METHODS FOR DETECTION OF TOXEMIA
VERFAHREN ZUM NACHWEIS EINER TOXÄMIE
PROCÉDÉS POUR LA DÉTECTION DE LA TOXÉMIE

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The present invention relates generally to analysis of extracellular fluids that contain carrier proteins, and, more particularly, but not by way of limitation, to methods for prognosis and detection of toxemia in a patient by analyzing a serum sample from the patient.

1. Field of the Invention

[0001] The present invention relates generally to analysis of extracellular fluids that contain carrier proteins, and, more particularly, but not by way of limitation, to methods for prognosis and detection of toxemia in a patient by analyzing a serum sample from the patient.

2. Description of Related Art

[0002] For illustration, but without limiting the scope of the invention, the background is described with respect to analyzing the blood of a human patient.

[0003] In the early stages of toxemia, the ability of a patient's body to evacuate toxins from the blood stream may become compromised. That is, the patient's biological systems for evacuating toxins from the patient's blood stream may stop functioning properly and begin to permit toxins to build in the patient's body. In later stages of toxemia, these toxins generally reach relatively high levels (as compared to normal toxin levels in a healthy patient), and the toxins may begin to cause noticeable symptoms such as illness, cell damage, organ failure, and the like. Currently known methods of diagnosing toxemia may not permit recognition or diagnosis of toxemia until later stages when symptoms are already noticeable. In these later stages, treatment may be less effective and may not be effective enough to prevent the death of the patient.

[0004] Analyzing hematologic parameters and/or measuring the concentration of various metabolites in blood samples from a patient are known in the art and may be widely-used methods of diagnosing toxemia (which may also be known in the art as toxaemia) in a patient, such as, for example, in a clinical setting. However, these known methods suffer a number of shortcomings and/or drawbacks.

[0005] One example of a known method may be referred to in the art as the "mean mass molecules" evaluation of a sample of a patient's blood serum. However, in the "mean mass molecules" evaluation, generally only a fraction of unbound, free endogenous toxins in the serum may be detected. This fraction may also be limited in that it may contain mostly toxins that are hydrophilic. "Absorbed" or "bound" hydrophobic toxins, which are respectively adsorbed on biological membranes or bound on carrier proteins, may not be detected. The failure to detect absorbed hydrophobic toxins can be detrimental to the treatment and recovery of the patient because these toxins may subsequently damage, or may have already damaged, cells, organs or systems in the patient. Such damage, caused by these absorbed and/or bound hydrophobic toxins, may subsequently cause changes in hematological parameters and/or other symptoms of toxemia.

[0006] Because the "mean mass molecules" evaluation (as well as other known methods of detecting toxemia) may not detect hydrophobic toxins, toxemia may not be detected until cells have been significantly damaged and/or hematologic changes or failures have already occurred. Stated otherwise, known methods of detecting toxemia may not detect toxemia in its early stages, and instead, may only detect toxemia in later stages of its development when it may be substantially harder to treat it effectively. In these later stages of toxemia, treatment may be inhibited by factors such as, for example, reduced capacity of the carrier proteins to evacuate toxins, and/or reduced capacity of the patients liver to detoxify or remove the toxins from the carrier proteins.

[0007] Soviet Union Patent, SU 1,459,656, published February 23, 1989, describes a method of diagnosing endogenous toxemia by an evaluation of the ability of erythrocyte membranes to bind the fluorescent probe ANS. This method may permit detection of damage to erythrocyte membranes that has already occurred due to erythrocyte interaction with toxins (either hydrophilic or hydrophobic). This method may permit relatively-earlier detection of toxemia in a patient, such as, for example, at a stage in which toxins have already damaged cell membranes but before the damage is extensive enough to cause failure of organs and/or systems of the patient. Practically speaking, this method may permit detection of toxemia as much as approximately 6 to 12 hours before other hematological parameters show detectable changes or before other symptoms may appear. This method therefore still suffers from the shortcomings that it may not detect toxemia before toxins have damaged cells of the patient, and therefore may not permit treatment early enough during the development of the disease to prevent or minimize the effects, complications, or symptoms of toxemia.

[0008] United States Patent No. 7,166,474 describes a method of detecting changes in transport properties of albumin by using electron paramagnetic resonance (EPR) spectroscopy (which may also be known in the art as electron spin resonance “ESR” spectroscopy) to evaluate a sample that contains albumin (an albumin-containing sample). This method can include evaluating the albumin transport function with respect to long chain fatty acids by using a spin probe represented by a spin-labeled fatty acid. Specifically, according to this method, the EPR-spectra of the spin probe can be measured in at least three aliquots of the sample, where each aliquot is mixed with significantly different concentrations.

SUMMARY OF THE INVENTION

The present invention is related to a method for detecting toxemia in a subject patient, comprising the steps of: (a) mixing a labelled 16-DOXYL-stearic acid probe having a site to bind to albumin with an aliquot of a subject patient's extracellular fluid containing albumin, the amount of probe such that the molar ratio of the probe to the albumin is in the range of 0.3 to 1.5; (b) mixing ethanol with the mixture of the aliquot and the probe, wherein the ethanol when added to the mixture increases the solubility of the probe in the aliquot, and wherein the amount of ethanol added causes a portion of the probe to dissociate from the albumin without causing toxins to dissociate from the albumin; (c) analyzing the mixture comprising the aliquot, the probe, and the ethanol to determine the binding efficiency of the albumin in the patient's extracellular fluid, wherein the binding efficiency is the concentration ratio of bound probe and unbound probe adjusted for the concentration of the added ethanol in the mixture and where the binding efficiency is calculated using the formula: 1/BE = (Sc/S-1) x (F/B) x P, where BE is binding efficiency, F is the concentration of bound probe, B is the concentration of bound probe, Sc is the critical solvent concentration at which all probe is dissociated from the albumin and dissolved in the solution, S is the solvent concentration and P is the concentration of albumin; and (d) comparing the subject binding efficiency to at least one control binding efficiency of the albumin in the extracellular fluid of at least one non-toxemic control patient, wherein the amount of ethanol mixed with the aliquot and probe does not induce conformational changes in the albumin, and wherein the volume of ethanol mixed with the aliquot makes up less than 30% of the volume of the aliquot, and optionally less than 10% of the volume of the aliquot. Various embodiments of the present description can be suitable for detecting variations in carrier-protein functions, characteristics, and/or parameters by mixing various substances with a sample of extracellular fluid (that contains carrier proteins) from a subject patients, and analyzing the mixture. Examples of such extracellular fluids include blood serum, lymph fluid, and spinal fluid. Some embodiments of the present methods are suitable for prognosis (predicting the onset of), detection, or diagnosis of toxemia in its early or precursor stages. The present invention may be suitable, for example, for diagnosis and/or monitoring of toxemia in patients in clinical settings such as intensive care units. By way of further examples the present invention may be suitable for such diagnosis, prognosis, or monitoring of patients in post-surgery recovery, or patients with or suffering from trauma, infection, sepsis, systemic inflammatory response syndrome (SIRS), stroke or brain attack, infarction, or the like.
the serum sample with a labeled hydrophobic probe having a long-hydrocarbonchain. Specifically, parameters of binding efficiency of a hydrophobic probe having a long-hydrocarbon chain to serum carrier proteins can correlate to the amount of toxic substances bound to the binding sites of the carrier proteins. This correlation can, for example, be caused by competitive binding on specific protein sites for substances with long hydrocarbon chains, and indirectly due to allosteric modification of the affinity of these sites upon ligand binding on other protein sites. Stated otherwise, when high levels of toxins are present in the subject patient’s blood, the toxins may crowd and/or modify the binding sites of the carrier proteins, thereby preventing the hydrophobic probe from binding to those binding sites. In this way, hydrophobic toxins can be detected as their levels rise, and even before they damage cells.

Some embodiments of the present method can comprise obtaining an aliquot from a sample of a subject patient’s extracellular fluid containing carrier proteins. Some of the present methods can comprise mixing a labeled hydrophobic probe with the aliquot. In some of these methods, the amount of probe can be such that the molar ratio of probe capable of binding to (with) carrier proteins to the carrier proteins is between about 0.3 and about 1.5. Some methods can comprise mixing a solvent into the aliquot. In some of these methods, the solvent can be such that when added to the aliquot and probe the solubility of the probe is increased in the aliquot. In some of these methods, the amount of solvent mixed with the aliquot can be sufficient to dissociate a portion of the probe from the carrier proteins without causing significant dissociation of toxins from the carrier proteins. In some methods, the amount of solvent mixed with the aliquot can be described as sufficient to dissociate a portion of the probe from the carrier proteins without causing dissociation of toxins from the carrier proteins.

Some methods can comprise obtaining an aliquot from a sample of the subject patient’s extracellular fluid containing carrier proteins. Some of the present methods can comprise mixing a labeled hydrophobic probe with the aliquot. In some of these methods, the amount of probe can be such that the molar ratio of probe capable of binding with (to) carrier proteins to the carrier proteins is between about 0.3 and about 1.5. Some of the present methods can comprise obtaining a solvent. In some of these methods, the amount of solvent mixed with the aliquot can be sufficient to dissociate a portion of the probe from the carrier proteins without causing significant dissociation of toxins from the carrier proteins. In some of these methods, the amount of solvent can be such that when mixed with the aliquot and probe the solubility of the probe is increased in the aliquot. In some of these methods, the amount of solvent mixed with the aliquot can be described as sufficient to dissociate a portion of the probe from the carrier proteins without causing dissociation of toxins from the carrier proteins.

