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(54) ANTIBODY IMMOBILIZED IN A ACRYLAMIDE COPOLYMER MATRIX

(71) We, CHANDON INVESTMENT PLANNING LTD., a company organised under the laws of the Cayman Islands (British West Indies), of West Wind Building, P.O.B. 111 Grand Cayman, Cayman Island, British West Indies, 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:—

The invention relates to antibodies immobilised in a polymer matrix based on acrylamide for use, in particular, in the radioimmunological determination of hormones, pharmaceuticals and vitamins.

In the radioimmunological determination of hormones, pharmaceuticals and vitamins, the hormones, pharmaceuticals or vitamins react with an antibody. The specificity of the reaction depends on immunological complementing, i.e., a cross reactivity may exist with substances exhibiting a similar chemical configuration. In an estriol-C_6 conjugate, the esteriol is present in an immunodeterminate configuration, in which nearly all of the important functional groups of the esteriol are exposed to the bonding range of the antibody. In spite of this, esteriol conjugates with the sulfate or glucuronide residue occupying the position at the phenolic C_3 are bonded to a considerable degree by the antibody. This is disadvantageous for the determination of free steroids, especially if the conjugate concentration exceeds the steroid concentration by orders of magnitude, as is true for the estrogens in the serum of pregnant women.

For these reasons heretofore, methods were essentially used in which specificity was ensured by solvent extraction prior to the radioimmune assay. Solvent extraction interferes, however, with continuous and mechanised sample processing.

In experiments to simplify radioimmunological methods by way of solid phase techniques, antibodies were bonded covalently to different matrices, e.g., agar, cellulose, glass particles, polyamides and polyacrylamides; see for example United States Patent No. 3,793,445 to Updike et al. The advantages of antibodies enclosed in a matrix consist of the exclusion of interfering molecules of higher molecular weight, the savings of pipetting and centrifuging and the extended stability of the immobilised antibodies at room temperature.

It has been found that the bonding specificity of the antibody, when immobilised in the known manner in a matrix, does not satisfy desirable requirements.

It has been found surprisingly that a variation and increase in bonding specificity of the antibody thus preventing the occurrence of undesirable cross reactions can be obtained through the use of polymer matrices of certain copolymers of acrylamide. According to the present invention there is provided an antibody immobilised in a polymer matrix which comprises a matrix of acrylamide copolymer and an antibody immobilised in the matrix, the acrylamide copolymer being a copolymer of acrylamide and at least one monomer chosen from acrylic acid, methacrylic acid, methacrylamide, salts and esters of acrylic acid or methacrylic acid, acrylamide or methacrylamide substituted at the nitrogen atom, and N,N'-dialkyltartardiamides.

With the aid of the immobilised antibodies of the invention, highly accurate, in particular radioimmunological, determinations of hormones, pharmaceuticals and vitamins are possible, because the fluctuation of the values determined attributable to non-specific bonds between steroid molecules and the matrix is minimised by the copolymerisation.

The micro-environment of the polymer matrix is affected by the copolymerisation of acrylamide with the comonomer. The hydrophobic and hydrophilic effects and electrostatic effects within the polymer matrix are found to be dependent upon the acrylamide copolymer and provide the particularly desirable bonding specificity. By varying the polymer matrix through copolymerisation, it is possible to achieve a substantial increase in the bond-

ing specificity of the antibody and to suppress undesirable cross activities. The polymer matrix is varied through a suitable copolymerisation in order to assure a specific reaction of haptens with the immobilised antibody and to prevent the cross section of haptens. Because of the increased specificity, the polymer matrices of the invention permit measurements directly in the non-extracted serum.

In various determinations, such as, e.g., of the thyroid hormones, thyroxine and tri-iodothyronine, competitive substances are used, such as anilinenaphtholsulfonic acid, merthoilate or salicylate, in order to displace the hormones from their bond with the different bonding proteins in the serum. It is known, however, that in the case of higher concentrations of these competitive substances, the bond between the hormone and the antibody is affected. With the use of the immobilised antibodies of the invention, it is possible to substantially increase the concentration of the competitive substances, i.e. by a factor of 100, without weakening the hormone-antibody bond. Without fully stating the reason for this result, it is presumed that electrostatic interactions are responsible.

