Consumer and Corporate Affairs Canada — Consommation et Corporations Canada

(11) (A) No. 1 157 374

(45) ISSUED 831122

(52) CLASS 167-47
C.R. CL. 167-114

(51) INT. CL.^{3} A61K 49/02

(19) (CA) CANADIAN PATENT (12)

(54) CHEMICAL METHOD OF LABELING PROTEINS WITH THE RADIONUCLIDES OF TECHNETIUM AT PHYSIOLOGICAL CONDITION

(72) Wong, Dennis W.,
U.S.A.

(21) APPLICATION No. 358,150

(22) FILED 800813

No. OF CLAIMS 33 - NO DRAWING

Canada

DISTRIBUTED BY THE PATENT OFFICE, OTTAWA.
CCA-274 (11-82)

ABSTRACT

A novel rapid chemical method of labeling plasma proteins, other compounds and/ or substances containing proteins with radionuclides of Technetium such as ^{95m}Tc, ^{99m}Tc or ^{99}Tc at physiologic pH 7.4 condition, producing a sterile non-pyrogenic radioactive tracer material suitable for biological and medical uses. These radiolabeled protein substances are not denatured by the labeling process but retain their natural physiological and immunological properties. This novel labeling technique provides a simple and rapid means of labeling plasma proteins such as human serum albumin, fibrinogen, antibodies, hormones and enzymes with ^{95m}Tc or ^{99m}Tc for scin-
10 tigraphic imaging which may allow visualization of thrombi, emboli, myocardial infarcts, infectious lesions or tumors.

SPECIFICATION

This invention relates to the development of radiolabeled protein substances useful for biological and medical applications. It is a chemical labeling process for incorporating a suitable radionuclide of Technetium such as ^{95m}Tc, ^{99m}Tc or ^{99}Tc with plasma proteins, other compounds and/or substances containing proteins. These protein substances are labeled with the radionuclides at optimal physiologic pH 7.4 conditions, thus avoiding harsh treatment of the proteins and preserving their biological properties. The invention further relates to a packaged instant
20 non-radioactive labeling reagent kit based on the same chemical labeling process and a simple method of using said labeling kit with generally available ^{99m}Tc-pertechnetate normal saline solution.

Proteins are essential constituents in plants and animals. As a class, proteins have great functional versatility. They may be classified according to biological

function as the enzymes, the antibodies or immunoglobulins, the clotting proteins and the toxins. Other proteins that have less intense biological activity are the transport proteins, the storage proteins, the contractile and structure proteins. A simple and reliable means of labeling these substances with a suitable radionuclide without denaturation offers unlimited potential in biomedical applications.

Protein denaturation and complete loss of biological activity are the primary concerns in the labeling of human plasma proteins with a radionuclide. Various methods of labeling plasma proteins such as serum albumin and fibrinogen with ^{125}I, ^{123}I and ^{131}I have been published in the literature. The most commonly used chemical means is radio-iodination of the protein in the presence of chloramine-T or iodine monochloride. The labeling yield, however, is low and varies from 50-70%. In order to be clinically useful, the desired radiolabeled protein must undergo a long and tedious separation and purification process. The radionuclide ^{131}I has other disadvantages. Among these are: emission of high energy beta and gamma photons which are not compatible with existing commercial display means; a long physical half life of 8 days resulting excessive irradiation to the patients; and finally, the dosage of any ^{131}I-labeled radiopharmaceuticals must be given to patients in very minute microcuries quantities.

Protein substances labeled with ^{125}I have wide range of applications in the field of radio-immuno assays. However, due to its low energy 35 KeV gamma photon flux and a 60 days long physical half life, ^{125}I-radiopharmaceuticals are not useful as scintillation imaging agents. The radionuclide ^{123}I possess a more desirable radioisotopic characteristics, but its radionuclidic purity is less than 94%. It contains many undersirable high energy and long half life radiocontaminants that interfere with labeling and purification processes. Imaging agents labeled with ^{123}I are still in experimental stages and are not available for general medical use.

Compounds labeled with ^{99m}Tc which eliminate most of the undesirable properties of the radioiodinated radiopharmaceuticals have been found extremely useful in biological studies and medical diagnosis. The radionuclide ^{99m}Tc has many advantages. It is a pure gamma emitter with a relatively short half life of 6 hours. The gamma photon of 140 KeV energy is compatible with existing conventional scintillation imaging equipments. Technetium-99m labeled radiopharmaceuticals can be safely administered to patients with a much large dose than radioiodinated compounds but produces a minimal amount of radiation health hazard. Human proteins such as serum albumin labeled with ^{99m}Tc has been used clinically in placenta localization, cardiac scan
10 and cisternography. More recently, there is increasing scientific and medical interest in ^{99m}Tc-labeled human fibrinogen, enzymes, hormones and antibodies for the localization and detection of thromboembolism, infectious lesions, myocardial infarction and neoplasm. Unfortunately, a more wide spread use of these radioactive tracer materials has been restricted because: 1) a simple and reliable chemical method of labeling protein substances with ^{99m}Tc at physiological condition which preserves the biological properties of the proteins has not been develop; 2) current ^{99m}Tc labeling technology using acid reduction of the radionuclide in the presence of a strong reducing agent causes complete denaturation of the protein; 3) the labeling yield is low with many radioactive impurities as well as free or unbound ^{99m}Tc; 4)
20 the possibility of hepatitis transmission and antigenic reactions.

