RADIOIMMUNOASSAY OF PENICILLOYL GROUPS IN BIOLOGICAL FLUIDS

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1. Introduction

Studies of penicillin allergies give a preponderant role to penicilloyl–protein conjugates [1,2]. Penicilloyl groups, derived from various penicillins by rupture of the β lactam ring, bind to proteins by covalent bonds to form compounds which have lost all antibacterial activity, but possess immunogenic potentiality. Another method of forming penicilloyl–protein conjugates by way of penicillenic acid intermediary is described [1,3] (fig.1). These compounds may be formed in man or animals after therapeutic treatment but cannot be detected by the classical microbiologic assay methods used for penicillin. Allergies have been noted [4,5] in consumers of products coming from animals treated with penicillin (milk particularly). Although these compounds present a risk to public health, they passed unnoticed through hygienic food inspection. Thus, it was of interest to devise a specific and sensitive method for the assay of these derivatives in biological fluids where they can be present in trace amounts.

An iodine-125 labelled conjugate has been prepared

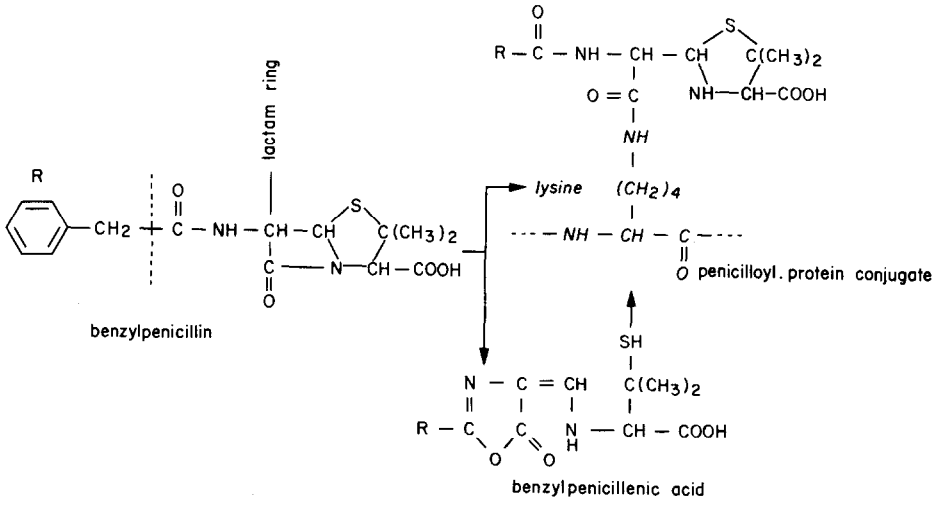

Fig.1. Formation of the penicilloyl–protein conjugate.

[6]. Already usable for the detection of antipenicilloyl antibodies in sera of penicillin allergic patients, it has been used for the development of a radioimmunoassay of penicilloyl groups. Assay is done directly on milk, urine and serum without previous extraction and with a detection limit of a few ppb. It permits a rapid, specific and easy to handle determination in the sera of hospital patients as well as in the inspection of animal products at the slaughter house and at the dairy.

2. Material and methods

2.1. *Preparation of immune-serum*

The immunogen is obtained by coupling penicillin to bovine γ-globulins (BGG), a penicillin–protein conjugate being formed. The conjugation reaction, involving the formation of covalent bonds between penicilloyl groups and available Σ-amino groups of BGG, is done in alkaline medium without using any activating agents (method adapted from Chase [7]). To a solution of 100 mg of dried and electrophoretically pure BGG (Boehringwerke A.G.) in 1 ml of 1M carbonate buffer pH 10.4, 1.2 g of penicillin G (crystallized sodium benzyl penicillinate, Specia) is added. After 12 hr of contact at +4°C, then again 12 hr later, further 600 mg of penicillin is added. The molar concentration of penicillin is then about 500 times greater than the available $-NH_2$ group concentration. After a total of 30–36 hr of incubation at +4°C, the reaction mixture is put on a Sephadex column G25 (K26/40 - Pharmacia) equilibrated with 0.15 M NaCl in 0.01 M phosphate buffer (pH 7.4). Elution is done with the same buffer at laboratory temperature. Fractions of 0.5 ml are recovered and tested by ultraviolet spectroscopy at 280 nm. The penicilloyl-BGG conjugate is thus entirely separated from the excess free penicillin.

