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[54] **FECAL SAMPLE IMMUNOASSAY METHOD TESTING FOR HEMOGLOBIN**

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[*] Notice: The portion of the term of this patent subsequent to Mar. 10, 2009 has been disclaimed.

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[22] Filed: **Sep. 23, 1991**

Related U.S. Application Data

[63] Continuation of Ser. No. 329,455, Mar. 28, 1989, Pat. No. 5,094,956, which is a continuation-in-part of Ser. No. 10,787, Feb. 4, 1987, abandoned.

[51] **Int. Cl.**[5] **G01N 33/72**

[52] **U.S. Cl.** **436/66**; 436/8; 436/17; 436/177; 436/815; 436/825

[58] **Field of Search** 436/66, 8, 17, 63, 177, 436/815, 825

[56] **References Cited**

U.S. PATENT DOCUMENTS

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0175326A2	3/1986	European Pat. Off. .

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Julkunen et al, *Scand J Infect Dis* 17:245–249 (1985).

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[57] **ABSTRACT**

A method for preparing a fecal sample for immunoassay testing comprising the steps of dispersing a sample of from 1 to less than 10 wt. % of a stool sample in an aqueous fecal test solution formulated with one or more preservatives, analyte stabilizing agents and endogenous interference reducing agents. The fecal solids are then permitted to settle to form a liquid phase substantially free from fecal solids, and the clarified liquid phase is removed to provide a test sample free from fecal solids. The fecal test solutions contain suitable stool stabilizers such as buffers and antimicrobial agents, analyte protecting agents such as proteolytic, reductive or oxidative enzyme inhibitors, endogenous assay interfering enzyme inhibitors such as a reducing agent, and non-specific binding inhibitors such as animal proteins. The stool sample should be fresh or be fresh frozen and thawed immediately before dispersion in the buffer solution. The sample is suitable for any solid-phase immunoassay determination of a fecal sample analyte. A method for determining analyte in the stool sample comprises conjugating anti-analyte antibody adhered to a insoluble support with analyte in the clarified sample, and determining the presence and extent of such conjugation. For determining human hemoglobin in a sample of human stool, the aqueous fecal test solution preferably contains a proteinase inhibitor, formaldehyde and an animal albumin.

4 Claims, 1 Drawing Sheet

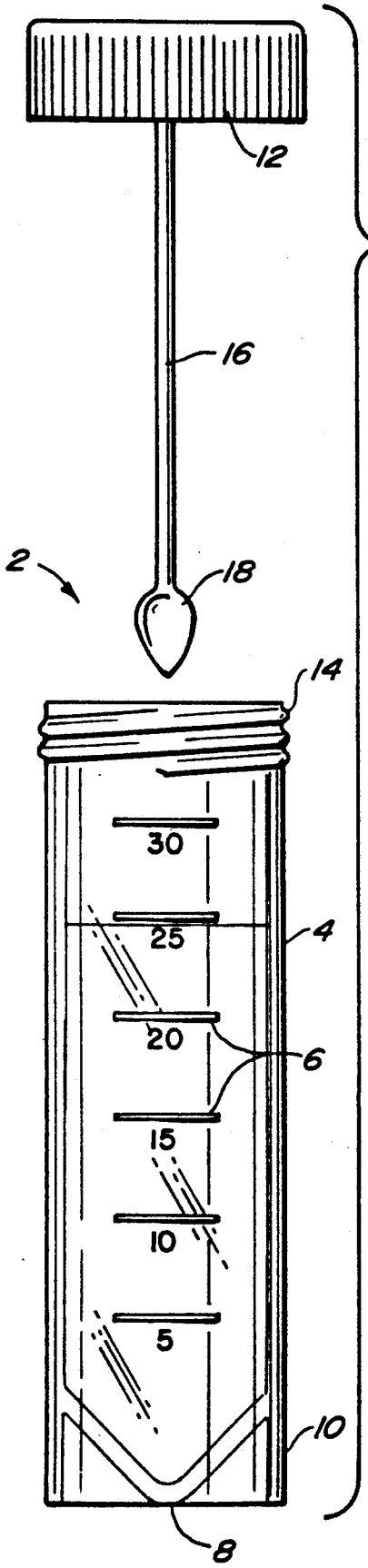

FIG._1.

