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(54) Title: CONTROLLED-RELEASE SOLID PHASE ASSAY DEVICE INCLUDING ENCAPSULATED REAGENTS FOR DETECTING CHEMICAL SUBSTANCES

(57) Abstract

The present invention provides a method and device for determining the presence of or detecting endogenous and exogenous chemical substances in fluids, especially in body fluids. At least one of the analytes is encapsulated to provide greater stability, and allow for delayed release of one or more of the reactants. Among the types of exogenous substances which can be assayed are central nervous system stimulants (*e.g.*, cocaine and amphetamine) and narcotics (*e.g.*, opiate alkaloids) in urine.

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CONTROLLED-RELEASE SOLID PHASE ASSAY DEVICE INCLUDING ENCAPSULATED REAGENTS FOR DETECTING CHEMICAL SUBSTANCES

BACKGROUND OF INVENTION

1. Field of Invention. This invention relates to a solid phase method, and device for practicing this method for the detection of endogenous and exogenous chemical substances in body fluids.

2. Background of Invention. A wide variety of analytical methods are known for the detection of chemical substances (both endogenous and exogenous) in body fluids. Among the endogenous substances for which rapid and sensitive assays are frequently required are sugar in urine, serum cholesterols and liver enzyme in blood. The detection of exogenous chemical substances in body fluids is often necessary as well. Among such exogenous substances frequently assayed are central nervous system stimulants (e.g., cocaine and amphetamines), depressants (e.g., barbiturates), psychopharmacological drugs (e.g., cannabis) and narcotics (e.g., opiate alkaloids). Many of the known analytical methods are complicated, costly and require a trained analyst or clinician. Gas-liquid-chromatography (GLC) coupled with flame ionization or thermionic detection systems is perhaps the most widely used instrumentation method for drug abuse testing. The sensitivity and selectivity of GLC make it the standard to which new

methods of analysis or screening are frequently compared. Thin layer chromatography (TLC) is also often applied to drug abuse testing. Separation of constituents of body fluids on a thin layer of silica gel or aluminum oxide using an optimized solvent mixture carrier can be achieved with the aid of a suitable sample preparation procedure. The separated constituents can be visualized with an appropriate staining reagent and quantified by reflectance densitometry. Immunological assays such as radioimmunoassay (RIA) and enzyme multiplied immunoassay technique (EMIT) have also been used for drug abuse testing. These latter techniques combine the use of antibodies that are highly specific to individual drugs with either radiometric or spectrophotometric measurements for quantitation. Gas chromatography coupled with mass spectrometry (GC/MS) is perhaps the most sophisticated analytical technique available at this time for drug abuse testing, but, is also the most costly and complex of the testing methods mentioned thus far. GC/MS, however, offers nearly complete characterization of the constituents in body fluids. Drug identification and detection is based not only on the chromatographic retention indices of constituent drugs, but also on their unique mass fragmentation pattern.

During the past forty years, the use of chemical test paper and solid solution chemistry has been widely accepted by medical practioners and patients in areas other than drug-testing, for example in the detection of glucose and ketones in urine and blood. The known paper tests, however, although quantitative and cost effective, do not lend themselves readily to testing substances of abuse because of their lack of specificity. Tests of this nature (i.e., color reactions) depend on the participation of analyte functional groups in the chemical reaction. Hence, the detection of common, and, in many cases, ubiquitous, functional groups provides information only about a characteristic

region of the analyte with consequent loss of specificity. In a clinical setting, the application of such paper tests to drug-testing would lead to a high frequency of false positives due to the similarities in chemical functional groups of the analyte of interest with those of constituents that are endogenous to biological function or drug therapy.

Substance abuse has reached epidemic proportions, affecting all levels of society. The most commonly abused substances today are CNS stimulants (e.g., cocaine and amphetamines), depressants (e.g., barbiturates), psychopharmacological drugs (e.g., cannabis) and narcotics (e.g., opiate alkaloids).

Some abused drugs are metabolized extensively by the liver. The metabolism of cocaine proceeds by hydrolysis to yield benzoylecgonine, the principal metabolite appearing in the urine. Though only between 1 to 12% of the total ingested dose is excreted in the first 24 hours as unchanged drug, the amount of unchanged cocaine appearing in the urine is dependent on urinary pH. The excretion of amphetamines is also influenced by urinary pH. Between 30 and 40% of amphetamine itself is excreted unchanged in the urine within 48 hours. After ingestion of large doses, however, unchanged amphetamine may be detected in urine for up to seven days. Metabolites of amphetamine, including p-hydroxyamphetamine and conjugated benzylmethylketone, also appear in urine in relatively small amounts.

Of the opiate alkaloids, morphine is the most widely used narcotic analgesic. Only a minor fraction (i.e., 7%) of the original morphine dose is excreted in the urine as unchanged drug. Approximately 45% is excreted as morphine-3-glucuronide.

Many of the endogenous and exogenous substances discussed herein give distinct colors when brought into contact with appropriate chemical reagents. Upon the application of a test substance to a filter paper impregnated with reagent solution, colored reaction products

become fixed on the surface of the paper and produce distinct colored rings. In this way, the products of chemical reactions may be separated from the reaction sphere and fixed in the surface of the test paper. The simplicity of such tests allows for a fast and effective identification procedure for drugs of abuse.

Generally, such colorimetric techniques are referred to as solid solution chemistry and differ considerably from conventional solution colorimetric methods which use dilute solutions of color-producing reactants. Interaction of the reactant with a functional group of the drug or metabolite in solution yields a color reaction identifiable by visual observation or spectroscopic absorbance. If, however, a molecule of drug or metabolite interacts with a reservoir or solid matrix of color-producing reactant at infinite concentration, that drug or metabolite may exhibit very different properties with regard to reactivity and specificity as compared to those obtained using conventional dilute solution chemistry. For example, Friedenberg et al. (Clin. Tox., 18(5), 619-633 (1981)) has observed that the detection sensitivity for alpha-amino acid residues of amino moieties with ninhydrin (1,3,5-triketohydrindene, a common diagnostic reagent) using solution chemistry techniques is typically on the order of one part per thousand, whereas solid solution chemistry will yield color reactions in the sensitivity range of one part per million to one part per ten million.

