Comparison of Radioimmunoassay with Enzyme-and Fluorescence-Immunoassays

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The essential criteria for a useful analytical technique are: specificity, sensitivity, accuracy, precision, simplicity, rapidity, applicability and being economical and non-hazardous. Hence, immunoassay based on a specific antigen-antibody reaction can be considered as a very sensitive and specific analytical technique. Immunoassay is subdivided into radioimmunoassay (RIA) and non-isotopic immunoassay according to markers chosen for detection of the antigen-antibody reaction. The present review compares and evaluates the characteristics, techniques and applications of the above method.

I. Characteristics of Radioimmunoassay and Non-isotopic Immunoassay

RIA developed by Berson and Yalow[1] based on a detection using radioisotope is sensitive, specific and has wide applications. For example in Japan, the number of in vitro tests using radioisotopes performed during past 10 years increased about 10 fold and reached about 60 million per year. However, RIA requires special facilities for its usage and for radioisotope disposal. Usually, physical separation of bound and free forms is necessary and the labeled substances have a short half-life.

Consequently, efforts have been made to develop a non-isotopic immunoassay or an alternative immunoassay that is based on antigen-antibody reaction but does not requires a radioisotope[2-4] Table 1 lists non-isotopic immunoassays subdivided into two groups. In the labeled method group, the antigen-antibody reaction is detected using labeled markers and in the non-labeled method group it is detected directly by such physical properties as turbidity and electric response.

The non-isotopic immunoassay procedure can be performed in a routine laboratory. The substance labeled with marker can be used for a long period, usually more than 5-6 months. Further separation of bound and free forms is not necessary in homogeneous system. However, in non-isotopic immunoassay, labeling and detection are rather complicated and the method is sometimes insensitive.

Table 2 shows the various kinds of markers and the sensitivity levels needed for their detection. A direct comparison of these values is difficult as there are large differences between theoretical

Table 1. Non isotopic (alternative) immunoassay.

Assay	Marker
Labeled method	
Enzyme immunoassay	Enzyme
Fluorescence immunoassay	Fluorescent
Phosphorescence immunoassay	Phosphorescent
Luminescence immunoassay	Luminescent
Spin immunoassay	Free radical
Viro immunoassay	Bacteriophage
Metal immunoassay	Metals
Particle immunoassay	Particles
Non-labeled method	
Nephelometry	Immunoprecipitin
Immunosenser	Electric response

Table 2. Sensitivity of various markers.

Markers	Sensitivity (mol/l)
Enzyme	4×10^{-17}
Luminescence	1×10^{-15}
Radioisotope	3×10^{-15}
Fluorescence	1×10^{-10}
Spin	2×10^{-8}

Table 3. Comparison of RIA, EIA and FIA.

	RIA	EIA	FIA
Facility	special	routine	
Labeling	simple	complicated	
Labeled substance	short lives	long lives	
B/F separation	necessary	not nescessary in homogeneous	
Detection	simple	complicated	simple
Interference	none	not so often	often
Sensitivity	sensitive	insensitive in some cases	

and practical estimations. Enzyme with a catalytic activity seems to be the most sensitive marker. However, the enzyme activity is determined by a biological reaction, thus some non-specific factors may interfere in the detection of the activity. Furthermore, the affinity of antigen-antibody reaction is frequently reduced when high molecular weight enzymes are coupled to antigens or antibodies. The sensitivity for detection of luminescent or fluorescent markers is lower than that of enzyme or radioisotope, but their small molecular size makes them useful markers when coupled to antigens or antibodies. However some non-specific factors usually interfere on detection of fluorescent or luminescent intensity. Such an interference is not observed in the determination of radioisotope.

Table 3 summarizes the characteristics of RIA, enzyme immunoassay (EIA) and fluorescence immunoassay (FIA).

II. Fluorescence-, Enzyme- and Radio-immunoassays of Thyroxine with Clonotype Antibodies Prepared by Chromatofocusing Technique

The sensitivity and specificity of an immunoassay depend on the quality of the antibody used. Monoclonal antibodies can distinguish one antigen from other antigens of structurally similar nature, but the affinity constants of monoclonal antibodies are usually less than those of the original polyclonal antibody[5]. Thus, competitive immunoassay with monoclonal antibody is generally specific but not so sensitive. On the other hand, it is well known that antiserum is heterogeneous with respect to its affinity.

Recently, we developed a new method for the separation of high affinity antibodies from polyclonal antiserum. It is based on the differences in the isoelectric points of individual clonotype antibodies. The separated antibodies were used in EIA, FIA and RIA of thyroxine (T_4)[6].

