leu-Ala-Lys-Thr-Tyr-Glu-Thr-Thr-Leu-Glu-Lys-Cys-Cys-Ala-Ala-Ala-Asp-Pro-His-Glu-Cys-Tyr-Ala-Lys-Val-Phe-Asp-Glu-Phe-Lys-Pro-Leu-Val-Glu-Glu-Pro-Gln-Asn-Leu-Ile-Lys-Gln-111-Asn-Cys-Glu-Leu-Phe-Glu-Gln-Leu-Gly-Glu-Tyr-Lys-Phe-Gln-Asn-Ala-Leu-Leu-Val-Arg-Tyr-Thr-Lys-Lys-Val-Pro-Gln-Val-Ser-Thr-Pro-Thr-Leu-Val-Glu-Val-Ser-Arg-Asn-Leu-Gly-Lys-Val-Gly-Ser-Lys-Cys-Cys-Lys-His-Pro-Glu-Ala-Lys-Arg-Met-Pro-Cys-Ala-Glu-Asp-Tyr-Leu-Ser-Val-Val-Leu-Asn-Gln-Leu-Cys-Val-Leu-His-Glu-222-Lys-Thr-Pro-Val-Ser-Asp-Arg-Val-Thr-Lys-Cys-Cys-Thr-Glu-Ser-Leu-Val-Asn-Arg-Arg-Pro-Cys-Phe-Ser-Ala-Leu-Glu-Val-Asp-Glu-Thr-Tyr-Val-Pro-Lys-Glu-Phe-Asn-Ala-Glu-Thr-Phe-Thr-Phe-His-Ala-Asp-Ile-Cys-Thr-Leu-Ser-Glu-Lys-Glu-Arg-Gln-Ile-Lys-Lys-Gln-Thr-Ala-Leu-Val-Glu-Leu-Val-Lys-His-Lys-Pro-Lys-Ala-Thr-111-Lys-Glu-Gln-Leu-Lys-Ala-Val-Met-Asp-Asp-Phe-Ala-Ala-Phe-Val-Glu-Lys-Cys-Cys-Lys-Ala-Asp-Asp-Lys-Glu-Thr-Cys-Phe-Ala-Glu-Glu-Gly-Lys-Lys-Leu-Val-Ala-Ala-Ser-Gln-Ala-Ala-Leu-Gly-Leu- (SEQ ID No.:1)

Figure 2: Amino Acid Sequence of [^{15}N $^{13}C_6$]-GLP1M Internal Standard and GLP-1 Specific Peptide Fragment

[^{15}N $^{13}C_6$]-GLP1M Internal Standard

H-His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-**Glu-Phe-Ile-Ala-Trp-[$^{15}N^{13}C_6$]Leu-Val-Lys**-Gly-Arg-OH (SEQ ID NO.:3)

[^{15}N $^{13}C_6$]-GLP1M Internal Standard Peptide Fragment

Glu-Phe-Ile-Ala-Trp-[$^{15}N^{13}C_6$]Leu-Val-Lys (SEQ ID NO.:2)

Figure 3: Structure of GLP-1 Specific Peptide Fragment

Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys (SEQ ID NO. 2)