Since sensitivity is essential in a practical drug screening device, solid solution chemical reactions (i.e., reactions of drugs or their metabolites with highly reactive color-forming species at infinite concentration) have tremendous potential value for drug testing. However, reproducibility and sensitivity in many currently used solid phase assays are often hampered by the instability of the reagent employed, as well as by the inability to carry out sequential reactions necessary to conduct many of such assays.

SUMMARY OF INVENTION

This invention provides a cost-effective, yet sensitive solid state method and screening device used in this method, to detect both endogenous and exogenous chemical substances in body fluids. More particularly, this invention provides solid phase assays for the detection of endogenous and exogenous chemical substances in body fluids, and means for conducting such assays, in which at least one reagent is present in the solid phase in encapsulated form. Encapsulation of one or more reagents provides greater stability for reagents which tend to decompose, thereby providing a longer shelf-life for the assay means when in the form of a kit. The encapsulation of certain reagents also permits delayed release of a reagent or reagents into the reaction sphere, thus permitting sequential or timed release of one or more reagents required in one or several assay steps.

The assay means of this invention can comprise a solid phase substrate, such as a chemical test paper, strip or spot, a glass or plastic tube, stick or bead, or the like, to which has been adhered at least one encapsulated target chemical immobilizing reagent, or a potentiating reagent or a visibly detectable marker. Such assay means can be used to assay chemical substances, including most commonly abused stimulants and narcotics in body fluids, such as urine, saliva, serum, perspiration and the like. The solid phase test system of the present invention is easily utilized and does not require sophisticated laboratory equipment or techniques.

In one embodiment, the present invention comprises a drug-screening test paper for the detection of abused drugs, such as, but not limited to, amphetamines, aporphines, catecholamines and various morphine alkaloids (e.g., morphine and codeine) in urine and other body fluids. In a preferred embodiment, the device of the present invention employs a metal complex in conjunction with controlled,

delayed release of a potentiating agent to produce a colorimetric reaction with drug-positive urine. Hyperflow kinetic encapsulation of one or more of the reagents adhered to a device prepared in accordance with this invention provides a delayed release mechanism for introducing any of the encapsulated reagents, such as a potentiating agent, at the appropriate time during the colorimetric chromatographic reaction.

This invention may be utilized to monitor any chemical substance in body fluids or in any other aqueous fluid. Endogenous substances such as hormones, lipoprotein-associated cholesterols (e.g., HDL- and LDL-cholesterol), or sugars may be monitored utilizing the concept of the present invention. In addition to detection of abused substances in body fluids, the present invention may also be utilized to monitor levels of prescribed or administered drugs.

DETAILED DESCRIPTION OF INVENTION

The present invention provides solid phase assays, and means for conducting such assays, in which at least one reagent is present in encapsulated form. The encapsulation of one or more reagents provides greater stability of reagents which tend to decompose, thereby, providing a longer shelf-life for the assay means when in the form of a kit. The encapsulation of certain reagents also provides a means to delay the release of certain reagents, allowing a sequential addition of the encapsulated reagent into the reaction sphere. The assay means comprises a solid phase substrate, such as a chemical test paper, strip or spot, a glass or plastic tube, stick or bead, or the like and one or more reagents selected from the group consisting of a target chemical immobilizing reagent, a potentiating reagent and a detectable marker, at least one of these reagents being encapsulated in a water swellable, pH- or heat-dependent or maleable polymer. The detectable marker is,

preferably, visually detectable. However, markers may also be selected from the group which are detectable by other means such as fluorescent markers, radioactive markers, chemiluminescent markers when the appropriate apparatus for detecting such markers is available to the assay user.

One of skill in the art can select the type of encapsulating material (encapsulating matrix) according to the type of release mechanism desired to release the encapsulated substance or reactant. Encapsulated materials can be released by mechanical rupture, thermal release or permeation. Choice of a combination of encapsulating materials can provide even further control of the release of the encapsulated materials. The encapsulating material can be selected, for instance, from a wide variety of natural and synthetic polymers, as well as natural gums, waxes and resins. Aqueous materials may be encapsulated in a variety of materials. For instance, in one embodiment, aqueous solutions may be encapsulated in a wax or polyvinyl alcohol shell and released by mechanical rupture. In another embodiment, chemical reactants may be encapsulated in a water swellable polymer such as gelatin or polyacrylate. Fatty acids may also be utilized as an encapsulating material. These useful encapsulating materials are pH dependent and will release the encapsulated contents by dissolution at pH below 7.0. Encapsulating materials such as saran are also useful when an application of heat is appropriate for the release of the encapsulated reactant. Other encapsulating materials useful in the present invention are known to those of skill in the art and their choice and use will be apparent to one of skill in the art.

The device of this invention comprises, in its broadest aspect, (1) a known solid substrate, (2) at least one known "reporter substance forming" reagent (i.e., one that interacts with an analyte to give a colored substance or some other indication of the analyte's presence)

for detecting the presence of an analyte. The reporter reagent is adhered to the solid substrate and is often one of the encapsulated reagents. In all cases, encapsulation protects the reagent(s) from atmospheric attack, i.e., provides storage stability. Therefore, it is evident that the assay device of this invention may be applied to the assay of any chemical wherein the stability of the reaction reagents will be increased by encapsulation.

The solid phase assay of this invention also provides encapsulation of reagents which, although stable to atmospheric conditions, are to be released in a delayed manner into the reaction sphere. In one aspect, this invention contemplates the use of microcapsules of different capsule thickness. Thus, by varying the composition and/or the thickness of the encapsulating material, one can control the time of release of the encapsulated material into the reaction sphere. For instance, if microcapsules of the same reagent are made using the same capsule-forming substance (polymer) but having different dissolution rates, for instance, by varying the polymer thickness, a single reagent can be released in stages over time ("timed release").