Some methods can comprise analyzing the mixture of the aliquot, probe, and solvent to determine the binding efficiency of the carrier proteins. Some of these embodiments can comprise analyzing the mixture of the aliquot, probe, and solvent to determine the subject binding efficiency to at least one control binding efficiency for at least one non-toxemic control patient.

Some methods can comprise analyzing the mixture of aliquot, probe, and solvent to determine a subject toxin-evacuation parameter of the carrier. Some of these methods can comprise analyzing the mixture of aliquot, probe, and solvent to determine a subject toxin-evacuation parameter to at least one control toxin-evacuation parameter for a non-toxemic control patient. Some of these methods, analyzing the mixture of aliquot, probe, and solvent can comprise analyzing the mixture to determine the concentrations of protein-bound and unbound probe in the mixture; deriving a subject binding efficiency of the carrier proteins from at least the concentrations of the protein-bound and unbound probe; and deriving a subject toxin-evacuation parameter of the carrier proteins as the square of the subject binding efficiency.

Some kits can comprise instructions for performing the various embodiments of the present methods using one or more elements of the present kits. For example, some kits can comprise instructions for: obtaining an aliquot having a predetermined volume of the extracellular fluid the molar ratio of probe capable of binding with (to) carrier proteins to the carrier proteins is between about 0.3 and about 1.5. Some of the present kits can comprise a solvent. In some of these kits, the solvent can be such that when mixed with the aliquot and probe the solubility of the probe is increased in the aliquot. In some of these kits, the amount of solvent mixed with the aliquot can be sufficient to dissociate a portion of the probe from the carrier proteins without causing significant dissociation of toxins from the carrier proteins. In some kits, the amount of solvent can be described as sufficient to dissociate a portion of the probe from the carrier proteins without causing dissociation of toxins from the carrier proteins.

Some methods can comprise obtaining an aliquot from a sample of the subject patient’s extracellular fluid containing carrier proteins. Some of the present methods can comprise obtaining an aliquot from a sample of the subject patient’s extracellular fluid containing carrier proteins; mixing a labeled hydrophobic probe with the aliquot, the amount of probe such that the molar ratio of probe capable of binding with (to) carrier proteins to the carrier proteins is between about 0.3 and about 1.5; mixing a solvent with the aliquot sufficient to dissociate a portion of the probe from the carrier proteins without causing significant dissociation of toxins from the carrier proteins; analyzing the mixture of the aliquot, probe, and solvent to determine the binding efficiency of the carrier proteins; comparing the subject binding efficiency to at least one control binding efficiency for a non-toxemic control patient; measuring the concentrations of protein-bound and unbound probe in the mixture; deriving a subject binding efficiency of the carrier proteins from at least the concentrations of the protein-bound and unbound probe; and deriving a subject toxin-evacuation parameter of the carrier proteins as the square of the subject binding efficiency.
induce significant conformational changes to the carrier proteins. In some methods and kits, the amount of solvent may be described as such that the solvent does not cause or induce conformational changes to the carrier proteins. In some, the amount of solvent is sufficient to increase the concentration of unbound probe in the mixture of aliquot, probe, and solvent to at least 5 times greater than the concentration of unbound probe in a mixture of aliquot and probe without solvent.

[0020] In some methods and kits, the solvent can comprise alcohol. In some methods and kits, the solvent can comprise ethanol. In some methods and kits, the volume of the amount of solvent can be less than about 30% of the volume of the aliquot. In some methods and kits, the volume of the amount of solvent can be less than about 25% of the volume of the aliquot. In some methods and kits, the volume of the amount of solvent can be less than about 20% of the volume of the aliquot. In some methods and kits, the volume of the amount of solvent can be less than about 15% of the volume of the aliquot. In some methods and kits, the volume of the amount of solvent can be less than about 10% of the volume of the aliquot. In some of these embodiments, the volume of the amount of solvent can be less than about 5% of the volume of the aliquot.

[0021] Some methods can comprise normalizing the subject binding efficiency to account for the reduction in carrier-protein concentration caused by the amount of solvent in the mixture. In some methods, the steps of deriving the subject binding efficiency and normalizing the subject binding efficiency can be performed simultaneously.

[0022] Some methods can comprise normalizing the subject binding efficiency to account for the reduction in carrier-protein concentration caused by medical conditions of the patient. In some embodiments, the at least one control binding efficiency can comprise a range of control binding efficiencies.

[0023] Some methods can comprise normalizing the subject binding efficiency to account for the reduction in carrier-protein concentration caused by medical conditions of the patient. In some embodiments, the at least one control binding efficiency can comprise a range of control binding efficiencies.

In some methods, the at least one control binding efficiency can comprise a range of control binding efficiencies. Some methods can comprise normalizing the subject binding efficiency to account for the reduction in carrier-protein concentration caused by medical conditions of the patient. In some embodiments, the at least one control binding efficiency can comprise a range of control binding efficiencies. In some methods, the extracellular fluid can be spinal fluid. In some methods, the extracellular fluid can be blood serum. In some methods, the extracellular fluid can be lymph fluid.

[0024] Some methods can comprise repeating one or more steps of the method for one or more additional aliquots. In some methods, the amount of solvent mixed with each of the one or more additional aliquots can be different from the amount of solvent mixed with the first or initial aliquot. In some methods, the subject binding efficiencies derived for the first or initial aliquot and the one or more additional aliquots can be averaged to derive an average subject binding efficiency. In some methods, the average subject binding efficiency can be compared in the step of comparing the subject binding efficiency to at least one control binding efficiency. In some methods and kits, the volume of the aliquot can be less than about 100 mL. In some of these embodiments, the volume of the aliquot can be about 50 mL. In some methods and kits, the volume of the aliquot can be less than about 100 mL. In some of these methods and kits, the volume of the aliquot can be about 50 mL.

[0025] In some methods and kits, the volume of the aliquot can be about 50 mL. In some of these methods and kits, the volume of the aliquot can be about 50 mL.

[0026] Some methods can comprise diagnosing, responsive to the subject binding efficiency being less than the at least one control binding efficiency, the subject patient with toxemia.

[0027] In some methods and kits, the probe can be labeled with a spin-label. In some methods and kits, the probe can be labeled with a radioactive label. In some methods and kits, the probe can be labeled with a radioactive label. In some methods and kits, the probe can be labeled with a fluorescent label. In some methods and kits, the probe can be labeled with a fluorescent label. In some methods, the extracellular fluid can be blood serum. In some methods, the extracellular fluid can be blood serum. In some methods, the extracellular fluid can be lymph fluid. In some methods, the extracellular fluid can be lymph fluid.

[0028] Any methods can consist of or consist essentially of - rather than comprise/include/contain/have - any of the described steps, elements, and/or features.

[0029] Details associated with the embodiments described above and others are presented below.

BRIEF DESCRIPTION OF THE DRAWINGS

[0030] The following drawings illustrate by way of example and not limitation. For the sake of brevity and clarity, every feature of a given structure is not always labeled in every figure in which that structure appears. Identical reference numbers do not necessarily indicate an identical structure. Rather, the same reference number may be used to indicate a similar feature or a feature with similar functionality, as may non-identical reference numbers.

FIGS. 1A-1B depict graphical representations of a number of labeled probes for use in various embodiments of the present methods.

FIG. 2 depicts a schematic representation of one exemplary procedure for evaluating a mixture containing an aliquot of serum and an amount of labeled probe by EPR spectroscopy that is suitable for use in embodiments of the present methods.

FIG. 3 depicts an exemplary EPR spectrum of a mixture of serum and a spin-labeled fatty acid probe (16-doxyl stearic acid) obtained by an EPR spectroscopy method of analysis that is suitable for use in embodiments of the present methods.

FIG. 4 depicts a graphical representation of the binding efficiency of serum carrier proteins, as assessed by the present methods, for a number of patients admitted to an intensive care unit (ICU).

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0031] The term "coupled" is defined as connected, although not necessarily directly, and not necessarily mechanically;
two items that are "coupled" may be integral with each other. The terms "a" and "an" are defined as one or more unless this disclosure explicitly requires otherwise. The terms "substantially," "approximately," and "about" are defined as largely but not necessarily wholly what is specified, as understood by a person of ordinary skill in the art.

The terms "comprise" (and any form of comprise, such as "comprises" and "comprising"), "have" (and any form of have, such as "has" and "having"), "include" (and any form of include, such as "includes" and "including") and "contain" (and any form of contain, such as "contains" and "containing") are open-ended linking verbs. As a result, a method that "comprises," "has," "includes" or "contains" one or more steps possesses those one or more steps, but is not limited to possessing only those steps. Likewise, a step of a method that "comprises," "has," "includes" or "contains" one or more features possesses those one or more features, but is not limited to possessing only those one or more features. For example, in a method that comprises the step of obtaining a sample of blood serum containing carrier proteins, the blood serum includes the specified features but is not limited to having only those features. For example, in such a method, the blood serum could also contain water.

Further, a device or structure that is configured in a certain way is configured in at least that way, but it can also be configured in other ways than those specifically described.

Various methods may include testing, analyzing, and/or evaluating of a sample of extracellular fluid from a subject patient. For clarity and brevity, embodiments are described below for testing blood serum of a subject patient. However, the embodiments, features, steps, and particulars described below can also be applied to other extracellular fluids such as, for example, lymph fluid, spinal fluid, and the like.

Some methods include obtaining a serum sample from a subject patient. A serum sample can be obtained, for example, by drawing blood from the patient and using centrifugation to substantially isolate the serum from the blood. Other suitable methods may also be used to obtain the serum sample from the subject patient. An aliquot can also be obtained from this subject sample. Such an aliquot can be separated from the sample or can include the entire sample.

