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An agency of Industry Canada

CA 2193344 C 2005/12/13

(11)(21) 2 193 344

(12) BREVET CANADIEN
CANADIAN PATENT

(13) C

(22) Date de dépôt/Filing Date: 1996/12/18

(41) Mise à la disp. pub./Open to Public Insp.: 1997/06/28

(45) Date de délivrance/Issue Date: 2005/12/13

(30) Priorité/Priority: 1995/12/27 (195 48 375.8) DE

(51) Cl.Int.^{6}/Int.Cl.^{6} G01N 33/543, G01N 33/576

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(54) Titre : METHODE DE DOSAGE IMMUNOLOGIQUE
(54) Title: IMMUNOLOGICAL DETERMINATION METHOD

(57) Abrégé/Abstract:
The present invention relates to a competitive immuno-chemical determination method in which the concentration of the analyte is determined in a 1-step process and the analyte in this case competes with a first and a second specific binding component for binding to a third specific binding component, the first specific binding component being bound to a water-insoluble support and the second specific binding component being provided with a signal-generating label.

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Abstract

Immunological determination method

The present invention relates to a competitive immunochemical determination method in which the concentration of the analyte is determined in a 1-step process and the analyte in this case competes with a first and a second specific binding component for binding to a third specific binding component, the first specific binding component being bound to a water-insoluble support and the second specific binding component being provided with a signal-generating label.

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Immunological determination method

The present invention relates to a competitive immunochemical determination method, in which the concentration of the analyte is determined in a 1-step process and the analyte in this case competes with a first and a second specific binding component for binding to a third specific binding component, the first specific binding component being bound to a water-insoluble support and the second specific binding component being provided with a signal-generating label.

Immunological determination methods have gained outstanding importance in many areas of clinical diagnosis since the first description of a radioimmunoassay (1959) and the first enzyme immunoassay.

In an enzyme immunoassay for the determination of an analyte, immunological binding and reaction components are used, such as, for example, haptens, antigens, antibodies or fragments of antibodies. The binding and reaction components used can in this case on the one hand be present bound to a solid phase or conjugated to a signal-generating label, e.g. a labeling enzyme via, for example, a covalent bond. Solid phases used are concave shaped articles, such as, for example, tubes or hollows in the form of microtiter plates, but also convex shaped articles, such as, for example, spheres. Planar solid phases, such as, for example, test strips, are also used. Alkaline phosphatase, β-galactosidase and horseradish peroxidase are frequently employed as labeling enzymes, chromogenic, fluorogenic or luminescent compounds being used as substrates. The composition of sample, incubation and washing buffers, as well as the various substrate/chromogen reagents, are known to the person skilled in the art.

In contrast to homogeneous enzyme immunoassays, in heterogeneous enzyme immunoassays unbound reaction components are removed from bound reaction components by a phase separation and subsequent washing steps. In this case, a differentiation is made between the 1-step and the 2-step process. Whereas in the 1-step process altogether only one separation step ("bound/free" separation) is carried out, this takes place in the 2-step process after each individual incubation or reaction step.

Generally, enzyme immunoassays according to the immunological reaction principles are divided into noncompetitive and competitive techniques. The noncompetitive techniques ("sandwich" tests) are distinguished in that solid phase-bound and signal-generating reaction components are present in a large molar excess in comparison with the analyte to be determined. For the formation of the "sandwich" complex, at least two binding sites of the analyte are necessary, which in each case are recognized by the solid phase-bound or signal-generating reaction components. The measured signal activity of the "sandwich" complex formed is in this case directly proportional to the analyte concentration.

In the competitive techniques, on the other hand, one of the reaction components is limiting for immune complex formation due to its low concentration, so that a competition takes place between reaction component and analyte around at least one binding site of the mutual binding component. Within the competitive techniques, these, for their part, can be subdivided into two groups. In the first group the number of insolubilized binding components is lower than the number of signal-generating reaction components and the analyte molecules to be determined, while in the second group the concentration of the signal-generating reaction component is lower than the number of

insolubilized binding components and free analyte molecules. In both cases, the signal activity of the complex formed is inversely proportional to the measured analyte concentration.