If microcapsules of more than one reagent are made using the same capsule-forming substance for each, but a different wall thickness for each, or using different capsule-forming substances, having different dissolution rates, for each, then several reagents can be released sequentially. Hence, one reagent can, for example, react first with one analyte and a second reagent can then react with another analyte, or one reagent can react first with an analyte and a second reagent can then react with the reaction product of the first reagent and the analyte.

Microcapsules can be made utilizing a variety of encapsulating materials. Microcapsules may also contain more than one encapsulated

reagent within the same microcapsule.

The encapsulating materials may be used singly or in combination. The choice of encapsulating material(s) will be dependent upon a number of factors.

In one aspect, the present invention comprises:

A solid substrate, a binding substance to bind the target substance to the assay theater and a reporter substance for detecting and/or indicating the presence of said target substance. In one embodiment, the assay of the present invention may also comprise an agent or agents for potentiating or enhancing the indicator or reporter substance signal. In a preferred embodiment, the signal is a visual signal and the signal is a color produced or generated by the substance(s) resulting from the reporter substance forming reagent's reaction with the analyte. Depending on the choice of reagents, one or more of the reagents are encapsulated. In one embodiment, the reporter substance is not encapsulated and the potentiating substance is encapsulated so that the reporter substance reacts with the analyte and the potentiating substance is then released to potentiate the indicia obtained from the reagent-analyte reaction.

In yet another embodiment both the reporting substance and the potentiating substance may be adhered to the substrate.

The foregoing aspects may be more fully understood by reference to the examples which follow. Thus, in Example 1, the substrate is filter paper, the reporter molecule is exemplified by potassium ferricyanide and the potentiating agent is encapsulated ninhydrin, with the ninhydrin being later released to potentiate the color of the hexacyanoferrate-drug complex formed by the earlier potassium ferricyanate-drug (analyte) reaction.

Example 2 illustrates the use of two encapsulated potentiating agents, ninhydrin and trichloroacetic acid. Thus, in Example 2, the

substrate is filter paper, the reporter agent is potassium ferricyanide and the potentiating agent is the primary- (trichloroacetic acid) and secondary (ninhydrin) encapsulated potentiating agents, whereby the primary agent is released at a greater rate than the secondary reagent to allow for denaturation of endogenous proteins followed by color potentiation of hexacyanoferrate-drug complex.

In Example 3, an ELISA assay, the substrate is the filter paper, the reporter molecule is an enzyme substrate, and the potentiating agent is the antigen-enzyme hapten.

A. Solid Substrates

Because interferences from drugs and metabolites of similar chemical structure or endogenous material present in a biological test material (e.g., urine) can hinder the sensitivity and specificity of the test, it is sometimes necessary that the interfering substances be separated from the reaction theater prior to reaction of the analyte of interest. The target chemical may interact with or be bound to a binding reagent on the solid substrate and then react with analyte detecting or visualizing reagent. In the alternative, the target chemical may be separated from interfering body fluid constituents or metabolites by a chromatographic or other separation step(s) on the solid substrate prior to reaction with the detection reagent or prior to contact with the solid substrate. For example, color reactions with chromatographic separations on an inert absorbent substrate capitalize on the interaction of color-producing reactant with drug or metabolite, but with the additional migration or segregation of drugs and metabolites within the contacted area or areas of test substance on the chemical test paper. In the presence of a highly potent color-producing reactant (e.g., ninhydrin) or other indicator reactant, interaction of drug or metabolite with reactant caused identifiable indicia of the

reaction product such as colored rings or bands to be formed as the molecules, metabolites and endogenous material migrate at different rates away from the application site of the test urine. Thus, this approach allowed one to differentiate the drug or metabolite derivative of interest from similarly structured derivatives or endogenous material derivatives. Among the solid substrates suitable for use in the device of the present invention are cellulose, nylon, and propylene.

B. Target Chemicals.

Almost any endogenous or exogenous chemical substance may be detected in body fluids by the method of the present invention. For instance, it is often necessary to monitor the levels of endogenous chemicals such as microbacteria, estrogen and androgen in body fluids. Most often the steroid hormones (estrogens and androgens), such as estradiol and testosterone, are monitored by radioimmunoassay (RIA). However, these methods often require prior extraction from the biological matrix and the shelf life of the available kits is limited by the radiochemical half-life of the iodinated (^{125}I) antigen.

The levels of exogenous chemicals such as antibiotics, chemotherapeutic agents and the like must often be monitored. Administration of the levels of many such drugs is often based on an average half-life rather than the known excretion or metabolism of the drugs. Utilization of the present invention allows an efficient, stable and cost effective method to monitor the levels of therapeutic drugs.

It is also desirable to monitor for the presence of exogenous substances such as central nervous system stimulants (e.g., cocaine and amphetamines), depressants (e.g., barbiturates), psychopharmacological drugs (e.g., cannabis) and narcotics (e.g., opiate alkaloids) in body fluids.

Thus, any target chemical substance in any fluid medium may be

assayed utilizing the assay of the present invention. While especially useful for the detection of chemicals in body fluids, the assay of the present invention may be utilized to detect chemical substances in any fluid medium. The assay of the present invention may be utilized to detect environmental pollutants, pesticides and the like.

C. Indicating Reagent

The choice of indicating reagent will be dependent on the type of chemical substance to be targeted. In the preferred embodiment, a color indicating reagent is utilized. Many color indicating reagents are organic color indicating reagents, the specificity of which depends on the participation and availability of a specific functional group in the chemical reaction. Detection of the group gives information only about the presence of that specific chemical functionality, but not its origin. Thus, the detected functional group may originate from the compound of interest, i.e., an abused drug or metabolite thereof, or from an interfering compound containing the same functional group. Thus, there is a loss of reliability as to the actual reactant detected, with consequent loss of sensitivity to the amounts of reactant actually present. In one aspect, the present invention provides a solution to this problem by incorporating color indicating metallic complexes whose specificity for the drug or metabolite of interest relies on the participation of more than one functional group, their relative orientation in space, and the size, nature and oxidation state of the central metal atom. In many cases, the metallic complex can chelate to more than one molecule of the drug or metabolite of interest, thereby enhancing the intensity of the colored products fixed in the surface of the paper. Metal complexes, for instance, $K_3[Fe(CN)_6]$, $K_3[Co(CN)_6]$, $Na_2[Fe(CN)_5NO]$, Na[Fe(EDTA)] and $Na_2[Cu(EDTA)]$, can be potentiated by agents such as ninhydrin, 5-sulfosalicylic acid, 2, 4-

Dinitrophenylhydrazine and phthalic dicarboxaldehyde, respectively.

