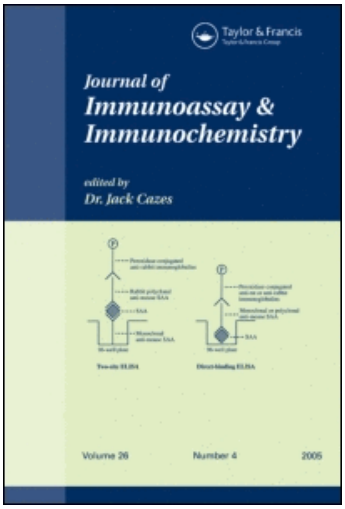


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# Simultaneous Immunoenzymometric Assay for Antibodies Against Human Interleukin-2 and Human Serum Albumin in Rat Serum

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# SIMULTANEOUS IMMUNOENZYMOMETRIC ASSAY FOR ANTIBODIES AGAINST HUMAN INTERLEUKIN-2 AND HUMAN SERUM ALBUMIN IN RAT SERUM.

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## ABSTRACT

A simultaneous immunoenzymometric assay for anti-human interleukin-2 antibody and anti-human serum albumin antibody in rat serum was developed. Two antigen-immobilized polystyrene balls were immersed in a diluted serum sample in an assay tube and then the antibodies on the balls were made to react with a horseradish peroxidase labelled anti-rat IgG antibody in the same tube after washing. The enzyme activity of each ball was measured by fluorometry. Not only were the sensitivity (70 ng/ml each), assay recovery (100-101%), and precision (C.V.= 5-13%) comparable to those of conventional immunoenzymometric assays using one antigen-immobilized ball but the assay was also much more feasible for mass routine assays. Thus, conventional immunoassays can be replaced by this convenient simultaneous method.
(KEY WORDS: Simultaneous immunoenzymometric assay, Polystyrene ball, Antibody determination, Recombinant human interleukin-2, Human serum albumin)

## INTRODUCTION

Simultaneous determination by immunological methods has become very important for mass screenings and clinical assays. However, it is difficult to measure some antigens (Ags) or antibodies (Abs) simultaneously in one reaction vessel by immunoassay (1). In most cases, as many reaction vessels as kinds of Ags

or Abs are needed with repetition of identical procedures of sampling, washing and dispensing for each vessel.

Some methods have been developed for simultaneous measurement of specific IgGs or IgEs using nitrocellulose paper (2,3) and cellulose thread (4) as solid phases. However, they are not as accurate as the conventional methods and require special equipment. Furthermore, their procedures are not suitable for small samples and are difficult to apply to a variety of detection methods, especially radioactivity measurement. Thus, they are unsuitable for routine assays and can not replace the usual immunoassays. A method for simultaneous determination of two Abs in one reaction vessel was reported by Ianconescu (5) using microtiter plates and Falcon assay screening test (F.A.S.T.) system lids as solid phases. However, problems arise here due to the different shapes of the solid phase for Ag-immobilization.



Here we describe a simultaneous immunoenzymometric assay (simultaneous IEMA) using polystyrene balls of the same shape as the solid phase. Two balls of immobilized Ags are placed together in one reaction tube and the assay is performed in the same manner as conventional IEMAs. We have developed a simultaneous IEMA for anti-recombinant human interleukin-2 Ab and anti-human serum albumin Ab in rat serum and estimated their production after the administration of recombinant human interleukin-2 (rIL-2) containing human serum albumin (HSA) as a vehicle.

## MATERIALS AND METHODS

### Antigens and Chemicals

rIL-2 and HSA were obtained from Shionogi (Osaka, Japan) and Green Cross (Osaka, Japan), respectively. All chemicals were of analytical grade, unless otherwise specified.

## Antibodies

Rat antisera against rIL-2 and HSA for assay standards were produced by immunizing rats with respective Ags emulsified with Freund's complete adjuvant in the usual way (6). The amounts of anti-rIL-2 Ab and anti-HSA Ab measured by the quantitative precipitation test with a ultraviolet absorption method (7) were 0.94 mg/ml in the anti-rIL-2 antiserum (A41-4) and 2.2 mg/ml in the anti-HSA antiserum (A46-4), respectively.