On comparison of the two immunological reaction principles, it can be determined that the known competitive techniques are inferior to noncompetitive techniques with regard to sensitivity, measuring range, specificity, robustness and incubation time (EKINS R. (1985) CURRENT CONCEPTS AND FUTURE DEVELOPMENTS: IN ALTERNATIVE IMMUNOASSAYS).

The differing affinity of the serum antibodies to be detected therefore very significantly determines whether the 1-step or 2-step process is employed. In the detection of low-affinity antibodies, the 2-step process has proven advantageous compared with the 1-step process, in particular if the serum incubation time is carried out overnight as the first step. However, care is to be taken that in the second incubation step an equilibrium is not established between the bound and unbound signal-generating reaction components, which would lead to a displacement of low-affinity analyte antibodies. The detection of low-affinity antibodies is thus in principle better feasible using the 2-step process than with the 1-step process, which is easier to carry out and shorter for the user.

The purity of the solid phase-bound antigen, for binding of which both the antibodies to be detected and the signal-generating reaction components compete in a competitive enzyme immunoassay, plays an important part. The epitope density of the insolubilized antigen is in this case crucial for the sensitivity of detection (KENNY G. et al. (1983) J. CLIN. MICROBIOL. 17, 655-665). If a protein mixture is employed, as is the case, for example, with a purified HAV virus antigen,

the sensitivity of detection can be considerably adversely affected if the virus-specific protein fractions represent only a small fraction of the total protein employed. In order to avoid this, in the known processes an appropriately highly purified antigen must therefore be used, the production of the necessary degree of purity being associated with a considerable expense and correspondingly turning out to be difficult, expensive and time-consuming (PURCELL R. et al. (1976) JOURNAL OF IMMUNOLOGY 116, 349-356). In particular in the detection of low-affinity anti-HAV antibodies which are formed immediately after inoculation has taken place, the various commercially available diagnostic tests show a poor sensitivity. Only by the use of purified HAV antigen or the vaccine antigen was it possible to improve the sensitivity in the detection of low-affinity antibodies (DELEM A. (1992) BIOLOGICALS 20, 289-291).

The object on which the present invention is based therefore consisted in developing a process which allows the detection of low-affinity antibodies with the aid of the 1-step process and in this case an only minimally purified antigen can be used.

This object was essentially achieved by the embodiments provided in the patent claims.

The immunochemical process according to the invention for the determination of an analyte by means of a heterogeneous competitive determination process includes the following steps:

a) incubation of the analyte with a first, a second and a third specific binding component, the first specific binding component being bound to a water-insoluble solid phase (solid phase-bound reaction component) and the second specific binding component being provided with a signal-generating label

(signal-generating reaction component), and the analyte and the first and the second specific binding components competing for binding to the third specific binding component (common binding component),

b) separation of the signal-generating label bound to the solid phase via the third specific binding component from the unbound fraction,

c) measurement of the signal which can be generated by the bound fraction of the label, and

d) determination of the analyte concentration by comparison of the values found in step c) with a standard curve plotted under identical conditions or calculated theoretically.

Analytes which can be determined by means of the process according to the invention are known per se to the person skilled in the art. Advantageously, the analyte is an antibody, in particular an antibody of human or animal origin, which is relevant in micro-biological diagnosis, in particular in the field of infection diagnosis such as is carried out in blood banks, such as, for example, anti-HAV antibodies, anti-HIV antibodies, anti-HCV antibodies or antibodies in the diagnosis of hepatitis B.

The first and the second specific binding components must be able to react specifically with the third specific binding component. Advantageously, the first and the second specific binding components are antibodies, monoclonal or polyclonal, or antibody fragments. To carry out the process according to the invention, however, lectins or synthetic/recombinant antibodies can also be employed.

Fundamentally, the process according to the invention

can also be used for the detection of antigens which are not antibodies, it then being possible for the first and the second specific binding components to be an antigen and the third specific binding component to be an antibody.

Solid phases as such as known per se to the person skilled in the art. Advantageously, water-insoluble solid phases are employed, such as, for example, latex particles, magnetically attractable particles or micro-titer plates.

