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(54) **URACIL-SPECIFIC FLUORESCENCE DETECTION REACTION AND METHOD FOR EXAMINING DIHYDROPYRIMIDINE DEHYDROGENASE DEFICIENCY**

REAKTION ZUR ERKENNUNG URACILSPEZIFISCHER FLUORESZENZ UND VERFAHREN ZUR UNTERSUCHUNG VON DIHYDROPYRIMIDIN-DEHYDROGENASE-MANGEL

RÉACTION DE DÉTECTION DE FLUORESCENCE SPÉCIFIQUE DES URACILES ET PROCÉDÉ D’EXAMEN D’UNE DÉFICIENCE EN DIHYDROPYRIMIDINE DÉSHYDROGÉNASE

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(56) References cited:
JP-A- 2001 112 472 US-B1- 6 927 035

- **KAWASAKI ET AL.: "Pyrimidine Enki o Shikibetsu Dekiru Shinki Keiko Hanno no Kaihatsu to Hanno Kiko no Kaimei", THE JAPAN SOCIETY FOR ANALYTICAL CHEMISTRY DAI 57 NENKAI KOEN YOSHISHU, 27 August 2008 (2008-08-27), page 370, XP002711551,**
- **KAWASAKI ET AL.: ’Pyrimidine Enki o Shikibetsu Dekiru Shinki Keiko Hanno no Kaihatsu to Hanno Kiko no Kaimei’ THE JAPAN SOCIETY FOR ANALYTICAL CHEMISTRY DAI 57 NENKAI KOEN YOSHISHU vol. 57, 27 August 2008, page 370, XP002711551**
- **TAKAYUKI SHIBATA ET AL.: ’A novel and specific fluorescence reaction for uracil’ ANALYTICA CHIMICA ACTA vol. 674, no. 2, 30 June 2010, pages 234 - 238, XP027187478**

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Description

Technical Field

5 **[0001]** The present invention relates to a method of detecting uracil-specific fluorescence, a reagent for uracil detection, and a method for examining dihydropyrimidine dehydrogenase deficiency by using the reaction.

Background Art

10 **[0002]** Fluorinated pyrimidine anti-cancer agents including 5-fluorouracil (5-FU) are representative anti-cancer agents frequently used for malignant tumors such as breast cancer, gastrointestinal cancer and the like. It is known, however, that administration of a fluorinated pyrimidine anti-cancer agent to a patient genetically defective in dihydropyrimidine dehydrogenase (DPD), which is a rate-determining enzyme in fluorinated pyrimidine decomposition, raises blood concentration of fluorinated pyrimidine, as well as causes serious side effects such as blood disorder, bone marrow suppression and the like, which could lead to death in the worst case. Since DPD is an enzyme that generally degrades uracil and thymine, DPD-defective patients are known to show high concentrations of uracil and thymine, particularly uracil, in urine or blood. Therefore, quantification of the concentration of uracil in urine or blood of patients before administration of a fluorinated pyrimidine anti-cancer agent to the patients enables diagnosis of DPD deficiency, based on which an accident due to the administration of a fluorinated pyrimidine anti-cancer agent to DPD defective patients can be prevented.

[0003] As a method of quantifying uracil in urine or blood, a method using high performance liquid chromatography (HPLC) (S. Sumi, K. Kidouchi, S. Ohba and Y. Wada, J. Chromatogr. B 1995, 672, 233-239), and an immunological measurement method using an anti-uracil monoclonal antibody (JP-A-2008-120824 and JP-A-2001-112472) have heretofore been developed. However, the HPLC method is associated with defects in that measurement of one sample takes time and multiple samples cannot be analyzed simultaneously, an expensive apparatus is necessary and the like. The immunological measurement method is also associated with defects in that substrate specificity is low and determination of DPD deficiency is difficult, it is costly and the like.

[0004] As a DPD activity measurement method other than quantification of uracil, a method of directly measuring the DPD activity of peripheral blood mononuclear cells (B.E. Harris, R. Song, S. Soong and R.B. Diasio, Cancer Res. 1990, 50, 197-201), and a method including administering uracil labeled with a radioisotope and measuring the content of metabolized labeled CO_2 in the breath (L.K. Mattison, H. Ezzeldin, M. Carpenter, A. Modak, M.R. Johnson and R.B. Diasio, Clin. Cancer Res., 2004, 10, 2652-2658) have been reported. However, they are associated with various problems in that the former does not permit simultaneous analysis of multiple samples since it requires HPLC separation, and the latter uses radioactive substances, may misdiagnose as DPD deficiency due to the influence of other enzymes and the like.

[0005] With such background, the DPD activity measurement methods developed heretofore are not used for routine examination in hospitals, and at present, the DPD activity of patient is measured only when a side effect such as nausea and the like or abnormality in blood examination is observed after administration of a fluorinated pyrimidine anti-cancer agent. Therefore, many cases of death have been reported in the world, which were caused by aggravation of side effects even though administration of fluorinated pyrimidine was discontinued after finding DPD deficiency (Tomoyuki Takaba, Jin Moriyama, Tsuyoshi Yokoyama, Shuichiro Matoba, Toshihito Sawada, The Japanese Journal of Gastroenterological Surgery, 2008, vol. 41, pages 2075 - 2080).

[0006] Therefore, the development of a uracil quantification method and a DPD deficiency examination method, which are capable of detecting uracil highly accurately and economically by a convenient method in a short time, has been desired.

45 **[0007]** Kawasaki et al., Japan Society for Analytical Chemistry, Dai 57 Nenkai Koen Yoshishu, 27 August 2008, page 370, Y1079, describes a fluorescence reaction specific to detection of cytosine and uracil.

[Document List]

50 [patent documents]

[0008]

Patent document 1: JP-A-2008-120824
55 Patent document 2: JP-A-2001-112472

[non-patent documents]

[0009]

Non-patent document 1: S. Sumi, K. Kidouchi, S. Ohba and Y. Wada, J. Chromatogr. B 1995, 672, 233-239
Non-patent document 2: B.E. Harris, R. Song, S. Soong and R.B. Diasio, Cancer Res. 1990, 50, 197-201
Non-patent document 3: L.K. Mattison, H. Ezzeldin, M. Carpenter, A. Modak, M.R. Johnson and R.B. Diasio, Clin. Cancer Res., 2004, 10, 2652-2658
Non-patent document 4: Tomoyuki Takaba, Jin Moriyama, Tsuyoshi Yokoyama, Shuichiro Matoba, Toshihito Sawada, The Japanese Journal of Gastroenterological Surgery, 2008, vol. 41, pages 2075 - 2080

SUMMARY OF THE INVENTION

Problems to be Solved by the Invention

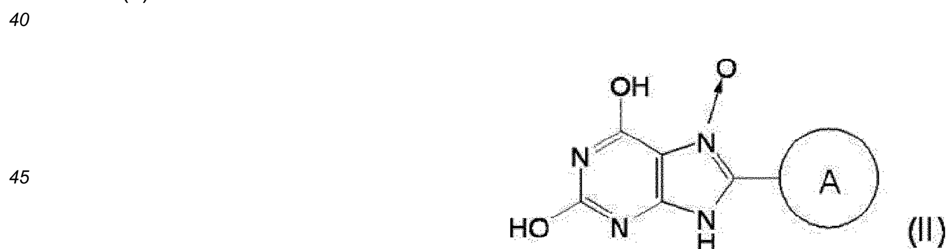
[0010] The present invention aims to provide a uracil detection method comprising a novel uracil-specific reaction, which is capable of detecting uracil highly accurately and economically by a convenient method in a short time. In addition, the present invention provides a reagent for the reaction, and an examination method of DPD deficiency using the reaction.