Other analyte indicators useful in the present invention include, but are not limited to, colbalt(II) thiocyanate, Dragendorff's reagent ($OBiNO_3$, KI and acetic acid), lead acetate, mercuric chloride, carbazone, and iodoplatinate.

D. Potentiating Agent

In addition to increasing sensitivity by solid solution chemistry, the present invention provides the use of a potentiating agent. The addition of trace quantities of a potentiating agent, subsequent to the indicating reaction, greatly enhances the detection or visualization of reaction product such as colored rings or bands on the substrate. However, the provision of the potentiating agent at the proper stage of the reaction provides reliability, specificity and sensitivity. Unless introduced in sequence, the use of the potentiating agent is nonselective since it can react with all reactive components, including those unrelated to the drug or metabolite of interest.

E. Controlled Release Mechanism

According to the present invention, microencapsulation of the potentiating agent or other indicator or color-producing reactants provides their sequential introduction into the reaction theater. For instance, the nascent drug-positive urine spot is propagated by the absorbing medium and visualized (detected) via sensitized release of a potentiating reagent or color-producing reactant at the appropriate time during the course of the colorimetric reaction. The delayed release mechanism is produced by the microencapsulation of the potentiating agent or color-producing reactant in a water swellable, pH-dependent or malleable polymer matrix. The microencapsulated formulation is adhered onto the surface of the solid phase support or chemical test

paper.

The controlled release mechanism provided by encapsulation of some or all of the reactants and incorporation in chemical test papers provides the sensitive and reliable device and method for detecting chemicals, metabolites or substances such as abused drugs and/or their metabolites in body fluids. The present invention may also be utilized for numerous other applications in diagnostic and medical screening procedures. The present invention may also be utilized to provide chemical test papers impregnated with encapsulated reagents for selective release of specific immuno-reagents and enzymes, both of which are time-dependent reactions involving more than one reagent. Equally important, microencapsulation is useful in preserving the potency of the agent(s), thereby prolonging the shelf-life of the diagnostic or medical test kit or chemical test paper.

Preferably, the potentiating agent or color-producing reactant is encapsulated in polymer microcapsules of approximately 100 micrometer in diameter. Encapsulation procedures useful in practicing the present invention are known to those skilled in the art of hyperflow kinetic encapsulation.

Briefly, in the manufacture of one embodiment of small capsule spheres (microspheres) useful in practicing the present invention, a slurry or emulsion containing the potentiating agent or color-producing reactant and a suitable polymer are pumped to the center of a rotating disk. An even distribution of the feed material, along with control of the rotational velocity of the disk, provides control over the size distribution of the microspheres. Optimum dynamical conditions allow disengagement of individual microspheres at the periphery of the disk. The disk is positioned at a distance above the collection surface to allow sufficient time for the microspheres to dry, cool and solidify before impact.

However, other encapsulating procedures known to those in the art may be used to provide the microspheres for the controlled, sustained or sequential release mechanism of reactant or reactants essential to the colorimetric reaction. For instance, the encapsulating procedure taught and disclosed in U.S. Patent No. 3,389,194 (Somerville), incorporated herein by reference, may be used to encapsulate reactants used in the present invention. This method produces filler material (reactant) that is contained within a seamless film material (shell). A centrifugal extrusion device is used to manufacture the microcapsules. The device consists of an encapsulation head with two or more nozzles and concentric feed tubes which enter the head through a seal arrangement. The device is attached to a rotating shaft such that the direction of rotation is around its vertical axis. Shell and fill materials are pumped separately through a feed tube into the head and to the nozzles which consist of concentric orifices. As the head rotates, shell material flows through the outer orifice of the nozzle and fill material flows through the inner orifice of the nozzle, thereby creating a rod of filler material surrounded by a sheath of shell material. This extruded rod of material eventually breaks into individual capsules which are collected by appropriate means.

Many different types of polymers may be used in preparing the microencapsulated reactants. Microcapsules or microspheres of varying diameter or capsule-wall thickness (i.e., microcapsule) may be prepared using polymers such as, but not limited to, cellulose acetate trimellitate (CAT), cellulose acetate phthalate (CAP), polyacrylate-polymethacrylate copolymers (Eudragit RL-100 and RS-100 and others) and polyisobutylene. In the preferred microencapsulation methods of the present invention, the mechanical parameters useful in varying the manufacture of microcapsules or microspheres, and which such

parameters consequently vary the release characteristics of the encapsulated reactant, are the feed rates of the fill and matrix materials, the rotational speed of the disc or extrusion head, and the diameter of the disc or extrusion orifices.

The release rate of reagent through the polymer wall (i.e., microcapsule) or matrix (i.e., microsphere) is controlled by varying the initial ratio of reagent to wall or matrix material. A decrease in this ratio causes a decrease in the release rate of the reagent. (Benita, et al., J. Microencapsulation, 2(3), 207-222 (1985)). However, the diffusion rate of the reagent is dependent upon its solubility and molecular size. Therefore, two different reagents microencapsulated by the same method and having approximately the same reagent to wall or matrix ratio may have considerably different release rates if the solubility or molecular size of either reagent is also different.

Microcapsules or microspheres may be adhered to any solid substrate by air drying, as described in the preferred form of the present invention, or by the use of polymer binders such as cold-water soluble polyvinylalcohols (PVA), water soluble cellulose derivatives (e.g., methylcellulose) and starch derivatives.

