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Maggio

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[54] **CHEMICALLY INDUCED FLUORESCENCE IMMUNOASSAY**

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[58] Field of Search ..... 23/230 B; 424/8, 12; 195/103.5 A, 103.5 L; 435/7, 8

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[57] **ABSTRACT**

A competitive protein binding method is provided for the determination of an analyte which is a member of an immunological pair consisting of ligand and receptor for the ligand. A chemiluminescent source is employed comprised of one or more individual members, one chemiluminescent source member being conjugated to one of the members of the immunological pair, so as to provide chemiluminescence adjacent to the site of conjugation. A quencher molecule is conjugated to a member of the immunological pair. When the members of the immunological pair bind, the quencher molecule is brought within quenching distance of the chemiluminescent source so as to inhibit the emission of light by the chemiluminescent source. The amount of analyte present in the assay medium affects the amount of binding between the members of the immunological pair which results in quenching of the chemiluminescence. By observing the light emitted from the assay medium, either from the chemiluminescent source of the quencher, the change in light emission in relation to the concentration of analyte present in the assay medium can be used to determine the amount of analyte present in the assay medium. By employing standards having known amounts of analyte, the amount of analyte in an unknown sample can be quantitatively determined.

Reagent kits can be provided having predetermined amounts of the reagents, so as to substantially optimize the sensitivity of the assay.

32 Claims, No Drawings

CHEMICALLY INDUCED FLUORESCENCE IMMUNOASSAY

BACKGROUND OF THE INVENTION

1. Field of the Invention

The clinical diagnostic field has seen a broad expansion in recent years, both as to the variety of materials which may be readily and accurately determined, as well as the methods for the determination. One broad category of techniques involves the use of an organic receptor which is able to specifically bind to a particular spatial and polar organization of another molecule. For the most part, these compounds are antibodies, which are able to distinguish between the compound or composition of interest, and other compounds of analogous structure. By virtue of the binding of the receptor to a labeled ligand, one is able to distinguish between labeled liqand which is bound to receptor and unbound lageled ligand.

The observed effect of binding by the receptor will depend upon the label. In some instances, the binding of the antibody merely provides for a differentiation in molecular weight between bound and unbound labeled ligand. In other instances, the presence of the receptor may affect the nature of the signal obtained from the label, so that the signal varies with the amount of receptor bound to labeled ligand. A further variation is that the receptor is labeled and the ligand unlabeled. Where receptors are labeled with two different labels which interact when in close proximity, the amount of ligand present affects the degree to which the labels on the receptor may interact.

In developing an assay, there are many considerations. One consideration is the signal response to changes in the concentration of analyte. A second consideration is the ease with which the protocol for the assay may be carried out. A third consideration is the variation in interference from sample to sample. Ease of preparation and purification of reagents, availability of equipment, ease of automation, and interaction with ligands, are additional considerations, which do not exhaust the various concerns in developing a useful assay.

There is therefore a continuing need for new and accurate techniques which can be adapted for a wide spectrum of different ligands or be used in specific cases where other methods may not be readily adaptable.

2. Brief Description of the Prior Art

U.S. Pat. No. 3,709,868 is exemplary of a radioimmunoassay. U.S. Pat. No. 3,960,834 is exemplary of a spin immunoassay. U.S. Pat. No. 3,654,090 and German Auslengungsschrift No. 2,223,385 are exemplary of enzyme immunoassays. Articles of interest include an article by Ludwig Brand and James R. Gohlke, Annual Review of Biochemistry, 41, 843–868 (1972) and Stryer, Science, 162, 526 (1968). Smith, FEBS Letters 77, 25, (1977) describes a fluorescent immunoassay, where thyroxine is bound to a fluorescer and quenches the fluorescer, the quenching being reversed by binding of antibody to thyroxine. See also, Ullman et al, J. Biol. Chem. 251, 4172 (1976).

An excellent review of chemiluminescence may be found in McCapra, Quarterly Reviews 20, 485 (1966).

SUMMARY OF THE INVENTION

A competitive protein binding assay is provided having as an analyte a member of an immunological pair

which consists of ligand and receptor for the ligand. The assay is predicated on the presence of the analyte in an assay medium affecting the degree to which a chemiluminescence source is quenched by energy transfer to a quencher, at relatively long distances. By conjugating the chemiluminescence source or where the chemiluminescence source requires a plurality of components, one component of the chemiluminescence source, with a member of the immunological pair and conjugating a quencher with a member of the immunological pair, reagents can be prepared which when combined in the assay medium will provide varying degrees of light emission, depending upon the amount of analyte present in the assay medium.

