(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 692 513 B1

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:
06.06.2012 Bulletin 2012/23

(21) Application number: **04797198.1**

(22) Date of filing: **10.11.2004**

(51) Int Cl.:
G01N 33/68 *(2006.01)*

(86) International application number:
PCT/CA2004/001945

(87) International publication number:
WO 2005/045436 (19.05.2005 Gazette 2005/20)

(54) **DIAGNOSTIC METHODS FOR CONGESTIVE HEART FAILURE**

DIAGNOSEVERFAHREN FÜR KONGESTIVES HERZVERSAGEN

PROCEDES DIAGNOSTIQUES POUR UNE INSUFFISANCE CARDIAQUE CONGESTIVE

(84) Designated Contracting States:
AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IS IT LI LU MC NL PL PT RO SE SI SK TR

(30) Priority: **11.11.2003 US 706599**

(43) Date of publication of application:
23.08.2006 Bulletin 2006/34

(73) Proprietor: **Nexus DX, Inc.**
San Diego, CA 92121 (US)

(72) Inventors:
- **JACKOWSKI, George**
  Kettleby, Ontario L0G 1J0 (CA)
- **VAN LIESHOUT, Tracy**
  Hamilton, Ontario L8P 2G4 (CA)
- **THATCHER, Brad**
  Casalnuovo de Napoli I-80013 Napoli (IT)
- **ZHANG, Rulin**
  Brampton, Ontario L7A 1Y5 (CA)
- **YANTHA, Jason**
  Toronto, Ontario M6G 2R5 (CA)
- **RASAMOELISOLO, Michele**
  Winnipeg, Manitoba R2N 1M6 (CA)

(74) Representative: **Taylor, Kate Laura et al**
Harrison Goddard Foote
Saviour House
9 St Saviourgate
York, YO1 8NQ (GB)

(56) References cited:
US-B- 6 210 976

- **RASAMOELISOLO M ET AL: "Fine characterization of a series of new monoclonal antibodies directed against glycophorin A." VOX SANGUINIS 1997, vol. 72, no. 3, 1997, pages 185-191, XP002462928 ISSN: 0042-9007**
- **CLASTER S ET AL: "Degradation of erythrocyte glycophorin results in increased membrane bound hemoglobin." ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 15 FEB 1991, vol. 285, no. 1, 15 February 1991 (1991-02-15), pages 147-152, XP002462927 ISSN: 0003-9861**
- **SANTOS-SILVA ALICE ET AL: "ERYTHROCYTE DAMAGE AND LEUKOCYTE ACTIVATION IN ISCHEMIC STROKE" CLINICA CHIMICA ACTA, AMSTERDAM, NL, vol. 320, no. 1-2, June 2002 (2002-06), pages 29-35, XP008069381 ISSN: 0009-8981**
- **SANTOS-SILVA ET AL.: 'Erythrocyte damage and leukocyte activation in ischemic stroke' CLINICA CHIMICA ACTA vol. 320, no. 1-2, June 2002, pages 29 - 35, XP008069381**
- **CAIMI ET AL.: 'Erythrocyte aggregation and erythrocyte membrane properties in type 2 diabetes mellitus and in vascular atherosclerotic disease' THROMB. HAEMOST. vol. 83, no. 3, March 2000, pages 516 - 517, XP008069391**
- **ROGERS ET AL.: 'Decrease in erythrocyte glycophorin sialic acid content is associated with increased erythrocyte aggregation in human diabetes' CLINICAL SCIENCE vol. 82, no. 3, March 1992, pages 309 - 313, XP008069390**
- **GOLDMAN ET AL.: 'Hematopoiesis/ eruthropoiesis in myocardial infarcts' MODERN PATHOLOGY vol. 14, no. 6, June 2001, pages 589 - 594, XP008069380**

EP 1 692 513 B1

Description

FIELD OF THE INVENTION

[0001] The instant invention relates generally to the field of immunology; particularly to the use of immunologic assays to diagnose abnormal or disease states and most particularly to a sandwich ELISA (enzyme-linked immunosorbent assay) assay for the quantification of a truncated glycophorin circulating in biological fluid which is diagnostic for congestive heart failure (CHF).

BACKGROUND OF THE INVENTION

[0002] The diagnosis of a given disease requires standard agreed-upon observations usually made by the attending physician of the sick patient. For some diseases, a single test is available which gives nearly definitive results sufficient for a correct diagnosis, for example, the glucose tolerance test for diabetes. However, most diseases require a number of sophisticated tests to arrive at a probable diagnosis. At the present time, therapeutic interventions are frequently initiated at late stages of disease, often resulting in only modest improvements in the quality and length of the affected patients life. Disease prevention is easier and more effective than disease therapy. Earlier diagnosis decreases disease-associated morbidities, increases the quality and length of life of the patient and decreases overall costs of health care. Thus, it is a goal of biomedical researchers to develop diagnostic tests which can correctly diagnose disease at the early stages.

[0003] Early diagnosis of congestive heart failure (CHF) is particularly beneficial since the cardiac re-structuring which occurs with progressive disease may be slowed or prevented with early therapeutic intervention. However, early diagnosis has proven elusive since symptoms generally do not appear until the heart has already suffered structural changes.

[0004] CHF is a serious condition with a high mortality rate affecting approximately five million Americans (see US 6,572,895 for a discussion of CHF). It is currently believed that CHF is not a distinct disease process in itself, but rather represents the effect of multiple abnormalities which interact together to ultimately produce the progressive loss of the ability of the heart to function as a circulatory pump. Major pathophysiologic abnormalities which occur in CHF are activation of the hypothalmic-pituitary-adrenal axis, systemic endothelial dysfunction and myocardial re-structuring. The progression of CHF can be initiated by an event such as myocardial infarction wherein the heart muscle is damaged or it can result from hypertension and/or cardiac malformations. Recently, it has been discovered that patients with certain conditions such as insulin resistance and Type II diabetes have a particularly high risk for heart failure and poor prognosis once they develop CHF (Soläng et al. European Heart Journal 20:789-795 1999).

[0005] Disease processes, such as those which occur in diabetes and CHF, often result in cellular and/or tissue damage followed by the release of cellular and/or tissue specific biopolymer markers into the bodily fluids of an individual. These biopolymer markers are harbingers of disease and/or disease progression. Association of such biopolymer markers with abnormal and/or disease states provides new diagnostic avenues which may allow identification of patients in the early stages of disease or patients at risk for developing disease. Identification of biopolymer markers diagnostic for CHF is particularly advantageous considering the progressive pathophysiology involved in CHF. What is lacking in the art is an efficient, easy to perform diagnostic method capable of identifying an individual suffering from CHF.

SUMMARY OF THE INVENTION

[0006] The instant invention provides an efficient, easy to perform diagnostic method capable of identifying an individual suffering from CHF, The method comprises a sandwich ELISA assay using mouse monoclonal antibodies recognizing a truncated abnormal form of glycophorin to quantify elevated glycophorin in biological fluids. Glycophorin is the major integral membrane protein of the mammalian red blood cell (RBC) and is highly glycosylated. The glycosylation of glycophorin is responsible for the overall negative charge of the RBC cellular surface leading to the normal electrostatic repulsion among red blood cells. In the disease processes of diabetes and CHF the red blood cell (RBC) membrane proteins, including glycophorins, are abnormally degraded, thus reducing the overall negative charge of the cellular surface leading to a decrease in the normal electrostatic repulsion among red blood cells. As a consequence, aggregation of red blood cells occurs in the pathogenesis of diabetes and CHF. Using the sandwich ELISA assay of the invention, the instant inventors identified an abnormal, circulating glycophorin in the plasma of CHF patients. This glycophorin had a lower molecular weight than that of normal glycophorin, thus it is predicted to be a truncated fragment which has been cleaved from the RBC membrane surface during the disease process.

[0007] Two mouse monoclonal antibodies are used in the ELISA assay of the instant invention; 3F4, and 6G4.

[0008] Monoclonal antibody 3F4 recognizes the extracellular portion of glycophorins A and B. The binding of the 3F4 antibody to its epitope is sugar-dependent whereas the binding of the 6G4 antibody is not. These two monoclonal antibodies are described in detail in Rasamoelisolo et al. Vox Sanguins 72 : 185-191 (1997).

[0009] Other objectives and advantages of this invention will become apparent from the following description taken in conjunction with the accompanying drawings wherein are set forth, by way of illustration and example, certain embodiments of this invention. The drawings constitute a part of this specification and include exemplary embodiments of the present invention and illustrate var-

ious objects and features thereof.

[0010] In a first aspect the invention provides method for diagnosing congestive heart failure (CHF) in a biological fluid obtained from a subject, comprising the steps of:

A) contacting a monoclonal antibody specific for a glycophorin antigen with the biological fluid under conditions such that an antibody-antigen binding complex forms between said monoclonal antibody and said glycophorin antigen present in said biological fluid, wherein said glycophorin antigen is present on a truncated abnormal form of glycophorin; and
B) detecting said antibody-antigen binding complex wherein the presence of said antibody-antigen binding complex is diagnostic for congestive heart failure (CHF).