Means of Solving the Problems

[0011] The present inventors have conducted intensive studies in an attempt to solve the aforementioned problems and found that a uracil-specific reaction takes place by using a compound represented by the following formula (I), which resulted in the completion of the present invention.
[0012] Accordingly, the present invention relates to the following.

[1] A method of detecting uracil, comprising reacting uracil with a compound represented by the formula (I):

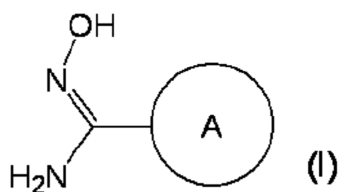
wherein A is C_{6-10} aryl group substituted by 1 to 3 C_{1-4} alkyl groups (hereinafter sometimes to be abbreviated as compound (I)), in the presence of an oxidant and a base, to give a fluorescent compound represented by the formula (II):



wherein A is as defined above (hereinafter sometimes to be abbreviated as compound (II)), wherein the amount of the base is 100-1000 equivalents relative to the compound represented by the formula (I).
[2] The method of the above-mentioned [1], wherein A is a 3-methylphenyl group.
[3] The method of the above-mentioned [2], comprising a step of extracting the fluorescent compound from a sample by using an organic solvent.
[4] The method of the above-mentioned [1], wherein the oxidant is potassium ferricyanide.
[5] The method of the above-mentioned [1], wherein the amount of the oxidant to be used is 0.001 - 3 equivalents relative to the compound represented by the formula (I).
[6] The method of the above-mentioned [1], wherein the base is potassium hydroxide.
[7] The method of the above-mentioned [1], wherein the reaction temperature is 50 - 120°C.

[8] The method of the above-mentioned [1], wherein the reaction time is 1 - 15 min.

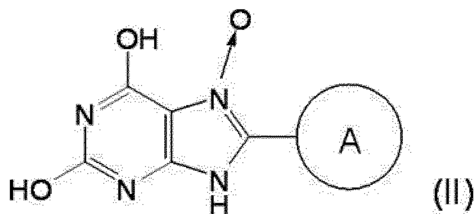
[9] A kit for uracil detection, comprising a reagent comprising a compound represented by the formula (I):



wherein A is C_{6-10} aryl group substituted by 1 to 3 C_{1-4} alkyl groups.

[10] The kit of the above-mentioned [9], wherein A is a 3-methylphenyl group.

[11] A fluorescent compound represented by the formula (II):



wherein A is C_{6-10} aryl group substituted by 1 to 3 C_{1-4} alkyl groups.

[12] The compound of the above-mentioned [11], wherein A is a 3-methylphenyl group.

[13] A method of examining dihydropyrimidine dehydrogenase deficiency, comprising detecting uracil in a sample by the method described in the above-mentioned [1].

[14] The method of the above-mentioned [13], wherein the sample is a blood sample and/or a urine sample derived from a human patient.

Effect of the Invention

[0013] According to the present invention, uracil can be detected highly accurately and economically by a convenient method in a short time.

Brief Description of the Drawings

[0014]

Fig. 1 shows the results of fluorometric analysis of a compound obtained by reacting uracil (each concentration) with 3-methylbenzamidoxime.

Fig. 2 shows the results of fluorometric analysis of a compound obtained by reacting uracil (each concentration) with 3-methylbenzamidoxime.

Fig. 3 shows the ^{1}H-NMR spectrum of a compound obtained by reacting uracil with 4-trifluoromethylbenzamidoxime.

Fig. 4 shows the results of fluorometric analysis of a compound obtained by reacting a sample other than uracil with 3-methylbenzamidoxime.

Fig. 5 shows the results of fluorometric analysis of a compound obtained by reacting uracil with 3-methylbenzamidoxime, or uracil with benzamidoxime, and treated under various conditions.

Fig. 6 shows the results of detection of uracil in urine with 3-methylbenzamidoxime.

Description of Embodiments

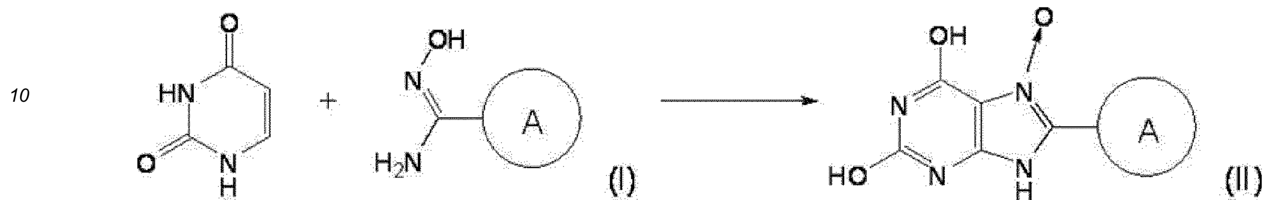
[0015] The definitions of the terms used in the present invention are described in detail in the following.

[0016] The "halogen atom" means a fluorine atom, a chlorine atom, a bromine atom or an iodine atom.

[0017] The "alkyl group" is a linear or branched chain alkyl group and, for example, a C_{1-4} alkyl group can be mentioned. Specific examples thereof include methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, tert-butyl and the like.

[0018] Specific examples of the C_{6-10} aryl group include phenyl, naphthyl and the like, and particularly preferred is phenyl.

[0019] The aryl group is substituted by 1 to 3 C_{1-4} alkyl groups at substitutable position(s). When two or more substituents are present, the respective substituents may be the same or different.
[0020] A preferable embodiment of the present invention is explained below.
[0021] The present invention provides a detection method of uracil, which comprises reacting uracil with a compound represented by the formula (I) to give a fluorescent compound represented by the formula (II).



[0022] In the above-mentioned formula (I) and the formula (II), A is a C_{6-10} aryl group substituted by 1 to 3 C_{1-4} alkyl groups. More preferred is 3-methylphenyl.
[0023] As compound (I), the following compounds (I-C) - (I-E) are preferable.

[Compound (I-C)]

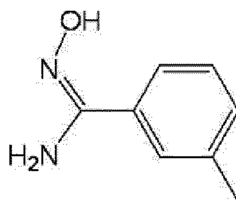
[0024] Compound (I) wherein A is a C_{6-10} aryl group substituted by 1 to 3 C_{1-4} alkyl groups.

[Compound (I-D)]

[0025] Compound (I) wherein A is 3-methylphenyl.

[Compound (I-E)]

[0026] A compound represented by the formula:



(namely, 3-methylbenzamidoxime).
[0027] Compound (I) is commercially available or can be produced by a method known per se.
[0028] As compound (II), the following compounds (II-C) - (II-E) are preferable.

[Compound (II-C)]

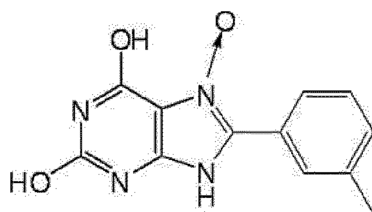
[0029] Compound (II) wherein A is a C_{6-10} aryl group substituted by 1 to 3 C_{1-4} alkyl groups.

[Compound (II-D)]

[0030] Compound (II) wherein A is 3-methylphenyl.

[Compound (II-E)]

[0031] A fluorescent compound represented by the formula:



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[0032] A fluorescent compound wherein, like compound (II-E), a hydrophobic functional group such as an alkyl group (preferably a methyl group) and the like is introduced into a phenyl group is particularly preferable since it can be extracted with an organic solvent (e.g., ethyl acetate). Since extraction from the urine or blood with an organic solvent is possible, multiple samples can be measured in a short time without being influenced by a fluorescent impurity in a sample (urine, blood).

