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(54) DIAGNOSTIC METHODS FOR CONGESTIVE HEART FAILURE

DIAGNOSEVERFAHREN FÜR KONGESTIVES HERZVERSAGEN

PROCEDES DIAGNOSTIQUES POUR UNE INSUFFISANCE CARDIAQUE CONGESTIVE

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

- **RASAMOELISOLO M ET AL:** "Fine characterization of a series of new monoclonal antibodies directed against glycoporphin A." **VOX SANGUINIS** 1997, vol. 72, no. 3, 1997, pages 185-191, XP002462928 ISSN: 0042-9007
- **CLASTER S ET AL:** "Degradation of erythrocyte glycoporphin 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 glycoporphin 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

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DescriptionFIELD 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 glycoporphin 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 hypothalamic-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 (Solång 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 glycoporphin to quantify elevated glycoporphin in biological fluids. Glycoporphin is the major integral membrane protein of the mammalian red blood cell (RBC) and is highly glycosylated. The glycosylation of glycoporphin 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 glycoporphins, 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 glycoporphin in the plasma of CHF patients. This glycoporphin had a lower molecular weight than that of normal glycoporphin, 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 glycoporphins 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 Rasamoeliso et al. Vox Sanguinis 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 glycoprotein antigen with the biological fluid under conditions such that an antibody-antigen binding complex forms between said monoclonal antibody and said glycoprotein antigen present in said biological fluid, wherein said glycoprotein antigen is present on a truncated abnormal form of glycoprotein; 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 glycoprotein.

FIGURE 4 shows the results of immunoprecipitation of glycoprotein from the plasma of CHF patients.

FIGURES 5A-C show chromatograms; FIGURE 5A shows captured glycoprotein from CHF patients; FIGURE 5B shows captured glycoprotein from healthy patients and FIGURE 5C shows captured purified glycoprotein.

FIGURE 6 shows chromatograms after deglycosylation treatment; the top chromatograph shows purified glycoprotein; the middle chromatograph shows captured glycoprotein from CHF patients and the bottom chromatograph is a control run without a glycoprotein 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 glycoprotein.

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

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

[0017] As used herein, the abbreviation "GP_{Ax2}" refers to the dimerized form of glycoprotein A.

[0018] As used herein, the abbreviation "GP_{Bx2}" refers to the dimerized form of glycoprotein B.

[0019] As used herein, the abbreviation "ELISA" refers to enzyme-linked immunosorbent 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 "glycoprotein" refers to the major integral glycoprotein of the mammalian erythrocyte membrane. Glycoprotein 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 glycoproteins).

[0027] As used herein, the term "circulating, truncated glycoprotein" refers to the abnormal glycoprotein fragment identified by the assay of the instant invention in the serum of CHF patients. The 3F4 mouse anti-glycoprotein monoclonal antibody which recognizes the extracellular portion of glycoprotein A and B binds to this circulating, truncated glycoprotein. This circulating, truncated glycoprotein is structurally different from the normal soluble glycoprotein 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 fluorophores, 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 (Martinez 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 glycoporphins 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 glycoporphins from the RBC membrane. These glycoporphin fragments are released into the bodily fluids where they stimulate the production of auto-antibodies. The decrease in glycoporphin 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 glycoporphin identified in the plasma of CHF patients using the sandwich ELISA assay described herein is an extracellular glycoporphin fragment which has been cleaved from the RBC membrane during the disease process. This circulating, truncated glycoporphin is structurally different from the normal soluble form of glycoporphin. The mouse anti-glycoporphin 3F4 monoclonal antibody also recognizes the circulating, truncated glycoporphin. The instant inventors have also shown by direct ELISA assay that CHF patients show an increase in anti-glycoporphin auto-antibodies. Thus, it is concluded that this circulating, truncated glycoporphin can be used as a new biopolymer marker for CHF diagnosis.

EXPERIMENTAL PROCEDURES

ANTIBODIES

[0044] The mouse anti-glycoporphin monoclonal antibodies used in the following experiments were purchased from BioAtlantic. Monoclonal antibody 6G4 recognizes amino acid residues 39-45 of glycoporphin A. Monoclonal antibody 5F4 recognizes the intracellular portion of glycoporphin A comprising amino acid residues 107-119. Monoclonal antibody 3F4 recognizes the extracellular portion of glycoporphins 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 Rasamoeliso et al. Vox Sanguinis 72:185-191 1997.

[0045] The mouse anti-glycoporphin 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 car-

bonate pH 9.4 was coated on ELISA plates (Nuc, Denmark) and set overnight at +4°C. Plates were then washed 3 times with 0.01M 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-glycoporphin 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 glycoporphins is detected by adding 100μl of TMB Tetramethyl Benzidine (Moss, Inc.). The reaction was stopped with 50μl of 1N H₂SO₄. 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. Glycoporphin captured from the plasma of CHF patients is shown on the left and the glycoporphin 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 glycoporphins in CHF plasma samples. The 3F4 MoAb recognizes the common sequence on both glycoporphins A and B (amino acid residues 5-25). This binding is sugar-dependent since this fragment of glycoporphin is highly glycosylated.

