Office de la Propriété Intellectuelle du Canada
Un organisme d'Industrie Canada

Canadian Intellectual Property Office
An agency of Industry Canada

CA 2351885 A1 2000/06/02

(21) 2 351 885

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 1999/11/16
(87) Date publication PCT/PCT Publication Date: 2000/06/02
(85) Entrée phase nationale/National Entry: 2001/05/14
(86) N° demande PCT/PCT Application No.: SE 99/02094
(87) N° publication PCT/PCT Publication No.: WO 00/31533
(30) Priorité/Priority: 1998/11/26 (9804069-4) SE

(51) Cl.Int.^{7}/Int.Cl.^{7} G01N 33/53, G01N 33/68, G01N 33/543

(71) Demandeur/Applicant:
EURODIAGNOSTICA AB, SE

(72) Inventeurs/Inventors:
APPELROS, STEFAN, SE;
BORGSTROM, ANDERS, SE

(74) Agent: FETHERSTONHAUGH & CO.

(54) Titre : ENSEMBLE DE TEST ET PROCEDE PERMETTANT DE DIAGNOSTIQUER UNE PANCREATITE AIGUE SEVERE

(54) Title: TEST KIT AND METHOD OF DIAGNOSING SEVERE ACUTE PANCREATITIS

(57) Abrégé/Abstract:
A method of in vitro diagnosing severe acute pancreatitis, monoclonal antibodies binding to carboxypeptidase B activation peptide (CAPAP) having SEQ ID NO:1 (aa 1 - 81) or fragments thereof, and a test kit, preferably a dipstick, are described. The method comprises A) bringing a sample of body fluid into contact with a) a first antibody binding to the CAPAP of SEQ ID NO:1, and b) a second antibody binding to the CAPAP of SEQ ID NO:1, other than the side in a), or c) a solid-phase bound CAPAP of SEQ ID NO:1 or fragments thereof, wherein one of the antibodies of a) and b) or the peptide of c) is labeled with a non-radioactive label, and wherein at least one of the antibodies of a) and b) is monospecific for CAPAP, and B) determining the level of antibody-antigen-antibody complex formed, or the level of an excess component in the competitive antigen-antibody reaction, and C) using the determined level for estimation of the amount of CAPAP in the sample for the diagnosis of severe acute pancreatitis in the patient.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : G01N 33/53, 33/543, 33/68 — A1

(11) International Publication Number: WO 00/31533

(43) International Publication Date: 2 June 2000 (02.06.00)

(21) International Application Number: PCT/SE99/02094

(22) International Filing Date: 16 November 1999 (16.11.99)

(30) Priority Data:
9804069–4 26 November 1998 (26.11.98) SE

(71) Applicant *(for all designated States except US)*: EURODIAGNOSTICA AB [SE/SE]; Ideon Malmö, S–205 12 Malmö (SE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BORGSTRÖM, Anders [SE/SE]; Bågängsvägen 21, S–216 20 Malmö (SE). APPELROS, Stefan [SE/SE]; Idunavägen 8A, S–216 19 Malmö (SE).

(74) Agents: BRITA, Nilsson et al.; AB Stockholms Patentbyrå, Zacco & Bruhn, Box 23101, S–104 35 Stockholm (SE).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: TEST KIT AND METHOD OF DIAGNOSING SEVERE ACUTE PANCREATITIS

(57) Abstract

A method of *in vitro* diagnosing severe acute pancreatitis, monoclonal antibodies binding to carboxypeptidase B activation peptide (CAPAP) having SEQ ID NO:1 (aa 1 – 81) or fragments thereof, and a test kit, preferably a dipstick, are described. The method comprises A) bringing a sample of body fluid into contact with a) a first antibody binding to the CAPAP of SEQ ID NO:1, and b) a second antibody binding to the CAPAP of SEQ ID NO:1, other than the side in a), or c) a solid–phase bound CAPAP of SEQ ID NO:1 or fragments thereof, wherein one of the antibodies of a) and b) or the peptide of c) is labeled with a non–radioactive label, and wherein at least one of the antibodies of a) and b) is monospecific for CAPAP, and B) determining the level of antibody–antigen–antibody complex formed, or the level of an excess component in the competitive antigen–antibody reaction, and C) using the determined level for estimation of the amount of CAPAP in the sample for the diagnosis of severe acute pancreatitis in the patient.

Test kit and method of diagnosing severe acute pancreatitis

The present invention relates to a test kit and a method of *in vitro* diagnosing severe acute pancreatitis with the aid of at least two antibodies each binding to a different site on the carboxypeptidase B activation peptide, CAPAP, or with one antibody specifically binding to CAPAP and a solid-phase bound epitope-containing peptide of CAPAP. Further, the invention relates to a monoclonal antibody binding to an antigenic site on CAPAP.

