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DESCRIPTION

FIELD OF THE INVENTION

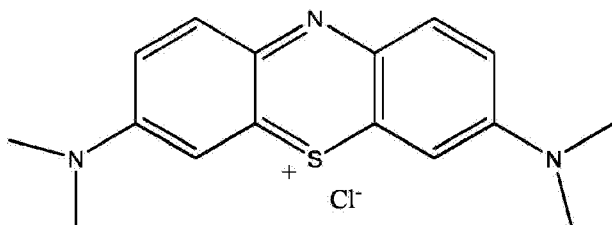
[0001] The invention relates to the purification of diaminophenothiazinium compounds and novel diaminophenothiazinium compounds formed during the purification process. In particular, the invention relates, but is not limited, to the purification of methylene blue and closely related compounds using novel intermediate complexes.

BACKGROUND TO THE INVENTION

[0002] Any reference to background art herein is not to be construed as an admission that such art constitutes common general knowledge in Australia or elsewhere.

[0003] The diaminophenothiazinium dyes are well known. In particular, the medical dye or antidote, methylthioninium chloride (3, 7-bis (dimethylamino) phenothiazin-5-ylum chloride), known commonly as "methylene blue", has in relatively recent times extended its use to a number of medical applications beyond its historic uses. Other common names for this dye include tetramethylthionine chloride, C.I.Solvent Blue, Swiss Blue, C.I.Basic Blue 8, Aniline Violet and Urolene Blue. It has the following structure (Formula I), although a person of skill in the art will appreciate the existence of certain resonance structures and tautomeric forms:

FORMULA I



[0004] Methylene blue has a long history of varied uses. Industrial applications have included its use as a colouring agent, redox indicator and dye, an immunological or microbiological stain, in photoelectronic imaging, as an environmental metal sequestrant, a leather dye and an antiseptic.

[0005] In the clinical field it has two primary established uses: firstly, as an antidote for methaemoglobinaemia, and occasionally for cyanide and carbon monoxide poisoning, and, secondly, as a chromodiagnostic or chromoendoscopic agent for a wide variety of clinical conditions, such as examining cellular dysplasia in for example Barret's Oesophagus and endoscopic polypectomy, and Fallopian tube patency and Fistula detection.

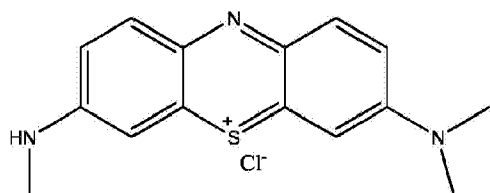
[0006] In 1891 Paul Ehrlich identified methylene blue as a treatment for Malaria. More recently it has been suggested that its use may be broadened to include treatment of tauopathies (or neurodegenerative diseases), viral infections, bipolar disorder and tracing of lymph nodes and lymphatic drainage. In dentistry, uses include finding small cracks in teeth and as a photodynamic dye for treating chronic peridontitis. Methylene blue is added to bone cements to provide discrimination between native and synthetic bone. It has also been used as an accelerant to harden bone cement, effectively increasing the speed at which the bone cement can be effectively applied. In species other than human, it has a wide range of uses, from treating "fin rot" in aquarium fish to methaemoglobinaemia in farm dogs caused by their inadvertent ingestion of toxic fox baits.

[0007] The original synthesis of methylene blue was developed in Germany in 1877 (German patent No.1886 to Badische Anilin-und Soda Fabrik) and since that time a number of other methodologies have been described or patented. A common thread to all the methodologies is the use of a range of metal catalysts including salts (or the metals themselves) of iron, manganese, copper, chromium (as chromate), aluminium and zinc, leaving a potential for metal ion contamination from the catalyst. Further metal contamination may come from the types of metal equipment used for the synthetic processes.

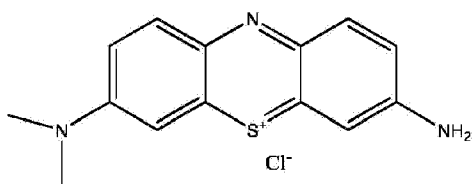
[0008] In addition to metal residues, the chemistry of methylene blue lends itself, during production, to the synthesis or inter-conversion of three other structurally and chemically similar organic entities. While these may have little effect on the industrial uses of methylene blue, and may also be used for similar cytological staining purposes, in clinical use these may be considered as undesired inclusions and are specifically cited in the United States Pharmacopoeia (USP) and British/European Pharmacopoeia (BP/EP) as contaminants.

[0009] These organic contaminants are collectively called "Azures" and come from some level of demethylation of the two dimethylamino groups at the 3 and 5 positions on the ring structure of methylene blue. Specifically they are: the trimethyl derivative known as Azure B (FORMULA II); the dimethyl derivative known as Azure A (FORMULA III) and monomethyl derivative known as Azure C (FORMULA IV).

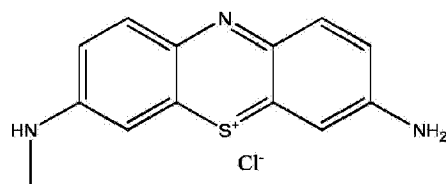
FORMULA II



FORMULA III



FORMULA IV



[0010] Due to the structural and chemical similarity of the Azures to methylene blue, these 'contaminants' are difficult to separate or remove from a mixture by standard means. It has also proven to be difficult to reduce their occurrence during synthesis of methylene blue. Nevertheless, numerous attempts have been made to remove or reduce the levels of Azures during the synthetic process, including re-purification by many means including recrystallisation of the final methylene blue product itself. Marshall and Lewis (1975) describe the purification of commercial methylene blue and Azure B by solvent extraction at a high pH of 9.5 with carbon tetrachloride and subsequent recrystallisation. They also describe metal ion removal by low temperature, low pH crystallisation. Lohr et al (1975) describe a purification process utilising column ion chromatography which is not practicable on a commercial scale.

[0011] More recently, in 2005 Storey et al (WO 2006/032879) described processes for the *de novo* manufacture and purification of methylene blue and derivatives thereof using a number of metal ions as catalysts within stepped processes (including chromate (IV) and copper (II) sulphate and iron (II) oxide) under controlled pH and temperature conditions. This is followed by washing/solvent extraction with organic solvents such as dichloromethane, 1,2-dichloroethane, chloroform, ethyl acetate, diethyl ether, chlorobenzene, petroleum ether, benzene, toluene and methyl acetate. Key steps in the final stage of the process include the addition of dimethyldithiocarbamate (DT), a sulphide, and a chloride salt such as sodium chloride, and carbonate such as sodium carbonate followed again by organic solvent washing and the addition of EDTA (ethylenediaminetetraacetic acid), followed again by organic solvent washing and recrystallisation and washing at a low pH in the presence of an organic solvent such as dichloromethane or tetrahydrofuran.

