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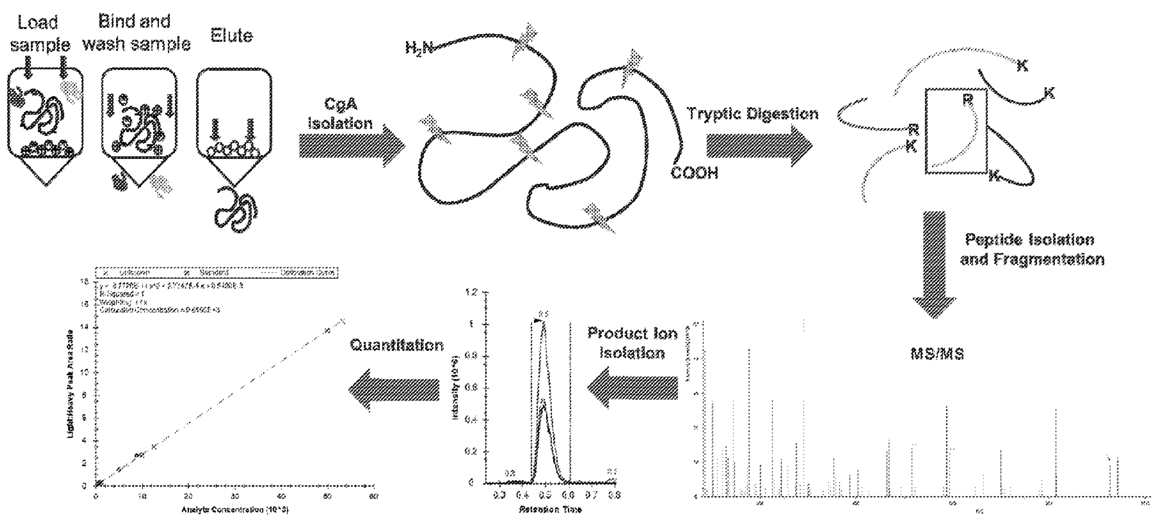
(54) Titre : PROCEDES DE DETECTION DE CHROMOGRANINE A PAR SPECTROMETRIE DE MASSE
(54) Title: METHODS FOR DETECTING CHROMOGRANIN A BY MASS SPECTROMETRY

FIGURE 1



(57) Abrégé/Abstract:
Provided are methods for detecting chromogranin A by mass spectrometry. In another aspect, provided herein are methods for quantitating chromogranin A by mass spectrometry. In another aspect, provided herein are methods for prognosis of or measuring the size of neuroendocrine tumors by mass spectrometry.

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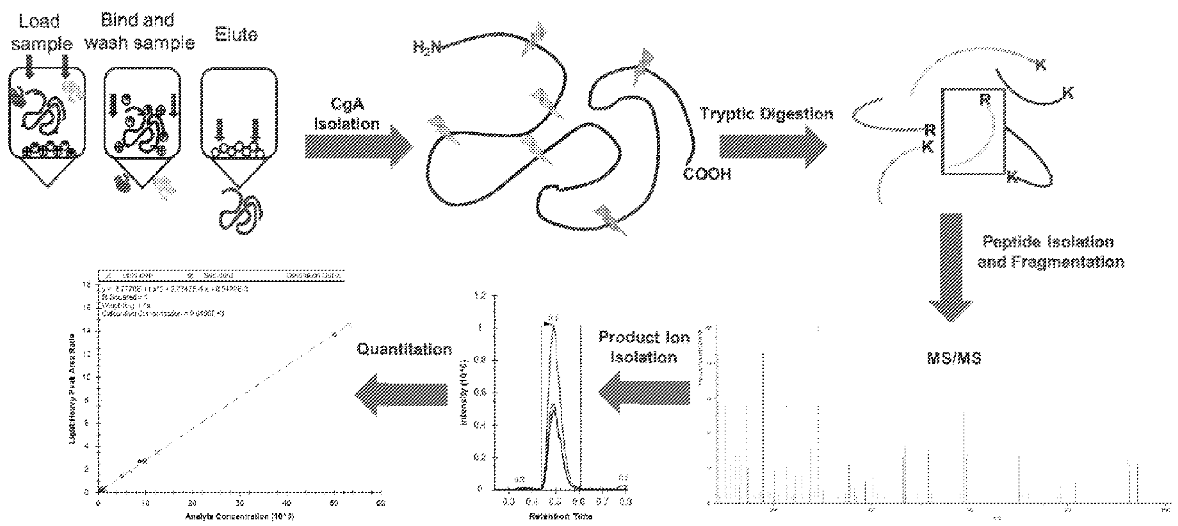
(54) **Title:** METHODS FOR DETECTING CHROMOGRANIN A BY MASS SPECTROMETRY

FIGURE 1



(57) **Abstract:** Provided are methods for detecting chromogranin A by mass spectrometry. In another aspect, provided herein are methods for quantitating chromogranin A by mass spectrometry. In another aspect, provided herein are methods for prognosis of or measuring the size of neuroendocrine tumors by mass spectrometry.

[Continued on next page]

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METHODS FOR DETECTING CHROMOGRANIN A BY MASS SPECTROMETRY

CROSS-REFERENCE TO RELATED PATENT APPLICATIONS

[0001] This application claims benefit of U.S. Provisional Application No. 62/644,210, filed March 16, 2018, which is incorporated by reference herein in its entirety.

BACKGROUND OF THE INVENTION

[0002] Chromogranin-A (CgA) is a 50kDa acidic glycoprotein expressed in the secretory granules of neuroendocrine tissue. Currently, blood levels of CgA are primarily measured using various immunoassays. However, as with any antibody-based assay, limitations arising from non-specific binding and a reduced dynamic range requiring sample dilution, pose hurdles in implementing such tests in diagnostic laboratories.

[0003] An accurate and sensitive assay for quantitating chromogranin A is needed.

SUMMARY OF THE INVENTION

[0004] Provided herein are methods for detecting or determining the amount of chromogranin A (CgA) in a sample by mass spectrometry, including tandem mass spectrometry.

[0005] In certain embodiments, the methods provided herein are for detecting or determining the amount of chromogranin A (CgA) comprising (a) purifying CgA in the sample; (b) ionizing CgA to produce ions detectable by mass spectrometry; and (c) detecting or determining the amount of the CgA ion(s) by mass spectrometry; wherein the amount of the CgA ion(s) is related to the amount of CgA in the sample.

[0006] In certain embodiments, the methods provided herein are for detecting or determining the amount of chromogranin A (CgA) comprising (a) subjecting the sample to solid phase extraction; (b) enzymatically digesting the CgA; (c) subjecting the CgA to liquid chromatography; (d) ionizing CgA to produce ions detectable by mass spectrometry; and (e) detecting or determining the amount of the CgA ion(s) by mass spectrometry; wherein the amount of the CgA ion(s) is related to the amount of CgA in the sample.

[0007] In certain embodiments, methods provided herein comprise selected reaction monitoring (SRM) mass spectrometry.

[0008] In some embodiments, the methods provided herein are fully automated.

[0009] In some embodiments, the methods provided herein are antibody-free methods.

[0010] In some embodiments, purifying provided herein comprises extraction of serum using solid phase extraction (SPE). In some embodiments, SPE is an anion exchange solid-phase extraction. In some embodiments, SPE is a mixed-mode anion exchange solid-phase extraction. In some embodiments, extracted samples are concentrated.

[0011] In some embodiments, purifying provided herein comprises liquid chromatography. In some embodiments, the liquid chromatography comprises high performance liquid chromatography (HPLC). In some embodiments, the liquid chromatography comprises high turbulence liquid chromatography (HTLC).

[0012] In some embodiments, extracted samples are enzymatically digested. In some embodiments, extracted samples are enzymatically digested by trypsin.

[0013] In some embodiments, the ionization comprises electrospray ionization (ESI). In some embodiments, the ionization comprises ionizing in positive mode. In some embodiments, the ionization comprises ionizing in negative mode.

[0014] In some embodiments, the ionization comprises atmospheric pressure chemical ionization (APCI). In some embodiments, the ionization comprises ionizing in positive mode. In some embodiments, the ionization comprises ionizing in negative mode.

[0015] In some embodiments, methods provided herein comprise measuring the amount of precursor ion having a mass-to-charge ratio of 593.2 ± 0.5.

[0016] In some embodiments, methods provided herein comprise measuring the amount of precursor ion having a mass-to-charge ratio of 729.6 ± 0.5.

[0017] In some embodiments, methods provided herein comprise measuring the amount of a fragment of chromogranin A. In some embodiments, the CgA fragment measured comprises a sequence ELQDLALQGA (SEQ ID NO:1). In some embodiments, the CgA fragment measured comprises a sequence RRPEDQELESLSAIEAELEK (SEQ ID NO:4).

[0018] In some embodiments, methods provided herein comprise measuring the amount of fragment ion having a mass-to-charge ratio of 516.3 ± 0.5 or 815.5 ± 0.5 or both.

[0019] In some embodiments, methods provided herein comprise measuring the amount of fragment ion having a mass-to-charge ratio of 831.5 ± 0.5 or 989.5 ± 0.5 or both.

[0020] In some embodiments, methods provided herein further comprise adding an internal standard. In some embodiments, the internal standard is isotopically labeled. In some embodiments, the internal standard comprises $C^{13}N^{15}$ labeled amino acids. In some embodiments, the internal standard is labeled on a leucine (L) or lysine (K). In some embodiments, the internal standard comprises a sequence

ILSILRHQNLLKELQDLAL*QGAK*ERAHQQK (SEQ ID NO:2), wherein * is a $C^{13}N^{15}$ labeled amino acid. In some embodiments, the internal standard comprises a sequence RRPEDQELESL*SAIEAELEK* (SEQ ID NO:5), wherein * is a $C^{13}N^{15}$ labeled amino acid.

[0021] In some embodiments, methods provided herein comprise measuring the amount of internal standard precursor ion having a mass-to-charge ratio of 600.8 ± 0.5 and/or product ion having a mass-to-charge ratio of 602.4 ± 0.5, 830.6± 0.5, or 958.7 ± 0.5.

[0022] In some embodiments, methods provided herein comprise measuring the amount of internal standard precursor ion having a mass-to-charge ratio of 600.8 ± 0.5 and/or product ion having a mass-to-charge ratio of 734.6 ± 0.5, 839.5 ± 0.5, or 997.6 ± 0.5.

[0023] In certain embodiments, the limit of quantitation of the methods is less than or equal to 100 ng/mL. In some embodiments, the limit of quantitation of the methods is less than or equal to 90 ng/mL. In some embodiments, the limit of quantitation of the methods is less than or equal to 80 ng/mL. In some embodiments, the limit of quantitation of the methods is less than or equal to 70 ng/mL. In some embodiments, the limit of quantitation of the methods is less than or equal to 60 ng/mL. In some embodiments, the limit of quantitation of the methods is less than or equal to 50 ng/mL.

[0024] In some embodiments, the limit of detection of the methods is less than or equal to 50 ng/mL. In some embodiments, the limit of detection of the methods is less than or equal to 40 ng/mL. In some embodiments, the limit of detection of the methods is less than or equal to 35.5 ng/mL.

[0025] In some embodiments, methods provided herein comprise linearity of quantitation across a range between 50 ng/mL to 50,000 ng/mL.

[0026] In some embodiments, methods provided herein comprise inter- and intra-assay reproducibility of CV ≤15%.