In one embodiment, the present invention describes a method and device for detecting the presence of morphine alkaloids, aporphine, catechol, amphetamine or metabolites thereof in urine or other body fluids. A solid substrate, such as, for instance, filter paper was treated with a pH-adjusted solution of a transition metal complex such as, for instance, potassium ferricyanide. After the impregnated papers have dried, a microencapsulated potentiating agent, such as, for instance, ninhydrin, was coated on the test paper. To assay for the presence of one of the target drugs of abuse, a drop (approximately 100 uL) of urine or another appropriate body fluid (e.g., saliva) is applied to the paper strips. However, timed-dipping may also be used. Drug or

metabolite constituents resembling morphine alkaloids, aporphine, catechol or amphetamine species migrated at different rates away from the application site of the absorbent substrate and immediately interacted with hexacyanoferrate ion to yield highly specific hexacyanoferrate-drug complexes. These complexes become immobilized within the fibers of the absorbent paper substrate. The controlled, delayed release of potentiating agent is activated when the polymer matrix of each individual microsphere comes in contact with the specimen. The polymers used for microencapsulation are capable of swelling and releasing active ingredients by diffusion. Thus, the release of the potentiating agent from the microcapsule is delayed for the period of time needed for the swelling of the microcapsule shell and the diffusion of agent through the shell. This delay period provides that sufficient time is allowed for migration of the drug, complex formation of hexacyanoferrate-drug, and immobilization of specific hexacyanoferrate-drug complexes (i.e., hexacyanoferrate-alkaloid, -aporphine, -amphetamine, and -catechol complexes). After a period of time the color reaction of immobilized drug complexes became intensified by the induction of potentiating agent to give a blue color for drug positive specimen and no color change for drug-free urine specimens.

The potentiating agent may be any agent that: 1) removes from the reaction theater any constituent endogenous to the sample specimen that may interfere with the exact determination of the target analyte; 2) manifests, through the action of chemical derivatization, complexation or enzymatic catalysis, the presence of a target analyte that is immobilized or fixed on a chemical substrate; or 3) intensifies a colorimetric reaction that has occurred on a chemical substrate. Preferably, the potentiating agent is selected from the group consisting of ninhydrin, trichloroacetic acid, dansyl chloride, fluorescamine,

2,4-dinitrophenylhydrazine, phthalic dicarboxaldehyde, and enzyme (e.g., alkaline phosphatase).

The following examples are offered by way of illustration and are not intended to limit the invention in any manner. In all examples, all percentages are by weight if for solids and by volume if for liquids, and all temperatures are in degrees Celsius unless otherwise noted.

EXAMPLE 1

Chemical test papers were prepared by saturating 6 x 46 cm strips of Whatman™ No. 3 filter paper with a 5% (w/v) solution of potassium ferricyanide ($K_3[Fe(CN)_6]$) prepared in 0.1 M phosphate buffer, pH 9.3. After allowing the test papers to air dry, microencapsulated potentiating agent, ninhydrin, was dispersed in a carrier and spray-coated onto the strips. Microspheres were applied to filter paper by dispersing them in a volatile carrier such as hexane in approximately 1% (w/v) concentration and immediately spray coating the mixture using a spray gun. Microspheres were kept dispersed during the coating process by constant agitation of the mixture in the solvent reservoir.

Because the microsphere matrix is slightly soluble in hexane (i.e., the dissolution time is 6 hours) the outer surface of the matrix softens and subsequently increases adhesion of the microspheres to the paper. Leakage of the encapsulated material was prevented by spray coating this mixture in a period of time that was less than the dissolution time of the matrix and by volatilizing the carrier solvent immediately after adhesion.

Substantial quantities of the potentiating agent, ninhydrin, were prepared in the form of microspheres (20 - 100 micrometers in diameter) using the spinning disk apparatus previously described for the preferred embodiment. Approximately 30 grams of milled ninhydrin was suspended in a slurry consisting of 10% (w/v)

methacrylate copolymer Eudragit RL-100 in chloroform. The slurry was fed to the center of a disk rotating at 4300 rpm. The resulting microspheres of ninhydrin were collected on paper and subsequently spray coated onto the pre-saturated paper strips.

Urine specimens were applied by means of a small bore pipette to previously treated test papers by permitting 100 microliters of the specimen to spread from the center point on the paper in a manner that is analogous to the capillary action of developing paper chromatograms. Once the urine specimen was applied to the paper strips, drug or metabolite constituents resembling morphine alkaloids, aporphine, catechol or amphetamine species migrated at different rates away from the application site of the absorbent substrate and immediately interacted with hexacyanoferrate ion to yield highly specific hexacyanoferrate-drug complexes which became immobilized within the fibers of the absorbent paper substrate. Coincidentally with the migration of sample specimen, the controlled, delayed release of potentiating agent was activated when the polymer matrix of each individual microsphere came in contact with the specimen. The polymers used are capable of swelling and releasing active ingredients by diffusion. This allowed for induction of the potentiating agent after immobilizing specific hexacyanoferrate-drug complexes (i.e., hexacyanoferrate-alkaloid, -aporphine, -amphetamine, and -catechol complexes). After a period of time the color reaction of immobilized drug complexes became intensified by the induction of potentiating agent to give a blue color for drug positive specimen and no color change for drug-free urine specimens.

Qualitative comparison study of positive donor urine samples tested by EMIT and GC/MS versus a novel test paper prepared in accordance with this invention is shown on Table 1.

Table 1.

