Analytical sandwich test for determining NT-proBNP

The present invention concerns an analytical sandwich test, in particular a test element and in particular in the form of an immunochromatographic test strip using the sandwich principle to determine N-terminal pro-brain natriuretic peptide (NT-proBNP).

NT-proBNP is a very promising marker for the diagnosis and management of heart failure. A review of heart failure and the importance of NT-proBNP as a marker substance for this is for example given in WO 00/45176 on pages 1 to 4 to which reference is herewith explicitly made. In addition the documents US 5,786,163, US 6,461,828, US 6,117,644, EP 1 151 304, WO 02/083913 and the EP Patent Application No. 03 010 591.0 dated 12.5.2003 (Klemt et al.) concern NT-proBNP, antibodies thereto and methods of determination.

At present the only NT-proBNP test that is available on the in-vitro diagnostic market is the fully automated Elecsys® NT-proBNP test from Roche Diagnostics which is based on a sandwich reaction with electrochemiluminescence detection. This test is designed to be used in large central laboratories and in addition to liquid reagents that have to be exactly dosed, requires a relatively complex instrument to dose the liquids and to detect the luminescence signal in order to carry out the test. A simple to use, rapid test for NT-proBNP which if needed can be evaluated visually without an evaluation instrument is presently not on the market.

Rapid tests for immunologically detectable substances have been known for a long time for numerous different parameters, for example from WO 97/06439, EP 0 291 194, US 5,591,645, US 4,861,711, US 5,141,850, US 6,506,612, US 5,458,852, US 5,073,484. In these cases the immunological detection reagents (essentially labelled and unlabelled antibodies or antigens) are usually provided in a dry form on a support which allows the transport of a sample liquid (in particular body fluids such as blood, serum, plasma, urine, saliva etc.) on or in the support. For this purpose the support is preferably capillary active, for example a membrane or a plastic support

provided with capillary channels. Among experts they are often referred to as immunochromatographic test strips or test devices.

In patients with acute respiratory distress it is advantageous to carry out a NT-proBNP determination as rapidly as possible in order to exclude or diagnose heart failure as a cause of the dyspnoea and to initiate appropriate treatment. Since the Elecsys® NT-proBNP test can only be carried out in a central laboratory, it is difficult to rapidly determine NT-proBNP outside the routine times. Hence it would be particularly advantageous for the emergency ward if a rapid test were available which could be carried out directly in the emergency ward outside of routine times. This rapid test should, however, ensure the same reference ranges and cut-offs as the reference method in the central laboratory (Elecsys® NT-proBNP) in order to enable a good comparability of the results independently of the type of test that is actually carried out.

The polyclonal antibodies (PAB) used in the Elecsys® NT-proBNP test recognize a very special fraction of NT-proBNP ("native" NT-proBNP; see EP Patent Application No. 03 010 591.0 dated 12.5.2003 from Klemt et al.; according to this the test recognizes the epitopes of NT-proBNP comprising the amino acids 1-21 (AA 1-21) and 39-50 (AA 39-50)). However, it has turned out that these polyclonal antibodies are unsuitable for NT-proBNP rapid tests that use particulate labels such as colloidal gold as a label since they exhibit a high undesired variability in the signal generation due to physico-chemical interactions with the components of the rapid test (such as the support materials, matrices etc.). This results in considerable fluctuations in the quality of the test from batch to batch.

Hence the object of the present invention was to eliminate the disadvantages of the prior art. In particular the aim is to provide a rapid test for determining NT-proBNP which can be reproducibly manufactured and has a good correlation to the laboratory method.

This object is achieved by the subject matter of the invention.

The invention concerns an immunological test, in particular in the form of a rapid test such as an analytical test element as characterized in the independent patent claims. Preferred embodiments are described in the dependent claims.

The inventive solution for producing an immunological test in a sandwich format and in particular a rapid test which correlates well with the Elecsys® reference method uses a special combination of antibodies comprising at least two antibodies to NT-proBNP where at least one antibody is a monoclonal antibody (MAB). Another antibody of the sandwich test according to the invention can either also be a MAB or a polyclonal antibody (PAB). In this connection one of these antibodies (abbreviated AB) is directed at least against parts of the epitope of NT-proBNP comprising amino acids 38 to 50 (in the following also abbreviated to AB (38-50) or MAB (38-50) or PAB (38-50)). At least one additional antibody is directed at least against parts of the epitope of NT-proBNP comprising amino acids 1 to 37 or 43 to 76 (in the following abbreviated to AB (1-37) or AB (43-76) or MAB (1-37) or MAB (43-76) or PAB (1-37) or PAB (43-76)). The epitopes recognized by the antibodies can slightly overlap preferably by less than 5 amino acids, especially preferably by less than 2 amino acids.

