

# PATENT SPECIFICATION

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## (54) SUBSTRATE COATED WITH RECEPTOR AND LABELLED LIGAND FOR ASSAYS

- (71) We, BECTON, DICKINSON & COMPANY, a corporation organised under the laws of the State of New Jersey, United States of America, of Rutherford, New Jersey, United States of America, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—
- This invention relates to the assay of ligands, and more particularly, to a new and improved article and process for assaying for ligands.
- The present procedure for assaying for ligands is based on the competition between a labelled form of a ligand the ligand for a limited number of sites on a receptor for the ligand. In brief, a known quantity of the labelled form of the ligand, a sample containing an unknown quantity of ligand and a known quantity of receptor for the ligand are combined, and the percentage of the labelled form of the ligand bound to the receptor will depend upon the quantity of ligand in the sample. After the receptor, including bound ligand, is separated from the sample the amount of the labelled form of the ligand bound to the receptor or remaining in the sample is determined and compared with a standard curve to determine the quantity of ligand which was present in the sample.
- In order to facilitate separation of the receptor from the sample, in many cases, the receptor is bound to a solid phase, in the form of a tube, insoluble particles or the like, whereby the receptor including bound ligand can be easily separated from a sample for making the determination as to the quantity of labelled ligand which is either bound to the receptor or remains free in the sample.
- The present invention is directed to an improvement in the article and procedures for effecting an assay in which the receptor is bound to an insoluble solid phase, and is related to the invention of our copending application No 28416/77 (Serial No. 1560860) which is directed to providing improvements in receptor coated plastics for assay of ligands.
- In accordance with the present invention there is provided an article for the assay of a ligand, comprising a solid, on said solid a receptor for both the ligand to be assayed and a labelled form of the ligand to be assayed, and a labelled form of the ligand to be assayed reversibly coated on at least 0.5% and no more than 25% of the surface of said receptor, whereby during a subsequent assay for the ligand, the labelled form of the ligand is removed from the receptor for re-equilibration with the receptor in competition with the ligand to be assayed. In this manner, there is provided an article containing both a receptor and a labelled form of the ligand to be assayed, whereby an assay can be effected by equilibrating a sample containing the ligand to be assayed with the solid substrate including the receptor and the labelled form of the ligand, thereby eliminating the necessity for separate addition of labelled ligand and sample during an assay.
- The ligand which can be coated on the receptor, in labelled form, is any one of a wide variety of ligands for which an appropriate receptor can be found, such as (1) antigens, which when introduced into the blood stream of a vertebrate, result in the formation of antibodies; (2) haptens, which when bound to an antigenic carrier and introduced into the blood stream of a vertebrate, produce antibodies specific for the hapten, or (3) ligands which have naturally occurring receptors which can be isolated in a form specific for the ligand. It is to be understood that a ligand can have naturally occurring receptors and also function as a hapten when bound to a protein.
- As representative examples of ligands to which the present invention is applicable, there may be mentioned: polypeptides, nucleotides, nucleosides and proteins, such as ACTH, oxytocin, luteinizing hormone, insulin, proinsulin, Bence-Jones protein,

chorionic gonadotropin, pituitary gonadotropin, growth hormone, renin, thyroxine binding globulin, bradykinin, angiotensin, follicle stimulating hormone; 5 cyclic AMP; choly glycine and cyclic GMP steroids, including: estrogens, gestrogens, androgens, adrenocortical hormones, bile acids, cardiotonic glycosides, aglycones as well as saponins. As specific examples, these 10 may be mentioned: thyroxine, triiodothyronine, testosterone, andosterone, equilenin, estrone, estriol, progesterone, pregnenolone, 17-hydroxydioxcorticosterone (compound S), 15 deoxycorticosterone, cortisone, corticosterone, cortisol, aldosterone, digoxin, digitoxin, etc.; vitamins, such as vitamin A, folic acid, the B vitamin group, vitamin C, the D vitamins, and vitamins E 20 and K; and miscellaneous ligands, such as, antigens for Viral Hepatitis A and B, Rubella, Herpes Simplex and  $\alpha$ -fetoprotein.