[0012] Further work by the same group (WO 2008/007074) describes purification of methylene blue by acyl derivatisation, at the N10 position (or a number of other organic derivatives including saturated aliphatic derivatives) and purification (with agents such as activated charcoal) and conversion by oxidation to the original methylene blue. The document also includes the concept of acylating a methylene blue precursor and purifying via the same processes.

[0013] A number of authors, in a similar fashion, have postulated that metal and organic residue removal may be achieved by further derivatisation of the methylene blue, post manufacture, so as to increase the chemical differences between the Azures and methylene blue and also to allow ease of metal residue removal. Buc et al 1959 (US Patent 2,909,520) described processes for the manufacture of acylated leuco methylene blue, in particular

benzoyl leuco methylene blue.

[0014] Gensler et al, 1966, describes the simple oxidative re-conversion of N-benzoyl leuco methylene blue to methylene blue. In fact all that was required was the presence of oxygen for an autoconversion.

[0015] Feraud et al (WO2008/006979) describe a method for industrial purification of methylene blue and other like compounds where both the Azures and metal levels are alleged to be reduced based on the formation of a large organic derivative of methylene blue by reaction to form a N-C bond at the N10 position of a diaminophenothiazinium compound.

[0016] This complex multi-step derivatisation of methylene blue (or a related derivative thereof) begins with reduction of the N followed by reaction of the resulting amine with certain derivative options. They then proceed to describe standard methods and/or concepts for purification of the complex organic derivative, including reduction of metal ion levels via filtration of the derivatised organic material through a support that retains metals, crystallisation from an appropriate solvent and other known methods. They use solvent washing or recrystallisation to reduce the Azure levels in this derivative. Again, the final process is reconversion or oxidation to methylene blue using quinones, nitric acid, perchloric acid iodine, hydrochloric acid, sulphuric acid, hydrogen peroxide or UV light, with a preference for 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ).

[0017] In summary, the levels of metals and/or organic impurities found within methylene blue samples is not unexpected when such historic synthetic approaches are considered, and may even be acceptable for the commercial product in use for many industrial and cytological purposes where these potential residues do not affect the use. However, with recent requirements for lower levels of these impurities in methylene blue used for pharmaceutical purposes, it has become necessary to address the removal of impurities in an efficient, simple and cost effective purification process.

OBJECT OF THE INVENTION

[0018] It is an aim of this invention to provide a purification method and intermediate which overcomes or ameliorates one or more of the disadvantages or problems described above, or which at least provides a useful alternative or simpler methodology.

[0019] Other preferred objects of the present invention will become apparent from the following description.

SUMMARY OF INVENTION

[0020] According to a first aspect of the invention, there is provided a process for the purification of a diaminophenothiazinium compound including the steps of:

1. (i) contacting the diaminophenothiazinium compound with a reducing agent at an acidic pH to form a protonated stabilised diaminophenothiazinium complex;
2. (ii) purifying the protonated stabilised diaminophenothiazinium complex and;
3. (iii) contacting the purified protonated stabilised diaminophenothiazinium complex with an oxidising agent to convert it back to the diaminophenothiazinium compound,

to thereby purify the diaminophenothiazinium compound.

[0021] The diaminophenothiazinium compound may be selected from the group consisting of methylene blue, Azure A, Azure B and Azure C.

[0022] In one embodiment, the contact with the reducing agent may form an N-10 protonated stabilised diaminophenothiazinium complex which is subsequently purified and oxidised back to the diaminophenothiazinium compound.

[0023] In one preferred embodiment of the first aspect, there is provided a process for the purification of methylene blue including the steps of:

1. (i) contacting the methylene blue with a reducing agent to form a stabilised protonated leucomethylene blue complex;
2. (ii) purifying the stabilised protonated leucomethylene blue complex; and
3. (iii) contacting the purified stabilised protonated leucomethylene blue complex with an oxidising agent to form methylene blue,

to thereby purify the methylene blue.

[0024] The reducing agent of the first aspect, and the preferred embodiment thereof, is a metal-free reducing agent.

[0025] In one embodiment, the reducing agent of the first aspect, and the preferred embodiment thereof, is selected from the group consisting of ascorbic acid, hydrogen, formic acid, oxalic acid, dithiothreitol, hydrazine, phosphites, hypophosphites and phosphorous acid.

[0026] Suitably, the reducing agent of the first aspect, and the preferred embodiment thereof, is ascorbic acid. Preferably it is L-ascorbic acid.

[0027] The diaminophenothiazinium compound or methylene blue is contacted with the reducing agent at an acidic pH. More preferably, the diaminophenothiazinium compound or methylene blue is contacted with the reducing agent at below pH 3. Even more preferably, the diaminophenothiazinium compound or methylene blue is contacted with the reducing agent at below pH 1.

[0028] Preferably, the reducing agent is in a solution made acidic with an acid chosen from a monoprotic or diprotic mineral acid.

[0029] Suitably, the acid is selected from the group consisting of hydrochloric, sulphuric, nitric, phosphoric, boric, hydrofluoric, hydrobromic and perchloric acids.

[0030] In one embodiment, the mineral acid is hydrochloric or sulphuric acid.

[0031] Suitably, the mineral acid is hydrochloric acid.

[0032] Preferably, the contacting with the reducing agent of the first aspect, and the preferred embodiment thereof, occurs in an organic solvent. Suitably, the organic solvent is a C1 to C8 alcohol or C1 to C4 alcohol. Preferably, the alcohol is methanol or ethanol.

[0033] The purification of the protonated stabilised diaminophenothiazinium complex (which in one embodiment may be an N-10 protonated stabilised diaminophenothiazinium complex) or stabilised protonated leucomethylene blue complex is preferably by recrystallisation.

[0034] In one embodiment, the recrystallisation is from a solvent selected from the group consisting of water, an alcohol and an ether.

[0035] If at least one of the recrystallisation solvents is an alcohol then it may be selected from the group consisting of C₁ to C₁₀ alcohols inclusive of methanol, ethanol, *n*-propanol and *iso*-propanol.

[0036] If at least one of the recrystallisation solvents is an ether it may be tetrahydrofuran or diethyl ether.

[0037] Suitably, the oxidising agent of the first aspect, and the preferred embodiment thereof, is a metal-free oxidising agent.

[0038] Preferred oxidising agents of the first aspect, and the preferred embodiment thereof, may be selected from the group consisting of oxygen, ozone, chlorine, fluorine, bromine, iodine, hydrogen peroxide, nitric acid and nitrate compounds, phosphoric acid, peroxydisulphuric acid, peroxymonosulphuric acid, sulphuric and sulphurous acids, chlorite, chlorate, perchlorate and other analogous halogen-containing compounds, hypochlorite and other hypohalite compounds, permanganates, nitrous oxide and quinones.

[0039] In one embodiment the oxidising agent of the first aspect, and the preferred embodiment thereof, is selected from the group consisting of oxygen and a quinone.