BRIEF DESCRIPTION OF THE FIGURES

[0011]

FIGURE 1 shows the data resulting from the sandwich ELISA using monoclonal antibody 3F4.
FIGURE 2 shows the data resulting from the sandwich ELISA using monoclonal antibodies 6G4, 5F4 and 3F4.
FIGURE 3 shows the data resulting from the direct ELISA evaluating the presence of an autoantibody to glycophorin.
FIGURE 4 shows the results of immunoprecipitation of glycophorin from the plasma of CHF patients.
FIGURES 5A-C show chromatograms; FIGURE 5A shows captured glycophorin from CHF patients; FIGURE 5B shows captured glycophorin from healthy patients and FIGURE 5C shows captured purified glycophorin.
FIGURE 6 shows chromatograms after deglycosylation treatment; the top chromatograph shows purified glycophorin; the middle chromatograph shows captured glycophorin from CHF patients and the bottom chromatograph is a control run without a glycophorin sample.

DEFINITIONS

[0012] The following list defines terms, phrases and abbreviations used throughout the instant specification. Although the terms, phrases and abbreviations are listed in the singular tense the definitions are intended to encompass all grammatical forms.

[0013] As used herein, the abbreviation "CHF" refers to congestive heart failure.

[0014] As used herein, the abbreviation "GP" refers to glycophorin.

[0015] As used herein, the abbreviation "GPA" refers to glycophorin A.

[0016] As used herein, the abbreviation "GPB" refers to glycophorin B.

[0017] As used herein, the abbreviation "GPAx2" refers to the dimerized form of glycophorin A.

[0018] As used herein, the abbreviation "GPBx2" refers to the dimerized form of glycophorin B.

[0019] As used herein, the abbreviation "ELISA" refers to enzyme-linked immumosorbent assay.

[0020] As used herein, the abbreviation "RBC" refers to red blood cell.

[0021] As used herein, the abbreviation "MoAb" refers to monoclonal antibody.

[0022] As used herein, the abbreviation "MS" refers to mass spectrometry.

[0023] As used herein, the abbreviation "SELDI" refers to a mass spectrometric technique; surface enhanced laser desorption ionization.

[0024] As used herein, the abbreviation "PBS" refers to phosphate buffered saline.

[0025] The terms "RBC", "red blood cell" and "erythrocyte" are used interchangeably herein.

[0026] As used herein, the term "glycophorin" refers to the major integral glycoprotein of the mammalian erythrocyte membrane. Glycophorin is highly glycosylated and occurs in isoforms A and B(see Concise Encyclopedia: Biochemistry and Molecular Biology, Third Edition, Revised and Expanded by Thomas A. Scott and E. Ian Mercer, Walter de Gruyter, Berlin-New York 1997, pages 201-202 and Instant Notes: BioChemistry, 2nd edition, B.D. Hames and N.M. Hooper, Springer-Verlag New York 2000, pages 125, 126 and 130 for an introduction to the RBC membrane and glycophorins).

[0027] As used herein, the term "circulating, truncated glycophorin" refers to the abnormal glycophorin fragment identified by the assay of the instant invention in the serum of CHF patients. The 3F4 mouse anti-glycophorin monoclonal antibody which recognizes the extracellular portion of glycophorin A and B binds to this circulating, truncated glycophorin. This circulating, truncated glycophorin is structurally different from the normal soluble glycophorin and is theorized to be a fragment cleaved from the RBC surface during disease processes.

[0028] As used herein, the term "biological fluid" refers to any bodily fluid. Illustrative, albeit non-limiting examples are blood, blood products, urine, saliva, cerebrospinal fluid and lymphatic fluid.

[0029] As used herein, the term "subject" refers to any mammalian organism. A particularly preferred subject is a human.

[0030] As used herein, the term "corresponding" is used generally with reference to antibody-antigen binding complexes, for example, an antibody corresponding to an antigen will bind to the antigen under physiologic conditions. The bound antibody-antigen is referred to as an antibody-antigen binding complex,

[0031] As used herein, the term "signal generating substance" refers to any material which undergoes a measurable reaction. Illustrative, albeit non-limiting examples are fluorophorea, enzymes and radioisotopes. A particularly preferred signal generating substance is per-

oxidase, the use of which is illustrated in the examples herein.

[0032] As used herein, the term "congestive heart failure" refers to a progressive, debilitating condition wherein the heart loses its ability to function as a circulatory pump.

[0033] As used herein, the term "antibody" refers to a protein secreted by B lymphocytes capable of binding specific molecules under physiologic conditions.

[0034] As used herein, the term "monoclonal antibody" refers to an antibody having single epitope specificity.

[0035] As used herein, the term "polyclonal antibody" refers to an antibody capable of binding with multiple epitopes.

[0036] As used herein, the term "antigen" broadly refers to any substance which induces an immune reaction; more particularly the term "antigen" refers to the corresponding binding partner of an antibody.

[0037] As used herein, the term "auto-antibody" refers to an antibody which recognizes self antigens, for example, antibodies produced by an organism which bind the organism's own proteins are referred to as auto-antibodies.

[0038] Specific antibodies can be used to quantify the amount of corresponding antigen in a biological sample. As used herein, the term "ELISA" refers to an enzyme-linked immunosorbent assay which can quickly detect and quantify minute amounts (less than a nanogram) of antigen in a biological sample. The test antibody is bound to an inert polymer support, such as a plastic tray with molded wells, and then exposed to the biological sample. Unbound proteins are washed away and a second antibody that reacts with the antigen at a different epitope than the test antibody reacts with is added. This second antibody has an enzyme attached to it that converts a colorless or nonfluorescent substrate into a colored or fluorescent product. The amount of second antibody bound, and hence the amount of protein antigen present in the original biological sample, is determined by the quantification of the intensity of color or fluorescence produced. This ELISA assay is also referred to as an "indirect ELISA" or a "sandwich ELISA". (see Instant Notes: Biochemistry, 2nd edition, B.D. Hames and N.M. Hooper, Springer-Verlag New York 2000, pages 112-114 for an introduction to the general principles of ELISA assays). There is also a form of ELISA assay that is referred to as "direct" wherein the antigen is bound to an inert polymer support and exposed to a biological sample containing the corresponding antibody.

DETAILED DESCRIPTION OF THE INVENTION

[0039] As a result of disease processes, damage to cells and tissues of the body occurs at the cellular and subcellular levels. Initially, these processes may only cause damage to the outer membranes of cells, causing a sloughing off of portions of the exterior cellular matrices, which process is broadly defined as reversible damage. As the length of time and/or the severity of the disease condition increases, the outer membranes begin to break down, resulting in membrane rupture followed by the release of intra-cellular components, which process is broadly defined as irreversible damage. When such damage occurs (reversible or irreversible), biopolymer markers are released into the circulation, causing the immune system to become activated, since these biopolymer markers are not normally present in the bodily fluids. The immune system views these biopolymer markers as invading pathogens or foreign bodies whose threat must be neutralized. In an effort to persevere against this perceived threat, auto-antibodies are formed to these biopolymer markers. These auto-antibodies can be characterized as sequela which are indicative of the original damaging insult to the organism. The presence of the auto-antibodies validates the theory that cellular damage acts as an initiator of an immune response leading to a cascade of auto-antibody production which ultimately manifests itself in a characteristic and often predictable disease state. The appearance of these biopolymer markers and their associated auto-antibodies are harbingers of disease and/or disease progression and are useful for diagnostic purposes.

[0040] Damage to the red blood cell membrane is known to occur in disease processes such as diabetes and CHF. In these diseases there is an increase in enzyme production and/or activation (neutrophil proteases, metalloproteases, sialidases and endopeptidases)that directly and/or indirectly leads to abnormal degradation of red blood cell membrane proteins (Gaczyńska et al. Cytobios 75:7-11 1993; Venerando et al. Blood 99(3) : 1064-1070 2002; Wegner et al. Cardiovascular Research 31:891-898 1996; Piwowar et al. Clinical Chemistry Lab Medicine 38(12):1257-1261 2000 and Santos-Silva et al. Clinica Chimica Acta 320:29-35 2002).

[0041] Additionally, it is well-documented that erythrocyte (RBC) aggregability is increased in diabetes and in vascular atherosclerotic disease (Caimi et al. Thromb Haemost 83:516-517 2000; Demiroglu et al. Experimental Clinical Endocrinol Diabetes 107(1):35-39 1999; Martinez et al. Clinical Hemorheology and Microcirculation 18:253-258 1998 and Ziegler et al. Metabolism 43(9) : 1182-1186 1994). Alterations in RBC membrane phospholipids are associated with RBC aggregability (Martínez et al. Clinical Hemorheology and Microcirculation 18: 253-258 1998). Sphingomyelin is the main phospholipid of the outer membrane and has been shown to contain a greater percentage of saturated fatty acids in diabetic patients than in non-diabetic patients. This increase in saturation is thought to reduce electrostatic repulsion between red blood cells, which in turn increases their aggregability.

[0042] Loss of glycophorins further reduces the electrostatic repulsion of red blood cells. Glycophorin is the major RBC integral membrane glycoprotein. The high sialylation of glycophorin is responsible for the negative surface charge which leads to the normal electrostatic

repulsion between red blood cells (Eylar et al. The Journal of Biological Chemistry 237(6):1992-2000 1962). The increase in enzyme production and/or enzyme activation in disease processes such as diabetes results in the loss of glycophorins from the RBC membrane. These glycophorin fragments are released into the bodily fluids where they stimulate the production of auto-antibodies. The decrease in glycophorin in turn leads to a decrease in the normal negative charge of the RBC membrane surface and thus decreases the overall electrostatic repulsion between blood cells. Loss of the electrostatic repulsion between red blood cells results with the aggregation of red blood cells seen in diabetes.