The above substances are only 25 representative, and it is understood that such substances can be used as appropriate analogs and the term labelled form of the ligand includes such analogs.

The ligand is coated on the antibody in 30 labelled form; i.e., the ligand coated on the antibody applied to a solid support contains a "label", "tag" or "tracer", (such terms are interchangeably used in the art) which can be for example a radioisotope, an enzyme or 35 a fluorescent material. The use of such labels or tags and the procedures for preparing a ligand containing such label, tag or tracer are well known in the art and no further details in this respect are needed for a complete understanding of the invention. 40 The preferred labelled ligand is radiolabelled, and as known in the art, such radioactive isotope is generally tritium or one of the radioisotopes of iodine.

The solid material on which the antibody 45 is coated may be any one of a wide variety of solid materials. As known in the art, such materials include suitable polymers, such as, polystyrene, polyethylene, polypropylene, polytetrafluoroethylene, polyamides and 50 polyacrylamides; glass; bacterial cells and ion exchange resins. Such solid carriers are known in the art and no further details in this respect are deemed necessary for a full understanding of the invention.

55 As known in the art, the receptor is coated on the solid phase, which can be in particulate form, sheet form or in the form of a tube, with a tube being particularly preferred. The antibody adheres to the solid 60 phase either by adsorption or by suitable covalent coupling to the solid phase, all of which procedures are known in the art. The preferred solid phase is a plastic tube and in particular a tube formed of polystyrene.

65 It has been found that a superior product

is formed if the antibody is coated onto a solid substrate to which the receptor adheres by adsorption by a technique in which the biologically receptive sites are 70 blocked with a blocking agent whereby the receptor is oriented with respect to the surface.

In accordance with such a technique, a receptor is coated on a plastic substrate in the presence of a deaggregation agent for 75 the receptor and a blocking agent for the ligand receptor sites in order to effect adherence of the receptor to the plastic substrate at other than the receptor sites.

More particularly, the blocking agent is a 80 substance which has a limited cross-reactivity with the receptor in order to effect blocking of the ligand receptor sites to thereby prevent adherence of the receptor to the plastic at the ligand receptor 85 sites, while also permitting subsequent binding of a ligand to the receptor. In general, the substance employed as a blocking agent for the ligand receptor sites has a cross-reactivity with the receptor of 90 from about 0.001 to about 5% and preferably from about 0.001 to about 1%. The upper limit of the cross-reactivity insures that in a subsequent assay the blocking agent can be displaced by the 95 ligand to be assayed whereby there can be competitive binding at the receptor sites. As should be apparent, the substance employed as a blocking agent should have an affinity for the plastic surface in order to 100 permit orientation of the receptor on the plastic. The substance employed as a blocking agent is preferably hydrophilic; however, it is to be understood that non-hydrophilic substances can also be 105 employed.

As representative examples of suitable blocking agents, there may be mentioned: cardiac glycosides, such as, ouabain, gitoxin gitaloxin and acetyl strophanthidin; 110 steriods, such as progesterol, testosterone and cortisol.

The selection of a suitable agent for blocking the receptor sites is deemed to be within the scope of those skilled in the art 115 from the teachings herein.

The coating of antibody on the plastic substrate is also effected in the presence of a deaggregating agent for the receptor in order to prevent adherence to the plastic 120 substrate in clumps or aggregates, which would limit the availability of receptor sites in the susequent assay. The deaggregating agent may be one of a wide variety of such agents which are used in the art, and as 125 representative examples of such agents, there may be mentioned: amino acids, such as, glycine and lysine; urea; guanidine; soluble inorganic salts; in particular, calcium salts. The selection of a suitable 130

deaggregating agent is deemed to be within the scope of those skilled in the art from the teachings herein.

