

performance is expected to be equivalent whether one uses analyte or analyte analogue. Unexpectedly, it has been found in the assay of this invention for digoxin that substitution of ouabain for digoxin as the immobilized antigen dramatically improves the sensitivity and precision of the assay. Although the exact mechanism responsible for this improvement is not known, it is believed to reside in the nature of the antigen-antibody interaction.

There is a need in the art for a heterogeneous immunoassay for digoxin, the sensitivity and precision of which are not limited by the affinity constant of the antibody.

DISCLOSURE OF INVENTION

The noncompetitive heterogeneous immunoassay of this invention is comprised of the following steps:

(a) forming a reaction mixture by contacting a molar excess of labeled, monovalent or divalent anti-digoxin antibody with a test sample suspected of containing digoxin, whereby a fraction of said antibody forms a complex with the digoxin and a fraction remains free;

(b) separating free antibody from the reaction mixture by contacting the reaction mixture with a solid phase comprising ouabain immobilized on a solid support, the ouabain being present in an amount capable of binding all of the free antibody; and

(c) measuring the amount of complex which elutes from the solid phase by measuring the label.

The amount of digoxin in the sample can then be determined by comparison with a standard curve.

In another aspect, the present invention is a competitive, heterogeneous immunoassay which is comprised of the following sequential steps:

(a) forming a reaction mixture by contacting a sample suspected of containing digoxin with a molar excess of ouabain immobilized on a solid phase;

(b) contacting the reaction mixture with a labeled, monovalent or divalent anti-digoxin antibody, said antibody being in molar excess over digoxin, but in molar deficiency relative to ouabain;

(c) allowing a reaction to take place whereby a fraction of the antibody forms a first complex with the digoxin and a second fraction forms a complex with the immobilized ouabain;

(d) separating the first fraction from the second fraction; and

(e) determining the amount of label present in either the first or the second fraction.

DESCRIPTION OF THE INVENTION

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, ascites fluid, or tissue culture medium and the methods for its immunopurification by affinity chromatography are known in the art. Briefly, an Ig fraction is prepared by ammonium sulfate precipitation. An IgG fraction is then prepared by ion exchange, gel filtration, or protein A chromatography. Affinity purification is effected by elution from an antigen column.

The antibody can be polyclonal or monoclonal. Monovalent antibodies are produced by methods known in the art. For example, Fab fragments are obtained by papain digestion of IgG; Fab' fragments are obtained by disulfide reduction of F(ab')₂ fragments obtained by pepsin digestion of IgG.

Any number of methods can be employed to couple the labeling substance to the antibody. The labeling substance can be enzyme, radioisotope, chromophore, fluorophore or any other substance which is capable of generating a detectable signal, either alone or in combination with other reagents. 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 and the activity of the labeling substance. The free sulfhydryl groups present on Fab' fragments provide specific reactive groups for covalent attachment of the label. Heterobifunctional crosslinking reagents having maleimido- or thiopyridyl-groups are useful for this purpose. Generally, it is desirable that the final step in the synthesis of the labeled antibody be an immunopurification step to ensure retention of immunoreactivity.

Ouabain or a conjugate thereof can be immobilized on a suitable support by methods known in the art, e.g. Smith, T. W., Butler, V. P., Haber, E., *Biochemistry*, Volume 9, p. 331 (1970). In general, the support is chosen for its flow characteristics and can include, for example, beaded agarose, beaded dextran, polyacrylamide, or glass. The ouabain can be coupled covalently to the support, either directly or through a spacer arm which can be a protein, polyamino acid, or synthetic linker. Usually, the affinity column material is discarded after one use, but it is possible to recycle it if desired. It is generally undesirable for the support to possess molecular sieving properties since, should the labeled antibody become dissociated from sample analyte, molecular sieving will tend to reduce the likelihood of their finding each other again.

In the noncompetitive mode, the assay of this invention can be performed as follows: A known volume of patient sample, usually 5 μ L to 500 μ L of serum, containing an unknown amount of digoxin is mixed with a solution containing an amount of labeled, monovalent or divalent antibody known to be in excess over digoxin. Usually the labeled antibody will be present in approximately 10-100 molar excess over digoxin. Digoxin and antibody are preincubated for a specified length of time, usually at least 5 minutes and not more than 30 minutes, at a fixed temperature between 4° C. and 45° C., usually 37° C. A known volume (usually 5 μ L to 500 μ L) of this solution containing digoxin-bound antibody and unbound antibody is passed through a column, preferably of dimensions 2 mm \times 10 mm, consisting of ouabain immobilized on a porous support. Sufficient ouabain-coupled support is used to bind all of the free labeled antibody. The column is eluted at a flow rate of 0.2-4.0 mL per minute with a suitable buffer, usually 1-5 mL total volume. The fraction which elutes from the column contains labeled antibody complexed with digoxin from patient serum. The activity of the label in this fraction is then measured. Alternatively, one can discard this fraction and elute the retained antibody from the column with a chaotropic agent or by extremes of pH. In the first instance, the amount of label is directly proportional to the concentration of digoxin in the sample. In the second, it is inversely proportional.

The assay of this invention can be performed manually or it can be adapted to a variety of automated or semi-automated instrumentation, such as the aca® discrete clinical analyzer manufactured by E. I. du Pont de Nemours and Company, Inc., Wilmington, Dela. In this case, patient sample and excess labeled, monovalent or divalent antibody are preincubated outside the in-