METHOD FOR PURIFYING CYS-LINKED ANTIBODY-DRUG CONJUGATES

FIELD OF THE INVENTION

The present invention relates to a method for purifying a mixture of cysteine (Cys)-linked antibody-drug conjugates (ADCs), in particular of a mixture wherein the amount of non-conjugated antibody is in the range of 10-40% by weight.

Such Cys-linked ADCs may have an important role in new targeted cancer treatments. Therefore, having an industrial (preparative) scale method for purifying a mixture of Cys-linked ADCs is a key requirement for the future commercial success of such ADCs.

BACKGROUND OF THE INVENTION

In recent years, dozens of ADCs have been taken into preclinical and clinical development and two ADCs have been approved for marketing in the last couple of years. Apart from more recent developments for conjugating linker-drugs to (monoclonal) antibodies (mAbs), the drug in most of the ADCs in (pre)clinical development and in the two currently marketed ADCs is either linked to the antibody through the N-atom of a lysine residue or through the S-atom of a cysteine residue. The marketed product Kadcyla® or ado-trastuzumab emtansine (Roche/Genentech ImmunoGen) is an example of a lysine-linked ADC and Adcetris® or brentuximab vedotin (Seattle Genetics/Takeda Millennium) is an example of a cysteine-linked ADC. One of the ADCs currently in (pre)clinical development is a cysteine-linked ADC of formula (II) shown herein below in which a duocarmycin drug is conjugated through a cysteine residue to trastuzumab.

Duocarmycins, first isolated from a culture broth of *Streptomyces* species, are members of a family of antitumor antibiotics that include duocarmycin A, duocarmycin SA, and

CC-1065. These extremely potent agents allegedly derive their biological activity from an ability to sequence-selectively alkylate DNA at the N3 position of adenine in the minor groove, which initiates a cascade of events that terminates in an apoptotic cell death mechanism.

In order to make Cys-linked ADCs, the antibody typically is partially reduced to convert one or more interchain disulfide bonds into two or more free cysteine residues. The thiol or sulfhydryl (SH) groups of the free cysteine residues are then subsequently conjugated with a linker-drug molecule to form a Cys-linked ADC. Typically, this conjugation process gives a random, heterogeneous mixture of antibodies loaded with 0, 2, 4, 6 and 8 linker-drugs. The lower is the average drug-to-antibody ratio (DAR), the higher is the amount of non-conjugated antibody (DAR0) in the reaction mixture.

Drug loading is known to have an effect on the antitumor activity of the ADC as described for example by K.J. Hamblett *et al.* in Clinical Cancer Research 10 (2004) 7063-7070. It also affects CMC (Chemistry, Manufacturing and Control) properties like aggregation.

WO2011/133039 of Applicant discloses a series of novel analogs of the DNA-alkylating agent CC-1065 and antibody-drug conjugates (ADCs) thereof. In Example 15, the preparation of a number of trastuzumab-duocarmycin conjugates has been described using 1.1 molar equivalents of a reducing agent to generate 2 free thiol groups per mAb. After quenching, the ADCs were purified using an r-Protein A column to give linker-drug conjugates having an average DAR of approx. 2.

The prior art discloses the use of hydrophobic interaction chromatography (HIC) as a polishing step in many monoclonal antibody (mAb) purification processes. It is mentioned that this mode of chromatography is particularly useful for aggregate removal, and it provides good clearance of other process-related impurities such as host cell protein(s), DNA, endotoxins, leached Protein A and endogenous viruses.

HIC is also a well-established method for the (analytical) determination of the DAR and drug load distribution for cysteine-linked ADCs (Laurent Ducry (ed.), Antibody-Drug Conjugates, Methods in Molecular Biology, 1045 (2013) 275-283). Chapter 17 of this book by Jun Ouyang depicts in Fig. 2 on page 276 a representative HIC chromatogram of a Cys-linked ADC (i.e., MC-VC-PABC-MMAE). It is mentioned that elution with a gradient of a decreasing salt concentration and an increasing organic modifier impacts the column retention of the drug-loaded species with the least hydrophobic, unconjugated form (i.e. non-conjugated antibody, DAR0) eluting first, and the most hydrophobic antibody with 8 linker-drugs (DAR8) eluting last. The data in Table 2 on page 279 show that with a weighted average DAR of 3.6 the mixture of Cys-linked ADCs only contains 4.7% of non-conjugated antibody.

US4771128 describes a method for isolating and purifying toxin conjugates using HIC, in particular for immunoglobulin (antibody) conjugated to the toxic ribosome-inactivating protein ricin A. The method involves first removing unconjugated ricin A and aggregates via sizing chromatography (i.e., size exclusion chromatography, SEC), followed by hydrophobic gel chromatography (i.e., HIC, using Phenyl Sepharose CL-4B, volume 70 ml), in which the conjugate mixture was separated by eluting with salt solutions of decreasing ionic strength. The non-conjugated immunoglobulin was eluted first. The buffer used in both the sizing step and the subsequent chromatographic separation step contained sodium chloride (1 M) at a flow rate of about 20-40 ml/h, cf. Example 1. In an alternative embodiment, a "fast flow" chromatographic separation and purification is provided (i.e., using Phenyl Sepharose CL-4B, column diameter 1 cm, volume 3.14 ml) wherein the unconjugated immunoglobulin is removed with the first column volume of phosphate buffer/sodium chloride (1.5 M) solution at a flow rate of about 0.13 ml/h, cf. Example 2, and the immunoconjugate is removed with a second column volume of phosphate buffer containing 10-60 vol. % of an organic solvent (i.e., 60 vol.% glycerol in Example 2).

The main disadvantage of the methods disclosed in the prior art is the use of an organic solvent which is neither desirable nor acceptable for an industrial scale process.

A problem that has not been addressed in the prior art to the best of Applicant's knowledge is the scaling up of the ADC purification process.

Having reviewed the prior art, there is clearly a need for a new method for purifying mixtures of Cys-linked ADCs. In particular, it would be desirable to have a method for the purification of mixtures of Cys-linked ADCs having an average DAR of about 2-3, which typically contain a relatively high amount of non-conjugated antibody, sometimes as much as 40% by weight, on an industrial preparative scale, and not having to use multiple chromatographic steps.

SUMMARY OF THE INVENTION

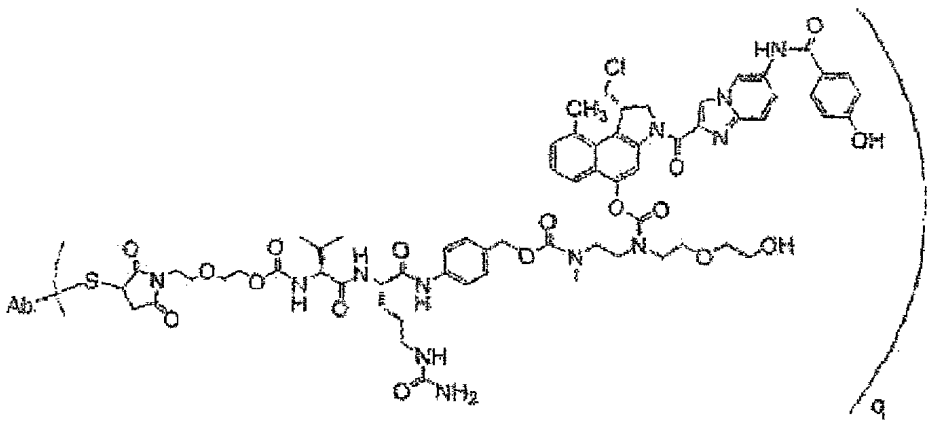
The present invention relates to a new method for the purification of a mixture of cysteine-linked antibody-drug conjugates, in particular of a mixture having an average DAR of about 2-3 wherein the amount of non-conjugated antibody is in the range of 10-40% by weight.

In a first aspect, the present invention provides for a method for purifying a mixture of cysteine-linked antibody-drug conjugates, wherein the amount of non-conjugated antibody is in the range of 10-40% by weight comprising:

a. providing the mixture in a 0.2-1.5 M aqueous salt solution;
b. loading said solution onto a preparative hydrophobic interaction chromatography column;
c. collecting a flow-through fraction that contains non-conjugated antibody;
d. washing said column with a 0.2-1.5 M aqueous salt solution while collecting the flow-through fraction; and

e. eluting said column with a 0-100 mM aqueous salt solution to obtain a purified mixture of cysteine-linked antibody-drug conjugates.

