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(54) Titre : PROCÉDE POUR LA PRÉPARATION SÉLECTIVE D'UN CONJUGUE ANTICORPS-MÉDICAMENT
 (54) Title: METHOD FOR SELECTIVELY MANUFACTURING ANTIBODY-DRUG CONJUGATE

(57) **Abrégé/Abstract:**

A method for producing an antibody-drug conjugate composition, comprising: (i) a step of reacting an antibody with a reducing agent in a buffer to reduce interchain disulfides, and (ii) a step of reacting drug linker intermediates with the antibody having thiol groups obtained in the step (i), wherein the reaction temperature in the step (i) is -10°C to 10°C, and the average number of bound drugs in the produced antibody-drug conjugate composition is 3.5 to 4.5, and the content of antibody-drug conjugates in which four drug linkers are bound to heavy-light interchain thiols, in the produced antibody-drug conjugate composition is 50% or more; and an antibody-drug conjugate composition, wherein the content of antibody-drug conjugates wherein the average number of bound drugs is 3.5 to 4.5, and the content of antibody-drug conjugates in which four drug linkers are bound to heavy-light interchain thiols, is 50% or more.

Abstract

A method for producing an antibody-drug conjugate composition, comprising: (i) a step of reacting an antibody with a reducing agent in a buffer to reduce interchain disulfides, and (ii) a step of reacting drug linker intermediates with the antibody having thiol groups obtained in the step (i), wherein the reaction temperature in the step (i) is -10°C to 10°C , and the average number of bound drugs in the produced antibody-drug conjugate composition is 3.5 to 4.5, and the content of antibody-drug conjugates in which four drug linkers are bound to heavy-light interchain thiols, in the produced antibody-drug conjugate composition is 50% or more; and an antibody-drug conjugate composition, wherein the content of antibody-drug conjugates wherein the average number of bound drugs is 3.5 to 4.5, and the content of antibody-drug conjugates in which four drug linkers are bound to heavy-light interchain thiols, is 50% or more.

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Description

Title of Invention: Method for selectively manufacturing antibody-drug conjugate

Technical Field

[0001]

The present invention relates to a method for producing an antibody-drug conjugate composition, in which the number of bound drugs and the binding sites are controlled, and an antibody-drug conjugate composition, in which the number of bound drugs and the binding sites are controlled.

Background Art

[0002]

It can be anticipated that an antibody-drug conjugate (hereinafter also referred to as "ADC") formed by binding a drug having cytotoxicity to an antibody that binds to an antigen capable of being expressed on the surface of a cancer cell, and by internalizing in the cell will be able to selectively deliver the drug to the cancer cell, so that it causes the drug to accumulate in the cancer cell and kill the cancer cell (see Non-Patent Literatures 1 to 3). As such an antibody-drug conjugate, an antibody-drug conjugate formed by binding exatecan

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that is a camptothecin derivative to an anti-B7-H3 antibody, or the like, is known (Patent Literature 1).
[0003]

An antibody has four interchain disulfides. These interchain disulfides are approached by solvents more easily than other disulfides, and are easily reduced. Hence, such an interchain disulfide can be used as a binding site to a drug (or a drug linker) in an antibody-drug conjugate. Such an interchain disulfide in an antibody is reduced, and a drug is then allowed to bind to thus generated thiol groups, so as to produce an antibody-drug conjugate in which 2 to 8 drugs bind to a single antibody molecule. Moreover, there is known a method for selectively producing an antibody-drug conjugate having four drug linkers bound to heavy-heavy interchain thiols, wherein the method comprises first completely reducing interchain disulfides in an antibody, re-oxidizing some of the generated interchain thiols to return them to disulfides, and then allowing drugs to bind to the remaining interchain thiols (Patent Literature 2). However, a method for selectively producing an antibody-drug conjugate having four drug linkers bound to heavy-light interchain thiols, is not yet known.

Citation List

Patent Literature

- 3 -

[0004]

Patent Literature 1: International Publication No.

WO2014/057687

Patent Literature 2: International Publication No.

WO2005/084390

Non-Patent Literature

[0005]

Non-Patent Literature 1: Ducry, L., et al., Bioconjugate Chem. (2010) 21, 5-13.

Non-Patent Literature 2: Alley, S. C., et al., Current Opinion in Chemical Biology (2010) 14, 529-537.

Non-Patent Literature 3: Damle N. K., Expert Opin. Biol. Ther. (2004) 4, 1445-1452.

Summary of Invention

Technical Problem

[0006]

An antibody-drug conjugate, in which 8 drugs are bound to a single antibody molecule, is excellent in terms of antitumor effects, but may cause problems in terms of safety, such as side effects and toxicity, in some cases. Hence, in order to reduce side effects or toxicity, while maintaining therapeutic efficacy, there are cases where an antibody-drug conjugate, in which the average number of bound drugs is less than 8, is used. Such an antibody-drug conjugate, in which the average

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number of bound drugs is less than 8, can be obtained, for example, by reacting drugs with an antibody, with controlling the amount of drugs per antibody molecule. The reaction products are antibody-drug conjugate compositions, in which the numbers of bound drugs are 2, 4, 6 and 8. Accordingly, there is the possible case where, even if antibody-drug conjugate compositions have the same average number of bound drugs as each other, their therapeutic efficacy and toxicity are different from each other, if they each have a different distribution of the number of bound drugs. That is to say, when the content of antibody-drug conjugates in which the number of bound drugs is 0 and 8 is high in an antibody-drug conjugate composition in which the average number of bound drugs is 4, its therapeutic efficacy may be reduced and strong toxicity may be expressed, when compared with the case where the content of antibody-drug conjugates in which the number of bound drugs is 4 is high. In addition, there is also the possible case where, even if the antibody-drug conjugates have the same number of bound drugs as each other, their therapeutic efficacy and toxicity are different from each other due to a difference in the binding sites of the drugs. Therefore, it has been desired to develop a method for producing an antibody-drug conjugate composition in which the number of bound drugs and the binding sites are controlled in the production of the antibody-drug conjugate composition.

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Solution to Problem

[0007]

As a result of intensive studies directed towards achieving the aforementioned object, the present inventors have found a method for producing an antibody-drug conjugate composition in which the number of bound drugs and the binding sites are controlled, with more simple operations. That is, the inventors have found that an antibody-drug conjugate composition, wherein the content of antibody-drug conjugates in which the average number of bound drugs is 3.5 to 4.5, and four drug linkers are bound to heavy-light interchain thiols, is 50% or more, can be produced by reducing an antibody using a reducing agent in a buffer at a temperature of -10°C to 10°C, and then reacting drug linker intermediates with the obtained antibody having thiol groups. Moreover, the present inventors have also found that the antibody-drug conjugate composition of the present invention has more excellent safety than antibody-drug conjugate compositions produced by the conventional production method (i.e., an antibody-drug conjugate composition, wherein the content of antibody-drug conjugates in which the average number of bound drugs is 3.5 to 4.5, and four drug linkers are bound to heavy-light interchain thiols, is 35% or less), thereby completing the present invention.

[0008]

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Specifically, the invention of the present application relates to the following (1) to (77):

(1)

A method for producing an antibody-drug conjugate composition, comprising:

(i) a step of reacting an antibody with a reducing agent in a buffer to reduce interchain disulfides; and

(ii) a step of reacting drug linker intermediates with the antibody having thiol groups obtained in the step (i), wherein

the reaction temperature in the step (i) is -10°C to 10°C , and

the average number of bound drugs in the produced antibody-drug conjugate composition is 3.5 to 4.5, and the content of antibody-drug conjugates in which four drug linkers are bound to heavy-light interchain thiols, in the produced antibody-drug conjugate composition is 50% or more.

(2)

The production method according to the above (1), wherein the average number of bound drugs in the produced antibody-drug conjugate composition is 4.0 to 4.1.

(3)

The production method according to the above (1) or (2), wherein the content of antibody-drug conjugates in which four drug linkers are bound to heavy-light

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interchain thiols, in the produced antibody-drug conjugate composition is in the range of 50% to 90%.

(4)

The production method according to the above (3), wherein the content of antibody-drug conjugates in which four drug linkers are bound to heavy-light interchain thiols, in the produced antibody-drug conjugate composition is in the range of 50% to 80%.

(5)

The production method according to the above (4), wherein the content of antibody-drug conjugates in which four drug linkers are bound to heavy-light interchain thiols, in the produced antibody-drug conjugate composition is in the range of 50% to 70%.

(6)

The production method according to the above (5), wherein the content of antibody-drug conjugates in which four drug linkers are bound to heavy-light interchain thiols, in the produced antibody-drug conjugate composition is in the range of 50% to 60%.

(7)

The production method according to any one of the above (1) to (6), wherein the content of antibody-drug conjugates in which four drug linkers are bound to heavy-heavy interchain thiols, in the produced antibody-drug conjugate composition is 5% or less.

(8)

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The production method according to the above (7), wherein the content of antibody-drug conjugates in which four drug linkers are bound to heavy-heavy interchain thiols, in the produced antibody-drug conjugate composition is 1% or less.

(9)

The production method according to any one of the above (1) to (8), wherein the content of antibody-drug conjugates in which two drug linkers are bound to heavy-heavy interchain thiols and two drug linkers are bound to heavy-light interchain thiols, in the produced antibody-drug conjugate composition is 5% or less.

(10)

The production method according to any one of the above (1) to (8), wherein the content of antibody-drug conjugates in which two drug linkers are bound to heavy-heavy interchain thiols and two drug linkers are bound to heavy-light interchain thiols, in the produced antibody-drug conjugate composition is 1% or less.

(11)

The production method according to any one of the above (1) to (10), wherein the reaction temperature in the step (i) is -5°C to 5°C .

(12)

The production method according to the above (11), wherein the reaction temperature in the step (i) is -3°C to 3°C .

(13)

The production method according to the above (12), wherein the reaction temperature in the step (i) is 0°C to 2°C.

(14)

The production method according to the above (13), wherein the reaction temperature in the step (i) is 0°C to 1°C.

(15)

The production method according to any one of the above (1) to (14), wherein the reaction temperature in the step (ii) is 0°C to 2°C.

(16)

The production method according to any one of the above (1) to (15), wherein the reducing agent is used in an amount of 2 to 3 molar equivalents per molecule of the antibody.

(17)

The production method according to any one of the above (1) to (16), wherein the reducing agent is tris(2-carboxyethyl)phosphine or a salt thereof.

(18)

The production method according to the above (17), wherein the salt of tris(2-carboxyethyl)phosphine is tris(2-carboxyethyl)phosphine hydrochloride.

(19)

- 10 -

The production method according to any one of the above (1) to (18), wherein the buffer is a histidine buffer.

(20)

The production method according to any one of the above (1) to (19), wherein the buffer comprises a chelating agent.

(21)

The production method according to the above (20), wherein the chelating agent is ethylenediaminetetraacetic acid.

(22)

The production method according to any one of the above (1) to (21), wherein the antibody is an anti-TROP2 antibody, an anti-CD98 antibody, an anti-B7-H3 antibody, or an anti-HER2 antibody.

(23)

The production method according to the above (22), wherein the antibody is an anti-TROP2 antibody.

(24)

The production method according to the above (22), wherein the antibody is an anti-CD98 antibody.

(25)

The production method according to the above (22), wherein the antibody is an anti-B7-H3 antibody.

(26)

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The production method according to the above (22),
wherein the antibody is an anti-HER2 antibody.

(27)

The production method according to any one of the
above (1) to (26), wherein the drug linker intermediate
has an N-substituted maleimidyl group.

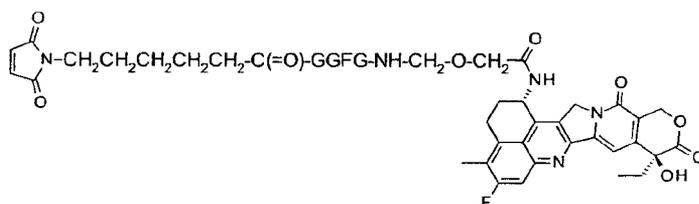
(28)

The production method according to the above (27),
wherein

the drug linker intermediate is

[0009]

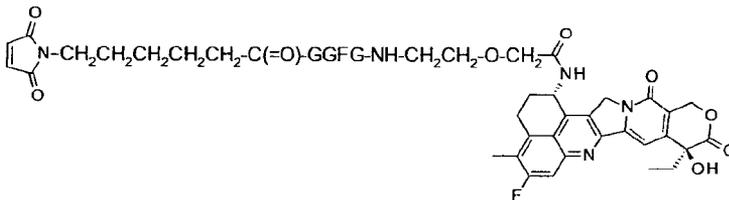
[Formula 1]



[0010]

[0011]

[Formula 2]



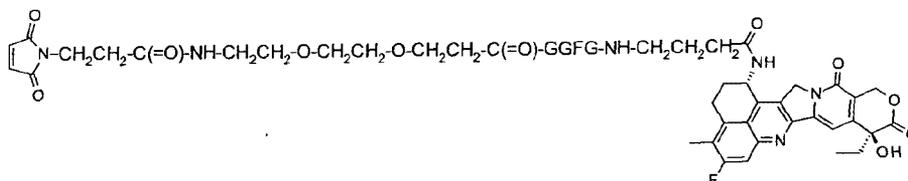
[0012]

, or

[0013]

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[Formula 3]



[0014]

wherein -GGFG- represents a tetrapeptide residue consisting of glycine-glycine-phenylalanine-glycine.

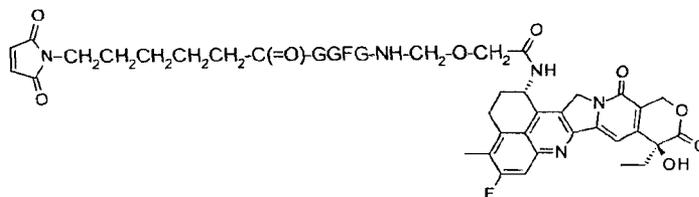
(29)

The production method according to the above (28), wherein

the drug linker intermediate is

[0015]

[Formula 4]



[0016]

wherein -GGFG- represents a tetrapeptide residue consisting of glycine-glycine-phenylalanine-glycine.

(30)

An antibody-drug conjugate composition produced by the production method according to any one of the above

(1) to (29).

(31)

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An antibody-drug conjugate composition, wherein the average number of bound drugs is 3.5 to 4.5, and the content of antibody-drug conjugates in which four drug linkers are bound to heavy-light interchain thiols is 50% or more.

(32)

The antibody-drug conjugate composition according to the above (31), wherein the average number of bound drugs is 4.0 to 4.1.

(33)

The antibody-drug conjugate composition according to the above (31) or (32), wherein the content of antibody-drug conjugates in which four drug linkers are bound to heavy-light interchain thiols, is in the range of 50% to 90%.

(34)

The antibody-drug conjugate composition according to the above (33), wherein the content of antibody-drug conjugates in which four drug linkers are bound to heavy-light interchain thiols, is in the range of 50% to 80%.

(35)

The antibody-drug conjugate composition according to the above (34), wherein the content of antibody-drug conjugates in which four drug linkers are bound to heavy-light interchain thiols, is in the range of 50% to 70%.

(36)

The antibody-drug conjugate composition according to the above (35), wherein the content of antibody-drug conjugates in which four drug linkers are bound to heavy-light interchain thiols, is in the range of 50% to 60%.
(37)

The antibody-drug conjugate composition according to any one of the above (31) to (36), wherein the content of antibody-drug conjugates in which four drug linkers are bound to heavy-heavy interchain thiols, is 5% or less.
(38)

The antibody-drug conjugate composition according to the above (37), wherein the content of antibody-drug conjugates in which four drug linkers are bound to heavy-heavy interchain thiols, is 1% or less.
(39)

The antibody-drug conjugate composition according to any one of the above (31) to (38), wherein the content of antibody-drug conjugates in which two drug linkers are bound to heavy-heavy interchain thiols and two drug linkers are bound to heavy-light interchain thiols, is 5% or less.
(40)

The antibody-drug conjugate composition according to the above (39), wherein the content of antibody-drug conjugates in which two drug linkers are bound to heavy-heavy interchain thiols and two drug linkers are bound to heavy-light interchain thiols, is 1% or less.

(41)

The antibody-drug conjugate composition according to any one of the above (31) to (40), wherein the antibody is an anti-TROP2 antibody, an anti-CD98 antibody, an anti-B7-H3 antibody, or an anti-HER2 antibody.

(42)

The antibody-drug conjugate composition according to the above (41), wherein the antibody is an anti-TROP2 antibody.

(43)

The antibody-drug conjugate composition according to the above (41), wherein the antibody is an anti-CD98 antibody.

(44)

The antibody-drug conjugate composition according to the above (41), wherein the antibody is an anti-B7-H3 antibody.

(45)

The antibody-drug conjugate composition according to the above (41), wherein the antibody is an anti-HER2 antibody.

(46)

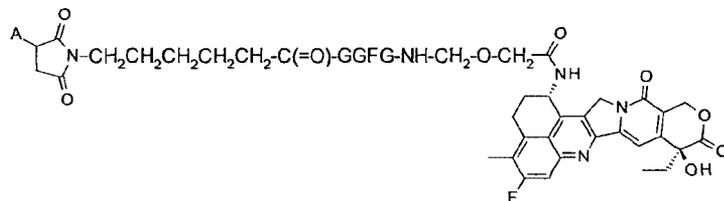
The antibody-drug conjugate composition according to any one of the above (31) to (45), wherein

the drug linker is

[0017]

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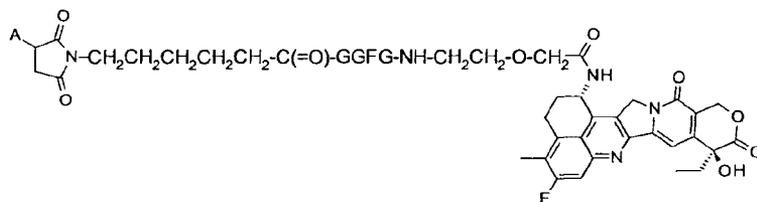
[Formula 5]



[0018]

[0019]

[Formula 6]

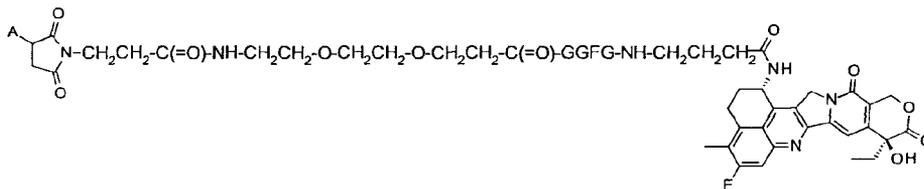


[0020]

, or

[0021]

[Formula 7]



[0022]

wherein A represents a binding site to the antibody, and -GGFG- represents a tetrapeptide residue consisting of glycine-glycine-phenylalanine-glycine.

(47)

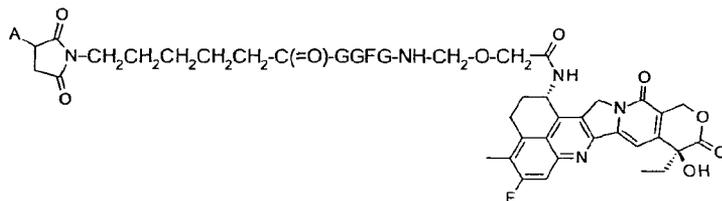
The antibody-drug conjugate composition according to the above (46), wherein

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the drug linker is

[0023]

[Formula 8]



[0024]

wherein A represents a binding site to the antibody, and -GGFG- represents a tetrapeptide residue consisting of glycine-glycine-phenylalanine-glycine.

(48)

A pharmaceutical composition comprising the antibody-drug conjugate composition according to any one of the above (30) to (47).

(49)

The pharmaceutical composition according to the above (48) for use in the treatment of tumor and/or cancer.

(50)

The pharmaceutical composition according to the above (49) for use in the treatment of lung cancer, kidney cancer, urothelial cancer, colon cancer, prostate cancer, glioblastoma multiforme, ovarian cancer, pancreatic cancer, breast cancer, melanoma, liver cancer, bladder cancer, stomach cancer, cervical cancer, uterine cancer, head and neck cancer, esophageal cancer, bile

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duct cancer, thyroid cancer, lymphoma, acute myelocytic leukemia, acute lymphocytic leukemia, chronic myelogenous leukemia, and/or multiple myeloma.

(51)

A method for treating tumor and/or cancer, comprising administration of the antibody-drug conjugate composition according to any one of the above (30) to (47).

(52)

The treatment method according to the above (51), which is a method for treating lung cancer, kidney cancer, urothelial cancer, colon cancer, prostate cancer, glioblastoma multiforme, ovarian cancer, pancreatic cancer, breast cancer, melanoma, liver cancer, bladder cancer, stomach cancer, cervical cancer, uterine cancer, head and neck cancer, esophageal cancer, bile duct cancer, thyroid cancer, lymphoma, acute myelocytic leukemia, acute lymphocytic leukemia, chronic myelogenous leukemia, and/or multiple myeloma.

(53)

A method for producing an antibody having thiol groups, comprising the step of reacting an antibody with a reducing agent in a buffer to reduce interchain disulfides, wherein

the reaction temperature is -10°C to 10°C , and

the produced antibody having thiol groups is used to produce an antibody-drug conjugate composition, wherein

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the average number of bound drugs is 3.5 to 4.5, and the content of antibody-drug conjugates in which four drug linkers are bound to heavy-light interchain thiols is 50% or more.

(54)

The production method according to the above (53), wherein the produced antibody having thiol groups is used to produce an antibody-drug conjugate composition, wherein the average number of bound drugs is 4.0 to 4.1.

(55)

The production method according to the above (53) or (54), wherein the produced antibody having thiol groups is used to produce an antibody-drug conjugate composition, wherein the content of antibody-drug conjugates in which four drug linkers are bound to heavy-light interchain thiols, is in the range of 50% to 90%.

(56)

The production method according to the above (55), wherein the produced antibody having thiol groups is used to produce an antibody-drug conjugate composition, wherein the content of antibody-drug conjugates in which four drug linkers are bound to heavy-light interchain thiols, is in the range of 50% to 80%.

(57)

The production method according to the above (56), wherein the produced antibody having thiol groups is used to produce an antibody-drug conjugate composition,

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wherein the content of antibody-drug conjugates in which four drug linkers are bound heavy-light interchain thiols, is in the range of 50% to 70%.

(58)

The production method according to the above (57), wherein the produced antibody having thiol groups is used to produce an antibody-drug conjugate composition, wherein the content of antibody-drug conjugates in which four drug linkers are bound to heavy-light interchain thiols, is in the range of 50% to 60%.

(59)

The production method according to any one of the above (53) to (58), wherein the produced antibody having thiol groups is used to produce an antibody-drug conjugate composition, wherein the content of antibody-drug conjugates in which four drug linkers are bound to heavy-heavy interchain thiols, is in the range of 5% or less.

(60)

The production method according to the above (59), wherein the produced antibody having thiol groups is used to produce an antibody-drug conjugate composition, wherein the content of antibody-drug conjugates in which four drug linkers are bound to heavy-heavy interchain thiols, is in the range of 1% or less.

(61)

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The production method according to any one of the above (53) to (60), wherein the produced antibody having thiol groups is used to produce an antibody-drug conjugate composition, wherein the content of antibody-drug conjugates in which two drug linkers are bound to heavy-heavy interchain thiols and two drug linkers are bound to heavy-light interchain thiols, is 5% or less.

(62)

The production method according to the above (61), wherein the produced antibody having thiol groups is used to produce an antibody-drug conjugate composition, wherein the content of antibody-drug conjugates in which two drug linkers are bound to heavy-heavy interchain thiols and two drug linkers are bound to heavy-light interchain thiols, is 1% or less.

(63)

The production method according to any one of the above (53) to (62), wherein the reaction temperature is -5°C to 5°C.

(64)

The production method according to the above (63), wherein the reaction temperature is -3°C to 3°C.

(65)

The production method according to the above (64), wherein the reaction temperature is 0°C to 2°C.

(66)

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The production method according to the above (65), wherein the reaction temperature is 0°C to 1°C.

(67)

The production method according to any one of the above (53) to (66), wherein the reducing agent is used in an amount of 2 to 3 molar equivalents per molecule of the antibody.

(68)

The production method according to any one of the above (53) to (67), wherein the reducing agent is tris(2-carboxyethyl)phosphine or a salt thereof.

(69)

The production method according to the above (68), wherein the salt of tris(2-carboxyethyl)phosphine is tris(2-carboxyethyl)phosphine hydrochloride.

(70)

The production method according to any one of the above (53) to (69), wherein the buffer is a histidine buffer.

(71)

The production method according to any one of the above (53) to (70), wherein the buffer comprises a chelating agent.

(72)

The production method according to the above (71), wherein the chelating agent is ethylenediaminetetraacetic acid.

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(73)

The production method according to any one of the above (53) to (72), wherein the antibody is an anti-TROP2 antibody, an anti-CD98 antibody, an anti-B7-H3 antibody, or an anti-HER2 antibody.

(74)

The production method according to the above (73), wherein the antibody is an anti-TROP2 antibody.

(75)

The production method according to the above (73), wherein the antibody is an anti-CD98 antibody.

(76)

The production method according to the above (73), wherein the antibody is an anti-B7-H3 antibody.

(77)

The production method according to the above (73), wherein the antibody is an anti-HER2 antibody.

[0025]

Advantageous Effects of Invention

[0026]

According to the present invention, there are provided a method for producing an antibody-drug conjugate composition in which the number of bound drugs and the binding sites are controlled, and an antibody-drug conjugate composition in which the number of bound drugs and the binding sites are controlled. The

antibody-drug conjugate composition of the present invention is excellent in terms of safety, and is useful as a medicament for the treatment of tumor and/or cancer. In addition, since an antibody-drug conjugate composition in which the number of bound drugs and the binding sites are controlled to be constant can be obtained, it is also excellent in terms of quality control and thus, is preferable.

Brief Description of Drawings

[0027]

[Figure 1] Figure 1 is a view showing the nucleotide sequence and amino acid sequence of a humanized anti-TROP2 antibody heavy chain (hTINA1-H1).

[Figure 2] Figure 2 is a view showing the nucleotide sequence and amino acid sequence of a humanized anti-TROP2 antibody light chain (hTINA1-L1).

[Figure 3] Figure 3 is a view showing the nucleotide sequence and amino acid sequence of a humanized anti-CD98 antibody heavy chain (h23M-H1).

[Figure 4] Figure 4 is a view showing the nucleotide sequence and amino acid sequence of a humanized anti-CD98 antibody light chain (h23M-L1).

[Figure 5] Figure 5 is a graph showing the peak area ratio (%) of each chain in a humanized anti-TROP2 antibody (hTINA1-H1L1) ADC composition produced by the conventional method.

[Figure 6] Figure 6 is a graph showing the distribution (%) of each number of bound drugs in a humanized anti-TROP2 antibody (hTINA1-H1L1) ADC composition produced by the conventional method.

[Figure 7] Figure 7 is a graph showing the peak area ratio (%) of each chain in a humanized anti-TROP2 antibody (hTINA1-H1L1) ADC composition produced by the method of the present invention.

[Figure 8] Figure 8 is a graph showing the distribution (%) of each number of bound drugs in a humanized anti-TROP2 antibody (hTINA1-H1L1) ADC composition produced by the method of the present invention.