[0043] Without being bound by any particular theory, the instant inventors propose that the circulating, truncated glycophorin identified in the plasma of CHF patients using the sandwich ELISA assay described herein is an extracellular glycophorin fragment which has been cleaved from the RBC membrane during the disease process. This circulating, truncated glycophorin is structurally different from the normal soluble form of glycophorin. The mouse anti-glycophorin 3F4 monoclonal antibody also recognizes the circulating, truncated glycophorin. The instant inventors have also shown by direct ELISA assay that CHF patients show an increase in anti-glycophorin auto-antibodies. Thus, it is concluded that this circulating, truncated glycophorin can be used as a new biopolymer marker for CHF diagnosis.

EXPERIMENTAL PROCEDURES

ANTIBODIES

[0044] The mouse anti-glycophorin monoclonal antibodies used in the following experiments were purchased from BioAtlantic. Monoclonal antibody 6G4 recognizes amino acid residues 39-45 of glycophorin A. Monoclonal antibody 5F4 recognizes the intracellular portion of glycophorin A comprising amino acid residues 107-119 . Monoclonal antibody 3F4 recognizes the extracellular portion of glycophorins A and B amino acid residues 5-25. The binding of the 3F4 antibody to its epitope is sugar-dependent whereas the binding of the 6G4 antibody is not. These monoclonal antibodies are described in detail in Rasamoelisolo et al. Vox Sanguinis 72:185-191 1997.

[0045] The mouse anti-glycophorin 3F4 monoclonal antibody was deposited with the American Type Culture Collection (ATCC) on April 23, 2000 as hybridoma NaM26-3F4D11A2 under Accession number PTA-5154. The American Type Culture Collection (ATCC) is located at 10801 University Boulevard, Manassas, Virginia 20110-2209.

QUANTIFICATION OF GLYCOPHORIN BY SANDWICH ELISA

[0046] One ug of each MOAb in 100μl of 50mM carbonate pH 9.4 was coated on ELISA plates (Nuc, Denmark) and set overnight at +4˚C. Plates were then washed 3 times with o.01M phosphate buffer 150mM NaCl pH 7,4 (PBS) purchased from Sigma containing 0.05% Tween 20 (PBST). Plates were then blocked with 200μl of PBST containing 0.5% BSA (Sigma) for 30 minutes at 37˚C. 100μl of CHF patient plasma (PRAISE 2 study) and healthy control plasma (Intergen) diluted 1/10 in PBST were then added per well in duplicate and incubated for 2 hours at room temperature. After 3 washes with PBST, 100μl of rabbit polyclonal anti-glycophorin A+B (BioAtlantic) were added and incubated for 1 hour at room temperature followed by the addition of 100μl of peroxidase labeled donkey polyclonal anti-rabbit IgG (H+L) diluted 1/50,000 in PBST containing 0.5% BSA (Jackson ImmunoResearch). The presence of the captured glycophorins is detected by adding 100μl of TMB Tetramethyl Benzidine (Moss, Inc.). The reaction was stopped with 50μl of 1N H_2SO_4. Plates were then read at 450nm on the BioRad microplate reader.

[0047] Figure 1 shows the result of the sandwich ELISA using the 3F4 monoclonal antibody. The absorbance at 450 nm is shown on the Y axis. Glycophorin captured from the plasma of CHF patients is shown on the left and the glycophorin captured from normal plasma (control, n=36) is shown on the right. The signal is significantly higher in CHF plasma than in controls (p<.001) calculated by an independent *t*- test indicating a higher amount of glycophorins in CHF plasma samples. The 3F4 MoAb recognizes the common sequence on both glycophorins A and B (amino acid residues 5-25) . This binding is sugar-dependent since this fragment of glycophorin is highly glycosylated.

[0048] In order to ascertain whether the assay is specific to the extracellular polypeptide of glycophorin or the oligosaccharide chains, the MoAbs 6G4 (recognizes amino acid residues 39-45 of glycophorin A and 5F4 (recognizes amino acid residues 112-129 of glycophorin A) were used. Both bind to the glycophorin A backbone independently of the sugar chains.

[0049] Eight CHF samples having the most elevated amount of glycophorin and 8 normal plasma samples having the'lowest amount of glycophorin were analyzed and the result is shown in Figure 2. Figure 2 shows results from sandwich ELISA assays comparing the glycophorin captured in plasma from CH2 patients and the glycophorin captured in normal control plasma (n=8). The top panel shows results using the 6G4 MoAb (p=o.001); the middle panel shows results using the 5F4 MoAb (p=0.36) and the bottom panel shows the results using the 3F4 MoAb (p=0.003). The Y axis represents the absorbance read at 450nm. Glycophorin captured from the plasma of CHF patients is shown on the left and the glycophorin captured from normal plasma is shown on the right in all three panels. The result shows that 6G4 detects elevated amount of glycophorin in CHF samples, while 5F4 shows no significant difference between both CHF and normal human plasma. This result indicates that glycophorin

may be cleaved from the red blood cell membrane during the progression of CHF since the fragments recognized by the antibodies are extracellular fragments. However, it is noted that a soluble form of glycophorin is present in normal as well as CHF patient plasma that is detected by the 5F4 monoclonal anti-intracellular domain of glycophorin.

DETECTION OF AUTO-ANTIBODY BY DIRECT ELISA

[0050] 0.5ug of purified glycophorin from blood group MM or asialoglycophorins from blood group MN (Sigma) in 50mM carbonate buffer pH 9.4 was adsorbed onto ELISA plates overnight at +4˚C. Plates washed 3 times with 0.01M Phosphate buffer 150mM NaCl pH 7,4 (PBS) from Sigma containing 0.05% Tween 20 (PBST). Plates were then blocked with 200µl of PBST containing 0.5% BSA (Sigma) for 30 minutes at 37˚C. 100µl of CHF plasma (PRAISE 2 study) and normal control plasma (Intergen) diluted 1/100 in PBST were then added per well in duplicate and incubated for 2 hours at room temperature. After 3 washes with PBST, 100µl of peroxidase labeled goat polyclonal anti-human IgG (H+L) diluted 1/10,000 in PBST (Jackson ImmunoResearch) were added. The presence of auto-antibody anti-glycophorins was detected by adding 100µl of TMB (Moss, Inc.) and the reaction was stopped with 50µl of 1N H_2SO_4. Plates were read at 450 nm on the BioRad microplate reader.

[0051] Glycophorin is known to be highly immunogenic due to the presence of a high amount of sugar chains. Once found in plasma it may induce an immune response generating anti-glycophorin auto-antibody.

[0052] To demonstrate the presence of CHF-induced auto-antibody against glycophorin, glycophorins from blood group MM and asialo glycophorins from blood group MN were coated on ELISA plates and plasma from healthy donors or from CHF patients were added. Figure 3 shows the results of the direct ELISA assay evaluating the presence of a CHF-induced auto-antibody in the plasma of normal and CHF patients (n=36). In the top panel, glycophorin from blood group MN was coated on the plate (p=0.01) and the bottom panel, desialylated glycophorin from blood group MN was coated on the plate (p=0.03). The Y axis represents the absorbance read at 450nm. Figure 3 shows the presence of auto-antibodies in CHF; independent to the blood group (M or N) and the heavy sialic acids on glycophorin.

IDENTIFICATION OF GLYCOPHORINS IN CHF PLASMA BY IMMUNOPRECIPITATION AND DETECTION BY IMMUNOBLOTTING

[0053] 1.2ml of pooled CHF plasma from the PRAISE 2 study was diluted v/v with PBS containing 0.5% Triton X-100. Then 2µl of 3F4 MoAb at 1.7 mg/ml were added. After overnight incubation at +4˚C, 25 µl of goat IgG anti-mouse IgG (H+L) coupled to SEPHAROSE-4B beads (Zymed) were added. The mixture was incubated for 5 hours at +4˚C and then the beads were washed 3 times with PBS containing 0.05% Tween 20. The captured (glyco)protein was eluted with 100µl of 0.1M glycine pH 2.5 then neutralized with 1M Tris pH 11. The eluate was concentrated on CentriVap Concentrator (Labconco), resuspended in 50µl of SDS-PAGE sample buffer, boiled 5 minutes at 100˚C and then loaded on 10% SDS-PAGE gel. At the end of the electrophoresis, proteins were transferred onto a nitrocellulose membrane and stained with 3F4 MoAb anti-GPA+B followed by a peroxidase labeled goat polyclonal anti-mouse IgG (H+L) diluted 1/50,000 in PBST (Jackson ImmunoResearch). The immunoblot was then developed using ECL (Amersham Pharmacia). To control the cross-reactivity of the secondary antibody to the 3F4 eluted from the column, the blot was incubated with the secondary antibody alone.

[0054] The molecules captured by 3F4-column were eluted and loaded on 10% SDS-PAGE gel and assessed on immunoblotting against the same MoAb. As shown in figure 4, the glycophorins found in CHF plasma have a molecular weight of 75, 45 and 40 kDa (lane 2, blot incubated with 3F4). Usually glycophorins run at 80 - 70 - 40 - 37 and 20kDa as dimer form of GPA, dimer GPA/GPB, dimer form of GPB, monomer form of GPA and monomer form of GPB, respectively as shown on lane 1 loaded with normal glycophorin purified from normal red blood cell membrane. Thus, the glycophorins found in the plasma of CHF patients have different molecular weights as compared to the normal glycophorin purified from RBC membranes. The immunoblot was incubated with the secondary antibody alone (control) or with the 3F4 antibody and then the secondary antibody. Lane 1 (in both blots) shows glycophorin purified from RBC membranes and Lane 2 (both blots) shows glycophorin from CHF patient plasma. Protein markers from 25 to 200 kDaltons are shown on the far left.