The immobilised antibodies of the present invention may in particular be used in the determination methods described and claimed in our copending Applications Nos. 16823/78 and 16824/78 (Serial Nos. 1603773 and 1603774).

The acrylamide copolymer of the present invention may be prepared according to known procedures for polymerizing acrylamide monomer and copolymerizable monomers. Formation of the desired copolymer matrix arrangement is described in greater detail hereinafter.

The proportion of acrylamide in the copolymer may be from 1 to 99 mole%, preferably from 5 to 95 mole% and, most preferably, from 20 to 80 mole%. Especially suitable are copolymers of acrylamide and methacrylic acid and, more particularly, those in which the proportion of methacrylic acid is 20 to 60 mole% based on the acrylamide. Methacrylic acid yields a hydrophobic matrix and provides a charge effect, permitting the extensive elimination of non-specific bonds of the substances to be determined.

Through the use of copolymers in accordance with the invention containing acrylic acid or methacrylic acid or salts thereof, a neutralization effect or a buffering action can be achieved for acid or alkaline solutions. This is a significant advantage in numerous determinations.

The preferred salts of acrylic acid or methacrylic acid are alkali metal or alkaline earth metal salts. Sodium and potassium salts are particularly preferred.

The preferred esters of acrylic acid or methacrylic acid are the methyl and ethyl esters.

The methacrylamide substituted at the nitrogen atom may be for example N-hydroxymethylmethacrylamide.

There may also be used, as comonomer with acrylamide, N,N'-dialkyltartardiamide.

The immobilization of the antibody takes place through inclusion in the polymer matrix and/or through covalent fixation in the polymer matrix in a known manner. A detailed description of the radioimmune assay is found, for example, in *Clinical Chemistry*, Vol. 19, No. 2, 1973, p. 145. In *Clinical Chemistry*, Vol. 19, No. 12, 1973, p. 1339, and *Clinical Chemistry*, Vol. 21, No. 7, 1975, p. 829, radioimmunological techniques are described in which immobilized antibodies are used.

The immobilized antibodies of the invention are suitable for the determination of different hormones, pharmaceuticals and vitamins, which are present in the serum or the plasma bonded in part to specific or non-specific bonding proteins. The hormones may consist of thyroid hormones, particularly thyroxine and tri-iodothyronine, the steroid hormones, such as cortisol, testosterone, progesterone, estron, estradiol and estriol and the heart glycosides, such as digitoxin and digoxin. Vitamins, particularly, Vitamin B12 and folic acid, also pharmaceuticals with strong protein bonds, such as, for example, anti-coagulants, dicumarol, analgesics and salicylates, may further be determined.

In addition to radioimmunological determinations, alternative methods of determination, such as, fluoroimmunological determination or determination with enzymatic marking, may also be considered.

The synthesis of antigens, the production of antisera, for example through the immunisation of anti-bodies, are known (see for example, *Clinical Chemistry*, Vol. 19, No. 2, 1973, p. 146 ff.).

The preparation of the antibody immobilized in a polymer matrix according to the invention may take place, for example, by adding a solution of the antibody to the monomer mixture. The initial mixture is, for example, polymerized by free radical polymerization and the polymer obtained comminuted, washed and dried.

The selection and the proportions of the copolymers added to the acrylamide are determined by the properties of the polymer desired with respect to specificity. The specificity may be varied over a wide range by altering the hydrophobicity and the charge of the matrix. The addition of the methylacrylamide increases hydrophobicity. The addition of acrylic acid or methacrylic acid or salts thereof varies the charge of the matrix. It is possible, both to add the salts of acrylic acid or methacrylic acid directly to

the monomer mixture or alternatively to polymerize the acids and subsequently form the salts in the polymer matrix through the exchange of ions.