[Figure 9] Figure 9 is a graph showing the peak area ratio (%) of each chain in a humanized anti-CD98 antibody (hM23-H1L1) ADC composition produced by the conventional method.

[Figure 10] Figure 10 is a graph showing the distribution (%) of each number of bound drugs in a humanized anti-CD98 antibody (hM23-H1L1) ADC composition produced by the conventional method.

[Figure 11] Figure 11 is a graph showing the peak area ratio (%) of each chain in a humanized anti-CD98 antibody (hM23-H1L1) ADC composition produced by the method of the present invention.

[Figure 12] Figure 12 is a graph showing the distribution (%) of each number of bound drugs in a humanized anti-

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CD98 antibody (hM23-H1L1) ADC composition produced by the method of the present invention.

[Figure 13] Figure 13 is a view showing the amino acid sequence of a humanized anti-B7-H3 antibody heavy chain (M30-H1).

[Figure 14] Figure 14 is a view showing the amino acid sequence of a humanized anti-B7-H3 antibody light chain (M30-L4).

[Figure 15] Figure 15 is a graph showing the peak area ratio (%) of each chain in a humanized anti-B7-H3 antibody (M30-H1-L4) ADC composition produced by the conventional method.

[Figure 16] Figure 16 is a graph showing the distribution (%) of each number of bound drugs in a humanized anti-B7-H3 antibody (M30-H1-L4) ADC composition produced by the conventional method.

[Figure 17] Figure 17 is a graph showing the peak area ratio (%) of each chain in a humanized anti-B7-H3 antibody (M30-H1-L4) ADC composition produced by the method of the present invention.

[Figure 18] Figure 18 is a graph showing the distribution (%) of each number of bound drugs in a humanized anti-B7-H3 antibody (M30-H1-L4) ADC composition produced by the method of the present invention.

[Figure 19] Figure 19 is a view showing the amino acid sequence of a humanized anti-HER2 antibody heavy chain.

[Figure 20] Figure 20 is a view showing the amino acid sequence of a humanized anti-HER2 antibody light chain.

[Figure 21] Figure 21 is a graph showing the peak area ratio (%) of each chain in a humanized anti-HER2 antibody ADC composition produced by the conventional method.

[Figure 22] Figure 22 is a graph showing the distribution (%) of each number of bound drugs in a humanized anti-HER2 antibody ADC composition produced by the conventional method.

[Figure 23] Figure 23 is a graph showing the peak area ratio (%) of each chain in a humanized anti-HER2 antibody ADC composition produced by the method of the present invention.

[Figure 24] Figure 24 is a graph showing the distribution (%) of each number of bound drugs in a humanized anti-HER2 antibody ADC composition produced by the method of the present invention.

[Figure 25] Figure 25 is a graph showing the tumor growth inhibitory effect of a humanized anti-TROP2 antibody ADC composition produced by the conventional method, and the tumor growth inhibitory effect of a humanized anti-TROP2 antibody ADC composition produced by the method of the present invention.

Description of Embodiments

[0028]

In the present description, the term "cancer" is used to have the same meaning as that of the term "tumor".

[0029]

In the present description, the term "gene" is used to include, not only DNA but also its mRNA and cDNA, and the cRNA thereof.

[0030]

In the present description, the term "polynucleotide" is used to have the same meaning as that of a nucleic acid, and it includes DNA, RNA, a probe, an oligonucleotide, and a primer.

[0031]

In the present description, the term "polypeptide" is used to have the same meaning as that of the term "protein".

[0032]

In the present description, the term "cell" includes cells in an individual animal, and cultured cells.

[0033]

In the present description, the term "interchain disulfide" is used to mean a disulfide located between two heavy chains in an antibody (a heavy-heavy interchain disulfide), or a disulfide located between a heavy chain and a light chain in an antibody (a heavy-light interchain disulfide).

[0034]

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In the present description, the term "interchain thiol" is used to mean a thiol group obtained by reducing an interchain disulfide of an antibody.

[0035]

In the present description, the term "heavy-heavy interchain thiol" is used to mean a thiol group obtained by reducing a heavy-heavy interchain disulfide of an antibody.

[0036]

In the present description, the term "heavy-light interchain thiol" is used to mean a thiol group obtained by reducing a heavy-light interchain disulfide of an antibody.

[0037]

In the present description, the term "tumor-associated antigen (TAA)" is used to mean an antigen which is expressed in both normal cells and tumor cells, but the expression thereof is relatively restricted to tumor cells.

[0038]

In the present description, the term "tumor-specific antigen (TSA)" is used to mean an antigen that is specific to tumor cells.

[0039]

In the present description, the term "TROP2" is used to have the same meaning as that of a TROP2 protein.

[0040]

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In the present description, the term "CD98" is used to have the same meaning as that of a CD98 protein. Since CD98 consists of a heavy chain and a light chain, the terms "CD98 heavy chain" and "CD98 light chain" are used to have the same meanings as those of a CD98 heavy chain protein and a CD98 light chain protein, respectively. Moreover, in the present description, the term "CD98" is used in a manner interconvertible with any one of "CD98 heavy chain" and "CD98 light chain," or "CD98 heavy chain" or "CD98 light chain," unless otherwise specified.

[0041]

In the present description, the term "anti-TROP2 antibody" is used to mean an antibody capable of binding to TROP2.

[0042]

In the present description, the term "anti-CD98 antibody" is used to mean an antibody capable of binding to CD98.

[0043]

In the present description, the term "anti-B7-H3 antibody" is used to mean an antibody capable of binding to B7-H3.

[0044]

In the present description, the term "anti-HER2 antibody" is used to mean an antibody capable of binding to HER2.

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[0045]

In the present description, the term "cytotoxic" is used to mean that a pathologic change is given to cells by any given way. It does not only mean a direct external injury, but also means all types of structural or functional damages given to cells, such as DNA cleavage, formation of a base dimer, chromosomal cleavage, damage to a cell mitotic apparatus, and a reduction in the activities of various types of enzymes.

[0046]

In the present description, the term "cytotoxicity" is used to mean an action to cause the above described cytotoxic phenomenon.

[0047]

In the present description, the term "antibody-dependent cellular cytotoxicity" is used to mean an "antibody dependent cellular cytotoxic (ADCC) activity", and this activity means the activity of NK cells to give a damage to target cells such as tumor cells, mediated by an antibody.

[0048]

In the present description, the term "complement-dependent cytotoxicity" is used to mean a "complement dependent cytotoxic (CDC) activity", and this activity means the activity of a complement to give a damage to target cells such as tumor cells, mediated by an antibody.

[0049]

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In the present description, the term "epitope" is used to mean the partial peptide or partial three-dimensional structure of an antigen that binds to a specific antibody. Such an epitope, which is a partial peptide of an antigen, can be determined by methods well known to a person skilled in the art, such as an immunoassay, for example, by the following method. First, various partial structures of an antigen are produced. For production of such partial structures, a known oligopeptide synthesis technique can be applied. For example, a series of peptides, in which an antigen has been successively cut at an appropriate length from the C-terminus or N-terminus thereof, are produced by genetic recombination techniques well known to a person skilled in the art, and thereafter, the reactivity of an antibody with such polypeptides is studied, and recognition sites are roughly determined. Thereafter, further shorter peptides are synthesized, and the reactivity thereof with the aforementioned peptides is then studied, so as to determine an epitope. Moreover, an epitope, which is a partial three-dimensional structure of an antigen that binds to a specific antibody, can be determined by specifying the amino acid residues of an antigen adjacent to the above described antibody by X-ray structural analysis.

[0050]

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In the present description, the phrase "antibody binding to the same epitope" is used to mean a different antibody binding to a common epitope. If a second antibody binds to a partial peptide or a partial three-dimensional structure, to which a first antibody binds, it can be determined that the first antibody and the second antibody bind to the same epitope. In addition, by confirming that a second antibody competes with the binding of a first antibody to an antigen (i.e., a second antibody prevents a first antibody from binding to an antigen), it can be determined that the first antibody and the second antibody bind to the same epitope, although the specific sequence or structure of the epitope has not been determined. Furthermore, when a first antibody and a second antibody bind to the same epitope and further, the first antibody has special effects such as antitumor activity, the second antibody can be expected to have the same activity as that of the first antibody.

[0051]

In the present description, the term "CDR" is used to mean a complementarity determining region (CDR). It is known that the heavy chain and light chain of an antibody molecule each have three CDRs. Such CDR is also referred to as a hypervariable domain, and is present in the variable region of the heavy chain and light chain of an antibody, in which the mutation of a primary structure

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is particularly high. The CDR is separated into three sites on the primary structure of a polypeptide chain in each of the heavy chain and light chain. In the present description, with regard to the CDR of an antibody, the CDRs of a heavy chain are referred to as CDRH1, CDRH2 and CDRH3, respectively, from the N-terminal side of the amino acid sequence of the heavy chain, whereas the CDRs of a light chain are referred to as CDRL1, CDRL2 and CDRL3, respectively, from the N-terminal side of the amino acid sequence of the light chain. These sites are located close to one another on the three-dimensional structure, and determine the specificity of the antibody to an antigen, to which the antibody binds.

[0052]

In the present description, the term "several" is used to mean a number from 2 to 10. The number is preferably 2 to 9, more preferably 2 to 8, even more preferably 2 to 7, further preferably 2 to 6, still further preferably 2 to 5, still further preferably 2 to 4, much further preferably 2 or 3, and much further preferably 2.

[0053]

In the present description, the term "antibody-drug conjugate composition" is used to mean a composition comprising, at any given ratio, an antibody-drug conjugate bound by two drug linkers, an antibody-drug conjugate bound by four drug linkers, an antibody-drug

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conjugate bound by six drug linkers, an antibody-drug conjugate bound by eight drug linkers, and an antibody not bound by any drug linker. In the present description, the "antibody-drug conjugate composition" is also referred to as an "ADC composition".

[0054]

In the present description, the "average number of bound drugs" is also referred to as a drug-to-antibody ratio (DAR), and the average number of bound drugs means the average number of drugs that bind to a single antibody molecule in an antibody-drug conjugate composition.

[0055]

In the present description, the term "content" is used to mean the content (molar % based on an antibody) of antibody-drug conjugates having a specific number of bound drugs and specific binding sites in an antibody-drug conjugate composition.

[0056]

In the present description, the term "identity" is used to have the same meaning as that of the term "homology".

[0057]

1. Antibody

The antibody used in the present invention can be generated with respect to an antigen of interest, for example, with respect to a tumor-specific antigen (TAA)

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or a tumor-associated antigen (TSA). Such an antibody has the property of being able to recognize tumor cells, the property of being able to bind to such tumor cells, and the property of being able to be incorporated into such tumor cells and then internalizing therein.

[0058]

The type of such an antigen of interest is not particularly limited, as long as it is a tumor cell-associated antigen. Examples of the antigen of interest include B7-H3, CD3, CD30, CD33, CD37, CD56, CD98, DR5, EGFR, EPHA2, FGFR2, FGFR4, FOLR1 (Folate Receptor 1), HER2, HER3, TROP2, and VEGF.

[0059]

The antibody used in the present invention can be obtained by the method described, for example, in WO2009/091048, WO2011/027808, or WO2012/133572. Specifically, a non-human animal is immunized with an antigen of interest, and lymph fluids, lymphoid tissues, blood cell samples or bone marrow-derived cells are then collected from the immunized animal. Thereafter, the plasma cells and/or plasmablasts of the non-human animal, which specifically bind to the antigen of interest, are selected. From the obtained plasma cells and/or plasmablasts, an antibody gene reacting against the antigen of interest is collected, and the nucleotide sequence of the antibody gene is then identified. Thereafter, the above described antibody or an antibody

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fragment thereof can be obtained based on the identified nucleotide sequence of the gene. The thus obtained antibodies are examined in terms of their binding activity to the antigen of interest, so that an antibody that is applicable to human diseases can be selected.

[0060]

Alternatively, according to known methods (e.g., Kohler and Milstein, *Nature* (1975) 256, pp. 495-497; Kennet, R. ed., *Monoclonal Antibodies*, pp. 365-367, Plenum Press, N.Y. (1980)), antibody-producing cells that produce an antibody reacting against the antigen of interest are fused with myeloma cells to establish hybridomas, so as to obtain a monoclonal antibody. Specific examples of such methods are described in WO2009/048072 (published on April 16, 2009) and WO2010/117011 (published on October 14, 2010).

[0061]

The antibody used in the present invention includes genetically recombinant antibodies, which are artificially modified for the purpose of reducing heterologous antigenicity against humans, for example, a chimeric antibody, a humanized antibody, and a human antibody. These antibodies can be produced according to known methods.

[0062]

The chimeric antibody is, for example, an antibody whose variable region and constant region are

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heterologous to each other, such as a chimeric antibody formed by conjugating the variable region of a mouse- or rat-derived antibody to a constant region derived from a human (see Proc. Natl. Acad. Sci. U.S.A., 81, 6851-6855, (1984)).

[0063]

Examples of the humanized antibody include an antibody formed by incorporating only CDR into a human-derived antibody (see Nature (1986) 321, pp. 522-525), and an antibody formed by transplanting the amino acid residues in some frameworks, as well as CDR, into a human antibody according to a CDR grafting method (International Publication No. WO90/07861).

[0064]

Preferred examples of the antibody of the present invention include an anti-TROP2 antibody, an anti-CD98 antibody, an anti-B7-H3 antibody, and an anti-HER2 antibody.

[0065]

An actual example of the humanized anti-TROP2 antibody can be any given combination of: a heavy chain comprising a heavy chain variable region consisting of any one of (1) an amino acid sequence consisting of amino acid residues at positions 20 to 140 of SEQ ID NO: 2, (2) an amino acid sequence having homology of at least 95% with respect to the amino acid sequence in the above (1), and (3) an amino acid sequence comprising a deletion,

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substitution or addition of one or several amino acids in the amino acid sequence in the above (1); and a light chain comprising a light chain variable region consisting of any one of (4) an amino acid sequence consisting of amino acid residues at positions 21 to 129 of SEQ ID NO: 4, (5) an amino acid sequence having homology of at least 95% with respect to the amino acid sequence in the above (4), and (6) an amino acid sequence comprising a deletion, substitution or addition of one or several amino acids in the amino acid sequence in the above (4).

[0066]

An example of the above described antibody comprising a preferred combination of the heavy chain and the light chain can be an antibody (hTINA1-H1L1), which consists of a heavy chain consisting of an amino acid sequence consisting of amino acid residues at positions 20 to 470 of SEQ ID NO: 2, and a light chain consisting of an amino acid sequence consisting of amino acid residues at positions 21 to 234 of SEQ ID NO: 4.

[0067]

The humanized anti-TROP2 antibody is not limited to a specific humanized antibody, as long as it retains CDRH1 consisting of the amino acid sequence shown in SEQ ID NO: 5 (TAGMQ), CDRH2 consisting of the amino acid sequence shown in SEQ ID NO: 6 (WINTHSGVPKYAEDFKG), CDRH3 consisting of the amino acid sequence shown in SEQ ID NO: 7 (SGFGSSYWYFDV), CDRL1 consisting of the amino acid

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sequence shown in SEQ ID NO: 8 (KASQDVSTAVA), CDRL2 consisting of the amino acid sequence shown in SEQ ID NO: 9 (SASYRYT), and CDRL3 consisting of the amino acid sequence shown in SEQ ID NO: 10 (QQHYITPLT), which are shown in the sequence listing.

[0068]

An example of the humanized anti-CD98 antibody can be any given combination of: a heavy chain comprising a heavy chain variable region consisting of any one of (1) an amino acid sequence consisting of amino acid residues at positions 20 to 135 of SEQ ID NO: 12, (2) an amino acid sequence having identity of at least 95% with respect to the amino acid sequence in the above (1), and (3) an amino acid sequence comprising a deletion, substitution or addition of one or several amino acids in the amino acid sequence in the above (1); and a light chain comprising a light chain variable region consisting of any one of (4) an amino acid sequence consisting of amino acid residues at positions 21 to 135 of SEQ ID NO: 14, (5) an amino acid sequence having identity of at least 95% with respect to the amino acid sequence in the above (4), and (6) an amino acid sequence comprising a deletion, substitution or addition of one or several amino acids in the amino acid sequence in the above (4).

[0069]

An example of the above described antibody comprising a preferred combination of the heavy chain and

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the light chain can be an antibody (hM23-H1L1), which consists of a heavy chain consisting of an amino acid sequence consisting of amino acid residues at positions 20 to 465 of SEQ ID NO: 12, and a light chain consisting of an amino acid sequence consisting of amino acid residues at positions 21 to 240 of SEQ ID NO: 14.

[0070]

The humanized anti-CD98 antibody is not limited to a specific humanized antibody, as long as it retains CDRH1 consisting of the amino acid sequence shown in SEQ ID NO: 15 (NYLIE), CDRH2 consisting of the amino acid sequence shown in SEQ ID NO: 16 (VINPGSGVTNYNEKFKG), CDRH3 consisting of the amino acid sequence shown in SEQ ID NO: 17 (AEAWFAY), CDRL1 consisting of the amino acid sequence shown in SEQ ID NO: 18 (KSSQSLLYSSNQKNYLA), CDRL2 consisting of the amino acid sequence shown in SEQ ID NO: 19 (WASTRES), and CDRL3 consisting of the amino acid sequence shown in SEQ ID NO: 20 (QRYYGYPWT), which are shown in the sequence listing.

[0071]

An example of the humanized anti-B7-H3 antibody can be any given combination of: a heavy chain comprising a heavy chain variable region consisting of any one of (1) an amino acid sequence consisting of amino acid residues at positions 20 to 141 of SEQ ID NO: 25, (2) an amino acid sequence having identity of at least 95% with respect to the amino acid sequence in the above (1), and

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(3) an amino acid sequence comprising a deletion, substitution or addition of one or several amino acids in the amino acid sequence in the above (1); and a light chain comprising a light chain variable region consisting of any one of (4) an amino acid sequence consisting of amino acid residues at positions 21 to 128 of SEQ ID NO: 26, (5) an amino acid sequence having identity of at least 95% with respect to the amino acid sequence in the above (4), and (6) an amino acid sequence comprising a deletion, substitution or addition of one or several amino acids in the amino acid sequence in the above (4).

[0072]

An example of the above described antibody comprising a preferred combination of the heavy chain and the light chain can be an antibody (M30-H1-L4), which consists of a heavy chain consisting of an amino acid sequence consisting of amino acid residues at positions 20 to 471 of SEQ ID NO: 25, and a light chain consisting of an amino acid sequence consisting of amino acid residues at positions 21 to 233 of SEQ ID NO: 26.

[0073]

The humanized anti-B7-H3 antibody is not limited to a specific humanized antibody, as long as it retains CDRH1 consisting of the amino acid sequence shown in SEQ ID NO: 27 (NYVMH), CDRH2 consisting of the amino acid sequence shown in SEQ ID NO: 28 (YINPYNDDVKYNEKFKG), CDRH3 consisting of the amino acid sequence shown in SEQ

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ID NO: 29 (WGYYGSPLYYFDY), CDRL1 consisting of the amino acid sequence shown in SEQ ID NO: 30 (RASSRLIYMH), CDRL2 consisting of the amino acid sequence shown in SEQ ID NO: 31 (ATSNLAS), and CDRL3 consisting of the amino acid sequence shown in SEQ ID NO: 32 (QQWNSNPPT), which are shown in the sequence listing.

[0074]

An example of the humanized anti-HER2 antibody can be an antibody, which consists of a heavy chain consisting of an amino acid sequence consisting of amino acid residues at positions 1 to 449 of SEQ ID NO: 33, and a light chain consisting of an amino acid sequence consisting of amino acid residues at positions 1 to 214 of SEQ ID NO: 34 (trastuzumab; U. S. Patent No. 5821337).

[0075]

Moreover, a CDR-modified humanized antibody, in which 1 to 3 amino acid residues in each CDR are substituted with other amino acid residues, is also included in the antibody used in the present invention, as long as the CDR-modified humanized antibody has binding activity to tumor cells.

[0076]

The antibody used in the present invention further includes a human antibody. Such a human antibody can be obtained by a method of using a human antibody-producing mouse having a human chromosomal fragment comprising the heavy chain and light chain genes of a human antibody

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(see Tomizuka, K. et. al., Nature Genetics (1997) 16, pp. 133-143; Kuroiwa, Y. et. al., Nucl. Acids Res. (1998) 26, pp. 3447-3448; Yoshida, H. et. al., Animal Cell Technology: Basic and Applied Aspects vol. 10, pp. 69-73 (Kitagawa, Y., Matsuda, T. and Iijima, S. eds.), Kluwer Academic Publishers, 1999.; Tomizuka, K. et. al., Proc. Natl. Acad. Sci. USA (2000) 97, pp. 722-727; etc.).

[0077]

Furthermore, there are known methods of obtaining a human antibody that is derived from a phage display selected from a human antibody library (see Wormstone, I. M. et. al, Investigative Ophthalmology & Visual Science. (2002) 43(7), pp. 2301-2308; Carmen, S. et. al., Briefings in Functional Genomics and Proteomics (2002), 1(2), pp. 189-203; Siriwardena, D. et. al., Ophthalmology (2002) 109(3), pp. 427-431; etc.).

[0078]

For instance, a phage display method comprising allowing the variable region of a human antibody to express as a single-chain antibody (scFv) on the surface of a phage, and then selecting a phage binding to an antigen, can be used (Nature Biotechnology (2005), 23, (9), pp. 1105-1116). By analyzing the gene of a phage selected based on its binding activity to an antigen, a DNA sequence encoding the variable region of a human antibody binding to an antigen can be determined. If the DNA sequence of scFv binding to an antigen were

elucidated, it would become possible to obtain a human antibody by producing an expression vector having the DNA sequence, and then introducing the expression vector into a suitable host (WO92/01047, WO92/20791, WO93/06213, WO93/11236, WO93/19172, WO95/01438, WO95/15388, Annu. Rev. Immunol (1994) 12, pp. 433-455, Nature Biotechnology (2005) 23(9), pp. 1105-1116).

[0079]

The binding activity of the above described antibody to an antigen of interest is evaluated, so that a preferred antibody can be selected. The dissociation constant between an antibody and an antigen can be measured by using Biacore[™] T200 (GE Healthcare Bioscience), which involves Surface Plasmon Resonance (SPR) as a detection principle. For instance, an antibody, which is adjusted to have a suitable concentration with respect to an antigen that is solid-phased as a ligand, is allowed to react with an analyte, and its association and dissociation are then measured, so as to obtain an association rate constant k_{a1} , a dissociation rate constant k_{d1} , and a dissociation constant (KD; $KD = k_{d1}/k_{a1}$). The binding activity to an antigen of interest can be evaluated, not only with the use of Biacore T200, but also by using an apparatus involving Surface Plasmon Resonance (SPR) as a detection principle, KinExA (Sapidyne Instruments) involving Kinetic Exclusion Assay as a detection principle, BLItz System (Pall) involving

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Bio-Layer Interferometry as a detection principle, an ELISA (Enzyme-Linked ImmunoSorbent Assay) method, or the like.

[0080]

The activity of internalizing in cells can be confirmed by applying (1) an assay of visualizing an antibody incorporated into cells under a fluorescence microscope, using a secondary antibody (fluorescent label) binding to a therapeutic antibody (Cell Death and Differentiation (2008) 15, 751-761), (2) an assay of measuring the amount of fluorescence incorporated into cells, using a secondary antibody (fluorescent label) binding to a therapeutic antibody (Molecular Biology of the Cell Vol. 15, 5268-5282, December 2004), or (3) a Mab-ZAP assay, in which immunotoxin that binds to a therapeutic antibody is used, and when the immunotoxin is incorporated into cells, toxin is released and cell growth is suppressed (BioTechniques 28: 162-165, January 2000). As such an immunotoxin, a recombinant conjugated protein consisting of a catalytic region of diphtheria toxin and a protein G can also be used.

[0081]

An example of another indicator used for a comparison of the properties of antibodies can be the stability of antibody. Differential scanning calorimetry (DSC) is a method capable of promptly and precisely measuring a thermal denaturation midpoint (T_m) serving as

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a good indicator for the relative structural stability of a protein. By using DSC to measure such T_m values and then comparing the obtained values, a difference in thermal stability can be compared. It is known that the preservation stability of an antibody shows a certain degree of correlation with the thermal stability of an antibody (Lori Burton, et. al., Pharmaceutical Development and Technology (2007) 12, pp. 265-273), and using thermal stability as an indicator, a preferred antibody can be selected. Examples of other indicators for selection of an antibody include a high yield in suitable host cells, and low cohesiveness in an aqueous solution. For example, since an antibody with the highest yield does not always exhibit the highest thermal stability, a comprehensive decision is made based on the aforementioned indicators, and an antibody, which is most suitably administered to humans, needs to be selected.

[0082]

Moreover, by regulating sugar chain modification binding to an antibody, antibody-dependent cytotoxicity can be enhanced. As techniques of regulating the sugar chain modification of an antibody, those described in WO99/54342, WO2000/61739, WO2002/31140, etc. are known, but the techniques are not limited thereto.

[0083]

When an antibody gene has been isolated and thereafter, the gene introduced into a suitable host to

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produce an antibody, a suitable combination of a host and an expression vector can be used. A specific example of the antibody gene can be a combination of a gene encoding the heavy chain sequence of the antibody described in the present description and a gene encoding the light chain sequence of the antibody described therein. For transformation of host cells, a heavy chain sequence gene and a light chain sequence gene can be inserted into a single expression vector, or these genes can also be inserted each into different expression vectors. When eukaryotic cells are used as hosts, animal cells, plant cells, eukaryotic microorganisms can be used. Examples of the animal cells include mammalian cells such as COS cells which are monkey cells (Gluzman, Y., Cell (1981) 23, pp. 175-182, ATCC CRL-1650), mouse fibroblasts NIH3T3 (ATCC No. CRL-1658), and a dihydrofolate reductase-deficient cell line of Chinese hamster ovary cells (CHO cells, ATCC CCL-61) (Urlaub, G. and Chasin, L. A. Proc. Natl. Acad. Sci. U.S.A. (1980) 77, pp. 4126-4220). On the other hand, when prokaryotic cells are used as hosts, *Escherichia coli* or *Bacillus subtilis* can be used, for example. An antibody gene of interest is introduced into these cells for transformation, and the transformed cells are then cultured *in vitro* to obtain an antibody. In the aforementioned culture method, there is the case where the yield is different depending on the sequence of an antibody, and thus, it is possible to select an antibody,

which is easily produced as a medicament, from antibodies having equivalent binding activity, using the yield as an indicator.

[0084]

Isotypes of the antibody used in the present invention are not limited, and examples of the isotype of the present antibody include IgG (IgG1, IgG2, IgG3, and IgG4), IgM, IgA (IgA1 and IgA2), IgD, and IgE. Among others, IgG or IgM is preferable, and IgG1, IgG2 or IgG3 is more preferable.

[0085]

Examples of the general function of an antibody include antigen-binding activity, activity of neutralizing the activity of an antigen, activity of enhancing the activity of an antigen, antibody-dependent cytotoxicity, complement-dependent cytotoxicity, complement-dependent cellular cytotoxicity, and internalization activity.

[0086]

Further, the antibody used in the present invention may be a multispecific antibody having specificity to at least two types of different antigens. In general, such a molecule binds to two types of antigen (i.e., a bispecific antibody). However, the "multispecific antibody" in the present invention includes an antibody having specificity to more antigens (e.g., 3 types of antigens).