A combination of antibodies is preferred comprising at least one polyclonal antibody (PAB) and one monoclonal antibody (MAB) (so-called PAB/MAB combination) to NT-proBNP.

The term PAB (X-Y) as used in this patent application means a polyclonal antibody which is directed against the epitope of NT-proBNP comprising the amino acids X to Y. MAB (X-Y) is a corresponding monoclonal antibody. AB (X-Y) generally denotes an antibody (e.g. PAB or MAB) which is directed against the epitope of NT-proBNP comprising the amino acids X to Y.

MAB a.b.c. (X-Y) is a monoclonal antibody directed against the epitope of NT-proBNP comprising the amino acids X to Y which is obtained from a deposited cell line a.b.c.

In order to guarantee a reproducible quality of the antibody-label conjugate, the MAB is preferably immobilized on a particulate label, in particular on a gold label.

Other suitable particulate labels are for example coloured latices, other metal sol labels, polymer labels or semiconductor nanocrystals (so-called quantum dots). The MAB-label conjugate is preferably provided on the rapid test device in such a manner that it can be detached from it by the sample liquid, for example by impregnating suitable support materials such as fleeces, membranes etc. It is, however, also possible to add the MAB-label conjugate as a solution to the rapid test.

The PAB which is preferably obtained by immunizing mammals, in particular sheep, goats or rabbits, is preferably provided in the rapid test as a biotin derivative and can be bound to an avidin or streptavidin detection line. However, it also possible to directly immobilize the PAB in the rapid test device, for example in the form of a detection line on a suitable chromatography membrane.

According to the invention it is also possible although less preferred, to use the labelled AB, in particular the labelled MAB, and the second antibody, in particular the second MAB or PAB in solution or in solutions for the rapid test. A binding partner which can capture the appropriately labelled AB is then located in a detection zone on the test device and thus binds the sandwich complex comprising first antibody, analyte and second antibody to a solid phase of the rapid test.

The MAB used according to the invention does not necessarily have to recognize the epitope (AA 1-21) that is detected in the reference system (Elecsys® test) in order to ensure good correlation with the reference test: The antibody combinations mentioned in claim 1 and in particular the MAB/PAB combinations MAB 17.3.1 (13-16)/PAB (39-50) and MAB 18.4.34 (27-31)/PAB (39-50) correlate well with the Elecsys® reference system which uses polyclonal antibodies to the epitopes AA 1-21 and AA 39-50 of NT-proBNP (PAB (1-21) and PAB (39-50)).

The polyclonal antibodies such as PAB (1-21) and PAB (39-50) can be obtained, characterized and identified by methods known to a person skilled in the art especially in analogy to example 2 of WO 00/45176.

The monoclonal antibodies such as MAB (38-42) and MAB (44-50) can be obtained, characterized and identified by methods known to a person skilled in the art

especially in analogy to example 3 of WO 00/45176 or example 3 of the EP Patent Application No. 03 010 591.0 dated 12.5.2003 (Klemt et al.).

The antibodies are labelled for example with gold or other labels, biotin etc. by methods known to a person skilled in the art (cf. also example 2 in WO 00/45176 and example 2 of the EP Patent Application No. 03 010 591.0 dated 12.5.2003 by Klemt et al.). Labelling with gold is for example described in detail in EP-A 0 898 170.

In particular the preferred monoclonal antibodies MAB 17.3.1 (13-16), MAB 16.1.39 (38-42), MAB 18.29.23 (64-67) and MAB 18.4.34 (27-31) can be obtained according to example 3 of the EP Patent Application No. 03 010 591.0 dated 12.5.2003 by Klemt et al. Corresponding cell lines are deposited at the "Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSZM) (accession numbers of the depository and date of deposition: DSM ACC2591 (7th May 2003) for MAB 17.3.1 (13-16); DSM ACC2590 (7th May 2003) for MAB 16.1.39 (38-42); DSM ACC2593 (7th May 2003) for MAB 18.29.23 (64-67) and DSM ACC2592 (7th May 2003) for MAB 18.4.34 (27-31).

The combination of MAB 18.4.34 (27-31) / PAB (39-50) results in a relatively good correlation with the reference test as does the combination MAB 17.3.1 (13-16) / PAB (39-50) (see also example 2).

In addition the combination MAB 18.4.34 (27-31) / PAB (39-50) proved to be particularly advantageous for the test according to the invention: This combination enabled a function curve to be adapted that is particularly suitable for rapid tests (see example 3). In comparison the combination MAB 17.3.1 (13-16) / PAB (39-50) exhibited a poorer test sensitivity.

