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(11)

EP 2 660 603 A1

(12)

EUROPEAN PATENT APPLICATION
published in accordance with Art. 153(4) EPC

(43) Date of publication:

06.11.2013 Bulletin 2013/45

(51) Int Cl.:

G01N 33/547 (2006.01) **G01N 33/53** (2006.01)
G01N 33/543 (2006.01)

(21) Application number: **11852495.8**

(86) International application number:

PCT/JP2011/007315

(22) Date of filing: **27.12.2011**

(87) International publication number:

WO 2012/090493 (05.07.2012 Gazette 2012/27)

(84) Designated Contracting States:

**AL AT BE BG CH CY CZ DE DK EE ES FI FR GB
GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO
PL PT RO RS SE SI SK SM TR**

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(30) Priority: **28.12.2010 JP 2010294126**

19.12.2011 JP 2011277597

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(54) **IMMUNOLOGICAL ASSAY METHOD**

(57) [Problem] To provide a so-called competitive immunological assay method capable of more uniformly immobilizing on a solid phase a substance equivalent to an antigen. [Solution] This immunological assay method measures an analyte substance in a sample by using: an equivalent substance immobilized on the solid phase during the execution of the assay, the equivalent substance being immunologically equivalent to the analyte substance; and a labeled antibody for specifically binding to the substance equivalent to the analyte substance.

The assay method is characterized in that the equivalent substance is immobilized on the solid phase using: a bond between the equivalent substance and a carrier substance, the carrier substance being a substance to which the labeled antibody does not bind; and a bond between the carrier substance and a specific binding substance immobilized on the solid phase, the specific binding substance being a substance that binds specifically to the carrier substance.

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Description

Field

5 **[0001]** The present invention relates to an immunological assay method for measuring an analyte substance in a sample using: an equivalent substance which is immunologically equivalent to the analyte substance and is immobilized on (bound to) a solid phase during the execution of the measurement; and a labeled antibody which specifically binds to the analyte substance and the equivalent substance.

10 Background

[0002] Specific substances present in the blood may be associated with specific diseases. For example, high levels or low levels of the specific substances out of a certain range may indicate development or seriousness of the specific diseases, and therefore such substances have been intensively measured in the field of clinical diagnosis.

15 **[0003]** There is homocysteine measurement in the blood, for example. Homocysteine is one of intermediate substances produced in the process of methionine metabolism in the body. Produced homocysteine is quickly metabolized through either of the pathways; conversion into methionine, or conversion into cysteine via cystathionine formation. High levels of homocysteine are considered as one of risk factors for cardiovascular disorders and thus the measurement thereof has attracted attention.

20 **[0004]** Immunological assay method is known as a method for measuring substances such as proteins, steroids, vitamins, and other immunologically antigenic substances (substances capable of artificially producing antibodies against the substances) in the blood. As the immunological assay method for homocysteine (Patent Literature 1), what is called a competitive assay method has been known. In the competitive assay method, the blood is used as a sample and homocysteine bound to the component in the blood by the S-S bond is first released. And next, homocysteine is converted

25 into S-adenosylhomocysteine which is an immunologically measurable derivative, by addition of adenosine with the enzyme. Subsequently, this S-adenosylhomocysteine is competitively bound to a labeled anti-S-adenosylhomocysteine antibody with S-adenosylhomocysteine which was prepared in advance and immobilized on a water-insoluble solid phase. Next, homocysteine was measured by measurement of the amount of the labeled antibody bound to the solid phase after what is called a B/F (bound/free) separation procedure.

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Citation List

Patent Literature

35 **[0005]**

Patent Literature 1: Japanese Patent No. 2870704

Summary

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Technical Problem

[0006] In the immunological assay method for homocysteine described in Patent Document 1, the water-insoluble solid phase with S-adenosylhomocysteine immobilized thereon was used. S-adenosylhomocysteine has been generally

45 immobilized on (bound to) the solid phase by: binding S-adenosylhomocysteine to a carrier substance such as bovine serum albumin; and making the carrier substance in this obtained complex to be physically adsorbed to the solid phase. However, it has been difficult to uniformly immobilize (bind) S-adenosylhomocysteine to the solid phase with such immobilization. Therefore, the amount of S-adenosylhomocysteine bound to the solid phase easily varies. The measured results vary as the amount of S-adenosylhomocysteine bound to the solid phase varies, thereby causing lower reproducibility in some cases. When the amount of S-adenosylhomocysteine bound to the solid phase is intended to be

50 controlled in order to avoid lower reproducibility, there has been a problem of complicated steps for quality control in preparation of the solid phase.

[0007] Further, there has been also a problem that some of S-adenosylhomocysteine is not involved in the immunoreaction with the antibody because of the binding to the carrier substance (for example, enclosed in the carrier substance), thereby hindering efficient immunoreaction with anti-S-adenosylhomocysteine labeled antibody. Such a problem may

55 result in lower reproducibility. When this is intended to be avoided, there has been also a problem that a monoclonal antibody or the like whose immunoreaction is not hindered by the binding between S-adenosylhomocysteine and the carrier substance must be used as the anti-S-adenosylhomocysteine labeled antibody.

5 [0008] Although the immunological assay for homocysteine is described above as a specific example, the above-mentioned problems are common problems in terms of an immunological assay method for measuring an analyte substance using: an equivalent substance which is immunologically equivalent to the analyte substance and is immobilized on a solid phase; and a labeled antibody which specifically binds to the analyte substance and the equivalent substance, and in particular, a method in which the equivalent substance is used with a carrier substance being physically adsorbed to the solid phase while the equivalent substance is bound to the carrier substance for immobilizing it on the solid phase.

10 Solution to Problem

[0009] The present inventors have intensively studied to solve the above-mentioned problems found in the conventional technique and completed the present invention.

[0010] The present invention to solve the above-mentioned problems is an immunological assay method, comprising:

15 measuring an analyte substance in a sample by using: an equivalent substance which is immunologically equivalent to the analyte substance and immobilized on a solid phase during the execution of the measurement; and a labeled antibody which specifically binds to the analyte substance and the equivalent substance, wherein the equivalent substance is immobilized on the solid phase by binding between the equivalent substance and a carrier substance which does not bind to the labeled antibody, and binding between the carrier substance and a specific binding substance which specifically binds to the carrier substance and is immobilized on the solid phase.