[0027] In some embodiments, CgA is not derivatized prior to mass spectrometry.

[0028] In certain embodiments, the sample is a body fluid. In some embodiments, the sample is cerebrospinal fluid (CSF). In some embodiments, the sample is plasma or serum. In some embodiments, the sample is whole blood. In some embodiments, the sample is saliva or urine.

[0029] In some embodiments, the methods may include adding an agent to the sample in an amount sufficient to deproteinate the sample.

[0030] In some embodiments, elevated levels of chromogranin A as compared to a reference range indicates increased risk of neuroendocrine tumors (NET). In some embodiments, quantitated levels of chromogranin A indicates the size of the neuroendocrine tumor. In some embodiments, quantitated levels of chromogranin A indicates the tumor burden of the neuroendocrine tumor. In some embodiments, quantitated levels of chromogranin A indicates the response to treatment of the neuroendocrine tumor. In some embodiments, quantitated levels of chromogranin A indicates the prognosis of the neuroendocrine tumor.

[0031] As used herein, unless otherwise stated, the singular forms "a," "an," and "the" include plural reference. Thus, for example, a reference to "a protein" includes a plurality of protein molecules.

[0032] As used herein, the term "purification" or "purifying" does not refer to removing all materials from the sample other than the analyte(s) of interest. Instead, purification refers to a procedure that enriches the amount of one or more analytes of interest relative to other components in the sample that may interfere with detection of the analyte of interest. Samples are purified herein by various means to allow removal of one or more interfering substances, *e.g.*, one or more substances that would interfere with the detection of selected CgA parent and daughter ions by mass spectrometry.

[0033] As used herein, the term "test sample" refers to any sample that may contain CgA. As used herein, the term "body fluid" means any fluid that can be isolated from the body of an individual. For example, "body fluid" may include blood, plasma, serum, bile, saliva, urine, tears, perspiration, and the like.

[0034] As used herein, the term "derivatizing" means reacting two molecules to form a new molecule. Derivatizing agents may include isothiocyanate groups, dinitro-fluorophenyl groups, nitrophenoxycarbonyl groups, and/or phthalaldehyde groups, and the like.

[0035] As used herein, the term "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 of the chemical entities as they flow around or over a stationary liquid or solid phase.

[0036] As used herein, the term "liquid chromatography" or "LC" means a process of selective retardation of one or more components of a fluid solution as the fluid uniformly percolates through a column of a finely divided substance, or through capillary passageways. The retardation results from the distribution of the components of the mixture between one or more stationary phases and the bulk fluid, (*i.e.*, mobile phase), as this fluid moves relative to the stationary phase(s). Examples of "liquid chromatography" include reverse phase liquid chromatography (RPLC), high performance liquid chromatography (HPLC), and high turbulence liquid chromatography (HTLC).

[0037] As used herein, the term "high performance liquid chromatography" or "HPLC" refers to liquid chromatography in which the degree of separation is increased by forcing the mobile phase under pressure through a stationary phase, typically a densely packed column.

[0038] As used herein, the term "high turbulence liquid chromatography" or "HTLC" refers to a form of chromatography that utilizes turbulent flow of the material being assayed through the column packing as the basis for performing the separation. HTLC has been applied in the preparation of samples containing two unnamed drugs prior to analysis by mass spectrometry. See, *e.g.*, Zimmer *et al.*, *J. Chromatogr.* A 854: 23-35 (1999); see also, U.S. Patents No. 5,968,367, 5,919,368, 5,795,469, and 5,772,874, which further explain HTLC. Persons of ordinary skill in the art understand "turbulent flow". When fluid flows slowly and smoothly, the flow is called "laminar flow". For example, fluid moving through an HPLC column at low flow rates is laminar. In laminar flow the motion of the particles of fluid is orderly with particles moving generally in straight lines. At faster velocities, the inertia of the water overcomes fluid frictional forces and turbulent flow results. Fluid not in contact with the irregular boundary "outruns" that which is slowed by friction or deflected by an uneven surface. When a fluid is flowing turbulently, it flows in eddies and whirls (or vortices), with more "drag" than when the flow is laminar. Many references are available for assisting in determining when fluid flow is laminar or turbulent (e.g., Turbulent Flow Analysis: Measurement and Prediction, P.S. Bernard & J.M. Wallace, John Wiley & Sons, Inc., (2000); An Introduction to Turbulent Flow, Jean Mathieu & Julian Scott, Cambridge University Press (2001)).

[0039] As used herein, the term "gas chromatography" or "GC" refers to chromatography in which the sample mixture is vaporized and injected into a stream of carrier gas (as nitrogen or helium) moving through a column containing a stationary phase composed of a liquid or a particulate solid and is separated into its component compounds according to the affinity of the compounds for the stationary phase.

[0040] As used herein, the term "large particle column" or "extraction column" refers to a chromatography column containing an average particle diameter greater than about 35 μm. As used in this context, the term "about" means ± 10%. In a preferred embodiment the column contains particles of about 60 μm in diameter.

[0041] As used herein, the term "analytical column" refers to a chromatography column having sufficient chromatographic plates to effect a separation of materials in a sample that elute from the column sufficient to allow a determination of the presence or amount of an analyte. Such columns are often distinguished from "extraction columns", which have the general purpose of separating or extracting retained material from non-retained materials in order to obtain a purified sample for further analysis. As used in this context, the term "about" means ± 10%. In a preferred embodiment the analytical column contains particles of about 4 μm in diameter.

[0042] As used herein, the term "on-line" or "inline", for example as used in "on-line automated fashion" or "on-line extraction" refers to a procedure performed without the need for operator intervention. In contrast, the term "off-line" as used herein refers to a procedure requiring manual intervention of an operator. Thus, if samples are subjected to precipitation, and the supernatants are then manually loaded into an autosampler, the precipitation and loading steps are off-line from the subsequent steps. In various embodiments of the methods, one or more steps may be performed in an on-line automated fashion.

[0043] As used herein, the term "mass spectrometry" or "MS" refers to an analytical technique to identify compounds by their mass. MS refers to methods of filtering, detecting, and measuring ions based on their mass-to-charge ratio, or "m/z". MS technology generally includes (1) ionizing the compounds to form charged compounds; and (2) detecting the molecular weight of the charged compounds and calculating a mass-to-charge ratio. The compounds may be ionized and detected by any suitable means. A "mass spectrometer" generally includes an ionizer and an ion detector. In general, one or more molecules of interest are ionized, and the ions are subsequently introduced into a mass spectrographic instrument where, due to a combination of magnetic and electric fields, the ions follow a path in space that is dependent upon mass ("m") and charge ("z"). *See, e.g.*, U.S. Patent Nos. 6,204,500, entitled "Mass Spectrometry From Surfaces;" 6,107,623, entitled "Methods and Apparatus for Tandem Mass Spectrometry;" 6,268,144, entitled "DNA Diagnostics Based On Mass Spectrometry;" 6,124,137, entitled "Surface-Enhanced Photolabile Attachment And Release For Desorption And Detection Of Analytes;" Wright *et al.*, *Prostate Cancer and Prostatic Diseases* **2**:264-76 (1999); and Merchant and Weinberger, *Electrophoresis* **21**:1164-67 (2000).

[0044] As used herein, the term "operating in negative ion mode" refers to those mass spectrometry methods where negative ions are generated and detected. The term "operating in positive ion mode" as used herein, refers to those mass spectrometry methods where positive ions are generated and detected.

[0045] As used herein, the term "ionization" or "ionizing" refers to the process of generating an analyte ion having a net electrical charge equal to one or more electron units. Negative ions are those having a net negative charge of one or more electron units, while positive ions are those having a net positive charge of one or more electron units.

[0046] As used herein, the term "electron ionization" or "EI" refers to methods in which an analyte of interest in a gaseous or vapor phase interacts with a flow of electrons. Impact of the electrons with the analyte produces analyte ions, which may then be subjected to a mass spectrometry technique.

[0047] As used herein, the term "chemical ionization" or "CI" refers to methods in which a reagent gas (*e.g.* ammonia) is subjected to electron impact, and analyte ions are formed by the interaction of reagent gas ions and analyte molecules.

[0048] As used herein, the term "fast atom bombardment" or "FAB" refers to methods in which a beam of high energy atoms (often Xe or Ar) impacts a non-volatile sample, desorbing and ionizing molecules contained in the sample. Test samples are dissolved in a viscous liquid matrix such as glycerol, thioglycerol, m-nitrobenzyl alcohol, 18-crown-6 crown ether, 2-nitrophenyloctyl ether, sulfolane, diethanolamine, and triethanolamine. The choice of an appropriate matrix for a compound or sample is an empirical process.

[0049] As used herein, the term "matrix-assisted laser desorption ionization" or "MALDI" refers to methods in which a non-volatile sample is exposed to laser irradiation, which desorbs and ionizes analytes in the sample by various ionization pathways, including photo-ionization, protonation, deprotonation, and cluster decay. For MALDI, the sample is mixed with an energy-absorbing matrix, which facilitates desorption of analyte molecules.

[0050] As used herein, the term "surface enhanced laser desorption ionization" or "SELDI" refers to another method in which a non-volatile sample is exposed to laser irradiation, which desorbs and ionizes analytes in the sample by various ionization pathways, including photo-ionization, protonation, deprotonation, and cluster decay. For SELDI, the sample is typically bound to a surface that preferentially retains one or more analytes of interest. As in MALDI, this process may also employ an energy-absorbing material to facilitate ionization.

[0051] As used herein, the term "electrospray ionization" or "ESI," refers to methods in which a solution is passed along a short length of capillary tube, to the end of which is applied a high positive or negative electric potential. Solution reaching the end of the tube is vaporized (nebulized) into a jet or spray of very small droplets of solution in solvent vapor. This mist of droplets flows through an evaporation chamber, which is heated slightly to prevent condensation and to evaporate solvent. As the droplets get smaller the electrical surface charge density increases until such time that the natural repulsion between like charges causes ions as well as neutral molecules to be released.

[0052] As used herein, the term "atmospheric pressure chemical ionization" or "APCI," refers to mass spectroscopy methods that are similar to ESI; however, APCI produces ions by ion-molecule reactions that occur within a plasma at atmospheric pressure. The plasma is maintained by an electric discharge between the spray capillary and a counter electrode. Then ions are typically extracted into the mass analyzer by use of a set of differentially pumped skimmer stages. A counterflow of dry and preheated N_2 gas may be used to improve removal of solvent. The gas-phase ionization in APCI can be more effective than ESI for analyzing less-polar species.

[0053] The term "Atmospheric Pressure Photoionization" or "APPI" as used herein refers to the form of mass spectroscopy where the mechanism for the photoionization of molecule M is photon absorption and electron ejection to form the molecular ion M+. Because the photon energy typically is just above the ionization potential, the molecular ion is less susceptible to dissociation. In many cases it may be possible to analyze samples without the need for chromatography, thus saving significant time and expense. In the presence of water vapor or protic solvents, the molecular ion can extract H to form MH+. This tends to occur if M has a high proton affinity. This does not affect quantitation accuracy because the sum of M+ and MH+ is constant. Drug compounds in protic solvents are usually observed as MH+, whereas nonpolar compounds such as naphthalene or testosterone usually form M+. Robb, D.B., Covey, T.R. and Bruins, A.P. (2000): *See, e.g.*, Robb *et al.*, Atmospheric pressure photoionization: An ionization method for liquid chromatography-mass spectrometry. *Anal. Chem.* 72(15): 3653-3659.