Human serum albumin has been labeled with ^{99m}Tc by an acidic chemical process, but the results have never been very satisfactory (Eckelman, U.S. Patent # 3725295, 4/73, Molinski, U.S. Patent # 4042676, 4/77 and Layne, et al, U.S. Patent # 4094965, 6/78). According to the labeling methodology, ^{99m}Tc (+7) in the stable form of sodium pertechnetate ($Na^{99m}TcO_4$) is first reduced to a chemically active +4 or +5 valence state with a reducing agent dissolved in 0.5-1 N hydrochloric acid solution

E

at a pH of less than 2. A diluted solution of the albumin is added to the reduced $^{99m}Tc/SnCl_2$ acidic mixture with subsequent binding of the radionuclide to the protein ligand. The final mixture is then adjusted to pH 6-7 with a suitable buffer. The exact labeling mechanism is not known. Since the optimal condition of preserving the physiobiological properties of the proteins is at a very narrow pH range of 7 to 7.4, proteins labeled by the above described chemical method are completely denatured.

The enzymes streptokinase and urokinase which are proteins, have been labeled with ^{99m}Tc using similar technique(Dugan,MA, U.S. Patent # 3812245, 5/74). The labeling yields of these proteins are extremely low. Purification of these radioactive
10 proteins requires a tedious process of removing large amount of free or unbound ^{99m}Tc, insoluble tin particles in the form of ^{99m}Tc-stannous hydroxide($^{99m}Tc\text{-}Sn(OH)_4$), and other protein degradation product. ^{99m}Tc-labeled streptokinase and urokinase have claimed to be effective in localizing preformed clots of the deep veins. However, they are ineffective in documenting early stage of accute thrombophlebitis. Streptokinase is highly antigenic in man.

An alternate approach for labeling proteins by chemical means has been patented in 1977(Abramovici, et al, U.S. Patent # 4057617, 11/77). According to this invention, the proteins antibody and fibrinogen are labeled with ^{99m}Tc at pH 11.6. A careful analysis of the labeling methodology reveals many flaws. Stannous chloride dissolved
20 in diluted hydrochloric acid or acetic acid is known to be a powerful reducing agent for the reduction of ^{99m}Tc-pertechnetate. Increasing the pH from 2 to 11.6 will not cause further reduction of ^{99m}Tc. On the contrary, during the process of pH adjustment, insoluble radioactive colloidal particles, stannous hydroxide, will form when alkali such as 0.1 N sodium hydroxide(NaOH) is added to a solution containing $SnCl_2$ and reduced ^{99m}Tc. The problems encountered by labeling proteins at alkaline pH condition is similar to the acidic chemical method, namely; protein denaturation, formation of insoluble stannous particles, protein degradation byproducts, free or unbound

^{99m}Tc and very low yield.

Protein substances that are labeled with ^{99m}Tc by the acidic method of Dugan (pH less than 2) or alkaline method of Abramovici(pH 11.6) are denatured with complete loss of physiological and immunological properties. This renders protein substances labeled by their methods unsuitable for biological and medical applications. This is evident by the formation of insoluble colloids, precipitates and protein degradation products after labeling process as described in their patents. The existence of protein degradation products is further proof that proteins labeled at extremes of the pH conditions have undergone drastic molecular alteration or transfor-

10 mation. Thus, these so called ^{99m}Tc-labeled proteins as claimed by Dugan and Abramovici, et al, do not have the same properties as they had prior to the labeling process. The use of denatured heterologous proteins in man carries risk of antigenic reaction and hepatitis transmission.

Significant protein denaturation occurs with earlier electrolytic method of labeling serum albumin and fibrinogen with ^{99m}Tc using zirconium electrodes in an acid medium(Dworkin, et al, U.S.Patent # 3784453, 1/74, Benjamin PP, Int. J. Appl. Rad. Isotopes 20: 187, 1969 and Wong,DW, et al, J. Nucl.Med. 16: 343, 1975). The labeling methodology requires the addition of the protein to be labeled to an acidic medium (pH 1.8) during electrolysis which leads to subsequent decomposition of the labeled

20 product. Recently, an improved electrolytic method of labeling plasma proteins has been developed by the present inventor(Wong, DW, J. Labeled Comp. and Radiopharmaceuticals 14: 603, 1978). These protein substances are labeled with ^{99m}Tc at physiologic pH 7.4 conditions, thus avoiding harsh treatment of the protein molecules and preserving their biological properties. The labeling mechanism is not well understood. The labeling of ^{99m}Tc to pure protein appears to involve a chemically active ^{99m}Tc-(Zr)citrate complex species with high protein binding capacity. The latter is

formed following initial reduction of ^{99m}Tc-pertechnetate by Zr^{++}ions as a result of electrolysis and by the addition of sodium citrate/NaOH solution during pH adjustment. In the presence of a pure protein such as fibrinogen or immunoglobulin, ^{99m}Tc firmly binds to the protein ligand. Whether the entire complex binds to the protein ligand or acts only as a transferring agent for reduced ^{99m}Tc for the final labeling has not been determined. Further investigation of the improved electrolytic technique indicates that similar complex species can be prepared by chemical means with stannous chloride or stannous tartrate under similar conditions. The resultant ^{99m}Tc-(Sn)citrate complex species is highly effective in labeling plasma proteins with su-
10 perior labeling efficiency and reproducibility. The labeling mechanism of the chemical method has not been determined. It is assumed that protein binding involves the reaction of ^{99m}Tc(Sn)citrate complex species with the protein ligand similar to the ^{99m}Tc(Zr)citrate reaction(Wong, DW, et al, Int. J. Appl. Rad. Isotopes 29: 251, 1978 and Wong, DW, el at, J. Nucl. Med. 20: 967, 1979).

