Journal of Immunological Methods, 40 (1981) 231—238

QUANTIFICATION OF β_2-MICROGLOBULIN BY INHIBITION ENZYME IMMUNOASSAY

YVES CARLIER [1], ALAIN COLLE [2], PIERRE TACHON [3], DANIEL BOUT [4] and ANDRÉ CAPRON [4]

[1] *Instituto Boliviano de Biologia de Altura, La Paz, Bolivia,* [2] *Centre d'Immunologie, Marseille Luminy,* [3] *Laboratoire d'Immunochimie, Institut Pasteur, Lyon and* [4] *Centre d' Immunologie et de Biologie Parasitaire, Institut Pasteur, Lille, France*

(Received 1 July 1980, accepted 14 September 1980)

Inhibition enzyme immunoassay was applied to β_2-microglobulin from urine. The technical conditions of the assay were determined. The detection limits of the assay were 5-500 ng/ml. Correlation coefficient obtained between radioimmunoassay and inhibition enzyme immunoassay was 0.97. Inhibition enzyme immunoassay provides an alternative, low cost method for the quantification of β_2-microglobulin.

INTRODUCTION

β_2-Microglobulin (β_2mG) is a small protein (MW 11,800) containing about 100 amino acid residues, which is found in most body fluids such as urine, serum, cerebrospinal fluid, saliva, colostrum, amniotic and seminal fluids (Berggard and Bearn, 1968; Peterson et al., 1969; Evrin and Peterson, 1971; Jonasson et al., 1974; Colle et al., 1976a). It presents large structural homologies with some regions of the light or heavy chains of immunoglobulins (Smithies and Poulik, 1972; Peterson and Cunningham, 1972), and with the two HLA polypeptide chains (Nakamuro et al., 1973; Peterson et al., 1974). In the mouse, β_2-microglobulin is reported to be associated not only with the major histocompatibility antigen (H-2), but also with the structurally and genetically related TL (Östberg et al., 1975; Vitetta et al., 1975) and Qa-2 (Michaelson et al., 1977) antigens, with the H-Y antigen (Fellous et al., 1978) and with a cell-cell interaction factor (Amerding et al., 1975), but its function in the immune response is still under investigation (Marx, 1974).

In clinical use, β_2mG determination can be helpful in many fields, particularly in gynecology/obstetrics and in nephrology. The determination of β_2mG in amniotic fluid can be used to determine foetal maturity or to study some pregnancy disorders (Cejka et al., 1973; Hall and Roux, 1974; Jonasson et al., 1974). In nephrology the determination of β_2mG in serum might be equivalent or superior to the determination of creatinine in the estimation of the glomerular filtration ratio and useful as a substitute for the more laborious inulin clearance determination (Wibell et al., 1973). The deter-

mination of β_2mG in urine can be used to evaluate the kidney's ability to reabsorb proteins in the proximal tubules, since β_2mG is able to pass through the glomerular membrane relatively easily, but the normal tubular reabsorption of filtered β_2mG is closed to 100%. The increased β_2mG content in the urine appears to be an approximate measure of the decreased tubular reabsorption of such proteins (Wibell et al., 1973).

The usual method for the determination of β_2mG level is a competitive solid-phase radioimmunoassay, from which a commercial kit was developed by Pharmacia ($\beta_2$microtest, Uppsala, Sweden). This assay was shown to be sensitive and reproducible, but its use involves expensive and sophisticated counting equipment, radioisotope licensing, and a radiolabelled reagent with a short half-life requiring special protective measures for safe handling.

Enzyme immunoassays, on the other hand, were shown to be equally sensitive and reproducible, do not have these inconveniences and are already applied in many fields (Widson, 1976; Carlier et al., 1979a, 1980). In previous studies we showed the inhibition enzyme immunoassay (IEIA) particularly adapted for antigen quantification, in its application for apolipoprotein B (Carlier et al., 1978). The aim of this investigation was to apply an IEIA, which was shown to be simple, rapid, sensitive and reproducible, to quantify β_2mG in urine.

MATERIALS AND METHODS

Antigens

β_2mG was purified using urine from patients with severe tubulopathies, following the technique described previously by Colle et al. (1976b). The purity of the obtained batches was found to be around 97% using sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Soluble antigen of *Toxoplasma gondii* was used as a non-related antigen to study the stability of the antigen coating. It was prepared as previously described (Carlier et al., 1980).

Anti-β_2mG serum

A rabbit anti-β_2mG serum, commercially available, was obtained from Sebia (Issy les moulineaux, France). Its specificity was verified using immunoelectrophoresis and gel immunodiffusion. Only one precipitation band, showing an identity reaction with pure β_2mG, was obtained using human serum and concentrated urine from a patient with tubulopathy as antigens.

