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(57) Abstract

A method of effecting an immunological determination of an analyte in a fluid sample, the method comprises the steps of (i) providing a column of a solid support material having a first binding agent which is covalently linked to the solid support material and which is capable of forming an immunological complex with said analyte, (ii) introducing the fluid sample onto the column to allow analyte (if present) therein to form an immunological complex with said first binding agent, (iii) providing a label bound to the immunological complex formed under (ii), and (iv) using the immobilised label in situ on the support to determine the analyte.

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IMMUNOLOGICAL DETERMINATIONS

The present invention relates to a method of effecting an immunological determination, e.g. an immunoassay.

Immunological techniques are well established for use in analytical procedures to determine, for example, the presence and/or amount of an antigen in a sample. The techniques generally rely on the binding of the antigen to an antibody which is specific therefor. Such techniques may be conducted wholly in the liquid phase but it is also know to immobilise antibody onto a solid support which, after the binding reaction has been effected, may be washed to remove unbound antigen. This then permits the detection of immobilised antigen-antibody complex.

The solid supports which have been used so far for such procedures have included so called dipsticks and also the surfaces of microtitre wells. Whilst these prior arrangements are effective for performing the immunological determination, they do suffer from a number of disadvantages, namely:

1. The assay could be conducted in the presence of inhibitors which may produce assay misreading.

2. The assay can not be used to enhance the signal level, i.e. the analyte can not be concentrated in the reaction vessel.

Furthermore, the immobilisation of the antibody on such solid supports is frequently by passive absorption which does not necessarily provide for stable immobilisation of the antibody. This has the disadvantage that the amount of antibody immobilised on the support for a given set of immobilisation conditions may not be a constant so that the support may be of unknown binding capacity (in terms of the amount of antigen which may be bound to immobilised antibody).

WO-A-91/13354 discloses a competitive immunological assay for detecting a target moiety using a column of a solid support material (e.g. tresyl chloride activated Sepharose) having antibody coupled thereto. In the method of WA-A-91/13354, the binding sites of the immobilised antibody are saturated with a labeled form of the target and then a liquid sample containing the target to be determined is flowed through the support thereby causing the target to displace the antigen (i.e. the labeled target). The

displaced antigen may then be detected so that the amount of the target in the liquid sample may be determined.

We believe that there is a disadvantage in the procedure of WO-A-91/13354 in that there is no means of verifying the result from the amount of target immobilised on the column. Thus if the result is to be verified it is necessary to repeat the entire determination.

According to the present invention there is provided a method of effecting an immunological determination of an analyte in a fluid sample, the method comprising the steps of

(i) providing a column of a solid support material having a first binding agent which is covalently linked to the solid support material and which is capable of forming an immunological complex with said analyte,

(ii) introducing the fluid sample onto the column to allow analyte (if present) therein to form an immunological complex with said first binding agent,

(iii) providing a label bound to the immunological complex formed under (ii), and

(iv) using the immobilised label in situ on the support to determine the analyte.

If the analyte to be determined is an antigen then the immobilised first binding agent will be an antibody capable of forming an immunological complex with the antigen. Alternatively if the analyte is an antibody then the immobilised first binding agent will be an antigen.

The immobilised label may be provided in a number of ways. For example, the label may be associated with the analyte so that formation of the immunological complex between the immobilised binding agent and the analyte results in the label becoming immobilised on the support. Alternatively the immobilised complex formed between the first binding agent and a non-labeled analyte may be treated with a labeled conjugate which is capable of binding to the complex formed between the first binding

agent and the analyte. The label of the conjugate thus becomes immobilised on the support and may be used for determining the immobilised analyte.

The method of the invention has a number of advantages.

Firstly, by using covalent bonding of the first binding agent to the support, a stable linkage is formed between this agent and the support. It is possible to control the amount of the binding agent on the support and therefore provide a support of known binding capacity. More specifically, the support (onto which the first binding agent is to be immobilised) will be provided with chemical groups which will react with chemical groups on the first binding agent to form covalent linkages whereby the first binding agent becomes immobilised on the support. The chemical reaction between the groups on the support and those on the first binding agent will occur preferentially (although not necessarily exclusively) to absorption of the latter on the former. Once the chemical reaction has been completed, the support may be treated to remove passively absorbed binding agent, to block absorption sites and to "block" any chemical groups of the support which have not reacted with a binding agent. Thus the analyte may only bind to the first binding agent and given that the quantity of chemical groups on the original support was known, it is possible to provide an immobilisation support of known binding capacity.