[0055] The IgG identified in control and 3F4 blots is the mouse monoclonal 3F4 used for the immunoprecipitation and released from the column. A band with a high MW > 200kDa is also detected. The instant inventors are not sure about the nature of this band. The band may be a complex form of IgM or IgG autoantibodies and the glycophorins.

IDENTIFICATION OF GLYCOPHORIN IN CHF PATIENT SAMPLE BY SELDI-TOF

[0056] The method of the instant invention can be carried out using the techniques of mass spectrometry. The PS20 chip (Ciphergen) was washed with pure Acetonitrile-190 (ACN) (Caledon) and allowed to air dry. 50 µg of Protein G (Pierce) was dissolved in 50µl UF water and 1ul was loaded to each spot containing 1µl of ACN. The mixture was incubated 1 hour in a humidity chamber and then the spot was blocked with 30µl of 0.5M Tris-HCl pH 7.4 (Caledon) for 15 minutes. The chip was then washed with UF water and allowed to air dry. Monoclonal antibody (MoAb) anti-GPA+GPB, the 3F4 at 1.7mg/ml

(BioAtlantic) was diluted 1/3 in PBS containing 0.1% TRITON X (Sigma) and 3μl of the MoAb solution was loaded per spot and incubated for 1 hour in a humidity chamber. Unbound MoAb was washed away from the chip by washing with PBS.

[0057] Purified glycophorin (Sigma), CHF plasma from PRAISE 2 study or normal plasma (Intergen) was added to the 3F4-coated chip as follows:

[0058] The glycophorin at 1mg/ml was diluted 1/5 in PBS; CHF and normal plasma samples were diluted 1/5 in PBS containing 0.05% Tween 20, and 2μl of each were loaded per spot. The chip was then incubated for 1 hour in a humidity chamber and washed twice with UF water.

[0059] The captured glycophorin was then treated with Endoproteinase GluC (Roche Diagnostics). For that, the GluC powder was dissolved in 50μl of UF water and a 1/10 dilution in 50mM Ammonium Carbonate pH 7.8 (BDH Laboratory Supplies) was prepared. 1μl of the GluC solution was added to each spot and incubated 2 hours in a humidity chamber. The spot was then allowed to dry and was either treated using Calbiochem deglycosylation kit or directly analyzed on SELDI after adding 1μl of saturated sinapinic acid (sigma) in 0.5% TFA 50% ACN. The chip was then read on SELDI (Ciphergen) at a Sensitivity=10, Intensity=180-190, range of 0-5000 Da (optimized for 0-5000 Da).

[0060] The (glyco)protein captured on the 3F4 chip was treated by GluC. Figure 5A shows data resulting from the on-chip treatment of the captured glycophorin from CHF. Figure. 5B shows data resulting from the on-chip treatment of the normal plasma samples. Figure 5C shows data resulting from the on-chip treatment of purified glycophorin. As shown in Figures 5A-C, a (glyco) peptide with a m/z of 2361+H is found in both CHF and glycophorin demonstrating that the (glyco)protein captured from CHF corresponds probably to the glycophorin. It is interesting to note that the chromatograms (Figures 5A-C) obtained from the purified glycoprhorin and the one from CHF plasma were not overlapped. This is due to the fact that the structure of the glycophorin in CHF is maybe slightly modified.

[0061] To further prove that the captured (glyco)protein is related to glycophorin, the captured (glyco)protein was deglycosylated on chip. Figure 6 shows on-chip deglycosylation treatment of the glycopeptides captured from either purified glycophorin or CHF plasma using the 3F4 monoclonal antibody coated on a PS20 chip. As shown in figure 6, at least 8 major peaks now matched to the peaks generated from the standard glycophorin. Also, it is noted that a lot more peaks were detected, they correspond not only to the peptides but also to the sugar chains released after the deglycosylation treatment.

[0062] In conclusion, the instant invention provides a sandwich ELISA assay for quantification of a truncated, glycophorin circulating in biological fluid which is diagnostic for CHF. It is important to note that glycophorin has not been previously recognized as a marker for congestive heart failure (CHF). The instant inventors are the first to document glycophorin as a marker for CHF and the assay described herein provides an efficient, easy to perform diagnostic method capable of identifying an individual suffering from CHF.

[0063] All patents and publications mentioned in this specification are indicative of the levels of those skilled in the art to which the instant invention pertains.

[0064] It is to be understood that while a certain form of the invention is illustrated, it is not to be limited to the specific form or arrangement of parts herein described and shown. It will be apparent to those skilled in the art that various changes may be made without departing from the scope of the invention and the invention is not to be considered limited to what is shown and described in the specification.

Claims

1. A method for diagnosing congestive heart failure (CHF) in a biological fluid obtained from a subject, comprising the steps of:

   A) contacting a monoclonal antibody specific for a glycophorin antigen with the biological fluid under conditions such that an antibody-antigen binding complex forms between said monoclonal antibody and said glycophorin antigen present in said biological fluid, wherein said glycophorin antigen is present on a truncated abnormal form of glycophorin; and
   B) detecting said antibody-antigen binding complex wherein the presence of said antibody-antigen binding complex is diagnostic for congestive heart failure (CHF).

2. The method in accordance with claim 1, wherein said biological fluid is selected from the group consisting of blood, blood products, urine, saliva, cerebrospinal fluid and lymphatic fluid.

3. The method in accordance with claim 1, wherein said monoclonal antibody is 3F4 and recognizes amino acid residues 5-25 of human glycophorin A and human glycophorin B.

4. The method in accordance with claim 1, wherein said monoclonal antibody is 6G4 and recognizes amino acid residues 39-45 of human glycophorin A.

5. The method in accordance with claim 1, wherein said detecting comprises the steps of:

   A)contacting said antibody-antigen binding complex with a polyclonal antibody corresponding to said glycophorin antigen under conditions such that a complex forms between said glyco-

phorin antigen and said polyclonal antibody;
B)attaching a label to a polyclonal antibody corresponding to the polyclonal antibody of step A;
C) contacting the complex formed in step A with the labeled polyclonal antibody formed in step B under conditions such that a complex forms between said labeled polyclonal antibody and said polyclonal antibody of step A; and
D) detecting the label on said labeled polyclonal antibody.

6. The method in accordance with claim 5, wherein the label on said labeled polyclonal antibody comprises a signal generating substance.

7. The method in accordance with claim 6, wherein said signal generating substance is peroxidase.

8. Use of a monoclonal antibody specific for a glycophorin antigen, wherein said glycophorin antigen is present on a truncated abnormal form of glycophorin, in a diagnostic method to quantify elevated levels of a truncated glycophorin in biological fluid obtained from a subject with CHF.

Patentansprüche

1. Verfahren zum Diagnostizieren von kongestiver Herzinsuffizienz (CHF) anhand eines einem Patienten entnommenen biologischen Fluids, das die folgenden Schritte beinhaltet:

A) Inkontaktbringen eines für ein Glycophorin-Antigen spezifischen monoklonalen Antikörpers mit dem biologischen Fluid unter solchen Bedingungen, dass ein Antikörper-Antigen-Bindungskomplex zwischen dem genannten monoklonalen Antikörper und dem genannten, in dem genannten biologischen Fluid vorhandenen Glycophorin-Antigen entsteht, wobei das genannte Glycophorin-Antigen auf einer verkürzten abnormalen Form von Glycophorin vorhanden ist; und
B) Nachweisen des genannten Antikörper-Antigen-Bindungskomplexes, wobei anhand der Anwesenheit des genannten Antikörper-Antigen-Bindungskomplexes kongestive Herzinsuffizienz (CHF) diagnostiziert wird.

2. Verfahren nach Anspruch 1, wobei das genannte biologische Fluid ausgewählt wird aus der Gruppe bestehend aus Blut, Blutprodukten, Urin, Speichel, Cerebrospinalflüssigkeit und Lymphflüssigkeit.

3. Verfahren nach Anspruch 1, wobei der genannte monoklonale Antikörper 3F4 ist und Aminosäurereste 5-25 von humanem Glycophorin A und humanem Glycophorin B erkennt.

4. Verfahren nach Anspruch 1, wobei der genannte monoklonale Antikörper 6G4 ist und Aminosäurereste 39-45 von humanem Glycophorin A erkennt.

5. Verfahren nach Anspruch 1, wobei das genannte Nachweisen die folgenden Schritte beinhaltet:

A) Inkontaktbringen des genannten Antikörper-Antigen-Bindungskomplexes mit einem polyklonalen Antikörper, der dem genannten Glycophorin-Antigen unter solchen Bedingungen entspricht, dass ein Komplex zwischen dem genannten Glycophorin-Antigen und dem genannten polyklonalen Antikörper entsteht;
B) Anbringen einer Markierung an einen polyklonalen Antikörper, der dem polyklonalen Antikörper von Schritt A entspricht;
C) Inkontaktbringen des in Schritt A gebildeten Komplexes mit dem in Schritt B gebildeten markierten polyklonalen Antikörper unter solchen Bedingungen, dass ein Komplex zwischen dem genannten markierten polyklonalen Antikörper und dem genannten polyklonalen Antikörper von Schritt A entsteht; und
D) Nachweisen der Markierung an dem genannten markierten polyklonalen Antikörper.

