United States Patent [19]

Litman et al.

[11] **4,391,904**

[45] * **Jul. 5, 1983**

[54] **TEST STRIP KITS IN IMMUNOASSAYS AND COMPOSITIONS THEREIN**

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[*] Notice: The portion of the term of this patent subsequent to Nov. 10, 1998, has been disclaimed.

[21] Appl. No.: **255,022**

[22] Filed: **Apr. 17, 1981**

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 106,620, Dec. 26, 1979, Pat. No. 4,299,916.

[51] **Int. Cl.**[3] **G01N 33/54**; G01N 21/00; G01N 1/48; G01N 21/06

[52] **U.S. Cl.** **435/7**; 435/188; 435/805; 435/810; 436/536; 436/537; 422/55; 422/56

[58] **Field of Search** ..... 424/1, 8, 12; 435/7, 435/188, 174, 178, 179, 180, 181, 810, 805; 23/230 B, 832; 422/55, 56

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[57] **ABSTRACT**

An assay method and compositions are provided for determining the presence of an analyte in a sample. The analyte is a member of an immunological pair (mip) of immunogens—ligand and receptor. The method has two basic elements: a solid surface to which one of the members of the immunological pair is bonded and a signal producing system, which includes a catalytic member bonded to a mip, which signal producing system results in a measurable signal on said solid surface related to the amount of analyte in the medium. The signal generating compound is produced without separation of the catalyst labeled mip bound to the solid surface from the catalyst labeled mip free in solution.

In a preferred embodiment, an enzyme is bonded to a mip which acts in conjunction with a solute to produce a signal generating product which binds preferentially to the solid surface when the enzyme is bound to the surface, resulting in a signal which is readily differentiated from signal generating compound produced by the catalyst and solute in the bulk solution.

9 Claims, No Drawings

TEST STRIP KITS IN IMMUNOASSAYS AND COMPOSITIONS THEREIN

REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of application Ser. No. 106,620, filed Dec. 26, 1979, now U.S. Pat. No. 4,299,916.

BACKGROUND OF THE INVENTION

1. Field of the Invention

There is continuing interest in developing new, simpler and more rapid techniques to measure the presence of an analyte in a sample suspected of containing an analyte. The analyte may be any of a wide variety of materials, such as drugs, naturally occurring physiological compounds, pollutants, fine chemicals, contaminants, or the like. In many cases, speed is important for the measurement, particularly with certain physiologically active compounds. In other situations, convenience can be a major consideration.

One convenient technique which has found wide application is the use of a "dip stick." Having a solid rod or film which can be dipped in a sample and then subsequently processed to produce a signal based on the amount of analyte in the original sample can provide many conveniences. There is ample instrumentation to measure a signal, such as light absorption or fluorescence, of a compound bound to a solid surface. Also, the dip stick allows for convenient handling, transfers, separations, and the like.

In developing an assay, it is desirable that there be a minimum number of steps and transfers in performing the assay, as well as a minimum number of separate reagents. Therefore, while a dip stick adds a convenience to separations, the separations in themselves are undesirable. Furthermore, the fewer the reagents that have to be packaged, added, and formulated, the fewer the errors which will be introduced into the assay and the greater economies and convenience of the assay.

It is therefore desirable to develop new assay methods, particularly employing rigid solid surfaces which may or may not be separated from the assay medium for measurement, where the signal may be developed without concern as to the presence of reagents in the solution affecting the observed signal on the solid surface.

2. Brief Description of the Prior Art

Patents concerned with various immobilized reagents in different types of test strips include U.S. Pat. Nos. 3,993,451; 4,038,485; 4,046,514; 4,129,417; 4,133,639; and 4,160,008, and Ger. Offen. No. 2,636,244. Patents disclosing a variety of methods involving separations of bound and unbound antigen include U.S. Pat. Nos. Re. 29,169; 3,949,064; 3,984,533; 3,985,867; 4,020,151; 4,039,652; 4,067,959*; 4,108,972; 4,145,406; and 4,168,146*.