In a particularly preferred embodiment of the present invention, the mixture of cysteine-linked antibody-drug conjugates is of the formula (II)



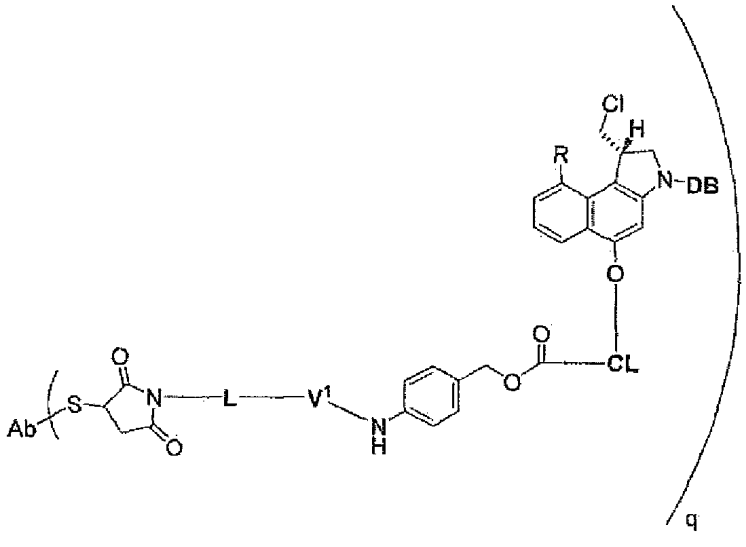
(II)

wherein

Ab is trastuzumab, and

q ranges from 0 to 8.

In another aspect, the present invention provides for a method for purifying a mixture of cysteine-linked antibody-drug conjugates of formula (I)

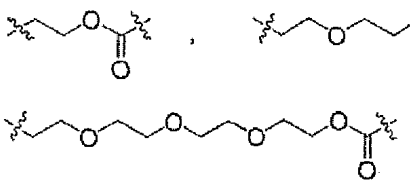


(I),

wherein

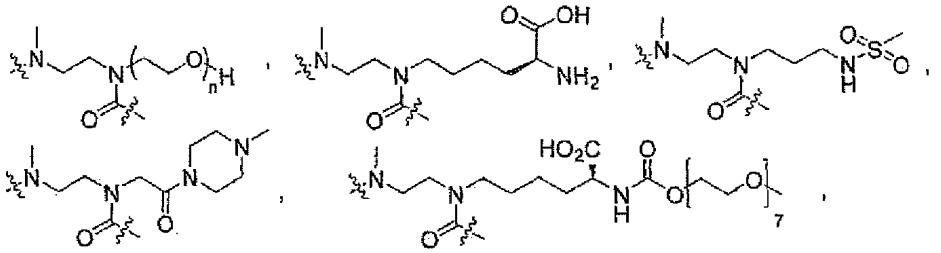
Ab is an antibody,

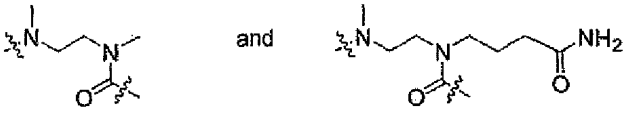
L is a linking group selected from

 and

V^1 is a conditionally-cleavable dipeptide of natural and/or unnatural amino acids,

CL is a cyclization linker selected from



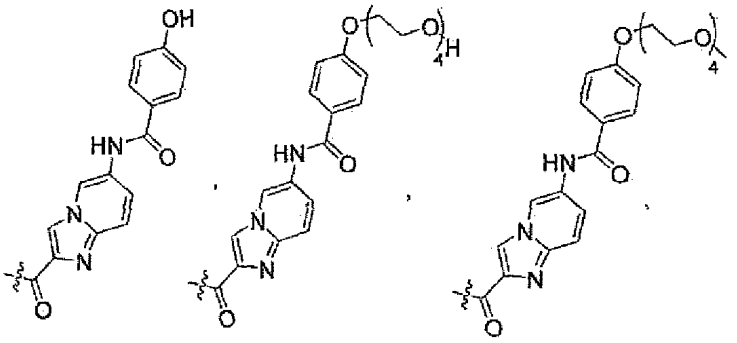
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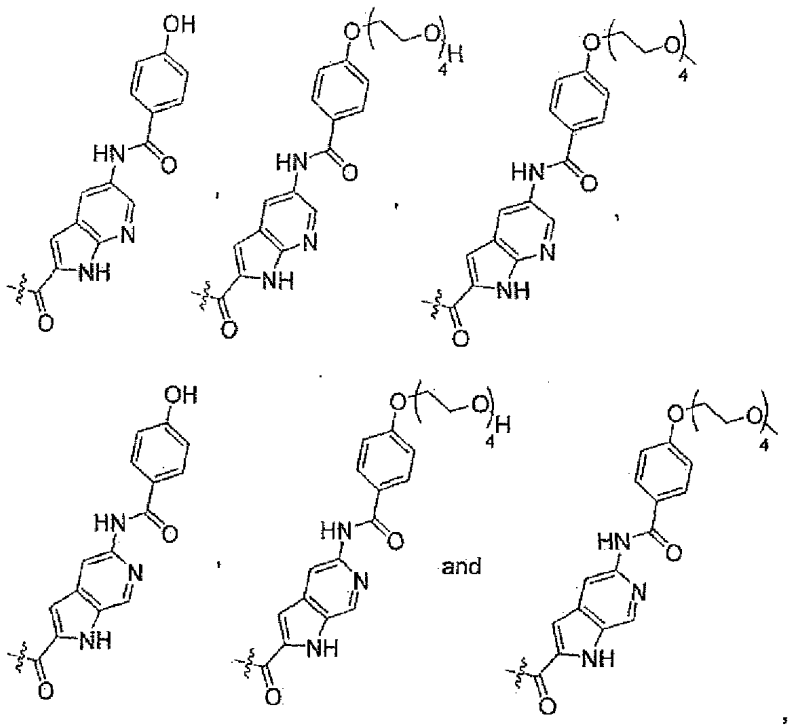
wherein n is an integer of from 1 to 16,

R is selected from H, CH_3, CH_2CH_3, OCH_3, OCH_2CH_3, CF_3, OCF_3, Cl, F,

q ranges from 0 to 8, and

DB is a DNA binding moiety selected from





wherein the amount of non-conjugated antibody is in the range of 10-40% by weight comprising:

a. providing the mixture in a 0.2-1.5 M aqueous salt solution;

b. loading said solution onto a preparative hydrophobic interaction chromatography column;

c. collecting a flow-through fraction that contains non-conjugated antibody;

d. washing said column with a 0.2-1.5 M aqueous salt solution while collecting the flow-through fraction; and

e. eluting said column with a 0-100 mM aqueous salt solution to obtain a purified mixture of cysteine-linked antibody-drug conjugates;

wherein no added organic solvent is used in either step a, b, d or e.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows an example of the influence of the amount of reductant on the distribution of DAR species. When using 1.0 equivalent of reductant, the percentage of non-conjugated trastuzumab antibody DARO is about 20% by weight.

Figure 2 depicts an analytical HIC chromatogram of a mixture of cysteine-linked antibody-drug conjugates according to formula (II) before and after HIC purification on a preparative scale according to the purification in Example 3.

Figure 3 depicts analytical HIC chromatograms of a mixture of cysteine-linked antibody-drug conjugates according to formula (II) before and after HIC purification on a preparative scale according to the purification in Example 4.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, it was found that a mixture of cysteine-linked antibody-drug conjugates (Cys-linked ADCs) wherein the amount of non-conjugated antibody is in the range of 10-40% by weight may be advantageously purified from non-conjugated antibody (DAR0) and non-conjugated linker-drug, which typically is quenched after completion of the conjugation reaction, by hydrophobic interaction chromatography. The method according to the present invention comprises:

a. providing the mixture in a 0.2-1.5 M aqueous salt solution;
b. loading said solution onto a preparative hydrophobic interaction chromatography column;
c. collecting a flow-through fraction that contains non-conjugated antibody;
d. washing said column with a 0.2-1.5 M aqueous salt solution while collecting the flow-through fraction; and
e. eluting said column with a 0-100 mM aqueous salt solution to obtain a purified mixture of cysteine-linked antibody-drug conjugates.

In the context of the present specification with "salt" is not meant a "buffer" (salt). Examples of suitable salts and buffers to be used in accordance with the method of the present invention are given herein below. Advantageously, in the method of the present invention buffered aqueous salt solutions are used.

In accordance with the method of the present invention, only aqueous solutions are used, hence, no added organic solvent is used in either step a, b, d or e. To be clear, step e can be carried out in the absence of salt.

The claimed method involves contacting a mixture of Cys-linked ADCs with HIC column packing material in an aqueous salt solution under column loading conditions that permit the mixture of antibodies loaded with 2 to 8 linker-drugs, the non-conjugated linker-

drug and impurities, typically aggregates, to bind to the column packing material, while the non-conjugated antibody does not bind and immediately is washed off/flows through the column under loading conditions. Elution with a lower concentration of the aqueous salt solution will separate the Cys-linked ADCs from the non-conjugated linker-drug and impurities which remain bound to the column packing material/stay on the column.