[0033] Since the above-mentioned reaction of uracil with compound (I) (hereinafter to be also referred to as the reaction in the present invention) proceeds in the presence of an oxidant and a base (particularly, strong base), further addition of an oxidant and a base is preferable.

[0034] As the oxidant, an oxidant known per se can be appropriately used. Examples of the oxidant include potassium ferricyanide, ferric chloride, cupric chloride, sodium iodate, potassium permanganate, potassium nitrate, ceric ammonium nitrate, potassium dichromate and the like. Particularly preferred is potassium ferricyanide. The amount of the oxidant to be used is, for example, 0.001 - 3 equivalents, preferably 0.5 - 2.5 equivalents, relative to compound (I).

[0035] As the base, a base known per se can be appropriately used. Examples of the base include potassium hydroxide, sodium hydroxide, lithium hydroxide, magnesium hydroxide, calcium hydroxide, potassium carbonate, sodium carbonate, lithium carbonate, potassium hydrogencarbonate, sodium hydrogencarbonate, lithium hydrogencarbonate and the like. Particularly preferred is potassium hydroxide. The amount of the base to be used is 100 - 1000 equivalents, relative to compound (I).

[0036] Since the reaction preferably proceeds by heating, the reaction in the present invention is preferably performed at a reaction temperature of 50 - 120°C, more preferably 80 - 120°C, particularly preferably 90°C.

[0037] Since the fluorescence intensity of compound (II) obtained by a reaction in an extremely short time or a reaction in a long time decreases, the reaction in the present invention is preferably performed in a reaction time of 1 - 15 min, more preferably 1 - 5 min, particularly preferably 2 min.

[0038] The reaction in the present invention can also be performed using a solvent. The solvent is not particularly limited as long as the reaction proceeds, and a solvent known per se can be used. Examples thereof include water, N,N-dimethylformamide, dimethyl sulfoxide, alcohols (e.g., methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, isobutanol, sec-butanol, tert-butanol etc.) and the like. Particularly preferred is water. In addition, water contained in the following sample may also function as a solvent.

[0039] Since compound (II) obtained by the reaction in the present invention is fluorescent, uracil can be detected by irradiating an excitation light in the excitation wavelength range to compound (II), and measuring the fluorescence intensity in the fluorescence wavelength range.

[0040] The excitation wavelength range is not particularly limited as long as compound (II) shows fluorescence by irradiating an excitation light, and those of ordinary skill in the art can properly select an appropriate wavelength range and perform the measurement. For example, irradiation of an excitation light in a 250 - 400 nm wavelength range is preferable, that in a 310 - 350 nm wavelength range is more preferable, and that at a 310 - 330 nm wavelength is particularly preferable.

[0041] The fluorescence wavelength range is not particularly limited as long as it is a fluorescence wavelength range, and those of ordinary skill in the art can properly select an appropriate wavelength range and perform the measurement. For example, measurement of fluorescence intensity in a 350 - 500 nm wavelength range is preferable, that in a 360 - 440 nm wavelength range is more preferable, and that at a 365 - 410 nm wavelength is particularly preferable.

[0042] The fluorescence intensity can be measured by a detection method known per se, and can be measured using, for example, a fluorescence spectrophotometer.

[0043] A reaction mixture containing compound (II) obtained by the reaction in the present invention is strong alkaline immediately after reaction. A treatment such as neutralization with an acid (e.g., acetic acid), salting out with a salt (e.g., sodium chloride), extraction with an organic solvent (e.g., ethyl acetate) and the like is preferably further performed to enhance fluorescence intensity.

[0044] Neutralization (pH 6 - 7) step → salting out step → extraction step is particularly preferably performed.

[0045] Compound (I) can be used as a reagent for uracil detection singly or together with other additives. Examples of the additive include an acid, an acidic reagent and the like.

[0046] As the "acid", an inorganic acid or organic acid can be mentioned. Examples thereof include hydrochloric acid, sulfuric acid, nitric acid, acetic acid, formic acid and the like.

[0047] As the "acidic reagent", an aqueous solution of the acid and the like can be mentioned.

[0048] A reagent for uracil detection can be used in a uracil detection kit singly or together with other items. Examples of the item include an oxidant, a base and a solvent, instructions, a packaging material, a reaction container, a pre-treatment filter for body fluid sample or waste product sample, and the like, which can aid advantageous progress of the reaction in the present invention.

[0049] As the oxidant, base and solvent, those similar to the examples recited above can be mentioned.

[0050] The instructions may describe a detection target, a sample to be used, a use method and a storage method (e.g., chilled storage, preservation in closed container etc.) and the like.

[0051] As the packaging material, reaction container and pre-treatment filter for body fluid sample or waste product sample, those known per se can be used.

[0052] The reaction in the present invention is specific to uracil. Being "specific to uracil" means reaction with uracil alone and no reaction with, for example, nucleic acid base, nucleoside, nucleotide and nucleic acid base derivatives (e.g., rare nucleic acid base such as pseudouridine and the like, fluorinated pyrimidine anti-cancer agent such as 5-fluorouracil and the like, etc.) and the like other than free uracil.

[0053] Since such reaction in the present invention is specific to uracil, dihydropyrimidine dehydrogenase deficiency can be examined by detecting uracil in a sample using the reaction in the present invention.

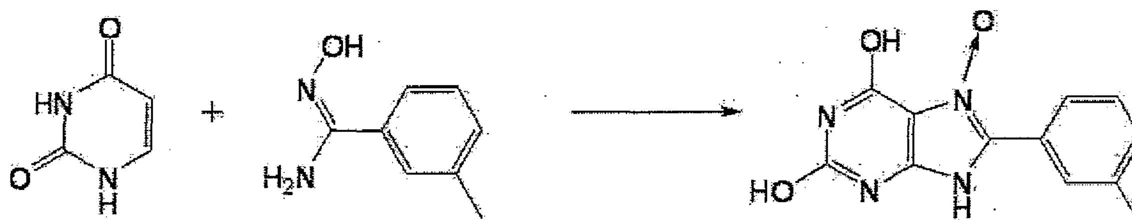
[0054] As the sample, for example, body fluid samples (e.g., blood sample etc.) and/or waste product samples (e.g., urine sample etc.) derived from mammals (e.g., human (particularly human patients) etc.) can be mentioned.

[0055] In addition, since the reaction in the present invention is specific to uracil, it is also useful for cell staining, sequencing of the base sequence of RNA and the like. Examples

[0056] The present invention is explained in more detail in the following by referring to Examples, Reference Examples and Experimental Examples, which are not to be construed as limitative.

Example 1: reaction of uracil with 3-methylbenzamidoxime

[0057]



[0058] Aqueous uracil solution (0 μm (Reference Example), 1 μM, 2 μM, 5 μM, 10 μm, 20 μm, 50 μm, 100 μm, 150 μm, 200 μm or 250 μm, each 0.25 ml), aqueous 3-methylbenzamidoxime solution (4 mM, 0.25 ml), aqueous potassium ferricyanide solution (8 mM, 0.25 ml) and aqueous potassium hydroxide solution (4 M, 0.25 ml) were mixed, and the mixture was heated at 90°C for 2 min. The obtained compound was subjected to fluorometric analysis under the following conditions. The measurement results of fluorometric analysis are shown in Table 1, Fig. 1, Fig. 2.