6. Verfahren nach Anspruch 5, wobei die Markierung an dem genannten markierten polyklonalen Antikörper eine Signalerzeugungssubstanz umfasst.

7. Verfahren nach Anspruch 6, wobei die genannte Signalerzeugungssubstanz Peroxidase ist.

8. Verwendung eines für ein Glycophorin-Antigen spezifischen monoklonalen Antikörpers, wobei das genannte Glycophorin-Antigen auf einer verkürzten abnormalen Form von Glycophorin vorliegt, in einem Diagnoseverfahren zum Quantifizieren erhöhter Werte eines verkürzten Glycophorins in biologischem Fluid, das einem Patienten mit CHF entnommen wurde.

Revendications

1. Procédé pour diagnostiquer une insuffisance cardiaque congestive (ICC) dans un liquide biologique prélevé d'un sujet, comprenant les étapes consistant à :

A) mettre en contact un anticorps monoclonal spécifique pour un antigène glycophorine avec le liquide biologique dans des conditions telles qu'un complexe antigène-anticorps se forme entre ledit anticorps monoclonal et ledit antigène

glycophorine présents dans ledit liquide biologique, ledit antigène glycophorine étant présent sur une forme anormale tronquée de glycophorine ; et à

B) détecter ledit complexe anticorps-antigène, la présence dudit complexe anticorps-antigène diagnostiquant une insuffisance cardiaque congestive (ICC).

2. Procédé selon la revendication 1, dans lequel ledit liquide biologique est sélectionné parmi le groupe comprenant le sang, les produits sanguins, l'urine, la salive, le liquide céphalorachidien et le liquide lymphatique.

3. Procédé selon la revendication 1, dans lequel ledit anticorps monoclonal est 3F4 et reconnaît les résidus d'acides aminés 5-25 de la glycophorine humaine A et.

4. Procédé selon la revendication 1, dans lequel ledit anticorps monoclonal est 6G4 et reconnaît les résidus d'acides aminés 39-45 de la glycophorine humaine A.

5. Procédé selon la revendication 1, dans lequel ladite détection comprend les étapes consistant à :

A) mettre en contact ledit complexe anticorps-antigène avec un anticorps polyclonal correspondant audit antigène glycophorine dans des conditions telles qu'un complexe se forme entre ledit antigène glycophorine et ledit corps polyclonal ;

B) attacher un traceur à un anticorps polyclonal correspondant à l'anticorps polyclonal de l'étape A ;

C) mettre en contact le complexe formé lors de l'étape A avec l'anticorps polyclonal marqué formé lors de l'étape B dans des conditions telles qu'un complexe se forme entre ledit anticorps polyclonal marqué et ledit anticorps polyclonal de l'étape A ; et à

D) détecter le traceur sur ledit anticorps polyclonal marqué.

6. Procédé selon la revendication 5, dans lequel le traceur sur ledit anticorps polyclonal marqué comprend une substance générant un signal.

7. Procédé selon la revendication 6, dans lequel ladite substance générant un signal est la peroxydase.

8. Utilisation d'un anticorps monoclonal spécifique pour un antigène glycophorine, ledit antigène glucophorine étant présent sur une forme anormale tronquée de glycophorine, dans un procédé diagnostique pour quantifier des niveaux élevés d'une glycophorine tronquée dans un liquide biologique prélevé sur un sujet atteint d'une ICC.

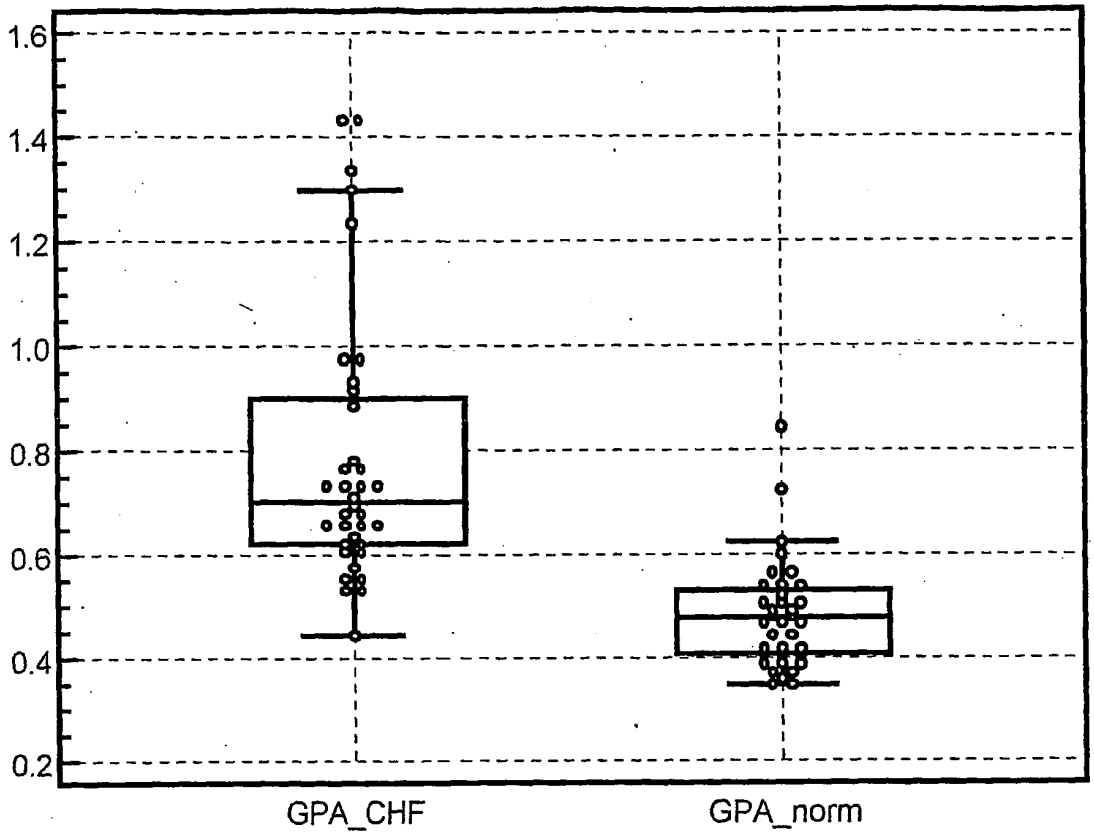

Figure 1

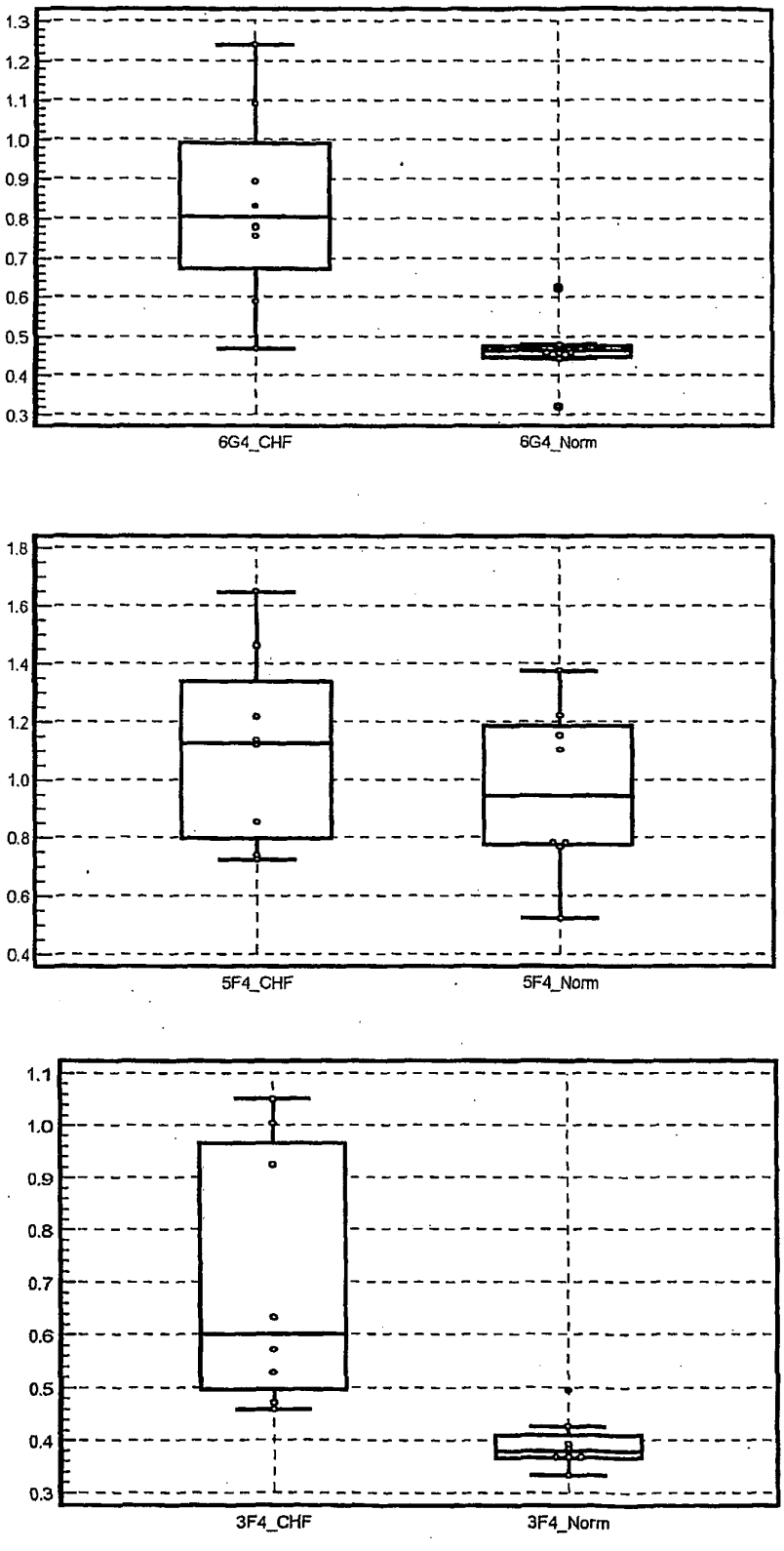
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
6G4_CHF
6G4_Norm
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
5F4_CHF
5F4_Norm
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
3F4_CHF
3F4_Norm