DETAILED DESCRIPTION OF THE INVENTION

Basically, the labeling methodology in the present invention requires the following sequential steps of: 1) initial reduction of ^{99m}Tc-pertechnetate by a stannous reducing agent; 2) the formation of a protein binding ^{99m}Tc(Sn)citrate complex species by the reaction of reduced ^{99m}Tc in +4 or +5 valence state with a solution of
20 sodium citrate; 3) raising the pH of the acidic radioactive admixture to 7.4 with 0.1-1 N sodium hydroxide solution; 4) covalent binding of the radionuclide ^{99m}Tc to the protein ligand by the addition of a source of pure protein. Thus, in the present invention, the actual labeling of the protein with ^{99m}Tc occurs at a pH 7.4 condition. The labeling yield is greater than 95% with less than 1% free or unbound ^{99m}Tc. The radiolabeled product is ready for immediate use without any additional purification process. To facilitate the labeling process, the chemical reactions in steps 2 and 3

can be combined into a single step using an alkaline pH 12.4 sodium citrate and NaOH solution. Since the chemical properties of ^{95m}Tc and ^{99}Tc are identical to that of ^{99m}Tc, the present labeling process can be used to label protein substances with these radionuclides with similar high yields. Both ^{95m}Tc and ^{99}Tc are commercially available in the form of ^{95m}Tc or ^{99}Tc-pertechnetate normal saline solution.

Any stannous salts such as stannous chloride($SnCl_2 \cdot 2H_2O$), stannous fluoride (SnF) or stannous tartrate can be used for the chemical reduction of ^{99m}Tc-pertechnetate to a chemically active ^{99m}Tc +4 or +5 valence state. In the present embodiment, stannous chloride is preferred. The stannous chloride reagent is freshly prepared by dissolving the desired amount of stannous chloride in 0.05 N hydrochloric acid(HCl) solution. The stannous reducing agent is sterilized by passage through a 0.22 nm biological filter and injected into individual sterile and non-pyrogenic serum vials. Alternatively, 0.5-1 ml of the stannous reducing agent containing 0.1-5 mg of $SnCl_2$ can be packaged in a sealed, nitrogen-purged ampule. The liquid stannous reducing agent can be stored under refrigeration at 2-8°C until needed. Preferably, the stannous reducing agent is prepared and packaged in the form of a freeze-dried solid which aids in shipping and storage and is more stable than in liquid reagent form. The lyophilized solid mixture of stannous chloride and 0.05 N HCl can be reconstituted with ^{99m}Tc-pertechnetate normal saline solution without loss of its reducing activity. The concentration of the reducing agent can be varied from 0.2-5 mg/ml depending upon the amount of ^{99m}Tc radioactivity used in the labeling process. The concentration of 0.1 mg $SnCl_2$ in 0.5 ml 0.05N HCl solution is adequate to reduce 60-100 mCi of ^{99m}Tc-pertechnetate.

The source of technetium should be water soluble, with preferred sources being alkali and alkaline earth metal pertechnetates. The technetium-99m is preferably obtained in the form of fresh sodium pertechnetate from a sterile technetium generator.

Any other sources of pharmacologically acceptable Tc-99m can be used, and a number of technetium generators are available. The present invention is not limited to the preparation of ^{99m}Tc-labeled protein substances. Other radionuclides of technetium such as ^{95m}Tc and ^{99}Tc are equally applicable in the labeling process. Depending on clinical applications, compounds labeled with either ^{95m}Tc or ^{99m}Tc are ideal scintigraphic imaging agents, whereas, ^{99}Tc-labeled protein substances may find wide range of applications in in vitro studies and radio-immuno assays.

Alkaline sodium citrate solution is prepared by dissolving 2% of trisodium citrate crystals in distilled water and adjusted to pH 12.4 with 1 N NaOH. One to 2 ml of this reagent is packaged in a sealed, sterile apyrogenic container. While it is preferred that an alkaline sodium citrate/NaOH solution be used to produce the ^{99m}Tc-(Sn)citrate complex species and to raise the pH to 7.4 condition prior to the addition of the protein, the same result can be achieved using two separate reagents, that is, a 2% sodium citrate solution and a 0.1-1 N NaOH solution. However, sodium citrate solution must be added first to react with the reduced ^{99m}Tc prior to pH adjustment with NaOH. The amount of NaOH solution needed can be determined by a simple routine experiment by those skilled in the art. The alkaline sodium citrate/NaOH solution is stable when kept in refrigeration at 2-8° C. To maintain a proper pH environment, it should be packaged in the form of a lyophilized solid. The freeze-dried powder is to be reconstituted with 1-2 ml distilled water at time of use.

The amount of protein substances that can be labeled with the radionuclides of technetium varies from 0.1- 100 mg. In the present invention, a concentration of 3 to 4 mg of protein in 1 ml diluent is adequate to bind up to 100 mCi of ^{95m}Tc, ^{99}Tc or ^{99m}Tc. Any pharmacologically acceptable diluents such as water for injection, normal saline, phosphate buffer having a neutral pH can be used to reconstitute or to dilute the protein to the desired concentration. The following proteins have been labeled with ^{95m}Tc, ^{99}Tc & ^{99m}Tc: 1) human fibrinogen; 2) human serum albumin, salt

poor; 3) the enzymes bovine thrombin, urokinase and streptokinase-activated human plasmin(plasmin-SK); 4) the hormone thyrotropin; 5) the transport proteins transferrin and lactoferrin; 6) human immune gamma globulin and antibacterial antibody (Rabbit, anti-Staphylococcus aureus antibody).

