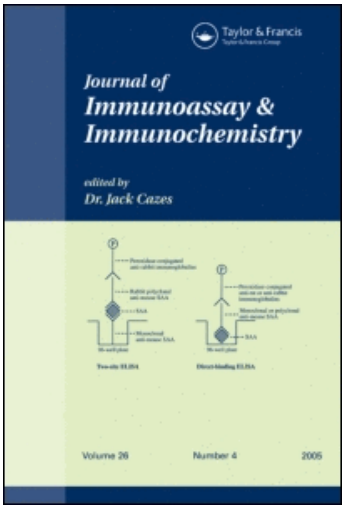

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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Online publication date: 09 February 2004

To cite this Article Chen, Xiaorui , Chen, Yueteng and Shen, Zhufang(2005) 'A Competitive ELISA for Albumin in Rat Urine', Journal of Immunoassay and Immunochemistry, 25: 1, 81 — 89
To link to this Article: DOI: 10.1081/IAS-120027228
URL: http://dx.doi.org/10.1081/IAS-120027228

JOURNAL OF IMMUNOASSAY & IMMUNOCHEMISTRY
Vol. 25, No. 1, pp. 81–89, 2004

A Competitive ELISA for Albumin in Rat Urine

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ABSTRACT

Nephropathy is characterized by urinary micro albumin. Rats are usually used in experimental studies. But, there was no specific and simple method for detecting rat urinary albumin. A specific, easily performed, and sensitive method for quantitatively determining rat urinary albumin is needed. Using rabbit anti-rat albumin polyclonal antibody, biotinylated goat anti-rabbit IgG, avidin conjugated peroxidase, and TMB (3,3′,5,5′-tetramethylbenzidine) as substrate, a competitive ELISA for rat albumin in urine was developed. With this method, the urinary albumin in diabetic and normal rats was detected. This method was sensitive to 30 ng/mL and reproducibly quantifies rat urinary albumin levels in the range of 0.05–5 μg/mL. The rabbit anti-rat albumin polyclonal antibody showed no cross reaction with bovine and human serum albumins and rat IgG, and showed little cross-reaction with mouse albumin. The intra-assay CV was

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DOI: 10.1081/IAS-120027228 1532-1819 (Print); 1532-4230 (Online)

less than 10%. The urinary albumin markedly increased in diabetic rats. Since quantifying urinary albumin was very important in the experimental study of diabetic nephropathy, the results from our experiments indicated that this competitive ELISA could be used for quantification of rat urinary albumin.

Key Words: Nephropathy; ELISA; Urinary albumin; Rats.

INTRODUCTION

Urinary albumin can directly reflect renal function. Albuminuria has been established as a marker which is strongly predictive of kidney disease, including diabetic nephropathy. Rats are usually used in experimental kidney disease studies. There are many methods for measuring human urinary albumin.[1,2] But, there is no accurate and easily performed method for measuring rat urinary albumin. A radioimmunoassay kit for human micro urinary albumin was even used to quantify rat urinary albumin. In this study, we have established a specific competitive ELISA method for quantifying rat urinary albumin.

EXPERIMENTAL

Chemicals and Reagents

Standard rat serum albumin and Tween-20 were purchased from Sigma Chemical Co., St. Louis, MO. Freund's complete adjuvant and Freund's incomplete adjuvant were obtained from Gibco Brl. Biotinylated goat anti rabbit IgG and peroxidase-conjugated streptoavidin were obtained from the Beijing Dingguo Biotechnology Development Center (Beijing, China). The chromogenic substrate was TMB (3,3′,5,5′-tetramethylbenzidine); 1 mg/mL substrate was dissolved in DMSO (dimethylsulfoxide). Just before using, 1 mL of the substrate was added to 4 mL of pH 5.9 acetate buffer, mixed with 5 mL of 0.03% hydrogen peroxide. Other reagents were of the best grade available.

Urine Collection

For collecting urine, rats were kept in metabolic cages for 24 hours. A suitable bacterial inhibitor was added to stop bacterial growth. Urine samples were stored at -20°C until use.