[0054] As used herein, the term "inductively coupled plasma" or "ICP" refers to methods in which a sample interacts with a partially ionized gas at a sufficiently high temperature such that most elements are atomized and ionized.

[0055] As used herein, the term "field desorption" refers to methods in which a non-volatile test sample is placed on an ionization surface, and an intense electric field is used to generate analyte ions.

[0056] As used herein, the term “desorption” refers to the removal of an analyte from a surface and/or the entry of an analyte into a gaseous phase.

[0057] As used herein, the term “limit of quantification”, “limit of quantitation” or “LOQ” refers to the point where measurements become quantitatively meaningful. The analyte response at this LOQ is identifiable, discrete and reproducible with a precision of 20% and an accuracy of 80% to 120%.

[0058] As used herein, the term “limit of detection” or “LOD” is the point at which the measured value is larger than the uncertainty associated with it. The LOD is defined arbitrarily as 2 standard deviations (SD) from the zero concentration.

[0059] As used herein, an “amount” of CgA in a body fluid sample refers generally to an absolute value reflecting the mass of CgAdetectable in volume of body fluid. However, an amount also contemplates a relative amount in comparison to another CgA amount. For example, an amount of CgAin a body fluid can be an amount which is greater than or less than a control or normal level of CgA normally present.

[0060] The term “about” as used herein in reference to quantitative measurements not including the measurement of the mass of an ion, refers to the indicated value plus or minus 10%. Mass spectrometry instruments can vary slightly in determining the mass of a given analyte. The term “about” in the context of the mass of an ion or the mass/charge ratio of an ion refers to +/- 0.5 atomic mass unit.

[0061] The summary of the invention described above is non-limiting and other features and advantages of the invention will be apparent from the following detailed description of the invention, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0062] Figure 1 shows the analytical workflow for the CgA LC-MS/MS assay**.** Negatively charged CgA binds through electrostatic, lipophilic, and hydrophilic interactions with the mixed-mode anion exchange resin under conditions where it is retained while other proteins are washed away. After elution, the isolated CgA is digested with trypsin and a representative peptide is quantified by LC-MS/MS analysis.

[0063] Figure 2 shows three calibration curves run over three separate days. Linear range was demonstrated to be 50 to 50,000 ng/mL. CV was less than 10%.

[0064] Figure 3 shows chromatograms corresponding to patient specimens with normal (top) and high (bottom) CgA values.

[0065] Figure 4 shows average ELISA immunoassay values as compared to the values obtained by LC-MS/MS.

[0066] Figure 5 shows a comparison between CisBio immunoassay and LC-MS/MS CgA assay (Passing & Bablok curve fit, 308 samples).

[0067] Figure 6 shows peak area graphs for normal and abnormal CgA levels determined by LC-MS/MS.

[0068] Figure 7 shows an example chromatogram for chromogranin internal standard.

DETAILED DESCRIPTION OF THE INVENTION

[0069] Levels of CgA are increased in the presence of neuroendocrine-derived tumors (NET), making CgA useful serum marker for monitoring patients with NETs. Circulating levels of serum CgA are proportional to tumor burden, providing prognostic information in treatment response. Here we describe a novel, fully automated, antibody-free LC-MS/MS assay to quantitate chromogranin-A out of serum. Exploiting the acidic properties of CgA, 100 uL of serum is extracted using an anion exchange solid-phase extraction plate followed by addition of internal standard. The extracted sample is then concentrated and enzymatically digested using trypsin. A unique peptide to CgA is then chromatographically resolved and analyzed by SRM on a Sciex 6500+ QTrap. The ratio of the analyte peak area to the isotopically labeled internal standard peak area is used to achieve quantitation. CgA shows linearity across a wide range (50-50000 ng/mL, $R^2 \geq 0.99$), as well as inter- and intra-assay reproducibility (CV $\leq 15\%$). A cohort of 300 patient samples was analyzed to compare CgA serum values measured by the Cisbio CGA-ELISA-US immunoassay to the described LC-MS/MS assay. When comparing immunoassay and LC-MS/MS measurements for CgA in this cohort, an R^2 of 0.71 was observed, showing a good correlation between the two assay platforms.

[0070] In certain embodiments, the methods provided herein are for detecting or determining the amount of chromogranin A (CgA) comprising (a) purifying CgA in the sample; (b) ionizing CgA to produce ions detectable by mass spectrometry; and (c) detecting or determining the amount of the CgA ion(s) by mass spectrometry; wherein the amount of the CgA ion(s) is related to the amount of CgA in the sample.

[0071] In certain embodiments, the methods provided herein are for detecting or determining the amount of chromogranin A (CgA) comprising (a) subjecting the sample to solid phase extraction; (b) enzymatically digesting the CgA; (c) subjecting the CgA to liquid chromatography; (d) ionizing CgA to produce ions detectable by mass spectrometry; and (e) detecting or determining the amount of the CgA ion(s) by mass spectrometry; wherein the amount of the CgA ion(s) is related to the amount of CgA in the sample.

[0072] In certain embodiments, methods provided herein comprise selected reaction monitoring (SRM) mass spectrometry.

[0073] In some embodiments, the methods provided herein are fully automated.

[0074] In some embodiments, the methods provided herein are antibody-free methods.

[0075] In some embodiments, purifying provided herein comprises extraction of serum using solid phase extraction (SPE). In some embodiments, SPE is an anion exchange solid-phase extraction. In some embodiments, SPE is a mixed-mode anion exchange solid-phase extraction. In some embodiments, extracted samples are concentrated.

[0076] In some embodiments, purifying provided herein comprises liquid chromatography. In some embodiments, the liquid chromatography comprises high performance liquid chromatography (HPLC). In some embodiments, the liquid chromatography comprises high turbulence liquid chromatography (HTLC).

[0077] In some embodiments, extracted samples are enzymatically digested. In some embodiments, extracted samples are enzymatically digested by trypsin.

[0078] In some embodiments, the ionization comprises electrospray ionization (ESI). In some embodiments, the ionization comprises ionizing in positive mode. In some embodiments, the ionization comprises ionizing in negative mode.

[0079] In some embodiments, the ionization comprises atmospheric pressure chemical ionization (APCI). In some embodiments, the ionization comprises ionizing in positive mode. In some embodiments, the ionization comprises ionizing in negative mode.

[0080] In some embodiments, methods provided herein comprise measuring the amount of precursor ion having a mass-to-charge ratio of 593.2 ± 0.5.

[0081] In some embodiments, methods provided herein comprise measuring the amount of precursor ion having a mass-to-charge ratio of 729.6 ± 0.5.

[0082] In some embodiments, methods provided herein comprise measuring the amount of a fragment of chromogranin A. In some embodiments, the CgA fragment measured comprises a sequence ELQDLALQGA (SEQ ID NO:1). In some embodiments, the CgA fragment measured comprises a sequence RRPEDQELESLSAIEAELEK (SEQ ID NO:4).

[0083] In some embodiments, methods provided herein comprise measuring the amount of fragment ion having a mass-to-charge ratio of 516.3 ± 0.5 or 815.5 ± 0.5 or both.

[0084] In some embodiments, methods provided herein comprise measuring the amount of fragment ion having a mass-to-charge ratio of 831.5 ± 0.5 or 989.5 ± 0.5 or both.

[0085] In some embodiments, methods provided herein further comprise adding an internal standard. In some embodiments, the internal standard is isotopically labeled. In some embodiments, the internal standard comprises $C^{13}N^{15}$ labeled amino acids. In some embodiments, the internal standard is labeled on a leucine (L) or lysine (K). In some embodiments, the internal standard comprises a sequence ILSILRHQNLLKELQDLAL*QGAK*ERAHQQK (SEQ ID NO:2), wherein * is a $C^{13}N^{15}$ labeled amino acid. In some embodiments, the internal standard comprises a sequence RRPEDQELESL*SAIEAELEK* (SEQ ID NO:5), wherein * is a $C^{13}N^{15}$ labeled amino acid.

[0086] In some embodiments, methods provided herein comprise measuring the amount of internal standard precursor ion having a mass-to-charge ratio of 600.8 ± 0.5 and/or product ion having a mass-to-charge ratio of 602.4 ± 0.5, 830.6± 0.5, or 958.7 ± 0.5.

[0087] In some embodiments, methods provided herein comprise measuring the amount of internal standard precursor ion having a mass-to-charge ratio of 600.8 ± 0.5 and/or product ion having a mass-to-charge ratio of 734.6 ± 0.5, 839.5 ± 0.5, or 997.6 ± 0.5.

[0088] In certain embodiments, the limit of quantitation of the methods is less than or equal to 100 ng/mL. In some embodiments, the limit of quantitation of the methods is less than or equal to 90 ng/mL. In some embodiments, the limit of quantitation of the methods is less than or equal to 80 ng/mL. In some embodiments, the limit of quantitation of the methods is less than or equal to 70 ng/mL. In some embodiments, the limit of quantitation of the methods is less than or equal to 60 ng/mL. In some embodiments, the limit of quantitation of the methods is less than or equal to 50 ng/mL.

[0089] In some embodiments, the limit of detection of the methods is less than or equal to 50 ng/mL. In some embodiments, the limit of detection of the methods is less than or equal to 40 ng/mL. In some embodiments, the limit of detection of the methods is less than or equal to 35.5 ng/mL.

[0090] In some embodiments, methods provided herein comprise linearity of quantitation across a range between 50 ng/mL to 50,000 ng/mL.

[0091] In some embodiments, methods provided herein comprise inter- and intra-assay reproducibility of CV ≤15%.

[0092] In some embodiments, CgA is not derivatized prior to mass spectrometry.

[0093] In certain embodiments, the sample is a body fluid. In some embodiments, the sample is cerebrospinal fluid (CSF). In some embodiments, the sample is plasma or serum. In some embodiments, the sample is whole blood. In some embodiments, the sample is saliva or urine.

[0094] In some embodiments, the methods may include adding an agent to the sample in an amount sufficient to deproteinate the sample.

[0095] In some embodiments, elevated levels of chromogranin A as compared to a reference range indicates increased risk of neuroendocrine tumors (NET). In some embodiments, quantitated levels of chromogranin A indicates the size of the neuroendocrine tumor. In some embodiments, quantitated levels of chromogranin A indicates the tumor burden of the neuroendocrine tumor. In some embodiments, quantitated levels of chromogranin A indicates the response to treatment of the neuroendocrine tumor. In some embodiments, quantitated levels of chromogranin A indicates the prognosis of the neuroendocrine tumor.

[0096] Suitable test samples include any test sample that may contain the analyte of interest. In some preferred embodiments, a sample is a biological sample; that is, a sample obtained from any biological source, such as an animal, a cell culture, an organ culture, etc. In certain preferred embodiments samples are obtained from a mammalian animal, such as a dog, cat, horse, etc. Particularly preferred mammalian animals are primates, most preferably male or female humans. Particularly preferred samples include blood, plasma, serum, hair, muscle, urine, saliva, tear, cerebrospinal fluid, or other tissue sample. Such samples may be obtained, for example, from a patient; that is, a living person, male or female, presenting oneself in a clinical setting for diagnosis, prognosis, or treatment of a disease or condition. The test sample is preferably obtained from a patient, for example, blood serum.