In accordance with the principles of this invention, 2-3 ml of ^{99m}Tc-pertechnetate normal saline solution providing 60-100 mCi of radioactivity is aseptically injected into the reaction vial containing 0.5 ml of a solution of 0.1 mg $SnCl_2$ in 0.05 N HCl. The radioactive content of the reaction vial is shaken for 1-10 minutes to allow complete reduction of ^{99m}Tc-pertechnetate. A sufficient amount of pH 12.4 sodium citrate/NaOH solution is added to the acidic reduced ^{99m}Tc solution (pH 1.8) to form the protein binding ^{99m}Tc(Sn)citrate complex species and to produce a pH 7.4 condition prior to the addition of the protein. This generally ranges from 0.5-1 ml. Immediately, inject 1 ml aqueous protein solution having 3-4 mg of protein content into the reaction vial slowly with gentle swirling to avoiding foaming. The radionuclide is quickly bound to the protein ligand and is stabilized by incubating the final radiolabeled product at 37°C or at room temperature for 10-30 minutes. The resulting ^{99m}Tc-labeled protein is stable for up to 48 hours without any evidence of radiochemical or biological decomposition. After incubation, the radioactive preparation is then intravenously injected into the patient.

Results from analysis of a series of at least 12 trials for each labeled protein indicate that an average binding efficiency of greater than 95%(range 95-99%) is achieved as assessed by radiochromatography with less than 1% free or unbound ^{99m}Tc-pertechnetate. Protein precipitation determinations using trichloroacetic acid demonstrate the existence of a reduced unbound species, presumably, ^{99m}Tc(Sn) complex which accounts for 3-4% of the radioactivity. Electrophoresis protein profiles are identical for both labeled and unlabeled protein fractions. Greater than 95%

of the radionuclide is firmly bound to the protein. In vitro assays demonstrate no significant loss of biological, enzymatic or immunological activity after labeling process. The final labeled product is sterile, apyrogenic up to seven days without any evidence of microorganism contamination.

Procedures for labeling protein substances with the radionuclide ^{95m}Tc are identical to that of ^{99m}Tc labeling technique. Instead of ^{99m}Tc-pertechnetate, 60-100 mCi of ^{95m}Tc-pertechnetate normal saline solution is used in the labeling process. Protein substances labeled with ^{95m}Tc are useful scintigraphic imaging agents. The gamma photon of ^{95m}Tc is compatible with the conventional scintigraphic equipments but with

10 a half life of 61 days. The longer half life of ^{95m}Tc-labeled compounds makes them attractive alternatives for imaging requiring observation periods of days rather than hours.

For purposes of in vitro assays or biological studies, ^{99}Tc-labeled tracer materials are preferred. Technetium-99 is a beta emitter with a half life of 2.12 X 10^5 years. Its beta photon energy of 200 KeV is compatible with existing beta liquid scintillation counters. When prepared in lyophilized form, ^{99}Tc-labeled compounds have useful shelf life in years rather months. The present invention offers an additional advantage over radioiodination reaction. It can produce a radioactive tracer material with high specific activity in terms of millicurie(mCi) of ^{99}Tc/ug protein

20 as compared to microcuries(uCi) of ^{125}I/mg of protein.

In vitro assays require highly pure radioactive tracer materials. Additional purification steps are needed in order to remove unbound ^{99}Tc(Sn) complex species and free ^{99}Tc-pertechnetate from the labeled protein. These radioactive impurities which are less than 5%, can easily be removed by gel filtration using high molecular weight dextran(Sephadex G-100) or polyacrylamide(Bio-Gel P-100) gel columns. The labeled product is added to the gel column and is eluted with pH 7.4 phosphate buffer. Pure ^{99}Tc-labeled protein having higher molecular weight will be eluted from the gel

column and collected in the first 10-20 ml fractions. Unbound $^{99}Tc(Sn)$ complex species and free ^{99}Tc will be retained in the column. The eluent which contains the pure radiolabeled protein is then lyophilized by conventional freeze-drying technics to remove water content. The lyophilized product is assayed for specific activity (mCi ^{99}Tc/ug or mg of protein content) and repackaged to the desired amount of radioactivity in sealed, sterile apyrogenic containers. Alternatively, the radiolabeled protein solution eluted from the column can be concentrated by precipitating the protein from solution with 1:1 ratio of 4 M ammonium sulfate solution. The protein precipitate is separated from the supernatant by ultracentrifugation. The superna-
10 tant is discarded. The protein precipitate is washed with pH 7.4 phosphate buffer and redissolved in the same buffer or normal saline to the desired specific concentration or specific activity.

The present invention is far superior to previous patented labeling technics. Based on the chemical labeling process described above, a simple instant non-radioactive labeling reagent kit can be prepared in advance with individual components packaged separately in sealed, sterile non-pyrogenic containers. The labeling kit is comprised of two basic components: a solution of stannous chloride dissolved in 0.05 N HCl and a pH 12.4 sodium citrate/NaOH solution packaged in lyophilized forms. The labeling kit is to be used in conjunction with a source of ^{95m}Tc-, ^{99}Tc- or ^{99m}Tc-
20 pertechnetate and a source of protein solution desired to be labeled. With the exception of human immunoglobulins or antibodies, exogenous protein preparations are commercially available in sterile apyrogenic solution or in lyophilized forms. These commercial protein preparations can be repackaged in small quantities and incorporated in the labeling kit.