The monomer concentration in the starting solution may be varied to obtain a suitable pore size of the polymer matrix. A monomer concentration in the range of approximately 20 mole% results in a pore size of approximately 7 to 10 Å.

An advantageous copolymer consists, for example, of acrylamide and 20 to 60 mole%, based on the acrylamide, acrylic acid and/or methacrylic acid, prepared from an approximately 20 mole% monomer solution, where at least part of the acid groups are converted into the corresponding alkali metal or alkaline earth metal salts.

The invention will now be explained in more detail with the aid of Examples.

Example 1.

In this Example, the effect of the use, as polymer matrix, of acrylamide homopolymer and acrylamide copolymer according to the invention on the cross reactivity (K) of estriol-3-glucoronide and estriol-3-sulfate is compared.

The antiestriol antibody chosen displays high cross reactivity of the 3-glucuronide conjugate and the 3-sulfate conjugate.

For each polymerisation mixture, the concentration was adjusted so that the total monomer concentration amounted to 2.9 mole%.

For one mixture, for example, 5 g acrylamide and 1.25 g N,N'-methylenebisacrylamide were dissolved in a glass beaker in 24 ml of a phosphate buffer with a pH value of 7.2. After the addition of the antibody in 1 ml phosphate buffer, the reaction was initiated with 0.15 g riboflavin and 0.10 ml N,N,N',N' - tetramethylethylenediamine and irradiated with UV light. The yellow block was subsequently comminuted, washed with distilled water and dried. In this way antiestriol antibody immobilised in a cross-linked acrylamide homopolymer matrix was obtained.

In addition antiestriol antibody immobilised in an acrylamide copolymer matrix in accordance with the following Table was also prepared.

The cross-reactivity was determined as follows:

Twenty-two mg of dry antiestriol antibody was weighed into small columns and agitated with 0.150 ml of an incubating solution. This incubating solution contained ^{3}H-estriol and unmarked hapten (estriol, estriol-3-glucuronide or extriol-3-sulfate). The temperature of the reaction was kept constant at 0°C. After an incubation period of 30 minutes, the free hapten was separated from the hapten bonded to the antibody by elution with phosphate buffer containing albumin. The eluate was collected in on a scintillation glass and diluted with 15 ml of a scintillation liquid and the radioactivity measured in a liquid scintillator. The concentration of the free indicator hapten ^{3}H-estriol was calculated from the impulses.

The results of the determinations in the aqueous system and with the use of polymer matrices of acrylamide homopolymer and acrylamide copolymers are summarised in the following Table. The values given are the values of cross reactivity K which is calculated from hundred times the amount of the mass of the corresponding hapten for y = 50, divided by the required mass of the cross-reacting hapten for y = 50. The values are calculated on the basis of ID_{50}.

TABLE

	Aqueous System	Acrylamide Homopolymer	Sodium Salt of Acrylic Acid/Acrylamide (For indicated Mole Ratio)			Sodium Salt of Methacrylic Acid/Acrylamide (For indicated Mole Ratio)		
			10:90	30:70	50:50	20:80	40:60	60:40
Estriol-3-glucuronide	61	29	19	10	14	14	4	21
Estriol-3-sulfate	47	33	30	12	24	27	6	23

The results show that with the use of the copolymers of the invention, a significant increase in bonding specificity was obtained as compared with aqueous systems and acrylamide homopolymers outside this invention. Through the suitable selection of the copolymer, an increase in specificity by a factor of 15 can be achieved compared with aqueous systems and by a factor of 7 with respect to acrylamide homopolymers. This is demonstrated by a comparison of the values of the aqueous system and the acrylamide homopolymer with the 40/60 mole ratio copolymer of methacrylic acid salt and acrylamide.

Example 2.

This Example shows the use of an antibody immobilised in a polymer matrix of the invention in a total determination of thyroxine by the enzymatic hydrolysis with pepsin.