Figure 2

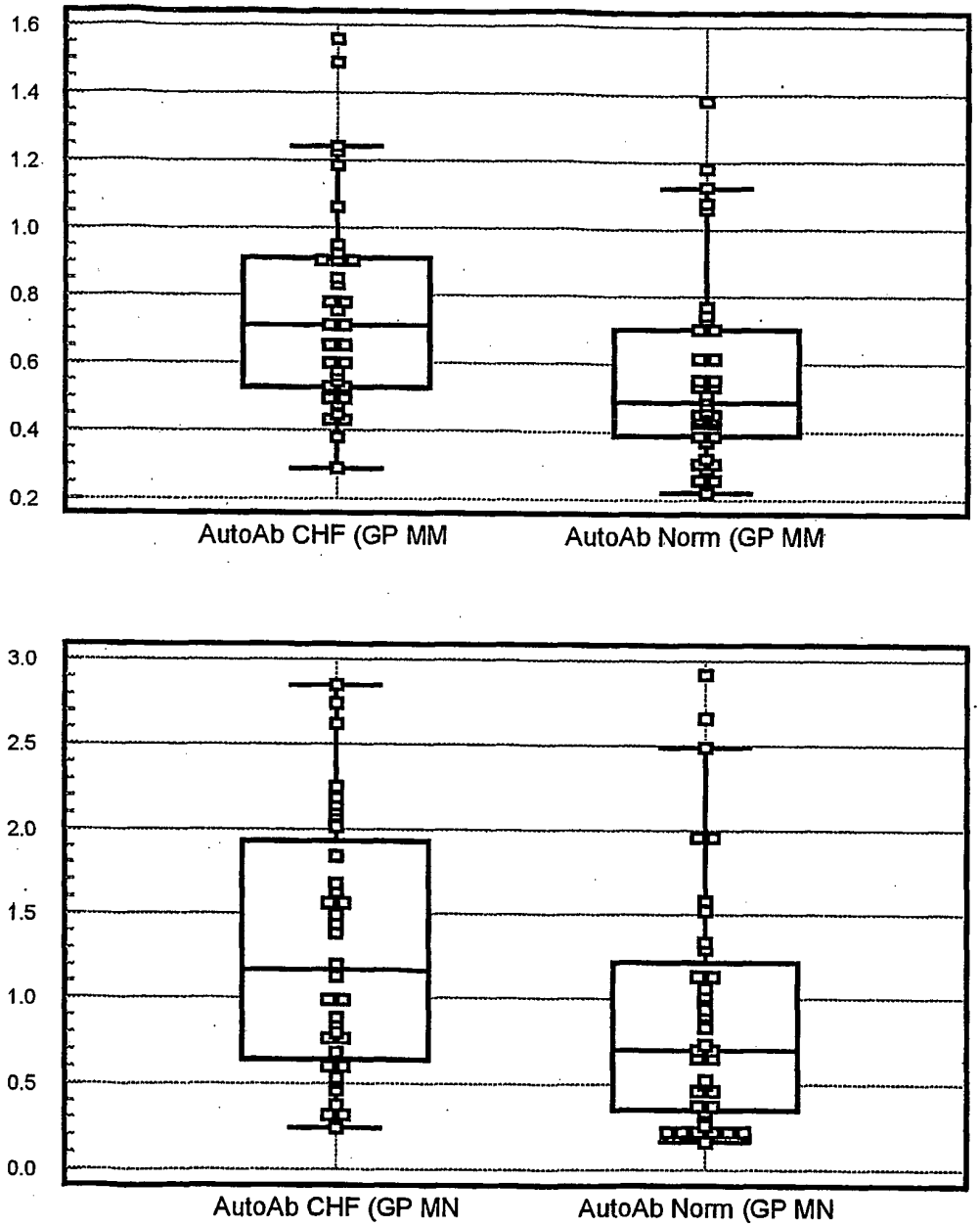
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
AutoAb CHF (GP MM
AutoAb Norm (GP MM
3.0
2.5
2.0
1.5
1.0
0.5
0.0
AutoAb CHF (GP MN
AutoAb Norm (GP MN

Figure 3

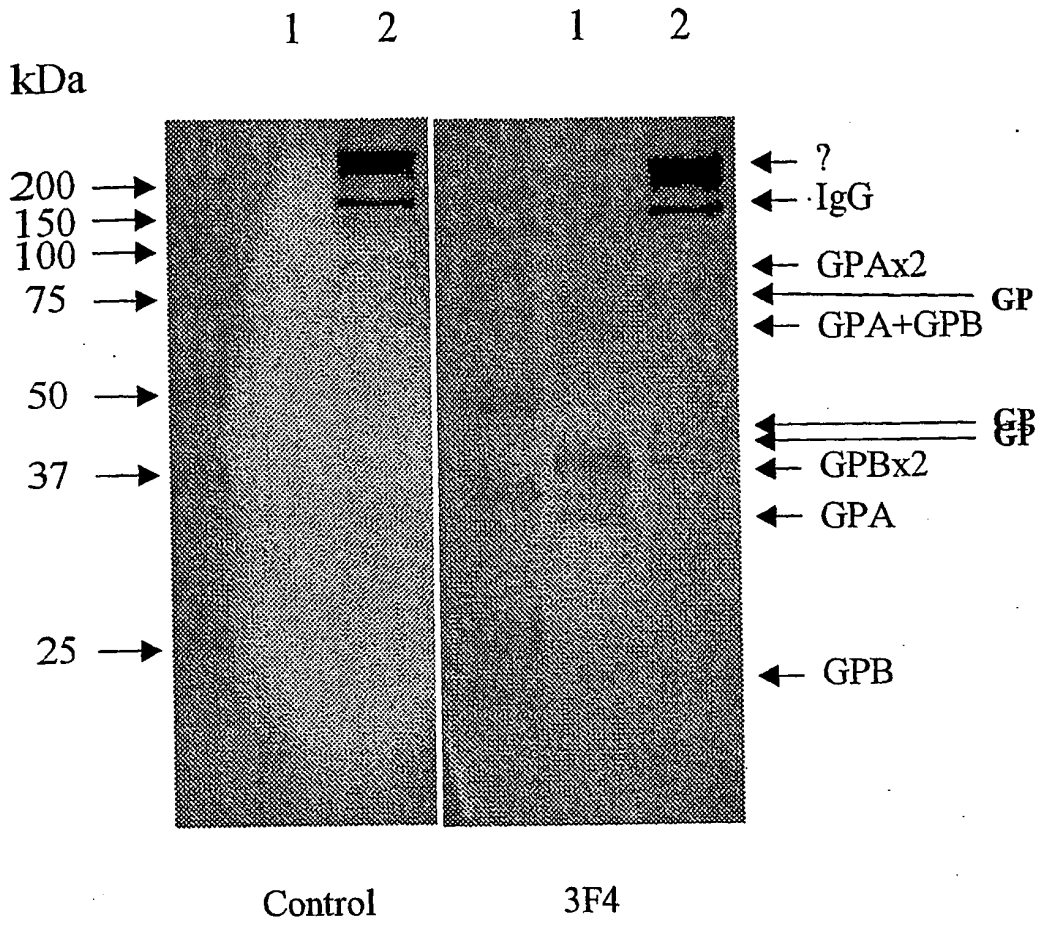
1
2
1
2
kDa
200
150
100
75
50
37
25
?
IgG
GPAx2
GP
GPA+GPB
GP
GP
GPBx2
GPA
GPB
Control
3F4