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Preparation of Polyclonal Anti-Rat Albumin Antibody

To raise the anti-rat albumin antiserum, 1 mg rat albumin in 50% Freund's complete adjuvant was intradermally injected into each rabbit at 20–30 skin sites. Animals were subjected to boosters 30 days later with the same amount of rat albumin in 50% Freund's incomplete adjuvant, followed by three additional booster injections every week. Each rabbit was finally intradermally and intramuscularly injected with 2 mg of the same antigen suspended in 50% Freund's incomplete adjuvant. The serum was taken on the 10th day after the final injection. The antiserum was directly used without further purification. The animals' care was in accordance with our institutional guidelines.

Assay Procedure

The assay was performed in 96-well plastic immuno plates (Nalge Nunc International Maxisorb). Antibody, samples and standards were diluted with phosphate buffered saline (PBS), pH 7.4, containing 1% normal goat serum. First, the plate was coated with 100 μL (1 μg/mL) rat albumin, incubated at 37°C for 2 hours. After washing the wells three times with PBS containing 0.05% Tween-20 (PBST), the plate was blocked with PBS containing 1% normal goat serum. It was washed three times with PBST. Then, 50 μL sample or standard and 50 μL rabbit anti-rat albumin antibody (1 : 5000) were added. After incubation at 37°C for 2 hours and washing three times with PBST, biotinylated goat anti rabbit IgG was added and the mixture was incubated at 37°C for 2 hours. Then, it was washed three times as above, avidin conjugated peroxidase was added, followed by incubation at 37°C for 1 hour and washing four times with PBST. Finally, the substrate TMB (3,3′,5,5′-tetramethylbenzidine) was added, and the reaction was kept for 10 mins, using 50 μL 1 M sulfuric acid to stop the reaction. The absorbance at 450 nm was read on a μ-Quant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Inc.).

RESULTS

Standard Curve of the Assay

All concentrations referred to the actual concentration in urine samples or aqueous standards. Because this method was very sensitive and the urine albumin concentrations were variable, the urine samples should be diluted

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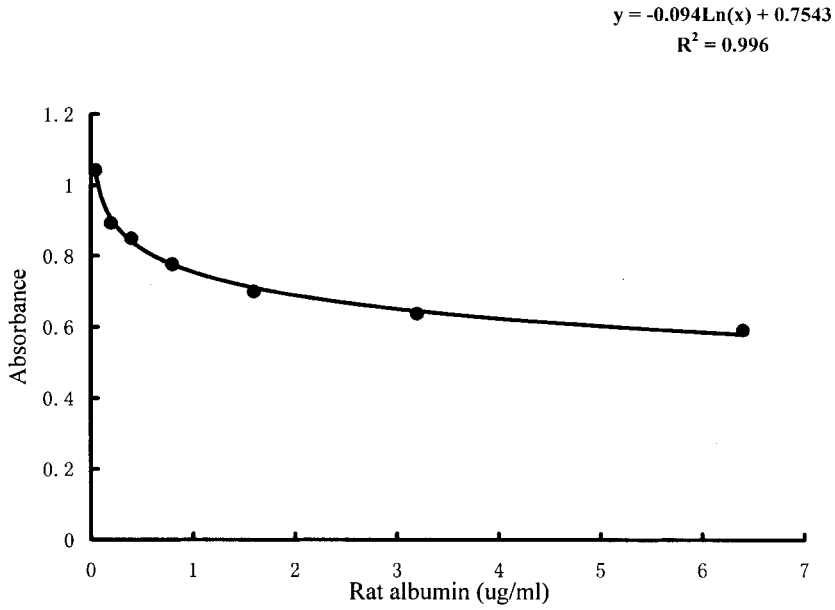

Figure 1. Standard curve of rat albumin in the assay.

20–500 fold. So, some samples should be tested twice. All measurements are carried out in triplicate.

As shown in Fig. 1, the standard curve of rat albumin is a hyperbola. The absorbance and the common logarithm of the concentration assume a linear shape ($R^2 = 0.996$).

Antibody's Competitive Binding to Other Proteins

In order to make sure that the method is specific, the cross-reaction of the antibody for rat albumin with different species' albumins and other rat proteins were studied. The result shown in Fig. 2 confirmed that the rabbit anti-rat albumin polyclonal antibody showed no cross-reaction with bovine and human albumin, and little cross-reaction with mouse albumin. Figure 3 shows that the rabbit anti-rat albumin polyclonal antibody also has no cross-reaction with rat IgG.