[0040] Preferably, the oxidising agent of the first aspect, and the preferred embodiment thereof, is 2, 3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) or, more preferably, *p*-benzoquinone.

[0041] The stabilised protonated leucomethylene blue complex may be a salt or other ion complex.

[0042] In one embodiment, the stabilised protonated leucomethylene blue complex may be a leucomethylene blue:ascorbate complex.

[0043] According to a second aspect of the disclosure there is provided a protonated stabilised diaminophenothiazinium:ascorbate complex.

[0044] In one embodiment of the second aspect, the protonated stabilised diaminophenothiazinium complex is a stabilised protonated leucomethylene blue:ascorbate complex.

[0045] In one embodiment, the protonated stabilised diaminophenothiazinium complex is an N-10 protonated stabilised diaminophenothiazinium complex.

[0046] A third aspect of the disclosure resides in the protonated stabilised diaminophenothiazinium:ascorbate complex of the second aspect when produced by the process of contacting the diaminophenothiazinium compound with ascorbic acid to form the protonated stabilised diaminophenothiazinium:ascorbate complex.

[0047] In one embodiment of the third aspect, the protonated stabilised diaminophenothiazinium:ascorbate complex is an N-10 protonated stabilised diaminophenothiazinium:ascorbate complex.

[0048] In one embodiment of the third aspect, the protonated stabilised diaminophenothiazinium:ascorbate complex is the stabilised protonated leucomethylene blue:ascorbate complex of the second aspect when produced by the process of contacting methylene blue with ascorbic acid to form the stabilised protonated leucomethylene blue:ascorbate complex.

[0049] The various conditions for the process of the third aspect may be as described for the first aspect.

[0050] According to a fourth aspect of the disclosure there is provided a use of a protonated stabilised diaminophenothiazinium complex in the purification of a diaminophenothiazinium compound.

[0051] In one embodiment, the protonated stabilised diaminophenothiazinium complex is an N-10 protonated stabilised diaminophenothiazinium complex.

[0052] In one embodiment of the fourth aspect, the protonated stabilised diaminophenothiazinium complex is a stabilised protonated leucomethylene blue complex used

in the purification of methylene blue.

[0053] In one embodiment, the stabilised protonated leucomethylene blue complex is a stabilised protonated leucomethylene blue salt.

[0054] In one embodiment, the stabilised protonated leucomethylene blue complex is the stabilised protonated leucomethylene blue:ascorbate complex of the second aspect.

[0055] The various features and embodiments of the present invention, referred to in individual sections above apply, as appropriate, to other sections, *mutatis mutandis*. Consequently features specified in one section may be combined with features specified in other sections as appropriate.

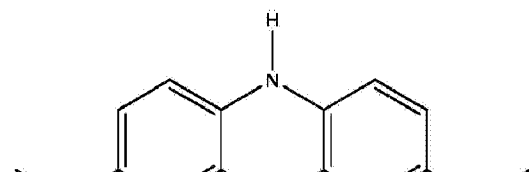
[0056] Further features and advantages of the present invention will become apparent from the following detailed description.

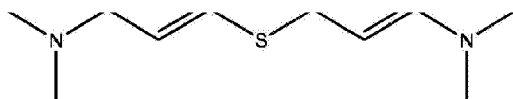
DETAILED DESCRIPTION

[0057] The present invention is predicated, at least in part, on the surprising finding that methylene blue can be simply and effectively purified by reducing a post-synthesis or commercially available sample to form a reduced stabilised protonated leucomethylene blue complex, which may be a salt or other ion complex, which can then be purified by, for example, recrystallisation before being allowed to oxidise back to methylene blue. Crystallisation of methylene blue itself, as a purification method to remove both Azure type impurities and metal contaminants, has proven to be ineffective on its own in achieving even close to pharmaceutical grade material and so it is particularly surprising that recrystallisation of the reduced leucomethylene blue form, even with the close structural similarities to the Azures still remaining, is an effective means for separation of that leucomethylene blue form from both the Azures and metal contaminants. A simple oxidation step then provides the methylene blue but in a highly purified form.

[0058] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as would be commonly understood by those of ordinary skill in the art to which this invention belongs.

[0059] As used herein, the term "leucomethylene blue" (also referred to herein as 'LMB') refers to the well known N-10 protonated reduced form of methylene blue which may also be referred to as N,N,N',N'-Tetramethyl-10H-phenothiazine-3,7-diamine, which has the chemical formula $C_{16}H_{19}N_3S$, CAS number 613-11-6, and which is shown in the structure below.





[0060] As used herein, the terms "complex", "complexes" and "complexed" refer generally to leucomethylene blue chemically associated with an ion or electrically neutral molecule. The complex may be a salt. While not wishing to bound to any particular theory, in a preferred embodiment it is believed the complex is a stabilised protonated leucomethylene blue associated with ascorbic acid which may be referred to as a stabilised protonated leucomethylene blue:ascorbate complex. The nature of the stabilised protonated leucomethylene blue complex may be affected by the process conditions used in the reduction step, including the pH and ionic strength which may influence salt formation.

[0061] As used herein, the terms "recrystallisation" and "recrystallising" and "crystallisation" and "crystallising" are used interchangeably to refer to the process of dissolving and subsequently crystallising from solution a substance such as methylene blue or, particularly, the stabilised protonated leucomethylene blue complex formed during the reduction step in the present inventive process.

[0062] The terms "diaminophenothiazinium compound" and "methylene blue", being a preferred embodiment of the diaminophenothiazinium compound, may be used interchangeably herein when describing elements of the purification process.

[0063] According to a first aspect of the invention, there is provided a process for the purification of a diaminophenothiazinium compound including the steps of:

1. (i) contacting the diaminophenothiazinium compound with a metal-free reducing agent at an acidic pH to form a protonated stabilised diaminophenothiazinium complex;
2. (ii) purifying the protonated stabilised diaminophenothiazinium complex; and
3. (iii) contacting the purified protonated stabilised diaminophenothiazinium complex with an oxidising agent to convert it back to the diaminophenothiazinium compound,

to thereby purify the diaminophenothiazinium compound.

[0064] The diaminophenothiazinium compound may be selected from the group consisting of methylene blue, Azure A, Azure B and Azure C.

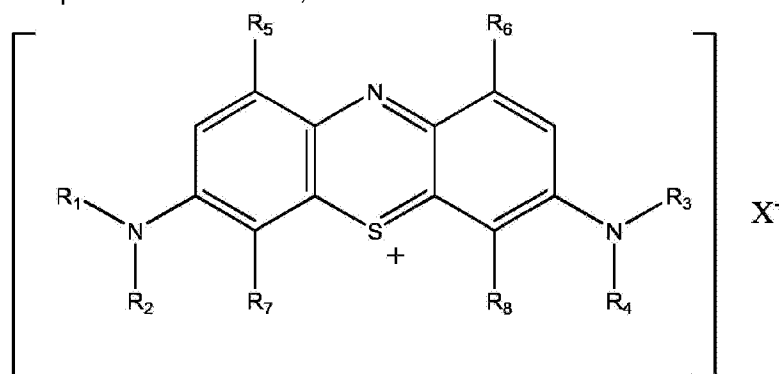
[0065] In one embodiment, the contact with the reducing agent may form an N-10 protonated stabilised diaminophenothiazinium complex which is subsequently purified and oxidised back to the diaminophenothiazinium compound. This is the case when the compound is methylene blue and may be the case for most diaminophenothiazinium compounds with substituents which allow protonation of the N-10 nitrogen upon contact with the reducing agent.