The invention is further elucidated on the basis of the following examples and figures.

Figure 1 shows a diagram of a preferred embodiment of a rapid test device according to the invention in the form of an immunochromatographic test strip.

Figure 2 shows the correlation of the antibody combination MAB 17.3.1 (13-16) / PAB (39-50) in the Elecsys® wet test format with the Elecsys® reference method PAB (1-21) / PAB (39-50).

Figure 3 shows the correlation of the antibody combination MAB 18.4.34 (27-31) / PAB (39-50) in the Elecsys® wet test format with the Elecsys® reference method PAB (1-21) / PAB (39-50).

Figure 4 shows function curves of NT-proBNP test strips according to example 1 with different antibody combinations.

Figure 5 shows the correlation of an NT-proBNP test strip with the antibody combination: Au-MAB 18.4.34 (27-31) / Bi-PAB (39-50) to the Elecsys® NT-proBNP test kit.

The numbers in the figures denote:

1 sample application zone
2 erythrocyte separation zone
3 detection zone
4 suction zone
5 support material
6 sample application matrix ("biotin fleece" and "gold fleece")
7 erythrocyte separation matrix
8 detection matrix
9 line-shaped immobilization zone
10 suction matrix

Examples

1) **Preparation of a test device for determining NT-proBNP from whole blood (cf. Fig. 1)**

The test device (fig. 1) consists of a support material (5) on which a sample application zone (1), an erythrocyte separation zone (2), a detection zone (3) and a

suction zone (4) are mounted. A sample application matrix (6) which partially overlaps an erythrocyte separation zone (7) is located in the sample application zone (1). The erythrocyte separation matrix (7) in turn slightly overlaps the detection matrix (8) (detection zone) on which an immobilized substance is applied in the form of a line (9). A suction matrix (10) slightly overlaps the detection matrix (8). All reagents that are necessary to form a complex with the analyte to be detected are accommodated in the sample application matrix (6). In the present case the sample application zone is composed of two fleeces on top of one another where the first ("gold fleece") is impregnated with a gold-labelled antibody to NT-proBNP (MAB 18.4.34 (27-31)) and the second fleece ("biotin fleece") contains a biotinylated antibody to NT-proBNP (PAB (39-50)). A line (9) made of streptavidin is applied within the detection zone.

A polyester foil (Pütz) of 350 µm thickness is used as the support layer (5). A polyester fleece (Roche Diagnostics) of 360 µm thickness is used as the "gold fleece" or "biotin fleece" of the sample application matrix (6). A glass fibre fleece (Roche Diagnostics) of 1.8 mm thickness is used as an erythrocyte separation matrix (7). A nitrocellulose membrane (Sartorius) of 140 µm thickness is used as the detection matrix (8). A glass fibre fleece (Roche Diagnostics) of 1.8 mm thickness is used as the suction matrix (10). The individual components (6, 7, 8, 10) are glued slightly overlapping on the support layer (5) by means of hot-melt adhesive as shown in fig. 1.

The impregnation formulation of the "gold and biotin fleeces" is:

"biotin fleece":	100 mM Hepes pH 7.4, 0.1 % Tween®, 20 µg/ml biotinylated PAB (39-50)
"gold fleece":	100 mM Hepes pH 7.4, OD 4 MAB 18.4.34 (27-31) gold conjugate

2) **Correlation of the epitope / antibody combination MAB 17.3.1 (13-16) / PAB (39-50) and MAB 18.4.34 (27-31) / PAB (39-50) in the Elecsys® format to the Elecsys® NT-proBNP test kit (cf. fig. 2 and 3)**

The correlation of the MAB / PAB combinations MAB17.3.1 (13-16) / PAB (39-50) and MAB 18.4.34 (27-31) / PAB (39-50) to the Elecsys® test kit (PAB (1-21) / PAB (30-50)) was examined in an electrochemiluminescence immunoassay on an Elecsys® 2010 (Roche Diagnostics). For this the PAB (39-50) was used as a biotinylated capture reagent and ruthenylated $F(ab')_2$ fragments of the MABs were used as the detection reagent. 20 µl sample or standard material was in each case incubated with 75 µl of the two antibody reagents for 9 minutes at 37°C. Afterwards 35 µl streptavidin-coated magnetic polystyrene particles were added and it was incubated for a further 9 minutes at room temperature. The electroluminescence signal of an aliquot of the incubation solution was measured routinely on the Elecsys® 2010 and converted into a concentration signal by means of a standard curve.