Urine samples

Nineteen urine samples adjusted to pH 7.0 were obtained from patients with clinically evident tubulopathies with a β_2mG level between 800 and 6700 μg/l as determined by the Pharmacia radioimmunoassay (see below). Ten urine samples with a β_2mG level $\leqslant$240 μg/l were used as controls.

Radioimmunoassay (RIA) for β_2mG evaluation

A commercially available RIA kit (Phadebas-$\beta_2$microtest; Pharmacia, Uppsala, Sweden) was used as reference method to quantify β_2mG in the urine samples. The test principle is based on antigenic competition between the standard ^{125}I-labelled β_2mG and the β_2mG of the studied samples, for an anti-β_2mG antibody solid phase (Sephadex particles). The competitive capacity was then compared with that of standard β_2mG preparations of known concentration. A centrifugation step was necessary to separate bound and free β_2mG, and the radioactivity bound to the sedimented Sephadex particles was measured.

Inhibition enzyme-immunoassay (IEIA) for β_2mG determination

IEIA was performed in disposable polystyrene tubes as previously described for the quantification of apolipoprotein B (Carlier et al., 1978), with some modifications, particularly in the coating step (see below). Briefly, β_2mG-coated tubes were incubated with 0.5 ml of diluted urine samples or β_2mG preparations of known concentrations (for the reference inhibition curve), and 0.5 ml of anti-β_2mG rabbit serum at a suitable dilution (see below) for 4 h at room temperature. The tubes were then emptied by suction, washed three times, and incubated overnight at 4°C with 1 ml of peroxidase-labelled sheep antibodies to rabbit immunoglobulins (conjugate: Institut Pasteur Production, Paris) at a suitable dilution (see below). Excess conjugate was then thoroughly washed and the amount of peroxidase fixed to the tubes determined using H_2O_2 as substrate and *o*-dianisidine as the hydrogen donor. After 1 h at room temperature, the reaction was stopped by addition of a drop of 5 N HCl and the yellow colour development was measured in a spectrophotometer at 405 nm. All the dilutions were performed in phosphate-buffered saline (PBS) (0.01 M, pH 7.2) and the washing steps with the same PBS containing 0.05% Tween 20.

RESULTS

Study of the coating conditions for β_2mG

Preliminary assays using a coating procedure similar to that used for apolipoprotein B (Carlier et al., 1978) lead to weak inhibition curves (Fig. 1a). In order to stabilize β_2mG on the polystyrene surface, the tubes were previously incubated for 3 h at 56°C with 1 ml of a bifunctional reagent, glutaraldehyde (Merck) at a 0.1% concentration in 0.1 M carbonate/bicarbonate buffer, pH 9.0. The tubes were washed three times and then incubated with 1 ml of β_2mG at a suitable concentration (see below) for 3 h at 37°C. After a further three washings, 1 ml of 0.5 M 4-aminobutyric acid (Merck) in 0.05 M carbonate/bicarbonate buffer, pH 10.0, was incubated in the tubes for 2 h at room temperature in order to block unused reactive groups of glutaraldehyde, to avoid non-specific binding of anti-β_2mG antibodies or conjugate. After washing, such glutaraldehyde-coated tubes were

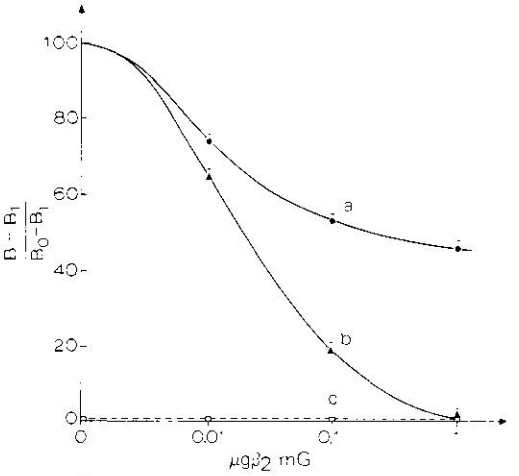

Fig. 1. β_2mG inhibition curves in IEIA using different conditions of coating: ●——●, β_2mG (1 µg) without glutaraldehyde; ▲——▲, β_2mG (1 µg) with glutaraldehyde; □- - - - -□, *Toxoplasma gondii* (1 µg) with glutaraldehyde; anti-β_2mG serum at 1/250 dilution; 500 ng of peroxidase-labelled antibodies. B = absorption of the studied samples; B_1 = absorption using 1 µg of free β_2mG; B_0 = absorption without free β_2mG; ⧳ = standard deviation.

further used for the inhibition reaction and gave an excellent inhibition curve with a 50% increase in sensitivity (Fig. 1b).