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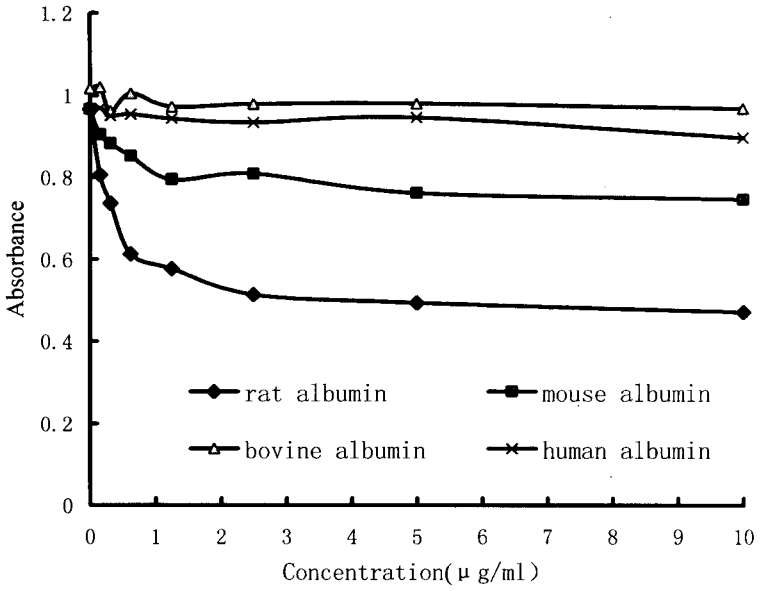

Figure 2. Curves of competitive binding of different species albumin with rabbit anti-rat albumin antibody produced in our lab.

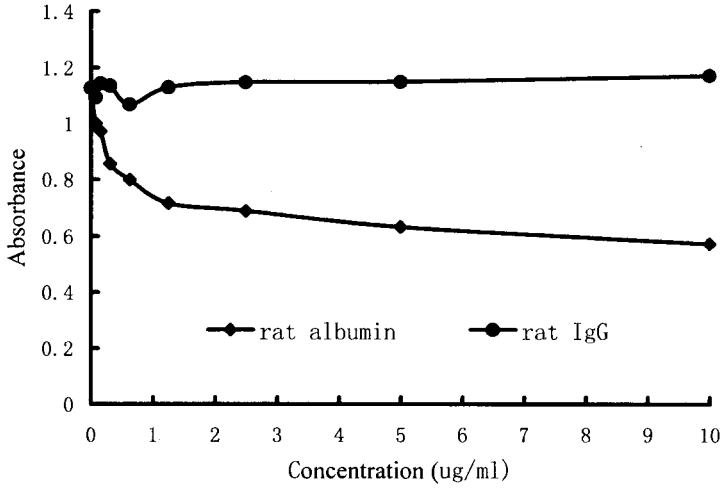

Figure 3. Curves of competitive binding of rat IgG with rabbit anti-rat albumin antibody.

Methodology

The assay sensitivity was 30 ng/mL, and the measurable range was 0.05–5 μg/mL. Urine samples should be diluted 20–500 fold. Analytical recovery is about 95%. Intra-assay coefficients of three rat albumin concentrations were 9%, 6%, and 6%. A urine sample diluted 8, 16, 32, 64, and 128 fold were measured as 1.240, 0.631, 0.258, 0.112, and 0.054 μg/mL.

Detection of Urinary Albumin in Normal and Diabetic Rats

Using this method, the urinary albumin excretion during 24 hours in normal Wistar rats and 1.5-year-old OLETF (Otsuka Long-Evans Tokushima Fatty) rats, which were known as a spontaneous type 2 diabetic model, was measured. As shown in Fig. 4, it is obvious that OLETF rats with severe hyperglycemia showed very high urinary albumin excretion when compared with normal Wistar rats. These findings indicate that these diabetic rats might suffer from renal complications.