Clinical samples from patients with cardiac failure were now measured with the two MAB/PAB test variants and the Elecsys® kit. The results are shown in figures 2 and 3. A very good correlation to the Elecsys® kit (r=0.978 and r=0.957) was obtained with both MAB/PAB variants.

3) **Function curve of an NT-proBNP test strip with two different MAB/PAB combinations**

An NT-proBNP test strip was prepared according to example 1. The following impregnation formulation for the reagent fleeces was used:

"biotin fleece": 100 mM Hepes pH 7.4, 0.1 % Tween®,
20 µg/ml biotinylated PAB (39-50)

"gold fleece": 100 mM Hepes pH 7.4, OD 4 MAB 18.4.34 (27-31) or MAB 17.3.1 (13-16) gold conjugate

Heparinized blood samples from healthy donors were spiked with sera containing NT-proBNP from heart failure patients and aliquoted. 150 µl of the spiked blood samples was pipetted onto the test strips and measured in a CARDIAC Reader® (Roche Diagnostics). The reaction time after sample detection was 12 minutes. In order to determine the NT-proBNP concentration of the samples, plasma was centrifuged from one aliquot and measured with an Elecsys® NT-proBNP kit (Roche Diagnostics). Function curves obtained in this manner of the two test strip variants MAB 17.3.1 (13-16) / PAB (39-50) and MAB 18.4.34 (27-31) / PAB (39-50) are shown in figure 4. The variant MAB 18.4.34 (27-31) / PAB (39-50) has a considerably steeper standard curve and is thus a more sensitive test.

4) **Correlation of an NT-proBNP test strip with the AB combination: Au-MAB 18.4.34 (27-31) / Bi-PAB (39-50) to the Elecsys® NT-proBNP test kit**

Sera containing NT-proBNP from patients with cardiac failure were added to heparinized blood samples from healthy donors and aliquoted. 150 µl of these "spiked" blood samples was pipetted onto the test strips and measured in a CARDIAC Reader® (Roche Diagnostics) according to the standard method. Plasma was centrifuged from the same sample and measured with the Elecsys® NT-proBNP kit on an Elecsys® 1010 analytical system (Roche Diagnostics). 60 samples were prepared in this manner and measured with both systems. Figure 5 shows the measured values for both systems. The correlation is very good at r=0.95.

CLAIMS

1. Immunological sandwich test for determining NT-proBNP, characterized in that
at least two antibodies to NT-proBNP are used where at least one of these antibodies is an MAB,
and one of these antibodies is directed at least against parts of the epitope of NT-proBNP comprising the amino acids 38 to 50 and
one of these antibodies is directed at least against parts of the epitope of NT-proBNP comprising the amino acids 1 to 37 or 43 to 76 where the epitope recognized by the antibodies can slightly overlap.

2. Immunological test as claimed in claim 1, characterized in that it uses one of the following antibody combinations:
AB (39-50) with: AB (1-21) or AB (13-16) or AB (22-38) or AB (27-31) or AB (51-76) or AB (64-67); or
AB (38-42) with: AB (1-21) or AB (22-37) or AB (43-76); or
AB (44-50) with: AB (1-21) or AB (22-43) or AB (51-76).

3. Immunological test as claimed in claim 1 or 2, characterized in that it uses one of the following PAB/MAB combinations:
PAB (39-50) with: MAB (1-21) or MAB (13-16) or MAB (22-38) or MAB (27-31) or MAB (51-76) or MAB (64-67); or
PAB (38-42) with: MAB (1-21) or MAB (22-37) or MAB (43-76); or
PAB (44-50) with: MAB (1-21) or MAB (22-43) or MAB (51-76).

4. Immunological test as claimed in claim 1, characterized in that it preferably uses the following antibody combinations:
MAB 18.4.34 (27-31) with: PAB (39-50) or PAB (38-42) or PAB (44-50) or
MAB 17.3.1 (13-16) with: PAB (39-50) or PAB (38-42) or PAB (44-50).

5. Immunological test as claimed in claim 1, characterized in that it uses the following antibody combinations:
MAB 18.4.34 (27-31) with MAB (38-42); or
MAB 18.9.8 (27-31) with MAB (38-42).

6. Immunological test as claimed in claim 5, characterized in that MAB (38-42) is the MAB 16.1.39 (38-42).

7. Immunological test as claimed in one of the claims 1 to 6, characterized in that it is an immunological rapid test.

8. Immunological rapid test as claimed in claim 7, characterized in that one of the antibodies is present as an antibody-gold conjugate.

9. Immunological rapid test as claimed in claim 7, characterized in that one of the antibodies is present as a biotinylated antibody.

10. Method for detecting NT-proBNP, characterized in that an immunological test as claimed in one of the claims 1 to 9 is used.