[0011] The present invention is also an immunological assay reagent for measuring an analyte substance in a sample, comprising;

25 an equivalent substance which is immunologically equivalent to the analyte substance and immobilized on a solid phase during the execution of the measurement; and

a labeled antibody which specifically binds to the analyte substance and the equivalent substance, wherein the equivalent substance is immobilized on the solid phase by binding between the equivalent substance and a carrier substance which does not bind to the labeled antibody, and binding between the carrier substance and a specific binding substance which specifically binds to the carrier substance and is immobilized on the solid phase.

[0012] The present invention is a method for manufacturing an immunological assay reagent for measuring an analyte substance in a sample, including an equivalent substance which is immunologically equivalent to the analyte substance and immobilized on a solid phase during the execution of the measurement; and a labeled antibody which specifically binds to the analyte substance and the equivalent substance, in which the equivalent substance being immobilized on the solid phase by binding between the equivalent substance and a carrier substance which does not bind to the labeled antibody, and binding between the carrier substance and a specific binding substance which specifically binds to the carrier substance and is immobilized on the solid phase, the method comprising:

40 freezing the solid phase with the specific binding substance immobilized thereon in a container; adding a conjugate of the equivalent substance and the carrier substance to the container in which the solid phase is frozen, and further freezing; and freeze-drying a mixture of the frozen solid phase and the frozen conjugate of the equivalent substance and the carrier substance in the container.

45 Advantageous Effects of Invention

[0013] According to the present invention, the reproducibility in the measurement of the analyte substance can be improved. Description of Embodiments

[0014] The immunological assay method of the present embodiment will be described below in detail.

50 [0015] A sample in the present embodiment refers to one containing an analyte substance described below. Examples of the sample include: biological samples containing the analyte substance, exemplified by body fluids such as blood, saliva, and urine; environmental water containing the analyte substance from river, lake, sewage or the like; and food containing the analyte substance. Since the assay method of the present embodiment utilizes the reactions in the liquid phase system, more specifically, the sample as used in the present embodiment refers to a liquid containing the analyte substance. When the analyte substance is originally included in non-liquid materials, the sample can be prepared by: suspension in a liquid and others; or extracting the analyte substance by well-known procedures, and dispersing or dissolving it in the liquid. It may be anticipated that an interfering substance which interferes a binding reaction (hereinafter, also referred to as an immunoreaction in this specification) of the labeled antibody to the analyte substance and the

equivalent substance may be present in the sample. In this case, the interfering substance present together with the analyte substance is preferably reduced until it does not have any influence on the immunoreaction by removing the interfering substance or purifying the analyte substance prior to the assay method of the present embodiment.

5 [0016] The analyte substance is not particularly limited as long as it has a low molecular weight (preferably, molecular weight of 2000 or less) and specifically binds to the labeled antibody described below. Examples of the analyte substance include amino acids such as homocysteine; vitamins such as folic acid, vitamin B₁₂ (cyanocobalamine), and vitamin D (cholecalciferol, calcidiol); proteins such as a thyroid stimulating hormone receptor (TSHR); hormones such as triiodothyronine (T3), thyroxine (T4), 3,5-diiodo-L-thyronine (T2), estrone (E1), estradiol (E2), estriol (E3), progesterone, and cortisol; biopolymers such as carbohydrate chains and lipoids; virus; chemical substances having pharmacological activity; and endocrine disruptors such as bisphenol A.

10 With respect to the analyte substance as used herein, for example, when homocysteine is an analyte substance, the analyte substance conceptually includes the homocysteine which is measured via its derivatives such as S-adenosylhomocysteine.

15 [0017] The equivalent substance used in the assay method of the present embodiment is a substance that is immunologically equivalent to the analyte substance and immobilized on the solid phase. The "immunologically equivalent" means that it is equal to the analyte substance in reactivity with the labeled antibody described below. More specifically, the equivalent substance refers to a substance which the labeled antibody binds at substantially the same ratio as the analyte substance, when the analyte substance and the equivalent substance are present at the same ratio. For example, when analyte substance is a protein, its partial peptide, its subunit, its derivatives, or others are included as examples of the equivalent substance. Among these, the same kind of substance as the analyte substance, which is prepared in advance (hereinafter, may be expressed as "artificially prepared"), for example, by being extracted from the same material as the sample, is particularly preferably used as the equivalent substance. For example, when homocysteine is converted into S-adenosylhomocysteine and the assay method of the present embodiment is applied with the S-adenosylhomocysteine as the analyte substance, artificially prepared S-adenosylhomocysteine is particularly preferably used as the equivalent substance. When the analyte substance is folic acid, artificially prepared folic acid is particularly preferably used as the equivalent substance.

20 [0018] The solid phase on which the equivalent substance is immobilized is not particularly limited as long as having insolubility to the liquid phase which can be separated by a B/F (bound/free) separation procedure generally carried out in the immunological assay method. The solid phase can be made from, for example, natural materials such as agarose and dextran, synthetic polymer materials such as polyethylene and polystyrene, metal materials, and other various water-insoluble materials. The solid phase can be in a particle form, a rod form, or a plate (sheet) form. Also, for example, the inner wall of the container for carrying out the assay method of the present embodiment can be utilized as the solid phase. Among these, a spherical solid phase which can provide a relatively large surface area is preferable because a large amount of a specific binding substance described below can bind thereto. Although the size of the solid phase is not particularly limited, it is preferable to use a small solid phase in order to improve the reactivity with the analyte substance and shorten the time required for the binding reaction of the labeled antibody to the analyte substance and the equivalent substance. Particularly preferably, the solid phase is in the form of fine particles having a particle diameter (particle diameter at an integrated value of 50% in the particle diameter distribution obtained by the laser diffraction/scattering method or other methods) of about 0.3 μm to 10 μm. The solid phase may enclose a magnetic substance for stirring by magnetic force or a B/F separation procedure.