Materials and Methods

Chromatofocusing:

The IgG fraction of rabbit anti T_4 serum was fractionated by chromatofocusing using a "Fast Protein Liquid Chromatography (FPLC)" system. The protein in the eluate from a Mono P^{TM} column (Pharmacia Fine Chemicals AB., Uppsala, Sweden) was monitered at 280 nm. The anti T_4 antibody titer was measured by inhibition in solid phase RIA using a kit of Eiken Immunochemical Laboratory (Tokyo, Japan).

Four major and several minor peaks of IgG protein were detected in the pH range of 6 to 8 on FPLC fractionation. The titer of anti T_4 antibody was also observed in the same pH region. The fractions were pooled and used for RIA, EIA and FIA (Fig 1).

RIA of T_4:

A mixture of 50 ul of T_4, 20 ul of diluted antibody and 50 ul of ^{125}I-labeled T_4 in a final volume of 0.3 ml of phosphate buffer (pH 9.0) was incubated for 20 h at room temperature.

Then, 4 ul of normal rabbit serum IgG and an appropriate amount of goat anti-rabbit IgG were added to the incubation mixture. After 24 h, each tube was centrifuged, and the resulting precipitate was counted in a gamma-counter.

EIA of T_4:

T_4-β-D-galactosidase conjugate was prepared using 4-(maleimido-methyl) cylohexane-1-

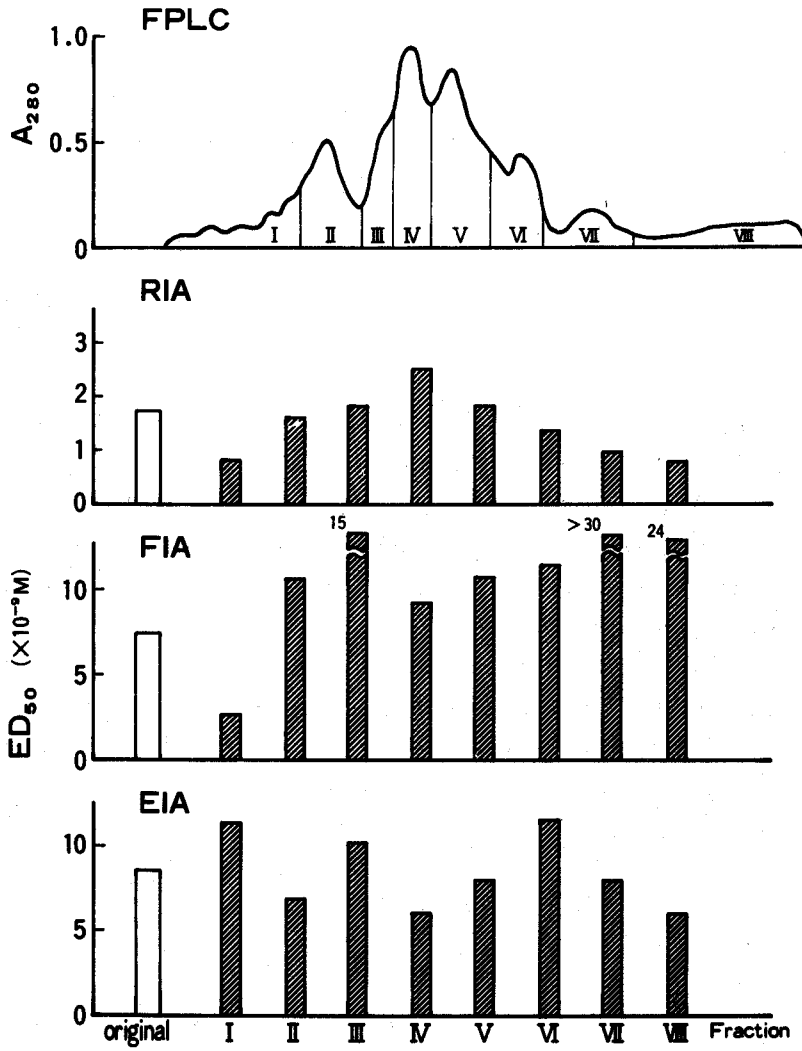

Fig. 1. The ED_{50} values (thyroxine concentration corresponding to 50% of bound form at zero concentration of thyroxine) in thyroxine immunoassays using clonotype anti-thyroxine antibody prepared by Fast Protein Liquid Chromatography (FPLC).