A further advantage is that the label is immobilised on the support. Therefore, once the analyte has become bound to the support and the immobilised label has been provided, the immobilised label may be used for more than one determination of the amount of analyte immobilised on the support. This is an advantage for the purpose of checking the result of determination from the amount of analyte bound to the column without the need to repeat the entire experimental procedure. This is not possible with the procedure of WO-A-91/13354 since the labeled antigen is eluted from the column.

The column may, for example, have a bed volume (i.e. the volume occupied by the support material) of 1-100 microlitres.

The solid support (to which the first binding agent is covalently bound) is preferably a particulate material, e.g. having a size in the range of 3-200 microns (preferably 100 to 200 microns).

The use of a flow through column and the particulate supports (with first binding agent immobilised thereto) have a number of advantages. In particular, the particulate supports provide a relatively high surface area (e.g. in excess of $1m^2\ g^{-1}$) which allows relatively large amounts of first binding agent available for binding with an analyte in the sample under investigation. Therefore a relatively large volume of a sample containing only a low concentration of analyte may be passed through the column to build up readily detectable quantities of immobilised immunological complexes (e.g. antibody-antigen). Therefore the apparatus is capable of handling relatively large sample volumes and/or relatively low analyte concentrations.

A further advantage of the invention is that, when desired, the column may be washed to ensure a "clean" column on which procedures may be effected. Thus, for example, after the sample has been introduced onto the column (to form the immunological complexes), the column may be washed to remove any unbound analyte species so that these do not act as interferents in a subsequent detection operation. If the label is provided on a conjugate of the complex formed between the analyte and the first binding agent then the column may be washed after treatment with the conjugate to remove non-immobilised label.

Various chemistries for the covalent immobilisation of the first binding agent (which will generally be an antigen or an antibody) are already known, see for example WO-A-92/15674 (Tepnel) the disclosure of which is incorporated by reference.

It is particularly preferred, in accordance with the invention, that the support to which the first binding agent is to be covalently linked comprises epoxy groups. Preferred supports for use in immobilising the first binding agent are of silica and have a siloxane matrix to which the epoxy groups are covalently linked. Suitable supports (having a siloxane matrix to which are linked epoxy groups) are disclosed in WO-A-93/13220 (Tepnel).

Supports provided with epoxy groups are particularly convenient for use in immobilising the first binding agent by means of a condensation reaction between the epoxy groups and functional groups on the first binding agent. Thus, for example (as will often be the case) where the first binding agent has amino group then the latter

groups may react with epoxy groups of the support under relatively mild conditions, e.g. in buffered solution at ambient temperature.

Irrespective of the method of covalent immobilisation used, it is highly preferred to treat the support with a blocking agent to displace any passively absorbed blocking agent and to block any unreacted functional groups of the support. A particularly convenient blocking agent is a partially hydrolysed gelatin, e.g. BycoA as available from Sigma. An alternative blocking agent is BSA.

The label which is immobilised to the support is preferably an enzyme label, e.g. Horse Radish Peroxidase. It will however be appreciated that a wide range of other enzymes may be used as well understood by those skilled in the art. Using an enzyme label, the amount of immobilised antigen is determined by introducing a substrate for the enzyme onto the column, allowing a detectable change (e.g. a colour change) to develop and then measuring the change.

It is of course possible to use other types of label, e.g. fluorophores, chromophores, and radiolabels.

The method of the invention may be used for the determination of a wide range of antibodies. Non-limiting example of antigens include Gliadin, Thyroid Hormone, Pituitory Hormone, Leutenising Hormone (LTH), and Follicle Stimulating Hormone (FSH). Many other examples will be apparent to those skilled in the art.

The invention will be further described by way of example only with reference to the accompanying drawings, in which:

Fig. 1 schematically illustrates one embodiment of apparatus for effecting the method of the invention;

Fig. 2 illustrates (to a much enlarged scale) one of the particles incorporated in the column of Fig. 1;

Fig. 3 indicates the formation of antigen-antibody complexes on the particle of Fig. 2; and

Fig. 4 illustrates a detection procedure.

Referring to Fig. 1, there is illustrated a flow through column 1 packed with particles 2. The column has inlet lines 3 along which reagents, for wash solutions and

samples may be provided as necessary. An outlet line 4 from the column selectively communicates with a waste line 5 or a detector 6. A heater 7 is provided for heating the column, if necessary.

The particles 2 may for example be of solid (non-porous) silica and have a size in the range 100 to 200 microns. The particles 2 are each provided with a siloxane matrix 8 (as depicted by the dashed line) which may be formed by treating the particles with 3-glycidoxypropyl trimethoxy silane as described more fully in WO-A-93/13220. The resultant siloxane matrix has free epoxy groups which may be used for immobilising antibodies 9 onto the particles 2 (see the enlarged schematic view of the particle 2 in Fig. 3). In this respect, the immobilisation may be effected by reaction of amino, hydroxyl or carboxyl groups on the protein.