Degree of coupling was determined by spectrofluorimetric measurement in the presence of fluorescamine (fluram Roche [8]). About 20 penicilloyl groups are bound per mole of BGG.

The method of immunization is derived from those described by Josephson [9] and Thiel [10]. Six rabbits were injected in the foot-pad with 11 mg of the immunogen (about 400 μg of bound penicillin) dissolved in 1 ml saline and emulsified 50:50 with Freund's complete adjuvant (Difco). Intramuscular (in gluteal muscle) and subcutaneous booster shots of 2 mg of the immunogen prepared in the same way were given 1 month later.

The antisera recovered were tested for antibody titer by passive hemagglutination of red blood cells coated with penicilloyl groups [9,10] and also estimated by measuring inhibition of penicilloylated T4 bacteriophage ability to lyse bacteria [11].

The strongest anti-serum was cleared of anti-BGG antibodies in batchwise operation with glutaraldehyde cross-linked BSA (bovine serum albumin)–BGG immunoadsorbent [12].

2.2. *Preparation of labelled antigen*

The radioactive tracer was obtained by coupling the penicilloyl group to BSA (Boehringwerke A.G.) previously labelled with iodine-125 [6]. The iodinated BSA was prepared with a specific activity of about 330 Ci/mmol (5.5 μCi/μg of BSA). The coupling of the penicillin to the ^{125}I-BSA was then done under conditions similar to the preparation of the immunogen. The degree of coupling, determined as previously, is 14–15 penicilloyl groups per mol of BSA. The specific activity of the prepared derivative, calculated from iodination yield and degree of coupling, is about 25 Ci/mmol of bound penicillin, or 70 mCi/mg of benzylpenicilloic acid.

Immunoreactivity (IR), expressed as percentage of the total radioactivity bound as antibody–(^{125}I-BSA-penicilloyl) complex, in presence of an excess of antibodies (antiserum diluted to 1/1000), ranges between 90 and 95%.

2.3. *Radioimmunoassay procedure*

Competition reaction is performed in hemolysis tubes and albuminous medium. The labelled immunogen is diluted by 0.15 M NaCl in 0.01 M phosphate buffer (pH 7.4) containing 0.5% HSA (human serum albumin, CNTS-Paris). The total radioactivity introduced (AT) is about 3000 cpm in 200 μl. The immune-serum is diluted in the same buffer up to 1/100. For higher dilutions, 50 mg of rabbit IgG is added per 100 ml. The dilution used is that which, in absence of unlabelled hapten, gives about 50% (dilution at 1/15 000) of bound radioactivity (Bo) (fig.2). Standard solutions of unlabelled hapten, as well as dilutions of milk, urine and serum, are made with saline buffered at pH 7.4. The total 400 μl reaction mixture (100 μl of diluted anti-serum, 100 μl of sample, 200 μl of diluted

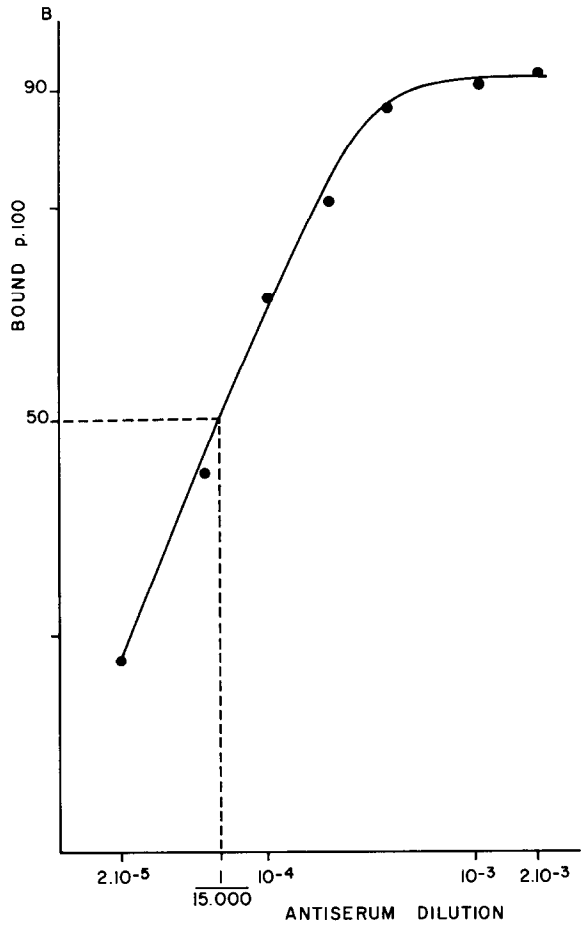

Fig.2. Antiserum dilution curve.