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[0087]

The antibody used in the present invention may be an antibody having identity (or homology) of 80% to 99% to the heavy chain and/or light chain of the above described antibody. By combining sequences having high homology to the above described heavy chain amino acid sequence and light chain amino acid sequence, an antibody having antigen-binding activity and internalization activity that are equivalent to those of each of the above described antibodies can be selected. Such homology is homology of generally 80% or more, preferably 90% or more, more preferably 95% or more, and most preferably 99% or more. In addition, by combining amino acid sequences comprising a substitution, deletion and/or addition of one to several amino acid residues with respect to the amino acid sequences of a heavy chain and/or a light chain, an antibody having various types of actions that are equivalent to those of each of the above described antibodies can be selected. The number of amino acid residues to be substituted, deleted and/or added is generally 10 or less amino acid residues, preferably 9 or less amino acid residues, more preferably 8 or less amino acid residues, more preferably 7 or less amino acid residues, even more preferably 6 or less amino acid residues, further preferably 5 or less amino acid residues, still further preferably 4 or less amino acid residues, still further preferably 3 or less amino acid

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residues, still further preferably 2 or less amino acid residues, and most preferably 1 amino acid residue.

[0088]

It is known that the lysine residue at the carboxyl terminus of the heavy chain of an antibody produced in cultured mammalian cells is deleted (Journal of Chromatography A, 705: 129-134 (1995)), and also, it is known that the two amino acid residues at the heavy chain carboxyl terminus, glycine and lysine, are deleted, and that the proline residue positioned at the carboxyl terminus is newly amidated (Analytical Biochemistry, 360: 75-83 (2007)). However, deletion and modification of these heavy chain sequences do not have an influence on the antigen-binding activity and effector function (activation of a complement, antibody-dependent cytotoxicity, etc.) of an antibody. Accordingly, the present invention also includes an antibody that has undergone the aforementioned modification, and specific examples of such an antibody include a deletion mutant comprising a deletion of 1 or 2 amino acids at the heavy chain carboxyl terminus, and an a deletion mutant formed by amidating the aforementioned deletion mutant (e.g., a heavy chain in which the proline residue at the carboxyl terminal site is amidated). However, deletion mutants regarding a deletion at the carboxyl terminus of the heavy chain of the antibody according to the present invention are not limited to the above described deletion

mutants, as long as they retain antigen-binding activity and effector function. Two heavy chains constituting the antibody according to the present invention may be any one type of heavy chain selected from the group consisting of a full length antibody and the above described deletion mutants, or a combination of any two types selected from the aforementioned group. The amount ratio of individual deletion mutants can be influenced by the types of cultured mammalian cells that produce the antibody according to the present invention, and culture conditions. The main ingredient of the antibody according to the present invention can be the case where one amino acid residue is deleted at each of the carboxyl termini of the two heavy chains.

[0089]

Homology between two types of amino acid sequences can be determined by using the default parameter of Blast algorithm version 2.2.2 (Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schaffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs," *Nucleic Acids Res.* 25: 3389-3402).

It is to be noted that, using the above described Blast algorithm, two types of percentages values, namely, Identity (or Identities) and Positively (or Positivities), can be

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calculated. The former is a value obtained when amino acid residues are identical between two types of amino acid sequences, the homology of which is to be obtained. The latter is a numerical value, for which amino acid residues having a similar chemical structure are also considered. In the present description, the value of identity when amino acid residues are identical to each other is defined as the value of homology.

[0090]

The obtained antibody can be purified until it becomes homogenous. For separation and purification of the antibody, ordinary separation and purification methods, which are applied to proteins, may be used. An antibody can be separated and purified by appropriately selecting methods, for example, from column chromatography, filtration, ultrafiltration, salting-out, dialysis, preparative polyacrylamide gel electrophoresis and isoelectric focusing, and then combining the selected methods (Strategies for Protein Purification and Characterization: A Laboratory Course Manual, Daniel R. Marshak et. al., eds., Cold Spring Harbor Laboratory Press (1996); Antibodies: A Laboratory Manual. Ed Harlow and David Lane, Cold Spring Harbor Laboratory (1988)), but the separation and purification methods are not limited thereto.

[0091]

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Examples of the chromatography include affinity chromatography, ion exchange chromatography, hydrophobic chromatography, gel filtration chromatography, reverse phase chromatography, and adsorption chromatography.

[0092]

These chromatographic methods can be carried out by using liquid chromatography such as HPLC or FPLC.

[0093]

Examples of a column used in affinity chromatography include a protein A column and a protein G column.

[0094]

2. Drug

The drug used in the present invention is not particularly limited, as long as it is a compound having antitumor effects and also having a substituent or a partial structure capable of binding to a linker structure. Regarding such a drug, a part of or the entire linker is cleaved in a tumor cell, and an antitumor compound portion is released, so that antitumor effects are exhibited. If the linker is cleaved at a binding portion to the drug, an antitumor compound is released while having its original structure, so that the original antitumor effects are exhibited. The drug is allowed to bind to the antibody via a linker portion having a specific structure. In the present description, a drug linker including this drug and this linker portion is also referred to a "drug".

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[0095]

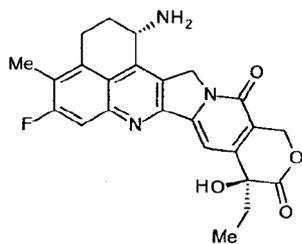
Examples of the antitumor compound include calicheamicin, doxorubicin, daunorubicin, mitomycin C, bleomycin, cycloctidine, vincristine, vinblastine, methotrexate, cisplatin or a derivative thereof, auristatin or a derivative thereof, maytansine or a derivative thereof, taxol or a derivative thereof, and camptothecin or a derivative thereof. Among these compounds, exatecan or monomethyl auristatin E are preferable.

[0096]

Exatecan ((1S,9S)-1-amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione), which is a camptothecin derivative, is a compound represented by the following formula.

[0097]

[Formula 9]



[0098]

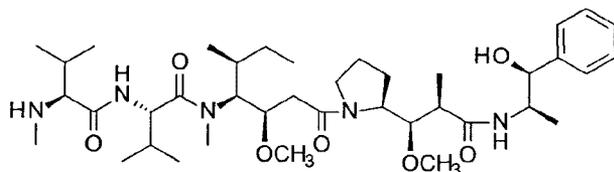
Monomethyl auristatin E ((2R,3R)-N-[(1R,2S)-1-methyl-2-hydroxy-2-phenylethyl]-2-methyl-3-[(2S)-1-[(3R,4S,5S)-3-methoxy-4-[(N-methyl-L-Val-L-Val-

- 56 -

) (methyl) amino]-5-methylheptanoyl]-2-pyrrolidinyl]-3-methoxypropanamide) is a compound represented by the following formula.

[0099]

[Formula 10]



[0100]

3. Linker

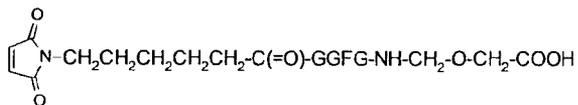
The drug used in the present invention can be bound to an antibody via a linker. The linker used in the present invention preferably has an N-substituted maleimidyl group. Examples of the linker include a cleavable linker and a non-cleavable linker. An example of the cleavable linker is a peptide linker cleaved by intracellular protease such as lysosomal protease or endosomal protease.

[0101]

The linker used in the present invention preferably has a structure induced from any one of the following formulae:

[0102]

[Formula 11]



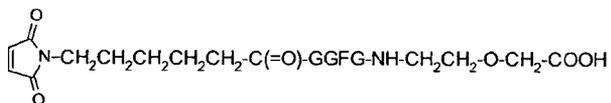
- 57 -

[0103]

,

[0104]

[Formula 12]

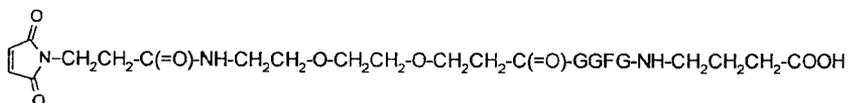


[0105]

, and

[0106]

[Formula 13]

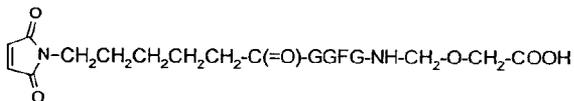


[0107]

, and preferably has a structure induced from the following formula:

[0108]

[Formula 14]



[0109]

.

[0110]

The term "GGFG" means a tetrapeptide residue consisting of glycine-glycine-phenylalanine-glycine.

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[0111]

The linker used in the present invention can be prepared, for example, according to the method described in Example 58 of WO2014/057687.

[0112]

4. Drug linker intermediate

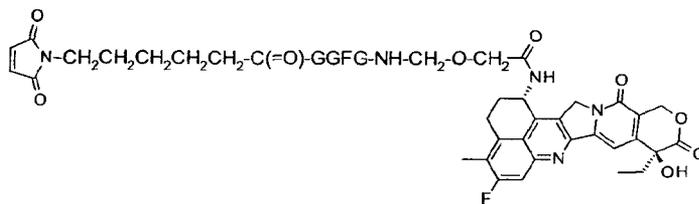
The drug linker intermediate used in the present invention can be produced by reacting the carboxyl group of the above described linker compound with the amino group of an antitumor compound, using a condensing agent, etc.

[0113]

The drug linker intermediate used in the method of the present invention is not particularly limited, as long as it is a compound subjectable to a reaction with an interchain thiol of an antibody. The present drug linker intermediate is preferably a compound having an N-substituted maleimidyl group, and it is more preferably,

[0114]

[Formula 15]

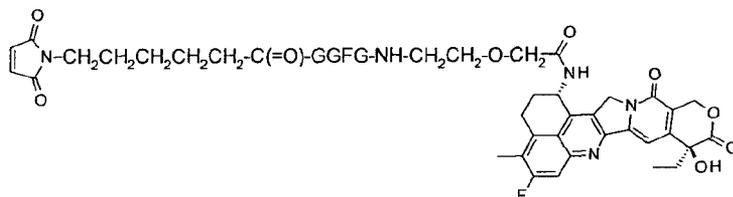


[0115]

[0116]

- 59 -

[Formula 16]

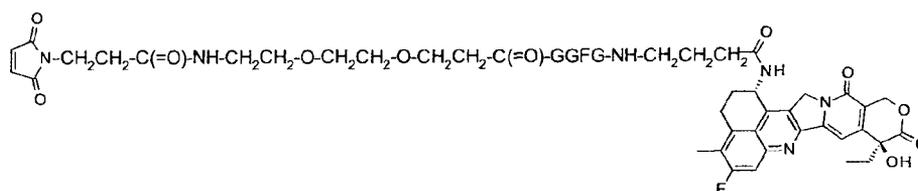


[0117]

, or

[0118]

[Formula 17]

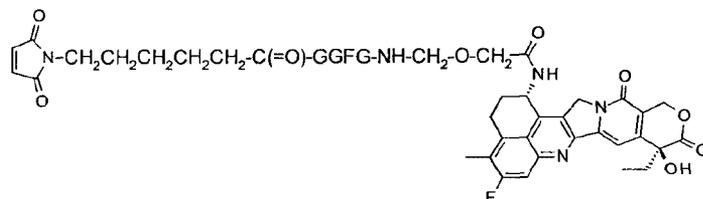


[0119]

, and it is further preferably,

[0120]

[Formula 18]



[0121]

.

[0122]

The drug linker intermediate used in the present invention can be prepared, for example, according to the

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methods described in Example 58, Example 43 and Example 14 of WO2014/057687.

[0123]

If the present drug linker intermediate is a drug linker intermediate having an N-substituted maleimidyl group, it is allowed to react with an interchain thiol generated by reduction of an interchain disulfide of an antibody, so that the antibody can be allowed to bind to the drug via the linker. Accordingly, the drug linker intermediate is preferably a compound having an N-substituted maleimidyl group. However, the drug linker intermediate used herein is not limited to such a compound, and all types of drug linker intermediates can be applied to the production method of the present invention, as long as they have a functional group for promoting the reaction with an interchain thiol of an antibody.

5. Antibody-drug conjugate

Regarding an antibody-drug conjugate, the number of drugs binding to a single antibody molecule is an important factor that has an influence on efficacy and safety. Since an antibody has four interchain disulfides and such a disulfide is constituted with two thiol groups, the number of drug binding to a single antibody molecule is 2, 4, 6, or 8.

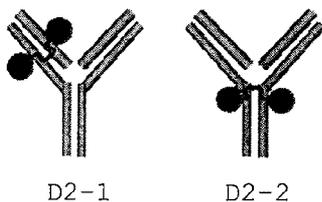
[0124]

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Examples of an antibody-drug conjugate in which two drugs bind to a single antibody molecule (hereinafter also referred to as "D2") include an antibody-drug conjugate in which two drug linkers are bound to heavy-light interchain thiols (hereinafter also referred to as "D2-1"), and an antibody-drug conjugate in which two drug linkers are bound to heavy-heavy interchain thiols (hereinafter also referred to as "D2-2").

[0125]

[Formula 19]



D2-1

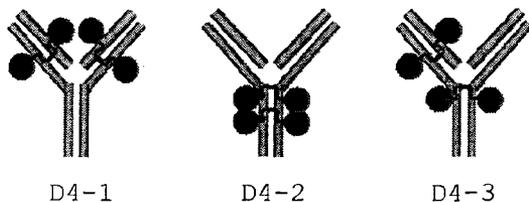
D2-2

[0126]

Examples of an antibody-drug conjugate in which four drugs bind to a single antibody molecule (hereinafter also referred to as "D4") include an antibody-drug conjugate in which four drug linkers are bound to heavy-light interchain thiols (hereinafter also referred to as "D4-1"), an antibody-drug conjugate in which four drug linkers are bound to heavy-heavy interchain thiols (hereinafter also referred to as "D4-2"), and an antibody-drug conjugate in which two drug linkers are bound to heavy-light interchain thiols and two drug linkers are bound to heavy-heavy interchain thiols (hereinafter also referred to as "D4-3").

[0127]

[Formula 20]

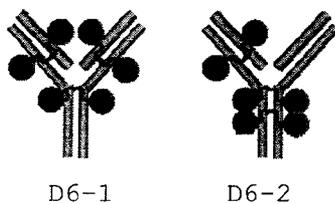


[0128]

Examples of an antibody-drug conjugate in which six drugs bind to a single antibody molecule (hereinafter also referred to as "D6") include an antibody-drug conjugate in which four drug linkers are bound to heavy-light interchain thiols and two drug linkers are bound to heavy-heavy interchain thiols (hereinafter also referred to as "D6-1"), and an antibody-drug conjugate in which two drug linkers are bound to heavy-light interchain thiols and four drug linkers are bound to heavy-heavy interchain thiols (hereinafter also referred to as "D6-2").

[0129]

[Formula 21]



[0130]

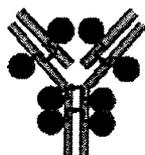
An example of an antibody-drug conjugate in which eight drug linkers are bound to a single antibody

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molecule (hereinafter also referred to as "D8") is an antibody-drug conjugate in which four drug linkers are bound to heavy-light interchain thiols and four drug linkers are bound to heavy-heavy interchain thiols.

[0131]

[Formula 22]



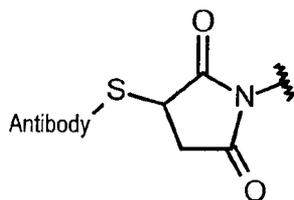
D8

[0132]

The interchain thiol of an antibody forms a thioether, for example, with the 3-position of the N-substituted maleimidyl group of a drug linker intermediate, and binds thereto. That is, the binding portion of an antibody with a drug linker is represented, for example, by the following formula:

[0133]

[Formula 23]



[0134]

wherein "Antibody-S-" is derived from the antibody.

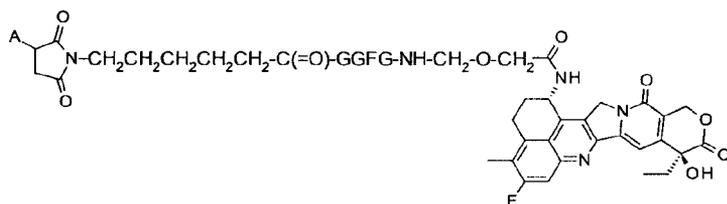
[0135]

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The drug linker is preferably,

[0136]

[Formula 24]

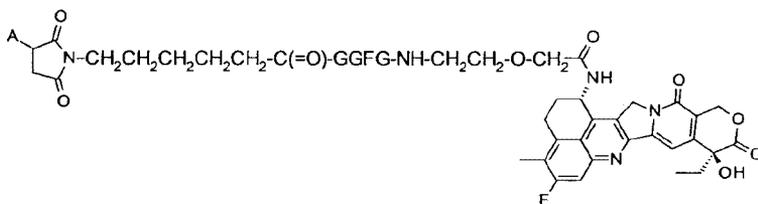


[0137]

,

[0138]

[Formula 25]

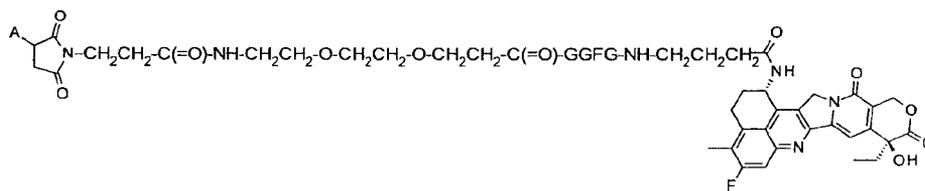


[0139]

, or

[0140]

[Formula 26]



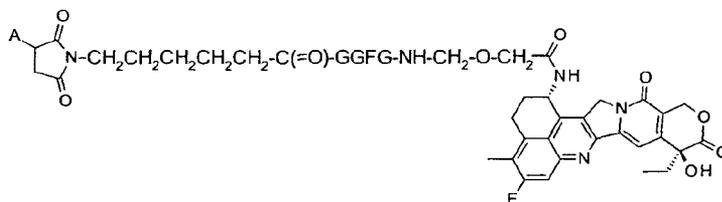
[0141]

wherein A represents the binding site to the antibody,
and it is more preferably,

[0142]

- 65 -

[Formula 27]



[0143]

wherein A represents the binding site to the antibody.

[0144]

An antibody-drug conjugate is produced, while determining reaction conditions such as the amounts of raw materials and/or reagents used in the reaction, so that the number of bound drugs can be controlled. Differing from the chemical reaction of a low molecular weight compound, the antibody-drug conjugate is generally obtained in the form of a mixture to which different numbers of drugs bind. The number of drugs binding to a single antibody molecule is specified and indicated as a mean value, namely, the average number of bound drugs.

[0145]

The production method of the present invention is a method for producing an antibody-drug conjugate composition in which the number of bound drugs and the binding sites are controlled, and the present production method consists of a first step of selectively reducing heavy-light interchain disulfide(s) of an antibody to convert them to thiol groups, and a second step of reacting drug linker intermediates with the antibody

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having thiol groups, so as to produce an antibody-drug conjugate composition in which the number of bound drugs and the binding sites are controlled. Hereafter, each step will be described in detail.

[0146]

(First step) Reduction of antibody

An antibody having thiol groups can be produced by reacting an antibody with a reducing agent in a buffer at a temperature of -10°C to 10°C .

[0147]

The reaction temperature is preferably -5°C to 5°C , more preferably -3°C to 3°C , even more preferably 0°C to 2°C , and further preferably 0°C to 1°C .

[0148]

The amount of the reducing agent is 1 to 4 molar equivalents, and preferably 2 to 3 molar equivalents, based on the amount of a single antibody molecule.

As such a reducing agent, for example, tris(2-carboxyethyl)phosphine or a salt thereof, dithiothreitol, or 2-mercaptoethanol can be used. The reducing agent is preferably tris(2-carboxyethyl)phosphine or a salt thereof, and more preferably tris(2-carboxyethyl)phosphine hydrochloride.

[0149]

As a buffer, a histidine buffer, a phosphate buffer, a borate buffer, an acetate buffer, a HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer or

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the like can be used, and among others, a histidine buffer is preferable.

[0150]

The buffer preferably comprises a chelating agent. Examples of the chelating agent that can be used herein include ethylenediaminetetraacetic acid (hereinafter also referred to as "EDTA") and diethylenetriaminepentaacetic acid. Among others, ethylenediaminetetraacetic acid is preferable. Such a buffer may be used in a concentration of 1 to 20 mM.

[0151]

The reaction time is preferably 1 to 15 hours, more preferably 3 to 10 hours, and even more preferably 5 to 7 hours.

[0152]

The pH applied for the reaction is pH 5 to 9, preferably pH 6 to 8, and more preferably pH 6.8 to 7.2.

[0153]

(Second step) Conjugation of antibody to drug linker intermediate

Drug linker intermediates are allowed to react with the antibody having thiol groups obtained in the first step to produce an antibody-drug conjugate composition. The drug linker intermediates are used in an amount of 2 to 10 molar equivalents, and preferably 4 to 6 molar equivalents, based on the amount of a single antibody molecule.

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[0154]

Specifically, a solution in which the drug linker intermediate has been dissolved is added to a buffer comprising the antibody having thiol groups obtained in the first step, so that they may be allowed to react with each other.

[0155]

Examples of the solvent in which the drug linker intermediate is dissolved, which can be used herein, include organic solvents such as a 50% acetone aqueous solution, a 80% ethanol aqueous solution, a 80% methanol aqueous solution, a 80% isopropanol aqueous solution, a 80% dimethyl sulfoxide aqueous solution, dimethyl sulfoxide (DMSO), dimethylformamide (DMF), dimethylacetamide (DMA), and N-methyl-2-pyrrolidone (NMP). Among these solvents, a 50% acetone aqueous solution or an 80% dimethyl sulfoxide aqueous solution is preferable.

[0156]

The organic solvent solution, in which the drug linker intermediate has been dissolved, is added in an amount of 1% to 20% v/v to a buffer comprising the antibody having thiol groups, so that they may be allowed to react with each other.

[0157]

The reaction temperature is preferably -10°C to 10°C , more preferably -5°C to 5°C , and even more preferably 0°C to 2°C .

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[0158]

The reaction time is preferably 0.5 to 2 hours.

[0159]

The conjugation reaction can be terminated by deactivating the reactivity of unreacted drug linker intermediates with a thiol group-containing reagent.

[0160]

As such a thiol group-containing reagent, for example, cysteine or N-acetyl-L-cysteine can be used. More specifically, the conjugation reaction can be terminated by adding N-acetyl-L-cysteine in an amount of 1 to 2 molar equivalents to the used drug linker intermediate, and then reacting them at room temperature for 10 to 30 minutes.

[0161]

After completion of the conjugation reaction, purification can be carried out using a commercially available ultrafiltration membrane or the like. While an acetate buffer, a histidine buffer, a phosphate buffer or the like is added to the reaction product, a low molecular weight portion can be removed by using the ultrafiltration membrane. As such an ultrafiltration membrane, an appropriate ultrafiltration membrane may be used. An ultrafiltration membrane having a molecular weight of 1 kDa to 100 kDa may be used, and an ultrafiltration membrane having a molecular weight of 30 kDa is preferable.

[0162]

6. Identification of antibody-drug conjugate composition

The produced antibody-drug conjugate composition is subjected to concentration, buffer exchange, purification, and the measurement of an antibody concentration and the average number of bound drugs, according to the following operations, so that the antibody-drug conjugate composition can be identified.

[0163]

(1) Concentration of antibody or antibody-drug conjugate aqueous solution

An antibody or an antibody-drug conjugate solution is placed in a vessel of Amicon Ultra (50,000 MWCO, Millipore Corporation), and is then subjected to a centrifugal operation (centrifuged at 2000 G to 3800 G for 5 to 20 minutes), using a centrifuge (Allegra X-15R, Beckman Coulter, Inc.), so that the antibody or the antibody-drug conjugate solution can be concentrated.

[0164]

(2) Measurement of antibody concentration

Using a UV measurement apparatus (Nanodrop 1000, Thermo Fisher Scientific Inc.), the concentration of the antibody can be measured according to the method provided by the manufacturer. During the measurement, 280 nm absorption coefficients ($1.3 \text{ mLmg}^{-1}\text{cm}^{-1}$ to $1.8 \text{ mLmg}^{-1}\text{cm}^{-1}$), which are different depending on individual antibodies, are used.

[0165]

(3) Exchange of buffer for antibody

A NAP-25 column (Cat. No. 17-0852-02, GE Healthcare Japan Corporation), in which a Sephadex[™] G-25 carrier is used, is equilibrated with a phosphate buffer containing sodium chloride (137 mM) and ethylenediaminetetraacetic acid (5 mM) (10 mM, pH 6.0; hereinafter also referred to as "PBS6.0/EDTA") according to the method provided by the manufacturer. Thereafter, 2.5 mL of an antibody aqueous solution is applied to a single NAP-25 column, and a fraction (3.5 mL) eluted with 3.5 mL of PBS6.0/EDTA is then fractionated. This fraction is concentrated by the same method as that described in the above (1), and the concentration of the antibody is then measured by the same method as that described in the above (2). Thereafter, the concentration of the antibody can be adjusted using PBS6.0/EDTA.

[0166]

(4) Purification of antibody-drug conjugate composition

A NAP-25 column is equilibrated with an acetate buffer containing sorbitol (5%) (10 mM, pH 5.5; hereinafter also referred to as "ABS"). To this NAP-25 column, an antibody-drug conjugate reaction aqueous solution (2.5 mL) is applied, and it is then eluted with a buffer in an amount determined by the manufacturer, so as to fractionate an antibody fraction. This fraction is applied again to the NAP-25 column, and then, a gel

- 72 -

filtration purification operation involving elution with a buffer is repeated two or three times in total, so as to obtain an antibody-drug conjugate composition, from which unbound drug linker intermediates or low molecular weight compounds (tris(2-carboxyethyl)phosphine hydrochloride, N-acetyl-L-cysteine and dimethyl sulfoxide) have been removed.

[0167]

(5) Separation method of using hydrophobic column chromatography for antibody-drug conjugate composition

(5-1) HPLC measurement method

An HPLC analysis was carried out under the following measurement conditions.

[0168]

HPLC system: Shimadzu Science HPLC System

Detector: Ultraviolet absorption spectrometer

(measurement wavelength: 280 nm)

Column: TSKgel Butyl-NPR (4.6 × 100 mm, 2.5 μm; TOSOH CORPORATION)

Column temperature: 30°C

Mobile phase A: 25 mM Phosphate buffer (pH 7.0) aqueous solution comprising 1.5 M ammonium sulfate

Mobile phase B: Mixed solution comprising 75% of 25 mM phosphate buffer (pH 7.0) and 25% of isopropyl alcohol

Gradient program: 20% - 60% (0 min - 20 min), 20% - 80% (20 min - 20.1 min), 80% - 80% (20.1 min - 23 min),

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80% - 20% (23 min - 23.1 min), 20% - 20% (23.1 min - 40 min)

Injected sample amount: 2 μ L

[0169]

(5-2) Data analysis

Regarding the present data, since antibody-drug conjugates are eluted in the order of increasing the number of bound drugs based on a difference in the salt concentration because of the characteristics of the column, the distribution in the number of bonds can be assumed by measuring individual area values. The peaks are D0 (an antibody not bound by any drug linker), D2, D4-1, D4-2, D6 and D8 in the order of elution, and thus, the distribution condition can be grasped.

[0170]

The content of antibody-drug conjugates, in which the number of bound drugs is 4, in the antibody-drug conjugate composition produced by the production method of the present invention is 50% or more.