In particular, the chemiluminescence source or component thereof and the quencher may be conjugated to either the ligand or the receptor and the resulting reagent combined in an aqueous, normally buffered medium at a mild temperature, and the amount of light emitted determined. By comparison with assay media having known amounts of analyte, a quantitative relationship can be developed between the quanta of emitted light and the amount of analyte in the assay medium.

Kits can be provided, where the reagents are included in premeasured amounts, so that they may be used directly or may be readily diluted to assay reagent solutions to provide concentrations which substantially optimize the sensitivity and performance of the assay.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

In accordance with the subject invention, chemiluminescence is employed to provide a signal related to the amount of analyte in an assay medium. The analyte is a member of an immunological pair which includes liqand and receptor. By conjugating the chemiluminescence source or where the source is comprised of more than one component, one component of the chemiluminescence source, with a member of the immunological pair and a quencher with a member of the immunological pair, the presence of analyte affects the amount of quencher which is within quenching distance of the conjugated chemiluminescence source. By combining the chemiluminescence source reagent and the quencher reagent where the two labels are on different molecules, and additional immunological pair members, as required, with the analyte in an assay medium, including any ancillary reagents necessary for the chemiluminescence, and determining the amount of light emitted from the assay medium, at a particular wavelength or a range of wavelengths from the assay medium, in relation to an assay medium having a known amount of analyte, the amount of analyte in the sample can be determined.

The method is predicated on the observation that when a dye is within a limited distance from a chemiluminescer in the excited state, the chemiluminescer may transfer its energy to the quencher without collision and without emitting radiation. The quencher may then emit radiation of a higher wavelength than the chemiluminescer or may lose the energy by radiationless decay. One can conjugate the member of the chemiluminescence source and the quencher to either ligand or receptor, so that when the two conjugates are brought together the amount of quencher within quenching distance of the chemiluminescer is affected by the amount of analyte present in the assay medium.

The nature and amount of light emitted from the assay medium will therefore be a function of the analyte present in the assay medium. By performing assays with known amounts of analyte, one can develop a quantative relationship between the amount of analyte in the assay medium and the amount of radiation emitted from the assay medium at one or more wavelengths.

Definitions

Analyte—the compound or composition to be measured, which may be a ligand which is mono- or polyepitopic, antigenic or haptenic, a single or plurality of compounds which share at least one common epitopic site or a receptor.

Ligand—any compound for which a receptor naturally exists or can be prepared.

Ligand analog—a modified ligand which can compete with the analogous ligand for receptor, the modification providing means to join to a label or to a hub nucleus.

Poly(ligand analog)—a plurality of ligand analogs joined together covalently, normally to a hub nucleus, to provide a compound having a plurality of epitopic sites capable of competing with the analogous ligand for receptor.

Label—either a component of a chemiluminescence source or a quencher dye, which form a light emitting reciprocal pair, where the quencher dye has a high transition probability of absorbing energy from the chemiluminescence source.

(a) chemiluminescer label—a compound which by itself or in combination with other compounds produces a molecule in an electronically excited state, which molecule can decay to a lower energy state by the emission of light and the total process results in a chemical change in one or more of the compounds.

(b) quencher—a molecule capable of inhibiting the chemiluminescent emission of light, when within a short but non-colliding distance, usually less than about 100 A, of the chemiluminescer molecule, by accepting the energy which would otherwise be emitted as chemiluminescent light. In effect, the quencher need not be the nearest neighbor to the chemiluminescer to effect quenching.

Label-conjugate—the label, either a compound of the chemiluminescence source or the quencher, is bonded, either by a bond or linking chain, to a member of the immunological pair but not both to the same molecule. The conjugate will have at least one label and may have a plurality of labels bonded to the member of the immunological pair or a plurality of such members bonded to the label or a plurality of ligands and labels i.e. poly(ligand analog)-polylabel. In particular, where an enzyme is the component of the chemiluminescence source employed as the label, a plurality of ligand analogs may be conjugated to the enzyme to form a poly(ligand analog) label.