The primary purpose of the labeling kit is to provide a simple means of labeling any protein substances so desired by the user with any radionuclides of Technetium based on the present labeling process. With availibility of such a kit, the

user can also label autologous plasma proteins such as human fibrinogen and human immunoglobulins(IgG, IgM, IgE, IgA and IgD) isolated from the patients. Antibodies or immunoglobulins are found only in patients who are afflicted with infection or tumors. Technetium-99m or ^{95m}Tc-labeled autologous antibodies provide a unique means of making a specific diagnosis of these diseases using scintillation imaging techniques. The use of autologous proteins also eliminate the risk of hepatitis transmission and antigenic reactions. Thus, such a simple instant labeling kit will provide the user an easy access to the present chemical labeling process and to produce a radioactive diagnostic composition suitable for use in scintillation imaging when-
10 ever is needed without risk of the ill effects, such as serum hepatitis, carried by heterologous human proteins.

In use, the labeling reagent kit of the invention is mixed with a source of ^{95m}Tc- or ^{99m}Tc-pertechnetate normal saline solution to form an efficiently labeled ^{95m}Tc-or ^{99m}Tc-protein suitable for scintigraphic imaging in the field of Nuclear Medicine. The radiolabeled protein of the present invention is prepared and readied for injection in a simple three-steps procedure. In the first step, using an aseptic technique, 2-3 ml of ^{95m}Tc or ^{99m}Tc-pertechnetate in normal saline solution providing 60-100 mCi of radioactivity is drawn into a syringe and is injected into the reaction vial containing the stannous reducing agent. Reduction of the radionuclide to
20 a chemically active +4 or +5 valence state occurs when the lyophilized powder is dissolved by ^{95m}Tc or ^{99m}Tc-pertechnetate normal saline solution. The content of the reaction vial is shaken for 1-10 minutes to allow complete reduction of the radionuclide. In the second step, a sufficient amount of reconstituted pH 12.4 sodium citrate/NaOH solution is added to the reduced ^{95m}Tc or ^{99m}Tc solution to form the protein binding ^{95m}Tc or ^{99m}Tc(Sn)citrate complex species and to produce a pH 7.4 condition prior to the addition of the protein. This generally ranges from 0.5 to 1 ml. In the third step, 1 ml of the reconstituted protein solution providing 3-4 mg of

protein is aseptically injected into the reaction vial containing the radioactive admixture resulting from step 2 and is allowed to incubate at 37°C or at room temperature for 10-30 minutes. After incubation, the radioactive preparation is ready for intravenous injection in patient without any additional purification process.

The same labeling kit can be used to label protein substances with ^{99}Tc according to the labeling procedure described above. In addition to the three basic labeling steps, the radiolabeled protein is purified by passage through a high molecular weight gel column to remove radioactive impurities. Pure ^{99}Tc-labeled protein is eluted from the gel column and collected in sterile container. The protein solution is then sterilized by ultrafiltration technics and packaged in sealed, sterile nonpyrogenic vials. To obtain high specific concentration, the protein solution can be lyophilized to remove water content and sterilized by gas autoclaving technics. This aids in shipping and storage and to produce a stable product with longer shelf life. The lyophilized product is assayed and packaged in sealed sterilized serum vials. Alternatively, the protein solution obtained from gel filtration can be concentrated by salting out the pure labeled protein with ammonium sulfate solution. Following centrifugation, separation and washing procedures, the protein precipitate is redissolved in pH 7.4 phosphate and sterilized by ultrafiltration technics. The purified product is then assayed and packaged in liquid or lyophilized form.

Plasma proteins perform many vital biological functions in plants and animals. A physiological chemical method of labeling these substances with a suitable radionuclide offers unlimited potential in biological investigation and medical application. Technetium-95m or 99m labeled human fibrinogen or plasmin-SK, for example, is extremely useful for localizing and detecting fibrinogen or fibrin depositions in thromboembolic disease, myocardial infarction or neoplasm using scintigraphic imaging technics. Similarly, infections lesions and tumors can be specifically detected with

^{95m}Tc-or ^{99m}Tc-labeled autologous immunoglobulins. Radiolabeled serum albumin and transferrin are useful in placenta localization, cardiac scan and cisternography. ^{95m}Tc-or ^{99m}Tc-labeled hormones and lactoferrin may find useful application in detecting hormone producing tumors found in the breast, the thyroid and adrenal glands. A dose of 3-25 mCi of radiolabeled protein substances administered intravenously to patient is sufficient to detect these lesions. Whole body scans are taken at various time intervals, e.g. 0.5-24 hours post administration of the dose using a rectilinear scanner or an Anger scintillation camera. Increased radioactivity at the sites of these lesions indicates the presence of thrombi, emboli, myocardial infarcts, infectious foci, tumors or other abnormalities.