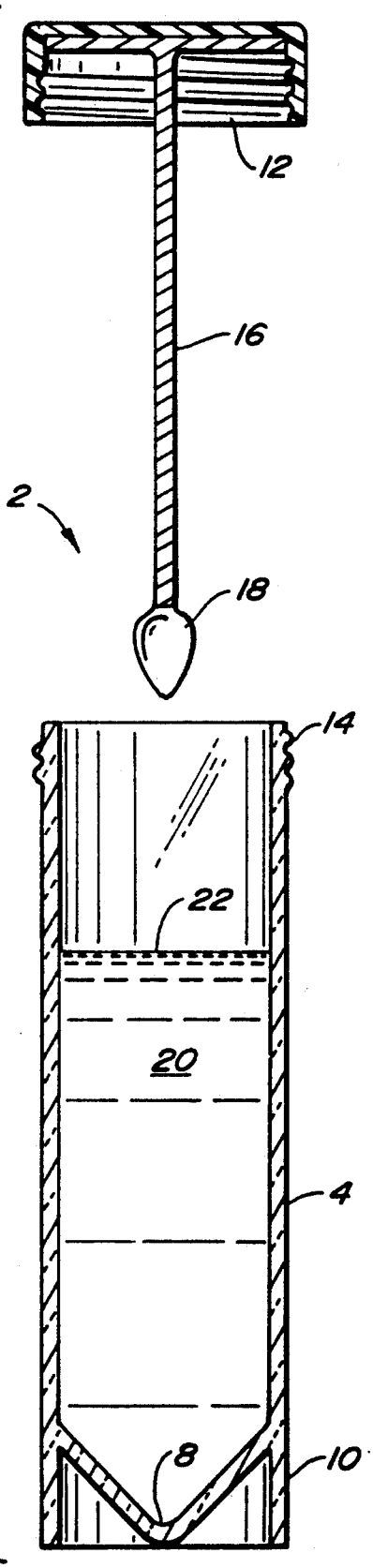

FIG._2.

FECAL SAMPLE IMMUNOASSAY METHOD TESTING FOR HEMOGLOBIN

CROSS REFERENCE TO RELATED APPLICATION

This application is a continuation of application Ser. No. 07/329,455, filed Mar. 28, 1989, now U.S. Pat. No. 5,094,956 which is a continuation-in-part of application Ser. No. 07/010,787 filed Feb. 4, 1987, now abandoned and incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

This invention relates to an improved method for analyzing fecal samples. In particular, this invention relates to a method for preparing fecal samples for immunoassays, the prepared fecal sample compositions and to solid-phase immunoassays for determining the presence and quantity of analytes in the samples.

BACKGROUND OF THE INVENTION

Stool or fecal samples are routinely tested for the presence of parasites, fat, occult blood, viruses, bacteria and other organisms and chemicals in the diagnosis for various diseases. The stool is usually collected, placed in a clean container and processed for testing.

Stool collection is usually non-invasive and theoretically ideal for testing pediatric or geriatric patients, for testing away from a clinical site, for frequently repeated tests and for determining the presence of analytes which are likely to be found in the digestive tract. Stool can also be collected with a swab or finger cot during examination and applied directly to a test surface. For microscopic examination or occult blood testing, the sample can be spread directly on a test surface. For other tests, such as testing for fat, the stool may be suspended in a liquid medium such as water.