The aqueous salt solution used for loading (step b) and washing (step d) can be the same or different. Advantageously, the aqueous salt solution used for loading (step b) and washing (step d) is the same.

As is known to the person skilled in the art, and as described in paragraph [0057] of US20100069617, optimal loading/binding and elution conditions on a HIC column depend on a number of factors. Therefore, variation in the individual retention characteristics of different mixtures of ADCs, e.g., due to variations in antibody, linker and drug, makes it desirable to customize/optimize the operating conditions of the HIC column in accordance with the present invention. This optimization primarily involves determining the hydrophobicity of the mixture of ADCs which is to be purified, e.g., by determining the (relative) hydrophobicity of the DAR2 species of any specific ADC, and selecting the (hydrophobicity of the) column packing material. It further involves choosing/optimizing the loading/binding aqueous salt concentration, the eluting aqueous salt concentration, the concentration of any buffering salt, and the pH.

Mixtures of Cys-linked ADCs of the formulae (I) and (II) in accordance with the present invention have the linker-drug conjugated to the antibody through the S-atom of a cysteine residue, i.e., they are cysteine-linked antibody-drug conjugates. Typically, the cysteine residue is a natural cysteine residue which is present in the heavy and/or light chain of the antibody (Ab) and forms interchain disulfide bonds. The present invention is particularly drawn to the purification of ADC compounds wherein the linker-drug is conjugated

through interchain disulfide bonds of Abs, more particularly mAbs. For example, IgG1 antibodies typically have four interchain disulfide bonds, all four located in the hinge region of antibodies, and after (partial) reduction of the disulfide bonds the linker-drug is randomly attached to free thiol groups.

Mixtures of Cys-linked ADC compounds of the formulae (I) and (II) in accordance with the present invention can be obtained according to methods and procedures that are well known to a person skilled in the art. Conjugation through interchain disulfide bonds can occur after complete or partial reduction of said disulfide bonds. Suitable methods for preparing such compounds can be found in the description and examples of Applicant's WO2011/133039. In particular, Example 15 of WO2011/133039 describes the partial reduction of trastuzumab to generate 2 free thiol groups per mAb and conjugation with a number of linker-drugs to ADCs having an average DAR of approx. 2. Examples 7 and 8 of WO2005/084390 describe partial reduction, partial reduction/partial reoxidation, and complete reduction strategies for (partial) loading of antibodies with the linker-drug vcMMAE.

The mixture of cysteine-linked antibody-drug conjugates (Cys-linked ADCs) to be purified in accordance with the present invention contains an amount of non-conjugated antibody in the range of 10-40% by weight, more particularly in the range of 10-35% by weight, even more particularly in the range of 15-35% by weight. It is well-known in the art that the amount of non-conjugated antibody present after conjugation decreases with increasing average drug-to-antibody ratio (DAR). As an example, the present inventors have found that when using more than 1.5 equivalents of a reducing agent to reduce the interchain disulfide bridges of the monoclonal antibody trastuzumab, less than 10% by weight of non-conjugated antibody (DAR0) is present in the mixture of conjugates. When using 1.0 equivalent of a reducing agent, a maximum amount of approx. 50% by weight of DAR2 is present in the mixture of conjugates and the percentage of non-conjugated antibody (DAR0)

trastuzumab is about 20% by weight (see Figure 1). It is to be noted that the distribution of DAR species with the ratio of reductant : mAb varies depending on the reactants and reaction conditions used.

The method in accordance with the present invention is particularly advantageous when striving to have an average DAR of about 2-3, more particularly of from 2.6 to 2.9, even more particularly of from 2.7 to 2.9.

The preparative HIC column to be used in accordance with the method of the present invention can be any preparative column which is commercially available. Examples of suppliers of such columns and/or of suitable column packing materials include Tosoh Bioscience, GE Healthcare, Bio-Rad and Merck Millipore.

Said HIC column can be packed with Fractogel EMD propyl (Merck), Fractrogel EMD phenyl (Merck Millipore), Butyl-S sepharose (GE Healthcare), Octyl Sepharose (GE Healthcare), Capto Octyl (GE Healthcare), Capto Butyl (GE Healthcare), Capto Phenyl ImpRes (GE Healthcare), Capto Butyl ImpRes (GE Healthcare), Toyopearl PPG-600M (Tosoh Bioscience), Toyopearl Hexyl-650 (Tosoh Bioscience), Toyopearl Butyl-650 (Tosoh Bioscience), Toyopearl Phenyl-650 (Tosoh Bioscience), Toyopearl Ether-650 (Tosoh Bioscience), Macroprep t-Butyl (Bio-Rad), Macroprep phenyl (Bio-Rad), Cellufine Butyl (JNC Corporation), Cellufine Phenyl (JNC Corporation) or Poros HP2 (Applied Biosystems).

Advantageously, said HIC column is packed with GE Healthcare's resins Butyl-S Sepharose 6 Fast Flow (FF), Capto Octyl, Octyl Sepharose 4 Fast Flow, Phenyl Sepharose 6 Fast Flow, Capto Butyl, Butyl Sepharose 4 Fast Flow or Capto Butyl ImpRes, or Tosoh Bioscience's resin Toyopearl PPG-600M. The relative hydrophobicity and many other characteristics of the various column packing materials/resins can be derived from information leaflets on said resins which can be obtained from the suppliers. Preferably, in accordance with the method of the present invention, the HIC column is packed with Butyl-S

Sepharose 6 FF, Capto Butyl, Butyl Sepharose 4 FF, Capto Butyl ImpRes or Toyopearl PPG-600M, more preferably it is packed with Butyl Sepharose 4 FF, Capto Butyl ImpRes or Toyopearl PPG-600M, most preferably it is packed with Butyl Sepharose 4 FF or Toyopearl PPG-600M.

Typically, in accordance with the method of the present invention, the column bed height is about 20-25 cm, advantageously about 20 cm, and the pressure on the column is kept below 2 bar.

The column dimensions are dictated by the amount of ADC material that one desires or needs to load onto the HIC column. As is well known to the person skilled in the art, the amount of ADC material that can be loaded increases with column internal diameter and column length.

The preparative HIC column to be used in accordance with the method of the present invention typically has a diameter in the range of 4.0-2,000 mm, preferably 15-2,000 mm, more preferably 80-2,000 mm, most preferably 400-2,000 mm. The larger the diameter of the column, the more ADC material can be loaded onto the top of the column. Advantageously, because the column loading and washing conditions are so-chosen that the non-conjugated antibody (DAR0) flows through the column, the capacity of the column increases. For example, if the amount of non-conjugated antibody present in the mixture of Cys-linked ADCs is 30% by weight, the purification process in accordance with the present invention allows for an approximate 30% higher loading of said column.

The amount of ADC material that is loaded on the preparative column used in accordance with the method of the present invention typically is in the range of 5-50 g/L, preferably in the range of 5-40 g/L, more preferably 10-40 g/L, even more preferably 30-40 g/L of column packing material.

In accordance with the method of the present invention, advantageously batch amounts of from 20 to 2,000 g can be purified, making the presently claimed HIC purification process suitable for an industrial scale production of GMP (Good Manufacturing Practice) ADC material.

Apart from the column diameter and length, also the average particle size ($d_{50,volume}$, median particle size of the cumulative volume distribution) of the column packing material is of relevance.

In accordance with the method of the present invention, the particle size chosen allows for a good separation at a minimal flow rate. In accordance with the method of the present invention the column packing material has a particle size in the range of 30-180µm. Preferably, the column packing material has a particle size in the range of 35-100µm; even more preferably, the column packing material has a particle size in the range of 45-90µm.

In accordance with the method of the present invention, the flow rate is in the range of 50-300 cm/h. Preferably, the flow rate is in the range of 80-250 cm/h, more preferably 100-220 cm/h, most preferably about 100-110 cm/h.

In accordance with the method of the present invention, the elution in step e is either performed in a regular mode (i.e., flow during elution is in the same direction as flow during loading and washing) or in a reverse mode (i.e., flow during elution is in the opposite direction as flow during loading and washing). The reverse-mode elution of the purified mixture of Cys-linked ADCs is particularly advantageous in case the (conjugation reaction) mixture of ADCs is purified from unconjugated linker-drug, e.g., by subjecting said (conjugation reaction) mixture to (e.g., activated carbon) filtration before applying the claimed method of purification.