(*Patents of particular interest)

SUMMARY OF THE INVENTION

A method is provided employing a relatively rigid insoluble, preferably bibulous, surface to which is conjugated a member of an immunological pair (abbreviated as "mip") the immunological pair consisting of ligand and a receptor which specifically binds to the lignd or their functional equivalent for the purposes of this invention. In addition to the surface, a signal producing system is provided which has as one member a catalyst, normally an enzyme, which is conjugated to a mip. Depending upon the amount of analyte present, the catalyst labeled mip will be partitioned between the bulk solution of the assay medium and the surface. The signal producing system provides a signal generating compound at the surface which generates a signal which is not significantly affected by any signal generating compound produced or present in the bulk solution. Therefore, the signal generating compound may be generated in the assay medium in the presence of unbound catalyst labeled mip. When the only catalyst in the signal producing system is the catalyst-labeled-mip, various expedients can be employed to enhance the difference in the rate of formation of the signal generating compound at the surface as compared to the bulk solution, e.g. enhancing the catalyst turnover rate at the surface. In addition to enhance the simplicity of this protocol, the last of the components of the signal generating system will be added at about the time of or before the addition of the catalyst bound to the mip.

Compositions are provided for performing the assay comprising combinations of the surface and various reagents in relative amounts for optimizing the sensitivity and accuracy of the assay.

The subject assay provides for a convenient method for detecting and measuring a wide variety of analytes in a simple, efficient, reproducible manner, which can employ visual inspection or conventional equipment for measuring a spectrophotometric property of a product bound to a surface.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

In accordance with the subject invention, an assay method and compositions are provided for measuring a wide variety of analytes, where the analyte is a member of an immunological pair (mip), the pair consisting of a ligand and a receptor (antiligand) which specifically binds to the ligand, or their functional equivalent for the purposes of the assay. The assay method has two essential elements: a surface to which is conjugated a mip; and a signal producing system which results in a signal generating compound associated with the surface, producing a detectible signal in an amount related to the amount of analyte in the assay medium. Preferably, the signal producing system will effect a two or more step conversion involving one or more compounds to produce, block or destroy the signal generating compound, where the rate of change in the concentration of the signal generating compound is related to the average distance between two molecules on the surface. The molecules may be the same or different. The immunological binding at the surface allows for localized enhanced concentrations of compounds of the signal producing system at the surface. Also, one may employ a scavenger as a third component which acts to inhibit the operation at the signal producing system in the bulk solution by scavenging an intermediate, catalyst or signal generating compound in the bulk solution.

The surface may be any convenient structure which substantially retains its form and may be separable from or part of the container. The manner of binding of the mip to the surface is not a critical aspect of this invention, so long as a sufficient amount of the mip is exposed to allow for binding to its homologous partner.

The signal producing system has at least two members: A catalyst, normally an enzyme, conjugated to a mip; and a solute which undergoes a reaction with a

substance bound to the surface, and thereby directly or indirectly enhances or inhibits the production of a detectible signal. The association of a member of the signal producing system with the surface may be as a result of insolubilization, complexation with a compound on the surface or interaction, including reaction, with a compound on the surface.

Where an intermediate material is produced by the signal producing system in soluble form, both in the bulk solution and at the surface, a scavenger can advantageously be employed, so as to substantially minimize the interaction of the intermediate material produced in the bulk solution with the surface.

A wide variety of different systems may be employed for altering the degree of production of the product at the surface as compared to the bulk solution and for inhibiting intermediates or product produced in or migrating into the bulk solution from interacting with the surface. Depending upon the particular protocols, various additions, incubation steps, and reagents will be employed.

By providing for the production of a detectible signal generating material on the surface that is related to the amount of analyte in a sample, one can relate the signal level detected from the surface to the amount of analyte in the solution. By employing standards having known amounts of analyte under the same or substantially the same conditions as with an unknown, one can quantitate the detected signal level with the amount of analyte in the sample.

In accordance with the subject invention, the method is performed without requiring a separation of bound and unbound catalyst-bound-mip, nor requiring a separation of analyte from the remainder of the sample, although the latter may be desirable. This provides substantial advantages in the convenience of the protocol and in avoiding the difficulties in achieving a clean separation.

The subject invention achieves a precise, specific and sensitive technique for detecting and measuring ligands and ligand receptors. The method provides for the preferential production, inhibition of production or destruction of a compound at a rigid surface, which compound is involved with the generation of a signal at the surface. The signal generating compound associated with the surface will be of a sufficient depth or on in the surface to provide a measurable signal.