Signal-generating labels as such are known per se to the person skilled in the art. Such a label can either be conjugated directly to the specific binding component concerned or by means of a reversible coupling known per se to the person skilled in the art, such as, for example, biotin-streptavidin, fos-jun or antibody-antigen bonds.

Signal-generating labels which are employed are preferably components which are capable of chemo-luminescence or fluorescence or enzymes which can convert the luminogenic, fluorogenic or chromogenic substrates. In the case of the enzymes, horseradish peroxidase is particularly preferred. In the case of the chemoluminescent labels, the compounds described in European Patent Applications EP-A-0 257 541 and EP-A-0 330 050 are particularly preferred.

Customarily, in the immunochemical processes described, after the separation of the solid and the liquid phase the signal is measured either on the solid phase or in the supernatant separated off. From the measured signal, the analyte concentration is determined in a manner known per se to the person skilled in the art by means of a so-called standard curve. To plot the standard curve, the signals are measured using the respective determination process for known analyte

concentrations and converted into a curve form either graphically or mathematically. Such a standard curve can also be calculated theoretically with a certain accuracy on account of the known physical and chemical properties of the specific binding components.

The process according to the invention can be used in immunochemical methods in which competition takes place between the analyte to be determined and the signal-generating reaction components and/or insolubilized reaction components for binding to a common binding component.

The process according to the invention is furthermore distinguished in that the common binding component only has to be minimally purified. It has turned out to be significant that the common binding component should have at least two different binding sites, one binding site being recognized by the insolubilized reaction components, while the signal-generating reaction components bind to the second binding site. In the presence of the analyte to be detected, specific competition of the analyte molecules with the signal-generating and/or the insolubilized reaction components then takes place for binding to the common binding component. In this case, it has proven to be essential for the process according to the invention that in the absence of an analyte the formation of a signal-forming "sandwich" complex is not prevented by the competition between insolubilized and signal-generating reaction components.

Surprisingly, it was found in the process according to the invention for the detection of antibodies against the hepatitis A virus that in the absence of an analyte (= negative control) no competition between the signal-generating reaction components (= monoclonal anti-HAV-specific antibody conjugate) and the insolubilized reaction components (= polyclonal anti-HAV-specific

antibodies) occurred for binding to the common binding component (= HAV antigen) and as a result the formation of a signal-generating "sandwich" complex is made possible. This was all the more surprising because the known monoclonal antibodies against HAV can almost completely block the binding of polyclonal antibodies to the virus in various competitive immunoassays (LEMON S. et al. (1993) VIROLOGY 4, 285-295; HUGHES J. et al. (1984) J. VIROL. 52, 465-473; STAPLETON J. et al. (1987) J. VIROL. 61, 491-498). However, in the presence of analyte molecules (= anti-HAV-specific antibodies = positive control) it was just as surprisingly possible to find that specific competition for the common binding component can take place between the analyte and the insolubilized reaction components and/or signal-generating reaction components.

The finding was also surprising that the common binding component only had to be purified minimally or not at all in order to be able to detect, even using the 1-step process, low-affinity antibodies which can occur in the early phase of an infection or after inoculation has taken place. In the process according to the invention, possibly due to the presence of solid phase-bound reaction components, conditions are created which allow particularly effective and specific competition between the analyte and the signal-generating and/or solid phase-bound reaction components for the binding sites of the common binding component. Minimally purified in the sense of the present invention in this case means that the proportion of specific protein is less than 80, preferably less than 50 %, very preferably less than 20 %.

Reaction components preferably used in the context of the invention are antibodies or defined fragments of antibodies. The preparation of polyclonal or monoclonal antibodies (KÖHLER G. and MILSTEIN C. (1975) NATURE 256, 495-497) is carried out by a method known per se to the

person skilled in the art. Besides polyclonal antibodies, monoclonal antibodies or fragments thereof ($F(ab')_2$ or Fab') can also be employed. According to the process according to the invention, in this case one reaction component is bound to the water-insoluble solid phase, while the second reaction component is employed as a signal-generating component. Preferably, in the process according to the invention a polyclonal antibody is bound to the solid phase and a monoclonal antibody which is conjugated to a labeling enzyme is used as a signal-generating reaction component. The preparation of the monoclonal conjugate used in the invention is likewise known to the person skilled in the art (review article: ISHIKAWA E. et al. (1983) J. IMMUNOASSAY 4, 209-327).