Figure 4

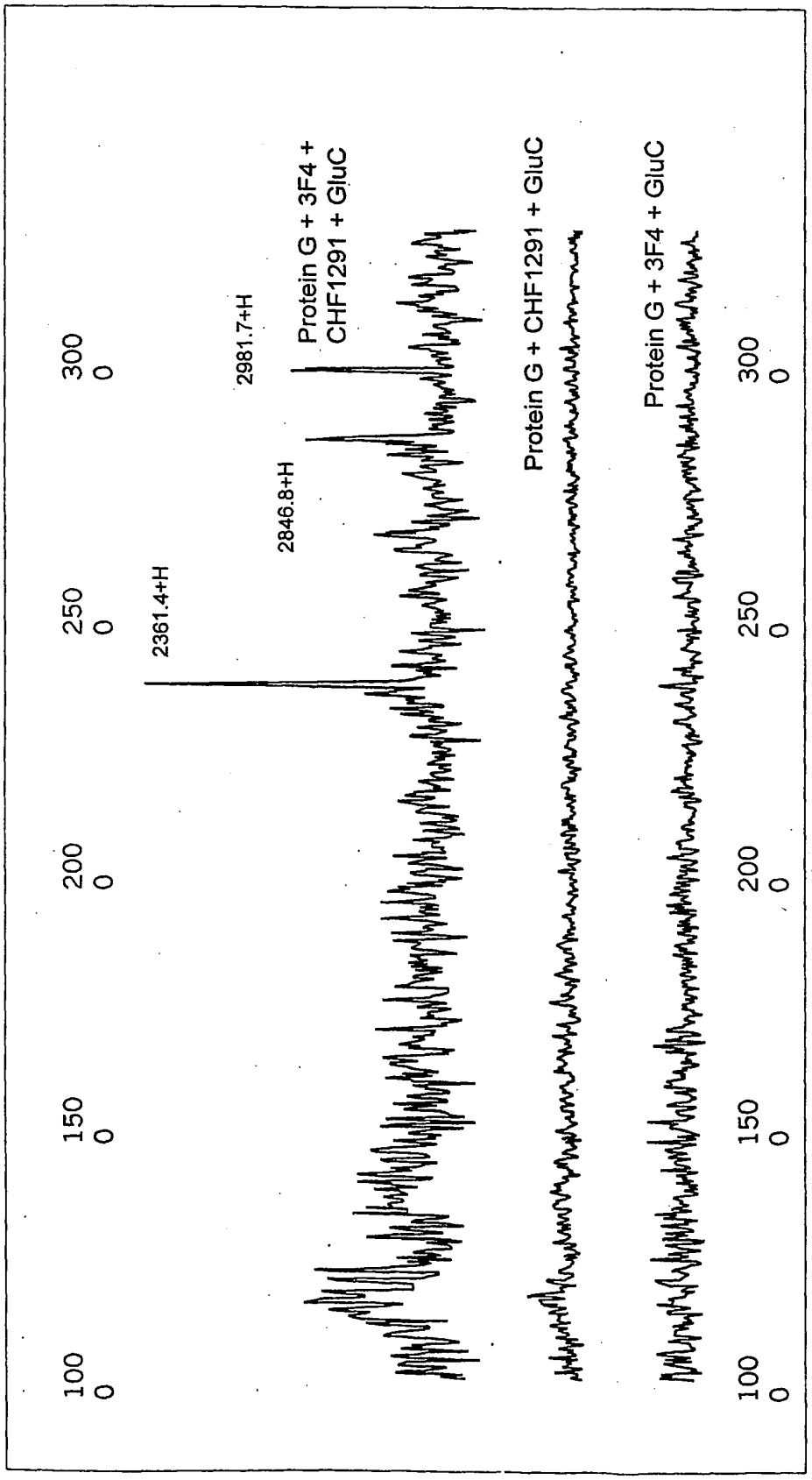
2361.4+H
2846.8+H
2981.7+H
Protein G + 3F4 + CHF1291 + GluC
Protein G + CHF1291 + GluC
Protein G + 3F4 + GluC
100
150
200
250
300

Figure 5A

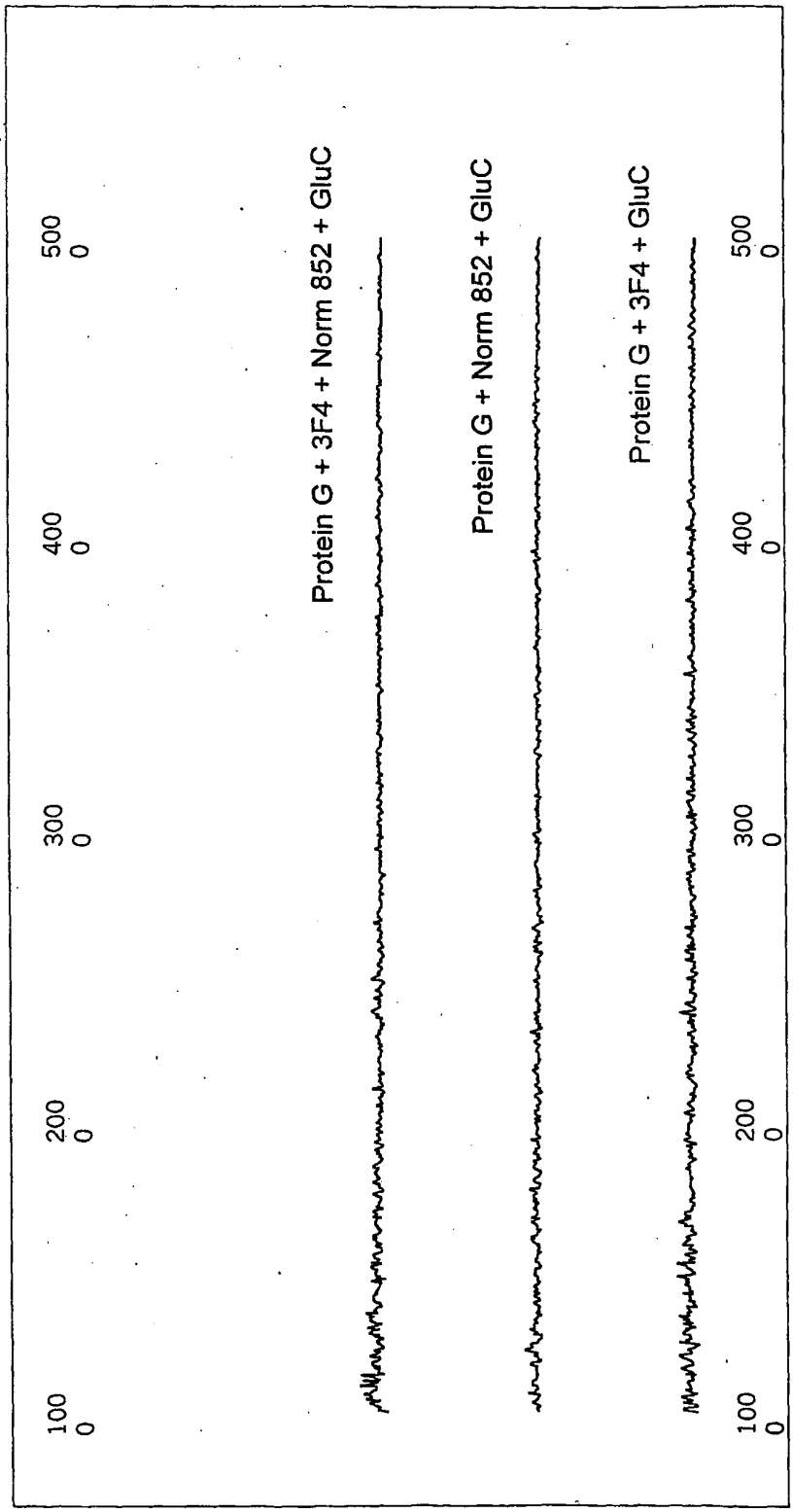
Protein G + 3F4 + Norm 852 + GluC
Protein G + Norm 852 + GluC
Protein G + 3F4 + GluC
100
200
300
400
500

Figure 5B

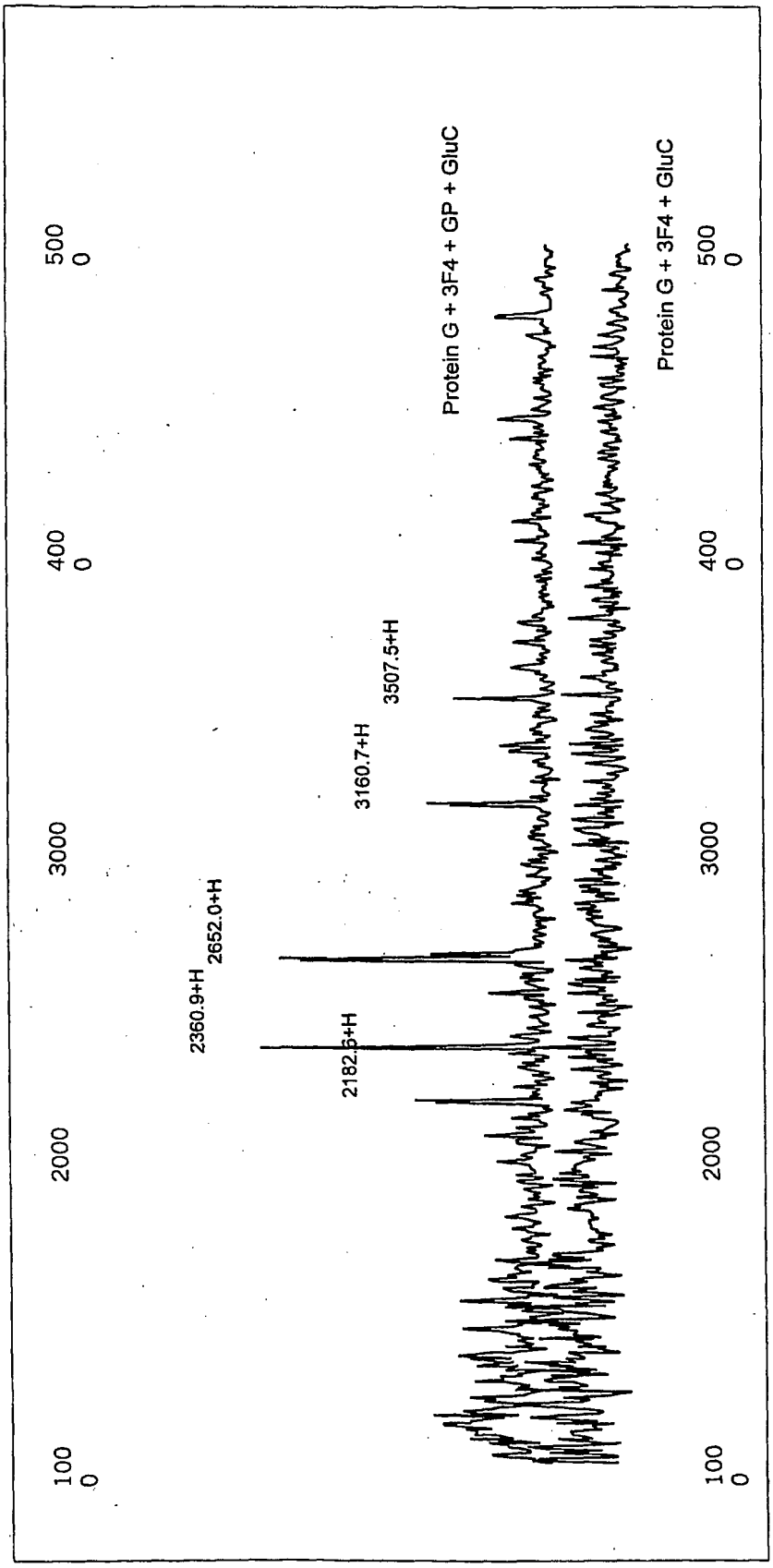
Protein G + 3F4 + GP + GluC
Protein G + 3F4 + GluC
2360.9+H
2652.0+H
2182.6+H
3160.7+H
3507.5+H
100
0
2000
3000
400
0
500
0