[0171]

The content of D4-1 in the antibody-drug conjugate composition produced by the production method of the present invention is 50% or more, or in the range of 50% to 90%, 50% to 80%, 50% to 70%, or 50% to 60%.

[0172]

The content of D4-2 in the antibody-drug conjugate composition produced by the production method of the

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present invention is preferably 5% or less, and more preferably 1% or less.

[0173]

The content of D4-3 in the antibody-drug conjugate composition produced by the production method of the present invention is preferably 5% or less, and more preferably 1% or less.

[0174]

(6) Measurement of antibody concentration and the average number of bound drugs in antibody-drug conjugate composition (UV method)

The concentration of bound drugs in the antibody-drug conjugate composition can be calculated by measuring the UV absorbance of an antibody-drug conjugate aqueous solution at two wavelengths, 280 nm and 370 nm, and then carrying out the following calculation.

[0175]

Since the total absorbance at a certain wavelength is equal to a sum of the absorbances of all absorbing chemical species in the system [additivity of absorbance], if it is assumed that the molar absorption coefficients of an antibody and a drug are not changed before and after conjugation of the antibody to the drug, the antibody concentration and the drug concentration in the antibody-drug conjugate composition are represented by the following relational expressions.

- 75 -

$$A_{280} = A_{D,280} + A_{A,280} = \epsilon_{D,280}C_D + \epsilon_{A,280}C_A \quad \text{Expression (1)}$$

$$A_{370} = A_{D,370} + A_{A,370} = \epsilon_{D,370}C_D + \epsilon_{A,370}C_A \quad \text{Expression (2)}$$

In the above expressions, A_{280} represents the absorbance of an antibody-drug conjugate aqueous solution at 280 nm, A_{370} represents the absorbance of an antibody-drug conjugate aqueous solution at 370 nm, $A_{A,280}$ represents the absorbance of an antibody at 280 nm, $A_{A,370}$ represents the absorbance of an antibody at 370 nm, $A_{D,280}$ represents the absorbance of a conjugate precursor at 280 nm, $A_{D,370}$ represents the absorbance of a conjugate precursor at 370 nm, $\epsilon_{A,280}$ represents the molar absorption coefficient of an antibody at 280 nm, $\epsilon_{A,370}$ represents the molar absorption coefficient of an antibody at 370 nm, $\epsilon_{D,280}$ represents the molar absorption coefficient of a conjugate precursor at 280 nm, $\epsilon_{D,370}$ represents the molar absorption coefficient of a conjugate precursor at 370 nm, C_A represents the antibody concentration in an antibody-drug conjugate composition, and C_D represents the drug concentration in an antibody-drug conjugate composition.

[0176]

Herein, for the values represented by $\epsilon_{A,280}$, $\epsilon_{A,370}$, $\epsilon_{D,280}$ and $\epsilon_{D,370}$, previously prepared values (estimated calculation values, or measured values obtained by the UV measurement of a compound) are used. For example, the value $\epsilon_{A,280}$ can be assumed from the amino acid sequence of

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the antibody according to a known calculation method (Protein Science, 1995, vol. 4, 2411-2423). The value $\epsilon_{A,370}$ is generally zero. The values $\epsilon_{D,280}$ and $\epsilon_{D,370}$ can be obtained by measuring the absorbance of a solution, in which the used conjugate precursor is dissolved in a certain concentration, according to the Lambert-Beer law (absorbance = molar concentration \times molar absorption coefficient \times cell light path length). The values A_{280} and A_{370} of the antibody-drug conjugate aqueous solution are measured, and the obtained values are then substituted into the formulae (1) and (2) to solve the simultaneous equations, thereby obtaining C_A and C_D . Moreover, C_D is divided by C_A to obtain the average number of bound drugs per antibody.

[0177]

(7) Measurement of the average number of bound drugs per single antibody molecule in antibody-drug conjugate composition (RPC method)

The average number of bound drugs per single antibody molecule in an antibody-drug conjugate composition can also be obtained by the below-mentioned high performance liquid chromatography (HPLC) analysis using a reversed phase chromatography (RPC) method, instead of the aforementioned UV method.

[0178]

(7-1) Preparation of sample used for HPLC analysis (reduction of antibody-drug conjugate)

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An antibody-drug conjugate solution (approximately 1 mg/mL, 60 μ L) is mixed with a dithiothreitol (DTT) aqueous solution (100 mM, 15 μ L). The mixture is incubated at 37°C for 30 minutes, so as to obtain a sample, in which interchain disulfides of the antibody-drug conjugate have been cleaved, and thereafter, the obtained sample is used in an HPLC analysis.

[0179]

(7-2) HPLC analysis

An HPLC analysis is carried out under the following measurement conditions.

[0180]

HPLC system: Agilent 1290 HPLC system (Agilent Technologies)

Detector: Ultraviolet absorption spectrometer
(measurement wavelength: 280 nm)

Column: PLRP-S (2.1 \times 50 mm, 8 μ m, 1000 \AA ; Agilent Technologies, P/N PL1912-1802)

Column temperature: 80°C

Mobile phase A: 0.04% Trifluoroacetic acid (TFA) aqueous solution

Mobile phase B: Acetonitrile solution containing 0.04% TFA

Gradient program: 29% - 36% (0 min - 12.5 min), 36% - 42% (12.5 min - 15 min), 42% - 29% (15 min - 15.1 min), 29% - 29% (15.1 min - 25 min)

Injected sample amount: 15 μ L

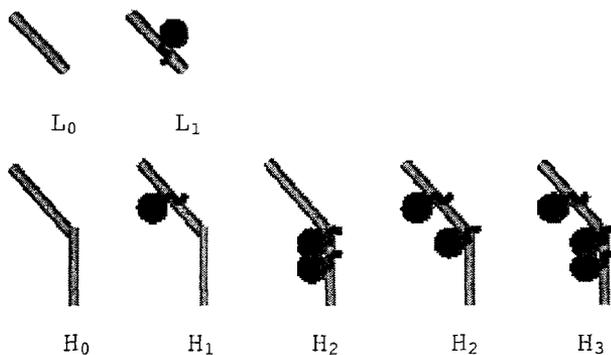
[0181]

(7-3) Data analysis

(7-3-1) When compared with the light chain (L_0) and heavy chain (H_0) of an antibody to which any drug is not bound, in the case of a light chain to which a drug is bound (a light chain to which one drug is bound: L_1) and heavy chains to which a drug(s) is(are) bound (a heavy chain to which one drug is bound: H_1 , a heavy chain to which two drugs are bound: H_2 , and a heavy chain to which three drugs are bound: H_3), hydrophobicity is increased in proportion to the number of bound drugs, and the retention time is prolonged. Thus, elution takes place in the order of L_0 , L_1 , H_0 , H_1 , H_2 , and H_3 . As a result of making a comparison in terms of the retention time between L_0 and H_0 , the detection peak can be assigned to any one of L_0 , L_1 , H_0 , H_1 , H_2 , and H_3 .

[0182]

[Formula 28]



[0183]

(7-3-2) Since a drug linker absorbs UV, peak area values are corrected according to the following expressions, using the molar absorption coefficients of a light chain, a heavy chain, and a drug linker, depending on the number of bound drug linkers.

[0184]

[Expression 1]

Light chain peak area correction value (L_i)

= Peak area

$$\times \frac{\text{Molar absorption coefficient of light chain}}{\text{Molar absorption coefficient of light chain} + \text{the number of bound drugs} \times \text{molar absorption coefficient of drug linker}}$$

[0185]

[Expression 2]

Heavy chain peak area correction value (H_i)

= Peak area

$$\times \frac{\text{Molar absorption coefficient of heavy chain}}{\text{Molar absorption coefficient of heavy chain} + \text{the number of bound drugs} \times \text{molar absorption coefficient of drug linker}}$$

[0186]

Herein, with regard to the molar absorption coefficients (280 nm) of the light chain and heavy chain of each antibody, values assumed from the amino acid sequences of the light chain and heavy chain of each antibody according to a known calculation method (Protein Science, 1995, vol. 4, 2411-2423) can be used. Moreover, with regard to the molar absorption coefficient (280 nm) of a drug linker, the actually measured molar absorption coefficient (280 nm) of a compound prepared by reacting each drug linker intermediate with mercaptoethanol or N-

- 80 -

acetyl cysteine and then converting an N-substituted maleimidyl group to a succinimide thioether can be used.
[0187]

(7-3-3) The peak area ratio (%) of each chain to a total of peak area correction values is calculated according to the following expression.

[0188]

[Expression 3]

$$\text{Light chain peak area ratio} = \frac{A_{L_i}}{A_{L_0} + A_{L_1}} \times 100$$

$$\text{Heavy chain peak area ratio} = \frac{A_{H_i}}{A_{H_0} + A_{H_1} + A_{H_2} + A_{H_3}} \times 100$$

Peak area correction value of each of A_{L_i} , A_{H_i} : L_i , H_i

[0189]

If L_1 and H_1 have been preferentially generated, it can be assumed that the heavy-light interchain disulfides have been selectively reduced. On the other hand, if L_0 and H_2 have been preferentially generated, it can be assumed that the heavy-heavy interchain disulfides have been selectively reduced.

[0190]

(7-3-4) The average number of bound drugs in an antibody-drug conjugate composition is calculated according to the following expression.

[0191]

The average number of bound drugs = (L_0 peak area ratio $\times 0$ + L_1 peak area ratio $\times 1$ + H_0 peak area ratio \times

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$0 + H_1 \text{ peak area ratio} \times 1 + H_2 \text{ peak area ratio} \times 2 + H_3$
 $\text{peak area ratio} \times 3) / 100 \times 2$

[0192]

The average number of bound drugs in the antibody-drug conjugate composition produced by the production method of the present invention is preferably 3.5 to 4.5, and more preferably 4.0 to 4.1.

[0193]

7. Medicament comprising antibody-drug conjugate composition

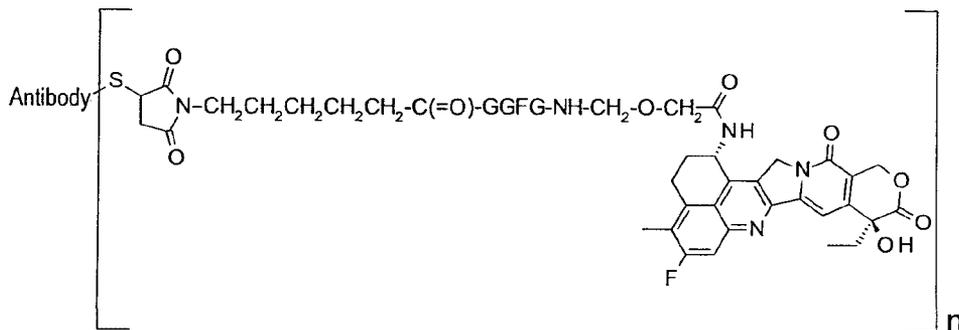
After the antibody-drug conjugate composition obtained by the present invention has been transferred into tumor cells, the linker portion thereof is cleaved, and the drug is released in the tumor cells.

[0194]

In the case of an antibody-drug conjugate represented by the following formula:

[0195]

[Formula 29]



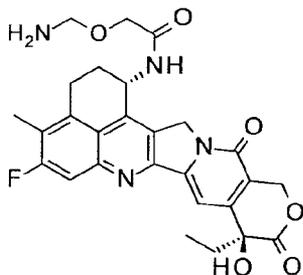
[0196]

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a compound represented by the following formula:

[0197]

[Formula 30]

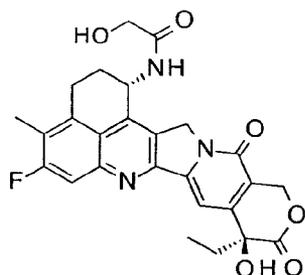


[0198]

is released. Since this compound has an unstable aminal structure, it is further hydrolyzed, so that a compound represented by the following formula:

[0199]

[Formula 31]



[0200]

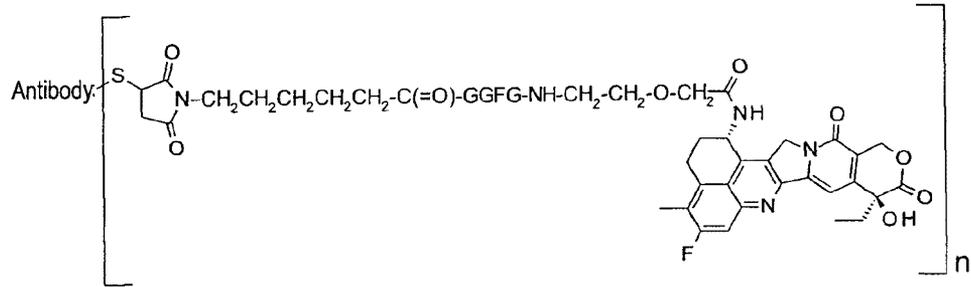
can be generated.

[0201]

In the case of an antibody-drug conjugate represented by the following formula:

[0202]

[Formula 32]

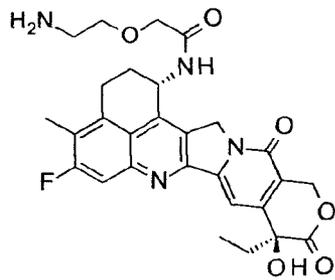


[0203]

a compound represented by the following formula:

[0204]

[Formula 33]



[0205]

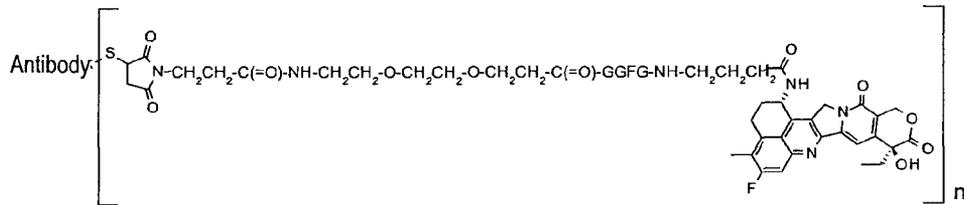
is released.

[0206]

In the case of an antibody-drug conjugate represented by the following formula:

[0207]

[Formula 34]

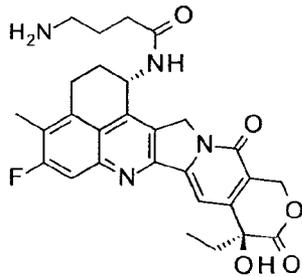


[0208]

a compound represented by the following formula:

[0209]

[Formula 35]



[0210]

is released.

[0211]

Since the antibody-drug conjugate composition obtained by the present invention exhibits cytotoxicity on cancer cells, it can be used as an active ingredient of a pharmaceutical composition for treating and/or preventing cancer.

[0212]

That is to say, the antibody-drug conjugate composition obtained by the present invention can be selected and used as an agent for chemotherapy that is a

principal treatment method in cancer therapy, and as a result of the use thereof, the growth of cancer cells can be retarded, the proliferation thereof can be suppressed, and further, cancer cells can be destroyed. By doing so, the release from symptoms caused by cancer or the improvement of QOL can be achieved for cancer patients, and the life of the cancer patients can be kept, so that therapeutic effects can be achieved. Even in the case where the destroying of cancer cells cannot be achieved, higher QOL of cancer patients can be achieved by the suppression or control of the growth of the cancer cells, so that the survival of the patients can be achieved for a longer period of time.

[0213]

The antibody-drug conjugate composition obtained by the present invention can be used, not only in the form of a drug alone in such drug therapy, but also in the form of an agent that is combined with other therapies in adjuvant therapy. The present antibody-drug conjugate composition can be combined with surgical operation, radiation therapy, hormone therapy, etc. Moreover, the present antibody-drug conjugate composition can also be used as an agent used for drug therapy in neoadjuvant therapy.

[0214]

In addition to the aforementioned therapeutic use, the antibody-drug conjugate composition obtained by the

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present invention can be expected to have the effect of suppressing the growth of very small metastatic cancer cells and further destroying such metastatic cancer cells. For instance, the present antibody-drug conjugate composition can be expected to have the effect of suppressing and destroying cancer cells in a body fluid in a metastatic process, or the effect of suppressing and destroying very small cancer cells, which are immediately after having adhered to any tissues. Therefore, the present antibody-drug conjugate composition can be expected to have the effect of suppressing and preventing cancer metastasis, particularly after the removal of cancer by surgical operation.

[0215]

The antibody-drug conjugate composition obtained by the present invention is not only administered to a patient by systemic therapy, but it can also be expected that the antibody-drug conjugate composition will be topically administered to cancer tissues and will exhibit therapeutic effects thereon.

[0216]

Examples of the type of cancer include lung cancer, kidney cancer, urothelial cancer, colon cancer, prostate cancer, glioblastoma multiforme, ovarian cancer, pancreatic cancer, breast cancer, melanoma, liver cancer, bladder cancer, stomach cancer, cervical cancer, uterine cancer, head and neck cancer, esophageal cancer, bile

duct cancer, thyroid cancer, lymphoma, acute myelocytic leukemia, acute lymphocytic leukemia, chronic myelogenous leukemia, and multiple myeloma, but are not limited thereto.

[0217]

The antibody-drug conjugate composition obtained by the present invention can be used as an active ingredient of a pharmaceutical composition for treating autoimmune disease, or a pharmaceutical composition for suppressing a rejection reaction against transplantation.

[0218]

When a pharmaceutical composition comprising the antibody-drug conjugate composition obtained by the present invention is administered to a mammal (e.g., a human, a horse, a bovine, a swine, etc., and preferably, a human), it can be administered systemically or topically, and preferably by parenteral administration.

[0219]

Examples of the administration route for parenteral administration include intradermal, intramuscular, intraperitoneal, intravenous and subcutaneous routes, but are not limited thereto. Examples of the administration method include injection and bolus injection, and the administration method is preferably injection.

[0220]

The pharmaceutical composition of the present invention can be prepared by selecting a suitable form

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depending on the administration method, and then applying a commonly used method of preparing various types of preparations. For instance, the antibody-drug conjugate composition obtained by the present invention is mixed with a solvent such as a sterilized liquid (including water and oil (oil derived from petroleum, animals, vegetables, or synthetic oil (e.g., peanut oil, soybean oil, mineral oil, sesame oil, etc.)), a saline, a dextrose aqueous solution, or a glycerol aqueous solution, and additives such as a moisturizer, an emulsifier, or a pH buffer, which are described in "Remington's Pharmaceutical Sciences" written by E. W. Martin, and the like, so as to prepare the pharmaceutical composition of the present invention.

[0221]

The pharmaceutical composition of the present invention may comprise a solubilizer, a local anesthetic (e.g., lignocaine) for relieving pain at an injection site, etc. The pharmaceutical composition of the present invention may be supplied in an aspect in which an active ingredient, a solvent and the like are each placed in different vessels. Moreover, when the pharmaceutical composition of the present invention is administered by injection, it may be administered, for example, in the form of an injection bottle containing an active ingredient and a sterilized-drug-grade water or saline. When the pharmaceutical composition of the present

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invention is administered by injection, the active ingredient may be mixed with sterilized water for injection or saline, before the administration thereof.

[0222]

The pharmaceutical composition of the present invention may comprise an antibody-drug conjugate composition and at least one cancer therapeutic agent other than the aforementioned antibody-drug conjugate composition. The antibody-drug conjugate composition obtained by the present invention can also be administered together with other cancer therapeutic agents, and the present antibody-drug conjugate composition can thereby enhance anticancer effects. Other anticancer agents used for such a purpose may be administered to an individual subject, simultaneously, separately, or continuously with the present antibody-drug conjugate composition. Otherwise, such other anticancer agents may also be administered at administration intervals that are different from those of the present antibody-drug conjugate composition. Examples of such a cancer therapeutic agent include carboplatin, cisplatin, gemcitabine, irinotecan (CPT-11), paclitaxel, pemetrexed, sorafenib, vinblastin, the agents described in International Publication No. WO2003/038043, and further, LH-RH analogs (leuprorelin, goserelin, etc.), estramustine-phosphate, estrogen antagonists (tamoxifen, raloxifene, etc.), and aromatase inhibitors (anastrozole,

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letrozole, exemestane, etc.). However, the type of the cancer therapeutic agent is not limited, as long as it is an agent having antitumor activity.

[0223]

The pharmaceutical composition of the present invention may also be provided in the form of a freeze-dried preparation or a liquid preparation. When the present pharmaceutical composition is provided as a freeze-dried preparation, it may be a preparation comprising suitable preparation additives that are used in the concerned technical field. Also, when the present pharmaceutical composition is provided as a liquid preparation, it may be a preparation comprising suitable preparation additives that are used in the concerned technical field.

[0224]

The composition of the pharmaceutical composition of the present invention and the concentration of an active ingredient thereof are changed depending on an administration method. In the case of the antibody-drug conjugate composition comprised in the pharmaceutical composition of the present invention, as the affinity of the antibody-drug conjugate for the antigen is increased, namely, as the affinity is increased in terms of the dissociation constant (K_d value) of the antibody-drug conjugate to the antigen (i.e., as the K_d value is decreased), the antibody-drug conjugate composition is

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able to exhibit medicinal effects, although it is administered in a small amount. Accordingly, for determination of the applied dose of the antibody-drug conjugate composition, the dose can be determined based on the condition of the affinity of the antibody-drug conjugate for the antigen. When the antibody-drug conjugate composition produced by the production method of the present invention is administered to a human, the present antibody-drug conjugate composition may be administered, for example, at a dose of approximately 0.001 to 100 mg/kg to the human, once, or divided over several administrations, at intervals of once per 1 to 180 days.

[0225]

The present invention will be specifically described in the following Examples. However, these examples are not intended to limit the scope of the present invention. In addition, the following Examples are not restrictively interpreted in any sense. Moreover, the reagents, solvents and starting materials described in the present description can be easily acquired from commercially available supply sources, unless otherwise specified.

Examples

[0226]

(Example 1) Construction of humanized anti-TROP2 antibody expression vector and production of antibody

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(i) Construction of humanized anti-TROP2 antibody heavy chain (hTINA1-H1) expression vector

A DNA fragment comprising a DNA sequence encoding the variable region of a humanized anti-TROP2 antibody heavy chain (hTINA1-H1) that is represented by nucleotide numbers 36 to 437 in the nucleotide sequence of a humanized anti-TROP2 antibody heavy chain (hTINA1-H1) shown in SEQ ID NO: 2 in the sequence listing was synthesized (Artificial Gene Synthesis Service, GENEART). Using the synthesized DNA fragment as a template, a DNA fragment comprising a DNA sequence encoding the variable region of a humanized anti-TROP2 antibody heavy chain (hTINA1-H1) was amplified with KOD-Plus- (TOYOBO) and the following primer set. Thereafter, an expression vector for chimeric and humanized antibody IgG1-type heavy chains, pCMA-G1, was cleaved with the restriction enzyme BspI, and the DNA fragment was then inserted into the cleaved site, using In-Fusion HD PCR cloning kit (CLONTECH), so as to construct a humanized anti-TROP2 antibody heavy chain (hTINA1-H1) expression vector. The obtained expression vector was named "pCMA-G1/hTINA1-H1".

Primer set:

5'-agctcccagatgggtgctgagc-3' (SEQ ID NO: 21: primer EG-Inf-F)

5'-gggcccttggtggaggctgagc-3' (SEQ ID NO: 22: primer EG1-Inf-R)

[0227]

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(ii) Construction of humanized anti-TROP2 antibody light chain (hTINA1-L1) expression vector

A DNA fragment comprising a DNA sequence encoding the variable region of a humanized anti-TROP2 antibody light chain (hTINA1-L1) that is represented by nucleotide numbers 38 to 402 in the nucleotide sequence of a humanized anti-TROP2 antibody light chain (hTINA1-L1) shown in SEQ ID NO: 4 in the sequence listing was synthesized (Artificial Gene Synthesis Service, GENEART). Using the synthesized DNA fragment as a template, a DNA fragment comprising a DNA sequence encoding the variable region of a humanized anti-TROP2 antibody light chain (hTINA1-L1) was amplified with KOD-Plus- (TOYOBO) and the following primer set. Thereafter, an expression vector for chimeric and humanized antibody light chains, pCMA-LK, was cleaved with the restriction enzyme BsiWI, and the DNA fragment was then inserted into the cleaved site, using In-Fusion HD PCR cloning kit (CLONTECH), so as to construct a humanized anti-TROP2 antibody light chain (hTINA1-L1) expression vector. The obtained expression vector was named "pCMA-LK/hTINA1-L1".

Primer set:

5'-ctgtggatctccggcgcgtacggc-3' (SEQ ID NO: 23: primer CM-LKF)

5'-ggagggggcggccaccgtacg-3' (SEQ ID NO: 24: primer KCL-Inf-R)

[0228]

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(iii) Production of humanized anti-TROP2 antibody (hTINA1-H1L1)

FreeStyle 293F cells (Invitrogen) were subcultured and cultured according to the instruction manual. Specifically, 1.2×10^9 FreeStyle 293F cells (Invitrogen), which were in the logarithmic growth phase, were inoculated in a 3 L Fernbach Erlenmeyer Flask (CORNING), and were then diluted with a FreeStyle293 expression medium (Invitrogen) to adjust to 1.0×10^6 cells/mL. Thereafter, the cells were subjected to shaking culture at 37°C in an 8% CO₂ incubator at 90 rpm for 1 hour. Thereafter, Polyethyleneimine (Polyscience #24765; 3.6 mg) was dissolved in Opti-Pro SFM (Invitrogen; 20 mL), and thereafter, a light chain expression vector (0.8 mg) and a heavy chain expression vector (0.4 mg), which had been prepared using PureLink HiPure Plasmid kit (Invitrogen), were added to Opti-Pro SFM (Invitrogen; 20 mL). The expression vector/Opti-Pro SFM mixed solution (20 mL) was added to the Polyethyleneimine/Opti-Pro SFM mixed solution (20 mL), and thereafter, the obtained mixture was gently stirred and was then left for 5 minutes. Thereafter, the FreeStyle 293F cells were added to the reaction mixture. The thus obtained mixture was subjected to shaking culture at 37°C in an 8% CO₂ incubator for 7 days at 90 rpm, and the obtained culture supernatant was then filtrated with Disposable Capsule Filter (ADVANTEC #CCS-045-E1H).

[0229]

The humanized anti-TROP2 antibody obtained by the combination of pCMA-G1/hTINA1-H1 with pCMA-LK/hTINA1-L1 was named "hTINA1-H1L1".