Some methods include mixing a labeled hydrophobic probe with the aliquot. The labeled hydrophobic probe may also be referred to herein as the "labeled probe," the "hydrophobic probe," or the "probe." The probe is (or comprises) an organic molecule, and/or is selected to be capable of binding with (to) carrier proteins (e.g., albumin) of the extracellular fluid (e.g., serum or plasma). The probe can comprise a suitable number of Carbon atoms. For example, the probe can comprise between 8 and 28 Carbon atoms, including 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, or 28 Carbon atoms, or any smaller range between 8 and 28 Carbon atoms, e.g., between 14 and 22, between 16 and 20, or between 18 and 22 Carbon atoms. In another example, the probe can comprise less than 8 Carbon atoms or more than 28 Carbon atoms. In some embodiments, the probe can comprise a hydrocarbon chain such as, for example, a hydrocarbon chain having any suitable number of Carbon atoms. The probe can comprise a hydrocarbon molecule whose structure is branched and/or whose structure comprises a ring structure. The probe can comprise a hydrocarbon molecule comprising elements other than Carbon or Hydrogen, such as, for example, Chlorine, Phosphorous, and/or Nitrogen.

The probe can comprise a hydrocarbon chain such as, for example, a fatty acid, a long-chain fatty acid, a medium-chain fatty acid, or the like. Examples of probes suitable for some embodiments of the present method include: 16-DOXYL-stearic acid, free radical; 5-DOXYL-stearic acid, free radical; 16:0-16 PC DOXYL, free radical (1- Palmitoyl-2-S tearyl-(16-DOXYL)- sn-Glycero-3-Phosphocholine); 18:0 PC C13 (1,2- Di stearoyl [1-13C] - sn-Glycero-3-Phosphocholine), Carbon-13 labeled; and Oleic acid-1,2,3,7,8,9,10-13C7, Carbon-13 labeled. Such labeled probes are available from commercial suppliers including, for example, (1) Sigma-Aldrich, Inc., St. Louis, Missouri, U.S.A., www.sigmaaldrich.com; (2) TCI America, Portland, Oregon, U.S.A. and Wellesley Hills, Massachusetts, U.S.A., www.tciamerica.com; (3) Fluorochem, Derbyshire, U.K., www.fluorochem.co.uk; and (4) Avanti Polar Lipids Inc., Alabaster, Alabama, U.S.A., www.avantilipids.com.

The probe may be suspended in a volume of liquid so as to, for example, enable accurate measurement of the amount of the probe. This liquid is referred to herein as the "suspension liquid" or "liquid" so as not to be confused with the solvent described herein (even though the suspension liquid may be or comprise a solvent as that term is used in a more general sense). In some of these examples, the suspension liquid may be (or comprise) the solvent, which is described below. In some of these examples, the probe may be suspended in the solvent in an amount desired for mixing the aliquot, probe, and solvent without further addition of solvent; or may be suspended in an amount of solvent smaller than is desired, such that additional solvent must be separately added to the mixture. For example, if 10 20.65 μL of solvent are mixed with a 50 μL aliquot: (1) the probe may be suspended in 10 μL of solvent and the suspension added to the aliquot; or (2) the probe may be suspended in 5 μL of solvent, the suspension added to the aliquot, and 5 μL of solvent (without probe) also mixed with the aliquot. Examples of suitable solution liquids, for use where the probe solution and solvent are mixed while both are liquid, include the materials described below for the solvent.

In others of these (in which the probe may be suspended in a volume of liquid), the probe solution may be added to a container first and the liquid portion of the liquid/probe solution permitted to evaporate, leaving substantially-only the probe in the container. In such embodiments, the aliquot of serum may then be added such that the probe then dissolves into the serum. Examples of suitable liquids for this suspension/evaporation method include both the liquids...
described below for the solvent, as well as other liquids (including solvents known in the art as non-polar solvents) that are not miscible with water, such as, for example, methyl ethyl ketone, methyl acetate, diethyl ether, dichloro methane, benzene, pentane, cyclo pentane, etc.

The amount of probe mixed with the aliquot is sufficient to permit a representative level of binding of the probe to the carrier proteins, i.e., a level of binding that is sufficient to be representative of the binding ability of the carrier proteins in the subject patient's body at the time the sample was taken from the subject patient. The amount of probe is also small enough to prevent (or to not permit) saturation of the fatty acid binding sites on the carrier proteins. The amount of probe is such that the molar ratio of probe (that is capable of binding with carrier proteins) to the carrier proteins is between about 0.3 and about 1.5 so as to, for example, encourage binding of the probe carrier proteins in a manner that is representative of the circulatory system of the patient. In some examples, the molar ratio of the probe to the carrier proteins is greater than, less then, or between any of about 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.1, 1.2, 1.3, 1.4, or 1.5. In other examples, the molar ratio of the probe concentration to the carrier-protein concentration can be any suitable ratio that permits the present methods to function as described.

The concentration of carrier proteins in the serum can be approximated from various texts known in the art.

The concentration of carrier proteins can be approximated as the expected concentration of carrier proteins in a healthy patient. In such examples, results can later be adjusted (as described below) to account for any reduction in carrier-protein concentration expected as a result of the medical conditions of the patient (e.g., toxemia). In some examples, the concentration of carrier proteins can be approximated as the expected concentration of carrier proteins in a patient with the same medical conditions of the subject patient (e.g., toxemia, cancer, and/or the like), and the amount of probe adjusted accordingly so as to, for example, reduce or eliminate the need to adjust results later to account for a reduction in carrier-protein concentration caused by medical conditions of a subject patient.

Some methods include mixing a solvent with the aliquot to, for example, increase the solubility of the hydrophobic probe in the water of the aliquot. As used specifically herein, the "solvent" mixed with the aliquot is a substance (or combination of substances) capable of increasing the solubility of the probe in the mixture (of at least aliquot, probe, and solvent), and can comprise one or more substances generally known in the art as solvents (e.g., polar solvents) and/or one or more substances not generally considered to be solvents (e.g., solids, water-based or other solutions, and the like). In some examples, the solvent may include a mixture of two or more substances. In some examples, the solvent is selected to be capable of causing some dissociation of the probe from the carrier proteins (e.g., albumin) in the aliquot. In some examples, the solvent can be (or comprise) a liquid that is miscible with, or soluble in, water (and/or that may be known in the art as a polar solvent), such as, for example, methanol, ethanol, acetonitrile, dimethyl sulfoxide (DMSO), tetrahydrofuran (THF), acetic acid, formamide, ethylene glycol, glycerin, water, and the like. In some examples, the solvent may be added at any suitable stage or in any suitable manner, including, for example, to the aliquot alone, to the probe alone, to a mixture of only the aliquot and probe, or to the complete mixture of aliquot, probe, and solvent.

Some examples of the present methods include mixing water with any component of the mixture or the entire mixture at any suitable stage, including, for example, to the aliquot alone, to the probe alone, to a mixture of only the aliquot and probe, or to the complete mixture of aliquot, probe, and solvent. Some methods include mixing additives with the water, with the solvent described above, with any other component of the mixture, and/or with the complete mixture, to adjust or modify the isotonic properties of the sample mixture. For example, some methods include mixing an amount of NaCl with water to achieve a concentration of about 0.9% NaCl to simulate the isotonic properties of blood and the normal cells of the body, as may be done in other areas of medicine and biological sciences.

In some examples where the solvent is selected to be capable of causing dissociation of the probe from the carrier proteins (e.g., albumin), the amount of solvent added to the aliquot is such that a portion of the probe is dissociated from (caused to not bind or stop binding with) the carrier proteins without causing significant dissociation of toxins from the carrier proteins. As used here, "significant dissociation of toxins" is dissociation from carrier proteins that substantially affects the representative nature of the binding properties exhibited by the carrier proteins in the aliquot (relative to the binding properties the same carrier proteins exhibited in the subject patient’s body at the time the sample was obtained from the subject patient). This lack or relatively minimal amount of dissociation of toxins may also be described as, simply, "without causing dissociation of toxins." As used here, "without causing dissociation of toxins" does not necessarily mean that no toxins are dissociated from carrier proteins, and in fact, some toxins may be dissociated from carrier proteins. Instead, as used here, "without causing dissociation of toxins" means that the binding properties exhibited by the carrier proteins in the aliquot are still representative of the binding properties the same carrier proteins exhibited in the subject patient's body at the time the sample was obtained from the subject patient). For example, a conformational change would substantially affect the
representative nature of the binding properties if it caused the carrier proteins to release toxins so as to bind a measurably greater amount of probe than the carrier proteins would have bound without such conformational changes. This lack or relatively minimal amount of conformational changes in carrier proteins may also be described as, simply, "without causing (or inducing) conformational changes to the carrier proteins" or "does not cause (or induce) conformational changes to the carrier proteins." As used here, this does not necessarily mean that no conformational changes are caused to or induced in the carrier proteins, and in fact, some conformational changes may occur. Instead, as used here, "without causing conformational" and/or "does not cause conformational" mean that the binding properties exhibited by the carrier proteins in the aliquot are still representative of the binding properties the same carrier proteins exhibited in the subject patient’s body at the time the sample was obtained from the subject patient).