## Buffers

A 0.1 mol/l phosphate buffer, pH 7.4, containing 0.05 mol/l sodium chloride and 0.01%(w/v) kanamycin sulfate (Sigma, St. Louis, MO) was used as a dilution buffer of Ag-immobilization (coating buffer). The coating buffer containing 1%(w/v) gelatin (Nippi Peptide PA-100, Osaka, Japan) and the coating buffer containing 0.5%(v/v) Tween 20 (Nacalai Tesque, Kyoto, Japan) were used as an assay buffer and a washing buffer, respectively. Throughout this assay, reagents or samples were diluted with the assay buffer unless otherwise specified.

## Antigen-Immobilized Ball

White polystyrene balls (1/4 inch in diameter, Sekisui, Osaka, Japan) were immersed in the coating buffer solution of rIL-2, 5 $\mu$ g/ml, and stood for 16 h at 15-25° C. The balls were then washed twice with the assay buffer and immersed again in the assay buffer for 2 h at 37° C. HSA was immobilized on green polystyrene balls (1/4 inch diameter, PACS, Kobe, Japan) in a similar manner, using 100 $\mu$ g/ml solution of HSA.

Immunoenzymometric Assay

The antisera against rIL-2 (A41-4) and HSA (A46-4) were diluted to various concentrations, 0.5-100 ng/ml, for standard solutions of the Abs with the assay buffer containing 2% normal rat serum.

The rIL-2-immobilized ball (white) and the HSA-immobilized ball (green) were placed in a reaction tube (polypropylene tube, 12.5 mm x 7 mm) which contained 600 $\mu$ l of a diluted rat serum sample (12 $\mu$ l of sample serum plus 588 $\mu$ l of the assay buffer) or the standard solution in duplicate. A series of the tubes sealed by sealing tape (3M, St. Paul, MN) were incubated for 5 h at 37° C. After washing three times with 600 $\mu$ l of the washing buffer, 600 $\mu$ l of the horseradish peroxidase labelled anti-rat IgG (H+L) Ab (ICN, Lisle, IL) (diluted 1:15,000) was added to each tube containing the two balls. The tubes were incubated for 16 h at 37° C and the balls were washed 4 times before measurement of the enzyme bound to each ball.



Enzyme Activity

Each ball was transferred into a tube for the enzyme reaction and 300 $\mu$ l of the substrate solution [0.6%(w/w) p-hydroxyphenylpropionic acid (Dojindo Lab., Kumamoto, Japan) and 0.015%(v/w) hydrogen peroxide in the coating buffer containing 0.1%(w/v) gelatin] was dispensed onto it. After incubation at 37° C for 30 min, 2.5 ml of 0.1 mol/l glycine-sodium hydroxide buffer, pH 10.5, was added to stop the reaction. The enzyme activity of each solution was found by measuring the fluorescent intensity with excitation at 320 nm and fluorescence monitoring at 405 nm using a fluorescence spectrophotometer (Hitachi MPF-4, Tokyo, Japan) fitted with a sample changer (Gilson, model 222, Middleton, WI).

### Measurement of rat serum samples

Serum samples from 58 male rats which were subjected to daily intravenous injection of 1, 5, or 25 $\mu$ g/kg of rIL-2 containing 2.5 mg/kg of HSA as a vehicle for 84 days were kindly gifted by Dr. Y. Harada of our institute and were measured by this method.

## RESULTS

### Analytical Conditions

The usual conditions for IEMAs were chosen according to our recent assays and no problem was encountered in this study. However, the assay time was rather longer, over one day, and we think that it can be shortened to less than 4 h (8).

From the preliminary investigations for preparation of Ag-immobilized balls, binding properties on the standard curves using 10 and 5 $\mu$ g/ml of rIL-2 solution for rIL-2-immobilization were similar but those using 2 $\mu$ g/ml were a little smaller. In the case of HSA-immobilization the properties did not changed at 1,000, 500 and 100 $\mu$ g/ml of HSA solution but became smaller at 50 $\mu$ g/ml. Thus, we used the 5 $\mu$ g/ml of rIL-2 solution and 100 $\mu$ g/ml of HSA solution for Ag-immobilization.