[0230]

(iv) Purification of humanized anti-TROP2 antibody (hTINA1-H1L1)

An antibody was purified from the culture supernatant obtained in the above (iii) by a two-step process, namely, by rProtein A affinity chromatography (4°C - 6°C) and ceramic hydroxyapatite (room temperature). After the purification by rProtein A affinity chromatography and after the purification using ceramic hydroxyapatite, a buffer substitution step was carried out at 4°C to 6°C. First, the culture supernatant was applied to MabSelect SuRe (HiTrap™ column, manufactured by GE Healthcare Bioscience), which had been equilibrated with PBS. After the entire culture supernatant had been placed in the column, the column was washed with PBS in an amount of two times or more of the volume of the column. Subsequently, elution was carried out using a 2 M arginine hydrochloride solution (pH 4.0), so as to collect a fraction comprising the antibody. The fraction was substituted with PBS according to dialysis (Slide-A-Lyzer Dialysis Cassette, Thermo Scientific), and thereafter, an antibody solution, which had been five times diluted with a buffer consisting of 5 mM sodium

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phosphate and 50 mM MES (pH 7.0), was applied to a ceramic hydroxyapatite column (Bio-Scale CHTType-I Hydroxyapatite Column, JAPAN Bio-Rad Laboratories K. K.), which had been equilibrated with a buffer consisting of 5 mM NaPi, 50 mM MES and 30 mM NaCl (pH 7.0). Subsequently, linear concentration gradient elution was carried out using sodium chloride, and a fraction comprising the antibody was collected. The fraction was substituted with HBSor (25 mM histidine/5% sorbitol, pH 6.0) according to dialysis (Slide-A-Lyzer Dialysis Cassette, Thermo Scientific). Finally, the resultant was concentrated using Centrifugal UF Filter Device VIVASPIN20 (cut-off molecular weight: UF10K, Sartorius, 4°C), and the IgG concentration was adjusted to 20 mg/mL or more, so as to prepare a purified sample.

[0231]

(v) Buffer exchange for humanized anti-TROP2 antibody (hTINA1-H1L1) and adjustment of concentration

A NAP-25 column (Cat. No. 17-0852-02, GE Healthcare Japan Corporation), in which a Sephadex G-25 carrier was used, was equilibrated with a phosphate buffer containing sodium chloride (137 mM) and ethylenediaminetetraacetic acid (5 mM) (10 mM, pH 6.0; hereinafter also referred to as "PBS6.0/EDTA") according to the method provided by the manufacturer. 2.5 mL of an antibody aqueous solution comprising the humanized anti-TROP2 antibody (hTINA1-H1L1) produced in the above (iv) was applied to a single

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NAP-25 column described above, and a fraction (3.5 mL) eluted with 3.5 mL of PBS6.0/EDTA was collected. This fraction was placed in a vessel of Amicon Ultra (50,000 MWCO, Millipore Corporation), and was then subjected to a centrifugation operation (centrifuged at 2000 G to 3800 G for 5 to 20 minutes) using a centrifuge (Allegra X-15R, Beckman Coulter, Inc.), so as to concentrate the antibody solution. Using a UV measurement apparatus (Nanodrop 1000, Thermo Fisher Scientific Inc.), the concentration of the antibody was measured according to the method provided by the manufacturer. For the measurement, the concentration of the antibody was measured using a 280 nm absorption coefficient ($1.54 \text{ mLmg}^{-1}\text{cm}^{-1}$), and thereafter, using PBS6.0/EDTA, the antibody concentration was adjusted to 21.8 mg/mL.

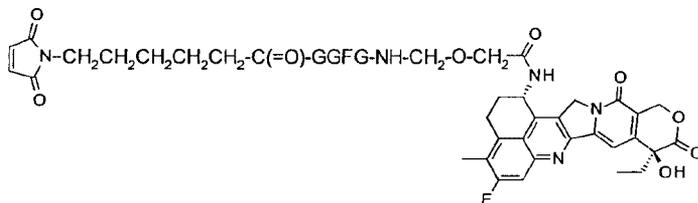
(Example 2) Production of drug linker intermediate

N-[6-(2,5-Dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]glycylglycyl-L-phenylalanyl-N-[(2-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino)-2-oxoethoxy)methyl]glycinamide, which is represented by the following formula, was synthesized by the method described in Example 58 of WO2014/057687.

[0232]

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[Formula 36]



[0233]

(Example 3) Production of humanized anti-TROP2 antibody

ADC composition

(Example 3-1) Production of humanized anti-TROP2 antibody

(hTINA1-H1L1) ADC composition according to conventional method

(i) Reduction of antibody

A humanized anti-TROP2 antibody (hTINA1-H1L1) (15 mL: corresponding to 327 mg, concentration: 21.8 mg/mL; 25 mM histidine buffer) was placed in a glass reaction vessel, and further, a 25 mM histidine buffer (18 mL, pH 5.0) was added thereto. To the present reaction solution, a 0.5 M EDTA aqueous solution (0.027 mL; 6 equivalents based on the antibody) was added, and thereafter, a 0.1 g/mL polysorbate 20 aqueous solution (0.033 mL; 0.01% based on the antibody) was added thereto. Thereafter, a 0.3 M disodium hydrogen phosphate aqueous solution was added to the reaction mixture, so that the mixture was adjusted to pH 7.12. Under stirring at 24°C, a 1.00 mg/mL tris(2-carboxyethyl)phosphine hydrochloride aqueous solution (1.58 mL; 2.45 equivalents per single antibody molecule) was added to the reaction mixture, and the

- 99 -

obtained mixture was then heated for 3 hours to result in an internal temperature of 35°C to 36°C, so that interchain disulfides of the antibody were reduced.

[0234]

(ii) Conjugation of antibody to drug linker intermediate

The solution obtained in the above (i) was cooled, and a 6.67 mg/mL 50% acetone aqueous solution (1.71 mL; 5.2 equivalents per single antibody molecule) of the compound obtained in Example 2 was then added to the reaction solution at an internal temperature of 16°C to 17°C under stirring over 60 minutes. The obtained mixture was stirred at the same temperature as described above for 20 minutes, so that the drug linker intermediate was allowed to bind to the antibody. Subsequently, a 50 mM N-acetylcysteine aqueous solution (0.135 mL; 3 equivalents per single antibody molecule) was added to the reaction mixture, and the thus obtained mixture was further stirred at the same temperature as described above for 20 minutes. The reaction of the drug linker intermediate was terminated, and the pH of the reaction mixture was then adjusted to pH 5.0 using a 10% acetic acid aqueous solution.

[0235]

(iii) Purification

Employing Pellicon XL (Millipore Japan, 50 cm²), the solution obtained in the above (ii) was circulated, while a 10 mM histidine buffer (pH 5.0) was added thereto using

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a roller pump, and a washing operation was carried out until the amount of water discharged became 500 mL, so that low molecular weight substances were removed. Thereafter, the remaining solution was concentrated to obtain 17.6 mL of a solution containing a humanized anti-TROP2 antibody (hTINA1-H1L1) ADC composition.

[0236]

(iv) Evaluation of properties

[Common operation A] Measurement of the average number of bound drugs per single antibody molecule in antibody-drug conjugate composition

The average number of bound drugs per single antibody molecule in an antibody-drug conjugate composition was obtained by a high performance liquid chromatography (HPLC) analysis of using the following method.

[0237]

1. Preparation of sample used in HPLC analysis (reduction of antibody-drug conjugate)

An antibody-drug conjugate solution (approximately 1 mg/mL, 60 μ L) was mixed with a dithiothreitol (DTT) aqueous solution (100 mM, 15 μ L). The mixture was incubated at 37°C for 30 minutes to prepare a sample in which the disulfides between the heavy chain and the light chain and between the heavy chain and the heavy chain of the antibody-drug conjugate were cleaved. The obtained sample was used in an HPLC analysis.

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[0238]

2. HPLC analysis

An HPLC analysis was carried out under the following measurement conditions.

[0239]

HPLC system: Shimadzu Science HPLC System

Detector: Ultraviolet absorption spectrometer

(measurement wavelength: 280 nm)

Column: PLRP-S (2.1 × 50 mm, 8 μm, 1000 Å; Agilent Technologies)

Column temperature: 80°C

Mobile phase A: 0.05% Trifluoroacetic acid (TFA) aqueous solution

Mobile phase B: Acetonitrile solution containing 0.04% TFA

Gradient program: 29% - 36% (0 min - 12.5 min), 36% - 42% (12.5-15 min), 42% - 29% (15 min - 15.1 min), 29% - 29% (15.1 min - 25 min)

Injected sample amount: 15 μL

[0240]

4. Data analysis

When compared with the light chain (L_0) and heavy chain (H_0) of an antibody to which a drug did not bind, in the case of a light chain to which a drug bound (a light chain to which one drug bound: L_1) and heavy chains to which a drug(s) bound (a heavy chain to which one drug bound: H_1 , a heavy chain to which two drugs bound: H_2 ,

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and a heavy chain to which three drugs bound: H₃), hydrophobicity was increased in proportion to the number of bound drugs, and the retention time was prolonged. Thus, elution took place in the order of L₀, L₁, H₀, H₁, H₂, and H₃. As a result of making a comparison in terms of the retention time between L₀ and H₀, the detection peak was assigned to any one of L₀, L₁, H₀, H₁, H₂, and H₃.

[0241]

Since a drug linker absorbed UV, peak area values were corrected according to the following expressions, using the molar absorption coefficients of a light chain, a heavy chain, and a drug linker, depending on the number of bound drug linkers.

[0242]

[Expression 4]

Light chain peak area correction value (L_i)

= Peak area

$$\times \frac{\text{Molar absorption coefficient of light chain}}{\text{Molar absorption coefficient of light chain} + \text{the number of bound drugs} \times \text{molar absorption coefficient of drug linker}}$$

[0243]

[Expression 5]

Heavy chain peak area correction value (H_i)

= Peak area

$$\times \frac{\text{Molar absorption coefficient of heavy chain}}{\text{Molar absorption coefficient of heavy chain} + \text{the number of bound drugs} \times \text{molar absorption coefficient of drug linker}}$$

[0244]

Herein, with regard to the molar absorption coefficients (280 nm) of the light chain and heavy chain of each antibody, values assumed from the amino acid

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sequences of the light chain and heavy chain of each antibody according to a known calculation method (Protein Science, 1995, vol. 4, 2411-2423) were used. In the case of the humanized anti-TROP2 antibody (hTINA1-H1L1), based on its amino acid sequence, the number 27640 was used as an estimated value for the molar absorption coefficient of the light chain, and the number 83810 was used as an estimated value for the molar absorption coefficient of the heavy chain. Moreover, with regard to the molar absorption coefficient (280 nm) of a drug linker, the actually measured molar absorption coefficient (280 nm) of a compound prepared by reacting each drug linker intermediate with mercaptoethanol or N-acetyl cysteine and then converting an N-substituted maleimidyl group to a succinimide thioether was used.

[0245]

The peak area ratio (%) of each chain to a total of peak area correction values was calculated according to the following expression.

[0246]

[Expression 6]

$$\text{Light chain peak area ratio} = \frac{A_{Li}}{A_{L0} + A_{L1}} \times 100$$

$$\text{Heavy chain peak area ratio} = \frac{A_{Hi}}{A_{H0} + A_{H1} + A_{H2} + A_{H3}} \times 100$$

Peak area correction value of each of A_{Li} , A_{Hi} : L_i , H_i

[0247]

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The average number of bound drugs per single antibody molecule in an antibody-drug conjugate composition was calculated according to the following expression.

[0248]

The average number of bound drugs = $(L_0 \text{ peak area ratio} \times 0 + L_1 \text{ peak area ratio} \times 1 + H_0 \text{ peak area ratio} \times 0 + H_1 \text{ peak area ratio} \times 1 + H_2 \text{ peak area ratio} \times 2 + H_3 \text{ peak area ratio} \times 3) / 100 \times 2$

The concentration of the antibody was found to be 16.49 mg/mL, the yield of the antibody was found to be 290 mg (86%), and the average number of bound drugs (n) per single antibody molecule, which was measured by the common operation A, was found to be 4.4. The HPLC chromatograph representing the peak area ratio (%) of each chain is shown in Figure 5.

[0249]

[Common operation B] Separation method involving hydrophobic column chromatography for antibody-drug conjugate composition

1. HPLC measurement method

An HPLC analysis was carried out under the following measurement conditions.

[0250]

HPLC system: Shimadzu Science HPLC System

Detector: Ultraviolet absorption spectrometer

(measurement wavelength: 280 nm)

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Column: TSKgel Butyl-NPR (4.6 × 100 mm, 2.5 μm;
TOSOH CORPORATION)

Column temperature: 30°C

Mobile phase A: 25 mM Phosphate buffer (pH 7.0)
aqueous solution comprising 1.5 M ammonium sulfate

Mobile phase B: Mixed solution comprising 75% of 25
mM phosphate buffer (pH 7.0) and 25% of isopropyl alcohol

Gradient program: 20% - 60% (0 min - 20 min), 20% -
80% (20 min - 20.1 min), 80% - 80% (20.1 min - 23 min),
80% - 20% (23 min - 23.1 min), 20% - 20% (23.1 min - 40
min)

Injected sample amount: 2 μL

[0251]

2. Data analysis

Regarding the present data, since antibody-drug
conjugates were eluted in the order of increasing the
number of bound drugs based on a difference in the salt
concentration because of the characteristics of the
column, a distribution in the number of bonds was assumed
by measuring individual area values. The peaks were D0
(an antibody not bound by any drug linker), D2, D4-1, D4-
2, D6 and D8 in the order of elution, and the
distribution conditions were the following: D0: 4.8%, D2:
16.8%, D4-1: 24.6%, D4-2: 13.1%, D6: 24.8%, and D8: 12.6%
(Figure 6).

[0252]

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(Example 3-2) Production of humanized anti-TROP2 antibody (hTINA1-H1L1) ADC composition according to the method of the present invention

(i) Reduction of antibody

A humanized anti-TROP2 antibody (hTINA1-H1L1) (22.9 mL; corresponding to 500 mg, concentration: 21.8 mg/mL; 25 mM histidine buffer) was placed in a glass reaction vessel, and further, a 25 mM histidine buffer (22 mL, pH 5.0) was added thereto. To the present reaction solution, a 0.5 M EDTA aqueous solution (0.0344 mL; 5 equivalents based on the antibody) was added, and thereafter, a 0.1 g/mL polysorbate 20 aqueous solution (0.050 mL; 0.01% based on the antibody) was added thereto. Thereafter, a 0.3 M disodium hydrogen phosphate aqueous solution was added to the reaction mixture, so that the mixture was adjusted to pH 7.12, and then, the mixture was cooled. Under stirring, a 1.00 mg/mL tris(2-carboxyethyl)phosphine hydrochloride aqueous solution (2.54 mL; 2.58 equivalents per single antibody molecule) was added to the reaction mixture at an internal temperature of 0°C to 1°C, and the obtained mixture was then stirred for 6 hours at an internal temperature of 0°C to 1°C, so that interchain disulfides of the antibody were reduced.

[0253]

(ii) Conjugation of antibody to drug linker intermediate

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A 6.03 mg/mL 50% acetone aqueous solution (2.99 mL; 5.1 equivalents per single antibody molecule) of the compound obtained in Example 2 was added to the solution obtained in the above (i) at an internal temperature of 0°C to 2°C under stirring over 10 minutes. The obtained mixture was stirred at the same temperature as described above for 40 minutes, so that the drug linker intermediate was allowed to bind to the antibody. Subsequently, a 50 mM N-acetylcysteine aqueous solution (0.206 mL; 3 equivalents per single antibody molecule) was added to the reaction mixture, and the thus obtained mixture was further stirred at the same temperature as described above for 10 minutes. The reaction of the drug linker intermediate was terminated, and the pH of the reaction mixture was then adjusted to pH 5.0 using a 10% acetic acid aqueous solution.

[0254]

(iii) Purification

Employing Pellicon XL (Millipore Japan, 50 cm²), the solution obtained in the above (ii) was circulated, while a 10 mM histidine buffer (pH 5.0) was added thereto using a roller pump, and a washing operation was carried out until the amount of water discharged became 700 mL, so that low molecular weight substances were removed. Thereafter, the remaining solution was concentrated to obtain 23.6 mL of a solution containing a humanized anti-TROP2 antibody (hTINA1-H1L1) ADC composition.

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[0255]

(iv) Evaluation of properties

The average number of bound drugs was measured in the same manner as the common operation A described in Example 3-1, (iv). In the case of the humanized anti-TROP2 antibody (hTINA1-H1L1), based on its amino acid sequence, the number 27640 was used as an estimated value for the molar absorption coefficient of the light chain, and the number 83810 was used as an estimated value for the molar absorption coefficient of the heavy chain.

[0256]

The concentration of the antibody was found to be 19.63 mg/mL, the yield of the antibody was found to be 463 mg (92%), and the average number of bound drugs (n) per single antibody molecule, which was measured by the common operation A, was found to be 4.1. The HPLC chromatograph representing the peak area ratio (%) of each chain is shown in Figure 7.

[0257]

The area value of the number of bound drugs was measured in the same manner as the common operation B described in Example 3-1, (iv). The distribution condition of the number of bound drugs was as follows: D0: 1.9%, D2: 19.5%, D4-1: 53.3%, D6: 18.5%, and D8: 5.5% (Figure 8).

[0258]

(v) Results

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The average number of bound drugs in the humanized anti-TROP2 antibody ADC composition produced by the conventional method (Example 3-1) was 4.4, and the content of D4-1 therein was 24.6%. On the other hand, the average number of bound drugs in the humanized anti-TROP2 antibody ADC composition produced by the method of the present invention (Example 3-2) was 4.1, and the content of D4-1 therein was 53.3%.

[0259]

(Example 4) Construction of humanized anti-CD98 antibody expression vector and production of antibody

(i) Construction of humanized anti-CD98 antibody heavy chain (hM23-H1) expression vector

A DNA fragment (nucleotide numbers 36 to 422) comprising a DNA sequence encoding the variable region of a humanized anti-CD98 antibody heavy chain (hM23-H1) that is represented by nucleotide numbers 58 to 405 in the nucleotide sequence of a humanized anti-CD98 antibody heavy chain (hM23-H1) shown in SEQ ID NO: 11 was synthesized (Artificial Gene Synthesis Service, GENEART). Using the synthesized DNA fragment as a template, a DNA fragment comprising a DNA sequence encoding the variable region of hM23-H1 was amplified with KOD-Plus- (TOYOBO) and the following primer set. Thereafter, an expression vector for chimeric and humanized antibody IgG1-type heavy chains, pCMA-G1, cleaved with the restriction enzyme BspI, and the DNA fragment was then inserted into

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the cleaved site, using In-Fusion HD PCR cloning kit (CLONTECH), so as to construct a humanized anti-CD98 antibody heavy chain (hM23-H1) expression vector. The obtained expression vector was named "pCMA-G1/hM23-H1".

Primer set:

5'-AGCTCCCAGATGGGTGCTGAGC-3' (SEQ ID NO: 21: primer EG-Inf-F)

5'-GGGCCCTTGGTGGAGGCTGAGC-3' (SEQ ID NO: 22: primer EG1-Inf-R)

[0260]

(ii) Construction of humanized anti-CD98 antibody light chain (hM23-L1) expression vector

A DNA fragment (nucleotide numbers 38 to 420) comprising a DNA sequence encoding the variable region of a humanized anti-CD98 antibody light chain (hM23-L1) that is represented by nucleotide numbers 61 to 405 in the nucleotide sequence of a humanized anti-CD98 antibody light chain (hM23-L1) shown in SEQ ID NO: 13 was synthesized (Artificial Gene Synthesis Service, GENEART). Using the synthesized DNA fragment as a template, a DNA fragment comprising a DNA sequence encoding the variable region of a humanized anti-CD98 antibody light chain (hM23-L1) was amplified with KOD-Plus- (TOYOBO) and the following primer set. Thereafter, an expression vector for chimeric and humanized antibody light chains, pCMA-LK, was cleaved with the restriction enzyme BsiWI, and the DNA fragment was then inserted into the cleaved site,

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using In-Fusion HD PCR cloning kit (CLONTECH), so as to construct a humanized anti-CD98 antibody light chain (hM23-L1) expression vector. The obtained expression vector was named "pCMA-LK/hM23-L1".

Primer set:

5'-CTGTGGATCTCCGGCGCGTACGGC-3' (SEQ ID NO: 23: primer CM-LKF)

5'-GGAGGGGGCGGCCACCGTACG-3' (SEQ ID NO: 24: primer KCL-Inf-R)

[0261]

(iii) Production of humanized anti-CD98 antibody (hM23-H1L1)

A humanized anti-CD98 antibody was produced by the same method as that applied in Example 1, (iii). A humanized anti-CD98 antibody obtained by the combination of pCMA-G1/hM23-H1 with pCMA-LK/hM23-L1 was named "hM23-H1L1".

[0262]

(iv) Purification of humanized anti-CD98 antibody (hM23-H1L1)

An antibody was purified from the culture supernatant obtained in the above (iii) by the same method as that applied in Example 1, (iv).

[0263]

(v) Buffer exchange for humanized anti-CD98 antibody (hM23-H1L1) and adjustment of concentration

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The humanized anti-CD98 antibody (hM23-H1L1) purified in the above (iv) was subjected to the exchange of the buffer and the adjustment of the concentration thereof by the same method as that applied in Example 1, (v). During the operation, the concentration of the antibody was measured using a 280 nm absorption coefficient ($1.65 \text{ mLmg}^{-1}\text{cm}^{-1}$), and thereafter, the antibody concentration was adjusted to 40 mg/mL using PBS6.0/EDTA.

[0264]

(Example 5) Production of humanized anti-CD98 antibody
ADC composition

(Example 5-1) Production of humanized anti-CD98 antibody
(hM23-H1L1) ADC composition according to conventional
method

(i) Reduction of antibody

A humanized anti-CD98 antibody (hM23-H1L1) (12 mL: corresponding to 480 mg, concentration: 40 mg/mL; 25 mM histidine buffer) was placed in a glass reaction vessel, and further, a 25 mM histidine buffer (36 mL, pH 5.0) was added thereto. To the present reaction solution, a 0.5 M EDTA aqueous solution (CALBIOCHEM; 0.0394 mL; 6 equivalents based on the antibody) was added, and thereafter, a 0.1 g/mL polysorbate 20 (NOF CORPORATION) aqueous solution (0.048 mL; 0.01% based on the antibody) was added thereto. Thereafter, a 0.3 M disodium hydrogen phosphate aqueous solution was added to the reaction

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mixture, so that the mixture was adjusted to pH 7.10. Under stirring at 21°C, a 1.00 mg/mL tris(2-carboxyethyl)phosphine hydrochloride (Nacalai Tesque, Inc.) aqueous solution (2.17 mL; 2.31 equivalents per single antibody molecule) was added to the reaction mixture, and the obtained mixture was then heated for 3 hours to result in an internal temperature of 35°C to 36°C, so that interchain disulfides of the antibody were reduced.

[0265]

(ii) Conjugation of antibody to drug linker intermediate

The solution obtained in the above (i) was cooled, and a 6.20 mg/mL 50% acetone aqueous solution (2.74 mL; 5.2 equivalents per single antibody molecule) of the compound obtained in Example 2 was then added to the reaction solution at an internal temperature of 17°C to 18°C under stirring over 7 minutes. The obtained mixture was stirred at the same temperature as described above for 40 minutes, so that the drug linker intermediate was allowed to bind to the antibody. Subsequently, a 50 mM N-acetylcysteine (Kishida Chemical Co., Ltd.) aqueous solution (0.197 mL; 3 equivalents per single antibody molecule) was added to the reaction mixture, and the thus obtained mixture was further stirred at the same temperature as described above for 30 minutes. The reaction of the drug linker intermediate was terminated,

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and the pH of the reaction mixture was then adjusted to pH 5.0 using a 10% acetic acid aqueous solution.

[0266]

(iii) Purification

Employing Pellicon XL (Millipore Japan, 50 cm²), the solution obtained in the above (ii) was circulated, while a 10 mM histidine buffer (pH 5.0) was added thereto using a roller pump, and a washing operation was carried out until the amount of water discharged became 600 mL, so that low molecular weight substances were removed. Thereafter, the remaining solution was concentrated to obtain 21.6 mL of a solution containing a humanized anti-CD98 antibody (hM23-H1L1) ADC composition.

[0267]

(iv) Evaluation of properties

The average number of bound drugs was measured in the same manner as the common operation A described in Example 3-1, (iv). In the case of the humanized anti-CD98 antibody (hM23-H1L1), based on its amino acid sequence, the number 41370 was used as an estimated value for the molar absorption coefficient of the light chain, and the number 77810 was used as an estimated value for the molar absorption coefficient of the heavy chain.

[0268]

The concentration of the antibody was found to be 20.8 mg/mL, the yield of the antibody was found to be 449 mg (91%), and the average number of bound drugs (n) per

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single antibody molecule, which was measured by the common operation A, was found to be 4.0. The HPLC chromatograph representing the peak area ratio (%) of each chain is shown in Figure 9.

[0269]

The area value of the number of bound drugs was measured in the same manner as the common operation B described in Example 3-1, (iv). The distribution condition of the number of bound drugs was as follows: D0: 4.2%, D2: 24.2%, D4-1: 27.8%, D4-2: 13.3%, D6: 20.8%, and D8: 7.6% (Figure 10).

[0270]

(Example 5-2) Production of humanized anti-CD98 antibody (hM23-H1L1) ADC composition according to the method of the present invention

(i) Reduction of antibody

A humanized anti-CD98 antibody (hM23-H1L1) (12.5 mL: corresponding to 500 mg, concentration: 40 mg/mL; 25 mM histidine buffer) was placed in a glass reaction vessel, and further, a 25 mM histidine buffer (27.5 mL, pH 5.0) was added thereto. To the present reaction solution, a 0.5 M EDTA aqueous solution (CALBIOCHEM; 0.041 mL; 6 equivalents based on the antibody) was added, and thereafter, a 0.1 g/mL polysorbate 20 (NOF CORPORATION) aqueous solution (0.050 mL; 0.01% based on the antibody) was added thereto. Thereafter, a 0.3 M disodium hydrogen phosphate aqueous solution was added to the reaction

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mixture, so that the mixture was adjusted to pH 7.10. The reaction solution was cooled, and under stirring at an internal temperature of 0°C to 1°C, a 1.00 mg/mL tris(2-carboxyethyl)phosphine hydrochloride (Nacalai Tesque, Inc.) aqueous solution (2.75 mL; 2.80 equivalents per single antibody molecule) was added to the reaction mixture, and the obtained mixture was then stirred for 6 hours to result in an internal temperature of 0°C to 1°C, so that interchain disulfides of the antibody were reduced.

[0271]

(ii) Conjugation of antibody to drug linker intermediate

A 6.08 mg/mL 50% acetone aqueous solution (3.14 mL; 5.4 equivalents per single antibody molecule) of the compound obtained in Example 2 was added to the solution obtained in the above (i) at an internal temperature of 0.7°C to 1.2°C under stirring over 10 minutes. The obtained mixture was stirred at the same temperature as described above for 50 minutes, so that the drug linker intermediate was allowed to bind to the antibody. Subsequently, a 50 mM N-acetylcysteine (Kishida Chemical Co., Ltd.) aqueous solution (0.205 mL; 3 equivalents per single antibody molecule) was added to the reaction mixture, and the thus obtained mixture was further stirred at the same temperature as described above for 30 minutes. The reaction of the drug linker intermediate was terminated, and the pH of the reaction mixture was

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then adjusted to pH 5.0 using a 10% acetic acid aqueous solution.

[0272]

(iii) Purification

Employing Pellicon XL (Millipore Japan, 50 cm²), the solution obtained in the above (ii) was circulated, while a 10 mM histidine buffer (pH 5.0) was added thereto using a roller pump, and a washing operation was carried out until the amount of water discharged became 600 mL, so that low molecular weight substances were removed.

Thereafter, the remaining solution was concentrated to obtain 23.6 mL of a solution containing a humanized anti-CD98 antibody (hM23-H1L1) ADC composition.

[0273]

(iv) Evaluation of properties

The average number of bound drugs was measured in the same manner as the common operation A described in Example 3-1, (iv). In the case of the humanized anti-CD98 antibody (hM23-H1L1), based on its amino acid sequence, the number 41370 was used as an estimated value for the molar absorption coefficient of the light chain, and the number 77810 was used as an estimated value for the molar absorption coefficient of the heavy chain.