The suitability of the antibodies used can be determined, for example, by experiments known per se to the person skilled in the art.

A binding component which can be employed in the context of the invention is any macromolecule which has at least two separate binding sites for the insolubilized and the signal-generating reaction components. Suitable macromolecules in this case are proteins - optionally modified by carbohydrates and/or lipids-, carbohydrates, lipids, synthetic polymers and nucleic acids. The molecular weight of the binding component is preferably between 50,000 and 2 million.

The process according to the invention can be used in all immunological detection methods in which, in heterologous immunoassays, specific competition can take place between the analyte and solid phase-bound reaction component and/or signal-generating reaction component for at least two binding sites of a common binding component.

Description of the figures

The detection limit of the process according to the invention was determined with the aid of defined dilutions of an anti-HAV standard (WHO standard). In Fig. 1, the measured extinctions of various antibody concentrations are shown in a semilogarithmic presentation. Owing to the competitive assay construction, the extinctions decrease with increasing antibody concentration.

The calculated analytical sensitivity is 13.4 IU/l, the extinction of the negative control as a cut-off having been halved.

It was possible to show with the aid of inoculation seroconversions that low-affinity antibodies against HAV are detectable at a very early stage using the process according to the invention. In Fig. 2 is shown the time course of a typical anti-HAV seroconversion (0193). Serum samples were taken from inoculation subjects at various times and the latter investigated using the process according to the invention. The ratios shown in this case correspond to quotients from the measured extinctions and the cut-off. Ratios of < 1 indicate that, with the detectable occurrence of anti-HAV antibodies, a seroconversion has taken place. As shown in Fig. 2, it was even possible after inoculation had taken place using the process according to the invention to detect a seroconversion from the second week.

In Fig. 3, the measured extinctions of the seroconversion 0193 investigated were quantified with the aid of the calibration curve of Fig. 1. Even from the 2nd week, an antibody concentration was determined which clearly exceeds the inoculation protection threshold value of 20 IU/l.

The following examples are intended to illustrate the invention:

Abbreviations:

HAV: hepatitis A virus

POD: peroxidase

SH: sulfhydryl groups

ATCC™: American tissue cell culture™

Examples

Example 1

a): Conjugation of antibodies

Monoclonal antibodies against HAV are reacted with a heterobifunctional reagent (TARRIMORE et al. (1983) J. IMM. METH. 62, 123-131), then incubated with SH-activated peroxidase (KING et al. (1978) BIOCHEMISTRY 17, 1499-1506) and subsequently purified by gel chromatography.

b): Preparation of HAV antigen

To prepare the HAV antigen, commercially available cells, such as, for example, human diploid embryonic lung fibroblasts, are infected with a characteristic hepatitis A virus strain, such as, for example, ATCC™ HM-175. After several days, the removed cell supernatant is centrifuged, the cell pellet obtained is taken up in a customary storage buffer and the antigen is inactivated according to a process known to the person skilled in the art. The inactivated antigen can be used further without additional purification.
The process according to the invention is in this case not restricted to the abovementioned method for the preparation of HAV antigen, but other preparation processes for antigen isolation known to the person skilled in the art can also be used. Moreover,

commercially available HAV antigen preparations can also be employed in the process according to the invention.

c): Coating of the hollows of microtiter plates

A determined amount of human polyclonal anti-HAV antibody (16 µg/ml) is added with gentle stirring to a coating solution (sodium carbonate 0.01 mol/l pH 9.6) and the mixture is homogenized for about 30 minutes. The coating of the individual hollows of a microtiter plate was carried out subsequently using a coating volume of 150 µl. After an incubation overnight at room temperature, the coating solution is aspirated and the individual hollows of the microtiter plate are washed twice with a wash solution (0.25 mol/l of citric acid/0.05 mol/l of tris pH 7.4). Following the last washing step, the individual hollows of the microtiter plate are sucked empty and it is sealed into aluminum films together with packed drying agent (e.g. silica gel). The coated microtiter plates are stored at 4°C until they are used.