Advantageously, the salt of the aqueous salt solution is selected from the group consisting of potassium thiocyanate, sodium chloride, potassium chloride, ammonium

chloride, sodium sulphate, potassium sulphate and ammonium sulphate. Preferably, the salt is sodium chloride or ammonium sulphate. More preferably, the salt is ammonium sulphate.

In accordance with the method of the present invention, the salt of the aqueous salt solution for loading (step b) and washing (step d) may be the same or different from the salt of the aqueous salt solution for eluting (step e). Advantageously, the same salt is used for steps b, d and e.

In accordance with the method of the present invention, the aqueous salt solution for loading (step b) and washing the column (step d) has a concentration of 0.2-1.5 M. Preferably, the aqueous salt solution has a concentration of 0.2-1.0 M, more preferably 0.45-0.9 M, most preferably 0.55-0.9 M.

In accordance with the method of the present invention, the aqueous salt solution for eluting the column (step e) has a concentration of 0-100 mM. Preferably, the aqueous salt solution has a concentration of 0-90 mM, more preferably 0-80 mM, even more preferably 0-70 mM, most preferably 0-55 mM.

In accordance with the method of the present invention, preferably the aqueous salt solution further contains a buffer. Advantageously, when the aqueous salt solution has a concentration of 0 mM (step e), it contains a buffer. Advantageously, the buffer is selected from the group consisting of sodium phosphate, potassium phosphate, ammonium phosphate, sodium acetate, potassium acetate, sodium citrate, potassium citrate, ammonium citrate and mixtures thereof. Preferably, the buffer is a phosphate, acetate or citrate or a mixture thereof such as a citrate-phosphate buffer. More preferably, the buffer is sodium phosphate or sodium acetate.

In accordance with the method of the present invention, the buffer for loading (step b), washing (step d) and eluting the column (step e) has a concentration of 0-100 mM, preferably

0-50 mM, more preferably 20-30 mM. Advantageously, a buffered aqueous salt solution is used in all steps (step a to e) of the method of the present invention.

The buffered aqueous salt solution that is advantageously used in accordance with the method of the present invention preferably is buffered to a pH of from about 4 to about 8, more preferably from about 5 to about 7, most preferably from about 5.0 to about 5.5.

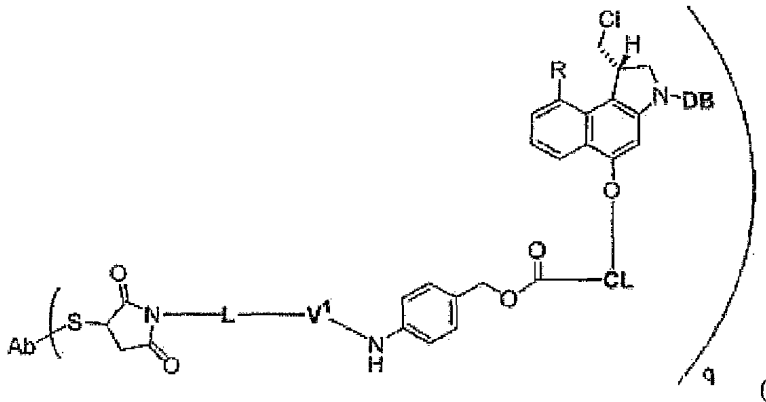
Hydrophobic interaction chromatography of ADCs in accordance with the method of the present invention makes use of differences in hydrophobic properties of non-conjugated antibodies, antibodies loaded with up to 8 linker-drugs, non-conjugated linker-drug and impurities, i.e., aggregates, in order to achieve separation and isolation of a purified mixture of Cys-linked ADCs. The more hydrophobic is the antibody, ADC, linker-drug or impurity, the stronger it will interact with the column packing material.

In accordance with the method of the present invention, the hydrophobicity of the desired ADC comprised in the mixture of cysteine-linked antibody-drug conjugates is measured by determining the retention time on an analytical HIC column relative to a reference, i.e., the retention time of the commercially available mAb trastuzumab (Herceptin®, Roche/ Genentech). To measure the hydrophobicity, an ADC sample is prepared having a final concentration of 1 mg/mL of cysteine-linked antibody-drug conjugates in 0.8 M ammonium sulphate and the analytical HIC column used is a TSKgel Butyl-NPR column (Tosoh Bioscience). The ADC sample is eluted using a linear gradient from 100% Buffer C (25 mM sodium phosphate, 1.5 M ammonium sulphate, pH 6.95) to 100% Buffer D (25 mM sodium phosphate, pH 6.95, 20% isopropanol) at 0.4 ml/min over 20 minutes and the retention time of the DAR2 species in the ADC sample is measured at 214 nm absorbance relative to trastuzumab (Herceptin®).

In accordance with the method of the present invention, using the analytical HIC column and method described in the previous paragraph, the DAR2 Cys-linked ADC species

has a relative hydrophobicity in the range of 0.1-0.6, particularly 0.2-0.5, more particularly 0.2-0.45, trastuzumab (Herceptin®) having a retention time (Rt) of 6.7 minutes.

The method according to the present invention is particularly suitable for the purification of a mixture of cysteine-linked antibody-drug conjugates of the formula (I)

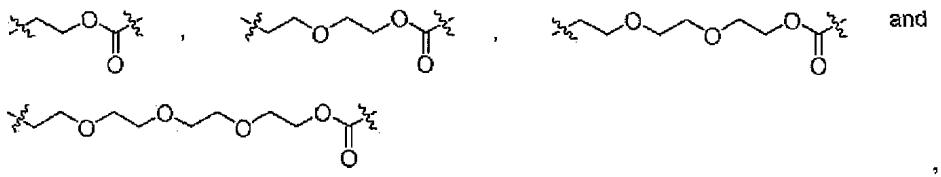


(I)

wherein

Ab is an antibody,

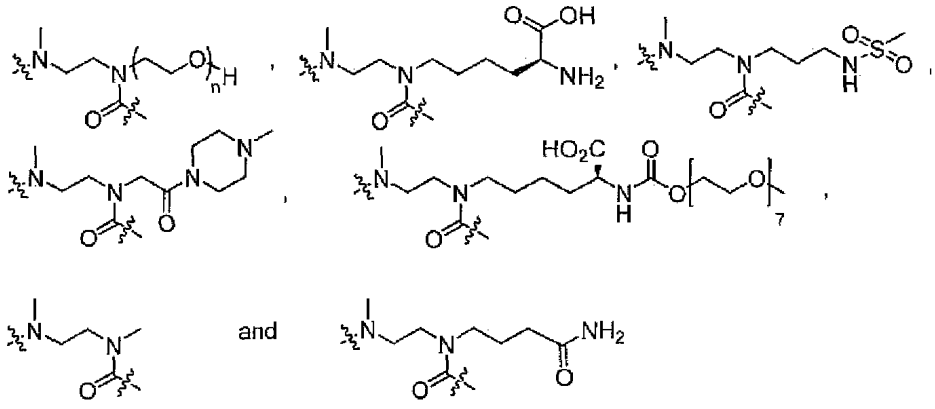
L is a linking group selected from



,

V^1 is a conditionally-cleavable dipeptide of natural and/or unnatural amino acids,