25 [0019] In the present embodiment, in order to immobilize the above-described equivalent substance to the solid phase, both are not directly bound to each other via chemical bonding or physical adsorption. Specifically, the equivalent substance is bound to the carrier substance, while the specific binding substance which specifically binds to the carrier substance is bound to the solid phase. Then, the binding between the carrier substance and the specific binding substance allows the equivalent substance to indirectly bind to the solid phase. The carrier substance and the specific binding substance can be referred to as immobilizing substances which are substances for immobilizing the equivalent substance to the solid phase. As used herein, the immobilization of the equivalent substance to the solid phase via the carrier substance and the specific binding substance is also referred to as indirect immobilization to the solid phase.

30 [0020] The carrier substance is not particularly limited as long as it does not bind to the labeled antibody described below and can bind to the equivalent substance. Examples of the carrier substance include bovine serum albumin (BSA), avidin, biotin, receptor proteins, fluorescein, and DNA. Among these, fluorescein or BSA which is easily available and cheap can be exemplified as suitable carrier substances. The carrier substance preferably binds to the equivalent substance via chemical bonding. The reason is that the binding sites of the carrier substance and the equivalent substance can be artificially controlled. Note that the carrier substance does not need to be a single and uniform molecule and may be a mixture of two or more kinds thereof.

35 [0021] The specific binding substance is not particularly limited as long as it can specifically bind to the carrier substance, and biotin for avidin, receptors for hormones and the like can be exemplified. Among these, when an antigenic substance such as fluorescein and BSA is used as the carrier substance, the antibody for the above carrier substance, which is

easily manufactured and moreover has sufficient specificity, is preferably used. Note that the specific binding substance does not need to be a single and uniform molecule and may be a mixture of two or more kinds thereof. Further, it is not necessary for the specific binding substance to specifically bind to the carrier substance itself. For example, the carrier substance is fluoresceinated, while a substance (for example, anti-fluorescein antibody) which specifically binds to fluoresceine can be used as the specific binding substance. The solid phase and the specific binding substance bind to each other, for example, by chemical means such as a covalent bond, or by adsorption.

[0022] The equivalent substance may be indirectly immobilized on the solid phase in advance before use. Or the sample is subjected to the measurement while the carrier substance is not bound to the specific binding substance, and then the equivalent substance may be indirectly immobilized on the solid phase during the execution of the assay method of the present embodiment. Specifically, the equivalent substance may be immobilized on the solid phase during the reaction in which the analyte substance and the equivalent substance are brought into contact with the labeled antibody in a free state to cause specific binding, or after the reaction in which the analyte substance and the equivalent substance are specifically bound to the labeled antibody.

As used herein, "the equivalent substance is immobilized on the solid phase during the execution of the measurement" conceptually includes the following cases: where the equivalent substance is immobilized on the solid phase before the measurement; where the equivalent substance is immobilized on the solid phase during the reaction in which the analyte substance and the equivalent substance are brought into contact with the labeled antibody in a free state to cause specific binding; and where the equivalent substance is immobilized on the solid phase after the reaction in which the analyte substance and the equivalent substance are specifically bound to the labeled antibody.

[0023] In the present embodiment, the labeled antibody is used, in which a labeling substance producing a detectable signal is bound to the antibody which specifically binds to the analyte substance and the equivalent substance. As described above, when the analyte substance itself is used as the equivalent substance, the labeled antibody can be easily prepared by using the analyte substance as immunogen. Further, the labeled antibody may be a mixture of two or more kinds thereof or may be a single and uniform antibody, and a polyclonal antibody or a monoclonal antibody can be used. Among these, a monoclonal antibody is particularly preferable. The reason are that the manufacturing method itself is already established; a desired amount of antibody can be obtained only by culturing a hybridoma, an antibody-forming cell, once the hybridoma is established; and also the reactivity is uniform.

[0024] The labeling substance producing a signal is a substance that can be detected by, for example, optical detection or radioactive detection of itself, or can be, for example, optically detected by acting on other substance(s). Specific examples of the labeling substance may include enzymes, chemiluminescent substances, bioluminescent substances, radioisotopes, and color substances. The binding between the labeling substance and the antibody can be formed, for example, by chemical means such as a covalent bond, or by physical binding between affinity substances bound to the both. In the present embodiment, the labeling substance and the antibody can be bound to each other in advance and used as the labeled antibody, or both can also be bound to each other by the binding of affinity substances such as avidin-biotin while the present embodiment is carried out.

[0025] When the analyte substance, the equivalent substance, and the labeled antibody which are described above are mixed, the analyte substance and the equivalent substance competitively bind to the labeled antibody. If the analyte substance derived from the sample is present in a large amount, the amount of the equivalent substance bound to the labeled antibody is relatively reduced. Since the equivalent substance is indirectly bound to the solid phase at the end, the B/F separation procedure is carried out after the immunoreaction for a certain period of time, and then a signal depending on the amount of the labeling substance present in the liquid phase, or depending on the amount of the labeling substance forming a complex with the solid phase is measured. The measurement of the signal enables, for example, detection of the analyte substance, or measurement of the amount, the concentration, and others of the analyte substance in the sample. In the present embodiment, preferably, the analyte substance, the equivalent substance, and the labeled antibody are brought into contact with each other in a free state (without the equivalent substance bound to the carrier substance being bound to the solid phase) to cause the immunoreaction. Preferably, during this process, or after their immunoreaction, the carrier substance bound to the equivalent substance is bound to the specific binding substance bound to the solid phase, thereby immobilizing the equivalent substance on the solid phase. This allows the analyte substance, the equivalent substance, and the labeled antibody to react with each other quickly and can improve the reproducibility of the measured value as a result. In order to carry out such measurement, the following processes can be exemplified: first supplying only the analyte substance, the equivalent substance and the labeled antibody to a reaction container; and subsequently supplying the solid phase with the specific binding substance bound thereto.

In addition, the assay method can be exemplified, in which a reagent prepared by freezing the equivalent substance and the solid phase without being bound to each other, more preferably a reagent prepared by freeze-drying after the freezing is used as an assay reagent; a sample including the analyte substance is supplied to dissolve a dried product; and thus the reaction between the analyte substance, the equivalent substance, and the labeled antibody coincides with the reaction between the equivalent substance and the solid phase.

The reagent prepared by freeze-drying after the freezing can be manufactured by: for example, freezing the solid phase

with the specific binding substance immobilized thereon in the container; adding a conjugate of the equivalent substance and the carrier substance to the container in which the solid phase is frozen, and further freezing it; and subsequently freeze-drying a mixture of the frozen solid phase and the frozen conjugate of the equivalent substance and the carrier substance in the container.