10

Protein substances labeled with ^{99}Tc have wide range of clinical applications such as in radioimmuno assays or other in vitro determinations. In infectious diseases, for example, the invading microorganism can be quickly and specifically identified with ^{99}Tc-antibacterial or antiviral antibodies within hours rather than in days as with standard laboratory tests. In this radioimmuno assay procedure, blood sample is obtained from patient by venipuncture and centrifuged to separate the plasma from the form elements. One to two-tenth milliliters(0.1-0.2 ml) of the plasma sample is added to a series of test tubes each containing 1 ml normal saline and 1-10 uCi of ^{99}Tc-antibacterial antibody specific against a known strain of bacteria.

20

If the matching bacteria is present in the plasma sample, an insoluble radioactive antigen-antibody complex is formed and precipitated from solution. After incubation at room temperature for 30 minutes and centrifuged, the supernatant is discarded. The precipitate is washed thoroughly with normal saline and retained for radioactivity assay using a beta liquid scintillation counter. The test is negative when the precipitate is absent in the test tube as evidenced by lack of radioactivity. Depending on the availability of antibacterial antibodies, many strains of bacteria can be

identified at the same time with the same plasma sample. This simple in vitro testing procedure can also be applied to detect varieties of virus in viral diseases and virus causing neoplasm with ^{99}Tc-antiviral antibodies.

The following examples illustrate the simplicity and efficacy of the present invention for labeling different types of plasma protein with the radionuclides of Technetium:

EXAMPLE 1

Protein formulations

1. Human serum albumin, 25% salf poor, diluted with pH 7.4 phosphate buffer or normal saline to a concentration of 1-25 mg/ml.
2. Human fibrinogen, reconstituted with pH 7.4 phosphate buffer to a concentration of 1-10 mg/ml.
3. Streptokinase-activated human plasmin, reconstituted with pH 7.4 phosphate buffer to a concentration of 5000 units/ml.
4. Human urokinase, reconstituted with pH 7.4 phosphate buffer to a concentration to 5000 units/ml.
5. Bovine thrombin, reconstituted with pH 7.4 phosphate buffer or normal saline to a concentration of 1000 units/ml.
6. Thyrotropin, reconstituted with pH 7.4 phosphate buffer or normal saline to a concentration of 20-100 units/ml.
7. Transferrin, reconstituted with pH 7.4 phosphate buffer to a concentration of 1-10 mg/ml.
8. Lactoferrin, reconstituted with pH 7.4 phosphate buffer to a concentration of 1-10 mg/ml.
9. Human immune gamma globulins or antibodies, diluted with pH 7.4 phosphate buffer to a concentration of 1-20 mg/ml.

Protein formulation(Cont.)

10. Antibacterial antibody(Rabbit, anti-Staphylococcus aureus), diluted to 1-20 mg/ml with pH 7.4 phosphate buffer.

EXAMPLE 2

A general procedure for labeling protein substances with ^{95m}Tc or ^{99m}Tc

1. Inject up to 2 ml (60-100 mCi) of ^{95m}Tc- or ^{99m}Tc-pertechnetate normal saline solution into a sterile evacuated serum vial containing 0.5 ml of a solution of 0.1 mg stannous chloride in 0.05 N hydrochloric acid. Mix the content of the reaction vial vigorously for 1-10 minutes for the complete reduction of
10 ^{95m}Tc- or ^{99m}Tc-pertechnetate.
2. Raise the pH of the mixture of Step (1) to 7.4 by adding 0.5-0.75 ml 2% sodium citrate solution previously adjusted to pH 12.4 with 1 N sodium hydroxide.
3. Immediately inject 1 ml(3-4 mg) of any protein solution desired to be labeled into the vial slowly with gentle swirling to avoid foaming.
4. Incubate the content of the vial at room temperature for 30 minutes. The final labeled product is clear, sterile and ready for use. Additional purification process of the labeled protein is unnecessary.
5. Perform complete qualitative and quantitative radioactive assays. The final
20 concentration should be in the range of 15-25 mCi labeled protein per ml.
6. For scintigraphic imaging, a dose of 3-25 mCi of the radiolabeled protein is sufficient to detect various types of lesions by scanning the patient with a rectilinear scanner or an Anger scintillation camera and by observing areas of increased radioactivity at the sites of these abnormalities as seen in the scintigraphic scans.

EXAMPLE 3

A general procedure for labeling protein substances with ^{99}Tc-pertechnetate

1. Inject 2-5 ml(60-5000 mCi) of ^{99}Tc-pertechnetate normal saline solution into a sterile evacuated serum vial containing 0.5 ml of a soluton of 0.1-5 mg stannous chloride in 0.05N hydrochloric acid. Mix the content of the reaction vial vigorously for 1-10 minutes for the complete reduction of ^{99}Tc-pertechnetate.
2. Raise the pH of the mixture of Step (1) with sufficient amount of pH 12.4 sodium citrate/sodium hydroxide solution to 7.4 condition.
3. Immediately inject 1-2 ml(0.1-100mg)of any protein solution to be labeled into the vial slowly with gentle swirling to avoid foaming.
4. Incubate the content of the vial at room temperature for 30 minutes.
5. Purify the labeled product by passage through an inert high molecular weight dextran or polyacrylamide gel column using normal saline or pH 7.4 phosphate buffer as the eluent.
6. After collecting the first 10-20 ml eluate from the gel column, pure ^{99}Tc-labeled protein is then concentrated to the desired specific activity by lyophilization technic and repackaged as such or by salting-out method with equal volume of 4 M ammonium sulfate solution. The radioactive protein precipitate is undergo further purification steps to remove ammonium sulfate solution, washed and redissolved in pH 7.4 phosphate buffer to the disired concentration.