### Selection of Labelled Second Ab

We obtained three kinds of the horseradish peroxidase labelled second Abs that were commercially available: rabbit anti-rat IgG (H+L) Ab (ICN, Lisle, IL), $F(ab')_2$ fragments of affinity purified Abs of goat anti-rat IgG(H+L) (Cappel Lab, Downingtown, PA) and affinity isolated Ag specific Ab of goat anti-rat IgG (whole molecule) (Sigma, St. Louis, MO). The labelled second Ab from ICN showed a

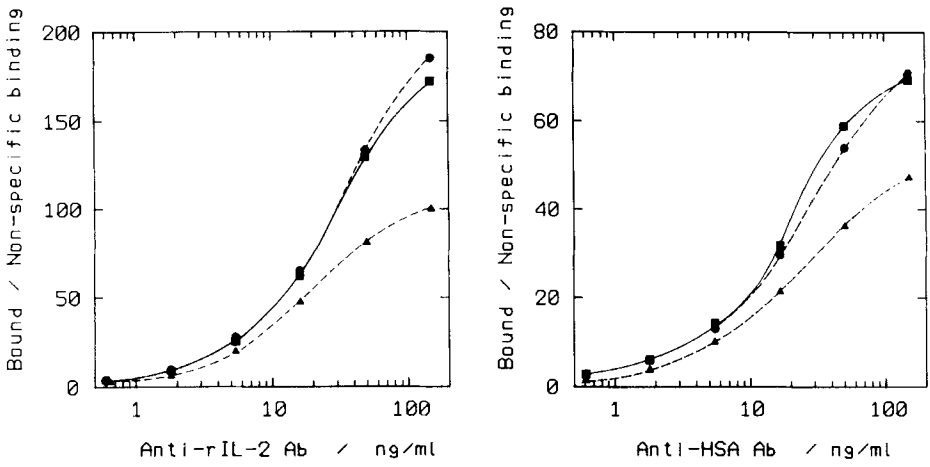


FIGURE 1. Standard curves of the simultaneous IEMA for anti-rIL-2 Ab (left) and anti-HSA Ab (right) with various concentrations of the labelled second Ab. Dilutions of the labelled second Ab are 1:10,000 (●), 1:15,000 (■), and 1:30,000 (▲). Vertical axes are the ratios of fluorescent intensity at a certain concentration of the Ab to that at zero concentration (non-specific binding).



comparatively good response on our IEMA standard curves (data not shown) and we used it for the study described below.

Fig. 1 shows the IEMA standard curves of anti-rIL-2 Ab and anti-HSA Ab with varying amounts of the labelled second Ab. The best IEMA responses were observed at a dilution of 1:15,000 which was thus used for the labelled second Ab.

## Effect of Rat Serum on the IEMA Standard Curve

Standard curves from the standard solutions prepared with the assay buffer were compared to those with the assay buffer containing normal rat serum. The differences were not large but similar profile changes were observed with both Ab assays on addition of normal rat serum, that is, increase of non-specific binding and

decrease of the maximum binding (data not shown). In order to cancel the serum interference when measuring unknown samples of rat sera, an equal amount of normal rat serum (2%) had to be added to the standard solution.

Standard Curves of Simultaneous IEMA and its Reliability

The absence of interference from the other analytes must be confirmed in simultaneous assays. As shown in Fig. 2, upper panel, the IEMA standard curve for anti-rIL-2 Ab was not affected by the addition of various concentrations of the anti-HSA antiserum. Furthermore, the IEMA standard curve for anti-HSA Ab did not change with an increase of the anti-rIL-2 antiserum (Fig. 2, lower panel). Each point of Fig. 2, lower panel, was plotted after correction of anti-HSA Ab content in the anti-rIL-2 antiserum (A41-4), since the anti-rIL-2 antiserum was found to contain some anti-HSA Ab (560 $\mu$ g/ml).

The range of the IEMA for anti-rIL-2 Ab was 70-2,000 ng/ml in rat serum (0.84-24 ng/tube) and that for anti-HSA Ab was 70-5,000 ng/ml (0.84-60 ng/tube).

Recovery and Precision

The assay recovery of this simultaneous IEMA was assessed by rat serum samples that were prepared by the addition of known amounts of the standard antisera. The relationships between added (x ng/ml) and measured (y ng/ml) concentrations were linear and the recoveries, 100-101%, were estimated from these slopes: for anti-rIL-2 Ab, $y = 1.01\ x + 0.0$, coefficient of variation (C.V.) = 5.3%, $n = 21$, and for anti-HSA Ab, $y = 1.00\ x - 8.0$, C.V. = 5.5%, $n = 30$. The differences between the added and measured values were not significant ($p<0.05$) according to regression analyses of both Ab assays.