5 The blocking agent is employed in an amount effective for blocking the receptor sites of the receptor for the ligand, which will vary with the amount of receptor and the particular receptor employed. In general, the blocking agent is employed in 10 an amount of from 0.0001 to 0.01%, preferably from 0.0005 to 0.005%, all weight percent of solution. Similarly, the amount of deaggregating agent employed is dependent on the receptor and amount thereof, with 15 the deaggregating agent generally being employed in an amount of from 0.01 M to 0.9 M, preferably from 0.25 M to 0.35 M. The amount of blocking agent and deaggregating agent to be employed is 20 deemed to be within the scope of those skilled in the art from the teachings herein. The receptor coated substrate is generally further treated with a protein containing buffer to reduce or eliminate non-specific 25 binding in the assay.

It has been found that by coating the plastic substrate with antibody in the presence of both a blocking agent and a 30 deaggregating agent, the amount of receptor required for the coating is significantly lower than that required in prior art teachings. It has also been found that the use of such lower amounts of antibody facilitates re-equilibration of the 35 tracer.

The antibody coated on the solid surface is then coated with the labelled ligand. The labelled ligand is applied to only a portion of the receptor surface: the labelled ligand is 40 applied to no more than 25% but at least 0.5% of the receptor surface. It has been found that re-equilibration of the labelled receptor is facilitated by limiting the application thereof to only a portion of the 45 receptor surface. Such a result can be readily achieved by limiting the surface contact area between receptor and labelled ligand in coating the receptor with the labelled ligand. Thus, for example, where a receptor 50 coated tube is employed, the surface area of receptor coated with labelled ligand is limited by placing only a small volume of the labelled ligand solution in the tube whereby only the bottom portion of the tube 55 is contacted with the labelled ligand and only the bottom portion of the receptor coated on the tube is coated with labelled ligand, with the upper portion of the coated receptor being free of labelled ligand. In 60 conducting the assay in the tube, the labelled ligand is removed from the coated receptor and is free to re-equilibrate with the coated receptor. It has been found that notwithstanding the fact that the labelled 65 ligand has been coated on only the lower

portion of the tube, upon completion of the assay, the labelled ligand is evenly distributed over the entire portion of the coated receptor.

The labelled ligand is applied to the receptor as an unbuffered water solution, preferably distilled water, thereby eliminating the possibility of a remaining residue upon drying of the tube. 70

The labelled form of the ligand may be applied to the receptor coated solid substrate at room temperature; however, higher and lower temperatures could be employed, with it being understood that the temperature employed is one which does 75 not adversely affect the ligand or receptor. Thus, there is provided a solid substrate which includes an appropriate receptor and only the labelled form of a ligand to be assayed, with the labelled form being free to 80 re-equilibrate with the receptor in competition with the ligand to be assayed. 85

The solid substrate coated with antibody and having a small portion of the surface area of the antibody coated with a labelled 90 form of a ligand to be assayed may now be employed for assays by procedures known in the art, except that only a sample containing the ligand to be assayed need be 95 added to the system.

Thus, if the labelled form of the ligand is radiolabelled, the presence of ligand in a sample is determined by a radioimmunoassay, with the radiolabelled 100 form of the ligand being removed from the antibody and re-equilibrating with the antibody in an amount dependent upon the quantity of unlabelled ligand to be assayed in the sample. The amount of the radiolabelled form of the ligand present on the solid substrate or in the solution is 105 determined by a suitable counter, as known in the art.

Similarly, if the labelled form of the ligand is enzyme labelled, the presence of 110 ligand in a sample is determined by an enzyme amplification procedure as known in the art.

The present invention will be further described by reference to the following 115 examples; however, the scope of the invention is not to be limited thereby.

#### EXAMPLE I

Digoxin antibody, elicited in sheep with a digoxin human serum albumin conjugate, in 120 an aqueous buffer solution [0.05M carbonate/bicarbonate, pH 9.6, 0.3M glycine, 0.001% ouabain and 0.005% chloramphenicol] is added (1.0ml) to a polystyrene tube, at room temperature, and 125 maintained at room temperature for two hours, followed by aspiration of the fluid from the tube. A buffer solution (0.1 M

phosphate pH 7.0, with 0.9% NaCl and 0.1% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, including 1 g of lysozyme per liter, is added to the tube and held at room temperature for about 10 minutes. The fluid is aspirated from the tube, and the tubes dried *in vacuo* at room temperature.