Traditional sample examinations have used complex chemical or microbiological procedures. These procedures are being rapidly replaced with immunoassay methods. Immunoassay techniques are highly sensitive and require only a small sample. Solid-phase techniques such as latex agglutination and enzyme immunoassays have been developed to such a stage of simplicity that they can be performed at home, at the doctor's office or other test sites without the need for highly trained laboratory technicians or expensive instruments. Application of solid-phase immunoassay procedures to the analysis of stool samples is thus highly desirable.

Application of immunoassay techniques to fecal analysis has proven to be difficult for several reasons. Stool handling is disagreeable and biohazardous, and sanitary and inoffensive procedures for processing stool have proven to be awkward and frequently complex. Analytes in stool samples are frequently unstable. Weighing, extracting and centrifuging, and storing samples are difficult except in a clinical laboratory equipped with suitable apparatus and skilled technicians.

Constituents of stool are known to interfere with solid-phase immunoassays. Immunoreactants immobilized on solid-phase are desorbed by stool constituents. Non-specific reactions occur.

To increase the commercial use of immunoassay techniques for measuring analytes in stool, a number of problems must be solved. Instability of the analyte in the stool, interference from stool constituents, needs for extensive handling of the stool, equipment contamination, and instrumentation needs must be minimized.

Simple preparation steps avoiding the use of expensive equipment and instruments are required to extend the use of immunoassay testing procedures to sites outside hospital and clinical laboratory environments.

DESCRIPTION OF THE PRIOR ART

Assay procedures including the preparation of concentrated suspensions of 10 and 25 wt. % stool in water or buffer solution have been described by Vellacott et al, *The Lancet* p 18 (Jan. 3, 1981) and Jilkunen et al, *Scand.J.Infec.D.* 17:245 (1985). These were centrifuged and sterile-filtered to provide a sample for testing.

Desorption of immunoreactants has been reduced by either heat treatment of the sample or by mixing 50 vol. % fetal calf serum or acid-protein buffer containing 5 vol. % bovine serum albumin (BSA) with the test sample.

Non-specificity problems have been overcome by heat-treating samples in the presence of a reducing agent.

Current stool handling procedures include storing and transporting stool samples in clean containers and reducing deterioration of analyte by maintaining the sample at low temperatures. Problems of non-uniformity are resolved by forming a suspension of an entire sample or by assaying several samples; the suspension is then treated by centrifugation, filtration, extraction and sterilization.

Current techniques for measuring hemoglobin in stool exemplify the problems. A widely used semi-quantitative procedure for measuring hemoglobin uses guaiac resin paper on which the stool is reacted with hydrogen peroxide. The reaction of hemoglobin with guaiac resin forms a blue color, the intensity of which is a function of the quantity of hemoglobin in the sample. This method does not distinguish hemoglobin derived from animal blood in food from human hemoglobin. Because this method is subject to variables derived from chemical and biochemical interference with the hemoglobin-guaiac resin reaction and variations in water content of the paper and stool, it is not truly quantitative.

A quantitative method for measuring hemoglobin in stool described in U.S. Pat. No. 4,378,971 involves heating a small amount of stool in a reducing acid milieu. Porphyrin, free from other contaminating fluorescent compounds, is extracted from the mixture. This procedure provides a very sensitive, quantitative measurement of hemoglobin in stool. However, it requires extensive handling, does not differentiate human and animal hemoglobins, and cannot be carried out rapidly.

The radialimmunodiffusion (RID) procedure described by Barrows, G.H. et al, *Am.J.Clin.Path.* 69:342-346 (1977) uses antibodies to human hemoglobin in conjunction with calibrators of known hemoglobin concentrations. A disk is punched out from filter paper, stool sample is applied to the disk, and the disk is placed on a RID plate. There it is allowed to react for 24 hours with a disk impregnated with the calibrators. This test has a detection limit of 0.3 mg of hemoglobin in 8 mg of stool. It requires overnight incubation. Use of filter paper limits sensitivity since all hemoglobin placed on the paper is not made available for the antigen-antibody reaction. Irreversible protein absorption may permit the release of as little as 5 to 10 percent of the hemoglobin placed on the paper.