[0066] It will be appreciated that the diaminophenothiazinium compound which is purified and

that which is the final desired compound may be different. For example, it may be desirable to purify a diaminophenothiazinium compound such as Azure B and once purification is complete to convert this by methylation into methylene blue. Thus, the first aspect explicitly considers interconversion between different diaminophenothiazinium compounds either subsequent to step (iii) or between steps (i) and (ii) or between steps (ii) and (iii).

[0067] Therefore, in one embodiment, the process may further include the step of converting the diaminophenothiazinium compound to a second, related diaminophenothiazinium compound.

[0068] In one embodiment, the diaminophenothiazinium compound of the first aspect is a compound of formula V, below:



Formula V

wherein R_1 , R_2 , R_3 and R_4 are independently selected from hydrogen, C_1 - C_6 alkyl and C_1 - C_6 alkenyl wherein each alkyl or alkenyl group may be substituted with hydroxy, halo or alkoxy;

R_5 , R_6 , R_7 and R_8 are independently selected from hydrogen, nitro, halo, haloalkyl, C_1 - C_6 alkyl and C_1 - C_6 alkenyl; and

X is an anionic counterion.

[0069] In one embodiment, the alkyl or alkenyl group, if substituted, is substituted with chloro, fluoro, bromo or iodo.

[0070] Preferably, R_1 , R_2 , R_3 and R_4 are independently selected from methyl, ethyl, propyl (including n-propyl and isopropyl), butyl (including n-butyl, isobutyl, sec-butyl and tert-butyl), pentyl, iso-amyl and hexyl.

[0071] Preferably, R_5 , R_6 , R_7 and R_8 are independently selected from hydrogen, nitro, haloalkyl and C_1 - C_4 alkyl.

[0072] Preferably, X is selected from the group consisting of a halide anion, or the conjugate

base of a mineral acid such as sulphuric, nitric, hydrochloric and phosphoric acids.

[0073] It will be appreciated, and would be understood by one of skill in the art, that the structure encompassed by formula V may also be represented by a number of related resonance structure and tautomeric form representations. For example, a double bond may be not be next to the sulphur and may instead be represented as linking one of the nitrogens to the ring. In this instance the positive charge would not sit on the sulphur atom but rather would be represented on the doubly bonded nitrogen atom. All such resonance and tautomeric structures are explicitly considered to be encompassed by the structure of formula V.

[0074] In one preferred embodiment of the first aspect, there is provided a process for the purification of methylene blue including the steps of:

1. (i) contacting the methylene blue with a reducing agent to form a stabilised protonated leucomethylene blue complex;
2. (ii) purifying the stabilised protonated leucomethylene blue complex by recrystallisation; and
3. (iii) contacting the purified stabilised protonated leucomethylene blue complex with an oxidising agent to form methylene blue,

to thereby purify the methylene blue.

[0075] The reducing agent of the first aspect, and the preferred embodiment thereof, is a metal-free reducing agent.

[0076] Any reducing agent known in the art to have sufficient reducing power to reduce methylene blue to LMB may be suitable.

[0077] In one embodiment, the reducing agent is selected from the group consisting of ascorbic acid, hydrogen, formic acid, oxalic acid, dithiothreitol, hydrazine, phosphites, hypophosphites and phosphorous acid. Suitably, the ascorbic acid is L-ascorbic acid.

[0078] It is highly preferred that the reducing agent is ascorbic acid, most preferably L-ascorbic acid since it is postulated that reduction of methylene blue with L-ascorbic acid forms a protonated LMB:ascorbate complex which is particularly stable and which allows for purification by simple recrystallisation means.

[0079] The diaminophenothiazinium compound is contacted with the reducing agent at an acidic pH. More preferably, the diaminophenothiazinium compound is contacted with the reducing agent at below pH 3. Even more preferably, the diaminophenothiazinium compound is contacted with the reducing agent at below pH 1.

[0080] The reduced diaminophenothiazinium complex may be a salt. When the reducing agent is ascorbic acid and the diaminophenothiazinium compound is methylene blue then the reduced diaminophenothiazinium complex is a stabilised protonated leucomethylene

blue:ascorbate complex.

[0081] The purification of the reduced diaminophenothiazinium complex may be achieved by a range of techniques which are known in the art such as chromatography, for example through a column of silica or modified silica or using HPLC, ion-exchange techniques, filtration, washing, recrystallisation and others. However, in a highly preferred embodiment, the purification of the reduced diaminophenothiazinium complex is by recrystallisation.

[0082] Filtration may be used in addition to the recrystallisation, particularly to remove metal ions, and can be effected by filtration through metal binding filters, for example filtration supports including silica gel, activated charcoal, neutral, basic or acidic alumina gel, micro porous membranes, resins grafted with metal-capturing groups, and fibres grafted with metal-capturing groups. Metal ions can also be removed by passage through or over any known metal binding absorbent such as silica or diatomaceous earth or other commercial substrate manufactured for the purpose of binding or removing metals.

[0083] In one embodiment, the recrystallisation is from a solvent selected from the group consisting of water, an alcohol and an ether. It may be preferable that all three solvent classes are used, in varying degrees, for the recrystallisation of the LMB complex. In one embodiment, when all three classes of solvents are employed in the recrystallisation then they may be used in a ratio of water:alcohol:ether of 1:2-4:6-10, preferably about 1:3:8 on a volume basis.

[0084] If at least one of the recrystallisation solvents is an alcohol then it may be selected from the group consisting of C₁ to C₁₀ alcohols or C₁ to C₄ alcohols inclusive of methanol, ethanol, *n*-propanol and *iso*-propanol.

[0085] If at least one of the recrystallisation solvents is an ether it may be tetrahydrofuran or diethyl ether.

[0086] The stabilised protonated leucomethylene blue complex may be a salt or other ion complex.

[0087] In one embodiment, the stabilised protonated leucomethylene blue complex may be a stabilised protonated leucomethylene blue:ascorbate complex.

[0088] The diaminophenothiazinium compound or methylene blue is contacted with the reducing agent at an acidic pH. An acidic environment is required for the reduction reaction to proceed at a useful rate and to produce optimal conversion of the methylene blue to LMB. More preferably, the methylene blue is contacted with the reducing agent at below pH 3. Even more preferably, the methylene blue is contacted with the reducing agent at below pH 1.