In order to verify the stability of such a coating, the following protocol was used: firstly, a non-related soluble antigen (*Toxoplasma gondii*) was coated using the procedure described above; secondly, the tubes were incubated for 3 h at 37° C with β_2mG; the inhibition reaction was then performed normally. Using these conditions, it was not possible to obtain any inhibition curve, thus showing that the antigen link to the plastic surface was strong and impossible to break (Fig. 1c). The inverse protocol, using β_2mG-coated tubes, coated secondly with *Toxoplasma* antigen gave an excellent inhibition curve, identical to that obtained with tubes coated only with β_2mG.

Determination of optimal coating concentration of β_2mG

The tubes were coated with different concentrations of β_2mG according to the procedure described above and then incubated with anti-β_2mG serum at a 1/250 dilution. After incubation with the conjugate and colorimetric quantitation of peroxidase fixed to the tubes, the absorbance values were plotted against concentration (µg/ml) of β_2mG used for coating (Fig. 2). The optimal coating concentration of 1 µg/ml was selected for further use.

Determination of the optimal dilution of anti-β_2mG serum

Serial dilutions of the antiserum were incubated with a fixed amount of β_2mG (1 µg/ml) coated on polystyrene tubes and the samples processed as described in Methods. A dilution curve is shown in Fig. 3. There was a

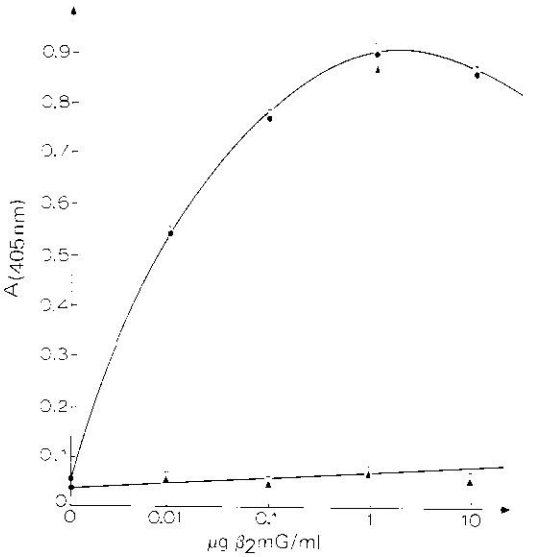

Fig. 2. Binding of anti-β_2mG serum to polystyrene tubes coated with various concentrations of β_2mG: ●——●, with anti-β_2mG serum at 1/250 dilution; ▲——▲, control without anti-β_2mG serum; arrow indicates the optimal coating antigen concentration; ♦ = standard deviation.

maximal absorbance for antiserum dilutions smaller than 1/100. Without antiserum or coated β_2mG, non-specific binding was reduced to less than 1 and 7% respectively. Optimal sensitivity of IEIA was achieved by using

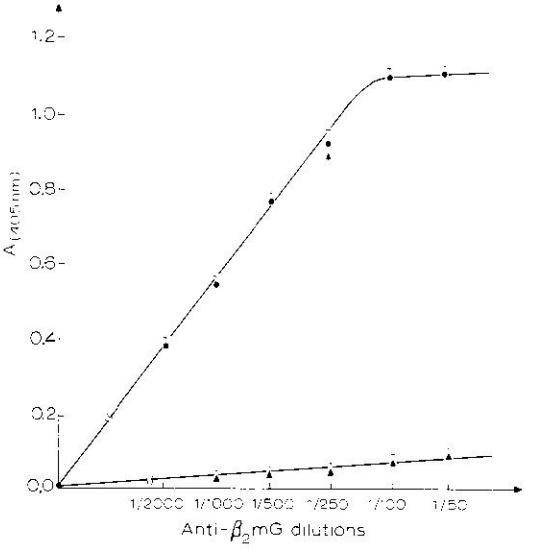

Fig. 3. Binding of different anti-β_2mG serum dilutions to β_2mG-coated polystyrene tubes: ●——●, with 1 μg/ml of coated β_2mG; △——△, control without coated β_2mG; arrow indicates the optimal dilution to be used; ♦ = standard deviation.

the antiserum dilution yielding 87% binding. This dilution could vary from one batch of antiserum to another, but was generally 1/250 and this dilution was further used.