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

[0049] Eight CHF samples having the most elevated amount of glycoporphin and 8 normal plasma samples having the lowest amount of glycoporphin were analyzed and the result is shown in Figure 2. Figure 2 shows results from sandwich ELISA assays comparing the glycoporphin captured in plasma from CHF patients and the glycoporphin captured in normal control plasma (n=8). The top panel shows results using the 6G4 MoAb (p=0.001); 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. Glycoporphin captured from the plasma of CHF patients is shown on the left and the glycoporphin captured from normal plasma is shown on the right in all three panels. The result shows that 6G4 detects elevated amount of glycoporphin in CHF samples, while 5F4 shows no significant difference between both CHF and normal human plasma. This result indicates that glycoporphin

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 (Inter-gen) 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 H₂SO₄. 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% TRI-TON X (Sigma) and 3 μ l 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 glycoprophin (Sigma), CHF plasma from PRAISE 2 study or normal plasma (Intergen) was added to the 3F4-coated chip as follows:

[0058] The glycoprophin 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 2 μ l 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 glycoprophin was then treated with Endoproteinase GluC (Roche Diagnostics). For that, the GluC powder was dissolved in 50 μ l of UF water and a 1/10 dilution in 50mM Ammonium Carbonate pH 7.8 (BDH Laboratory Supplies) was prepared. 1 μ l 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 1 μ l 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 glycoprophin 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 glycoprophin. As shown in Figures 5A-C, a (glyco) peptide with a m/z of 2361+H is found in both CHF and glycoprophin demonstrating that the (glyco)protein captured from CHF corresponds probably to the glycoprophin. It is interesting to note that the chromatograms (Figures 5A-C) obtained from the purified glycoprophin and the one from CHF plasma were not overlapped. This is due to the fact that the structure of the glycoprophin in CHF is maybe slightly modified.

[0061] To further prove that the captured (glyco)protein is related to glycoprophin, the captured (glyco)protein was deglycosylated on chip. Figure 6 shows on-chip deglycosylation treatment of the glycopeptides captured from either purified glycoprophin 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 glycoprophin. 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, glycoprophin circulating in biological fluid which is diagnostic for CHF. It is important to note that glycoprophin has not been previously recognized as a marker for congestive heart failure (CHF). The instant inventors are the

first to document glycoprophin 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.

5 **[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

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

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

30 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.

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

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

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

50 A) contacting said antibody-antigen binding complex with a polyclonal antibody corresponding to said glycoprophin 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 glycoprotein antigen, wherein said glycoprotein antigen is present on a truncated abnormal form of glycoprotein, in a diagnostic method to quantify elevated levels of a truncated glycoprotein 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

ste 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). 5
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. 10
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. 15
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. 20
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 ; 30
- B) attacher un traceur à un anticorps polyclonal correspondant à l'anticorps polyclonal de l'étape A ; 35
- 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 40
- D) détecter le traceur sur ledit anticorps polyclonal marqué. 45
6. Procédé selon la revendication 5, dans lequel le traceur sur ledit anticorps polyclonal marqué comprend une substance générant un signal. 50
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 glycophorine étant présent sur une forme anormale tronquée de glycophorine, dans un procédé diagnostique pour quantifier des niveaux élevés d'une glyco- 55
- phorine tronquée dans un liquide biologique prélevé sur un sujet atteint d'une ICC.