The above examples and detailed described procedures are for illustration purposes only and are not intended to be limiting of the scope of the invention. It will be apparent to those skilled in the art that both may be modified within the scope of the invention defined in the following claims.

EXAMPLE 4

Formulation of the non-radioactive labeling reagent kit for preparing ^{99m}Tc-labeled plasma proteins.

Essentially, the labeling reagent kit consists of three basic components each aseptically prepared and packaged separately in sterile non-pyrogenic serum vials. When properly prepared, lyophilized and stored at 2° to 8°C, such a labeling reagent kit is stable for more than two years.

Vial #1. Stannous reducing reagent: Each vial contains 0.1-5 mg of stannous chloride, stannous tartrate or stannous fluoride dissolved in 0.05 N
10 hydrochloric acid (HCl) solution. The content of the vial is lyophilized and stored under nitrogen.

Vial #2. Citrate complexing reagent: Each vial contains 1-2 ml of an aqueous solution of 2% sodium citrate made alkaline to pH 12.4 with 1 N NaOH solution. The content of the vial is lyophilized and stored under nitrogen. This reagent is to be reconstituted with 1-2 ml Water for Injection at time of use.

Vial #3. Plasma protein solution: Each vial contains 0.1 to 25 mg of any of the plasma proteins listed in Example 1 in lyophilized form and properly preserved with any pharmaceutically acceptable preservatives or
20 stabilizing agents.

EXAMPLE 5

Procedure for preparing ^{99m}Tc-labeled plasma protein injection utilizing the labeling reagent kit of Example 4.

The directions outlined below must be carefully followed for optimum preparation of ^{99m}Tc-labeled plasma protein injections.

1. Remove the kit from the refrigerator and warm to room temperature before continuing.
2. Reconstitute the citrate complexing reagent of vial #2 with 1-2 ml Water for injection until completely dissolved.
3. Reconstitute the content of vial #3 which contains the plasma protein desired to be labeled with ^{99m}Tc in Water for Injection or normal saline to a concentration of 0.1-25 mg/ml.
4. Aseptically inject 2-3 ml of ^{99m}Tc-pertechnetate in normal saline providing up to 100 mCi of radioactivity into the reaction vial #1 containing the
10 stannous reducing reagent and withdraw an equal volume of air.
5. Shake the contents of vial #1 vigorously for 1 minute and incubate at room temperature for additional 5-10 minutes to allow complete reduction of ^{99m}Tc.
6. Inject 0.5-1 ml of the reconstituted pH 12.4 citrate complexing reagent of vial #2 into the reaction vial #1 to bring the pH of the admixture to 7.4.
7. Immediately, inject 1 ml of the reconstituted plasma protein solution into the reaction vial #1 slowly with gentle swirling.
8. Incubate the contents of vial #1 at room temperature for 10-30 minutes after mixing to allow maximum labeling.
20 9. Do not use the preparation after 8 hours from time of formulation.

The above examples and detailed described procedures are for illustration purposes only and are not intended to be limiting of the scope of the invention. It will be apparent to those skilled in the art that both may be modified within the scope of the invention defined in the following claims.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A method of labeling human or animal plasma proteins, compounds or substances containing proteins with the radionuclides of Technetium at physiological pH 6-8 condition which comprises the sequential steps of:

a. treating a solution of the radionuclide of Technetium with a reducing agent;

b. reacting the reduced radionuclide of Technetium with a citrate complexing agent at pH 6-8 condition;

10 c. binding said radionuclide to the protein ligand and incubating the radioactive protein mixture at $37^{\circ}C$ or at room temperature for 10-30 minutes.

2. A method according to claim 1, wherein said radionuclides of Technetium(Tc) is selected from the group consisting ^{99m}Tc, ^{95m}Tc and ^{99}Tc.

3. A method according to claim 2, wherein said radionuclides of Tc is an aqueous solution of ^{99m}Tc-, ^{95m}Tc- or ^{99}Tc-pertechnetate of sodium, potassium or ammonium providing from 1 to 5000 millicurie(mCi) of radioactivity.

4. A method according to claim 1, wherein said reducing agent is stannous ions selected from the group consisting of stannous chloride, stannous fluoride or stannous tartrate.

20 5. A method according to claim 4, wherein said stannous reducing agent is present in the amount of 0.1-10mg dissolved in 0.05-0.1 N hydrochloric acid(HCl) solution.

6. A method according to claim 5, wherein said stannous reducing agent is present in the amount of 0.2-5 mg/ml 0.05 N HCl solution.

7. A method according to claim 1, wherein said citrate complexing agent is an aqueous solution of 0.5-10% by weight of sodium citrate made alkaline to pH above 8 with 1 N sodium hydroxide(NaOH)solution.

8. A method according to claim 7, wherein 0.5-20 ml of a 2% sodium citrate solution at pH 12.4 is present as the citrate complexing agent.

9. A method according to claim 1, wherein said plasma protein is selected from the group consisting of human serum albumin, human fibrinogen, human urokinase, streptokinase-activated human plasmin, human transferrin, human lactoferrin, human immunoglobulins, bovine thrombin, bovine thyrotropin and antibacterial antibody (Rabbit, anti-Staphylococcus aureus).

10. A method according to claim 9, wherein said plasma protein is present in the amount of 0.001-1000 mg dissolved in an aqueous medium together with any pharmaceutically acceptable preservatives or stabilizing agents.

11. A method according to claim 10,wherein said plasma protein is present in the amount of 1-25 mg dissolved in 1 ml distilled water or normal saline.