Determination of the optimal dilution of the conjugate

Different conjugate concentrations were used with 1 μg/ml of β_2mG coated on polystyrene tubes and antiserum at 1/250 dilution (Fig. 4). The optimal concentration which gave a high range of extinction values and a saturation of the antigenic sites of the anti-β_2mG antibodies was 500 ng/ml.

Inhibition reference curve

Different concentrations of β_2mG were used to obtain an inhibition reference curve (Fig. 5). Each sample was assayed in triplicate and the mean value was calculated. The results were expressed in percent as $(B - B_1)/(B_0 - B_1)$ where B is the absorption of the studied sample, B_1 the absorption obtained with 1 μg of β_2mG, giving a maximal inhibition reaction and B_0 the absorption obtained without free β_2mG, i.e. without inhibition reaction. Figure 5 shows a representative standard inhibition curve for β_2mG. As the amount of added soluble β_2mG was increased, the binding of anti-β_2mG

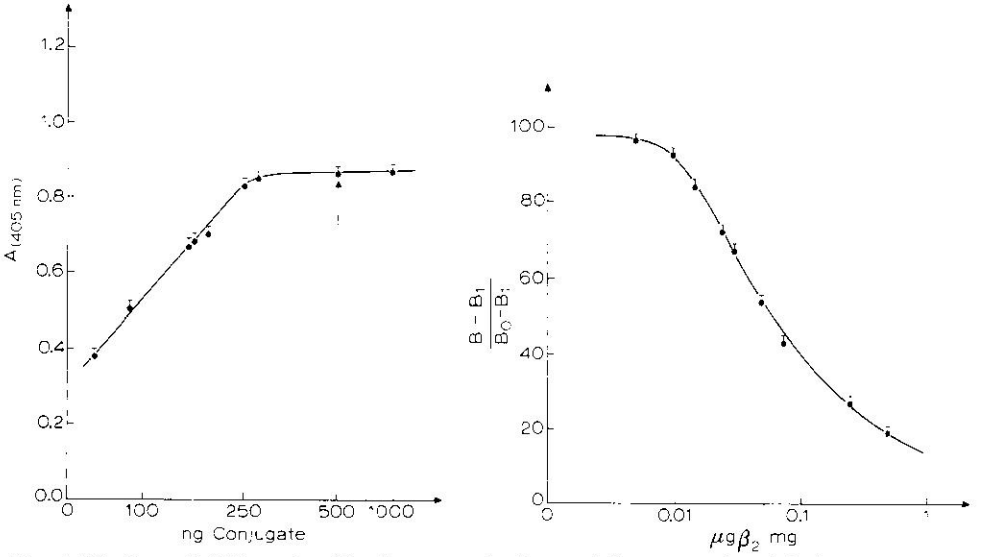

Fig. 4. Binding of different antibody concentrations of the peroxidase-labelled conjugate to β_2mG-coated polystyrene tubes (1 μg), incubated with anti-β_2mG serum (1/250); arrow indicates the optimal dilution to be used; ⊢ = standard deviation.

Fig. 5. β_2mG inhibition reference curve in IEIA. B = absorption of the studied samples; B_1 = absorption using 1 μg of free β_2mG; B_0 = absorption without free β_2mG; ⊢ = standard deviation.

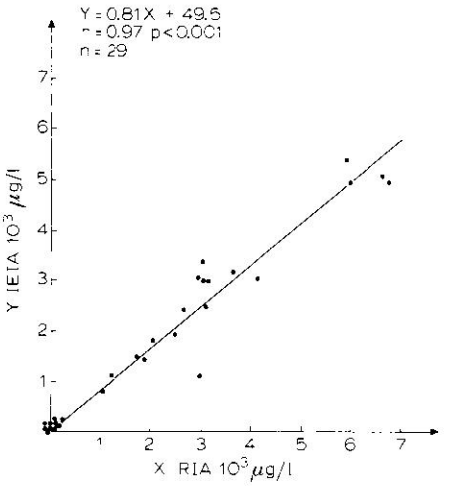

Fig. 6. Correlation between IEIA and RIA for β_2mG determination.

antibodies to β_2mG solid phase was inhibited and the intensity of the color reaction decreased progressively. The detection limits of the assay were 5—500 ng/ml, but the optimal working range in the linear part of the curve was from 10 to 200 ng/ml.

Sensitivity and precision of assay

The minimum detectable concentration (10 ng/ml in the linear part of the curve) is in excess of that required to measure β_2mG in urine and urine dilutions were routinely used. Some urines from patients with severe tubulopathies must be diluted by as much as 1/100, but generally 1/10 and 1/100 dilutions in PBS (0.01 M, pH 7.2) were sufficient to study tubular urines.