Figure 5C

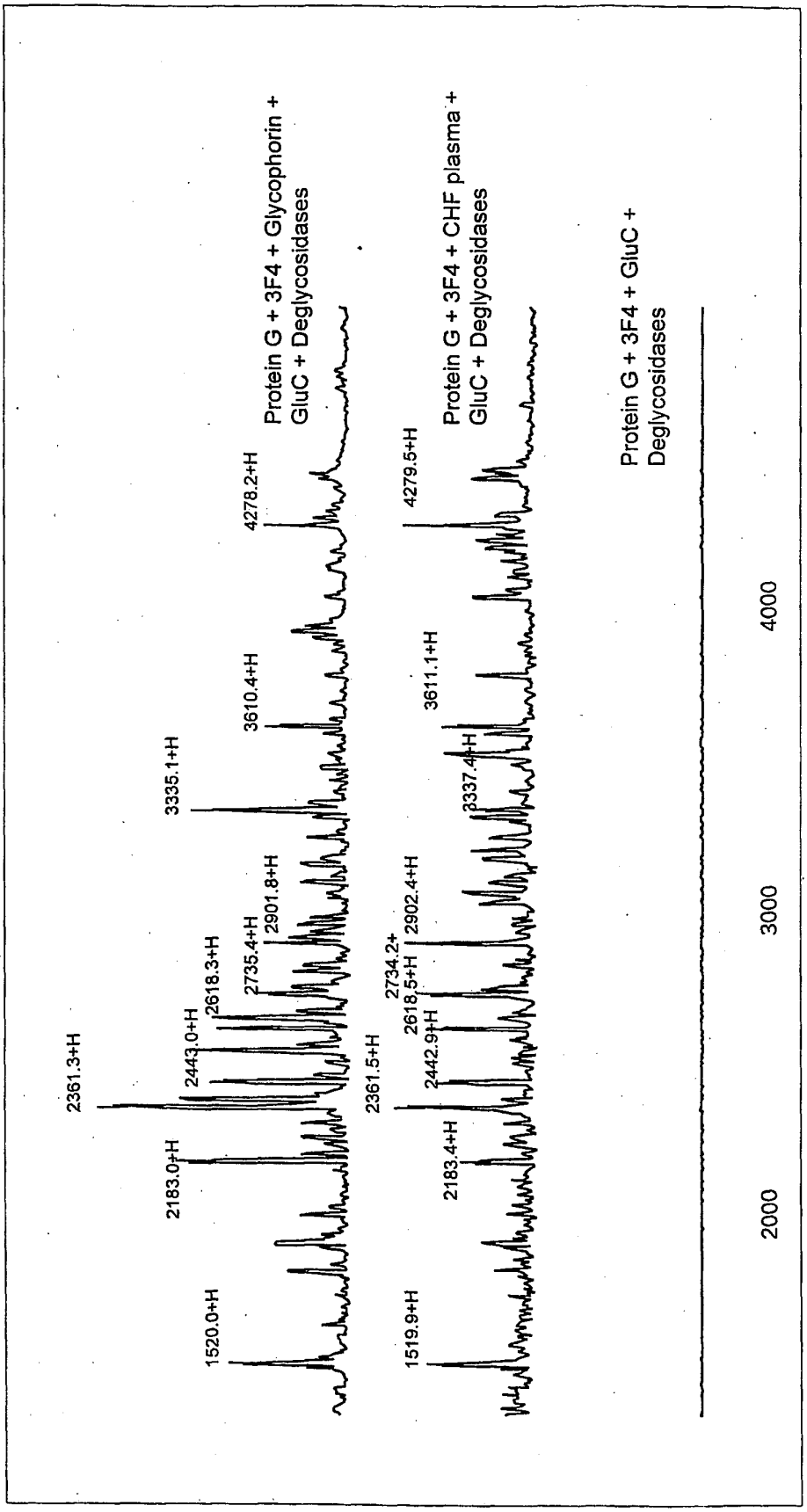
2361.3+H
2183.0+H
2443.0+H
2618.3+H
2735.4+H
2901.8+H
3335.1+H
3610.4+H
4278.2+H
Protein G + 3F4 + Glycophorin + GluC + Deglycosidases
1520.0+H
2361.5+H
2734.2+
2618.5+H
2442.9+H
2902.4+H
3337.4+H
3611.1+H
4279.5+H
Protein G + 3F4 + CHF plasma + GluC + Deglycosidases
1519.9+H
2183.4+H
Protein G + 3F4 + GluC + Deglycosidases
2000
3000
4000

Figure 6

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- US 6572895 B **[0004]**

Non-patent literature cited in the description

- **Soläng et al.** *European Heart Journal,* 1999, vol. 20, 789-795 **[0004]**
- **Rasamoelisolo et al.** *Vox Sanguins,* 1997, vol. 72, 185-191 **[0008]**
- **Thomas A. Scott ; E. Ian Mercer ; Walter de Gruyter.** Concise Encyclopedia: Biochemistry and Molecular Biology. 1997, 201-202 **[0026]**
- **B.D. Hames ; N.M. Hooper.** Instant Notes: BioChemistry. Springer-Verlag, 2000, 125, 126, 130 **[0026]**
- **B.D. Hames ; N.M. Hooper.** Instant Notes: Biochemistry. Springer-Verlag, 2000, 112-114 **[0038]**
- **Gaczyńska et al.** *Cytobios,* 1993, vol. 75, 7-11 **[0040]**
- **Venerando et al.** *Blood,* 2002, vol. 99 (3), 1064-1070 **[0040]**
- **Wegner et al.** *Cardiovascular Research,* 1996, vol. 31, 891-898 **[0040]**
- **Piwowar et al.** *Clinical Chemistry Lab Medicine,* 2000, vol. 38 (12), 1257-1261 **[0040]**
- **Santos-Silva et al.** *Clinica Chimica Acta,* 2002, vol. 320, 29-35 **[0040]**
- **Caimi et al.** *Thromb Haemost,* 2000, vol. 83, 516-517 **[0041]**
- **Demiroglu et al.** *Experimental Clinical Endocrinol Diabetes,* 1999, vol. 107 (1), 35-39 **[0041]**
- **Martinez et al.** *Clinical Hemorheology and Microcirculation,* 1998, vol. 18, 253-258 **[0041]**
- **Ziegler et al.** *Metabolism,* 1994, vol. 43 (9), 1182-1186 **[0041]**
- **Martínez et al.** *Clinical Hemorheology and Microcirculation,* 1998, vol. 18, 253-258 **[0041]**
- **Eylar et al.** *The Journal of Biological Chemistry,* 1962, vol. 237 (6), 1992-2000 **[0042]**
- **Rasamoelisolo et al.** *Vox Sanguinis,* 1997, vol. 72, 185-191 **[0044]**

专利名称(译)	充血性心力衰竭的诊断方法		
公开(公告)号	EP1692513A4	公开(公告)日	2008-02-20
申请号	EP2004797198	申请日	2004-11-10
申请(专利权)人(译)	SYN X制药，INC.		
当前申请(专利权)人(译)	SYN X制药，INC.		
[标]发明人	JACKOWSKI GEORGE VAN LIESHOUT TRACY THATCHER BRAD ZHANG RULIN YANTHA JASON RASAMOELISOLO MICHELE		
发明人	JACKOWSKI, GEORGE VAN LIESHOUT, TRACY THATCHER, BRAD ZHANG, RULIN YANTHA, JASON RASAMOELISOLO, MICHELE		
IPC分类号	G01N33/577 G01N33/53 G01N33/543 G01N33/68		
CPC分类号	G01N33/6893 G01N2800/325		
优先权	10/706599 2003-11-11 US		
其他公开文献	EP1692513A1 EP1692513B1		
外部链接	Espacenet		

摘要(译)

本发明提供了用于定量生物流体样品中循环血型糖蛋白的测定法。通过该测定法测量的循环血型糖蛋白是截短的血型糖蛋白，其诊断为充血性心力衰竭（CHF）。