<u>Sample Preparation for Mass Spectrometry</u>

[0097] Methods that may be used to enrich in CgA relative to other components in the sample (e.g. protein) include for example, filtration, centrifugation, thin layer chromatography (TLC), electrophoresis including capillary electrophoresis, affinity separations including immunoaffinity separations, extraction methods including ethyl acetate extraction and methanol extraction, and the use of chaotropic agents or any combination of the above or the like.

[0098] Protein precipitation is one preferred method of preparing a test sample. Such protein purification methods are well known in the art, for example, Polson *et al.*, *Journal of Chromatography B* **785**:263-275 (2003), describes protein precipitation techniques suitable for use in the methods. Protein precipitation may be used to remove most of the protein from the sample leaving CgA in the supernatant. The samples may be centrifuged to separate the liquid supernatant from the precipitated proteins. The resultant supernatant may then be applied to liquid chromatography and subsequent mass spectrometry analysis. In certain embodiments, the use of protein precipitation such as for example, acetonitrile protein precipitation, obviates the need for high turbulence liquid chromatography (HTLC) or other on-line extraction prior to HPLC and mass spectrometry. Accordingly in such embodiments, the method involves (1) performing a protein precipitation of the sample of interest; and (2) loading the supernatant directly onto the HPLC-mass spectrometer without using on-line extraction or high turbulence liquid chromatography (HTLC).

[0099] In some preferred embodiments, HPLC, alone or in combination with one or more purification methods, may be used to purify CgA prior to mass spectrometry. In such embodiments samples may be extracted using an HPLC extraction cartridge which captures the analyte, then eluted and chromatographed on a second HPLC column or onto an analytical HPLC column prior to ionization. Because the steps involved in these chromatography procedures can be linked in an automated fashion, the requirement for operator involvement during the purification of the analyte can be minimized. This feature can result in savings of time and costs, and eliminate the opportunity for operator error.

[00100] It is believed that turbulent flow, such as that provided by HTLC columns and methods, may enhance the rate of mass transfer, improving separation characteristics. HTLC columns separate components by means of high chromatographic flow rates through a packed column containing rigid particles. By employing high flow rates (e.g., 3-5 mL/min), turbulent flow occurs in the column that causes nearly complete interaction between the stationary phase and the analyte(s) of interest. An advantage of using HTLC columns is that the macromolecular build-up associated with biological fluid matrices is avoided since the high molecular weight species are not retained under the turbulent flow conditions. HTLC methods that combine multiple separations in one procedure lessen the need for lengthy sample preparation and operate at a significantly greater speed. Such methods also achieve a separation performance superior to laminar flow (HPLC) chromatography. HTLC allows for direct injection of biological samples (plasma, urine, etc.). Direct injection is difficult to achieve in traditional forms of chromatography because denatured proteins and other biological debris quickly block the separation columns. HTLC also allows for very low sample volume of less than 1 mL, preferably less than .5 mL, preferably less than .2 mL, preferably .1 mL.

[00101] Examples of HTLC applied to sample preparation prior to analysis by mass spectrometry have been described elsewhere. See, *e.g.*, Zimmer *et al.*, *J. Chromatogr.* A **854**:23-35 (1999); see also, U.S. Patents Nos. 5,968,367; 5,919,368; 5,795,469; and 5,772,874. In certain embodiments of the method, samples are subjected to protein precipitation as described above prior to loading on the HTLC column; in alternative preferred embodiments, the samples may be loaded directly onto the HTLC without being subjected to protein precipitation. The HTLC extraction column is preferably a large particle column. In various embodiments, one of more steps of the methods may be performed in an on-line, automated fashion. For example, in one embodiment, steps (i)-(v) are performed in an on-line, automated fashion. In another, the steps of ionization and detection are performed on-line following steps (i)-(v).

[00102] Liquid chromatography (LC) including high-performance liquid chromatography (HPLC) relies on relatively slow, laminar flow technology. Traditional HPLC analysis relies on column packings in which laminar flow of the sample through the column is the basis for separation of the analyte of interest from the sample. The skilled artisan will understand that separation in such columns is a diffusional process. HPLC has been successfully applied to the separation of compounds in biological samples but a significant amount of sample preparation is required prior to the separation and subsequent analysis with a mass spectrometer (MS), making this technique labor intensive. In addition, most HPLC systems do not utilize the mass spectrometer to its fullest potential, allowing only one HPLC system to be connected to a single MS instrument, resulting in lengthy time requirements for performing a large number of assays.

[00103] Various methods have been described for using HPLC for sample clean-up prior to mass spectrometry analysis. See, *e.g.*, Taylor *et al.*, *Therapeutic Drug Monitoring* **22**:608-12 (2000); and Salm *et al.*, *Clin. Therapeutics* **22** Supl. B:B71-B85 (2000).

[00104] One of skill in the art may select HPLC instruments and columns that are suitable for use with CgA. 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 include a bonded surface that interacts with the various chemical moieties to facilitate separation of the chemical moieties. One suitable bonded surface is a hydrophobic bonded surface such as an alkyl bonded surface. Alkyl bonded surfaces may include C-4, C-8, C-12, or C-18 bonded alkyl groups, preferably C-18 bonded groups. The chromatographic column includes an inlet port for receiving a sample and an outlet port for discharging an effluent that includes the fractionated sample. In one embodiment, the sample (or pre-purified sample) is applied to the column at the inlet port, eluted with a solvent or solvent mixture, and discharged at the outlet port. Different solvent modes may be selected for eluting the analyte(s) of interest. For example, liquid chromatography may be performed using a gradient mode, an isocratic mode, or a polytyptic (i.e. mixed) mode. During chromatography, the separation of materials is effected by variables such as choice of eluent (also known as a "mobile phase"), elution mode, gradient conditions, temperature, *etc*.

[00105] In certain embodiments, an analyte may be purified by applying a sample to a column under conditions where the analyte of interest is reversibly retained by the column packing material, while one or more other materials are not retained. In these embodiments, a first mobile phase condition can be employed where the analyte of interest is retained by the column, and a second mobile phase condition can subsequently be employed to remove retained material from the column, once the non-retained materials are washed through. Alternatively, an analyte may be purified by applying a sample to a column under mobile phase conditions where the analyte of interest elutes at a differential rate in comparison to one or more other materials. Such procedures may enrich the amount of one or more analytes of interest relative to one or more other components of the sample.

[00106] In one preferred embodiment, the HTLC may be followed by HPLC on a hydrophobic column chromatographic system. In certain preferred embodiments, a TurboFlow Cyclone P® polymer-based column from Cohesive Technologies (60 µm particle size, 50 x 1.0 mm column dimensions, 100Å pore size) is used. In related preferred embodiments, a Synergi Polar-RP® ether-linked phenyl, analytical column from Phenomenex Inc (4 µm particle size, 150 x 2.0 mm column dimensions, 80Å pore size) with hydrophilic endcapping is used. In certain preferred embodiments, HTLC and HPLC are performed using HPLC Grade Ultra Pure Water and 100% methanol as the mobile phases.

[00107] By careful selection of valves and connector plumbing, two or more chromatography columns may be connected as needed such that material is passed from one to the next without the need for any manual steps. In preferred embodiments, the selection of valves and plumbing is controlled by a computer pre-programmed to perform the necessary steps. Most preferably, the chromatography system is also connected in such an on-line fashion to the detector system, *e.g.*, an MS system. Thus, an operator may place a tray of samples in an autosampler, and the remaining operations are performed under computer control, resulting in purification and analysis of all samples selected.

[00108] In certain preferred embodiments, CgA or fragments thereof in a sample may be purified prior to ionization. In particularly preferred embodiments the chromatography is not gas chromatography.

<u>Detection and Quantitation by Mass Spectrometry</u>

[00109] In various embodiments, CgA or fragments thereof may be ionized by any method known to the skilled artisan. Mass spectrometry is performed using a mass spectrometer, which includes an ion source for ionizing the fractionated sample and creating charged molecules for further analysis. For example ionization of the sample may be performed by electron ionization, chemical ionization, electrospray ionization (ESI), photon ionization, atmospheric pressure chemical ionization (APCI), photoionization, atmospheric pressure photoionization (APPI), fast atom bombardment (FAB), liquid secondary ionization (LSI), matrix assisted laser desorption ionization (MALDI), field ionization, field desorption, thermospray/plasmaspray ionization, surface enhanced laser desorption ionization (SELDI), inductively coupled plasma (ICP) and particle beam ionization. The skilled artisan will understand that the choice of ionization method may be determined based on the analyte to be measured, type of sample, the type of detector, the choice of positive versus negative mode, *etc*.

[00110] In preferred embodiments, CgA or a fragment thereof is ionized by electrospray ionization (ESI) in positive or negative mode.

[00111] After the sample has been ionized, the positively charged or negatively charged ions thereby created may be analyzed to determine a mass-to-charge ratio. Suitable analyzers for determining mass-to-charge ratios include quadrupole analyzers, ion traps analyzers, and time-of-flight analyzers. The ions may be detected using several detection modes. For example, selected ions may be detected *i.e.*, using a selective ion monitoring mode (SIM), or alternatively, ions may be detected using a scanning mode, e.g., multiple reaction monitoring (MRM) or selected reaction monitoring (SRM). Preferably, the mass-to-charge ratio is determined using a quadrupole analyzer. For example, in a “quadrupole” or “quadrupole ion trap” instrument, ions in an oscillating radio frequency field experience a force proportional to the DC potential applied between electrodes, the amplitude of the RF signal, and the mass/charge ratio. The voltage and amplitude may be selected so that only ions having a particular mass/charge ratio travel the length of the quadrupole, while all other ions are deflected. Thus, quadrupole instruments may act as both a “mass filter” and as a “mass detector” for the ions injected into the instrument.

[00112] One may enhance the resolution of the MS technique by employing “tandem mass spectrometry,” or “MS/MS”. In this technique, a precursor ion (also called a parent ion) generated from a molecule of interest can be filtered in an MS instrument, and the precursor ion is subsequently fragmented to yield one or more fragment ions (also called daughter ions or product ions) that are then analyzed in a second MS procedure. By careful selection of precursor ions, only ions produced by certain analytes are passed to the fragmentation chamber, where collisions with atoms of an inert gas produce the fragment ions. Because both the precursor and fragment ions are produced in a reproducible fashion under a given set of ionization/fragmentation conditions, the MS/MS technique may provide an extremely powerful analytical tool. For example, the combination of filtration/fragmentation may be used to eliminate interfering substances, and may be particularly useful in complex samples, such as biological samples.

[00113] The mass spectrometer typically provides the user with an ion scan; that is, the relative abundance of each ion with a particular mass/charge over a given range (*e.g.*, 100 to 1000 amu). The results of an analyte assay, that is, a mass spectrum, may be related to the amount of the analyte in the original sample by numerous methods known in the art. For example, given that sampling and analysis parameters are carefully controlled, the relative abundance of a given ion may be compared to a table that converts that relative abundance to an absolute amount of the original molecule. Alternatively, molecular standards may be run with the samples, and a standard curve constructed based on ions generated from those standards. Using such a standard curve, the relative abundance of a given ion may be converted into an absolute amount of the original molecule. In certain preferred embodiments, an internal standard is used to generate a standard curve for calculating the quantity of CgA. Methods of generating and using such standard curves are well known in the art and one of ordinary skill is capable of selecting an appropriate internal standard. For example, an isotope of CgA may be used as an internal standard. Numerous other methods for relating the amount of an ion to the amount of the original molecule will be well known to those of ordinary skill in the art.