12. A method of labeling human or animal plasma proteins, compounds or substances containing proteins with ^{99m}Tc-pertechnetate at physiological pH 6-8 condition comprising the sequential steps of:

a. treating 2-10 ml (50-500 mCi) of ^{99m}Tc-pertechnetate in normal saline with 0.5-1 ml of a solution of 0.1-5 mg stannous chloride, stannous tartrate or stannous fluoride in 0.05 N HCl solution at room temperature for 10 minutes;

b. raising the pH of the acidic mixture of step (a) to 7.4 with a sufficient amount of a pH 12.4 sodium citrate/NaOH solution;

c. adding from 0.1 mg to 100mg of the desired protein to be labeled in 1-5 ml diluents to the admixture of step (b) and incubating said admixture at 37°C or at room temperature for 30 minutes.

13. A method according to claim 12, wherein said plasma protein is selected from the group consisting of human serum albumin, human fibrinogen, human urokinase, streptokinase-activated human plasmin, human transferrin, human lactoferrin, human immunoglobulin, bovine thrombin, bovine thyrotropin and antibacterial antibody (Rabbit, anti-Staphylococcus aureus).

14. A method according to claim 12, wherein said human serum albumin labeled with ^{99m}Tc.

15. A method according to claim 12, wherein said human fibrinogen labeled with ^{99}[illegible].

16. A method according to claim 12, wherein said human urokinase labeled with ^{99m}Tc.

17. A method according to claim 12, wherein said streptokinase-activated human plasmin labeled with ^{99m}Tc.

18. A method according to claim 12, wherein said human transferrin labeled with ^{99m}Tc.

19. A method according to claim 12, wherein said human lactoferrin labeled with ^{99m}Tc.

20. A method according to claim 12, wherein saim human immunoglobulin labeled with ^{99m}Tc.

21. A method according to claim 12, wherein said bovine thrombin labeled with ^{99m}Tc.

22. A method according to claim 12, wherein said bovine thyrotropin labeled with ^{99m}Tc.

23. A method according to claim 12, wherein said antibacterial antibody (Rabbit, anti-Staphylococcus aureus) labeled with ^{99m}Tc.

24. A kit for labeling protein substances with the radionuclides of Technetium at physiological pH 6-8 condition comprising a stannous reducing agent, an alkaline citrate complexing agent and a protein solution desired to be labeled aseptically prepared and packaged separately in sealed, sterile, apyrogenic containers and admixing with the active ingredients of said kit with a solution of a radionuclide of Techentium at time of use, said radionuclide of Technetium is selected from the group consisting ^{99m}Tc-, ^{95m}Tc-, or ^{99}Tc-pertechnetate providing 1 -5000 mCi of the radioactivity.

25. A kit of claim 24, wherein said stannous reducing agent is selected from the group consisting of stannous chloride, stannous fluoride or stannous tartrate.

26. A kit of claim 24, wherein said stannous reducing agent is present in the amount of 0.1-10 mg dissolved in 0.05N to 0.1 N HCl solution.

27. A kit of claim 24, wherein said stannous reducing agent is present in the amount of 0.2-5 mg per ml 0.05 N HCl solution packaged as a freeze-dried solid.

28. A kit of claim 24, wherein said alkaline citrate complexing agent is an aqueous solution of 0.5-10% by weight of sodium citrate made alkaline to pH above 8 with 1 N NaOH solution.

29. A kit of claim 24, wherein 1-20 ml of an queous solution of 2% sodium citrate at pH 12.4 packages in the form of a freeze-dried solid as said alkaline citrate complexing agent.

30. A kit of claim 24, wherein said protein substance is selected from the group consisting of human serum albumin, human fibrinogen, human urokinase, streptokinase-activated human plasmin, human transferrin, human lactoferrin, human immunoglobulin, bovine thrombin, bovine thyrotropin and antibacterial antibody (Rabbit, anti-Staphylococcus aureus).

31. A kit of claim 24, wherein said protein substance is present in the amount of 0.001-1000 mg dissolved in an aqueous medium together with any pharmaceutically acceptable preservatives or stabilizing agents.

32. A kit of claim 24, wherein said protein substance is present in the amount of 1-25 mg dissolved in 1 ml distilled water or normal saline together with any pharmaceutically acceptable preservatives or stabilizing agents, including packaging said protein solution in the form of a freeze-dried solid.

33. A method of preparing ^{99m}Tc-labeled protein substance at physiologic pH 6-8 condition suitable for use in radiologic testing comprises the sequential steps of:

a. reconstituting the alkaline citrate complexing agent with 1-20 ml Water for Injection until completely dissolved;

b. redissolving the freeze-dried protein substance with Water for Injection,, normal saline or any pharmaceutically acceptable carrier to a concentration of 1-25 mg/ml;

c. dissolving the freeze-dried powder of 0.2-5 mg stannous reducing agent with 2-3 ml (50-5000 mCi) ^{99m}Tc-pertechnetate in normal saline for about 1-10 minutes;

d. reacting the mixture of step (c) with 0.5-2 ml of the alkaline citrate complexing solution to a pH of 7.4 condition;

e. adding to the admixture of step (d) 1 ml (1-25 mg) of the reconstituted protein solution and incubating said radioactive protein admixture at 37°C or at room temperature for 10-30 minutes.

SUBSTITUTE

REMPLACEMENT

SECTION is not Present

Cette Section est Absente