Receptor—any compound or composition capable of recognizing a particular spatial and polar organization of a molecule i.e. epitopic site. Illustrative receptors include naturally occurring receptors, antibodies, enzymes, lectins, Fab fragments and the like. The receptor may be monovalent or polyvalent in receptor sites usually polyvalent e.g. antibodies. For any specific ligand, the receptor will be referred to as "antiligand". The receptor-antiligand-and its reciprocal liqand form an immunological pair.

Poly(ligand analog)-label—a composition in which a plurality of ligand analogs and one or a plurality of labels are bonded together whereby the ligand analog and label are in juxtaposition, so that when receptor is bound to ligand analog, label on the labeled receptor is in within quenching distance of the reciprocal label. Where an enzyme is part of the chemiluminescence source and the ligand is haptenic, a plurality of ligand analogs may be bonded to the enzyme. Alternatively, a plurality of ligand analogs and one or more labels may be conjugated to a water soluble polyfunctionalized hub nucleus.

Assay

The subject assay is carried out in an aqueous, normally homogeneous, zone normally, but not necessarily at a moderate pH, generally close to optimum assay sensitivity. The assay zone for the determination of analyte is prepared by employing in an appropriate assay solution, usually buffered, the unknown sample, which may have been subject to prior treatment, the chemiluminescer labeled reagent and the quencher labeled reagent (includes poly(ligand analog)-polylabel), and as appropriate ligand or antiligand.

The presence of antiligand or ligand in combination with a predetermined amount of antiligand in the assay medium controls the degree to which the quencher comes within quenching distance of the chemiluminescer.

There are four basic variations in the preparation of the quencher and chemiluminescer reagents. The four variations are:

(1) chemiluminescer conjugated to ligand as chemiluminescer labeled ligand and quencher conjugated to receptor as quencher labeled antiligand;

(2) quencher conjugated to ligand as quencher labeled ligand and chemiluminescer conjugated to receptor as chemiluminescer labeled antiligand; and

(3) chemiluminescer conjugated to receptor as chemiluminescer labeled antiligand and quencher conjugated to receptor as quencher labeled antiligand.

(4) chemiluminescer conjugated to ligand as chemiluminescer labeled ligand and quencher conjugated to ligand as quencher labeled ligand.

With the first two combinations, when the reagents are combined, the quencher will be in quenching distance of the chemiluminescer. The presence of analyte, either ligand or antiligand, will serve to reduce the amount of energy transfer between the chemiluminescer and quencher by diminishing the number of quencher molecules within quenching distance of the chemiluminescer. In the third combination, a polyepitopic ligand (includes poly(ligand analog)) must be added for either antiligand or monoepitopic ligand as analyte. Where the ligand is polyepitopic, increased quenching will be observed as the concentration of the polyepitopic ligand increases to a maximum quenching, followed by decreased quenching as the concentration of polyepitopic ligand continues to increase. Thus, a biphasic response is obtained, so that one must know on which portion of the curve one is operating in order to obtain a discrete result. By contrast, with poly(ligand analog), the presence of monoepitopic ligand will serve to diminish quenching. With receptor as analyte, increasing concentrations of receptor will also serve to diminish quenching.

Where the chemiluminescer and the quencher are both conjugated to ligand, an assay for either ligand or polyvalent antiligand may be performed. Where the assay is for ligand, the two label-conjugates are employed in conjunction with antiligand which brings the chemiluminescer and quencher together into quenching distance of each other. The addition of ligand reduces the amount of chemiluminescer and label which are within quenching distance. For the determination of antiligand the two label-conjugates are employed. With increasing amounts of antiligand, there will be a decrease of chemiluminescence to a minimum and then an increase as the concentration of antiligand increases. If one is uncertain as to which portion of the biphasic curve is involved, one or more sample dilutions will indicate the particular concentration.

It should be understood, that in referring to quenching, all that is intended is that there be transfer of energy from the chemiluminescer to the quencher. The result of this transfer will be that light of a single or range of wavelengths which might otherwise have been emitted by the chemiluminescer will be transferred to the quencher, which may then fluoresce, emitting light of a higher wavelength than the energy absorbed. Depending upon the quantum efficiency of emission of the chemiluminescer, the efficiency of energy transfer from the chemiluminescer to the quencher, and the quantum efficiency of emission of the quencher, as well as the wavelength range which is monitored, one may observe greater or lesser amounts of light due to the quenching. Therefore, when referring to quenching, it is not intended that there necessarily be a diminution of the signal which is observed. In fact, if one is observing the light emitted by the quencher, increasing quenching will result in an increasingly large signal.