CL is a cyclization linker selected from

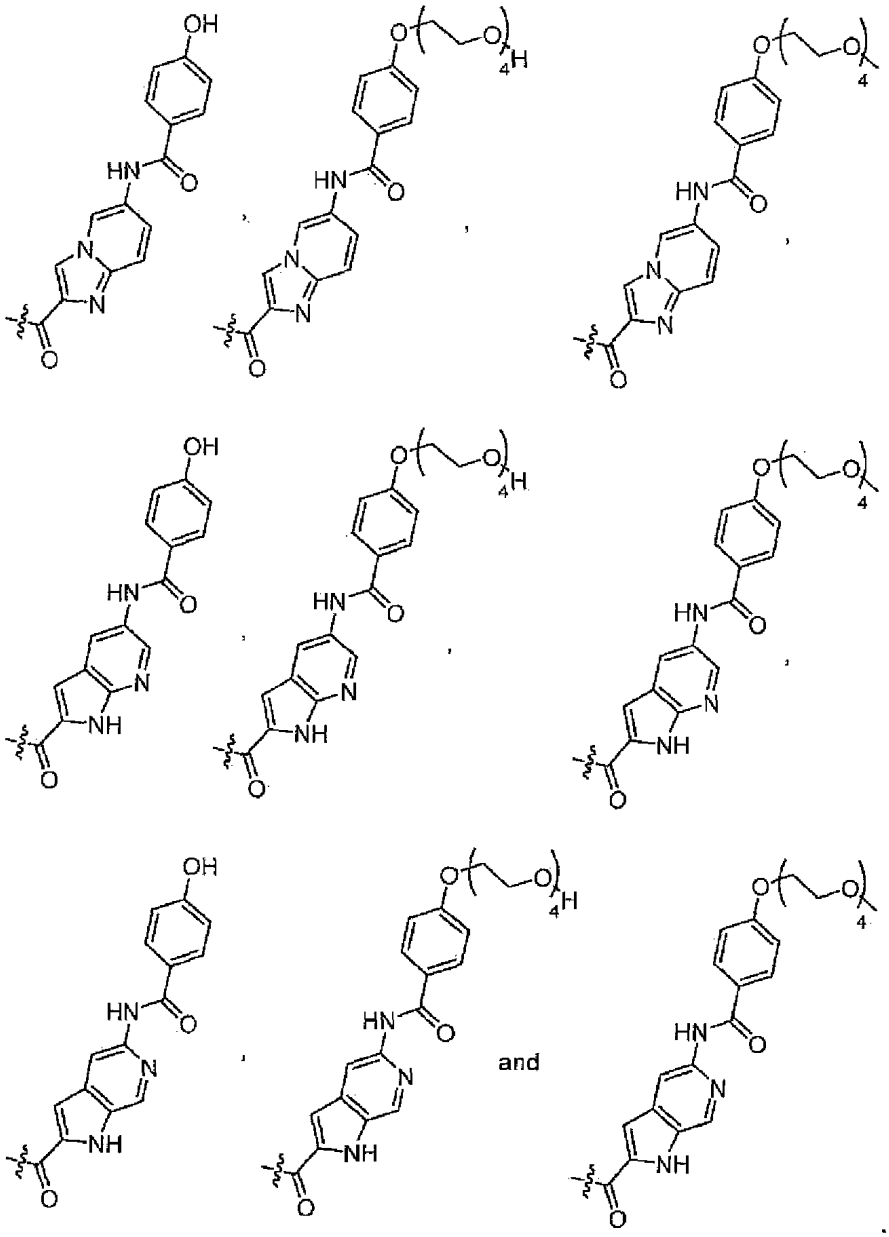


wherein n is an integer of from 1 to 16,

R is selected from H, CH_3, CH_2CH_3, OCH_3, OCH_2CH_3, CF_3, OCF_3, Cl, F,

q ranges from 0 to 8, and

DB is a DNA binding moiety selected from



and

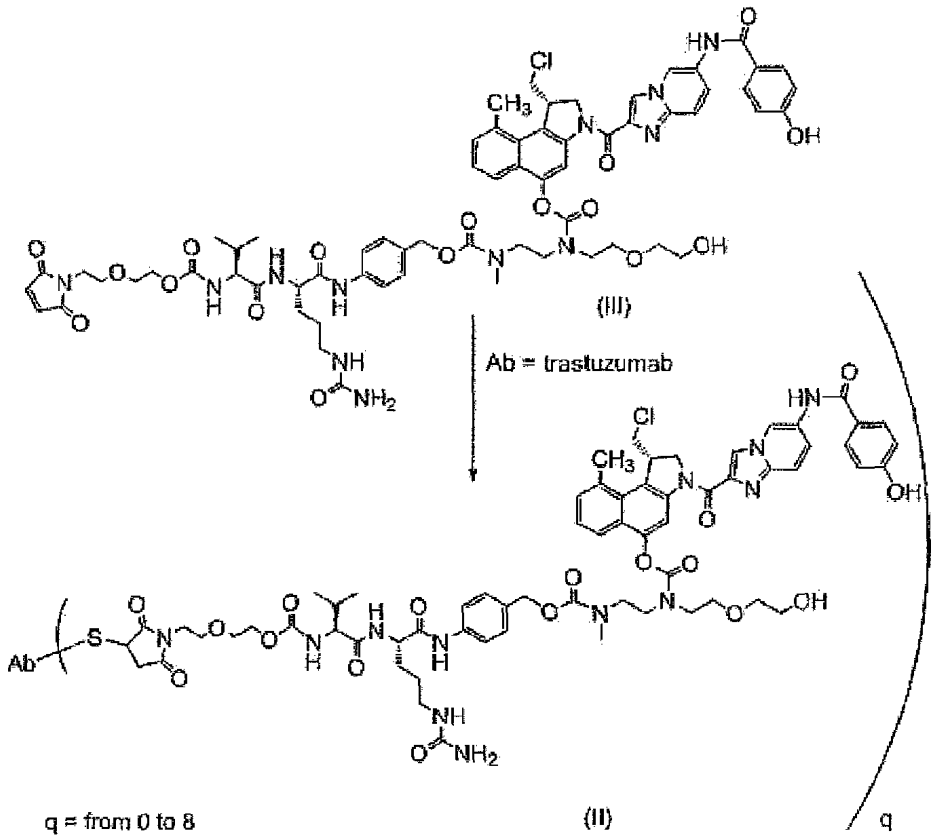
Such mixtures of Cys-linked ADCs have been described in detail in WO2010/062171 and WO2011/133039 of Applicant.

In accordance with the method of the present invention, the conditionally-cleavable dipeptide of natural and/or unnatural amino acids advantageously is selected from the group consisting of phenylalanyllysine, valyllysine, valylalanine, alanyllysine, valylcitrulline, N-methylvallylcitrulline, phenylalanylcitrulline, isoleucylcitrulline, tryptophanyllysine, trypto-phanylcitrulline, phenylalanylarginine, phenylalanylalanine, phenylalanyl-N^9-tosylarginine, phenylalanyl-N^9-nitroarginine, leucyllysine, leucylcitrulline and phenylalanyl-O-benzoyl-threonine. Preferably, the dipeptide is phenylalanyllysine, valyllysine or valylcitrulline.

In accordance with the method of the present invention, the Ab is selected from the group consisting of an anti-CD19 antibody, an anti-CD22 antibody, an anti-CD30 antibody, an anti-CD33 antibody, an anti-CD56 antibody, an anti-CD70 antibody, an anti-CD74 antibody, an anti-CD138 antibody, an anti-CLL-1 antibody, an anti-5T4 antibody, an anti-CD303 antibody, an anti-Tag 72 antibody, an anti-Lewis A like carbohydrate antibody, an anti-EphB3 antibody, an anti-HMW-MAA antibody, an anti-CD38 antibody, an anti-Cripto antibody, an anti-EphA2 antibody, an anti-GPNMB antibody, an anti-integrin antibody, an anti-MN antibody, an anti-HER2 antibody, an anti-PSMA antibody, an anti-EGFR antibody, an anti-CD203c antibody, an anti-SLC44A4 antibody, an anti-Nectin-4 antibody, an anti-mesothelin antibody, an anti-CD44 antibody, an anti-CD79 antibody, an anti-FcRL5 antibody, an anti-MUC16 antibody, an anti-NaPi2b antibody, an anti-STEAP-1 antibody, an anti-ETBR antibody, an anti-TF antibody, an anti-MUC1 antibody, anti-HGFR antibody, an anti-CD37 antibody, an anti-FOLR1 antibody, an anti-CEACAM antibody, an anti-TROP2 antibody, an anti-GCC antibody, an anti-Lewis Y antibody, an anti-LIV1 antibody, an anti-DLL3 antibody, and an anti-EPCAM antibody. The antibody preferably is a monoclonal antibody (mAb).

In accordance with the method of the present invention, the Ab, or preferably mAb, is an anti-HER2 antibody. More preferably, the antibody is an anti-HER2 monoclonal antibody, particularly trastuzumab or a biosimilar thereof.

In a specific embodiment of the method of the present invention, a mixture of Cys-linked ADCs according to formula (II) is prepared by using the antibody trastuzumab or a biosimilar thereof, which antibody is reduced with tris(2-carboxyethyl)phosphine (TCEP, 1.1 molar equivalents per mole antibody) and is reacted with the linker-drug of formula (III) (1.3 molar equivalents per free thiol group). The conjugation typically is carried out in N,N-dimethylacetamide (DMAc) or dimethyl sulfoxide (DMSO), preferably in DMAc.



In certain embodiments of the method of the present invention, the conjugation reaction mixture is treated with an N-acetyl cysteine stock solution (1 molar equivalent per linker-drug conjugate) to block the reactive groups of non-conjugated linker-drug of formula (III).

In certain embodiments of the method of the present invention, the conjugation reaction mixture is subjected to a filtration step to remove insoluble excess of linker-drug of formula (III), Removing excess linker-drug before loading the reaction mixture onto the column in-

creases the capacity of the column. Filters well known to those skilled in the art can be used. Typically, the filtration step involves use of a prefilter followed by a filter with an absolute pore size rating. Suitable prefilters are depth filters containing activated carbon. Preferred are filters such as ZetaCarbon SLP (3M).