Fluorometric analysis:

[0059]

Type of equipment: JASCO FP-6300 Spectrofluorometer
Ex/Em = 330 nm/410 nm
Slit width: 5 nm, 5 nm
Sensitivity: medium
Response: medium

Table 1

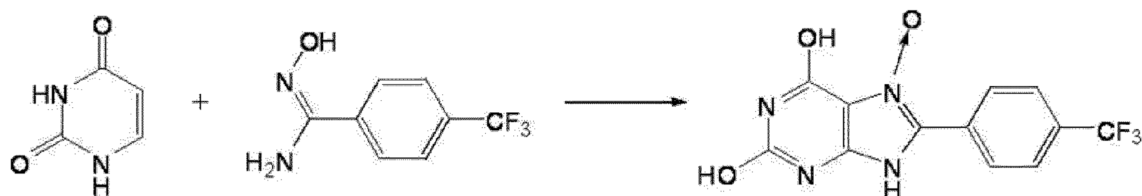
[0060]

Table 1.uracil concentration and fluorescence intensity of obtained compounds

uracil concentration (μM)	fluorescence intensity (RFU)
0 (Reference Example)	0.2
1	9.8
2	17.5
5	42.0
10	86.0
20	179
50	391
100	668
150	950
200	1113
250	1310

Example 2: (Reference) reaction of uracil with 4-trifluoromethylbenzamidoxime

[0061]



[0062] Aqueous uracil solution (1 mM, 100 ml), 4-trifluoromethylbenzamidoxime (4 mM, 100 ml), aqueous potassium ferricyanide solution (8.0 mM, 100 ml) and aqueous potassium hydroxide solution (2 M, 100 ml) were mixed, and the mixture was heated at 100°C for 10 min. After the reaction, the reaction mixture was extracted with ethyl acetate, and the obtained organic layer was concentrated. This operation was repeated 13 times, and the obtained residues were mixed and purified by silica gel column chromatography (eluent: ethyl acetate/methanol=9/1→8/2→7/3) to give the object compound (25 mg) as a white powder (yield: 6%). The obtained compound was subjected to ^{1}H-NMR. The results are shown in Fig. 3. Measuring machine: varian UNITY plus 500(500MHz)
^{1}H NMR (DMSO-d_6, 400MHz) δ=8.89 (broad s, 0.25H), 8.29 (d, J=8.0 Hz, 0.5H), 8.08 (d, J=7.8Hz, 0.5H), 7.72 (d, J=8.0Hz, 0.5H), 7.68 (d, J=7.8Hz, 0.5H), 6.68 (broad s, 0.25H).

[0063] From the above, it has been found that the reaction of uracil and compound (I) produces compound (II) (Fig. 3), and compound (II) is a fluorescent compound (Table 1, Fig. 1). In addition, it has been found that the uracil concentration and the fluorescence intensity are correlated (Table 1, Fig. 1). Furthermore, it has been found that the fluorescence intensity becomes the highest at a 410 nm fluorescence wavelength (Fig. 2).

Reference Example 1: reaction of sample other than uracil with 3-methylbenzamidoxime

[0064] In the same manner as in Example 1 except that uracil was changed to the measurement samples shown in the following Table 2, fluorometric analysis was performed. The results are shown in Fig. 4.

Table 2

[0065]

Table 2. Measurement sample

sample No.	measurement sample	
1	nucleic acid base	cytosine
2		thymine
3		adenine
4		guanine
5	nucleoside	uridine
6		cytidine
7		thymidine
8		adenosine
9		guanosine
10	nucleotide	5'-UMP
11		5'-CMP
12		5'-dTMP
13		5'-AMP
14		5'-GMP
15	nucleic acid base derivative	pseudouridine
16		5-fluorouracil
17		1-methyluracil
18		6-methyluracil
19		5,6-dihydrouracil
20	saccharide	glucose
21		fructose
22		lactose
23		ribose
24		sucrose
25	amino acids	mixture of amino acids (20 kinds) constituting living organisms

[0066] From the above, it has been found that the reaction in the present invention has very high substrate specificity, and does not provide fluorescence to nucleic acid base, nucleoside, nucleotide, nucleic acid base derivative (e.g., rare nucleic acid base such as pseudouridine and the like, fluorinated pyrimidine anti-cancer agent such as 5-fluorouracil and the like, uracil metabolite such as dihydrouracil and the like, methyluracil etc.) and the like, other than free uracil (Fig. 4) .

Example 3: quantification of urinal uracil concentration (direct quantification of urinal uracil)

(Reagent: total 1 mL)

Reagent composition A (test reagent)

[0067]

(1)8% diluted urine (urine sample: healthy volunteer (37-year-old, male)): 125 μL
(2) Water or uracil standard solution: 125 μL
(3) 4 mM aqueous 3-methylbenzamidoxime solution: 250 μL
(4) 8 mM aqueous potassium ferricyanide solution: 250 μL

(5) 2 M aqueous potassium hydroxide solution: 250 μL

Reagent composition B (fluorescent blank derived from urine)

[0068]

(1) 8% diluted urine (urine sample: healthy individual volunteer (37-year-old, male)): 125 μL
(2) Water: 125 μL
(3) Water: 250 μL
(4) 8 mM aqueous potassium ferricyanide solution: 250 μL
(5) 2 M aqueous potassium hydroxide solution: 250 μL

Reagent composition C (fluorescent blank derived from reagent)

[0069]

(1) Water: 125 μL
(2) Water: 125 μL
(3) 4 mM aqueous 3-methylbenzamidoxime solution: 250 μL
(4) 8 mM aqueous potassium ferricyanide solution: 250 μL
(5) 2 M aqueous potassium hydroxide solution: 250 μL

(Operation)

[0070]

(1) The reagents with the above-mentioned compositions were sequentially added to reaction containers
(2) The mixture was heated at 90°C for 2 min and
(3) Cooled in ice bath for 2 min
(4) The fluorescence intensity of the reaction mixture was measured (measurement conditions followed those in Example 1)
(5) Creatinine concentration of the same urine was measured

[0071] The creatinine concentration was measured by either of the following two kinds of methods. Both methods were performed according to the attached instructions for the detail of the steps.

Method 1

[0072] In this method, AUTION sticks 10PA (ARKRAY Factory, Shiga, Japan), which is urine test paper, was used.

(Operation)

[0073]

(1) Test paper was immersed in urine for 2 seconds
(2) Excess urine on the test paper was gently wiped, and the test paper was horizontally maintained and stood for 60 seconds
(3) Creatinine concentration was determined based on the color change of the test paper

Method 2

[0074] In this method, Creatinine Assay Kit (Cayman, MI, USA) was used.

(Operation)

[0075]

(1) Alkaline Picrate Solution (150 μL) attached to the kit was added to 15 μL of 10-fold diluted urine, and the mixture

was incubated at room temperature for 10 min.
(2) The absorbance at 490-500 nm was measured (Initial absorbance: I_{abs})
(3) Acid Solution (5 μL) attached to the kit was added to the solution, and the mixture was incubated at room temperature for 20 min.
(4) The absorbance at 490-500 nm was measured (Final absorbance: F_{abs})
(5) Using the analytical curve prepared using a creatinine standard solution, the creatinine concentration was calculated from F_{abs}-I_{abs} values.

(Results)

[0076] The fluorescence intensity derived from urinal uracil produced by a fluorescent derivatization reaction is obtained by subtracting each value of fluorescence intensity obtained with reagent composition B and reagent composition C from the intensity obtained by the reaction with reagent composition A, and an addition analytical curve is drawn, from which urinal uracil concentration can be quantified. By creatinine correction of the value obtained from the analytical curve, the value of 14.1 μmol/g Cre was obtained. This value is within the range of average urinal uracil concentration of Japanese people. The creatine correction was performed to avoid a urine volume error of urinal uracil. From the above results, it has been clarified that the method of the present invention can quantify urinal uracil.