It will be appreciated that each particle 2 will be bonded to a large number of antibodies 9 (e.g. there may be nano to micromoles of bound protein per gram). Moreover the particles together provide a surface area of the order of about 1 m^2/gram so that there are very large numbers of antibodies available for use in the analysis procedure described below.

The antibodies 9 will be capable of forming an antibody-antigen complex with a specific antigen, e.g. a virus or a hormone (such as thyroid hormone) and as such the column 1 (containing the particles 2) may be used for detecting the presence of that specific antigen in a sample using the following procedure.

Initially, the particles 2 may be washed by introducing a suitable wash solution into the column to ensure that the column is "clean" and does not contain any unwanted interferents.

The sample to be investigated may now be prepared using known procedures and introduced onto the column so that antigen 10 (if present in the sample) forms antibody-antigen complexes with the antibodies 9.

Subsequently the column is washed to remove any non-bound material which could act as interferent in the subsequent steps of the procedure. The antibody-antigen complexes (immobilised on the particles 2) are depicted schematically in Fig. 3.

There may now be introduced onto the column a conjugate is for the antibody-antigen complex described above. The conjugate (which is linked to an enzyme as shown - see Fig. 4) becomes bound to the previously form antibody-antigen complex (to which the conjugate is in effect an antibody).

The column may be washed once again to remove any unbound material.

In the next step, a substrate for the enzyme is introduced onto the column. The enzyme substrate combination is such that a detectable change will be produced. After a predetermined length of time, liquid present in the column may be passed to a microtitre tray or well, optionally treated with a "stop" solution so as to stabilise the change and subjected to detection by known procedures. The change may, for example, be one which can be detected spectroscopically and may be one allowing a quantitative determination of the amount of antigen present in the original sample.

It will be appreciated that if antigen was present in the original sample then the conjugate (with its linked enzyme) will be retained on the column so that the reaction with the substrate can take place. Conversely, if antigen was not present in the column then the conjugate (with its linked enzyme) will not be retained on the column so that the change cannot take place.

The invention is illustrated by the following non-limiting Example.

Example

The following materials were used

(1) A solid silica support prepared as disclosed in Example 1 of WO-A-93/13220 so as to have a siloxane matrix linked to epoxy groups.

(2) A bicarbonate buffer prepared from

(i) a solution of 0.212 g Na_2CO_3 in 20 ml water, and

(ii) a solution of 1.680 g $NaHCO_3$ in 200 ml water

To prepare the bicarbonate buffer, 180 ml of solution (ii) was admixed with 20 ml of solution (i)

(3) Rabbit Anti-Gliadin (ex Sigma)

(4) Gliadin-HRP conjugate (HRP = Horse Radish Peroxidase)

(5) 1% solution of BycoA (commercial blocking agent (partially hydrolysed gelatin) (ex Sigma) in the bicarbonate buffer (2).

(6) Phosphate Buffered Saline 1% BSA Tween 20 0.05%.

(7) TMPa solution prepared from 1.6 mM tetramethyl benzidine in 26% DMF/H_2O.

(8) TMPb solution - 0.022% H_2O_2 in water

Procedure

Rabbit Anti-Gliadin was diluted with the bicarbonate buffer at a ratio of RAG:buffer of 1:1000. 50 mg of the support was then mixed with the RAG solution and reaction between the epoxy groups of the supports and amino groups of the RAG allowed to proceed for 2.5 hours at ambient temperature.

Approximately 10 mg of the support was then provided (between two frits) in a column having a volume of 200 µl. The amount of the support was such that the volume of the support (between the frits) was about 50 µl. The column was then washed with the bicarbonate buffer at a rate of 15 mls per minute for 15 seconds. Subsequently the supports on the column were treated overnight at 4°C with 100 µl of the BycoA solution (i.e. (5) above). The column was then washed with the bicarbonate buffer at a rate of 2.5 ml/minute for 15 seconds.

100 µl of the Gliadin-HRP conjugate was then aspirated into the column which was then incubated for 10 minutes followed by washing with the Phosphate Buffered Saline.

50 µl each of TMPa and TMPb (the substrate for HRP) were then aspirated into the column and incubated for 2 minutes.

A volume of 100 µl was then dispersed from the column into a microtitre well and 100 µl of 0.1 m sulphuric acid (acting as a stop solution to provide a stable colour), were added to the well.

The optical density of the solution in the well was determined at 490 nm and was found to be 0.54 as compared to a volume of 0.24 obtained on a "blank" experiment conducted as set out above save that the Gliadin-HRP conjugate was not added to the column.

The Example demonstrates that the RAG had become covalently linked to the support and could be used for the determination of Gliadin.