[0274]

The concentration of the antibody was found to be 19.91 mg/mL, the yield of the antibody was found to be 470 mg (94%), and the average number of bound drugs (n)

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per single antibody molecule, which was measured by the common operation A, was found to be 4.1. The HPLC chromatograph representing the peak area ratio (%) of each chain is shown in Figure 11.

[0275]

The area value of the number of bound drugs was measured in the same manner as the common operation B described in Example 3-1, (iv). The distribution condition of the number of bound drugs was as follows: D0: 2.2%, D2: 18.1%, D4-1: 51.0%, D6: 20.6%, and D8: 7.6% (Figure 12).

[0276]

(v) Results

The average number of bound drugs in the humanized anti-CD98 antibody ADC composition produced by the conventional method (Example 5-1) was 4.0, and the content of D4-1 therein was 27.8%. On the other hand, the average number of bound drugs in the humanized anti-CD98 antibody ADC composition produced by the method of the present invention (Example 5-2) was 4.1, and the content of D4-1 therein was 51.0%.

(Example 6) Production of humanized anti-B7-H3 antibody ADC composition

(Example 6-1) Production of humanized anti-B7-H3 antibody (M30-H1-L4) ADC composition according to conventional method

(i) Reduction of antibody

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A humanized anti-B7-H3 antibody (M30-H1-L4) (produced according to the method described in Reference Example 1 of WO2014/057687, 12.4 mL: corresponding to 250 mg, concentration: 20.1 mg/mL; 25 mM citrate buffer) was placed in a glass reaction vessel, and a 25 mM histidine buffer (18 mL, pH 7.5) was further added thereto. To the present reaction solution, a 0.5 M EDTA aqueous solution (CALBIOCHEM; 0.018 mL; 5 equivalents based on the antibody) was added, and thereafter, a 0.1 g/mL polysorbate 80 (NOF CORPORATION) aqueous solution (0.013 mL; 0.01% based on the antibody) was added thereto. Thereafter, a 0.3 M disodium hydrogen phosphate aqueous solution was added to the reaction mixture, so that the mixture was adjusted to pH 7.02. Under stirring at 35°C, a 1.00 mg/mL tris(2-carboxyethyl)phosphine hydrochloride (Nacalai Tesque, Inc.) aqueous solution (1.05 mL; 2.15 equivalents per single antibody molecule) was added to the reaction mixture, and the obtained mixture was then heated for 2 hours to result in an internal temperature of 35°C to 36°C, so that interchain disulfides of the antibody were reduced.

[0277]

(ii) Conjugation of antibody to drug linker intermediate

The solution obtained in the above (i) was cooled, and a 6.22 mg/mL 50% acetone aqueous solution (1.36 mL; 4.8 equivalents per single antibody molecule) of the compound obtained in Example 2 was then added to the

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reaction solution at an internal temperature of 15°C to 16°C under stirring over 4 minutes. The obtained mixture was stirred at the same temperature as described above for 20 minutes, so that the drug linker intermediate was allowed to bind to the antibody. Subsequently, a 50 mM N-acetylcysteine (Kishida Chemical Co., Ltd.) aqueous solution (0.102 mL; 3 equivalents per single antibody molecule) was added to the reaction mixture, and the thus obtained mixture was further stirred at the same temperature as described above for 20 minutes. The reaction of the drug linker intermediate was terminated, and the pH of the reaction mixture was then adjusted to pH 5.0 using a 10% acetic acid aqueous solution.

[0278]

(iii) Purification

Employing Pellicon XL (Millipore Japan, 50 cm²), the solution obtained in the above (ii) was circulated, while a 10 mM histidine buffer (pH 5.0) was added thereto using a roller pump, and a washing operation was carried out until the amount of water discharged became 300 mL, so that low molecular weight substances were removed. Thereafter, the remaining solution was concentrated to obtain 13.1 mL of a solution containing a humanized anti-B7-H3 antibody (M30-H1-L4) ADC composition.

[0279]

(iv) Evaluation of properties

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The average number of bound drugs was measured in the same manner as the common operation A described in Example 3-1, (iv). In the case of the humanized anti-B7-H3 antibody (M30-H1-L4), based on its amino acid sequence, the number 30160 was used as an estimated value for the molar absorption coefficient of the light chain, and the number 87250 was used as an estimated value for the molar absorption coefficient of the heavy chain.

[0280]

The concentration of the antibody was found to be 18.4 mg/mL, the yield of the antibody was found to be 241 mg (94%), and the average number of bound drugs (n) per single antibody molecule, which was measured by the common operation A, was found to be 3.8. The HPLC chromatograph representing the peak area ratio (%) of each chain is shown in Figure 15.

[0281]

The area value of the number of bound drugs was measured in the same manner as the common operation B described in Example 3-1, (iv). The distribution condition of the number of bound drugs was as follows: D0: 6.5%, D2: 30.5%, D4-1: 27.9%, D4-2: 12.3%, D6: 17.7%, and D8: 4.9% (Figure 16).

[0282]

(Example 6-2) Production of humanized anti-B7-H3 antibody (M30-H1-L4) ADC composition according to the method of the present invention

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(i) Reduction of antibody

A humanized anti-B7-H3 antibody (M30-H1-L4) (produced according to the method described in Reference Example 1 of WO2014/057687, 27.1 mL: corresponding to 500 mg, concentration: 18.5 mg/mL; 10 mM histidine buffer) was placed in a glass reaction vessel, and further, a 10 mM histidine aqueous solution (25 mL) was added thereto. To the present reaction solution, sucrose (MERCK; 1.25 g) and a 0.5 M EDTA aqueous solution (CALBIOCHEM; 0.041 mL; 6 equivalents based on the antibody) were added, and thereafter, a 0.1 g/mL polysorbate 80 (NOF CORPORATION) aqueous solution (0.050 mL; 0.01% based on the antibody) was added thereto. Thereafter, a 0.3 M disodium hydrogen phosphate aqueous solution was added to the reaction mixture, so that the mixture was adjusted to pH 7.08. The reaction solution was cooled, and under stirring at an internal temperature of 0°C to 1°C, a 1.00 mg/mL tris(2-carboxyethyl)phosphine hydrochloride (Nacalai Tesque, Inc.) aqueous solution (2.08 mL; 2.13 equivalents per single antibody molecule) was added to the reaction mixture, and the obtained mixture was then stirred for 5.5 hours to result in an internal temperature of 0°C to 1°C, so that interchain disulfides of the antibody were reduced.

[0283]

(ii) Conjugation of antibody to drug linker intermediate

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A 6.04 mg/mL 50% acetone aqueous solution (2.82 mL; 4.8 equivalents per single antibody molecule) of the compound obtained in Example 2 was added to the solution obtained in the above (i) at an internal temperature of 0°C to 1°C under stirring over 20 minutes. The obtained mixture was stirred at the same temperature as described above for 20 minutes, so that the drug linker intermediate was allowed to bind to the antibody. Subsequently, a 50 mM N-acetylcysteine (Kishida Chemical Co., Ltd.) aqueous solution (0.205 mL; 3 equivalents per single antibody molecule) was added to the reaction mixture, and the thus obtained mixture was further stirred at the same temperature as described above for 20 minutes. The reaction of the drug linker intermediate was terminated, and the pH of the reaction mixture was then adjusted to pH 5.0 using a 10% acetic acid aqueous solution.

[0284]

(iii) Purification

Employing Pellicon XL (Millipore Japan, 50 cm²), the solution obtained in the above (ii) was circulated, while a 10 mM histidine buffer (pH 5.0) was added thereto using a roller pump, and a washing operation was carried out until the amount of water discharged became 800 mL, so that low molecular weight substances were removed. Thereafter, the remaining solution was concentrated to

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obtain 23.6 mL of a solution containing a humanized anti-B7-H3 antibody (M30-H1-L4) ADC composition.

[0285]

(iv) Evaluation of properties

The average number of bound drugs was measured in the same manner as the common operation A described in Example 3-1, (iv). In the case of the humanized anti-B7-H3 antibody (M30-H1-L4), based on its amino acid sequence, the number 30160 was used as an estimated value for the molar absorption coefficient of the light chain, and the number 87250 was used as an estimated value for the molar absorption coefficient of the heavy chain.

[0286]

The concentration of the antibody was found to be 19.4 mg/mL, the yield of the antibody was found to be 455 mg (89%), and the average number of bound drugs (n) per single antibody molecule, which was measured by the common operation A, was found to be 4.1. The HPLC chromatograph representing the peak area ratio (%) of each chain is shown in Figure 17.

[0287]

The area value of the number of bound drugs was measured in the same manner as the common operation B described in Example 3-1, (iv). The distribution condition of the number of bound drugs was as follows: D0: 2.7%, D2: 22.3%, D4-1: 58.4%, D6: 14.1%, and D8: 2.4% (Figure 18).

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[0288]

(v) Results

The average number of bound drugs in the humanized anti-B7-H3 antibody ADC composition produced by the conventional method (Example 6-1) was 3.8, and the content of D4-1 therein was 27.9%. On the other hand, the average number of bound drugs in the humanized anti-B7-H3 antibody ADC composition produced by the method of the present invention (Example 6-2) was 4.1, and the content of D4-1 therein was 58.4%.

[0289]

(Example 7) Production of humanized anti-HER2 antibody ADC composition

[0290]

(Example 7-1) Production of humanized anti-HER2 antibody ADC composition according to conventional method

(i) Reduction of antibody

A humanized anti-HER2 antibody (trastuzumab; U.S. Patent No. 5821337) (22.3 mL: corresponding to 500 mg, concentration: 22.4 mg/mL; 25 mM histidine buffer) was placed in a glass reaction vessel, and a 25 mM histidine buffer (27 mL, pH 5.0) was further added thereto. To the present reaction solution, a 0.5 M EDTA aqueous solution (0.034 mL; 5 equivalents based on the antibody) was added, and thereafter, a 0.1 g/mL polysorbate 20 aqueous solution (0.050 mL; 0.01% based on the antibody) was added thereto. Thereafter, a 0.3 M disodium hydrogen

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phosphate aqueous solution was added to the reaction mixture, so that the mixture was adjusted to pH 7.12. Under stirring at 22°C, a 1.00 mg/mL tris(2-carboxyethyl)phosphine hydrochloride aqueous solution (2.12 mL; 2.15 equivalents per single antibody molecule) was added to the reaction mixture, and the obtained mixture was then stirred for 3 hours to result in an internal temperature of 22°C to 25°C, so that interchain disulfides of the antibody were reduced.

[0291]

(ii) Conjugation of antibody to drug linker intermediate

The solution obtained in the above (i) was cooled, and a 6.15 mg/mL 50% acetone aqueous solution (2.77 mL; 4.8 equivalents per single antibody molecule) of the compound obtained in Example 2 was then added to the reaction solution at an internal temperature of 11°C to 13°C under stirring over 20 minutes. The obtained mixture was stirred at the same temperature as described above for 20 minutes, so that the drug linker intermediate was allowed to bind to the antibody. Subsequently, a 50 mM N-acetylcysteine aqueous solution (0.206 mL; 3 equivalents per single antibody molecule) was added to the reaction mixture, and the thus obtained mixture was further stirred at the same temperature as described above for 20 minutes. The reaction of the drug linker intermediate was terminated, and the pH of the

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reaction mixture was then adjusted to pH 5.0 using a 10% acetic acid aqueous solution.

[0292]

(iii) Purification

Employing Pellicon XL (Millipore Japan, 50 cm²), the solution obtained in the above (ii) was circulated, while a 10 mM histidine buffer (pH 5.0) was added thereto using a roller pump, and a washing operation was carried out until the amount of water discharged became 600 mL, so that low molecular weight substances were removed.

Thereafter, the remaining solution was concentrated to obtain 22.7 mL of a solution containing a humanized anti-HER2 antibody ADC composition.

[0293]

(iv) Evaluation of properties

The average number of bound drugs was measured in the same manner as the common operation A described in Example 3-1, (iv). In the case of the humanized anti-HER2 antibody (trastuzumab), based on its amino acid sequence, the number 26150 was used as an estimated value for the molar absorption coefficient of the light chain, and the number 81290 was used as an estimated value for the molar absorption coefficient of the heavy chain.

[0294]

The concentration of the antibody was found to be 20.39 mg/mL, the yield of the antibody was found to be 462 mg (90%), and the average number of bound drugs (n)

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per single antibody molecule, which was measured by the common operation A, was found to be 3.9. The HPLC chromatograph representing the peak area ratio (%) of each chain is shown in Figure 21.

[0295]

The area value of the number of bound drugs was measured in the same manner as the common operation B described in Example 3-1, (iv). The distribution condition of the number of bound drugs was as follows: D0: 3.6%, D2: 26.1%, D4-1: 34.1%, D4-2: 13.6%, D6: 17.6%, and D8: 5.0% (Figure 22).

(Example 7-2) Production of humanized anti-HER2 antibody ADC composition according to the method of the present invention

[0296]

(i) Reduction of antibody

A humanized anti-HER2 antibody (trastuzumab; U.S. Patent No. 5821337) (22.3 mL; corresponding to 500 mg, concentration: 22.4 mg/mL; 25 mM histidine buffer) was placed in a glass reaction vessel, and a 25 mM histidine buffer (25 mL, pH 5.0) was further added thereto. To the present reaction solution, a 0.5 M EDTA aqueous solution (0.034 mL; 5 equivalents based on the antibody) was added, and thereafter, a 0.1 g/mL polysorbate 20 aqueous solution (0.050 mL; 0.01% based on the antibody) was added thereto. Thereafter, a 0.3 M disodium hydrogen phosphate aqueous solution was added to the reaction

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mixture, so that the mixture was adjusted to pH 7.13, and then the mixture was cooled. Under stirring, a 1.00 mg/mL tris(2-carboxyethyl)phosphine hydrochloride aqueous solution (2.37 mL; 2.40 equivalents per single antibody molecule) was added to the reaction mixture at an internal temperature of 0°C to 1°C, and the obtained mixture was then stirred at an internal temperature of 0°C to 1°C for 6 hours, so that interchain disulfides of the antibody were reduced.

[0297]

(ii) Conjugation of antibody to drug linker intermediate

A 6.14 mg/mL 50% acetone aqueous solution (2.84 mL; 4.9 equivalents per single antibody molecule) of the compound obtained in Example 2 was added to the solution obtained in the above (i) at an internal temperature of 0°C to 2°C under stirring over 10 minutes. The obtained mixture was stirred at the same temperature as described above for 40 minutes, so that the drug linker intermediate was allowed to bind to the antibody. Subsequently, a 50 mM N-acetylcysteine aqueous solution (0.206 mL; 3 equivalents per single antibody molecule) was added to the reaction mixture, and the thus obtained mixture was further stirred at the same temperature as described above for 50 minutes. The reaction of the drug linker intermediate was terminated, and the pH of the reaction mixture was then adjusted to pH 5.0 using a 10% acetic acid aqueous solution.

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[0298]

(iii) Purification

Employing Pellicon XL (Millipore Japan, 50 cm²), the solution obtained in the above (ii) was circulated, while a 10 mM histidine buffer (pH 5.0) was added thereto using a roller pump, and a washing operation was carried out until the amount of water discharged became 600 mL, so that low molecular weight substances were removed. Thereafter, the remaining solution was concentrated to obtain 21.7 mL of a solution containing a humanized anti-HER2 antibody ADC composition.

[0299]

(iv) Evaluation of properties

The average number of bound drugs was measured in the same manner as the common operation A described in Example 3-1, (iv). In the case of the humanized anti-HER2 antibody (trastuzumab), based on its amino acid sequence, the number 26150 was used as an estimated value for the molar absorption coefficient of the light chain, and the number 81290 was used as an estimated value for the molar absorption coefficient of the heavy chain.

[0300]

The concentration of the antibody was found to be 21.2 mg/mL, the yield of the antibody was found to be 459 mg (89%), and the average number of bound drugs (n) per single antibody molecule, which was measured by the common operation A, was found to be 4.0. The HPLC

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chromatograph representing the peak area ratio (%) of each chain is shown in Figure 23.

[0301]

The area value of the number of bound drugs was measured in the same manner as the common operation B described in Example 3-1, (iv). The distribution condition of the number of bound drugs was as follows: D0: 2.8%, D2: 23.8%, D4-1: 55.2%, D6: 15.0%, and D8: 3.3% (Figure 24).

[0302]

(v) Results

The average number of bound drugs in the humanized anti-HER2 antibody ADC composition produced by the conventional method (Example 7-1) was 3.9, and the content of D4-1 therein was 34.1%. On the other hand, the average number of bound drugs in the humanized anti-HER2 antibody ADC composition produced by the method of the present invention (Example 7-2) was 4.0, and the content of D4-1 therein was 55.2%.

[0303]

(Test Example 1) Therapeutic efficacy of antibody-drug conjugate composition

[0304]

Mice: 5- to 6-week-old female BALB/c-nu/nu mice (Charles River Laboratories International, Inc.) were acclimatized under SPF conditions for 4 to 7 days, before the use for experiments. The mice were fed with a

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sterilized solid feed (FR-2, Funabashi Farms Co., Ltd) and sterilized tap water (prepared by adding a 5-15 ppm sodium hypochlorite solution to tap water).

[0305]

Measurement and calculation expression: The major axis and minor axis of a tumor were measured two or more times a week, using electronic digital calipers (CD-15C, Mitutoyo Corp.), and the volume (mm^3) of the tumor was then calculated. The applied calculation expression is as follows.

[0306]

Tumor volume (mm^3) = $1/2 \times \text{major axis (mm)} \times [\text{minor axis (mm)}]^2$

[0307]

A human pancreatic adenocarcinoma cell line CFPAC-1 (4×10^6 cells), which had been purchased from ATCC, was suspended in a normal saline. Thereafter, the obtained solution was subcutaneously implanted into the female BALB/c-nu/nu mice (Day 0), and the mice were then randomly divided into groups on Day 11. After completion of the grouping, the humanized anti-TROP2 antibody ADC composition produced according to the conventional method (Example 3-1) and the humanized anti-TROP2 antibody ADC composition produced according to the method of the present invention (Example 3-2) were each administered to the mice, via caudal vein, at a dose of 0.3 mg/kg, 1 mg/kg, or 3 mg/kg. The antibody-drug conjugate

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compositions were all diluted with Acetate-Buffered Saline (pH 5.5) (Nacalai Tesque, Inc.), and thereafter, the obtained solution was then administered in a liquid amount of 10 mL/kg to each mouse. The therapeutic efficacy was determined, using the minimum dose capable of regression of the tumor volume (regression dose) as an indicator. The regression dose of the humanized anti-TROP2 antibody ADC composition produced by the conventional method was 1 mg/kg, whereas the regression dose of the humanized anti-TROP2 antibody ADC composition produced by the method of the present invention was also 1 mg/kg (Figure 19). Thus, it was demonstrated that the antibody-drug conjugate composition produced by the production method of the present invention has therapeutic efficacy equivalent to that of the antibody-drug conjugate composition produced by the conventional production method.

[0308]

(Test Example 2) Safety of antibody-drug conjugate composition

[0309]

The humanized anti-TROP2 antibody ADC composition produced according to the conventional method (Example 3-1) and the humanized anti-TROP2 antibody ADC composition produced according to the method of the present invention (Example 3-2) were each administered to cynomolgus monkeys of cross-species at intervals of once per three

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weeks, a total of three times. The monkeys were observed until the day following the final administration, and the maximum dose that did not provide severe toxicity (HNSTD) was analyzed. As a result, the HNSTD of the humanized anti-TROP2 antibody ADC composition produced by the conventional method was 10 mg/kg, whereas the HNSTD of the humanized anti-TROP2 antibody ADC composition produced by the method of the present invention was 30 mg/kg. Hence, it was demonstrated that the antibody-drug conjugate composition produced by the production method of the present invention has safety that is more excellent than that of the antibody-drug conjugate composition produced by the conventional production method.

[0310]

(Consideration 1)

From the results of Examples 3, 4, 6 and 7, the average number of bound drugs in the antibody-drug conjugate composition produced by the conventional production method, and the average number of bound drugs in the antibody-drug conjugate composition produced by the production method of the present invention, were both found to be 3.5 to 4.5. On the other hand, the content of antibody-drug conjugates in which four drug linkers are bound to heavy-light interchain thiols, in the antibody-drug conjugate composition produced by the conventional production method was 35% or less, whereas

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the same content as described above in the antibody-drug conjugate composition produced by the production method of the present invention was 50% or more. As such, it was demonstrated that an antibody-drug conjugate composition wherein the average number of bound drugs is 3.5 to 4.5, and the content of antibody-drug conjugates in which four drug linkers are bound to heavy-light interchain thiols is 50% or more, can be selectively produced by using the production method of the present invention.

[0311]

(Consideration 2)

From the results of Test Example 2, it was demonstrated that the antibody-drug conjugate composition produced by the production method of the present invention has safety that is more excellent than that of the antibody-drug conjugate composition produced by the conventional production method.

From the aforementioned results, it was demonstrated that the antibody-drug conjugate composition of the present invention (an antibody-drug conjugate composition wherein the average number of bound drugs is 3.5 to 4.5, and the content of antibody-drug conjugates in which four drug linkers are bound to heavy-light interchain thiols is 50% or more) has safety that is more excellent than that of the antibody-drug conjugate composition produced by the conventional production method (an antibody-drug

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conjugate composition wherein the average number of bound drugs is 3.5 to 4.5, and the content of antibody-drug conjugates in which four drug linkers are bound to heavy-light interchain thiols is 35% or less).

Sequence Listing Free Text

[0312]

SEQ ID NO: 1: The nucleotide sequence of humanized anti-TROP2 antibody heavy chain (hTINA1-H1)

SEQ ID NO: 2: The amino acid sequence of humanized anti-TROP2 antibody heavy chain (hTINA1-H1)

SEQ ID NO: 3: The nucleotide sequence of humanized anti-TROP2 antibody light chain (hTINA1-L1)

SEQ ID NO: 4: The amino acid sequence of humanized anti-TROP2 antibody light chain (hTINA1-L1)

SEQ ID NO: 5: The amino acid sequence of anti-TROP2 antibody (TINA1) CDRH1

SEQ ID NO: 6: The amino acid sequence of anti-TROP2 antibody (TINA1) CDRH2

SEQ ID NO: 7: The amino acid sequence of anti-TROP2 antibody (TINA1) CDRH3

SEQ ID NO: 8: The amino acid sequence of anti-TROP2 antibody (TINA1) CDRL1

SEQ ID NO: 9: The amino acid sequence of anti-TROP2 antibody (TINA1) CDRL2

SEQ ID NO: 10: The amino acid sequence of anti-TROP2 antibody (TINA1) CDRL3

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SEQ ID NO: 11: The nucleotide sequence of humanized anti-CD98 antibody heavy chain (hM23-H1)

SEQ ID NO: 12: The amino acid sequence of humanized anti-CD98 antibody heavy chain (hM23-H1)

SEQ ID NO: 13: The nucleotide sequence of humanized anti-CD98 antibody light chain (hM23-L1)

SEQ ID NO: 14: The amino acid sequence of humanized anti-CD98 antibody light chain (hM23-L1)

SEQ ID NO: 15: The amino acid sequence of anti-CD98 antibody (M23) CDRH1

SEQ ID NO: 16: The amino acid sequence of anti-CD98 antibody (M23) CDRH2

SEQ ID NO: 17: The amino acid sequence of anti-CD98 antibody (M23) CDRH3

SEQ ID NO: 18: The amino acid sequence of anti-CD98 antibody (M23) CDRL1

SEQ ID NO: 19: The amino acid sequence of anti-CD98 antibody (M23) CDRL2

SEQ ID NO: 20: The amino acid sequence of anti-CD98 antibody (M23) CDRL3

SEQ ID NO: 21: The nucleotide sequence of primer EG-Inf-F

SEQ ID NO: 22: The nucleotide sequence of primer EG1-Inf-R

SEQ ID NO: 23: The nucleotide sequence of primer CM-LKF

SEQ ID NO: 24: The nucleotide sequence of primer KCL-Inf-R

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SEQ ID NO: 25: The amino acid sequence of humanized anti-B7-H3 antibody heavy chain (M30-H1)

SEQ ID NO: 26: The amino acid sequence of humanized anti-B7-H3 antibody light chain (M30-L4)

SEQ ID NO: 27: The amino acid sequence of anti-B7-H3 antibody (M30) CDRH1

SEQ ID NO: 28: The amino acid sequence of anti-B7-H3 antibody (M30) CDRH2

SEQ ID NO: 29: The amino acid sequence of anti-B7-H3 antibody (M30) CDRH3

SEQ ID NO: 30: The amino acid sequence of anti-B7-H3 antibody (M30) CDRL1

SEQ ID NO: 31: The amino acid sequence of anti-B7-H3 antibody (M30) CDRL2

SEQ ID NO: 32: The amino acid sequence of anti-B7-H3 antibody (M30) CDRL3

SEQ ID NO: 33: The amino acid sequence of humanized anti-HER2 antibody heavy chain

SEQ ID NO: 34: The amino acid sequence of humanized anti-HER2 antibody light chain

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

[Claim 1]

A method for producing an antibody-drug conjugate composition, comprising:

(i) a step of reacting an antibody with a reducing agent in a buffer to reduce interchain disulfides; and

(ii) a step of reacting drug linker intermediates with the antibody having thiol groups obtained in the step (i), wherein

the reaction temperature in the step (i) is -10°C to 10°C , and

the average number of bound drugs in the produced antibody-drug conjugate composition is 3.5 to 4.5, and the content of the antibody-drug conjugates in which four drug linkers are bound to heavy-light interchain thiols, in the produced antibody-drug conjugate composition is in the range of 50% to 90%.

[Claim 2]

The production method according to claim 1, wherein the average number of bound drugs in the produced antibody-drug conjugate composition is 4.0 to 4.1.

[Claim 3]

The production method according to claim 1 or 2, wherein the reaction temperature in the step (i) is -5°C to 5°C .

[Claim 4]

The production method according to claim 3, wherein the reaction temperature in the step (i) is -3°C to 3°C .

[Claim 5]

The production method according to claim 4, wherein the reaction temperature in the step (i) is 0°C to 2°C .

[Claim 6]

The production method according to claim 5, wherein the reaction temperature in the step (i) is 0°C to 1°C .

[Claim 7]

The production method according to any one of claims 1 to 6, wherein the reducing agent is used in an amount of 2 to 3 molar equivalents per molecule of the antibody.

[Claim 8]

The production method according to any one of claims 1 to 7, wherein the reducing agent is tris(2-carboxyethyl)phosphine or a salt thereof.

[Claim 9]

The production method according to claim 8, wherein the salt of tris(2-carboxyethyl)phosphine is tris(2-carboxyethyl)phosphine hydrochloride.

[Claim 10]

The production method according to any one of claims 1 to 9, wherein the buffer is a histidine buffer.

[Claim 11]

The production method according to any one of claims 1 to 10, wherein the buffer comprises a chelating agent.

[Claim 12]

The production method according to claim 11, wherein the chelating agent is ethylenediaminetetraacetic acid.

[Claim 13]

The production method according to any one of claims 1 to 12, wherein the antibody is a humanized antibody.

[Claim 14]

The production method according to any one of claims 1 to 13, wherein the antibody is an anti-TROP2 antibody, an anti-CD98 antibody, an anti-B7-H3 antibody, or an anti-HER2 antibody.