A special situation exists with small haptens, those of from about 125 to 2000 molecular weight. With these haptens a substantially reduced chemiluminescence can be achieved i.e. quenching without quencher bonded to receptor, particularly where the receptor is an antibody. While the reduction in signal will not be as great as when quencher is conjugated to receptor, a sufficient reduction may be achieved to have an acceptable assay. Except for using receptor without quencher, the assay will be performed in the same manner, reading the light emitted by the chemiluminescer.

In carrying out the assay, an aqueous medium will normally be employed. Other polar solvents may also be employed, usually oxygenated organic solvents of from one to six, more usually from one to four carbon atoms, including alcohols, ethers and the like. Usually, these cosolvents will be present in less than about 40 weight percent, more usually in less than about 20 weight percent.

The pH for the medium will usually be in the range from about 5 to 12, more usually in the range from about 7 to 10, and when enzymes are employed as part of the chemiluminescence source, 7 to 9. Various buffers may be used to achieve the desired pH and maintain the pH during the determination. Illustrative buffers include borate, phosphate, carbonate, Tris, barbital and the like. The particular buffer employed is not critical to this invention, but in individual assays, one buffer may be preferred over another.

Moderate temperatures are normally employed for carrying out the assay and usually constant temperatures during the period of the assay will be employed. The temperatures will normally range from about 10° to 50° C., more usually from about 15° to 40° C.

The concentration of analyte which may be assayed will generally vary from about 10^{-4} to 10^{-15} M, more usually from about 10^{-6} to 10^{-13} M. Stated another way, the concentration ranges of interest will generally be from about 10^{-3} to 10^{-14} g/ml.

In addition to the concentration range of analyte of interest, considerations such as whether the assay is qualitative, semi-quantative or quantitative, the equipment employed, and the characteristics of the reagents will normally determine the concentration of the reagents. While the concentration of analyte will determine the range of concentrations of the other reagents, normally to optimize the sensitivity of the assay, individual reagent concentrations will be determined empirically. Since the binding constant and binding profile of receptors will vary, for example, with antibodies from bleed to bleed, each new batch of antibodies may require different concentration ratios for the different reagents.

Normally, for mono- and polyepitopic ligand analytes, the concentration of antiligand based on binding sites will be about equal to the minimum concentration of interest based on binding sites and not more than about 50 times the maximum concentration of interest based on binding sites, usually about 1 to 10 times, and more usually about 1 to 3 times the maximum concentration of interest based on binding sites.

For polyepitopic ligand receptor analytes, the equivalent ratios of labeled ligand or ligand to receptor analyte will generally be in the range of about 0.01 times the minimum concentration of interest and not more than about 100 times the maximum concentration of interest based on binding sites. The labeled receptor employed in conjunction with the labeled ligand or ligand will generally be present in from about 0.01 to 100 times the concentration of ligand or labeled ligand based on binding sites.

For polyepitopic ligand analytes, where labeled ligand is employed, the concentration of labeled ligand will generally be not less than about 10^{-4}, more usually not less than about 10^{-2} times the minimum concentration of interest and usually in the range of about equal to the minimum concentration of interest and not exceeding about the maximum concentration of interest. The ratio of labeled receptor will generally be not less than about 0.1 times the concentration of labeled ligand based on binding sites and not greater than about 100 times the concentration of labeled ligand based on binding sites.

For monoepitopic ligand analytes and monoepitopic ligand receptor analytes, when employing labeled ligand (includes poly(ligand analog)-label), the concentration of labeled ligand based on binding sites will usually be not less than 10^{-4} times the minimum concentration of interest, more usually not less than 10^{-2} times the minimum concentration of interest and usually in the range of about the minimum concentration of interest to the maximum concentration of interest. When poly(ligand analog) is employed with labeled antiligand, the concentration of poly(ligand analog) will fall within the same ranges as indicated for the labeled ligand and the concentration of antiligand has been indicated previously.