Suitable absolute pore size filters are made of polyether sulphone (PES), cellulose acetate (CA) or polyvinylidene difluoride (PVDF). Preferred filters are PVDF or PES filters, typically with an absolute pore size of 0.2 μm.

In certain embodiments of the method of the present invention, the conjugation reaction mixture is prepared for HIC column purification by using sodium phosphate and ammonium sulphate, adjusted to a final concentration of 20-30 mM of sodium phosphate and 0.55-0.65 M ammonium sulphate at pH 6.0-6.5 (Buffer A).

In alternative embodiments of the method of the present invention, the conjugation reaction mixture is prepared for HIC column purification by using sodium acetate and ammonium sulphate, adjusted to a final concentration of 20-30 mM of sodium acetate and 0.55-0.9 M ammonium sulphate at pH 5.0-5.5 (Buffer A).

In a specific embodiment of the method of the present invention, the method involves the use of a HIC column (8cm x 20cm, Butyl Sepharose 4 Fast Flow), which is first equilibrated with three column volumes of Buffer A (20-30 mM sodium phosphate, 0.55-0.65 M ammonium sulphate, pH 6.0-6.5) at a flow rate of 100 cm/h, followed by loading onto the column of the conjugation reaction mixture in Buffer A (step b) and collecting a flow-through fraction containing non-conjugated antibody (step c).

Step d involves washing the HIC column with three column volumes of the same Buffer A (20-30 mM sodium phosphate, 0.55-0.65 M ammonium sulphate, pH 6.0-6.5) at a flow rate of 100 cm/h while collecting the flow-through fraction, thereby removing the residual amounts of non-conjugated antibody.

Step e involves eluting the HIC column with three column volumes of Buffer B (20-30 mM sodium phosphate, 45-55 mM ammonium sulphate, pH 6.0-6.5) at a flow rate of 100 cm/h to obtain the purified mixture of Cys-linked ADCs. Said eluting can be performed either in a regular mode or in a reverse mode (as explained hereinabove).

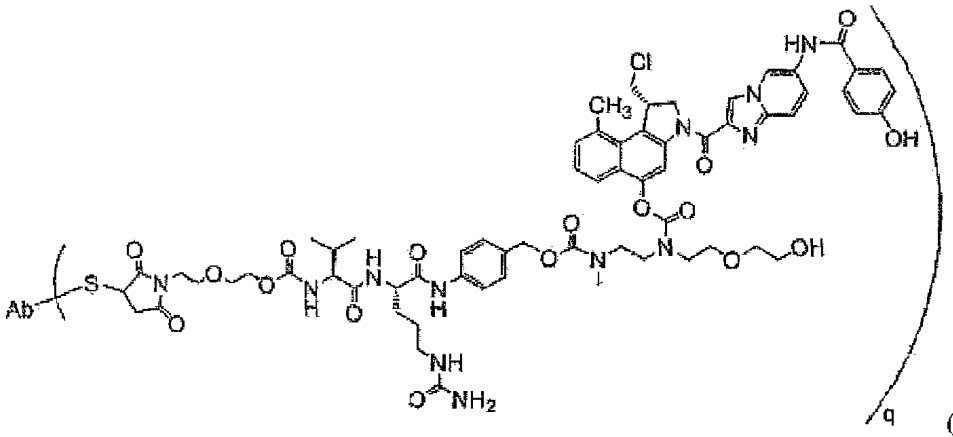
In another specific embodiment of the method of the present invention, the method involves the use of a HIC column (1cm x 20cm, Toyopearl PPG-600M), which is first equilibrated with three column volumes of Buffer A (20-30 mM sodium acetate, 0.55-0.9 M ammonium sulphate, pH 5.0-5.5) at a flow rate of 100 cm/h, followed by loading onto the column of the conjugation reaction mixture in Buffer A (step b) and collecting a flow-through fraction containing non-conjugated antibody (step c).

After loading, the HIC column is washed with three column volumes of the same Buffer A (20-30 mM sodium acetate, 0.55-0.9 M ammonium sulphate, pH 5.0-5.5) at a flow rate of 100 cm/h while collecting the flow-through fraction, thereby removing the residual amounts of non-conjugated antibody.

Step e involves eluting the HIC column with three column volumes of Buffer B (20-30 mM sodium acetate, pH 5.0-5.5) at a flow rate of 50-100 cm/h to obtain the purified mixture of Cys-linked ADCs. Said eluting can be performed either in a regular mode or in a reverse mode (as explained hereinabove).

As a result, the mixture of Cys-linked ADCs is purified to predominantly give the desired DAR2 and DAR4 species. Under the above conditions, most of the DAR6 and DAR8 species, the non-conjugated linker-drug as well as any aggregate impurities remain on the HIC column. By washing the HIC column with water-for-injection (WFI) the DAR6 and DAR8 species as well as the non-conjugated linker-drug can be eluted from the column.

The method according to the present invention is particularly suitable for the purification of a mixture of cysteine-linked antibody-drug conjugates of the formula (II)



(II)

wherein

Ab is trastuzumab and

q ranges from 0 to 8.

As a result of using the method for purifying a mixture of Cys-linked ADCs in accordance with the present invention, notably removing non-conjugated antibody from said mixture of ADCs, the average DAR increases. For example, as shown below in Example 3, the average DAR of a Cys-linked ADC compound according to formula (II) is increased from 1.75 to 2.5 after HIC purification.

After HIC purification, the buffer of the purified Cys-linked ADC typically is changed into a lyophilization buffer and subsequently the Cys-linked ADC is freeze-dried to give a lyophilized cake using conventional methods and equipment.

EXAMPLES

Example 1 - Preparation of the linker-drug solution of compound of formula (III)

In the protective environment of an isolator (glove box), a sufficient amount of solid of the compound of formula (III) was weighed into a bottle. The solid was dissolved in 100% DMAc to a concentration of approx. 20 mM. Then, the bottle was taken out of the isolator and stored at room temperature, but protected from light, in a fume hood.

After determining the exact concentration, the linker-drug solution was diluted to 40 mM.

Example 2 - Conjugation of linker-drug with trastuzumab

The anti-HER2 monoclonal antibody (mAb) trastuzumab was conjugated to the linker-drug of formula (III) giving a mixture of cysteine-linked antibody-drug conjugates of formula (II).

All handlings were performed under continuous stirring in a fume hood.

Immediately prior to conjugation, a solution of 60 mg/mL trastuzumab in 4.2 mM histidine, 50 mM trehalose, 0.01% polysorbate 20, pH 6 was mixed 2:1 with reduction buffer (4.2 mM histidine, 50 mM trehalose, 3 mM EDTA (ethylenediaminetetraacetic acid) and 1 mM TCEP, pH 6). TCEP is the reducing agent and is added in a molar ratio of 1.15 molar equivalents for 1 equivalent of trastuzumab to generate 2 free thiol groups per mAb. After incubation at room temperature for 60 min, N,N-dimethylacetamide (DMAc) solution (100%) and linker-drug of formula (III) (10 mM in DMAc, 2.2 equivalents with respect to mAb) were added such that the final concentration of DMAc was 2.5% v/v.

After overnight conjugation, the mixture was filtered through an activated carbon filter (ZetaCarbon SLP, 3M) followed by a 0.2 µm polyether sulfone (PES) filter to remove the insoluble excess of linker-drug of formula (III).

Figure 2A and 3A show the chromatogram of the obtained conjugation reaction mixture of two different batches on an analytical HIC column (described herein below). No DAR8 was detectable. The average DAR was calculated to be 1.75.

Example 3 - Purification using HIC

All chromatographic steps were performed at room temperature.

The conjugation reaction mixture obtained above was prepared for HIC column purification by mixing with a buffer of sodium phosphate (84 mM) and ammonium sulphate (2.21 M) in a ratio of 1 volume of buffer to 2 volumes of conjugation reaction mixture to a final concentration of sodium phosphate (26 mM) and ammonium sulphate (0.62 M) at pH 6.5.

A preparative 8cm x 20cm column was packed with Butyl Sepharose 4 Fast Flow (GE Healthcare). The column was equilibrated with 3 column volumes of Buffer A (26 mM sodium phosphate, 0.62 M ammonium sulphate, pH 6.5) at a flow rate of 100 cm/h. The conjugation reaction mixture was loaded onto the column up to 10 g/L column packing material/resin. The flow rate was set at 100 cm/h. Under these conditions, the non-conjugated antibody (i.e., trastuzumab) did not bind to the column/flowed through and was further washed off the column with 3 column volumes of Buffer A (26 mM sodium phosphate, 0.62 M ammonium sulphate, pH 6.5) at a flow rate of 100 cm/h. The flow-through fraction of loading and washing was collected and combined. Elution of the DAR2 and DAR4 species of cysteine-linked antibody-drug conjugates was realized by eluting with 3 column volumes of Buffer B (25 mM sodium phosphate, 50 mM ammonium sulphate, pH 6.2) at a flow rate of 100 cm/h. Under these conditions, any left non-conjugated linker-drug and most of the DAR6 cysteine-linked antibody-drug conjugates remained on the column. Washing the column with 2 column volumes of Water for Injection (WFI) at a flow rate of 100 cm/h eluted any left non-conjugated linker-drug and most of the DAR6 cysteine-linked antibody-drug conjugates.

