

than does the analyte itself. It is believed that the dissociation constant, K_d , of the antibody-(analyte-analogue) complex should be at least ten to one hundred times that of the antibody-analyte complex. In the embodiment in which an affinity column is used to effect the separation step, it is believed that the rate at which antibody dissociates from analyte-analogue must be fast compared to the rate at which sample elutes from the column. For example, in an embodiment of this invention used to detect digoxin, ouabain is chosen as the analyte-analogue because, while it can still bind to anti-digoxin antibody, the dissociation constant of the resulting complex is sufficiently greater than that of a digoxin complex so as to permit the assay to be performed rapidly and without the need for inclusion of a chaotropic agent. Other examples of useful analyte/analyte-analogue pairs include procainamide/N-acetylprocainamide and theophylline/caffeine. It is expected that one should also be able to utilize partially denatured or chemically modified antigen as analogue; see, for example, Landsteiner, *The Specificity of Serological Reactions*, Cambridge: Harvard Press, (1945).

In the preferred embodiment, an affinity column is pre-saturated with an amount of labeled monovalent antibody known to be in molar excess over analyte. In this way, one is assured that every molecule of analyte will be able to displace one molecule of labeled antibody.

Because the labeled antibody can be present in excess, there is no need for very precise loading of the column, thus simplifying its synthesis. Slight variations in the amount of antibody on the column will not affect the accuracy or reproducibility of the measurements made.

Alternatively, one can perform the present invention in a batch mode rather than a column mode. In the batch mode, a slurry of affinity column packing material having analyte-analogue immobilized thereon and labeled, anti-analyte antibody bound thereto is added to a reaction vessel containing liquid sample. After a suitable incubation period, the vessel will be centrifuged and the supernatant fluid containing displaced antibody will be aspirated and analyzed.

In the preferred mode, the assay of this invention is performed as follows: A known volume of liquid sample, usually 5 μ L to 500 μ L, containing an unknown quantity of analyte is percolated through a column, preferably of dimensions 2 mm \times 10 mm, containing analyte-analogue immobilized on a column packing material and pre-saturated with an excess of labeled, monovalent anti-analyte antibody. The sample is eluted from the column at a flow rate of 0.2-5.0 mL per minute with a suitable buffer such as phosphate buffered saline, usually 1-5 mL total volume. The fraction which elutes from the column contains labeled antibody complexed with analyte from liquid sample. The activity of the label in this fraction is then measured and correlated by means of a standard curve to the concentration of analyte in the sample.

The assay of this invention can be performed manually or can be adapted to a variety of semi-automated or automated instrumentation, such as the acaTM discrete clinical analyzer available from E. I. du Pont de Nemours and Company, Wilmington, Del. The following examples illustrate the invention.

EXAMPLE I

Fluorescent Immunoassay for Digoxin

Fluoresceinated α -digoxin antibodies were purchased from Cappel Laboratories as immunoglobulin (IgG) fraction. Divalent digoxin monospecific antibodies were purified from this fraction by affinity chromatography on a ouabain-affinity resin.

(A) Synthesis of Affinity Column Packing Material Having Ouabain Immobilized Thereon

Ouabain was attached to an agarose matrix through human serum albumin, HSA, as follows:

(1) A ouabain-HSA conjugate was synthesized. Ouabain (0.56 mmole dissolved in 20 mL of water) was oxidized with sodium metaperiodate (1.02 mmole) for 1 hour at room temperature in the dark. Quantitative oxidation was verified by thin layer chromatography on silica gel G plates developed in ethyl acetate:methanol:H₂O (75:25:1 by volume). The excess periodate was removed by passing the aqueous mixture through a 3 mL column of DOWEX AG-1X8 which is a strong basic anion exchange resin with quaternary ammonium exchange groups attached to a styrene-divinylbenzene copolymer lattice. Quantitative recovery of ouabain was verified by following radiolabeled (tritiated) ouabain. The solution of oxidized ouabain was buffered to pH 9.5 with the addition of 0.4 mL of 5% Na₂CO₃ and combined with 20 mL of HSA solution (28 mg/mL). After 45 minutes, the conjugate was reduced with the addition of 0.3 gm of sodium borohydride freshly dissolved in 20 mL of water. Three hours later, 8 mL of 1 M formic acid was added to lower the pH to 6.5. After 1 hour at pH 6.5, the pH was raised to pH 7.5 with 1 M NH₄OH. The entire reaction mixture was dialyzed extensively against distilled water, and then finally against 0.015 M sodium phosphate buffer, pH 7.8, 0.15 M NaCl. The conjugate was concentrated on an Amicon PM-30 membrane (inert, nonionic, polymeric membrane with macrosolute retention cut-off of 30,000 daltons) to 4.2 mg/mL. Protein concentration was determined by the method of Lowry. [Lowry, O H., et al.(1951) J. Biol. Chem., 193, 265.]

(2) The ouabain-HSA conjugate was immobilized on material sold under the trade name Affi-Gel®10 (Bio-Rad Laboratories). This material is a 6% beaded agarose matrix having a long hydrophilic chemical spacer arm terminating in a N-hydroxy succinimidyl ester for chemical attachment of ligands such as proteins via their amino groups. The immobilization procedure was as follows: 25 mL of Affi-Gel® was washed with 75 mL of ice-cold water. The gel was added to the concentrated ouabain-HSA conjugate and allowed to mix on a rocker overnight at 4° C. The excess active ester groups were blocked by adding 0.1 mL of 1 M ethanolamine, pH 8.0, for 1 hour at room temperature. Finally, the gel was washed extensively with distilled water, and then in turn with 500 mL of 0.5 M NaCl; 400 mL of 0.1 M glycine, pH 2.5; 300 mL of 2.5 M NH₄SCN; 1000 mL of phosphate buffered saline. The column packing material (also referred to as "resin") was stored at 4° C. in the presence of 0.02% sodium azide.

(B) Affinity Purification of Anti-Digoxin

Digoxin-specific antibodies were immunopurified directly from an IgG fraction using the following representative protocol: The column packing material as prepared in A(2) above was packed into a column (0.7 cm \times 15 cm) to a bed volume of 6 mL and equilibrated