5 The amount, the ratio, and others of the components included in the reagent can be appropriately set according to the type of a target sample and others.

[0026] According to the present embodiment, the analyte substance in the sample can be reproducibly measured. Therefore, the present embodiment provides the assay method, the reagent, and the manufacturing method therefor which enable the measurement of, for example, the analyte substance in a low concentration with good reproducibility and good accuracy.

10 [0027] Also, in the present embodiment, in order to bind the equivalent substance to the surface of the solid phase, the carrier substance is not directly bound to the solid phase, but bound to the solid phase via the specific binding substance. As a result, this reduces the contact area of the carrier substance on the surface of the solid phase and enables binding of more equivalent substances. Such binding of the equivalent substances allows the equivalent substance to bind to the solid phase while being involved in the immunoreaction. In this way, according to the present embodiment, the effect associated with the above reproducibility is achieved and furthermore the amount of the equivalent substance required for the measurement can be also reduced.

15 [0028] The present embodiment is effective particularly for the case where, for example, the analyte substance is S-adenosylhomocysteine or folic acid, in which only binding of the equivalent substance to the carrier substance causes a problem of difficulty in uniformly immobilizing the equivalent substance to the surface of the solid phase. Application of the present embodiment, for example, can uniform the amount of S-adenosylhomocysteine (or folic acid) bound to the surface of the solid phase and avoid variation of the measured results associated with variation of the bound amount, thereby achieving good reproducibility. Conventionally, in order to avoid this problem, S-adenosylhomocysteine (or folic acid) more than necessary has been consumed to control the amount of S-adenosylhomocysteine (or folic acid) bound to the surface of the solid phase, and moreover complicated steps for quality control have been required in preparation of the solid phase, which can be skipped in the present embodiment.

EXAMPLE

30 [0029] Hereinafter, although the present invention will be further described by way of Examples of homocysteine, an example of amino acids, and folic acid, an example of vitamins, the present invention is not limited thereto. In the following description, a fluoresceinated S-adenosylhomocysteine solution may be expressed as a solution (A), beads with anti-fluorescein antibodies immobilized thereon as beads (B), an ALP-labeled anti-S-adenosylhomocysteine antibody solution as a solution (C), beads with S-adenosylhomocysteine directly immobilized thereon as beads (D), a fluoresceinated folic acid solution as a solution (E), an ALP-labeled anti-folic acid antibody solution as a solution (F), and beads with folic acid directly immobilized thereon as beads (G).

Example 1: Assay Reagent for Homocysteine, Manufacture Thereof, and Measurement Using Same

40 (1) Preparation of Fluoresceinated

S-adenosylhomocysteine Solution

45 [0030] Zero point five mg of a conjugate (SAH-BSA) of commercially available S-(5'-adenosyl)-L-homocysteine (produced by Sigma-Aldrich Corporation) (hereinafter, referred to as S-adenosylhomocysteine or SAH) and bovine serum albumin (BSA) was dissolved in 0.25 mL of borate buffer (50 mmol/L, pH 9.0). Five equivalents of 6-(Fluorescein-5-carboxamide) hexanoic acid succinimidyl ester (5-SFX) dissolved in N,N-dimethylformamide was added thereto and allowed to react at 37°C for 3 hours to make fluoresceinated S-adenosylhomocysteine. This was diluted with 0.1 mol/L Tris buffer (pH 7.5) including BSA to produce a fluoresceinated S-adenosylhomocysteine solution (A). S-adenosylhomocysteine in the solution (A) specifically reacts with the solution (C) prepared below and its reactivity was equal to that of S-adenosylhomocysteine which was obtained by treating a blood sample. BSA in the solution (A) did not react with the solution (C) prepared below.

55 (2) Preparation of Solid Phase with Anti-fluorescein Antibody Immobilized thereon

[0031] Spherical plastic beads having a diameter of about 2 mm were used as a solid phase. Per plastic bead, 0.1 μg of anti-fluorescein antibodies which specifically bind to fluorescein were added and incubated at 30°C for 17 hours, allowing the antibodies to be adsorbed to the beads. The washed beads were placed in a 1% BSA solution controlled

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at 53°C for 3 hours for blocking and thus beads (B) with anti-fluorescein antibodies immobilized thereon were obtained.

(3) Preparation of Labeled Antibody Solution for Detection

5 **[0032]** A mouse was immunized with commercially available S-adenosylhomocysteine as an antigen to prepare anti-S-adenosylhomocysteine antibodies which specifically bind to S-adenosylhomocysteine. The prepared antibodies were bound to alkaline phosphatase (ALP), which were then diluted with 0.1 mol/L Tris buffer (pH 7.5) including BSA to produce an ALP-labeled anti-S-adenosylhomocysteine antibody solution (C).

10 (4) Measurement of Standard Solution

[0033] Commercially available S-adenosylhomocysteine was diluted with 0.1 mol/L Tris buffer (pH 7.5) to prepare standard solutions for homocysteine measurement of the concentrations shown in Table 1. The buffer without S-adenosylhomocysteine was used as a standard solution of zero concentration. The solution (A), the standard solution, and the solution (C) were added to the beads (B), and the mixture was set in a commercially available immunological assay analyzer (trade name: AIA-600II, manufactured by Tosoh Corporation) and allowed to react at 37°C for 10 minutes. After a B/F separation procedure, 4-methylumbelliferyl phosphate (4MUP), a substrate of ALP, was added thereto, and the increasing rate (nmol/L/second) of 4-methylumbelliferone (4MU), which was produced by decomposition of 4MUP by ALP, was calculated by measuring the fluorescence intensity of 4MU. About 0.08 µg of the solution (A) was used per reaction.

20 **[0034]** The results are shown in Table 1. The results in Table 1 represent the mean values obtained by repeating the same procedure 5 times for the standard solution of each concentration.