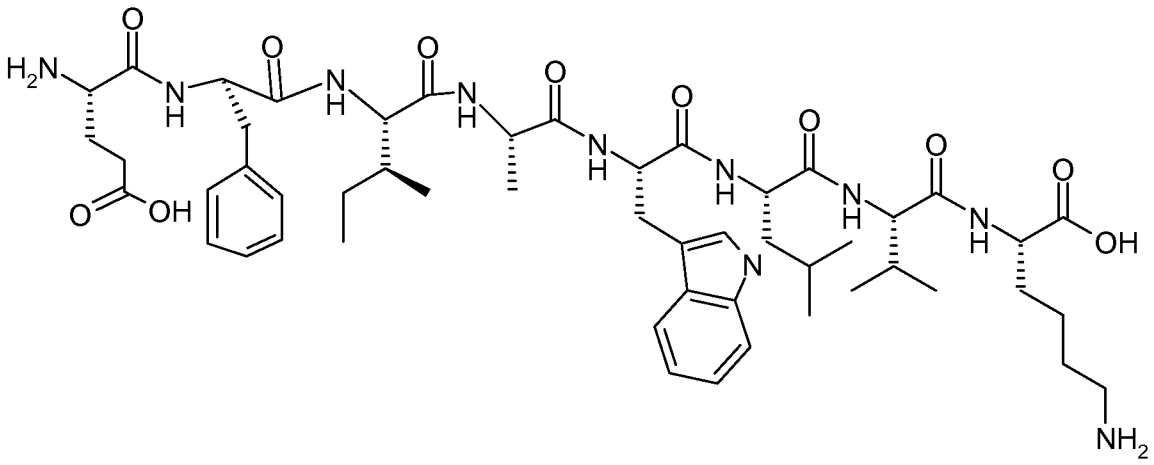
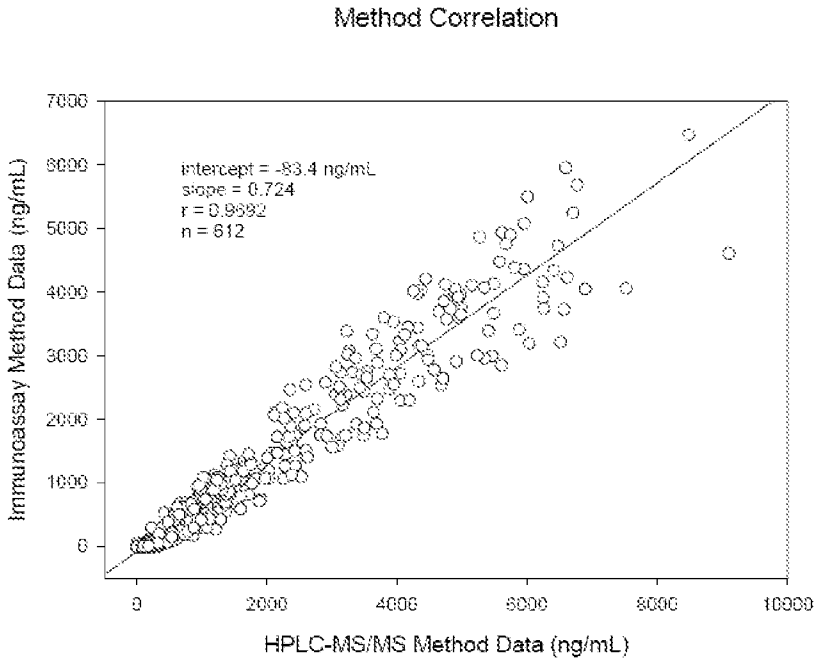


Figure 4: Method correlation plot for data generated using high performance liquid chromatography and mass spectroscopy methods and the ELISA methods.



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 08/66661

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/00 (2008.04)
USPC - 435/4
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)
USPC: 435/4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 435/7.1, 23, 24, 212; 436/2, 161, 173, 815; 530/350, 380, 399, 407, 412 (text search-see terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST(USPT,PGPB, USOC, EPAB,JPAB); Google Patents; Google Scholar- citerone david, hottenstein charles, kehler jonathan, method, detect$, determin$, identif$, protein, plasma, protease, proteinase, high performance liquid chromatography, mass spectroscopy, HPLC, MS, GLP-1, GLP, standard peptide, protease$, proteinase$

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2006/0141528 A1 (AEBERSOLD et al.) 29 June 2006 (29.06.2006), abstract, [0044], [0049], [0071], [0072], [0199], [0197], [0061], [0038], [0115], [0041], [0139], [0040], [0042], [0026], [0146], [0013], [0075], [0046]	1-3, 13, 15-32 --- 4-12, 14
Y	WO 2003/059934 A2 (ROSEN et al.) 24 July 2003 (24.07.2003), para [0042]; table 2 fusion no. 62, fusion no. 90, fusion no. 139; SEQ ID NO:611	4-12, 14

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"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
"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
"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
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 September 2008 (24.09.2008)	02 OCT 2008
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

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