[0089] The acidic environment may be achieved by the addition of one of a variety of acids such as non-interfering organic acids or mineral acids. Preferably, the reduction solution is made acidic with an acid chosen from the monoprotic and diprotic mineral acids such as

hydrochloric, sulphuric, nitric, phosphoric, boric, hydrofluoric, hydrobromic and perchloric. More preferably, the mineral acid is hydrochloric or sulphuric acid. Most preferably, the mineral acid is hydrochloric acid

[0090] The oxidising agent used to convert the purified LMB complex back into methylene blue is not particularly limited and wide range of known and commercially available oxidising agents may be suitable for use. In fact, once the reducing agent is removed the LMB complex it will oxidise back to methylene blue on standing due to contact with air.

[0091] Suitably, the oxidising agent is a metal-free oxidising agent.

[0092] Preferred oxidising agents may be selected from the group consisting of oxygen, ozone, chlorine, fluorine, bromine, iodine, hydrogen peroxide, nitric acid and nitrate compounds, phosphoric acid, peroxydisulphuric acid, peroxymonosulphuric acid, sulphuric and sulphurous acids, chlorite, chlorate, perchlorate and other analogous halogen-containing compounds, hypochlorite and other hypohalite compounds, permanganates, nitrous oxide and a quinone.

[0093] In one embodiment the oxidising agent is selected from the group consisting of oxygen and a quinone.

[0094] Preferably, the oxidising agent is 2, 3-dichloro-5, 6-dicyano-1,4-benzoquinone (DDQ) or, more preferably, p-benzoquinone.

[0095] In addition to the recrystallisation step other purification methods may be used to further purify the reduced stabilised protonated LMB complex. Filtration may be used, particularly to remove metal ions, and can be effected by filtration through metal binding filters, for example filtration supports including silica gel, activated charcoal, neutral, basic or acidic alumina gel, micro porous membranes, resins grafted with metal-capturing groups, and fibres grafted with metal-capturing groups. Metal ions can also be removed by passage through or over any known metal binding absorbent such as silica or diatomaceous earth or other commercial substrate manufactured for the purpose of binding or removing metals.

[0096] Preferably in the purification process of the invention, one or more of the steps may be conducted under controlled and stepped temperature conditions. Reactions may also be preferably performed under an inert atmosphere. Solvents, acids and reagents used in the processes are preferably degassed and are ultra pure containing only very low levels of metal ions. All of these measures can assist in stabilising the intermediates, including the reduced stabilised protonated LMB complex and the final methylene blue product, and products of the reaction steps. Particularly, it is beneficial to form the reduced stabilised protonated LMB complex under controlled temperature conditions of 15 to 30°C, more preferably 20 to 25°C for the period of the entire reaction. The liquid volume of the reaction is subsequently reduced under vacuum distillation at 35 to 45°C, preferably about 40°C.

[0097] Further, purification and drying of the reduced stabilised protonated LMB complex preferably occurs under a nitrogen atmosphere and controlled temperature conditions at 15 to 30°C, preferably 20 to 25°C. In addition, the oxidation of the stabilised protonated LMB complex to convert it back to methylene blue is preferably carried out under stepped temperature conditions between, initially, 5 to 20°C, preferably 10 to 15°C and, subsequently, 15 to 30°C, preferably 20 to 25°C and under a nitrogen atmosphere.

[0098] A final purification of the so-formed methylene blue by recrystallisation may be optionally performed and, if employed, may be carried out under controlled staged temperature conditions of, initially, 50 to 75°C, preferably 60 to 65 °C, followed by a reduction in temperature to 55 to 60°C, followed by cooling to 30 to 50°C, preferably 40 to 45 °C and finally cold conditions of 0 to 5 °C. The control of temperature within the limitations described above for the various process steps reduces the formation of various impurities, for example to reduce the likelihood of demethylation, and also optimises yield.

[0099] In one embodiment the methylene blue starting material is first passed through a purification step prior to reduction. This may be desirable depending on the purity of the commercially available starting material. Recrystallisation from an acidic solution may be suitable.

[0100] Thus, in one embodiment of the first aspect, there is provided a process for the purification of methylene blue including the steps of:

1. (i) recrystallising the methylene blue from an acidic solution;
2. (ii) contacting the recrystallised methylene blue with a reducing agent to form a stabilised protonated leucomethylene blue complex;
3. (iii) purifying the stabilised protonated leucomethylene blue complex by recrystallisation;
4. (iv) contacting the purified stabilised protonated leucomethylene blue complex with an oxidising agent to form a purified methylene blue; and
5. (v) recrystallising the purified methylene blue from an acidic solution,

to thereby purify the methylene blue.

[0101] The various elements and conditions for this embodiment are as previously described, *mutatis mutandis*, for the first aspect.

[0102] Preferably in the purification process of the invention, one or more of the steps may be conducted in an inert atmosphere, such as nitrogen. This can assist in stabilising the intermediates, particularly the reduced stabilised protonated LMB complex, and products of the reaction steps.

[0103] In a starting mixture of methylene blue, Azure A, B and C (and other contaminants such as metals), reduction of the methylene blue in the process of the invention to a stabilised protonated LMB complex allows simplified separation from the azures as, in the reduced form, the difference in the chemical properties between the stabilised protonated LMB complex and

the Azures has in some manner been amplified. Purification of the stabilised protonated LMB complex enables simplified removal of metal contaminants.

[0104] The process described significantly reduces metal ions present, however, if the starting material is particularly high in metal contaminants then the stabilised protonated LMB complex may be further cleaned of any metal contaminants by any of many known processes and then the purified and concentrated complex can be simply converted back to methylene blue.

[0105] One of the advantages of the process of the invention over known processes is the simplicity whereby the synthesis of a stabilised protonated LMB complex, which is then subjected to the main purification step, allows purification without the need for the preparation of a complex organic derivative of methylene blue which additional synthesis and deprotection steps often add to residual contamination.

[0106] The process of the invention is a reliable and consistent process applicable to any manufacturers "raw" material, for example methylene blue, which substantially reduces metal and organic contaminants as required and provides for a high chemical purity, in an economically viable manner which is applicable on an industrial scale. Reduction of most metals is greater than three fold using the process of the invention, and the final methylene blue product contains less than 3% Azure B and in preferred embodiments provides for less than 2.5% Azure B, by weight, in the final product. The introduction of additional recrystallisation steps can reduce the level of Azure B to less than 2%.

[0107] Repeating the recrystallisation and washing steps can result in even lower levels of contaminants, as can the introduction of any specific metal absorbent technology as an additional step within the process. Similarly too, increasing the amount of water used at this stage can also substantially reduce the Azure levels.

[0108] For the process of the invention, any practicable equipment can be used. Preferably non-metallic vessels and equipment is used, such as glass or ceramic or plastic lined equipment, to ensure that additional metal contamination is not introduced.