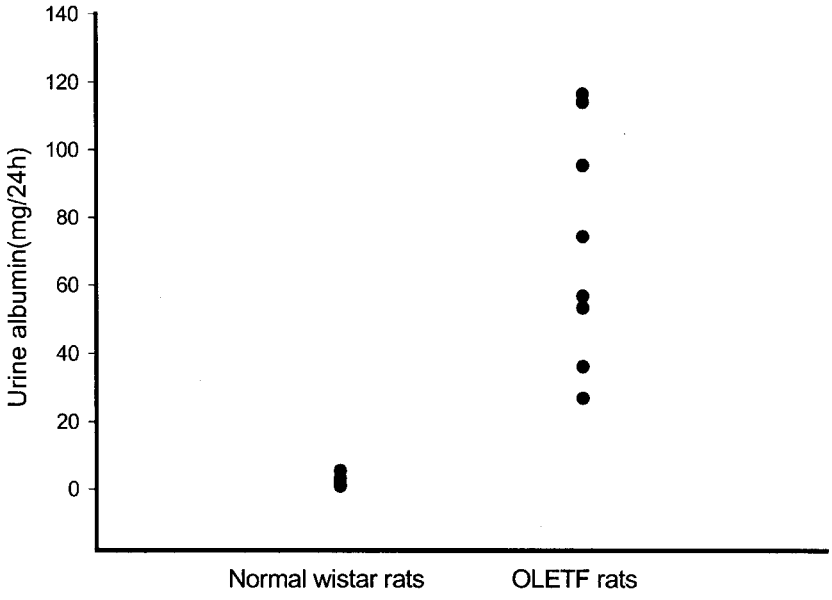

Figure 4. Urinary albumin in normal rats ($n = 8$) and OLETF rats ($n = 8$).

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DISCUSSION

Kidney disease is now attracting increasing attention. Not only is primary kidney disease very common, but many other diseases such as diabetes and hypertension also present kidney complications. Rats are generally used in experimental kidney disease studies. Urinary albumin can directly reflect kidney filtration function, so, in many studies, urinary albumin must be measured quantitatively. Radio-immuno assay or rocket immunoelectrophoresis have been used to measure rat urinary albumin. But, these methods are not convenient and yield radioactive contaminants. For these reasons, this competitive ELISA method was developed. From the results described above, it is clear that the concentration of rat urinary albumin was variable.

It should be noted that the radio-immuno kit for measuring human urinary albumin cannot be used to measure rat urinary albumin because of no cross-reactivity between human albumin antibody and rat albumin. We have found that the radio-immuno kit that measures human albumin cannot detect rat urinary albumin (results not shown here). From our results, one can conclude that the antibody used in this study showed no cross-reaction with human albumin. Similarly, many immuno-assay kits that measure a protein in one species cannot necessarily be directly used to detect the related protein in other species, since they may react differently.

Except for urinary albumin, urinary protein has been used to reflect kidney function. We found that the colorimetric methods usually used to measure protein cannot be used directly to quantify urinary protein, because urine is very complex. Directly using these methods to measure urinary protein may result in very high concentrations. A feasible method should choose an appropriate agent to precipitate the protein and dissolve it in an appropriate solvent, then using colorimetric methods to quantify the protein concentration.

ELISA techniques, in general, offer several important advantages in comparison with RIA.[3] There are no radioactive materials to be handled, the shelf life of the reagents is not limited by the half-life of the radioisotope, and only small quantities of antibody are required. There are no manual separation procedures. The wide range of albumin concentrations observed in urine samples is in agreement with other authors' findings.[4–6]

The method established in this study is easy to use, except that one should prepare the antibody needed. Other reagents were low cost and could be easily purchased from the market. Finally, we come to the conclusion that this method is suitable for inexpensively measuring large numbers of rat urine samples in the laboratory.

Although this method has the merits mentioned above, we should point out that the polyclonal antibody binds equally well to whole serum albumin as

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it does to proteolysed serum albumin fragments. As is generally known, urine can contain many proteins, and one protein may have different degradation fragments.[7] The finding of albumin in rats' urine is an indication of increased porosity of the glomeruli, whilst the presence of albumin fragments is indicative of the abnormal occurrence of proteolysis occurring in the kidneys, which is common in the nephritic syndrome which occurs among diabetics. Fractionated rat urinary albumin could be measured by SDS gel electrophoresis followed by immunostaining assay for the fractions, or measured by injecting radiolabeled rat albumin and size exclusion chromatography for urine, as shown by Tanya et al.[6] Our methods could not be used to measure fractionated rat urinary excretion. It detects overall rat albumin excretion rate, reflecting kidney filtration function.

ACKNOWLEDGMENT

The authors would like to thank Professor Mingzhi Xie for her assistance.

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Received November 30, 2003
Accepted May 5, 2003
Manuscript 3086

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