d): Enzyme immunoassay for the determination of anti-HAV antibodies

The process according to the invention is a competitive enzyme immunoassay by the 1-step process. Sample to be investigated (25 µl), conjugate (= POD-conjugated anti-HAV-specific monoclonal antibody, 50 µl) and HAV antigen (50 µl) are added successively to the hollows of a microtiter plate which are coated with human polyclonal anti-HAV-specific antibodies. The coated microtiter plate, conjugate and HAV antigens were taken from the Enzygnost® anti-HAV test kit (order code OQEC Behringwerke AG, Marburg). If the sample to be investigated in this case contains the anti-HAV antibodies to be determined, these compete with the conjugate molecules and/or insolubilized polyclonal

anti-HAV-specific antibodies for binding to the HAV antigen. After an incubation time of 2 hours at 37°C, excess conjugate and unbound reactants are removed by aspiration and washing four times, e.g. with the Behring ELISA Processor II or Behring ELISA Processor III (Behringwerke AG, Marburg), and the amount of the bound conjugate is determined (30 min, room temperature, protected from light) by the addition of 100 µl of substrate/chromogen solution (Behringwerke AG, order code OUVP). The enzymatic reaction of the chromogen tetramethylbenzidine dihydrochloride is interrupted by addition of 100 µl of 0.5 N sulfuric acid and the extinction at 450 nm is determined photometrically. The measured extinction is in this case indirectly proportional to the anti-HAV-antibody concentration contained in the sample.

e): Comparison of the process according to the invention with an alternative, fully monoclonal process.

The process according to the invention is an enzyme immunoassay by the 1-step process which contains a polyclonal anti-HAV-specific antibody of human origin bound to the solid phase and, as conjugate antibody, a monoclonal anti-HAV-specific mouse antibody. The comparison process differs from the process according to the invention in that the polyclonal solid-phase antibody is replaced by a monoclonal antibody.

In Table 1, the process according to the invention is shown with regard to signal intensity of the negative control and measured detection limit with the alternative, fully monoclonal process.

Table 1: Comparison of the process according to the invention with a fully monoclonal process

	Extinction of the negative control	Detection limit
Process according to the invention	1427 mE	13 IU/l
Fully monoclonal process	543 mE	28 IU/l

The higher signal intensity of the negative control and the improved detection limit of the process according to the invention developed from a polyclonal and a monoclonal anti-HAV-antibody clearly show the superiority of the process according to the invention compared with an alternative, fully monoclonal process.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. An immunochemical process for the determination of an analyte by means of a heterogeneous competitive determination process, which includes the following steps:

a) incubation of the analyte with a first, a second and a third specific binding component, the first specific binding component being bound to a water-insoluble solid phase and the second specific binding component being provided with a signal-generating label, and the analyte and the first and the second specific binding components competing for binding to the third specific binding component,

b) separation of the signal-generating label bound to the solid phase via the third specific binding component from the unbound fraction,

c) measurement of the signal which is generated by the bound fraction of the label, and

d) determination of the analyte concentration by comparison of the values found in step c) with a standard curve plotted under identical conditions or calculated theoretically,

wherein the analyte is a hepatitis A virus (HAV) specific antibody, in that the first specific binding component used is a polyclonal anti-hepatitis A virus antibody or corresponding antibody fragments, the second specific binding component used is a monoclonal anti-hepatitis A virus antibody or corresponding antibody fragments and the third specific binding component used is an hepatitis A virus antigen, and in that the first and second specific binding components recognize different binding sites on the third binding component.

2. The process as claimed in claim 1, wherein the immunochemical determination method used is a 1-step process.

3. The process as claimed in claim 1, wherein an enzyme is used as a signal-generating component.

4. The process as claimed in claim 3, wherein horseradish peroxidase is used as the enzyme.

5. The process as claimed in claim 1, wherein the analyte to be determined is an anti-hepatitis A virus-specific antibody of the subclasses IgM and/or IgG.

6. The process as claimed in claim 1, wherein the third specific binding component is employed in a minimally purified form which has a specific protein content of less than 80%.

7. The process as claimed in claim 6, wherein the third specific binding component is employed in a minimally purified form which has a specific protein content of less than 50%.