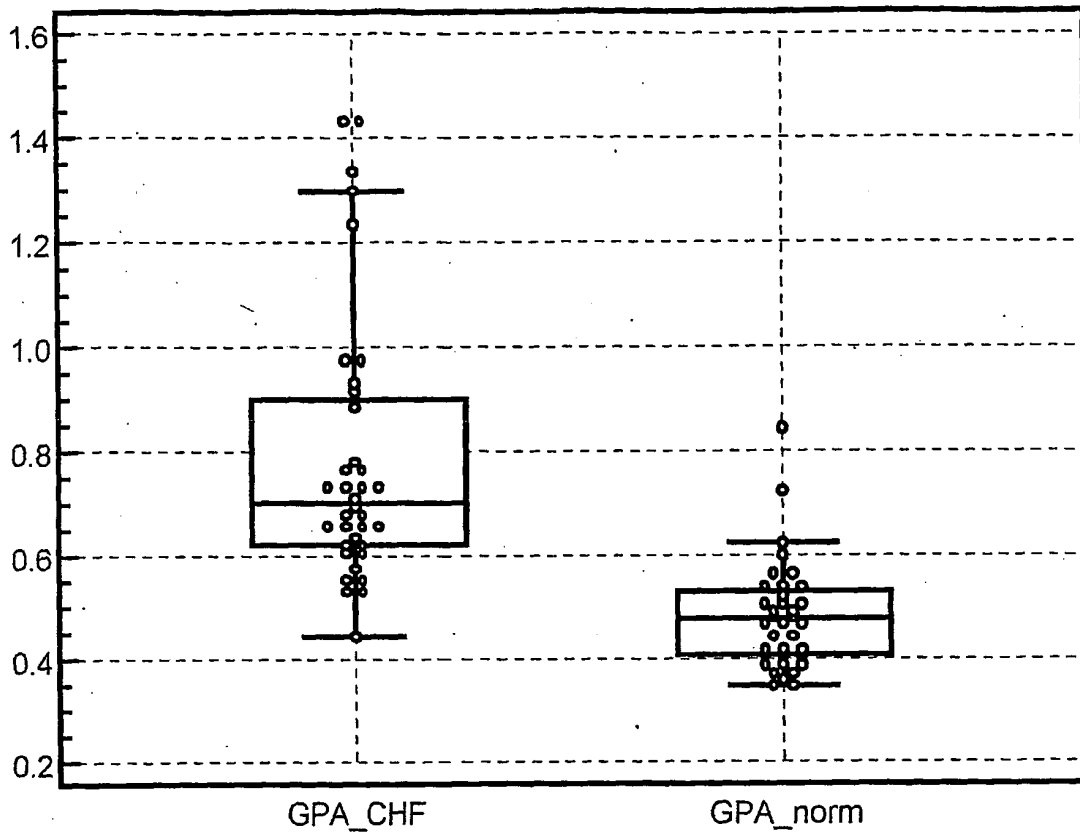


Figure 1

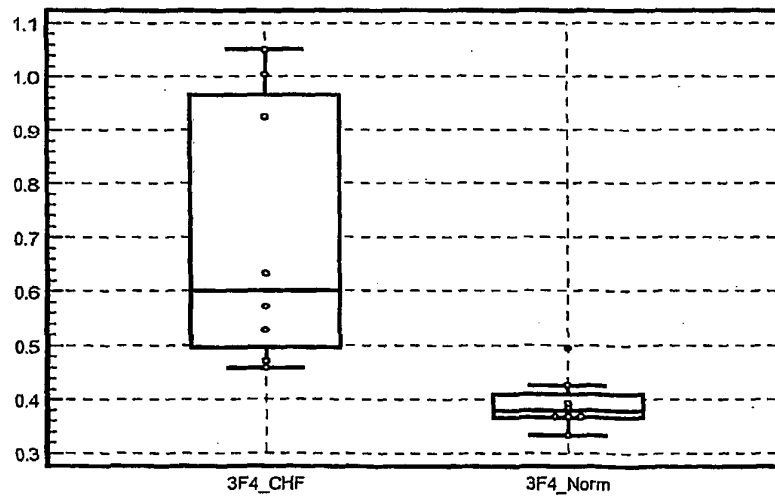
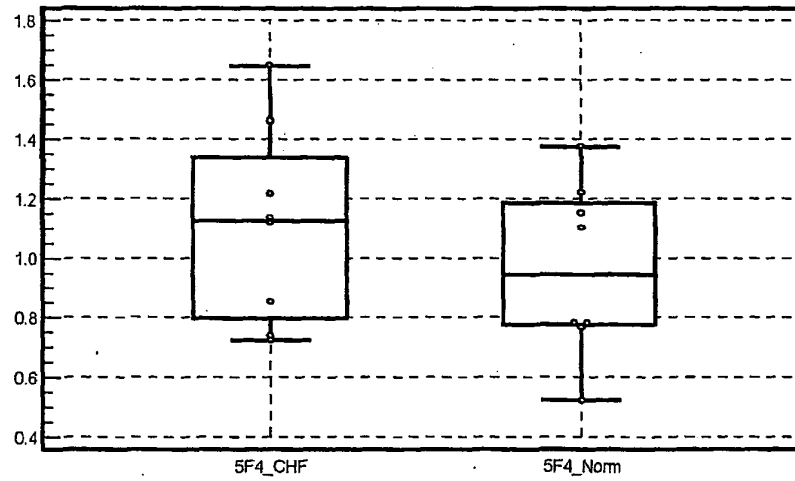
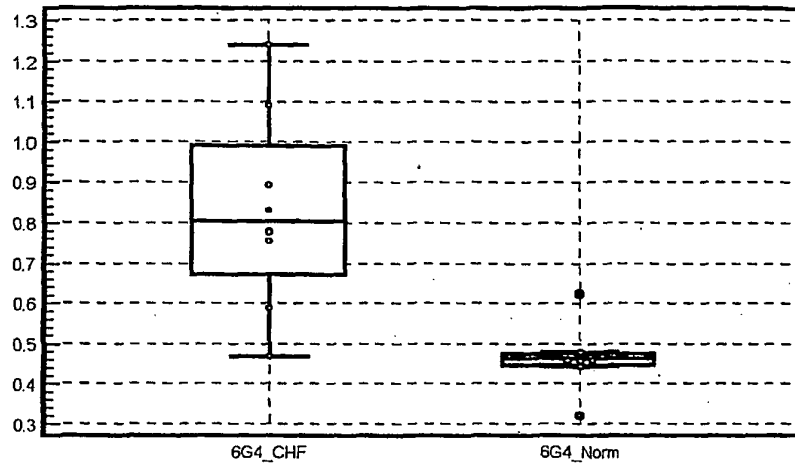