Background

The clinical rationale for assessment of severity of an attack of acute pancreatitis has not become obvious until recently when more specific treatments have emerged. Anticytokines, antibiotics and endoscopic sphincterotomy have shown promising results but only when the treatment has been instituted within 24-48 hours of onset of pain. Furthermore, four out of five cases of acute pancreatitis are mild and do not benefit from any expensive specific treatment.

During recent years many new laboratory variables have been proposed as early single tests for severity prediction in acute pancreatitis.

Measurement of substances that mirror the activation of trypsinogen like the trypsinogen activation peptide, TAP, the carboxypeptidase B activation peptide, CAPAP, and trypsin-α_1-antitrypsin complexes seem to be able to predict severity already within 24 hours of onset of acute pancreatitis. Inflammatory mediators like interleukin-6 and PNM-elastase are also useful.

The carboxypeptidase B activation peptide (CAPAP) is the largest activation peptide (MW 9400) released from any pancreatic proenzyme. The activation occurs normally in the intestine during the digestion of food. Serum and urine normally contain no or very low levels of immunoreactive CAPAP. During the initial phase of acute pancreatitis this activation occurs prematurely within and around the pancreatic gland. CAPAP then leaks to the circulation from where it will be eliminated trough glomerular filtration and excreted in the urine. Only one small clinical study has been published so far [Appelros S, Thim L, Borgström A. The activation peptide of carboxypeptidase B in serum and urine. Gut 1998; 42:97-102].

CAPAP is very stable in serum and urine, and the determination of the protein is enabled by a CAPAP RIA kit, marketed by EuroDiagnostica AB, Malmö, Sweden. However, handling of reagents comprising a radioactive label require specialized laboratories and

trained personnel. Once the sample to be tested reaches the laboratory, the test may be performed in approximately two hours.

The US patent 5,356,781 discloses an immunological method for detecting the activation of pancreatic zymogens for diagnosing or monitoring the progress of pancreatic disease. The method comprises detection of the absence or presence in a sample of peptides having the same carboxy-terminal pentapeptide sequence as the activation peptides of pancreatic zymogens. Among these procarboxypeptidases A and B are mentioned. However, no antibodies binding to procarboxypeptidases are disclosed.

There are good reasons for an early assessment of severity in acute pancreatitis since this could discount the majority of the attacks which are mild and have a self limiting course and do not require any treatment other than general support and parenteral fluid. Thus, there is a need for a rapid method for identification of patients with severe attack of acute pancreatitis already at the time of admission to the Emergency room. The perfect method for determination of severity in regular clinical practice does not yet exist, and a simple and reliable test that can be used to predict the severity in acute pancreatitis would be desirable.

Description of the invention

The present invention provides a method of diagnosing severe acute pancreatitis, a test kit and monoclonal antibodies binding to CAPAP.

The CAPAP used in the present invention is the human carboxypeptidase B activation peptide of 81 amino acids, which results from the N-terminal 95-amino acid activation peptide after further degradation of the C-terminal end by the dual action of active carboxypeptidase B and trypsin. The amino acid sequence of this 81 amino-acid CAPAP is disclosed in the Sequence listing part of this description and it is nominated SEQ ID NO:1.

More precisely, the present invention provides a method of *in vitro* diagnosing severe acute pancreatitis in a patient comprising the steps of

A) bringing a sample of body fluid from the patient into contact with a sufficient amount of

a) at least one first antibody binding to an antigenic site on the carboxypeptidase B activation peptide (CAPAP) having the amino acid sequence SEQ ID NO:1, and

b) at least one second antibody binding to an antigenic site on the CAPAP having the amino acid sequence SEQ ID NO:1,other than the site in a), or

c) a solid-phase bound CAPAP having the amino acid sequence SEQ ID NO:1 or a peptide fragment thereof containing an epitope,

wherein at least one antibody of a) and b) or the peptide of c) is labeled with a non-radioactive label, and

wherein at least one of the first and second antibodies is monospecific for CAPAP, and

B) determining the level of the antibody a) - CAPAP - antibody b) complex formed, or the level of an excess component in the competitive reaction of antibody a) – CAPAP and antibody a) – peptide c) with the aid of the label used, and

C) using the determined level for estimation of the amount of CAPAP in the sample for the diagnosis of severe acute pancreatits in the patient.

The amount of CAPAP in the sample of body fluid which will be regarded as cut-off level for severe acute pancreatitis will be determined emprically for each specific assay set-up, but will normally be in the range of 60 to 100 nmol/L , such as 80 nmol/L, for a urine sample, and 6 to 10 nmol/L , such as 8 nmol/L, for a blood or serum sample.