25 (5) Measurement of Blood Sample

[0035] A reducing agent, an enzyme for adding adenosine, and adenosine were added to a blood sample taken from a healthy person with his/her consent, and the mixture was incubated at 37°C for 10 minutes to convert homocysteine in the sample into S-adenosylhomocysteine, followed by the immunological assay. The solution (A), the treated sample, and the solution (C) were added to the beads (B), which was set in the commercially available immunological assay analyzer and allowed to react at 37°C for 10 minutes. After a B/F separation procedure, 4MUP was added thereto and the increasing rate (nmol/L/second) of 4MU was calculated by measuring the fluorescence intensity of 4MU.

30 **[0036]** The results are shown in Table 1. The results in Table 1 represent the mean values obtained by repeating the same procedure 5 times. The concentrations of the samples in the table are the values measured by the HPLC method.

35 Comparative example 1

(1) Preparation of Solid Phase with Equivalent Substance Directly Immobilized thereon

[0037] Per spherical plastic bead having a diameter of about 2 mm, 0.01 µg of the SAH-BSA and 0.2 µg of BSA were added and incubated at 30°C for 17 hours, allowing S-adenosylhomocysteine to be adsorbed to the beads. The washed beads were placed in a 0.1% blocking agent (trade name: Block Ace, produced by DS Pharma Biomedical Co., Ltd.) solution controlled at 53°C for 3 hours for blocking and thus beads (D) with S-adenosylhomocysteine directly immobilized thereon were obtained.

45 (2) Measurement of Standard Solution

[0038] The same standard solution as that in Example 1 and the solution (C) were added to the beads (D), which was set in the commercially available immunological assay analyzer and allowed to react at 37°C for 10 minutes. After a B/F separation procedure, 4MUP was added thereto and the increasing rate (nmol/L/second) of 4MU was calculated by measuring the fluorescence intensity of 4MU. About 0.12 µg of the SAH-BSA was used per reaction.

50 **[0039]** The results are shown in Table 1. The results in Table 1 represent the mean values obtained by repeating the same procedure 5 times for the standard solution of each concentration.

55 (3) Measurement of Blood Sample

[0040] A sample treated as described in Example 1 (5) and the solution (C) were added to the beads (D), which was set in the commercially available immunological assay analyzer and allowed to react at 37°C for 10 minutes. After a B/F separation procedure, 4MUP was added thereto and the increasing rate (nmol/L/second) of 4MU was calculated by

measuring the fluorescence intensity of 4MU.

[0041] The results are shown in Table 1. The results in Table 1 represent the mean values obtained by repeating the same procedure 5 times.

[Table 1]

| | | Solid Phase | | |
|---|--|-------------------------------|--|-------------------------------|
| | | Example 1 | | Comparative Example 1 |
| Concentration of Standard Solution | Mean Value of Increasing Rate of Fluorescence Intensity (mean) | Coefficient of Variation (CV) | Mean Value of Increasing Rate of Fluorescence Intensity (mean) | Coefficient of Variation (CV) |
| $\mu\text{mol/L}$ | nmol/L/s | % | nmol/L/s | % |
| 0 | 30.62 | 0.97 | 33.16 | 2.66 |
| 2.10 | 20.50 | 1.38 | 22.54 | 3.03 |
| 4.12 | 15.43 | 1.74 | 16.62 | 2.35 |
| 8.14 | 10.06 | 1.29 | 10.66 | 4.78 |
| 15.1 | 6.217 | 2.37 | 6.972 | 3.62 |
| 50.2 | 2.498 | 1.69 | 2.955 | 3.40 |
| | | Solid Phase | | |
| | | Example 1 | | Comparative Example 1 |
| Concentration of Sample | Mean Value of Increasing Rate of Fluorescence Intensity (mean) | Coefficient of Variation (CV) | Mean Value of Increasing Rate of Fluorescence Intensity (mean) | Coefficient of Variation (CV) |
| $\mu\text{mol/L}$ | nmol/L/s | % | nmol/L/s | % |
| 9.9 | 9.641 | 2.78 | 10.18 | 14.3 |
| 11.7 | 8.435 | 5.18 | 8.759 | 3.34 |
| 24.9 | 4.640 | 5.93 | 5.168 | 9.52 |
| 13.5 | 7.144 | 8.80 | 7.911 | 9.60 |
| 7.0 | 11.68 | 2.37 | 12.17 | 1.62 |
| CV: Standard Deviation of Increasing Rate / mean \times 100 | | | | |

[0042] Table 1 shows that the coefficients of variation of the measured results for the standard solution in Example 1, in which the assay method of the present embodiment was carried out, are smaller than those in the method of Comparative Example 1, and thus, the reproducibility of repetitive measurements is improved. For the sample, although the coefficients of variation of some measured results in Example 1 are larger than those in the method of Comparative Example 1, there is no sample with more than 10% of coefficient of variation. Accordingly, it is said that the reproducibility of the assay method of the present embodiment is improved in total. This indicates that the equivalent substance is uniformly immobilized on the entire surface of the solid phase in the method of the present embodiment, in which the equivalent substance is bound to the solid phase via the specific binding substance such as an antibody. In addition, the amount of the equivalent substance used per measurement in the method of the present embodiment is about two thirds of the amount used in the immobilization method without anti-fluorescein antibodies, which shows that even small amount of the equivalent substance can cause the immunoreaction efficiently.

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Example 2: Freeze-Dried Assay Reagent for Homocysteine, Manufacture Thereof, and Measurement Using Same

(1) Manufacture of Freeze-Dried Reagent

5 **[0043]** First, the beads (B) and the solution (C) were placed in a plastic container and frozen. Subsequently, the solution (A) was further added thereto and frozen. The obtained frozen reagent was dried thereby to manufacture a reagent (freeze-dried product) for homocysteine measurement.

(2) Measurement

10 **[0044]** The standard solution of zero concentration was added to the freeze-dried reagent manufactured as described above. The increasing rate (nmol/L/second) of 4MU, which was produced by decomposition of 4MUP by ALP, was calculated by measuring the fluorescence intensity of 4MU with the commercially available immunological assay analyzer as in Example 1.

15 **[0045]** The results are shown in Table 2. The results in Table 2 represent the mean values obtained by repeating the same procedure 5 times.

Example 3

(1) Manufacture of Freeze-Dried Reagent

20 **[0046]** First, the solution (A) and the beads (B) were placed in a plastic container and bound to each other, which was then frozen. Subsequently, the solution (C) was further added thereto and frozen. The obtained frozen reagent was dried thereby to manufacture a reagent (freeze-dried product) for homocysteine measurement.