3-O-succinyl digoxigenin tyrosine [<sup>125</sup>I] is diluted with distilled water to 15—20,000 cpm/50μl and 50μl introduced into the antibody coated tube, followed by drying *in vacuo* to provide an antibody coated tube including tracer added thereto.

**EXAMPLE II**

The antibody coated tubes, including tracer, prepared as in Example I are used for digoxin assay follows:

1. At room temperature, 50μl of Digoxin standard or Patient Sample and 1000μl of phosphate buffered saline (PBS) are pipetted into the tubes as follows:

Tube No.	Digoxin in Standard ng/ml
1,2	0.0
3,4	0.5
5,6	1.0
7,8	1.5
9,10	2.0
11,12	3.0
13,14	5.0
15,16	Patient Samples

The tubes are incubated in a water bath at 37°C for 60 minutes.

2. The liquor is aspirated from the tubes, followed by addition of 1.0—1.5 ml PBS and aspiration. The tubes now contain labelled and unlabelled digoxin bound to antibody.

3. The tubes are counted in sequence for 0.5—5 minutes with a gamma-counter and a standard curve is prepared which covers the range of 0.5—5 ng digoxin per ml of patient sample.

4. The digoxin concentration of the sample is obtained from the standard curve.

The present invention is particularly advantageous in that an assay may be effectively performed without the necessity for separate addition of tracer during the assay.

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, within the scope of the appended claims, the invention may be practiced otherwise than as particularly described.

**WHAT WE CLAIM IS:—**

1. An article for the assay of a ligand, comprising a solid, on said solid a receptor for both the ligand to be assayed and a labelled form of the ligand to be assayed, and a labelled form of the ligand to be assayed reversibly coated on at least 0.5% and no more than 25% of the surface of said receptor, whereby during a subsequent assay for the ligand, the labelled form of the ligand is removed from the receptor for re-equilibration with the receptor in competition with the ligand to be assayed.

2. An article as claimed in Claim 1, wherein the labelled form of the ligand is labelled with a radioactive isotope.

3. An article as claimed in Claim 1 or Claim 2, wherein the solid is a plastics material.

4. An article as claimed in any one of the preceding claims wherein the solid is in the form of a plastics tube and only the bottom portion of the receptor on the tube has the labelled ligand coated thereon.

5. An article as claimed in any one of the preceding claims wherein the receptor is an antibody.

6. An article as claimed in any one of the preceding claims wherein the ligand to be assayed is digoxin and the labelled form of the ligand is a radiolabelled form of digoxigenin.

7. An article as claimed in any one of the preceding claims wherein the solid is polystyrene.

8. An article as claimed in any one of the preceding claims wherein the labelled form of the ligand is 3-O-succinyl digoxigenin tyrosine [<sup>125</sup>I].

9. An article as claimed in any one of the preceding claims wherein the receptor on the solid was applied in the presence of a blocking agent for the receptor and a deaggregating agent.

10. A process for preparing an article according to any one of the preceding claims wherein a receptor for the ligand to be assayed is coated on a tube and the labelled form of the ligand to be assayed is coated on only the bottom portion of the receptor on the tube whereby the labelled form of the ligand is reversibly coated on at least 0.5% and no more than 25% of the surface of the receptor for re-equilibration with the receptor in competition with the ligand to be assayed.

11. A process for the assay of a ligand comprising: equilibrating a sample including

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the ligand with an article as defined in any one of Claims 1 to 9.

5 12. An article for the assay of a ligand according to Claim 1 and as described in Example 1.

13. A process for the assay of a ligand as described with reference to Example II.

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Reference has been directed in pursuance of section 9, subsection (1) of the Patents Act 1949, to patent No. 1488532.

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