Experimental Example 1: extraction of compound (II)

(fluorescent compound) with organic solvent

[0077]

(i) Aqueous uracil solution (40 μm, 0.25 ml), (ii) compound (I) [aqueous 3-methylbenzamidoxime solution or aqueous benzamidoxime solution] (4 mM, 0.25 ml), (iii) aqueous potassium ferricyanide solution (8 mM, 0.25 ml) and aqueous potassium hydroxide solution (2 M, 0.25 ml) were mixed, and the mixture was heated at 90°C for 2 min. The reaction mixture was treated under the conditions shown in Fig. 5, and fluorometric analysis was further performed under the following conditions. The measurement results of fluorometric analysis are shown in Fig. 5.

Fluorometric analysis:

[0078]

Type of equipment: JASCO FP-6300 Spectrofluorometer
Ex/Em = 315 nm/365 nm
Excitation band width: 5 nm
Fluorescence band width: 5 nm

[0079] When 3-methylbenzamidoxime was used as compound (I), a remarkable increase in the fluorescence intensity was observed by extraction with an organic solvent after neutralization.

Example 4: quantification of urinal uracil concentration (quantification of urinal uracil via solvent extraction)

(Reagent: total 1 mL)

[0080]

(1) 8% diluted urine (urine sample: healthy individual volunteer (23-year-old, male)): 125 μL
(2) Water or uracil standard solution: 125 μL
(3) 4 mM aqueous 3-methylbenzamidoxime solution: 250 μL
(4) 8 mM aqueous potassium ferricyanide solution: 250 μL
(5) 2 M aqueous potassium hydroxide solution: 250 μL

(Operation)

[0081]

(1 The reagents with the above-mentioned compositions were sequentially added to reaction containers
(2) The mixture was heated at 90°C for 2 min and
(3) Cooled in ice bath for 2 min
(4) Acetic acid (30 μL) was added and blended
(5) Sodium chloride (400 mg) was added and the mixture was vigorously blended
(6) Ethyl acetate (1 mL) was added and the mixture was vigorously blended
(7) The fluorescence intensity of the upper layer was measured (measurement conditions followed those in Example 1)
(8) Creatinine concentration of the same urine was measured (measurement conditions followed those in Example 3)

(Results)

[0082] The results are shown in Fig. 6. By creatinine correction of the value obtained from the analytical curve, the value of 40.1 μmol/g Cre was obtained. This value is within the range of average urinal uracil concentration of Japanese people. The creatine correction was performed to avoid a urine volume error of urinal uracil.

[0083] From the above results, it has been clarified that, when urine contains a large amount of fluorescent impurity substances, the method of the present invention can properly quantify urinal uracil by extraction with an organic solvent.

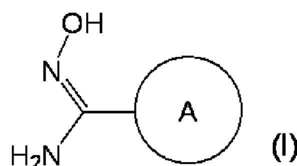
Industrial Applicability

[0084] Since the reaction in the present invention is specific to uracil, uracil can be detected highly accurately and economically by a convenient method in a short time by using the reaction in the present invention. In addition, since the uracil concentration and the fluorescence intensity of the compound obtained by the reaction in the present invention are correlated, uracil can be quantified by the reaction in the present invention, without using a special technique. Therefore, using the method of the present invention, DPD deficiency can be conveniently examined, which is advantageous for the chemical therapy of cancer.

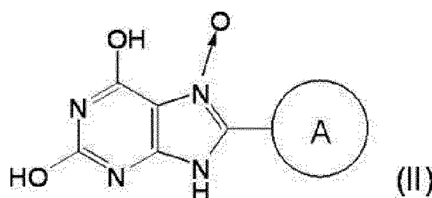
[0085] This application is based on patent application No. 2010-44610 filed in Japan.

Claims

1. A method of detecting uracil, comprising reacting uracil with a compound represented by the formula (I):



wherein A is C_{6-10} aryl group substituted by 1 to 3 C_{1-4} alkyl groups, in the presence of an oxidant and a base, to give a fluorescent compound represented by the formula (II) :



wherein A is as defined above, wherein the amount of the base is 100 - 1000 equivalents relative to the compound represented by the formula (I).

2. The method according to claim 1, wherein A is a 3-methylphenyl group.

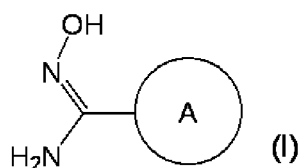
3. The method according to claim 2, comprising a step of extracting the fluorescent compound from a sample by using an organic solvent.

4. The method according to claim 1, wherein (i) the oxidant is potassium ferricyanide and/or (ii) the base is potassium hydroxide.

5. The method according to claim 1, wherein the amount of the oxidant to be used is 0.001 - 3 equivalents relative to the compound represented by the formula (I).

6. The method according to claim 1, wherein (i) the reaction temperature is 50 - 120°C and/or (ii) the reaction time is 1 - 15 minutes.

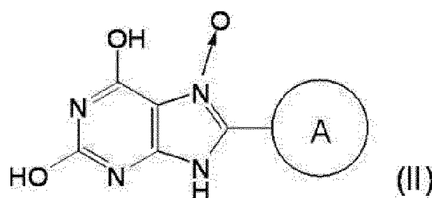
7. A kit for uracil detection, comprising a reagent comprising compound represented by the formula (I):



wherein A is C_{6-10} aryl group substituted by 1 to 3 C_{1-4} alkyl groups.

8. The kit according to claim 7, wherein A is a 3-methylphenyl group.

9. A fluorescent compound represented by the formula (II):



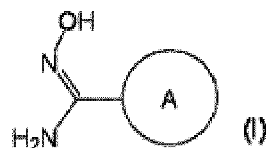
wherein A is C_{6-10} aryl group substituted by 1 to 3 C_{1-4} alkyl groups.

10. The compound according to claim 9, wherein A is a 3-methylphenyl group.

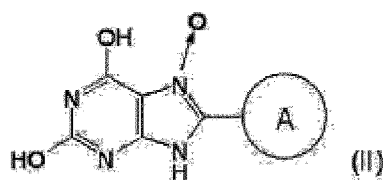
11. A method of examining dihydropyrimidine dehydrogenase deficiency, comprising detecting uracil in a blood sample and/or a urine sample derived from a human patient, by the method according to claim 1.

Patentansprüche

1. Verfahren zum Nachweis von Uracil, umfassend das Umsetzen von Uracil mit einer Verbindung, die durch die Formel (I) dargestellt wird:



wobei A eine C_{6-10}-Arylgruppe ist, die mit 1 bis 3 C_{1-4}-Alkylgruppen substituiert ist, in Gegenwart eines Oxidationsmittels und einer Base unter Bildung einer fluoreszierenden Verbindung, die durch die Formel (II) dargestellt wird:



wobei A wie oben definiert ist, wobei die Menge der Base 100 bis 1000 Äquivalente beträgt, bezogen auf die durch die Formel (I) dargestellte Verbindung.

2. Verfahren gemäß Anspruch 1, wobei A eine 3-Methylphenylgruppe ist.

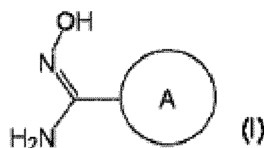
3. Verfahren gemäß Anspruch 2, umfassend einen Schritt des Extrahierens der fluoreszierenden Verbindung aus einer Probe unter Verwendung eines organischen Lösungsmittels.

4. Verfahren gemäß Anspruch 1, wobei es sich (i) bei dem Oxidationsmittel um Kaliumhexacyanoferrat(III) und/oder (ii) bei der Base um Kaliumhydroxid handelt.

5. Verfahren gemäß Anspruch 1, wobei die zu verwendende Menge des Oxidationsmittels 0,001 bis 3 Äquivalente beträgt, bezogen auf die durch die Formel (I) dargestellte Verbindung.