For a large number of analytes, the concentration range of interest will fall between 100 μg to one pg per ml. For many analytes, the concentration range of interest will vary from about two-fold to 100-fold so that a quantitative determination will require the ability to distinguish small differences in the concentration of the analyte in the assay medium. Immunoassays are predicated on detecting the complexation between ligand and receptor, where one or both may be labeled. The lower the concentration of the analyte, the fewer the number of complexes which are formed. Therefore, in order to be able to accurately determine the number of labeled complexes which are formed, either the label must provide a signal which can be efficiently counted at an extremely low level of events, e.g. radioactive emission, or the complex must permit amplification or multiplication, e.g. fluorescence or a catalyzed reaction.

When employing an amplification system, many problems are encountered. One serious problem is signal resulting from other than labeled complexes, namely background. Background signal can result from materials in the sample; labeled contaminants when labeling the member of the immunological pair, and unbound labeled member. In developing an assay, the signal generated by labeled complexes must not be obscured by the signal from the background and must be substantially greater than the background signal. Therefore any amplification achieved by the signal generating system must be primarily, if not solely, associated with the labeled complex rather than with background label.

In many assay techniques a clean separation of labeled immune complex and background label is required, where careful attention must be given to non-specific effects. For example, where a fluorescent label is employed in a heterogeneous system, e.g. dipstick, after combining all of the reagents with the dipstick, the dipstick must be removed and carefully washed to remove any fluorescer which is non-specifically bound. Furthermore, the number of fluorescers involved with a complex is limited to the number which can be conveniently conjugated to a member of an immunological pair, although further amplification can be obtained by employing a second labeled receptor which binds to a first receptor which binds to a ligand analyte. This step requires an additional reagent, another addition and a careful separation to avoid non-specific interactions.

The subject invention obviates or minimizes many of the shortcomings of other methods. For each complex a plurality of signal generating events are achieved by employing a catalyst. The catalyst is partitioned between the bulk solution and a surface in proportion to the amount of analyte in the assay medium. The production of signal generating product resulting from the catalyzed reaction at the surface is substantially independent of concurrent production of signal generating product, if any, produced in the bulk solution. Thus, the assay operates with the catalyst present in the bulk solution during the time the modulation of the amount of signal generating compound at the surface is occurring. The need for separating the surface from the bulk solution, whether careful or not, for measuring the signal is avoided in the subject invention, although the separation may be preferable.

Furthermore, in the subject invention, the signal generating compound can be of substantial depth on or in the surface. The presence of the catalyst at the surface allows for the deposition or conversion of a large number of signal generating compounds to provide a strong signal. This is of great importance when the measurement is visual inspection, particularly where the signal generation involves the absorption of light.

Before further describing the invention, a number of terms will be defined.

DEFINITIONS

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

Specific binding pair—two different molecules, where one of the molecules has an area on the surface or in a cavity which specifically binds to a particular spatial and polar organization of the other molecule. The members of the specific binding pair are referred to as ligand and receptor (antiligand). These will be referred to in the subject application as members of an immunological pair, abbreviated as "mip". Homologous or complementary mips are ligand and receptor, while

analogous mips are either ligands or receptors, which are differentiated in some manner, e.g. labeling.

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

Receptor (antiligand)—any compound or composition capable of recognizing a particular spatial and polar organization of a molecule i.e. epitopic or determinant site. Illustrative receptors include naturally occurring receptors, e.g. thyroxine binding globulin, antibodies, enzymes, Fab fragments, lectins, nucleic acids and the like.

Ligand Analog—a modified ligand which can compete with the analogous ligand for a receptor, the modification providing means to join a ligand analog to another molecule. The ligand analog will usually differ from the ligand by more than replacement of a hydrogen with a bond which links the ligand analog to a hub or label, but need not.

Poly(ligand-analog)—a plurality of ligands or ligand analogs covalently joined together, normally to a hub nucleus. The hub nucleus is a polyfunctional material, normally polymeric, usually having a plurality of functional groups e.g. hydroxy, amino, mercapto, ethylenic, etc. as sites for linking. The hub nucleus is normally water soluble or at least dispersible and will usually be at least about 35,000 daltons, but generally not exceeding about 600,000 daltons. Illustrative hub nuclei include polysaccharides, polypeptides, including proteins, nucleic acids, ion exchange resins and the like.