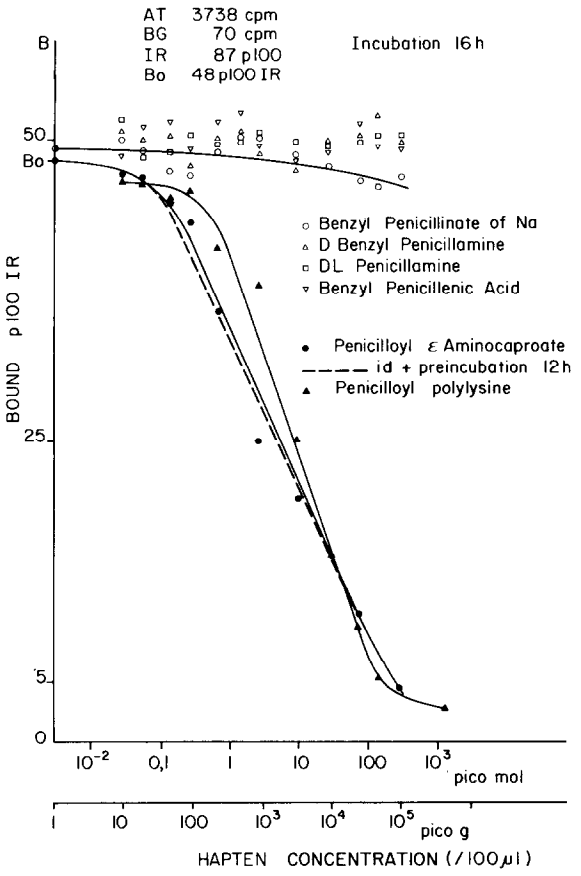

Fig.3. Standard curves obtained with penicillin G and various derivatives.

labelled antigen) is left overnight at room temperature. The antibody–(penicilloyl-iodinated BSA) complex is precipitated by addition of 50 µl of anti-rabbit sheep serum, then 4 hr incubation at +4°C. After addition of 2 ml buffered saline the precipitate is centrifuged. The supernatant is discarded and the pellet radioactivity is measured with an 'Autogamma' scintillation spectrometer (Packard).

3. Results and discussion

3.1. *Assay characteristics – Specificity – Sensitivity*

The standard curves (fig.3) represent the binding percentage of the fraction of immunologically active radioactivity introduced versus logarithm of unlabelled hapten added. They indicate that there is no crossreaction between penicilloyl groups and penicillin or derivatives such as D and DL penicillamine, benzylpenicillenic acid.

The average association constant has been calculated; its value is 0.8×10^7 L/M.

Identical curves are obtained after 12 hr preincubation of antiserum with unlabelled hapten alone, or without preincubation. Also, penicilloyl Σ-aminocaproate and penicilloyl-poly-L-lysines, at equal concentration in penicilloyl groups, cause the same binding inhibition.

These observations suggest that the association

constant might be identical no matter in what form the penicilloyl groups are present. Another fact confirms this assumption. We calculated tracer specific activity using an immunological method adapted from Mitchell [13].

This method postulates that the association constant is the same for labelled and unlabelled antigens. Two successive overdoses (11 nCi) of ^{125}I-BSA conjugate have been added. The amounts of penicilloyl Σ amino caproate which cause the same binding inhibition are twice 270 pg. The specific activity thus determined is then 40 mCi/mg of penicilloic acid. The agreement between this result and that obtained directly from iodation yield and coupling degree thus confirms the starting hypothesis. As a result the abscissa can be graduated in penicilloyl group quantities and the standard curves presented may be used for the assay of penicilloyl groups, irrespective of the chemical or conjugated (to seric or tissue protein) form in which they are present.