[00114] One or more steps of the methods may be performed using automated machines. In certain embodiments, one or more purification steps are performed on-line, and more preferably all of the purification and mass spectrometry steps may be performed in an on-line fashion.

[00115] In certain embodiments, such as MS/MS, where precursor ions are isolated for further fragmentation, collision activation dissociation is often used to generate the fragment ions for further detection. In CAD, precursor ions gain energy through collisions with an inert gas, and subsequently fragment by a process referred to as “unimolecular decomposition”. Sufficient energy must be deposited in the precursor ion so that certain bonds within the ion can be broken due to increased vibrational energy.

[00116] In particularly preferred embodiments, CgA is detected and/or quantified using MS/MS as follows. The samples are subjected to liquid chromatography, preferably HPLC, the flow of liquid solvent from the chromatographic column enters the heated nebulizer interface of an MS/MS analyzer and the solvent/analyte mixture is converted to vapor in the heated tubing of the interface. The analyte is ionized by the selected ionizer. The ions, e.g. precursor ions, pass through the orifice of the instrument and enter the first quadrupole. Quadrupoles 1 and 3 (Q1 and Q3) are mass filters, allowing selection of ions (i.e., “precursor” and “fragment” ions) based on their mass to charge ratio (m/z). Quadrupole 2 (Q2) is the collision cell, where ions are fragmented. The first quadrupole of the mass spectrometer (Q1) selects for molecules with the mass to charge ratios of CgA. Precursor ions with the correct mass/charge ratios of CgA are allowed to pass into the collision chamber (Q2), while unwanted ions with any other mass/charge ratio collide with the sides of the quadrupole and are eliminated. Precursor ions entering Q2 collide with neutral argon gas molecules and fragment. This process is called collision activated dissociation (CAD). The fragment ions generated are passed into quadrupole 3 (Q3), where the fragment ions of CgA are selected while other ions are eliminated.

[00117] The methods may involve MS/MS performed in either positive or negative ion mode. Using standard methods well known in the art, one of ordinary skill is capable of identifying one or more fragment ions of a particular precursor ion of CgA that may be used for selection in quadrupole 3 (Q3).

[00118] As ions collide with the detector they produce a pulse of electrons that are converted to a digital signal. The acquired data is relayed to a computer, which plots counts of the ions collected versus time. The resulting mass chromatograms are similar to chromatograms generated in traditional HPLC methods. The areas under the peaks corresponding to particular ions, or the amplitude of such peaks, are measured and the area or amplitude is correlated to the amount of the analyte of interest. In certain embodiments, the area under the curves, or amplitude of the peaks, for fragment ion(s) and/or precursor ions are measured to determine the amount of CgA. As described above, the relative abundance of a given ion may be converted into an absolute amount of the original analyte, using calibration standard curves based on peaks of one or more ions of an internal molecular standard.

[00119] The following examples serve to illustrate the invention. These examples are in no way intended to limit the scope of the methods.

EXAMPLES

Example 1: CgA quantitation by mass spectrometry

[00120] Reagent summary:

Reagents	Supplier & Catalog Number	Quantity
Chromogranin-A	Abcam, ab85486	250 ug
Chromogranin-A IS (See CgA Internal Standard Prep below for sequence)	New England Peptide, Custom Synthesis ≥95% purity	6 mg
Biocell Charcoal Stripped and Delipidated Serum	Biocell, 1101-00	1 L
Ammonium Bicarbonate (AmBic)	Sigma, A Millipore, FX0440-56141-500G	500 g
Triethylammonium bicarbonate, 1.0M	Sigma, T7408-500mL	500 mL
Dithiothreitol	Sigma, 43819-25G	25g
Iodoacetamide	Sigma, I1149-25G	25g
Trypsin	Sigma, T1426-500MG	0.5 g
Formic Acid, 98%	Millipore, FX0440-5	0.5 L
Ammonium hydroxide, 28-30%	Sigma, 221228-500ML-A	500 mL
HPLC Water	Burdick & Jackson, 365-4	4 L
Acetonitrile	Burdick & Jackson, 015-4	4 L
Methanol	Fisher Scientific, A454-4	4 L
Bovine Serum Albumin	Sigma, A2153-500G	500 g
MicroAmp Clear Adhesive Film	Thermo Fisher, 4306311	100 films

Thermo Scientific Accucore C18 100 x 2.1 mm, 2.6u	Themro, 17126-102130	1 column
Oasis MAX 96-well 30mg SPE Plate	Waters, 186000373	1 plate

[00121] Sciex 6500+ QTrap Mass Spectrometer, Thermo Fisher Aria Cohesive TLX4 with Agilent Pumps, Hamilton Microlab Star, SPEware IP8 were used to detect CgA.

[00122] Patient serum (100 µL) was added to 600 µL of 120 mM ammonium bicarbonate, and the entire volume was extracted using a mixed-mode anion exchange plate. (Waters Oasis® MAX 30 mg). Samples are then washed twice with 3% ammonium hydroxide and water, respectively. Next, CgA was eluted, internal standard (IS) added, and the sample was evaporated under heated nitrogen.

[00123] After dry-down, samples were reconstituted, reduced, alkylated, and tryptically digested for 2 hours in a rapid enzyme digestion microwave system (Hudson Technology).

[00124] Post-digestion, samples were acidified and analyzed by LC-MS/MS on an Aria TLX-4 Transcend UPLC. Peptides were resolved using a Thermo Accucore C18 100×2.1mm, 2.6 micron HPLC column using water/acetonitrile /0.1% formic acid gradients. The MS detector was a Sciex 6500+ Qtrap. Quantitation of CgA was based on the chromatographic peaks of fragment ions produced during MS/MS corresponding to a representative tryptic peptide and its corresponding heavy-labeled IS. The analytical workflow is summarized in Figure 1.

[00125] Multiple reaction monitoring was used for detecting both the analyte and the internal standard (IS).

[00126] Concentrations of Chromogranin A (CgA) were determined using peak area ratio and a calibration curve.

In assays detecting ELQDLALQGAK (SEQ ID NO: 1), a labeled winged peptide internal standard was used: **ILSILRHQNLLKELQDLAL*QGAK*ERAHQQK** (SEQ ID NO: 2): *$C^{13}N^{15}$ labeled amino acids. After digestion, the resulting peptide was ELQDLAL*QGAK* (SEQ ID NO: 3).

In assays detecting RRPEDQELESLSAIEAELEK (SEQ ID NO: 4), a labeled winged peptide internal standard was used: **EGSANRRPEDQELESL*SAIEAELEK*VAHQL** (SEQ ID NO: 5); *$C^{13}N^{15}$ labeled amino acids. After digestion, the resulting peptide was RRPEDQELESL*SAIEAELEK* (SEQ ID NO: 6).

[00127] Table 1: CgA fragment used to quantitate CgA levels in the sample and precursor and product ions (mass-to-charge m/z ratios)

[00128]

Analyte	Precursor Ion (m/z)	Product Ion (m/z)
CgA: ELQDLALQGAK	593.2	516.3, 815.5
CgA IS: ELQDLAL*QGAK*	600.8	602.4, 830.6, 958.7

[00129] Table 2: CgA fragment used to quantitate CgA levels in the sample and precursor and product ions (mass-to-charge m/z ratios)

[00130]

Analyte	Precursor Ion (m/z)	Product Ion (m/z)
CgA: RRPEDQELESLSAIEAELEK	729.6	831.5, 989.5
CgA: RRPEDQELESL*SAIEAELEK*	734.6	839.5, 997.6

[00131] Table 3: The values obtained were compared to the values quantitated by ELISA immunoassay. See also Figure 4.

[00132]

Sample ID#	Mayo Result	ARUP Result	Dawn Result	Average (Mayo, ARUP, Dawn)	LCMS
Ref Ranges	**<93**	**0-95**	**N/A**	**N/A**	**N/A**
Units	*ng/mL*	*ng/mL*	*ng/mL*	*ng/mL*	*ng/mL*
1	1654	2286	3070	1753	1402
2	872	2172	1724	1192	2084
3	1286	1603	1680	1142	1774
4	793	1151	1220	791	730
5	499	1077	1285	715	827
6	588	639	657	471	4021
7	42	479	482	251	237
8	207	302	297	202	589
9	277	284	285	212	677
10	156	212	187	139	412
11	114	181	182	119	94
12	120	145	136	101	342
13	82	120	130	83	115
14	64	105	101	68	50
15	63	96	101	65	62
16	57	88	86	58	<50
17	62	80	80	56	<50
18	47	74	76	49	<50
19	41	60	60	40	<50
20	<20	36	45	40	<50

[00133] Samples from 308 patients were analyzed to compare CgA serum values measured by the Cisbio CGA-ELISA-US immunoassay (Codolet, France) with values from our LC-MS/MS assay.

[00134] After natural logarithm-transformation of the measurements, a normal distribution was seen in the 308 patients. A paired t-test was performed on these data and the concordance between the assays was measured by the Pearson correlation coefficient and Passing & Bablok curve fitting. All analyses were performed in Analyse-it v2.30.

[00135] The assay was validated. Performance characteristics are presented in Table 4:

Characteristics	Values
Intra-assay precision	5.2-15.7%

Inter-assay precision	7.4-10.1%
Recovery of CgA spiked into patient samples	90-110%
Analytical sensitivity (Limit of Detection)	35.5 ng/mL
Analytical sensitivity (Limit of Quantitation)	50 ng/mL
Linearity	50-50,000 ng/mL
Reference Range	<140 ng/mL (n=160)

[00136] Typical results for patient samples with low and high concentrations of circulating CgA are shown in Figure 3.

[00137] Measured CgA levels of the LC-MS/MS were on average comparable to the CisBio assay, although there was substantial scatter (Pearson's correlation 0.76) (Figure 5).

[00138] Conclusion: We have developed and validated a fully automated LC-MS/MS assay for CgA from serum

[00139] The contents of the articles, patents, and patent applications, and all other documents and electronically available information mentioned or cited herein, are hereby incorporated by reference in their entirety to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference. Applicants reserve the right to physically incorporate into this application any and all materials and information from any such articles, patents, patent applications, or other physical and electronic documents.

[00140] The methods illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including," containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof. It is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the invention embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[00141] The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the methods. This includes the generic description of the methods with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[00142] Other embodiments are within the following claims. In addition, where features or aspects of the methods are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

THAT WHICH IS CLAIMED IS:

1. A method for determining the amount of chromogranin A (CgA) in a sample, said method comprising:

(a) purifying CgA in the sample;

(b) ionizing CgA to produce one or more ion(s) of CgA detectable by mass spectrometry;

(c) determining the amount of the ion(s) from step (b) by mass spectrometry, wherein the amount of ions is related to the amount of CgA in the sample.

2. The method of claim 1, wherein said purifying comprises extraction by solid phase extraction (SPE).

3. The method of claim 2, wherein the SPE is an anion exchange solid phase extraction.

4. The method of claim 2, wherein the SPE is a mixed-mode anion exchange solid phase extraction.

5. The method of claim 2, wherein extracted samples are enzymatically digested.

6. The method of claim 5, wherein the digestion comprises trypsin digestion.

7. The method of claim 1, wherein said purifying comprises liquid chromatography.

8. The method of claim 7, wherein said liquid chromatography comprises high performance liquid chromatography (HPLC).

9. The method of claim 7, wherein said liquid chromatography comprises high turbulence liquid chromatography (HTLC).

10. The method of claim 1, wherein said ionization comprises electrospray ionization (ESI).

11. The method of claim 1, further comprising adding an internal standard.

12. The method of claim 11, wherein said internal standard is isotopically labeled.

13. The method of claim 1, wherein the sample is serum.

14. The method of claim 1, wherein the sample is cerebrospinal fluid (CSF).

15. The method of claim 1, wherein the method comprises measuring the amount of precursor ion having a mass-to-charge ratio of 729.6 ± 0.5.