[0109] The resultant purified methylene blue product, after oxidation of the stabilised protonated LMB complex, can be further purified by filtration, recrystallisation or other methods as discussed above. In one embodiment of the invention, a methylene blue product is provided comprising (i) from about 2-5 waters of hydration; (ii) a solubility in water of up to about 2%; and (iii) metal levels below the levels specified in the European Pharmacopoeia 5.0. The methylene blue product may further comprise from about 5-25% moisture content.

[0110] According to a second aspect of the disclosure there is provided a protonated stabilised diaminophenothiazinium:ascorbate complex.

[0111] In one embodiment, the protonated stabilised diaminophenothiazinium complex is an N-10 protonated stabilised diaminophenothiazinium complex.

[0112] In one embodiment of the second aspect, the protonated stabilised diaminophenothiazinium complex is a stabilised protonated leucomethylene blue:ascorbate complex.

[0113] The stabilised protonated leucomethylene blue:ascorbate complex has been found to have particularly advantageous properties for use in the process of purification described herein. The stabilised protonated leucomethylene blue:ascorbate complex shows excellent stability and appears to represent a form of stabilised protonated LMB complex which has surprisingly good differentiation from the Azures thereby allowing for excellent purification in a single simple recrystallisation step. The need for post-synthesis or other synthetic modification of methylene blue with organic groups is thus avoided. The stabilised protonated leucomethylene blue:ascorbate complex is also formed and then converted back to methylene blue using only simple reagents which are subsequently easily removed without contributing to further impurities within the sample.

[0114] A third aspect of the disclosure resides in the protonated stabilised diaminophenothiazinium:ascorbate complex of the second aspect when produced by the process of contacting the diaminophenothiazinium compound with ascorbic acid to form the protonated stabilised diaminophenothiazinium:ascorbate complex.

[0115] In one embodiment of the third aspect, the protonated stabilised diaminophenothiazinium:ascorbate complex is an N-10 protonated stabilised diaminophenothiazinium:ascorbate complex.

[0116] In one embodiment of the third aspect, the protonated stabilised diaminophenothiazinium:ascorbate complex is the stabilised protonated leucomethylene blue:ascorbate complex of the second aspect when produced by the process of contacting methylene blue with ascorbic acid to form the stabilised protonated leucomethylene blue:ascorbate complex.

[0117] The various conditions for the process of the third aspect may be as described for the first aspect.

[0118] According to a fourth aspect of the disclosure there is provided a use of a protonated stabilised diaminophenothiazinium complex in the purification of the diaminophenothiazinium compound.

[0119] In one embodiment, the protonated stabilised diaminophenothiazinium complex is an N-10 protonated stabilised diaminophenothiazinium complex.

[0120] In one embodiment of the fourth aspect, the protonated stabilised diaminophenothiazinium complex is a stabilised protonated leucomethylene blue complex used in the purification of methylene blue.

[0121] In one embodiment, the stabilised protonated leucomethylene blue complex is a stabilised protonated leucomethylene blue salt.

[0122] In one embodiment, the stabilised protonated leucomethylene blue complex is the stabilised protonated leucomethylene blue:ascorbate complex of the second aspect.

[0123] Although the embodiments exemplified herein relate to the purification of methylene blue, it will be appreciated that, as described for the broad form of the present invention, the inventive concept described may be extended to other diaminophenothiazinium compounds.

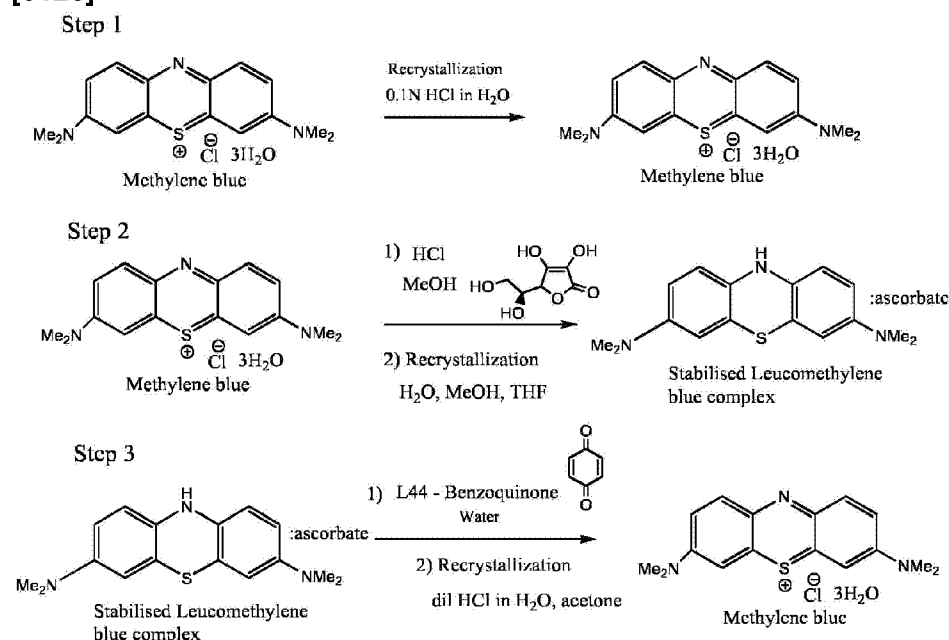
[0124] The following Example is provided by way of illustration of the invention and is in no way limiting of the scope of the invention.

EXPERIMENTAL

[0125] In this Example, a commercially available methylene blue is purified in accordance with the following steps:

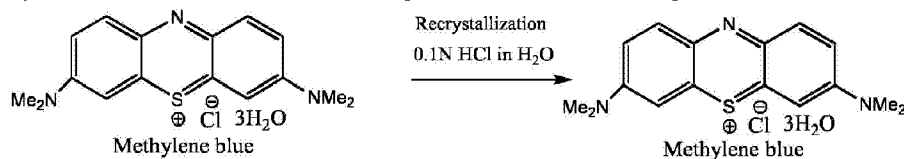
Overall Manufacturing Processing Scheme

[0126]



Step 1: Recrystallization of commercially available methylene blue starting material

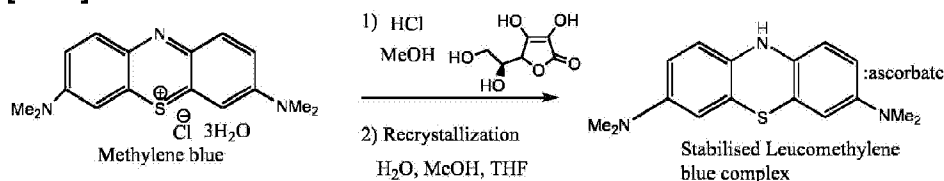
[0127] It will be understood that this initial recrystallisation step is optional and its use may depend on the purity level of the commercially available starting material. Its use provides optimal results when the starting material contains significant levels of impurities.