[0046] In some examples, the amount of solvent is such that the concentration of unbound (free) probe in the mixture (of aliquot, probe, and solvent) is at least a multiple greater than (e.g., 5, 6, 7, 8, 9, 10, 20, 50, 100, 500, 1000, 5000, 10000, or 100000 times greater than) the concentration of unbound (free) probe before the addition of the solvent (e.g., in a mixture of only the aliquot and probe). In other embodiments, the amount of solvent is such that the concentration of unbound (free) probe in the mixture (of aliquot, probe, and solvent) is in a range between about a lower multiple and a higher multiple greater than (e.g., between about 5 and about 10, between about 10 and about 100, or between about 100 and about 1000 times greater than) the concentration of unbound (free) probe before the addition of the solvent (e.g., in a mixture of only the aliquot and probe).

[0047] In some examples, the volume of the amount of solvent mixed with the aliquot is a percentage of the volume of the aliquot. For example, for a 50 µL aliquot, the volume of solvent added may be between about 1% and about 30%, i.e., between about 0.5 µL and about 15 µL, or in any individual percentage or range of percentages within this range, e.g., about 10%, about 20%, between about 5% and about 20%, between about 10% and about 20%, or the like. In some examples, the volume of solvent is such that the concentration of unbound (free) probe is greater than and/or less than about any of: 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29% and 30%. In some examples, a specific volume of solvent mixed with the aliquot is within this range, such as, for example, for an aliquot of 50 µL, about 1 µL, about 5 µL, about 10 µL, about 15 µL, about 20 µL, or any other suitable volume of solvent can be added.

[0048] The addition of solvents in such amounts can reduce variations and/or errors by largely overcoming the influence of stochastic factors such as, for example, influences caused by various trace substances in the aliquot. For example, an aliquot of serum may have trace amounts of alcohol that vary significantly relative to trace amounts of alcohol in a different aliquot of serum. Such significant relative differences may introduce variations that can affect the repeatability and reliability of the results achieved. By adding the solvent (in an amount to achieve the effects described above) prior to analyzing the mixture, and then adjusting the results to account for the concentration of the added solvent (e.g., to normalize the results by negating the effects on bound-and unbound-probe concentrations caused by the added solvent), the relative significance of such variations can be reduced. This reduction in relative significance can increase the repeatability and reliability of the results achieved by the present methods.

[0049] In some examples, the probe and the solvent can be mixed together prior to mixing either of the probe or solvent with the aliquot. In some examples, a surfactant may also be added to the mixture to promote, encourage, or assist the binding of probe to the carrier proteins in the aliquot of serum. In such examples, the surfactant may be added at any suitable stage or in any suitable manner, including, for example, to the aliquot alone, to the probe alone, to a mixture of only the aliquot and probe, or to the complete mixture of aliquot, probe, and solvent. Examples of suitable surfactants include nonionic detergents such as Tween 20 and Triton X-100, which may be available from suppliers such as Sigma-Aldrich, Inc., St. Louis, Missouri, U.S.A., www.sigmaaldrich.com. Some methods may include incubating the mixture (of probe, solvent, and aliquot of serum) for a period of time including, for example, greater than or less than about any of 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 minutes. Some methods may include agitation of the mixture (e.g., by shaking, or by shaking at between, greater than, less than, or between about 5 and 8 Hz for a period of time that can be separate from or at least partially (including wholly) concurrent with the incubation, including, for example, less than about any of 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 minutes. Additionally, some methods may include incubating and/or agitating the mixture at about a predetermined temperature such as, for example, 37° C (approximate human body temperature), or the like. In other methods, the mixture can be agitated and/or incubated for any suitable period of time, at any suitable frequency, and/or at any suitable temperature.

[0050] As described in more detail below, some methods include analyzing the mixture of the aliquot, probe, and solvent to determine the binding efficiency of carrier proteins including, for example, as a function of concentrations of residual-free and protein-bound fractions of the probe in the aliquot (adjusted for the added solvent by multiplying by the factor inversely proportional to solvent concentration). As an example, some methods include measuring the concentration of each of the protein-bound and unbound (free) fractions of the probe, and the binding efficiency derived from these concentrations (and adjusted for the change in concentration caused by the addition of the solvent). The presence of toxemia is indicated if, in the subject sample (aliquot), the binding efficiency of carrier proteins is reduced relative to a control range exhibited by carrier proteins in non-toxemic control subjects, and if the degree of reduction in
binding efficiency is higher than the reduction of carrier-protein concentration. In some methods, the reduction in binding efficiency can be normalized to account for the reduction in carrier-protein concentration, such that the normalized binding efficiency can be compared directly to the control range. The control subjects may be any one subject or group of subjects suitable for comparison, including, for example, one or more non-toxicemic ICU patients, non-toxicemic subjects, healthy subjects, or the like.

[0051] Investigations of solutions of carrier proteins have revealed that the binding constant $K_b$ (which may also be known in the art as the inverse of the dissociation constant or as the ratio of the association and dissociation coefficients) of carrier proteins (e.g., albumin) in hydrophilic solution, e.g., serum, with respect to hydrophobic substances (e.g., binding of long chain fatty acids to serum albumin), can be reduced by the addition of a solvent (e.g., alcohol), as illustrated by the linear function of the inverse of solvent concentration:

$$K_b = K' (S_c/S - 1)$$  \hspace{1cm} (1)

where $K_b$ is the binding constant, $S$ is the solvent concentration, $S_c$ is the critical concentration of the solvent in a solution at which the probe is completely soluble (will completely dissociate from the carrier proteins and dissolve in the solution), and $K'$ is the constant equal to binding constant $K_b$ in a solution containing the solvent at concentration of $S_c/2$. One possible explanation for this is that hydrophobic forces in hydrophilic solutions are functions of the entropy and the enthalpy of the solution, and the introduction of such solvents can modify each of the entropy and the enthalpy of the solution, in some instances, for example, as a linear function of solvent concentration.

[0052] Without the addition of solvent to the aliquot, other factors such as, for example, concentrations of various metabolites and serum proteins, temperature, pH variations, and the like, can influence the entropy of the water medium and cause significant variations in the hydrophobic forces in the aliquot. This can result in variations in the concentration of the unbound (free) fraction of the hydrophobic probe. In contrast, the addition of an amount of solvent that increases the concentration of unbound probe in the mixture (of aliquot, probe, and solvent) to at least 5 times greater than the concentration of unbound probe before the addition of the solvent (e.g., a mixture with only the aliquot and probe) can overcome the influence of stochastic factors on the aliquot, and thereby significantly reduce variations in measured results down, for example, to 20% or less.

[0053] Concentrations of protein-bound and unbound (free) fractions of the probe can be measured by any suitable methods or techniques. For example, the concentrations of protein-bound and unbound (free) fractions of the labeled probe in the mixture of the aliquot, probe, and solvent can be measured by: measurement of radioactivity (e.g., where the probe has a radioactive label, such as Carbon-13), fluorescence spectroscopy (e.g., where the probe has a fluorescent label), EPR-spectroscopy (e.g., where the probe has a spin label), luminescent spectroscopy (e.g., where the probe has a luminescent label), or the like. However, without the addition of polar solvent at a relatively low concentration in the aliquot, the concentration of unbound-free probe in the aliquot may be below a minimal concentration measurable with such methods of measurements. Some methods include mixing a solvent with the aliquot, where the amount (volume, concentration, or the like) is such that the solvent does not induce significant conformational changes to carrier proteins in the aliquot and/or significant dissociation of protein-bound metabolites and toxins. In some methods, the amount of solvent is such that the concentration of unbound probe in the mixture is increased to a level sufficient for accurate measurement by the method of measurement used. For example, in some methods, the amount of solvent mixed with the aliquot is the smallest amount necessary to increase the concentration of unbound probe in a mixture of probe, aliquot, and solvent to at least about five times greater than the concentration of unbound probe in a mixture of only the same probe and aliquot.

[0054] Some methods can include one or more intermediate or preparation steps for preparing the mixture after mixing the aliquot, probe, and solvent but before measuring the concentrations of protein-bound and unbound (free) fractions of the probe. For example, for probes having radioactive or fluorescent labels, the mixture can be incubated in a dialysis tube divided into two volumes by a membrane that is permeable to the probe but not permeable to carrier proteins in the mixture so as to separate the mixture into two portions, a first portion containing carrier proteins and unbound probe, and a second portion containing unbound probe but substantially no carrier proteins. After this incubation, the probe concentration can be measured for each of the carrier-protein-containing and carrier-protein-free portions of the mixture. Measurement of the concentrations of protein-bound probe and/or unbound probe depends of the type of labeled probe used. The first separated portion can be analyzed using either measurement of radioactivity or fluorescence, as appropriate to the type of labeled probe. If the measurement method is unsuitable for direct measurement of free probe (e.g., spectroscopy of some fluorescent labels), the second separated portion can be mixed with a portion of carrier proteins and the concentration of the previously-unbound probe analyzed similar to the portion of protein-bound probe as it is described above.

[0055] By way of another example, for a spin-labeled probe, the mixture can be analyzed using an EPR-spectrometer
to obtain an EPR spectrum of the probe such that the EPR spectrum is simultaneously indicative of different spectral components corresponding to protein-bound and free fractions of the probe. More specifically, spectral analysis of the EPR-spectrum can permit separation of single spectral components, identification of their relation to protein-bound and unbound fraction of spin probe, and approximation or estimation of their relative concentrations.