25 **[0047]** The standard solution of zero concentration was added to the freeze-dried reagent manufactured as described above. The increasing rate (nmol/L/second) of 4MU, which was produced by decomposition of 4MUP by ALP, was calculated by measuring the fluorescence intensity of 4MU with the commercially available immunological assay analyzer as described above.

30 **[0048]** The results are shown in Table 2. The results in Table 2 represent the mean values obtained by repeating the same procedure 5 times.

[0049]

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[Table2]

| Solid Phase | Increasing Rate of Fluorescence Intensity nmol/L/s | | | | | Mean of Increasing Rate (mean) nmol/L/s | Standard Deviation (SD) - | Coefficient of Variation (CV) % |
|-------------|---|-------|-------|-------|-------|---|------------------------------|------------------------------------|
| Example 2 | 34.87 | 36.21 | 31.66 | 32.10 | 34.32 | 33.83 | 1.915 | 5.66 |
| Example 3 | 33.27 | 40.68 | 36.94 | 32.12 | 34.15 | 35.43 | 3.430 | 9.68 |

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50 **[0050]** Table 2 shows that in the case such as Example 2 in which the reagent is manufactured by the manufacturing method where the equivalent substance is not immobilized on the solid phase in a manufacturing stage of the reagent and the equivalent substance is immobilized to the solid phase during the measurement, the coefficients of variation of the measured results are smaller than those in the method of Example 3 where the equivalent substance is bound to the solid phase in advance and subsequently freeze-dried, and thus, the reproducibility of repetitive measurements is improved. This indicates that when the equivalent substance is in a free state without being immobilized on the solid phase at the time when the immunoreaction is initiated by addition of the sample (in the case of Examples 2 and 3, the standard solution of zero concentration) to the freeze-dried reagent, the reaction between the labeled antibody and the analyte substance and the reaction between the labeled antibody and the equivalent substance are accelerated.

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Example 4: Immunoreaction Reagent for Folic Acid, Manufacture Thereof, and Measurement Using Same

(1) Preparation of Fluoresceinated Folic Acid Solution

5 **[0051]** Ten mg of a conjugate (BSA-FOL) of commercially available folic acid (produced by Wako Pure Chemical Industries, Ltd.) and bovine serum albumin were dissolved in 4.4 mL of borate buffer (50 mmol/L, pH 9.0). Five equivalents of 5-SFX dissolved in N,N-dimethylformamide was added thereto, which was allowed to react at 37°C for 3 hours to make fluoresceinated folic acid. This was diluted with 0.05 mol/L Tris buffer (pH 7.5) including BSA to produce a fluo-
10 resceinated folic acid solution (E).

(2) Preparation of Labeled Antibody Solution for Detection

[0052] A mouse was immunized with commercially available folic acid as an antigen to prepare anti-folic acid antibodies which specifically bind to folic acid. The prepared antibodies were bound to ALP, which were then diluted with 0.05
15 mol/L Tris buffer (pH 7.5) including BSA to produce an ALP-labeled anti-folic acid antibody solution (F).

(3) Measurement of Standard Solution

[0053] Commercially available folic acid (produced by Wako Pure Chemical Industries, Ltd.) was diluted with 0.01
20 mol/L phosphate buffer (pH 7.5) to prepare standard solutions for folic acid measurement of the concentrations shown in Table 3. The buffer without folic acid was used as a standard solution of zero concentration. The solution (E), the standard solution, and the solution (F) were added to the beads (B), which was set in the commercially available immunological assay analyzer and allowed to react at 37°C for 40 minutes. After a B/F separation procedure, 4MUP was
25 added thereto and the increasing rate (nmol/L/second) of 4MU was calculated by measuring the fluorescence intensity of 4MU. The results are shown in Table 3. The results in Table 3 represent the mean values obtained by repeating the same procedure 3 times for the standard solution of each concentration.

(4) Measurement of Blood Sample

30 **[0054]** A reducing agent was added to a blood sample taken from a healthy person with his/her consent, and the mixture was incubated at 37°C for 10 minutes to release folic acid from a folate-binding protein in the sample, followed by the immunoreaction. The solution (E), the treated sample, and the solution (F) were added to the beads (B), which was set in the commercially available immunological assay analyzer and allowed to react at 37°C for 40 minutes. After
35 a B/F separation procedure, 4MUP was added thereto and the increasing rate (nmol/L/second) of 4MU was calculated by measuring the fluorescence intensity of 4MU.

[0055] The results are shown in Table 3. The results in Table 3 represent the mean values obtained by repeating the same procedure 3 times. The concentrations of the samples in the table are the values measured by AIA-PACK FOLATE (produced by Tosoh Corporation) and the commercially available immunological assay analyzer (AIA-600II, manufac-
40 tured by Tosoh Corporation).

Comparative example 2

(1) Preparation of Solid Phase with Folic Acid Directly Immobilized thereon

45 **[0056]** Per spherical plastic bead having a diameter of about 2 mm, 0.03 μg of BSA-FOL and 0.2 μg of BSA were added and incubated at 30°C for 17 hours, allowing folic acid to be adsorbed to the beads. The washed beads were placed in a 0.1% blocking agent (trade name: Block Ace, produced by DS Pharma Biomedical Co., Ltd.) solution controlled at 53°C for 3 hours for blocking and thus beads (G) with folic acid immobilized thereon were obtained.

(2) Measurement of Standard Solution

[0057] The same standard solution as that in Example 3 and the solution (F) were added to the beads (G), and the mixture was set in the commercially available immunological assay analyzer and allowed to react at 37°C for 40 minutes. After a B/F separation procedure, 4MUP was added thereto and the increasing rate (nmol/L/second) of 4MU, which was
55 produced by decomposition of 4MUP by ALP, was calculated by measuring the fluorescence intensity of 4MU.

[0058] The results are shown in Table 3. The results in Table 3 represent the mean values obtained by repeating the same procedure 3 times for the standard solution of each concentration.