[0128] To a glass-lined, closed and jacketed reactor was charged methylene blue (5.5 kg, 1.0 eq.) and sterile purified water (54 kg). Then 0.6 Kg of 31 % HCl solution was charged. The batch was adjusted to a temperature of 60 to 65°C and agitated until dissolution. Next, the batch was cooled over a minimum of 9 hours to 0 to 5 °C. The suspension was filtered at 0 to 5°C and the filtercake was washed with 3.8 kg of cold water (0 to 5 °C), followed by a rinse with 7.3 kg of cold acetone (0 to 5 °C). The filtercake was dried under a flow of nitrogen to give 5.16 Kg of product.

Step 2: Process for reduction of methylene blue to leucomethylene blue

[0129]



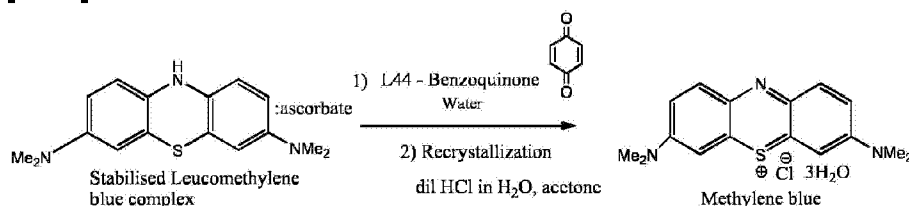
[0130] To a 50 L glass-lined reactor was charged 2.8 kg of purified methylene blue from step 1, 1.3 kg of L-ascorbic acid and 32.7 kg of methanol. 1 kg of 31 % hydrochloric acid was charged to the batch under N₂ with agitation. A rinse of 0.5 kg of methanol was done on the charging equipment to complete transfer. The batch was agitated at 20 to 25°C for a minimum of 15 minutes. The pH was checked (should be below 1) and the batch was agitated for a minimum of 18 hours. An IPC sample was taken and residual methylene blue with respect to stabilised protonated leucomethylene blue complex in the batch was less than 2%. The batch was then distilled under vacuum to 5.0 to 5.5 parts (14 to 15 L) with respect to the starting methylene blue, while applying an external temperature of NMT 40°C. With agitation, 2.2 kg of water was added and then the temperature of the batch was adjusted to 20 to 25°C. 19.5 kg of THF was added over 1.5 hours with agitation while maintaining the batch temperature of 20 to 25°C. The batch (suspension) was agitated at 20 to 25°C for a minimum of 16 hours. The suspension was filtered under a constant flow of nitrogen. A mixture of 0.4 Kg of methanol and 4.4 kg of THF (10% MeOH/THF) was used to wash the filtercake, followed by a rinse with 2.5 kg of THF. The cake was dried under a flow of nitrogen to give 1.805 kg of crude stabilised protonated leucomethylene blue complex. A second loop was done on a similar scale and the

products from the first and second loop were used in the recrystallization step.

[0131] To a 50 L glass reactor was charged 3.3 kg of crude stabilised protonated leucomethylene blue complex, and 3.7 kg of degassed SP water and the mixture was agitated at 20 to 25 °C for 10 to 15 minutes. Then 8.8 kg of degassed methanol was added and the batch was stirred at 20 to 25 °C for 10 to 15 minutes and a solution was confirmed. Next, 26.1 kg of degassed THF was added over a minimum of 1.5 hours. The batch was agitated for a minimum of 24 hours at 20 to 25 °C to complete crystallization. The suspension was filtered under nitrogen atmosphere. A mixture of 0.6 kg of methanol and 5.9 kg of THF (10% MeOH/THF) was used to wash the filtercake, followed by a rinse of 3.2 kg THF. The cake was dried under a flow of nitrogen to give 3.2 kg of light blue to white solid of purified stabilised protonated leucomethylene blue complex.

Step 3: Oxidation of purified stabilised protonated leucomethylene blue complex to methylene blue

[0132]



[0133] To a 50 L glass reactor was charged 3.2 kg purified leucomethylene blue and 16.1 kg of SP water. The batch was cooled to 10 to 15°C. Via a carboy, a solution of 1 kg p-benzoquinone and 4.1 kg of acetone was added into the batch while keeping the temperature at 10 to 15°C. The carboy was rinsed with 0.9 kg of acetone for complete transfer. The batch was agitated at 10 to 15°C for 35 minutes and then allowed to warm to 20 to 25°C and further agitated at this temperature for 5 hours. The batch was sampled for the completion of the reaction by HPLC (typically very little to no leucomethylene blue was detected). With vigorous agitation, 20.2 kg of acetone was added to the mixture and the suspension was stirred at 20 to 25°C for 5 hours. The suspension was filtered and the filtercake was washed with 6.8 kg of acetone at 20 to 25 °C. The filtercake was dried under a flow of nitrogen until transferrable. The solid was transferred back into the 50 L reactor and 25.3 kg of acetone was added and the temperature was adjusted to 20 to 25 °C and the batch was agitated for 3 hours. The suspension was filtered and the filtercake was washed with 7.8 kg of acetone at 20 to 25 °C. The filtercake was dried under a flow of nitrogen until transferrable.

Recrystallization

[0134] To the 50 L reactor was charged all of the above methylene blue, 43.1 kg of SP water

and 34.5 kg of hydrochloric acid solution. The batch was adjusted to 60 to 65 °C and agitated for 10 to 15 minutes. The batch temperature was then adjusted to 55 to 60 °C and the batch was subjected to final clarification filtration at this temperature into a second 50 L reactor. A rinse with 3.4 kg of the HCl solution at 55 to 60°C was done to complete the filtration. With agitation, the batch was cooled to 40 to 45 °C and 16.3 kg of acetone was charged to the reactor over 30 minutes. The batch was then agitated for 25 minutes at 40 to 45°C. With agitation, the batch was cooled to 0 to 5°C over a minimum of 8 hours. The batch was further agitated at 0 to 5 C for 3 hours. The suspension was filtered cold at 0 to 5°C and the filtercake was washed with 2.1 kg of cold water (0 to 5°C.), followed by a rinse of 190 kg of cold acetone (0 to 5°C.). The filtercake was dried under a flow of heated nitrogen to give 3.1 kg of purified methylene blue.

[0135] The exemplified process provides the purity data for a series of 3 batches of methylene blue product, purified according to the present process, with purity levels relative to the Azures listed in table 1. Table 2, below, compares this product to the commercially available methylene blue starting material and to metal impurity levels required by the European Pharmacopoeia.

Table 1: levels of the Azure contaminants in three representative batches of methylene blue prepared by the present process.

	Batch 1	Batch 1	Batch 1
	1305308	1305309	1305310
Azure A&C	0.2%	0.2%	0.1%
Azure B	2.3%	2.3%	2.4%

Table 2 - Metal contamination limits for methylene blue (European Pharmacopoeia).