Surface—the surface will be non-dispersed and of a dimension of at least about 1 μm^2 and generally greater, often at least about 1 mm^2, frequently from about 0.5 cm^2 to 10 cm^2, usually being on a support when less then about 0.5 cm^2; and may be of any material which is insoluble in water and provides the necessary properties for binding of a mip and a detectible signal generating compound to provide a desired signal level. Desirably, the surface will be gelatinous, permeable, porous or have a rough or irregular structure, which may include channels or indentations, generally having a substantial void volume as compared to total volume. Depending upon the nature of the detectible signal generating compound, the surface will be adsorbent or non-adsorbent, preferably being weakly or non-adsorbent. The surface may be transparent or opaque, a single material or a plurality of materials, mixtures or laminates. A wide variety of materials and shapes may be employed. The surface will be capable of substantially retaining its integrity under the conditions of the assay so that substances which are bound to the surface will remain bound to the surface and not diffuse into solution.

Signal producing system—the signal producing system has at least two members: (1) a catalytic member; and (2) a solute, which undergoes a reaction catalyzed by the catalytic member, which leads directly or indirectly to a product on or in the surface which provides a detectible signal. Desirably, a third compound will be present which provides for enhanced rate of change of the signal generating compound at the surface as compared to the bulk solution. This can be as a result of the component being bound to the surface or interacting with another member of the signal producing system.

The catalytic member may be enzymatic or non-enzymatic, preferably enzymatic. Whther one or more than one enzyme is employed, there will be at least one enzyme bound to a mip. (An enzyme acting as a catalyst should be distinguished from an enzyme acting as a receptor.)

The solute can be any compound which is capable of undergoing a reaction catalyzed by a catalytic member of the signal producing system, which reaction results either directly or indirectly in modulating the formation of a detectible signal generating compound associated with the surface. The association of the signal generating compound to the surface may be as a result of insolubilization of the product produced when solute undergoes the catalyzed reaction, complexation of the product with a compound on the surface or reaction or interaction of a compound on the surface with the product of the catalyzed reaction.

The signal generating compound will provide an electromagnetic signal, e.g. a spectrophotometric or visible, electrochemical or electronic detectible signal. The signal generating compound will be associated with the surface due to its insolubility, or covalent or non-covalent binding to the surface. The observed detectible signal from the surface will be related to the amount of catalyst bound to the surface through the binding of the catalyst-bound-mips to the mip-bound-surface.

Various techniques and combinations of reagents may be employed to enhance the production of the detectible signal at the surface, while minimizing interference from materials in the bulk solution.

Label—the label may be any molecule conjugated to another molecule where each of the molecules has had or can have had a prior discrete existence. For the most part, labels will be compounds conjugated to a mip. In referring to a catalyst conjugated to an antiligand, the reagent will be referred to as a catalyst-bound-antiligand, while for a ligand conjugated to a surface, the reagent will be referred to as ligand-bound surface.

METHOD

The subject assay is carried out in an aqueous zone or medium, where the final assay medium may be the result of prior individual additions of reagents or combinations of reagents and incubations, prior separations involving removal of the surface from an aqueous medium and transfer to a different aqueous medium having one or more reagents, or combinations thereof. The subject method, however, does not require a separation of catalyst-bound-mip which is unbound from that which is bound to its homologous partner bound to the surface (mip-bound-surface). The medium consists of a liquid phase and a non-fluid phase which is the "surface."

In carrying out the assay, the mip-bound surface will be contacted by the sample, and by the members of the signal producing system, and any ancillary materials in an aqueous medium, either concurrently or stepwise, to provide a detectible signal associated with the surface. The detectible signal will be related to the amount of the catalyst-bound-mip bound to the surface, which in turn will be related to the amount of analyte in the sample. Depending upon the nature of the signal producing system and the desired method for detecting the signal, the surface may be read in the assay medium or will be read separate from the assay medium.

In carrying out the assay, an aqueous medium will normally be employed. Other polar solvents may also be included, usually oxygenated organic solvents of from 1–6, more usually from 1–4 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 of about 4–11, more usually in the range of about 5–10, and preferably in the range of about 6.5–9.5. The pH is chosen so as to maintain a significant level of specific binding by the receptor while optimizing signal producing efficiency. In some instances, a compromise will be made between these two considerations. 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. Constant temperatures during the period of the measurement are generally required only if the assay is performed without comparison with a control sample. The temperatures for the determination will generally range from about 10°–50° C., more usually from about 15°–45° 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. Considerations such as whether the assay is qualitative, semi-quantitative or quantitative, the particular detection technique and the concentration of the analyte of interest will normally determine the concentration of the other reagents.