[0056] Binding efficiency (BE) of the carrier proteins is the concentration ratio of bound probe and unbound probe. In some embodiments of the present methods, binding efficiency is derived, calculated, or otherwise determined once the mixture of aliquot, probe, and solvent has reached binding equilibrium (with respect to the binding of probe to carrier proteins). Binding efficiency (BE) and/or BE parameters of serum carrier proteins can be calculated using the protein-bound probe concentration (B) and unbound probe concentration (F), and solvent concentration (S) in the mixture. For example, BE can be calculated with either:

\[
\frac{1}{BE} = (Sc / S - 1) \cdot (F / B) \cdot P \\
\]

OR

\[
\frac{1}{BE} = (Sc / S - 1) \cdot (F / B) \cdot P \cdot (N - B / P) \\
\]

where Sc (specified above), P is the concentration of carrier proteins in the sample, and N is the number of binding sites for the probe on the carrier protein molecule.

[0057] Formula (3), above, differs from Formula (2) by a factor (N-B/P) that adjusts for the effect of binding saturation. For some serum samples, in which carrier proteins are mostly presented by serum albumin, generally N=7 and the factor (N-B/P) is generally close to 6.

[0058] In some methods, a toxin evacuation parameter (TEP) can be derived, calculated, or otherwise determined for the carrier proteins, as an alternative or in addition to the binding efficiency BE. The toxin evacuation parameter can be indicative to the ability of the carrier proteins to bind toxins relative to the ability of the carrier proteins to release bound toxins, and can be calculated as the square of binding efficiency (BE):

\[
TEP = BE \cdot BE = BE^2 \\
\]

[0059] The present methods were performed for non-toxemic control patients to determine control values for non-toxemic (and/or healthy) patients, including control values such as mean and a range of normal variation. The mean BE of the control patients was assigned a value of 100%. The control range of BE variation in healthy control patients was between about 40% and about 160%. In some examples, the control range used for comparison to the BE of the subject patient may be any suitable value or subset range between about 40% and about 160% including, for example, between about 50% and about 150%, between about 40% and about 100%, or the like. In some examples, a control value may be used (where a subject patient’s BE below the control value indicated the presence or likely onset of toxemia). In such embodiments, the control value used may be any suitable value between about 20% and about 160%, including, for example, 20%, 25%, 30%, 35%, 40%, 43%, 45%, 50%, 55%, 60%, 65%, or any other suitable value in the disclosed range. In some examples, different control values can be used to diagnose, varying degrees of risk. For example, a BE above 40% can indicate a low risk of having or developing toxemia, a BE between 20% and 40% can indicate a moderate risk of having or developing toxemia, and a BE below 20% can indicate a high risk of having or developing toxemia. In some examples, BE can be calculated relative to (as a percentage of) the mean value of the control range.

[0060] As discussed above, the presence of toxemia can be indicated if, in the subject aliquot, the binding efficiency (BE) of carrier proteins is reduced relative to the control range or value, and if the degree of reduction in BE is higher than the reduction of carrier-protein concentration from a carrier-protein concentration that would be expected in control patients. For example, the concentration of carrier proteins may be reduced by the addition of solvent; and the accuracy of the derived BE can be improved if the derived BE is adjusted for this reduction. By way of another example, the concentration of carrier proteins may be reduced (relative to a healthy patient) in a subject patient with toxemia; and the accuracy of the derived BE can be improved if the derived BE is adjusted for this reduction. Reduction in carrier-protein concentration will not always be known exactly, and adjustment for reduction in carrier-protein concentration (that may result from several sources) can be approximated from various data, e.g., expected concentrations of carrier proteins for healthy and/or toxemic patients.

[0061] Some methods can comprise diagnosing a patient with toxemia response to, for example, the BE of the subject
Some methods may include using the results for triage purposes such as determining whether and when to assign in medical wards or toxic cleanup activities where the risk of exposure toxins may be high. Some methods can comprise testing or evaluating the health of a subject patient to assess the patient’s readiness or suitability for a task, assignment, and/or deployment, such as, for example, submarine duty, military deployment, specific line of employment, and/or specific tasks or group of tasks, deployment or assignment in medical wards or toxic cleanup activities where the risk of exposure toxins may be high.

Some methods may include using the results for triage purposes such as determining whether and when to treat various patients relative to each other. For example, where there are three patients, one with a BE of 2%, one with a BE of 10%, and one with a BE of 15%; the patient with a BE of 2% may be too ill to have a reasonable likelihood of recovery; and the patients with BEs of 10% and 20% may both have a reasonable likelihood of recovery, but the patient with a BE of 10% may have a more immediate need for treatment to capitalize on the reasonable likelihood of recovery. In such a situation, if resources are limited, the patient with a BE of 10% can be treated first, the patient with a BE of 20% can be treated second, and the patient with a BE of 2% can be treated last (if sufficient resources are available) or may be comforted with pain killers or the like.

Some methods can comprise evaluating the viability of donor organs (e.g., liver, kidney, or the like) by testing a sample of the donor’s blood serum or other extracellular fluid. Some methods can comprise evaluating the competence (health and/or functionality) of transplanted organs (e.g., liver, kidney, or the like) in patient’s body. Some methods may comprise evaluating a competence of patient’s protective (immune) system in response to exotoxins (e.g., snake venom, poisons, food poisoning, or the like). Some methods may comprise evaluating blood derivatives (e.g., donated whole blood, plasma, serum, or the like) for carrier-protein competence (health or functionality), carrier-protein deficiencies, and/or the like.

Example of EPR Spectroscopy

Some methods can use EPR spectroscopy to analyze the mixture of aliquot, probe, and solvent (some of which probe is generally bound to carrier proteins in the aliquot). One example of a suitable labeled probe is depicted in FIG. 4, which depicts a suitably labeled stearic acid molecule, 16-doxyl stearic acid. The mixture can be placed into the EPR spectrometer and exposed to both a high magnetic field and microwave power, and various properties (such as the properties described herein) of the mixture measured (directly or indirectly).

1. Binding of Spin-Labeled Probe to Carrier Proteins in the Sample

Spin-labeled fatty acid probes (e.g., as shown in FIG. 1A-1B) can be used to study carrier proteins by EPR spectroscopy. Spin-labeled fatty acid probes may also be referred to herein as “fatty acid probes” (and more generally “labeled probes” or “probes”). One exemplary procedure includes mixing an amount of labeled fatty acid probe (e.g., 16-doxyl stearic acid, a fatty acid labeled with a nitroxide radical) with a small (i.e., 50 μl) amount of serum or plasma. The molar ratio of the probe to carrier proteins (e.g., albumin) can be in the range between about 0.3 and about 1.5 so as to, for example, permit a level of binding that is sufficient to be representative of the binding ability of the carrier proteins in the subject patient’s body at the time the sample was taken from the subject patient while preventing saturation of the fatty acid binding sites on the carrier proteins. The labeled probe can also be mixed with a polar solvent such as, for example, ethanol. The binding affinity of carrier proteins for the labeled probe can be reduced by the ethanol to increase the number of unbound probe molecules in the mixture, as described above. After mixing the probe and solvent with the aliquot of serum, the resultant mixture can be incubated with constant agitation (e.g., at 5-8 Hz) for 10 min at 37°C.

This exemplary procedure is schematically depicted in FIG. 2. The embodiment of the present methods depicted in FIG. 2 includes: (1) placing the probe into a container, (2) mixing an aliquot of serum with the probe in the container, (3) mixing solvent with the aliquot and probe in the container, (4) placing the mixture (of aliquot, probe, and solvent) into a pipette (before or after incubation), (5) placing the pipette into, and analyzing the mixture with, the EPR spectrometer to obtain EPR spectra of the mixture, and (6) processing the measurements to obtain the concentrations of protein-bound and unbound probe, and determining the binding efficiency of the carrier proteins in the serum of the aliquot. The steps and/or order of steps depicted and/or described in this embodiment of the present methods are not intended to be limiting. Other embodiments of the present methods may omit any of these steps, and/or may include other steps,
may include any combination of these or other steps in any suitable order.

[0067] Interaction between the stearic acid probe and serum carrier proteins may be mostly specific to albumin. The affinity of albumin for 16-doxyl stearic acid (the labeled probe) may generally be similar to its affinity for unlabeled stearic acid (which may be relatively high, e.g., a binding constant of about 10^9 mol^{-1}). In blood serum of a healthy patient (and in the absence of binding site saturation), the abundance of albumin relative to other serum proteins and the presence of several high-affinity binding sites for long chain fatty acids may result in 99% or more of the stearic acid probe being bound exclusively to albumin.

2. Instrumentation

[0068] Following incubation of the probe with the sample, an amount of the mixture can be placed into a glass capillary tube. The tube can then be inserted into an EPR spectrometer (e.g., Model No. EPR 01-08 available from MedInnovation GmbH, Wildau, Germany). In the EPR spectrometer, the mixture is exposed to both a high magnetic field and microwave power. This exposure induces resonance of the spin label and absorption of microwave power. An EPR spectrum can thereby be generated by scanning measurements of the magnetic field strength and absorption of microwave power. Other EPR spectrometers, such as conventional X-Band EPR spectrometers, or other EPR spectrometers operating with a microwave frequency of approximately 9-10 GHz, can be also used for obtaining these measurements. The sample can be maintained at 37°C during the measurement process to mimic physiologic conditions.

3. Data processing

[0069] The EPR spectrum obtained with the spin probe can be analyzed using a simulation process. Simulation can be performed using least-square fitting of a model spectrum to the measured spectrum. In this way, the EPR spectrum of the spin probe can be calculated using the appropriate model and parameters of the site where the spin probe is situated.

[0070] The EPR spectrum obtained will generally consists of a large set of data points containing some amount of measurement noise or error. If the parameters of the binding site model are accurately established, an ideal experimental spectral curve can generally be derived. This task may be more complex when there are several sites that can bind the spin probe. In this situation, these different binding sites can be considered to improve accuracy when deriving the model spectrum. A number of different tools have been developed that enable the derivation of a composite model spectrum for compounds that possess several binding sites for the spin probe.