Metal	European Pharmacopoeia Limits (ppm)	Present product (ppm)	Commercial Sample of Methylene Blue (ppm)
Aluminium	100	5.1	18
Copper	300	0.14	7.4
Iron	200	3.6	46
Zinc	100	0.69	91
Nickel	10	0.09	2.5
Chromium	100	0.91	5.0
Molybdenum	10	0.81	3.5
Manganese	10	0.09	0.81
Tin	10	0.04	0.11
Lead	10	0.13	0.2
Cadmium	1.0	<0.02	<0.1
Mercury	1.0	0.35	1.0

[0136] It can be seen that in every instance the level of metal impurity is significantly lower

than that prescribed as acceptable by the European Pharmacopoeia 5.0 and, in some instances, the level of the metal approaches the limits of detection by standard methods. The combined level of the 3 Azure dye impurities is about 2.5% by weight or less.

[0137] The above description of various embodiments of the present invention is provided for purposes of description to one of ordinary skill in the related art. It is not intended to be exhaustive or to limit the invention to a single disclosed embodiment. As mentioned above, numerous alternatives and variations to the present invention will be apparent to those skilled in the art of the above teaching. Accordingly, while some alternative embodiments have been discussed specifically, other embodiments will be apparent or relatively easily developed by those of ordinary skill in the art. Accordingly, this patent specification is intended to embrace all alternatives, modifications and variations of the present invention that have been discussed herein, and other embodiments that fall within the scope of the above described invention.

[0138] In the claims which follow and in the preceding description of the invention, except where the context clearly requires otherwise due to express language or necessary implication, the word "comprise", or variations thereof including "comprises" or "comprising", is used in an inclusive sense, that is, to specify the presence of the stated integers but without precluding the presence or addition of further integers in one or more embodiments of the invention.

REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- [DE1886 \[0007\]](#)
- [WO2005A \[0011\]](#)
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P a t e n t k r a v

1. Fremgangsmåde til oprensning af en diaminophenothiazinium-forbindelse, indbefattende trinnene:

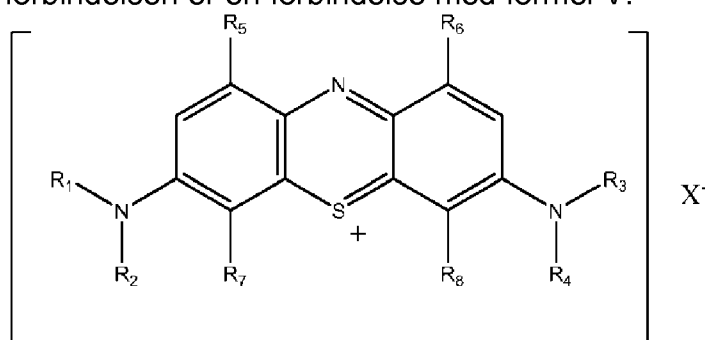
5 (i) at bringe diaminophenothiazinium-forbindelsen i kontakt med et metalfrit reduktionsmiddel ved en sur pH-værdi til dannelse af et protoneret stabiliseret diaminophenothiazinium-kompleks;

(ii) at oprense det protonerede stabiliserede diaminophenothiazinium-kompleks; og

10 (iii) at bringe det oprensede protonerede stabiliserede diaminophenothiazinium-kompleks i kontakt med et oxidationsmiddel, således at det konverteres tilbage til diaminophenothiazinium-forbindelsen, således at diaminophenothiazinium-forbindelsen derved oprenses.

15 **2.** Fremgangsmåde ifølge krav 1, hvor det protonerede stabiliserede diaminophenothiazinium-kompleks er et N-10 protoneret stabiliseret diaminophenothiazinium-kompleks, som efterfølgende oprenses og oxideres til dannelse af diaminophenothiazinium-forbindelsen.

20 **3.** Fremgangsmåde ifølge krav 1 eller krav 2, hvor diaminophenothiazinium-forbindelsen er en forbindelse med formel V:



Formel V

hvor R_1 , R_2 , R_3 og R_4 uafhængigt er udvalgt blandt hydrogen, C_1 - C_6 -alkyl og C_1 - C_6 -alkenyl, hvor hver alkyl- eller alkenylgruppe kan være substitueret med hydroxy, halogen eller alkoxy;

25 R_5 , R_6 , R_7 og R_8 uafhængigt er udvalgt blandt hydrogen, nitro, halo, haloalkyl, C_1 - C_6 -alkyl og C_1 - C_6 -alkenyl; og

X er en anionisk modion.

4. Fremgangsmåde ifølge et hvilket som helst af de foregående krav, hvor diaminophenothiazinium-forbindelsen er udvalgt fra gruppen bestående af methylenblåt, methylengrønt, azur A, azur B og azur C, fortrinsvis hvor diaminophenothiazinium-forbindelsen er methylenblåt.
- 5
5. Fremgangsmåde ifølge krav 1, hvor det metalfri reduktionsmiddel er udvalgt fra gruppen bestående af ascorbinsyre, hydrogen, myresyre, oxalsyre, dithiothreitol, hydrazin, phosphitter, hypophosphitter og phosphorsyrling.
- 10
6. Fremgangsmåde ifølge et hvilket som helst af de foregående krav, hvor diaminophenothiazinium-forbindelsen bringes i kontakt med reduktionsmidlet ved en pH-værdi på under 3, eventuelt en pH-værdi på under 1.
- 15
7. Fremgangsmåde ifølge et hvilket som helst af de foregående krav, hvor det metalfri reduktionsmiddel er i en opløsning, der er gjort sur med en syre udvalgt blandt en monoprotisk eller diprotisk mineralsk syre, eventuelt saltsyre eller svovlsyre.
- 20
8. Fremgangsmåde ifølge et hvilket som helst af de foregående krav, hvor oprensningen af det protonerede stabiliserede diaminophenothiazinium-kompleks sker ved en fremgangsmåde udvalgt fra gruppen bestående af kromatografi, ionbytning, filtrering, vask og rekrystallisering.
- 25
9. Fremgangsmåde ifølge et hvilket som helst af de foregående krav, hvor oxidationsmidlet er et metalfrit oxidationsmiddel, eventuelt oxygen eller et quinon.
- 30
10. Fremgangsmåde ifølge krav 1, hvor det protonerede stabiliserede diaminophenothiazinium-kompleks er et stabiliseret protoneret leucomethylenblåt-kompleks i form af et leucomethylenblåt:ascorbat-kompleks.
- 35
11. Fremgangsmåde ifølge et hvilket som helst af de foregående krav, yderligere indbefattende trinnet, før trin (i), at rekrystallisere diaminophenothiazinium-forbindelsen ud fra en sur opløsning.

12. Fremgangsmåde ifølge et hvilket som helst af de foregående krav, yderligere indbefattende trinnet, efter trin (iii), at rekrystallisere den oprensede diaminophenothiazinium-forbindelse ud fra en sur opløsning.