6. Verfahren gemäß Anspruch 1, wobei (i) die Reaktionstemperatur 50 bis 120 °C beträgt und/oder (ii) die Reaktionszeit 1 bis 15 Minuten beträgt.

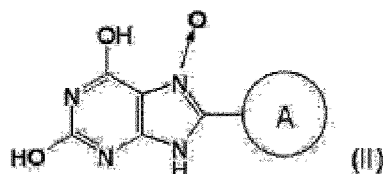
7. Kit zum Nachweis von Uracil, umfassend ein Reagens, das eine Verbindung umfasst, die durch die Formel (I) dargestellt wird:



wobei A eine C_{6-10}-Arylgruppe ist, die mit 1 bis 3 C_{1-4}-Alkylgruppen substituiert ist.

8. Kit gemäß Anspruch 7, wobei A eine 3-Methylphenylgruppe ist.

9. Fluoreszierende Verbindung, die durch die Formel (II) dargestellt wird:



wobei A eine C_{6-10}-Arylgruppe ist, die mit 1 bis 3 C_{1-4}-Alkylgruppen substituiert ist.

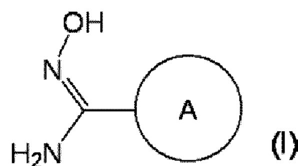
10. Verbindung gemäß Anspruch 9, wobei A eine 3-Methylphenylgruppe ist.

11. Verfahren zum Untersuchen eines Dihydropyrimidin-Dehydrogenase-Mangels, umfassend das Nachweisen von Uracil in einer Blutprobe und/oder einer Urinprobe, die von einem menschlichen Patienten stammt, nach dem Verfahren gemäß Anspruch 1.

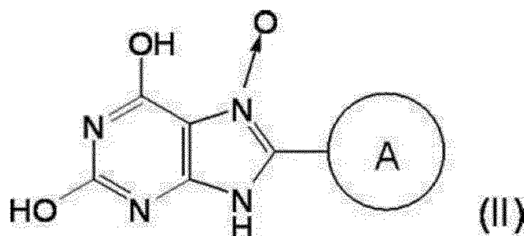
Revendications

1. Procédé de détection d'uracile, comprenant la mise en réaction d'uracile avec un composé représenté par la formule

(I) :



dans lequel A est un groupe C_{6-10} aryle substitué par 1 à 3 groupes C_{1-4} alkyle, en présence d'un antioxydant et d'une base, pour obtenir un composé fluorescent représenté par la formule (II) :



dans lequel A est tel que défini ci-dessus, dans lequel la quantité de la base est de 100 à 1 000 équivalents relativement au composé représenté par la formule (I).

2. Procédé selon la revendication 1, dans lequel A est un groupe 3-méthylphényle.

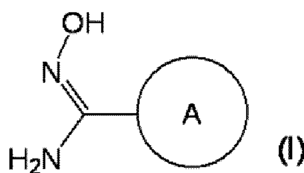
3. Procédé selon la revendication 2, comprenant une étape d'extraction du composé fluorescent d'un échantillon en utilisant un solvant organique.

4. Procédé selon la revendication 1, dans lequel (i) l'antioxydant est le ferricyanure de potassium et/ou (ii) la base est l'hydroxyde de potassium.

5. Procédé selon la revendication 1, dans lequel la quantité de l'oxydant à utiliser est de 0,001 à 3 équivalents relativement à un composé représenté par la formule (I).

6. Procédé selon la revendication 1, dans lequel (i) la température de réaction est de 50 à 120 °C et/ou (ii) la durée de réaction est de 1 à 15 minutes.

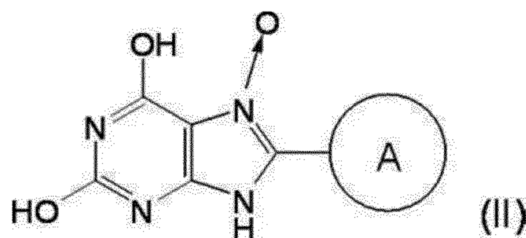
7. Kit de détection d'uracile, comprenant un réactif comprenant un composé représenté par la formule (I) :



dans lequel A est un groupe C_{6-10} aryle substitué par 1 à 3 groupes C_{1-4} alkyle.

8. Kit selon la revendication 7, dans lequel A est un groupe 3-méthylphényle.

9. Composé fluorescent représenté par la formule (II) :



dans lequel A est un groupe C_{6-10} aryle substitué par 1 à 3 groupes C_{1-4} alkyle.

10. Composé selon la revendication 9, dans lequel A est un groupe 3-méthylphényle.

11. Procédé d'examen d'un déficit en dihydropyrimidine déshydrogénase, comprenant la détection d'uracile dans un échantillon de sang et/ou un échantillon d'urine provenant d'un patient humain, par le procédé selon la revendication 1.