16. The method of claim 1, wherein the method comprises measuring the amount of a fragment of CgA.

17. The method of claim 16, wherein the CgA fragment measured comprises a sequence RRPEDQELESLSAIEAELEK (SEQ ID NO: 4).

18. The method of claim 1, wherein the method comprise measuring the amount of fragment ion having a mass-to-charge ratio of 831.5 ± 0.5 or 989.5 ± 0.5.

19. The method of claim 1, wherein the method comprises adding an internal standard.

20. The method of claim 19, wherein the internal standard is isotopically labeled.

21. The method of claim 19, wherein the internal standard comprises a $C^{13}N^{15}$ labeled amino acid.

22. The method of claim 19, wherein the internal standard is labeled on a leucine (L) or lysine (K).

23. The method of claim 19, wherein the internal standard comprises a sequence EGSANRRPEDQELESL*SAIEAELEK*VAHQL (SEQ ID NO: 5), wherein L* and K* is each a $C^{13}N^{15}$ labeled amino acid.

23. The method of claim 19, wherein the method comprises measuring the amount of internal standard precursor ion having a mass-to-charge ratio of 734.6 ± 0.5 or product ion having a mass-to-charge ratio of 839.5 ± 0.5 or 997.6 ± 0.5.

24. The method of claim 1, wherein the limit of quantitation of the methods is less than or equal to 50 ng/mL.

25. The method of claim 1, wherein the limit of detection of the methods is less than or equal to 35.5 ng/mL.

26. The method of claim 1, wherein the method has a linearity of quantitation across a range between 50 ng/mL to 50,000 ng/mL.

27. The method of claim 1, wherein the method has an inter- and intra-assay reproducibility of CV ≤15%.

28. The method of claim 1, wherein the mass spectrometry is selected reaction monitoring (SRM) mass spectrometry.

29. The method of claim 1, wherein the method is fully automated.

30. The method of claim 1, wherein the method is antibody-free.

FIGURE 1

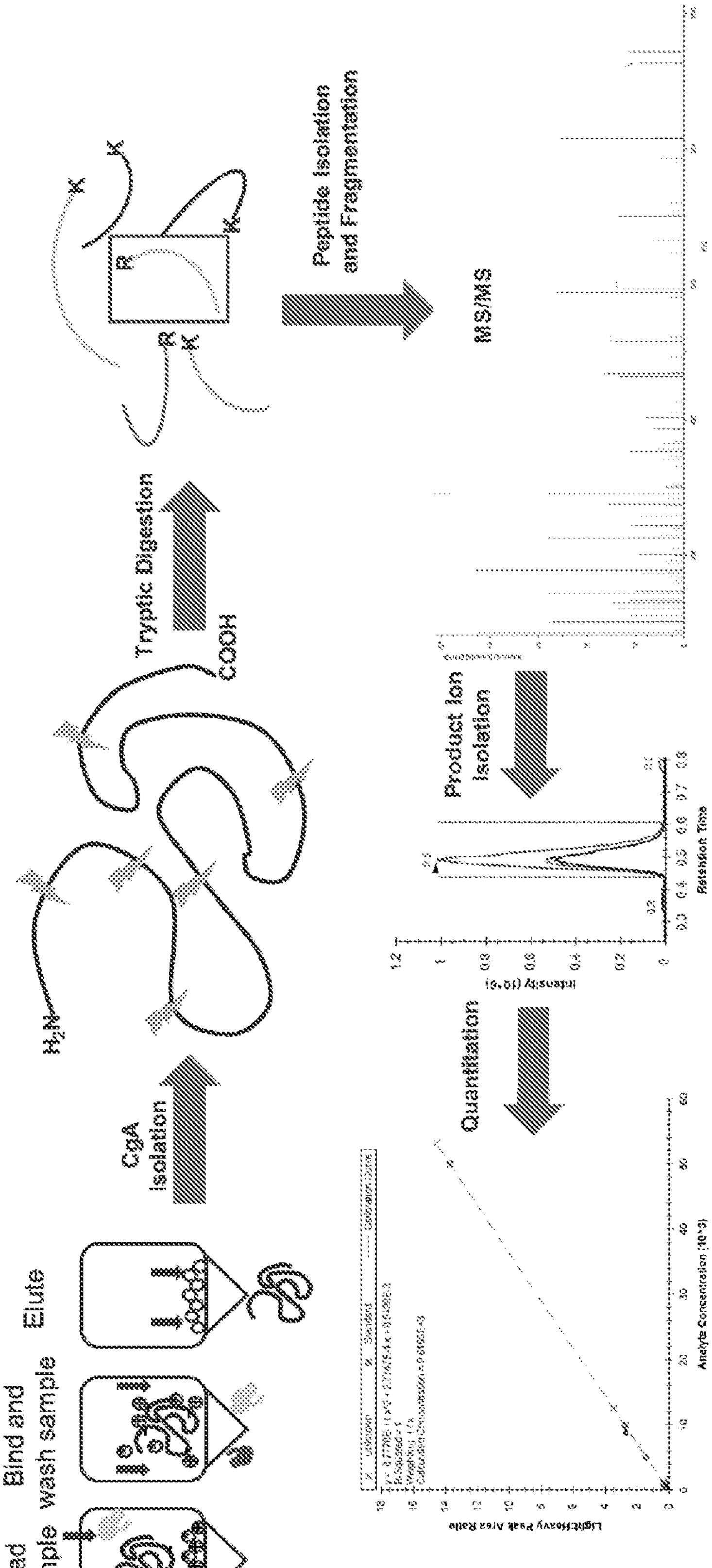

Load sample
Bind and wash sample
Elute
CgA Isolation
H_2N
COOH
Tryptic Digestion
R
K
Peptide Isolation and Fragmentation
MS/MS
Product Ion Isolation
Quantitation
Retention Time
Analyte Concentration (10^3)

FIGURE 2

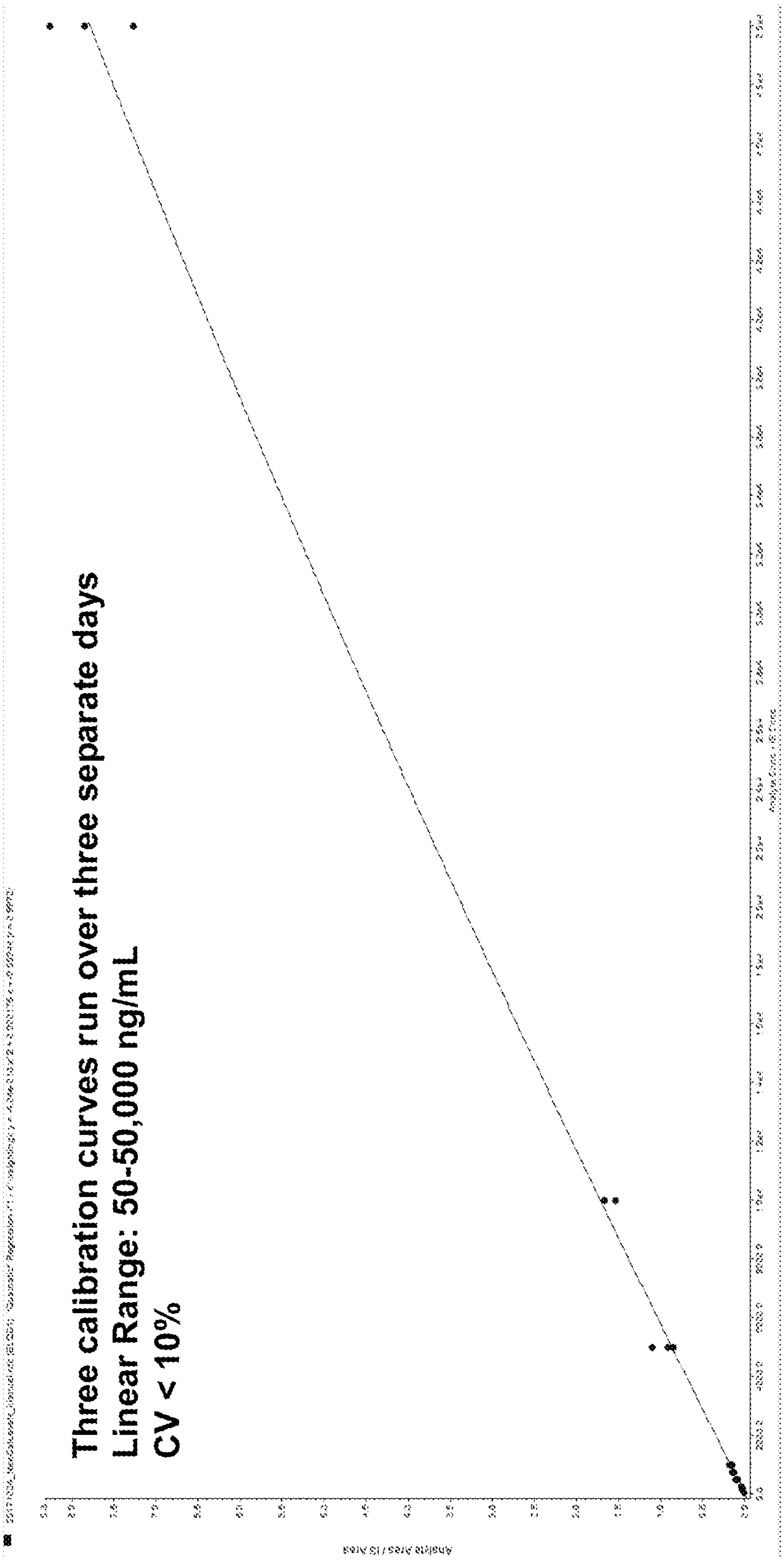

Three calibration curves run over three separate days
Linear Range: 50-50,000 ng/mL
CV < 10%