The concentrations of the various reagents will vary widely depending upon which protocols are employed, the nature of the analyte, the mip which is bound to the surface and the mip which is bound to the catalyst, the required sensitivity of the assay, and the like. In some instances, large excesses of one or the other of the mips may be employed, while in some protocols the sensitivity of the assay will be responsive to variations in the mip ratios.

By way of illustration, if the analyte is a polyepitopic antigen, one could have excesses of antiligand as antiligand-bound-surface and as catalyst-bound-antiligand, without seriously affecting the sensitivity of the assay, provided that the surface is first contacted by the sample, followed by contact with the signal producing system. Where antiligand is the sample and the protocol involves the combination of the analyte and catalyst-bound-antiligand prior to contacting the antigen-bound-surface, the sensitivity of the assay will be related to the ratios of the analyte and catalyst-bound-antiligand concentration.

In addition to the considerations involving the protocol, the concentration of the reagents will depend on the binding constant of the antiligand, the binding constant profile for a particular antisera, as well as the required sensitivity of the assay. Also, when all of the signal producing system is present in the liquid phase, the catalyst substrates and ancillary reagents should be at a concentration which allows for substantial immunological pair binding before a large amount of signal producing product is formed. Where the sensitivity of the assay is concentration related, frequently the particular concentrations will be determined empirically. When the sample is combined with the homologous catalyst-bound-mip, generally the total binding site concentration of the catalyst-bound-mip will be not less than about 0.1 times the minimum concentration of interest based on binding sites of analyte and usually not more than about 1,000 times the maximum concentration of interest based on analyte binding sites, usually about 0.1 to 100 times, more usually about 0.3–10 times the maximum concentration of interest. When the analyte is preadsorbed to the mip-bound-surface, the concentration of catalyst-bound-mip will depend on the desired rate of binding to the surface, the production of interfering signal generating compound in the liquid phase, the cost of the reagent, etc.

The concentration of catalyst-bound-mip will be chosen so that the amount of catalyst-bound-mip in the void volume-liquid immediately adjacent to and occluded in the surface will not significantly interfere with the measurement of the change in concentration of the signal generating compound at the surface as a result of catalyst-bound-mip bound to the surface. The chosen concentration will be affected by the sensitivity of the measurement, the degree of quantitation desired, the accuracy with which one must distinguish the lowest concentration of interest and the like.

In most situations, the ratio of concentration in the void volume of catalyst-bound-mip unbound to the surface to catalyst-bound-mip bound to the surface should be not greater than about 100 fold, usually not greater than about 10 fold at the maximum concentration of interest of the analyte, preferably at the mid-range concentration range of interest of the analyte.

The combination of the solid surface with the sample may be prior to, concomitant with, or subsequent to combining the catalyst-bound-mip with the sample. By employing a single unit or entity as the surface, one can use the surface to concentrate the analyte in a large sample. Also, the surface allows for removal of the analyte from other materials in the sample which could interfere with the determination of the result. Therefore, a preferred embodiment will be to combine the surface with the sample, followed by removal of the surface from the sample containing medium and transfer to the assay medium.

Alternatively, one could leave the surface in contact with the sample and add the remaining reagents. It is also feasible, although in some instances not desirable, to combine the sample with the catalyst-bound-mip, followed by introduction of the surface into the assay medium. For example, with a ligand analyte, enzyme-bound-antiligand and ligand-bound-surface, this last technique could be effectively used.

Frequently, the last of the components of the signal producing system will be added at about the same time as the catalyst-bound-mip, without any intermediate step, such as separating or washing the surface.

Where a receptor is the analyte, instead of having a single immunological pair, one may employ two immunological pairs, where the receptor acts as the ligand in one pair and the receptor in the other. For example, with IgE, one could bind the allergen or antigen to the surface and bind the catalyst to anti-IgE. In this way, the IgE acts as a bridge between two mips which in themselves cannot interact. In referring to a mip, this situation should be considered a special case which is intended to be included.

In developing protocols for the method, certain basic considerations will govern the order of addition and the combinations of reagents. The first consideration is that preferably where the surface-bound-mip and the catalyst-bound-mip are different members e.g. one is ligand and one is antiligand, the two will be brought together prior to or substantially concomitant with combination with the surface. The catalyst-bound-mip and solute will preferably be combined as a single reagent, except

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