Fig.1

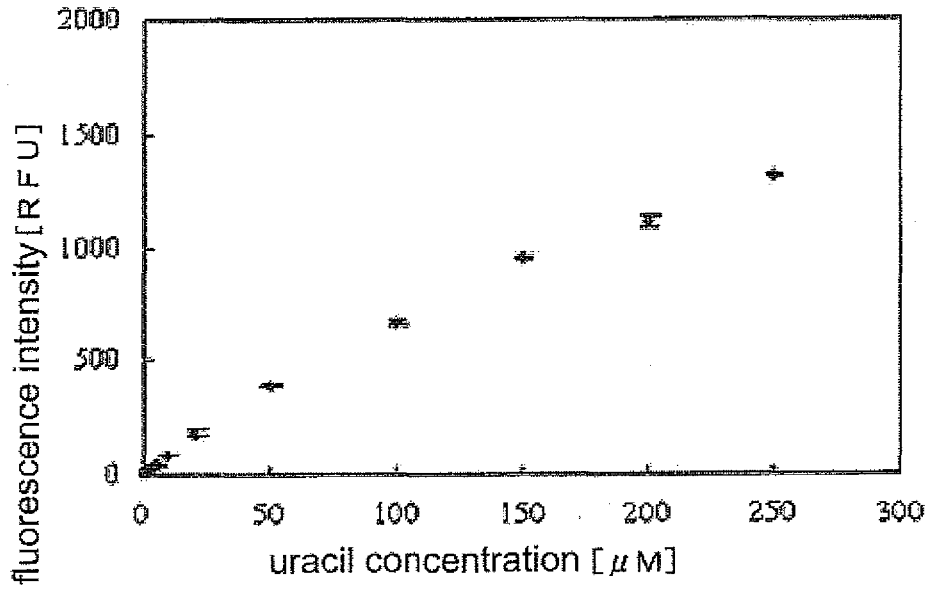


Fig.2

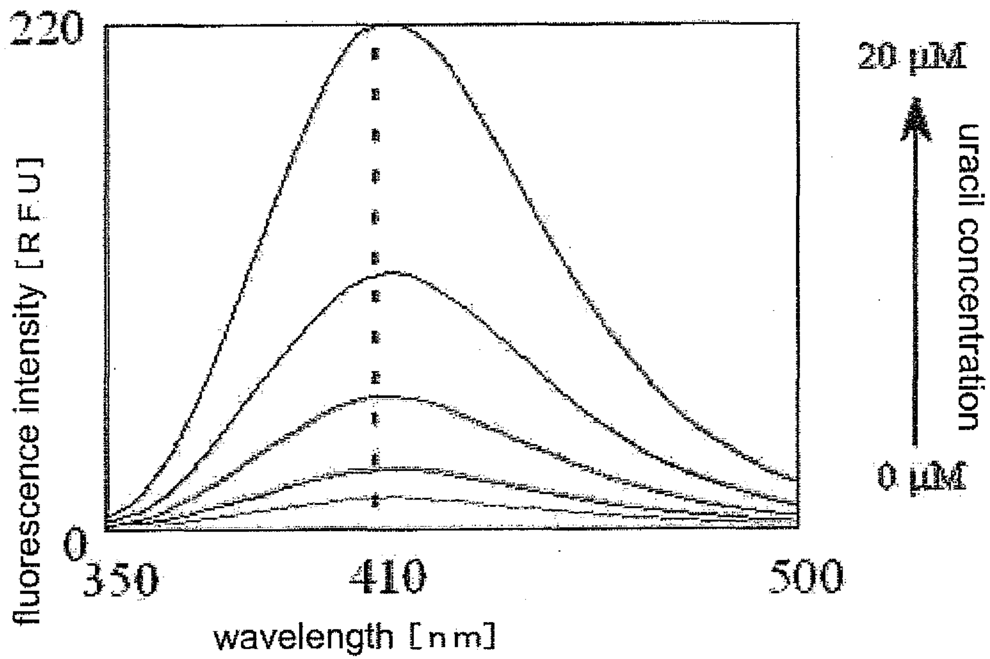


Fig.3

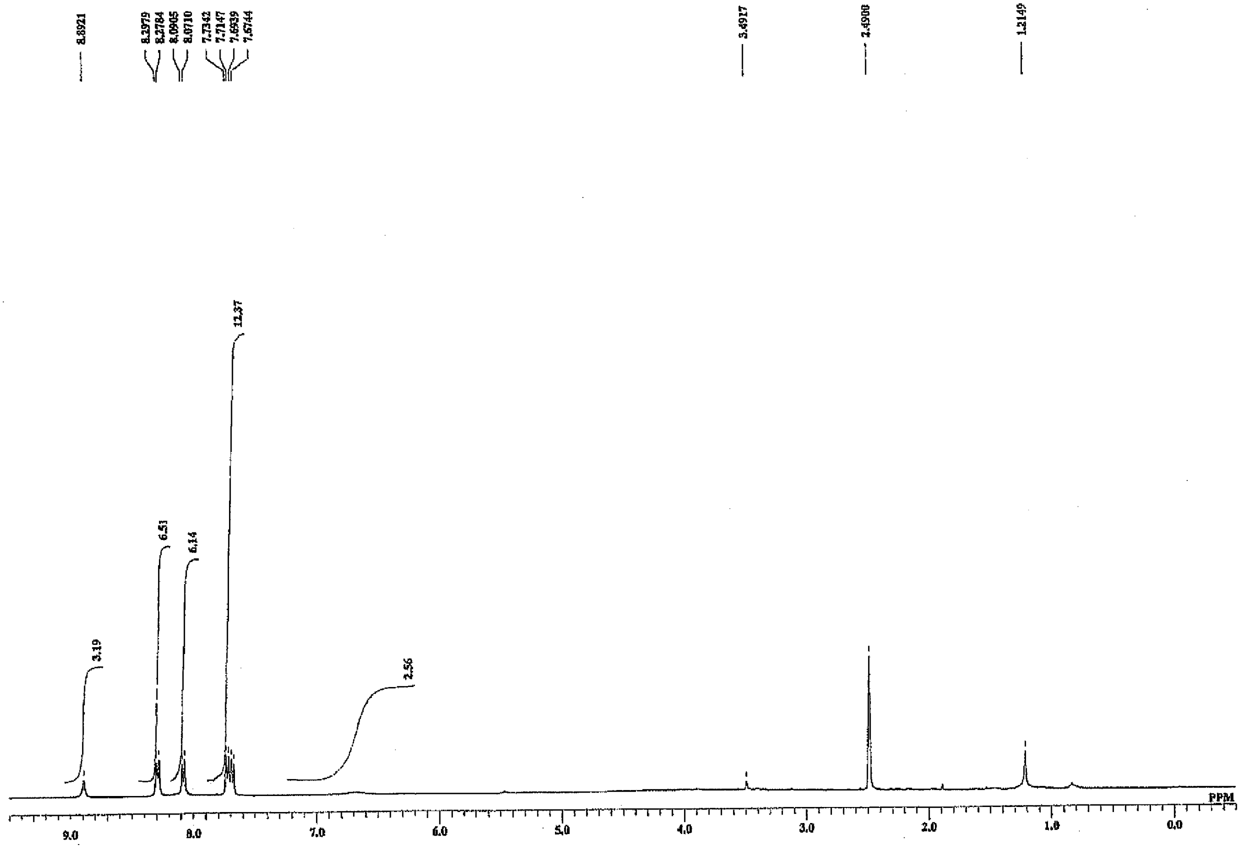


Fig.4

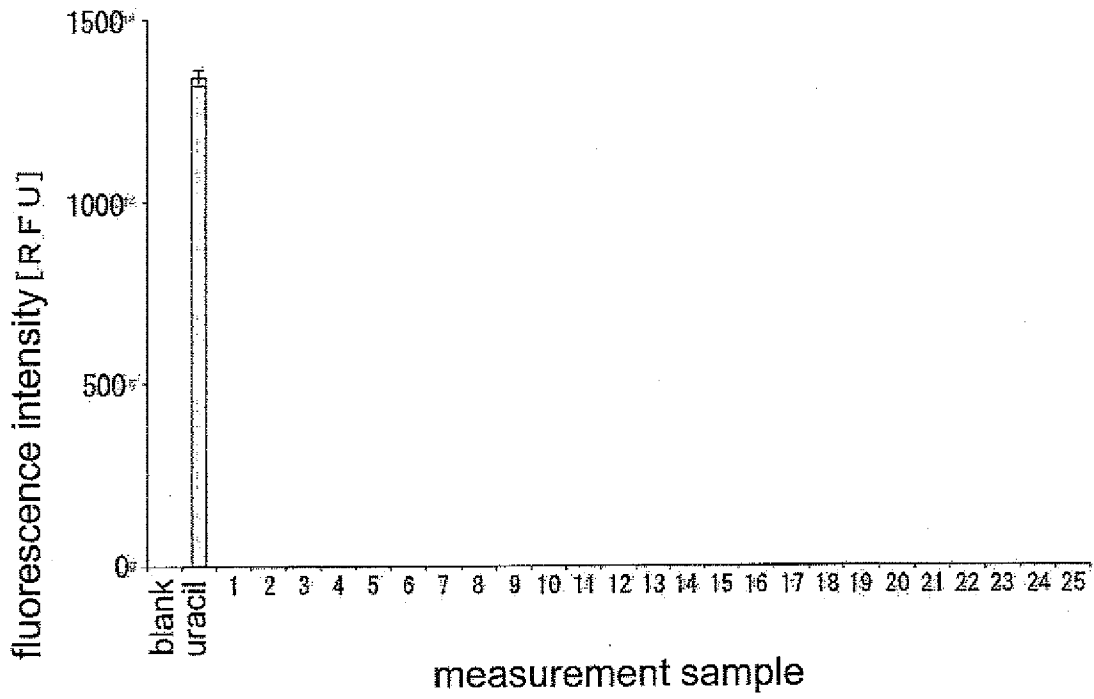


Fig.5

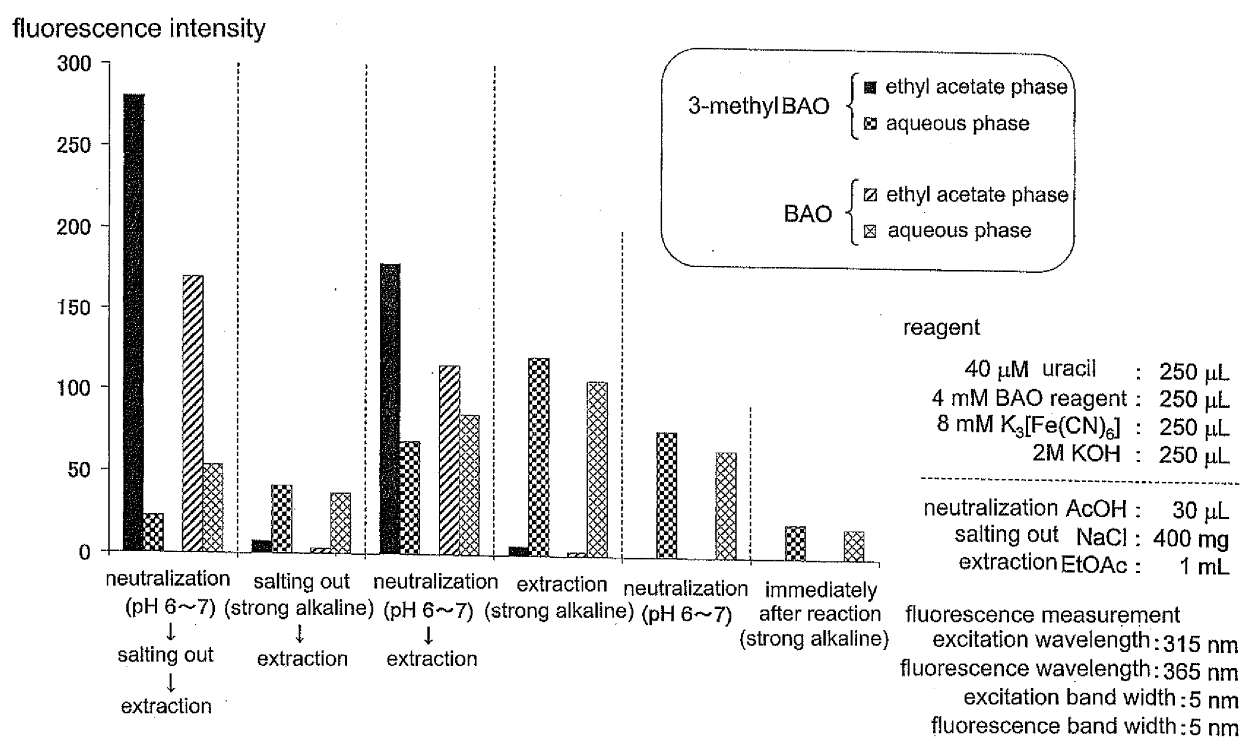
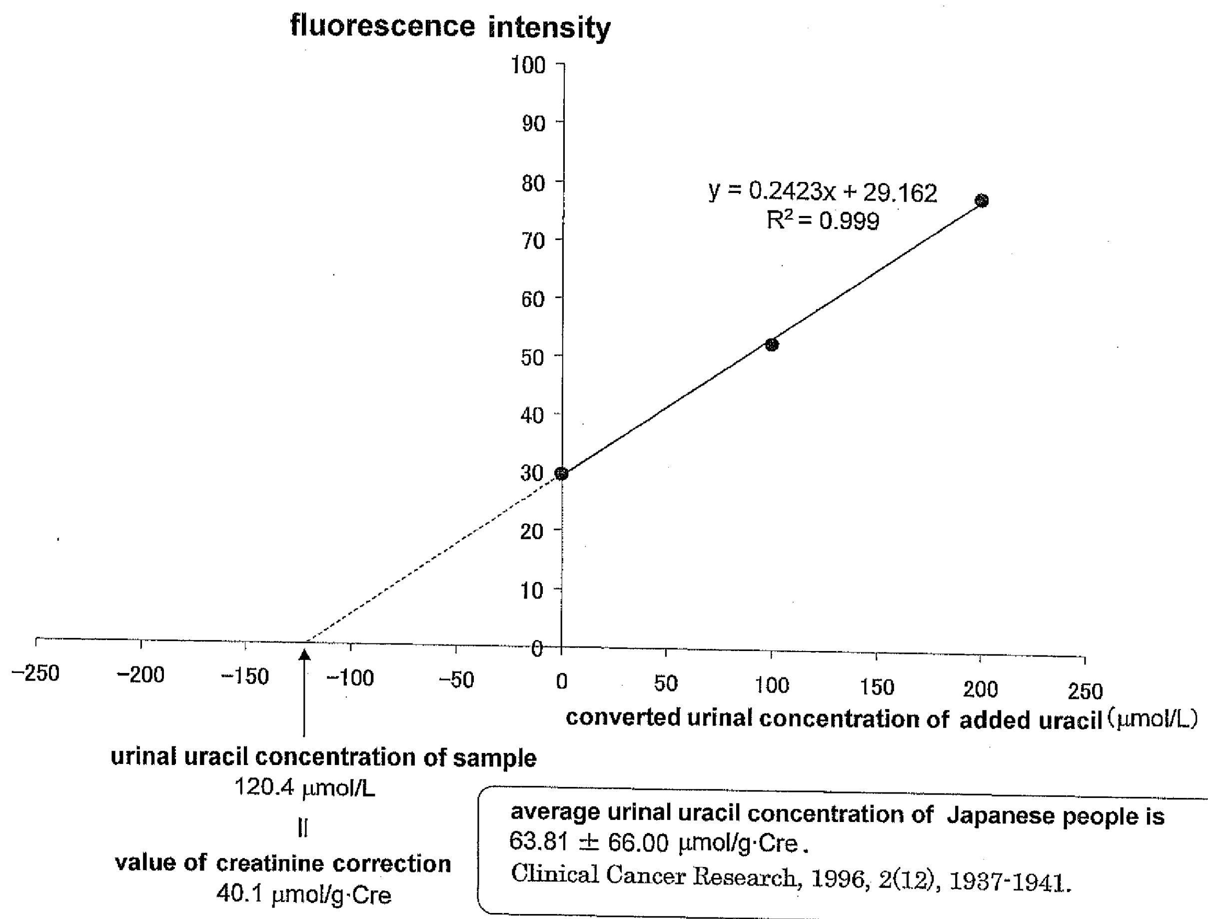


Fig.6



REFERENCES CITED IN THE DESCRIPTION

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patsnap

专利名称(译)	尿嘧啶特异性荧光检测反应和检测二氢嘧啶脱氢酶缺乏的方法		
公开(公告)号	EP2543991B1	公开(公告)日	2018-05-09
申请号	EP2011750654	申请日	2011-03-01
申请(专利权)人(译)	长崎大学		
当前申请(专利权)人(译)	长崎大学		
[标]发明人	KAI MASAAKI SHIBATA TAKAYUKI		
发明人	KAI, MASAAKI SHIBATA, TAKAYUKI		
IPC分类号	G01N21/78 C09K11/06 G01N33/58 G01N33/533		
CPC分类号	C09K11/06 C09K2211/1074 C12Q1/32 G01N21/6428 G01N33/533 G01N2333/90206 Y10T436/14 Y10T436/145555 Y10T436/147777		
代理机构(译)	J A KEMP		
优先权	2010044610 2010-03-01 JP		
其他公开文献	EP2543991A1 EP2543991A4		
外部链接	Espacenet		

摘要(译)

本发明提供一种尿嘧啶的检测方法，其能够在短时间内通过方便的方法高精度且经济地检测尿嘧啶。本发明涉及尿嘧啶的检测方法，其包括使尿嘧啶与式（I）表示的化合物反应，得到式（II）表示的荧光化合物。