Within-batch precision was calculated from the difference between triplicate measurements of 29 urines. The coefficient of variation was found to be $\leqslant 2\%$.

Comparison between IEIA and RIA for β_2mG determination

IEIA was more accurate than Phadebas $\beta_2$micro-test since the within-assay coefficient of variation was 2% instead of 4.7—8.4% (Pharmacia handbook information). Results obtained by the two methods correlated well ($r = 0.97$; $P < 0.001$) (Fig. 6). The mean levels found by IEIA were sometimes lower than those obtained by RIA.

DISCUSSION

IEIA was shown to be a specific, precise and sensitive method for the quantification of β_2mG and its results equaled those of the Phadebas

$\beta_2$micro test since a good correlation was obtained for the β_2mG urinary levels determined by both methods.

Only urine samples were studied in the present work and further studies will be necessary to evaluate IEIA with serum or amniotic fluid.

The main advantage of IEIA over RIA, besides its higher precision and the elimination of a centrifugation step, is the avoidance of a radioisotope-labelled reagent, and hence the accompanying sophisticated counting equipment, radioisotope licensing and special protective measures for isotope manipulations.

IEIA provides an alternative, low cost method, that can easily be automated (Carlier et al., 1979b) for quantification of β_2-microglobulin in biological fluids.

ACKNOWLEDGEMENTS

The authors wish to thank Miss S. Carton and Mr. L. Hornez for their excellent technical assistance and Miss M. del Carmen Burgoa A. for her contribution to the preparation of the manuscript.

REFERENCES

Amerding, D., R.T. Kubo, H.M. Grey and D.H. Katz, 1975, Proc. Natl. Acad. Sci. U.S.A. 72, 4577.
Berggard, I. and A.G. Bearn, 1968, J. Biol. Chem. 243, 4095.
Carlier, Y., D. Bout, J.C. Fruchart, C. Desreumaux, P. Dewailly, G. Sezille and J. Jaillard, 1978, J. Immunol. Methods 21, 317.
Carlier, Y., D. Bout and A. Capron, 1979a, Ann Inst. Pasteur (Lyon) 12, 25.
Carlier, Y., D. Bout and A. Capron, 1979b, J. Immunol. Methods 31, 237.
Carlier, Y., D. Bout, J.P. Dessaint, A. Capron, F. Van Knapen, E.J. Ruitenberg, R. Bergquist and G. Huldt, 1980, Bull. WHO 58, 99.
Cejka, J., F. Cohen and K. Kithier, 1973, Clin. Chim. Acta 47, 59.
Colle, A., R. Guinet, M. Leclercq and Y. Manuel, 1976a, Clin. Chim. Acta 67, 93.
Colle, A., C. Tonnelle, Y. Manuel, L. Rivat and C. Ropatz, 1976b, Eur. J. Immunol. 6, 660.
Evrin, P.E. and P.A. Peterson, 1971, Scand, J. Clin. Lab. Invest. 28, 439.
Fellous, M., E. Günther, R. Kemler, J. Wiels, R. Berger, J.L. Guente, H. Jacob and F. Jacob, 1978, J. Exp. Med. 148, 58.
Hall, P.W. and J.F. Roux, 1974, Am. J. Obstet. Gynec. 120, 56.
Jonasson, L.E., P.E. Evrin and L. Wibell, 1974, Acta Obstet. Gynec. Scand. 53, 49.
Marx, J.L., 1974, Science 185, 428.
Michaelson, J., L. Flaherty, E. Vitetta and M.D. Poulik, 1977, J. Exp. Med. 145, 1066.
Nakamuro, K., N. Tanigaki and D. Pressman, 1973, Proc. Natl. Acad. Sci. U.S.A. 70, 2863.
Ostberg, L., L. Rask, H. Wigzell and P.A. Peterson, 1975, Nature 253, 735.
Peterson, P.A. and B.A. Cunningham, 1972, Proc. Natl. Acad. Sci. U.S.A. 69, 1967.
Peterson, P.A., P.E. Evrin and I. Berggard, 1969, J. Clin. Invest. 48, 1189.
Peterson, P.A., L. Rask and J.B. Lindbolm, 1974, Proc. Natl. Acad. U.S.A. 71, 35.
Smithies, O. and M.D. Poulik, 1972, Science 175, 187.
Vitetta, E.S., J.W. Uhr and E.A. Boyse, 1975, J. Immunol. 114, 252.
Wibell, L., P.E. Evrin and I. Berggard, 1973, Nephron 10, 320.
Widson, G.B., 1976, Clin. Chem. 72, 1243.