The phrase "at least one first antibody" is used to define that there may be several different antibodies which, however, do not bind to the same sites on the protein as the second antibody. The phrase " at least one second antibody" is used to define that there may be several different antibodies which, however, do not bind to the same sites on the protein as the first antibody.

The phrase " antibody monospecific for CAPAP" is used to define that the antibody binds to only one specific site on the CAPAP, and examples of monospecific antibodies are monoclonal antibodies, monospecific polyclonal antibodies and recombinant modified antibodies having specific binding to the protein.

In a preferred embodiment of the invention the first and the second antibodies are monoclonal antibodies.

The solid phase used for the solid-phase bound CAPAP having the amino acid sequence SEQ ID NO:1 or a peptide fragment thereof containing an epitope may be any solid phase used in the art of immunoassays, such as cellulose particles, glass surfaces, plastics surfaces, magnetic beads, etc.

In an embodiment of the invention the determination B) is performed with a double sandwich enzyme-linked immunosorbent assay (ELISA) or a competitive immunosorbent assay.

In another embodiment the label in b) is selected from the group consisting of gold, carbon, and latex particles, and enzymes.

In yet another embodiment the sample of body fluid in A) is a urine sample, and when the estimated amount of CAPAP in C) is > 60 -100 nmol/L of urine, the patient is diagnosed positive for severe acute pancreatitis.

In still another embodiment the sample of body fluid in A) is a blood sample, and when the estimated amount of CAPAP in C) is > 6 - 10 nmol/L of blood, the patient is diagnosed positive for severe acute pancreatitis.

The present invention further provides a monoclonal antibody binding to an antigenic site on CAPAP having the amino acid sequence SEQ ID NO:1 or a peptide fragment thereof containing an epitope.

To our knowledge, no monoclonal antibodies against CAPAP have been previously reported. However, now several different antibodies against CAPAP having the amino acid sequence SEQ ID NO:1 are produced for selection of at least two antibodies binding to different sites on CAPAP. These may then be used in the method and test kit of the invention.

The present invention also provides a test kit for acute pancreatitis comprising

a) at least one first antibody binding to an antigenic site on the carboxypeptidase B activation peptide (CAPAP) having the amino acid sequence SEQ ID NO:1, and

b) at least one second antibody binding to an antigenic site on the CAPAP having the amino acid sequence SEQ ID NO:1, other than the site in a), or

c) a solid-phase bound CAPAP having the amino acid sequence SEQ ID NO:1 or a peptide fragment thereof containing an epitope,

wherein at least one antibody of a) and b) or the peptide of c) is labeled with a non-radioactive label, and

wherein at least one of the first and second antibodies is monospecific for CAPAP.

In an embodiment the label is selected from the group consisting of gold, carbon and latex particles, and enzymes.

In a preferred embodiment the test kit is a dipstick.

Preparation of monoclonal antibodies

Immunization

Balb/c mice were inoculated with CAPAP purified protein having the amino acid sequence SEQ ID NO:1, covalent linked to BSA. All injections were performed with the antigen mixed with RIBI adjuvant (MPL + TDM). Each mouse was injected with 10 μg conjugate, followed one and two weeks later by injections of 1 μg per mouse and then a resting period of seven weeks. The mice now received a booster dose of 1 μg per mouse and

10 days later blood samples were collected and analyzed for antibodies against CAPAP in ELISA. Pure CAPAP was coated at a concentration of 1 µg/ml in carbonate buffer pH 9.0, 60 µl per well in Maxisorp ELISA plates at room temperature over night.

Fusion

The mouse with the highest concentration of antibodies in the serum was injected with 1 µg of CAPAP having the amino acid sequence SEQ ID NO:1 conjugated to BSA in RIBI adjuvant intraperitoneally. Three days later the mouse was killed by cervical dislocation and the spleen was removed. The spleen cells were harvested as a single cell suspension and washed three times with serum free medium and counted. The spleen cells were mixed with half their number of SP 2/0 mouse myeloma cells similarly washed in serum free medium and pelleted together by centrifugation at 250 x g and after careful removal of all liquid placed in a 37°C water-bath. The fusion procedure is performed at this temperature and with added reagents pre-warmed to 37°C. The cell pellet is gently broken with the tip of a pipette and 1 ml of 50% poyethylene glycol 1500 solution (PEG 1500, Boehringer Mannheim) is slowly added during the period of one minute and gentle mixing. The reaction mixture is gently mixed for one minute. During continued gentle mixing 1 ml of serum free medium is added during one minute, 2 ml during the second minute, 4 ml during the third and 8 ml during the fourth minute. The cells are centrifuged at 200 x g for seven minutes, the supernatant removed and the cell pellet with minimal disturbance transferred to 100 ml of pre-warmed medium supplemented with 17% FCS and HAT for selection of hybridomas. The cells were distributed to ten 96-well plates, the previous day seeded with intraperitoneal lavage cells, 3000 per well, as feeder cells. Supernatants from wells with growing hybridomas were tested in ELISA as above when the cells covered approximately 50% of the bottom area.