TABLE 1. COMPARATIVE QUANTITATIVE TESTING OF DONOR URINE SAMPLES

POSITIVE DONOR URINE TESTED BY EMIT AND GC/MS	TEST PAPER DRUG IDENTIFICATION
1	AMPHETAMINE
2	"
3	"
4	"
5	"
6	"
7	"
8	"
9	"
10	"
11	"
12	"
13	PENTOBARBITAL
14	"
15	BUTALBITAL
16	"
17	"
18	"
19	"
20	PENTOBARBITAL
21	"
22	"
23	"
24	"
25	MORPHINE
26	"
27	"
28	"
29	DIHYDROCODEINE
30	"
31	"
32	"
33	MYDROCODONE
34	"
35	"
36	"

a) SAMPLE 1 THRU 12 TESTED POSITIVE FOR AMPHETAMINES IN RANGES OF 0.6 to 6.0 MICROGRAMS/ML OF URINE.

b) SAMPLES 13 THRU 24 TESTED POSITIVE FOR BARBITUDES IN RANGES OF 1.9 to 19.0 MICROGRAMS/ML.

c) SAMPLES 25 THRU 36 TESTED POSITIVE FOR OPIATES IN RANGES OF 0.9 to 8.2 MICROGRAMS/ML.

This study and subsequent specificity studies indicate a correlation of 90 percent positive results when compared to the GC/MS method. The other ten percent were positives; however, the specific chemical compound was not comparative. This reaction could have been caused by multiple metabolites in the sample or other drugs taken by the donor which have similar but unknown side chains.

The results of a study using 31 spiked samples of drug free urine reagent in pre-test paper prepared in accordance with this invention at three different levels of concentration is shown on Table 2.

Table 2.

FIGURE 2 RESPONSE TO TEST PAPERS TO DRUG-SPIKED URINE (USING TWO DIFFERENT MICROENCAPSULATED POTENTIATING AGENTS)

		RESPONSE					
		10 ug/mL		5 ug/mL		1 ug/mL	
Substance		(-)	(+)	(-)	(+)	(-)	(+)
Acetaminophen	a		x	x		x	
	b		x	x		x	
Amobarbital	a		x	x		x	
	b		x	x		x	
Apomorphine	a		x	x		x	
	b		x		x	x	

Table 2 (con't)

Substance		RESPONSE 10 ug/mL (-)	10 ug/mL (+)	5 ug/mL (-)	5 ug/mL (+)	1 ug/mL (-)	1 ug/mL (+)
Atropine	a	x		x		x	
	b	x		x		x	
Barbital	a		x	x		x	
	b		x		x		x
Benzoyl ecgonine	a		x	x		x	
	b		x		x	x	
Cocaine	a	x		x		x	
	b		x		x		x
Codeine	a	x		x		x	
	b		x		x		x
Dextromethorphan	a	x		x		x	
	b		x	x		x	
Dihydromorphone	a		x	x		x	
	b		x		x	x	
d-Methamphetamine	a	x		x		x	
	b		x		x		x
Ephedrine	a	x		x		x	
	b		x		x		x
Hydromorphone	a		x	x		x	
	b		x	x		x	
Hydroxamphetamine	a		x	x		x	
	b		x		x	x	
Methadone	a	x		x		x	
	b	x		x		x	
6-monoacetylmorphine	a	x		x		x	
	b		x		x		x

Table 2 (con't)

Substance		RESPONSE 10 ug/mL (-)	10 ug/mL (+)	5 ug/mL (-)	5 ug/mL (+)	1 ug/mL (-)	1 ug/mL (+)
Morphine	a		x	x		x	
	b		x	x		x	
Morphine glucuronide	a		x		x		x
	b	x		x		x	
Nalorphine	a	x		x		x	
	b	x		x		x	
Naloxone	a		x	x		x	
	b		x	x		x	
Sodium pentobarbital	a	x		x		x	
	b	x		x		x	
N-norcodeine	a		x	x		x	
	b		x	x		x	
Oxycodone	a		x	x		x	
	b		x	x		x	
Oxymorphone	a	x		x		x	
	b		x		x	x	
Phenobarbital	a		x		x	x	
	b	x		x		x	
Phenylephrine	a	x		x		x	
	b	x		x		x	
Phenylpropanolamine	a	x		x		x	
	b	x		x		x	
Pseudomorphine	a		x	x		x	
	b		x	x		x	
Salicylic acid	a	x		x		x	
	b	x		x		x	

Table 2 (con't)

Substance		RESPONSE 10 ug/mL (-)	10 ug/mL (+)	5 ug/mL (-)	5 ug/mL (+)	1 ug/mL (-)	1 ug/mL (+)
Secobarbital	a	x		x		x	
	b	x		x		x	
Thebaine	a	x		x		x	
	b	x		x		x	
Drug Free	a	x		x		x	
	b	x		x		x	

Two controlled release potentiating agents were used. It is noted that only three potential abused drugs (secobarbital, sodium pentobarbital and methadone) were not positively detectable at the level of 10 micrograms/mL. This indicates that additional standards varying the pH of the isolation of the test paper and modification of the reagents may be necessary to determine these materials. However, the other abused drugs which showed a positive response demonstrated that this diagnostic technique is specific, quantitative and effective.

EXAMPLE 2

Test papers described in Example 1 were further developed by spray-coating a primary and secondary microencapsulated potentiating agent onto the absorbent chemical substrate. Microspheres of ninhydrin and trichloroacetic acid were prepared using the method and apparatus of the previous example. However, ninhydrin was encapsulated in a polymer matrix consisting of the methacrylate copolymer Eudragit RS-100, which is less permeable than the RL-100 polymer matrix of trichloroacetic acid. The present embodiment, therefore, consisted of an absorbent substrate saturated with 5% (w/v) solution of potassium ferricyanide ($K_3[Fe(CN)_6]$) prepared in 0.1 M, pH

9.3 phosphate buffer onto which is spray-coated microspheres of ninhydrin (RS-100) and microspheres of trichloroacetic acid (RL-100).

Urine specimens were applied to the surface of the test papers as described in the previous example. Urine constituents resembling morphine alkaloids, aporphines, amphetamine or catechol species migrated at different rates from the application site of the specimen and formed hexacyanoferrate-drug complexes which were again immobilized within the fibers of the paper substrate. Controlled, delayed release of both potentiating agents (i.e., ninhydrin and trichloroacetic acid) was activated when the polymer matrix of each individual microsphere came in contact with the specimen. However, trichloroacetic acid was released at a greater rate than ninhydrin because the polymer matrix of trichloroacetic acid microspheres is more permeable than that of ninhydrin microspheres. After a period of time the color reaction of immobilized hexacyanoferrate-drug complexes became intensified by the induction of trichloroacetic acid for the denaturation of endogenous proteinaceous material present in the sample specimen that can interfere with the simultaneous induction of ninhydrin for color intensification.