(3) Measurement of Blood Sample

[0059] A sample treated as described in Example 3 (4) and the solution (F) were added to the beads (G), and the mixture was set in the commercially available immunological assay analyzer and allowed to react at 37°C for 40 minutes. After a B/F separation procedure, 4MUP was added thereto and the increasing rate (nmol/L/second) of 4MU was calculated by measuring the fluorescence intensity of 4MU.

[0060] The results are shown in Table 3. The results in Table 3 represent the mean values obtained by repeating the same procedure 3 times.

[0061]

[Table 3]

| | | Solid Phase | | |
|--|--|-------------------------------|--|-------------------------------|
| | | Example 4 | | Comparative Example 2 |
| Concentration of Standard Solution | Mean Value of Increasing Rate of Fluorescence Intensity (mean) | Coefficient of Variation (CV) | Mean Value of Increasing Rate of Fluorescence Intensity (mean) | Coefficient of Variation (CV) |
| ng/mL | nmol/L/s | % | nmol/L/s | % |
| 0 | 65.36 | 0.457 | 41.94 | 33.5 |
| 1.29 | 49.84 | 1.76 | 31.22 | 88.7 |
| 3.18 | 37.08 | 1.23 | 20.47 | 71.7 |
| 6.51 | 23.63 | 1.37 | 42.44 | 20.3 |
| 13.1 | 15.43 | 0.499 | 27.92 | 15.8 |
| 25.7 | 8.568 | 1.05 | 28.06 | 51.0 |
| | | Solid Phase | | |
| | | Example 4 | | Comparative Example 2 |
| Concentration of Sample | Mean Value of Increasing Rate of Fluorescence Intensity (mean) | Coefficient of Variation (CV) | Mean Value of Increasing Rate of Fluorescence Intensity (mean) | Coefficient of Variation (CV) |
| ng/mL | nmol/L/s | % | nmol/L/s | % |
| 1.80 | 45.29 | 0.579 | 17.09 | 90.4 |
| 2.15 | 39.85 | 2.15 | 28.20 | 44.4 |
| 3.46 | 37.86 | 0.700 | 23.30 | 48.7 |
| 6.60 | 25.10 | 1.01 | 11.53 | 40.1 |
| 15.5 | 11.02 | 1.92 | 21.39 | 25.4 |
| CV: Standard Deviation of Increasing Rate / mean×100 | | | | |

[0062] Table 3 shows that the competitive reaction of folic acid occurs in the standard solutions and the samples and the increasing rate of the fluorescence intensity of 4MU depends on the concentration of folic acid in Example 3. The coefficients of variation also fall within the acceptable range of this measurement system. This indicates that in the method of the present embodiment where the equivalent substance is bound to the solid phase via the specific binding substance (antibody), the equivalent substance is uniformly immobilized on the entire surface of the solid phase and involved in the immunoreaction. On the other hand, in Comparative Example 2 where the equivalent substance is bound to the carrier substance and this carrier substance is directly bound to the solid phase, the competitive reaction does not occur. Thus, it was found that Comparative Example 2 was not applicable to the measurement of folic acid in the sample.

Claims

- 5 1. An immunological assay method, comprising
 measuring an analyte substance in a sample by using: an equivalent substance which is immunologically equivalent
 to the analyte substance and immobilized on a solid phase during the execution of the measurement; and a labeled
 antibody which specifically binds to the analyte substance and the equivalent substance,
 wherein the equivalent substance is immobilized on the solid phase by binding between the equivalent substance
 and a carrier substance which does not bind to the labeled antibody, and binding between the carrier substance
 and a specific binding substance which specifically binds to the carrier substance and is immobilized on the solid
 10 phase.
- 15 2. The assay method according to claim 1, wherein the analyte substance is S-adenosylhomocysteine which is derived
 from a blood component, the equivalent substance is artificially prepared S-adenosylhomocysteine, and the carrier
 substance is fluorescein or bovine serum albumin.
- 20 3. The assay method according to claim 1, wherein the analyte substance is folic acid which is derived from a blood
 component, the equivalent substance is artificially prepared folic acid, and the carrier substance is fluorescein or
 bovine serum albumin.
- 25 4. The assay method according to any one of claims 1 to 3, wherein the equivalent substance is immobilized on the
 solid phase during a process in which the analyte substance and the equivalent substance are brought into contact
 with the labeled antibody in a free state to cause binding, or after the analyte substance and the equivalent substance
 are bound to the labeled antibody.
- 30 5. An immunological assay reagent for measuring an analyte substance in a sample, comprising:
 an equivalent substance which is immunologically equivalent to the analyte substance and immobilized on a
 solid phase during the execution of the measurement; and
 a labeled antibody which specifically binds to the analyte substance and the equivalent substance,
 wherein the equivalent substance is immobilized on the solid phase by binding between the equivalent substance
 and a carrier substance which does not bind to the labeled antibody, and binding between the carrier substance
 and a specific binding substance which specifically binds to the carrier substance and is immobilized on the
 solid phase.
- 35 6. The assay reagent according to claim 5, wherein the analyte substance is S-adenosylhomocysteine which is derived
 from a blood component, the equivalent substance is artificially prepared S-adenosylhomocysteine, and the carrier
 substance is fluorescein or bovine serum albumin.
- 40 7. The assay reagent according to claim 5, wherein the analyte substance is folic acid which is derived from a blood
 component, the equivalent substance is artificially prepared folic acid, and the carrier substance is fluorescein or
 bovine serum albumin.
- 45 8. A method for manufacturing an immunological assay reagent for measuring an analyte substance in a sample,
 including an equivalent substance which is immunologically equivalent to the analyte substance and immobilized
 on a solid phase during the execution of the measurement; and a labeled antibody which specifically binds to the
 analyte substance and the equivalent substance, in which the equivalent substance being immobilized on the solid
 phase by binding between the equivalent substance and a carrier substance which does not bind to the labeled
 antibody, and binding between the carrier substance and a specific binding substance which specifically binds to
 the carrier substance and is immobilized on the solid phase, the method comprising:
 50 freezing the solid phase with the specific binding substance immobilized thereon in a container;
 adding a conjugate of the equivalent substance and the carrier substance to the container in which the solid
 phase is frozen, and further freezing; and
 freeze-drying a mixture of the frozen solid phase and the frozen conjugate of the equivalent substance and the
 carrier substance in the container.
- 55 9. The manufacturing method according to claim 8, wherein the analyte substance is S-adenosylhomocysteine which
 is derived from a blood component, the equivalent substance is artificially prepared S-adenosylhomocysteine, and

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the carrier substance is fluorescein or bovine serum albumin.