[0071] Analysis of the EPR spectrum generated from the stearic acid probe bound to albumin (as described above) reveals four distinct spectral components. The major portion of the spectrum is represented by two components, as represented by lines B and C in FIG. 3. Each of these major spectra components is representative of the portion of fatty acid probe bound to carrier proteins (e.g., albumin) (as may correspond to the pictorial representation of carrier proteins capable of binding with (to) the probe). The two remaining components are representative of free or unbound fatty acids present in the solution. The unbound fatty acids may be present singularly in solution, as represented by line D in FIG. 3 (as may correspond to the pictorial representation of a carrier protein that is saturated by toxins), or may be organized into clusters of fatty acid micelles, as represented by line E in FIG. 3. The process of simulation generally determines the values of ideal spectrum parameters representing the equation that provides the best curve fit of the simulated and measured spectra. These parameters can include the intensity of each spectral component as well as specific EPR parameters determining the position, width, and shape of spectrum lines.

[0072] Each EPR spectrum reflects the structural and functional characteristics of the protein that impact the binding of the probe to albumin. One technique that is employed for the generation of EPR spectra includes a sample mixture (of serum aliquot, probe, and solvent). The characteristics of albumin that can be assessed from the EPR spectrum that is generated can include the concentration of the fatty acid probe that is bound to albumin, and the concentration of unbound probe. Another characteristic that can be generated is an estimation of changes in protein conformation (significant conformational changes are prevented and/or minimized in the present methods) at the albumin binding site for fatty acids (certain parameters of the EPR spectrum indicate the mobility of the fatty acid probe at its binding site on albumin; such mobility can be influenced by several parameters, and those parameters can be used to prove an absence of significant conformational changes of albumin molecule due to excessive concentration of a polar solvent in a sample mixture).

Exemplary Data Obtained Through Testing of the Present Methods

[0073] The present methods have been tested on ICU-patients suffering from post-surgery diseases and on healthy subjects. The common methods employed in this testing are discussed first, and the results of this testing on several specific cases are discussed below. The samples tested were obtained at the intensive care unit (ICU) of the Blokhin Russian Oncological Scientific Center of the Russian Academy of Medical Science, Moscow, Russia. These samples
were then frozen at -30° C, and later investigated by the inventor of the present methods.

Blood serum was obtained by whole blood centrifugation. An aliquot of 50 μl of serum from each patient was used for each test. A spin probe of 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinioxy (purchased from Fluorochem Ltd., Derbyshire, UK) was mixed into the aliquot at a concentration of 0.58 mmol/l. A solvent, 10 μl of ethanol, was mixed into the aliquot. The mixture was then incubated for 10 min at 37° C with continuous agitation in a standard shaker operated at about 5-8 Hz.

After incubation, the probe was placed into a glass capillary (e.g., Model No. RM-40, available from KABE LABOTECHNIK GmbH, Numbrecht-Elsenroth, Germany).

The EPR spectrum of the mixture was then measured, as described below. The above-described capillary was placed into the resonator of an EPR spectrometer for spectroscopic analysis. The spectroscopy parameters were as follows: microwave power 15 mW at frequency 9.32 GHz; magnetic field 3325 G with scan range 120 G; modulation amplitude 2 G; data accumulation by three scans each with 4096 measured points and a sweep time 60 s. The capillary temperature was 37° C, and was controlled within +/- 0.2° C.

The EPR spectrum was analyzed by computer using an EPR-spectrum simulation with nonlinear least-squares fits. The spectrum model included five components. The first two are the S and W components which represent portions of the probe that were bound differently on carrier proteins. As shown here, the S and W components primarily differed by spectral parameters of A tensors (hyperfine splitting tensor): A∥(S) = 30.02 Oe, A⊥(S) = 9.02 Oe, All (W) = 21.5 Oe, and A⊥(W) = 13.35 Oe. Parameters of the g tensor used in the spectral calculations of these two components were: g∥(S) = 1.9983, g⊥(S) = 2.0019, g∥(W) = 1.9990, and g⊥(W) = 2.0013. The third F component represented the unbound spin probe residing free in the sample. The parameters of the F component were: A∥(F) = A⊥(F) = 15.6 Oe, and g∥(F) = g⊥(F) = 2.0008. The width of spectrum line for the F component, L(F) = 0.42 Oe, was significantly different from the width of the spectrum line for the S and W components, L(S) = L(W) = 3.45 Oe. The fourth and fifth components represented minor fractions of the probe that were not related to protein-bound or unbound-free probe, but to the probe molecules aggregated into micelles (M) and ones associated with free-lipids (P). The parameters of the M component were: A∥(M) = A⊥(M) = 0, g∥(M) = g⊥(M) = 2.0014, and L(M) = 11.96 Oe. The parameters of the P component were: A∥(P) = A⊥(P) = 14.2 Oe, g∥(P) = g⊥(P) = 2.0012, and L(P) = 1.1 Oe. The considering of the M and F components in the spectrum model can improve accuracy of the analysis of concentrations of protein-bound and unbound-free probes, but is not necessarily required in embodiments of the present methods.

During the EPR-spectrum simulation with nonlinear least-squares fits, relative concentrations of all spectral components as well as precise values of spectral parameters were determined. At the specified ethanol concentration, the majority of the (spin) probe, i.e., 90 to 99%, was found to be bound on carrier proteins (mostly on serum albumin, which generally makes up about 90% of the total carrier proteins in serum). The unbound fraction of the spin probe was found to have a relative concentration of 0.5 to 10%, and was found to vary somewhat among samples of different patients, and among different samples taken under different clinical conditions (clinical statuses) of the same patient.

FIG. 4 depicts the results of this analysis of binding efficiency of carrier proteins in serum of eight different patients (A - H, described below) suffering from post-surgery disease, observed during the time the patients were administered in the ICU.

**A Patient with septic shock**

Clinical diagnosis: Patient A was diagnosed with lymphoma, septic shock, acute respiratory failure, and acute kidney failure.

Microbiological data: Candida alb and Candida spp were discovered in pleural cavity.

From the time of Patient A entered the ICU, binding efficiency (BE) was drastically reduced to the range of about 2-10%, and remained very low (as illustrated by line A in FIG. 4). Dysfunction of the toxin evacuation of serum carrier proteins in Patient A was observed in the early stages of the sepsis-related toxemia. Patient A expired.

**B Patient with severe sepsis**

Clinical diagnosis: Patient B was diagnosed with severe sepsis, general peritonitis and intestinal haemorrhage.

Microbiological data: Pseudomonas aeruginosa was discovered in bronchoscopy and drainage.

BE for patient B was initially reduced and gradually decreased (as illustrated by line B in FIG. 4). Patient B expired.

**C Patient with sepsis**

Clinical diagnosis: Patient C was diagnosed with sepsis and hepatic failure.

Microbiological data: Cholangiostoma -E. faecium was discovered in drainage.

Binding efficiency (BE) was drastically reduced compared the control range. Specifically, BE for Patient C was

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reduced to about 10% as a result of sepsis-related toxemia (as illustrated by line C in FIG. 4). Patient C was treated with antibiotic therapy, and was significantly rehabilitated by the sixth (6th) day following admission to the ICU. BE for Patient C varied (and largely correlated to) the course of treatment, e.g., BE was lower initially and increased with the reduction of toxemia caused by the antibiotic therapy. Patient C was successfully discharged from the ICU on the tenth (10th) day after admission.

(D) Patient with severe sepsis

[0089] Clinical diagnosis: Patient D was diagnosed with severe sepsis, peritonitis, bilateral pneumonia, chronic renal failure, and liver failure.

[0090] Microbiological data: Sputum - Ps. aeruginosa, Acinetobacter, Candida alb were discovered.

[0091] Drastic reduction of binding efficiency (BE) was observed on the first day of admission to the ICU (as illustrated by line D in FIG. 4). Patient D was treated with antibiotic therapy, and was partially rehabilitated by the eighth (8th) day following admission to the ICU, however, at the ninth (9th) day, acute exacerbation from liver failure and thrombocytopenia occurred that debilitated the patient. Patient D expired.

(E) Patient who did not exhibit outward signs of toxemia

[0092] Clinical diagnosis: Patient E was diagnosed with partially compensated respiratory failure.

[0093] Binding efficiency (BE) remained relatively high (compared to toxemic patients) during the time of observation (as illustrated by line E in FIG. 4). Patient E was successfully discharged from the ICU on the fourth (4th) day after admission.

(F) Patient who did not exhibit outward signs of toxemia

[0094] Clinical diagnosis: Patient F did not show complications during the period of time of his administration in the ICU.

[0095] Binding efficiency (BE) remained relatively high (compared to toxemic patients) during the time of observation (as illustrated by line F in FIG. 4). Patient F was successfully discharged from the ICU.

(G) Patient who did not exhibit outward signs of toxemia

[0096] Clinical diagnosis: Patient G did not show complications during the period of time of his administration in the ICU.

[0097] Reducing binding efficiency (BE) observed at second day after admission, then parameter BE showed gradual increase during following period of time and remained relatively high (compared to toxemic patients) from fourth (4th) day on (as illustrated by line G in FIG. 4). Patient G was successfully discharged from the ICU on the sixth (6th) day after admission.

(H) Patient who did not exhibit outward signs of toxemia

[0098] Clinical diagnosis: Patient H diagnosed with nephropathy and partially compensated respiratory failure combined with Chronic Obstructive Pulmonary Disease.