RIA: Double antibody radioimmunoassay

FIA: Fluorescence polarization immunoassay

EIA: Enzyme immunoassay

(Cited from Miyai, K. et al., Proceedings, XII International Federation of Clinical Chemistry, Rio de Janeiro, 1984.)

carboxylic acid succinimide ester. A mixture of 5 ul of T_4-β-D-galactosidase conjugate, 50 ul of T_4, 5 to 37 ul of anti T_4 IgG solution, and 300 ul of 20 mM Tris-HCl buffer (pH 9.0) containing ANS, salicylic acid and NaN_3 was incubated for 3 h at room temperature. Then 4 ul of normal rabbit IgG and 30 ul goat anti rabbit IgG was added and the mixture were further incubated overnight at room temperature. The mixtures were centrifuged and the resulting precipitates were suspended in 0.8 ml of 3 mM o-nitrophenyl-galactopyranoside in buffer and incubated for 2 h at 37°C. Then 0.1 ml of 15% sodium carbonate was added and the absorbance was measured at 405 nm.

FIA of T_4:

T_4-fluorescence conjugate used in this experiment was a reagent in a kit TDX® obtained from Abbot Laboratories, As a fundamental experiment, 75 ul of T_4-fluorescence conjugate was mixed with 1.25 ml of 90 mM barbital buffer (pH 8.5) containing 0.5 M NaCl and its fluorescence polarization (FP) was measured with a fluorescence polarization fluorimeter. Then the anti T_4 was added and the FP was measured for about 1 h until it reached a contstant value. The quantity of anti T_4 was adjusted to obtain the same FP_0 (FP at zero concentration of T_4) as the original anti T_4-IgG. Then standard T_4 was added cumulatively, and the FP was measured at each standard level.

Results and Discussion

The sensitivity around the mean doses of the assay was expressed as ED_{50} which is the concentration of unlabeled T_4 corresponding to 50% of bound form at zero concentration of T_4. Fig. 1 shows the ED_{50} values of RIA, EIA and FIA using FPLC fractions. The most sensitive assay were obtained using fractions I and VIII for RIA, fraction I for FIA and fraction VIII for EIA. As shown in Fig. 2, there were no correlation between ED_{50} values of FIA and RIA or those of FIA and EIA.

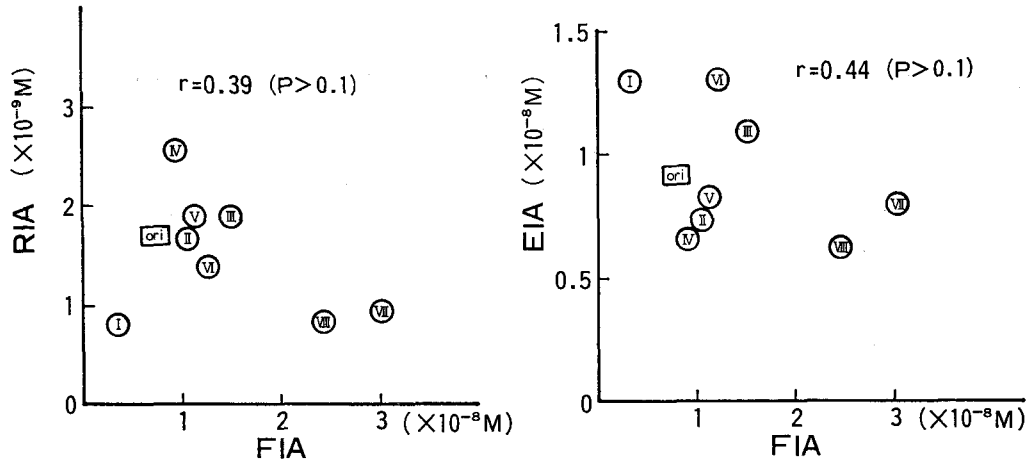

Fig. 2. Correlation between ED_{50} of FIA, RIA and EIA for thyroxine (ED_{50}, FIA, RIA, EIA: see Fig. 1).

Conclusions

The sensitivity of immunoassay can be improved by an appropriate combination of clonotype antibody and the assay system.

III. Application of Radioimmunoassay and Enzyme Immunoassay to Mass Screening for Neonatal Hypothyroidism.

During the past 20 years, neonatal screening programs for inborn errors of metabolism have become wide-spread, as early diagnosis and treatment can prevent irreversible mental retardation resulting from various disorders. A recent development in this field is the introduction of immunoassay. The screening techniques used include measurements of T_4 or thyrotropin (TSH)

Table 4. RIA and EIA for measuring TSH in dried blood samples.