[Claim 15]

The production method according to claim 14, wherein the antibody is an anti-TROP2 antibody.

[Claim 16]

The production method according to claim 15, wherein the anti-TROP2 antibody retains CDRH1 consisting of the amino acid sequence shown in SEQ ID NO: 5 (TAGMQ), CDRH2 consisting of the amino acid sequence shown in SEQ ID NO: 6 (WINTHSGVPKYAEDFKG), CDRH3 consisting of the amino acid sequence shown in SEQ ID NO: 7 (SGFGSSYWYFDV), CDRL1

consisting of the amino acid sequence shown in SEQ ID NO: 8 (KASQDVSTAVA), CDRL2 consisting of the amino acid sequence shown in SEQ ID NO: 9 (SASYRYT), and CDRL3 consisting of the amino acid sequence shown in SEQ ID NO: 10 (QQHYITPLT).

[Claim 17]

The production method according to claim 15, wherein the anti-TROP2 antibody consists of a heavy chain consisting of an amino acid sequence consisting of amino acid residues at positions 20 to 470 of SEQ ID NO: 2, and a light chain consisting of an amino acid sequence consisting of amino acid residues at positions 21 to 234 of SEQ ID NO: 4.

[Claim 18]

The production method according to claim 17, wherein the lysine residue at the carboxyl terminus of the heavy chain of the anti-TROP2 antibody is deleted.

[Claim 19]

The production method according to claim 14, wherein the antibody is an anti-B7-H3 antibody.

[Claim 20]

The production method according to claim 19, wherein the anti-B7-H3 antibody retains CDRH1 consisting of the amino acid sequence shown in SEQ ID NO: 27 (NYVMH), CDRH2 consisting of the amino acid sequence shown in SEQ ID NO: 28 (YINPYNDDVKYNEKFKG), CDRH3 consisting of the amino acid sequence shown in SEQ ID NO: 29 (WGYYGSPLYYFDY),

CDRL1 consisting of the amino acid sequence shown in SEQ ID NO: 30 (RASSRLIYMH), CDRL2 consisting of the amino acid sequence shown in SEQ ID NO: 31 (ATSNLAS), and CDRL3 consisting of the amino acid sequence shown in SEQ ID NO: 32 (QQWNSNPPT).

[Claim 21]

The production method according to claim 19, wherein the anti-B7-H3 antibody consists of a heavy chain consisting of an amino acid sequence consisting of amino acid residues at positions 20 to 471 of SEQ ID NO: 25, and a light chain consisting of an amino acid sequence consisting of amino acid residues at positions 21 to 233 of SEQ ID NO: 26.

[Claim 22]

The production method according to claim 21, wherein the lysine residue at the carboxyl terminus of the heavy chain of the anti-B7-H3 antibody is deleted.

[Claim 23]

The production method according to claim 14, wherein the antibody is an anti-HER2 antibody.

[Claim 24]

The production method according to claim 23, wherein the anti-HER2 antibody consists of a heavy chain consisting of an amino acid sequence consisting of amino acid residues at positions 1 to 449 of SEQ ID NO: 33, and a light chain consisting of an amino acid sequence

consisting of amino acid residues at positions 1 to 214 of SEQ ID NO: 34.

[Claim 25]

The production method according to claim 23, wherein the anti-HER2 antibody consists of a heavy chain consisting of an amino acid sequence shown in SEQ ID NO: 33, and a light chain consisting of an amino acid sequence shown in SEQ ID NO: 34.

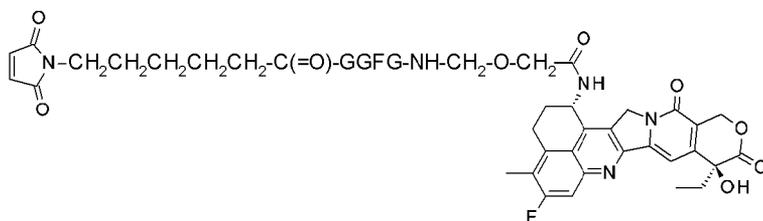
[Claim 26]

The production method according to any one of claims 1 to 25, wherein the drug linker intermediate has an N-substituted maleimidyl group.

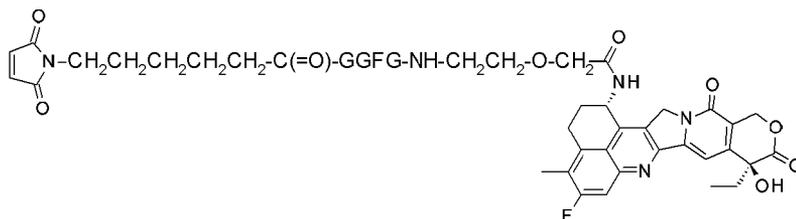
[Claim 27]

The production method according to claim 26, wherein the drug linker intermediate is

[Formula 1]

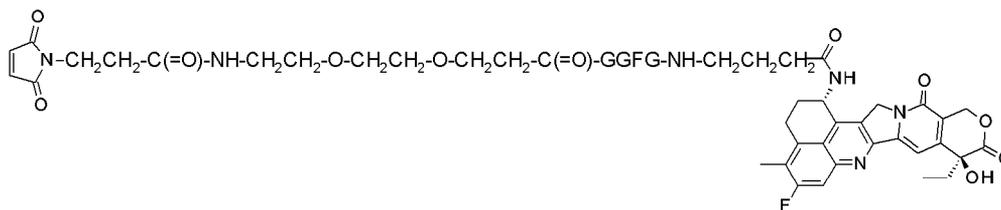


[Formula 2]



, or

[Formula 3]

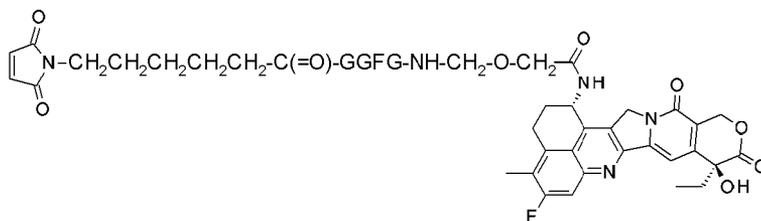


wherein -GGFG- represents a tetrapeptide residue consisting of glycine-glycine-phenylalanine-glycine.

[Claim 28]

The production method according to claim 27, wherein the drug linker intermediate is

[Formula 1]



wherein -GGFG- represents a tetrapeptide residue consisting of glycine-glycine-phenylalanine-glycine.

[Claim 29]

The production method according to any one of claims 1 to 28, wherein the content of the antibody-drug conjugates in which four drug linkers are bound to heavy-light interchain thiols, in the produced antibody-drug conjugate composition is in the range of 50% to 80%.

[Claim 30]

The production method according to any one of claims 1 to 28, wherein the content of the antibody-drug conjugates in which four drug linkers are bound to heavy-

light interchain thiols, in the produced antibody-drug conjugate composition is in the range of 50% to 70%.

[Claim 31]

The production method according to any one of claims 1 to 28, wherein the content of the antibody-drug conjugates in which four drug linkers are bound to heavy-light interchain thiols, in the produced antibody-drug conjugate composition is in the range of 50% to 60%.

[Claim 32]

An antibody-drug conjugate composition produced by the production method as defined in any one of claims 1 to 31.

[Claim 33]

An antibody-drug conjugate composition, wherein the content of antibody-drug conjugates in which the average number of bound drugs is 3.5 to 4.5, and four drug linkers are bound to heavy-light interchain thiols, is in the range of 50% to 90%.

[Claim 34]

The antibody-drug conjugate composition according to claim 33, wherein the average number of bound drugs is 4.0 to 4.1.

[Claim 35]

The antibody-drug conjugate composition according to claim 33 or 34, wherein the antibody is a humanized antibody.

[Claim 36]

The antibody-drug conjugate composition according to any one of claims 33 to 35, wherein the antibody is an anti-TROP2 antibody, an anti-CD98 antibody, an anti-B7-H3 antibody, or an anti-HER2 antibody.

[Claim 37]

The antibody-drug conjugate composition according to claim 36, wherein the antibody is an anti-TROP2 antibody.

[Claim 38]

The antibody-drug conjugate composition according to claim 37, wherein the anti-TROP2 antibody retains CDRH1 consisting of the amino acid sequence shown in SEQ ID NO: 5 (TAGMQ), CDRH2 consisting of the amino acid sequence shown in SEQ ID NO: 6 (WINTHSGVPKYAEDFKG), CDRH3 consisting of the amino acid sequence shown in SEQ ID NO: 7 (SGFGSSYWYFDV), CDRL1 consisting of the amino acid sequence shown in SEQ ID NO: 8 (KASQDVSTAVA), CDRL2 consisting of the amino acid sequence shown in SEQ ID NO: 9 (SASYRYT), and CDRL3 consisting of the amino acid sequence shown in SEQ ID NO: 10 (QQHYITPLT).

[Claim 39]

The antibody-drug conjugate composition according to claim 37, wherein the anti-TROP2 antibody consists of a heavy chain consisting of an amino acid sequence consisting of amino acid residues at positions 20 to 470 of SEQ ID NO: 2, and a light chain consisting of an amino

acid sequence consisting of amino acid residues at positions 21 to 234 of SEQ ID NO: 4.

[Claim 40]

The antibody-drug conjugate composition according to claim 39, wherein the lysine residue at the carboxyl terminus of the heavy chain of the anti-TROP2 antibody is deleted.

[Claim 41]

The antibody-drug conjugate composition according to claim 36, wherein the antibody is an anti-B7-H3 antibody.

[Claim 42]

The antibody-drug conjugate composition according to claim 41, wherein the anti-B7-H3 antibody retains CDRH1 consisting of the amino acid sequence shown in SEQ ID NO: 27 (NYVMH), CDRH2 consisting of the amino acid sequence shown in SEQ ID NO: 28 (YINPYNDDVKYNEKFKG), CDRH3 consisting of the amino acid sequence shown in SEQ ID NO: 29 (WGYYGSPLYYFDY), CDRL1 consisting of the amino acid sequence shown in SEQ ID NO: 30 (RASSRLIYMH), CDRL2 consisting of the amino acid sequence shown in SEQ ID NO: 31 (ATSNLAS), and CDRL3 consisting of the amino acid sequence shown in SEQ ID NO: 32 (QQWNSNPPT).

[Claim 43]

The antibody-drug conjugate composition according to claim 41, wherein the anti-B7-H3 antibody consists of a heavy chain consisting of an amino acid sequence consisting of amino acid residues at positions 20 to 471

of SEQ ID NO: 25, and a light chain consisting of an amino acid sequence consisting of amino acid residues at positions 21 to 233 of SEQ ID NO: 26.

[Claim 44]

The antibody-drug conjugate composition according to claim 43, wherein the lysine residue at the carboxyl terminus of the heavy chain of the anti-B7-H3 antibody is deleted.

[Claim 45]

The antibody-drug conjugate composition according to claim 36, wherein the antibody is an anti-HER2 antibody.

[Claim 46]

The antibody-drug conjugate composition according to claim 45, wherein the anti-HER2 antibody consists of a heavy chain consisting of an amino acid sequence consisting of amino acid residues at positions 1 to 449 of SEQ ID NO: 33, and a light chain consisting of an amino acid sequence consisting of amino acid residues at positions 1 to 214 of SEQ ID NO: 34.

[Claim 47]

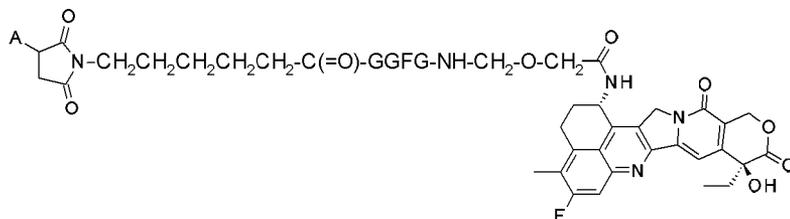
The antibody-drug conjugate composition according to claim 45, wherein the anti-HER2 antibody consists of a heavy chain consisting of an amino acid sequence shown in SEQ ID NO: 33, and a light chain consisting of an amino acid sequence shown in SEQ ID NO: 34.

[Claim 48]

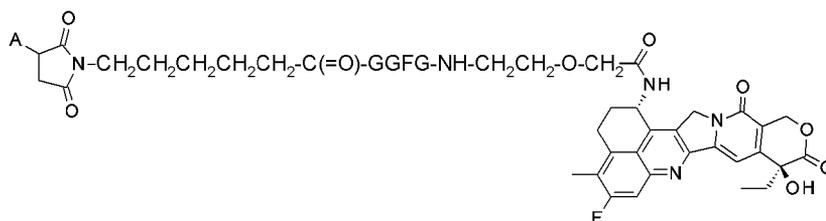
The antibody-drug conjugate composition according to any one of claims 33 to 47, wherein

the drug linker is

[Formula 4]

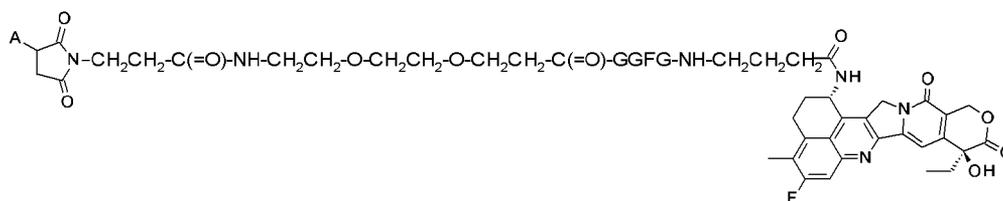


[Formula 5]



, or

[Formula 6]



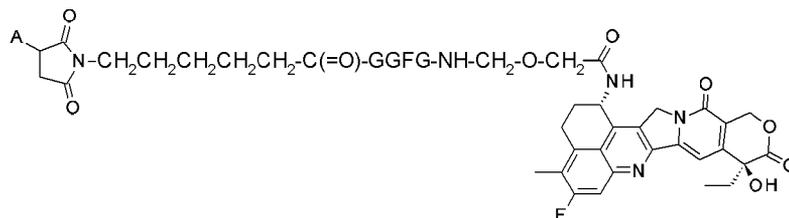
wherein A represents a binding site to the antibody, and -GGFG- represents a tetrapeptide residue consisting of glycine-glycine-phenylalanine-glycine.

[Claim 49]

The antibody-drug conjugate composition according to claim 48, wherein

the drug linker is

[Formula 4]



wherein A represents a binding site to the antibody, and -GGFG- represents a tetrapeptide residue consisting of glycine-glycine-phenylalanine-glycine.

[Claim 50]

The antibody-drug conjugate composition according to any one of claims 33 to 49, wherein the content of the antibody-drug conjugates in which four drug linkers are bound to heavy-light interchain thiols, in the produced antibody-drug conjugate composition is in the range of 50% to 80%.

[Claim 51]

The antibody-drug conjugate composition according to any one of claims 33 to 49, wherein the content of the antibody-drug conjugates in which four drug linkers are bound to heavy-light interchain thiols, in the produced antibody-drug conjugate composition is in the range of 50% to 70%.

[Claim 52]

The antibody-drug conjugate composition according to any one of claims 33 to 49, wherein the content of the antibody-drug conjugates in which four drug linkers are bound to heavy-light interchain thiols, in the produced

antibody-drug conjugate composition is in the range of 50% to 60%.

[Claim 53]

A pharmaceutical composition comprising the antibody-drug conjugate composition as defined in any one of claims 33 to 52, and a pharmaceutically acceptable carrier, excipient or diluent.

[Claim 54]

The pharmaceutical composition according to claim 53, wherein the antibody-drug conjugates target tumor and/or cancer cells.

[Claim 55]

The pharmaceutical composition according to claim 54, wherein the cancer is lung cancer, kidney cancer, urothelial cancer, colon cancer, prostate cancer, glioblastoma multiforme, ovarian cancer, pancreatic cancer, breast cancer, melanoma, liver cancer, bladder cancer, stomach cancer, cervical cancer, uterine cancer, head and neck cancer, esophageal cancer, bile duct cancer, thyroid cancer, lymphoma, acute myelocytic leukemia, acute lymphocytic leukemia, chronic myelogenous leukemia, or multiple myeloma.

[Figure 1]

SEQ ID NO: 1: Nucleotide sequence of humanized anti-TROP2 antibody heavy chain (hTINA1-H1)

```
atgaaacacctgtggttcttctcctgctggtggcagctcccagatgggtgctgagccaggtgca
gctggtgacagtctggcgccgaagtgaagaaaccaggcgccagcgtgaaggtgctcctgcaaggcca
gcggtacacctttaccaccgcccgcagtcagtggtgcccaggtcctggacagggcctggaa
tggatgggctggatcaacacccacagcggcgctgccaaatacgccgaggacttcaagggcagagt
gaccatcagcgcgcgacaccagcacctccacagcctacctgcagctgagcagcctgaagtccgagg
acaccgccgtgtactactgcgccagaagcggcttcggcagcagctactggtacttcgacgtgtgg
ggccagggcaccctcgtgaccgtcagctcagcctccaccaagggcccaagcgtcttccccctggc
accctcctccaagagcacctctggcggcacagccgctgggtgcctggtcaaggactacttcc
ccgaacccgtgaccgtgagctggaactcaggcgcctgaccagcggcgtgcacaccttccccgct
gtcctgcagtcctcaggactctactccctcagcagcgtggtgaccgtgccctccagcagcttggg
caccagacctacatctgcaacgtgaatcacaagcccagcaaacaccaaggtggacaagagagttag
agcccaaatcttgtgacaaaactcacacatgccaccctgccagcacctgaactcctgggggga
ccctcagcttctccttccccccaaaacccaaggacaccctcatgatctccgggaccctgaggt
cacatgctggtggtggacgtgagccacgaagaccctgaggtcaagttcaactggtacgtggacg
gctgaggtgcataatgccaagacaaagccccgggaggagcagtacaacagcagctaccgggtg
gtcagcgtcctcaccgtcctgcaccaggactggctgaatggcaaggagtacaagtgaaggtctc
caacaaagcctcccagccccatcgagaaaaccatctccaaagccaaaggccagccccgggaaac
cacaggtgtacaccctgccccatccccgggaggagatgaccaagaaccaggtcagcctgacctgc
ctggtcaaaggcttctatcccagcgacatcgccgtggagtgggagagcaatggccagcccagaaa
caactacaagaccaccctcccgtgctggactccgacggctccttcttctctacagcaagctca
ccgtggacaagagcaggtggcagcagggcaacgtcttctcatgctccgtgatgcatgaggctctg
cacaaccactacaccagaagagcctctcctgtctcccggcaaa
```

Signal sequence (1-57), variable region (58-420), constant region (421-1410)

SEQ ID NO: 2: Amino acid sequence of humanized anti-TROP2 antibody heavy chain (hTINA1-H1)

```
MKHLWFFLLLVAAAPRWVLSQVQLVQSGAEVKKPGASVKVSKASGYTFITAGMQWVRQAPGQGLE
WMGWINTHSGVPKYAEDFKGRVTISADTSTSTAYLQLSSLKSEDTAVYYCARSFGSSYWFYFDVW
GQGLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSVHTFPA
VLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPPELLGG
PSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRV
VSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIISKAKGQPREPQVYTLPPSREEMTKNQVSLTC
LVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFNFSVMHEAL
HNHYTQKSLSLSPGK
```

Signal sequence (1-19), variable region (20-140), constant region (141-470)

[Figure 2]

SEQ ID NO: 3: Nucleotide sequence of humanized anti-TROP2
antibody light chain (hTINA1-L1)

atggtgctgcagaccagggtgttcattcctctcctgctgctgtggatctccggcgctacggcgacat
ccagatgaccagagccctagcagcctgagcgccagcgtggcgacagagtgaccatcacatgca
agccagccaggacgtgtccacagccgtggcctggatcagcagaagcctggcaaggccccaag
ctgctgatctacagcgccagctaccggtacaccggcgctgccagcagatcttctggcagcggctc
cggcaccgacttcaccctgacaatcagcagcctgcagcccaggacttcgccgtgtactactgcc
agcagcactacatcacccccctgaccttggccagggcaccaagctggaaatcaagcgtacgggtg
gccgccccctcctgtgttcattccccccctccgacgagcagctgaagtccggcaccgcctcctg
gggtgctgctgaataacttctaccccagagaggccaaggtgcagtggaaggtggacaacgccc
tgcagtcgggaactcccaggagagcgtgaccgagcaggacagcaaggacagcacctacagcctg
agcagcacctgaccctgagcaaaagccgactacgagaagcacaaggtgtacgcctgagaggtgac
ccaccagggcctgagctcccccgctaccaagagcttcaacaggggggagtg

Signal sequence (1-60), variable region (61-387), constant region
(388-702)

SEQ ID NO: 4: Amino acid sequence of humanized anti-TROP2
antibody light chain (hTINA1-L1)

MVLQTVFISLLWISGAYGDIQMTQSPSSLSASVGDRVTITCKASQDVSTAVAWYQQKPKAPK
LLIYSASYRYTGVPSRFSGSGSDFTLTISLQPEDFAVYYCQQHYITPLTFGQGTKLEIKRTV
AAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSL
SSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Signal sequence (1-20), variable region (21-129), constant region
(130-234)

[Figure 3]

SEQ ID NO: 11: Nucleotide sequence of humanized anti-CD98 antibody heavy chain (hM23-H1)

atgaaacacctgtggttcttctctctgctggtggcagctcccagatgggtgctgagccaggtgca
gctggtgcagctctggcgccgaagtgaagaaaccaggcgccagcgtgaaggtgctctgcaaggcca
gcggtacgccttcagcaactacctgatcgagtggtgcccaggccctggacagggactggaa
tggatgggctgatcaaccctggcagcggcgtgaccaactacaacgagaagttcaagggcagagt
gaccatcacggccgacaccagcacctccaccgcctacatggaactgagcagcctgcccagcaggg
acaccgcccgtgtactattgtgccagagccgaggcttggttgcctactggggccagggaaacctc
gtgaccgtcagctcagcctccaccaagggcccaagcgtcttccccctggcaccctcctccaagag
cacctctggcggcacagccgcccctgggctgcctggtaaggactacttccccgaaccctgtagccg
tgagctggaactcagggcccctgaccagcggcgtgcacaccttccccgctgtcctgcagtcctca
ggactctactccctcagcagcgtggtgaccgtgccctccagcagctgggcaaccagacctacat
ctgcaacgtgaatcacaagcccagcaacaccaaggtggacaagagagttgagcccaaatcttgtg
acaaaactcacacatgcccaccctgcccagcacctgaactcctggggggaccctcagctcttccct
ttccccccaaaacccaaggacaccctcatgatctcccggaccctgaggtcacatgctgtggtggt
ggacgtgagccacgaagaccctgaggtcaagttcaactggtacgtggacggcgtggaggtgcata
atgccaagacaaagccccgggagggagcagtaaacagcacgtaccgggtggtcagcgtcctcacc
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agcccccatcgagaaaaccatctccaaagccaaagggcagccccgggaaccacaggtgtacacc
tgcccccatccccgggagggagatgaccaagaaccaggtcagcctgacctgcctggtcaaaggcttc
tatcccagcgacatcgccgtggagtgaggagcaatggccagcccgagaacaactacaagaccac
ccctcccgtgctggactccgacggctccttcttctctacagcaagctcaccgtggacaagagca
ggtggcagcagggcaacgtcttctcatgctccgtgatgcatgaggctctgcacaaccactacacc
cagaagagcctctccctgtctcccggcaaa

Signal sequence (1-57), variable region (58-405), constant region (406-1395)

SEQ ID NO: 12: Amino acid sequence of humanized anti-CD98 antibody heavy chain (hM23-H1)

MKHLWFFLLLVAAPRWVLSQVQLVQSGAEVKKPGASVKVSKASGYAFSNYLIEWVRQAPGGGLE
WMGVINPGSGVTNYNEKFKGRVTITADTSTSTAYMELSSLRSEDVAVYYCARAEAWFAYWGQGLT
VTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSVHTFPAVLQSS
GLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCKDKTHTCPPCPAPPELLGGPSVFL
FPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT
VLHQDWLNGKEYKCKVSNKALPAPIEKTIISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGF
YPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFCSSVMHEALHNHYT
QKSLSLSPGK

Signal sequence (1-19), variable region (20-135), constant region (136-465)

[Figure 4]

SEQ ID NO: 13: Nucleotide sequence of humanized anti-CD98
antibody light chain (hM23-L1)

```
atggtgctgcagaccaggtgttcactcctgctgctgtggatctccggcgcgtacggcgacat
cgtgatgaccagagccctgacagcctggcctgtctctgggagagagagccaccatcaactgca
agagcagccagagcctgctgtactccagcaaccagaagaactacctggcctggtatcagcagaag
cccggccagcctccaagctgctgatctactgggccagcaccagagaaagcggcgtgcccgatag
attcagcggcagcgggaagcggcaccgacttcaccctgacaatcagctccctgcaggccgaggacg
tggcctgtactactgccagcgtactacggctaccctggacctttggccagggcaccaaggtg
gaaatcaagcgtacggtggccgccccctccgtgttcacttccccccctccgacgagcagctgaa
gtccggcaccgctccgtggtgtgcctgctgaataacttctaccccagagaggccaaggtgcagt
ggaaggtggacaacgcctgcagtccgggaactcccaggagagcgtgaccgagcaggacagcaag
gacagcacctacagcctgagcagcacctgaccctgagcaaagccgactacgagaagcacaaggt
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agtgt
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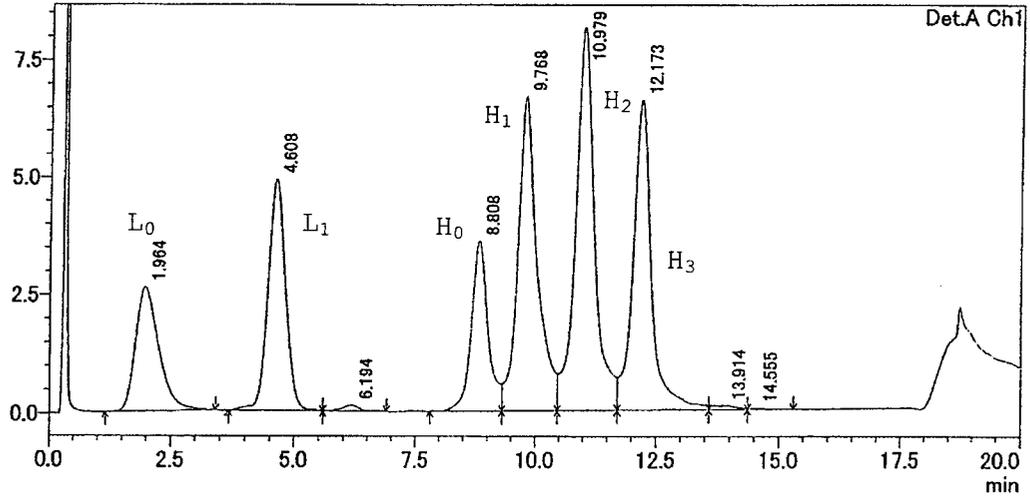
Signal sequence (1-60), variable region (61-405), constant region
(406-720)

SEQ ID NO: 14: Amino acid sequence of humanized anti-CD98
antibody light chain (hM23-L1)

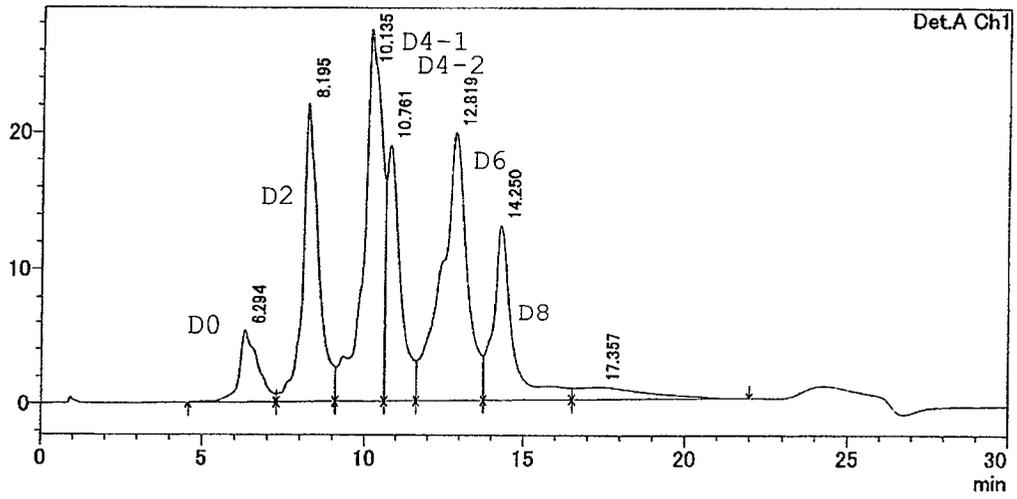
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MVLQTQVFISLLLWISGAYGDIVMTQSPDSLAVSLGERATINCKSSQSLLYSSNQKNYLAWYQQK
PGQPPKLLIYWASTRESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQRYYGYPWTFGQGTKV
EIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSK
DSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
```