Figure 2

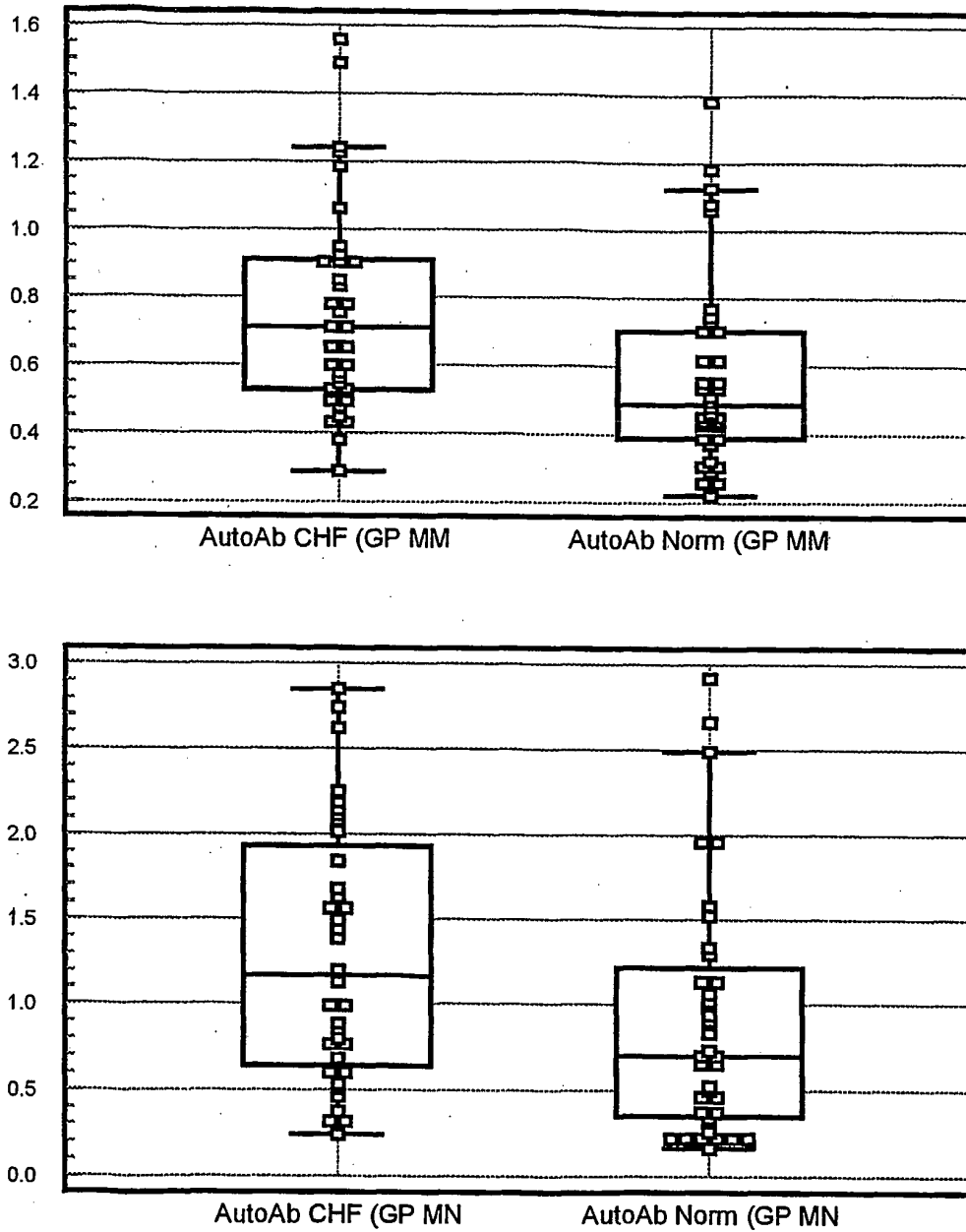


Figure 3

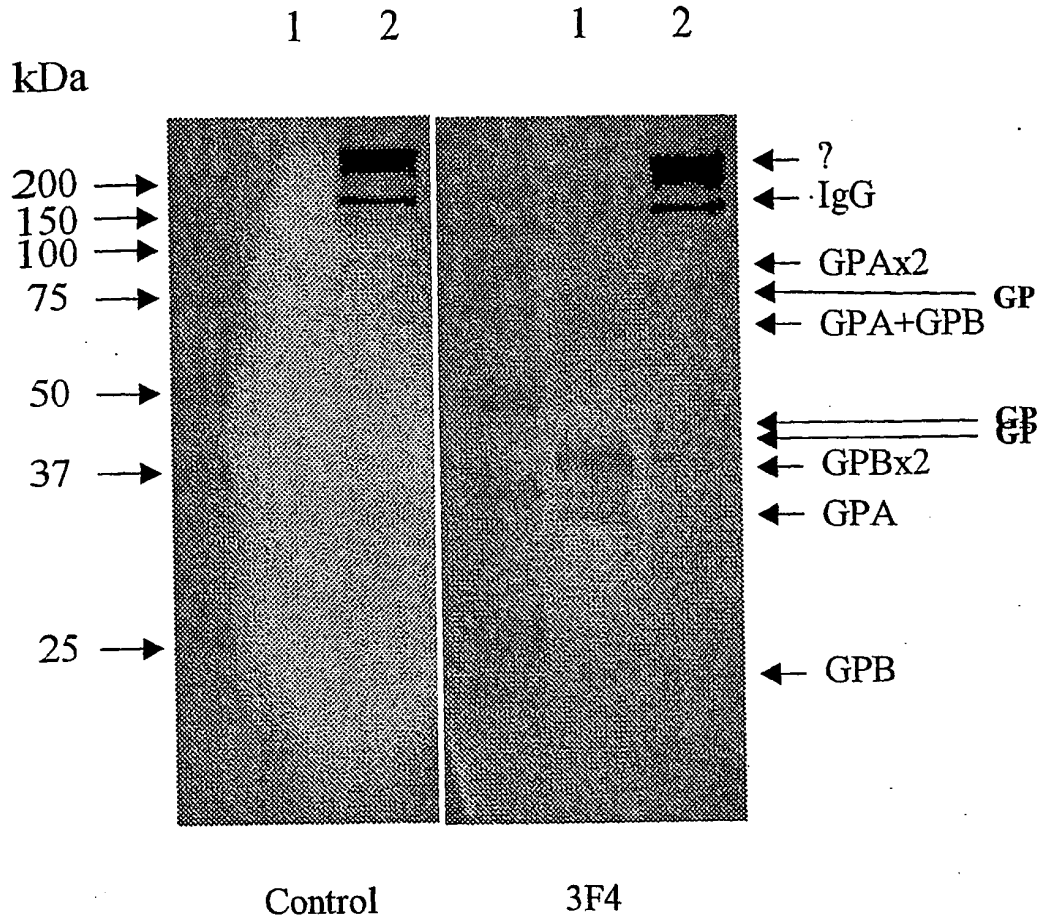


Figure 4

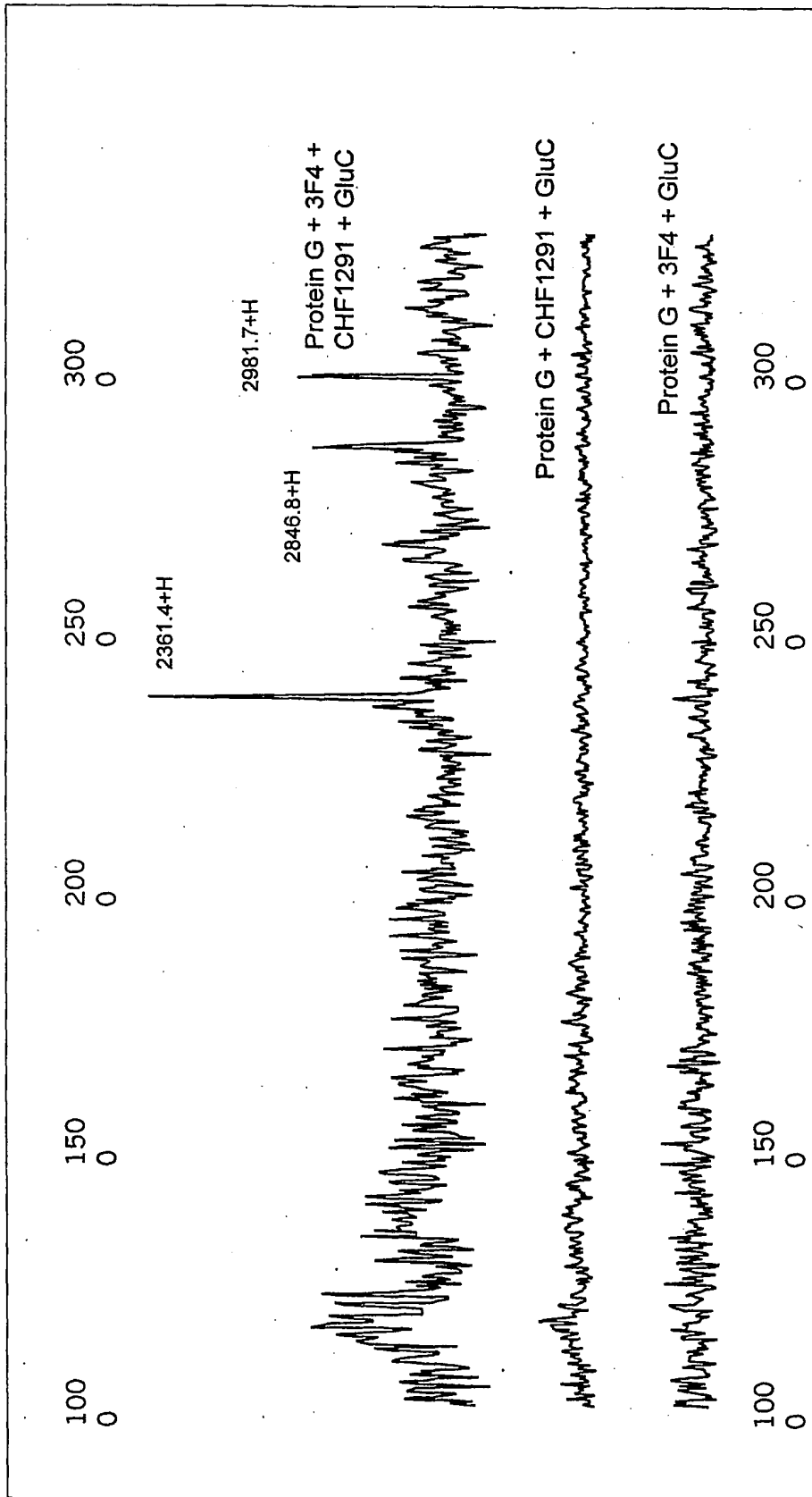


Figure 5A

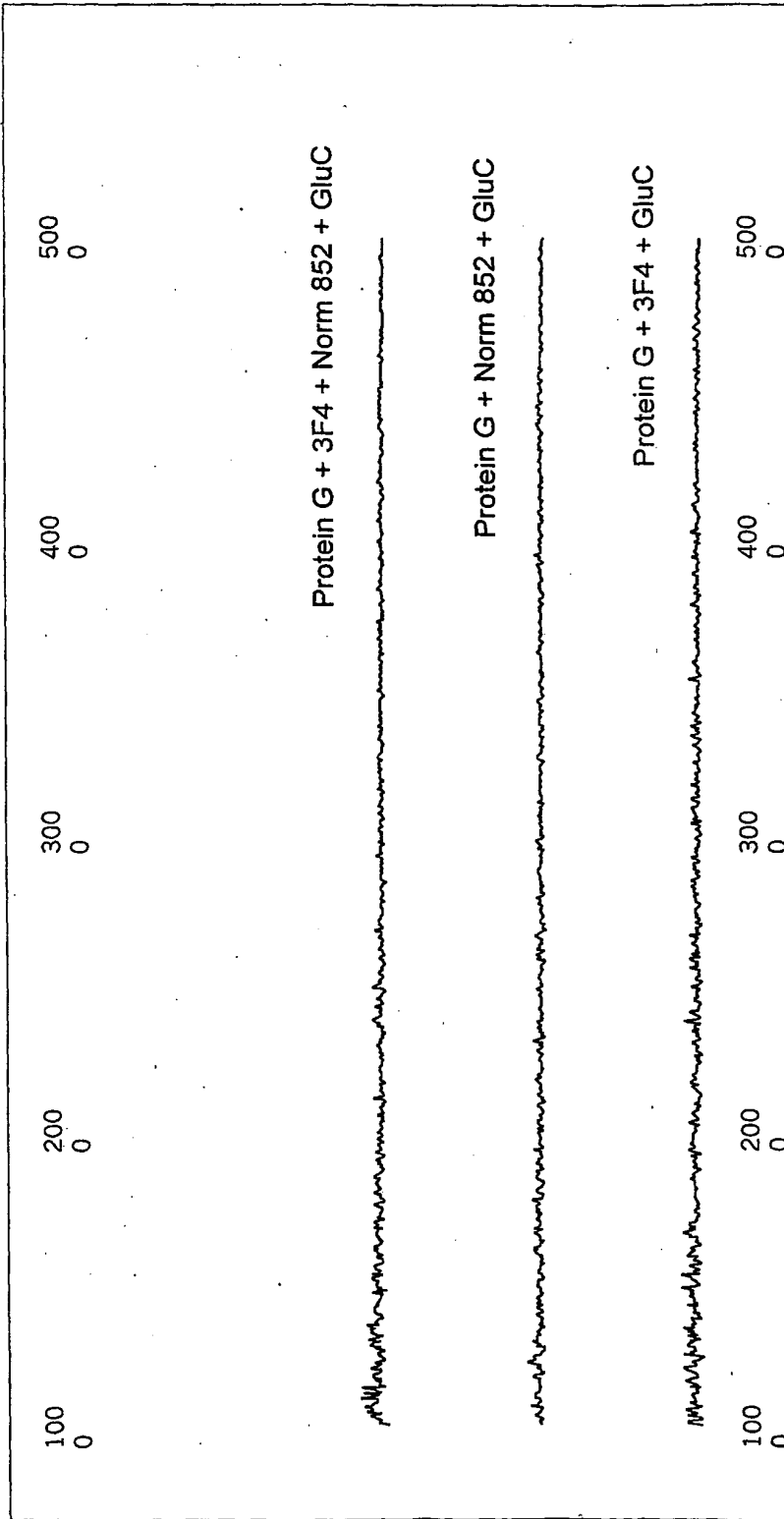