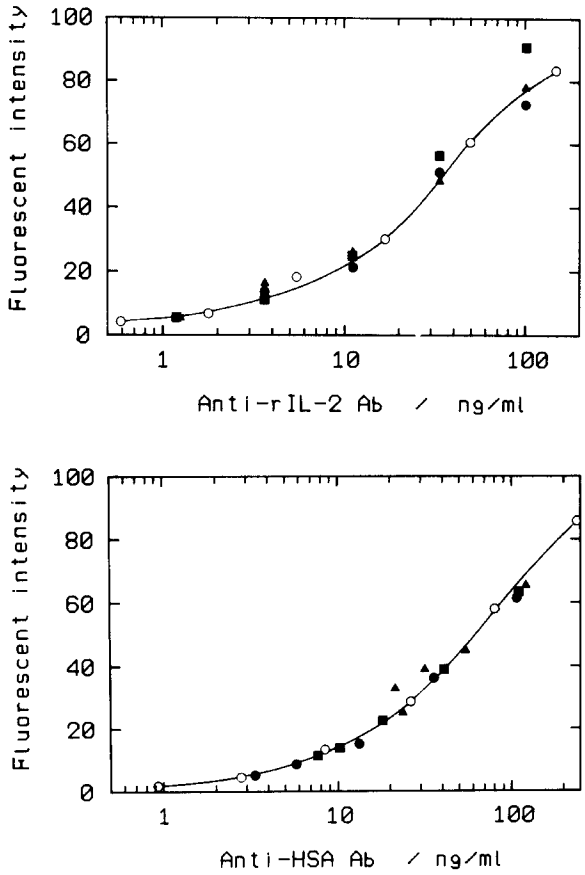


FIGURE 2. Standard curve of the simultaneous IEMA for anti-rIL-2 Ab with various concentrations of anti-HSA Ab (upper) and that for anti-HSA Ab with various concentrations of anti-rIL-2 Ab (lower).
Anti-HSA Ab concentrations in the upper panel are 0 (○), 3.70 (●), 11.1 (■), and 33.4 (▲) ng/ml. Anti-rIL-2 Ab concentrations in the lower panel are 0 (○), 3.70 (●), 11.1 (■), and 33.4 (▲) ng/ml. Concentrations of the standard solutions are shown in these horizontal axes and the measured values of rat serum samples from these standard curves need to be corrected by a dilution factor of 50.

TABLE 1

Precision of the Simultaneous IEMA for Anti-rIL-2 Antibody and Anti-HSA Antibody in Rat Serum.

| Ab | Ab Added, ng/ml | Ab Measured, ng/ml | | | |
|---|---|---|---|---|---|
| | | Mean | (n) | S.D. | (C.V.%) |
| Anti-rIL-2 | 75 | 77 | (6) | 5.5 | ( 6.9) |
| | 650 | 600 | (6) | 40 | ( 6.7) |
| | 1950 | 1710 | (6) | 215 | (13 ) |
| Anti-HSA | 70 | 73 | (6) | 3.5 | ( 4.8) |
| | 600 | 605 | (6) | 34 | ( 5.6) |
| | 5500 | 4940 | (4) | 505 | (10 ) |



The precision was examined using samples of Ab-added rat sera. As shown in Table 1, the intra-assay variation on both Ab assays, C.V. = 5-13%, was similar to those of conventional IEMAs. However, precision and recovery became worse around maximum concentration of each assay range.

Comparison with Conventional IEMA

In this study, we also developed respective IEMAs using one Ag-immobilized ball. The conditions of the reaction volume (300 $\mu$ l) and dilution of the enzyme-labelled second Ab (1:30,000) was optimized in a similar manner to that described above. The other conditions were the same as the simultaneous IEMA. Also, the sensitivity, reliability and precision were similar to those of the simultaneous IEMA (data not shown).

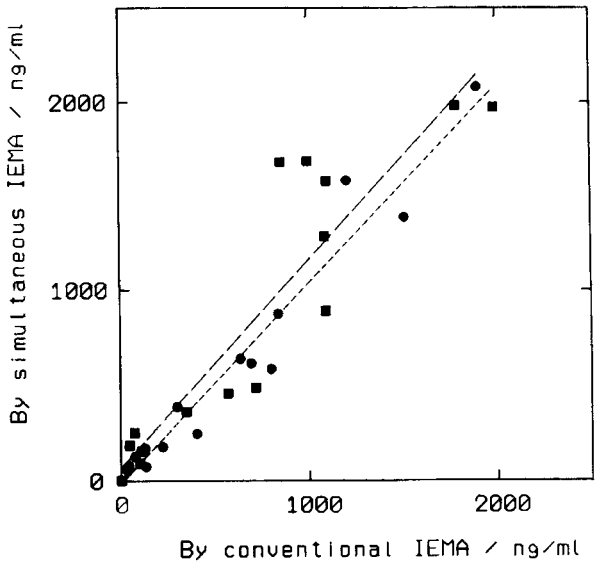


FIGURE 3. Correlation between conventional IEMA and simultaneous IEMA.
For anti-rIL-2 Ab (--●--); y = 1.05 x - 4.37, r = 0.979, n = 20. For anti-HSA Ab ( —■—); y = 1.10 x + 76, r = 0.976, n = 17.