Figure 2B shows the chromatogram of the conjugation reaction mixture on an analytical HIC column (described herein below) after HIC purification on a preparative scale. No DAR0 was detectable. The average DAR was calculated to be 2.50.

Example 4 - Alternative purification using HIC

All chromatographic steps were performed at room temperature.

A separate batch of a conjugation reaction mixture as obtained above was prepared for HIC column purification by mixing with a buffer of sodium acetate (75 mM) and ammonium sulphate (2.4 M) in a ratio of 1 volume of buffer to 2 volumes of conjugation reaction mixture to a final concentration of sodium acetate (25 mM) and ammonium sulphate (0.8 M) at pH 5.3.

A preparative 1cm x 20cm column was packed with Toyopearl PPG-600M (Tosoh Bioscience). The column was equilibrated with 3 column volumes of Buffer A (25 mM sodium acetate, 0.8 M ammonium sulphate, pH 5.3) at a flow rate of 100 cm/h. The conjugation reaction mixture was loaded onto the column up to 35 g/L of column packing material/resin. The flow rate was set at 100 cm/h. Under these conditions, the non-conjugated antibody (i.e., trastuzumab) did not bind to the column/flowed through, and was further washed off the column with 3.5 column volumes of Buffer A (25 mM sodium acetate, 0.8 M ammonium sulphate, pH 5.3) at a flow rate of 100 cm/h. The flow-through fraction of loading and washing was collected and combined. Elution of the DAR2 and DAR4 species of cysteine-linked antibody-drug conjugates was realized by eluting with 3.5 column volumes of Buffer B (25 mM sodium acetate, pH 5.3) at a flow rate of 100 cm/h. Under these conditions, any left non-conjugated linker-drug and most of the DAR6 cysteine-linked antibody-drug conjugates remained on the column. Washing the column with 2 column volumes of 40% isopropanol at a flow rate of 100 cm/h eluted any left non-conjugated linker-drug and most of the DAR6 cysteine-linked antibody-drug conjugates.

Figure 3B shows the chromatogram of the conjugation reaction mixture on an analytical HIC column (described herein below) after HIC purification on a preparative scale. No DAR0 was detectable. The average DAR was calculated to be 2.80.

Example 5 - Analysis using analytical HIC

The analysis of cysteine-linked antibody-drug conjugates was performed by analytical hydrophobic interaction chromatography (HIC). The sample was prepared by diluting 10 µL of cysteine-linked antibody-drug conjugate with 90 µL 0.89 M aqueous ammonium sulphate solution resulting in a final concentration of 1 mg/mL of cysteine-linked antibody-drug conjugate in 0.8 M ammonium sulphate. 10 µL of the sample was injected onto a TSKgel Butyl-NPR column (Tosoh Bioscience). The elution method consisted of a linear gradient from 100% Buffer C (25 mM sodium phosphate, 1.5 M ammonium sulphate, pH 6.95) to 100% of Buffer D (25 mM sodium phosphate, pH 6.95, 20% isopropanol) at 0.4 ml/min over 20 minutes. A Waters Acquity H-Class UPLC system equipped with PDA-detector and Empower software was used. Absorbance was measured at 214 nm and the retention time of cysteine-linked antibody-drug conjugates was determined.

The same analytical method was applied on a sample of trastuzumab/Herceptin®, which sample was prepared as described above and of which sample the retention time was measured at 214 nm.

Example 6 - Determination of relative hydrophobicity

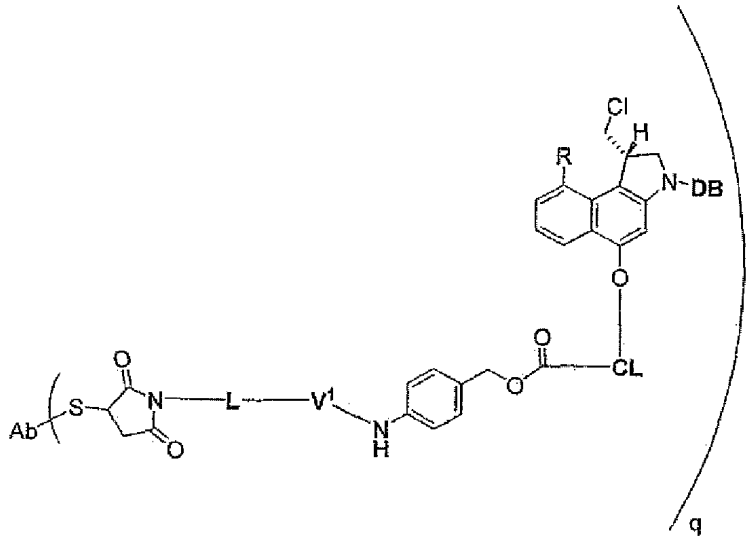
The relative hydrophobicity of a DAR2 cysteine-linked antibody-drug conjugate species was calculated using the retention time (Rt) of said DAR2 species in the mixture of Cys-linked ADCs and the retention time of trastuzumab/ Herceptin® using the following formula:

[Rt(DAR2)-Rt(trastuzumab/ Herceptin®)]/Rt(trastuzumab/Herceptin®).

The DAR2 species of the cysteine-linked antibody-drug conjugate of formula (II) showed a retention time of 9.6 minutes and a relative hydrophobicity of 0.4 on the analytical HIC column described above, when the retention time of trastuzumab/Herceptin® was 6.7 minutes.

CLAIMS

1. A method for purifying a mixture of cysteine-linked antibody-drug conjugates of formula (I)

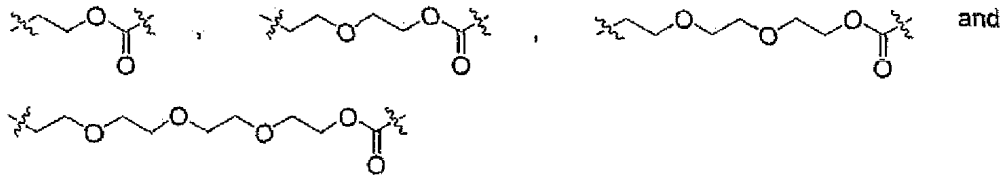


(I),

wherein

Ab is an antibody,

L is a linking group selected from

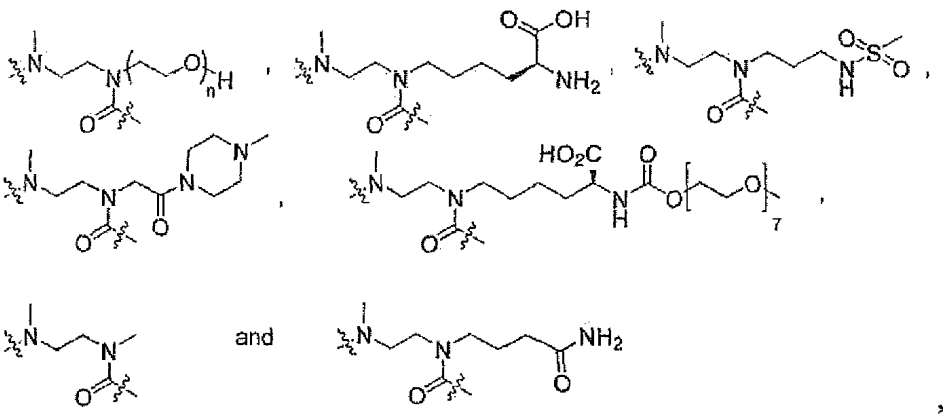


and

,

V^1 is a conditionally-cleavable dipeptide of natural and/or unnatural amino acids,