CLAIMS

1. A method of effecting an immunological determination of an analyte in a fluid sample, the method comprising the steps of

(i) providing a column of a solid support material having a first binding agent which is covalently linked to the solid support material and which is capable of forming an immunological complex with said analyte,

(ii) introducing the fluid sample onto the column to allow analyte (if present) therein to form an immunological complex with said first binding agent,

(iii) providing a label bound to the immunological complex formed under (ii), and

(iv) using the immobilised label in situ on the support to determine the analyte.

2. A method as claimed in claim 1 wherein the label is associated with the analyte.

3. A method as claimed in claim 1 wherein the label is associated with a conjugate which will bind to the complex formed between the first binding agent and the analyte.

4. A method as claimed in any one of claims 1 to 3 wherein the label is an enzyme and the determination is effected by treatment of the immobilised enzyme with a substrate therefore, said substrate being one in which a detectable change is produced by the enzyme.

5. A method as claimed in any one of claims 1 to 4 wherein the first binding agent is an antibody and the analyte comprises an antigen.

6. A method as claimed in any one of claims 1 to 4 wherein the first binding agent is an antigen and the analyte comprises an antibody.

7. A method as claimed in any one of claims 1 to 6 wherein the covalent linkage of the first binding agent to the support is effected by reaction of epoxy groups on the support with functional groups on the first binding agent.

8. A method as claimed in claim 7 wherein the functional group of the binding agent is an amino group.

9. A method as claimed in any one of claims 1 to 8 wherein the support with covalently linked binding agent has been treated with a blocking agent prior to step (ii).

10. A method as claimed in claim 9 wherein the blocking agent is a partially hydrolysed gelatin.

11. A method as claimed in any one of claims 1 to 10 wherein the support comprises particles.

12. A method as claimed in claim 11 wherein the particle size is 3-200 microns.

13. A method as claimed in any one of claims 1 to 12 wherein the support comprises silica.

14. A method as claimed in any one of claims 1 to 13 wherein the support is provided with a siloxane matrix to which the first binding agent is covalently linked.

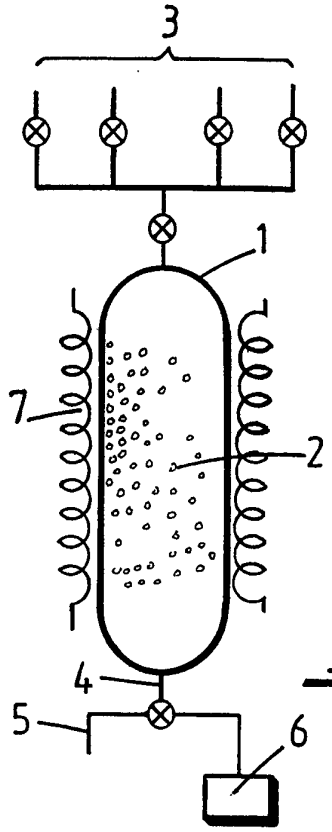

FIG. 1

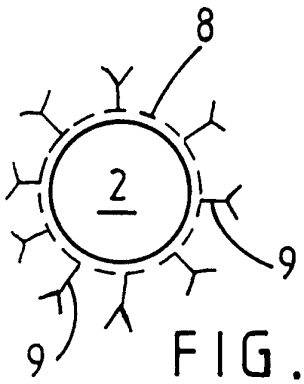

FIG. 2

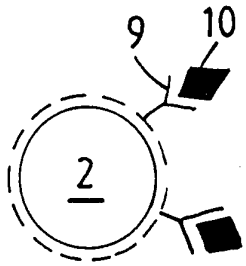

FIG. 3

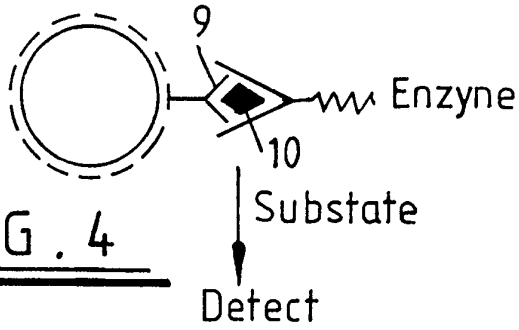

FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 96/01393

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/543 G01N33/538

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	WO,A,87 07384 (QUIDEL) 3 December 1987 see the whole document ---	1-6,11, 13
X	WO,A,86 03589 (PHARMACIA AB.) 19 June 1986 see page 14, line 17 - page 17, line 12; claims 1-5 ---	1-6,11, 12
Y	WO,A,92 15674 (TEPNEL MEDICAL LIMITED) 17 September 1992 cited in the application see the whole document --- -/--	7,13,14

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
5 November 1996	26.11.96

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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