The order of addition of the various reagents may vary widely, depending upon whether an equilibrium or rate measurement is involved, the nature of the rea-

gents, the rate at which equilibrium is achieved between the ligand and antiligand, and the nature of the chemiluminescence source. Where the chemiluminescence source has a plurality of components, with one of the components being a label, the chemiluminescence can be initiated at any time by the addition of the other components of the chemiluminescence source. In those situations where the chemiluminescence source involves more than one component, the labeled reagents and the unknown may be combined simultaneously, followed by the addition of the other components of the chemiluminescence source. Alternatively, one could combine the analyte with the labeled antiligand, followed by the addition of labeled ligand, as appropriate, followed by the addition of the remaining components of the chemiluminescence source. The various additions may be interrupted by incubation. In those instances where the chemiluminescence source is a single component, normally the labeled receptor will be combined with the analyte, followed by the addition of the labeled ligand, as appropriate.

Depending on the mode employed, equilibrium or nonequilibrium, the rate of binding of the antiligand to ligand and labeled ligand and the relative concentrations of the ligand, labeled ligand and labeled antiligand, one or more incubation steps may be involved. Normally, times between additions may vary from a few seconds to many hours, usually not exceeding 16 hrs, more usually not exceeding 6 hrs. Usually, incubation times will vary from about 0.5 min to 1 hr, more usually from about 0.15 min to 30 min. Since the ultimate result will be dependent upon the result obtained with standard(s) treated in substantially the same manner, and when possible in the identical manner the particular mode and periods of time are not critical, so long as significant reproducible differentiations are obtained with varying concentrations of analyte.

Depending upon the choice of assay protocol, the equipment employed and the concentration of analyte involved, assay volumes may be as small as about 1 μl, more usually being about 25 μl, and will usually not exceed 5 ml, more usually not exceeding 2 ml.

The assay measurement will depend upon counting the quanta of light emitted from the assay medium. Various instruments may be used, such as scintillation counters, photocells or the like, which are capable of measuring light at a single or over a range of wavelengths.

Materials

The primary components in the subject assay for analyte, which may or may not be employed in every case are: labeled ligand (includes poly(ligand analog)-label); labeled antiligand, ligand; antiligand; and additional components as required for the chemilumiinescence source.

Analyte The ligand analytes of this invention are characterized by being monoepitopic or polyepitopic. The polyepitopic ligand analytes will normally be poly(amino acids) i.e. polypeptides and proteins, polysaccharides, nucleic acids, and combinations thereof. Such combinations of assemblages include bacteria, viruses, chromosomes, genes, mitochondria, nuclei, cell membranes, and the like.

For the most part, the polyepitopic ligand analytes employed in the subject invention will have a molecular weight of at least about 5,000, more usually at least about 10,000. In the poly(amino acid) category, the poly(amino acids) of interest will generally be from about 5,000 to 5,000,000 molecular weight, more usually from about 20,000 to 1,000,000 molecular weight; among the hormones of interest, the molecular weights will usually range from about 5,000 to 60,000 molecular weight.

The wide variety of proteins may be considered as to the family of proteins having similar structural features, proteins having particular biological functions, proteins related to specific microorganisms, particularly disease causing microorganisms, etc.

The following are classes of proteins related by structure:

protamines
histones
albumins
globulins
scleroproteins
phosphoproteins
mucoproteins
chromoproteins
lipoproteins
nucleoproteins
glycoproteins
unclassified proteins, e.g. somatotropin, prolactin, insulin, pepsin

A number of proteins found in the human plasma are important clinically and include:

Prealbumin
Albumin
α_1-Lipoprotein
α_1-Acid glycoprotein
α_1-Antitrypsin
α_1-Glycoprotein
Transcortin
4.6 S-Postalbumin
Tryptophan-poor
α_1-glycoprotein
α_1X-Glycoprotein
Thyroxin-binding globulin
Inter-α-trypsin-inhibitor
Gc-globulin
(Gc 1-1)
(Gc 2-1)
(Gc 2-2)
Haptoglobin
(Hp 1-1)
(Hp 2-1)
(Hp 2-2)
Ceruloplasmin
Cholinesterase
α_2-Lipoprotein(s)
α_2-Macroglobulin
α_2HS-glycoprotein
Zn-α_2-glycoprotein
α_2-Neuramino-glycoprotein
Erythropoietin
β-lipoprotein
Transferrin
Hemopexin
Fibrinogen
Plasminogen
β_2-glycoprotein I
β_2-glycoprotein II
Immunoglobulin G (IgG) or γG-globulin
Mol. formula:

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