[0099] Binding efficiency (BE) remained relatively high (compared to toxemic patients) during the time of observation (as illustrated by line H in FIG. 4). Patient H was successfully discharged from the ICU on the third (3rd) day after admission.

(J) Healthy person

[0100] Volunteer J observed to be without evidence of any diseases.

[0101] Binding efficiency (BE) for volunteer J is omitted from FIG. 4 for clarity. However, BE for volunteer J remained at approximately 120% at the time of observation.

(K) Healthy person

[0102] Volunteer K observed to be without evidence of any diseases.

[0103] Binding efficiency (BE) for volunteer K is omitted from FIG. 4 for clarity. However, BE for volunteer K remained at approximately 100% at the time of observation.

[0104] Analysis of the clinical relevance of the parameter BE (as measured for these patients with the present methods) indicated that significant reduction of binding efficiency (BE) indicated a high probability of a subject patient developing toxemia two days earlier than other known laboratory parameters and clinical indices. The changes in BE observed in
the course of patient administration in the ICU correlated with the clinical condition and the course of disease for every observed patient.

Some embodiments of the description include a kit including materials for performing the various steps of the present methods. In one example of a kit for detecting toxemia in a subject patient from a sample of the subject patient’s blood serum containing carrier proteins, the kit includes an amount of labeled probe and an amount of solvent. The amounts of probe and solvent are such that when mixed with aliquot of serum having about a predetermined volume (e.g., 50, 60, 70, 80, 90, 100, or more uL), the mixture will achieve the results described above, and/or have the proportions of ingredients described above.

The kit can include components for performing the various steps and/or portions of the present methods, as described herein. The kit can further include instructions for performing the various steps or portions of the present methods, as described above.

The methods can comprise or be limited to any combination of the steps and/or features characteristics described, unless the context explicitly or necessarily precludes the combination. For example, the method can include mixing probe with an aliquot and measuring the concentrations of bound and unbound probe; and another embodiment can include mixing probe and solvent with the aliquot, measuring the concentrations of bound and unbound probe, and normalizing the concentrations to substantially negate the changes in concentrations caused by the added. By way of another example, the kits can include a pipette and an amount of probe; another example can include an amount of probe and an amount of solvent; and another example can include an amount of probe, an amount of liquid solution, and an amount of solvent.

Claims

1. A method for detecting toxemia in a subject patient, comprising the steps of:

   (a) mixing a labelled 16-DOXYL-stearic acid probe having a site to bind to albumin with an aliquot of a subject patient’s extracellular fluid containing albumin, the amount of probe such that the molar ratio of the probe to the albumin is in the range of 0.3 to 1.5;
   (b) mixing ethanol with the mixture of the aliquot and the probe, wherein the ethanol when added to the mixture increases the solubility of the probe in the aliquot, and wherein the amount of ethanol added causes a portion of the probe to dissociate from the albumin without causing toxins to dissociate from the albumin;
   (c) analyzing the mixture comprising the aliquot, the probe, and the ethanol to determine the binding efficiency of the albumin in the patient’s extracellular fluid, wherein the binding efficiency is the concentration ratio of bound probe and unbound probe adjusted for the concentration of the added ethanol in the mixture and where the binding efficiency is calculated using the formula: 1/BE= (Sc/S-1) x (F/B) x P, where BE is binding efficiency. F is the concentration of unbound probe, B is the concentration of bound probe, Sc is the critical solvent concentration at which all probe is dissociated from the albumin and dissolved in the solution, S is the solvent concentration and P is the concentration of albumin; and
   (d) comparing the subject binding efficiency to at least one control binding efficiency of the albumin in the extracellular fluid of at least one non-toxemic control patient, wherein the amount of ethanol mixed with the aliquot and probe does not induce conformational changes in the albumin, and wherein the volume of ethanol mixed with the aliquot makes up less than 30% of the volume of the aliquot, and optionally less than 10% of the volume of the aliquot.

2. The method of claim 1, wherein step (c) comprises:

   (c1) measuring the concentrations of protein-bound and unbound probe in the mixture;
   (c2) deriving the subject binding efficiency of the albumin from at least the concentrations of the protein-bound and unbound probe; and optionally,
   (c3) deriving a subject toxin-evacuation parameter of the albumin as the square of the subject binding efficiency.

3. The method of any of claims 1 to 2, wherein the amount of ethanol mixed with the aliquot and probe increases the concentration of unbound probe in the mixture of the aliquot, the probe, and the ethanol to at least 5 times greater than the concentration of unbound probe in a mixture of the aliquot and the probe without the ethanol.

4. The method of any one of claims 1 to 3, further comprising the step of:
(e) normalizing the subject binding efficiency to account for the reduction in albumin concentration caused by
the ethanol in the mixture;
optionally, wherein steps (c2) and (e) are performed simultaneously.

5. The method of any one of claims 1 to 4, further comprising the step of:

(f) normalizing the subject binding efficiency to account for the reduction in albumin concentration caused by
medical conditions of the patient.

6. The method of any one of claims 1 to 5, wherein the at least one control binding efficiency comprises a range of
control binding efficiencies of the albumin in the extracellular fluid of each of a plurality of control patients.

7. The method of claim 2, further comprising:

(e1) repeating steps (a), (b), (c1), and (c2) for each of two or more aliquots, wherein a different amount of ethanol
is used for each of the two or more repetitions;
optionally, wherein the subject binding efficiencies derived for the two or more repetitions are averaged to derive
an average subject binding efficiency, and wherein the average subject binding efficiency is utilized in step (d).

8. The method of any one of claims 1 to 7, wherein the volume of the aliquot is less than 100 μL, and optionally, less
than 50 μL.

9. The method of any one of claims 1 to 8, wherein the probe is labelled with a spin-label, a radioactive label, or a
fluorescent label.

10. The method of any one of claims 1 to 9, wherein the extracellular fluid includes: blood serum, blood plasma, lymph
fluid, or spinal fluid.

11. The method of any one preceding claim, where the binding efficiency is calculated using the formula: 1/BE=(Sc/S-
1) x (F/B) x P x (N-B/P), where BE is binding efficiency, F is the concentration of unbound probe, B is the concentration
of bound probe, Sc is the critical solvent concentration at which all probe would be dissociated from the albumin
and dissolved in the solution, P is the concentration of albumin, S is the solvent concentration and N is the number
of binding sites for the probe on the albumin molecule.

Patentansprüche

1. Ein Verfahren zum Nachweis einer Toxämie bei einem Patienten, bestehend aus den folgenden Schritten:

(a) der Mischung einer markierten 16-DOXYL-Stearinsäure-Sonde, die eine Stelle zur Anbindung an Albumin
aufweist, mit einem Aliquot eines Albumins, das Extrazellulärflüssigkeit eines Patienten enthält, die Sonden-
menge ist dabei so, dass das Volverhältnis der Sonde zum Albumin im Bereich von 0,3 bis 1,5 liegt;
(b) der Mischung von Ethanol mit der Mischung des Aliquot und der Sonde, wobei das Ethanol, wenn es der
Mischung beigefügt wird, die Löslichkeit der Sonde im Aliquot erhöht und wobei die Menge des hinzugefügten
Ethanols dazu führt, dass sich ein Teil der Sonde vom Albumin dissoziiert, ohne dass sich Toxine vom Albumin
dissoziierten;
(c) der Analyse der Mischung, bestehend aus dem Aliquot, der Sonde und dem Ethanol, um die Bindungseffizienz
des Albumin in der Extrazellulärflüssigkeit des Patienten zu bestimmen, wobei die Bindungseffizienz das Konz-
zentrationsverhältnis von gebundener und ungebundener Sonde, angepasst an die Konzentration des hinzu-
gefügten Ethanols in der Mischung, bedeutet und wobei die Bindungseffizienz unter Verwendung der folgenden
Formel berechnet wird: 1/BE= (Sc/S-1) x (F/B) x P, wobei BE die Bindingseffizienz, F die Konzentration der
ungebundenen Sonde, B die Konzentration der gebundenen Sonde, Sc die kritische Lösungskonzentration ist,
bei der alle Sonden vom Albumin dissoziiert und in der Lösung gelöst werden, S ist die Lösungskonzentration
und P die Konzentration des Albumin; und
(d) dem Vergleich der Patienten-Bindungseffizienz mit mindestens einer Kontroll-Bindungseffizienz des Albumin
in der Extrazellulärflüssigkeit mindestens eines nicht-toxämischen Kontrollpatienten,
wobei die mit dem Aliquot und der Sonde gemischte Menge des Ethanols nicht die konformativen Änderungen
im Albumin blockiert, und
wobei das Volumen des mit dem Aliquot gemischten Ethans weniger als 30% des Aliquot-Volumens und optional weniger als 10% des Aliquotvolumens ausmacht.

2. Das Verfahren in Anspruch 1, wobei Schritt (c) Folgendes umfasst:
   (c1) die Messung der Konzentrationen der protein-gebundenen und ungebundenen Sonde in der Mischung;
   (c2) die Ableitung der Patienten-Bindungseffizienz des Albumin von mindestens den Konzentrationen der protein-gebundenen und ungebundenen Sonde; und optional,
   (c3) die Ableitung eines Toxin-Evakuierungs-Parameters des Albumin im Patienten als Quadrat der Patienten-Bindungseffizienz.