- 5 **10.** The manufacturing method according to claim 8, wherein the analyte substance is folic acid which is derived from a blood component, the equivalent substance is artificially prepared folic acid, and the carrier substance is fluorescein or bovine serum albumin.

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2011/007315

| A. CLASSIFICATION OF SUBJECT MATTER G01N33/547(2006.01) i, G01N33/53(2006.01) i, G01N33/543(2006.01) i | | |
|--|--|---|
| According to International Patent Classification (IPC) or to both national classification and IPC | | |
| B. FIELDS SEARCHED | | |
| Minimum documentation searched (classification system followed by classification symbols) G01N33/547, G01N33/53, G01N33/543 | | |
| Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Jitsuyo Shinan Koho 1922-1996 Jitsuyo Shinan Toroku Koho 1996-2012 Kokai Jitsuyo Shinan Koho 1971-2012 Toroku Jitsuyo Shinan Koho 1994-2012 | | |
| Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) JSTPlus/JMEDPlus/JST7580 (JDreamII) | | |
| C. DOCUMENTS CONSIDERED TO BE RELEVANT | | |
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| Y/A | Doncheva N, Penkov A, Velcheva A, Boev M, Popov B, Niagolov Y., Study of homocysteine concentration in coronary heart disease patients and comparison of two determination methods., Ann Nutr Metab., 2007, Vol.51, No.1, Page.82-87 | 1, 4, 5/2, 3, 6-10 |
| Y/A | JP 2870704 B1 (Axis Research A/S), 17 March 1999 (17.03.1999), entire text & US 6063581 A & GB 9204922 D & EP 623174 A & EP 726322 A1 & WO 1993/015220 A1 | 1, 4, 5/2, 3, 6-10 |
| <input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex. | | |
| * Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "I" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family | | |
| Date of the actual completion of the international search 22 February, 2012 (22.02.12) | | Date of mailing of the international search report 06 March, 2012 (06.03.12) |
| Name and mailing address of the ISA/ Japanese Patent Office | | Authorized officer |
| Facsimile No. | | Telephone No. |

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2011/007315

| C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT | | |
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|----------------|--|---------|------------|
| 专利名称(译) | 免疫学测定方法 | | |
| 公开(公告)号 | EP2660603A1 | 公开(公告)日 | 2013-11-06 |
| 申请号 | EP2011852495 | 申请日 | 2011-12-27 |
| [标]申请(专利权)人(译) | 东曹株式会社 | | |
| 申请(专利权)人(译) | 东曹公司 | | |
| 当前申请(专利权)人(译) | 东曹公司 | | |
| [标]发明人 | FURUTA YUKI SHINTANI KOJI | | |
| 发明人 | FURUTA, YUKI SHINTANI, KOJI | | |
| IPC分类号 | G01N33/547 G01N33/53 G01N33/543 | | |
| CPC分类号 | G01N33/543 G01N33/54306 | | |
| 代理机构(译) | TBK | | |
| 优先权 | 2011277597 2011-12-19 JP 2010294126 2010-12-28 JP | | |
| 其他公开文献 | EP2660603B1 EP2660603A4 | | |
| 外部链接 | Espacenet | | |

摘要(译)

[问题]提供一种所谓的竞争性免疫学测定方法，其能够在固相上更均匀地固定相当于抗原的物质。[解决方案]该免疫学测定方法通过使用以下方法测量样品中的分析物物质：在测定执行期间固定在固相上的等效物质，等效物质与分析物物质在免疫学上等同；和用于特异性结合相当于分析物物质的物质的标记抗体。该测定方法的特征在于使用等效物质和载体物质之间的键将等同物质固定在固相上，载体物质是标记抗体不结合的物质；和载体物质与固定在固相上的特异性结合物质之间的键，特异性结合物质是与载体物质特异性结合的物质。

| INTERNATIONAL SEARCH REPORT | | International application No. EP2011852495 |
|--|---|---|
| A. CLASSIFICATION OF SUBJECT MATTER G01N33/547(G01N33/53), G01N33/53(2006.01), G01N33/543(2006.01) | | |
| According to International Patent Classification (IPC) or to both national classification and IPC | | |
| B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) G01N33/547, G01N33/53, G01N33/543 | | |
| Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched JSTARS Shintani Hoho 1999-2012 JSTARS Shintani Hoho 1999-2012 JSTARS Shintani Hoho 1999-2012 Tokoku Jitsuyou Shintani Hoho 1999-2012 | | |
| Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) JSTARS/SPIDER/SPIDER/SPIDER/SPIDER | | |
| C. DOCUMENTS CONSIDERED TO BE RELEVANT | | |
| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| Y/A | Dencheva H, Benkov A, Velichova A, Bover M, Popov B, Hladikova M, Study of homocysteine concentration in coronary heart disease patients and comparison of two determination methods. Ann Nutr Metab. 2007, Vol.51, No.1, Page. 62-67 | 1, 4, 5/2, 3, 6-10 |
| Y/A | JF 2870704 B1 (Axis Research A/S), JF H04 1 999 (17.03.1999) with the text * US 603383 A * GB 9204922 D * EP 623174 A * EP 726322 A1 * WO 1993/015220 A1 | 1, 4, 5/2, 3, 6-10 |
| <input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex. | | |
| * Special requirement of cited document: ** Date of publication of cited document: the international filing date or priority date and date to which the application has been extended to be considered to be prior art in the event of a subsequent filing date | | |
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| * "V" document cited in the international search report is not considered to be prior art in the event of a subsequent filing date | | |
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| * "X" document cited in the international search report is not considered to be prior art in the event of a subsequent filing date | | |
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| * "Z" document cited in the international search report is not considered to be prior art in the event of a subsequent filing date | | |
| Date of the actual completion of the international search: 22 February, 2012 (22.02.12) | | |
| Date of mailing of the international search report: 06 March, 2012 (06.03.12) | | |
| Name and mailing address of the ISA: INTERNATIONAL PATENT OFFICE | | |
| Authorized officer | | |
| E-mail: No. Telephone No. | | |
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