FIGURE 3

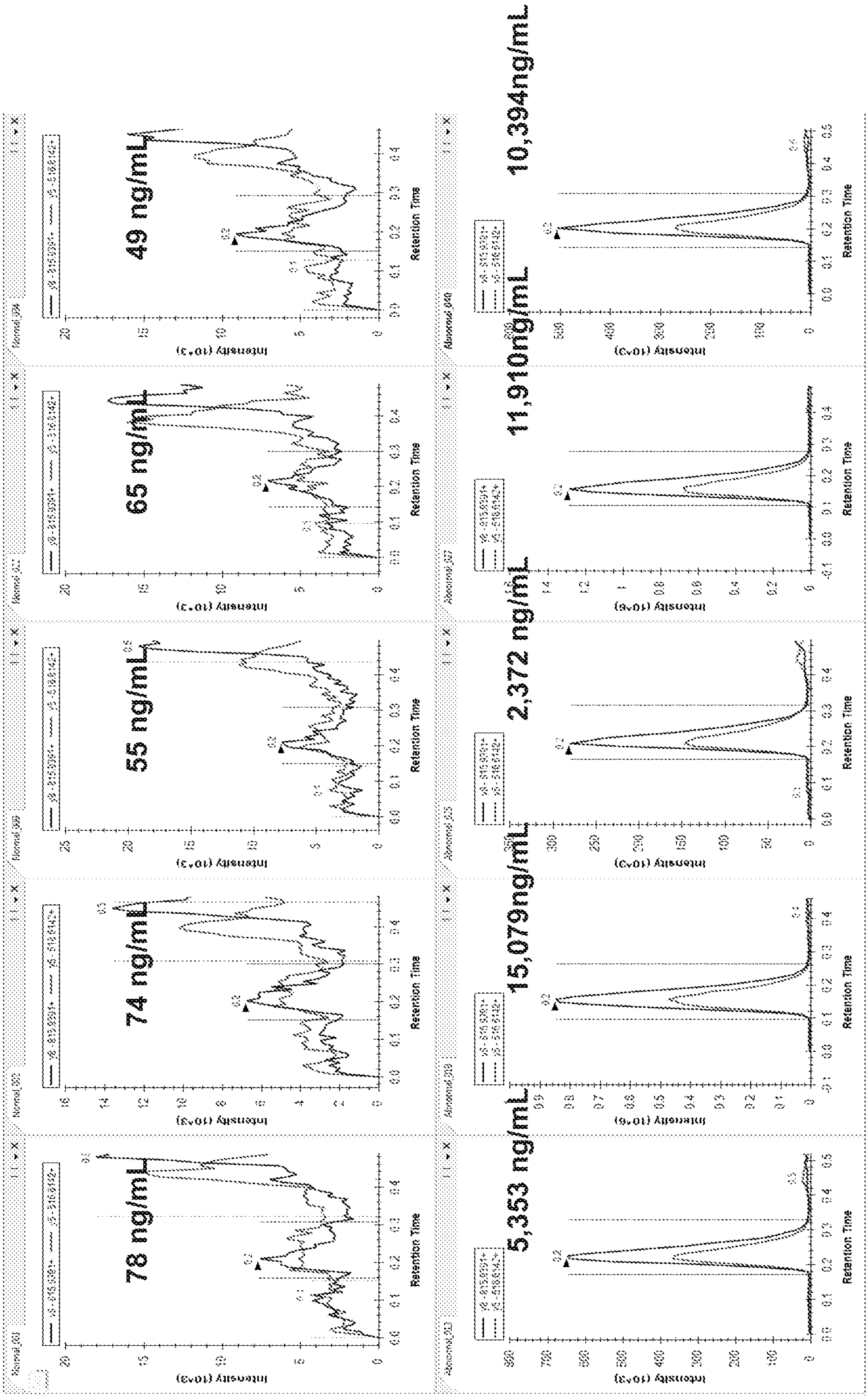
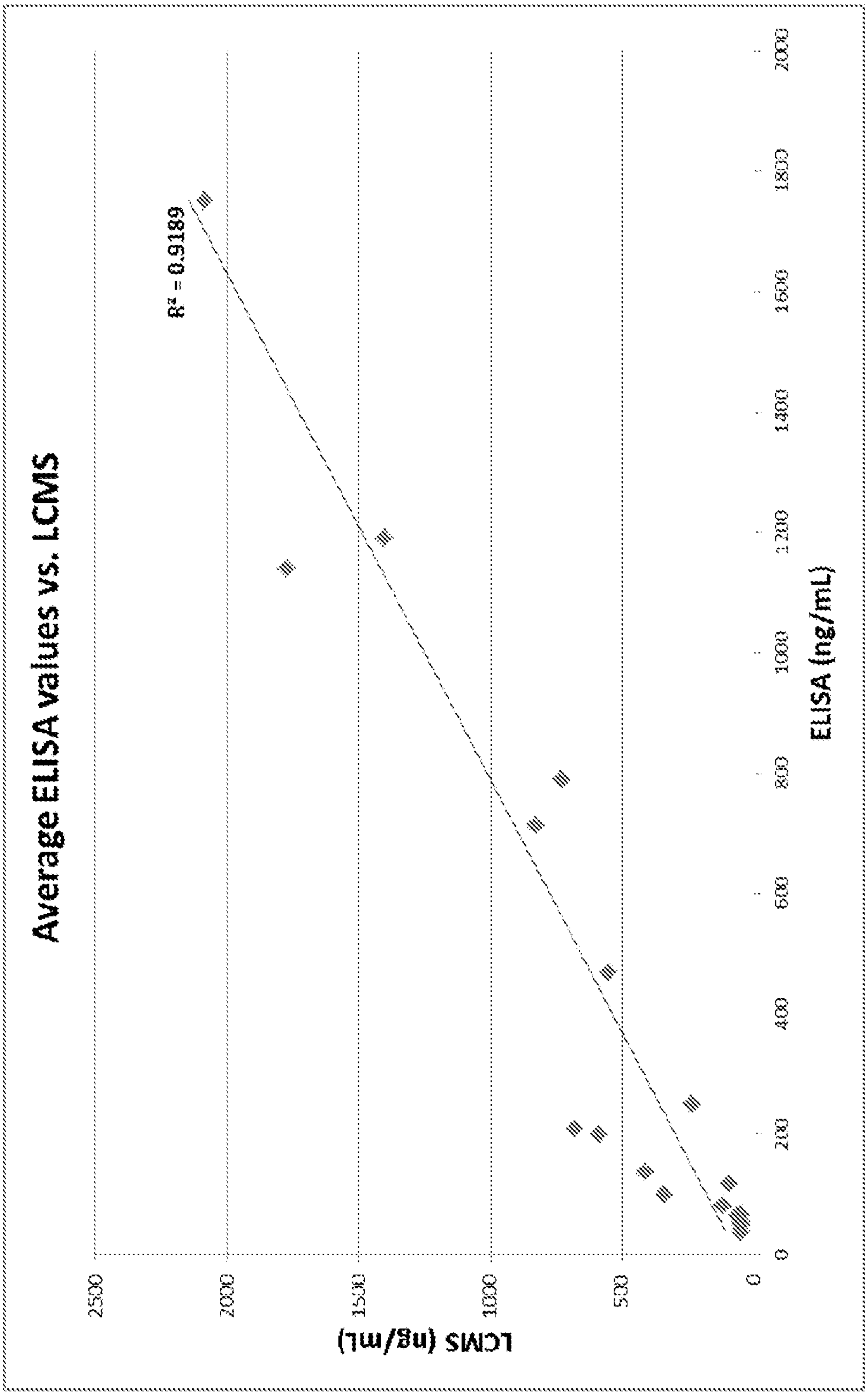

78 ng/mL
74 ng/mL
55 ng/mL
65 ng/mL
49 ng/mL
5,353 ng/mL
15,079ng/mL
2,372 ng/mL
11,910ng/mL
10,394ng/mL
Intensity
Retention Time

FIGURE 4


Average ELISA values vs. LCMS
R² = 0.9189
LCMS (ng/mL)
2500
2000
1500
1000
500
0
ELISA (ng/mL)
0
200
400
600
800
1000
1200
1400
1600
1800
2000

5/7

FIGURE 5

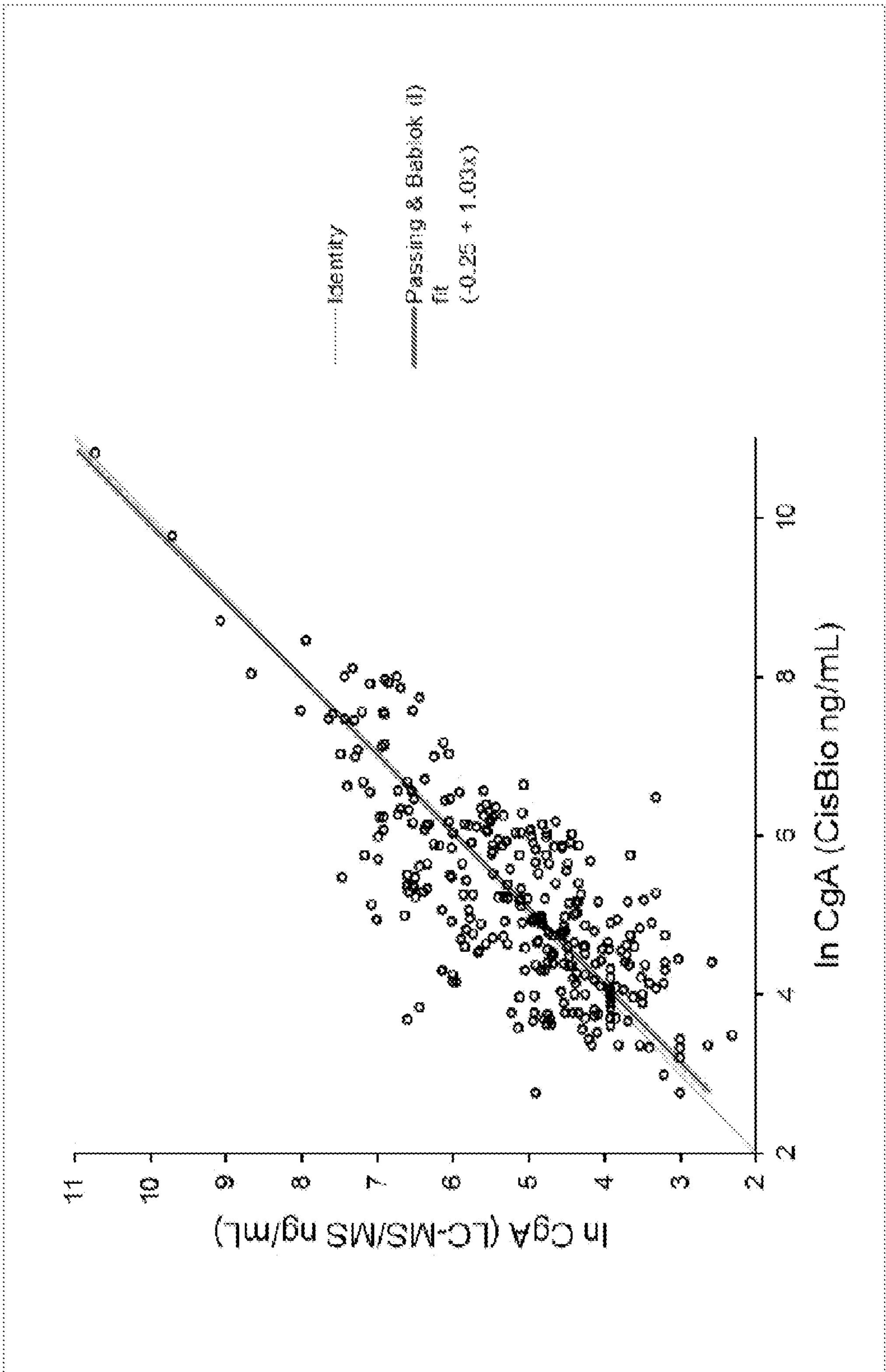

Identity
Passing & Bablok
fit
(-0.25 + 1.03x)
ln CgA (LC-MS/MS ng/mL)
ln CgA (CisBio ng/mL)
2
3
4
5
6
7
8
9
10
11