As shown in Fig. 3, good correlations were found between the conventional IEMA and the simultaneous IEMA for both Abs.

## DISCUSSION

We developed a novel simultaneous IEMA for two Abs using two Ag-immobilized balls in an assay tube. By use of our simultaneous IEMA, the Ab levels in rat sera were observed after repetitive administration of rIL-2 containing HSA as a vehicle. As shown in Fig. 4, the serum concentrations of anti-rIL-2 Ab increased with the dose of rIL-2 but those of anti-HSA Ab did not. The details of further results will be given elsewhere.

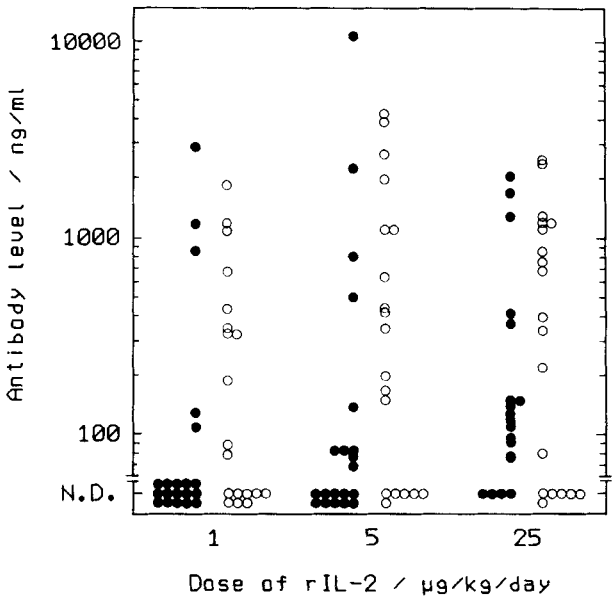


FIGURE 4. Concentrations of anti-rIL-2 Ab (●) and anti-HSA Ab (○) in rat sera after repetitive administration of rIL-2 containing HSA (2.5 mg/kg/day) as a vehicle. N.D.: <70 ng/ml (both Abs)



This method was not only comparable to a conventional IEMA using one Ag-immobilized ball with respect to assay performance, i.e., sensitivity, reliability, reproducibility, and precision, but also much more feasible for mass routine assays. We believe that this simultaneous IEMA can replace conventional IEMAs. The assay results of Fig. 4 offer proof of the value of this method.

During mass routine assay, transferring each ball to a tube for the enzyme assay can be tedious. We recommend rolling one ball into the tube held at a suitable angle while the other ball remains at the bottom due to surface tension. This manipulation makes it easy to dispense balls to the appropriate tubes.

Ianconescu developed a simultaneous IEMA using the F.A.S.T. system (5). As this assay uses two solid phases with different shapes, wells and lids, and the standard curve profile may change due to the differences in surface breadth. Furthermore, the F.A.S.T. system is not suitable for measurement of radioactivity. Our method overcame these problems. The use of solid phases of the same shape eliminates differences of Ag binding potential of the solid phases. Also, if necessary, we can easily count radioactivities and measure the enzyme activities by various methods, such as colorimetry and chemiluminometry using commonly available equipment. In this study, we tried to measure only two kinds of Ab but the number can be increased using the respective Ag-immobilized balls, preferably 1/8 inch diameter.



Our procedure should be applicable to usual IEMAs and immunoradiometric assays for Ags by combining labelled Abs against each Ag. Moreover, simultaneous competitive enzyme immunoassays or radioimmunoassays can be performed in a similar manner using Ab-immobilized balls and mixtures of labelled Ags.

In simultaneous assays, samples are not always diluted to their optimum concentrations for each analyte. The sensitivity of some assays may be decreased by the addition of the unlabelled second Ab. We are now working for the development of other simultaneous assays.

## ACKNOWLEDGEMENTS

We thank Dr. Y. Harada, who gave us the samples of rat sera.

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