

molar excess over the analyte, wherein the dissociation constant between said antibody and the analyte-analogue is greater than the dissociation constant between said antibody and the analyte, whereby said antibody is displaced from the immobilized analyte-analogue as said antibody forms a complex with the analyte from the liquid sample;

- (2) separating said complex from the solid phase; and
- (3) measuring the amount of said complex which is related to the amount of analyte initially present in the liquid sample.

In a preferred embodiment, the anti-analyte antibody is monovalent, and the solid phase is a material such as beaded agarose or cross-linked dextran, packed in a column through which sample containing analyte is percolated. This assay is applicable to analytes having antigenic or haptenic properties, requires no pre-incubation of sample with labeled antibody, and is readily automated.

BRIEF DESCRIPTION OF THE DRAWING

FIG. 1 shows the results of a digoxin assay performed according to the invention using a fluorescent anti-digoxin antibody.

FIG. 2 shows the results of a digoxin assay performed according to the invention using an enzyme-labeled anti-digoxin antibody.

DETAILED DESCRIPTION OF THE INVENTION

For the purposes of this disclosure the following terms are defined as follows: analyte is an antigenic or haptenic substance, or group of substances, whose presence or the amount thereof in a liquid medium is to be determined; analyte-analogue is any substance, or group of substances, whose affinity for anti-analyte antibody is less than that of the analyte; monovalent antibody is an antibody or antibody fragment having only one binding site for antigen and can include Fab and Fab' fragments and half-molecules (heavy chain-light chain dimers) as described in co-pending application Ser. No. 374,971 filed May 5, 1982 incorporated herein by reference.

The labeling substance can be an enzyme, chromophore, fluorophore, chemiluminescent material, radioisotope, coenzyme, pigment particle, latex particle, or any other substance which is capable of generating a signal either by itself or in combination with other reagents.

In general, the labeling substance is chosen so as not to require overly complicated instrumentation for its detection and so as to be relatively immune to interference from normal constituents of body fluids. For these reasons, enzymes are generally preferred. Among those enzymes which have been widely used as labeling substances are β -galactosidase, horseradish peroxidase, and alkaline phosphatase. β -Galactosidase is generally preferred because it is not usually found in serum, has numerous chromogenic and fluorogenic substrates, and has a relatively high turnover number.

Analytes of interest can include proteins, peptides, hormones, drugs, vitamins, cell and tissue antigens, bacteria, viruses, etc. These are usually found in biological fluids such as whole blood, blood serum, blood plasma, urine, saliva, and cerebrospinal fluid, but can also be found on cells and in tissues.

Monovalent antibodies are produced by known methods. For example, Fab fragments are obtained by papain digestion of IgG [Porter, *Biochem. J.*, Volume 73, 119

(1959)]; Fab' fragments are obtained by disulfide reduction of F(ab')₂ fragments obtained by pepsin digestion of IgG [Nisonoff, *Methods Med. Res.*, Volume 10, 132 (1964)]; half-molecules are formed by sulfitolysis of IgG as described in co-pending application Ser. No. 374,971, filed May 5, 1982, incorporated herein by reference. Intact IgG is sulfitolyzed with sodium sulfite (100 mM per mg IgG) in the presence of 5,5'-dithiobis(2-nitrobenzoic acid) (2.5 mM per mg IgG), preferably in a buffered medium at room temperature under nitrogen, to yield S-sulfonated half molecules of IgG. In general, it is desirable to immunopurify the antibody prior to its use in an immunoassay. Again, the methods for isolation of IgG from animal serum and the methods for its immunopurification by affinity chromatography are known. [Jaton, et al., *Immunological Methods*, Lefkovits & Perris, eds., New York: Academic Press, 1979, 44].

Although divalent antibodies can be used in the assay of this invention, sensitivity is improved by the use of monovalent antibodies, and the latter are, therefore, preferred. This is due to the fact that monosaturated divalent antibodies cannot be distinguished from unsaturated antibodies in terms of their binding behavior. If monovalent antibodies are employed, there will be a one-to-one correspondence between binding of sample analyte and displacement of labeled antibody from the solid phase.

Methods for coupling the labeling substance to the antibody are known. See, for example, *Methods in Enzymology*, van Vunakis & Langone (eds.), Volume 70 (1980), and references contained therein. In general, one should couple at least one label to each antibody, preferably covalently, and in such a manner as to preserve the immunoreactivity of the antibody. The free sulfhydryl groups present on Fab' fragments and the sulfonate group present on half-molecules provide specific reactive groups for covalent attachment of the label. Labeling of the antibody using these groups is known not to affect the immunoreactivity of the antibody. Heterobifunctional crosslinking reagents having maleimido or thiopyridyl groups are useful for the labeling [O'Sullivan et al., *Methods in Enzymology*, Volume 73, 147 (1981)]. Generally, it is desirable that the final step in the synthesis of the labeled antibody be an immunopurification step.

Analyte or analyte-analogue can be immobilized on a suitable solid phase by known methods. [Kiefer, *Immunological Methods*, Lefkovits & Perris, eds., New York: Academic Press, 1979, 137]. In the preferred mode, the solid phase is an affinity column packing material which is chosen for its flow characteristics and can include, for example, beaded agarose, polyacrylamide, glass, cellulose and cross-linked dextran. The analyte-analogue can be coupled covalently to the solid phase, either directly or through a spacer arm. The spacer arm can be, for example, a protein or polyamino acid.

Although the column packing material of an affinity column serves as the solid phase in the preferred embodiment of this invention, other solid phases are contemplated. For example, the solid phase can be the wall of a plastic test tube or microtiter plate. In these cases, the analyte-analogue can be immobilized by physical means, and the complexed displaced, labeled, anti-analyte antibody will be separated from the solid phase by decanting the reaction mixture.

The analyte-analogue should be chosen so as to have a substantially lower affinity for anti-analyte antibody