EXAMPLE 3

This example illustrates the use of the invention as an enzyme-linked immunoassay technique to determine drugs of abuse with a high degree of specificity. The present example consists of the following components: a) a solid substrate as in Examples 1 and 2, except that the said substrate is saturated with an enzyme substrate (e.g., 5-bromo-4-chloro-3-indolyl phosphate); b) a controlled, delayed release mechanism imparted by water swellable microspheres or microcapsules; c) a hapten comprising of antigen (e.g., morphine, benzoylecgonine, tetrahydrocannabinol, barbiturate, amphetamine) conjugated (covalently linked) with enzyme (e.g., alkaline phosphatase);

and d) an antigen specific mono- or polyclonal antibody. The hapten of the present example is analogous to the potentiating reagent of the previous examples in its action upon the substrate to produce a color change for a target drug positive specimen.

Microspheres or microcapsules containing hapten bound to antigen specific antibody are manufactured in accordance with the method of hyperflow kinetic encapsulation or any other encapsulation method known to those in the art. The hapten-antibody coupling pair may be prepared by incubating the two components prior to microencapsulation. Subsequent to manufacture of protected, immobilized and immunochemically active hapten- antibody coupling pair, the microencapsulated product was applied to the surface of the solid phase substrate which was previously saturated with enzyme substrate as described in previous examples.

In the method of the present invention, a narrow strip (6 x 0.5 cm) of the coated paper substrate is contacted with a urine specimen containing an antigen of one of the drugs of abuse. The hapten-antibody coupling pair is sensitized upon contact of the polymer matrix of each microsphere with the specimen. The immunochemical and enzymatic activity of the hapten-antibody coupling pair is delayed for a period of time determined by the characteristics of the polymer matrix selected for the encapsulating microsphere. This delay allows sufficient time for the antigen to migrate and become separated from endogenous constituents. As the microspheres become fully activated (fully swelled), constituents interact with the hapten-antibody coupling pair in the pores and confines of each individual microsphere. Within this space, the antigen, in the case of drug-positive specimen, competes with hapten for antigen-specific antibody. Consequently, antigen becomes bound competitively to antibody binding sites in the microspheres and unbound, enzymatically active hapten is released

from the microspheres. A distinctive color is then produced when the released hapten comes in contact with the enzyme substrate which is present in the absorbent substrate. When no target antigen or drug is present, no color change is observed since no antigen is available to compete with the hapten.

EXAMPLE 4

This example illustrates the use of the invention as a multi-enzyme-catalyzed assay for serum triglycerides. The technique capitalizes on the sequential or delayed release of multiple enzymes contained in microspheres or microcapsules of varying polymer-matrix compositions to produce a dye on a solid substrate that can be detected and quantitated visually for various concentrations of serum triglycerides. The release of multiple enzymes and reagents is achieved by exploiting different porosities and ratios of reagent or enzyme to polymer matrix material of the polyacrylate- polymethacrylate copolymers type as described elsewhere. The present example consists of the following components: a) a solid substrate as in previous examples, except that the said substrate is saturated with adenosine triphosphate (ATP), surfactant (e.g., sodium dodecylsulfate) and a leuco dye (e.g., 2-(3,5-dimethoxy-4-hydroxyphenyl)-4,5-bis (4-dimethylaminophenyl)imidazole); b) controlled, delayed release mechanisms imparted by water swellable microspheres or microcapsules; c) lipase enzyme; d) glycerol kinase enzyme; e) L-α-glycerophosphate oxidase enzyme; and f) peroxidase enzyme.

Microspheres or microcapsules of individual enzymes, lipase (LP), glycerol phosphatase (GP), L-α-glycerophosphate oxidase (GPO), and peroxidase (PO), are manufactured in accordance with the methods of the previous examples. However, the porosity of the enzyme-polymer matrix and ratio of enzyme to polymer matrix composition is chosen such that the rate of release of each enzyme follows the order: LP >

GP > GPO > PO. Preferably, the rate of release of enzyme from its corresponding polymer matrix is formulated such that induction of each enzyme into the reaction theater occurs sequentially. The protected and enzymatically active enzymes are applied to the surface of the solid phase substrate which has been previously saturated with ATP, surfactant and leuco dye as described in previous examples.

Thus, in the method of the present invention, a narrow strip of coated paper substrate is contacted with a serum specimen containing endogenous lipoproteins. The release of each enzyme is sensitized upon contact of the corresponding microsphere- or microcapsule polymer matrix with the serum specimen. During this sensitization period, the serum specimen migrates through the absorbent substrate to allow for separation of lipoproteins into their triglyceride and protein constitutive components. As LP is released from the corresponding microspheres or microcapsules at a rate greater than GP, GPO and PO, the separated triglycerides are hydrolyzed catalytically by LP in the presence of water (from serum) to glycerol and fatty acids. After a period of time, induction of GP into the reaction theater occurs at a rate greater than GPO and PO. In the presence of ATP (saturated on the substrate), glycerol formed from the previous step is phosphorylated catalytically by GP to L-α-glycerophosphate and adenosine diphosphate (ADP). Subsequent to the release of GP, induction of GPO occurs at a rate that is greater than PO. The L-α-glycerophosphate that is formed from the previous step is oxidized catalytically in the presence of air by GPO to dihydroxyacetone phosphate and hydrogen peroxide. In the final step of the colorimetric reaction, PO is released from the corresponding microspheres or microcapsules. A distinctive color is produced, with intensity proportional to the concentration of triglycerides originally present in serum specimen, when the leuco dye that is saturated in the absorbent substrate is oxidized catalytically by

PO to form a dye that can be detected by visual observation.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The components, methods, procedures and techniques described herein are presently representative of the preferred embodiments, are intended to be exemplary, and are not intended as limitations on the scope of the present invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention and are defined by the scope of the appended claims.