U.S. Pat. No. 4,582,811 describes a procedure including binding hemoglobin in a sample with antibody impregnated in paper, and then reacting the product with a substrate to measure pseudoperoxidase activity of the hemoglobin.

U.S. Pat. No. 4,427,769 describes an immunological method involving extraction of hemoglobin applied to a guaiac resin coated paper, and measuring it with a sandwich enzyme immunoassay technique. Kim et al, *Clin.-Chim.Acta.* 152:175 (1985) describes a still further approach wherein a stool sample applied to a glass fiber filter is placed on a gel and allowed to incubate for 2–4 hours. Hemoglobin is quantitatively determined down to a limit of 0.2 mg of hemoglobin per gram of stool based on the presence or absence of a visible band.

The procedures describe above require the direct transfer of a stool specimen to the test system. Transfer of hemoglobin from the sample to the test system is only partial. Undesirable reactions caused by stool constituents are difficult to control with reagents due to their uniform distribution throughout the sample. Most of the procedures require a well equipped laboratory and trained technicians.

Adams and Layman, *Ann.Clin.Labs.Sci.* 4:343 (1974) describe a latex agglutination test involving blending 1 gm of stool in 100 ml of buffer solution and filtering the suspension. This test can detect hemoglobin down to a level of 10 ml of blood per gram of stool.

Vellacott, et al, *The Lancet.* 1:18 (1981) have described a fluorescent immunoassay method in which a 20% suspension of stool in water is used as a test sample. This sample is sonicated and centrifuged prior to testing.

Japanese Patent Application 60173471 (Dialog Derwent World Patent Acc. No. 85-259806/42) describes applying stool containing an analyte to a porous material. The porous material contains a carrier to which an antibody which binds with the analyte is attached. The sample is washed and contacted with further reagents to provide a change in spectroscopic characteristics.

European Patent Application 70366 (Dialog Derwent World Patent Acc. No. 83-12612K/06) describes an immunoperoxidase sandwich test method for determining hemoglobin, albumin or globulin in stool samples using beads upon which antibodies for the analyte are immobilized and a peroxidase labeled secondary antibody.

SUMMARY AND OBJECTS OF THE INVENTION

The method of this invention for preparing a fecal sample composition for immunoassay testing comprises forming a dispersion of 1 up to 10 wt. % and preferably from 1 to 5 wt. % of a stool sample in an aqueous fecal test solution. The aqueous fecal test solution can contain preservatives and endogenous interference reducing agents to protect the test sample components against assay related deterioration and the assay from interference. The aqueous fecal test solution of this invention contains at least one stool stabilizing agent or at least one analyte stabilizing agent. The aqueous fecal test solution also preferably contains agents which facilitate the immunoassay such as at least one inhibitor of endogenous enzymes which may interfere with the immunoassay and one or more non-immune animal proteins or polyamino acid polymers to reduce non-specific binding. Fecal solids in the dispersion are permitted to settle to form a clarified liquid phase substantially free from fecal solids. The clarified liquid phase is removed to provide a test sample free from fecal solids.

The preferred stool stabilizing agents include buffering agents, and antimicrobial agents such as antibacterial and/or antimycotic agents. The preferred analyte protecting agents include inhibitors of proteolytic, reductive and/or oxidative enzymes. For immunoassays using alkaline phosphatase enzyme labels, the preferred endogenous enzyme inhibitor inhibits the activity of endogenous alkaline phosphatase, such as formaldehyde or equally effective enzyme inhibitors.

The stool sample is preferably freshly collected or has been chilled to a temperature below −20° C. immediately upon collection. Frozen stool is preferably then raised to a temperature within the range of from 2° to 6° C. immediately before being dispersed in the aqueous fecal test solution.