CL is a cyclization linker selected from

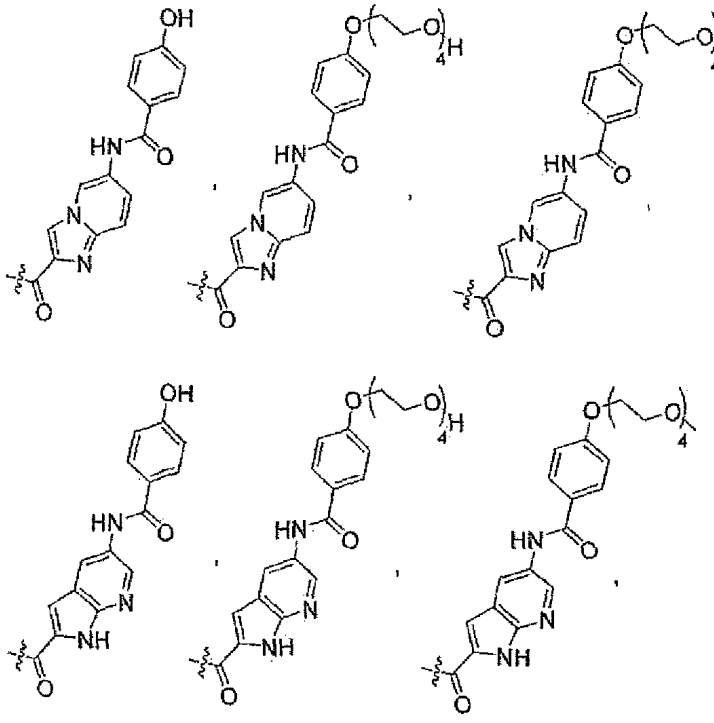


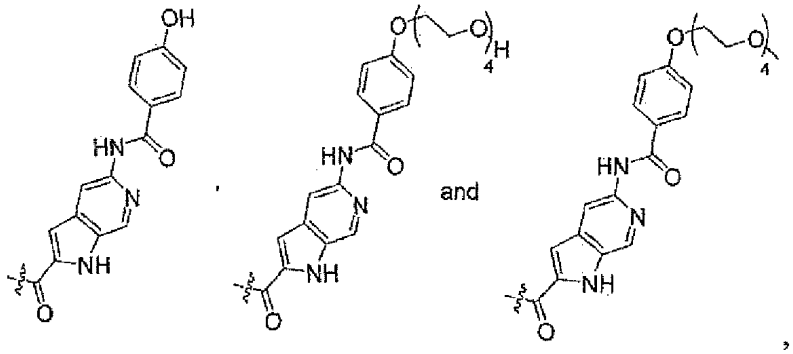
wherein n is an integer of from 1 to 16,

R is selected from H, CH_3, CH_2CH_3, OCH_3, OCH_2CH_3, CF_3, OCF_3, Cl, F,

q ranges from 0 to 8, and

DB is a DNA binding moiety selected from





wherein the amount of non-conjugated antibody is in the range of 10-40% by weight comprising:

a. providing the mixture in a 0.2-1.5 M aqueous salt solution;

b. loading said solution onto a preparative hydrophobic interaction chromatography column;

c. collecting a flow-through fraction that contains non-conjugated antibody;

d. washing said column with a 0.2-1.5 M aqueous salt solution while collecting the flow-through fraction; and

e. eluting said column with a 0-100 mM aqueous salt solution to obtain a purified mixture of cysteine-linked antibody-drug conjugates;

wherein no added organic solvent is used in either step a, b, d or e.

2. The method according to claim 1, wherein said column is packed with Fractogel EMD propyl, Fractrogel EMD phenyl, Butyl-S sepharose, Octyl Sepharose, Capto Octyl, Capto Butyl, Capto Phenyl ImpRes, Capto Butyl ImpRes, Toyopearl PPG-600M, Toyopearl Hexyl-650, Toyopearl Butyl-650, Toyopearl Phenyl-650, Toyopearl Ether-650, Macroprep t-Butyl, Macroprep phenyl, Cellufine Butyl, Cellufine Phenyl or Poros HP2.

3. The method according to claim 1 or 2, wherein said column has a diameter in the range of 4.0-2,000 mm, preferably 15-2,000 mm.

4. The method according to any one of claims 1 to 3, wherein the column loading is in the range of 5-50 g/L, preferably 5-40 g/L of column packing material.

5. The method according to any one of claims 1 to 4, wherein the column packing material has an average particle size in the range of 30-180 μm.

6. The method according to any one of claims 1 to 5, wherein the salt of the aqueous salt solution in step a, d or e is selected from the group consisting of potassium thiocyanate, sodium chloride, potassium chloride, ammonium chloride, sodium sulphate, potassium sulphate and ammonium sulphate, preferably the salt is sodium chloride or ammonium sulphate.

7. The method according to any one of claims 1 to 6, wherein the aqueous salt solution in step a, d or e further contains a buffer.

8. The method according to claim 7, wherein the buffer is selected from the group consisting of sodium phosphate, potassium phosphate, ammonium phosphate, sodium acetate, potassium acetate, sodium citrate, potassium citrate, ammonium citrate and mixtures thereof, preferably the buffer is sodium phosphate or sodium acetate.

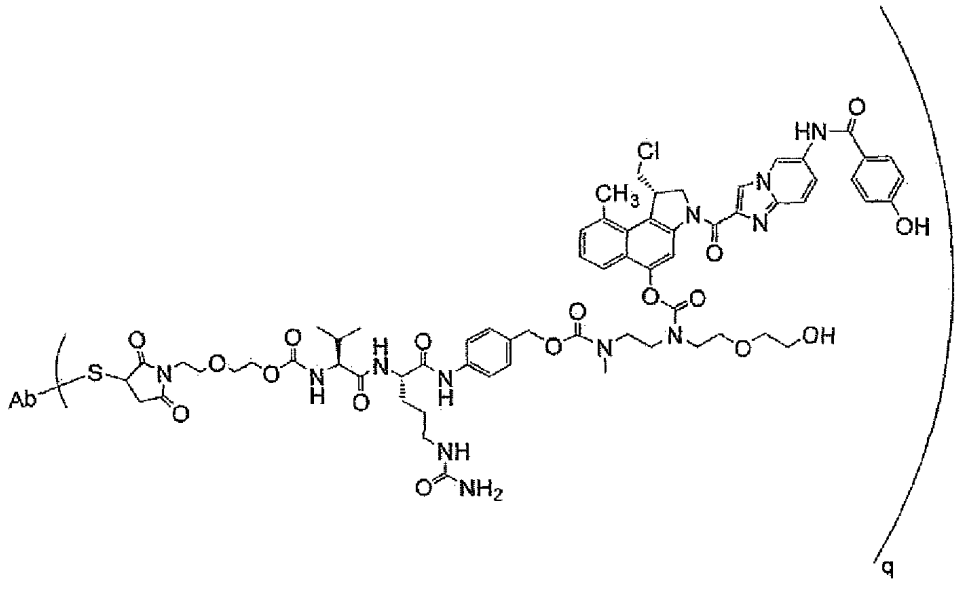
9. The method according to claim 7 or 8, wherein said aqueous salt solution is buffered to a pH of from about 4 to about 8.

10. The method according to any one of claims 1 to 9, wherein the elution in step e is performed in a reverse mode.

11. The method according to any one of claims 1 to 10, wherein the Ab is selected from the group consisting of an anti-CD19 antibody, an anti-CD22 antibody, an anti-CD30 antibody, an anti-CD33 antibody, an anti-CD56 antibody, an anti-CD70 antibody, an anti-CD74 antibody, an anti-CD138 antibody, an anti-CLL-1 antibody, an anti-5T4 antibody, an anti-CD303 antibody, an anti-Tag 72 antibody, an anti-Lewis A like carbohydrate antibody, an anti-EphB3 antibody, an anti-HMW-MAA antibody, an anti-

CD38 antibody, an anti-Cripto antibody, an anti-EphA2 antibody, an anti-GPNMB antibody, an anti-integrin antibody, an anti-MN antibody, an anti-HER2 antibody, an anti-PSMA antibody, an anti-EGFR antibody, an anti-CD203c antibody, an anti-SLC44A4 antibody, an anti-Nectin-4 antibody, an anti-mesothelin antibody, an anti-CD44 antibody, an anti-CD79 antibody, an anti-FcRL5 antibody, an anti-MUC16 antibody, an anti-NaPi2b antibody, an anti-STEAP-1 antibody, an anti-ETBR antibody, an anti-TF antibody, an anti-MUC1 antibody, anti-HGFR antibody, an anti-CD37 antibody, an anti-FOLR1 antibody, an anti-CEACAM antibody, an anti-TROP2 antibody, an anti-GCC antibody, an anti-Lewis Y antibody, an anti-LIV1 antibody, an anti-DLL3 antibody, and an anti-EPCAM antibody.

12. The method according to any one of claims 1 to 11, wherein the mixture of cysteine-linked antibody-drug conjugates is of the formula (II)



(II)

wherein

Ab is trastuzumab and

q ranges from 0 to 8.

13. The method according to claim 12, wherein the purified mixture of said cysteine-linked antibody-drug conjugates of the formula (II) has an average drug-to-antibody ratio (DAR) of from 2.6 to 2.9.

14. The method according to claim 13, wherein the average DAR is 2.80.