3. Das Verfahren in Anspruch 1 bis 2, wobei die mit dem Aliquot und der Sonde gemischte Ethanol-Menge die Konzentration der ungebundenen Sonde in der Mischung aus Aliquot, Sonde und Ethanol um mindestens das 5-fache gegenüber der Konzentration der ungebundenen Sonde in einer Mischung des Aliquot und der Sonde, ohne Ethanol, erhöht.

4. Das Verfahren eines der Ansprüche 1 bis 3, darüberhinaus bestehend aus dem folgenden Schritt:
   (e) der Normalisierung der Patienten-Bindungseffizienz, die sich durch die Reduzierung in der Albumin-Konzentration, aufgrund des Ethans im Gemisch, erklärt; optional, wobei die Schritte (c2) und (e) gleichzeitig ausgeführt werden.

5. Das Verfahren eines der Ansprüche 1 bis 4, darüberhinaus bestehend aus dem folgenden Schritt:
   (f) der Normalisierung der Patienten-Bindungseffizienz, die sich durch die Reduzierung in der Albumin-Konzentration, aufgrund des medizinischen Zustandes des Patienten, erklärt;

6. Das Verfahren eines der Ansprüche 1 bis 5, wobei die mindestens eine Kontroll-Bindungseffizienz einen Bereich für die Kontroll-Bindungseffizienz des Albumin in der Extrazellulärfüssigkeit jedes einer Vielzahl von Kontroll-Patienten umfasst.

7. Das Verfahren in Anspruch 2, darüberhinaus bestehend aus:
   (e1) der Wiederholung der Schritte (a), (b), (c1) und (c2) für zwei oder mehr Aliquote, wobei eine unterschiedliche Menge Ethanol für jede der zwei oder mehr Wiederholungen verwendet wird; optional, wobei die für die zwei oder mehr Wiederholungen abgeleiteten Patienten-Bindungseffizienzen gemittelt werden, um eine durchschnittliche Patienten-Bindungseffizienz abzuleiten, und wobei der Durchschnitt der Patienten-Bindungseffizienz in Schritt (d) verwendet wird.

8. Das Verfahren eines der Ansprüche 1 bis 7, wobei das Volumen des Aliquot unter 100 µL und optional unter 50 µL liegt.

9. Das Verfahren eines der Ansprüche 1 bis 8, wobei die Sonde mit einem Spin-Label, einem radioaktiven Label oder einem fluoreszierenden Label markiert ist.

10. Das Verfahren eines der Ansprüche 1 bis 9, wobei die Extrazellulärfüssigkeit Folgendes einschließt: Blutserum, Blutplasma, Lymphflüssigkeit oder Rückenmarksflüssigkeit.

11. Das Verfahren eines der vorhergehenden Ansprüche, wobei die Bindungseffizienz unter Verwendung der folgenden Formel berechnet wird: BE = (Sc/(S-1)) x (F/B) x P x (N-B/P), wobei BE die Bindungseffizienz, F die Konzentration der ungebundenen Sonde, B die Konzentration der gebundenen Sonde, Sc die kritische Lösungskonzentration ist, bei der alle Sonden vom Albumin dissoziiert und in der Lösung gelöst werden, P ist die Konzentration des Albumin, S die Lösungskonzentration und N die Anzahl der Bindungsstellen für die Sonde am Albumin-Molekül.

**Revendications**

1. Un procédé de détection de la toxémie chez un patient sujet, comprenant les opérations suivantes :
(a) le mélange d'une sonde 16-DOXYL-acide stéarique marquée possédant un site à lier à de l'albumine avec une aliquote d'un fluide extracellulaire d'un patient sujet contenant de l'albumine, la quantité de sonde étant telle que le rapport molaire de la sonde sur l'albumine se situe dans la plage de 0,3 à 1,5,
(b) le mélange d'éthanol avec le mélange de l'aliquote et de la sonde, où l'éthanol, lorsqu'il est ajouté au mélange, augmente la solubilité de la sonde dans l'aliquote et où la quantité d'éthanol ajoutée amène une partie de la sonde à se dissocier de l'albumine sans amener des toxines à se dissocier de l'albumine,
(c) l'analyse du mélange contenant l'aliquote, la sonde et l'éthanol de façon à déterminer l'efficacité de liaison de l'albumine dans le fluide extracelulaire du patient, où l'efficacité de liaison est le rapport de concentration de sonde liée et de sonde non liée ajusté en fonction de la concentration de l'éthanol ajouté dans le mélange et où l'efficacité de liaison est calculée au moyen de la formule : \( 1/BE = (Sc/S-1) \times (F/B) \times P \), où BE est l'efficacité de liaison, F est la concentration de sonde non liée, B est la concentration de sonde liée, Sc est la concentration de solvant critique à laquelle la totalité de la sonde est dissociée de l'albumine et dissoute dans la solution, S est la concentration de solvant et P est la concentration d'albumine, et
(d) la comparaison de l'efficacité de liaison du sujet à au moins une efficacité de liaison témoin de l'albumine dans le fluide extracelulaire d'au moins un patient témoin non toxémique,

2. Le procédé selon la Revendication 1, où l'opération (c) comprend :
   (c1) la mesure des concentrations de sonde liée à une protéine et de sonde non liée dans le mélange,
   (c2) la dérivation de l'efficacité de liaison du sujet de l'albumine à partir d'au moins les concentrations de la sonde liée à une protéine et de la sonde non liée, et éventuellement,
   (c3) la dérivation d'un paramètre d'évacuation de toxine chez le sujet de l'albumine sous la forme du carré de l'efficacité de liaison du sujet.

3. Le procédé selon l'une quelconque des Revendications 1 à 2, où la quantité d'éthanol mélangée à l'aliquote et à la sonde augmente la concentration de sonde non liée dans le mélange de l'aliquote, de la sonde et de l'éthanol jusqu'à au moins 5 fois la concentration de sonde non liée dans un mélange de l'aliquote et de la sonde sans éthanol.

4. Le procédé selon l'une quelconque des Revendications 1 à 3, comprenant en outre l'opération suivante :
   (e) la normalisation de l'efficacité de liaison du sujet de façon à prendre en compte la réduction de concentration en albumine provoquée par l'éthanol dans le mélange, éventuellement, où les opérations (c2) et (e) sont exécutées simultanément.

5. Le procédé selon l'une quelconque des Revendications 1 to 4, comprenant en outre l'opération suivante :
   (f) la normalisation de l'efficacité de liaison du sujet de façon à prendre en compte la réduction de concentration en albumine provoquée par des états pathologiques du patient.

6. Le procédé selon l'une quelconque des Revendications 1 à 5, où la au moins une efficacité de liaison témoin comprend une plage d'efficacités de liaison témoins de l'albumine dans le fluide extracellulaire de chaque patient d'une pluralité de patients témoins.

7. Le procédé selon la Revendication 2, comprenant en outre :
   (e1) la répétition des opérations (a), (b), (c1) et (c2) pour chaque aliquote parmi les deux ou plus aliquotes, où une quantité différente d'éthanol est utilisée pour chaque répétition des deux ou plus répétitions, éventuellement, où les efficacités de liaison du sujet dérivées pour les deux ou plus répétitions sont moyennées de façon à dériver une efficacité de liaison du sujet moyenne, et où l'efficacité de liaison du sujet moyenne est utilisée à l'opération (d).

8. Le procédé selon l'une quelconque des Revendications 1 à 7, où le volume de l'aliquote est inférieur à 100 µL, et éventuellement inférieur à 50 µL.
9. Le procédé selon l’une quelconque des Revendications 1 à 8, où la sonde est marquée avec un marqueur de spin, un marqueur radioactif ou un marqueur fluorescent.


11. Le procédé selon l’une quelconque des Revendications précédentes, où l’efficacité de liaison est calculée au moyen de la formule : \( \frac{1}{BE} = \frac{(Sc/S-1)}{F/B} \times P \times (N-B/P) \), où BE est l’efficacité de liaison, F est la concentration de sonde non liée, B est la concentration de sonde liée, Sc est la concentration de solvant critique à laquelle la totalité de la sonde serait dissociée de l’albumine et dissoute dans la solution, P est la concentration d’albumine, S est la concentration de solvant et N est le nombre de sites de liaison pour la sonde sur la molécule d’albumine.
a) 16-DOXYL-stearic acid, free radical

b) 5-DOXYL-stearic acid, free radical

c) 16:0-16 PC DOXYL, free radical (1-Palmitoyl-2-Stearoyl-(16-DOXYL)-sn-Glycero-3-Phosphocholine)

FIG. 1A
d) 18:0 PC C13 (1,2-Distearoyl[1-13C]-sn-Glycero-3-Phosphocholine), Carbon-13 labeled

\[
\text{CH}_3(\text{CH}_2)_9\text{CH}_2^\text{13C} = \text{13C}^\text{13C} \cdots \text{13C} = \text{13C}^\text{13C} \cdots \text{13C}^\text{13C} \text{CH}_3
\]

\[
\text{O} - \text{H}
\]

\[
\text{e) Oleic acid-1,2,3,7,8,9,10-13C}_7, \text{ Carbon-13-labeled}
\]

f) 12-N-Methyl-7-nitrobenzo-2-oxa-1,3-diazolamino stearate [12-NBDS or NBD-stearate], fluorescent probe (Fluorescent labeled stearic acid). CAS Registry: 117056-67-4

\[
\text{HO}
\]

\[
\text{g) 12-(9-anthroyl)stearic acid [anthroylstearic acid], fluorescent probe (Fluorescent labeled stearic acid). CAS Registry: 37469-99-1}
\]

**FIG. 1B**
FIG. 4
REFERENCES CITED IN THE DESCRIPTION

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