The solid-phase immunoassay method of this invention for determining analyte in a fecal sample comprises contacting the clarified liquid phase with a solid support to which an anti-analyte is adhered for a time sufficient to permit antibody conjugation with analyte; and determining hemoglobin adhering to the insoluble support. For determining human hemoglobin in a sample of human stool according to this method, the aqueous fecal solution preferably contains an inhibiting amount of a proteinase inhibitor, from 0.02 to 0.5 wt. % of formaldehyde and from 0.01 to 10 wt. % of a non-immune animal protein.

It is an object of this invention to provide an improved procedure for preparing stool samples for solid-phase immunoassay which is simple, quick and can be carried out in the home or other non-laboratory site by a technically unskilled person with simple, inexpensive resources. It is a further object of this invention to provide a procedure for preparing a stool sample composition which provides reduced interference with solid immunoassay procedures. A still further object of this invention is an improved immunoassay method for fecal occult blood testing of stool samples.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a view of a sample preparation vial suitable for use in the method of this invention

FIG. **2** is a cross-sectional view of the sample preparation vial of FIG. **1**.

DETAILED DESCRIPTION OF THE INVENTION

This invention is a method for preparing a fecal or stool sample for analysis using immunological techniques. This procedure is described hereinafter in conjunction with an immunoassay procedure for determining human hemoglobin in a stool sample, by way of example, not by way of limitation. The sample preparation procedure described hereinbelow is equally suitable for preparing stool samples for determining other stool analytes by immunoassay methods, and the use of this procedure for all such applications is intended to be included within the scope of this invention.

In general, the method of this invention provides a stabilized stool sample solution which yields an improved result in immunoassays. The stool is suspended in an aqueous fecal test solution in a concentration of less than 10 wt. %. The fecal test solution contains agents which stabilize the stool and protect the analyte from deterioration. The solution also preferably contains agents which reduce endogenous sources of immu-

noassay interference and proteins to reduce non-specific binding. The clarified liquid from the stool suspension contains sufficient analyte for a highly accurate immunoassay determination. We have discovered that because the stool and analyte are diluted to low levels, the protective and inhibiting functions can be achieved with such low concentrations of reagents that the reagents do not significantly interfere with the immunoassay. Prior to this invention, effective stool stabilization and analyte protection with chemical or biochemical reagents was incompatible with immunoassay methods. The high reagent concentrations required greatly interfered with the immunoassays. Accordingly, expensive, time-consuming non-reagent purification and stabilization techniques were found necessary to prepare fecal samples for immunoassay analysis.

The method of this invention for preparing a fecal sample composition for immunoassay testing involves a first step of forming a dispersion of from 1 up to less than 10 wt. % and preferably from 1 to 5 wt. % of a stool sample in an aqueous fecal test solution. The aqueous fecal test solution of this invention contains at least one stool stabilizing agent and/or at least one analyte stabilizing agent. The aqueous fecal test solution also preferably contains agents which facilitate the immunoassay such as at least one inhibitor of endogenous enzymes which may interfere with the immunoassay and one or more non-immune animal protein or polyamino acid polymer to reduce non-specific binding.

For stabilizing the stool, the aqueous fecal test solution contains buffering agents and/or antimicrobial agents. The aqueous fecal test solution can be buffered to a pH selected to increase the stability of the stool. For hemoglobin immunoassays, a buffer solution having a pH of from 7.0 to 8.0 is desirable. Any conventional buffering agents can be used to prepare the test buffer solution, if they do not interfere with the later assay procedure and assay reagents. One example of a suitable buffering solution is standard phosphate buffered saline, pH 7.2 to 7.6 and preferably about 7.4.

The stool stabilizing agent can include any biocidal or biostatic agents which will inhibit microbial growth in the sample but will not interfere with the immunoassay. Any conventional biocidal or biostatic agent can be used which will not interfere with the later assay procedure and assay reagents. An example of suitable biocidal agents are antibiotics such as penicillin or streptomycin, and antimycotic agents such as fungizone. A commercial buffer solution containing these antimicrobial agents is available from Gibco Co., New York, N.Y. The concentration of the antibiotic and antimycotic agents is adjusted based on the activity of the reagent selected. In general, the level is sufficient to suppress the reproduction of the microbes and preferably is sufficient to kill a majority of them.