8. The process as claimed in claim 7, wherein the third specific binding component is employed in a minimally purified form which has a specific protein content of less than 20%.

9. The process as claimed in claim 1, wherein the third specific binding component is a hepatitis A virus antigen raised in cell culture.

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FIG.1

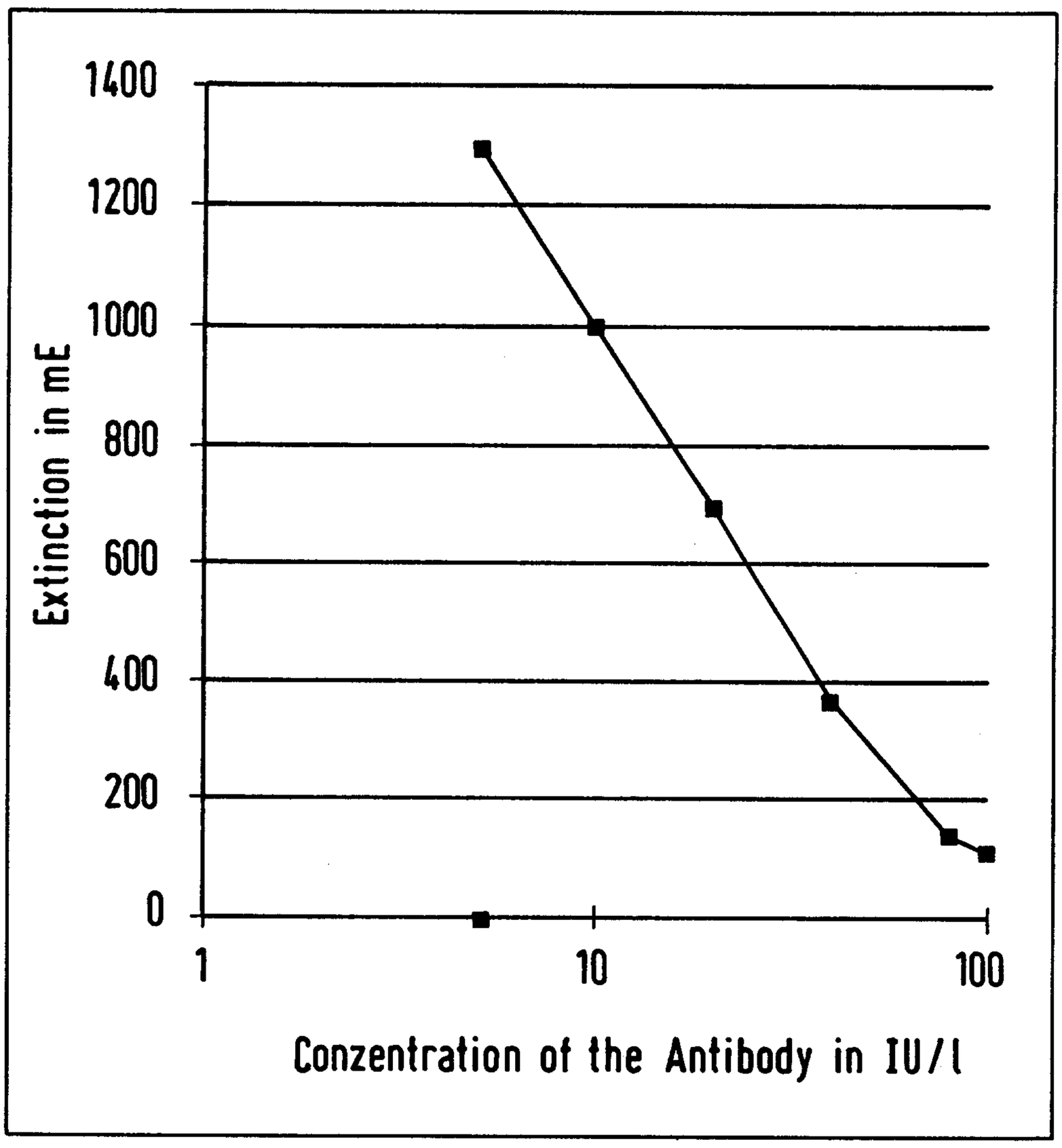

FIG.2

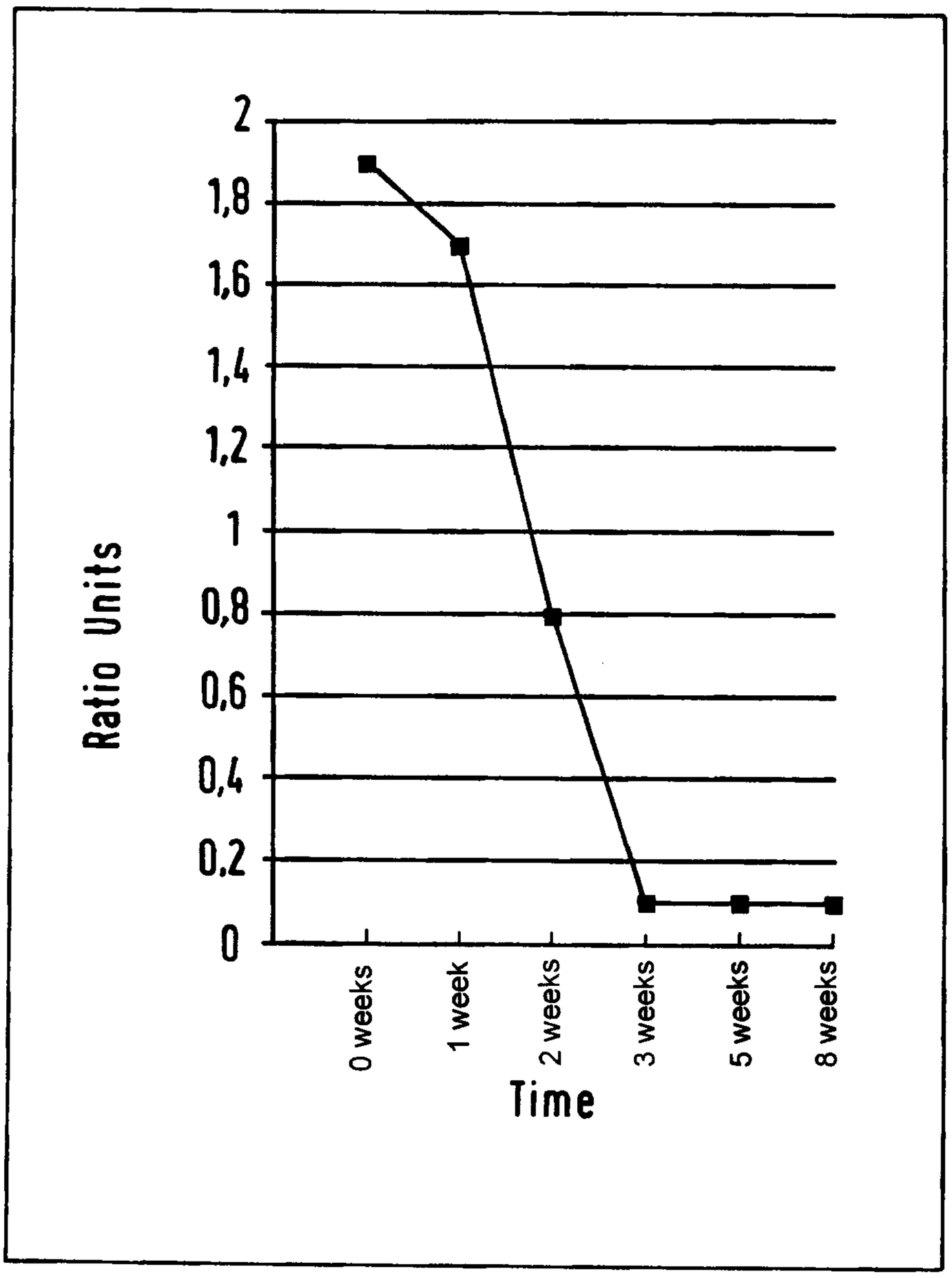
Seroconversion 0193
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
Ratio Units
0 weeks
1 week
2 weeks
3 weeks
5 weeks
8 weeks
Time

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FIG.3 Quantification of Seroconversion 0193