Figure 5B

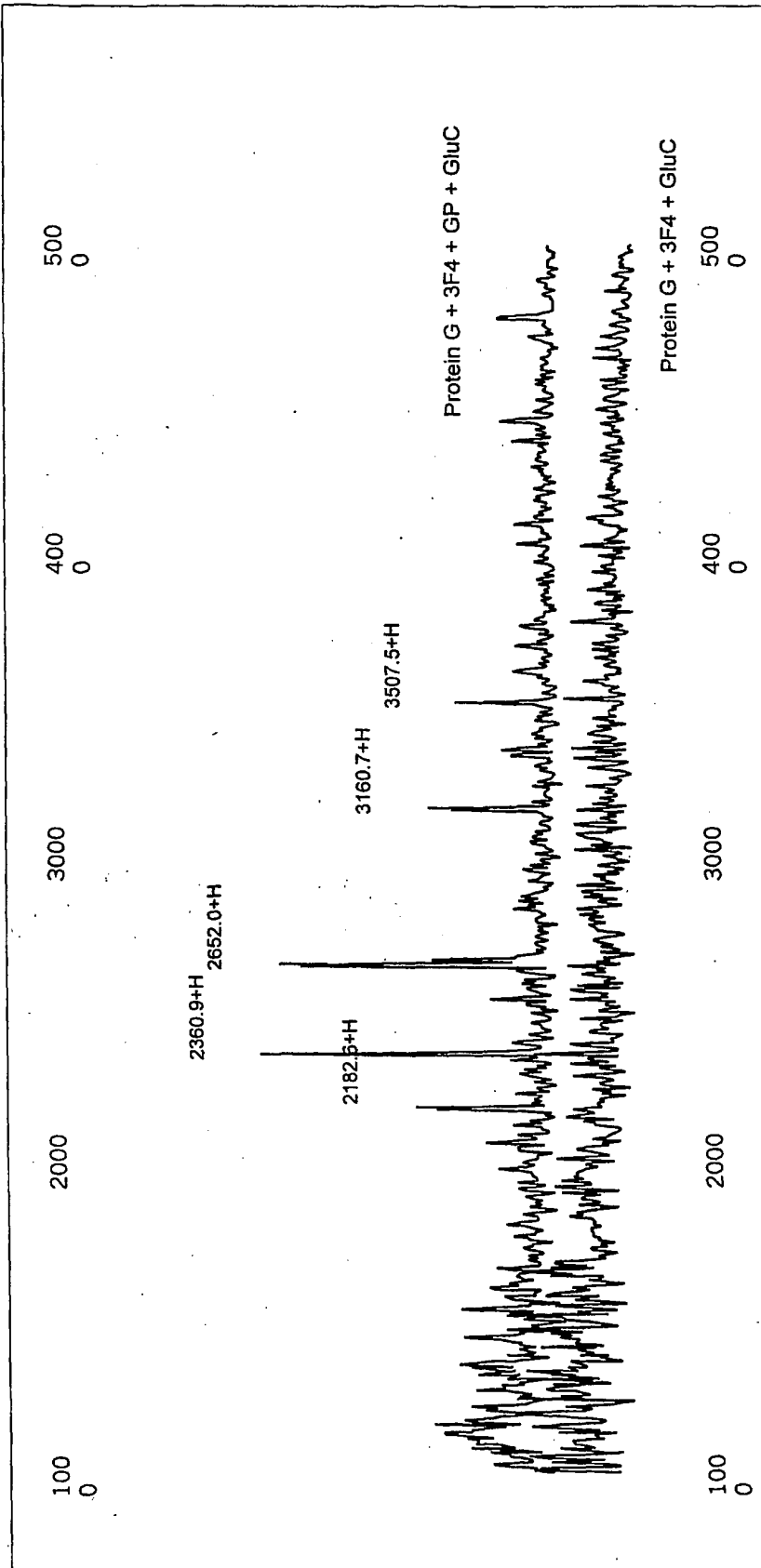


Figure 5C

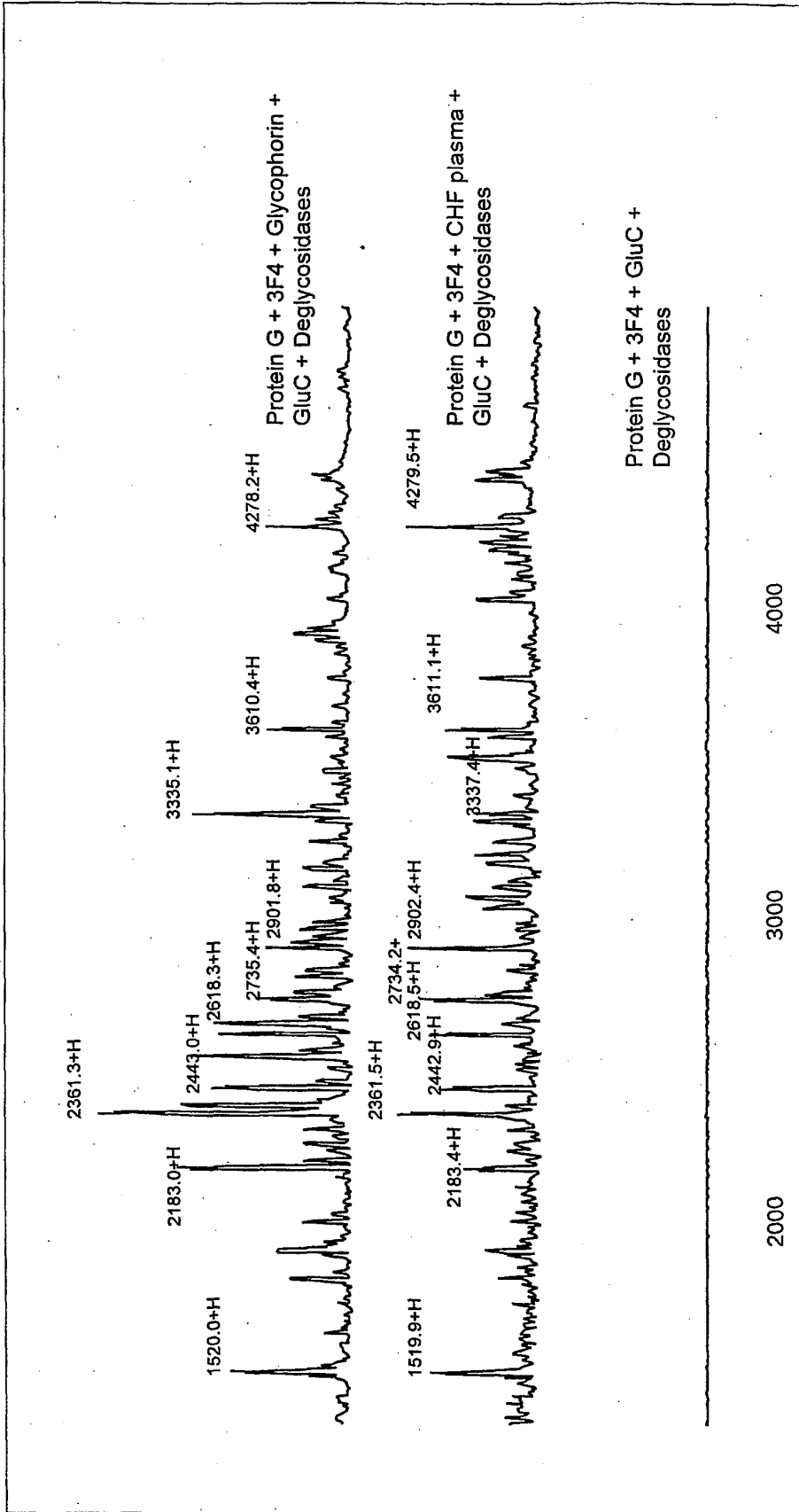


Figure 6

REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- US 6572895 B [0004]

Non-patent literature cited in the description

- **Solång et al.** *European Heart Journal*, 1999, vol. 20, 789-795 [0004]
- **Rasamoeliso et al.** *Vox Sanguinis*, 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]
- **Piowar 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]
- **Martinez 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]
- **Rasamoeliso 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		
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其他公开文献	EP1692513A4 EP1692513A1		
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)。

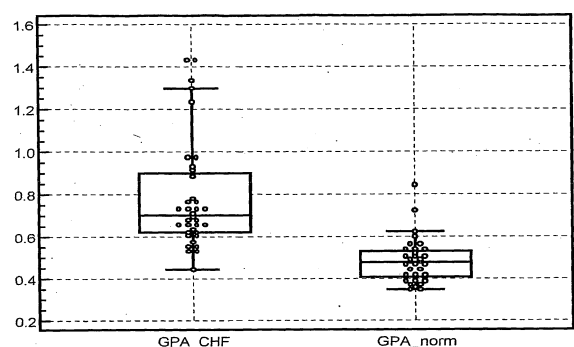


Figure 1