What is claimed is:

1. A device for assay of a target chemical substance in aqueous solution comprising:

(a). a solid substrate;

(b). a binding reagent to immobilize said target chemical to said solid substrate;

(c). a reporter substance capable of visually demonstrating the presence of said target chemical, at least one of (b) and (c) being encapsulated and adhered to the surface of said solid support.

2. The device of claim 1 further comprising a potentiating agent.

3. The device of claim 1 wherein said binding agent is an antibody and said target chemical is an antigen.

4. The device of claim 1 wherein said binding agent is an antigen and said target chemical is an antibody.

5. The device of claim 1 wherein said solid substrate is selected from the group consisting of nylon, propylene and paper.

6. The device of claim 1 wherein said binding agent is a metallic complex.

7. The device of claim 6 wherein said metallic complex is selected from the group consisting of $K_3[Fe(CN)_6]$, $K_3[Co(CN)_6]$, $Na_2[Fe(CN)_5NO]$, Na[Fe(EDTA)] and $Na_2[Cu(EDTA)]$.

8. The device of claim 7 wherein said metallic complex is $K_3[Fe(CN)_6]$.

9. The device of claim 1 wherein said potentiating reagent is selected from the group consisting of ninhydrin, trichloroacetic acid, dansyl chloride, fluorescamine, 5-sulfosalicylic acid, 2,4-dinitrophenylhydrazine, phthalic dicarboxaldehyde, and alkaline phosphatase.

10. The device of claim 1 wherein said encapsulated substance is encapsulated in a material selected from the group consisting of a water swellable polymer, a malleable polymer and a pH sensitive polymer.

AMENDED CLAIMS

[received by the International Bureau on 31 August 1992 (31.08.92);
original claims 1,3-6,9 and 10 amended;
new claims 11-17 added; other claims unchanged (3 pages)]

1. A device for assay of a target chemical substance in aqueous solution comprising:

a solid substrate; and

a reporter substance capable of visually demonstrating the presence of said target chemical, wherein said reporter substance is encapsulated and adhered to said solid substrate.

2. The device of claim 1 further comprising a potentiating agent.

3. The device of claim 1 wherein said reporter substance is an antibody and said target chemical is an antigen.

4. The device of claim 1 wherein said reporter substance is an antigen and said target chemical is an antibody.

5. The device of claim 1 wherein said solid substrate is selected from the group consisting of glass, plastic, nylon, propylene and paper.

6. The device of claim 1 wherein said reporter substance is a metallic complex.

7. The device of claim 6 wherein said metallic complex is selected from the group consisting of $K_3[Fe(CN)_6]$, $K_3[Co(CN)_6]$, $Na_2[Fe(CN)_5NO]$, Na[Fe(EDTA)] and $Na_2[Cu(EDTA)]$.

8. The device of claim 7 wherein said metallic complex is $K_3[Fe(CN)_6]$.

9. The device of claim 2 wherein said potentiating agent is selected from the group consisting of ninhydrin, trichloroacetic acid, dansyl chloride, fluorescamine, 5-sulfosalicylic acid, 2,4-dinitrophenylhydrazine, phthalic dicarboxaldehyde, and alkaline phosphatase.

10. The device of claim 1 wherein said encapsulated reporter substance is encapsulated in a material selected from the group consisting of a water swellable polymer, a malleable polymer and a pH sensitive polymer.

11. The device of claim 1 wherein said encapsulated reporter substance is adhered to said solid support by physical means.

12. The device of claim 11, wherein said physical means is air drying.

13. The device of claim 1 wherein said encapsulated reporter substance is adhered to said solid support by the use of polymer binders such as cold-water soluble polyvinylalcohols, water soluble cellulose derivatives and starch derivatives.

14. A device for assay of a target chemical substance in aqueous solution, comprising:

a solid substrate;

a reporter substance capable of visually signalling the presence of said target chemical; and

at least one potentiating agent enhancing reporter substance's signal, wherein said potentiating agent is encapsulated and adhered to said solid substrate.

15. The device of claim 14 wherein said potentiating reagent is selected from the group consisting of ninhydrin, trichloroacetic acid, dansyl chloride, fluorescamine, 5-sulfosalicylic acid, 2,4-dinitrophenylhydrazine, phthalic dicarboxaldehyde, and alkaline phosphatase.

16. A kit assaying a target chemical substance in aqueous solution, comprising:

a device comprising a solid substrate and a reporter substance capable of visually demonstrating the presence of said target chemical, wherein said reporter substance is encapsulated and adhered to said solid substrate;

a sample applicator for applying a test sample to said solid substrate; and

instructions for using said device.

17. A kit assaying a target chemical substance in aqueous solution, comprising:

a device comprising a solid substrate, a reporter substance capable of visually signalling the presence of said target chemical, and at least one potentiating agent enhancing reporter substance's signal, wherein said potentiating agent is encapsulated and adhered to said solid substrate;

a sample applicator for applying a test sample to said solid substrate; and

instructions for using said device.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/02393

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): G01N 21/78, 33/543, 33/94
US CL : 422/56; 436/92, 98, 169, 815, 816, 829, 901; 435/7.92, 805, 970

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	422/56-58; 436/92, 98, 169, 170, 518, 810, 815, 816, 817, 829, 901; 435/7.9, 7.92, 805, 970

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
X/Y	US, A, 4,939,098 (SUZUKI ET AL) 03 July 1990, see the entire document.	1-4,10/5-9
Y,P	US, A, 5,075,078 (OSIKOWICZ ET AL) 24 December 1991, see the entire document.	5
Y	US, A, 4,844,866 (WALLACE ET AL) 04 July 1989, see the entire document.	6-9

* Special categories of cited documents:[15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
23 June 1992	30 JUN 1992
International Searching Authority[1]	**Signature of Authorized Officer**[20]
ISA/US	ROBERT J. HILL, JR.