Signal sequence (1-20), variable region (21-135), constant region
(136-240)

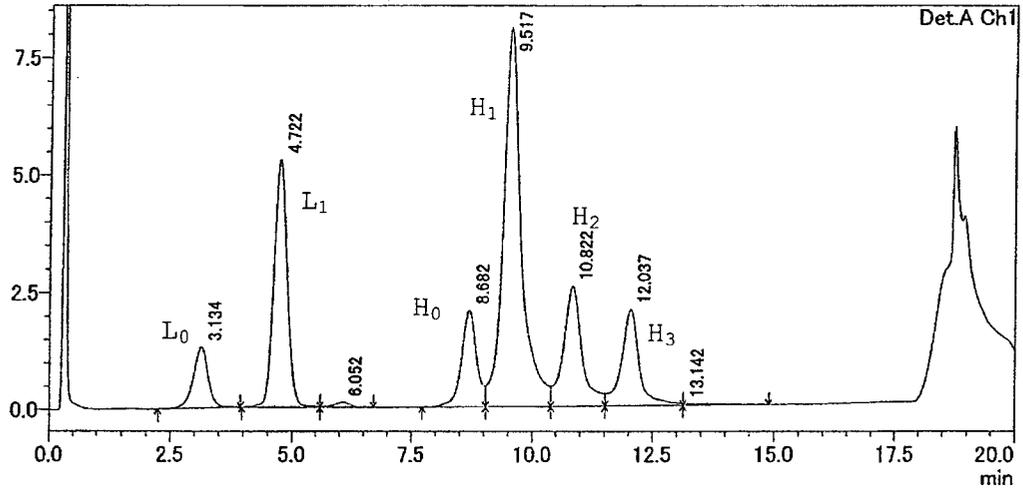
[Figure 5]



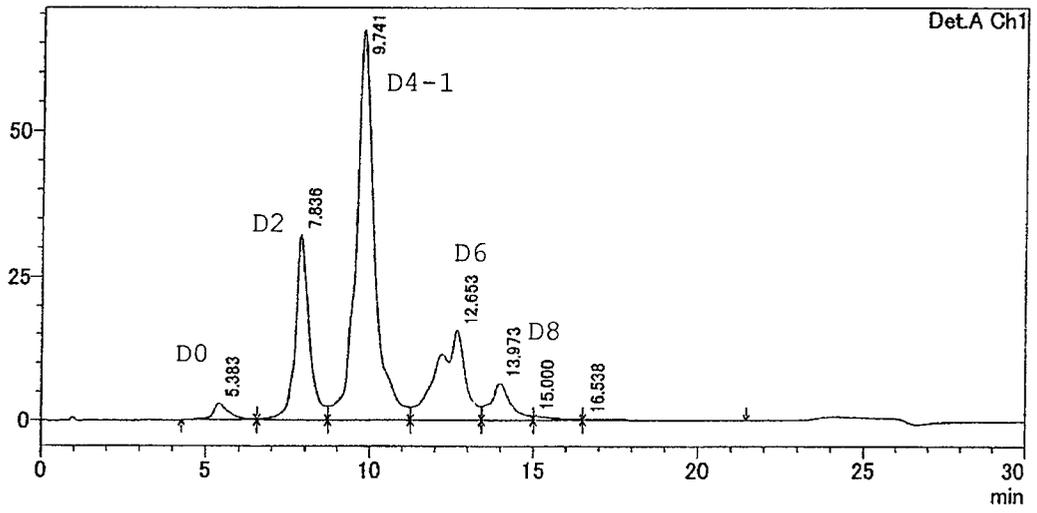
[Figure 6]



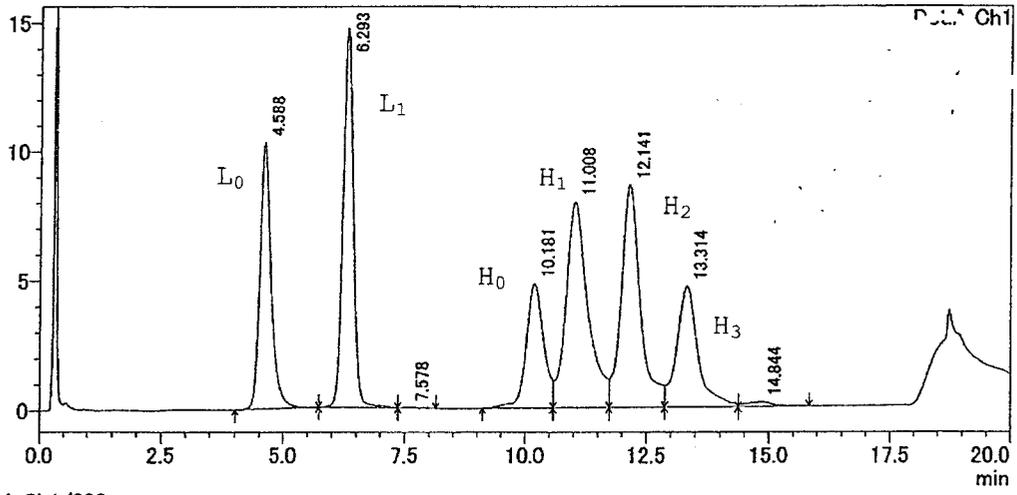
[Figure 7]



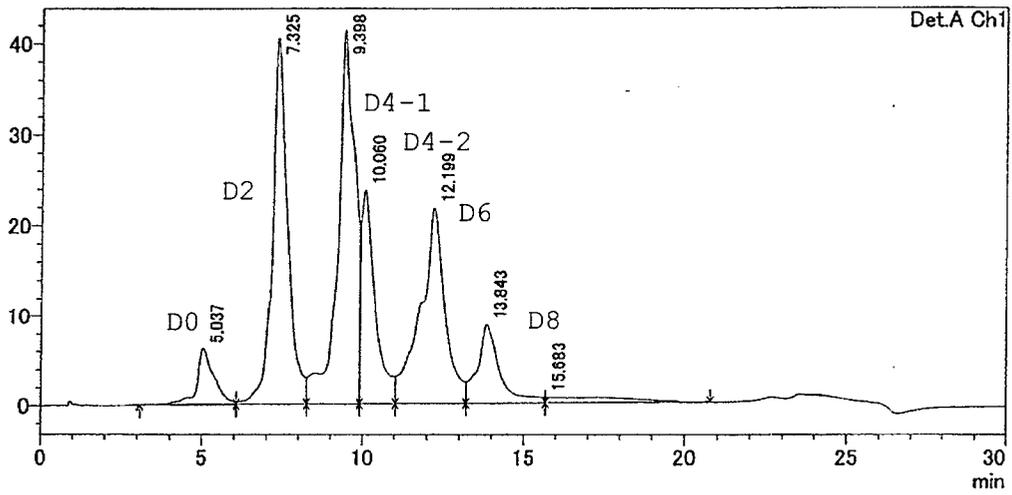
[Figure 8]



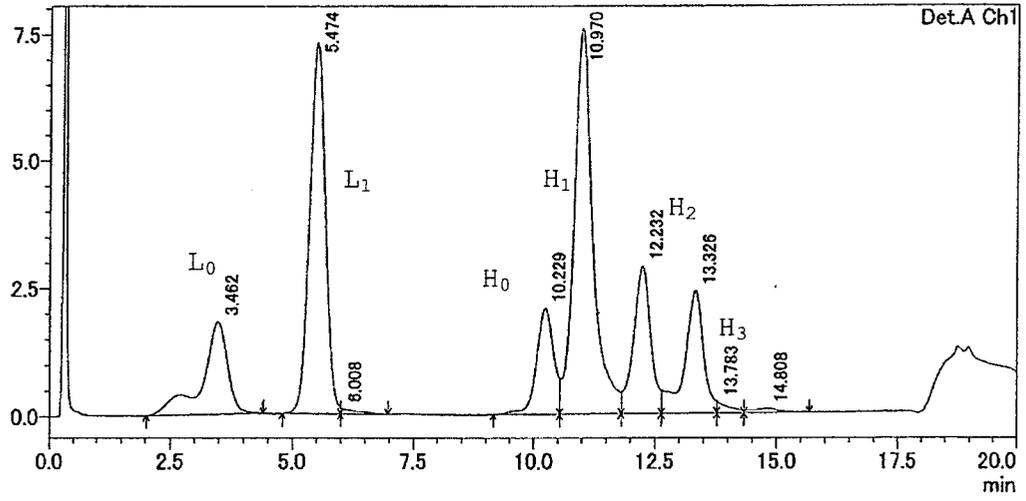
[Figure 9]



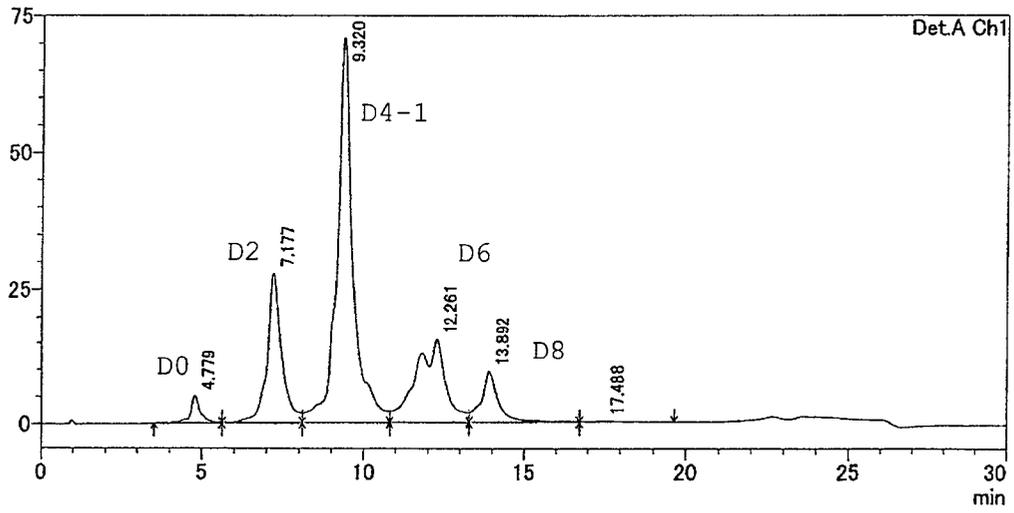
[Figure 10]



[Figure 11]



[Figure 12]



[Figure 13]

SEQ ID NO: 25: Amino acid sequence of humanized anti-B7-H3 antibody heavy chain (M30-H1)

MKHLWFFLLLVAAAPRWVLSQVQLVQSGAEVKKPGSSVKVCKASGYTFITNYVMHWVR
QAPGQGLEWMGYINPYNDDVKYNEKFKGRVTITADESTSTAYMELSSLRSEDTAVYY
CARWGYGSPLYYFDYWGGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVK
DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH
KPSNTKVKDKRVEPKSCDKHTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTC
VVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKE
YKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPS
DIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEAL
HNHYTQKSLSLSPGK

Signal sequence (1-19), variable region (20-141),
constant region (142-471)

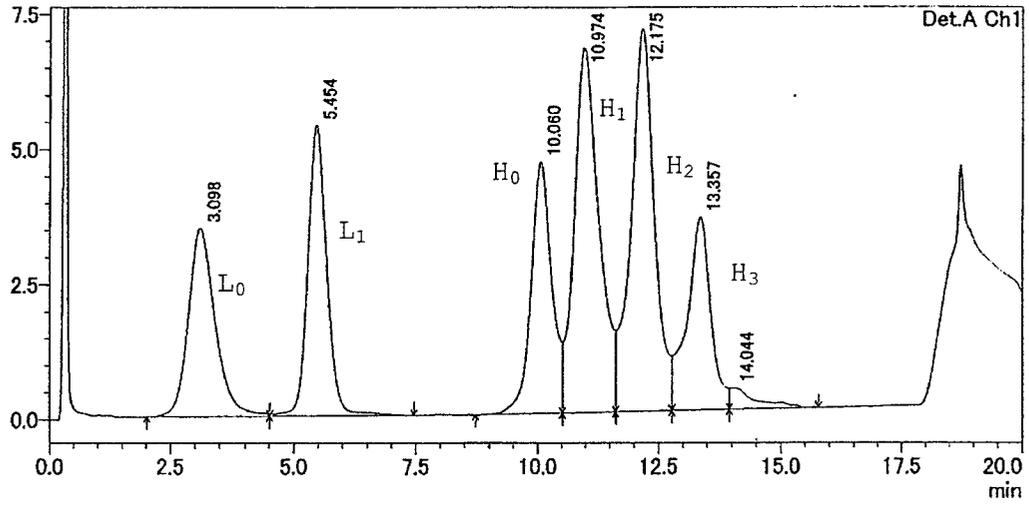
[Figure 14]

SEQ ID NO: 26: Amino acid sequence of humanized anti-B7-H3 antibody light chain (M30-L4)

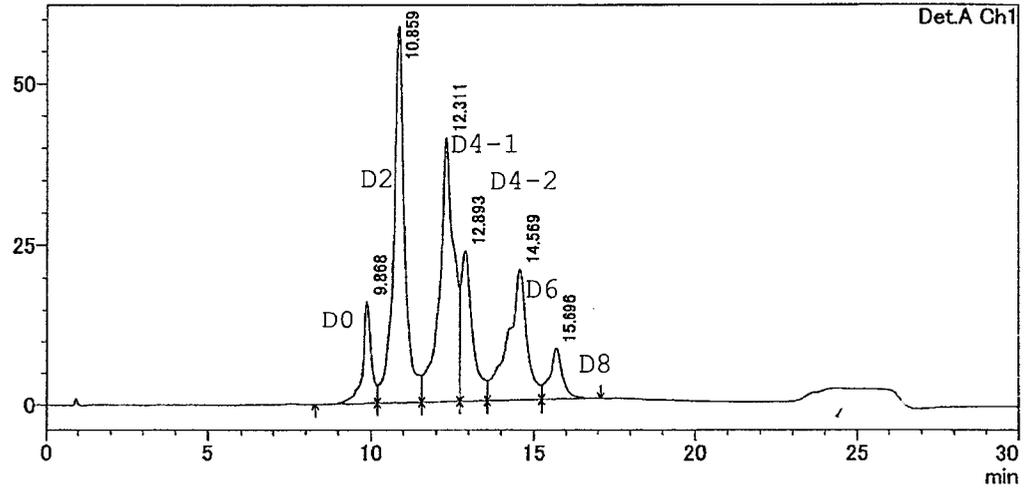
MVLQTQVFISLLLWISGAYGEIVLTQSPATLSLSPGERATLSCRASSRLIYMHWYQQ
KPGQAPRPLIYATSNLASGIPARFSGSGSGTDFTLTISSELPEDFAVYYCQQWNSNP
PTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDN
ALQSGNSQESVTEQDSKDSTYLSSTLTLSKADYEEKHKVYACEVTHQGLSSPVTKSF
NRGEC

Signal sequence (1-20), variable region (21-128),
constant region (129-233)

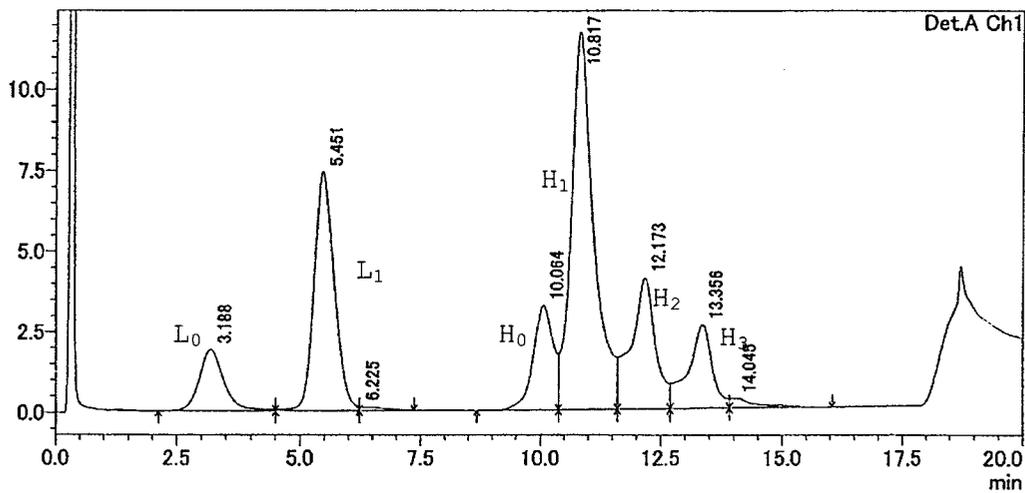
[Figure 15]



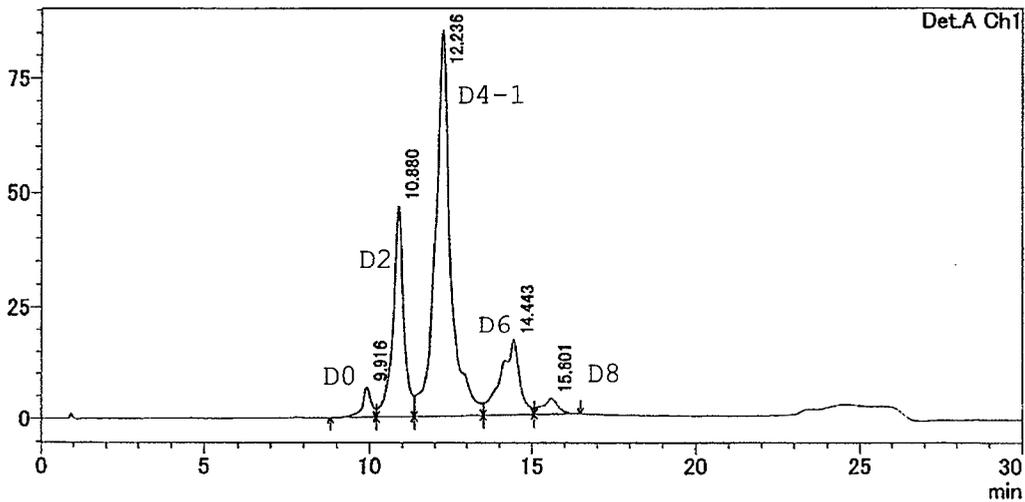
[Figure 16]



[Figure 17]



[Figure 18]



[Figure 19]

SEQ ID NO: 33: Amino acid sequence of humanized anti-HER2 antibody heavy chain

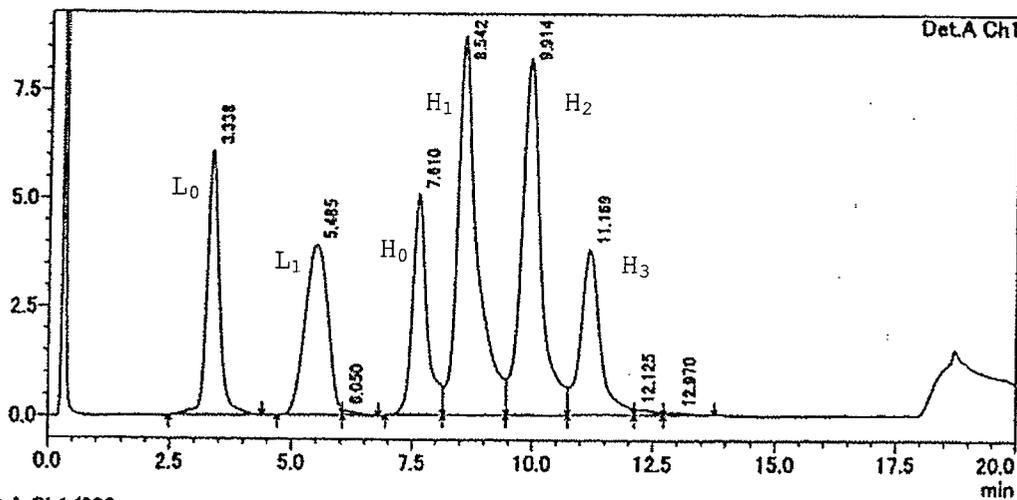
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGY
TRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT
LVTVSSASTKGPSVFLPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH
TFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT
CPPCPAPELLGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGV
EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA
KGQPREPQVYITLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPP
VLDS DGSFFLYSKLTVDKSRWQQGNV FSCSVMHEALHNHYTQKSLSLSPGK

[Figure 20]

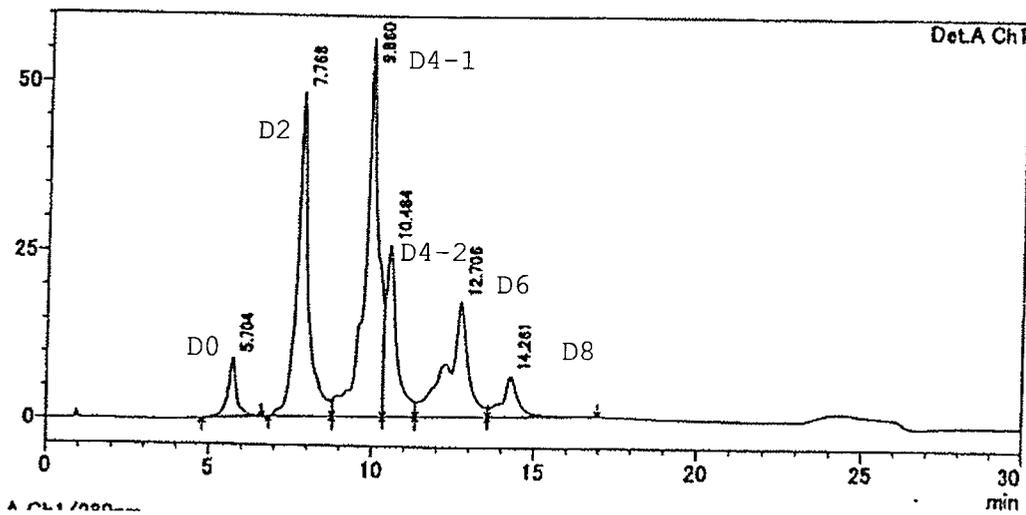
SEQ ID NO: 34: Amino acid sequence of humanized anti-HER2 antibody light chain

DIQMTQSPSSLSASVGDRTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSG
VPSRFGSRSGTDFTLTISLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVAAPS
VFIFPPSDEQLKSGTASVVCLLNMFYPREAKVQWKVDNALQSGNSQESVTEQDSKDS
TYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

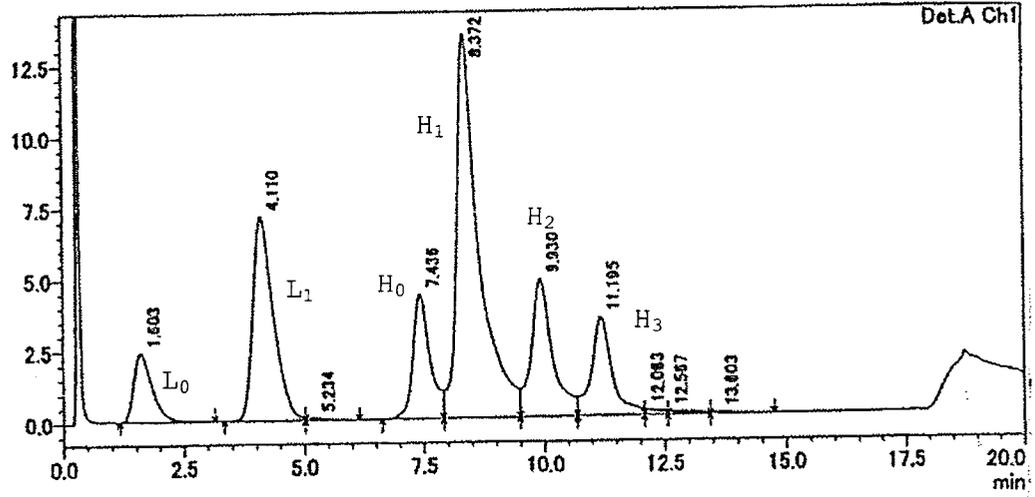
[Figure 21]



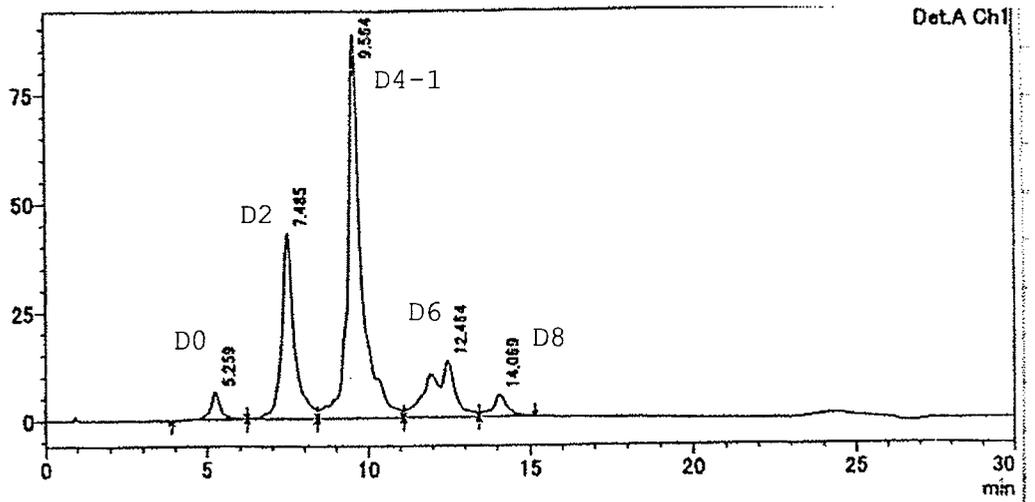
[Figure 22]



[Figure 23]



[Figure 24]



[Figure 25]