Particularly for proteinaceous analytes, the analyte stabilizing or protecting agent can be an inhibitor of enzyme activity which would affect the analyte. Inhibitors of proteolytic, reductive and oxidative enzymes are useful. Any conventional inhibitors of proteolytic, reductive or oxidative enzyme activity can be used to prepare the aqueous fecal test solution, if they do not interfere with the later assay procedure and assay reagents. Examples of suitable proteolytic activity inhibitors include phenylmethylsulfonylfluoride (PMSF), pepstatin A, Bestatin, and chymostatin (Sigma Chemical Co.). A suitable commercial product is the proteolytic activity inhibitor aprotinin, a derivative of bovine lung described by Kraut, H. et al, *Z.Physiol.Chem.* 198:97–101 (1930). The proteolytic activity inhibitor is present in a concentration sufficient to inhibit a major proportion of the proteolytic activity. Aprotinin can be present in concentrations of from 10,000 to 30,000 kallikrein inactivator units per liter of fecal test solution.

The aqueous fecal test solution also preferably contains reagents which inhibit or deactivate enzymatic activity which may interfere with the particular immunoassay procedure used. Particularly when the immunoassay uses alkaline phosphatase labeled reagents, endogenous alkaline phosphatase naturally present in the sample presents substantial interference. If alkaline phosphatase levels in the sample are not adequately suppressed by other reagents, the broadly active enzyme inhibitors or deactivators are useful. The preferred test system uses formaldehyde as an inhibitor of alkaline phosphatase in the sample. Other enzyme inhibitors include metal chelating agents, heavy metal ions, certain amino acids such as tyrosine and phenylalanine, and high concentrations of zinc or inorganic phosphates. Any conventional enzyme inhibitor can be used to prepare the aqueous fecal test solution, if it does not interfere with the later assay procedure and particularly any enzyme reactions used in the immunoassay procedure. The level of enzyme inhibitor or deactivator is selected to be sufficient to achieve the level of inhibition required for the sample. For the general stool samples, alkaline phosphatase inhibitors such as formaldehyde can be used in concentrations of from 0.01 to 0.5 wt. % and preferably in concentrations of from 0.01 to 0.2 wt. %.

A non-specific binding inhibitor is preferably present in the fecal test solution. Suitable non-specific binding inhibitors are non-immune water-soluble animal proteins and polyamino acids which would not interfere with the later assay procedure and particularly any protein measurements in the immunoassay procedure. Suitable animal proteins are include bovine (BSA), human (HSA), rabbit (RSA), goat (GSA), sheep (SHA), and horse (HOSA) serum albumins, for example; serum gamma globulin, of the previously described animals and other animal proteins such as ovalbumin, fibrinogen, thrombin, transferin, glycoproteins, etc. Suitable water-soluble amino acid polymers include polylysine, polyglutamic acid, polyalanine, polyhistidine, polymethionine, polyproline, and the like. For assays where non-specific binding presents a problem, the concentration of the non-specific binding inhibitor can be from 0.1 to 1.0 wt. % and is preferably from 1 to 5 wt. %.

The preserved aqueous fecal test solution can also contain other protective agents including proteins, carbohydrates, salts and the like which provide a protective function.

Microbial and chemical changes in the sample should be inhibited and preferably completely arrested immediately after the sample is obtained. The stool is preferably dispersed in the aqueous fecal test solution immediately after being obtained, where the preservatives and other reagents in the buffer solution will stabilize the sample. If the stool is to be stored or shipped before testing, it should be quickly frozen to a temperature below −20° C. immediately after being obtained to prevent chemical and microbial changes, and the sample should be maintained at this temperature until the sample is to be dispersed in the aqueous fecal test solution. Immediately before being dispersed, the frozen

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