The antibody immobilised in a polymer matrix consisted of an antibody immobilised in a copolymer matrix of acrylamide and 40 mole%, based on the acrylamide, of the sodium salt of methacrylic acid, prepared from a 20 mole% monomer solution and antibody prepared by synthesis of thyroxine-ethylester and coupling on alubumin on carbodiimide. The antibody was produced by the immunisation of rabbits.

Ten µl serum with a thyroxine content of 18 µg per 100 ml were diluted with 160 µl of an enzyme solution, said enzyme solution consisting of 2 mg/ml pepsin dissolved in 0.1 N hydrochloric acid.

The enzyme reaction was performed at room temperature in 30 minutes.

To this, 170 µl subsequently 150 µl, of a tracer solution with a content of 5.2 ng/ml of reactively marked thyroxine were added.

The entire solution was then placed on 60 mg antibody gel using the technique of Example 1. The incubation period was 30 minutes, the temperature 22°C.

Evaluation resulted in a recovery of 98%.

Example 3.

This Example demonstrates the use of an antibody immobilised in a polymer matrix of the invention in the total determination of cortisol.

The antibody immobilised in a polymer

matrix consisted of antibody immobilised in a copolymer matrix of acrylamide and 40 mole%, based on the acrylamide, of a mixture of sodium and calcium salts of methacrylic acid prepared from a 20 mole% monomer solution and antibody prepared by synthesis of cortisol-C3-oxime and coupling on albumin; the antibody production being by the immunisation of rabbits.

Ten μl serum or plasma with a cortisol content of 15 μg per 100 ml were prepared. To this was added 500 μl of a tracer solution consisting of ^{125}I-cortisol in an aqueous citrate buffer of pH 3.5.

The entire solution was then placed on 100 mg immobilised antibody using the technique of Example 1. The incubation period was 20 minutes, the temperature 25°C.

Evaluation showed a recovery of 99%.

Example 4.

This Example demonstrates the use of an antibody immobilised in a polymer matrix of the invention in the determination of thyroxine with the application of competitive substances.

The antibody immobilised in a polymer matrix consisted of a copolymer of acrylamide and 40 mole%, based on the acrylamide, of a mixed calcium-sodium salt of methacrylic acid prepared from a 20 mole% monomer solution and antibody prepared as described in Example 2.

A tracer solution (500 μl) consisting of ^{125}I-thyroxine in a phosphate buffer at pH 9.6 was placed on 80 mg of the immobilised antibody.

Further, 500 μl of the same tracer solution containing 6 mg/ml anilinenaphtholsulfonic acid, were placed on 80 mg of the immobilised antibody.

Both samples were incubated at a temperature of 22°C for 20 minutes.

Evaluation showed no interference with the bond of thyroxine with the antibody in spite of the high concentration of the competitive substance present, i.e. anilinenaphtholsulfonic acid.

WHAT WE CLAIM IS:—

1. An antibody immobilised in a polymer matrix which comprises a matrix of acrylamide copolymer and an antibody immobilised in the matrix, the acrylamide copolymer being a copolymer of acrylamide and at least one monomer chosen from acrylic acid, methacrylic acid, methacrylamide, salts and esters of acrylic acid or methacrylic acid, acrylamide or methacrylamide substituted at the nitrogen atom, and N,N'dialkyltartardiamides.

2. An antibody immobilised in a polymer matrix according to claim 1 wherein the copolymer is of acrylamide and 20 to 60 mole%, based on the acrylamide, of methacrylic acid.

3. An antibody immobilised in a polymer matrix according to claim 1 wherein the copolymer is of acrylamide and an alkali metal salt or alkaline earth metal salt of acrylic acid or methacrylic acid.

4. A process for preparing an antibody immobilised in a polymer matrix which process comprises polymerising acrylamide and at least one monomer as defined in claim 1 in the presence of the antibody to the immobilised.

5. Antibody immobilised in a polymer matrix prepared by the process claimed in claim 4.

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