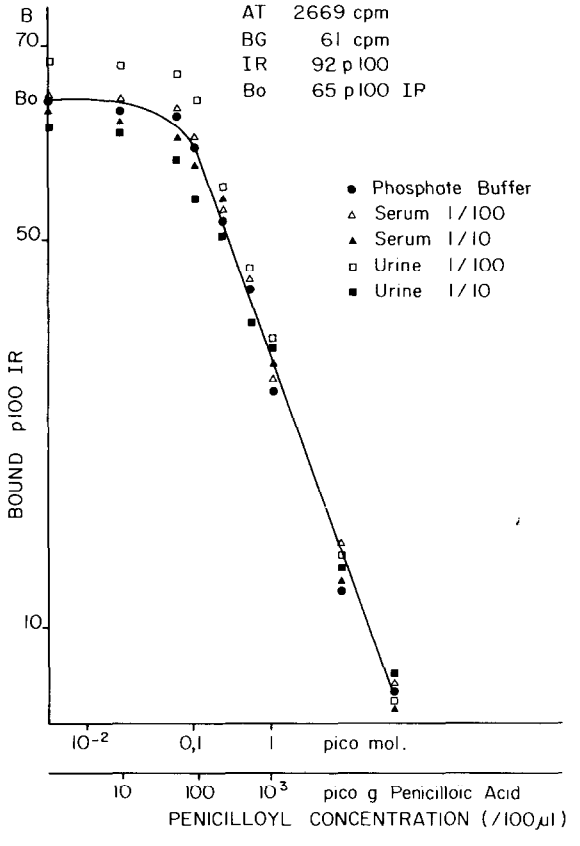

Fig.4. Standard curves obtained in milk, urine and serum at different dilutions.

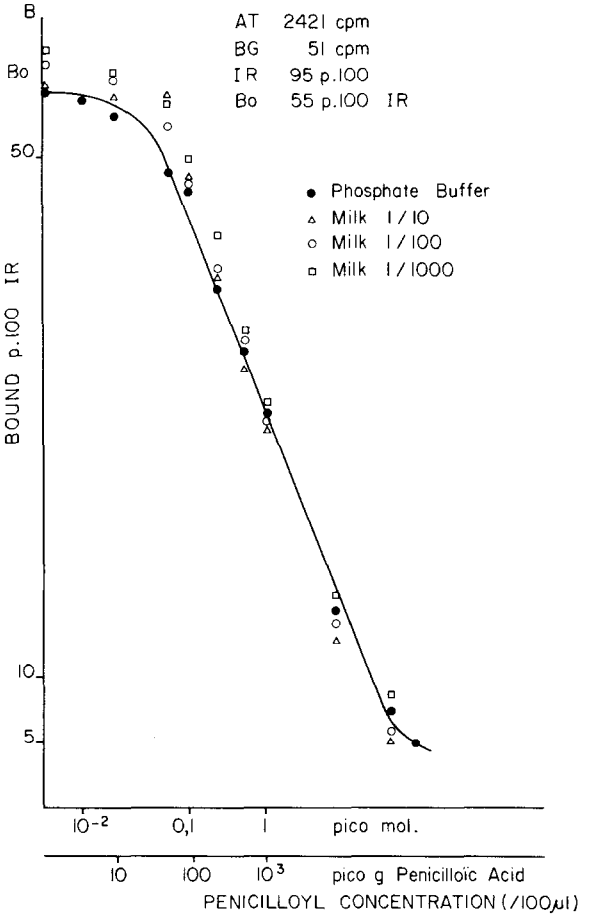

Fig.5. Standard curves obtained in milk, urine and serum at different dilutions.

The detection limit is about 50 pg of benzyl penicilloic acid. The binding inhibition expressed in B/Bo varies from 90 to 10% when the amount of penicilloyl introduced varies between 50 pg and 50 ng. The mid-point (B/Bo = 50%) is found at 1 ng. The non-specific binding (BG) obtained with normal rabbit serum is lower than 2% of the total activity introduced (AT). Tracer utilization life is 5 weeks. However, at the end of this time, non-specific binding may be 10 to 12% of the total activity.

All these characteristics are perfectly reproducible with different tracer preparations.

The sensitivity seems low compared to those usually obtained in radioimmunoassay using iodinated tracers.

However, the detection limit is low enough for the intended applications, and we purposely prepared a tracer of moderate specific activity in order to increase its reliability and shelf life.

3.2. *Characteristics of the assay in biological samples*

Standard curves (fig.4 and 5) were established in various biological fluids (cow milk, pig serum and urine) at different dilutions showing no effect of the biological material (protein or interfering substances, such as salts or urea, concentration) on the assay. Penicilloyl group determination can thus be performed directly in these biological samples without previous extraction, after suitable dilution. Detection threshold is in the range of a few ppb.

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