	RIA	EIA
Facility	special	routine
Sample size (3 mm disc)	2	2
Procedure time (days)	3	2
Measurable range (U/ml)	5 - 160	5 - 160
C.V. (%)		
within assay	11 - 19	10.6 - 13.6
between assays	5 - 11	11.6 - 18.3
Correlation		
EIA (y)/RIA (x)	y = 1.1 x -6.3, r = 0.94	

Table 5. Comparison of results of pilot screening for neonatal hypothyroidism by RIA and EIA methods.

	RIA	EIA
Total infants screened	17,160	17,160
Recalled for examination	52 (1/330)	83 (1/206)
Abnormal cases with elevated TSH	6	6
Primary hypothyroidism	5 (1/3, 400)	5 (1/3, 400)
Transient infantile hyperthyrotropinemia	0	1
Severe anemia with normal TSH in serum but elevated TSH in blood	1	0

for hypothyroidism, 17 -oH-progesterone for congenital adrenal hyperplasia, trypsin for cystic fibiosis, and alpha-fetoprotein for nural tube defect. These substances are usually measured by RIA. However, the number of samples taken in a mass screening program is so large that a non-isotopic method is preferable. Recently, new RIA[7,8] and EIA[9-11] methods for measurement of TSH in dried blood samples on filter paper for use in mass screening were developed in Japan. Our results of testing new born babies by both the RIA and EIA methods are described here.

Materials and Methods

Procedure of RIA:

In the early experiment, paired TSH assay method was used[12]. A 9-mm dried blood disc from each subject was soaked in 500 ul of phosphate buffered saline. Equal parts (200 ul) of the extracts of blood discs from two babies were combined and TSH concentration of the mixture was determined by the two-step double antibody method of RIA using a kit of Daiichi Radio-isotope Laboratory (Tokyo, Japan). In the latter experiment, single TSH assay method was used. TSH in two 3-mm dried blood discs was determined by the two-step double antibody method of RIA using an Eiken Cretin-kit (Eiken Immunochemical Laboratory, Tokyo, Japan).

Procedure of EIA:

Semiautomated one step sandwich EIA was used.[12,13] All reagents were supplied from Eiken Immunochemical Laboratory. Two 3-mm dried blood discs and 150 ul of normal rabbit serum IgG solution were placed in anti-TSH IgG coated tube and the mixture was allowed to stand for 40 min at room temperature. Then 100 ul of anti-TSH IgG- -D-galactosidase complex which was prepared with N,N'-o-phenylendimaleimide was added. The mixture was incubated for 20 to 24 h at 25°C. After 2 ml of washing solution was poured into each tube, the contents of the tubes were discarded. This washing step was repeated three times. The enzyme activity in the tubes was measured in an analyzer specifically manufactured for this purpose (Shimazu Co., Kyoto, Japan). The substrate (250 ul of 4-methylumbelliferyl- -D-galactopyranoside) was added to each tube and after incubation for 40 min at 37°C, 25 ml of 0.1 M glycine buffer (pH 0.3) was added to stop the enzyme activity. Fluorescence intensity was measured in a flow fluorescence spectrometer at 450 nm with excitation at 350 nm. The data were linked to a computer and the calibration curve, measured values and distribution of the values were automatically computed.

Cut-off point:

When the TSH values of the babies were above the cut-off point in the first determination, the TSH concentrations in the remaining samples from these babies were measured concomitantly with other babies in the second determination. When the values were again above the cut-off point, the babies were recalled for examination. The cut-off point was set at the 4th percentile in the paired TSH assay and the 2nd percentile in the single RIA and EIA assays.

Results and Discussion

Fundamental studies:

Table 4 shows a comparison of RIA and EIA of TSH for measuring TSH in dried blood samples on filter paper. The sensitivity, reproducibility of EIA are comparable with those of RIA. A good correlation was observed between the TSH values determined by RIA and EIA.

Results of screening[13]:

Among 247,500 babies in Osaka area screened by the paired TSH assay between 1975 and 1982, 30 patients had a typical primary hypothyroidism. Table 5 shows a comparison of results on the pilot screening for neonatal hypothyroidism by the single RIA and EIA methods. The recall rate was slightly greater in EIA than in RIA. One case of normal serum TSH and severe anemia, which may cause increasing TSH concentration in dried blood samples, was detected by RIA but not by EIA. On the other hand, a case of transient hyperthyrotropinemia with slightly elevated TSH was detected by EIA but not by RIA.

Conclusions

Though the EIA method had a slightly higher recall rate, its performance as an analytical technique makes it useful and reliable for mass screening.

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