FIGURE 6

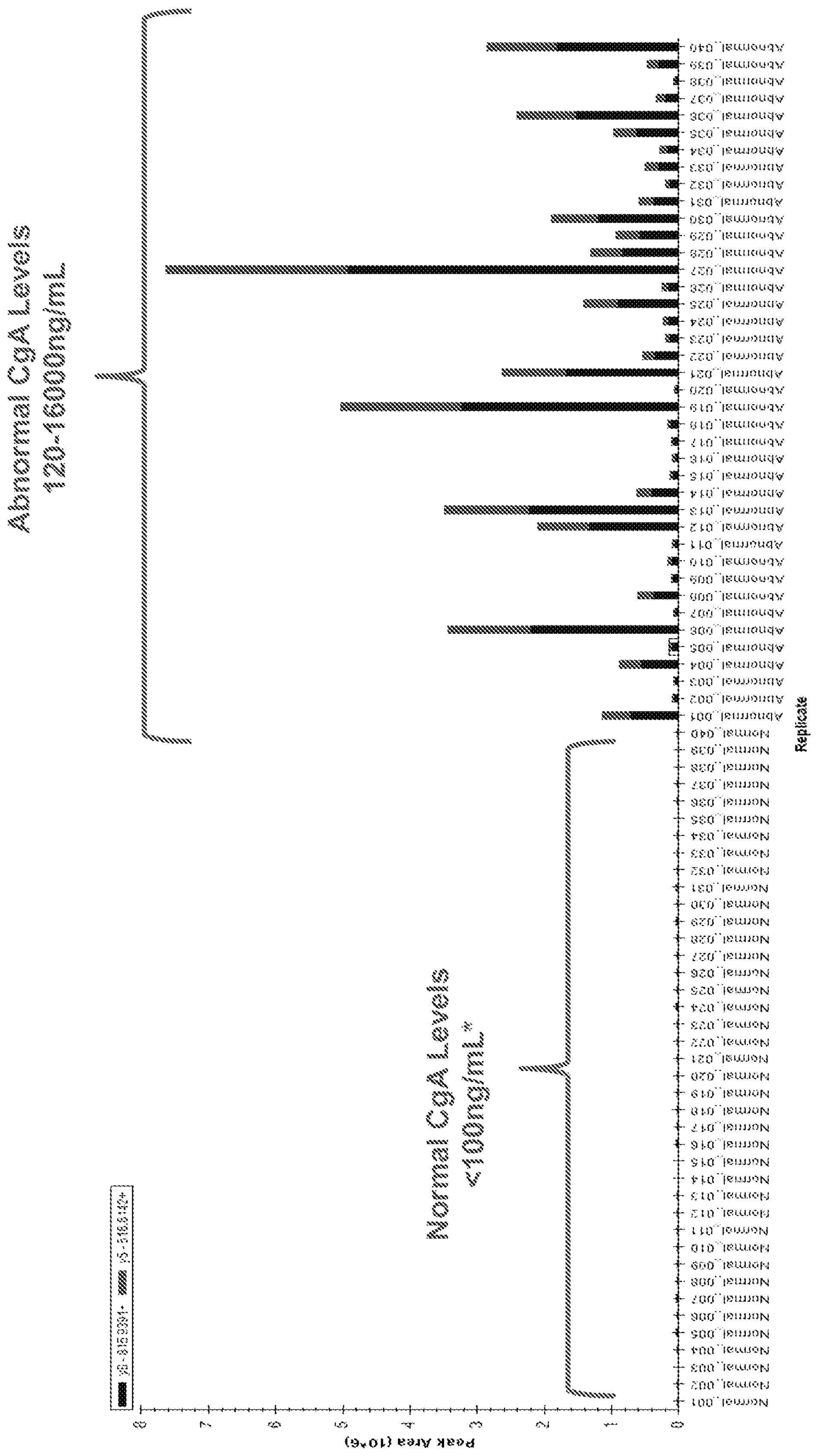

Abnormal CgA Levels
120-16000ng/mL
Normal CgA Levels
<100ng/mL*
Peak Area (10^6)
Replicate

FIGURE 7

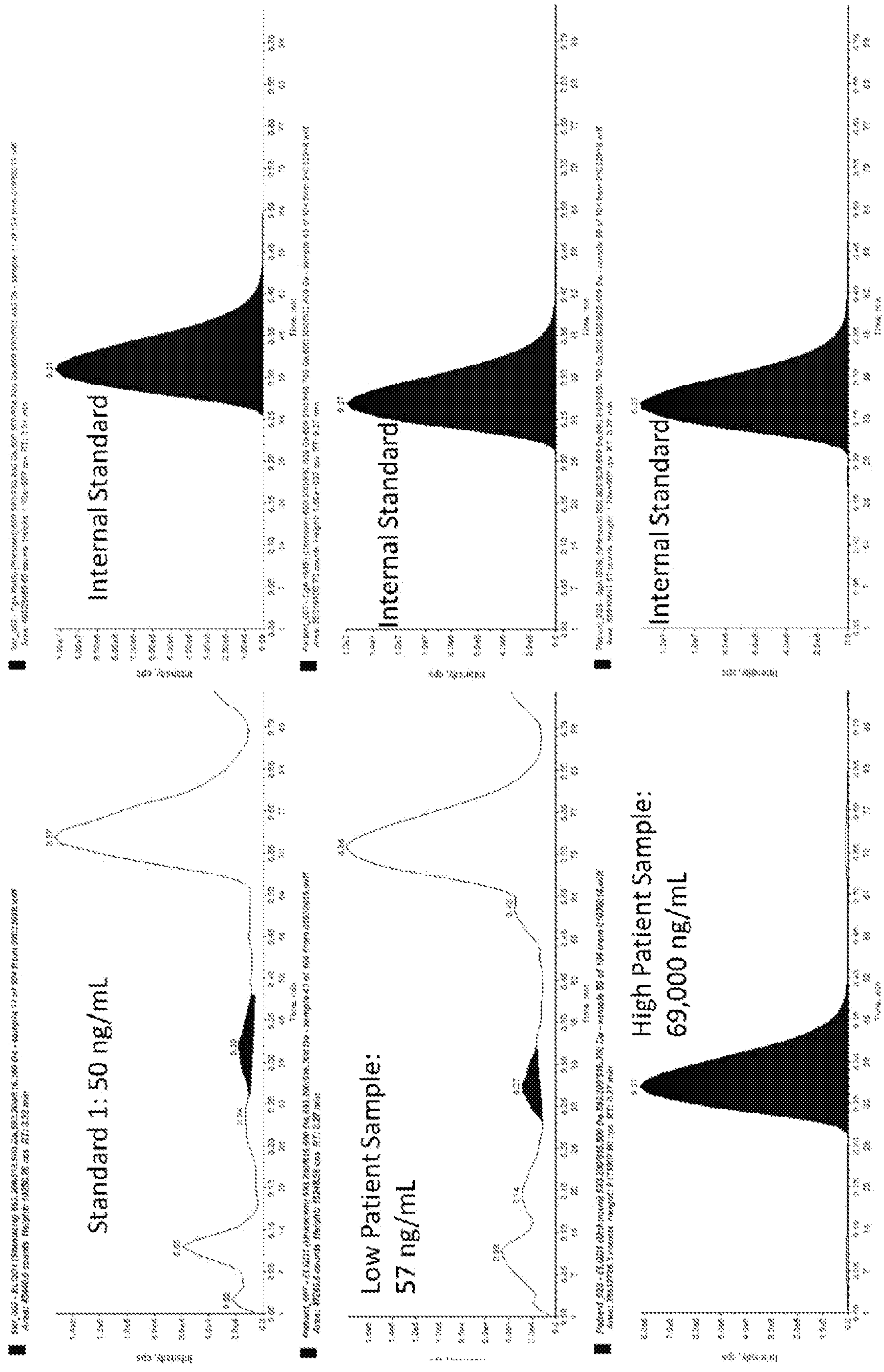
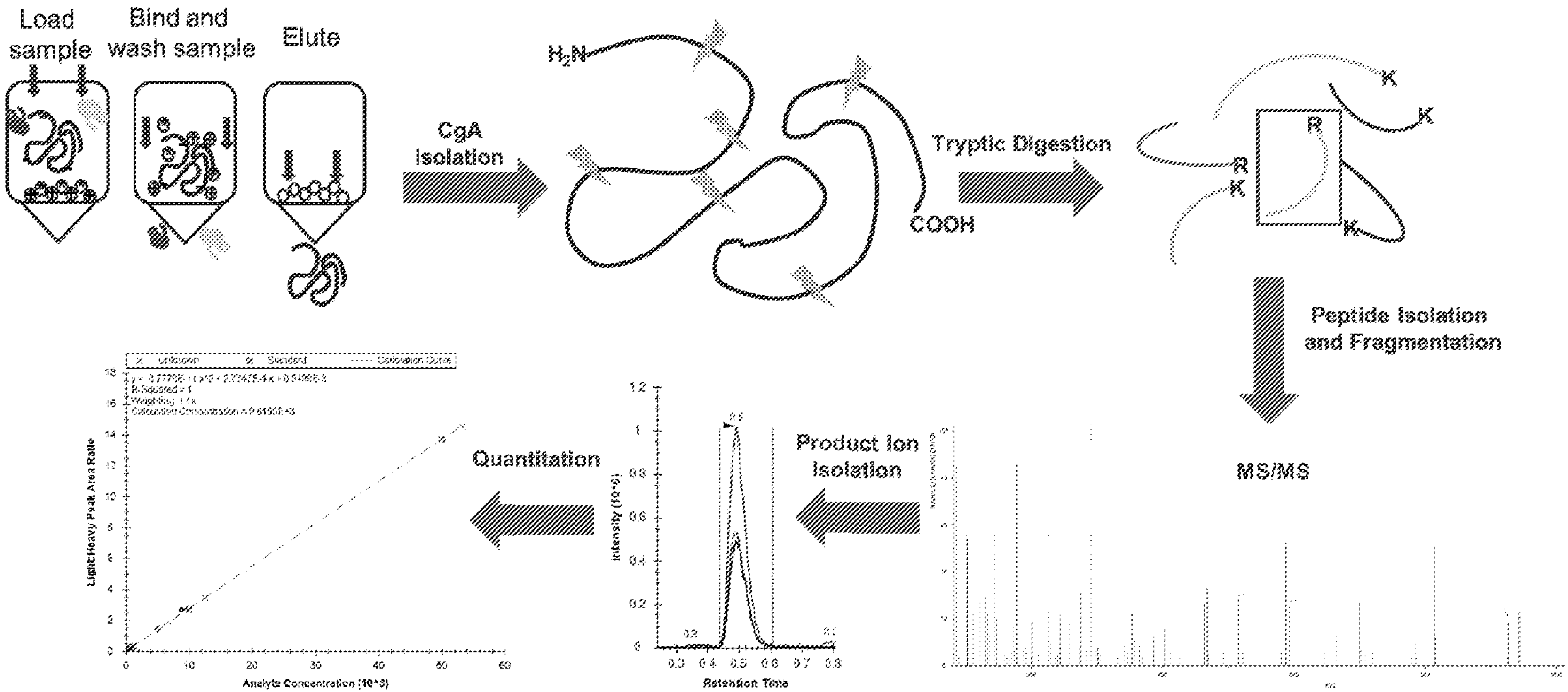

Standard 1: 50 ng/mL
Internal Standard
Low Patient Sample:
57 ng/mL
Internal Standard
High Patient Sample:
69,000 ng/mL
Internal Standard

FIGURE 1



Load sample
Bind and wash sample
Elute
CgA Isolation
H_2N
COOH
Tryptic Digestion
K
K
R
R
K
K
Peptide Isolation and Fragmentation
MS/MS
Product Ion Isolation
Quantitation
Retention Time
Intensity (10^6)
Analyte Concentration (10^3)
Light:Heavy Peak Area Ratio