Selection of monoclonal antibodies

Positive and negative screening procedures are performed on the 20 possible hybridoma cell lines to select monoclonal antibodies binding to different sites on the CAPAP protein.

One-step rapid tests

At least two of the produced monoclonal antibodies binding to different sites on the CAPAP having the amino acid sequence SEQ ID NO:1 are used for dipsticks. In this regard, use may be made of a commercially available test set-up, e.g. Clear View from Johnson &

Johnson or Test Pac from ABBOTT in accordance with the recommendations of the manufacturer. Both these test systems give a visual reading within seconds to minutes.

SEQUENCE LISTING

<110> EURODIAGNOSTICA AB

<120> Test kit and method of diagnosing acute severe pancreatitis.

<130> 192962200/BN

<140>

<141>

<150> SE 9804069-4

<151> 1998-11-26

<160> 1

<170> PatentIn Ver. 2.1

<210> 1

<211> 81

<212> PRT

<213> Homo sapiens

<400> 1

His His Gly Gly Glu His Phe Glu Gly Glu Lys Val Phe Arg Val Asn
1 5 10 15

Val Glu Asp Glu Asn His Ile Asn Ile Ile Arg Glu Leu Ala Ser Thr
20 25 30

Thr Gln Ile Asp Phe Trp Lys Pro Asp Ser Val Thr Gln Ile Lys Pro
35 40 45

His Ser Thr Val Asp Phe Arg Val Lys Ala Glu Asp Thr Val Thr Val
50 55 60

Glu Asn Val Leu Lys Gln Asn Glu Leu Gln Tyr Lys Val Leu Ile Ser
65 70 75 80

Asn

192962201/BN

Claims

1. A method in *in vitro* diagnosing severe acute pancreatitis in a patient comprising the steps of

A) bringing a sample of body fluid from the patient into contact with a sufficient amount of

a) at least one first antibody binding to an antigenic site on the carboxypeptidase B activation peptide (CAPAP) having the amino acid sequence SEQ ID NO:1, and

b) at least one second antibody binding to an antigenic site on the CAPAP having the amino acid sequence SEQ ID NO:1,other than the site in a), or

c) a solid-phase bound CAPAP having the amino acid sequence SEQ ID NO:1 or a peptide fragment thereof containing an epitope,

wherein at least one antibody of a) and b) or the peptide of c) is labeled with a non-radioactive label, and

wherein at least one of the first and second antibodies is monospecific for CAPAP, and

B) determining the level of the antibody a) - CAPAP - antibody b) complex formed, or the level of an excess component in the competitive reaction of antibody a) – CAPAP and antibody a) – peptide c) with the aid of the label used, and

C) using the determined level for estimation of the amount of CAPAP in the sample in the diagnosis of severe acute pancreatits in the patient.

2. The method according to claim 1, wherein the determination B) is performed with a double sandwich enzyme-linked immunosorbent assay (ELISA) or a competitive immunosorbent assay.

3. The method according to claim 1 or 2, wherein the label in b) is selected from the group consisting of gold, carbon, and latex particles, and enzymes.

4. The method according to any one of claims 1 - 3, wherein the sample of body fluid in A) is a blood sample, and when the estimated amount of CAPAP in C) is > 6 - 10 nmol/L of urine, the patient is diagnosed positive for severe acute pancreatitis.

5. The method according to any one of claims 1 - 3, wherein the sample of body fluid in A) is a urine sample, and when the estimated amount of CAPAP in C) is > 60 - 100 nmol/L of blood, the patient is diagnosed positive for severe acute pancreatitis.

6. A monoclonal antibody binding to an antigenic site on CAPAP having the amino acid sequence SEQ ID NO:1 or a peptide fragment thereof containing an epitope.

AMENDED SHEET

7. Test kit for severe acute pancreatitis comprising

a) at least one first antibody binding to an antigenic site on the carboxypeptidase B activation peptide (CAPAP) having the amino acid sequence SEQ ID NO:1, and

b) at least one second antibody binding to an antigenic site on the CAPAP having the amino acid sequence SEQ ID NO:1, other than the site in a), or

c) a solid-phase bound CAPAP having the amino acid sequence SEQ ID NO:1 or a peptide fragment thereof containing an epitope,

wherein at least one antibody of a) and b) or the peptide of c) is labeled with a non-radioactive label, and

wherein at least one of the first and second antibodies is monospecific for CAPAP.

8. Test kit according to claim 7, wherein the label is selected from the group consisting of gold, carbon and latex particles, and enzymes.

9. Test kit according to claim 7 or 8, wherein the kit is a dipstick.
